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Expression of TLR2, TLR3, TLR4, and TLR7 on pulmonary lymphocytes of *Schistosoma japonicum*-infected C57BL/6 mice

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Abstract

Despite the paramount role of TLRs in the induction of innate immune and inflammatory responses, there is a paucity of studies on the role of TLRs in *Schistosoma japonicum* infection. Here, we observed obvious infiltration of inflammatory cells in *S. japonicum*-infected C57BL/6 mouse lungs. Expression and release of IFN-γ, IL-4, and IL-17 were significantly higher in pulmonary lymphocytes from infected mice compared with control mice in response to anti-CD3 plus anti-CD28 mAbs. Higher percentages of TLR2, TLR3, TLR4, and TLR7 were expressed on such lymphocytes, and the TLR agonists PGN, Poly I:C, LPS, and R848 induced a higher level of IFN-γ. However, a higher level of IL-4 was found in the supernatant of pulmonary lymphocytes from infected mice stimulated by these TLR agonists plus CD3 Ab. Only R848 plus anti-CD3 mAb could induce a higher level of IFN-γ in such lymphocytes. TLR expressions were then compared on different pulmonary lymphocytes after infection, including T cells, B cells, NK cells, NKT cells, and $\gamma\delta$T cells. The expression levels of TLR3 on T cells, B cells, NK cells, and $\gamma\delta$T cells were increased in the lungs after infection. NK cells also expressed higher levels of TLR4 after infection of control mice. Collectively, these findings highlight the potential role of TLR expression in the context of *S. japonicum* infection.

Keywords

Schistosoma japonicum, lung, TLRs, cytokines, ligands

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Introduction

Schistosoma japonicum is one of the major infectious agents of schistosomiasis, which causes significant morbidity and mortality, especially in developing countries.[1,2] Schistosomula and its egg migration in the lung can trigger immune pathological reactions, including the formation of granulomas, fibrosis, and interstitial pulmonary disease.[3] The outcome of schistosome infection relies on both innate and adaptive immune responses,[4] including immune cell cytokines, adhesion molecules, and chemokines.[5] Additionally, granulomatous inflammation against parasite eggs is the hallmark of schistosome infection.

TLRs, which are a class of PRRs, are germline-encoded innate immune receptors.[6,7] Mammalian TLRs, central innate receptors, are able to distinguish distinct pathogen-associated molecular patterns (PAMPs) from viruses, bacteria, and parasites. Binding of TLRs with their specific ligands induces a signaling cascade resulting in the induction of type I

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IFNs and other cytokines, which drive an inflammatory response and activate the adaptive immune system.[8,9] The central role is to protect the host by perceiving danger and detecting the presence of invading pathogens. They are expressed on extensive immune cell types, such as T and B cells, NK cells, macrophages, and dendritic cells (DCs), and also by a number of non-immune cells.[7–9] Although *in vivo* functions of TLRs during bacterial, viral, and, to a lesser extent, during fungal and protozoan parasitic infections have been extensively reported,[10] finite studies have addressed the role of TLRs in the immune response to *S. japonicum* infections.[11]

In mice and humans combined there are 13 paralogous TLRs; 10 in humans and 12 in mice.[12] They are trans-membrane receptors that are found either on the cell membrane (TLR1, 2, 4, 5, and 9) or on intracellular organelles (TLR3, 7, and 8).[13,14] It is well known that each TLR family member recognizes a specific pathogen component and, upon activation, triggers a signaling cascade leading to cytokine production and adaptive immune response.[15,16] Among potential signaling PRRs, TLR2, TLR3, and TLR4 have been identified as central sensors of parasite and egg components during *S. mansoni* infection by some scholars.[17–19] Moreover, Wang et al. have demonstrated that TLR7/8 ligands could enhance the protective efficacy of DNA vaccines against schistosomiasis.[8] In our study, we tested the response to TLR ligands PGN, Poly I:C, LPS, and R848 as measured by IFN-γ and IL-4 secretion *in vitro*.

In this study, the expressions of TLRs in different kinds of innate immune cells were compared by flow cytometry between normal and *S. japonicum*-infected mice, and TLR agonists were used to detect the function of TLRs in the progress of *S. japonicum* infection.

Materials and methods

Mice

Female C57BL/6 mice (6–8 wk old) were acquired from Zhongshan University Animal Center (Guangzhou, China) and fed in a specific-pathogen-free facility at Guangzhou Medical University. All animal experiments were performed in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (1988.11.1). The animal protocols were approved by the Committee on the Ethics of Animal Experiments of Guangzhou Medical University.

Parasite infection

S. japonicum cercariae were shed from naturally infected *Oncomelania hupensis* snails, which were purchased from Jiangsu Institute of Parasitic Disease (Wuxi, China). Female C57BL/6 mice were divided into two groups, 30 as control (normal group) and 30 infected with 40 $\pm$ 5 cercariae of *S. japonicum* per mouse (infected group) and killed at 6 wk after infection.

Abs

The FITC-conjugated anti-mouse CD3 (17A2), PE-Cy7-conjugated anti-mouse NK1.1 (PK136), FITC-conjugated anti-mouse TCR-cdCR (17A2), Alexa Fluor-conjugated anti-mouse TLR2 (6C2), APC-conjugated anti-mouse TLR3 (118F), PE-conjugated anti-mouse TLR4 (TF901), PE-conjugated anti-mouse TLR7 (A94B10), and isotype-matched control mAb (X39, G155-178) were purchased from BD/Pharmingen (San Diego, CA). The neutralizing rat anti-mouse TLR2 (clone TC11-18H10.1) and an isotype-matched rat IgG2a mAb (clone RTK2758) were purchased from BioLegend (San Diego, CA).

