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Characteristics of IL-17 induction by *Schistosoma japonicum* infection in C57BL/6 mouse liver

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Summary

Schistosomiasis japonica is a severe tropical disease caused by the parasitic worm *Schistosoma japonicum*. Among the most serious pathological effects of *S. japonicum* infection are hepatic lesions (cirrhosis and fibrosis) and portal hypertension. Interleukin-17 (IL-17) is a pro-inflammatory cytokine involved in the pathogenesis of many inflammatory and infectious conditions, including schistosomiasis. We infected C57BL/6 mice with *S. japonicum* and isolated lymphocytes from the liver to identify cell subsets with high IL-17 expression and release using flow cytometry and ELISA. Expression and release of IL-17 was significantly higher in hepatic lymphocytes from infected mice compared with control mice in response to both non-specific stimulation with anti-CD3 monoclonal antibody plus/anti-CD28 monoclonal antibody and PMA plus ionomycin. We then compared IL-17 expression in three hepatic T-cell subsets, T helper, natural killer T and γδT cells, to determine the major source of IL-17 during infection. Interleukin-17 was induced in all three subsets by PMA + ionomycin, but γδT lymphocytes exhibited the largest increase in expression. We then established a mouse model to further investigate the role of IL-17 in granulomatous and fibrosing inflammation against parasite eggs. Reducing IL-17 activity using anti-IL-17A antibodies decreased infiltration of inflammatory cells and collagen deposition in the livers of infected C57BL/6 mice. The serum levels of soluble egg antigen (IL) -specific IgGs were enhanced by anti-IL-17A monoclonal antibody blockade, suggesting that IL-17 normally serves to suppress this humoral response. These findings suggest that γδT cells are the most IL-17-producing cells and that IL-17 contributes to granulomatous inflammatory and fibrosing reactions in *S. japonicum*-infected C57BL/6 mouse liver.

Keywords: interleukin-17; liver; natural killer T cell; *Schistosoma japonicum*; T helper type 17; γδT cell.

Introduction

Schistosomiasis is a tropical parasitic disease caused by blood-dwelling worms of the genus *Schistosoma*. The main species pathogenic to humans are *S. mansoni*, *S. japonicum* and *S. haematobium*.[1] Schistosomiasis caused by *S. japonicum* is endemic in China and the Philippines. Disease symptoms are due predominantly to the host immune response to schistosome eggs (ova) and the granulomatous reaction evoked.[2–4] Granulomas destroy the eggs and sequester or neutralize otherwise pathogenic egg antigens but also lead to fibrogenesis in host tissues.[4] In Schistosomiasis japonica, pathology develops at sites of highest egg accumulation, most often the intestines and liver. Infection by *S. japonicum*, a multi-cellular parasite with an extremely diverse repertoire of antigens, induces the production of multiple cytokines that mediate the immune response. These cytokines are therefore potential therapeutic targets for schistosomiasis treatment.

Lymphocytes are white blood cells of uniform appearance but varied function. Subtypes include T, B and natural killer cells. The T cells are divided into αβ T cells and γδT cells according to the subunit combination of the T-cell receptor.[5] Natural killer T (NKT) cells are a small but essential subset of T lymphocytes with characteristics of both T and NK cells.[6,7] These NKT and γδT cells

represent approximately 20% and 1–2%, respectively, of all T lymphocytes in the liver, higher than in spleen or mesenteric lymph nodes. We took advantage of the high proportion of NKT and γδT cells in the liver to characterize the cellular sources of interleukin-17 (IL-17), a critical pro-inflammatory cytokine in the pathogenic response to *S. japonicum*.

Interleukin-17 activates neutrophils by inducing the release of other cytokines important in granulopoiesis (granulocyte colony-stimulating factor) and neutrophil chemotaxis (CXCL1/KC and CXCL8/IL-8).[8] It is essential not only for the development of autoimmune disease but also for protection against pathogens,[9] including *S. japonicum*.[10] In fact, several studies have concluded that IL-17 is most directly associated with the severity of hepatic granulomatous inflammation.[11–14] We analysed IL-17A expression by flow cytometry and IL-17 release by a specific ELISA in both infected and control mice. Previous reports concluded that IL-17 was produced mainly by T helper type 17 (Th17) cells after infection by *S. japonicum*.[15] However, there is limited information about other lymphocyte subsets that secret IL-17 in schistosomiasis. Multiple studies have demonstrated that Th17 is not the only IL-17-producing T-cell population; $CD4^+$ T cells, NKT cells and γδT cells are also IL-17-producing T cells in many infections, such as *Listeria monocytogenes*,[16] *Nocardia asteroides*[17] and *Salmonella enterica* serovar Typhi.[18]

The aim of the current study was to characterize the role of IL-17 in the pathogenic processes of the *S. japonicum*-infected liver. We compared IL-17 expression and secretion from three lymphocyte subsets, $CD4^+$ T, NKT and γδT cells in response to non-specific stimulation with PMA and ionomycin and found that γδT lymphocytes were the largest IL-17-producing cell population in the mouse liver. Methods developed and validated in patients with chronic hepatic diseases have identified direct biomarkers for fibrosis, indirect multi-test batteries and physical methods to evaluate the severity of liver damage induced by *S. japonicum* and other pathogens.[19] Among these fibrosis markers, pro-collagen type III (PC-III) and type IV collagen (IV-C) are sensitive and accurate fibrosis markers as measured by ELISA. Serum PC-III concentration reflects the difference between collagen production and elimination and is more a marker of active fibrogenesis than fibrosis.[20] In this study, we also show that decreasing IL-17 with a neutralizing anti-IL-17A monoclonal antibody (mAb) increased schistosome-specific antibody levels and partially protected against *S. japonicum* infection in mice.

