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Structural characteristics of immunogenic liver-stage antigens derived from *P. falciparum* malarial proteins

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ABSTRACT

A fully effective antimalarial vaccine must contain multiple proteins from the different development stages of *Plasmodium falciparum* parasites involved in host-cell invasion or their biologically active fragments. It must therefore include sporozoite molecules able to induce protective immunity by blocking the parasite's access to hepatic cells, and/or proteins involved in the development of this stage, amongst which are included the Liver Stage Antigen-1 (LSA-1) and the Sporozoite and Liver Stage Antigen (SALSA).

Our studies have focused on the search for an association between the structure of high activity binding peptides (HABPs), including both conserved native and their modified analogues, and their ability to bind to the MHC Class II HLA-DR molecules during formation of the MHCII-peptide–TCR complex leading to inducing the appropriate immune response. These studies are part of a logical and rational strategy for developing multi-stage, multi-component, minimal subunit-based vaccines, mainly against the *P. falciparum* malaria.

Introduction

Malaria, mainly the one caused by the *Plasmodium falciparum* parasite, is one of the main causes of morbidity and mortality in tropical countries, mainly in sub-Saharan Africa. The infection initiates when sporozoites (larvae-like structures of the *Plasmodia*) transmitted via the bite of an infected female *Anopheles* mosquito invade liver cells, in a process mediated through a still unknown number of parasite proteins. It has thus become necessary to identify and study those proteins involved in the invasion process to hepatic cells in order to use them as targets for developing a vaccine capable of blocking invasion and controlling the spread of this devastating disease; which annually afflicts 500 million people and kills around 3 million of them, being children under the age of 5 the most affected population [1].

The idea of developing malarial vaccines with sporozoite antigens was first considered following the observation that immunization with radiation-attenuated sporozoites could induce complete protective immunity against experimental challenge with fully-infective non-irradiated sporozoites [2]. Strategies were then developed aimed at generating an antibody response capable of neutralizing sporozoites and thereby prevent them from invading the hepatocyte, and/or elicit a cell-mediated immune response that will inhibit their development as intra-hepatic parasites.

After the identification of the Circumsporozoite Protein (CSP) and the Thrombospondin-Related Protein (TRAP) present on the membrane of this parasite's stage and their suggestion as probable targets of protective immunity against the sporozoite, efforts were concentrated on molecules expressed by the *P. falciparum* parasite during development of the liver stage. The first was the Liver Stage Antigen-1 (LSA-1), a protein of about 240 kDa composed of 1909 amino acids whose N- and C-terminal regions are conserved among parasite strains from different parts of the world [3].

LSA-1 contains a characteristic 17 amino-acid-long sequence (EQQSDLEQERRAKEKLQ) repeated 86 times between residues 154 and 1629 that displays an amphipathic α-helical structure [4]. LSA-1 synthesis begins soon after sporozoite invasion to the hepatic cell and increases throughout the liver stage. It localizes around developed hepatic schizont pseudocytomers and associates with flocculent material in the parasitophorous vacuole surrounding hepatic merozoites. LSA-1 has not been found in the cytoplasm or on the surface of infected hepatocytes; however, it has been suggested that peptide fragments from this protein may be exported from the parasitophorous vacuole to hepatocyte surface [5].

Based on these observations, 20-mer-long non-overlapped synthetic peptides covering the entire protein (28 in total) were analyzed using a highly robust, specific and sensitive binding assay

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to identify amino acid sequences with high binding capacity to Hep-G2 cells and Red Blood Cells (RBCs) in the search for minimal host-cell binding protein fragments. As a result, three high activity binding peptides (HABPs) were found inside the variable region and therefore discarded from subsequent immunological studies. One peptide denoted as 20,630 (INGKIIKNSEKDEIIKSNLRY) located towards the conserved N-terminal region was found to bind with high capacity to both cells [6].

The Sporozoite and Liver Stage Antigen (SALSA) was also analyzed using this same methodology. SALSA is a short protein expressed on the sporozoite membrane surface and also during the *P. falciparum* liver stage. It is encoded by a 249 bp open reading frame (ORF) expressing a 70 kDa protein for which only the first 83 residues are reported in Genbank (Accession No. AAB36334). SALSA synthesis is initiated during the sporozoite stage and increases throughout the whole hepatic stage [7]. According to ultrastructural studies using colloidal gold-labeled antibodies, SALSA is regularly distributed over the parasite's surface and not present inside the parasite's cytoplasm. It also seems to be confined to the granular material present in the parasitophorous vacuole in mature hepatic merozoite forms [7]. Preliminary studies have indicated that SALSA antibodies significantly reduce sporozoite invasion, suggesting that this protein could be involved in sporozoite invasion to hepatic cells.

