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The *Schistosoma mansoni* cyclophilin A epitope 107-121 induces a protective immune response against schistosomiasis

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ABSTRACT

Great efforts have been made to identify promising antigens and vaccine formulations against schistosomiasis. Among the previously described *Schistosoma* vaccine candidates, cyclophilins comprise an interesting antigen that could be used for vaccine formulations. Cyclophilin A is the target for the cyclosporine A, a drug with schistosomicide activity, and its orthologue from *Schistosoma japonicum* induces a protective immune response in mice. Although *Schistosoma mansoni* cyclophilin A also represents a promising target for anti-schistosome vaccines, its potential to induce protection has not been evaluated. In this study, we characterized the cyclophilin A (SmCyp), initially described as Smp17.7, analyzed its allergenic potential using *in vitro* functional assays, and evaluated its ability to induce protection in mice when administered as an antigen using different vaccine formulations and strategies. Results indicated that SmCyp could be successfully expressed by mammalian cells and bacteria. The recombinant protein did not promote IgE-reporter system activation *in vitro*, demonstrating its probable safety for use in vaccine formulations. T and B-cell epitopes were predicted in the SmCyp sequence, with two of them located within the active isomerase site. The most immunogenic antigen, SmCyp (107–121), was then used for immunization protocols. Immunization with the SmCyp gene or protein failed to reduce parasite burden but induced an immune response that modulated the granuloma area. In contrast, immunization with the synthetic peptide SmCyp (107–121) significantly reduced worm burden (48–50%) in comparison to control group, but did not regulate liver pathology. Moreover, the protection observed in mice immunized with the synthetic peptide was associated with the significant production of antibodies against the SmCyp (107–121) epitope. Therefore, in this study, we identified an epitope within the SmCyp sequence that induces a protective immune response against the parasite, thus representing a promising antigen that could be used for vaccine formulation against schistosomiasis.

1. Introduction

Schistosomiasis, a debilitating disease caused by flatworm parasites of the *Schistosoma* genus, affects more than 200 million individuals worldwide and causes 1.9 million DALYs (disability-adjusted life years) globally (GBD 2016 DALYs and HALE Collaborators, 2017GBD 2016 DALYs and HALE Collaborators, 2017; WHO, 2018). Due to the inadequacy of the current control strategy, which is based on chemotherapy with praziquantel (PZQ) to limit disease transmission, vaccination represents a promising alternative. In fact, a recent study based

Abbreviations: ADCC, antibody-dependent cell mediated cytotoxicity; CsA, cyclosporin A; Cyps, cyclophilins; GST, glutathione S-transferase; NFAT, nuclear factor of activated T-cells; PZQ, praziquantel; ROC curve, receiver operating characteristic curve; SmCyp, *Schistosoma mansoni* cyclophilin A

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on a mathematical model demonstrated that even a vaccine resulting in partial protection could interrupt transmission within approximately 18 years (Stylianou et al., 2017).

In searching for antigens that could be used in vaccine formulations, the cyclophilins have arisen as candidates. Cyclophilins (Cyps) or peptidyl-prolyl cis-trans isomerases are highly conserved ubiquitous proteins that carry out a wide range of functions including protein folding, trafficking and assembly (Fischer et al., 1989; Schönbrunner et al., 1991; Trandinh et al., 1992; Göthel and Marahiel, 1999).

Cyps are present in different compartments of the cell such as the cytosol, mitochondria, nucleus, or extracellular matrix in many different isoforms (Handschumacher et al., 1984; Rassow et al., 1995; Hoffmann and Schiene-Fischer, 2014; Rajiv and Davis, 2018). In *Schistosoma mansoni*, Kiang et al. (1996) showed that cyclophilin is located in the tegument, muscle layers, intestinal epithelium, and interior of the worm, therefore, it is in contact with the host immune system. Unlike the majority of species in which cyclophilin A is the major isoform, *S. mansoni* cyclophilin A is less abundant than the cyclophilin B isoform (Bugli et al., 1998).

In general, cyclophilins are specific protein targets for the immunosuppressive drug cyclosporin A (CsA). This drug can facilitate the complete elimination of a wide range of parasites including schistosomes, even if administered only once during experimental infection (Bueding et al., 1981; Chappell et al., 1987;Chappell and Thomson, 1988). In *S. mansoni*, the cyclosporin/cyclophilin complex inhibits translocation of the parasite nuclear factor protein, homolog to the human NFAT (nuclear factor of activated T-cells) to the nucleus, thereby inhibiting parasite development (Serra et al., 1999). Despite its role in parasite development and its accessibility to the host immune system, *S. mansoni* cyclophilin has never been tested in vaccine formulations against schistosomiasis.

However, Han et al. (2012) demonstrated that *Schistosoma japonicum* cyclophilin A induces a protective immune response in immunized mice (Han et al., 2012). Further, the immunization of mice with a recombinant form of *S. japonicum* cyclophilin A provided further support for the importance of this protein in parasite development, since the immune response elicited by rSjCypA immunization in mice was found to impair *S. japonicum* development (Han et al., 2012).

Although *S. mansoni* cyclophilin A (SmCyp) is thought to be a potential target for anti-schistosome vaccines, its ability to induce protection is not clear. Here, we characterized this antigen, analyzed its allergenic potential based on an *in vitro* functional assay, and evaluated its ability to induce protection in mice when used as an antigen with different vaccine formulations and strategies. The safety of SmCyp was confirmed by its inability to promote IgE reporter assay activation. Immunization with DNA or recombinant protein failed to induce protection in mice, although these formulations regulated liver granuloma area in immunized mice. However, an immunogenic epitope identified in the SmCyp sequence, namely SmCyp (107–121), induced a significant reduction in worm burden (48–50%), which was associated with the significant production of antibodies against this epitope. Our results indicated that SmCyp (107–121) is a promising vaccine candidate.

2. Materials and methods

2.1. In silico characterization of SmCyp

The *S. mansoni* cyclophilin A (access number: XP_018652799.1; Smp_040130.1) amino acid sequence was analyzed using SOSUI Engine version 1.11 (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html) to predict transmembrane domains and solubility. EULOC (http://euloc.mbc.nctu.edu.tw/output.php), CELLO2 (http://cello.life.nctu.edu.tw/) and PSORT II (https://psort.hgc.jp/form2.html) was used to predict cellular localization. SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptides in the protein sequence. The molecular weight and isoelectric point of SmCyp was predicted using the compute pi/MW tool (https://web.expasy.org/cgi-bin/capute_pi/pi_tool). To assess the similarity between human or helminthic proteins, the amino acid sequences were compared with the National Center for Biotechnology Information protein database using the BlastP (BLAST - Basic Local Alignment Search Tool) algorithm (blast.ncbi.nlm.nih.gov/Blast.cgi), with the default parameters of this algorithm.

T cell and B cell epitope prediction was performed using Rankpep (http://imed.med.ucm.es/Tools/rankpep.html) and BCPred (http://ailab.ist.psu.edu/bcpred/), respectively. In Rankpep, T cell epitope prediction was performed using default parameters selecting IaB to predict C57BL-6 T cell epitopes in the protein sequence. While for BCPred, B cell epitope predictions were performed using Flexible Length Prediction (FLP) and 75% specificity. Potential SmCyp allergenicity was searched using the Allergen Online database (http://www.allergenonline.org/databasefasta.shtml) and full-length Fasta analysis.

