Tropical Biomedicine 38(1): 172-179 (2021)
https://doi.org/10.47665/tb.38.1.029

RESEARCH ARTICLE

Dendritic cell TLR4 induces Th1-type immune response against *Cryptosporidium parvum* infection

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ARTICLE HISTORY

Received: 20 October 2020
Revised: 20 December 2020
Accepted: 23 December 2020
Published: 25 March 2021

ABSTRACT

The objective of this study was to investigate the mechanism of Toll-like receptor (TLR4)-mediated dendritic cell (DC) immune against *Cryptosporidium parvum* infection. *C. parvum* sporozoites were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester. Murine bone marrow-derived DCs were isolated, and divided into TLR4 antibody blocking (TAB; infected with 2×10^5 labeled sporozoites and 0.5 μg TLR4 blocking antibody), TLR4 antibody unblocking (TAU; infected with 2×10^5 labeled sporozoites), and blank control (BC; with 1.5 mL Roswell Park Memorial Institute 1640 medium) groups. The adhesion of *Cryptosporidium* sporozoites to DCs and $CD11c^+$ levels were examined by fluorescence microscopy and flow cytometry. Male KM mice were orally injected with *C. parvum*. The proliferation of T lymphocytes in spleen, expression of cytokines in peripheral blood, and TLR4 distribution features in different organs were further determined by immunohistochemistry. A significantly higher expression of $CD11c^+$ and higher *C. parvum* sporozoite adhesion were found in the TAU group compared with other groups. The expression of $CD4^+CD8^-/CD8^+CD4^-$ in the spleen were obviously differences between the TAB and TAU groups. The expression of TLR4, interleukin IL-4, IL-12, IL-18 and IFN-γ improved in the TAU group compared with TAB group. Higher expression of TLR4 was detected in the lymph nodes of mice in the TAU group, with pathological changes in the small intestine. Hence, TLR4 could mediate DCs to recognize *C. parvum*, inducing Th1 immune reaction to control *C. parvum* infection.

Keywords: *Cryptosporidium parvum*; Dendritic cells; Toll-like receptor; Th-1 type immune reaction.

INTRODUCTION

Cryptosporidiosis is a parasitic disease caused by *Cryptosporidium* spp. found in both humans and animals. It can cause acute diarrhea and is life-threatening for both immune-competent and immunocompromised individuals (Šlapeta, 2013). Although the infection is self-limiting in immunocompetent hosts, immunocompromised and malnourished individuals can develop a chronic disease (Caccia̋ *et al.*, 2016). No effective therapeutic or preventive interventions have been developed for AIDS patients infected with *C. parvum*, in whom this disease can be fatal (Kaushik *et al*., 2008). Nevertheless, the exact mechanism underlying the host immune response to *C. parvum* infection is still poorly understood. Several studies have indicated that dendritic cells (DCs) are essential in recognizing, processing and presenting antigens to naïve T cells. Therefore, it is necessary to further elucidate the roles of DCs against *C. parvum* infection via Toll-like receptor 4 (TLR4).

As one of the most powerful professional antigen-presenting cells (APCs), DCs have dual roles in T cell activation (Wu *et al*., 2016). Immature DCs from peripheral tissues can capture external antigens and recognize them through pathogen-associated molecular patterns (PAMPs) (Acton *et al*., 2016; Ferreira *et al*., 2016). Consequently, immature DCs can migrate into lymph nodes and other immune organs, reach the maturation state, and in turn upregulate the expression of the related factors, including major histocompatibility complex (MHC)-I, MHC-II, CD86 and other co-stimulatory factors, as well as the expression of pro-

inflammatory cytokines (Li *et al*., 2015). CD11c is one of the most important marker molecules present on the surface of DCs, and its expression is correlated with the maturation degree of DCs (Bedi *et al*., 2014). Several studies have suggested that cytokines induced by DCs are essential for the host defense against *C. parvum* infection. However, little is known about the potential role of pattern recognition receptors (PRRs) to cryptosporidiosis (Liu *et al*., 2014).

In innate immunity, Toll-like receptors (TLRs) are among the most important PRRs which can recognize a wide range of pathogens or PAMPs, including virus, bacteria, fungi and parasites (Kawai *et al*., 2010). For instance, TLR4 can recognize lipopolysaccharide (LPS) as the major component of the outer membrane from Gram-negative bacteria (Schneider *et al*., 2012). It can also identify some parasite antigens such as *Plasmodium'*s and *Toxoplasma*'s glycosylphosphatidylinositol (GPI), and produce a strong immune reaction. Therefore, it is considered critical in initiating defense responses to mobilize immune effector cells and activate adaptive immunity. Further, *C. parvum* can expose a number of glycoprotein antigens on its surface, providing the substantial basis for TLR recognition (Bogert *et al*., 2008; Yang *et al*., 2015).

