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Original article

Role of T lymphocytes and papain enzymatic activity in the protection induced by the cysteine protease against *Schistosoma mansoni* in mice

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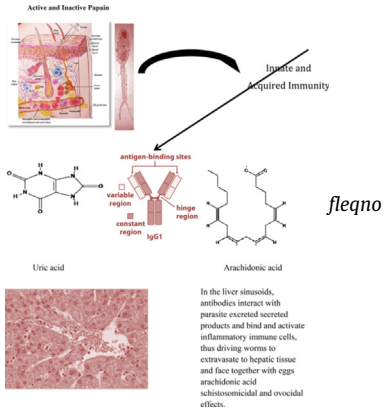
HIGHLIGHTS

- Papain use deciphered the protection mechanism(s) of the schistosomiasis vaccine.
- Papain stimulation of innate immunity induced parasite egg attrition.
- Papain enzymatic and non-enzymatic sites activated T cells and innate immunity.
- IgG1 antibodies and liver uric acid and ARA levels correlated with protection.
- Identification of type 2 immunity-inducing cysteine peptidases motifs is required.

GRAPHICAL ABSTRACT



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ABSTRACT

Papain, an experimental model protease, was used to decipher the protective mechanism(s) of the cysteine peptidase-based schistosomiasis vaccine. To examine the role of T lymphocytes, athymic nude (nu/nu) and immunocompetent haired (nu/+) mice were subcutaneously (sc) injected with 50 μg active papain two days before percutaneous exposure to 100 cercariae of *Schistosoma mansoni*. Highly significant ($P < 0.005$) reductions in worm burden required competent T lymphocytes, while significant increases ($P < 0.05$) of >80% in dead parasite ova in the small intestine were independent of T cell activity and likely relied on the innate immune axis. To investigate the role of enzymatic activity, immunocompetent mice were sc injected with 50 μg active or E-64-inactivated papain two days before exposure to cercariae. The reductions in worm burden were highly significant ($P < 0.0001$), reaching >65% and 40% in active and inactivated papain-treated mice, respectively. Similar highly significant ($P < 0.0001$) decreases of 85% in the viability of parasite ova in the small intestine occurred in both active and inactivated papain-treated mice. These findings indicated that immune responses elicited by one or more papain structural motifs are necessary and sufficient for induction of considerable parasite and egg attrition. Correlates of protection included IgG1-dominated antibody responses and increases in the levels of uric acid and arachidonic acid in the lung and liver upon parasite migration in these sites. Identification of the shared patterns or motifs in cysteine peptidases and evaluation of their immune protective potential will pave the way to the development of a safe, efficacious, storage-stable, and cost-effective schistosomiasis vaccine.

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Introduction

Schistosomiasis is a parasitic disease endemic in 74 developing countries; approximately 500 million people, mostly children, are infected, and 800 million are at risk of infection [1–4]. Praziquantel is the only commercially available drug recommended for mass treatment campaigns because of its low cost and limited side effects. However, cure is often incomplete, reinfection is not prevented, and treatment must be repeated, increasing the threat of inducing parasite resistance to the drug [1–4]. Of note, transmission in countries near river estuaries, such as Egypt, can never be interrupted, regardless of the approach applied, until residents in countries along the river source and bed are all infection-free and until countries near the estuary stop receiving infected snails (http://www.cornwallriversproject.org.uk/education/ed_cd/background/river_system.htm). Therefore, to prevent parasite transmission and infection of children in all developing countries, development of a safe and validated vaccine is critically needed.

It has been discovered that a safe and efficacious schistosomiasis vaccine can be readily formulated with larval or developing worm excretory-secretory products (ESP), e.g., *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (SG3PDH), 2-Cys peroxiredoxin (TPX), calpain etc. [5,6], provided that the ESP is combined with type 2 immunity cytokines such as thymic stromal lymphopoietin (TSLP), interleukin (IL)-25 or IL-33 or a type 2 immunity-inducing molecule such as papain [7,8]. Indeed, ESP are able to both induce vigorous immune responses and interact with immune effector antibodies and cells. A predominant type 2 immune environment is required for the development, recruitment and activation of eosinophils and basophils. These innate immune cells are the sources of the most potent toxic radicals and inflammatory mediators, targeting the parasite as well as affecting host blood capillary endothelium integrity, especially in the lung and liver. In support of this proposed mechanism, lung- and liver-stage schistosomula have been reported to be the most susceptible stages to immune attack *in vivo* [7,9–12].

Evidence has been provided for the hypothesis stating that the vaccine formula for an effective schistosomiasis vaccine should use larval ESP in the context of a polarized type 2, not type 1, cytokine environment. Immunizing outbred, akin to man, mice with recombinant SG3PDH (rSG3PDH) and TPX-derived peptides in a multiple antigen peptide (MAP) construct in combination with the type 2-inducing papain or the type 2 cytokine TSLP, IL-25, or IL-33 reproducibly and consistently elicits highly significant ($P < 0.0001$) 60–75% reductions in challenge worm burden and worm egg counts in the liver and small intestine [7]. The hypothesis was fully confirmed as outbred mice immunized with helminth cysteine peptidases, which are schistosome molecules that are both ESP and type 2 immune responses-inducing, consistently and reproducibly demonstrated highly significant ($P < 0.0001$) reductions (60–83%) in challenge *S. mansoni* and *S. haematobium* worm burden and worm egg load in the liver and small intestine compared to unimmunized mice and hamsters [13–17]. Moreover, the cysteine peptidase papain, used alone for two vaccinations or as a single injection before the challenge of CD-1 mice and hamsters with *S. mansoni* and *S. haematobium*, respectively, induced highly significant ($P < 0.005$) reductions in worm burden and egg load [7,15,18].

It is imperative to examine the basis and mechanism(s) of the anti-schistosomiasis protective effect of cysteine peptidases, particularly the role of thymus (T)-derived lymphocytes and protease enzymatic activity. The experimental model protease papain was used to decipher the effects of cysteine peptidases on parasitological parameters; levels of serum antibody responses; uric acid, a main product of cysteine peptidase catabolic activity [19,20]; and the endoschistosomicide, arachidonic acid (ARA) [21–23]. The data together revealed differential effects of the innate and T-dependent immune axis and papain enzymatic activity and structural motifs on *S. mansoni* worm burden, parasite egg viability, humoral antibody responses, and lung and liver uric acid and ARA levels.

Material 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.

Mice and parasites

Three athymic homozygous male nude (*Foxn1nu*$^{-}$/*Foxn1nu*$^{-}$, herein referred to as nude, nu/nu) and six heterozygous female (*Foxn1nu*$^{-}$/*Foxn1nu*$^{+}$, herein referred to as half-nude or nu/+) mice (Swiss Nu/Nu, Charles River Laboratories, Paris, France) were obtained through the courtesy of Professor Dr. Mohamed Ghoneim, Urology and Nephrology Center, Mansoura, Egypt and were housed (three per cage) in sterilized polycarbonate cages on a 12 h light/dark cycle under aseptic conditions. Food and sterile water were given *ad libitum*. Approval for housing and breeding was obtained from the Mansoura Medical Research Ethics Committee of the University of Mansoura. Notably, the mice are outbred, not inbred; in addition, the homozygous nude mice lack a thymus, are unable to produce T cells or to mount many types of adaptive immune responses, especially antibody formation, requiring $CD4^{+}$ helper T cells, and lack hair (nude). The heterozygous mice are immunocompetent and haired (albino).

Cercariae of an Egyptian strain of *S. mansoni* were obtained from the Schistosome Biological Materials Supply Program, Theodore Bilharz Research Institute (SBSP/TBRI), Giza, Egypt, and used for infection immediately after shedding from *Biomphalaria alexandrina* snails. Infection of the mice was performed via whole body exposure to viable cercariae as described previously [7,13,16].

Papain

Papain from *Carica papaya* (>3 units/mg) was obtained from Sigma-Aldrich, Merck (St. Louis, MO, USA). Papain (21 μM) was inactivated as described previously [24] by incubation for 30 min at room temperature with 200 μM of an irreversible inhibitor of cysteine peptidases, E-64 (L-trans-epoxysuccinylleucylamide-(4-guanidino)-butane; Sigma-Aldrich).

