Parasites & Vectors

Protection against Schistosoma mansoni infection using Fasciola hepatica-derived fatty acid binding protein from different obtaining systems

--Manuscript Draft--

Manuscript Number:	
Full Title:	Protection against Schistosoma mansoni infection using Fasciola hepatica-derived fatty acid binding protein from different obtaining systems
Article Type:	Research
Section/Category:	Helminths and Helminthic Disease
Funding Information:	Regional Government of Castile and Leon (SA342U13) — Not applicable Instituto de Salud Carlos III (RICET VI PN I+D+I 2008-2011, ISCIII FEDER (RD12/0018/0002)) — Not applicable Banco de Santander — Jose Rojas-Caraballo
Abstract:	Background: This study reports the immunoprotection induced by cross-reacting Fasciola hepatica fatty acid binding proteins, native (nFh12) and recombinantly expressed using two different expression systems Escherichia coli (rFh15) and baculovirus (rFh15b) against the Schistosoma mansoni infection. Methods. BALB/c mice were vaccinated with native or recombinant FABP formulated in adjuvant adaptation (ADAD) system with natural or chemical synthesized immunomodulators (PAL and AA0029) and then challenged with 150 S. mansoni cercariae. Parasite burden, hepatic lesion and antibody response was studied in vaccination trials. Furthermore differences between rFh15 and rFh15b immunological responses (cytokine production, splenocyte population and antibody levels) were studied. Results: Vaccination with nFh12 induced significant reductions in worm burden (83%), eggs in tissues (82-92%) and hepatic lesions (85%) compared to infected controls using PAL. Vaccination with rFh15 showed less total worm (56-64%), eggs in liver (21-61%), eggs in the gut (30-77%) and hepatic damage (67-69%) using PAL and AA0029 as immunomodulators. In contrast mice vaccinated with rFh15b showed only reductions in eggs trapped in liver and intestine (53% and 60% respectively), and hepatic lesion (45%). We observed significant rise of TNFα, IL-6, IL-2, IL-4 and high antibody response (IgG, IgG1, IgG2a, IgM and IgE) in mice immunised with either rFh15 or rFh15b. Moreover, immunisation with rFh15b showed increase of IFNγ and decrease of B220 cells compared to untreated mice, and less production of IgG1 and IgM than immunised by rFh15. Conclusions: Higher level of protection is obtained using Fasciola hepatica-derived fatty acid binding protein against Schistosoma mansoni infection. The percentage of protection varies depending on the expression system used.
Corresponding Author:	Julio López-Abán, PhD Universidad de Salamanca Salamanca, Salamanca SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universidad de Salamanca
Corresponding Author's Secondary Institution:	
First Author:	Belén Vicente
First Author Secondary Information:	
Order of Authors:	Belén Vicente Julio López-Abán, PhD

	Jose Rojas-Caraballo, PhD
	Esther del Olmo, PhD
	Pedro Fernández-Soto, PhD
	Antonio Muro, PhD
Order of Authors Secondary Information:	

Protection against *Schistosoma mansoni* infection using *Fasciola hepatica*-derived fatty acid binding protein from different obtaining systems

Belén Vicente[1], Julio López-Abán[1]*, Jose Rojas-Caraballo[1], Esther del Olmo[2], Pedro Fernández-Soto[1], Antonio Muro[1]*.

16th November 2015

[1]Parasite and Molecular Immunology Laboratory. Tropical Disease Research Centre, Universidad de Salamanca (IBSAL-CIETUS). Avda. Licenciado Méndez Nieto s/n 37007 Salamanca, Spain.

Abstract

Background: This study reports the immunoprotection induced by cross-reacting *Fasciola hepatica* fatty acid binding proteins, native (nFh12) and recombinantly expressed using two different expression systems *Escherichia coli* (rFh15) and baculovirus (rFh15b) against the *Schistosoma mansoni* infection.

Methods. BALB/c mice were vaccinated with native or recombinant FABP formulated in adjuvant adaptation (ADAD) system with natural or chemical synthesized immunomodulators (PAL and AA0029) and then challenged with 150 *S. mansoni* cercariae. Parasite burden, hepatic lesion and antibody response was studied in vaccination trials. Furthermore differences between rFh15 and rFh15b immunological responses (cytokine production, splenocyte population and antibody levels) were studied.

Results: Vaccination with nFh12 induced significant reductions in worm burden (83%), eggs in tissues (82-92%) and hepatic lesions (85%) compared to infected controls using PAL. Vaccination with rFh15 showed less total worm (56-64%), eggs in liver (21-61%), eggs in the gut (30-77%) and hepatic damage (67-69%) using PAL and AA0029 as immunomodulators. In contrast mice vaccinated with rFh15b showed only reductions in eggs trapped in liver and intestine (53% and 60% respectively), and hepatic lesion (45%). We observed significant rise of TNFα, IL-6, IL-2, IL-4 and high antibody response (IgG, IgG1, IgG2a, IgM and IgE) in mice immunised with either rFh15 or rFh15b. Moreover, immunisation with rFh15b showed increase of IFNγ and decrease of B220 cells compared to untreated mice, and less production of IgG1 and IgM than immunised by rFh15.

Conclusions: Higher level of protection is obtained using *Fasciola hepatica*-derived fatty acid binding protein against *Schistosoma mansoni* infection. The percentage of protection varies depending on the expression system used.

Key words: *Schistosoma mansoni*, *Fasciola hepatica*, PAL, AA0029, Fatty acid binding protein, vaccine.

Background

The blood flukes *Schistosoma mansoni*, *S. haematobium* and *S. japonicum* are the main responsible of schistosomiasis in humans in Africa, Asia and South America. The Word Health Organization (WHO) estimated that 261 million people living in 78 countries required treatment in 2013, of whom 121 millions were school-age children and 92 % of them live in Africa [1]. Presently, the main strategy against schistosomiasis involves the use praziquantel to reduce worm burden and morbidity presenting good efficacy, affordable cost, operational convenience and limited side effects [2]. High rates of reinfection and the reduced susceptibility of schistosomula leads to sub-optimal cure rates. After decades of continuous treatment the concern of resistant linage selection or spreading of native tolerant strains is an important threat [3]. The use of artemisinin derivatives and combinations with praziquantel could be used to improve cure rate in endemic areas [4, 5]. Many researchers believe that immunoprophylaxis could be an attractive tool together with chemotherapy, safe water supply, adequate sanitation, hygiene education or snail control [6]. Reduction of parasite burden, amelioration of pathology and blocking of transmission are considered desirable features of the vaccine [7]. The basis of vaccines against schistosomes are demonstrated by the partial resistance developed against natural infection and the high protection induced by irradiated cercariae reaching worm reductions of 41-75% depending on the total number of immunising parasites [8].

A plethora of proteins have been proposed as potential vaccines against schistosomiasis using different methods of discovering: cDNA library screening with sera raised against whole or fractions of schistosomes, PCR amplification from a cDNA library, identification of membrane protein signal sequences, mining the genome to identify membrane o secretory proteins by reverse vaccinology [9, 10]. Only a little number of vaccines have reached Phase I clinical trials and only the glutathione-S transferase rSh28GST (Bilhvax) have reached Phase III against urinary schistosomiasis [12]. The protein Sm14 from *S. mansoni*, derived from a cloned gene exhibited affinity to fatty acids was able to protect outbreed mice and rabbits against the challenge with *S. mansoni* cercariae. Further works leaded to *Pichia pastoris* expression and the use of the synthetic adjuvant GLA-SE, which has been utilised in Phase I clinical trials [13]. Also, Sm14 shows significant homology to rFh15 from *Fasciola hepatica*, identical basic three-dimensional structure and shared discontinuous epitopes. Moreover, Sm14 induces abolition of liver damage in mice sheep and goats against the experimental infection by *F. hepatica* [13-15]. The native nFh12 and the recombinant rFh15 FABP from *F. hepatica* have shown protections in terms of worm and liver lesion reductions using Freund's adjuvant in C57/BL6 mice against *S. bovis* infection [16-17]. Moreover, large parasite burden reduction, liver lesion amelioration and anti-fecundity effect were observed in BALB/c mice and golden hamster vaccinated with the rFh15 using the ADAD (adjuvant adaptation) vaccination system against the *S. bovis* challenge [18-19]. Furthermore a FABP of 14.6 kDa purified from *Fasciola gigantica* has proved reductions in parasite measurements and liver lesion against *S. mansoni* infection in CD1 mice [20].

New expression systems are needed to allow a better conservation of post-translational modifications than in prokaryotic production systems. Baculovirus based expression system is a safe, versatile and powerful cloning tool for production of recombinant proteins in eukaryotic cells that could be interesting to test against the *S. mansoni* challenge and study the immunological response [21,22].

