JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Expression of Toll-like receptor (TLR) 2 and TLR4 in the livers of mice infected by *Clonorchis sinensis*

Chao Yan*, Xiang-Yang Li*, Bo Li, Bei-Bei Zhang, Jiang-Tao Xu, Hui Hua, Qian Yu, Zhuan-Zhuan Liu, Lin-Lin Fu, Ren-Xian Tang, Kui-Yang Zheng

Department of Pathogenic Biology and Immunology, Laboratory of Infection and Immunity, Xuzhou Medical College, Xuzhou, Jiangsu Province, China

* These authors contributed equally to this work.

Abstract

Introduction: *Clonorchis sinensis* is one of the most important foodborne pathogens in humans, and can cause biliary diseases such as gallstones, cholecystitis, cholangitis, and cholangiocarcinoma. Toll-like receptors (TLRs) as sensors are crucial to initiating both innate and adaptive immune defenses against pathogens. However, little is known about the hepatic expression of TLRs of hosts induced by *C. sinensis* infection.

Methodology: In the present study, the expression and distribution of TLR2 and TLR4 were investigated in a mouse model of clonorchiasis on days 28, 56, 84, and 112 post-infection (PI) using real-time quantitative reverse transcription polymerase chain reaction (PCR) and immunohistochemically staining, respectively. The levels of cytokines that are mediated by TLR2 and TLR4 were also evaluated using a cytometric bead array.

Results: Results showed that the transcripts of TLR2 and TLR4 were upregulated on day 28 PI in *C. sinensis*-infected mice compared with non-infected ones ($p < 0.01$). In addition, their proteins were strongly immunohistochemically positive in the cytoplasm and membrane of endothelial cells, fibroblasts, and biliary epithelium cells of *C. sinensis*-infected mice. The levels of interleukin (IL)-4, IL-10, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) were increased with activation of TLR2 and TLR4.

Conclusions: The expression of TLR2 and TLR4 is upregulated against *C. sinensis* infection, which suggests that TLR2 and TLR4 might be involved in immune responses during *C. sinensis* infection.

Key words: *Clonorchis sinensis*; Toll-like receptor 2; Toll-like receptor 4; C3H/HeN mice.

J Infect Dev Ctries 2015; 9(10):1147-1155. doi:10.3855/jidc.6698

(Received 07 February 2015 – Accepted 08 June 2015)

Introduction

Clonorchiasis, caused by the liver fluke *Clonorchis sinensis*, is a foodborne parasitic zoonosis affecting approximately 15 million people in endemic Asia including China, Korea, Vietnam, and Japan [1]. It has been claimed that about 85% of these people are in China [1-3]. Such disease is characterized by cholangitis with remarkable recruiting inflammatory cells in the early stage of infection, followed by hyperplasia of the intrahepatic bile ducts, cholangiofibrosis, and cholelithiasis in the host liver during the chronic stage [4]. Moreover, as a group I carcinogen, *C. sinensis* can induce human cholangiocarcinoma in chronically infected people [5-7].

The family of Toll-like receptors (TLRs) is one of the most important pattern recognition receptors (PRRs) that plays a key role in recognition of pathogen-associated molecular patterns (PAMPs) derived from a variety of pathogens, as well as in mediating the innate immune response and adaptive immune response of the host [8-10]. TLRs are members of type I transmembrane proteins divided into three domains: ectodomains containing 18–31 leucine-rich repeats recognizing PAMPs derived from a wide range of viral, bacterial, and parasite ligands; transmembrane domains; and intracellular Toll-interleukin 1 receptor (TIR) domains that induce downstream signal transduction. Once PAMPs are recognized by leucine-rich domains, the signal cascades are initiated, and remarkable pro-inflammatory cytokines and chemokines, as well as costimulatory molecules, are ultimately induced through the activation of a variety of transcriptional complexes including NF-κB and AP-1 [11-15]. Additionally, host susceptibility to infectious diseases

may be associated with the TLRs' polymorphisms [16-18].

Like other helminthiases, clonorchiasis is characterized by the predominant Th2-associated cytokine production with a suppression of Th1-associated cytokines during chronic infection. However, the factors and molecular mechanisms underlying polarization of the immune response are largely unknown [19-21]. Recent studies have shown that the expression of TLR2 and TLR4 is upregulated in other helminth infections, such as *Fasciola* spp., *Schistosoma* spp., *Taenia* spp., and *Brugiamalayi*, suggesting that TLRs might play an important role during helminth infections [22-27]. For example, Gao *et al*. demonstrated that TLR2 but not TLR4 is critical to mediating interleukin (IL)-10 secretion from dendritic cells in response to *Schistosome japomicum* antigens [28]. The expression of TLR2 and TLR4 can be upregulated by excretory/secretory products derived from another liver fluke, *Opisthorchis viverrini*, suggesting that TLR2 and TLR4 might participate in immune response against *O. viverrini* [29,30]. However, the expression and distribution of TLR2 and TLR4, which are probably engaged in immune responses against *C. sinensis* infection, are not clear. In view of this background, the objective of present study was to identify such TLR expression and distribution in the livers of mice following infection with *C. sinensis*.

