Journal of Immunology Research

Differentiation and Function of T Cell Subsets in Infectious Diseases

Lead Guest Editor: Yuejin Liang
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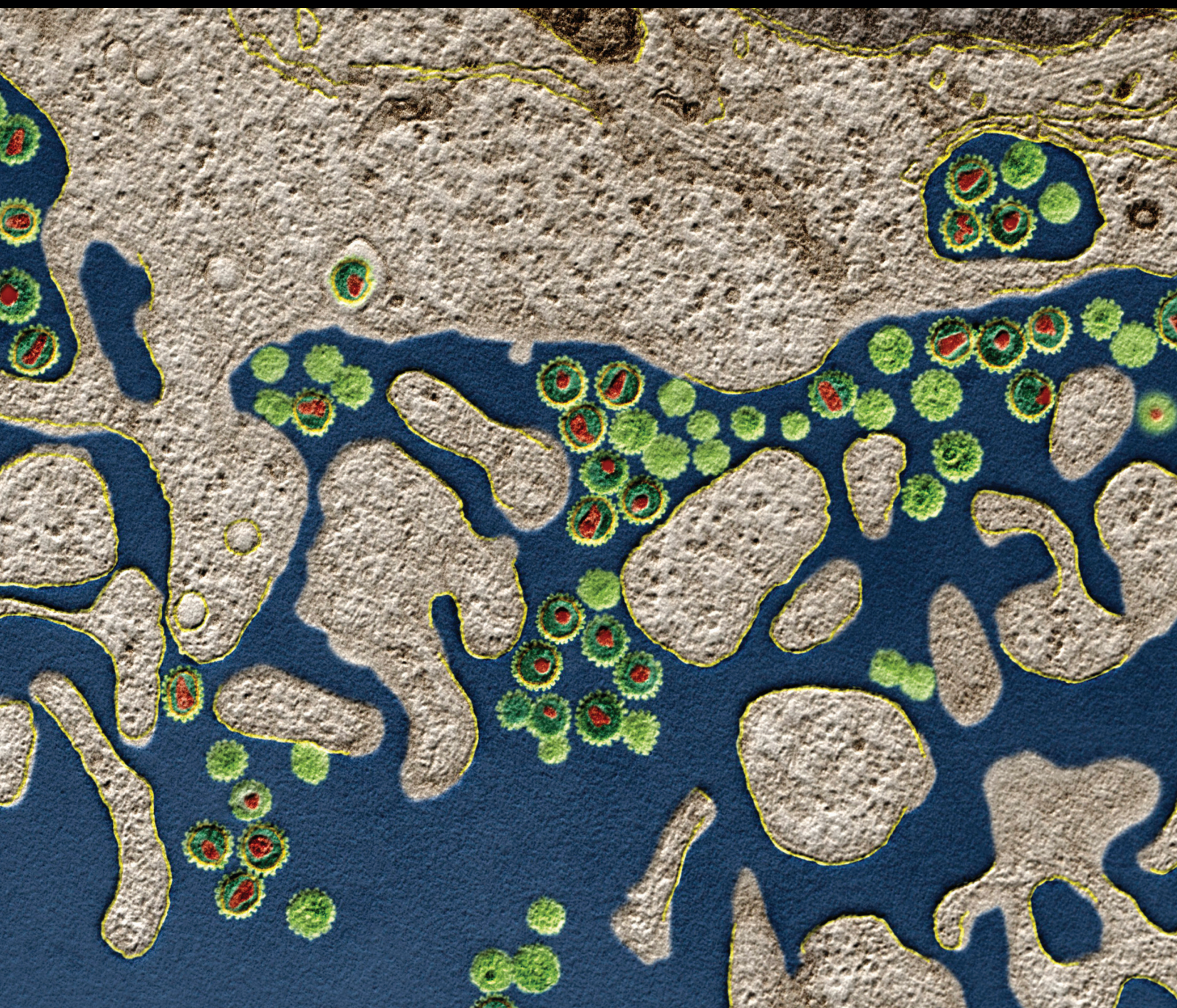

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Editorial Board

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Editorial

Differentiation and Function of T Cell Subsets in Infectious Diseases

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Infectious diseases remain a public health problem in the world, regardless of the continued effort at control. The aim of the host immune response during infection is to clear invading pathogens with limited tissue damage. Both innate and adaptive T cells play a key role in direct pathogen clearance through proinflammatory cytokine and cytotoxic T lymphocyte (CTL) activity. In addition, T helper (Th) cells and regulatory T (Treg) cells are required for plasma cell-secreted antibodies and immunomodulatory cytokines (*e.g.*, IL-10), respectively. In recent years, the role of novel Th cell subsets, including Th17, Th22, and T follicular cells, in regulating anti-infectious immunity, has gained much importance, since they play a crucial role in the development and outcome of diseases. In addition to adaptive T cells, there are unconventional T cells, such as $\gamma\delta$ T cells and NKT cells, which are defined as different subpopulations (*e.g.*, IFN-$\gamma^+\gamma\delta$ T cells vs. $\gamma\delta$17 T cells and IFN-γ^+ NKT cells vs. NKT17 cells) based on their cytokine production signatures. These innate-like T cells rapidly respond to pathogens and display effector functions without undergoing extensive clonal expansion. An understanding of T cell differentiation and function in infectious diseases, as well as their underlying mechanisms, may contribute to the development of potential therapies for emerging infectious diseases in the future.

In this special issue, a total of 15 manuscripts were received and eight manuscripts have been accepted for publication after several rounds of review. Three of these publications are review articles that provide comprehensive information regarding the role of innate and adaptive T cells in infectious diseases. R. Zheng et al. generated a meta-analysis by searching published articles identified as relating to the clinical features of human brucellosis in China. They described a significant decrease of $CD4^+$, but increase of $CD8^+$ T cells, as well as a reduction of $CD4^+/CD8^+$ ratio in the blood of human brucellosis patients compared to healthy subjects. D. A. Cronkite and T. M. Strutt focus on the regulation of inflammation by lymphocytes. The recent advancements in our understanding of inflammatory triggers, imprinting of the innate immune responses, and the role of T cell memory in regulating inflammation are discussed in this review paper. In particular, the field of innate immune cell memory must be further investigated in order to effectively implement this new insight into vaccine design and clinical therapy for infectious diseases. Y. Zhao et al. explore the role of $\gamma\delta$ T cells in different infections and their potential application in the clinic. They focus on various subsets of $\gamma\delta$ T cells, which play a critical role in regulating host immunity against pathogens, including bacteria, viruses, and parasites. Since $\gamma\delta$ T cells are involved in the elimination of pathogens, this cell type might have promising implications for the treatment of infectious diseases in preclinical studies.

Among the five research articles in this special issue, three manuscripts from Chinese research groups are associated with *Schistosoma* or *Schistosoma*/HIV coinfection. Y. Zhu et al. demonstrated that the administration of pioglitazone, which is an agonist of peroxisome proliferator-activated receptor- (PPAR-) γ, reduces splenic and hepatic immunopathogenesis in *Schistosoma japonicum*-infected

mice through inducing Treg cells. Moreover, PPAR-γ-agonist can promote Foxp3 expression through both macrophage-dependent and independent manners. Y. Yang et al. demonstrate that the $CD4^+/CD8^+$ T cell ratio is lower in *Schistosoma*/HIV coinfection patients compared with levels in patients with HIV- or *Schistosoma*-infection only. This result suggests significant immune suppression in coinfection patients. X. Chen et al. determine that Good's syndrome (GS) patients have an inverted ratio of $CD4^+/CD8^+$ T cells, more Treg cells, and a lower percentage of Vγ2 subpopulation in $\gamma\delta$ T cells in the blood. In particular, $CD4^+$ T cells from GS patients show an insufficient ability to proliferate and express higher levels of PD-1, explaining the impaired $CD4^+$ T cell responses in GS patients.

There are two manuscripts that are not directly linked to the study of T cells but highlight the important immune components that can regulate T cell responses. R. C. Araújo et al. detected an increased level of human leukocyte antigen E (HLA-E) in hepatocytes and Kupffer cells of HCV patients compared with that in health people. Since HLA-E can bind and present peptides to antigen-specific $CD8^+$ T cells, it may play a role in regulating T cell activation in HCV patients. J. Qu et al. underscore the immunomodulatory role of Toll-like receptor (TLR3) in NK cells. They demonstrate that $TLR3^+$ NK cells display activated phenotypes as evidenced by upregulated surface activation markers and increased cytokine production. Although TLR3 expression is comparable on T cells after *Schistosoma* infection, TLR3-dependent NK cell activation may regulate T cell responses through cell-cell interaction and the cytokine microenvironment in lymphoid organs.

In summary, this special issue covers several important aspects of T cell functions related to infectious diseases. We hope that these articles can provide guidance for further research on T cell functions and effective therapeutics.

Conflicts of Interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

Acknowledgments

We would like to thank all the authors who submitted their high-quality manuscripts to this special issue and provided new insights into T cell study. We would like to thank the reviewers and editors, who made great efforts to improve the quality of this special issue. We also thank Dr. Linsey Yeager for assistance with manuscript preparation.

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Research Article

TLR3 Modulates the Response of NK Cells against *Schistosoma japonicum*

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Natural killer (NK) cells are classic innate immune cells that play roles in many types of infectious diseases. NK cells possess many kinds of TLRs that allow them to sense and respond to invading pathogens. Our previous study found that NK cells could modulate the immune response induced by *Schistosoma japonicum (S. japonicum)* in C57BL/6 mice. In the present study, the role of TLRs in the progress of *S. japonicum* infection was investigated. Results showed that the expression of TLR3 on NK cells increased significantly after *S. japonicum* infection by using RT-PCR and FACS ($P < 0.05$). TLR3 agonist (Poly I:C) increased IFN-γ and IL-4 levels in the supernatant of cultured splenocytes and induced a higher percentage of IFN-γ- and IL-4-secreting NK cells from infected mouse splenocytes ($P < 0.05$). Not only the percentages of MHC II-, CD69-, and NKG2A/C/E-expressing cells but also the percentages of IL-4-, IL-5-, and IL-17-producing cells in TLR3$^+$ NK cells increased significantly after infection ($P < 0.05$). Moreover, the expression of NKG2A/C/E, NKG2D, MHC II, and CD69 on the surface of splenic NK cells was changed in *S. japonicum*-infected TLR3$^{-/-}$ (TLR3 KO mice, $P < 0.05$); the abilities of NK cells in IL-4, IL-5, and IL-17 secretion were decreased too ($P < 0.05$). These results indicate that TLR3 is the primary molecule which modulates the activation and function of NK cells during the course of *S. japonicum* infection in C57BL/6 mice.

1. Introduction

Schistosomiasis japonica is a chronic helminth infection of humans caused by *S. japonicum* [1, 2]. The eggs of *S. japonicum* are deposited in the liver, lung, and intestinal wall and induce granulomatous inflammation and progressive fibrosis, which are the primary clinical pathological changes. There are many types of cells involved in the fight against invading *S. japonicum* and its eggs, including Th cells, natural killer (NK) cells, NKT cells, myeloid-derived suppressor cells (MDSCs), and macrophages [3–6]. Thus, obvious changes could be detected in the immune organs, such as the spleen and local lymph nodes [7, 8].

NK cells are innate lymphocytes that respond rapidly to invading pathogens by exerting a direct cytotoxic effect or secreting various cytokines, particularly interferon-gamma (IFN-γ) [7] Recent studies have reported that NK cells are able to survive long enough to take part in the adaptive immune response [9], and NK cells could play an important role in the immune response of host against pathogen and tumor [10]. In parasite infection, both activated and inhibitory receptors such as CD16, CD69, NKG2D, and Ly49a on

NK cells were downregulated after a 16-day post-*Angiostrongylus cantonensis* infection in mice [11]. The decrease of circulating frequency of $CD56^{+}CD161^{+}$ NK cells in human visceral leishmaniasis [12] and the downmodulation of effector functions in NK cells upon *Toxoplasma gondii* infection were both found too [13]. The negative regulatory role of NK cells in *S. japonicum* egg-induced liver fibrosis was found [14]. Our previous research has found that Th2-like response was induced in the splenic NK cells of *S. japonicum*-infected mice [7].

Toll-like receptors (TLRs) are evolutionarily conserved molecules that were originally identified in vertebrates on the basis of their homology with Toll [15] mammalian TLRs are a family of at least 12 membrane proteins that trigger innate immune responses. The TLR family members are pattern recognition receptors (PRRs) that recognize lipid, carbohydrate, peptide, and nucleic acid structures collectively, which are expressed widely by different groups of microorganisms [16]. TLR2, TLR3, and TLR4 could response to helminth antigens and modulate the activation of dendritic cells during *S. japonicum* infection [17, 18]. TLR3 was reported to modulate immunopathology during *Schistosoma mansoni* egg-driven Th2 responses in the lung [19].

NK cells possess many kinds of TLRs that allow them to sense and respond to invading pathogens. It was reported that in healthy controls, TLR2 and TLR4 of NK cells are mainly intracellular expressed which is similar to TLR9 [20]. TLRs could mediate activation of NK cells in bacterial/viral immune responses in mammals [21]. TLR3 and TLR7 activation in uterine NK cells might play important roles in nonobese diabetic (NOD) mice [22]. Immune response modifiers (IRMs) could modulate NK cell function both in vitro and in vivo, and human NK cell activation was controlled in distinct indirect pathways by TLR7 and TLR8 agonists [23]. In this study, the roles of TLRs on NK cells from the *S. japonicum*-infected mouse spleen were investigated.

2. Materials and Methods

2.1. Ethics Statement. Six- to eight-week-old female C57BL/6 mice (wild-type, Laboratory Animal Centre of Sun Yat-sen University, China) were used for the experiments. Experiments were also performed by using $TLR3^{-/-}$ mice (B6; 129S1-$Tlr3^{tm1Fl}$v/J) purchased from Model Animal Resource Information Platform (Nanjing, China; strains: J005217). All mice were maintained in a specific pathogen-free microenvironment at the Laboratory Animal Centre, Guangzhou Medical University. Animal experiments were performed in strict accordance with the regulations and guidelines of the institutional animal care and use committee of Guangzhou Medical University. All protocols for animal use were approved to be appropriate and humane by the institutional animal care and use committee of Guangzhou Medical University (2012-11). Every effort was made to minimize suffering.

2.2. Mice, Parasites, and Infection. The *Schistosoma japonicum* cercariae used in experiments were obtained from *Oncomelania hupensis*-infected snails (Jiangsu Institute of Parasitic Disease, China). 30 C57BL/6 mice and 10 $TLR3^{-/-}$ mice were percutaneously infected with 40 ± 5 cercariae, and an equal number of uninfected normal mice were used as control. All mice were sacrificed 6 weeks after *S. japonicum* infection as reported before [5].

2.3. Antibodies. The following monoclonal antibodies were used for these studies: PE-conjugated rat IgG1 (R3-34), APC-conjugated rat IgG1 (R3-34), APC-cy7-conjugated anti-mouse CD3 (145-2C11), Alexa Fluor 647-conjugated anti-mouse TLR2 (6C2), PE-conjugated anti-mouse TLR4 (MTS510), PE-conjugated anti-mouse TLR7 (A94B10), PerCP-Cy5.5-conjugated anti-mouse CD4 (RM4-5), APC-conjugated anti-mouse CD8 (RPAT8), FITC-conjugated anti-mouse $\gamma\delta$TCR (GL3), APC-cy7-conjugated anti-mouse CD11b (M1/70), PE-conjugated anti-mouse Ly6G (1A8), FITC-conjugated anti-mouse CD94 (20d5), PE-conjugated anti-mouse CD314 (XMG1.2), APC-conjugated anti-mouse IFN-γ (XMG1.2), PE-conjugated anti-mouse IL-4 (11B11), PE-conjugated anti-mouse IL-17A (TC11-18H10), and APC-conjugated anti-mouse IL-5 (TRFK5). All antibodies were purchased from BD Pharmingen (San Diego, CA, USA). FITC-conjugated rat IgG1 (G0114F7), FITC-conjugated anti-mouse MHC II (M5/114.15.2), FITC-conjugated anti-mouse CD94 (Kp43), PE-cy7-conjugated rat IgG1 (G0114F7), PE-cy7-conjugated anti-mouse F4/80 (EMR1, Ly71), PE-cy5-conjugated anti-mouse CD19 (6D5), PE-cy7-conjugated anti-mouse NK1.1 (PK136), APC-conjugated rat IgG1 (G0114F7), APC-conjugated anti-mouse TLR3 (11F8), PE-conjugated anti-mouse TLR3 (11F8), PE-conjugated anti-mouse NKG2D (A10), and APC-conjugated anti-mouse CD69 (H1.2F3) antibody were purchased from BioLegend (San Diego, CA, USA).

2.4. Preparation of Splenocytes and NK Cells. Mice were sacrificed after infection for 6 weeks. The spleens were mechanically dissociated and processed through a 100 μm cell strainer (BD Falcon). After erythrocyte was removed by RBC lysis buffer (NH_4Cl 8.29 g, $KHCO_3$ 1 g, and Na_2EDTA 37.2 mg per liter), the cells were washed twice in Hanks' balanced salt solution and resuspended in complete RPMI-1640 medium, which contained 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 50 μM 2-mercaptoethanol. Splenocytes were counted under microscope and then diluted to a final concentration of 2×10^6 cells/ml for cell culture. For cell staining, splenocytes were stained with fluorescent-labeled anti-mouse CD3 and NK1.1 antibodies for 30 min, followed by washing twice and resuspending in sterile PBS with 0.5 Bull Serum Albumin (BSA). Then, $CD3^{-}NK1.1^{+}$ NK cells were sorted by using flow cytometry (MoFlo XDP, Beckman, USA). The purity of NK cells was above 90%, which was used immediately after sorting.

2.5. Cell Surface and Intracellular Staining. Splenic lymphocyte stimulation was performed as previously described [7]. Briefly, for cell surface staining, single splenic lymphocyte suspensions were washed twice and stained with anti-CD3, NK1.1, TLR2, TLR4, CD8, CD4, $\gamma\delta$T, CD19, Ly6G, F4/80,

MHC II, CD69, CD94 (NKG2A/C/E), or CD314 (NKG2D) antibodies for 30 min at 4°C in the dark. Stained cells were washed twice and detected by using flow cytometry. For intracellular cytokine staining, single splenic lymphocyte suspensions were incubated for 5 h in the presence of phorbol 12-myristate 13-acetate (PMA) (20 ng/ml, Sigma) and ionomycin (1 μg/ml, Sigma) at 37°C under a 5% CO_2 atmosphere; brefeldin A (10 μg/ml, Sigma) was added 1 hour after stimulation. Cells were washed twice and stained with antibodies of cell surface markers for 30 min at 4°C in the dark. Cells were fixed with 4% paraformaldehyde and permeabilized overnight at 4°C in PBS buffers containing 0.1% saponin (Sigma), 1% BSA, and 0.05% NaN_3. In the next day, cells were stained with conjugated antibodies specific for TLR3, TLR7, IL-4, IFN-γ, IL-17, and IL-5 for 30 min. Stained cells were washed twice and detected by using flow cytometry (CytoFLEX, Beckman Coulter, USA), and data were analyzed by the program CytExpert 1.1 (Beckman Coulter, USA).

2.6. RNA Preparation for Real-Time PCR. Total RNA of splenic lymphocytes or NK 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). The following primers were synthesized by Invitrogen (Shanghai, China): for TLR2, 5-CTCTCCGTC CCAACTGATGA-3 (forward) and 5-GGTCTGGTTGC ATGGCTTTT-3 (reverse); for TLR3, 5-ATTCGCCCTCC TCTTGAACA-3 (forward) and 5-TCGAGCTGGGTGAG ATTTGT-3 (reverse); for TL4, 5-AGGTTGAGAAGTCC CTGCTG-3 (forward) and 5-GGTCCAAGTTGCCGTTTC TT-3 (reverse); for TLR7, 5-GCATTCCCACTAACAC CACC-3 (forward) and 5-ACACACATTGGCTTTGGAC C-3 (reverse); for β-actin, 5-CCGTAAAGACCTCTAT GCCAAC-3 (forward) and 5-GGGTGTAAAACGCAGC TCAGTA-3 (reverse). mRNA expression was analyzed with RT-qPCR by using Takara SYBR Premix Ex Taq II (RR820A). PCR conditions were listed as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Amplification was carried out in triplicate. β-Actin mRNA was used for normalization. Amplification was performed by using the CFX96 touch qPCR system (Bio-Rad, Hercules, CA, USA), and quantitative mRNA levels were normalized to β-actin mRNA expression levels. The levels of TLR transcripts were normalized to β-actin transcripts by using the relative quantity $(RQ) = 2^{-\triangle\triangle Ct}$ method. qPCR products were analyzed on a 1.0% multi-welled agarose gel. Electrophoresis was performed in 1× TAE buffer at 400 V for 30 min. The gel was visualized in a ChemiDoc XRS Universal Hood II (Bio-Rad Laboratories).

2.7. ELISA Detection. Cells were cultured with different stimulations in 96-well plates at 37°C under a 5% CO_2 atmosphere for 72 h. Supernatant was collected, and the levels of IFN-γ and IL-4 were detected by using ELISA according to the manufacturer's instructions (IFN-γ: 551866, BD; IL-4: 555232, BD). The lower detection limit for IFN-γ is 3.126 pg/ml and 7.8 pg/ml for IL-4. Samples were read at 450 nm by using a microplate reader (Moder ELX-800, BioTek).

2.8. Statistical Analysis. Data were analyzed with SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Differences in mean values between groups were assessed by using Mann–Whitney U test. One-way ANOVA was used to analyze data of TLR3 KO mouse in Figure 1. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Increasing TLR3 Expression on Splenic NK Cells in S. japonicum-Infected Mice. To explore the role of TLRs on NK cells in the progress of *S. japonicum* infection, the expression of TLRs on NK cells from *S. japonicum*-infected mice was detected firstly. As shown in Figure 2, the spleens from normal and infected mice were isolated, and single cell suspensions were prepared, and different fluorescent-labeled anti-mouse CD3 and NK1.1 antibodies were used to sort $CD3^-NK1.1^+$ NK cells by FACS. The purity of sorted NK cells was above 95%. Moreover, mRNA was extracted from both splenocytes and splenic NK cells, respectively. cDNAs were synthesized, and qPCR was performed. The results demonstrated that the expressions of TLR2, TLR3, TLR4, and TLR7 mRNA in splenic NK cells significantly increased after *S. japonicum* infection ($P < 0.05$). TLR3 mRNA expression increased almost fivefold when compared to normal mice ($P < 0.01$). Moreover, splenocytes were stained and the expression of TLR2, TLR3, and TLR4 on $CD3^-NK1.1^+$ NK cells was detected (Figure 2(c)). Results (Figure 2(d)) showed that the percentages of TLR2 and TLR3 in splenic NK cells in the infected group were higher than the normal group (TLR2: 12.80 ± 2.442 versus 21.23 ± 2.409, $P < 0.05$; TLR3: $13.75 \pm 1.623\%$ versus $1.558 \pm 0.266\%$, $P < 0.01$). However, there were no significant differences in the expressions of TLR4 and TLR7 on NK cells between normal and infected mice ($P > 0.05$).

3.2. Poly I:C Promotes IL-4 Secretion from Infected Mouse Splenic NK Cells. To further explore the roles of TLRs on NK cells, splenocytes from normal and infected mice were cultured with PGN, poly I:C, or LPS, respectively. Supernatants were collected 72 h later, and IFN-γ and IL-4 levels were detected by using ELISA. As shown in Figure 3(a), the IL-4 level in the supernatants of infected mice was significantly higher than normal mice ($P < 0.05$). After poly I:C stimulation, IL-4 level was significantly higher than the nonstimulated controls ($38.76 \pm 4.45\%$ versus $23.88 \pm 2.33\%$, $P < 0.05$).

Furthermore, splenocytes from both infected mice and normal mice were stimulated by using PGN, Poly I:C, LPS, or PMA plus ionomycin, respectively. The expression of IFN-γ and IL-4 in NK cells was detected via intracellular cytokine staining (Figure 3(b)). As shown in Figure 3(c), the percentage of $IL\text{-}4^+$ cells in splenic NK cells from infected mice was significantly increased (unst: $2.52 \pm 2.00\%$ versus $9.37 \pm 1.22\%$; PGN: $2.32 \pm 1.37\%$ versus $13.03 \pm 4.11\%$; Poly I:C: $2.54 \pm 1.97\%$ versus $14.60 \pm 4.53\%$; LPS: $2.44 \pm 1.67\%$

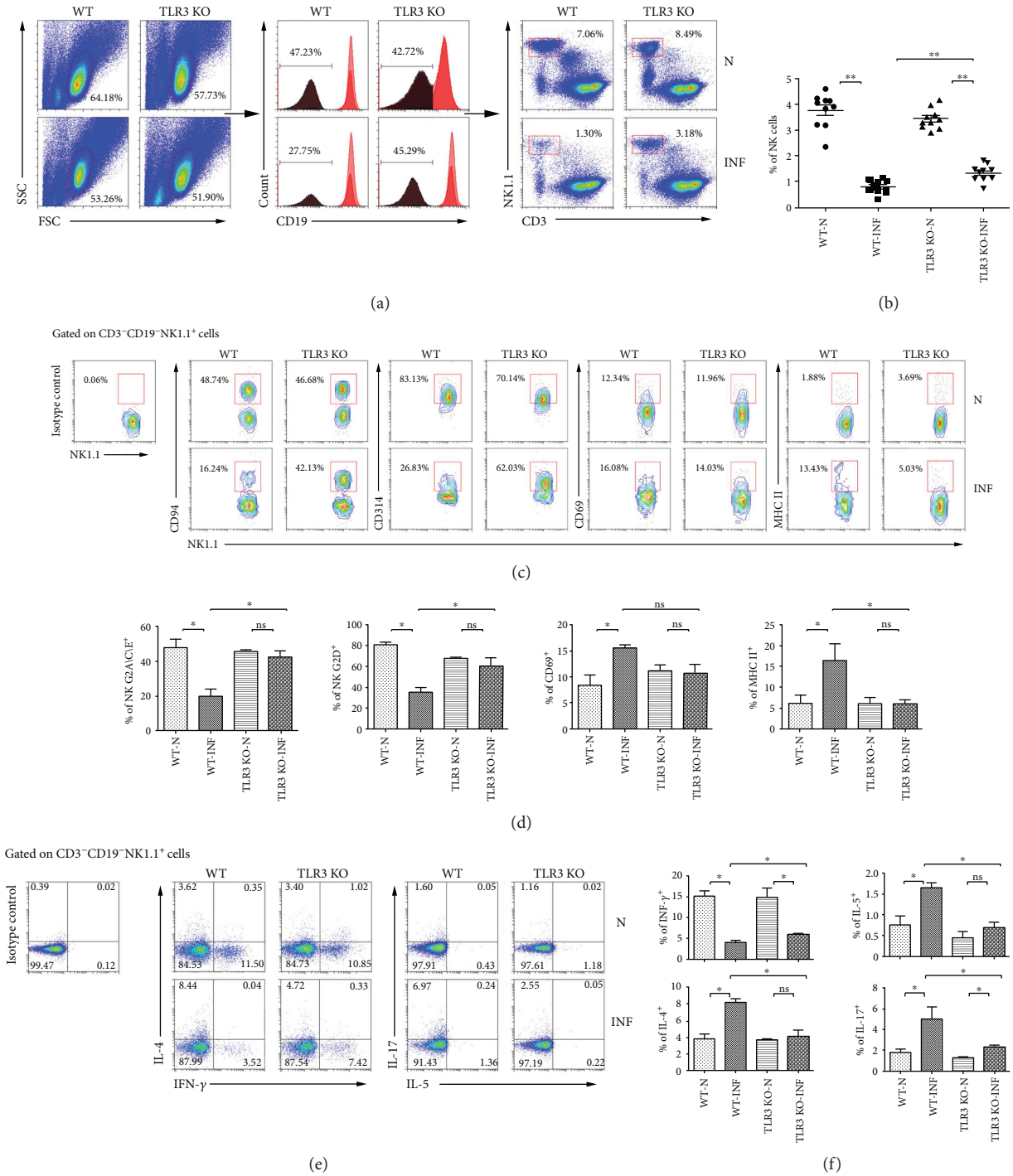


Figure 1: Characteristics of NK cells in *S. japonicum*-infected TLR3$^{-/-}$ mice. Female C57BL/6 wild-type mice (WI) and TLR3$^{-/-}$ mice (TLR3 KO) were infected with *S. japonicum* cercariae or not and sacrificed after 5 weeks. Splenic lymphocytes were isolated and stained by different fluorescent-labeled antibody and analyzed by FACS. (A/B) CD19^{-} cell population was gated firstly. The percentage of CD3^{-}NK1.1^{+} NK cells was analyzed. (a) A representative result is shown. (b) Statistical results of the percentage of NK cells in the spleen were calculated from FACS data. One syndrome represents an independent experiment. (c, d) The expression of MHC II, CD69, NKG2A/C/E, and NKG2D on NK cells of mice from both the normal and infected WT and TLR3 KO mouse spleen were detected. (c) A representative result is shown. (d) Statistical results of at least three independent experiments are shown. (e, f) Splenic lymphocytes were stimulated with PMA plus ionomycin for 5 hours, stained by the means of intracellular staining, and analyzed by flow cytometry. The expressions of IFN-γ, IL-4, IL-5, and IL-17 on NK cells from different groups are shown. (e) A representative result is shown. (f) The statistical results of at least three independent experiments are shown. Data are shown as mean + SEM of 3–5 samples from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$; ns for $P > 0.05$. N: normal; INF: infected.

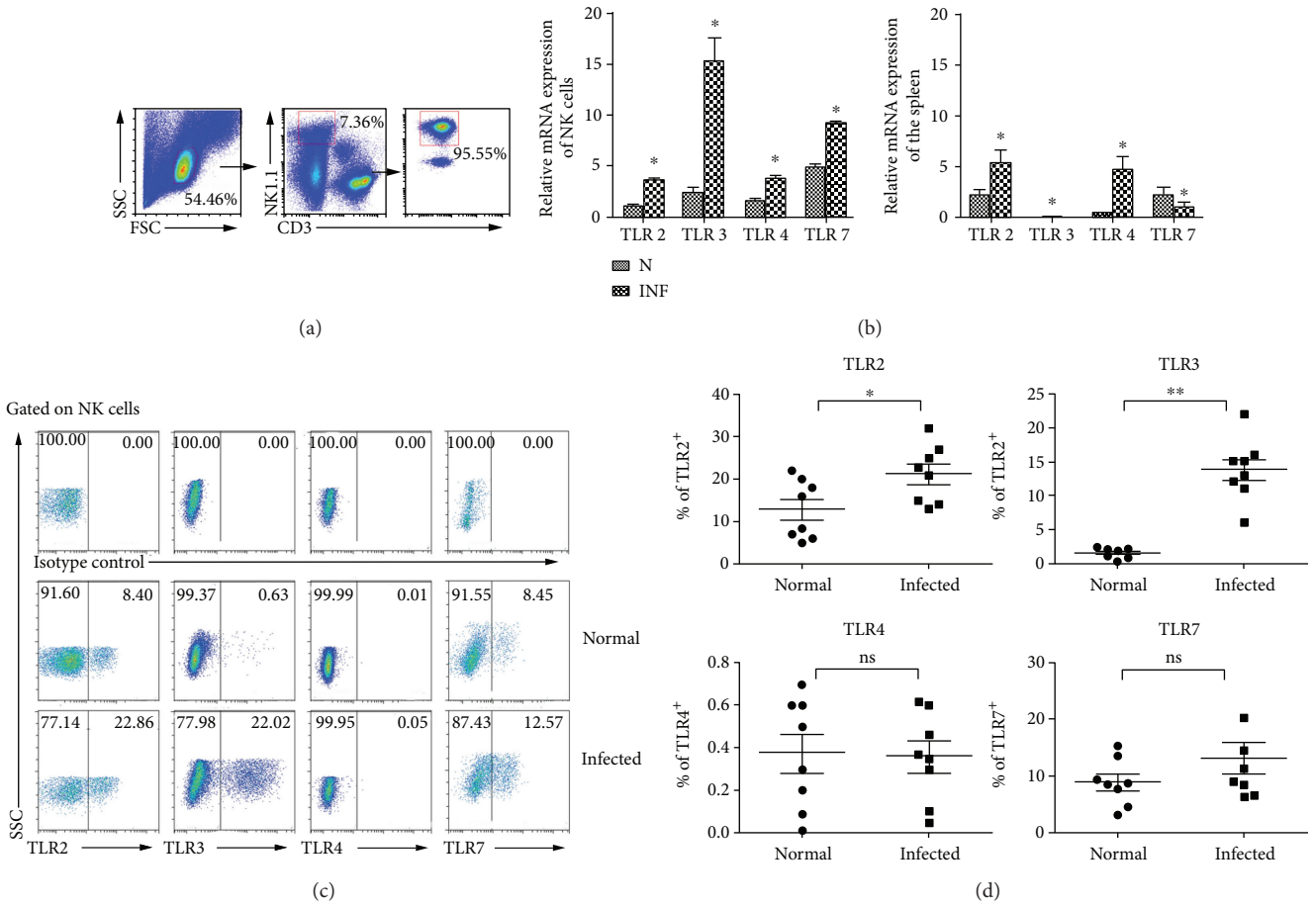
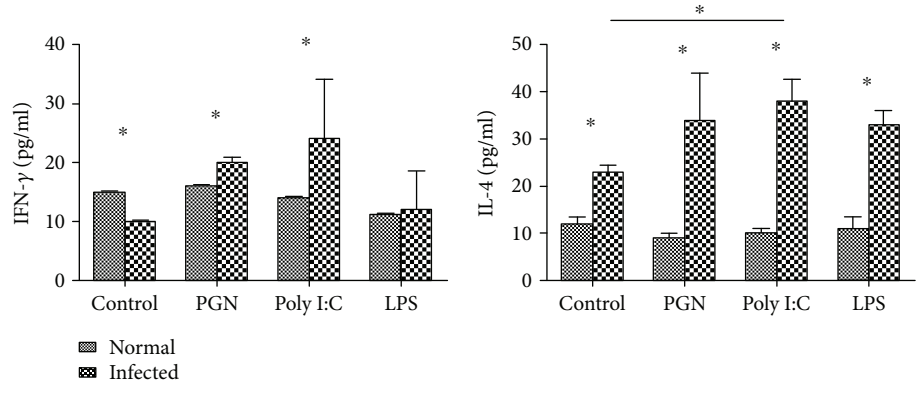


FIGURE 2: Expression of different TLRs in mouse splenic lymphocytes and NK cells after *S. japonicum* infection. (a) Splenic lymphocytes were separated from normal and *S. japonicum*-infected wild-type mice, and $CD3^{-}NK1.1^{+}$ NK cells were isolated from splenic lymphocytes by using flow cytometry and the purity of isolated splenic NK cells was identified by FACS. (b) Total RNA of splenic lymphocytes and NK cells was harvested, respectively. The accumulation of TLR2, TLR3, TLR4, and TLR7 mRNA was quantified by using qPCR. The levels of TLR transcripts were normalized to β-actin transcripts by using the relative quantity (RQ) $= 2^{-\Delta\Delta Ct}$ method. Data represent means ± SEM of at least three experiments. (c, d) Expression of TLR2, TLR3, TLR4, and TLR7 on splenic NK cells was assessed by using flow cytometry. (c) A representative result is shown. (d) Statistic results of 6 to 8 independent results are shown. N: normal; INF: infected; $^{*}P < 0.05$, comparison between the normal and infected groups.

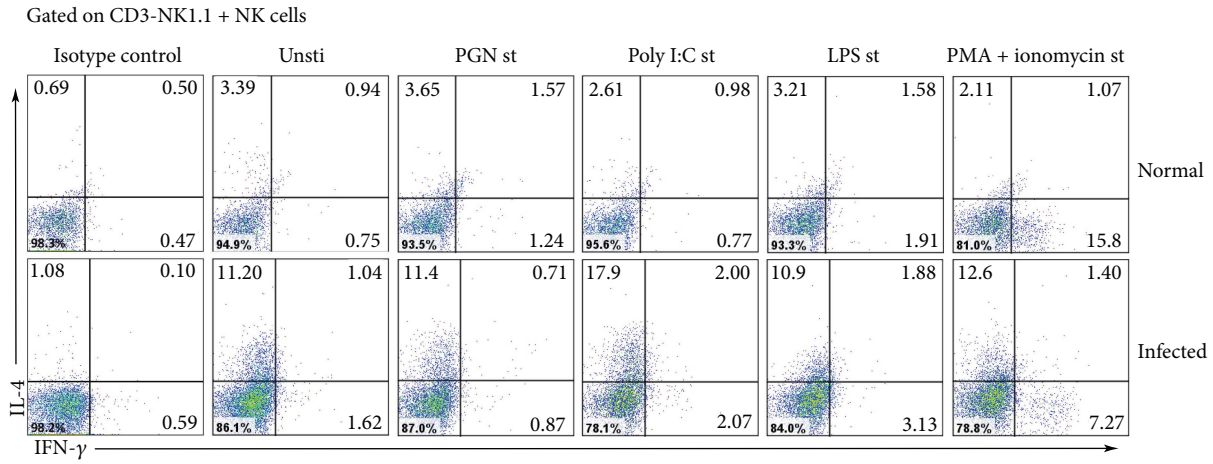
versus 12.17 ± 6.00%; PI: 3.04 ± 1.55% versus 9.6 ± 4.15%, $P < 0.05$). The percentage of $NK^{+}IL\text{-}4^{+}$ cells induced by poly I:C was significantly higher than the control group ($P < 0.05$). The percentage of IFN-γ^{+} cells in splenic NK cells from infected mice was higher than normal mice (unst: 4.54 ± 0.95%; PGN: 2.42 ± 1.08%; Poly I:C: 6.01 ± 1.50%; LPS: 4.02 ± 1.80%; PI: 6.11 ± 0.47%, $P < 0.05$), but the percentage of IFN-γ^{+} NK cells induced by these TLRs was not higher than the unstimulated controls in infected mice ($P > 0.05$).

3.3. Cellular Source of TLR3 in the Spleens of S. japonicum-Infected Mice. Single splenocyte suspensions were prepared from normal and *S. japonicum*-infected mice to investigate the cellular source of TLR3 in the *S. japonicum*-infected mouse spleen. TLR3 expression was detected by intracellular staining in different cell subsets, including $CD3^{+}CD4^{+}$ Th cells, $CD3^{+}CD8^{+}$ Tc cells, $CD3^{+}NK1.1^{+}$ NKT cells, $CD3^{+}\gamma\delta TCR^{+}\gamma\delta T$ cells, $CD3^{-}CD19^{+}$ B cells, and $CD3^{-}CD19^{-}NK1.1^{+}$ NK cells in the mononuclear cell population. Figures 4(a) and 4(b) showed that the proportion of $TLR3^{+}$ NK cells was increased approximately threefold after infection (normal: 2.07 ± 0.63%; infected: 5.65 ± 0.78%, $P < 0.01$). There were no significant differences in detection of Th cells, Tc cells, B cells, NKT cells, or $\gamma\delta$T cells ($P > 0.05$).

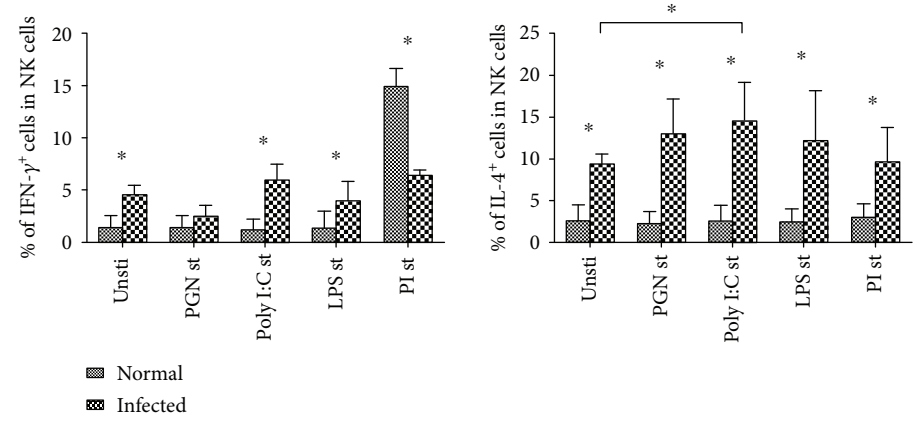
At the same time, TLR3 expression was detected in the myeloid cell populations, including $CD11b^{+}Ly6G^{+}$ neutrophils, $CD11b^{+}F4/80^{+}$ macrophages, $CD11c^{+}B220^{+}CD11b^{-}$ plasmacytoid dendritic cells (pDCs), and $CD11c^{+}B220^{-}CD11b^{+}$ conventional dendritic cells (cDCs). As shown in Figures 4(c) and 4(d), TLR3 was highly expressed on (the surface of?) neutrophils from normal mice (8.03 ± 0.97%), but the proportion declined after *S. japonicum* infection (4.46 ± 0.51%, $P < 0.05$, Figures 4(c) and 4(d)). Similar trend was also found on macrophages and cDCs, but there was no statistical difference ($P > 0.05$). No significant difference between pDCs and cDCs was detected ($P > 0.05$).



