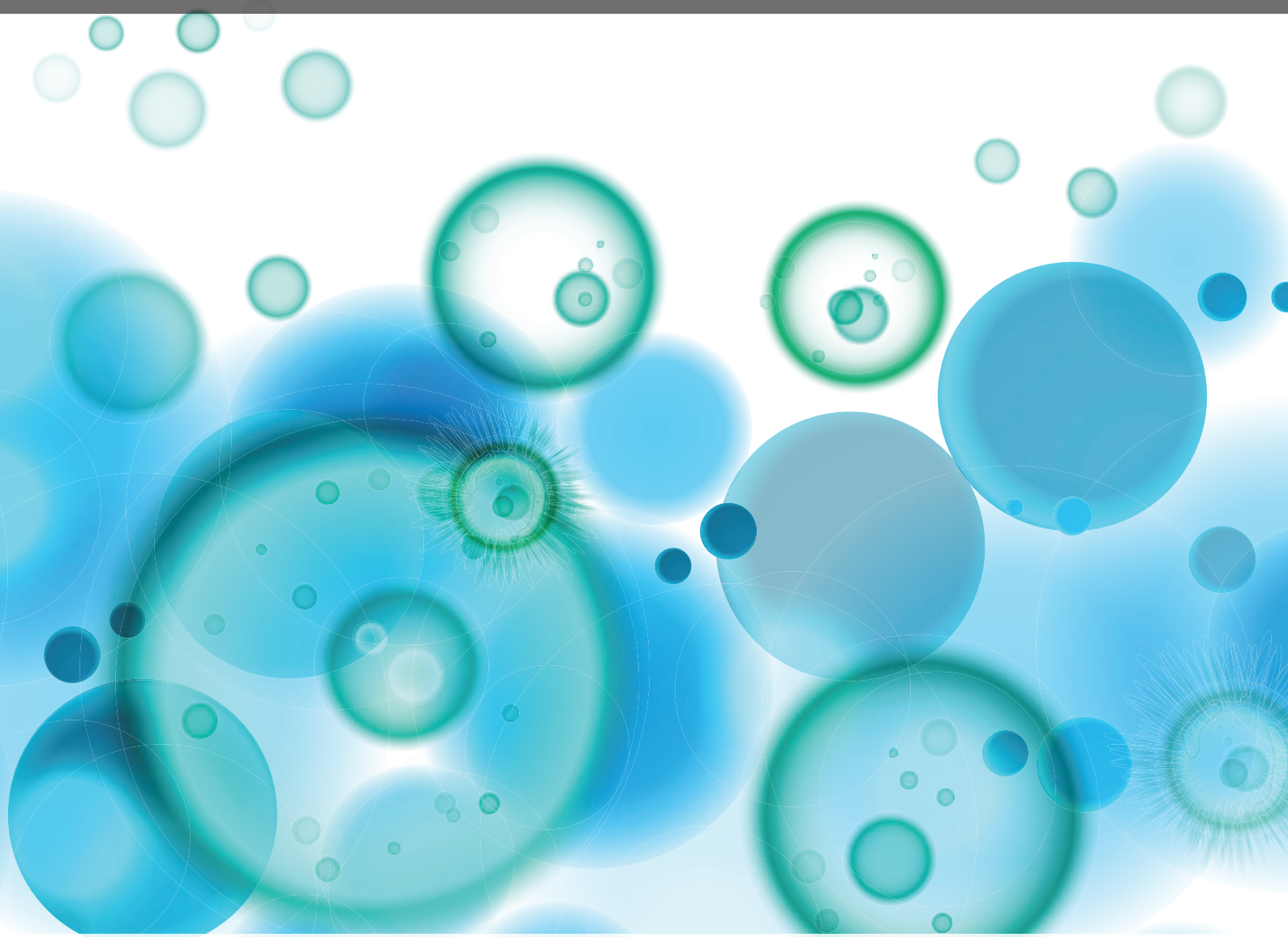
THE SCHISTOSOMIASIS VACCINE – IT IS TIME TO STAND UP

EDITED BY: Rashika El Ridi, Ahmad Ali Othman and Donald McManus
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88919-741-5
DOI 10.3389/978-2-88919-741-5

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THE SCHISTOSOMIASIS VACCINE – IT IS TIME TO STAND UP

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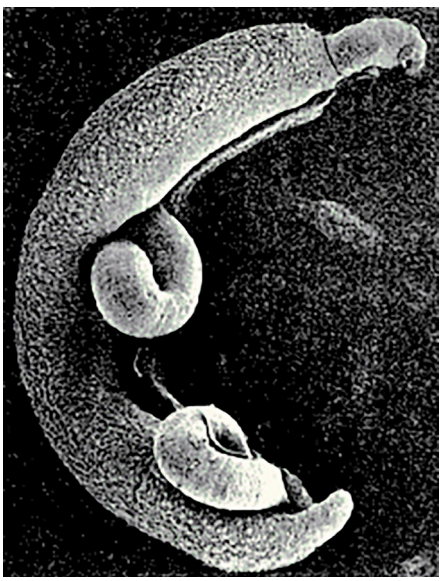

Male schistosome carrying the female shower the host with ailments, sickness and morbidity.

Image by Angelica Matos, available at: http://www.ebah.com.br/content/ABAAAezr0AF/aula-8-schistosoma-mansoni-fasciola-sp

Schistosomiasis is a severe parasitic disease, endemic in 74 developing countries with up to 600 million people, including many children, infected and 800 million at risk of contracting the disease following infection with *Schistosoma mansoni, S. haematobium or S. japonicum*. Disease burden is estimated to exceed 70 million disability-adjusted life-years, and leads to remarkably high YLD (years lived with disability) rates. Even more importantly, people with schistosomiasis are highly susceptible to malaria, tuberculosis and hepatic and acquired immunodeficiency viruses. There is only one drug, praziquantel, currently available for treatment and it has high efficacy, low cost, and limited side effects. However, only 13% of the target population has received the drug, and those treated are at continuous risk of reinfection necessitating repeated drug administration and the emergence of drug resistant parasites is a constant threat. There currently is no vaccine. While the target of >40% protection has been achieved with some molecules such as excretory-secretory proteins including calpain, glyceraldehyde 3-phosphate dehydrogenase, and cysteine peptidases, very recent articles reiterate the findings published during the last 2 decades of the last century, contradicting the established data of the pioneers of schistosome biology. A consensus should be reached without delay, in order to propose collaborative independent experiments and proceed ahead to pre- and clinical trials with efficacious candidate vaccine molecules. The proposed plan aims to finally

antigens, adjuvants, and approaches for immunization against *S. mansoni, S. haematobium, and S. japonicum*. It is hoped that the forum will end with a very few candidate antigens and a consensus approach regarding target immune responses, thus leading to encouraging the World Health Organization and other international foundations to sponsor the development and implementation of the urgently required, yet still elusive, vaccine for preventing and eliminating the transmission of schistosomiasis.

Citation: El Ridi, R., Othman, A. A., McManus, D., eds. (2015). The Schistosomiasis Vaccine – It is Time to Stand Up. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-741-5

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frontiers in Immunology

EDITORIAL
published: 30 July 2015
doi: 10.3389/fimmu.2015.00390

Editorial: The schistosomiasis vaccine – it is time to stand up

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Keywords: ***Schistosoma mansoni***, ***Schistosoma haematobium***, **schistosomiasis vaccine, paramyosin, Sm14, schistosome peptidases, type 1 and type 2 immunity**

Schistosomiasis is a severe parasitic disease, endemic in 74 developing countries with up to 600 million people infected and 800 million, mostly children, at risk of contracting the disease following infection predominantly with *Schistosoma mansoni*, *Schistosoma haematobium,* or *Schistosoma japonicum*. The disease burden is estimated to exceed 70 million disability-adjusted life-years, and leads to remarkably high YLD (years lived with disability) rates. Even more importantly, people with schistosomiasis are highly susceptible to malaria, tuberculosis, and hepatic and acquired immunodeficiency viruses. There is only one drug, praziquantel, currently available for treatment and it has high efficacy, low cost, and limited side effects. However, only 13% of the target population has received the drug, and those treated are at continuous risk of reinfection necessitating repeated drug administration and the emergence of drug-resistant parasites is a constant threat (1). Currently there is no vaccine. The *a priori* requirements for discovery of a vaccine formulation include the following: identification of protective key immune players in humans; characterization and isolation of target antigens; establishment of efficacy in terms of reduction of parasite burden as well as amelioration of immunopathology; establishment of safety; and finally, provision of considerable funds along with physical infrastructure and qualified personnel to carry out clinical trials.

The target of >40% protection has been achieved with some schistosome molecules such as fatty acid binding protein (Sm14), paramyosin, calpain large subunit (Sm80), superoxide dismutase (SOD), glutathione S-transferase (GST), glyceraldehyde 3-phosphate dehydrogenase, and cysteine peptidases (2). Furthermore, Pearson et al. (3) identified the antigens selectively recognized by serum IgG1 and IgE of *S. haematobium* patients who acquired praziquantel-induced resistance (DIR) to the infection, or self-cured macaques following *S. japonicum* infection. The probed antigens were derived from *S. mansoni* and *S. japonicum*, likely because of the documented antigen conservation among the three main clinically important species, and were selected among those known to be secreted or localized to the tegument. The tegument is at the host–parasite interface, but its access by host effector antibodies is entirely prevented in healthy schistosomes, otherwise they would not survive a day, not to mention decades, in the host bloodstream. Anyhow, the study identified once again calpain, SOD, and GST as vaccine candidates together with surface membrane-associated antigens such as tetraspanins and glucose transporters, as well as an array of newly discovered target antigens. A remarkable finding in the study was the implication that type 2 (IgG1 and IgE) and not type 1-related antibodies are critical for human resistance against *S. haematobium* reinfection. Besides the worm tegument, which may not be accessed by host effector antibodies, the digestive tract is the other major interface between host and parasite. Schistosome peptidases responsible for digesting blood-born cells, components, and nutrients may be targeted, and possibly neutralized and blocked, by host antibodies and, thus, represent potential vaccine candidates. The timely study of Figueiredo et al. (4) reviewed what is known about the properties and vaccine potential of proteins secreted by the esophagus, and the lining (gastrodermis) of the blind-ended gut, namely Sm14, Sm10.3, venom allergen-like (VAL) protein, Cu–Zn SOD, cathepsin B, and cathepsin L.

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology

Received: *09 July 2015*
Accepted: *16 July 2015*
Published: *30 July 2015*

Citation:
El Ridi R, Othman AA and McManus DP (2015) Editorial: The schistosomiasis vaccine – it is time to stand up. Front. Immunol. 6:390. doi: 10.3389/fimmu.2015.00390

It is reassuring we have convened on a handful of promising vaccine candidates and several reviews in this issue illustrate the advances that have been made. Kurtis et al. (5) reviewed the discovery, gene cloning, and expression of paramyosin; its localization in muscles, just below the tegument, and in the gut lining of adult worms; its protective potential in rodents against *S. mansoni* (24–53% protection without adjuvant, associated with induction of interferon-gamma, IFN-γ) and against *S. japonicum* (62–86% protection without adjuvant); its immunogenicity in humans, whereby *S. japonicum* paramyosin was found to be the target of protective type 2-biased cytokine and antibody responses; and plans to move it toward phase I clinical trials. The history of the discovery, gene cloning and expression trials, vaccine potential, and outcomes of completed phase I clinical trials were reported for the fatty acid binding protein, Sm14, by Tendler et al. (6). Cost-effective, large-scale production of recombinant Sm14 expressed in *Pichia pastoris* is currently in place, and the protein will be formulated with glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) adjuvant. This synthetic adjuvant has been selected as it enhances type 1, namely IFN-γ, responses, identified as the basis of the Sm14-mediated protective immunity in animal models and humans. The protective potential of other prominent vaccine candidates, the antioxidant enzymes Cu–Zn SOD and glutathione S peroxidase formulated as plasmid cDNA and recombinant protein preparations, has been assessed in the Olive Baboon (7). The vaccine formulations were entirely safe and strongly immunogenic but, in accord with a plethora of previous vaccine trials involving type 1 immune response-inducing adjuvants or plasmid cDNA constructs, induced limited and/or variable protection in non-human primates against *S. mansoni* challenge infection.

Despite the fact that protective immunity to *S. mansoni* and *S. haematobium* infection in humans is documented to be dependent on type 2 immune responses (2, 3, 5, and references therein), formulations of schistosomiasis vaccines destined for use in humans still aim to induce predominant type 1-related cytokines and antibodies, clearly indicating we have not yet reached a consensus regarding the type of immune responses an anti-schistosomiasis vaccine should elicit. The review by Fonseca et al. (8) is, thus, particularly well timed as it seeks to find the optimal immune weapons generating vaccination-mediated resistance against schistosome infection via identifying the immune responses associated with protective immunity elicited by several vaccine candidates namely GST, Sm14, calpain (Sm80), tetraspanins, and Sm29 in mono- and multivalent formulations. The review emphasized and documented the importance of specific antibodies and strong IFN-γ production in parasite elimination regardless of the vaccine candidate used.

Since currently available vaccine candidate formulations mediate type 1-biased protective immunity, which is limited or partial at best, it is important to revisit the lessons of the radiation-attenuated (RA) cercarial vaccine (9). In this respect, a meta-analysis of the experimental studies undertaken with the RA cercarial vaccine in mice (755 observations from a total of 105 articles) was performed by Fukushige et al. (10), who reported that the RA vaccine has the potential to induce protection as high as 78% with a single dose of vaccine. While major predictors of protection were the immunizing cercarial number (antigen dose) and interval between the last vaccination and challenge (duration of immune memory), the study emphasized the importance of host immunization with more than a single schistosome molecule in order to achieve protection. The early pioneers studying schistosome biology helped devise an efficacious schistosomiasis vaccine by demonstrating that the physiological and reproductive status of *S. mansoni* is strongly influenced by the microenvironment of the host and that the lung and liver are the sites of innate and acquired immunity-mediated parasite attrition in permissive (mice, hamsters) and non-permissive (rats) hosts (11).

To compile a road map for the successful development of a schistosomiasis vaccine: (1) It appears we have at hand a plethora of well-characterized, ready for use vaccine candidates (2–11). (2) As noted by Fonseca et al. (8), 24 h and older schistosomula are refractory to killing by antibody-dependent complement-mediated attrition, and this fully applies to antibody-dependent cell-mediated cytotoxicity (ADCC) as well. (3) Specific antibodies may access the worm gut lumen and those that escape immediate digestion might be able to neutralize and interfere with enzymes critical for worm feeding and fecundity, but not survival, as these processes by definition impact on juvenile and adult worms not schistosomula migrating in the lung capillaries and liver sinusoids (4). (4) We are left then with the hunt and chase theory, whereby immune antibodies and cells interact with excreted–secreted parasite products in the vicinity of migrating schistosomula, alarming and activating effector immune cells (2, 9, 12). (5) Eosinophils and basophils would be particularly effective immune cells but need a type 2 immune environment for recruitment and activation (2, 9, 12, 13). (6) Protective immunity against reinfection with *S. mansoni* and *S. haematobium* in humans is documented to be associated with type 2 responses (2, 3, 5, 8). (7) There is considerable evidence demonstrating that immunization of outbred, akin to man, mice with selected vaccine candidates in conjunction with type 2 immune response-inducing cysteine peptidase, papain, or cytokines (namely interleukin-25, interleukin-33, or thymic stromal lymphopoietin) can elicit a reduction in *S. mansoni* worm burdens consistently higher than 50% and reaching the 78% level achieved by vaccination with the RA cercarial vaccine (14). (8) These molecules inducing type 2 immunity were replaced by *S. mansoni* cysteine peptidases, leading to consistent and highly significant ($P < 0.0001$) 50–83% protection of outbred mice against *S. mansoni* challenge infection (15). (9) It has been demonstrated that this approach, incorporating a cysteine peptidase-based vaccine, is effective in protecting hamsters and mice against *S. haematobium* as well (16). (10) A consensus should be reached without delay in order that independent, collaborative experiments could be devised and undertaken that would result in the development of a near sterilizing protective immunity-inducing schistosomiasis vaccine (2, 9).

In conclusion, discovery of a successful vaccine for a host as complex as man against a parasite as complex as *Schistosoma* is a monumental scientific challenge with many factors at play including parasite strain; intensity, duration, and frequency of infection; genetic make-up and immunological status of the host; perinatal sensitization; host nutritional status; and co-infections with other infectious pathogens. Insights of protective immune responses

generated by vaccination have been deduced from experiments with rodents or, more importantly, non-human primates, but data and experience with humans are still much needed. Important considerations such as vaccine efficacy, safety, and cost, all count in the development of a successful human vaccine. It is highly unlikely that the vaccine, when available, would stand alone, but it could be a major element in an integrated control package. A primary goal should be the vaccination of children in endemic regions at an age as early as possible on the path to the elimination of schistosomiasis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Immunology

ORIGINAL RESEARCH
published: 05 May 2015
doi: 10.3389/fimmu.2015.00213

Of monkeys and men: immunomic profiling of sera from humans and non-human primates resistant to schistosomiasis reveals novel potential vaccine candidates

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Schistosoma haematobium affects more than 100 million people throughout Africa and is the causative agent of urogenital schistosomiasis. The parasite is strongly associated with urothelial cancer in infected individuals and as such is designated a group I carcinogen by the International Agency for Research on Cancer. Using a protein microarray containing schistosome proteins, we sought to identify antigens that were the targets of protective IgG1 immune responses in *S. haematobium*-exposed individuals that acquire drug-induced resistance (DIR) to schistosomiasis after praziquantel treatment. Numerous antigens with known vaccine potential were identified, including calpain (Smp80), tetraspanins, glutathione-*S*-transferases, and glucose transporters (SGTP1), as well as previously uncharacterized proteins. Reactive IgG1 responses were not elevated in exposed individuals who did not acquire DIR. To complement our human subjects study, we screened for antigen targets of rhesus macaques rendered resistant to *S. japonicum* by experimental infection followed by self-cure, and discovered a number of new and known vaccine targets, including major targets recognized by our human subjects. This study has further validated the immunomics-based approach to schistosomiasis vaccine antigen discovery and identified numerous novel potential vaccine antigens.

Keywords: schistosomiasis, protein microarray, vaccine, human, drug-induced resistance

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology

Received: *16 March 2015*
Accepted: *18 April 2015*
Published: *05 May 2015*

Citation:
Pearson MS, Becker L, Driguez P, Young ND, Gaze S, Mendes T, Li X-H, Doolan DL, Midzi N, Mduluza T, McManus DP, Wilson RA, Bethony JM, Nausch N, Mutapi F, Felgner PL and Loukas A (2015) Of monkeys and men: immunomic profiling of sera from humans and non-human primates resistant to schistosomiasis reveals novel potential vaccine candidates. Front. Immunol. 6:213. doi: 10.3389/fimmu.2015.00213

Introduction

The carcinogenic blood fluke, *Schistosoma haematobium*, infects more than 100 million people throughout Africa and is the most prevalent of the human schistosomes, causing more than half

of all infections (1). *S. haematobium* adult flukes migrate to the vasculature of the organs of the pelvis. Severe morbidity results from host immune responses to eggs in tissues and includes periportal fibrosis, portal hypertension, and hepato-splenic disease (2). Formerly known as urinary schistosomiasis, *S. haematobium* infection was recently renamed "urogenital schistosomiasis" in recognition that the disease affects both the urinary and genital tracts of women and men. Female *S. haematobium* lay between 20 and 200 eggs daily (3), which penetrate the vessel wall and move toward the lumen of the bladder. Some of the eggs become sequestered in the tissue of the pelvic organs such as the urinary bladder, ureters, cervix, vagina, prostate gland, and seminal vesicles, where they cause chronic inflammation, pelvic pain, bleeding, and an altered cervical epithelium in women (4). *S. haematobium* is unique among the schistosomes in its recognition as a group I carcinogen by the International Agency for Research on Cancer because of its robust association with urothelial carcinoma (5). *S. haematobium* infection also increases susceptibility to infection with HIV-1, progression to disease, and results in a higher likelihood of transmitting infection to others (6).

Praziquantel (PZQ) is widely used to treat human schistosome infections and has two main effects on schistosomes – paralysis and tegument damage (7). An added benefit of PZQ treatment is that it mediates destruction of flukes thereby exposing antigens on the worm surface to the host immune system. This release of surface antigens induces and/or enhances parasite-specific immune responses (8), resulting in immune-mediated killing of the parasite. Early studies reported modifications in T-cell proliferative responses (9), whereas recent studies noted modifications in the levels and types of antibody (10–13) and cytokine responses (14–16) following PZQ treatment. The immune response triggered by PZQ treatment is thought to last for more than 1 year (14, 17–19) and confer at least some level of resistance to re-infection. This phenomenon is referred to as "drug-induced resistance" (DIR) (20). The mechanisms behind DIR differ significantly from those of putative natural resistance (PR, resistant individuals who have not received PZQ therapy) and can be related to the origin (developmental stage) and concentration of the released antigen, as well as the type of antigen-presenting cells (APCs) involved. PZQ treatment introduces a large amount of adult fluke antigen directly into the bloodstream as a result of many worms dying at once (21), whereas naturally acquired resistance in the absence of PZQ treatment (PR) is stimulated by the introduction of smaller quantities of adult antigen due to a more gradual worm death. The process of PR is additionally stimulated by the release of antigens from naturally dying larval schistosomes (schistosomula) primarily through the skin and pulmonary vasculature, thus inducing different APCs and resulting in different interactions between the antigens and the immune system (22). This additional stimulus does not appear to factor significantly in DIR due to the ineffectiveness of PZQ against schistosomula (7, 8). Whatever the mechanism, it is important that an antigen threshold is reached in order to sufficiently stimulate anti-schistosome immunity (23, 24).

Studies with car washers in schistosome-infected waters of Lake Victoria in Kenya showed that a subset of the men developed resistance to re-infection after PZQ therapy while others remained susceptible despite treatment (25, 26). It was found that IgE production to soluble worm antigen preparation (SWAP) paralleled the development of resistance, and did not occur in those who remained susceptible to re-infection (25). Additionally, our own immuno-proteomic studies have used *S. haematobium* SWAP to identify a number of antigens that are released by PZQ treatment and/or are the target of DIR immune responses (27, 28). However, despite the power of these proteomic studies in identifying individual parasite proteins, the utilization of SWAP (where worms are homogenized and solubilized under native conditions in the absence of detergents that will solubilize the cell membranes) does not result in full representation of the *S. haematobium* proteome. Indeed, numerous abundantly expressed proteins with multiple membrane spanning domains that are released from the tegument with detergents (29, 30) are accessible to chemical labeling on the surface of live worms (30), are recognized by sera from PR individuals, and are lead vaccine antigens against schistosomiasis (31–33).

A third mechanism of resistance to schistosomiasis is seen in the rhesus macaque (*Macaca mulatta*). It is unique among animal models of schistosomiasis in that, once an infection reaches patency, worm death starts to occur from week 10 (34) and egg output diminishes over time until the infection is eliminated (35, 36). This phenomenon only occurs above a threshold worm burden (35, 36), presumably as sufficient immune stimulus is required for this process to occur (23, 24). This self-cure mechanism is thought to be antibody-mediated because of a strong inverse association between the rapidity and intensity of the IgG response and the number and morphology of surviving worms (34). Two-dimensional immunoblotting of worm extracts showed the immune response to be directed at gut digestive enzymes, tegument surface hydrolases, and anti-oxidant enzymes (34).

The use of protein microarrays to profile the immune response to pathogens has become widespread over recent years and offers significant advantages over the conventional immuno-proteomic approaches described above. In parasitology, protein array studies have been used extensively in malaria (37) to compare antibodies from un-protected and protected subjects, identifying the antibodies (and their cognate antigens) that confer immunity (38–40). For schistosomes (37), similar studies have profiled antibody responses in *S. japonicum*- and *S. mansoni*-infected rodents (41, 42) and human subjects who are naturally resistant or susceptible to *S. mansoni* (20).

Based on the success of our previous immunomics approach which analyzed antibody signatures of PR and chronically infected (CI) individuals from an *S. mansoni*-endemic area of Brazil (20), we decided to use the same experimental approach to identify antigens which are the targets of humoral immune responses in (1) DIR human subjects from an *S. haematobium*-endemic area in Africa and (2) rhesus macaques that had undergone self-cure after experimental *S. japonicum* infection. Given the extensive similarities in protein-coding gene sequences between the three major human schistosomes (86–92%) (43), as well as the extensive recognition of *S. japonicum* proteins on our array by sera from *S. mansoni*-infected individuals (20), we reasoned that sera from *S. haematobium*-infected individuals would strongly recognize many of the arrayed *S. mansoni* and *S. japonicum* proteins. Moreover, these cross-reactive antigens would potentially form

the basis of a pan-schistosome vaccine that protects against all three human species. Leveraging existing protein arrays from our previous study, which contain antigens primarily from the antibody-accessible teguments of the adult fluke and the immunologically vulnerable schistosomulum stage, we show that DIR individuals and self-curing rhesus macaques make robust antibody responses to a number of tegument-associated proteins, including novel and previously described schistosome vaccine candidates.

Materials and Methods

Ethical Statement

Ethical and institutional approval was granted by the Medical Research Council of Zimbabwe and the University of Zimbabwe Institutional Review Board. Local permission for the study was granted by the Provincial Medical Director. The study design, aims, and procedures were explained in the local language, Shona, prior to enrollment. Participants were free to drop out of the study at any time and informed written consent was obtained from all participants prior to taking part in the study and to receiving anthelmintic treatment. As routine, all participants were offered treatment with the standard dose of PZQ (40 mg/kg) at the end of the study. All work involving experimental procedures with Rhesus macaques was approved by the Ethics Committee of Kunming Institute of Zoology, Chinese Academy of Sciences (CAS) (ID: SYDW-2011017).

Study Cohort

The study participants were residents of a *S. haematobium*-endemic rural village in Murewa in the Mashonaland East Province of Zimbabwe (31°94′E; 17°67′S). The village was selected because health surveys regularly conducted in the region showed little or no infection with soil-transmitted helminths (STH) and a low *S. mansoni* prevalence (<2%). Serum samples were provided from a cohort of *S. haematobium*-infected individuals ($n = 106$) aged 5–14 years who had never been treated with PZQ prior to this study and were free from co-infection with other helminths, *Plasmodium*, and HIV (14, 44). At the start of the study (baseline), subjects who were positive for *S. haematobium* eggs (at least one egg found in at least one of three urine samples, each collected on a separate day) following urinalysis were treated with PZQ by weight (40 mg/kg) and then assessed by urinalysis at 6 weeks to confirm clearance of the infection (no eggs found in any of three urine samples, each collected on a separate day). Individuals were followed for 18 months and maintained regular water contact throughout this period. Subjects were assessed for infectivity with *S. haematobium* at 6 months and at the end of the study. Individuals who were egg-positive at 18 months post-treatment ($n = 32$) were deemed CI and those who were egg-negative ($n = 74$) were categorized as DIR (**Figure 1**). Serum samples were obtained from both 0- and 18-month timepoints.

For this study, we selected a subset of subjects as follows: CI subjects that had the highest post-treatment egg burdens (eggs/10 ml 10–104; $n = 10$) and DIR subjects that had some of the highest egg burdens at baseline (eggs/10 ml 44–743; $n = 10$), reasoning that these individuals represented extremities of the DIR and CI spectrums and therefore would maximize the likelihood of identifying differences in antibody signatures between CIs and DIRs. Subject ages (in years) were as follows: CIs (5, 8, 8, 9, 10, 10, 11, 11, 12, 14), range = 5–14, mean = 9.8, median = 10; DIRs (6, 8, 8, 8, 8, 9, 9, 10, 11, 12), range = 6–12, mean = 8.9, median = 8.5.

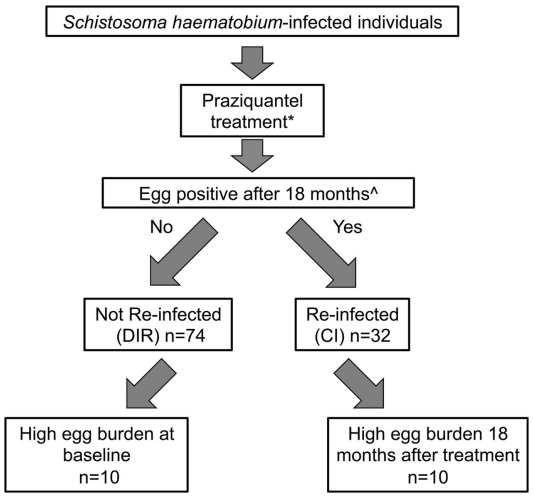

FIGURE 1 | Characterization of study cohort and sub-cohort used for the study described herein. *Treatment efficacy was assessed by urinalysis 6 weeks after praziquantel therapy – all subjects were egg-negative (no eggs found in any of three urine samples, each collected on a separate day). ^Subjects remained in the endemic study area and had regular water contact for the study duration.

Infection of Self-Curing Rhesus Macaques

The study used six captive-bred adult male rhesus macaques (*M. mulatta*; mean age 9.67 ± 0.82 years, mean weight 7.98 ± 0.85 kg) from the Kunming Primate Research Center, CAS. Macaques were group-housed prior to the experiment but then singly after infection for fecal sampling. Cercariae of *S. japonicum* were shed from patent snails (*Oncomelania hupensis*) provided by the Jiangsu Institute of Parasitic Diseases (Wuxi, China), collected from the water surface using a bacteriological loop and placed on glass cover slips for infection. Rhesus macaques anesthetized with ketamine hydrochloride (6 mg/kg body weight, Gutian Pharmaceutical Corporation, Fujian, China) were infected percutaneously with 600 cercariae via the shaved abdominal skin for 30 min. Blood was obtained by intravenous sampling prior to infection (week 0) and at 12 and 20 weeks after exposure. Elimination of infection was confirmed at week 20 by assessment of eggs per gram of feces using both the Percoll technique (45) and Kato-Katz method (46).

Probing of Protein Microarrays with Human and Macaque Sera

Protein microarrays were leveraged from a previous study by us (20) and contained both *S. mansoni* ($n = 45$) and *S. japonicum* ($n = 172$) proteins which were either (1) known or predicted to be localized to the tegument and/or (2) expressed in the schistosomulum (41), which is vulnerable to immune attack. Human IgG1 and IgE responses to antigens were determined by probing arrays with sera as previously described (20). Macaque antibody responses

were determined by probing of arrays with sera as described for human sera with the exception that a goat anti-monkey IgG-biotin (1:500) (Sigma) secondary antibody was used.

Protein Array Data Analysis and Bioinformatics

Array data analysis was conducted using the "group average" method (20), where the mean signal intensity (SI) of the negative control (empty vector) spots for all sera were subtracted from the SI of each protein spot. The following reactivity cut-offs (calculated as one standard deviation above the negative control spots for all groups) were used: human IgG1 – 8239; human IgE – 1861; macaque IgG – 3210. Statistical analyses (Student's *t*-test) were conducted with Graphpad Prism 6 to determine significant differences between samples for a given reactive protein.

The transcription of genes in the adult and egg stages of *S. haematobium* was assessed for *S. haematobium* orthologs of all arrayed *S. mansoni* and *S. japonicum* proteins that were the targets of significantly different IgG responses between DIR and CI post-treatment sera using publicly available RNA-seq data (43). These data were filtered for quality (PHRED score of >30) using Trimmomatic (47) and aligned to the open reading frames of the published gene set (43) using Bowtie (v2.1.0) (48). Normalized levels of gene transcription were calculated using the software package RSEM (v1.2.11) (49) and reported as the numbers of transcripts per million reads sequenced (TPMs). The TPM value of each gene was $\log_2$-transformed and subjected to heat map visualization using R (v3.1.2)[1], and utilizing the heatmap.plus (v1.3)[2] package.

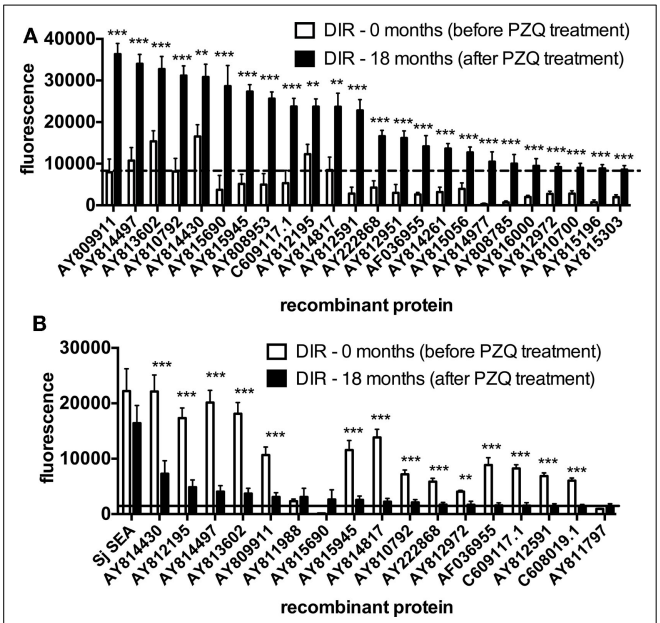

FIGURE 2 | Antibody responses to arrayed antigens differ in *Schistosoma haematobium*-infected humans before and after praziquantel treatment. **(A)** IgG1. **(B)** IgE. Average adjusted signal intensity values depicting the antibody response to each reactive antigen are shown for the drug-induced resistant (DIR) cohort before and after praziquantel treatment. The dashed and solid lines are the respective cut-offs for IgG1 (8239) and IgE (1861) reactivity, calculated as one standard deviation of the mean of the no-DNA control spots on the array. Statistical analysis was performed using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Results

Antibody Signatures of DIR Human Subjects Differ Before and After PZQ Treatment

To investigate the difference in antibody responses to arrayed antigens of the DIR cohort before and after PZQ treatment (therefore identifying antigens which are putatively exposed by drug therapy), sera from this group at baseline and 18 months after drug therapy were used to probe protein microarrays. IgG1 responses were significantly higher in DIRs at 18 months post-treatment compared to baseline for all 24 reactive proteins. Antigens which were the target of the most significantly different ($p < 0.0001$) responses pre- and post-drug treatment included AY810700 (glucose transporter), AY815303 [glutathione-*S*-transferase (GST)], and AY809911 (Ig domain-containing, sensory guidance protein) (**Figure 2A**). In contrast, IgG1 responses of the CI cohort to reactive proteins before and after PZQ treatment were not significantly different for any protein (data not shown). Additionally, IgE responses in the DIR group were significantly lower at 18 months post-PZQ treatment compared to baseline for the majority (78%) of the 18 reactive antigens (**Figure 2B**). Arrayed antigens that were the targets of IgE in post-treatment DIRs included AY814430 (calpain), AY812195 [extracellular superoxide dismutase (SOD)], and AY814497 (Na^+/K^+ ATPase β subunit – SNaK1β).

IgG1 Profiles Differ Between *S. haematobium*-Infected Humans Who Do and Do Not Acquire DIR After PZQ Treatment

In order to analyze changes in antibody signatures to arrayed antigens related to the acquisition of DIR (thereby identifying proteins which are potential inducers of a protective antibody response), arrays were interrogated with sera from post-treatment CIs and DIRs and probed for IgG1 reactivity. IgG1 responses were significantly elevated in DIRs compared to CIs at 18 months to 20 of the 24 (83%) reactive proteins. The three antigens that were targets of the most significantly different ($p < 0.0001$) IgG1 responses were AY810792 (butyrylcholinesterase), AY812951 (mastin), and AY815196 [a homolog of human tetraspanin (TSP)-33] (**Figure 3**). Homologs and/or family members of known schistosome vaccine candidates such as calpain (50) (AY814430), a 28-kDa GST – *Sh*28GST (51) (AY815303), and the TSPs *Sm*-TSP-1 and *Sm*-TSP-2 (33, 52) (AY815196) were also identified. **Table 1** lists all of the antigens depicted in **Figure 3** along with their *S. haematobium* orthologs as we reasoned that these were probably the native parasite antigens that our DIR and CI sera were targeting during the course of *S. haematobium* infection. Of the 20 antigens that were targets of significantly elevated IgG1 responses in post-treatment DIRs compared to CIs, only 7 (35%) were targets of IgE responses that were deemed to be above the reactivity cut-off (**Figure 4**).

[1]http://www.R-project.org
[2]http://cran.r-project.org/web/packages/heatmap.plus

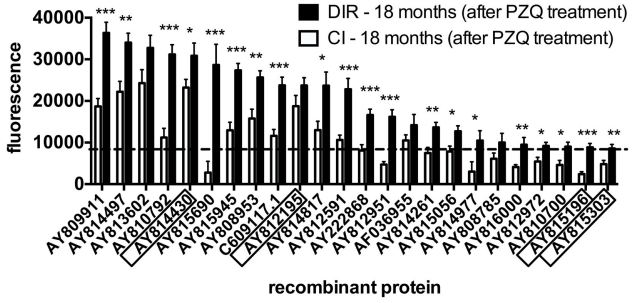

FIGURE 3 | IgG1 antibody profiles to arrayed antigens differ between *Schistosoma haematobium*-infected humans who do and do not acquire drug-induced resistance after praziquantel treatment. Average adjusted signal intensity values depicting IgG1 antibody responses to each reactive antigen are shown for the drug-induced resistant (DIR) and chronically infected (CI) cohorts after praziquantel treatment. Boxed antigens indicate homologs of known vaccine candidates. The dashed line is the cut-off for IgG1 reactivity (8239), calculated as one standard deviation of the mean of the no-DNA control spots on the array. Statistical analysis was performed using Student's *t*-test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Transcription Analysis

The transcription of genes in the adult and egg stages of *S. haematobium* was assessed for orthologs of all 20 arrayed *S. mansoni* and *S. japonicum* proteins that were the target of significantly different DIR IgG responses post-treatment using publicly available RNA-seq data. We did not find any significant difference in the level of transcription between life stages for a given protein. MS3_02176 (the gene encoding microsomal GST-3) was expressed most highly and relatively constitutively in all developmental stages examined (**Figure 5**).

IgG Profiles of *S. japonicum*-Infected Self-Curing Rhesus Macaques Differ During the Course of Infection

To investigate IgG responses of rhesus macaques to arrayed proteins during the course of a self-curing infection, protein arrays were probed with sera taken at week 0 (primary infection), week 12, and week 20 (after parasite elimination). Antibody responses to all (eight proteins – **Table 1**) but one reactive protein (AY812195 – extracellular SOD) were significantly elevated between 0 and 12 weeks post-infection (p.i.), with the three most robust and highly significant responses being aimed at proteins of unknown function (AY815838 and AY812161) and a MARVEL domain-containing lipid-raft-associated protein (AY815056). The IgG reactivity of only one protein (AY812195 – extracellular SOD) was elevated at 20 weeks compared to 12 weeks p.i. (**Figure 6**; **Table 1**).

Three Different Disease Models of Resistance to Schistosomiasis Reveal Common Reactivity to Some Arrayed Proteins

We searched for reactive proteins common to DIR human subjects, *S. japonicum*-infected self-curing rhesus macaques (both described herein), and humans living in an *S. mansoni*-endemic area of Brazil who, unlike DIRs, have never been treated with PZQ but are putatively resistant to infection (20). Three reactive proteins were common targets of "protective" antibody responses in the DIR and macaque models: a MARVEL domain-containing lipid-raft-associated protein; a glucose transporter (SGTP1); and an extracellular SOD (although the IgG response to this protein was not significantly elevated between DIRs and CIs after PZQ treatment). Two reactive antigens were commonly recognized by both DIRs and PRs: ribosome-binding protein 1 and the beta subunit of Na^+/K^+ ATPase (SNaK1β) (**Figure 7**).

