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FULL PAPER

Parasitology

# Hepatoprotective immune response during *Trichinella spiralis* infection in mice

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**ABSTRACT.** Infections with gastrointestinal nematodes provoke immune and inflammatory responses mediated by cytokines released from T-helper type-2 (Th2) cells. Infections with *Trichinella* species have been reported to differ by the host species. Previously, in rats, we observed acute liver inflammation in response to infection with *Trichinella spiralis*, and the rat hosts showed a series of biochemical changes characterized by a decrease in serum paraoxonase (PON) 1 activity associated with the down-regulation of hepatic PON1 synthesis. In the present study, we investigated the effect(s) of species differences on the immune response against *T. spiralis* infection by analyzing serum PON1 activity and the associated inflammatory/anti-inflammatory mediators in mice. There were inconsistent changes in the serum PON1 activity of mice infected with *T. spiralis*, and these changes were associated with significant increases in the serum levels of interleukin (IL)-2, IL-4, IL-10, IL-12 (p70), granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor α during the enteric phase of the infection, while the levels of IL-5 and interferon γ were significantly increased throughout the entire experimental period. Moreover, *T. spiralis* infection in mice was associated with little inflammatory cell infiltration in hepatic tissues. Given the zoonotic prevalence of *T. spiralis*, further mechanistic research in this area is warranted.

**KEY WORDS:** inflammation, liver, paraoxonase-1, *Trichinella spiralis*, zoonotic nematode

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Gastrointestinal parasites may be considered the most significant infectious agents in the world in terms of their global prevalence and their ability to cause diseases. In addition to causing harmful effects in humans, gastrointestinal parasites are responsible for extensive diseases and economic losses in domestic animals [36, 47]. It is estimated that two billion individuals around the world are infected with parasitic worms, and almost one billion children live in regions of high transmission [25]. Virtually all vertebrates are susceptible to infection by at least one member of the genus *Trichinella*; however, by all accounts, humans seem to stand out as they are particularly susceptible to the clinical illnesses caused by the most far-reaching zoonotic parasitic nematodes in the world [18]. In humans, trichinellosis, which is caused by oral infection with *Trichinella* species, can be categorized into three stages according to parasitic growth: 1) the presence of muscle larvae inside skeletal striated muscle cells; 2) the presence of adult worms in the small intestine; and 3) the presence of newborn larvae in the lymphatic vessels and bloodstream. During the different stages of parasitic growth, *Trichinella spiralis* deals with the host immune response by releasing various proteins in attempts to avoid or restrain the host defense mechanisms; these are considered to be crucial for the successful invasion and survival of the parasite within the host [11, 52]. The expulsion of *T. spiralis* worms from the small intestine is a complex immune-mediated process that involves the activation of T-helper type-2 (Th2) cells. Th2-related cytokine levels rise immediately after the invasion of *T. spiralis* larvae into the intestine, and the levels of interleukin (IL)-4 and IL-13 peak before the development of mature muscle larvae [27]. It is well known that cytokines released by Th1 and Th2 cells act antagonistically and they mutually regulate each other; however, it remains unclear which factors are responsible for balancing Th1 and Th2 cytokines over the course of an immune response [20, 33].

Several previous works suggest a genetic basis for the variation in the host response to parasitic infections observed in natural

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residents. Therefore, the use of strains of laboratory animals with defined genetic background, it became possible to examine, whether the responses seen in the wild could be reproduced experimentally in populations of animals with strictly controlled genetic constitutions [5]. Such variations may include innate (non-immunological) and acquired (immunologically mediated) resistance mechanisms [49]. Consequently, research with small animal models has provided much insight into the *in vivo* defense reactions against infections, but the immune reactions observed against infections differ considerably depending on the species of the animal host used [48]. Mice are permissive for the full growth, development, and sexual maturation of some parasites. In contrast, rats are less vulnerable or only semi-permissive as they do not provide an appropriate microenvironment for parasite development [41]. The response that limits the progression of infection is usually related to the presence of natural antibodies against the parasite and other humoral and/or cellular immune responses [48]. This may explain why the reported severity of *Trichinella* infection varies according to the host species [1]. Furthermore, the variable immune responses between species might have subsequent effects on other organs, including the liver. Although *T. spiralis* rarely induces liver disease [30], we observed acute hepatitis in response to infection with *T. spiralis* in rats, and the rat hosts showed a series of biochemical changes characterized by a decrease in serum paraoxonase (PON1) activity associated with the down-regulation of hepatic PON1 synthesis, especially during the enteric phase [13]. This response is an innate immune reaction, which plays a critical role in limiting tissue injury [39]. A key component of the acute phase response is the altered hepatic synthesis of a wide array of proteins, including PON1.