(a)



(b)



(c)

Figure 3: IFN-γ and IL-4 secretion induced by TLR agonists. (a) Splenic lymphocyte suspensions from normal and infected mice were prepared and cultured with PGN, Poly I:C, or LPS. Seventy-two hours later, IFN-γ or IL-4 production was assessed by using ELISA. (b) Splenic lymphocyte suspensions from normal and infected mice were prepared and stimulated with PGN, Poly I:C, LPS, or PMA plus ionomycin for 5 hours. IFN-γ and IL-4 production by splenic NK cells was assessed by using flow cytometry. Results of IFN-γ and IL-4 produced by splenic NK cells are calculated and shown (c). Data are shown as mean + SEM of 5 samples from three independent experiments. $^*P < 0.05$, comparison between the normal and infected groups.

3.4. Phenotypic and Functional Changes in TLR3$^+$ Splenic NK Cells Induced by S. japonicum. Moreover, splenocytes from normal and infected mice were isolated and stained with different fluorescent-labeled antibodies, including anti-CD19, CD3, NK1.1, TLR3, MHC II, CD69, NKG2A/C/E, and NKG2D to investigate the role of *S. japonicum* on TLR3$^+$ splenic NK cells. The CD19$^-$ mononuclear cells were gated firstly; the expressions of MHC II, CD69,

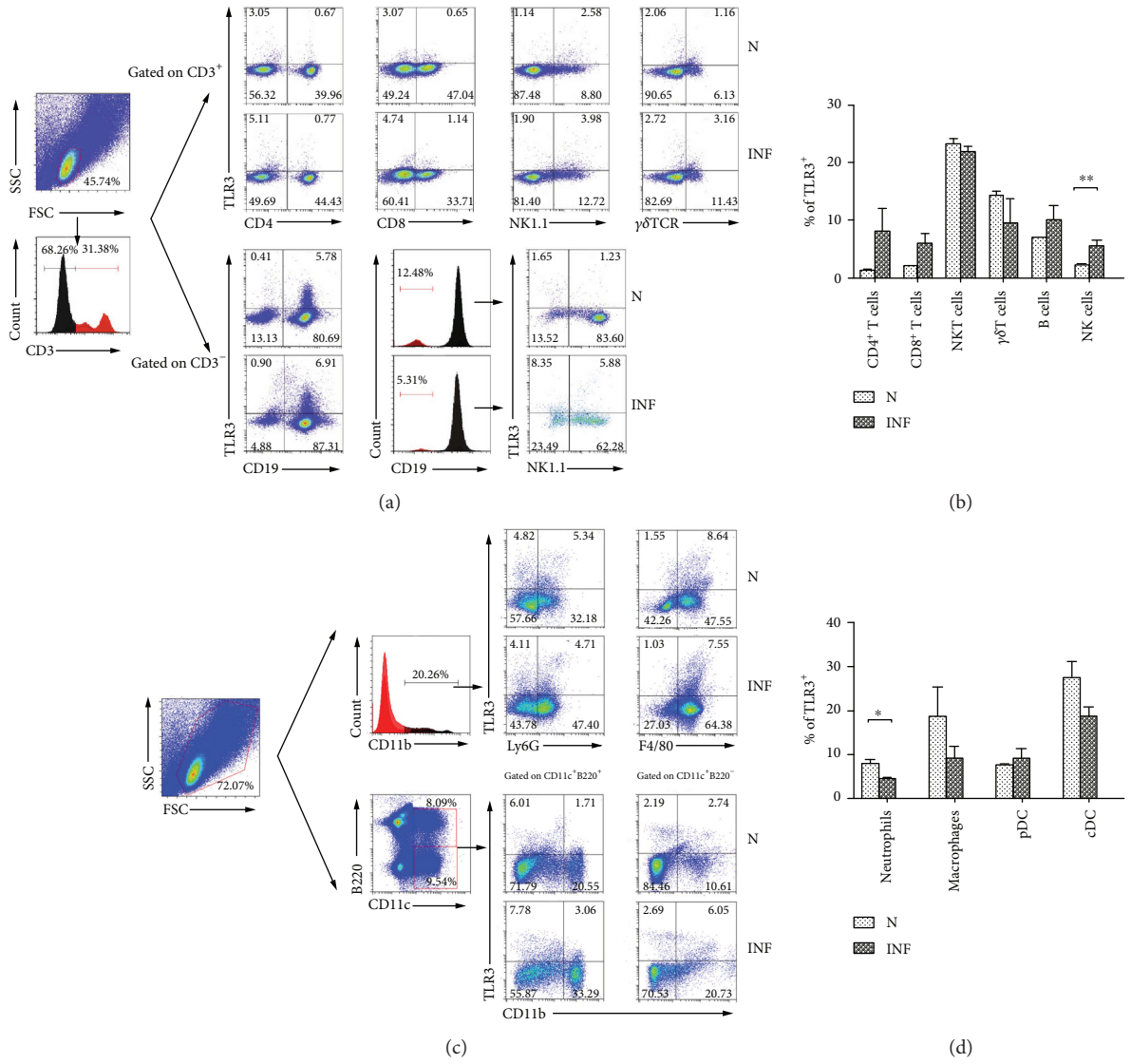


FIGURE 4: Expression of TLR3 on different spleen cell subsets. (a) Single spleen cell suspensions were separated, the population of small size live cell was gated firstly, and the expression of TLR3 on $CD4^+$ T cells, $CD8^+$ T cells, NKT cells, and $\gamma\delta$T cells in $CD3^+$ cell population, $CD3^-CD19^+$ B cells, and $CD3^-CD19^-NK1.1^+$ NK cells was analyzed by flow cytometry. Statistical analysis of expression of TLR3 on different lymphocyte subsets of the spleen is shown in (b). (c) Splenic lymphocytes were gated on the total live cell population firstly; the expression of TLR3 on different myeloid subsets containing $CD11b^+Ly6G^+$ neutrophils, $CD11b^+F4/80^+$ macrophages, $CD11c^+B220^+CD11b^-$ plasmacytoid dendritic cells (pDCs), and $CD11c^+B220^-CD11b^+$ conventional dendritic cells (cDCs) is shown in (d). Data represent means ± SD of three independent experiments with 3–5 mice per group. $^{**}P < 0.01$; $^{*}P < 0.05$. N: normal; INF: infected.

NKG2A/C/E, and NKG2D were detected on $TLR3^{-/+}$ NK cells (Figure 5(a)). The results (Figure 5(b)) demonstrated that the percentages of MHC II-, CD69-, and NKG2A/C/E-expressing cells in $TLR3^+$ NK cells significantly increased after infection (MHC II: 76.59 ± 3.054% versus 92.92 ± 1.770%; CD69: 17.72 ± 1.257% versus 22.30 ± 0.3512%; NKG2A/C/E: 77.80 ± 2.498% versus 90.48 ± 1.626%, $P < 0.05$). On the contrary, the percentage of NKG2D on $TLR3^+$ NK cells was significantly decreased after infection (47.43 ± 4.974% versus 12.99 ± 1.155%, $P < 0.05$). The expression of NKG2A/C/E on $TLR3^-$ NK cells significantly increased after infection (70.23 ± 0.6203% versus 77.18 ± 1.348%, $P < 0.05$), while the percentage of NKG2D on $TLR3^-$ NK cells was significantly decreased after infection (61.08 ± 3.683 versus 13.64 ± 1.682, $P < 0.05$). No significant difference was found in the expression of MHC II and CD69 on the surface of $TLR3^-$ NK cells ($P > 0.05$).

At the same time, splenocytes from normal and *S. japonicum*-infected mice were isolated and stimulated by PMA plus ionomycin, and intracellular cytokine staining was done as described in Materials and Methods. The expression of IFN-γ, IL-4, IL-5, and IL-17 was investigated on $TLR3^{-/+}$ splenic NK cells (Figure 5(c)). Results (Figure 5(d)) showed that the percentages of IL-4-, IL-5-, and IL-17-producing cells in $TLR3^+$ NK cells population significantly increased after infection (IL-4: 14.73 ± 1.968% versus 8.787.97581%;

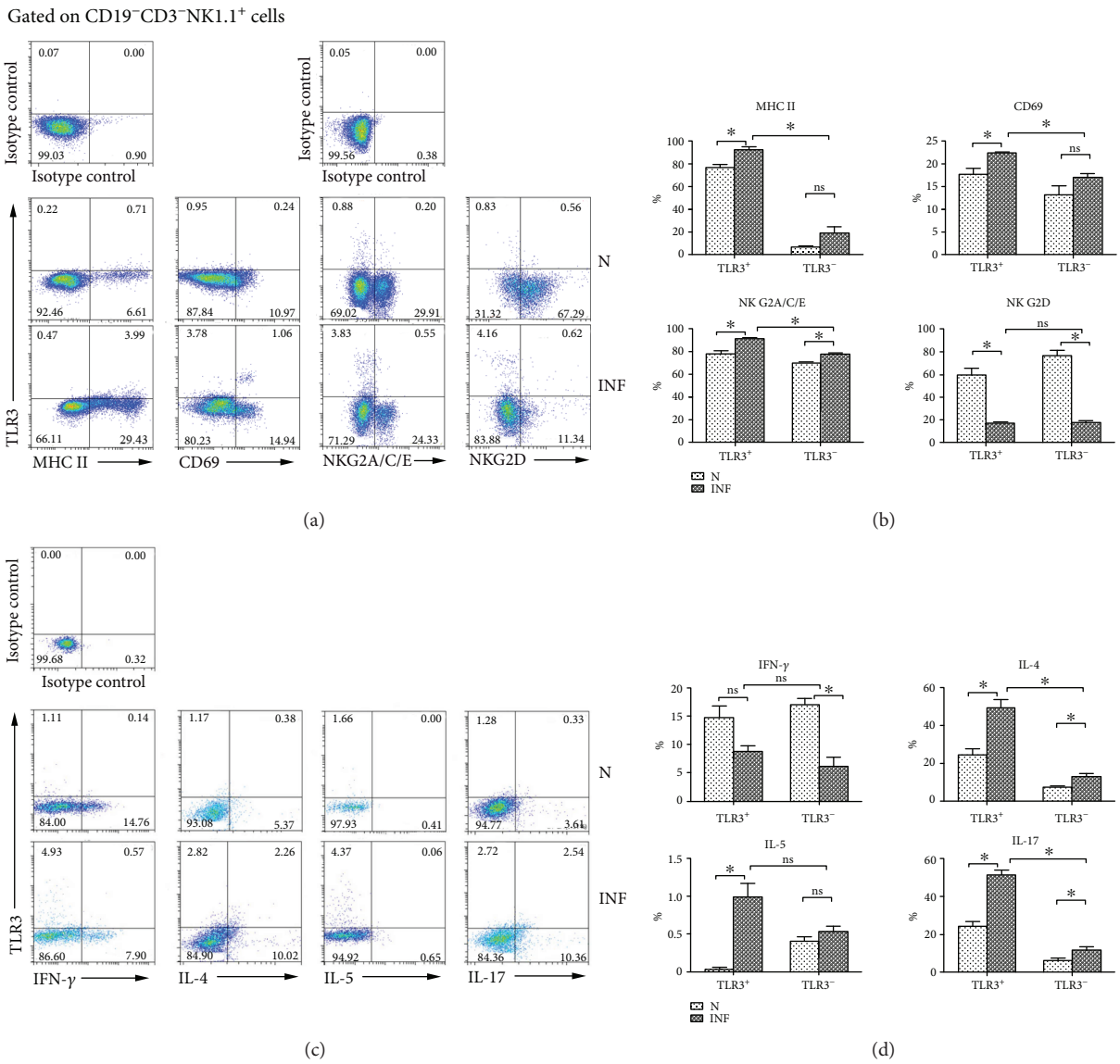


Figure 5: Phenotypic and functional changes in TLR3$^+$ splenic NK Cells. (a, b) Splenic lymphocytes were gated on CD19$^-$CD3$^-$NK1.1$^+$ cells firstly, and cells were stained with monoclonal antibodies against mouse NKG2A/C/E, NKG2D, MHC II, and CD69. A representative result is shown in (a). The percentage of activated molecule expression in TLR3$^+$ NK cells and TLR3$^-$ NK cells was calculated from FACS data (b). (c, d) Splenic lymphocytes were stimulated with PMA plus ionomycin for 5 hours and fixed and stained by using monoclonal antibodies. (c) A representative result is shown. (d) The percentage of cytokine production in TLR3$^+$ NK cells and TLR3$^-$ NK cells was calculated from FACS data. Data was from three independent experiments with 3–5 mice per group and shown as mean ± SEM. $^*P < 0.05$; ns for $P > 0.05$. N: normal; INF: infected.

IL-5: 0.03333 ± 0.03333% versus 0.9833 ± 0.1922%; IL-17: 24.50 ± 2.466% versus 51.76 ± 2.258%, $P < 0.05$). However, no significant difference was found in the percentage of IFN-γ-producing cells in TLR3$^+$ NK cells ($P > 0.05$). In the population of TLR3$^-$ NK cells, the percentages of IL-4- and IL-17-producing cells significantly increased (IL-4: 7.150 ± 1.028% versus 12.85 ± 1.627%; IL-17: 6.223 ± 1.309% versus 11.98 ± 1.534%, $P < 0.05$). However, the percentage of IFN-γ-secreting cells significantly decreased (16.96 ± 1.190% versus 6.120 ± 1.609%, $P < 0.05$). No significant difference was found in the percentage of IL-5-producing NK cells (0.4067 ± 0.05812% versus 0.5267 ± 0.08192%, $P > 0.05$).

3.5. TLR3 Mediates the Function of Splenic NK Cells during S. japonicum Infection. Furthermore, wild-type (WT) and TLR3 knockout (TLR3 KO) mice were infected with *S. japonicum* to confirm the role of TLR3 on NK cells. Splenocytes were isolated 5 weeks after infection, and the noninfected wild-type and TLR3 knockout mice were sacrificed as the control groups. As shown in Figure 1(a), the percentage of CD3$^-$NK1.1$^+$ NK cells in CD19$^-$ mononuclear cells

from different group of mice was explored by FACS. The results demonstrated that the percentage of NK cells from infected WT mice ($0.80 \pm 0.28\%$) was much less than that of uninfected WT mice ($3.76 \pm 0.56\%$, $P < 0.01$). So was the comparison between infected ($1.33 \pm 0.14\%$) and noninfected ($3.43 \pm 0.13\%$, $P < 0.01$) TLR3 KO mice. It was notable that the percentage of NK cells from TLR3 KO infected mice increased compared with WT infected mice ($P < 0.01$, Figure 1(b)).

Next, the expression of MHC II, CD69, NKG2A/C/E, and NKG2D on NK cells of mice from four groups was detected by using flow cytometry (Figure 1(c)). The expression of MHC II on NK cells from WT mice increased significantly after *S. japonicum* infection ($6.15 \pm 1.95\%$ versus $16.36 \pm 2.93\%$, $P < 0.05$, Figure 1(d)). MHC II expression on NK cells of TLR3 KO mice, by contrast, did not change after infection in statistics ($5.94 \pm 2.25\%$ versus $5.97 \pm 0.94\%$, $P > 0.05$, Figure 1(d)). The percentage of MHC II-expressing NK cells from TLR3 KO mice was less than WT after infection in statistics ($P < 0.05$, Figure 1(d)). Similar to MHC II, CD69 expression on NK cells from WT mice also increased significantly after infection ($8.34 \pm 2.18\%$ versus $15.39 \pm 0.69\%$, $P < 0.05$, Figure 1(d)), and there was no difference between infected and noninfected TLR3 KO mice ($11.10 \pm 2.04\%$ versus $10.66 \pm 3.37\%$, $P > 0.05$, Figure 1(d)). However, expression of CD69 on NK cells from infected TLR3 KO mice did not change obviously compared with their matched control group ($P > 0.05$, Figure 1(d)). Consistent with our previous study, the expression of NKG2A/C/E and NKG2D on NK cells from WT mice decreased significantly after *S. japonicum* infection (NKG2A/C/E: $47.88 \pm 8.12\%$ versus $19.93 \pm 3.69\%$; NKG2D: $80.62 \pm 2.21\%$ versus $35.35 \pm 3.65\%$, $P < 0.05$, Figure 1(d)). As we expected, there was no obvious change between infected and noninfected TLR3 KO mice (NKG2A/C/E: $45.25 \pm 2.42\%$ versus $42.35 \pm 6.65\%$; NKG2D: $67.17 \pm 1.62\%$ versus $60.51 \pm 13.67\%$, $P > 0.05$, Figure 1(d)). Notably, expression of NKG2A/C/E and NKG2D on NK cells from infected TLR3 KO mice was much higher when compared with infected WT mice ($P < 0.05$, Figure 1(d)).

We also measured cytokine production of NK cells from infected TLR3 KO mice. Figures 1(e) and 1(f) showed that the percentage of IFN-γ^+ NK cells from WT mice decreased obviously after *S. japonicum* infection (noninfected: $15.19 \pm 2.62\%$; infected: $4.03 \pm 0.25\%$, $P < 0.05$, Figure 1(f)) as our previous study, and so was the TLR3 KO group (noninfected:$14.73 \pm 4.4\%$; infected: $5.94 \pm 0.6\%$, $P < 0.05$, Figure 1(f)). The percentages of IL-4^+ and IL-5^+ NK cells in noninfected WT mice were $3.86 \pm 0.26\%$ and $0.76 \pm 0.44\%$, respectively. These percentages increased significantly to $8.27 \pm 0.70\%$ ($P < 0.05$, Figure 1(f)) and $1.66 \pm 0.22\%$ ($P < 0.05$, Figure 1(f)), respectively, after *S. japonicum* infection. These numbers decreased significantly to $4.07 \pm 1.77\%$ and $0.70 \pm 0.16\%$ from infected TLR3 KO mice ($P < 0.05$, Figure 1(f)), which were equal to the noninfected TLR3 KO mice group (IL-4: $3.78 \pm 0.09\%$; IL-5: 0.45 ± 0.13, $P > 0.05$, Figure 1(f)). Unexpectedly, the percentages of IL-17^+ NK cells increased significantly after infection from both WT (noninfected: $1.74 \pm 0.09\%$; infected: $5.06 \pm 1.71\%$, $P < 0.05$, Figure 1(f)) and TLR3 KO mice (noninfected: $1.31 \pm 0.05\%$; infected: $2.31 \pm 0.38\%$, $P < 0.05$, Figure 1(f)). Still, there were significant differences between WT and TLR3 KO infected mice ($P < 0.05$, Figure 1(f)).

4. Discussion

It was reported that NK cells could express many kinds of TLRs, which could play an important role in the progress of NK cell activation in response to bacterial and viral infection [21] and tumors [24]. Here, roles of TLRs on NK cells in the progress of *S. japonicum* infection were investigated in the spleen of C57BL/6 mice after infection for 6 weeks, which is the acute phase of infection in previous report [6–8]. As shown in Figure 2, splenic NK cells expressed a higher level of TLR3 than TLR2, TLR4, TLR7, and TLR9 from *S. japonicum*-infected mouse ($P < 0.05$). It suggested that TLR3 might be involved in modulating the activation of *S. japonicum* infection-induced NK cell.

As we know that *S. japonicum* infection could induce a Th2-dominant immune response in the body [25]. Our previous research has found that the ability of splenic NK cells in secreting IFN-γ from *S. japonicum*-infected mouse is decreased while the IL-4 secretion is increased [7]. Here, our results demonstrated that Poly I:C could induce a higher level of IL-4 from infected mouse splenocytes (Figure 3(a), $P < 0.05$) and the percentage of IL-4^+ NK cells in poly I:C cocultured infected splenocytes was extremely higher ($P < 0.05$). It suggested that *S. japonicum* infection-induced TLR3 on NK cells was involved in modulating the function of NK cells. However, it was reported that poly I:C is not only a TLR3 ligand but also can interact with RIG-I [26, 27], which might influence the expression of IL-4 [28].

On the other hand, it was reported that TLR3 could modulate immunopathology during *Schistosoma mansoni* egg-driven Th2 responses in the lung [19]. Many types of immune cells express TLR3, such as DC cells, macrophages, NK cells, T cells, and B cells [29–32]. In this study, the expression of TLR3 was examined on different types of splenocytes from normal and *S. japonicum*-infected mice. Results showed that TLR3 expression only increased obviously in NK cell from infected mice ($P < 0.01$, Figure 4(a)). It suggested that NK cells might be the main target cells which could respond to materials through TLR3 in the spleen in the course of *S. japonicum* infection. Although the engagement of both TLR2 and TLR3 by schistosome eggs is important for the production of inflammatory cytokines and interferon-stimulated genes, such as some chemokines, by DCs [18], no significant differences of TLR3 expressions were observed in pDCs, cDCs, neutrophils, and macrophages ($P > 0.05$, Figure 4(b)). It might relate to the fact that myeloid cells mainly play function on the site of local inflammation [33].

MHC II, CD69, NKG2A/C/E, and NKG2D are activation- and function-associated molecules which were expressed on the surface of NK cells [34–37]. Our results demonstrated that the percentages of MHC II-, CD69-, and NKG2A/C/E-expressing cells in TLR3$^+$ NK cells increased

significantly after infection ($P < 0.05$, Figures 5(a) and 5(b)). It indicated that *S. japonicum* infection could induce the activation of $TLR3^+$ NK cell in the spleen. Moreover, it was reported that NK lineage may develop into cytokine-producing/APCs which affect the priming and progress of systemic autoimmune disease [37]. And many cytokines, such as IFN-γ, IL-4, IL-5, and IL-17, were reported to be secreted by NK cells [7, 38]. Our results showed a significant increase in the percentage of IL-4-, IL-5-, and IL-17-producing cells in $TLR3^+$ NK cells population from infected mice ($P < 0.05$, Figures 5(c) and 5(d)). It indicated that TLR3 might mediate the Th2-like immune response of splenic NK cells in the progress of *S. japonicum* infection.

Additionally, $TLR3^{-/-}$ mice were infected with *S. japonicum* to further evaluate the role of TLR3 on NK cells during infection. Results showed that the expression of NKG2A/C/E, NKG2D, MHC II, and CD69 on NK cells was decreased significantly in *S. japonicum*-infected $TLR3^{-/-}$ mice (Figures 1(c) and 1(d)). These results suggested that TLR3 mediated the activation of NK cells during *S. japonicum* infection. Meanwhile, our results showed the significant decrease of IFN-γ-producing NK cells and increase of IL-4-, IL-5-, and IL-17-producing NK cells in *S. japonicum*-infected $TLR3^{-/-}$ mice ($P < 0.05$, Figures 1(e) and 1(f)). It confirmed that TLR3 could modulate the function of NK cells in the course of *S. japonicum* infection.

In summary, our study demonstrated that splenic NK cells from *S. japonicum*-infected C57BL/6 mice expressed a higher level of TLR3, and TLR3-expressing NK cells were more sensitive, both in vitro and in vivo. Our results suggested that TLR3 might be involved in modulating the immune response of NK cells in the course of *S. japonicum* infection in C57BL/6 mice.

Abbreviations

S. japonicum:	*Schistosoma japonicum*
TLR:	Toll-like receptor
NK:	Nature killer
IL:	Interleukin
Th:	T helper cell
IFN:	Interferon
KO:	Knockout
PRRs:	Pattern recognition receptors
PMA:	Phorbol 12-myristate 13-acetate.

Data Availability

All data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

All protocols for animal use were approved to be appropriate and humane by the institutional animal care and use committee of Guangzhou Medical University (2012-11). Every effort was made to minimize suffering.

Conflicts of Interest

The authors declare that there are no conflicts of interest in this study.

Authors' Contributions

Design of the research was conducted by Jun Huang and Nuo Dong; performing experiments was executed by Jiale Qu, Lu Li, and Hongyan Xie; data interpretation and analysis was facilitated by Xiaona Zhang, Quan Yang, Huaina Qiu, Yuanfa Feng, and Chenxi Jin; drafting the manuscript or revising it critically for important intellectual content was performed by Nuo Dong and Jun Huang. All authors read and approved the final manuscript. Jiale Qu, Lu Li, and Hongyan Xie contributed equally to this work.

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Research Article

PPAR-γ Agonist Alleviates Liver and Spleen Pathology via Inducing Treg Cells during *Schistosoma japonicum* Infection

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Background. Peroxisome proliferator-activated receptor- (PPAR-) γ plays critical roles in human metabolic disorders and has recently been implicated as a regulator of cellular proliferation and inflammatory responses. Regulatory T cells (Tregs), which express high levels of PPAR-γ protein, have the ability to maintain immune tolerance to self-antigens and regulate immune response to *Schistosoma* infection. However, mechanisms involved in the resolution of these responses are elusive. *Methods*. Liver and spleen tissue samples in *Schistosoma japonicum*-infected mice after administration of pioglitazone (a PPAR-γ agonist) were collected. The hepatic and splenic pathologies were detected by H&E and Masson staining. The percentages of Th1/2 and Treg cells in the liver and spleen of each mouse were determined using flow cytometry. Levels of gene expression of PPAR-γ and Foxp3 in tissues or cells were determined using real-time PCR (RT-PCR). Macrophages were treated with pioglitazone *in vitro* or cocultured with normal purified $CD4^+$ T cells for detecting Treg cells by flow cytometry. The interactions of PPAR-γ with Foxp3 in $CD4^+$ T cells were detected by coimmunoprecipitation. *Results*. Administration of pioglitazone resulted in the prevention of the development of hepatic and splenic pathologies. Activation of PPAR-γ by pioglitazone resulted in increased percentages of $CD4^+CD25^+Foxp3^+$ Treg cells and decreased percentages of $CD3^+CD4^+IFN\text{-}\gamma^+$ and $CD3^+CD4^+IL\text{-}4^+$ cells in the liver and spleen of *Schistosoma japonicum*-infected mice. In addition, the PPAR-γ agonist can induce Treg cells *in vitro* directly or by modulating the macrophage's function indirectly. Furthermore, through interaction with Foxp3 in $CD4^+$ T cells, the PPAR-γ agonist can promote the expression of Foxp3; however, the inhibitor of PPAR-γ weakened the expression of Foxp3 by modifying the coexpression of Foxp3 and PPAR-γ. *Conclusions*. Our study reveals a previously unrecognized role for PPAR-γ/Foxp3 signaling in regulating the immunopathology that occurs during *Schistosoma* infection through induction of Treg cells.

1. Introduction

Schistosomiasis, caused by schistosomes, remains a major public health problem in many countries in Asia and Europe [1, 2]. The most serious schistosomiasis immune pathogenesis is the hepatic granuloma formation around deposited eggs and subsequent fibrosis, which are orchestrated by the $CD4^+$ T cell response [3, 4]. During infection, an initial proinflammatory Th1-type polarized response is continuously triggered by schistosome-soluble adult worm antigen fractions, with elevated interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-12 (IL-12) levels. After a large number of deposited schistosome eggs, the process of granuloma formation in the liver is triggered, which undergoes an obvious shifting from an initial restrictive Th1-type response to a late Th2-dominant response [5]. Importantly, schistosome infections are renowned for their ability to induce powerful regulatory networks including regulatory T cells that regulate schistosome egg-induced pathology [5–7]. However, the mechanism underlying regulatory cell-controlled liver pathology is still largely unknown.

Peroxisome proliferator-activated receptor- (PPAR-) γ, an important fatty acid-activated nuclear receptor, plays a critical role in human metabolic disorders, such as

dyslipidemia and insulin resistance [8, 9]. PPAR-γ also plays an important role in the regulation of immune response-related fibrosis. One of the PPAR-γ agonists (rosiglitazone) has been reported to prevent murine hepatic fibrosis, which was accompanied by the induction of the expression of TNF-α and IL-6 but a reduction of the expression of TGF-β1 and α-SMA in liver tissue [10]. In the CCL4-induced rat liver fibrosis model, infection with the recombinant lentiviral expression vector carrying the rat PPAR-γ gene resulted in suppressing hepatic stellate cell (HSC) proliferation and hepatic fibrosis and inhibiting the expression of α-SMA and type I collagen [11]. However, the potential mechanisms of the PPAR-γ agonist on the development of *Schistosoma* egg-induced liver pathology are still not fully understood.

Evidence suggested that PPAR-γ is a crucial transcription factor for regulating Treg cell accumulation and function, and the specific ablation of PPAR-γ in Treg cells greatly reduced the population of Treg cells accumulated in visceral adipose tissue (VAT) [12]. PPAR-γ agonists (ciglitazone and 15-deoxy-$\Delta^{12,14}$-PG J2) as molecules could significantly increase Foxp3 expression in human iTregs [13]. PPAR-γ agonist- (pioglitazone) attenuated upper airway allergic inflammation may be mediated by the induction of Tregs [14]. In addition, our previous data showed that pioglitazone could increase regulatory T cells in the VAT of high-fat-diet mice [15]. In view of the importance of PPAR-γ in the regulation of immune response, we attempt to examine not only the effectiveness of pioglitazone but also shed light on the significance of the PPAR-γ/Foxp3 axis to regulate schistosome egg-induced liver pathology during *S. japonicum* infection.

2. Materials and Methods

2.1. Ethics Statement, Animal, Parasites and Antigen Preparation. Six-week-old C57BL/6 mice were obtained from the Model Animal Research Center of Nanjing University and kept in specific pathogen-free environments in the Animal Care Facility of Nanjing Medical University. All experiments were performed in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (1988-11-01). All of the animal experiments were approved by the Nanjing Medical University Animal Ethics Committee (number 1601004).

Cercariae were collected from *S. japonicum*-infected snails, which were bought from the Jiangsu Institute of Parasitic Diseases (Wuxi, China).

Soluble egg antigen (SEA) was prepared carefully to prevent endotoxin contamination. The concentration of SEA was measured using the Bicinchoninic Acid Protein Assay Kit (Pierce, USA). The concentration of endotoxin in SEA was below 0.03 EU/ml using a timed gel endotoxin detection kit (Sigma-Aldrich, USA).

2.2. Experimental Mice Model. Mice were divided into four groups randomly as follows: group I was identified as normal control (uninfection) and group II was treated with pioglitazone only (uninfection + PIO). Group III was infected with 10 ± 1 *S. japonicum* cercariae through the skin (infection), and group IV was treated with pioglitazone 4 weeks post *S. japonicum* infection (infection + PIO). Pioglitazone (10 mg/kg) was given by intragastric means every other day for 5 weeks in groups of uninfection + PIO and infection + PIO. Meanwhile, mice in the uninfection group and infection group were given normal saline for 5 weeks. All mice were sacrificed at 9 weeks post infection.

2.3. Histopathological Examination. The sections of livers were examined with hematoxylin and eosin (H&E) staining and Masson staining. The sectioned liver tissue was fixed in 4% paraformaldehyde, embedded in paraffin and stained according to standard protocols. Single-egg granulomas were examined and sizes were calculated using AxioVision Rel 4.7 (Carl Zeiss GmbH, Jena, Germany). Additionally, the degree of hepatic fibrosis was evaluated using a professional image analysis software (Image Pro Plus).

Eggs in the liver were calculated after putting 0.2 mg of liver in 10% KOH overnight and counting the number of eggs by taking 10 μl three times.

Hepatic mass index (HMI) was calculated by the liver weight and body weight. The same was done with the spleen index.

2.4. Cell Preparation and Stimulation. Spleen lymphocytes were prepared as in a previous study [16]. Briefly, single cell suspensions were prepared by collecting the cells after the spleen was ground in incomplete RPMI 1×1640 medium (Gibco, USA), followed by red blood cell (RBC) lysis, washing by staining buffer which contained PBS with 1% FBS, and then filtering through a 200 gauge mesh.

Hepatic lymphocytes were prepared as described before [16]. Briefly, mouse livers were perfused and then the excised liver was cut into small pieces and incubated in digestion buffer (collagenase IV/dispase mix, Invitrogen, Carlsbad, CA). The digested liver tissue was then homogenized using a MediMachine with 50 mm Medicons (Becton Dickinson, San Jose, CA) for 5 min at low speed. The liver suspension was then centrifuged at low speed to sediment the hepatocytes. The remaining cells were separated on a 35% Percoll (Sigma-Aldrich) gradient by centrifuging at 600 ×g. The lymphocyte fraction was resuspended in red cell lysis buffer and then washed in 10 ml of complete RPMI 1640.

For $CD4^+$ T cell preparation of the spleen, purified $CD4^+$ T cells were acquired by MACS (magnetic-activated cell sorting) according to the manufacturer's protocols (Miltenyi Biotec). $CD4^+$ T cells were then stimulated with PIO (10 μg/ml), PIO + GW9662 (a potent antagonist of PPAR-γ) (10 μg/ml + 2 ng/ml), SEA (25 μg/ml), SEA + GW9662 (25 μg/ml + 2 ng/ml), and PIO + SEA (10 μg/ml + 25 μg/ml) for 24 h.

For peritoneal macrophage preparation, peritoneal exudate cells (PECs) from each mouse were harvested using lavage with ice-cold PBS with 1% FBS. PECs were resuspended in RPMI medium containing 10% FBS and 1% penicillin streptomycin (PS). Nonadherent cells were removed after 2 hours of incubation. Adherent macrophages were incubated with 5 mM EDTA/PBS for 10 min at 37°C,

and the cell purity marked with F4/80 was detected by flow cytometry (~98%) [16]. Then, peritoneal macrophages were treated with the above stimulants for 24 h. After that, macrophages were scoured off and cultured with purified $CD4^+$ T cells for 24 h, and the cells were collected for Treg cell analysis by flow cytometry.

2.5. Flow Cytometry. For Th1/Th2 analysis, 2×10^6 cells per ml were stimulated for 6 h at 37°C in 5% CO_2 with 0.7 μl of GolgiPlug (BD Biosciences, USA), 25 ng/ml of PMA (Sigma-Aldrich), and 1 μg/ml of ionomycin (Sigma-Aldrich). After the staining of surface markers (CD3-APC, CD4-FITC), cells were then washed with staining buffer and fixed and permeabilized with the Fix&Perm Cell Permeabilization Kit (Medium A/Medium B) (Life Technologies) and then stained with antibodies against INF-γ-PE or IL-4-PE. Finally, cells were washed twice and resuspended in staining buffer prior to flow cytometry analysis.

For Treg analysis, 2×10^6 cells were stained with CD4-FITC and CD25-PE-CY7 for 30 minutes, and then cells were washed with staining buffer and fixed and permeabilized with the Fix&Perm Cell Permeabilization Kit (Life Technologies) according to the manufacturer's instructions. Cells were incubated for 40 min at 4°C in the dark. Subsequently, cells were blocked by Fc and then stained with Foxp3-PE. Finally, cells were washed twice and resuspended in staining buffer prior to flow cytometry analysis.

2.6. Real-Time PCR Analysis. For real-time PCR analysis, total RNA was extracted using the Total RNeasy kit (TaKaRa, Tokyo) and cDNA was prepared using random hexamer primers (GeneWiz, Grand Island). Primer sequences used were as follows: Foxp3 [17], forward: GGCCCTTCT CCAGGACAGA, reverse: GCTGATCAT GGCTGGGTTGT; PPAR-γ, forward: GCGGCTGAGAA ATCACGTTC, reverse: GAATATCAGTGGTTCACCGCT TC; and GADPH, forward: AACTTTGGCATTGTGG AAGG, reverse: GGATGCAGGGATGATGTTCT. Gene expression was quantified using the LightCycler® 96 System (Roche Life Science, Switzerland). Amplification was performed in a total volume of 20 μl for 40 cycles and products were detected using SYBR Green I dye (Roche Life Science, Switzerland). The relative expression of mRNA was calculated using a comparative method ($2^{-\Delta\Delta ct}$) according to the ABI Relative Quantification Method.

2.7. Coimmunoprecipitation. Coimmunoprecipitation was performed for the determination of the interaction between Foxp3 and PPAR-γ in $CD4^+$ T cells. Briefly, the lysates (~200 μg of protein) of purified $CD4^+$ T cells were incubated with rocking at 4°C with anti-Foxp3 (rabbit anti-mouse; cat: 222298-1-AP used at 1 : 500 dilution, Proteintech) or anti-PPAR-γ (rabbit anti-mouse; cat: 16643-1-AP used at 1 : 500 dilution, Proteintech). After 2 h, the immune complexes were incubated with protein A/G-plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The immunopurified proteins were washed and immunoblotted using specific antibodies.

2.8. Statistical Analyses. All analyses were carried out with the SPSS 21.0 software. Data were shown as mean ± SD. Multiple comparisons were performed by one-way ANOVA, and followed by LSD posttest for comparison between two groups. Significance was considered when P values < 0.05. We used GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA) for all graphical representations.

3. Results

3.1. PPAR-γ Agonist Alleviates Hepatic and Splenic Pathology. To investigate the impact of pharmacological modulation of pioglitazone on hepatic pathology, we first detected the expression of PPAR-γ in the liver after five weeks post treatment. Results showed that pioglitazone treatment could significantly increase hepatic PPAR-γ expression ($P < 0.001$) (Figure 1(a)). Additionally, we weighed the liver, spleen, and body weight of each mouse and calculated the hepatic mass index (HMI) and spleen index. Results showed that the HMI and spleen index decline when infected mice received pioglitazone treatment ($P < 0.001$; $P < 0.001$) (Figures 1(b)–1(d)). We also calculated the number of eggs in the liver. Results showed that pioglitazone had little impact on egg production (Figure 1(e)). In addition, H&E and Masson staining results showed that treatment with pioglitazone significantly reduced the average area of single-egg granulomas (Figures 1(f) and 1(g)) and ameliorated liver fibrosis (Figures 1(h) and 1(i)). Together, these data showed that pioglitazone played an important role in moderating hepatic and splenic pathologies during *S. japonicum* infection.

3.2. PPAR-γ Agonist Increases the Treg Cells and Inhibits Effector Cell Response. As $CD4^+$ T cells orchestrate the development of immunopathology in schistosomiasis [5], we first investigated whether $CD4^+$ T cells were regulated after pioglitazone treatment during *S. japonicum* infection (Figure 2(a)). Our results showed that the absolute numbers of $CD4^+$ T cells both in the liver and spleen of pioglitazone-treated mice were not changed after *S. japonicum* infection. Previous studies showed that regulatory T cells play critical roles in regulating the development of immunopathology in schistosomiasis [18, 19], our results showed that the proportion of Treg cells both in the liver and spleen of pioglitazone-treated mice was apparently increased after pioglitazone treatment in *S. japonicum*-infected mice when compared with untreated infected mice ($P < 0.001$; $P < 0.001$) (Figure 2(b)). Altogether, these results suggested that pioglitazone may participate in regulating immunopathology through inducing Treg cells after *S. japonicum* infection.

