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RESEARCH ARTICLE

Specific binding of aspartic protease and enterocytes promotes *Trichinella spiralis* invasion of murine intestinal epithelium cells

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ABSTRACT

Trichinella spiralis is an important foodborne zoonotic parasite and it is necessary to develop vaccine to prevent *T. spiralis* infection in food animals. *T. spiralis* aspartic protease-2 (TsASP2) has been demonstrated to play a crucial role in larval invasion of intestinal epithelium cells (IECs). The purpose of this study was to assess the interaction between TsASP2 and IECs and to investigate the immune protection elicited by vaccination with rTsASP2. The results showed that the enzymatic activity of native aspartic protease was detected in crude proteins of all *T. spiralis* development stages other than NBL stage, the highest activity was observed in the IIL stage. The results of Western blot showed that TsASP2 protein was expressed at ML, IIL and AW but not NBL, and the TsASP2 expression level at IIL stage was significantly higher than those of other three worm stages ($P < 0.05$). The specific binding between rTsASP2 and IECs was observed by immunofluorescence test (IFT) and confocal microscopy, and the binding site was localized at the IEC membrane and this binding ability was inhibited by aspartic protease specific inhibitor pepstain A. The results of ELISA showed that the binding ability was protein dose-dependent. Vaccination with rTsASP2 triggered a mixed Th1/Th2 humoral and mucosal immune responses, as demonstrated by the elevation levels of Th1/Th2 cytokines (IFN-γ and IL-4) secreted by the spleen and mesenteric lymph nodes (MLNs) of immunized mice. The mice vaccinated with rTsASP2 exhibited a 54.17% reduction in enteral adult worms and a 54.58% reduction in muscle larvae after *T. spiralis* challenge. The results demonstrated that TsASP2 might be a potential molecular target for anti-*Trichinella* vaccines.

Keywords: *Trichinella spiralis*; aspartic protease; interaction; invasion; intestinal epithelium.

INTRODUCTION

Trichinella spiralis is an important foodborne pathogen that has been found to infect many kinds of mammalian species (Pozio, 2007). Human *Trichinella* infection is mainly caused from ingestion of raw or poorly cooked meat containing the infective larvae of *Trichinella* (Cui *et al.*, 2013a). Outbreaks of trichinellosis have been reported in many countries, especially in developing countries. From 2004 to 2009, 15 trichinellosis outbreaks were reported in mainland China (Cui *et al.*, 2011). The main infection source of trichinellosis is domestic pigs (Jiang *et al.*, 2016; Rostami *et al.*, 2017). This disease has become an important public health problem and gained an increasing attention worldwide. Therefore, it is necessary to develop the vaccines to block *Trichinella* infection transmission in food animals (Bai *et al.*, 2017; Qi *et al.*, 2018).

After the infected muscle tissue is ingested and digested by the digestive enzymes, the muscle larvae (ML) are released and then activated into intestine infective larvae (IIL) by exposure to intestinal contents or bile (Ren *et al.*, 2013). The IIL penetrate into the enteric epithelium and grow to adult worms (AWs). The IIL invasion of intestinal epithelium cells (IECs) is crucial for the establishment of *Trichinella* infection (Liu *et al.*, 2013). Intestinal epithelium is not only the first host's native innate defense barrier but also the principal interaction location between *T. spiralis* and the host (Long *et al.*, 2015; Wang *et al.*, 2017). Therefore, analysis of the interaction between *T. spiralis* proteins and the IECs is helpful to elucidate the mechanism of the *Trichinella* invasion of intestinal epithelium (Han *et al.*, 2020; Hu *et al.*, 2020b).

Trichinella spiralis excretory/secretory (ES) proteins play an essential role in parasite invasion and regulating host immune responses (Bolas-Fernandez *et al.*, 2006; Bien *et al.*, 2012). In our previous studies, a *T. spiralis* aspartic protease-2 (TsASP2; GenBank: 339237490) was identified from ES proteins of ML and IIL. Recombinant TsASP2 (rTsASP2) was

expressed and purified and it possessed the aspartic protease activity, cleaved several host proteins, facilitated *T. spiralis* invasion of murine IECs (Xu *et al.*, 2020a). The purpose of this study was to assess the interaction between TsASP2 and IECs and to investigate the immune protection elicited by vaccination with rTsASP2.

MATERIALS AND METHODS

Parasites, experimental animals

T. spiralis strain (ISS534) utilized in this experiment was collected from domestic pigs in central China (Wang *et al.*, 2012). Female BALB/c mice aged 5 weeks old were purchased from the Experimental Animal Center of Henan Province. All animal experimental procedures were approved by the Life Science Ethics Committee of Zhengzhou University (No. SCXK 2017-0001).

Preparation of worm crude protein and rTsASP2 protein

T. spiralis ML from infected mice were obtained using artificial digestion (1% pepsin and 0.75% HCl) at 35 days post infection (dpi) (Jiang *et al.*, 2012). The IIL were isolated from the infected mouse intestines at 6, 12 and 24 hours post infection (hpi) and the AWs were obtained from mouse intestines at 36, 48, 60 hpi and 3 dpi, and 6 dpi (Liu *et al.*, 2015c; Sun *et al.*, 2015). The AW were cultured in DMEM for 24 h at 37°C and the NBL were harvested (Li *et al.*, 2015; Wu *et al.*, 2016). *T. spiralis* worms were homogenized with tissue grinder and ultrasonication, then the supernatant containing worm crude proteins was collected after centrifugation (Yang *et al.*, 2015). The concentration of crude proteins was measured by the Bradford method. The rTsASP2 protein was prepared in our laboratory (Xu *et al.*, 2020a). The *Escherichia coli* BL21 (DE3) containing the recombinant plasmid pMAL-c2x/ TsASP2 was induced with 0.1 mM IPTG at 16°C for 20 h, the rTsASP2 was purified with an Amylose Prepacked Column (NEB, China) (Cui *et al.*, 2015b; Xu *et al.*, 2018). The anti-rTsASP2 serum was prepared as described (Long *et al.*, 2014; Sun *et al.*, 2018b).

Analysis of aspartic protease activity in different stages of *T. spiralis*

The fluorescent substrate MCA-Gly-Lys-Pro-IleLeu-Phe-Phe-Arg-Leu-Lys (DNP)-D-Arg- amide (synthesized by Sangon Biotech Co., Shanghai, China) was used to assess the aspartic protease enzymatic activity of *T. spiralis* soluble crude proteins. Total reaction volume was 100 µl, including 50 µg/ml crude proteins and 5 µM fluorescent substrate. After mixing the crude proteins and substrate for 30 min, the reaction termination fluid (35% methyl alcohol, 30% ethyl alcohol, 35% ddH_2O) was added, and the fluorescent intensity was continuously detected with spectrophotofluorometry (Synergy H1, BioTek, USA) using excitation wavelength 320 nm and emission wavelength 390 nm, respectively (Mantilla-Olea *et al.*, 2018).