Same as before, 5 non-overlapping 20 amino-acid-long peptides covering this entire protein were synthesized and analyzed regarding their binding capacity to hepatic cells and RBCs. Two of these peptides, namely 20,608 (IWSAEKKDEKEASEQGEESHY) and 20,610 (TNEKKDDGKTDKVQEKVLEKY), were found to bind with high activity to HepG2 cells but not to RBCs [8].

Since it has been found that conserved HABPs are not antigenic nor immunogenic unless they are modified by replacing their critical binding residues (previously identified by glycine analogue scanning) [6,8] for others having similar volume and surface but opposite polarity, thus rendering them immunogenic and protection inducers [9], modified LSA-1 and SALSA HAPBs were synthesized and assessed regarding their immunologic and structural properties.

LSA-1 and SALSA have been extensively studied due to their suggested importance in infection and their potential use as vaccines and for them a strong genetic restriction mediated by the Major Histocompatibility Complex (MHC) molecules has been described as discussed herein. Our work has focused on searching for an association between the three dimensional (3D) structure of these native and modified HABPs with their ability to bind to the MHC Class II (HLA-DR$\beta1^*$) molecules during formation of the MHCII-peptide–TCR complex necessary to induce an appropriate immune response, with the purpose of developing multistage, multi-component, minimal subunit-based synthetic vaccines capable of controlling malaria, mainly the one caused by *P. falciparum*.

Materials and methods

Synthetic peptides. Peptides were synthesized by solid-phase *t*-Boc chemistry [10] and numbered according to our Institute's sequential numbering system (modified peptides are written in bold throughout the manuscript). The final product was purified by analytical HPLC and characterized by RP-HPLC and mass spectrometry (MS). Peptide polymers for immunization studies were obtained after Cys and Gly residues were added at the N- and C-termini. High molecular weight polymers within the 8–24 kDa were obtained for immunization using the cysteine oxidation method and assessed by size exclusion chromatography (SEC).

Circular dichroism (CD) analysis. The CD spectra of the peptides were collected in 50 mM phosphate buffer, pH 7.0, and trifluoroethanol/H_2O solution (70/30) using a JASCO J810 spectropolarimeter with 1-mm path length cell. CD data were expressed as mean residue ellipticity [θ] in deg·cm^2·dmol^{-1} [11].

Animals and immunization. Groups of 6–8 *Aotus* monkeys, kept in our field station in Leticia (Amazonas, Colombia) according to the National Institute of Health guidelines for animal handling [12] and under strict supervision of CORPOAMAZONIA, were subcutaneously immunized with 125 μg polymerized peptide on days 1 and 20. Peptides were previously homogenized with Freund's complete adjuvant for the first dose and with Freund's incomplete adjuvant for the second dose. Controls received only Freund's adjuvant and saline solution on the same days. Blood samples were drawn pre-immunization (P0), 20 days after the first (I_{20}), and 15 (II_{15}) and 20 (II_{20}) days after the second dose.

All monkeys were previously tested for the presence of antibodies against air-dried fixed *P. falciparum* sporozoites and infected RBC schizonts at a 1:20 dilution. Monkeys testing positive were returned to the jungle without further manipulation.

Indirect immunofluorescence assays (IFA). P. falciparum 3D7 strain sporozoite air-dried slides, kindly provided by Dr. Patricia De la Vega, and air-dried RBCs infected with the FCB-2 *P. falciparum* strain were used to evaluate the presence of SALSA in the different parasite stages. Slides were incubated for 30 min with monkey sera dilutions, starting at 1:40 for antibody analysis. Reactivity was observed by fluorescence microscopy using the F(ab)2 fragment of a goat affinity purified IgG anti-monkey IgG:FITC conjugate diluted 1:100. Pre-immune sera from all monkeys were used as negative controls.