2.2. Model building and sequence alignment

The amino acid sequence of *S. mansoni* SmCyp was retrieved from the NCBI database (Accession ID XP_018652799.1) and submitted to build a three-dimensional homology model by automated comparative modeling using Protein Homology/analogY Recognition Engine V 2.0 (Phyre^2) using Intensive Modelling Mode (Kelley et al., 2015). The resulting structure was predicted with 100% confidence with 96% sequence coverage (161 residues), or > 90% confidence for 100% coverage. The resulting predicted image was generated using UCSF Chimera (v 1.13.1) Modeling software (Pettersen et al., 2004)

Multiple amino acid sequence alignment of *S. mansoni* SmCyp (Accession ID XP_018652799.1), and *S. mansoni* cyclophilin A (Accession ID AAC47317.1) or *S. mansoni* SmCyp (Accession ID XP_018652799.1), *Mus musculus*, peptidyl-prolyl cis-trans isomerase (Accession NP_032933.1), and *Homo sapiens,* peptidyl-prolyl isomerase A (Accession AAH05982.1) was performed using Clustal Omega (Sievers and Higgins, 2018) and EMBOSS Water was used for amino acid pairwise sequence alignment between *S. mansoni* SmCyp and *P. falciparum* chain A, peptidyl-prolyl cis-trans isomerase.

2.3. Mice and parasites

All procedures performed using mice were approved by the FIOCRUZ's Ethics Commission in animal use (CEUA) under the license numbers LW26/12. C57BL/6 mice (6–8-weeks-old) were purchased from the Instituto René Rachou (IRR) facility. The parasite life cycle (LE strain) was routinely maintained by passage through *Biomphalaria glabrata* snails and hamsters (Lombardo et al., 2019). Cercariae shedding was induced by exposing infected snails to light for 1–2 h and were provided by the Lobato Paraense snail facility from IRR.

2.4. SmCyp recombinant protein expression

The *SmCyp* synthetic gene containing the coding region sequence of SmCyp (access number:XP_002575376.1) was designed using ApE software (www.apesoftware.com). The mini-gene was designed with restriction sites for BamHI (GGATCC) at the 5′ end and XhoI (CTCGAG) followed by AgeI (ACCGGT) at the 3′ end. The gene encoding SmCyp was synthesized and cloned into the pIDT Smart cloning vector by IDT Technologies and subsequently subcloned into the bacterial expression vector pET28a TEV (Carneiro et al., 2006) using the BamHI and XhoI restriction sites and into the mammalian expression vector pcDNA 3.1 V5/His A (Invitrogen) using BamHI and AgeI restriction sites. The constructs were used to transform *Escherichia coli* BL21(DE3plys) and DH5α strains, respectively, and clones containing inserts were selected. The presence of the gene and its correct insertion into the plasmid were confirmed by sequencing. The expression of the 6× histidine-tag fusion

protein in *E.coli* were induced with 1 mM isopropyl-B-D-galactopyranoside for 3 h at 37 °C with shaking at 250 rpm using BL21/PET28a TEV/*SmCyp* clones. The bacteria were lysed in a buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 40 mM imidazole) containing 1 μg/ml of lysozyme and 250 U of Benzonase Nuclease (Qiagen) for 30 min on ice. After centrifugation (14,000 x g, 30 min), the supernatant was used for the purification of recombinant protein, under native conditions, by affinity chromatography using the QIAexpress® Ni-NTA Fast Start Kit (Qiagen) according to the manufacturer's instructions. The pcDNA 3.1 V5/His A and pcDNA 3.1 V5/His A/*Smcyp* plasmid DNA purification was performed using EndoFree Plasmid Giga kit (Qiagen), according to manufacturer's instructions. To confirm the production of rSmCyp in mammalian cells, the construct pcDNA 3.1 V5/His A/*SmCyp* was transfected into HEK293 T cells using Lipofectamine 2000 (Invitrogen). The plasmid pcDNA 3.1 V5/His B, containing a non-related gene with a C-terminal 6× His-tag was used as the positive control, and the empty plasmid pcDNA3.1/V5-His A and non-transfected cells were used as negative controls. In each well from a 24-well culture plate, 2.0×10^5 cells were seeded in 500 μL of DMEM with 10% FBS. The plate was maintained at 37 °C and 5% CO_2 for 24 h. Each sample of plasmid DNA (0.8 μg) and Lipofectamine 2000 (2 μL) was diluted separately in 50 μL of DMEM, and after a 5-min incubation at room temperature, the diluted Lipofectamine 2000 was mixed with each DNA sample. This mixture was incubated for 20 min at room temperature, facilitating the formation of DNA–liposome complexes. Each mixture was added to the cells, which were maintained for approximately 5 h at 37 °C in 5% CO_2 and then washed in DMEM containing 10% FBS. After 48 h, transfected cultures were lysed for total protein extraction in 150 μL of 2× Laemmli buffer (BioRad) with 5% β-mercaptoethanol. The samples were then boiled at 100 °C for 5 min and stored at −20 °C until use. The quality of the recombinant proteins obtained using bacteria and mammalian cells was analyzed by SDS-Page and Western blotting using an anti-6×his tag monoclonal antibody (Ge Healthcare Life Sciences). Briefly, protein samples were separated by SDS-PAGE (15%) and electrotransferred onto nitrocellulose blotting membranes (Amersham™ protran) at 300 mA for 2 h on ice. Then membranes were blocked overnight with 5% skimmed milk powder in TBS (20 mM tris, 500 mM NaCl, pH7.5). To detect the fusion protein, the nitrocellulose membrane was probed with a mouse monoclonal anti-6×his tag antibody (1:3000; GE Healthcare Life Sciences) as the primary antibody, and subsequently incubated with HRP-conjugated goat anti-mouse IgG antibody (1:5000; Southern Biotech) as the secondary antibody. Immunoreactivity was detected with ECL Plus Western Blotting Detection System (GE Healthcare life sciences) and images were captured using ImageQuant LAS 4000 (GE Healthcare life sciences) by chemiluminescent method.

2.5. IgE Reporter system activation in vitro

The ability of rSmCyp to promote IgE-mediated basophil activation degranulation was assessed using the humanized rat basophilic leukemia cell line (RS-ATL8 and NFAT-DsRed reporters) as previously described (Wan et al., 2014b) with some modifications. Briefly, 1×10^5 cells/well were seeded in a white 96-well flat plate (RS-ATL-8 reporter) or a clear 96-well flat plate (NFAT-DsRed reporter); then, 50 μL of serum from individuals infected with *Schistosoma mansoni* (diluted 1:50) was added to each well. Then, cells were incubated for 16 h in a 37 °C/5% CO_2 incubator. Recombinant SmCyp was used to stimulate sensitized cells at three different concentrations (0.1, 0.001, and 0.0001 μg/mL). Unstimulated sensitized cells were used as a negative control and anti-human IgE antibody stimulation (1 μg/mL, goat-anti-human polyclonal, Merck) was used as a positive control. After 4 h of incubation with stimuli at 37 °C/5% CO_2, 50 μL of ONE-Glo Luciferase substrate (Promega) was added to RS-ATL8 cells and luminescence was measured using Tecan Spark 10 M multimode microplate reader. Each condition was analyzed in triplicates. Fluorescence was observed in NFAT-DsRed cells 24 h after stimulation using a fluorescence microscope (Evos FL Digital Inverted Fluorescence Microscope) at 10× magnification, 530 nm excitation, and 593 nm emission. Receiver operating characteristic curves (ROC curves) were plotted to calculate the cutoff point, using the negative control (unstimulated cells) and the positive control (cells stimulated with anti-human IgE antibody). Samples of schistosomiasis patients were kindly provided by Colin Fitzsimmons and David Dunne. Samples used in this study are from a subgroup of participants of a study performed in Piida Village in Uganda described in Naus et al., 2003. Sixteen serum samples from individuals living in Piida Vilage (4 female/12 male), selected based on their high IgE antibodies levels against SmTal-1 or Swap, was used in this assay. Additionally, among the 16 sera analyzed, a pool of sera from individuals with the highest levels of IgE against SmTal-1 and another pool of sera from individuals with moderate levels of IgE against SmTal-1 were evaluated and treated as additional samples. The median epg determined by Kato-Katz in feces samples from these 16 individuals were 646.7 epg.

