

VACCINATION OF MICE WITH AN ANTIGENIC SERINE PROTEASE-LIKE PROTEIN ELICITS A PROTECTIVE IMMUNE RESPONSE AGAINST *TRICHINELLA SPIRALIS* INFECTION

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ABSTRACT: Trichinellosis has major economic impacts on animal husbandry and food safety, and the control and elimination of trichinellosis is a major objective of veterinary medicine. A gene encoding serine protease of *Trichinella spiralis* (*Ts-Adsp*) was identified by immunoscreening an adult *T. spiralis* cDNA library. In this study, the recombinant *Ts-Adsp* protein (*rTs-Adsp*) was cloned and expressed in a prokaryotic expression system and purified by Ni-affinity chromatography. To determine whether the purified *rTs-Adsp* is a potential vaccine candidate for the control of *T. spiralis* infection, we immunized BALB/c mice with this protein in combination with an alum adjuvant and subsequently challenged with *T. spiralis* larvae. The results showed that mice vaccinated with *rTs-Adsp* exhibited an average reduction in the muscle larvae burden of 46.5% relative to the control group. Immunization with the *rTs-Adsp* antigen induced both humoral and cellular immune responses, which manifested as elevated specific anti-*rTs-Adsp* IgG and IgE antibodies and a mixed Th1–Th2 response, as determined by Th1 (IFN- γ and IL-2) and Th2 (IL-4, IL-10, and IL-13) cytokine profiling, with the Th2 predominant. Thus, purified *rTs-Adsp* is able to limit the invasion of *T. spiralis*, and this protein could be an effective vaccine candidate for trichinellosis.

Trichinella spiralis is one of the most widespread zoonotic pathogens in the world (Dupouy-Camet, 2009). The host specificity of *T. spiralis* is extremely poor and this parasite can infect almost all mammals. The complete life cycle of *T. spiralis* takes place in a single host. There are 3 major antigenic stages, muscle larvae (ML), adult worms (Ad), and newborn larvae (NBL). Trichinellosis remains a serious public health threat in many countries. The global prevalence of trichinellosis is difficult to evaluate but it is estimated that approximately 11 million people may be infected (Dupouy-Camet, 2000). Although this disease is well controlled in Europe and the United States, it is now emerging in both developed and developing countries such as China, Argentina, and some eastern European countries (Gottstein et al., 2009). In China for example, there were 15 reported outbreaks of human trichinellosis including 1,387 cases and 4 deaths between 2004 and 2009 (Cui et al., 2011).

In naturally immunized mammals, muscle invasion could be prevented if the invasive stages of *T. spiralis* (Ad and NBL) could be interrupted. *T. spiralis* affects the immune system of the host by expressing a variety of proteins that influence immune regulation (Zocevic et al., 2011). If we can find potential vaccine candidate molecules that can trigger an appropriate immune response, these molecules will be good targets to disorder parasite biology. Although several researchers are dedicated to the study of protection against *T. spiralis*, there is still no reliable vaccine that can be widely used (Abdel Rahman et al., 2005; Wang et al., 2009; Yang et al., 2010; Wei et al., 2011).

Crude antigen preparations of *T. spiralis* from 3 life-cycle stages can induce protection against larval challenge in mice or pigs, and Ad and NBL antigens have been shown to be immunogenic in pigs (Zhu et al., 2005). The vaccination of mice demonstrated that the Ad antigen provided the best protection by reducing the

number of adult worms (89%) and muscle larvae (80%) (Darwish et al., 1996). When assessing candidates for vaccine antigens, proteins are usually the first option because proteins are easier to identify and modify than are other compounds and can be produced economically in large quantities (Hein and Harrison, 2005). A recombinant vaccine against *Taenia solium* infection in pigs was successfully developed, and this vaccine represented the peak of research efforts spanning many years on the use of recombinant antigens as vaccines against taeniid cestode infection in their intermediate hosts (Lightowers, 2006). Lustigman et al. (2002) reported that one basic strategy to identify and clone target antigens of *Onchocerca volvulus* larvae as possible vaccine candidates was the immunoscreening of cDNA libraries using immune sera from humans or animals. Other methods to identify vaccine candidates include the cloning of enzymes that could protect a host from injury and of proteins that might be related with immune evasion (Lustigman et al., 2002). Parasite proteases are interesting targets for the treatment and prevention of parasitic diseases due to their indispensable function in modulating the interactions between parasites and hosts (Trap et al., 2006).