Immunity adjuvants are recognised to have crucial importance in vaccine development. Adjuvant Adaptation (ADAD) vaccination systems was developed as an alternative to Freund's adjuvant in vaccination against trematodes as *F. hepatica* and schistosomes, since its side effects do not permit the use in commercial vaccine. ADAD combines the antigen together with non-haemolytic saponins from *Quillaja saponaria* and a natural or synthetic immunomodulator, forming an emulsion with the non-mineral oil Montanide ISA 763AVG to obtain a long-term delivery system [22]. The natural immunomodulator PAL is a hydroalcoholic extract from the rhizome of the fern *Phlebodium pseudoaureum* able to down-regulate the Th-response in mice immunised with *Anisakis simplex*, *Trichinella spiralis* and *F. hepatica* antigens [23]. The synthetic diamine AA0029 inhibits lymphoproliferation, modulates of delayed type hypersensivity in a *T. spiralis* model, modifies ratios of CD8+, CD4+ and MHC Class II cells, and increases nitric oxide production in LPS pre-stimulated rat alveolar macrophages [24]. Experiments using 14-3-3 protein from *S. bovis*, and FABP from *F. hepatica* formulated in ADAD system have yielded high protection in terms of parasite burden and liver damage [18, 19, 25].

The aim of this study is to show the immunoprophylactic properties of three FABP from *Fasciola hepatica* (nFh12, rFh15 and rFh15b) using the ADAD vaccination system against *Schistosoma mansoni* infection in BALB/c mice. Also immunological response to immunisation is studied using one recombinant obtained in *Escherichia coli* (rFh15) and one produced in baculovirus transformed *Trichoplusia ni* caterpillars (rFh15b).

Methods

Animals, ethics statement and parasites

Animal procedures used in this study complied with the Spanish (L32/2007, L6/2013 and RD53/2013) and the European Union (Directive 2010/63/EU) regulations on animal experimentation. University of Salamanca's Ethics Committee approved procedures used in the present study (protocol 48531). SPF female CD1 and BALB/c mice obtained from Charles River (Lyon, France) weighing 19-26 g used in this work were maintained in a temperature and humidity controlled environment with a 12 hours light/dark cycle with water and food *ad libitum* in the University of Salamanca's Animal Experimentation facilities. The animals' health status was monitored throughout the experiments by a health surveillance program according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines. Mice were humanely euthanized with an intraperitoneal injection of pentobarbital (100 mg/kg), according to protocols supplied by the University of Salamanca's animal facilities at the

end of the experimental procedures or when any deterioration of mice health status was evidenced. Size of groups was calculated by power analysis using "size.fdr" package for R and following the 3Rs recommendations [26,27]. All efforts were made to minimise suffering. LE strain of *S. mansoni* was maintained in our laboratory in *Biomphalaria glabrata* snails as intermediate host and CD1 mice as definitive host. The number of cercariae and their viability were determined using a stereoscopic microscope.

S. mansoni soluble adult worm antigen and *F. hepatica* native nFh12 obtaining

Soluble adult worm antigens from *S. mansoni* (SoSmAWA) used for ELISA were prepared as previously described [16]. Twenty adult worms were suspended in 1 mL of sterile phosphate-buffered saline (PBS) containing 1mM phenyl methyl sulphonyl fluoride (PMSF; Sigma, St Louis, MO), homogenised, frozen and thawed thrice and then sonicated thrice (70 kHz) for 1 min each. The suspension was centrifuged at 20000 g for 30 min at 4 ºC. Native 12 kDa *F. hepatica* antigen (nFh12) was purified as described by Hillyer [28] by a combination of gel filtration using Sephadex G-50 and two-step iso-electric focusing runs with 3–10 and 4–6 ampholytes. A rabbit monospecific, polyclonal anti-Fh12 antiserum was then used in SDS-PAGE and immunoblot to confirm that the purified polypeptide was Fh12.

Recombinant rFh15 and rFh15b protein expression and purification

The recombinant fatty-acid binding proteins from *F. hepatica* were produced as recombinants using two different expression systems. The first one of them was based on the use of *E. coli* BL21 bacteria (rFh15). The obtaining of such recombinant protein was manufactured following Rodríguez-Pérez et al. [29]. Briefly, total RNA from one *F. hepatica* adult worm was isolated and used for cDNA synthesis. The rFh15 gene (GeneBank M95291.1) was amplified using the following primer sequences: forward 5´-GGATCCATGGCTGACTTTG TGGG-3' and reverse 5'-CTCGAGCGCTTTGAGCAGAGTG-3' and restriction sites for BamHI and XhoI were added. PCR products were then purified and cloned into pGEX-4T2 vector with a *S. japonicum* glutathion S-transferase sequence for further detection and purification. The resulting recombinant DNA plasmid was purified and then sequenced to verify integrity of the cloned insert. Transformed *E. coli* BL21 cells were grown in Luria Bertani medium with ampicillin until reaching an optical density of 0.6 and then induced by the addition of isopropyl β-tiogalactopyranoside (IPTG). The cell pellet was recovered by centrifugation of the culture at 18000 *g* for 30 min, suspended in PBS with 1 mM PMSF and 1 % Triton X-100 then sonicated and centrifuged. Solubilised protein was purified by affinity chromatography with a glutathione Sepharose 4B resin. Non-retained proteins were eluted with PBS whilst rFh15 was eluted by addition of PBS plus thrombin.

The second method to obtain the recombinant rFh15 protein was based on the use of a baculovirus expression vector system, manufactured by

ALGENEX (Madrid, Spain) using standardised protocols. Briefly, nucleotide sequence from 15 kDa fatty acid binding protein (GeneBank M95291.1) was synthesized and a Kozak sequence was inserted into the N-terminus extreme, together with *BamH*I and *Xba*I restriction sites at N- and C-terminus, respectively and used to be cloned into the pFasBacHis vector. The plasmid pMA (ampR) with the cloned Fh15 gene between KpnI / SacI sites was used to amplify DNA by transformation of *E. coli* (DH5alpha) cells and isolating ampicillin-resistant colonies. The resulting amplified DNA together with the cloning vector (pFasBacHis) were cut with restriction enzymes BamHI and XbaI and the corresponding band (412 bp) from Fh15 insert was isolated and purified. pFasBacHis vector was then dephosphorylated with alkaline phosphatase treatment and the Fh15 insert was then ligated. The resulting product was then used to transform *E. coli* (DH5alpha) cells and ampicilline and gentamicine resistant colonies were then isolated. The DNA from this isolated colonies was then isolated and characterised by using the restriction enzymes for BamHI and XbaI sites, respectively, and automated sequence was performed to verify the sequence of the insert. The resulting vector and the sequence of the Fh15 insert is depicted in Figure1A. To obtain the recombinant baculovirus, *E. coli* special competent (DH10B) cells were transformed starting from previously generated vector (pFBFh15His). These cells carry the receptor bMON14272 that contains a beta-galactosidase codifying gene. Upon incorporation in the same cell the vector and the receptor, the recombinant baculovirus presents resistance to kanamycin, tetracyclin and geneticin and losses its beta-galactosidase activity. One colony resistant to the three antibiotics was selected, the DNA isolated and used to transfect insect cells sf21 using the cellfectin reagent (Invitrogen). Seventy-two hours after the transfection the so-called progeny 1 from the recombinant baculovirus was collected and stored until its use. Finally, thirty *Trichoplusia ni* larvae were inoculated with the previously obtained recombinant virus. Larvae were harvested during the next 48 – 96 hours and the expression of the recombinant protein was then assessed by using both Coomassie blue staining and Western blot with monoclonal anti-6His antibodies.

ADAD vaccination system

The rFh15 protein was formulated in a micelle composed by non-haemolytic saponins from *Quillaja saponaria* (Qs; Sigma, St Louis, Missouri) and natural (PAL) or the synthetic aliphatic diamine (AA0029) as immunomodulator. Then, this micelle was emulsified in a non mineral oil (Montanide ISA763A, SEPPIC, Paris, France) as an oil/water 70/30 and subcutaneously injected into BALB/c mice. The ADAD vaccination system consists of a set of two subcutaneous injections. The first injection, called "Adaptation", contains Qs and PAL or AA0029 emulsified in the non-mineral oil. The second injection, administered 5 days after the adaptation, contains the rFh15 antigen with Qs and PAL or AA0029 in the emulsion oil. Individual doses per injection included in each case were as follows: 20 μg of Qs, 600 μg of PAL or 100 μg of AA0029, and 10 μg of nFh12, rFh15 or rFh15b in a final volume of a 200 μL injection of emulsion in the non-mineral oil [18, 25].