Discussion

The critical role that antibodies play in resistance to schistosomiasis resistance has been well established in animal models by numerous passive transfer studies [e.g., Ref. (59, 60)], and there is evidence that some mechanisms of protective immunity in humans are antibody-mediated, both in individuals naturally resistant to schistosomiasis (20) and those who acquire resistance after PZQ therapy (25). Herein, we describe the antibody reactivity profiling of a schistosome protein array with sera from *S. haematobium*-exposed DIR and CI individuals and rhesus macaques self-cured of a *S. japonicum* experimental infection (34) in an effort to identify schistosome antigens that might be the targets of resistant human and non-human primate hosts. We previously utilized this protein microarray to define the antibody signatures of individuals that are either naturally resistant to or CI with *S. mansoni* in a schistosomiasis endemic area of Brazil (20). We restricted our antibody isotype analyses to IgG1 and IgE. IgG1 is one of the main drivers of the protective humoral response to schistosomiasis (23, 24), an observation supported by studies showing that key tegument vaccine antigens like Smp80 (calpain), *Sm*-TSP-2, and Sm29 are the targets of these responses in schistosome-resistant individuals (32, 33, 61). IgE is thought to be critical in resistance to schistosomiasis, including the DIR process (25, 62, 63), but caution is warranted in development of anti-helminth vaccines that drive IgE responses due to potential anaphylactic responses in individuals who are pre-sensitized from chronic helminth infection/exposure (64).

Significantly elevated IgG1 responses were detected to 24 antigens in DIR subjects 18 months after therapy compared to pre-treatment responses. In stark contrast, we did not detect elevated IgG1 responses to any proteins in CI subjects at 18 months post-treatment compared to pre-treatment levels. None of these antigens were recognized in a previous study by us in which pooled sera from *S. haematobium*-exposed individuals before and after PZQ treatment were used to probe 2D gels containing *S. haematobium* SWAP (27), likely because the majority of proteins on the array are membrane-associated tegument proteins and might not be well represented in SWAP due to the very mild solubilizing nature (Tris) of the preparation.

It is noteworthy that IgG1 reactivity to a further 105 (48%) arrayed antigens was significantly higher in post- compared to pre-treatment DIRs but signal intensities were below the cut-off, so the proteins were deemed non-reactive. This decreased level of reactivity possibly reflects the heterogeneity of the antigen–antibody interaction, i.e., antibodies to *S. haematobium* proteins are reacting with a protein array containing *S. mansoni* and *S. japonicum* antigens. Indeed, significant differences in

TABLE 1 | Arrayed proteins significantly reactive to *S. haematobium*-infected DIR post-treatment sera and *S. japonicum*-infected, self-curing rhesus macaque sera.

Array ID (GenBank accession number)	Description	Reactivity difference[a] (*p* value)	Frequency of recognition (%)	*S. haematobium* homolog; amino acid identity with arrayed antigen				Therapeutic use
				GenBank accession number	Description/aa homology	Length (aa)	TM domains[b]	
DIR-reactive proteins								
AY810792	Butylcholinesterase (*S. japonicum*)	6.97×10^{-6}	100	MS3_01257	Acetylcholinesterase; 86%	745	1	IgG to *S. mansoni* AchE drives complement-mediated killing of somules by 75–95% (53)
AY812951	Mastin (*S. japonicum*)	7.10×10^{-6}	90	MS3_04920	Plasminogen; 70%	492	1	
AY815196	Similar to NM_079585 tetraspanin 86D in *Drosophila melanogaster* (*S. japonicum*)	1.40×10^{-5}	50	MS3_02232	Tetraspanin-33; 81%	259	4	Vaccination with members of this family (*Sm*-TSP-1 and *Sm*-TSP-2) induces 65–69% protection in a mouse model of schistosomiasis (33). Vaccination with a member of this family (Sj23) induces 35% protection in a mouse model of schistosomiasis (54)
AY815945	SJCHGC09124 protein (*S. japonicum*)	2.29×10^{-5}	100	MS3_10649	Hypothetical protein; 73%	141[c]	3	
AY809911	SJCHGC02149 protein (*S. japonicum*); putative immunoglobulin domain superfamily (sensory guidance protein) (*S. mansoni*); 90%	3.06×10^{-5}		MS3_07405	Hypothetical protein; 87%	574[c]	1	
C609117.1	Succinate dehydrogenase (*S. mansoni*)	1.37×10^{-4}	100	MS3_03684	Succinate dehydrogenase cytochrome b560 subunit, mitochondrial; 93%	379	2	
AY815690	Myosin-7 (*S. japonicum*)[d]	2.25×10^{-4}	80	MS3_09744	Ribosome-binding protein 1; 90%	775	0	
AY812591	SJCHGC04069 protein (*S. japonicum*)	4.25×10^{-4}	100	MS3_01313	Hypothetical protein (RNA binding); 71%	392	0	
AY222868	SJCHGC06654 protein (*S. japonicum*)	4.45×10^{-4}	90	MS3_04717	Large subunit ribosomal protein; 48%	150	1	
AY808953	Zinc finger CCCH domain-containing protein 3 (*S. japonicum*)	0.0023	100	MS3_10292	Hypothetical protein; 41%	201	0	
AY814497	SJCHGC02432 protein (*S. japonicum*)	0.0027	100	MS3_04817	Hypothetical protein; 58%	351	0	
AY814261	Ectonucleotide pyrophosphatase/phosphodiesterase family member 5 (*S. japonicum*)	0.0033	90	MS3_08684	Ectonucleotide pyrophosphatase/phosphodiesterase family member 5; 67%	452	1	Suppression of *S. mansoni* ortholog (SmNPP-5) impairs the parasite's ability to establish infection *in vivo* (55)
AY816000	Cytochrome b-561 (*S. japonicum*)	0.0081	60	MS3_10028	Cytochrome b-561; 85%	242	6	
AY815303	Similar to microsomal glutathione *S*-transferase in *Oryctolagus cuniculus* (*S. japonicum*)	0.0099	50	MS3_02176	Microsomal glutathione *S*-transferase 3; 85%	151	3	A member of this protein family (*Sh*28GST) is undergoing clinical trial as a vaccine against *S. haematobium* (51)

(Continued)

TABLE 1 | Continued

Array ID (GenBank accession number)	Description	Reactivity difference[a] (*p* value)	Frequency of recognition (%)	*S. haematobium* homolog; amino acid identity with arrayed antigen				Therapeutic use
				GenBank accession number	Description/aa homology	Length (aa)	TM domains[b]	
AY810700	Solute carrier family 2 protein (*S. japonicum*)	0.0100	50	MS3_02545	Solute carrier family 2, facilitated glucose transporter member 1; 85%	522	12	Suppression of *S. mansoni* ortholog (SGTP1) impairs the parasite's ability to establish infection *in vivo* (56)
AY812972	SJCHGC02374 protein (*S. japonicum*)	0.0106	60	MS3_11481	Hypothetical protein; 90%	71[c]	0	
AY814817	SJCHGC06849 protein (*S. japonicum*)	0.0130	90	MS3_05945	Hypothetical protein (TATA-box binding); 71%	416[c]	0	
AY815056	SJCHGC06191 protein (*S. japonicum*), marvel-containing potential lipid-raft-associated protein (*S. mansoni*); 90%	0.0155	80	MS3_07473	Hypothetical protein; 91%	215	4	
AY814977	Nervana 2 (*S. japonicum*)	0.0381	70	MS3_03655	Sodium/potassium-transporting ATPase subunit beta-2; 87%	293	1	Suppression of *S. mansoni* ortholog (SNaK1β) impairs the parasite's ability to establish infection *in vivo* (57)
AY814430	Calpain (*S. japonicum*)	0.0497	100	MS3_02003	Calpain; 83%	2028	0	*S. mansoni* ortholog (Smp80) induces 64% protection in a baboon model of schistosomiasis (50)
Macaque-reactive proteins								
AY815838	SJCHGC05998 protein (*S. japonicum*)	1.18×10^{-8}[e], 2.29×10^{-8}[f]	100	N/A				
AY812161	UPF05056 protein (*S. japonicum*)	8.72×10^{-4}[e], 1.02×10^{-6}[f]	100	N/A				
AY815056	SJCHGC06191 protein (*S. japonicum*), marvel-containing potential lipid-raft-associated protein (*S. mansoni*); 90%	5.80×10^{-8}[e], 2.36×10^{-4}[f]	100	N/A				
AY810700	Solute carrier family 2 protein (*S. japonicum*)	0.0071[e]	33	N/A				Suppression of *S. mansoni* ortholog (SGTP1) impairs the parasite's ability to establish infection *in vivo* (56)
AY812195	Extracellular superoxide dismutase (Cu–Zn) (*S. japonicum*)	0.0071[f]	83	N/A				*S. mansoni* ortholog (SmCT-SOD) induces 39% protection in a mouse model of schistosomiasis (58)
AY814158	Major egg antigen (p40) (*S. japonicum*)	0.0444[e]	67	N/A				
AY808379	SJCHGC09517 protein (*S. japonicum*)	0.0454[e]	17	N/A				
AY09526	SJCHGC09219 protein (*S. japonicum*)	0.0504[e], 0.0067[f]	67	N/A				

[a] *For DIR-reactive proteins, difference is in elevation of IgG1 response of DIRs compared to CIs post-treatment.*
[b] *Transmembrane (TM) domains predicted by TMHMM 2.0. For full-length proteins, first TM domain contains an N-terminal signal sequence.*
[c] *Proteins lack start methionine.*
[d] *We believe the sequence represented by AY815690 ["myosin-7 (S. japonicum)"] has been incorrectly annotated due to its high degree of homology with other parasite orthologs of ribosome-binding protein 1 and lack of hits with any form of myosin.*
[e] *Difference in elevation of IgG response between 0 and 12 weeks p.i.*
[f] *Difference in elevation of IgG response between 0 and 20 weeks p.i.*

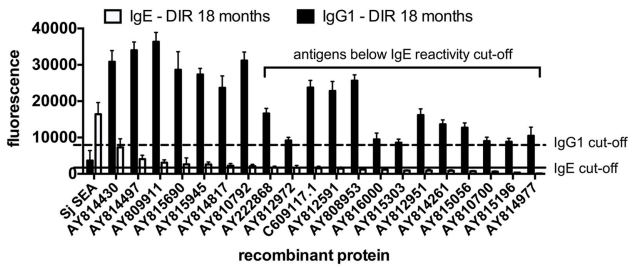

FIGURE 4 | Some arrayed antigens that induce IgG1 responses in *Schistosoma haematobium*-infected humans who acquire drug-induced resistance after praziquantel treatment are not the targets of IgE. Average adjusted signal intensity values depicting IgG1 and IgE antibody responses to each IgG1 antigen reactive to post-treatment sera from drug-induced resistant (DIR) humans. The dashed and solid lines are the respective cut-offs for IgG1 (8239) and IgE (1861) reactivity, calculated as one standard deviation of the mean of the no-DNA control spots on the array. *Schistosoma japonicum* SEA is included for comparative purposes.

antibody recognition patterns were observed when using sera from *S. haematobium*-exposed people to probe crude antigen preparations from the closely related *S. bovis*, and vice versa (65). Moreover, sequence variation in the epitopes of *Sh*28GST, and its homologs from *S. mansoni* and *S. bovis* significantly altered the immune response generated by the host (66).

Twenty reactive arrayed antigens were the targets of significantly greater IgG1 responses in DIRs compared to CIs post-treatment. A further 72 (33%) proteins were the target of significantly different IgG1 recognition profiles between DIRs and CIs but were below the reactivity cut-off. We hypothesize that at least some of these IgG1-reactive proteins are major targets of protective immunity, engendering resistance to schistosomiasis through an antibody-mediated neutralization of the cognate antigen, the role of which is essential to the survival of the parasite within the host (e.g., nutrient acquisition, immune evasion) such that disruption of its function results in worm impairment. Indeed, some of these antigens are protective in animal challenge models of schistosomiasis; for example, vaccination with and the Ca^{2+}-activated protease, calpain (AY814430), induces 64% in baboons (50). *Sh*28GST (a homolog of the arrayed immunoreactive protein AY815303) is a multi-functional enzyme present in the tegument and sub-tegument of adult (67) and larval (68) schistosomes and the current focus of vaccine trials in humans (51). Its exact function is unknown [studies suggest it may aid in immune evasion by the parasite through its role in fatty acid metabolism and prostaglandin D2 synthesis (69), but vaccine efficacy has been attributed to the induction of antibodies that neutralize enzyme activity (70)]. Other extracellular enzymes were prominent amongst the IgG1-reactive proteins, including proteases (calpain, mastin), esterases, and superoxide dismutase, so it is tempting to speculate that antibodies to these enzymes neutralize key physiological processes (71, 72), and this now warrants further investigation. Members of the TSP family in schistosomes (*Sm*-TSP-1 and *Sm*-TSP-2) are four-transmembrane domain proteins located within the tegument of larval and adult worms that have functions in membrane biogenesis (73). TSP-based vaccines have shown to be efficacious against schistosomiasis with *Sm*-TSP-1 and *Sm*-TSP-2 (33, 52) and *Sj*23 (54, 74) conferring protection in animal challenge models.

Other significant IgG1 responses were aimed at tegument-associated proteins that play fundamental roles in parasitism. Surface-associated acetylcholinesterase (AChE) (AY810792) has been implicated in the regulation of glucose scavenging from host blood (75) and anti-AChE antibodies facilitate complement-mediated killing of larval schistosomes (53). Genes encoding the glucose transporter SGTP1 (AY810700), Na^+/K^+ ATPase subunit SNaK1β (AY814977) and ectonucleotide pyrophosphatase/phosphodiesterase SmNPP-5 (AY814261) have all been functionally silenced within schistosomes using RNAi (55–57), resulting in impairment of the worm's ability to establish infection in the host and highlighting their importance to parasite survival.

Significantly IgG1-reactive proteins whose therapeutic potential has not yet been examined include mastin (AY812951) and a MARVEL domain-containing lipid-raft-associated protein (AY815056). Mastin is a trypsin-like serine protease and, in schistosomes, proteases of this class are known as cercarial elastases (CEs) for their role in skin degradation to facilitate penetration of the free-living cercaria into the definitive host (76). Mastin, however, differs in structural homology to CEs and has been assigned to a group of "non-CE" serine proteases (77). The five members of this group are yet to be functionally characterized in terms of their roles in parasitism, but mastin is unique in that it is highly upregulated in the intra-mammalian schistosomula and adult stages [60 and 150% relative to the constitutively expressed *smcox1*, respectively (77) compared to the free-living stages of the parasite (78, 79), alluding to a fundamental parasitic function]. MARVEL domains have four-transmembrane helix architecture and proteins containing these motifs associate with membrane micro-domains and have been implicated in membrane biogenesis (80). In a pathogenesis context, the MARVEL domain-containing protein Nce102 regulates actin organization and invasive growth of *Candida albicans*, with Nce102 deletion mutants showing decreased virulence in mice (81). Antigens such as mastin and the MARVEL domain protein are attractive vaccine candidates for the reasons described herein as well as the successful use of proteases (82–84) and membrane structural proteins as anti-helminth vaccines [e.g., Ref. (33, 54, 85, 86)].

A group of ribosome-associated proteins were also the targets of significantly higher IgG1 responses in DIRs compared to CIs post-treatment and included ribosome-binding protein 1. Ribosome-associated proteins have received attention in the field of parasite immunology because of their classification as "patho-antigens" – conserved intracellular molecules capable of inducing an immunopathological response (87). Patho-antigens such as acidic ribosomal protein P0 conferred protection as vaccines against the intracellular parasites *Leishmania major* (87) and *Plasmodium yoelii* (88) in mouse challenge models of infection, and antibodies to *P. falciparum* P0 have been detected in individuals who are immune to malaria (89). The roles of these antigens, such as ribosome-binding protein 1, in the induction of anti-schistosome immunity is unclear, but it is possible that these intracellular molecules are stimulating host immune effectors through exosome-mediated pathways [recently identified in related helminths (90, 91)]. It should also be noted that

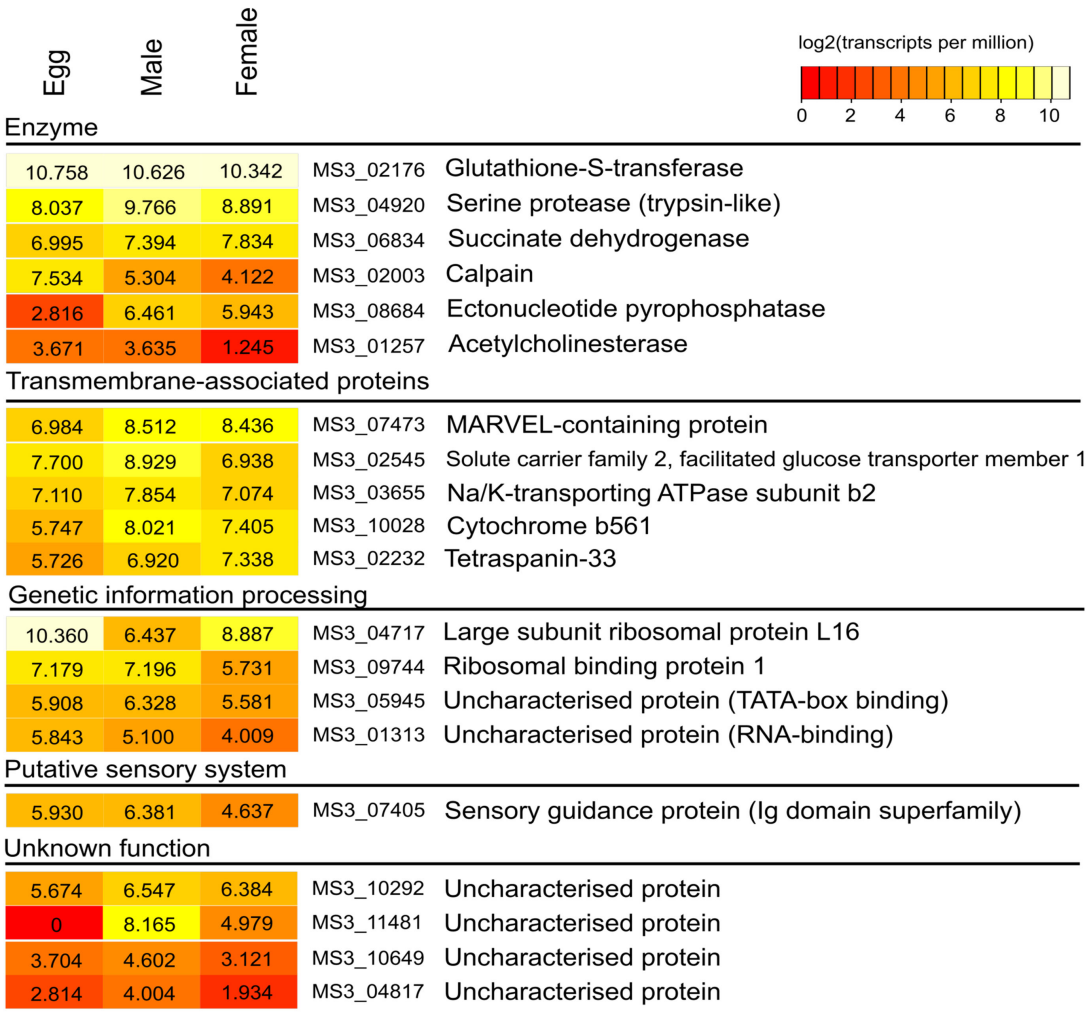

FIGURE 5 | Gene transcription in the adult and egg stages of *S. haematobium* for all arrayed proteins inducing significantly different and reactive IgG responses to DIR post-treatment sera. Data were assembled from publicly available RNA-seq databases (43). These data were filtered for quality (PHRED score of >30) using Trimmomatic (8) and aligned to the open reading frames of the published gene set (7) using Bowtie (v2.1.0) (9). Normalized levels of gene transcription were calculated using the software package RSEM (v1.2.11) (10) and reported as the numbers of transcripts per million reads sequenced (TPMs). The TPM value of each gene was $\log_2$-transformed and subjected to heat map visualization using R.

ribosome-binding protein 1 was one of the two antigens recognized by both *S. mansoni*-exposed PR subjects in Brazil and *S. haematobium*-exposed DIR subjects in Africa (**Figure 6**), possibly highlighting a common role in different mechanisms of schistosomiasis resistance.

IgE responses to arrayed antigens were, for the most part, significantly weaker in post-therapy DIRs compared to pre-treatment responses, which appears to be in contrast to the positive association between IgE levels and the process of acquiring DIR status (25, 62). This could be likely for two reasons: (1) these earlier studies on DIR employed soluble antigen preparations to detect IgE responses, whereas the majority of arrayed proteins are membrane-associated and therefore would not have been present in buffer-soluble parasite extracts or (2) the DIR cohort, being egg-negative, does not receive the IgE-inducing stimulus of egg antigens (92). The latter explanation may be supported by the case of extracellular SOD (AY812195); the IgE response to this protein was significantly lower in egg-negative, post-treatment DIRs (**Figure 2B**) but significantly higher in egg-positive, post-treatment CIs (data not shown). Indeed, a recent study describing the prediction of IgE-binding antigens in *S. mansoni*-infected individuals reported no significant change in the IgE response to extracellular SOD before and 5 weeks after PZQ treatment (93), which lends support to the observation that the waning IgE response to some antigens in DIRs might be due to the reduced amount of IgE-inducing stimulus received by this cohort. Less than half of the antigens that were significantly reactive for DIR post-treatment IgG1 compared to pre-treatment levels were reactive (above the cut-off) for IgE responses.

IgE poses somewhat of a conundrum for helminth vaccinologists due to its clear association with naturally acquired protection (22, 63), but the accompanying risk of vaccinating people with a recombinant protein that is the target of pre-existing IgE responses and poses the risk of inducing atopy (64), or potentially

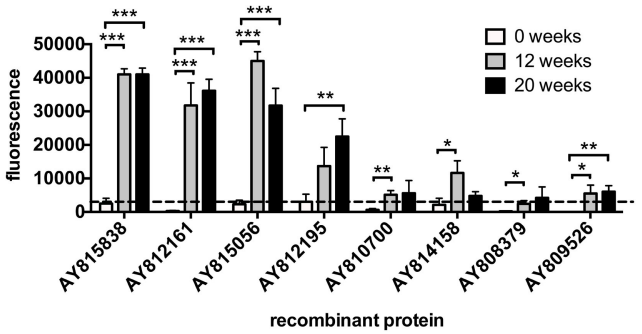

FIGURE 6 | IgG antibody profiles to arrayed antigens differ in *Schistosoma japonicum*-infected, self-curing rhesus macaques during the course of infection from exposure to perfusion. Average adjusted signal intensity values depicting IgG antibody responses to each significantly reactive antigen are shown at baseline (0 weeks), 12 weeks post-infection, and elimination (20 weeks post-infection). The dashed line is the cut-off for IgG reactivity (3210), calculated as one standard deviation of the mean of the no-DNA control spots on the array. Statistical analysis was performed using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

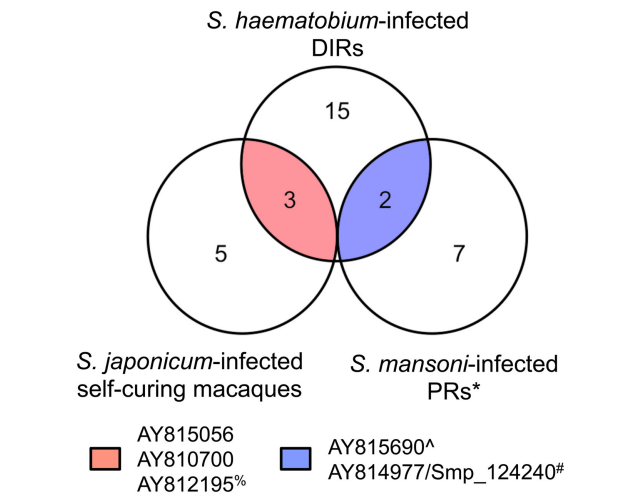

FIGURE 7 | Different disease models of schistosomiasis resistance show common IgG responses to some arrayed antigens. Venn diagram depicting common IgG reactive proteins between *Schistosoma haematobium*-infected humans from an endemic area in Africa who acquire drug-induced resistance (DIR) after praziquantel treatment, *Schistosoma japonicum*-infected self-curing rhesus macaques, and *Schistosoma mansoni*-infected humans from an endemic area of Brazil who are naturally resistant (PRs). *Data from Gaze et al. (20); %IgG1 response to AY812195 is significantly different between DIRs before and after praziquantel treatment but not between DIRs and CIs post-treatment; #AY814977 and Smp_124240 are the respective *S. japonicum* and *S. mansoni* orthologs of SNaK1β. ^We believe the sequence represented by AY815690 ["myosin-7" (*S. japonicum*)] has been incorrectly annotated due to its high degree of homology with other parasite orthologs of ribosome-binding protein 1 and lack of BlastP hits with any form of myosin.

anaphylaxis. Instead of excluding potentially protective IgG1-inducing antigens that are the targets of parasite-derived IgE in exposed individuals from further vaccine development, we propose that the molecules be assessed for allergenicity through the use of basophil-activation studies, given that the induction of IgE and clinical manifestation of allergy are not mutually inclusive events (94). Another strategy aimed at minimizing potential allergenicity of helminth proteins involves their fusion to Fcγ, thereby directing the chimeric protein to the negative signaling receptor FcγRIIb expressed on pro-allergic cells (95).

The IgG1 response in *S. japonicum*-infected self-curing macaques to the majority of reactive antigens was significantly higher at 12 weeks p.i. [around the time that worm death starts to occur (34)] compared to week 0. Proteins that were the target of these antibodies included a protein with weak sequence homology to a bacterial hydrolase (AY815838), extracellular SOD (AY812195), and the previously discussed glucose transport and MARVEL domain-containing proteins. Extracellular SOD is thought to facilitate the parasite's evasion of the immune response by neutralizing the effects of reactive oxygen and nitrogen species and has proven efficacious in murine vaccine trials (58). Moreover, both hydrolases and anti-oxidant enzymes were suggested to be the targets of IgG-mediated worm elimination in a previously established macaque self-cure model of schistosomiasis (34).

Given the cognate recognition of antigen by both B and helper T cells in the immune response, we hypothesize that the best antigens for a recombinant protein vaccine are those that elicit responses by both antibodies and T cells during the acquisition of DIR. The antigens described herein should now be subjected to further refinement by assessing their ability to drive T-cell proliferation *ex vivo*. T-cell profiling of B-cell antigens has been conducted for the vaccinia virus (discovered using protein array profiling) where plasmids encoding arrayed proteins were expressed as inclusion bodies and screened for T-cell reactivity in a high-throughput format (96).

In this pilot study, we have described the screening of a schistosome protein array to identify potential targets of protective immunity in *S. haematobium*-infected people who acquire DIR after PZQ treatment, with the hypothesis that these antigens are responsible for essential parasitic functions such that antibody-mediated neutralization of these molecules result in worm impairment or death. While the modest number of targets identified from this work may be reflective of the heterogeneity between the antigens and sera used in the study, a benefit of this approach is the identification of proteins that are cross-reactive between *S. haematobium*, *S. japonicum*, and *S. mansoni*, a desirable feature of a vaccine antigen if it is to be protective against all medically important schistosome species. If a pan-schistosome vaccine is developed, it will likely be part of a control program that integrates a vaccination cocktail of multiple recombinant antigens with chemotherapy, and so a comprehensive portfolio of the targets of DIR is a crucial component of the vaccine discovery strategy. Future iterations of our protein array will be expanded to represent even more of the schistosome proteome, ensuring that an extensive complement of DIR-reactive vaccine antigens will be available for progression into further development.

Acknowledgments

We would like to thank the residents and children of Chipinda village as well as the teachers from Chipinda primary and secondary school for their participation and generous and kind support

of this study. We are also very grateful for the co-operation of the Ministry of Health and Child Welfare in Zimbabwe. We like to thank the Provincial Medical Director of Mashonaland East, the Environmental Health Workers, and nursing staff at Chitate Clinics and Murehwa Hospital. We are grateful to the members of the National Institutes of Health in Harare and the Biochemistry Department at University of Zimbabwe for technical and logistical support. This research received financial support from the National Health and Medical Research Council of Australia (NHMRC) (Program Grant no: APP1037304), the Wellcome Trust UK (Grant no: WT082028MA), the Cunningham Trust, the Carnegie Trust for the Universities of Scotland, and the Shanghai Science and Technology Commission (Grant no: 15ZR1444300). DM is an NHMRC Senior Principal Research Fellow, AL and DD are NHMRC Principal Research Fellows. NY is an NHMRC Early Career Research Fellow.

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frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 28 January 2015
doi: 10.3389/fimmu.2015.00022

Kicking in the guts: *Schistosoma mansoni* digestive tract proteins are potential candidates for vaccine development

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Schistosomiasis is a debilitating disease that represents a major health problem in at least 74 tropical and subtropical countries. Current disease control strategies consist mainly of chemotherapy, which cannot prevent recurrent re-infection of people living in endemic area. In the last decades, many researchers made a remarkable effort in the search for an effective vaccine to provide long-term protection. Parasitic platyhelminthes of *Schistosoma* genus, which cause the disease, live in the blood vessels of definitive hosts where they are bathed in host blood for many years. Among the most promising molecules as vaccine candidates are the proteins present in the host–parasite interface, so numerous tegument antigens have been assessed and the achieved protection never got even close to 100%. Besides the tegument, the digestive tract is the other major site of host–parasite interface. Since parasites feed on blood, they need to swallow a considerable amount of blood for nutrient acquisition. Host blood ingested by schistosomes passes through the esophagus and reaches the gut where many peptidases catalyze the proteolysis of blood cells. Recent studies show the emergence of antigens related to the parasite blood feeding, such as esophageal gland proteins, proteases, and other proteins related to nutrient uptake. Herein, we review what is known about *Schistosoma mansoni* digestive tract proteins, emphasizing the ones described as potential vaccine candidates.

Keywords: *Schistosoma mansoni*, vaccine, digestive tract, esophageal gland, gastrodermis, proteases

INTRODUCTION

Schistosomiasis is considered the most important human helminth infection in terms of global mortality in developing countries. It is estimated that schistosomiasis causes 280,000 deaths in Sub-Saharan Africa alone (1, 2). The morbidity rates are also high and this disease is responsible for the loss of up to 4.5 million DALYs (disability adjusted life years) annually (3). Many countries have been investing in intervention strategies based on mass drug administration (4, 5). Nonetheless, the massive treatment has not decreased the endemicity due to constant re-infection of people and low quality of sanitary conditions in endemic areas (6–8) and yet, around 800 million people live in risk areas in 74 developing countries, of which at least 200 million people are infected (3, 9).

Many scientists search for a feasible vaccine against schistosomiasis; they believe this is the best strategy for reducing the disease transmission and morbidity. If a vaccine induced even a partial reduction in worm burdens it would considerably reduce pathology and limit parasite transmission (10, 11). Preventive vaccination would clearly overcome re-infection problems avoiding the need for repeated treatments of people living in endemic areas. As a result, vaccine strategies represent an essential component as an adjunct to chemotherapy for the control of schistosomiasis. The improvement in understanding of the immune response to schistosomes suggests that development of a vaccine is possible (5). In the search for vaccine targets, proteins located at the parasite/host interface are likely to be the most important, since they are commonly associated with mechanisms of escape from the host immune system or other adaptation to parasitism (12). The two major surfaces that constitute these interfaces are the outer tegument and the gastrodermis – gut lining (13). The tegument has been the major target of vaccine development. Recently, however, scientists are also focusing on schistosome gut and its secreted products. Protection studies demonstrate the potential of an esophageal gland secreted protein, gut proteases, and other gastrodermis proteins to be exploited for vaccine development. Throughout this work, we will review the digestive tract of *Schistosoma mansoni* and also present and discuss recent studies in vaccine development.

DIGESTIVE TRACT

The digestive tract of *S. mansoni* consists of the oral sucker, the esophagus, which is surrounded by the esophageal gland in its posterior portion and the blind-ended gut. Schistosome mouth is subapical and opens through the oral sucker, which is the beginning of the digestive tract in the anterior extremity of the worm.

The oral sucker is a funnel-shaped vent covered by the tegument and bears thousands of spines, which extend up to the esophagus (14–16). The esophagus immediately succeeds the mouth opening and connects the sucker to the gut. It is a short tube that is invested by longitudinal and circular muscle fibers and two stronger circular muscle act as sphincters that control the entrance of blood (14, 17, 18). The esophagus is covered by an extension of the syncytial tegument with higher specialized surface architecture, specially focused in blood processing, and also an enormous membrane expanded into plates (17). The posterior half of the esophagus is surrounded by the esophageal gland that releases secretions into its lumen to process ingested host blood (17). The esophagus ends into the beginning of the gut. Then, posterior to the ventral sucker, the gut bifurcates into two lateral portions, which run either side of the reproductive organs and rejoin posterior to them, forming again a single portion that continues to the posterior end of the worm body. Since the gut is blind-ended, secretion of products might occur by regurgitation through the mouth (19, 20). The surface of the gut is a syncytial epithelial layer known as gastrodermis. Although gastrodermis is syncytial like the tegument, its cytoplasm differs by the presence of numerous mitochondria, nuclei, and biosynthetic machinery, with an active Golgi apparatus, a well-developed granular endoplasmic reticulum and numerous vesicles (19–21). Besides that, the gut syncytium is extended by the presence of fine cytoplasmic lamellae that characterize its absorptive nature (22, 23).

The primary function of the schistosome digestive tract is to digest macromolecules acquired from the blood of the mammalian host, and to absorb the soluble products (24). To live and reproduce inside the host, the mature, blood feeding worms use amino acids derived from degradation of serum proteins and lysed erythrocytes. Previous studies suggest that lysosomes directly secrete their contents into the gut lumen to digest incoming plasma (22, 24). The digestion process is presumed to be predominantly extracellular with the final stages possibly intracellular (22, 24, 25). Several peptidases are employed in processing and acquisition nutrients provided by host blood (26–28). Carrier proteins sequester essential organic and inorganic nutrients for uptake into the gastrodermis and the accumulated products are periodically eliminated from the blind-ending gut by regurgitation (22, 24). The structure of the gastrodermis has been well documented by transmission and scanning electron microscopic studies (19–21), but its protein composition has yet to be fully explored.

PROTEOMIC ANALYSES

While the tegument has been the major target of characterization studies of *S. mansoni* for many years, the schistosome gastrodermis is a difficult target since it is inaccessible, what makes it pretty hard to collect samples. An alternative technique to have some evidences about digestive proteins is the collection of worms' vomit, what is also difficult because the worms do not open their mouth easily in culture (22). The very first proteomic study on the vomit induced regurgitation in adult worms by osmotic shock with distilled water. Although this technique presents some contaminations from other tissues, it provides with at least an idea of gut contents. Some host proteins (hemoglobin, immunoglubolin (Ig)G, and serum albumin) were identified as well as worm antioxidants [superoxide dismutase (SOD) and thioredoxin] and proteins related to fatty acid absorption (lysophospholipase and fatty acid binding protein Sm14) (29). Biochemical analyses on vomit obtained either by osmotic or temperature shock demonstrated enzymatic activity, confirming that schistosomes utilize proteases to digest hemoglobin and host serum proteins (28, 30). Another proteomic study extended significantly the list of constituents secreted into the schistosome gut lumen as part of the blood feeding process. The authors induced the regurgitation by both temperature shock and protein starvation and the vomit contents were analyzed through electrophoresis followed by mass spectrometry. A total of 71 distinct proteins were identified, among them many proteases (cathepsins B1, C and S/L, asparaginyl peptidase and two proline carboxypeptidases, pro-X carboxylpeptidase, and dipeptidylpeptidase II), protease inhibitors (serpin and α2 macroglobulin), a series of lysosomal proteins (lipid-binding saposins and cholesterol-binding NPC-2), ion transporters (ferritins and calumenin), and also the previously identified antioxidants (SOD and thioredoxin) (24). The authors suggest that, due to the morphological changes in the gastrodermis and presence of cytoplasmic proteins (such as Sm14), parasite vomitus contents have contamination as a result of worm damage (24). Besides vomitus analyses, Nawaratna and colleagues (23) used a novel technique, the laser capture microdissection to recover the gut epithelium from tissue sections for analysis of gene expression by RT-PCR. This study identified 121 up-regulated transcripts, as expected, proteases (cathepsins A, B, D2, and L), lysosomal proteins (saposins, NPC-2, LAMP acid lipase, and phospholipase A2), transporters (Ca^{2+} ATPase, phospholipid transporter, and amino acid transporter), and the antioxidant SOD were identified, in agreement to proteomic investigations (23).

TARGETING THE GUT

The relationship between the parasite and its host is largely nutritional, involving the unidirectional transfer of nutrients. In this context, schistosome seeking for food has been the primary selection pressure to initiate the evolution of host–parasite association (31, 32). Schistosomes not only feed on blood, but are bathed in their food. Adult male *S. mansoni* is estimated to ingest some 39,000 erythrocytes hourly, while the female, due to egg production, requires 10 times more, 330,000 erythrocytes hourly (33). In *S. mansoni*, the lysis of blood cells takes place exclusively in the digestive tract; it begins in the esophagus, where the esophageal gland secrets digestive enzymes and erythrocytes are rapidly lysed (15, 17). Then, peristaltic movements pass lysed cells down into the anterior gut and, finally the gut lumen takes up the essential nutrients (20).

Blood is essential to worm survival since it provides a consistent and renewable nutrient resource (31). Eventual impairments to blood processing would compromise parasite development inside the host leading them to death through starvation. There are at least two evidences showing this phenomenon. First, the treatment of infected mice with cysteine protease inhibitors not only reduces significantly worm burden but it also inhibits egg production by females. Authors believe that inhibitors arrest hemoglobin degradation causing obstruction of the feeding activity and nutrient uptake (34, 35). Besides, the elimination of *S. mansoni* adult worms

by rhesus macaques seems to be related to gut damage. Surviving moribund worms had alterations in their intestinal epithelium and absence of food in the gut lumen, pointing to worm starvation. The authors suggest that blocking antibodies impact on nutrient uptake by both gut and tegument (36). In this context, interventions that block blood processing reveal new anti-schistosome targets for schistosomiasis control and so, the digestive tract and its secretions appear to be a great source of key antigens.

ESOPHAGEAL GLAND AND Sm10.3 AS VACCINE CANDIDATE

The esophageal gland is a bi-lobed structure that lies around the posterior esophagus. The first morphology study on the esophageal gland, in the late 1970s, suggested its function in initial blood digestion (15). Recently, a detailed morphological and functional study on this gland confirmed the central role of esophagus in blood processing, not acting simply as a conduit (17). The architecture of the esophageal gland comprises as many as 1000 cell bodies, each connected to posterior esophagus by microtubule-lined extensions. Cell bodies are specialized in protein production and export, they synthesize large numbers of crystalloid vesicles and release their contents into the lumen (17, 37). Esophageal gland secretions are responsible, among other activities, for initiating hemolysis and blood digestion. Erythrocytes are lysed upon their entry to the posterior esophagus and very few intact cells are seen beyond that point (17, 24). Besides, these secretions may also act neutralizing host immune effectors and contributing to parasite survival. Leukocytes are somehow tethered in the posterior lumen in variable states of degeneration (13, 17, 19).