However, the mechanism by which TLR4 mediates DCs response to *C. parvum* infection is still not clear. This study investigated the interaction between DCs, *C. parvum* and TLR4. The mouse bone marrow DCs were isolated, blocked with TLR4 antibody, and further stimulated with *C. parvum* to analyze its maturation degree *in vitro*. Moreover, a mouse model infected with *C. parvum* was established to evaluate the TLR4 function that resists *C. parvum in vivo*.

MATERIAL AND METHODS

Animals and parasites

Male KM mice, aged 4 weeks, were purchased from Laboratory Animal Center of Anhui Medical University, China. *C. parvum* oocysts were collected and purified from bovine stool using sucrose discontinuously.

All the animals were maintained at a temperature of 22°C ± 1°C and relative humidity of 50% ± 1% under a 12h-light/dark cycle. All animal studies (including the mice euthanasia procedure) were performed in compliance with the regulations and guidelines of Anhui Medical University institutional animal care, and according to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the Institutional Animal Care and Use Committee (IACUC) guidelines, approval number is 2018-029.

Reagents

Fetal bovine serum was purchased from Gibco (Grand Island, NY, USA); Murine granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) were obtained from Peprotech (London, U.K.); Anti-Mouse CD11c fluorescein isothiocyante (FITC), Anti-Mouse CD3 P-phycoerythrin (PE), Anti-Mouse CD4 Allophycocyanin (APC) and Anti-Mouse-CD8 FITC were purchased from eBioscience (San Diego, CA, USA); 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) were obtained from Dojindo (Japan); Anti-TLR4 antibody was obtained from Abcam (Toronto, Canada).

DCs generation from bone marrow

Bone marrow-derived DCs (BMDCs) were generated as previously described (Inaba *et al*., 2000). Briefly, BMDCs were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37°C with 5% CO_2. Consequently, cells were induced by 5 ng/mL GM-CSF and IL-4. The culture medium was replaced every 2 days. Adherent DCs were observed after 24 h.

Excystation of *C. parvum* and CFSE staining

C. parvum oocysts were suspended in 0.8% sodium hypochlorite for 7 min and then incubated at 37°C for 30 min. The cells were then washed twice in sterile phosphate-buffered Saline (PBS, pH 7.2) and suspended in PBS (pH 7.2) containing 0.8% Na-taurocholate (Solarbio, China) at 37°C for 30 min. Then, excystated sporozoites were labeled with 10 μM of CFSE for 30 min, and then finally washed three times with PBS (pH 7.2) (Perez-cordon *et al*., 2014).

Infection of DCs with *C. parvum*

DCs (1×10^6) were plated into six-well plates and cultured for 6 h. They were then divided into three groups: blank control (BC) group, TLR4 antibody blocking (TAB) group, and TLR4 antibody unblocking (TAU) group. The mice in the TAB group were infected with 2×10^5 labeled sporozoites and 0.5 μg TLR4 antibody, in the TAU group with 2×10^5 labeled sporozoites, and in the BC group with 1.5 mL RPMI 1640 medium. The adhesion of *Cryptosporidium* sporozoites to DCs was examined using fluorescence microscopy 24h after infection. All experiments were performed in triplicate.

Determination of CD11c expression

After 2 h of infection with sporozoites, 2×10^5 DCs were stained with 5 μL anti-mouse CD11c FITC for 30 min, washed with ice-cold PBS three times, and analyzed using flow cytometry (FACSCalibur) and CellQuest software.

C. parvum infection *in vivo*

The mice were divided into three groups: TLR4 antibody seal (TAS) group, treated with 10 μL (20 mg/mL) of anti-TLR4 antibody, TLR4 antibody unsealing (TAU) group, and control group. In addition, the mice in the TAS and TAU groups were administrated with 2×10^5 *C. parvum* oocysts using oral gavage, while the control group received the same amount of saline.

Proliferation situation of CD3/CD4/CD8 and qPCR analysis

To analyze the proliferation of $CD4^+CD8^+$, lymphocytes were isolated from spleen cells on the 5, 8 and 12 dpi. Cell surface staining was performed using PE anti-mouse CD3, APC anti-mouse CD4, and FITC anti-mouse CD8a for 30 min. After washing in PBS, the cells were fixed with 4% Para-formaldehyde and analyzed using FACSCalibur.

Total RNA was extracted from leukocyte using TRIzol reagent (Invitrogen, Toronto, Canada), and then reversed transcribed into cDNA using PrimeScript RT reagent Kit (TaKaRa, Japan). To examine the expression of immune-related factors (IL-4, IL-6, IL-12, IL-18, IFN-γ and TLR4) after infection with *C. parvum* mediated by TLR4, specific primers were designed, as shown in Table 1. Real-time quantitative polymerase chain reaction (PCR) was carried out using a 7500 Real-time PCR System (Applied Biosystems, Warrington, UK) and the SYBR Green kits (Qiagen, Germany). The amplified volume contained 10 μL of 2×SYBR premix, 0.8 μL of cDNA, 0.5 μL of 25 μM p1, 0.5 μL of 25 μM p2 and 8.2 μL of ddH_2O, and β-actin served as the reference gene.