RT-PCR and Western blotting analysis of TsASP2 expression in various *T. spiralis* phases

Total RNAs from ML, IIL, 3 day AW, 6 day AW and NBL were extracted using Trizol reagent (Invitrogen, USA). RT-PCR was performed to ascertain TsASP2 gene transcription in different *T. spiralis* stages as previously reported (Zhang *et al.*, 2013). A control gene GAPDH from *T. spiralis* (GenBank: AF452239) was also amplified (Wang *et al.*, 2013a).

The crude proteins of *T. spiralis* at various stages (ML, IILs, AW at 3 dpi, AW at 6 dpi and NBL) were separated by SDS-PAGE (Wang *et al.*, 2013b), and these proteins were transferred onto the polyvinylidene difluoride (PVDF) membrane. The membrane was first incubated with 5% skimmed milk in Tris-buffered saline- 0.5% Tween-20 (TBST) at 37°C for 2 h, and then reacted with anti-rTsASP2 serum (1:100 dilution) at 37°C for 1 h. After washing with TBST 3 times, the membrane was incubated with HRP-conjugated anti-mouse IgG (Sigma-Aldrich, USA) at 37°C for 1 h. After washing again, the membrane was visualized using an enhanced chemiluminescent Kit (Beyotime Biotech, China) (Liu *et al.*, 2014; Wang *et al.*, 2014; Zhang *et al.*, 2016). The mouse anti-GAPDH antibody was used as an internal control after the membrane was washed with the stripping buffer (Beyotime Biotech, China) (Liu *et al.*, 2016; Yang *et al.*, 2019a) .

Immunofluorescence test (IFT)

The location of TsASP2 at *T. spiralis* worms was observed by IFT as previously reported (Liu *et al.*, 2018; Cui *et al.*, 2019). *T. spiralis* worms (ML, IIL, AW at 3 dpi and NBL) and intestine isolated from infected mice at 6 hpi and 3 dpi were embedded in paraffin and sliced into 2 µm-thick sections. The sections were blocked with 5% normal goat serum at 37°C for 30 min and then probed by anti-rTsASP2 serum. The sections were also incubated with infection serum or normal serum as positive or negative control. The FITC-conjugated anti-mouse IgG (1:100; Santa Cruz, USA) was used to stain the section, and the results were observed under fluorescent microscopy (Olympus, Japan) (Li *et al.*, 2018; Song *et al.*, 2018b)

ELISA analysis of specific binding between TsASP2 and IEC protein

The IECs were isolated from normal mouse intestine in our laboratory (Ren *et al.*, 2011). The binding between TsASP2 and IECs was measured by ELISA (Sun *et al.*, 2018a; Guo *et al.*, 2020). Briefly, the ELISA plate was coated with various concentrations of IEC proteins overnight at 4°C. After blocking with 5% skimmed milk in PBST, the ELISA plate was incubated with different concentrations of rTsASP2 at 37°C for 2 h. Anti-rTsASP2 serum and normal serum (1:100) were added in the plate at 37°C for 1 h and then HRP labelled anti-mouse IgG was added as the secondary antibodies. The result was colored with 3, 3', 5, 5'-tetramethylbenzidine (TMB, Solarbio, China) and the optical density (OD) at 450 nm was measured as previously reported (Xu *et al.*, 2020b).

Confocal microscopy

The interaction between IECs and TsASP2 was also examined by IFT and confocal microscopy (Ren *et al.*, 2018; Lei *et al.*, 2020). IECs were grown on the cell culture plate until the confluence. rTsASP2 or MBP proteins (20 µg/ml) was added on the IECs and cultured for 2 h. The IECs were fixed with paraformaldehyde, and following blocked with 5% normal goat serum. After incubating with anti-rTsASP2 serum, immune serum against maltose-binding protein (MBP) tag or normal serum for 1 h, the IECs were stained with FITC-conjugated anti-mouse IgG for another hour. To analyze influence of rTsASP2 protease activity on the interaction, an aspartic protease specific inhibitor-pepstain A was used to pre-incubate with rTsASP2. The IEC nuclei were stained with propidium iodide (PI). The results were observed under confocal microscopy (Song *et al.*, 2018a; Yan *et al.*, 2020).

Immunization regimen and evaluation of systemic immune response

Four groups of male BALB/c mice (40 animals each group) were used in this study. The first group of mice was immunized subcutaneously with 20 µg of rTsASP2 emulsified in ISA201 adjuvant and two booster immunization was performed using the same method at a 2- week-interval. Other three groups were subcutaneously immunized with MBP protein emulsified in ISA201, only ISA20 adjuvant or PBS

alone using the same vaccination scheme (Long *et al.*, 2014; Yue *et al*., 2020). To evaluate the induced systemic responses, serum samples were collected from vaccinated mice at weeks 0, 2, 4 and 6 after immunization.

The levels of anti-rTsASP2 antibodies (total IgG, IgG1 and IgG2a) in serum samples of immunized mice were determined by ELISA with IIL crude proteins (Sun *et al.*, 2019a; Hu *et al.*, 2020a). Briefly, the plates were coated with IIL crude proteins (0.25 μg/ml) at 4°C overnight. After blocking with 5% skimmed milk, the plates were incubated with immune serum (1:100 dilution). Then HRP-conjugated goat anti-mouse IgG, IgG1 or IgG2a were added and incubated at 37°C for 1 h. O-phenylenediamine dihydrochloride (OPD) was used as substrate and the reaction was terminated with 2 M H_2SO_4. The absorbance at 450 nm was measured using a microplate reader (Xu *et al.*, 2020b).

ELISA determination of intestinal total and specific sIgA

To determine total and TsASP2-specific sIgA in enteric fluid, enteric washings were collected as previously reported (Bermudez-Cruz *et al.*, 2016; Sun *et al.*, 2019b). In brief, a 20-cm long of enteric segment was dissected, and the enteric interior was eluted with cold PBS with 1% protease inhibitor PMSF (Sangon Biotech, Shanghai, China). The washing were recovered, centrifuged at 5000 *g* for 5 min, and the supernatants were collected (Li *et al.*, 2018). Enteral total and specific sIgA was measured as described before (Zhang *et al.*, 2020a).

Cytokine determination

To ascertain the specific cellular immune responses against rTsASP2 immunization, the spleens and mesenteric lymph nodes (MLNs) were isolated from immunized mice at 0, 2, 4, 6 weeks post vaccination (Qi *et al.*, 2018; Zhang *et al.*, 2020a). The splenocytes and MLN cells were prepared in the DMEM medium and their density were adjusted to 2×10^5 cells/ml. After the cells were stimulated with rTsASP2 at a concentration of 3 μg/ml at 37°C for 72 h, the culture supernatant was collected and the concentrations of cytokines (IFN-γ and IL-4) were measured using a sandwich ELISA method (Pompa-Mera *et al.*, 2014; Zhang *et al.*, 2020b).

Larval challenge experiment

Two weeks after the last immunization, all vaccinated mice were challenged orally with 300 *T. spiralis* ML. Intestinal AWs were recovered from ten mice each group 6 days after challenge. The muscle larvae were examined in remaining 10 mice from each group at 35 dpi (Liu *et al.*, 2015a). The worm reduction rate was calculated based on the number of AW or larvae per gram (LPG) of skeletal muscles collected from immunized group versus those from the PBS control group (Wang *et al.*, 2018; Yang *et al.*, 2020).

