



Effects of TLR agonists on immune responses in *Trichinella spiralis* infected mice

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Abstract

Human trichinellosis is acquired by eating raw or undercooked meats carrying muscle larvae of *Trichinella* spp. Toll-like receptors (TLRs) are essential components of the innate immune system. However, little is known about the potential application of TLR agonists for immunotherapy against *Trichinella spiralis* (*T. spiralis*) infection. Here, we evaluated the effects of four TLR agonists (i.e., TLR3, TLR4, TLR8, and TLR9 agonists) on *T. spiralis* infection in mice. The reduction rate of worm burden showed that TLR3 agonist poly(I:C) significantly reduced *T. spiralis* infection rather than TLR4, TLR8, and TLR9 agonists ($p < 0.05$). Moreover, TLR3 showed a continuous high-level of expression during 6–35 days post infection (dpi). The levels of interferon-gamma (IFN- γ), interleukin (IL)-2, and IL-6 increased significantly in mice serum compared with control group after treatment with TLR3 agonist at 0, 3, 6, 9, 12, 15, 18, 21, 28, and 35 dpi ($p < 0.05$). A significant decreasing trend was also detected in levels of IL-10 and IL-4 after treatment with TLR3 agonist compared with control group at 0, 3, 6, 9, 12, 15, 18, 21, 28, and 35 dpi ($p < 0.05$). Overall, this study suggested that TLR3-targeted therapies might be effective on worm burden reduction by regulation of the cytokine levels in the mice infected with *T. spiralis*.

Keywords *Trichinella spiralis* · Toll-like receptor · TLR agonist · Cytokine

Introduction

Human trichinellosis has been listed as a neglected tropical disease, reported in 55 countries worldwide, and seriously affected the public health, slaughtering industries as well as the import and export trade (Pozio 2007; Shimoni and Froom 2015; Wang et al. 2012). The severity of human trichinellosis can range from subclinical to fatal (Gomez-Morales et al. 2018). Indeed, the early diagnosis and treatment of trichinellosis are of great importance in reducing major public health risk.

The genus *Trichinella* is a unique nematode that the adult worms and the larvae parasitize in the same host (Ashour 2013). *Trichinella spiralis* (*T. spiralis*) infection begins when larvae are released from cysts and invade the small intestine, and then the larvae mature into adult worms. After copulation, the female adult worms release newborn larvae which travel through the circulatory system to skeletal muscle cells where they encyst and develop into muscle larvae (Lee and Best 1983). Toll-like receptors (TLRs) are widely expressed in various immune cells, including dendritic cells, macrophages, B cells, specific types of T cells, and even in non-immune cells, such as epithelial, endothelial, and fibroblast cells (Lester and Li 2014; Manicassamy and Pulendran 2009; Sun et al. 2011; Zakeri et al. 2016). TLR protective immunity against some parasitic helminths conferred by T helper type 1 or 2 (Th1 or Th2, respectively) cytokine responses (Perrigou et al. 2008). During the intestinal phase, the immune response is mixed, and the Th1 response characterized by increased interferon gamma (IFN- γ), interleukin(IL)-2, and IL-12 was predominant at the initial stage, and the Th2 response characterized by increased IL-4, IL-5, IL-9, IL-10, and IL-13 was elevated subsequently (Aranzamendi et al. 2012; Ding et al. 2017; Ishikawa et al. 1998). The expulsion of *T. spiralis* is

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dependent upon the polarized Th2 immune response regulated by TLRs (Nutman 2015). In fact, downstream TLRs negatively correlated with the development of Th2 cytokine responses of parasitic helminths (Perrigou et al. 2008). In mouse embryonic fibroblast cells, TLR4 and TLR9 expression was significantly increased and TLR3 expression significantly decreased after treatment with ES protein of *T. spiralis* (Kim et al. 2015). Therefore, a dual effect on TLRs could be exerted by activation or negative regulation of these receptors by *T. spiralis* (Ilic et al. 2012). The study of TLRs should shed more light on the role played by TLRs dysregulation and provide new application studies for therapeutic strategy and vaccine development (Venugopal et al. 2009). TLR immunotherapy can prevent antigen-specific and independent generalized immunosuppression in a susceptible population (Dasgupta et al. 2014). Further studies are needed for the screening of potential TLR agonists for immunotherapy against *T. spiralis* infection.

