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ORIGINAL PAPER

Evaluation of protective immune response in mice by vaccination the recombinant adenovirus for expressing *Schistosoma japonicum* inhibitor apoptosis protein

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Abstract Schistosomiasis is a worldwide parasitic disease, and while it can be successfully treated with chemotherapy, this does not prevent reinfection with the parasite. Adenovirus vectors have been widely used for vaccine delivery, and a vaccination approach has the potential to prevent infection with *Schistosoma*. Here, we developed a recombinant adenoviral vector that expresses *Schistosoma japonicum* inhibitor apoptosis protein (Ad-SjIAP) and assessed its immunoprotective functions against schistosomiasis in mice. Murine immune responses following vaccination were investigated using enzyme-linked immunosorbent assays (ELISA), lymphocyte proliferation, and cytokine assays. The protective immunity in mice was evaluated by challenging with *S. japonicum* cercariae. Our results indicated that immunization with the Ad-SjIAP in mice induced a strong serum IgG response against IAP including IgG1, IgG2a, and IgG2b. In addition, lymphocyte proliferation experiments showed that mice treated with Ad-SjIAP significantly increased the lymphocyte response upon stimulation with recombinant *Schistosoma japonicum* inhibitor apoptosis protein (rSjIAP). Moreover, cytokine assays indicated that vaccination of Ad-SjIAP significantly increased the production of interferon (IFN)-γ and IL-2 as compared to the corresponding control group. Furthermore, following the challenge with *S. japonicum* cercariae, the vaccine conferred moderate protection, with an average rate of 37.95 % for worm reduction and 31.7 % for egg reduction. Taken together, our preliminarily results suggested that schistosoma IAP may be a potential vaccine against *S. japonicum* and that adenoviral vectors may serve as an alternative delivery vehicle for schistosome vaccine development.

Keywords Adenovirus · *Schistosoma japonicum* · Inhibitor apoptosis protein · Vaccine

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Introduction

Schistosomiasis japonica caused by *Schistosoma japonicum* is a major public health problem in China and South Asia (Gray et al. 2008). Despite the significant advances in the prevention and control of schistosomiasis, current strategies primarily depend on snail eradication and the application of intensive chemotherapy (McManus 2000). Praziquantel (PZQ) is the only drug widely used for treatment, but it does not prevent reinfection and also has no effect on larval worms (Doenhoff and Pica-Mattoccia 2006). In addition, there is an increasing concern that heavy use of praziquantel will lead to drug resistance. Vaccine strategies represent an essential component as an adjunct to chemotherapy for the future control of schistosomiasis (McManus and Loukas 2008).

Vaccination against schistosomiasis in various animal models using irradiated Schistosoma cercariae or schistosomula engendered high levels (around 90 %) of immunity to an infective challenge (Ford et al. 1984), validating the concept of vaccine development for treatment of

schistosomiasis. However, delivery problems, the need of large quantities of attenuated parasites, and safety considerations combine to make this approach impractical for wide use (McManus and Loukas 2008). Recombinant vaccines against cestode platyhelminths have been developed successfully and applied in practice (Craig et al. 2007). In this regard, it is encouraging that a large number of schistosome antigens and additional candidates have been discovered through molecular biology studies (Berriman et al. 2009; Cheng et al. 2005; Zhou et al. 2009).

Despite numerous reports of potentially promising vaccine derived from schistosome antigens, the level of protection conferred by these antigens is much lower than that achieved with the radiation-attenuated vaccine model (McManus and Loukas 2008; Siddiqui et al. 2008). Consequently, there is a need to identify novel vaccine candidates and/or to develop alternative delivery strategies to improve vaccine efficacy. Several types of vaccines including DNA (Zhu et al. 2011), recombinant protein (Lv et al. 2009), synthetic peptide (Yang et al. 2000), and multivalent vaccines have been developed and tested against *S. japonicum* infection (Zhou and Zhuge 2007). However, each of these vaccines induces partial protection against challenge infection as measured by reduction in total worm burdens (Zhou and Zhuge 2007). To further enhance vaccine efficacy, some of the candidates were immunized in combination with adjuvants such as IL-18 (Wei et al. 2008, 2009), cimetidine (a chemical agent) (Li et al. 2011), or CpG-ODN (Teixeira de Melo et al. 2013). These approaches have enhanced the efficacy of vaccines to some extent.

