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Conservation of CD4⁺ T cell-dependent developmental mechanisms in the blood fluke pathogens of humans

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Abstract

Schistosoma blood flukes are trematode parasites with a cosmopolitan distribution that infect over 200 million people globally. We previously showed that *Schistosoma mansoni* growth and development in the mammalian host is dependent on signals from host CD4⁺ T cells. To gain insight into the mechanisms that underlie this dependence, we sought to determine the evolutionary origins and limits of this aspect of the host-pathogen relationship. By infecting RAG-1^{-/-} mice with a range of different schistosome species and strains, we tested several hypotheses concerning the time during *Schistosoma* evolution at which this dependence arose, and whether this dependence is specific to *Schistosoma* or is also found in other blood flukes. Our data indicate that the developmental dependence on CD4⁺ T cells previously described for *S. mansoni* is conserved in the evolutionarily basal species *Schistosoma japonicum*, suggesting this developmental adaptation arose early in *Schistosoma* evolution. We also demonstrate that the development of the more evolutionarily-derived species *Schistosoma haematobium* and *Schistosoma intercalatum* are dependent on adaptive immune signals. Together, these data suggest that the blood fluke parasites of humans utilize common mechanisms to infect their hosts and to co-opt immune signals in the coordination of parasite development. Thus, exploitation of host-schistosome interactions to impair or prevent parasite development may represent a novel approach to combating of all the schistosome pathogens of humans.

Keywords

Blood fluke; Schistosomatidae; *Schistosoma*; *Schistosomatium*; CD4⁺ T cells; Host-parasite interactions; Host-parasite co-evolution; Parasite developmental biology

1. Introduction

Schistosoma parasites infect more than 200 million people in 74 countries worldwide (Chitsulo et al., 2000). The African schistosomes *Schistosoma mansoni* and *Schistosoma intercalatum*,

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and the Asian species *Schistosoma japonicum* and *Schistosoma mekongi*, establish chronic infections that can lead to severe and life-threatening hepatosplenic disease. *Schistosoma haematobium*, the causative agent of urinary schistosomiasis, is found throughout Africa. Praziquantel is the only anthelmintic drug approved for the treatment of all schistosome species. High re-infection rates, the threat of drug resistance and rebound morbidity following treatment and re-infection (Ross et al., 2002; McManus, 2005) make the development of alternate treatments and vaccines highly desirable.

Deposition of schistosome eggs in host tissues induces granuloma formation that is driven by CD4⁺ T helper 2 (Th2) responses to egg antigens (Pearce and MacDonald, 2002). In addition to constituting the dominant cause of pathology during schistosome infection, egg-induced granulomas also facilitate the transit of eggs across the bowel wall and their egress from the body (Doenhoff et al., 1978). Interestingly, we have previously shown that normal *S. mansoni* growth, sexual maturation and fecundity are also dependent on host CD4⁺ T cells (Davies et al., 2001) and thus, *S. mansoni* appears to exploit the activities of host CD4⁺ T cells to facilitate parasite development and transmission (Davies and McKerrow, 2003). Together, these findings may explain why schistosomiasis patients who are co-infected with human immunodeficiency virus (HIV) excrete fewer eggs than those who are HIV-negative (Karanja et al., 1997). The origin of, and basis for, this dependence on host immune signals remains unclear but attenuation of parasite development in immunocompromised hosts may provide a selective advantage to the parasite, preventing premature death of an already stressed host (Davies and McKerrow, 2003).

Our studies on parasite development in the vertebrate host have focused primarily on *S. mansoni*, but there are compelling reasons to extend these studies to include the range of human schistosome pathogens. In addition to the different disease manifestations associated with each *Schistosoma* species, these pathogens also differ significantly with respect to host range, speed and success of schistosomulum migration and duration of the pre-patent period (Loker, 1983; Gui et al., 1995; Rheinberg et al., 1998; He et al., 2002). *Schistosoma japonicum* parasitizes the broadest range of natural definitive hosts among the Schistosomatidae (Loker, 1983). In contrast, *S. haematobium* is essentially a human pathogen, without significant animal reservoirs in nature (Loker, 1983). With regard to schistosomulum migration, *S. japonicum* experimental infections are characterized by greater speed and success of parasite migration (summarized in Ruppel et al., 2004). It has been suggested that the rapid migration and extended host range of *S. japonicum* are due to distinct cercarial enzymes which facilitate rapid passage through the mammalian host (Ruppel et al., 2004; Chlichlia et al., 2005). Conversely, studies of lung migration in the mouse model show that *S. haematobium* is slower in its migration to, and egress from, the lung than other species (Rheinberg et al., 1998). Finally, the pre-patent period of infection and the associated host immune response to worm antigens alone is of shortest duration in *S. japonicum* infection and most prolonged in *S. haematobium* infection (Loker, 1983). Amongst the human schistosome pathogens, *S. mansoni* and *S. intercalatum* are intermediate in terms of vertebrate host range, migration patterns and duration of the pre-patent period.

