Dynamics of Th17 Cells and Their Role in *Schistosoma japonicum* Infection in C57BL/6 Mice

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Abstract

**Background:** The current knowledge of immunological responses to schistosomiasis, a major tropical helminthic disease, is insufficient, and a better understanding of these responses would support vaccine development or therapies to control granuloma-associated immunopathology. CD4⁺ T cells play critical roles in both host immune responses against parasitic infection and immunopathology in schistosomiasis. The induction of T helper (Th)1, Th2 and T regulatory (Treg) cells and their roles in schistosome infections are well-illustrated. However, little in vivo data are available on the dynamics of Th17 cells, another important CD4⁺ T cell subset, after *Schistosoma japonicum* infection or whether these cells and their defining IL-17 cytokine mediate host protective responses early in infection.

**Methodology:** Levels of Th17 and the other three CD4⁺ T cell subpopulations and the cytokines related to induction or repression of Th17 cell generation in different stages of *S. japonicum* infection were observed. Contrary to reported in vitro studies, our results showed that the Th17 cells were induced along with the Th1, Th2, Treg cells and the IFN-γ and IL-4 cytokines in *S. japonicum* infected mice. The results also suggested that *S. japonicum* egg antigens but not adult worm antigens preferentially induced Th17 cell generation. Furthermore, decreasing IL-17 with a neutralizing anti-IL-17 monoclonal antibody (mAb) increased schistosome-specific antibody levels and partial protection against *S. japonicum* infection in mice.

**Conclusions:** Our study is the first to report the dynamics of Th17 cells during *S. japonicum* infection and indicate that Th17 cell differentiation results from the integrated impact of inducing and suppressive factors promoted by the parasite. Importantly, our findings suggest that lower IL-17 levels may result in favorable host protective responses. This study significantly contributes to the understanding of immunity to schistosomiasis and may aid in developing interventions to protect hosts from infection or restrain immunopathology.

Introduction

CD4⁺ T cells play an important role in the initiation of immune responses against an infection by providing help to other cells and by taking on a variety of effector functions during immune reactions. Upon antigenic stimulation, naïve CD4⁺ T cells activate, expand and differentiate into different effector subsets termed T helper (Th)1 and Th2 cells. The appropriate induction and balance between Th1 and Th2 cellular responses to an infectious agent can influence both pathogen growth and immunopathology [1]. Th17 cells recently emerged as a third independent effector cell subset differentiated from CD4⁺ T cells upon antigenic stimulation [2–5]. Although the functions of these cell subtypes are not completely understood, emerging data suggest that by producing their defining cytokine IL-17, Th17 cells play an important role in host defenses against extracellular pathogens, such as *Klebsiella pneumoniae* [6], *Pseudomonas aeruginosa* [7], *Porphyromonas gingivalis* [8] and *Bacteroides fragilis* [9], which are not efficiently cleared by Th1-type and Th2-type immunity. Meanwhile, several studies have shown that Th17 cells and IL-17 also play important roles in immunopathology in some infectious diseases, such as pulmonary tuberculosis [10], toxoplasmosis [11] and schistosomiasis [12–17].

CD4⁺ T cells can also be induced to differentiate into CD4⁺CD25⁺ T regulatory (Treg) cells with immunosuppressive activities that down-regulate immune responses, thereby inhibiting immunopathology while promoting parasite survival via direct repression of the induction and responses of the other CD4⁺ subsets, Th1, Th2 and Th17 cells [18–22].

Since the functional analysis of IL-17 produced by Th17 cells has suggested an important and unique role for this cytokine in...
Treg cells during multicellular pathogenic infection. Recent studies linked IL-17 with the severity of liver inflammation and suggested that Treg cells contribute to the pathology in schistosomiasis, a serious disease caused by parasitic worms such as Schistosoma japonicum widespread in vertebrates including humans. However, the role of Treg cells in protection against S. japonicum infection is still unclear. For the first time, we describe here the changes in Th17 cell levels during S. japonicum infection and suggest that the schistosome egg antigens are primarily responsible for stimulating the generation of host Th17 cells after S. japonicum infection. We further show that the level of Th17 cells in the host is determined by a combination of factors, namely exposure to complex parasitic antigens that either induce or suppress their generation. We also suggest that lowering IL-17 levels may favor the host’s protective responses against S. japonicum infection. Our findings help to better understand the relationship between the host and parasite in terms of immune protection and pathology in schistosomiasis and may contribute to the future development of vaccination and therapeutic strategies.