Methodology

Ethics statement

This study strictly followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. All animals were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China. The study was approved by the animal ethics committee of Xuzhou Medical College (No. SCXK<SU>2010-0003). The animals used in this study were euthanized by overdose of MS-222 and sodium pentobarbital anesthesia, and all efforts made were to minimize the suffering of the animals.

Preparation of C. sinensis metacercariae

Metacercariae of *C. sinensis* used for infection were obtained from naturally infected fresh fish, *Pseudorasbora parva*. The fish in this experiment, which were not an endangered or protected species, were collected under the permission of the government of Guangxi Autonomous Region, China, and there was no specific permission required during this collection. The fish containing *C. sinensis* metacercariae were immediately homogenated and digested through artificial gastric juice (0.7% pepsin in 1% HCl solution, PH 2.0) as described by Chen [31]. *C. sinensis* metacercariae were morphologically identified under a dissecting microscope by experienced staff before intragastric administration.

Mice, experimental infection, and sampling

C3H/HeN mice between six and eight weeks of age at the commencement of the experiment were purchased from the Animal Center of Xuzhou Medical College, China, and were provided food and water *ad libitum*. One hundred and ten mice were inoculated with 35 metacercariae of *C. sinensis* suspended in approximate 0.3 mL phosphate-buffered saline (PBS) by gavage intragastric intubation. Tissue samples in the portal area of liver removed from six to eight mice at each time point were immediately sampled, fixed in PBS with 10% formaldehyde for 24 hours, and paraffin embedded for hematoxylin and eosin (H&E) staining and specific immunohistochemistry. The segments of left lobe of the liver were also immediately placed in a 2 mL microcentrifuge tube containing 1 mL RNAstore (Tiangen Inc., Beijing, China) and stored at -80°C until RNA extraction. The same protocol was applied in six non-infected control mice, which were used for the basal TLR2 and TLR4 levels.

RNA extraction

About 50 mg of mice specimens were removed from RNAstore reagent (Tiangen Inc., Beijing, China), and total RNA was extracted using commercially TRiZoL reagent (Invitrogen, San Diego, USA) according to the manufacturer's recommendations. Total RNA was dissolved with 50 μL of RNase-free water and stored at -80°C. The RNA integrity was examined by electrophoresis on 2% fresh agarose gel. Total RNA concentration of each sample was adjusted to 1 μg/μL.

Real-time reverse transcription (RT)-PCR

A commercial TIANScript RT kit (Tiangen Inc., Beijing, China) was used for first-strand cDNA synthesis. In brief, an equal amount of total RNA (3 μg) from each tissue sample was incubated with 2 μL oligo $(dT)_{15}$ primer (10 μM), 2 μL dNTPs mix (2.5 mM each) and nuclease-free water up to 14.5 μL at 72°C for 5 minutes, and immediately chilled on ice. Subsequently, 5.5 μL of M-MLV RT 5× reaction

buffer containing 0.5 μL of 0.1M DTT, 1 μL TIANScript M-MLV (200 U/μL) and 1 μL RNasin were mixed homogeneously. The mixtures were reacted at 42°C for 50 minutes, and inactivated by heating at 95°C for 5 minutes. The reaction mixtures were aliquoted and stored at -20°C.

PCR reactions (20 μL) of the gene TLR2, TLR4, and β-actin were performed using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol, including 0.40 μM of each primer and 1 μL of cDNA under the following conditions: after an initial denaturation at 94°C for 5 minutes, 40 cycles of 94°C for 30 seconds (denaturation), 60°C for 30 seconds (annealing), and 72°C for 30 seconds (extension). Before analysis of the relative expression level of each gene, the threshold cycle at which the fluorescent signal reached an arbitrarily set threshold near the middle of the log-linear phase of the amplification for each reaction was calculated, and the relative quantity of mRNA was determined. Quantification of target gene expression was evaluated in the terms of the comparative cycling threshold (C_t) normalized by β-actin with the $2^{-\Delta\Delta Ct}$ method. The primers used in this study are given in Table 1.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues from each mouse were prepared for immunohistochemical staining following the conventional procedure. Briefly, serial 4-μm thick sections were incubated with 3% H_2O_2 in PBS for blocking endogenous peroxidase. Nonspecific antigenic sites in sections were subsequently hampered by 4% bovine serum albumin in PBS-T for 20 minutes. Immunostaining for TLR2 and TLR4 protein was performed using specific TLR2 and TLR4 primary antibodies (Abcam, Cambridge, USA, cat. no. ab47093 and ab24192) for 4°C overnight at concentrations of 2 μg/mL and 10 μg/mL, respectively. Immunostaining was then detected with the Polink-2 Plus Polymer HRP Detection System with 3,3-diaminobenzidine (Gold Bridge International, Inc., Mukilteo, USA) according to the procedural instructions. Sections were then washed in distilled H_2O and counterstained with hematoxylin. Negative control sections were processed with IgG isotype instead of primary antibodies for evaluating their specificity. Immunohistochemistry-stained sections were checked by an inverted microscope (Olympus, Tokyo, Japan), and positive staining was identified when a brown pigmentation was microscopically observed and the images were analyzed using Image-Pro Plus software.