Parasitological parameters

Worm burden and total egg load in the liver and intestine of individual mice were evaluated using the following formula: % change = [mean number in untreated control mice − mean number in papain-treated mice/mean number in untreated control mice] × 100. The percentages of eggs at each developmental stage were evaluated using 5 fragments of the ileum and the large intestine as previously described [16,17]. Liver paraffin sections from each control and test mouse were stained with haematoxylin and eosin and examined for the number and diameter of granulomas surrounding eggs. Of note, data are presented as liver granuloma number and diameter (μm) mean ± SE of five fields per each of 2 sections for five mice per group [16,17].

Humoral antibody assays

Papain (AAB02650.1) shows 30% identity and 41% positives with *S. mansoni* cathepsin B1, SmCB1 [Accession: 4I04_A, GenInfo Identifier (GI): 582045207] with several notable stretches of shared amino acids. Accordingly, SmCB1 was used as a putative enzyme-linked immunosorbent assay (ELISA) target to analyse humoral immune responses in nu/nu mice at 40 days post infection (PI). At every test interval, serum from individual immunocompetent nu/+ mice untreated or pre-treated with active or inactivated papain before infection with *S. mansoni* was tested in duplicate by ELISA at 1:500 and 1:1000 dilutions for binding to 250 ng/well SmCB1, a gift from Professor John P. Dalton (Queen University at Belfast, North Ireland). Horseradish peroxidase-labelled anti-mouse IgG (H + L) conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was diluted 1:5000. At 17, 31, and 49 days after infection, serum samples from each mouse group were diluted 1:250 to estimate the level of IgM and IgG class antibodies and 1:25 to analyse the binding of IgE and IgA antibodies to SmCB1. The conjugate dilutions were 1:1000 for alkaline phosphatase (AKP)-labelled monoclonal antibody to IgM, IgG1, IgG2a and IgG2b (Pharmingen, San Diego, CA), 1:500 for biotin-labelled rat monoclonal antibody to IgA and IgE (BioLegend, San Diego, CA, USA), and 1:3000 for AKP-labelled streptavidin. The reaction was measured spectrophotometrically following incubation with p-nitrophenyl phosphate substrate (Calbiochem, San Diego, CA).

Role of T lymphocytes

The contribution of T cells was assessed in two independent experiments. In each experiment, female nu/nu and nu/+ mice were injected subcutaneously (sc) at the tail base region with 0 or 50 μg papain in 100 μL of Dulbecco's phosphate-buffered saline (D-PBS), pH 7.0. Two days later, all mice (10 mice per group) were percutaneously exposed to 100 cercariae of *S. mansoni*. Parasitological parameters and humoral responses were evaluated 40 days PI (Fig. 1A).

Role of papain enzymatic activity

The effect of cysteine peptidase activity was assessed in two independent experiments. For each experiment, of a total of eighty-five female nu/+ mice were used. Ten were left unimmunized and uninfected and were considered naïve animals. The remaining 75 mice were randomly distributed into three equal groups of 25 mice each; these mice were injected sc at the tail base region with 0 or 50 μg active or inactivated papain in 100 μL D-PBS. Two days later, the mice were percutaneously exposed to 200 (first experiment) or 100 (second experiment) cercariae of *S. mansoni*. Serum, lung and liver pieces were obtained from 4 mice per group at 7, 17, 24, 31, and 43 or 49 days PI for assessment of immune and biochemical correlates of protection. Parasitological parameters in five to ten mice were evaluated at 43 days (first experiment) or 49 days (second experiment) PI (Fig. 1B).

Serum uric acid and lipid assays

At each test interval, serum samples of individual mice were subjected in duplicate to fluorometric (Victor™ X4 Multilabel Plate Reader, PerkinElmer, Waltham, MA) determination of uric acid levels using an Amplex® Red Uric Acid/Uricase Assay Kit (Molecular Probes, Invitrogen Detection Technologies, Paisley, UK) and colorimetric (Multiskan EX, Labsystems, Helsinki, Finland) enzymatic determination of total cholesterol (Cholesterol-LQ kit, Chronolab Systems, S.L., Barcelona, Spain) and triglycerides (triglycerides kit, Chronolab) following the manufacturer's instructions. Levels of circulating unbound (free) ARA were evaluated by competitive ELISA using an AA (Arachidonic Acid) ELISA Kit (E-EL-0051, Elabscience Biotechnology Co., Ltd, Wuhan, China) as per the manufacturer's protocol. The absorbance readings of the ARA standard dilutions were plotted against the concentration values using an Excel scatter plot and formula, and the serum sample concentrations are expressed as ng/mL.

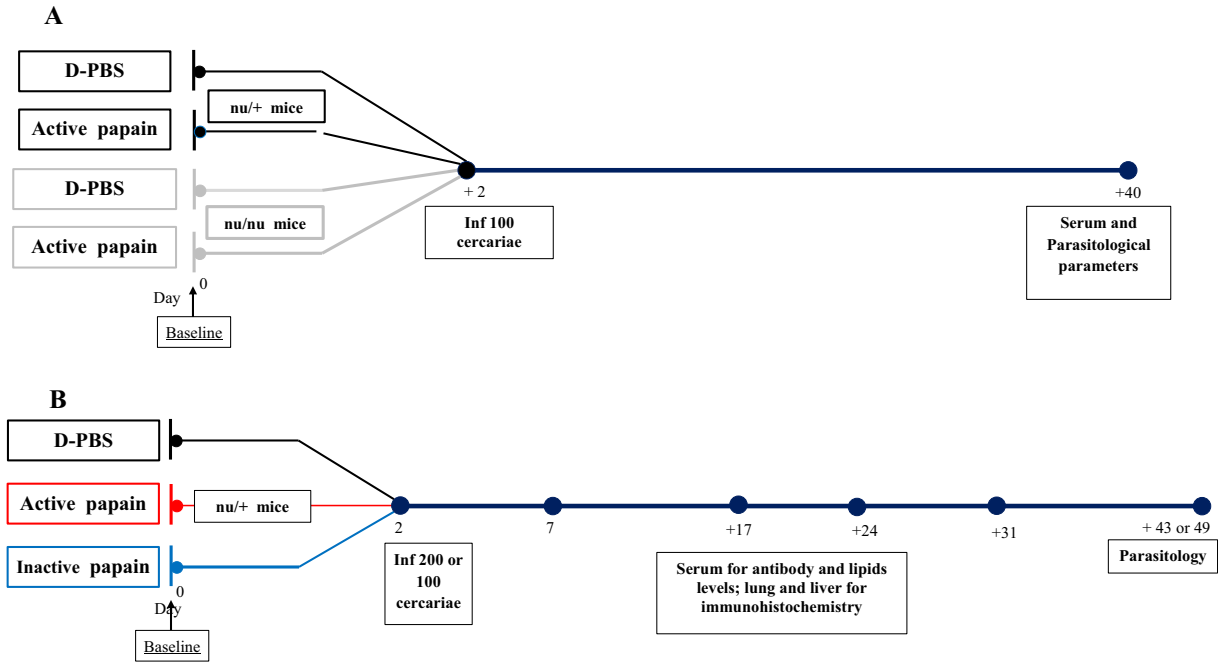

Fig. 1. Diagrammatical representation of the experimental design. (A) Comparison of the effects of papain on *S. mansoni* infection in immunocompetent (nu/+) versus athymic (nu/nu) mice. (B) Evaluation of the effects of active and inactive papain on *S. mansoni* infection in immunocompetent (nu/+) mice. Each diagram represents two separate experiments.