Statistical analysis

Data were represented as the means ± standard deviation (SD). The statistical analyses were performed with One-way ANOVA and student's *t*-test using SPSS version 19.0 software. $P < 0.05$ was considered as statistically significant.

RESULTS

Aspartic protease activity of *T. spiralis* at different stages

To determine the enzymatic activity of native aspartic protease in various *T. spiralis* worm stages, a specific fluorogenic synthetic substrate was incubated with crude proteins of various *T. spiralis* worm stages. The results showed that aspartic protease activities in intestinal stage worms (6 hours-3 days) were significantly higher than that in the ML stage (Figure 1) ($P<0.05$). The aspartic protease activity was scarcely detected in NBL stage.

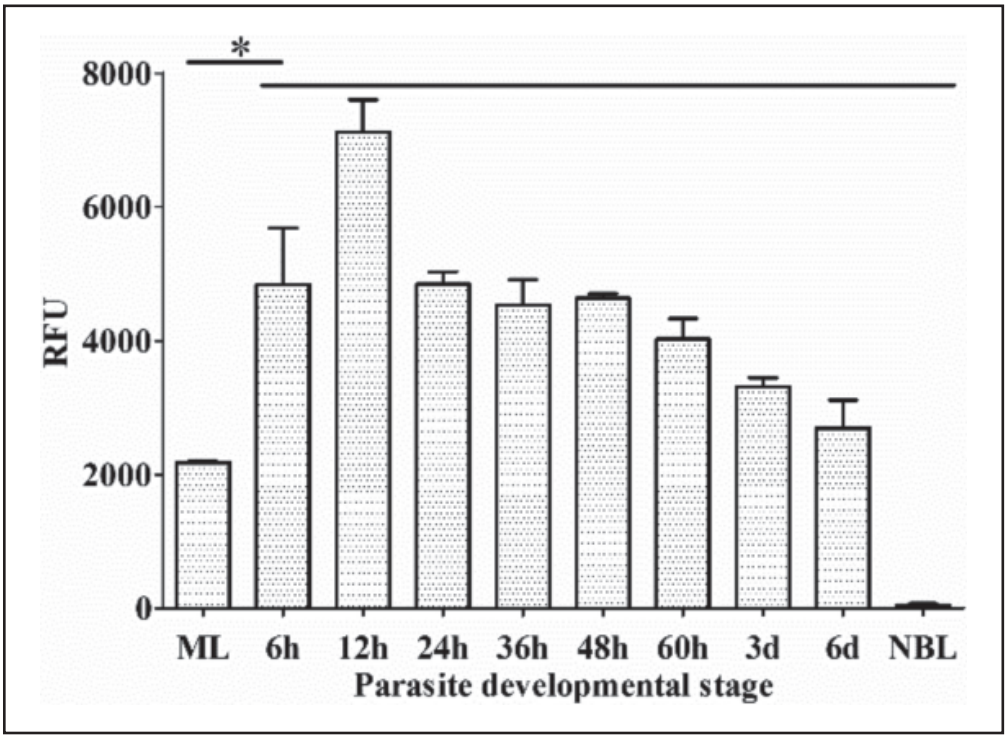

Figure 1. Analysis of aspartic protease activity in crude protein of *T. spiralis* at various stages. Asterisks indicate a statistically significant difference compared with the ML stage ($P < 0.05$). RFU, relative fluorescence units.

TsASP2 expression level at different *T. spiralis* stage worms

TsASP2 mRNA transcript (1280 bp) at different *T. spiralis* stage worms was amplified by using RT-PCR method (Figure 2A). The control gene GAPDH (580 bp) was also observed at diverse worm phases (Figure 2B). Surprisingly, Western blot analysis revealed that TsASP2 protein was expressed in ML, IIL and AW stages but not in NBL stage. The TsASP2 expression level at IIL stage was significantly higher than those in other worm stages (ML, 3-day and 6-day adults, and NBL) ($P < 0.05$) (Figure 2C).

Expression and Localization of TsASP2 at different stage worms by IFT

Expression and localization of TsASP2 in different *T. spiralis* phases were investigated using IFT. The results showed that native TsASP2 was localized at the muscle cells, stichosome and midgut of ML and IIL, and around the embryos in the female AWs, but no immunostaining was detected in NBL cross-section (Figure 3).

Specific binding between TsASP2 and IECs protein measured by ELISA

The binding between rTsASP2 and IEC protein was confirmed by ELISA. The results showed that the OD values have a dose-dependent relationship with IEC proteins and revealed an elevating trend with the increasing IEC coating concentration. Furthermore, the OD values were also dose dependent on rTsASP2 and displayed an elevating trend with increasing rTsASP2 concentration (Figure 4).

Localization of rTsASP2 binding with IECs

The binding and cellular localization of rTsASP2 with IECs were detected by IFT with anti-rTsASP2 serum, but not by normal serum. No immunostaining was detected between MBP proteins and IECs, and rTsASP2 and C2C12 cells (Figure 5). Confocal microscopy showed that the binding between rTsASP2 and IECs was located at the cytomembrane of IECs (Figure 6). After rTsASP2 was pre-incubated with pepstain A, the staining intensity became weaken, suggesting that the binding of rTsASP2 and IECs was inhibited by the aspartic protease specific inhibitor pepstain A.

Humoral and mucosal responses

Serum specific anti-rTsASP2 antibody IgG level was obviously elevated following the first immunization compared with three control groups ($P<0.05$) (Figure 7A), demonstrating that rTsASP2 possesses good immunogenic. The IgG1 levels on weeks 2, 4 and 6 post immunization was significantly higher than IgG2a (t_{2w}=8.438, t_{4w} = 16.451, t_{6w} =12.489, $P < 0.0001$) (Figure 7B), suggesting that the Th1/Th2-mixed type of immune response was elicited by rTsASP2 immunization, and the Th2 response was dominant.

Furthermore, rTsASP2 also elicited the enteral mucosal sIgA response. Total sIgA level was evidently higher in TsASP2-immunized mice than that in mice inoculated with MBP, ISA201 and PBS alone (Figure 7C) (F_{2w} = 525.778, F_{4w} = 278.873, F_{6w} = 106.814, $P < 0.0001$) (Fig. 7C). Similarly, TsASP2-specific sIgA level was significantly higher than the three control groups (Figure 7D) (F_{2w} = 67.589, F_{4w} = 149.162, F_{6w} = 300.25, $P < 0.0001$).

Cytokine responses

To examine the cytokines secreted by MLN and spleen of mice immunized with rTsASP2, the cytokine levels of IFN-γ and IL-4 were measured by ELISA. Compared with MBP, ISA201 and PBS control groups, the IFN-γ levels in spleen and MLNs of rTsASP2 immunized mice were significantly increased at 4 and 6 weeks after first immunization (P <0.0001), and IL-4 levels were evidently elevated at 2, 4 and 6 weeks following immunization (P <0.0001) (Figure 8).

