Non-encapsulated *Trichinella* spp., *T. papuae*, diminishes severity of DSS-induced colitis in mice

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Summary

Background: **Helminths use various mechanisms to avoid host immunity and protect themselves from being eliminated. Despite evading host immune responses, immunosuppression and regulation mechanisms elicit functions that diminish the adverse effects of unrelated inflammatory diseases.**

Objective: **We investigated whether helminthic infections can ameliorate inflammatory diseases.**

Methods: **Mice were infected with *Trichinella papuae* and then subjected to induced colitis through the oral administration of dextran sulfate sodium (DSS). Macroscopic and microscopic examinations measured weight loss, stool consistency, gross bleeding, colon length, and tissue inflammation. In addition, cytokine expression was observed in colon tissue by SYBR real-time RT-PCR to investigate the Th1, Th2, and regulatory cytokines.**

Result: **The results showed that *T. papuae* infection decreased the severity of DSS-induced colitis, including weight loss, bloody diarrhea, shortening of colon, and colon tissue damage in mice (p <0.05). The expression level of IL-4 was high in the colons of DSS-treated mice without helminthic infection, while infected mice with DSS treatment had lower IL-4 levels (p <0.05). Uninfected DSS-treated mice failed to produce IL-10 mRNA in colon tissue, which may cause more severe colitis. In contrast, prior *T. papuae* infection DSS-treated mice had IL-10 levels in the colon significant lower than the normal and infected control groups.**

Conclusion: **Our data provide the evidence that prior *T. papuae* infection can ameliorate DSS-induced colitis in mice and may be considered for a novel therapeutic strategy against immunological diseases in the future.** ***(Asian Pac J Allergy Immunol 2013;31:106-14)***

Key words: *Trichinella papuae, colitis, inflammatory bowel diseases, Th1 and Th2 cytokines, regulatory cytokines*

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Introduction

Inflammatory bowel disease (IBD) is an idiopathic chronic condition causing severe inflammation of the gastrointestinal tract. The disease has two major forms, Crohn's disease (CD) and ulcerative colitis (UC), which can be distinguished by the location and nature of the inflammation.[1] The incidence of IBD is gradually increasing in Western countries, especially Europe and North America, while the prevalence and incidence of the diseases are lower in Eastern countries.[2] According to the hygiene hypothesis, helminthic infections in early childhood that are common in developing countries may stimulate and modulate host immune responses to protect against IBD. Recently, helminths and helminth-derived products have been studied for their potential to prevent colitis complications in experimental animal and human trials.[3-7] Preliminary data analyzed by Summers et al.[8] produced fascinating results using the eggs of the pig whipworm, *Trichuris suis*, to alleviate colitis in IBD patients.

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Moreover, clinical trials have been performed to evaluate the advantages of *T. suis* on IBD.[3,9] Studies evaluating the treatment efficacy in colitis in the experimental mouse model have been carried out using not only *T. suis*, but also several other parasitic helminths. Previously, *Trichinella spiralis* has exhibited its capability in effecting the regression of IBD and other immune disorders.[10,11] In addition to *T. spiralis*, another species in the genus *Trichinella* shows therapeutic potential against immune diseases as well. A study of infection with *T. pseudospiralis*, a non-encapsulated species, in animal models with experimental autoimmune encephalopathy (EAE) found that the infected animals developed less severe symptoms than the non-infected group.[12,13]

T. papuae, another non-encapsulated *Trichinella* spp., is the parasitic helminth we investigated in this study. Wu et al. demonstrated strong evidence that supports the efficacy of non-encapsulated *Trichinella*, *T. pseudospiralis*, in amelioration of inflammatory diseases.[13] Regarding reduction of inflammation in the intestines and muscle of the host, low pathogenicity, induction of high levels of total IgE, and delay in rejection of intestinal worms exhibiting during infection[13] support the immunomodulatory roles of the parasite. Thus, *T. papuae* is another non-encapsulated *Trichinella* that could be a candidate modulator of the host immune response. However, evaluating the therapeutic potential of *T. papuae* in inflammatory diseases including colitis has yet to be performed. Therefore, the aim of this study was to investigate the therapeutic potential of *T. papuae* on dextran sulfate sodium (DSS)-induced colitis in mice. Here, we infected mice with *T. papuae* prior to the administration of DSS for 7 days. Macroscopic and microscopic examinations were carried out to measure the disease activity index (DAI). Furthermore, the expression levels of Th1, Th2 and Treg cytokines in colon tissues were determined using SYBR real-time RT-PCR.

