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Antigenicity and immunogenicity of a novel chimeric peptide antigen based on the *P. vivax* circumsporozoite protein

Nora Céspedes[a,b,c], Myriam Arévalo-Herrera[a,c], Ingrid Felger[e], Steve Reed[f], Andrey V. Kajava[g], Giampietro Corradin[b], Sócrates Herrera[a,d,*]

[a] *Malaria Vaccine and Drug Development Center (MVDC), Carrera 37 2Bis No. 5E-08, A.A. 25574 Cali, Colombia*
[b] *Biochemistry Department, University of Lausanne, 155 Ch. des Boveresses, 1066 Epalinges, Lausanne, Switzerland*
[c] *Faculty of Health, University of Valle, Calle 4B No. 36-00 Cali, Colombia*
[d] *Caucaseco Scientific Research Centre, A.A. 760042 Cali, Colombia*
[e] *Swiss Tropical and Public Health Institute, Socinstrasse 57, Basel, Switzerland*
[f] *Infectious Disease Research Institute, 1124 Columbia Street Suite 400, Seattle, WA, USA*
[g] *CRBM, CNRS, University of Montpellier, Montpellier, France*

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ABSTRACT

Background: Plasmodium vivax circumsporozoite (*Pv*CS) protein is a major sporozoite surface antigen involved in parasite invasion of hepatocytes and is currently being considered as vaccine candidate. *Pv*CS contains a dimorphic central repetitive fragment flanked by conserved regions that contain functional domains.
Methods: We have developed a chimeric 137-mer synthetic polypeptide (*Pv*CS-NRC) that includes the conserved region I and region II-plus and the two natural repeat variants known as VK210 and VK247. The antigenicity of *Pv*CS-NRC was tested using human sera from PNG and Colombia endemic areas and its immunogenicity was confirmed in mice with different genetic backgrounds, the polypeptide formulated either in Alum or GLA-SE adjuvants was assessed in inbred C3H, CB6F1 and outbred ICR mice, whereas a formulation in Montanide ISA51 was tested in C3H mice.
Results: Antigenicity studies indicated that the chimeric peptide is recognized by a high proportion (60–70%) of residents of malaria-endemic areas. Peptides formulated with either GLA-SE or Montanide ISA51 adjuvants induced stronger antibody responses as compared with the Alum formulation. Sera from immunized mice as well as antigen-specific affinity purified human IgG antibodies reacted with sporozoite preparations in immunofluorescence and Western blot assays, and displayed strong *in vitro* inhibition of sporozoite invasion (ISI) into hepatoma cells.
Conclusions: The polypeptide was recognized at high prevalence when tested against naturally induced human antibodies and was able to induce significant immunogenicity in mice. Additionally, specific antibodies were able to recognize sporozoites and were able to block sporozoite invasion *in vitro*. Further evaluation of this chimeric protein construct in preclinical phase *e.g.* in *Aotus* monkeys in order to assess the humoral and cellular immune responses as well as protective efficacy against parasite challenge of the vaccine candidate must be conducted.

1. Introduction

Plasmodium vivax, the second most important *Plasmodium* species in terms of epidemiological significance, causes ~70–80 million clinical cases of malaria annually with 2.85 billion people living at risk of infection [1]. Although this parasite has been considered benign, during the last decade lethal *P. vivax* malaria cases have been documented [2,3] and chloroquine-resistant strains have recently emerged [4,5]. Furthermore, this parasite species produce hypnozoites forms that upon periodic reactivation induce clinical relapses, even in individuals who have emigrated from endemic regions.

During the last 2–3 decades, significant efforts have been invested in the development of *Plasmodium falciparum* subunit vaccines [6–8] and currently the RTS,S construct based on the *P. falciparum* circumsporozoite (CS) protein is the most advanced malaria vaccine candidate [9]. The CS protein is abundantly

* Corresponding author at: Malaria Vaccine and Drug Development Center (MVDC), Carrera 37 2Bis No. 5E-08, A.A. 25574, Cali, Colombia. Tel.: +57 2 521 6228; fax: +57 2 557 0449.
E-mail address: sherrera@inmuno.org (S. Herrera).

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expressed on the sporozoite (spz) surface of all *Plasmodium* species. It is one of the antigens that is predominantly recognized by sera of individuals rendered immune after vaccination with irradiated spz [10], and by individuals from endemic areas who have developed clinical immunity [11,12]. In *P. vivax*, the central repeat domain (90–261 aa) is composed of 19 blocks of 9 amino acids each, with two allelic forms circulating in nature: the VK210 or common type (GDRADGQPA) [13] and the VK247 or variant type (ANGAGNQPG) [14]. Flanking regions contain small stretches of highly conserved sequences designated as region I (85–89 aa) and region II-plus (338–355 aa) that represent ligand domains for invasion of hepatocytes [15,16].

It has been shown that the CS protein is proteolytically cleaved at region I (RI) [17,18], and that this processing is required for parasite invasion. Antibodies to RI as well as those against the repeat region are able to block the cleavage process [15,17], thus, an effective vaccine targeting the CS protein must be able to elicit antibodies specific to the RI as well as to the two repeats variants regions.

