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Abstract

Title of Dissertation:

Blood Fluke Exploitation of Innate-Adaptive Immune Interactions to Facilitate Parasite Development

Erika W. Lamb

Thesis directed by:

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Schistosoma trematodes are parasitic blood flukes of mammals that develop in the portal system vasculature and cause significant morbidity and mortality in humans. It was previously shown that the development of *Schistosoma mansoni* is dependent on signals from host $CD4^+$ T cells. Our goal was to elucidate the mechanisms of $CD4^+$ T cell facilitation of schistosome development and to evaluate the conservation of this developmental mechanism in other medically important *Schistosoma*. We provide evidence that the development of *S. japonicum*, *S. haematobium*, and *S. intercalatum* rely on signals from the host adaptive immune system for normal development and that these signals are mediated by host $CD4^+$ T cells. However, the development of a

distantly related schistosomatid, *Schistosomatium douthitti*, is not dependent on host adaptive immune signals, suggesting that this is a *Schistosoma*-specific adaptation. We have also investigated the mechanism by which $CD4^+$ T cells mediate effects on schistosome development. While T cell homeostasis mediated by IL-7 and by IL-2 appear to be critical, neither of these cytokines affect parasite development directly. Indeed, no role has been identified for any of the major effector cytokines produced by $CD4^+$ T cells. We now show that conventional antigen-specific activation of $CD4^+$ T cells is not required for schistosome maturation and development. Further, the mere presence of naïve $CD4^+$ T cells alters expression of innate immune system genes in the liver. We propose that this transcriptional modulation at the site of parasite development may make the intra-host environment more conducive to blood fluke development and contribute to the pathogenesis of schistosomiasis. Finally, we show that direct stimulation of the innate immune system bypasses the requirement for $CD4^+$ T cells in parasite development. Together these results indicate that $CD4^+$ T cells facilitate schistosome development indirectly by priming innate immune responses. Further characterization of innate immune system exploitation by schistosomes may identify interactions that can be exploited for therapeutic and prophylactic purposes.

Blood Fluke Exploitation of Innate-Adaptive Immune Interactions to Facilitate Parasite Development

By

Erika White Lamb

Dissertation submitted to the Faculty of the

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requirements for the degree of

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For Douglas, Derek and Kiera;

I love you with all of my heart

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Chapter 1

Introduction

Schistosomiasis

History and Epidemiology

Schistosomiasis has plagued humans for thousands of years. Not only have descriptions of this illness been deciphered from ancient hieroglyphs, but schistosome eggs and circulating antigens have been found in ancient Chinese and Egyptian mummies (1, 2). Therefore, it may seem extraordinary to describe schistosomiasis as an "Emerging Infectious Disease." However, the spread of schistosomes to new areas (3, 4), the emergence and rise of urban transmission of schistosomiasis (5, 6) and increasing reports of treatment failure and resistance (7-10) argue that continued diligence against this disease is essential.

Schistosomiasis is caused by trematode flatworms, or blood flukes, of the genus *Schistosoma*, and is endemic in 74 developing countries (Fig. 1). There are five schistosomes that cause human disease: *S. mansoni*, *S. japonicum*, *S. mekongi*, and *S. intercalatum*, which are the causative agents of intestinal schistosomiasis, and *S. haematobium*, the causative agent of urinary schistosomiasis. *S. mansoni* is found in Africa and is the only species found in Latin America. *S. japonicum* is found throughout the Pacific region, especially in China and the Philippines, though it has been eradicated from Japan. *S. mekongi* has a restricted geographic range and is found in foci along the Mekong River Valley in Laos and Cambodia. *S. intercalatum* is also restricted to geographical foci, but in Africa, and may actually be composed of two separate species: *S. intercalatum*, found in the Democratic Republic of Congo and the

recently described *S. guineensis*, found in Lower Guinea (11, 12). Finally, *S. haematobium*, the causative agent of urinary schistosomiasis, is the most prevalent and widespread species in both Africa and the Middle East (13).

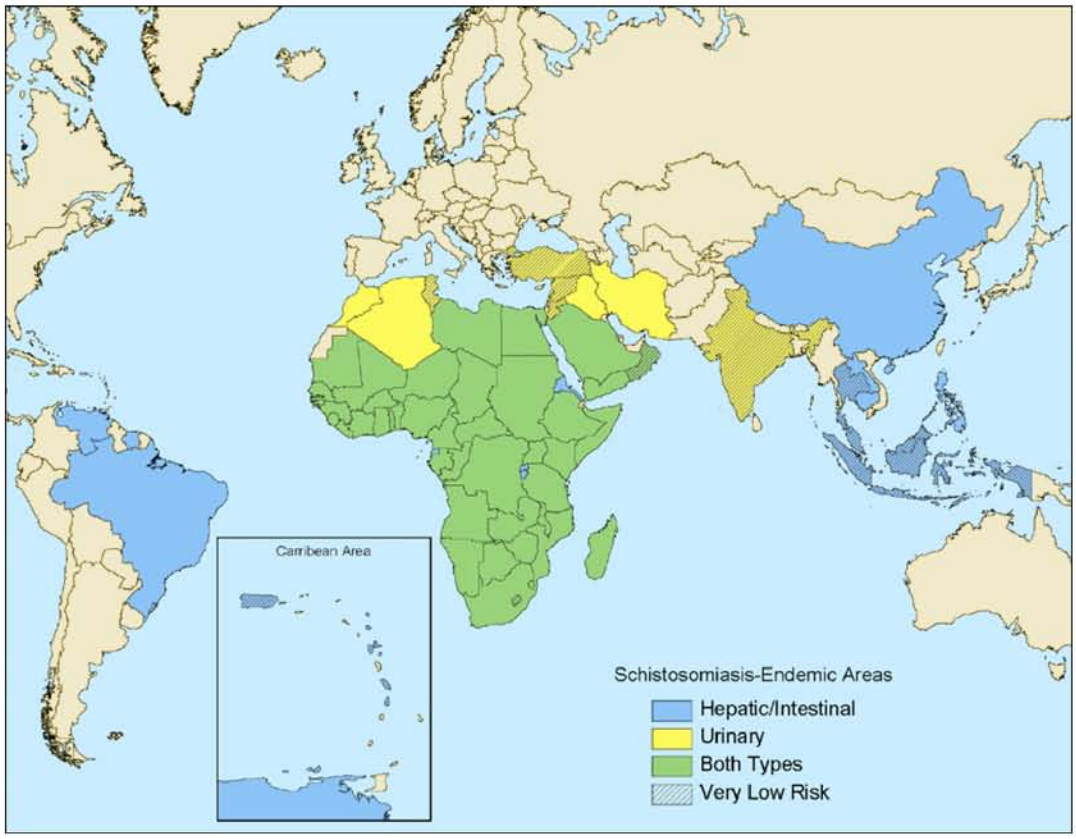
Humans have played important roles in the dissemination of schistosomiasis throughout the world. *S. mansoni* was introduced to the New World from Africa by the African slave trade, where the presence of a suitable snail intermediate host, *Biomphalaria glabrata*, led to the establishment of the disease in the Caribbean and Brazil (14). More recently, the introduction of the intermediate snail host *Biomphalaria tenagophila* from South America to Lower Zaire was concurrent with the emergent transmission of *S. mansoni* in this previously non-affected region (15). Recent environmental changes, usually associated with water resource development, have led to the spread of schistosomiasis to previously non-endemic areas (16, 17). For example, construction of the Diama Dam on the Senegal River led to the establishment and spread of *S. mansoni* into Mauritania and Senegal (3, 18). Development of the Aswan Dam on the Nile River in Egypt led to a decrease in *S. hematobium* in the Nile Delta but introduced *S. mansoni* transmission in Upper Egypt (3). Additionally, development of the Three Gorges Dam on China's Yangtze River is expected to increase schistosome transmission to previously non-endemic areas (19).

Despite knowledge of the disease and years of treatment and control efforts, there has been no diminution in the number of people infected or the number at risk for infection (16). Schistosomiasis has been targeted for control

by the Special Programme for Research and Training in Tropical Diseases (TDR) of the United Nations Development Program, the World Bank, and the World Health Organization. Schistosomiasis is listed as a category II disease, indicating that despite an available control strategy, a heavy disease burden persists. It is estimated that 200 million people worldwide are infected with schistosomes, with 85% of those infected residing in Sub-Saharan Africa (20, 21). Of those infected, 120 million are symptomatic and an estimated 20 million people suffer from severe disease (20). The Disability Adjusted Life Years (DALYs) due to schistosomiasis is conservatively estimated at 1,760,000, with mortality estimates of 15,000 deaths annually (22). New estimates of schistosome-related morbidity and mortality suggest that both factors have been severely underestimated (23) and that the global burden of the disease is closer to 4.5 million DALYs (18), with 150,000 deaths per year due to kidney failure caused by *S. hematobium* and 130,000 deaths annually due to portal hypertension caused by *S. mansoni* (21). Schistosomiasis ranks second only to malaria in terms of socio-economic and public health importance in tropical regions (24, 25).

Figure 1: Geographical distribution of hepatic/intestinal and urinary schistosomiasis.

Hepatic/intestinal schistosomiasis (blue), caused by *S. mansoni* and *S. japonicum* and urinary schistosomiasis (yellow), caused by *S. haematobium* are found throughout the tropics and often overlap in distribution (green). (From: http://www2.ncid.cdc.gov/travel/yb/utils/ybGet.asp?section=dis&obj=schisto.htm&cssNav=browseoyb)


Carribean Area
Schistosomiasis-Endemic Areas
Hepatic/Intestinal
Urinary
Both Types
Very Low Risk

Schistosome Life Cycle

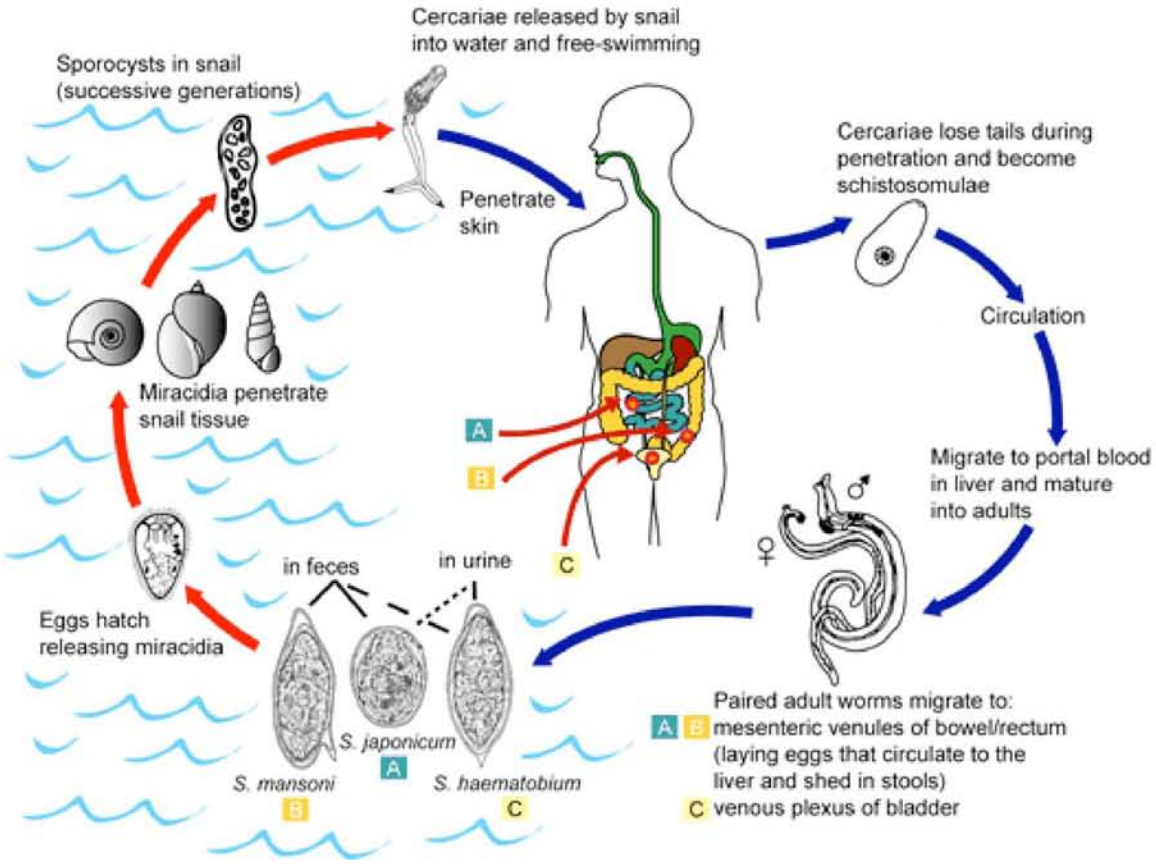
Schistosoma are digenean blood flukes, whose life cycle alternates between an asexual reproductive phase in molluscan intermediate hosts and a sexual reproductive phase in vertebrate definitive hosts. Maintenance of the schistosome life cycle is dependent upon the availability of the appropriate intermediate freshwater snail hosts, which differ for each *Schistosoma* species. The major human schistosome pathogens *S. mansoni*, *S. japonicum* and *S. hematobium* infect *Biomphalaria*, *Oncomelania*, and *Bulinus* snails, respectively. Additionally, the *Schistosoma* have different requirements for vertebrate definitive hosts. *S. japonicum* parasitizes the broadest range of vertebrate hosts, and is considered a zoonotic disease, while *S. hematobium* is primarily a human parasite (26). Control efforts targeting both the molluscan and vertebrate hosts of *Schistosoma* have been employed, including application of molluscicides and introduction of snail predators to achieve mollusk control, and the development of a possible zoonotic vaccine against *S. japonicum* to achieve control of infection in animal reservoirs (27).

Snails are infected after eggs deposited in freshwater streams or lakes hatch, releasing ciliated miracidia, which are the infective form for the snail hosts (Fig. 2). Following infection, the miracidium transforms into a primary sporocyst and two generations of asexual amplification occur, ending with the production of cercariae. In response to environmental stimuli, such as temperature or light, cercariae are released from the snail into the water. Cercariae penetrate the skin of their vertebrate hosts and drop their bifurcated tails, forming schistosomula.

Schistosomula enter the host's circulatory system and travel through the capillary beds of the lungs at approximately seven days following infection. Little or no growth is evident at this point as lung stage larvae are the same size as cercariae. The schistosomula reach the hepatic portal vein eight days after infection and begin a rapid period of development, with anastomosis of the growing gut caeca at day fifteen, sex organ development during the third week and gametogenesis and pairing at 28 days post-infection (28). Adult pairs migrate to the superior and inferior mesenteric veins (*S. mansoni* and *S. japonicum*, respectively) or the vesical plexus and veins draining the ureter (*S. haematobium*). The eggs traverse the walls of the intestine or ureters and are excreted in the feces or urine. If the eggs are excreted into freshwater containing the appropriate snail hosts, the life cycle is maintained. A pair of worms produces between 300 (*S. mansoni*) to 3500 (*S. japonicum*) eggs per day over an average life span of 5 years, though productive infections of more than 30 years have been described (29).

Figure 2: *Schistosoma* Life Cycle.

Eggs are passed in feces (*S. mansoni and S. japonicum*) or urine *(S. haematobium*) and hatch in fresh water, releasing miracidia. Miracidia infect the appropriate snail host (*Biomphalaria*, *Oncomelania*, and *Bulinus*, respectively) and undergo successive sporocyst generations. Cercariae are released from intermediate snail hosts and penetrate the skin of vertebrate hosts. Schistosomulae enter the host vasculature and migrate to the hepatic portal system, where parasites undergo a rapid period of growth and development. Paired adult worms migrate to the mesentery or ureter venules and lay eggs. Eggs pass through the intestinal or ureter wall and are released in the feces or urine. (From: http://www.dpd.cdc.gov/dpdx/HTML/Schistosomiasis.htm)


Cercariae released by snail into water and free-swimming
Sporocysts in snail (successive generations)
Penetrate skin
Cercariae lose tails during penetration and become schistosomulae
Circulation
Migrate to portal blood in liver and mature into adults
Miracidia penetrate snail tissue
A
B
C
in feces
in urine
Eggs hatch releasing miracidia
S. japonicum
A
S. mansoni
B
S. haematobium
C
Paired adult worms migrate to:
A B mesenteric venules of bowel/rectum (laying eggs that circulate to the liver and shed in stools)
C venous plexus of bladder

Disease

In order for adult parasites to co-exist with their hosts for such long periods of time, the *Schistosoma* must evade immune detection or elimination and minimize damage to the host. Indeed, adult worms are not responsible for the symptoms or pathology associated with schistosomiasis. Instead, both the acute and chronic forms of schistosomiasis are the result of egg-induced immunopathology.

Acute schistosomiasis, or Katayama fever, occurs 4-10 weeks following a heavy exposure to cercariae and is caused by a systemic hypersensitivity to migrating schistosomula or antibody-egg antigen complexes and is marked by fever, malaise, abdominal pain and diarrhea (3, 30). This febrile illness is mediated through the production of tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 (31). Though Katayama Fever occurs in endemic populations in response to *S. japonicum*, acute schistosomiasis caused by *S. mansoni* and *S. hematobium* is usually seen in travelers and migrants from non-endemic areas who travel to schistosome endemic regions (30). It is possible that the absence of the acute phase of this disease in endemic areas is due to *in utero* exposure to schistosome antigens (32) and development of a helper T (T_H)2 biased response, which may block development of the T_H1 mediated Katayama fever response (31).

The chronic form of both intestinal and urinary schistosomiasis is caused by a local immune response to eggs that embolize to downstream tissues. Chronic schistosomiasis caused by the “intestinal” schistosome species, *S.*

mansoni, *S. japonicum*, and *S. mekongi*, can manifest as mild intestinal, hepatointestinal, or severe hepatosplenic forms (33). Chronic disease associated with *S. intercalatum* is limited to mild intestinal disease (30). In intestinal schistosomiasis, a local granulomatous response against eggs trapped in the gut wall causes pain, loss of appetite and diarrhea that sometimes contains blood (3, 30, 33). Hepatic schistosomiasis occurs when eggs which do not pass into the intestinal lumen are carried by the blood to the liver, where eggs become trapped in the liver sinusoids and eventually die. In response to egg antigens, a T_H2 $CD4^+$ T cell dependent granulomatous reaction is induced, in which, macrophages, eosinophils, and $CD4^+$ T cells surround the trapped egg (34, 35) and IL-13- and IL-21- mediated fibrotic lesions progressively develop (35-37). Over time, fibrotic tissue may lead to congestion within the liver and severe sequelae such as development of porto-systemic venous shunts (31, 36). Rupture and bleeding from venous shunts is a major cause of mortality in *S. mansoni* and *S. japonicum* infected individuals (30). Occasionally, eggs embolize to other organs, such as the spleen, lungs or central nervous system (CNS) and the resulting disease and symptoms result from granulomatous responses at these sites.

The symptoms of urinary schistosomiasis are provoked by the granulomatous response against eggs in the vesical and ureteral walls (30). The primary symptom is hematuria, especially in children. Chronic urinary schistosomiasis may lead to renal failure and is associated with the development of squamous-cell carcinoma of the bladder (3, 30). Another severe consequence

of *S. hematobium* infection is the development of genital schistosomiasis, when eggs embolize to the cervix or testes and induce local granulomatous reactions (31).

Importantly, the development of the granuloma response to schistosome eggs embedded in the tissue plays an essential protective role in the host. In animals with an altered T_H2 response, protective granulomas do not develop and the hosts suffer from hepatotoxic liver damage, possibly due to schistosome egg hepatotoxins and excessive production of pro-inflammatory cytokines (31). Thus, paradoxically, the immune response necessary for protection against toxic damage is also responsible for the immunopathology associated with chronic schistosomiasis.

Treatment and vaccine development

Praziquantel, an antihelminthic drug, is the drug of choice for the treatment of schistosomiasis. It is effective against all forms of human disease-causing schistosomes and has an overall cure rate of 60-90% in patients from endemic areas (7). However, praziquantel is effective only against the adult worm, and does not prevent infection, migration of the schistosomula or re-infection. The exact mechanism of action on the adult parasites is unknown, though host antibodies are necessary (3, 38). Importantly, both treatment failure (7, 9, 10, 39) and parasite resistance (8, 40) to praziquantel have been reported. Oxamniquine is an alternative drug available for the treatment of *S. mansoni*, but is not effective against the other schistosomes and is not readily available (41).