Materials and methods

Mice, parasites and infection

Female C57BL/6 mice, 6–8 weeks old, were purchased from Zhongshan University Animal Centre (Guangzhou, China) and maintained in a specific-pathogen-free facility at Guangzhou Medical College. Cercariae of *S. japonicum* were shed from naturally infected *Oncomelania hupensis* snails collected from fields in Anhui Province, China. Mice were infected percutaneously with 40 ± 5 cercariae and killed at 5–7 weeks after infection. 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) then at the same dose every third day until 2 days before killing. Animal experiments were performed in strict accordance with the regulations for the Administration of Affairs Concerning Experimental Animals, and all efforts were made to minimize suffering.

Antibodies

The FITC-conjugated anti-mouse CD3 (17A2), allophycocyanin-Cy7-conjugated anti-mouse CD3 (145-2C11), Peridinin chlorophyll protein-Cy5.5-conjugated anti-mouse CD4 (RM4-5), phycoerythrin-Cy7-conjugated anti-mouse NK1.1 (PK136), FITC-conjugated anti-mouse T-cell receptor-γδCR (17A2), phycoerythrin-conjugated anti-mouse IL-17A (TC11-18H10), allophycocyanin-conjugated anti-mouse interferon-γ (IFN-γ; XMG1.2), and isotype-matched control mAb (X39, G155-178) were purchased from BD/Pharmingen (San Diego, CA). The neutralizing rat anti-mouse IL-17A mAb (clone TC11-18H10.1) and an isotype-matched rat IgG2a mAb (clone RTK2758) were purchased from BioLegend (San Diego, CA).

Isolation of lymphocytes

Mice were anaesthetized and immobilized from weeks 5 and 7 after infection. The precava was cut and sterile normal saline was injected to remove blood from the liver through the ventriculus sinister. The liver was removed, pressed through 200-gauge stainless-steel mesh, and suspended in Hanks' balanced salt solution. Lymphocytes were isolated by Ficoll–Hypaque density gradient centrifugation. Isolated cells were washed twice in Hanks' balanced salt solution and resuspended at 2×10^6 cells/ml in complete RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 50 μM 2-mercaptoethanol.

ELISA detection of cytokines

Single-cell suspensions were prepared and plated in 96-well micro-titre plates at 4×10^5 cells/200 μl medium per well. Anti-CD3 mAb (1 μg/ml) and anti-CD28 mAb (1 μg/ml) were added to each well and plates were incubated overnight at 4°. Supernatants were collected

72 hr later and released cytokines were measured using mouse cytokine multiplex assay kits for IFN-γ (R&D Systems Inc., Minneapolis, MN) and IL-17 (BD Pharmingen, Franklin Lakes, NJ). ELISAs were performed in accordance with the manufacturer's instructions. The optical density of each well was read at 450 nm using a microplate reader (Model ELX-800; BioTek Instruments Inc., Winooski, VT).

Detection of cell surface markers and intracellular cytokine expression

Single-cell suspensions from the livers of control mice and mice infected with *S. japonicum* were stimulated with 20 ng/ml PMA plus 1 μg/ml ionomycin for 5 hr at 37° under a 5% CO_2 atmosphere. Brefeldin A (10 μg/ml; Sigma-Aldrich, St. Louis, MO) was added during the last 4 hr of incubation. Cells were washed twice in PBS, fixed with 4% paraformaldehyde and permeabilized overnight at 4° in PBS buffer containing 0·1% saponin (Sigma-Aldrich), 0·1% BSA and 0·05% NaN_3. Cells were then stained for 30 min at 4° in the dark with conjugated antibodies specific for the cell surface antigens CD3, CD4, NK1.1, and T-cell receptor-$\gamma\delta$ or the cytokines IFN-γ and IL-17A. The expression phenotypes of antibody-labelled lymphocytes (200 000–300 000 cells per run) were analysed by flow cytometry (BD Calibur and Aria II) and results were analysed with FLOWJO version 6·0 (Tree Star Inc., Ashland, OR). Isotype-matched controls for cytokines were included in each staining protocol.

Histology studies

Livers were removed from the mice, perfused three times with 0·01 M PBS (pH 7·4), fixed in 10% formalin, embedded in paraffin, and sectioned. The sections were then examined by light microscopy under 100 × and 400 × magnification after standard haematoxylin & eosin staining for visualization of cellular changes or Masson's trichrome staining for collagen deposition.

Granuloma size was measured by computer-assisted morphometric analysis using DP-2BSW software (Olympus, Shinjuku, Tokyo, Japan). Granulomas were measured under a microscope in 80−100 visual fields of liver sections (mounted on coded slides) by an observer blind to treatment history. To more accurately reflect the true shape and dimensions of the granulomas in thin sections, only cross-sections containing a visible central egg were counted. Granuloma size is expressed as mean area in $\mu m^2 \pm$ SD.

ELISA detection of collagens

The PC-III and IV-C in serum were analysed by ELISA according to the manufacturer's instructions (BIOVALUE). Samples were read at 450 nm using a microplate reader (Model ELX-800, BioTek).