Material and methods

Animals and parasites

T. spiralis strains were identified by OIE Collaborating Center on Foodborne Parasites in Asian-Pacific Region in August 2014 and maintained in Institute of Zoonoses, Jilin University. Female C57BL/6 mice, 6–8 weeks old, were purchased from Jilin University experimental animal center (Jilin, China), with the certificate No. 2016–0001 in conformity of SCXK (Jilin). The muscle larvae of *T. spiralis* were recovered from infected mice by artificial digestion method as previously described (Friend et al. 1996).

Infection of *T. spiralis* and determination of parasite burden

All mice were infected with 300 muscle larvae per os. Experimental mice infected with *T. spiralis* were given the reported TLR3 agonist poly(I:C) (InvivoGen) 7.5 mg/kg, TLR4 agonist LPS (InvivoGen) 2 mg/kg, TLR8 agonist TL8-506 (InvivoGen) 5 mg/kg, and TLR9 agonist ONDM362 (InvivoGen) 5 mg/kg by tail vein injection at 0, 2, 5, 8, 11, 14, 17, 20, and 27, 34 days post infection (dpi). Meanwhile, the control group of mice was only injected with PBS. Parasite burden was calculated as the total number of larvae per gram (LPG) of muscle tissue at 35 dpi. The reduction percentage of LPG in the treated animals was calculated as follows: $\text{LPG reduction rate} = (\text{control group mean LPG} - \text{treated group mean LPG}) / \text{control group mean LPG} \times 100\%$ (Garcia et al. 2013; Gurish et al. 2004)

Experimental infection and treatment with poly(I:C)

Two groups of 10 mice were infected with 300 muscle larvae of *T. spiralis* (T1, ISS534) per os. The experimental mice infected with *T. spiralis* were given poly(I:C) 300 µg per mouse by tail vein injection at 0, 2, 5, 8, 11, 14, 17, 20, 27, and 34 dpi. The serum was collected from each mouse at 0, 3, 6, 9, 12, 15, 18, 21, 28, and 35 dpi (Bruschi et al. 2014).

Real-time PCR

Total RNA was extracted from splenocyte with Trizol reagent (Invitrogen) at 0, 3, 6, 9, 12, 15, 18, 21, 28, and 35 dpi. In total, 1 µg RNA was reverse transcribed to cDNA using RNA simple total RNA Kit according to the manufacturer's instructions (Tiangen Biochemical Technology). Real-time PCR experiments were carried out in triplicate with SYBR premix Ex Taq™ II (Promega) on an ABI 7500 Fast Real-Time PCR System (ABI). The PCR primers were designed by the Primer-BLAST tool. TLR3 primers: forward primer 5'-caaacccccgggtggtcccggtt-3' and reverse primer 5'-aaggcggcccgaaaacatcct-3'. For normalization of target gene expression, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5'-atgacatcaagaagggtggaag-3' and reverse primer 5'-tccttgaggccatgtagg-3' was taken for the calculation of a reference gene. Relative mRNA expression was analyzed by using the Applied Biosystems 7500 Software and calculated by the comparative Ct method (Schmittgen and Livak 2008).

ELISA

Serum samples were collected from the mice in control and treated group at 0, 3, 6, 9, 12, 15, 18, 21, 28, 35 dpi. Serum cytokine was assayed for levels of IFN-γ, IL-2, IL-4, IL-6, and IL-10 using ELISA Kit (eBioscience) (Mansson Kvarnhammar et al. 2013).

Statistics

The results are expressed as the mean ± standard error, SE. Comparisons between control and treated groups were performed with paired-samples *t* tests by SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant at a 5% significance level ($p < 0.05$).

