T cell-mediated immunity in CBA mice during *S. japonicum* infection

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Abstract

Characterisation of the cellular immune response to schistosomiasis is well established for *Schistosoma mansoni* but a comprehensive description of T cell-mediated immune responses against *S. japonicum* infection is lacking. In this study, twenty CBA mice were infected with cercariae of *S. japonicum* and the immune response at different time points (3, 4, 5 and 6 weeks post infection) determined. Mouse spleen and liver cells were isolated from the mice and stimulated with schistosomal adult worm antigen (SWAP) and schistosomal egg antigen (SEA). Flow cytometry was undertaken to evaluate the immune response generated against these antigens. There was a relatively higher Th1 immune response to SWAP at the early phase of infection (Weeks 3-5 post-infection). However, a Th2 immune response directed against SEA was dominant at week 6 post-infection, a time point when the highest IgG response against both SWAP and, especially, SEA was generated. Furthermore, there was a consistent increase of IL-17 producing $CD4^+$ cells over the course of the infection in both spleen and liver with gamma-delta T cells being the main producers in the latter organ. The regulatory immune response was highest at the early phase of the immune response followed by a rapid decline at week 6-post infection. Prior to egg-laying, *S .japonicum* induced a regulatory T cell immune response which may limit the early Th1-mediated immune response that is believed to be protective in murine schistosomiasis. Following egg laying, the immune response was polarized to a Th2 immune response mainly directed against the eggs and this may contribute to parasite survival.

Keywords: Schistosomiasis, T cell immune response, Th1-Th2 shift, IL-17, Regulatory T cells

Introduction

Schistosomiasis is a chronic parasitic disease and the cause of significant residual morbidity; consequently it is of considerable public health importance, having substantial socioeconomic impacts on many impoverished communities [1]. The pathogenesis in schistosomiasis is due mainly to the immunological response against schistosome eggs [1]. Many previous studies on the immunology of schistosomiasis showed that a Th1 polarized immune response occurs at the early phase of infection followed by switch to a Th2-type response [2]. However, this model has only been clearly demonstrated using *Schistosoma. mansoni* but a full and detailed description of the Th1-Th2 shift in regard to *S. japonicum infection* is lacking although the currently available information suggests that this paradigm broadly holds true for this species as well [3, 4]. In contrast to infection with *S. mansoni*, neutrophils have been implicated as the main cell type involved in hepatic granulomatous pathology in schistosomiasis japonica [5] and higher egg output (at least four times greater) and greater severity of immunophathology is also observed [6]. Thus, even though both schistosomes likely share many common antigenic determinants, it is probable that there are unique species-specific antigens which led early investigators to suggest there are differences in the host immune response to the two bloodflukes [7, 8].

A recent study by Xu et al. [3] showed that the Th1-Th2 shift appears to operate in the low pathology C57BL/6 mouse strain infected with *S. japonicum* [3] but, to our knowledge, it is not known whether the shift occurs in the high pathology CBA mouse which exhibits a stronger splenic proliferative response and a lesser suppressor T cell response after a patent schistosome infection [9, 10]. Therefore, we sought to analyse the immune responses generated in CBA mice during the course of infection with S. *japonicum* particularly as this

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McManus DP, Dunne DW, Sacko M, Utzinger J, Vennervald BJ, Zhou X-N. Schistosomiasis. *Nat Rev Dis Primers* 2018; **4**:13.

strain has been used widely in schistosomiasis vaccine trials to screen vaccine candidates [11-17].

Until recently, Th1 and Th2 cells were considered to be the predominant T cells involved in the immune response to schistosomiasis. However, following the discovery of Th17, there has been a paradigm shift from the CD4 Th1/Th2 cell paradigm to include Th17, and endeavours to uncover the role of Th17 cells in schistosome immunity, especially in relation to the development of schistosomiasis immunopathology. Limited studies in C57BL/6 mice showed generally that there is a correlation between a high level of IL-17 and disease severity [18, 19]. A limitation of much research conducted to date on the immunology of schistosomiasis has been the dependence on the C57BL/6 mouse [20], due to the availability of various genetically modified strains on this background. Accordingly, studies on the Th1–Th2 shift and the involvement of Th17 in murine schistosomiasis japonica with other mouse strains such as high pathology CBA mice are limited or have not been undertaken. Such studies are very important in that a wider view of the area will help further understanding of the immune response to schistosomiasis.

Studies, mainly focussing on *S. mansoni,* showed that naturally occurring regulatory T cells (Tregs) play an essential part in the immune response to schistosomiasis by controlling both Th1 and Th2 effector cells [21-23]. For example, one report revealed that Tregs can suppress Th2 cytokine production in C57BL/6 mice induced by immunization with *S. mansoni* eggs [24]. In contrast, another study showed that Tregs bring about Th2 polarization by inhibiting the Th1 immune response [25]. As knowledge on Tregs in schistosomiasis japonica is limited, we sought to determine the effector and regulatory T cell immune responses in CBA mice at different time points following *S. japonicum* infection.