Detection of antibodies by ELISA

Immunoglobulin G and IgE antibodies to soluble egg antigen (SEA) were measured by ELISA. Briefly, SEA was dissolved in coating buffer (0·05 M sodium bicarbonate buffer, pH 9·6) at a concentration of 80 μg/ml. Then, 100 µl of this solution was added to each well and the plate was incubated overnight at 4°. After removing the solution, the plate was blocked by adding 200 μl/well blocking solution (5% skimmed milk power in 0·02 M PBS with 0·05% Tween-20, pH 7·2) at 37° for 1 hr. After the wells were emptied, 100 μl of 10-fold serially diluted serum (1 : 10 or 1 : 100 for IgE; 1 : 1000, 1 : 10 000, or 1 : 100 000 for IgG) was added to each well and incubated at 37° for 1 hr. After four washes, 100 μl horseradish peroxidase-conjugated goat anti-mouse IgG (ZB2305; ZSGB-Bio, Beijing, China) and horseradish peroxidase-conjugated goat anti-mouse IgE (40411-250; Alpha Diagnostic International, San Antonio, TX) solution diluted in PBS/Tween-20 was added and incubated at 37° for 1 hr. The plate was washed as above and the reaction was visualized by adding 100 μl TMB Substrate Reagent (BD) to each well for 10 min in the dark at room temperature. The reaction was stopped by adding 100 μl/well stop solution (1 M H_2SO_4) and the absorbance of each well was measured at 450 nm using an ELISA plate reader (Model ELX-800; BioTek).

Statistics

Statistical evaluation of differences between means was performed by unpaired, two-tailed Student's *t*-tests. $P < 0{\cdot}05$ was considered significant.

Results

Elevated IL-17 release from cultured hepatic lymphocytes isolated from control and S. japonicum-infected mice in response to anti-CD3 mAb plus anti-CD28 mAb stimulation

Interleukin-17 may be a major contributor to schistosomiasis-associated liver pathology.[13,21] To investigate whether *S. japonicum* infection promotes IFN-γ and IL-17 production in liver lymphocytes, we compared IFN-γ and IL-17 secretion from $CD3^+$ T cells isolated from blood-free normal or infected liver. Release of these cytokines into the supernatant was barely detectable in cultures of unstimulated lymphocytes from normal and infected livers, but was significantly induced in both cell populations by anti-CD3 mAb plus anti-CD28 mAb stimulation. However, release was substantially higher in lymphocytes

from infected livers (IFN-γ: 13·96 ± 0·37 ng/ml; IL-17: 1·14 ± 0·12 ng/ml), suggesting that the production of IFN-γ and IL-17 in the liver is markedly enhanced by infection (*$P < 0·05$; Fig. 1).

The proportion of IL-17$^+$ cells in the total T-cell population isolated from infected and uninfected liver

Interleukin-17 expression by lymphocytes is a marker of disease severity in schistosomiasis.[22] To estimate the IFN-γ and IL-17A release capacity of CD3$^+$ cells isolated from blood-free normal and infected C57BL/6 mouse livers, expression of both cytokines in response to PMA + ionomycin stimulation was measured by FACS (Fig. 2a). These experiments revealed significantly higher percentages of IL-17$^+$ CD3$^+$ and IFN-γ^+ CD3$^+$ cells in the lymphocyte population isolated from infected livers compared with uninfected livers following stimulation (IFN-γ: 17·02 ± 6·22% versus 4·11 ± 2·75%, $P < 0·05$, Fig. 2b; IL-17: 4·03 ± 1·27% versus 2·02 ± 0·85%, $P < 0·01$, Fig. 2c). Hence, consistent with IFN-γ and IL-17 release (Fig. 1), a significantly higher percentage of T cells from infected livers expressed these cytokines following activation. Moreover, in parallel with the result obtained with CD3$^+$ cells, the proportion of IL-17$^+$ cells and IFN-γ^+ cells in CD3$^-$ cells increased after infection (IFN-γ: 6·95 ± 5·11% versus 2·00 ± 1·58%, $P < 0·05$, Fig. 2b; IL-17: 1·49 ± 0·85% versus 0·35 ± 0·17%, $P < 0·01$, Fig. 2c). Additionally, the proportions of both IL-17$^+$ CD3$^+$ and IFN-γ^+ CD3$^+$ cells were significantly higher than of CD3$^-$ cells in both normal control and infected group ($P < 0·05$).

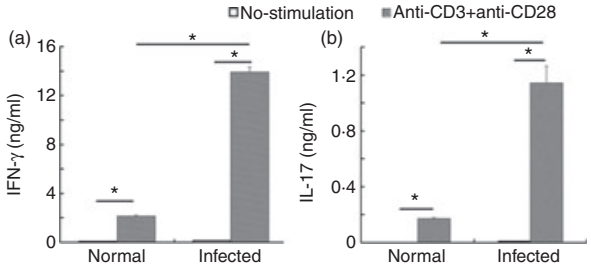

Figure 1. Elevated interleukin-17 (IL-17) release from cultured hepatic lymphocytes isolated from control or *Schistosoma japonicum*-infected mice in response to anti-CD3 monoclonal antibody (mAb) plus anti-CD28 mAb stimulation. Single liver cell suspensions of normal and infected mice were prepared and then cultured in the presence of anti-CD3 mAb plus anti-CD28 mAb. The culture supernatants were collected after 72 h of incubation for detection of interferon- γ (IFN-γ) and IL-17 by ELISA. The data are representative of four experiments, each with three or four replicates per group (*$P < 0·05$, the error bars indicate SD).