In a previous study, we cloned and analyzed a cDNA encoding serine protease that was identified from an Ad3 cDNA library of *T. spiralis* (Li et al., 2008; Wu et al., 2009). In this study the cDNA encoding serine protease was expressed, purified, and used to immunize BALB/c mice, with alum adjuvant, to evaluate the protective effects in a murine model of *T. spiralis* infection.

MATERIALS AND METHODS

Parasites and animals

Trichinella spiralis (ISS534) was maintained in Wistar rats in our laboratory by serial passage infections. Muscle larvae were recovered from infected mice with artificial digestion solution (1% pepsin/HCl). Female BALB/c mice aged 6–8 wk were obtained from Norman Bethune University of Medical Science (NBUMS), China. They were kept under regulated conditions of humidity and temperature and fed with water and mouse feed ad libitum.

Expression and purification of recombinant *Ts-Adsp* protein (*rTs-Adsp*)

A cDNA encoding serine protease was immunoscreened from an Ad3 cDNA library of *T. spiralis* (Wu et al., 2009). The sequences were analyzed

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and nucleotide and protein were compared to existing databases in GenBank by BLAST searching (GenBank EU263328). The cDNA which encoded serine protease was named *Ts*-Adsp. The full-length cDNA was amplified by PCR from a *Ts*-Adsp/pBluescript phagemid with the following primers: forward, 5'-CCGAATTCATTTTCAGGAGGCTCT-3' and reverse, the T7TER primer. The PCR product was digested with *Eco*RI and *Xho*I and subcloned into the prokaryotic expression vector pET-28a. After transformation of *Escherichia coli* BL21 (DE3) (Invitrogen, Carlsbad, California), the expression of the recombinant protein was induced by IPTG at 37 °C for 4 hr. Following sonication and centrifugation, the recombinant protein was found in inclusion bodies and then purified by Ni-affinity chromatography (Qiagen, Valencia, California) according to the manufacturer's instructions. Subsequently, the bound protein was washed, eluted, and dialyzed. The protein was then assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Immunization schedule and challenge infection

The mice were divided into 3 groups of 20 animals each. Pre-immune sera were collected by tail bleeding before immunization. The mice in the first group were subcutaneously injected with 20 µg of r*Ts*-Adsp formulated with an alum adjuvant in a total volume of 200 µl. The mice were boosted twice with half the initial dose of the antigen at an interval of 2 wk. The last 2 groups were given phosphate-buffered saline (PBS) containing the same volume adjuvant or PBS only as the control animals. Two weeks after the last booster, each group of mice was challenged with 200 *T. spiralis* muscle larvae.

Analysis of IgG, IgG1, IgG2a, and IgE by enzyme-linked immunosorbent assay (ELISA)

The sera were collected by tail bleeding at 0, 1, 3, 5, 7, 9, and 11 wk post-immunization. The IgG, IgG1, IgG2a, and IgE against r*Ts*-Adsp in the sera were detected by ELISA. Briefly, a 96-well ELISA plate (Costar, Cambridge, Massachusetts) was coated with 1 µg of purified r*Ts*-Adsp in 100 µl/well bicarbonate buffer (pH 9.6) at 4 °C overnight. After blocking with 1% bovine serum albumin (Sigma, Saint Louis, Missouri) for 1 hr at 37 °C, the serum samples diluted with PBS were added and the plate was incubated at 37 °C for 1 hr. Then the wells were washed and HRP-conjugated goat anti-mouse IgG, IgG1, and IgG2a (Sigma) were added at 37 °C for another 1 hr. After extensive washing, the binding was visualized with the substrate o-phenylene-diamine (Sigma). The absorbance at 492 nm was measured. Specific IgE levels were measured with an ELISA kit (BD Biosciences Pharmingen, San Diego, California) as described previously (Deville et al., 2005).