In addition to these life cycle differences, the schistosome species that infect humans are not closely related and are traditionally distributed into three separate groups based on egg morphology (Rollinson and Southgate, 1987), along with *Schistosoma* species that parasitize other mammals: the “*japonicum*” group (*S. japonicum*, *S. mekongi*, *Schistosoma malayensis*, *Schistosoma sinensium*); the “*mansoni*” group (*S. mansoni*, *Schistosoma rodhaini*); and the “*haematobium*” group (*S. haematobium*, *S. intercalatum*, *Schistosoma guineensis*, *Schistosoma curassoni*, *Schistosoma bovis*). Thorough phylogenetic analyses based on combinations of morphological and molecular data support these traditional groupings (Lockyer et al., 2003). These analyses also indicate the genus *Schistosoma* originated in Asia,

with the Asian “*japonicum*” group of parasites being basal or ancestral to the African “*mansoni*” and “*haematobium*” groups. This model is further supported by analysis of mitochondrial genome sequences (Le et al., 2000; Lockyer et al., 2003). The “*japonicum*” group possesses a mitochondrial gene order identical to that of other digenetic flukes and cestodes, whereas representatives of the “*mansoni*” and “*haematobium*” groups exhibit a unique gene arrangement that is different from all other flatworms. After arising in Asia, subsequent spread of *Schistosoma* to Africa gave rise to the two distinct “*mansoni*” and “*haematobium*” lineages (Morgan et al., 2001; Lockyer et al., 2003). Of these two, the “*haematobium*” group of schistosomes represents a separate and more derived lineage of African parasites than the “*mansoni*” group. Thus far, our data on the developmental dependence of schistosomes on host CD4⁺ T cells is restricted to the “*mansoni*” group, which lies between the more basal “*japonicum*” group and the more derived “*haematobium*” group.

An understanding of how schistosomes interact with host CD4⁺ T cells to complete their development may prove important for the development of more effective vaccines and other immunotherapies aimed at interrupting parasite development in the mammalian host. To gain insight into the mechanisms that underlie these interactions, we sought to determine when developmental dependence on host CD4⁺ T cells first arose during *Schistosoma* evolution and whether it is conserved throughout the genus. Firstly, we hypothesized that if developmental dependence on host CD4⁺ T cells arose early during *Schistosoma* evolution, it would be detectable in the most ancestral taxa such as *S. japonicum*. To test this hypothesis, we examined the development of *S. japonicum* in recombination activating gene-1 (RAG-1)-deficient (RAG-1^{-/-}) mice, which lack all B and T cells, and specifically evaluated the effect of CD4⁺ T cells on schistosome development in this immunodeficient context using an adoptive transfer approach. Because of its fragmented geographic distribution and the existence of distinct populations of *S. japonicum* that exhibit genotypic and phenotypic differences (Sobhon et al., 1986; Woodruff et al., 1987; Hope et al., 1996), we analyzed parasite development in a range of geographic strains to provide a thorough analysis of this species’ response to host signals. Second, we hypothesized that if developmental dependence on CD4⁺ T cells had been broadly conserved during *Schistosoma* evolution, it would also be detectable in more derived taxa, such as the “*haematobium*” group of parasites. To test this hypothesis, we examined the development of *S. haematobium* and *S. intercalatum* in RAG-1^{-/-} mice. Third, we hypothesized that if developmental dependence on host CD4⁺ T cells arose prior to the divergence of *Schistosoma* from other schistosomes, evidence of this adaptation would be found in other schistosome genera. To test this hypothesis, we examined the development of the distantly related schistosome *Schistosomatium douthitti* in RAG-1^{-/-} animals. Our results suggest that schistosome dependence on host CD4⁺ T cells is specific to, and conserved throughout, the genus *Schistosoma* and that our findings are broadly applicable to all the major schistosome pathogens of humans.

2. Materials and methods

2.1. Mice

Wild type C57BL/6 mice were purchased from National Cancer Institute (NCI), (Frederick, MD). Breeding pairs of RAG-1^{-/-} mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in-house to generate sufficient numbers for experiments. All studies involving animals were performed in accordance with protocols approved by the relevant Institutional Animal Care and Use Committees.

2.2. Schistosome maintenance and mouse infections

The Chinese strain of *S. japonicum* was originally isolated by Dr. H. Vogel in 1928 (precise location unknown; likely An-huei Province) and the stock replenished by Dr. Henry van der

