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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

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HIGHLIGHTS

- rFh15 induces protection against the challenge by *S. bovis* in BALB/c mice and *Mesocricetus auratus* models.
- Immunomodulation with synthetic compounds is useful to induce immunoprotection against *S. bovis* infection.
- ADAD system using AA0029 as immunomodulator offers a new vaccination strategy.
- rFh15 formulated in ADAD system with AA0029 induces high level of TNFα, IL-2, IL-6 and IL-4.

GRAPHICAL ABSTRACT

Protection of mice and hamsters against *Schistosoma bovis* (mean±SEM) using rFh15 formulated with AA0029 with adjuvant adaptation (ADAD) vaccination system

Groups	Worms			EPG		EPG/Female		Lesion score
	Total	Females	Males	Liver	Intestine	Liver	Intestine	
BALB/c mice								
Infected	12.4±1.0	5.3±0.6	7.1±0.6	1107±275	709±238	209±52	134±45	2.75±0.49
AA0029+Qs	9.6±1.4	4.4±0.7	5.2±0.7	630±98	415±78	143±22	94±18	2.59±0.28
AA0029+Qs+rFh15	4.4±1.4*	2.0±0.7*	2.4±0.7*	377±141*	163±35*	189±71	82±18	1.60±0.51*
Golden hamsters								
Infected	56.3±11.3	23.2±5.0	33.2±8.6	9333±2881	15125±2308	402±179	652±166	2.87±0.40
AA0029+Qs	12.7±7.6*	6.2±2.8*	6.5±2.7*	2044±598*	6894±3844*	330±73	1112±197	2.00±0.41
AA0029+Qs+rFh15	9.3±1.7*	4.5±0.7*	4.8±0.7*	950±391*	581±397*	211±54*	129±136*	1.25±0.25*

EPG eggs per gram. SEM: standard error of the mean. * Significant reduction compared to their respective infection controls ($p<0.05$) .

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ABSTRACT

Fatty acid binding proteins (FABP) from *Fasciola hepatica* have demonstrated immune cross-protection against schistosomes. The present study was conducted to develop a new formulation of the recombinant FABP rFh15 with the synthetic immunomodulator AA0029 in the adjuvant adaptation (ADAD) vaccination system and to evaluate its ability to induce immune response and protection against the challenge with *Schistosoma bovis* cercariae. Immunization of BALB/c mice showed high levels of TNFα, IFNγ, interleukin (IL)-2, IL-6, and IL-4 in splenocyte supernatant culture and also high levels of serum specific anti-rFh15 IgG, IgG1, IgG2a IgE and IgM antibodies suggesting a mixed Th1/Th2 immune response. Using this approach, high levels of protection against experimental challenge with *S. bovis* cercariae were observed in the mouse and hamster models. A marked reduction up to 64% in worm burden, as well as in the number of eggs retained in liver (66%) and intestine (77%) and hepatic lesions (42%), was achieved in vaccinated BALB/c mice. Golden hamsters vaccinated and challenged in similar conditions had reductions in recovered worms (83%), liver eggs (90%), intestine eggs (96%), liver lesions (56%) and worm fecundity (48–80%). These data suggest that formulation of rFh15 in the ADAD vaccination system using the AA0029

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immunomodulator could be a good option to drive an effective immunological response against schistosomiasis.

1. Introduction

The blood fluke *Schistosoma bovis* is an important cause of disease in domestic ruminants in Africa, Southwest Asia and the Mediterranean Europe. *S. bovis* belongs to the *S. haematobium* group which has species affecting humans (*S. haematobium*, *S intercalatum*, *S. guineensis*) and domestic animals (*S. mattheei*, *S. magrebowiei*, *S. leiperi* and *S. curassoni*) (Webster et al., 2013). The infective stage, cercariae, spin around in freshwater seeking the skin of a suitable final host, they penetrate the dermis and transform into schistosomula. Then, they enter into the blood vessels and migrate to lungs, heart, liver and finally the portal–mesenteric system in which they mature into adult males and females, and live for years despite the intense immune response displayed by the host. Embryonated eggs are either eliminated in feces or trapped in tissues developing severe intestinal and liver chronic disease. Most *S. bovis* infections in grazing ruminants in endemic areas occur at a subclinical level causing significant losses due to long-term effects on ruminants as well as to an increased susceptibility to other pathogens (de Bont and Vercruysse, 1998; Vercruysse and Gabriel, 2005). Natural infections elicit a concomitant immunity acting through a reduction of female fecundity observed with reductions in fecal and tissue eggs without effects in worm burden that seems produced by serum factors (Vercruysse and Gabriel, 2005). Also, high levels of IL-4 were observed in mice with primary infections by *S. bovis* (Uribe et al., 2007). For many years, animal and human schistosomiasis control strategies have been based on control of intermediate freshwater snail and mass continuous treatment of final hosts, in particular with praziquantel. These measures have not represented a definitive solution due the high rate of post-treatment reinfections and its limited effect on morbidity and mortality reductions (Doenhoff et al., 2009; Pérez del Villar et al., 2012). A vaccine appears as a very valuable additional complement to mass chemotherapy in long-term disease control strategy. Vaccination is based on the partial resistance developed against the natural infection, the protection induced by irradiated cercariae and the cross-resistance stimulated by other flukes as *Fasciola hepatica* (Hewitson et al., 2005; Rodríguez-Osorio et al., 1993; Vercruysse and Gabriel, 2005).