In vitro splenocyte proliferation assay

One week after the last immunization, 5 mice from each group were sacrificed. Spleen cells were separated (Yang and Cook, 2003) and cultured in RPMI 1640 with 10% fetal bovine serum (Sigma) and 100 U/ml antibiotics (penicillin and streptomycin). After being counted, the cells at a density of 2×10^6 /ml were cultured in a 96-well cell culture plate (Costar) with 100 µl of RPMI 1640 per well. The cells were stimulated with 20 µg/ml r*Ts*-Adsp at 37 °C for 48 hr in the presence of 5% CO₂ followed by the addition of 10 µl/well CCK-8 (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan) solutions. The plates were then incubated for 4 hr in the dark. Splenocytes cultured in medium alone and stimulated with 4 µg/ml concanavalin A (Con A, Sigma) served as negative and positive controls, respectively. Then the absorbance of the samples at 450 nm was measured.

Detection of cytokine production

Spleen cells were separated, cultured as described above, and seeded into a 24-well cell culture plate at a density of 2×10^6 /ml in 400 µl of RPMI 1640. The cells were stimulated with 20 µg/ml r*Ts*-Adsp at 37 °C for 48 hr in an incubator containing 5% CO₂. Cells were stimulated with 4 µg/ml Con A and RPMI 1640 alone as positive and negative controls, respectively. After incubation for 48 hr, the supernatants were collected and the levels of IFN-γ, IL-2, IL-4, IL-10, and IL-13 were measured with an ELISA kit (eBioscience, San Diego, California) according to the manufacturer's instructions.

Flow cytometry analysis of T lymphocytes

One week after the final immunization, blood was collected from 5 mice in each group. Peripheral blood lymphocytes were prepared after removal of erythrocytes using lysing solution (Beckman, Fullerton, California). The cells were stained with optimal concentrations of FITC-conjugated anti-mouse CD3ε (0.5 mg/ml), PE/Cy5-conjugated anti-mouse CD4 (0.2 mg/ml), and PE-conjugated anti-mouse CD8a (0.2 mg/ml) (Biollegend, San Diego, California). Finally, the cells were fixed in PBS with 1% paraformaldehyde and the samples were analyzed by a BD FACSCalibur™ flow cytometer (BD Biosciences, Heidelberg, Germany).

Analysis of worm reduction

Ten mice from each group were sacrificed 35 days after challenge to estimate the number of muscle larvae (Kapel and Gamble, 2000). The level of protective immunity was calculated based on the mean number of muscle larvae collected from the vaccinated group compared with the number collected from the control group. The protection rate was calculated as follows:

Muscle larvae reduction (%) = $(1 - \frac{\text{average number of muscle larvae in immunized mice}}{\text{average number of muscle larvae in PBS control mice}}) \times 100\%$.

Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed using GraphPad Prism 5 (GraphPad InStat® Software, San Diego, California). Comparisons among the groups were performed with one-way or two-way analysis of variance. Data were expressed as the mean ± standard deviation (SD). *P*-values are denoted as follows for comparisons with the control group: ** = *P* < 0.01 and *** = *P* < 0.001.

RESULTS

Expression and purification of r*Ts*-Adsp

The gene of the encoding sequence of *Ts*-Adsp was cloned into pET-28a vector and the recombinant protein was expressed and induced by IPTG. After purification with Ni-affinity chromatography, the r*Ts*-Adsp was analyzed by SDS-PAGE. Purified recombinant proteins appeared as a single band (Fig. 1). The molecular mass of r*Ts*-Adsp was approximately 47 kDa, which is consistent with the expected size of the gene product. The pET-28a vector was also expressed as a control.

Humoral immune responses induced by vaccination

The vaccination of mice with r*Ts*-Adsp formulated with alum induced significantly higher levels of r*Ts*-Adsp-specific IgG than did the control treatment (Fig. 2A). The levels of IgG were greatly increased following the second and third immunizations. The levels of IgG subtypes were measured to further assess the efficacy of the vaccine. The results showed that the levels of IgG1 and IgG2a were significantly elevated after the last 2 immunizations, with IgG1 predominating (Fig. 2B). The levels of specific IgE were significantly increased after the second and third boost in immunized mice (Fig. 2C). Control groups did not express specific IgE.

Evaluation of splenocyte proliferation

The number of spleen cells from mice immunized with recombinant protein significantly increased when stimulated with r*Ts*-Adsp or Con A (Fig. 3). Stimulation with r*Ts*-Adsp had little effect on spleen cells from adjuvant or PBS control mice (*P* > 0.05). The data suggest that proliferation of splenocytes from immunized mice can be induced in vitro in response to stimulation with r*Ts*-Adsp.