PON1 is a high-density lipoprotein (HDL)-associated enzyme that is a free radical scavenger involved in the acute phase response [15]; it exerts anti-atherogenic and anti-inflammatory effects through the hydrolysis of lipid peroxides and the protection of low-density lipoprotein (LDL) from oxidation. In addition, PON1 inhibits not only the oxidation of LDL but also that of HDL [3]. Nonetheless, the mechanisms responsible for regulating PON1 activity during *T. spiralis* infection in different animal species have yet to be fully understood. Given the medical significance of *Trichinella* species, there is a critical need to better understand new points of interest of the host-parasite interaction. In this study, we used mice to investigate the effect(s) of species differences on the immune response against *T. spiralis* infection by analyzing serum PON1 activity and the associated inflammatory/anti-inflammatory mediators.

## MATERIALS AND METHODS

### *Experimental animals*

Six-week-old male C57BL/6J mice (18−25 g) were purchased from Charles River Japan, Inc. (Yokohama, Japan). All animals were housed in clean cages and given a diet and water *ad libitum*. Their environmental conditions were controlled in terms of light (12 hr. light-dark cycle starting at 8:00 a.m.) and room temperature (23 ± 3°C). All protocols were approved by the Institutional Review Board for Animal Experiments of the University of Miyazaki (Approval No. 2015-004), in compliance with the lows of Japan 'Act on Welfare and Management of Animals'. All animal procedures were conducted in BSL2 room.

### *Parasitological techniques*

To infect the experimental mice, infectious *T. spiralis* larvae were recovered from the muscles of mice that had been infected for more than 4 weeks, as previously described by Crum *et al.* [10] and Farid *et al*. [13]. In brief, larva donor mice were euthanized by cervical dislocation. The skinned and eviscerated carcasses were minced in a meat grinder and digested for 1–2 hr. at 37°C in distilled water (10 m*l*/g) containing 1% (w/v) pepsin (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 1% (v/v) hydrochloric acid. Larvae were collected by pouring the digestion fluid through a mesh and washed several times with 0.85% NaCl. The animals randomized to the *T. spiralis* group were administered 600 first-stage larvae by oral gavage. In order to count the muscle stage larvae, whole carcasses of infected mice were digested as previously described and larvae were counted under microscope after washing. Intestinal *T. spiralis* stages were obtained from the intestine of infected mice from 2 days post-infection (p.i.) until the end of the experiment using Baermann technique.

### *Serum PON1 activity*

Serum PON1 activity was assayed using three synthetic substrates: paraoxon (diethyl-p-nitrophenyl phosphate; Sigma Chemical Co.), phenyl acetate (Nacalai Tesque, Inc., Kyoto, Japan) and dihydrocoumarin (Sigma Chemical Co.) as described by Aviram and Rosenblat [2]. All activities were measured at 25°C.

### *Measurement of serum cytokine concentrations*

Twenty-three inflammatory mediators were analyzed in the serum of the experimental mice with a multiplex bead-based immunoassay kit (Bio-Rad Laboratories, Inc., Tokyo, Japan). These mediators included eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1)/monocyte chemotactic and activating factor (MCAF), macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation, normal T cell expressed and secreted (RANTES), and tumor necrosis factor (TNF)-α. Data from the measurements were acquired and analyzed using the Bio-Plex suspension array system (Luminex 100 system; Bio-Rad Laboratories, Inc.), and the concentrations of each cytokine were determined using Bio-Plex Manager Version 4.1 software [21]. Data are expressed in terms of picogram of cytokine per milliliter of serum (*p*g/m*l*).

