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Oral administration with attenuated *Salmonella* encoding a *Trichinella* cystatin-like protein elicited host immunity

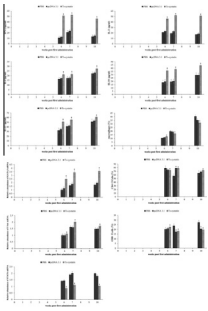
X.D Liu [a,1], X.L. Wang [a,1], X. Bai [a,1], X.L. Liu [a,1], X.P. Wu [b,*], Y. Zhao [a,*], S.M. Sun [a], L. Yu [a], X.Z. Su [d], Z.Q. Wang [e], F. Wang [a], M.Y. Liu [a,c,*]

[a] *Key Lab for Zoonoses Research, Ministry of Education, Institute of Zoonosis, Jilin University, Changchun 130062, PR China*
[b] *National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, 207 Rui Jin Er Road, Shanghai 200025, PR China*
[c] *Jiangsu Co-Innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, PR China*
[d] *Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA*
[e] *Department of Parasitology, Medical College, Zhengzhou University, 40 Daxue Road, Zhengzhou 450052, PR China*

HIGHLIGHTS

- A eukaryotic expression system was used to ensure the similarity of the expressed protein and the native protein.
- The recombinant *Salmonella* invasion process was similar to the natural course of infection with *Trichinella*.
- Th1- and Th2-specific cellular transcription factors and the cytokine profile were evaluated.
- T lymphocytes and macrophages were detected by flow cytometry.

GRAPHICAL ABSTRACT



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ABSTRACT

Trichinellosis is a public health problem and is regarded as an emergent/re-emergent disease in various countries. The cDNA encoding a cystatin-like protein (Ts-cystatin) was identified by immunoscreening intestinal muscle larvae cDNA libraries with serum from pigs experimentally infected with 20,000 *Trichinella spiralis* muscle larvae. To study its impact on host immunity, we chose a eukaryotic expression system based on several comparisons of immunogenicity between the two *Salmonella typhimurium* administration schemes, which indicated that the eukaryotic expression system was superior. Humoral IgG and mucosal IgA were measured to determine the antibody response. To explore whether Th1 and Th2 responses were responsible for the induced protection, Th1- and Th2-specific cellular transcription factors and the cytokine profile were examined. Changes in the T lymphocyte and macrophage populations were detected by flow cytometry. Lastly, parasitological examination was examined. The results showed that Ts-cystatin induced a Th1/Th2-mixed type of immune response and decreased STAT6 transcription. The intestinal adult recovery increased by 10.9% in the Ts-cystatin group, the Ts-cystatin group fecundity rate was decreased by 91%. Furthermore, the number of muscle larvae did not change compared with the control group. In conclusion, our results suggest that Ts-cystatin plays an important role in *Trichinella* resistance to rapid expulsion by the host and is worth further study.

* Corresponding authors at: Key Lab for Zoonoses Research, Ministry of Education, Institute of Zoonosis, Jilin University, Changchun 130062, PR China. Fax: +86 431 87836702.
E-mail addresses: xpwu@jlu.edu.cn (X.P. Wu), zhaoyingsohu@sohu.com (Y. Zhao), liumy@jlu.edu.cn, mingyuanliu16@aliyun.com (M.Y. Liu).
[1] These authors contributed equally to the work.

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1. Introduction

Trichinosis is caused by ingesting undercooked meat containing the larvae of the nematode *Trichinella spiralis*. This parasite has a nearly worldwide distribution in both developing and developed countries, and it can cause serious illness and even death in humans. A dramatic decline in human trichinosis cases has been observed due to the regulation of the food industry; however, the infection rate in swine remains high in prevalent areas and poses a great threat to human health (Liu and Boireau, 2002). *Salmonella*-carried targeted genes are extremely effective at inducing both cellular and humoral immunity (Kwon et al., 2007; Petavy et al., 2008a,b; Cazorla et al., 2008; Moreno et al., 2010; Galen et al., 2009; Pompa-Mera et al., 2011). Administering a *Salmonella*-carried targeted gene orally can mimic the natural process of *Salmonella* infection, which targets the intestinal mucosa, where professional antigen-presenting cells (APCs) are abundant in the intestinal Peyer's patches (PPs). This feature makes *Salmonella*-carried targeted genes extremely advantageous for investigating immune response with a gastrointestinal stage (Jepson and Clark, 2001; Rescigno et al., 2001a,b; Cochlovius et al., 2002; Urashima et al., 2000). In addition, live attenuated *Salmonella* can induce the secretion of several cytokines and proinflammatory mediators that enhance early innate immunity and create a local environment conducive to antigen presentation (Dietrich et al., 2003). All of the above indicate the potency of using *Salmonella* as target gene vector to characterize the gene involved in immune regulation. In *S. typhimurium*, one such regulon is modulated by the PhoP (transcriptional activator) and PhoQ (sensor kinase) proteins, which are essential to *S. typhimurium* pathogenesis and survival within macrophages (Miller et al., 1989; Groisman and Lipps, 1989). These regulators control the transcription of multiple unlinked phoP-activated and phoP-repressed genes (Belden and Miller, 1994; Behlau and Miller, 1993; Pulkkinen and Miller, 1991). Originally characterized in the *S. typhimurium*-BALB/c mouse model of typhoid fever, this operon can modulate important virulence functions, including survival within macrophages (Miller et al., 1989; Fields et al., 1986) and resistance to endogenous antimicrobial peptides of the defensin-cryptdin family (Fields et al., 1986; Miller et al., 1990a,b). PhoP/PhoQ null mutants are markedly attenuated in BALB/c mice and are effective vaccines in these animals (Miller et al., 1989; Galan and Curtiss, 1989; Miller et al., 1990a,b; Seyed et al., 2012). So phoP/phoQ deletion mutants are attenuated strain of *Salmonella* and provide security guarantees for host. Currently, phoP/phoQ mutants were widely used in a variety of pharmacology experimental studies in mice and immune models.

Trichinella has a complex life cycle that involves both enteral and parenteral phases, which stimulate both mucosal and systemic immune responses. Although much remains unknown about the mechanism of *Trichinella* invasion, recent studies have successfully focused on some of the immune regulate antigens of this parasite. In our previous works, the cDNA encoding a cystatin-like protein (Ts-cystatin) was identified by immunoscreening intestinal muscle larvae cDNA libraries with serum from pigs experimentally infected with 20,000 *T. spiralis* muscle larvae. Further, immunoscreening cDNA libraries of all *Trichinella* life stages that it is a highly immunogenic/antigenic gene (Wu et al., 2009). The main goal of this work was to determine the ability of this gene to induce an immune response, when expressed by live attenuated *Salmonella*. Although *Salmonella* is reported to be competent at carrying both prokaryotic and eukaryotic plasmids and then provoking an immune response, many research groups have targeted gene expression in *E. coli* and have collected the fusion protein. Although this method can provide a large amount of protein, the expressed protein may differ from the native active protein in terms of structure and function. We chose a eukaryotic expression system based on several comparisons of the immunogenicities of two *Salmonella* administration schemes, which indicated that the eukaryotic expression system is superior (Darji et al., 2000; Pasetti et al., 1999; Zhao et al., 2009). Humoral IgG and mucosal IgA were measured to examine the antibody response, and a splenocyte proliferation assay was performed to evaluate cellular responses. To explore whether the Th1 or Th2 response was induced, Th1- and Th2-specific cellular transcription factors were examined in addition to the cytokine profile. Furthermore, changes in T lymphocyte and macrophage populations were detected by flow cytometry. Lastly, parasitological examination was examined.