Micro exon gene (MEG) proteins 4.1, 4.2, and 14 (38) and also venom allergen-like (VAL) protein 7 (39) are some of the proteins identified in the esophageal gland (17). Recent study demonstrated preliminary evidence that MEG-4.1, also termed Sm10.3, induces erythrocyte agglutination *in vitro*, what could be related to erythrocyte digestion (40). Besides proteomic studies, a transcriptional analysis of esophageal gland revealed that more than 120 genes are differentially up-regulated there. The great majority of transcripts identified code for proteins related to binding or hydrolase activities, which may be important to nutrient uptaking and red cell lysis and catabolism (41).

Sm10.3 antigen was used in vaccine development against schistosomiasis in the murine model. When formulated with Freund's adjuvant, this antigen induced a mixed Th1/Th2-type response, as IFN-γ, TNF-α, and low levels of IL-5 were detected in the supernatant of cultured splenocytes (40). The vaccination also reduced in 32% the worm burden and ameliorated liver pathology, since 43.6% less eggs were found in the liver and there was a significant reduction in the number, size and fibrosis of granulomas, 23.8, 11.8, and 39.8%, respectively. This data suggest that Sm10.3 is a potential candidate for vaccine development (40). These findings, together with the detection of host IgG binding in schistosome esophagus, suggest that esophageal gland proteins are attractive vaccine candidates, not only because they mediate initial feeding process, but also because antibodies targeting such proteins would not face the hostile acid proteolytic environment in the gut (17).

GUT PROTEASES

Schistosome proteases are involved in a vast range of essential processes such as invasion, migration, feeding, reproduction, activation, and evasion of immune system [reviewed in Ref. (26, 42)]. Genomic studies identified at least 250 peptidases in the genome of *S. mansoni* (43, 44). In the feeding context, many proteases have been identified in the regurgitated gut contents by biochemical and proteomic analysis (24, 30). In addition, several studies confirm the presence of proteases in the gastrodermis by immunocytochemistry and point out the roles of cysteine endopeptidases, cathepsin L, cathepsin D, cathepsin B, asparaginyl endopeptidase, and metalloproteases in hemoglobin and serum albumin degradation and processing (45–48). The great amount of proteases in the gut provides significant redundancy in blood protein degradation (27). Since *S. mansoni* gut proteases have a vital role in blood processing researchers believe that they are promising vaccine candidates and some of them have already been tested (26, 42, 49, 50).

Schistosoma mansoni cathepsin-B1 (SmCB1) is the most abundant papain-like cysteine peptidase in the parasite gastrodermis (51), it was first localized in the parasite gut lumen, however, another study hypothesized that this protein could be also expressed in the cecum and protonephridia of cercariae (52). Immunological studies with schistosomiasis patients suggested that SmCB1 is an immunodominant target of the immune response during pre-patent schistosome infection since authors demonstrated that SmCB1 is targeted by IgG and IgE specific antibodies (53). El Ridi and colleagues (50) evaluated the potential of active SmCB1 as vaccine with its inbuilt adjuvanticity or as adjuvant to another known candidates (54). In the first situation, SmCB1 reduces significantly worm burden (66–73%), eggs in liver (51%), and in small intestine (25%). However, when SmCB1 is incubated with proteinase-inhibitor prior to immunization, the levels of protection decrease significantly, pointing out the importance of the peptidase activity in protective potential. The immunization with active form of SmCB1 by itself also induces the production of high levels of IL-4, IL-5, and IL-13 and high titers of IgG, IgG1, and IgG2b (54). Besides being a promising vaccine target, SmCB1 also acts as an adjuvant, since it increases the protection provided by immunization with other proteins, such as glyceraldehyde 3-phosphate dehydrogenase and peroxiredoxin-multiple antigen peptide, from <10%, formulated in Th1 adjuvants, to up to 84%, formulated with SmCB1 (54, 55).

The protein Sm32 is an asparaginyl peptidase (SmAE) member of the legumain family that, probably, cleaves zymogens of proteinases involved in hemoglobin degradation (56–58). Its activation is an autocatalytic event and it is related to a loss of a C-terminal portion and an N-terminal pro-domain, what reduces the protein from 50 kDa to approximately 32 kDa (59). Several peptides of SmAE were chemically synthesized and, when administrated in a Freund's formulation, showed relative immunogenicity in rabbits and mice, in particular the hydrophilic regions of the molecule (56, 57). When SmAE was evaluated as DNA vaccine, it elicited a significant humoral response and egg count reduction (32%), but it failed to reduce the entire worm burden (60).

Cathepsin D (SmCD), an aspartic protease, seems to be involved in hemoglobin process in *S. mansoni* (28, 61). Schistosomula treated with RNAi to SmCD were unable to develop and survive in mice, indicating the crucial functions of this protease in parasite maintenance (48). Two SmCD peptides formulated with lipid core scaffold (LCS) elicit a humoral response in mice. The anti-SmCD antibodies recognize the native SmCD in adult worms protein extracts, and almost abrogate its enzymatic activity *in vitro*, pointing out the potential of this protein as a vaccine target (62).

OTHER GUT PROTEINS AS VACCINE TARGETS

SUPEROXIDE DISMUTASE

Immunolocalization assays demonstrated the antioxidant enzyme Cu/Zn SOD in the two major host–parasite interfaces: schistosome tegument and gastrodermis. The localization of this protein suggests the development of parasite antioxidant mechanisms to protect themselves against the host cellular response and also against hemoglobin oxidation products, respectively (63). Schistosome SOD was also detected in proteomic analyses of worm's vomit and transcriptional analysis of digestive tract (23, 24, 29). DNA vaccination strategies using Cu-Zn SOD cDNA protected mice from *S. mansoni* infection reducing the worm burden up to 54%; two SOD peptides were also tested and reduced worm burden from 31 to 51% (64). In another vaccination experiment, one SOD peptide, CT-SOD, also formulated as DNA vaccine, induced a Th1-immune response and significantly decreased worm burden (36–43%) after surgical transfer of adult worms into the mesenteric veins of immunized mice (65). A cross-reactivity study demonstrated that mice anti-SmSOD antibodies or infected individual sera do not recognize human SOD in its native form, meaning that SmSOD could serve as a basis for developing a vaccine against schistosomiasis (66).

SYNTENIN

In a recent study, schistosome syntenin (SmSynt), a PDZ-domain protein, was localized in the gastrodermis of *S. mansoni*, even though none of the proteomic and transcriptional studies identified this protein at this location (67). The role the protein plays in the natural biology of the parasites remains unclear since knocking down the expression of the SmSynt gene yielded no clear phenotype *in vitro*. However, syntenin is involved in many cellular processes in mammalian cells, among them, cellular trafficking and biogenesis of small extracellular vesicles, what could be also its function in schistosome digestive tract. The recombinant SmSynt seems to be a relevant vaccine target since it induces the production of IgG antibodies and Th1-cytokines likely important in disease control. Mice vaccinated with the recombinant protein induced 30–37% of worm burden reduction. A reduction in liver damage was also noted in vaccinated versus control mice, 38–43% reduction in the number, and 35–37% reduction in the area of liver granulomas (67).

SAPOSIN

Four proteins possessing the characteristic saposin domain were identified in schistosome vomit (24). Saposins bind sphingolipids, facilitating their degradation by ceramidases and they also bind other lipids, sequestering them in the gut lumen for transport/uptake into the cells (24, 68). A gut saposin-like protein (SmSLP-1) has proven to be immunogenic since antibodies from infected individuals recognized its recombinant form, however, it was ineffective as a vaccine in the mouse model. SmSLP-1 formulated with Freund's adjuvant was utilized in a vaccination trial, and despite the presence of high antibody titers in immunized mice, adult worms, and eggs recovered from vaccinated animals were the same as in control group (68). Unfortunately, not all gut proteins tested as vaccine were successful.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Hematophagous parasite *S. mansoni* succeeds in the tough job of surviving inside human host and this success can be attributed, among other abilities, to their capacity of actively feed via the digestive tract. Blood processing and nutrient uptake are critical

Table 1 | Digestive tract antigens.

Localization	Antigen	Vaccine formulation	Functions likely affected by vaccination	Worm burden reduction
Esophageal gland	Sm10.3	Recombinant protein	Initial blood processing	32%
Gut lumen	Cathepsin B	Recombinant protein	Hemoglobin processing	Up to 84%
	SmAE	DNA vaccine	Hemoglobin processing	None[a]
	Cathepsin D	Synthetic peptides	Apical processing of hemoglobin	–
Gastrodermis	Superoxide dismutase[b]	DNA vaccine	Antioxidant mechanisms; protection from hemoglobin oxidation products	Up to 54%
	Syntenin	Recombinant protein	Cellular trafficking and biogenesis of small extracellular vesicles	Up to 37%
	Saposin	Recombinant protein	Lipid binding, transport, and uptake	None

[a] *32% reduction in recovered eggs.*

[b] *Also identified in the tegument.*

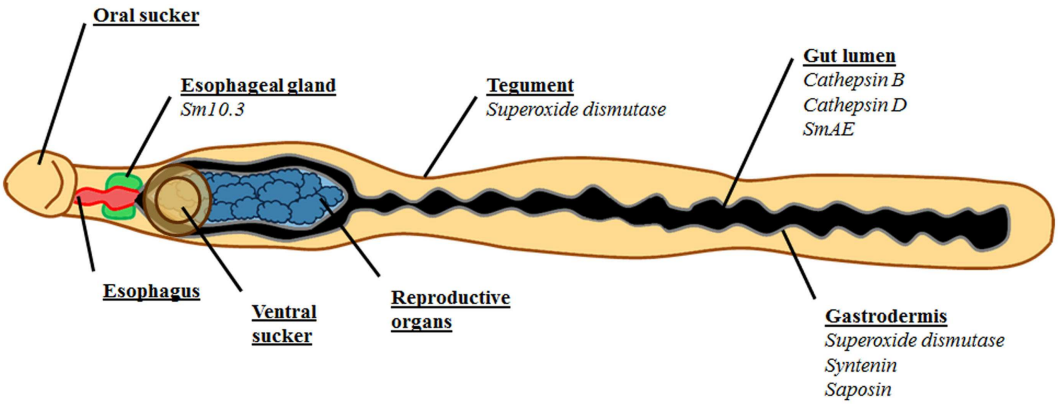

FIGURE 1 | Schematic representation of *S. mansoni* emphasizing the digestive tract and its antigens tested as vaccine. Esophagus is represented in red, esophageal glands are green, gut lumen is black, and gastrodermis is gray. The reproductive organs, which are surrounded by the bifurcated gut, are represented in blue. The tegument is brown, as well as the oral and ventral suckers. The antigens are listed under the location they were identified in italics. Superoxide dismutase was identified both in the tegument and in the digestive tract.

for the survival of schistosomes and direct or indirect interruption of these processes may represent a realistic strategy for vaccine development. These interventions would probably lead worms to starvation, and consequently death, since insufficient supply of energy impairs growth, pairing, maturation, and fecundity. Here, we described the physiological function of *S. mansoni* alimentary tract and also reported some examples of successful vaccines formulated with digestive tract proteins, summarized in **Table 1** and **Figure 1**.

The efficacy of these vaccines seems to remain on the generation of antibodies that are able to bind to the target enzyme and inhibit its enzymatic function in the parasites, compromising essential aspects of their biology. The gut lumen seems a hostile site for antibodies due to its low pH and the presence of multiples proteases. These features are probably the reasons why not all antigens conferred protection. However, despite the aggressive environment of the gut, immunization experiments showed that targeting molecules from digestive tract successfully yielded some protection against schistosomes. We are still in the beginning of understanding the complex feeding mechanisms by which schistosomes obtain nutrients from their host and we have no clue if antibodies are able to affect this process. However, we do know that infected patients generate high levels of antibodies against the gut secreted polysaccharide antigens circulating anodic and cathodic antigens (CCA and CAA), used in diagnostic tests (69), and also against the gut protease SmCB1 (53). These data indicate that gut antigens are somehow available to antigen presentation.

Proteomic analyses on schistosomes gut secretions revealed many cathepsins and other peptidases involved in the proteolytic pathways of host nutrients. The proteolytic pathways for hemoglobin and albumin have been described and they showed that a large number of proteases provide some redundancy (28). Functional redundancy is an issue for the development of vaccines based on gut proteases; however, this issue could be amended by formulating a vaccine comprising either a combination of proteases or a chimeric antigen consisting of epitopes of different enzymes (26). The better understanding of nutrient uptake and the advancement of new vaccination approaches are key points for the development of intervention strategies, specially the development of a vaccine.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 November 2014; accepted: 09 January 2015; published online: 28 January 2015.
*Citation: Figueiredo BC, Ricci ND, de Assis NRG, de Morais SB, Fonseca CT and Oliveira SC (2015) Kicking in the guts: Schistosoma mansoni digestive tract proteins are potential candidates for vaccine development. Front. Immunol. **6**:22. doi: 10.3389/fimmu.2015.00022*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.
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frontiers in Immunology

MINI REVIEW
published: 21 July 2015
doi: 10.3389/fimmu.2015.00347

Development of paramyosin as a vaccine candidate for schistosomiasis

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Schistosomiasis, caused by three principal species of diecious trematodes (flatworms), currently afflicts over 250 million individuals, results in an estimated 2–15% chronic disability, and contributes to poor health and economic stagnation in endemic areas. Although schistosomiasis is effectively treated with praziquantel, rapid reinfection with rebound morbidity precludes effective control based on chemotherapy alone and justifies current efforts to develop vaccines for these parasites. Paramyosin (Pmy), an invertebrate muscle-associated protein, has emerged as a promising vaccine candidate for both *Schistosoma mansoni* and *Schistosoma japonicum*. Herein, we discuss the discovery of Pmy, its development as a vaccine candidate in rodents and bovines, as well as studies of naturally occurring immune responses to Pmy in prospective, observational human studies. We conclude with a proposed developmental plan to move Pmy toward Phase I clinical trials.

Keywords: *Schistosoma japonicum*, vaccines, paramyosin, zoonoses, preclincal

OPEN ACCESS

Edited by:
Ahmad Ali Othman, Tanta Faculty of Medicine, Egypt

Reviewed by:
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Specialty section:
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology

Received: *13 April 2015*
Accepted: *23 June 2015*
Published: *21 July 2015*

Citation:
Jiz MA, Wu H, Olveda R, Jarilla B and Kurtis JD (2015) Development of paramyosin as a vaccine candidate for schistosomiasis. Front. Immunol. 6:347. doi: 10.3389/fimmu.2015.00347

Schistosomiasis, caused by three principle species of diecious trematodes (flatworms), currently afflicts over 250 million people worldwide (1, 2) and results in 1.53 million DALYs lost per annum (3), although this is likely a considerable underestimate. A recent reassessment of the global burden of schistosomiasis suggests that the actual health burden is 4–30 times greater than the previous WHO estimate (1, 4). Infection is characterized by the presence of adult worms within the portal and mesenteric veins for *Schistosoma japonicum* and *Schistosoma mansoni* or within the veins draining the urinary bladder for *S. haematobium*. Chronic infection results in reduced childhood growth and nutritional status, anemia, hepatosplenomegaly, hepatic or urinary bladder fibrosis, and bleeding esophageal varices. End organ and systemic pathology result from granulomatous inflammation and fibrosis induced by parasite eggs trapped in host tissues.

Efforts to mitigate the impact of schistosomiasis in endemic regions have focused on chemotherapy of active infections with praziquantel (PZQ), control of intermediate snail hosts, and improved sanitation.

In longitudinal cohort studies in the Philippines, we have demonstrated that improvements in nutritional status and hemoglobin levels associated with PZQ treatment are transitory; both nutritional status and hemoglobin returned to their pre-treatment morbid levels within 9–12 months (5). In addition, "rebound" morbidity, in which repeated cycles of annual treatment and reinfection result in more aggressive morbidity, has been described (6). Therefore, annual treatment

programs with PZQ are insufficient to reverse these morbidities, while resource requirements to shorten the treatment interval are prohibitive. Because current control strategies employing chemotherapy with PZQ have not reduced transmission and morbidity to acceptable levels, there is an urgent need for complementary approaches, such as vaccines for schistosomiasis control.

Discovery of Paramyosin

In *S. mansoni*, Sher and colleagues demonstrated that mice vaccinated intradermally with schistosomula or adult worm extracts adjuvanted with BCG were protected from cercarial challenge (7). The sera from these mice were strikingly monospecific, recognizing a 97 kDa antigen in adult worm extracts and immunoprecipitating a 97 kDa antigen from detergent extracts of metabolically labeled worms (8). Size-fractionation analysis of adult worm extracts indicated that only fractions containing a protease sensitive 97 kDa antigen induced protection in this model (7). Monoclonal antibodies against Sm97 were developed from mice vaccinated intradermally with adult worm extract and BCG (8). Indirect immunofluorescence using monoclonal anti-Sm97 localized the antigen to regions just below the tegument and in the gut syncitia of adult worms. Sm97 was weakly recognized by chronically infected mouse sera and not recognized by sera from mice infected with irradiated cercariae (8). Immunoaffinity purified Sm97 was shown to elicit delayed-type hypersensitivity in intradermally vaccinated mice, suggesting that this molecule is also capable of evoking cell-mediated responses.

A rabbit anti-Sm97 serum immunoprecipitated a 97 kDa antigen from *in vitro* translation products of adult worm mRNA and identified a 1317 base pair clone, which encoded approximately 50% of the native protein (9). The clone had 36% homology with nematode myosin, but was designated paramyosin based on amino acid composition and cross reactivity of anti-Sm97 sera with other native paramyosins. Structural analysis of the deduced amino acid sequence and electron microscopy of purified schistosome paramyosin indicated that the protein adopts an alpha helical coiled-coil conformation with a seven residue repeat (10).

Paramyosin was localized to the contents of membrane bound elongate bodies within the tegument and subtegumental cell bodies using immunoelectron gold microscopy and polyclonal anti-paramyosin antisera (11). Paramyosin was in a non-filamentous form within these elongate bodies and staining was rarely observed in the thick filaments of cortical muscle.

A full length cDNA clone of *S. mansoni* paramyosin was sequenced and revealed significant homology to antigen B of *Taenia solium* and the IgG Fc γ-binding protein of *Taenia crassiceps* (12, 13). These homologous proteins bind the Fc region of IgG and collagen, and inhibit complement activation. Based on these homologies, schistosome paramyosin was shown to inhibit complement C1 and C9, and to bind polymeric collagen, and IgG (14–17). These immune-related properties may explain the tegumental localization of an otherwise muscle associated protein.

Murine Protection Studies in *S. mansoni*

Passive transfer of monoclonal or polyclonal anti-paramyosin antibodies did not protect mice from cercarial challenge (18); however, immunization with 4 μg of native or 40 μg of a partial recombinant paramyosin fragment with BCG induced 39 and 26% protection, respectively. These data suggested that protection induced by paramyosin was cell mediated and not antibody dependent. Schistosome myosin and heterologous nematode paramyosin did not induce protection, suggesting the requirement of schistosome paramyosin specific epitopes for protection. In other experiments, 5 μg of native paramyosin induced 24–53% protection in mice without adjuvant (19).

Native and partial recombinant paramyosin stimulated IFN-γ production and macrophage killing of schistosomula in vaccinated mice. In addition, splenocytes from mice vaccinated with paramyosin/BCG were stimulated to produce IFN-γ by 3-h-old and 7-day-old schistosomula in a dose dependent fashion (18).

Pmy Development in *S. japonicum*

In contradistinction to the *S. mansoni* model, a mouse monoclonal IgE antibody, subsequently demonstrated to recognize paramyosin (20), was shown to confer 19–58% protection against *S. japonicum* cercarial challenge following passive transfer (21, 22). In addition, the monoclonal stimulated eosinophill-mediated killing of schistosomula *in vitro* (22).

To determine if paramyosin would also induce protection against *S. japonicum*, we biochemically purified paramyosin from *S. japonicum* adult worms. SDS-PAGE demonstrated a single protein with a molecular weight of 97 kDa. In four separate experiments, vaccination of mice with *S. japonicum* paramyosin without adjuvant induced significant resistance (62–86%, $p < 0.001$) against cercarial challenge as compared to controls. These data suggest that *S. japonicum* paramyosin represents a promising candidate vaccine (23). Paramyosin has also been evaluated as a component of a multi-antigen DNA-based vaccine in mice; however, delivery in this format has not augmented the level of protection seen with recombinant or biochemically purified native paramyosin (24).

Based on these encouraging protection data, McManus and colleagues cloned the full length paramyosin gene from an *S. japonicum* cDNA library probed with a hyperimmune rabbit sera (25), while Nara et al. independently isolated the full-length cDNA of Sj97 by screening a distinct library using a mouse monoclonal IgE antibody, which recognizes a 97 kDa surface molecule on *S. japonicum* larvae (26). Alignment of the predicted amino acid sequences of *S. mansoni* and *S. japonicum* paramyosins revealed 95% identity.

Sj97 is the Target of Protective Th2 Biased Cytokine Responses in Humans

We conducted a longitudinal treatment–reinfection study design with 616 *S. japonicum* infected participants, 7–30 years of age. We evaluated the relationship between cytokine responses made by PMBCs in response to *S. japonicum* soluble adult worm extract (SWAP), Sj97, Sj67, and Sj22.6, measured 4 weeks after treatment

with PZQ, and resistance to reinfection in a population from Leyte, The Philippines. Reinfection was measured every 3 months for a total of 18 months by duplicate Kato–Katz assessment on three stools per person per timepoint. We employed repeated measures models adjusted for both repeated measures within person, clustering by household and several potential confounders of reinfection intensity including: directly observed water contact, socio-economic status, age, sex, village, and baseline intensity of infection.

Schistosoma japonicum transmission was high: 54.8 and 91.1% were reinfected within 6 and 18 months, respectively. A Th2 bias in the following cytokine ratios, IL-5/IL-12, IL-13/IL-12, IL-4/IFN-g, IL-5/IFN-γ, and IL-13/IFN-γ, in response to Sj97, predicted a 30–41% lower intensity of reinfection (all $p < 0.05$) after adjustment for potential confounders. An example of one of these protective relationships (for IL-13/IFN-γ ratio) is presented in **Figure 1A** (27). Similar results were found for responses to SWAP.

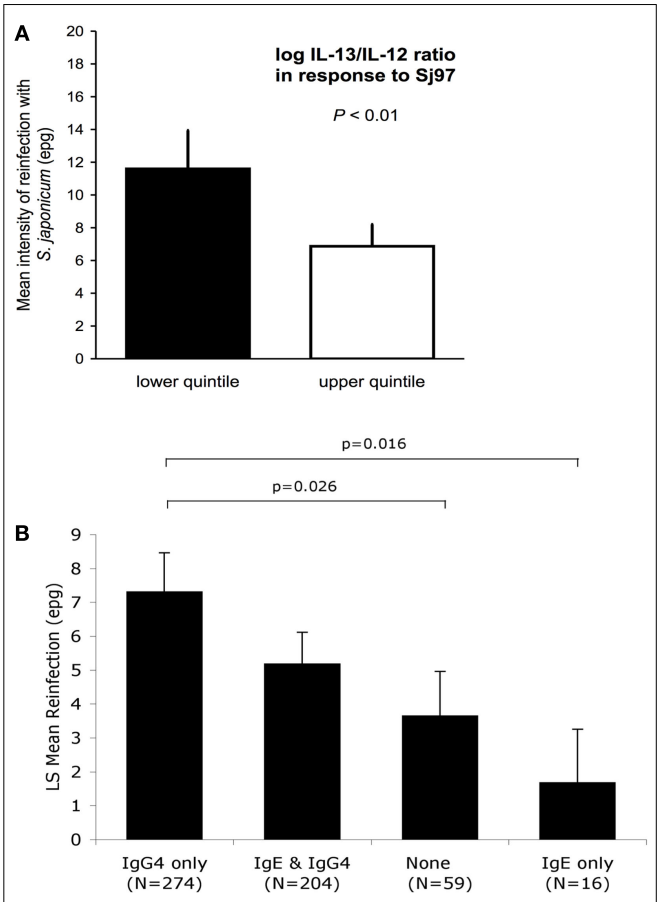

FIGURE 1 | (A) Intensity of reinfection with *S. japonicum* 18 months after treatment with PZQ, as predicted by cytokine ratios in responses to SWAP ($N = 493$). White and black bars represent the back-transformed LS mean intensity of reinfection for the upper and lower quintile of IL13/IL12 log cytokine-ratio distribution. Error bars represent SEs. LS mean estimates, SEs, and *p*-values for difference in means between quintiles are adjusted for confounders and clustering. Adapted from Ref. (26). **(B)** IgE responses to rSj97 (paramyosin) predict resistance to *S. japonicum* reinfection at 12 months post-treatment, and are attenuated by IgG_4. Least square (LS) means represent the mean reinfection egg burden after adjusting for potential confounders and clustering by household in a repeated measures model using the combined Sj97 IgE and IgG_4 response variable ($p = 0.023$ for time by combined IgE–IgG_4 variable interaction). Confounders in this model include age, gender, village of residence, exposure, and baseline intensity. *p*-values are for detailed comparisons between "IgG_4 only" and the rest of the groups. Error bars represent SEs. Reprinted from Ref. (27).

Sj97 is the Target of Protective IgE Responses in Humans

Using the same study population and analytic approach described for the cytokine analyses, we have evaluated the relationship between antibodies to rSj97 and resistance to reinfection in humans. We obtained serum 4 weeks post-treatment and measured anti-rSj97 IgE, IgA, and IgG subclass specific antibody levels using a bead-based assay. Responders were defined based on the mean +2 SD of the fluorescence values obtained in a group of 10 unexposed North American controls. In repeated measures models, individuals with IgE but not IgG4 responses to rSj97 had a 77% lower intensity of reinfection at 12 months compared to individuals with IgG4 but not IgE responses, even after adjusting for potential confounders including directly observed water contact, village, age, sex, and baseline intensity of infection ($p = 0.016$) (28), see **Figure 1B**.

Bovine Vaccine Trials

Using partially purified rSj97, McManus et al. (29) demonstrated that Chinese buffalo immunized with rSj97 in Quill A developed specific anti-Sj97 antibodies and had 34% fewer worms after laboratory challenge compared to controls. Importantly, the authors also acknowledged that, "*whereas the yields of rec-Sj97 are sufficient for small trials, the expression levels are very low and are inadequate for large-scale use*."

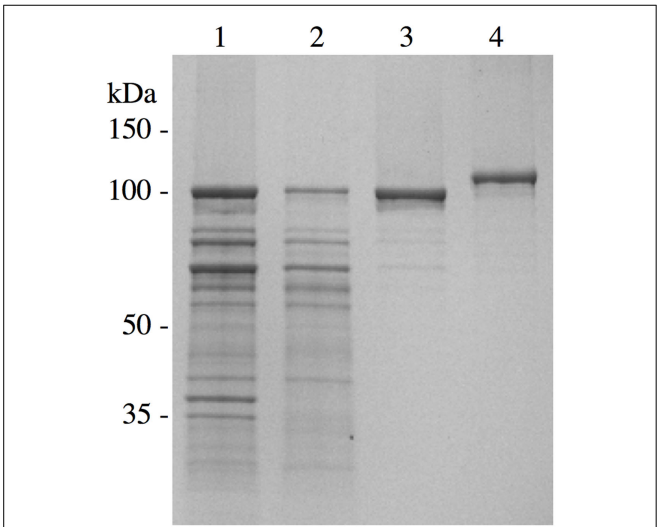

FIGURE 2 | Chromatographic purification of *S. japonicum* paramyosin. A pET-30 plasmid containing paramyosin was expressed and purified similarly as described (28). Lane 1, inclusion body preparation; lane 2, anion exchange chromatography; lane 3, size exclusion chromatography; lane 4, purified *S. japonicum* paramyosin with a thioredoxin fusion tag. Reprinted from Ref. (30).

Pilot Scale Expression, Purification, and Lyophilization of rSj97

As indicated above, recombinant Sj97 has not been successfully produced at pilot scale despite considerable efforts (29, 30). After discovering that Th2 and IgE responses to Sj97 were associated with marked resistance to reinfection in *humans*, we have focused our efforts on overcoming this scale-up challenge (31). Our typical yield from a single 10 L fermentation is 600 lyophilized vials at >0.250 mg rSj97/vial.

Characterization of rSj97

We have conducted extensive functional, biochemical, biophysical, and immunologic analyses on rSj97 to demonstrate that our recombinant protein is free of concerning contaminants, is correctly folded, and has appropriate functional properties. Electrophoretic mobility and purity (**Figure 2**), sterility, endotoxin level (by FDA approved test), residual SDS concentration, identity (LC-MS), secondary and tertiary structural analysis (CD), collagen binding, IgG binding, and stability (>12 months) gave expected results (31).

Future Product Development for rSj97

Together with colleagues, we are actively pursuing vaccination trials in both murine and bovine models with encouraging preliminary results. These experiments are designed to evaluate several doses of antigen as well as several adjuvants suitable for both veterinary and human use. Because *Schistosomiasis japonica* is a zoonosis, with bovines playing a key role in transmission to humans (32, 33), we are also evaluating the impact of bovine vaccination on human incidence of infection in endemic communities. Sj97 is a target of protective human IgE responses; therefore, we are also evaluating the potential for inducing hypersensitivity reactions by skin testing with rSj97 in infected bovines, again with encouraging preliminary results. If successful, these data, together with our GMP ready process for expression and purification, will form a compelling argument to move recombinant paramyosins toward Phase I trials in humans.

Acknowledgments

This work was supported by grants from the US National Institutes of Health (R01AI101274, R21AI097963, R56AI091753, R01AI48123, and TMRC P50 AI098481).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Immunology

MINI REVIEW
published: 12 May 2015
doi: 10.3389/fimmu.2015.00218

Development of the Brazilian anti Schistosomiasis vaccine based on the recombinant fatty acid binding protein Sm14 plus GLA-SE adjuvant

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Data herein reported and discussed refer to vaccination with the recombinant fatty acid binding protein (FABP) family member of the schistosomes, called Sm14. This antigen was discovered and developed under a Brazilian platform led by the Oswaldo Cruz Foundation, from the Health Ministry in Brazil, and was assessed for safety and immunogenicity in healthy volunteers. This paper reviews past and recent outcomes of developmental phases of the Sm14-based anti schistosomiasis vaccine addressed to, ultimately, impact transmission of the second most prevalent parasitic endemic disease worldwide.

Keywords: vaccine, schistosomiasis, Sm14, disease of poverty, FABP, Brazil

OPEN ACCESS

Edited by:
Rashika El Ridi, Cairo University, Egypt

Reviewed by:
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Ramaswamy Kalyanasundaram, University of Illinois, USA
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Specialty section:
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology

Received: *11 February 2015*
Accepted: *21 April 2015*
Published: *12 May 2015*

Citation:
Tendler M, Almeida M and Simpson A (2015) Development of the Brazilian anti Schistosomiasis vaccine based on the recombinant fatty acid binding protein Sm14 plus GLA-SE adjuvant. Front. Immunol. 6:218. doi: 10.3389/fimmu.2015.00218

Introduction

Schistosomiasis is considered by the World Health Organization to be 1 of 17 neglected tropical diseases (1). With 800 million people at risk, and 200 million infected in 74 countries, schistosomiasis is the second most prevalent human, parasitic disease in the world after malaria. Some 7.1 million people are infected with *Schistosoma mansoni* in the Americas, of whom 95% are in Brazil (1). It is estimated that 25 million people are exposed to the risk of schistosomiasis in the Americas (2). The WHO estimated that the morbidity of schistosomiasis resulted in the annual loss of 1.7 million disability-adjusted life years (DALYs), while mortality was estimated to be 41,000 deaths per year (3). Control measures aim to reduce morbidity through treatment with praziquantel, improved sewerage, access to potable water, and snail control (1–4). Vaccination, even if not 100% effective, could contribute to the long-term reduction of egg-excretion from the host, and thus controls transmission. An effective vaccine would also contribute to a positive trade-off regarding the aggressive inflammatory response that has been observed following interrupted chemotherapy in children living in high-transmission areas (5–7). The underlying reason for this "rebound morbidity" is unclear, but is thought to be due to an interruption of the natural down-regulating process of specific immunological mechanisms typical for this disease. This outcome results from the typically high-level re-infection after chemotherapy and is a direct result of chemotherapy being primarily directed against morbidity and less against transmission of the disease. This effect needs to be taken seriously, as the observed aggravated gross symptoms reflect long-term pathology, which is difficult to remedy (8).

There have been initiatives in several countries to develop a vaccine against schistosomiasis. The Brazilian Sm14-based anti schistosomiasis vaccine is the sole technology that emerged from an endemic country, and that is at an advanced stage of development toward a safe highly innovative product.

In this review, we present the evolution of the Sm14 anti-schistosome vaccine from the initial gene cloning to the results of the recently completed phase I clinical trials.

Demonstration that Adult Schistosome Saline Extracts Contain Protective Antigens

The experimental background for the development of anti-schistosome vaccines lies with the use of animal models of infection that showed that an initial parasite infection resulted in partial immunity against re-infection (9–11). Levels of resistance achievable in laboratory models ranged from 60% in mice up to 90% in rabbits. In an attempt to establish whether vaccine development was feasible, extracts of adult parasites were utilized to immunize experimental hosts to investigate whether such antigen preparations also possessed the capacity to protect against infections. In two independent lines of investigation, it was demonstrated that simple saline extracts of live adult worms, which are enriched in surface associated molecules, were indeed capable of inducing protection comparable to that achieved with live infection (12–14).

Adult Worm Antigen Gene Cloning

Once gene-cloning technology became incorporated into the vaccine research field, the genes for a number of major antigens released by adult schistosomes briefly cultured in saline were cloned and sequenced (15–17). One of these proved to be a fatty acid binding protein (FABP) termed Sm14, which subsequently became the basis of the experimental vaccine herein discussed. The protein derived from the cloned gene exhibited significant homologies with a family of related polypeptides, which bind hydrophobic ligands, and purified recombinant protein exhibited an affinity to fatty acids. Antibodies to the purified protein were shown to bind to tubercles, which are structures located on the dorsal surface of adult male schistosome and known to contain lipids (17). In addition, the protein was localized to the muscle layers as well as in the body of the parasite. As the schistosome cannot synthesize fatty acids *de novo*, and is dependent on the uptake of lipids from serum, the available data supported a role for Sm14 in the transport of fatty acids. Following transfer of the Sm14 gene to a high level expression vector, subsequent experiments demonstrated that the recombinant rSm14 was able to protect outbred Swiss mice by up to 66% and New Zealand White rabbits by up to 89% against challenge with *S. mansoni* cercariae. It was thus demonstrated that rSm14 could provide the basis of an anti-schistosome vaccine (18).

Vaccine Development

The Sm14 project has been mostly funded by public funds at Oswaldo Cruz Institute/FIOCRUZ, belonging to the Brazilian Ministry of Health. Since the early stage of the development, there was a critical concern to reduce the cost of production at its lowest level and to ensure the use of non-proprietary components in the production process in order to get a low final price for the vaccine. The strategy adopted to reduce the cost of production of the human Sm14 vaccine involved the following steps:

i. Scaling up steps of production process: this started in 2003 with the primary target being the investigation of Sm14 stability. New constructs were developed with highly stable novel molecular design (19), beginning thus to pave the way for the ultimate goal of achieving large-scale production of highly purified vaccine at both high yield and low cost.
ii. Replacement of more expensive reagents seeking a royalty and proprietary free route of components: as presented in **Figures 1** and **2**, two substitutions were successfully achieved, which were the replacement of IPTG for lactose or salt (NaCl), for the steps of induction of protein expression in culture. Expression vectors were also constructed to avoid commercial ones. Furthermore, the Sm14 vaccine is based only on two highly purified and well-characterized components: the protein itself and the glucopyranosyl lipid adjuvant stable emulsion (GLA-SE) adjuvant produced and supplied by the Infectious Disease Research Institute (IDRI, Seattle). Our partner, IDRI, is a non-profit organization fully committed to the support of the development of technologies for the control of the so-called neglected diseases.
iii. Production process of the protein in large-scale is presently in place at Ourofino, the partner for the veterinary vaccine. There is currently pilot scale production of the vaccine in 5 L fermentor, which is being scaled-up to 50 and 100 L fermentors, with final cost already estimated to be approximately US $1,00) for one 50 μg dose.

A series of modifications of the prototypic experimental Sm14 vaccine, which consisted of a fusion protein presented with RIBI adjuvants (oil-in-water emulsions derived from bacterial and mycobacterial cell wall components), were undertaken to gradually convert the original laboratory-bench protein into a clinical product. First, it was demonstrated that rSm14 could be produced in a non-fused form while retaining its protective immunogenicity (20). A genomic polymorphism was identified in the Sm14 gene whereby the conserved methionine at position 20 is polymorphic, being exchangeable with threonine (M20T) (21). Both forms were found to be protectively immunogenic to adopt the same three dimensional structure in solution and to be functional in that they were able to bind fatty acids. The M20 isoform was found to exhibit superior stability, however, and was adopted for further vaccine development. A variety of approaches to vaccine formulation were explored in which it was demonstrated that short peptides derived from the C-terminal of sm14 were capable of conferring equivalent levels of protective immunity to experimental animals as intact rSm14 (22). rSm14 was found to be protective when presented in a live vaccine form within recombinant *Mycobacterium bovis* BCG (23, 24), and that rSm14 expressed as a fusion with tetanus toxin fragment C induced immunoprotection against schistosomiasis in mice (25). Last, a modified version of the protein with improved stability due to the avoidance of dimerization and subsequent aggregation was engineered by Cys62 replacement. The latter version was adopted as the lead compound for vaccine development (19).