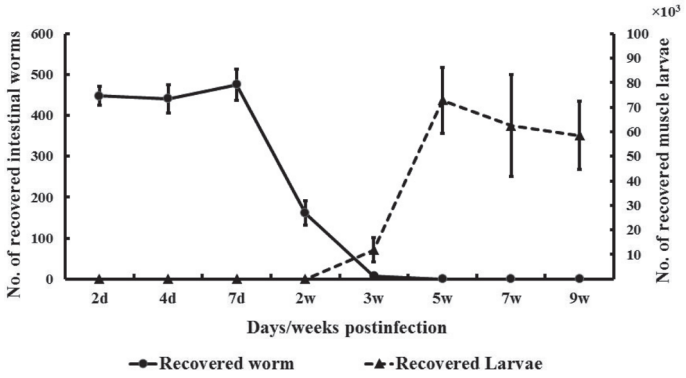


**Fig. 1.** Number of recovered intestinal-phase worms and muscle-stage larvae in male mice after infection with 600 first-stage larvae of *T. spiralis*. Bars represent the means ± SEM (*n*=5).

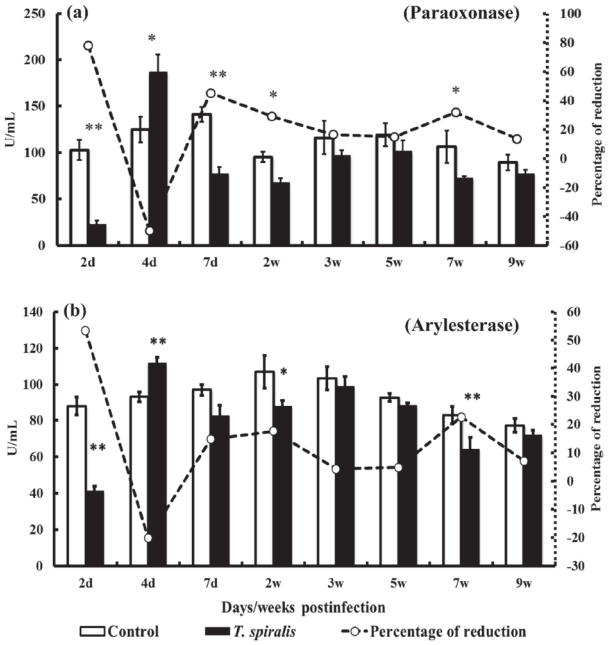


**Fig. 2.** Effect of *T. spiralis* infection on the serum PON1 (paraoxonase (a) and arylesterase (b)) activity in male mice on days 2, 4, and 7 as well as weeks 2, 3, 5, 7, and 9 p.i. **P*<0.05 and ***P*<0.01 when compared with control values. Bars represent means ± SEM (*n*=5).

### *Histopathological analysis*

The right lobes of the liver were collected from the control and *T. spiralis*-infected mice and fixed in neutral buffered formalin (pH 7.2; Nacalai Tesque, Inc.). Sections from each specimen were stained with hematoxylin and eosin (HE) to examine the liver tissues for inflammatory cell infiltration (lymphocyte, neutrophil, and eosinophil infiltration), histopathological changes in the liver cells, as well as amyloid deposits.

### *Experimental design*

Eighty male C57BL/6J mice were randomly allocated into two groups: a control group and a *T. spiralis*-infected group (n=40 each). Blood for serum samples was collected under terminal anesthesia from the abdominal vein of five animals from each group on days 2 and 4, and weeks 1, 2, 3, 5, 7, and 9 p.i.

### *Statistics*

Statistical analysis was performed using the statistical software package SPSS for Windows (Version 16.0; SPSS Inc., Chicago, IL, U.S.A.). The significance of differences between groups was evaluated by nonparametric tests (Mann-Whitney *U* test). Results are expressed as the mean ± standard error of the mean (SEM). A *P* value <0.05 was considered to be significant.