Schalie (University of Michigan) in 1966 using parasites provided by the same laboratory in Germany. Another Chinese isolate obtained in 1977 and provided by Dr. Mao, also likely from An-huei Province, was subsequently mixed with the Vogel strain. The Japanese strain of *S. japonicum* was obtained by Dr. van der Schalie in 1968, from Ko-fu, Yamanashi, Japan. Since then, this stock has been maintained in the laboratory and replenished with parasites obtained from Ko-fu during the years 1977–85 by Dr. Hosaka (NIH, Japan). The Formosan strain of *S. japonicum* was isolated in Pu-yen, Taiwan in 1962 by Dr. van der Schalie. The Philippine strain of *S. japonicum* is composed of parasites isolated from Leyte by Dr. van der Schalie in 1969, and by Dr. Sano (Hamamatsu University) in 1977, that were subsequently mixed. *Schistosoma japonicum* strains were maintained using appropriate subspecies of *Oncomelania hupensis* as intermediate hosts – *Oncomelania hupensis hupensis* for the Chinese strain, *Oncomelania hupensis nosophora* for the Japanese strain, *Oncomelania hupensis formosana* for the Formosan strain, and *Oncomelania hupensis quadrasi* for the Philippine strain. For infections, mice were anesthetized with 50 mg/kg ketamine/6 mg/kg xylazine and the skin of the ventral abdomen shaved and moistened with water. *Schistosoma japonicum* cercariae were collected from crushed snails, counted under a dissecting microscope and applied to the shaved abdominal skin of the anaesthetized mice using a hair loop. Approximately 35 cercariae were applied to each mouse. *Schistosoma japonicum* infections were allowed to proceed for 28 days.

Schistosoma haematobium parasites were originally isolated around 1950 by Dr. Elmer Berry from an unknown location in Egypt. This laboratory stock was subsequently mixed with an isolate from NMRU III, another Egyptian isolate from Arawash (Cairo) in 1977. *Schistosoma haematobium* were maintained using *Bulinus truncatus* as intermediate hosts. Cercarial shedding was induced by placing infected *Bulinus* in the light for 1 h. Mouse tails were exposed to approximately 1,000 *S. haematobium* cercariae per mouse for 40 min. *Schistosoma haematobium* infections were allowed to proceed for 79 days.

Schistosoma intercalatum, an isolate from Cameroon originally obtained from Dr. A. Théron (Centre de Biologie et d'Ecologie Tropical et Méditerranéenne, Perpignan), was maintained using *Bulinus crystallinus* as intermediate hosts. Cercarial shedding was induced by placing snails in the light for 1–2 h, as for *S. haematobium*. Mouse tails were exposed to approximately 150 *S. intercalatum* cercariae per mouse for 40 min. *Schistosoma intercalatum* infections were allowed to proceed for 42 days.

Schistosomatium douthitti-infected *Stagnicola elodes* were generously provided by Dr. E. S. Loker (University of New Mexico) and subsequently maintained using *S. elodes* as intermediate hosts. Cercarial shedding was induced by placing infected *S. elodes* in the dark for 1–2 h. Cercariae were counted and collected using a wire loop and applied to the shaved abdominal skin of anaesthetized mice. Approximately 100 *S. douthitti* cercariae were applied to each mouse. *Schistosomatium douthitti* infections were allowed to proceed for 42 days.

In all experiments, groups of wild type and RAG-1^{-/-} mice were exposed at the same time to parasites from the same cercarial pool. Numbers of mice studied for each schistosome species and strain are summarized in Table 1.

2.3. Parasite recovery and measurement of parasitological parameters

Parasites were recovered from the portal system by perfusion (Smithers and Terry, 1965), immediately fixed in 4% neutral-buffered formaldehyde and photographed using a Nikon Coolpix 4500 4.0 megapixel digital camera connected to a Vistavision trinocular dissecting microscope at 20× magnification. Length of male parasites was determined from digital images using ImageJ software (<http://rsb.info.nih.gov/ij>). Quantitative analysis of parasite length was performed on male worms as male schistosomes always outnumber females in experimental infections and female growth is significantly influenced by pairing with males (Hernandez et

al., 2004). Liver tissue was digested in 0.7% trypsin (50 ml) in PBS for 2–3 h at 37°C and eggs were counted under a dissecting microscope.

2.4. Reconstitution of RAG-1^{-/-} mice with wild type CD4⁺ lymphocytes

Lymph nodes and spleens from wild type C57BL/6 mice were dispersed through a 70-µm nylon strainer. Cells were incubated with anti-CD4 coated microbeads (Miltenyi Biosciences) and separated using Midi-Macs magnetic columns (Miltenyi Biosciences). Four times 10⁶ cells were transferred into RAG-1^{-/-} mice by i.v. injection into a lateral tail vein. Recipient animals were then infected with cercariae 24 h later, as described in section 2.2. To verify the efficacy of adoptive transfers at necropsy, splenocytes from reconstituted RAG-1^{-/-} mice were surface labeled with allophycocyanin (APC)-Cy7-conjugated antibodies to CD4, fluorescein isothiocyanate (FITC)-conjugated antibodies to CD8, phycoerythrin (PE)-conjugated antibodies to NK1.1 and PerCp-Cy5.5-conjugated antibodies to CD19 (BD Biosciences) and analyzed using a LSR II Optical Bench flow cytometer with FACSDiva and Winlist software, version 5.0 (Verity Software House).