Glutathione-S transferase (GST), 14-3-3ζ from *S. bovis* and cross-reacting fatty acid binding proteins (FABP) from *F. hepatica* have been proposed as potential vaccine candidates. GST and FABP have been used with Freund's adjuvant in several experimental infection models showing reductions in fluke burden, liver damage or egg hatchability (Abán et al., 1999; Abáné et al., 2000; Boulanger et al., 1999; Bushara et al., 1993; da Costa et al., 1999). However Freund's adjuvant cannot be used in a hypothetical commercial vaccine due to its side effects. Maximum protection in experimental vaccines depends on both humoral and cellular mechanisms; therefore new adjuvant systems should be introduced. An alternative approach to the classical Freund's adjuvant is the adjuvant adaptation (ADAD) vaccination system, that combines the vaccine antigen together with non-hemolytic saponins from *Quillaja saponaria* and an immunomodulator, forming an emulsion with the non-mineral oil Montanide ISA 763AVG for vaccination against *F. hepatica* with FABP (Martínez-Fernández et al., 2004). Trials with the 14-3-3ζ protein from *S. bovis* and FABP from *F. hepatica* formulated in ADAD system have been previously done (Uribe et al., 2007; Vicente et al., 2014). In this work we include the synthetic diamine AA0029, that has demonstrated immunomodulatory properties such as inhibition of lymphoproliferation, modulation of delayed type hypersensivity, modified ratios of CD8+, CD4+, and MHC-Class II cells, and increased nitric oxide production in LPS pre-stimulated rat alveolar macrophages (del Olmo et al., 2006). Furthermore, vaccination with FABP from *F. hepatica* formulated in ADAD with AA0029 showed less hepatic damage after the challenge with the liver fluke and resistance to lethal infection (López-Abán et al., 2012). Mouse is the most common model in vaccine development against schistosomiasis and golden hamster is a suitable host to maintain life cycle of *S. bovis* and preclinical studies before to test in sheep (Oleaga and Ramajo, 2004).

The objective of this article is to characterize both humoral and cellular immune responses induced by FABP recombinant protein from *F. hepatica* formulated in ADAD vaccination system using the new synthetic immunomodulator AA0029 in BALB/c mice. Moreover immunoprotection levels will be studied using two *S. bovis* experimental infection models, BALB/c mice and *Mesocricetus auratus*.

2. Materials and methods

2.1. Animals and parasites

Fifty-four 7-week-old female BALB/c mice (Charles River, Lyon, France) weighing 18–20 g were used in the study. Animals were maintained in a temperature and humidity controlled environment with a 12 h light/dark cycle with free access to water and food at the University of Salamanca's Animal Experimentation facilities. Eighteen 7-week-old female golden hamsters (*Mesocricetus auratus*, Charles River) weighing 100–120 g were housed at the Animal Experimentation Unit of IRNA-CSIC. Animal procedures used in this study complied with the Spanish (L32/2007, L6/2013 and RD53/2013) and the European Union (Di 2010/63/CE) regulations on animal experimentation. The University of Salamanca's Ethics Committee approved procedures used in the present study (protocol 48531). A strain of *S. bovis* from Salamanca (Spain) was maintained in the Department of Animal Pathology of IRNASA-CSIC in *Planorbarius metidjensis* as intermediate host and sheep as definitive host (Oleaga and Ramajo, 2004). The number of cercariae and their viability were determined using a stereoscopic microscope.

2.2. Antigens

Soluble adult worm antigens from *S. bovis* (SoSbAWA) used for ELISA were prepared as previously described (Abán et al., 1999). Twenty adult worms were suspended in 1 mL of sterile phosphate-buffered saline (PBS) containing 1 mM phenyl-methyl-sulphonyl fluoride (PMSF; Sigma, St. Louis, MO), homogenized, frozen and thawed thrice and then sonicated thrice (70 kHz) for 1 min each. The suspension was centrifuged at 20,000 g for 30 min at 4 °C. A recombinant FAPB from *F. hepatica* (rFh15) was used for immunizations and ELISA, and it was prepared in accordance with López-Abán et al. (2012). Briefly, total RNA from one *F. hepatica* adult worm was isolated and used for cDNA synthesis. The rFh15 gene (accession number M95291.1) was amplified using the following primer sequences: forward 5′-GGATCCATGGCTGACTTTGTGGG-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* GST sequence for further detection and purification. The resulting recombinant DNA plasmid was purified and then sequenced to verify integrity of the cloned insert. Transformed *Escherichia 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 18,000 *g* for 30 min, suspended in PBS with 1 mM PMSF and 1% Triton X-100 sonicated and centrifuged. Solubilized protein was purified by affinity chromatography with a glutathione Sepharose 4B resin. Non-retained proteins were eluted with PBS and the rFh15 protein was eluted adding PBS plus thrombin. Protein purity was assessed by SDS–PAGE and quantified by bicinchoninic acid (BCA) method.

2.3. ADAD vaccination system

The rFh15 protein was formulated in a micelle composed by non-hemolytic saponins from *Q. saponaria* (Qs; Sigma, St. Louis, Missouri) and the synthetic aliphatic diamine AA0029. Then, this micelle was emulsified in a non mineral oil (Montanide ISA763A, SEPPIC, Paris, France) as an oil/water 70/30 and subcutaneously injected. The ADAD vaccination system consists of a set of two subcutaneous injections. The first injection, called "Adaptation", contains AA0029 and Qs emulsified in the non-mineral oil. The second injection, administered 5 days after the adaptation, contains the rFh15 antigen with AA0029 and Qs in the emulsion oil. In control adjuvant group (AA0029 + Qs) two injections of AA0029 and Qs were administrated. Individual doses per injection in mice included in each immunization, 100 µg of AA0029, 20 µg of Qs and 10 µg of rFh15 in a final volume of a 200 µL injection of emulsion with the non-mineral oil. In hamsters each dose contained 100 µg of AA0029, 20 µg of Qs and 20 µg of rFh15 in a final volume of a 200 µL was used (Martínez-Fernández et al., 2004; Uribe et al., 2007). The lipidic diamine AA0029 was obtained from the corresponding 2-amino-hexadecanoic acid (which was also properly obtained from diethyl acetamidomalonate and 1-bromotetradecane); the amino group was protected as a *tert*-butyl carbamate (*Boc*) and the acid group reduced to an alcohol (the acid was transformed into a mixed hydride and then reduced with sodium borohydride). Afterwards, the hydroxyl group was first mesylated, then transformed into the corresponding azide and further reduced to a diamine, resulting in the diamine AA0029 [*tert*-butyl (1-aminohexadecan-2-yl)carbamate] (del Olmo et al., 2006).

2.4. Immunological assessment of rFh15 using AA0029 in ADAD vaccination system in BALB/c mice

Three groups of six female BALB/c each were used for characterization of immunological response: Untreated, Injected with AA0029 + Qs as adjuvant control, and Immunized with rFh15 formulated in ADAD system (AA0029 + Qs + rFh15). Firstly, mice were immunized on week 0 and identical booster doses were administered 2 and 4 weeks after. Two weeks after the immunization schedule the mice were anesthetized 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 (IgG, IgG1, IgG2a, IgM and IgE) detection from each animal before each immunization and at the necropsy.

