Regulation of host immune cells and cytokine production induced by *Trichinella spiralis* infection

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**Abstract** – The nematode *Trichinella spiralis* can cause immunoregulation during the early phase of infection. However, previous studies are still insufficient for a full understanding of this phenomenon and its underlying mechanism. In this study, immune cells and cytokine profiles of *T. spiralis* infected mice were examined by Meso Scale Discovery (MSD) and flow cytometry. The MSD results of the spleen showed that Th1 immunity was inhibited from 6 h to 6 days post-infection (dpi) and the level of Th2 immune response was significantly increased at 6 dpi. The mesenteric lymph node showed a Th1/Th2 mixed immune response from 3 dpi to 6 dpi with a downtrend of Th1 at 6 dpi. Flow cytometry analysis showed that the proportion of Th1 cells of T cells was decreased significantly at 6 h after infection, the proportion of Th2 cells was markedly increased, indicating that Th1 immunity was significantly inhibited at 6 h after infection, and a hybrid immune response based on Th2 type was presented from 30 h to 6 dpi. The immunoregulation effects observed during this study have provided a better understanding of the development of the immune response induced by *Trichinella* infection.

**Key words:** *Trichinella spiralis*, Meso Scale Discovery, Cytokines, Immunoregulation.

Introduction

Trichinellosis is a common zoonosis with a global distribution that has a great socioeconomic impact on human and animal health [12]. Although *Trichinella spiralis* is well controlled in Europe and the United States, it is still prevalent in developing and developed countries, including China, Argentina, and Eastern Europe [10]. After its successful isolation in 1835 [4], there has been a large number of studies on *T. spiralis* infection and parasitism [19]. It has been found that *T. spiralis* invasion is not just a simple case of mechanical penetration [23], and that immune suppression has been observed in the intestinal phase of mice [20, 21]. Continuous infection with *T. spiralis* can stimulate the body to produce...
acquired immune effects, but the host cannot completely inhibit
the growth of *T. spiralis*, indicating that it can inhibit, and
escape from, the host’s immune response [8]. This phenomenon
further supports this study that the host’s immune mechanisms
play an important role in the development, invasion, and
parasitism of *T. spiralis* [26].

During the early stage of *T. spiralis* infection, cellular
immunity is inhibited, while in the late stage, cellular immune
function has recovered, and humoral immunity began to play a
role in resisting *T. spiralis* infection [1]. During *T. spiralis*
infection, Th1/Th2 cells play an important role in balancing
immune system function, and once that balance is broken, the
host will become infected [17]. Numerous studies have shown
that *T. spiralis* infection affects its host’s immune system,
inhibiting Th1/Th17 cell response and inducing Treg cells to
reduce the inflammatory response [28]. When *T. spiralis* infects
the host, different phase-specific antigens are produced in
different developmental stages of the parasite life cycle, which
induce the host to produce a specific immune response [25, 27].
To account for this characteristic, we have carried out experi-
ments to verify the four key stages of *T. spiralis* infection
during the intestinal phase. By examining the expression and
regulation of host immune cells and cytokines, our goal
is to find a breakthrough point in the immune regulation of
*T. spiralis* which could lead to new approaches to the treatment
of trichinellosis.

### Materials and methods

#### Ethics

All mice protocols were reviewed and approved by the Ethics Committee of Jilin University affiliated with the Provincial Animal Health Committee, Jilin Province, China (Ethical Clearance number IZ-2009-08).

#### Obtaining of muscle larvae and infection of BALB/c mice

*Trichinella spiralis* (ISS534) was provided by the Institute of Zoonosis of Jilin University. Female BALB/c mice, aged 6 weeks, were purchased from the Animal center of the medical department of Jilin University.

Muscle larvae were obtained from infected mice by artificial digestion performed using the latest version of the magnetic stirrer protocol, according to the OIE standard protocol. After a series of precipitation and washing steps, muscle larvae suitable for sample addition were finally obtained. The number of *T. spiralis* larvae obtained by digestion was counted under a microscope.

Forty-eight BALB/c mice were divided randomly into two
groups, an infected group and a control group. Each mouse in
the infected group was infected with 250 muscle larvae at the
same time. Under aseptic conditions, samples of peripheral
blood, spleens, and mesenteric lymph nodes of the infected
group and the control group (six mice per group) were collected
for MSD and flow cytometry analysis at 6 h, 30 h, 3 d, and 6 d
after infection.

### Meso scale discovery (MSD) determination of cytokines

Based on the following advantages, MSD technology was
selected for this experiment. Since the sensitivity of MSD is
up to 0.05 pg/mL, it is more effective in finding a difference
between the infected group and the control group. Only when
the MSD plate is fed into the instrument and stimulated by
electrodes can the signal be generated. The excitation time of
each well is unified with the signal acquisition time, and the
stability of data can be high.