2.6. Immunization of mice

Female C57BL/6 mice (10 mice per group) were used for immunization protocols. For DNA vaccination, mice received four doses of 100 μg of purified DNA (pcDNA 3.1 V5/His A or pcDNA 3.1 V5/His A/*SmCyp*) in the quadriceps muscle, with an interval of 15 days between each dose. Five days before the first immunization, mice received 50 μL of cardiotoxin (10 μM)/quadriceps muscle. For rSmCyp, SmCyp (94–108) and SmCyp (107–121) peptide mixture, or SmCyp (107–121) immunization, mice were subcutaneously immunized with 25 μg of protein or 10 μg of synthetic peptides three times with a 15-day interval between each dose. Complete Freund's adjuvant was used in the first immunization dose and incomplete Freund's adjuvant was used for the following boosts. Physiological saline plus Freund's adjuvant was used for control groups. Fifteen days after the last boost, mice were challenged through percutaneous exposure of the abdominal skin for 1 h to water containing 100 cercariae (LE strain) as described by Smithers and Terry (1965). Fifty days after challenge, adult worms were recovered by perfusion of the portal system and mesenteric veins according to Pellegrino and Siqueira (1956). The intestine and liver from each mouse were also collected 50 days post-infection. These organs were weighed and digested with 10% KOH for 16 h at 4 °C and for 2 h at 37 °C. The eggs were obtained by centrifugation at 900 × *g* for 10 min and resuspended in 1 mL of saline. Egg numbers were counted using a light microscope. Blood samples were collected from each mouse with an interval of 15 days beginning 15 days after the first immunization. Serum samples were collected and stored at −20 °C until use. Two independent experiments were performed.

2.7. Measurement of hepatic granuloma area

Liver sections of animals from control (pcDNA 3.1 V5/His A and Freund) and experimental groups (pcDNA 3.1 V5/His A/*SmCyp*, rSmcyp, and SmCyp (107–121) were collected 50 days post-infection to evaluate the effect of immunization on granuloma formation. The liver sections removed from the central part of the left lateral lobe were fixed in 10% buffered formaldehyde in PBS. Histological sections were obtained using a microtome and the slides were stained with hematoxylin and eosin (H&E). To perform area measurements, 100 granulomas from each group with a single well-defined egg at the exudative-productive stage were randomly selected and analyzed with a 10× objective lens using a JVC TK-1270/RGB microcamera. Using a digital pad, the total area of each granuloma was measured and the results were expressed as square micrometers (μm^2).

2.8. Measurement of specific antibodies levels

The measurement of specific anti-SmCyp, anti-SmCyp (94–108), or

anti-SmCyp (107–121) IgG, IgG1 and IgG2c antibodies levels were performed by ELISA. MaxiSorp 96-well microtiter plates (Nunc) were coated with 5 µg/mL (rSmCyp) or 1 µg/mL (peptides) of antigens in carbonate-bicarbonate buffer, pH 9.6, for 16 h at 4 °C. The plates were then blocked for 2 h at 4 °C with 300 µL/well of PBST (phosphate-buffered saline, pH 7.2 with 0.05% tween-20) plus 10% FBS (fetal bovine serum; GIBCO, USA). One hundred microliters of each serum sample diluted 1:50 in PBST was added per well and incubated for 1 h at room temperature. Plate-bound antibody was detected using 100 µL/well of a peroxidase-conjugated anti-mouse IgG (1:10,000), IgG1 (1:2000) or IgG2c (1:4000) (Southern Biotech, USA). Color reactions were developed by adding 100 µL of TMB (Microwell Peroxidase Substrate System) per well and the reaction was stopped with 50 µL of 5% sulfuric acid per well. Absorbance was measured at 450 nm using an ELISA microplate reader.

2.9. Statistical analysis

GraphPad Prism 7.0 (Graph-Pad Software, San Diego, CA, USA) was used to perform statistical analyses. Normal distribution was tested using the Shapiro-Wilk normality test. ELISA analysis was performed using two-way ANOVA and Tukey's multiple comparison test. Parasitological and histological analyses were performed using a Student's *t*-test ROC curves were used to calculate the cutoff point, which was used to determine basophil activation of RS-ATL8 RBL cells stimulated with rSmCyp. Cutoff point determinations were based on the relative unit values of luciferase activity observed in the negative (RS-ATL-8 cells RBL + serum) and positive (RS-ATL-8 RBL cells + serum + anti-IgE) controls.

3. Results

3.1. Generation of recombinant SmCyp

The *SmCyp* gene was inserted into a mammalian (pcDNA3.1V5/HIS A) and bacteria (pET28a TEV) expression vector as described in the methods section. Before starting the DNA immunization protocol, we evaluated *SmCyp* expression in mammalian cell cultures. Western blotting analysis of proteins extracts of HEK293 T transiently-transfected cells, using an antibody against the 6 × His tag, demonstrated the successful expression of the rSmCyp with the expected molecular weight of 17.1 kDa (Fig. 1A).

A recombinant form of SmCyp was also obtained using bacteria. Fig. 1B demonstrates the electrophoretic profile of the purified rSmCyp with the expected molecular weight. Protein recognition by the 6 × His tag monoclonal antibody (Fig. 1C) indicated the production of rSmCyp in frame with the 6 × His tag. This recombinant protein was used for immunization protocols and other assays (Fig. 1C).