Immunohistochemistry

To detect the expression of TLR4 in different tissues infected with *C. parvum*, the spleen, lymph nodes and small intestine were collected from different groups on the 5, 8 and 12 dpi. All tissues were fixed with 10% formaldehyde for 24 h and cut into 5 μm paraffin-embedded sections. A two-step immunohistochemical staining technique EnVision was used in this study. Anti-TLR4 antibody (diluted at 1:1000) was used as the primary antibody. The density of diaminobenzidine

Table 1. Primer sequence and its optimum annealing temperatures for PCR amplifications

Mouse target gene	Primer	Primer sequence	Annealing temperature (°C)	Size of amplicons (bp)
IL-4	IL-4-F IL-4-R	CTCTCTGTGGTGTTCTTCG ATCCTGCTCTTCTTTCTCG	60	174
IL-6	IL-6-F IL-6-R	TGCTGCTATCAATCCATGA ACCTCCTGTTTGGCTCCA	60	150
IL-12	IL-12-F IL-12-R	TGCTGCTATCAATCCATGA ACCTCCTGTTTGGCTCCA	58	125
IL-18	IL-18-F IL-18-R	ACTCTTGCGTCAACTTCAA CCTCGAACACAGGCTGTC	54	135
β-Actin	β-Actin-F β-Actin-R	CTGTCCCTGTATGCCTCTG ATGTCACGCACGATTTCC	58	178
IFN-γ	IFN-γ-F IFN-γ-R	GCTCTGAGACAATGAACG TTCTTCCACATCTATGCC	60	145
TLR4	TLR4-F TLR4-R	CTTTATTCAGAGCCGTTGG TGTCATCCTGCTCTTCTTT	54	180

(DAB) with analyzed using ImageJ software and compare using average optical density.

Statistical analysis

All data were analyzed using SPSS19.0 and one-way analysis of variance. F-test was used to evaluate the differences. *P* value less than 0.05 was considered statistically significant.

RESULTS

Expression of CD11c$^+$ in DCs after infection with *C. parvum*

CD11c$^+$ is considered one of the key markers for DC maturation (Poncini *et al*., 2010; Bedi *et al*., 2014). In this study, the expression of CD11c$^+$ was examined by flow cytometry. The expression level of CD11c$^+$ was 83.37 ± 3.73 and 67.67 ± 1.80 in the TAB and TAU group, respectively, while it was 7.06 % in BC group (Figure 1). Moreover, a significant difference was observed between the groups ($P < 0.05$). These results suggested that *C. parvum* could significantly promote the maturation of DC.

***C. parvum* sporozoites adhesion to DCs**

C. parvum sporozoites labeled with CFSE were co-cultured with DCs for 24 h. As shown in Figure 2, the adhesion was clearly visible in the TAB and TAU groups (Figure 2D and 2E), while no signal was detected in the BC group (Figure 2F). Nevertheless, a lower signal was observed in the TAB group compared with the TAU group.

The proliferation of CD3$^+$CD4$^+$CD8$^-$ / CD3$^+$CD8$^+$CD4$^-$ T cells

CD4$^+$ T cells have an important role in resistance to *C. parvum* (McDonald *et al*., 2013). To examine the role of TLR4 in *C. parvum* infection, the expression levels of CD3/CD4/CD8 were detected at different time points, that is, on 5, 8 and 12 dpi. As shown in Figure 3, in the group analysis, the expression of CD4$^+$ was not the same on 5 d, 8 d and 12 d after infection with *C. parvum*. Also, the expression of CD4$^+$ CD8$^-$ was observed on the 8th day after infection with *C. parvum*, but the difference was not significant ($P > 0.05$). The expression of CD3$^+$CD4$^+$CD8$^-$ was significantly higher in the TAU group compared with the BC and TAB group ($P < 0.05$) on the 5 and 8 dpi (highest peak), and then it decreased. Moreover, on the 5 and 8 dpi, the expression of CD3$^+$CD8$^+$CD4$^-$ was significantly lower in the

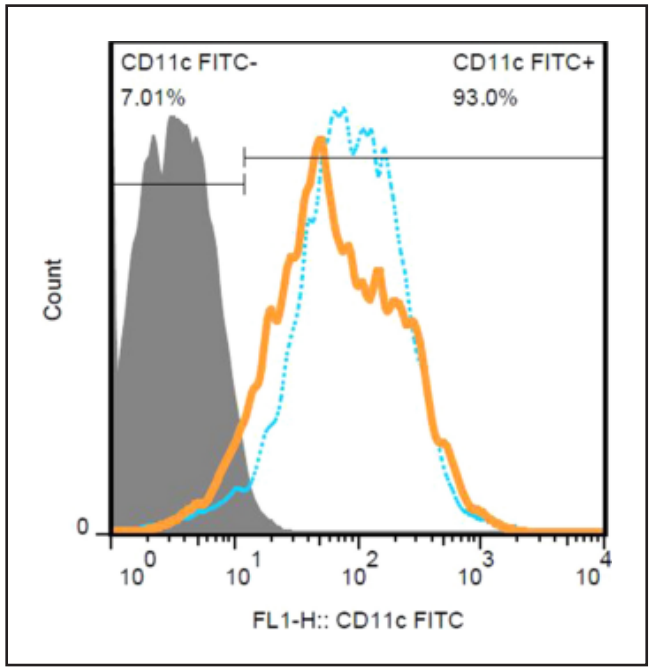

Figure 1. Expression of CD11c$^+$ in DCs after infection with *C. parvum*. Blue resprents TAU; orange resprents TAB; shadow resprents BC.