2.5. Cytokine measurement

Splenocytes obtained from individual mice were cultured in a 6-well plate at 1×10^6 cells per well concentration in complete RPMI 1640 medium containing 10% heat-inactivated fetal calf serum L-glutamine, 5 mM and antibiotics: 100 units/mL penicillin and 100 µg/mL streptomycin (López-Abán et al., 2007). Splenocytes from immunization and control mice were stimulated *in vitro* with rFh15 at a final concentration of 10 µg/mL for 72 hours at 37 °C in a humidified atmosphere with 5% CO_2. Control wells were also prepared containing splenocytes from untreated mice. Culture supernatants were recovered for cytokine determination. A flow cytometry-based technique was used for interferon γ (IFNγ), and tumor necrosis factor α (TNFα), interleukin (IL)-1α, IL-2, IL-4, IL-6, IL-10 and IL-17 cytokine quantitation was carried out in each of the groups of mice used in this study. A FlowCytomix Mouse Th1/Th2 10plex kit (Bender MedSystems GmbH, Vienna, Austria) was used, according to the manufacturer's instructions. Briefly, different sized 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 h at room temperature. The specific antibodies bind to the cytokines captured by the first antibodies. After washing the tubes with PBS plus 2% fetal 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 were 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 were analyzed using FlowCytomix Pro 3.0 software (Bender MedSystems, Vienna, Austria). Each cytokine concentration was determined from standard curves using known mouse recombinant cytokine concentrations. Results were expressed as mean and standard error of the mean (SEM).

2.6. Flow cytometry analysis of T- cell splenocyte populations

Splenocytes from untreated, AA0029 + Qs treated and rFh15 immunized mice were incubated with the blocking anti-CD16/CD32 monoclonal antibody for 5 minutes at room temperature and stained with commercial fluorochrome-conjugated antibodies at 1/50 dilution in PBS plus 2% fetal calf serum for 30 minutes at 4 °C. 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% fetal calf serum and then centrifuged at 1000 *g* for 5 min and the supernatant was discarded. Then cells were fixed with 100 µL of a 2% paraformaldehyde solution for 1 h at 4 °C. Phenotypic analyses were performed in a FACScalibur flow cytometer at the University of Salamanca's Flow Cytometry Central Service. Data were collected on 30,000 events (gated by forward and side scatter) and analyzed using Gatelogic Flow Cytometry Analysis Software (INIVAI Technologies Pty Ltd). Results were expressed as mean and SEM.

2.7. Vaccination experiment schedules

Two independent experiments were carried out with mice and golden hamsters for the vaccination challenge. BALB/c mice were randomly divided in four groups of 9 animals each as follows: Untreated and uninfected, *S. bovis* infected, Injected with ADAD with AA0029 + Qs and infected, Vaccinated with ADAD plus AA0029 + Qs + rFh15 and infected. Animals were vaccinated and boosted 2 weeks after the first immunization. Two weeks after the immunization schedule, each mouse was challenged with *S. bovis* cercariae by the "ring method". Mice were restrained with a mixture of ketamine 50 mg/kg, diazepam 5 mg/kg and atropine 1 mg/kg injected intraperitoneally and then a suspension of 150 cercariae in 1 mL of mineral water was poured on the abdominal region during 45 min. Eight weeks post-infection all mice were euthanized with intraperitoneal injection of sodium pentobarbital (60 mg/kg) and then perfused by intra-cardiac injection of PBS plus heparin, and the number of recovered *S. bovis* adult worms from the portal and mesenteric veins was recorded. In addition, the number of parasite eggs per gram (EPG) of liver and intestine was counted after digestion with 25 mL of 5% KOH (16 h at 37 °C with gentle shaking) using a McMaster camera. The relationship between eggs and the number of females was evaluated as a measurement of a possible anti-fecundity effect. Macroscopic lesions of liver were quantified considering changes in size, consistency, color, blood vessels, and presence of schistosomal pigment and scored as follows (0) no lesions, (1) mild, (2) moderate, and (3) intense. Protection rates were calculated with the following formula: (mean infected control group recovered worms – mean experimental group recovered worms) × 100/mean infected control group recovered worms. Blood samples were collected from each animal before immunization, infection and necropsy for humoral immune response studies.

A second experiment was performed using golden hamsters. Animals were randomly divided into three groups of 6 each as follows: *S. bovis* infected, Injected with ADAD with AA0029 + Qs and infected, Vaccinated with ADAD with AA0029 + Qs + rFh15 and infected. Two weeks after the first immunization animals were boosted with the same doses. Two weeks after the immunization schedule, each animal was challenged with 200 *S. bovis* cercariae for 45 min as above. All animals were euthanized using an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and perfused 8 weeks after the infection as above. Adult worm burden, number of parasite eggs in liver and intestine, fecundity rates were recorded and percentages of reduction were calculated. Macroscopic lesions of liver were quantified as earlier criteria. Serum samples were collected during experiment for humoral immune response study.

2.8. Anti-rFh15 and SoSbAWA specific antibody production

Specific anti-soluble *S. bovis* adult worm antigens (SoSbAWA) or anti-rFh15 antibodies profiles were measured using an indirect ELISA as described by Abán et al. (1999). Briefly, 96-well polystyrene plates (Costar) were coated with 2.5 µg of SoSbAWA or 2.0 µg of rFh15 antigen for 12 h 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 h at 37 °C, followed by the addition