Vaccination experiment schedules

BALB/c mice were randomly allocated in groups of 9 animals each as follows: Untreated and uninfected; *S. mansoni* infected; control adjuvant (injected with ADAD with Qs and the natural immunomodulator PAL or the synthetic AA0029) and Vaccinated groups (Vaccinated with ADAD with the corresponding FABP nFh12, rFh15 or rFh15b formulated with the corresponding immunomodulator PAL or AA0029 and infected). Two weeks after the first immunisation animals were boosted with the same doses. Two weeks after the second immunisation, each mouse was exposed to 150 *S. mansoni* cercariae for 45 min. Eight weeks post-infection all mice were euthanized with intraperitoneal injection of sodium pentobarbital (100 mg/kg) and then perfused by intra-cardiac injection of PBS plus heparin, and the number of recovered *S. mansoni* adult worms from the portal and mesenteric veins was recorded. In addition, the number of parasite eggs in liver and intestine was counted using a McMaster camera after digestion with 25 mL of 5% KOH for 16 hours at 37 ºC with gentle shaking. Macroscopic lesions of liver were quantified as granuloma affected surface per 100 mm^2 in each mouse using ImageJ 1.45s software [30]. Protection percentage was calculated for all parasitological and pathological magnitudes as follows: (mean in the infected control group – mean in experimental group) x 100 / mean in infected control group. Blood samples were collected from each animal before immunisation, infection and necropsy for humoral immune response studies.

Specific antibody response against FABP and SoSmAWA

Specific anti-rFh15 or anti-SoSmAWA antibodies profiles were measured using an indirect ELISA as described by Abán et al. [16]. Briefly, 96-well polystyrene plates (Costar) were coated with 2.0 µg of nFh12, rFh15, rFh15b or 2.5 µg of SoSmAWA antigen for 12 hours in carbonate buffer (pH 9.0) and then blocked with 2 % bovine serum albumin in PBS. Sera were then added at 1:100 dilutions and incubated for 1 hour at 37 ºC, followed by the addition of goat peroxidase-labelled anti-mouse IgG, IgG1, IgG2a, IgM or IgE antibodies at 1:1000 dilution (Sigma, St. Louis MO). The reaction was developed with H_2O_2 and ortophenilenediamine (OPD, Sigma) in citrate buffer (pH 5.0) and absorbance was measured at 492 nm with an Ear400FT ELISA reader (Lab Instruments).

Immune response in BALB/c mice immunised with the recombinant FABP rFh15 and rFh15b

Four groups of six female BALB/c each were used for the characterisation of immunological response. Untreated; Injected with ADAD only with AA0029+Qs as adjuvant control; Immunised with rFh15 formulated in ADAD system with AA0029 (AA0029+Qs+rFh15); and Immunised with rFh15b formulated in ADAD system with AA0029 (AA0029+Qs+rFh15b). Mice were immunised and two booster doses were given after 2 and 4 weeks respectively. Two weeks after the immunisation schedule all the mice were anesthetised with isoflurane and euthanized by cervical dislocation. Spleens

were then aseptically removed for obtaining splenocytes by perfusion with sterile PBS to study cytokine profile and to quantify T-cell subpopulations. Blood samples were collected for antibody detection from each animal before each immunisation and at the necropsy.

Cytokine measurement

Splenocytes obtained from individual mouse were cultured in a 6-well plate at $1x10^6$ cells per well in complete RPMI 1640 medium containing 10% heat-inactivated foetal calf serum, 5 mM L-glutamine and antibiotics: 100 units/mL penicillin and 100 µg/mL streptomycin as previously described [31]. Cells were *in vitro* stimulated with rFh15 or rFh15b at a final concentration of 10 µg/mL for 72 hours at 37 ºC in a humidified atmosphere with 5% CO_2. Culture supernatants were recovered for cytokines determination. Splenocytes belonging to untreated mice were used as controls. A flow cytometry-based technique was used for interferon γ (IFNγ), tumor necrosis factor α (TNFα), interleukin (IL) 1α, IL-2, IL-4, IL-6, IL-10 and IL-17 quantitation in each of the groups of mice used in this study. The FlowCytomix Mouse Th1/Th2 10plex kit (Bender MedSystems GmbH, Vienna, Austria) was used according to the manufacturer's instructions. Briefly, different size fluorescent beads, coated with capture antibodies specific for the aforementioned cytokines were incubated with mouse splenocyte samples and with biotin-conjugated secondary antibodies for 2 hours at room temperature. The specific antibodies bind to the analytes captured by the first antibodies. After washing the tubes with PBS plus 2% foetal calf serum, Streptavidin-Phycoerythrine (S-PE) solution was added and incubated at room temperature for 1 h. S-PE binds to the biotin conjugate and emits fluorescent signals. Flow cytometry data was collected using a FACSCalibur flow cytometer (BD Biosciences) at the University of Salamanca's Flow Cytometry Central Service; 8000 events were collected (gated by forward and side scatter) and data was analysed using FlowCytomix Pro 3.0 software (Bender MedSystems, Vienna, Austria). Each cytokine concentration was determined from standard curves using known mouse recombinant cytokines concentrations.

Flow cytometry analysis of splenic B and T-cell populations

Splenocytes from untreated, AA0029+Qs-treated, rFh15-immunised and rFh15b-immunised mice were incubated with the blocking anti-CD16/CD32 monoclonal antibody for 5 min at room temperature and stained with commercial fluorochrome-conjugated antibodies at 1/50 dilution in PBS plus 2 % foetal calf serum for 30 min at 4 ºC. Rat anti mouse CD45-peridinin chlorophyll protein (PerCP)- cyanine dye (Cy5.5), CD4-fluorescein isothyosanate (FITC), CD8-phycoerythrin (PE), CD45R/B220-allophycocyanin (APC), CD197-PE (CCR7), CD62L-APC and hamster anti-mouse CD27 APC (BD Pharmingen, USA) were used. After incubation, cells were washed in PBS with 2% foetal calf serum and then centrifuged at 1000 *g* for 5 min and the supernatant was discarded. The cells were then fixed with 100 µL of a 2% paraformaldehyde solution for 1 hour at 4 ºC. Phenotypic analyses were performed in a FACScalibur flow cytometer. Data

were collected on 30000 events (gated by forward and side scatter) and analysed using Gatelogic Flow Cytometry Analysis Software (INIVAI technologies Pty Ltd).

Statistical analysis

The results were expressed as mean and standard error of the mean (SEM). Normal distribution of data was studied by Kolmogorov-Smirnov test. Significant differences among groups were found using one-way ANOVA test and post hoc Tukey's honest significance test (HSD) or Kruskal-Wallis test. All statistical analyses were considered significant at $p<0.05$. Software SPSS 21 sofware (IBM) was used for data analysis.

Results

Recombinant expression and detection of antigens

Expression and purification of *F. hepatica*-derived recombinant proteins nFh12 and rFh15 was previously reported. Here, we used a baculovirus expression vector system that improves the production of recombinant proteins compared to the classical expression systems based on the use of bacteria or yeast, which also retains recombinant proteins native configuration along the production and purification steps to produce a *F. hepatica*-derived fatty acid binding protein (Figures 1A and 1B). Starting from 30 *T. ni* larvae inoculated with recombinant virus, cells were recovered during the next 48-96 hours to assess recombinant protein expression, which was confirmed using both Coomassie blue staining and western blot using anti-6His monoclonal antibody as shown in Figure 1C. As it can be seen, Coomassie blue staining detected a majority band with an estimated molecular weight of 15.7 kDa in the crude extract. Specific detection with monoclonal antibody confirms the presence of one single band with the same molecular weight. Upon detection of the recombinant protein, it was on-column purified by affinity chromatography using a Ni-NTA column (Figure 1D). As depicted, a single band with a molecular weight of 15 kDa was detected using Coomassie blue staining, coming from pooled column-retained fractions, dialysed against ammonium carbonate (50 mM), lyophilized and resuspended in high-purity distilled water. Western blot from the same fraction also reveals the presence of one single band with the same molecular weight (Figure 1D). Protein quantitation revealed the recovery of 5 mg of pure recombinant protein.

Vaccination with the native nFh12 formulated in ADAD with PAL triggers protection against *S. mansoni* infection

Significant reductions in recovered total worms (83%), males (87%) and females (82%) were observed in BALB/c mice immunised with nFh12 formulated in ADAD with the natural immunomodulator PAL (PAL+Qs+nFh12) compared to the infected control group (Table 1). Also, a significant decrease in the number of eggs present in liver (82%) was detected, but not in the number of eggs in intestine (20%) in comparison with infected group (Table 1). In concordance, hepatic damage extension was

significantly reduced (85%) compared to infection control group (Table 1. Figure 2). Furthermore, mice injected only with PAL+Qs showed not significant protection in terms of parasite burden or hepatic lesions (Table 1). A significantly higher production of specific anti-nFh12 IgG was observed in nFh12 vaccinated group compared to uninfected, infected or adjuvant controls after the second immunisation which remains until the end of the experiment (Figure 3A). Also all infected groups showed significant production of IgG, IgG1 at 8 week post-infection against SoSmAWA but only vaccinated with PAL+Qs+nFh12 showed significant IgG2a production (Figure 4A).