In addition to the various attempts using different antigens alone or in combination with various adjuvants, different delivery methods were also shown to increase the protective efficacy against schistosomiasis (Cao et al. 2013; Chen et al. 2011; Dai et al. 2009; Wei et al. 2010). For example, Dai et al. showed that mice vaccinated with cocktail DNA vaccines (the mixture of DNA plasmids encoding Sj23 and *S. japonicum* triosephosphate isomerase and sixfold-repeated genes of the complementarity-determining region 3) significantly reduced both worm reduction by 45.00 % and liver egg reduction by 50.88 % (Dai et al. 2009). In addition, Chen et al. used an attenuated *Salmonella typhimurium* strain VNP20009 that produced the Sj23-GST bivalent antigen from *S. japonicum* and demonstrated that this antigen was highly effective against *S. japonicum* infection in orally immunized mice (41.69 % worm reduction and 57.71 % egg reduction) (Chen et al. 2011). In a similar approach, Wei et al. used recombinant pseudorabies virus (PRV) Bartha-K61 vaccine strains to express *S. japonicum* 26 kD glutathione *S*-transferase and 14 kD fatty acid-binding protein. This strategy also enhanced protection in mice, with a reduction in worms of 39.3 % and a decrease in hepatic egg reduction of 45.5 %. Similar effects were also observed in sheep, with reductions of 48.5 % and 51.2 %, respectively (Wei et al. 2010).

Adenovirus vector, which was originally developed for gene therapy applications, has been widely used for vaccine delivery (Kamimura et al. 2011; St George 2003). Adenovirus has a broad host range such as human, mice, rats, rabbits, pigs, and nonhuman primates (Kamimura et al. 2011), and it can induce innate and adaptive immune responses (Nazir and Metcalf 2005). Recently, the use of adenovirus for delivering *S. japonicum* triosephosphate isomerase has been shown to provide a relatively higher protective effect in mice against schistosomiasis (Dai et al. 2014). In our previous study, we cloned a complementary DNA (cDNA) encoding an inhibitor of apoptosis (IAP) into *S. japonicum* and found that IAP likely has vital roles in the parasite life cycle and in development in the final host (Peng et al. 2010). In the present study, we constructed a recombinant adenovirus expressing SjIAP (Ad-SjIAP) and further tested their abilities to induce protective immunity against *S. japonicum* challenge.

Materials and methods

Parasites and animals

The life cycle of *S. japonicum* (Jiangxi isolate, China) was maintained in New Zealand rabbits and *Oncomelania hupensis* in Shanghai Veterinary Research Institute of China. Seven-week-old BALB/c mice were purchased from the Shanghai Experimental Animal Centre of Chinese Academy of Sciences (China). Human 293 T cells, kindly provided by Prof. Zejun Li from the Shanghai Veterinary Research Institute, were grown in Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 5 % (*v*/*v*) heated and incubated fetal calf serum (Invitrogen, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, USA) at 37 °C in a 5 % CO_2 incubator.

Recombinant adenovirus

The recombinant shuttle plasmids encoding SjIAP were constructed according to the standard molecular procedures. Briefly, the recombinant plasmids were constructed by PCR-based amplification (forward primer: 5′ GAG GAT CCC CGG GTA CCG GTC GCC ACC ATG TCT TAT TTT CAG AAC CTA TCA AAT GC3′; reverse primer: 5′ TCA TCC TTG TAG TCG CTT TTT GGA ACA TTA TTG CTG TGA G 3′) based on the recombinant plasmid constructed previously as template (Peng et al. 2010). The purified PCR production was cloned into the shuttle vector by In-Fusion^{TM} PCR Cloning Kit (Clontech, China). Then, the recombinant shuttle plasmids and the plasmid pBHGlox Δ E13Cre (Microbix Biosystems) were co-transfected into 293 T cells for adenoviral package. At 10 days of post-transfection, when approximately 50 % cells showed cytopathogenic effect (CPE), the cells were

collected by low-speed centrifugation. The viruses were released by using the freeze-thaw method.