TAU group compared with the BC group ($P < 0.05$). On the 12 dpi, no difference was found between groups. These data suggested that CD3$^+$CD4$^+$CD8$^-$ could be upgraded in the early stage of *C. parvum* infection.

Differences in expression of immune-related factors mediated by TLR4

CD4$^+$ T cells have been implicated as key components in immune response to *C. parvum* infection. The expression of immune-related factors was also associated with resistance to infection. Nevertheless, the effect mechanisms responsible for recovery from infection are poorly understood (Robinson *et al*., 2001). Futher, evidence to illustrate the role of TLR4 against *C. parvum* was not available. Therefore, the

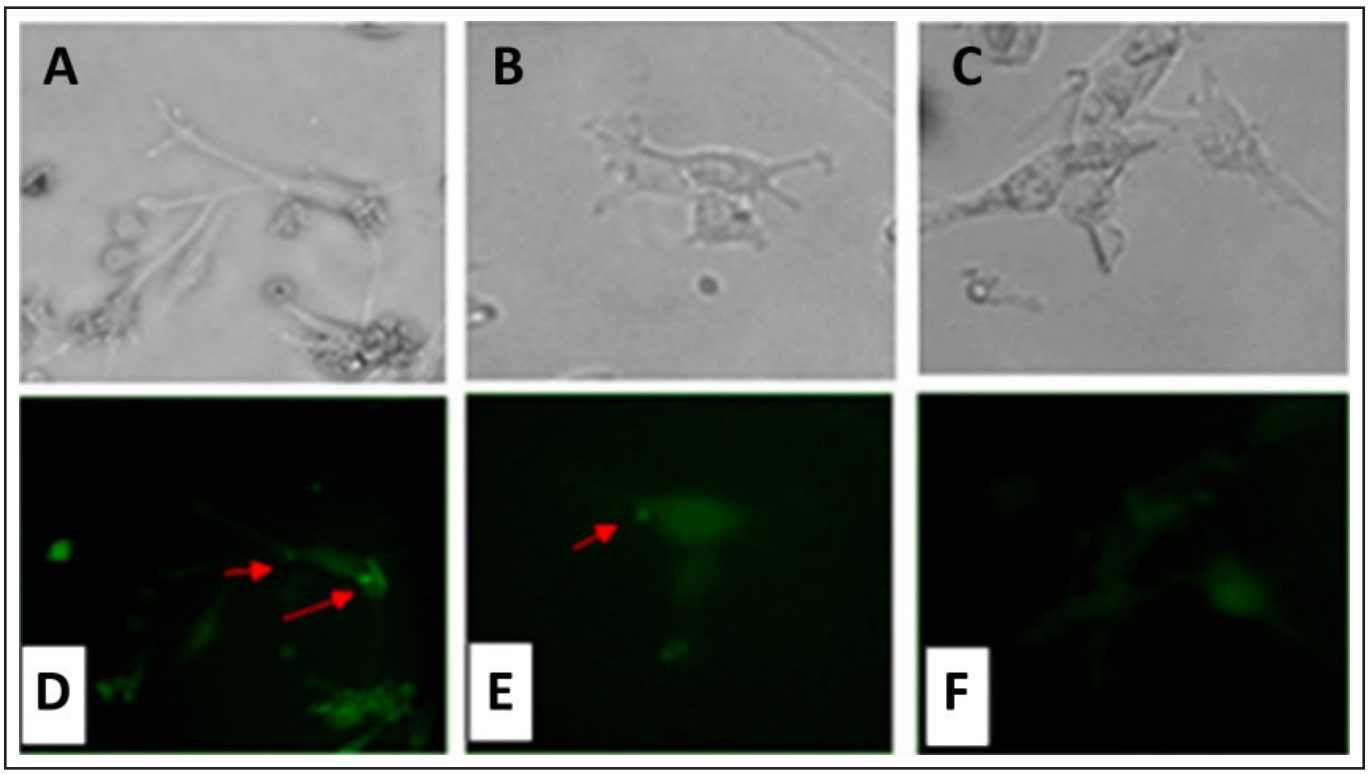

Figure 2. Adhesion between DCs and the tagged-CFSE *C. parvum* sporozoites. (A and D) TAU group, (B and E) TAB group, and (C and F) BC group (400×).