Vaccination with the recombinant rFh15 formulated with ADAD using PAL stimulates high protection against *S. mansoni* infection

Mice vaccinated with rFh15 formulated in ADAD with the natural immunomodulator PAL (PAL+Qs+rFh15) induced significant reduction in worm burden (56% in total worms, 63% in females and 49% in males) compared to infected controls (Table 1). Slight but significant decreases in the number of eggs present in the liver (21%) and the gut (30%) of the vaccinated group were observed in comparison to infected group in concordance with the reduction in worm burden (Table 1). Moreover, liver surface damage showed significant reduction (69%) compared to infected group (Table 1, Figure 2B). Mice injected with PAL+Qs showed not significant reductions in parasite burden or hepatic lesions (Table 1). A significantly higher production of specific anti-rFh15 IgG, was observed in rFh15 vaccinated group compared to uninfected control group (Figure 3B). Also all infected groups showed significant increasing of IgG, IgG1 against SoSmAWA at 8 weeks post-infection, but only vaccinated with PAL+Qs+Fh15 showed statisticaly significant IgG2a increase (Figure 4B)

Vaccination with rFh15 induces more protection than rFh15b against *S. mansoni* infection in BALB/c mice using ADAD vaccination system with the immunomodulator AA0029

Vaccination with rFh15 formulated in ADAD with the synthetic immunomodulator AA0029 (AA0029+Qs+rFh15) induces significant reduction in worm burden (64% in total worms, 69% in females and 58% in males) in comparison with infected controls (Table 1). Also significant decreases in the number of eggs recovered from the liver (61%) and the gut (77%) of the vaccinated group were observed in comparison with infected group agreeing with the reduction in worm burden. Moreover, liver surface damage showed significant reduction (67%) compared to infected mice (Table 1, Figure 2). Vaccination with rFh15b obtained from *T. ni* larvae (AA0029+Qs+rFh15b) showed significant protection in terms of recovered females (44%) eggs confined in liver (53%), eggs in the gut (60%) and hepatic lesion (75%). However, no significant reduction was observed in the recovery of total and male adult parasites (Table 1, Figure 2). Adjuvant controls treated with AA0029+Qs showed no-protection against the

S. mansoni challenge (Table 1). A significantly higher production of specific anti-rFh15 and anti-rFh15b IgG, were observed against their respective vaccinated group compared to uninfected control group at the time of infection and the end of the experiment particularly in mice vaccinated with rFh15 (Figure 3C). Also all infected groups showed significant increasing of IgG, IgG1 against SoSmAWA at 8 weeks post-infection but not IgG2a (Figure 4C).

Cell immune response induced by rFh15 and rFh15b using ADAD vaccination system with AA0029 as immunomodulator

Cytokine levels were measured in cultured splenocyte supernatants to analyse Th1, Th2, Treg and Th17 T-cell responses. It was observed that mice immunised with AA0029+Qs+rFh15 showed a significant increase of TNFα, IL-6, IL-2 and IL-4 compared to untreated and adjuvant controls (Table 2). Similarly, mice treated with AA0029+Qs+rFh15b, had high levels of TNFα, IL-2 and IL-4 compared to untreated and adjuvant controls (Table 2). Additionally, we observed less IL-6 production and high significant levels of IFNγ than mice vaccinated with AA0029+Qs+rFh15 (Table 2). We observed that untreated mice and adjuvant controls (PAL+Qs) showed similar cytokine patterns. Also, no differences were found in IL-17 and IL-10 cytokine levels neither rFh15 nor rFh15b immunised mice. Regarding to the percentage of splenocyte populations only mice vaccinated with AA0029+Qs+rFh15b showed a significant reduction of B220 cells compared with untreated and PAL+Qs treated animals (Table 3). No differences in T and B splenocyte population were observed between untreated mice and those treated with PAL+Qs.

Differential antibody patterns in mice vaccinated with rFh15 vs rFh15b

Antibody response of rFh15 and rFh15b-immunised mice were studied to know the intensity of the humoral response elicited by the two recombinant proteins, due to the importance of antibodies in resistance to schistosomiasis and in an attempt to explain the different protection observed between these molecules. Two weeks after the immunisation schedule a significant high production of specific IgG, IgG1, IgG2a, IgM, IgE anti-rFh15 or anti-rFh15b was observed in AA0029+Qs+rFh15 and in AA0029+Qs+rFh15b vaccinated respectively, compared to adjuvant and untreated controls (Figure 5). Furthermore, we observed significant higher levels of IgG1 and IgM in vaccinated with rFh15 than those mice vaccinated with rFh15b (Figure 5).

Discussion

Many efforts have been focused on schistosomiasis vaccine development because the potential contribution to control or eradication the disease. FABP from *F. hepatica* have demonstrated a valuable cross-protection against *S. bovis* in experimental models [16-19] as well as FABP of 14.6 kDa from *F. gigantica* [20]. Also the *S. mansoni* FABP Sm14 have reached Phase I studies [15]. However, the immunoprotective potential of *F. hepatica* FABPs have not been tested against *S. mansoni* infection until now. In this

study, we present the immunoprotective potential of FABP obtained from *F. hepatica* represented by the native form (nFh12) and two recombinants (rFh15, rFh15b) against the *S. mansoni* infection in BALB/c mice. These molecules have been expressed in prokaryotic and eukaryotic systems. This inbreed mice has a biased Th2 genetic background considered of choice since resembles the immunological profile observed in people living in endemic areas [32] (Alves et al., 2015). In this study, we used the adjuvant adaptation (ADAD) vaccination system using natural (PAL) and synthetic (AA0029) immunomodulators developed by our research group for vaccination against fasciolosis and schistosomiasis to improve limitations of the classical Freund's adjuvant [22, 25, 31, 33].

We observed high protection in terms of worm recovery, eggs trapped in tissues and hepatic damage in mice vaccinated with the native nFh12 and the *E. coli* recombinant rFh15. These results are close to those obtained in vaccination against *S. bovis* with both antigens formulated in ADAD vaccination system with PAL as well as AA0029 [18-19]. These results are comparable to those shown using the FABP Sm14 obtained from *S. mansoni* in experimental models [15] or using the *F. gigantica* 14.6 kDa molecule [20]. These results together reinforce the value of FABPs in schistosomiasis vaccination development. We observed a high production of specific IgG by ELISA against the three antigens used for vaccination indicating an intense immunological response. A vigorous humoral response is found in natural resistance to infection of people living in hyperendemic areas [33, 34, 35] and experimental models [36]. Also, vaccinated animals generated high levels in both IgG and IgG1 against SoSmAWA at week 8 post-challenge but there was significant production of IgG2a only using the natural immunomodulator PAL. This effect has been observed in previous works related with the use of PAL in vaccination against *F. hepatica* and *S. bovis* and it was associated with protection and down regulation of the dominant Th2 established in schistosomes or *F. hepatica* infections [18,19, 37, 38]. An appropriate adjuvant system able to induce an adequate immune response is recognised as an important tool for developing vaccines and a good feature is the specific adjuvant activity driving the immunological response together with the antigen [32, 39]. We did not find any protection induced in mice treated neither with PAL+Qs or AA0029+Qs after the challenge with *S. mansoni* when compared with infection controls. This indicates the specific activity of both adjuvants in our experiments.

Additionally, we observed that the antigen obtained using baculovirus as vector (rFh15b) formulated with AA0029 in ADAD showed high reduction in egg tissues and liver damage, but there was slight not-significant reduction in total worm burden compared to AA0029+Qs+rFh15 vaccinated mice after the challenge. So we studied the immune response to rFh15 and rFh15b. Mice immunised with the synthetic immunomodulator AA0029 with *E. coli* recombinant rFh15 in ADAD vaccination system promotes an early potent mixed Th1/Th2 and pro-inflammatory immune response with significant production of TNFα, IL-6, IL-2, IL-4 and high level of specific antibodies that could explain the protection against the *S. mansoni* challenge as it was

pointed out in a previous work of experimental protection against *F. hepatica* and *S. bovis* using AA0029 formulated in ADAD [19, 31, 32]. Vaccination with the protein produced in *T. ni* (AA0029+Qs+rFh15b) showed high levels of TNFα, IL-6, IFNγ IL-2, IL-4 and antibodies, but reduction in B220 cells percentage compared to untreated mice. Moreover, we observed less IL-6, IgG1 and IgM compared to immunised with AA0029+Qs+rFh15. This indicates a potent proinflamatory and Th1/Th2 mixed response with an impairment of humoral response involving B memory cells and immunoglobulins that could be responsible of the low protection in terms of worm recovery [40]. Another possible explanation of the differences in protection could be the post-translational modifications that happen in the different expression systems involving glycoxilation [41].