3.2. SmCyp characterization

The amino acid sequence of SmCyp (access number: XP_018652799.1) was used for bioinformatics analysis. SOSUI analysis indicated that SmCyp is a soluble protein with an average hydrophobicity of − 0.3434. Protein localization predicted by EULOC, CELLO2 and PSORT II indicated that SmCyp is a cytoplasmic protein. Also, SinalP 4.1 also indicated that this protein has no signal peptide, this result gives additional support to localization predictions. SmCyp had a predicted molecular weight of 17,660 Da and a pI 8.26. BlastP analysis showed that this protein was similar to the peptidyl-prolyl cis-trans isomerase from other helminths (Supplementary Table 1); among them, *Schistosoma haematobium* and *S. japonicum,* species that cause schistosomiasis in Africa and Asia, respectively. The SmCyp shares 67% sequence identity with mouse and 68% with human peptidyl-prolyl cis-trans isomerase (Supplementary Table 1 and Supplementary Fig. 1

To evaluate the structure of SmCyp, we performed homology modeling of SmCyp (XP_018652799.1) using the Phyre2. A three-dimensional model for SmCyp was successfully built with high confidence with Protein Homology/analogY Recognition Engine V 2.0 (Phyre2) using Intensive Modelling Mode. The resulting structure was predicted using six templates (4I9Y: e3 sumo-protein ligase ranbp2; 1VDN: Cyclophilin from *Saccharomyces cerevisiae*, variant A; 1W8M: Cyclophilin from *Homo sapiens*, variant A; 1IHG: bovine cyclophilin 40, 3K2C: peptidyl-prolyl cis-trans isomerase 2 from *Encephalitozoon cuniculi*; 1XO7: Cyclophilin from *Trypanosoma cruzi*the bovine Cyclophilin 40 (PDB code: c1ihgA) with 100% confidence with 96% sequence coverage (161 residues), or > 90% confidence for 100% coverage.

The amino acids comprising the active sites for cyclophilins had been previously identified in another isoform of *S. mansoni* cyclophilin A (AAC47317.1), which had its three-dimensional structure solved by X-ray crystallography in reduced and oxidized states (Gourlay et al., 2007). To identify SmCyp active site amino acids, alignment was performed using *S. mansoni* cyclophilin A (AAC47317.1) and SmCyp (XP_018652799.1) amino acids sequences. The result showed that the amino acids sequences are closely related, and that the amino acids of the active site are conserved among them (Fig. 2B). The amino acids comprising the SmCyp active site are highlighted in the three-dimensional model of SmCyp (Fig. 2A).

The T and B cells epitopes predicted by RankPep and BCpred with the highest scores are indicated in Fig. 2B and Supplementary Table 2. Among the predicted epitopes, two regions, namely SmCyp (94–108) and SmCyp (107–121), contain T cell epitopes predicted with higher

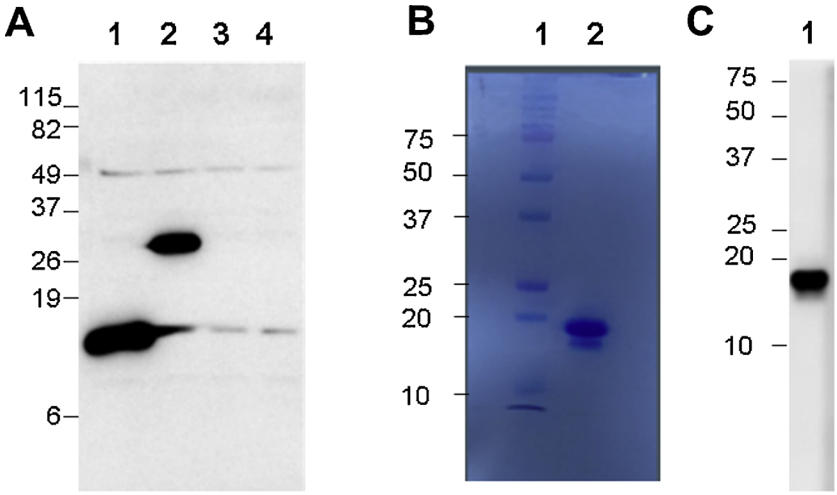

Fig. 1. Evaluation of recombinant *Schistosoma mansoni* cyclophilin A (SmCyp) expression in mammalian and bacterial systems. **(A)** *SmCyp* expression in mammalian cell cultures was evaluated by Western blotting, after the transient transfection of HEK293 T cells, using an antibody against the 6 × His tag. Protein extracts of cells transfected with the pcDNA3.1/V5-His A plasmid containing the coding region of the *SmCyp* gene of *S. mansoni* fused to a 6 × His codon (lane 1), pcDNA3.1/V5-His B plasmid containing the coding region of a non-associated gene from *S. mansoni* fused to the 6 × His codon (lane 2), the empty pcDNA3.1/V5-His A plasmid (Lane 3), and non-transfected cells (Lane 4) were analyzed. **(B)** Recombinant SmCyp (rSmCyp) obtained in the bacterial system was evaluated by SDS-PAGE (15%) stained by Coomassie blue; Dual Color, BioRad Molecular weight marker (kDa) was loaded in lane 1 purified rSmCyp in lane 2. **(C)** Recognition of rSmCyp by the anti-6 × His tag monoclonal antibody was confirmed by Western blotting against the purified protein (Lane 1).

