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Passive transfer of *Plasmodium falciparum* MSP-2 pseudopeptide-induced antibodies efficiently controlled parasitemia in *Plasmodium berghei*-infected mice

Paola A. Martínez [a], Nubia Yandar [a], Liliana P. Lesmes [a], Martha Forero [a], Oscar Pérez-Leal [a], Manuel Elkin Patarroyo [a,b], José Manuel Lozano [a,b,c,*]

[a] *Fundación Instituto de Inmunología de Colombia-FIDIC, Bogotá, Colombia*
[b] *Departamento de Farmacia and Facultad de Medicina, Universidad Nacional de Colombia, Bogotá, Colombia*
[c] *Facultad de Medicina, Universidad Colegio Mayor de Nuestra Señora del Rosario, Bogotá, Colombia*

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ABSTRACT

We have developed monoclonal antibodies directed against the pseudopeptide ψ-130, derived from the highly conserved malarial antigen *Plasmodium falciparum* merozoite surface protein 2 (MSP-2), for obtaining novel molecular tools with potential applications in the control of malaria. Following isotype switching, these antibodies were tested for their ability to suppress blood-stage parasitemia through passive immunization in malaria-infected mice. Some proved totally effective in suppressing a lethal blood-stage challenge infection and others reduced malarial parasitemia. Protection against *P. berghei* malaria following Ig passive immunization can be associated with specific immunoglobulins induced by a site-directed designed MSP-2 reduced amide pseudopeptide.

1. Introduction

Merozoite surface proteins (MSP) have been considered as potential vaccine candidates against *Plasmodium* spp. blood-stages due to their easy accessibility which turns them into excellent immune system's targets [40]. Among these surface proteins interacting with erythrocyte membrane during invasion, the MSP-2 has gained notable attention as a source of vaccine candidate peptides, since the reactivity of antibodies acquired by humans during natural infection against this protein is associated with clinical immunity against malaria [40]. The MSP-2 is a 35–56 kDa glycoprotein whose size and isoelectric point vary depending on the parasite strain. It is displayed over the surface of merozoites and in residual bodies from all *Plasmodium falciparum* strains studied so far [6,45]. It possess a highly polymorphic central region flanked by dimorphic sequences of either ICI or FC27 allelic families, which are in turn flanked by conserved sequences [13,46,50] as observed in Fig. 1A.

The isotype of antibodies produced in response to *P. falciparum* MSP-2 during infection generally reflects the type of parasite allele causing the infection and the length of exposure [36,49,52]. Some studies have shown that monoclonal antibodies directed against MSP-1 and MSP-2 surface antigens inhibit *in vitro* parasite growth [10,25,39], while others have established that immunizing mice with peptides from the *P. falciparum* MSP-2 N-terminal region makes them capable of protecting themselves against *P. berghei* and *P. yoelii* strains [24,43]. It has been reported that a MSP-2 N-terminal region peptide (namely 4044) bound with high specificity to human erythrocytes and possesses three binding motifs [34]. Bearing in mind that humoral immunity plays an important role in the defense against blood-stage infections [3–5,11,14,27,30,37,41,44], passive immunization is considered to be a potential strategy in the search for epitopes and in studying the mechanisms by which antibodies provide a protective effect against a pathogen-causing disease. Steric interference in merozoite invasion of erythrocytes, inhibition of intra-erythrocyte development or processing of merozoite surface proteins, antibody-depend cell inhibition and phagocytosis are among the mechanisms proposed to date for explaining how antibodies achieve their effect [9,28,39,54,55]. Studies on mice have shown that passive immunization with antibodies directed against *P. yoelii* AMA-1, CS, MSP-1, proteins confer protection [31,32,47]. It has been demonstrated *in vitro* that IgG antibodies from African adults immune to *P. falciparum* have inhibited parasite growth in cooperation with monocytes; which was correlated with reduced *in vivo* parasitemia [3].

* *Corresponding author at*: Fundación Instituto de Inmunología de Colombia-FIDIC, Carrera 50 No. 26-00, ZC 020304, Bogotá, Colombia. Tel.: +57 1 315 8920; fax: +57 1 481 5269.
E-mail addresses: jm_lozano@fidic.org.co, jmlozanom@unal.edu.co (J.M. Lozano).

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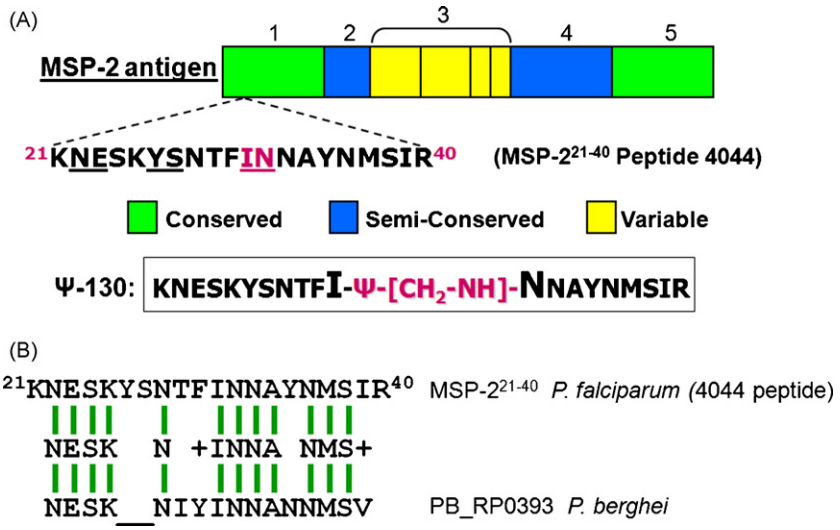

Fig. 1. Structural characteristics of the MSP-2-derived peptide 4044. Schematic representation of the *Plasmodium falciparum* merozoite surface protein-2 in which the peptide 4044 primary structure is shown, as well as its genetic organization containing conserved domains (blocks 1 and 5), non-repetitive dimorphic domains (blocks 2 and 4), and the central polymorphic repeats (block 3) (A). Amino acid alignment between peptide 4044 and the whole genome shotgun sequence of *Plasmodium berghei* ANKA strain as reported in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) (B).

Since eliciting protection-inducing antibodies depends upon the antigen's nature, previous work has established that immunizations with *P. falciparum* MSP-1 reduced amide pseudopeptides led to producing monoclonal antibodies able of inhibiting *P. falciparum in vitro* invasion of and growth into human red blood cells (RBCs) compared to specific antibodies against the native peptide [25]. These malaria pseudopeptides' immuno-modulating properties result from replacing a naturally produced peptide-bond by a reduced amide form, thus inducing new structure features in the target molecule as a result of replacing the normal trigonal sp2 planar geometry of an amide bond by four new sp3 hybridized bonds which modifies the entire molecule's 3D structure. A desirable side-effect of such a geometrical change is the new molecule's increased stability to sera endo-proteases. Modifying the peptide bond thus produces a less biodegradable peptide bond, influencing molecules' immuno-biological properties without modifying the amino acids' side-chains so important for appropriate molecular recognition and thus transforming these molecules into potential transition-state analogues as efficient protease blockers [25].

Bearing in mind that proteolytic processing of specific parasite surface antigens is essential during infection, then blocking such processing could be a strategy leading to evading certain infections [22].

This work was thus aimed at producing specific site-directed antibodies against the ψ-130 isoster-bond pseudopeptide derived from the native sequence of the High Activity Binding Peptide (HABP) coded 4044 peptide and the subsequent assessment of these antibodies protection-inducing ability by passive immunization experiments conducted in mice. Further use of passive immunization with site-directed and specifically designed antibodies could be a useful immuno-therapeutic strategy in areas where malarial strains are resistant to antibiotics and other pharmacological agents for achieving control of this deadly disease.

2. Materials and methods

2.1. Bioinformatics analysis

Both the *P. falciparum* MSP-2 protein sequence (gi: 1222687) as well as the peptide 4044 *P. falciparum* MSP-2-derived sequence were aligned against the whole genome shotgun sequence of *Plasmodium berghei* ANKA strain reported in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/), by using the TBLASTN tool.

