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Immunization with rP22 induces protective immunity against *Schistosoma mansoni*: Effects on granuloma down-modulation and cytokine production

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ABSTRACT

Schistosomiasis remains a significant public health problem in tropical countries and it is recognized as the most important human helminth infection in terms of morbidity and mortality. Although the existing antischistosomal drugs are highly effective, they do not prevent against reinfection or granuloma formation. Therefore, vaccine strategies are essential for the control of schistosomiasis. Our group recently identified the recombinant (r) P22 protein, a component of the adult worm protein fraction PIII that has been shown to engender protective and immunomodulatory effects on murine schistosomiasis. A cDNA clone encoding rP22 was isolated from a *Schistosoma mansoni* adult worm cDNA library using anti-PIII rabbit serum; it exhibited complete identity with *S. mansoni* Sm21.7 EF-hand antigen. Confocal microscopy revealed that rP22 is a tegument protein localized on the surface of *S. mansoni* miracidia and adult worms. Mice immunized with rP22 exhibited a 51% and 22.5% decrease in adult worm burden and in hepatic eggs, respectively. Additionally, rP22 vaccine produced a reduction in 60% of liver granuloma size and 71% of fibrosis in mice, suggesting that rP22 might contribute to down-modulate granulomatous hypersensitivity to *S. mansoni* eggs. Protective immunity in mice was associated with high titers of rP22-specific IgG antibodies; elevated production of IFN-γ, TNF-α and IL-10; and a reduced level of IL-4. In conclusion, these findings indicate that rP22-based vaccines could be useful to elicit protection and reduce pathology associated to schistosomiasis.

1. Introduction

Schistosomiasis is a human parasitic disease caused by trematodes of the genus *Schistosoma* that affects more than 207 million individuals worldwide [1] and causes up to 250,000 deaths per year [2]. Furthermore, the impact of the severe and debilitating effects of schistosomiasis account for the loss of 4.5 million disability adjusted life years (DALY) annually [3].

Currently, schistosomiasis control strategies are predominately based on the treatment of infected individuals with safe and effective drugs [4]. However, mass treatment has been proven to be insufficient to stop disease transmission, prevent reinfection or reduce parasite-induced morbidity [1,5,6]. Therefore, a protective anti-schistosomiasis vaccine would be valuable to current disease control programs [7].

Desirable characteristics of a schistosomiasis vaccine candidate include not only the capacity to reduce worm burden and fecundity, but also the capacity to down-regulate granulomatous responses to eggs that become trapped in the host liver and intestines and cause morbidity [8]. Although many antigens have already been described as promising vaccine candidates against schistosomiasis, the protection level achieved has been approximately 50%. However, there is common agreement that even a partial reduction in worm burden could considerably reduce pathology and limit parasite transmission [9].

Another important aspect of potential vaccine antigens is the determination of the type of immune response is elicited by immunization [8]. Protective immunity against *Schistosoma mansoni* has been the subject of numerous studies. In the murine model, the immunological mechanisms involved in protection are related to antibody responses and cellular immune response dependent on $CD4^+$ T cell-mediated IFN-γ responses [10–13]. Among cells and cytokines involved in immunomodulation, $CD4^+$ T cells and IL-10 play an important role in granuloma formation and regulation [14–17].

Abbreviations: SWAP, *Schistosoma mansoni* soluble worm antigen preparation; PIII fraction or PIII, anionic protein fraction obtained from SWAP.

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Our group has been studying PIII, an anionic protein fraction obtained from the *S. mansoni* soluble worm antigen preparation (SWAP), which has been shown to reduce worm burden and granuloma formation in PIII-immunized mice after challenge infection [18,19]. PIII also decreased *in vitro* granuloma reactions by human peripheral blood mononuclear cells from chronic schistosomiasis patients [20]. Previous works have demonstrated that some antigenic components of PIII, such as P24 [16] and recombinant RP44 [21], possess both protective and immunomodulating properties against *S. mansoni* infection after mice immunization. However, other PIII components and their individual immunological activities against schistosomiasis remain unknown.

In this study, we describe and characterize the recombinant protein rP22, a component of the PIII fraction. rP22 was identified as a tegument protein and it is identical to Sm21.7, a member of the EF-hand-containing parasite protein family that also exhibits dynein light chain (DLC) domain [22]. Further, we evaluated the ability of rP22 immunization in mice to induce protection against *S. mansoni* infection, and its capacity of down-regulate granulomatous hypersensitivity to parasite eggs.

2. Materials and methods

2.1. Mice and parasites

Female C57BL/6 mice aged six to eight weeks were purchased from the Federal University of Minas Gerais (UFMG) animal facility. All protocols involving animals were approved by the local Ethics Committee on Animal Care (CETEA–UFMG # 043/2008). *S. mansoni* cercariae were maintained routinely on *Biomphalaria glabrata* snails at the Centro de Pesquisas Rene Rachou-Fiocuz (CPqRR); for infections, cercariae were prepared by exposing infected snails to light for 1 h to induce shedding. Cercarial numbers and viability were determined using a light microscope prior to infection.

2.2. Antigen preparation

SWAP and PIII preparations were produced and purified as previously described [20]. Briefly, SWAP was prepared as soluble supernatant fluids from buffered saline homogenates of adult *S. mansoni* worms. PIII was obtained from SWAP by FPLC using Q-Sepharose anion-exchange chromatography with 5 mm × 90 mm glass columns packed with Q-Sepharose beads sized between 45 and 165 mm (Pharmacia, Uppsala, Sweden).

2.3. Cloning and sequencing of rP22 cDNA

The rP22 DNA sequence was obtained from a cDNA expression library constructed from mRNA of *S. mansoni* adult worms. The expression library was screened using polyclonal rabbit IgG antibodies against PIII; identified clones were sequenced using the DYEnamic™ ET dye terminator cycle sequencing MegaBACE™ kit and the MegaBACE 1000 capillary sequencer (Amersham Biosciences, Buckinghamshire, England) as described previously [23]. The sequences obtained were compared with those already deposited in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm available at the NCBI website (http://www.ncbi.nlm.nih.gov/Blast/).

Specific oligonucleotides were designed to amplify the cDNA clone coding rP22 sequence. The 5′ and 3′ oligonucleotides, GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GGA TAG TCC AAT GGA AAA ATT TAT TC and GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC GTT ACT TGG TGT ACG CCA AG, were used in a PCR reaction to amplify the complete open reading frame of rP22. PCR was performed using Platinum Pfx enzyme (Invitrogen, Carlsbad, USA); the reaction was initiated with one cycle of 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 52 °C, and 3 min at 68 °C. PCR products were cloned by a BP recombination reaction into pDONR™221 cloning vector (Invitrogen, USA), according to manufacturer specifications; plasmids were sequenced to confirm proper insertion. After producing and sequencing an entry clone, a LR recombination reaction was performed with pDONR-rP22 and pETDEST42 to clone the full-length cDNA sequence of rP22 into an expression vector (Invitrogen, USA).