Artemether, an anti-malarial drug, is effective against the immature stages of *S. mansoni*, *S. japonicum* and possibly *S. hematobium* and is currently being evaluated for treatment in combination with praziquantel (3, 30, 42-45).

Treatment with praziquantel is the mainstay of the control effort against schistosomiasis (3, 46). The World Health Assembly adopted a resolution in 2001 to regularly treat 75% of school-age children at risk for schistosomiasis in endemic countries by the year 2010 (47) and various national and global control programs have been established in attempts to meet those goals. Importantly, evaluation of ongoing treatment and control efforts suggests that treatment leads to reduced morbidity, despite high re-infection rates (13). Treatment may lead to immunologic changes which increase both resistance and immunoregulatory mechanisms, leading to higher protection against re-infection and reduced morbidity (48).

There is no evidence of immune-mediated clearance of adult worms in *S. mansoni*, *S. japonicum* or *S. hematobium* mouse infection models (49). However, analysis of human patients suggests that an age-dependent partial protection against schistosome infections exists. The concept of age-dependent resistance is based on two observations. First, pre-pubescent children carry a heavier worm burden than adults in endemic areas (31, 50). Second, adults are more refractory to re-infection following praziquantel treatment than children (31). This resistance to re-infection is correlated with immunoglobulin E (IgE) responses to worm antigens (31). Importantly, IgE responses require both humoral (B cell) and cell mediated (T_H2) immune responses. Resistance to re-

infection may also be influenced by hormonal changes at puberty, which may alter the immune response (50). Both the age-dependent increase in resistance to re-infection and the immunological response of “endemic normals”, who are frequently exposed to schistosome infected water but are resistant to infection, share several characteristics of immunity. For example there are increased ratios of IgE to IgG4 and increased levels of IgM against *S. mansoni* adult worm antigens (51, 52) associated with both types of resistance. Interestingly, despite an increase in IgE specific against adult worm antigens, the cellular response of endemic normals is associated with elevated IFNγ, a T_H1 effector cytokine, which suggests differences in the cellular and humoral arms of protection (51, 53).

In addition to age-dependent resistance, genetic correlates of protection have been identified. Intensity of infection is correlated with polymorphisms at the SM1 locus, which maps to a region that encodes T_H2 cytokines, again implicating IgE in protective responses (31). The age-dependent increase in resistance to schistosomes and the identification of endemic normals suggests that the quest for a suitable schistosome vaccine is potentially achievable.

There are currently no vaccines against any of the human schistosomiases, despite continued efforts within the schistosome research community to develop a suitable vaccine. Nonetheless, the irradiated cercariae vaccine model, which is highly successful in mice, provides an interesting and informative model in the search for a suitable vaccine. Gamma-irradiated cercariae penetrate the skin but have reduced migration in the host, compared to non-irradiated cercariae, which leads to the retention of the immature

schistosomes in the skin, lymph nodes and lung, the stimulation of the host's immune system, and the development of a protective immune response (54). Challenge infection with non-irradiated cercariae results in a 90% decrease in worm burden, demonstrating the effectiveness of the immune response against the migrating schistosomules. The protective immune response in vaccinated mice is mediated by T_H1-driven antibody responses (54), though more recent studies have suggested that the polarity of the $CD4^+$ response may not be important (55). Despite the success of the irradiated cercariae vaccine in mice, expansion of the vaccine into humans is unfeasible. Therefore, human vaccine efforts have focused on developing vaccines against schistosome antigens that are immunogenic in mice vaccinated with irradiated cercariae. One such antigen, Glutathione S-transferase (GST), is the target of a vaccine currently in Phase II clinical trials (56, 57). In addition to interest in the development of a human vaccine against the major schistosome pathogens, there has been recent effort into the development of a transmission blocking vaccine against *S. japonicum*, for use in water buffalo in China (27).

Host-Schistosome Interactions

Immune response to blood flukes

Experimental analysis of the immune response during *Schistosoma* infections has been dominated by research of the T_H2-mediated granulomatous response to schistosome egg antigens. However, there is a body of work that describes the immune response to the schistosome larvae during the initial phases of infection. Within hours after penetration of the skin by schistosome cercariae, a local inflammatory response occurs, mediated by the production of pro-inflammatory cytokines IL-1β, IL-12, TNFα, MIP1α, and IL-6, and a concurrent influx of polymorphonuclear and mononuclear cells. Following production of pro-inflammatory cytokines, regulatory factors such as IL-1 receptor agonist (IL-1RA), IL-10, prostaglandins (PG) E_2 and D_2 are produced, which down-regulate the initial inflammatory response (29). Production of immune regulatory factors soon after infection suggests that schistosomules may produce immunomodulatory molecules. Excretory-secretory (ES) products produced by skin-stage schistosomules have been implicated in immune modulation, through activation of mast cells, induction of $CD4^+$ T cell apoptosis and/or alteration of the activation status of antigen presenting cells (29). It has been hypothesized that invading schistosomules actively downmodulate the immune response in order to circumvent protective immunity, thus allowing schistosomules to exit the skin (29).

There are little data regarding the host immune response to the migrating schistosomule or the adult worm during pre-patent infection. However, evaluation of the cellular immune response during acute schistosomiasis in humans suggests that the immune response is dominated by the production of T_H1 cytokines (31, 58) though other work suggests a mixed T_H1/T_H2 (or T_H0) response develops during the acute phase (59). Regardless, following the onset of egg production and deposition, a dramatic shift in the immune response occurs and a strongly T_H2-polarized immune response develops in response to egg antigens (60) directing granuloma development around the schistosome eggs (61, 62). T_H2 mediated granulomas are made up of collagen, macrophages, eosinophils and $CD4^+$ T cells and are necessary for host survival as hepatoxicity leads to increased morbidity in the absence of a T_H2-mediated granulomatous response (31). Typically, during the chronic infection, an increase in T_H1 cytokine production is concurrent with a downmodulation of the T_H2 response, resulting in a decrease in granuloma size (63). Failure to downmodulate the T_H2 response results in increased fibrosis and fibrosis-mediated sequelae and is mediated through the actions of the T_H2 cytokines IL-13 and IL-21 (35-37). IL-10 plays a critical role in the immunoregulation of the acute and chronic responses, preventing the detrimental effects of strongly polarized T_H1 or T_H2 responses (64, 65).

Schistosome development in the host is dependent upon CD4$^+$ mediated signals

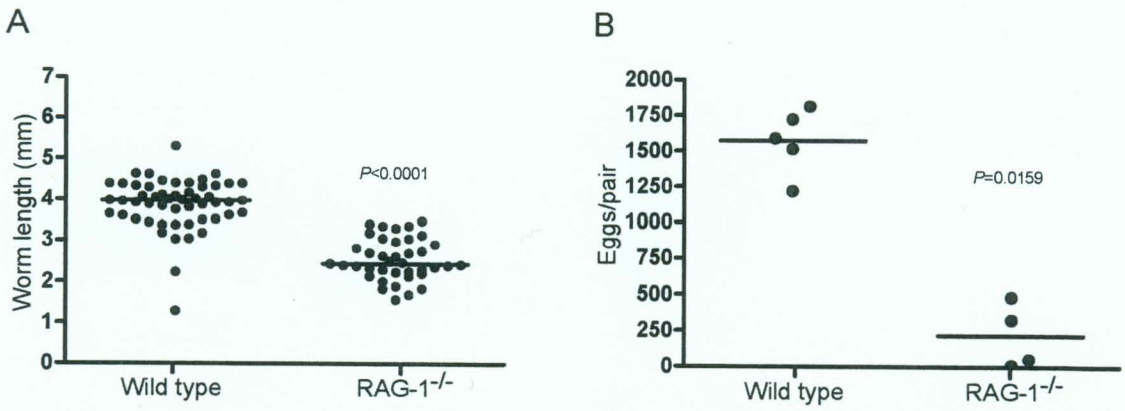
Interestingly, CD4$^+$ T cell-mediated granulomas around eggs in the intestinal wall facilitate the transit of eggs through the tissue and into the intestinal lumen (66, 67), suggesting that the host adaptive immune response plays an important role in completing the schistosome life cycle by facilitating transmission back to the molluscan intermediate host. Several lines of evidence suggest that other aspects of the intramammalian phase of the life cycle are also dependent on the adaptive immune system. First, maintenance of the schistosome life cycle from the schistosomule phase to the growth, maturation, pairing and egg production of adult worms, has not been achieved under *in vitro* conditions (31). Therefore, it seems likely that schistosome parasites require host-derived signals for full development, beyond the nutritional requirements that are provided under various *in vitro* culture conditions. Second, there are numerous reports of impaired schistosome development in hosts that are immunosuppressed or immunodeficient for various reasons. For example, fewer *S. mansoni* worms develop in mice following hydrocortisone treatment, indicating a possible role for host immune signals in schistosome development (68, 69). Immunosuppression, through either hydrocortisone treatment or T cell depletion, also leads to reduced or delayed *S. mansoni* fecundity (70) though a similar reduction in *S. japonicum* fecundity was not observed in a similar model system (71). When *Schistosoma* infections were evaluated in severe combined immunodeficiency (*Prkdc*$^{scid/scid}$) mice, which mostly lack functional B and T cells, reduced *S. mansoni* and delayed *S. japonicum* egg-laying was observed (72,

73), suggesting a requirement for either B or T cells in facilitating schistosome development.

Because *Prkdc*$^{scid/scid}$ mice have a “leaky” phenotype in which both B and T cells are detectable, especially on the CB17 background used in some of these studies (74), the role of the host adaptive immune response in schistosome development was further investigated through infections of recombination activation gene (RAG) deficient mice. In the absence of either RAG-1 or RAG-2, V(D)J rearrangement does not occur, leading to an inability to rearrange immunoglobulin and T cell receptor genes and early arrest in both B and T lymphocyte differentiation. Thus, RAG-deficient animals do not possess any mature B or T cells (75, 76). *S. mansoni* development, as assessed through measurements of parasite growth, pairing and egg production, is attenuated in the absence of B and T cells (77) (Fig. 3). Further studies unambiguously identified CD4$^+\alpha\beta$ T cells as the component of the adaptive immune system that is critical for parasite development. First, adoptive transfer of CD4$^+\alpha\beta$ T cells fully restores parasite development in RAG-deficient animals, whereas transfer of CD8$^+$ T cells or B cells does not. Second, specific depletion of CD4$^+\alpha\beta$ cells in wild type mice attenuates parasite development, whereas depletion of CD8$^+$ T cells, B cells or $\gamma\delta$T cells does not affect parasite development (77). Thus, CD4$^+\alpha\beta$ T cells are necessary for normal *S. mansoni* development in the murine host.

Figure 3: *S. mansoni* development is attenuated in mice lacking adaptive immune components.

Six weeks following laboratory infection with *S. mansoni* cercariae, parasite growth and development were assessed in wild type and RAG-$1^{-/-}$ mice through evaluation of (A) parasite length and (B) egg production.


A
Worm length (mm)
7
6
5
4
3
2
1
0
P<0.0001
Wild type
RAG-1-/-
B
Eggs/pair
2000
1750
1500
1250
1000
750
500
250
0
P=0.0159
Wild type
RAG-1-/-

T cell mediated immune responses

A principle feature of the adaptive immune response is the ability to direct a specific and tailored response against the antigens present on an invading microorganism. T lymphocytes (T cells) are responsible for coordinating the specific response to bacterial, viral and helminthic pathogens. There are two types of T cells which are functionally and phenotypically identifiable. Cytotoxic T cells (CTLs) express the CD8 cell surface molecule and recognize infected cells through interactions between the T cell receptor (TCR) and antigen presented in the context of major histocompatability complex (MHC) class I molecules. T helper cells (T_H) cells express the CD4 cell surface molecule and respond to antigen presented in the context of MHC class II molecules by antigen presenting cells. T_H cells produce cytokines which drive inflammatory and allergic responses. Subsets of T_H cells are further defined based on the production of different cytokines. T_H1 cells produce interferon gamma (IFNγ) and direct the inflammatory response against bacterial and viral pathogens. T_H2 cells produce interleukin (IL) -4, IL-5 and IL-13 and are the primary effector cells in the response to parasitic helminth infections and also mediate allergic responses. Regulatory T cell (T_{reg}) responses are mediated through the effects of immunomodulatory cytokines such as IL-10.

Hypotheses and specific aims

Several lines of evidence imply that the mechanism by which $CD4^+$ T cells facilitate schistosome development does not involve the development of polarized helper T cells (T_H) responses. First, schistosomes develop normally when either T_H1 or T_H2 development is blocked by deletion of STAT-4 and STAT-6, respectively (77, 78). Second, no role has been found for any of the key mediators of the T_H1, T_H2, or regulatory T cell (T_{reg}) effector responses, including IFNγ, TNF, IL-4, IL-5, IL-13, and IL-10 (77). Based on the finding that $CD4^+$ T cells, in the absence of any obvious effector function, are necessary for the development and maturation of schistosomes, we sought to investigate whether early events in the activation of T cells by antigen are involved in the development of schistosomes. First, we sought to evaluate the extent to which the evolutionary dependence upon host CD4-mediated signals is conserved in other medically important *Schistosoma* species. Secondly, we investigated the role of the common gamma chain (γ_C) cytokines IL-7 and IL-2 on schistosome development, since these cytokines play important roles in the homeostatic maintenance of T cells and the autocrine activation of antigen-stimulated T cells, respectively. Finally, we investigated whether T cell activation by antigen through the TCR is required for normal parasite development.

The central goal of this dissertation is to further elucidate the mechanism through which host $CD4^+$ T cells influence schistosome development and to evaluate the extent to which this host-parasite relationship is conserved in other

medically important schistosoma species. We hypothesize that dependence on host $CD4^+$ T cell signals is conserved throughout the *Schistosoma* genus. Further, we hypothesize that the ability of $CD4^+$ T cells to promote normal schistosome development is dependent on the ability of these cells to specifically respond to schistosome antigens. To test these hypotheses, our specific aims are as follows:

Specific Aim 1: To evaluate the evolutionary dependence upon host CD4-mediated signals among *Schistosoma* genus. *S. 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, the evolutionary origins and limits of this aspect of the host-pathogen relationship are examined through evaluation of the development of a range of different schistosome species and strains in immunodeficient mice.

Specific Aim 2: To determine whether the common gamma chain cytokine interleukin-2 plays a significant role in modulating schistosome development. Preliminary data suggest that interleukin-2 (IL-2), a critical regulatory and effector cytokine produced and utilized by $CD4^+$ T cells, may play a role in determining the outcome of schistosome development. Because IL-2 mediates pleiotropic effects, the requirement for IL-2 production by $CD4^+$ T cells in modulating *S. mansoni* development is evaluated.

Specific Aim 3: To evaluate the role of antigen-specific $CD4^+$αβ T cell activation in *S. mansoni* development. It is possible that the ability of $CD4^+$ T cells to promote schistosome development is independent of T cell responses to antigen and is related instead to normal homeostatic processes such as naïve T cell survival (79). To address this possibility, schistosome development in an *in vivo* model system where $CD4^+$ T cells are present but are unable to respond to schistosome antigens is examined.

Chapter 2

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 *S. 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 when during *Schistosoma* evolution 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 *S. japonicum*, suggesting this developmental adaptation arose early in *Schistosoma* evolution. We also demonstrate that the development of the more evolutionarily derived species *S. haematobium* and *S. intercalatum* is 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 all the schistosome pathogens of humans.

Introduction

Schistosoma parasites infect more than 200 million people in 74 countries worldwide (20). The African schistosomes *Schistosoma mansoni* and *S. intercalatum*, and the Asian species *S. japonicum* and *S. mekongi*, establish chronic infections that can lead to severe and life-threatening hepatosplenic disease. *S. haematobium*, the causative agent of urinary schistsosomiasis, is found throughout Africa. Praziquantel is the only anthelminthic 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 (3, 27) 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 (T_H2) responses to egg antigens (31). In addition to constituting the dominant cause of pathology during schistosome infection, egg-induced granulomas also facilitate transit of eggs across the bowel wall and their egress from the body (67). Interestingly, we have previously shown that normal *S. mansoni* growth, sexual maturation and fecundity are also dependent on host $CD4^+$ T cells (77) and thus, *S. mansoni* appears to exploit the activities of host $CD4^+$ T cells to facilitate parasite development and transmission (78). 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 (80). 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 (78).

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 (26, 81-83). *S. japonicum* parasitizes the broadest range of natural definitive hosts among the Schistosomatidae (26). In contrast, S*. haematobium* is essentially a human pathogen, without significant animal reservoirs in nature (26). With regard to schistosomulum migration, *S. japonicum* experimental infections are characterized by greater speed and success of parasite migration (summarized in(84)). It has been suggested the rapid migration and extended host range of *S. japonicum* are due to distinct cercarial enzymes which facilitate rapid passage through the mammalian host (84, 85). 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 (81). 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 (26). 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 (86), along with *Schistosoma* species that parasitize other mammals: the "*japonicum*" group (*S. japonicum*, *S. mekongi*, *S. malayensis*, *S. sinensium*); the "*mansoni*" group (*S. mansoni*, *S. rodhaini*); and the "*haematobium*" group (*S. haematobium*, *S. intercalatum*, *S. guineensis*, *S. curassoni*, *S. bovis*). Thorough phylogenetic analyses based on combinations of morphological and molecular data support these traditional groupings (87). 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 (87, 88). 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 (14, 87). 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. First, 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 ($RAG\text{-}1^{-/-}$) 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 (89-91), 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 $RAG\text{-}1^{-/-}$ 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.

Materials and methods

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.

Schistosome maintenance and mouse infections

S. japonicum strains were maintained using appropriate subspecies of *Oncomelania hupensis* as intermediate hosts – *O. h. hupensis* for the Chinese strain, *O. h. nosophora* for the Japanese strain, *O. h. formosana* for the Formosan strain, and *O. h. 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. *S. 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. *S. japonicum* infections were allowed to proceed for 28 days. *S. haematobium* were maintained using *Bulinus truncatus* as intermediate hosts. Cercarial shedding was induced by placing infected *Bulinus* in the light for 1 hour. Mouse tails were

exposed to approximately 1000 *S. haematobium* cercariae per mouse for 40 minutes. *S. haematobium* infections were allowed to proceed for 79 days. *S. intercalatum*, an isolate from Cameroon originally obtained from the Théron group, was maintained using *B. crystallinus* as intermediate hosts. Cercarial shedding was induced by placing snails in the light for 1-2 hours, as for *S. haematobium*. Mouse tails were exposed to approximately 150 *S. intercalatum* cercariae per mouse for 40 minutes. *S. intercalatum* infections were allowed to proceed for 42 days. *Schistosomatium douthitti* was maintained using *Stagnicola elodes* as intermediate hosts. Cercarial shedding was induced by placing infected *S. elodes* in the dark for 1-2 hours. 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. *S. 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.

Parasite recovery and measurement of parasitological parameters

Parasites were recovered from the portal system by perfusion (92), 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 (93). Liver tissue was digested in 0.7% trypsin (50 ml) in phosphate-buffered saline (PBS) for 2-3 hours at 37°C, and eggs were counted under a dissecting microscope.

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). 4×10^6 cells were transferred into RAG-1$^{-/-}$ mice by intravenous injection into a lateral tail vein. Recipient animals were then infected with cercariae 24 hours later, as described above. To verify the efficacy of adoptive transfers at necropsy, splenocytes from reconstituted RAG-1$^{-/-}$ mice were surface labeled with APC-Cy7-conjugated antibodies to CD4, FITC-conjugated antibodies to CD8, 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).

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.

Results

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. 4-6). First, parasites recovered from RAG-1$^{-/-}$ mice were considerably smaller than those obtained from wild type controls (Fig. 4A). To quantify these differences, parasite length was determined from digital micrographs. Male worms of all 4 *S. japonicum* strains recovered from RAG-1$^{-/-}$ mice were significantly reduced in length ($P<0.0001$ for all strains) when compared to male worms recovered from wild type mice (Fig. 4B). The percent reduction of mean worm length in RAG-1$^{-/-}$ mice compared to 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 post-infection.

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 to infection of wild type mice, (Fig. 5). 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. 6). 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 ± SEM) for the Chinese strain to 3369 ± 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. 5), egg production by this strain was still ablated in RAG-1$^{-/-}$ mice, making the loss of fecundity even more striking.