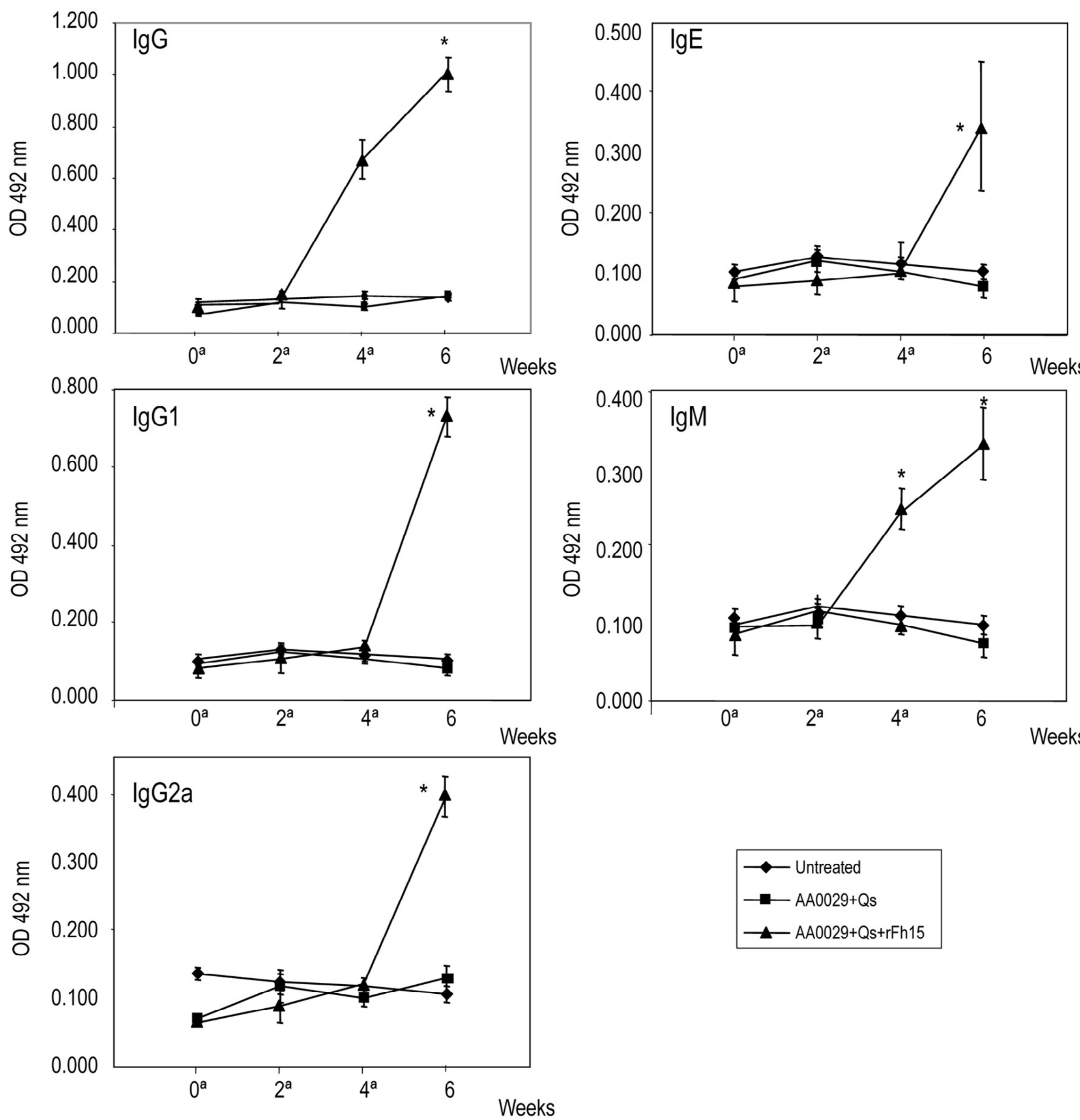

Fig. 1. Serum anti-rFh15 specific IgG, subtypes IgG1 and IgG2a, IgE and IgM antibody levels (mean ± SEM) during the immunization experiment by ELISA. BALB/c mice were immunized with AA0029 + Qs + rFh15 using the adjuvant adaptation (ADAD) vaccination system. Untreated and AA0029 + Qs treated controls were used. OD optical density *$p < 0.05$ compared to untreated control. [a]Immunization.

of goat peroxidase-labeled anti-mouse, IgG1, IgG2a, IgM or IgE antibodies at 1:1000 dilution (Sigma) or anti-hamster IgG antibodies at 1:1000 dilution (Sigma). The reaction was developed with H_2O_2 and ortophenilenediamine (Sigma) in citrate buffer (pH 5.0) and the absorbance was measured at 492 nm with an Ear400FT ELISA reader (Lab Instruments). Results were expressed as mean and standard error of mean (SEM).

2.9. Statistical analysis

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 when appropriate. All statistical analyses were considered significant at the $p < 0.05$. SPSS 21 software (IBM) was used for data analysis.

3. Results

3.1. Immune response induced by immunization with rFh15 in ADAD system using AA0029 as immunomodulator

Innate pro-inflammatory (TNFα, IL-6), Th1 (IFNγ, IL-1α, IL-2), Th2 (IL-4), regulatory (IL-10) and Th17 (IL-17) cytokine levels was measured in cultured splenocyte supernatants. It was observed that the AA0029 + Qs + rFh15 combination stimulated significant increase of TNFα ($p = 0.049$), IL-6 ($p = 0.001$), IL-2 ($p = 0.036$) and IL-4 ($p = 0.001$) compared to untreated control group. However no differences were