2.4. Sequence analysis of rP22

The amino acid sequence of rP22 was obtained and used as a query in Blast and PSI-Blast searches against the non-redundant protein sequence database to identify rP22 orthologs (http://blast.ncbi.nlm.nih.gov/Blast) and to search for conserved domains using the CDART program [24]. Primary structure analysis of rP22 was performed using the Expasy (Expert Protein Analysis System, http://ca.expasy.org/tools) computer program. Molecular weight (MW) and isoelectric point (pI) were calculated with the ProtParam tool (http://expasy.org/tools/protparam.html). The identification of signal peptide (SignalP 3.0, http://www.cbs.dtu.dk/services/SignalP), and transmembrane protein prediction (TMHMM, http://www.cbs.dtu.dk/services/TMHMM-2.0) were also performed by bioinformatics.

2.5. Expression of recombinant protein

Escherichia coli BL21 (DE3) (Invitrogen, USA) were transformed with pETDEST42-rP22 expression vector to produce a recombinant P22 that contained a C-terminal hexahistidine tag. For protein expression, transformed cells were grown in 1L LB plus ampicilin (OD600 = 0.6). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.5 mM, and cells were incubated for 3–4 h at 37 °C. Cells were harvested by centrifugation and resuspended in 30 ml of binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20–40 mM imidazole, pH 7.4). Cell suspensions were then frozen and thawed three times. Next, the lysates were submitted to three 30 s cycles of sonication to release the target protein. The fusion protein obtained in the supernatant after centrifugation was then purified by metal affinity chromatography using the Akta Prime system (Amersham Biosciences, England) under native conditions. Briefly, the sample was loaded onto a Ni^{2+}-NTA columns (5 ml bed volume) pre-equilibrated with the same buffer. The columns were washed with 10 bed volumes of the binding buffer and were then eluted with 20–500 mM imidazole linear gradient. The main peak was pooled, and the protein purity of fractions was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Additionally, the elution buffer was exchanged with Phosphate Buffered Saline pH 7.4 (PBS) before use of this protein.

2.6. Polyclonal antibody production

Polyclonal rabbit sera were produced against preparations of PIII, recombinant P22 or native P24. Rabbits were inoculated with 100 μg of rP22, P24 or PIII preparation by i.m. injections at 15-day intervals with a total of three injections. PIII and recombinant proteins were formulated with Freund's adjuvant (complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant (IFA) for the subsequent injections). Fifteen days after the last inoculation, rabbits were exsanguinated [19]. The sera were assayed for Western blot analysis and for the indirect immunofluorescence assays.

2.7. Western blot analysis

Purified rP22, SWAP or pellet of an *E. coli* clone expressing rP24 fragment (another PIII component) were submitted to SDS-PAGE, transferred to nitrocellulose membranes and blocked for 16 h at room temperature with PBS casein (1.6% of casein in PBS 1×). The membranes were washed three times with PBST (PBS 1×, 0.05% Tween 20) and probed with anti-PIII, anti-rP22 or anti-rP24 rabbit serum diluted 1:500, 1:10,000 and 1:500, respectively, in PBS with 0.25% casein for 1 h at room temperature. Next, the membranes were washed three times and probed for 1 h with anti-rabbit IgG conjugated to horseradish peroxidase (Sigma, St. Louis, MO, USA) diluted 1:10,000 in PBS–0.25% casein. After three washes, the membranes were treated with a solution containing 3,3′aminobenzidine (DAB) 600 μg/ml, $NiCl_2$ 10 μl/ml and H_2O_2 1 μl/ml, diluted in PBS 1×; the color reaction was stopped with distilled water.

2.8. Immunization of mice

Eight to ten week old female C57BL/6 mice were divided into five groups of seven mice each. Mice were subcutaneously injected in the nape of the neck with either 50 μg of rP22 or SWAP. First and second boosts were given 2 and 4 weeks, respectively, after the initial immunization. SWAP and rP22 protein concentrations for vaccination were determined with a Micro BCA Protein Assay Kit (Thermo Scientific Co., Waltham, MA, USA). The antigens rP22 and SWAP were formulated with 100 μg of *Corynebacterium parvum* and 1 mg of aluminum hydroxide ($Al(OH)_3$) as adjuvant [16]. PBS 1× or PBS 1× with *C. parvum* and $Al(OH)_3$) were administered to saline and adjuvant control groups, respectively, following the same immunization protocol. To distinguish the two different time points of the vaccination/infection protocol, mice group that received rP22 + adjuvant were named rP22-Vac. Mice that were vaccinated with rP22 + adjuvant and subsequently challenged were labeled rP22-VacCha group. rP22-Vac group mice were sacrificed 15 days after the third immunization; all other groups were challenged with cercariae after the last immunization and mice sacrificed after 8 weeks of challenge infection. Three immunization experiments were performed independently as well as subsequent analysis of protection, antibody response and cytokine profile.

2.9. Challenge infection and worm burden assessment

Fifteen days after the last immunization, mice were challenged through percutaneous exposure of abdominal skin to water containing 25 cercariae (LE strain) for one hour. Sixty days after challenge, mice were sacrificed and adult worms were perfused from their portal veins [21]. Three independent experiments were performed to determine protection levels. Protection was calculated by comparing the number of worms recovered from each vaccinated group compared with its respective control group using the formula:

$$\%\ \text{Protection} = \left[\frac{(C-I)}{C}\right] \times 100$$

In this formula, *C* represents the worms recovered from the adjuvant control group and *I* represents the worms recovered from the experimental group.

2.10. Immunolocalization of rP22 in S. mansoni adult worms and egg

Adult worms were recovered from perfused mice, and the livers of *S. mansoni*-infected mice were also removed to perform confocal microscopy studies. Parasites and tissues fixed in Paraplast Plus (Sigma, USA) were used in section assays. Slices of 7 μm-thickness were deparaffinized with a xylol series and were blocked with 1% BSA in PBST (Tween 20 0.05%) for 1 hr. Sections were next incubated with anti-rP22 rabbit serum diluted 1:50 in blocking buffer. Serum from a non-immunized rabbit was used as a negative control. Samples were washed three times with PBST and were incubated with anti-rabbit IgG antibodies conjugated to Alexa Fluor 488 (Invitrogen, USA) diluted 1:100 in blocking buffer containing Hoescht dye (Invitrogen, USA) to stain cellular nuclei. The samples were washed three times and were mounted onto the glass slides using Hydromount (National Diagnostics, Atlanta, USA). Parasites were then visualized using a Zeiss 510 Meta confocal microscope with an immersion objective. All parameters and microscope settings used were maintained for all samples.