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. 7A). Measurements revealed that male worms from reconstituted RAG-1$^{-/-}$ mice were twice the length of those recovered from control RAG-1$^{-/-}$ mice (5.46 mm +/- 0.12, compared to 2.72 mm +/- 0.089) (Fig 7B). Though sexual maturation as measured by the percentage of females in pairs was not significantly increased by adoptive transfer of CD4^{+} T cells (Fig. 7C), egg production by the pairs that did form was restored to wild type levels (Fig 7D; 44.35 +/- 9.5 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.

S. 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 (94), 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. 8A). 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. 8B). There was a 44% reduction in mean *S. haematobium* worm length in RAG-1$^{-/-}$ mice. *S. 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. 8A and B). *S. haematobium* sexual maturation was significantly attenuated in RAG-1$^{-/-}$ mice, as demonstrated by reduction in percent pairing among female parasites (Fig. 8C). 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 6610 ± 558.6 in wild type mice)(Fig. 8D). 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.

Schistosomatium 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 *Schistosomatium douthitti* cercariae and evaluated the development of the worms at 6 weeks post infection. Visually, *Schistosomatium douthitti* worms recovered from RAG-1$^{-/-}$ mice were similar in appearance to those recovered from wild type mice (Fig. 9A). Careful measurement of *S. douthitti* male worms revealed a slight reduction in the length of worms from RAG-1$^{-/-}$ mice compared to those recovered from wild type mice (Fig. 9B). However, development of *Schistosomatium 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. 9C) or the number of eggs produced per pair (Fig. 9D) in RAG-1$^{-/-}$ mice. Because female *Schistosomatium 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 *Schistosomatium douthitti*.

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 (77)). Our data clearly show that, similar to *S. mansoni* (77) the adaptive immune system is necessary to facilitate worm development and sexual maturation in all of the human pathogens we examined (Figs. 4-8). 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. 7). 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 (87). 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 (73). 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 (74). 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. 8) was indistinguishable from that of *S. mansoni* (77) and *S. japonicum* (Figs. 4-6). These results suggest that the developmental dependence on adaptive immune signals described in *S. mansoni* and *S. japonicum* (Figs. 4-6) 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. 7;(77)), 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 requires $CD4^+$ 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 *Schistosomatium douthitti* was not significantly impaired by lack of an adaptive immune system (Fig. 9). While *Schistosomatium 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 (87, 95). 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 (87). 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 (26). By contrast, the natural definitive hosts for *Schistosomatium douthitti* are small short-lived rodents such as muskrats,

meadow voles and the redback mouse (summarized in (96)). 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 (26). Expanding upon 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 (77). 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 (78). 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 (26). 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 (76). However, we show that $RAG^{-/-}$ animals can support the normal development of the distantly related schistosome *Schistosomatium douthitti* (Fig. 9), 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 (97), and animals that possess defects in NK cell function, such as NOD-SCID mice (77).

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 T helper 1 (T_H1) and T_H2 responses (77, 98). Rather, more fundamental aspects of $CD4^+$ T cell biology, including homeostatic maintenance of $CD4^+$ T cells by interleukin-7 (IL-7) and production of IL-2 by $CD4^+$ T cells, appear to play an indirect role in creating a permissive environment for parasite development (97).

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. First, 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 (87, 99), 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.

Acknowledgements

This work was supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases grants K22 AI053054 and R01 AI066227 (to SJD). *S. japonicum* and *S. haematobium* parasites were provided through NIH/NIAID contract N01 AI30026. E. S. Loker generously provided *Schistosomatium douthitti*-infected *Stagnicola elodes* snails and insightful discussion. We thank Cara H. Olsen for assistance with statistical analyses.

Table 1: Schistosome infections. Number of mice infected with each schistosome species.

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

Figure 4: *S. 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 post-infection. (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.

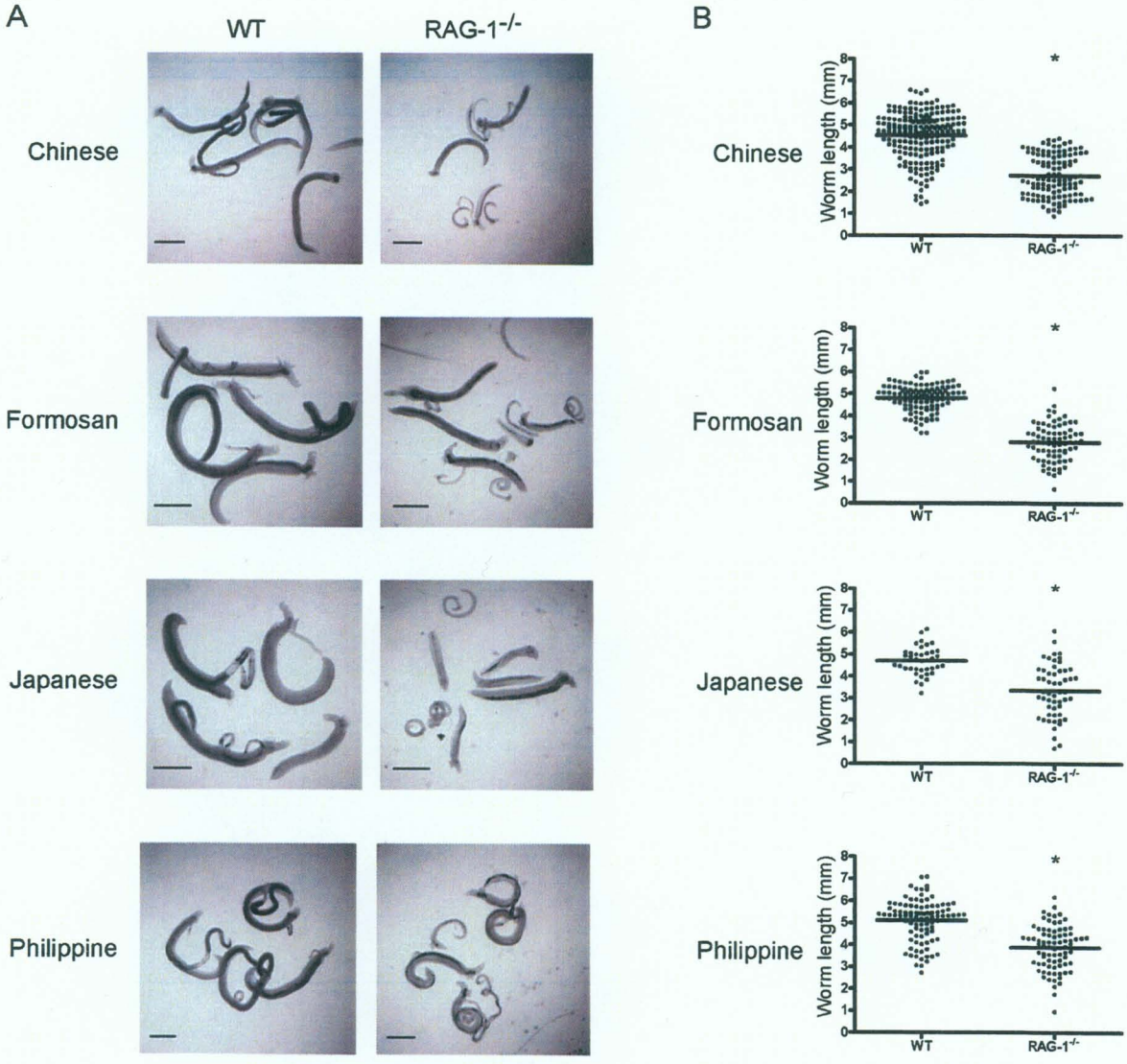

A
WT
RAG-1-/-
Chinese
Formosan
Japanese
Philippine
B
Chinese
Formosan
Japanese
Philippine
Worm length (mm)
WT
RAG-1-/-
*

Figure 5: Pairing of *S. 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 post infection was quantified for each mouse as: (the number of paired females/total number of females) × 100. Percent paired females worms recovered per mouse are shown for 8-15 mice per group. Horizontal bars represent the median value for each experimental group. Mann-Whitney P values are indicated on the graphs.

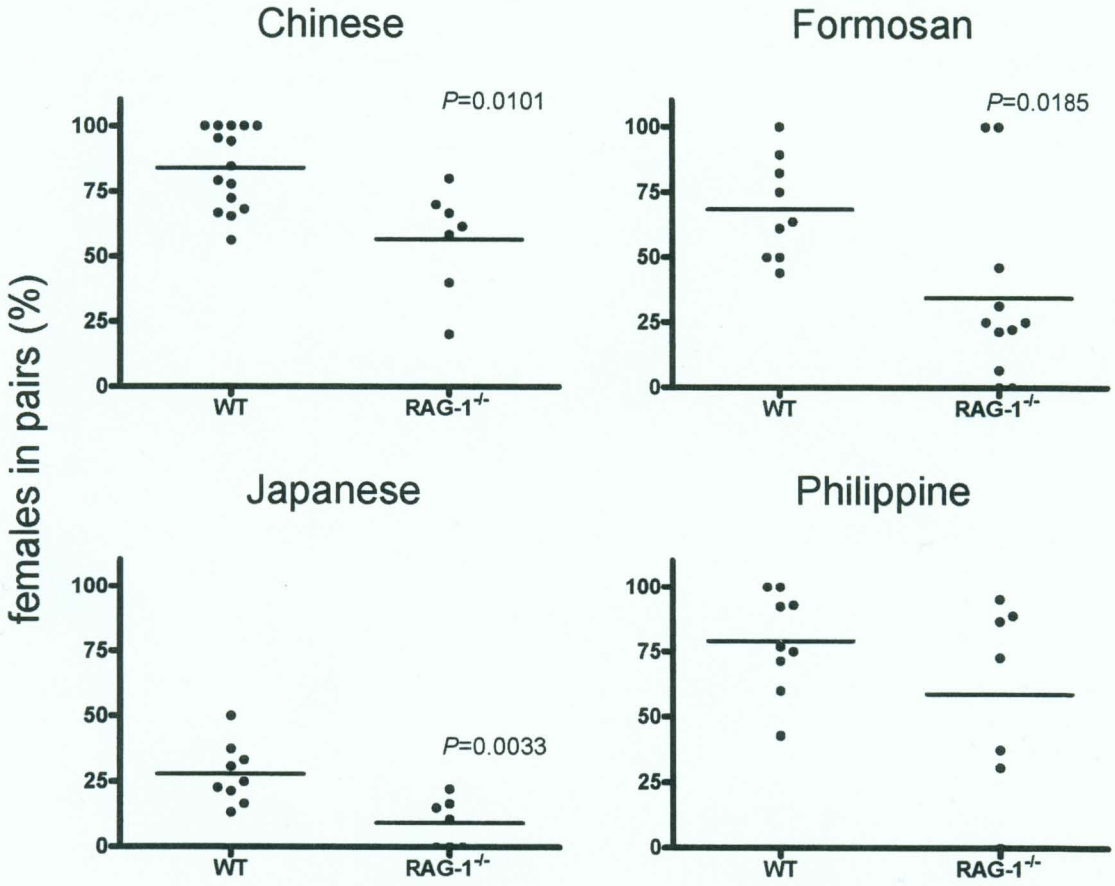

Chinese
Formosan
Japanese
Philippine
females in pairs (%)
P=0.0101
P=0.0185
P=0.0033
100
75
50
25
0
WT
RAG-1-/-

Figure 6: *S. 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 (8-15 mice per group.) Horizontal bars represent the median value for each experimental group. Mann-Whitney P values are indicated on the graphs.

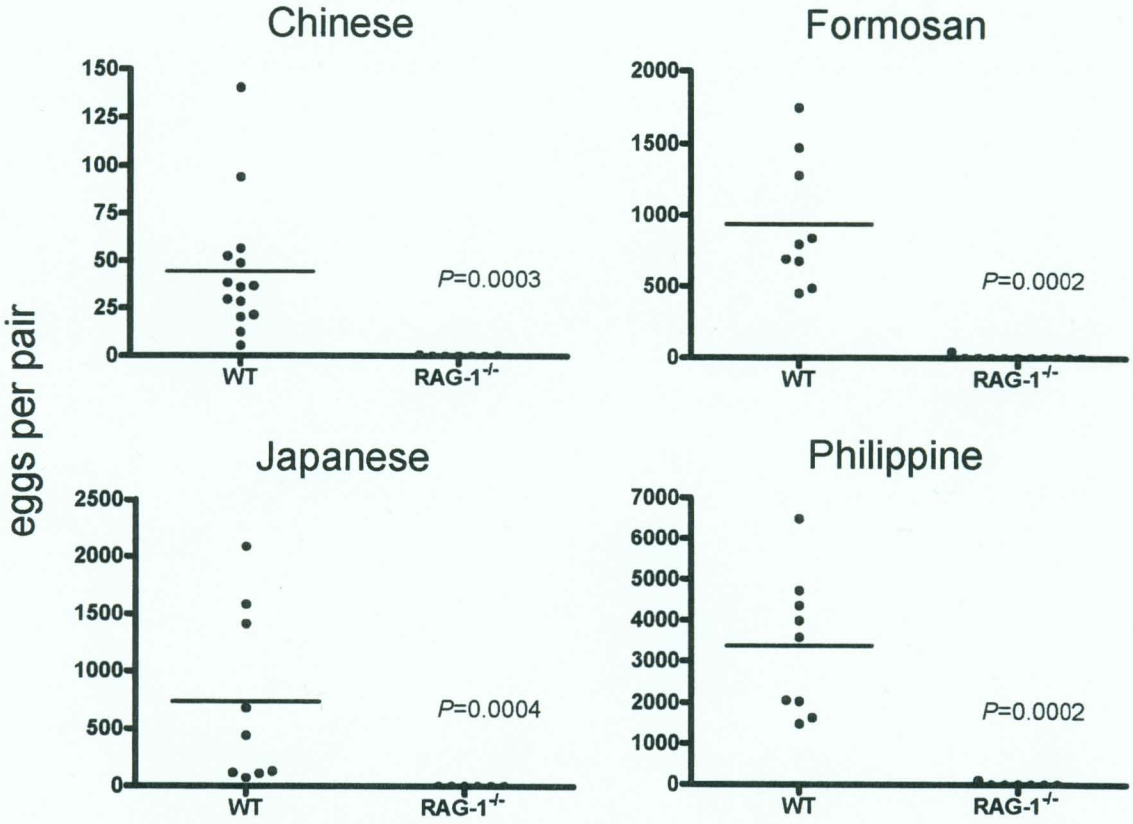

Chinese
Formosan
Japanese
Philippine
eggs per pair
150
125
100
75
50
25
0
2000
1500
1000
500
2500
7000
6000
5000
4000
3000
P=0.0003
P=0.0002
P=0.0004
P=0.0002
WT
RAG-1-/-

Figure 7: $CD4^+$ lymphocytes restore *S. japonicum* development in RAG-$1^{-/-}$ mice.

4×10^6 $CD4^+$ wild type T cells were transferred into RAG-$1^{-/-}$ recipient mice. RAG-$1^{-/-}$, wild type and $CD4^+$-reconstituted RAG-$1^{-/-}$ mice were infected with 35 *S. japonicum* (Chinese strain) cercariae two days after the transfer. (A) Micrographs of representative parasites from RAG-$1^{-/-}$, wild type (WT) 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 females 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.

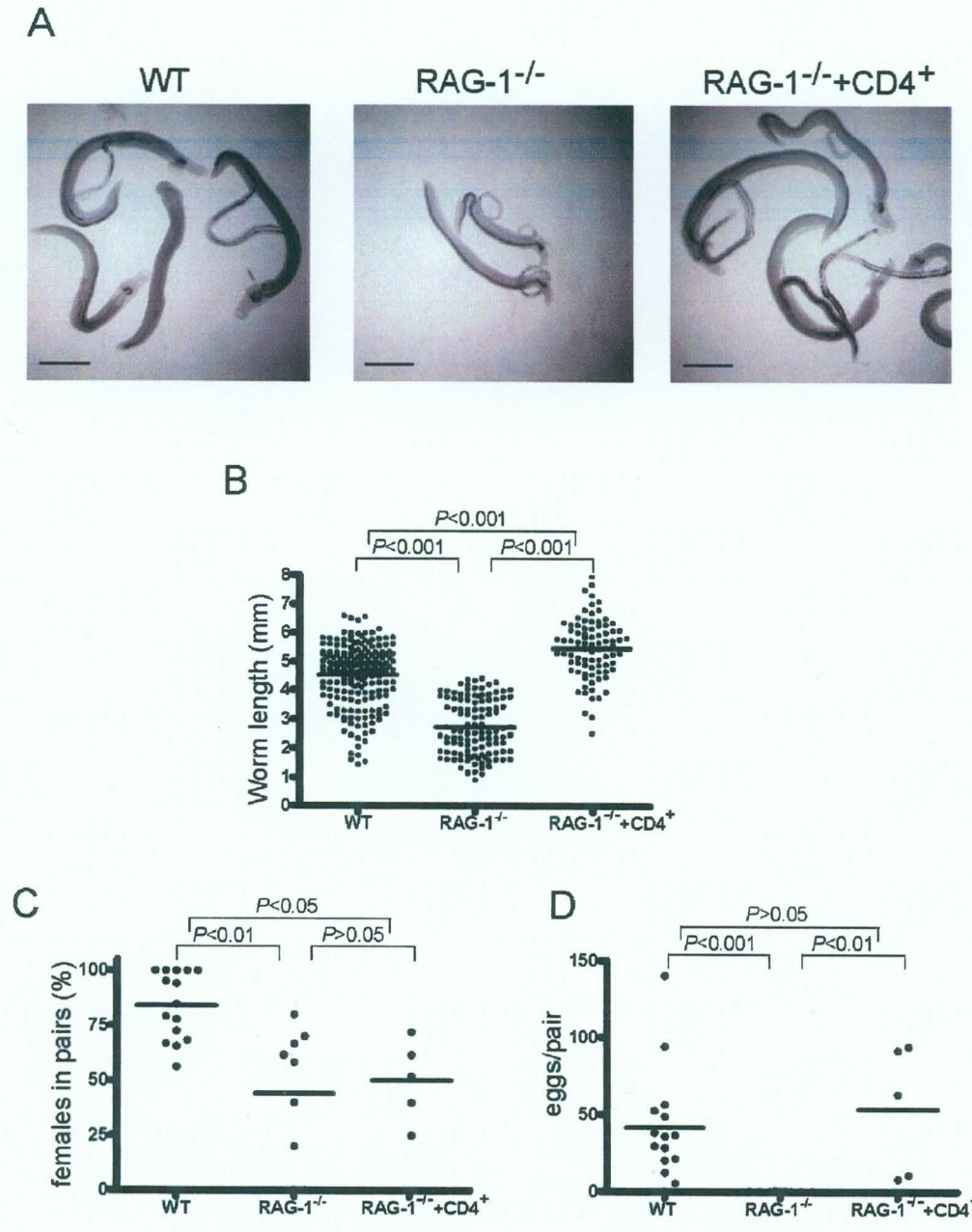

A
WT
RAG-1-/-
RAG-1-/-+CD4+
B
P<0.001
P<0.001
P<0.001
Worm length (mm)
WT
RAG-1-/-
RAG-1-/-+CD4+
C
P<0.05
P<0.01
P>0.05
females in pairs (%)
WT
RAG-1-/-
RAG-1-/-+CD4+
D
P>0.05
P<0.001
P<0.01
eggs/pair
WT
RAG-1-/-
RAG-1-/-+CD4+

Figure 8: *S. haematobium* and *S. intercalatum* development in RAG-1$^{-/-}$ mice.

Wild type (WT) and RAG-1$^{-/-}$ mice were infected with 1000 *S. haematobium* cercariae and evaluated 77 days post-infection (A-D) or 100 *S. intercalatum* cercariae and evaluated 42 days post infection. (A) Micrographs of representative *S. haematobium* and *S. intercalatum* parasites from RAG-1$^{-/-}$ and wild type mice at 20× magnification. Scale bar = 1mm. (B) *S. 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) *S. 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.

