

Original Article

Expression of toll-like receptor 2, 4 and related cytokines in intraperitoneally inoculated Balb/C mice with *Echinococcus multilocularis*

Shadike Apaer^{1,2*}, Tuerhongjiang Tuxun^{1,2,4*}, Hai-Zhang Ma³, Heng Zhang¹, Hao Zhang¹, Jiangduosi Payiziwula², Pei-Ji Zhao², Aizimaiti Aihaiti², Yu-Peng Li², Tao Li², Jin-Ming Zhao^{2,4}, Ren-Yong Lin^{1,4}, Hao Wen^{1,2,4}

¹State Key Laboratory Incubation Base of Xinjiang Major Diseases Research and Xinjiang Key Laboratory of Echinococcosis, ²Departments of Liver Transplantation & Laparoscopic Surgery, Digestive and Vascular Surgery Centre, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang Uyghur Autonomous Region, China; ³Department of General Surgery, Qilu Hospital, Jinan, China; ⁴WHO Collaborating Center for Prevention and Care Management of Echinococcosis, First Affiliated Hospital of Xinjiang Medical University and Xinjiang Centers for Disease Control, Urumqi, Xinjiang Uyghur Autonomous Region, China. *Equal contributors.

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Abstract: Immune response pattern between host and *Echinococcus multilocularis* (*E. multilocularis*) is considered as a crucial point in development of alveolar echinococcosis (AE). In this study, we are aiming to study the expression patterns of TLR2 and TLR4 with related cytokines and transcription factors in secondary *E. multilocularis* infected murine model. The murine model of AE was developed by using intraperitoneal inoculation of *E. multilocularis* protoscolices and albendazole (*E. m*+ABZ group) or carboxy methyl cellulose (CMC; *E. m*+CMC group) administration via gastric tube was initiated in the third month and continued for one month. Mice with CMC administration served as negative controls (C+CMC group). The splenic cells and peritoneal exudates cells (PECs) were prepared and the levels of IFN- γ , IL-10, and IL-5 in splenic cells and PECs culture supernatants were detected using enzyme linked immune-sorbent assay (ELISA). Besides, the mRNA expression levels of TLR2, 4, transcription factors and cytokines were detected by using real-time fluorescent quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The concentration levels of IFN- γ , IL-10, and IL-5 in PECs and splenic cell supernatants were extremely lower, however, significantly elevated after stimulated with Concanavalin A (ConA) for 36 h with higher concentrations in *E. m*+CMC group comparing to both *E. m*+ABZ and C+CMC group. The mRNA levels of TLR2, 4 and GATA3, IFN- γ , IL-10 in splenic cells were significantly increased in *E. m*+CMC group comparing with other groups. Simultaneously, T-bet mRNA expressions were elevated in *E. m*+ABZ and C+CMC group compared to *E. m*+CMC group. In addition, T-bet/GATA3 ratios was higher in *E. m*+ABZ group compared to *E. m*+CMC group and were higher in C+CMC group than those in *E. m*+CMC group. TLR2 mRNA expression in splenic cells showed a positive correlation with IL-10 concentration levels in splenic cell culture supernatants. The present study provides evidence on the possible role of TLR2 in the process of immune tolerance during *E. multilocularis* infection and suggests albendazole treatment might reverse the immune tolerance situation and improve parasite clearance process.

Keywords: Toll-like receptor, immune modulation, albendazole, *echinococcus multilocularis*

Introduction

Alveolar echinococcosis (AE) caused by the larval stage of tapeworm *Echinococcus multilocularis* (*E. multilocularis*) continues to be a major public health issue around the world, especially to pastoral and/or semi-pastoral area in China, Central Asia, Middle East, South America and some part of Europe [1]. Radical resection and autologous liver transplantation (ALT) along with long-term albendazole (ABZ) administra-

tion were the treatments of choice [2]. The distinct immune response profile induced during host-*E. multilocularis* crosstalk determines the abortion or infiltrative growth of parasite [3]. Both clinical and experimental studies claimed the impaired dendritic cell functions and imbalanced T cell subsets including Th1/Th2/Th17/Treg immune response [4-6]. Increasing number of studies indicated that an appropriate polarized T helper cell response could be driven by the interaction with parasite and/or parasite