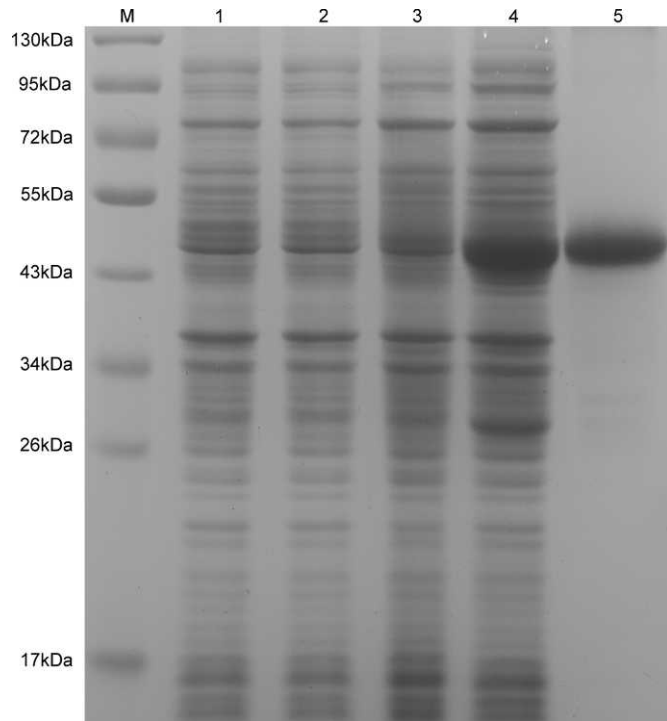


FIGURE 1. Expression and purification of *rTs-Adsp*. Proteins were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. M, Prestained Protein Marker (Genview, Houston, Texas); lanes 1 and 2, *Escherichia coli* lysate control (pET-28a expression vector) before and after IPTG induction, respectively; lanes 3 and 4, uninduced and IPTG-induced *E. coli* lysate (pET-28a-*Ts-Adsp*), respectively; lane 5, purified *rTs-Adsp* protein.

Evaluation of cytokine production

The spleen cells from immunized mice produced obviously higher levels of cytokines than did those from the control mice (Fig. 4). Spleen cells from the control groups showed extremely low expression of cytokines when stimulated with *rTs-Adsp*. No detectable cytokine expression was observed for unstimulated splenocytes.

Evaluation of the percent change of CD4⁺ and CD8⁺ T cells

The results showed that the percent of CD4⁺ T cells in the immunized group was higher than those in the control group (alum or PBS) ($P < 0.05$; Fig. 5). The CD4⁺:CD8⁺ ratio of *rTs-Adsp*-immunized mice ($74.93:21.86 = 3.43$) showed a significant increase compared to the alum- ($68.98:27.55 = 2.50$) or PBS-immunized mice ($67.08:30.42 = 2.20$). The results indicated that CD4⁺ lymphocytes played a major role in protection against *T. spiralis* infection.

Protective immunity

The immunized group exhibited a 46.5% reduction in the muscle larvae burden 35 days after challenged relative to the control group (Fig. 6). There was no difference in the muscle larvae burden between the alum-immunized and PBS-vaccinated control mice.