Cytometric bead array

The concentrations of IL-4, IL-10, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) cytokines in the sera from *C. sinensis*-infected mice and normal control mice were determined using a cytometric bead array (CBA) kit (BD Biosciences, Franklin Lakes, NJ, USA). In brief, 50 μL capture beads were mixed with 50 μL of mouse Th1/Th2 PE detection reagent II and 50 μL of sera from *C. sinensis*-infected mice. Simultaneously, a 10-point calibration curve was obtained using cytokine standards and beads for each cytokine. The samples were incubated for three hours at room temperature and kept away from light. They were then washed and centrifuged at 200 g for five minutes and analyzed in a flow cytometer (FACSCanto II; BD Biosciences, Franklin Lakes, NJ, USA). Positive and negative serum controls, a standard reference curve (mouse inflammation standard or mouse Th1/Th2 cytokine standards) provided in the cytometric bead array kit was used to interpolate picograms per microliter levels of each cytokine from the sera.

Histological observation

All mice were assayed for histological observation

Table 1. Primers for polymerase chain reaction amplification used in this experiment

Gene	Primer	Sequence	GenBank accession no.	Product size (bp)
TLR2	TLR2-F	5'-TGTTTGCTCCTGCGAACTCCTA-3'	NT_039240.8	160
	TLR2-R	5'- AGCCTGGTGACATTCCAAGACG-3'		
TLR4	TLR4-F	5'- GTGCCAATTTCATGGGTCT -3'	NM_021297.2	150
	TLR4-R	5'- CATCGAAGTCAATTTTGGTGTT -3'		
β-actin	β-actin-F	5'-CGTGGGCCGCCCTAGGCACCA-3'	NM_007393.3	243
	β-actin-R	5'-TTGGCCTTAGGGTTCAGGGGGG-3'		

by H&E staining and Masson's trichrome staining. The procedures were routinely performed based on the manufacturer's recommendations (Beyotime Inc., Haimen, Jiangsu Province, China for H&E staining, and Kaiji Inc., Nanjing, Jiangsu Province, China for trichrome staining). Images were digitized and analyzed in Image-Pro Plus software.

Statistical analysis

Data obtained from 6~8 mice were expressed as mean ± standard error of the mean (SEM); differences between infection groups and non-infected control group were analyzed by one-way analysis of variance (ANOVA). SPSS version 16.0 and GraphPad Prism software performed the statistical analyses. A statistically significant difference was observed when $p < 0.05$.

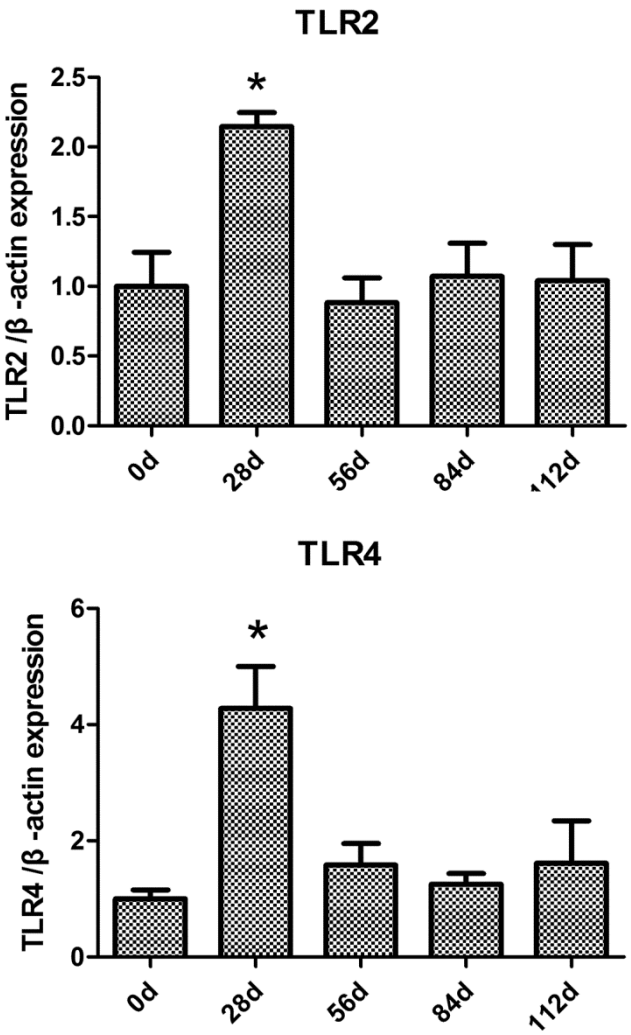
Results

Expression and distribution of TLR2 and TLR4 in livers of normal and C. sinsensis*-infected mice*

In the present study, mRNA transcripts of TLR2 and TLR4 at different time points during *C. sinensis* infection were investigated by relative quantitative PCR. Figure 1 shows the relative expression of TLR2 and TLR4 at different time points during *C. sinensis* infection. Upon infection, the expression of both TLR2 and TLR4 mRNA in *C. sinensis*-infected mice were significantly upregulated on day 28 post-infection (PI) compared with those of non-infected mice ($p < 0.01$, Figure 1). However, both the level of TLR2 and TLR4 gene expression subsequently returned to almost normal baseline levels until the experiment concluded (Figure 1).