Table 1
Effect of pretreatment with active papain on parasitological parameters of *S. mansoni* infection in nu/+ and nu/nu mice.[*]

Parameter counts	nu/+ controls	nu/+ papain	nu/nu controls	nu/nu papain
Total worm burden				
Mean ± SD	20.8 ± 3.7	7.8 ± 2.7	12.2 ± 5.2	8.6 ± 4.4
P value		0.0001		NS
Reduction (%)[a]		62.5		
Male worm burden				
Mean ± SD	10.2 ± 2.2	4.0 ± 1.8	6.0 ± 2.1	4.8 ± 2.6
P value		0.0011		NS
Reduction (%)		60.7		
Female worm burden				
Mean ± SD	10.7 ± 2.3	3.8 ± 1.2	6.2 ± 3.5	3.8 ± 1.9
P value		0.0001		NS
Reduction (%)		64.4		
Liver egg counts				
Mean ± SD	8585 ± 4367	4071 ± 1884	3202 ± 1788	2002 ± 1002
P value		0.0217		NS
Reduction (%)		52.5		
Intestine egg counts				
Mean ± SD	3613 ± 2014	1574 ± 764	2120 ± 1164	922 ± 93
P value		0.0168		NS
Reduction (%)		56.4		
% Immature ova[b]				
Mean ± SD	46.5 ± 20.0	46.1 ± 17.3	74.0 ± 13.3	44.5 ± 23.5
P value		NS		0.038
Reduction (%)				41.3
% Mature ova				
Mean ± SD	45.3 ± 20.1	19.8 ± 11.5	16.4 ± 9.4	8.2 ± 4.6
P value		0.036		NS
Reduction (%)		56.3		
% Dead ova				
Mean ± SD	6.7 ± 1.9	33.9 ± 21.9	9.5 ± 6.3	49.2 ± 26.7
P value		0.003		0.027
Increase (%)		80.2		80.6
Granuloma number[c]				
Mean ± SD	15.3 ± 1.1	2.5 ± 0.7	5.0 ± 2.3	1.3 ± 0.5
P value		0.0008		0.0432
Reduction (%)		83.6		73.4
Granuloma diameter				
Mean ± SD	362.7 ± 53.3	111.5 ± 68.1	146.0 ± 66.1	160.7 ± 83.5
P value		0.0001		NS
Reduction (%)		69.2		

[*] The data are typical of two independent experiments. Papain-injected immunocompetent (nu/+) and athymic (nu/nu) mice were exposed two days later with 100 cercariae of *S. mansoni* in parallel with untreated mice (controls), and assessed (5–10 per group) for parasitological parameters 40 days post infection. Differences between papain-treated and control mice were assessed for significance using Mann-Whitney test.

[a] Reduction % = mean number in untreated control mice – mean number in active papain- pretreated mice/ mean number in untreated control mice × 100.

[b] Ova developmental stages in small intestine. NS = not significant, as assessed by the Mann-Whitney test (two-tailed *P* value), comparing controls and papain- treated mice.

[c] Liver granuloma number and diameter (μm) are mean ± SE of five fields per each of 2 sections for five mice per group.

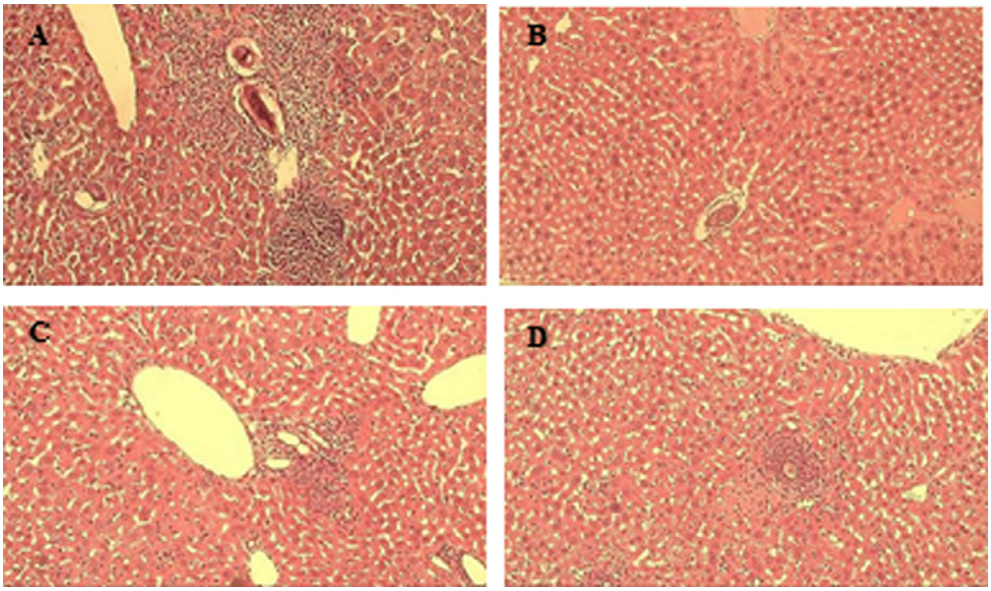

Fig. 2. Haematoxylin-eosin-stained paraffin liver sections 40 days after *S. mansoni* infection. Immunocompetent (A, B) and athymic (C, D) mice were treated with 0 (A, C) or 50 (B, D) μg papain two days before percutaneous exposure to 100 cercariae. Each figure is representative of 5 mice per group. Magnification: 200x.