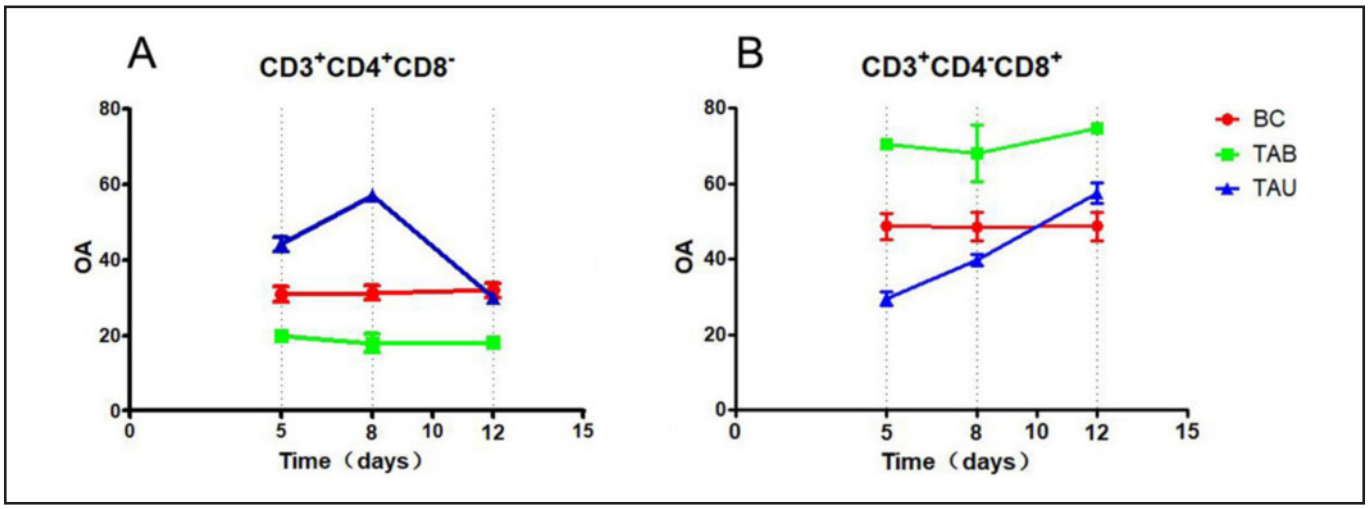

Figure 3. Proliferation of CD3/CD4/CD8 from spleen T lymphocytes after infection with *C. parvum.* (A) Changes in $CD3^+CD4^+CD8^-$ among the BC, TAB and TAU groups 5, 8 and 12 days after infection with *C. parvum*. (B) Changes in $CD3^+CD4^-CD8^+$. Three samples were compared with PBS-injected controls. The difference analysis was performed by flow cytometry (mean ± standard error of the mean of six biological replicates).

effects of TLR4 on the secretion of cytokines from peripheral blood examined on the 5, 8 and 12 dpi. As shown in Figure 4, the expression of immune-related factors was significantly upregulated due to *C. parvum* infection. Significantly higher expression levels of IL-4, IL-12, IL-18, IFN-γ and TLR4 were observed in the TAU group compared with the TAB group ($P < 0.05$), while no difference in the expression of IL-6 was found between the TAU and TAB groups. For all cytokines, the highest peak was detected on the 8 dpi. These data suggested that *C. parvum* induced IL-4, IL-12, IL-18, IFN-γ and TLR4 production. Most cytokines involves in Th1 immune response had the tendency of upregulation, but only IL-4 involved in Th2 immune response was upregulated.

Immunohistochemistry reaction

Villous damage, atrophy and reduction were observed in the intestine on 12 dpi. The surface of the intestine mucosa had the form of a crater, and a large number of shedding tissue fragments were found in the intestines. However, evidence to illustrate the changes in the expression of TLR4 in intestine after the infection of *C. parvum* was not sufficient. Hence, immunohistochemical analysis was performed to observe the expression of TLR4 in spleen, intestine and lymph node separately.

As shown in Figure 5, the highest expression of TLR4 was found in the intestine (Figure 5D-F) and lymph nodes (Figure 5H-I) of mice in the TAU group. The expression was