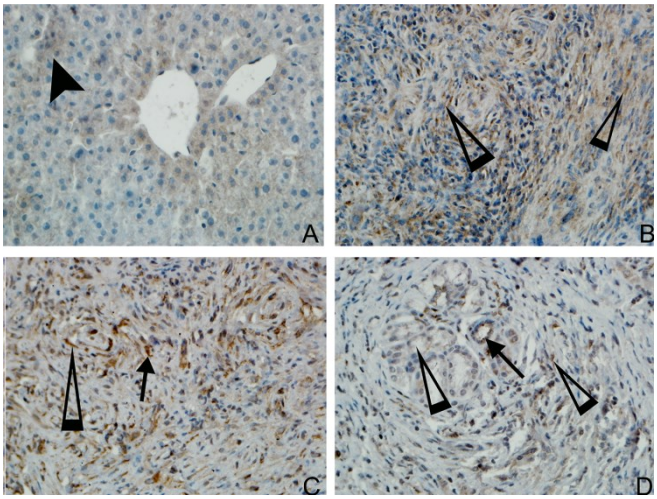
To observe the distribution of TLR2 and TLR4 protein in the livers of *C. sinensis*-infected mice, immunohistochemical staining was employed to exhibit the localization of TLR2 (Figure 2) and TLR4 (Figure 3) protein specifically in the livers of *C. sinensis*-infected mice and non-infected ones. In non-infected control mice, TLR2 and TLR4 proteins were slightly stained in the hepatocytes (indicated as up arrow in Figure 2A and Figure 3A). In contrast, both TLR2 and TLR4 were strongly stain ed in cytoplasm and membrane of fibroblasts (indicated as ∆) and on the lateral and luminal surfaces of biliary epithelium cells (indicated as →) in *C. sinensis*-infected mice. Among these inflammatory cells, Kupffer cells appeared to express TLR2 and TLR4 proteins in responses to *C. sinensis* infection during the whole infection, but TLR2 and TLR4 in other cells, such as eosinophils and lymphocytes, were expressed weakly or negatively in the infection. As expected, most TLR2 and TLR4 proteins were located in the cytoplasm and membrane of the cells, suggesting that they might be processed *en route* to the apical cell membrane.

Figure 1. mRNA expression of TLR2 and TLR4 in livers of C3H/HeN mice followed by *C. sinensis* infection.



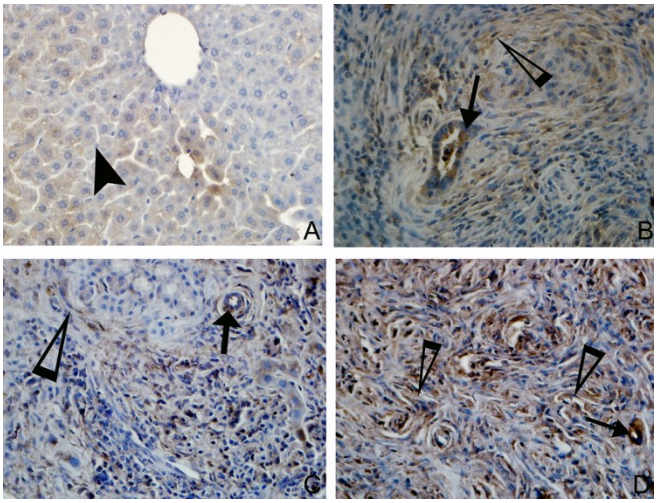
Relative levels of TLR2 and TLR4 mRNA expression on days 28, 56, 84, and 112 post-infection (PI) were analyzed by quantitative real-time polymerase chain reaction (PCR); values were rectified by mRNA expression of β-actin for each sample. The data obtained from at least six mice by two-independent experiments are expressed as mean ± standard error of mean (SEM).; * $P < 0.01$, compared with non-infected mice

Figure 2. Immunohistochemical staining of TLR2 in livers of normal and *C. sinensis* infected mice.



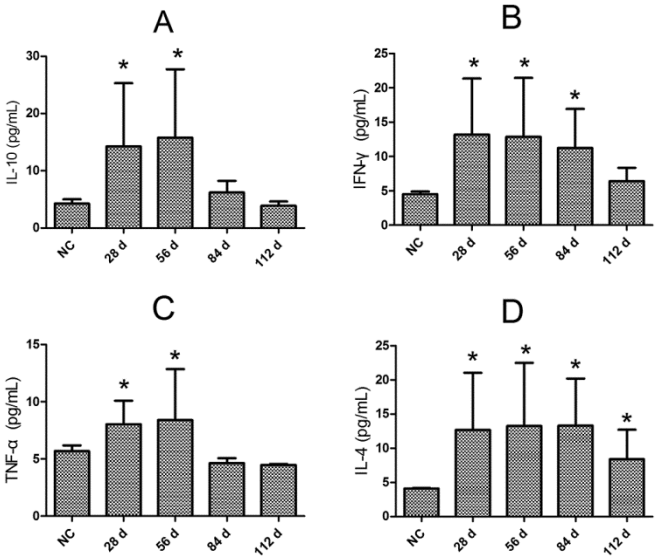
A–D represent TLR2 in the livers of A) healthy control mice, and B) mice infected by *C. sinensis* on day 28 post-infection (PI), C) day 84 PI, and D) day 112 PI. TLR2 protein was highly stained in biliary epithelium cells (indicated as → in C and D) and fibroblasts (indicated as △ in B, C, and D) but only slightly stained in hepatic cells (indicated as up arrow) of normal control mice. The original magnification is 400×.