Materials and methods

Parasites

Oncomelania hupensis hupensis, naturally infected with *S. japonicum*, were obtained from an endemic area in Anhui Province, PR China, and transported to the QIMR Berghofer Medical Research Institute in Australia. Cercariae were shed from the infected snails and collected as described [26].

Mice

Twenty female CBA mice (6–8 weeks old) were anaesthetised by intraperitoneal injection (IP) with ketamine (100mg/kg) and Xylazin-20 (30 mg/kg), their abdomens were shaved and exposed for 40 minutes to 34 *S. japonicum* cercariae. Five CBA mice were culled and perfused with PBS at weeks 3, 4, 5 and 6 post–challenge infection. Fifty µl of blood was collected from each mouse by tail bleed prior to culling. The livers and spleens of the individual mice were collected for cell isolation. Moreover, a piece of liver was cut from each mouse and used for liver egg counts. Five naive mice were also used as controls.

Worm and liver egg counts

Adult *S. japonicum* worms were collected by perfusion at weeks 4, 5 and 6 post infection and the total worm count and worm pair numbers were determined. Liver egg counts were performed to determine the number of eggs per gram (EPG) in the liver as described [27]. Briefly, a weighed liver portion was digested with 4% (w/v) KOH and incubated overnight at 37^0C. Then the homogenate was centrifuged at 2000g for 10 minutes, washed with 4% KOH (w/v), the supernatant was discarded and the pellet containing eggs was resuspended and fixed in 10% (v/v) paraformaldeyde. Counting of the eggs was performed four times for each sample and the average was taken to calculate the liver EPG.

Adult worm soluble antigen preparation (SWAP)

Freshly purfused adult worms of *S. japonicum* were washed with Tris buffer PH 7.4, and homogenised for 5 minutes on ice using a motor cord-less homogeniser (Kimble Chase, USA) and the homogenate was the centrifuged at 16,000g [28] for 30 minutes. Protein concentration was measured by standard Bradford protein assay (Biorad, Hercules, CA, USA) using bovine serum albumin as standard.

Soluble egg antigen (SEA)

Livers from mice infected with *S. japonicum* were chopped and digested with 100 mg/ml Collagenase B (Roche, Mannheim, Germany) overnight at 37^0C on a rocker. Following washing by spinning at 2000 rpm for 5 minutes and resuspending with ice cold 1× PBS, the preparation was passed through 250µm and 150µm sieves. The isolation of eggs was achieved by centrifuging the preparation at 3000rpm for 5 minutes over a percoll (MA, USA)-0.25M sucrose gradient [29]. The isolated eggs were homogenized in PBS, examined under a microscope to confirm they were completely disrupted, centrifuged at 10,000g for 2 hours at 4^0C and the supernatant was used as SEA. Protein concentration was measured by standard Bradford protein assay.

Lipopolysaccharide (LPS) removal from the SWAP and SEA

LPS removal from SWAP and SEA was undertaken using Triton X-114 (Sigma-Aldrich, St Louis USA) as described [30]. Briefly, 1% Triton×-114 was added to the SWAP or SEA preparations and the mixture was incubated for 30 minutes at 4^0C on a wheel shaker, followed by 10 minutes incubation at 37^0C. The samples were then centrifuged at room temperature at 20,000×g for 10 minutes and the upper aqueous phase containing the SWAP or SEA preparation was retained. Residual endotoxin removal was confirmed using a Pierce

LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assays (ELISAs)

100 µl of 1µg/ml of SWAP or SEA in buffer (Bicarbonate buffer PH 9.6) were coated separately on immunoplates (NUNC, Roskilde, Denmark) at 4^0C overnight. The plates were blocked with 5% (v/v) skim milk in PBS for an hour at 37^0C and then washed 3 times with PBS containing 0.05% (v/v) Tween 20 (PBST). Mouse serum samples, serially diluted (1:100-1:12,800) with 5% (w/v) skim milk in PBST (100µl/well), were added to the plates which were incubated at 37^0C for 2 hours. Sheep anti- mouse IgG antibody conjugated with peroxidise (diluted 1: 20, 000), used as secondary antibody, was added to the plates which were incubated for 1 hour at 37^0C. PBST washes were applied five times after each step, 2 minutes between each wash. Finally, reactions were developed with 3,3′,5,5′-tetramethylbenzidine substrate solution (TMB) (Aldrich, St Louis, Mo, USA) (100µl/well) for 5 minutes, and stopped using 2N sulphuric acid (50 µl /well). Optical density (OD) values were read at 450 nm using a microplate reader, and all tests were run in duplicate for each test plate. A positive antibody response was defined as an OD value higher than 2 times the mean OD values of serum samples from the naive, control mice.

Spleen and liver cell preparation and stimulation

Spleens were gently pressed through a 70µm cell strainer to produce a single cell suspension. Red blood cells were removed from the cell suspension using RBC lysis buffer (Sigma, St.Louis, MO, USA), before it was washed and resuspended in Iscove's Modified Dulbeccco's Medium (IMDM; Gibco,Grand Island, NY,USA) to enable counting of viable cells.