Western blot analysis. One hundred and twenty five microgram of recombinant *P. falciparum* LSA-1 and SALSA (kindly provided by Dr. Pierre Druilhe, Pasteur Institute, Paris) were separated by discontinuous SDS-PAGE using 12% acrylamide (w/v) and transferred to nitrocellulose membranes. Nitrocellulose strips were individually incubated with monkey sera diluted 1:100 in blocking solution, washed several times and incubated with goat anti-*Aotus* IgG conjugated to alkaline phosphatase (AP) at a 1:1000 dilution and developed with NBT/BCI.

NMR analysis. The 3D structures were determined based on ^{1}H NMR studies where samples are dissolving in 500 μL of a 2,2,2-trifluoroethanol-d_3 (TFE-d_3)/H_2O mixture (30/70 v/v). Studies have shown that nascent structures have an inherent propensity to acquire their native structures in the presence of solvents such as 2,2,2-trifluoroethanol (TFE), which stabilizes well-ordered conformations [13].

^{1}H NMR spectra were acquired on a BRUKER DRX-600 spectrometer. Proton spectra were assigned by using double quantum filter correlation spectroscopy (DQF-COSY) [14], total correlation spectroscopy (TOCSY) [15] and nuclear Overhauser enhancement spectroscopy (NOESY) experiments [16]. Two dimensional NMR data were processed using TOPSPIN software. NOESY spectra recorded at different temperatures (285–315 K) were used for obtaining amide temperature coefficients for predicting hydrogen bonds ($-\Delta\delta H^N/\Delta T$ ppm/K).

Structure calculations. Peptide structure was determined using Molecular Simulations Inc. (MSI) software. NOEs were grouped into three categories (strong, medium and weak) and then converted into distance restrictions (1.8–2.8, 2.8–3.5 and 3.5–5.0 Å). Hydrogen bond constraints were introduced for NH proton temperature coefficients; distance ranges involving these likely NH–O hydrogen bonds were set at 1.8–2.5 Å. A set of 50 structures was obtained using the DGII software and refined using a restrained simulated annealing protocol.

HLA-DR molecule affinity purification. Human molecules were purified from DR1 WT100BIS (DR$\beta1^*$0101), DR3 COX (DR$\beta1^*$0301), DR4 BSM (DR$\beta1^*$0401) and DR11 BM21 (DR$\beta1^*$1101) homozygous EBV-B cell lysates by affinity

chromatography using anti-HLA-DR mAb L-243 cross-linked to protein-A Sepharose CL-4B (Amersham, Pharmacia Biotech, AB) as affinity support.

Competition binding assays. Peptide binding competition assays were carried to measure unlabeled peptide's relative binding affinity to compete with biotinylated indicator peptides for purified HLA-DR molecules, where a good competitor peptide was able to inhibit binding of indicator peptide to any HLA molecule being tested by more than 50% [17].

Results and discussion

Peptide characterization

Analytical chromatography results showed that peptide purity after semi-preparative HPLC was sufficiently high to be analyzed by NMR. MS assays determined the following experimental masses (theoretical masses appear indicated in parenthesis). For LSA-1 peptides: 2323.60 (2325.10) for 20,630; 2281.60 (2283.00) for **24,282**; and 2522.10 (2523.70) for **24,404**. Experimental masses of SALSA peptides were: 2368.11 (2369.80); for peptide 20608; 2272.73 (2273.50) for **24,276**; 2498.90 (2495.80) for **24,488**; 2333.27 (2334.60) for peptide 20610 and 2517.30 (2519.30) for **24,402** (data not shown). Moreover, the polymers used in immunization assays had molecular weights in the 8–24 kDa range as assessed by SEC.

The CD spectra of monomeric and polymeric peptides collected in 30% TFE showed that both peptide forms had similar secondary structures, suggesting that the configuration adopted by the polymers used for immunizing *Aotus* monkeys was quite similar to the structure of their monomer analogues used for structural analysis (CD, ^{1}H NMR), and in biological (HLA-DRβ1* binding) and immunological assays (IFA and Western blot). All peptides presented two negative minima at 208 and 222 nm, suggesting a tendency for these peptides to present an α-helix structures (Supplementary material).

Table 1A
Amino acid sequences of native conserved HABPs corresponding to the original *P. falciparum* LSA-1 and SALSA peptides, as well as immunogenic modified HABPs (shown in bold) with their corresponding modifications. The table contains the number of *Aotus* monkeys (5–8 per group) developing high antibody titers against the whole parasite, as assessed by IFA on days 0 (PI), 20 days after the first immunization (I_{20}), 10 (II_{10}), 15 (II_{15}) and 20 (II_{20}) days after the second immunization.

