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Roles of Th17 cells in pulmonary granulomas induced by *Schistosoma japonicum* in C57BL/6 mice

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ABSTRACT

In schistosomiasis, limited information is available about the role of interleukin-17 (IL-17) in lung, despite the fact that this cytokine plays a crucial role during pro-inflammatory immune responses. In our study, we observed $CD4^+$T cells changed after the infection. Furthermore, ELISA and FACS results revealed that *Schistosoma japonicum* infection could induce a large amount of IL-17 in mouse pulmonary lymphocytes. IL-17-producing cells, including Th17 cells, $CD8^+$T (Tc) cells, γδT cells and natural killer T cells, was also associated with the development of lung inflammatory diseases. FACS results indicated that Th17 cell was the main source of IL-17 in the infected pulmonary lymphocytes after phorbol-12-myristate-13-acetate (PMA) and Ionomycin stimulation. Moreover, FACS results revealed that the percentage of Th17 cells continued to increase as over the course of *S. japonicum* infection. Additionally, cytokines co-expression results demonstrated that Th17 cells could express more IL-4 and IL-5 than IFN-γ. Reducing IL-17 activity by using anti-IL-17 ameliorated the damage and decreased infiltration of inflammatory cells in infected C57BL/6 mouse lungs. Collectively, these results suggest Th17 cells is the major IL-17-producing cells population and IL-17 contributes to pulmonary granulomatous inflammatory during the *S. japonicum* infection.

1. Introduction

Schistosomiasis is a worldwide, chronic, parasitic disease caused by blood flukes and causes significant morbidity and mortality especially in developing countries [1]. The main human species are *Schistosoma mansoni*, *S. japonicum* and *S. haematobium* [2] and the *S. japonicum* is endemic in Asian developing countries, including China [3]. Schistosomula and its eggs migrate through a variety of tissue including skin, lung, liver, intestinal and vesical mucosa [4], which may induce pathological changes and immune response. Moreover, granulomatous inflammation against parasite eggs is the pathological hallmarks of schistosome infection [5]. Immune cells, such as T cells, B cells, macrophages and DCs, are involved in the process of granulomatous inflammation, with eosinophils the most frequent cells [6].

Infection of *S. japonicum*, a multi-cellular parasite which has an extremely diverse repertoire of antigens, induces the production of bulk cytokines that play important roles in the immune response to infection [7]. Interleukin-17 (IL-17) is a major proinflammatory mediator that works through several mechanisms, including the production of chemokines, cytokines, and growth factors [8]. It has been reported to participate in host defense against various types of pathogens [9,10] and thought to be an important cytokine in the immune response against *S. japonicum* infection [7,11].

In addition to Th17 cells, $CD8^+$T (Tc) cells [12], γδT cells and natural killer (NK) T cells have also been shown to produce IL-17 in the lung [13]. Multiple studies have demonstrated that IL-17 is largely expressed by Th17 cells [8,14], whereas some reports showed that

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IL-17 production was dominated by γδT cells rather than $CD4^+$T cells during some infections, such as *Mycobacterium tuberculosis* [15] and *Escherichia coli* [16] infection. However, there is limited information to know which population is the main source of producing IL-17 cells during the *S. japonicum* infected lung. Therefore, the aim of current study was to observe lymphocyte subpopulations that produce IL-17 in the pathogenic processes of the *S. japonicum* infected lung and the characteristics of main IL-17-producing cells.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice, 6–8 weeks old, were purchased from Zhongshan University Animal Center (Guangzhou, China) and maintained in an animal care facility under pathogen-free conditions. Animal experiments were performed in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (1988.11.1). All protocols for animal use were approved to be appropriate and humane by institutional animal care and use committee of Guangzhou Medical University (2011–44).

2.2. Parasite infection

S. japonicum cercariae were shed from naturally infected *Oncomelania hupensis* snails, which were purchased from Jiangsu Institute of Parasitic Disease (Wuxi, China). Twenty mice were infected percutaneously with 40 ± 5 cercariae. At 4, 5, 6 and 7 weeks post-infection, five mice were randomly chosen and sacrificed. Five pathogen-free mice constituted the control group.