2.11. rP22 and SWAP specific antibody production

Following immunization, serum samples from seven mice from each experimental group were collected at two-week intervals. A measurement of specific anti-rP22 or anti-SWAP antibodies was performed using an indirect ELISA. Maxisorp 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml of either rP22 or SWAP in carbonate–bicarbonate buffer, of pH 9.6 for 16 h at 4 °C. Plates were washed with PBS 0.05% Tween 20 and were then blocked for 1 h at room temperature with 200 μl/well of PBS–casein (phosphate buffer saline, pH 7.2 with 1.6% casein). Next, 100 μl of each serum sample diluted 1:800 (IgG) or 1:400 (IgG1 and IgG2a) in PBS 0.25% casein was added to each well and was incubated for 1 h at room temperature. Plate-bound antibodies were detected by peroxidase-conjugated anti-mouse IgG, IgG1 and IgG2a (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) diluted in PBS–0.25% casein 1:5,000. The plates were developed by the addition of 100 μl of detection solution (R&D systems, Minneapolis, USA) containing tetramethylbenzidine (Thermo Scientific Pierce) and H_2O_2 in each well; after 20 min, reactions were stopped with the addition of 50 μl of 5% sulfuric acid per well. The plates were read at 450 nm in an ELISA plate reader (ELX 800 BIO-TEK Instruments Inc.).

2.12. Cytokine analysis

Cytokine experiments were performed using splenocyte cultures from individual mice immunized with either rP22 or SWAP plus *C. parvum*/$Al(OH)_3$ ($n = 7$ for each group). Splenocytes were isolated from macerated spleens of individual mice 10 days after the third immunization or at 8 weeks post-infection. Cells were washed twice with sterile PBS and were plated at a concentration of 1×10^6 cells per well in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U/ml of penicillin G sodium, 100 μg/ml of streptomycin sulfate, and 250 ng/ml of amphotericin B. Splenocytes were maintained in culture with medium alone or stimulated with rP22 (25 μg/ml), SWAP (25 μg/ml) or ConA (1.6 μg/ml), as previously described [25]. The 96-well plates (Nunc, Denmark) were maintained in an incubator at 37 °C with 5% CO_2. For cytokine assays polymyxin B (30 μg/ml) was added to the cultures and this treatment completely abrogate the cytokine response to LPS as previously described [26]. Culture supernatants were collected after 24 h of rP22 or SWAP stimulation for TNF-α analysis and after 72 h for IL-10, IL-4, TGF-β and IFN-γ analysis. IL-4, IL-10, IFN-γ, TGF-β and TNF-α concentrations were measured using Duoset ELISA kit (R&D Systems, USA) according to the manufacturer's directions.

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rP22        MDSPMEKFIQTYLTLLRDGDETVETSKLSESCRKEKLDMKQVNEWIALFDVDKDQKITFE  60
Sm21.7      MDSPMEKFIQTYLTLLRDGDETVETSKLSESCRKEKLDMKQVNEWIALFDVDKDQKITFE  60
Sj[I(H)A]   MASTMEQFITAYLTIYRDGDEVVDKKELVDYCRKEKLDMKMVDTWLSHFDVDKDNRITLE  60

rP22        EFCRGLGLKQNEMRIERNHIKTVQSGREQSLPEGVSIIASTMPKPKQVEVTQLFKDIY-N  119
Sm21.7      EFCRGLGLKQNEMRIERNHIKTVQSGREQSLPEGVSIIASTMPKPKQVEVTQLFKDIY-N  119
Sj[I(H)A]   EFCRGLGLKANEMRIERNHIKAVQSGRVPKIPDGVEIIASTMPLPRQVEVTQLYKDLLPK  120

rP22        EVKKDPDMNKVVKTFKSELERRYGRVWQVNAVTHSYWASFSHEPFQSIQFQYDNKIILAW  179
Sm21.7      EVKKDPDMNKVVKTFKSELERRYGRVWQVNAVTHSYWASFSHEPFQSIQFQYDNKIILAW  179
Sj[I(H)A]   SGGHEADMRKVANDFKIKLEERYERVWQVVLLTGSYWMNFSHEPLQSIQFKVDKNIILAW  180

rP22        RTPSN  184
Sm21.7      RTPSN  184
Sj[I(H)A]   RTPNN  185
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Fig. 1. *In silico* analysis of *Schistosoma* EF-hand- and dynein light domain-containing proteins. The full amino acid sequences of rP22, Sm21.7 (XP_002569898) and *S. japonicum* tegument antigen [I(H)A] (CAX72713.1) are aligned to show homologous domains (CDART). Numbers after each row indicate the residue position relative to the amino acid position of each protein. EF-hand motifs are boxed in light grey, dynein domains are boxed in dark grey (Pfam) and provisional calmodulin domains are underlined.

2.13. Analysis of egg count in the liver tissue, granuloma area and fibrosis

Livers from mice (7 mice per group) of control and experimental groups immunized with rP22 or SWAP and infected with 25 cercariae were collected 8 weeks post-infection in order to evaluate the effect of immunization on granuloma formation and determine the number of eggs present in tissue. Livers fragments were fixed with DMSO-methyl alcohol in a proportion of 1:5. Fragments were processed for Paraplast (Sigma, USA) embedding and histopathological sections were cut using a microtome at 5 μm. Sections were stained on a slide with hematoxilin–eosin (HE) or with Masson's trichrome to measure granuloma area and fibrosis, respectively. The areas of individual granulomas were obtained through the MacBiophotonics ImageJ software analyzer. Fifteen granulomas from each mouse with a single well-defined egg were randomly chosen using a microscope with the 10× objective lens; granulomas were then scanned using JVC TK-1270/RGB microcamera. Using a digital pad, the total area of each granuloma measured, and the results were expressed in square micrometers (μm^2). To calculate the area of fibrosis in the granulomas, the pixels of the collagen areas were selected through the real image with subsequent creation of a binary image and obtain the area in μm^2. To count eggs trapped in liver, a sample of the left lateral hepatic lobe of each animal was weighted and subsequently digested in 5% KOH [27]; three 1-ml subsamples of the digestion fluid were counted for each sample. The mean count was used to determine eggs per gram (EPG) in the liver.

2.14. Statistical analysis

Statistical analysis was performed with the ANOVA test using the GraphPad Prism software package. The Bonferroni test was used to compare subgroups.

3. Results

3.1. Cloning and molecular characterization of S. mansoni rP22

The full-length sequence of the *S. mansoni* cDNA encoding rP22 was obtained from an adult worm cDNA library using PCR with specific oligonucleotides. The resulting full-length cDNA displayed an ORF of 555 bp, encoding a protein of 184 amino acids with a predicted molecular mass of approximately 21.7 kDa and an isoelectric point of 6.85. BlastP comparisons of the deduced *S. mansoni* protein sequence in GenBank showed that the best match (*E*-value = 2×10^{-105}) was to *S. mansoni* Sm21.7 antigen; over 184 amino acids, rP22 exhibited complete identity and similarity to Sm21.7 (Fig. 1). The next best match was to a *Schistosoma japonicum* tegument antigen [I(H)A] with 76% identity. This protein most likely represents an rP22 ortholog. Once rP22 was identical Sm21.7 antigen, the signature EF-hand motif sequence present in Sm21.7 was also identified in rP22 (residues 41–66, outlined by a light grey box in Fig. 1) as well as the presence of a dynein-light domain (residues 81–181). In contrast, *S. japonicum* tegument antigen [I(H)A] possesses a multi-domain region with an EF-hand motif inside a provisional calmodulin domain (residues 17–66) and does not possess dynein-light domain. Additionally, as we did not identify a putative signal peptide or transmembrane domain in the rP22 sequence, it was predicted as a soluble protein.