2.2. Isoster peptide bond-immunogen synthesis

Based on the MSP-$2^{21–40}$ (peptide 4044) primary structure observed in Fig. 1, a set of reduced amide pseudopeptides was designed and synthesized as described bellow. The non-modified as well as its pseudopeptide surrogates were obtained in both monomer and polymer forms. All molecules were manually synthesized using a Merrifield's previously reported protocol for t-Boc-based solid-phase peptide synthesis (SPPS) [28], this procedure was later modified for multiple-peptide synthesis [17]. Each amino acid residue was placed on the pseudopeptide backbone as has been described elsewhere [7,16,42]. The ψ-[CH_2NH] surrogate was introduced by the deprotected N^{α}-amino group's resin-bound reductive alkylation with the t-Boc-protected amino acid aldehyde (0.576 mmol) in DMF containing 0.5% HOAc followed by portion-wise addition of $NaBH_3CN$ (0.67 mmol) for 40–60 min. The resin was checked for a totally allowed coupling by the Ninhydrin test and, when necessary, the coupling reaction was repeated until completeness. Coupling was allowed to proceed for 5 h with constant stirring, followed by *N*,*N*′-dimethylformamide (DMF), isopropanol and dichloromethane washing. Standard solid phase peptide synthesis was carried out to introduce the remaining t-Boc amino-acids to the N-terminal final residue. Protected pseudopeptide-resin batches were treated with TFA and cleaved from the resin by treatment with low concentrations of anhydrous HF containing 10% anisole at 0 °C for 60 min. After HF evaporation in an N_2 stream, each pseudopeptide-resin product was washed with cold diethyl ether, then extracted with 5% HOAc and lyophilized. The crude products obtained for each ψ-[CH_2NH] surrogate were further analyzed by analytical RP-HPLC, purified by preparative RP-HPLC and identified by MALDI-TOF mass spectrometry. A full procedure for peptide-based polymer production is elsewhere described [23,25].

2.3. Mice immunization for poly- and monoclonal antibody production

The pseudopeptide ψ-130 having a methylene amine isoster bond between residues ^{31}I-N^{32} from preliminary tests was further

employed for inducing antibodies [26]. As it was previously reported, the reactive hybridomas were obtained by successively immunizing BALB/c female mice with 4044 pseudopeptide analogues' polymer forms. Briefly, mice were intra-peritoneally (i.p.) inoculated 6 times with 50 μg of each molecule emulsified in Complete Freund's Adjuvant (v/v) for the first inoculation and in Incomplete Freund's Adjuvant for the subsequent immunizations. Mice were bled before the first immunization and weekly after each immunization; they were boosted with the same 4044 pseudopeptide analogue in saline solution on the fifth and fourth days prior to cellular fusion. Spleen cells obtained from mice showing high antibody titers to modified pseudopeptide analogue were then fused with X63Ag8 myeloma cells using 3000–3700 polyethylene glycol (Sigma Chemical Co., St Louis, MO), following the Kohler and Milstein's method reported elsewhere [11]. Supernatants from wells growing positive hybridomas for the ψ-[CH_2NH] 4044 analogue peptide were tested by ELISA and the ones reacting positively were cloned twice by limiting dilution. The most promising reactive hybridoma clones from immunoassays were then expanded in larger culture volumes following preliminary specificity evaluation by Western blot [26].

Likewise, two female BALB/c mice were i.p. immunized with a *P. falciparum* mature schizont-lysate for obtaining *P. falciparum* reactive sera, which was inoculated same as above and the so obtained mice serum was used as positive control for all immunochemical techniques.

2.4. Hybridoma sub-cloning and culturing

The original 1A8/A8 hybridoma was sub-cloned until obtaining reactive hybridomas encoded as E7, B9 and C1 recognizing *P. falciparum* MSP-2 and having different immunoglobulin isotypes, which were then cultured at 37 °C in 5% CO_2 on RPMI 1640 medium supplemented with 10% bovine fetal serum; supernatants were periodically collected until completing about 1-L volume.

2.5. Immunoglobulin purification

In vitro culture supernatants were 10X concentrated with saccharose on a Spectra-pore® dialysis membrane (Houston, Texas, USA) at 4 °C after being precipitated with 50% ammonium sulfate and dialyzed overnight against PBS and concentrated five-fold with saccharose.

A Pharmacia glass column (Uppsala, Sweden, Belgium) was packaged with resin volumes ranging from 100 mL to 300 mL to allow Ig isolation. Weak anionic exchange chromatography was performed with the antibody-enriched supernatants from the above reactive hybridoma cultures using a DEAE-Sephadex resin (Pharmacia, Uppsala, Sweden, Belgium) previously equilibrated with 10 mM Tris–HCl buffer at pH 8.5 and to which antibody was led to bind at room temperature overnight with constant shaking. 10 mL fractions, each containing Igs from the resin, were collected by progressively step increasing of the buffer ionic strength from 50 mM, 100 mM and 500 mM NaCl; the resin was then re-equilibrated with 10 mM Tris–HCl buffer at pH 8.5. The chromatography was performed at a constant 0.625 mL/min flow-rate. All eluted fractions were characterized by direct dot blot for Igs detection and positive fractions were submitted to indirect dot blot against ψ-130. Reactive chromatographically eluted fractions from direct and indirect dot blots were pooled according to ionic strength order of elution. Pooled fractions were subsequently dialyzed against saline solution (125 mM NaCl) at 4 °C. Each Ig pooled fraction purity was proved according to SDS-PAGE analysis (data not shown). Further characterization was performed to define each Ig pool isotype composition, dot blot reactivity, and Western blot reactivity against *P. falciparum* lysate, *E. coli* MSP-2 expressed fragment and protein concentration.

2.6. Ig protein determination

Each Ig pool's protein concentration was determined using the micro-bicinchoninic acid technique (BCA) (Pierce, Rockford, IL, USA). Obtained antibodies were diluted 1/10 to 1/100 times from pure Ig pooled samples' original stocks in saline solution and the calibration curve was performed by using a standard of 2 mg/mL bovine serum albumin (BSA) protein, ranging from 0 to 8 μg of total protein in PBS. Sample dilutions were tested in duplicate. 200 μL of the mixture of reagents A (bicinchoninic acid) and B (Cu^{2+}) were then added according to manufacturer's instructions. Optical density (OD) was then read at 570 nm following incubation at 37 °C for 30 min and the quantity of protein from each sample was obtained from the calibration curve as observed in Table 2.

2.7. Immunochemical techniques

2.7.1. Direct and indirect dot blotting

With the aim of determining specific Ig to their inducer pseudopeptide, direct as well as indirect immuno-dot assays were conducted over each Ig isolation step. Volumes of 5 μL of each Ig-containing sample were spotted onto nitrocellulose membrane for direct dot blotting (Bio-Rad Laboratories, Hercules, CA). For indirect dot blotting 5 μg of pseudopeptide ψ-130 were first spotted before Ig sampling. Membranes were blocked with 0.1% Tween-20–5% skimmed milk (w/v) in PBS for 1 h at room temperature. Indirect dot blotting membranes were incubated with primary antibody (Ig pooled samples) overnight at 4 °C. Horse Alkaline Phosphatase Anti-Mouse IgG (H + L) (Vector Laboratories, Inc., Burlingame, CA) was added to direct and indirect dots at 1/1000 dilution for 1 h at room temperature with continuous shaking. An activating solution ($MgCl_2$ 5 mM, NaCl 0.1 M, Tris–HCl 0.1 M pH 9.5) was then added for 40 min and revealed with an NBT/BCIP solution (Promega, Madison. WI, USA).

2.7.2. ELISA tests

Enzyme-linked immuno-sorbent assay (ELISA) were performed for detecting antibody presence and measuring Ig pools' isotypes once being purified. Briefly, each ELISA microplate-well (Nunc, Inter, Med, Denmark) was coated with 100 μL 0.005 μg/μL of pseudopeptide as well as *P. falciparum* lysate dissolved in bicarbonate buffer, pH 9.63. 100 μL of 1:100 sera dilution of pure Ig pool were tested in duplicate after blocking with 3% skimmed milk in 0.05% PBS–Tween-20. Specific anti-tested compound activity was detected using a 1/1000 goat anti-mouse IgG (H + L) peroxidase conjugate (Vector Laboratories, Inc. Burlingame, CA.). A TMB developing solution (KLP, Gaithersburg. USA) was used as substrate. The reaction was stopped by adding 50 μL 1 M H_3P0_4 per well. Results were expressed as optical densities (OD) obtained at 450 nm.

2.7.3. Immunoblotting

P. falciparum-schizont lysate and recombinant MSP-2 were dissolved in Laemmli's buffer, using β-mercaptoethanol as reducing agent and resolved on 13% SDS-poly-acrylamide gels [14]. The resolved proteins were electrophoretically transferred to a nitrocellulose membrane [44]. The antibody Ig pools or the mice sera were used as primary antibodies and detected using goat anti-mouse IgG (H + L) alkaline phosphatase conjugate (Vector Laboratories, Inc., Burlingame, CA). A BCIP/NBT solution (Promega, Madison, WI, USA) was used as reaction substrate.