Results

Effects of TLR agonists on parasite burden

Parasite burdens of muscle larvae were compared among the groups which were given with poly(I:C), LPS, TL8-506, ONDM362, respectively, and control group. The maximum

reduction rate of parasite burden was observed in poly(I:C) treatment group ($p < 0.05$) (Table 1).

Expression of TLR3 in splenocyte

The statistical analysis of TLR3 expression in spleen between control group and poly(I:C) treatment group during 0–3 dpi showed no significant differences. Compared to control group, TLR3 exhibited a continuous high-level expression in poly(I:C) treatment group during 6–35 dpi (Fig. 1).

Cytokine production stimulated by poly(I:C) treatment

Th1 cytokines (i.e., IFN- γ , IL-2) and Th2 cytokine IL-6 in mice serum significantly increase after poly(I:C) treatments at 0, 3, 6, 9, 12, 15, 18, 21, 28, and 35 dpi compared with control group ($p < 0.05$; Fig. 2 a, b, and c). Significant decreases in levels of Th2 cytokines (i.e., IL-4 and IL-10) were detected in control and treatment group at 0, 3, 6, 9, 12, 15, 18, 21, 28, and 35 dpi (Fig. 2d, e; $p < 0.05$).

Discussion

TLRs represent one of the most studied pattern recognition receptor (PRR) families (Harris et al. 2006; Tartey and Takeuchi 2017). Qu demonstrated that TLR3 is the primary molecule which modulates the activation and function of NK cells during *Schistosoma japonicum* (*S. japonicum*) infection in mice (Qu et al. 2018). The expression levels of TLR3 on different pulmonary lymphocytes were increased after *S. japonicum* infection (Chen et al. 2019). *T. spiralis* can also modulate the immune response by regulating the expression of TLRs and their signaling pathways (Yu et al. 2013). Here, we analyzed the effects of TLR agonist on mice infected with *T. spiralis*. After treatment, the TLR3 agonist poly(I:C) was considered as the best candidate among the four kinds of TLR agonists. Our study provided evidence that the TLR3 agonist was able to interfere with the parasitism of *T. spiralis*. According to our results, experimental mice infected with *T. spiralis* after poly(I:C) treatment showed a consistently

high-level expression of TLR3 comparing to that in control group during 6–35 dpi. These results indicated that TLR3 agonists could activate TLR3 at different stages of *T. spiralis* infection and suggested that TLR3 agonists might affect the infection of *T. spiralis*. The activation of TLRs promotes both innate immunity responses and the induction of adaptive immunity (Cui et al. 2014). As Toll-like receptor expression level changes during *T. spiralis* infection, Th1 and Th2 responses could be regulated and induce T lymphocyte proliferation and cytokine production which also affected the function of antigen-presenting cells (Brown et al. 2014; Mansson Kvamhammar et al. 2013). In the previous study, it is indicated that *T. spiralis* can induce a complex Th1/Th2 response with predominant polarization to Th2 during intestinal and muscle phase (Ding et al. 2017). As reported in the “Material and methods” section, the expression levels of IFN- γ , IL-2, IL-4, IL-6, and IL-10 were detected by ELISA. We observed that *T. spiralis* could regulate the cytokine expression of the host at different stages. The proinflammatory cytokines (i.e., Th1 type cytokines IFN- γ , IL-2, and Th2 type cytokine IL-6) in mice significantly increased after poly(I:C) treatment compared with control group (Fig. 2 a, b, and c). Meanwhile, significant decreases of the anti-inflammatory cytokines (i.e. Th2 cytokines IL-4 and IL-10) were observed in experimental group (Fig. 2d, e; $p < 0.05$). Therefore, the reduction of the parasite burden may be related to these proinflammatory responses caused by Th1 cytokine increase. Indeed, many preclinical and clinical studies have demonstrated that the use of purified TLR agonists or TLR ligands as adjuvants represents a considerable potential TLR-targeted therapy against a variety of inflammatory diseases and autoimmune conditions (Kamdar et al. 2013; Majewska and Szczepanik 2006). It is clear that TLR agonists can be useful for several purposes, including signal therapy to accelerate and enhance the induction of vaccine-specific responses (Dowling and Mansell 2016). Above all, TLRs could be considered as a “Swiss Army” knife of the immune system with the capability of applying to the development of vaccines against trichinellosis. In this study, we evaluated the effects of four TLR agonists on *T. spiralis* infection in mice and found that TLR3