2. Materials and methods

2.1. Experimental animals

Six- to eight-week-old female BALB/c mice were housed under specific pathogen-free conditions. All of the experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication No. 85-23, revised 1996). The protocol was approved by the Ethics Committee of the Institute of Zoonosis, Jilin University, China (Ref number 20080106).

2.2. Bacterial strain and parasite

The attenuated *S. enterica Typhimurium* phoP/phoQ strain was used as a carrier of the eukaryotic expression vector harboring the Ts-cystatin antigen. The bacteria were grown in Luria broth (LB) containing 2% NaCl (Darji et al., 2000; Galan and Curtiss, 1990) in a rotary shaker at 250 rpm at 37 °C until the optical density at 600 nm (OD600) reached 0.5–1. For the preparation of bacterial suspensions for administration to mice, cultures of the *S. enterica Typhimurium* phoP/phoQ strains were precipitated by centrifugation (6000*g*, 10 min), and the pellet was resuspended in phosphate-buffered saline (PBS).

The *T. spiralis* (ISS 534) parasites used in this study were maintained in Wistar rats in our laboratory by serial passage. Each mouse was orally infected with 400 *T. spiralis* larvae. Adult worms were collected from the intestines of infected mice, and muscle larvae were recovered from the muscle of infected mice by a standard pepsin–hydrochloric acid digestion method (Wang et al., 2009).

2.3. Plasmid construction and transformation

The full-length Ts-cystatin gene was obtained by PCR amplification using the following primers: 5′ GAGGTACCGCCACCATGGGT TTTATGCACTGTATTT-3′ (forward) and 5′-CCCCTCGAGTTAACATTCA ACAGTTGACTT-3′ (reverse). The KpnI and XhoI restriction sites are underlined. The amplified DNA fragment was cloned into the eukaryotic expression vector pcDNA3.1 using the cytomegalovirus promoter. The recombinant plasmid was named pcDNA3.1(+)-Ts-cystatin. To make competent cells, the attenuated *S. typhimurium* strain was grown in LB broth at 37 °C overnight to an OD600 of 0.6–0.8. Following centrifugation, the bacterial pellet was washed three times in sterile ice-cold water and one time in 10% glycerol and was finally resuspended in water. The recombinant plasmid pcDNA3.1(+)-Ts-cystatin and the negative control plasmid pcDNA3.1 were subsequently electroporated into the bacteria in a 2-mm cuvette under the following conditions: 2.5 kV, 25 μF, and 200 Ω (Gene Pulser Xcell, Bio-Rad, Hercules, CA). This procedure produced the strains phoP/phoQ-pcDNA 3.1(+)-Ts-cystatin (A1) and phoP/phoQ-pcDNA3.1 (A0). The positive transformants

were selected on LB agar containing 50 μg/ml ampicillin and were identified by PCR amplification and restriction enzyme digestion. The PCR products were sequenced (Sangon, China) to further confirm the introduction of plasmids in SL7207 and verify the reading frame of the cloned gene.

2.4. Administration protocol

Ninety BALB/c mice were randomly divided into 3 groups, with 30 animals per group. The administrations were performed at weeks 0, 2, and 4 with 1×10^9 PQ-pcDNA3.1(+)-Ts-cystatin or PQ-pcDNA3.1 cells in 100 μl of PBS. The third group of mice was given 100 μl of PBS only. Subsequently, the mice were boosted with the same dose at weeks 2 and 4. Thirty minutes prior to oral inoculation, the mice were given 100 μl of 10% sodium bicarbonate to neutralize stomach acidity.

2.5. Challenge infection and parasitological examination

Two weeks after the final administration, mice from the three groups were orally challenged with 300 *T. spiralis* larvae. Ten mice from each group were sacrificed 6 days after the challenge, adult worms were recovered from the intestine, as described by McGuire et al. (2002). The muscle larvae were examined from another 10 mice from each group 35 days after the challenge using a routine digestion method described previously. Parasitological examination was calculated based on the mean number of adult worms or muscle larvae collected from the group administrated with A0 or A1 compared with those collected from the PBS group. Female fecundity was calculated 24 h after the adult worms were harvested at day 6 after the challenge. Briefly, 5 adult worms from each mouse were placed in a 24-well plate in RPMI-1640 containing 20% fetal bovine serum (FBS) with penicillin and streptomycin. After incubation at 37 °C for 24 h, the number of Newborn larvae (NBL) was counted in each well and compared with those in the control group.

2.6. Reverse transcriptase polymerase chain reaction (RT-PCR) and indirect immunofluorescence assays (IFAs) to detect Ts-cystatin transcription and expression in vivo

The spleen and mesenteric lymphoid nodes (MLNs) of the mice were harvested 2 weeks after the final administration. For RT-PCR assays, total RNA was extracted from the homogenized tissue using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. cDNA samples were obtained using a cDNA First Strand Synthesis Kit (Bioer Technology, China). Transcription of the Ts-cystatin gene in the spleen and MLNs was analyzed by RT-PCR using specific primers. Mouse β-actin was also amplified as a quality control.

For IFA analysis, tissues were fixed in 4% neutral-buffered formalin, embedded in paraffin wax, sectioned at 6 μm, and transferred onto poly-l-lysine-pretreated slides. The sections were washed three times with cold PBS and blocked with 5% normal goat serum (5%) at room temperature for 30 min. After incubation of the sections with Ts-cystatin protein (rTs-cystatin) with immunized mouse serum at a 1:200 dilution in PBS plus 5% normal goat serum at 4 °C overnight, tissue sections were incubated with FITC-conjugated goat anti-mouse IgG (Sigma) at a 1:400 dilution. A section incubated with serum from a non-immunized mouse at the same dilution served as a negative control. The sections were examined and photographed using a fluorescence microscope (Olympus, Japan).

2.7. Antibody detection by enzyme-linked immunosorbent assay (ELISA)

Specific total IgG antibodies against the rTs-cystatin were determined by ELISA. Briefly, 96-well microtiter plates (Costar, USA) were coated with 100 μl of rTs-cystatin protein at a concentration of 1.0 μg/ml in bicarbonate buffer (pH 9.6). After washing with PBS-Tween20 (PBST), the plates were blocked with 100 μl of PBST containing 1% bovine serum albumin and incubated with serum samples diluted with PBS (1:100) at 37 °C for 2 h. The plates were then washed and incubated with 1:4000 diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma). The reactions were detected by addition of the substrate 3,3′,5,5′,-tetramethylbenzidine (TMB) and stopped with 50 μl of 1 N H_2SO_4. Optical density at 450 nm was measured with a microplate reader (Tecan Sunrise, Switzerland). All samples were run in triplicate.

