

***Trichinella spiralis*: impact on the expression of Toll-like receptor 4 (TLR4) gene during the intestinal phase of experimental trichinellosis**

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Abstract

Introduction: Toll-like receptors (TLRs) play a key role in the rapid activation of the innate immune response to a variety of pathogens. The aim of this study was to evaluate the effect of *Trichinella spiralis* infection on the level of expression of the *tlr4* gene in mouse intestines during the intestinal phase of experimental trichinellosis. **Material and Methods:** The experimental material consisted of the small and large intestines of BALB/c mice infected with *Trichinella spiralis* sampled at 4, 8, and 16 days post infection (dpi). **Results:** A statistically significant increase was demonstrated in the *tlr4* mRNA level isolated from the infected mice jejunum at 4, 8, and 16 dpi over the uninfected control. Moreover, at 4, 8, and 16 dpi in the jejunum of infected mice, a strong positive reaction for the presence of TLR4 protein compared with that of uninfected mice was observed. **Conclusion:** Infection with *T. spiralis* changes the expression of the *tlr4* gene in the small intestine of the mouse host.

Keywords: mouse, intestines, trichinellosis, toll-like receptor 4, q-PCR, immunohistochemical staining.

Introduction

Trichinellosis is still one of the important parasitic diseases in Poland and some other countries. This zoonosis is predominantly caused by *Trichinella spiralis* larvae which are present in the host's muscle tissue. In its adult stage this nematode inhabits the epithelial layer of the small intestine of the host where it induces an immune-mediated inflammatory response reflected in intestinal pathology (1, 2, 6, 8). It is well known that mice infected with *T. spiralis* develop enteropathy during the early phase of infection, comprising villus atrophy, crypt hyperplasia, goblet and Paneth cell hyperplasia, and infiltration of the mucosa by a variety of inflammatory cells. The goal of this study was the evaluation of expression level of TLR4 during the

intestinal stage of *T. spiralis*. For this reason investigation of the small intestine was necessary. Different parts of the intestines were examined and results were compared to non-infected tissue such as the large intestine.

Toll-like receptors (TLRs) are a family of protein receptors responsible for recognition of pathogens by detecting different pathogen-associated molecular patterns (PAMPs). It was demonstrated that TLRs participated in intestinal epithelial immune responses to a variety of invading pathogens including parasites (13). Relatively little is known about the role of TLRs in the host response to helminth parasites that usually induce the host Th2-response. In this context, we selected one of the best known and most frequently analysed members of the TLR family, TLR4. TLR4 is a transmembrane protein whose expression is found

on the cell surface. Since its discovery, TLR4 has gained much attention due to its high capacity for identifying a diverse array of pathogenic ligands: bacterial, viral, fungal, protozoan, and also those of helminth parasites (14). TLRs activate a signalling pathway *via* four various adaptor proteins. Two examples of this are TLR4 mediating nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) activation through myeloid differentiation factor 88 (MyD88) and dependent and independent mechanism interferon regulatory factor3 (IRF3) activation being mediated by a Toll receptor-associated activator of interferons (TRIF) (21). Different stages of *T. spiralis* had varying effects on the expression of TLRs (9). It is now known, that at the intestinal stage of *T. spiralis* infection, the mRNA expression levels of *tlr* 1, 2, 3, 4, and 9 are upregulated and those of *tlr* 5 and 6 are down regulated (9). Recently, Zhang *et al.* (23) presented that TLR2 and TLR4 play important roles in heat shock protein 70 (Ts-Hsp70)-induced protective immunity against *T. spiralis* infection. Also, helminth-derived molecules have been shown to be involved in TLR4 signalling in the case of *Brugia malayi* and *Onchocerca volvulus*, and it is suggested that the pathogenesis of filarial infections (at least in a murine model) seems to depend on TLR4 signalling (17, 15, 18). Moreover, Helmby and Grencis (3) provided evidence that TLR4 plays a pathogenic role in the development of chronic intestinal nematode infection (in a murine *Trichuris* model). Then, Kerepesi *et al.* (7) revealed that TLR4 was essential for adaptive protective immunity to larval *Strongyloides stercoralis* in mice and this receptor was required for killing this parasite during the adaptive immune response.

The aim of this study was to examine the influence of *T. spiralis* infection on the expression of the *tlr4* gene in mouse in experimental trichinellosis. We focused on the intestinal phase of trichinellosis, which is initially dominated by the Th1 type of response (4). TLR4 is involved in activation mainly of the early (innate) immune response during helminth infections, which coincides with activation of the Th1 type response. Our research contributes to better understanding the significant changes in TLR expression (at the mRNA level and protein level) during the intestinal stage of *T. spiralis* infection, an infection which threatens human health from sources not only in wild animals but also in domestic ones.

Material and Methods

Animals and *T. spiralis*. Male BALB/c mice, 8–10 weeks old with body weights of 20–25 g were used. The experimental material consisted of the small and large intestines of the mice infected with *Trichinella spiralis* strain ISS003 (400 larvae per

mouse) sampled at 4, 8, and 16 dpi. The methods of obtaining infective larvae and of euthanising animals were described previously (19). Each experimental group consisted of six animals.