As to adenovirus amplification and purification, human 293 T cells were seeded in the T25 culture flask, when cell confluence reach 60 %, then adding in 2 ml virus crude extracts in the fresh cultured medium. When 50 % of cells showed CPE, the cells were collected and the released viruses were used for the next round. The adenovirus viruses were purified by using the Adeno-X^{TM} Virus Purification Kit according the manufacturer's instruction (Clontech). The titers of the recombinant adenoviruses were determined on human 293 T cells by using the Adeno-X^{TM} Rapid Titer Kit (Clontech).

Western blot analysis of IAP expression in 293 T cells transduced with recombinant adenovirus

Human 293 T cells were cultured in a 12-well cell culture plate (Corning) and then were transduced with the control adenovirus and recombinant adenovirus (Ad-SjIAP) at a multiplicity of infection (MOI) of 10 plaque forming units (PFU)/cell, respectively. Then, the transduced cells were further cultured for 48 h and the CPE was observed by a microscopy. The expression of SjIAP in the transduced cells was analyzed by Western blot with the rabbit anti-SjIAP polyclonal antibody prepared previously (Peng et al. 2010). Briefly, the cell lysates were separated by 12 % SDS-PAGE and transferred to a nitrocellulose membrane. Then, the membrane was blocked with 5 % nonfat milk in phosphate-buffered saline with Tween (PBST) for 1 h at room temperature and further incubated with anti-rSjIAP serum (1:500 dilution) for 2 h at room temperature. Then, the membrane was rinsed with phosphate-buffered saline with Tween 20 (PBST) three times, followed by incubation with Horseradish peroxidase (HRP)-conjugated goat anti-rabbits IgG (1:10000 dilution) (Co Win Biotech, China) for 1 h and then washed three times with PBST. The membranes were developed with enhanced chemiluminescence reagent (Millipore, USA) and then captured by Luminescent Image Analyzer (GE Healthcare, USA).

Immunization schedule

In experiment #1, forty mice were randomly divided into three groups: 13 in the Ad-SjIAP group, 12 in the Ad group, and 15 in the phosphate-buffered saline (PBS) group. Two groups were intramuscularly injected with 0.1 ml PBS containing Ad-SjIAP (1×10^7 PFU) and containing control adenovirus (1×10^7 PFU). The control group received 0.1 ml PBS. Two identical doses were given, with a 3-week interval. In experiment #2, twenty-seven mice were randomly divided into three groups: nine in the Ad-SjIAP group, nine in the control adenoviruses group, and nine in the PBS group. Three identical doses were given: the second and third injections were given at 3 and 7 weeks, respectively, after the first injection.

Antibody assay

Enzyme-linked immunosorbent assays (ELISA) and Western blot were used to analyze antibody response in the immunized mice. Serum samples from each group were collected pre-vaccination, 3 and 6 weeks following the prime immunization. Total IgG antibodies were tested by ELISA in both experiment #1 and experiment #2. Briefly, a 96-well ELISA plate (Costar, USA) was coated with 100 μl of rSjIAP (10 μg/ml) in carbonate-bicarbonate buffer (pH = 9.6) per well overnight at 4 °C. The plate was then washed with PBST and blocked with 1.5 % bovine serum albumin (BSA) in PBST for 1 h at 37 °C. After washing, 100 μl of each serum sample (1:50 dilution) was added and incubated for 1.5 h at 37°C. Plate-bound antibody was detected with HRP-conjugated goat anti-mouse IgG (1:5000 dilution) (Proteintech, China). The detection was used 3, 3′, 5, 5′-tetramethyl benzidine dihydrochloride (TMB) (Tiangen, China) as a substrate solution. Finally, the reaction was stopped with 2 M H_2SO_4 (50 μl/well), and the absorbance was measured at 450 nm (BioTek Synergy HT, USA). Western blot was also used to determine the IgG antibodies after the second immunization with the serum dilution (1:10 dilution) and secondary antibody dilution (1:5000 dilution). In addition, ELISA was also performed to detect the titer of specific IgG, IgG1, IgG2a, IgG2b, and IgG3 antibodies against rSjIAP as described above except for the serum dilution (1:100 dilution) and secondary antibody dilution (1:2500 dilution) (Proteintech, China).