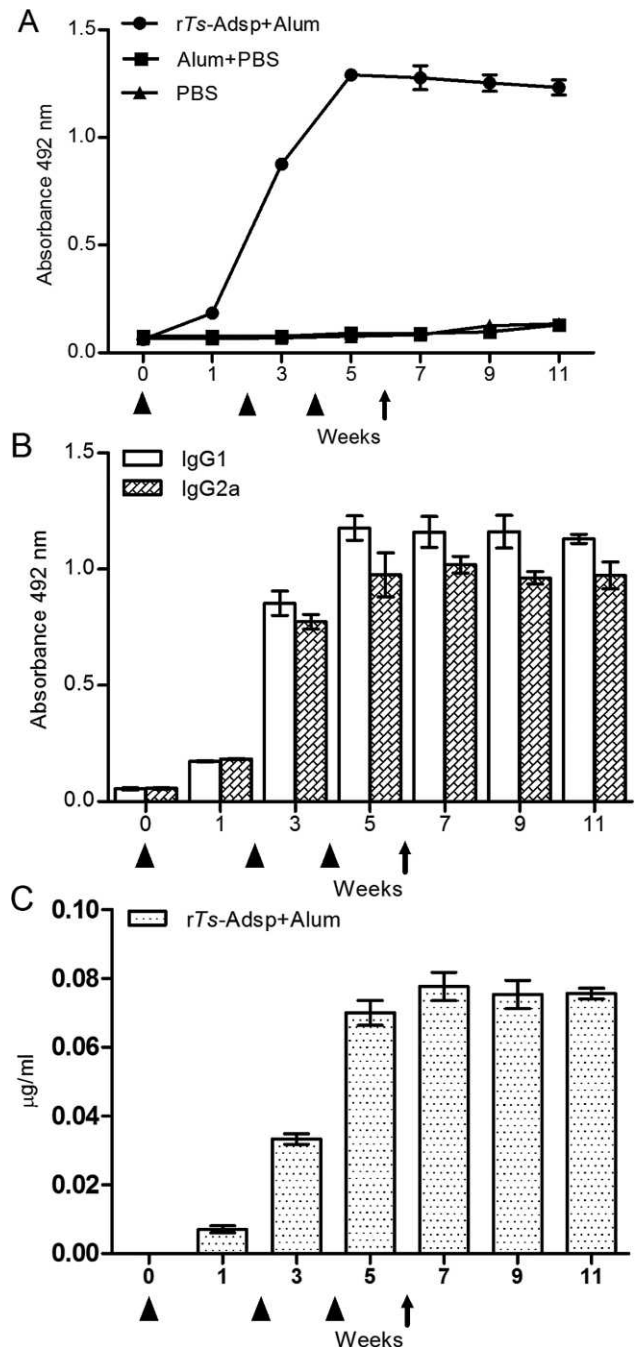


FIGURE 2. Analysis of antibody responses in mice. Anti-*rTs-Adsp* IgG levels in the sera of immunized mice or control (alum or PBS) mice were measured by ELISA (A). The IgG subclass responses to *rTs-Adsp*-immunized mice were detected at different time points (B). *rTs-Adsp*-specific IgE levels in the sera of immunized mice were measured (C). The values shown for each group are the mean $\pm$ SD of the antibody levels ($n = 10$). The immunization time points are marked with solid triangles ($\blacktriangle$) and the challenge day is marked with a solid arrow ($\uparrow$).

DISCUSSION

Because most of the serine protease-like antigens are present during the Ad and NBL stages, serine proteases may play important roles in *T. spiralis* development, invasion, and

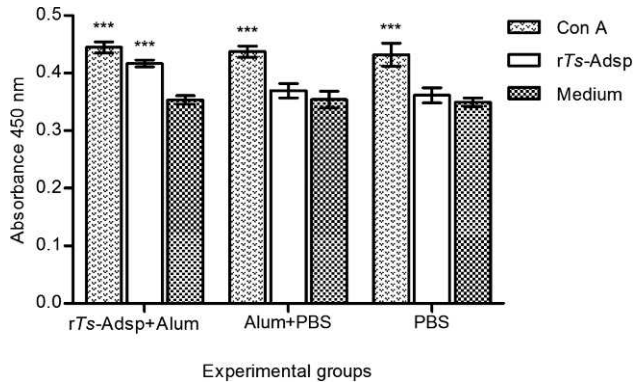


FIGURE 3. Splenocyte proliferative responses. Spleen cells from rTs-Adsp-vaccinated and control mice were collected 1 wk after the last immunization. Splenocytes were stimulated in vitro with rTs-Adsp, concanavalin A, or cultured with medium alone. Cellular proliferation was estimated using the Cell Counting Kit-8 (CCK-8) assay; values are presented as the mean $\pm$ SD ($n = 5$). *** $P < 0.001$ versus the medium-only culture.