Real-time PCR and immunohistochemical staining. The expression of the *tlr4* gene at the mRNA and protein level was examined using quantitative real-time PCR (q-PCR), immunohistochemical staining (IHC) using the Rabbit ABC staining system (Santa Cruz Biotechnology, USA), and specific primary polyclonal antibodies against TLR4 (Santa Cruz Biotechnology). Detection was performed as reported previously (20). The housekeeping gene *pbgd* (porphobilinogen deaminase) was amplified as the reference gene for mRNA quantification. Primers for the *tlr4* gene were forward 5'-TTC TTC TCC TGC CTG ACA CC-3' and reverse 5'-CTT TGC TGA GTT TCT GAT CCA T-3' (a 94 bp product), and for the *pbgd* gene were forward 5'-TGG ACC TAG TGA GTG TGT TG-3' and reverse 5'-GGT ACA GTT GCC CAT CTT TC 3' (a 138 bp product). The relative quantification of target gene expression was calculated based on the E-method algorithm. All results were normalised to expression of the reference housekeeping gene (*pbgd*) and compared to appropriate control experiments.

Statistical analysis. Data were analysed using Statistica 6.1 for Windows (StatSoft, now Tibco, USA). All variables were expressed as mean \pm standard deviation (SD). Differences between groups were analysed by the Mann-Whitney U test. A value of $P < 0.05$ was considered statistically significant.

Results

In the jejunum from *T. spiralis*-infected mice examined at 4, 8, and 16 dpi, the level of expression of the *tlr4* gene at the mRNA level revealed a statistically significant increase over that of uninfected animals (Table 1). This increase peaked on 8 dpi at 306% of the control value. However, in the colon removed from control and infected mice, changes were not significant (Table 1). Moreover, an increase in the expression of the *tlr4* gene at the protein level was observed in the jejunum exsected from *T. spiralis*-infected mice. TLR4 protein was localised by IHC. In the crypts of small intestines of infected mice a strong positive reaction (brown stain) indicating the presence of TLR4 protein (Figs 2 and 3) was found on 4 and 8 dpi. At the same time, no positive reaction for the presence of TLR4 protein was observed in the jejunum from uninfected mice, but only single positive cells were seen (Fig. 1).

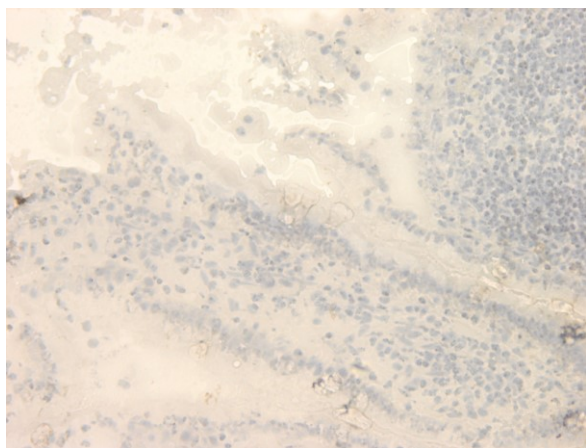


Fig. 1. No positive expression of TLR4 at protein level in a jejunum of an uninfected mouse (control group); TLR4 protein was visualised by IHC using primary antibodies against TLR4. Brown pigmentation indicates immunopositive cells with TLR4 protein

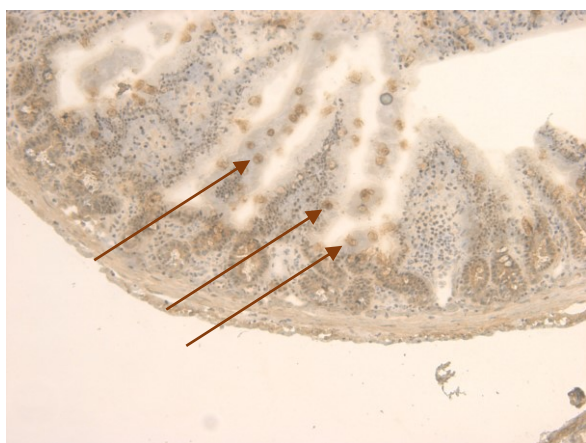


Fig. 2. Expression of the TLR4 at the protein level in a jejunum of a *Trichinella spiralis*-infected mouse on 4 dpi with a strong positive reaction for presence of TLR4 protein. Objective magnification 20 \times ; TLR4 protein localised by IHC using primary antibodies against TLR4. Brown pigmentation indicates immunopositive cells with TLR4 protein (arrows)