Immune protection

To investigate the protective efficacy of rTsASP2 immunization against *T. spiralis* infection, the intestinal adult worm, NBL production and muscle larval burden were examined in all vaccinated mice. Compared with the PBS group, the rTsASP2-immunized mice showed a 54.58% reduction in adult worms at 6 dpi (F = 26.72, $P < 0.0001$) and 54.17% reduction in muscle larvae at 35 dpi (Figure 9) (F = 37.811, $P < 0.0001$). Furthermore, the production of NBL by the female adults from rTsASP2-immunized mice was evidently decreased compared with the other three control groups (F = 20.476, $P < 0.0001$).

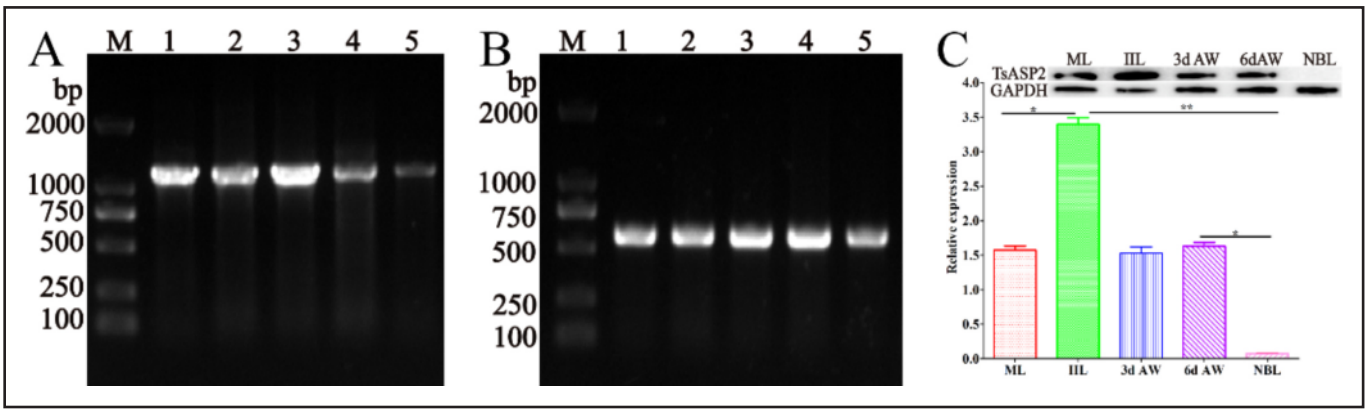

Figure 2. RT-PCR and Western blotting analysis of TsASP2 expression level at diverse *T. spiralis* phases. **A, B**: RT-PCR analysis of TsASP2 transcription. **A**: TsASP2 gene (1780 bp); **B**: GAPDH (570 bp); **M**: DL2000 DNA marker; lane 1: ML; lane 2: IIL; lane 3: 3 d AWs; lane 4: 6 d AWs; lane 5: NBL. **C:** Western blotting of TsASP2 expression in diverse *T. spiralis* stages based on three independent densitometry measurement. The relative expression level of TsASP2 in IIL the stage was significantly higher than those in other worm stages (*$P < 0.05$).

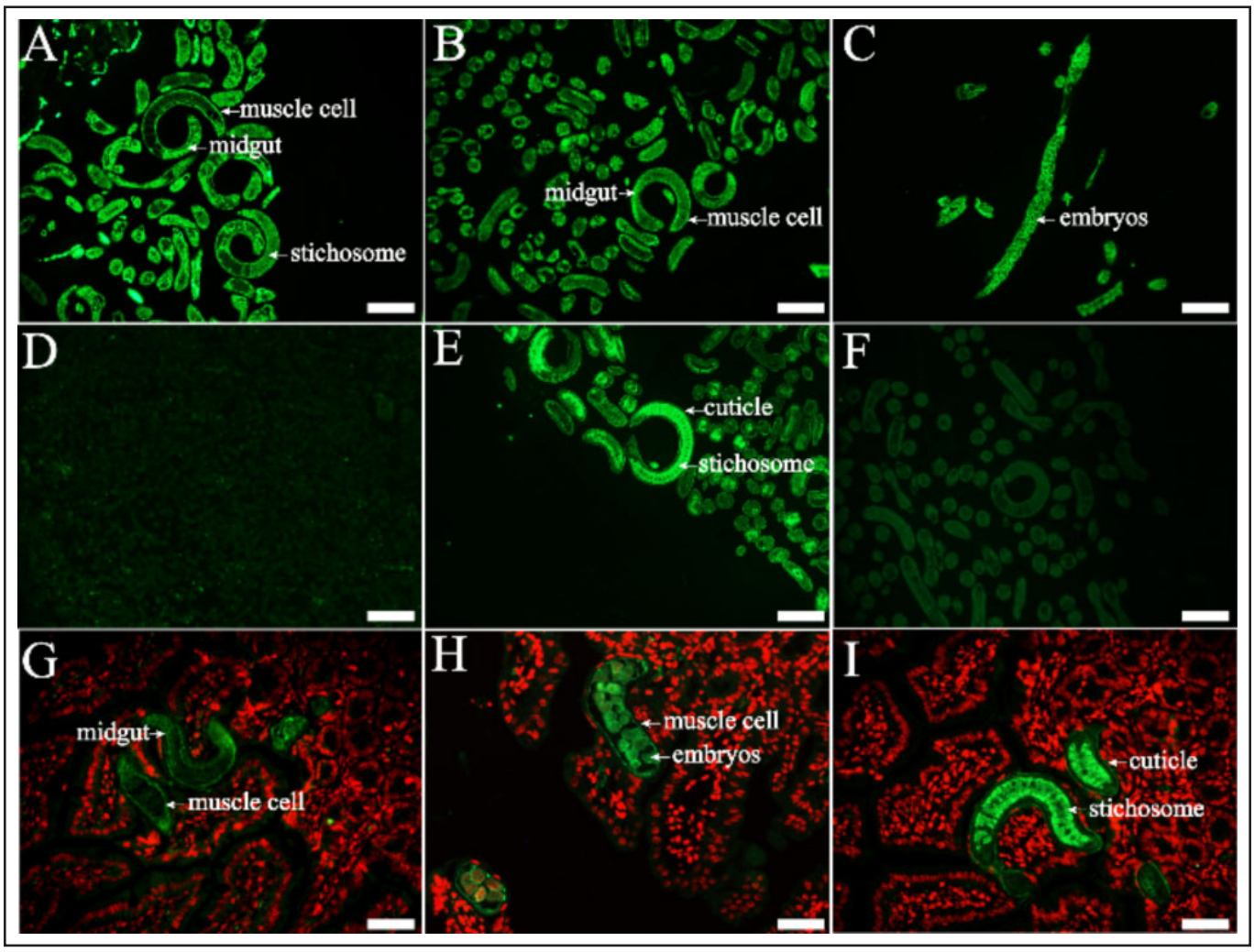

Figure 3. Immunolocalization of TsASP2 at various *T. spiralis* phases. The cross-sections of various *T. spiralis* phases including ML (A), IIL (B), AW (C) and NBL (D) and intestinal sections from infected mice at 6 hpi (G) and 3 dpi (H) were probed by anti-rTsASP2 serum. Fluorescence stating was observed at stichosome, muscle cells and midgut of ML and IIL, and surround the embryos of female AW. ML (E) and intestinal section at 6 hpi (I) were incubated by infection serum as a positive control; ML (F) incubated with normal serum as a negative control. Scale bars: 100 µm.