2.5. Statistical Analysis

Because unequal variances were observed among some of the groups analyzed in this study, stringent non-parametric tests were used throughout to test the significance of differences between experimental groups. For two groups, significance of differences between experimental groups was tested using Mann-Whitney tests, and for three groups the significance of differences was tested using Kruskal-Wallis tests followed by Dunns' multiple comparison tests. Statistical analyses were performed with GraphPad Prism Version 4.0 software (GraphPad Software, Inc., San Diego, CA). *P* values of less than 0.05 were considered significant.

3. Results

3.1. *Schistosoma japonicum* growth and development is attenuated in RAG-1-deficient mice

To determine whether developmental dependence on immune signals is conserved in the ancestral “*japonicum*” group of *Schistosoma* parasites, we infected groups of wild type and RAG-1^{-/-} mice with four different geographic strains of *S. japonicum* to assess the development of each strain in the absence of an adaptive immune system. Parasite development was evaluated by analyzing three separate parasitological parameters: worm length; the proportion of female parasites participating in pairs; and the number of eggs deposited in the liver by each parasite pair. Worm length was used as a measure of parasite growth during the pre-patent period, whereas pairing and egg production were used to assess parasite sexual maturation and subsequent reproductive activity, respectively. Of these parameters, worm length and egg production are the most robust and reproducible in identifying differences in parasite development, while parasite pairing appears more variable because although males generally always outnumber females in all schistosome infections, the male:female ratio does vary between species, between strains and from one experiment to another. For this reason, we argue that accurate assessment of parasite development is best accomplished by assessing multiple parasitological parameters (worm size, egg production and female pairing).

Similar parasite recovery rates were measured in wild type and RAG-1^{-/-} mice for all of the *S. japonicum* strains examined (data not shown), indicating that, as for *S. mansoni*, CD4⁺ T cells are not required for migration of *S. japonicum* to the portal vasculature. However, for all strains examined, *S. japonicum* parasites recovered from infected RAG-1^{-/-} mice exhibited marked alterations in development when compared with parasites from wild type animals (Figs. 1–3). Firstly, parasites recovered from RAG-1^{-/-} mice were considerably smaller than those obtained from wild type controls (Fig. 1A). To quantify these differences, parasite length was

determined from digital micrographs. Male worms of all four *S. japonicum* strains recovered from RAG-1^{-/-} mice were significantly reduced in length ($P < 0.0001$ for all strains) when compared with male worms recovered from wild type mice (Fig. 1B). The percent reduction of mean worm length in RAG-1^{-/-} mice compared with wild type mice varied from one strain to another, ranging from a 24% reduction for the Philippine strain to a 42% reduction for the Formosan strain. Therefore, in the absence of an adaptive immune system, there is a highly significant decrease in *S. japonicum* worm growth as measured at 28 days p.i.

To determine whether absence of the adaptive immune system affected the sexual maturation of *S. japonicum*, the numbers of parasite pairs recovered from RAG-1^{-/-} mice were compared with those obtained from wild type controls. For all four geographic strains, infection of RAG-1^{-/-} mice led to a reduced number of paired worms compared with infection of wild type mice, (Fig. 2). These differences were statistically significant for all strains except the Philippine strain. For the other strains, the differences in the means of percent females paired, between wild type and RAG-1^{-/-} mice, ranged from 18.7 for the Japanese strain to 34.1 for the Formosan strain. From these data we conclude that the absence of the adaptive immune system significantly delayed sexual maturation for all of the *S. japonicum* strains with the exception of the Philippine strain.

To assess whether the presence or absence of an adaptive immune system influenced the fecundity of each of the *S. japonicum* strains, the numbers of eggs that accumulated in the livers of infected RAG-1^{-/-} mice were compared with those in wild type controls (Fig. 3). Virtually no eggs were found in livers of RAG-1^{-/-} mice, while egg production in wild type mice varied among the *S. japonicum* strains, ranging from 44.4 ± 9.4 eggs/pair (mean $\pm$ S.E.M.) for the Chinese strain to $3,369 \pm 567$ eggs/pair for the Philippine strain. Consequently, the adaptive immune system is essential for normal parasite reproduction in all of the *S. japonicum* strains examined. Interestingly, despite normal pairing by the Philippine strain in RAG-1^{-/-} mice (Fig. 2), egg production by this strain was still ablated in RAG-1^{-/-} mice, making the loss of fecundity even more striking.