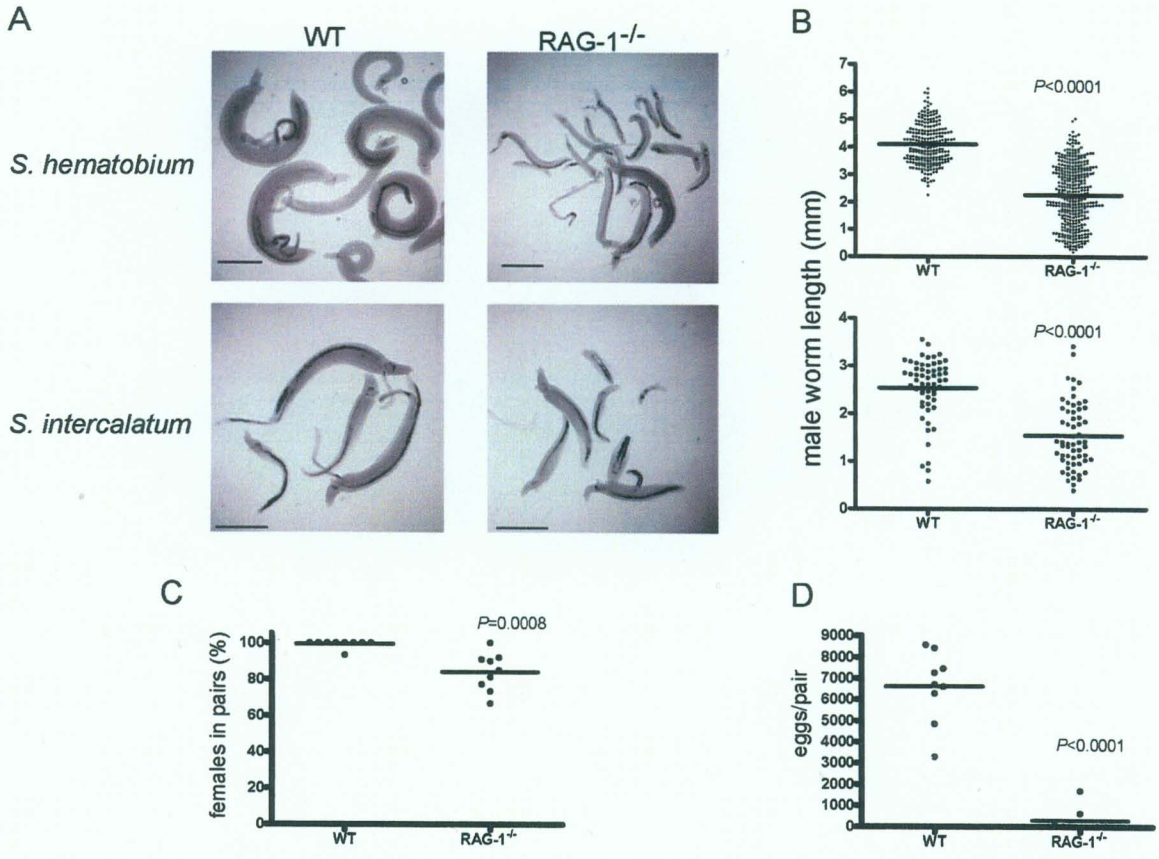
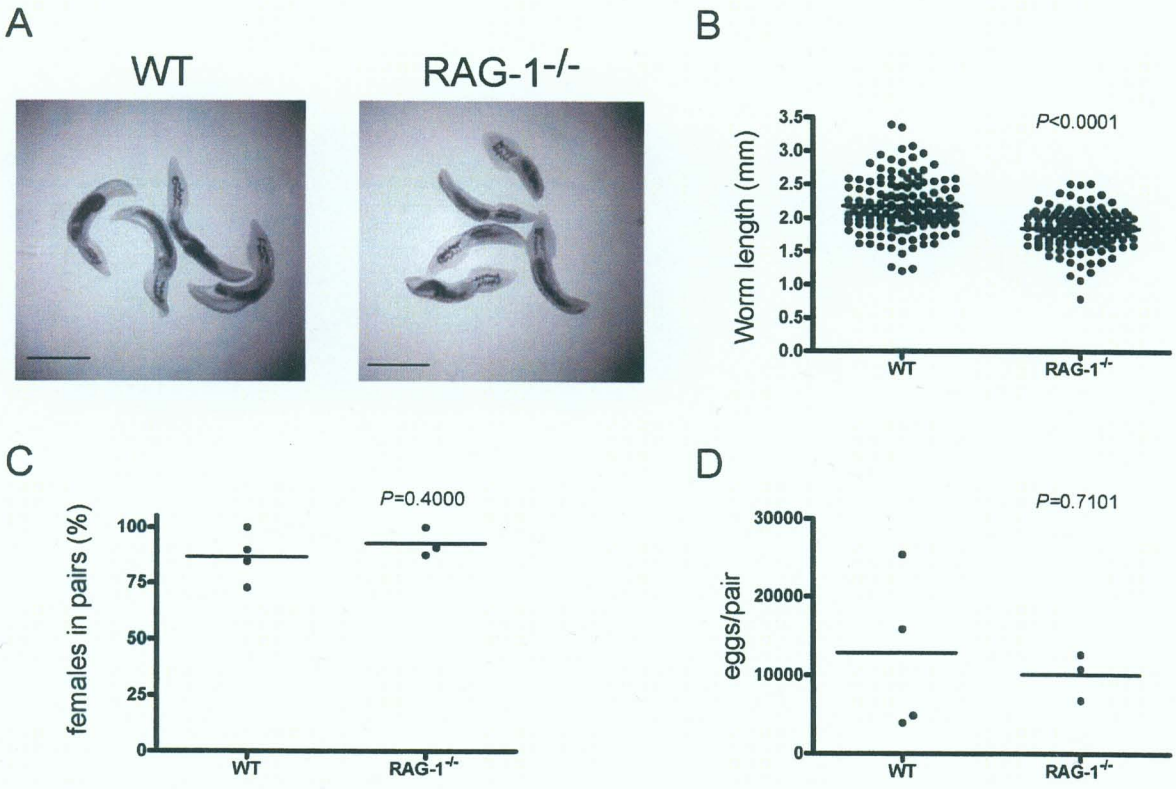

A
WT
RAG-1-/-
S. hematobium
S. intercalatum
B
male worm length (mm)
P<0.0001
P<0.0001
WT
RAG-1-/-
C
females in pairs (%)
P=0.0008
WT
RAG-1-/-
D
eggs/pair
P<0.0001
WT
RAG-1-/-

Figure 9: *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 post-infection. (A) Micrographs of representative *S. douthitti* parasites recovered from RAG-1$^{-/-}$ and wild type mice at 20× magnification. Scale bar = 1mm. (B) *S. 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.


A
WT
RAG-1-/-
B
Worm length (mm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
P<0.0001
WT
RAG-1-/-
C
females in pairs (%)
100
75
50
25
0
P=0.4000
WT
RAG-1-/-
D
eggs/pair
30000
20000
10000
0
P=0.7101
WT
RAG-1-/-

Chapter 3

The Role of IL-2 Production by $CD4^+$ T Cells in *S. mansoni* Growth and Development

All of the figures shown in this chapter reflect the work of Erika Lamb, as contributed to the published work:

Rebecca B. Blank, Erika W. Lamb, Anna S. Tocheva, Emily T. Crow, K. C. Lim, James H. McKerrow, and Stephen J. Davies. 2006. The Common gamma Chain Cytokines Interleukin (IL)-2 and IL-7 Indirectly Modulate Blood Fluke Development via Effects on $CD4^+$ T Cells. *The Journal of infectious diseases* 194:1609-1616.

Dr. Davies contributed to the design of the experiments and interpretation of the data as well as the preparation of the manuscript.

Introduction

Schistosoma mansoni development is severely impaired in recombination activating gene deficient ($RAG^{-/-}$) mice which lack B and T lymphocytes and is restored by adoptive transfer of $CD4^+$ T cells (77). These data and other lines of evidence indicate that $CD4^+$ T cells play a critical role in facilitating schistosome development in the definitive mammalian host. Impaired *S. mansoni* development has also been reported in IL-7-deficient ($IL\text{-}7^{-/-}$) mice (100). Worms recovered from $IL\text{-}7^{-/-}$ mice display decreased parasite migration, stunted growth, and reduced parasite pairing and fecundity, suggesting that this host factor may also play a direct role in promoting parasite development. Since IL-7 is not expressed by T lymphocytes but is produced by endothelial and epithelial cells such as thymic stromal cells, it may represent a non-$CD4^+$ T cell-mediated mechanism for host modulation of schistosome development. However, IL-7 is critical for the thymic development of T lymphocytes and their subsequent survival in the periphery, as demonstrated by the severely lymphopenic phenotype of $IL\text{-}7^{-/-}$ (101) and $IL\text{-}7R^{-/-}$ (102) mice. It was therefore hypothesized that the defective schistosome development reported in $IL\text{-}7^{-/-}$ mice was the indirect result of impaired T cell development and T lymphopenia in the absence of IL-7 signaling, rather than the result of a direct effect of IL-7 on the parasites. To test this hypothesis, schistosome infections were examined in mice that lack components of the IL-7R complex (IL-7Rα and the common γ (γ_C) chain), because IL-7 signaling is abrogated in these mice (102, 103) but IL-7 synthesis is

intact, as demonstrated by their ability to sustain homeostatic proliferation of adoptively transferred IL-7Rα-expressing T cells (104).

Infection of IL-7Rα$^{-/-}$ and $\gamma_C^{-/-}$ mice revealed that schistosome development is compromised in the absence of IL-7R signaling, indicating that the effect of IL-7 on parasite development is indirect and likely the result of T lymphopenia in the absence of IL-7 signals (97). Interestingly, deletion of the γ_C chain severely impaired schistosome development, with parasites that were even smaller than those recovered from IL-7Rα$^{-/-}$ mice. Therefore, signaling by other γ_C chain - dependent cytokines (IL-2, IL-4, IL-9, IL-15 and IL-21) might also influence schistosome development. Because there is currently no evidence that parasite development is altered in IL-4$^{-/-}$ (105, 106), IL-9$^{-/-}$ (107), IL-15$^{-/-}$ (JHM and SJD, unpublished data), or IL-21-signaling-deficient mice (37), attention focused on IL-2, which, in addition to its long recognized autocrine role in the proliferation and function of T_H1 cells (108, 109), is now known to be critical in the survival and functionality of $CD4^+CD25^+$ T_{reg} cells (110) and to the early differentiation of T_H2 cells (111). To test the role of IL-2 in schistosome development, *S. mansoni* development was examined in mice where IL-2 signaling is specifically disrupted. Although considerably less severe than those observed in IL-7Rα$^{-/-}$, $\gamma_C^{-/-}$ and RAG-1$^{-/-}$ mice, defects in the development of *S. mansoni* worms were detected in IL-2$^{-/-}$ mice (97). To determine whether IL-2 acted directly on the parasite or whether IL-2 signaling via its receptor was required for normal schistosome development, parasite development was exaimined in IL-2Rα$^{-/-}$ (CD25$^{-/-}$) mice (112) in which IL-2 is produced, but transduction of IL-2 signals is impaired. The

phenotype of *S. mansoni* worms isolated from IL-2R$\alpha^{-/-}$ mice was similar to that of worms from IL-2$^{-/-}$ mice, with evidence of developmental impairment that was less pronounced than that observed in IL-7R$\alpha^{-/-}$ mice (97). These data indicate that, in addition to IL-7, IL-2 signals are also required for normal schistosome development and that these signals, like IL-7, have an indirect effect on the developing parasites (97).

The diversity of roles played by IL-2 in CD4$^+$CD25$^+$ T_{reg} cell function (110) and CD4$^+$ T helper responses (108, 109, 111) suggests that the indirect effect that IL-2 has on schistosome development may be mediated by a number of different mechanisms. To test whether the defective parasite development observed in IL-2$^{-/-}$ and IL-2R$\alpha^{-/-}$ mice could be specifically attributed to a lack of CD4$^+$CD25$^+$ T_{reg} cells, the ability of these cells to rescue schistosome development in RAG-1$^{-/-}$ mice was assessed. Interestingly, transfer of purified CD4$^+$CD25$^+$ T_{reg} cells or CD4$^+$CD25$^-$ T cells both rescued schistosome development in RAG-1$^{-/-}$ mice, indicating that, although CD4$^+$CD25$^+$ T_{reg} cells have the ability to restore parasite development, these cells are not specifically required and other CD4$^+$ T cells can mediate the same effects (97).

Because we could not identify a specific role for CD4$^+$CD25$^+$ T_{reg} cells in schistosome development, we tested whether expression of IL-2 by CD4$^+$ T cells is required to rescue schistosome development by adoptively transferring IL-2$^{-/-}$ CD4$^+$ T cells into RAG-1$^{-/-}$ mice.

Materials and Methods

Experimental Mice

Wild type mice were purchased from National Cancer Institute (NCI), (Frederick, MD). Breeding pairs of IL-$2^{-/-}$ (113) and RAG-$1^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in-house to generate sufficient numbers for experiments. All mice possessed the C57BL/6 genetic background. Mice were infected percutaneously via the tail skin with 150 *S. mansoni* cercariae (Puerto Rican strain) shed from infected *Biomphalaria glabrata* snails (92). All studies were performed in accordance with protocols approved by the relevant Institutional Animal Care and Use Committees.

Cell isolation

Lymph nodes and spleens from wild type C57BL/6 mice or IL-$2^{-/-}$ 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). 4×10^6 Cells were transferred into RAG-$1^{-/-}$ mice by intravenous injection into a lateral tail vein. Recipient animals were then infected with cercariae 24 hours later, as described above. To verify the efficacy of adoptive transfers at necropsy, splenocytes from reconstituted RAG-$1^{-/-}$ mice were surface labeled with APC-Cy7-conjugated antibodies to CD4, FITC-conjugated antibodies to CD8, 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).

Parasite recovery and measurement of parasitological parameters

Parasites were recovered from the portal system by perfusion (92), 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 (93). Liver tissue was digested in 0.7% trypsin (50 ml) in phosphate-buffered saline (PBS) for 2-3 hours at 37°C, and eggs were counted under a dissecting microscope.

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.

Results

To test whether IL-2 production by $CD4^+$ T cells is required to rescue schistosome development in $RAG^{-/-}$ mice, $RAG\text{-}1^{-/-}$ mice were reconstituted with $CD4^+$ T cells isolated from either $IL\text{-}2^{-/-}$ or wild-type $IL\text{-}2^{+/+}$ donors. Parasite growth was not restored in $RAG^{-/-}$ recipients of $IL\text{-}2^{-/-}$ T cells (Fig. 10A). Male parasites recovered following the adoptive transfer of $IL\text{-}2^{-/-}$ T cells were identical in size to those from non-reconstituted $RAG^{-/-}$ mice and significantly smaller than those from $RAG^{-/-}$ mice reconstituted with $IL\text{-}2^{+/+}$ $CD4^+$ T cells (Fig. 10A). Additionally, egg production was not restored in the presence of $IL\text{-}2^{-/-}$ $CD4^+$ cells (Fig. 10B). Worm pairs recovered from recipients of $IL\text{-}2^{-/-}$ $CD4^+$ T cells produced similar numbers of eggs to those from non-reconstituted recipients and significantly less than pairs from $RAG\text{-}1^{-/-}$ recipients of wild-type $CD4^+$ T cells, indicating that $IL\text{-}2^{-/-}$ $CD4^+$ T cells could not restore the fecundity of schistosome pairs in $RAG\text{-}1^{-/-}$ mice.

When the activation phenotype of wild type and $IL\text{-}2^{-/-}$ $CD4^+$ cells was assessed prior to transfer, an unusually high percentage of $CD44^{hi}CD62L^{low}$ cells were found amongst $CD4^+$ T cells from naïve $IL\text{-}2^{-/-}$ donors,compared to wild type donors (Fig. 11A). However, flow cytometric analysis of all recipients at the time of necropsy revealed that $IL\text{-}2^{-/-}$ $CD4^+$ T cells repopulated $RAG\text{-}1^{-/-}$ recipients to the same levels as wild type $CD4^+$ T cells (Fig. 11B).

Discussion

The data presented here demonstrate that IL-2 expression by $CD4^+$ T cells is crucial for the ability of these cells to facilitate schistosome development. However, the role of IL-2 in this process cannot be ascribed to any of the generally recognized functions of this cytokine. For instance, while $CD4^+CD25^+$ T_{regs} are capable of facilitating parasite growth, they are not specifically necessary, as adoptive transfer of CD25-depleted cells leads to normal parasite development (97), indicating that the effect of IL-2 on schistosome growth is not mediated through the T_{reg} population. Additionally, attenuation of parasite growth and development can not be explained though a defect in either T_H1 or T_H2 mediated immunity, since schistosome development proceeds normally in both STAT-$4^{-/-}$ and STAT-$6^{-/-}$ mice, which are deficient in T_H1 and T_H2 differentiation, respectively, and in a variety of other mutant animals that lack critical T_H1 and T_H2 effector cytokines (77, 78, 98). Therefore, the role of IL-2 in schistosome development may relate to more basic aspects of T cell function, such as homeostatic maintenance mechanisms. Although IL-2 is required for the homeostasis of $CD4^+CD25^+$ T_{reg} cells (110), a similar role has not been identified for non-regulatory populations. Alternatively, IL-2 production from T cells may have a currently unidentified effect on another host cell population and this secondary cell population may play a role in schistosome development. This possibility could be assessed by examining the ability of adoptively transferred IL-2R$\alpha^{-/-}$ $CD4^+$ cells to restore parasite development in RAG-$1^{-/-}$ mice. Another

possibility is since transcription of the IL-2 gene is an early and vital step in the response of T cells to antigen (114), there may yet be a role for early antigen-specific responses of T cells in parasite development. Finally, it is possible that the altered activation phenotype on transferred IL-$2^{-/-}$ $CD4^{+}$ cells (Fig. 11A) affects the ability of the cells to exert the required schistosome enhancing effect. Since the expression of co-stimulatory molecules on the surface of activated or memory $CD4^{+}$ T cells differs from that of naïve $CD4^{+}$ cells, the activated phenotype may alter cell-cell interactions that lead to augmented schistosome growth.

Figure 10: *Schistosoma mansoni* development is not restored by IL-2$^{-/-}$ CD4^{+} cells

One day before infection, RAG-1$^{-/-}$ recipients were reconstituted with CD4^{+} T cells from 4 X10^{6} wild type (IL-2$^{+/+}$) or IL-2$^{-/-}$ donors, and *S. mansoni* worm development and egg production were assessed at 6 weeks after infection. A) Length of male *S. mansoni* worms isolated from RAG-1$^{-/-}$ recipients reconstituted with PBS alone, IL-2$^{+/+}$ CD4^{+} T cells, or IL-2$^{-/-}$ CD4+ T cells; overall $P<0.0001$, Kruskal-Wallis test. B) Number of eggs deposited per pair of worms in the livers of RAG-1$^{-/-}$ recipients reconstituted with PBS alone, IL-2$^{+/+}$ CD4^{+} T cells, or IL-2$^{-/-}$ CD4^{+} T cells; overall $P=0.0474$, Kruskal-Wallis test. In panels A, and B, *P* values for each experimental group pair were calculated using Dunn's multiple comparison tests.