Mouse liver was chopped and then digested with 5 ml collagenase (5 mg collagenase Type IV (Sigma), 5 mL Hank's balanced salt solution HBSS (Sigma) and 200 µg DNase (Sigma)) and the preparation was then pressed through 100µm cell strainers. The filtered solution was then washed with 2% (v/v) foetal bovine serum in phosphate buffered saline (FBS/PBS), the sedimented pellet resuspended, Percoll density gradient medium added and the suspension centrifuged at 1700 rpm for 12 minutes at room temperature. The resulting supernatant containing hepatocytes, debris and the Percoll medium was discarded, the pellet was resuspended in 1ml of RBC lysis buffer (Sigma) and the preparation was incubated for 5 minutes at room temperature. Then, 9 ml of (2% v/v) FBS/PBS was added, the preparation was centrifuged at 1200 rpm for 6 minutes at 4^0 C, the supernatant discarded and the pellet resuspended in IMDM.

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The purified spleen and liver cells were counted and their viability assessed using the trypan blue exclusion method [31]. Once counted, cells were resuspended at a concentration of 5 x 10^6/mL and used to plate flat bottom 96 well plates. The cells were stimulated with SEA (10µg/ml), SWAP (10µg/ml), Concanavalin A (Con A) (0.5 µg/ml), as a positive control; IMDM only was added to cells plated as negative controls. After cell culture for 68 hours, Brefeldin A (BFA), at a final concentration of 200 µg/ml, was added to the plates. Cells were harvested 4 hours after the addition of BFA.

Flow cytometryic analysis

Effector T cell assessment

Cells were first surface stained with Brilliant Violet 421 conjugated anti CD3 (Clone 17A2, Biosearch, CA, USA), Alexa Fluor 700 conjugated anti CD4 (Clone: RM4-5, BD Bioscience, New Jersey, USA), Brilliant Violet 650 conjugated anti CD8 (Clone: 53-6.7, Biosearch) and Alexa Fluor 488 conjugated anti γδ TCR (Clone: GL3, Biosearch). Following washing with

2% (v/v) FBS, cells were fixed by fixation/permeabilization solution (BD Biosearch) and washed with BD perm/wash (BD, Biosciences). Intracellular staining was performed using PerCpCy5.5 conjugated anti IFNγ (Clone: XMG 1.2, Biosearch), Phycoerythrin (PE)-CF594 conjugated anti IL-4 (Clone: 11B11, BD Bioscience) and Alexa Fluor 647 conjugated anti IL-17 (Clone: TC11-18H10.1, Biosearch).

Regulatory T cell assessment

Cells were first surface stained with Brilliant Violet 421 conjugated anti CD3 (Clone: 17A2, Biosearch), Alexa Fluor 700 conjugated anti CD4 (Clone: RM4-5, BD Bioscience) and PerCP5.5 conjugated anti CD25 (Clone: PC61, BD Bioscience). Following washing with 2% FBS, cells were fixed by transcription factor (TF) Fix/Perm buffer (BD Bioscience) and washed with BD TF perm/wash (BD, Biosciences). Intracellular staining was performed using PE-CF594 conjugated anti FoxP3 (Clone: MF23, BD Bioscience).

Fixable viability stain 780 (BD, Horizon TM), for discrimination of viable from non-viable cells and purified rat anti-mouse CD16/CD32 (mouse CD Fc Block TM) (2.4G2, BD Pharmingen) for blocking non specific binding, were used for both effector T cell and regulatory T cell assessment. Fluorescent Minus One (FMO), single stained cells and anti-rat and anti-hamster Ig k and negative control compensation beads (BD, Bioscience) were also prepared to act as controls and to allow for fluorochrome compensation. The stained cells were analysed using Fortessa 4A and FACS Diva software (BD, Bioscience). Analysis of the data was carried out using Flowjo version 10 software.

Statistical analysis

All data are presented as the mean ± SEM. Differences between groups were assessed for statistical significance using the two-tailed t-test. A statistically significant difference for a

particular comparison was defined as P value ≤ 0.05 using GraphPad Prism software (Version 7.02).

Results

Worm and egg burdens in *S. japonicum*-infected CBA mice

CBA mice infected with *S. japonicum* were perfused at weeks 3, 4, 5 and 6 post- infection and worms were collected and counted. A small number of immature worms were observed at 3 weeks post- infection. At week 6 post- infection, the average total number of worms and worm pairs were 27±2 and 12+2, respectively. Liver egg burdens determined for the different time points showed a consistent increase over the course of infection, commencing at week 4 post-infection (Figure 1).

Figure 1. Worm and liver egg burden in *S. japonicum*- infected CBA mice. At each time point post-challenge infection, five infected CBA mice were culled and perfused A. Number of worms collected at the different time points post infection. B. Number of paired worms collected at the different time points post infection. C. Number of eggs per gram of liver. The error bars in the panels represent the standard error of the mean (SEM).

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Increased immune cell numbers in spleen and liver cells after *S. japonicum* infection
Isolation and counting of immune cells from the spleen and liver of infected and control mice revealed the total number of the cells in both organs increased in *S. japonicum*-infected animals over the course of the infection (Figure 2).