3.2. Reconstitution with wild type CD4⁺ T cells restores *S. japonicum* growth and development in RAG-1^{-/-} mice

To test whether CD4⁺ T cells are sufficient to rescue the development of *S. japonicum* in the absence of all other adaptive immune system components, we transferred wild type CD4⁺ T cells into RAG-1^{-/-} recipients and evaluated the development of a representative strain (Chinese) of *S. japonicum* in these animals. Flow cytometric analysis of the secondary lymphoid tissues of RAG-1^{-/-} recipient mice at the time of necropsy demonstrated that adoptive transfer of CD4⁺ T cells resulted in selective reconstitution of the CD4⁺ T cell compartment to levels comparable with wild type mice, whereas CD8⁺ T cells and B cells were not detectable (data not shown). Parasites from RAG-1^{-/-} mice reconstituted with CD4⁺ T cells were visibly larger than those recovered from RAG-1^{-/-} mice that did not receive cells (Fig. 4A). Measurements revealed that male worms from reconstituted RAG-1^{-/-} mice were twice the length of those recovered from control RAG-1^{-/-} mice ($5.458 \text{ mm} \pm 0.115$, compared with $2.715 \text{ mm} \pm 0.089$) (Fig. 4B). Though sexual maturation as measured by the percentage of females in pairs was not significantly increased by adoptive transfer of CD4⁺ T cells (Fig. 4C), egg production by the pairs that did form was restored to wild type levels (Fig. 4D; 44.35 ± 9.441 eggs/pair for wild type mice, 0.14 ± 0.096 eggs/pair for control RAG-1^{-/-} mice and 53.61 ± 18.8 eggs/pair for reconstituted RAG-1^{-/-} mice). Identical results were obtained for the Japanese strain of *S. japonicum* (data not shown). Thus, transfer of CD4⁺ T lymphocytes from wild type donors to RAG-1^{-/-} mice was sufficient to restore *S. japonicum* development.

3.3. *Schistosoma haematobium* and *S. intercalatum* development is attenuated in RAG-1^{-/-} mice

To examine whether more derived *Schistosoma* species also exhibit the same developmental dependence on the adaptive immune system, we analyzed the development of two human pathogens from the “*haematobium*” group, *S. haematobium* and *S. intercalatum*, in RAG-1^{-/-} mice. While the laboratory mouse does not constitute an ideal host for *S. haematobium*, we found that, comparable with the findings of others (Cheever et al., 1983), sufficient cercariae (approximately 5% of the infectious dose) reached adulthood in the portal system to make comparison between wild type and RAG-1^{-/-} mice feasible. Numbers of parasites recovered from wild type and RAG-1^{-/-} mice were comparable, with slightly more parasites found in RAG-1^{-/-} animals (data not shown). Interestingly, *S. haematobium* growth was visibly reduced in RAG-1^{-/-} mice (Fig. 5A). Measurement of male worms revealed a significant decrease in worm size in RAG-1^{-/-} mice compared with that in wild type mice ($P < 0.0001$) (Fig. 5B). There was a 44% reduction in mean *S. haematobium* worm length in RAG-1^{-/-} mice. *Schistosoma intercalatum*, a closely related human pathogen, demonstrated a similar and significant reliance on the presence of an adaptive immune system for optimal growth, with a 40% reduction in mean worm length in RAG-1^{-/-} mice (Fig. 5A and B). *Schistosoma haematobium* sexual maturation was significantly attenuated in RAG-1^{-/-} mice, as demonstrated by reduction in percent pairing among female parasites (Fig. 5C). The number of eggs produced per *S. haematobium* pair was also significantly reduced in RAG-1^{-/-} mice (285.6 ± 188.9 in RAG-1^{-/-} versus $6,610 \pm 558.6$ in wild type mice) (Fig. 5D). Few female worms were recovered from either wild type or RAG-1^{-/-} mice infected with *S. intercalatum* parasites, preventing analysis of pairing and egg production in this species. However, taken together our data indicate that developmental dependence on adaptive immune signals is clearly conserved in the more derived “*haematobium*” group of *Schistosoma* parasites.

3.4. *Schistosoma douthitti* development is not dependent on the adaptive immune system

To test whether the adaptive immune system plays a role in the growth and development of a distantly related schistosome from outside the genus *Schistosoma*, we infected wild type and RAG-1^{-/-} mice with *S. douthitti* cercariae and evaluated the development of the worms at 6 weeks p.i. Visually, *S. douthitti* worms recovered from RAG-1^{-/-} mice were similar in appearance to those recovered from wild type mice (Fig. 6A). Careful measurement of *S. douthitti* male worms revealed a slight reduction in the length of worms from RAG-1^{-/-} mice compared with those recovered from wild type mice (Fig. 6B). However, development of *S. douthitti* was not affected by the absence of an adaptive immune system, as there was no reduction in either the proportion of paired females (Fig. 6C) or the number of eggs produced per pair (Fig. 6D) in RAG-1^{-/-} mice. Because female *S. douthitti* can also produce eggs by facultative parthenogenesis, we also analyzed the number of eggs produced per female worm, but again no differences in egg production were detected in RAG-1^{-/-} and wild type mice (data not shown). In summary, these data indicate that developmental responsiveness to adaptive immune signals is not conserved in *S. douthitti*.