Polimerized Peptide No.	Peptide Sequence	PI	I_{20}	II_{10}	II_{15}	II_{20}
Pf-LSA1	**P_1 P_4 P_6 P_9**					
20,630	I N G K **I** I K N **S** E K **D** **E** **I** I K S N L R Y	0/5	0/5	0/5	0/5	0/5
24,282	– – – – **N** – – – **A** – – **P** **M** – – – – – – – –	0/8	6(640)	3(640)	1(320)	1(320)
24,404	– – – – **N** – – – **A** – – **P** **M** **A** – – – – – – –	0/8	0/8	0/8	1(160)	1(320)
Pf-SALSA						
20,608	I W S A E K K D E K E A S E Q G E E S H Y	0/5	0/5	0/5	0/5	0/5
24,276	– – – **S** **M** – **M** – – – **M** – **A** **M** – – – – – – –	0/8	3(640)	1(320)	1(320)	1(320)
24,488	– – – **S** **V** – **M** – – – **V** – **A** **V** – – – – – – –	0/8	1(320)	0/8	0/8	0/8
20,610	T N E **K** K **D** D G K **T** **D** **K** V Q E K V L E K Y	0/5	0/5	0/5	0/5	0/5
24,402	– – – **M** – **N** – – – **V** **A** **M** – – – – – – – – –	0/8	0/8	0/8	1(320)	1(320)

Immunological studies

A large number of studies carried out during the last 15 years in our institution with hundreds of native or HABPs modified in their critical binding residues has allowed us to break the immunological code of silence (i.e. the non-antigenicity nor immunogenicity of conserved HABPs) and lead us to the conclusion that critical binding residues of these HABPs (previously identified by glycine analogue scanning) had to be replaced by others having similar mass and volume but opposite polarity to render them into highly immunogenic and protection inducing molecules. Consistently with these observations, when the only conserved LSA-1 peptide binding with high capacity to hepatocytes and RBCs (thereby named HABP 20,630) was modified in its critical hepatocyte binding residues yielding peptides **24,282** and **24,404**, very high antibody titers were induced against the *P. falciparum* sporozoite as assessed by IFA (Table 1A, Fig. 1A), with the later peptide inducing only comparable antibodies titers after the second immunization. These same sera recognized by Western blot the recombinant LSA-1 protein (with an apparent molecular weight of 77.5 kDa) (Fig. 1C) as well as in an ELISA (data not shown).

Similarly, SALSA conserved HABPs 20,608 and 20,610 were not immunogenic but induced long-lasting antibody titers against the sporozoite when they were modified into peptide **24,276** and **24,402**, respectively, as assessed by IFA (Table 1A, Fig. 1B1), as well as against blood-stage structures (Fig. 1B1-B4). These data agree with previous studies indicating that SALSA is expressed in sporozoite forms (pre-erythrocytic development stage) as well as in all erythrocytic stages.

Modified HABP **24,488** induced short-lived antibodies, which agrees with a phenomenon previously described by us [18]. The 20,610 modified SALSA conserved HABP named **24,404** also induced high antibody titers recognizing structures in both parasite stages but at a later time.

All these antibodies recognized by Western blot a set of proteins of about 47 and 42 kDa, probably corresponding to cleavage fragments of the recombinant SALSA protein, which has a theoretical molecular mass of 70 kDa.

Table 1B
Summary of native and modified HABP's structural features (as determined by ^{1}H NMR) and biological properties (as assessed by their ability to bind to purified HLA-DRβ1* allele molecules).

Protein	Peptide	Structural features	Rmsd Å	Maximum NOE violations Å	Maximum angular violations °	Distance (Å)	Haplotypes: DR1 % Binding HLA-DRβ1* alleles 0101°	DR52 0301	DR52 1101	DR53 0401
Pf LSA1	20,630	α-helix G3-N18	0.3	0.2	1.2	18.1	-18	44	30	12
	24,282	α-helix K7-E10 and P12-N18	0.30	0.4	1.3	19.7	-17	41	**60**	17
	24,404	α-helix K4-E10 and P12-I15	0.1	0.2	1.0	19.8	36	17	**50**	35
Pf SALSA	20,608	α-helix W2-E18	0.2	0.2	1.1	19.8	6	16	26	8
	24,276	α-helix S3-G16	0.3	0.1	1.0	19.5	-8	**47**	43	19
	24,488	α-helix S3-K6 and D8-H20	0.2	0.1	1.0	16.9	0	**50**	32	31
	20,610	α-helix K4-Y20	0.3	0.1	1.1	21.4	-11	26	31	12
	24,402	α-helix K5-K20	0.3	0.2	1.2	19.8	-2	**52**	24	24