Methods

Mice and Parasite infection

Laboratory strains of *T. papuae* were maintained in the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Thailand, by serial infection into female ICR mice. Muscle stage larvae were obtained from the muscle tissue of the mice at 60-75 days post-infection (dpi) using artificial digestion with pepsin solution (0.7% pepsin (BDH, UK), 0.7% HCl). The larvae were obtained by the Baermann technique[14] and washed thoroughly with 0.85% normal saline solution (NSS). The six-week-old female BALB/c mice (6-8/ group) used for the experiment were purchased from the National Laboratory Animal Center, Mahidol University, Salaya Campus, Nakhon Pathom, Thailand. The mice were orally infected with 100 larvae and kept for 45 days before DSS treatment. Mice were maintained in the Laboratory Animal Science Center, Faculty of Tropical Medicine, Mahidol University and all animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the Faculty of Tropical Medicine, Mahidol University.

Induction of colitis

Colitis was induced in parasite-infected and non-infected mice by administering DSS (35-50,000 kDa; MP Biomedicals, OH) according to the method used in a previous experiment.[5] In brief, mice were given 5% DSS dissolved in sterile water for 7 days and then changed to sterile water alone for 3 days. Mice were euthanized and their colons were collected for further analysis. Mice not treated with DSS, with and without *T. papuae* infections, were used as controls.

Evaluation of colitis

After administration of DSS, mice were observed every day for 7 days. Macroscopic evaluations were done according to the method used in a previous experiment.[15] The parameters used for the evaluation of pathogenesis were weight loss, stool consistency, and the presence of blood in stools.[15] All parameters were evaluated and graded to define the disease activity index (DAI).

Histopathological examination

To determine the magnitude of colon inflammation and tissue damage, colon samples were dissected, the colon length was measured,[16] and the tissue was preserved in 10% formalin saline (10% formalin in 0.85% NSS). Colons were processed, embedded in paraffin, and cut into 5 µm thin slices with a microtome. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) in order to examine their histopathology. The histopathology was evaluated according to the method used in a previous experiment.[15] The histological grading of colitis is shown in the Figure 1.

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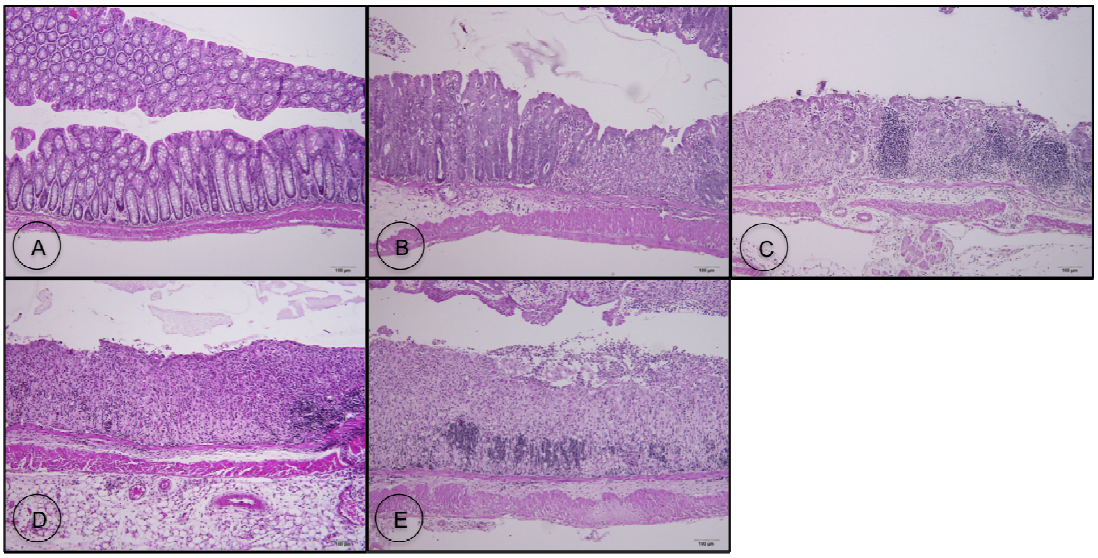

Figure 1. Histopathological grading of the colon in DSS-induced colitis with or without *T. papuae* infection. (A) Grade 0: Normal colonic mucosa. (B) Grade 1: Loss of one-third of the crypts. (C) Grade 2: Loss of two-thirds of the crypts. (D) Grade 3: Lamina propria covered with a single layer of epithelial cells with mild inflammatory cell infiltration. (E) Grade 4: Erosion of colon tissue with marked inflammatory cell infiltration. Scale bars, 100 μm.