In conclusion our data show the ability of FAPB obtained from *F. hepatica* to induce protection against protection against the infection of *S. mansoni* in BALB/c mice. Also the use of PAL seems to induce an increase of Th1 like immune response during infection. ADAD formulation with the immunomodulator AA0029 showed an intense pro-inflamatory and mixed Th1/Th2 immune response. These molecules could have valuable effects on reduction of pathology and transmission of the disease. These studies warrant further studies in other animal models closer to human beings to state the actual protection ability of FABP against *S. mansoni* infection.

List of abbreviations

ADAD: Adjuvant adaptation vaccination system; ANOVA: analysis of variance. FABP: fatty acid binding proteins; nFh12: native FABP of 12 kDa; Qs: non haemolytic saponines from *Quillaja saponaria*. rFh15: recombinant FABP of 15 kDa expressed in *Escherichia coli*; rFh15b: recombinant FABP of 15 kDa expressed in *Trichoplusia ni*; SoSmAWA: soluble adult worm antigens from *Schistosoma mansoni*.

Competing interests

Sponsors had no role in study design, or collection, analysis and interpretation of data. Authors are the only responsible in writing and submitting for publication.

Author's contributions

BV, JL-A and AM, conceived and designed the study. BV, JL-A, JR-C and EO performed experiments. BV, JL-A, JR-C PF-S and AM analyzed the data. BV JR-C and JL-A drafted the first manuscript. AM critically revised the manuscript. All authors read and approved the final manuscript.

Aknowledgements

Regional Government of Castile and Leon, Ref. SA342U13 and RICET VI PN I+D+I 2008-2011, ISCIII FEDER (RD12/0018/0002), Governement of Spain supported this research. Banco de Santander supported JRC. We are grateful to ASAC Pharmaceutical International AIE (Alicante, Spain) for kindly providing PAL, to D.J. Lora Sanz (Safic-Alcan

España S.L.) and SEPPIC (Barcelona, Spain) for providing Montanide ISA 763A GV and to G.V. Hillyer for providing the native nFh12 protein.

Autor Details:

[1]Parasite and Molecular Immunology Laboratory, Tropical Disease Research Centre, Universidad de Salamanca (IBSAL-CIETUS), Avda. Licenciado Méndez Nieto s/n 37007 Salamanca, Spain. Tel. +34 923294535 Fax +34 923294515. [2]Department of Pharmaceutical Chemistry. Faculty of Pharmacy. University of Salamanca, IBSAL-CIETUS. Salamanca, Spain. Jose Rojas-Caraballo current address: Centro de Investigación en Salud para el Trópico (CIST), Carretera Troncal del Caribe, Sector Mamatoco, Santa Marta, Magdalena, Colombia. Facultad de Medicina, Universidad Cooperativa de Colombia, Carretera Troncal del Caribe, Sector Mamatoco, Santa Marta, Magdalena, Colombia

References

[1] WHO Schistosomiasis: number of people treated worldwide in 2013. Wkly Epidemiol Rec. 2015;90:25-32.

[2] Cioli D, Pica-Mattoccia L, Basso A, Guidi A. Schistosomiasis control: praziquantel forever? Mol Biochem Parasitol. 2014;195:23-9.

[3] Greenberg RM. New approaches for understanding mechanisms of drug resistance in schistosomes. Parasitology. 2013;140:1534-46

[4] Pérez del Villar L, Burguillo FJ, López-Abán J, Muro A. Systematic review and meta-analysis of artemisinin based therapies for the treatment and prevention of schistosomiasis. PLoS One. 2012;7:e45867

[5] Liu YX, Wu W, Liang YJ, Jie ZL, Wang H, Wang W, Huang YX. New uses for old drugs: the tale of artemisinin derivatives in the elimination of schistosomiasis japonica in China. Molecules. 2014;19:15058-74.

[6] Grimes JE, Croll D, Harrison WE, Utzinger J, Freeman MC, Templeton MR. The roles of water, sanitation and hygiene in reducing schistosomiasis: a review. Parasit Vectors. 2015;8:156.

[7] El Ridi R, Othman AA, McManus DP. Editorial: the schistosomiasis vaccine - it is time to stand up. Front Immunol. 2015;6:390.

[8] Fukushige M, Mitchell KM, Bourke CD, Woolhouse ME, Mutapi F. A Meta-analysis of experimental studies of attenuated *schistosoma mansoni* vaccines in the mouse model. Front Immunol. 2015;6:85.

[9] McWilliam HE, Driguez P, Piedrafita D, McManus DP, Meeusen EN. Novel immunomic technologies for schistosome vaccine development. Parasite Immunol. 2012;34:276-84.

[10] Fonseca CT, Braz Figueiredo Carvalho G, Carvalho Alves C, de Melo TT. *Schistosoma* tegument proteins in vaccine and diagnosis development: an update. J Parasitol Res. 2012;2012:541268.

[12] Riveau G, Deplanque D, Remoué F, Schacht AM, Vodougnon H, Capron M, Thiry M, Martial J, Libersa C, Capron A. Safety and immunogenicity of rSh28GST antigen in humans: phase 1 randomized clinical study of a vaccine candidate against urinary schistosomiasis. PLoS Negl Trop Dis. 2012;6:e1704.

[13] Tendler M, Almeida M, Simpson A. Development of the Brazilian anti-schistosomiasis vaccine based on the recombinant fatty acid binding protein Sm14 plus GLA-SE adjuvant. Front Immunol. 2015;6:218.

[14] Hillyer GV. Fasciola antigens as vaccines against fascioliasis and schistosomiasis. J Helminthol. 2005;79:241-7.

[15] Mossallam SF, Amer EI, Ewaisha RE, Khalil AM, Aboushleib HM, Bahey-El-Din M. Fusion protein comprised of the two schistosomal antigens, Sm14 and Sm29,

provides significant protection against *Schistosoma mansoni* in murine infection model. BMC Infect Dis. 2015;15:147.

[16] Abán JL, Ramajo V, Arellano JL, Oleaga A, Hillyer GV, Muro A. A fatty acid binding protein from *Fasciola hepatica* induced protection in C57/BL mice from challenge infection with *Schistosoma bovis*. Vet Parasitol. 1999;83:107-21.

[17] Abáné JL, Oleaga A, Ramajo V, Casanueva P, Arellano JL, Hillyer GV, Muro A. Vaccination of mice against *Schistosoma bovis* with a recombinant fatty acid binding protein from Fasciola hepatica. Vet Parasitol. 2000;91:33-42.

[18] Vicente B, López-Abán J, Rojas-Caraballo J, Pérez del Villar L, Hillyer GV, Martínez-Fernández AR, Muro A. A *Fasciola hepatica*-derived fatty acid binding protein induces protection against schistosomiasis caused by *Schistosoma bovis* using the adjuvant adaptation (ADAD) vaccination system. Exp Parasitol. 2014;145:145-51.

[19] Vicente B, López-Abán J, Rojas-Caraballo J, del Olmo E, Fernández-Soto P, Ramajo-Martín V, Muro A. The combination of the aliphatic diamine AA0029 in ADAD vaccination system with a recombinant fatty acid binding protein could be a good alternative for the animal schistosomiasis control. Exp Parasitol. 2015;154:134-42.

[20] Rabia Aly I, Diab M, El-Amir AM, Hendawy M, Kadry S. *Fasciola gigantica* fatty acid binding protein (FABP) as a prophylactic agent against *Schistosoma mansoni* infection in CD1 mice. Korean J Parasitol. 2012;50:37-43.

[21] Sokolenko S, George S, Wagner A, Tuladhar A, Andrich JM, Aucoin MG. Co-expression *vs*. co-infection using baculovirus expression vectors in insect cell culture: Benefits and drawbacks. Biotechnol Adv. 2012;30:766-81.

[21] Barford D, Takagi Y, Schultz P, Berger I. Baculovirus expression: tackling the complexity challenge. Curr Opin Struct Biol. 2013;23:357-64.

[22] Martínez-Fernández AR, Nogal-Ruiz JJ, López-Abán J, Ramajo V, Oleaga A, Manga-González Y, Hillyer GV, Muro A. Vaccination of mice and sheep with Fh12 FABP from *Fasciola hepatica* using the new adjuvant/immunomodulator system ADAD. Vet Parasitol. 2004;126:287-98.

[23] López-Abán J, Esteban A, Vicente B, Rojas-Caraballo J, del Olmo E, Martínez-Fernández AR, Hillyer GV, Muro A. Adaptive immune stimulation is required to obtain high protection with fatty acid binding protein vaccine candidate against *Fasciola hepatica* in BALB/C mice. J Parasitol. 2012;98:527-35.