## RESULTS

### *Monitoring of T. spiralis infection at the intestinal and muscle stages*

Infection with *T. spiralis* larvae was monitored as shown in Fig. 1. After oral infection of male C57BL/6J mice with 600 first-stage larvae of *T. spiralis*, the worm number at the intestinal stage was high from day 2 until day 7 p.i.; subsequently, the number gradually declined through weeks 2 and 3 p.i., and was zero by week 5 p.i. Infection at the muscle stage was detectable by the digestion method in body musculature starting from week 2 p.i., and the number of larvae gradually increased through week 3, reaching a plateau from weeks 5 to 9 p.i.

### *Serum enzyme activities*

Infection with *T. spiralis* induced a statistically significant reduction in the paraoxonase activity of PON1, which was measured with paraoxon as a substrate, on days 2 and 7 p.i. as well as weeks 2 and 7 p.i. when compared to the control group (Fig. 2a). Relative to the control group, *T. spiralis*-infected mice showed a statistically significant decrease in the arylesterase activity of PON1, which was measured with phenyl acetate as a substrate, on days 2 as well as weeks 2 and 7 p.i. (Fig. 2b). Interestingly, there were statistically significant increases in both paraoxonase and arylesterase activities on day 4 p.i.

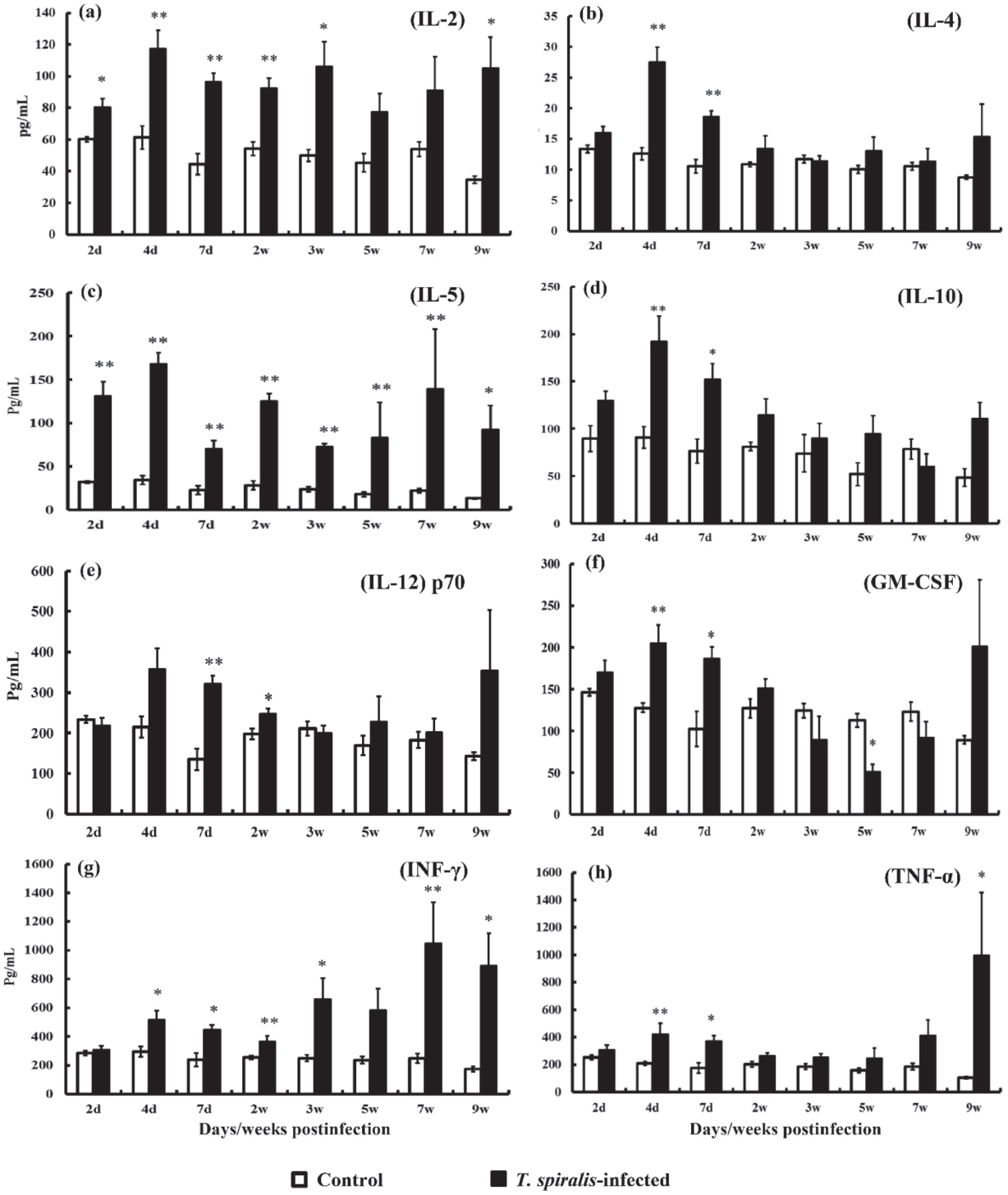


**Fig. 3.** Serum concentrations of IL-2 (a), IL-4 (b), IL-5 (c), IL-10 (d), IL-12 (p70) (e), GM-CSF (f), INF-γ (g), and TNF-α (h) in response to infection with 600 first-stage larvae of *T. spiralis* in male mice on days 2, 4, and 7 as well as weeks 2, 3, 5, 7, and 9 p.i. **P*<0.05 and ***P*<0.01 when compared with control values. Bars represent means ± SEM (*n*=5).

*Changes in serum cytokine levels among mice infected with T. spiralis*

The serum levels of eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, KC, MCP-1, MCAF, MIP-1α, MIP-1β, RANTES, and TNF-α of the control and *T. spiralis*-infected mice were examined on days 2, 4, and 7 p.i. as well as weeks 2, 3, 5, 7, and 9 p.i. (Fig. 3). Eotaxin, G-CSF, IL-1α, IL-1β, IL-3, IL-6, IL-9, IL-12 (p40), IL-13, IL-17A, KC, MCP-1, MCAF, MIP-1α, MIP-1β, and RANTES were undetectable in both the control and *T. spiralis*-infected groups throughout the experimental period. The level of IL-2 (Fig. 3a) showed statistically significant