The final steps toward a clinically applicable formulation of rSm14 were taken by developing a *Pichia pastoris* based expression

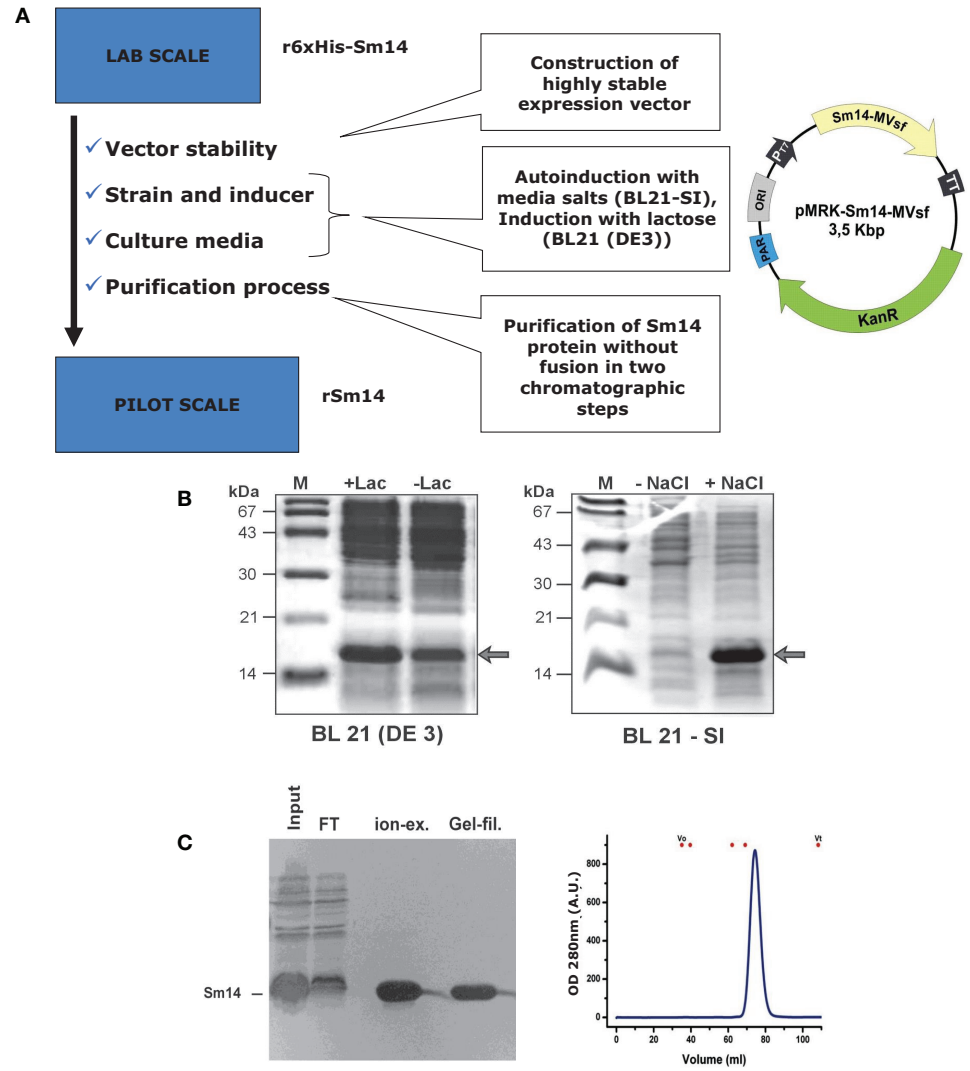

FIGURE 1 | Expression of Sm14 in *Escherichia coli*. (A) Steps taken to improve the production process of Sm14 in *E. coli*. **(B)** Induction/expression systems with lactose (left) and salt (right). **(C)** Final purification steps involving ion exchange chromatography or gel filtration.

system for the protein (26), and further process development resulted in modifications that avoided any proprietary genetic structures or media requirements enabling low-cost manufacturing. In addition, the synthetic adjuvant GLA-SE was selected for incorporation into the final product, which has now been utilized in Phase I clinical trials since this adjuvant enhances the Th-1 type responses such as gamma-interferon production that have been identified as representing the basis of Sm-14 mediated protective immunity both in human patients and animal models (see below) (27). The good manufacturing practice (GMP) production of rSm14 for clinical trials was undertaken at the LICR protein production facility at Cornell University in Ithaca, NY.

Immune Response to Sm14

The collaborative research group based at the *Fundação Oswaldo Cruz* has focused exclusively on the stepwise development of schistosomiasis vaccine guided solely by molecular considerations together with assays of protective immunity in animal models. On the other hand, other groups have investigated the nature of the immune response elicited by the antigen particularly in the context of natural human infection and the development of resistance to infection. As a first step, it was demonstrated that the sera of schistosomasis patients contain significant amounts of IgG1 and IgG3 subclass antibodies, whereas low levels of IgM, IgA, or IgE were measurable (28, 29). Specifically, the cellular immune responses to rSm14 were examined in chronic, treated patients and uninfected individuals living in an endemic area for schistosomiasis. Lymphocyte proliferative responses to rSm14 were detectable in the peripheral blood mononuclear cells of all groups studied with the highest proliferation index to rSm14 being detected in uninfected endemic normal (EN) individuals who are naturally resistant to schistosomiasis (28). This result provides direct evidence that the immune response to Sm14 may contribute to protective immunity in man. Moreover, it was determined that lymphocyte proliferation in the uninfected group was dependent

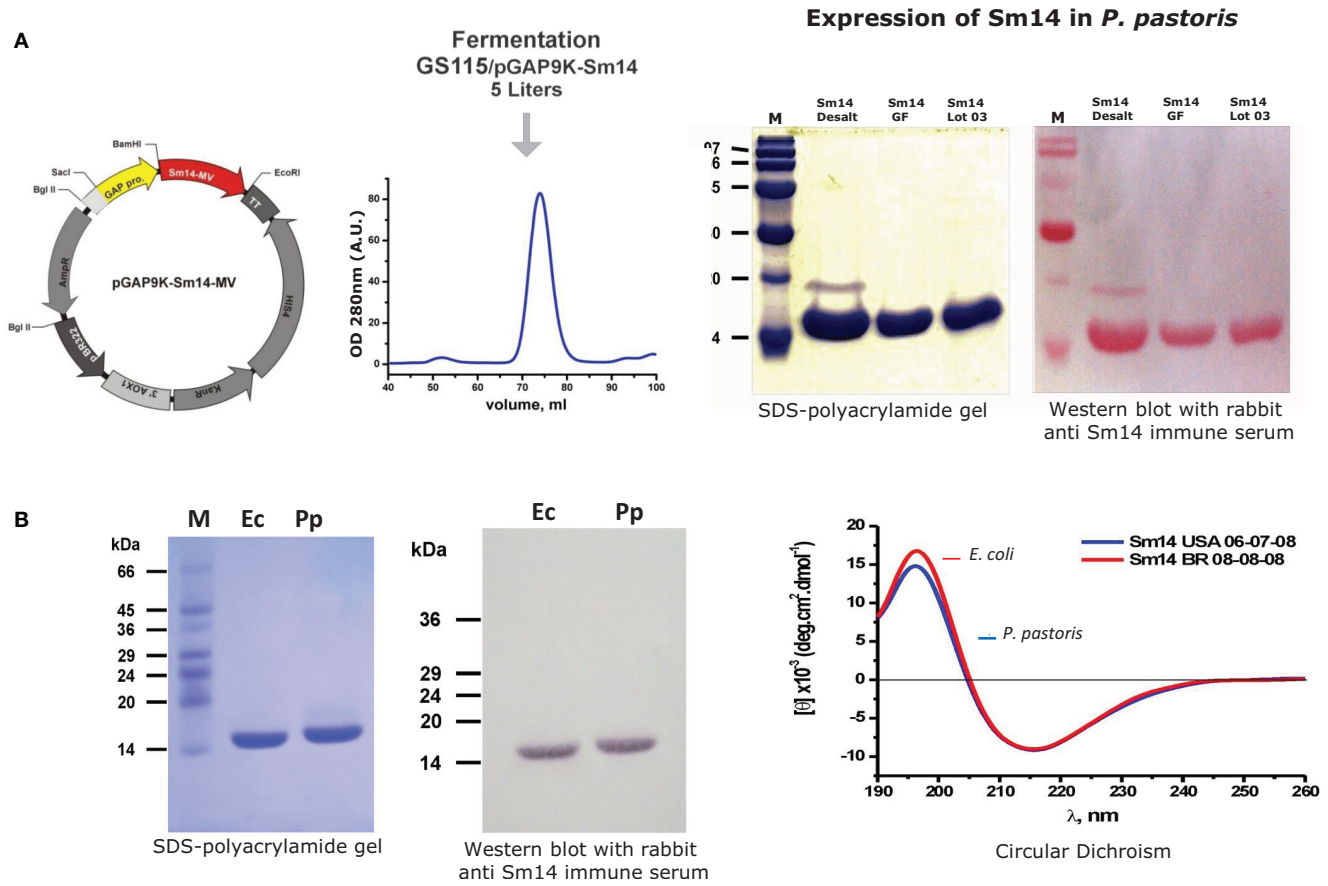

FIGURE 2 | Expression of Sm14 in *Pichia pastoris*. (A) Purification and detection of Sm14 expressed in *P. pastoris*. **(B)** Comparison between Sm14 protein batches purified using both platforms; *E. coli* (Ec) versus *P. pastoris* (Pp).

on IFN-gamma suggesting that the Th1 might be associated with resistance to infection. Furthermore, analyses in mice suggested that the same immune effector mechanism may be responsible for the protective immunity stimulated by rSm14 vaccination, i.e., that the schistosome vaccine based on Sm14 may reproduce naturally occurring protective immunity in man (30). Interestingly, the strong parallel relationship between naturally occurring immunity and rSm14 vaccination could be extrapolated to the molecular level. T-cell epitopes were identified within the molecule that are recognized by T-cells producing gamma interferon from resistant individuals. Furthermore, the peptide epitopes from Sm14, but not from another schistosome antigen (paramyosin), stimulated protective immunity and gamma-interferon producing T-cells in vaccinated mice (31, 32).

Phase I Clinical Trials

Following approval by the local Ethics Committee and the Brazilian Regulatory Agency, ANVISA, two separate Phase I clinical trials of the rSm14/GLA-SE vaccine have been undertaken with healthy volunteers. The first trial involved 20 males and the second involved 10 females (www.clinicaltrials.gov) (Number NCT01154049). These trials demonstrated that the vaccine is safe and immunogenic. The vaccine was administered intramuscularly in three 0.5 mL doses, each containing 50 μg of both rSm14 and GLA-SE. The second dose was administered 8 weeks after the first one, while the third dose was given 1–2 months later. There were no serious adverse events reported with the only side effects being mild local pain at the site of vaccination in some individuals. With the support of Infectious Disease Research (IDRI, Seattle, USA) clinical trial team, cells and sera from Brazilian volunteers were shipped to Seattle and extensively screened for the immune response generated by vaccination with Sm14 + GLA-SE toward the identification of the immunological signature of human immunization. Vaccination stimulated anti-Sm14 IgG antibodies as well as a Th1 T-cell response, resulting in gamma-interferon production in the vaccinated individuals. (manuscript submitted to Vaccine).

rSm14 as a Multi-Specific Anti-Helminth Vaccine

It has long been known that there is cross reactive protective immunity between the animal parasite *Fasciola hepatica* and schistosomes. Analysis of the molecular basis of this protective response identified a cross reactive antigen present in *F. hepatica*, termed Fh15 (33). The cloning and sequencing of Sm14 revealed this to be the corresponding protein in *S. mansoni*.

Molecular models showed that Fh15 and Sm14 adopt the same basic three-dimensional structure, consisting of a barrel-shaped molecule, and also identified shared discontinuous epitopes principally derived from amino acids in the C-terminal portions of the molecules. Moreover, rSm14 provided complete protection against challenge with *F. hepatica* metacercariae in a mouse model, suggesting that it may be possible to produce a single vaccine that would be effective against at least two parasites, *F. hepatica* and *S. mansoni*, of veterinary and human importance, respectively (18). Further analysis confirmed these initial findings and also demonstrated that vaccination with rSm14 can protect the natural host of *F. hepatica*, the sheep, against experimental parasite challenge resulting in complete abolition of liver pathology (34). In independent experiments undertaken in Spain, protection against *fasciola* infection was later also achieved in goats immunized with rSm14, where again significantly reduced liver damage was recorded (35). Several groups around the world have been undertaking studies to evaluate the potential of FABPs homologous to Sm14 derived from various organisms as vaccines against a number of different helminth diseases. Those include diseases caused by *F. hepatica* (18, 36–41), *S. mansoni* (18, 42), *Schistosoma japonicum* (43, 44), *Echinoccus granulosus* (45), and *Clonorchis sinensis* (46) in both experimental and natural veterinary hosts. The published reports from these groups provide a robust dataset, indicating the widespread potential efficacy of vaccines based on Sm14. Although experimental work has been undertaken with the FABPs from many parasites, only Sm14 has reached the stage of GMP production and clinical trials.

Future Perspectives

To conclude all pre-clinical stages, Sm14 project has overcome bottlenecks of a vaccine development, scaled-up the production, formulated the product, and fulfilled all regulatory requirements to start clinical study phase. The national regulatory authority (ANVISA) approved the results of phase 1 clinical studies, and Fiocruz has licensed Sm14 technology to a Brazilian company, Ourofino, for final development and commercialization of Sm14 vaccine for use in cattle herds against *fasciola*.

Field based immunogenicity and safety phase 2 trials of the Brazilian Sm14 + GLA-SE anti schistosomiasis vaccine are planned to start in 2015 in endemic areas (Brazil and Africa).

Acknowledgments

We thank our research team from Experimental Schistosomiasis Laboratory/Fiocruz and nurses from staff study from INI/Fiocruz for conducting the study; Donald De Roo from LICR for technical support for setting up the Fill Finish procedures at Florida Biologics and Quality Control Panel at PPD Inc; Flavia Parra for the organization of Data Bank; Tom Vedvick, from Infectious Disease Research Institute, for most valuable advices and support for formulation of Sm14with GLA; Tatiane dos Santos for support with preparation of Brochure of Investigator and Toxicology Tests; Carlos Henrique Henrique, from Ourofino Animal Health, for support at all levels with operational steps and preclinical animal tests; Jorge Bermudez, Vice President of Innovation and Production at Fiocruz and Robert Bergquist and PHI/WHO(World Health Organization), for strategic support. *Funding*: This study was funded by FINEP (*Financiadora de Estudos e Projetos* – Governmental Funding Agency); FAPERJ(Funding Agency from State of Rio de Janeiro); IOC/IOCRUZ(Oswaldo Cruz Institute/Oswaldo Cruz Foundation, Brazilian Health Ministry); and OUROFINO Saude Animal, SP, Brazil.

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frontiers in Immunology

ORIGINAL RESEARCH
published: 02 June 2015
doi: 10.3389/fimmu.2015.00273

OPEN ACCESS

Edited by:
Rashika El Ridi, Cairo University, Egypt

Reviewed by:
Mohamed Labib Salem, Tanta University, Egypt
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Specialty section:
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology

Received: *23 January 2015*
Accepted: *16 May 2015*
Published: *02 June 2015*

Citation:
Carvalho-Queiroz C, Nyakundi R, Ogongo P, Rikoi H, Egilmez NK, Farah IO, Kariuki TM and LoVerde PT (2015) Protective potential of antioxidant enzymes as vaccines for schistosomiasis in a non-human primate model. Front. Immunol. 6:273. doi: 10.3389/fimmu.2015.00273

Protective potential of antioxidant enzymes as vaccines for schistosomiasis in a non-human primate model

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Schistosomiasis remains a major cause of morbidity in the world. The challenge today is not so much in the clinical management of individual patients, but rather in population-based control of transmission in endemic areas. Despite recent large-scale efforts, such as integrated control programs aimed at limiting schistosomiasis by improving education and sanitation, molluscicide treatment programs and chemotherapy with praziquantel, there has only been limited success. There is an urgent need for complementary approaches, such as vaccines. We demonstrated previously that anti-oxidant enzymes, such as Cu–Zn superoxide dismutase (SOD) and glutathione S peroxidase (GPX), when administered as DNA-based vaccines induced significant levels of protection in inbred mice, greater than the target 40% reduction in worm burden compared to controls set as a minimum by the WHO. These results led us to investigate if immunization of non-human primates with antioxidants would stimulate an immune response that could confer protection as a prelude study for human trials. Issues of vaccine toxicity and safety that were difficult to address in mice were also investigated. All baboons in the study were examined clinically throughout the study and no adverse reactions occurred to the immunization. When our outbred baboons were vaccinated with two different formulations of SOD (SmCT-SOD and SmEC-SOD) or one of GPX (SmGPX), they showed a reduction in worm number to varying degrees, when compared with the control group. More pronounced, vaccinated animals showed decreased bloody diarrhea, days of diarrhea, and egg excretion (transmission), as well as reduction of eggs in the liver tissue and in the large intestine (pathology) compared to controls. Specific IgG antibodies were present in sera after immunizations and 10 weeks after challenge infection compared to controls. Peripheral blood mononuclear cells, mesenteric, and inguinal node cells from vaccinated animals proliferated and produced high levels of cytokines and chemokines in response to crude and recombinant antigens compared with controls. All together, these data demonstrate the potential of antioxidants as a vaccine in a non-human primate model.

Keywords: ***Schistosoma mansoni*, antioxidants, vaccine, anti-fecundity, baboon**

Introduction

Schistosomiasis remains a major cause of morbidity in the world. The disease is endemic in 76 countries of the world where it affects some 200 million people (1). Substantial research has established that school-aged children in endemic areas are at significant risk of serious disease resulting from infection with schistosomes (2, 3). Lack of access to clean water and sanitation and inadequate personal hygiene are important shared risk factors for these infections (4).

Currently, chemotherapy with praziquantel (PZQ) is the preferred treatment for schistosomiasis (5–8). Control programs based on mass chemotherapy are complicated by rapid and frequent re-infection and the difficulties and expense of maintaining these programs over a long term (9–12). There is evidence that schistosomes develop drug resistance against PZQ in some regions (13, 14), and there is additional evidence for serious rebound morbidity if regular and periodic treatments are interrupted (15–17). Despite recent large-scale efforts, integrated control programs aimed at limiting schistosomiasis by improving education and sanitation, molluscicide treatment programs to reduce the population of the intermediate snail host, and chemotherapy have had limited success (18). Effective control of schistosomiasis could prevent up to 130,000 deaths and avert up to 25 million disability adjusted life years lost annually (19). The challenge today is not so much in the clinical management of individual patients, but rather in population-based control of transmission (and consequently, of morbidity) in endemic areas. Because current control strategies employing chemotherapy with PZQ have not reduced transmission and morbidity to acceptable levels, there is an urgent need for complementary approaches, such as vaccines for schistosomiasis (20, 21).

Our own studies have focused on antioxidant enzymes as vaccine candidates [recently reviewed by Huang et al. (22)]. We hypothesized that antioxidants play a role in protecting the adult worms from damage derived from reactive oxygen species (23–26). To begin to test this hypothesis, we demonstrated that expression of the schistosome antioxidant enzymes [Cu-Zn superoxide dismutase (SOD); glutathione S peroxidase (GPX)] is developmentally regulated such that the lowest levels of gene expression (as measured by transcription) and enzyme specific activity were in the larval stages, the most susceptible to immune killing, and highest in adult worms, the least susceptible to immune elimination (23, 25, 27–29). To provide direct evidence that antioxidant enzymes were important in immune evasion (25) and thus were viable candidate vaccines, we used DNA vaccination strategies to demonstrate the efficacy of DNA constructs encoding either Cu/Zn cytosolic superoxide dismutase (SmCT-SOD), signal peptide-containing SOD (SmSP-SOD, also known as SmEC-SOD), or glutathione peroxidase (SmGPX) to be protective against *Schistosoma mansoni* infection in a murine challenge model. Employing different doses of plasmid cDNA constructs, mice exhibited a significant level of worm burden reduction when challenged with *S. mansoni* cercariae after immunization with SmCT-SOD (54%) and SmGPX (43.4%) from six independent experiments (29).

The WHO has identified several candidate vaccine antigens for independent evaluation, but none fulfilled the required standards in trials with mice. One of the criteria was a worm burden reduction of >40% in a murine challenge model (30). We have demonstrated that both SmCT-SOD and SmGPX were each capable as DNA-based (plasmid or vaccinia virus vehicles) vaccines to consistently induce significant levels of protection in an *S. mansoni* murine-challenge model, greater than the target 40% reduction in worm burden compared to control set as a minimum by the WHO. We also demonstrated in a mouse model that DNA encoding SmCT-SOD as a vaccine is able to significantly reduce worm burden by targeting adult *S. mansoni* worms 21 days and older (31).

The above results with the antioxidant enzymes in a murine model of *S. mansoni* encouraged us to investigate if immunization of non-human primates with antioxidants would stimulate an immune response that would be safe and that could confer protection.

Materials and Methods

Animals and Screening

Young wild caught Olive Baboons (*Papio cynocephalus anubis*), both males and females aged 4–5 years, were from schistosomiasis non-endemic areas and habituated and quarantined for 90 days in accordance with good animal welfare standards. The baboons were screened for the presence of several infections and parasites (such as malaria, tuberculosis, leishmaniasis, nematodes, etc.) before they were assigned to the study. For assessing the presence of current *Schistosoma* infections, the fecal material was examined for the presence of eggs and the serum from each animal was checked for the presence of antibodies against soluble egg antigens (SEA). Positive animals were excluded from our study or when necessary successfully treated using ivermectin and/or metro-nidazole as appropriate, months before the start of immunization. Detailed hematological tests certified all animals were in excellent health during their quarantine period. The animals were housed isolated at the facilities of the Institute of Primate Research (IPR), Karen, Nairobi, Kenya. Ethical clearance for these studies was obtained from the Institute for Primate Research IACUC (IPR/SRP3/2004) and the University of Texas Health Science Center at San Antonio IACUC (08039x).

Preparation of the Antioxidant Vaccines

cDNA containing the entire open reading frames of SmCT-SOD, SmEC-SOD, SmGPX were previously cloned into the eukaryotic expression vectors pcDNAI/Amp (pc) (31, 32) and VR1055 (Vical, San Diego, CA, USA) (33). Each plasmid preparation was then purified by double gradient centrifugation in $CsCl_2$, dialyzed against PBS to remove the $CsCl_2$, ethanol precipitated, and re-suspended in sterile sucrose (25% in PBS). The last boost consisted of the respective recombinant proteins incorporated into microspheres made of polylactic acid (PLA). Only recombinant proteins were incorporated into PLA microspheres, as previously described by us (32). Of note, the following terms are used interchangeably throughout the study: SmCT-SOD or CTSOD; SmEC-SOD or ECSOD; SmGPX or GPX; and Vector Only or Control.

Immunizations and Challenge

Two vaccination experimental protocols were carried out: Experiment 1 (**Figure 1A**) was for safety and toxicity evaluation after vaccination, while Experiment 2 (**Figure 2A**) for assessment of the efficacy of vaccination upon challenge with *S. mansoni*. In Experiment 1 (**Figure 1A**), groups of five olive baboons received 500 μg of purified DNA via two intramuscular (quadriceps) injections with a 26-gage needle in each leg. Each group received a second and third 500 μg dose of their respective DNA, 4 weeks apart. A fourth dose of 500 μg of recombinant proteins encapsulated in PLA microspheres as described (32) was given 4 weeks after the third injection. The control animals were primed and boosted with empty vector DNA, while the last boost consisted of empty PLA microspheres in PBS instead of recombinant proteins.

In Experiment 2 (**Figure 2A**), groups of five 4- to 5-year-old olive baboons were primed with 1000 μg of purified DNA, boosted once with 1000 μg doses of their respective purified DNA, and once with 1000 μg of the respective recombinant protein encapsulated in PLA microspheres, with all immunizations 4 weeks apart from each other. The control animals were primed and boosted with empty vector DNA, while the last boost consisted of empty PLA microspheres in PBS instead of recombinant proteins. The baboons were then anesthetized using a mixture of xylazine (2% Rompun™) and Ketamine hydrochloride (100 mg/ml; Agrar Holland BV, Soest, The Netherlands) at a dose of 10 mg/kg body weight. After shaving the groin areas of the baboons, 600 cercariae (Kenyan strain) in suspension from individual beakers were poured into a groin pouch and left to penetrate via the skin for 30 min.

Blood Collection for Hematology, Cell, and Serum Isolation

All animals were anesthetized with ketamine hydrochloride (10 mg/kg body weight) prior to blood collection. Then, a 10 ml syringe with a 21-gauge needle was used to draw blood from the femoral vein, where it was placed into an EDTA treated tube for hematology (Experiment 1) or used for serum collection (Experiment 2). In addition, 50 ml syringes containing 20 ml of Alsever's anti-coagulant solution (Sigma) and fitted with a 21-gauge needle were used to draw 20 ml of blood to a total volume of 40 ml blood mixture from the opposite limb (Experiment 2).

In the Experiment 1, as depicted in **Figure 1A**, the blood was drawn every 2 weeks (with exception of the last time point in which blood was taken 3 weeks after last boost). The eight time points were: week 0 (pre-immune, PI), week 2 (2 weeks post-priming), week 4 (pre-1st boost), week 6 (2 weeks post-1st boost), week 8 (pre-2nd boost), week 10 (2 weeks post-2nd boost), week 12 (pre-3rd boost), and week 15 (3 weeks post-3rd Boost). In the Experiment 2 (**Figure 2A**), the blood was drawn at three time-points: week 0 (pre-immune, PI), week 12 (pre-challenge, PC), and week 22 (pre-perfusion, PP).

Clinical Assessment for Safety and Toxicity

The baboons were monitored daily for appetite, behavior, and demeanor, and tested at 2-week intervals for temperature, weight measurements, complete blood counts, and leukocyte differentials at baseline (week 0) and after all immunizations.

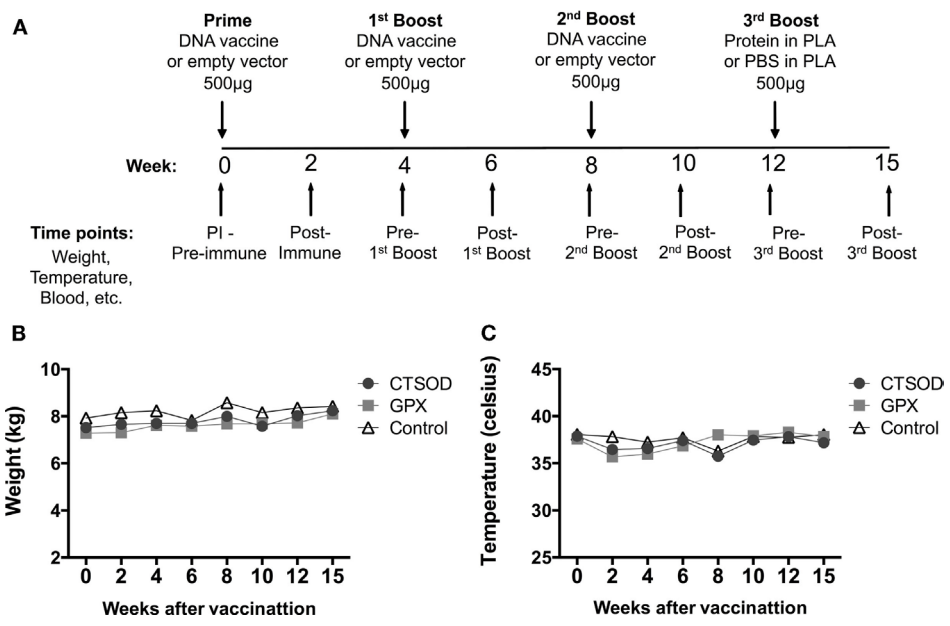

FIGURE 1 | Experiment 1 scheduling and clinical assessment of safety and toxicity. In **(A)**, the schematic representation of Experiment 1 protocol where olive baboons ($n = 5$ animals per vaccine group, $n = 10$ controls) were immunized with CTSOD, ECSOD, and GPX DNA-protein based vaccine. For safety and toxicity, the animals were monitored daily for appetite, behavior, and demeanor observations (data not shown) and bi-weekly for weight and temperature. In **(B,C)**, the average results of the bi-weekly monitoring of weight **(B)** and temperature **(C)** from baboons before and after immunizations with antioxidant vaccines during safety and toxicity evaluation. Animals were euthanized at week 26 post-immunization.

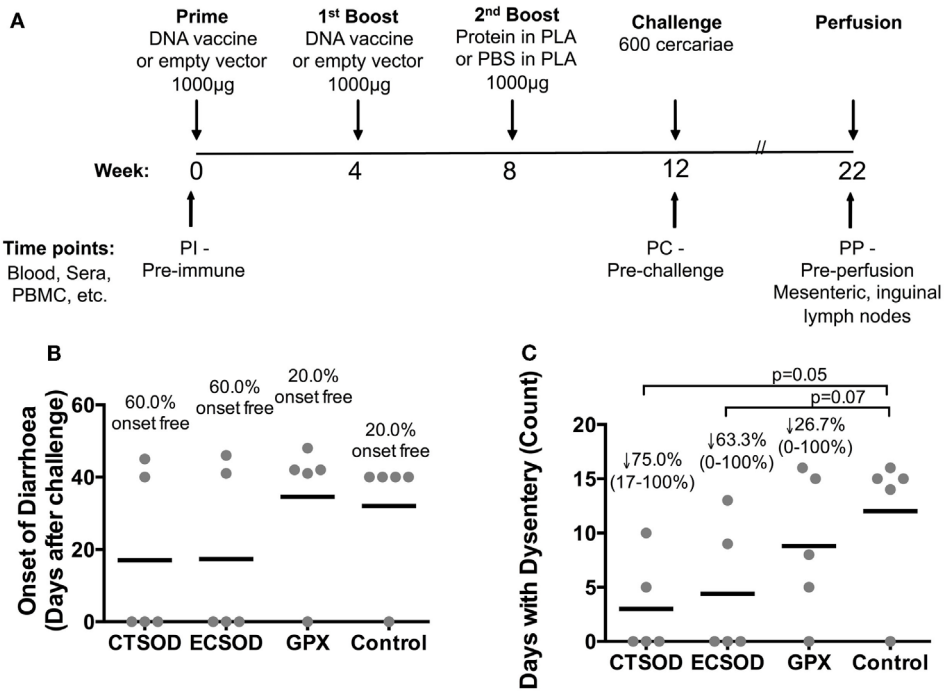

FIGURE 2 | Experiment 2 scheduling and clinical assessment after challenge infection. In **(A)**, the schematic representation of the Experiment 2 protocol where olive baboons were immunized with CTSOD, ECSOD, and GPX DNA-protein based vaccine ($n = 5$ animals per each group), and further challenged with 600 *S. mansoni* cercariae. Animals were also assessed for safety and toxicity of vaccination (data not shown). In **(B,C)**, commence of diarrhea and duration of dysentery in days after challenge infection. Individual values (sphere); mean average (dash). The percentage of animals that did not suffer diarrhea is represented in **(B)**. In **(C)**, the percentage of reduction (represented by the arrow), the range (minimal-maximal, represented between brackets), and the statistical *p*-value were expressed in comparison to the mean average of the Control group. Non-parametric (Kruskal–Wallis, Mann–Whitney) tests were applied. Values were considered statistically significant when $p < 0.05$.

Perfusion

Approximately 10 weeks post-challenge, the baboons were euthanized by intravenous administration of heparinized sodium pentobarbital. For each animal, the viscera were exposed after a median incision. The gross pathology of the liver, intestines, and mesenteric lymph nodes was evaluated. Perfusion of worms from the mesenteric vasculature and the liver was achieved after the administration of citrated saline through the abdominal aorta, as described previously (34), and adult worms were counted. To ensure that all worms were recovered, during the perfusion, the mesenteric veins were manually searched for schistosome worms. The worms were counted, and as an indicator of efficacy, the mean percent protection observed in the experimental groups was determined as the decrease in worm burden of each animal compared to the mean recovery of the control groups (35).

Parasitological Parameters

The weekly assessment of eggs per gram of feces (EPG) from vaccinated and control baboons commencing 6 weeks after *S. mansoni* cercariae challenge was determined by the thick smear Kato-Katz microscope technique (36). For extraction and determination of eggs present in organs, 10% tissue samples (by weight) of liver and intestines were digested overnight in 5% KOH, as described previously (37). The eggs were counted and the percentage of reduction in egg excretion in stool or tissues calculated in relation to the mean average of the control group.

Enzyme-Linked Immunosorbent Assay

The titers of IgG antibodies in the baboon sera against recombinant antigens derived from *S. mansoni* were determined by ELISA as described with minor modifications (32). Flat-bottom 96-well polystyrene plates (Maxisorp Nunc, GIBCO, Scotland) were coated overnight at 4°C with 50 µl of antigens diluted in PBS 0.15 M, pH 7.2 (PBS) at concentration of 5 µg/ml. After washing (automatic ELISA Washer – MR 5000, Dynatech) and blocking, 50 µl/well of doubling dilution of individual serum or pooled sera diluted in PBS-Tween 20, 0.05% PBS-T were added to each plate and the plates incubated overnight at 4°C. After washing, 50 µl/well of unconjugated goat anti-monkey IgG antibodies (Abd Serotec) were added and the plates were incubated 1 h at 37°C. After the incubation, the plates were washed, and 50 µl of alkaline-phosphatase conjugated donkey anti-goat IgG antibodies (Abd Serotec) were added to those plates with unconjugated antibody, and the plates were incubated for another 1 h at 37°C. Detection of reactivity was performed by using 100 µl/well of pNPP in diethanolamine (DEA) buffer (pNPP Microwell Substrate System, KPL) and the absorbance measured at intervals at 405 nm by an automatic ELISA Reader (BIORAD). All the

assay conditions were previously set up for optimal concentrations through checkerboard and titration curves and all the reagents for antibody detection used in this assay were shown to be clear of non-specific reactions.

Isolation of Peripheral Blood Mononuclear Cells and Tissue Cells for Recall Proliferation and Cytokine Culture

The mixture of blood/Alsever's fluid was layered in two tubes containing 10 ml of density gradient solution (Ficoll-Paque™ Research Grade, Pharmacia, Uppsala, Sweden) and centrifuged at 2000 rpm for 20 min at room temperature. The peripheral blood mononuclear cells (PBMCs) were recovered from the gradient interphase of plasma and red blood cells, washed twice in RPMI 1640 medium containing 80 μg/ml gentamycin (Life Technologies, UK) by centrifuging 2000 rpm for10 min for the first time, and 1500 rpm for 10 min for the second time. The pellets were re-suspended in 10 ml of RPMI 1640 containing 10 μg/ml of Polymixin B, 10% FCS (GIBCO, Paisley, UK), 80 μg/ml gentamycin solution, 1% glutamine (200 mM), and 25 mM HEPES. For cells derived from spleen, mesenteric, and inguinal lymph nodes, the tissues were disrupted after having been pressed over a 40 μm cell strainer with a help of a syringe plunge. The cells were washed once in RPMI 1640 medium containing 80 μg/ml gentamycin (Life Technologies, UK) by centrifuging 2000 rpm for 10 min before the red blood cells were lysed for 10 min with lysing solution. After two more washes, the pellet was also re-suspended in 10 ml of RPMI 1640 containing 10 μg/ml of Polymixin B, 10% FCS (GIBCO, Paisley, UK), 80 μg/ml gentamycin solution, 1% glutamine (200 mM), and 25 mM HEPES. Viable cells were counted in a hemocytometer chamber using Trypan Blue dye exclusion (Sigma, St Louis, MO, USA). After cell count adjustment, 2×10^5 cells were dispensed with 200 μl of RPMI 1640 containing 10% FCS (GIBCO, Paisley, UK), 80 μg/ml gentamycin solution, 1% glutamine (200 mM), and 25 mM Hepes into triplicate wells of a 96-well microtiter plates containing 20 μl of parasite antigen at 5 μg/ml (SWAP, SEA), 10 μg/ml (recombinant antigens), or 5 μg/ml of Con A. Plates were cultured at 37°C, 5% CO_2 for 72 h, when 20 μl of 0.5 μCi of ^{3}H thymidine was added per well. After incubation for another 18–20 h, the cells were harvested onto fiberglass filter papers before the thymidine incorporation was measured using 2 ml of scintillation fluid per filter in a Beta counter. Results were expressed as stimulation index (S.I.), the ratio of the mean counts per minute (cpm) of triplicate culture cells taken up in the presence of the antigen over those obtained with medium alone.

For cytokine cultures, 2×10^6 cells were dispensed with 1000 μl of RPMI 1640 containing 10% FCS (GIBCO, Paisley, UK), 80 μg/ml gentamycin solution, 1% glutamine (200 mM), and 25 mM HEPES into each well of 48-well tissue culture plates containing 20 μl of parasite antigen at 25 μg/ml (Schistosome Soluble Worm Antigen Preparation, SWAP; Soluble Egg Antigen, SEA), 50 μg/ml (recombinant antigens), or 25 μg/ml of Con A. After 72 h of incubation at 37°C, 5% CO_2, about 800 μl of supernatant/well were harvested and stored at −70°C until use. Freezing medium as 80%FCS/20% dimethyl sulfoxide (DMSO) was used in a drop wise manner in order to keep the remaining cells in cryo-freezing vials at 1×10^7/ml concentration inside a liquid nitrogen tank.

Analysis of Soluble Cytokines and Chemokines Secretion After Antigenic Stimulation

Harvested supernatants derived from PBMC cultures after stimulation with crude and recombinant antigens were assayed simultaneously using a 23-plex Non-Human Primate Cytokine/Chemokine Immunoassay Milliplex Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions, including quality controls. Briefly, 50 μl of standards or samples were incubated with multi-cytokine beads for 2 h in the dark, and following washes with a vacuum manifold (Millipore, Billerica, MA, USA) with biotinylated reporter for 1.5 h. The plates were then incubated with Streptavidin–Phycoerythrin for 30 min before the reaction was stopped for data collection in the Luminex 200 instrument using Luminex IS 2.3 software (Luminex Corporation, Austin, TX, USA) with a minimum of 50 beads per analyte. The resulting mean fluorescence intensity (MFI) was normalized and analyzed through the BeadView Multiplex Data Analysis Software version 1.0 (Millipore, Billerica, MA, USA), and expressed in pg/ml. The following cytokines and chemokines were assayed: IFN-γ, IL-12, IL-4, IL-6, IL-17A, CCL3 (MIP-1α), IL-5, IL-13, CCL2 (MCP-1), IL-1β, IL-2, IL-15, CCL4 (MIP-1β), TNF-α, IL-10, TGF-α, IL-1Ra, IL-8, GM-CSF, sCD40L, VEGF, G-CSF, IL-18.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (Prism 6 for Mac OS X) software. Parametric tests (ANOVA, student's *t*-tests) were used after log-transformation with corrections for multiple analyses. Otherwise, non-parametric (Kruskal–Wallis, Mann–Whitney) tests were applied. Values were considered statistically significant when $p < 0.05$, and assigned $^*p = 0.01$–0.05; $^{**}p = 0.001$–0.01; and $^{***}p = <0.001$.

Results

Safety and Toxicity (Experiment 1)

As noted in Section "Immunizations and Challenge" and in **Figure 1A**, the Experiment 1 was for safety and toxicity evaluation after vaccination. All animals in the study were examined clinically throughout the study. The animals appeared healthy, eating normally without any physical signs of toxicity. The weight (**Figure 1B**) and temperature (**Figure 1C**) of each animal were normal throughout the study. In addition, as shown in **Tables 1** and **2**, the hematological values of the vaccinated animals remained within the normal limits throughout the observation period and there were no other clinical adverse signs. In addition, we examined the vaccine injection sites following immunization and did not observe any significant irritation at the vaccination site i.e., baboons did not experience any muscle transient induration, granulomas, abscesses, ulcers, cutaneous erythema, inguinal lymphadenopathy,or skin swelling at the vaccination sites (data not shown). In a similar manner, we examined baboons for adverse events due to vaccination in Experiment 2. There were none.

Diarrhea, Duration of Dysentery, and Egg Excretion (Experiment 2)

The safety of our vaccine candidates encouraged us to proceed with Experiment 2 (outlined in **Figure 2A**), where groups of five

TABLE 1 | Summary of effects of vaccination in baboons (Experiment 1)[a].