Author Summary
Th17 immune cells secrete the IL-17 cytokine and contribute to host defenses against certain infections. Recent studies linked IL-17 with the severity of liver inflammation and suggested that Treg cells contribute to the pathology in schistosomiasis, a serious disease caused by parasitic worms such as Schistosoma japonicum widespread in vertebrates including humans. However, the role of Treg cells in protection against S. japonicum infection is still unclear. For the first time, we describe here the changes in Th17 cell levels during S. japonicum infection and suggest that the schistosome egg antigens are primarily responsible for stimulating the generation of host Th17 cells after S. japonicum infection. We further show that the level of Th17 cells in the host is determined by a combination of factors, namely exposure to complex parasitic antigens that either induce or suppress their generation. We also suggest that lowering IL-17 levels may favor the host’s protective responses against S. japonicum infection. Our findings help to better understand the relationship between the host and parasite in terms of immune protection and pathology in schistosomiasis and may contribute to the future development of vaccination and therapeutic strategies.

Materials and Methods

Ethics statement
Animal experiments were performed in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (1988.11.1), and all efforts were made to minimize suffering. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University for the use of laboratory animals (Permit Number: NJMU 07-0137).

Mice, parasites and infection
Female 8-week old C57BL/6 mice were purchased from SLAC Laboratory (Shanghai, China) and bred in university facilities. All animal experiments were performed in accordance with the Chinese laws for animal protection and in adherence to experimental guidelines and procedures approved by the Institutional Animal Care and Use Committee (IACUC), the ethical review committee of Nanjing Medical University, for the use of laboratory animals. Oncomelania hupensis harboring S. japonicum cercariae (Chinese mainland strain) were purchased from the Jiangxi Institute of Parasitic Diseases (Nanchang, China).

For kinetic analysis of T cell populations and cytokines, each mouse was infected with 12 cercariae of S. japonicum through the abdominal skin. At 3, 5, 8 and 13 weeks post-infection, four mice were randomly chosen from the infected and normal control groups and sacrificed for further study. For challenge experiments, each mouse was infected with 40 cercariae of S. japonicum by abdominal skin exposure.

Preparation of antigens and immunization schedule
S. japonicum SWA were prepared by harvesting the soluble fraction obtained from sonicated S. japonicum adult worms as previously described [42]. S. japonicum eggs were extracted from the livers of infected rabbits and enriched. The S. japonicum soluble egg antigens (SEA) were then prepared from the homogenized eggs as...
previously described [43]. SWA and SEA were diluted with PBS to a final concentration of 10 mg/ml for immunization.

Three independent experiments were carried out in the same manner. In each experiment, C57BL/6 mice were divided randomly into three groups (two test and one control) consisting of eight mice per group. Each mouse was injected subcutaneously in the back with 100 µl of a solution containing 50 µg of SEA, 50 µg of SWA or PBS emulsified in incomplete Freund’s adjuvant (IFA, Sigma-Aldrich, St. Louis, MO) [44]. Each mouse was immunized two times with a 14-day interval. Two weeks after the last immunization, serum samples were collected, and mice were sacrificed for further study.

Cell culture

Single cell suspensions of splenocytes and lymphocytes were prepared by mincing the mouse spleens and mesenteric lymph nodes in PBS containing 1% FBS (Gibco, Grand Island, NY) and 1% EDTA. Red blood cells were lysed using ACK lysis buffer.

For preparation of single cell suspensions of hepatic lymphocytes, mouse livers were perfused via the portal vein with a PBS/heparin mixture (75 U/ml, Sigma Chemical Co., St. Louis, MO). The excised liver was cut into small pieces and incubated in 10 ml of digestion buffer (collagenase IV/dispase mix, Invitrogen Life Technologies, Carlsbad, CA) for 30 min at 37°C. The digested liver tissue was then homogenized using a MediMachine with 50 µm Medicons (Becton Dickinson, San Jose, CA) for 3 min at low speed [45]. The liver suspension was then centrifuged at low speed to sediment the hepatocytes. The remaining cells were separated on a 35% Percoll gradient by centrifuging at 600 x g. The lymphocyte fraction was resuspended in 2 ml of red cell lysis buffer and then washed in 10 ml of complete RPMI 1640 with 0.1 M EDTA.