Figure 2. Number of immune cells in *S. japonicum* -infected CBA mice at different time points post-infection. At each time point, five infected mice were culled and the spleens and livers removed and five naive mice were used as controls. A. Number of splenocytes B. Number of liver lymphocytes.

The serum IgG response was higher against egg-derived antigens than against adult worm-derived antigens

Generally, there was a trend of increased IgG response against both SWAP and SEA in the sera of mice over the course of the infection (Figure 3). At week 6 post-infection, the IgG response was significantly more pronounced (p= 0.0159) against SEA (titre of 1: 3200) than SWAP (titre of 1:200) in infected animals whereas at weeks 5 and 6 post- infection the IgG response against SEA was significantly higher (p=0.0357) than that from uninfected controls (Figure 3).

Figure 3. Levels of anti-SWAP and anti-SEA-IgG antibodies determined by ELISA in sera from *S. japonicum*-infected CBA mice at different time points post-infection and from uninfected control animals. At each time point, blood was taken from five *S. japonicum* infected mice and five naive (uninfected) animals. The OD values determined for anti-SEA and anti-SWAP IgG serum antibodies at different time points were compared; *p< 0.05 is significant. **SWAP-ELISA**: Schistosome soluble adult worm antigen preparation-enzyme-linked immunosorbent assay; **SEA-ELISA**: Schistosome soluble egg antigen-enzyme-linked immunosorbent assay.

The Th1 immune response is higher during the early phase *S. japonicum* infection and is generated against adult worm derived antigens.

The percentage of $CD4^+$ T splenocytes producing IFN-γ increased consistently up to week 5 post-infection but this was followed by a dramatic decrease by week 6 (Figure 4A; Supplementary Figure 1A). The highest proportion of IFN-γ-producing $CD4^+$ splenocytes was observed when cells were stimulated with SWAP (Figure 4A).

The production of IFN-γ by liver $CD4^+$ lymphocytes after culture with SEA and SWAP declined substantially at week 6 post-infection (Figure 4B), similar to that observed with the splenocytes, following a consistent increase over the previous weeks (Figure 4A;

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Supplementary Figure 1B). However, the proportion of IFN-γ-producing liver lymphocytes was highest when restimulated with SEA (Figure 4B).

Figure 4. Frequency of IFN-γ producing CD4$^+$ splenocytes and liver lymphocytes of *S. japonicum*-infected CBA mice at different time points post-infection following stimulation with different antigens in vitro. A. The change in percentage of IFN-γ-producing CD4+ splenocytes (Th1 cells) over the course of the infection. B. The change in percentage IFN-γ-producing CD4$^+$ liver lymphocytes (Th1 cells) over the course of the infection.

A Th2 immune response follows egg-laying by *S. japonicum*

Unlike IFN-γ-producing splenocytes, the percentage of IL-4 producing CD4$^+$ splenocytes was low and showed a slightly decreasing trend at the early phase of *S. japonicum* infection but a dramatic increase in percentage of these cells occurred at week 6 post-infection. This time point coincided with the highest egg burden in the liver, likely correlating with a Th1/Th2 shift (Figure 5A; Supplementary Figure 2A). The highest proportion of IL-4-producing CD4$^+$ splenocytes occurred when these cells were stimulated with SEA (Figure 5A). Similarly, the percentage of CD8$^+$ splenocytes producing IL-4 increased sharply at week 6 even though the number was relatively low compared with the CD4$^+$ splenocytes (Figure 5B).

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The trend of percentage of IL-4 producing liver lymphocytes was similar to that of splenocytes in that we observed a low percentage up to week 5 followed by a sharp increase by week 6 (Figure 5C; Figure 6B; Supplementary Figure 2B).

Figure 5. Frequency of IL-4 producing cells by *S. japonicum*-infected CBA mice at different time points post-infection following stimulation with different antigens in vitro. A. Change in IL- producing $CD4^+$ splenocytes (Th2 cells) over the course of infection. B. Change in IL-4-producing $CD4^+$ liver lymphocytes (Th2 cells) over the course of infection. C. Change in IL-4-producing $CD8^+$ liver lymphocytes (Th2 cells) over the course of infection.

Figure 6. Flow cytometry analysis of IFN-γ producing cells (Th1) and IL-4 producing cells (Th2) at different time points post-*S. japonicum* infection of CBA mice. The histogram shows the percentage change of Th1 and Th2 cells over the course of the *S. japonicum* infection in CBA mice. A. Frequency of $CD4^+$ splenocyte producing IFN-γ (Th1 cells) and IL-4 (Th2 cells). B. Frequency of $CD4^+$ liver lymphocytes producing IFN-γ (Th1 cells) and IL-4 (Th2 cells).

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Supplementary Figure 1. Flow cytometry analysis of IFN-γ- producing splenocytes and liver lymphocytes (Th1 cells) of CBA mice at different time points post *S. japonicum* infection. A. Flow cytometry chart showing percentage changes of Th1 ($CD3^+$ CD4+ $IFN\gamma^+$) cells in the spleen of infected mice at different time points. B. Flow cytometry chart showing percentage changes of Th1 ($CD3^+$ $CD4^+$ IFNγ+) cells in the liver of infected mice at different time points. The percentage of INF-γ- producing cells in both panels was estimated from the total number of $CD4^+$ T cells.