During the last decade, the *P. vivax* CS (*Pv*CS) has been extensively characterized [19–21], and both preclinical studies and Phase 1 clinical trials using long synthetic peptides (LSP) corresponding to the N, R and C regions of the protein have indicated its potential as a vaccine candidate [22–25]. In addition, a recombinant chimeric *Pv*CS construct containing both the VK210 and the VK247 repeat variants was recently produced in *Escherichia coli* and its immunogenicity tested in mice. Different formulations of the construct were found to elicit antibodies that agglutinated live sporozoites [26,27].

Herein, we describe studies on novel formulations of the chimeric *Pv*CS-NRC synthetic vaccine containing critical epitopes in a single construct joined together by the non-immunogenic modified diethylene glycol linker (DEG), selection of fragments was based on previous studies on the characterization of the *Pv*CS protein (identification of B, CD4+, CD8+ epitopes) and on the immunogenicity of a pools of individuals peptides tested in mice, monkeys and humans [19–25].

2. Materials and methods

2.1. Vaccine

The polypeptide *Pv*CS-NRC was synthesized using solid-phase fluorenylmethoxycarbonyl (F-moc) chemistry [28]. Pseudoproline (oxazolidine) dipeptides were introduced at critical points in the C-terminal region and DEG (Merck Chemicals Ltd., Nottingham, UK) was inserted between the three fragments RI+VK210, RI+VK247 and C (Fig. 1A). Pre-purified tertiary-butylthiolated precursors were oxidized and folded in solution accordingly [29]. The resulting construct was HPLC-purified and the purity (>85%) confirmed by analytic C18 HPLC (Fig. 1B) and mass spectrometry (Fig. 1C) (MALDI-TOF; Applied Biosystem). A polypeptide model was built using the INSIGHT II program (Accelrys, San Diego) [30] (Fig. 1D).

Additionally, seven 20-aa long synthetic peptides identified as B-cell and T-helper (Th) epitopes located in the N-terminal (p8, p9 and p40) and central repeat (p10, p11, p32 and p43) regions of the *P. vivax* CS protein, and a LSP representing the carboxyl-terminal region (301–372 aa) designated as C-peptide, were synthesized and used to characterize antibody responses.

2.2. Human sera

Human serum samples of adults from malaria-endemic areas of Colombia and Papua New Guinea (PNG) as well as from a non-endemic area (Switzerland) were used to assess peptide antigenicity. PNG sera ($n = 42$) were collected in a cross-sectional study carried out in a highly malaria-endemic region of Maprik District, East Sepik Province [31]. The Colombian samples ($n = 32$) were obtained in the Buenaventura region where *P. vivax* is endemic with a constant low level of transmission [32]. Ethical clearance was obtained from the PNG Medical Research Advisory Committee as well as from the IRB of the Malaria Vaccine and Drug Development Center (MVDC) in Colombia. Negative control samples were obtained from Swiss adult donors with no malaria history and no previous travel to malaria-endemic areas and used as control in ELISA and IFA test.

2.3. Immunogenicity studies in mice

Groups of five mice corresponding to two syngeneic strains (C3H, CB6F1) and a group of seven outbred mice (ICR) were injected with *Pv*CS-NRC formulated in alhydrogel (1 mg/mouse) and GLA-SE (20 μg/mouse) [33,34], respectively, whereas a Montanide ISA51 (Seppic Inc., Paris, France) formulation was assessed in C3H mice according to manufacturer's recommendations. All mice were injected subcutaneously with 20 μg of peptide in 50 μL volume at the base of the tail on days 0, 20 and 40. Animals care was performed in accordance with institutional guidelines.

2.4. Humoral immune response

Antibody responses to *Pv*CS-NRC as well as to the other peptides in mice and human were measured by ELISA; *Pv*CS-NRC was used at 1 μg/mL, whereas the constituent peptides were used at 5 μg/mL, and the ELISA performed as described previously [19]. Additionally, parasite recognition by anti-peptide antibodies was determined by IFAT using *P. vivax* sporozoites as described previously [35]. Cut-off points for ELISA were calculated as three SD above the mean absorbance value at 405 nm of normal mouse sera or from healthy volunteers who had never been exposed to malaria. IFAT antibody titers were determined as the reciprocal of the end-point dilution that showed positive fluorescence [23].

2.5. Reactivity of affinity-purified human antibodies

Reactivity of purified human antibodies with the polypeptide as well as the different constituents was determined by ELISA competition assay, using *Pv*CS-NRC (1 μg/mL) as the antigen substrate. Human IgG specific to *Pv*CS-NRC were purified from pooled sera (50 mL) obtained from adults living in malaria-endemic areas of PNG as described previously [36]. The affinity-purified antibodies to *Pv*CS-NRC were incubated 30 min at room temperature together with the constituent polypeptides (RI, VK210, VK247 or C) prior to adding to the peptide-coated ELISA plates. Reactivity of the adsorbed antibody preparations was then determined as described elsewhere (Section 2.6).

2.6. Western blot analysis

Protein extracts from lysed *P. vivax* sporozoites were separated on 12% SDS-polyacrylamide gel and electro-transferred to nitrocellulose (0.2 mm, Whatman Schleicher + Schuell, Florham Park, NJ) under cooling conditions overnight at 30 V. The membrane was blocked at room temperature, incubated with the primary antibody solution (purified IgG or sera from immunized mice), washed and incubated with an alkaline phosphatase-conjugated goat anti-mouse or anti-human IgG (1:1000, Sigma) [37]. Bound antibodies were visualized using BSIP/NBT substrate.