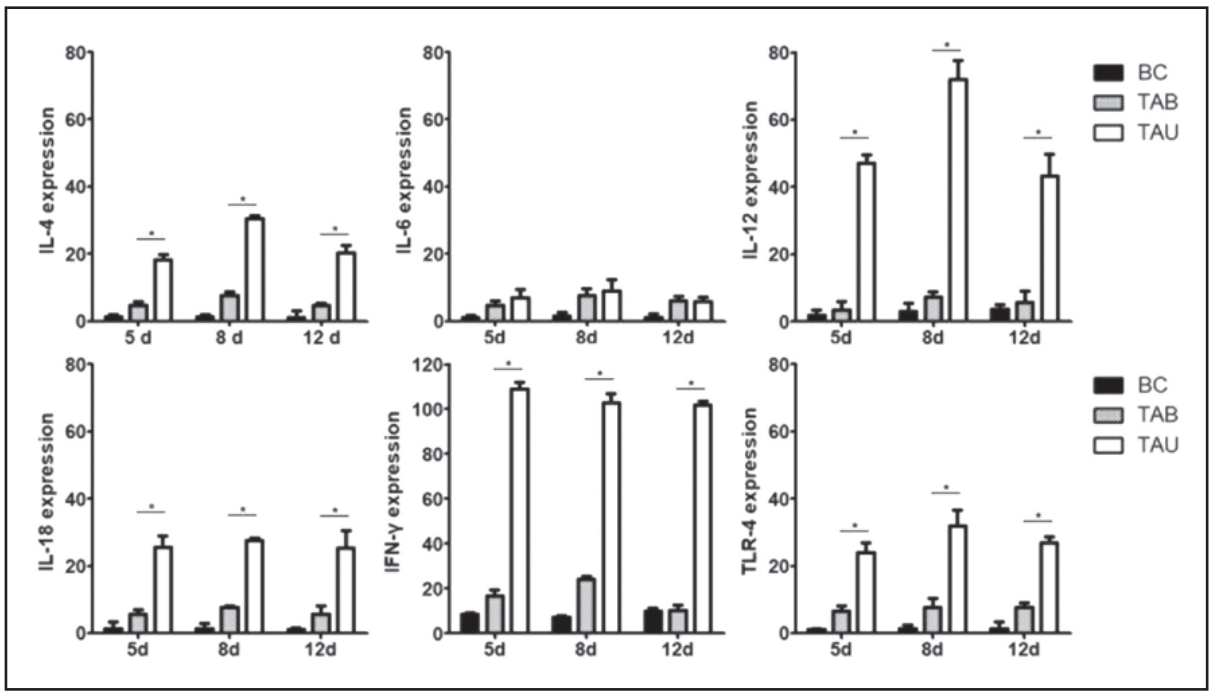

Figure 4. The expression levels of immune-related factors at different stages and in different groups after infection with *C. parvum*. The controls were treated with PBS 5, 8, 12 dpi. The T cells from peripheral blood were isolated, and the expression of IL-4, IL-6, IL-12, IL-18, IFN-γ and TLR4 was detected using q-PCR (mean ± standard error of the mean of six biological replicates).