driven component and Toll-like receptors (TLRs) in various parasitic infections, and thus, enables parasite escape from the immune system's attack and guarantee long-period survival [7-11]. Our previous studies indicated the involvement of elevated TLR2 and TLR4 in the process of immune tolerance both in cystic and alveolar echinococcosis [7, 12]. However, very little is known as both the alteration and possible role of TLRs in murine model to date. In addition, no related reports, to our knowledge, were found about the possible role of ABZ treatments on the TLRs alterations. Therefore, in current study, secondary *E. multilocularis* infection murine model was developed to further assess the alteration of TLR2, 4 mRNA expressions and related cytokines before and after ABZ administration, if any, as well as to study their relationship with relative cytokines.

Materials and methods

Biochemicals and drugs

If not otherwise stated, all tissue culture media were purchased from Gibco-BRL (Zurich, Switzerland), biochemical reagents were from Sigma (MO, USA), and ABZ was purchased from GlaxoSmithKline (Tianjin, China). All reagents used for drug analysis were of analytical or research grade.

Mice

Female 8-week-old BALB/C mice were purchased from the experimental animal center of Xinjiang Medical University for secondary infection with *E. multilocularis* and as control animals. All mice were housed in a temperature-controlled, light-cycle room in animal facilities according to the Chinese animal protection guidelines, with food and water *ad libitum*.

Maintenance and isolation of E. multilocularis metacestode Echinococcus

The *E. multilocularis* metacestode were maintained by serial transplantation passages through *i.p.* injection in gerbils. Four to 10 weeks after *i.p.* injection, gerbils were sacrificed, and the parasite tissue was removed from the peritoneal cavity under aseptic conditions, placed into 0.9% normal saline (NS), and was washed several times. After grinding the tissue through a sterile 50- μ m sieve, 100-freshly prepared acephalic vesicular cysts were suspended in 100 μ L 0.9% NS and injected intra-

peritoneally into a mouse for secondary infection. Control mice received 100 μ L of 0.9% NS.

ABZ administration started at the time point of chronic infection

ABZ suspensions were prepared in carboxy methyl cellulose (CMC) 0.5% (w/v) in water. ABZ suspensions were freshly prepared each week and stored at -20°C for a period of 7 days maximum. The control suspensions containing only CMC were treated identically. Treatments were initiated on the same day, and were repeated daily for 35 consecutive days. Mice were categorized into three groups of 8 in each: (1) *E. multilocularis* infection treated with ABZ (*E. m*+ABZ) group; (2) *E. multilocularis* infection with CMC (*E. m*+CMC) group; (3) Control+CMC (C+CMC) group.

Cell preparations

Mice were sacrificed 35 days post-treatment with ABZ. Spleen cell suspensions were prepared from both infected and control BALB/C mice. The cell suspensions were depleted of erythrocytes by treatment with 0.83% NH₄Cl in 0.01 M Tris-HCl (pH 7.2) and subsequently resuspended in RPMI-1640 complete medium containing heat-inactivated 10% fetal calf serum (FCS, Gibco), 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco). PECs from control and infected mice were collected by peritoneal rinsing with 10 mL RPMI-1640. Cells were subsequently washed twice with HBSS and resuspended in RPMI-1640.

Cell cultures

Spleen cells and PECs were cultured in 48-well round-bottom plates at 2×10^6 /well for 36 hours and the supernatants were collected. The cell cultures were stimulated with Concanavalin A (Con A) (5 μ g/ml; Sigma) or were left unstimulated as negative controls. All tests were performed in quadruplicates.

Real-time fluorescent quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

TLR2 and TLR4 mRNA expressions were all determined by using a commercial QuantiFast SYBR Green PCR Kit (QIAGEN, Germany) according to the manufacturer's instructions.