Determination of cytokine mRNA expression in colon

Colon RNA was extracted using the TriZol reagent (Invitrogen, CA) according to manufacturer's instructions. Five micrograms of RNA were treated with DNase I to eliminate any contaminating genomic DNA. After that, first-strand cDNAs were constructed from the RNA template using Oligo dT primer and RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, MD). The target cytokine transcribes were analyzed by SYBR real-time RT-PCR. The reactions were set up with 2 μl of first-strand cDNA, 1X SsoFast™ Evagreen® Supermix (Biorad, CA), and 500 nM of each forward and reverse primers. Primers for amplification of mouse cytokines were designed using the Primer3 program (http://frodo.wi.mit.edu/ primer3/). The primers are described in Table 1. Amplification was performed with Rotor-Gene™ 6000 (Corbett Life Science) with cycles of 95°C for 5 min and 45 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. All samples were analyzed in duplicate. The Ct value of each cytokine in colon tissue was used to calculate gene expression levels in comparison with the housekeeping gene using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Gene expression levels were calculated using the formula of $2^{-\Delta CT}$. The calculated values are represented in arbitrary units (AU).

Statistical analysis

All sets of data were collected and analyzed for statistical significance using one-way ANOVA and the Mann-Whitney U test. Colon length and cytokine expression profiles were analyzed with one-way ANOVA. DAI and PAI were analyzed with the Mann-Whitney U test, with a p value of ≤ 0.05 considered statistically significant.

Results

Uninfected BALB/c strain mice treated with DSS initially developed signs of colitis on the fourth day with progressive weight loss, loose stools, and positive results on fecal occult blood testing. The severity of the diseases progressively increased until the seventh day with a high DAI. The mice exhibited severe weight loss and bloody diarrhea. In contrast, *T. papuae*-infected mice treated with 5% DSS showed a significantly diminished severity of colitis with a lower DAI than DSS-treated uninfected mice (p <0.05) (Figure 2). DSS-untreated control mice both infected and not infected with *T. papuae* did not spontaneously develop any signs or symptoms of colitis (Figure 2). After administering DSS, mice were euthanized on the tenth day. Colons were then removed and their lengths measured before being histopathologically examined. A comparison of colon length between DSS-treated and untreated groups found that the colon length of

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Table 1. Primers for detecting mouse cytokines

Gene	Accession no.	Primers (5'-3')	Length (nt)	Product size (bp)
GAPDH	NM_008084.2	*Fw*: 5'ACCCAGAAGACTGTGGATGG	20	171
		Rw: 5'CACATTGGGGGTAGGAACAC	20	
IFN-γ	NM_008337.3	*Fw*: 5'ACTGGCAAAAGGATGGTGAC	20	211
		Rw: 5'ACCTGTGGGTTGTTGACCTC	20	
IL-4	NM_021283.2	*Fw*: 5'TCAACCCCCAGCTAGTTGTC	20	227
		Rw: 5'AAATATGCGAAGCACCTTGG	20	
IL-10	NM_010548.2	*Fw*: 5'CCAAGCCTTATCGGAAATGA	20	162
		Rw: 5'TTTTCACAGGGGAGAAATCG	20	

uninfected mice treated with DSS was significantly shorter than the untreated controls (p <0.05) (Figure 3A, B). A shorter colon length was also noted in infected mice with DSS treatment. The colon length of the infected group was not significantly different (p >0.05) from the untreated control mice (Figure 3A, B).