The mesenteric lymph nodes and spleens were obtained at
6 h, 30 h, 3 dpi, and 6 dpi from the infected and control mice,
under aseptic conditions, and stored at $-80 \, ^\circ C$ prior to use in
the detection of cytokines IL-2, IL-4, IL-10, IL-17A, IFN-γ,
and TGF-β. For the cytokine determination test, 300 μL of
linker was added to 200 μL of biomarker antibody
(K15069L-1, Univ. Biotechnology, China) and incubated for
30 min at room temperature, then 200 μL of termination
solution was added and the solution incubated at room temperature
for 30 min. A volume of 50 μL of antibody was added to each
well of the MSD plate and the plate was incubated for 1 h at
room temperature. The MSD plate was rinsed three times with
1× PBS (0.05% Tween-20), and 25 μL each of the diluent and
sample were added. The plate was sealed and shaken at room
temperature for 1 h. After washing the MSD plate three times
again, 50 μL of the detection antibody was added to each well,
and the plate shaken at room temperature for 1 h. After incuba-
tion, the MSD plate was rinsed three times with 150 μL of
washing solution. The MSD plate was rinsed three times, 150 μL of the readout liquid was added. All data were analyzed
with MSD software (QuickPlex SQ120, MSD, USA).

#### Flow cytometry analysis of Th1, Th2, Th17, and Treg cells

An amount of 100 μL of peripheral blood was taken at 6 h,
30 h, 3 dpi, and 6 dpi from 12 mice from the infected and
control mice, under aseptic conditions, and placed in an antico-
agulant tube prior to further tests. Th1 cells (CD3, CD4, IFN-γ),
Th2 cells (CD3, CD4, IL-4), Th17 cells (CD3, CD4, IL-17),
and Treg cells (CD4, CD25, Foxp3) were analyzed and identified.
The cellular subpopulations were detected using fluorescently labelled antibodies with samples incubated for 15 min. All the steps were carried out at $2–8 \, ^\circ C$. The FITC anti-mouse CD4 antibody (No. 11-0042-
81, Biolegend, USA) was used on all samples. Cy5 anti-mouse
CD3 antibody (No. 15-0031-82, eBioscience, USA) and PE
anti-mouse IFN-γ antibody (No. 12-7311-81, eBioscience,
USA) to label Th1 cells; Cy5 anti-mouse CD3 antibody
(No. 15-0031-82, eBioscience, USA) and APC anti-mouse
IL-4 antibody (No. 17-7041-81, eBioscience, USA) to label
Th2 cells; Cy5 anti-mouse CD3 antibody (No. 15-0031-82,
eBioscience, USA) and PE anti-mouse IL-17 antibody
(No. 12-7177-81, eBioscience, USA) to label Th17 cells; and
APC anti-mouse CD25 antibody (No. 17-0251-81, eBio-
sience, USA) and PE anti-mouse Foxp3 antibody (No. 13-
5773-82, eBioscience, USA) to label Treg cells. All data were
analyzed using the CellQuest software on the BD FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany).

Data processing and statistical analysis

Data differences were analyzed by ordinary one-way ANOVA with GraphPad Prism 8.1.2 software. \( p < 0.05 \) was considered to be statistically significant. The replications of the experiments (MSD and flow cytometry) were performed three times.

Results

MSD detection of cytokine results

When compared with the control group, the following observations were made in the mesenteric lymph nodes of infected mice: IL-2 levels significantly decreased at 6 h after infection, increased significantly at 3–6 dpi, and were not significantly different at 30 h; IL-4 levels were increased from 3 dpi to 6 dpi. IL-10 levels decreased (albeit not significantly) at 6 h and 30 h after infection, and increased significantly at 3–6 dpi, and IL-17A levels decreased significantly at 6 dpi, and significantly increased at 30 h and 3 dpi. IFN-\( \gamma \) levels were significantly increased at 3–6 dpi, and TGF-\( \beta \) levels were increased at 30 h after infection (Fig. 1).

When compared with the control group, the following observations were made in the spleens of infected mice: levels of IL-2 were significantly decreased at 6 h, 30 h, and 3 d after infection, IL-4 levels were significantly decreased at 6 h after infection and increased at 6 dpi, and IL-10 levels were significantly decreased at 30 h and 3 d after infection. IFN-\( \gamma \) levels were decreased at 30 h after infection and TGF-\( \beta \) levels were decreased at 3 dpi, significantly increased at 6 h, and slightly increased at 6 dpi (Fig. 2).