2.7.4. Indirect immunofluorescence (IFA)

Synchronized-*P. falciparum* mature schizont-infected RBCs were fixed with methanol at −20 °C on multi-well slides (ICN, Pharmaceuticals Inc., Irvine, CA, USA). They were incubated with a 1/100 dilution of pre-immune and post-immune mouse sera for 1 h at room temperature and a 1/1200 dilution of goat anti-mouse IgG (H + L) isothyocyanate–fluoresceine conjugated was added (Vector Laboratories, Inc. Burlingame, CA) to each well and let react for 1 h at room temperature. The slides were visualized under fluorescence microscope (Olympus AXS 50, Tours, France).

2.7.5. P. falciparum recombinant MSP-2 expression and purification

The gene encoding PfMSP-2 was cloned in the pQE-30 expression vector, which adds a 6-histidine tail to its N-terminal region. The recombinant plasmid was transfected in *E. coli* RG and the strain was cultivated in Terrific Broth medium (TB) (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 2.31 g/L KH_2PO_4 and 12.54 g/L K_2HPO_4), supplemented with ampicillin (0.1 mg/L) and chloramphenicol (0.034 mg/L) for 12 h at 37 °C with constant shaking. 950 mL TB medium were then inoculated with continuous shaking at 37 °C until reaching 0.6–0.8 OD at 600 nm. Recombinant protein expression was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) at 1 mM final concentration and incubating for 5 h at 37 °C. After centrifuging at 10,000 rpm for 30 min, the pellet was treated with denaturing agents (6 M urea, 10 mM Tris–HCl, 100 mM NaH_2PO_4 and 15 mM imidazol) and lysozyme (1 mg/mL). The sample was sonicated and centrifuged at 12,000 rpm for 35 min at 4 °C for collecting the supernatant and subsequently submitting it to SDS-PAGE and Western blotting to ascertain recombinant protein expression.

2.7.6. Malarial parasites

Murine parasite *P. berghei* ANKA was thawed and subsequently grown *in vivo* for infecting BALB/c mice through tail-vein inoculation. Slides for microscopic analysis were prepared with *P. berghei*-parasitized RBC thin smears. Infected erythrocytes were collected in phosphate-buffered saline (PBS)-heparin. The chloroquine resistant Colombian *P. falciparum*-FCB-2 strain was used for the *in vitro* assays [12].

2.7.7. P. berghei infection kinetics standardization on BALB/c mice

In order to establish a malarial *in vivo* infection in BALB/c mice (animals obtained from the Universidad Nacional de Colombia mouse-breeding facility), a previously cryo-preserved *P. berghei* ANKA rodent malarial strain stored in 30% glycerol containing 100 UI/mL heparin RPMI medium was thawed and heated at 37 °C and subsequently washed with 1× non-supplemented RPMI medium and the so obtained sample was submitted to a spin-down at 1500 rpm for 5 min. The pellet was then resuspended in 1 mL RPMI for immediately inoculating five BALB/c mice intraperitoneally. Parasitemia in all infected animals was monitored by blood-smear staining with Giemsa, evidencing that mice became parasitized by the second post-infection day and that parasitemia increased slowly until the animals' death between 10 and 12 days after infection. *P. berghei* blood-stage life cycle did not developed synchronically in the 24 h following inoculation, as it was elsewhere described [18]. In addition, animals had not reached a 20% parasitemia by the sacrifice day.

2.7.8. BALB/c mice passive immunization and murine malarial experimental challenge

Groups of five 6–8-week-old BALB/c female mice were immunized with purified antibody or saline solution (control group) on days −1, 0, 1 and 5 (being day 0 the day of *P. berghei* ANKA infection) following previously described protocols [1,47]. In order to determine the infective parasite dose, two *P. berghei* concentrations were tested by intravenously inoculating mice in the tail lateral vein. A first set of five groups of five mice each were inoculated with 5×10^3 infected Red Blood Cells (iRBC) while a second set of six groups of five mice was treated with 2×10^4 iRBC per mouse. Parasitemia was monitored daily until day 22 by counting 1000 peripheral blood RBCs (healthy and infected) on a Giemsa-stained blood-smear, according to protocols described elsewhere [4,27]. The data so obtained were recorded in a database for later analysis. Blood samples were also taken for sera extraction by retro-ocular puncture on days −1 and on the day of sacrifice.

2.7.9. Malarial invasion inhibition by antibodies

Antibodies were tested for their ability to inhibit *P. falciparum* (FCB-2 strain) invasion of human RBCs in *in vitro* assays. Ring-stage-iRBCs (>5% parasitemia) were synchronized using the sorbitol technique [20] and incubated in complete RPMI 1640 media supplemented with 25 mM HEPES buffer, 1 mg/mL hypoxantine, 40 μg/mL gentamycin, 5 U/mL penicillin, 2 g/L glucose, 5% $NaHCO_3$ and 10% 0+ plasma. When parasites had reached the

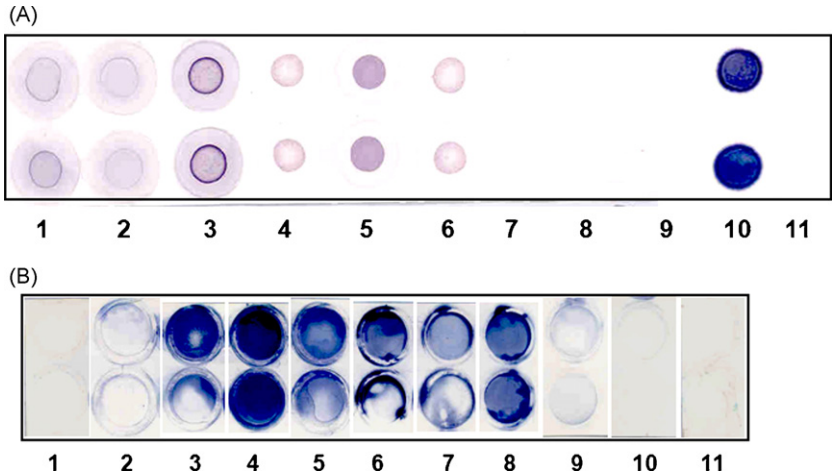

Fig. 2. Reactivity of purified antibodies from E7, B9 and C1 culture supernatants. Direct (A) and indirect (B) dot blotting of precipitates and supernatants following precipitation with ammonium sulfate. Ig ammonium sulfate precipitates E7 in lanes 1 and 4; B9 in lanes 2 and 5; and C1 in lanes 3 and 6. Ig ammonium sulfate supernatants E7 in lane 7, B9 in lane 8 and C1 in lane 9 (A). Ig ammonium sulfate precipitates E7 in lanes 1 and 4, B9 in lanes 2 and 5 and C1 in lanes 3 and 6. Ig ammonium sulfate supernatants E7 in lane 7, B9 in lane 8 and C1 in lane 9. Positive control in lane 10 and negative control in lane 11 (B).

Table 1
Fractions composing each Ig pool.

Hybridoma supernatant code	Column eluted fractions	Pool number	Ionic strength (nM)
E7	6–13	1	50
E7	25–30	2	50
E7	41–57	3	100
E7L1	78–95	4	100
E7L1	96–102	5	500
B9	5–12	6	50
B9	39–45	7	100
B9L1	40–45	8	100
B9L1	46–54	9	100
B9L1	55–63	10	500
B9L1	64–69	11	500
B9L2	35–54	12	100
B9L2	59–88	13	500
C1	2–10	14	50
C1	31–37	15	100
C1	42–46	16	500
C1L1	31–54	17	100
C1L1	55–78	18	500
C1L1	79–96	19	500
C1L2	27–44	20	100
C1L2	51–68	21	500

L1: first wash and L2: second wash.

Table 2
Protein concentration for Ig pools after immunoglobulin isolation.