	Week	Time point	WBC (×10³)	Neu (%)	Eos (%)	Baso (%)	Lymp (%)	Mono (%)
CTSOD	0	PI	8.84 ± 2.53	61.2 ± 3.7	1 ± 0.71	0 ± 0	34.2 ± 0.96	2.6 ± 1.14
	2	Post-I	14.98 ± 5.09	59.2 ± 6.94	1.8 ± 1.30	0 ± 0	35 ± 9.08	3.8 ± 2.17
	4	Pre-boost 1	12.66 ± 2.0	58.8 ± 12.58	1.2 ± 1.30	0 ± 0	38.8 ± 11.99	1.2 ± 0.45
	6	Post-boost 1	11.48 ± 2.74	45.6 ± 10.78	1.2 ± 2.17	0 ± 0	49.8 ± 10.92	2.6 ± 1.34
	8	Pre-boost 2	10.68 ± 2.95	38.2 ± 15.75	2.2 ± 2.17	0 ± 0	59 ± 16.73	0.6 ± 0.89
	10	Post-boost 2	16.86 ± 4.77	41.6 ± 9.18	3.2 ± 1.64	0 ± 0	55 ± 8.34	0.2 ± 0.45
	12	Pre-boost 3	12.52 ± 4.04	50.4 ± 17.56	2.4 ± 1.67	0 ± 0	46.4 ± 16.52	0.6 ± 0.89
	15	Post-boost 3	9.08 ± 2.48	47 ± 14.02	1 ± 0	0.4 ± 0.55	51.2 ± 13.99	0.4 ± 0.89
GPX	0	PI	11.62 ± 3.71	57.8 ± 14.97	1 ± 1.0	0 ± 0	37 ± 15.44	2.8 ± 0.84
	2	Post-I	10.32 ± 2.66	46.5 ± 7.05	0.7 ± 0.5	0.25 ± 0.5	49.5 ± 7.72	2.75 ± 0.96
	4	Pre-boost 1	10.46 ± 2.37	44.4 ± 14.71	0.2 ± 0.45	0.2 ± 0.45	53 ± 14.18	2.2 ± 0.84
	6	Post-boost 1	12.92 ± 3.27	36.2 ± 4.44	0.6 ± 0.55	0 ± 0	59.8 ± 4.09	3.4 ± 1.52
	8	Pre-boost 2	12.56 ± 2.17	51.2 ± 10.92	0.8 ± 1.30	0 ± 0	44.6 ± 9.94	2.2 ± 0.84
	10	Post-boost 2	18.9 ± 5.03	30.8 ± 13.54	0.6 ± 0.89	0 ± 0	67.6 ± 11.93	1 ± 1.73
	12	Pre-boost 3	13.52 ± 2.53	40.4 ± 20.44	1 ± 0.71	0 ± 0	57.6 ± 20.01	1 ± 2.24
	15	Post-boost 3	15.54 ± 4.58	66.2 ± 5.17	1 ± 2.24	0 ± 0	32 ± 4.53	0.6 ± 0.55
Control		Normal range	6–25.3	21–81	0–6	0–1	17–83	0–5

[a]*CTSOD n = 5, GPX n = 5, Control n = 10.*

Values are expressed as mean average ± SD.

PI, pre-immune; Post-I, post-immune; WBC, total leukocyte count; Neu, neutrophils; Eos, eosinophils; Bas, basophils; Lymp, lymphocytes; Mono, monocytes.

TABLE 2 | Summary of effects of vaccination in baboons (Experiment 1)[a].

	Week	Time point	RBC (×10⁶)	Hb (g/dl)	PVC (%)	MCV (fl)	MCH (pg)	MCHC(g/dl)
CTSOD	0	PI	4.302 ± 0.3	12.94 ± 0.93	38.8 ± 2.77	90.72 ± 11.5	30.26 ± 3.83	33.3 ± 0
	2	Post-I	4.06 ± 0.17	12.58 ± 0.36	33.5 ± 1.52	82.4 ± 1.12	30.98 ± 1.2	37.52 ± 1.48
	4	Pre-boost 1	4.626 ± 0.29	13.64 ± 0.15	37.86 ± 2.48	82.04 ± 1.66	29.54 ± 1.91	36 ± 2.29
	6	Post-boost 1	4.166 ± 0.23	13 ± 0.19	33.46 ± 2.69	80.3 ± 3.46	31.24 ± 1.81	39.02 ± 3.55
	8	Pre-boost 2	4.398 ± 0.35	13.56 ± 0.48	35.42 ± 4.46	80.34 ± 4.57	30.94 ± 2.58	38.7 ± 4.68
	10	Post-boost 2	4.634 ± 0.19	14.56 ± 0.21	39.04 ± 2.47	84.2 ± 2.37	31.4 ± 1.63	38.6 ± 2.58
	12	Pre-boost 3	4.248 ± 0.26	11.94 ± 1.15	35.42 ± 2.75	83.32 ± 3.24	27.92 ± 2.3	33.54 ± 3.04
	15	Post-boost 3	5.022 ± 0.19	12.34 ± 1.1	34.42 ± 1.20	68.5 ± 2.96	24.56 ± 2.12	35.88 ± 3.82
GPX	0	PI	4.264 ± 0.51	13.14 ± 0.76	40.34 ± 1.93	95.24 ± 9.91	31.28 ± 3.57	33.3 ± 0
	2	Post-I	4.59 ± 0.39	12.77 ± 0.55	38.25 ± 3.29	83.35 ± 0.17	27.9 ± 2.03	33.55 ± 2.6
	4	Pre-boost 1	4.79 ± 0.24	14.6 ± 0.86	39.92 ± 2.23	83.08 ± 2.98	31.08 ± 1.87	36.6 ± 2.95
	6	Post-boost 1	4.532 ± 0.43	13.78 ± 0.74	38.36 ± 6.42	88 ± 14.44	30.48 ± 1.46	35.58 ± 6.35
	8	Pre-boost 2	4.596 ± 0.26	14.64 ± 0.88	36.48 ± 2.06	79.36 ± 0.96	31.8 ± 0.23	40.08 ± 0.57
	10	Post-boost 2	4.348 ± 0.23	14.86 ± 0.96	36.42 ± 2.25	83.7 ± 1.46	34.14 ± 1.32	40.78 ± 1.99
	12	Pre-boost 3	4.554 ± 0.26	11.92 ± 0.34	39.82 ± 4.49	87.14 ± 4.91	26.18 ± 1.92	30.12 ± 3.99
	15	Post-boost 3	5.286 ± 0.52	13.58 ± 0.89	35.9 ± 3.23	68.1 ± 1.81	25.76 ± 2.17	37.96 ± 3.51
Control		Normal range	3.64–5.36	11.3–14.5	27.7–49.3	65.4–102	23.5–35.2	28.9–44.4

[a]*CTSOD n = 5; GPX n = 5; Control n = 10.*

Values are expressed as mean average ± SD.

PI, pre-immune; Post-I, post-immune; RBC, erythrocyte count; Hb, hemoglobin concentration; PVC, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

baboons each were vaccinated with CTSOD and GPX, as well as ECSOD, and challenged with 600 *S. mansoni* cercariae. Weekly clinical analysis demonstrated that all three vaccinated groups had higher percentage of animals without diarrhea (CTSOD = 60%, ECSOD = 60%, and GPX = 40%) upon the onset of egg deposition when compared to only 20% in the Control group (**Figure 2B**). In addition, animals vaccinated with antioxidants showed fewer mean days of dysentery compared to controls (**Figure 2C**), where CTSOD group presented with a mean reduction of 75% (ranging from 100 to 17%), ECSOD with 63% (ranging from 100 to 0%), and GPX with 25% (ranging from 100 to 0%).

The weekly assessment of the EPG commencing 6 weeks after challenge showed that vaccinated groups had an overall reduction of eggs when compared to the Control group (**Figures 3A–F**). This reduction was statistically significant within ECSOD at week 8 (**Figure 3C**) when compared to the unvaccinated Control group. When the total EPG over a 5-week period was analyzed, all vaccinated groups showed reduction of excreted eggs, specially

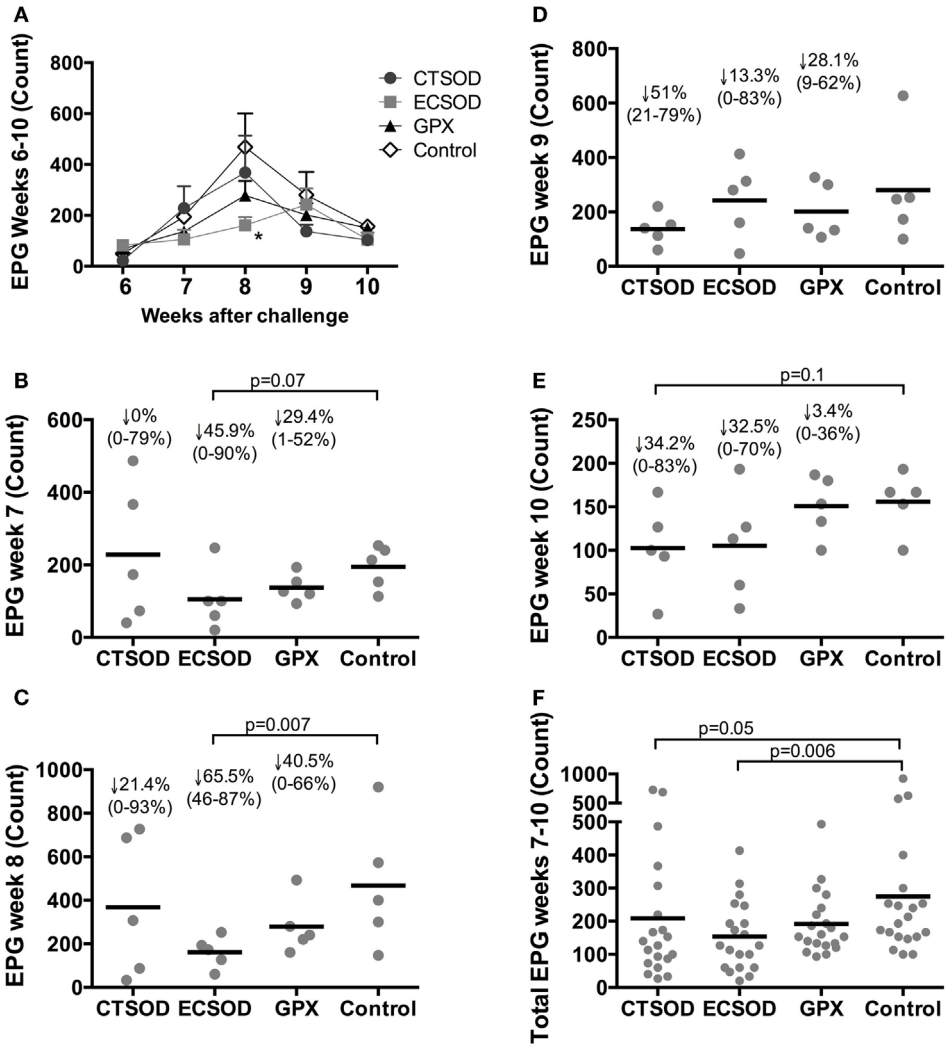

FIGURE 3 | Dynamics of weekly egg excretion per gram of feces (EPG) from Experiment 2. Assessment of eggs per gram of feces (EPG) from vaccinated and control baboons ($n = 5$ animals per group) commencing 6 weeks after *S. mansoni* cercariae challenge: weekly overview **(A)**; week 7 **(B)**; week 8 **(C)**; week 9 **(D)**; week 10 **(E)**; and the total EPG excreted over 5 weeks period **(F)**. The statistical p-value was expressed in comparison to the mean average of the Control group. Results were log-transformed and corrected for multiple analyses when applying ANOVA and student's t-tests. Values were considered statistically significant when $p < 0.05$.

ECSOD vaccinated animals who showed statistically significant reduction when compared to the Control (**Figure 3F**).

Tissue Eggs from Liver, Small Gut, and Large Gut (Experiment 2)

The distribution of eggs recovered in harvested organs after KOH digestion from vaccinated and control baboons 10 weeks after challenge at perfusion time point is shown in **Figure 4**. There was an overall reduction of eggs in all vaccinated groups in the liver, small gut, and large gut when compared to the Control group. The egg reduction in the liver (**Figure 4A**) ranged from 56 to 0% for CTSOD, 84 to 3% for ECSOD, and 56 to 0% for GPX, while in the small gut (**Figure 4B**), the reduction in CTSOD group ranged from 64 to 0%, ECSOD ranged from 88 to 0%, and GPX ranged from 100 to 0%. When the large gut was evaluated (**Figure 4C**), egg reduction ranged from 70 to 0% for CTSOD, 93 to 0% for ECSOD, and 89 to 0% for GPX, when compared to the mean average of Control group.

Worm Burden/Percentage of Protection (Experiment 2)

When the distribution of worms (**Figure 5A**), males (**Figure 5B**), and females (**Figure 5C**) recovered from outbred vaccinated and control baboons 10 weeks after challenge with *S. mansoni* cercariae was evaluated at the perfusion time point, ECSOD vaccinated group showed a reduction of total worms that ranged from 89 to 0% (mean protection 19.5%), while GPX group showed a reduction that ranged from 47 to 0% (mean protection 17.1%)

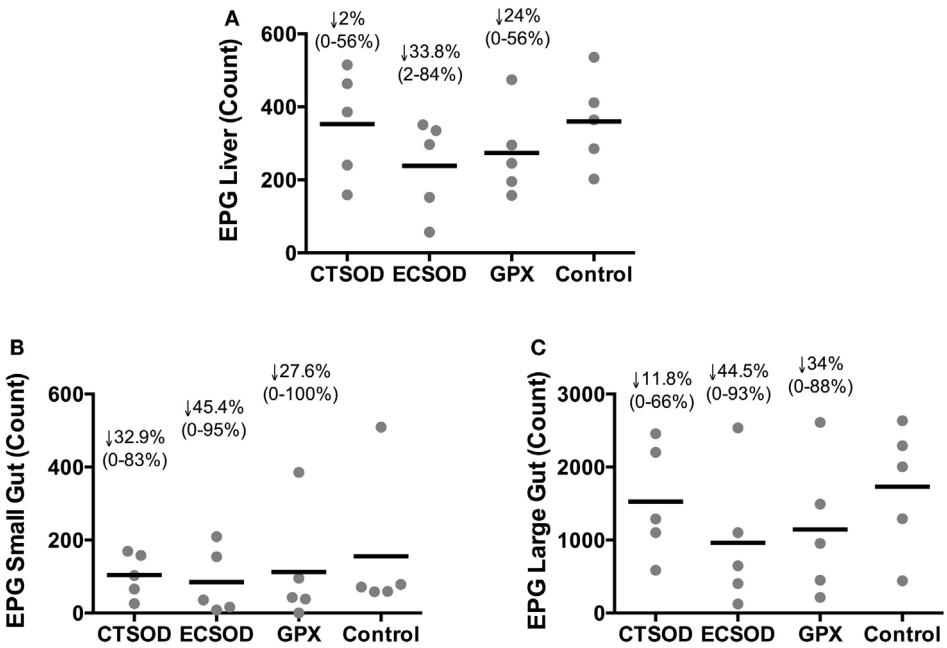

FIGURE 4 | **Distribution of eggs recovered in tissues from Experiment 2**. Liver **(A)**, small gut **(B)**, and large gut **(C)** eggs after KOH digestion from vaccinated and control baboons ($n = 5$ animals per group) 0 weeks after challenge with *S. mansoni* cercariae (perfusion time point). Individual values (sphere); mean average (dash). The percentage of reduction (represented by the arrow), the range (minimal–maximal, represented between brackets), and the statistical p-value were expressed in comparison to the mean average of the Control group. Non-parametric (Kruskal–Wallis, Mann–Whitney) tests were applied. Values were considered statistically significant when $p < 0.05$.

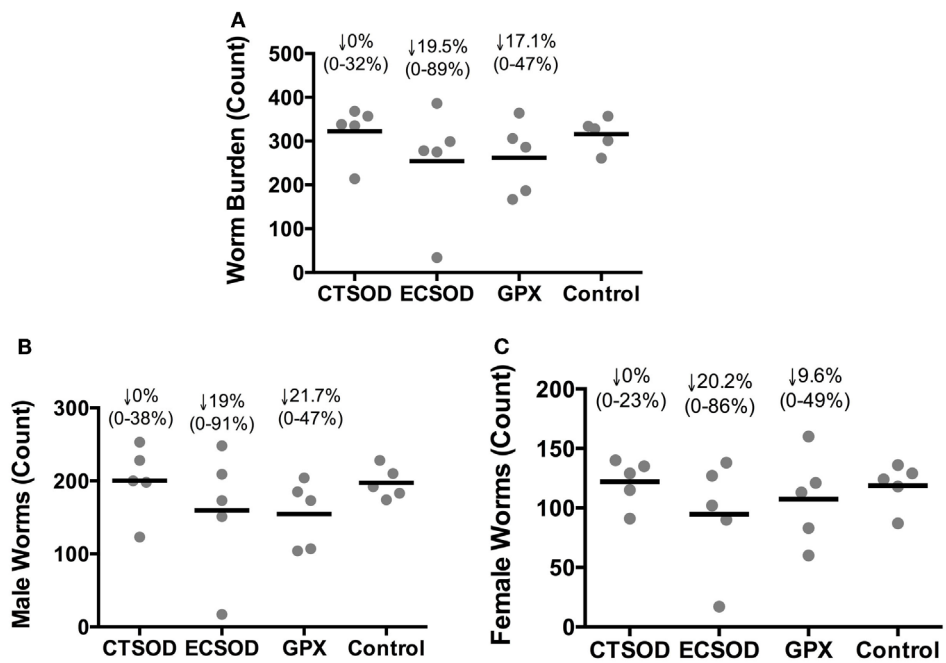

FIGURE 5 | **Distribution of worms recovered (worm burden) from Experiment 2**. Total worm burden **(A)**, male **(B)** and female **(C)** worm count. Worms from vaccinated and control baboons ($n = 5$ animals per group) 10 weeks after challenge with *S. mansoni* cercariae (perfusion time point). Individual values (sphere); mean average (dash). The percentage of reduction (represented by the arrow), the range (minimal–maximal, represented between brackets), and the statistical p-value were expressed in comparison to the mean average of the Control group. Non-parametric (Kruskal–Wallis, Mann–Whitney) tests were applied. Values were considered statistically significant when $p < 0.05$.

of worms when compared to the Control group (**Figure 5A**). The same pattern was observed when worms were stratified into males (**Figure 5B**) and females (**Figure 5C**). The CTSOD group failed to show a mean reduction in worms, although reduction in two vaccinated baboons (out of five) is visible.

Antibody Response (Experiment 2)

Titration of baboon sera against recombinant antigens showed that levels of specific IgG antibodies were stimulated after immunizations (PC) with CTSOD (**Figure 6A**) as well as with GPX (**Figure 6D**), and to a lesser degree with ECSOD (**Figure 6B**), when compared to control sera (**Figures 6C,E**). Antibody levels continued to be elevated 10 weeks after challenge infection (PP) for CTSOD and GPX groups.

CMI Proliferation/Recall Responses (Experiment 2)

Recall proliferation assays from PBMCs (**Figures 7A–C**) were performed against crude and recombinant antigens before (pre-immune, PI), after immunizations (pre-challenge, PC), and after challenge with *S. mansoni* at the perfusion time point (PP). The recall proliferation assays for mesenteric (MLN) and inguinal (ILN) lymph nodes (**Figures 7D–E**) were performed only at the time of the perfusion (PP). All results were expressed as Stimulation Index (S.I.): the ratio of the mean cpm of triplicate culture cells taken up in the presence of the antigen over those obtained with medium alone. PBMC (**Figures 7A,B**) and MLN cells (**Figures 7D,E**) from both CTSOD and ECSOD groups proliferated in response to recombinant CTSOD antigens when compared with Control. Similarly, PBMC (**Figure 7C**), mesenteric, and inguinal node

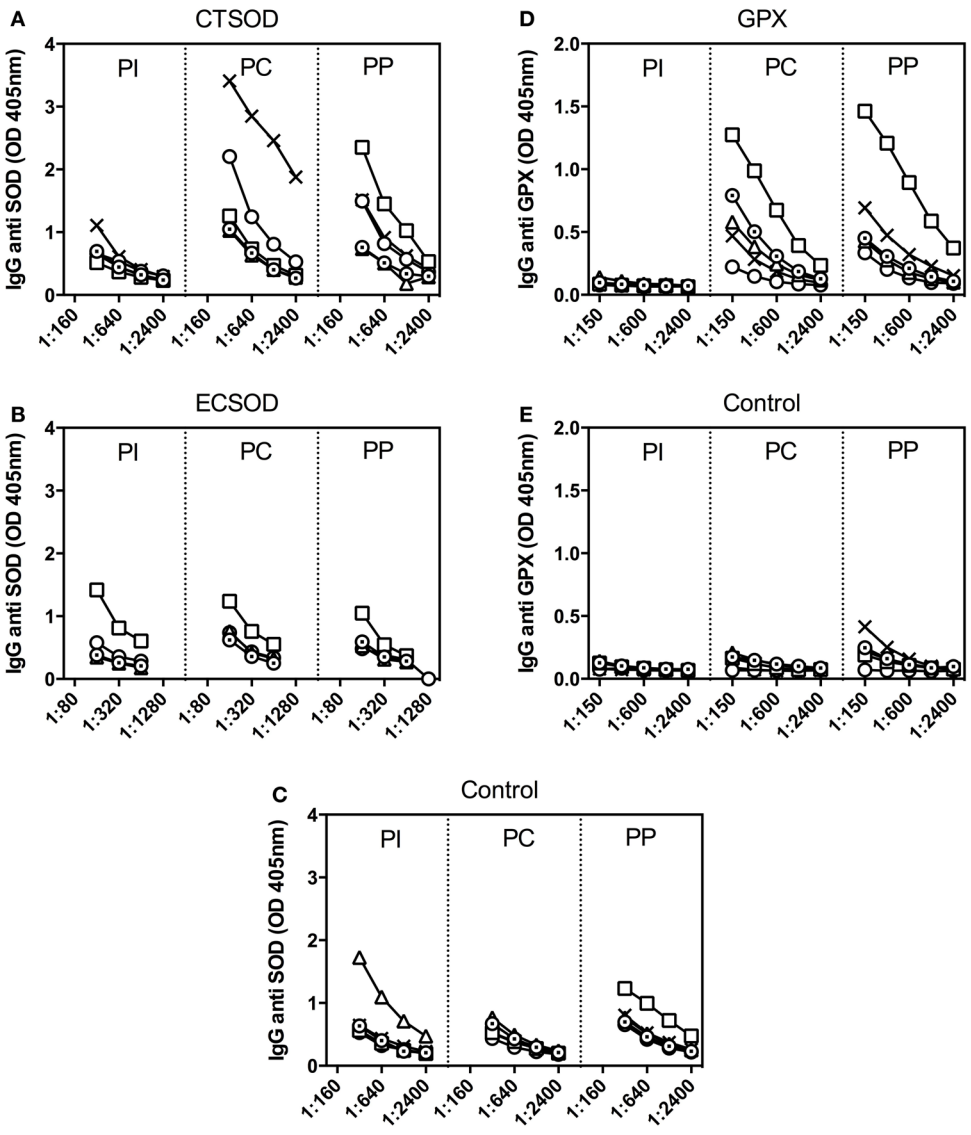

FIGURE 6 | **Antibody response from Experiment 2**.Titration of baboon IgG antibodies against recombinant SOD **(A–C)** and GPX **(D–E)** from CTSOD **(A)**, ECSOD **(B)**, GPX **(D)**, and Control **(C,E)** groups (n = 5 animals per group) before (PI), after immunizations (pre-challenge, PC), and before perfusion (pre-perfusion, PP).

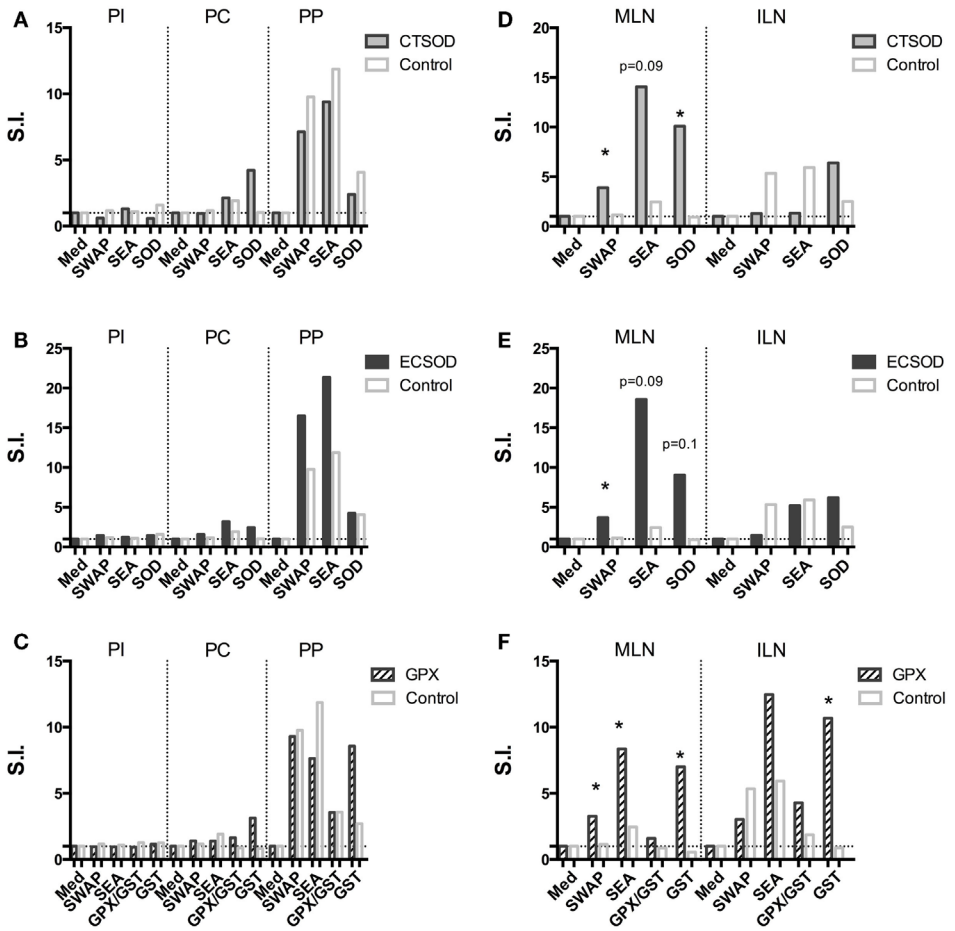

FIGURE 7 | **Recall proliferation assays from Experiment 2**. PBMC **(A–C)** and mesenteric (MLN) and inguinal (ILN) lymph nodes **(D–F)** against crude and recombinant antigens from CTSOD **(A,D)**, ECSOD **(B,E)**, GPX **(C,F)**, and Control groups ($n = 5$ animals per group) before (PI), after immunizations (pre-challenge, PC), and after challenge with *S. mansoni* at the perfusion time point (PP). Results were expressed as Stimulation Index (S.I.), the ratio of the mean counts per minute (cpm) of triplicate culture cells taken up in the presence of the antigen over those obtained with medium alone. Non-parametric (Kruskal–Wallis, Mann–Whitney) tests were applied. Values were considered statistically significant when $p < 0.05$.

cells (**Figure 7F**) from GPX vaccinated animals proliferated in response to recombinant antigens in comparison with Control group. PBMC and MLN from all groups responded to SEA and SWAP after challenge with *S. mansoni* cercariae (**Figures 7D–F**).

Secretion of Cytokines and Chemokines upon Stimulation (Experiment 2)

A panel of 23 multiplexed IFN-γ, IL-12, IL-4, IL-6, IL-17A, CCL3 (MIP-1α), IL-5, IL-13, CCL2 (MCP-1), IL-1β, IL-2, IL-15, CCL4 (MIP-1β), TNF-α, IL-10, TGF-α, IL-1Ra, IL-8, GM-CSF, sCD40L, VEGF, G-CSF, IL-18 cytokines and chemokines (**Figures 8–11**; Figures S1 and S2 in Supplementary Material) was used to investigate secretion from individual supernatants derived from PBMCs from CTSOD (A, D, G), ECSOD (B, E, H), and GPX (C, F, I) groups after 72 h stimulation with crude and recombinant antigens at all time points: before vaccination (PI), before challenge (PC), and at perfusion (PP). Values were expressed as median of cytokine secretion from five individual supernatants.

In general, vaccination with ECSOD and CTSOD induced variable levels of cytokines before challenge (PC) upon stimulation with recombinant antigens when compared to the baseline levels (PI), although these levels were somewhat discrete. However, vaccination with GPX induced higher levels of IL-5 (**Figure 8F**) and IL-17A (**Figure 9I**) cytokines, and reduction of IL-8 (**Figure 10F**) and chemokines such as CCL2, CCL3, and CCL4 (**Figures 11C,F,I**) when compared to Control. CTSOD vaccination induced increase in the IL-17A levels (**Figure 9G**), while a reduction of IL-1β, IL-18 (**Figures 10A,B,G,H**) cytokines, and CCL3 and CCL4 (**Figures 11C,I**) chemokines in CTSOD and ECSOD groups was also observed, when compared to Control.

After challenge, several cytokine and chemokine levels increased in the vaccinated groups after the natural boost provided from the infection with *S. mansoni* (**Figures 8–11**). For instance, high levels of IL-4, IL-5, IL-13 (**Figures 8A–I**), IFN-γ (**Figures 9A–C**) cytokines were stimulated after challenge (PP) against SWAP and SEA in the CTSOD, ECSOD, and GPX groups when compared

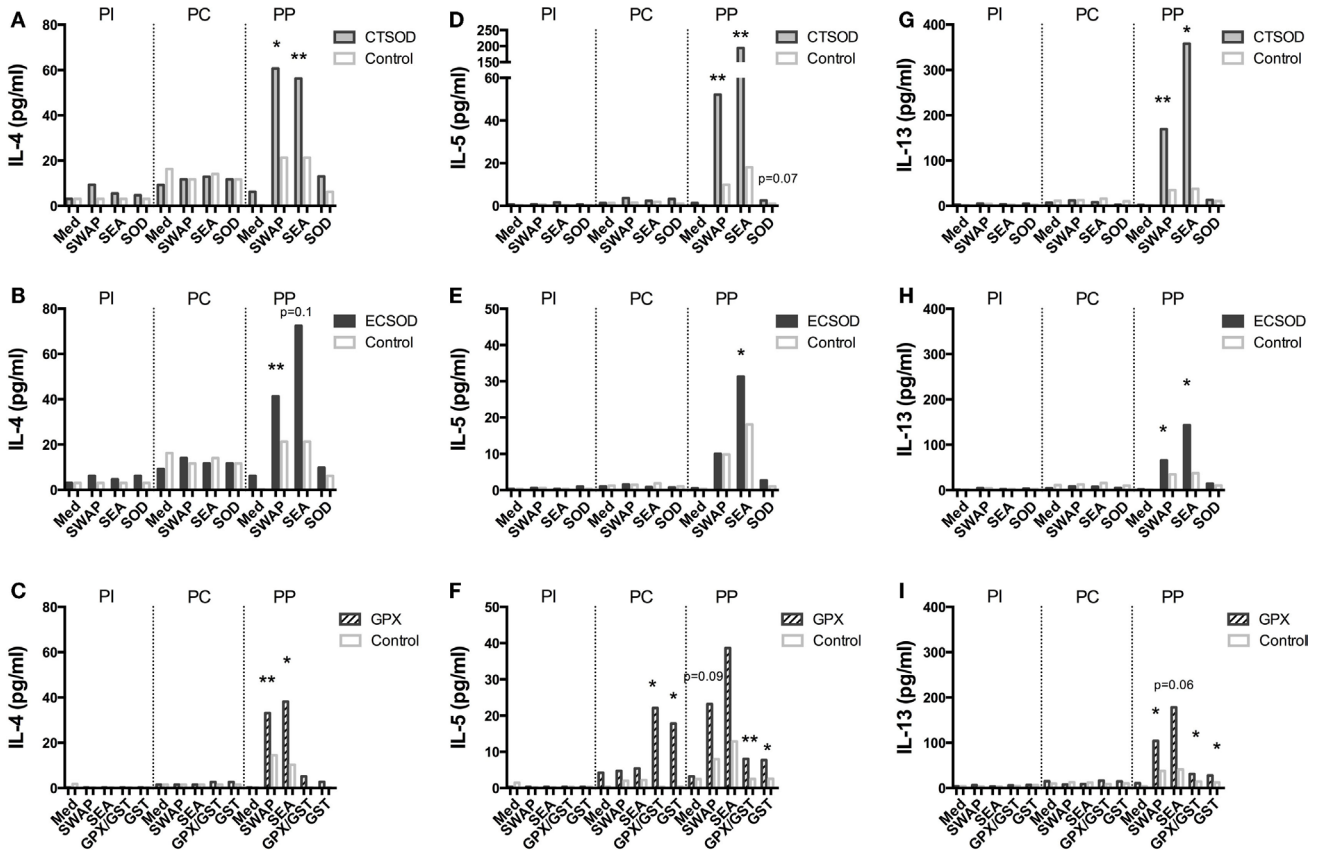

FIGURE 8 | **Secretion of cytokines in supernatants from Experiment 2**. PBMCs from CTSOD **(A,D,G)**, ECSOD **(B,E,H)**, and GPX **(C,F,I)** groups (n = 5 animals per group) were stimulated 72 h with crude and recombinant antigens and secreted IL-4, IL-5, and IL-13 levels determined, before vaccination (PI), before challenge (PC), and at perfusion (PP). Values were expressed as median of cytokine secretion in five individual supernatants in relation to the Control baboons. Non-parametric (Kruskal–Wallis, Mann–Whitney) tests were applied. Values were considered statistically significant when $p < 0.05$, and assigned $^{*}p$ = 0.01–0.05; $^{**}p$ = 0.001–0.01; and $^{***}p$ = <0.001.

to (PC) levels, and this response was higher in the vaccinated groups when compared to the control group. The levels of IL-15 (**Figures 9D–F**) were also increased, especially in the GPX group, before perfusion (PP) when compared to control.

Discussion

As mentioned previously, our laboratory has demonstrated that vaccination of inbred mice with naked DNA constructs containing Cu/Zn cytosolic superoxide dismutase (CT-SOD), signal-peptide containing SOD (EC-SOD), or glutathione peroxidase (GPX) derived from *S. mansoni* showed significant levels of protection compared to a control group (29). Screening of candidate antigens in mice is an important first step in vaccine development but it is unclear whether protective efficacy can be translated directly to humans (38). Therefore, our murine results encouraged us to investigate if immunization of non-human primates with these antioxidants would stimulate an immune response that could be correlated with protection as a prelude study for human trials. Baboons are an excellent model for schistosomiasis, as they are similar to humans in ontogeny, immune response (including human-like IgG subclasses), reproductive physiology, etc., and develop a human-like acute disease after natural or experimental exposure to *S. mansoni* (38–40). In addition, since issues of vaccine safety are also difficult to address in mice, another advantage of testing schistosome vaccines in baboons is the opportunity to address many of the deficiencies of mouse studies (38–41). We have also previously used baboons to successfully investigate the immune mechanisms associated with other schistosome vaccine candidates (42). Overall, our data indicate that no adverse reactions or abnormal animal demeanor occurred to the immunization of baboons with antioxidants, as our behavioral, clinical, and hematological evaluations of the vaccinated animals remained within the normal limits throughout the observation period for both experiments. Thus the vaccines are safe and well tolerated.

The next step was to check the antigenicity of the antioxidants after vaccination with baboons, as the dose and nature of the antigen as well as the route or kind of adjuvant used can ultimately dictate the outcome of an immunization, among other factors. Plasmid/naked DNA vaccines are less toxic, but also less immunogenic, therefore potent and safe adjuvants that can be used as vaccine delivery systems and as immunostimulatory adjuvants are necessary (43). Biodegradable and biocompatible polyester polymers such as PLA to encapsulate antigens (44–46) have been successfully

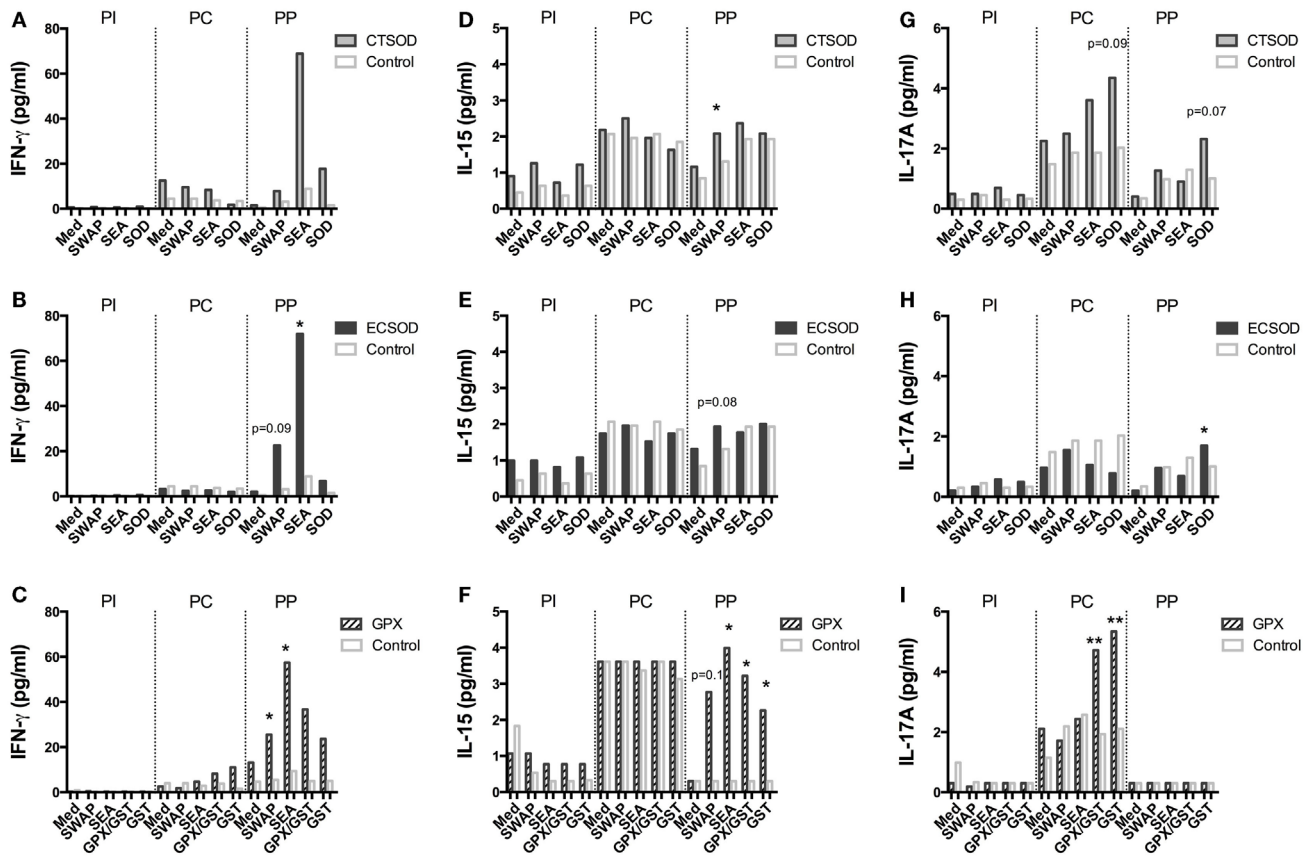

FIGURE 9 | **Secretion of cytokines in supernatants from Experiment 2**. PBMCs from CTSOD **(A,D,G)**, ECSOD **(B,E,H)**, and GPX **(C,F,I)** groups (n = 5 animals per group) were stimulated 72 h with crude and recombinant antigens and secreted IFN-γ, IL-15, and IL-17A levels determined, before vaccination (PI), before challenge (PC), and at perfusion (PP). Values were expressed as median of cytokine secretion in five individual supernatants in relation to the Control baboons. Non-parametric (Kruskal–Wallis, Mann–Whitney) tests were applied. Values were considered statistically significant when $p < 0.05$, and assigned $^{*}p$ = 0.01–0.05; $^{**}p$ = 0.001–0.01; and $^{***}p$ = <0.001.

used in applications ranging from cancer therapy to infectious diseases (47–50). The enhanced adjuvant effect of such microparticles appears to be a consequence of efficient and controlled delivery of the adsorbed proteins into dendritic cells and macrophages at the injection site and local lymph nodes (51, 52). In the murine model, we showed that along with DNA immunization, one single dose of SmCT-SOD proteins encapsulated in PLA microspheres, for instance, was able to induce high titers of specific antibodies in immunized mice (32). Therefore, in our baboon studies, we evaluated if the immunization protocol of priming with naked DNA and boosting with the respective antioxidant proteins encapsulated in PLA microspheres could meet or even enhance the immunogenicity and protection levels achieved by prime-boost immunization with DNA only. All three antioxidant vaccines were immunogenic to different degrees, stimulating both humoral and cellular responses (including cytokines and chemokines). As observed previously by us and by other studies, the specific antibody and cellular responses primed by DNA vaccination were boosted by infection. This natural boosting is believed to be beneficial in endemic areas where individuals are continuously exposed to the parasite (31, 53, 54).