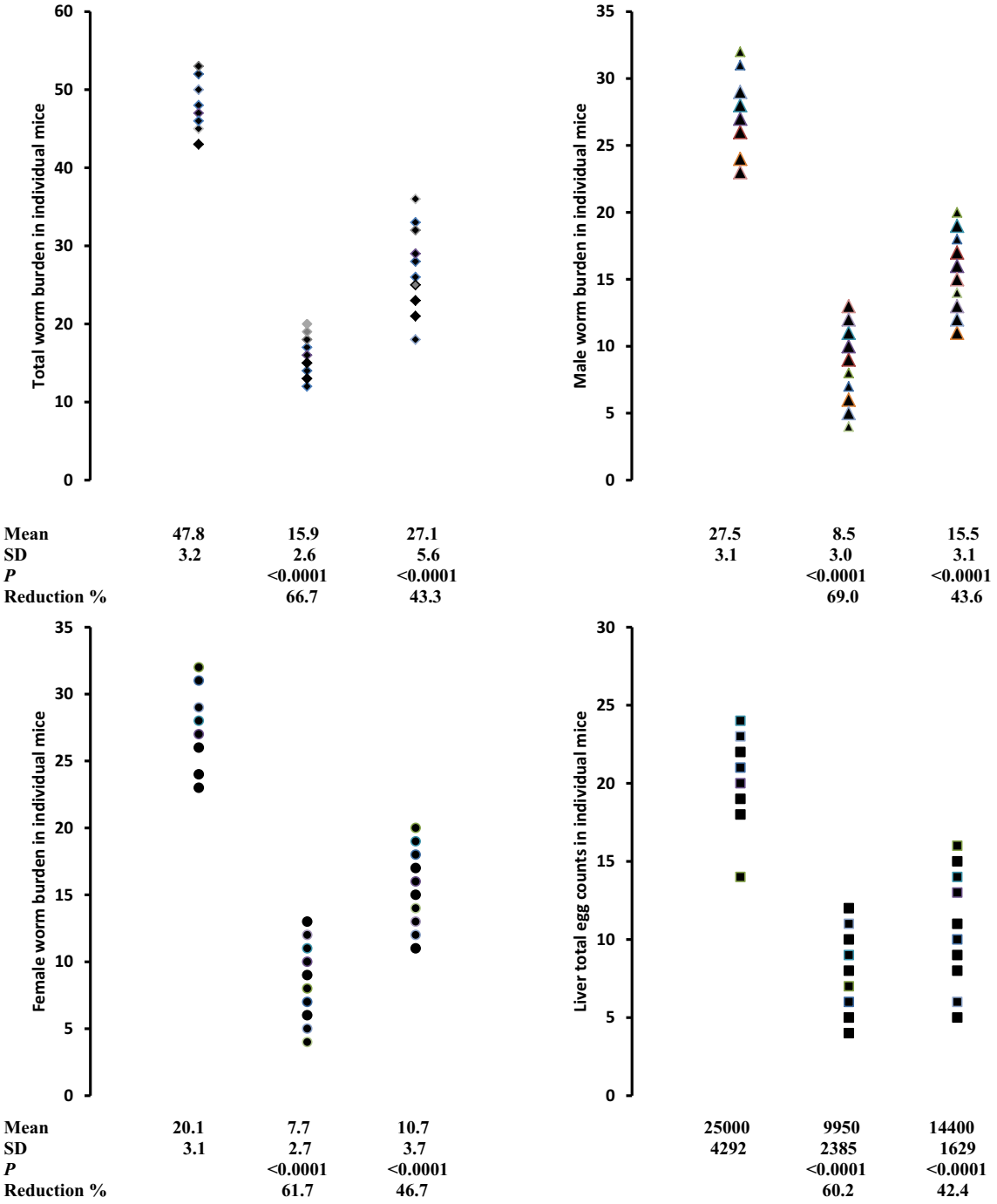

Fig. 3. Effect of pretreatment with active or inactivated papain on parasitological parameters in immunocompetent nu/+ mice. Mice (8 to 10 per group) were pretreated with 0 (controls, left column in every panel) or 50 μg active (papain-pretreated, middle column in every panel) or E-64-inactivated (inactivated papain-pretreated, right column in every panel) papain before infection with 200 cercariae of *S. mansoni*. Parasitological parameters in individual mice were assessed 43 days after infection. Reduction % = mean number in untreated control mice – mean number in papain- pretreated mice/mean number in untreated control mice × 100. Significance of differences between the 3 groups was assessed by ANOVA and found to be highly significant ($P < 0.0001$) for all parameters tested. Significance of differences between papain-treated and control mice, assessed using One-Way Anova with post test and Mann-Whitney test, are shown for every parameter (P). Reduction in worm burden and liver egg counts in mice pretreated with inactivated papain was significantly ($P < 0.005$) lower than in mice pretreated with active papain.

Immunohistochemistry

Immunohistochemical analyses of uric acid and ARA content in the lung and liver of naïve and test mice were performed using 5 μm paraffin sections. Briefly, lung and liver sections were incubated with D-PBS supplemented with 1% bovine serum albumin (Sigma-Aldrich) to block non-specific sites for 30 min and were then incubated overnight at 10 °C with a 1:250 dilution rabbit polyclonal antibody to uric acid (ab53000, Abcam, Cambridge, MA, USA) or 100 μL blocking solution containing 0 (negative controls) or 1 μg of a rabbit polyclonal antibody to ARA (MBS2003715, MyBioSource, San Diego, CA). The sections were washed with D-PBS and incubated with 0.5 μg of AKP-labelled goat anti-rabbit immunoglobulin [Goat F(ab')2 Anti-Rabbit IgG - H&L

Table 2
Effect of pretreatment with active or inactivated papain on parasitological parameters of *S. mansoni* infection in immunocompetent nu/+ mice.[*]

Parameter counts	Pretreatment		
	Controls	Active papain	Inactivated papain
Total worm burden			
Mean ± SD	25.8 ± 3.9	8.2 ± 2.7	12.4 ± 1.6
P value		< 0.0001	< 0.0001
Reduction (%)[a]		68.2	51.9
Male worm burden			
Mean ± SD	13.0 ± 3.2	4.2 ± 1.3	6.2 ± 1.3
P value		< 0.0001	0.0017
Reduction (%)		67.7	52.3
Female worm burden			
Mean ± SD	12.8 ± 1.3	4.0 ± 1.7	6.2 ± 0.4
P value		< 0.0001	< 0.0001
Reduction (%)		68.7	51.5
Liver egg counts			
Mean ± SD	12714 ± 4060	7222 ± 2526	5800 ± 2280
P value		0.0050	0.0066
Reduction (%)		43.2	54.3
Intestine egg counts			
Mean ± SD	11428 ± 5480	7850 ± 5744	5600 ± 2264
P value		NS	NS
Reduction (%)			
% Immature ova[b]			
Mean ± SD	41.1 ± 11.3	24.4 ± 10.7	25.8 ± 12.3
P value		0.0071	0.0390
Reduction (%)		40.6	37.2
% Mature ova			
Mean ± SD	53.3 ± 10.5	30.4 ± 13.1	36. 0 ± 11.5
P value		0.001	NS
Reduction (%)		42.9	
% Dead ova			
Mean ± SD	5.5 ± 2.5	45.2 ± 15.3	38.1 ± 14.5
P value		< 0.0001	0.0001
Increase (%)		87.8	85.5
Granuloma number[c,d]			
Mean ± SD	26.6 ± 2.3	9.8 ± 3.8	8.1 ± 1.0
P value		0.002	0.0002
Reduction (%)		63.1	69.5
Granuloma diameter			
Mean ± SD	391.9 ± 84.3	285.8 ± 68.1	254.6 ± 59.1
P value		0.0008	0.0002
Reduction (%)		27.0	35.0

[*] Competent nu/+ mice were injected with 0 (Controls) or 50 μg active or inactivated papain in100 μL D-PBS, percutaneously exposed two days later to 100 cercariae of *S. mansoni*, and assessed (six per group) for parasitological parameters 49 days post infection.

[a] Reduction % = mean number in untreated control mice – mean number in papain- pretreated mice/ mean number in untreated control mice × 100.

[b] Ova developmental stages in small intestine. NS = not significant, as assessed by the Mann-Whitney test (two-tailed *P* value).

[c] Liver granuloma number and diameter (μm) were evaluated in five fields per each of two sections for five mice per group.

[d] Significance of differences between the 3 groups was assessed by ANOVA and found to be highly significant ($P < 0.0001$) for all parameters tested. Significance of differences between papain-treated and control mice, assessed using One-Way Anova with post test and Mann-Whitney test, are shown for every parameter (*P* value). Reduction of total, male, and female worm burden values in mice treated with inactivated papain was significantly ($P < 0.05$) lower than in mice pretreated with active papain. The effects of active and inactivated papain on egg counts and developmental stages were not significantly different.

(AP), preadsorbed, ab98505, Abcam] in 100 μL D-PBS/1% bovine serum albumin for 1 h at room temperature. After thorough washing in 10 mM Tris-HCl, pH 8.0, the reaction was visualized with HistoMark RED Phosphatase Substrate Kit from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA). HistoMark RED reagents form a brilliant scarlet reaction product that is stable in organic solvents. Photographs were acquired by light microscopy (Olympus, Tokyo, Japan).

Statistical analysis

One Way Analysis of Variance (ANOVA), Student's 2-tailed *t*-, and/or Mann-Whitney tests were used to analyse the statistical significance of differences between selected values, and differences were considered significant at $P < 0.05$.

Results

Role of T lymphocytes

In two independent experiments, papain pretreatment of immunocompetent nu/+ mice elicited highly significant ($P < 0.005$) reductions of more than 60% in total, male, and female worm burden and an approximately 50% decrease ($P < 0.05$) in parasite egg counts in the liver and small intestine. In athymic nu/nu mice, papain pretreatment did not elicit significant differences in worm burden and parasite egg counts in the liver and small intestine in comparison to untreated mice (Table 1). However, papain-pretreated nu/+ or nu/nu mice similarly displayed significant ($P < 0.05$) changes in egg development in the small intestine, notably an increase of >80% in the percentage of dead ova and decreases in the number of granulomas (Table 1; Fig. 2). Together, the data suggest that an intact thymus and competent T lymphocytes are required for papain to induce significant reductions in worm and parasite egg burden but are NOT required for papain-mediated attrition of the majority of parasite eggs in the small intestine.

Notably, nu/nu mice, whether untreated or pretreated with papain before infection, were able to produce only low levels of IgM antibodies to the cysteine peptidase SmCB1 at 40 days PI (data not shown).

Role of papain enzymatic activity

Worm burden parameters

Two consecutive experiments (Fig. 3, Table 2) demonstrated that in immunocompetent nu/+ mice, papain pretreatment elicited highly significant ($P < 0.0001$) reductions of more than 60% (approximately 68%) in total, male, and female worm burdens. Immunocompetent mice pretreated with inactivated papain also exhibited highly significant ($P < 0.005$) decreases of approximately 50% in total, male, and female worm burdens; however, the reduction in worm burden was significantly ($P < 0.005$, Fig. 3; and $P < 0.05$, Table 2) lower than that in mice pretreated with active papain. The data suggest that inactivated cysteine peptidase may be associated with protection against *S. mansoni* infectivity, while enzymatic activity is required to potentiate the protective effect, especially in a heavy-infection model.