For the determination of specific IgA, the interior of the small intestine of mouse was washed twice with a total of 1 ml of cold PBS. After centrifugation at 800*g* for 10 min, the supernatants of the intestinal washes were harvested. Specific IgA antibodies was analyzed as describe above. Total IgA was quantified by a sandwich ELISA using rabbit anti-mouse IgA antibody (Abcam, UK) as the capture antibody and HRP-conjugated goat anti-mouse IgA antibody as the detection antibody. To compensate for variations in the efficiency of the recovery of secretory antibodies among animals, the antigen-specific IgA in each sample was normalized to the total IgA present in the lavage.

2.8. Cytokine analysis

For cytokine quantification, spleen cells were aseptically removed from immunized and control mice at 6, 7, and 10 weeks post-infection. The spleen cell suspensions were washed in RPMI-1640 medium (Invitrogen) and treated for 5 min with erythrocyte lysing buffer (9 volumes of 0.16 M NH_4Cl and 1 volume of 0.17 M Tris–HCl, pH 7.5). The cells were plated in 12-well tissue culture plates (Nunc) at 2×10^6 cells per well in 1 ml of RPMI-1640 containing 5% FBS and supplemented with penicillin G, streptomycin, and amphotericin B (1×10^4 U/ml, 1×10^4 μg/ml, and 25 μg/ml, respectively) and cultured in the presence of r Ts-cystatin (5 μg/mL), incubated at 37 °C in a humidified 5% CO_2 incubator for 72 h. Then, the supernatant was collected. IL-2, -4, -10, and -13 as well as IFN-γ were quantified in the supernatant using a sandwich ELISA kit (eBioscience) according to the manufacturer's instructions. Cytokine concentrations were determined by comparison with standard curves constructed with known amounts of the respective mouse recombinant cytokines.

2.9. mRNA quantification of GATA-binding protein-3 (GATA-3), T-box expressed in T cells (T-bet), and STAT6

Spleen total RNA was extracted 2 weeks after the last administration and challenge, as described above. Real-time PCR was performed using the SYBR-Green method, and the relative quantification was performed by the delta Ct method. The primers used for PCR amplification were as follows: for GATA-3, 5′-AA GAAAGGCATGAAGGACGC-3′ (forward) and 5′-GTGTGCCCATTTG GACATCA-3′ (reverse); for T-bet, 5′-CACTAAGCAAGGACGGCGAA-3′ (forward) and 5′-CCACCAAGACCACATCCACA-3′ (reverse); for Stat-6, 5′-TGGAGAGCATCTATCAGAGGGA-3′ (forward) and 5′-GCG GAACTCTTCTATAACAGCTT-3′ (reverse); for β-actin, 5′-AGCTGCGT TTTACACCCTTT-3′ (forward) and 5′-AAGCCATGCCAATGTTGTCT-3′ (reverse).

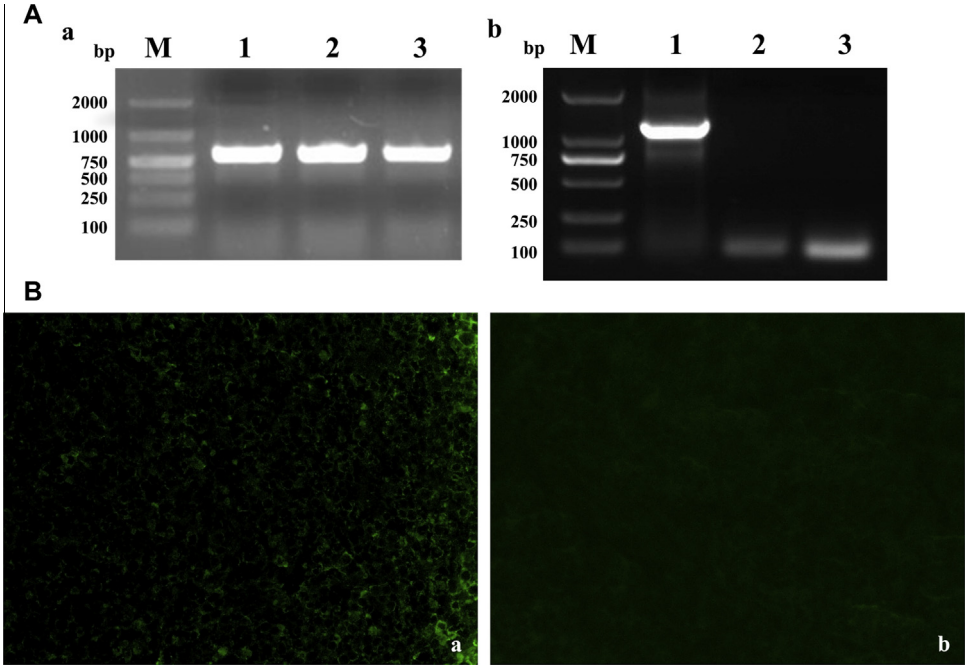

Fig. 1. Transcription and expression of the target gene in vivo. (A) The transcription of Ts-cystatin in vivo was spleen was analyzed by RT-PCR. β-Actin gene was amplified as an internal control. PCR products were analyzed on a 1% agarose gel with ethidiumbromide staining. Ts-cystatin amplified fragment was observed in Ts-cystatin group, but not observed in the PBS and pcDNA3.1 groups. M: DL2000 Marker; 1: Ts-cystatin group of the mouse spleen RNA; 2: PBS group of the mouse spleen RNA, 3: pcDNA3.1group of the mouse spleen RNA. (B) Expression of target genes in spleen was detected by indirect immunofluorescence using mouse serum against rTs-cystatin. (a) Anti-rTs-cystatin serum (100×). (b) Normal serum (100×).

2.10. Detection of T lymphocytes and macrophages by flow cytometry

Blood samples were obtained at 6, 7, and 10 weeks with the use of the anticoagulant heparin. FITC-conjugated anti-mouse CD3ε antibody and Cy5-conjugated anti-mouse CD4 antibody were used to label CD4+ T cells, FITC-conjugated anti-mouse CD3ε antibody and PE-conjugated anti-mouse CD8a antibody were used to label CD8+ T cells, and FITC-conjugated anti-mouse CD14+ was used to label macrophages. The procedures followed the manufacturer's instructions (BioLegend). T lymphocytes and macrophages were detected using an Elite ESP Flow Cytometer (Coulter, USA).

2.11. Statistical analysis

Data were expressed as the mean ± standard error (S.E.) and evaluated by one-way ANOVA analysis using SPSS 11.5 software. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Transcription and expression of the target gene in vivo

The transcription of Ts-cystatin in vivo was spleen was analyzed by RT-PCR. β-Actin gene was amplified as an internal control. PCR products were analyzed on a 1% agarose gel with ethidiumbromide staining. Ts-cystatin amplified fragment was observed in Ts-cystatin group, whereas the amplified gene fragment was not observed in the PBS and pcDNA3.1 groups (Fig. 1A).

Expression of target genes in spleen was detected by indirect immunofluorescence using mouse serum against rTs-cystatin. The target gene was identified in the mouse spleen (Fig. 1Ba) and was not detected in the negative control (Fig. 1Bb).