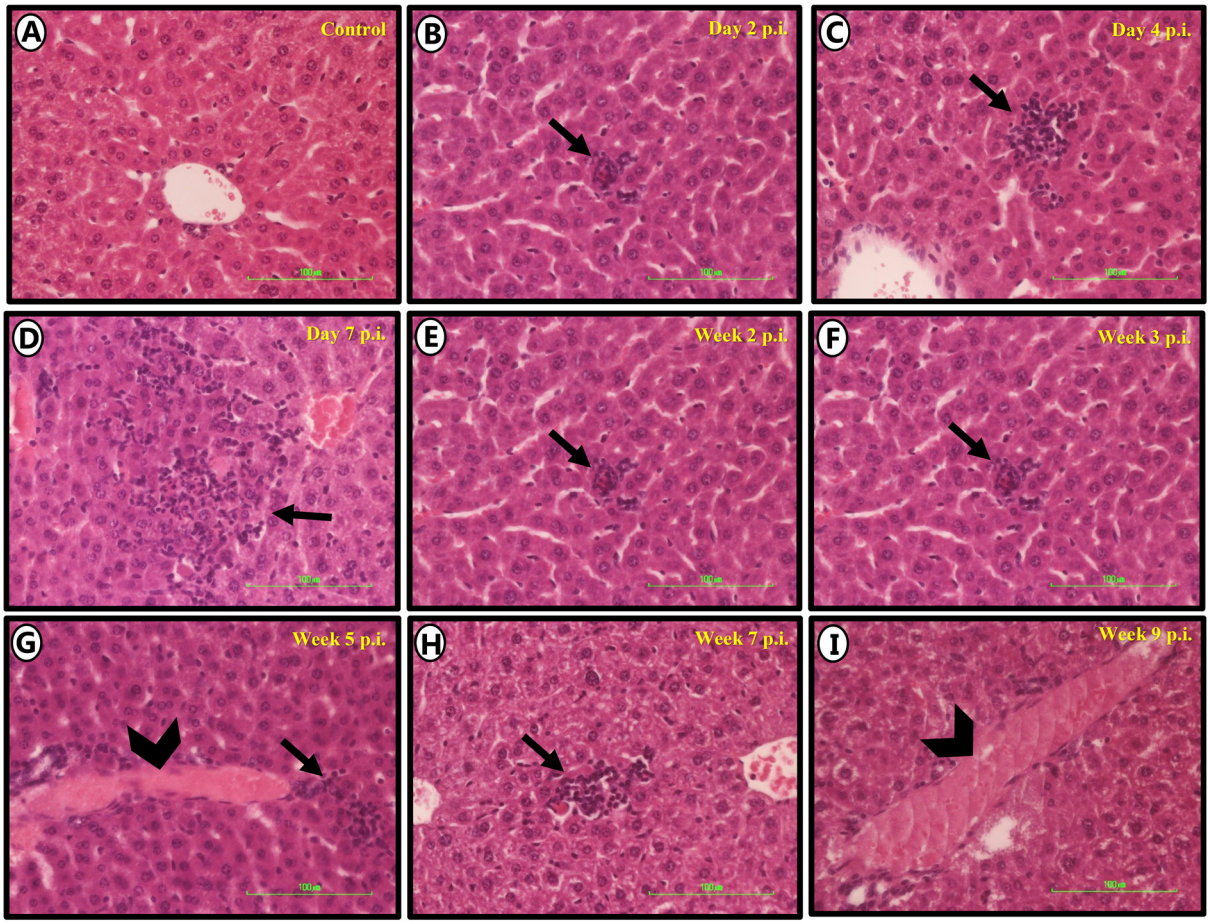


**Fig. 4.** Histopathological examination of mouse liver sections on different days following infection with 600 first-stage larvae of *T. spiralis*. The liver sections of mouse tissue were deparaffinized, stained with HE, and examined under light microscopy. Bold arrows indicate inflammatory cell accumulation (lymphocytes, neutrophils, and eosinophils) on days 2 (B), 4 (C), and 7 (D) as well as weeks 2 (E), 3 (F), 5 (G), 7 (H), and 9 (I) p.i. Arrowheads indicate sections with parasites at the muscle stage. (A) Control liver section with no abnormalities. Scale bars, 100 $\mu$m.

increases significantly increased on days 2, 4, and 7 p.i. as well as weeks 2, 3, and 9 p.i. Statistically significant increases in the level of IL-4 (Fig. 3b) was detected on days 4 and 7 p.i., while the level of IL-5 (Fig. 3c) was significantly increased throughout the experimental period, including on days 2, 4, and 7 p.i. as well as weeks 2, 3, 5, 7, and 9 p.i. Moreover, we reported statistically significant increases in the level of IL-10 (Fig. 3d) on days 4 and 7 p.i. The level of IL-12 (p70; Fig. 3e) was significantly increased on day 7 p.i. and week 2 p.i. Additionally, the level of GM-CSF (Fig. 3f) was significantly increased on days 4 and 7 p.i. Also, a statistically significant increase in the level of INF-γ (Fig. 3g) was detected on days 4 and 7 p.i. as well as weeks 2, 3, 7, and 9 p.i. The level of TNF-α (Fig. 3h) was significantly increased on days 4 and 7 p.i. as well as week 9 p.i.