Table 1. Primer sequence and amplicon of genes

Gene	Primer sequences	Amplicon (bp)
GATA3	F-5'-ACAGAAGGCAGGGAGTGTC-3' R-5'-GGTAGAGTCCGCAGGCATT-3'	109
T-bet	F-5'-GGACCCAAGTGTCAACTGCT-3' R-5'-AACTGTGTTCCCGAGGTGC-3'	154
TLR2	F-5'-CCAAGACCTACCTGGAGTGG-3' R-5'-AGGAACTGGGTGGAGAACCT-3'	101
TLR4	F-5'-TTCAGAGCCGTTGGTGTATCT-3' R-5'-TGTCCTCCATTCCAGGTAG-3'	174
IL-10	F-5'-CCAAGACCCAGACATCAAGG-3' R-5'-AAGGCATTCTTACCTGCTC-3'	110
INF- γ	F-5'-CTGCTGATGGGAGGAGATGT-3' R-5'-TTTGTCAATCGGGTGTAGTCA-3'	176
β -actin	F-5'-CGTTGACATCCGTAAGACC-3' R-5'-AACAGTCCTcctAGAAGCAC-3'	100

F: Forward; R: Reverse.

The primers were synthesized by Shengggong Biotech (Shanghai, China) shown as in **Table 1**. β -actin was analyzed as an internal control and gene expression was normalized to it. The quantitative PCR analyses of the data were performed using SYBR Green program on i-Q 5.0 Real-time PCR system (Bio-Rad, Foster City, CA, USA). The relative amounts of PCR products were determined using the relative standard curve method. mRNA expression level fold changes were calculated as described by the SYBR Green I protocol.

Enzyme-linked immune-sorbent assay (ELISA)

Concentrations of IFN- γ (assay sensitivity was 4 pg/mL), IL-5 (4 pg/mL), and IL-10 (2 pg/mL), were determined from splenic cell supernatants and PECs' by ELISA using a commercial mice ELISA kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. Cytokine concentrations were calculated by using the mean optical density of two wells and comparison with a standard curve.

Statistical analysis

All the quantitative data were expressed as Median [interquartile (IQR)] in the text. Statistical analysis was performed using statistical software (SPSS, version 17.0, Chicago, IL, USA). Mann-Whitney (M-W) test was used to determine the differences between groups. Spearman correlation analysis was applied as a test of correlation between two continuous

variables, and determined by Spearman correlation coefficients. Probable values of 0.05 and below were considered to be statistically significant.

Results

Evaluation/Assessing of secondary *E. multilocularis* experimental model and parasite burden

The overall success rate of secondary AE infection model establishment is 100% (16/16). All mice were sacrificed 17 weeks after *E. multilocularis* metacestode inoculation, and lesion tissues and masses were removed for monitoring parasite burden in the peritoneal cavity (**Figure 1A**). Mice treated with ABZ administration resulted in a significantly reduced median parasite weight of 1.04 (IQR, 0.9-1.3) g which was lower when compared to *E. m*+CMC mice 5.2 (IQR, 4.4-5.8) g as shown in **Figure 1B**.

TLR2, TLR4 mRNA expression levels in splenic cells

The mRNA expression levels of TLR2 and TLR4 were detected in spleen cells using qRT-PCR technique. TLR2 mRNA levels were significantly elevated in *E. m*+CMC group when comparing with C+CMC group and *E. m*+ABZ group ($P<0.05$, $P<0.01$ respectively). TLR4 mRNA levels were slightly higher in *E. m*+CMC group compared to both *E. m*+ABZ and C+CMC group, albeit no statistical differences were found between them ($P>0.05$) as shown in **Figure 2**.