The histopathology of colon was examined and the degree of inflammation and tissue damage was scored (Figure 1). The colon histology of DSS-treated, uninfected mice showed extensive inflammatory cell infiltration. Additionally, tissue erosion was observed in several areas of the colon tissue. Examination of colon tissue from *T. papuae*-infected mice with DSS treatment found that the degree of inflammation and tissue damage was less than in the uninfected mice treated with DSS. The pathology was quantified into histological scores as described previously. Infected mice treated with DSS exhibited significantly lower histological scores (p <0.05) than uninfected mice treated with DSS (Figure 4). The colon tissues of the DSS-untreated control groups with and without *T. papuae* infection did not show any evidence of inflammation and tissue damage (Figure 4).

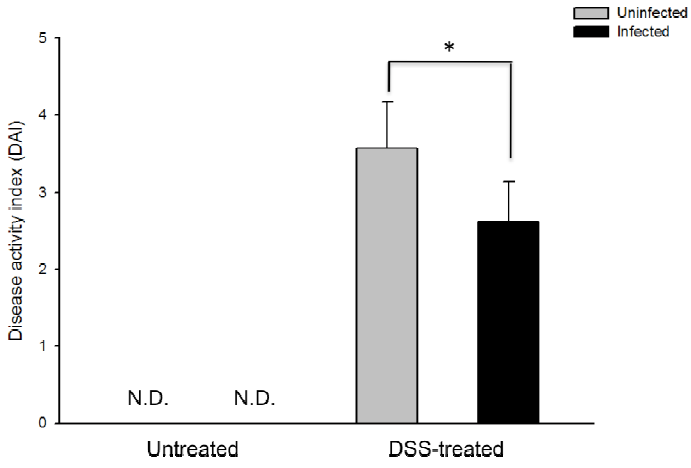

Figure 2. Disease activity index (DAI) calculated from macroscopic examination scores of colitis. The clinical manifestations were weight loss, stool consistency, and occult/gross bleeding. Non-treated control mice with and without *T. papuae* infection showed no signs and symptoms of colitis. N.D. = not detected. Infection of *T. papuae* significantly reduced DAI in DSS-treated mice, * p <0.05, compared with the non-infected group. Data are presented as means ± SE; n = 6 to 8 mice per group.

Expression levels of the colon cytokine mRNA were analyzed with SYBR real-time RT-PCR. Initially, conventional RT-PCR was performed to optimize the annealing temperature and verify the specificity of the primers. PCR products of each target were amplified as single predominant bands at the expected size. Cytokine transcription levels revealed that the DSS-induced colitis with and without *T. papuae* infection had no detectable IFN-γ mRNA in the colon tissue while IFN-γ mRNA was present in the colon tissue of the untreated control mice (Figure 5A). Expression levels of IL-4 in both infected and non-infected mice treated with DSS markedly up-regulated during colitis development. However, mice with DSS-induced colitis with prior infection expressed lower levels of IL-4 than the uninfected mice with DSS treatment (p <0.05) (Figure 5B). Moreover, there was no detectable expression of IL-10, which is the regulatory cytokine, in the colon tissue of uninfected mice treated with DSS. Interestingly, IL-10 mRNA was still present in the colon of *T. papuae*-infected mice treated with DSS although the levels of expression were significantly less than those of the untreated control mice (p <0.05) (Figure 5C).

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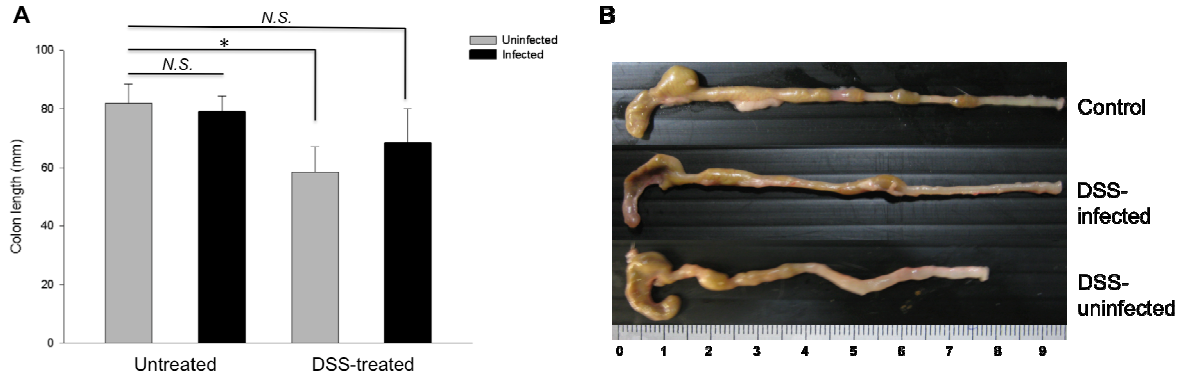