4. Discussion

In the present study, we examined developmental dependence on adaptive immune signals in every major schistosome pathogen of humans except *S. mansoni* (on which we have reported previously - see Davies et al., 2001). Our data clearly show that, similar to *S. mansoni* (Davies et al., 2001) the adaptive immune system is necessary to facilitate worm development and sexual maturation in all of the human pathogens we examined (Figs. 1–5). Further, our studies indicate that, similar to *S. mansoni*, CD4⁺ T cells are the critical immune element for normal

parasite development in *S. japonicum*, because adoptive transfer of wild type CD4⁺ T cells into RAG-1^{-/-} animals was sufficient to restore *S. japonicum* development to normal levels (Fig. 4). Because *S. japonicum* and the other *Schistosoma* of the “*japonicum*” group occupy a more basal or ancestral position within the genus relative to *S. mansoni*, these data suggest that developmental dependence on CD4⁺ T cells arose early in *Schistosoma* evolution, prior to diversification within the genus (Lockyer et al., 2003). An alternative explanation is that developmental dependence on CD4⁺ T cells arose multiple times during *Schistosoma* evolution, but the hypothesis that this adaptation evolved once and was subsequently conserved through speciation events is more parsimonious.

Our results from *S. japonicum* infection of immunodeficient hosts are in agreement with previous studies which suggested that development of this parasite is influenced by immune signals. For example, a delay in onset of *S. japonicum* egg production was previously reported in severe combined immunodeficiency (*Prkdc^{scid/scid}*) mice (Cheever et al., 1999). However, no defects in parasite growth or pairing were reported, perhaps because of the “leaky” phenotype of *Prkdc^{scid/scid}* mice, in which both B and T cells are detectable (Nonoyama et al., 1993). The data presented here clearly show that *S. japonicum* growth and sexual maturation are drastically attenuated in the complete absence of CD4⁺ T cells and that CD4⁺ T cells are sufficient to rescue *S. japonicum* development to wild type levels in RAG^{-/-} mice.

Our data also show that the development of blood flukes belonging to the more evolutionarily derived “*haematobium*” group of *Schistosoma* is also dependent on adaptive immune signals, as the development of *S. haematobium* and *S. intercalatum* in RAG-1^{-/-} mice (Fig. 5) was indistinguishable from that of *S. mansoni* (Davies et al., 2001) and *S. japonicum* (Figs. 1–3). These results suggest that the developmental dependence on adaptive immune signals described in *S. mansoni* and *S. japonicum* has been conserved in the more evolutionarily derived “*haematobium*” group, and possibly throughout the entire genus. An alternative explanation is that developmental dependence on adaptive immune signals arose separately in the “*haematobium*” group, but given that this adaptation is conserved in the two other *Schistosoma* groups, including the most ancestral “*japonicum*” group, the most parsimonious explanation is that this developmental dependency arose early during *Schistosoma* evolution and was subsequently conserved through speciation events, even in the most derived members of the genus. Because we have not yet performed adoptive transfer studies with “*haematobium*” group parasites to conclusively demonstrate that their development is also dependent on CD4⁺ T cells, we cannot exclude the possibility that *S. haematobium* and its closest relatives are dependent on other components of the adaptive immune system, such as CD8⁺ T cells or B cells. However, given that development in the “*japonicum*” and “*mansoni*” groups is dependent on CD4⁺ T cells (Fig. 4; Davies et al., 2001), and that both groups occupy more basal evolutionary positions within *Schistosoma* relative to the “*haematobium*” group, the most parsimonious explanation for our results is that this mechanism arose early during *Schistosoma* evolution and has been conserved in the “*haematobium*” group, such that development of *S. haematobium*, *S. intercalatum* and possibly other members of the “*haematobium*” group also require CD4⁺ T cells. Thus we propose that developmental dependence on immune signals is conserved throughout the genus *Schistosoma*, having arisen early during *Schistosoma* evolution and been conserved through evolution of the most derived taxa.

That growth and development of *Schistosoma* blood flukes is dependent on the adaptive immune system, and specifically on CD4⁺ T cells, suggests that extensive host-pathogen co-evolution has given rise to a complex relationship between *Schistosoma* blood flukes and their hosts. In contrast, development of the more distantly related schistosome *S. douthitti* was not significantly impaired by lack of an adaptive immune system (Fig. 6). While *S. douthitti* and the closely related *Heterobilharzia americana* are parasites of mammals, phylogenetic analyses

of the Schistosomatidae place these two parasites within the clade of bird schistosomatids, a grouping that lies basal to *Schistosoma* and comprises the bulk of the family Schistosomatidae (Snyder et al., 2001; Lockyer et al., 2003). The finding that *Schistosomatium* development is normal in immunodeficient hosts suggests that developmental dependence on immune signals is not conserved in parasites basal to the *Schistosoma* and that dependence on host adaptive immune signals marks an evolutionary departure that occurred after the split of *Schistosoma* from a common bird-infecting ancestor. Further, the placement of *Schistosomatium* and *Heterobilharzia* within the avian parasite clade suggests these parasites and *Schistosoma* acquired mammalian hosts in separate evolutionary events (Lockyer et al., 2003). That *Schistosomatium* development was normal in $RAG^{-/-}$ mice suggests that developmental dependence on immune signals is exclusively a feature of *Schistosoma* and is consistent with the potentially disparate origins of mammalian parasitism in *Schistosomatium* and *Schistosoma*. This hypothesis could be tested further by examining the development of other non-*Schistosoma* parasites in immunodeficient settings. This would be possible for *Heterobilharzia* because this parasite will infect mice, but similar testing of avian schistosomes is complicated by lack of immunodeficient model hosts.