GATA3, IFN- γ , T-bet and IL-10 mRNA expression levels in splenic cells

Th1/Th2 cell related transcription factors and cytokines GATA3, IFN- γ , T-bet and IL-10 mRNA expression levels were measured by using qRT-PCR technique. As shown in **Figure 3**, relative mRNA expression levels of GATA3 and IFN- γ in *E. m*+CMC group were found to be elevated compared to *E. m*+ABZ and C+CMC group, however, without statistical differences ($P>0.05$, $P>0.05$ respectively). T-bet mRNA expression levels were increased in *E. m*+ABZ group compared to both *E. m*+CMC group and C+CMC group, nevertheless, no statistical differences between them ($P>0.05$). IL-10 mRNA expressions were significantly higher in *E. m*+CMC group than those in *E. m*+ABZ and C+CMC

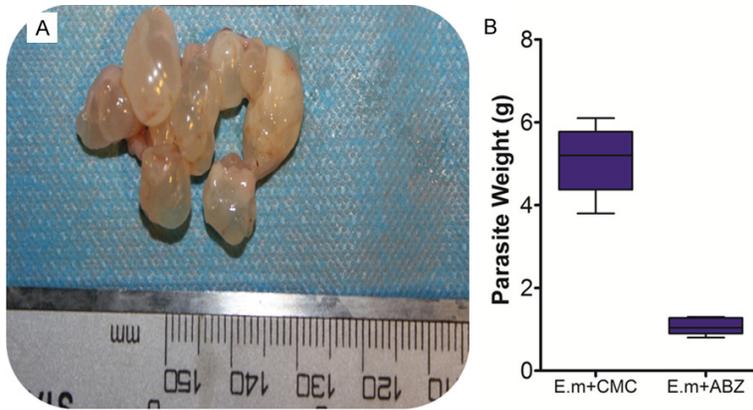


Figure 1. Assessment of parasite load in intraperitoneal cavity of mice infected with *E. multilocularis*. All mice were sacrificed, and lesion tissues and masses were removed for monitoring parasite burden in the peritoneal cavity (A). Mice treated with ABZ administration resulted in a significantly reduced median parasite weight which was lower when compared to *E. m+CMC* mice (B).

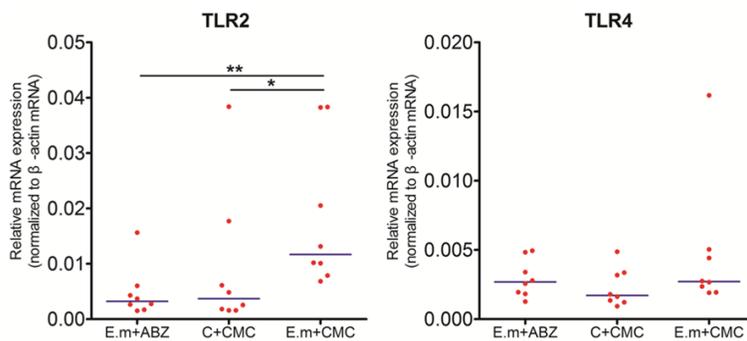


Figure 2. qRT-PCR analyses of TLR2 and TLR4 mRNA expressions in different groups. The relative expressions of TLR2 and TLR4 were calculated, and results showed TLR2 mRNA expressions were significantly elevated in *E. m+CMC* group compared to C+CMC and *E. m+ABZ* group with statistical differences ($P < 0.05$, $P < 0.01$ respectively). Relative mRNA expressions of TLR4 were higher in *E. m+CMC* group comparing with both *E. m+ABZ* and C+CMC group, albeit no statistical differences were found between them ($P > 0.05$). P value < 0.001 with marker ***; P value < 0.01 with marker **; P value < 0.05 with marker *.

group with a statistical differences between *E. m+CMC* and *E. m+ABZ* groups ($P < 0.05$). We also detected the T-bet/GATA3 ratio and found it significantly elevated in *E. m+ABZ* group compared to both *E. m+CMC* and C+CMC group ($P < 0.05$).

Concentration levels of cytokines in PECs supernatants

The concentration levels of cytokines before and after Con A stimulation were detected by using ELISA techniques. Con concentrations of

IFN- γ and IL-10 were extremely lower (data not shown), and elevated markedly after stimulating with Con A, resulting in the higher level in *E. m+CMC* group than those in *E. m+ABZ* and C+CMC group, nevertheless no statistical differences were found between them ($P > 0.05$). IL-5 concentration levels were also too lower, and interestingly, then elevated markedly with the stimulation of Con A. It is increased significantly in *E. m+CMC* and *E. m+ABZ* group compared to C+CMC group with statistical differences ($P < 0.01$) as shown in **Figure 4**.