Figure 3. Colon length of mice. All mice were euthanized to remove the colon. The colons were measured to determine their length prior to histopathological examination. (A) The colon lengths of DSS-treated mice without *T. papuae* infection were significantly shorter than those of control mice, * $p < 0.05$. Colon length of DSS-treated mice with *T. papuae* infection did not significantly differ from those of control mice. *N.S.* = not significant. Data are presented as means ± SE; n = 6 to 8 mice per group. (B) Representative colons are shown. Colons from the DSS-treated mice without *T. papuae* infection are shorter than those from the control groups.

Discussion

The burden of IBD on patients depends on the severity of the disease and the availability of a successful medical treatment. To improve treatment and thus, the quality of life in IBD patients, several novel therapeutic approaches have been studied including helminth therapy.[8,17] *Trichinella spiralis* has been found to have the potential to reduce the severity of colitis in a mouse model. In our experiment, we demonstrated the potential of another helminth belonging to the same genus, showing that *T. papuae* can ameliorate the morbidity of colitis in a mouse model. We decided to use *T. papuae* because this particular parasite can survive for a long time in the host even though there is no capsule to protect it from the host immune response. Additionally, it has been observed that low levels of inflammatory cells infiltrate the host tissue surrounding parasite-infected muscle cells.[18] This suggests that *T. papuae* might elicit an immunomodulatory mechanism to suppress host inflammatory responses, which could be beneficial for alleviating the clinical manifestations of colitis.

Inducing colitis in mice with DSS is a well-established model that is widely used for studying the clinical manifestations of IBD, including abdominal pain, progressive weight loss, bloody diarrhea, severe inflammation of intestinal tissue, and lesions of the mucosa and the submucosa, as observed in humans. In our study, previous *T. papuae* infection (45 dpi) reduced the disease

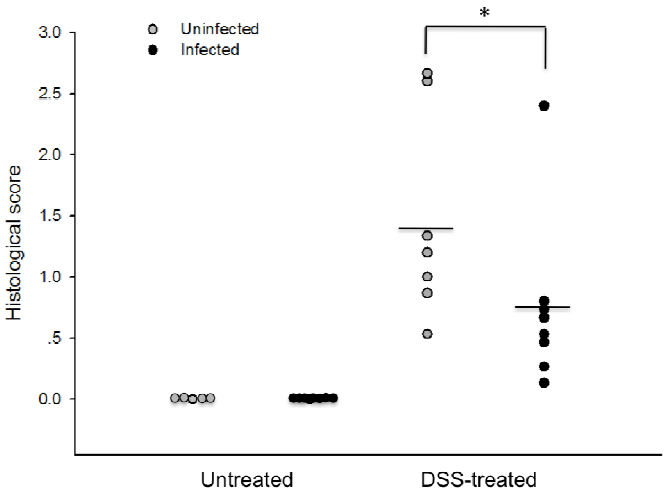

Figure 4. Histological scores for tissue inflammation. Colons were sectioned to be 5 μm thick and stained with H&E. The histopathological examination results were analyzed and used to determine a histological score. Colon tissue inflammation in DSS-treated mice with a *T. papuae* infection was significantly less than that for DSS-treated mice without *T. papuae* infection. * $p < 0.05$. The mean for each group is shown as a bar. n = 6 to 8 mice per group.