3.2. Production of recombinant rP22

The full-length DNA sequence of rP22 was cloned by recombination into the expression vector pET-DEST42 to produce a protein that contained a C-terminal hexahistidine tag. Protein expression was induced in transformed *E. coli* and resulted in a band at approximately 26 kDa (Fig. 2A, lanes 2–5). Bacteria were then lysed by sonication, and the lysate was separated into soluble and insoluble fractions. The soluble fraction was bound to a nickel-charged column, and after affinity chromatography through an imidazole linear gradient was performed, the eluted fractions containing rP22 were pooled. Protein yield after purification was estimated to be approximately 6.0 mg of rP22/L culture (Fig. 2B, lane 2). For vaccination experiments, the purified protein was dialyzed to remove the imidazole.

3.3. rP22 is a component of the PIII fraction

Western blot assays were performed to determine the presence of rP22 in the protective PIII protein fraction. Immunoblotting of purified rP22 using anti-PIII polyclonal antibodies revealed a band at 26 kDa that corresponds to rP22 and also demonstrating that native P22 is a protein component of the PIII fraction (Fig. 2C, lane 2). Anti-PIII rabbit serum also revealed other proteins from SWAP

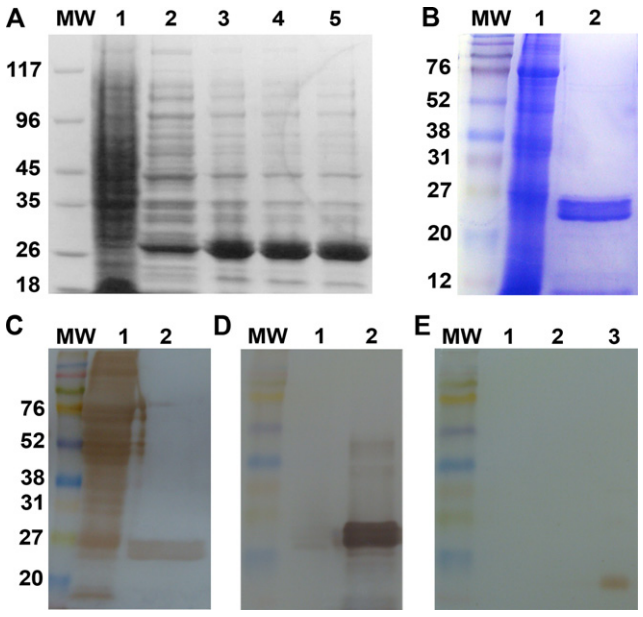

Fig. 2. Expression, purification and identification of rP22 as a component of the PIII fraction. (A) SDS–PAGE of the supernatant of an *E. coli* clone expressing rP22. Lanes: MW, molecular weight marker; 1, negative control (BL21 not transformed); 2–5, induction time (h) following the addition of 0.5 mM of IPTG to the cultures. (B) SDS–PAGE of purified rP22. The culture supernatant was purified through a Ni-column. Lanes: MW, molecular weight marker; 1, soluble worm antigen preparation (SWAP); and 2, sample eluted from rP22 purification. Western blot analysis of SWAP and rP22 probed with anti-PIII serum (C); anti-rP22 serum (D) or anti-P24 serum (E). Two micrograms of SWAP (lane 1), purified rP22 (lane 2) and pellet of *E. coli* expressing rP24 fragment (E-lane 3) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Next, the membranes were probed with serum from a PIII-, rP22- or P24-vaccinated rabbit diluted 1:500, 1:10,000 and 1:500, respectively. Lanes: MW, molecular weight marker; SWAP; 2, sample eluted from rP22 purification; and 3, pellet of *E. coli* expressing rP24 fragment. The molecular weight (kDa) of molecular marker proteins is showed on the left.

(Fig. 2C, lane 1). To determine the capacity of anti-rP22 serum recognize native P22 in the SWAP preparation, rP22 and SWAP were probed with anti-rP22 rabbit serum. A positive reaction was detected for rP22 band (Fig. 2D, lane 2) and for a band at approximately 22 kDa that corresponds to the native P22 present in SWAP preparation (Fig. 2D, lane 1). To demonstrate that P24, and rP22 are not the same protein, an immunoblotting of PIII, rP22 and P24-expressing clone using anti-P24 rabbit serum was performed. No reaction was detected to rP22 (Fig. 2E, lane 2) and a positive reaction was detected to a band at approximately 19 kDa that corresponds to the expressed fragment of recombinant P24 (Fig. 2E, lane 3), demonstrating that rP22 is a PIII-component different from P24. No significant reaction between PIII and anti-P24 serum was detected (Fig. 2E, lane 1).

3.4. rP22 is a tegument protein of S. mansoni

Immunofluorescence staining using rabbit serum raised against rP22 revealed through confocal microscopy that native P22 is located on the surface of *S. mansoni* adult worms (Fig. 3D–N). The tegument-associated antigen P22 appeared to be highly expressed on the surface of both male and female adult worms and it was not found in internal tissues of the parasite. No anti-native P22 antibody staining was observed in sections incubated with pre-immune rabbit serum (Fig. 3A–C).

In the present study, we also demonstrated P22 localization in parasite eggs. Liver sections of *S. mansoni* infected mice were incubated with rabbit serum against rP22. Liver sections containing granulomas show miracidia inside eggshell stained by anti-rP22, as it can be seen by green band running around miracidia surface (Fig. 3T–V), suggesting this antigen to be at the miracidium tegumental layer. No staining was observed in liver granuloma sections incubated with naive rabbit serum (Fig. 3O–R).

3.5. Antibody profile following mice immunization

The specific antibody response to either rP22 or SWAP was evaluated by ELISA. After three rounds of vaccination, significant levels of anti-rP22 IgG and anti-SWAP IgG were detected in the sera of rP22- and SWAP-immunized mice, respectively (Fig. 4A and B). Sera from SWAP vaccinated mice did not present significant antibody levels for rP22. Conversely, sera from rP22-immunized mice presented significant IgG levels for SWAP (Fig. 4B). To determine the IgG isotype profile induced by immunization, specific IgG1 and IgG2a to rP22 or SWAP were also analyzed. Significant levels of rP22-specific IgG1 and SWAP-specific IgG1were detected in the sera of rP22- and SWAP-vaccinated mice, respectively (Fig. 4C and D). Considerable anti-rP22 IgG2a and anti-SWAP IgG2a were also detected in the sera of the mice immunized with rP22 and SWAP, respectively, which remained almost constant after second immunization (Fig. 4E and F). In addition, sera from rP22-immunized mice presented considerable IgG2a levels for SWAP (Fig. 4F). There was no significant production of IgG or isotypes by mice primed with PBS or adjuvant alone.