2.7. Inhibition of sporozoite invasion (ISI) assay

Affinity-purified human IgG antibodies specific to *Pv*CS-NRC as well as sera of immunized mice were tested for the capacity to inhibit *P. vivax* sporozoite invasion of HepG2-A16 hepatoma-cells *in vitro* as described previously [22].

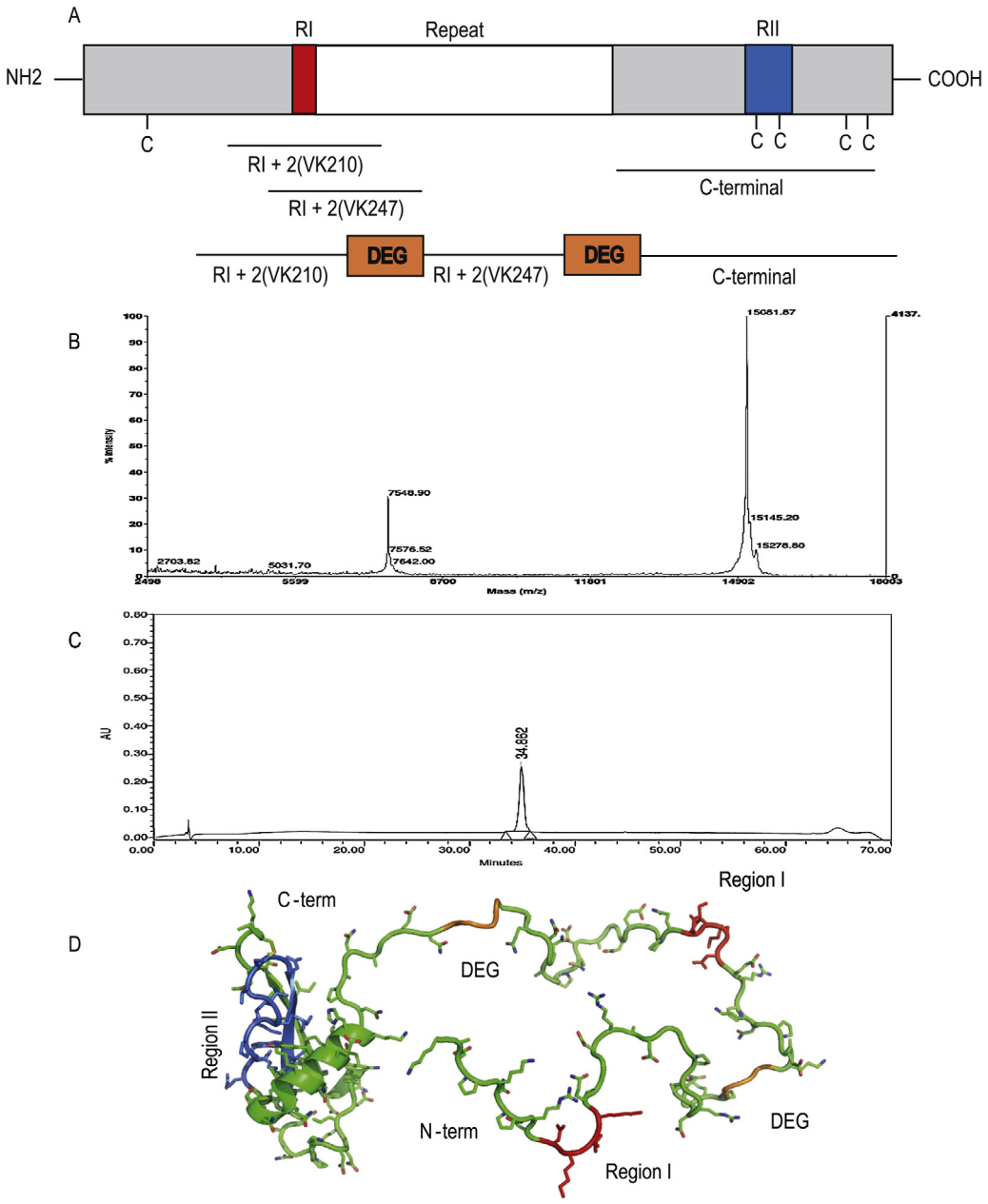

Fig. 1. *Pv*CS-NRC structure and production. (A) Schematic representation of the *Pv*CS and localization of epitopes included in the *Pv*CS-NRC construct. DEG: diethylene glycol derivative. Sequence: KAEPKNPREN KLKQPGDRAD GGPAGDRADG QPA-DEG-KAEPKNPREN KLKQPGENGA GDQPGANGAG NQPG-DEG-NEGANAPNEK SVKEYLDKVR ATVGTEWTPC SVTCGVGVRV RRRVNAANKK PEDLTLNDLE TDVCTMDKCA. (B) MALDI TOF mass spectrum of *Pv*CS-NRC. After size exclusion chromatography pre-purified (G75) samples were subject to folding of Cys(StBu) induced by a 100-fold molar excess of cysteine at 37 °C, pH 7.2, for 24 h. The main impurity corresponds to an incomplete oxidation of one 1 Stbu (*m*/*z*: 15145). The peak at *m*/*z* 15278 corresponds to the sinapinic acid addition product generated by the laser treatment of the sample during the mass spectrometry analysis. (C) HPLC chromatogram of *Pv*CS-NRC. (D) Structural model of peptide *Pv*CS-NRC. The N-terminal half of the peptide that includes two RI regions (red) and DEG cross-linkers (orange) is unfolded [49] while the C-terminal half form globular structure that was modeled by using the crystal structure of the homologous domain from *P. falciparum* (pdb code 3VDJ) [50]. The RII region is shown in blue.