IL-17 expression in specific T-cell subsets isolated from infected and uninfected mouse liver

Interleukin-17-producing T cells, including $\gamma\delta$T cells, NKT cells and CD4$^+$ Th cells, have been implicated in the development of granulomatous disease.[23–25] Therefore, we investigated whether expression of IL-17 was elevated in $\gamma\delta$T cells, NKT cells, and CD4$^+$ helper T cells during *S. japonicum* infection. Lymphocytes were isolated from livers of control and infected C57BL/6 mice, counted, stimulated by PMA + ionomycin, and IL-17 expression detected by FACS. The IL-17$^+$ fraction was higher in each subtype from *S. japonicum*-infected mice (Fig. 3a–c), with $\gamma\delta$T cells showing the highest percentage (Fig. 3c) and the highest MFI of IL-17$^+$ cells (Fig. 3d). The proportion of IL-17$^+$ cells in the uninfected $\gamma\delta$T-cell population was 9·11 ± 6·39% after stimulation and 25·40 ± 4·76% in the infected $\gamma\delta$T-cell population (Fig. 3c, $P < 0·01$). The percentage of IL-17$^+$ cells in the $\gamma\delta$T cells was significantly higher than that CD4$^+$ T cells (3·28%±1·83%) and NKT cells (2·72 ± 1·38%) after *S. japonicum* infection (Fig. 3c, **$P < 0·01$). There was no significant difference between the proportion of IL-17$^+$ $\gamma\delta$T cells (0·89 ± 0·38%) and IL-17$^+$ CD4$^+$ T (1·05 ± 0·64%) cells in the total infected and stimulated T-cell population, whereas they were both higher than infected/stimulated IL-17$^+$ NKT cells (0·49 ± 0·31%, Fig. 3b, $P < 0·05$). $\gamma\delta$T lymphocytes also exhibited the highest geometric mean fluorescence intensity (MFI) of the three subsets ($\gamma\delta$T cells: 206·24 ± 45·81; CD4$^+$ T cells: 108·22 ± 21·10; NKT cells: 62·09 ± 15·78; $\gamma\delta$T versus CD4$^+$ T cells, $P < 0·05$; $\gamma\delta$T versus NKT cells, $P < 0·01$; CD4$^+$T versus NKT cells, $P < 0·05$.). Hence, we conclude that all three subpopulations of T lymphocytes in infected liver are sources of IL-17 after non-specific stimulation, but that $\gamma\delta$T lymphocytes produce the most IL-17.

Reduction of hepatic granulomatous inflammation by anti-IL-17 mAb *in vivo*

Control, infected and infected/anti-IL-17 mAb-treated mice were killed and the morphological and histopathological differences in livers were evaluated. Normal liver was light red with a smooth surface following blood removal (Fig. 4a), whereas the infected liver was darker red with many small white spots on the surface, indicating severe inflammation and many pyogenic granulomas. In contrast, livers from infected/anti-IL-17 mAb-treated mice resembled control livers, with few small white spots, indicating reduced inflammation and granuloma response (Fig. 4a). Haematoxylin and eosin staining of liver sections revealed the normal cellular organization of uninfected hepatic lobules, with the typical actinomorphous distribution of hepatic cord centred around central veins.

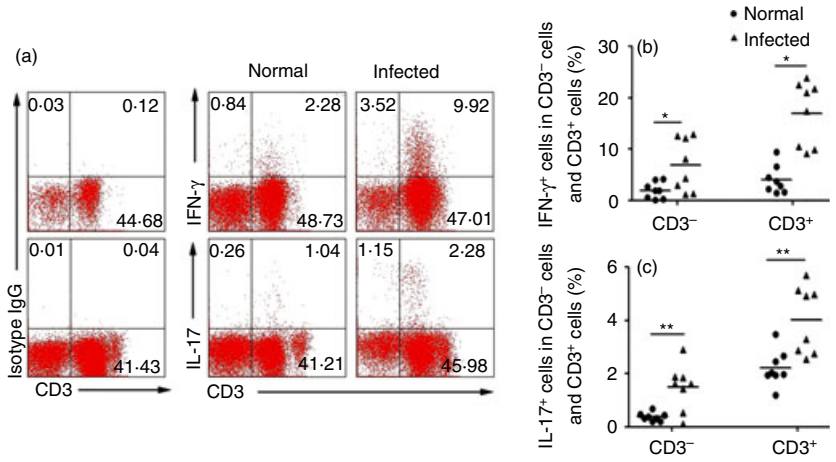

Figure 2. The proportion of interleukin-17-positive (IL-17$^+$) cells in the CD3$^-$ cells and CD3$^+$ cell population isolated from control or infected liver. Six weeks after the infection, the mice were killed. Single cell suspensions of liver cells were stimulated with PMA, ionomycin and brefeldin A. Cells were stained with anti-CD3-FITC and then intracellularly stained with phycoerythrin-conjugated antibodies against IL-17 and allophycocyanin-conjugated anti-mouse interferon-γ (IFN-γ) and also isotype IgG2a control antibody. (a) The IFN-γ and IL-17 expressions of CD3$^-$ cells and CD3$^+$ cells from control or infected liver were analysed by flow cytometry. Flow cytometric analysis from one representative experiment. (b, c) Average percentages of IFN-γ^+ T cells and IL-17$^+$ T-cell populations in normal and infected mice were calculated from FACS data (*$P < 0{\cdot}05$).