Suppementary Figure 2. Flow cytometry analysis of IL-4-producing splenocytes and liver lymphocytes (Th2 cells) of CBA mice at different time points post-*S. japonicum*

infection. A. Flow cytometry chart showing percentage changes of Th2 ($CD3^+$ $CD4^+$ $IL\text{-}4^+$) cells in the spleen of infected mice at different time points. B. Flow cytometry chart showing percentage changes of Th2 ($CD3^+$ $CD4^+$ IL- 4^+) cells in the liver of infected mice at different time points. The percentage of IL-4- producing cells in both panels was estimated from the total number of $CD4^+$ T cells.

Gamma delta T cells are the major cells producing IL-17 in the liver

The frequency of IL-17-producing $CD4^+$ cells in both splenocytes and liver lymphocytes consistently increased over the course of the *S. japonicum* infection (Figure 7A; Figure 7B). However, gamma delta T cells were the main cells producing IL-17 in the liver and similar to $CD4^+$ cells, they generally showed a consistent increase over the course of the infection with a sharp increase at week 6 post-infection. Furthermore, a higher proportion of IL-17-producing cells was found in the liver (20%) compared with the spleen (2%) (Figure 7A; Figure 7C).

Figure 7. **Frequency of IL-17-producing cells in *S. japonicum*-infected CBA mice at different time points post-infection following stimulation with different antigens in vitro**. A. Frequency of IL-17-producing $CD4^+$ splenocytes. B. Frequency of IL-17-producing $CD4^+$ liver lymphocytes. C. Frequency of IL-17-producing gamma delta T cells ($CD3^+$ CD4-CD8-$\gamma\delta TCR^+$) in the liver.

The regulatory T cell immune response is higher during the early phase of infection

There was a consistent gradual increase in the frequency of regulatory T splenocytes over the course of the infection but a sharp decrease occurred at week 6 post-infection (Figure 8A; Supplementary Figure 3). Examination of the regulatory T cell immune response in the liver was not undertaken at weeks 3 and 4 post-infection due to the low number of immune cells at

these time points but a decrease in the number of regulatory T cells was observed from week 5 to 6 post-infection (Figure 8B).

Figure 8. Frequency of regulatory T splenocytes in S. *japonicum*-infected CBA mice over the different time points following stimulation with different antigens in vitro. A. Frequency of regulatory T cells ($CD3^+$ $CD4^+$ $CD25^+$ $FoxP3^+$) in the spleen. B. Frequency of regulatory T cells in the liver ($CD3^+$ $CD4^+$ $CD25^+$ $FoxP3^+$).

Supplementary Figure 3. Flow cytometry chart showing percentage changes of regulatory T cells ($CD3^+CD4^+CD25^+FoxP3^+$) in the spleen of CBA mice at different time points post-*S. japonicum* infection. The percentage of regulatory T cells was estimated from the total $CD4^+$ T cells present. Five naive mice were used as controls.

Discussion

Studies on understanding immune response mechanisms against schistosomes have focused predominantly on *S. mansoni* and have provided convincing evidence that a Th1-type immune response dominates against this species in the early phase of infection but is followed by a Th1/Th2 shift following egg deposition [2, 32]. In contrast, similar detailed information on the immune response against *S. japonicum* is lacking. In addition, much of our accumulated knowledge on the immunology of schistosomiasis comes from data obtained with the commonly used C57BL/6 mouse strain. However, use of the CBA mouse, in which schistosomiasis pathology is more severe, may reflect distinct differences in immunological features as it has been shown that this strain produces a stronger splenic proliferative response and a reduced suppressor T cell response after a patent *S. mansoni* infection than do infected C57BL/6 mice [9, 33]. We report here the results of a similar in depth study with *S. japonicum*-infected CBA mice which, to our knowledge, had not been undertaken hitherto.

Experimental helminth models have shown how the interaction between Th1, Th2, Th17 and regulatory T cells (Tregs) can affect resistance to infection [34, 35]. Accordingly we also examined the effector T cell and regulatory T cell immune responses in the spleen and liver of CBA mice at different time points post-infection with *S. japonicum*. In general, there was an increase in the number of splenocytes and liver immune cells over the time course of the infection. There was consistent increased Th1 immune response up until week 5 post-infection and it was higher against the adult parasite derived protein (SWAP) than egg derived protein (SEA) suggesting the immune response was directed primarily against the adult worm. The Th1 immune response observed at the early phase of the infection may be targeting killing of the schistosomulum stage as it has clearly been shown previously that the Th1 response in murine schistosomiasis is crucial in killing these larvae and/or the adult parasites by inducing macrophages and B cells to produce complement fixing and opsonising antibodies which eventually result in host resistance [3, 36-40]. However, in the current study, the Th1 immune response declined sharply at week 6 post-infection, a time point when considerable numbers of eggs are being laid, and was replaced by a Th2 immune response, a finding in concordance with other studies showing a Th1/Th2 shift after infection of mice with both *S. japonicum* and *S. mansoni* [2, 3, 41].