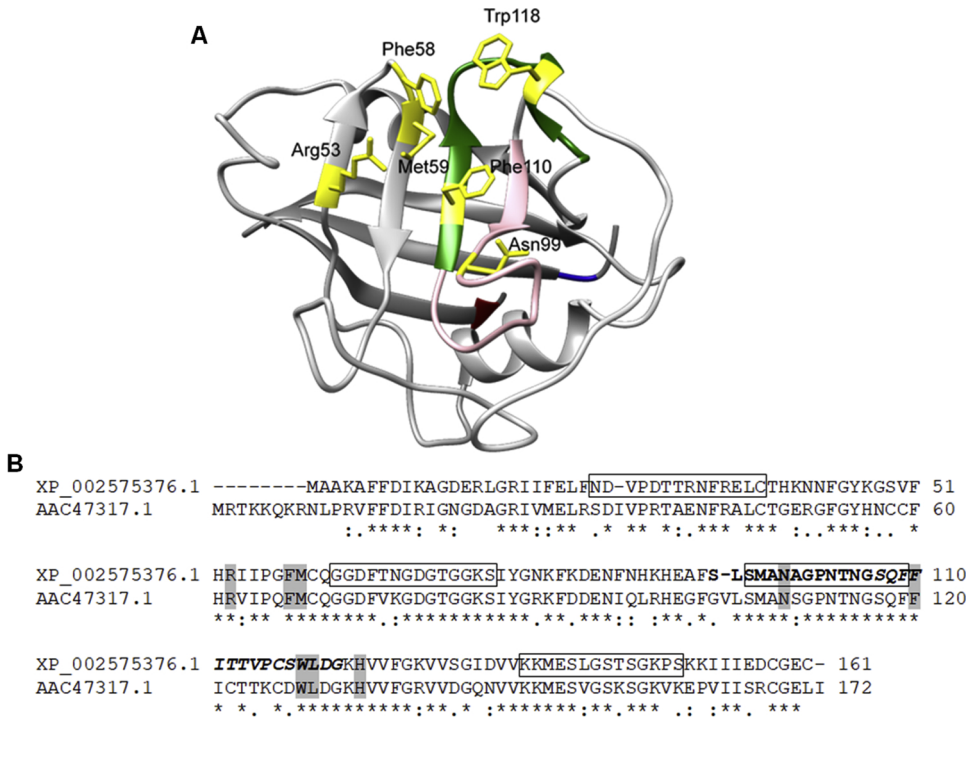

Fig. 2. Predicted three-dimensional model of *Schistosoma mansoni* cyclophilin A (SmCyp). **(A)** Phyre^2-predicted three-dimensional structure model of *S. mansoni* SmCyp based on six templates (4I9Y: e3 sumo-protein ligase ranbp2; 1VDN: Cyclophilin from *Saccharomyces cerevisiae*, variant A; 1W8M: Cyclophilin from *Homo sapiens*, variant A; 1IHG: bovine cyclophilin 40, 3K2C: peptidyl-prolyl cis-trans isomerase 2 from *Encephalitozoon cuniculi*; 1XO7: Cyclophilin from *Trypanosoma cruzi*) with 100% confidence. The active site amino acids (Arg-53, Phe-58, Met-59, Asn-99, Phe-110, Trp-118, Leu-199, and His-123) are shown in yellow, the predicted T cell epitope SmCyp (94–108) is colored pink, SmCyp (107–121) is colored green, C-term GLU160 is red, and N-term ALA3 is blue. **(B)** Amino acid sequence alignment of *S. mansoni* SmCyp (Accession ID XP_018652799.1) and *S. mansoni* cyclophilin A (Accession ID AAC47317.1) was produced using Clustal Omega. The amino acids of the active site are highlighted in grey. The predicted B cell epitopes are shown in boxes. The predicted T cell epitope SmCyp (94–108) is indicated in bold, and SmCyp (107–121) is indicated in italic bold. Identical residues are indicated by asterisks, conserved bases by double dots, and semi-conserved bases by single dots.

scores. As shown in the Fig. 2A and B, the SmCyp (107–121) sequence comprises three of eight amino acids of the active site of SmCyp. In contrast, the SmCyp (94–108) sequence contains only one amino acid (ASN-99) of the SmCyp active site, but also contains an amino acid sequence that was predicted to be a B cell epitope (Supplementary Table 2).

3.3. Assessment of potential rSmCyp allergenicity

The cyclophilin family has been described as a pan-allergen (Flückiger et al., 2002). Therefore, we analyzed SmCyp allergenicity using the allergen online database based on the SmCyp full-length sequence. In this analysis, proteins that have a percent identity greater than 50% and an e-value score less than 1^{e-7} with a known allergen are likely to cause an allergic reaction. The results indicated that SmCyp could possibly cross-reacts with an allergen from *Malassezia sympodialis*, Der f Mal f 6 allergen from *Dermatophagoides farinae*, and with peptidylprolyl cis-trans isomerases from *Aspergillus fumigatus*, *Catharanthus roseus*, *Daucus carota*, and *Betula pendula* (Supplementary Table 3).

To further evaluate rSmCyp allergenicity, we performed an *in vitro* assay using the humanized rat basophilic leukemia cell lines RS-ATL8 and NFAT-DsRed, as described (Wan et al., 2014a, 2014b). Even with different concentrations, rSmCyp was unable to activate basophils sensitized with IgE from sera of *S. mansoni*-infected individuals (Fig. 3), since the luminescence detected in the reporter cell line stimulated with the recombinant protein was below the cutoff.

3.4. Protective immunity induced by SmCyp immunization in mice

To assess the ability of SmCyp to induce protection in a murine model, three different vaccine formulations (DNA immunization, recombinant protein, and synthetic peptide) were evaluated. Sera from mice immunized with a mixture of SmCyp (94–108) and SmCyp (107–121) showed higher levels of antibodies against SmCyp (107–121) but not SmCyp (94–108) (Supplementary Fig. 2); therefore, for immunization protocols we used a vaccine formulation containing only the synthetic peptide SmCyp (107–121). Immunization with pcDNA 3.1/His-A/*SmCyp* or rSmCyp neither significantly reduced the worm burden nor the number of eggs trapped in the intestine and liver (Table 1), but significantly reduced the area of liver granulomas (Fig. 4). In contrast, the vaccine formulation containing SmCyp (107–121) resulted in a significant reduction in worm burden (48–50%) and the number of eggs trapped in the liver (47–51%) and intestine (48%) (trial 2) (Table 1), but had no effect in pathology modulation (Fig. 4).

3.5. Specific humoral immune response induced by vaccine formulations

Antibody levels were measured in serum samples from animals immunized with the different vaccine formulations. In animals immunized with pcDNA 3.1/His-A/*SmCyp* significant production of antibodies against rSmCyp or SmCyp (107–121) was not observed (Fig. 5C and D). However, immunization with recombinant SmCyp induced a significant increase in the production of specific SmCyp IgG antibodies after the second and third immunization dose as compared to that in control and SmCyp (107–121) groups (Fig. 5A). In contrast, after the third vaccine dose, mice immunized with SmCyp (107–121) produced higher levels of anti-SmCyp (107–121) IgG antibodies when compared to those in control and rSmCyp groups (Fig. 5B).

In the SmCyp (107–121) group, a significant increase in the levels of anti- SmCyp (107–121) specific antibodies was observed after the second boost, whereas the rSmcyp group did not produce antibodies against this epitope. Although, an increase in the levels of anti-SmCyp specific IgG antibodies was observed in the rSmCyp group after the first boost with no additional production was induced by the second boost.

Regarding IgG subclasses, mice immunized with rSmCyp produced significant levels of IgG1 and IgG2c against rSmCyp after the second and third immunization doses in comparison to the levels of these antibodies observed in Freund and SmCyp (107–121) groups (Fig. 6 A and B). In animals immunized with SmCyp (107–121) peptide, after the third dose of the vaccine, increased levels of IgG1 and IgG2c antibodies against SmCyp (107–121) epitope were observed in comparison to the levels of antibodies observed in Freund and rSmCyp groups (Fig. 6 C and D). In rSmCyp group, boosters induced an increase in the levels of