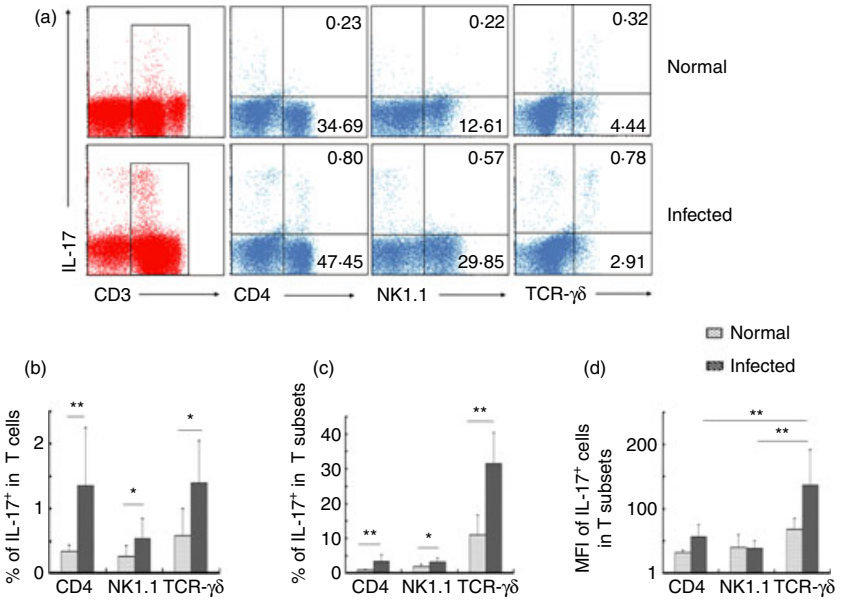

Figure 3. Interleukin-17 (IL-17) expression in specific T-cell subsets isolated from control or infected mouse liver. Female C57BL/6 mice were infected with 40 $\pm$ 5 *Schistosoma japonicum* cercariae per mouse. Six weeks after the infection, the mice were killed. Single-cell suspensions of liver cells were stimulated with PMA, ionomycin and brefeldin A. Cells were stained with anti-CD3-allophycocyanin-Cy7, anti-T-cell receptor-$\gamma\delta$-FITC, anti-CD4-Peridinin chlorophyll protein-Cy5.5, anti-NK1.1-phycoerythrin-Cy7 and then intracellularly stained with anti-IL-17A-phycoerythrin for FACS analysis. (a) Intracellular IL-17 expression by gated populations of CD4$^+$ T cells, natural killer T (NKT) cells and $\gamma\delta$T cells isolated from normal and infected mice, respectively. The data are representative of eight experiments giving similar results. (b) Percentage of the IL-17$^+$ NK1.1$^+$, IL-17$^+$ $\gamma\delta$TCR$^+$, IL-17$^+$ CD4$^+$ cells in CD3$^+$ T cells were calculated from eight independent experiments with similar results. (c) The percentage of IL-17$^+$ cells in CD4$^+$ T cells, NKT cells and $\gamma\delta$T cells were shown. (d) The percentage of mean fluorescence intensity of IL-17$^+$ cells in CD4$^+$ T cells, NKT cells and $\gamma\delta$T cells (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, the error bars indicate SD).

Infection by *S. japonicum* markedly altered the histological structure of the mouse liver. There were many more inflammatory cells in the infected liver lobules than in uninfected controls, while anti-IL-17A neutralizing mAb markedly reduced numbers of inflammatory cells in infected liver (Fig. 4b). The extent of hepatic granulomatous

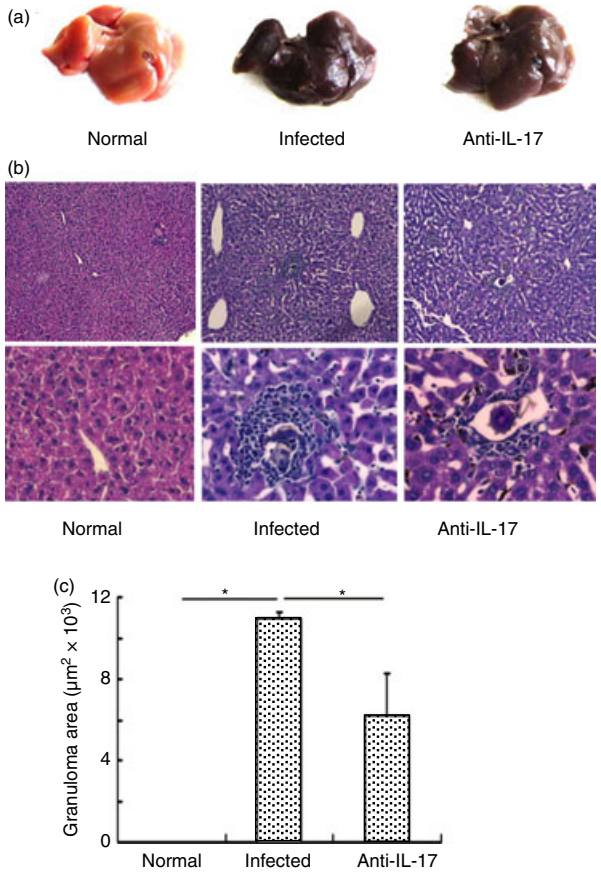

Figure 4. Reduction of hepatic granulomatous inflammation by anti-interleukin-17 (IL-17) monoclonal antibody (mAb) *in vivo*. Thirty female C57BL/6 mice were divided into three groups, normal group, infected group, anti-mouse IL-17 group. The infected group and anti-mouse IL-17 group were infected with 40 ± 5 cercariae of *Schistosoma japonicum* per mouse. For the infected and anti-IL-17 group, 62·5 μg control IgG mAb or anti-IL-17 mAb per mouse were administered intraperitoneally every 3 days, a total of four times, respectively. Six weeks after the infection, the mice were killed. (a) The gross appearance of the three groups. (b) Livers were flushed with 0·01 M PBS three times, fixed in 10% formalin, embedded in paraffin and sectioned. Sections of the liver of normal mice (left panels), infected mice (middle panels) and anti-mouse IL-17 mAb mice (right panels) were examined by haematoxylin & eosin staining (original magnification × 100 for upper panels and × 400 for lower panels). In the control IgG mAb group and anti-IL-17 mAb group, granuloma could be observed. (c) Sizes of the granulomas were measured by computer-assisted morphometric analysis. Only granulomas with a visible central egg were analysed for accuracy. Between 80 and 100 granulomas for each group were measured under a microscope (*$P < 0·05$, the error bars indicate SD).

inflammation around schistosome eggs was measured by computer-assisted morphometric analysis. Infected livers developed large liver granulomas (mean cross-sectional area, 10·95 ± 0·31 $\mu m^2 \times 10^3$), whereas these granulomas were significantly smaller in infected/anti-IL-17 mAb-treated mice (6·18 ± 2·09 × 10^3 μm^2; Fig. 4c; $P < 0·05$). Taken together, these results suggest that generation of IL-17 during *S. japonicum* infection may enhance hepatic granulomatous inflammation, consistent with previous studies implicating IL-17 in hepatic immunopathology associated with schistosomiasis.