Histology studies

Lungs were removed from the mice and perfused with 0.01 M PBS (pH 7.4) for three times, fixed in 10% formalin, paraffin embedded, and serially sectioned. Standard hematoxylin and eosin staining was done. The slices were examined by light microscopy under 100$\times$ magnification.

Lymphocyte isolation

Mice were anesthetized and fixed from wk 5 and 7 after infection. The excised lung was cut to small pieces and incubated in 5 mL of digestion buffer (collagenase IV/DNase I mix, Invitrogen Corporation) for 30 min, at 37 and 5% carbon dioxide. The digested lung tissue was pressed through 200-gauge stainless-steel mesh, and then was suspended in Hank's balanced salt solution (HBSS). Lymphocytes were isolated by Ficoll-Hypaque (DAKEWE, China) density gradient centrifugation. Isolated cells were washed twice in HBSS and re-suspended in complete RPMI 1640 medium supplemented.

Cytometric bead array (CBA)

Single-cell suspensions were cultured in 96-well microtiter plates at 4×10^5 cells/200 μl medium per well in the presence of anti-CD3 mAb plus anti-CD28 mAb and supernatants were collected 72 h later. Levels of the released cytokines in supernatants were determined by using Mouse Th1/Th2 Kit FlowCytomix (eBioscience). CBA kit was performed in accordance with the manufacturer's instructions. The samples were analyzed on flow cytometry (BD Calibur and Aria II).

RNA preparation for real-time PCR

Total RNA of pulmonary cells was isolated by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). 1 μg of total RNA was transcribed to cDNA by using a SuperScript III Reverse Transcriptase Kit (Qiagen, Valencia, CA). Primers were synthesized by Invitrogen (Shanghai, China) and were TLR2 sense 5-CTCTCCGTCCCAACTGATGA-3, antisense 5-GGTCTGGTTGCATGGCTTTT-3; TLR3 sense 5-ATTCGCCCTCCTCTTGAACA-3, antisense 5-TCGAGCTGGGTGAGATTTGT-3; TL4 sense 5-AGGTTGAGAAGTCCCTGCTG-3, antisense 5-GGTCCAAGTTGCCGTTTCTT-3; TLR7 sense 5-G CATTCCCACTAACACCACC-3, antisense 5-ACACA CATTGGCTTTGGACC-3 (reverse); β-antisense 5-CC GTAAAGACCTCTATGCCAAC-3, antisense 5-G GGTGTAAAACGCAGCTCAGTA-3. mRNA expression was analyzed with RT-qPCR by using Takara SYBR Premix Ex Taq II (RR820A). Reaction mixtures were incubated for 95°C for 30 s, followed by 95°C for 5s and 60°C for 30s (40 cycles) . In all cases, amplification was tested in triplicate. Amplification was performed by using the CFX96 touch qPCR system (Bio-Rad, Hercules, CA, USA), and the levels of TLR transcripts were normalized to β-actin transcripts by using the relative quantity $(RQ) = 2^{-\Delta\Delta Ct}$ method.

ELISA detection

Single-cell suspensions were cultured in 96-well microtiter plates at 4×10^5 cells/200 μl medium per well and challenged with either PGN, Poly I:C, LPS, R848 or together with anti-CD3 Ab, respectively. Then, supernatants were collected, and concentrations of the different cytokines were determined by using mouse cytokine kits for IFN-γ (BD Pharmingen) and IL-4 (BD Biosciences). ELISAs were performed in accordance with the manufacturer's instructions. The optical density of each well was read at 450 nm by using a microplate reader (Model ELX-800, BioTek).

FACS detection

Single cell suspensions from the lungs of control mice and mice infected with *S. japonicum* were collected as described before. Cells were stained with conjugated Abs specific for the cell surface Ags CD3, CD19, NK1.1, and $\gamma\delta$TCR, respectively. After washing in PBS, cells were fixed with 4% paraformaldehyde, permeabilized overnight at 4°C in PBS buffer containing 0.1% saponin (Sigma), 0.1% BSA, and 0.05% NaN_3, then stained with conjugated Abs specific for mouse TLRs, including TLR2, TLR3, TLR4, and TLR7. Ab-Labeled lymphocytes (200,000–300,000 cells per run) were acquired on flow cytometry (BD Calibur and Aria II) and data were analyzed by using Cell Quest software (BD Biosciences). Isotype-matched controls were included in each staining protocol.

Statistics

Data was expressed as mean $\pm$ SD and statistical evaluation of difference between means was performed by unpaired, two-tailed t tests. All statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) and $P < 0.05$ was considered significant.

Results

Pathological inflammation in the infected mice

Granulomatous and fibrosing inflammation against parasite eggs are the pathological characters of schistosome infection. To examine the pathological changes in the lungs of *S. japonicum*-infected mice, 6–8-wk-old female C57BL/6 mice were infected with *S. japonicum* and were sacrificed 6 wk after infection. Lung tissues were cut into 5 μm sections and stained with hematoxylin and eosin to observe the effects of infection on lung microstructure. The pathologic damage and the infiltration of large amounts of inflammatory cells were observed in infected lung compared to normal control mice (Figure 1a). IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-17, and GM-CSF in the supernatant, which were detected by CBA, were associated with schistosomiasis-associated lung pathology. The releases of IFN-γ, IL-4, IL-5, and IL-17 were barely detectable in cultures of unstimulated lymphocytes from normal and infected lung. However, the levels of these cytokines were substantially higher by anti-CD3 mAb plus anti-CD28 mAb stimulation from normal infected lung than that in normal mice (IFN-γ: 695.4 ± 611.6 pg/mL versus 142.8 ± 236.0 pg/mL, $P > 0.05$; IL-4: 2392.4 ± 928.4 pg/mL versus 11.2 ± 0.8 pg/mL, $P < 0.05$; IL-5: 944.6 ± 657.1 pg/mL versus 3.8 ± 6.6 pg/mL, $P > 0.05$; IL-17: 8443.4 ± 206.1 pg/mL versus 1000.4 ± 1404.6 pg/mL, $P < 0.05$, Figure 1b). Consistent with them, a higher level of IL-6 from infected lung was observed, although not significantly ($P > 0.05$). Taken together, these results suggest that infection by *S. japonicum* markedly altered the histological structure of the mouse lung and induced the cytokines production.