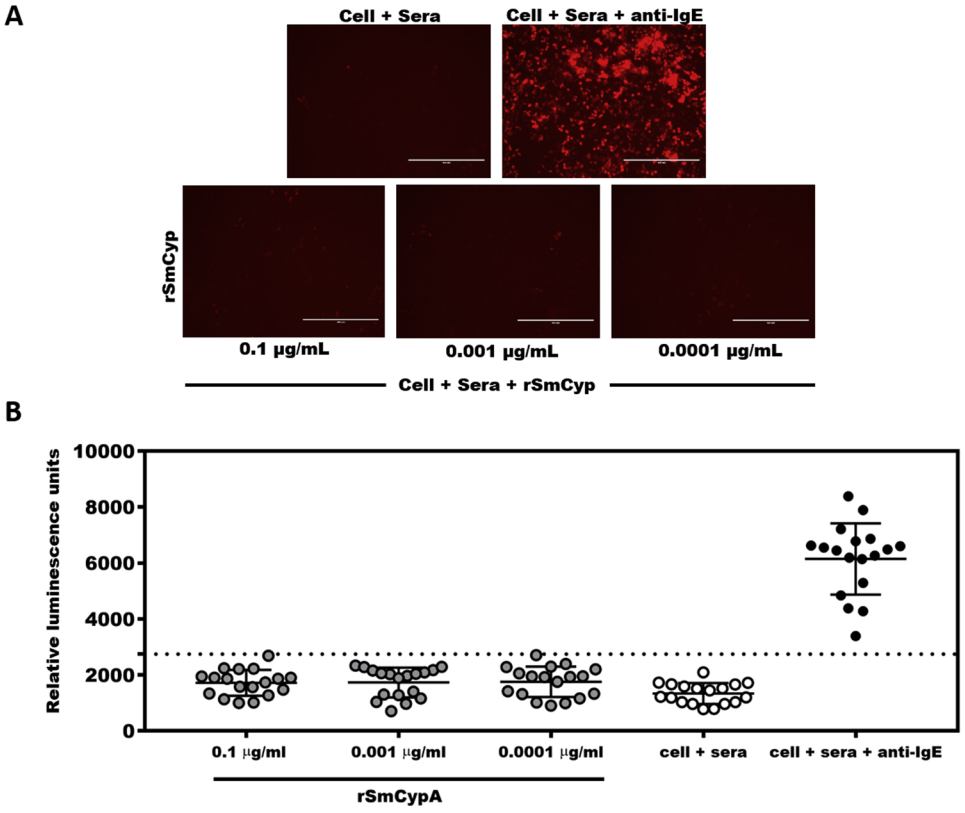

Fig. 3. Evaluation of humanized basophils activation by recombinant *Schistosoma mansoni* cyclophilin A (rSmCyp). NFAT-DsRed **(A)** and RS-ATL8 **(B)** IgE reporter systems cell strains were sensitized with sera from individuals infected with *S. mansoni* and stimulated with rSmCyp, using three different concentrations, specifically 0.1, 0.001, or 0.0001 µg/mL (gray circles), with 1 µg/mL of polyclonal goat anti-human IgE as a positive control (closed circles) or unstimulated cells as a negative control (open circles). **(A)** Representative images of NFAT-DsRed cells 24 h after stimulation. **(B)** Data represent relative luminescence unit values obtained for each condition. Receiver operating characteristic curves (ROC curves) were used to calculate the cutoff point, using the negative (unstimulated cells) and positive (cells stimulated with anti-human IgE antibody) controls, which is denoted by the dotted line.

Table 1
Protective immune response in immunized mice.

	Worm burden recovery							
	Female Mean±SD	Male Mean±SD	Total Mean ±SD	% of protection*	Egg/gram of liver Mean ±SD	% of reduction#	Egg/gram of intestine Mean ±SD	% of reduction#
DNA Vaccine								
Trial 1								
pcDNA 3.1 V5/His A	18±4	18±3	36±5		24803±3577		16467±4776	
pcDNA 3.1 v5/His A/ *SmCyp*	17±5	20±4	37±8	(NS)	28504±12200	(NS)	14459±5534	12%(NS)
Trial 2								
pcDNA 3.1 V5/His A	22±5	24±4	46±7		9859±2457		10226±5599	
pcDNA 3.1 v5/His A/ *SmCyp*	20±7	24±5	44±10	4.3% (NS)	12420±5302	(NS)	6668±2521	35%(NS)
Recombinant protein								
Trial 1								
PBS	16±8	18±11	34±18		12752±6195		24953±11787	
rSmCyp	15±7	19±7	34±14	(NS)	10883±4625	15%(NS)	25109±13440	(NS)
Trial 2								
PBS	13±3	17±4	30±5		9303±7418		13468±1342	
rSmCyp	15±3	17±5	32±8	(NS)	12851±7325	(NS)	11543±6247	14%(NS)
Synthetic peptide								
Trial 1								
PBS	12±2	13±2	25±3		16470±6075		8114±1335	
SmCyp (107-121)	5±5#	8±5	13±10	48%(p = 0.02)	8019±5575	51%(p = 0.03)	5984±5217	26%(NS)
Trial 2								
PBS	9±4	9±4	18±8		11939±3764		9357±3037	
SmCyp (107-121)	5±3#	4±4#	9±7	50%(p = 0.02)	6350±3173	47%(p = 0.004)	4858±3813	48%(p = 0.01)

*Reduction of total worms of immunized group compared to control group.
#Reduction of eggs in tissue (liver and intestine) of immunized group compared to control group.
NS- Not significant.

IgG2c antibodies against this protein in comparison to the first dose of the vaccine (Fig. 6 B). The first boost also increased the production of IgG1 anti-rSmCyp in animals immunized with this recombinant protein, but, after the second boost, a decrease in the levels of these antibodies was observed (Fig. 6 A). In SmCyp (107–121) group, boosters increased the levels of IgG1 and IgG2c antibodies produced against SmCyp (107–121) epitope (Fig. 6 C and D).

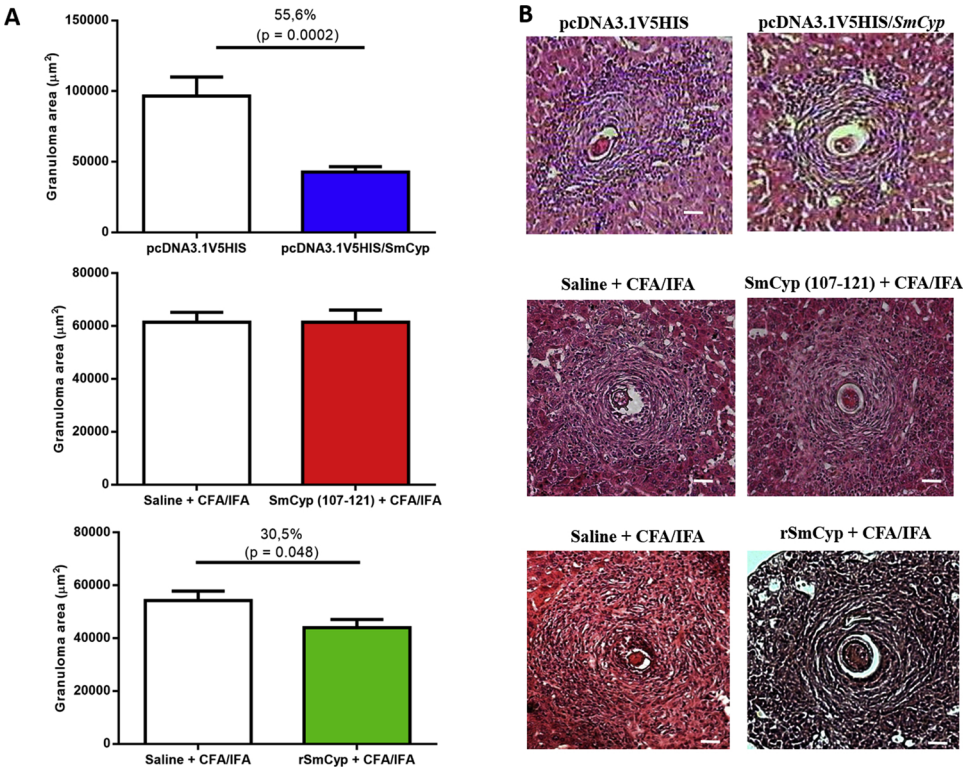

Fig. 4. Hepatic granuloma in immunized mice. One hundred granulomas from each group with a single well-defined egg and at the exudative-productive stage were randomly selected and analyzed using a 10× objective lens **(A)**. The total diameters of granulomas were measured, and the results are expressed as mean square micrometers (μm^2) ± standard deviation (SD). Statistically significant differences are denoted in the graph based on p values. **(B)** Representative histological sections of liver granuloma.