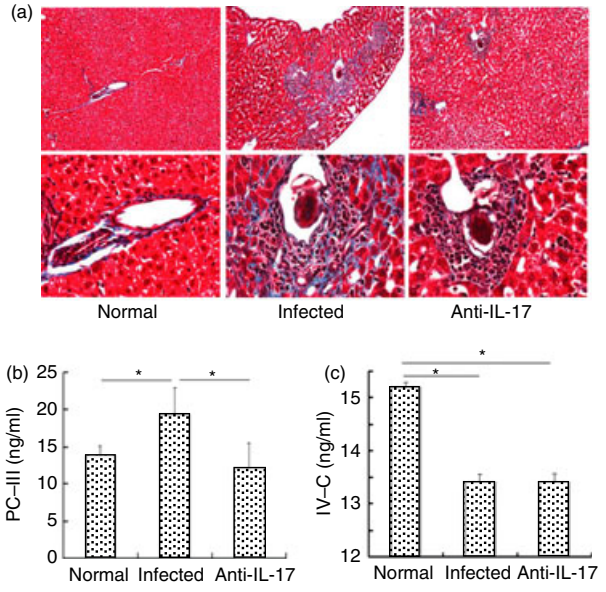

Figure 5. Reduction of hepatic fibrosing inflammation by anti-interleukin-17 (IL-17) monoclonal antibody (mAb) *in vivo*. Female C57BL/6 mice were divided into three groups, normal group, infected group and anti-mouse IL-17 mAb group. The infected and anti-IL-17 mAb groups were infected with 40 ± 5 cercariae of *Schistosoma japonicum* per mouse. For infected and group, 62·5 μg of control IgG mAb or anti-IL-17 mAb per mouse were administered intraperitoneally every three days, for a total of four times, respectively. Six weeks after the infection, the mice were killed. (a) Livers were flushed with 0·01 M PBS three times, fixed in 10% formalin, embedded in paraffin, and sectioned. Sections of the liver of normal, control IgG mAb and anti-IL-17 mAb mice were examined by Masson's trichrome staining (× 100 for upper panels, × 400 for lower panels). (b and c) Levels of pro-collagen type III (PC-III) and type IV collagen (IV-C) in the serum of three groups were detected by ELISA (*$P < 0·05$, the error bars indicate SD).

Reduction of hepatic fibrosing inflammation by anti-IL-17 mAb *in vivo*

Granulomatous and fibrosing inflammation against parasite eggs are the pathological hallmarks of schistosome infection.[13] To measure interstitial fibrosis, liver tissues were cut into 4-mm sections and stained with Masson's trichrome, which stains collagen deposits blue. Masson's trichrome-positive fibrosis (indicative of collagen deposition) and infiltration of inflammatory cells were significantly elevated in infected livers compared with uninfected control and infected/anti-IL-17A mAb-treated livers (Fig. 5a). Serum levels of PC-III and IV-C were detected by ELISA (Fig. 5a,b). Serum levels of PC-III

were significantly higher in infected mice compared with both uninfected control and infected/anti-IL-17 mAb-treated mice (Infected: 19·31 ± 3·63 ng/ml; Control: 13·86 ± 1·28 ng/ml; Anti-IL-17: 12·10 ± 3·43 ng/ml; $P < 0·05$ for both control and anti-IL-17A mAb-treated livers compared with infected livers, Fig. 5b). In contrast, IV-C levels were significantly higher in uninfected control mice than in both infected and infected/anti-IL-17 mAb-treated mice (Control: 15·24 ± 0·87 ng/ml; Infected: 12·90 ± 1·44 ng/mL; Anti-IL-17: 12·83 ± 1·56 ng/ml; $P < 0·05$ for both treatment groups compared with control mice; Fig. 5c), whereas there was no significant difference in IV-C levels between infected and infected/anti-IL-17 mAb-treated mice (Fig. 5c). Hence, IL-17 moderately decreased IV-C expression during *S. japonicum* infection. Interleukin-17 appears to differentially regulate the expression of collagen isoforms and contributes to hepatic fibrosis by promoting PC-III synthesis and deposition.

Role of IL-17 in SEA-specific IgG and IgE production

The evolving cellular response to *S. japonicum* infection is gradually accompanied by production of non-complement fixing IgG and IgE antibodies.[26] There were significant differences in the serum levels of SEA-specific IgG and IgE antibodies between uninfected control mice, mice infected with *S. japonicum*, and infected mice treated with anti-IL-17A mAb as detected by ELISA. Infected mice exhibited significantly elevated SEA-specific IgG (Fig. 6a; $P < 0·05$) and IgE (Fig. 6b; $P < 0·05$) levels compared with control mice. Levels of SEA-specific IgG were even higher in the anti-IL-17 group, although not significantly, while IgE levels were not altered by anti-IL-17A mAb. These effects held across different serum dilutions, suggesting that there was no saturation of ELISA binding sites. Hence, IL-17 normally serves to suppress SEA-specific IgG expression, but has little effect on SEA-specific IgE expression.