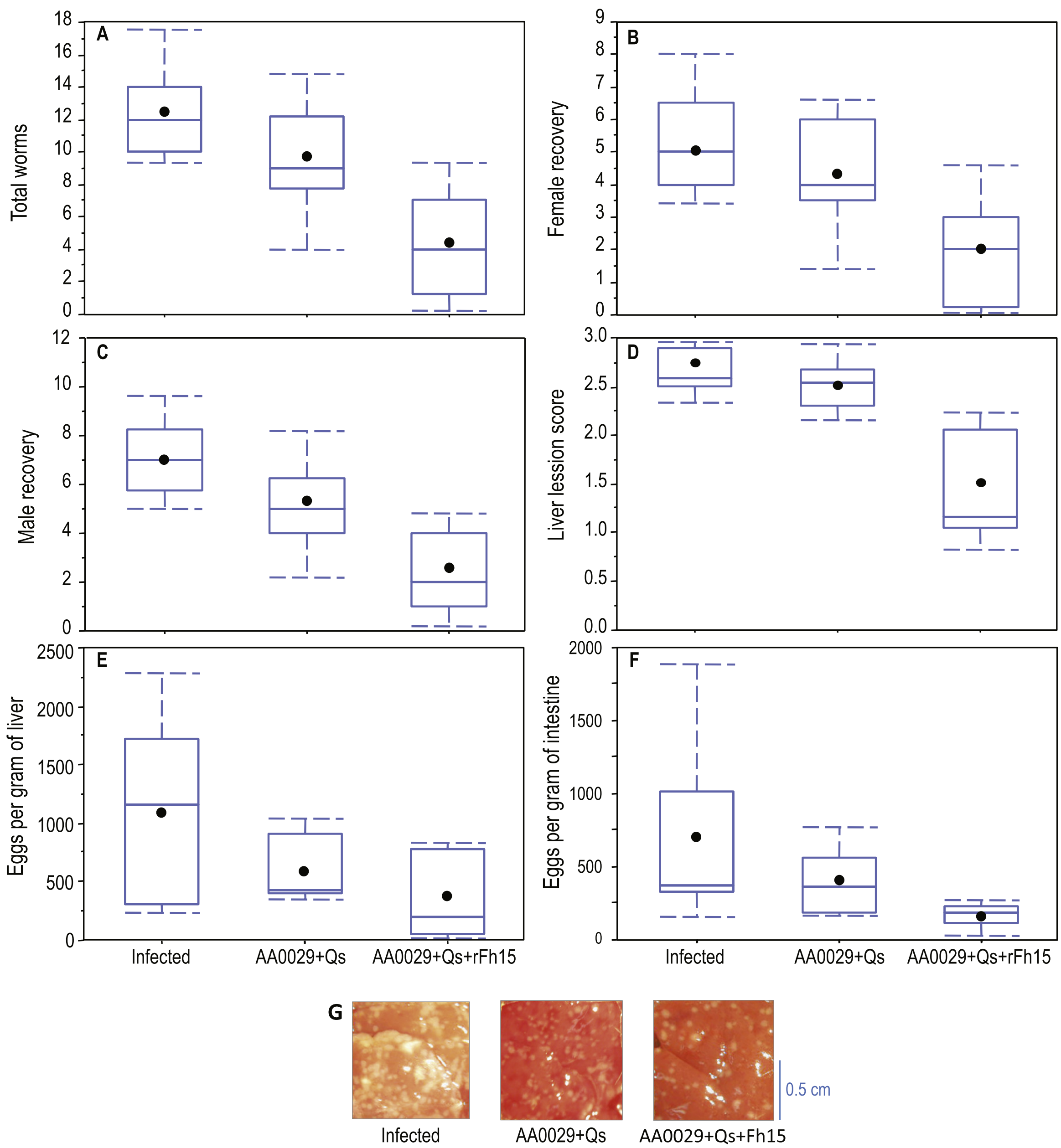

Fig. 2. Boxplots with number of total recovered worm (A), females (B), males (C), hepatic damage (D), eggs per gram of liver (E) and egg per gram of intestine (F), and representative photographs of the hepatic damage in the groups (G) in BALB/c mice vaccinated with AA0029 + Qs + rFh15 using the adjuvant adaptation (ADAD) vaccination system, challenged with 150 cercariae of *Schistosoma bovis* and perfused 8 weeks post-challenge. Data are represented in boxplots with mean (solid symbol), median, Q1 and Q3 (box), percentiles 10 and 90 (error bars).

Table 1
Interleukin (IL)-6, TNF-α, IL-1α IFNγ, IL-2, IL-4, IL-10, IL-17 production by splenocytes and percentages of CD45, CD4, CD8, CD197, CD62L, CD27 and B220 splenocyte populations 2 weeks after immunization (mean ± SEM). BALB/c mice were immunized with PAL+Qs + Fh15 using the adjuvant adaptation (ADAD) vaccination system. Untreated mice and mice treated with AA0029 + Qs were used as controls.

	Untreated	AA0029 + Qs	AA0029 + Qs + rFh15
Cytokine concentration (pg/mL)			
TNFα	313 ± 98	214 ± 20	937 ± 130*
IL-6	964 ± 118	1318 ± 137	2755 ± 226*
IL-1α	527 ± 65	368 ± 32	448 ± 23
IFNγ	543 ± 35	643 ± 16	735 ± 23
IL-2	592 ± 74	774 ± 84	1025 ± 47*
IL-4	1138 ± 101	1508 ± 82	2078 ± 145*
IL-10	481 ± 46	485 ± 39	424 ± 7
IL-17	1724 ± 167	2048 ± 43	2053 ± 46
Cell percentages			
CD45	75.7 ± 3.4	77.0 ± 0.7	75.5 ± 2.8
CD4	21.1 ± 1.3	20.7 ± 0.4	21.3 ± 0.5
CD8	8.4 ± 0.5	8.4 ± 0.6	10.2 ± 0.6
CD197	16.9 ± 1.7	18.0 ± 2.1	12.6 ± 2.8
CD62L	23.2 ± 3.2	20.1 ± 5.0	17.2 ± 0.9
CD27	19.4 ± 1.9	18.0 ± 1.6	16.9 ± 0.8
B220	35.9 ± 3.2	39.4 ± 0.6	23.2 ± 1.7

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

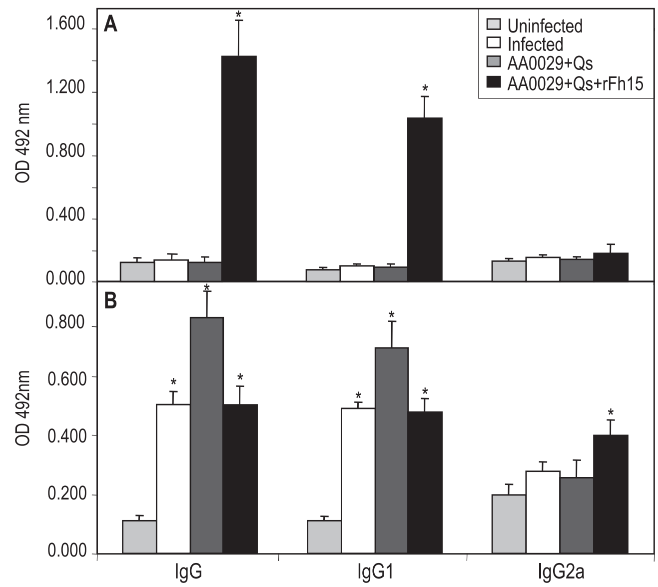

Fig. 3. Serum specific IgG, IgG1 and IgG2a antibody levels by ELISA 8 weeks post-challenge against rFh15 (A), and against soluble adult worm antigens from *S. bovis* (SoSbAWA) (B). BALB/c mice were vaccinated with rFh15 using the adjuvant adaptation system (ADAD), challenged with 150 cercariae of *Schistosoma bovis* and perfused 8 week post-challenge. OD optical densities *$p < 0.05$ in comparison with uninfected controls.

