Protection against *Schistosoma haematobium* infection in hamsters by immunization with *Schistosoma mansoni* gut-derived cysteine peptidases, SmCB1 and SmCL3

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**A R T I C L E I N F O**

Article info
Received 13 June 2017
Received in revised form 15 September 2017
Accepted 20 October 2017
Available online 6 November 2017

Keywords:
Schistosomiasis
*Schistosoma haematobium*
Hamsters
Cysteine peptidases
Vaccine
Type 2 immune responses
Oogram

**A B S T R A C T**

We examined the immunogenicity and protective potential of SmCB1 and SmCL3 cysteine peptidases, alone and in combination, in hamsters challenged with *S. haematobium*. For each of two independent experiments, eight Syrian hamsters were immunized twice with a three week-interval with 0 (controls), 20 μg SmCB1, 20 μg SmCL3, or 10 μg SmCB1 plus 10 μg SmCL3, and then percutaneously exposed eight weeks later to 100 *S. haematobium* cercariae. Hamsters from each group were assessed for humoral and whole blood culture cytokine responses on day 10 post challenge infection, and examined for parasitological parameters 12 weeks post infection. At day 10 post-infection we found that SmCB1 and SmCL3 elicited low antibody titres and weak but polarized cytokine type 2 responses. Nevertheless, both cysteine peptidases, alone or in combination, evoked reproducible and highly significant reduction in challenge worm burden (>70%, *P* < 0.02) as well as a significant reduction in worm egg counts and viability. The data support our previous findings and show that *S. mansoni* cysteine peptidases SmCB1 and SmCL3 are efficacious adjuvant-free vaccines that induce protection in mice and hamsters against both *S. mansoni* and *S. haematobium*.

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1. Introduction

Schistosomiasis is a severe parasitic disease caused by trematode blood flukes of the genus *Schistosoma*, predominantly *Schistosoma mansoni* and *Schistosoma haematobium*. The disease is widespread in many countries in the Middle East, Africa, and South America [1,2]. Cercariae released by compatible freshwater snails invade the host through the skin and transform into schistosomula that move into the dermal blood capillaries en route to the lung. Maturation begins in the liver sinusoids and is completed in the lower mesenteric vein (*S. mansoni*) or the peri-vesical venous plexus (*S. haematobium*). Migrating and mature parasites induce weak immune responses and inflict limited harm to the host, but eggs produced by fecund female worms cause the major clinical manifestations of schistosomiasis. Eggs exit the blood capillaries to the exterior with the stool or urine by impairing the integrity of the tissues of the wall of the distal colon or rectum (*S. mansoni*) or lower urinary tract (*S. haematobium*). However, many eggs drift with the blood circulation and become trapped in the host tissues, especially the liver, where they provoke vigorous granulomatous inflammatory reactions with an aim to destroy the egg and contain the spread of the egg-derived antigens. These egg-induced immune reactions result into local edema, congestion, nodules, tubercles and polyps formation, neo-angiogenesis, fibrosis, scarring, and calcification in the liver and other tissues [1–3].

Praziquantel is the only drug available to treat schistosomiasis and despite its low cost, limited side-effects, and efficacy in curing light and moderate infection in humans, the number of people treated with praziquantel in 2013 and 2014 represented only 20.7% of the targeted population requiring chemotherapy [3,4]. A vaccine that is effective against intestinal/hepatic and urinary disease is needed to reduce, or even eliminate the disease burden, especially in Africa [5,6].

We recently demonstrated that functionally active recombinant forms of two major gut-derived cysteine peptidases of *S. mansoni* administered subcutaneously without adjuvant effectively protect...
mice against infection with *S. mansoni* and *S. haematobium* [7–10]. While second-order (primary adaptive) spleen cell and humoral immune responses to experimental infection in mice are consistently weak and skewed towards the type 1 axis prior egg deposition in tissues [11–18], immunization with cathepsin B1 (SmCB1) and cathepsin L3 (SmCL3) cysteine peptidases modulate innate immune responses by inducing the secretion of cytokines that initiate type-2 anti-schistosome immune responses. These vaccine-induced responses resulted in highly significant reduction of worm burden (>60%, *P* < 0.005), egg production and the viability of ova trapped in the liver and intestine [7–11].