Since Treg cells play important roles in suppressing both Th1 and Th2 responses during *Schistosoma* infection [20, 21], we next examined whether Th1 and Th2 cells were regulated by pioglitazone treatment during *S. japonicum* infection. Results showed that Th1 response ($CD3^+CD4^+$ IFN-γ^+) was significantly decreased both in the liver and spleen of pioglitazone-treated mice post *S. japonicum* infection ($P < 0.001$; $P < 0.001$) (Figure 2(c)). In addition,

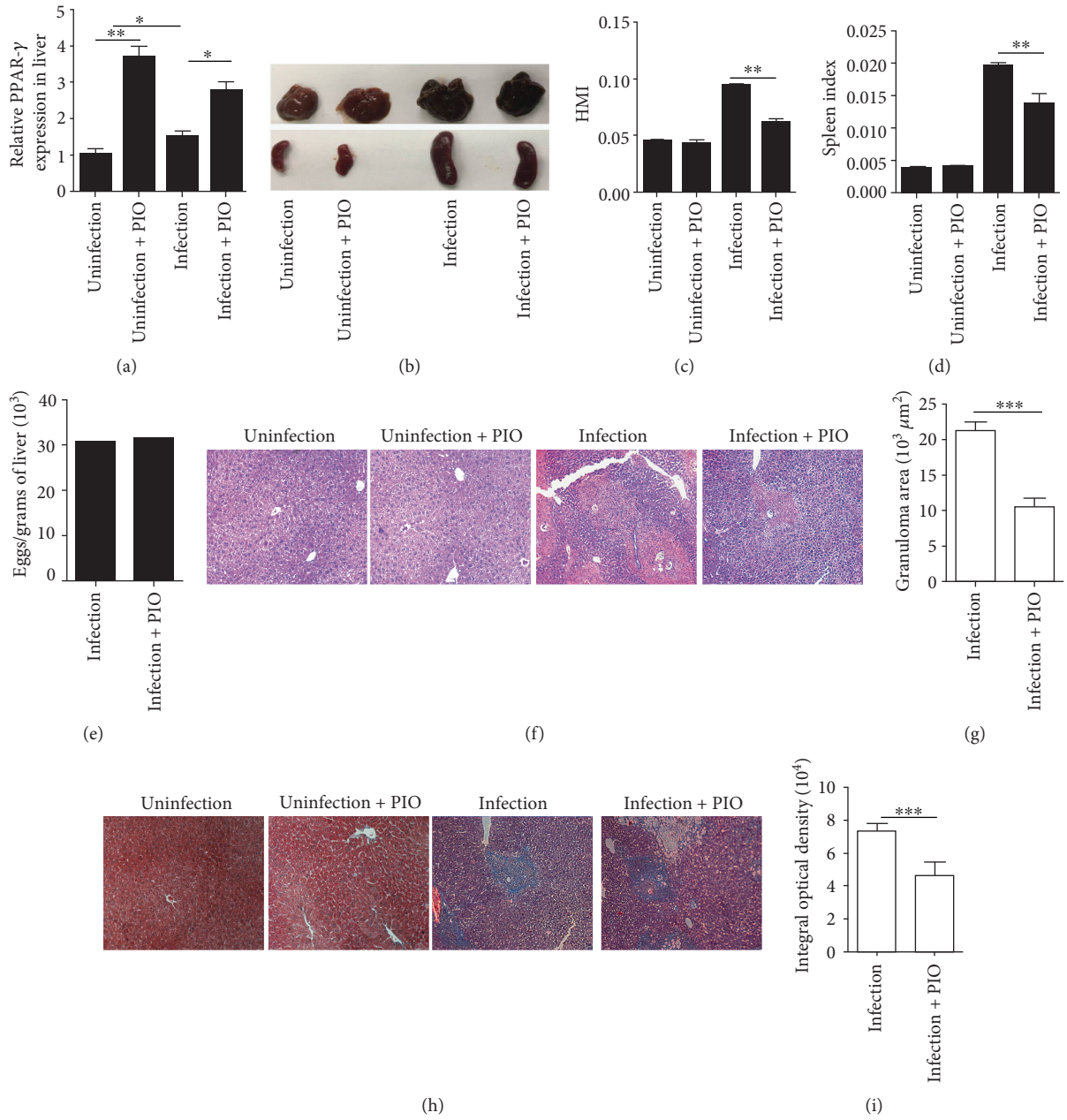
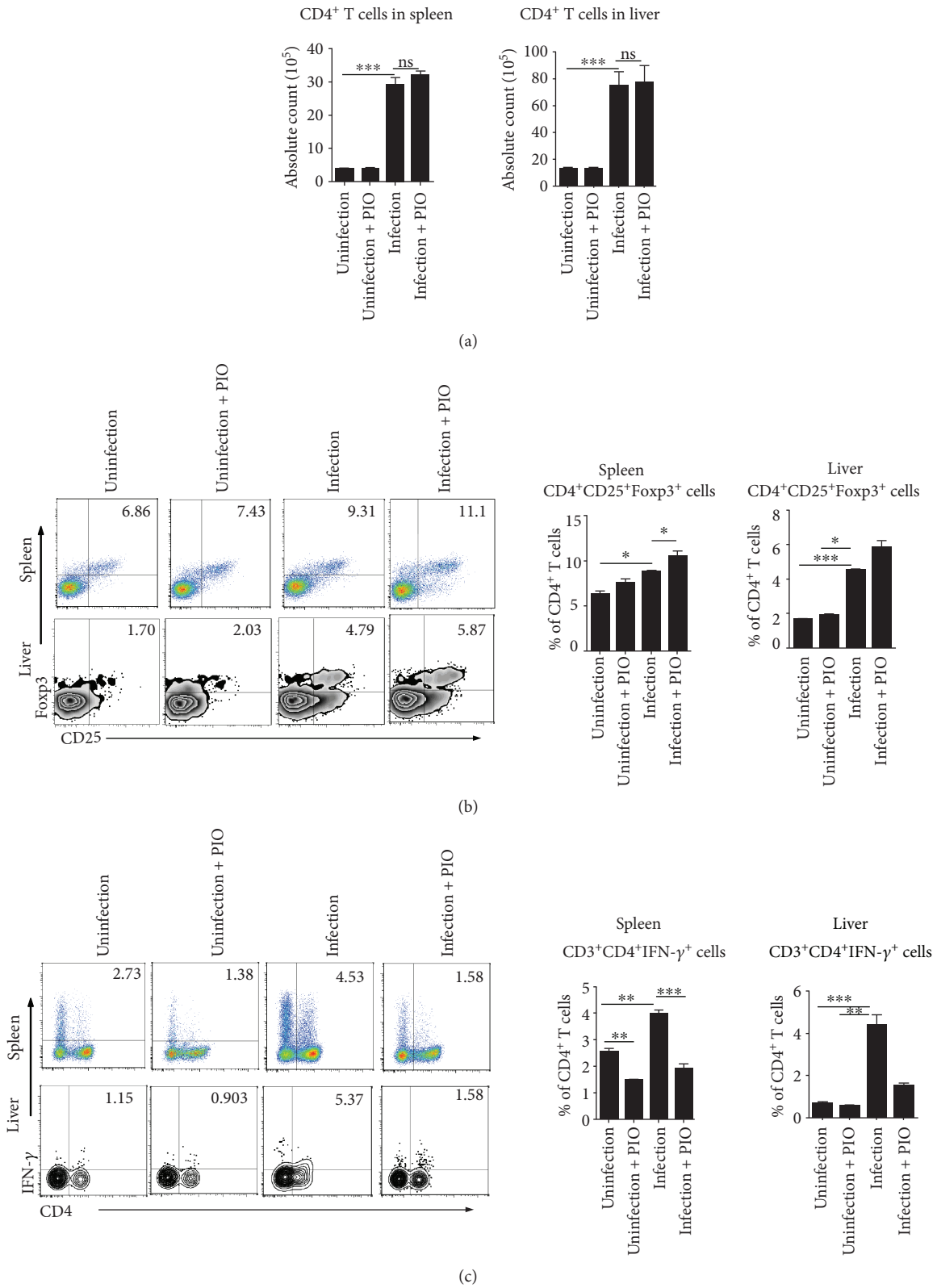


Figure 1: The role of the PPAR-γ agonist in hepatic and splenic pathologies. (a) The mRNA level of PPAR-γ in the liver of mice in each group after continuous intragastric administration with pioglitazone for 5 weeks. Data are expressed as the mean ± SD of 6 mice for each group. (b–d) Hepatic mass index (HMI) and spleen index were calculated by the ratio of the liver or the spleen weight to the body weight, respectively. (e) The numbers of eggs extracted from the liver (1 gram) in each mice were determined by microscopic examination. (f, h) Paraffin-embedded liver sections stained with H&E or Masson; original magnification, ×100. (g) For each mouse, the sizes of 20 liver granulomas around single eggs were quantified with AxioVision Rel 4.7. Paraffin-embedded sections were stained with Masson; original magnification, ×100; (i) the mean optical density of collagen fibers by Masson staining was digitized and analyzed on Image Pro Plus software. Data are expressed as the mean ± SD of 12 mice for each group, which resulted from two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (ANOVA/LSD).

our data also showed that Th2 ($CD3^{+}CD4^{+}IL\text{-}4^{+}$) cells were significantly reduced in the liver but only slightly decreased in the spleen of pioglitazone-treated mice when compared with untreated infected mice ($P < 0.001$) (Figure 2(d)).

3.3. PPAR-γ Agonist Induces Foxp3 and Augmented Treg Cells In Vitro. Given that PPAR-γ is a crucial transcription factor for promoting Treg cell expression and accumulation [22], we used pioglitazone and GW9662 (PPAR-γ antagonist) to stimulate splenic mononuclear lymphocytes under SEA stimulation *in vitro*, and detected the expression of Foxp3, an important transcription factor of Treg cells. Results showed that treatment with the PPAR-γ agonist or SEA induced the mRNA levels of Foxp3 in the spleen cells;



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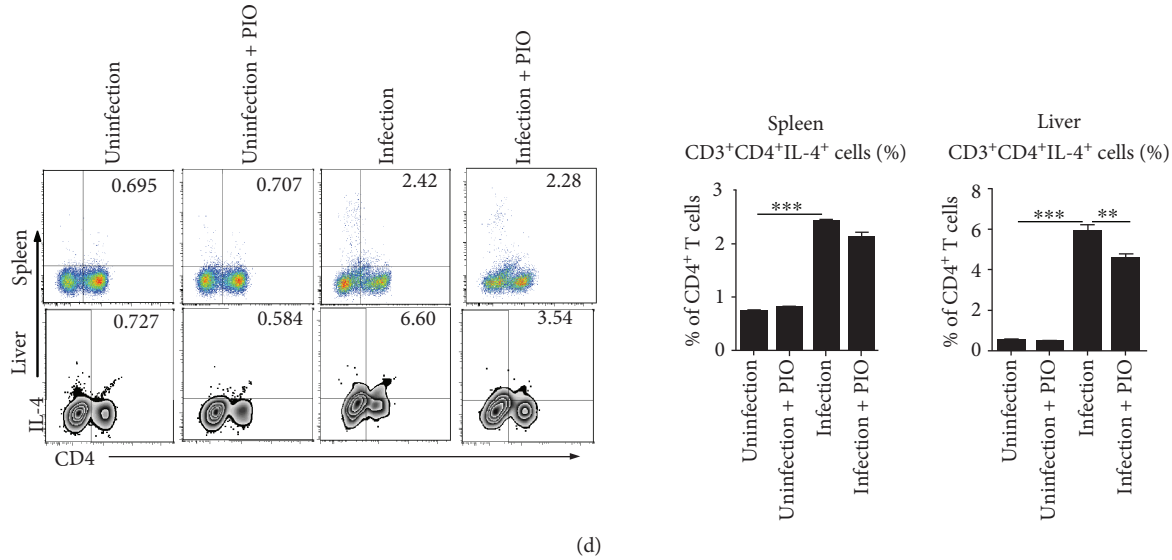

Figure 2: The expression of regulatory cells and effector Th1/Th2 cells after pioglitazone treatment of *S. japonicum* infection. (a–d) Single cell suspensions of mouse spleen or liver from pioglitazone-treated mice infected with or without *S. japonicum* were prepared. (a) The absolute numbers of $CD4^+$ T cells both in the liver and spleen of mice were calculated as the proportion of $CD3^+CD4^+$ T cells in the leukocyte gate multiplied by the total cell count. (b) Cells were stained with CD25-APC and CD4-FITC and then intracellularly stained with PE-conjugated antibodies against Foxp3 for FACS analysis of $CD4^+CD25^+Foxp3^+$ (Treg). (c, d) Cells were stained with CD3-APC and CD4-FITC and then intracellularly stained with PE-conjugated antibodies against IFN-γ or IL-4 for FACS analysis of $CD3^+CD4^+IFN\text{-}\gamma^+$ (Th1) or $CD3^+CD4^+IL\text{-}4^+$ (Th2) cells, respectively. Data are expressed as the mean ± SD of 12 mice for each group from three independent experiments (ANOVA/LSD), $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

however, the expression of Foxp3 was decreased after PPAR-γ antagonist treatment (Figures 3(a) and 3(b)). In addition, we also found that the percentage of Treg cells was increased after PPAR-γ agonist or (and) SEA treatment, while PPAR-γ antagonist blocked this enhancement (Figures 3(c) and 3(d)). Taken together, these data suggested that the activation of PPAR-γ signaling could promote the production of $CD4^+CD25^+Foxp3^+$ Treg cells.

3.4. PPAR-γ Agonist Promotes Treg Cell Differentiation through Activating Macrophages. It is given that macrophages serve as professional antigen-presenting cells (APCs) and play a key role in regulating adaptive immune responses [23]. To investigate whether PPAR-γ signaling in macrophages could affect the differentiation of $CD4^+$ T cells, we firstly treated macrophages with pioglitazone with or without SEA, and analyzed the activation of macrophages. Results showed that pioglitazone-stimulated macrophages significantly increased the expression of CD80 or MHCII in macrophages (Figures 4(a) and 4(b)). In addition, the activated macrophages by pioglitazone or SEA were then cocultured with normal purified $CD4^+$ T cells (Figure 4(c)). Results showed that pioglitazone pretreated macrophages significantly promoted the percentage of Treg cells with or without SEA stimulation; however, PPAR-γ inhibitor-pretreated macrophages could inhibit the proportion of Treg cells ($P < 0.001$) (Figures 4(c)–4(e)).

As PPAR-γ can be expressed in alternatively activated (M2) macrophages [24, 25], which are the main cellular constituents of granulomas [5] and important regulators in schistosomiasis [26, 27], we investigated whether PPAR-γ signaling regulates the M2 macrophage polarization. Results showed that pioglitazone stimulation increased $CD206^+$ macrophages; however, the PPAR-γ antagonist dampened the expression of $CD206^+$ macrophages (Figure 4(d)). Altogether, these results suggested that pioglitazone promotes $CD4^+$ T cell differentiation into Treg cells through the interaction with macrophages.

3.5. PPAR-γ Promotes Treg Cell Differentiation through Interaction with Foxp3. Considering that PPAR-γ was preferentially expressed in T cells [28], we next investigated whether pioglitazone could act on $CD4^+$ T cells directly and promote Treg cell differentiation. Results showed that the percentage of Treg cells was significantly increased after direct pioglitazone stimulation ($P < 0.01$). However, the percentage of Treg cells was significantly decreased when the PPAR-γ inhibitor was used ($P < 0.01$) (Figure 5(a)). However, SEA-treated $CD4^+$ T cells alone failed to cause an increased proportion of Treg cells, suggesting that SEA-induced $CD4^+$ T cell differentiation required the presence of antigen-presenting cells. These results indicated that the activation of PPAR-γ by an agonist could directly promote differentiation of $CD4^+$ T cells into Treg cells.

To determine whether PPAR-γ promoted $CD4^+$ T cell differentiation through interaction with Foxp3, $CD4^+$ T cells were prepared and stimulated with pioglitazone *in vitro*. Results showed that treatment with a PPAR-γ agonist (pioglitazone) resulted in enhanced coexpression of PPAR-γ and Foxp3 (Figure 5(b)), both of which are key transcription factors for Treg cells. On the other hand, treatment with a PPAR-γ inhibitor (GW9662) suppressed the interaction

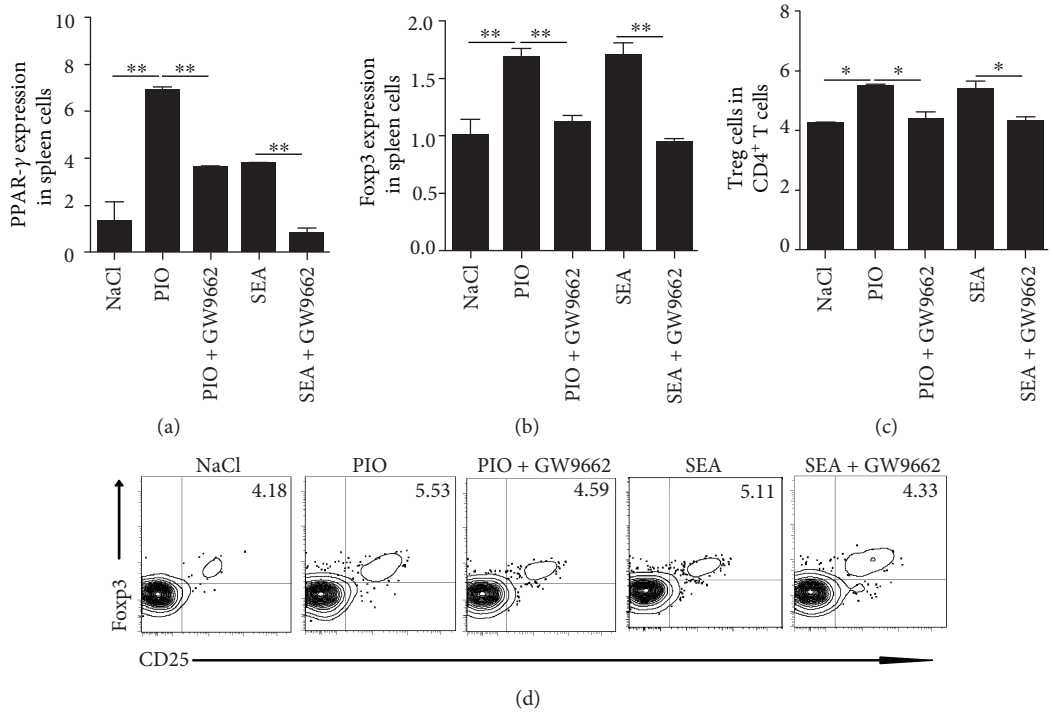

Figure 3: The expression of Foxp3 and Treg cells after pioglitazone or (and) SEA stimulation *in vitro*. (a, b) Splenocytes from WT mice were prepared and stimulated by pioglitazone (PIO, 10 μg/ml) and a PPAR-γ antagonist (GW9662, 2 ng/ml) with or without SEA (25 μg/ml) stimulation for 24 h, and then the mRNA level of PPAR-γ and Foxp3 were analyzed by RT-PCR. (c, d) The percentage of Treg was detected after pioglitazone treatment in spleen cells with or without SEA stimulation for 24 h. All experiments were repeated three times with similar results (ANOVA/LSD). $^{*}P < 0.05$ and $^{**}P < 0.01$.

between PPAR-γ and Foxp3 in CD4$^+$ T cells (Figure 5(c)), which is accompanied by decreasing Treg cells. Altogether, these results demonstrated that pioglitazone modulates PPAR-γ interaction with Foxp3 in CD4$^+$ T cells, which subsequently induced the percentage of Treg cells and regulated the immune function.

4. Discussion

Although it has been well recognized that regulatory T cells play important roles in regulating immune response to infection, the mechanism involved in the regulatory function to schistosome infection has remained largely unclear. In this study, we for the first time report that PPAR-γ interacts with Foxp3 to promote the differentiation of CD4$^+$ T cells into Treg cells and subsequently attenuating immunopathology during schistosome infection.

The main pathology in schistosomiasis results from eggs deposited in the liver, which impacts on the hosts' living quality, health status, or even mortality [5]. Interestingly, our results found that pioglitazone treatment could significantly improve the liver and spleen pathologies by HMI, spleen index, HE, and Masson staining. In addition, treatment with pioglitazone had little impact on egg production, while praziquantel reduced the number of eggs in the liver. These data suggested that pioglitazone played an important role in moderating hepatic and splenic pathologies but had no effect on the killing of adult worms during *S. japonicum* infection.

CD4$^+$ T cells are the key factors involved in the development of immunopathology in schistosomiasis [5]; however, pioglitazone treatment did not influence the numbers of CD4$^+$ T cells in *S. japonicum*-infected mice. Treg cells play a pivotal role in maintaining immune homeostasis [29, 30] and regulating the development of immunopathology in schistosomiasis [18, 19, 31]. Studies had showed that *Schistosoma* eggs can induce a marked Treg cell response, which prevents Th1 development and Th2 response [21]. Our results showed that pioglitazone treatment increased the expression of Treg cells, but decreased Th1/Th2 responses in *S. japonicum*-infected mice. Consistently, *in vitro* treatment of splenocytes with the PPAR-γ agonist and (or) SEA also induced the mRNA levels of Foxp3 and the percentage of Treg cells, while the PPAR-γ antagonist blocked this enhancement. Our results are supported by a study regarding inflammatory bowel disease (IBD) which used PPAR-γ deficient mice [32].

PPAR-γ can be expressed in macrophages [24, 25, 33], which are also the main cellular constituents of granulomas and important regulators in schistosomiasis [26, 27]. It is clear that PPAR-γ plays an important role in the regulation and maintenance of M2-type polarization [34–37]. Our results showed that PPAR-γ signaling is involved in the activation of macrophages and induction of CD206$^+$

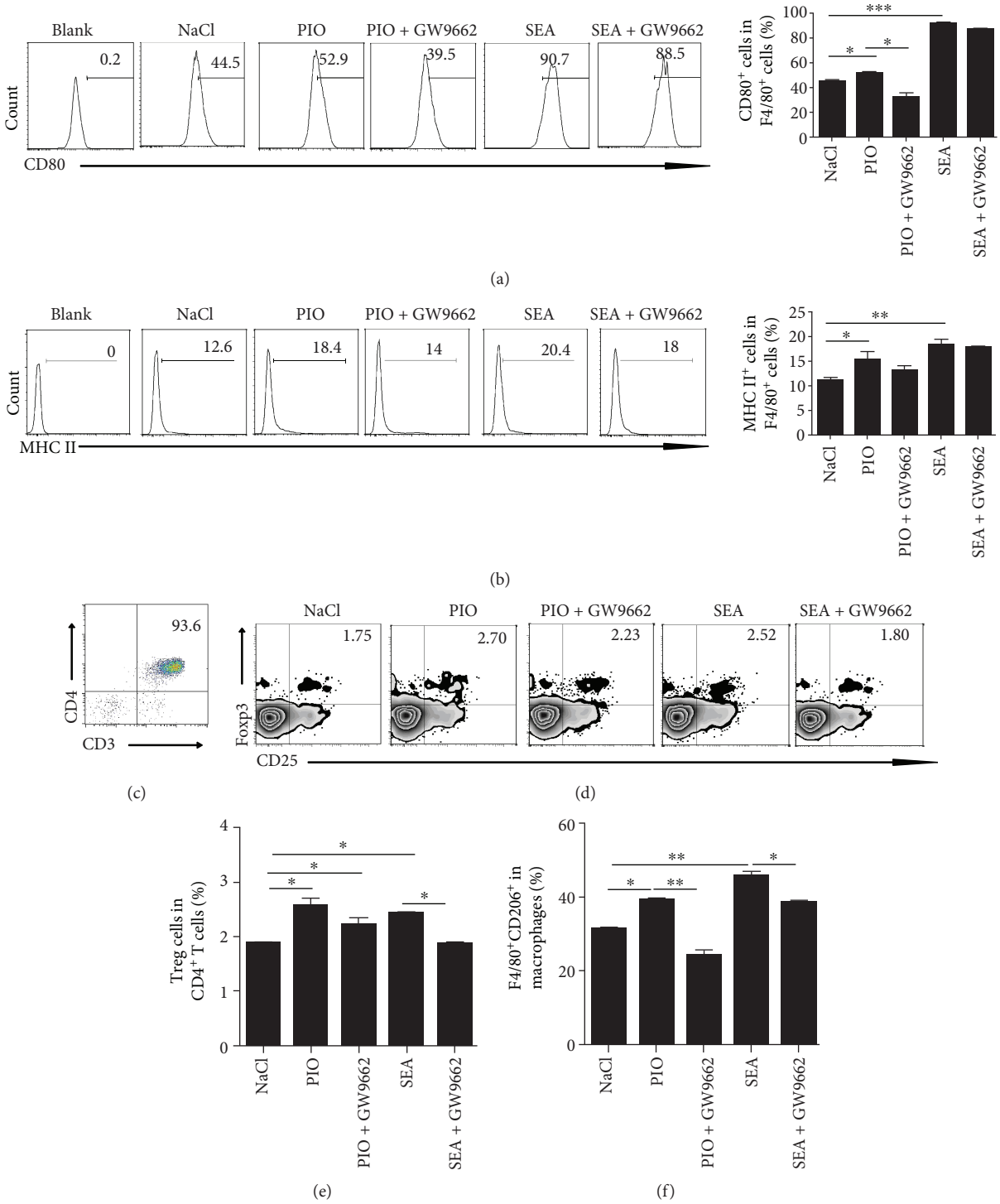

Figure 4: PPAR-γ signaling promotes Treg differentiation involved in macrophages. (a, b) Peritoneal macrophages (1×10^6) were treated with pioglitazone (10 μg/ml) with or without SEA (25 μg/ml). The expression of $CD80^+F4/80^+$ or $MHCII^+F4/80^+$ was evaluated by flow analysis. (c) $CD4^+$ ($CD3^+CD4^+$) T cells from normal mice were prepared by magnetic-activated cell sorting. Flow analysis results show that purity was ~94%. (d, e) Macrophages were treated with pioglitazone (10 μg/ml) with or without SEA (25 μg/ml) and cocultured with purified $CD4^+$ T cells for 24 h, then Treg ($CD4^+CD25^+Foxp3^+$) cells were analyzed by FACS. (f) After peritoneal macrophages (1×10^6) were treated with pioglitazone (10 μg/ml) with or without SEA (25 μg/ml), the percentage of M2 macrophages ($CD206^+F4/80^+$) was evaluated by flow analysis. All experiments were repeated three times with similar results (ANOVA/LSD), $^*P < 0.05$ and $^{**}P < 0.01$.

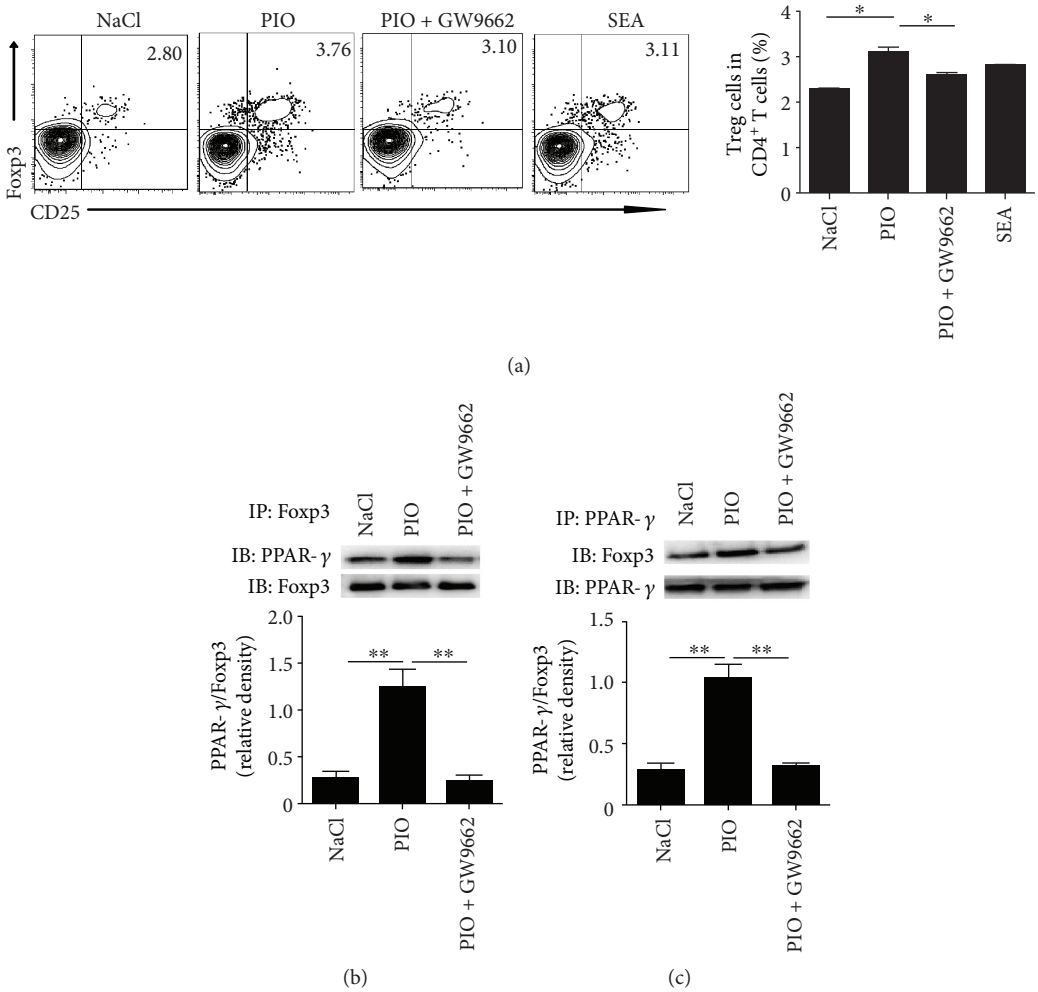

Figure 5: PPAR-γ/Foxp3 interaction promotes Treg differentiation. (a) $CD4^+$ T cells (1×10^6) were treated with pioglitazone (10 μg/ml), SEA (25 μg/ml), or (and) GW9662 (2 ng/ml) for 24 h, and then Treg ($CD4^+CD25^+Foxp3^+$) cells were analyzed by FACS. (b) Normal purified $CD4^+$ T cells of WT mice were stimulated with pioglitazone or GW9662 for 24 h, and then $CD4^+$ T cell fractions were subjected to immunoprecipitation (IP) with an anti-PPAR-γ antibody, and the immunoprecipitates were analyzed by immunoblotting (IB) with anti-Foxp3 or anti-PPAR-γ antibody. (c) The association of PPAR-γ and Foxp3 was confirmed by a reciprocal immunoprecipitation assay using anti-Foxp3. All experiments were repeated three times with similar results (ANOVA/LSD), $^*P < 0.05$ and $^{**}P < 0.01$.

macrophages by using pioglitazone agonists and antagonists. In addition, we also found that activating PPAR-γ signaling in macrophages promoted Treg cell differentiation when cocultured with $CD4^+$ T cells. This is supported by Savage et al.'s study, which indicated that human anti-inflammatory M2 macrophages induce $Foxp3^+GITR^+$ $CD25^+$ regulatory T cells [38].

To further clarify whether PPAR-γ activation could affect Treg cell differentiation directly, we used pioglitazone to stimulate purified spleen $CD4^+$ T cells, and we found that PPAR-γ signaling could induce the expression of Foxp3 and increase the percentages of Treg cells. It has been reported that visceral adipose tissue- (VAT-) resident Tregs specifically express PPAR-γ, which appears to interact with Foxp3 in VAT-Treg cells. Indeed, ectopic coexpression of Foxp3 and PPAR-γ in conventional T cells induces a VAT-Treg-type gene-expression profile [22]. Our results showed that through interaction with Foxp3 in $CD4^+$ T cells, PPAR-γ can induce the percentage of Treg cells and regulate immune response.

5. Conclusions

Our data showed that pioglitazone could induce $CD4^+$ $CD25^+Foxp3^+$ T cells by activating PPAR-γ/Foxp3 signaling, and subsequently suppressing the Th2-mediated immunopathology during *S. japonicum* infection. This study will deepen our understanding of the mechanism of the PPAR-γ/Foxp3 axis in the immunological effects of schistosome infection.

Data Availability

The authors declare that the data supporting the findings of this study are available within the article, or from the authors on reasonable request.

Disclosure

The funders had no role in study design, data collection and analysis, preparation of the manuscript, or decision to publish.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Zhipeng Xu and Minjun Ji conceived and designed the experiments. Yuxiao Zhu, Zhipeng Xu, Ran Liu, Yangyue Ni, Hongzhi Sun, Min Hou, Bingya Yang, and Jingwei Song carried out the animal experiments. The manuscript was written by Yuxiao Zhu, Zhipeng Xu, and Minjun Ji. All authors read and approved the final version of the paper.

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Review Article

Protective Role of γδ T Cells in Different Pathogen Infections and Its Potential Clinical Application

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γδ T cells, a subgroup of T cells based on the γδ TCR, when compared with conventional T cells (αβ T cells), make up a very small proportion of T cells. However, its various subgroups are widely distributed in different parts of the human body and are attractive effectors for infectious disease immunity. γδ T cells are activated and expanded by nonpeptidic antigens (P-Ags), major histocompatibility complex (MHC) molecules, and lipids which are associated with different kinds of pathogen infections. Activation and proliferation of γδ T cells play a significant role in diverse infectious diseases induced by viruses, bacteria, and parasites and exert their potential effector function to effectively eliminate infection. It is well known that many types of infectious diseases are detrimental to human life and health and give rise to high incidence of illnesses and death rate all over the world. To date, there is no comprehensive understanding of the correlation between γδ T cells and infectious diseases. In this review, we will focus on the various subgroups of γδ T cells (mainly Vδ1 T cells and Vδ2 T cells) which can induce multiple immune responses or effective functions to fight against common pathogen infections, such as *Mycobacterium tuberculosis*, *Listeria monocytogenes*, influenza viruses, HIV, EBV, and HBV. Hopefully, the gamma-delta T cell study will provide a novel effective way to treat infectious diseases.

1. Introduction

Infectious diseases are mainly caused by pathogen infection (including viruses, bacteria, and parasites). Many types of infectious diseases are detrimental to human life and health and give rise to high incidence of illnesses and death rate all over the world [1]. Dual infection by different types of viruses and a secondary infection is a common clinical phenomenon, which threatens the health of human beings [2–4]. At the beginning, major focus has been put on pathogens instead of host immune response [5]. But pathogens develop chemical resistance which causes a decrease in curative effect [6, 7]. Therefore, more and more researchers are focusing on conventional T cells and their subpopulations with different phenotypes [8–11]. However, the study on the function and immune response of unconventional T cells (γδ T cells) is still neither enough nor systematic. In this review, we will introduce the direct and indirect effector function and immunity of γδ T cells in detail in a variety of pathogen infections in the hope to provide more information for clinical treatment based on the better understanding of the function of different subsets of gamma-delta T cells.

γδ T cells, a subgroup of T cells based on the different T cell receptor (TCR), when compared with conventional T cells (αβ T cells), make up a very small proportion of T cells. They are widely distributed in different parts of the human body [12]. γδ T cells are mainly divided into three subgroups according to the expression of γ (including 2/3/4/5/8/9) and δ (including 1/2/3/5) chains: Vδ1 T cells, Vδ2 T cells, and Vδ3 T cells [13]. Specifically, Vδ1 gene is paired with

different Vγ elements (including Vγ2/3/4/5/8), Vδ2 gene is paired with Vγ9 chain, and Vδ3 gene is associated with Vγ2 or Vγ3 [14]. The distribution and function of different subgroups of $\gamma\delta$ T cells are strikingly different.

Vδ1 T cells are mostly found in the mucosal epithelium and are in connection with infection of many pathogens [15], such as *Listeria monocytogenes*, human immunodeficiency virus (HIV), and cytomegalovirus (CMV) [16–21]. Vδ2 T cells are primarily enriched in circulating blood. Vδ2 T cells are uniquely matched with Vγ gene usage of Vγ9 (termed Vγ9Vδ2) and they make up the majority of $\gamma\delta$ T cells in the peripheral blood [22, 23]. Vδ2 T cells also exhibit their effective immune response to bacteria and viruses (like mycobacteria, influenza viruses, and Epstein–Barr virus) like Vδ1 T cells [24–27]. Vδ2 T cells based on expressing CD27 and CD45RA are segmented into four different functional subsets with respective characteristic: $CD45RA^+CD27^+$ (naïve), $CD45RA^-CD27^+$ (central memory without effector function which are rich in lymph nodes), $CD45RA^-CD27^-$ (effector memory), and $CD45RA^+CD27^-$ (terminal differentiation which massively appears in the inflammatory site) [28, 29]. They play a significant role via their effector functions and memory responses during infections [28]. The natural killer cell receptor (NKG2D) and Toll-like receptors (TLRs) are also expressed on the surface of both Vδ1 T cells and Vδ2 T cells to exert their effector function during infections even in tumor immunity [30–32]. In contrast with Vδ1 T cells and Vδ2 T cells, Vδ3 T cells, the smallest subset of $\gamma\delta$ T cells, are abundant in the liver and are mainly involved in the process of chronic viral infections [33, 34].

In addition, $\gamma\delta$ T cells are categorized into a suite of multiple functional populations as follows: IFN-γ-producing $\gamma\delta$ T cells, IL-17A-producing $\gamma\delta$ T cells, and antigen-presenting $\gamma\delta$ T cells. They indirectly promote immune response against pathogen infection by $\gamma\delta$ T cells themselves or other immune cells (like $CD8^+$ T cell and B cells) [35–37].

Murine $\gamma\delta$ T cells also have various subsets on the basis of characteristic Vγ usage (including1/2/3/4/5/6/7): Vγ1 combined with Vδ6.3, Vγ5 paired with Vδ1, Vγ6 paired with Vδ1, and Vγ7 paired with three diverse Vδ elements (including Vδ4/5/6) [38]. Interestingly, human Vδ1 cells are the primary subtypes found at mucosal surfaces and share certain characteristics with murine $\gamma\delta$ intraepithelial lymphocytes (which are associated with Vγ7) [39]. On the contrary, Vγ9Vδ2 T cells are restricted to certain species and are found only in humans and higher primates [39].

2. $\gamma\delta$ T Cells Recognize Antigens

$\alpha\beta$ T cells which depend on antigen presentation and restrictive major histocompatibility complex (MHC) molecules recognize antigens. $\gamma\delta$ T cells, however, can recognize various types of antigens (including nonpeptide antigens and stress-induced ligands) without restrictive MHC molecules [40]. Mounting evidence indicates that $\gamma\delta$ T cells exert their protective function in elimination of pathogens and tissue repair via producing cytokines, chemokines, and lytic enzymes, cytotoxic and noncytolytic antiviral activities, and so on [41].

Based on the diverse subtypes, $\gamma\delta$ T cells could recognize different types of antigens. Vδ1 T cells could recognize MHC class I chain-related antigens A and B (MICA/B) and stress-induced molecules frequently expressed on epithelial cell in a $\gamma\delta$ TCR-dependent manner [40, 42–44]. Activated Vδ1 T cells could exert their effector function against bacterial infection and kill infected cells by production of interleukins and interferons [45]. Interestingly, MICA/B expressed on cancer cell are recognized by both Vδ1 T cells and Vδ2 T cells but in a NKG2D-dependent manner [46, 47]. In addition, Vδ1 T cells respond to MICA-related UL16-binding proteins (ULBPs) based on their ability to combine with human cytomegalovirus (HCMV) glycoprotein UL16 in the same manner [48, 49]. ULBPs are a family of MHC class I-related human cell surface molecules and ligands of NKG2D which play a key role in regulation of innate and adaptive immune responses [50, 51]. Lipids and glycolipid which are relevant to various bacteria (like mycobacteria) are required for the presentation of MHC-related class Ib molecules which are expressed on antigen-presenting cells (APCs), and thus, the bacteria-derived antigens can be recognized by Vδ1 T cells [52–55].

Vδ2 T cells, in particular, are activated by low molecular weight nonpeptidic antigens (also called phosphoantigens (P-Ags)) which are produced by transformed cells or cells infected by pathogens (such as viruses and bacteria) [56, 57]. IPP (isopentenyl pyrophosphate) and HMBPP ((E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate) are the most prominent ones. In general, P-Ags associated with infected or transformed cells are produced by way of the mevalonate pathway (like IPP) when compared with the microbes in the isoprenoid pathway (like HMBPP) [58, 59]. In other words, P-Ags generated by diverse cells and different metabolic pathways are different to each other. For example, HMBPP primarily comes from *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and so on [57]. Some clinical medicines can alter the intracellular level of P-Ags to some degree. Nitrogen-containing bisphosphonates (N-BPs) and statins (a kind of anticholesterol drugs) are the most common medicines to increase or decrease the P-Ag level via inhibiting the P-Ag-relevant enzyme [60]. The level of P-Ags also has an obvious trend of increase during stress and infection. Antigen presentation to Vδ2 T cells is independent of restrictions of MHC molecules [60]. Early studies suggested that Vδ2 T cells recognize P-Ags by presentation of CD1d which is expressed on APCs (such as dendritic cells) and monocytes [52, 61]. Butyrophilin 3A1 (BTN3A1) is involved in the process of presenting P-Ags [62]. BTN3A1 binds with P-Ags by its B30.2 domain, and finally, P-Ags are recognized by Vδ2 TCR [63, 64]. Besides, BTN3A1 combined with P-Ags also plays an import role in the process of activation of Vδ2 T cells following N-BP treatment [63]. Like $\alpha\beta$ T cells, the activation and proliferation of Vδ2 T cell also need the second signals which depend on costimulators including CD40-CD40L, CD28-B7.1/7.2, CD137 (4-1BB), and CD2 [65, 66]. Toll-like receptors, as the most common pathogen recognition receptors, have the capacity to recognize infectious pathogen-associated molecule patterns [32]. Activated

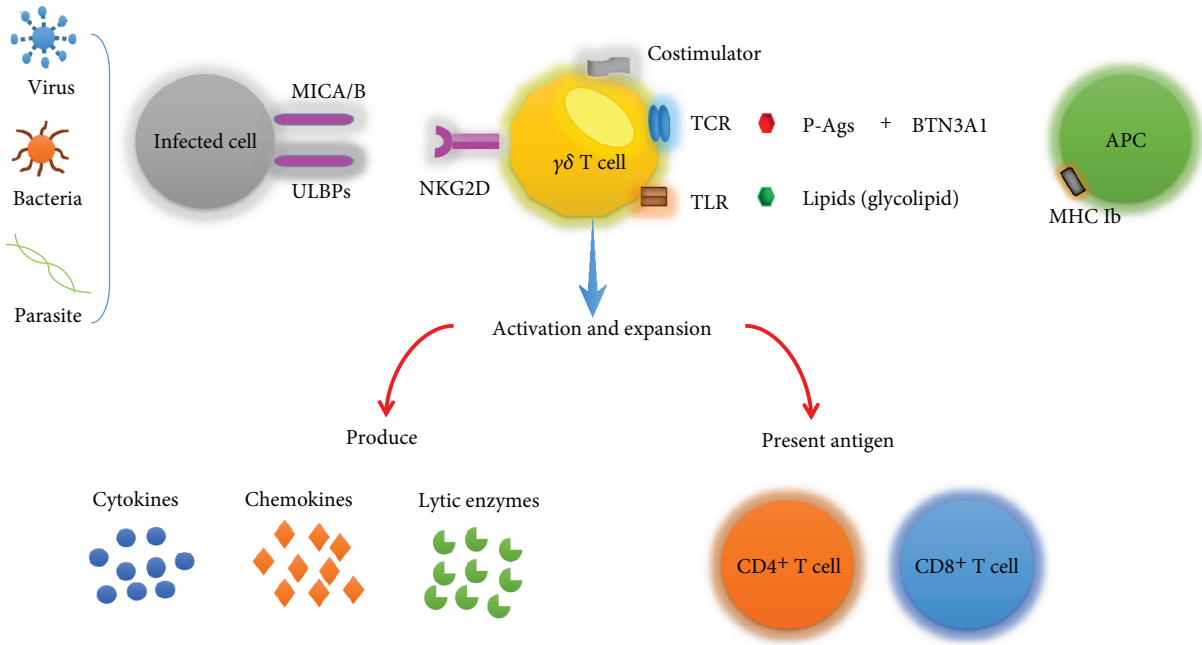

Figure 1: $\gamma\delta$ T cells recognize antigens. Diverse subtypes of $\gamma\delta$ T cells could recognize different types of antigens. $\gamma\delta$ T cells (both Vδ1 and Vδ2) could recognize stress-induced molecules MICA/B and ULBPs which are expressed in cancer and transformed and infected cells in a NKG2D-dependent manner. Vδ1 T cells could recognize bacteria-derived antigens (including lipids and glycolipid) via MHC-related class Ib molecules which are expressed on antigen-presenting cells. Vδ2 T cells recognize phosphoantigens via forming tight complexes following binding with BTN3A1, and in the context of costimulators, Vδ2 T cells are activated and expanded. Vδ3 T cells can be activated by CD1d which may combine with glycolipid and kill CD1d target cells. Activated Vδ2 T cells and Vδ1 T cells could activate the expression of Toll-like receptors which have the capacity to recognize infectious pathogen-associated molecule patterns. Activation and proliferation of $\gamma\delta$ T cells exert their potential effector functions via producing cytokines, chemokines, and lytic enzymes, performing cytotoxic and noncytolytic antiviral activities, presenting antigens to $CD4^+$ and $CD8^+$ T cells, inducing maturation of dendritic cells (DCs), providing B cell help, and so on.