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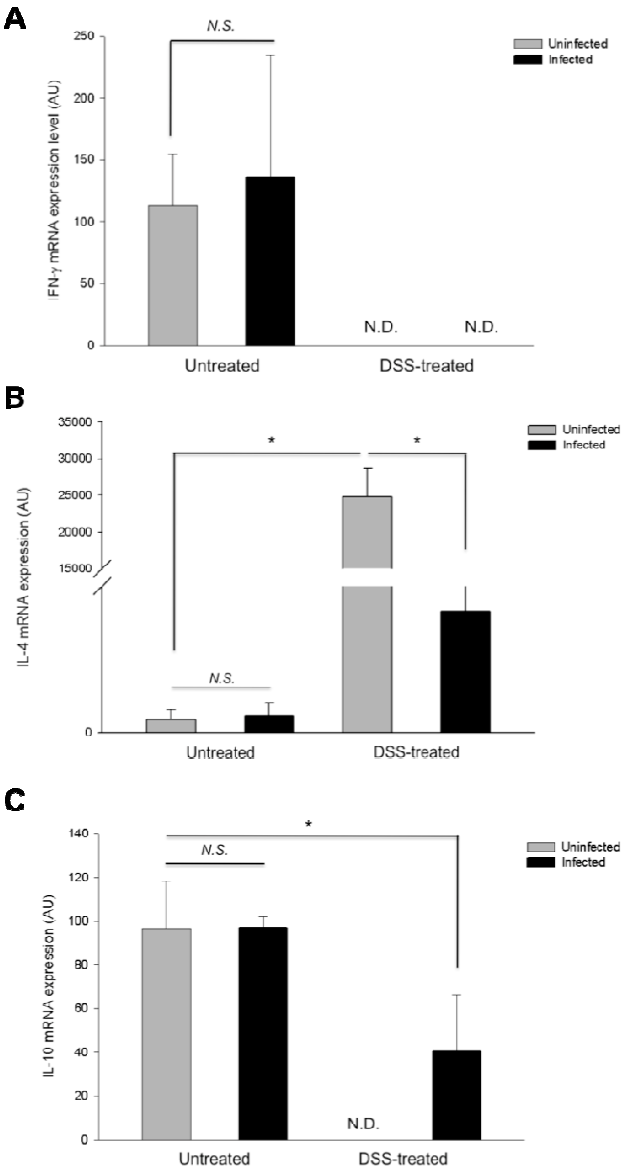

Figure 5. SYBR real-time RT-PCR showing cytokine expression levels from the colon tissue of mice. (A) mRNA expression levels of IFN-γ (B) mRNA expression levels of IL-4. (C) mRNA expression levels of IL-10. Data are shown in arbitrary units (AU). * p <0.05. *N.S.* = not significant. Data are presented as means ± SE; n = 6 to 8 mice per group.

morbidity in this animal model. The use of previous *T. papuae* infection (45 dpi) as the model differs from the study performed by Khan et al. 2002, which showed prior *T. spiralis* infection at 21 dpi diminished colitis in mice.[10] In relation to the host reactions against *Trichinella* spp, the immune response to non-encapsulated *Trichinella* spp. differs from that for encapsulated species.[19] Infection with *T. pseudospiralis*, mice elicited high levels of IL-10 (regulatory cytokine) at 45 and 60 dpi respectively, while *T. spiralis* and *T. britovi* infected mice up-regulated IL-10 levels to 15 dpi. Therefore, we anticipated that infection with *T. papuae* just prior to DSS (45 dpi) could generate high levels of regulatory cytokines, as previously described in *T. pseudospiralis*[19] and might elicit an effective immunomodulatory response at that time. In addition, we infected mice with a different number of *T. papuae* (100 muscle stage larvae), compared to *T. spiralis* infection (375 muscle stage larvae) in the previous study.[10] Our previous investigation suggests that infection with more than 150 muscle stage larvae of *T. papuae* causes morbidity and mortality in mice (unpublished data). DSS treatment in early infections (15 dpi) was performed in a preliminary study but we found that the mice developed severe colitis with a 50% mortality starting on the fifth day until the seventh day of the experiment (data not shown). These findings may be due to the disruption of intestinal barriers by invasion of the adult parasite and newborn with early infection,[20] which synergizes with the effect of DSS in the induction of mucosal injury and inflammation.