Concentration levels of cytokines in splenic cell supernatants

We have measured cytokines in spleen cells using ELISA technique. IFN- γ concentration levels were extremely lower, and elevated after stimulating with Con A. Concentrations of IFN- γ were higher in *E. m+CMC* group than those in *E. m+ABZ* and C+CMC group, but with no statistical differences ($P > 0.05$). IL-10 levels were significantly elevated in *E. m+CMC* group compared to both *E. m+ABZ* and C+CMC group ($P < 0.05$). IL-5 concentrations were decreased in *E. m+CMC* group compared to

both *E. m+ABZ* and C+CMC group, however, there were no statistical differences between them ($P > 0.05$) as shown in **Figure 4**.

Correlation analysis between TLR2 mRNA expression levels and IL-10 concentration levels

Spearman correlation coefficients indicated that TLR2 mRNA expressions in splenic cells had a positive correlation with spleen cell IL-10 concentration levels ($r = 0.4344$, $P = 0.0339$), when no correlations were found between spleen cell TLR4 mRNA expressions and IL-10

Toll-like receptors in mice with *Echinococcus multilocularis*

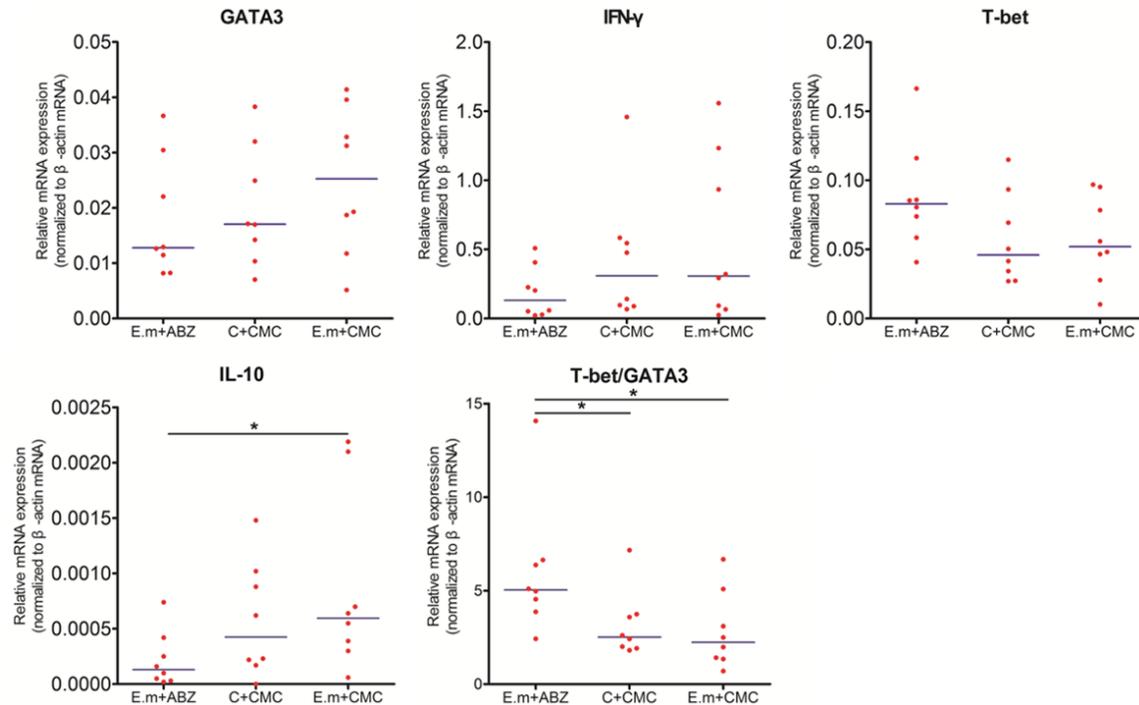


Figure 3. Relative mRNA expressions of cytokines and transcription factors in splenic cells. Relative cytokines and transcription factors GATA3, IFN- γ , T-bet, and IL-10 mRNA expression levels were calculated by using qRT-PCR technique. GATA3, IFN- γ and IL-10 mRNA expression levels were elevated in *E. m*+CMC group compared to both *E. m*+ABZ and C+CMC group. Relative mRNA expressions of T-bet were found to be increased in *E. m*+ABZ group comparing with both *E. m*+CMC and C+CMC group. Similarly, T-bet/GATA3 was significantly elevated in *E. m*+ABZ group comparing with both *E. m*+CMC and C+CMC group. *P* value <0.001 with marker ***; *P* value <0.01 with marker **; *P* value <0.05 with marker *.

concentrations ($r=0.2153$, $P=0.3124$) as shown in **Figure 5**.