Ig (pool)	[μg/μL]	Ig (pool)	[μg/μL]
1	0.23	20	0.22
2	0.42	21	0.64
3	**0.26**	22	0.16
4	0.30	23	0.02
5	0.22	24	**0.24**
6	0.20	25	0.10
7	0.30	26	0.03
8	0.25	27	0.05
9	0.30	28	0.12
10	0.25	29	0.14
11	**0.46**	30	0.10
12	0.30	31	0.10
13	0.57	32	**0.27**
14	0.25	33	0.18
15	**0.48**	34	0.26
16	**0.52**	35	0.14
17	0.17	36	0.17
18	0.60	37	0.37
19	**0.86**	38	0.45

schizont stage, 96-well plates containing three antibody dilutions (1/2, 1/4 and 1/8) were seeded with cultured iRBCs and additional non-iRBCs for completing a final 100 μL volume per well at 1.5% hematocrit and 0.3% parasitemia. All antibodies were assessed in triplicate, being incubated for 18 h at 37 °C in 5% O_2, 5% CO_2, and 90% N_2. Cells were harvested after centrifugation and 50 μL supernatant were removed and replaced by 100 μL 15 μg/mL hydroethidine [53]. The pellet was resuspended in 300 μL PBS following further incubation at 37 °C for 30 min and washes with PBS, then poured into polystyrene tubes and for quantifying parasitemia by flow cytometry using FCAScan equipment (Becton Dickinson, San José, CA). The sequence of events was recorded and analyzed using Cell Quest software. An FSC × FL_2 profile was used for establishing an inclusion gate for non-iRBC. Parasitized-RBCs were then quantified by quadrant analysis. Normal RBCs and parasitized RBCs in 15 μg/mL EGTA were used as controls. Invasion inhibition was calculated as being 100 × (% parasitemia in control − % parasitemia in test)/(% parasitemia in control).

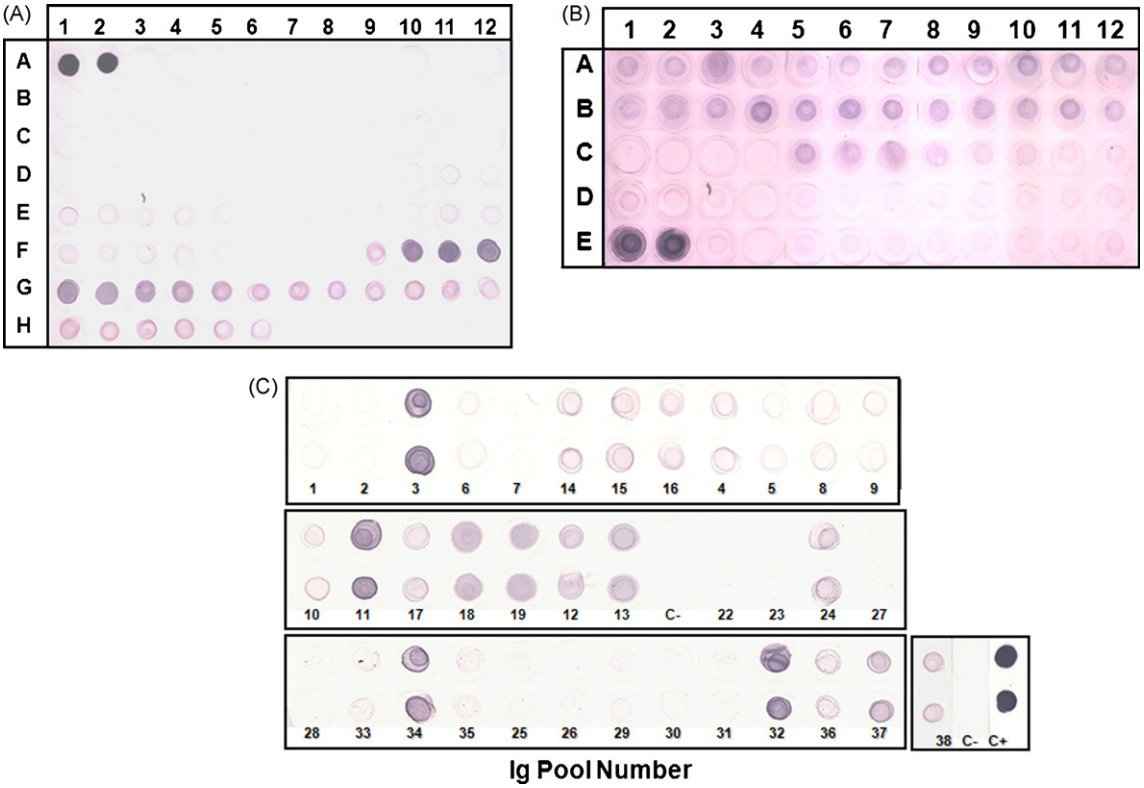

Fig. 3. Reactivity of ionic-exchange chromatography Ig fractions obtained from column B9. Direct dot blot of fractions 1–86 (A5–H6), positive controls A1 and A2, negative controls A3 and A4 (A). Indirect dot blot of fractions 40–86 which had positive direct dots, positive controls E1 and E2 (B). Direct dot blot of the 38 Igs pools (C).

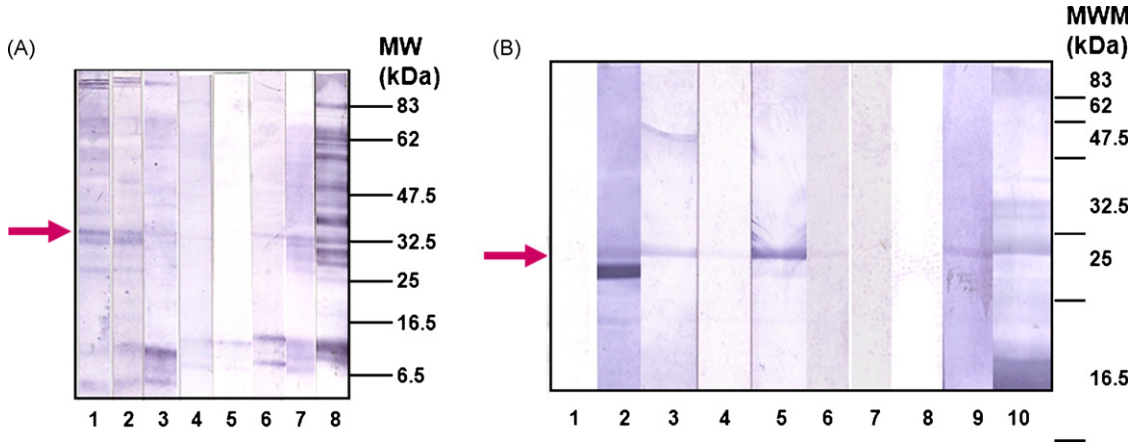

Fig. 4. Reactivity of pools selected for passive immunization against *P. falciparum* and recombinant MSP-2 expressed in *E. coli*. Western blot against *P. falciparum:* Ig pool 3 in lane 1, Ig pool 24 in lane 2, Ig pool 11 in lane 3, Ig pool 32 in lane 4, Ig pool 15 in lane 5, Ig pool 16 in lane 6, Ig pool 19 in lane 7, positive control sera against *P. falciparum* in lane 8 (A). Western blot against recombinant MSP-2 expressed in *E. coli*: negative control is shown in lane 1, positive monoclonal anti-histidine control in lane 2, Ig pool 3 in lane 3, Ig pool 24 in lane 4, Ig pool 11 in lane 5, Ig pool 32 in lane 6, Ig pool 15 in lane 7, Ig pool 16 in lane 8, Ig pool 19 in lane 9 and positive control sera against *P. falciparum* in lane 10 (B).

3. Results

3.1. Pseudopeptide rational designing and bioinformatics analysis

After a first TBLASTN performed analysis with the aim of comparing the *P. falciparum* MSP-2 sequence (gi: 1222687) and the *P. berghei* ANKA genome reported in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) no consensus sequence was detected between them (data not shown). However, when a more specific TBLASTN was carried out between the 4044 native peptide whose amino-acid sequence ^{21}KNESKYSNTFINNAYNMSIR^{40} belonging to *P. falciparum* MSP-2 (gi: 1222687) and the *P. berghei* ANKA genome reported in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/), a 66% identity was determined between peptide 4044 and the *P. berghei* genome within a contig, coded PB_RP0393. Such alignment allowed comparing 18 residues within which 12 were totally conserved between both species and among which I^{12} and N^{13} are found (Fig. 1B). In Fig. 1A, the MSP-2 sequence targeted for the ψ-130 pseudopeptide design is displayed. The 4044 peptide sequence belongs to a highly conserved region of the MSP-2 *P. falciparum* antigen. As previously reported for our group this sequence monomer and polymeric forms were synthesized and physicho-chemically characterized [26].

3.2. Antibody isolation and characterization

After treating the sub-clones coded E7, B9 and C1 culture supernatants produced against *P. falciparum* MSP-2 protein ψ-130 peptide with ammonium sulfate, precipitates and supernatants presenting high and low reactivity respectively, in both direct and indirect dot blotting were obtained (Fig. 2 A and B). An average of 100 fractions were obtained after submitting E7 (IgM), B9 (92% IgM + 8% IgG1) and C1 (75% IgM + 25% IgG isotypes) ammonium sulfate precipitated Igs to anionic exchange chromatography. Those fractions presenting a positive reactivity in both direct and indirect dot blotting were used for forming homogeneous Ig pools as illustrated in Table 1 and Fig. 3, thus obtaining a total of 38 Ig pools having protein concentrations ranging from 0.02 to 0.86 mg/mL (Table 2). Direct dot blotting (Fig. 3) was used as a criterion for evaluating specific antibody presence in the isolated protein fractions. Further specific assessment of the above isolated Ig reactivity against both, a protein band having a relative electrophoretical movility about 45 kDa in a *P. falciparum* lysate and a strong reactivity against a band between 25 and 30 kDa in a recombinantly *E. coli* MSP-2 expressed fragment by Western blot demonstrating these Igs recognition patterns.