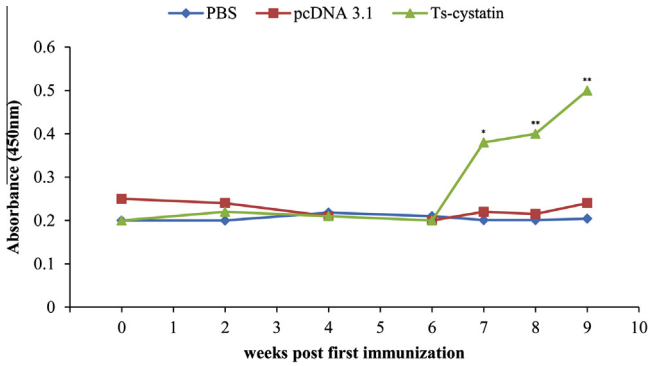

Fig. 2. Detection of serum IgG. Mouse IgG responses to pcDNA3.1 or pcDNA 3.1(+)-Ts-cystatin were measured by ELISA. The values shown for each group are the mean ± standard error of the antibody levels ($n = 10$). ($^{*}P < 0.05$; $^{**}P < 0.01$).

3.2. Detection of serum IgG

As shown in Fig. 2, the ELISA results demonstrated that the Ts-cystatin -specific antibody concentration increased with the number of oral administration.

3.3. Detection of intestinal IgA

As shown in Fig. 3, Ts-cystatin-specific IgA antibodies were detected in intestinal lavage fluid.

3.4. Cytokine analysis

An ELISA assay was used to detect IFN-γ, IL-2, IL-4, IL-10, and IL-13 secreted by the splenocytes of mice immunized with PQ-pcDNA3.1(+)-Ts-cystatin. Compared with the vector alone and

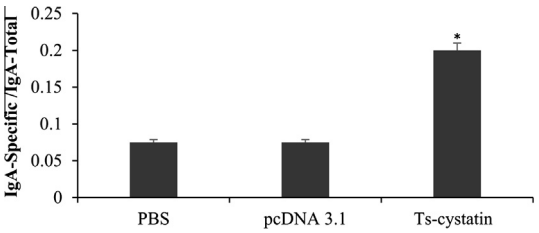

Fig. 3. Detection of intestinal IgA. IgA-specific/IgA-total in the intestinal washes of mice immunized with pcDNA3.1, pcDNA 3.1(+)-Ts-cystatin or PBS control. The results are the mean ± standard error for 10 mice per group. (*$P < 0.05$).

PBS control groups, IFN-γ, IL-2, IL-4, IL-10, and IL-13 secretion was significantly increased at week 6 ($P < 0.05$) and remained high at weeks 7 and 10 (Fig. 4).

3.5. The detection of T lymphocytes and macrophages by flow cytometry

At the 6th week, when compared with the control group (PBS and pcDNA3.1(+)), the CD4+ T lymphocyte count was significantly reduced, the CD8+ T lymphocyte count was increased, and the CD4+/CD8+ ratio was decreased in the Ts-cystatin group (Figs. 5 and 6). Interestingly, compared with control group at the CD4+ T lymphocyte count in the Ts-cystatin group was increased, the CD8+ T lymphocyte count was decreased, and the ratio was increased significantly at weeks 7 and 10 (Table 1).

At the 6th week, compared with the PBS and pcDNA3.1 groups, the CD14+ macrophages counts in the Ts-cystatin group were increased. In the 7th and 10th weeks, the CD4+ cell counts were higher than those in the 6th week for all groups. Compared with the PBS and pcDNA3.1 groups, the CD14+ cell counts were reduced in the Ts-cystatin group (Figs. 7 and 8).

3.6. Transcription factor analysis

Because T-bet and GATA-3 are well-characterized transcription factors that play roles in the differentiation of Th1 and Th2 cells, respectively, the mRNA levels for both of these genes were analyzed. In the Ts-cystatin group, Both T-bet and GATA3 transcription increased with administration and challenge; compared with the PBS control group, the difference was significant. STAT6 transcription did not increase with administration or challenge, similar to the PBS control group (Figs. 9).

3.7. Parasite burden assessment

To evaluate the efficacy of the recombinant *Salmonella* strategy, the adult worm burden, the muscle larvae burden, and female fecundity were examined. As shown in Fig. 10, in the Ts-cystatin group, the intestinal adult worm recovery increased by 23.9%;