The cells were cultured in triplicate in complete RPMI 1640 medium (Gibco) containing 10% FBS, 2 mM pyruvate, 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U of penicillin/ml and 0.1 mg/ml streptomycin. Subsequently, 2 x 10^5 cells per well in 200 µl of complete media were cultured in 96 well plates (Nunc, Roskilde, Denmark) for 72 h at 37°C in the presence of 25 ng/ml ionomycin (Sigma-Aldrich) in complete RPMI 1640 medium in the presence of 0.66 µl/ml Golgistop (BD Biosciences Pharmingen) for 6 h at 37°C in 5% CO_2. After 6 h, the cells were collected and surface stained with anti-CD3-APC (eBioscience) and anti-CD4-FITC (eBioscience). Subsequently, the cells were washed, fixed, permeabilized with Cytofix/Cytoperm buffer and intracellularly stained with PE conjugated antibodies against IL-17A, IFN-γ or IL-4 (or isotype IgG2a control antibody) (eBioscience) for detection of Th17, Th1 or Th2 cells, respectively, according to the manufacturer’s protocol and analyzed with a FACS Calibur flow cytometer. Cells were gated on the CD3^+CD4^+ population for analysis of Th17, Th1 or Th2 cells.

For detection of Treg cells, the Mouse Regulatory T Cell Staining Kit (eBioscience) was used. A single cell suspension of splenocytes from each mouse was prepared and 1 x 10^6 cells from each sample were stimulated with 25 ng/ml PMA and 1 µg/ml ionomycin (Sigma-Aldrich) in complete RPMI 1640 medium in the presence of 0.66 µl/ml Golgistop (BD Biosciences Pharmingen) for 6 h at 37°C in 5% CO_2. After 6 h, the cells were collected and surface stained with anti-CD3-PerCP mAbs (eBioscience), anti-CD4-FITC mAbs and anti-CD25-APC mAbs, followed by fixation and permeabilization with Cytofix/Cytoperm and intracellular staining with anti-Foxp3-PE or IgG2a-PE rat immunoglobulin control antibody, according to the manufacturer’s protocol. Cells were gated on the CD3^+CD4^- population for analysis of Treg cells.

rmIL-17A and neutralizing anti-mouse IL-17A mAb administration and S. japonicum infection

The recombinant mouse IL-17A (rmIL-17A), the neutralizing rat anti-mouse IL-17A mAb (clone 50104) and its control IgG2a mAb were purchased from R&D Systems, Inc. Two independent experiments were carried out in the same manner. In each experiment, 16 mice were randomly assigned in four groups (four mice per group). Each mouse was challenged with 40 cercariae of S. japonicum as described above. For two groups of mice, 70 µg of mAb or its control IgG2a mAb per mouse were administered intraperitoneally (i.p.) four days before S. japonicum infection, and the administration of the same dose of mAb was repeated every four days during the infection until two days before the mice were sacrificed [12]. Simultaneously, for the other two groups of mice, 300 ng/mice of rmIL-17A or PBS were administered i.p. two days before S. japonicum infection and repeated every 48 h during the infection until two days before the mice were sacrificed [49]. Forty-two days after the challenge infection (two days after the last injection of rmIL-17A or anti-IL-17A), all four mice in each group were sacrificed. Serum samples were collected for ELISA detection of the levels of SEA or SWA specific antibodies, and the organs were isolated for histopathological examination. The splenocytes were prepared for incubation as previously mentioned for detection of cytokines in the culture supernatant or intracellular staining for detection of Th17, Th1, Th2 and Treg cells.
Histopathological examination and estimation of worm and egg burdens in liver

Forty-two days after the challenge, all mice injected with neutralizing mAb or rmIL-17A and their controls were sacrificed, and perfusion was performed with saline containing heparin to recover the adult worms. Two grams of each liver were digested with 5% KOH at 37°C overnight, and the numbers of eggs were determined by microscopic examination. The remaining parts of the livers were dissected and immediately fixed in 10% buffered formalin. Liver sections were embedded in paraffin and stained with hematoxylin and eosin (H&E) for microscopic examination. The lesions were assessed on coded slides by an observer unaware of the experimental setting. The sizes of the granulomas were measured by computer-assisted morphometric analysis as previously described [44], and 30 visual fields in the liver section of each mouse (ten sections for each mouse and five random microscope fields for each section) were measured under a microscope (magnification: 100×) (Olympus, Tokyo, Japan). Granuloma sizes are expressed as means of areas measured in μm² ± SD. The percentages of neutrophils, eosinophils, lymphocytes and macrophages in the same granulomas were determined by microscopic examination [1000× magnification] of 200 randomly selected cells (not including hepatocytes) in each granuloma. Ten sections for each mouse and five microscope fields for each section were counted. Percentages of cells were calculated from microscopic analysis of the same granulomas analyzed for lesion size [14,50,51].