Parasite egg parameters

The effects of active and inactivated papain on egg counts in the liver and small intestine and on egg developmental stages in the small intestine were not significantly different, as mice receiving either treatment exhibited highly significant ($P < 0.005$) increases in dead ova in the intestine and decreases in the number and diameter of granulomas in the liver compared to untreated controls (Table 2, Fig. 4A–C). The data indicated that enzymatic activity was not required for papain to have an impact on parasite egg numbers and development, especially regarding the attrition of eggs in the small intestine and changes in the number and diameter of granulomas in the liver (Table 2, Fig. 4A–C).

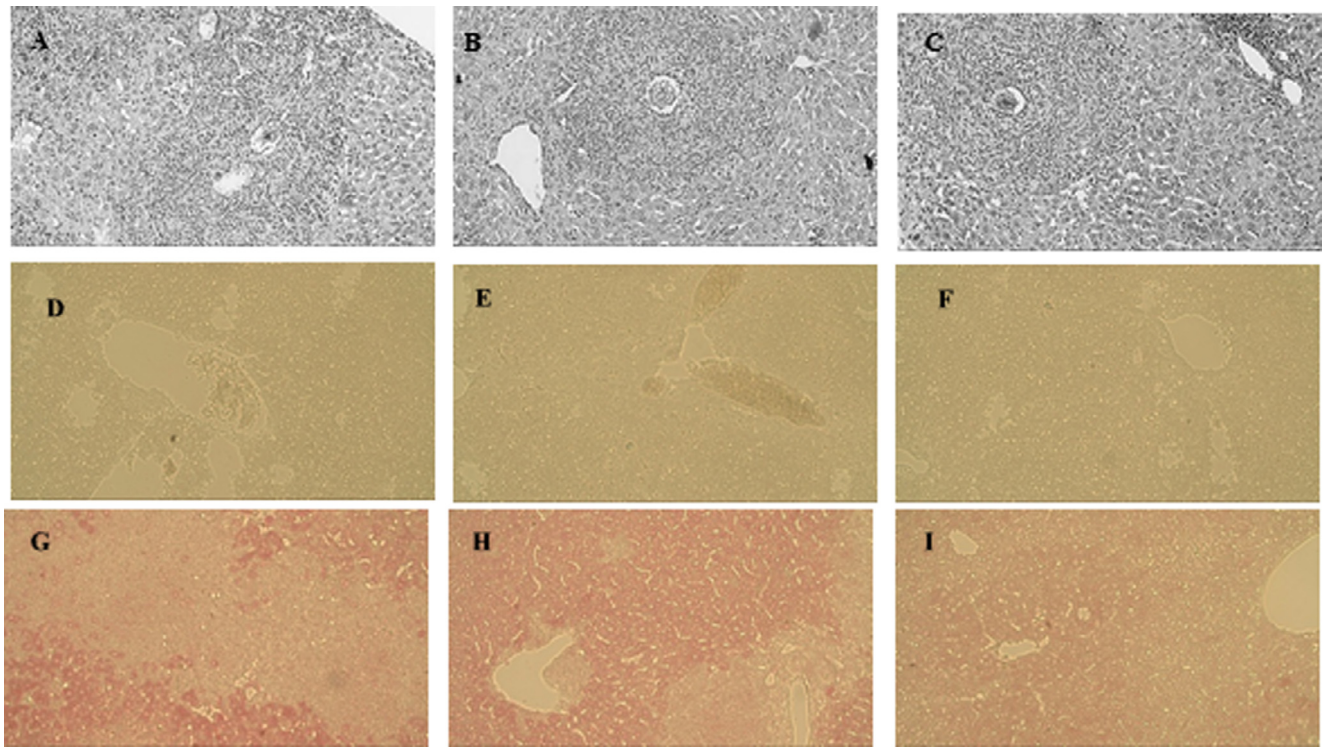

Fig. 4. Paraffin liver sections 49 days after *S. mansoni* infection. Immunocompetent mice were treated with 0 (left vertical panel, A, D, G) or 50 μg active (middle vertical panel, B, E, H) or E-64-inactivated (right vertical panel, C, F, I) papain two days before percutaneous exposure to 100 cercariae. Each image showing staining with haematoxylin-eosin (A–C), irrelevant rabbit IgG (D–F), or anti-uric acid (G–I) antibody is representative of 5 mice per group. Magnification: 200x.

Humoral immune responses

The serum total antibodies binding to SmCB1 gradually increased with infection time in untreated nu/+ mice and were more readily detectable at 7, 17, and 24 days PI than in active and inactivated papain-treated mice (Fig. 5A). At later intervals, the antibodies were a mixture of IgM, IgG1, IgG2a, IgG2b, and IgA antibodies. Conversely, SmCB1-specific antibodies in the serum of papain-pretreated mice appeared to be IgG1 polarized, a notable correlate of protection (Fig. 5B–F). In inactivated papain-pretreated mice, SmCB1-binding antibodies of the IgG1 isotype were predominant, but IgG2a antibodies were also readily detectable, together with low amounts of IgG2b, and IgA antibodies (Fig. 5B–F).

Serum and organ uric acid

Upon schistosome infection, serum uric acid and lipid levels in control and papain-pretreated mice decreased in comparison to naïve mice, interval 0 (Fig. 6A–D). Serum uric acid levels in control and active papain-pretreated mice were similar at every interval PI and were elevated compared to those of inactivated papain-pretreated mice from day 24 until the end of the experiment. Serum uric acid levels in inactivated papain-pretreated mice were significantly ($P < 0.005$) lower than those of naïve and infection-control mice on day 31 and at perfusion (Fig. 6A).

Immunohistochemical procedures were validated using liver sections obtained from mice 49 days PI (with 100 cercariae) and an anti-uric acid antibody. The staining of liver cells appeared entirely negative following treatment with a control rabbit antibody (Fig. 4D–F). Liver sections obtained from untreated and papain-treated mice incubated with the anti-uric acid antibody revealed differential uric acid distribution; uric acid was absent in the circumoval granulomas but readily detectable in liver cells elsewhere (Fig. 4G–I).

Repeated immunohistochemistry assays using lung sections of naïve mice and mice 7 days PI and an anti-uric acid antibody revealed an absence of uric acid in the lungs of naïve and untreated infected mice and moderate reactivity in the lungs of papain-pretreated mice (Fig. 7A–D). Among liver sections obtained from naïve mice and mice 17 days PI, uric acid was detectable only in sections from papain-pretreated mice (Fig. 8A). At 24 days PI, uric acid levels were considerably increased in the livers of papain-pretreated mice compared to naïve and infection control mice (Fig. 8B). At the time of perfusion, uric acid appeared to accumulate in the livers of untreated and active papain-pretreated mice, while uric acid was weakly distributed in liver sections from mice pretreated with inactivated papain, consistent with the serum levels at that time interval (Fig. 8C).

Serum cholesterol and triglycerides

The levels of serum cholesterol and total triglycerides were essentially similar in untreated and papain-pretreated *S. mansoni*-infected mice (Fig. 6C and D).

Serum and organ arachidonic acid

Post-infection changes and fluctuations in free ARA serum levels followed a pattern similar to that of serum uric acid until day 24. The levels of free, unesterified serum ARA in active papain-pretreated mice were significantly ($P < 0.05$) higher than those in infection-control mice from day 24 until the end of the experiment. Serum ARA levels in inactivated papain-pretreated mice were significantly ($P < 0.05$) higher on days 24 and 31 and lower at perfusion than those in infection-control mice (Fig. 6B).