Seven out of the 38 Ig pools obtained were selected based on protein concentration, dot and Western blot reactivity and pool's volume, which presented average immunoglobulin isotype percentages as follows: 45.9% IgM; 13.1% IgG1; 4.5% IgG2a; 4.3% IgG2b; 6.5% IgG3 (see Table 3). These seven chosen pools recognized both a band corresponding to the MSP-2 protein in the *P. falciparum* lysate (Fig. 4A) as well as in the *Escherichia coli* MSP-2 recombinantly expressed product between 25 and 30 kDa by Western blotting (Fig. 4B).

Table 3
Characteristics of Ig pools used for passive immunization trials.

Pool	Inoculate amount (μg)	%IgM	%IgG1	%IgG2a	%IgG2b	%IgG3	No infected/no tested	Protection%
(A)								
3	52	62.1	20.9	4.3	4.2	8.5	1/5	80
11	92	63.9	21.5	3.3	3.8	7.5	4/5	20
15	96	62.7	17.8	6.0	5.1	8.4	1/5	80
32	54	63.9	21.5	3.3	3.8	7.5	0/5	100
(B)								
15	96	62.7	17.8	6.0	5.1	8.4	5/5	0
16	104	68.5	15.4	4.0	4.1	7.9	5/5	0
19	172	65.5	21.0	3.8	3.2	6.5	4/5	20
24 (culture)	48	62.1	20.9	4.3	4.2	8.5	5/5	0
S/N	ND	58.0	21.0	7.0	4.0	10.0	4/5	20

A: first passive immunization assay; B: second passive immunization assay; S/N: supernatant; ND: not determined.

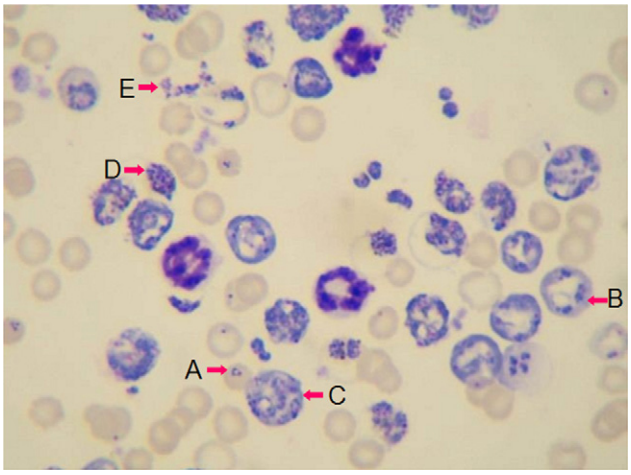

Fig. 5. BALB/c mice *in vivo* infection with *P. berghei-ANKA*. Giemsa-stained peripheral blood slides from BALB/c infected mice observed by light microscopy at 100×. Arrows indicate *P. berghei* asexual blood stages. (A) Ring-stages, (B) mature trophozoites, (C) EARLY schizonts, (D) mature schizonts and (E) free merozoites.

3.3. P. berghei in vivo infection

Following the intravenous inoculation of female BALB/c mice with a lethal dose of a *P. berghei* ANKA strain, animals were followed-up over a fixed time period in order to evaluate appearance and establishment of parasitemia. It was observed that 24 h after being infected, all animals had reached a mean 0.2% parasitemia as evidenced by the presence of immature *Plasmodium* stages such as ring-stages in all analyzed mice blood samples as can be observed in Fig. 5. During the first 5 days of infection the parasitemia had progressed allowing for the presence of different parasite stages such as rings, trophozoites and immature schizonts. From day 6 on, the malarial infection had become asynchronic as it can be observed in Fig. 5. All animals had reached a 26.3% parasitemia by day 8, presenting all the previously mentioned forms plus mature schizonts and free merozoites. Therefore after successfully infecting BALB/c mice, freshly obtained *P. berghei* inoculums were used for passive immunization experiments.

3.4. Passive immunization experiments with defined antibodies against the ψ-130 pseudopeptide

According to *P. berghei* growth kinetics described above, the BALB/c mouse animal model was available for malarial infection experiments. Animals infected with 5000 iRBCs presented average $0.5 \pm 0.3\%$, $3.5 \pm 6.4\%$ and $0.2 \pm 0\%$ parasitemia on days 6, 14 and 22 respectively compared to those infected with 25,000 iRBCs presenting $0.5 \pm 0.3\%$, $22 \pm 15\%$ and $32 \pm 24\%$, respectively. Controls for both groups presented $1.3 \pm 1.3\%$ and $0.4 \pm 0.7\%$ average parasitemia on day 6; control animals infected with 5000 iRBCs did not survive up to day 14 and those infected with 25,000 iRBCs survived up to day 18 (Fig. 6).

Regarding groups infected with 5000 iRBCs, it was observed that Ig pool 11 had no evident protection-inducing effect on animals since 80% of mice developed infection and did not survive until the end of trial. Ig pools 3 and 15 conferred protection on 80% of the animals and 20% survived up to day 13, reaching 30% and 51% parasitemia, respectively. Ig pool 32 had an evident

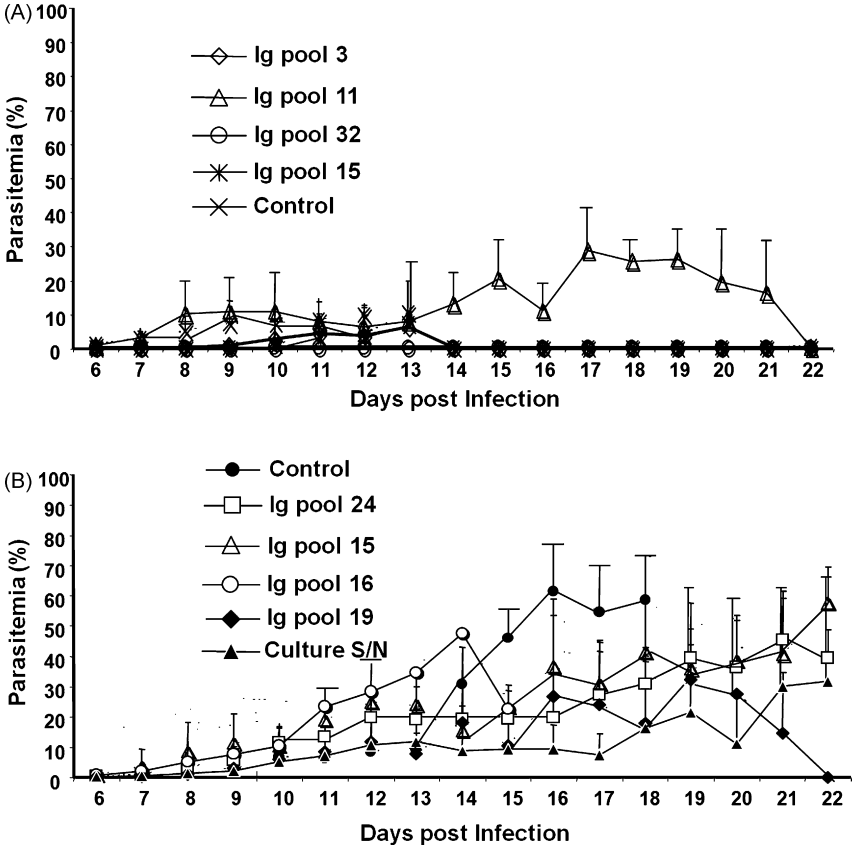

Fig. 6. Course of parasitemia in *P. berghei* infected animals. Administrated infection dose: 5000 iRBCs (A). Administrated infection dose: 25,000 iRBC (B). Each line represents mean parasitemia of each animal group with its corresponding S.D.

protection-inducing effect on all animals since average parasitemia was 0.3% on day 14 for this group (Table 3 and Fig. 6). Mice immunized with the antibody Ig pool 32, having an isotype composition of 64% IgM and 36% IgG, as well as with antibody Ig pool 15 whose isotype composition was 63% IgM and 37% IgG displayed very low parasitemia levels regarding the control group (*P*-value 0.0010). These two tested animal groups also got the highest level of survival regarding the whole set of tested mice groups (Table 3).