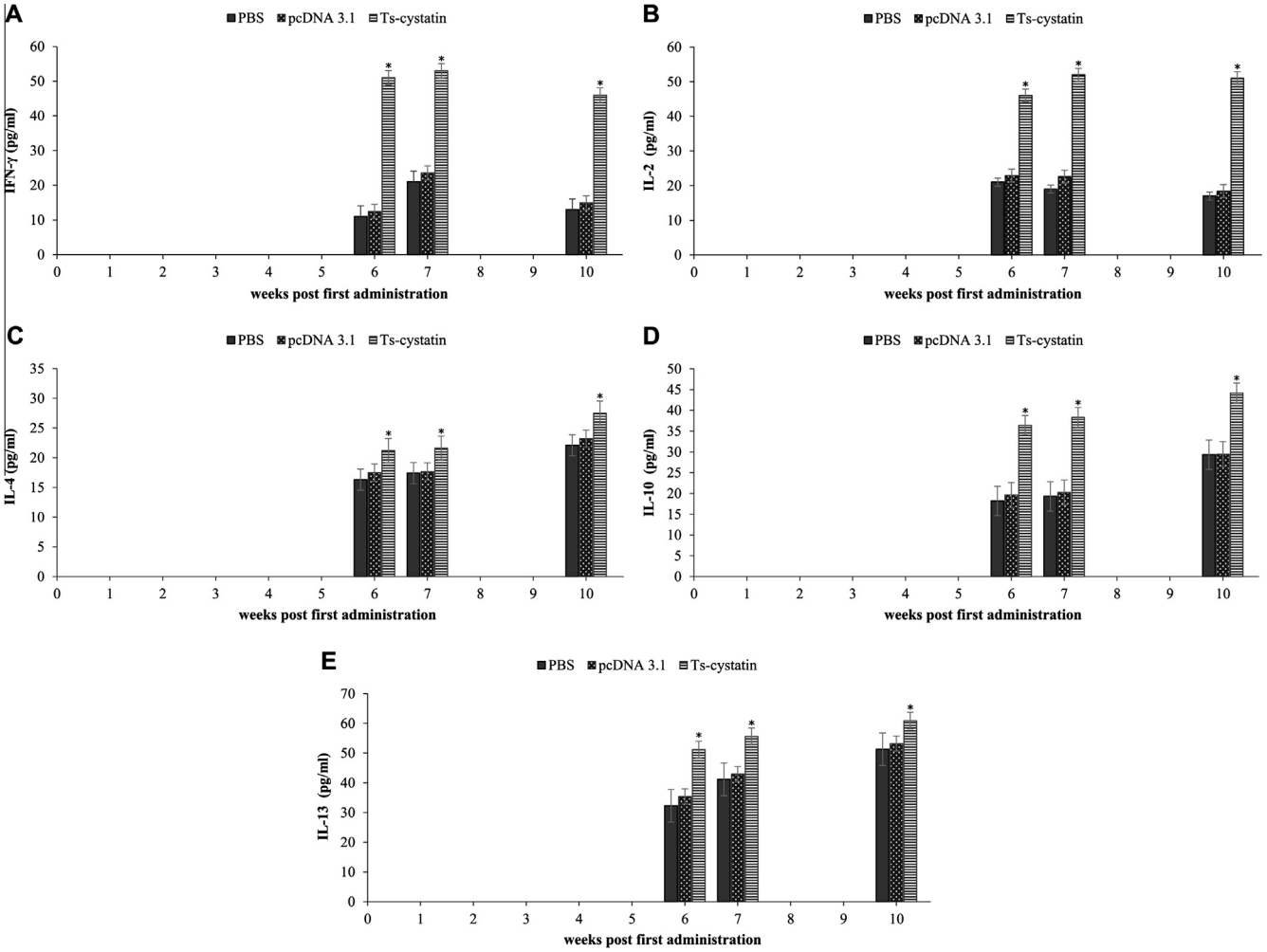

Fig. 4. IFN-γ (A), IL-2 (B), IL-4 (C), IL-10 (D), and IL-13 (E) secreted by splenocytes isolated from pcDNA3.1/pcDNA 3.1-Ts-cystatin-treated and PBS control mice were detected by ELISA assays. Spleen cells were cultured for 48 h. The number of cytokine-producing cells is expressed as SFU/2 × 10^5 cells after background subtraction. (*$P < 0.05$).

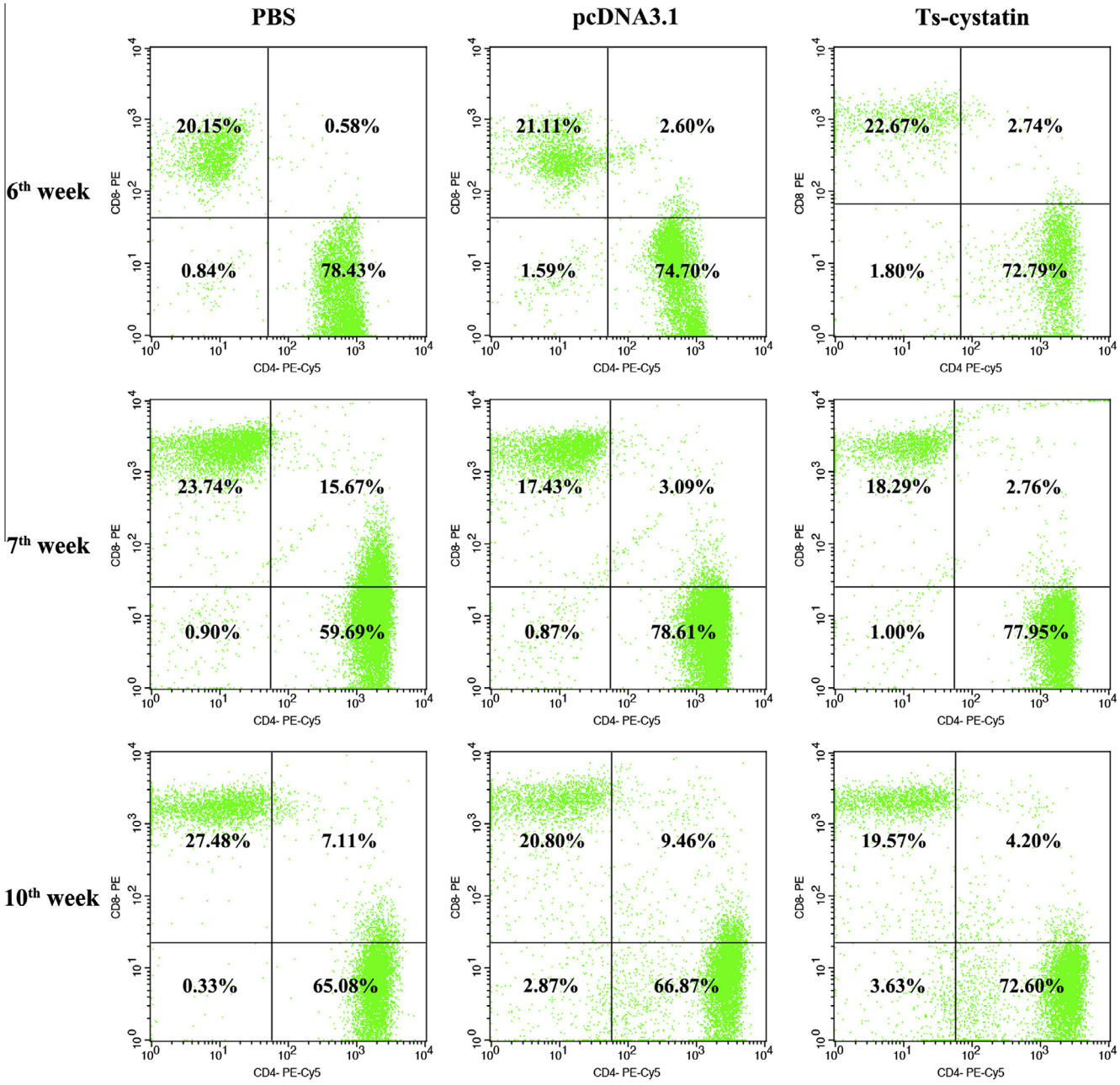

Fig. 5. The detection of peripheral blood T lymphocytes by flow cytometry. The percent change of CD4+ and CD8+ T cells in peripheral blood lymphocytes from PBS control, pcDNA3.1 and pcDNA 3.1(+)-Ts-cystatin group at 6, 7, and 10 weeks post-infection. The upper left and lower right quadrants of each panels show the CD3+/CD4+ and CD3+/CD8+ double positive cells, respectively.

the Ts-cystatin group female fecundity rate was significantly lower, and decreased by 91%. However, the number of muscle larvae in the Ts-cystatin group remained similar to that of the control group.

4. Discussion

The *Trichinella spiralis* gene Ts-cystatin has been proven to encode a highly immunogenic antigen in previous work in our lab by immunoscreening cDNA libraries of all *Trichinella* life stages (Wu et al., 2009). The main goal of this work was to determine the ability of this gene Ts-cystatin to induce an immune response, when expressed by live attenuated *Salmonella*. We detected Ts-cystatin mRNA in the spleen, and recombinant Ts-cystatin was expressed in the spleen as determined by immunofluorescence staining with specific antisera, suggesting that the Ts-cystatin gene was transcribed and expressed in the tissues of experimental mice after administration with Ts-cystatin DNA delivered by attenuated *S. typhimurium*. Humoral IgG and mucosal IgA were measured to determine the antibody response. To explore whether Ts-cystatin regulate the Th1 and Th2 responses, Th1- and Th2-specific cellular transcription factors were measured, and cytokine profiles were also examined. Changes in the T lymphocyte and macrophage populations were detected by flow cytometry. Lastly, the rate of infection in Ts-cystatin treatment and no-Ts-cystatin group was examined. The results showed that this gene induced a Th1/Th2-mixed type of immune response and decreasing the transcription of STAT6. The intestinal adult worm recovery from the Ts-cystatin group increased 10.9%, and the Ts-cystatin group fecundity rate was decreased by 91%. However, when compared with the control