[24] del Olmo E, Plaza A, Muro A, Martínez-Fernández AR, Nogal-Ruiz JJ, López-Pérez JL, Feliciano AS. Synthesis and evaluation of some lipidic aminoalcohols and diamines as immunomodulators. Bioorg Med Chem Lett. 2006;16:6091-5.

[25] Uribe N, Muro A, Vieira C, Lopez-Aban J, del Olmo E, Suárez L, Martínez-Fernández AR, Siles-Lucas M. Genetic and immunological characterization of the 14-3-3 molecule from *Schistosoma bovis.* J Parasitol. 2007;93:964-9.

[26] Charan J, Kantharia ND. How to calculate sample size in animal studies? J Pharmacol Pharmacother. 2013;4:303-6.

[27] Festing MF, Altman DG. Guidelines for the design and statistical analysis of experiments using laboratory animals. ILAR J. 2002;43:244-58.

[28] Hillyer GV. Comparison of purified 12 kDa and recombinant 15 kDa *Fasciola hepatica* antigens related to a *Schistosoma mansoni* fatty acid binding protein. Mem Inst Oswaldo Cruz. 1995;90:249-53.

[29] Rodríguez-Pérez J, Rodríguez-Medina JR, García-Blanco MA, Hillyer GV. *Fasciola hepatica*: molecular cloning, nucleotide sequence, and expression of a gene encoding a polypeptide homologous to a *Schistosoma mansoni* fatty acid-binding protein. Exp Parasitol. 1992;74:400-7.

[30] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9:671-5.

[31] Rojas-Caraballo J, López-Abán J, Pérez del Villar L, Vizcaíno C, Vicente B, Fernández-Soto P, del Olmo E, Patarroyo MA, Muro A. In vitro and in vivo studies for assessing the immune response and protection-inducing ability conferred by *Fasciola hepatica*-derived synthetic peptides containing B- and T-cell epitopes. PLoS One. 2014;9:e105323.

[32] Alves CC, Araujo N, dos Santos VC, Couto FB, Assis NR, Morais SB, Oliveira SC, Fonseca CT. Sm29, but not Sm22.6 retains its ability to induce a protective immune response in mice previously exposed to a *Schistosoma mansoni* infection. PLoS Negl Trop Dis. 2015;9:e0003537.

[33] Siles-Lucas M, Uribe N, López-Abán J, Vicente B, Orfao A, Nogal-Ruiz JJ, Feliciano AS, Muro A. The *Schistosoma bovis* Sb14-3-3zeta recombinant protein cross-protects against *Schistosoma mansoni* in BALB/c mice. Vaccine. 2007;25:7217-23.

[34] McManus DP, Loukas A. Current status of vaccines for schistosomiasis. Clin Microbiol Rev. 2008;21:225-42.

[35] Melo TT, Sena IC, Araujo N, Fonseca CT. Antibodies are involved in the protective immunity induced in mice by *Schistosoma mansoni* schistosomula tegument (Smteg) immunization. Parasite Immunol 2014;36:107–111.

[36] El-Shabasy EA, Reda ES, Abdeen SH, Said AE, Ouhtit A.Transmission electron microscopic observations on ultrastructural alterations in *Schistosoma mansoni* adult worms recovered from C57BL/6 mice treated with radiation-attenuated vaccine and/or praziquantel in addition to passive immunization with normal and vaccinated rabbit sera against infection. Parasitol Res. 2015;114:1563-80.

[37] López-Abán J, Nogal-Ruiz JJ, Vicente B, Morrondo P, Diez-Baños P, Hillyer GV, Martínez-Fernández AR, Feliciano AS, Muro A. The addition of a new immunomodulator with the adjuvant adaptation ADAD system using fatty acid binding proteins increases the protection against *Fasciola hepatica*. Vet Parasitol. 2008;153:176-81.

[38] López-Abán J, Casanueva P, Nogal J, Arias M, Morrondo P, Diez-Baños P, Hillyer GV, Martínez-Fernández AR, Muro A. Progress in the development of *Fasciola hepatica* vaccine using recombinant fatty acid binding protein with the adjuvant adaptation system ADAD. Vet Parasitol. 2007;145:287-96.

[39] Stephenson R, You H, McManus DP, Toth I. Schistosome Vaccine Adjuvants in Preclinical and Clinical Research. Vaccines (Basel). 2014;2:654-85.

[40] Rodig SJ, Shahsafaei A, Li B, Dorfman DM. The CD45 isoform B220 identifies select subsets of human B cells and B-cell lymphoproliferative disorders. Hum Pathol. 2005;36:51-7.

[41] Lu HY, Chen YH, Liu HJ. Baculovirus as a vaccine vector. Bioengineered. 2012;3:271-4.

Table 1: Protection levels (% of reduction, R) in worm recovery (total counts, female and male), hepatic damage extension (mm^2/100 mm^2) and in number of eggs per gram (EPG) in tissues in vaccinated BALB/c mice using natural and recombinant FABP (nFh12, rFh15 or rFh15b) formulated with the adjuvant adaptation (ADAD) vaccination system with the natural immunomodulator PAL or the synthetic AA0029.

Groups	Total worms (mean±SEM)	R (%)	Females (mean±SEM)	R (%)	Males (mean±SEM)	R (%)	Hepatic lesion (mean±SEM)	R (%)	EPG in liver (mean±SEM)	R (%)	EPG intestine (mean±SEM)	R (%)
Experiment 1												
Infected	36.3±4.9	-	19.7±2.9	-	16.6±2.0	-	64.1±7.1	-	17432±3586	-	14812±3934	-
PAL+Qs	23.4±2.5	36	12.4±1.4	37	11.1±1.0	33	74.4±6.4	NR	16551±2620	5	17367±2277	NR
PAL+Qs+nFh12	6.0±1.7*	83	3.6±1.0*	82	2.1±0.8*	87	9.4±3.0*	85	3089±1001*	82	1186±523*	92
Experiment 2												
Infected	49.0±6.1	-	24.0±7.3	-	25.0±8.8	-	61.2±8.3	-	18008±2362	-	18197±2079	-
PAL+Qs	31.6±3.4	35	18.6±2.1	22	13.5±1.2	46	71.0±6.2	NR	17098±2706	5	21700±2968	NR
PAL+Qs+rFh15	21.8±2.5*	56	9.0±1.2*	63	12.8±1.5*	49	18.7±2.2*	69	14247±668	21	12724±488	30
Experiment 3												
Infected	34.5±6.9	-	18.0±3.6	-	16.5±3.4	-	61.8±14.4	-	9986±2360	-	7748±1315	-
AA0029+Qs	42.5±8.0	NR	20.0±4.4	NR	22.5±3.9	NR	77.0±19.0	NR	13242±1597	NR	8084±775	NR
AA0029+Qs+rFh15	12.5±3.8*	64	5.5±2.0*	69	7.0±2.0*	58	20.6±14.0*	67	3872±1814*	61	1800±730*	77
AA0029+Qs+rFh15b	25.1±7.8	27	10.1±3.2*	44	15.0±4.8	9	15.2±7.1*	75	4692±1181*	53	3098±800*	60

NR no-reduction. $p < 0.05$ in comparison with infected controls

Table 2. Cytokine production (TNF-α, IL-6, IL-1α IFNγ, IL-2, IL-4, IL-10, IL-17) in supernatants of splenocyte cultures in untreated BALB/c mice, treated with AA0029+Qs and immunised with AA0029+Qs+rFh15 and AA0029+Qs+rFh15b two weeks after immunisation schedule. (Mean ± standard error of the mean)

	Untreated	AA0029+Qs	AA0029+Qs+rFh15	AA0029+Qs+rFh15b
Cytokine (pg/ml)				
TNFα	313 ±98	214 ± 20	937 ± 130*	1074±89*
IL-6	964 ±118	1318 ± 137	2755 ± 226*	1613±137*†
IL-1α	527 ± 65	368 ± 32	448 ± 23	581±142
IFNγ	543 ± 35	643 ± 16	735 ± 23	890±79*
IL-2	592 ± 74	774 ± 84	1025 ± 47*	888±41*
IL-4	1138 ± 101	1508 ± 82	2078 ± 145*	1653±18*
IL-10	481 ± 46	485 ± 39	424 ± 7	459±21
IL-17	1724 ± 167	2048 ± 43	2053 ± 46	1988±268

* $p < 0.05$ in comparison with untreated controls and treated with AA0029+Qs. † $p<0.05$ compared to mice treated with AA0029+Qs+rFh15

Table 3. Percentages of splenocyte populations (CD45, CD4, CD8, CD197, CD62L, CD27, B220) in untreated BALB/c mice, treated with AA0029+Qs and immunised with AA0029+Qs+rFh15 and AA0029+Qs+rFh15b two weeks after immunisation schedule. (Mean ± standard error of the mean)

	Untreated	AA0029+Qs	AA0029+Qs+rFh15	AA0029+Qs+rFh15b
Cell percentages				
CD45	75.7 ± 3.4	77.0 ± 0.7	75.5 ± 2.8	66.7±1.3
CD4	21.1 ± 1.3	20.7 ± 0.4	21.3 ± 0.5	21.7±4.0
CD8	8.4 ± 0.5	8.4 ± 0.6	10.2 ± 0.6	9.5±1.3
CD197	16.9 ± 1.7	18.0 ± 2.1	12.6 ± 2.8	14.9±0.6
CD62L	23.2 ± 3.2	20.1 ± 5.0	17.2 ± 0.9	15.2±5.0
CD27	19.4 ± 1.9	18.0 ± 1.6	16.9 ± 0.8	16.7±3.6
B220	35.9 ±3.2	39.4 ± 0.6	23.2 ± 1.7	21.3±0.7*

* $p < 0.05$ in comparison with untreated controls and treated with AA0029+Qs.