Repeated immunohistochemistry assays using lung sections of naïve mice and mice 7 days PI and an anti-ARA antibody revealed an absence of ARA in the lungs of naïve and untreated infected

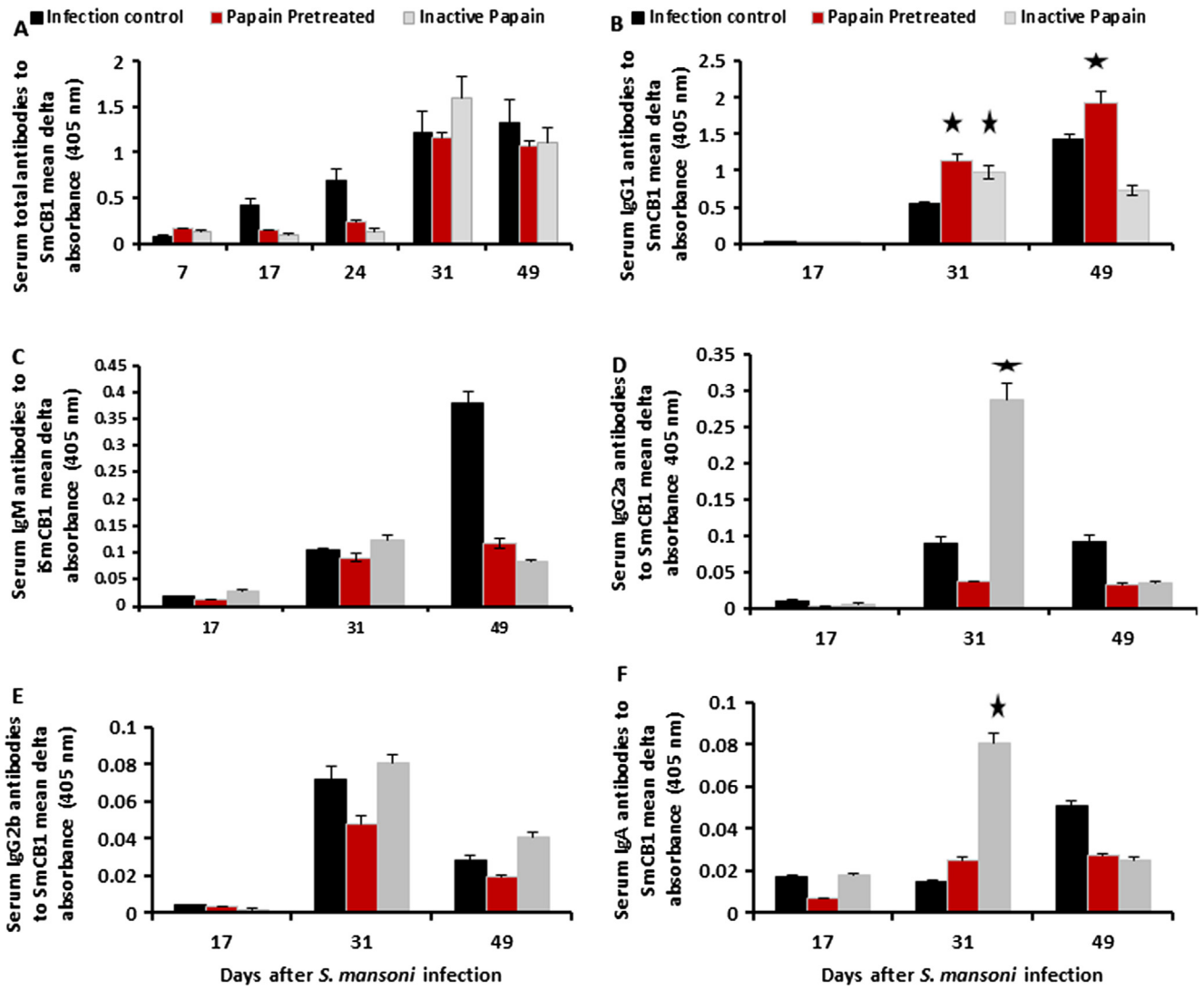

Fig. 5. The serum antibody isotype response to SmCB1 is representative of two experiments. Serum samples from three to five untreated (Infection Control), and active (Papain Pretreated) and inactivated (Inactive Papain) papain-pretreated mice were tested PI for binding to SmCB1 in replicate ELISAs. The serum samples were diluted 1:500, 1:250 and 1:25 to detect total, IgG class, and IgA antibodies, respectively. The columns represent the mean delta absorbance (with the mean values of serum samples from 3 naïve mice subtracted), and the vertical bars denote the SD about the mean. Significant differences (Student's *t*-test) from control values at $P < 0.05$ are indicated by an asterisk (*). IgE antibodies were not detected in serum samples diluted 1:25.

mice and moderate reactivity in the lungs of papain-pretreated mice (Fig. 7E–H). Among liver sections obtained from naïve mice and mice 17 days PI, ARA was readily detectable only in those from inactivated papain-pretreated mice (Fig. 8D). At 24 days PI, ARA levels were considerably higher in the livers of active papain-pretreated mice than in the livers of naïve and infection-control mice (Fig. 8E). At the time of perfusion, ARA appeared to have accumulated in the livers of untreated and papain-pretreated mice, especially in circumoval granulomas (Fig. 8F).

Discussion

Nude mice cannot generate mature T lymphocytes and are unable to mount many types of adaptive immune responses because they lack a thymus. However, the parasitological parameters of *S. mansoni* in athymic nu/nu and comparably infected age- and sex-matched immunologically intact mice did not significantly differ, as has been previously observed [25]. Studies in mice lacking T and B cells (Scid, severe combined immunodeficient) or lacking T cells only (nude) have indicated that the absence of B cells profoundly hampers the development and pairing of *S. japonicum* [26]. Additionally, several reports have revealed that *S. mansoni* worms, especially females, co-opt innate immune signals to facilitate their development in the absence of CD4+ T cells [27–29]. This study on nu/nu mice in parallel with nu/+ immunocompetent mice revealed that papain administration before schistosome infection differentially impacts the immune responses to worms and ova. Papain pretreatment led to a highly significant ($P < 0.005$) reduction of >60% in *S. mansoni* worm burden in immunocompetent mice only, indicating the involvement of T-dependent immune responses. This finding is in accord with the fact that the action of papain has been shown to predominantly stimulate type 2 helper T cell responses via interaction with basophils [30] or B lymphocytes [31] or through basophil-dendritic cell cooperation [32]. However, a significant ($P < 0.05$) attrition of parasite ova of >80% in the small intestine appeared to rely on papain-induced T cell-independent immune responses. Papain has been shown to directly activate basophils in an FcεR1-independent manner to produce IL-2, IL-4, IL-5, and IL-13 [30], to access B cells independently of the B cell receptor (BCR) [31], and, when sc