3.6. Protective effect of rP22 vaccination

To investigate the protective activity induced by vaccination with rP22 plus *C. parvum*/Al(OH)$_3$ in murine model of *S. mansoni* infection, mice were challenged with cercariae. The number of adult worms recovered in the adjuvant control group and in the experimental groups was determined and %protection was calculated (Table 1). Animals immunized with rP22 plus *C. parvum*/Al(OH)$_3$ and challenged with 25 cercariae showed a 51% reduction in adult worm burden compared with the adjuvant control group in three independent experiments (Table 1). The number of eggs in liver was also assessed and rP22 vaccination led a 22.5% reduction in hepatic egg burden compared to adjuvant control group, demonstrating its protective capacity against *S. mansoni* challenge.

3.7. rP22 vaccination reduces hepatic granuloma size and fibrosis

To evaluate the effect of rP22 on reducing granuloma reactions and fibrosis, histological analysis was performed by digital morphometry. Ten days after the third immunization, mice were challenged with 25 cercariae. After 8 weeks of challenge infection, rP22- or SWAP-immunized mice were sacrificed and liver samples were taken for histological analysis. Hematoxilin and eosin stained liver sections were then used to measure the size of individual granulomas. rP22 vaccination reduced liver granuloma area by 60% compared with mice that were immunized with adjuvant alone (Fig. 5A and Table 1). Smaller granulomas were also in mice immunized with SWAP; however these granulomas were not as small as those observed in the rP22 immunized group. Liver sections were then stained by Masson's trichrome to measure fibrosis. Liver from mice immunized with rP22 showed a 71% reduction of fibrosis compared with adjuvant control group (Fig. 5B; Table 1). A significant reduction of fibrosis was also detected in the hepatic tissue of SWAP-vaccinated mice, but not as noteworthy as the fibrosis reduction observed in rP22-immunized group.

3.8. Cytokine profiles following mice immunization

Cytokine experiments were performed using splenocyte cultures from individual mice immunized with rP22 plus *C.*

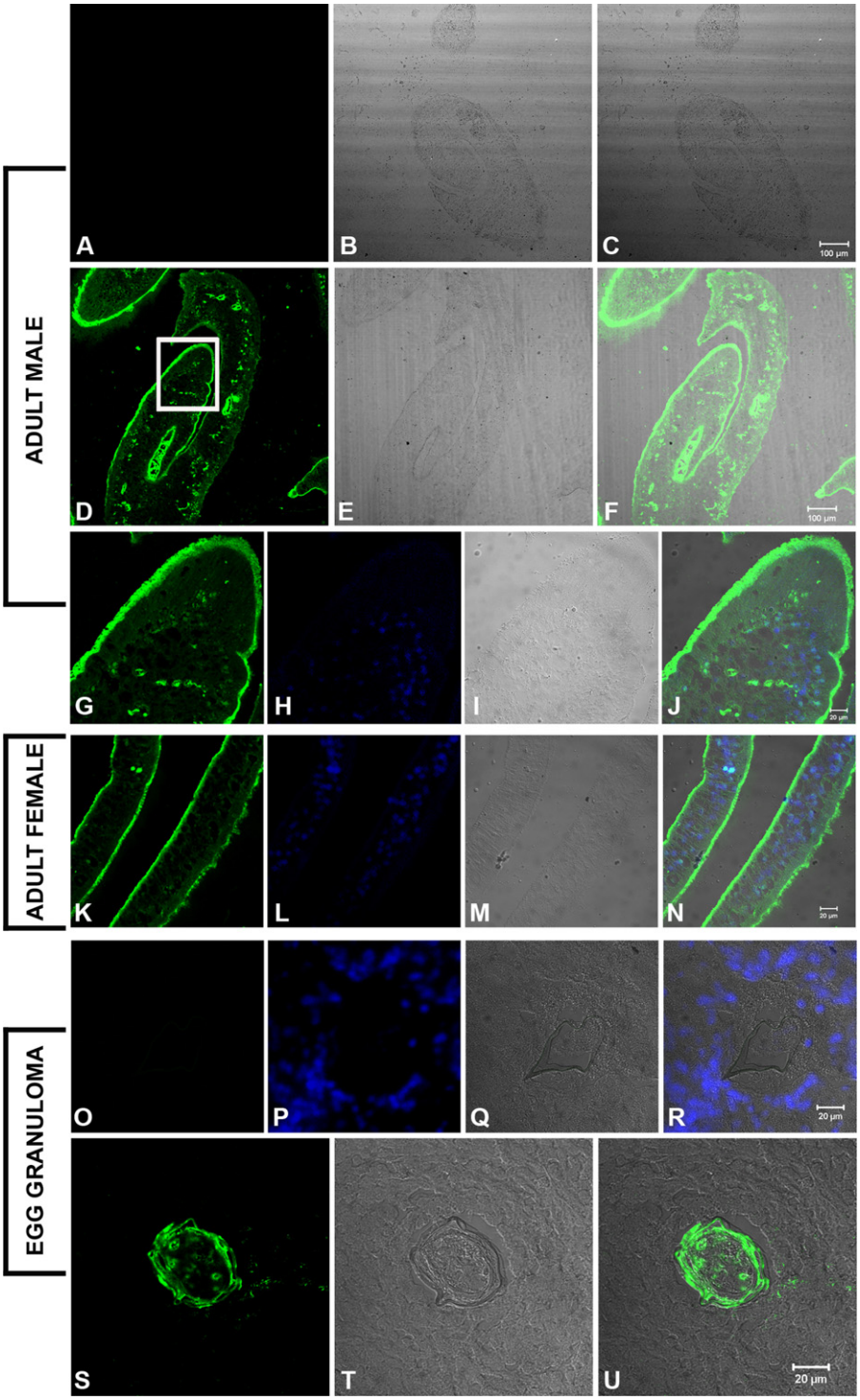

Fig. 3. Immunolocalization of rP22 in the *S. mansoni* tegument. Fluorescence confocal microscopy images and corresponding differential interface contrast (DIC) images of adult male (A–J) and female worms (K–N), and miracidia inside the egg (O–U) of *S. mansoni* are shown. Polyclonal anti-rP22 and secondary goat anti-rabbit antibodies coupled to Alexa 488 (green) were used for fluorescence detection of rP22. Serum from a naive rabbit was used as negative control for adult worm (A–C) and miracidium (O–R). Hoescht (blue) was used for nucleus visualization (H, J, L, N, P and R). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

parvum/$Al(OH)_3$ ($n = 7$ for each group) or from mice immunized with SWAP-adjuvant. Splenocytes from rP22-vaccinated mice were isolated either 15 days after the last vaccination (rP22-Vac) or after 60 days of infection (rP22-VacCha). Splenocytes from SWAP-immunized mice were isolated after 60 days of infection. Production (IFN-γ, TNF-α, IL-4, IL-10 and TGF-β) were measured in the supernatants of spleen cells cultured only in RPMI medium containing fetal bovine serum or in the presence of rP22 or SWAP. High levels of IFN-γ and the proinflammatory cytokine TNF-α were produced by rP22-stimulated splenocytes from the rP22-immunized groups compared with the control or SWAP-stimulated splenocytes (Fig. 6A and B). Additionally, higher levels of the