Lymphocyte proliferation and cytokine assay

For splenocyte proliferation assays, splenocyte suspensions were prepared from mice at 3 weeks after the final injection in experiment #1 using the EZ-Sep Mouse 1 × Lymphocyte separation medium (Dakewe, China) according to the manufacturer's instructions with four mice per group, and 2×10^5 cells/200 μl/well were cultured in 96-well plates in triplicate in RPMI-1640. Cultures were stimulated with 5 μg/ml rSjIAP that was obtained in our previous study (Peng et al. 2010) or RPMI-1640 and incubated at 37 °C with 5 % CO_2. Unstimulated control cultures were run concurrently. After 72 h of incubation, 100 μl supernatant was removed for estimation of cytokine production and to the remainder, 20 μl of methyl thiazolyl tetrazolium (MTT, 5 mg/ml; Sigma) was added and incubated for another 4 h at 37 °C. The contents of the plate were centrifuged at 1000 g for 10 min and then the supernatants were removed. The addition of 100 μl dimethyl sulfoxide (DMSO) of each well followed and then incubated for 30 min at 37 °C to dissolve the formazan salt crystals, and the absorbance was measured at

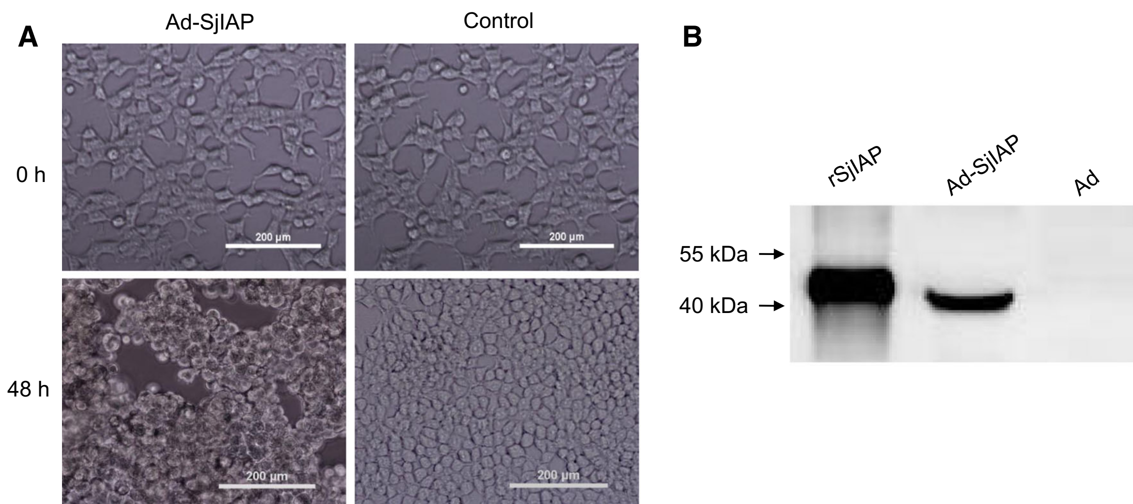

Fig. 1 Evaluation of SjIAP expression in human 293 T cells transduced with recombinant adenovirus (Ad-SjIAP). **a** Microscopic observations of human 293 T cells transduced with recombinant adenovirus (Ad-SjIAP). **b** Western blot analysis of the SjIAP expression in the human 293 T cells transduced with Ad-SjIAP

570 nm. To quantify the cytokine production (interferon (IFN)-γ, IL-2, IL-4, IL-10) by the proliferating splenocytes, the medium collected above was used in a Th1/Th2 cytokine ELISA kit (Ebiosciences, CA, USA) according to the manufacturer's instructions.

Challenge infection

Four weeks after the final vaccination, mice were challenged percutaneously with 40±2 *S. japonicum* cercariae by the cover glass method (Kamiya et al. 1993).

Evaluation of worm and egg burdens

Thirty-eight days post-challenge, all the mice were euthanized, then perfused through the abdominal aorta using PBS after the hepatic portal was cut, and the worm burdens were determined (Cheng et al. 2006, 2009). The live egg counts were determined as described elsewhere (Cheng et al. 2009).

Statistical analysis

Data were analyzed by Student's *t* test using the software GraphPad Prism (GraphPad Software, Inc. La Jolla, CA, USA).