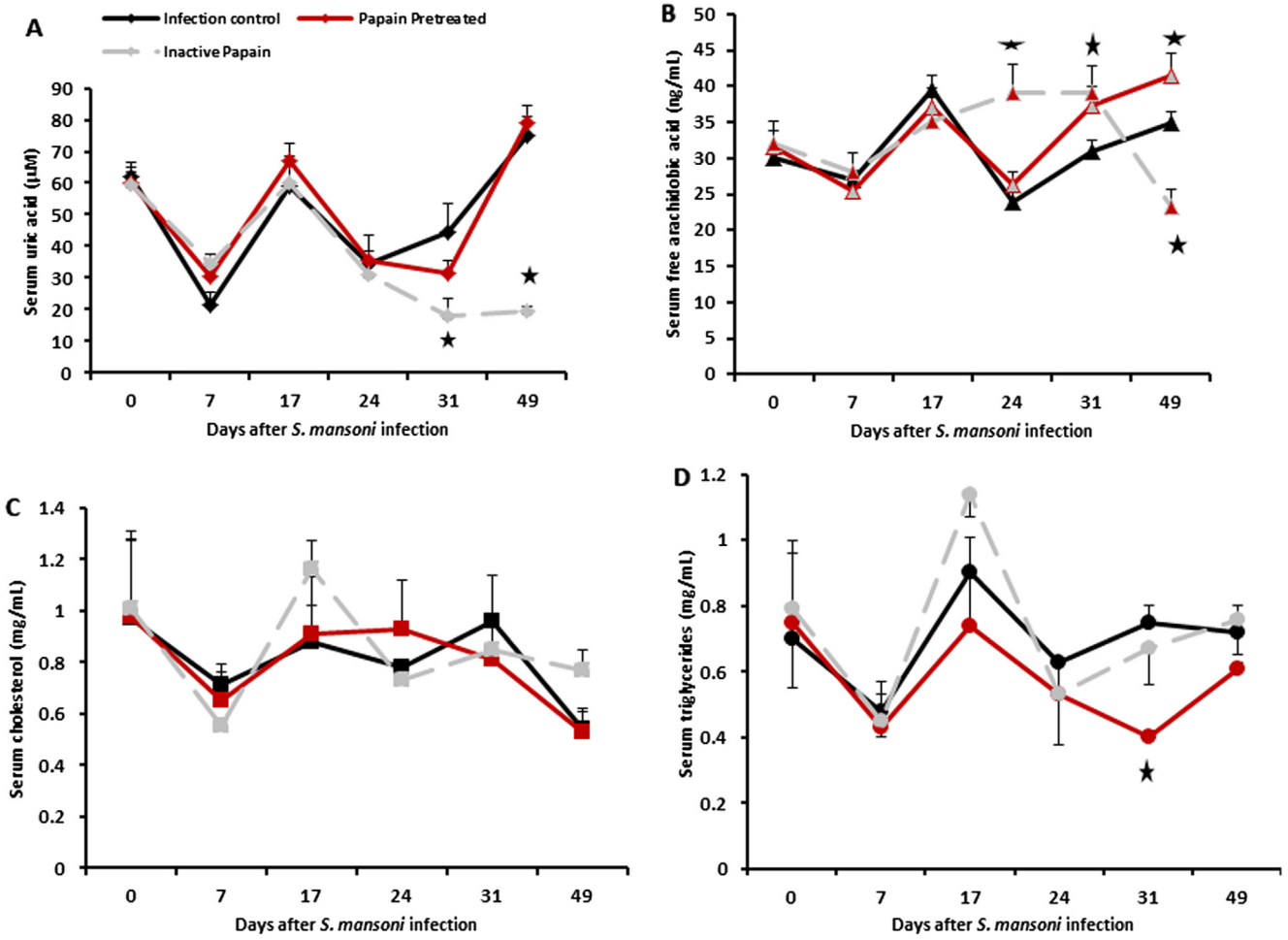

Fig. 6. Each point represents the mean serum uric acid or lipid levels for five individual mice per group ± SD. Interval 0 represents the values recorded in naïve mice. Significant differences (One Way ANOVA and Student's *t*-test) at $P < 0.05$ are indicated by an asterisk (*).

injected, to elicit the production of reactive oxygen species (ROS), which lead to lipid oxidation and barrier disruption in epithelial cells with subsequent production of TSLP, IL-25, and IL-33 [19,32]. These alarmins stimulate innate lymphoid cell type 2 (ILC2) proliferation and the production of type 2 cytokines [33,34]. Papain has previously been found to induce asthma-like airway inflammation, especially eosinophil infiltration and mucus hyper production, in T and B cell-deficient (Rag -/-) mice, further indicating that papain operates via T cell-independent mechanism(s) [33,34]. Additionally, even low levels of SmCB1-specific IgM antibodies produced at patency may interact with the excreted-secreted enzyme, and activate complement cascade-dependent inflammatory reactions, leading to parasite egg attrition. Thus, independently of T cells and vigorous humoral antibody production, papain may skew innate immune responses to invading parasites towards an environment conducive to attrition of the parasite ova. It may thus be concluded that stimulation of T lymphocytes and innate immune cells could be major mechanisms cooperating in cysteine peptidase-mediated protection against schistosomes. Experiments in progress aim to investigate the identity of the innate immune cells cooperating with T cells in induction of protection, and evaluate the nature and amounts of cytokines produced.

Four independent experiments revealed that a single subcutaneous injection of active papain in immunocompetent mice before exposure to cercariae of *S. mansoni* generates highly significant (up to $P < 0.0001$) reduction in worm burden and impairment of egg load and viability, as indicated by significant ($P < 0.05$) decreases in liver granuloma number and diameter and highly significant ($P < 0.0001$) increase in the percentage of dead ova. These data support previous findings of papain-related protection against *S. mansoni* in CD-1 mice [7,18] and against *S. haematobium* in hamsters [15]. Exposure to enzymatically active cysteine peptidases consistently induces epithelial barrier disruption, with subsequent conversion of dying cell-derived purines into uric acid, a major amplifier of type 2 immune responses [19,35–40] and modulator of lipid metabolism, especially lipid metabolism involving ARA, which is needed for cell membrane synthesis and repair [20,22,41]. In support of this hypothesis, papain injection skewed innate and acquired immune responses to the antigens of invading *S. mansoni* towards the type 2 axis, preceding the effects of egg-derived soluble antigens, with preponderance of IgG1 antibodies and accumulation of uric acid and ARA in the lung 7 days PI and in the liver starting 17 days PI. Type 2-related antibodies to ESP, especially cysteine peptidases, and ARA, a documented schistosomicide [42–45] and a putative endoschistosomicide [21–23], appear to join forces to mediate the attrition of developing worms in the lung capillaries and liver sinusoids. Repeat experiments have demonstrated that ARA impairs the viability and hatchability of *S. mansoni* eggs *ex vivo* (El Ridi, personal communication) and may well be responsible for parasite ovum attrition in the liver and small intestine via accumulation in the liver, especially in papain-pretreated mice.

Papain inactivated with E-64 elicited considerable protection against *S. mansoni* infection. The reductions in total, male, and female worm burden were highly significant ($P < 0.0001$) in the