Because mice are poorly permissive to *S. haematobium* infection, we felt it was important to investigate the effectiveness of the SmCB1/SmCL3 cysteine peptidase vaccine in a more permissive model (hamster) as an important step towards advancing our vaccine towards trials in non-human primates and pre-clinical studies. To reduce the possibility of our vaccine inducing responses that may protect in an immunologically non-specific manner, as Wilson and colleagues [19] suggest take place in murine models, our challenge infection were administered eight weeks following the second immunization.

2. Materials and methods

2.1. 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 numbers CUF5 F PHY 21 14 and CUF5-F-Imm-5-15.

2.2. Parasites and animals

Female Syrian hamsters (*Mesocricetus auratus*) were raised at the Schistosome Biological Materials Supply Program, Theodore Bilharz Research Institute (SBSP/TBRI), Giza, Egypt, and when 6 week-old, were 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 immediately after shedding from *Bulinus truncatus* snails. For challenge infections, hamsters were anesthetized, the abdomen shaved and wetted with sterile deionized water, and then exposed to 100 cercariae in 100 μL deionized water which was protected from spreading by a sterile steel ring.

2.3. Cysteine peptidases

Functionally active *S. mansoni* cathepsin B1 (SmCB1) and cathepsin L3 (SmCL3) were produced in the methylotrophic yeast *Pichia pastoris* GS115 (Invitrogen) and PichiaPink™ (Thermo Fisher) strain, respectively, as described previously by our laboratory [7–10].

2.4. Experimental design

For each of two consecutive experiments, three female Syrian hamsters were left unimmunized and uninfected and considered naïve animals. A total of 32 hamsters were divided into four groups of eight hamsters. Hamsters were subcutaneously injected twice at the tail base region, with a three-week interval, with 200 μL Dulbecco’s phosphate-buffered saline, pH 7.1 (D-PBS) containing 0 (control group), 20 μg SmCB1, 20 μg SmCL3, or 10 μg SmCB1 plus 10 μg SmCL3. Eight weeks after the second immunization, the control and immunized hamsters were exposed to 100 cercariae of *S. haematobium*. On day 10, when a large proportion of migrating larvae are expected to be in the lung capillaries, serum and whole blood cultures supernatants from three hamsters/group were assessed for immunological responses to the immunogen. Sera were separated on an individual hamster basis, and stored at −20 °C until use. Parasitological parameters were evaluated for five hamsters per group 12 weeks after the challenge infection.

2.5. Serum antibody assays

Hamster serum antibody titers were assessed by indirect enzyme-linked immunosorbent assay (ELISA) for binding to 250 ng/well SmCB1 or SmCL3 in duplicate wells as described [9–11]. Alkaline phosphatase (AKP)-labeled anti-hamster IgG (H + L) conjugate was diluted 1:1000 (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA).

2.6. Whole blood cultures

Heparinized whole blood (150 μL) was diluted 1:4 with RPMI-1640 medium supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 25 mM HEPES, and 20 μg/ml polymyxin B (Sigma Chemical Company, St. Louis, Missouri) as an inhibitor of lipopolysaccharide. Diluted blood samples were incubated in duplicate in 48-well culture plates (Corning Costar, Corning, NY, USA) in the presence of 0 or 10 μg SmCB1 or SmCL3 for 72 h at 37 °C/3% CO₂. Plates were then centrifuged at 400g for 10 min and the cell-free supernatants transferred into wells of a sterile plate and stored at −76 °C until assayed for cytokine release.

2.7. Cytokine assays

Serum and culture supernatants were assayed by capture ELISA in duplicate for levels of hamster IL-4, IL-5, IL-13, IL-17, and IFN-γ following the manufacturer’s instructions (MyBioSource, Inc., San Diego, CA, USA).