### *Histopathological examination of the hepatic tissues*

We examined the HE-stained liver sections to identify correlations between the changes in hepatic functions as a result of the reduced serum PON1 activity and the hepatic innate immune response against *T. spiralis* infection. The examined sections showed little inflammatory cell infiltration (lymphocyte, neutrophil and eosinophils), especially near the portal vein and in the Glisson's sheath (Fig. 4).

## DISCUSSION

Parasitic infections induce a variety of reactions to circumvent the immune response. *T. spiralis* is a natural parasite of rodents, providing controlled life cycle for the investigation of these mechanism [23]. In this study, we found that *T. spiralis* infection in mice, unlike in rats, significantly decreased serum PON1 activity in an inconsistent pattern and for limited periods of time (Fig. 2). The observed reduction in serum PON1 paraoxonase activity occurred on days 2 and 7 p.i. as well as weeks 2 and 7 p.i., whereas that for serum PON1 arylesterase activity occurred on day 2 p.i. as well as weeks 2 and 7 p.i.; subsequently, the levels of both paraoxonase and arylesterase activity unexpectedly increased significantly on day 4 p.i., coinciding with the maximum serum levels of IL-2, IL-4, IL-10, and GM-CSF. Regarding the correlation between serum enzyme activity and the mouse immune response

against *T. spiralis* infection, we reported a detectable increase in the levels of IL-2, IL-4, IL-5, IL-10, IL-12 (p70), GM-CSF, INF-γ, and TNF-α in the serum of mice infected with *T. spiralis*. However, the peak serum level of each of these cytokines was reached at different periods of the infection: the levels of the cytokines were markedly high during the enteric phase of parasite infection, especially IL-4, IL-10, IL-12 (p70), GM-CSF, and TNF-α, and the levels of IL-2, IL-5, and INF-γ remained high throughout almost the entire experimental period (Fig. 3).

It is known that chronic helminth infections are often associated with Th2-polarized responses predominantly involving IL-4 and IL-10 [34]. IL-4 is involved in tissue repair and remodeling, the control of Th1 responses, and worm expulsion via the alternative activation of macrophages [32]. Thus, the increased serum levels of IL-4 during *T. spiralis* infection might have anti-inflammatory effects against hepatic inflammation and can explain the reduced recruitment of inflammatory cells to hepatic tissue that is associated with the infection (Fig. 4). IL-10, also known as cytokine synthesis inhibitory factor (CSIF), is a cross-regulation factor of Thl and Th2 responses; it is produced by the Th2 subset of cells [37]. In this report, we found that the serum level of IL-4 in mice infected with *T. spiralis* was very low, while that of IL-10 was high. Our data are in accordance with the concept proposed by Bogdan and Nathan [7] that IL-4 strongly causes a decrease in IFN-γ synthesis, since the addition of anti-IL-4 monoclonal antibody causes an increase in IFN-γ synthesis to levels higher than those of the controls. We concluded that in *T. spiralis* infection, the level of IFN-γ is high and long-lasting because the level of IL-4 is very low. Notably, IL-10 has a pivotal role in regulating liver inflammation during *T. spiralis* infection in mice, as evidenced by hepatic necrosis in IL-10 knock-out mice [6]. Upon infection with *T. spiralis*, mice readily mount an innate and adaptive IL-10 response to limit tissue injury and to reduce immune-mediated inflammation caused by migrating new born larvae; this response coincidentally facilitates the intracellular infection by *T. spiralis* [22]. Both IL-4 and IL-10 may act as anti-inflammatory mediators against necroinflammatory lesions in liver tissue [12].

In our study, we found significant elevations in the serum levels of IL-5 during *T. spiralis* infection throughout the experimental period. In contrast, intestinal expression of Th2-associated cytokine IL-5 is one of the first events that occurs during nematode or helminth infection, and high levels of IL-5 protein are produced by cells isolated from the mesenteric lymph nodes within a few days after *T. spiralis* infection [19]. IL-5 is thought to be particularly important for IgA synthesis, and for the proliferation, growth, activation, and survival of eosinophils *in vivo* [29, 45].