Results

Construction and identification of recombinant adenovirus

Recombinant adenoviral vectors encoding SjIAP were constructed as described above and then were verified by PCR using specific primers for SjIAP (data not shown). Electron microscopy showed cytopathogenic effects in HEK 293 T cells transduced with Ad-SjIAP (Fig. 1a). Next, the expression of SjIAP in transduced cells was determined using Western blot. As shown in Fig. 1b, a single band with the predicted

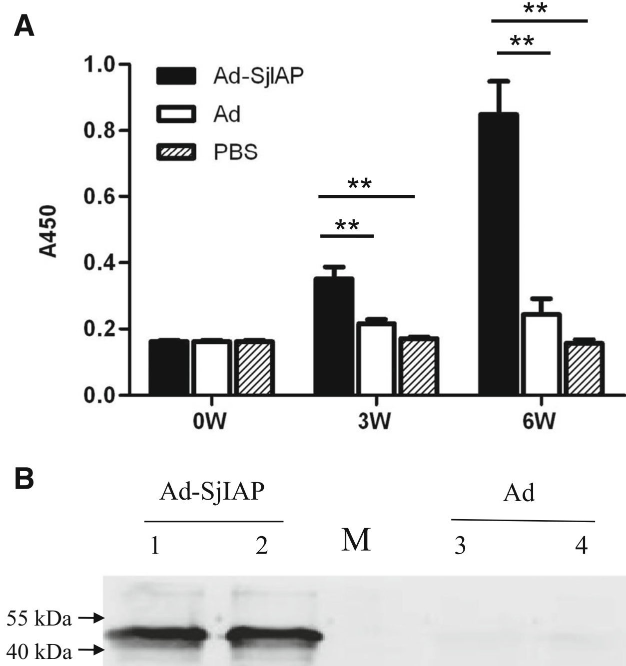

Fig. 2 The IgG antibody responses in the mice injected with Ad-SjIAP. **a** The total antibody responses in mice injected with Ad-SjIAP. Absorbance values (450 nm) are shown as mean±SD of eight mice in each group. **Statistical significance (P<0.01). **b** Western blot analysis of the IgG antibody responses to soluble worm antigens (the serum was diluted with 1:20) from the mice injected with Ad-SjIAP and control adenovirus (Ad). *1* serum from one of mice injected with Ad-SjIAP, *2* serum from another mice injected with Ad-SjIAP, *M* protein marker, *3* serum from one of mice injected with control adenoviruses, *4* serum from another mice injected with control adenoviruses