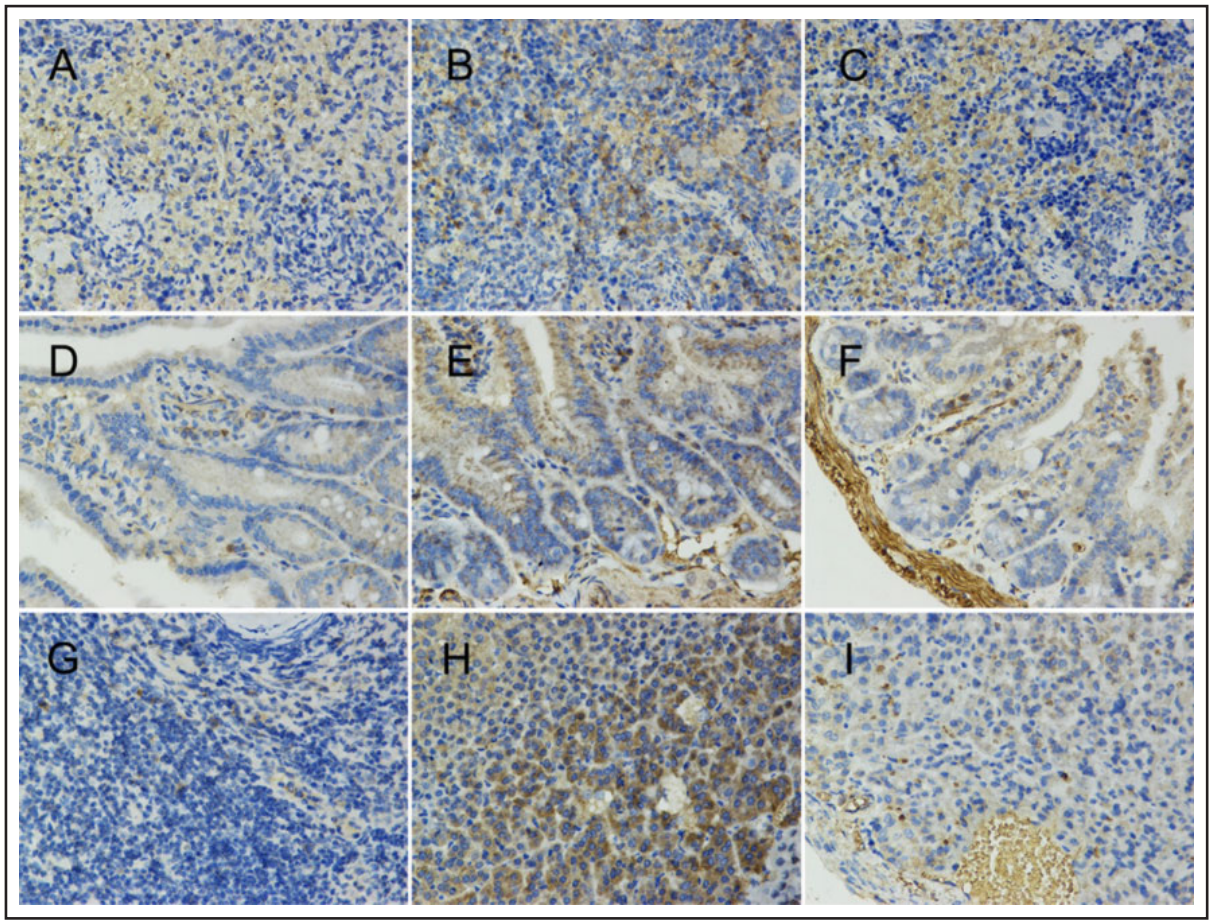

Figure 5. Expression of TLR4 (brown granules) in the spleen, intestine, and lymph nodes in different groups by immunohistochemical analysis. (A-C) The spleen samples of (A) TBA group, (B) TAU group, and (C) blank control. (D-F) The intestine samples of (D) TBA group, (E) TAU group, and (F) blank control. (H and I) The lymph node samples of (H) TBA group, (G) TAU group, and (I) blank control (200×).

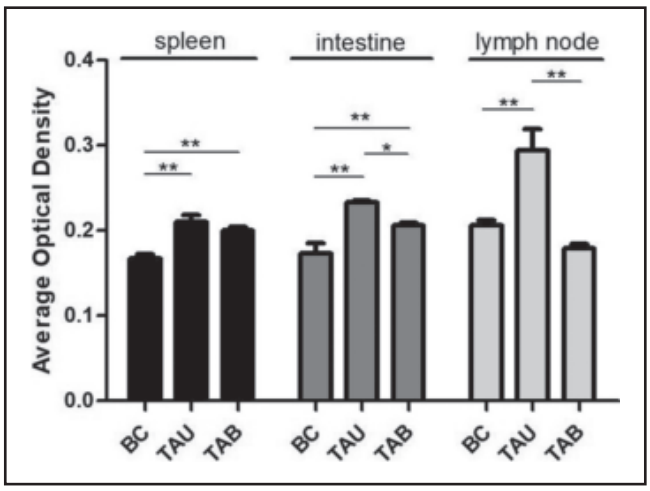

Figure 6. Quantitative analysis of TLR4 expression. Immunostaining was visualized with DAB, representing the expression of TLR4. The density of DAB was analyzed with ImageJ, using the average optical density for comparison.

the lowest in the control group, while no difference was found in the spleen (Figure 5 A-C).

Futhermore, on the 12 dpi, pathological changes was observed in the small intestine in two infection groups; in the TAB group, intestinal mucosal epithelial cells were widely damaged due to villous atrophy (Figure 5 D-E).

To quantitatively analysis the expression of TLR4 in spleen, intestine, and lymph nodes on the 12 dpi, ImageI was used to determine the density of DAB. As shown in Figure 6, the optical density of TLR4 from the spleen of mice in the BC, TAU and TAB groups was 0.160, 0.211, 0.202, respectively. The expression of TLR4 of was higher in the TAU and TAB groups than in the BC group, with significantly differences ($P < 0.01$), but no difference was found between the TAU and TAB groups ($P > 0.05$). The optical density was 0.172, 0.232, and 0.201 in the intestine. The expression of TLR4 was higher in the TAU and TAB groups than that in the BC group, with obvious difference ($P < 0.01$). In lymph nodes, the optical density was 0.293. The expression of TLR4 in the TAU group was noticeably different from that in the TAB and BC groups ($P < 0.01$), but no difference was observed between the TAB and BC groups ($P > 0.05$).

DISCUSSION

Several studies have indicated that some parasites, such as *Toxoplasma gondii* and *C. parvum,* have the ability to migrate into DCs and exploit these cells to disseminate through the body (Lantier *et al*., 2013; Perez-cordon *et al*., 2014). In this study, CD11c marker was used to investigate the maturation of DCs *in vitro*. *C. parvum* could promote mouse DC maturation in the presence of TLR4; the expression of CD11c was five times higher in DCs expressing TLR4 (TAU group) compared with those in which TLR4 was blocked (TAB group). However, the expression of CD11c was not completely blocked in the TAB group, suggesting the presence of another receptor associated with the activation, such as DC-Sign. In addition, after infecting DCs for 24 h, a higher adhesion of *C. parvum* sporozoites to DCs was observed in the TAU group compared with the TAB group. However, once again, the adhesion of *C. parvum* sporozoites to DC was not completely inhibited in the TAB group, further suggested that TLR4 might not be the only mediator in activation of these cells.