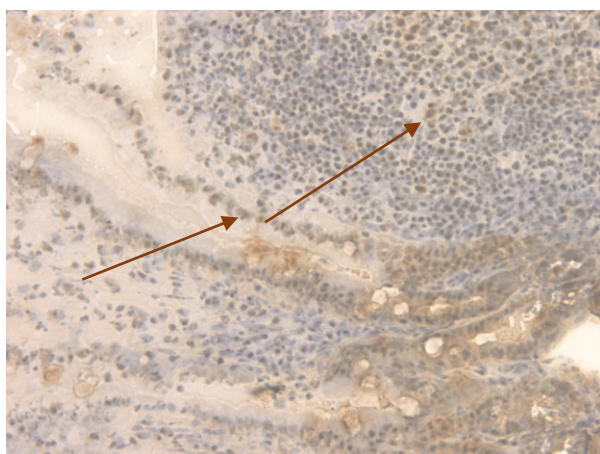


Fig. 3. Expression of the TLR4 at the protein level in a jejunum of a *Trichinella spiralis*-infected mouse at 8 dpi with a strong positive reaction for presence of TLR4 protein. Objective magnification 20 \times ; TLR4 protein localised by IHC using primary antibodies against TLR4. Brown pigmentation indicates immunopositive cells with TLR4 protein (arrows)

Table 1. Expression of the *tlr4* gene at the mRNA level in the jejunum and colon of uninfected and *Trichinella spiralis*-infected mice determined using q-PCR

Days post infection (dpi)	Relative mRNA expression of <i>tlr4</i> gene, determined by qPCR	
	Jejunum	Colon
0 (uninfected mice)	0.59 \pm 0.09	0.8 \pm 0.1
4	1.51 \pm 0.32*	0.85 \pm 0.07
8	1.81 \pm 0.12*	0.82 \pm 0.07
16	1.27 \pm 0.13*	0.82 \pm 0.06

Data represent mean \pm SD and are representative of 6 animal groups.

* $P < 0.05$, compared with the control value derived from uninfected mice (Mann–Whitney U test)

Discussion

Our results indicated a significantly increased level of the *tlr4* gene expression in the small intestine of *T. spiralis*-infected mice, which may suggest the contribution of this receptor to the host defence mechanisms during the intestinal phase of experimental trichinellosis. Demonstration of *tlr4* mRNA and TLR4 protein in the jejunum of infected mice confirms the mooted involvement of this TLR in the recognition of *Trichinella spiralis* PAMPs. Therefore, we may conclude that TLR4 participates in the activation of the early (innate) immune response against *T. spiralis*. Helminth infections usually stimulate the host Th2 cell response, but Ishikawa *et al.* (4) showed that during the intestinal phase of *T. spiralis* infection the immune response is mixed Th1/Th2, with initial predominance of the Th1 type of response and subsequent domination of the Th2 type. However, determination of the precise role of TLR4 in the control of trichinellosis requires further detailed investigation.

To date, a few studies have investigated the effect of *T. spiralis* infection on the level of expression of the *tlr4* gene in host cells. Kim *et al.* (9) concluded that *tlr4* gene expression level is upregulated in tissues of infected mice, but they examined gene expression only at the mRNA level and not at the protein level. This study and ours demonstrate the maximum increase in *tlr4* expression at a similar time after infection. On the other hand, difference also exists between these authors' and our results. Kim *et al.* (9) showed that the level of expression of the *tlr4* gene in the infected small intestine, examined at the end of the first week after infection, was lower than in the control (uninfected) intestine. This difference is probably caused by use of a different *T. spiralis* strain, a different mouse strain, and a lower dose of infective larvae (250 larvae per mouse).

Similarly, Yu *et al.* (22) confirmed that the mRNA expression of *tlr4* was upregulated in the early stage (intestinal phase) of *T. spiralis* infection. These authors also demonstrated that the level of expression of *tlr4* gene was statistically significantly higher at 3 days after infection with *T. spiralis* than in the uninfected control. The difference between these authors and our results was possibly caused by the use of a different *T. spiralis*

strains, a different mouse strain such as C57BL/6, and different tissues.

Recent studies by Zhang *et al.* (23) have shown that Ts-Hsp 70 activated dendritic cells through TLR2 and TLR4. Furthermore, the results of Ilic *et al.* (5) indicated that *T. spiralis* excretory-secretory antigens engage TLR2 and TLR4 and induce tolerogenic properties in human dendritic cells *via* these receptors.

Considering parasitic helminth infections, Mishra *et al.* (11, 12) showed that expression of some TLR genes (including TLR4 gene) was upregulated in the brain during murine neurocysticercosis caused by *Mesocostoides corti* larvae. Then Shan *et al.* (16) demonstrated a significant increase in the level of *tlr4* gene expression in peripheral blood mononuclear cells from patients with chronic cystic echinococcosis. These findings suggest the involvement of this receptor in the recognition of *Echinococcus granulosus* PAMPs, and that altered TLR4 expression might promote chronic cystic infection (*via* cytokine modulation). Kosik-Bogacka *et al.* (10) described a significant increase in the level of *tlr4* gene expression in intestinal epithelial cells isolated from a rat infected with *Hymenolepis diminuta* during the early stage of experimental hymenolepidosis.

In summary, determination of the exact role of TLRs in helminth infections requires further analysis. In conclusion, we can postulate that trichinellosis may be associated with changes in TLR expression in the host intestinal epithelium. It is evident that further studies are needed to specify the expression of other members of the TLRs during the intestinal phase of trichinellosis.

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