T. papuae infection suppressed the clinical manifestation of colitis in DSS-treated mice, which was shown using both macroscopic and microscopic parameters. Another *Trichinella* species, *T. spiralis*, has demonstrated the ability to diminish DNBS-induced colitis previously.[10] These findings imply that *Trichinella* infections with both capsulated and non-capsulated helminths have the potential to ameliorate colitis in animal models. In addition to colitis, the relationship between *Trichinella* species infections and other inflammatory disease models has been studied. *T. spiralis* and *T. pseudospiralis* infections reduced the morbidity of experimental autoimmune encephalomyelitis (EAE) in animal models through the suppression of Th1 and Th17 cytokines.[13,21] Advantages of *Trichinella* infection were also observed in mice with allergic inflammation in their airways. *T.* spiralis-infected mice challenged with OVA reduced the airway hyper-responsiveness and infiltration of inflammatory cells into the peribronchial tree.[11]

Helminthic infections are characterized by a polarization of the Th2 response that strongly induces the production of Th2 cytokines such as IL-4, IL-5, and IL-13. In *Trichinella* infection, the emergence of Th2 cytokines was observed in both encapsulated and non-encapsulated species.[22,23] In the mouse model of colitis, levels of Th1 cytokine production in colon and spleen cells was related to

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the severity of the disease. *T. spiralis* infection in DNBS-treated mice had the potential to alleviate colitis through increasing the Th2 immune response, which in turn antagonizes the production of Th1 cytokines.[17] We found that the expression of IFN-γ could not be detected in the colon samples of mice with DSS-induced colitis, with and without prior *T. papuae* infection. In addition, levels of IL-4 expression in the colon tissue of the DSS-treated mice were markedly increased, especially in the non-infected group. We believe that DSS-induced colitis in our mouse model was dependent on the imbalance of the Th1/Th2 response and an uncontrolled elevation of IL-4 in the colon. This suggests that the presence of high-levels of Th2 cytokines in the colon is not refractory to DSS-induced colitis, as mentioned elsewhere. This conclusion is supported by a previous study in which a Th2 response was induced by injecting *Schistosoma mansoni* eggs into DSS-treated mice but this failed to decrease the severity of colitis.[5] However, the BALB/c strain mice used in the experiment contain a genetic background that biases the immune response toward Th2 production.[24,25] In another case, using C57/B6 strain mice predominantly producing Th1, high levels of Th1 and Th17 immune responses, that relate to the clinical manifestation of colitis, were elicited.[26,27] This suggests that the Th1, Th2 and Th17 responses have all been shown to influence the development of the disease, depending on the genetic background of the host. The interaction of genetic background and cytokine environment probably explains the development of the two main forms of IBD, Crohn's disease and ulcerative colitis, which are different in their cytokine responses.[28,29]

Protection against colitis in animal models with helminthic infection has been characterized by the Th2 response that neutralizes Th1-associated colitis. Immune regulatory responses have an important role in ameliorating colitis as well.[5] IL-10 is the regulatory cytokine that suppresses and regulates the function of several immune cells, such as cytotoxic and helper T cells, B cells, NK cells, macrophages and mast cells as well as pro-inflammatory mediators such as TNF-α, IL-12, IL-1, nitric oxide, and several chemokines.[30,31] The results of IL-10 gene therapy in mice with DNBS-induced colitis suggest an important role for this cytokine in decreasing macroscopic and histological manifestations of colitis.[32] Additionally, administering human recombinant IL-10 into the same bacteria cell wall polymers that induce colitis in rats managed to suppress granulomatous inflammation.[33] In helminthic infections, inducing regulatory cells and IL-10 production are two mechanisms that the parasites use to evade the host immune responses.[34] These characteristics may offer an alternative therapy against inflammatory diseases in animal models and human trials.[3,5,7,8] In this study, DSS-treated mice with *T. papuae* infection expressed IL-10 in colon tissue in small amounts, while DSS treated mice without *T. papuae* infection failed to produce any IL-10 at all. This suggests that the remaining the IL-10 in the colon tissue may cause the amelioration effect on DSS-induced colitis in mice with *T. papuae* infection. However, in other helminthic infections shows IL-10 does not have any protective effect in colitis. In *Heligmosomoides polygyrus* infections, the parasite diminished colitis without the effect of IL-10.[35] Thus, the role of helminth-induced IL-10 and its effects on colitis need to be investigated further in future studies.

Trichinella infection modulates and regulates the host microenvironment and the immune response. Although these parasites elicit the potentially therapeutic effects for immune diseases including IBD, the infection can also cause morbidity and mortality in human hosts. Therefore, identification and characterization of helminth-derived immunomodulatory molecules would more desirable and may be an advantageous treatment for incurable inflammatory and autoimmune diseases in the future.

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