2.8. Parasitological parameters

Worm burden and total egg load in liver and intestine in individual hamsters were evaluated using the formula: % change = | mean number in infected controls -mean number in infected, treated hamsters/mean number in infected controls | × 100. Percentages of egg developmental stages were evaluated using 3 to 5 fragments of the ileum and the large intestine. After washing in saline solution and slight drying on absorbent paper, each intestinal fragment was placed between two slides and analyzed by light microscopy to count and classify the eggs. For each fragment, up to 100 eggs were counted and classified according to their developmental stage as immature, viable eggs/mature, viable eggs/non-viable calcified eggs as previously described [20]. Liver sections from each control and test hamster were stained with haematoxylin and eosin and examined for the number and diameter of granulomas surrounding eggs. Of note, all ova counts, viability estimates and granuloma counts/diameters were recorded for each hamster, based on a minimum of three replicates. The mean values for the hamsters for every group was then calculated and differences from unimmunized infected hamsters statistically analyzed by 2-tailed Student t and/or Mann-Whitney tests.
3. Results

3.1. Cytokine and humoral responses to the vaccines

Immune responses of hamsters were examined 10 days after the challenge infection with *S. haematobium*. Whole blood cultures from all groups of hamsters released negligible amounts of IL-4 following incubation in antigen-free or antigen-supplemented medium (data not shown). Whole blood cells from animals immunized with SmCB1, SmCL3 or SmCB1 plus SmCL3, cultured 10 days after infection, secreted lower IL-5, IL-13 and IL-17 levels than unimmunized controls, with a dramatic decrease in IFN-γ levels (Fig. 1). Significantly heightened memory responses to SmCB1 and/or SmCL3 were predominantly recorded for IL-5 and IL-13, notably in hamsters immunized with SmCB1 plus SmCL3 (Fig. 1).

Antibodies to SmCB1 were detected in the serum of hamsters (titer ≥ 1:1600) immunized with this antigen either alone or in combination with SmCL3 using (Fig. 2). By contrast, significant antibody titers were not observed against SmCL3 in the serum of hamsters immunized with this antigen alone or combined with SmCB1.

3.2. Parasitological findings

SmCB1 and SmCL3 alone elicited highly significant (*P* < .008) reduction in total worm burden, with the highest decrease observed in hamsters immunized with SmCB1. The highest reduction (*P* < .005) in parasite egg load in the large intestine was recorded in hamsters immunized with the cysteine peptidases in combination (Table 1). Immunization with the cysteine peptidases, alone or in combination, did not alter the number of eggs recovered from the whole liver tissue (Table 1) but was associated with highly significant decrease (~50%, *P* < .02) in the diameter of
circumoval granulomas (Table 2, Fig. 3) as well as death of the majority of ova (up to 90.0%, \( P < 0.009 - <0.001 \)) that reached the small and large intestine (Table 3).

### 4. Discussion

In the present study, we have demonstrated that immunization of hamsters, a permissive laboratory model for *S. haematobium* infection, with SmCB1 and SmCL3, alone or in combination and without formulation in adjuvant, induces high-level (60–75%) protection against challenge infection. This data supports our previous results using murine models \([7–10]\) for both *S. haematobium* and *S. mansoni*; thus, we have shown that these proteases are capable of eliciting cross-species protection. Whereas in our previous reports challenge infections were administered four or six weeks after the second immunization, here the period was extended to eight weeks in consideration of the study of Wilson and colleague \([19]\) that suggested spurious protection may result from non-specific immune responses that hamper the passage of schistosomules.
through the lungs of their hosts. Extending the time-frame between vaccination and challenge was suggested as a means of avoiding these non-specific effects [19], lending more strength to the idea that a vaccine is acting via immune-dependent factors.