TLR expression on pulmonary cells

To explore the expression of TLR mRNA and protein in pulmonary cells, cells were isolated from normal and infected C57BL/6 mice lungs and were examined by qPCR and FACS, respectively. As shown in Figure 2a, the results demonstrated that the expression

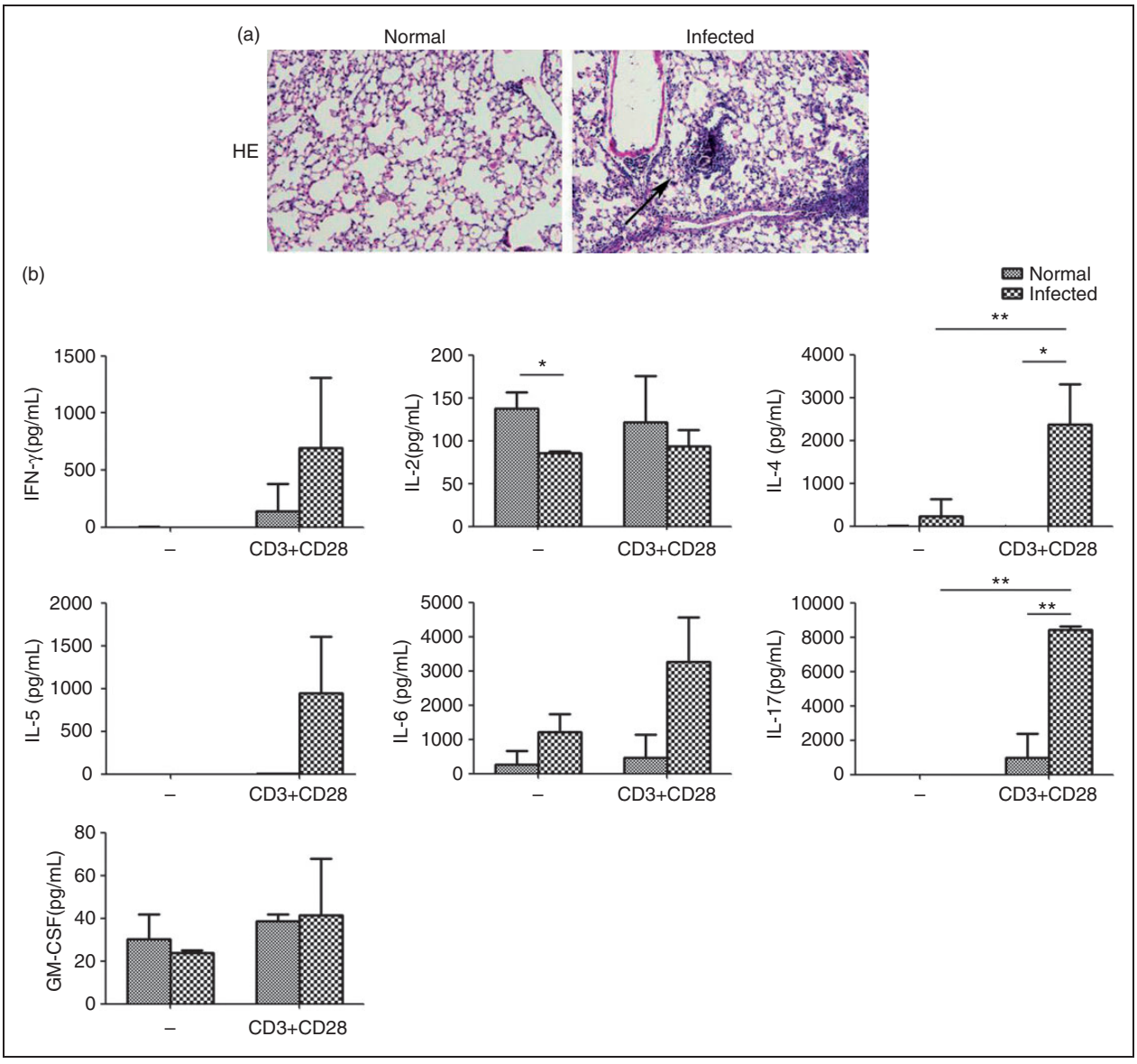

Figure 1. The histopathological changes in the lung of infected C57BL/6 mice. (a) Sections of the lung of normal mice (left panels) and infected mice (right panels) were examined by H&E staining (×100). The multi-cellular granuloma could be observed in the infected group. (b) The levels of IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-17, and GM-CSF were detected by CBA. The data are representative of six experiments, each with three or four replicates per group (*$P < 0.05$, **$P < 0.01$; the error bars indicate SD).

of TLR2 mRNA decreased after *S. japonicum* infection ($P < 0\ 05$), whereas there was no significant difference in the expression of TLR3, TLR4, and TLR7 mRNA between normal and infected mice. Of the normal pulmonary mononuclear cells, the percentages of cells expressing TLR2 and TLR4 protein comprised $18.04 \pm 8.99\%$ and $8.10 \pm 2.67\%$, respectively. After infection, their protein expressions were significantly elevated compared with uninfected lungs (TLR2: $36.40 \pm 16.01\%$, TLR4: $17.55 \pm 8.52\%$; *$P < 0.05$, **$P < 0.01$, Figure 2c). Likewise, the cells expressing TLR3 and TLR7 showed an increase in the infected group, although not significantly compared to normal mice (TLR3: $22.06 \pm 13.32\%$ versus $25.11 \pm 16.15\%$, TLR7: $19.08 \pm 14.26\%$ versus $14.14 \pm 9.16\%$, $P > 0.05$). Thus, we concluded that the infection might induce the expression of TLR2, TLR3, TLR4, and TLR7 protein in infected lung.