migration (Wu et al., 2009). Adult *T. spiralis* secrete primarily serine proteinases, which degrade immunoglobulin and facilitate the penetration of the parasites into host enterocytes in addition to promoting the evasion of the host immune responses (Todorova et al., 1995; Todorova and Stoyanov, 2000). A trypsin-like serine protease could be involved in host immune suppression by influencing the outcome of the host and parasite relationship and may contribute to parasites' infection and survival (Balasubramanian et al., 2010). The humoral immune responses induced by infection with *T. spiralis* could inhibit serine

proteinases activity, and the inactivation of the proteinases by antibodies plays a crucial role in the survival of *T. spiralis* and may minimize the direct damage of host tissues (Todorova, 2000; Todorova and Stoyanov, 2000). Consequently, these proteinases are good targets for anti-helminth vaccines. A previous study argued that *Dictyocaulus viviparus* proteinases (including serine proteinases) can limit parasite-mediated tissue damage by functioning as targets for the antibody response, and the proteinases were the active component in anti-*D. viviparus* vaccine (Britton et al., 1992). In this study, we expressed and purified a gene encoding serine protease that was obtained from an adult *T. spiralis* cDNA library to investigate a vaccine candidate for *T. spiralis*.

The mice were subcutaneously injected with the recombinant protein formulated with alum to determine whether it could be a suitable vaccine candidate for the control of *T. spiralis* infections. The antibody response is an important indicator of whether a vaccine works (Harris and Gause, 2011). When developing recombinant anti-helminth vaccines, most antigens are selected based on the level of the antibody response induced (Maizels et al., 1999), and vaccines eliciting strong protection are usually relevant to an efficient antibody response (Diemert et al., 2008; McManus and Loukas, 2008). In this study, the IgG antibody titer was significantly higher in the vaccine group than in the control group. The strong immune response continued for several weeks until the end of the experiments. Simultaneously, we measured the serum levels of rTs-Adsp-specific IgG1 and IgG2a to elucidate the possible mechanisms of protection. IgG1 antibodies may reflect a Th2 response and IgG2a antibodies reflect Th1-cell activation (Else and Finkelman, 1998). The results showed that rTs-Adsp induced a mixed IgG1–IgG2a antibody