Discussion

Human AE caused by the larval stage of metacystode of *E. multilocularis* is a lethal neglected tropical disease with “cancer-like” infiltrative growth pattern. After successful entering the portal blood stream, *E. multilocularis* reside itself primarily in liver and develop a granulomatous lesion with potential to invade adjacent organs and metastasize to remote organs [13]. Early diagnosis and radical resection are challenged due to insidious and asymptomatic growth. Radical resection varies from partial hepatectomy to ALT is optimal method along with long-term ABZ treatment [14, 15].

It is widely accepted that immune interaction between *E. multilocularis* and host’s immune system was pivotal during disease progression [16, 17]. *E. multilocularis* has evolved a broad spectrum of ability to actively modulate, even

escape from the host’s immune system for successful survival [18, 19]. Different subtypes of T helper (Th) cells such as Th1, Th2, Th17 and Th9, as well as Treg cells are involved in the interaction and related with the ability of parasite clearance and/or immune tolerance [4, 20, 21]. Identifying of parasite and/or parasite driven components through pathogen associated molecular patterns (PAMPs) are crucial for achieving an appropriate polarized T helper cell immune response that would enable the parasite to escape from the immune system’s attack and guarantee long-period survival.

TLRs are firstly discovered and mostly studied among pattern recognition receptors (PRRs). They take a significant role in antigen recognition and play as powerful immune-stimulant in innate immunity which in turn develops antigen-specific acquired immunity as well [22, 23]. The cellular localization of TLRs and respective PAMPs they identify will determine, to a large extent, the nature of the T cell polarization. It is showed that Th2 based responses orchestrate