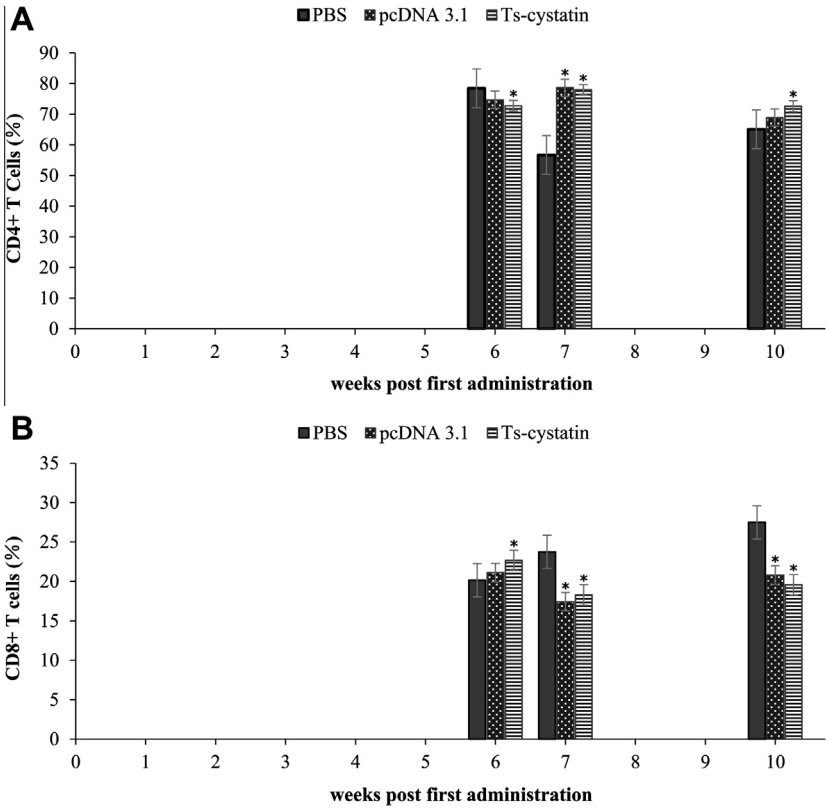

Fig. 6. The percentage of CD4+ (A) and CD8+ (B) T cells at different time points post-administration. (*$P < 0.05$).

Table 1
Dynamic changes of CD4+/CD8+ cells.

Weeks post first immunization	PBS	pcDNA 3.1	T626-55
6	3.89 ± 0.062	3.54 ± 0.071	3.21 ± 0.054*
7	2.51 ± 0.035	4.51 ± 0.042**	4.26 ± 0.043**
10	2.37 ± 0.046	3.31 ± 0.045*	3.71 ± 0.065**

* $P < 0.05$.
** $P < 0.01$.

group, the muscle larvae burden remained unchanged. Thus, our results suggest that the *Trichinella* gene Ts-cystatin plays a role in *Trichinella* resistance to rapid expulsion by the host and that it is worth further study.

A live attenuated *Salmonella* was used as an in vivo *Trichinella* gene expression system. As a result of the eukaryotic expression vector carrying the plasmid, the maximum degree of similarity between the expressed proteins and the native proteins was obtained. In addition, recombinant attenuated *Salmonella* is delivered orally, and it can invade intestinal lymphoid tissue; thus, the gene can be expressed in antigen-presenting cells. This process stimulates natural infection with *Trichinella* and was the starting point for invasion of the gut. Reverse transcription PCR and indirect immunofluorescence experiments showed that one week after the first administration, the gene was targeted to the spleen for transcription and expression.

This study found that recombinant bacteria can stimulate the body's cell-mediated immune response. Previously, host cell proliferative responses have been reported to a filarial cysteine protease inhibitor (Schierack et al., 2003). Filarial cystatins have biological activity. The Ts-cystatin gene sequence analysis showed that the gene encoding the amino acid sequence has three repeated domains, of which the first two are 74% homologous; the third domain showed 37% homology. These three domains are different from other nematode cysteine protease inhibitor domains, and they lack the highly conserved nematode cysteine protease inhibitor motif QXVXG. These results suggested that Ts-cystatin may not have protease inhibitor activity. Therefore, *Trichinella* may affect the immune response of host cells through different mechanisms.

In our study, in mice treated with the recombinant attenuated *Salmonella* vector, we found that Ts-cystatin generated a serum IgG antibody response, although this response was not strong. After natural infection with *T. spiralis*, the immune response differed with that induced by administration with Ts-cystatin. *Trichinella* infection in pigs was detected in serum using antibodies against the gene product. Ts-cystatin is a TSL-1 family antigen, with a tyvelose-containing structure. Early studies found that the tyvelose composition has very strong antigenicity; for example, anti-tyvelose monoclonal antibodies can protect host intestinal cells from *Trichinella* invasion (McVay et al., 2000). However, tyvelose can only be synthesized in *Trichinella* rhabdites. The antigen obtained from the parasite crude extracts differs from the same antigen collected from the ES antigens. This indicates that tyvelose has important implications in protein antigenicity. In our study, the fact that the expressed gene did not induce a strong antigenic response may be related to *Salmonella's* inability to synthesize tyvelose in mice. Therefore, the antigenicity of the recombinant gene is less than that of the native protein. In addition, Petavy et al. used *Salmonella* as a vector for the oral administration of dogs against the *Echinococcus* tapeworm; serum IgG and its subtypes were examined, and no difference was found between the experimental group and the control group (Petavy et al., 2008a,b).

Parasitological examination in our study demonstrated that the Ts-cystatin-treated mice had increased intestinal recovery, that female fecundity decreased significantly in the experimental group compared to the control group, and that no significant changes in