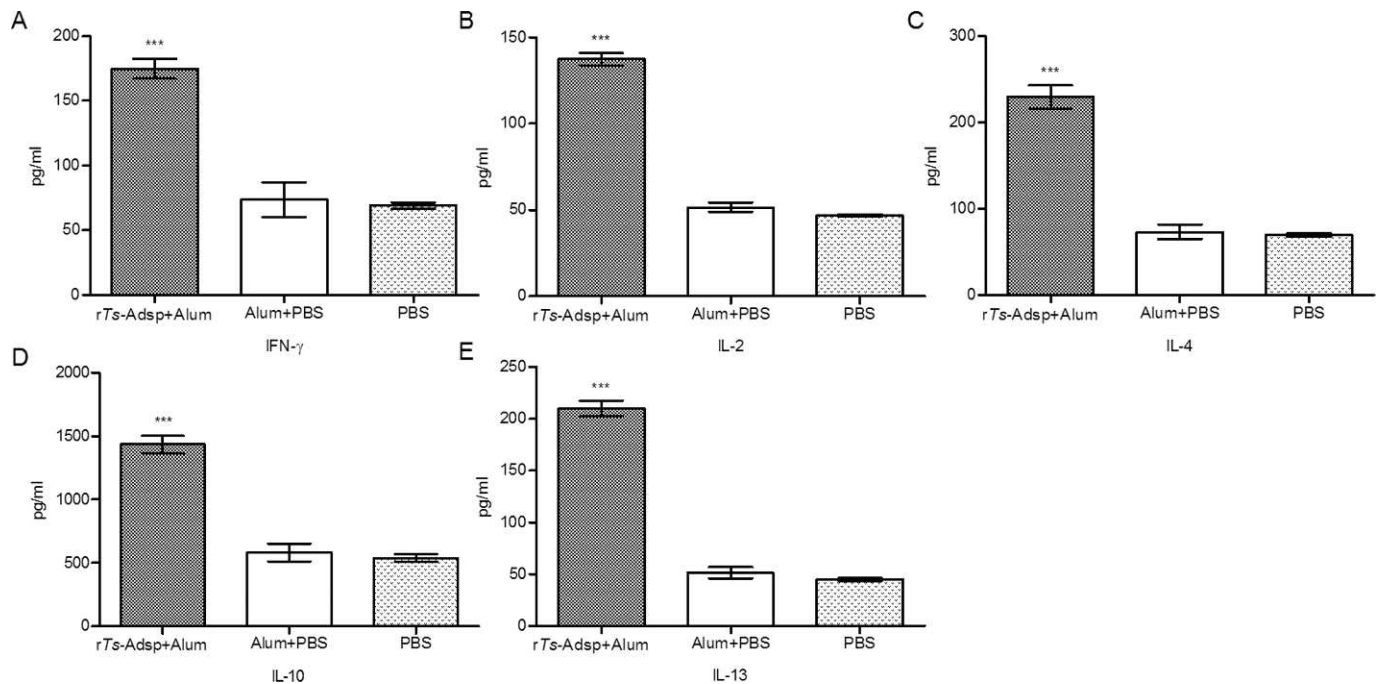


FIGURE 4. Production of cytokines from splenocytes after rTs-Adsp stimulation in vitro. One week after the last immunization, spleen cells were obtained from each group of mice. Analysis was performed on cells stimulated for 48 hr with 20 μ g/ml rTs-Adsp. The levels of (A) IFN- γ , (B) IL-2, (C) IL-4, (D) IL-10 and (E) IL-13 are presented as the mean $\pm$ SD ($n = 5$). *** $P < 0.001$ indicates that the production of cytokines by immunized mice was significantly different from that by PBS-treated mice.

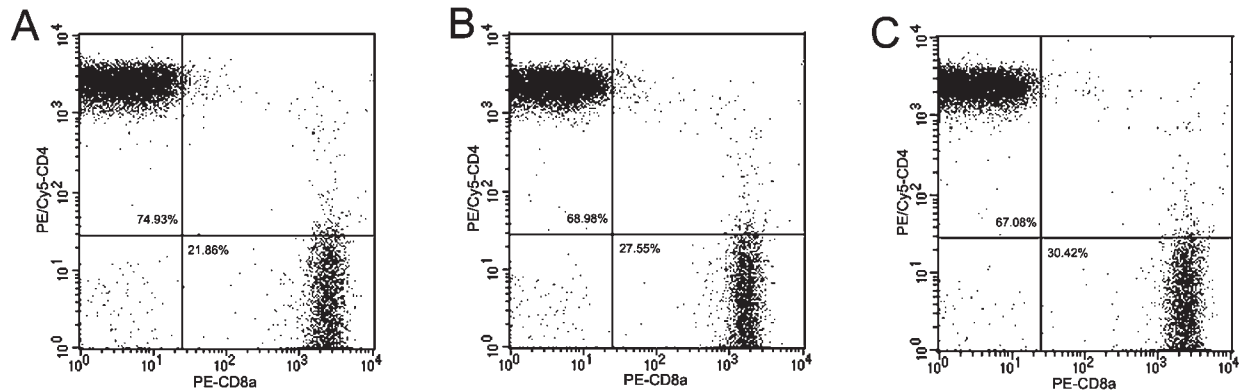


FIGURE 5. Flow cytometry analysis of T lymphocytes. The percent change of CD4⁺ and CD8⁺ T cells in peripheral blood lymphocytes from (A) immunized mice, (B) alum-, and (C) PBS-control mice. A gating strategy was used to analyze CD4 and CD8 expression. CD3⁺ T cells were gated, getting rid of myeloid cells, B cells, and dead cells. The upper left and lower right quadrants of each panel show the CD3:CD4 and CD3:CD8 double positive cells, respectively.

response, with IgG1 predominating. The data showed that the levels of IgE response were elevated in immunized mice. IgG1 and IgE antibodies are under the control of a Th2-type response. It is important to analyze the expression of IgE because the elevated levels of IgE antibodies are implicated in the clearance of *T. spiralis* worms at the intestinal mucosa level (Watanabe et al., 2005).