Several mouse models have been used for investigating *C. parvum* infection and exploring its immune reaction *in vivo*. In addition, younger mice have shown to be more susceptible to *C. parvum* (Sayed *et al*., 2016; Schaefer *et al*., 2016). In this study, 4-week old mice were infected with *C. parvum* to investigate the role of TLR4 in inducing the immune response against *C. parvum* infection. The obtained data indicated that the discharge of the oocysts in mouse feces was the highest 5 dpi. In fact, the host infected with *C. parvum* displayed elongation when the function of TLR4 was inhibited, in turn suggesting that TLR4 was directly related to the chronic infection of *C. parvum in vivo*.

T cells have an important role in defending the host against *C. parvum* infection. $CD4^+$ and $CD8^+$ T cells appears to be an important component in host's response to *C. parvum*. CD4 T lymphocytes have been shown to be essential in preventing and controlling *Cryptosporidium* (Adjei *et al*., 2000; Mcnair *et al*., 2013). In this study, the expression of $CD3^+$, $CD4^+$ and $CD8^+$ from spleen lymphocytes after infection with *C. parvum* was detected by flow cytometry. Briefly, significantly higher expression of $CD4^+$ and lower expression of $CD8^+$ were found after infection, while significantly lower expression of $CD4^+$ and higher expression of $CD8^+$ were found in cells in which TLR4 was blocked. Therefore, the hypothesis that TLR4 is a multifunctional protein participating in the proliferation of T cells during *C. parvum* infection and their growth was proposed.

The induction of IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-15, IFN-γ and other cytokines have been reported as relative to *C. parvum* infection (Mateen *et al*., 2016). Among these cytokines, IFN-γ and IL-12 have shown to be essential in the host's defense against *C. parvum* infection in mice and humans. Ehigiator *et al*. demonstrated that mice with variants of IL-12 could develop chronic infection with *C. parvum* (Ehigiator *et al*., 2005). IFN-γ knockout mice suffered a more severe infection of *C. parvum* compared with the control mice (Ehigiator *et al*., 2007). Additionally, activated DCs have shown the ability to release IL-12 and IFN-γ in response to various infections (Xu *et al*., 2017). Robinson *et al.* indicated that TLR4, IL-4 and IL-6 were involved in the clearance of *Cryptosporidium* (Robinson *et al*., 2001). In this study, the expression levels of TLR4, IL-6, IL-4, IL-12, IFN-γ and IL-18 in the peripheral blood were detected after infection using q-PCR. The expression of these factors was significantly upregulated after infection with *C. parvum*. Further, significantly higher expression levels of IL-4, IL-12, IL-18, IFN-γ and TLR4 were observed in the TAU group compared with the TAB group ($P<0.05$), while no difference in the expression of IL-6 was found between the TAU and TAB groups. These data suggested that *C. parvum* induced TLR4 to enhance the expression of IL-12, IL-18 and IFN-γ, thus activating Th1-type immune response, and it was predominant. In addition, IL-4 also could be induced at the higher expression level. The findings also showed that Th2-type immune response could be activated.

C. parvum is a well-known intestinal parasite that can cause severe pathological changes in the gastrointestinal tract of the host (Kosik-Bogacka *et al*., 2012). In this study, an immunohistochemical approach was used to further investigate the pathological changes in murine gut infected with *C. parvum*. Briefly, serious damage to the mucosal structure, shorter villus, and a large number of shedding intestinal epithelial cells were found in small intestine compared with uninfected control mice. Further, high expression of TLR4 was observed in the spleen, intestine and lymph nodes of mice infected with *C. parvum*.

CONCLUSIONS

In conclusion, following activation with TLR4, TLR4-mediated DCs recognize *C. parvum* once it invades the host, playing a fundamental role in host innate and adaptive immunity instead of capturing and submitting antigens. Moreover, activated DCs promote the proliferation of $CD4^+$ T cells to release Th1-type cytokines and resist *C. parvum* infection.

ACKNOWLEDGMENTS

This study was supported by the key research project of National Foundation for Science and Technology (Grant No. 2018YFD0500100, 2018YFD0502006 and 2016YFC1202700), the Natural Science Foundation of Anhui (Grant No. KJ2016A838), the Natural Science Foundation of Shanghai (Grant No. 18ZR1443500), the General Program Shanghai Municipal Commission of Health and Family Planning of China (No. 201840286 and 201640278), the Youth Science Foundation of Chinese Center for Disease Control and Prevention (No. 2018A105), Shenzhen San-Ming Project for prevention and research on vector-borne diseases (SZSM201611064), the Fifth Round of Three-Year Public Health Action Plan of Shanghai (Grant No. GWV-10.1-XK13), the Open project of Key Laboratory of Tropical Disease Control (Sun Yat-sen University), Ministry of Education (Grant No. 2019kfkt02), and the Key Laboratory of Echinococcosis Prevention and Control (Project No. 2020WZK2002).

Conflicts of interest

The authors declare no conflicts of interest with regards to this study or the manuscript prepared for publication.

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