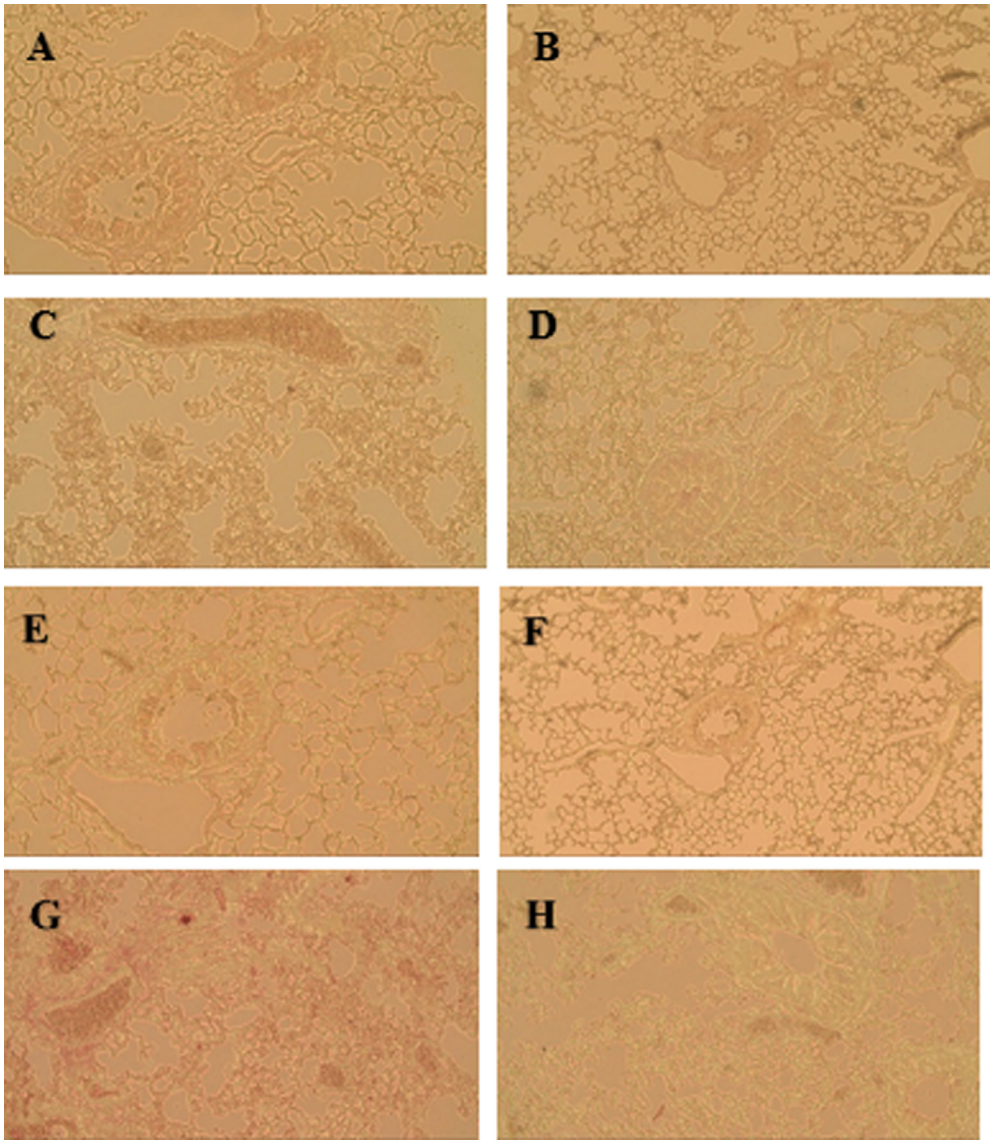

Fig. 7. Immunohistochemical assays of lung uric acid and ARA. Lung sections 7 days PI were reacted with an anti-uric acid (A–D) or anti-ARA (E–H) antibody in repeated immunohistochemical assays. Lung sections of naïve (A, E) and infected control (B, F) mice were negative. Sections of lung of active (C, G) and inactivated (D, H) papain-treated mice showed moderate reactivity. The images shown are representative of the consistently recorded reactivity for each mouse group at the specified interval. Magnification: 200x.

range of 45%, markedly lower than the >60% consistently obtained with active papain. Active SmCB1 uniformly elicited highly significant ($P < 0.005$ and up to <0.0001) reduction in challenge *S. mansoni* worm burden in repeat experiments, but the values decreased with time from approximately 60% to approximately 50%, likely due to decreased enzymatic activity upon extended storage at −70 °C [13,16,17]. On the other hand, the significant differences and the percentage reduction in challenge *S. mansoni* elicited in CD-1 mice after vaccination with active and E-64-inactivated SmCB1 or enzymatically active and inactive (catalytic site mutant) *Fasciola hepatica* cathepsin L1 (FhCL1) [13] were similar to those recorded after administration of enzymatically active and inactivated papain. These observations stress the need to elucidate the cysteine peptidase-induced protection mechanism(s) and the validity of papain as an experimental cysteine peptidase model and prototype.

The enzymatic activity of cysteine peptidases is required for cleavage of protease-activated receptors on basophils and epithelial cells [46–49], cell damage, epithelial barrier breakdown, and tissue injury with subsequent release of uric acid as well as for the induction of T-dependent and T-independent type 2 immune responses [24,30–40,46–49]. As is the case for model allergens, papain catalytic site activity is, however, not required for uptake by B cells [31], release of high levels of uric acid [40], or interaction with epithelial, stromal, or endothelial cell toll-like receptors (TLRs) with subsequent activation of innate and acquired type 2 immune responses to concomitantly injected antigens [39,40,50]. The induction of a T helper 2 cell-biased antibody response by papain via the skin has been found to be independent of its enzymatic activity [50]. Similarly, the production of type 2-related antibodies in *S. mansoni*-challenged mice was elevated following treatment with inactivated papain, and organ uric acid and ARA accumulation were evident; however, the antibody production was less polarized and the uric acid and ARA accumulation was lower at some intervals for inactivated papain-administered mice than for active papain-administered mice. This may explain the lower reduction in challenge worm burden recorded in inactivated papain-treated mice than in active papain-treated mice. However, the production of a mixture of IgG1 and IgG2a antibodies and mod-

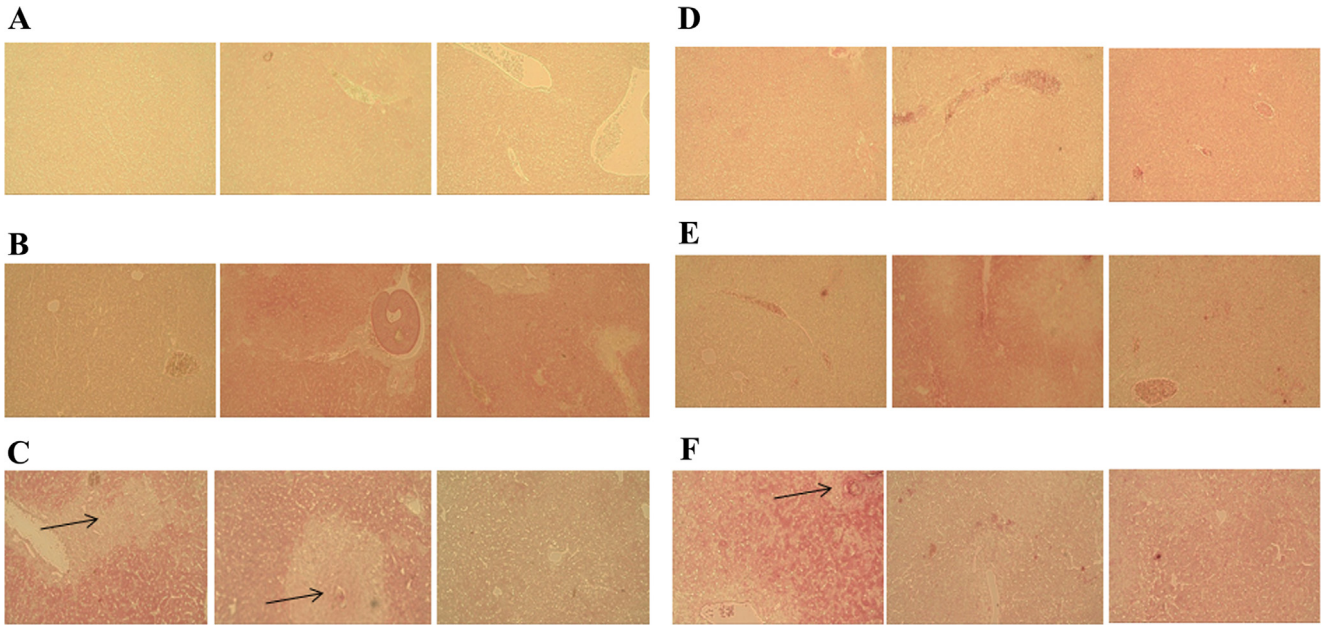

Fig. 8. Immunohistochemical assays of liver uric acid and ARA. Liver sections were reacted with an anti-uric acid (A–C) or anti-ARA (D–F) antibody in repeated immunohistochemical assays, 17 (A, D), 24 (B, E) days PI, and at perfusion (C, F) Sections of naïve mice were consistently negative (not shown), in contrast to sections from infected (left figure in each panel), active papain-treated (middle figure in each panel), and inactivated papain-treated (right figure in each panel) mice. The images shown are representative of the consistently recorded reactivity for each mouse group in the two experiments at the specified interval. The arrows point to intragranulomar eggs. Magnification: 200x.

erate amounts of uric acid and ARA in the host lung and liver were adequate to induce considerable decreases in parasite ovum counts and viability and in granuloma number and diameter in the inactivated papain-treated mice. Thus, in addition to or independently of catalytic site activity, linear or conformational motifs in the papain polypeptide are likely responsible for its protective capacity. These putative structural patterns are still unidentified but are likely shared by the polypeptide chains of SmCB1, SmCL3, FhCL1, and cysteine peptidase allergens. Identification of these putative patterns or motifs will pave the way to the development of a safe, efficacious, storage-stable, and cost-effective schistosomiasis vaccine.

Conclusions

The role of thymus (T)-derived lymphocytes and protease enzymatic activity in the protection induced by the cysteine peptidase–based schistosomiasis vaccine was addressed using papain, the model experimental protease. The protective effect of cysteine peptidase against schistosomiasis relies on activation of both the innate and adaptive arms of immunity. The cysteine peptidase catalytic and non-enzymatic sites elicit protection via induction of preponderant IgG1 antibody response and high levels of uric acid and arachidonic acid in the lung and liver at the time of developing worm migration in these sites. These findings are crucial for the development of a safe, efficacious, and cost-effective schistosomiasis vaccine.

Conflict of interest

The authors have declared no conflict of interest.

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