The worm/egg reduction rate (percentage of protection) was calculated according to the following formula: (1 - mean of worms/eggs in injected mice/mean of worms/eggs in control mice)×100% [52].

Statistical analysis

Statistical analysis was performed using the SPSS version 10.1 (Statistical Package for Social Sciences, Chicago, IL) software. Statistical significance was determined by Student’s t-test and P<0.05 was considered significant.

Results

Kinetic analysis of the proportions of Th17, Th1, Th2 and Treg cells among CD4⁺ T cells during S. japonicum infection in C57BL/6 mice

Consistent with previous studies, the granulomas began to form from five weeks after infection in the mouse liver after egg deposition and continued to develop (Fig. 1A). As shown in Fig. 1B and 1C, in parallel with the development of the granulomas, the proportion of Th17 cells in splenic CD4⁺ T cells increased very slowly during the first five weeks post-infection compared to that before infection (week 0) and increased rapidly thereafter. Meanwhile, the proportion of the Treg cells in the total splenic CD4⁺ T cell population showed a continuous increase after infection. Additionally, the proportions of both Th1 and Th2 cells in CD4⁺ T cells also increased. During the first three weeks post-infection, the proportion of Th1 cells rose much more quickly than that of the Th2 cells. However, after egg deposition, the number of Th2 cells kept increasing rapidly, while the number of Th1 cells reached a plateau by eight weeks post-infection (Fig. 1B and 1C). Compared to the CD4⁺ T cells responses in the spleen, that of the mesenteric lymphocytes showed a more rapid increase of Th17 cells during the first three weeks post-infection and a weaker Th1 response throughout infection (Fig. 1D and 1E). Meanwhile, stronger Th2 but weaker Treg responses were observed in the liver (Fig. 1F and 1G). These results indicated that all of the CD4⁺ T cell subsets (Th17, Th1, Th2 and Treg cells) increased as over the course of infection.

ELISA detection of cytokines in the supernatant of cultured splenocytes

To further investigate the kinetics of cytokines which affect the differentiation, development and proliferation of Th17 cells during S. japonicum infection in C57BL/6 mice, the splenocytes of infected mice were cultured, and the cytokine levels in the supernatants were detected by ELISA. The results in Figure 2 show that, consistent with the generation of Th17 cells, the level of IL-17 increased very slowly in the first five weeks post-infection compared to that before infection (week 0). However, IL-17 increased rapidly after five weeks post-infection. Meanwhile, both the inducing cytokines (TGF-β, IL-6, IL-23 and IL-21) and the inhibitory cytokines (IFN-γ and IL-4) of Th17 cell generation all increased after infection. Taken together, these results suggest that the generation of Th17 cells during infection with S. japonicum may occur as a net effect of the inducing and inhibitory factors.

FACS analysis of the proportions of Th17, Th1, Th2 and Treg cells in CD4⁺ T cells in mice immunized with SWA or SEA

Schistosome parasitic worms are multicellular pathogens which have three different life cycle stages (schistosomula, adult worm and egg) in definitive hosts including humans. Among the multitude of schistosome antigens that stimulate host immune responses, the adult worm and egg are two important sources of antigens that are involved induction of different types of Th cell responses or Tregs at different infection stages. Studies show that Schistosoma mansoni eggs induce egg antigen-specific Th17 responses and contribute to the severe immunopathology in murine schistosomiasis [12–17]. Consistent with these studies, our data also suggest that the S. japonicum egg antigens have the ability to significantly induce egg antigen-specific Th17 responses (Figure S1A, S1B and S1C). To further investigate the roles of these two major types of S. japonicum antigens on Th17 cell generation, SWA and SEA were used to immunize C57BL/6 mice or to induce CD4⁺ T cells to differentiate in vitro. As shown in Figure 3A and 3B, a significantly higher percentage of Th17 cells was only observed in the SEA immunized group by FACS analysis, suggesting that repeated vaccinations of mice with SEA, instead of SWA, preferentially induced Th17 cells in vitro. Furthermore, the data also suggests that the eggs produced by adult worms in hosts, compared to the adult worms themselves, may more rapidly induce Th17 cells during S. japonicum infection. Meanwhile, additionally, SEA preferentially induced a significant increase of Th2 cells and Tregs, while SWA preferentially induced a significant increase of Th1 cells and caused only a slight increase of Treg and not of Th17 or Th2 cells (Figure 3A and 3B). The profiles of CD4⁺ T cells differentiation induced by SEA or SEA stimulation in vivo also confirmed the above findings (Figure S2A and S2B).