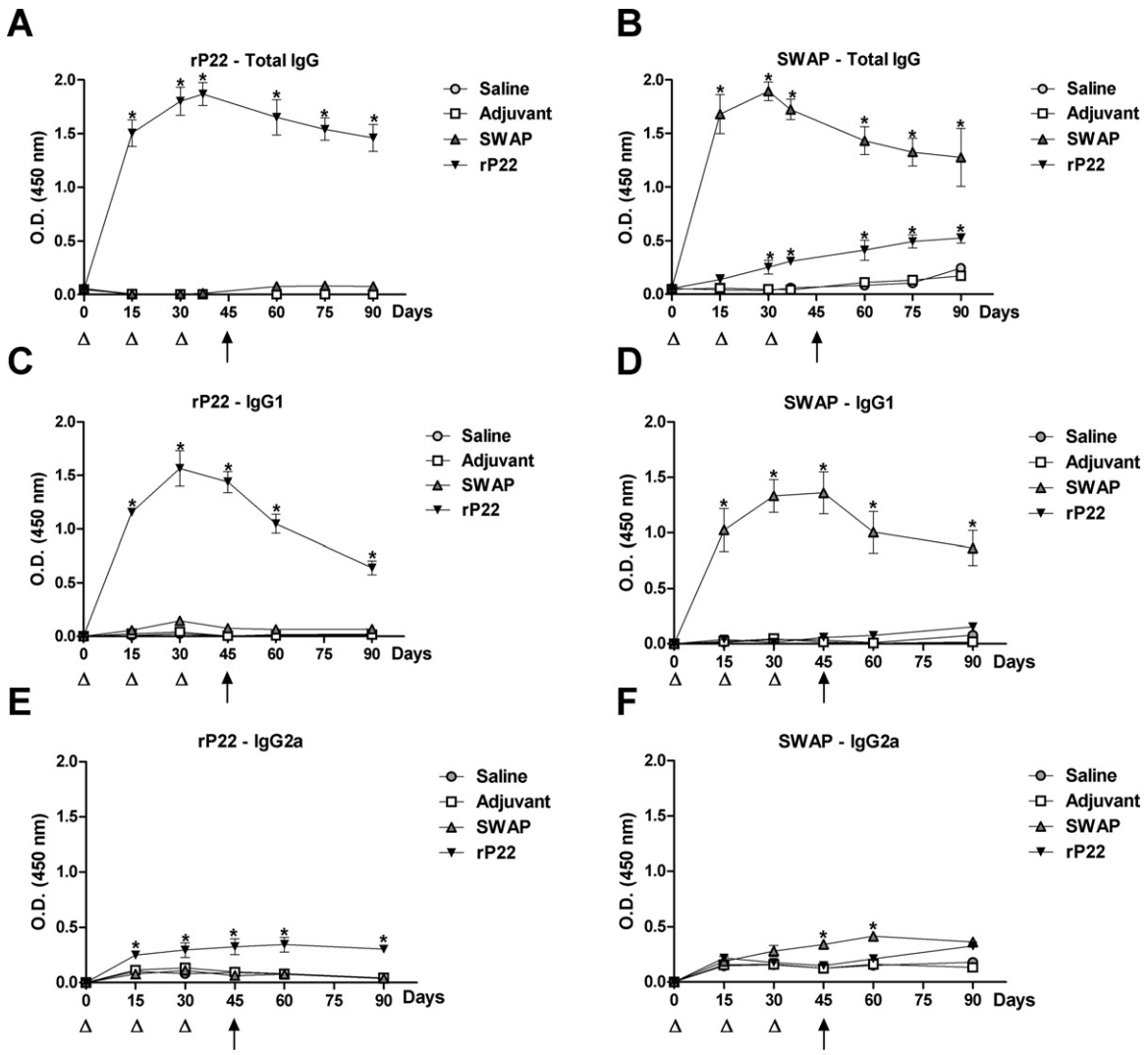

Fig. 4. Specific IgG and isotype responses to rP22 and SWAP. Mice were immunized with either rP22 or SWAP at days 0, 15 and 30; and challenged with normal cercariae at day 45. Triangles and arrows show the times of immunization and infection, respectively. rP22-specific IgG (A), IgG1 (C) and IgG2a (E); and SWAP-specific IgG (B), IgG1 (D) and IgG2a (F) were determined by ELISA. Sera were diluted 1/800 for total IgG and 1/400 for IgG1 and IgG2a. Saline, group that received PBS; adjuvant, group that received *C. parvum*/Al(OH)$_3$ alone; SWAP, mice immunized with SWAP plus adjuvant; and rP22, mice vaccinated with rP22 plus adjuvant. The results are presented as the mean absorbance measured at 450 nm for each group. *Statistically different from saline or adjuvant groups ($p < 0.05$). Data are representative of three independent experiments with similar results ($n = 7$).

immunomodulatory cytokine IL-10 and lower secretion of IL-4 were also produced by rP22-stimulated splenocytes compared to SWAP-stimulated splenocytes (Fig. 6C and D). High levels of IFN-γ, IL-4 and the IL-10 were produced by SWAP-stimulated splenocytes from the SWAP-immunized groups compared with the control. rP22-stimulated splenocytes from SWAP-vaccinated mice produced higher levels of IFN-γ and TNF-α combined with lower levels of IL-4 when compared with SWAP-stimulated splenocytes from the same group. As seen in the rP22-VacChan group, significant IFN-γ production was detected after infection of rP22-vaccinated mice compared to rP22-Vac. IFN-γ, TNF-α and IL-10 levels remained high in the rP22-VanChan group, while IL-4 levels remained low compared to rP22-Vac group. No significant difference on TGF-β production was detected between rP22 and SWAP-stimulated splenocytes of all groups (data not shown). These results indicate that the immunization of mice with rP22 associated with *C.*

Table 1
Protective effect and liver granuloma size induced by C57BL/6 mice vaccination with rP22 and challenged with 25 *S. mansoni* cercariae.

Groups	Total worms Mean ± SD	Number of eggs per gram liver tissue Mean ± SD	Hepatic granuloma area (μm^2) Mean ± SD	Fibrosis area (μm^2) Mean ± SD
Adjuvant	10.8 ± 1.6	5783 ± 800.5	152,550 ± 15,444	151,780 ± 16,267
SWAP	6.8 ± 1.6* (37%)†	5299 ± 869* (8.4%)†	109,304 ± 15,258* (28.3%)†	100,960 ± 39,611* (33.5%)†
rP22-VacCha	5.3 ± 1.8* (51%)†	4481 ± 857.2* (22.5%)†	61,082 ± 16,556*,# (60%)†	44,157 ± 11,482*,# (71.0%)†

† Worm reduction rate (%); egg reduction rate (%) in liver tissue; granuloma or fibrosis area reduction rate (%).
* Statistically significant compared with the adjuvant control group ($p < 0.05$).
# Statistically significant compared with SWAP group ($p < 0.05$).