Table 1 Effects of TLR agonists on parasite burden of mice

Group	Dose (mg/kg)	Number of mice	LPG (mean \pm SD)	LPG reduction (%)
Control	7.5	10	1076.2 \pm 28.00	–
TLR3	7.5	10	614.0 \pm 24.20	42.94*
TLR4	2	10	852.1 \pm 26.04	20.82
TLR8	5	10	765.1 \pm 24.98	28.90
TLR9	5	10	944.1 \pm 24.03	12.27

* $p < 0.05$; vs the control group

Fig. 1 Expression of TLR3 in the spleen of mice infected with *Trichinella spiralis*. The histogram represents TLR3 mRNA expression level in splenocyte of mice infected with *T. spiralis*. Results are expressed as mean \pm SE. Control and TLR3 group mice were treated with PBS and poly(I:C), respectively

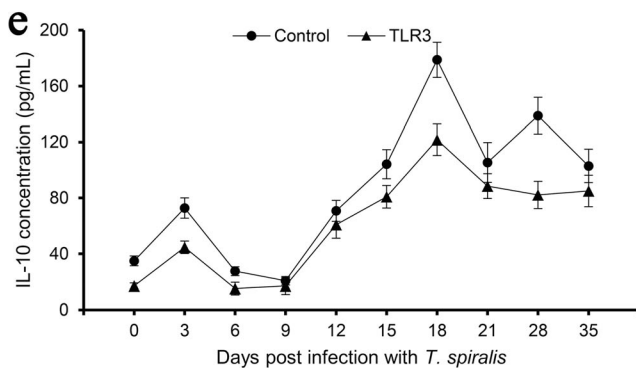
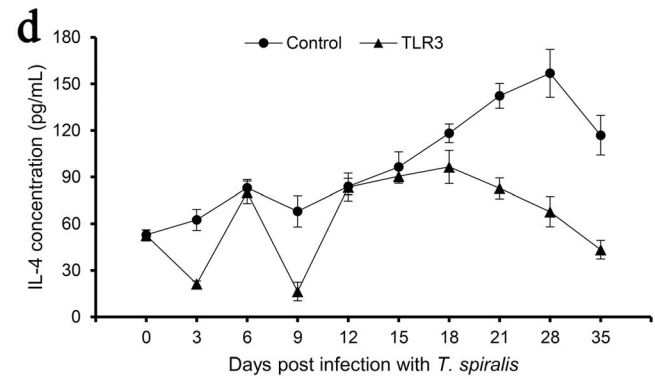
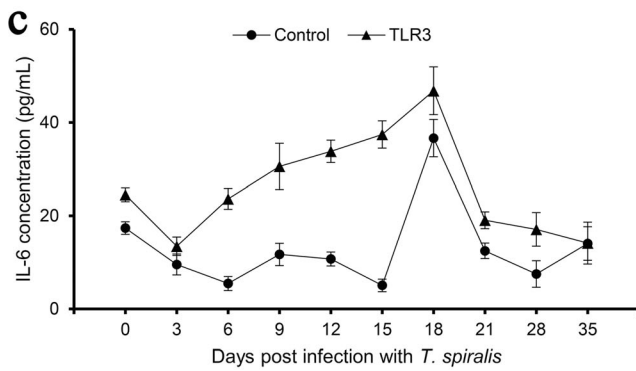
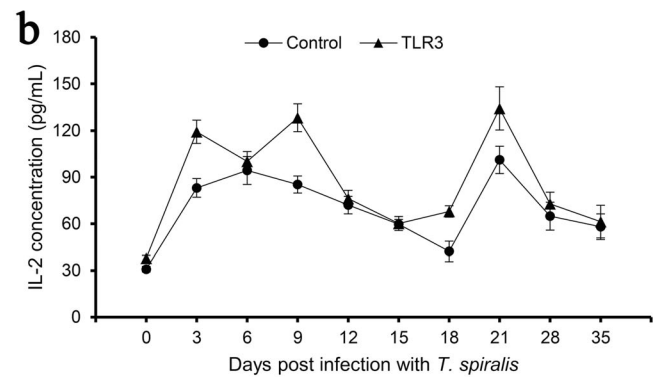
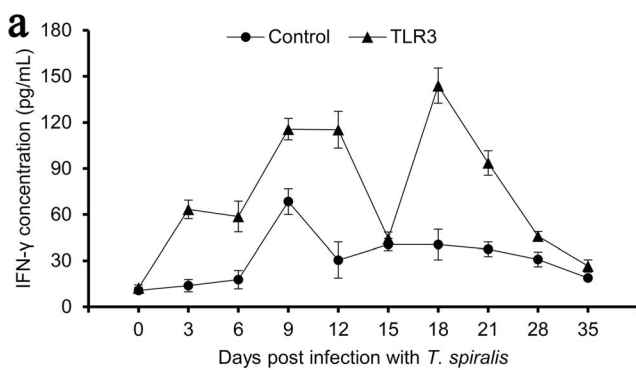
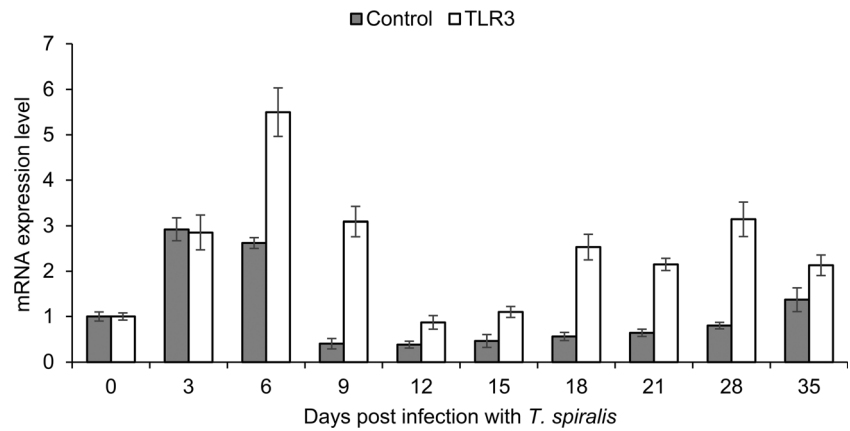