A successful prophylactic vaccine should trigger both humoral and cellular immune responses in immunized individuals. A lymphoproliferative assay performed after the stimulation of spleen cells with rTs-Adsp showed that the number of splenocytes from immunized mice increased substantially. The results indicated that the vaccine induced a cell-mediated immune response. Cytokines are regulatory proteins that modulate the immune response by lymphocyte activation, proliferation, differentiation, survival, and apoptosis which can affect the immune system (Giulietti et al., 2001; Zaros et al., 2007). After stimulation with rTs-Adsp, the splenocytes produced elevated levels of IL-2 and IFN- γ , which are markers of the Th1 response, and of IL-4, IL-10, and IL-13 which are the markers of the Th2 response. In accordance with the antibody responses, the immunization

induced both Th1- and Th2-like responses, with the Th2 type prominent. Among CD4⁺ T cells, Th2-dominated responses are critical in host protective immune responses to many gastrointestinal nematodes (Foster and Elsheikha, 2012). Th2-type cytokines are associated with humoral immune responses and can lead to the aggregation and activation of eosinophils and mast cells, thus inhibiting the parasite infection (Bruschi and Chiumiento, 2012). IL-4 and IL-13 are important in the development of resistance to infection with *T. spiralis*, and the worm will survive for a prolonged period when the effects of these cytokines are suppressed (Patel et al., 2009). IL-10 plays a predominant role in promoting resistance to parasites and in simultaneously protecting infected animals from developing fatal immunopathologies (Schopf et al., 2002). The findings are consistent with those of previous reports which demonstrated that both Th1 and Th2 immune responses are important in protective immunity against *T. spiralis* infection; however, Th2-type antibodies and cytokines play more important roles (Scales et al., 2007; Yang et al., 2010).

The ratio of CD4⁺:CD8⁺ T cells may be used as an indicator of cell-mediated immune response. Results from this study showed that the CD4⁺:CD8⁺ ratio was increased significantly in immunized mice, with increased CD4⁺ T cells while CD8⁺ T cells decreased. These results are consistent with the antibody responses and cytokine production described above. The immune protection against *T. spiralis* challenge is elicited by rTs-Adsp and mediated through induction of CD4⁺ T cells in which a Th2-type immune response predominated.

Protective immunity is ultimately associated with a reduction in the muscle larvae burden. The data showed that the vaccine induced partial protection against *T. spiralis* larval challenge. As we know, many factors affect the immune response to vaccines such as antigen composition and dose, the type of vaccine, the immunization route, and the adjuvant used. Alum is a good candidate for the preparation of anti-parasite vaccines because it can preferentially induce a Th2 immune response that is similar to the response stimulated by some helminths (Gupta, 1998). Because *T. spiralis* is an antigenically complex parasite, the immune responses induced by single-antigen vaccination may lead to stage-limited protection. It has been reported that an effective vaccine could consist of multiple antigens that can elicit immune responses targeting the same life cycle stage (Lightowlers et al.,

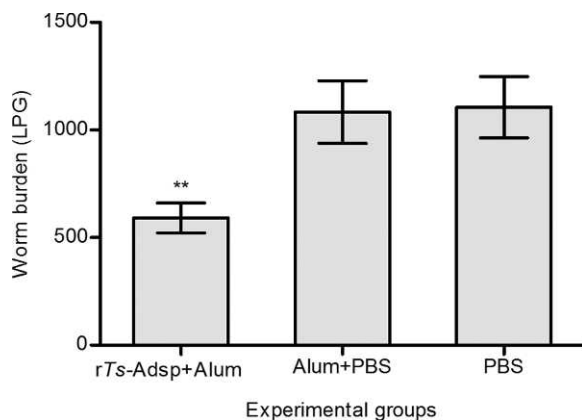


FIGURE 6. Protective immunity of rTs-Adsp-vaccinated mice after being challenged with 200 *Trichinella spiralis* larvae. The results are presented as the mean $\pm$ SD (n = 10). ** P < 0.01 indicates statistically significant differences with respect to muscle larvae burden relative to the PBS control group.

2003). Alternatively, the composition of proteins should be effective on different genetic backgrounds and could generate strong protection against parasite invasion (Dziadek et al., 2011). Therefore, we will optimize the conditions to achieve the desired level of protection.

In conclusion, the serine protease gene of adult *T. spiralis* can induce strong humoral and cellular immune responses. The vaccinated mice showed partial protection against challenge with *T. spiralis* larvae. The findings provide a valuable component of a future vaccine against trichinellosis, and we will exploit a better scheme to improve the protection or will mix with other effective stage-specific antigens to enhance the protective response.

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