2.8. Statistical analysis

Main experimental comparisons involved analysis of differences between adjuvants and strains of mice. Antibody titers were compared among adjuvants and strains of mice and a descriptive analysis was performed to evaluate trends in humoral immune responses within each group of mice. Additionally, the Mann–Whitney test was performed with OD data obtained after the third immunization to compare the antibody response of different groups of adjuvants, and the Kruskal–Wallis test was used to compare different strains of mice. Non-parametric p values ≤ 0.05 were considered statistically significant. Finally, the capacity to inhibit *P. vivax* sporozoite invasion of HepG2-A16 hepatoma-cells *in vitro* was compared between adjuvants in the different strains of mice using the Mann–Whitney test.

3. Results

3.1. Antigenic recognition

Table 1 shows the prevalence of seropositive reactivity using serum samples from PNG and Colombia endemic areas, tested against *Pv*CS-NRC and other CS fragments. In general, all polypeptides used in this study were more frequently recognized by PNG

Table 1
Prevalence of antibodies to *Pv*CS derived synthetic peptides in donors from Colombian and PNG.

Peptide[a]	Amino acids sequence[a]	Length	Location	Colombia ($n = 36$)[b]			PNG ($n = 38$)[b]		
				% >control + 3SD[c]	Ratio[d] (%)	Mean OD	% >control + 3SD[3]	Ratio[d] (%)	Mean OD
*Pv*CS-NRC	KAEPKNPREN KLKQPGDRAD GQPAGDRADG QPA-PEG-KAEPKNPREN KLKQPGENGA GDQPGANGAG NQPG-PEG-NNEGANAPNEK SVKEYLDKVR ATVGTEWTPC SVTCGVGVRV RRRVNAANKK PEDLTLNDLE TDVCTMDKCA	137	81–113, 287–346	58	50	0.34	69	50	0.38
P8	GDAKKKKDGK KAEPKNPREN	20	71–90	6	6	0.15	76	60	0.18
P9	KAEPKNPREN KLKQPGDRAD	20	81–100	44	44	0.60	68	55	0.18
P10	KLKQPGDRAD GQPAGDRADG	20	91–110	6	6	0.22	76	63	0.37
P11	GDRADGQPAG DRADGQPA	16	96–113	17	6	0.22	67	57	0.36
P32	ENGAGDQPGA NGAGNQPG	16	96–113	11	11	0.20	65	50	0.3
P40	KAEPKNPREN KLKQPGENGA	20	81–100	44	31	0.35	63	63	0.4
P43	KLKQPGENGAG DQPGANGAG	20	91–110	19	3	0.14	38	23	0.2
C-terminal	NEGANAPNEK SVKEYLDKVR ATVGTEWTPC SVTCGVGVRV RRRVNAANKK PEDLTLNDLE TDVCTMDKCA	70	301–372	30	19	0.17	59	43	0.23

[a] Peptides P32, P40 and P43 correspond to VK247 region.
[b] Human sera sample were tested at 1:200 dilution.
[c] Percentage of positive responses evaluated as OD values higher than the mean negative control + 3SD. Sera samples obtained from Swiss adult donors with no malaria history and no previous travel to malaria-endemic areas were used as negative control.
[d] Percentage of OD ratio higher than 2 between the mean duplicate experimental and mean negative control OD.

sera than by Colombian sera. More than 65% of adult donors from PNG and 58% Colombian adults recognized the *Pv*CS-NRC antigen. Additionally, most of the polypeptide fragments were also recognized by up to 76% of PNG samples and up to 44% of Colombian samples (Table 1). Peptides P9 and P40, containing the *Pv*CS-RI region, were the epitopes most frequently recognized by Colombian sera (44%); whereas PNG sera were most reactive to P8 and P10 peptides (76%). The prevalence of antibodies specific for P11 and P32 peptides corresponding to the two repeat variants (VK210 and VK247, respectively) were also higher in PNG (>65%) with similar frequency of recognition within the same region (Table 1). Finally, the C-terminus was recognized by 59% and 30% of PNG and Colombian sera, respectively.

3.2. Reactivity of purified IgG antibodies

Reactivity and specificity of the affinity-purified IgG were confirmed by ELISA and IFA tests. Antibodies showed reactivity to *Pv*CS-NRC by ELISA at dilutions to 0.1 μg/mL, and recognized the native protein on *P. vivax* sporozoites by IFAT at a concentration of 16 μg/mL. Moreover, anti-*Pv*CS-NRC antibodies recognized each of the individual constituent fragments by ELISA inhibition assay; antibodies reacted mainly with RI and C-regions followed by both VK210 and VK247 repeat regions (Fig. 2A). Finally, Western blot analysis of specific anti-*Pv*CS-NRC purified antibodies recognized a double band at approximately 50 kDa typical of the CS protein (Fig. 2B).