TLR expression regulates IFN-γ and IL-4 secretion

These PRRs recognize PAMPs in various cell compartments and trigger the release of inflammatory cytokines and type I IFNs for host defense. Therefore, we sought

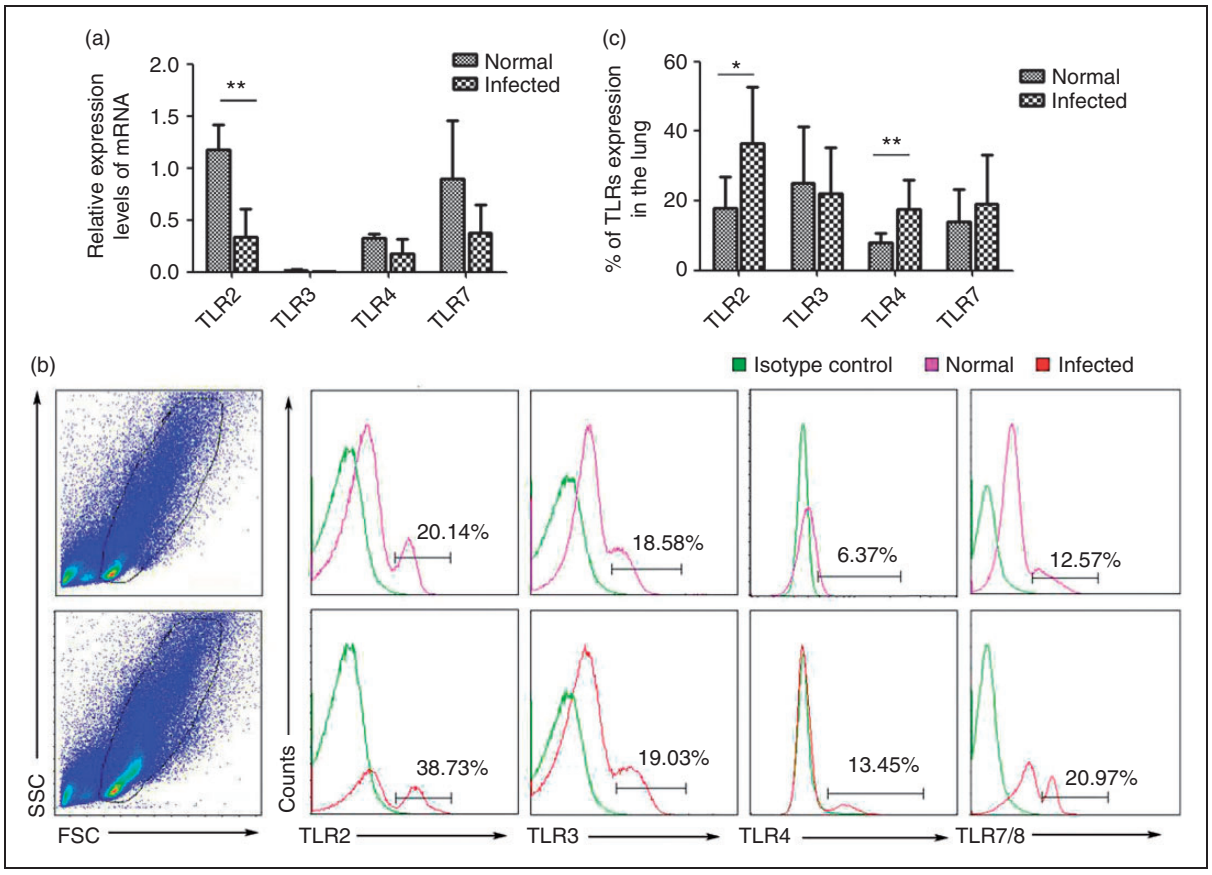

Figure 2. TLR expressions in lymphocytes isolated from control or infected mouse lung. (a) The accumulation of TLR2, TLR3, TLR4, and TLR7 mRNA was quantified by using qPCR. (b) The expression of TLR2, TLR3, TLR4, and TLR7 protein from control or infected lung were analyzed by flow cytometry. Flow cytometric analysis from one representative experiment. (c) Average percentages of TLRs in normal and infected mice were calculated from FACS data ($n = 6$) (*$P < 0.05$, **$P < 0.01$; the error bars indicate SD).

to analyze whether TLRs upon stimulation by specific ligands (PGN for TLR2, Poly I:C for TLR3, LPS for TLR4, and R848 for TLR7/8) initiate downstream signaling events that induce secretion of IFN-γ and IL-4. The single mononuclear lung cell suspensions of normal and infected mice were cultured in the presence of different ligands and anti-CD3 plus ligand. 72 h later, the culture supernatants were collected, and IFN-γ and IL-4 levels were detected by ELISA. As shown in Figure 3, we noted that increased IFN-γ secretion from infected lung in response to PGN, Poly I:C, LPS, and R848 (*$P < 0.05$, **$P < 0.01$), while there was no significant difference for IL-4 (Figure 3a and c). We noted that the levels of these two cytokines in the supernatant were considerably low in cultures of ligands-stimulated lymphocytes from normal and infected lungs, which was in the range 10–30 pg/mL. However, release of IFN-γ and IL-4 from pulmonary lymphocytes was significantly increased in normal and *S. japonicum* infected group by anti-CD3 plus specific ligands stimulation, especially IL-4. Thus, we concluded that PGN, Poly I:C, LPS, and R848 in infected lung could induce higher levels of IL-4 compared to the normal control (PGN: 1289.82 ± 295.86 pg/mL versus 13.80 ± 2.98 pg/mL; Poly I:C: 1340.63 ± 342.53 pg/mL versus 13.80 ± 2.98 pg/mL; LPS: 1281.41 ± 323.51 pg/mL versus 22.81 ± 8.46 pg/mL; R848: 1780.65 ± 830.27 pg/mL versus 15.40 ± 1.84 pg/mL; **$P < 0.01$) and infected mice exhibited significantly elevated IFN-γ^+ cells in response to R848 plus CD3 compared with control mice.