Discussion

Interleukin-17 is a pro-inflammatory cytokine that facilitates mucosal neutrophil recruitment by inducing the expression of downstream cytokines that mediate granulopoiesis as well as activating the local production of CXCR2 ligands that are chemoattractants for neutrophils.[27] It is a major inflammatory cytokine during infection, including infection by *S. japonicum*.[9,21,28] Schistosomes are parasitic trematodes that infect and cause disease in many vertebrate species. The main adaptive immune response against schistosomes is mediated by MHC class II-restricted $CD4^+$ T cells. An initial pro-inflammatory Th1-polarized response lasts into the period of early oviposition at around 5 weeks post-infection, at which time peri-oval granulomatous inflammation begins. However, within the next 1–2 weeks, granuloma formation increases concomitant with a dramatic change in the cytokine environment, which under normal circumstances becomes dominated by anti-inflammatory Th2-type cytokines.[26] Interleukin-4, IL-5 and IL-13 have previously been implicated in *S. japonicum*-associated liver fibrosis in mice and in humans.[29–32] Moreover, the granulomas that formed around the eggs in the liver were reported to be positively regulated by IL-17.[13,21,22]

We measured IL-17 production from mononuclear cells isolated from healthy and infected mouse livers by ELISA and FACS. Stimulation by anti-CD3 mAb plus anti-CD28 mAb induced much greater IL-17 release (and IFN-γ release) from lymphocytes of infected mice than from lymphocytes of uninfected mice ($P < 0·05$) (Fig. 1). Moreover, non-specific stimulation by PMA and ionomycin induced a much greater increase in the proportion of $IL\text{-}17^+$ cells in the $CD3^+$ T-cell population from infected livers than in the $CD3^+$ T-cell population from uninfected livers (Fig. 2). Hence, secretion of IL-17 by liver lymphocytes was significantly enhanced by *S. japonicum* infection.

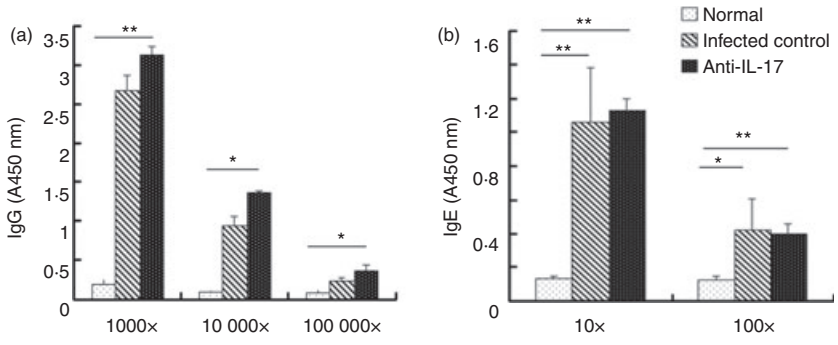

Figure 6. Role of interleukin-17 monoclonal antibody (IL-17 mAb) in soluble egg antigen (SEA) -specific IgG and IgE production. SEA-specific IgG and IgE antibodies in serum from normal, infected and anti-IL-17 mAb treated mice were detected by ELISA. All the samples were collected from 6-week-infected mice. The sera were serially diluted (1 : 10 or 1 : 100 for IgE; 1 : 1000, 1 : 10 000, or 1 :100 000 for IgG). IgG and IgE levels are expressed as the A_{450} values of the individual sera (*$P < 0·05$, **$P < 0·01$, the error bars indicate SD).

The normal liver contains significant numbers of resident lymphocytes, including T cells, B cells, dendritic cells, and natural killer cells. Recent reports have suggested that IL-17 is produced by natural killer cells, NKT cells, $\gamma\delta$T cells, neutrophils and $CD8^+$ T cells in addition to $CD4^+$ T (Th17) cells, at least in some diseases.[23–25] Our results (Fig. 2) demonstrated that *S. japonicum* infection could induce a small fraction of $CD3^-$ lymphocytes to produce IL-17, too. The $CD3^-$ lymphocytes may include B cells, dendritic cells, natural killer cells and neutrophils, so further investigation is required.

Previous reports indicated that $CD4^+$ T cells are the dominant IL-17 producers.[33,34] Moreover, Xiaoyun Wen *et al.* reported that the proportion of Th17 cells in the spleen, mesenteric lymph nodes and liver increased during *S. japonicum* infection, whereas Yuxia Zhang *et al.* reported that $CD4^+$ T (Th17) cells isolated from the hepatic granulomatous cell clusters of mice 6 weeks post-infection are the major IL-17-producing cells.[15,34] However, $\gamma\delta$T cells are thought to be the main source of IL-17 in several infectious conditions.[35–37] Studies of infected mice also found that the number of IL-17-producing $\gamma\delta$T cells far exceeded the number of IL-17-producing $CD4^+$ T cells.[38,39] In the current study, we compared IL-17 expression in $CD4^+$ T, NKT and $\gamma\delta$T lymphocyte subsets from normal liver and infected liver in response to non-specific stimulation with PMA and ionomycin. As shown in Fig. 3, NKT cells and $\gamma\delta$T cells were also sources of IL-17, and the proportions of IL-17^+ CD4, NKT and $\gamma\delta$T cells were significantly higher in infected mice than in uninfected control mice ($P < 0{\cdot}05$). The IL-17^+ $CD4^+$ T and IL-17^+ $\gamma\delta$T fractions accounted for $1{\cdot}05 \pm 0{\cdot}64\%$ and $0{\cdot}89 \pm 0{\cdot}38\%$ of the total $CD3^+$ T population after infection, respectively, both significantly higher than the IL-17^+ NKT fraction ($0{\cdot}49 \pm 0{\cdot}31\%$). However, IL-$17^+$ $\gamma\delta$T cells emitted the highest immunofluorescence of the three T-cell subtypes. We conclude that all three cell types are sources of IL-17 during *S. japonicum* infection, but that of these cell subtypes, $\gamma\delta$T cells produced the most IL-17. These results indicate that $\gamma\delta$T cells may be a first line of defence inflammation before $CD4^+$ T-cell responses during *S. japonicum* infection. These $\gamma\delta$T cells are a unique T-cell population because they function as part of the innate immune response and are capable of responding to cytokine signals in the absence of antigen,[40] demonstrating antigen specificity but requiring neither priming nor recruitment to the site of infection. Further investigations are required to elucidate the full spectrum of effects of this cell type during infection.