The activation of macrophages during *T. spiralis* infection may lead not only to the production of TNF-α but also to the generation of IFN-γ, which has been reported to be effective for killing some parasites, such as *Leishmania* [35]. Moreover, IFN-γ produced by T cells activates macrophages to secrete TNF-α, which in turn cooperates with IL-12 to sensitize the same or other macrophages to secrete even more IFN-γ and reactive oxygen species that rapidly destroy parasites through a process of lipid peroxidation [4]. In addition, TNF-α, in combination with IFN-γ, activates murine macrophages for the elimination of parasitic infections. As mentioned above, our data are in accordance with the concept proposed by Bogdan and Nathan [7] that IL-4 strongly causes a decrease in IFN-γ synthesis [17]. We conclude that in our *T. spiralis* infection model, the level of IFN-γ was high because the level of IL-4 was low.

Based on the knowledge that DCs and activated Kupffer macrophages are the main cell populations producing IL-12 early after infection, and that T cells associated with such DCs are likely to produce the IL-2 necessary for NK cell activation following some parasitic infections [28, 38, 43], in many systems, the early production of IL-12 from phagocytic cells results in the development of a Th1-polarized response [16]. Moreover, the administration of IL-12 to mice infected with *Nippostrongylus brasiliensis* inhibited the Th2-dependent responses and altered the cytokine profile in mesenteric lymph node cells from one dominated by IL-4, IL-5, and IL-9 to one dominated by IFN-γ [24, 44]; this suggests that in our study, NK cells participated in the early development of the Thl response after *T. spiralis* infection by producing cytokines, such as IFN-γ, TNF-α, and GM-CSF [43]. In turn, IFN-γ maximizes the parasiticidal capacity of Kupffer cells [40]. This suggests that IL-12 acts as a hepatoprotective agent with anti-fibrotic effects likely via the activation of signal transducer and activator of transcription 4 (STAT4) in immune cells as well as the activation of natural killer (NK) and natural killer T (NKT) [31, 51]. Interestingly, our results with regard to IL-10 and IL-12 were not consistent with the results of previous studies reporting that the elevated levels of IL-10 suppressed the production of IL-12 (p70) [26, 42].

GM-CSF is a cytokine expressed by activated T cells and macrophages that is required for the Th1 and Th2 cytokine responses [8]; as such, the increased level of TNF-α in the serum of the infected mice was consistent with the results of Cohn *et al.* [9] who proposed that TNF-α increases Th2 cell transendothelial migration, thereby potentiating mucosal Th2 responses during enteric helminth infection [14].

Interestingly, we found in our previous work that male Wistar rats infected with *T. spiralis* mounted an immune response that was associated with hepatic inflammation and a subsequent decrease of serum PON1 activity [13]. However, in our current study with *T. spiralis* infection, the absence of liver inflammation (Fig. 4) and the inconsistent changes in serum PON1 activity support the notion that the defense against *Trichinella* is a parasite–host interaction, which differs from the host–*Trichinella* models and is not necessarily comparable [50]. It is worth mentioning that in our previous study [13], we measured transcriptional alterations of some immune mediators that were associated with hepatic inflammation in a rat model, while in the current study we evaluated the serum concentrations of immune mediators to reveal the hepatoprotective mechanism in a standpoint of immune responses of a mouse model. Notably, the correlation between the transcriptional and protein expression profiles could be different since there are many processes between transcription and translation as well as protein stability [46]. Therefore, a comprehensive account of liver-specific transcriptional and circulating cytokine/chemokine dynamics in *T. spiralis*-infected models should be considered in further studies. In addition to this, using KO mice targeting a series of genes related to immune regulations may be helpful to understand what mechanism (s) alter liver injury during *T. spiralis*-infection.

We conclude from the current study that the initial predominance of IFN-γ during *T. spiralis* infection in mice may result in

the preferential expansion of Th1-like cells and the simultaneous inhibition of Th2-like cells [29]. Future investigations aimed at determining the nature of the protective immune response in the liver itself and its relation to other immune organs during *T. spiralis* infection in mice are warranted.

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