Flow cytometry analysis results

Evaluation of the percent change of T lymphocytes

The ratio of CD4\(^+\)/CD8\(^+\) reflects the immune regulation status and immune level of the host. In infected mice at the intestinal stage of infection, levels of CD4\(^+\) T cells at 6 dpi were markedly increased, while CD8\(^+\) T cells at 6 dpi were decreased. According to the trends, CD4\(^+\) T levels were decreasing at 30 h after infection, while CD8\(^+\) T levels were on the rise (Fig. 3A and B). Compared with the ratio of CD4\(^+\)/CD8\(^+\) at 6 h, it was significantly decreased at 30 h after infection (Fig. 3C).

Evaluation of the percent change of IFN-\( \gamma \) detection

As far as the overall trend is concerned, the expression level of IFN-\( \gamma \) was fluctuated. The secretion of IFN-\( \gamma \) by Th1 cells is involved in cellular immune regulation, mediates the immune response, and has an immunosuppressive effect on the worms invading the intestine of mice. In the infected group, the expression of IFN-\( \gamma \) at 6 h was significantly lower, while at 30 h, it was slightly higher (Fig. 4). Compared with the 6 h infection group, the expression of IFN-\( \gamma \) in the infected group was significantly increased at 6 dpi.

Evaluation of the percent change of IL-17

IL-17 mediates the inflammatory response. Expression in the infected group was gradually elevated at the four time points of infection (Fig. 5). Compared with the 6 h infection group, the expression of IL-17 in the infected group was increased at 6 dpi.

Evaluation of the percent change of Foxp3\(^+\)

Foxp3\(^+\) was expressed at a high level at each time point (Fig. 6). The infected group expression levels were only higher.
Figure 2. Cytokine expression levels of IL-2, IL-4, IL-10, IFN-γ, and TGF-β in the spleen in mice infected with 250 muscle larvae of *Trichinella spiralis*.

Figure 3. Percentage and expression levels of CD4+ and CD8+ T cells in mice infected with 250 muscle larvae of *Trichinella spiralis* compared to uninfected (control) mice from 6 h to 6 dpi. (A) Comparison of the mean percentage (±SD) of CD4+ T cells. (B) Comparison of the mean percentage (±SD) of CD8+ T cells. (C) Comparison of the expression (±SD) of CD4+/CD8+ T cells. *p < 0.05 and **p < 0.01 indicating a statistically significant difference.

Figure 4. Percentage and expression levels of IFN-γ of Th1 cells in mice infected with 250 muscle larvae of *Trichinella spiralis* compared to uninfected (control) mice from 6 h to 6 dpi. *p < 0.05 and **p < 0.01 indicating a statistically significant difference.

Figure 5. Percentage and expression of IL-17 of Th17 cells in mice infected with 250 muscle larvae of *Trichinella spiralis* compared to uninfected (control) mice from 6 h to 6 dpi. *p < 0.05 indicating a statistically significant difference.
at 30 h and 6 dpi, and no significant differences were seen at the other two time points. Treg cell numbers were highest at 6 dpi.

**Evaluation of the percent change of IL-4**

Compared with the 30 h infection group, the expression of IL-4 in the infected group was significantly increased at 6 dpi. With regards to the overall trend, levels of IL-4 expression fluctuated greatly at 6 h and 3 dpi (Fig. 7). IL-4 activates mast cells to release histamine and serotonin to increase intestinal peristalsis and causes diarrhea, thereby stimulating the discharge of parasites. Changes in IL-4 levels during intestinal invasion may be due to immune escape by *T. spiralis*.

**Discussion**

Trichinella spiralis infection has an immunosuppressive effect on the host’s innate immune system [2, 24, 29]. At this stage, the worms develop rapidly, the morphology, and excretion change constantly, and the newborn larvae begin to be developed at 4 dpi. Ultimately, through complex developmental processes, the newborn larvae migrate rapidly with blood or lymph and invade the skeletal muscle. It is speculated that the immune regulation of the host at different time points up to 6 dpi is complex and volatile. Most studies have tested the immune regulation above 1 dpi. In this study, different views on this detection time were presented, and mice were used as experimental animals to detect the immune response of mesenteric lymph and invade the skeletal muscle. It is speculated that the immune regulation of the host at different time points up to 6 dpi is complex and volatile. Most studies have tested the immune regulation above 1 dpi. In this study, different views on this detection time were presented, and mice were used as experimental animals to detect the immune response of mesenteric lymph nodes and spleen during the four stages. The four stages were: the invasion period of muscle larvae into mesenteric lymph nodes and spleen during the four stages.

The four stages were: the invasion period of muscle larvae into mesenteric lymph nodes and spleen during the four stages. The initial stage of adult *T. spiralis* (6 h), the initial stage of adult *T. spiralis* (30 h), the mating period of the adult males and females (3 dpi), and the growth period of newborn larvae (6 dpi).