Figure 3. Distribution of TLR4 in livers of normal and *C. sinensis* infected mice.



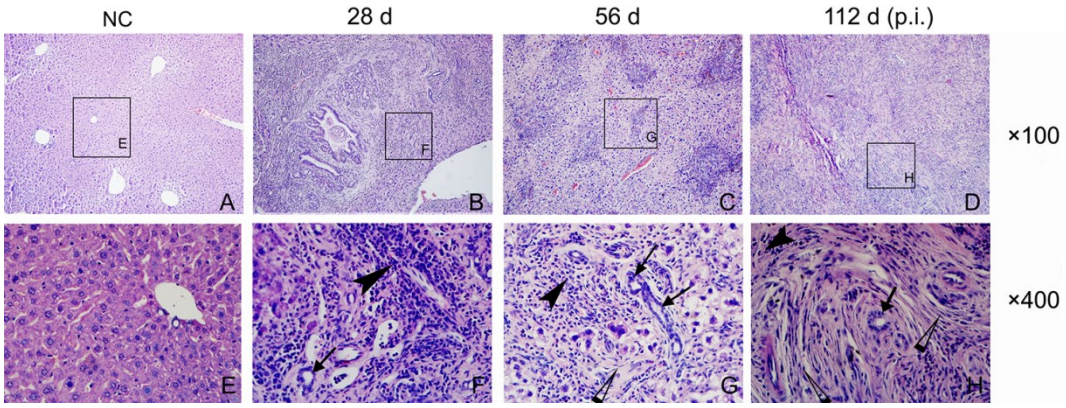
3A represents TLR4 protein slightly stained in the liver of normal-control mice (indicated as up arrow). TLR4 protein was highly stained in biliary epithelium cells (indicated as →) and fibroblasts (indicated as △) in the livers of *C. sinensis*-infected mice on days 28, 84, and 112 112 PI in B, C, and D, respectively. All pictures are shown enlarged ×400.

Figure 4. Levels of IL-10, IFN-γ, TNF-α, and IL-4 and in the sera of *C. sinensis*-infected C3H/HEN mice.



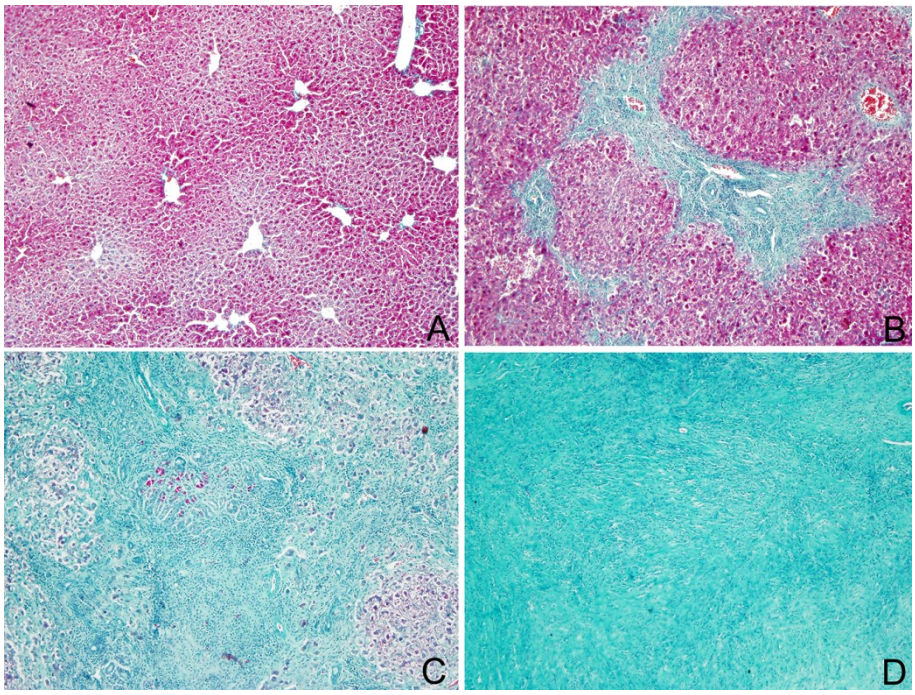
Compared with the control mice, the concentrations of A) IL-10, B) IFN-γ, C) TNF-α, and D) IL-4 were increased on day 28 post-infection (PI) and day 56 PI.; * $P < 0.05$ compared with corresponding cytokines of the control mice.

Figure 5. Representative pathologic changes in the livers of *C. sinensis*-infected C3H/HeN mice at different points in time.