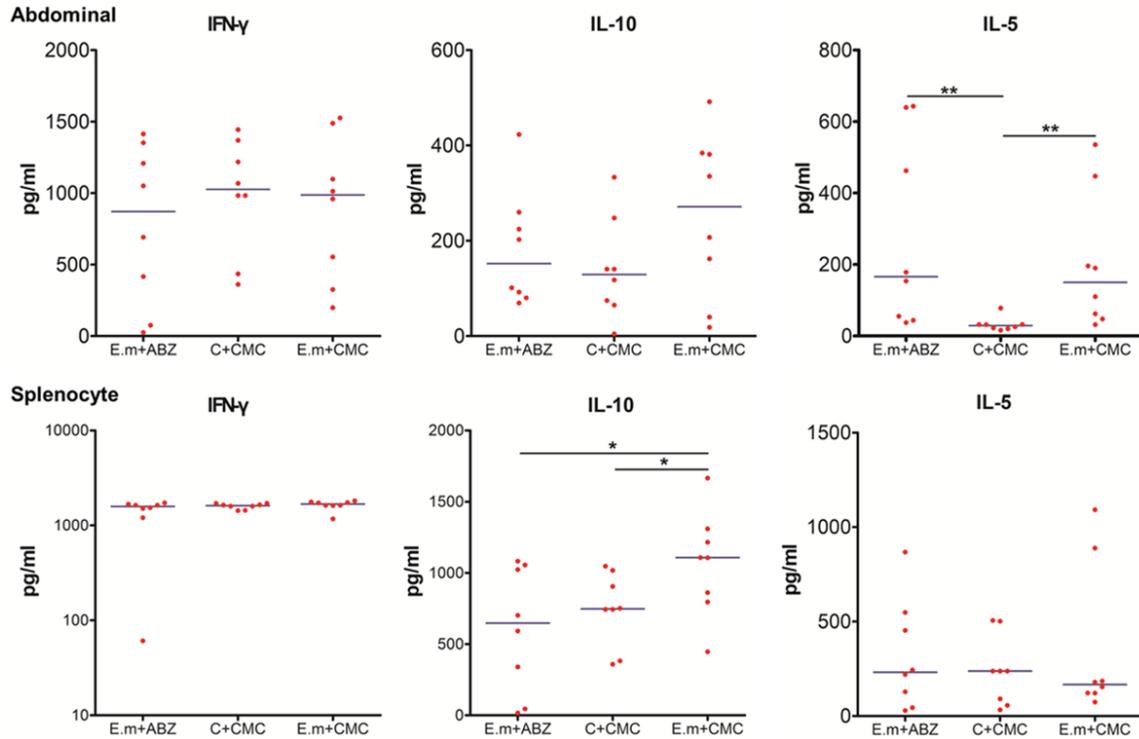


Figure 4. Concentration levels of cytokines in PECs and spleen cell supernatants. Cytokine concentration levels before and after Con A stimulation were detected by using ELISA technique. After stimulating with Con A, concentrations of IFN- γ from both PECs and spleen cell supernatants were similar between all three groups. IL-10 concentration levels were elevated after Con A stimulation and significantly decreased in *E. m*+ABZ group when comparing with *E. m*+CMC group. Concentration levels of IL-5 were significantly increased in *E. m*+CMC and *E. m*+ABZ group compared to C+CMC group in PECs, however, it was higher than that in Control group in spleen cell supernatants with no statistical differences. *P* value <0.001 with marker ***; *P* value <0.01 with marker **; *P* value <0.05 with marker *.

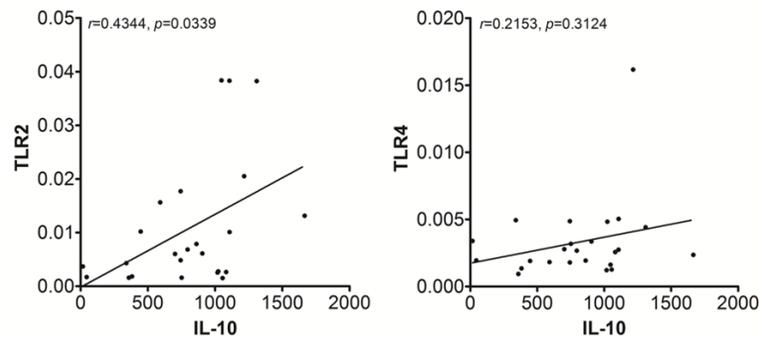


Figure 5. Correlation analysis between TLR2 mRNA expression levels and IL-10 concentration levels. Spearman correlation coefficients indicated that TLR2 mRNA expressions in splenic cells had a positive correlation with spleen cells IL-10 concentration levels ($r=0.4344$, $P=0.0339$). No correlations were found between spleen cell TLR4 mRNA expressions and IL-10 concentrations ($r=0.2153$, $P=0.3124$).

ed by TLRs signaling pathways are beneficial for the pathogen survival and disease progression [24, 25]. The potent critical role of them in helminthic infections such as leishmaniasis,

malaria, trypanosomiasis and filariasis were reported [26-29]. TLR2 and TLR4 are also capable to bind helminthes and/or their products, thus induce inflammatory response by secreting pro-inflammatory cytokines from DCs [22, 30]. Growing evidences indicated that TLRs may trigger effector DCs and macrophages that promote Th2-type responses [23, 30]. In filarial nematodes infection, TLR4 is involved in anti-inflammatory/Th2 biased immune response that prolong survivability of the parasite inside host [31]. Likewise,

TLR2 and TLR4 have been reported to be expressed during helminthic infection that uphold and maintain Th2 type-immune responses to make “worm favorable” conditions

[23, 32]. In our previous study, we observed elevated expression of TLR2/4 both in peripheral blood and hepatic tissue and their correlations to anti-inflammatory cytokines [7, 12].