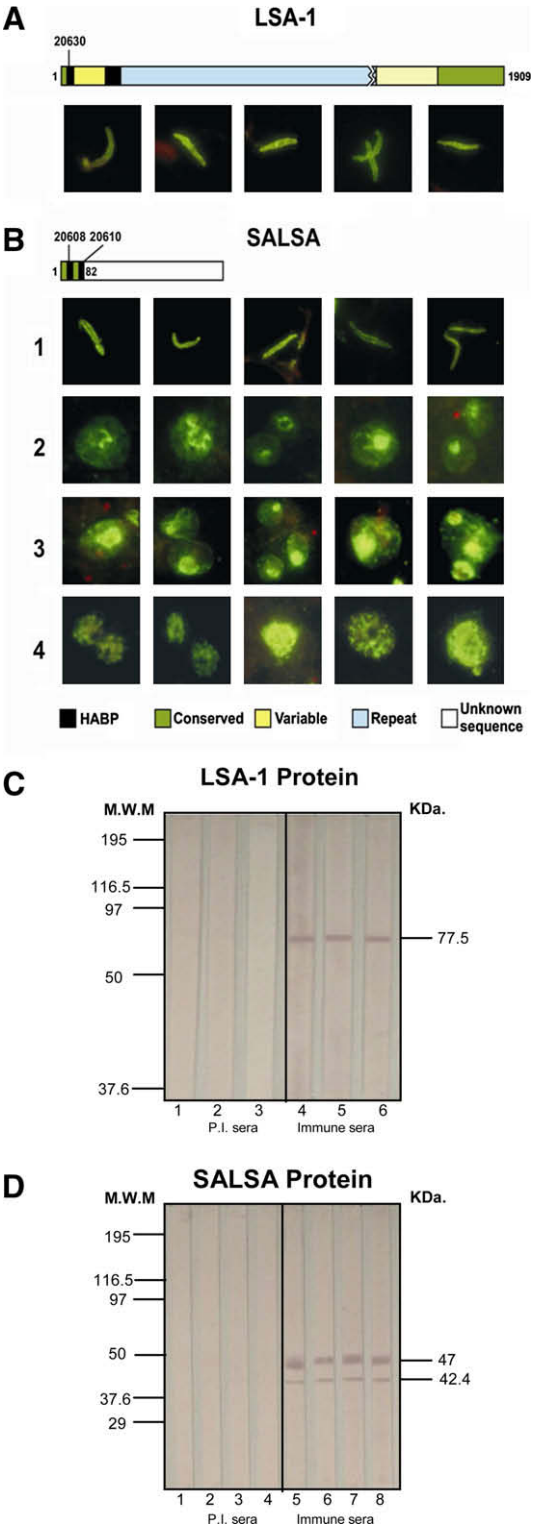

Fig. 1. (A) Schematic representation showing the structure and localization of conserved HABP 20,630 in LSA-1 and the granular immunofluorescence pattern on sporozoites membrane as assessed by IFA, determined using sera of *Aotus* monkeys immunized with modified **24,282**. (B) SALSA structure showing the localization of conserved HABPs 20,608 and 20,610. B1 to B4 show the IFA patterns shown by antibodies induced in *Aotus* monkeys against SALSA modified HABP **24,276** reacting with a protein in sporozoite membrane (B1) and erythrocytic stages: early rings (B2), trophozoites (B3) and schizonts (B4). (C) Western blot analysis showing the reactivity of sera collected from monkeys immunized with modified peptides **24,282** and **24,404** recognizing the LSA-1 recombinant protein (ca. 77.5 kDa). (D) Same analysis performed with sera collected from monkeys immunized with modified peptides **24,276** and **24,402** recognizing the recombinant SALSA protein (47 and 42.4 kDa).