Representative tissue sections were prepared from **A)** normal control mice (NC), as well as *C. sinensis* infected mice on **B)** day 28 PI, **C)** day 84 PI, and **D)** day 112 PI (n = 3~4). The pathology caused by *C. sinensis* is clearly shown by the hyperplastic change or bile duct obstruction of the infected bile duct epithelium (indicated as → in F, H), collagen fibers (indicated as △ in G and H), inflammatory cell infiltrations (indicated as up arrow in F and H). A, B, C, and D are enlarged 100×while E, F, G**,** and H are magnified 400×.

Figure 6. Masson's trichrome-stained sections for detection of collagen during the time course for the progression of liver damage caused by *C. sinensis*.



Representative tissue sections were prepared from **A)** normal control mice (NC), as well as *C. sinensis* infected mice on **B)** day 28 post-infection (PI), **C)** day 84 PI, and **D)** day 112 PI (n = 3~4). Green streaks represent collagen deposition. Original magnification in photomicrographs is 100×.

The dynamics of cytokines in sera from C. sinensis-infected C3N/HEN mice

To underline the significance of activation of TLR2 and TLR4 in the host immune system, cytokines in the sera of *C. sinensis*-infected mice such as IL-4, IL-10, IFN-γ, and TNF-α, which are closely relative with TLR2 and TLR4, were further detected. As shown in Figure 4, the levels of these cytokines were all increased as the TLR2 and TLR4 were activated on days 28 PI and 56 PI, and the levels of IL-4 (Figure 4D) and IFN-γ (Figure 4B) were still higher than in control mice on day 84 PI; however, IL-10 (Figure 4A), IFN-γ (Figure 4D), and TNF-α (Figure 4C) appeared to be normal levels on day 112 PI.

Histological findings

To further explore the potential roles of TLR2 and TLR4 in the disease outcomes of clonorchiasis, histological changes in the livers of *C. sinensis*-infected mice were examined. As shown in Figure 5 and Figure 6, extensive inflammatory cell infiltrations in portal areas of livers were observed accompanied by part of the liver cell degeneration, and bile duct obstruction of the infected liver appeared on day 28 PI (Figure 5). However, inflammatory cell infiltration in the livers of infected animals appeared to be reduced, and collagen fibers gradually increased with the infection prolonged from day 56 PI; these pathological changes did not disappear until the experiment concluded (Figure 5 and Figure 6).

Discussion

Among a number of animal models, hamsters are considered good candidates for the study of the relationship between host immune response and *C. sinensis* infection [32,33]. However, this animal model is restricted for use because commercial antibodies and other reagents against hamsters are not available. In addition, rabbits and rats are not appropriate hosts for this purpose because they have no gallbladders. Therefore, mice are substitutable for hamsters to study host-parasite interaction of *C. sinensis* since they have gallbladders, and their antibodies or other reagents are commercially available [19-21]. Importantly, C3H/HeN together with C3H/HeJ mice (a deficient function of TLR4) will provide contrastive data for further exploring the role of TLR4 in *C. sinensis* infection. Thus, C3H/HeN mice were chosen for investigating the expression and distribution of TLR2 and TLR4 in the liver of C3H/HeN mice following *C. sinensis* infection.

Specific bacterial PAMPs such as lipopolysaccharides could stimulate innate immune responses in the hemocoel of *Biomphalaria glabrata*, which subsequently promotes cell proliferation in the amebocyte-producing organ (APO) and may contribute to the resistance to infection with larval trematodes [34]. Furthermore, previous studies also have shown that up-regulation of TLR4, TLR2, or both are critical to recognition of PAMPs derived from helminths and for induction of innate immune response, ultimately leading to a protective Th2-promoting phenotype through several signal pathways [35]. However, the expression and distribution of TLR2 and TLR4 in response to the infection of *C. sinensis in vivo* have not yet been reported so far. The results of present study showed that the transcripts of both TLR2 and TLR4 gene were increased on day 28 PI, which is interestingly consistent with the increased in cytokines including Th1 and Th2 types immune responses, suggesting that TLR2 and TLR4 might be engaged in the production of cytokines such as IL-4, IL-10, IFN-γ, and TNF-α in mice with *C. sinensis* infection [36]. In addition, TLR2-mediated immune response via inflammatory/anti-inflammatory cytokines may be involved in the imbalance of oxidants/antioxidants in patients re-infected by *O. viverrini* [37].

Several studies demonstrated that TLRs could be expressed by almost cell types in murine livers, even under normal physiological conditions, but different expression levels of TLRs can be regulated depending on the cell type in response to various exposures to PAMPs. The distribution of TLR2 and TLR4 in the liver of mice infected by *C. sinensis* is not clear. In our study, we found that both TLR2 and TLR4 proteins were mainly located in the cytoplasm and membrane of endothelial cells, fibroblast, and biliary epithelium cells (BEC) in *C. sinensis*-infected mice, although eosinophils, lymphocytes, and hepatic cells were slightly stained during the infection of *C. sinensis* compared to normal-control mice. Notably, the great density of TLR2 and TLR4 proteins were constantly stained in the fibroblast of fibrotic tissues, suggesting that the TLR signaling pathway might provide a new mechanism to account for persistent fibrogenesis [14,38].