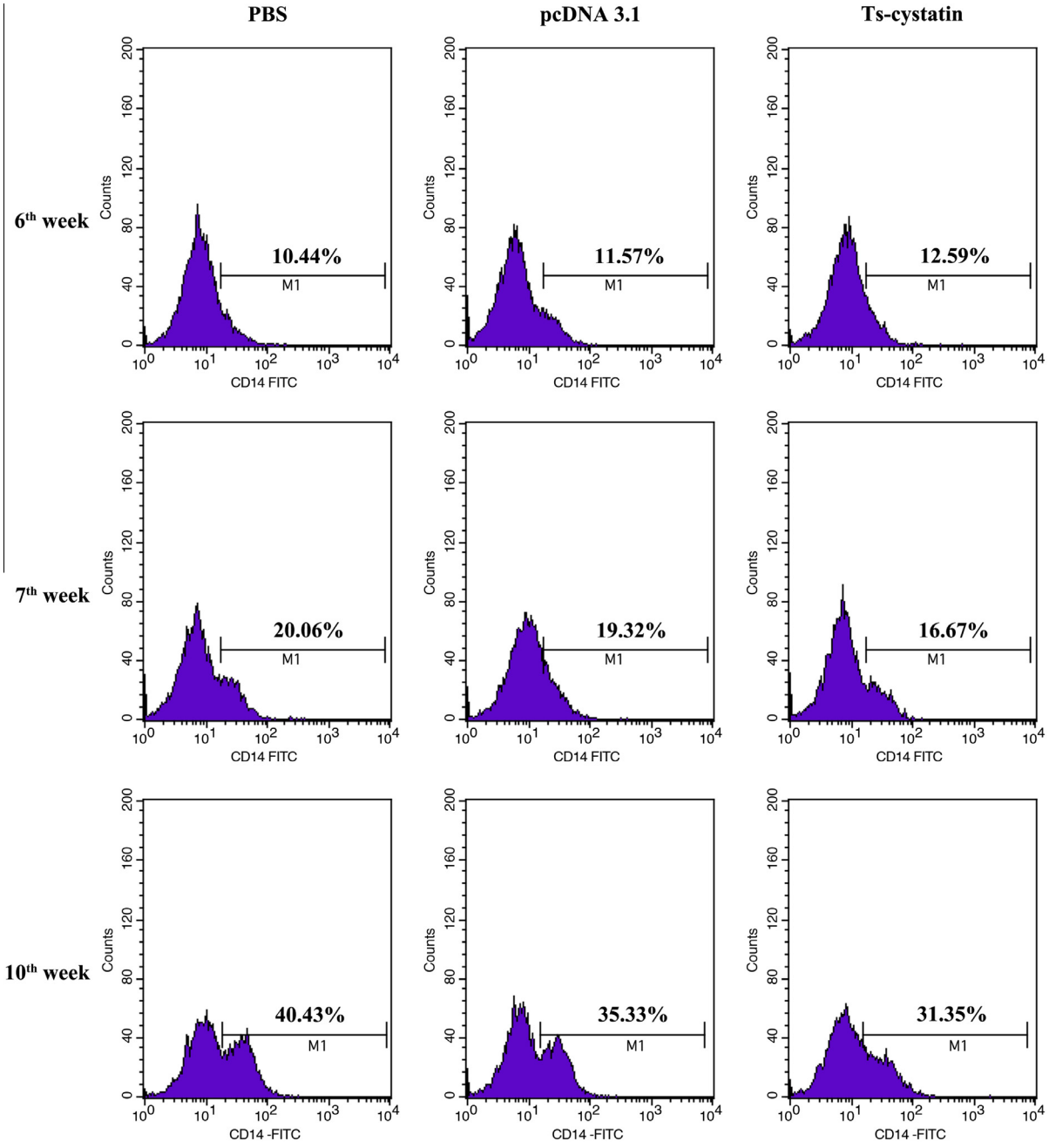

Fig. 7. The detection of peripheral blood macrophages by flow cytometry. The percent change of peripheral blood macrophages from PBS control, pcDNA3.1 and pcDNA 3.1(+)-Ts-cystatin group at 6, 7, and 10 weeks post-infection.

the number of muscle larvae were observed. Ts-cystatin plays an important role in host intestinal invasion by *Trichinella*. It may be involved in the invasion of intestinal epithelial cells by *Trichinella*, and the development of newborn larvae and larvae output have a very negative impact. Our previous gene transcription and expression studies showed that Ts-cystatin was transcribed at different developmental stages; however, the differences in the transcript levels were significant. The peak was observed in the muscle larvae (muscle L1 and intestinal L1) and at the adult stage (Ad2, Ad3, and Ad5). Little or no gene transcription was observed in the NBL. This gene delivered from newborn larvae had very little impact. This may be the result of the activation of protease or regulation of immune response in the newborn larvae during parturition by the gene in the female body, thus interfering with normal females during childbirth. Another possibility is that intestinal mucosal IgA directed against females and newborn larvae had an impact on parasite (Jacqueline et al., 1981).

This study found that after oral administration with a recombinant attenuated *Salmonella* carrying the Ts-cystatin expression plasmid, Both T-bet and GATA3 transcription increased. T-bet and GATA-3 were used as Th1- and Th2-specific cellular transcription factors to indicate the balance between Th1/Th2 responses. Recently, it has been proved that both T-bet and GATA-3, as T cell differentiation-specific transcription factors, have determined the polarization differentiation of Th1 and Th2. As a member of the Tbr1 subfamily of T-box genes, T-bet plays a key role in Th1 cell differentiation. However, GATA-3 promotes the generation of Th2 specific cell factor (such as IL-4 et al.), which is a Th2specific

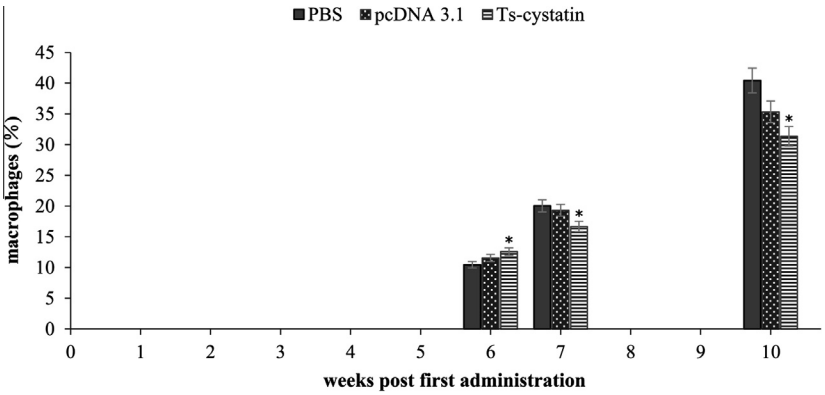

Fig. 8. The percentage of CD14+ macrophages at different weeks post administration. (*$P < 0.05$).