The increased Th2 response may be triggered by the increased numbers of eggs trapped in the liver at week 6 post-infection as the immune response was higher against SEA than SWAP (Figure 5) and there was also high liver egg burden at this time point (Figure 1). Such a polarized Th2 immune response may lead to hepatic pathology and favour the development of pathogenic IgE [38]. It is clear that a Th2 type of immune response induces humoral immunity by inducing the production of IgG1 [34]. The current study also demonstrated that there was a consistent increase in IgG titre over the time course of the *S. japonicum* infection with a sharp increase at week 6 post-infection. Moreover, a significantly

higher IgG response was observed against the egg derived antigen (SEA) than worm derived antigen (SWAP) indicating the predominant Th2 immune response is directed against parasite eggs.

Even though there was a trend towards an increased Th1 immune response at the early phase of infection (up to week 5 post-infection), the frequency of IFN-γ-producing cells (Th1 immune response) was lower compared with the generated Th2 immune response at the later phase of the infection. This may be the reason why most of the schistosomula were able to survive and mature into adult parasite (more than 70% of the cercariae used to infect the CBA mice were recovered as adult worms) (Figure 1). Moreover, we also demonstrated a trend of increasing T regulatory immune response at the early phase of infection which likely suppressed the potentially parasite-damaging Th1 immune response [34, 39, 42-46]. Similar to the current report, there is some prior evidence that schistosomes can induce T regs. Sm29, a membrane-bound glycoprotein found on the tegument of *S. mansoni*, with homology to some unknown *S. japonicum* proteins, has been shown to increase the production of immune regulatory cytokines, particularly IL-10, which can inhibit the Th1 immune response [47]. However, in contrast, another study showed that Foxp3(+) Treg cells did not play a prominent role in regulating immunity to *S. mansoni* larvae, [48]; consequently, the different factors accounting for these contradictory findings require further investigation.

Surprisingly, there was a sharp decline of Treg cells at week 6 post-infection in both the spleen and liver which may have contributed to the increased Th2 immune response at this time point. This type of unregulated Th2 immune is one of the reasons for the severe fibrosis characteristic of murine schistosomiasis [49]. There is also evidence showing that a deficiency of T regs can result in immunopathology and that $CD25^+$ T regs can prevent this immunopathological response directed against eggs [22, 34, 50]. The reasons why this phenomenon (high Th2 immune response with low T reg response) was observed in the

present study may be because: a). High pathology CBA mice were used rather than the more common C57BL/6 strain in which pathology is less severe [51]; and b). The CBA mice were infected with a relatively high cercarial dose (34) of *S. japonicum* resulting in a higher liver egg burden and a more pronounced chronic infection than reported in other studies.

We additionally examined the IL-17 mediated immune response against *S. japonicum* and showed a consistent increase in the percentage of IL-17 producing cells over the time course of the infection in both the spleen and liver. This IL-17-mediated immune response could have been directed against *S. japonicum* eggs trapped in the liver [20]. IL-17 also plays an important role in contributing to the granulomatous inflammatory and fibrosing reactions in schisotosome-infected mice [19]. Moreover, IL-17 mediated immunity and a Th2 immune response recruit eosinophils and neutrophils, respectively, for the formation of liver granulomas [35]. In the present study, the main IL-17 producing cells in the liver were gamma delta T cells and a sharp increase in the numbers of these cells was observed at week 6 post-infection, supporting a previous study with *S. japonicum*-infected C57BL/6 mice [19].

In summary, we demonstrated a Th1/Th2 shift in the immune response over the course of a *S. japonicum* infection in CBA mice. Moreover, we showed a significantly high involvement of gamma delta T cells in the infected liver in producing IL-17 late on in the immune response at a time point coinciding with the accumulation of high numbers of eggs in the liver. T regulatory cells were relatively highly engaged at the early phase of the infection and were probably induced by the parasite to suppress the disadvantageous Th1 immune response that could potentially clear the infection. Hence, down regulating the T regulatory response at an early phase of infection may help the host clear the infection even though this may also contribute to host injury by the generation of a pro-inflammatory Th1 immune response. Generally, the Th2 immune response observed in the present study, which was predominant at the later phase of the infection, seems more of a Type II inflammatory

type rather than a Type II regulatory response [35] as the former is more common in allergic and fibrotic reactions of the type evident in CBA mice which produce a severe form of schistosome-induced fibrosis. We also speculate that anti-schistosome vaccine candidates that can primarily induce a pronounced Th1 immune response before the parasite commences egg-laying would result in parasite clearance and that enhanced host resistance to schistosomiasis might be feasible.

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References

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1. Wilson, M.S., et al., *Immunopathology of schistosomiasis.* Immunology and cell biology, 2007. **85**(2): p. 148-154.
2. Pearce, E.J. and A.S. MacDonald, *The immunobiology of schistosomiasis.* Nature Reviews Immunology, 2002. **2**(7): p. 499-511.
3. Xu, X., et al., *Activation-induced T helper cell death contributes to Th1/Th2 polarization following murine Schistosoma japonicum infection.* J Biomed Biotechnol, 2010. **2010**: p. 202397.
4. Farwa, A., et al., *Immune modulation of Th1, Th2, and T-reg transcriptional factors differing from cytokine levels in Schistosoma japonicum infection.* Parasitol Res, 2018. **117**(1): p. 115-126.