Conclusions

The present study is the first report on TLR expression in a mouse model of clonorchiasis; it showed that expression of TLR2 and TLR4 were up-regulated during the infection of *C. sinensis*, indicating

that both TLR2 and TLR4 probably participate in the stimulation of the innate immune response during *C. sinensis* infection. The roles that TLR2 and TLR4 play in *C. sisensis* infection require further investigation.

Acknowledgements

Project support was provided, in part, by the National Natural Science Foundation of China (Grant Nos. 81171590, 31302077 and 81572019), the Open Funds of the State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Grant No. SKLVEB2013KFKT005), a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the Starting Foundation for Talents of Xuzhou Medical College (Grant No. 53591317), the Natural Science Foundation of Xuzhou Medical College (Grant Nos. 2014KJ08 and 2014KJ10). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Coordinating Office of the National Survey on the Important Human Parasitic Diseases (2005) A national survey on current status of the important parasitic diseases in human population. Chin J Parasitol Parasit Dis 23 Suppl: 332-340 (in Chinese).
2. Qian MB, Chen YD, Liang S, Yang GJ, Zhou XN (2012) The global epidemiology of clonorchiasis and its relation with cholangiocarcinoma. Infect Dis Poverty 1: 4.
3. Chen J, Xu MJ, Zhou DH, Song HQ, Wang CR, Zhu XQ (2012) Canine and feline parasitic zoonoses in China. Parasit Vectors 5: 152.
4. Lun ZR, Gasser RB, Lai DH, Li AX, Zhu XQ, Yu XB, Fang YY (2005) Clonorchiasis: a key foodborne zoonosis in China. Lancet Infect Dis 5: 31-41.
5. Lee JH, Yang HM, Bak UB, Rim HJ (1994) Promoting role of Clonorchis sinensis infection on induction of cholangiocarcinoma during two-step carcinogenesis. Korean J Parasitol 32: 13-18.
6. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, Cogliano V (2009) WHO International Agency for Research on Cancer Monograph Working Group. A review of human carcinogens-Part B: biological agents. Lancet Oncol 10: 321-322.
7. Arji N, Busson M, Iraqi G, Bourkadi JE, Benjouad A, Bouayad A, Mariaselvam C, Salah S, Fortier C, Amokrane K, Marzais F, Boukouaci W, Krishnamoorthy R, Charron D, El Aouad R, Tamouza R (2014) Genetic diversity of TLR2, TLR4, and VDR loci and pulmonary tuberculosis in Moroccan patients. J Infect Dev Ctries 8: 430-440. doi:10.3855/jidc.3820.
8. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. Annu Rev Immunol 21: 335-376.
9. Hoebe K, Janssen E, Beutler B (2004) The interface between innate and adaptive immunity. Nat Immunol 5: 971-974.
10. Tuon FF, Amato VS, Bacha HA, Almusawi T, Duarte MI, Amato Neto V (2008) Toll-like receptors and leishmaniasis. Infect Immun 76: 866-872.
11. Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, Janeway CA Jr (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell 2: 253-258.
12. O'Neill LA, Greene C (1998) Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. J Leukoc Biol 63: 650-657.
13. Cario E, Rosenberg IM, Brandwein SL, Beck PL, Reinecker HC, Podolsky DK (2000) Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. J Immunol 164: 966-972.
14. Takeuchi O, Takeda K, Hoshino K, Adachi O, Ogawa T, Akira S (2000) Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. Int Immunol 12: 113-117.
15. Bhattacharyya S, Kelley K, Melichian DS, Tamaki Z, Fang F, Su Y, Feng G, Pope RM, Budinger GR, Mutlu GM, Lafyatis R, Radstake T, Feghali-Bostwick C, Varga J (2013) Toll-Like Receptor 4 Signaling Augments Transforming Growth Factor-β Responses: A Novel Mechanism for Maintaining and Amplifying Fibrosis in Scleroderma. Am J Pathol 82: 192-205.
16. Junpee A, Tencomnao T, Sanprasert V, Nuchprayoon S (2010) Association between Toll-like receptor 2 (TLR2) polymorphisms and asymptomatic bancroftian filariasis. Parasitol Res 107: 807-816.
17. Senhaji N, Diakité B, Serbati N, Zaid Y, Badre W, Nadifi S (2014) Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms: new data and a meta-analysis. BMC Gastroenterol 14: 206.
18. Hart BE, Tapping RI (2012) Genetic diversity of Toll-Like receptors and immunity to *M. leprae* infection. J Trop Med 2012: 415057.
19. Choi YK, Yoon BI, Won YS, Lee CH, Hyun BH, Kim HC, Oh GT, Kim DY (2003) Cytokine responses in mice infected with *Clonorchis sinensis*. Parasitol Res 91: 87-93.
20. Kim EM, Bae YM, Choi MH, Hong ST (2012) Cyst formation, increased anti-inflammatory cytokines and expression of chemokines support for *Clonorchis sinensis* infection in FVB mice. Parasitol Int 61: 124-129.
21. Guo QQ, Fu LL, Tang RX, Xia H, Zheng KY, Liu YS, Du WP, Cao LL, Zhao K, Dai QF (2009) The dynamic Th1/Th2 cytokines in the spleens of mice infected with *Clonorchis sinensis*. Chin Trop Med 9: 1187-1189 (in Chinese).
22. Venugopal PG, Nutman TB, Semnani RT (2009) Activation and regulation of toll-like receptors (TLRs) by helminth parasites. Immunol Res 43: 252-263.
23. Goodridge HS, Marshall FA, Else KJ, Houston KM, Egan C, Al-Riyami L, Liew FY, Harnett W, Harnett MM (2005) Immunomodulation via novel use of TLR4 by the filarial nematode phosphorylcholine-containing secreted product, ES-62. J Immunol 174: 284-293.
24. Mishra BB, Mishra PK, Teale JM (2006) Expression and distribution of Toll-like receptors in the brain during murine neurocysticercosis. J Neuroimmunol 181: 46-56.
25. Hise AG, Daehnel K, Gillette-Ferguson I, Cho E, McGarry HF, Taylor MJ, Golenbock DT, Fitzgerald KA, Kazura JW, Pearlman E (2007) Innate immune responses to endosymbiotic *Wolbachia bacteria* in *Brugia malayi* and