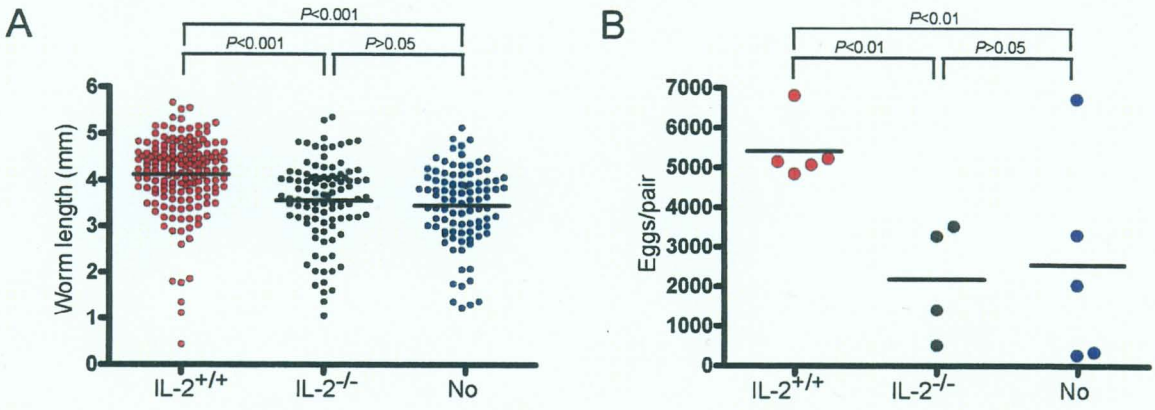
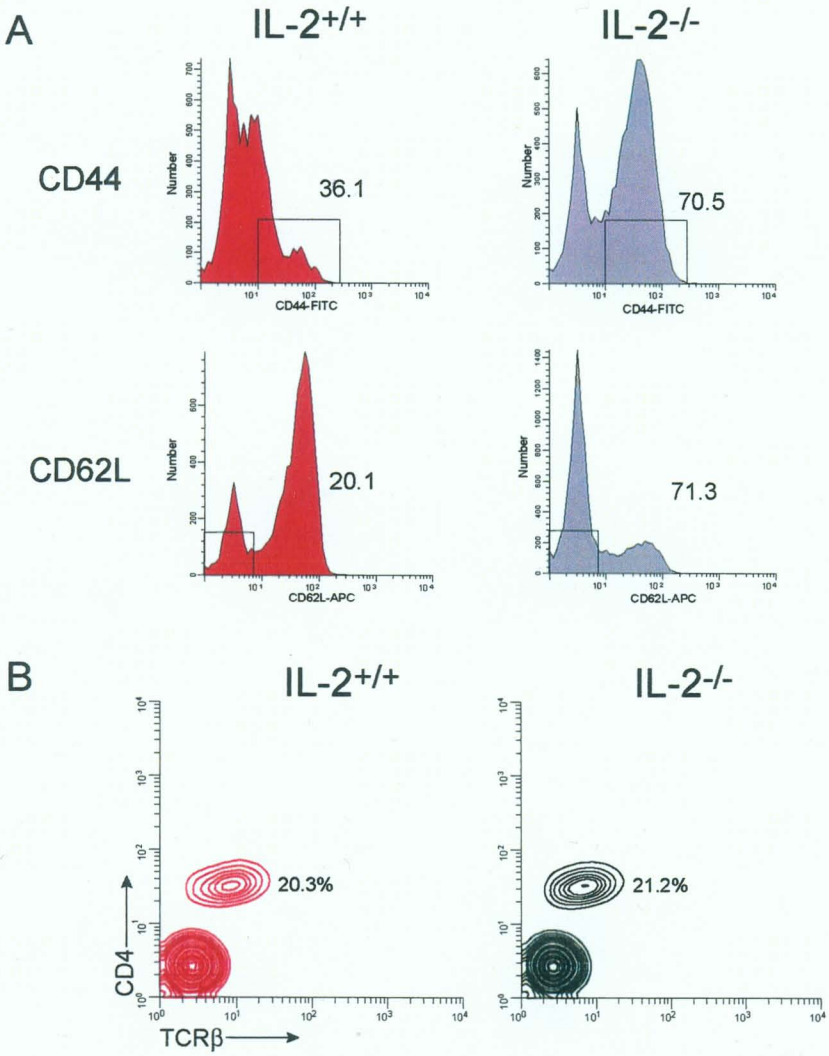

A
P<0.001
P<0.001
P>0.05
Worm length (mm)
0
1
2
3
4
5
6
IL-2+/+
IL-2-/-
No
B
P<0.01
P<0.01
P>0.05
Eggs/pair
0
1000
2000
3000
4000
5000
6000
7000
IL-2+/+
IL-2-/-
No

Figure 11: Activation phenotype and engraftment of transferred $CD4^+$ T cells.

$CD4^+$ T cells were isolated from IL-$2^{+/+}$ or IL-$2^{-/-}$ mice and transferred into RAG-$1^{-/-}$ recipients. (A) Prior to transfer, the activation phenotype was analyzed through measurement of CD44 and CD62L expression on gated $CD4^+TCR\beta^+$ lymphocytes. Percentage of lymphocyte cells with $CD44^{hi}$ or $CD62L^{low}$ is indicated on each histogram. (B) Six weeks post-infection, CD4 and TCRβ expression was measured on $NK1.1^-$ lymphocytes recovered from the spleens of from RAG-$1^{-/-}$ recipients of IL-$2^{+/+}$ and IL-$2^{-/-}$ cells. Percent $CD4^+TCR\beta^+$ cells is indicated on each histogram.


A
IL-2+/+
IL-2-/-
CD44
36.1
70.5
Number
CD44-FITC
CD62L
20.1
71.3
CD62L-APC
B
IL-2+/+
IL-2-/-
20.3%
21.2%
CD4
TCRβ

Chapter 4

Blood Fluke Exploitation of Innate-Adaptive Immune Interactions to Facilitate Parasite Development

(manuscript in preparation)

Note: all of the figures and tables shown reflect the work of Erika Lamb. Dr. Davies contributed to the design of the experiments and interpretation of the data as well as the preparation of the manuscript.

Abstract

$CD4^+$ T cells play an important role in the development of *Schistosoma* larvae into egg-producing adult worms. Our recent studies have focused on the mechanism by which $CD4^+$ T cells mediate this effect. While T cell homeostasis mediated by IL-7 and by IL-2 appear to be critical, neither of these cytokines affect parasite development directly. Indeed, no role has been identified for any of the major effector cytokines produced by $CD4^+$ T cells. We have therefore focused on whether activation of $CD4^+$ T cells via the T cell receptor (TCR) is involved in promoting schistosome development. Mutations that block T cell receptor signaling, through deletion of the PKC-θ or Bcl10 signal transduction molecules, have no effect on worm development, suggesting that activation of $CD4^+$ T cells through the antigen receptor is dispensable for normal schistosome development. Further, using TCR-transgenic $RAG^{-/-}$ mice which possess monospecific populations of $CD4^+$ T cells that only recognize irrelevant antigens, we demonstrate that recognition of schistosome antigens by $CD4^+$ T cells is also not required for parasite development. Together, these results suggest that conventional antigen-specific activation of $CD4^+$ T cells is not required for schistosome maturation and development. We propose a model in which the presence of $CD4^+$ T cells, with or witout concomitant TCR activation, modulates the intra-host environment, making it more conducive to blood fluke development and contributing to the pathogenesis of schistosomiasis. Consistent with this hypothesis, microarray analyses reveal that the presence of naïve $CD4^+$ T cells

alters gene expression at the site of parasite development in the liver, with approximately 20% of the differentially expressed genes involved in acute phase responses. Furthermore, we show that acute phase responses are induced by *S. mansoni* infection only in immunocompetent mice. Finally, we show that induction of concomitant acute phase responses is associated with a significant increase in *S. mansoni* growth and development in RAG-$1^{-/-}$ mice in the absence of $CD4^{+}$ T cells.

Introduction

Extensive co-evolution of parasitic organisms and their hosts has given rise to complex host-parasite relationships in which exploitation of host responses to infection by parasites is a recurring theme. Nowhere is the complexity of host-parasite relationships better exemplified than by the parasitic helminths, which infect a third of the world's human population by establishing chronic infections that persist for years, often in the face of vigorous immune responses. The blood flukes – pathogenic trematodes of the genus *Schistosoma* – account for a significant proportion of these helminth infections, causing considerable morbidity and mortality. These parasites initiate infection by direct skin penetration and then migrate in the blood stream to hepatic pre-sinusoidal venules, where rapid growth and development ensues. We and others previously showed that the human pathogen *S. mansoni* co-opts $CD4^+$ T cell-dependent mechanisms to facilitate parasite development and egg excretion (66, 67, 77). The latter requires formation of T_H2-dependent granulomas in the bowel wall to allow passage of eggs from the portal vasculature into the intestinal lumen (66, 67). However, the mechanisms by which $CD4^+$ T cells facilitate development of schistosome worms have not been elucidated. Previous studies suggested that, while homeostatic maintenance of peripheral $CD4^+$ T cell populations through the action of γ_c cytokines are required to provide a permissive environment for schistosome development (97), effector T_H1 and T_H2 responses and the elaboration of effector cytokines are not required for normal

parasite development (77, 78, 98). In this study, we therefore sought to test whether initial activation of $CD4^+$ T cells through the T cell antigen receptor (TCR) by schistosome antigens was required for schistosome development to proceed. We evaluated the role of $CD4^+$ T cell activation by examining schistosome development in an *in vivo* context where $CD4^+$ T cells are present but are unable to respond to schistosome infection through analyses of *S. mansoni* infections in (i) mice with defects in the TCR signaling pathway and (ii) TCR-transgenic $RAG^{-/-}$ mice.

Naïve T cells become activated following specific interactions between the TCR and antigenic peptide, presented in the context of major histocompatability complex (MHC) molecules on antigen presenting cells (APC) (115). This interaction and other co-stimulatory signals leads to the initiation of signaling cascades, resulting in the growth and proliferation of the T cell, mediated primarily through the production of IL-2, and upregulation of high affinity IL-2R. IL-2 acts in an autocrine manner, leading to the growth, proliferation and differentiation of antigenically stimulated T cells. Transcription of the IL-2 gene is mediated through the binding of the NF-κB, NF-AT and AP-1 transcription factors to the IL-2 promoter region (115). Binding of these transcription factors to the IL-2 promoter is cooperative and all three proteins must be present for IL-2 transcription and subsequent T cell activation to occur. Thus, a blockade in any of the signaling cascades leading to NF-κB, NF-AT or AP-1 will prevent, or severely impair, T cell activation.

The function of various proteins in transmitting signals from the TCR to NF-κB has been investigated by deleting the corresponding genes in laboratory mice. These studies have revealed essential roles for both PKC-θ and Bcl10 in the transmission of signals from the TCR to NF-κB (116, 117). T lymphocytes from PKC-θ-deficient mice have reduced proliferation and IL-2 production in response to both anti-CD3/anti-CD28 stimulation and in mixed lymphocyte reactions (116). PKC-θ-deficient T cells also have reduced cell surface expression of IL-2Rα (CD25) and CD69 (an early T cell activation marker), further demonstrating the reduced activation status of these T cells (116). Bcl10 is an adaptor molecule which plays important roles in the signal transduction pathways of both B and T cell receptors (117). Bcl10-deficient T cells have impaired proliferation and IL-2 secretion in response to signaling through the TCR following treatment with anti-CD3/anti-CD28 (117). Consequently, Bcl10-deficient mice exhibit impaired humoral and cellular immune responses in response to both lymphocytic choriomeningitis virus (LCMV) and vesicular stomatis virus (VSV) infection (117). Thus, Bcl10 is necessary for the *in vitro* and *in vivo* activation of T cells. Importantly, Bcl10 and PKCθ are specific for the TCR mediated pathway of NF-κB activation, as TNFα, IL-1, and lipopolysaccharide (LPS) treatment lead to normal NF-κB activation in both PKC-θ– and Bcl10- deficient mice (118). The impaired T cell activation phenotypes of $Bcl10^{-/-}$ and PKC-$\theta^{-/-}$ mice thus provide an opportunity to evaluate schistosome development in hosts where $CD4^{+}$ T cell responses are significantly compromised.

As a complementary approach, *S. mansoni* development was analyzed in TCR-transgenic RAG$^{-/-}$ mice which possess monospecific populations of CD4^{+} T cells that only recognize irrelevant non-schistosome antigens. In these mice, development of all endogenous B and T cells is blocked by mutation of the RAG genes, but development of a homogeneous, monospecific population of CD4^{+} T cells is permitted by transgenic expression of previously rearranged MHC class II-restricted TCRs. Three different transgenic TCRs were used for this purpose. The OT-II TCR is specific for the chicken ovalbumin peptide $OVA_{323-339}$ when presented in the context of the murine I-A^{b} MHC class II molecule (119). The DO11.10 (120) TCR recognizes the same $OVA_{323-339}$ peptide in the context of the I-A^{d} MHC class II molecule. Finally, the Cyt-5CC7 TCR is specific for the pigeon cytochrome c peptide, (PCC_{81-104}) presented by the MHC class II molecule I-E$^{a/k}$ (121).

Through evaluation of *S. mansoni* development in the presence of CD4^{+} T cells but in the absence of antigen-specific T cell activation, we demonstrate that the ability of CD4^{+} T cells to promote schistosome development is independent of T cell responses to antigen. We propose a model in which the presence of CD4^{+} T cells, with or without concomitant TCR activation, modulates the intra-host environment, making it more conducive to blood fluke development and contributing to the pathogenesis of schistosomiasis. Consistent with this hypothesis, we demonstrate that the presence of naïve CD4^{+} T cells alters gene expression at the site of parasite development in the liver by priming the innate immune system to mount acute phase responses. Finally, we demonstrate that

the requirement for $CD4^+$ T cells in blood fluke development can be bypassed by stimulating the innate immune system directly. These data suggest that blood flukes exploit a fundamental link between $CD4^+$ T cells and the innate immune system to coordinate their development within the mammalian host.

Materials and Methods

Experimental Mice

RAG-1$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in-house to generate sufficient numbers for experiments. Bcl10$^{-/-}$ mice (117) were kindly provided by Dr. Tak Mak. PKCθ$^{-/-}$ mice (116) were kindly provided by Dr. Abraham Kupfer. OT-II mice (119) were the kind gift of Dr. Philippa Marrack, with permission from Dr. Francis Carbone. Bcl10$^{-/-}$, PKC-θ$^{-/-}$, and OT-II mice, originally with a mixed 129/C57BL/6 background, were backcrossed for several generations to the C57BL/6 background. OT-II mice were then bred with RAG-1$^{-/-}$ in house, to generate OTII/RAG-1$^{-/-}$ mice. C57BL/6 (National Cancer Institute, Frederick, MD) and 129 x C57BL/6 F1 hybrid wild type mice (Taconic) were used as positive controls, although no differences in parasitological parameters were found in parasites recovered from either wild type strain (data not shown). BALB/cTac-TgN(DO11.10)-Rag2^{tm1} (DO11.10/RAG-2$^{-/-}$) mice (120) and B10.A/AiTac-[Tg]TCRCyt5CC7-I-[KO]-Rag2^{tm1} (Cyt/RAG-2$^{-/-}$) mice (121) were kindly provided by Dr. D. Jankovic. RAG-2$^{-/-}$ and wild type mice on the BALB/c and B10.A2 backgrounds (Taconic) were used as controls, respectively.

Mice were infected percutaneously via the tail skin with 150 *S. mansoni* cercariae (Puerto Rican strain) shed from infected *Biomphalaria glabrata* snails (92). All studies were performed in accordance with protocols approved by the relevant Institutional Animal Care and Use Committees.

Parasite recovery and measurement of parasitological parameters

Parasites were recovered from the portal system by perfusion (92), 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 (93). Liver tissue was digested in 0.7% trypsin (50 ml) in phosphate-buffered saline (PBS) for 2-3 hours at 37°C, and eggs were counted under a dissecting microscope.

Cell isolation and Adoptive Transfer

Lymph nodes and spleens from wild type C57BL/6, Bcl10$^{-/-}$ or PKC-$\theta^{-/-}$ 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). 3×10^6 cells were transferred into RAG-1$^{-/-}$ mice by intravenous injection into a lateral tail vein. Recipient animals were then infected with cercariae 24 hours later, as described above. To verify the efficacy of adoptive transfers at necropsy, splenocytes from reconstituted RAG-1$^{-/-}$ mice were surface labeled with APC-Cy7-conjugated

antibodies to CD4, FITC-conjugated antibodies to CD8, 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).

Analysis of cytokine production

$CD11c^+$ cells were isolated from wild type spleens through incubation of cell suspensions with anti-CD11c coated microbeads (Miltenyi Biosciences and separated using Midi-Macs magnetic columns (Miltenyi Biosciences). $CD4^+$ cells were isolated from spleens and livers of wild type, $Bcl10^{-/-}$ and OTII/RAG-$1^{-/-}$ mice as described above. $CD4^+$ T cells and $CD11c^+$ cells, pulsed with 50 μg/ml schistosome worm antigen preparation (SWAP)(122, 123), 5 μg /ml OVA or 1 μg / μg anti-CD3 (BD Bioscience), were cultured for 72 hours in 96 well plates at a ratio of 5×10^5 $CD4^+$ cells to 5×10^4 $CD11c^+$ cells. Culture supernatants were assessed for IFNγ and IL-10 by ELISA using BD Opt EIA Mouse IL-10 IFNγ and IL-10 antibody pairs and ELISA reagents (BD Bioscience) and analyzed using a Spectramax M2 Plate reader (Molecular Devices).

Analysis of cell surface molecule expression

T cell activation on cells recovered from spleen and liver was performed by analysis of $CD44^{hi}CD62L^{low}$, CD69, and CD25 expression gated on $CD4^+TCR\beta^+NK1.1^-$ cells. Cells isolated from spleens and livers were surface labeled with FITC-conjugated antibodies to CD44 or CD69, PE-conjugated

antibodies to TCRβ or NK1.1, PerCp-Cy5.5-conjugated antibodies to NK1.1 or CD4, APC-conjugated antibodies to CD62L, and APC-Cy7-conjugated antibodies to CD4 or CD25, (BD Biosciences) and analyzed using a LSR II Optical Bench flow cytometer with FACSDiva and Winlist software, version 5.0 (Verity Software House).

Microarray

Livers from naïve male wild type, $RAG^{-/-}$, and OT-II/$RAG^{-/-}$ (N=9) mice were pooled into groups of three and prepared for cDNA microarray analysis. Briefly, RNA was isolated via RNAzol (Tel-Test, Friendswood, TX) and the RNeasy protocol (Qiagen) and analyzed for purity and concentration on a NanoDrop® ND-1000 Spectrophotometer (Wilmington, DE). cDNA was prepared from two 30μg aliquots of each pooled sample and labeled with either Cy3 or Cy5 fluorescent probes. One 30μg aliquot from each pool was used to create a background control pool, while the second aliquot was used as the comparative sample. For further isolation and labeling protocol details please refer to http://www.niaid.nih.gov/dir/services/rtb/microarray/protocols.asp. Samples were hybridized as described by Schaupp(124) to Mmbe custom arrays manufactured by the NIAID Microarray Facility. Further information about these arrays can be found at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL1057. Hybridizations were performed in triplicate. Images were scanned by GenePix4000B Scanner (Axon Instruments/Molecular Devices, Sunnyvale, CA) and analyzed using the mAdb program (http://madb.niaid.nih.gov/). Signals were

calculated as mean intensity – median background. Data were analyzed using Significance Analysis of Microarrays (SAM), version 2.11 (Stanford University) and Student's *t* tests (EXCEL) with *P* values <0.05 considered significant. We applied supervised average-linkage hierarchical clustering on differentially regulated genes, as implemented in the program Cluster version 2.11 (M. Eisen; http://www.microarrays.org/software), separately to both the genes and arrays. The results were analyzed, and figures generated, using TreeView version 1.60 (http://www.microarrays.org/software). Initial gene annotation was performed through GoMiner (http://discover.nci.nih.gov/gominer/) with more rigorous annotation performed through interrogation of PubMed and online inheritance of man (OMIM) databases (http://www.ncbi.nlm.nih.gov/entrez/).

Induction and analysis of acute phase responses

A cohort of *S. mansoni* infected RAG-$1^{-/-}$ mice with opportunistic bacterial co-infections were identified at necropsy based on gross and histopathological examination of abcesses primarily in the respiratory tract and associated tissues. Microbiological examination of a sample of these animals identified *Pseudomonas aeruginosa* as the etiologic agent, a common opportunistic pathogen of immunodeficient and immunosuppressed mice. In other wild type and RAG-$1^{-/-}$ mice, acute phase responses were induced by intraperitoneal injection of low doses of lipopolysaccharide (LPS) (E.coli 055:B5) (Difco Laboratories,Michigan) at 2μg LPS/mouse, either as a single dose or repeatedly, depending on experiment. Acute phase responses were monitored by evaluating plasma concentrations of serum amyloid A by ELISA (Biosource).

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.