Numerous studies on individuals in endemic areas for *Schistosoma* showed that parasite-specific humoral and cellular responses vary in their correlation with the development of resistance and/or susceptibility/morbidity to infection/re-infection (55–57). And an increasing body of evidence indicates that a balance between innate cells and CD4+ T helper (Th) cells Th1, Th2, Th17, and Treg responses (which cross-regulate one another during infection) rather than a polarizing effect (e.g., Th1 vs Th2) is likely beneficial in the development of protection against *S. mansoni* infections, both in humans and experimental models (58–61). Moreover, it is becoming evident that the interaction between these responses rather than just the levels of individual cytokines alone may influence outcomes such as resistance to reinfection (62, 63).

In our study, a mixed cytokine/chemokine response was observed, where the *S. mansoni* infection stimulated a Th2 response in all groups as well as an inflammatory profile that differed from controls. Although it was clear that vaccination with ECSOD, CTSOD, and GPX stimulated different responses when compared to the control group. The specific role of antibodies or cellular responses and the development of protection against

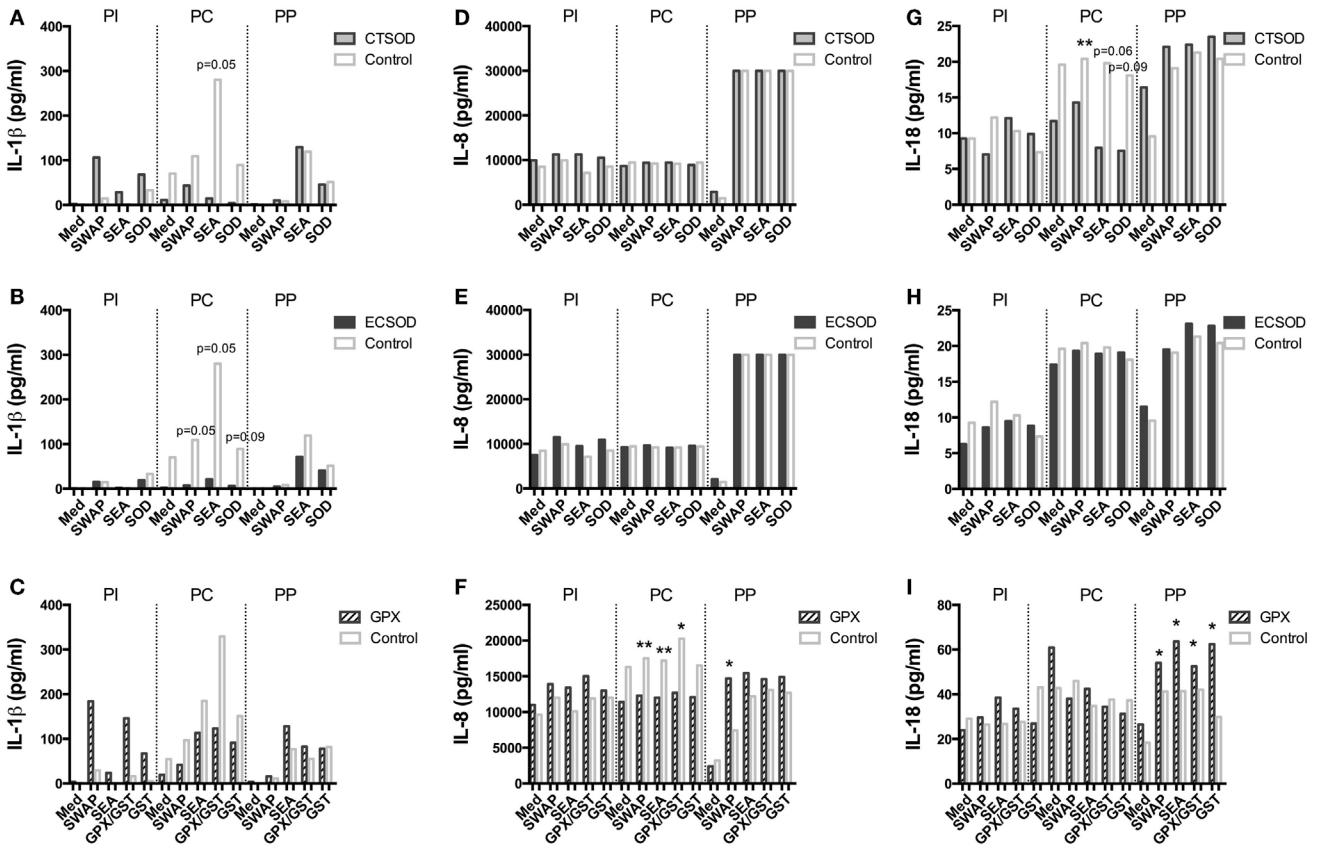

FIGURE 10 | Secretion of cytokines in supernatants from Experiment 2. PBMCs from CTSOD **(A,D,G)**, ECSOD **(B,E,H)**, and GPX **(C,F,I)** groups (n = 5 animals per group) were stimulated 72 h with crude and recombinant antigens and secreted IL-1β, IL-8, and IL-18 levels determined, before vaccination (PI), before challenge (PC), and at perfusion (PP). Values were expressed as median of cytokine secretion in five individual supernatants in relation to the Control baboons. Non-parametric (Kruskal–Wallis, Mann–Whitney) tests were applied. Values were considered statistically significant when $p < 0.05$, and assigned $^{*}p$ = 0.01–0.05; $^{**}p$ = 0.001–0.01; and $^{***}p$ = <0.001.

challenge with *S. mansoni* is still unknown, and currently under investigation. As shown by Mola et al. (64), the levels of most cytokines produced by PBMC or antibodies vary depending on whether the baboons experienced a primary infection compared to re-infection with *S. mansoni*. Although the young baboons used in this study were caught in the wild from non-endemic areas and were quarantined and checked for the presence of current schistosomiasis infection (and other infections), we cannot rule out the possibility that some of these animals were infected prior to the beginning of our study, therefore influencing the cytokine responses. However, this in fact would most likely resemble the situation in endemic areas, where people in endemic areas would be exposed to one or multiple infections or none at all before vaccination with antioxidants, and in many instances, such exposure to worm antigens in humans would have occurred very early in life during childhood or even *in utero* (65, 66), which makes outbred baboons ideally suited for such studies.

In schistosomiasis, pathogenesis is mainly caused by the immunological reaction of the host to the eggs (and their secretions) released by adult worm pairs that inhabit the portal circulation. Consequently, the severity of the disease is directly related to the worm burden and the inflammatory response to deposited eggs (67). For instance, as recently reviewed (68), the chemokines CCL2, CCL3, and CCL4 correlate with the severity of *S. mansoni*, where CCL3 is related to the recruitment of eosinophils and induction of granulomatous pathology (69), while CCL2 is associated with glomerulopathy (70). A more "traditional" vaccine concept would target a reduction of the worm number or even prevent infection. However, since the released eggs are responsible for both transmission and pathology, a vaccine targeted at parasite fecundity and egg viability has become relevant (71, 72). The vaccination of baboons with antioxidants in the present study stimulated a partial reduction in the worm burden, which was in sharp contrast with our previous studies in mice (29). However, vaccination with antioxidants, especially ECSOD, promoted a strong anti-fecundity and anti-pathology effect, by means of an overall reduction of eggs in all vaccinated groups in the feces, liver, small gut, and large gut, as well as reduction of diarrhea (dysentery) when compared to the Control group. One of the hallmarks of pathology, diarrhea normally occurs during moderate and heavy *S. mansoni* infections due to eggs released by the worms, which in turn induce an immunological response leading to pathology

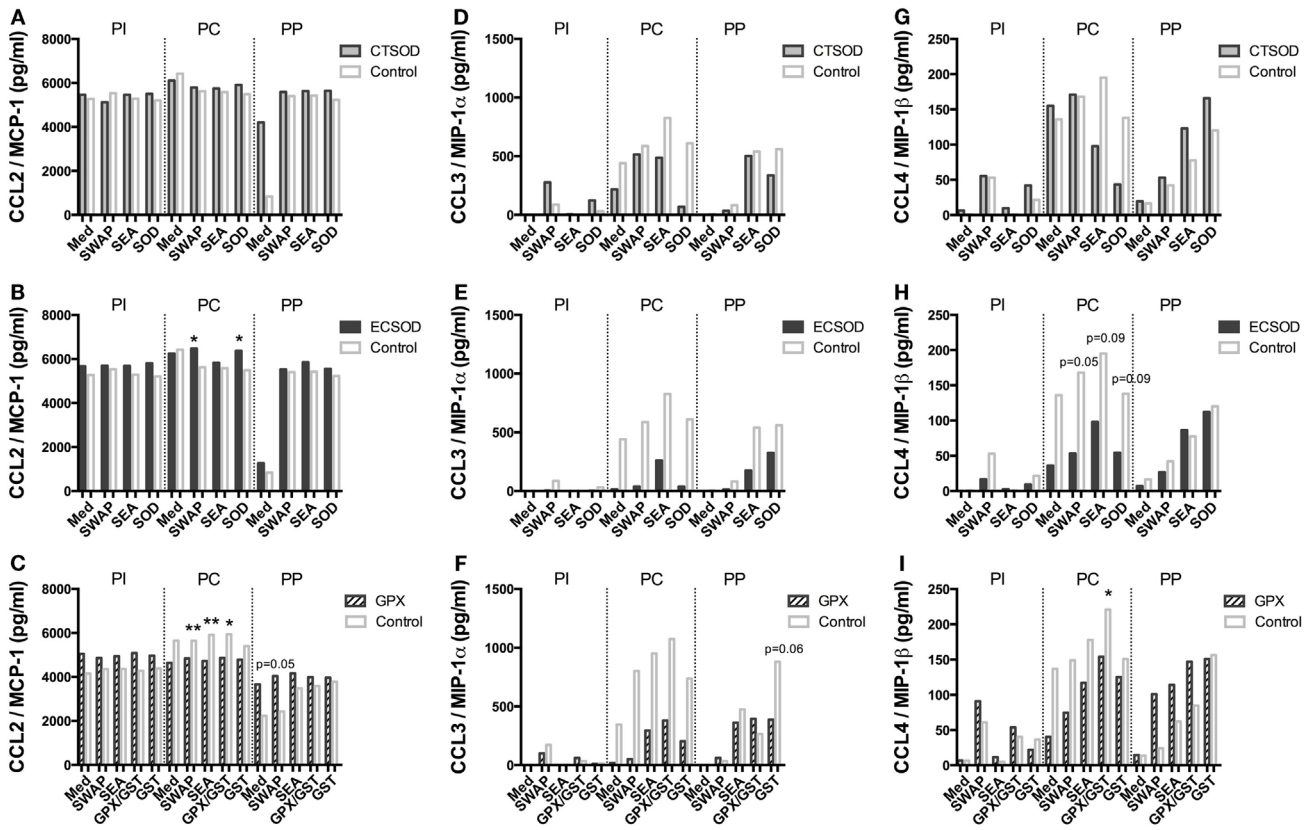

FIGURE 11 | Secretion of chemokines in supernatants from Experiment 2. PBMCs from CTSOD **(A,D,G)**, ECSOD **(B,E,H)**, and GPX **(C,F,I)** groups (n = 5 animals per group) were stimulated 72 h with crude and recombinant antigens and secreted CCL2 (MCP-1), CCL3 (MIP-1α), and CCL4 (MIP-1β) levels determined, before vaccination (PI), before challenge (PC), and at perfusion (PP). Values were expressed as median of cytokine secretion in five individual supernatants in relation to the Control baboons. Non-parametric (Kruskal–Wallis, Mann–Whitney) tests were applied. Values were considered statistically significant when $p < 0.05$, and assigned $^{*}p$ = 0.01–0.05; $^{**}p$ = 0.001–0.01; and $^{***}p$ = <0.001.

in the intestine wall (67). In our study, diarrhea was reduced in all vaccinated groups, in particular in the CTSOD and ECSOD groups. Other studies had reported both immune responses and anti-fecundity effects against female worms with glutathione-S-transferase, SmGST (73, 74), as well as the large subunit of calpain, Sm-p80 (75). Interestingly, the significant reduction in fecundity in this study was in spite of the small reduction in worm burden. The mechanism of the anti-fecundity effect by antioxidants or any other vaccine candidate, which seems to be independent of worm burden, remains to be determined.

Taken together, our results challenge the common concept for markers and correlates of protection, since in despite of the lower than 40% worm reduction, as set by WHO, the high anti-fecundity/anti-pathology effect observed by means of reduction of eggs and overall pathology indicates that these antioxidants vaccines could prevent intestinal pathology and therefore warrants further investigation. In addition, the use of outbred juvenile baboons more accurately reflects the human situation as regards response to vaccination and subsequent challenge infection. Experiments with different vaccine regimens (including multivalent immunizations), delivery modes, and adjuvants in the baboon model are planned for the near future.

Acknowledgments

We would like to thank Gregory Sonnenberg for the excellent assistance in preparing GPX recombinant proteins. We also would like to thank the excellent technical support of Fred Nyundo, Simon Kiarie, and Sammy Kisara who assisted with parasite assays. We also thank Habib Saibulu, James Ndungu, Zachariah Maheli, and the team from Animal Sciences at IPR, who routinely offered professional husbandry, veterinary, and surgical care to the study animals as required. This research was supported by NIAID grant AI18867.

Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00273/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 09 March 2015
doi: 10.3389/fimmu.2015.00095

Eliminating schistosomes through vaccination: what are the best immune weapons?

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The successful development of vaccines depends on the knowledge of the immunological mechanisms associated with the elimination of the pathogen. In the case of schistosomes, its complex life cycle and the mechanisms developed to evade host immune system, turns the development of a vaccine against the disease into a very difficult task. Identifying the immunological effector mechanisms involved in parasite attrition and the major targets for its response is a key step to formulate an effective vaccine. Recent studies have described some promising antigens to compose a subunit vaccine and have pointed to some immune factors that play a role in parasite elimination. Here, we review the immune components and effector mechanisms associated with the protective immunity induced by those vaccine candidates and the lessons we have learned from the studies of the acquired resistance to infection in humans. We will also discuss the immune factors that correlate with protection and therefore could help to evaluate those vaccine formulations in clinical trials.

Keywords: schistosome vaccine, protective immunity, schistosomiasis, effector mechanisms, immune response

INTRODUCTION

Vaccination is a great strategy to control and eradicate diseases (1) and there is no doubt that the development of a vaccine against schistosomiasis would have a massive impact on disease control and would be useful as a complementary tool to the disease eradication (2). Schistosomes in contrast to viruses and bacteria are complex parasites that pass through different life stages in different anatomic sites of its definitive host (3). The parasite has evolved to live for decades in the host, developing interesting strategies to evade host immune system [reviewed in Ref. (4)]. So developing an effective vaccine against schistosomes is a difficult task. But since the parasite does not replicate in its definitive host, even a partial reduction in parasite burden is believed to have an impact on disease control and eradication.

For decades, scientists have tried to develop an effective vaccine formulation against schistosomes, and although two candidates are under clinical trials, the search for new candidates and vaccine formulations are far from ending. During all those years of studies on schistosome vaccine development, some immunological mechanisms involved in parasite elimination have been proposed. Complement activation has been suggested to be involved in parasite elimination, but from what we know so far, only recently transformed schistosomes are killed by complement and 24 h schistosomula became refractory to death induced by complement activation (5). Since most of these studies on complement activation and parasite death were performed *in vitro*, the significance of complement activation to parasite death *in vivo* can be questioned. Schistosomes only get to host vessels 72 h after penetrating the host and by this time the parasite is already resistant to death induced by complement (6). Indeed, many evasion mechanisms developed by the parasite have been described and give support to the idea that the activation of the membrane attack complex might not be the major mechanism involved in parasite elimination (7–10).

Antibody-dependent cellular cytotoxicity (ADCC) is another immune mechanism that has been associated with parasite elimination. In individuals living in endemic areas for schistosomiasis, ADCC involving IgE, IgG, eosinophils, monocytes, and platelets was associated with the acquisition of resistance to reinfection (11–13). In mice, ADCC has been highlighted as the immune mechanism involved in parasite death in animals immunized with Smp-80 and GST (14–16). However, eosinophils may not be the major cell involved in ADCC in mice, since deficiency in this cell did not result in any changes in worm and egg burden after infection, demonstrating that eosinophils do not play major roles in parasite death (17).

Regardless of the mechanisms involved, antibodies are key players in the protective immunity induced by vaccines. Immunization of mice deficient in B cells impaired the protective response induced in wild-type animals by vaccination with irradiated cercariae (18). Also, transference of sera from mice immunized with schistosomula tegument (Smteg) or Smp-80 to a naïve recipient induce partial protection against challenge infection (19, 20). Other evidence of the importance of antibodies in the protective immune response induced by vaccination comes from the studies of Hewiston and coworkers (21). They demonstrated that the protective immune response induced by attenuated cercariae was abrogated in CD154-deficient mice. CD40–CD154 interaction is involved in eliciting a humoral immune response dependent on

T cells (22). The inoculation of IL-12 together with the vaccine in these deficient mice restored all the cellular immune parameters in mice lung but failed to restore protection and antibody production (21).

Cellular immune responses are also important in parasite elimination. Immunization of C57BL-6 mice deficient on IFN-γ, and TNFRI impair or abrogate protection induced by vaccine (18, 23, 24). The role of IFN-γ and TNF-α in parasite killing seems to be related to nitric oxide production by macrophage. Immunization of mice deficient in the TNFRI with irradiated cercariae abrogates protection and impairs nitric oxide synthase (iNOS) expression in lung macrophages (24). Nevertheless, immunization of mice deficient in iNOS result only in partial reduction on the protective immunity induced by irradiated cercariae, indicating that nitric oxide is not the major factor involved in parasite death (25).

In BALC-c mice, deficiency in IL-4R expression abrogates protection induced by irradiated cercariae that can be restored by wild-type serum transference (26). Recently, protective immunity-associated Th2 profile was observed in outbred mice immunized with glyceraldehyde 3-phosphate dehydrogenase (SG3PDH) and peroxiredoxin (TPX) (27). IL-10 and IL-17 production seems to correlate negatively with protection. Blocking IL-10 with neutralizing antibodies enables protection against challenge infection in mice previously infected with *Schistosoma mansoni* and treated with praziquantel (28). In *S. japonicum* infection, blocking IL-17 with neutralizing antibodies enhances antibody production and protection in infected mice (29).

Although CD8+ cells are classically related to immune responses against intracellular pathogens, its role in schistosome elimination has been recently described (30). Immunization of mice with the *S. japonicum* 22.6/26GST coupled to Sepharose 4B bead induced a significant reduction in parasite burden that was associated with an increase in the number of activated CD8+ cells (30). These activated CD8+ cells were able to promote death of parasite carrying host MHCI molecules in its surface (30).

Coulson and Wilson (31) suggested that the major mechanism involved in parasite elimination after immunization with the irradiated cercariae vaccine was in fact the generation of an inflammatory focus in the lung of immunized mice that impairs parasite migration and therefore its transformation into adult worms (31). Evidence that support this hypothesis is given by histological examination of mouse's lungs which demonstrates that the parasites in the inflammatory foci were alive and when recovered from the lung and transferred to a naïve recipient they developed into adult worms (31, 32).

Besides all the knowledge generated and described so far, the majority of the studies on immune mechanisms involved in schistosome elimination have been performed using the irradiated cercariae strategy that for security reason are not used in human trials. Currently, researchers are developing vaccine formulations based on one or a cocktail of parasite antigens and although there are many studies on different vaccine strategies, little is known about the effective protective mechanisms. In this review, the immune components and mechanisms elicited by different vaccine strategies using subunit formulations containing promising parasite antigens will be described. We will evaluate whether there is (are) immune factor(s) that correlates with protection. And this information might be used to rationally design a vaccine formulation and to suggest a strategy that better elicits these protective responses. These correlates of protection can also help to evaluate whether those vaccines are effective during clinical trials.

THE IMMUNE RESPONSE ELICITED BY PROMISING SCHISTOSOME ANTIGENS

Some of the schistosome antigens tested in pre-clinical trials emerged as promising candidates to compose an anti-schistosomiasis vaccine due to their ability to consistently induce protective immune responses in different animal models, under different formulations and vaccine strategies.

Two schistosome antigens are under clinical trials, the fatty acid-binding protein of 14 kDa from *S. mansoni*, Sm14 (33), and the glutathione-*S*-transferase of 28 kDa from *S. haematobium* (34). The *S. mansoni* tetraspanin 2, TSP-2, is now been produced under good manufacture practices (GMP) to soon be evaluated in Phase I clinical Trial (35).

GLUTATHIONE *S*-TRANSFERASE

The 28 kDa glutathione *S*-transferase of *S. mansoni*, Sm28GST, is one of the most promising vaccine candidates. Recognized as the enzyme glutathione *S*-transferase (36), Sm28GST was identified in tegument, parenchyma, and genital organs of schistosomula and adult worm (16, 37). In the vaccine protocol, Sm28GST purified protein was able to significantly reduce the worm burden in rats and mice (16). *In vitro* experiments suggested that this protection was related to the cytotoxic response since in the presence of anti-Sm28GST antibodies and mouse eosinophils, schistosomula can be killed through ADCC (15, 16). The importance of antibodies in worm elimination was evaluated by the passive transfer of specific antibodies which were able to induce protection against challenge infection (16). A study performed by Boulanger and colleagues (38) reinforced the immunoprotective potential of Sm28GST protein, by demonstrating that immunization of baboons with the recombinant protein together with aluminum hydroxide (Alum) confers up to 80% of protection.

Several studies demonstrated the importance of antibodies in parasite elimination in animals immunized with Sm28GST. Mouse immunization with one dose of rSm28GST plus bacillus Calmette-Guérin (BCG) or Alum, as adjuvants, conferred protection against *S. mansoni* infection, and induced significant production of specific IgG, IgA, and IgE (39). In another study, mice immunization with Sm28GST produced in recombinant *Mycobacterium bovis* BCG, regardless of the immunization route induced a vigorous production of IgG1, IgG2a, and IgG2b levels, which was associated with the neutralization of the Sm28GST enzymatic activity (40). Intradermal immunization with a DNA encoding Sm28GST, also induced a significant production of anti-Sm28GST IgG antibodies, mainly IgG2a and IgG2b, with an ability to kill schistosomula through ADCC mechanism (41). In addition to the antibodies, the cellular immunity is also critical to *Schistosoma* elimination. Mice immunized with the recombinant Sm28GST protein or with peptide derived from C-terminal region showed reduction in worm burden, in fibrosis, and in the number of eggs in the liver that were associated with high levels of IFN-γ (42, 43). In a DNA vaccine strategy, immunization of mice with *Sm28GST* co-delivered with

an IL-18-encoding plasmid, also induced a strong IFN-γ production and result in a significant reduction in egg and worm burden, reinforcing the importance of the Th1 response to the protection induced by Sm28GST (44).

One important feature of Sm28GST is the existence of cross-reactivity with other *Schistosoma* species, including *S. haematobium*, *S. japonicum*, and *S. bovis* (45). This property can be explored in the context of vaccine development which can act to eliminate different *Schistosoma* species at the same time. In this sense, it was demonstrated that immunization of primates with rSm28GST protect against heterologous infection with *S. haematobium* (46). The Sh28GST (GST protein derived from *S. haematobium*) has the ability to confer protection in monkeys that showed a reduction in worm fecundity (47). The results of the studies with schistosome GST as antigen in vaccine formulations clearly demonstrate the importance of antibodies for anti-fecundity effect and parasite elimination, through neutralization and ADCC mechanisms, respectively. It is important to note that this antigen can induce protection by reducing worm burden or female fecundity and thus this vaccine formulation is efficient to limit infection and pathology.

Sm14

Sm14 is a *S. mansoni* fatty acid-binding protein that might be involved in lipid uptake from the host (48). Due to its predicted function, Sm14 represent an interesting target for vaccines against schistosomes. Schistosomes are unable to synthesize fatty acids and sterols through *de novo* pathway and therefore require host lipids to maintain its complex membrane system and physiological function (49, 50). Sm14 is expressed in the cercariae, schistosomula, adult worm, and eggs and located in the parasite tegument and gut, both tissue that represent the interface between parasite and host (51).

The ability of rSm14 to protect against schistosomiasis was first demonstrated by Tendler and coworkers (52). In their study, immunization of mice with rSm14 alone or formulated with Freund's adjuvant (FA) induced protection levels ranging from 50 to 68% (52). In rabbits, rSm14 plus FA elicited 89% protection against challenge infection (52). Interestingly, Sm14 was also able to protect mice from *Fasciola hepatica* infection thus demonstrating its potential to be used in a vaccine formulation against both parasites (52).

In the case of Sm14, the protective immune response is dependent on IFN-γ and TNF-α production since in mice deficient in those cytokines, immunization with the recombinant form of Sm14 fails to induce protection (23). The importance of cellular components in the protective immune response elicited by Sm14 is also shown by the ability of epitopes from Sm14 to induce proliferative response in lymphocytes from resistant individuals and to induce protection in mice (53, 54). Immunization of mice with *Sm14* gene also induced significant protection associated with antibody production and increased production of IFN-γ by spleen cells and lung lavage cells (55). In a DNA vaccine strategy, the use of *IL-12* as adjuvant induced a significant production of IL-10 and nitric oxide and failed to induce antibody production and protection (55). The role of antibodies in the protective immunity induced by Sm14 cannot be ruled out. Although so far no direct role for antibodies in parasite elimination have been described, all the successful vaccine formulation containing Sm14 induce significant antibody production (23, 54, 55). Therefore, the role of antibodies in the protective immunity induced by Sm14 is a key question that has to be addressed if they are to be used as correlates of protection in future clinical trials.

TETRASPANINS

Tetraspanins are members of membrane-spanning proteins containing four transmembrane domains, three short intracellular domains, and two extracellular loops (EC1 and EC2) (56). In schistosomes, tetraspanins are located in the outer tegument, thus in contact with host immune system (57). The EC2 loop mediates protein–protein interactions (58) that are important to tetraspanin role in the molecular organization of cell membranes. Through interaction with many proteins, tetraspanins form a complex termed as tetraspanin-enriched microdomains (TEM) (59). The importance of tetraspanins to parasite survival was recently demonstrated using interference RNA technique (RNAi). Silencing *Tsp-1* and *Tsp-2* transcription resulted in significant reduction in the number of worm that reaches maturity in the mammalian host. Also, schistosomula treated with *Sm Tsp-2* double strand RNA displayed vacuolated and thinner tegument, demonstrating TSP-2 role in maintaining tegument integrity (60).

Immunization of mice with a recombinant form of TSP-1 and TSP-2 resulted in 29–38 or 53–61% reduction in worm burden, respectively. The protection was associated with an increased titer for IgG1 and IgG2a antibody (61). Immunization of mice with a vaccine formulation containing TSP-2, Alum, and CpG as adjuvant elicited a protection level ranging from 25 to 27%. In this vaccine formulation, although significant titers of IgG antibodies were observed, there was no clear association between antibody levels and parasite burden and also there was no significant increased production of cytokine specific for TSP-2 immunized group. In contrast, an increased level of IFN-γ, IL-4, and IL-10 were observed in mice immunized with a chimerical protein containing TSP-2 and the 5B region of the hookworm aspartic protease Na-APR-1 (62). Besides the great result observed against *S. mansoni* infection, TSP-2 orthologs in *S. japonicum* do not represent a good vaccine candidate, since in this species TSP-2 is extremely polymorphic (63).

Smp-80

Another promising antigen is the large subunit of calpain, Smp-80. Schistosome calpain is composed of a smaller subunit of 28 kDa and a larger subunit of 78 kDa (64). The large subunit was described to be localized in the parasite surface (64). This subunit has proteolytic activity in the presence of Ca2+ (64) and plays an important role in immune evasion. Smp-80 is involved in the renewal/recycling of the parasite surface, an important evasion mechanism used by the parasite to escape from host immune system (65).

The large subunit of calpain has been evaluated in different vaccine formulations and strategies and the immune components produced in response to immunization have also been described. This antigen induced significant protection in mice, baboon, and hamster and protects against *S. mansoni*, *S. japonicum*, and

S. haematobium (66–68). Immunization of mice with naked DNA containing *Smp-80* gene resulted in 39% of reduction in worm burden, the use of *IL-2* and *IL-12* gene as adjuvant increased the protection level to 57 and 45%, respectively. This increased protection was associated with an increased production of specific IgG2a and IgG2b, increased proliferative response, and IFN-γ production (69, 70). The use of *GM-CSF* and *IL-4* gene as adjuvant resulted in 42 and 44% reduction in worm burden associated with increased production of IL-4, IgG, IgG1, and IgG2b in *GM-CSF* immunized animals and IL-4 and IgG3 in *IL-4* immunized group (70, 71). When the recombinant form of Smp-80 is used as antigen either in its recombinant form or in a prime-boost regimen, higher titers of antibodies and significant production of IL-2 and IFN-γ are observed and this immune profile is associated with a reduction of 51 and 49% in worm burden (66). Therefore, the protective immune response induced by Smp-80 seems to correlate to a Th1 profile with increased IFN-γ and antibody production especially IgG2a.

Indeed in mice immunized with Smp-80, antibodies play an important role in the elimination of the parasite. Transference of sera from *Smp-80* immunized mice to a naïve recipient result in 31–45% reduction in worm burden after a challenge infection (20). Complement seems not to play a major role in the parasite elimination once immunization of mice deficient in C3 factor with Smp-80 did not result in significant reduction of the protection level observed in wild-type mice (72). ADCC instead seems to be the immune mechanism involved in parasite death, increased number of dead schistosomula was observed *in vitro* when these parasites were incubated with sera from Smp-80 plus CpG immunized mice in the presence of lung lavage cells or lung cells (14). The increased percentage of dead parasites was associated with production of nitric oxide suggesting that the production of this molecule might be involved in ADCC-induced parasite killing (14).

Sm29

Sm29, other promising vaccine antigen, was identified by Cardoso and coworkers (73) using *in silico* analysis to identify in the *S. mansoni* transcriptome putative expressed proteins localized in the parasite tegument. The characterization of Sm29 demonstrated that this protein is expressed in the tegument of schistosomula and adult worm (74). The ability of Sm29 to induce protective immunity was assessed in mice with a vaccine formulation containing FA which resulted in a significant reduction of 51% in worm burden, 60% in intestinal eggs, and 50% in liver granuloma area, associated with a significant production of IgG, IgG1- and IgG2a-specific antibody, IFN-γ, TNF-α, and IL-10 cytokine production (74).

IMMUNE RESPONSE INDUCED BY MULTIVALENT FORMULATIONS

Schistosomes are complex parasites and thus the design of a multivalent vaccine against the parasite might enhance subunit vaccine efficacy. Recently, these multivalent vaccine formulations containing promising antigens have been tested. Sm29 was tested together with the SmTSP-2 in a multivalent chimeric recombinant vaccine in mice, aiming to enhance the single antigen vaccination efficacy. The vaccine formulation with a chimeric protein containing TSP-2 and the C-terminal part of Sm29 resulted in a small increase in the protection level induced by rSm29 alone from 20.36 to 34.83% (75), or by rTSP-2 alone from 27 to 34.83% (62, 75) when formulated with CpG-ODN plus alum. The chimeric protein induced a significant production of IgG and IgG2a-specific antibodies and a Th1 immune profile (75).

Another chimeric formulation combining Sm29 and Sm14 recombinant proteins was tested in vaccine protocol associated or not with poly (I:C)-adjuvant. Although immunization of Swiss mice with a subunit vaccine containing rSm14 or rSm29 alone did not induce significant reduction in adult worm, the vaccine formulation containing both rSm14 and rSm29 elicited 31 or 40% protection when it was formulated without adjuvant or with Poly (I:C), respectively (76).

The results observed in these multivalent formulations demonstrate that it is a promising strategy, but the choice of antigens that induce similar protective immune profile is a key step for the success of the vaccine formulation.

WHAT CAN WE LEARN FROM THE STUDIES IN HUMANS?

In endemic areas, the existence of naturally resistant individuals (77) that present persistently negative stool examination even if they are in constant contact with contaminated water enable the search for immune factors and biomarkers involved in resistance to *Schistosoma* infection.

Studies on human immune responses to Sm14, demonstrate that CD4+ T cells from naturally resistant individuals mounted a Th1-type of immune response to rSm14, based on IFN-γ and TNF-α production (78). Moreover, T-cell proliferative responses to rSm14 from these individuals were totally abrogated after treatment with anti-IFN-γ antibodies. These individuals also produce significant levels of IgG1 and IgG3 antibodies against Sm14, subclasses associated with parasite killing (79). Significant production of IgG1 and IgG3 specific for Sm29 and TSP-2 were also observed in individuals naturally resistant to *S. mansoni* infection (61, 80).

Human antibody responses to the large subunit of schistosomal calpain have also been associated with resistance. In humans infected with *S. japonicum*-presenting light infections, a strong reactivity to calpain was observed whereas in individuals with stronger infection, low reactivity to calpain was observed (81). In a study in an endemic area for schistosomiasis in Egypt, IFN-γ production in response to Sm14 and IgE and IgA antibodies against Sm28GST correlated with resistance to infection (82).

Grzych and colleagues (83) reinforce the pivotal role of the antibodies in protection. They show that IgA specific to Sm28GST were present in the sera from infected individuals before and after treatment with praziquantel and that these immunoglobulins have a key role in neutralizing the GST enzyme activity which resulted in impaired female fecundity.

Since 1998, Sh28GST recombinant protein plus aluminum hydroxide adjuvant has been tested in the human population. Partial results of the phase I clinical trials were published by Riveau and colleagues (34), in their study, no relevant side effects or toxicity following vaccine administration was observed. Humoral immune responses generated by the vaccine were characterized by high levels of IgG1 and IgG3 production and low levels of IgG2 and IgA. The cellular response profile was characterized

Table 1 | Summary of the protection levels and immune components elicited by immunization.

Antigen	Immunization strategy	Adjuvant	Protection level (%)	Humoral response	Cellular response	Reference
	DNA vaccine	None	39		Prolif. IFN-γ, IL-4	(67–69)
		IL-2	57	↑ IgG, IgG2a and b	↑ Prolif. ↑ IFN-γ ↓ IL-4	
		IL-12	45	↑ IgG, IgG2a and b	↑ Prolif. ↑ IFN-γ ↓ IL-4	
		IL-4	44	↑ IgG3	Prolif. IFN-γ ↑ IL-4	
		GM-CSF	42	↑ IgG, IgG1	Prolif. IFN-γ ↑ IL-4	
Smp-80	Prime E boost	Resiquimod (R848)	49	↑ IgG, IgG1, IgG2a and b, IgG3, IgA, IgM	IL-2 and IFN-γ	(70)
	Recombinant protein	Resiquimod (R848)	51	↑ IgG, IgG1, IgG2a and b, IgG3, IgA, IgM	IL-2 and IFN-γ	
		CpG-ODN	52.86	IgG, IgG1, IgG2b, IgG3, IgM	ADCC AND NO production	(14, 71)
	Recombinant protein	None	25			(22)
		FA	25	↑ IgG2a		
Sm14		rIL-12	42.2	↑ IgG2a	Protection is dependent on IFN-γ and TNF-α production	
	DNA vaccine	None	40.5	IgG	IFN-γ by SC and BAL cells	(53)
	Synthetic peptides	FA + Padre	26–36.7	IgG1, IgG2a	IFN-γ IL-10	(52)
Sm29	Recombinant protein	FA	51	IgG1, IgG2a	IFN-γ, TNF-α, IL-10	(73)
		CpG-Alum	20	IgG, IgG1, IgG2a	IFN-γ	(74)
TSP-1	Recombinant protein	FA	29–38	IgG1, IgG2a	Not reported	(59)
TSP-2	Recombinant protein	FA	53–61	IgG1, IgG2a	Not reported	(59)
		Alum + CpG	25–27	IgG, IgG1[a]	IL-4, IFN-γ, and IL-10[b]	(60)
	Purified protein	FA	40–68.3	Not reported	Eosinophils (ADCC)	(16)
Sm28GST	Recombinant protein	Alum	46	Not significant	IL-2 and IFN-γ	(41)
	DNA vaccine	IL-18	23	Not significant	IFN-γ	(42)
Sh28GST	Recombinant protein	FA	77 (fecundity)	IgG and IgA	Not reported	(45)
		BCG	60	IgG		

[a] *No association with protection;*

[b] *production in response to infection and not to immunization; SC, spleen cells; BAL, broncho alveolar lavage; ↑ compared to vaccine formulation without adjuvant.*

by significant production of IL-5 and IL-13, resulting in a Th2 predominant response. The ability of antibodies to inhibit the enzymatic Sh28GST activity was also observed, corroborating with experimental studies (34).

CONCLUSION AND FUTURE PERSPECTIVE

Understanding the immunological mechanisms involved in parasite elimination during an infection is a key step toward the development of an effective vaccine. Here, we reviewed the immune components activated under different formulations containing antigens described as promising candidates to compose an anti-schistosomiasis vaccine (summarized in **Table 1**). Although for some of the antigens the immune mechanism involved in parasite death have been demonstrated, for others they are still to be identified. The biological role the target antigen plays in the survival of the parasite and the immunological components elicited by a protective formulation gives us an indication of what immune mechanisms might be involved in parasite death. Yet determining their involvement in protective immunity is still necessary. To address this question, the use of animals deficient in components of the immune system represents an interesting tool that should be further explored. Once those immune factors that correlate with protection are identified, they can be used as a biomarker of resistance in clinical trials.