3.3. Immunogenicity in mice

Antibody titers were determined in all inbred and outbred immunized mice (Table 2). Stronger antibody titers were generally observed when the polypeptide was formulated in GLA-SE and Montanide ISA51 than in Alum ($p = 0.0001$). Moreover, antibody responses were more consistent for the GLA-SE vaccine formulation, while high variability in number of responders and antibody titers was observed for mice inoculated with the Alum formulation. Although differences in response to *Pv*CS-NRC formulated in GLA-SE or Alum were observed between the different strains of mice (Table 2), no significant differences were observed among the two syngeneic (C3H, CB6F1) strains and the outbred (ICR) strain ($p = 0.220$).

CB6F1 and ICR mouse strains showed higher ELISA antibody titers, ranging from 6.5×10^5 to 5.9×10^6, when they were immunized with the *Pv*CS-NRC formulated in GLA-SE. In both cases seroconversion was detected after the second immunization, with boosting observed after the third immunization (Fig. 3). In the case of C3H mice, ELISA titers were highly variable, ranging from 8.1×10^3 to 1.9×10^6, with the exception of one animal that required three vaccine doses before seroconversion, all other mice seroconverted after the second immunization. Regarding *Pv*CS-NRC-Alum formulations, most of the animals seroconverted with the second immunization dose, yet others required the third dose to seroconvert. The highest response was observed in the CB6F1 strain of mice with ELISA titers ranging between 3.0×10^2 and 6.6×10^5, whereas the lowest response was shown in ICR and C3H mouse strains with ELISA titers ranging between 9.0×10^2 and 7.2×10^4, and 3.0×10^2 and 8.1×10^3, respectively (Fig. 3).

Similar to GLA-SE, the *Pv*CS-NRC polypeptide formulated in Montanide ISA 51 induced potent antibody responses in C3H mice with average ELISA titers between 6.6×10^5 and 5.9×10^6 and all mice seroconverted after the second immunization (Fig. 3).

Antibodies to *Pv*CS-NRC recognized the entire polypeptide and the individual peptide fragments, although some variability was observed depending on the adjuvant or mouse strain used (Fig. 4A). In general, the C-terminal fragment and the RI-containing peptide

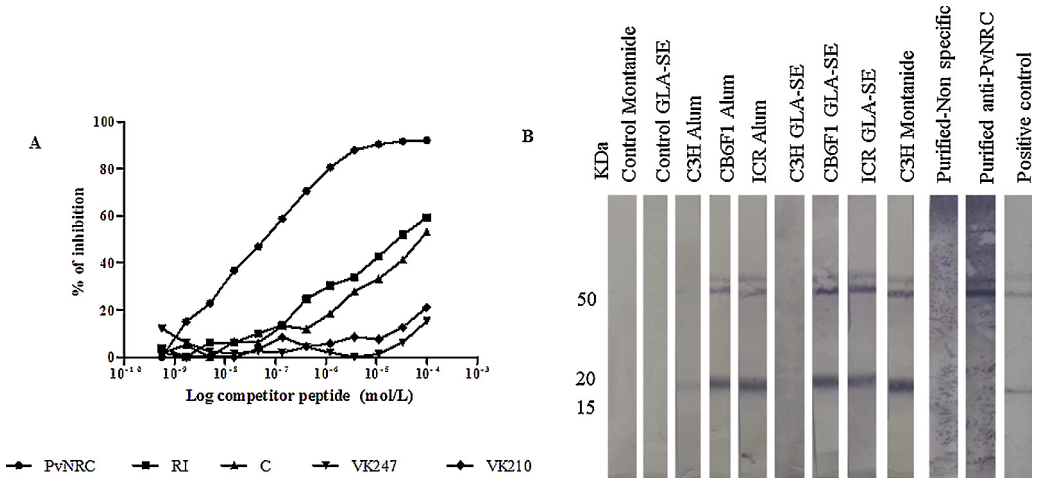

Fig. 2. Antibodies reactivity. (A) ELISA competition assay using anti-human specific purified antibodies to *Pv*CS-NRC. Human affinity-purified antibodies to *Pv*CS-NRC were incubated together with the constituent polypeptides (RI, VK210, VK247 or C) at serial dilutions starting at 1 mg/mL and the reaction incubated for 30 min at room temperature, prior their addition to the ELISA peptide-coated plates. (B) Immunoblot assays: lysed *P. vivax* sporozoites electrophoresed under non-reducing conditions were probed with affinity-purified human α-*Pv*CS-NRC or antibodies produced in mice at 1:100 dilution. Relative size standards are indicated on the left in kDa. As negative control non-specific human purified antibodies from Swiss donors or sera of mice immunized with a *P. falciparum* erythrocytic antigen formulated in GLA-SE [46] and the homologous in *P. vivax* in Montanide were used. As a positive control a pool of serum samples from adults immunized with a mixture of *Pv*CS LSP in a Phase 1b clinical trial were used [25].

(P9) were the most frequently recognized, followed by the VK210 repeat. The VK247 repeat was recognized only by sera of mice immunized with *Pv*CS-NRC formulated in GLA-SE.

All immunized mice recognized *P. vivax* sporozoites by IFA, regardless of the adjuvant used, albeit at different antibody titers (Fig. 4B). Higher responses were observed in CB6F1 and ICR mice immunized with the GLA-SE formulation (IFA titers: 1:10,240), followed by responses in sera of C3H mice immunized with the Montanide ISA51 formulation (IFA titers: 1:5000), and then by sera of CB6F1 mice immunized with Alum (IFA titer: 1:1280).