ELISA detection of cytokines secreted in culture by splenocytes from SEA or SWA immunized mice

To further investigate the cytokines that were reported to affect the differentiation, development and proliferation of Th17 cells, splenocytes from mice after vaccination with SWA or SEA were isolated and cultured as described in Materials and Methods, and the levels of cytokines in the supernatants were detected by ELISA. As shown in Figure 4, compared to the SWA and PBS control groups, a significantly higher level of IL-17 was observed in the SEA group, suggesting that repeated vaccination with SEA
preferentially induced the production of IL-17. Compared to the PBS control group, the increase of IL-4 was mainly observed in the SEA group, while the increase of IFN-γ was only observed in the SWA group. Compared to the PBS control group, the levels of TGF-β, IL-6, IL-23 and IL-21, which are associated with the generation of Th17 cells, were significantly increased in both the SEA and SWA groups. However, when comparing the SEA group with the SEA group, the data showed that repeated vaccination with SEA preferentially induced higher levels of IL-23 and IL-21, and further supports that egg antigens are possibly more important in the increase of Th17 cells during *S. japonicum* infection.

**Protection against *S. japonicum* infection by administration of recombinant mouse (rm) IL-17A or anti-IL-17A neutralizing mAb in C57BL/6 mice**

To evaluate the role of IL-17 in the host protective responses against *S. japonicum* infection, C57BL/6 mice were injected with rmIL-17A or anti-IL-17A neutralizing mAb to increase or decrease the level of IL-17 *in vivo*, respectively, and then challenged with *S. japonicum*. The protection was measured by the reduction in the worm and egg burden [52] compared between groups injected with rmIL-17A or anti-IL-17A neutralizing mAb and their respective controls. Compared to the control IgG2a mAb group, injection of mice with neutralizing anti-IL-17A mAb led to a 26.61% reduction (*P*<0.01) in worm burden (Fig. 5A). On the other hand, compared to the PBS control group, no reduction of worm (*P>0.05*) or egg (*P>0.05*) burden was observed in the rmIL-17A group.

Consistent with other reports [12–16,36], our results in Figure 5B and 5C also show that elevating IL-17 *in vivo* by injecting mice with rmIL-17A led to slightly enhanced hepatic granulomatous inflammation (without statistical significance), while decreasing the level of IL-17 by use of an anti-IL-17A neutralizing mAb led to decreased hepatic immunopathology. The percentages of neutrophils and eosinophils in the granulomas, which are thought to be the important populations [14], were increased when *S. japonicum* infected mice were injected with rmIL-17A. However, injection with an anti-IL-17A neutralizing mAb led to decreases in the percentages of neutrophils and eosinophils in the granulomas (Fig. 6). In addition, the results in Figure 6 also show that rmIL-17A decreased while anti-IL-17A neutralizing mAb increased the percentages of lymphocytes and macrophages in *S. japonicum* infected mice.

**Effect of rmIL-17A or anti-IL-17A neutralizing mAb on CD4+ T cells, cytokines and antibody responses in *S. japonicum* infected mice**

To further investigate the possible mechanism underlying the effects of IL-17 on the response to anti-schistosome infection and the hepatic immunopathology, we detected the levels of CD4+ T cells, cytokines and antibody responses in mice after administration of rmIL-17A or anti-IL-17A neutralizing mAb. The proportions of Th1/Th2/Th17/Treg cell subsets in mouse spleens and liver were measured. Single cell suspensions of splenocytes, lymphocytes or liver cells were stimulated with PMA and ionomycin in the presence of Golgistop for 6 h. Cells were surface stained with anti-CD3-APC and anti-CD4-FITC and then intracellularly stained with PE-conjugated antibodies against IL-17A, IFN-γ, IL-4 or type IgG2a control antibody for FACS analysis of Th17, Th1 or Th2 cells. Tregs were detected in single cell suspensions of splenocytes, lymphocytes or liver cells using the Mouse Regulatory T Cell Staining Kit. A, Histopathology in the livers (magnification: 400×). Results are representative of three independent experiments. B, Flow cytometric analysis of CD4+ T cells from mouse spleens and liver (400×). C, Mesenteric lymph nodes and livers. Data are expressed as the mean ± SD of 12 mice from three independent experiments. doi:10.1371/journal.pntd.0001399.g002