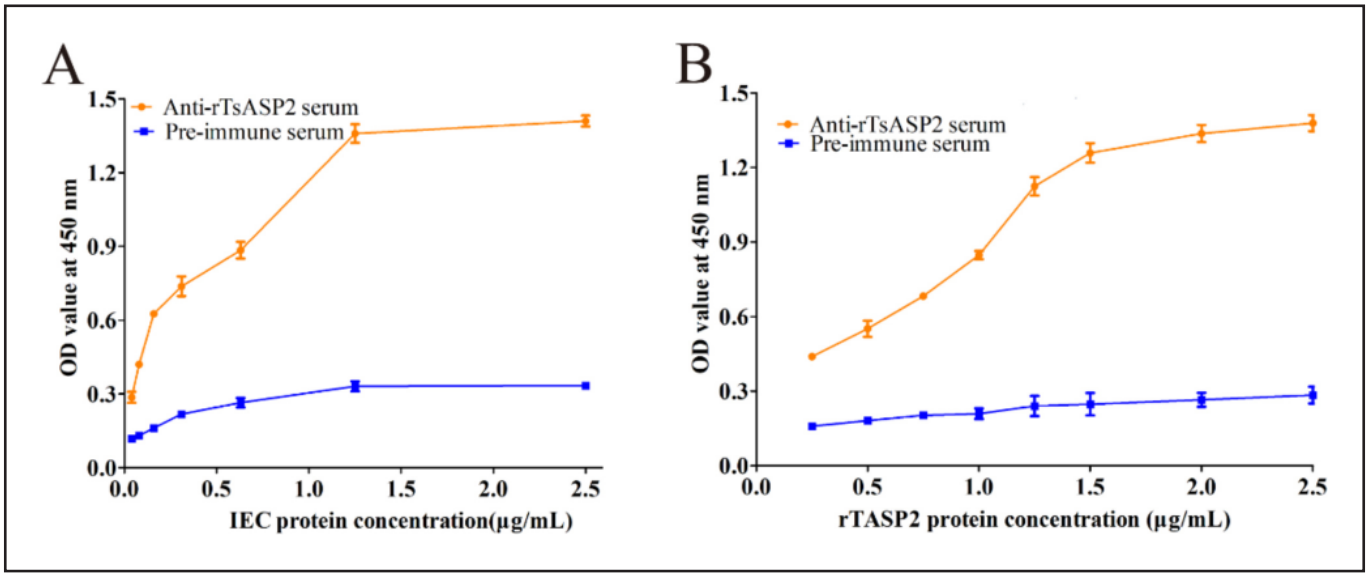

Figure 4. Binding ability of rTsASP2 with IECs measured by ELISA. **A**. Binding between 10 µg/ml rTsASP2 and various concentrations of IEC proteins. **B.** Binding between IEC proteins (5 µg/ml) and various concentrations of rTsASP2. The binding of rTsASP2 with IEC protein is dose-dependently related to rTsASP2 and IEC protein.

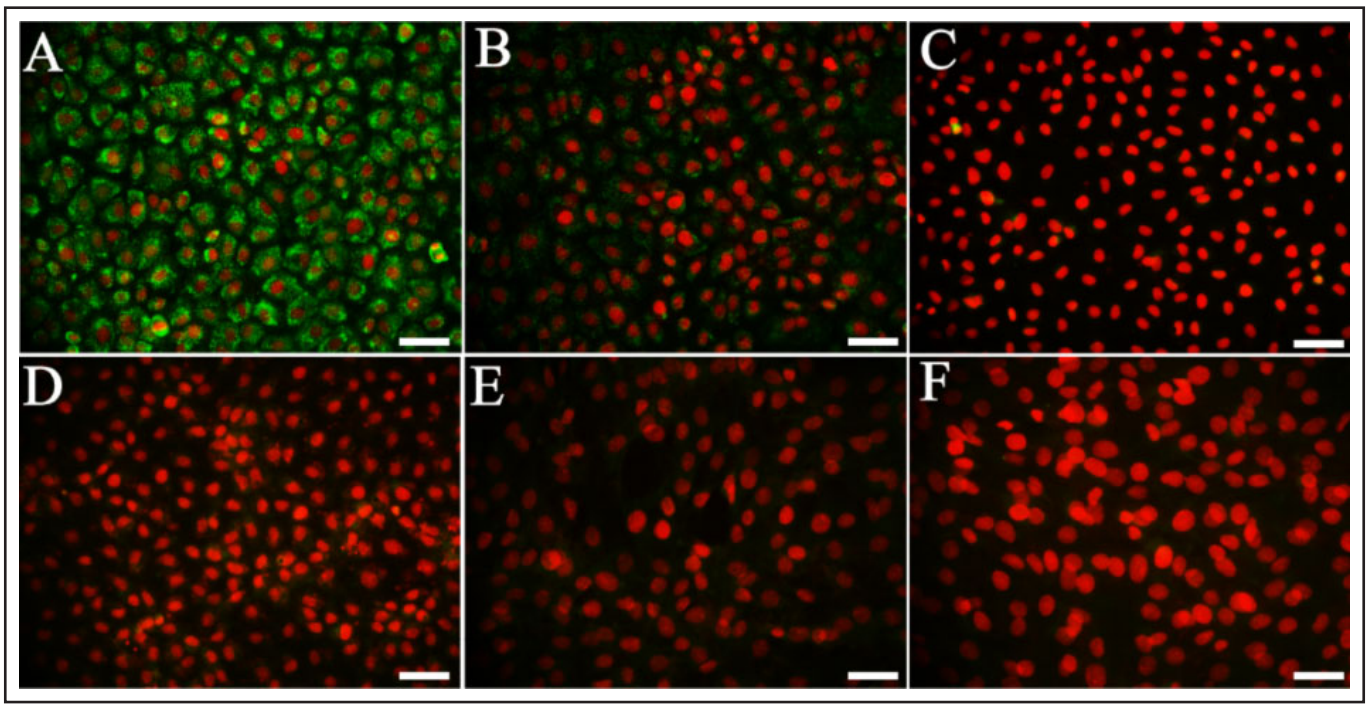

Figure 5. Specific binding between rTsASP2 and IEC analyzed by IFT. **A-D**: IECs; **E-F:** C2C12 cells. **A**: IECs were incubated with rTsASP2 and then probed with anti-rTsASP2 serum. **B**: IECs were incubated with rTsASP2 pre-incubated with pepstain A and then probed with anti-rTsASP2 serum; **C**: IECs were incubated with rTsASP2 and then probed with normal serum. **D:** IECs were incubated with MBP protein and probed with anti-MBP serum. **E**: C2C12 cells were with rTsASP2 and probed with anti-rTsASP2 serum. **F**: C2C12 cells were incubated with MBP protein and probed with anti-MBP serum. Scale bars: 50 µm.