detected regarding Th17 and Treg cytokine levels (Table 1). Mice treated only with AA0029 + Qs showed similar cytokine levels to the untreated controls (Table 1). With regard to the percentage of splenocyte populations in AA0029 + Qs + rFh15 immunized mice, a slight reduction was observed in B220, even though the differences between groups were not statistically significant. No differences in T and B splenocyte populations were observed in mice treated only with AA0029 + Qs (Table 1). Specific antibodies (IgG, IgG1, IgG2a, IgM and IgE), which increased against rFh15 antigen, were evaluated by ELISA in sera of mice along the immunization experiment (Fig. 1). A significant IgG and IgM enhancement was observed at week 4 of the experiment in AA0029 + Qs + rFh15 immunized mice compared to untreated mice or treated with AA0029 + Qs. Additionally, significant IgE, IgG1 and IgG2a were only observed at the endpoint of the experiment ($p < 0.05$).

3.2. Evaluation of vaccination in BALB/c mice

It was observed that the AA0029 + Qs + rFh15 combination elicited a significant reduction in parasitological magnitudes. The adult worm burden became reduced by 64% (4.4 ± 1.4 *cf* 12.4 ± 1.0 $p = 0.001$), in females by 62% (2.0 ± 0.7 *cf* 5.3 ± 0.6; $p = 0.016$), in males by 66% (2.4 ± 0.7 *cf* 7.1 ± 0.6; $p = 0.001$), eggs in liver by 66% (377 ± 141 EPG *cf* 1107 ± 275 EPG; $p = 0.005$), eggs in intestine by 77% (163 ± 35 EPG *cf* 709 ± 238 EPG; $p = 0.048$), eggs per female in liver 10% (189 ± 71 EPG/female *cf* 209 ± 52 EPG/female; n.s., not significant-), eggs per female in intestine 39% (82 ± 18 EPG/female *cf* 134 ± 45 EPG/female; n.s.) and liver injuries by 42% (Score 1.60 ± 0.51 *cf* 2.75 ± 0,49; $p = 0.048$) compared to infected controls (Fig. 2). Recovered worms were all mature adults and the male/female ratio was not altered. A significant reduction of fecundity rate was not observed in vaccinated group. Moreover, mice injected only with AA0029 + Qs showed slight not-significant reduction rates regarding adult worm recovery (23% in total worms, 9.6 ± 1.4 *cf* 12.4 ± 1.0; 17% in females 4.4 ± 0.7 *cf* 5.3 ± 0.6, 27% in males 5.2 ± 0.7 *cf* 7.1 ± 0.6), liver damage (6% in score 2.59 ± 0.28 *cf* 2.75 ± 0.49), egg in tissues (43% in liver, 630 ± 98 EPG *cf* 1107 ± 275 EPG; 42% in intestine 415 ± 78 EPG *cf* 709 ± 238 EPG), and tissue eggs per female (31% in liver 143 ± 22 EPG/female *cf* 209 ± 52 EPG/female; 29% in intestine 94 ± 18 EPG/female *cf* 134 ± 45 EPG/female) compared to infected controls (Fig. 2). Antibody response along the experiment was monitored by ELISA using rFh15 and SoSbAWA antigens. A strong IgG response against rFh15 was detected in vaccinated mice compared to either control or AA0029 + Qs treated groups, after the immunization schedule and during the experiment (week 8 p.i. OD 1.556 ± 0.235 *cf* 0.145 ± 0.050; $p < 0.05$) (Fig. 3A). Increases in specific IgG and IgG1 against SoSbAWA were found only 8 weeks p.i. in all infected mice, but only AA0029 + Qs + rFh15 vaccinated mice showed a significant increase of IgG2a compared to other groups (Fig. 3B).

3.3. Evaluation of vaccination in Mesocricetus auratus

Vaccination with AA0029 + Qs + rFh15 in golden hamsters showed reductions of 83% in adult worm burden (9.3 ± 1.7 *cf* 56.3 ± 11.3; $p = 0.004$), 81% in females (4.5 ± 0.7 cf 23.2 ± 5.0; $p = 0.001$), 85% in males (4.8 ± 0.7 *cf* 33.2 ± 8.6; $p = 0.002$), 90% in liver eggs (EPG 950 ± 391 *cf* 9333 ± 2881; $p = 0.021$), 96% in intestine eggs (EPG 581 ± 397 *cf* 15,125 ± 2308; $p = 0.002$), 48% in liver eggs per female (EPG/female 211 ± 54 *cf* 404 ± 179; $p = 0.049$), 80% in intestine eggs per female (EPG/female 129 ± 136 *cf* 651 ± 166; $p = 0.027$) and 56% hepatic injuries (score 1.25 ± 0.25 *cf* 2.87 ± 0.40 $p < 0.001$) when compared with infected controls (Fig. 4). Additionally, hamsters treated only with AA0029 + Qs showed significant reduction in total adult worms (78%, 12.7 ± 7.6 *cf* 56.3 ± 11.3; $p = 0.012$), females (73%, 6.2 ± 2.8 *cf* 23.2 ± 5.0; $p = 0.032$), males (80%, 6.5 ± 2.7 *cf* 33.2 ± 8.6; $p = 0.030$), eggs in liver (EPG 78%, 2044 ± 598 *cf* 9333 ± 2881; $p = 0.039$) and eggs in intestine (EPG 54%, 6894 ± 3844 *cf* 15,125 ± 2308; $p = 0.041$). However, no-significant reduction was found in worm fecundity or liver lesion (Fig. 4). However, no reduction was observed in eggs per female in intestine (1112 ± 197 *cf* 651 ± 166) and no-significant reduction was found in eggs per female in liver (18%, 330 ± 73 *cf* 402 ± 179) or liver lesion score (30%, 2.00 ± 0.41 *cf* 2.87 ± 0.40) (Fig. 4). Using rFh15 as antigen in ELISA we observed significant increased IgG at the time of the infection and after 8 weeks p.i. in AA0029 + Qs + rFh15 vaccinated animals compared to non-vaccinated indicating that all animals were correctly vaccinated (Fig. 5). High levels of specific IgG antibodies anti-SoSbAWA were observed in all infected animals at week 8 p.i. (Fig. 5).