Fig. 2 The level of cytokine production in serum after stimulation with poly(I:C). The serum samples were collected from mice infected with *T. spiralis* after poly(I:C) and PBS treatment ($n = 10$). The production

levels of IFN- γ (a), IL-2 (b), IL-6 (c), IL-4 (d), and IL-10 (e) were detected at 0, 3, 6, 9, 12, 15, 18, 21, 28, and 35 days post infection.

agonist administration could reduce the parasite burden significantly, and the levels of the proinflammatory cytokines (IFN- γ , IL-2, and IL-6) increase and the levels of anti-proinflammatory cytokines (IL-4 and IL-10) decrease after TLR3 agonist treatment.

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Author contribution Bin Tang and Xue Bai performed parasite burden determination. Xiaolei Liu performed ELISA; Yang Wang performed real-time PCR; Jing Ding performed *T. spiralis* infection and treatment with poly(I:C), and Mingyuan Liu analyzed the data. Xuelin Wang conceived of or designed the study. Xuelin Wang and Bin Tang wrote the paper. All the authors have read and approved the final version of the manuscript.

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Compliance with ethical standards All the animal husbandry and experimental procedures were performed in accordance with the Chinese Animal Management Ordinance (People's Republic of China Ministry of Health Document No. 55 in 2001). All the experimental procedures were reviewed and approved by the ethical committee in Jilin University for the care and use of laboratory animals.

Conflict of interest The author declares that there is no conflict of interest.

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