2.3. Antibodies

APC-cy7-conjugated anti-mouse CD3 (145-2C11), FITC-conjugated anti-mouse CD3 (17A2), FITC conjugated anti-mouse CD8 (53–6.7), PerCP-cy5.5-conjugated anti-mouse CD4 (RM4-5), FITC-conjugated anti-mouse γδTCR (17A2), PE-cy7-conjugated anti-mouse NK1.1 (PK136), PE-conjugated anti-mouse IL-17 (TC11-18H10), PE-conjugated anti-mouse IL-4 (11B11), APC-conjugated anti-mouse IL-5 (TRFK5), APC-conjugated anti-mouse IL-9 (D9302C12), APC-conjugated anti-mouse IL-10 (JES5-16E3), APC-conjugated anti-mouse IFN-γ (XMG1.2) and isotype-matched control monoclonal antibodies (X39, G155-178) were purchased from BD/Pharmingen (San Diego, CA). The neutralizing rat anti-mouse IL-17A mAb (TC11-18H10.1) and an isotype-matched rat IgG2a mAb (RTK2758) were purchased from BioLegend.

2.4. Lymphocytes isolation

Mice were narcotized and fixed on weeks 0, 4, 5, 6, 7 after infection. The excised lung was cut to small pieces and incubated in 5 ml of digestion buffer (collagenase IV/DNase I mix, Invitrogen Corporation) for 30 min at 37 °C. The digested lung tissue was pressed through 200-gauge stainless-steel mesh, and then was suspended in Hank's balanced salt solution (HBSS). Lymphocytes were isolated by Ficoll-Hypaque (DAKEWE) density gradient centrifugation. Isolated cells were washed twice in HBSS and resuspended at 2×10^6 cells/ml in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 50 μM 2-mercaptoethanol.

2.5. ELISA detection of cytokines

Microtiter plates were coated with anti-CD3 (1) and anti-CD28 (1 μg/ml) and incubated overnight at 4 °C. Single-cell suspensions were cultured in 96-well microtiter plates at 4×10^5 cells/200 μl medium per well and supernatants were collected 72 h later. Levels of the released cytokines in supernatants were determined by using mouse cytokine multiplex assay kits for IFN-γ (BD Pharmingen), IL-4 (BD Biosciences) and IL-17 (R&D Systems). ELISAs were performed in accordance with the manufacturer's instructions. The optical density of each well was read at 450 nm by using a microplate reader (Model ELX-800, BioTek).

2.6. Detection of cell surface markers and intracellular cytokine expression

Single cell suspensions from the lungs of control mice and mice infected with *S. japonicum* were incubated with 20 ng/ml phorbol-12-myristate-13-acetate (PMA) plus 1 μg/ml ionomycin at 37 °C under a 5% CO_2 atmosphere. One hour later, cells were treated with brefeldin A (10 mg/ml, Sigma) and incubated for an additional 4 h. Cells were stained with conjugated antibodies specific for the cell surface antigens CD3, CD4, CD8, NK1.1, and γδTCR, respectively. After washing in PBS, cells were fixed with 4% paraformaldehyde, and permeabilized overnight at 4 °C in PBS buffer containing 0.1% saponin (Sigma), 0.1% BSA, and 0.05% NaN_3, then stained with conjugated antibodies specific for the cytokines, including IFN-γ, IL-4, IL-5, IL-9, IL-10 and IL-17. Antibody-labeled lymphocytes (200,000–300,000 cells per run) were acquired on flow cytometry (BD Calibur and Aria II) and data were analyzed by using CellQuest software (BD Biosciences). Isotype-matched controls for cytokines were included in each staining protocol.

2.7. Neutralizing anti-mouse IL-17A mAb administration and S. japonicum infection

10 mice were randomly assigned in two groups (five mice per group). Each mouse was challenged with 40 cercariae of *S. japonicum* as described above. Neutralizing rat anti-mouse IL-17A mAb or an isotype-matched rat IgG2a mAb was first administered intraperitoneally 3 weeks after *S. japonicum* infection (62.5 μg per mouse) at the same dose every third day until 2 days before sacrifice.

2.8. Histology studies

Lungs were removed from the mice, perfused three times with 0.01 M phosphate-buffered saline (pH = 7.4), fixed in 10% formalin, embedded in paraffin, and sectioned. Paraffin tissue sections (5 μm) of mice in different groups were stained with hematoxylin and eosin. Briefly, lung tissue sections were immersed in xylene to remove paraffin, then in consecutive ethanol concentrations from 100% to 80% (v/v). The tissue sections were immersed in hematoxylin and eosin. Subsequently, sections were dehydrated in ethanol and immersed in xylene. The sections were then examined by light microscopy under 100× and 400× magnification after standard hematoxylin-eosin (H&E) staining for visualization of cellular changes.

2.9. Statistics

Statistical evaluation of differences between means was performed by unpaired two-tailed *t* tests. $P < 0.05$ was considered significant.