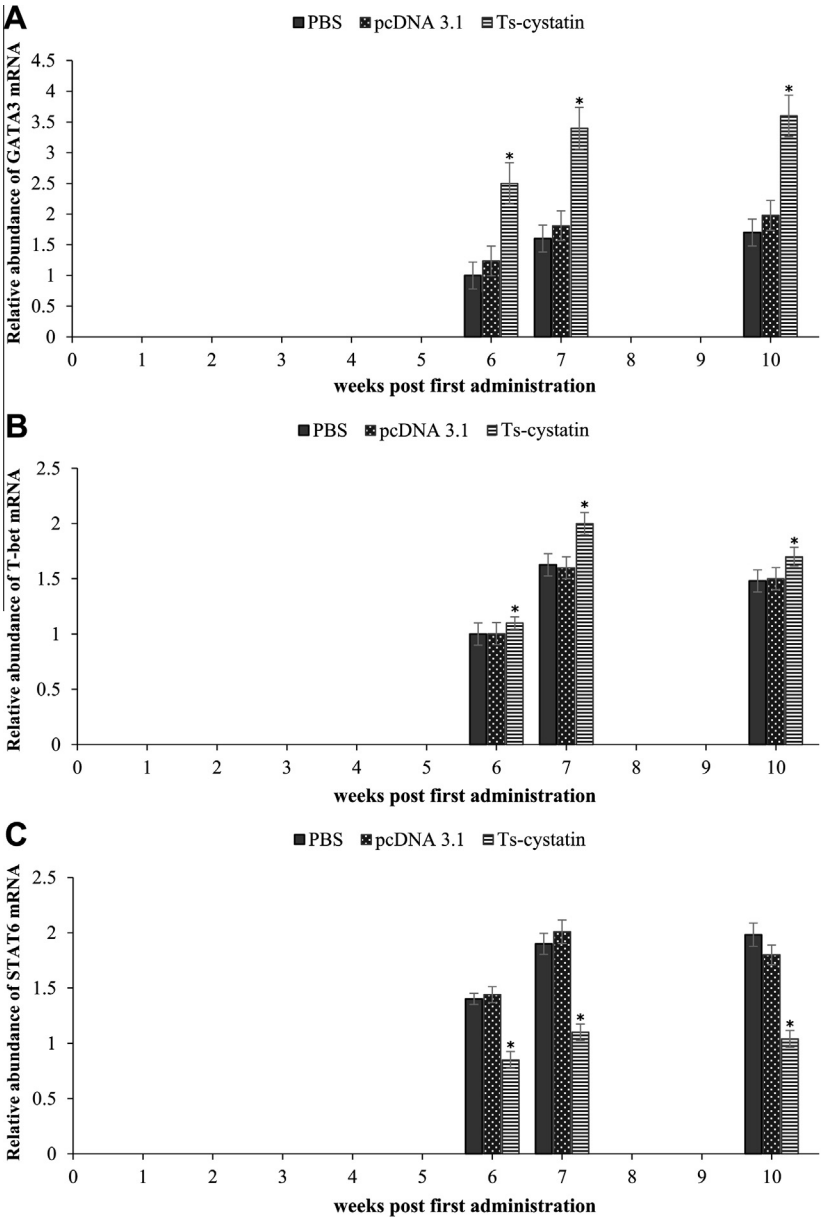

Fig. 9. Real-time PCR of the Transcription Factors mRNA was extracted from the spleens of pcDNA3.1/pcDNA 3.1(+)-Ts-cystatin and PBS-treated mice. The relative abundance of mRNA in each group was detected using real-time PCR. (*$P < 0.05$). (A) GATA-3 transcription level; (B) T-bet transcription level; (C) STAT6 transcription level.

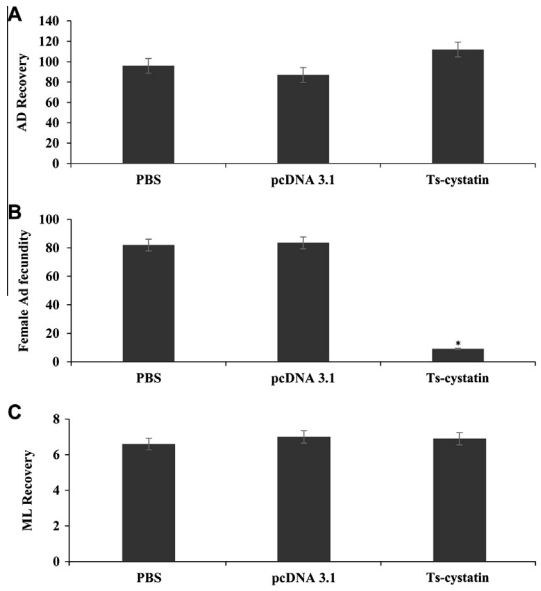

Fig. 10. Adult worm burden reduction (A), female worm fecundity reduction (B), and muscle larva burden reduction (C) in treated mice following challenge with 300 *T. spiralis* larvae per mouse. The results are presented as the arithmetic mean of 10 mice per group ± standard error. (*$P < 0.05$).

transcription factor (Yates et al., 2004). Szabo et al. (2000) first identified the transcription factor T-bet and demonstrated that T-bet was selectively expressed in Th1 cells. Subsequent studies have shown that T-bet is activated by IFN-γ and the IL-12-specific induction of Th1 cell differentiation. GATA-3 is a zinc finger transcription factor and a GATA family member and was discovered in 1993; it is a Th2-specific cellular transcription factor. GATA-3, through activation by IL-5 and the IL-4 promoter, can be regulate on a number of levels to induce the Th2 differentiation of naïve CD4+ T cells (Hidekazu et al., 2004). These results suggest that this gene induced a Th1/Th2-mixed type of immune response.

In this study, IFN-γ, IL-2, IL-4, IL-10, and IL-13 secretion by the splenocytes of mice immunized with PQ-pcDNA3.1-Ts-cystatin significantly increased after administration and continued to increase at weeks 7 and 10. These results also suggest that Ts-cystatin induced a Th1/Th2-mixed type of immune response.

In mice administrated orally with Ts-cystatin recombinant *Salmonella*, the CD4+ T lymphocyte count was significantly reduced compared with the control group; moreover, the CD8+ T lymphocyte count was increased, and the CD4+/CD8+ ratio decreased. After the challenge infection, in the Ts-cystatin group, the CD4+ T lymphocyte count increased, the CD8+ T lymphocyte count decreased, and the CD4+/CD8+ ratio increased significantly. T lymphocyte levels in the blood, in the same animal, the same age group is relatively stable, its changes often indicate changes in host immune function. T cells are divided into functions CD4+ T lymphocyte and CD8+ T lymphocyte, respectively, indicating the Th and Ts/Tc cell subsets. Th/Ts ratio of dynamic equilibrium reacts the level of immune regulation and immune status of the body. Decrease in its ratio indicates the inhibition of host immune response (Haggqvist and Hultman, 2003). The results suggest after oral administration with Ts-cystatin recombinant *Salmonella*, the mice were immunosuppressed. After the challenge infection, the mice in Ts-cystatin group were easier to be infected. Expulsion response was inhibited. So parasitological examination in our study demonstrated that the Ts-cystatin-treated mice had increased intestinal recovery.

At the 6th week, the CD14+ cell number was increased in the Ts-cystatin-immunized group when compared with the PBS group; however, in the 7th and 10th weeks, it was lower than that of the PBS group. It is well known that the body activates the adaptive immune response using antigen-presenting cells to induce T cell activation and those macrophages and DCs are antigen-presenting cells that can sense pathogen invasion and present different types of antigens to induce a host Th1- or Th2-type immune response. Many studies have shown that worms can inhibit the host immune response or induce a host immune response to complete its life cycle to achieve symbiosis with the host; the most effective means is through the use of excretory-secretory antigens to modulate the host immune system, which does not recognize the antigen; the host is often subsequently infected with other pathogens, resulting in death or other serious consequences (Maizels and Yazdanbakhsh, 2008). Thus, Ts-cystatin may reduce the number of macrophages to inhibit the host immune response to *Trichinella*.

After the oral administration of a recombinant attenuated *Salmonella*, we examined STAT6 transcription levels. Previously, a study found that a rapid expulsion process occurs in the host after *Trichinella* infection and that host STAT6 expression increases. STAT6 is related to the increased intestinal smooth muscle contractility observed following infection by *Trichinella* (Akiho et al., 2002), and it promotes goblet cell and mast cell proliferation (Khan et al., 2001; Urban et al., 2000). These changes are conducive to a rapid expulsion response, and they are necessary for the establishment of host protective immunity. However, our results showed that after oral administration with Ts-cystatin attenuated *Salmonella*, STAT6 transcription did not increase during natural infection with *Trichinella*. This gene did not induce trichinosis or rapid expulsion of the main reaction gene. In contrast, Ts-cystatin decreased STAT6 transcription, suggesting that this gene plays a role in *Trichinella* resistance to rapid expulsion by the host.

In conclusion, we found that administration of attenuated *Salmonella*-delivere Ts-cystatin induced a Th1/Th2-mixed type of immune response and decreased the transcription of STAT6. Furthermore, changes in the T lymphocyte and macrophage populations were observed. We therefore speculate that Ts-cystatin may be an important gene involved in the immunosuppression induced by *Trichinella*. Further studies are required to elucidate the roles of this gene.

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