Results

Blockade of TCR signals to NF-κB does not impair parasite development

At 42 days post infection, *S. mansoni* worms recovered from both Bcl10$^{-/-}$ and PKC-θ$^{-/-}$ mice were indistinguishable in size from those recovered from wild type mice (Fig. 12A) and did not exhibit the stunted phenotype of schistosomes recovered from RAG-1$^{-/-}$ mice. Parasite pairing, a marker of parasite development, was similar in Bcl10$^{-/-}$, PKC-θ$^{-/-}$ and wild type mice (Fig. 12B). Further, parasite pairs from Bcl10$^{-/-}$, PKC-θ$^{-/-}$ and wild type mice deposited comparable numbers of eggs (Fig. 12C), unlike pairs from RAG-1$^{-/-}$ mice which show greatly reduced egg production. Compared to wild type animals, CD4^{+} T cell responses to *S. mansoni* were impaired by deletions in the TCR-NF-κB pathway. During the pre-patent infection (28 days post-infection) fewer Bcl10$^{-/-}$ CD4^{+} T cells acquired an activated phenotype in response to *S. mansoni* infection, as measured by loss of CD62L and acquisition of CD25, CD44 or CD69 expression in both the spleen (Fig. 13A) and the liver (Fig. 13B). Additionally, CD4^{+} T cells isolated from spleens and livers of infected Bcl10$^{-/-}$ mice produced less IFN-γ and IL-10 in response to schistosome worm antigens compared to CD4^{+} T cells from infected wild type mice (Fig. 13C). Very similar results were obtained with CD4^{+} T cells from infected PKC-θ$^{-/-}$ (data not shown). Interestingly, a non-antigen specific IFN-γ response is elicited from CD4^{+} cells recovered from Bcl10$^{-/-}$ mice, perhaps due to the absence of regulatory T cells in mice deficient in Bcl10$^{-/-}$ (125). Very similar T cell activation phenotypes and cytokine production

patterns were measured in response to a patent infection at 42 days post-infection (data not shown). Importantly, normal *S. mansoni* development in both Bcl10$^{-/-}$ and PKC-$\theta^{-/-}$ mice confirms that neither T_{regs} (97) nor IL-10 (Davies, SJ, unpublished observation) are necessary to facilitate parasite development. Together these data suggest that activation of CD4$^+$ T cells through the TCR and subsequent CD4$^+$ T cell effector responses are not required for normal schistosome development.

Bcl10$^{-/-}$ CD4$^+$ T cells facilitate parasite development

To directly test whether TCR-to-NF-κB signaling is required for CD4$^+$ T cells to facilitate schistosome development, the ability of Bcl10$^{-/-}$ CD4$^+$ T cells to rescue parasite development in RAG-1$^{-/-}$ mice was examined using an adoptive transfer approach. Adoptive transfer of Bcl10$^{-/-}$ CD4$^+$ T cells prior to infection partially restored worm growth and egg production, resulting in a phenotype that was intermediate between RAG-1$^{-/-}$ recipients that received wild type CD4$^+$ T cells and negative controls injected with PBS, in terms of parasite length (Fig. 14A), percent pairing (Fig. 14B) and egg production (Fig. 14C). Presumably because TCR signals are required for T cell homeostasis, Bcl10$^{-/-}$ CD4$^+$ T cells failed to undergo vigorous homeostatic proliferation upon transfer to lymphopenic RAG-1$^{-/-}$ mice and recipients of Bcl10$^{-/-}$ T cells therefore exhibited variable levels of engraftment that were consistently lower than those of wild type cells (Fig. 14D). We therefore tested for any correlation between levels of CD4$^+$ T cell engraftment and worm length in individual recipients. Strikingly, this analysis

revealed a significant positive correlation between the number of Bcl10$^{-/-}$ CD4^{+} T cells in each recipient and the mean length of the worms recovered from that recipient (Fig. 14E). Together, these data suggest that CD4^{+} T cells do not require intact antigen receptor signaling to facilitate parasite development and that a requirement for sufficient *numbers* of CD4^{+} T cells, rather than their ability to transduce TCR signals, is the critical factor for allowing parasite development to proceed. Further, these data predict a threshold of approximately 2 x 10^{6} splenic CD4^{+} T cells as the minimum number required to restore parasite growth to the levels observed in wild type mice.

Antigen non-responsive T cells facilitate S. mansoni development

Despite the lack of schistosome-specific cytokine responses by CD4^{+} T cells in Bcl10$^{-/-}$ mice, a small increase in the number of activated CD44^{+} CD62L^{-} cells was detected in the livers of Bcl10$^{-/-}$ animals in response to infection (Fig. 13B). Also, non-infected Bcl10$^{-/-}$ mice possessed significant numbers of hepatic CD4^{+} T cells with an effector/memory phenotype (Fig. 13B), suggesting that transduction of TCR signals is not completely blocked by loss of Bcl10. We therefore examined *S. mansoni* development in RAG$^{-/-}$ mice that transgenically express MHC class II-restricted TCRs specific for irrelevant antigens, to provide a context where only naïve CD4^{+} T cells are present and where these cells are unable to respond to schistosome antigens by virtue of their highly restricted TCR repertoire. In all three TCR-transgenic RAG$^{-/-}$ lines examined, schistosome development was enhanced relative to RAG$^{-/-}$ controls that lacked the TCR, as

determined by assessment of parasite size (Fig. 15A) and egg production (Fig. 15B). In OT-II/RAG-$1^{-/-}$ and Cyt/RAG-$2^{-/-}$ mice, there was partial restoration of *S. mansoni* development, while DO11.10/RAG-$2^{-/-}$ mice supported parasite development that was indistinguishable from wild type control mice. Analysis of $CD4^+$ T cell activation revealed that TCR-transgenic $RAG^{-/-}$ mice possessed $CD4^+$ T cells that were almost exclusively naïve prior to infection and that *S. mansoni* infection resulted in only very small or no increases in the numbers of activated cells (Fig. 16A). $CD4^+$ T cells from *S. mansoni*-infected OT-II/RAG-$1^{-/-}$ mice were completely unresponsive to schistosome antigens, though these cells retained the ability to respond to the appropriate antigen, $OVA_{323-339}$ peptide (Fig. 16B), *in vitro*. Similar results were obtained for DO11.10/RAG-$1^{-/-}$ and Cyt/RAG-$2^{-/-}$ mice (data not shown). Together, these data demonstrate that $CD4^+$ T cells require neither antigen stimulation through the TCR nor activation in order to provide the positive signals necessary to facilitate parasite development.

$CD4^+$ T cells modulate gene expression in the liver

Because naïve and non-responsive $CD4^+$ T cells appear to influence schistosome development without responding to schistosome infection, and because direct physical interaction between these cells and developing schistosomes in portal venules seems unlikely, we hypothesized that naïve $CD4^+$ T cells in TCR-transgenic $RAG^{-/-}$ mice mediate their effects on schistosome development indirectly, by interacting with other liver components and influencing gene expression to make this environment more permissive for parasite

development. To assess whether the presence of naïve $CD4^+$ T cells influences hepatic gene expression, an oligonucleotide array representing the entire murine transcriptome was used to compare transcript levels in RAG-$1^{-/-}$ and OT-II/RAG-$1^{-/-}$ liver tissue from non-infected mice. A provisional list of differentially expressed genes from this analysis was further filtered by comparison with genes that are differentially expressed between wild type and RAG-$1^{-/-}$ livers, to eliminate artifactual differences between RAG-$1^{-/-}$ and OT-II/RAG-$1^{-/-}$ livers. Through microarray analysis, we found that 175 genes were differentially expressed in livers of OT-II/RAG-$1^{-/-}$ mice compared to RAG-$1^{-/-}$ controls (Fig. 17A). Interestingly, only one of these differentially expressed genes (cell differentiation antigen CD2) could be identified as T cell-specific, and transcripts of genes known to be T cell-specific were not over-represented in the livers of OT-II/RAG-$1^{-/-}$ mice. These data suggest that the naïve TCR-transgenic $CD4^+$ T cells contribute relatively little to the overall composition of total liver RNA from OT-II/RAG-$1^{-/-}$ mice, presumably because these cells are relatively few in number and are transcriptionally quiescent in the absence of cognate antigen. In contrast, approximately 20% of the differentially expressed genes encode proteins that are involved in the acute phase response (Fig. 17b), including complement C4B (stk19), Jun-B, colony stimulating factor-1 (csf1), and prostaglandin E2 synthase (ptges3). We identified a number of differentially regulated genes involved in the metabolic and neuroendocrine changes associated with the acute phase response, including insulin growth factor binding protein 4 (Ifgbp4), low density lipoprotein receptor-related protein 2, transcript

variant 5 (Lrp2), phosphatidylcholine transfer protein (Pctp) and cryptochrome 1 (Cry1). Additionally, a number of genes were identified which play important roles in hepatocyte, macrophage or dendritic cell function (Fig. 17c), such as fibroblast growth factor 1 (Fgf1), tyrosine kinase 2 (Tyk2), and nitric oxide synthase interacting protein (Nosip). These genes are of particular interest as they are expressed in innate immune system cells that may interface with $CD4^+$ T cells. Other categories of differentially expressed genes were also potentially linked to the ability of hepatocytes to elaborate acute phase proteins, including genes involved in vesicular trafficking and cytoskeletal rearrangement (ADP-ribosylation factor interacting protein 2 (arfip2), kinesin family member 13B, transcript variant 1(Kif13b), and microtubule-associated protein 4 (Mtap4)) and protein folding and processing (heat shock protein 40 (DNAjb9) and transporter 2 (Tap2)) (Fig. 17D). Overall, these data suggest that naïve $CD4^+$ T cells modulate gene expression in the liver by raising baseline levels of acute phase gene transcription.

A defective acute phase response to S. mansoni in $RAG^{-/-}$ mice can be circumvented through toll like receptor ligands

Mediated primarily by the action of pro-inflammatory cytokines on hepatocytes, the acute phase response consists of alterations in the plasma concentrations of acute phase proteins and the induction of catabolic processes that mobilize energy stores. This results in decreased gluconeogenesis and alterations in lipoprotein and cholesterol components, together with various

neuroendocrine changes (126, 127). On the basis of our transcriptional analysis, we hypothesized that the broad changes in hepatic gene expression that underlie the acute phase response may participate in creating an environment that is permissive to schistosome development and that priming of the acute phase response by $CD4^+$ T cells was the basis of the ability of these cells to facilitate schistosome development. To test this hypothesis, we first examined whether the presence of schistosome worms in the portal system induced an acute phase response. Consistent with previous work that examined acute phase responses to schistosome infection (128), wild type mice exhibited elevated plasma levels of serum amyloid A (SAA), a signature acute phase protein, at 4 weeks post infection, indicating that schistosome worms do induce an acute phase response. However, elevated plasma levels of SAA were not detected in RAG-$1^{-/-}$ mice infected for an equivalent period of time, indicating that this response requires priming by the adaptive immune system (Fig. 18A). Despite their non-responsiveness to schistosome infection, RAG-$1^{-/-}$ mice retain the ability to mount an acute phase response when stimulated with a potent acute phase stimulus, the toll like receptor- (TLR-) 4 ligand, lipopolysaccharide (LPS) (Fig. 18B). In light of these results, we tested whether the requirement for $CD4^+$ T cells in schistosome development could be bypassed by inducing acute phase responses with other pro-inflammatory stimuli. First, we examined schistosome development in a cohort of RAG-$1^{-/-}$ mice that exhibit acute phase responses due to concurrent bacterial infection. While these mice do not develop any B or T cells and remain immunodeficient, opportunistic bacterial infections cause acute

phase responses in these animals, as measured by plasma levels of SAA (data not shown). Of 9 infected animals with gross or histologic evidence of co-infection, significant enhancement of schistosome growth (Fig. 19A) and parasite fecundity (Fig. 19B) were observed when compared to worms from infection-free RAG-1$^{-/-}$ animals. Secondly, treatment of RAG-1$^{-/-}$ mice with low doses of LPS led to a significant increase in parasite length (Fig. 19A) and an increase in egg production, the latter narrowly failing to reach statistical significance (Fig. 19B). These data suggest that acute phase responses, whether induced by adaptive immune system priming or other inflammatory processes, are required for normal schistosome development. Alternatively, the induction of the acute phase response may serve as a marker of an unidentified innate inflammatory response effector mechanism that is required for normal schistosome development.

Discussion

$CD4^+$ T cells play an important role in the development of *Schistosoma* larvae into egg-producing adult worms (77, 97, 129). Our recent studies have focused on the mechanism by which $CD4^+$ T cells mediate this effect. While T cell homeostasis mediated by IL-7 and IL-2 production appear to be critical, neither of these cytokines affect parasite development directly (97) nor has any role been identified for $CD4^+$ T cell effector cytokines (77, 78, 98). We therefore focused on evaluating whether activation of $CD4^+$ T cells via the TCR is involved in promoting schistosome development. Here we show that *S. mansoni* parasites develop normally in mice with defects in the TCR signal transduction pathway, demonstrating that TCR- mediated T cell activation is not necessary for normal parasite development (Fig. 13). Additionally, normal *S. mansoni* development in TCR-transgenic $RAG^{-/-}$ mice (Fig. 15), in the complete absence of a schistosome-mediated T cell response (Fig. 16) demonstrates that TCR specificity to schistosome antigens is also not required for $CD4^+$ T cells to facilitate schistosome development. Taken together, these results indicate that TCR-antigen interactions and subsequent TCR-mediated T cell activation are unnecessary for *S. mansoni* development. Furthermore, we show that parasite growth has a positive correlation with the number of $CD4^+$ T cells (Fig. 14), suggesting a requirement for a sufficient number of $CD4^+$ T cells to facilitate schistosome development.

The lack of requirement for TCR-mediated T cell activation in schistosome development, together with the observation that normal blood fluke development appears to require $CD4^+$ T cell homeostasis, led us to hypothesize that $CD4^+$ T cells play an indirect role in facilitating schistosome development, through modulation of the host environment during homeostatic interactions with other cells and tissues. Maintenance of naïve peripheral T cells is an active process that involves interactions between self peptide-expressing APCs and T cells that alter T cell gene expression and longevity (130, 131). Thus, we hypothesized that T cell-APC interactions might induce changes in APCs and other cells. The results from our microarray analysis show that the presence of a naïve population of $CD4^+$ T cells is sufficient to alter global gene expression in the liver (Fig. 17A). Interestingly, many of the differentially regulated genes we identified as upregulated by the presence of naïve $CD4^+$ T cells are involved in the acute phase response (Fig. 17B). Furthermore, several of the genes that are differentially expressed in the presence of OT-II TCR-expressing $CD4^+$ T cells, such as diaphanous homolog 1(Diap1), transporter 2 (Tap2), Cry1, related RAS viral oncogene (rras2) and betaine-homocysteine methyltransferase (bhmt), are also differentially regulated in mice following induction of acute phase responses by LPS injection (127), while many others share functional homology with genes induced by LPS treatment (127).

The microarray data predict that acute phase responses may require priming by $CD4^+$ T cells and are impaired in $RAG^{-/-}$ mice. To test this prediction, we evaluated the development of acute phase responses in both wild type and

RAG$^{-/-}$ mice in response to *S. mansoni.* Interestingly, acute phase responses in response to pre-patent schistosome infection are induced only in immunocompetent mice, suggesting that the adaptive immune system is required to prime acute phase responses to schistosome worms. These results are consistent with previous studies which reported altered acute phase responses during patent *S. mansoni* infections in thymectomized mice (128). However, acute phase responses can be induced in RAG-1$^{-/-}$ mice treated with potent acute phase stimuli such as LPS and thus CD4^{+} T cells are required only for priming of acute phase responses to helminthic pathogens such as schistosomes, which are generally not believed to express TLR ligands.

To test whether CD4^{+} T cell- dependent acute phase responses induced by schistosome infection play a role in facilitating schistosome development, we circumvented the requirement for CD4^{+} T cells by inducing acute phase responses in schistosome-infected RAG-1$^{-/-}$ mice with classical T cell-independent inducers such as bacterial infection and LPS. Importantly, induction of concomitant acute phase responses, through co-infection with bacteria or through direct stimulation with LPS, was associated with a significant increase in *S. mansoni* growth and development in RAG-1$^{-/-}$ mice in the absence of CD4^{+} T cells (Fig 19). Whether acute phase proteins directly affect parasite development or whether the acute phase response serves as an indicator of other innate immune responses which affect parasite development remains to be elucidated. Nonetheless, collectively our data indicate that naïve CD4^{+} T cells facilitate parasite development by priming the innate immune system without responding

to the infection themselves and that the requirement for $CD4^+$ T cells for schistosome development can be bypassed by stimulating the innate immune system directly through TLR ligands.

Our data may explain several seemingly incongruent reports about the host signals necessary to facilitate schistosome development. First, tumor necrosis factor (TNF) has been shown to stimulate parasite egg laying in spontaneously immunodeficient *Prkdc*$^{scid/scid}$ mice(72). TNF is produced by macrophages and monocytes in response to inflammatory stimuli and plays a role in the induction of acute phase responses. Thus, treatment of *Prkdc*$^{scid/scid}$ mice with exogenous TNF may bypass the requirement for T cells to prime the innate response necessary for schistosome development. However, TNF does not restore parasite growth and development in $RAG^{-/-}$ mice, perhaps because, unlike *Prkdc*$^{scid/scid}$ mice, these animals never develop any peripheral T cells and consequently TNF alone is not sufficient to induce the innate responses required to trigger full schistosome development.

Second, normal parasite development is observed in β_2-microglobulin (β_2-m)$^{-/-}$/$MHCII^{-/-}$ mice, which lack most $CD4^+$ T cells but retain a population of non-MHC II-restricted $CD4^+$ T cells in the liver (77). As our results show that neither antigen-mediated T cell activation nor antigen-specificity is necessary for normal schistosome development, this largely liver-specific population of T cells may still prime the innate immune components that facilitate parasite development even in the absence of MHC class II expression. This is especially interesting in context of a study which reports that functional immunological synapses form between

dendritic cells and T cells in the absence of antigen and MHC class II, causing alterations in gene expression and increased *in vitro* survival, probably through an LFA1-ICAM interaction (132, 133). Such interactions could thus alter gene expression in dendritic cells or APCs that ultimately contribute to facilitating schistosome development.

Third, IL-7 and thyroxine have been implicated in schistosome development (100, 134, 135) with synergistic effects that may be mediated through alterations in host glucose metabolism (136). Our results show that the number of peripheral T cells is positively correlated with schistosome development (Fig. 14E) and it has been previously shown that thyroxine increases the number of peripheral $CD4^+$ cells (137), suggesting that the synergistic effect of IL-7 and thyroxine may be mediated through an increase in the number of peripheral $CD4^+$ T cells. Furthermore, thyroxine treatment increases the gene expression and synthesis of the acute phase reactants ceruloplasmin (138) and mannan-binding lectin (139). Thus, thyroxine may mediate schistosome development through induction of acute phase components. As host glucose metabolism is modified extensively during the acute phase response, our results may also explain the positive effects of IL-7 and thyroxine on glucose metabolism.

Finally, dehydroepiandrosterone (DHEA) and the bloodstream form DHEA-sulfate (DHEA-S) play protective roles in murine and baboon models of schistosome infection (50, 140, 141). Intriguingly, DHEA levels are low during childhood, when schistosome primary and reinfection rates are highest, but rise

sharply at the onset of puberty, at which time schistosome reinfection rates sharply decline (50). DHEA suppresses IL-6 production (142, 143) and as IL-6 is a key mediator of the acute phase response, it is possible that the increase in DHEA at the onset of puberty dampens the acute phase response and the production of schistosome trophic factors.

In conclusion, we show that schistosome development is dependent on acute phase responses or other innate responses which are primed by $CD4^+$ T cells. Importantly, $CD4^+$ T cells can play an essential role in this process without responding to schistosome antigens. This suggests that a previously unrecognized interaction between the innate and adaptive immune systems is exploited by blood flukes to facilitate their development in the mammalian host.

Figure 12: *S. mansoni* development is normal in Bcl10- and PKCθ- deficient mice.

Wild type, Bcl10$^{-/-}$ and PKC-θ$^{-/-}$ mice were infected with *S. mansoni* cercariae and worms were collected 42 days post-infection. (A) Male worm lengths were measured from digital micrographs. (B) Percent pairing was quantified for each mouse as: (the number of paired females/total number of females) X 100. (C) Egg production per schistosome pair was determined by dividing the total number of eggs calculated for each mouse liver by the number of parasites recovered. Horizontal bars represent the mean value for each experimental group. A representative experiment is shown, containing 4-5 mice per group.