4. Discussion

Great efforts have been made to identify promising antigens and vaccine formulations against schistosomiasis. Among the already described *S. mansoni* vaccine candidates, including tetraspanin, Sm14, Smp80, and Sm29 (Tendler et al., 1996; Hota-Mitchell et al., 1997; Tran et al., 2006; Cardoso et al., 2008), cyclophilins have arisen as an interesting antigen for use in vaccine formulations, since this protein is the target of the schistosomicide drug cyclosporin A (Bueding et al., 1981). The demonstration that *S. japonicum* cyclophilin A induces a protective immune response in a murine model (Han et al., 2012) provided additional support for the use of this protein in vaccine formulations against *S. mansoni*. Schistosomes produce different isoforms of cyclophilins, and in this study, we evaluated the ability of cyclophilin A (SmCyp), originally described by Kiang and coworkers as Smp17.7 (Kiang et al., 1996), to induce protection in mice when used as an antigen with different vaccine formulations and strategies.

The characterization of this protein using *in silico* tools indicated that SmCyp is a soluble protein of approximately 18 kDa and is predicted to be a cytoplasmic protein. Immunolocalization assays identified SmCyp in the tegument, muscle layer, and gut epithelium of the *S. mansoni* (Kiang et al., 1996). The parasite tegument and gut epithelium represent the interface between the host and parasite (Wilson and Coulson, 2009). Many vaccine formulations containing antigens described as located in the tegument and gut were previously found to induce protection in mice (Fonseca et al., 2012; Figueiredo et al., 2015).

In the SmCyp sequence, the amino acids 94–122 are predicted to be a region containing T cell epitopes, whereas four B cell epitopes were predicted in different regions of the protein sequence (Supplementary Table 2 and Fig. 2B). The three-dimensional structure of SmCyp

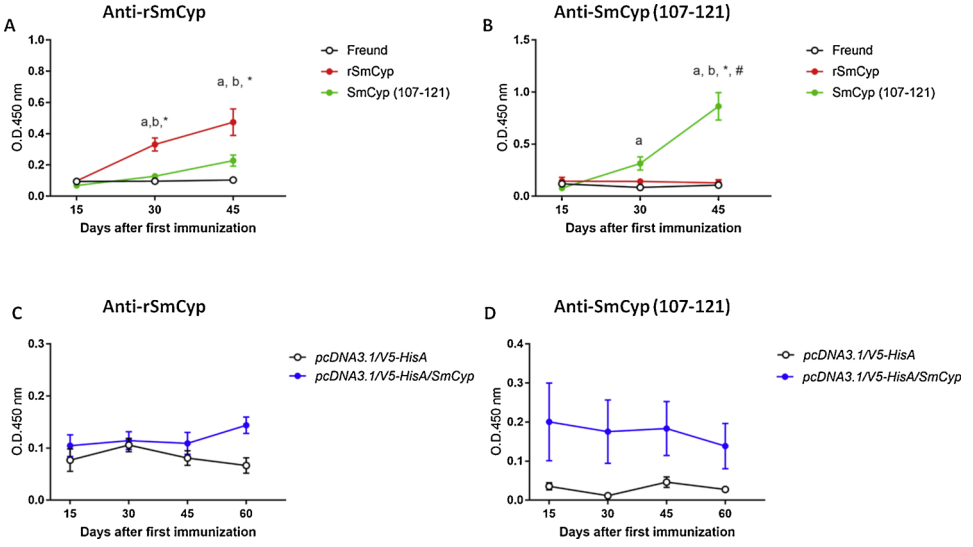

Fig. 5. IgG kinetics after mouse immunization. Mice were immunized with three doses of recombinant protein or synthetic peptide vaccine **(A and B)** or with four doses of DNA vaccine **(C and D)** and assessed to determine the kinetics of specific anti-recombinant *Schistosoma mansoni* cyclophilin A (rSmCyp) **(A and C)** or anti-SmCyp (107–121) **(B and D)** IgG antibody production. Results represent the mean absorbance ± SD measured at 450 nm. Significant differences between experimental and control groups are denoted by "a"; differences between rSmCyp and SmCyp (107–121) groups are denoted by "b"; * denotes significant differences compared to the first dose; # denotes significant differences compared to the second dose.

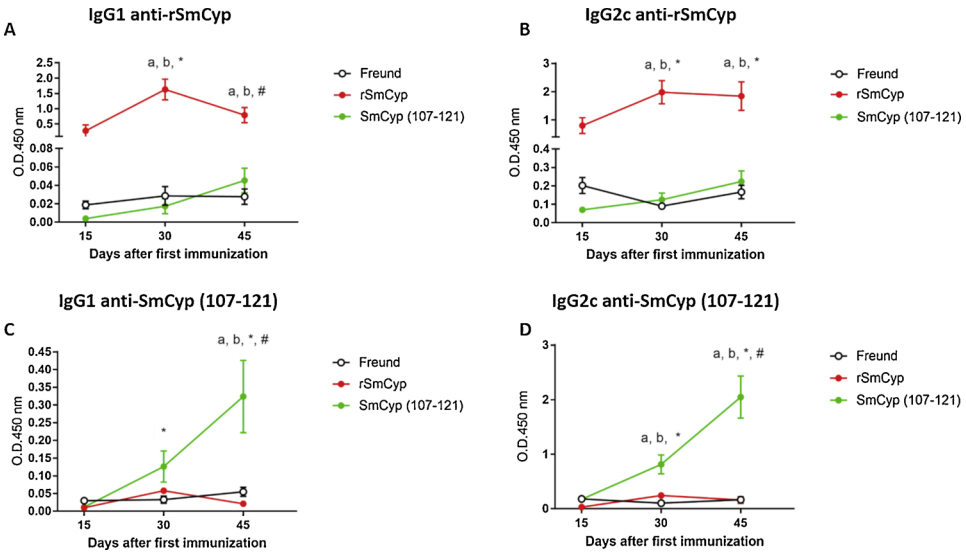

Fig. 6. Kinetics of specific IgG 1 and IgG2c production after immunization with rSmCyp and SmCyp (107–121). Sera from immunized mice were assessed to determine the production of IgG1 (A and C) and IgG2c (B and D) specific to anti-rSmCyp (A and B) or anti-SmCyp (107–121) (C and D). Results represent the mean absorbance + standard error of mean (SEM) measured at 450 nm. Significant differences between experimental and control groups are denoted by "a"; differences between rSmCyp and SmCyp (107–121) groups are denoted by "b"; * denote significant differences compared to the first dose; # denote significant differences compared to the second dose.

obtained by computational modeling demonstrated that this protein has a β-barrel core (Fig. 2) structure similar to the canonical structure of cyclophilins (Gourlay et al., 2017). Among the amino acids that comprise the SmCyp active site, Asn-99, Phe-110, Trp-118, and Leu-199 are in a region of predicted epitopes. However, from the two synthetic peptides produced containing the amino acid sequences 94–108 or 107–121, only SmCyp (107–121) was found to be immunogenic, since significant production of serum antibodies against this peptide was observed in mice immunized with a mixture of both peptides (Supplementary Fig. 2).

Cyclophilins are well conserved among organisms. SmCyp demonstrated high similarity to other cyclophilins, including cyclophilins from other helminths that infects humans, and also to the cyclophilin from mice and humans. Sharing epitopes with cyclophilins from other helminths could be an additional feature of SmCyp to be used as a vaccine candidate, since a vaccine containing this protein as an antigen could potentially protect against other helminthic parasites. However, the similarity to host proteins is not beneficial because the immune response to self-antigens is highly regulated and limits immune system activation upon vaccination (Bendle et al., 2005; Higgins et al., 2009). The poor immunogenicity observed for SmCyp (94–108) could be a consequence of the high-level of conservation of amino acids from this region between the parasite and the host (*Mus musculus* and *Homo sapiens*), as observed in Supplementary Fig. 1.