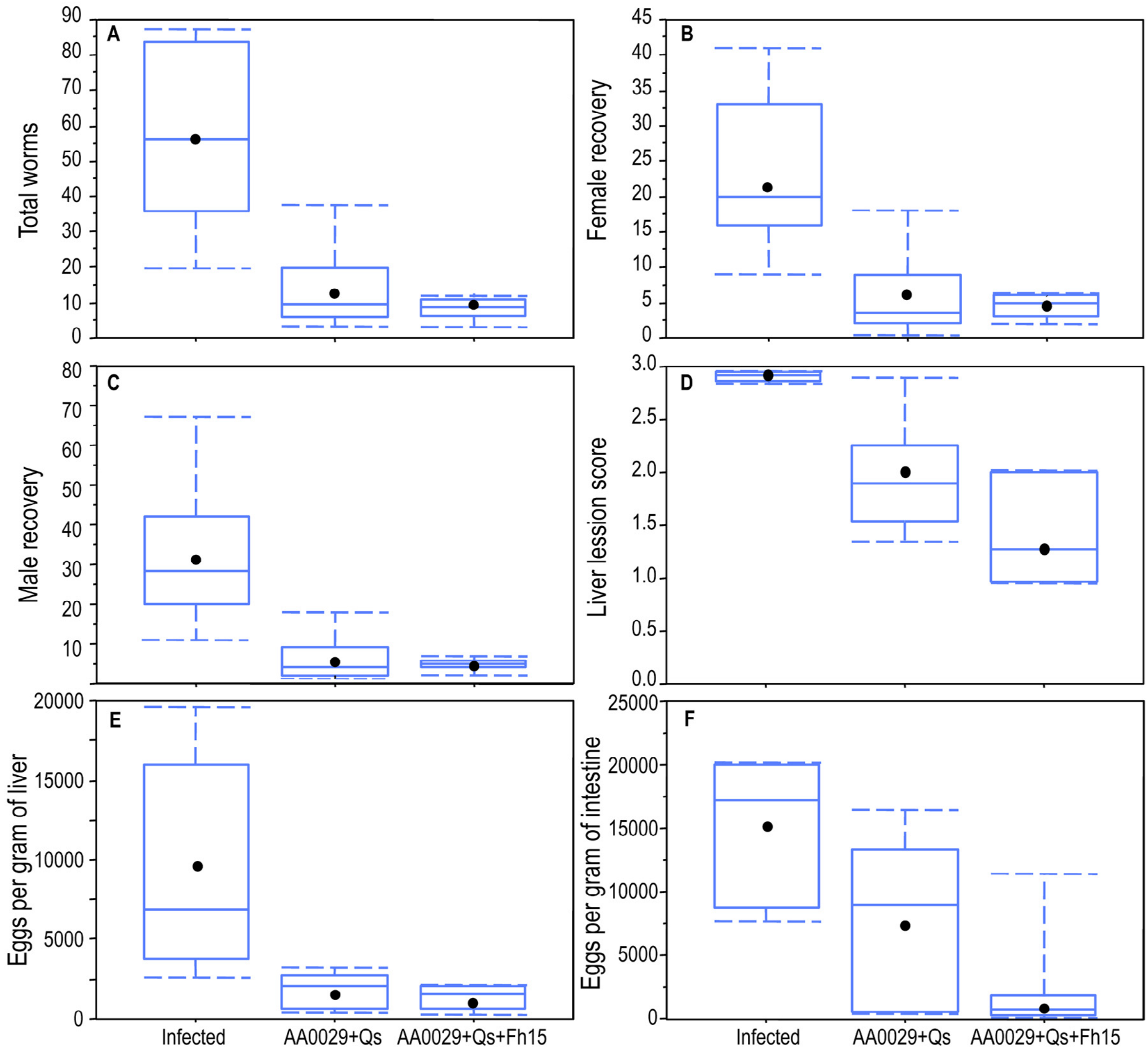

Fig. 4. Boxplots with number of total recovered worm (A), females (B), males (C), hepatic damage (D), eggs per gram of liver (E) and egg per gram of intestine (F) in *Mesocicetus auratus* vaccinated with AA0029 + Qs + rFh15 using the adjuvant adaptation (ADAD) vaccination system, challenged with 150 cercariae of *Schistosoma bovis* and perfused 8 weeks post-challenge. Data are represented in boxplots with mean (solid symbol), median, Q1 and Q3 (box), percentiles 10 and 90 (error bars).

4. Discussion

Fatty acid binding proteins (FABP) have demonstrated to be reliable vaccine candidates in schistosome and *F. hepatica* vaccine development (Hillyer, 2005; McManus and Loukas, 2008). In the progress of an effective vaccine against schistosomes the use of immunogenic antigens together with appropriate adjuvant systems, which are able to induce an adequate immunological response represents an important goal. The ADAD vaccination system is a new adjuvant system proposed as alternative to Freund's in vaccination against *F. hepatica* and schistosomes including immunomodulators as the hydroalcoholic extract from the rhizome of *Phlebodium pseudoaureum* (PAL) or chemically synthesized aliphatic diamines and amino-alcohols (AA0029, AA2829, OA0012) with promising results in vaccines against these trematodes (Martínez-Fernández et al., 2004; Uribe et al., 2007; Vicente et al., 2014). In this work we studied the effects of the synthetic immunomodulator AA0029 in the immunological response induced by the recombinant FABP rFh15 formulated in ADAD vaccination system prior to use it in a vaccination trial against the infection by *S. bovis* in BALB/c mice. Immunization with ADAD plus AA0029 + Qs + rFh15 elicited increased levels of TNFα and IL-6 innate pro-inflammatory cytokines, IL-2, representative of Th1 response and IL-4 of the Th2 response compared to mice from the adjuvant control group (AA0029 + Qs) or untreated controls. No significant changes in Treg or Th17 cytokines and in percentages of splenocyte populations were found. There is a broad consensus that associates protection against *Schistosoma* spp. in the mouse model with high levels of IFNγ, TNFα (Wilson and Coulson, 2009) frequently associated with the presence of other cytokines as IL-12 (Cardoso et al., 2008), IL-10 (Rezende et al., 2011), IL-6, and IL-17 (Torben et al., 2011). Beside pro-inflammatory and Th1 cytokines, we observed significant levels of IL-4 as it was observed using as adjuvants, the combination of ODN and R848 (Wang et al., 2013), alum and ODN (Teixeira de Melo et al., 2013), alum (Zhang et al., 2011), peptidoglycan or thymic stromal lymphopoietin (El-Ridi and Tallima, 2012) as adjuvants. These data indicate that AA0029 formulated in ADAD with Qs and rFh15 promotes an intense mixed Th1/Th2 response. An early, intense and balanced cytokine immune response before the challenge seems determinant in the success of the rFh15 formulation with AA0029 in ADAD in concordance with experimental immunoprotection against *Fasciola hepatica* observed using synthetic peptides with ADAD (Rojas-Caraballo et al., 2014). In addition, we observed an intense specific response of IgG, IgG1, IgG2a, IgM and IgE against the rFh15 antigen in immunized