Infection with *S. japonicum* results in a granulomatous inflammatory and fibrosing reaction in liver and intestine against parasite eggs.[22] Granuloma formation is the result of a host adaptive immune response mediated by $CD4^+$ T cells specific for SEAs[3,22,26] that damages hepatocytes and destroys the normal histological structure of the liver (Fig. 4a). The main pathological lesions of hepatic schistosomiasis are the granuloma formed around schistosome eggs at the acute stage of the infection and subsequent liver fibrosis at chronic and advanced stages.[41] Hepatic fibrosis during schistosomiasis results from a massive deposition of extracellular matrix in the periportal spaces, leading to blockage of the portal veins with ensuing portal hypertension, as well as splenomegaly, portocaval shunting and gastrointestinal varices.[2] Persistent fibrosis in chronic schistosomiasis may cause hepatic cirrhosis and liver cancer, both associated with high mortality. The severity of liver pathogenesis may be correlated with IL-17 activity, possibly released by inflammatory cells such as neutrophils and eosinophils in the granulomas.[22] We administered an anti-IL-17 mAb to infected mice to evaluate the role of IL-17 in the host protective responses against *S. japonicum* infection. Gross examination of livers excised from control, infected and infected/anti-IL-17 mAb-treated mice (Fig. 4a) indicated that infection caused inflammatory cell infiltration that was partially blocked by anti-IL-17 mAb, strongly implicating IL-17 in this response. Moreover, schistosome infection also produced hepatic granulomatous inflammation, which was significantly reduced by anti-IL-17 mAb ($P < 0{\cdot}05$ compared with infected mice) as measured by granuloma cross-sectional area. Taken together, these results are consistent with previous reports demonstrating a positive relationship between IL-17 activity and the severity of liver pathogenesis and reports implicating IL-17 in the granulomatous inflammatory reaction during *S. japonicum* infection.

Histological observations using Masson's trichrome staining also revealed liver fibrosis (collagen deposition) and inflammatory cell infiltration in infected mouse liver, responses that were also suppressed by anti-IL-17 mAb (Fig. 5a). Both pro-peptide and mature collagen type III can be used as a biomarker for liver fibrosis, and serum levels of type IV collagen predict the state of liver fibrosis,[42] which in turn reflects collagen degradation.[43] Hence, the levels of PC-III and IV-C may reflect the current pathological progress of schistomiasis.[44] Comparison of infected and infected/anti-IL-17 mAb-treated mice suggested that IL-17 may increase PC-III expression, possibly by inducing type III collagen synthesis, whereas anti-IL-17 mAb may attenuate fibrosis by reducing extracellular collagen III deposition in the liver (Fig 5b). In contrast to PC-III, the difference in hepatic IV-C expression between infected and infected/anti-IL-17 mAb-treated mice was minimal (Fig 5c), suggesting that IL-17 does not regulate collagen degradation. An alternative hypothesis is that the level of IV-C may be affected by other pathological conditions, such as lung or intestine failure, but this requires further investigation. Taken together, these data demonstrated that IL-17 contributes to the formation and development of fibrosis during

S. japonicum infection and that targeting IL-17 may have therapeutic potential for treatment of schistosomiasis.

Numerous *in vitro* studies on the immunology of schistosomiasis have clearly demonstrated the essential role of antibodies in various effector or regulatory mechanisms.[45] Abundant productions of non-complement fixing IgG and IgE antibodies were measured after infection. In our study, the levels of SEA-specific IgG and IgE antibodies in mouse serum were measured to examine associations between schistosome-specific antibody responses and anti-IL-17 mAb treatment during *S. japonicum* infection. The serum titres of SEA-specific IgGs and IgEs increased rapidly during infection. The level of SEA-specific IgG was actually increased further by anti-IL-17 mAb, whereas there was no significant effect of this treatment on IgE titres, which is consistent with the results of Xiaoyun Wen *et al.*[15] As the humoral response plays an important role in anti-schistosome immunity,[46,47] IL-17 may be a target for therapeutic intervention in schistosomiasis patients, as it is in inflammatory bowel disease and mycoplasma pneumonia.[48,49]

In conclusion, we demonstrate that the IL-17$^+$ lymphocyte population expands significantly in the liver of C57BL/6 mice during infection by *S. japonicum*. In particular, $\gamma\delta$T lymphocytes represented the largest population of IL-17-producing cells. Moreover, we found that IL-17 was involved in granuloma formation, infiltration of inflammatory cells and collagen deposition. Anti-IL-17 mAb therapy may be helpful in preventing inflammation-mediated liver damage caused by schistosomiasis.

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Disclosures

The authors have no financial conflict of interest.

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