Structural analysis

NOESY spectra of peptides 20,608**, 24,276**, **24,488**; 20,610, **24,402**; 20,630, **24,282** and **24,404** showed sequential, short and medium range $d_{NN}(i,i+1)$ $d_{\alpha\beta}(i,i+3)$, $d_{\alpha N}(i,i+3)$, and $d_{\alpha N}(i,i+4)$ NOE connectivities, which together with the low amide proton chemical shift temperature coefficients found for some of their amino acids, suggests the presence of an α-helix structure (Supplemental material).

Families of 22, 23, 22, 26, 24, 24, 26 and 30 low energy conformers chosen out of the initial set of 50 structures were obtained for peptides 20608, **24,276**, **24,488**; 20,610, **24,402**; 20,630, **24,282** and **24,404**, respectively. The chosen structures had no distance violation larger than 0.40 Å or ω angles greater than 1.3°. Average root mean square deviations (RMSD) for main-chain atoms were obtained by superimposing such structures between amino acids W2-E18 in peptide 20,608, S3-G16 in peptide **24,276**, S3-K6 and D8-H20 in peptide **24,488**, K4-K20 in peptide 20,610, K5-K20 in peptide **24,402**, G3-N18 in peptide 20,630, K7-E10 and P12-N18 in peptide **24,282** and K4-E10 and P12-I15 in peptide **24,404** (Table 1B). The DSSP programme [19] displayed a clear α-helical structure in all these peptides, as shown in Table 1B.

Immunogenetic analysis

Peptide binding assays to HLA-DRβ1* isolated molecules showed that LSA-1 modified HABPs bind with high capacity to HLA-DRβ1* 1101 molecules, displaying the characteristics binding motifs and register for this molecule. In peptide **24,282**, I6 fits in Pocket 1, S9 or A in Pocket 4, K11 in Pocket 6 and I14 in Pocket 9 (Table 1A, B and Fig. 2), suggesting that the most appropriate conformation for this peptide was with I14 in Pocket 9 showing an increase from 18.1 to 19.7 Å between these two residues; and probably that modifications in N5I, P12D, and M13E putative contact TCR residues had also a marked influence in shifting the immunological properties of conserved HABP 20,630 to render it highly immunogenic, as determined by the different methods. Similarly, the A14I modification with same increase in distance done to **24,404** seemed to have delayed the appearance of detectable antibodies (Table 1A, B and Fig. 2).

In the same way, 20,608 modified HABPs **24,276** and **24,488** showed high binding capacity to HLA-DRβ1*0301 isolated molecules, displaying the classical binding motifs and registers for this molecule: M5 in Pocket 1, D8 in Pocket 4, K10 in Pocket 6 and A13 in Pocket 9 (Table 1A, B and Fig. 2). Same as before, the immunological properties of these HABP analogues were modify by the S4A, M7E, M11E and M14E replacements since these residues were now properly oriented towards the TCR as shown by the induction of permanent high antibody titers with **24,276**. In this case, the distance between Pocket 1 and Pocket 9 was the same but the orientation of TCR contacting residues was dramatically different; further modifications performed on **24,488** (E5V, E11V, E14V), induced only short-lived antibodies that appear after the first dose but disappear later on, a phenomenon previously described [18], attributable to a dramatic reduction in the distance between residues fitting into Pockets 1 and 9, as clearly observed in this case.

These immunogenic and structural data extend and confirm our previous findings on the functional and structural compartmentalization of *P. falciparum* parasite molecules involved in invasion to host cells, since HABPs derived from soluble proteins such as LSA-1 and SALSA display an α-helical structure and bind high activity to molecules of HLA-DR52 haplotype (HLA-DRβ1*0301 and HLA-DRβ1*1101) [20].

It has been shown that LSA-1 contains potent B and T cell epitopes both in repeat and non-repeated regions [3–5]. Protection induced in volunteers vaccinated with X-radiated sporozoites