Finally, Western blot analysis of the anti-*Pv*CS-NRC pool of sera corresponding to C3H mice immunized with Montanide, and ICR and CB6F1 mice with GLA-SE formulation or Alum formulation recognized two bands at approximately 50 kDa and ~15 kDa (Fig. 2B), while no response was observed with serum pool of C3H mice immunized with *Pv*CS-NRC formulated in GLA-SE or Alum.

3.4. Inhibition of sporozoite invasion (ISI) assay

Functional activity of specific *Pv*CS-NRC-purified human antibodies and of whole antisera obtained after the third immunization was tested in ISI assays to determine the capacity of anti-peptide antibodies to inhibit *P. vivax* sporozoite invasion of human hepatoma-cells (HepG2À-16) *in vitro*. Human antibodies to the entire *Pv*CS-NRC polypeptide resulted in inhibitory activity up to 70%. Likewise, mouse sera produced strong inhibition (>65%), although the strongest inhibition (>75%) was observed in sera of mice immunized with *Pv*CS-NRC formulated in GLA-SE. No significant differences were observed among the different adjuvant groups ($p = 0.08$) (Fig. 4C).

4. Discussion

In the process of developing a pre-erythrocytic *P. vivax* vaccine for human use, we have developed a chimeric LSP derived from the *P. vivax* CS protein which includes an N-fragment-containing region I; the two repeat variants found in nature (VK210 and VK247); and the C fragment, all considered to play an important functional roles in sporozoite invasion of hepatocytes [15,17]. It is encouraging that the chimeric polypeptide construct, named *Pv*CS-NRC, and its individual peptides, were widely recognized by sera of individuals naturally exposed to malaria and additionally induced significant response in immunized mice. Of particular interest antibodies elicited recognized the protein on sporozoites and blocked sporozoite invasion *in vitro*.

All CS protein fragments studied were recognized by ELISA when tested with sera collected from both PNG and Colombian donors although, as expected each one was recognized with different frequencies, most likely due to the varying *P. vivax* endemicity in the respective countries [38,39]. Although, in some cases the response to individual peptides seem to be greater than the full construct, especially for PNG samples, it is not possible to compare the additive or synergistic response since the amount of antigen used to test the *Pv*CS-NRC and their corresponding antigens was different. As previously shown [19], peptides corresponding to N-terminal flanking regions as well as to the repeat regions were more frequently recognized by human sera than that corresponding to the C-region. In fact, under natural conditions, the C-region appears to be masked by the N-fragment of the protein, and therefore it may be less exposed to the immune system [17]. However, in previous studies we have observed that even when peptides containing nearly complete N and C regions when administered

Table 2
Immunogenicity of *Pv*CS-NRC formulated in different adjuvants in mice.

Adjuvant	C3H	Responders	CB6F1	Responders	ICR	Responders
GLA-SE	1.54 ± 0.91	5/5	2.26 ± 0.34	5/5	2.66 ± 0.53	6/6
Alum	0.09 ± 0.05	3/5	0.36 ± 0.20	5/5	0.28 ± 0.18	5/6
p	0.0061		0.0061		0.001	

Data represented as OD media. Mann–Whitney test was performed with OD data obtained after the third immunization to compare the antibody response of different groups of adjuvants. *p* values ≤0.05 were considered significant.