The percentage and absolute numbers of T cells, B cells, NK cells, NKT cells, and γδT cells

Accumulating evidence indicates that many cells of the adaptive immune system, including T and B cells, but also the innate immune cells, including NK cells and so on, may contribute to immunoregulatory effects during the infection. Therefore, we sought to investigate the effect of *S. japonicum* infection on the percentage of T

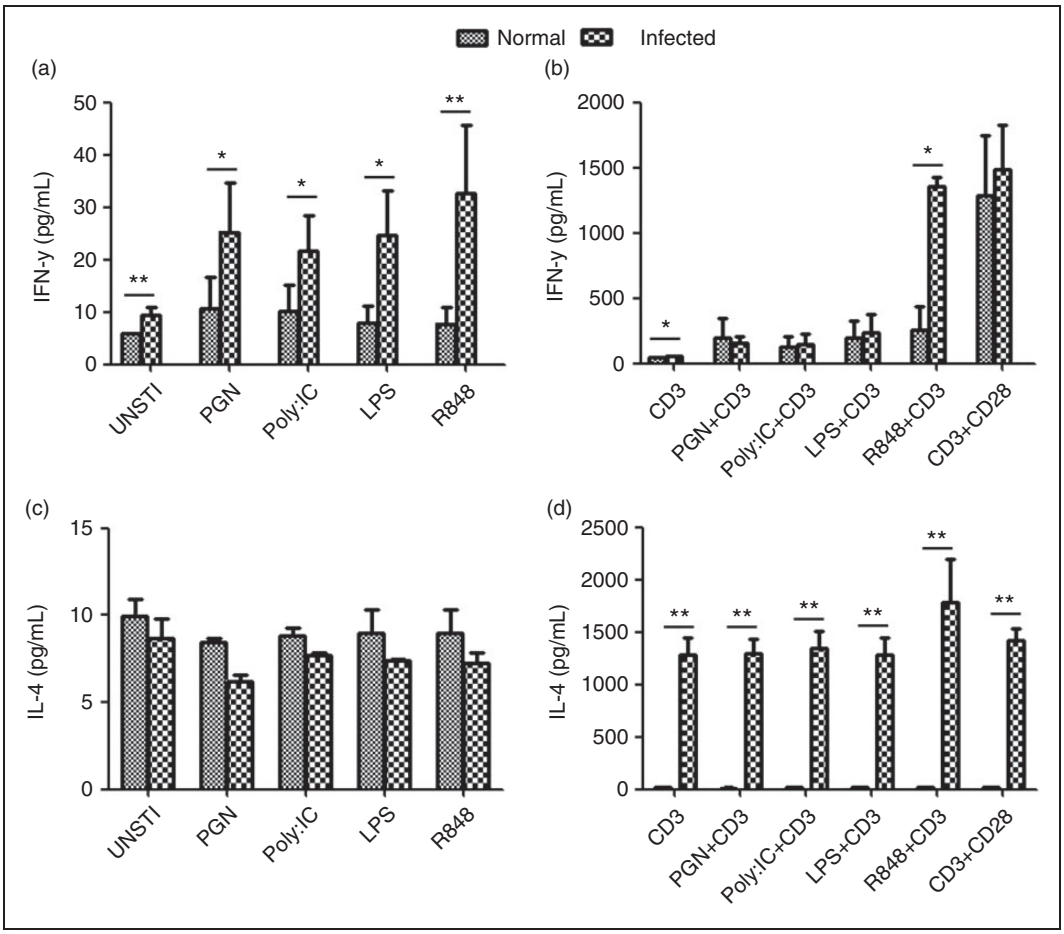

Figure 3. TLRs regulate IFN-γ and IL-4 secretion. Single lung cell suspensions of normal and infected mice were prepared and then cultured in the presence of different ligands plus anti-CD3 mAb. The culture supernatants were collected after 72 h of incubation for detection of IFN-γ and IL-4 by ELISA. The data are representative of four experiments, each with three or four replicates per group (*$P < 0.05$; the error bars indicate SD).

cells, B cells, NK cells, NKT cells, and $\gamma\delta$T cells in the lungs of C67BL/6 female mice by FACS. As shown in Figure 4, there was no significant increase the proportion of T cells ($33.56 \pm 9.17\%$) in the total infected lymphocyte compare to the normal, whereas the absolute number was higher in infected lung ($13.92 \pm 3.81 \times 10^5$ versus $5.88 \pm 1.48 \times 10^5$, $P < 0.01$). Moreover, it indicated that the percentages of NK cells and $\gamma\delta$T cells from infected mice lung were $19.53 \pm 3.72\%$ and $3.71 \pm 1.09\%$, respectively, which were obviously higher than that from normal mice ($14.18 \pm 3.76\%$ and $2.04 \pm 0.32\%$, respectively) ($P < 0.05$, Figure 4). Conversely, the percentages of B cells and NKT cells were $19.60 \pm 8.35\%$ and $5.76 \pm 0.42\%$, respectively, in infected mice, which were less than that of normal mice ($29.71 \pm 7.93\%$, $P > 0.05$; $10.80 \pm 4.13\%$, $P < 0.05$). In addition, considering the dramatically increased number of pulmonary mononuclear cells in response to infection ($4.15 \pm 0.99 \times 10^6$ to $1.91 \pm 0.23 \times 10^6$), the differences between the number of B cells and NKT cells in the normal and infected lungs were not obvious.