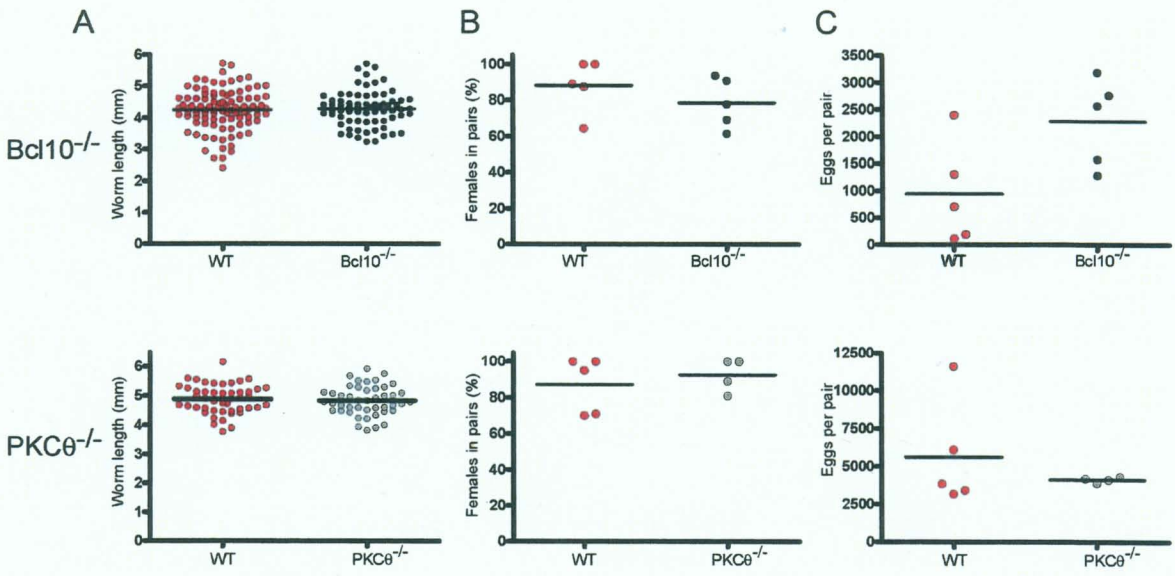

A
B
C
Bcl10-/-
PKCθ-/-
Worm length (mm)
Females in pairs (%)
Eggs per pair
WT
Bcl10-/-
PKCθ-/-

Figure 13: $CD4^+$ T cell activation and T cell responses are impaired in Bcl10-deficient mice.

T cell activation in response to pre-patent *S. mansoni* infection was measured 28 days post-infection. Surface expression of CD44, CD62L, CD69 and CD25 on $CD4^+TCR\beta^+NK1.1^-$ (A) spleen and (B) liver cells from uninfected wild type, infected wild type, uninfected $Bcl10^{-/-}$ and infected $Bcl10^{-/-}$ mice is shown. (C) $CD4^+$ cells were isolated from the spleen and liver of infected or control wild type and $Bcl10^{-/-}$ mice, and co-cultured with dendritic cells pulsed with schistosome worm antigen (SWAP). After 72 hours, culture supernatants were collected and IFNγ and IL-10 were measured via ELISA.

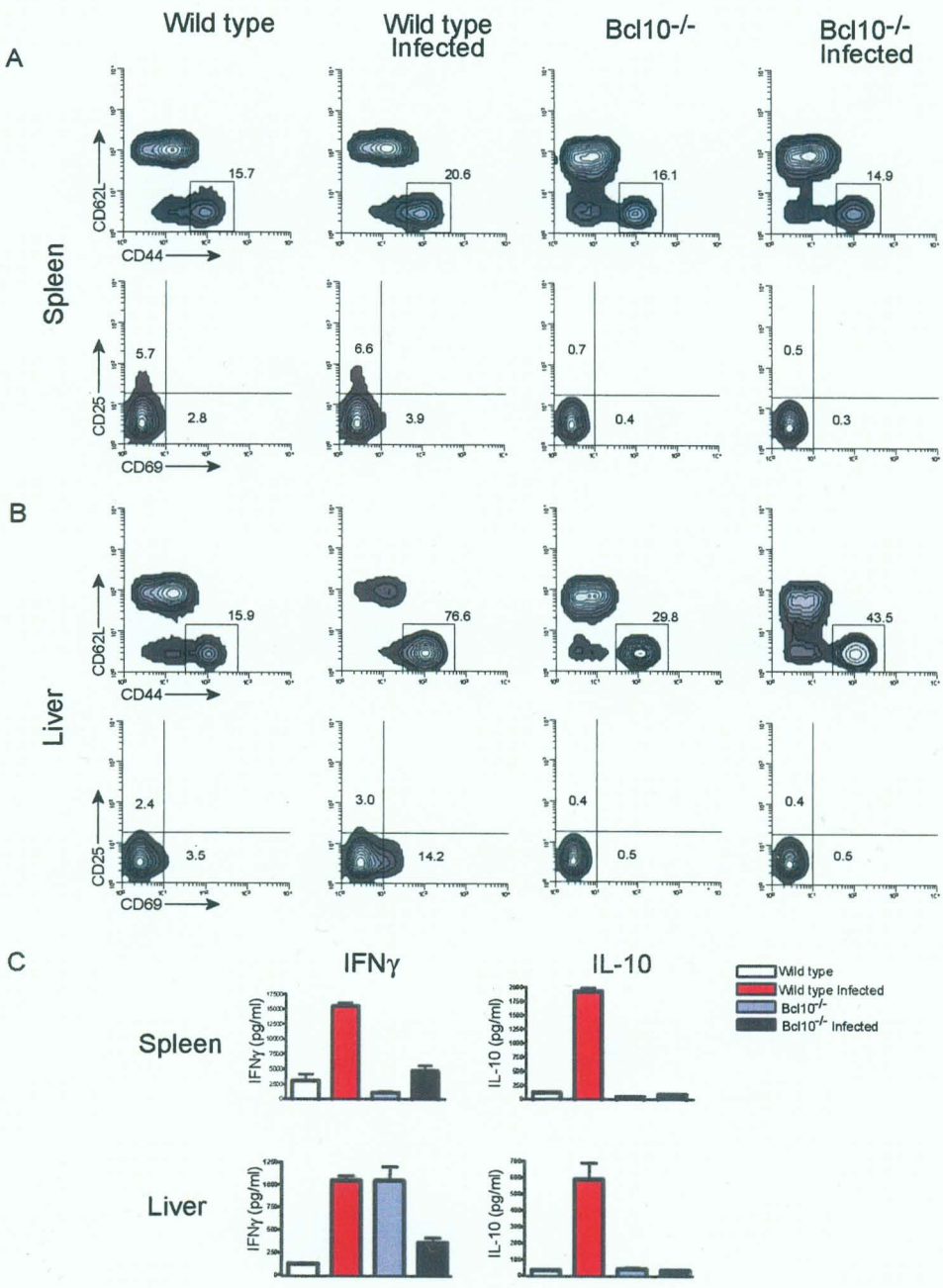

A
Wild type
Wild type Infected
Bcl10-/-
Bcl10-/- Infected
Spleen
CD62L
CD44
15.7
20.6
16.1
14.9
CD25
CD69
5.7
2.8
6.6
3.9
0.7
0.4
0.5
0.3
B
Liver
15.9
76.6
29.8
43.5
2.4
3.5
3.0
14.2
0.4
0.5
0.4
0.5
C
IFNγ
IL-10
Spleen
Liver
IFNγ (pg/ml)
IL-10 (pg/ml)
Wild type
Wild type Infected
Bcl10-/-
Bcl10-/- Infected

Figure 14: Parasite development in RAG-1$^{-/-}$ mice is partially restored by transfer of Bcl10$^{-/-}$ CD4^{+} cells.

RAG-1$^{-/-}$ mice were infected with *S. mansoni* cercariae 24 hours after adoptive transfer of 3 X10^{6} wild type or Bcl10$^{-/-}$ CD4^{+} cells. Parasites were collected 42 days post-infection and assessed for (A) worm length (B) parasite pairing and (C) egg production. (D) Homeostatic proliferation was determined by evaluating the number of CD4^{+}TCRβ^{+} cells recovered from the spleens of infected animals. (E) For each individual recipient animal, CD4^{+} T cell numbers at the time of necropsy were correlated with the average worm length for parasites recovered from that animal. WT transfer and No Transfer bars represent the average length of parasites recovered from control mice that received wild type CD4^{+} T cells or no cells, respectively.

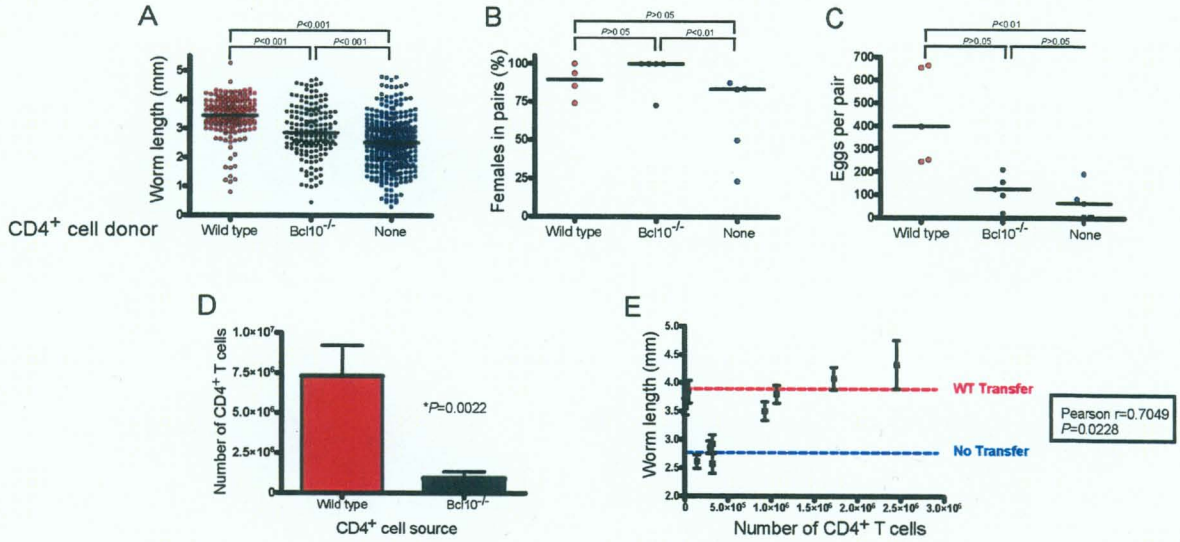

A
B
C
D
E
Worm length (mm)
Females in pairs (%)
Eggs per pair
CD4+ cell donor
Wild type
Bcl10-/-
None
P<0.001
P>0.05
P<0.01
Number of CD4+ T cells
*P=0.0022
CD4+ cell source
WT Transfer
No Transfer
Pearson r=0.7049
P=0.0228
Number of CD4+ T cells

Figure 15: *S. mansoni* development is partially restored in the presence of naïve and non-responsive TCR-transgenic T cells.

OT-II/RAG-1$^{-/-}$, DO11.10/RAG-2$^{-/-}$, Cyt/RAG-1$^{-/-}$ and appropriate genetically matched wild type and RAG$^{-/-}$ controls were infected with *S. mansoni* cercariae and parasites were collected 42 days post-infection. (A) Worm length and (B) egg production were determined as described in Figure 12.

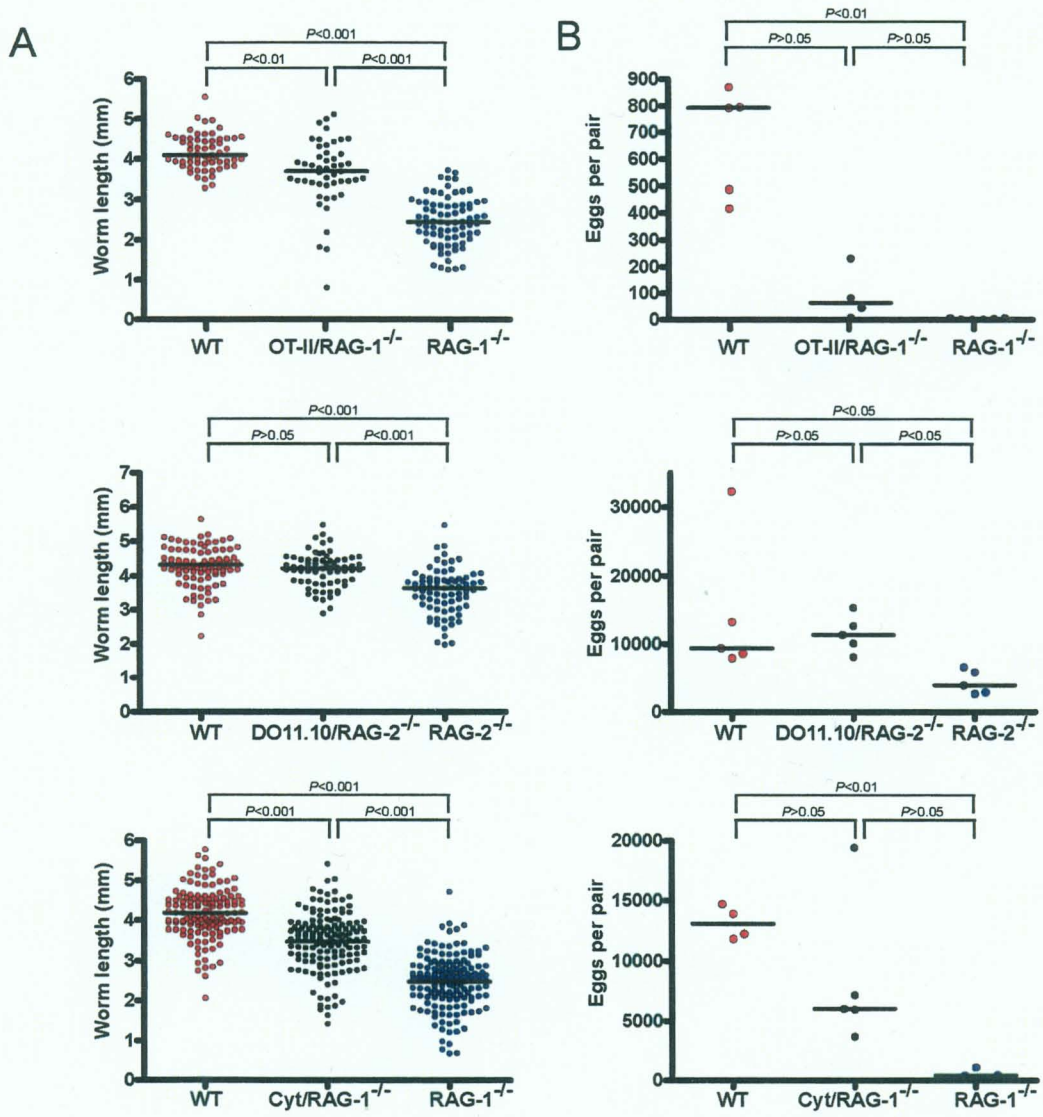

A
B
P<0.001
P<0.01
P<0.001
Worm length (mm)
WT
OT-II/RAG-1-/-
RAG-1-/-
P<0.01
P>0.05
P>0.05
Eggs per pair
P<0.001
P>0.05
P<0.001
DO11.10/RAG-2-/-
RAG-2-/-
P<0.05
P>0.05
P<0.05
P<0.001
P<0.001
P<0.001
Cyt/RAG-1-/-
P<0.01
P>0.05
P>0.05

Figure 16: T cell activation in response to schistosome infection is blocked in OTII/RAG-1$^{-/-}$ mice.

OTII/RAG-1$^{-/-}$ mice were infected with *S. mansoni* cercariae and T cell responses were measured 28 days later. (A) Surface expression of CD44 and CD62L on spleen and liver CD4^{+}TCRβ^{+}NK1.1^{-} cells from uninfected wild type, infected wild type, uninfected OTII/RAG-1$^{-/-}$ and infected OTII/RAG-1$^{-/-}$ mice is shown. CD4^{+} cells were isolated from the spleen and liver of infected or control wild type and OTII/RAG-1$^{-/-}$ mice, and co-cultured with dendritic cells pulsed with OVA or SWAP. (B) After 72 hours, culture supernatants were collected and IFNγ and IL-10 were measured via ELISA.

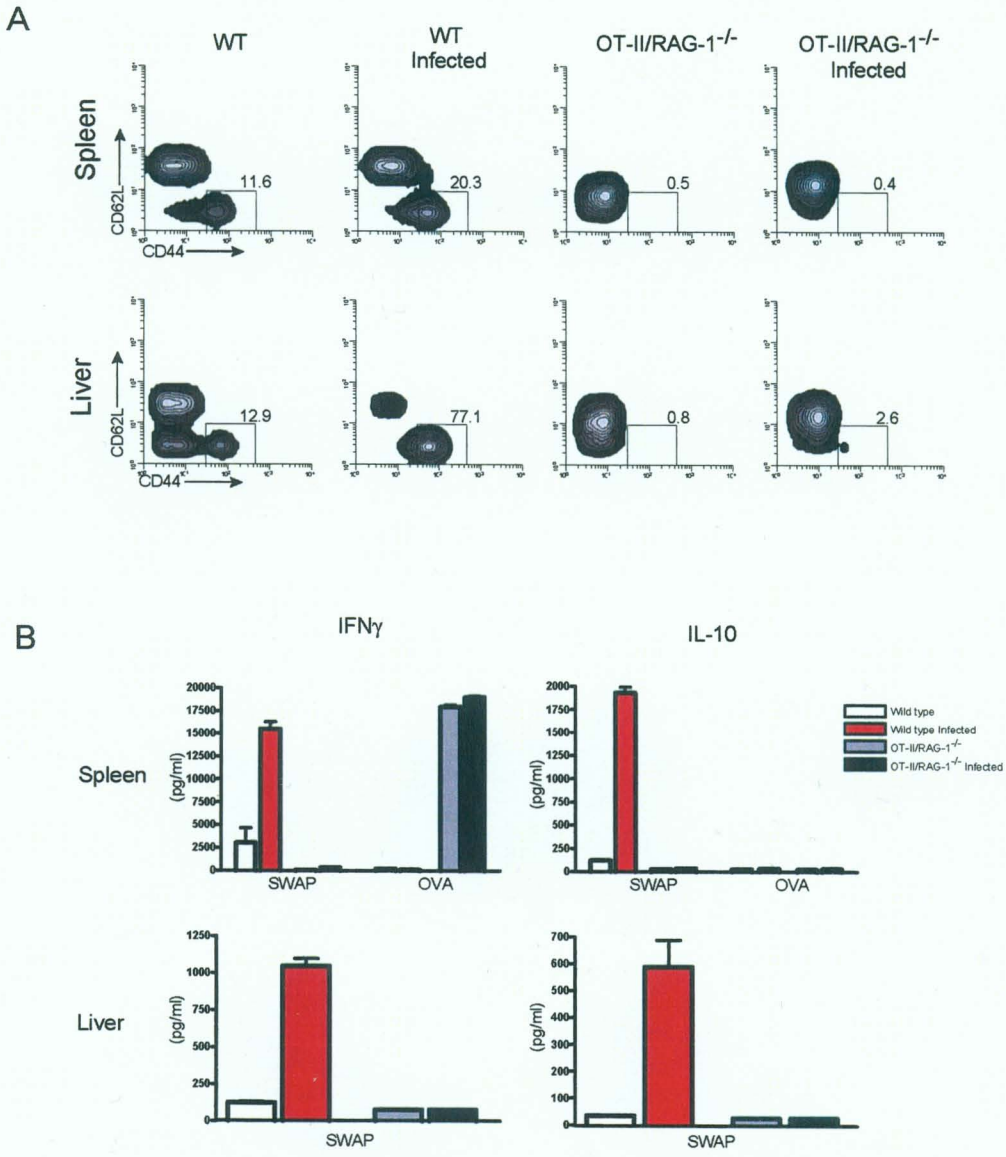

A
WT
WT Infected
OT-II/RAG-1-/-
OT-II/RAG-1-/- Infected
Spleen
Liver
CD62L
CD44
11.6
20.3
0.5
0.4
12.9
77.1
0.8
2.6
B
IFNγ
IL-10
Spleen
Liver
(pg/ml)
SWAP
OVA
Wild type
Wild type Infected
OT-II/RAG-1-/-
OT-II/RAG-1-/- Infected

Figure 17: Differential gene expression in RAG$^{-/-}$ and OT-II/RAG$^{-/-}$ livers. Microarray analysis revealed 175 genes that are expressed at statistically different levels between RAG$^{-/-}$ and OT-II/RAG$^{-/-}$ genes. Supervised clusters of (A) all 175 differentially expressed genes, (B) acute phase genes, (C) hepatic and APC genes, and (D) cytoskeleton, vesicular transport and protein processing genes are shown. Red corresponds to overexpression and green to underexpression, compared to the pooled reference sample.