The MSD results of the spleen in the early stages of *T. spiralis* infection showed that the Th1 immune response was inhibited from 6 h after infection to 3 dpi, until it returned to normal levels at 6 dpi, whereas the immunosuppression of the Th2 response only occurred at 6 h after infection. In addition, the level of Th2 immune response induced by IL-4 was significantly increased in the spleen at 6 dpi, which was beneficial to the stimulation of the humoral immune response. In 1198, Lawrence et al. [17] have also found that intestinal lesions are related to IL-4 and the immune response mediated by Th2 cells, agreeing with our results. These results suggest that the excreta of *T. spiralis* play an immunosuppressive role in peripheral immune organs (mesenteric lymph nodes and spleen). Th1 cells have an important role in the resistance to intracellular pathogens (viruses, bacteria, and parasites) and in
the case of intracellular infection, Th1 cells can effectively trigger a cellular immune-mediated host defense response. However, the muscle larvae are still able to invade the intestinal epithelial cells and develop into adult parasites. Inhibition of Th1-type immunity in this period will provide sufficient time for the invasion, development, and the production of larvae of *T. spiralis*. It is beneficial therefore, for *T. spiralis* to establish infection [14, 22], evade the host immune system, and to reduce the resistance of the host and obtain the chance of parasite parasitism [6]. The level of immunosuppressive TGF-β expression in the spleen was significantly increased at the 6 h after infection, indicating that it plays an important role in inducing Th1-type and Th2-type immunosuppression. This suggests that there must be some molecules in the excreta of *T. spiralis* that have a strong immunomodulatory effect that can stimulate the expression of TGF-β [13]. Isolation and identification of these molecules will be part of a future study.

The immune mechanism of parasitic diseases mainly involves the interaction of Th1 and Th2 reactions and related cytokines, which inhibits their reproduction and amplification [11]. Th1 cells mainly secrete IFN-γ to regulate cellular immunity and mediate cellular immune response. According to the results of this experiment, the expression of IFN-γ fluctuates at different infection times, which indicates that the content of IFN-γ secreted by Th1 cells changes, participates in the regulation of cellular immunity, mediates the immune response, and regulates cellular immunity, and invasiveness. *Trichinella spiralis* infecting the intestinal tract of mice has certain immunosuppressive effects. IL-4 produced by the Th2 subgroup mainly acts as an immune response against extracellular multicellular parasites. Mast cells are activated by IL-4 to release histamine and serotonin, which can increase intestinal peristalsis and diarrhea and stimulate the discharge of parasites. As a whole, the level of IL-4 fluctuates greatly at 30 h and 6 dpi, the changes may be caused by immune escape of *T. spiralis*. Th17 cells are one of the subgroup cells differentiated from CD4+ T cells, which specifically secrete cytokine IL-17. Even though Th17 cells complete differentiation, they will be limited by many complex factors. The differentiation of Th17 cells was inhibited by the production of IFN-γ and IL-4, which were secreted by Th1 and Th2 cells [7]. In this experiment, the level of IL-17 in the infected group increased at 6 dpi compared with 6 h, indicating that Th17 secreted IL-17 to control infection. The number of Th17 and Treg cells would remain at a stable level to maintain the stability of the immune system. *Trichinella spiralis* most likely down-regulates host immune response through signaling to increase the number of Treg cells [16]. Many studies have shown that both the rapid and slow stages of *Trichinella* infection can increase the number of Treg cells. The experimental results support this statement, Foxp3+ was expressed at a high level at each time point. The main effect of Foxp3+ on host immune suppression is to maintain immune tolerance and control excessive inflammatory reactions [5, 15]. In this study, we found that the mesenteric lymph nodes in mice after *Trichinella* infection showed a significant Th1/Th2 mixed immune response from 3 dpi, and before this time, *T. spiralis* had already established infection and developed into adulthood. The inflammatory and immune responses after 3 dpi helped to reduce the damage caused by parasites and establish long-term parasitism. The level of Th2 response in the spleen from 6 dpi was also significantly increased, which was consistent with published studies on *T. spiralis* immunology which show that infection induces a Th2-type-dominated immune response in the host [3, 18]. This reflects the complexity of host immune regulation in the early stages of *Trichinella* infection. *Trichinella* has achieved long-term parasitism in the host by inducing a Th2-type immune response produced by the body [9]. The results of this study will help to elucidate the immunosuppressive mechanism induced by *T. spiralis* in early induction of the host, and provide a firm foundation for the study of related diseases.

**Conflict of interest**

No financial or personal relationships are maintained with other authors or organizations that could inappropriately influence or bias this paper entitled “Regulation of host immune cells and cytokine production induced by *Trichinella spiralis* infection”.

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