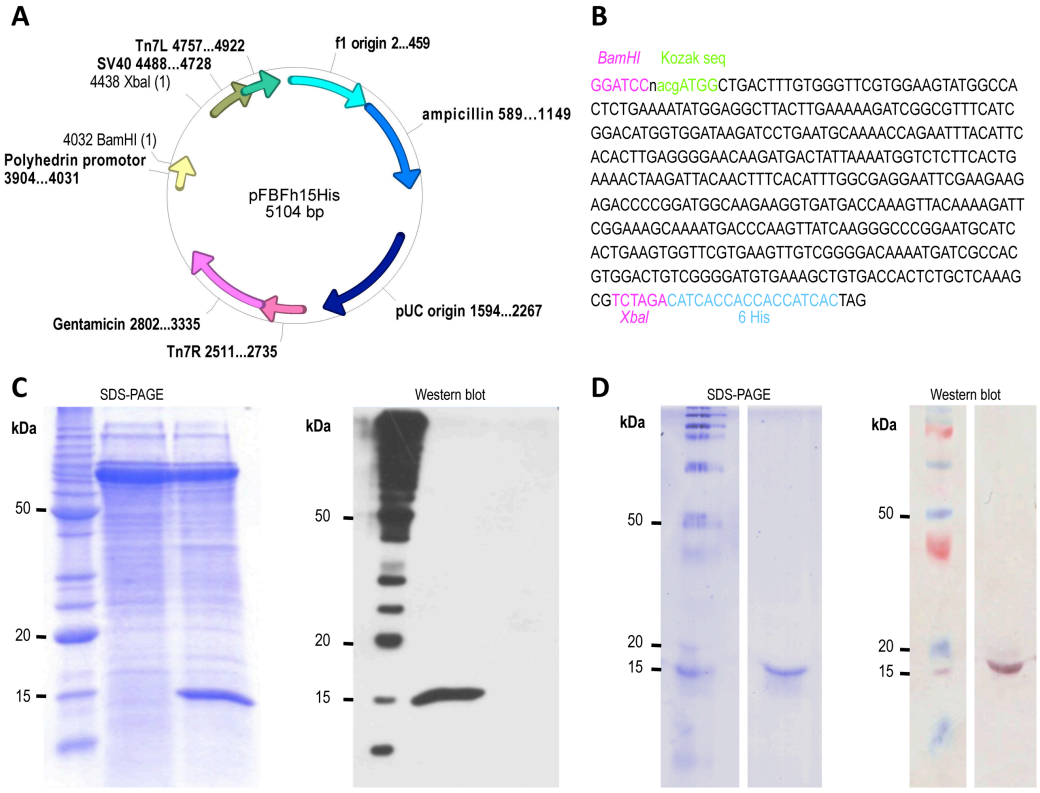

Figure 1. The expression and purification of rFh15b using the baculovirus system. A. The generated vector pFBFh15His. B. The nucleotide sequence from Fh15, including the Kozak sequence, the C-terminus 6-His tag and the restriction sequences for BamHI and Xbal, C. The expression of rFh15b detected with Coomassie blue staining (lane 1, molecular weight marker; lane 2: non-induced baculovirus; lane 3, induced baculovirus) and Western blot using anti-6His monoclonal antibody (lane 1, molecular weight marker; lane 2, non-induced baculovirus; lane 3: IPTG induced baculovirus). D. Purification of rFh15b by affinity chromatography detected with Coomassie blue staining and Western blot using anti-6His monoclonal antibody

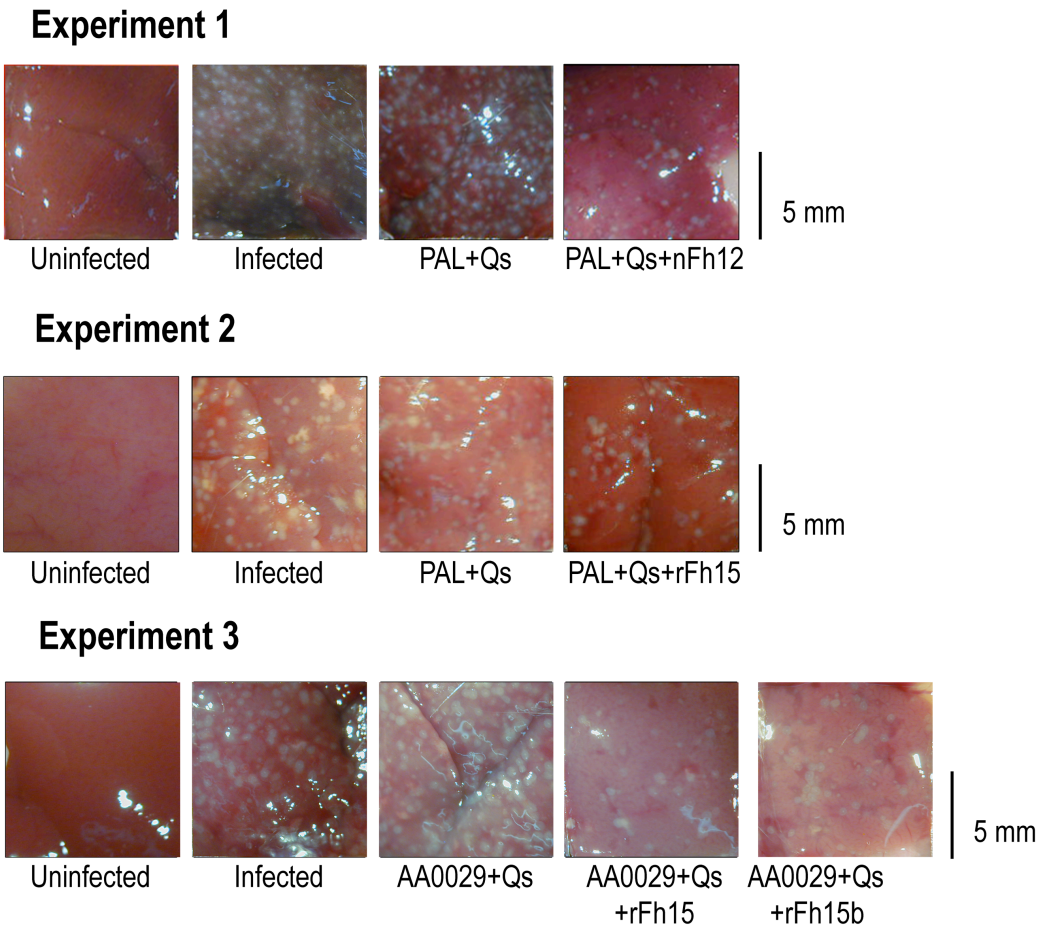

Figure 2. Representative hepatic lesion area reduction in BALB/c mice after vaccination. Natural and recombinant FABP (nFh12, rFh15 or rFh15b) formulated with the adjuvant adaptation (ADAD) vaccination system were used with the natural immunomodulator PAL or the synthetic AA0029 and challenged with 150 cercariae of *S. mansoni,* in three separated experiments.