Regarding groups infected with 25,000 iRBC, it was observed that Ig pools 24 and 15 did not confer protection but did have an effect on the course of parasitemia and animal survival, since 80% and 60% respectively presented 40% and 58% average parasitemia on day 22 as can be observed in Fig. 7C. Ig pool 19 and culture S/N protected 20% of the animals; reaching a maximum parasitemia of 0.6% and 0.5% on days 18 and 15, respectively (Table 3).

When comparing groups immunized with Ig pool 15 and inoculated with different doses, it was observed that the greatest percentage reached by animals was 10% with 5000 iRBC and 58% with 25,000 iRBC. The course of parasitemia for groups having 5000 iRBC and 25,000 iRBC was $0.3 \pm 0.1\%$ and $1.0 \pm 1.6\%$ on day 6, $0.4 \pm 0.2\%$ and $25 \pm 8.3\%$ on day 14 and $0.2 \pm 0.1\%$ and $53.2 \pm 11.8\%$ on day 22, respectively (Fig. 7A).

Western blotting analyses of sera from protected animals showed that they recognized the MSP-2 protein in the *P. falciparum* lysate at 45 kDa and the recombinant expressed in *E. coli* at 30 kDa. all mice's recognition of the recombinant protein was equal whereas mice 4, 10, 33 and 60 recognition in the *P. falciparum* lysate was stronger (Fig. 8).

3.5. Treated animals' sera reactivity recognized P. falciparum by immunofluorescence

As observed in Fig. 9, all protected animals and those delaying course of parasitemia recognized *P. falciparum*-mature schizonts, showing the characteristic "bunch of grapes" reaction pattern of antigens such as MSP-2 [38]. It is worth stating that pre-immune sera from the same animals did not recognized malarial parasites.

3.6. Antibodies from treated animals displays in vitro neutralizing activity

Most obtained Ig pools inhibited *P. falciparum* human RBC-invasion at some degree. The Ig pools used in passive immunization assays inhibited invasion by approximately 53% at a 0.12 μg/mL of Ig concentration while Ig pools 3, 15, 24, 32, 11 and 16 inhibited invasion by 84%, 87%, 27%, 44%, 68% and 66% respectively. Ig pool 19 reached maximum 60% inhibition at a 0.06 μg/mL of Ig concentration; however, only Ig pools 24, 32, 15 and 19 seemed to have a dose-dependent inhibition pattern (Fig. 10).

Cytokine levels associated to a Th immune response were indistinctively detected by flow cytometry in all immunized animal groups. However, high levels of TNF-α were observed (≥2000 pg/mL) (data not shown).

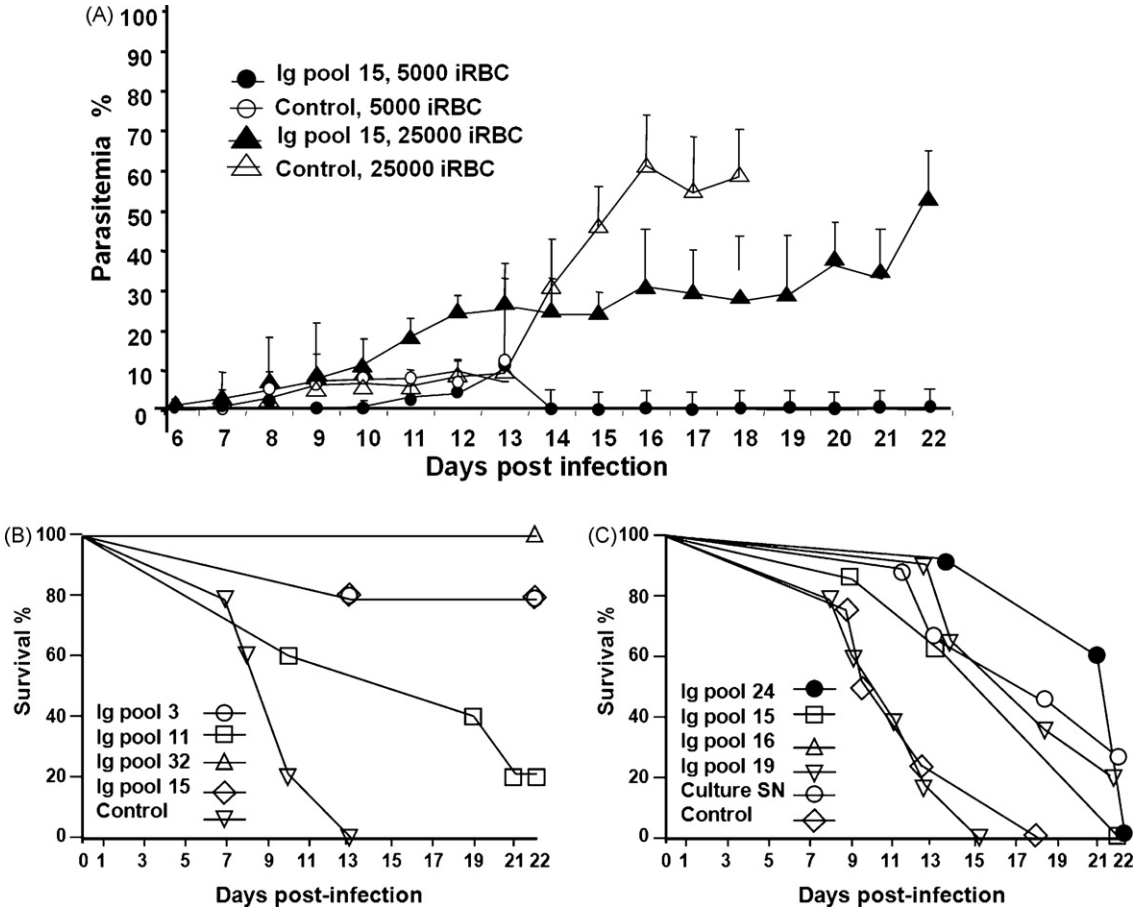

Fig. 7. Comparing percentage parasitemia of animals being passively transferred with Ig pool 15 upon infection with 5000 iRBCs and 25,000 iRBCs. A. Each line represents the mean parasitemia of each animal group. Vertical bars represent the standard deviation (S.D.). Survival profile of malarial infected animals after being passively transferred with specific anti-ψ-130 pseudopeptide antibodies in a first trial (B) and a second trial (C).