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Th17 in *Schistosome japonicum* Infection in Mice

![Graph A](chart1.png)

![Graph B](chart2.png)
Discussion

During the past several years, the Th1/Th2 paradigm has been updated to include a third helper subset called Th17. Through the induction of chemokines and the recruitment of other effector T cell populations [53–55], the responses of Th17 cells dominate in response to certain defined pathogens and play important roles in both host defense against pathogens and immunopathogenesis [11,56]. Schistosomiasis is a typical chronic infectious disease. Infection induces the generation of Th1, Th2 and Treg cells, as well as Th17 cells that are involved in the formation of hepatointestinal perioval granulomas. In this study, we investigated the kinetics of the generation of Th17 cells induced by parasite antigens from different stages of S. japonicum infection in mice as well as the role of Th17 cells in the host protective responses.

In our study, as the parasites began to produce eggs, the granulomas formed in the mouse liver and developed continuously. After the eggs were deposited into the liver and the granulomas were beginning to form, the proportion of Th17 cells in the spleen, mesenteric lymph nodes and liver CD4+ T cells increased slowly up to five weeks post-infection but then increased more rapidly between five and eight weeks post-infection while accompanied by the development of the granulomas. Meanwhile, the proportions of Th1, Th2 and Treg cells in the CD4+ T cells also increased. These findings suggested that the schistosomal antigens induced the simultaneous generation of both Th17 cells and the other CD4+ subsets that are thought to suppress the generation of Th17 cells during infection as reported in many previous studies [2,5,32]; however, these factors seem to have failed to suppress the generation of Th17 cells in our S. japonicum infection experiments. In addition, the results also suggested it may be the egg antigens of S. japonicum that were responsible for the more rapid increase in the proportion of Th17 cells in total CD4+ T cells from five weeks onward after infection.

Schistosome eggs and adult worms are two important sources of antigens exposed to the host during S. japonicum infection [57]. They both have the potential to induce Th1, Th2, Th17 and Treg cells and the corresponding cytokines. Therefore, we confirmed the above hypothesis by using to SEA or SWA to immunize mice as well as to stimulate CD4+ cells in vitro, and our data showed that it was SEA that preferentially induced the generation of Th17 cells and production of IL-17.

The generation and suppression of Th17 cells by Th1, Th2 and Treg cells and/or their cytokines have been demonstrated in numerous studies of in vivo and/or in vitro induction of T cells under defined polarizing conditions [25,27,32–34]. Based on these reports, the current widely accepted differentiation mode of the Th17 cell subset is as follows. In the presence of TGF-β and IL-6, CD4+ T cells are induced to express the transcription factor RORγt by stimulating the STAT3 and Smad signaling pathway,

Figure 3. Proportions of Th17, Th1, Th2 and Treg cells in mice immunized with SEA or SWA. For each of three independent experiments, C57BL/6 mice (8 per group) were each injected subcutaneously in the back with 100 μl of an IFA emulsified solution containing 50 μg of SEA, 50 μg of SWA or PBS. The immunizations were repeated two times with a 14-day interval. Two weeks after the final vaccination, the mice were sacrificed, and single cell suspensions of splenocytes were prepared. The splenocytes were stimulated with PMA/ionomycin in the presence of Golgistop for 6 h, followed by surface staining with anti-CD3-APC and anti-CD8-FITC and then intracellular staining with PE-conjugated antibodies against IL-17A, IFN-γ, IL-4 or isotype IgG2a control antibody for FACS analysis of Th17, Th1 or Th2 cells. Splenocytes were stained with the Mouse Regulatory T Cell Staining Kit for detection of Treg cells.

Results are expressed as mean ± SD of 24 mice from three independent experiments. *P<0.05; **P<0.01; ***P<0.001, compared to control mice injected with PBS.