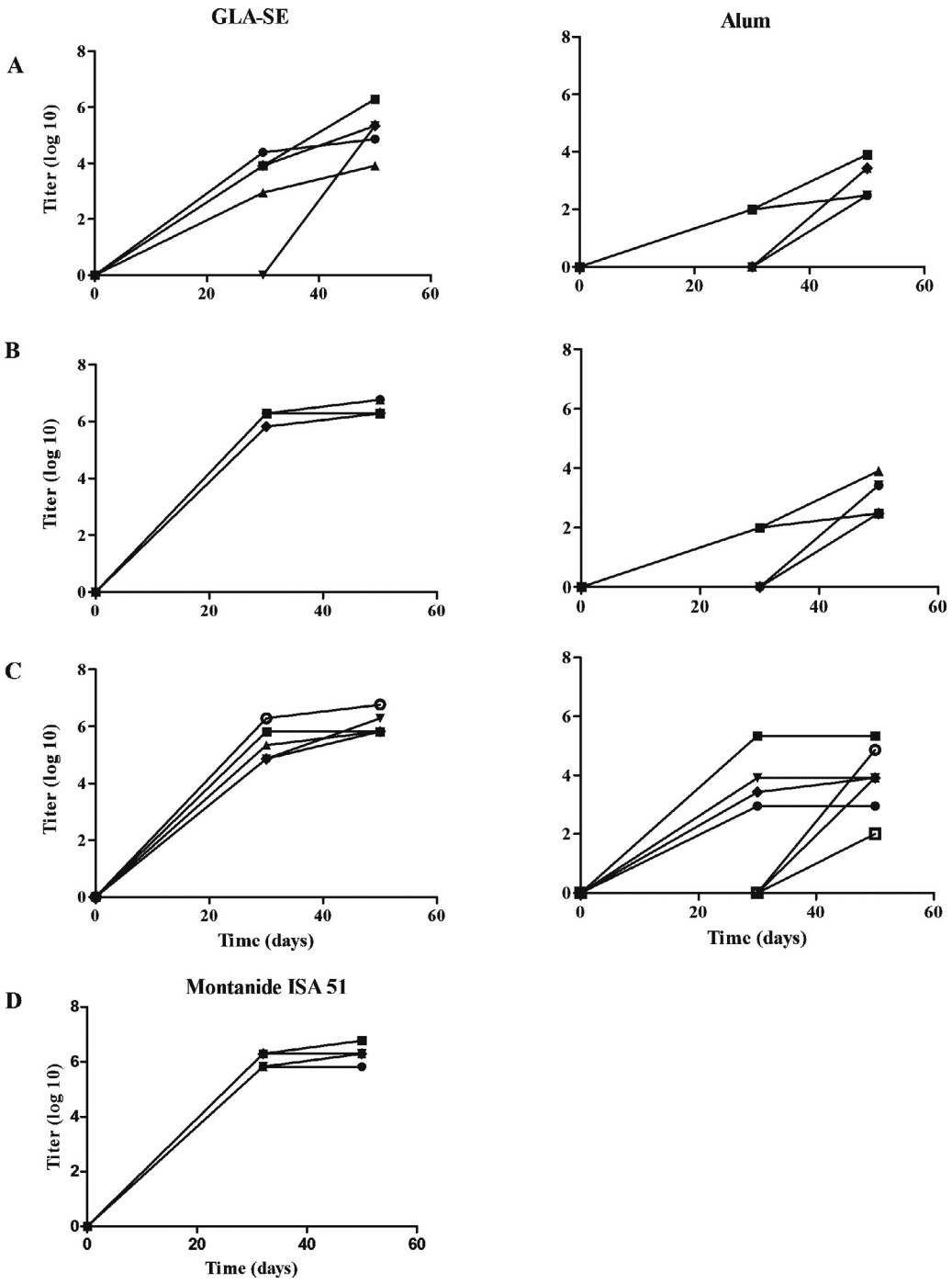

Fig. 3. Humoral response to *Pv*CS-NRC peptide formulated in Alum, GLA-SE or Montanide, determined in (A) C3H mice, (B) CB6F1 mice and (C) ICR mice. (D) C3H with PvNRC formulated in Montanide. Antibody titers are expressed in $\log_{10}$ of immune sera determined as the last dilution with an OD higher than that of the control serum + 3SD. The induced antibody responses were measured by ELISA ten days after second and third immunizations. ELISA was performed using threefold serial dilutions of the sera, starting from 1:100 to 1: 1.7×10^7. Results were considered positive when absorbance of the test sera was greater than or equal to the mean value of control sera + 3SD. Normal mice sera samples obtained from naïve mice were used as control sera at 1:200 dilution.

independently, the N-fragment is more immunogenic in monkeys and humans than the C-fragment, with the latter more immunogenic in mice [22]. This observation is a critical consideration for human use vaccines development.

Herein, although *Pv*CS-NRC displayed varying immunogenicity depending on the different vaccine formulations, reactivity of all formulations in both inbred and outbred mouse strains confirms the presence of multiple T-cell epitopes along the entire length of *Pv*CS-NRC. Regarding helper efficiency, C and RI regions were the most frequently recognized by specific *Pv*CS-NRC antibodies. The lower immunogenicity of VK247 peptide is noteworthy; however we had also found that in endemic Colombian regions where VK247 is the prevalent phenotype [40], antibodies to VK210 were more frequent than those to VK247, probably indicating its greater immunogenicity [41].

It was interesting to confirm that purified human IgG specific to the polypeptide recognized the individual epitopes comprised in *Pv*CS-NRC in ELISA, and were reactive to parasite in IFA and Western blot. Even more meaningful was the capacity displayed by the specific anti-*Pv*CS-NRC antibodies to inhibit sporozoite