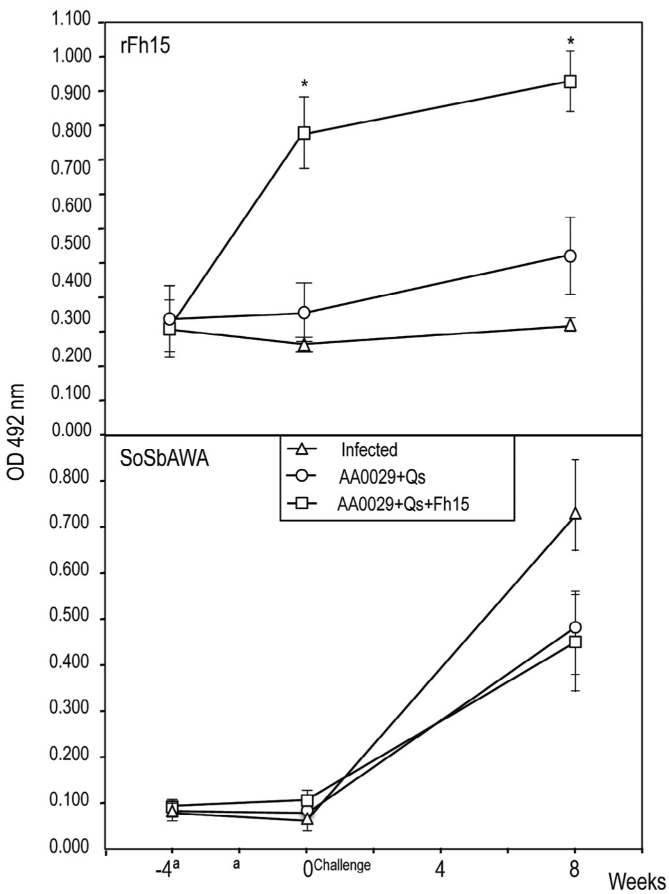

Fig. 5. Serum antibody level detection (mean ± SEM) of IgG against rFh15 and SoSbAWA antigen in ELISA of *Mesocricetus auratus* vaccinated with AA0029 + Qs + Fh15 using the adjuvant adaptation (ADAD) vaccination system and challenged with 200 cercariae of *Schistosoma bovis* percutaneously. OD optical density $^{*}p < 0.05$ compared to infected controls. [a]Immunization.

mice. Antibodies have been related with *in vitro* killing of schistosomula (Torben et al., 2012) and worm burden reduction in mice infected with *S. mansoni* after passive transfer of serum or purified IgG from Smp80 vaccinated baboons or mice (Torben et al., 2011) and antibodies have been related with self cure in the rhesus macaque (Wilson and Coulson, 2009).

Furthermore, we evaluated the ability of the formulation of AA0029 + Qs + rFh15 to induce protection against the cercarial challenge by *S. bovis* in terms of worm recovery, eggs in tissues and hepatic damage in two models BALB/c mice and golden hamster. These magnitudes are in concordance with protection studies in natural ruminant hosts to estimate protection (Vercruysse and Gabriel, 2005). The level of protection achieved was significantly high as compared to infected controls, and it was comparable to the previous results of vaccination against *S. bovis* using rFh15 formulated Freund's adjuvant in C57/BL6 mice (Abáné et al., 2000) or with ADAD vaccination system using PAL, the hydro-alcoholic extract from *Phlebodium pseudoaureum*, in BALB/c mice (Vicente et al., 2014), and better than protection achieved by 14-3-3 protein from *S. bovis* formulated in ADAD with PAL or AA0029 (Uribe et al., 2007). Recovered adult worms in both models did not show an altered sex ratios, which suggests that vaccination did not selectively affect one sex or the other. As would be expected the lower level of infection in vaccinated mice and hamsters was accompanied by a reduced pathology, as evidenced by a lower number of eggs found in their livers; the livers were also less injured. Reduction of disease is considered a desirable feature of a schistosomiasis vaccine candidate (Siddiqui et al., 2011). Moreover, we observed that vaccination seems to reduce the eggs trapped in tissues per female in hamsters pointing an impairment of the oviposition capacity of the surviving females as happens in repetitive natural infections (Vercruysse and Gabriel, 2005). The anti-fecundity effect along with reduction of egg viability is considered useful for reduction transmission in schistosomiasis (Ahmad et al., 2009; Dai et al., 2014). Furthermore, a significant increase of IgG2a against SoSbAWA was observed in protected mice in vaccination experiment. Regarding the adjuvant control groups treated only with AA0029 + Qs and infected, we observed reductions in tissue eggs only in hamsters. This effect could be related to immunomodulatory activity of AA0029 due to short time between immunization schedule and infection. A similar situation was observed in vaccination against experimental strongyloidosis using AA0029 as adjuvant (Vlaminck et al., 2010). It was also observed that chitosan nanoparticles are able to modulate the granuloma area reducing liver injuries and induce a moderate protection against *S. mansoni* infection (Oliveira et al., 2012).

This work demonstrates that the use of the synthetic immunomodulator AA0029 with rFh15 in ADAD vaccination system promotes an early potent mixed Th1/Th2 type of immune response with significant production of TNFα, IL-6, IL-2, IL-4 and antibodies, and corroborates the immunoprophylactic properties of rFh15 against *S. bovis* with reduction in parasite burden and morbility in two models. A defined molecule as AA0029 with immunomodulatory properties could contribute to drive immune response representing an innovative approach for the designing and implementation of trematode vaccines.

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