Some members of the cyclophilin family are described as allergens and cross-recognition by anti-cyclophilin IgE has been observed among different species (Flückiger et al., 2002). Additionally, immunization with *S. japonicum* cyclophilin A induces a strong Th2 type immune response (Han et al., 2012), which could potentiate an allergic reaction. The use of potential allergenic antigens in vaccine formulations against helminths is a safety concern that needs to be addressed before moving forward to pre-clinical and clinical studies. An example of a promising vaccine that failed a phase I clinical trial due to generalized urticarial reactions induced post vaccination is the *Necator americanus* Na-ASP-2 (Diemert et al., 2012). In silico analysis of SmCyp suggests that this antigen is a potential allergen (Supplementary Table 3), but evaluation of the recombinant form of SmCyp in a functional *in vitro* assay failed to activate basophils sensitized with sera from individuals infected with *S. mansoni* (Fig. 3).

Since SmCyp has characteristics of a good vaccine candidate, we evaluated this antigen using different formulations and vaccine strategies. In mice immunized with the gene encoding SmCyp, no significant reduction in the parasite burden or the number of eggs trapped in the liver and intestine was observed (Table 1). This lack of protection could not be attributed to unsuccessful expression of the protein in mammalian cells since *in vitro* assays demonstrated its production by HEK293 T cells (Fig. 1). However, poor transfection efficiency in host cells of immunized mice could have impaired SmCyp production and resulted in a low vaccine immunogenicity accompanied by insignificant anti-SmCyp antibody production.

The use of recombinant proteins together with a strong adjuvant might overcome the low immunogenicity observed after immunization with a DNA vaccine containing *SmCyp*. Adjuvants are key components of vaccine formulations that promote immune responses by recruiting professional antigen presenting cells (APCs) to the vaccination site, increasing the delivery of antigens to APCs, or activating cytokine production by APCs, which provides additional signals for T cell activation and differentiation (McKee et al., 2010). Indeed, mice immunization with recombinant SmCyp in association with Freund's adjuvant resulted in the significant production of anti-SmCyp antibodies but failed to reduce parasite and egg burden. In contrast, the immunization of mice with a synthetic peptide containing the SmCyp (107–121) epitope significantly reduced worm burden and the number of eggs trapped in the liver (Table 1).

Many studies have demonstrated the important role of humoral immune responses in the elimination of schistosome parasites. B cell-deficient mice vaccinated with radiation-attenuated cercariae of *S. mansoni* showed significantly diminished protection against challenge infection when compared to that in wild-type vaccinated mice (Jankovic et al., 1999). Moreover, serum transfer studies performed in rabbits, rats, and mice showed that serum from vaccinated animals could protect against *S. mansoni* infection when transferred to non-immunized recipients (Ford et al., 1984; Bickle et al., 1984; Torben et al., 2011; Melo et al., 2014). In our study, we observed a significant production of antibodies against SmCyp epitopes when mice were immunized with rSmCyp and SmCyp (107–121). However, only mice immunized with SmCyp (107–121) exhibited a protective immune response. These animals produced significantly higher levels of antibodies against the epitope containing amino acids 107–121. In contrast, mice immunized with the recombinant protein produced substantial levels of antibodies against other epitopes of the SmCyp protein, but not against SmCyp 107–121 epitope (Fig. 5).

The evaluation of IgG subclasses in the sera from immunized mice, indicate that mice immunized with SmCyp (107–121) produce antibodies against this epitope of both IgG1 and IgG2c subclasses. IgG1 antibodies in mouse are known to bind with high affinity to FcγRIIB, which is an inhibitor receptor and with low affinity to FcyRIII. In contrast, IgG2c antibodies bind to FcγRI, FcγRIV and FcyRIII with high,

intermediate and low affinity, respectively. In consequence, while IgG1 is a weaker inducer of ADCC and complement activation, IgG2c efficiently activate these immune effectors (Nimmerjahn and Ravetch, 2005; Bruhns and Jönsson, 2015)

Different mechanisms of parasite elimination involve antibodies against targets in the parasite. Antibody-dependent cell mediated cytotoxicity (ADCC) is an important mechanism involved in *Schistosoma* elimination. *In vitro* studies demonstrated that ADCC is associated with schistosomula killing in mice immunized with 28-kDa glutathione S-transferase (GST) and Smp-80 (Balloul et al., 1987; Torben et al., 2012). Moreover, antibodies are involved in the reduction of female fecundity after GST immunization (Xu et al., 1991). In our study, the association of high levels of antibodies against the SmCyp 107–121 epitope and protection, suggests that these specific antibodies could block the enzymatic site of SmCyp, preventing peptidyl-prolyl cis-trans isomerase activity and consequently resulting in the death of the parasites at some point during their development. However, ADCC active elimination of the parasite mediated by anti-SmCyp (107–121) IgG2c antibodies cannot be ruled out, and additional studies are necessary to determine which IgG subclass and effector mechanisms are involved in the protective immunity induced by SmCyp (107–121) epitope.

A vaccine against schistosomiasis can act to eliminating the parasite, reducing its fecundity, or reducing the pathology associated with egg deposition in the liver. Therefore, we also evaluated liver pathology in mice immunized with different vaccine formulations containing SmCyp or one of its epitopes. The granulomatous reaction represents the major pathology associated with schistosomiasis, and the intensity of this reaction correlates with host morbidity (Wynn and Cheever, 1995; Stadecker and Hernandez, 1998; Abath et al., 2006). In murine schistosomiasis, it has been shown that pathology is induced by $CD4^+$ Th2 cells. These cells, through the production of IL-4 and IL-13, mediate the granulomatous response against schistosome eggs embedded in the host liver (Fallon et al., 2000; Wilson et al., 2007), whereas IL-10, IFN-γ, and inducible regulatory T-cells act to limit schistosome-induced pathology (Hesse et al., 2004). Analysis of the granulomatous response in immunized mice showed that immunization with the entire protein, as a DNA or recombinant protein subunit vaccine, significantly reduced the granuloma area. However, in mice immunized with the SmCyp (107–121) synthetic peptide, no significant reduction in granuloma area was observed (Fig. 4). Recently, Floudas and co-workers (2017) described some immunomodulatory proprieties of SmCyp (Smp17.7). They demonstrated that this protein modulates the production of pro-inflammatory cytokines by LPS-activated BMDCs (Bone Marrow-derived Dendritic cells), reduces T cell proliferation, and increases the proportion of $CD4^+$ $Foxp3^+$ regulatory cells (Floudas et al., 2017).

Therefore, immunization with the SmCyp protein or gene would make the immune system prone to a modulatory profile that leads to changes in the granulomatous reaction, whereas immunization with the synthetic peptide SmCyp (107–121) induces a significant production of specific antibodies that target important amino acids of the active sites of this protein, potentially inhibiting protein activity and leading to parasite death. The precise mechanisms of parasite elimination and granuloma modulation still require further clarification. However, our study demonstrates that SmCyp can regulate hepatic granuloma formation and we identified an epitope from this protein that could represent a target to induce a protective immune response against the parasite. The lack of activation of the RS-ATL8 IgE-reporter system suggests that this could be safe to use.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.04.021.

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