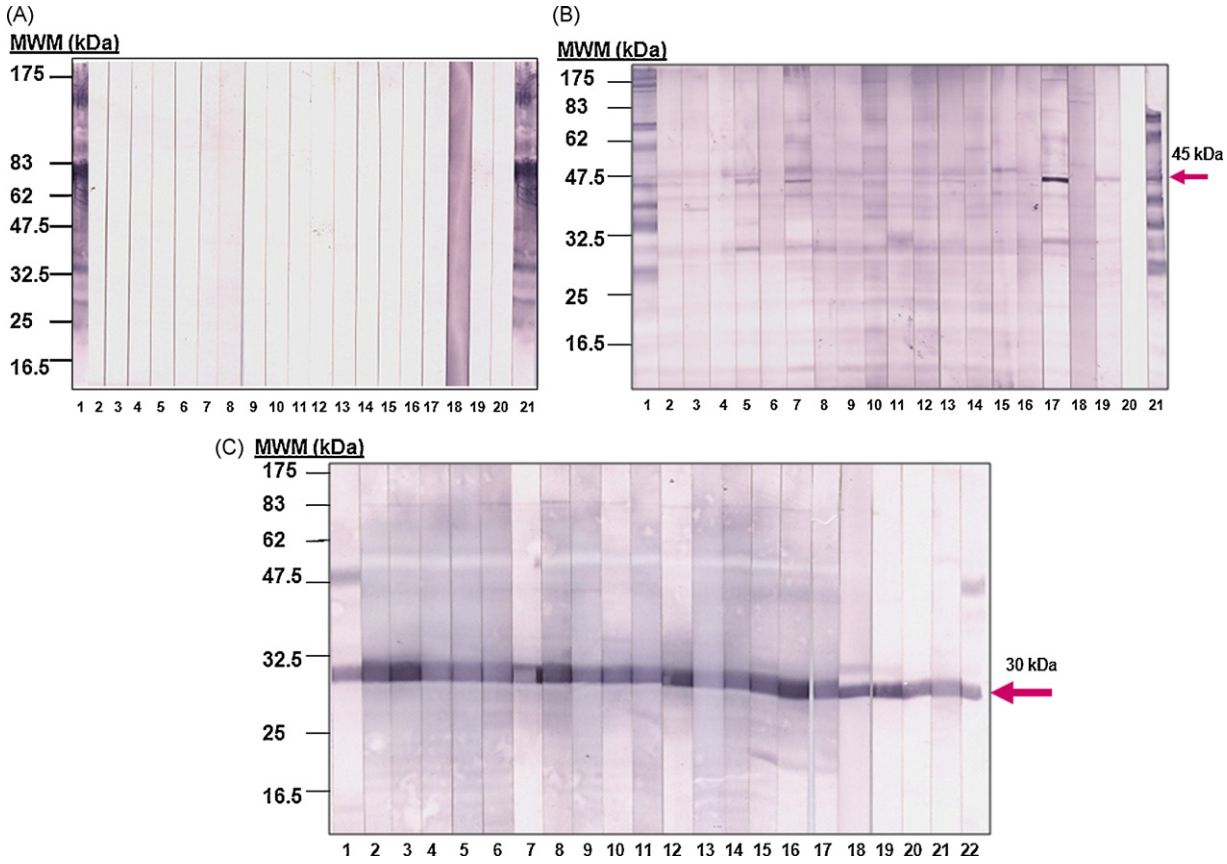

Fig. 8. Immuno-reactivity of sera from protected mice. Western blot using pre-immune (A) and post-immune (B) sera against *P. falciparum* lysate and the recombinant MSP-2 expressed in *E. coli* (C). Mice 1, 2, 3 and 4 immunized with Ig pool 3 in lanes 2, 3, 4, 5; mice 7 and 10 immunized with Ig pool 11 in lanes 6 and 7; mice 11, 12, 13, 14 and 15 immunized with Ig pool 32 in lanes 8, 9, 10, 11 and 12; mice 21, 22, 24, 25 immunized with Ig pool 15 in lanes 13, 14, 15 and 16; mouse 33 immunized with Ig pool 24 in lane 17; mouse 51 immunized with Ig pool 19 in lane 18; mouse 60 immunized with E2 culture supernatant lane 19; positive controls consisting in *P. falciparum* reactive sera in lane 1 and 21, negative control in lane 20 (A and B). Positive monoclonal anti-histidine control (lanes 1 and 22) (C).

4. Discussion

Different studies have established that the natural immune response developed by people exposed to malaria is directed against MSP-2 dimorphic and polymorphic regions; it is thus a species-specific response [21,48,51] which has been associated to clinical immunity only in subjects living in malarial endemic areas [29,36]. As we have proposed in our researching, pseudopeptide chemistry has been proposed as a feasible alternative for obtaining novel site-directed structural proves able to induce an immune response against conserved *P. falciparum* polymorphic protein regions [25]. Even though this protein's N-terminal region is highly conserved among *Plasmodium* species, a homologue has still not been identified in *P. berghei*. However, other studies have evidenced that immunizing with peptides from the *P. falciparum* MSP-2 N-terminal region conferred protection in mice against infection with heterologous species such as *P. yoelii* and *P. berghei* [19,24,43], suggesting that antibodies raised against *P. falciparum* MSP-2 cross-react with an orthologuos gene presented in *P. berghei*. Such statement is further supported by the bioinformatics analysis here reporting a 66% identity between peptide 4044 and the PB_RP0393 *P. berghei* contig and by antibody-mediated protection conferred against a lethal *P. berghei* infection. The molecular scheme followed up by this study is in complete agreement with such findings, since peptide 4044 containing the Ile (I^{12}) and Asn (N^{13}) amide-reduction targeted residues was used for designing the pseudopeptide as it localizes within the conserved region and is also included within this peptide's HABPs. Previous studies have demonstrated that passive immunization with monoclonal antibodies directed against *P. chabaudi* and *P. yoelii* lysate or *P. yoelii* MSP-1, AMA-1 and CS malarial antigens have suppressed parasitemia in murine malaria models [4,27,31,47]. This study corroborated the protection-inducing ability of antibodies produced against 4044 peptide reduced amide pseudopeptide (*Pf* MSP-2^{21-40}). Seven Ig pools of antibodies from culture supernatant with a defined composition of isotypes were tested, four of which inhibited *in vitro* invasion (Fig. 10) to some degree of which three Ig pools suppressed *in vivo* parasitemia in passive immunization assays (Table 3).

Fig. 7 B and C shows survival profiles for all malarial infected animals being passively immunized with Ig pools. The high survival percentage demonstrates the potential of using site-directed-designed pseudopeptide for inducing functional antibodies as a novel immunotherapeutic procedure for controlling malaria. As it can be observed for the first performed trial (Fig. 7B), Ig pool 32 conferred 100% protection against malarial infection. All animals belonging to this experimental group had lower malarial parasitemias and survived malarial infection, compared to the control group receiving only saline solution, in which all animals reached high parasitemia levels and none of them survived. Ig pools 3 and 15 displayed a similar behavior protecting 80% of animals, since four out of five animals were protected and only one died due to malarial infection by day 13. On the other hand, the Ig pool 11 conferred only a 20% protection.

Regarding the second experimental trial, the Ig pool 19 and the Ig-culture supernatant treated groups had a

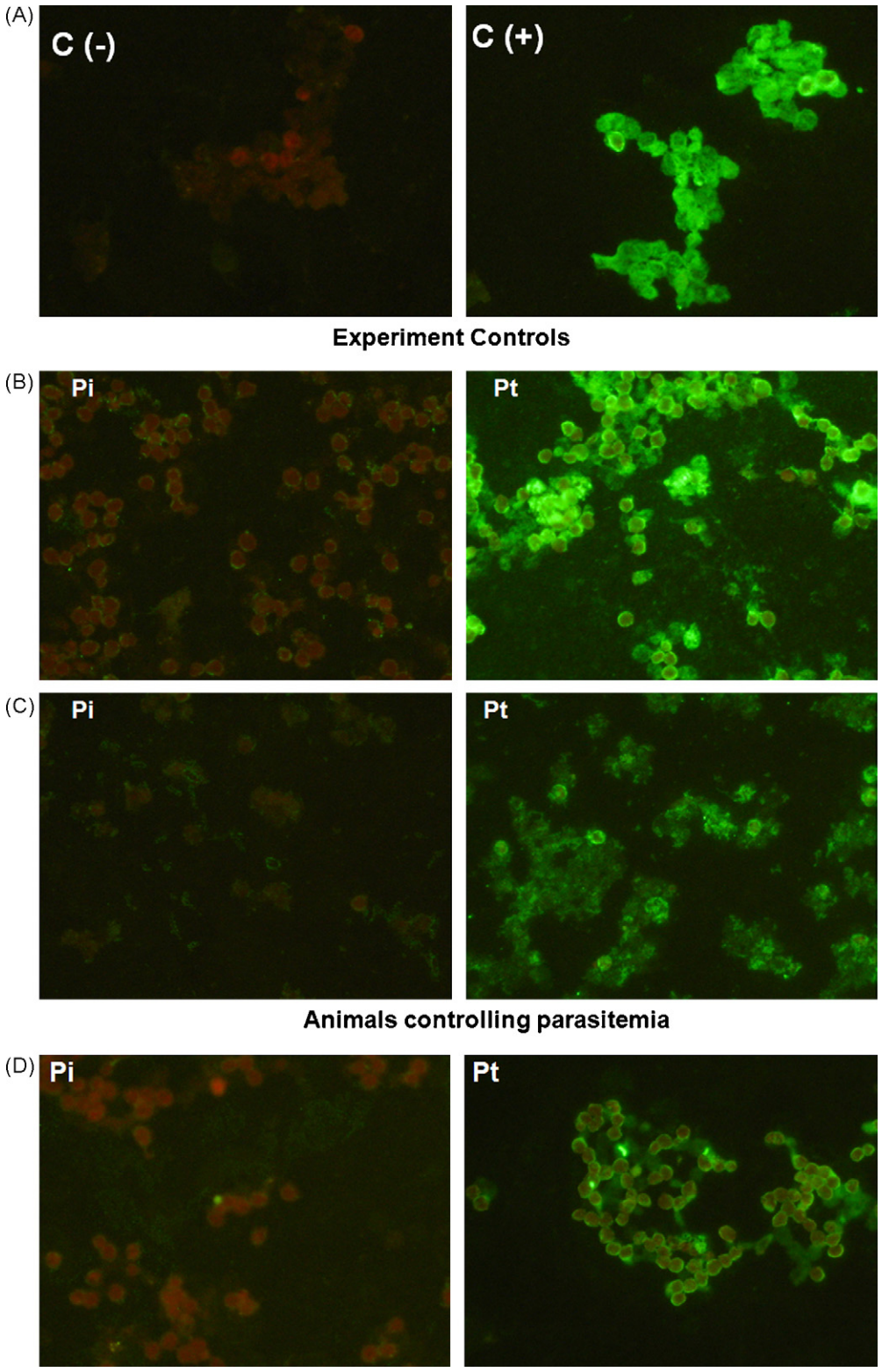

Fig. 9. Recognition of *P. falciparum* native proteins by sera from Ig passively transferred mice. Immunofluorescence of pre-immune sera (left-hand side) and post-Ig passive immunization sera (right-hand side) against *P. falciparum* mature schizonts. Controls (A). Non-protected mouse 10 inoculated with pool 11 which presented a reduced parasitemia (B). Non-protected mouse 33 inoculated with pool 24 which presented reduced parasitemia (C). Protected mouse 15 immunized with pool 32 (D). Protected mouse 60 immunized with culture supernatant (E).

similar behavior since both conferred 20% protection. However Ig pools 15, 16 and 24 had no protecting effect against malarial experimental challenge as it can be seen in Fig. 7.