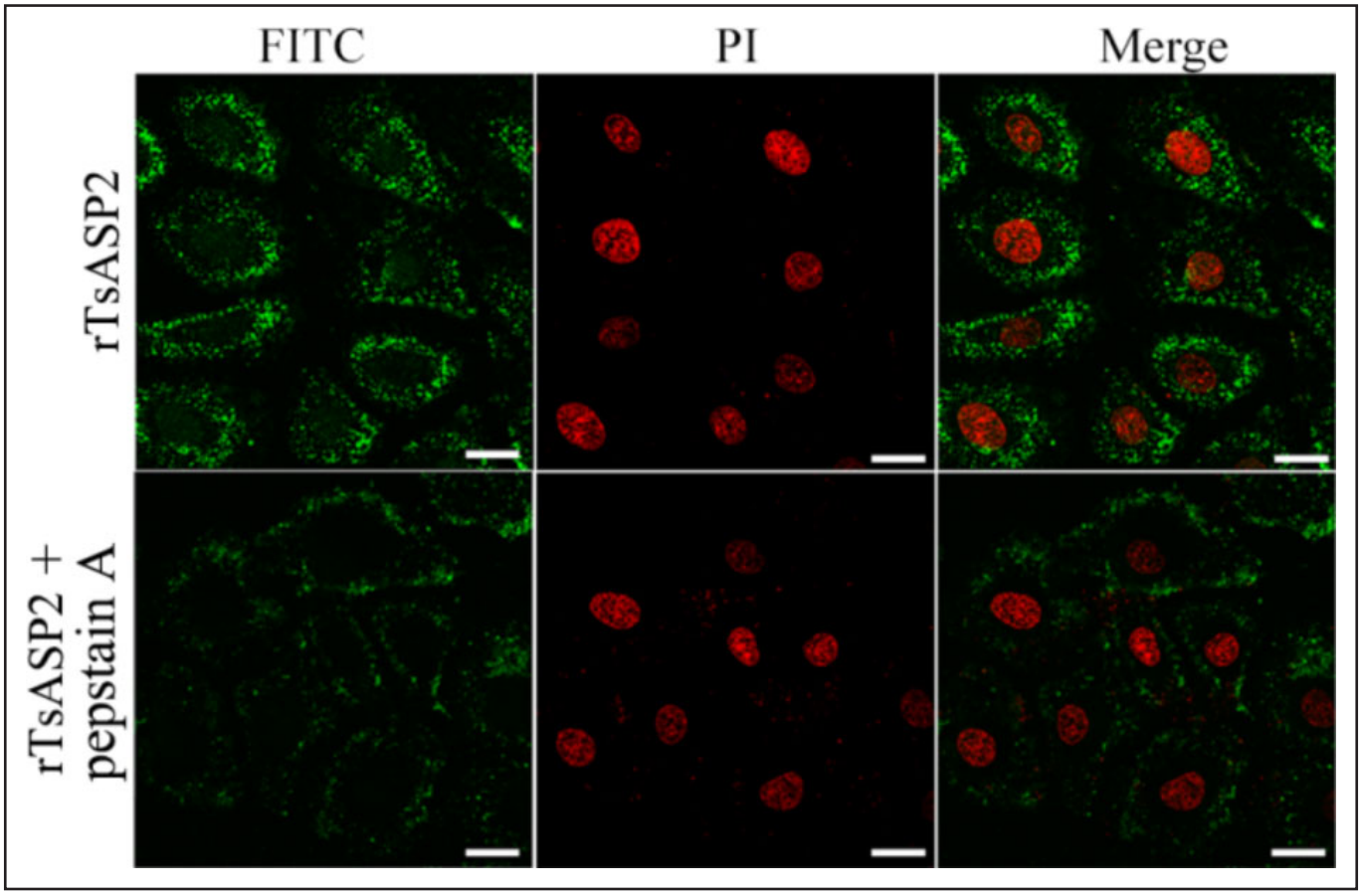

Figure 6. Cellular localization of rTsASP2 binding with IECs under confocal microscopy. The IECs were first incubated with rTsASP2 alone or rTsASP2 pre-treated with pepstain A, and then probed with anti-rTsASP2 serum. Immunostaining was observed in the cytomembrane and partial cytoplasm in IECs when the IECs were incubated with rTsASP2. When the IECs were incubated with rTsASP2 pre-treated with pepstain A, less immunostaining was observed. Scale bars: 20 µm.