5. Chuah, C., et al., *Spatial and temporal transcriptomics of Schistosoma japonicum-induced hepatic granuloma formation reveals novel roles for neutrophils.* J Leukoc Biol, 2013. **94**(2): p. 353-65.
6. Mitchell, G.F., W.U. Tiu, and E.G. Garcia, *Infection characteristics of Schistosoma japonicum in mice and relevance to the assessment of schistosome vaccines.* Adv Parasitol, 1991. **30**: p. 167-200.
7. Erickson, D.G., C.E. Jones, and D.B. Tang, *Schistosomiasis mansoni, haematobia, and japonica in hamsters: Liver granuloma measurements.* Experimental parasitology, 1974. **35**(3): p. 425-433.
8. Von Lichtenberg, F., D.G. Erickson, and E.H. Sadun, *Comparative histopathology of schistosome granulomas in the hamster.* Am J Pathol, 1973. **72**(2): p. 149-78.
9. Tran, M.H., et al., *Tetraspanins on the surface of Schistosoma mansoni are protective antigens against schistosomiasis.* Nat Med, 2006. **12**(7): p. 835-40.
10. Lewis, F.A. and E.M. Wilson, *Strain differences in lymphocyte responses and in vitro suppressor cell induction between Schistosoma mansoni-infected C57BL/6 and CBA mice.* Infection and Immunity, 1981. **32**(1): p. 260-267.
11. Ranasinghe, S.L., et al., *Kunitz-type protease inhibitor as a vaccine candidate against schistosomiasis mansoni.* International Journal of Infectious Diseases, 2018. **66**: p. 26-32.
12. You, H., et al., *Suppression of the insulin receptors in adult Schistosoma japonicum impacts on parasite growth and development: further evidence of vaccine potential.* PLoS neglected tropical diseases, 2015. **9**(5): p. e0003730.
13. You, H., et al., *The insulin receptor is a transmission blocking veterinary vaccine target for zoonotic Schistosoma japonicum.* Int J Parasitol, 2012. **42**(9): p. 801-7.

14. Nawaratna, S.S., et al., *Lysosome-associated membrane glycoprotein (LAMP)–preliminary study on a hidden antigen target for vaccination against schistosomiasis.* Scientific reports, 2015. **5**: p. 15069.

15. Zhang, W., et al., *Inconsistent protective efficacy and marked polymorphism limits the value of Schistosoma japonicum tetraspanin-2 as a vaccine target.* PLoS neglected tropical diseases, 2011. **5**(5): p. e1166.

16. Don, T.A., J.M. Bethony, and A. Loukas, *Saposin-like proteins are expressed in the gastrodermis of Schistosoma mansoni and are immunogenic in natural infections.* International Journal of Infectious Diseases, 2008. **12**(6): p. e39-e47.

17. Verity, C.K., D.P. McManus, and P.J. Brindley, *Cellular responses to Schistosoma japonicum cathepsin D aspartic protease.* Parasite Immunol, 2002. **24**(7): p. 363-7.

18. Zhang, Y., et al., *IL-17 neutralization significantly ameliorates hepatic granulomatous inflammation and liver damage in Schistosoma japonicum infected mice.* Eur J Immunol, 2012. **42**(6): p. 1523-35.

19. Chen, D., et al., *Characteristics of IL-17 induction by Schistosoma japonicum infection in C57BL/6 mouse liver.* Immunology, 2013. **139**(4): p. 523-32.

20. Larkin, B.M., et al., *Induction and regulation of pathogenic Th17 cell responses in schistosomiasis.* Seminars in Immunopathology, 2012. **34**(6): p. 873-88.

21. Taylor, J.J., M. Mohrs, and E.J. Pearce, *Regulatory T cell responses develop in parallel to Th responses and control the magnitude and phenotype of the Th effector population.* J Immunol, 2006. **176**(10): p. 5839-47.

22. Turner, J.D., et al., *CD4+CD25+ regulatory cells contribute to the regulation of colonic Th2 granulomatous pathology caused by schistosome infection.* PLoS Negl Trop Dis, 2011. **5**(8): p. e1269.

23. Dewals, B., et al., *Control of Schistosoma mansoni egg-induced inflammation by IL-4-responsive CD4(+)CD25(-)CD103(+)Foxp3(-) cells is IL-10-dependent.* Eur J Immunol, 2010. **40**(10): p. 2837-47.

24. Baumgart, M., et al., *Naturally occurring CD4+Foxp3+ regulatory T cells are an essential, IL-10-independent part of the immunoregulatory network in Schistosoma mansoni egg-induced inflammation.* J Immunol, 2006. **176**(9): p. 5374-87.

25. McKee, A.S. and E.J. Pearce, *CD25+CD4+ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development.* J Immunol, 2004. **173**(2): p. 1224-31.

26. Gobert, G.N., et al., *Developmental gene expression profiles of the human pathogen Schistosoma japonicum.* BMC genomics, 2009. **10**(1): p. 128.