Vδ2 T cells and Vδ1 T cells could activate the expression of Toll-like receptors in reverse [32]. After activation, Vδ2 T cells exert their potential effector functions in the following ways: producing cytokines, chemokines, and lytic enzymes; performing cytotoxic and noncytolytic antiviral activities; inducing maturation of dendritic cells (DCs); providing B cell help; and presenting antigens to $CD4^+$ and $CD8^+$ T cells (Figure 1).

Vδ3 T cells can be activated by CD1d which may combine with glycolipid and kill CD1d target cells and release different kinds of cytokines (includingTh1, Th2, and Th17) and even promote maturation of DC into APCs [33].

3. Function of $\gamma\delta$ T Cells in Infectious Diseases

In early report, researchers pay more attention on $\alpha\beta$ T cells' protective immunity during infectious diseases. But there is no systematic understanding on $\gamma\delta$ T cells' direct or indirect protective ability to fight against pathogens. This review will summarize the diverse functions of $\gamma\delta$ T cells in various infectious diseases.

3.1. Bacteria

3.1.1. Mycobacterium tuberculosis (MTB). $\gamma\delta$ T cells play a significant role in MTB infection. Interestingly, Vγ9Vδ2 T cells which exist in humans and the vast majority of nonhuman primates carry huge weight in mycobacterial infections [67]. On the contrary, Vδ1 T cells seem to be more relevant to other infectious diseases, such as HIV diseases [68].

Vγ9Vδ2 T cells recognize HMBPP via forming tight complexes following binding with BTN3A1 during MTB infection. In the presence of costimulators, Vγ9Vδ2 T cells are subsequently activated and expanded [69]. Recently, a number of studies show that phosphoantigen HMBPP and many cytokines participate not only in expansion but also in recall-like expansion and effector functions of Vγ9Vδ2 T cells after MTB infection [24]. Compared with $CD4^+$ T cells, HMBPP-activated Vγ9Vδ2 T cells produce a speck of IL-2 which contributes to the proliferation of unconventional T cells. It has been demonstrated in cynomolgus monkeys that low-dose IL-2 could synergize with nitrogen-containing bisphosphonate or pyrophosphomonoester drugs to expand Vγ9Vδ2 T cells [70]. Similarly, in nonhuman primate models, HMBPP together with IL-2 maximizes its stimulating effect [71]. Besides, T cell growth cytokines (like IL-15 and IL-21) and Th17-related cytokines are also involved in the above process [24]. After Vγ9Vδ2 T cells are activated and proliferated, they take part in the process to fight against MTB. In early years, Gercken et al. [72] have already proven that the mononuclear phagocytes as accessory cells infected by MTB could activate $\gamma\delta$ T cells and rest upon costimulators

to show a number of functions, especially secretion of cytokine and expression of cytolytic effectors. Generally, MTB phosphoantigen-activated $\gamma\delta$ T cell produces TNF-α and IFN-γ to enhance the protective responses to MTB [73]. Meanwhile, cytolytic effector function based on granulysin and perforin is essential for $\gamma\delta$ T cell to defend against the MTB infections. There is direct evidence that $\gamma\delta$ T cell inhibits and even kills the intracellular MTB by granulysin and perforin with bactericidal ability in macaque models [74]. In addition to the above anti-MTB effects of $\gamma\delta$ T cell, it is newly discovered that activated $\gamma\delta$ T cell may stimulate the maturation of DCs to modulate other cells (like CD4 T helper cells and B cells) to enhance immune response to MTB [75–77]. Phenotype differentiation of Vγ9Vδ2 T cells also help to strengthen the effective function of $\alpha\beta$ T cells to fight against MTB, like promoting $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells to secrete TNF-α and IFN-γ to kill MTB [78]. Research evidence also suggests that memory response of Vγ9Vδ2 T cells may be based on its phenotype differentiation, and further research is needed to unveil the exact mechanism [25].

Overall, the immune response of HMBPP-activated Vγ9Vδ2 T cells to fight against MTB is dependent on secretion of cytokine, expression of cytolytic effector function, and maturation of DCs.

3.1.2. Listeria monocytogenes. *Listeria monocytogenes (L. monocytogenes)* is an intracellular bacterium and exists in food (like meat and other dairy products). It can cause a wide range of foodborne diseases in both animals and human [79]. *L. monocytogenes* can cross the blood-brain barrier, intestinal barrier, or feto-placental barrier and lead to serious infectious illness and death in different populations [80].

IL-17A is mainly produced by $\gamma\delta$ T cells during *L. monocytogenes* infection to promote innate and adaptive immune responses, and it promotes host function of effective elimination of infection by producing cytokines and CXC chemokines [81–84]. Herein, the proliferation and accumulation of neutrophils depending on cytokines and CXC chemokines induced by IL-17A are involved in cross-priming to $CD8^+$ T cells during *L. monocytogenes* infection [85]. In the early infective stage in the liver of mouse models, IL-17A produced by $\gamma\delta$ T cells enhances the antibacterial activity of nonphagocytic cells infected by *L. monocytogenes*, which is involved in promoting antimicrobial peptide mouse β-defensin (mBD) gene expression [86]. Besides, the IL-17A-producing $\gamma\delta$ T cells which are activated rapidly following *L. monocytogenes* infection mediate its antibacterial immune response via IL-23 production by pathogen-activated macrophages/DCs during the early phase of infection [86]. Moreover, in the IL-$17A^{-/-}$ mouse model, following *L. monocytogenes* infection, the bacterial burden in the spleen and liver was significantly higher than that of control mice within the stipulated time [87, 88]. Therefore, it can be concluded that IL-17A plays a significant role in the innate immune response to *L. monocytogenes*. Subsequently, IL-17A has been proven to be indispensable in cytotoxic T cell response against primary *L. monocytogenes* infection. It can also promote the expansion of cytotoxic T cell ($CD8^+$ T cell). Collectively, innate IL-17A produced mainly by $\gamma\delta$ T cells could induce the proliferation of cytotoxic T cell and play their effective cytotoxic T cell response to eliminate *L. monocytogenes* [87, 88].

IL-17A also plays a crucial role in controlling intestinal pathogens during secondary *L. monocytogenes* infection. In the mouse model infected with the internalin A mutant recombinant strain of *L. monocytogenes* (which simulate human intestinal invasion conditions), V$\gamma4^+$ memory $\gamma\delta$ T cells are confirmed as resident memory (Trm) population in the mesenteric lymph nodes (MLNs) [18]. $\gamma\delta$ Trms exert effective elimination of bacteria by early IL-17A secretion to mediate the process in which $\gamma\delta$ Trms contain the bacteria within granulomas in the liver and form large clusters with myeloid cells (including neutrophil) at the sites of *L. monocytogenes* replication foci [18].

3.2. Viruses

3.2.1. Influenza Virus. Due to annual cocirculation and rapid spreading, influenza viruses lead to a large amount of global morbidity and mortality. Influenza viruses widely spread not only from children to the elderly but also to the diverse crowds [89]. Influenza viruses could be divided into the following categories: influenza A viruses (IVA), influenza B viruses (IVB), and influenza C viruses (IVC). IVA show a much more severe infection when compared with IVB and IVC viruses. IVA are derived from swine and avian species and can infect the human respiratory tract through several ways of virus transmission. Recently, researchers are increasingly focusing on the establishment of mouse models following avian influenza H5N1 infection to explore a nicely controlled mechanism of influenza virus infection by gamma-delta T cells [90].

Innate immunity acts as a frontline defense to eliminate virus by interferon and at the same time enhance the adaptive immune response [91]. Phosphoantigen-activated $\gamma\delta$ T cells secret substances associated with killing cells infected by influenza viruses to fight against viruses, such as perforin, granzyme B, and granulysin [92, 93]. In humanized mouse models, phosphoantigen treatment significantly decreased weight loss and mortality associated with IVA infection and could control human IVA infection possibly via the selective activation and expansion of human Vδ2 T cells. Thus, phosphoantigen-activated $\gamma\delta$ T cells have a significant ability to clear human and avian influenza viruses [90]. In addition, $\gamma\delta$ T cells also assist in strengthening the activity of APCs by providing significant signal molecules. After that, APCs play their antigen-presenting role to present influenza antigens to acquire T cells (like $CD8^+$ T cells and $CD4^+$ T cells) and influenza viruses will finally be cleared by these antigen-specific T cell responses. Moreover, phosphoantigen-activated and expanded $\gamma\delta$ T cells also induce the expression of CCR1 [94]. CCRs are inflammatory chemokine receptors that promote the ability of elimination of viruses [92, 93].

The number of activated and proliferating $\gamma\delta$ T cells, however, varies from person to person after influenza vaccination. Studies compared the number of activated and proliferating $\gamma\delta$ T cells between young and elderly healthy human measured by flow cytometry following vaccination. It has

been discovered that elderly individuals have lower number and slower kinetics changes of activated and proliferating $\gamma\delta$ T cells than young men. It can be concluded from the study that age serves as an important factor to affect the efficiency of T cell response and may make vaccination have a severe drop-off in effectiveness [95].

Besides phosphoantigen and age, type I IFNs and other cytokines could also influence $\gamma\delta$ T cell immune response against influenza infection [96, 97]. In the mouse model infected with IVA, researchers exposed IVA-infected mice to smoke or air. Mice exposed to chronic cigarette smoke recovered poorly from primary influenza A pneumonia but recruited $\gamma\delta$ T cells to the lungs that predominantly expressed IL-17A. Depletion of IL-17A significantly increased T-bet expression in $\gamma\delta$ T cells and improved recovery from acute IVA infection [97]. Collectively, cytokines and phosphoantigen play a crucial part in $\gamma\delta$ T cell-mediated antiviral immune response during influenza virus infection.

3.2.2. Human Immunodeficiency Virus (HIV). HIV infection is different from other viral infections that it does not depend on any one $\gamma\delta$ T cell subset alone but need two primary subsets of $\gamma\delta$ T cells to participate together [98]. The percentage of two subsets of $\gamma\delta$ T cells, however, can be changed or reversed during HIV infections [99]. Vδ1 and Vδ2 T cells in good proportion would play a key role in HIV infections. It has been reported that increasing Vδ1 during HIV infection correlated with the proliferation of $CD8^+$ T cells [100]. Recently, researchers found that the changes in $\gamma\delta$ T cell and $CD8^+$ T cell in primary and chronic stages of HIV infection (PHI and CHI) are different. Specifically, in untreated chronic HIV infection (UT-CHI), researchers found a positive correlation between $\gamma\delta$ T cell frequency and $CD8^+$ T cell activation. In contrast, in primary HIV Infection (PHI) patients, a negative correlation was found [101]. In addition to Vδ1 and $CD8^+$ T cells, there is a correlation between Vδ2 T cells and $CD4^+$ T cell and they are inversely associated with viral loads [102]. Moreover, inversion of the Vδ2-to-Vδ1 ratio was detected before the inversion of the CD4-to-CD8 ratio, which suggests that the abnormal percentage of Vδ1 and Vδ2 T cells also affected the $CD4^+$ to $CD8^+$ T cell ratio [103]. Recent studies highlight that the CD4/CD8 ratio may serve as a better biomarker for assessing disease progression and HIV's immune suppression in HIV-infected population [104]. It is also supported by another finding that there is a significant relationship between early levels of soluble biomarkers and exhausted CD4/activated CD8 T cells via systematic analysis of correlation between soluble inflammatory biomarker expression and CD4/CD8 T cells at the different stages of HIV infection (including PHI, CHI, and UT-CHI) in HIV-infected Mozambican adults [105]. The lopsided proportion of Vδ1 and Vδ2 T cells causes a negative response against HIV with inhibited cytotoxicity of $\gamma\delta$ T cells to kill HIV-infected cells, inhibited secretion of proinflammatory cytokines which is associated with antiviral ability, inhibited ability to block coreceptors for HIV entry, inhibited activation of innate and acquired immunity, and imbalance between cell activation and killing [106, 107]. Thus, dysfunction of $\gamma\delta$ T cells leads to HIV immune evasion and finally causes chronic infection [98] (Figure 2). Recently, it was reported that in acute HIV-1 infection, the phenomenon of the lopsided proportion of Vδ1 and Vδ2 T cells can be reversed by syphilis coinfection.

The effects of both Vδ1 and Vδ2 T cells to defend against HIV have been identified in past years [19]. Expansion of Vδ1 T cells was associated with microbial translocation which has relevance to immune activation [108]. Recently, researchers found that HIV-infected patients have a higher percentage (but not absolute numbers) of Vδ1 T cells [109]. Interestingly, according to the expression of the ε chain of the CD3 protein which is used for TCR signaling, Vδ1 T cells can be segmented into two subsets: $CD3\varepsilon^{lo}$ Vδ1 T cells and $CD3\varepsilon^{hi}$ Vδ1 T cells [109]. $CD3\varepsilon^{lo}$ and $CD3\varepsilon^{hi}$ T cells have diverse phenotypes and functions. $CD3\varepsilon^{lo}$ cells frequently express terminally differentiated (TD) cells, exhausted phenotypes, and programmed death-1 (PD-1) and fail to produce IL-17, suggesting that $CD3\varepsilon^{lo}$ Vδ1 T cells have a lower responsiveness to antigenic stimulation than $CD3\varepsilon^{hi}$ Vδ1 T cells [109]. This study indicates that HIV may partially induce Vδ1 T cell inactivation and inhibit their effector functions to control virus during HIV infection. Vδ2 T cells exhibited their functions in multiple ways when compared with Vδ1 T cells. Phosphoantigen-activated Vδ2 T cells have direct cytotoxicity for HIV-infected cells even for tumor cells and exhibit B helper T cell function [110–112]. Besides, activated Vδ2 T cells have immune response by producing type 1 cytokines or chemokines including IFN-γ, TNF-α, RANTS, and MIP [106, 113, 114]. In the context of diverse kinds of chemokines (especially β-chemokine), Vδ2 T cells can inhibit coreceptors for HIV entry [110, 115]. Vδ2 T cells, in addition to being immune cells, are also confirmed as APCs [116]. Interestingly, antigen-stimulated $\gamma\delta$ T cells costimulate NK cells and increase NK cell killing of autologous DC (editing) which is impaired in HIV^+ patients [117]. Interaction between DC and $\gamma\delta$ T cells also plays a key role in immune response to pathogen infections and virus-induced immune evasion [118]. Especially, in HIV-1 infection, exposure of DC to HIV-1 leads to its dysfunction but inversely stimulates $\gamma\delta$ T cell proliferation and IFN-γ secretion via CCR5-mediated mechanism and plays a crucial role in controlling of HIV-1 replication, virus dissemination within DC via CCL4-mediated mechanism, and HIV-1 transfer to susceptible $CD4^+$ T cells [119].

Effector function of Vδ2 T cells and Vδ1 T cells at different stages of HIV infection, namely, PHI and CHI, is remarkably different. Vδ2 T cells are reported as potential regulatory T cells (Tregs) and play a crucial role in controlling immune activation by anti-inflammatory cytokine secretion during P-HIV [101]. Compared with C-HIV, both mucosal Vδ2 T cells and Vδ1 T cells exert more effective antiviral response in P-HIV [115].

Above all, Vδ2 T cells act as a bridge between innate and acquired immunity to eliminate HIV. However, study shows that the number and function of Vδ2 T cells are depleted during HIV infection [120]. Depletion of Vδ2 T cells is caused by activation of the p38-caspase pathway via combination of HIV and CC chemokine receptor (CCR5) and

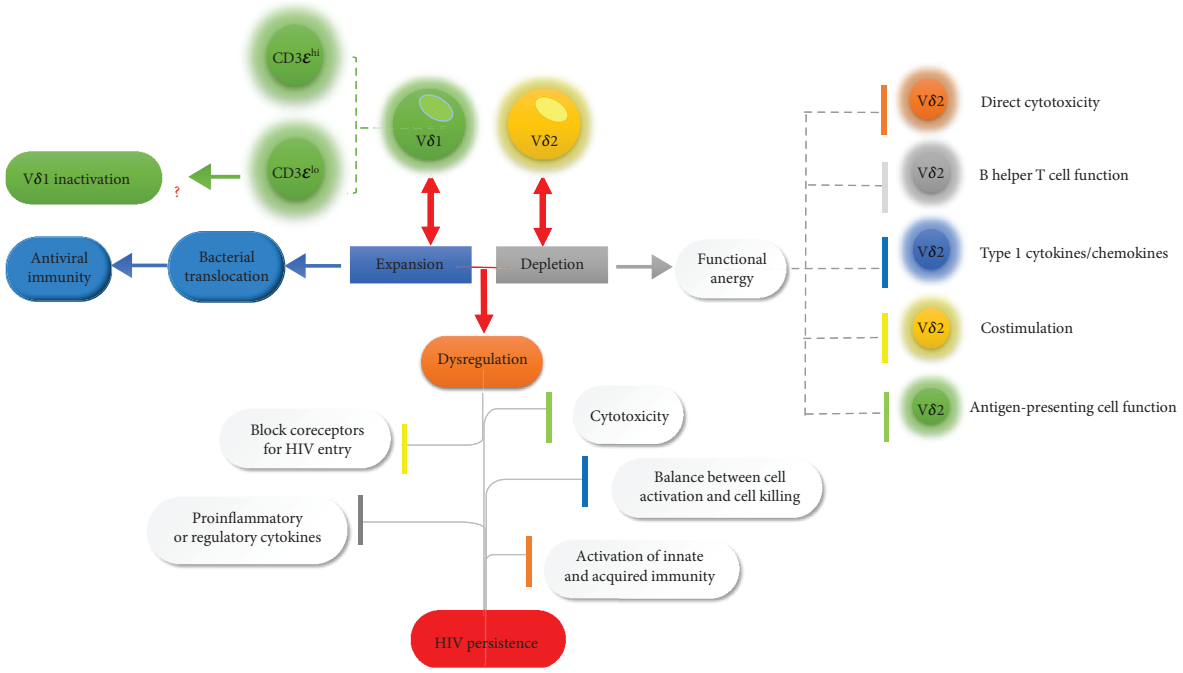

Figure 2: Dysregulation of $\gamma\delta$ T cells during human immunodeficiency virus (HIV) infection. Expansion of Vδ1 T cells during HIV infections was associated with microbial translocation which has relevance to immune activation and exhibited its antiviral immune response. Recently, Vδ1 T cells are segmented into two subsets: CD3εlo Vδ1 T cells and CD3εhi Vδ1 T cells, and CD3εlo Vδ1 T cells may at least partially induce Vδ1 T cell inactivation based on its lower responsiveness to antigenic stimulation. However, the number and function of Vδ2 T cells are depleted during HIV infection. Depletion of Vδ2 T cells leads to inefficient immune response to HIV with inhibited direct cytotoxicity, B helper T cell function, type 1 cytokine or chemokine secretion, antigen-presenting cell function, and costimulation of NK cells. The lopsided proportion of Vδ1 and Vδ2 T cells causes a negative response against HIV infection with inhibited cytotoxicity, coreceptor for HIV entry, proinflammatory or regulatory cytokine release, activation of innate and acquired immunity, and imbalance between cell activation and killing. Thus, dysfunction of $\gamma\delta$ T cells leads to HIV immune evasion and finally causes chronic infection.

integrin a4β7 [121]. There is no doubt that the depletion of Vδ2 T cells leads to the inefficient immune response to HIV.

Though the majority of Vδ2 T cells are decreased in HIV infection, activated CD16$^+$ Vγ9Vδ2 T cells as a subset of Vγ9Vδ2 T cells (based on expression of Fc receptor for IgG, also called CD16) have the capacity to induce antibody-dependent cell-mediated cytotoxicity (ADCC) and exert their antiviral functions in HIV type 1 disease [122]. In an earlier report, Vδ2 T cells expanded by zoledronate (one kind of bisphosphonates) and IL-2 are capable of enhancing ADCC cytotoxic effectors in HIV patients [107].

3.2.3. Epstein–Barr Virus (EBV). EBV, a virus related to transformation of B cell, could cause severe infections in individuals and more likely cause diseases including acute infectious mononucleosis, chronic active EBV infection, Burkitt lymphoma, and tumor (nasopharyngeal carcinoma) [123–125]. There were initial reports that cytotoxic lymphocytes have important influence on anti-EBV action, such as adaptive CD8$^+$ T cell responses [126, 127]. Recently, it has been reported that innate cytotoxic lymphocyte participates in EBV infections [128]. NK cells and Vγ9Vδ2 T cells also exert their cytotoxic lymphocyte function against EBV infection [128]. Furthermore, latent EBV infection shows much a more significant increase in the expansion of both natural killer cells and Vγ9Vδ2 T cells when compared with lytic EBV infection [129]. Expanded Vγ9Vδ2 T cells interact with P-Ag which is produced by the mevalonate pathway by TCR of Vγ9Vδ2 T and BTN3A1 in EBV-infected individuals [129, 130]. In acute infectious mononucleosis, the expression of $\gamma\delta$ TCR and the number of $\gamma\delta$ T cells were increased analyzed by whole transcriptome profiling [27]. Overexpression of HSP60, HSP70, HSP90, and ULBPs, as protein ligands, can strengthen the recognition and effective cytotoxicity function of $\gamma\delta$ T cells against virus-infected cells or malignant host cells [131, 132]. Human MutS homologue (including hMSH2/3/6), which is one kind of protein for DNA mismatch repair and also as a stress-induced protein ligand, is overexpressed in B lymphoblastic cells. This improves the recognition and effective cytotoxicity function of $\gamma\delta$ T cells as well as protein ligands [133]. Besides, EBNA1 as nuclear antigen (also called latency I) is expressed on EBV-infected memory B cells and is indispensable for replication of viral genome. It can be recognized by Vγ9Vδ2 T cells and leads to Vγ9Vδ2 T cell expansion [128, 134]. Finally, activated Vγ9Vδ2 T cells could fight against EBV latency. In addition, activated Vγ9Vδ2 T cells which are based on FasL and TRAIL may exert effective elimination function of EBV-transformed lymphoblastoid cell lines [128]. Indeed, P-Ag-stimulated Vγ9Vδ2 T cells were able to prevent outgrowth of adoptively transferred EBV-transformed lymphoblastoid cell lines *in vivo* [135]. And adoptive transfer of Vγ9Vδ2 T

cells could prevent tumorigenesis in mice in which EBV-associated lymphoma formation was induced by EBV infection [136]. In summary, Vγ9Vδ2 T cells combined with other cytotoxic innate lymphocyte subsets (NK T cells) can target various stages of EBV infection.

3.2.4. Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV). HBV and HCV are involved in liver damage and can lead to viral hepatitis and even liver cancer [137, 138]. The liver is rich with multiple innate immune cells (like natural killer cells and $\gamma\delta$ T cells) and plays an important role in innate immunity in the various stages of liver diseases [139–141]. Hepatic $\gamma\delta$ T cells occupy a small proportion in total liver lymphocytes [139]. At the beginning, the number of Vδ2 T cells, which account for a considerable proportion of $\gamma\delta$ T cells in the liver, tends to decline accompanied by disease progression [142, 143]. Nevertheless, Vδ1 T cells are expanded in liver diseases (especially acute-on-chronic liver failure infected by hepatitis B virus) when compared with Vδ2 T cells and defense against liver damage by producing increased cytotoxicity and inflammatory cytokine [144]. Researchers recently revealed that the frequency of $\gamma\delta$ T cell subsets (both Vδ1 and Vδ2) has increased in HBV-infected patients without symptoms. In HBV-infected patients, increased effector memory Vδ2 T cells play a protective role by producing interferon-γ [145]. But in chronic HCV-infected patients, activation and differentiation of Vδ2 T cells exert cytotoxicity via acquisition and expression of cytotoxic natural killer-like phenotype to eradicate the virus instead of producing interferon-γ [146]. Interestingly, $\gamma\delta$ T cells could strengthen TNF-α production (induce IFN-γ expression) and CD107a expression (a functional marker for cytotoxicity) with antiviral drug interferon-α treatment. In other words, interferon-α can enhance cytotoxic function of $\gamma\delta$ T cells in chronic HBV infection [147]. Moreover, peripheral Vδ2 T cells activated by nonpeptidic antigens (such as pyrophosphomonoesters) can inhibit the replication of HCV via noncytolytic antiviral ability [148]. In contrast, it has been reported that in HBV-infected immunocompetent mice, $\gamma\delta$ T cells mediated $CD8^+$ T cell exhaustion by mobilizing myeloid-derived suppressor cell (MDSC) infiltration to the liver in HBV-induced tolerance [149].

3.3. Parasite

3.3.1. Plasmodium. Malaria caused by *Plasmodium* occurs in tropical and subtropical regions and endangers the physical health. An earlier report demonstrated that conventional T cells ($CD4^+$ and $CD8^+$ T cells) exhibit a protective role in the elimination of *Plasmodium falciparum* [150]. Accumulating findings indicate that $\gamma\delta$ T cells play a key role in defending against *Plasmodium* infection. $\gamma\delta$ T cells are found increased during *Plasmodium* infection [151]. In $\gamma\delta$ T cell depletion mice, the level of protective antibody (IgG2a) which eradicates the malaria parasite exhibits an apparent decline when compared with control [152]. In mouse models without sufficient $\gamma\delta$ T cell, it was discovered that, in the context of agonistic anti-CD40 antibody, $\gamma\delta$ T cells are involved in controlling *Plasmodium berghei* XAT (PbXAT). Afterwards, DCs can be activated by unconventional T cells by means of CD40 ligand expression, and whereafter, helper T lymphocyte 1 cells exert their effector response defending against *Plasmodium* via Th1 differentiation during PbXAT infection [152–154]. In addition, cytokines such as IL-12 and TNF are also crucial for controlling *Plasmodium* infection and decrease the risk of fever, clinical malaria, and parasitemia [155]. IL-12 and IL-18 are essential for expression of TIM3 (T cell immunoglobulin domain and mucin domain 3), one member of the TIM protein family, in $\gamma\delta$ T cell, which could offer clinical malaria important opportunities for risk reduction [156]. Especially, IL-17A, which is largely produced by $\gamma\delta$ T cells, could slow down the course of diverse pathogen infections. According to the report, IL-17A-producing $\gamma\delta$ T cells in combination with monocytes are involved in the early process of fighting against parasites [157]. Some cytokines and chemokines (such as TNF and MIP-1β/1α) which increase the risks of severe malaria, however, are derived from $\gamma\delta$ T cell [158]. Collectively, cytokines and chemokines have dual effects on *Plasmodium* infections.

Different subgroups of $\gamma\delta$ T cell play various roles in controlling *Plasmodium* infections. Vγ9Vδ2T cells activated by *P. falciparum* antigens produce cytotoxic granules to kill merozoites and control parasite density during the blood stage of infection [159]. The proportion of $V\delta2^+$ $\gamma\delta$ T cells increased in previously naïve adults following malaria infection. But children with repeated malaria were associated with reduced percentages of $V\delta2^+$ $\gamma\delta$ T cells and cytokine secretion and increased expression of immunoregulatory genes. Taken together, the loss and dysfunction of $V\delta2^+$ $\gamma\delta$ T cells in children with repeated malaria may lead to clinical tolerance of the parasite [160]. Moreover, the diminished $V\delta2^+$ $\gamma\delta$ T cell proinflammatory cytokine production in this situation was associated with expression of the immunoregulatory markers TIM3 and CD57. Higher $V\delta2^+$ $\gamma\delta$ T cell proinflammatory cytokine production was associated with protection from subsequent *P. falciparum* infection [161]. Recently, it was discovered that both reduction and dysfunction of $V\delta2^+$ $\gamma\delta$ T cells promote the expression of CD16 which causes $V\delta2^+$ $\gamma\delta$ T cells to exhibit inefficient recognition of nonpeptidic antigens [162]. $V\gamma1^+$ $\gamma\delta$ T cells are also important for defense against *Plasmodium* infection. During early *Plasmodium berghei* XAT (PbXAT) infection stage, expanding $V\gamma1^+$ $\gamma\delta$ T cells promotes CD40 ligand expression and IFN-γ secretion. CD40 ligand- (CD40L-) CD40 signaling activates DCs to induce protective immunity. It was manifested that the $V\gamma1^+$ $\gamma\delta$ T cell response is dependent on IFN-γ-activated DCs [163]. Nonetheless, at the late stage, the IFN-γ positivity of $V\gamma1^+$ $\gamma\delta$ T cells is reduced due to $\gamma\delta$ T cell dysfunction. Indeed, $V\gamma1^+$ $\gamma\delta$ T cells promote inhibitory receptor expression, such as PD-1, LAG-3, and TIM3 at the late stage [163].

4. Possible $\gamma\delta$ T Cell-Based Clinical Application

Bisphosphonates (also called aminobisphosphonates (ABP)) are commonly used to activate Vγ9Vδ2 T cells via accumulating and elevating the level of cellular IPP and its

metabolites [164]. Pamidronate (PAM) and zoledronate (Zol) are bisphosphonates that can inhibit the IPP-metabolizing enzyme farnesyl diphosphate synthase (FDPS) which is a key enzyme of the mevalonate pathway [165, 166]. PAM is considered as an economical and practical way to activate Vγ9Vδ2 T cells [167]. In humanized mouse models, it is reported that PAM reduces disease severity and mortality and controls lung inflammation and viral replication after human influenza virus infection [168]. Zol is broadly exploited to enhance adoptive cancer immunotherapy and stimulate effector $\gamma\delta$ T cells with antitumor activity [169, 170]. However, ABP as an anti-infection agent have certain limitations in clinical use. Intravenous infusion of ABP gives rise to immune-mediated diseases (such as persistent autoimmune syndromes) because of TNF-α and IFN-γ release by Vγ9δ2 T cells which will induce inflammatory response or acute clinical response [171]. ABP affect oral absorption and inhibit bone resorption and even lead to bone side effects in cancer treatment [172]. Tetrakis-pivaloyloxymethyl 2-(thiazole-2-ylamino) ethylidene-1,1-bisphosphonate (PTA) as a synthetic bisphosphonate prodrug can also inhibit FDPS. It can get inside the cells where it is converted into acid enzymes with activity by intracellular esterases [173]. PTA could activate the expansion of peripheral blood Vγ9δ2 T cells which are separated from cancer patients (prostate and breast cancer) [174]. Compared with Zol, PTA activates $\gamma\delta$ T cell expansion more effectively and produces more cytokines (TNF-α and IFN-γ) [173].

Besides P-Ag-induced activation of $\gamma\delta$ T cells, BTN3A-specific monoclonal antibody (mAb) 20.1 can also activate Vγ9Vδ2 TCR by CDR3 of Vγ9 and Vδ2 chain responsiveness to mAb 20.1 [175]. Meanwhile, mAb 20.1 can interfere with the P-Ags-response [175]. Thus, BTN3A-specific antibody may be useful agents against pathogen infections.

Adoptive transfer of $\gamma\delta$ T cells by intravenous infusion is the most common way for the clinical trials of patients [176, 177]. Adoptive transfer therapy is confirmed as a safe way without requiring preconditioning to expand Vγ9Vδ2 T cells and has been reported in many studies [178, 179]. Researchers recently pay more attention to not only the safety but also the clinical effects of *in vitro* expanded $\gamma\delta$ T cells in multiple ways including DNA copy number and negative conversion rate of HbeAg during active HBV infections (https://www.clinicaltrials.gov/). In nonhuman primate models infected by *Mycobacterium tuberculosis*, adoptive transfer of Vγ9Vδ2 T cells has no or reduced tuberculosis dissemination when compared with control [180]. Vγ9Vδ2 T cells by adoptive transfer therapy display central/effector memory and exert their effector function defense against MTB infections via secreting anti-*M. tuberculosis* cytokines and inhibiting intracellular bacteria [180]. Adoptive transfer therapy based on $\gamma\delta$ T cells is also applicable for treatment of a range of cancers including renal cancer, breast and cervical cancer, and non-small-cell lung cancer [181, 182]. Interestingly, it is more vulnerable to accomplish successfully adoptive transfer of $\gamma\delta$ T cells following ABP treatment [183].

An earlier study reports that low-dose IL-2 could synergize with nitrogen-containing bisphosphonate or pyrophosphomonoester drugs to expand Vγ9Vδ2 T cells [71]. Phosphoantigens combined with IL-2 are an efficient method to activate and expand Vγ9Vδ2 T cells both *in vitro* and *in vivo* [74, 184]. Expression of NO synthase (NOS2) exerts profound influence on $\gamma\delta$ T cell properties, including IL-2 secretion, its expansion, and glycolysis metabolism. Recently, there is a report that IL-2 is not completely necessary for Vγ9Vδ2 T cells in adoptive immunotherapy [174]. IL-18 represents a new potential treatment for HIV-positive individuals since it activates Vγ9Vδ2 T cell responses to phosphoantigen [185].

Broadly speaking, $\gamma\delta$ T cell-based clinical application has both advantages and limits in controlling and even eliminating pathogen infections. $\gamma\delta$ T cells have the following extraordinary advantages: firstly, $\gamma\delta$ T cell-based clinical application emphasizes the importance of host immune response instead of pathogens themselves. Secondly, $\gamma\delta$ T cells rapidly gather at the site of infection and exert effective function of elimination of pathogens. Thirdly, $\gamma\delta$ T cells play multiple roles in controlling infection on the basis of different subsets of $\gamma\delta$ T cells with different functions and $\gamma\delta$ T cells act as functionally diversified cells such as APC and potential regulatory T cells. Fourthly, though $\gamma\delta$ T cells make up a very small proportion of T cells in the human body, they can be directly activated by phosphoantigens or indirectly activated by drugs that induce IPP accumulation or monoclonal antibody, both of which are economical and practical. Fifthly, there is a relatively safe way for the clinical trials of patients: adoptive transfer of $\gamma\delta$ T cells by intravenous infusion. However, current application of conventional therapy also has certain limitations in clinical use. It has been reported that phosphoantigen reapplication may lead effector cells to an incapable, exhausted, and even dead condition [186]. Irrational drug use like overdoses may lead to autoimmune diseases. Moreover, activated $\gamma\delta$ T cells by drugs like ABP release many proinflammatory cytokines and may also give rise to immune-mediated diseases such as persistent autoimmune syndromes. Therefore, it is important to confirm both the safety and the dose of clinical medication in the future and $\gamma\delta$ T cell-based immune therapy still needs further discussion and research.

Above all, though the mentioned potential therapeutic methods have some limitations, it put forward ideas and methods for further clinical research. To achieve an effective and safe treatment on infected patients, no doubt, we need a broader and deeper understanding of effector function of different subgroups of human $\gamma\delta$ T cells.

5. Summary

Since the diverse subpopulations of $\gamma\delta$ T cells possess different biological characteristics, they play different roles in various infectious illnesses induced by bacteria, viruses, and parasites. Different kinds of antigens associated with various pathogen infections including nonpeptidic antigens (P-Ags), MHC molecules, and lipids could be directly or indirectly recognized by $\gamma\delta$ T cells. Some $\gamma\delta$ T cells are immediately activated, while some $\gamma\delta$ T cells also need a second signal costimulation. The activation and expansion of $\gamma\delta$ T cells

exert their effector function during pathogen infections. Growing evidence suggests that γδ T cells act as a link to connection innate with adaptive immunity. It is intriguing to find that γδ T cells can also work as APC to present pathogen infection-associated antigen to $CD4^+$ and $CD8^+$ T cells. In addition, γδ T cells exert their protective function in the elimination of pathogens and tissue repair via producing cytokines, chemokines, and lytic enzymes and cytotoxic and noncytolytic antiviral activities. γδ T cells can also promote DC maturation and provide B cell help to produce antibody. Collectively, γδ T cells play a significant role in the elimination of pathogens. In view of the promising implications of γδ T cells to treat infectious diseases in preclinical studies, it is hoped that γδ T cells will provide a potentially effective new way to treat infectious diseases.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Yueshui Zhao and Ling Lin contributed equally to this manuscript.

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Research Article

Immune Dysfunction and Coinfection with Human Immunodeficiency Virus and *Schistosoma japonicum* in Yi People

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Objective. To explore the association between infections with HIV and *Schistosoma japonicum*, and to determine the influences of the HIV-*S. japonicum* coinfections on the immune system of Yi people. *Methods*. A block design study was conducted in a Yi county in southwestern China, one of the endemic areas of both HIV/AIDS and *S. japonicum* in China. All participants were screened for HIV antibodies and *S. japonicum* antibodies (SjAb) and were classified into four groups: HIV(+)/*S. japonicum*(−), HIV(−)/*S. japonicum* (+), HIV(+)/*S. japonicum*(+), and HIV(−)/*S. japonicum*(−). *Results*. There were significant differences among the four groups in both $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes, but no significant difference in $CD3^+$ T lymphocytes. Both the $CD4^+$ T lymphocyte counts and the ratio of $CD4^+/CD8^+$ were lower in HIV-infected people compared with those uninfected. People infected with *S. japonicum* had increased $CD4^+$ T lymphocyte counts but reduced $CD8^+$ T lymphocyte counts. Similarly, the ratio of $CD4^+/CD8^+$ was higher in *S. japonicum*-infected people compared with those uninfected. People coinfected with HIV and *S. japonicum* had lower $CD4^+$ T lymphocyte counts, lower ratio of $CD4^+/CD8^+$, and higher $CD8^+$ T lymphocyte counts compared with those infected with HIV only or *S. japonicum* only. People infected with HIV only and those coinfected with HIV and *S. japonicum* had a higher level of IFN-γ compared with people with no infection. There were no significant differences between people infected with HIV only and with *S. japonicum* only in the levels of IFN-γ and IL-10. *Conclusions*. People coinfected with HIV and *S. japonicum* might have a suppressed immune function because of a decrease in $CD4^+$ T lymphocyte counts, a lowered ratio of $CD4^+/CD8^+$, and an increase in $CD8^+$ T lymphocyte counts. Coinfection with HIV and *S. japonicum* would alter the level of IFN-γ in plasma.

1. Introduction

Human immunodeficiency virus (HIV) is a global health problem, as are helminth infections. Schistosomiasis is one of the chronic, water-borne helminth diseases [1], and it is a risk factor for HIV infection [2]. People infected with *S. mansoni* or *S. haematobium* are more susceptible to HIV infection due to common high-risk behaviors, such as having multiple sexual partners and other exposures to sexually transmitted diseases [3–5]. There is also an overlap of multiple risk factors associated with the HIV and *S. mansoni* infections in the same geographical setting or the biological interaction between them to increase the risk of individuals to be coinfected with both [3, 4, 6–8]. Epidemiological studies have reported that there is an association between HIV infection and schistosomiasis [8–11].