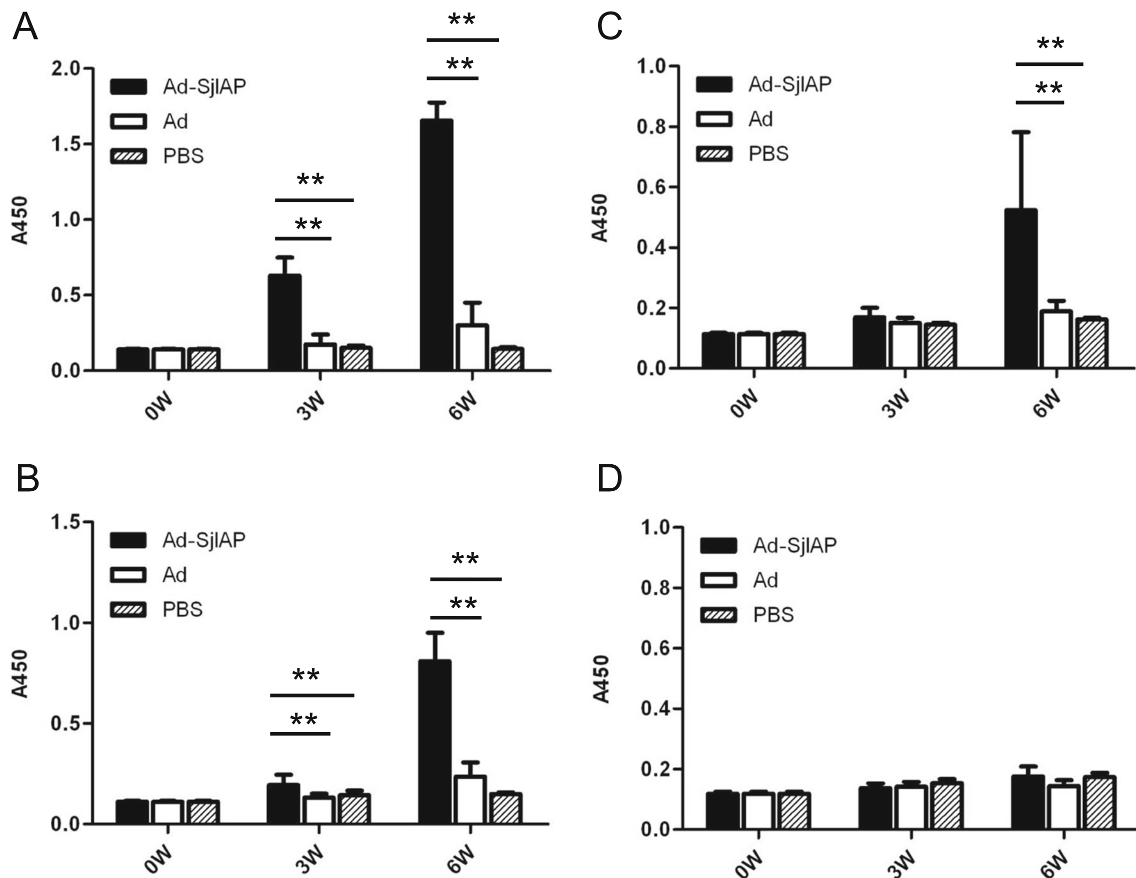

Fig. 3 The IgG subclass profiles in the mice injected with Ad-SjIAP. **a** IgG 1. **b** IgG 2a. c IgG 2b. **d** IgG 3. Absorbance values (450 nm) are shown as mean±SD of six mice in each group. **Statistical significance ($P<0.01$)

molecular weight of SjIAP was recognized; however, there was no detection in the cells transduced with control adenovirus.

Antibody response

The results of ELISA antibody titers of sera from treated mice are summarized in Fig. 2a. The titers of IgG antibodies in response to rSjIAP were significantly elevated following the first injection of Ad-SjIAP, and they were further increased with administration of the second injection (Fig. 2a). To confirm that the antibodies against rSjIAP were specific, we further used Western blotting to examine whether sera from immunized mice were able to recognize proteins from worm lysates. As shown in Fig. 2b, the sera from Ad-SjIAP immunized mice yielded a single band when incubated with worm protein lysates. Expectedly, the results were not observed with sera from control adenovirus-immunized mice (Fig. 2b).

The IgG subclass profiles in the sera of treated mice were also determined by ELISA at 3 weeks after both the first and second injections. Vaccination of Ad-SjIAP significantly increased the levels of these IgG subclasses (Fig. 3a–c), with the exception of IgG3 (Fig. 3d). From these data, we calculated the ratio of IgG1 to IgG2a isotypes before challenging with *S. japonicum* cercaria (Fig. 3). The ratios of IgG1 to IgG2a in the Ad-SjIAP and Ad-immunized mice were 2 and 1.3,

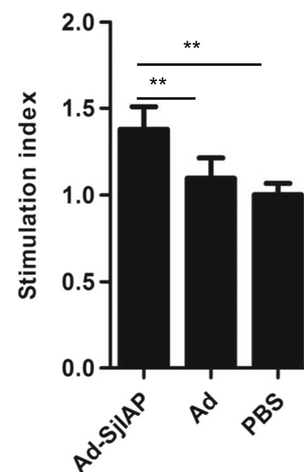

Fig. 4 Stimulation index of analyses of murine splenocytes with Ad-SjIAP treatment after rSjIAP stimulation in vitro. Absorbance values (450 nm) are shown as mean±SD of four mice in each group. **Statistical significance ($P<0.01$)

respectively. Similar results were also observed in experiment #2 (data not shown).

Splenocyte proliferation

Cell-mediated immune function was assessed by measuring splenocyte proliferation in response to the treatment of rSjIAP. As shown in Fig. 4, mice treated with the Ad-SjIAP significantly increased the lymphocyte response upon stimulation with rSjIAP (Fig. 4).

Cytokine production

Cellular immunity produced in the immunized mice was indirectly evaluated by measuring cytokines released in the supernatants from the cultures of rSjIAP-stimulated spleen cells. Vaccination of Ad-SjIAP significantly increased the production of IFN-γ and IL-2 when compared with the corresponding control group (Fig. 5a and b). Moreover, IFN-γ showed the highest level of production among the four cytokines examined (Fig. 5).