In current secondary *E. multilocularis* infection murine model, TLR2, 4 expression in splenic cells and related cytokines in splenic and PECs culture supernatant and their alterations after ABZ administration were detected and discussed. Significantly increased TLR2 mRNA expression levels were observed in *E. m*+CMC subjects when compared with C+CMC subjects. Meanwhile, decreased level of TLR2 was detected after ABZ treatment. Relative TLR4 mRNA expressions were slightly increased in *E. m*+CMC subjects and decreased after ABZ treatment, however, no statistical differences were found among three groups. Increased TLR2 and TLR4 expressions in intraperitoneally infected mice in our study might be associated with the role of TLRs in the recognition of *E. multilocularis* PAMPs. Such increased recognition by TLR2, 4 may play a critical role in initiating different immune response that might help the parasite to maintain its survival by inducing immune tolerance.

Besides, the Th1/Th2 subset immune response profile have been studied by detecting Th1 related transcription factor and cytokine T-bet, IFN- γ as well as Th2 related transcription factor and cytokine GATA3, IL-5, meanwhile, immune suppressive cytokine IL-10 was also detected, if any, and its correlation with TLRs were analyzed. The increased levels of IL-10 and IL-5 both in PECs and spleen cell supernatants are shown in infected mice and decreased after ABZ administration, especially in spleen cells with statistical significance. This might be due to enhanced TLR2 and TLR4 recognition skewed the naïve CD4⁺ T cells towards Th2 and Treg that are favor for parasite survival. However, the levels of IFN- γ have shown no significance among these groups. Furthermore, supported by our previous findings, expression levels of TLR2 showed a positive correlation with IL-10 levels in splenic cells supernatants [7, 12]. Such an interesting result might be translated into overwhelming anti-inflammatory immune response and over expression of TLR2 and TLR4 in peripheral and regional milieu. [3, 21, 32]. The immune tolerance induced by parasitic infection may help it to grow under the umbrella with compromised threat by host's

immune system and thus develop an occupying lesion. However, the anti-helminthic agent ABZ administration may abate the vitality of the parasite, thus consequently, the cross-talk between *E. multilocularis* and host's immune system is attenuated and resulted in clearance of parasite or abortion of the lesion.

Conclusions

Collectively, expression levels of TLR2 mRNA significantly increased in intraperitoneally *E. multilocularis* infected mice and displayed a positive correlation with IL-10 levels that is critical for immune tolerance. On the other hand, decreased level of TLR2 mRNA and IL-10 were observed and indicates that ABZ administration might play a role to reverse the immune tolerant situation and parasite clearance.

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Disclosure of conflict of interest

None.

Address correspondence to: Hao Wen, State Key Laboratory Incubation Base of Xinjiang Major Diseases Research and Xinjiang Key Laboratory of Echinococcosis, Digestive & Vascular Surgery Center, The First Affiliated Hospital of Xinjiang Medical University, 137 Liyushan South Road, Xinshi District, Urumqi 830054, Xinjiang Uyghur Autonomous Region, China; Departments of Liver Transplantation & Laparoscopic Surgery, Digestive & Vascular Surgery Center, The First Affiliated Hospital of Xinjiang Medical University, 137 Liyushan South Road, Xinshi District, Urumqi 830054, Xinjiang Uyghur Autonomous Region, China; WHO Collaborating Center for Prevention and Care Management of Echinococcosis, First Affiliated Hospital of Xinjiang Medical University and Xinjiang Centers for Disease Control, 137 Liyushan South Road, Xinshi District, Urumqi 830054, Xinjiang Uyghur Autonomous Region, China. Tel: (+86) 991 436 2844; Fax: (+86) 991 436 0051; E-mail: dr.wen-hao@163.com

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