TLR expression in different lymphocytes isolated from infected and uninfected mouse lung

To characterize TLR distribution in different lymphocytes, changes in TLR expression were determined after infection. Lymphocytes were isolated from normal and infected C57BL/6 mice lungs. Firstly, cells were stained with different fluorophore conjugated Abs for CD3, CD19, NK1.1, and $\gamma\delta$TCR for FACS analysis. T cell, B cell, NK cell, and $\gamma\delta$T cell populations all significantly expressed higher levels of TLR3 ($2.45 \pm 0.90\%$, $12.87 \pm 6.63\%$, $10.27 \pm 1.37\%$, and $7.20 \pm 3.04\%$, respectively) in the infected lung than that in the infected cell population ($8.48 \pm 4.57\%$, $32.05 \pm 14.18\%$, $17.41 \pm 2.30\%$, and $18.48 \pm 8.58\%$, respectively; Figure 5b, *$P < 0.05$, **$P < 0.01$). In contrast,

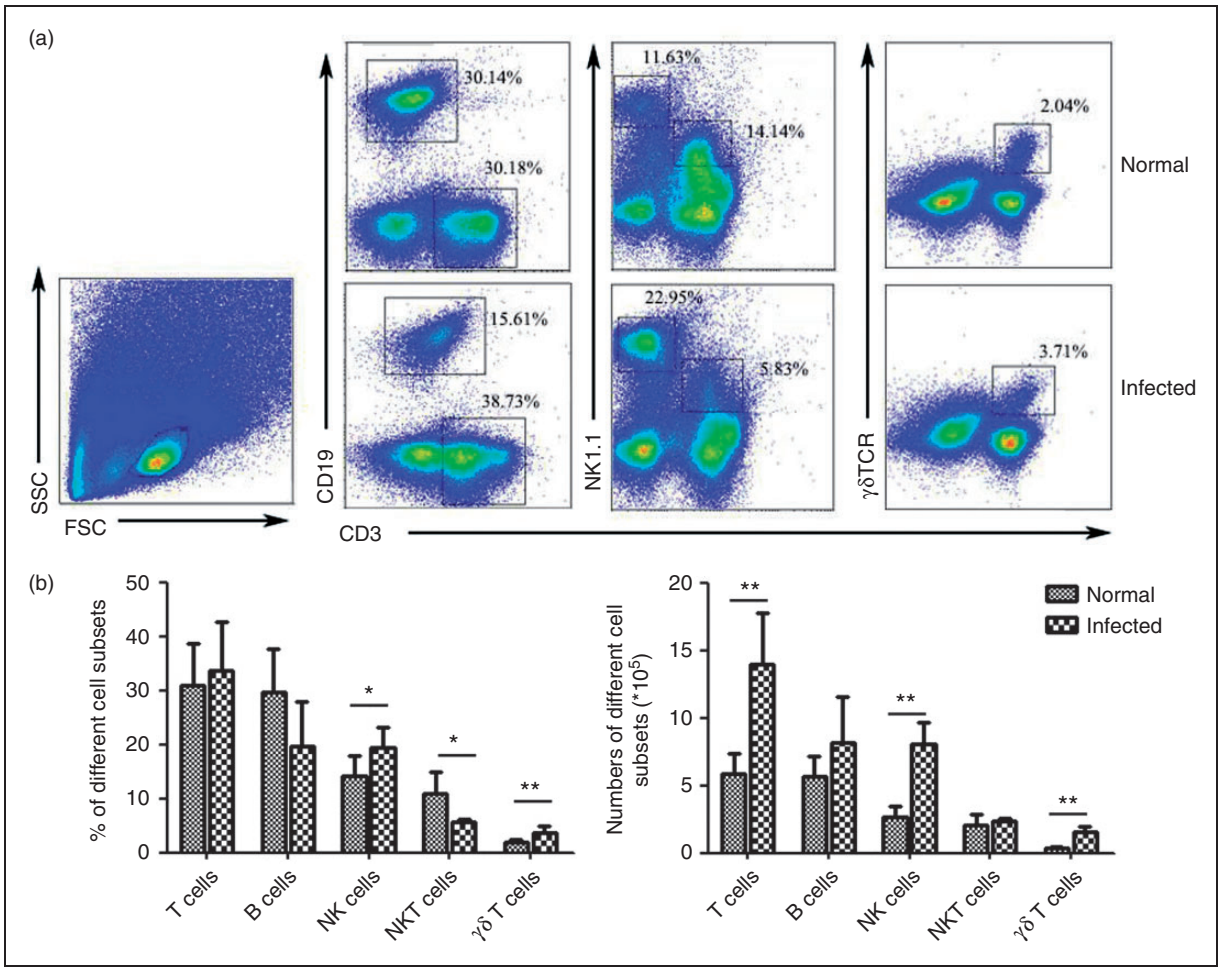

Figure 4. The percentages and absolute numbers of T cells, B cells, NK cells, and $\gamma\delta$T cells. (a) Flow cytometric analysis of CD3, CD19, NK1.1, and $\gamma\delta$TCR expression on normal and infected mice lung lymphocytes is shown. Representative FACS plots are shown ($n = 6$). The numbers represent the percentage of cells in each subset. (b) Average percentages of T cells, B cells, NK cells, NKT cells, and $\gamma\delta$T cells were calculated from FACS with the number of lymphocytes counted under microscope. Cell numbers are from different cell subsets (*$P < 0.05$; the error bars indicate SD).

there was no significant difference in NKT cells between normal and infected mice ($14.39 \pm 1.44\%$ versus $15.35 \pm 2.81\%$). Additionally the proportion of TLR4$^+$ NK1.1$^+$ cells in the lymphocyte population isolated from infected lung was significantly higher compared with uninfected lung ($8.02 \pm 0.67\%$ versus $2.90 \pm 2.37\%$, $P < 0.01$, Figure 5).