Onchocerca volvulus are dependent on TLR2, TLR6, MyD88, and Mal, but not TLR4, TRIF, or TRAM. J Immunol 178: 1068-1076.
26. Dowling DJ, Hamilton CM, Donnelly S, La Course J, Brophy PM, Dalton J, O'Neill SM (2010) Major secretory antigens of the helminth Fasciola hepatica activate a suppressive dendritic cell phenotype that attenuates Th17 cells but fails to activate Th2 immune responses. Infect Immun 78: 793-801.
27. Gundra UM, Mishra BB, Wong K, Teale JM (2011) Increased disease severity of parasite-infected TLR2$^{-/-}$ mice is correlated with decreased central nervous system inflammation and reduced numbers of cells with alternatively activated macrophage phenotypes in a murine model of neurocysticercosis. Infect Immun 79: 2586-2596.
28. Gao Y, Zhang M, Chen L, Hou M, Ji M, Wu G (2012) Deficiency in TLR2 but not in TLR4 impairs dendritic cells derived IL-10 responses to schistosome antigens. Cell Immunol 272: 242-250.
29. Ninlawan K, O'Hara SP, Splinter PL, Yongvanit P, Kaewkes S, Surapaitoon A, LaRusso NF, Sripa B (2010) *Opisthorchis viverrini* excretory/secretory products induce toll-like receptor 4 upregulation and production of interleukin 6 and 8 in holangiocyte. Parasitol Int 59: 616-621.
30. Pinlaor S, Tada-Oikawa S, Hiraku Y, Pinlaor P, Ma N, Sithithaworn P, Kawanishi SL (2005) *Opisthorchis viverrini* antigen induces the expression of Toll-like receptor 2 in macrophage RAW cell line. Int J Parasitol 35: 591-596.
31. Chen XW (1984) Isolation and observation of excystation for Clonorchis sinensis and Metorchis spp. Chin J Zool 4: 3235 (in Chinese).
32. Lee JH, Rim HJ, Bak UB (1993) Effect of *Clonorchis sinensis* infection and dimethylnitrosamine administration on the induction of cholangiocarcinoma in Syrian golden hamsters. Korean J Parasitol 31: 21-30.
33. Lee JH, Rim HJ, Sell S (1997) Heterogeneity of the "oval-cell" response in the hamster liver during cholangiocarcinogenesis following *Clonorchis sinensis* infection and dimethylnitrosamine treatment. J Hepatol 26: 1313-1323.
34. Sullivan JT, Belloir JA (2014) Activation of an innate immune response in the schistosome-transmitting snail Biomphalaria glabrata by specific bacterial PAMPs. Dev Comp Immunol 42: 256-260.
35. Kerepesi LA, Hess JA, Leon O, Nolan TJ, Schad GA, Abraham D (2007) Toll-like receptor 4 (TLR4) is required for protective immunity to larval *Strongyloides stercoralis* in mice. Microbes Infect 9: 28-34.
36. Zhang M, Gao Y, Du X, Zhang D, Ji M, Wu G (2011) Toll-like receptor (TLR) 2 and TLR4 deficiencies exert differential in vivo effects against *Schistosoma japonicum*. Parasite Immunol 33: 199-209.
37. Yongvanit P, Thanan R, Pinlaor S, Sithithaworn P, Loilome W, Namwat N, Techasen A, Dechakhamphu S (2012) Increased expression of TLR-2, COX-2, and SOD-2 genes in the peripheral blood leukocytes of opisthorchiasis patients induced by *Opisthorchis viverrini* antigen. Parasitol Res 110: 1969-1977.
38. Yang L, Seki E (2012) Toll-like receptors in liver fibrosis: cellular crosstalk and mechanisms. Front Physiol 3: 138.

Corresponding author

Kui-Yang Zheng, MD; Ren-Xian Tang, MD
Department of Pathogenic Biology and Immunology, Laboratory of Infection and Immunity, Xuzhou Medical College, 209 Tongshan Road ,Xuzhou, Jiangsu Province, China
Phone: +00 86 0516 83262615
Fax: +00 86 0516 83262615
Email: ZKY02@163.com; Tangrenxian-t@163.com

Conflict of interests: No conflict of interests is declared.