Evaluation of the life cycles of the schistosome parasites in context of their definitive hosts points to another possible explanation for the differences between the requirements of *Schistosoma* and *Schistosomatium* for host immune signals. *Schistosoma* species infect a variety of mostly large mammals, including long-lived ungulates and primates (Loker, 1983). By contrast, the natural definitive hosts for *S. douthitti* are small short-lived rodents such as muskrats, meadow voles and the redback mouse (summarized in Raiczuk and Hall, 1988). The short pre-patent period of *S. douthitti* and its ability to produce viable eggs by facultative parthenogenesis may therefore be adaptations that maximize the likelihood of transmission between relatively short-lived definitive hosts (Loker, 1983). Expanding on these observations, we suggest there would be little selective pressure to delay parasite development based on the immune status of short-lived hosts. In contrast, there may be selective advantages for the parasite in delaying development in immunocompromised hosts that are longer-lived (Davies et al., 2001). Thus for *Schistosoma*, reduced rates of egg production in animals deficient in an immune response may prolong the survival of the host animal, and therefore extend the opportunity for parasite transmission to new hosts (Davies and McKerrow, 2003). If this hypothesis is correct, we predict that development of *Schistosoma* species that parasitize small, short-lived hosts such as *S. rodhaini*, will not be influenced by immune signals. Conversely, development of parasites from the *Schistosomatium* clade that infect larger hosts such as *H. americana*, which parasitizes carnivores, will be modulated by immune signals. These possibilities could be tested experimentally as both *S. rodhaini* and *H. americana* have been reported to infect mice (Loker, 1983). Finally, we do not see this explanation for the differences between *Schistosoma* and *Schistosomatium* as mutually exclusive to the phylogenetic argument presented above and hypothesize that both factors may have shaped the host-parasite relationships exhibited by these parasites.

A possible explanation for *Schistosoma* developmental attenuation in $RAG^{-/-}$ mice is that parasite development is inhibited by the large numbers of natural killer (NK) lymphocytes that $RAG^{-/-}$ animals possess in the absence of a normal complement of B and T cells (Mombaerts et al., 1992). However, we show that $RAG^{-/-}$ animals can support the normal development of the distantly related schistosome *S. douthitti* (Fig. 6), arguing that the attenuated development of *Schistosoma* species reported here is not due to non-specific effects mediated by NK cells. This conclusion is further supported by the fact that attenuated *Schistosoma* development is still observed in immunodeficient mice that lack NK cells, such as common γ chain-deficient ($\gamma c^{-/-}$) mice (Blank et al., 2006), and animals that possess defects in NK cell function, such as NOD-SCID mice (Davies et al., 2001).

While the mechanism by which CD4⁺ T cells modulate *Schistosoma* development remain unclear, previous studies with *S. mansoni* indicate it is not dependent on the expression of classical effector functions such as Th1 and Th2 responses (Davies et al., 2001, 2004). Rather, more fundamental aspects of CD4⁺ T cell biology, including homeostatic maintenance of CD4⁺ T cells by IL-7 and autocrine production of IL-2 by CD4⁺ T cells, appear to play an indirect role in creating a permissive environment for parasite development (Blank et al., 2006).

In conclusion, the results we present here provide insights into the function and evolutionary origins of *Schistosoma* dependence on adaptive immune signals to coordinate their development within the definitive mammalian host. These findings are significant for several reasons. Firstly, while we have not yet examined the developmental phenotype of all the schistosomes that infect humans, including the predominantly human parasites *S. mekongi* and *S. malayensis*, the close evolutionary relationship of these specific parasites to *S. japonicum*, together with our finding that developmental dependence on immune signals is likely conserved throughout the genus *Schistosoma*, suggests that all *Schistosoma* species will display similar requirements for normal development. Second, our results show that, although the *Schistosoma* have apparently acquired humans as hosts multiple times throughout evolution (Combes, 1990; Lockyer et al., 2003), the schistosome pathogens of humans share a common reliance on immune signals for their development. Thus, a molecular understanding of the host-parasite interactions that occur in one species of *Schistosoma* is likely to be applicable to the other human pathogens, despite the fact that most of the schistosome pathogens of humans are more closely related to non-human-infecting schistosomes than to each other. Finally, our results indicate that approaches aimed at interfering with interactions between schistosomes and the adaptive immune system to disrupt parasite development may be of therapeutic and prophylactic value in combating all the schistosome infections of humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *Schistosoma japonicum* development is impaired in RAG-1^{-/-} mice. Wild type (WT) and RAG-1^{-/-} mice were infected with the Chinese, Formosan, Philippine and Japanese strains of *S. japonicum* and worms were collected by perfusion at 28 days p.i. A) Digital micrographs obtained at 20 × magnification. Scale bar = 1 mm. B) Male worm length was determined from digital micrographs using parasites collected from groups of eight to ten animals. Horizontal bars represent the median value for each experimental group. * $P < 0.0001$, as determined by Mann-Whitney tests.