In early 1990, some researchers found that animals infected with *Schistosoma mansoni* could produce antibodies that was specific to one protein of HIV, the regulatory protein virion infectivity factor (VIF), and the VIF could identify a

170 kDa peptide of *S. mansoni* [12]. In humans, a study conducted in rural Tanzanian villages near Lake Victoria found that *S. mansoni* infection predicted HIV infection among reproductive age women [13]. Besides, urogenital schistosomiasis may be a risk factor for HIV infection [14, 15]. Furthermore, some studies have reported that HIV increases the risk of parasite infection as HIV attacks the human immune system and causes cellular immunity dysfunction [16]. People infected with parasites are also at higher risk for HIV infection compared with those uninfected [16, 17]. Infections of schistosomiasis and with HIV can be mutually promoted through the immunological interactions [18, 19]. Cytokines play an important role in both antiviral and antiparasitic diseases. The HIV- (nonenvelope) specific antiviral T-cell immune response is dominated by the secretion of IFN-γ, TNF-α (Th1 profile) [4], and IL-17 (Th17 profile), whereas *S. mansoni* infection in humans is predominantly characterized by the secretion of IL-4, IL-5, IL-13 (Th2 profile), and IL-17 (Th17 profile) in the acute phase and a regulatory phenotype (T regs) in the chronic phase [20]. IL-17 is also a critical mediator of liver fibrosis in *S. japonicum*-infected mice [21]. Both HIV and schistosome infection cause increased levels of IFN-γ and IL-10 [22–25]. After HIV and schistosome infection, the balance of the immune state is maintained by upregulating the expression of IFN-γ [26, 27].

$CD4^+$ T cells expressing the chemokine receptor CCR5 are the predominant targets of HIV during initial infection, and specific $CD4^+$ T helper (Th) subsets are particularly susceptible to HIV [28–30]. *In vitro* studies demonstrated that patients with active schistosomiasis displayed higher cell surface densities of chemokine receptors CCR5 and CXCR4, making the cells more susceptible to HIV than those from helminth-free individuals [31]. As HIV infection is associated with reduced $CD4^+$ T lymphocyte counts, it was previously reported that the destruction of helper $CD4^+$ lymphocytes by the HIV virus in coinfected individuals could affect granuloma formation of *S. mansoni* infection and alter the egg excretion efficiency [10, 32]. Granuloma formation in *S. mansoni* infection is a $CD4^+$ T lymphocyte-dependent process [32]. Some earlier studies have hypothesized that the destruction of helper $CD4^+$ T lymphocytes (Th2) by HIV, coupled with the significant importance of $CD4^+$ cells in the formation of granuloma, may lead to a decreased ability of the Th2 aiming to produce proinflammatory cytokines, and hence lead to severe hepatic morbidity [33, 34]. HIV-infected patients with *S. mansoni* coinfection also displayed a significantly higher number of Gag-specific IL-10-positive $CD8^+$ T cells. Immunological studies have also found the biological mechanisms through which chronic HIV infection could affect *S. mansoni*-related morbidities [12, 31]. These mechanisms could result in differences in the prevalence and intensity of *S. mansoni* infection, the efficiency of parasite egg excretion, morbidity patterns, and the response to anthelmintic treatment among HIV-infected and noninfected people [9, 31, 35]. Similar observations on the influence of egg excretion were reported in HIV-1-positive individuals coinfected with *S. mansoni* or *S. haematobium* in Ethiopia, Kenya, and Uganda [9, 36, 37]. However, some studies found that *S. mansoni* infection was not associated with HIV acquisition. *S. haematobium* causes urogenital schistosomiasis and poses a risk for HIV acquisition through the urogenital lesions [15, 38]. As opposed to *S. haematobium*, *S. mansoni* is unlikely to cause genital lesions to have an impact on HIV-1 acquisition [39]. Other studies suggested that systemic immune modulation by *S. mansoni* might not significantly increase the susceptibility to HIV acquisition [9, 39].

To our knowledge, the impact of *S. japonicum* and HIV coinfection on immune responses has not been fully assessed. Hence, in this study, we investigate the possible association between the infections with HIV and *S. japonicum* by exploring the alteration of $CD4^+$ and $CD8^+$ T lymphocyte counts and cytokine levels in coinfected individuals compared with those uninfected or infected with HIV or *S. japonicum* alone.

2. Materials and Methods

2.1. Study Field. This study was conducted in Puge County of the Liangshan Yi Prefecture, southwestern China, from July 22nd to August 11th, 2015. It is an underdeveloped region inhabited mainly by the Yi people, an ethnic minority group in China. In this mountainous region of southwestern China, the high risk of HIV infection is mainly due to drug abuse and the casual sexual behavior of the "Yi people" [40]. This "Yi" county has a complex topography with mountains and valleys with an average elevation of 1800 meters. The local climate is subtropical monsoon with mild winters and warm and humid summers. These special geographical and environmental conditions are suitable for *S. japonicum* to grow and spread. Infections with *S. japonicum* are also common due to poor sanitation conditions and weak health infrastructure.

2.2. Participants. A total of 90 Yi individuals was recruited from the county above. Participants had to be Yi people aged >3 years without metabolic or autoimmune disease. Pregnant women and those with severe organic or mental diseases or any known medical problem were excluded. Individuals who did not have T lymphocyte or cytokine test or *Schistosoma* antibody test results were also excluded.

2.3. Study Procedures. Figure 1 showed the research process. Health workers who participated in this investigation were trained under the guidance of a unified protocol. They informed all the potential participants and explained the objectives, procedures, and potential risks of the study. Written consent was obtained from all adult participants and from the parents or legal guardians of children. A data collection sheet covered information on sociodemographic factors, details of HIV and *S. japonicum* infections, and other conditions.

All participants were asked to provide a 5 ml blood specimen for measurements of $CD4^+$ and $CD8^+$ T lymphocyte counts and cytokine levels like IL-10, IL-17, and IFN-γ. T lymphocyte counts were enumerated in EDTA blood by using Becton Dickinson (BD) FACScount (version 1.5; BD FACScount™ controls, catalogue number: 340166; BD

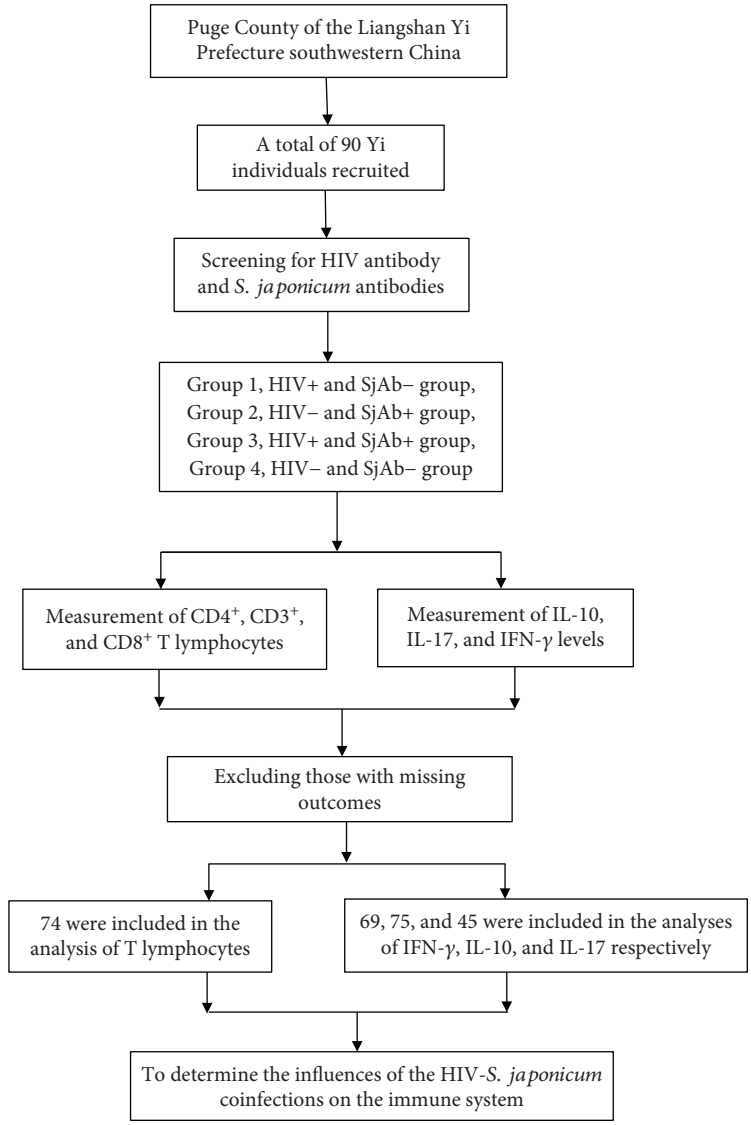

Figure 1: The research process.

FACScount reagents, catalogue number: 340167). Plasma was stored at −80°C for cytokine quantification, which was performed by the use of a human Multiplex Immunoassay (ProcartaPlex Mix&Match Human 5-plex, eBioscience Inc., Vienna, Austria (with the headquarters at San Diego, CA), catalogue number: EPX050-15122-801). A total of 50 μl of plasma sample was used to quantify the related cytokines in the samples.

All the participants were screened for HIV antibody by using the Diagnostic Kit for Antibody to HIV (colloidal gold) (a product of InTec Products Inc., Xiamen, P. R. China, batch number: 20150420, 100 persons per kit). Those positive of HIV antibody were further tested with HIV RNA by using the diagnostic kit for the quantification of HIV RNA (PCR Fluorescence Probing) (a product of the Da An Gene Group Inc., Zhongshan, P. R. China, batch number: 2015002, 48 persons per kit). Participants were also screened for *S. japonicum* antibodies (SjAb) by using the Indirect Hemagglutination Assay (IHA) kit (a product of the Anji Pharmaceutical Group Co., Ltd, Anhui, P. R. China, batch number: 20150200, 100 persons per kit). Those positive for SjAb were asked to provide a faecal sample of at least 30 g collected in the morning at home, and an oral description and specific instructions for handling and contamination avoidance of the stool sample were given. All the samples were sent to the laboratory of the local CDC for examination as soon as possible after they were collected. The faecal samples were processed within 12 h postcollection by using the stool hatching method for the detection of *S. japonicum* miracidia. Every sample was initially read by two examiners and reviewed by a third examiner if there was a disagreement.

2.4. Statistical Analysis. Data were double-entered and cross-checked by using the EpiData software (version 3.1; The EpiData Association, Odense, Denmark). Kruskal-Wallis rank tests were used to compare median $CD4^+$ and $CD8^+$ T lymphocyte counts and the levels of cytokines IL-10, IL-17,

Table 1: Characteristics of individuals included in the analysis of T lymphocytes.

Characteristic		Group 1 ($n = 24$)	Group 2 ($n = 12$)	Group 3 ($n = 11$)	Group 4 ($n = 27$)	z/χ^2	P
Age, median (interquartile range), years		33.5 (24.00, 39.25)	36.00 (15.25, 44.75)	36.00 (33.00, 40.00)	35.00 (18.00, 45.00)	1.920*	0.559
Sex	Male	17	9	5	15		0.348**
	Female	7	3	6	12		
Education	Illiterate	12	5	8	9		0.208
	Elementary school and above	12	7	3	17		
Occupation	Peasant	21	9	10	18		0.341**
	Else	3	3	1	8		

Note. Group 1, the HIV+ and SjAb− group; Group 2, the HIV− and SjAb+ group; Group 3, the HIV+ and SjAb+ group; Group 4, the HIV− and SjAb− group; *Kruskal-Wallis rank test, **Fisher's exact test.

and IFN-γ. A two-sided P value of 0.05 or less was regarded as significant. Statistical analyses were carried out with SPSS (version 20.0; IBM SPSS Institute Inc., USA).

3. Results

A total of 90 participants were recruited and they were classified into four groups: HIV+/SjAb− ($n = 32$), HIV−/SjAb+ ($n = 13$), HIV+/SjAb+ ($n = 16$), and HIV−/SjAb− ($n = 29$). After excluding those with missing outcomes, 74 were included in the analysis of T lymphocytes (Table 1) and 69, 75, and 45 were included in the analyses of IFN-γ, IL-10, and IL-17, respectively (Table 2). All of the SjAb-positive participants were examined for *S. japonicum* miracidia by a stool-hatching method, and no active miracidia were found.

The median of the participants was 34.5 years and interquartile range (IQR) was 24.7–41.2. There were no significant differences among the four groups in age, gender, occupation, and education (Tables 1 and 2). Figure 2 showed that there were significant differences among the four groups in both CD4 and $CD8^+$ T lymphocytes but no significant differences in $CD3^+$ T lymphocytes. These results suggested that infection with HIV might reduce the $CD4^+$ T lymphocyte counts but increase the counts of $CD8^+$ T lymphocytes. The ratio of CD4/CD8 was lower in HIV-infected people compared with those who were uninfected. Infection with *S. japonicum* was associated with increased $CD4^+$ T lymphocyte counts but with reduced $CD8^+$ T lymphocyte counts. Similarly, the ratio of $CD4^+/CD8^+$ was higher in people infected with *S. japonicum* compared with those uninfected. Coinfection with HIV and *S. japonicum*, similar to HIV infection alone, was significantly associated with lymphocyte counts. People with coinfection had lower $CD4^+$ T lymphocyte counts, a lower ratio of CD4/CD8, and higher $CD8^+$ T lymphocyte counts compared with those infected with HIV only or *S. japonicum* only.

Figure 3 presents the levels of IFN-γ, IL-10, and IL-17 and comparisons among the four groups. There was no significant difference in IL-17 among the four groups ($Z = 5.255$, $P = 0.154$), while there were significant differences among the four groups for both IFN-γ and IL-10. People infected with HIV and those who were coinfected with HIV and *S. japonicum* had a higher level of IFN-γ compared with people with no infection. There were no significant differences between people infected with HIV only and with *S. japonicum* only in the levels of IFN-γ and IL-10.

4. Discussion

Studies have suggested that HIV infection damages the immune system and decreases $CD4^+$ T lymphocyte counts of the human body [16]. We found that people infected with HIV had higher $CD8^+$ T lymphocyte counts compared with those who were uninfected. In our study, participants were relatively young (33.5 (24.00, 39.25) years) with a short average infection duration of HIV (42.65 ± 21.52 months). Consistent with previous findings, HIV-infected people had higher levels of $CD8^+$ T lymphocytes compared with uninfected people [41]. In the early stage of HIV infection, the immune system may not be seriously damaged and may be in the condition of stress response state. During such phase, uncontrolled replication of HIV-1 infection leads to activations of the $CD8^+$ T lymphocytes (which can inhibit HIV replication by cytolytic and noncytolytic responses) and increases the concentration of cytokines such as IFN-γ and IL-10 [42, 43]. Earlier studies demonstrated a correlation of maintaining the $CD8^+$ T lymphocyte immune profile with a slow progression of HIV-1 infection [44, 45].

$CD4^+$ T lymphocytes also play an important role in the human body for the balance of immune responses, and is a widely used marker of HIV immune impairment [46]. We found that people who were coinfected with HIV and *S. japonicum* had a lower level of $CD4^+$ T lymphocyte counts and a higher level of $CD8^+$ T lymphocyte counts. Coinfection with HIV and *S. japonicum* might speed up the disease progression of HIV infection. Destruction of the $CD4^+$ T cell pool increases susceptibility of the host to other infectious diseases [17].

We did not find that infection with *S. japonicum* exerted a significant impact on the level of T lymphocytes. People infected with *S. japonicum* had a slightly higher level of $CD4^+$ T lymphocyte counts and a lower level of $CD8^+$ T lymphocyte counts compared with people with no infection, but these were not statistically significant. The $CD4^+$ T lymphocyte responses are central to the development of

Table 2: Characteristics of individuals included in the analysis of IFN-γ, IL-10, and IL-17 levels.

IFN-γ							
Characteristic		Group 1 ($n = 19$)	Group 2 ($n = 12$)	Group 3 ($n = 15$)	Group 4 ($n = 23$)	z/χ^2	P
Age, median (interquartile range), years		34 (8.00, 40.00)	36.00 (16.00, 44.75)	36.00 (33.00, 40.00)	36.00 (23.00, 45.00)	2.161*	0.540
Sex	Male	12	10	8	11		0.216**
	Female	7	2	7	12		
Education	Illiterate	10	4	9	7	4.009	0.260
	Elementary school and above	9	8	6	15		
Occupation	Peasant	16	9	13	17		0.843**
	Else	3	3	2	5		
IL-10							
Characteristic		Group 1 (n=22)	Group 2 (n=12)	Group 3 (n=14)	Group 4 (n=27)	z/χ^2	P
Age, median (interquartile range), years		34.00 (19.25, 40.50)	36.00 (16.00, 44.75)	36.00 (32.75, 40.25)	35.00 (18.00, 45.00)	1.074*	0.783
Sex	Male	15	10	9	15		0.419**
	Female	7	2	5	12		
Education	Illiterate	11	4	9	9	4.460	0.216
	Elementary school and above	11	8	5	18		
Occupation	Peasant	18	9	12	19		0.776**
	Else	4	3	2	8		
IL-17							
Characteristic		Group 1 ($n = 16$)	Group 2 ($n = 8$)	Group 3 ($n = 7$)	Group 4 ($n = 14$)	z/χ^2	P
Age, median (interquartile range), years		32.00 (7.25, 41.50)	29.50 (9.75, 44.75)	35.00 (30.75, 38.00)	25.50 (7.75, 34.50)	3.612*	0.307
Sex	Male	11	7	4	8		0.363**
	Female	5	1	3	6		
Education	Illiterate	9	2	5	4		0.324**
	Elementary school and above	7	6	2	9		
Occupation	Peasant	12	5	6	9		0.869**
	Else	4	3	1	4		

*Kruskal-Wallis rank test, **Fisher's exact test.

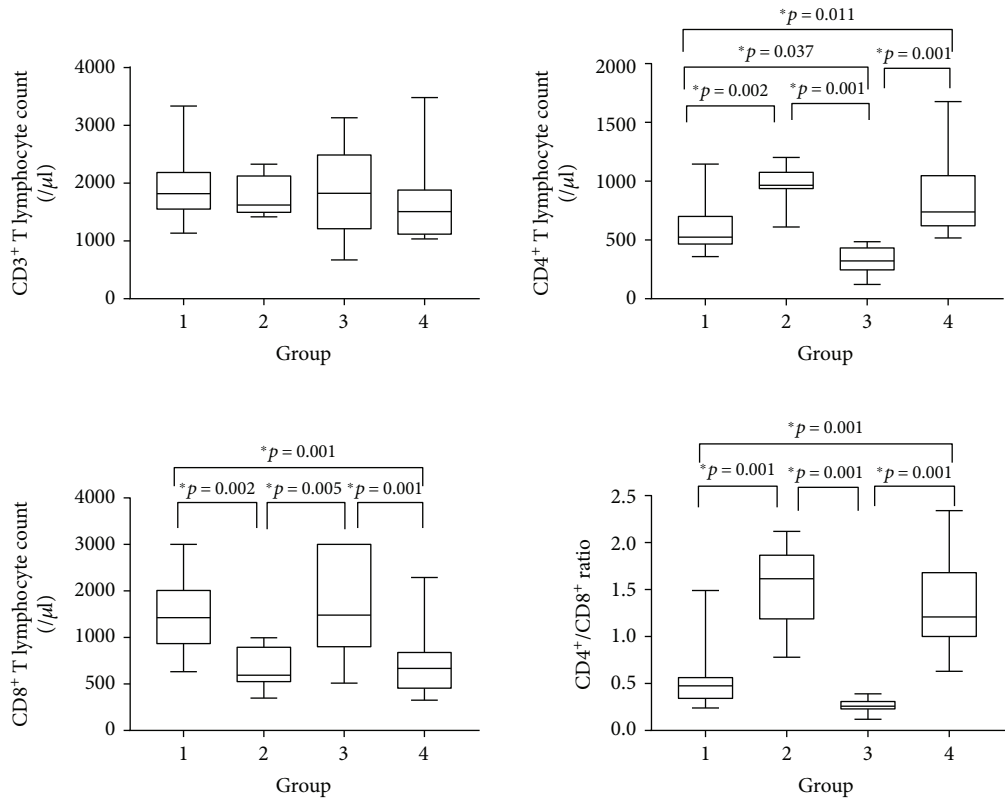

Figure 2: Comparison of T lymphocytes among 4 groups. Data are presented as the min to max.

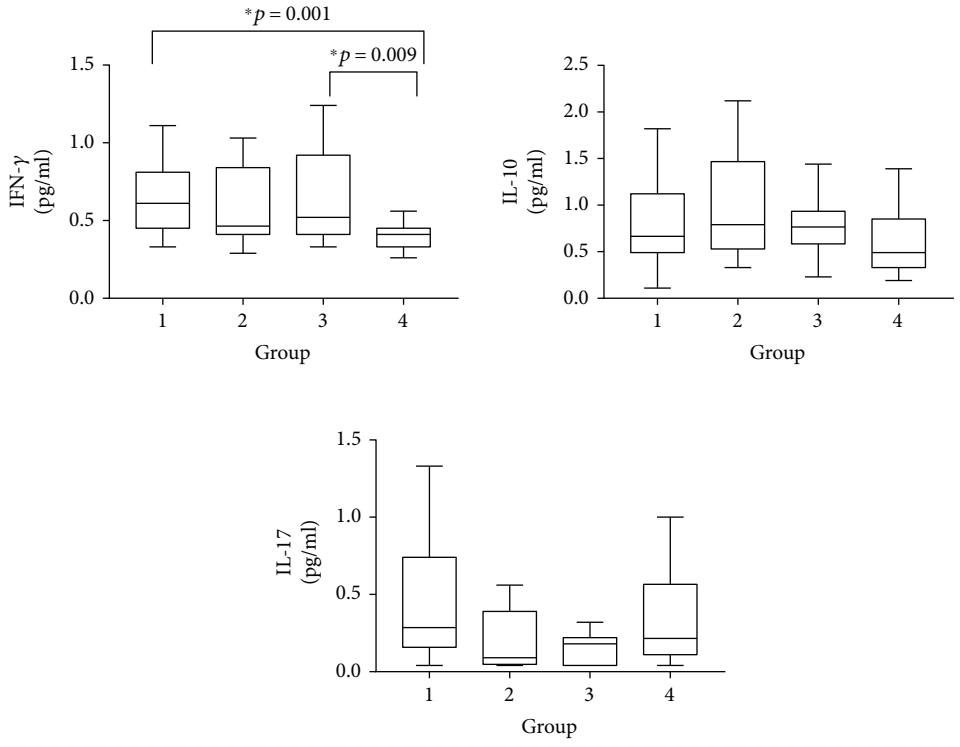

Figure 3: Comparison of IFN-γ, IL-10, and IL-17 levels among 4 groups. Data are presented as the min to max.

immunopathology in *S. mansoni* or *S. japonicum* infections [47, 48]. At a chronic phase of infections, the Th1 response will shift to a Th2 response (characterized by the upregulation of IL-10 and other cytokines), which downregulates the production and function of the Th1 response [49], accompanied by a dysfunction of cellular immunity, like the apoptosis of $CD4^+$ T lymphocytes and activation of $CD8^+$ T lymphocytes [50, 51]. A possible explanation of this inconsistency might be that the SjAb+ subjects in this research were all asymptomatic and had no active miracidia. Therefore, they might be in an early stage of infection or were previously infected. At the early stage of schistosome infections, immunity dysfunction might not occur and the level of T lymphocytes did not change substantially. A standard treatment of schistosome infection could switch the immune system back to normal. It was reported that Th1 type responses, which are characterized by producing IFN-γ, were predominated by other than Th2 type responses in the early stage of schistosome infections [52]. Consistently, we found that SjAb+ individuals had a higher level of IFN-γ. Since Th1 cells primarily mediate cellular immunity, arousing the increase of $CD4^+$ T lymphocytes while $CD8^+$ T lymphocytes are not yet activated or just in the stress state [51, 53], no significant changes in the levels of $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes were shown in the current study.

In our study, people infected with HIV only had a higher level of IFN-γ compared with people with no infection with HIV and *S. japonicum*. Individuals coinfected with both HIV and *S. japonicum* had similar results when compared with people with no infection with HIV and *S. japonicum*; however, they had no higher levels of IFN-γ or IL-10 compared with individuals infected with HIV or *S. japonicum* alone. A possible explanation for this was that people might be in the early stage of infection with *S. japonicum* or taking praziquantel for the treatment of schistosomiasis that could impact the levels of IL-10 and IFN-γ in plasma [10]. We found no significant difference in the level of IL-17 among the four groups.

The small sample size is a main limitation for the study, largely because of the challenging conditions in the study field (i.e., due to the effective measurements for the prevention and control *Schistosoma japonicum*, it is rather difficult to find enough cases to conduct this research), and we were not able to explore the interactions between the infections in different stages. We could not control for potential impacts from other pathogens on the results. The levels of three cytokines in the study were lower compared with those previously reported.

5. Conclusions

People coinfected with HIV and *S. japonicum* might have a suppressed immune function because of a decrease in $CD4^+$ T lymphocyte counts, a lowered ratio of $CD4^+/CD8^+$, and an increase in $CD8^+$ T lymphocyte counts. Coinfection with both HIV and *S. japonicum* would alter the level of IFN-γ in plasma. No difference in the level of IL-17 was detected among the four study groups.

Abbreviations

HIV: Human immunodeficiency virus
AIDS: Acquired immunodeficiency symptom
SjAb: *Schistosoma japonicum* antibody
IHA: Indirect hemagglutination assay
RNA: Ribonucleic acid
CD: Cluster of differentiation
Th: T helper lymphocyte
IFN: Interferon
IL: Interleukin
CI: Confidence interval
OR: Odds ratio.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

This study was evaluated and approved by the Ethics Review Committee of the Ethical Institute of The School of Public Health, Fudan University and written informed consent was obtained from all participants. The potential participants who agreed to attend the study were asked to sign a written informed consent by the staff of the local CDC. If participants were less than 18 years of age, their parents were asked to sign a written parental permission. At the completion of the study and in accordance with the local treatment policies, these participants with positive results were informed of the results and provided with appropriate medical consultations and treatment.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Yu Yang and Peng-Lei Xiao participated in the data collection, statistical analysis, interpretation of the data, and preparation of the manuscript. Yi-Biao Zhou, Yue Chen, and Qing-Wu Jiang participated in the interpretation of the data and in the critical review and revision of the manuscript draft. Xiu-Xia Song, Ya Yang, Yan Shi, and Jian-Chuan Gao performed data collection for the study and participated in the preparation of the manuscript. Ya Yang and Wan-Ting Chen were responsible for data management and read all the smears. All authors read and approved the final manuscript.

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Review Article

The Regulation of Inflammation by Innate and Adaptive Lymphocytes

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Inflammation plays an essential role in the control of pathogens and in shaping the ensuing adaptive immune responses. Traditionally, innate immunity has been described as a rapid response triggered through generic and nonspecific means that by definition lacks the ability to remember. Recently, it has become clear that some innate immune cells are epigenetically reprogrammed or "imprinted" by past experiences. These "trained" innate immune cells display altered inflammatory responses upon subsequent pathogen encounter. Remembrance of past pathogen encounters has classically been attributed to cohorts of antigen-specific memory T and B cells following the resolution of infection. During recall responses, memory T and B cells quickly respond by proliferating, producing effector cytokines, and performing various effector functions. An often-overlooked effector function of memory CD4 and CD8 T cells is the promotion of an inflammatory milieu at the initial site of infection that mirrors the primary encounter. This memory-conditioned inflammatory response, in conjunction with other secondary effector T cell functions, results in better control and more rapid resolution of both infection and the associated tissue pathology. Recent advancements in our understanding of inflammatory triggers, imprinting of the innate immune responses, and the role of T cell memory in regulating inflammation are discussed.

1. Introduction

Advances on several research fronts have significantly broadened our understanding of the triggers and modulators of inflammation. Of importance to this review, we now appreciate that at sites of infection, adaptive immune memory cells regulate innate inflammatory responses that contribute to the control of pathogens. Herein, potential means to modulate inflammation for the optimal generation of protective immunity through vaccination are discussed.

The ultimate goal of vaccination is to stimulate the generation of long-lived protective immunity without causing adverse clinical symptoms. Traditional vaccination strategies employing inactivated or attenuated pathogens or pathogen-derived protein antigens primarily target the generation of neutralizing antibody responses from B cells that act to prevent infection upon pathogen reencounter [1]. These regimes have been remarkably effective at mitigating the morbidity and mortality of a number of infectious diseases in vaccinated populations and most notably have led to the complete eradication of smallpox [2]. However, intracellular pathogens like influenza viruses (IAV) [3], human immunodeficiency virus (HIV) [4], and *Mycobacterium tuberculosis* [5, 6] have yet to be effectively controlled by neutralizing antibody-based vaccine approaches. Such pathogens either rapidly mutate external proteins that are targets for antibody or are not likely seen by antibody and are more effectively controlled by cell-mediated immune responses. The generation of protective T cell-mediated immunity through vaccination is appealing for pathogens like IAV that undergo antigenic shifts to evade neutralizing antibody given that T cells can recognize antigenic targets that are more conserved between strains. T cell-based vaccines against IAV may thus have the benefit of mediating universal protection against

unforeseen and emergent pandemic strains of the virus [7], and they may potentially also eliminate the need for annual IAV vaccine reformulation. Inflammatory enhancing adjuvants have the potential to boost the efficacy of novel neutralizing antibody-based and T cell-based vaccines [8–11]. In order for such adjuvanated T cell-based vaccines to be efficacious and safe, they will need to target the induction of both pathogen-specific inflammation and adaptive immunity at relevant sites of infection.

2. There: The Regulation of Innate Inflammatory Responses by Pathogen

When a pathogen breaches the initial barriers of the skin or a mucosal surface, both soluble and cellular innate defense mechanisms are encountered and an inflammatory response is rapidly initiated. Some of the most potent soluble antimicrobial factors encountered include complement, lysozymes, defensins, mucins, lectins, cathelicidins, and lipocalins [12–15]. Several of these soluble antimicrobial mediators, such as activated complement components and lipocalin-2, are pluripotent, and in addition to performing antimicrobial functions, they amplify the inflammatory response triggered in resident sentinel immune cells upon pathogen sensing [13, 16, 17]. Within minutes to hours of detection of alarm signals, a "heightened alert" inflammatory transcriptional program ensues in sentinel innate immune cells, which include tissue-resident macrophages and dendritic cells. The result of this program is the generation of an antipathogen state and the production of a myriad of inflammatory cytokines, chemokines, biogenic amines, and eicosanoids [18] that induce a similar state in neighboring tissue cells.

Soluble inflammatory chemokines [19] and activated complement [20, 21] produced in response to pathogen sensing contribute to the attraction of additional innate immune cells such as neutrophils, NK cells, and monocytes to the site of infection [19, 22]. The recruited inflammatory cells encircle the damaged or infected cells and release more proinflammatory cytokines including tumor necrosis factor (TNF), IL-6, IL-12, and type I and II interferons (IFNs). Neutrophils also release DNA nets to trap free extracellular pathogens [23, 24], and NK cells attempt to lyse infected host cells through cytotoxic means [25, 26]. The innate inflammatory cytokine and cellular swarm attempts to contain the pathogen until highly specific, activated cells of the adaptive immune response are recruited to ultimately clear the infection [27]. If coordinated recruitment of innate and adaptive immunity fails to effectively control the pathogen, clinical disease will ensue. A major challenge for vaccine design is to mimic this inflammatory environment, which is needed to stimulate the generation of effective and robust immunity, without causing the immunopathology and tissue damage associated with clinical infection.

2.1. Pathogen Sensing. In order for the inflammatory events discussed above to occur, pathogens must be detected in compromised tissues. Many different subsets of classic dendritic cells, plasmacytoid dendritic cells, and macrophages [28, 29] are distributed throughout tissues in a network that facilitates immediate detection of both invading pathogens and the associated tissue damage [30, 31]. These sentinel innate cells sense pathogens and pathogen-associated tissue damage in a generic way through multiple distinct pathways [32]. They employ germ-line encoded pattern recognition receptors (PRRs) that recognize pathogen-associated molecular pattern (PAMPs) [32] and damage-associated molecular pathogens (DAMP) [33, 34] to detect changes in their environment [35, 36]. Recognition of pathogen-derived products such as lipopolysaccharide (LPS) by Toll-like receptors (TLR) 1, 2, and 4; flaggelin by TLR5; single stranded (ss) by TLRs 7 and 8; double-stranded (ds) RNA by TLR 3; and CpG DNA by TLR9 occurs either at the surface of the cell or within endoplasmic vesicles [37]. Host cell-derived danger signals or alarmins such as heat shock proteins, uric acid crystals, high-mobility group box 1, S100 proteins, serum amyloid A, and products of purine metabolism released from damaged or stressed cells are sensed by DAMP receptors such as RAGE, TLRs, and purinergic receptors [38, 39]. Recognition of PAMPs and DAMPs triggers the activation of signaling pathways that ultimately leads to the expression of the transcription factors NF-κb, AP-1, and interferon regulatory factors (IRFs) [32, 40, 41]. These transcription factors control the expression of hundreds of immune defense response genes [18, 40, 42]. An attractive means to both tailor and enhance the generation of vaccine-induced immunity is through the use of adjuvants that selectively trigger PRR and DAMP receptors. Such adjuvants are currently being explored to improve the generation of adaptive immune responses to inactivated pathogen and protein-based vaccines [8–11].

Advancements in our knowledge of intracellular sensors of pathogens and host-derived stress products have revealed novel targets to modulate and improve vaccine efficacy [43, 44]. A number of intracellular sensors, including the nucleotide-binding-domain and leucine-rich-repeat- (NLR-) containing proteins [45, 46] and the AIM-like-receptor (ALR) proteins [47], trigger the inflammasome pathway. The activation of the inflammasome complex and the activation of caspase-1 enzymatic activity are best known for triggering maturation of the proforms of the cytokines IL-1 and IL-18 [48]. However, alternative outcomes such as phagosome maturation, autophagy, glycolysis, lipid metabolism, and oxidation of arachidonic acid to generate eicosanoid signaling molecules, as well as inflammatory pyroptotic cell death, can also be triggered [44]. IL-1 and IL-18, in their mature forms, are potent proinflammatory cytokines [49]. The importance of the inflammasome-sensing pathway and the production of IL-1 and IL-18 to effective pathogen defense is highlighted by the fact that many infectious organisms, such as viruses, that gain access to the cytosol encode proteins that attempt to evade detection by intracellular sensors [50].

Intracellular sensors interact with adaptor proteins such as apoptosis-associated speck-like protein containing a C-terminal caspase activation and recruitment domain (ASC) [51] to trigger the activation of the proteolytic functions of the caspase-1 enzyme. Triggers of caspase enzymatic activity are extensively reviewed elsewhere [44, 46, 52]. The discovery of noncanonical activation pathways involving

caspases other than caspase-1 [44, 53], as well as the ability of the type I IFN, a pro- and anti-inflammatory cytokine [54], to both prime cells for cytosolic sensing [44] and inhibit NLR signaling [55] emphasizes the need to more fully understand the workings of the inflammasome complex before targeted modulators [56] can be employed to enhance the generation of vaccine-induced memory CD4 and CD8 T cell immune responses.

2.2. Inflammatory "Rheostats." Under normal circumstances, inhibitory "innate immune rheostats" act to prevent unnecessary inflammation at barrier surfaces [57, 58]. Inflammatory responses in tissues are tempered in many ways via recognition of soluble as well as cell surface ligands. This includes the blockade of activating DAMP receptor signaling by tissue-derived factors such as surfactant proteins and mucins [59–62]. Inhibitory DAMP receptor and inhibitory cytosolic receptor triggering by host-derived ligands such as DNA is an additional example of how inflammatory responses are kept in check [63–65]. Ligation of cell surface receptors on monocytes and dendritic cells, such as CD200R by CD200 ligand, that trigger dampening signals [66] is yet an additional means by which inflammation is regulated. Lastly, suppression of NF-κb activation by the release of mitochondrial H_2O_2 in lung APC [67] and the production of the anti-inflammatory cytokines IL-10 and TGF-beta by both regulatory T cells and tissue cells [68–70] mitigate inflammatory responses. The potent efficiency of IL-10 and TGB-beta in counterregulating inflammatory cytokine production as well as in inhibiting both costimulatory and major histocompatibility complex (MHC) molecule expression on antigen-presenting cells (APCs) likely explains why many pathogenic viruses encode homologues of inhibitory cytokines and inhibitory ligands to evade the innate immune response. Expression of IL-10 by Epstein-Barr virus (EBV) [71] and expression of the inhibitory ligand CD200 by cytomegalovirus (CVM) [72] are prime examples.

Safely overcoming these "rheostats" by targeted blockade of inhibitory molecules or by employing novel adjuvant formulations that facilitate the generation of protective local immunity through vaccination without causing damaging adverse effects is of paramount importance [73–75]. Indeed, the generation of overzealous inflammatory responses following pathogen or adjuvant stimulation has the potential to cause severe inflammatory disease [34, 76]. Individuals who possess well-characterized genetic polymorphisms in numerous inflammatory mediators and signaling molecules, such as those associated with chronic inflammatory diseases like psoriasis, ulcerative colitis, and Crohn's disease [77], are at increased risk for developing undesired inflammatory complications following vaccination. In addition to genetic predispositions, environmental factors, such as the microbiome, may also play a role in setting the inflammatory "rheostat" at mucosal surfaces [78–82]. Interestingly, individual-specific microbiota signatures have been shown to impact both disease susceptibility and severity via either innate or adaptive immune pathways [83].

The control of immune response gene expression by long noncoding (lnc) RNAs [84] is another recently described homeostatic mechanism that could be targeted to improve vaccine efficacy as well as for therapeutic control of inflammation. Depending on the cell type involved, binding of specific lnc RNAs to regulatory regions of immune response genes and the subsequent control of nucleosome positioning can either promote or actively repress inflammatory gene expression [85]. A number of long noncoding RNAs are dysregulated during viral infection [86, 87], and changes in their expression are being assessed for use as biomarkers of disease severity [88]. The control of inflammatory responses by noncoding RNAs could have an exciting future in tailoring host inflammatory responses.

In addition to the homeostatic mechanisms and negative feedback loops discussed above, which preserve vital functions of organs such as the lung and intestine, the timing of vaccination administration may also need to be taken into account. Patterns of expression of proteins such as IL-6, inflammatory monocyte chemokine ligand (CCL2), as well as Toll-like receptor (TLR) 9, which are regulated by circadian clock proteins [89], may explain why morning vaccine administration appears more effective than afternoon administration at inducing specific antibody in older adults [90]. Differences in the magnitude of inflammatory responses across seasons may also influence the efficacy of vaccination. A recent study found that the magnitude of the inflammatory cytokine response detected following stimulation of monocytes with different pathogen-derived products, including those from influenza A virus, differs in different seasons [91]. In the individuals studied, inflammatory cytokine responses were maximal during the summer months of June and July and weakest in winter months [91]. The authors speculate that the tendency to produce reduced levels of inflammatory cytokines such as IL-1, TNF, and IL-6 during the winter may impact an individual's susceptibility to pathogens such as influenza A during the flu season. How the efficiency of vaccination is affected by the seasonal changes warrants further investigation.

3. Inflammation and the Generation of Adaptive Immune Responses

To successfully generate protective immunity through vaccination, antigen-specific T cells must interact with activated APC displaying cognate antigen in the context of MHC. Such interactions result in the receipt of signal 1, the specific antigen, and signal 2, the costimulatory molecule-dependent signals, required for full T cell activation. Recognition of inflammatory cytokines by their corresponding cytokine receptors constitutes signal 3 that can amplify proliferation as well as effector functions in activated cells.

Foreign antigens introduced by vaccination must reach the secondary lymphoid organs in order for T cell activation to occur. Antigen is delivered to draining lymph nodes via the lymph in particulate form or within migrating tissue-resident antigen-presenting cells that have egressed from the inflammatory site [92]. Particulate antigens in the lymph are captured by specialized APCs that are strategically poised in the draining lymph nodes [93]. Larger-sized antigens are captured by lymph node dendritic cells that reside within the lymphatic sinus endothelium [93] or by subcapsular

sinus macrophages [94, 95]. Smaller-sized antigens are transferred to lymph node follicle dendritic cells and B cells via a conduit system [96]. Once engulfed and processed, antigens are presented by antigen-presenting macrophages, dendritic cells, and/or B cells to naïve CD4 and CD8 T cells on MHC class II and class I molecules, respectively. Antigens that gain access to the circulation are delivered to the spleen via the blood and are detected in a similar fashion by the APCs that reside there.

Exposure to and engulfment of pathogen-derived products at the site of vaccination or infection activates APCs and triggers their production of inflammatory cytokines. Cohorts of APC, once activated, will begin to migrate towards lymphoid organ chemokines CCL19 and CCL21 in a CCR7-dependent fashion [97, 98]. Egress of tissue-resident APC from sites of infection is a rapid event, and migratory subsets can be detected in lymphoid organs within 14 to 24 hrs of antigen administration [99, 100]. Both tissue-resident dendritic cells and macrophages display migratory behavior upon activation [29, 101–103]. Interestingly, following infection with respiratory viruses such as influenza A virus, one APC subset, alveolar macrophages, becomes undetectable in the infected lung tissue until recruited monocytes are able reestablish the population [104]. It remains unclear, however, whether the inability to detect alveolar macrophages following influenza is the result of their complete egress out of the tissue, a switch in their surface marker phenotype in response to the inflammatory milieu, or because of their elimination by the viral infection [29, 102].