Regarding invasion inhibition assays, there was no-evident correlation between *in vitro* inhibition and *in vivo* protection-inducing effect, since Ig pools 11, 19 and 32 inhibited *in vitro* invasion by 68%, 37% and 44% while inhibiting *in vivo* invasion by

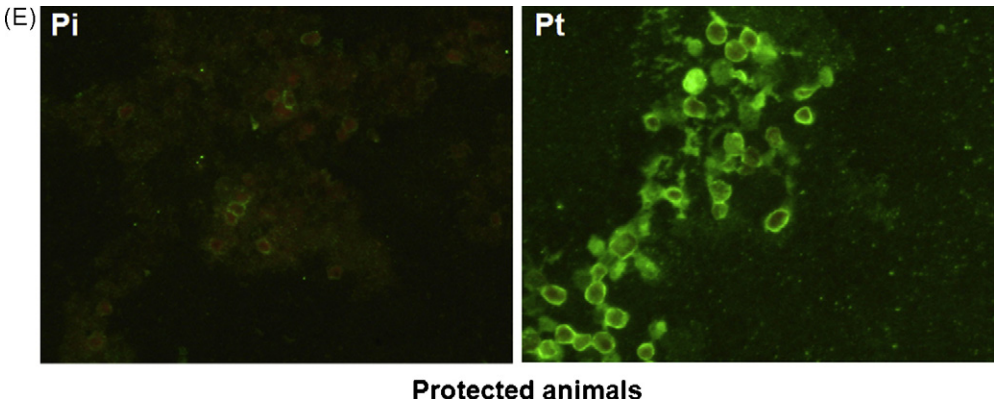

Fig. 9. (*Continued*).

20%, 20% and 100%, respectively (Figs. 6 and 9); however, there was an appreciable correlation between Ig pools 3 and 15 inhibiting *in vitro* 84% and 87%, respectively and both 80% *in vivo*. It should be considered that different strains were used for *in vitro* and *in vivo* assays and therefore such observed correlation could arise due to recognition of highly conserved epitopes within both species by both Ig pools. Whereas the remaining pools could have been contained antibodies recognizing conserved epitopes and thus did not show cross-reactivity.

According to the results presented herein, a sequential or structurally related or functional *P. falciparum* MSP-2 molecular homolog in *P. berghei* thus becomes evident, agreeing with the *P. falciparum* MSP-2-pseudopeptide induced Igs' passive immunization protection-inducing effect observed after infecting BALB/c mice with a *P. berghei* strain.

Given the nature of the antigen from which the antibodies came, processing merozoite surface proteins represents the most appropriate mechanism for explaining the protection-inducing effect observed, without disregarding the steric interference effect [2,15,37]; however, the differences found in, *in vivo* and *in vitro* assays suggest the participation of other mechanisms such as: antibody-dependent cell inhibition (ADCI), antibody-dependent phagocytosis (ADPh) and complement fixation, although some of them have only been described in primate and human models up to the moment [8,9,30,35,55]. Nevertheless, other experiments must be done to confirm these hypotheses.

As expected, our results suggest that when a higher concentration of antibodies is present, then the protection-inducing effect also increases (Figs. 4 and 6 Table 2), possibly in response to any of the above-mentioned mechanisms.

Correlation was not seen between the percentage of inoculated isotypes and the suppressed parasitemia. Most antibody isotypes were IgM (60–65%) and IgG1 (15-20%). An evident protection-inducing effect appeared in this study, irrespective of the isotype, thus differing with other researchers reporting a correlation between antibody isotype and protection [1,27,29,33,48,55], which lead to presume that the effects in this case were due to antibody specificity.

MSP-2 recognition of both *P. falciparum* lysate and recombinant in Western blot was seen in all Ig pools, even though Ig pools 3, 11 and 19 presented stronger recognition than 32 and 15 (Fig. 5B). However, only Ig pools 3, 32 and 15 suppressed parasitemia and the level of protection for Ig pools 11 and 19 was less, indicating that antibodies were found in Ig pools 3, 32 and 15 having greater affinity, managing to avoid parasite invasion of RBC.

In agreement to the results obtained when immunizing with Ig pool 26 and infecting with 5000 iRBCs and 25,000 iRBCs (Fig. 7A), it is presumed that the differences may be due to the infective

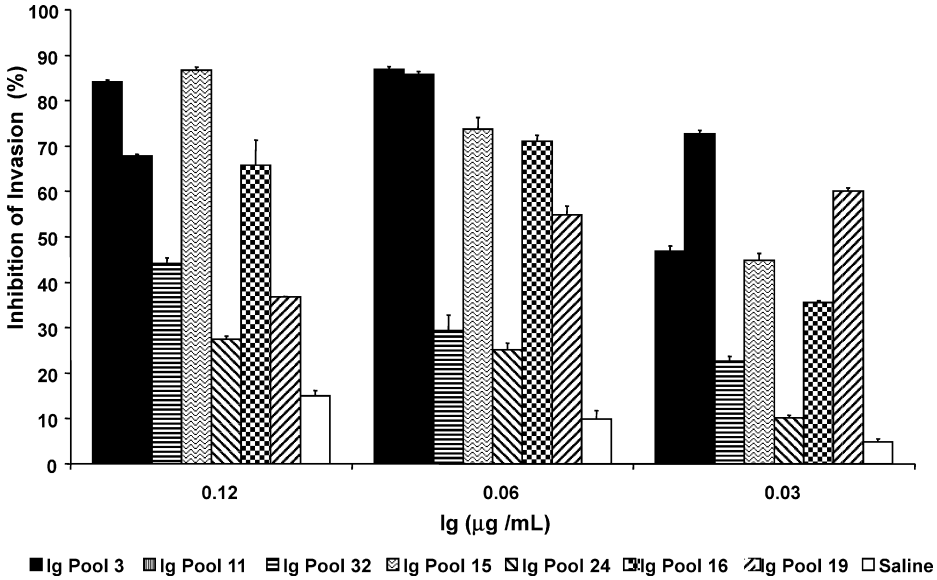

Fig. 10. *In vitro* invasion inhibition capacity of Ig pools 3, 8, 16, 24, 26, 27 and 30.

dose applied as it has been previously reported [1] as the same protein concentration, same isotype percentage and same antibody was used, in opposition to other studies reporting that antibody's isotype may be important for conferring protection [1,27,29,33,48,55].

This study has led proving that antibodies produced by Mab E7, B9 and C1 were capable of inhibiting parasite *in vitro* and *in vivo* invasion, regardless of the immunoglobulin isotype proportion present in each studied Ig pool. It would thus be interesting to find out whether there are any differences between protection achieved by passively immunizing with an specific isotype and whether interaction between different isotypes produced a synergistic effect, as has been recently reported [31].

The results presented herein provide for the first time clear evidence of the existence of a *P. falciparum* MSP-2^{21-40} homolog sequence in the *P. berghei* genome, since mice being challenged with the heterologous *P. berghei*-ANKA strain were capable of efficiently controlling and eliminating malarial induced infections. Additionally, structurally modified antigens, constituted by reduced amide pseudopeptides of *P. falciparum*, are efficient antibody inducers able of neutralizing malarial infection by blocking specific pathogen's ligands.

The therapeutical and neutralizing power of any of the MSP-2-pseudopeptide-antibodies could be attributed to individual Ig structural properties. Altogether the results presented in this work displays some evidence that selected site-directed designed pseudopeptides becomes a reliable strategy towards obtaining novel and versatile molecular tools for a sub-unit, multi-component anti-malarial vaccine formulation having all a potential immuno-therapeutic application.

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