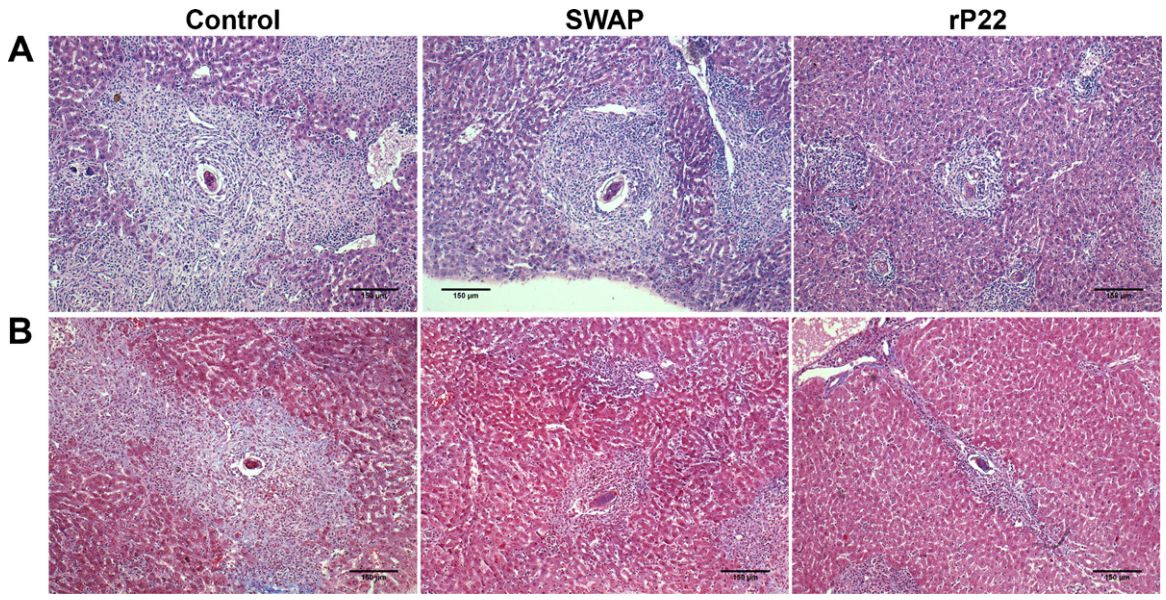

Fig. 5. Photomicrographs of hepatic granulomas in C57BL/6 mice 8 weeks after challenge infection. Histological sections were stained with hematoxylin–eosin (A) or Masson's trichrome (B) to show granuloma size and fibrosis, respectively, from mice groups immunized with adjuvant alone (control), SWAP + adjuvant, or rP22 + adjuvant.

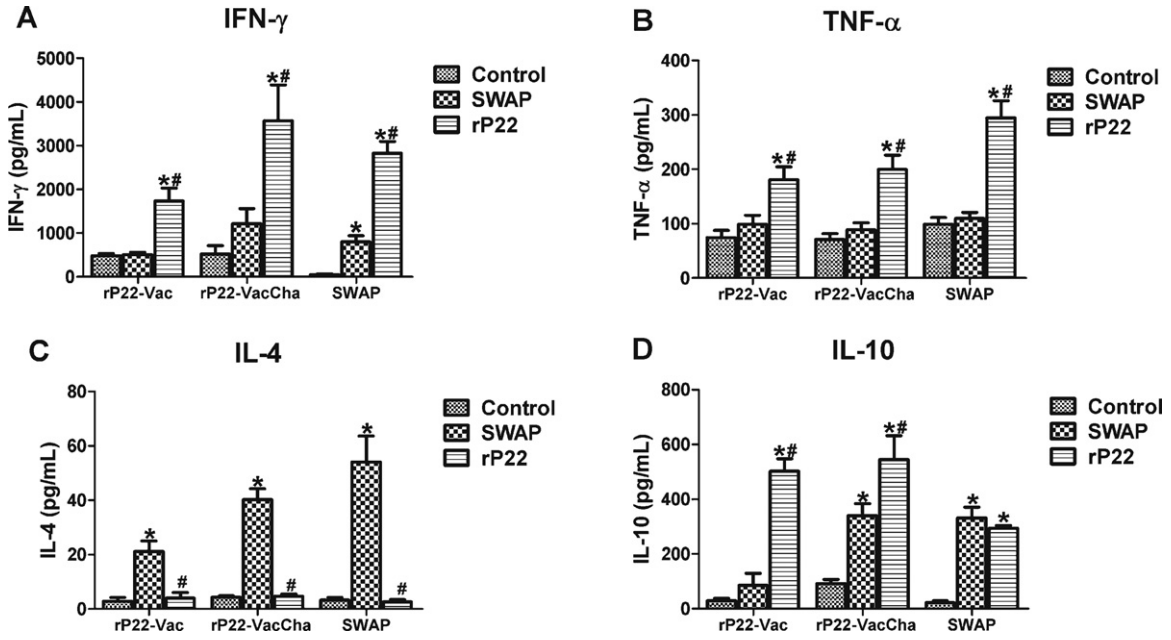

Fig. 6. Cytokine profiles of mice immunized with rP22 or SWAP. Splenocytes isolated from mice of rP22-Vac group (mice vaccinated with rP22), rP22-VacCha group (mice vaccinated with rP22 and subsequently challenged) or SWAP group (mice vaccinated with SWAP and subsequently challenged) were assayed for IFN-((A), TNF-((B), IL-4 (C) and IL-10 (D) production in response to *in vitro* stimulation with SWAP (25 μg/ml), rP22 (25 μg/ml) or medium alone as control. Results represent the mean ± SD of each group. *Statistically significant differences between cytokines produced after SWAP or rP22 stimulation compared with unstimulated splenocytes (control) ($p < 0.05$). #Statistically significant differences between rP22-stimulated splenocytes compared with SWAP-stimulated splenocytes ($p < 0.05$). Data are representative of three independent experiments with similar results ($n = 7$).

parvum/$Al(OH)_3$ adjuvants induced an immune response with persistent and increased levels of IFN-γ, TNF-α and IL-10 combined with reduced levels of IL-4.

4. Discussion

Schistosomiasis is a chronic debilitating parasitic disease that represents a major health problem in endemic areas, such as various parts of South America, Africa, and Southeast Asia [28,29]. Since current schistosomiasis control programs applied to reduce morbidity and mortality have been proven inadequate and many commonly employed measures have been ineffective, there is a consensus that the development of an appropriate protective vaccine would be a crucial part of the overall integrated control strategy.