Discussion

S. japonicum infections follow direct contact with water cercariae and penetrate the skin of human, then shed their bifurcated tails.[20] The resulting schistosomula enter capillaries and lymphatic vessels en route to the lung. The academics pointed out that even after the parasites have exited the lungs and entered the small intestine, systemic immune response in the lung is sustained.[21] In *S. japonicum*-infected mouse lung, the histopathological changes appear compared with healthy model in this study, as reported. Granuloma formation is the immediate product of complex cellular interactions with the participation of adhesion molecules, cytokines and chemokines. The early phase of schistosomiasis is characterized by a moderate Th1 response. The schistosome egg Ags could induce an immediate and robust inflammatory response after egg deposition. As the disease progressing, Th1 response switches to a dominant Th2 response in the host. Thus, we systemically detected the levels of these cytokines and their relationships with infection. Our results showed that *S. japonicum* infections are effective inducers of the above Th1 (IFN-γ), Th2 (IL-4, IL-5), and Th17 (IL-17) type cytokines response in the lung, which is consistent with our previous report.[20]

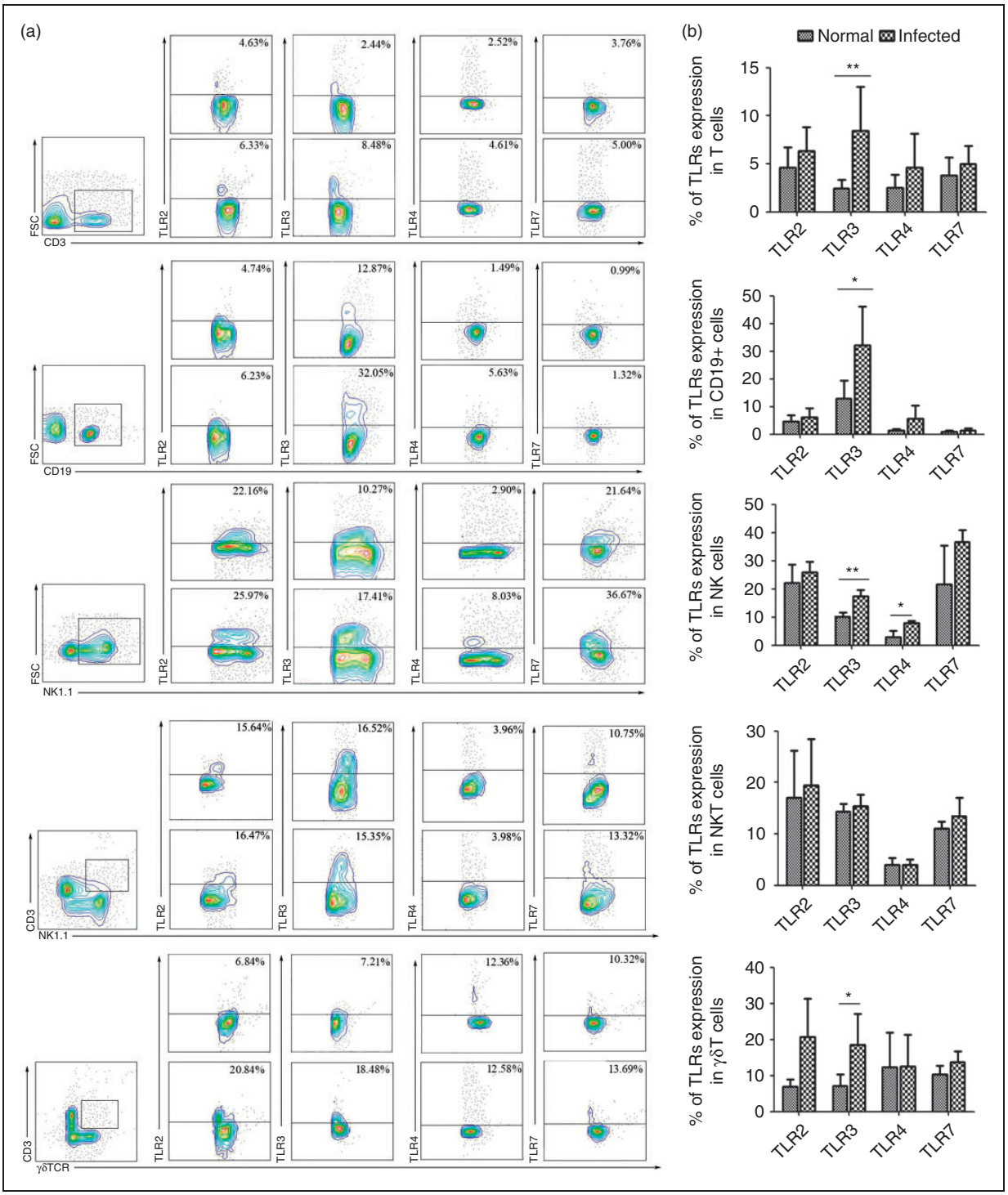

Figure 5. TLR expressions in different lymphocytes isolated from infected and uninfected mouse lung. (a) The percentages of TLR2^{+}, TLR3^{+}, TLR4^{+}, and TLR7^{+} expressed on T cells, B cells, NK cells, NKT cells, and $\gamma\delta$T cells, respectively. Flow cytometric analysis from one representative experiment. (B) The percentages of different TLRs expressed on T cells, B cells, NK cells, NKT cells, and $\gamma\delta$T cell in the lung were calculated. The results represent for ten independent experiments (*$P < 0.05$, **$P < 0.01$; the error bars indicate SD).