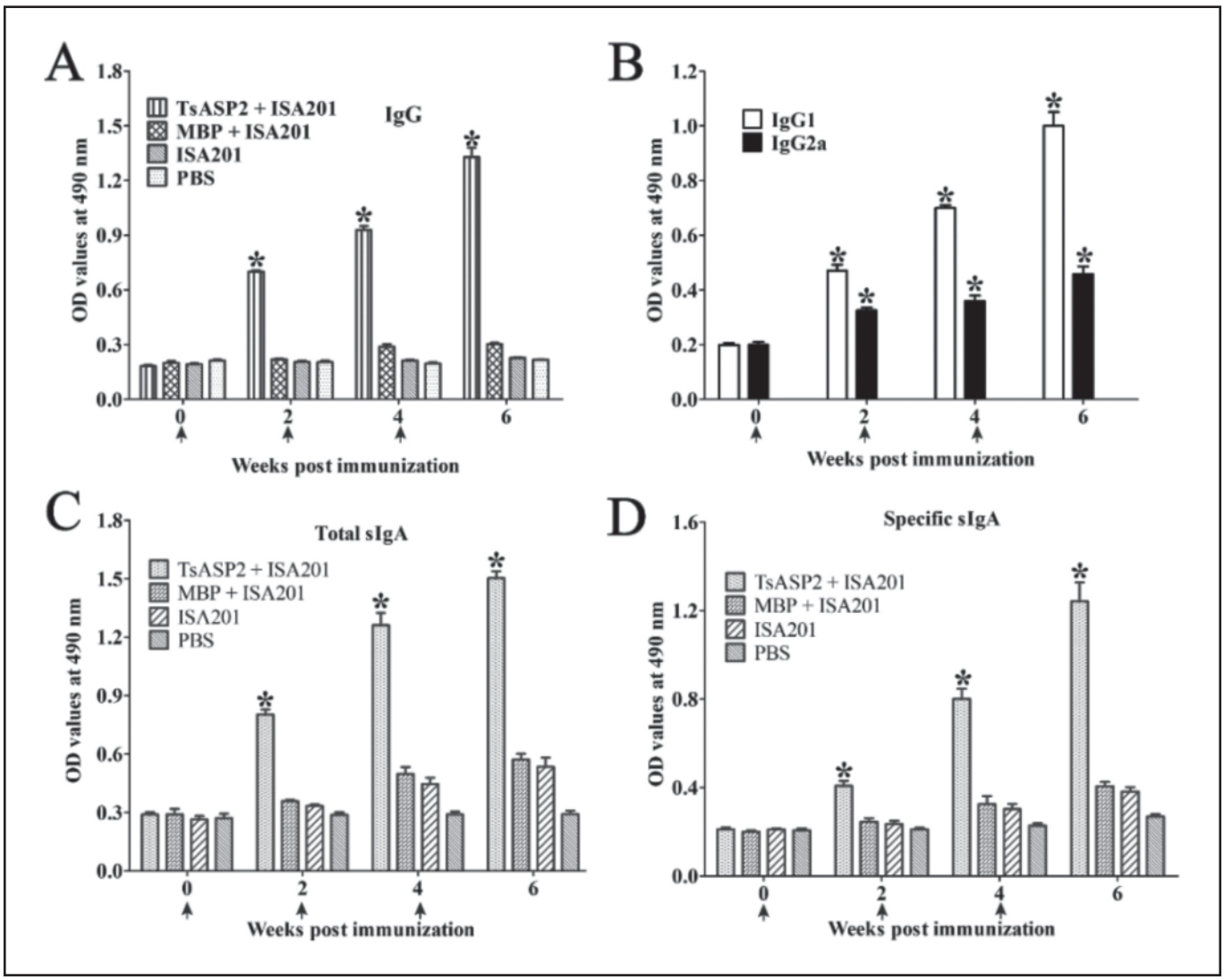

Figure 7. Analysis of anti-rTsASP2 IgG and sIgA responses in mice vaccinated with rTsASP2. **A:** anti-rTsASP1 IgG levels in serum of immunized mice at various time intervals after immunization. **B:** the IgG subclass response of immunized mice at diverse time intervals after immunization. **C:** total intestinal sIgA. **D:** TsASP2-specific sIgA. The data are shown as the mean OD values ± SD for 20 mice per group for IgG and IgG1/IgG2a, and 5 mice for total sIgA and TsASP1-specific sIgA. No detectable specific sIgA was observed in mice inoculated with only MBP, adjuvant or PBS. Arrows (↑) indicate the vaccination times. **P* <0.0001 compared with the MBP, adjuvant and PBS group.

DISCUSSION

Aspartic proteases are one of the most important families of proteases and have been identified in numerous parasites. They play the important roles in parasitic infection, such as larval invasion, digestion and proteolysis (Tcherepanova *et al.*, 2000; Jolodar *et al.*, 2004). The aspartic proteases in adult parasites of *Ancylostoma caninum* and *Necator camericanus* can degrade host skin macromolecules, hemoglobin and serum proteins in a host-specific mode (Williamson *et al.*, 2003a, 2003b). The *T. spiralis* aspartic protease had been identified in different worm phases (AW, ML, and IIL), and its function and enzymatic activity had also been characterized. The aspartic protease promoted the larval invasion of IECs and migration, suggesting that TsASP might play a key role for the degrading intercellular or other cytoplasmic proteins, and facilitating the larval invasion into IECs (Xu *et al.*, 2020a, 2021).

In the present study, the enzymatic activities of TsASP2 were detected by incubation with the specific fluorogenic synthetic substrate and crude proteins of various *T. spiralis* stages, the results revealed that the highest activities were detected in the IIL stage, which was similar with the previous study (Xu *et al.*, 2020a). This result was further confirmed by RT-PCR and Western blot, as both the TsASP2 mRNA and protein expression levels were the highest in IIL stage. Interestingly, the TsASP2 protein expression was scarcely detected in the NBL stage. The TsASP2 expression was observed by IFT at various stages of the nematode, and localized in the muscle cells and stichosome in the ML and IIL and around the embryos in the female AW, but no immunostaining was seen in the NBL. These results might imply that the TsASP2 could play important functions at the IIL stage of *T. spiralis*, but not in the NBL stage.

ELISA was also employed in this study to ascertain the interactions between TsASP2 and IEC proteins. The binding between TsASP2 and IEC proteins was the protein dose-dependent as showed in Figure 4. The results of IFT and confocal microscopy showed that rTsASP2 can specifically bind to the IEC cytomembrance, and this binding was inhibited when TsASP2 was suppressed by specific inhibitor pepstain A, suggesting that the enzymatic activities of TsASP2