Evaluation of the protective efficacy

Four weeks after the last immunization, all mice were challenged with *S. japonicum* cercariae. At 38 days post-challenge, all animals were sacrificed, and the reductions in worms and eggs were investigated. Mice vaccinated with Ad-SjIAP induced moderate protection against challenge infection (Table 1), resulting in a 37.95 % reduction in adult worm burden and a 31.7 % reduction in liver egg burden (mean values of both trials were combined) as compared to the PBS control group (Table 1).

Discussion

Improved understanding of the immune response to schistosome infection in animal models and humans implies that the development of a vaccine is possible. For this be realized, in addition to the discovery of new target antigens, it is also necessary to explore alternative vaccination strategies to improve the vaccine efficacy. Recombinant live vector vaccines

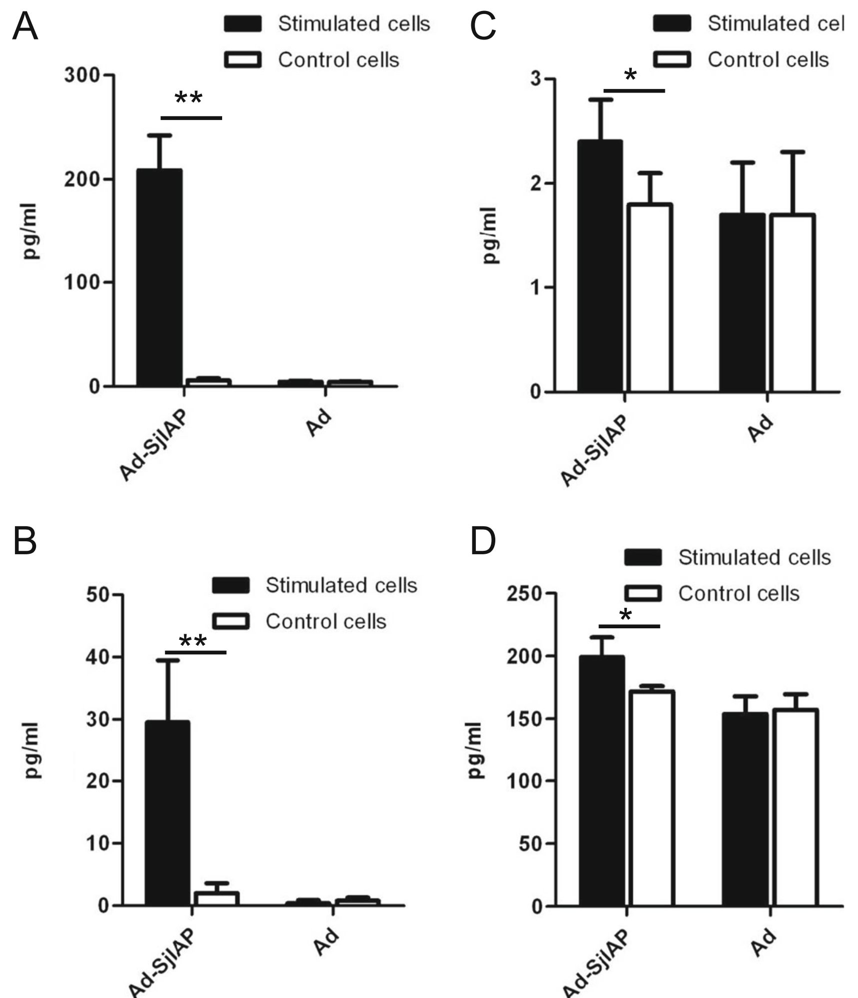

Fig. 5 Cytokine production by splenocytes of mice injected with Ad-SjIAP after rSjIAP stimulation in vitro. **a** IFN-γ. **b** IL-2. **c** IL-4. **d** IL-10. Absorbance values (450 nm) are shown as mean±SD of four mice in each group. $^{**}P<0.01$ and $^{*}P<0.05$

Table 1 Effect of Ad-SjIAP administration on *S. japonicum* challenge in BALB/c mice

Groups	No. of mice	Worm burden	Worm reduction (%)[a]	Liver eggs	Egg reduction (%)[a]
Experiment #1					
Ad-SjIAP	9	15.2±2.4	41.5 %** (40.6 %****)	62,230±22,084	39.6 %** (35.2 %***)
Ad	8	25.6±6.1	1.5 %	96,000±35,665	6.9 %
PBS	11	26.0±2.4	–	103,111±26,844	–
Experiment #2					
Ad-SjIAP	9	18.3±2.8	34.4 %** (29.6 %****)	100,606±10,472	23.8 %** (14.4 %)
Ad	9	26.0±2.4	6.8 %	117,521±27,527	10.9 %
PBS	9	27.9±2.3	–	131,962±13,927	–