The immunological mechanism by which the high level of protection is achieved in our vaccine system is still unclear and needs further exploration. Cytokine responses examined in whole blood cultures supernatants at 10 days post challenge infection revealed low but significant responses indicative of activation of type-2 responses. These findings confirm our previous data, which showed that pre-treatment of hosts with cysteine peptidase modulates schistosome infection-induced first- and second-order cytokine signals. Indeed, vaccination of mice with the cysteine peptidase papain, diminished early induction of type 1, type 17, and type 2 cytokines following Schistosoma mansoni infection, and skewed the immune system towards a polarized type 2 immune milieu [11]. The in vivo administration of cysteine peptidases has been shown to cause epithelial and endothelial barrier

Table 3
Effect of immunization with SmCB1 or SmCL3 alone or in combination on liver granulomas induced by challenge S. haematobium in hamsters.\(^a\)

<table>
<thead>
<tr>
<th>Granuloma parameters</th>
<th>Infected controls</th>
<th>SmCB1</th>
<th>SmCL3</th>
<th>SmCB1 + SmCL3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>15.3 ± 2.3</td>
<td>11.3 ± 3.0</td>
<td>13.5 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Reduction %</td>
<td>30.1</td>
<td>30.1</td>
<td>30.1</td>
</tr>
<tr>
<td></td>
<td>Diameter</td>
<td>306.5 ± 67.6</td>
<td>213.6 ± 19.9</td>
<td>217.5 ± 35.2</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Reduction %</td>
<td>50.2</td>
<td>48.6</td>
<td>48.6</td>
</tr>
</tbody>
</table>

Experiment 2

<table>
<thead>
<tr>
<th>Granuloma parameters</th>
<th>Infected controls</th>
<th>SmCB1</th>
<th>SmCL3</th>
<th>SmCB1 + SmCL3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>22.5 ± 4.3</td>
<td>12.2 ± 2.0</td>
<td>16.4 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.0021</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Reduction %</td>
<td>45.7</td>
<td>45.7</td>
<td>45.7</td>
</tr>
<tr>
<td></td>
<td>Diameter</td>
<td>395.0 ± 45.6</td>
<td>196.7 ± 46.3</td>
<td>199.7 ± 51.7</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.0029</td>
<td>0.0059</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Reduction %</td>
<td>50.2</td>
<td>49.4</td>
<td>49.4</td>
</tr>
</tbody>
</table>

\(^a\) Hamsters (5 per group) were vaccinated with SmCB1 or SmCL3 alone or in combination, challenged 8 weeks after second immunization with 100 cercariae of S. haematobium, and assessed for number and diameter of granulomas in liver. Granuloma counts and diameters were recorded for each hamster, based on a minimum of three sections. The mean of values for the hamsters for every group was then calculated and differences from unimmunized infected hamsters (infected controls) statistically analyzed by Student’s \(^t\)-test (two-tailed \(^t\) value). Reduction % = mean number in unimmunized hamsters – mean number in cysteine peptidase-immunized hamsters/mean number in unimmunized hamsters \(\times 100\).
disruption leading to release of alarmins [21] that bind to specific receptors on innate lymphoid cells type 2 (ILC2). Activation of ILC2 leads to release of type 2 cytokines, namely IL-5 and IL-13 [22,23]. Additionally, cytokine peptides may interact with protease-activated receptors (PAR) on naïve T cells, driving their differentiation towards the type 2 axis [24]. Our observed skewing of the immune responses towards the type 2 immunity axis is in accordance with our previous results obtained in the murine S. mansoni [7,8,10,11] and S. haematobium [9] models. However, in this hamster model of S. haematobium infection vaccine-induced immune responses exhibited a greater impact on the host protective responses to the parasite eggs and eggs-derived antigens which resulted in significantly (P < 0.02) smaller granuloma diameters compared to unimmunized S. haematobium–infected hamsters.

The role of immunogen-specific antibodies in protection is still unclear. For example, despite the high level of protection achieved by immunization with SmCL3 there was no detectable levels of specific antibodies at 10 days post challenge even a serum dilutions of 1:50 (although we cannot rule out the role of class-specific antibodies at 10 days post challenge even a serum dilu-

<ref>References:</ref>