ACKNOWLEDGMENTS

Authors acknowledge Dr. John Kusel for reviewing this manuscript. This work was supported by CNPq, INCT-DT/CNPq, Proep/CPqRR, FAPEMIG, and PapesVI/Fiocruz. Fellowship: C. C. Alves (Fapemig), C. T. Fonseca (PQ/CNPq), and S. C. Oliveira (PQ/CNPq).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 October 2014; accepted: 18 February 2015; published online: 09 March 2015.
*Citation: Fonseca CT, Oliveira SC and Alves CC (2015) Eliminating schistosomes through vaccination: what are the best immune weapons? Front. Immunol. **6**:95. doi: 10.3389/fimmu.2015.00095*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.
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frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 27 February 2015
doi: 10.3389/fimmu.2015.00085

A meta-analysis of experimental studies of attenuated *Schistosoma mansoni* vaccines in the mouse model

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Schistosomiasis is a water-borne, parasitic disease of major public health importance. There has been considerable effort for several decades toward the development of a vaccine against the disease. Numerous mouse experimental studies using attenuated *Schistosoma mansoni* parasites for vaccination have been published since 1960s. However, to date, there has been no systematic review or meta-analysis of these data. The aim of this study is to identify measurable experimental conditions that affect the level of protection against re-infection with *S. mansoni* in mice vaccinated with radiation attenuated cercariae. Following a systematic review, a total of 755 observations were extracted from 105 articles (published 1963–2007) meeting the searching criteria. Random effects meta-regression models were used to identify the influential predictors. Three predictors were found to have statistically significant effects on the level of protection from vaccination: increasing numbers of immunizing parasites had a positive effect on fraction of protection whereas increasing radiation dose and time to challenge infection had negative effects. Models showed that the irradiated cercariae vaccine has the potential to achieve protection as high as 78% with a single dose vaccination. This declines slowly over time but remains high for at least 8 months after the last immunization. These findings provide insights into the optimal delivery of attenuated parasite vaccination and into the nature and development of protective vaccine induced immunity against schistosomiasis, which may inform the formulation of human vaccines and the predicted duration of protection and thus frequency of booster vaccines.

Keywords: schistosomiasis, attenuated cercariae, protective immunity, random effects meta-regression, animal model, systematic review

INTRODUCTION

Schistosomiasis is a water-borne parasitic disease of major public health importance. More than 4.5 million disability adjusted life years (DALYs) are lost each year worldwide due to schistosome infection (1–4). Human schistosomiasis is mainly caused by three species: *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum* (5). More than 90% of reported cases are from sub-Saharan Africa where both *S. mansoni* and *S. haematobium* infections are endemic (6). The vast majority of control programs use the antihelminthic drug praziquantel for mass drug administration. This low-cost and efficacious drug has achieved a significant reduction in disease prevalence and infection intensity in many endemic areas (7–10). However, there are multiple reports of re-infection after chemotherapy (11–13). In addition, praziquantel can clear only adult worms and has little or no effect on existing eggs and immature worms (14). This means that there is need for additional complementary interventions, one of which is vaccination.

Slowly developing acquired immunity plays a crucial role in the reduction of infection prevalence and intensity in older age groups in endemic areas (15, 16). This suggests that exposure to schistosome antigens can promote protective immunity in humans; however, to date, there is no licensed schistosome vaccine (17, 18). Currently, the leading vaccine candidate is the 28 kDa *S. haematobium* GST (Sh28GST, Brand name: Bilhvax), which is now in phase 3 clinical trials (19–21). Alongside recombinant antigen vaccine studies, the attenuated live cercariae vaccine has been studied extensively in mouse models (22, 23). Properly prepared attenuated cercariae live long enough to invade the host skin and stimulate protective acquired immunity against subsequent challenge infection but die in the host's body before they mature into adult worms (24). Attenuated schistosome cercariae vaccination experiments in animals use cercariae, which are weakened by ionizing radiation (X-ray or gamma ray), ultraviolet, heat, or chemical treatment. Host animals are immunized with attenuated parasites either once or several times before challenge infection with non-attenuated pathogenic cercariae. A certain number of days after the challenge infection, immunized animals and control animals are perfused to quantify the level of protection due to immunization by comparing the number of adult worms recovered from both groups.

A large number of mouse experimental studies using attenuated *S. mansoni* cercariae for vaccination have been published since 1960s (25); however, such studies have never been systematically analyzed. The aim of this study was to conduct a meta-analysis to identify measurable experimental conditions (predictors) that affect the level of protection against challenge infection of vaccinated animals. In addition, levels of each predictor associated with maximum levels of protection were estimated.

MATERIALS AND METHODS

SYSTEMATIC REVIEW

An electronic literature search was performed using Science Citation Index Expanded, Conference Proceedings Citation Index and BIOSIS Citation Index, all of which were provided through Web of Knowledge[1]. Alongside these, EMBASE[2], OVID MEDICINE[3], and CAB abstracts, were searched simultaneously though OvidSP[4]. Reference lists of all articles identified by the electronic search were searched manually for additional relevant reference. In addition, ProQuest Dissertations and Thesis Full Text[5] was searched as a source of pre-published and gray literature. The search terms were chosen to be as inclusive as possible and were "cercaria*" AND ("irradiat*" OR "attenuat*") AND ("vaccin*OR schistosom*"). Also, we searched by "Attenuate*" AND "schistosome*" AND "vaccin*." This search was completed in July 2013. After duplicated articles were removed a total of 1,013 articles were identified. Titles and abstracts were screened by at least two independent reviewers to exclude those that were not relevant to an attenuated schistosome vaccine animal model. Full texts of potentially relevant articles were reviewed by two independent reviewers for further selection. Non-English articles were included, and several Chinese and German articles were identified and translated into English by a native Chinese speaker and German speaker, respectively, for the analysis.

A study was considered eligible if it met all of the following inclusion criteria: (1) vaccination with attenuated cercariae; (2) use of ionizing radiation for attenuation; (3) use of percutaneous immunization and challenge (i.e., the natural transmission route for schistosome infection); (4) challenge infection using normal (non-attenuated) cercariae; (5) worm burden measured after the challenge infection via perfusion; (6) outcome (fraction of protection) reported or could be calculated. In this study, fraction of protection means the proportion of reduction in worm burden in vaccinated mice compared to that of control mice group. For articles, which reported worm count after challenge infection, the following equation was used to calculate the outcome: fraction of protection = [(average number of worms per mouse retrieved from control group − average number of worms per mouse retrieved from vaccinated group)/average number of worms per mouse retrieved from control group]. In the case of articles, which failed to report worm counts (allowing calculation of this quantity), only those that stated that they used the same equation as above were included.

Studies were excluded if they met any of the following exclusion criteria: (1) immunizing attenuated cercariae developed to adulthood; (2) hosts were transgenic or genetically engineered; (3) hosts had an *in vivo* depletion of immune cells; (4) attenuated cercariae were prepared by any means other than ionizing irradiation; (5) a non-cercarial vaccine was used (e.g., adult worm, schistosomula, subunit); (6) an artificial infection was conducted prior to vaccination; or (7) hosts were treated with anthelmintic drugs.

Articles often reported results from multiple separate experiments such as use of different doses of attenuated parasite. In these cases, results from each experiment were recorded as an observation. A total of 755 observations from 105 articles (articles are listed in Supplementary Material) meeting searching criteria and also using mouse as a host and *S. mansoni* for vaccination and challenge infection. Although the mouse is not a natural host for schistosome infection, it is the most commonly used animal for attenuated schistosome parasite vaccine animal model. A list of potential predictors (given in **Table 1**) was drawn up and these quantities were extracted from each article. These potential predictors have been suggested their importance by review articles and also their quantities been reported by many experimental studies (26). When an article reported a dose range rather than an exact dose the mid-value was used for the analysis.

STATISTICAL ANALYSIS

Random effects meta-regression

Random effects meta-regression was used to identify the influential predictors and effect of dose on protection. Multiple observations (1–56) were recorded from single articles and therefore article was included as a random effect in the models. The models were built using a backwards stepwise procedure with eight potential predictors (listed in **Table 1**). The effect of the number of immunizing parasites was explored in two ways in the two separate models: as an average number of immunizing parasites per dose or as a total number of immunizing parasites. Correlations between variables were examined visually by scatter plot graphs for all possible predictor combinations (data not shown). Then,

Table 1 | Possible predictors investigated and their units/codes.

Variable name	Units/code
Number of immunizing parasites (total and number per dose)	Number of parasites log10 transformed
Number of challenge parasites	Number of parasites log10 transformed
Number of immunizations	Count
Irradiation dose	Krad
Host age	Weeks
Host sex	Male, female, mixed
Time between the last immunization and challenge	Days
Time between challenge and perfusion	Days

[1]http://www.webofknowledge.com
[2]http://www.elsevier.com
[3]http://www.ovid.com
[4]http://ovidsp.tx.ovid.com
[5]http://www.proquest.com

all the possible combinations of two-way interactions of potential predictors were examined using a random effects meta-regression model with two-way interactions. The outcome variable (fraction of protection) was transformed as −ln(1 − fraction of protection) to reduce the skewness of residuals (27). Although using confidence intervals and SE is the most common weighting method for meta-regression (28), many studies in our dataset failed to report either confidence intervals or SD and there were no comparable studies, which enabled us to justify imputing them. Two kinds of information were available on the size of the studies: the number of control animals and the number of vaccinated animals. The majority of studies used similar numbers of control and vaccinated animals; however, there were several articles, which used a higher number of vaccinated animals than control animals. To account for the impact of these unbalanced studies, the number of control animals was used as the more conservative weighting option.

Missing values and outliers

Several outliers were excluded from the analysis. They were six observations with animals kept longer than 300 days or <7 days after the last immunization and four observations that used more than 10,000 cercariae for immunization. After excluding outliers 745 observations were kept for further selection.

When the numbers of control animals were not reported in an article and only the numbers of vaccinated animals were given, numbers of control animals were then imputed by a linear regression imputation method between numbers of vaccinated and control animals for all studies (29). When the observation was missing for both the number of control animals and vaccinated animals (4 observations from 4 articles), the average number of control animals of the remaining data set was used for imputation, which was 10 control animals. Out of 745 observations, 725 observations from 100 articles reported all predictors and were used for the analysis.

Statistical software

Papers identified by systematic review were recorded by Thomson Reuters EndNote and the extracted data were entered on a Microsoft Excel 2010 spread sheet for further analysis. IBM SPSS Statistics Version 19.0 and Minitab. Inc., MINITAB 16 were used for statistical analysis. GraphPad Software GraphPad Prism version 6.03 was used for graphical expression.

RESULTS

Among eight potential predictors (**Table 1**), three predictors were found to have statistically significant effects ($P < 0.05$) on the outcome value −ln(1 − fraction of protection) following the backwards stepwise selection: the log10 transformed total number of immunizing parasites ($P < 0.001$), the irradiation dose ($P < 0.001$), and the time between the last immunization and challenge ($P = 0.04$) (**Table 2**). The reported ranges of each predictor were the total number of immunizing parasites (50–5,000 cercariae), the irradiation dose (3–160 krad), and the time between the last immunization and challenge (7–230 days). All identified predictors were significant ($P < 0.05$) in the model no matter with or without outliers in the model. The number of immunizing parasites was significant in the model regardless of the version of this variable used, i.e., the average number of immunizing parasites per dose or total number of immunizing parasites. In both cases, the models were initially considered with the number of immunizations. When the total number of immunizing parasites was used as a predictor, the number of immunizations was not significant. Therefore, for the final model, the total number of immunizing parasite was used as a predictor with number of immunizations excluded from the model.

Table 2 | Results from random effects meta-regression models.

Predictors	Coefficient	SE	*P*-value
Number of immunizing parasites per dose (log10 transformed)	0.4338	0.0661	<0.001
Irradiation dose	−0.0047	0.0008	0.04
Time between the last immunization and challenge	−0.0015	0.0007	<0.001

Positive coefficients indicate the predictor's positive dose effect on fraction of protection whereas negative coefficients indicate predictor's negative influence on fraction of protection.

The interaction between log10 transformed total number of immunizing parasites and the time between the last immunization and challenge was statistically significant ($P = 0.04$). However, this interaction was excluded from the final model for the following reasons: (1) the model with the interaction showed biologically implausible fitted values of fraction of protection for some predictors, (2) the model with/without interaction showed similar fitted values for the fraction of protection around the most frequent values of predictors.

Fitted graphs for each predictor are shown in **Figure 1** with the outcome variable back-transformed to fraction of protection. Fitted graphs for each predictor were generated by fixing other predictor values at their modes: 500 immunizing parasites, 28 days for the time between the last immunization and challenge, and 20 krad for irradiation dose (solid line in **Figure 1**). The fitted graph of total number of immunizing parasites and fraction of protection showed the lowest level of predicted protection was 41% with 50 cercariae, which increased up to 75% with 5,000 cercariae (solid line in **Figure 1A**). Similarly, the minimum level of protection predicted for 160 krad irradiation was 26% protection, which increased to 65% with 3 krad irradiation (solid line in **Figure 1B**). The estimated level of protection 7 days after the last immunization was 63%, which reduced to 49% by 230 days after the last immunization (solid line in **Figure 1C**). Fitted graphs showed that the total number of immunizing parasites had a positive impact on the fraction of protection whereas irradiation dose and the time between the last immunization and challenge had negative impacts (**Figure 1**). Besides this, to estimate the highest protection, fitted graphs for each predictor were generated with other predictor values at their optimal level: 5,000 immunizing parasites, 7 days for the time between the last immunization and challenge, and 3 krad for irradiation dose (dashed line in **Figure 1**). The models suggested that highest achievable protection was 78% at 7 days after the last immunization, with the mouse immunized with 5,000 cercariae, which were attenuated with 3 krad (dashed

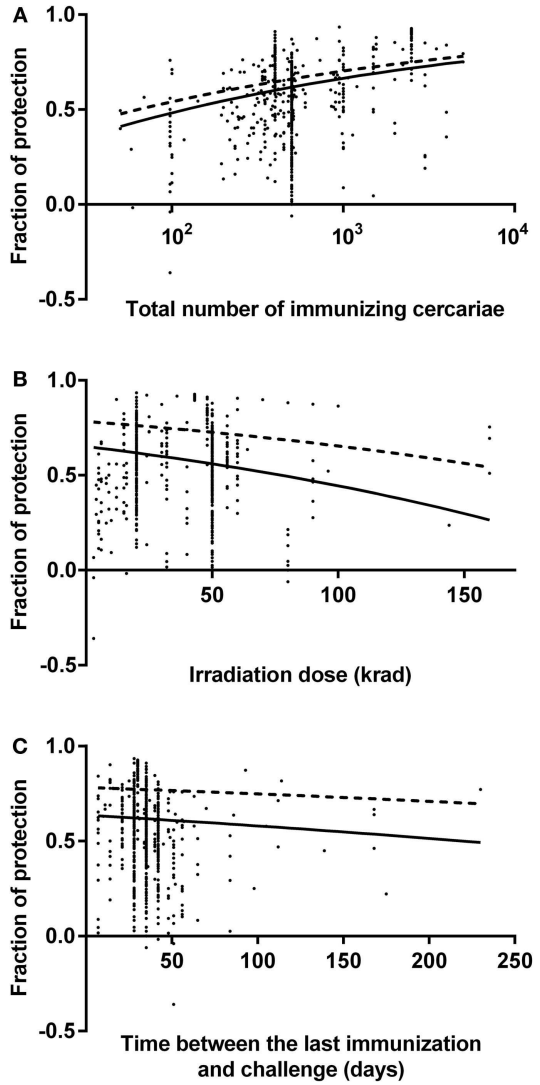

FIGURE 1 | Fitted graphs for identified predictors from a random effects meta-regression model. Identified predictors effects on fraction of protection in mouse model: **(A)** the number of immunizing cercariae over the range 50–5,000 cercariae, **(B)** the irradiation dose over the range 3–160 krad, **(C)** the time between the last immunization and challenge over the range 7–230 days. Data points indicate reported fraction of protection for each observation. Negative fractions indicate that vaccination was associated with increase of schistosome worm burden. Lines are fitted graphs generated from random effects meta-regression model (see text). Dashed lines in the graphs show the highest level of protection over range that could be achieved.

line in **Figure 1**). This 78% protection will decrease over time but would stay as high as 70% by 230 days after the last immunization (dashed line in **Figure 1C**).

DISCUSSION

Irradiated *S. mansoni* cercariae vaccines have been tested experimentally against schistosome infection for decades, with important insights obtained from the individual experiments (25). Although the translation of the irradiated parasites vaccine in humans has not been pursued for schistosomiasis, a precedent for this type of approach for human vaccination has been set by malaria vaccine, which uses live attenuated sporozoites (Sanaria® PfSPZ Vaccine) and has now completed phase 1/2a clinical trial (30). This study represents a meta-analysis of the experimental irradiated cercariae vaccine studies to identify robust variables that affect the levels of protection to inform human vaccine research and development.

The random effect meta-regression models identified three predictors of a reduction in worm burden: these were the total number of irradiated cercariae per immunization, the time between the last immunization and challenge, and the irradiation dose for parasite attenuation. We identified a positive correlation between the number of irradiated cercariae per immunization and the level of protection within the range of 50–5,000 cercariae used in the original studies. The models suggested that the optimally prepared irradiated cercariae vaccine could achieve a protection as high as 78% against challenge infection. As fitted graphs have shown, this is predicted for a single vaccination with 5,000 cercariae attenuated with 3 krad irradiation. This protection declines over time, but remains high for at least 8 months after the last immunization. Approximately 70% protection against challenge infection could be achieved after 8 months.

The number of immunizing cercariae represents the antigen dose, our results show a positive dose dependency of schistosome attenuated vaccine for higher protection. Studies of live attenuated vaccine for malaria infection also reported a similar positive correlation between the dose of immunizing parasites and the level of protection against future infection. Recently, as part of the phase 1 clinical trial of the human malaria vaccine using live attenuated sporozoites (Sanaria® PfSPZ Vaccine), a dose-escalation trial was conducted using 7,500–135,000 irradiated *Plasmodium falciparum* sporozoites per immunization. The participants group that received the highest dose per immunization achieved the highest levels of protection against challenge infection (31–33). Although the adequate number of immunizing schistosome parasites are needed to protect baboon hosts has not been well quantified yet, experimental studies have been conducted with up to 45,000 schistosome parasites and reported positive protections (34–36). These reports suggest that a large number of attenuated cercariae would be required for vaccination in humans. The intermediate host snails have been reported to shed a large number of cercariae that is approximately 3,600–6,000 cercariae per snail over the first 50 days of shedding (37). Schistosome infected snails and cercariae are commercially available from organizations such as the NIH-NIAID Schistosomiasis Resource Center (38) and Schistosomiasis Collection at the Museum at National History Museum, London for laboratory use (39). Clearly producing an adequate number of cercariae of a satisfactory quality to use in vaccinations is still highly challenging (18). Although we cannot directly translate animal vaccine study results into human use, their value is in highlighting the nature and development of vaccine induced protective immunity against schistosomiasis. For example, the dynamic relationships between vaccination dose and level of protection are informative for human studies, as has been alluded to by drug induced resistance against re-infection (40, 41). It is also worth mentioning that human

vaccination trials using infection or irradiated parasite vaccination have recently been conducted in human *P. falciparum* studies (42–44).

The result from the random effects meta-regression model showed a decrease in the fraction of protection with an increased time between the last immunization and challenge. This period between immunization and challenge represents the time to secondary encounter with the same antigen. When the initial encounter with the antigen takes place via infection or vaccination, the number of B and T cell produced against the antigen increases dramatically (45–48). Only a small fraction of those cells will survive as antigen-specific memory T and B cells or as long-lived plasma cells and they will be maintained for a long time (45–48). The duration of immune memory in humans after the vaccination is still controversial (49). However, there are several reports estimates for the length of immune memory after the last booster vaccination; 15 years for combined hepatitis A and B vaccine (50), 22 years for hepatitis B vaccine (51), over 30 years for poliovirus vaccine (49, 52), and over 60 years for small pox vaccine (49, 53). A longitudinal immuno-epidemiological study of schistosomiasis has been conducted by Butterworth et al., which reported that the protection induced by chemotherapy can remain for as long as 21 months after the treatment (54). However, other studies reported treated participants' re-infection within 1 year (12, 55). One of the difficulties in evaluating the length of protective immunity in humans is that, in contrast to experimental animals, humans encounter a variety of antigens that could stimulate their immune systems through their daily life. In addition, people infected and being treated for schistosomiasis normally live in schistosomiasis endemic areas. Regarding the influence of schistosome infection on vaccine efficacy, Kariuki et al. have shown that the protection levels induced by attenuated cercariae vaccination were high in baboon hosts in a group chronically infected and then treated after vaccination, as well as in a group that was infected and previously treated before vaccination (36). In addition, encounters with infectious cercariae by people in endemic areas may work as a "natural booster" to stimulate protective immunity. In our study, the times between the last immunization and challenge (7–230 days) were relatively short compared with the life span of humans and schistosome parasites. This reflects that the average lifespan of a mouse is much shorter than that of the schistosome parasite (56, 57). The decrease in the fraction of protection over time was captured with our models even within this relatively short time range. This result would suggest that boosting vaccines may be necessary for long lasting protection against schistosomes.

There are several different cercariae attenuation methods as we described in the introduction. Within these, ionizing radiation (X-ray and gamma ray) is the most commonly used attenuation method for attenuated schistosome cercariae preparation. Two relatively high irradiation doses around 20 or 50 krad have been reported as the optimal doses for parasite attenuation (58, 59) and, in fact, many past studies have applied these irradiation doses. However, our results suggest that a lower irradiation dose could improve protection. The lower irradiation doses enable attenuated parasites to live longer in the vaccinated host. After vaccination, irradiated cercariae have been reported to be present around the skin exposure site for approximately 4 days and then gradually moved to the lungs where they transformed from cercariae into lung-stage schistosomula (60). It has been reported that the immunizing parasite has to reach the lungs and transform to lung-stage schistosomula to elicit protective immunity against challenge infection (60, 61), which may not be the case for cercariae attenuated with high doses of ionizing radiation. Several studies have reported that non-attenuated challenge cercariae in vaccinated mice slowly move to the lungs and then gradually disappear (61, 62). Several studies report that cercariae exposed to extremely high irradiation doses will die in the host skin before they start to migrate inside the host body (60, 63). Mountford et al. reported that hosts needed to be exposed to both highly irradiated cercariae, which die in the host skin, and lung-stage schistosome parasites to elicit protective immunity (64). One of the possible reasons for the high levels of protection observed when using irradiated cercariae vaccine is that hosts are exposed to a wide variety of antigens, which are expressed by different parasite life stages. Parasites, which were attenuated with lower irradiation dose, can survive long enough to express a variety of antigens from different life stages (65). However, in practice, allowing parasites to live longer inside vaccinated people may not be well accepted or ethically approved. The results of our meta-analysis suggests that for recombinant vaccine development the combination of antigens, which are unique to the different schistosome life stages may be an important factor in achieving a better protection.

CONCLUSION

In this study, we identified three predictors for effective immunization against schistosome infection using attenuated cercariae: the total number of immunizing parasites, the irradiation dose, and the time between the last immunization and challenge. The study results suggested that the optimally prepared irradiated cercariae vaccine could achieve a protection as high as 78% against challenge infection. Within the reported dose range, the maximum protection could be achieved with the highest number of immunizing cercariae (5,000 cercariae) and the lowest irradiation dose (3 krad). This protection slowly declines but remains high for at least 8 months after the last immunization. This achievable protection is much higher than the partial protection reported by the *S. mansoni* purified antigens that failed to achieve consistent protection above 40% in mice (21, 66, 67). Although none of the radiation attenuated cercariae vaccine studies achieved complete protection against challenge infection, it is thought that partial protection induced by immunization can significantly reduce both schistosome related morbidity and parasite transmission (68, 69). This meta-analysis shows there is the high potential for an attenuated cercarial vaccine, while also providing insights helpful for schistosome vaccine development more generally.

AUTHOR CONTRIBUTIONS

The initial conception and design of the work: KM, CB, and FM. Performed the systematic review: MF, KM, and CB. Contributed to draft manuscript editing/reviewing: MF, KM, CB, MW, and FM. Statistical analyses of the data: MF, with inputs from MW, and FM. All authors contributed to the revisions and approved the final version of the manuscript.

ACKNOWLEDGMENTS

We would like to thank our colleagues from Epigroup and Parasite Immuno-epidemiology group (University of Edinburgh), especially Norman Nausch for discussions on the manuscript and Welcome M. Wami for advice on the statistical analysis. Over this research project period MF was funded by a studentship from Japan Student Services Organization, KM was supported by the Medical Research Council UK, CB was supported by the Biotechnology and Biological Sciences Research Council, FM was supported by the Thrasher Research Fund and the Wellcome Trust.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2015.00085/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 December 2014; accepted: 13 February 2015; published online: 27 February 2015.

*Citation: Fukushige M, Mitchell KM, Bourke CD, Woolhouse MEJ and Mutapi F (2015) A meta-analysis of experimental studies of attenuated Schistosoma mansoni vaccines in the mouse model. Front. Immunol. **6**:85. doi: 10.3389/fimmu.2015.00085*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

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frontiers in Immunology

MINI REVIEW
published: 21 April 2015
doi: 10.3389/fimmu.2015.00186

S. mansoni trapping in lungs contributes to resistance to reinfection

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Worm transplantation studies show that physiological and reproductive status of the worm is influenced by the microenvironment of the host and critical for vaccine design. Worm migration studies in rats with ^{75}Se-methionine labeled cercariae demonstrated that resistance to reinfection (R/R) requires a host immune response resulting in worm death. In permissive hosts, inflammation due to anti eggs immunity leads to host death, whereas in non-permissive hosts this is not the case due to reduced egg burdens. Eggs-induced pathology and inflammatory debris resulting from immune attack on worms are important for vaccine design. Protective immune responses are perhaps induced when naïve hosts are vaccinated with either schistosome-derived molecules or attenuated cercariae as suggested by the induction of protective anti-parasite antibodies and monoclonals. However, these immunological strategies rarely produce 85–90% R/R as is achievable by portal-caval shunting. Alternatively, induction of anti-schistosoma immunity may induce portacaval shunting, seems highly unlikely although not yet tested. Differential screening with sera from twice-infected rats, protective (F2x) from Fisher vs. non-protective (W2x) from Wistar–Furth rats, was used to identify candidate vaccine antigens.

Keywords: *S. mansoni*, portacaval shunt, self-cure, resistance-to-reinfection, F2x (sera from twice-infected Fisher rats), W2x (sera from twice-infected Wistar–Furth rats), permissive vs. non-permissive hosts

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology

Received: *16 February 2015*
Accepted: *04 April 2015*
Published: *21 April 2015*

Citation:
Knopf PM and Suri PB (2015) S. mansoni trapping in lungs contributes to resistance to reinfection. Front. Immunol. 6:186. doi: 10.3389/fimmu.2015.00186

Background

The authors' combined research careers of over 60 years has focused on a preventive vaccine against schistosomiasis. In 1970 while Dr. Knopf was a postdoctoral fellow at the Salk Institute (California), Harvard physicist John Platt, then incumbent "Scientist In Residence," presented a seminar entitled "What We Must Do"(1). Platt focused on the disparity between the funding and scientific resources invested on diseases of wealthier longer-lived populations such as: obesity, cancer, diabetes, and ischemic heart disease compared to diseases of the poor such as bacterial/viral and parasitic infections. Moreover, interests in anti-Vietnam war, woman's lib, and racial inequality were also predominant in that era. So, emotionally moved and stimulated by Platt's seminar, a group of young scientists and technicians formed study groups to explore their knowledge and potential of immunology to address this huge disparity. Topics chosen included birth control, food distribution, and infectious diseases such as schistosomiasis, malaria, and hookworm. Dr. Knopf, along with Alan Sher, Donato Cioli, and Italo Cesari, became further interested in schistosomiasis and in particular with the work of R. Smithers, R. Terry, and J. Clegg on *in vitro* culturing of Schistosoma worms (2). After visiting the schistosome research laboratory of A. MacInnis at UCLA, Dr. Knopf accepted a faculty position at Brown University in the Department of Molecular Biology and Immunology.

Dr. Alferd Senft, an established researcher in the field of schistosomiasis with connections to the Rockefeller Foundation, was already a faculty member at Brown. Dr. Knopf started his research career in schistosomiasis in 1972.

At Brown, Dr. Knopf set-up the *Schistosoma mansoni* life cycle and learned to perform animal infections with help from Don Harn and F. von Lichtenberg (both at Harvard). Dr. Suri joined the laboratory in 1982 and her dissertation focused on differential screening and recombinant DNA technology to identify candidate vaccine protein antigens of *S. mansoni*. After completing postdoctoral training in liver cirrhosis, she rejoined Dr. Knopf's laboratory as an assistant professor and continued research on the identification of T-cell epitopes on a *S. mansoni* candidate vaccine antigen using overlapping peptides and a novel prediction algorithm (3).

Results

Dr. Knopf's initial schistosome study (supported by Rockfeller) was an attempt to repeat data from mice (permissive hosts), in rats (non-permissive hosts). In rats, *S. mansoni* does not complete its life cycle (no eggs in stool, no worms in mesenteric veins)(4). Rats developed R/R, which was dependent on cercarial dose used in challenge infections (5). Both at 50 and 500 cercariae used for primary infection, there was significant R/R. At a primary and challenge dose of 500 cercariae/rat, there was significant R/R of 50%, but at the higher challenge dose of 5000 cercariae, no further increase in R/R was observed. We speculated this was due to either induction of immune tolerance or saturation of immune capacity by the secondary infection. In mice, however, a dose of 50 cercariae was lethal in <2 months; interestingly, a dose as high as 5000 cercariae/rat induced no morbidity at all. At 4 weeks post infection, worm yields by portal perfusion of once infected rats are maximal and so is their size. From weeks 4 to 8, worm attrition occurs with minimal worm pairing, low egg production, and poor embryo maturation to miracidia. About 50–60% of cercariae achieve this immature state, as confirmed by the use of radiolabeled worms (6). In mice, worms continue to grow rapidly until 6 weeks, with pairing occurring earlier and female worms laying eggs. The number of eggs is far fewer in infected rats as is worm yield and pairing by 6 weeks (~10% of mouse yields). We developed a modified assay to collect lung-stage worms, adding HEPES (7) before incubation of minced lungs, in order to neutralize acid generated by both worms and inflammatory responses in infected minced lung tissue. Increased somule yields were found and thus we confirmed data from mice studies (8) in rats. While worm yields increased, the percent R/R decreased from 80–90% to 50–60%.

Dr. Cioli spent a year in Dr. Knopf's laboratory and they performed a unique series of worm transfer experiments. They assessed the survival, growth, and egg laying capacity of *S. mansoni* worms surgically transplanted from mice (permissive hosts) into rats (non-permissive hosts) or from rats to hamsters (permissive hosts) (4). Following transplantation into rats, adult mouse worms regress in size, remain in the liver, and produce small numbers of incompletely developed eggs. Conversely, transplantation of rat worms to hamster, worms increase in size, localize in the mesenteric veins, and produce numbers of eggs approaching normal hamster-grown worms within 3 weeks. These studies indicate that the physiological and reproductive status of the worm is strongly influenced by the microenvironment of the host. Furthermore, the suppression of worm growth and egg laying capacity in a non-permissive host is a reversible phenomenon since on transfer into a permissive environment; stunted worms can resume growth and oviposition.

In collaboration with Cioli, Mangold, and Dean (6), parasite migration was studied using ^{75}Se-methionine radiolabeled cercariae obtained by exposing previously infected snails to the tracer a day prior to shedding. Infected snails take up >90% of radiolabeled methionine analog in 12 h thus resulting in labeled cercariae, which were then used to infect groups of young rats (~120 g). On day 0, animals received normal Fisher rat serum (FNRS) or serum from twice-infected Fisher rats (F2x). Over the next few days, abdominal skin (infection site), as well as lung and liver tissue, was removed. Autoradiographic analysis of the pressed tissue revealed that the number of liver foci observed were nearly equal to the number of worms collected by portal perfusion (**Figures 1A,B**). Results confirmed that ~70% of applied cercariae attached to skin. Within 3–5 days, parasites migrated from skin to lungs with high efficiency in both normal and passively immunized rats. The total number of labeled parasites detected in skin, lungs, and liver was constant through day 5, declined to about 60% of this value by day 11 in both groups. Over the next 10 days, the rate of decline decreased significantly in normal rats, but did not change in immune rats. By day 21 post infection, nearly 50% fewer foci (labeled parasites) were detectable in livers of immune rats. The kinetics of migration from lungs to the liver differed in the two groups. In passively immunized rats with F2x, the parasites are trapped in the lungs longer as evidenced from the increased number of foci in lungs (**Figure 1B**). In other words, the disappearance of labeled parasites from the lungs was delayed in passively immunized rats. Labeled parasites accumulated in the liver, reaching maximal values by 11 days post infection in both groups, and remaining constant through day 21. However, half the numbers of labeled parasites were in liver of immune rats. We conclude that a subpopulation of parasites in the lungs is the target of protective immunity conferred by the F2x serum used for passive immunization. Target parasites, retained longer in lungs, were probably prevented from migrating successfully to the liver. Another parasite subpopulation migrated to the liver with normal kinetics, but was shunted out of the liver pathway and was lost forever.

Next, lung schistosomula isolated from both FNRS and F2x passively immunized rats were transferred by intravenous injection into naïve recipient rats and their continued migration from lungs to liver compared. Similar worm yields by portal perfusion from FNRS or F2x recipients were noted. We conclude that the effects of immunity molecules in F2x on somules during 1 week post infection were insignificant or reversible.

Another interpretation of the worm transfer studies could indicate a defect in worm sexual maturation in rats (6, 9). Perhaps in permissive host species, there exists a host hormone that binds to a sex-hormone receptor present in worms, but this is accomplished poorly in rats. The hormone produced by rats could partially

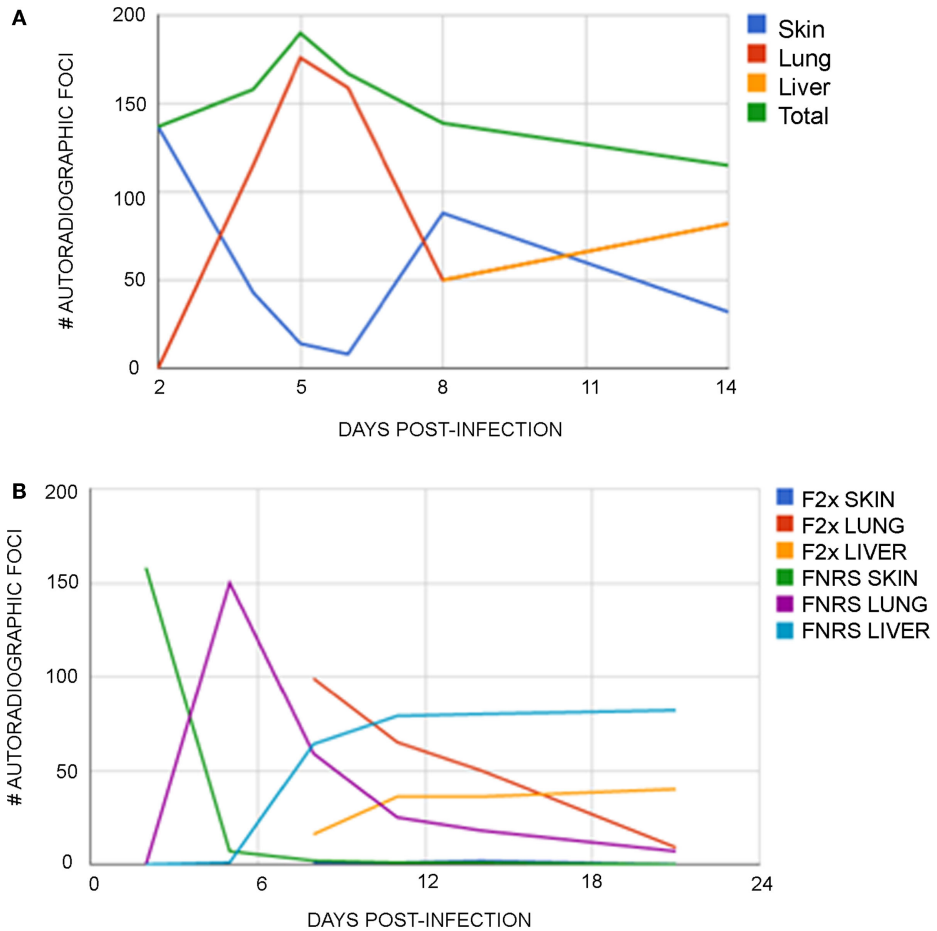

FIGURE 1 | (A) Distribution of auto radiographic foci in normal rats at various time post infection. Rats were exposed to 200 ^{75}Se-Selenomethionine-labeled cercariae on day 0. The mean number of auto radiographic foci numerated from two separate experiments are plotted. **(B)** Distribution of auto radiographic foci at various time post infection in normal and day 5 passively immunized rats. Rats were exposed to 200 ^{75}Se-Selenomethionine-labeled cercariae on day 0 and a subgroup was passively immunized with F2x on day 5 post infection. The mean numbers of auto radiographic foci numerated from two separate experiments are plotted.

inhibit worm maturation was studied by removal of pituitary (hypophysectomy or HYPOX) and then by removal of gonads, ovaries, thyroid, or adrenal glands. These rats were then infected with cercariae. Major finding was that worm yields and maturation improved in both *HYPOX* and in *THYROX* rats (10, 11). Thus, some step in thyroid hormone metabolism created limits on schistosome maturation and survival (**Table 1**).

The miracidia hatched from eggs produced in cercariae-infected THYROX rats were incubated with snails and a month later, shed cercariae collected and used to infect normal mice. About 4–6 weeks later, worms had paired and produced viable eggs. This is a Koch-like proof of the conclusion that the T3 is likely to generate, or be the source of, the inhibition of worm maturation in a non-permissive host.

During Dr. Knopf's sabbatical leave in D. McLaren's laboratory at Mill Hill (UK), in collaboration with G. Mitchell, worm migration was studied in inbred 129 strain of mice. Interestingly, although a permissive host, some of the inbred 129 strain of mice did not yield adult worms when infected with cercariae. Assessed at 8–10 weeks, the somules had moved from skin to lungs but failed to migrate to the portal circulation, and thus could not be collected by portal perfusion. Some worms were found in the lung capillaries involved with eosinophils. Concurrently, A. Wilson and P. Coulson described the "leaky liver" phenomenon that pre-existed in some of these 129 strain mice and was independent of infection. This was an anatomical anomaly of blood vessels that failed to yield properly located adult worms. We published our results in separately submitted papers (12, 13).

Next, our concerted efforts were focused on the use of differential screening to identify candidate vaccine antigens exclusively binding with antibodies in protective F2x but absent in non-protective W2x sera. A tegumental glycoprotein Sm25 was identified as a candidate vaccine antigen and corresponding gene (GP22) cloned. Codons 43–182 amplified by PCR and cloned in pET15b bacterial expression system. Recombinant protein r140, in combination with different adjuvants, was tested for its vaccination potential. We vaccinated mice and rats with recombinant products (3, 14–16). Despite high titer anti-r140 antibodies with

TABLE 1 | Normal (4–8 rats) or THYROX (6 rats) per group were infected at 270 cercariae/rat and maintained daily without thyroid hormone or with T3 or T4.