The lifespan of activated tissue-migratory APCs within draining lymph nodes, especially the dendritic cell subset, is relatively short [105], and optimal antigen presentation by such cells occurs within 24 hrs of tissue egress [99, 100]. In addition to functioning as APCs within the T cell zones [101, 106], migratory dendritic cells can also act as "cargo carriers" that deliver engulfed antigen to APC resident in lymphoid organs [107, 108]. Whether migratory or lymph node resident, APCs once activated express increased levels of MHC I and II molecules, as well as increased expression of costimulatory molecules, such as CD40, CD70, CD80, and CD86 [28]. Activated APCs also produce numerous proinflammatory cytokines including IL-12, IL-6, and type I IFN for the plasmacytoid dendritic cell subset [28]. The inflammatory mediators that these highly activated APCs produce and the surface costimulatory molecules that they express play a key role in shaping the ensuing adaptive immune response [109, 110]. Vaccine strategies that specifically target pathogen-derived antigens to APCs *in vivo* [111], that employ antigen-loaded dendritic cells themselves as the vaccine vehicles [112], or that additionally trigger specific PRR receptors to direct T cell polarization are actively being explored as means to amplify the generation of effective T cell responses [8–10]. Such strategies are of particular interest for vaccination regimes for the elderly and cancer patients where the generation of effective immunity is challenging because of their compromised or suppressed immune states [112, 113].

3.1. And Back Again: The Regulation of Early Innate Inflammatory Responses by Memory T Cells. Following an acute infection or vaccination, the activation and expansion of naïve pathogen-specific T cells and the generation of effector cells generally occur within 7 days. Under normal circumstances, the majority of expanded effector cells that migrate to sites of infection or antigen administration undergo contraction following subsequent pathogen or antigen clearance. A small cohort of the expanded effectors will, however, survive to memory [114]. These antigen-specific memory cells, which exist at a frequency higher than that found in the naïve state [115], mediate potent immunological protection upon secondary pathogen encounter.

Some antigen-specific memory T cells possess the ability to migrate throughout the body and are readily detected within tissues [116, 117]. This migration pattern is markedly different from that of naïve T cells, which only circulate through the blood and secondary lymphoid tissues [118, 119]. When compared to naïve T cells, memory T cells also have increased cytokine-producing potential [120, 121]. One subset of memory T cells, the tissue-resident memory T cell subset that does not circulate, is found exclusively within the tissues and may be strategically poised and specialized to perform sentinel functions [122–125]. Targeting the generation of tissue-resident memory T cells, especially for pathogens that infect mucosal tissues, is thus an attractive means to improve the efficacy of vaccines against pathogens that are not effectively controlled by traditional antibody-based approaches.

In addition to rapidly producing cytokines upon recognition of cognate antigen, memory T cells perform many other effector functions to protect the host against infection [126]. These functions are, for the most part, recalled independently of most costimulatory molecules [127]. This is one major way in which memory cells are distinct from naïve T cells that are dependent upon costimulatory signals for their full activation. For CD4 T cells, the best-known effector role is the provision of help for antigen-specific B [128] and cytotoxic CD8 T cell responses as reviewed elsewhere [126, 129, 130]. A novel effector role of memory T cells that is becoming more appreciated is the regulation of innate immune responses at sites of infection [126]. Of importance to this discussion, memory T cells mediate rapid production of effector cytokines akin to the responses elicited from innate immune cells upon cognate encounter with specific pathogen-derived antigen. Memory T cells thus have the potential to act as powerful antigen-specific sentinels that are able to initiate rapid inflammatory responses against pathogens [122, 131–133]. In fact, our studies in an influenza model showed that memory T cell-mediated inflammatory responses are induced faster, are bigger, and are better at containing virus than innate responses in naïve IAV-infected animals that are triggered through PRR-dependent mechanisms [133] (Figure 1).

Both memory CD4 [132, 133] and CD8 [131, 134, 135] T cells have the capacity to regulate and enhance the generation of early innate inflammatory responses within tissues upon cognate recognition of antigen. The antigen-specific regulation of inflammatory responses provides an additional means by which the immune response can generate alarm signals during infections with pathogens that possess

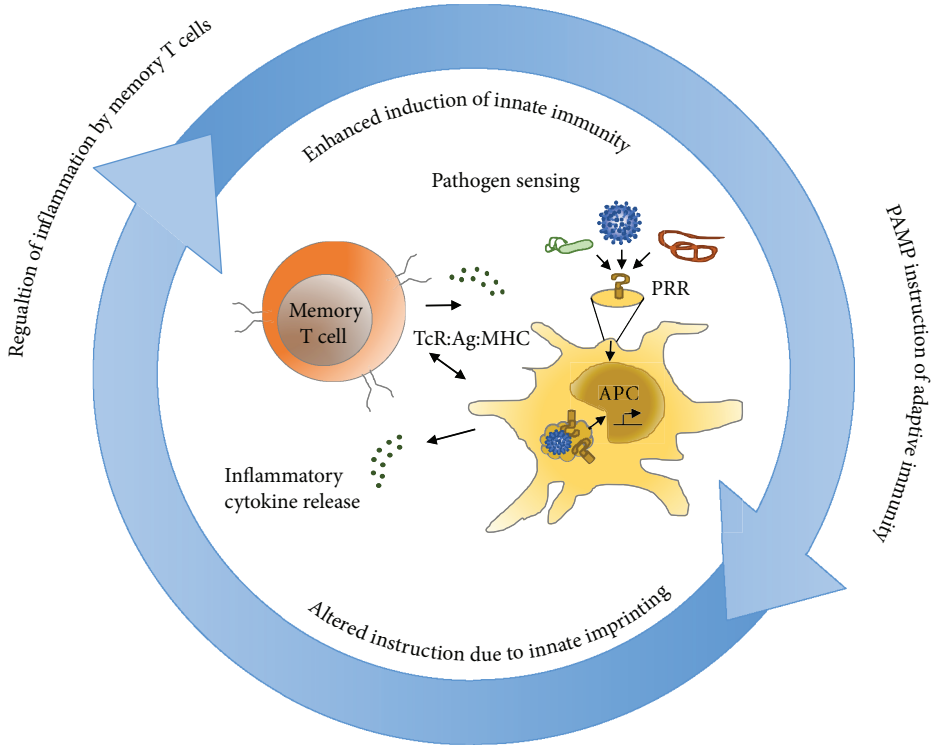

Figure 1: Memory T cells regulate inflammation at sites of infection. Traditional paradigms of innate instruction of adaptive immunity must now also appreciate that adaptive memory T cells regulate both the nature and shape of innate inflammatory responses. Memory CD4 T cell-mediated enhanced inflammatory responses are initiated independently of the classic PRR signaling and classic costimulatory molecule recognition and are better at containing virus than innate responses triggered in naïve hosts through PAMP-dependent mechanisms.

means to evade detection by the innate immune-sensing mechanisms discussed earlier [136]. It also provides a means whereby experienced memory cells can modulate the effector functions of the ensuing adaptive response of expanded secondary effector T cells that arise from resting memory T cell precursors during recall [137].

For memory CD4 T cells, enhanced inflammatory responses are initiated in the lung following IAV infection independently of the classic PRR signaling molecules MYD88 and TRIF [133]. Memory CD4 T cell-regulated enhanced inflammatory responses can also be initiated in the absence of infection. Indeed, the intranasal administration of cognate peptide antigen in the absence of any adjuvants or the administration of endotoxin-free protein that contains the epitope for which the cells are specific leads to the generation of potent early innate inflammatory responses [133]. This suggests that even though CD4 T cells themselves can express PRRs and produce inflammatory cytokines following PAMP recognition [138, 139], such PRR triggering is not required for the mediation of memory CD4 T cell sentinel functions [133].

The ability of memory CD4 T cells to induce inflammatory responses upon pathogen detection is also independent of their production of the classic proinflammatory cytokines TNF and IFN-γ and does not require the receipt of CD80, CD86, and CD40 costimulatory molecule signals [133]. That memory cells do not depend on signal 2 to perform sentinel functions within the lung is in fitting with the observation that the activation and early recall of memory CD4 T cells *in vivo* are not affected by blockade of the CD28 costimulatory pathway [140]. The sentinel capacity of memory CD4 T cells thus appears to be very different from the sentinel functions of CD8 T cells, which are dependent upon TNF [141], IFN-γ [142–144], GM-CSF [145], and potentially also the receipt of costimulatory signals *in vivo* [146]. Similarities and inherent differences in the priming and function of memory CD4 and CD8 T cell responses are additional factors that must be considered in the design of innovative vaccination strategies that target the generation of protective antigen-specific T cells.

Following secondary IAV infection, the earlier and more robust inflammatory response induced by memory CD4 T cells correlates with improved control of the virus in the lung [133]. Our recent findings show that one innate inflammatory cytokine involved in this response, IL-6, plays a central role in maximizing the multicytokine-producing potential of secondary $CD4^+$ T effector cells that accumulate in the lung at the peak of the recall response [137] (Figure 2). In murine and human systems, multicytokine-producing potential, or the ability to coproduce TNF, IL-2, and IFN-γ, is associated with the ability of memory T cell responses to protect against numerous viral, bacterial, and parasitic pathogens [120]. Multicytokine potential, as well as the ability to mediate effector functions such as help and cytotoxicity, correlates with superior protective capacity when secondary effector cells (derived from memory precursors) are compared on a per cell basis to primary effectors derived from naïve T cells [121, 147, 148]. Innovative vaccines thus not

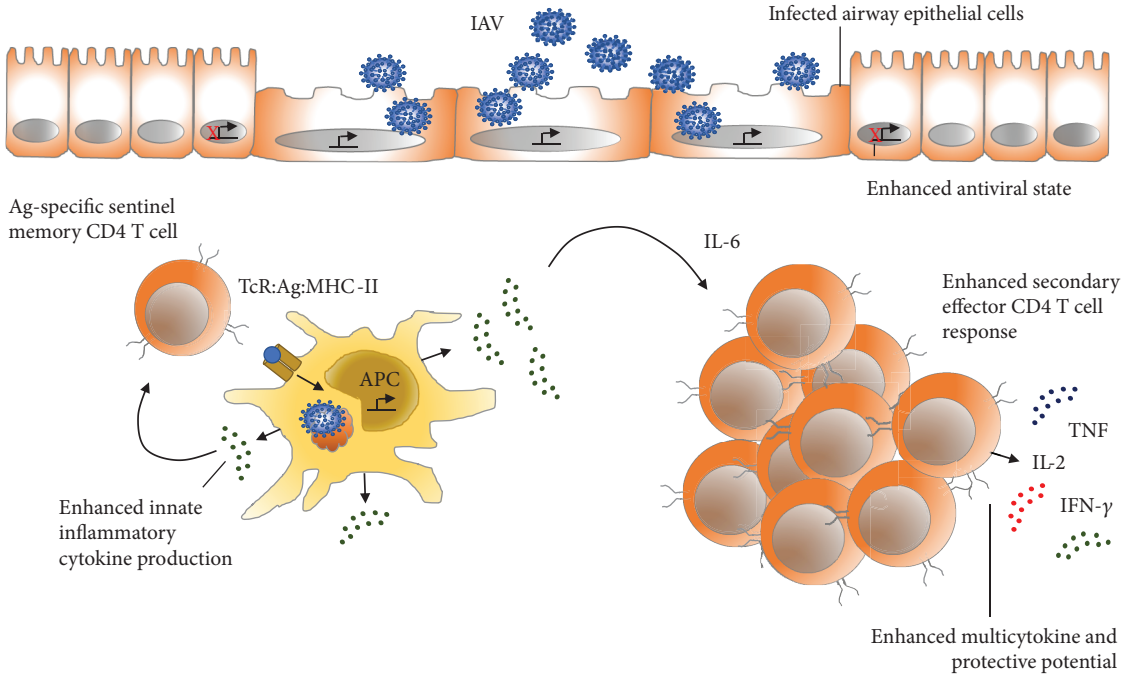

Figure 2: Memory T cells regulate the accumulation and functional potential of secondary effector T cells in the lung through antigen-specific upregulation of the inflammatory cytokine IL-6. Following secondary IAV infection, the earlier and more robust inflammatory response induced by memory CD4 T cells correlates with improved control of the virus in the lung. One innate inflammatory cytokine involved in this response, IL-6, plays a central role in maximizing the multicytokine-producing potential of secondary $CD4^+$ T effector cells that accumulate in the lung at the peak of the recall response. Experienced memory cells thus modulate the effector functions of recently expanded secondary effector T cells that arise from resting memory T cell precursors during recall responses by inducing potent inflammatory signals.

only should target the induction of large numbers of memory T cells but also should strive to generate cells that possess optimal functional potential. Current research employing high-dimensional mass cytometry that simultaneously measures over 40 parameters, including cell surface markers and intracellular proteins, as well as RNA expression at single-cell resolution [149], will further advance our understanding of strong correlates of protection in specific models of infection. Such correlates will, in turn, help facilitate the development of optimal vaccination strategies.

4. Training of the Innate Immune System

Another significant advance in our understanding of innate immunity is the knowledge that cells of the innate immune system are altered or "trained" by past experiences [150]. For the majority of innate immune cells, such imprinting results in a generic and nonspecific heightened inflammatory response that increases host antimicrobial defenses upon secondary infection. Responses by NK cells may be an exception to this as they have been shown to display some elements of antigen-dependent memory [151–153]. It should be noted, however, that trained innate immune responses are functionally distinct from the highly specific recall responses characteristic of adaptive immune memory mediated by specialized subsets of CD4 and CD8 T cells and of antibody-producing B cells.

It has been long been appreciated that organs such as the lung remain in an altered state for an extended period of time following infection or insult [154, 155]. The heightened inflammatory state that exists following the resolution of pathogen infection lasts for days, weeks, or even months and can provide a degree of nonspecific protection to unrelated pathogens. Examples of heightened protective immunity induced by infection or vaccination are many and are discussed in detail elsewhere [150, 156, 157]. A prime example is the ability of BCG vaccination, in mice as well as in humans, to increase resistance against a number of different pathogens [157–160]. Priming of innate immune cells resulting in increased nonspecific pathogen protection can also be caused by viral pathogens [161, 162] and even exposure to pathogen-derived molecular patterns [163–166].

The protection afforded by "imprinted" innate immunity is associated with the presence of increased numbers of activated macrophages [150, 156], dendritic cells [167], and other innate immune cells within the tissues that are characterized as being in a heightened antimicrobial state [150, 156]. In animal models, this nonspecific protection is transferrable to naïve hosts by the adoptive transfer of "trained" macrophages, and, notably, the transfer of protection does not require the presence of T cells [165, 168]. Recent studies have shown that this "imprinted" state is maintained by long-term translational and epigenetic changes within the "trained" monocytes and macrophages [165, 169, 170]. Signals generated through recognition of the microbiota that ultimately lead to the production of the inflammatory cytokine GM-CSF, which also has

colony-stimulating functions, is just one example of how heightened inflammatory "rheostats" can be established within mucosal tissues [171]. How conditioning of innate immune cells by the microbiota and infectious pathogens in human tissues influences the ability to generate protective immune responses following vaccination remains to be determined. However, some groups have begun to establish models using primary human monocytes to shed preclinical insight on the ability of pathogen-derived products to "imprint" human APC *in vitro* [172].

Pathogen-associated encounters may not be the only events capable of training innate immune cells. The engulfment of apoptotic host cells in the absence of infection has traditionally been considered an immunologically neutral event that fails to generate DAMP signals [33]. Recent observations, however, show that even this steady-state process can imprint macrophages for heightened inflammatory responses that mediate nonspecific resistance to microbial infection [173]. These and other findings in a murine model [174] suggest that most if not all tissue-resident macrophages become experienced during development by normal cellular turnover processes that educate them for future pathogen encounter.

The altered inflammatory state that exists following the resolution of infection can also have alternative and undesired outcomes. For example, conditioning of innate immune cells by prior infection can result in increased susceptibility to secondary infection [175]. Increased susceptibility to secondary bacterial infection occurs following many respiratory virus infections [176] and contributes markedly to the morbidity and mortality of disease [177]. Mechanisms underlying increased susceptibility to secondary infection are many and include deficiencies in bacterial scavenging receptors such as MARCO on macrophages [178], as well as the depletion of tissue-resident APC populations during primary infection [104]. Increased production of inflammatory dampening cytokines IL-10 and TGF-beta [179, 180] and attenuation of protective host defenses through diminished production of IL-1b [181], IL-27 [182], and antimicrobial peptides [181] can also contribute to increased susceptibility. Increased expression of inflammatory dampening receptors such as CD200R [66, 155] and differences in the chemotaxis, survival, phagocytic, and respiratory burst functions of neutrophils [183–185] may also lead to an inability of the innate immune system to contain and control secondary microbial threats following respiratory viral infection. In addition to regulating early inflammatory responses that facilitate pathogen control, vaccine-induced T cell immunity may also be able to prevent these deficiencies in innate immunity as experimental evidence suggests that susceptibility to secondary bacterial infections is mitigated in primed animals in models of IAV infection [186].

4.1. And Back Yet Again: Heterologous Infection, Memory T Cells, and Inflammation. While highly specific in nature, the adaptive immune response can also alter the outcome of infections with seemingly unrelated pathogens. This phenomenon, which has been termed heterologous immunity [187], is mediated by cross-reactive T cells with T cell receptors that have the potential to recognize more than one peptide-MHC complex. Heterologous immunity is long-lasting and much like "innate imprinting" it can be either beneficial or detrimental. For instance, in animal models of lymphocytic choriomeningitis virus (LCMV), cytomegalovirus (CMV), or IAV infection, prior virus-specific immunity has a beneficial impact on the outcome of subsequent vaccinia virus infection and results in improved viral clearance [188]. However, in the reverse scenario, prior IAV-specific immunity can increase the immunopathology of respiratory LCMV and murine CMV infection. Preexisting, heterologous immunity has been shown to alter protective T cell immunodominance hierarchies induced by primary infection. It is argued that the presence of cross-reactive T cells narrows the virus-specific T cell repertoire and drives the selection of viruses able to escape adaptive immunity. Conversely, the recall of cross-reactive memory T cells can also result in protective immune responses. Given the capacity of memory T cells to regulate inflammation [133, 135], beneficial heterologous immunity in the latter scenario likely also involves well-guided innate inflammatory responses that contribute to the initial control of pathogens. One can thus infer from these studies that the severity of disease is impacted not only by the past history of infections but also by the sequence of such infections. These observations have important implications for the design and timing of the delivery of vaccines [189].

5. Summary

Understanding of the impact of prior pathogen encounter on both innate and adaptive immunity is imperative for the design of innovative vaccination regimes. Exciting developments in the field of macrophage and monocyte biology are changing the way memory is typically perceived in innate immune cells. The "training" of innate immunity must be further investigated in order to effectively implement these insights into improved vaccines that are better able to promote durable memory states. In addition, traditional paradigms of innate instruction of adaptive immunity must now appreciate that memory T cells regulate both the nature and shape of innate inflammatory responses through antigen-specific means. Furthermore, memory-regulated inflammatory responses can impact the development and functional potential of secondary effector T cells. Every infection, commensal interaction, and immunogenic vaccine thus has the potential to change the host tissue microenvironment as well as the adaptive immune T cell repertoire. Such changes can impart lasting immunological consequences that are able to influence subsequent responses to infection both positively and negatively.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Liver HLA-E Expression Is Associated with Severity of Liver Disease in Chronic Hepatitis C

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Hepatitis C virus (HCV) can escape from innate and adaptive immunity, making the immune response ineffective. Human leukocyte antigen E (HLA-E) might regulate the antiviral function of immune response and contribute to the persistence of HCV and the severity of liver disease. This study aimed to evaluate the expression of HLA-E in the liver and its association with the severity of liver disease in HCV patients. We performed a retrospective analysis of liver biopsies from 125 HCV patients and from 20 control subjects without liver disease. Liver biopsies were reviewed and classified according to severity of fibrosis and inflammatory activity. The pathologist assessed the magnitude of HLA-E expression in a semiquantitative way, attributing scores from 0 to 3. Immunohistochemistry showed positive for HLA-E in hepatocyte and Kupffer cells. The rate of HLA-E positivity in hepatocytes and Kupffer cells was significantly higher in HCV patients compared to controls. The liver samples classified as severe fibrosis and necroinflammatory activity presented greater expression of HLA-E on Kupffer cells and hepatocytes, with a significant linear association. It indicates that HLA-E expression may have an immunomodulatory effect and a possible role in the severity of liver disease in chronic hepatitis C.

1. Introduction

HCV infection is one of the main causes of chronic liver disease worldwide. The long-term impact of HCV infection is highly variable, ranging from minimal histological changes to extensive fibrosis and cirrhosis with or without hepatocellular carcinoma (HCC) [1]. Approximately 80% of patients with HCV infection fail to eradicate the virus and carry a substantial risk to progress towards liver fibrosis/cirrhosis [2]. The exact mechanisms favouring persistent infection and leading to liver fibrosis are not completely known. Numerous studies suggest an association between an impaired immune response and clinical outcome, and there is evidence that immune cells modulate fibrogenesis of hepatitis C [3–5].

Natural killer cells (NK) are specialized lymphocytes that provide a first-line defence through their ability to kill pathogen-infected cells and transformed cells, and they exert an important antifibrotic activity on activated hepatic stellate cells (HSCs) [6–10]. NK cell function is regulated by a balance of inhibitory and activating signals, which are mediated by a diverse array of cell-surface receptors. Inhibitory receptors include a variety of killer cell immunoglobulin-like receptors (KIRs) and C-type-lectin receptors such as CD94/NKG2A. These receptors bind self-major histocompatibility complex (MHC) class I molecules, which are constitutively expressed in normal cells. If the self-class I molecule expression increases in a target cell, it can result in increased inhibitory signals and impaired NK cell activity [11]. Decreased

NK cell cytotoxic activity was reported in infections with human cytomegalovirus, Epstein-Barr virus, herpes simplex virus, and HCV. Therefore, altered function of NK cells might be a general mechanism by which viruses escape the immune system [5].

HLA-E is a nonclassical MHC class I molecule, virtually transcribed on all human tissues and cell lines. The role of this molecule in the innate immune response is to present signal sequence-derived peptides of other HLA class I molecules to inhibit NK-mediated cell lysis via recognition by CD94/NKG2A. However, HLA-E can also bind and present other peptide sequences, which can be self or pathogen derived and can be recognized by adaptive T-cells. HLA-E is considered to play a role in both innate and adaptive immunity, via interacting with both NK cells and presenting peptides to antigen-specific CD8+ T-cells [12].

In our study, we analyzed the expression of HLA-E in the liver and its association with the severity of liver disease in chronic hepatitis C patients.

2. Material and Methods

2.1. Patients. This is a retrospective study conducted through the revision of medical records of 125 patients diagnosed with chronic hepatitis C. All patients were serum HCV RNA positive and had undergone a liver biopsy. Twenty liver biopsies from subjects without liver disease, who died from cardiovascular complications, were used as control. The local ethics committee approved this study (Protocol number: 13812/2014).

2.2. Histological Analysis and Immunohistochemistry. The liver samples of the control subjects were reviewed to rule out the presence of inflammatory activity, fibrosis, fat, and iron deposit. The liver biopsies from HCV patients were reviewed and classified according to severity of fibrosis and inflammatory activity (METAVIR classification) [13].

The evaluation of hepatic expression of the HLA-E molecule was performed on liver specimens included in paraffin blocks using the immunohistochemistry technique. In brief, 4 μm-thick slices were made in the paraffin-embedded tissues and mounted on slides pretreated with poly-L-lysine. Antigenic recovery, blocking of endogenous peroxidase, and blocking of nonspecific reactions were performed. The slides were incubated at room temperature in a wet chamber overnight, with the anti-HLA-E antibody MEM-E/02 (EXBIO Antibodies, Czech Republic), at 1 : 100 dilution.

HLA-E expression was classified according to the percentage of HLA-E-positive cells, by manually counting 10 visual fields at ×200 magnification for each liver sample. The results were described in three categories: grade 0 (without positive cell expression), grade 1+ (<25%), 2+ (25–50%), and grade 3+ (>50% of positive cell expression).

2.3. Statistical Analysis. Continuous variables were expressed as mean values, standard deviation, and range. Categorical variables were expressed with frequency and percentage. A chi-square analysis and a chi-square for linear trend were used to compare categorical variables, and the continuous variables were analyzed using the Kruskal-Wallis test.

A P value less than 0.05 was considered to be significant. Statistical interpretation of data was performed using Statistical Package for Social Sciences (SPSS) version 17.0 for Windows.

3. Results

The mean age of HCV patients and control subjects was 49.7 (±11.7) and 58.7 (± 20) years, respectively. Most HCV patients were male (50.8%), and 55% of the control subjects were female. Genotype 1 was the most prevalent (68% of the patients). In HCV-infected samples, the METAVIR classification for liver biopsies showed the following: F0/1: 31%, F2: 33%, F3/F4: 36%, A0/1: 32%, A2: 42%, and A3: 26%. In the control subjects' liver samples, there was no inflammation activity, fibrosis, fat, or iron deposit.

Immunohistochemistry showed positive for HLA-E in hepatocyte cytoplasm and Kupffer cells. These cells were identified according to their morphology, and a costaining with CD68-specific antibodies, for Kupffer cells, was done (Figures 1(a) and 1(b)).

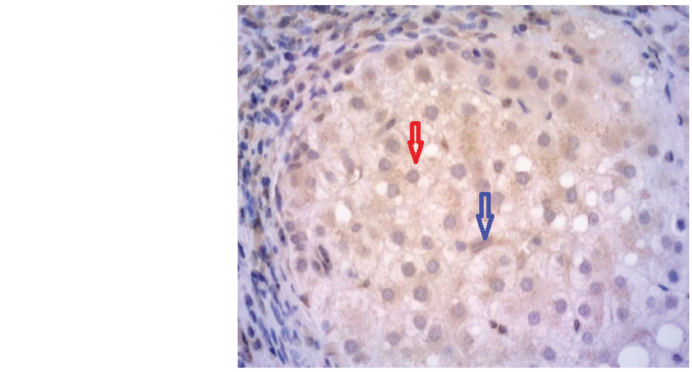
The rate of HLA-E positivity in hepatocytes and Kupffer cells was significantly higher in HCV patients compared to controls (58.6% × 20% and 45.3% × 10%, resp.; Table 1).

Patients older than 40 years and the male gender were associated with a higher frequency of HLA-E expression. Sixty-four percent of patients older than 40 years presented HLA-E expression on hepatocytes, compared to 36% of patients aged less than 40 years ($P = 0.04$). Fifty-five percent of males presented HLA-E expression in Kupffer cells, compared to only 34% of female patients ($P = 0.02$).

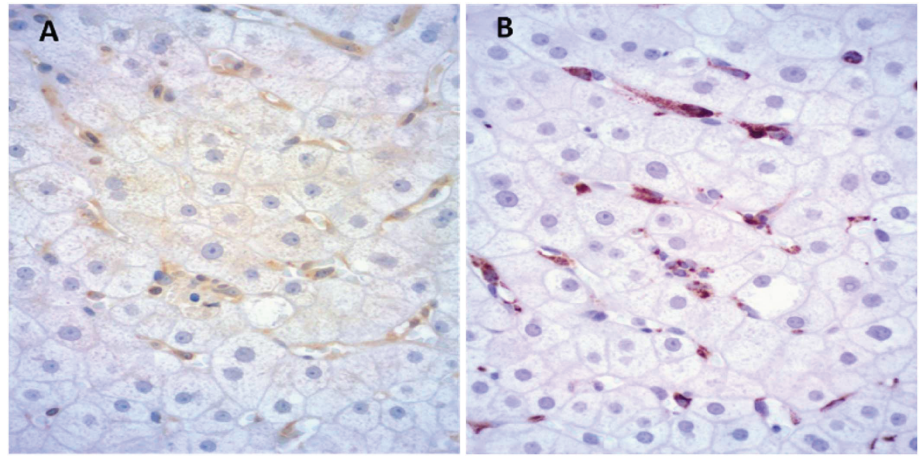
Significant association ($P < 0.05$) was found between degrees of necroinflammation and fibrosis and HLA-E expression in hepatocytes and Kupffer cells in HCV patients. The liver samples classified as severe fibrosis (METAVIR F3-F4) or necroinflammatory activity (METAVIR A3) presented greater expression of HLA-E in Kupffer cells and hepatocytes, with a linear association ($P < 0.05$). Figures 2–5 show the association between HLA-E expression and liver fibrosis/necroinflammatory activity.

4. Discussion

In this study, a significant upregulation of HLA-E expression was found in livers derived from HCV-infected individuals compared to HCV-negative subjects. Expression of HLA-E was detected on CD68(+) macrophages/Kupffer cells and hepatocytes. Nattermann et al. also found enhanced intrahepatic HLA-E expression in HCV-infected livers as compared to HCV-negative livers. Expression of HLA-E was detected in CD68(+) macrophages/Kupffer cells and CD31(+) sinusoidal endothelial cells, as well as in CD14(+) and CD83(+) cells [5]. They showed that, as a potential underlying mechanism, HCV gives rise to a peptide (HCV core amino acids 35–44) that binds to HLA-E, stabilizes its surface expression, and thereby impairs NK cell cytotoxicity [5, 14].



(a)



(b)

Figure 1: (a) HLA-E positivity in hepatocytes (red arrow) and Kupffer cells (blue arrow). (b) To analyze the identity of intrahepatic HLA-E-positive cells, liver samples were double-labeled for HLA-E and CD68. All sections were immunostained with the indirect immunoperoxidase method and counterstained with hematoxylin. In double-labeling experiments, we incubated the sections with CD68-specific antibodies after performing the HLA-E-specific immunoperoxidase reaction. A: HLA-E-specific immunoperoxidase reaction (yellow); B: anti-CD68 (red) to confirm that these positive cells were Kupffer cells.

Table 1: Frequency of HLA-E expression in hepatocyte cytoplasm and Kupffer cell in HCV patients and control subjects.

	HLA-E expression in hepatocyte cytoplasm							HLA-E expression in Kupffer cell						
	0*		1+**		2-3+****			0*		1+**		2+***		
	$N^{n.}$	%	$N^{n.}$	%	$N^{n.}$	%		$N^{n.}$	%	$N^{n.}$	%	$N^{n.}$	%	
HCV ($n = 125$)	52	41.6	47	37.6	26	20.8	P	69	55.2	38	30.4	18	14.4	P
Control ($n = 20$)	16	80	04	20	0	0	0.004	18	90	02	10	0	0	0.01

$N^{n.}$: number of individuals; %: percentage; P: P value. Grades of HLA-E expression: 0* (without positive cell expression), 1+** (<25%), 2+*** (25–50%), and 2-3+**** (>25% or >50% of positive cell expression). There was no 3+ HLA-E expression in Kupffer cell.

NK cells importantly contribute to the initial control of viral infections through their rapid and potent cytotoxic activity. They also exert an important antifibrotic activity on activated HSCs. Thus, an impaired function of NK cells can predispose the evolution to chronic hepatitis C infection and to the progression of liver fibrosis [5–10], the HLA-E molecule being a key component of this process via its interaction with inhibitory NK cell receptors. Nattermann et al. found a significantly increased proportion of inhibitory receptors, NKG2A, expressed in NK cells in HCV-positive patients, resulting in reduced cytolytic activity against cells incubated with the HLA-E-stabilizing peptide HCV core 35–44 [15]. Apart from NK cell inhibition, HLA-E upregulation may have further functional consequences. Increased HLA-E expression was seen particularly on hepatic CD68(+) macrophages/Kupffer cells, which should protect these cells against lysis by NK cells and thus prolong antigen presentation [5].

Epidemiological investigations have shown that fibrosis is accelerated in some situations, such as among older males [16]. Age is an independent factor, associated with a higher rate of fibrosis progression [17]. Studies have shown that

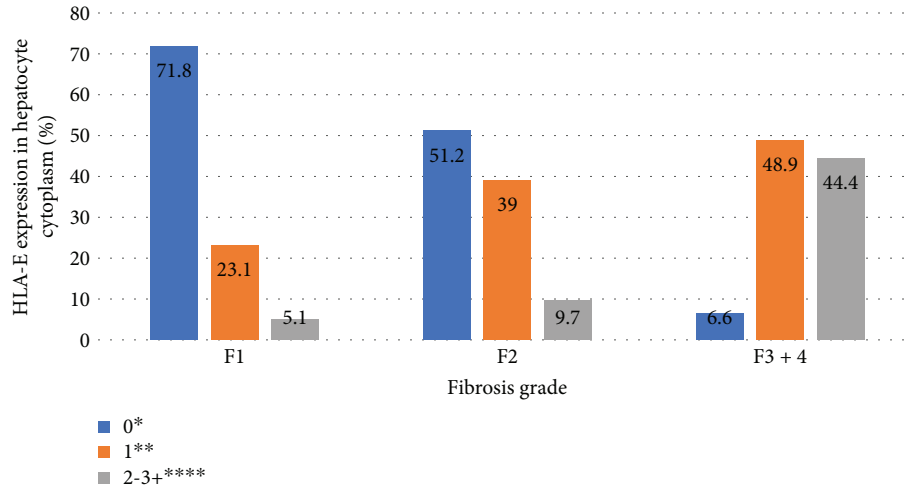


Figure 2: Association between HLA-E expression in hepatocyte cytoplasm and liver fibrosis. HLA-E expression was described in the following grades: 0* (without positive cell expression), 1+** (<25%), 2-3+**** (>25 or >50% of positive cell expression). Liver fibrosis was described according to the METAVIR classification: F1: portal fibrosis without septa, F2: portal fibrosis with rare septa, F3-4: numerous septa without and with cirrhosis. $P < 0.001$.

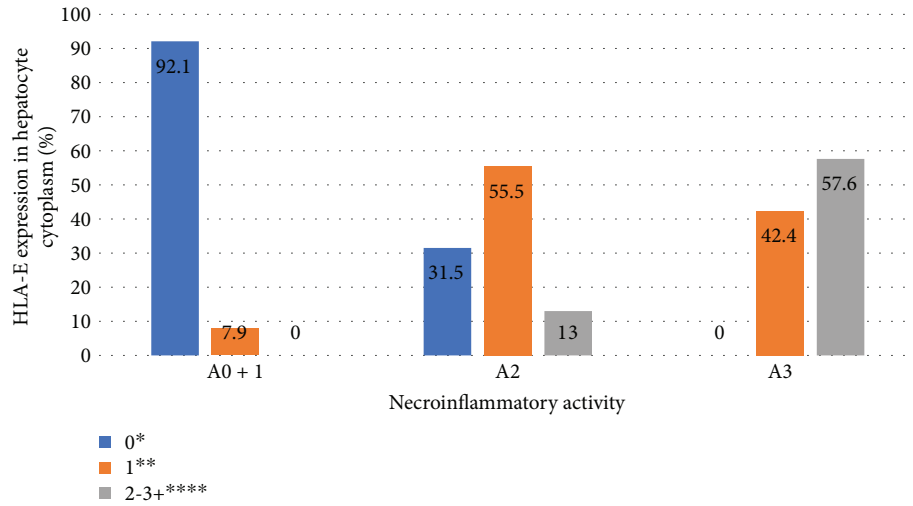


Figure 3: Association between HLA-E expression in hepatocyte cytoplasm and liver necroinflammatory activity. HLA-E expression was described in the following grades: 0* (without positive cell expression), 1+** (<25%), 2-3+**** (>25 or >50% of positive cell expression). Liver necroinflammatory activity was described according to the METAVIR classification: A0 + 1: no or mild activity, A2: moderate activity, A3: severe activity. $P < 0.001$.

the rate of progression can be 300 times greater in patients affected by the disease in their seventh decade of life compared to those affected in the third decade of life [18]. Similarly, male gender is also considered to be an important factor for the progression of hepatic fibrosis, increasing the rate of fibrosis up to 10 times, independent of age [18]. The aging of the immune system can also be related to these findings, since liver samples from HCV patients older than 40 years and male gender showed greater frequency of HLA-E expression on hepatocytes and Kupffer cells compared to younger female patients, as observed in the present study.

Liver fibrosis progression in HCV patients remains an important clinical issue, and it is a difficult phenotype to investigate. Since the precise date of HCV infection is often not available, it is confounded by environmental factors, which are difficult to quantify accurately, and there are limitations to the practicality and precision of longitudinal assessments [16].

We reported for the first time that HLA-E expression in the liver microenvironment was significantly higher in severe stages of fibrosis and necroinflammatory activity in HCV-infected patients. Persistent HCV infection is typically associated with chronic inflammatory changes within the liver, ultimately resulting in fibrosis and cirrhosis. Not only direct cytopathic effects but also immune-mediated mechanisms are likely to be involved in liver injury, and our data reinforces the participation of immune response in liver fibrosis progression. In our analyses, liver samples classified as severe

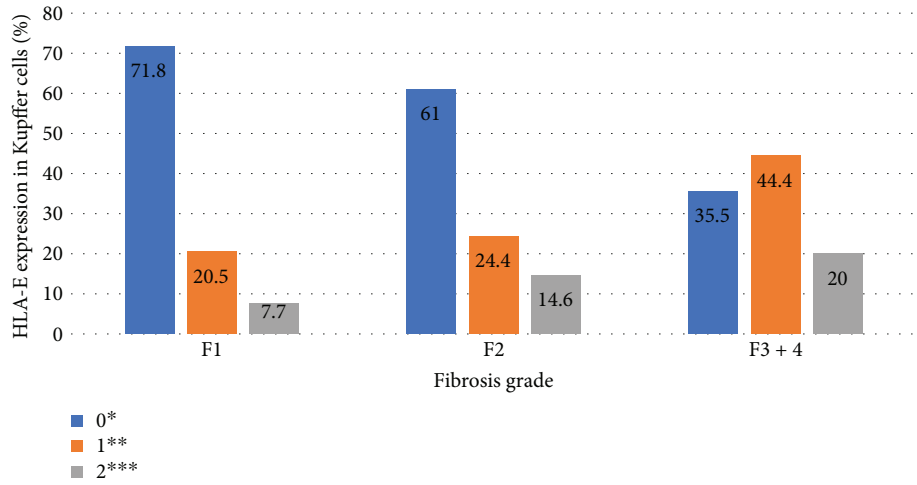


Figure 4: Association between HLA-E expression in Kupffer cells and liver fibrosis. HLA-E expression was described in the following grades: 0* (without positive cell expression), 1+** (<25%), 2+*** (25–50%). There was no grade 3+ expression in this case. Fibrosis was described according to the METAVIR classification: F1: portal fibrosis without septa, F2: portal fibrosis with rare septa, F3-4: numerous septa without and with cirrhosis. $P = 0.02$.

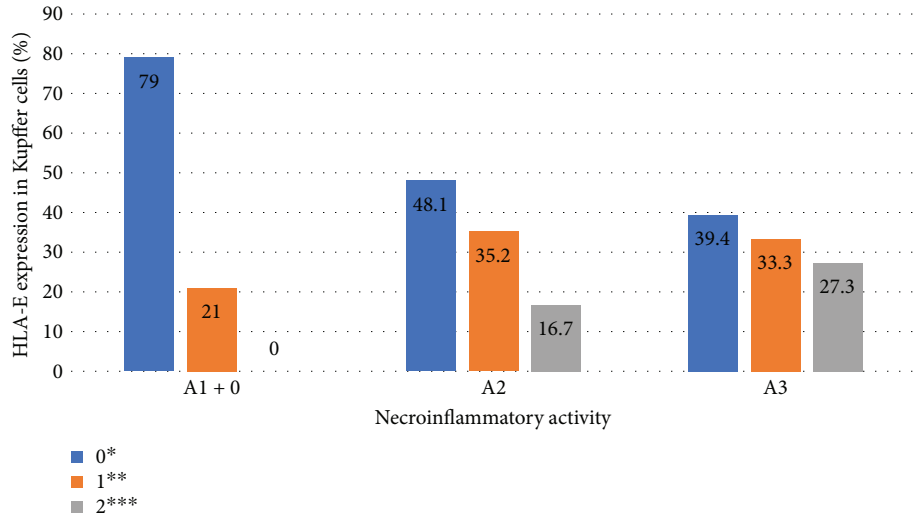


Figure 5: Association between HLA-E expression in Kupffer cells and liver necroinflammatory activity. HLA-E expression was described in the following grades: 0* (without positive cell expression), 1+** (<25%), 2+*** (25–50%). There was no grade 3+ expression in this case. Necroinflammatory activity was described according to the METAVIR classification: A0 + 1: no or mild activity, A2: moderate activity, A3: severe activity. $P = 0.002$.

fibrosis and necroinflammatory activity presented greater expression of HLA-E in Kupffer cells and hepatocytes, with a linear association.

5. Conclusions

The nonclassical HLA-E molecule might have an immunomodulatory effect and a possible role in the severity of liver disease in chronic hepatitis C. Knowledge of the expression profile of HLA-E may aid in the identification of HCV-infected patients with a worse prognosis and less favourable outcomes.

Data Availability

The data used to support the findings of this study can be requested from the author by email (rcaraujo@hcrp.usp.br).