Fig. 2.

Pairing of *Schistosoma japonicum* females is decreased in RAG-1^{-/-} mice. Pairing of the Chinese, Formosan, Philippine and Japanese strains of *S. japonicum* in RAG-1^{-/-} and wild type (WT) mice at 28 days p.i. was quantified for each mouse as: (the number of paired females/total number of females) $\times$ 100. Percent paired females worms recovered per mouse are shown for eight to 15 mice per group. Horizontal bars represent the median value for each experimental group. Mann-Whitney *P* values are indicated on the graphs.

Fig. 3.

Schistosoma japonicum egg production is impaired in RAG-1^{-/-} mice. Livers from wild type (WT) and RAG-1^{-/-} mice infected with the Chinese, Formosan, Philippine and Japanese strains of *S. japonicum* were homogenized and the eggs in each liver were counted. Egg production per schistosome pair was measured by dividing the total number of eggs in each mouse liver by the number of parasite pairs recovered from that mouse. Egg production per schistosome pair is shown for each mouse (eight to 15 mice per group.) Horizontal bars represent the median value for each experimental group. Mann-Whitney *P* values are indicated on the graphs.

Fig. 4.

CD4⁺ lymphocytes restore *Schistosoma japonicum* development in RAG-1^{-/-} mice. Four times 10⁶ CD4⁺ wild type T cells were transferred into RAG-1^{-/-} recipient mice. RAG-1^{-/-}, wild type (WT) and CD4⁺-reconstituted RAG-1^{-/-} (RAG-1^{-/-}+CD4⁺) mice were infected with 35 *S. japonicum* (Chinese strain) cercariae 2 days after the transfer. A) Micrographs of representative parasites from RAG-1^{-/-}, wild type and reconstituted RAG-1^{-/-} (RAG-1^{-/-}+CD4⁺) mice at 20 × magnification. Scale bar = 1 mm. B) Male worm length was determined from digital micrographs using parasites collected from groups of eight to ten animals. Overall Kruskal-Wallis *P* value < 0.0001. C) Percent paired female worms recovered per mouse. Overall Kruskal-Wallis *P* value = 0.0011. D) Egg production per schistosome pair is shown for each mouse. Overall Kruskal-Wallis *P* value = 0.0003. Horizontal bars represent the median value for each experimental group. Dunn's post-test comparison *P* values are indicated on the graphs.

Fig. 5. *Schistosoma haematobium* and *Schistosoma intercalatum* development in RAG-1^{-/-} mice. Wild type (WT) and RAG-1^{-/-} mice were infected with 1,000 *S. haematobium* cercariae and evaluated 77 days p.i. (A–D) or 100 *S. intercalatum* cercariae and evaluated 42 days p.i. A) Micrographs of representative *S. haematobium* and *S. intercalatum* parasites from RAG-1^{-/-} and wild type mice at 20 × magnification. Scale bar = 1 mm. B) *Schistosoma haematobium* and *S. intercalatum* male worm length was determined from digital micrographs using parasites collected from groups of nine to ten animals. C) Percent paired *S. haematobium* females recovered per mouse. D) *Schistosoma haematobium* egg production per pair is shown for each mouse. Horizontal bars represent the median value for each experimental group. Mann-Whitney *P* values are indicated on the graphs.

Fig. 6.

Schistosomatium douthitti development in RAG-1^{-/-} mice. Wild type (WT) and RAG-1^{-/-} mice were infected with 100 *S. douthitti* cercariae and evaluated 42 days p.i. A) Micrographs of representative *S. douthitti* parasites recovered from RAG-1^{-/-} and wild type mice at 20 × magnification. Scale bar = 1 mm. B) *Schistosomatium douthitti* male worm length was determined from digital micrographs using parasites collected from groups of three to five animals. C) Percent paired females recovered per mouse. D) Egg production per parasite pair is shown for each mouse. Horizontal bars represent the median value for each experimental group. Mann-Whitney P values are indicated on the graphs.

Table 1

Schistosome infections. Number of mice infected with each schistosome species.

Parasite	Mouse genotype		
	Wild type	RAG-1 ^{-/-}	RAG-1 ^{-/-} reconstituted with CD4 ⁺ T cells
<i>Schistosoma japonicum</i> (Chinese)	15	9	5
<i>S. japonicum</i> (Formosan)	17	15	
<i>S. japonicum</i> (Philippine)	10	7	
<i>S. japonicum</i> (Japanese)	9	7	3
<i>Schistosoma haematobium</i>	9	10	
<i>Schistosoma intercalatum</i>	10	10	
<i>Schistosomatium douthitti</i>	16	12	