Group	Time post primary infection	Total worm yields	Male worms length (mm)	Female worms length (mm)	Sex ratio (m/f)	Weight gain (g/week)
Normal	4 weeks (5)	19.2	9.6	9.4	1.0	
	5 weeks (6–8)					41.7
	6 weeks (4)	10.5	4.5	6.0	0.75	
	7 weeks (6)	6.3	1.8	4.5	0.4	
Thyrox	7 weeks (6)	16.3	7.8	8.5	0.9	8.9
Thyrox + T3	7 weeks (6)	5.3	2.2	3.2		31.3
Thyrox + T4	7 weeks (6)	1.5	0.8	0.7	0.8	27.8

Weighed weekly. Worms collected: number, length (mm), gender scored.

protective isotypes that detected a 25 kDa surface antigen, no protection against subsequent cercarial challenge or any effect on egg yield was observed. This has led us to conclude that not all molecules produced by worms are candidate target sites. However, the differential screening technique we developed in identifying candidate vaccine antigens has proven useful in another widespread tropical disease, malaria (17).

Conclusion

After a combined research career of 60 years, we strongly believe that we now understand something new about schistosome infection. Our research sheds light on the importance of host microenvironment in parasite attrition. Schistosomes have an enormous host range including several mammalian species such as, man and some primates, as well as rodents, and other phyla (snails for miracidia). Man and mouse appear to be good examples of *permissive* primary hosts (after infection with cercariae, viable eggs are found in feces); while *Rattus norvegicus* rats appear to be *non-permissive* hosts as cercarial infection results in absence of viable eggs in feces. Then, there are transient or *temporary permissive* hosts (permissive initially but subsequently become non-permissive) such as rhesus macaque, guinea pigs, and *Rattus rattus* (water rat, black rat). 129 strain mice are mixed. These "Transient permissive" hosts become that way by acquiring, e.g., portacaval shunts (before or during infection) or self-curing a primary infection. Also thyroidectomy can convert the rats from non-permissive to permissive state. Therefore, it seems appropriate to categorize the host in the vaccination study accordingly and be aware of the host defense mechanisms in vaccine objective.

In conclusion, one can predict two ways to end an infection by schistosoma parasites: either *via* immune attack or *via* portacaval shunting. Rats do both, *before* worms can form pairs, whereas others such as: mice, some monkey strains, and guinea pigs take longer before becoming non-permissive hosts and eliminating the parasites. Hence, the latter can be classified as *conditionally* permissive. The thyrox *R. norvegicus* rat should likely be classified as conditionally or transiently permissive.

Great leaps have been made toward schistosomiasis vaccine in the past decade, reviewed in Ref. (18–22). Calpain (Sm-p80) stands out as protective vaccine with cross species specificity, and already been tested as recombinant vaccine (hamsters and mice with Abs longevity 60 weeks in mice) and as DNA vaccine (baboons-Abs detected 5–8 years post vaccination). Coupled with purification to homogeneity, Sm-p80 is ready to be manufactured with GMP practices for clinical trials (23). Detection and testing of schistosome gut antigens related to nutrient uptake as potential vaccines is exciting (24). Use of nanoparticle gene delivery (25) and design of conformational and self-adjuvinating epitopes (26, 27) have taken the field of vaccine development to new levels. With the advances in Bioinformatics, availability of proteomic and genomic databases (28), a vaccine in the next decade could be a reality. While not identical to cases of pneumococcal bacterial or poliovirus infection, where multivalent vaccines resulted in successful vaccine, parasitic infections lead to multiple forms of the infectious agent: larval, juvenile, adult male and female worm, eggs etc., displaying unique and shared epitopes. Cocktail vaccines combining target antigens unique to different life cycle stages might give an additive protection. As pointed out by McManus and Loukas (18), putative resistant (PR) – a well defined cohort of individuals in Brazil, who display natural resistance to schistosome infection despite years of exposure to *S. mansoni*, should be studied further. They might hold a clue and give new insight to vaccine development.

Acknowledgments

We wish to thank Donato Cioli and Jonathan Kurtis for their invaluable insight in critiquing this manuscript; Sean Suri and Ami Shah with technical support. Without the financial grant support from Rockefeller Foundation, Edna McConnell Clark Foundation, WHO, NIH over the years, this project would not be possible.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 23 March 2015
doi: 10.3389/fimmu.2015.00130

Induction of protective immune responses against *Schistosomiasis haematobium* in hamsters and mice using cysteine peptidase-based vaccine

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One of the major lessons we learned from the radiation-attenuated cercariae vaccine studies is that protective immunity against schistosomiasis is dependent on the induction of T helper (Th)1-/Th2-related immune responses. Since most schistosome larval and adult-worm-derived molecules used for vaccination uniformly induce a polarized Th1 response, it was essential to include a type 2 immune response-inducing molecule, such as cysteine peptidases, in the vaccine formula. Here, we demonstrate that a single subcutaneous injection of Syrian hamsters with 200 μg active papain, 1 h before percutaneous exposure to 150 cercariae of *Schistosoma haematobium*, led to highly significant ($P < 0.005$) reduction of >50% in worm burden and worm egg counts in intestine. Immunization of hamsters with 20 μg recombinant glyceraldehyde 3-phosphate dehydrogenase (rSG3PDH) and 20 μg 2-cys peroxiredoxin-derived peptide in a multiple antigen peptide construct (PRX MAP) together with papain (20 μg/hamster), as adjuvant led to considerable (64%) protection against challenge *S. haematobium* infection, similar to the levels reported with irradiated cercariae. Cysteine peptidases-based vaccination was also effective in protecting outbred mice against a percutaneous challenge infection with *S. haematobium* cercariae. In two experiments, a mixture of *Schistosoma mansoni* cathepsin B1 (SmCB1) and *Fasciola hepatica* cathepsin L1 (FhCL1) led to highly significant ($P < 0.005$) reduction of 70% in challenge *S. haematobium* worm burden and 60% reduction in liver egg counts. Mice vaccinated with SmCB1/FhCL1/rSG3PDH mixture and challenged with *S. haematobium* cercariae 3 weeks after the second immunization displayed highly significant ($P < 0.005$) reduction of 72% in challenge worm burden and no eggs in liver of 8–10 mice/group, as compared to unimmunized mice, associated with production of a mixture of type 1- and type 2-related cytokines and antibody responses.

Keywords: *Schistosoma haematobium*, schistosomiasis vaccine, cysteine peptidases, papain, cathepsins, type 1 and 2 immune responses, larval excretory–secretory products

INTRODUCTION

Schistosomiasis is a debilitating parasitic disease that affects humans in 74 countries, mainly in the Middle East, sub-Saharan Africa, South America, and some regions of the Philippines, China, and Indonesia. Two species, *Schistosoma mansoni* and *Schistosoma haematobium*, are responsible for the majority of human infections. As a result of the insensitivity and unreliability of current diagnostic techniques and the paucity of sound epidemiological surveys, it is not clear whether the number of active *Schistosoma* infections is 209 (1), 230 (2), 252 (3), or 391–587 (4) million people worldwide. People infected with schistosomes react intensely to antigens derived from the huge numbers of parasite eggs that have failed to escape to the exterior via feces (*S. mansoni*) or urine (*S. haematobium*), and are trapped in the host tissues. These intense immunological reactions lead to fibrosis and dysfunction of the affected organs, namely liver, gut, and urinary bladder (1–5).

A single anti-schistosome drug, praziquantel (PZQ), is readily available. Despite its low cost and self-limiting side-reactions, the drug has only been offered to less than 13% of the target population (1). Innumerable persons are left untreated, suffering long-term disabilities and exacerbation of co-infections (1–6). Praziquantel is highly effective in treatment of light and moderate infections. However, in areas of high endemicity and transmission and/or intensive PZQ mass administration, PZQ cure rates are almost negligible [(7) and references therein]. A schistosomiasis vaccine could protect up to 600–780 million individuals, mostly children, living in endemic regions at risk of the infection. Articles in this topic and elsewhere have duly reported on the history and fate of a number of candidates and potential vaccine antigens, of which very few have shown satisfactory efficacy and none has reached the commercial level (8–10).

One of the main reasons hindering the development of a vaccine against schistosomiasis is the entrenched dogma stating

protection is dependent on the generation of type 1 immune responses. This belief was based on preponderance of interferon-gamma (IFN-γ) released by bronchoalveolar leukocytes, total lung tissue, and lung-draining lymph nodes in radiation-attenuated (RA) cercariae-vaccinated mice (11, 12). Lung schistosomula-derived antigens seeping in lung tissues or released from extravasated dying larvae expectedly induce preponderant type 1 immune responses [(13, 14) and references therein]. Yet, these immune responses might be irrelevant to parasite attrition, as it must be reiterated healthy schistosomes are exclusively intravascular and may not be directly affected by the immune events in lung alveoli, parenchyma, or draining lymph nodes. More importantly, several studies using knockout mice conclusively demonstrated that the optimal protection in the RA vaccine model is dependent on the induction of both type-1 and type-2-associated immune responses (15–17).

We have well-learned the lessons of the successful RA vaccine model and thought it is imperative to use type 2-, not type 1-inducing cytokines or molecules as adjuvants to the schistosome-derived antigens used for vaccination (14). The highly significant ($P < 0.0001$) and reproducible protection against challenge *S. mansoni* worms achieved in mice, immunized with larval antigens derived from excretory–secretory products (ESP), namely recombinant glyceraldehyde 3-phosphate dehydrogenase (rSG3PDH) and 2-cys peroxiredoxin-derived peptide in a multiple antigen peptide construct (PRX MAP) in conjunction with papain, interleukin (IL)-25, IL-33, or thymic stromal lymphopoietin (TSLP), supported our belief. Our proposal was particularly strengthened by the significant ($P < 0.02$) levels of protection obtained following immunization with papain, IL-25, or IL-33 alone [(18, 19) and references therein]. Therefore, we felt it was important to examine whether this approach could be applied to *S. haematobium*, and examined immunological and parasitological parameters in hamsters immunized with papain alone, or papain in conjunction with rSG3PDH and PRX MAP.

Since papain, IL-25, IL-33, or TSLP may not be readily used for human vaccination, we resolved the issue by replacing these type 2-inducing molecules by parasite-derived cysteine peptidase, namely *S. mansoni* cathepsin B1 (SmCB1). Immunization of outbred mice with SmCB1 alone generated a polarized type 2 immune response environment that was associated with highly significant ($P < 0.0001$) reduction of 83% of *S. mansoni* challenge worm burden; this supported our hypothesis stating that *S. mansoni* larvae will almost all succumb if met by a type 2 cytokine environment (18–21). To further improve the vaccine efficacy, we included another cysteine peptidase, *Fasciola hepatica* cathepsin L with the aim of inducing the production of anti-cathepsin L antibodies that would neutralize the *S. mansoni* homologous enzyme and inhibit its function. The highest level of worm burden reduction and decrease in worm egg counts in liver and small intestine of outbred mice were achieved when this peptide formulation was combined with rSG3PDH (22–25). It is important to note that SG3PDH is a larval and adult worm ESP (26), documented to be also associated with the larval surface membrane (22), and to induce polarized type 1 and type 17 immune responses (19).

Therefore, we proposed a novel schistosome cysteine peptidase-based formula that fulfills all requirements for an efficacious vaccine for schistosomiasis (24, 25). First, two immunizations are sufficient to induce highly significant ($P < 0.0001$) and highly reproducible (eight experiments) reduction of up to 66% in *S. mansoni* worm burden and egg counts in host liver and intestine. Second, the vaccine is adjuvant/chemical free, bypassing the insurmountable obstacle of adjuvant use in pre- and clinical trials in humans. Third, vaccine-induced protection is associated with generation of both type 1 and type 2 cytokines-related immune responses. Fourth, the vaccine was entirely safe in outbred mice and did not induce IgE antibodies or any adverse reaction during immunization and after challenge.

To proceed forward with and efficacious vaccine formula against *S. mansoni*, it is important that we demonstrate that these approaches apply to *S. haematobium* and *S. japonicum*. Indeed, most vaccine strategies applied to date have not shown cross-species efficacy. Moreover, vaccine studies in the *S. haematobium* model are rather rare and, thus, the present study represents an addition to this neglected field. Accordingly, we, herein, investigated whether our vaccine formulation of functional cysteine peptides without the addition of a chemical adjuvant is also effective in protecting mice against a challenge infection with *S. haematobium*.

MATERIALS AND METHODS

ETHICS STATEMENT

All animal experiments were performed following the recommendations of the current edition of the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, USA, and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Science, Cairo University, permit number CUFS F PHY 21 14.

ANIMALS AND PARASITES

Female Syrian hamsters (*Mesocricetus auratus*) and CD1 mice were raised at the Schistosome Biological Materials Supply Program, Theodore Bilharz Research Institute (SBSP/TBRI), Giza, Egypt, and maintained throughout experimentation at the animal facility of the Zoology Department, Faculty of Science, Cairo University. Cercariae of an Egyptian strain of *S. haematobium* were obtained from SBSP/TBRI, and used for infection immediately after shedding from *Bulinus truncatus* snails.

PAPAIN AND IMMUNOGENS

Papain from *Carica papaya* (BioChemika ≥3 units/mg) was obtained from BioChemika, and used in an active form or following inactivation by incubation in the presence of 5 μM of the irreversible inhibitor of cysteine peptidases, L-trans-epoxysuccinylleucylamide-(4-guanido)-butane (E-64, Calbiochem, San Diego, CA, USA), as described previously (24, 27). Recombinant *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (rSG3PDH) was prepared and purified to homogeneity, as described (23) and contained <0.06 Endotoxin Units/ml as judged by the Pyrogen Gel-Clot Limulus Amebocyte Lysate test. 2-Cys peroxiredoxin (28) (H-^{104}RKQEISKAYGVFDE EDGNA^{122}-OH)-derived peptide, showing lowest homology to the murine counterpart, was synthesized as a MAP (tetra branched multiple antigen peptide) construct (PRX MAP) and purified at AnaSpec

Inc. (San Jose, CA, USA). Functionally active *S. mansoni* cathepsin B1 (SmCB1) and *F. hepatica* cathepsin L1 (FhCL1) were prepared as described (24, 29).

These *S. mansoni*-derived molecules were used for vaccination of hamsters and mice against infection with *S. haematobium*, as they are remarkably conserved across *S. mansoni* and *S. haematobium*. *S. mansoni* cathepsin B1 and SG3PDH show 94–96% identities at the amino acid level with the corresponding enzyme of *S. haematobium* (GI:68596858 and GI:685936895, respectively). *S. mansoni* 2-Cys peroxiredoxin (PRX)-derived peptide used in the MAP construct shows 84% identities and 89% similarity with the corresponding peptide of *S. haematobium* PRX (GI:685965340). *F. hepatica* cathepsin L was readily recognized by outbred mice infected with *S. mansoni*.

INJECTION, INFECTION, AND ANALYSES IN HAMSTERS

Hamsters (10 per group) were injected subcutaneously (sc) into two sites with 200 μg active papain. One hour later, injected and untreated hamsters were anesthetized, abdomen-shaved, and then exposed percutaneously using the ring method (30) to 150 *S. haematobium* cercariae.

Hamsters (10 or11 per group) were immunized, twice at 3 weeks interval, sc on one side with 20 μg active or E-64-inactivated papain, and intramuscularly on the other side with 20 μg rSG3PDH and 20 μg PRX MAP. Four weeks later, naïve and immunized hamsters were exposed to 120 cercariae of *S. haematobium* as described above.

Serum was recovered from three hamsters per group per experiment on day 14 post infection. Sera were individually assessed by enzyme-linked immunosorbent assay (ELISA) for antibody binding to soluble *S. haematobium* adult worm antigen (SAWA, 1.0 μg/well) prepared as described (31), rSG3PDH (250 ng/well), and PRX MAP (1.0 μg/well). Alkaline phosphatase (AKP)-labeled anti-hamster IgG (H + L) conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was diluted 1:1000. For each experiment, antibody isotypes of individual sera, diluted 1:50, for each hamster group were determined using biotin-labeled monoclonal antibodies to hamster IgG classes, IgG1, and IgG2 (Pharmingen, San Diego, CA, USA), and AKP-labeled streptavidin from Promega (Madison, WI, USA).

Worm burden and liver and intestine worm egg load in individual hamsters (6–8 per group) were evaluated 12 weeks after challenge infection (19, 24). Mean values ± SE for each group were calculated. Percent change was evaluated by the formula: % change = mean number in infected controls - mean number in infected, treated mice/mean number in infected controls × 100.

IMMUNIZATIONS AND INFECTIONS IN MICE

In two experiments, mice (12–13 per group) were immunized sc at the base of the tail, twice with a 3-week interval with 10 μg SmCB1 and 10 μg FhCL1 alone or combined with rSG3PDH (10 μg/mouse). Three weeks after the second injection, untreated and immunized mice were infected percutaneously via whole body exposure to 100 ± 5 viable cercariae of *S. haematobium*. Spleen cells and serum were recovered from 2–3 mice per group per experiment on day 8 post infection. Worm burden and liver worm egg load were assessed in mice 12 weeks after the challenge infection as described for hamsters.

CYTOKINE AND HUMORAL RESPONSES

Spleen cells (SC) were harvested on day 8 after infection with *S. haematobium* cercariae, and cultured with 0 or 5 μg/ml immunogen as described (19, 24). At 48 and 72 h of incubation, cultured SC were thawed and frozen for release of intracellular cytokines, and supernatants stored at −76°C until assayed by capture ELISA for levels of IL-4, IL-5, IL-17A, IFN-γ (ELISA MAX^{TM} Set, BioLegend), and IL-13 (DuoSet ELISA Development System, R&D Systems Europe), following the manufacturer's instructions.

Sera were obtained from unimmunized and immunized mice 8 days following infection with cercariae of *S. haematobium*, and individually assessed by ELISA for humoral antibody titer reactivity to SAWA (1.0 μg/well) and SmCB1 (250 ng per well). Antibody isotypes in mouse sera (1:200 dilution) were analyzed using rat alkaline phosphatase-conjugated monoclonal antibodies to various mouse IgG classes (Pharmingen) and biotin-labeled monoclonal antibody to mouse IgA, and IgE (BioLegend) with sera diluted 1:25 (19, 24).

DATA ANALYSIS AND STATISTICS

All values were tested for normality. Mann–Whitney test was used to analyze the statistical significance of differences between experimental and control values and considered significant at $P < 0.05$.

RESULTS

EFFECT OF PRE-TREATMENT WITH THE CYSTEINE PEPTIDASE, PAPAIN ON HAMSTER HUMORAL IMMUNE RESPONSES, AND RESISTANCE TO *S. HAEMATOBIUM*

Hamster humoral antibody binding to soluble adult worm antigens (SAWA) 14 days after exposure to 150 cercariae of *S. haematobium* was negligible, and was not significantly enhanced by pre-infection treatment with active papain (data not shown). Yet, a single papain injection 1 h before hamster exposure to *S. haematobium* cercariae led to highly significant ($P < 0.005$) decrease in total worm burden of 55%. The decrease in worm burden was observed for both male and female worms (**Table 1**). Hamster pre-treatment with active papain before infection did not induce a significant decrease in worm egg counts in liver. However, the decrease in intestine egg counts was highly significant ($P < 0.002$), reaching 78.1% (**Table 1**).

EFFECTS OF THE CYSTEINE PEPTIDASE, PAPAIN AS ADJUVANT ON HAMSTER HUMORAL IMMUNE RESPONSES, AND RESISTANCE TO *S. HAEMATOBIUM*

Hamsters immunized with rSG3PDH and PRX MAP in conjunction with inactive or active papain were challenged with 120 *S. haematobium* cercariae 4 weeks later, and tested for serum antibody binding to SAWA and to the immunogens 14 days after infection. Highest antibody binding to SAWA was observed in hosts immunized with the vaccine in conjunction with active papain. The antibody binding to rSG3PDH and PRX MAP, 6 weeks after the boost immunization, is evidence for memory response to

the immunogens, and was again highest in hamsters immunized with the vaccine and active papain as adjuvant (**Figure 1**). No immunogen-specific IgG1 antibodies were detected, while about 10% of the bound antibodies were of the IgG2 isotype (data not shown).

Active papain used as an adjuvant to rSG3PDH/PRX MAP vaccination of hamsters against *S. haematobium* led to highly significant ($P = 0.0007$) decrease of 64% in total worm burden. The decrease in worm burden was observed for both male and female worms. Inactivation of papain with E-64 almost completely eliminated its protective effect (**Table 2**; **Figure 2**). Importantly, hamster immunized with rSG3PDH/PRX MAP and active papain as adjuvant showed significant decrease in challenge *S. haematobium* worm egg counts in both liver (32.8%, $P < 0.05$) and intestine (59.4%, $P < 0.01$) (**Table 2**; **Figure 2**).

Table 1 | Effect of papain pre-treatment on parasitological parameters of *S. haematobium*-infected hamsters.

Parameter	Infected controls	Active papain
Total worm burden		
Mean ± SE	48.8 ± 2.6	22.1 ± 0.9
P value (reduction %)		<0.005 (54.7)
Male worm burden		
Mean ± SE	30.5 ± 2.6	13.3 ± 0.8
P value (reduction %)		<0.005 (56.3)
Female worm burden		
Mean ± SE	17.8 ± 1.2	8.5 ± 0.7
P value (reduction %)		<0.005 (52.2)
Liver egg counts		
Mean ± SE	70450 ± 1646	60000 ± 5400
P value (reduction %)		Not significant
Intestine egg counts		
Mean ± SE	16564 ± 2210	3620 ± 616
P value (reduction %)		<0.002 (78.4)

Parasitological parameters were assessed 12 weeks after infection of untreated (infection controls), or active papain-injected hamsters (7 hamsters per group). Two-tailed P value as assessed by the Mann–Whitney test. Reduction % = mean number of untreated hamsters – mean number of papain treated hamsters/mean number in untreated hamsters × 100.

CYSTEINE PEPTIDASE-BASED VACCINE AGAINST MURINE *S. HAEMATOBIUM*

In two experiments, 9 out of 9 untreated/infected mice had 4–6 worms and 950–2400 eggs in liver. Vaccination with SmCB1/FhCL1 mixture led to highly significant ($P < 0.005$) reduction of 70% in challenge *S. haematobium* worm burden and 60% reduction in liver egg counts, as 5 out of 10 mice had 1–2 worms and 3 out of 10 mice showed 850–2400 eggs in liver. Mice vaccinated with SmCB1/FhCL1 and rSG3PDH displayed highly significant ($P < 0.005$) reduction of 72% in challenge worm burden and no eggs in liver of 8–10 mice/group, as compared to unimmunized mice.

Spleen cells obtained from unimmunized and immunized mice 8 days following *S. haematobium* infection, and stimulated *in vitro* with cysteine peptidase or rSG3PDH produced higher levels of IL-4, IL-5, IL-13, and also IL-17 and IFN-γ, compared to naïve and unimmunized infected mice, implying that cysteine peptidase-based protection against *S. haematobium* was associated with a mixture of type 1, type 2, and type 17 cytokines (**Figure 3**).

Serum antibody titer and isotype responses to SAWA and SmCB1 in unimmunized and immunized mice, 8 days after infection with *S. haematobium* are shown in **Figure 4**. Antibody responses to SAWA were observed only in mice immunized with rSG3PDH in conjunction with SmCB1 and FhCL1, and included IgG1, IgG2a, IgG2b, and IgA antibodies. No IgE antibodies were detected in sera diluted 1:25 (**Figures 4A,B**). High antibody responses to SmCB1 were observed in mice immunized with the cysteine peptidases and consisted predominantly of IgG1 antibodies. Serum antibody responses to SmCB1 were highest in

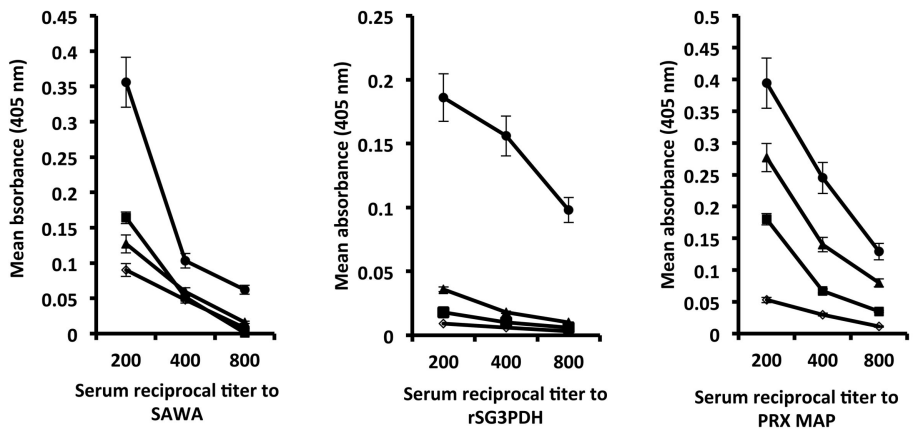

FIGURE 1 | Effect of combining rSG3PDH/PRX MAP vaccine with active papain on humoral responses of 14 day *S. haematobium*-infected hamsters. Each point represents mean ELISA absorbance (405 nm) of sera from three individual naïve (o), infected control (■) hamsters, and hamsters immunized with inactive (▲) and active (●) papain in conjunction with rSG3PDH and PRX MAP, tested in duplicate, and the horizontal bars depict the SE around the mean.

mice immunized with the cysteine peptidases in conjunction with rSG3PDH, and included IgG1, IgG2a, IgG2b, and IgA antibodies (**Figures 4C,D**).

Table 2 | Effect of combining rSG3PDH/PRX MAP vaccine with inactive or active papain on parasitological parameters of *S. haematobium*-infected hamsters.

Parameter	Infected controls	Inactive papain/Ag mix	Active papain/Ag mix
Total worm burden			
Mean ± SE	25.5 ± 2.0	18.7 ± 2.4	9.3 ± 2.8
P value (reduction %)		NS	0.0007 (63.5)
Male worm burden			
Mean ± SE	15.5 ± 1.1	11.8 ± 1.9	5.1 ± 0.6
P value (reduction %)		NS	0.0007 (67.1)
Female worm burden			
Mean ± SE	10.0 ± 0.9	6.8 ± 0.7	4.2 ± 0.4
P value (reduction %)		0.037 (32.0)	0.0007 (58.0)
Liver egg counts			
Mean ± SE	19833 ± 2438	14714 ± 937	13312 ± 1505
P value (reduction %)		NS	0.0481 (32.8)
Intestine egg counts			
Mean ± SE	5333 ± 840	4028 ± 566	2162 ± 301
P value (reduction %)		NS	0.0067 (59.4)

Ag mix = rSG3PDH + PRX MAP. NS = not significant, as assessed by the Mann–Whitney test (two-tailed P value). Reduction % = mean number in unimmunized hamsters − mean number in papain/Ag mix immunized hamsters/mean number in unimmunized hamsters × 100.

DISCUSSION

We have proposed that to achieve significant attrition of schistosomes during their journey in the lung blood capillaries and liver sinusoids, eosinophils and basophils must be recruited to the circulation and activated by type 2 cytokines, such as IL-4, IL-5, and IL-13, or via binding antibody-ESP complexes in the vicinity of the larvae (18–21). Worms-derived antigens induce preponderant type 1- and type 17- and limited or no type 2-related cytokines and antibodies. Accordingly, we have proposed that deviation of the host immune system toward the type 2 axis, via administering type 2 immune responses-inducing molecules, such as the cysteine peptidase, papain (32–34) before infection will lead to considerable decrease in worm burden, as compared to uninjected animals. In four consecutive experiments, 50 μg papain injected sc in outbred mice,1 h before exposure to 125 cercariae of *S. mansoni* consistently, and reproducibly elicited highly significant ($P < 0.0001$) reduction in worm burden of 70% ±3. The reduction was also highly significant for decrease in worm liver ($P < 0.0001$) and small intestine ($P < 0.001$) egg counts but only of approximately 50% (14).

Here, our studies with peptidases were extended to determine the validity of this approach for protection in the hamster model of *S. haematobium* (**Table 1**). We found that papain treatment prior to challenge infection caused a reduction in *S. haematobium* worm burden in hamsters which was not associated with significant humoral responses. We are currently exploring the molecular mechanism by which pre-treatment with active papain leads to such highly significant ($P < 0.005$) decrease in total, male and female worm burden. Second, while the reductions in intestine egg counts were considerable in papain-injected hamsters, there was no decrease in liver egg counts; this could suggest elevated fecundity in the surviving worms, perhaps due to increased levels

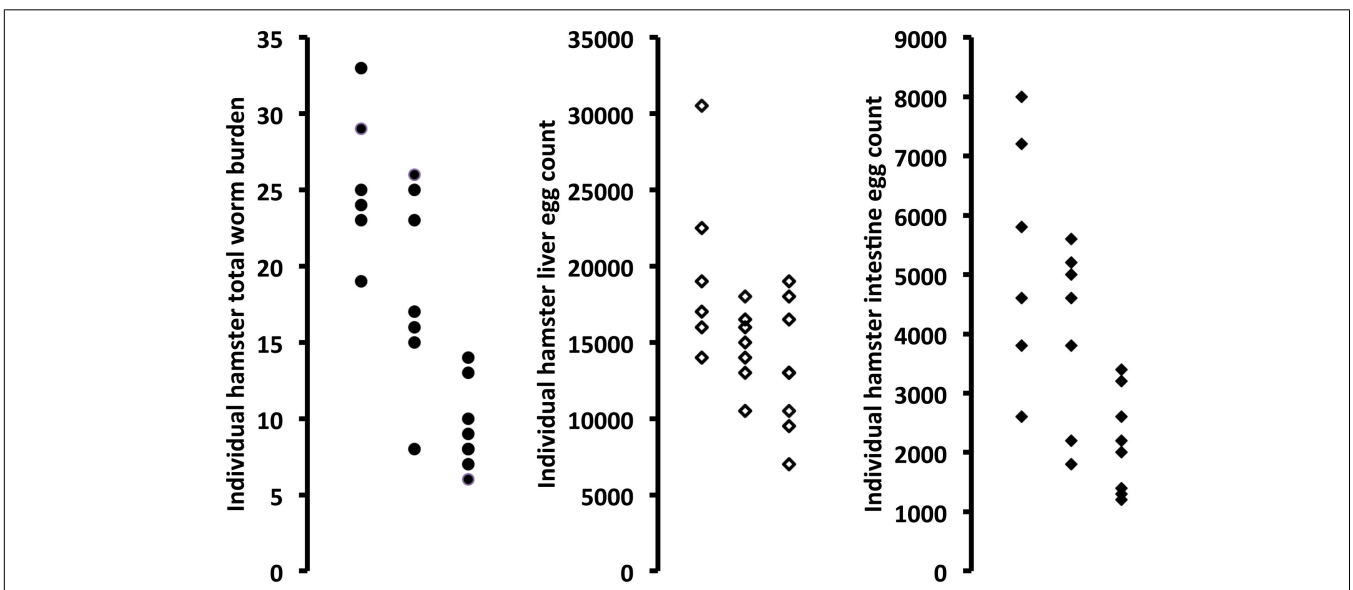

FIGURE 2 | Effect of combining rSG3PDH/PRX MAP vaccine with inactive (middle column in each panel) or active (right column in each panel) papain on parasitological parameters of *S. haematobium*-infected hamsters, as compared to unimmunized hamsters (left column in each panel). Each point represents parasitological parameter in individual hamsters, 12 weeks after infection with *S. haematobium* cercariae.

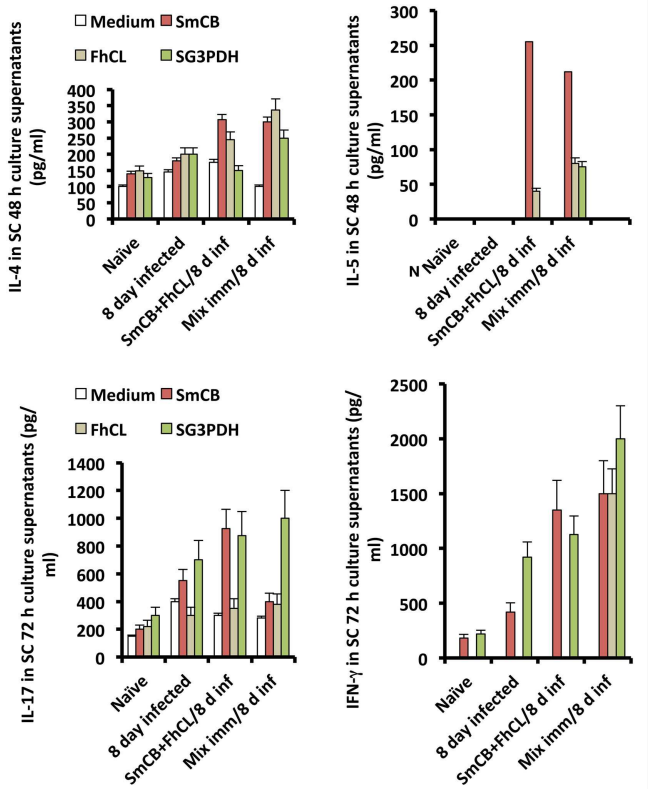

FIGURE 3 | Effects of immunization with SmCB1 + FhCL1 alone or in combination with rSG3PDH (Mix imm) on spleen cells cytokine release. Representative of two independent experiments whereby SC obtained from naïve mice, and unimmunized or immunized mice 8 days after infection (inf) with *S. haematobium* cercariae were stimulated *in vitro* with 0 (medium) or 5 μg immunogen/well of duplicate wells. Columns represent levels of cytokines in supernatants assessed 48 (IL-4 and IL-5) and 72 h (IL-13, not shown, IL-17, and IFN-γ) later, and vertical bars denote the SE about the mean values for 2–3 mice/group.

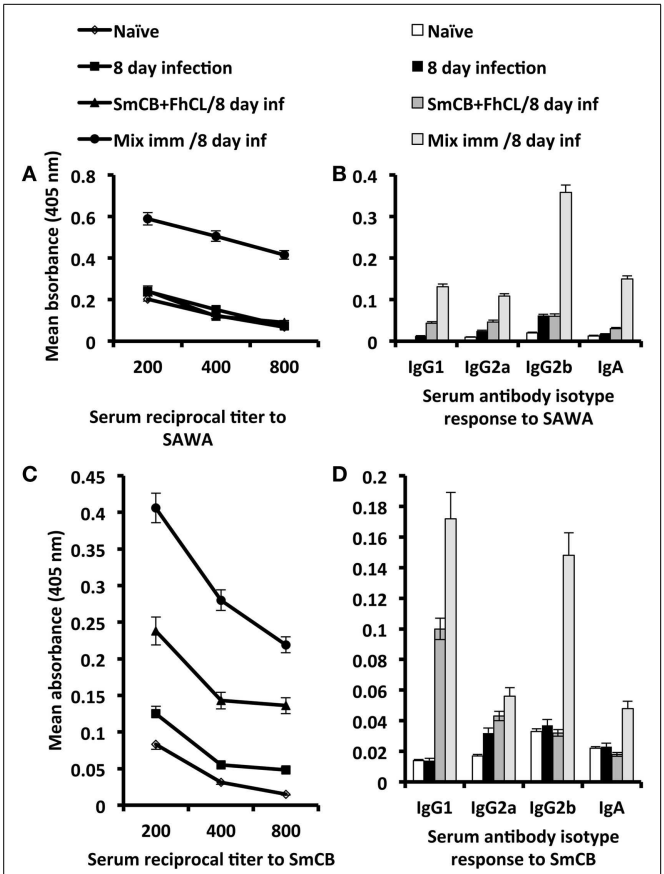

FIGURE 4 | Effects of immunization with SmCB1 + FhCL1 alone or in combination with rSG3PDH (Mix imm) on serum antibody responses. Representative of two independent experiments whereby sera obtained from 2–3 naïve mice, and unimmunized or immunized mice 8 days after infection (inf) with *S. haematobium* cercariae were tested for antibody titer and isotype response to SAWA **(A,B)** and SmCB1 **(C,D)**. Each point or column represent mean ELISA absorbance (405 nm) of 2–3 mice tested in duplicate, and vertical bars denote the SE around the mean. No IgE antibodies were detected in sera diluted 1:25.

of type 2 cytokines which has been reported to correlate with schistosome increased egg production (24, 35).

Vaccination of hamsters with our candidate vaccine mixture, rSG3PDH, and PRX MAP, in conjunction with papain led to highly significant ($P = 0.0007$) decrease of 64% in *S. haematobium* challenge worm burden provided using active not E-64-inactivated papain. These findings extend and confirm results recently obtained in mice immunized with the antigen mixture in conjunction with active papain and challenged with *S. mansoni*. It is of importance to note that active, but not inactivated papain, helped to generate immunogens-specific humoral antibody response that appeared to be essential for protection and decrease in worm egg load in liver. These findings suggest that the key to papain-mediated protective effect may be induction of active enzyme activity-dependent long-lived antibody-secreting cells, similarly to the proteases, natterins from the venom of *Thalassophryne nattereri* fish (36, 37).

Since papain derived from the plant *Carica papaya* could not be used for human vaccination, we sought to replace this by the *S. mansoni* cysteine peptidase, SmCB1, and the *F. hepatica* cathepsin L, both members of the papain-like peptidase family. As reported previously, immunization of outbred mice with SmCB1 + FhCL1 + rSG3PDH elicited highly significant ($P < 0.0001$) decrease of about 66% in challenge *S. mansoni* worm burden and worm egg counts in liver and intestine, distinctly higher than for SmCB1 and FhCL1, without rSG3PDH (24, 25). These results were reproduced here since by demonstrating protection of mice against challenge *S. haematobium* with similar formulations. Protection against murine *S. haematobium* appeared to be associated with induction of Th1, Th17, and Th2 cytokines and antibody responses, corroborating our suggestion that the generation of type 2-related immune responses is important in the design of an effective schistosomiasis vaccine (18–21, 24, 25).

The cysteine peptidases used in the study are purified as zymogens, which are stabilized by their propeptide segments. The schistosome and fasciola peptidases have been shown previously by Dalton and colleagues (38–41) to be extremely stable and not readily susceptible to breakdown. Jilkova et al. (42) recently

described how the propeptide of SmCB1 can stabilize the enzyme and resist auto-processing, and suggested that the enzyme can be activated when delivered *in vivo* by tissue glycoaminoglycans. Therefore, it is possible that following injection of the cysteine proteases, these become activated to mature enzymes by interaction with glycoaminoglycans under the skin.

Concerns may be raised for the future use of cysteine peptidases for vaccination of humans because of their potential to induce IgE antibodies. However, the cysteine peptidases used in the vaccine formula consistently failed to elicit production of IgE in mice despite booster immunizations with SmCB1 and FhCL1 alone or combined (this study and 24). Additionally, serum antibodies of *S. mansoni*-infected humans that bound to SmCB1 and FhCL1 were found to be essentially of the IgG and IgA isotype (24, 43, 44). Nonetheless, helminth cysteine peptidases-induced IgE may be irrelevant for children in rural areas of the developing world, to whom the vaccine is intended, as these usually harbor other parasites that stimulate IgE antibodies of diverse specificities, thus precluding harmful hypersensitive reactions. Pre-clinical studies in healthy volunteers must be performed before any positive or negative conclusion can be drawn regarding cysteine peptidase-based vaccine implementation in humans.

ACKNOWLEDGMENTS

Thanks are extended to Mr. Abdel Badih Foda for excellent and dedicated care of the experimental hamsters and mice. We acknowledge the contribution of the reviewers in improving the quality of the manuscript presentation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 December 2014; accepted: 10 March 2015; published online: 23 March 2015.
*Citation: Tallima H, Dalton JP and El Ridi R (2015) Induction of protective immune responses against Schistosomiasis haematobium in hamsters and mice using cysteine peptidase-based vaccine. Front. Immunol. **6**:130. doi: 10.3389/fimmu.2015.00130*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.
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