Disclosure

Part of the results discussed in this article has been presented before as a poster at the 67th Annual Meeting of the American Association for the Study of Liver Diseases: The Liver Meeting 2016 (https://aasldpubs.onlinelibrary.wiley.com/doi/abs/10.1002/hep.28799).

Conflicts of Interest

The authors have no conflict of interests to disclose.

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Review Article

Meta-Analysis of the Changes of Peripheral Blood T Cell Subsets in Patients with Brucellosis

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Brucellosis is one of the most prevalent zoonotic diseases in the world, but its pathogenesis is not very clear. At present, it is thought that it may be related to the immunity of T cells. The conclusions of related studies are inconsistent, and its clinical significance is not explicit. We searched published articles in electronic databases up to December 2017 identified as relating to the clinical features of human brucellosis in China. Only eight studies had sufficient quality for data extraction. Meta-analysis showed a significantly decreased proportion of CD4+ T cells in human brucellosis patients compared to healthy subject individuals. The frequency of CD8+ T cells was significantly higher in human brucellosis patients than that in the healthy control group. The pooled analysis presented a significant decrease of the CD4+/CD8+ ratio in human brucellosis patients compared to healthy subjects. There is immunologic dysfunction of T lymphocyte in patients with human brucellosis, the CD4+ and CD8+ T cells might be the important factors affecting the progress of brucellosis.

1. Introduction

Brucellosis is one of the most common zoonotic infections globally [1], which is a highly contagious zoonosis. Human brucellosis is transmitted to humans by direct/indirect contact with infected animals or through the consumption of raw meat and dairy products [2, 3]. Patients may have the symptoms of fever, sweating, fatigue, and osteoarthritis and even more serious conditions in different organ systems [4–6]. Brucellosis is often misdiagnosed as malaria, typhoid fever, rheumatic fever, osteoarthritis, and other diseases. Brucella has brought great harm to public health, food safety, and so on. The specific pathogenesis of brucellosis infection is not very clear. It is difficult to make a diagnosis by epidemiological and clinical symptoms. Human brucellosis is prone to multiple system complications, and once the brucellosis progresses to chronic phase, it will be difficult to cure [7]. Therefore, the pathogenesis of brucellosis is a hot research issue, and it is also believed that brucella infection is related to both innate immunity and adaptive immunity [8].

Studies have shown that the changes of T lymphocyte are crucial to the interpretation of the clinicopathological features of brucellosis in the process of chronic infection and recurrence [9, 10]. There are three mechanisms of acquired immunity in brucella infection: (1) the antimicrobial effect of macrophage induced by interferon-gamma secreted by CD4+, CD8+, γ, and β T lymphocyte [11]; (2) the hosts eliminate the macrophages infected by brucella through the cytotoxic effect of CD8+, γ, and β T lymphocytes [12]; and (3) the modulating effect of the antibody can enhance the phagocytosis of macrophages [13]. Activated CD8+ T lymphocytes and CD4+ Th1 type immune responses play an important role in the scavenging of intracellular pathogens. Treg has the function of inhibiting the proliferation of T lymphocyte and eliminating the pathogen. The changes in the number and function of Treg will inhibit the immune function of

the host, which may be one of the mechanisms that leads brucella infection progresses into chronicity [14]. However, most of the immunology-related studies of the interaction between the host and brucella are mostly derived from domesticated ruminants or mice. The immune mechanism of brucella infection in diverse hosts is different.

There are few reports about the changes of peripheral blood T cell subsets in patients with brucellosis, and the results reported are not consistent [15–22]. It is difficult to draw a conclusion because of the deviations in experiments, population difference, and single or small sample study. In order to reduce the differences and bias among the various research institutes, meta-analysis method was used to analyze the previous research results to investigate the changes in the frequency of T cell subsets in peripheral blood of patients and provide the direction for further exploration of the mechanism of the immune pathogenesis of brucellosis.

2. Methods

2.1. Search Strategy. We performed a systematic review of the literature to identify articles relating to the changes of peripheral blood T cell subsets in patients with brucellosis. With the assistance of a professional medical librarian, we electronically searched for the literature in Wanfang Data, PubMed, and EMbase with MESH and keyword subject headings "brucellosis," "Brucella," "Brucel*," and "malta fever," and "CD3," "CD4," "CD8," "Th17," "Th1," "Th2," "Treg," and "Regulatory T Cells" for entries published from databases' inception before December 2017. We did not restrict the types of studies and publication languages. Duplicate entries were identified by two investigators screening the title and abstract of the article, the author, the year of publication, and the volume, issue, and page numbers of the source, and reviewed potentially all relevant articles.

2.2. Selection Criteria. We systematically and inclusively reviewed articles by two investigators. The reviewers selected articles firstly by title and abstract, next by full text, and lastly by analyzing eligible studies in detail until demonstrated 100% agreement in articles are included and excluded by two investigators.

Studies with the following criteria were excluded such as (i) articles related to nonhuman brucellosis, (ii) reported data that overlapped with already included articles, (iii) articles could not provide original data of the patients, (iv) and articles addressing topics not related to the changes of peripheral blood T cell subsets in patients with Brucellosis.

Studies with the following criteria were included such as (i) designed as a case-control study, (ii) the literatures assessed the changes in peripheral blood T cell subsets in patients with brucellosis, and (iii) provided sufficient data, including mean and standard deviation of T cell from case and control to calculate the efficient size.

2.3. Data Extraction. Data was extracted by two reviewers independently including date of the first author's name, publication year, study design, study location, patient, the number of male and female patients, detection technology, and treatment status methods of diagnosis, and results of each study were recorded. Study quality was assessed using the Newcastle-Ottawa scale (NOS). The results of data extraction must reach an agreement and consensus among the reviewers. Due to the inconsistency of the standard of the staging of disease in the literature included, the total mean and total standard deviation of the case group in the literature consist of acute and chronic staging are combined with the following formulas:

$$x = \frac{n_1 x_1 + n_2 x_2 + \cdots + n_k x_k}{n},$$
$$S = \sqrt{\frac{\left(n_1 {x_1}^2 + n_2 {x_2}^2 + \cdots + n_k {x_k}^2\right) + \left[(n_1 - 1){S_1}^2 + (n_2 - 1){S_2}^2 + \cdots + (n_k - 1){S_k}^2\right] - nx^2}{n - 1}}, \quad (1)$$

where n_i, x_i, and S_i denote the number of samples, the mean, and the standard deviation of the ith group, respectively.

2.4. Statistical Analysis. Data were analyzed using the mean difference (MD) with 95% confidence intervals (CI) for continuous outcomes. The mean ± SD was extracted and calculated in all included publications. Cochran's Q test and Higgin's I^2 statistics were simultaneously adopted for the test of heterogeneity of combined MDs. A random effects model was adopted to aggregate the pooled MD when significant heterogeneity existed ($p < 0.1$ and/or $I^2 > 50\%$); on the contrary, a fixed effects model was employed ($p > 0.1$ and/or $I^2 < 50\%$). Publication bias was detected by Egger's regression asymmetry test when the number of included trials ≥ 7. Sensitivity analyses were performed by omitting each study to identify the stability of combined results.

3. Results

3.1. Systematic Review. Literature searches yielded 5118 potential articles, leaving 8 publications that met inclusion and exclusion criteria for data extraction and final analyses. Eight studies representing 396 patients with human brucellosis and 212 cases of healthy control were finally included in the meta-analysis. All 8 articles included in the analysis were case-control studies. Figure 1 illustrates the detailed search process.

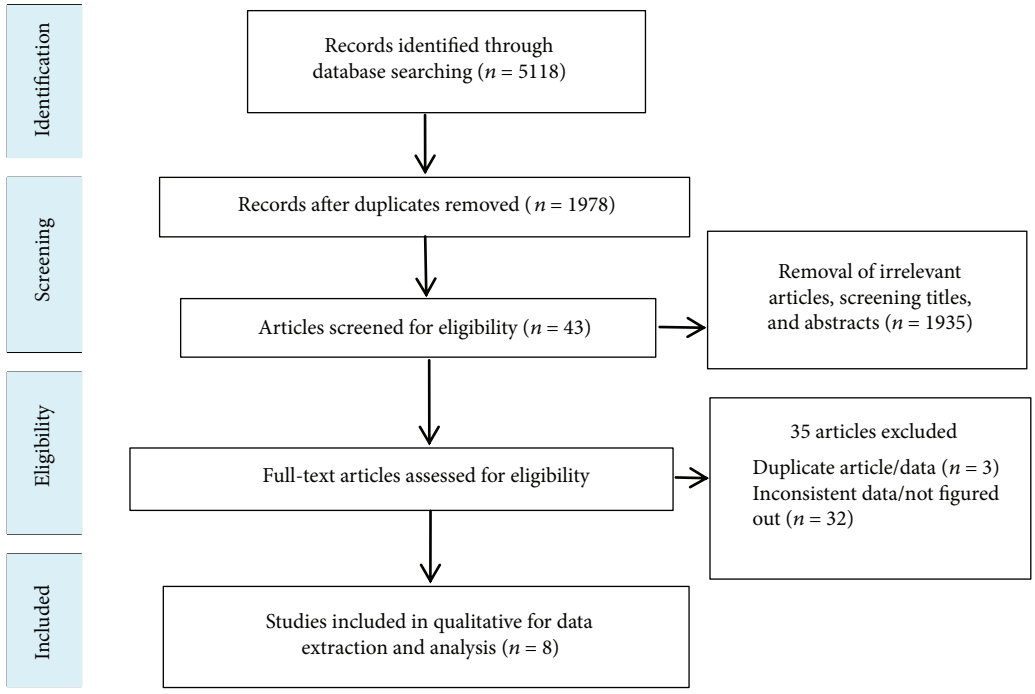

Figure 1: Procedure of the selection process.

In the 8 studies we selected, there are 6 articles in English and 2 in Chinese. Seven studies provided data about CD3+ T cells in peripheral blood [15, 17–22], 8 studies are related to CD4+ T and CD8+ T cells in peripheral blood [17–22], 4 studies are related to the ratio of CD4+/CD8+ in peripheral blood [15, 16, 19, 20], and 3 studies are related to Th1 cells and Th2 cell [16, 17, 22]. Only 2 studies are related to Treg cells in peripheral blood. The basic characteristics of the literature and patients are shown in Table 1.

3.2. Changes of Peripheral Blood CD3+ T Cell. Seven studies including 346 patients with human brucellosis and 162 cases of healthy control, in which provided the data of the changes of peripheral blood CD3+ T cells in human brucellosis patients. Two studies reported that the proportions of CD3+ T cells in human brucellosis patients were significantly increased compared to control individuals [15, 19], while another 5 studies [17, 18, 20–22] showed that there was no significant difference between the two groups. The heterogeneity test showed (I^2 = 85.6%, 95% CI (72.4%; 92.5%), $p < 0.0001$), indicating that there is statistical heterogeneity between studies. With regard to the heterogeneity results, random effects model was used. A meta-analysis showed that the proportions of CD3+ T cells in human brucellosis patients were increased but no significant difference between patients and control individuals ([MD = 1.6265, 95% CI (−1.8789; 5.1319), $p = 0.3631$]). The forest plot for these analyses was shown in Figure 2. Sensitivity analysis was included in the literature, and the effect of single study on the combined results was evaluated. The analysis showed that the results were not substantially altered, when any one study was deleted, as shown in Table 2. Egger's regression asymmetry test was used to evaluate publication bias, if any. No bias was found ($t = 0.0210$, df = 5, $p = 0.9841$).

3.3. Changes of Peripheral Blood CD4+ T Cell. Changes of peripheral blood CD4+ T cells were reported by 8 trials, containing 396 patients with human brucellosis and 212 cases of healthy control. In the 8 studies we selected, 6 studies reported that proportions of CD4+ T cells in human brucellosis patients were significantly decreased compared to control individuals [15–17, 19, 20, 22]; the other of 2 studies showed that there was no significant difference between the two groups [18, 21]. The result (I^2 = 92.1%, 95% CI (86.8%, 95.3%), $p < 0.0001$) is showed by the heterogeneity test in the meta-analysis. Regarding this conclusion, the random effects model is applied.

Results of the meta-analysis showed a significantly decreased proportion of CD4+ T cells in human brucellosis patients compared to healthy subject individuals ([MD = −9.03, 95% CI (−12.93; −5.14), $p < 0.0001$]). The forest plot for these analyses was shown in Figure 3. We also conducted sensitivity analysis to assess the influence of individual studies on the pooled results. The pooled results were not substantially altered, when any one study was deleted (Table 3). Egger's regression asymmetry test showed no evidence of publication bias ($t = 0.5995$, df = 6, $p = 0.5708$).

3.4. Changes of Peripheral Blood CD8+ T Cell. Eight trails with 396 patients with human brucellosis and 212 cases of healthy control reported changes of peripheral blood CD8+ T cell. These trials show homogeneity in the consistency of the trial results (I^2 = 77.2%, 95% CI (54.7%, 88.5%), $p < 0.0001$). Therefore, a random effects model should have been used for statistical analysis. Meta-analysis showed that

Table 1: Characteristics of studies included in the meta-analysis.

Year	Author(s)	Region	Treatment status	Study design	Number		Sex (M/F)		NOS scale
					Patient	Control	Patient	Control	
2015	Gao et al. [15]	China	No report	Case-control design	142	45	89/53	27/18	7
2016	Gu et al. [16]	China	No report	Case-control design	50	50	42/8	38/12	7
2010	Manuel et al. [17]	Spain	All patients were treated	Case-control and cohort design	24	24	17/7	18/6	8
2006	Panagiotis et al. [18]	Greece	No report	Case-control design	35	15	28/7	11/4	7
2005	Celik et al. [19]	Turkey	All patients were treated	Case-control and cohort design	43	20	19/24	11/9	8
1996	Pourfathollah et al. [20]	Iran	No report	Case-control design	56	26	—	15/11	6
1996	Zapata et al. [21]	Spain	All patients were treated	Case-control and cohort design	21	21	19/2	—	6
2005	Akbulut et al. [22]	Turkey	No report	Case-control design	25	11	16/9	7/4	7

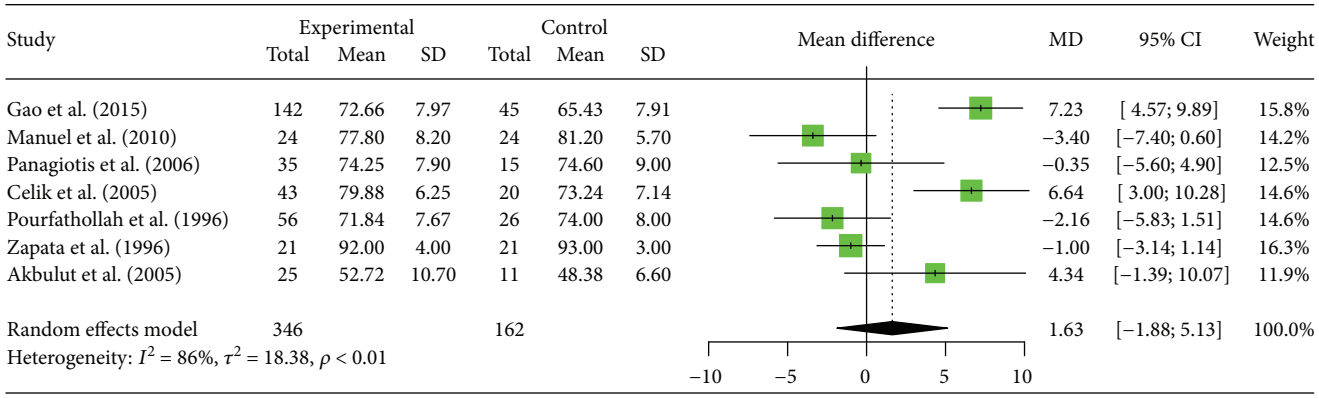

Figure 2: Forest plot of the changes of peripheral blood CD3+ T cell in human brucellosis patients compared with controls.

Table 2: The results of CD3+ T cell data sensitivity analysis.

	Influential analysis (random effects model)				
	MD	95% CI	p value	Tau^2	I^2
Omitting 1	0.5238	[−2.5631; 3.6106]	0.7395	10.5972	74.9%
Omitting 2	2.4580	[−1.2836; 6.1997]	0.1979	17.8897	85.7%
Omitting 3	1.9108	[−1.9972; 5.8188]	0.3379	20.2834	87.8%
Omitting 4	0.7666	[−2.9579; 4.4912]	0.6866	17.5758	85.1%
Omitting 5	2.2725	[−1.6304; 6.1755]	0.2538	19.6843	86.5%
Omitting 6	2.1258	[−1.9855; 6.2371]	0.3109	21.8144	84.7%
Omitting 7	1.2547	[−2.6029; 5.1123]	0.5238	19.8369	87.8%

there is a significantly increased proportion of CD8+ T cell in human brucellosis patients compared to healthy subject individuals ([MD = 5.24, 95% CI (2.99; 7.50), $p < 0.0001$]). Five studies reported significantly increased proportions of CD8+ T cells in human brucellosis patients compared to control individuals [15, 16, 19, 20, 22]; the other 3 studies showed that there were no significant difference between the two groups [17, 18, 21]. The forest plots for these analyses were shown in Figure 4. Sensitivity analysis indicated that the above meta-analysis results were relatively stable, as shown in Table 4. Egger's regression asymmetry test showed no evidence of publication bias ($t = -1.4357$, df = 6, $p = 0.2011$).

3.5. Changes of Peripheral Blood CD4+/CD8+ Ratio. Of the 8 included trials, 4 articles [15, 16, 19, 20] provided data of the changes of peripheral blood CD4+/CD8+ ratio in human brucellosis patients compared to controls including 291 patients with human brucellosis and 141 cases of healthy control. The trials showed homogeneity in the consistency of the trial results ($I^2 = 94.9\%$, 95% CI (90.0%, 97.4%), $p < 0.0001$). Therefore, a random effects model should have been used for statistical analysis. In the 4 studies we selected, each of them reported significantly decreased CD4+/CD8+ ratio in human brucellosis patients compared to control individuals. Results of the meta-analysis showed a significantly decreased proportion of CD4+/CD8+ ratio human brucellosis patients compared to healthy individuals ([MD = −0.6291, 95% CI (−0.99, −0.27), $p = 0.0006$]). The forest plot for these analyses was shown in Figure 5.

3.6. Changes of Peripheral Blood Th1 Cell. Changes of peripheral blood Th1 cell were also reported by 3 trials, which included 99 patients with human brucellosis and 85 cases of healthy control. In the 3 studies we selected, 1 reported significantly increased proportion of Th1 cells in human brucellosis patients compared to control individuals [16], 1 reported significantly decreased proportion of Th1 cell in

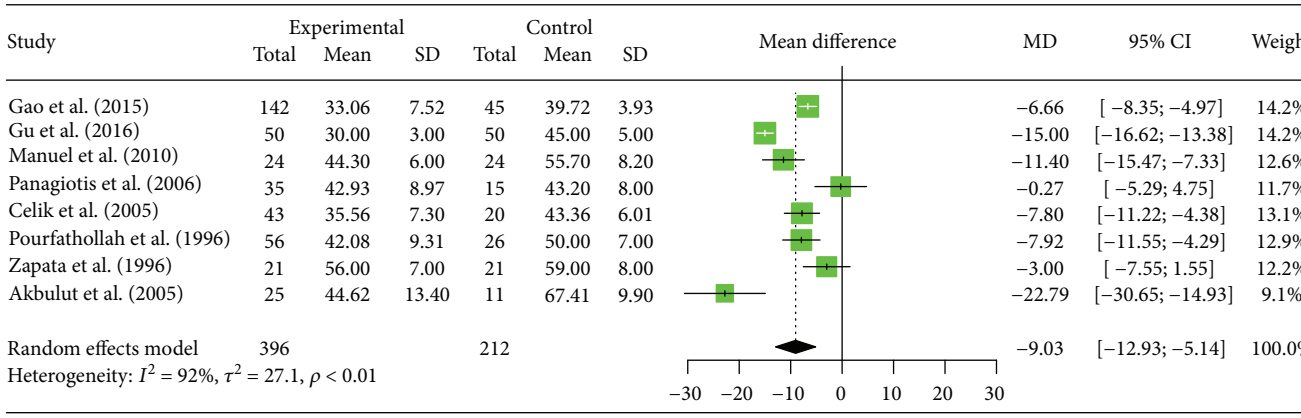

Figure 3: Forest plot of the changes of peripheral blood CD4+ T cell in human brucellosis patients compared with controls.

Table 3: The results of CD4+ T cell data sensitivity analysis.

	Influential analysis (random effects model)				
	MD	95% CI	p value	Tau^2	I^2
Omitting 1	−9.4574	[−14.0627; −4.8521]	<0.0001	33.4726	91.0%
Omitting 2	−7.8215	[−11.0090; −4.6339]	<0.0001	13.7059	80.3%
Omitting 3	−8.7126	[−13.0720; −4.3533]	<0.0001	30.0784	93.2%
Omitting 4	−10.1635	[−14.0935; −6.2334]	<0.0001	23.9963	91.9%
Omitting 5	−9.2435	[−13.6663; −4.8207]	<0.0001	30.8919	93.1%
Omitting 6	−9.2212	[−13.6181; −4.8242]	<0.0001	30.5423	93.1%
Omitting 7	−9.8610	[−13.9606; −5.7614]	<0.0001	26.2844	92.4%
Omitting 8	−7.6681	[−11.5829; −3.7532]	0.0001	24.6279	92.3%

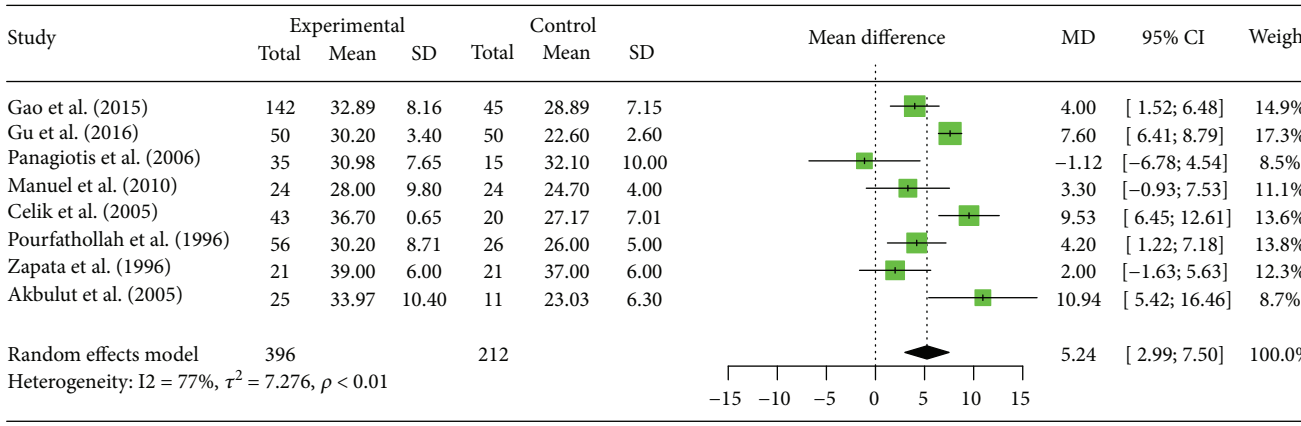

Figure 4: Forest plot of the changes of peripheral blood CD8+ T cell in human brucellosis patients compared with controls.

human brucellosis patients compared to control individuals [17], and the rest of the 1 study found that there were no significant difference between the two groups [22]. The results of heterogeneity test (I^2 = 98.1%, 95% CI (96.4%, 98.9%), p < 0.0001) indicate that there is statistical heterogeneity between studies. With regard to the heterogeneity results, random effects model was used. Results of the meta-analysis showed increased proportions of Th1 cells in human brucellosis patients but no significant difference between patients and control individuals ([MD = 4.51, 95% CI (−9.39, 18.40), p = 0.5249]). The forest plot for these analyses was shown in Figure 6.

3.7. Changes of Peripheral Blood Th2 Cell. There are 3 articles providing data of the changes of peripheral Th2 cells in human brucellosis patients compared to controls including 99 patients with human brucellosis and 85 cases of healthy control. In the 3 studies we selected, 1 reported significantly increased proportion of Th2 cells in human brucellosis patients compared to control individuals [16]; the rest of

Table 4: The results of CD8+ T cell data sensitivity analysis.

	Influential analysis (random effects model)				
	MD	95% CI	p value	Tau^2	I^2
Omitting 1	6.7050	[5.7618; 7.6482]	<0.0001	8.3543	77.5%
Omitting 2	4.8388	[3.5210; 6.1566]	<0.0001	8.5000	71.9%
Omitting 3	6.5501	[5.6575; 7.4427]	<0.0001	5.7473	74.8%
Omitting 4	6.5028	[5.6013; 7.4042]	<0.0001	7.5933	79.0%
Omitting 5	6.0810	[5.1607; 7.0012]	<0.0001	7.4068	77.1%
Omitting 6	6.5711	[5.6481; 7.4941]	<0.0001	8.3139	78.9%
Omitting 7	6.6377	[5.7287; 7.5466]	<0.0001	6.5298	75.8%
Omitting 8	6.2442	[5.3510; 7.1373]	<0.0001	7.1158	78.5%

the 2 studies found that there were no significant differences between the two groups [17, 22]. These trials show homogeneity in the consistency of the trial results [$I^2 = 98.1\%$, 95% CI (96.5%, 99.0%), $p < 0.0001$]. Therefore, a random effects model should have been used for statistical analysis. Results of the meta-analysis showed increased proportions of Th2 cell in human brucellosis patients but no significant difference between patients and control individuals ([MD = 0.93, 95% CI (−1.21, 3.07), p = 0.3925]). The forest plot for these analyses was shown in Figure 7.

4. Discussion

Although brucellosis is a disease that can be cured, there are still 5%~15% of brucellosis progression to chronicity with characteristics of a typical clinical manifestation, chronic fatigue syndrome, and recurrence [7, 23, 24]. Brucellosis is an infection-allergic zoonosis caused by brucella and has a worldwide distribution. There are more than 50 million new infections in the world every year. In recent years, the prevalence of brucellosis infection in China has also increased significantly [25]. Chinese CDC data showed that the annual incidence had increased from 5000 cases in 2002 to more than 60 thousand cases in 2015 [25]. In order to reveal the variation of T cell subsets such as CD3+ T cells, CD4+ T cells, CD8+ T cells, CD4+/CD8+ T cell ratios, Th1 cells, and Th2 cells in peripheral blood and further clarify its clinical significance, a total of 8 related articles were systematically evaluated in the study. All the researches were case-control studies. The quality of the research was evaluated by the NOS standard, and the overall score was 6~8. In general, the number of research in system analysis is not enough, the clinical studies of higher quality and large samples are needed to provide scientific and reliable evidence for the clinical application.

In recent years, the importance of T cells in brucella infection has been increasingly emphasized. Brucella was considered as an intracellular parasitic bacteria; in pathogenesis, cell-mediated immunity was the main reason [26, 27]. CD4+ and CD8+ T lymphocytes secrete cytokines such as interferon-gamma, TNF-alpha, and IL-2, which promote Th1 related to cytokine production, while the cytokine production and release by T lymphocytes can enhance the bactericidal ability of macrophages [28]. Interferon-gamma secreted by T lymphocytes not only activates the antibacterial ability of macrophages but also activates the cytotoxic effect of T lymphocyte [29]. Rafiei et al. found that the proportion of T lymphocytes secreting interferon-gamma in peripheral blood of chronic brucellosis patients was significantly lower than that in patients with acute stage, while the proportion of T lymphocytes producing IL-13 increased significantly [30]. Disproportions of Th1/Th2 and increases of cytokines secreted by Th2 cells in patients with chronic phase suggest that CD4+ T lymphocyte dysfunction is associated with chronic brucellosis. The proportions of CD4+ and CD8+ T lymphocytes in peripheral blood of patients with acute brucellosis were not significantly different from that of healthy controls, while the proportion of CD8+ T lymphocytes increased significantly in patients with chronic brucellosis, especially in patients with recurrent or symptomatic symptoms. But only a few CD8+ T lymphocytes secrete interferon [31]. Brucellosis mouse model inoculated with T lymphocytes could reduce the number of brucella in mice spleen, which indicates that CD4+ and CD8+ T lymphocyte immunity is involved in resistance to brucella infection [32]. MHC-II-deficient mice (without CD4+ T lymphocytes) had stronger ability to control S19 infection than MHC-I-deficient mice (without CD8+ T lymphocytes), indicating that CD8+ T lymphocytes play a major role in the immunity against brucellosis infection [33]. However, immunological studies on the interaction between host and brucella are mostly in cells or animal model experiments and cannot fully reflect the immune status of patients.

CD3+ T cells represent mature lymphocytes, which are the main active cells in cellular immunity, and CD3+ T cells represent the overall level of cellular immunity. By testing the changes of T lymphocyte subsets in 142 patients of brucellosis and 45 healthy controls, Gao et al. [15] has found that CD3+ T lymphocytes are increased in patients with brucellosis. Çelik et al. [19] reported that the proportion of CD3+ T lymphocytes in peripheral blood of patients with acute brucellosis was significantly increased. Some other researches showed that the numbers of peripheral blood CD3+ T lymphocytes had no significant difference between brucellosis patients and healthy controls. This study showed that CD3+ T cells in human brucellosis patients had increased but there were no significant difference between patients and control individuals. The conclusion that we obtained needs to be confirmed further.

CD4+ T cell is an auxiliary T lymphocyte that assists other cells to participate in the immune response. The decrease of CD4+ T cells can lead to a series of dysfunction in Tc, NK, macrophages, and B cells. CD8+ T cells are the main effector cells that play specific cytotoxic effects, specifically killing target cells, mainly involved in the resistance to intracellular infection [34] and antitumor [35] and participate in graft rejection [36]. CD8+ T cells are not just a homogeneous cell group, some part of them are immunosuppressive cells that inhibit the function of other immune cells [37]. The ratio of CD4+/CD8+ is related to the function of the effector T cells, usually keeping dynamic balance in order to maintain the stability of cellular immune function. The decrease in the number of CD4+ T cells and the ratio of CD4+/CD8+ can lead to the dysfunction of the effector T

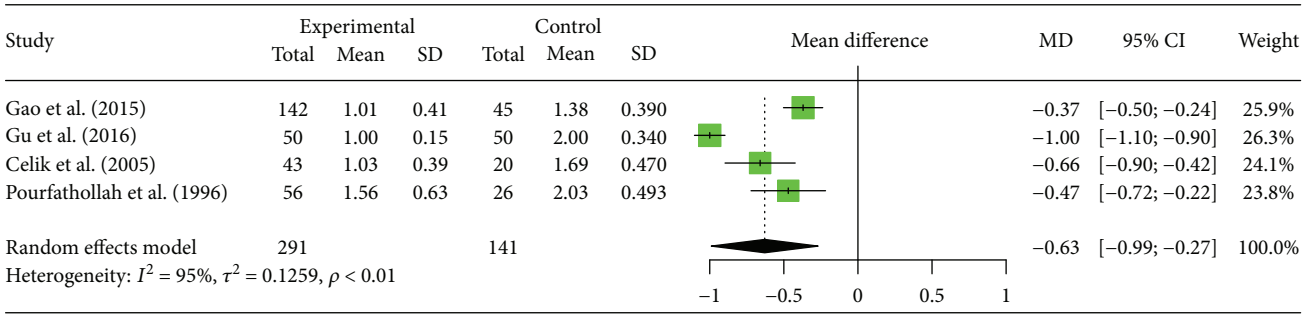

Figure 5: Forest plot of the changes of peripheral blood CD4+/CD8+ ratio in human brucellosis patients compared with controls.

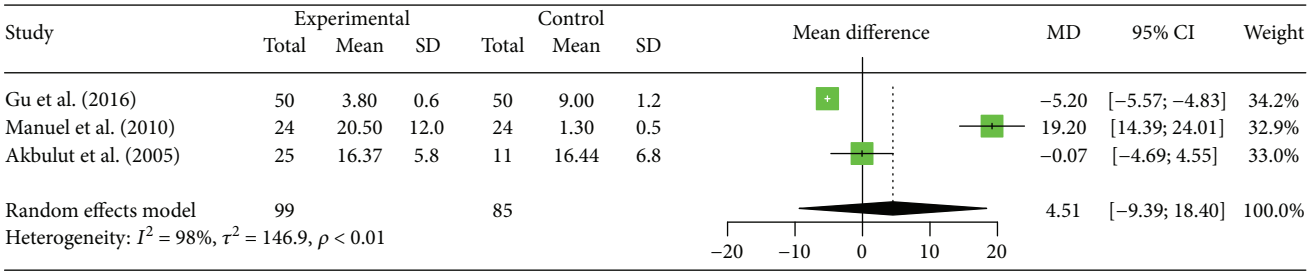

Figure 6: Forest plot of the changes of peripheral blood Th1 cell in human brucellosis patients compared with controls.

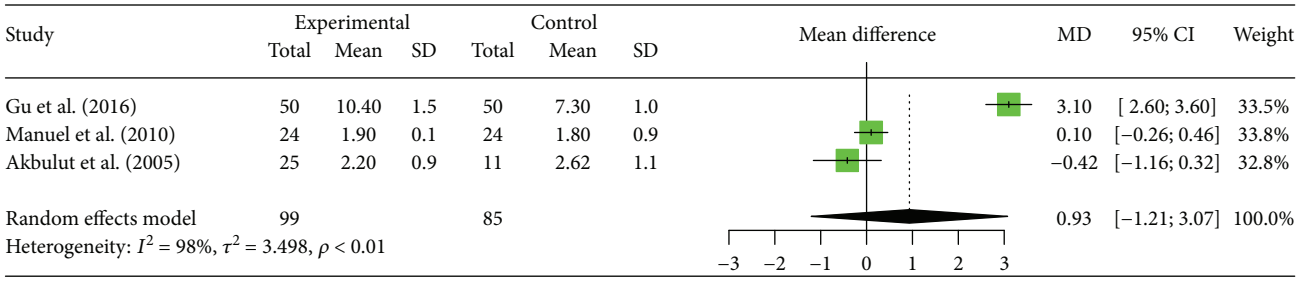

Figure 7: Forest plot of the changes of peripheral blood Th2 cell in human brucellosis patients compared with controls.

cells. The conclusions from different related researches of the changes in the percentage of CD4+ T, CD8+ T, and CD4+ T/CD8+ T ratios in peripheral blood in patients with brucellosis are not consistent. According to the results of the rat model, it is conjectured that the CD8+ T lymphocyte actives as CTL and plays the cytotoxic effect by secreting granulysin [38]. Some scholars [39] also believed that it may only compensate for the ineffective CD4+ T lymphocyte response by increasing the number of CD8+ T lymphocyte in patients with chronic brucellosis. Some studies reported that the CD4+ T lymphocyte proliferation reaction ability in patients with chronic brucellosis was significantly lower than that of the healthy controls and the acute patients [40]. This study shows that the frequency of CD4+ T cells in brucellosis patients is lower than that of healthy controls, while the CD8 cell frequency is higher than that of controls, and the ratio of CD4/CD8 is lower than the control group, which shows that there is immunologic dysfunction of the T lymphocyte in patients with brucellosis. Further research is needed to confirm the changes of CD4+ and CD8+ T cell function in peripheral blood of patients with brucellosis.

Th1 and Th2 cells are the first classified CD4+ T cell functional subgroups. Th1 cells mainly mediate cellular immunity and delayed hypersensitivity through secreted inflammatory cytokines. Th2 cells mainly mediate humoral immune response, and both of them must keep dynamic balance in order to maintain the stability of cellular immune function. The IFN-gamma secreted by Th1 cells antagonized the IL-4 secreted by Th2 cells, and IFN- gamma secreted by Th1 cells will destroy the macrophages infected with brucella [41–44]. Dorneles et al. [45] indicated that prime-immunized with *B. abortus* S19 or RB51 in cattle induce a strong and complex Th1 immune response characterized by proliferation of CD4+ Th1 cells and higher secretion of IFN-γ, which plays an important role in controlling the occurrence and development of brucella infection. Studies have shown that immune response induced by Th1 cells is necessary for highly effective vaccines to prevent brucellosis. Therefore, the increase of Th2 cell level in host cells may inhibit the immune response of Th1 cells and break the balance between Th1 and Th2 cells, leading the occurrence of brucellosis [46]. This study shows that proportions of Th1 and Th2 cells

increased in human brucellosis patients but neither of them has significant difference between patients and control individuals. The statistical power of the analysis was not enough since only 3 studies were used for this part of the analysis; the conclusion that we obtained still needs further research.

Treg cells play an important role in preventing immune responses against pathogens [47]. Abbas et al. found that the numbers of CD4+ Treg cells and their $CD25^{high}$ and $FoxP3^{high}$ subsets increase significantly in the peripheral blood of human brucellosis, with this increase being greater in the chronic group [48]. By contrast, Ganji at al. found a significantly lower percentage of CD25/FoxP3+ Treg cells in chronic patients than in the acute patients and control groups [49]. Only 2 eligible studies are related to Treg cells in peripheral blood, which is unable to carry out the meta-analysis.

Th17 cells have been found to be major stimulatory participants in the pathogenesis of human disease [50]. In mouse models, Pujol et al. found that the activation of Th17-related response was ineffective to control the *B. canis* infection [51]. There were no reports on Th17 in patients with brucellosis.

Our study has some limitations. First, heterogeneities exist among the included documents, which may be related to the diversities of race, age, diagnostic criteria and the staging of acute and chronic illness. Second, it is hard to unify the standard staging of acute and chronic illness with the included literatures; the results of the case group in the literature consist of acute and chronic staging are combined with the formulas, leading to the failure to do more accurate analysis for the different stages of the brucellosis patients. Last, due to the quantity of the included literature is not enough, the subgroup analyses cannot be tested.

In summary, we found that there was immunologic dysfunction of T lymphocyte in patients with human brucellosis, which may provide some pieces of evidence for immune regulation therapy of brucellosis. More high-quality and large sample experiments are needed to further confirm the relationship between the frequency and function of peripheral blood T cells subsets and the pathogenesis of brucellosis.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Research Article

Aberrant Peripheral Immune Function in a Good Syndrome Patient

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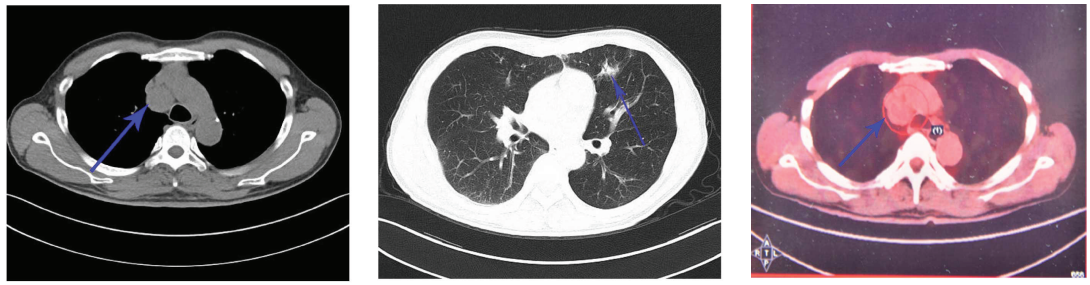
Good's syndrome (GS) is often accompanied by recurrent respiratory infections and chronic diarrhea. The main purpose was to evaluate the peripheral immune status of a GS patient after thymoma resection. Twenty healthy volunteers were recruited as healthy controls (HCs). Flow cytometry was applied to determine the proportions of circuiting $CD4^+$ T cells, $CD8^+$ T cells, $\gamma\delta$T cells, and regulatory T (Treg) cells in our GS patient. We also examined the proliferation capability of ex vivo $CD4^+$ T cells and detected the levels of cytokines interferon- (IFN-) γ and interleukin-17A secreted by ex vivo immune cells from this GS patient. Compared with healthy control subjects, this GS patient had fewer B cells, an inverted ratio of $CD4^+/CD8^+$ cells, and more Treg cells in his peripheral blood. Additionally, the patient's Vδ2 T cell levels were significantly decreased despite having a normal percentage of $\gamma\delta$T cells. Ex vivo peripheral $CD4^+$ T cells from the patient showed insufficient proliferation and division potential as well as excessive expression of PD-1. Moreover, IFN-γ was predominantly derived from $CD8^+$ T cells in this GS patient, rather than from $CD4^+$ T cells and $\gamma\delta$T cells. This GS patient had impaired T and B cell immunological alternations and cytokine disruptions after thymectomy. Detailed research should focus on therapies that can adjust the immune status in such patients for a better outcome.

1. Introduction

Thymoma is an epithelial tumor originating from the thymus, and it is linked to various immunological disorders according to different subtypes. GS, a subtype of thymoma firstly described in 1954 by Dr. Good [1], was a rare disease accompanied by respiratory system abnormality with recurrent bronchitis, sinusitis, pneumonia, and chronic diarrhea. To date, patients diagnosed with GS mainly distributed in Europe and America and totally 47 cases emerged in China [2]. GS is typically detected based on a diagnosis of thymoma in combination with characteristic clinical laboratory test results, such as a low number or even absence of peripheral B cells, hypogammaglobulinemia, $CD4^+$ T lymphopenia, and an inverted ratio of $CD4^+/CD8^+$ cells. This condition may also have autoimmune manifestations, such as myasthenia gravis or diabetes mellitus [3]. Thymectomy is an optimal treatment strategy that hinders locally aggressive growth and metastasis of the thymoma, and this procedure is widely applied clinically in conjunction with regular immunoglobulin infusions to restore immune balance [4].