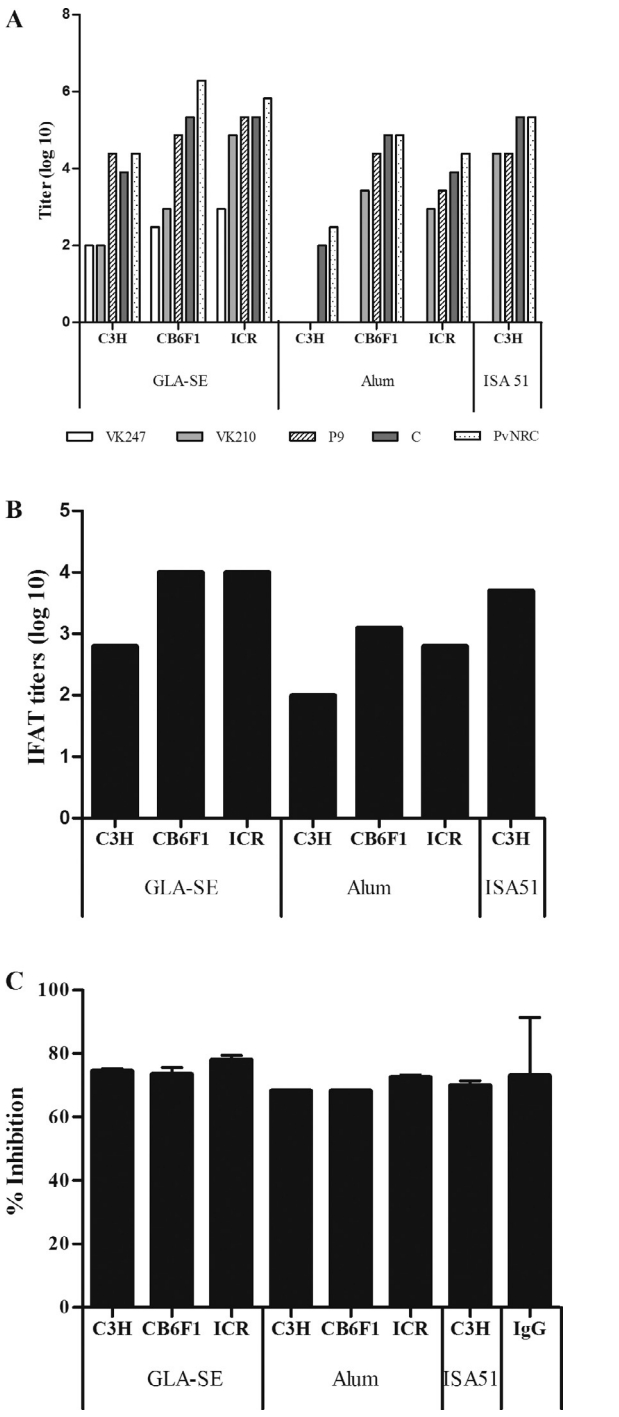

Fig. 4. Anti-*Pv*NRC produced in mice. (A) Comparison of mice ELISA-antibody titers to *Pv*CS-NRC and to individual components. A pool of sera for each mice group was used to determine the reactivity of anti-*Pv*CS-NRC antibodies to VK210, VK247 P9 and C fragments. (B) IFAT profiles of sera from different mice strains immunized with *Pv*CS-NRC vaccine formulations. Antibody titers are expressed in $\log_{10}$ of immune sera determined as the last dilution that showed positive fluorescence. (C) *In vitro* inhibition sporozoite invasion. The HepG2-A16 cells were at a density of 8×10^4 cells/0.3 mL of medium, 3×10^4 *P. vivax* sporozoites were used. A 100 μg/mL purified IgG antibodies or 1:20 dilution of sera from mice immunized were added to each well. Percentages of inhibition are shown as the mean ± SD of duplicate wells. Each experiment was performed twice with similar results. Percentage of invasion inhibition was calculated by the formula: ([mean schizont number of negative control − mean schizont number of sample/mean schizont number of negative control] × 100). Negative control was normal mouse sera or Colombian normal human sera from naïve donors. Percentage of invasion inhibition was compared between GLA-SE and Alum adjuvants in the different strains of mice using the Mann–Whitney test p values ≤0.05 were considered significant.

invasion *in vitro*. This might be associated with the significant clinical immunity observed in PNG [31]. Likewise, sera of immunized mice were also reactive by ELISA, IFA and WB and induced ISI >65%, and appeared to be associated with the adjuvant used, with GLA-SE and Montanide performing better than Alum. However, in terms of functional antibodies activity, no significant difference was observed among the three groups.

These results stress the complexity of adjuvants. While Alum is most commonly used with human vaccines [42], it has not been found successful against malaria since such vaccines induce Th2 responses that are poorly effective against malaria parasites where protection is mainly mediated by Th1 responses. Here, one of the mouse stains displayed a strong but variable antibody response when the polypeptide was formulated in Alum. In general, in this study *Pv*CS-NRC formulated in adjuvants Montanide ISA51 and GLA-SE induced strong antibody responses in most mouse strains tested. Immunogenicity of peptides formulated in Montanide ISA720 and ISA51 has been determined in previous studies on CS-LSP in *Aotus* monkeys [22,24] and human volunteers [23,25]. These formulations have been shown to be safe, well tolerated and immunogenic. However in other studies, Montanide formulations have been reported to induce reactogenicity as well as stability and reproducibility problems among different formulation batches [43–45].

We proposed to use a Glucopyranosyl lipid A stable oil-in-water emulsion (GLA-SE) previously shown to produce strong cellular and/or humoral immune responses in animal models [26,33,34,46]. In the present study, GLA-SE formulations induced the strongest humoral responses in both inbred and outbred mice, and antibodies obtained from immunized mice appeared to induce the strongest *in vitro* inhibition of sporozoite invasion, although no significant differences were observed regarding the formulation used. The GLA-SE formulation also induced antibodies that recognized all epitopes in the *Pv*CS-NRC polypeptide, including reactivity with the two repeat variants, and being the only groups of mice that recognized VK247 variant.

Antibodies obtained by immunization with the polypeptide formulated in all tested adjuvants recognized the native protein in IFAT and Western blot regarding to the strain used. Three main bands were observed, two bands at approximately 50 kDa are typical of the CS protein [47], whereas the band at 15 kDa could correspond to fragments of the processed CS protein.

This study confirms that the use of synthetic peptides represents a promising strategy for vaccine development. LSPs with lengths >100 amino acids can now be routinely and reproducibly synthesized [48]. Since for liver stage antigens where elicitation of a specific T-cell response is needed and linear peptides of limited length are required we argue that short peptides containing specific T-cell and B-cell epitopes inserted co-linearly into a long peptide chain with interposed non-immunogenic spacers to minimize the neo-epitopes formation could be enough to induce a protective immune response directed to the protein [37]. In conclusion, given the encouraging results obtained in this study, further studies are warranted for evaluation of *Pv*CS-NRC as a potential vaccine candidate.

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