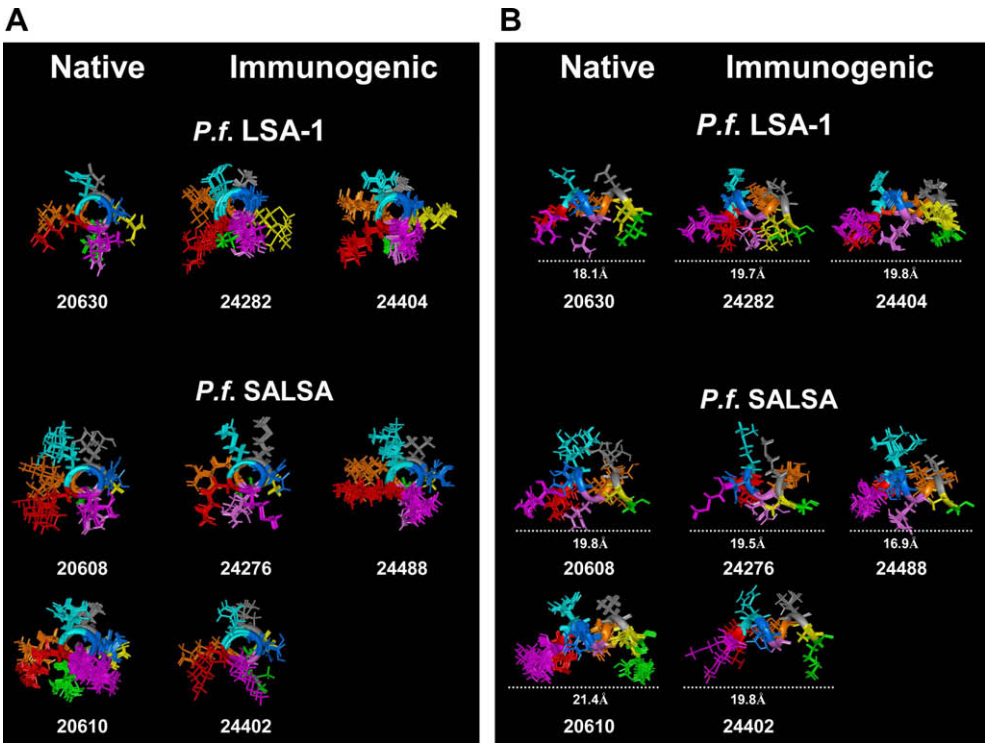

Fig. 2. (A) Lateral view and (B) frontal view of the three-dimensional structure of native and modified immunogenic HABPs, as determined by ^{1}H NMR. Residues were aligned according to the corresponding HLA-DRβ1* molecule's binding motif and reading register identified for the immunogenic modified HABP to which it binds. In all peptides shown, fuchsia corresponds to Pocket 1, red (P2), turquoise (P3), dark blue (Pocket 4), rose (P5) orange (Pocket 6), grey (P7), yellow (P8) and green (Pocket 9).

correlates with a proliferative response to 3 T-cell epitopes, the specially variable T1 epitope (residues 84–107) which contains our variable HABP 20,633 [6], T3 (residues 1813–1835) and T5 (1818–1909) [21,22]. None of these T-cell epitopes included conserved HABPs, which poses a tremendous problem for the development of an antimalarial vaccine with global coverage, due to the parasite's tremendous genetic variability.

Malaria patients from Gabon having Class II HLA-DRβ1*0201 and DQβ1*0301 alleles were more frequently found to produce IL-6 and INF-γ when their lymphocytes were stimulated with the LSA-Rep (residues 187–227) or LSA-CTL peptide (residues 1786–1794) but a clear association with HLA-markers and protection against malaria was not found [23].

A longitudinal study performed with Gabonese children under the age of 4 found associations between HLA-DQβ1*0501 and protection to malaria anaemia and malarial reinfection when LSA-J (corresponding to the degenerate repeat) and ls6 (KPIVQYDNF) peptides were used as antigens to induce production of INF-γ [24], suggesting a strong association with the genetic control of the immune response mediated by these MHC molecules.

Immunizations in chimpanzees with combinations of liver stage synthetic peptides of LSA-1, LSA-3, STARP and SALSA-1 (the later one completely containing our conserved HABP 20,608) and SALSA-2 (entirely containing our conserved HABP 20,610) showed that when these peptides are homogenized and administered together in a mineral oil emulsions (ISA51), they induce long-lasting CD8+ cytotoxic T lymphocytes, which lasted for at least 6 months [25].

Therefore, the here results presented show that our modified **24,276** and **24,402** SALSA HABPs together with LSA-1 modified **24,282**, could be excellent epitopes of a pre-erythrocytic vaccine to be included as liver-stage components of a multi-antigenic, multistage, minimal subunit-based, chemically synthesized antimalarial vaccine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.04.138.

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