In GS patients, an imbalance of immune cells and cytokines usually leads to tumorigenesis and recurrent chronic infections. Regulatory T (Treg) cells maintain self-tolerance and control autoimmunity, but they can also contribute to immunosuppression by restraining effective anti-infection immunity [5]. Interferon-gamma (IFN-γ) is an essential cytokine responsible for the cytotoxic effects exerted by T helper type 1 cells, and interleukin- (IL-) 17A, a proinflammatory cytokine, actively participates in chronic infection through the IL-17 and IL-23 signaling pathways. However, to date, little is known about the role of peripheral Treg cells



Chest X-ray (a) Chest X-ray (b) PET/CT (c)

Figure 1: Clinical imaging findings of this GS patient. (a) An anterior mediastinal mass (2.8 cm) in chest X-ray (blue arrow). (b) Signs of pneumonia in chest X-ray (blue arrow) when first hospitalized. (c) Lesion showed via PET/CT (blue arrow).

or the involvement of these two cytokines in GS patients. Here, we detected circulating immune cells, including Treg cells, and inflammatory cytokines to assess the peripheral immunological status of a GS patient. Our findings may provide the foundation for new treatment strategies for recurrent postthymectomy respiratory infections in GS patients.

2. Materials and Methods

2.1. Patient and Specimens. This study was approved by the Ethical Committee of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). All presurgery information of the GS patient was collected from previous medical histories. Age-matched donors with no family history of autoimmune disease or tumors were considered as HCs.

2.2. Flow Cytometry Analysis. Peripheral blood samples were collected in morning fasting status. The fluorochrome-conjugated monoclonal antibodies used in this study were as follows: FITC-conjugated anti-CD4, FITC-conjugated anti-CD8, APC-conjugated anti-CD25, APC-conjugated anti-CD28, BV421-conjugated anti-Foxp3, PE-conjugated anti-CD39, BV421-conjugated anti-TCR$\gamma\delta$, PE-conjugated anti-Vδ2, APC-conjugated anti-IL-17A, PE-Cy7-conjugated anti-IFN-γ (Biolegend, San Jose, CA, USA), PE-conjugated anti-PD-1 and PE-conjugated anti-PD-L1(BD Bioscience, San Jose, CA, USA), FITC-conjugated anti-Vδ1 (Abcam, USA), and relevant isotype controls. For extracellular staining, appropriate mAbs of surfacemarkers were added to samples in the dark at room temperature for 20 minutes. For intracellular staining, peripheral blood mononuclear cells (PBMCs) were stimulated with PMA (500 ng/mL), ionomycin (1 μg/mL), and BFA (2 μg/mL) at 37°C for 5 hours. For intranuclear staining, PBMCs were incubated with fixation/permeabilization buffer for 30 minutes at 4°C. Anti-Foxp3 antibody was added to the cell pellet for 30 minutes in the dark at 4°C. Labeled cells were then measured by a flow cytometer (BD Arial II; BD Bioscience, San Jose, CA, USA), and the data were analyzed via FlowJo 6.0 software (Tree Star).

2.3. Proliferation Assay. We performed proliferation assay in round-bottomed 96-well plates. PBMCs (1×10^6) isolated from the GS patient and healthy donors were labeled with CFSE (0.5 μM; Invitrogen) and incubated with anti-CD3 (2 μg/mL) and anti-CD28 (2 μg/mL) (eBioscience, San Diego, CA, USA) at 37°C incubator for 5 days. Cells were harvested and were subjected to flow cytometry (BD FACS Calibur). Data were analyzed by FlowJo 6.0 software (Tree Star).

2.4. Statistical Analysis. SPSS 17.0 software (IBM Corp., Armonk, NY, USA) was used to analyze all data. Data of HCs are shown as mean ± SEM.

3. Results

3.1. Clinical Characteristics of This GS Patient. A 62-year-old man attended a community hospital on April 16, 2012, due to a series of respiration symptoms such as cough and asthma with sputum. Chest X-rays showed a 2.8 cm anterior mediastinal mass and signs of pneumonia (Figures 1(a) and 1(b)). Afterwards, TBNA (transbronchial needle aspiration) failed to collect target tissues after two attempts. So he went to the First Affiliated Hospital of Nanjing Medical University for clear-cutting diagnosis on May 10, 2012. PET/CT scan was subsequently taken, and the SUV value of the lesion was as depicted by 2.4 (Figure 1(c)), which almost excluded the possibility of tumor. Considering his dissipating symptoms of pneumonia, the patient adopted the outpatient follow-up treatment for 7 months until November 27, 2012. In the meantime, the lesions showed no significant regression in computed tomography (CT) imaging during routine follow-ups. On December 4, 2012, the patient was admitted to the Department of Thoracic Surgery for lump resection surgery. The lesion was later pathologically diagnosed with thymoma, B2 type. On February 25, 2013, the patient was readmitted for recurrent respiratory infection. A series of detailed clinical analyses was further carried out, and the results were as follows: fasting blood glucose 7 mmol/l (3.9–6.1 mmol/L), HA1c 7.2% (4.0%–6.4%), positive autoantibodies of glutamic acid decarboxylase (GAD) and insulin autoantibody (IAA), globulin 19.7 g/L (20.0–40.0 g/L), and serum IgG 3.2 g/L (7.0–16.0 g/L), IgM 0.17 g/L (0.4–2.3 g/L), and IgA 0.37 g/L (0.7–4.0 g/L). Complement component 3 (C3) and complement component 4 (C4) results were within reference ranges. The results of routine blood test and blood biochemistry analysis were normal. Serum virus copy

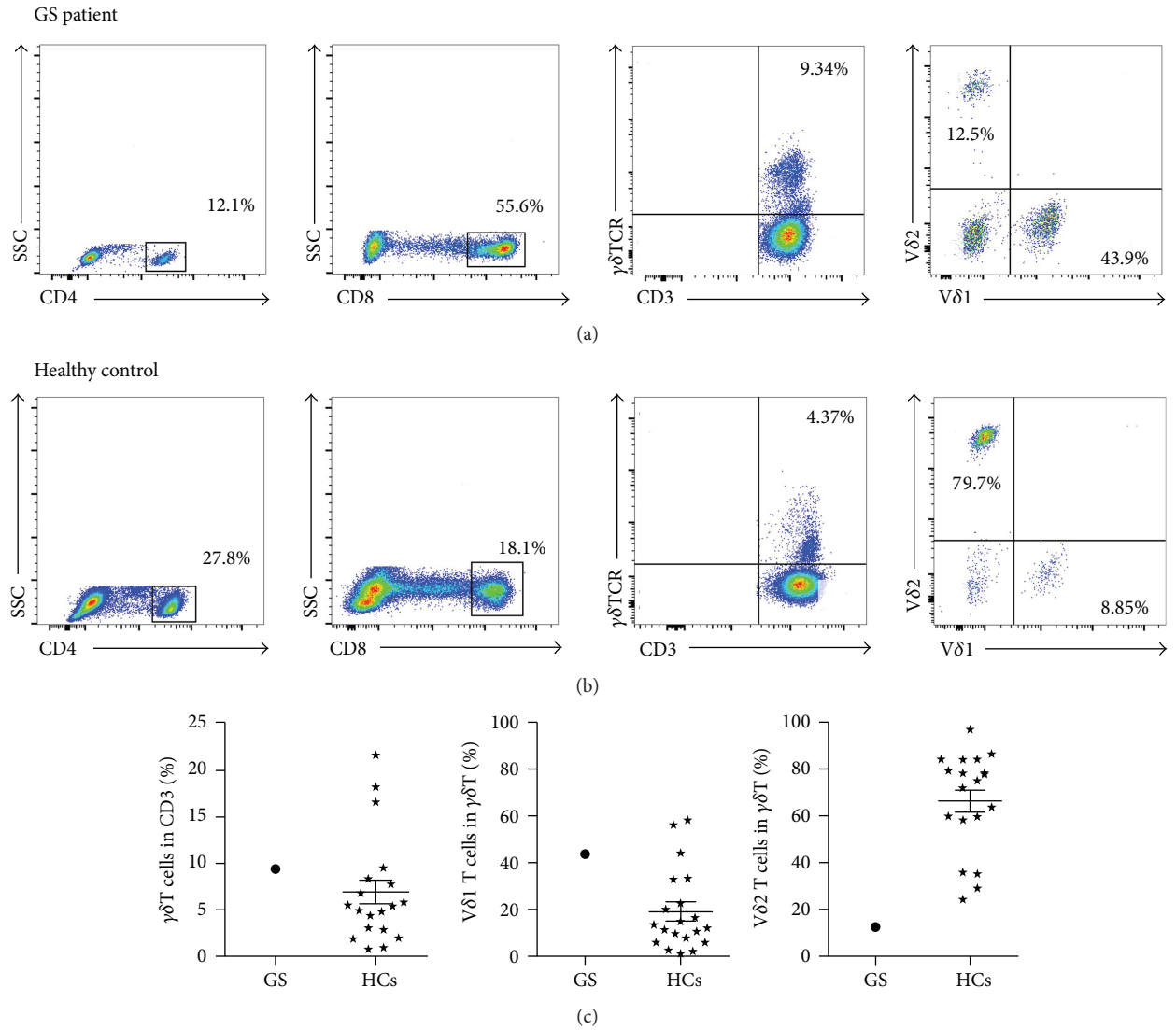


Figure 2: Distribution of peripheral blood immune cells in this GS patient and HCs. Representative of dot pots of $CD4^+$ T cell, $CD8^+$ T cell, $\gamma\delta$T cell, and its subgroups (Vδ1, Vδ2) in our GS patient (a) and HCs (b). Detailed statistical graphs of $\gamma\delta$T cells and its subgroups in this GS patient and HCs (c; $N = 20$). $\star$: HCs group.

number detection including CMV, EBV, HIV, and HBV was negative. But his peripheral lymphocyte subsets were abnormal with low $CD4^+$ T cells (16.2%; 30%–40%) and extremely low B cells (0.1%; 9%–14%). He also had slightly decreased muscle tension which improved after surgery. Moreover, this GS patient had mild diarrhea after thymoma resection, which improved without additional treatment. The combination of disrupted peripheral lymphocytes and immunoglobulin and a history of high blood sugar, as well as the diagnosis of thymoma, lead to the patient final diagnosis of GS. Immunoglobulin replacement was immediately and regularly applied to enhance postsurgery immunity supportive therapy until now. Between February 2012 and March 2017, the patient was readmitted several times to our hospital for recurrent respiratory infections due to high susceptibility to gram-negative bacilli or fungi of *Candida albicans* (July 7, 2013; August 19, 2013; May 14, 2015; and March 25, 2016). This GS patient often suffered from cough with sputum, fever of 39°C, and chest pain as well as signs of pneumonia identified via chest CT images. Doctors usually utilized combination therapy including antibiotics (levofloxacin tablets/imipenem/cefodizime) and antifungal (fluconazole) drug to prevent *Candida albicans*, as well as ambroxol for cough with sputum, which eliminated respiratory inflammation and prevented double infection (bacterial-fungal infections). Moreover, the routine monitoring of immunoglobulin, blood sugar, peripheral lymphocyte subsets, and immunoglobulin infusion is still ongoing every 3 months in our GS patient.

3.2. Overall Peripheral Immune Cell Distribution in a Postthymectomy GS Patient. A peripheral blood sample was collected on November 22, 2016 during a routine outpatient examination of a GS patient 4 years after thymectomy. At the time of sample collection, the patient did not show any obvious signs of respiratory infection, but he had a history of

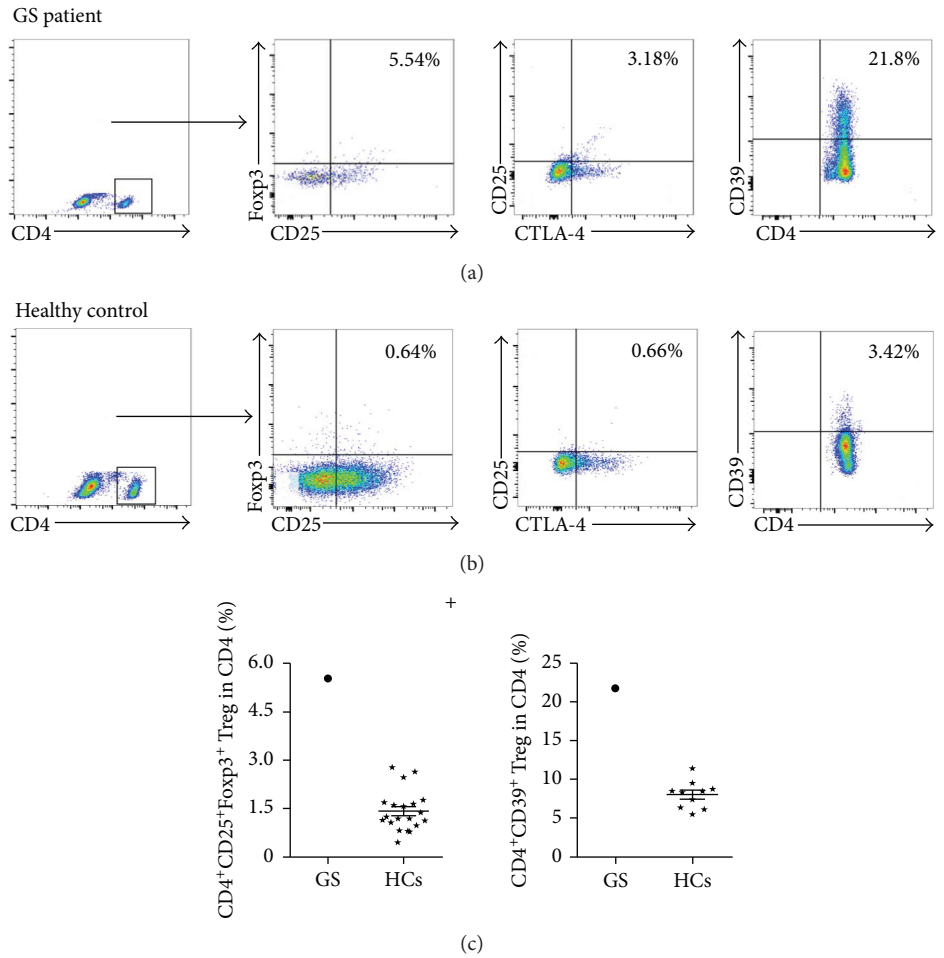


Figure 3: Elevated levels of peripheral $CD4^+$ Tregs in this GS patient and HCs. Representing plots of three main markers of $CD4^+$ Treg cell subgroups in our GS patient (a) and HCs (b), respectively. The percentages of $CD4^+CD25^+Foxp3^+$ Tregs ($N = 20$) and $CD4^+CD39^+$ Treg ($N = 10$) in this GS patient and HCs (c). $\star$: HCs group.

recurrent respiratory infections. The proportions of his peripheral immune cells were evaluated by flow cytometry. As shown in Figure 2, this GS patient had an elevated proportion of $CD8^+$ T cells (55.6%) and a reduced proportion of $CD4^+$ T cells (12.1%) compared with the corresponding levels in healthy control subjects (HCs); furthermore, B cells (1%) were nearly absent in this patient, and he also had an inverted ratio of $CD4^+/CD8^+$ cells. $\gamma\delta$T cells play a crucial role in preventing bacterial and fungal infection and act as the first protective barrier of innate immunity. These cells are split into two subgroups based on the classification of the δ ligand: Vδ1 T cells, which are almost located in the epithelial tissue, and Vδ2 T cells, which account for 70% of the total $\gamma\delta$T cells in the peripheral blood. Although the relative proportion of $\gamma\delta$T cells in our GS patient was similar to that in HCs, the percentage of Vδ2 T cells was particularly low (12.5% versus 66.45 $\pm$ 20.79%) in our GS patient. Accordingly, the percentage of Vδ1 T cells in this patient was higher than that in the HCs (43.9% versus 19.12 $\pm$ 17.20%) (Figure 2(c)). This different distribution of $\gamma\delta$T cell subsets may be related to the patient's recurrent respiratory infections. It is possible that the altered distribution of peripheral immune cells partly caused an inefficient peripheral immune response.

3.3. Evaluation of Treg Cells in Our GS Patient. Tregs modulate immune homeostasis that induced immune suppression effect in the peripheral blood. We further analyzed the proportions of five subtypes of Tregs with GS patient via flow cytometry. As shown in Figure 3, the percentages of $CD4^+CD25^+Foxp3^+$ Treg cells (5.54% versus 1.46 $\pm$ 0.71%) and $CD4^+CD25^+CTLA\text{-}4^+$ Treg cells (3.18% versus 0.66%) were higher in our GS patient compared with HCs. Surprisingly, the percentage of $CD4^+CD39^+$ Treg cells was 21.8% in our GS patient, which was much higher than that in the HCs (8.034 $\pm$ 1.868%). Recent studies indicated that $CD8^+$ Treg cells are increased and associated with tumor stages in human ovarian cancer [6]. As shown in Figure 4, the proportions of $CD8^+CD28^-$ Treg cells and $CD8^+Foxp3^+$ Treg cells in our GS patient were 88.8% (HCs 38.66 $\pm$ 6.93%) and 2.3% (HCs 0.71 $\pm$ 0.29%), respectively. Additionally, the CD28 expression in our GS patient's immune cells was significantly lower than that in HCs. Thus, the excessive populations of various Treg cells in our GS

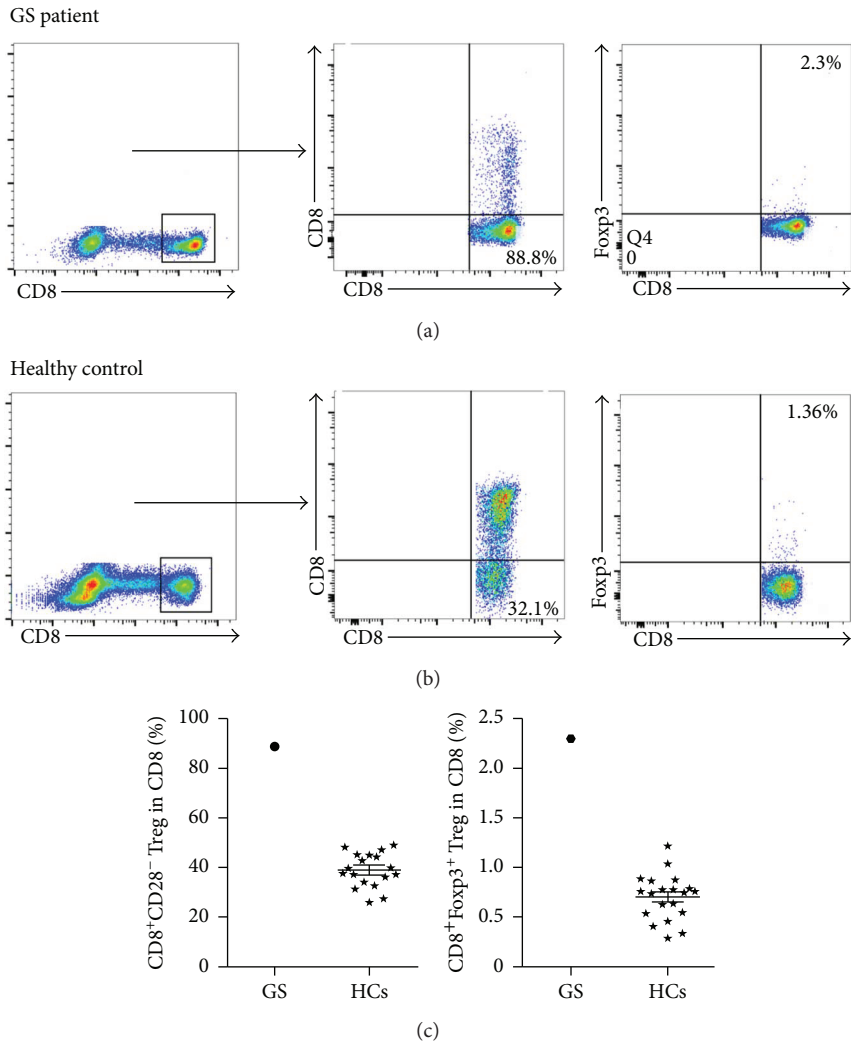


Figure 4: Elevated $CD8^+$ Treg infiltration in the peripheral blood of GS patient and HCs. Representative dot plots of peripheral $CD8^+$ Treg cells in this GS patient (a) and HCs (b), respectively. Statistical graphs of GS patient and HCs with $CD8^+CD28^-$ Tregs and $CD8^+Foxp3^+$ Tregs (c; $N = 20$, resp.). $\star$: HCs group.

patient's peripheral blood highlight the severe immune suppression status of this GS patient.

3.4. Aberrant Proliferation Capability of $CD4^+$ T Cells in Our GS Patient. Cell proliferation capacity is an essential index for assessing the presence of an effective immune response. We investigated the ex vivo proliferation of circuiting $CD4^+$ T cells from our GS patient via flow cytometry. The result shows that $CD4^+$ T cells from our GS patient underwent less proliferation than those from HCs (34.0% versus 80.8%) following stimulation by CD3/CD28; additionally, the cells from the GS patients had lower proliferation rates and fewer divisions (Figure 5). It has been widely reported that immune checkpoints, including programmed death receptor-1 (PD-1) and programmed death ligand-1 (PD-L1), participate in the progression of immune suppression, which also partly correlates with T cell activation and proliferation as well as cytokine secretion. In this study, our GS patient's peripheral $CD4^+$ T cells expressed markedly higher levels of PD-1

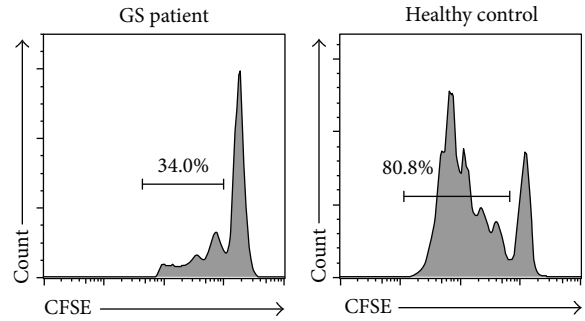


Figure 5: Cell proliferation capacity of peripheral $CD4^+$ T cells in this GS patient. Representative dot pots of proliferation assay in this GS patient and HCs.

(34.2% versus $4.2 \pm 1.8\%$) but lower levels of PD-L1 (6.61% versus $10.1 \pm 3.9\%$) than those reported for GS patients (Figure 6); this difference was similar to that reported by previous studies [7]. These results suggest that both poor

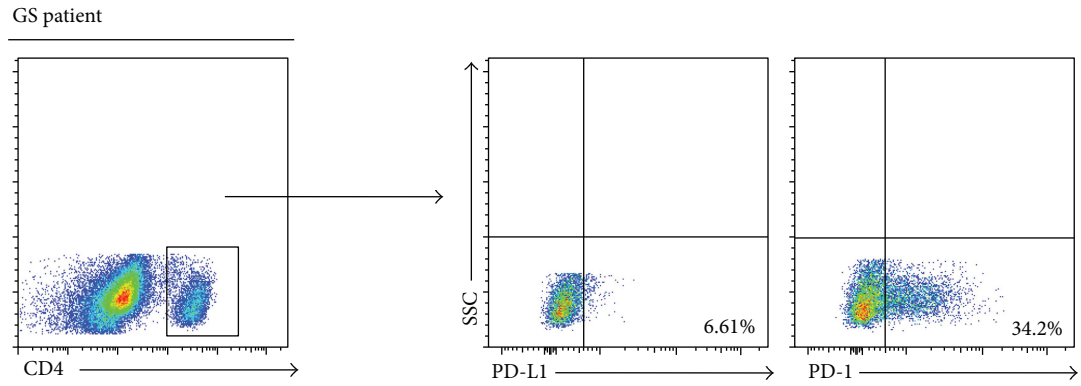


Figure 6: Expression of PD-1 and PD-L1 in this GS patient. Representative dot pots of PD-1 and PD-L1 in this GS patient.

proliferation and increased PD-1 expression of circulating $CD4^+$ T cells, along with a higher level of CTLA-4 expression, may be at least partly associated with the delayed immune reactions to infection in our GS patient.

3.5. Cellular Levels of IFN-γ and IL-17A in Our GS Patient. We detected the cellular IFN-γ levels produced by ex vivo $CD4^+$ T cells, $CD8^+$ T cells, and $\gamma\delta$T cells from our GS patient, and cells from 10 healthy volunteers were included as controls (Figure 7). The amount of IFN-γ produced by $CD4^+$ T cells was similar between our GS patient and the HCs (32.5% versus $31.08 \pm 12.50\%$). In contrast, the amount of IFN-γ secreted by $CD8^+$ T cells was higher in our GS patient than in the HCs (71.1% versus $44.15 \pm 12.49\%$), and the opposite trend was observed in $\gamma\delta$T cells (15.1% versus $33.98 \pm 12.26\%$). Additionally, we also found that the circuiting $CD4^+$ T cells from our GS patient secreted slightly more IL-17A compared with those from the HCs (4.3% versus $2.62 \pm 1.38\%$) (Figure 8). However, the levels of cellular IL-17A produced by $CD8^+$ T cells and $\gamma\delta$T cells were not significantly different between our GS patient and the HCs (1.20% versus $1.37 \pm 0.91\%$; 2.70% versus $1.42 \pm 0.41\%$, resp.). These results illustrate the abnormality of cellular cytokine levels in our GS patient, which may be linked to the recurrent respiratory infections suffered by this patient.

4. Discussion

The thymus is a vital organ responsible for the differentiation and maturation of the immune system in early childhood and adolescence. GS is a rare type of thymoma and has high susceptibility to encapsulated bacteria in the respiratory system, as well as opportunistic viral and fungal invasions, due to serious combined T and B cell immunodeficiency [8, 9]. GS patients often display $CD4^+$ T cell lymphopenia and an inverse ratio of $CD4^+/CD8^+$ [10]. The present case also showed this abnormality of circuiting lymphocytes, with lower than normal percentages of $CD4^+$ T cells and a reversed ratio of $CD4^+/CD8^+$ cells. Furthermore, we found that the patient's peripheral $CD4^+$ T cells had a lower proliferation capacity and a higher expression level of PD-1. Further experiments are needed to determine if the altered PD-1 levels in our GS patient contribute to the reduced proliferation of the patient's $CD4^+$ T cells. In addition, the higher expression of CTLA-4 in the $CD4^+$ T cells (Figure 3) of our GS patient may function to capture and remove CD80 and CD86; if so, these proteins would be unable to trigger CD28, further impairing T cell activation [11]. The altered peripheral immune milieu in our GS patient may at least partially explain his repeated respiratory infections.

$\gamma\delta$T cells participate vigorously in the first line of innate immunity [12, 13]. However, their functions in GS have not been fully elucidated yet. Here, although the percentage of peripheral $\gamma\delta$T cells in our GS patient was similar to that of HCs, the amount of Vδ2 T cells was extremely low, resulting in a relatively higher proportion of Vδ1 T cells in the peripheral blood. Previous studies have revealed that peripheral Vδ1 T cells exert strong anti-infection and antitumor effects through binding to MHC class I chain-related gene A/B (MICA/B) or UL16-binding proteins (ULBPs), and these cells display a strong cytotoxic ability upon stimulation with PHA (polyhydroxyalkanoates) and IL-7 *in vitro* [14–18]. Furthermore, upregulated levels of peripheral Vδ1 T cells followed by an inverse ratio of Vδ2/Vδ1 T cells were reported to participate in antiviral immunity during HIV infection [19]. However, the increased proportion of Vδ1 T cells in this GS patient did not seem to exert an effective function, given the repeated respiratory infections suffered by the patient. The role of these cells in GS is in need of further exploration.

There are no previous reports on the cellular expression of IL-17A in the peripheral blood of GS patients; however, since this cytokine acts as a culprit in the aggressive progressions of various chronic inflammatory diseases [20], we investigated its levels in our GS patient. We separately measured the IL-17A produced by $CD4^+$ T cells, $CD8^+$ T cells, and $\gamma\delta$T cells but did not detect any obvious differences between the cells from our GS patient and those from HCs. However, tissue-derived IL-17A, which can recruit myeloid-derived suppressor cells (MDSCs), was reported to energetically participate in the immunosuppression milieu in human colorectal cancer [21]. Therefore, future work should investigate the significance of IL-17A that has infiltrated into the lesions of our GS patient.

Peripheral Vδ2 T cells can secrete IFN-γ [15], but the level of IFN-γ secreted by $\gamma\delta$T cells from our GS patient was significantly lower compared with HCs, and this

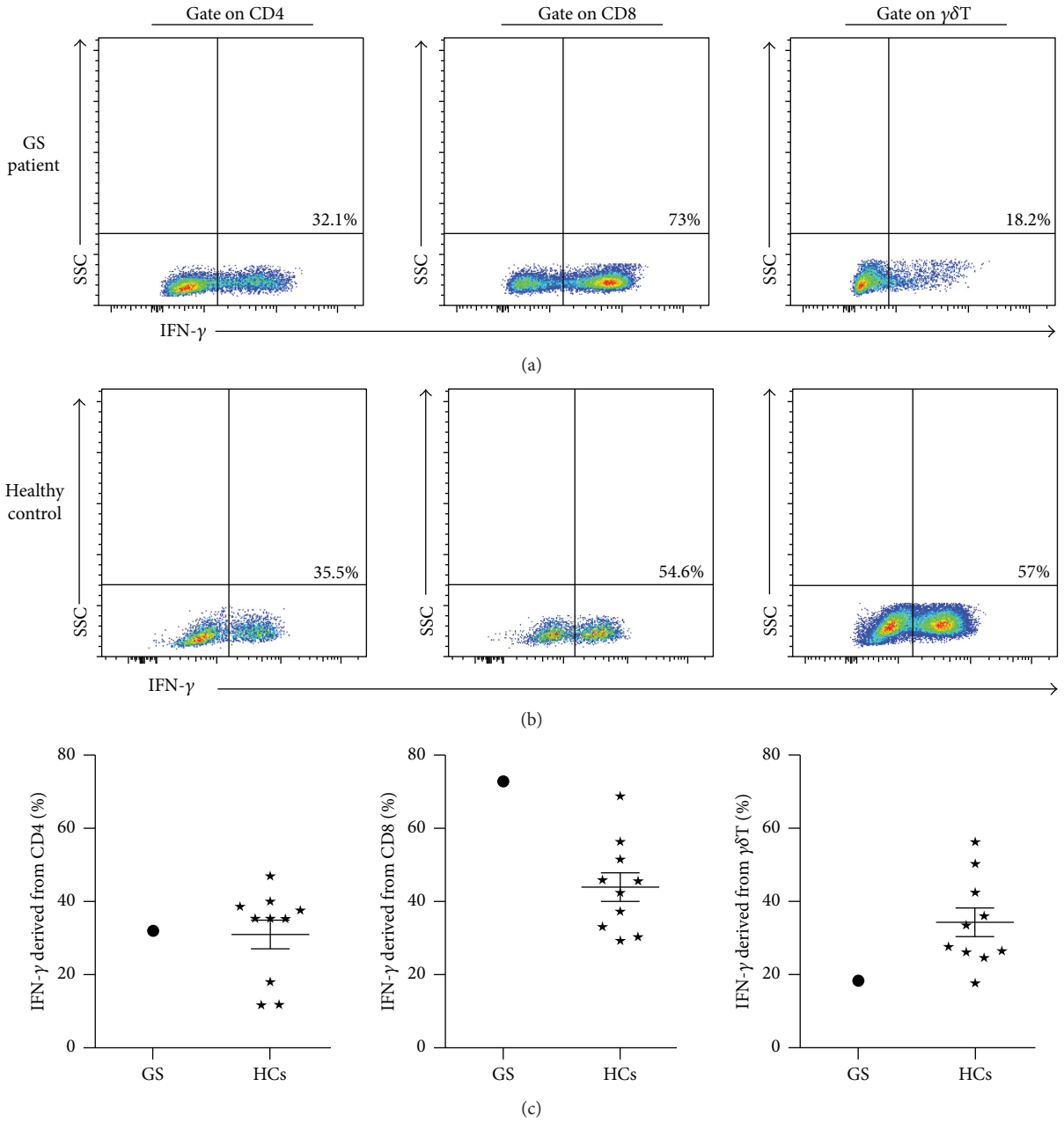


Figure 7: Cellular IFN-γ levels originated from the peripheral immune cells in this GS patient and HCs. Representative dot pots of IFN-γ derived from $CD4^+$ T cells, $CD8^+$ T cells, and $\gamma\delta$T cells in this GS patient (a) and 10 HCs (b). Detailed statistical graphs of GS patient and HCs with cellular IFN-γ (c; $N = 10$, resp.). $\star$: HCs group.

difference may be involved with the observed mild alternations in the $CD4^+$ T cell-derived IFN-γ levels in this patient (Figure 7). Because IFN-γ production by $CD8^+$ T cells may partially compensate for insufficient cellular IFN-γ [22], we also measured the expression levels of IFN-γ produced by these cells. Data from numerous cytokine profiles suggest that the recurrent respiratory infections suffered by our GS patient could be related to the intracellular expressions of IL-17A and IFN-γ. However, functional evaluations of immune elements, especially the conventional cytotoxic effect of IFN-γ expressed by $CD8^+$ T cells need to be performed in future work. Furthermore, the complete cytokine profiles of GS patients should be measured as part of a comprehensive assessment of GS patients.

Treg cells play an essential role in preserving self-immune tolerance through the effective suppression of self-recognizing immune cells. Multifunctional circulating Treg cells with lower Foxp3 expression have been reported in immunodeficient patients [23–28]. The levels of peripheral $CD4^+CD25^+Foxp3^+$, $CD4^+CD25^+CTLA\text{-}4^+$, $CD4^+CD39^+$, $CD8^+CD28^-$, and $CD8^+Foxp3^+$ Treg cells were all relatively higher in our GS patient, which indicate that the patient's peripheral blood had an immunosuppressed milieu. One study reported that CD28-deficient mice exhibit weakened

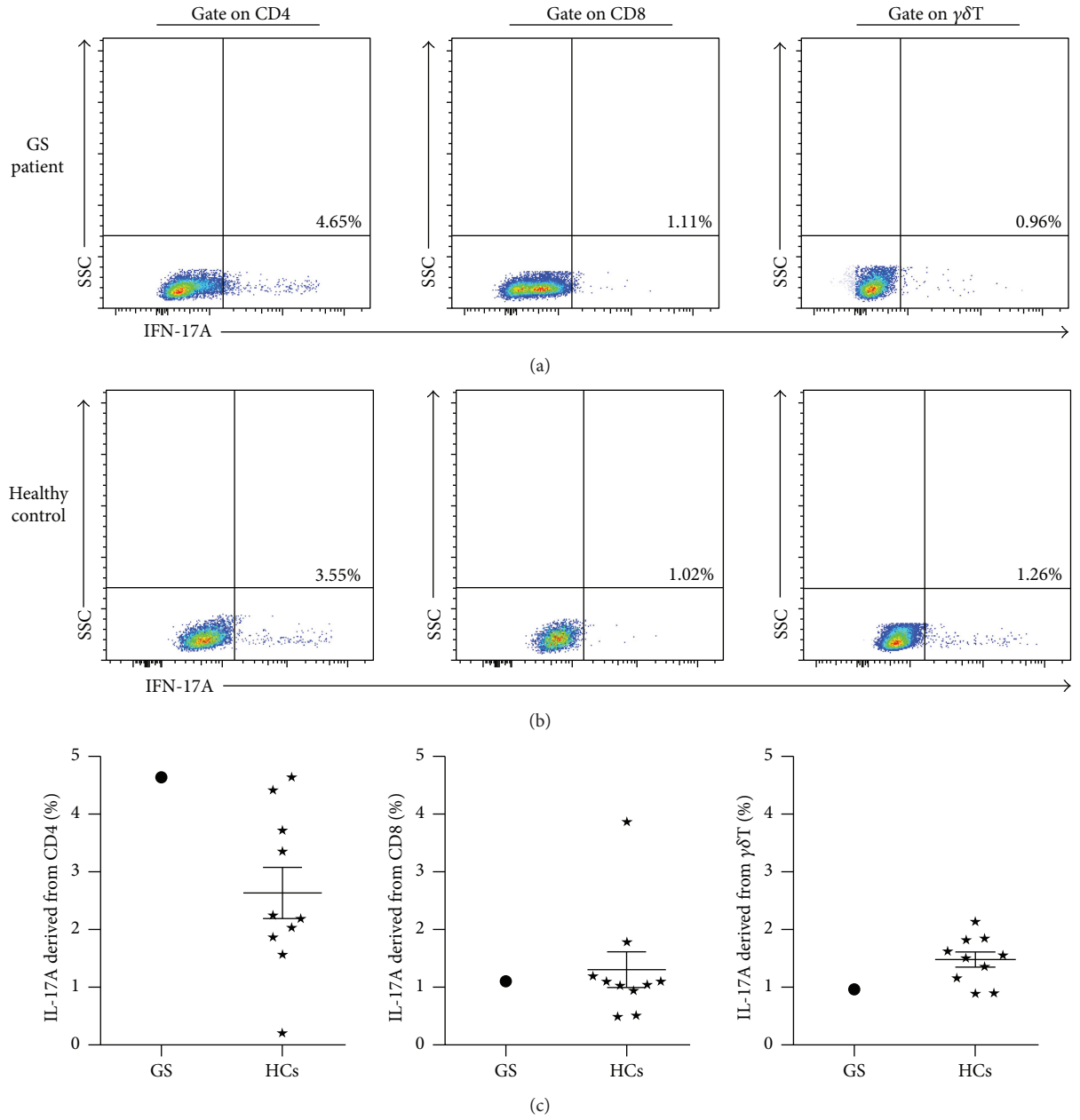


Figure 8: Cellular IL-17A levels derived from circuiting immune cells in this GS patient and HCs. Representative dot pots of IL-17A derived from $CD4^+$ T cell, $CD8^+$ T cell, and $\gamma\delta$T cell in this GS patient (a) and HCs (b). Statistical graphs of intracellular IL-17A in GS patient and HCs (c; $N = 10$, resp.). $\star$: HCs group.

T cell proliferation potential after TCR stimulation and a passive B cell response, despite retaining normal cytotoxic effects [29]. Therefore, it is possible that the lower expression of CD28 in our GS patient may have been partially responsible for the observed defects in his immune responses, particularly after the patient's thymus removal, which can eliminate the immature lymphocytes.

Notably, our GS patient had a higher level of $CD4^+CD39^+$ Treg cell infiltration (Figure 3). CD39 is an enzyme that hydrolyzes adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) with the assistance of CD73. However, the accumulation of adenosine is considered another metabolism-related mechanism of the immunosuppressive effect caused by alterations in Treg cell populations [30]. Given that our GS patient repeatedly suffered from respiratory infections, we plan to conduct a follow-up study detecting the levels of circulating and lesion-based adenosine and evaluating the potential correlation between adenosine levels and Treg cells in GS progression.

Some GS patients lack peripheral B cells, have stalled pre-B cells, and/or exhibit weakened maturation of erythroid and myeloid precursors due to bone marrow defects [31]. Interestingly, this abnormal lymphocyte distribution is not constrained to only within the bone marrow but is also observed in the lymph nodes, lymphoid tissue, and spleen

[9]. Unfortunately, it is impossible to exclude the effects of the surgery itself and the postsurgery immune replacement therapy on the results of our study because there is inadequate preoperative and postoperative information available to evaluate their effect on our laboratory data. Thus, we will continue to evaluate the peripheral immune status of this GS patient during follow-up visits over the coming year, and we will collect a broader set of data on any additional GS patients we encounter.

5. Conclusions

In summary, we determined the peripheral immune cell distribution and corresponding intracellular cytokine secretion levels of a GS patient. Although there are still no effective protocols for treating GS, except for immunoglobulin replacement, our work expanded knowledge of the post-thymectomy immunosuppressed status in this condition, which might provide potential targets for successive supportive treatment.

Conflicts of Interest

The authors declare no financial or commercial conflict of interest.

Authors' Contributions

Xian Chen and Jie-xin Zhang contributed equally to this work. Xian Chen and Wen-wen Shang performed the experiment. Xian Chen and Jie-xin Zhang wrote the paper and analyzed the data. Shu-xian Jin and Wei-ping Xie supported the clinical features of this GS patient and clinical blood samples. Fang Wang and Shu-xian Jin designed and supervised the study.

Acknowledgments

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Supplementary Materials

Figure S1(A): the statistical comparison of IFN-γ^+ IL-17A$^+$ cells in CD4$^+$ T cells with this GS patient and HCs ($N = 10$). (B): representative dot plot of IFN-γ^+ IL-17A$^+$ cells derived from peripheral CD4$^+$. T cells in this GS patient and HCs. *(Supplementary Materials)*

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