Although some experiments indicated that the results generated from the model differ from those of infection, the different results with cytokines seem to reflect their correlation with granuloma formation.

The potential contribution of TLRs in fighting parasitic infections has gained much attention in the last decade.[22] Among numerous TLRs, TLR2, and TLR4 are the most important sensors of parasite components during *S. japonicum* infection.[23] TLR3 interacts with dsRNA from *Schistosoma* eggs.[24] Ashour demonstrated that other TLR-related genes are generally decreased during the course of *Schistosoma* infection.[25] Consistent with it, we observed that the significantly reduced expression of TLR2 mRNA in the lung after *Schistosoma* infection. Our results showed that the higher levels of TLR2 and TLR4 protein existed in *S. japonicum*-infected lung tissue compared to normal mice, whereas levels of TLR3 and TLR7 were not changed significantly ($P > 005$). The results indicated that infection might induce the expression of TLR2 and TLR4 protein in the lung. TLRs are classic pattern recognition receptors, which bind virulent factors from pathogens and initiate synthesis of pro-inflammatory cytokines (e.g. CXCL8). Therefore, irrespective of tissue type, levels of TLR expression have profound impacts on cytokines secretion, no matter produce or impress. The higher expression of TLR2 and TLR4 in the infected lung may induce some kind of cytokines but further investigation is required. In agreement with that, in studies involving pulmonary inflammation in response to bacterial endotoxins in rabbits, blocking TLR4 reduced CXCL8 expression in bronchoalveolar lavage fluid.[26]

The infection by *S. japonicum* induces the production of multiple cytokines that mediate the immune response, which is a multi-cellular parasite with an extremely diverse repertoire of Ags.[27] It is known that TLRs are classic pattern recognition receptors that bind virulent factors from pathogens and initiate synthesis of pro-inflammatory cytokines.[28,29] In studies involving pulmonary inflammation in response to bacterial endotoxins in rabbits, blocking TLR4 could reduce CXCL8 expression in bronchoalveolar lavage fluid.[30] We observed that in the presence of TLR2, TLR3, TLR4, and TLR7/8 ligands, IFN-γ was induced in the infected lung compared to the control. Stimulation by anti-CD3 mAb plus R848 induced higher IFN-γ release from lymphocytes of infected mice than from lymphocytes of uninfected mice ($P < 005$). Release of IL-4 into the supernatant was barely detectable in cultures of different types of TLR ligands stimulating lymphocytes from normal and infected lung, but was significantly induced in cell populations by anti-CD3 mAb plus TLR ligands stimulation ($P < 0.01$). *In vitro* studies have shown that anti-CD3 mAb can also be potent activators of T cells. Consistently, we showed that secretion of Th1 cytokine (IFN-γ) by infected lung lymphocytes was significantly enhanced upon challenge with anti-CD3 mAb stimulation plus R848 *in vitro*. Moreover, in the infected lung, TLR ligands with anti-CD3 mAb could significantly induce Th2 cytokine (IL-4). Consequently, almost four kinds of TLRs (TLR2, TLR3, TLR4, and TLR7) were involved in immune response to induce Th2 cytokine, but the secretion of Th1 cytokine only could be effectively stimulated by R848.

B lymphocytes, as positive and negative regulators of immunity, have critical roles in both autoimmune and infectious diseases.[31] NK cells and NKT cells are classic innate immunologic lymphocytes, which participate in immunity to infective diseases, tumors,[32] and transplantation.[33] Our previous study reported that $\gamma\delta$T cells play a considerable role in fighting *S. japonicum* infection in the liver and mesenteric lymph nodes of C57BL/6 mice.[34,35] Numerous studies on the immunology of schistosomiasis have clearly pointed out the immune cells, such as T cells, B cells, NK cells, NKT cells, and $\gamma\delta$T cells, participate in the *S. japonicum* infection. In this report, we observed a significant accumulation of NK cells and $\gamma\delta$T cells in lung infected with *S. japonicum* (Figure 4). Combined, it is likely that NK cells and $\gamma\delta$T cells exert significant effects during the infection. These results are consistent with our previous reports, which implicated NK cells and $\gamma\delta$T cells in the lung during *S. japonicum* infection.

B Cells exert suppressive activity after activation and TLR are critical in this process.[31] Becker et al. reported that NK cells could be activated by *Leishmania* lipophosphoglycan through TLR2.[36] $\gamma\delta$T cells respond to conserved structures, such as PAMPs released during infection and danger-associated molecular patterns (DAMPs), generated in the context of cell damage and stress through their TCR, NK cell receptors (NKRs), and TLRs.[37–39] Fang et al. described TLR2, TLR3, TLR4, and TLR7 mRNA expression in splenic $\gamma\delta$T cells.[40] Expression of TLR1, TLR2, TLR4, and TLR6 can be up-regulated in $\gamma\delta$T cells in response to mitochondrial DAMPs, following tissue burn injury.[41] Our results showed that TLR3 expressed on the T cells, B cells, NK cells, and $\gamma\delta$T cells in the lung is up-regulated after infection compare to the control mice and TLR4 expression is higher in the NK cells. In addition, TLR3 activates Ag-presenting cells and bridges innate and adaptive immunity by coordinating responses of T cells, B cells, NK cells, and $\gamma\delta$T cells. TLR4 stimulation by infection might also lead to positive inflammatory signals on NK cells. Taken together, we can speculate that T cells, B cells, NK cells, and $\gamma\delta$T cells through different types of TLRs contribute to the inflammatory immune response.

In conclusion, our findings demonstrated that TLRs contribute to *S. japonicum* infection, which might provide basic scientific knowledge for the development of new therapeutic approaches for the treatment of schistosomiasis patients.

Declaration of conflicting interests

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