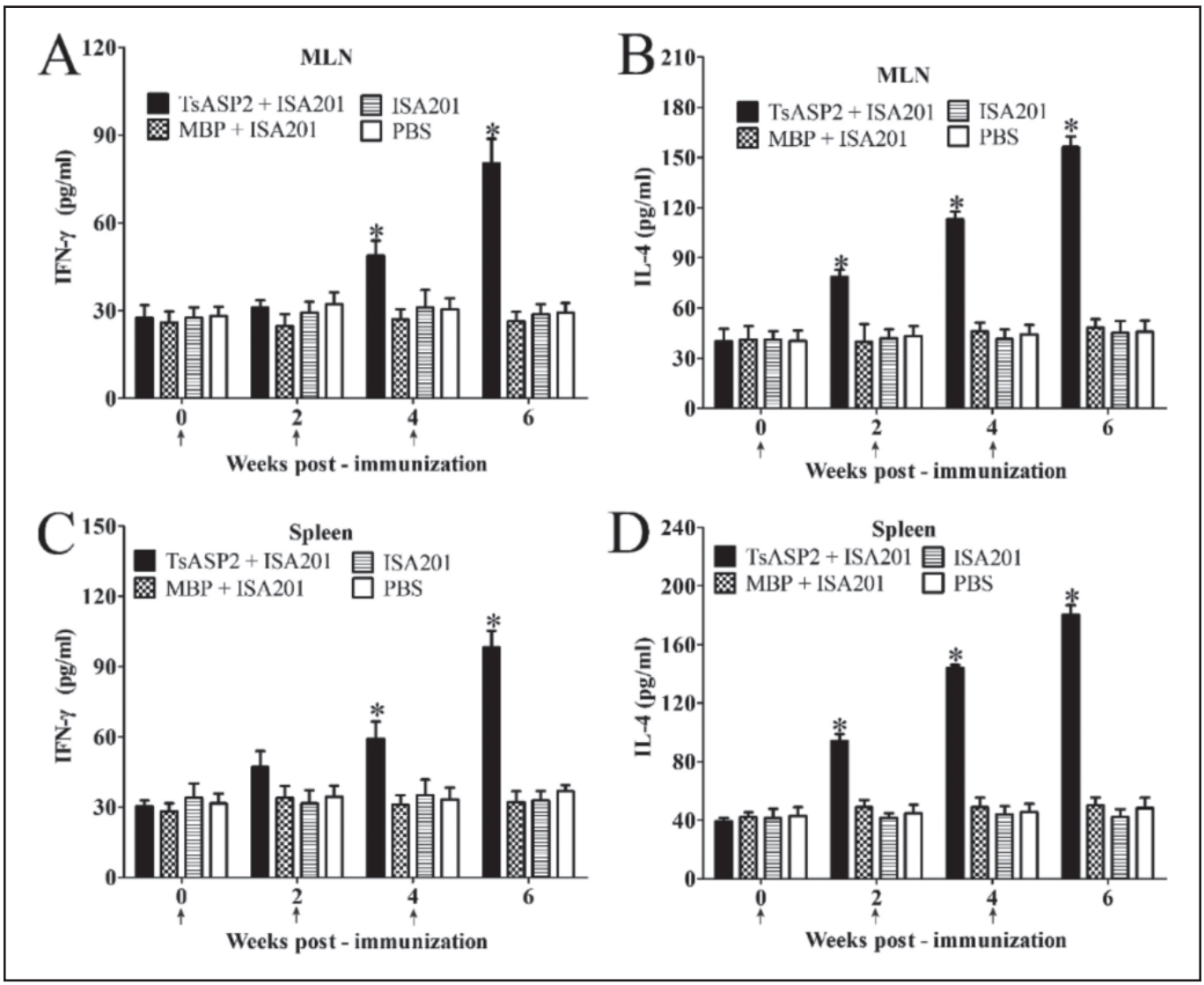

Figure 8. Cytokines secreted by splenocytes and MLN cells upon rTsASP2 re-stimulation. The concentrations of IFN-γ (**A, C**) and IL-4 (**B, D**) were determined in the supernatant after spleen and MLN cells were stimulated by 4 μg rTsASP2 for 72 h. Arrows (↑) indicate the vaccination times. The asterisks (*P < 0.05) indicate that statistically significant differences compared to the MBP, ISA201 and PBS control groups.

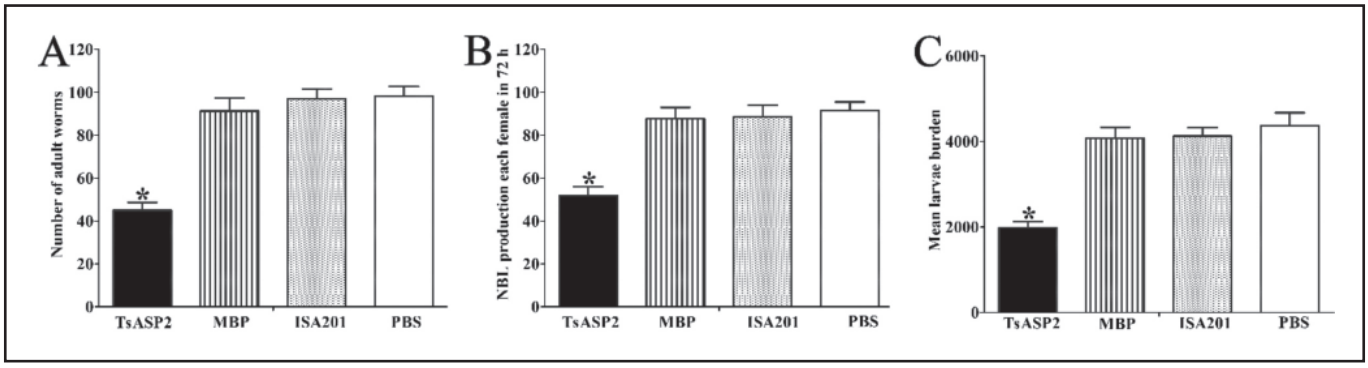

Figure 9. Immune protection of rTsASP2-immunized mice after being challenged with 300 *T. spiralis* larvae. **A:** Intestinal adult worm burden. **B:** The newborn larval production of each female cultured *in vitro* for 72h. **C:** Muscle larvae burden (larvae per gram, LPG). Result are presented as the mean ± SD of ten mice each group. * P < 0.0001 compared to the MBP, ISA201 and PBS groups.

also participated in the interaction between rTsASP2 and IEC proteins (Long *et al.*, 2015; Guo *et al.*, 2020; Yan *et al.*, 2020).

Vaccination of mice with rTsASP2 triggered specific humoral and mucosal immune responses, as demonstrated that both specific IgG and sIgA levels were significantly elevated two weeks post vaccination. The IgG1 level was significantly higher than IgG2a, suggested that rTsASP2 emulsified in ISA201 adjuvant elicited a mixed IgG1/ IgG2a antibody response (Yang *et al.*, 2019b; Zhang *et al.*, 2020b). The antibody response was important for the protective immunity, but the cellular immunity was also crucial to defend *T. spiralis* infection (Cui *et al.*, 2013b; Liu *et al.*, 2015b). In this study, the cytokines of IFN-γ and IL-4 secreted by the splenocytes and MLN cells of mice vaccinated with rTsASP2 were significantly elevated. The production of IFN-γ and IL-4 confirms that the immune responses induced by rTsASP2 vaccination were the mixed Th1/Th2 type. Previous studies have demonstrated that the concomitant Th1/Th2 immune responses were crucial for the protection against *T. spiralis* infection (Xu *et al.*, 2017; Cui *et al.*, 2019). Especially, the IL-4 is a critical mediator for the humoral immune response, and plays a crucial role in expulsion of *T. spiralis* by a Th2-type (Finkelman *et al.*, 2004; Madden *et al.*, 2004; Yang *et al.*, 2010).

Vaccination of mice with rTsASP2 also induced the significant protection, as intestinal adult worms, NBL production and muscle larval burden in vaccinated mice was distinctly reduced. The immune protection (e.g., muscle larval burden reduction) induced by vaccination of rTsASP2 could be comparable with that of several recombinant *T. spiralis* proteins, such as cathepsin B (50.90%; Cui *et al*., 2019), serine protease (52.10%; Sun *et al*., 2018a), serine proteinase (52.50%; Yue *et al*., 2020), peptidase (41.93%; Lei *et al*., 2020), elastase-1 (64.06%; Zhang *et al*., 2020), and TsASP1 (60.50%; Xu *et al*., 2021). The reduction of the worm burden might be related with the Th1/Th2 immune response which may interrupt larval invasion of enterocytes, impair larval establishment and development in enteric epithelium (McVay *et al.*, 2000; Song *et al.*, 2018b). Previous studies suggested that anti-*Trichinella* antibodies also participated in the damage and destruction of *T. spiralis* NBL through ADCC (Moskwa, 1999; Cui *et al.*, 2015a; Zhang *et al*., 2020a). Our results demonstrated that the rTsASP2 might be a potential molecular target for anti-*Trichinella* vaccines.

In conclusion, TsASP2 was expressed at various *T. spiralis* stages, the highest expression level in the IIL stage, but not in the NBL. TsASP2 was principally localized at the muscle cells and stichosome of the ML and IIL and around the embryos in the female AW. The rTsASP2 was specially bound to the cytomembrane of IECs. Vaccination of mice with rTsASP2 induced a specific humoral and cellular immune responses, and significant protection. The TsASP2 could be a candidate vaccine target molecule against *T. spiralis* infection.

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Conflicts of interest

The authors declare no conflicts of interest with regards to this study or the manuscript prepared for publication.

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