27. McManus, D.P., et al., *Recombinant paramyosin (rec-Sj-97) tested for immunogenicity and vaccine efficacy against Schistosoma japonicum in mice and water buffaloes.* Vaccine, 2001. **20**(5-6): p. 870-8.

28. Negrão-Corrêa, D., et al., *Association of Schistosoma mansoni-specific IgG and IgE antibody production and clinical schistosomiasis status in a rural area of Minas Gerais, Brazil.* PLoS One, 2014. **9**(2): p. e88042.

29. Dalton, J.P., et al., *A method for the isolation of schistosome eggs and miracidia free of contaminating host tissues.* Parasitology, 1997. **115 (Pt 1)**: p. 29-32.

30. Liu, S., et al., *Removal of endotoxin from recombinant protein preparations.* Clin Biochem, 1997. **30**(6): p. 455-63.

31. Strober, W., *Trypan blue exclusion test of cell viability.* Current protocols in immunology, 2001: p. A3. B. 1-A3. B. 3.

32. Grzych, J.M., et al., *Egg deposition is the major stimulus for the production of Th2 cytokines in murine schistosomiasis mansoni.* J Immunol, 1991. **146**(4): p. 1322-7.

33. Lewis, F.A. and E.M. Wilson, *Strain differences in lymphocyte responses and in vitro suppressor cell induction between Schistosoma mansoni-infected C57BL/6 and CBA mice.* Infect Immun, 1981. **32**(1): p. 260-7.

34. Allen, J.E. and R.M. Maizels, *Diversity and dialogue in immunity to helminths.* Nat Rev Immunol, 2011. **11**(6): p. 375-88.

35. Diaz, A. and J.E. Allen, *Mapping immune response profiles: the emerging scenario from helminth immunology.* Eur J Immunol, 2007. **37**(12): p. 3319-26.

36. Butterworth, A., *Immunological aspects of human schistosomiasis.* British medical bulletin, 1998. **54**(2): p. 357-368.

37. de Melo, T.T., et al., *Evaluation of the protective immune response induced in mice by immunization with Schistosoma mansoni schistosomula tegument (Smteg) in association with CpG-ODN.* Microbes and infection, 2013. **15**(1): p. 28-36.

38. Wynn, T. and K. Hoffmann, *Defining a schistosomiasis vaccination strategy–is it really Th1 versus Th2?* Parasitology today, 2000. **16**(11): p. 497-501.

39. Maizels, R.M. and M. Yazdanbakhsh, *Immune regulation by helminth parasites: cellular and molecular mechanisms.* Nat Rev Immunol, 2003. **3**(9): p. 733-44.

40. Pearce, E.J., et al., *Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, Schistosoma mansoni.* J Exp Med, 1991. **173**(1): p. 159-66.

41. Chen, S., et al., *Imbalance of Th1/Th2 and Th17/Treg promoting schistosome egg granuloma formation.* Int J Clin Exp Med, 2017. **10**(10): p. 14290-14300.

42. Jenkins, S.J., et al., *Modulation of the host's immune response by schistosome larvae.* Parasite Immunol, 2005. **27**(10-11): p. 385-93.

43. Alebie, G., *Immune Modulation by Schistosoma mansoni Infection and its Implication in Auto Immune Disorders and Allergic Diseases.* Journal of Bacteriology & Parasitology, 2014. **5**(3): p. 1.

44. Tang, C.L., et al., *Effect of CD4+ CD25+ regulatory T cells on the immune evasion of Schistosoma japonicum.* Parasitol Res, 2011. **108**(2): p. 477-80.

45. Tang, C.-l., et al., *Effect of CD4+ CD25+ regulatory T cells on the immune evasion of Schistosoma japonicum.* Parasitology research, 2011. **108**(2): p. 477-480.

46. Tan, M.J., et al., *[Suppressive effect of induced CD4+CD25+ regulatory T cells from mice infected with Schistosoma japonicum]*. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi, 2008. **26**(3): p. 166-9, 173.

47. Oliveira, S.C., et al., *A double edged sword: Schistosoma mansoni Sm29 regulates both Th1 and Th2 responses in inflammatory mucosal diseases.* Mucosal Immunol, 2016. **9**(6): p. 1366-1371.

48. Redpath, S.A., et al., *Schistosoma mansoni larvae do not expand or activate Foxp3+ regulatory T cells during their migratory phase.* Infection and immunity, 2015. **83**(10): p. 3881-3889.

49. Chiaramonte, M.G., et al., *Studies of murine schistosomiasis reveal interleukin-13 blockade as a treatment for established and progressive liver fibrosis.* Hepatology, 2001. **34**(2): p. 273-82.

50. Singh, K.P., et al., *Retroviral Foxp3 gene transfer ameliorates liver granuloma pathology in Schistosoma mansoni infected mice.* Immunology, 2005. **114**(3): p. 410-7.

51. Ponichtera, H.E., et al., *CD209a expression on dendritic cells is critical for the development of pathogenic Th17 cell responses in murine schistosomiasis.* J Immunol, 2014. **192**(10): p. 4655-65.