Discussion

The simultaneous generation of both Th17 cells and the other CD4+ subsets that are thought to suppress the generation of Th17 cells during infection as reported in many previous studies [2,5,32]; however, these factors seem to have failed to suppress the generation of Th17 cells in our S. japonicum infection experiments. In addition, the results also suggested it may be the egg antigens of S. japonicum that were responsible for the more rapid increase in the proportion of Th17 cells in total CD4+ T cells from five weeks onward after infection.

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Figure 5. Protection against *S. japonicum* infection in C57BL/6 mice administered rmIL-17A or anti-IL-17A neutralizing mAb. rmIL-17A, anti-mouse IL-17A mAb or controls were administered to mice, followed by challenge with 40 *S. japonicum* cercariae per mouse. A. Six weeks after the challenge, the mice were sacrificed and perfused to collect adult worms. The numbers of eggs extracted from the liver were determined by microscopic examination. Protection was measured by assessing the worm and egg burden. Values are given as mean ± SD of eight mice from two
which leads to the differentiation of Th17 cells. IL-21 together with TGF-β also stimulate the alternative pathway for Th17 differentiation. Meanwhile, the mature Th17 cells amplify themselves by autocrine IL-21. IL-23 also contributes to the maintenance of Th17 cell stability through engagement of the IL-23R, which is expressed by memory or activated T cells [58]. Simultaneously, IFN-γ and IL-4 can effectively inhibit the generation of Th17 cells [2, 5, 59].

Our study clearly showed that during the course of S. japonicum infection, in parallel with the increase of the proportion of Th17 cells, both the inducing (TGF-β, IL-6, IL-21 and IL-23) and inhibitory (IFN-γ, IL-4, Th1, Th2 and Treg cells) factors of Th17 cell generation increased as the infection progressed. These results suggested that a multicellular pathogen such as S. japonicum introduced complex sets of antigens at different stages of infection into the host that could promote both the inducible and the inhibitory factors of Th17 cell generation, but the overall net result was an increase in Th17 cells. In another words, the observed increase in Th17 cells during S. japonicum infection was probably due to the ability of the S. japonicum antigens to more strongly upregulate Th17 inducing factors than the Th17 suppressive factors. In addition, immunization of mice with SEA also preferentially induced Th17 cell generation and the production of higher levels of known factors involved in the generation of Th17 cells (TGF-β, IL-23 and IL-21), while accompanied by the increase in the reported inhibitory factors of Th17 cell generation (Treg and Th2 cells, as well as IL-4).

Many studies have shown that Th17 cells play important roles both in host defenses against extracellular pathogens [6], which are not efficiently cleared by Th1-type and Th2-type immunity and in immunopathogenesis caused by infection. In S. japonicum infection, it has been reported that Th17 may play an important role in the liver immunopathogenesis and in the formation and growth of granulomas around the eggs produced by the adult worm. The findings indicate that the development of severe murine schistosomiasis correlates with high levels of IL-17 and suggest that the exacerbated egg-induced immunopathology is largely mediated by the subset of SEA-induced Th17 cells which produces IL-17 [12, 13]. Our study also suggested that the IL-17 level was positively related to the severity of liver pathogenesis, which was possibly due to the enrichment of inflammatory cells including neutrophils and eosinophils in the granulomas. However, there has been no evidence reported yet to indicate whether Th17 and its product IL-17 either improve or impair the anti-schistosome immune response. Considering that the protection against S. japonicum infection is mainly based on the clearance of the schistosomulum at the early stage of infection, we investigated the potential protective effect of IL-17 levels during that period. Our results showed that administration of rmIL-17A to mice failed to reduce the worm and egg burdens, indicating that high levels of IL-17 did not contribute to the protective responses. Instead, decreasing the level of IL-17 in mice by injecting anti-IL-17A neutralizing mAb led to reductions of worm and egg burdens, suggesting that the decrease of IL-17 levels contributed to effective protective responses against S. japonicum infection. Our study further showed that administration of the anti-IL-17A neutralizing mAb increased the levels schistosome specific IgG1, IgG2a and/ or IgG, suggesting that increased antibody-dependent-cell-cytotoxicity (ADCC), one of the well accepted mechanisms of killing extra-cellular residing pathogens including schistosome [60], may at least partially contributed to the more effective protective responses against S. japonicum infection. However, the mechanism underlying the role of IL-17 in the protection against S. japonicum infection needs to be further investigated.