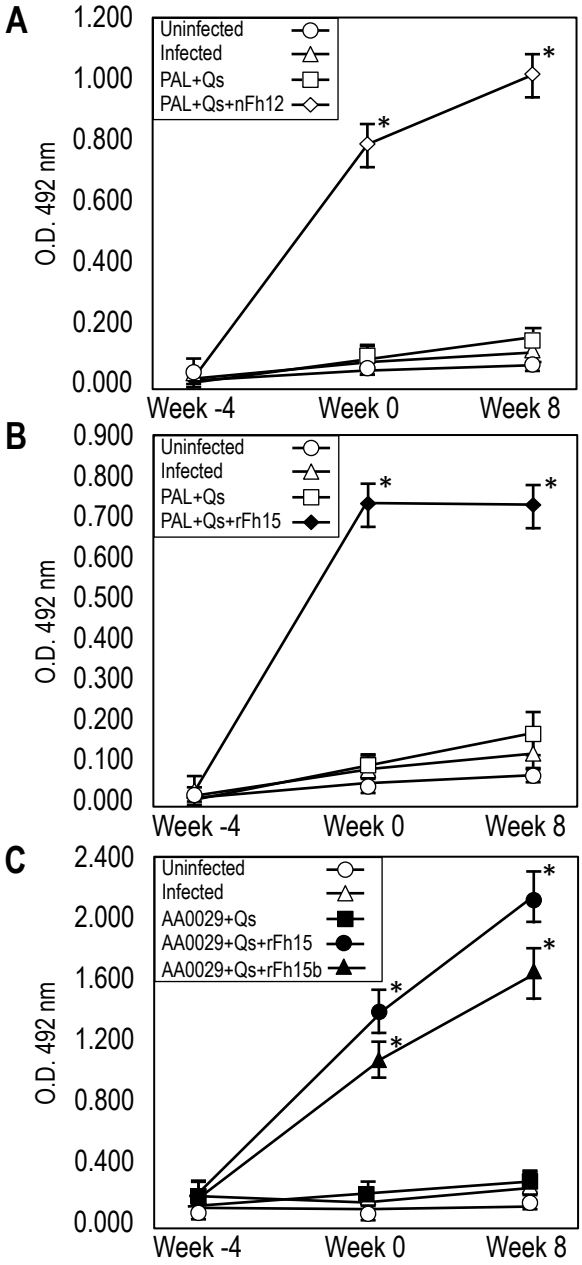

Figure 3. **Serum specific IgG antibody levels by ELISA during vaccination trials against nFh12, rFh15 or rFh15b**. BALB/c mice were vaccinated with their respective antigens formulated with the adjuvant adaptation (ADAD) vaccination system with the natural immunomodulator PAL or the synthetic AA0029 and challenged with 150 cercariae of *S. mansoni*. A, Vaccination using nFh12 formulated with PAL. B, Vaccination with rFh15 using PAL. C, Vaccination using rFh15 or rFh15b formulated with AA0029. O.D. optical densities. * $p<0.05$ compared to uninfected controls.

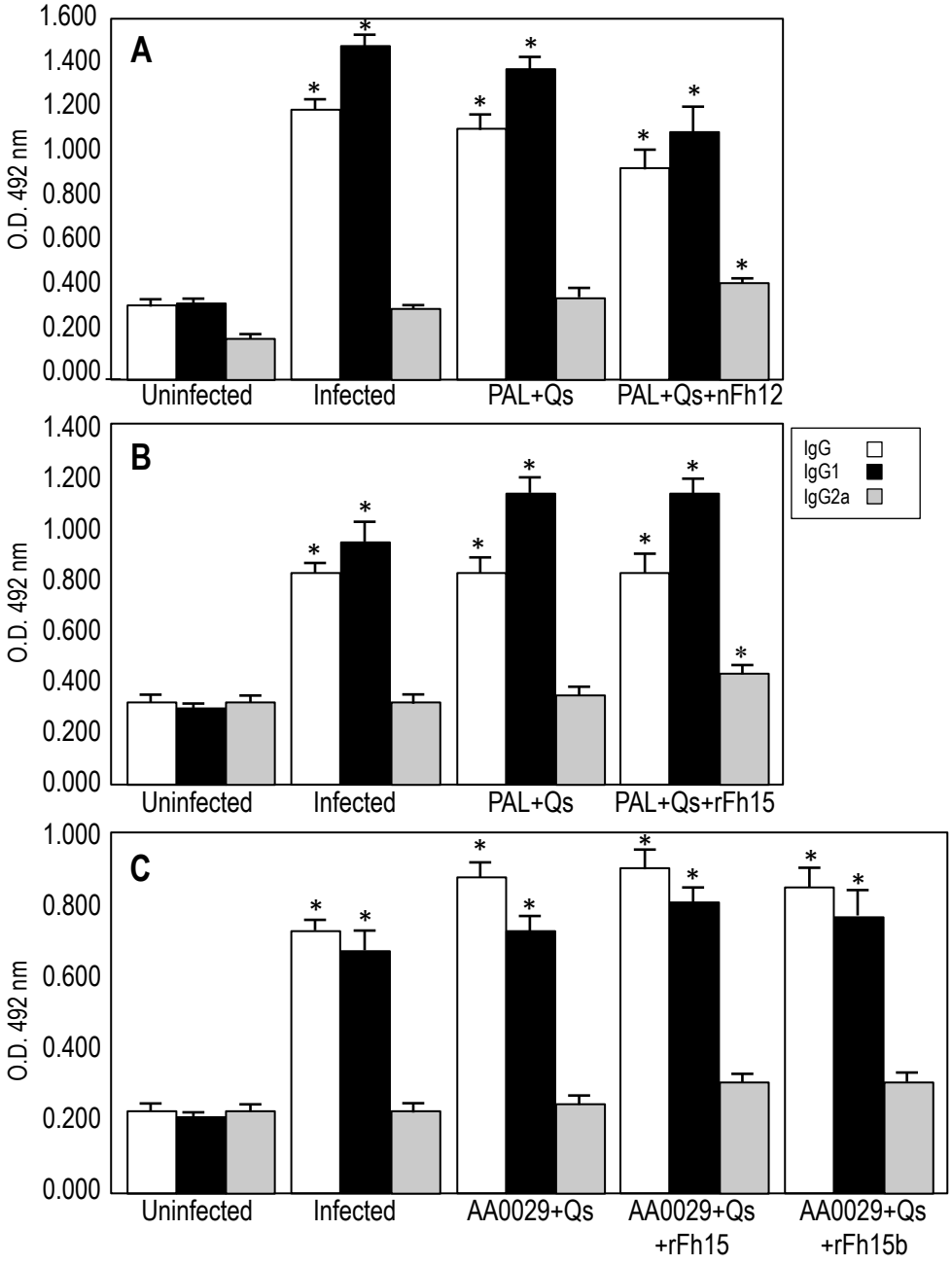

Figure 4. Serum specific IgG, IgG1 and IgG2a antibody levels by ELISA 8 weeks post-challenge against soluble adult worm antigens from *S. mansoni* (SoSbAWA). BALB/c mice were vaccinated with their respective antigens formulated with the adjuvant adaptation (ADAD) vaccination system with the natural immunomodulator PAL or the synthetic AA0029 and challenged with 150 cercariae of *S. mansoni*. A, Vaccination with PAL+Qs+nFh12+PAL. B, Vaccination with PAL+Qs+rFh15. C, vaccination using AA0029+Qs+rFh15 and AA0029+Qs+rFh15b. O.D. optical densities. * $p<0.05$ compared to uninfected controls.

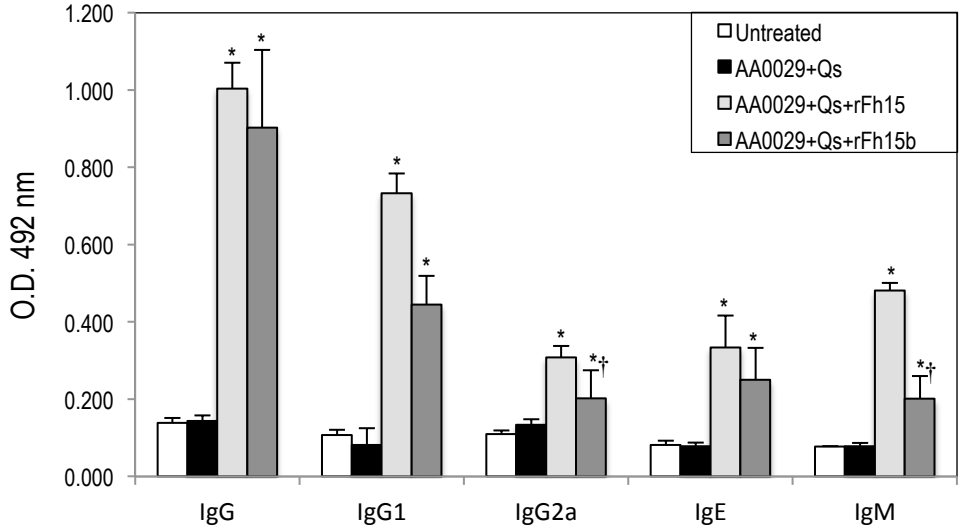

Figure 5. Antibody detection (IgG, IgG1, IgG2a, IgE and IgM) against rFh15 or rFh15b two weeks after immunisation schedule in BALB/c mice. Groups: Untreated, Treated with AA0029+Qs, Immunised with AA0029+Qs+rFh15 and Immunised with AA0029+Qs+rFh15b**.** (Mean ± standard error of the mean). O.D. optical density.v* $p < 0.05$ in comparison with untreated controls and treated with AA0029+Qs. † $p<0.05$ compared to mice treated with AA0029+Qs+rFh15.