Innumerous antigenic preparations and purified native and recombinant antigens from *S. mansoni* have been assayed for their capacity to elicit protection against schistosomiasis. Additionally, the recent publication of both the *S. mansoni* [30] and *S. japonicum* [31] genomes has brought to the scientific community an enormous amount of data to be mined in the search for new vaccine candidates. In this context, our laboratory has identified a protective protein fraction from adult worms, named PIII, which reduced worm burden and decreased granuloma size in a murine immunization model [18,20]. To identify PIII components and to

determine their role in the protective properties of this fraction, a cDNA library was constructed, and clones were screened using anti-PIII serum. Herein, we identified rP22 protein as a component of the protective PIII fraction and characterized the immune response induced following rP22 vaccination.

In this report, we determined that the rP22 amino acid sequence is identical to Sm21.7 antigen. Sm21.7 contains one EF-hand motif and a dynein light chain domain at amino acid positions 41–66 and 81–181, respectively. The EF-hand motif is present in a group of important parasite antigens, such as Sm22.6 and Sm21.6 [32,33]. These surface proteins were recognized by antibodies from protected mice and by serum from *S. mansoni* patients that are resistant to reinfection. Dynein light chain domain-containing proteins, such as rP22, are likely components of the cytoskeleton and are involved in the motility of vesicles and organelles along microtubules; they may also provide a structural scaffold for parasite tegument. rP22 showed 76% of identity with the *S. japonicum* tegument antigen [I(H)A].

Additionally, we confirmed by confocal fluorescence microscopy that rP22 is located on the tegument of *S. mansoni* adult worms and miracidia. This surface antigen was highly expressed on the tegument and absent in the internal tissues of the parasite body. It is interesting to notice that rP22 is a tegument antigen, and not a secreted or transmembrane protein, because neither a peptide signal nor a transmembrane domain was detected by bioinformatic approaches. In accordance with our confocal findings, Braschi et al. examined a great number of dynein-related proteins present in tegument extracts by proteomic analysis and concluded that the layer immediately beneath the plasma membrane is largely composed of dynein homologues which form a macromolecular complex [34].

Tegument proteins, such as rP22, are important sources of parasite antigens for the development of a schistosome vaccine, mainly because tegument is a dynamic host-interactive layer that is involved in innumerous parasite functions such as nutrition, excretion, osmoregulation, sensory reception, signal transduction and immune evasion and modulation [35,36]. Recently, publications have shown that some proteins located in the tegument, such as TSP-2 [27] and Sm-p80 [37], are able to induce high levels of protection against *S. mansoni* infection in the murine model. To determine if rP22 conferred protection against *S. mansoni* infection, immunized mice were challenged with cercariae and worm burdens were assessed. Immunization with rP22 induced a 51% reduction in worm burden. Although rP22 is a component of PIII, as confirmed by western blot analysis with anti-PIII serum, comparative analysis found that rP22 immunization was more efficient in reducing worm burden compared to vaccination with the entire PIII fraction. Another component of PIII fraction, a 24 kDa protein (P24) also induced high levels of protection against *S. mansoni* challenge [16]. Sera produced against P24 did not recognize rP22 band in western blot assay and confirmed that rP22 and P24 were not the same antigen but different PIII components.

Protective immunity against schistosomiasis involves both humoral and cellular immune responses [13]. In this report, rP22-immunized mice produced significant levels of anti-rP22 IgG, IgG1 and IgG2a after the first immunization. Additionally, sera from rP22 vaccinated mice presented significant antibody levels for SWAP, suggesting that rP22-specific antibodies were probably recognizing native P22 from SWAP. The dynamic IgG and isotype response patterns to SWAP were similar to the antibody profile elicited by rP22 immunization. Conversely, little antibody response to rP22 was detected in the sera from SWAP-immunized mice. These findings are opposed to the results observed in western blot analysis which confirmed a positive reaction for rP22 probed with anti-PIII rabbit serum, suggesting that range of native P22 concentration in the constitution of SWAP and PIII could explain the differences in rP22 recognition pattern.

There is lack of consensus regarding the desired type of immune response that should be elicited against *S. mansoni*. In the irradiated cercariae vaccination model, protection can be mediated through a Th1, a Th2, or a mixed Th1/Th2 immune response [38]. The involvement of IFN-γ in protective immunity to schistosomiasis is well described in mice exposed to the irradiated cercariae vaccine [39]. The treatment of vaccinated mice with monoclonal anti-IFN-γ antibodies totally abrogated the achieved protective immunity [11]. The use of IFN-γ knockout mice showed similar results and confirmed the essential role of IFN-γ in protective immunity against murine schistosomiasis [12]. We also demonstrated increased IFN-γ production in rP22-vaccinated mice that were challenged with *S. mansoni*, suggesting that the protective effect of rP22 vaccination could be related to the maintenance of high levels of IFN-γ.

In the murine model of *S. mansoni* infection, the $CD4^+$ Th cell response evolves from a Th1- to a Th2-dominated response following egg production by adult worms [40,41]. During chronic infection, the Th2-dominated response is downregulated at the same time as there is a slight increase in the Th1 response. This phenomenon marks the transition into the chronic stages of the infection, which is associated with the downmodulation of granuloma size and results in smaller, newly formed, periovular granulomas and reduced cytokine expression by $CD4^+$ T cells [42,43]. Herein, we demonstrated that rP22-vaccinated mice that were challenged with *S. mansoni* produced high levels of IFN-γ after immunization and during transition to chronic stage. The maintaining high levels of IFN-γ may have contributed to balance the Th2-dominated immune response during chronic stage of *S. mansoni*, since a significant decrease of fibrosis and granuloma size were detected in rP22-immunized mice.

In regards to new antigens in the schistosomiasis vaccine research field, the anti-morbidity effect is an important requirement to be evaluated [7]. The granulomatous reactions that occur around parasite eggs represent the major pathology associated with schistosomiasis; further, the intensity of these reactions correlates with host morbidity [17,44,45]. In murine schistosomiasis, pathology is induced by a $CD4^+$ Th2 driven granulomatous response directed against schistosome eggs lodged in the host liver. The Th2 cytokines IL-4 and IL-13 drive this response, whereas IL-10, IL13Rα2, IFN-γ and inducible regulatory T-cells act to limit schistosome induced pathology [46–49]. In our study, histopathological analysis revealed a significant reduction in granuloma size and fibrosis in the livers of mice vaccinated with rP22. This immunomodulatory effect might be associated in part to the maintaining high levels of IL-10 also observed for rP22-immunized mice. Similar results were described for PIII immunization, showing that native P22 could play an important role in the granuloma downmodulation promoted by PIII immunization.

Previous work has also demonstrated that PIII and P24, a PIII component, were able to reduce granulomatous reactions *in vitro* and *in vivo* after cercarial challenge of immunized mice [16,19]. Our results suggest that rP22 anti-morbidity effects might be correlated with the capacity of rP22 to elicit both IFN-γ and IL-10 production. We suggest that balance between IFN-γ and IL-10 may be crucial for the protective and immunomodulating properties of rP22. Thus, we believe that the combination of rP22 with multiple target antigens could induce effective protection against the *S. mansoni*, leading the development of an appropriate antischistosomiasis vaccine.

Conflict of interests

The authors declare that they have no competing interests.

Acknowledgements

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