In summary, for the first time our study reported on the kinetics of the generation of Th17 cells, which were likely preferentially induced by egg antigens, during S. japonicum infection. We also determined that the proportion of Th17 cells increased together with other CD4+ subsets reported to inhibit them, including Th1, Th2 and Treg cells, as well as their suppressive cytokines in a S. japonicum infection mouse model. These findings suggest that the generation of Th17 cell is determined by the integrated impact of the inducing and suppressive factors promoted by parasitic antigens. More importantly, our study for the first time indicates that a decrease in the level of IL-17 in the early stage of S. japonicum infection may contribute to the host protective responses.
Supporting Information

Figure S1 SEA-specific Th17 responses in S. japonicum infected mice. For each of two independent experiments, 6 female C57BL/6 mice were infected with 12 cercariae of S. japonicum per mouse. After eight weeks, single cell suspensions of splenocytes, lymphocytes or liver cells were in vitro stimulated with SEA or PBS for 48 h. A. Flow cytometric analysis of Th17, Th1, Th2 and Treg cells from one representative experiment. Single cell suspensions of splenocytes were stimulated with PMA/ionomycin in the presence of Golgistop for 6 h, followed by surface staining with anti-CD3-APC and anti-CD4-FITC and intracellularly staining with PE-conjugated antibodies against IL-17A or isotype IgG2a control antibody for FACS analysis of Th17, Th1 or Th2 cells. Splenocytes were also stained with the Mouse Regulatory T Cell Staining Kit for detection of Treg cells. Cells were gated on the CD3+ population for analysis of Th17, Th1, Th2 cells analysis, or gated on the CD3+CD4+ population for analysis of Treg cells. B. The proportions of Th17, Th1, Th2 and Treg cells in CD4+ T cells. Results are expressed as the mean ± SD of eight mice from two independent experiments. *P<0.05; **P<0.01; ***P<0.001, compared to control mice injected with PBS or isotype antibody. C. ELISA detection of cytokines in the supernatant produced by splenocytes from S. japonicum infected mice after administration of rmIL-17A or anti-IL-17A neutralizing mAb. Splenocytes (2×10^5/well in 200 μl of complete media) were cultured in 96 well plates with PMA/ionomycin. Culture supernatants were collected after 72 h, and cytokines were detected by ELISA. Results are expressed as mean ± SD of eight mice from two independent experiments. *P<0.05; **P<0.01; ***P<0.001, compared to control mice injected with PBS or isotype antibody. SEA (D) and SWA (E) specific IgG, IgG1 and IgG2a antibodies in serum from S. japonicum infected mice after administration of rmIL-17A or anti-IL-17A neutralizing mAb were detected by ELISA. Results are expressed as mean ± SD of eight mice from two independent experiments. *P<0.05; **P<0.01; ***P<0.001, compared to control mice injected with PBS or isotype antibody.

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in total CD4+ T cells from mouse spleens, mesenteric lymph nodes and livers. Data are expressed as the mean ± SD of 12 mice from two independent experiments. **P<0.01, compared to PBS control group. C. The culture supernatants were collected after 48 h of SEA in vitro stimulation for detection of IL-17 by ELISA. Data are expressed as the mean ± SD of 12 mice from two independent experiments. *P<0.05; **P<0.01; ***P<0.001, compared to PBS control group.

(TIF)

Figure S2 In vitro induction of CD4+T cell differentiation by SEA or SWA. Using a negative selection cell isolation kit, CD4+ T cells were isolated from naive mouse splenocytes in the

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negative fraction, while APCs were obtained from the positive fraction and irradiated with 30 Gy. The CD4+ T cells (2×10^6/well) were cultured in triplicate wells of 24-well plates with APCs (1×10^6/well) in the presence of 30 μg/ml of SEA, SWA or PBS as control in complete RPMI 1640 medium (2 ml/well). After 72 h, the cells were surface stained with anti-CD4 and anti-CD4-APC, FITC, and then intracellularly stained with PE-conjugated antibodies against IL-17A, IFN-γ, IL-4 or isotype IgG2a control antibody for FACS analysis of Th17, Th1, or Th2 cells. Splenocytes were also stained with the Mouse Regulatory T Cell antibody for FACS analysis of Th17, Th1 or Th2 cells. Proportions of Th17, Th1, Th2 and Treg cells in CD4+ T cells. Results are expressed as mean ± SD of nine samples from three independent experiments.

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