[a] The number in the brackets indicated the reductions as compared to the Ad group

**$P<0.01$ (Ad-SjIAP vs. PBS); ***$P<0.05$, ****$P<0.01$ (Ad-SjIAP vs. Ad)

have been shown to be capable of inducing effective immune responses by imitating natural infection and are considered to be promising vaccine types. Several studies by using *Streptococcus gordonii* (Wang et al. 2013), *Bacillus subtilis* (Li et al. 2009), and *Salmonella typhimurium* (Chen et al. 2011) as live recombinant vaccine carriers have been shown good immunogenicity with potential protection of mice challenged with *S. japonicum*.

In the present study, we explored the potential of adenoviruses as live vaccine vector against schistosomiasis. Adenoviruses offer several advantages, including their safety and the relative ease with which they can be manipulated, the ability to infect a wide range of actively dividing and nondividing mammalian cells, and the development of high levels of antigen-specific humoral and cell-mediated immune responses. In addition, adenovirus can serve as an adjuvant by activating innate immunity (Molinier-Frenkel et al. 2002; Vemula and Mittal 2010). This has made adenoviral vectors a clear choice for delivery vehicles in recombinant vaccination.

Here, we used a replication-deficient adenoviral vector to express *S. japonicum* IAP, a protein that may play vital roles in the parasitic life cycle and development (Peng et al. 2010). Both ELISA and Western blot analyses indicated that vaccination with Ad-SjIAP engendered a relatively higher titer antibody response to rSjIAP. It has long been recognized that T cell-mediated immunity is fundamental to acquired resistance to schistosomes in mice (McManus and Loukas 2008). Studies of putative resistant (PR) individuals in endemic *Schistosoma mansoni* (Correa-Oliveira et al. 2000) suggested that the Th1 response (particularly IFN-γ) to schistosomulum antigens is the key to disease resistance in these subjects. In addition, repeated vaccination with irradiated cercariae produced incremental increases in Th2-mediated (IL-4 and IL-5 predominance) protection (McManus and Loukas 2008). Moreover, studies using B cell-deficient and cytokine-deficient mice demonstrated that successful antischistosome vaccination required induction of strong Th1 and Th2 responses. Vaccination with Ad-SjIAP in BLAB/c mice significantly increases IFN-γ and IL-2 levels in splenocytes, suggesting that Ad-SjIAP may induce a Th1-dominant immune response upon the stimulation of rSjIAP. However, based on the results by calculating the IgG1/IgG2a ratios in the Ad-SjIAP immunized mice imply that there may be a Th2-dominant immune response. The difference was probably due to the antigen (rSjIAP) used for stimulation, which needs to be further investigated.

Animal experiments indicated that vaccination with Ad-SjIAP moderately induced protection against challenge infection, suggesting that SjIAP may be a potential vaccine antigen for further investigation for schistosomiasis control. In the present study, two independent animal experiments were carried out to evaluate the immune effect of Ad-SjIAP against challenge infection in mice. In experiment #1, we observed that mice vaccinated with Ad-SjIAP induced in a 41.5 % reduction in adult worm burden and a 39.6 % reduction in liver egg burden as compared to the PBS control group (Table 1). Then, we increased one more shot in experiment #2. However, the immune protection was not increased correspondingly, suggesting the complexity of the Ad-SjIAP-induced immune response. In addition, we used human replication-deficient adenoviral vectors (type 5), which are not able to replicate in the target cells. This type of adenoviral vector is advantageous in terms of safety and can be developed based on commercially available vectors. However, since this vector is not able to replicate in target cells, the amount of antigen presentation and the subsequent antigen-specific humoral and cell-mediated immune responses may to some extent be limited. Consequently, it is necessary to compare the immune effects of an antigen alone with the mixture of the antigen with adenovirus as well as the recombinant adenovirus for expressing the antigen in the future.

In conclusion, our preliminarily results suggested that schistosoma IAP may be a potential vaccine against *S. japonicum* and that adenoviral vectors may serve as an alternative delivery vehicle for schistosome vaccine development.

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