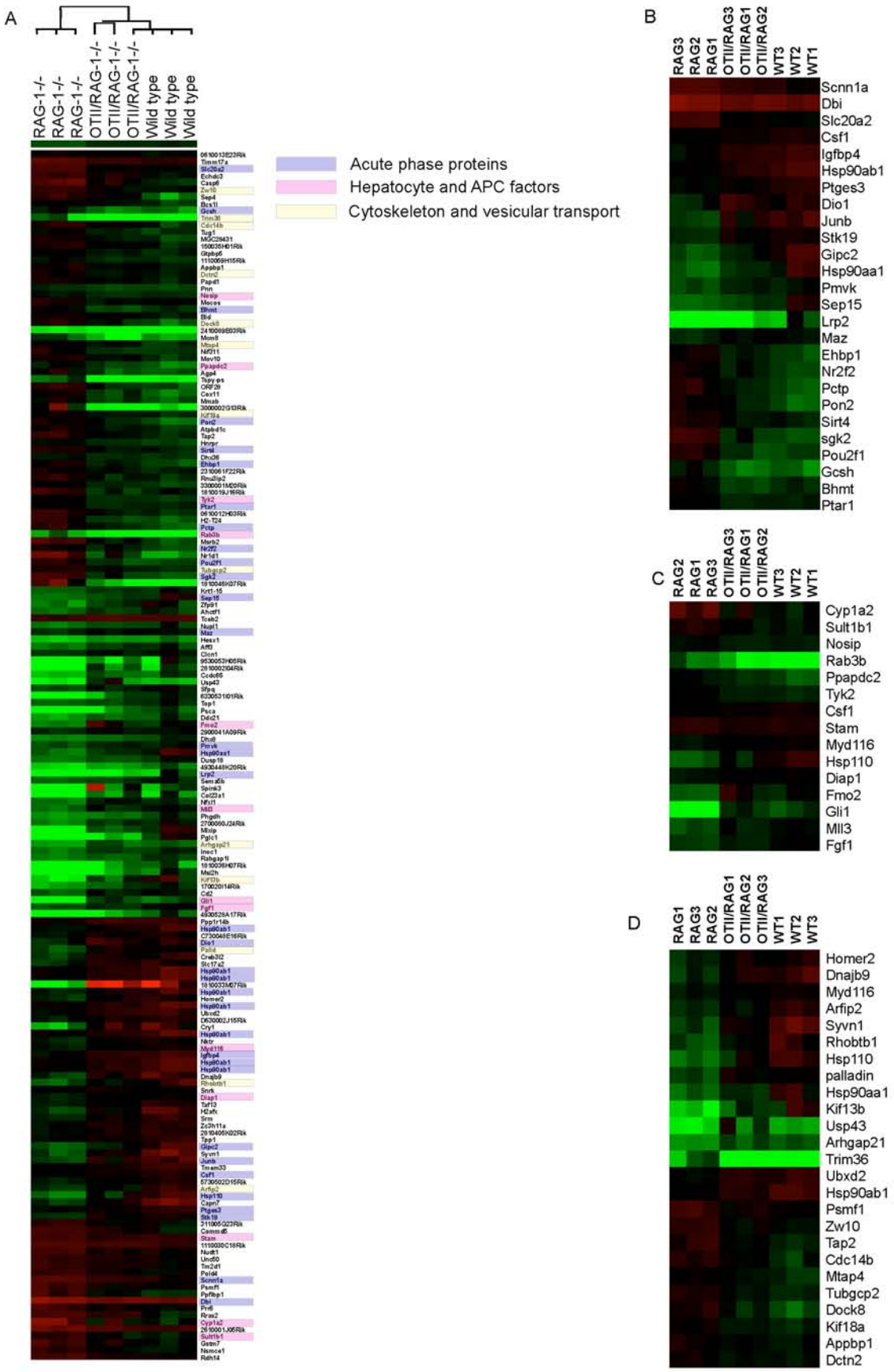

A
RAG-1-/-
RAG-1-/-
RAG-1-/-
OTII/RAG-1-/-
OTII/RAG-1-/-
OTII/RAG-1-/-
Wild type
Wild type
Wild type
Acute phase proteins
Hepatocyte and APC factors
Cytoskeleton and vesicular transport
B
RAG3
RAG2
RAG1
OTII/RAG3
OTII/RAG1
OTII/RAG2
WT3
WT2
WT1
Scnn1a
Dbi
Slc20a2
Csf1
Igfbp4
Hsp90ab1
Ptges3
Dio1
Junb
Stk19
Gipc2
Hsp90aa1
Pmvk
Sep15
Lrp2
Maz
Ehbp1
Nr2f2
Pctp
Pon2
Sirt4
sgk2
Pou2f1
Gcsh
Bhmt
Ptar1
C
RAG2
RAG1
RAG3
OTII/RAG3
OTII/RAG1
OTII/RAG2
WT3
WT2
WT1
Cyp1a2
Sult1b1
Nosip
Rab3b
Ppapdc2
Tyk2
Csf1
Stam
Myd116
Hsp110
Diap1
Fmo2
Gli1
Mll3
Fgf1
D
RAG1
RAG3
RAG2
OTII/RAG1
OTII/RAG2
OTII/RAG3
WT1
WT2
WT3
Homer2
Dnajb9
Myd116
Arfip2
Syvn1
Rhobtb1
Hsp110
palladin
Hsp90aa1
Kif13b
Usp43
Arhgap21
Trim36
Ubxd2
Hsp90ab1
Psmf1
Zw10
Tap2
Cdc14b
Mtap4
Tubgcp2
Dock8
Kif18a
Appbp1
Dctn2

Figure 18: Parasite-induced acute phase response is impaired in RAG-1$^{-/-}$ mice.

(A) Wild type and RAG-1$^{-/-}$ mice were infected with *S. mansoni* cercariae and serum amyloid A (SAA) levels were measured in plasma collected at 4 weeks post-infection. (B) Wild type and RAG$^{-/-}$ mice were injected with 2 μg LPS and serum amyloid A levels were measured in serum collected 24 hours after LPS injection.

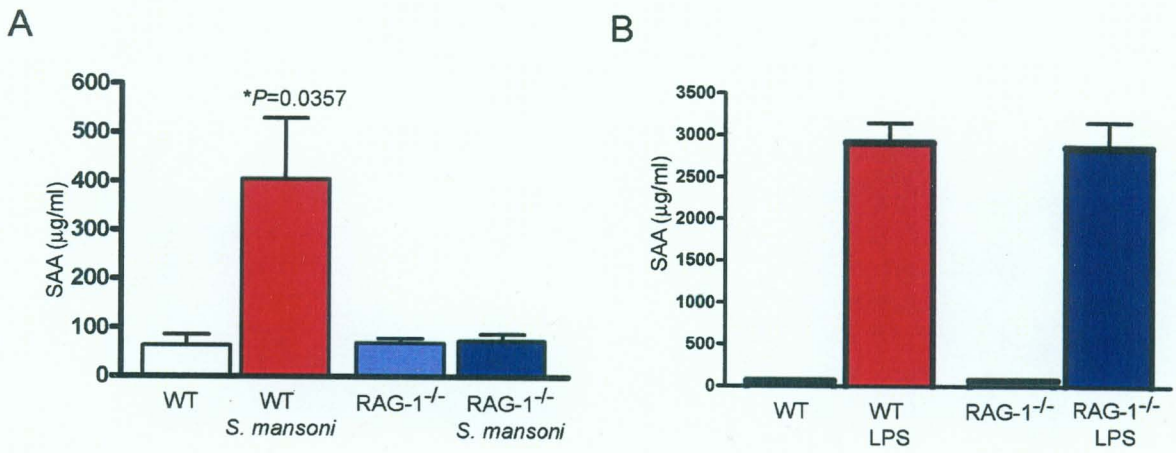
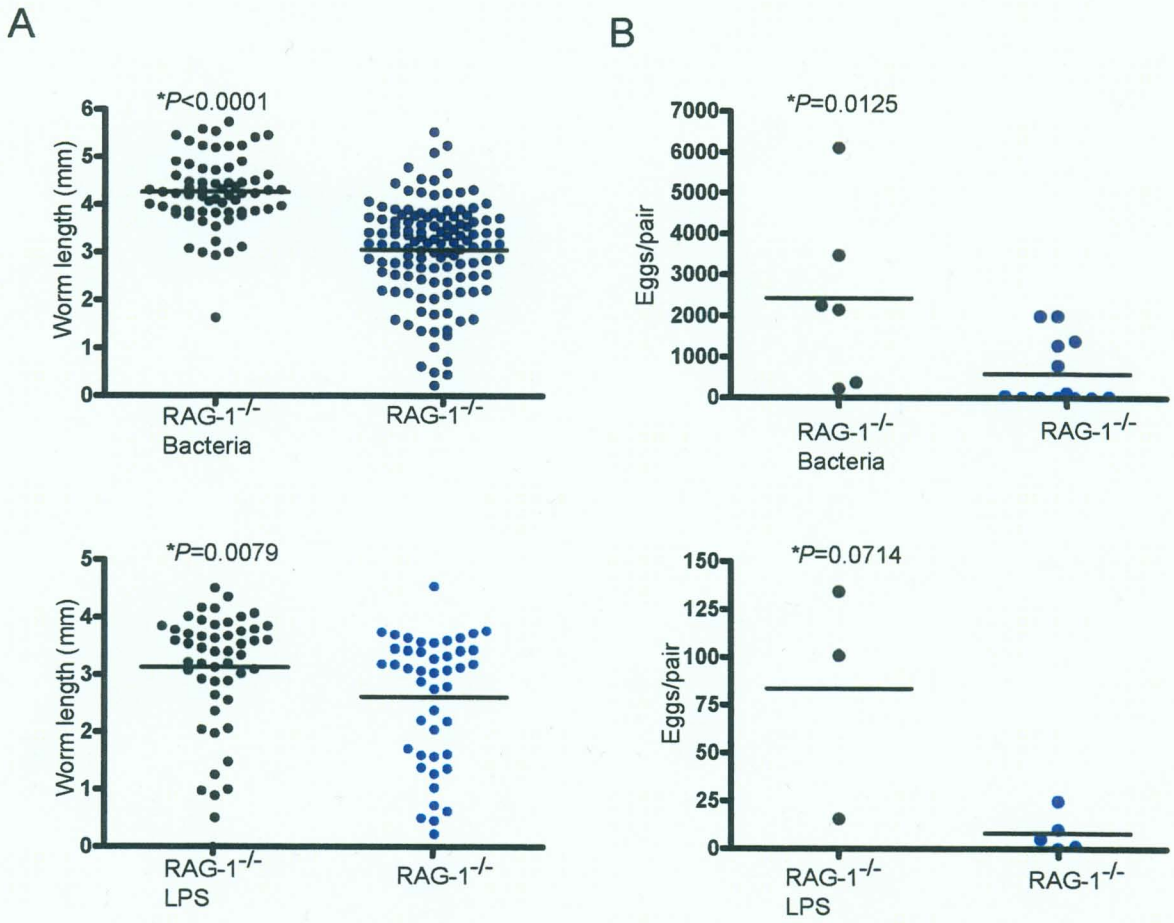

A
600
500
400
300
200
100
0
SAA (µg/ml)
*P=0.0357
WT
WT
S. mansoni
RAG-1-/-
RAG-1-/-
S. mansoni
B
3500
3000
2500
2000
1500
1000
500
0
SAA (µg/ml)
WT
WT
LPS
RAG-1-/-
RAG-1-/-
LPS

Figure 19: *S. mansoni* development is augmented in RAG-1$^{-/-}$ mice experiencing an acute phase response.

(A) Worm lengths and (B) egg production following infection with *S. mansoni,* as described in Figure 12, in a cohort of mice with bacterial co-infection (42 days post-infection) and in mice treated with LPS (39 days post-infection).


A
B
*P<0.0001
Worm length (mm)
RAG-1-/-
Bacteria
RAG-1-/-
*P=0.0125
Eggs/pair
RAG-1-/-
Bacteria
RAG-1-/-
*P=0.0079
Worm length (mm)
RAG-1-/-
LPS
RAG-1-/-
*P=0.0714
Eggs/pair
RAG-1-/-
LPS
RAG-1-/-

Chapter 5

Summary and Future Directions

Conservation of developmental mechanisms in the genus *Schistosoma*

We show that, similar to *S. mansoni* (77) the adaptive immune system is necessary to facilitate parasite development and maturation in all of the human pathogens we examined: *S. japonicum*, *S. haematobium* and *S. intercalatum* (Chapter 2 and (129)). Adoptive transfer of $CD4^+$ cells from wild type mice into RAG-$1^{-/-}$ restores *S. japonicum* development, demonstrating that, as for *S. mansoni*, $CD4^+$ T cells are necessary and sufficient for normal *S. japonicum* development. The role of $CD4^+$ T cells in the development of *S. haematobium* and *S. intercalatum* was not directly tested and thus development of these parasites may rely on other non-$CD4^+$ T cell components. This could be tested definitively through the evaluation of *S. haematobium* and *S. intercalatum* in RAG-$1^{-/-}$ recipients following adoptive transfer of $CD4^+$ T cells. Nonetheless, the most parsimonious explanation for the data we present in Chapter 2 is that developmental dependence on $CD4^+$ T cells arose early in *Schistosoma* evolution, as demonstrated by its presence in the most ancestral "japonicum group", and has been conserved throughout *Schistosoma* evolution, even in the most derived "*haematobium*" group. In contrast, development of the more distantly related schistosome, *Schistosomatium douthitti*, which is more closely related to the blood fluke parasites of birds and likely acquired mammalian definitive hosts in a separate evolutionary event to the *Schistosoma*, was not significantly impaired by the lack of an adaptive immune system suggesting that developmental dependence on immune signals is exclusively a feature of

Schistosoma. These results suggest that the medically important *Schistosoma* share a common mechanism of exploiting vertebrate host adaptive immune signals for parasite development. Thus, elucidation of the interactions between *S. mansoni* and murine hosts may be extrapolated to other human *Schistosoma* pathogens.

An alternative explanation for the differences between *Schistosoma* and *Schistosomatium* is that the size and longevity of the host species involved is drastically different, with *Schistosomatium* parasitizing small, short-lived rodents and *Schistosoma* parasitizing larger, longer-lived mammals (26). There may be selective advantages for *Schistosoma* parasites to delay development, and consequently delay egg production, in long-lived immunocompromised hosts, thus prolonging the survival of the host animal, and extending the opportunity for parasite transmission to new hosts (77, 78). Correspondingly, there may be little selective pressure for *Schistosomatium* to delay development in short-lived hosts. This hypothesis could be tested experimentally through examining the development of *S. rodhaini* and *Heterobilharzia americana* (26) in immunodeficient hosts. Genetically, *S. rodhaini* is the closest relative of *S. mansoni*(87), though *S. rodhaini* primarily infects rodents (26). Conversely, *H. americana*, the closest relative of *Schistosomatium douthitti*, infects larger, longer-lived mammals (26). If developmental dependence on the host immune system arose in separate events due to the longevity of the host species, *S. rodhaini* should develop normally in $RAG^{-/-}$ mice, whereas *H. americana* would be predicted to show impaired development. Interestingly, since viable offspring

have been reported from experimental and natural *S.rodhaini*/*S.mansoni* hybrids (144-147), differential dependence on host immune signals in these two species could allow for genetic analysis of this dependence. A differential dependence on adaptive immune signals between *H. americana* and *S. douthitti* and/or between *S. rodhaini* and *S. mansoni* would suggest that the host life span is an important selective pressure for the acquisition of parasite dependence on host adaptive immune signals.

The role of IL-7 and other γ_C-chain cytokines in schistosome development

While previous work has suggested a role for IL-7 in parasite development (100) our data show that the effect of IL-7 on parasite development is indirect and likely a result of the T cell lymphopenia in IL-$7^{-/-}$ and IL-$7R\alpha^{-/-}$ animals (97). Likewise, another γ_c cytokine, IL-2, also appears to be critical for schistosome development, but again our data indicate that, like IL-7, this cytokine influences parasite development indirectly. Since the requirement for IL-2 can not be ascribed to any of the generally recognized functions of this cytokine, such as promoting T_{reg}, T_H1 or T_H2 development or effector functions, we hypothesize that the role of IL-2 in schistosome development may relate, like IL-7, to more basic aspects of T cell function, such as homeostatic maintenance or early antigen-specific responses of T cells. For instance, naïve T cells may require autocrine IL-2 for homeostatic maintenance. Alternatively, IL-2 produced by $CD4^+$ T cells may be required for other cells or tissues to exert effects on schistosome growth. Evaluation of *S. mansoni* development in additional adoptive transfer models could address these possibilities. Assessment of parasite development following adoptive transfer of IL-$2R\alpha^{-/-}$ $CD4^+$ T cells into RAG-$1^{-/-}$ mice could be used to explore the requirement for another cell or tissue to respond to $CD4^+$ T cell produced-IL-2, since adoptively transferred IL-$2R\alpha^{-/-}$ $CD4^+$ T cells would be able to produce but not respond to IL-2. Conversely, normal *S. mansoni* development following adoptive transfer of IL-$2^{+/+}$ T cells into IL-$2R\alpha^{-/-}$ /$RAG^{-/-}$ or $\gamma_c^{-/-}$/$RAG^{-/-}$ mice would demonstrate the need for autocrine IL-

2 production and signaling. Alternatively, the inability of IL-$2^{-/-}$ $CD4^{+}$ cells to restore parasite development may not be due to a lack of the IL-2 molecule but rather to the highly activated phenotype of transferred IL-$2^{-/-}$ $CD4^{+}$ cells. Alterations in $CD4^{+}$ T cell surface molecule expression may prevent these activated T cells from forming the necessary cell-cell interactions necessary for schistosome development after adoptive transfer.

The mechanisms by which $CD4^+$ T cells influence schistosome development

We evaluated the role of $CD4^+$ T cell activation by examining schistosome development in an *in vivo* context in which $CD4^+$ T cells are present but are unable to respond to schistosome infection (Chapter 4). Together, our data demonstrate that $CD4^+$ T cells do not require activation through antigen mediated TCR stimulation in order to facilitate parasite development. Interestingly, previous results from adoptive transfer experiments in which both $CD4^+CD25^+$ T_{reg} and $CD4^+CD25^-$ T cell transfer restored parasite development (97) also suggest that neither T cell specificity nor effector function are important to mediate parasite development.

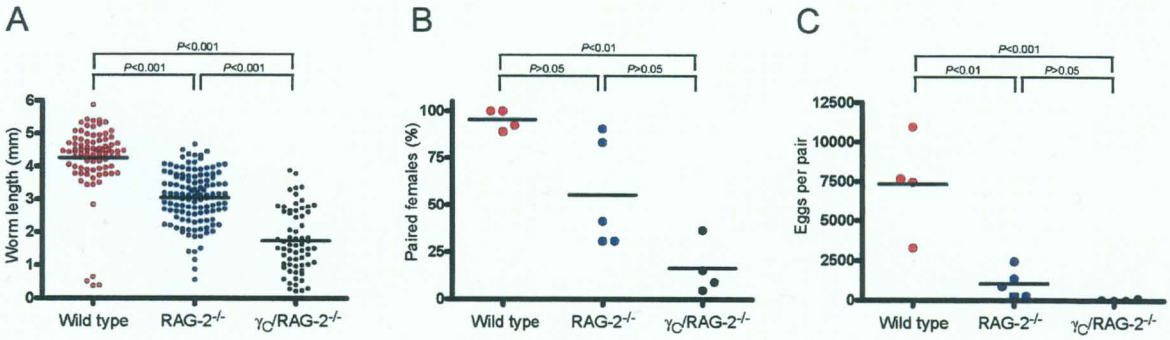
Because non-responsive $CD4^+$ T cells appear to influence schistosome development without responding to schistosome infection, we hypothesized that naïve $CD4^+$ T cells mediate their effects on schistosome development indirectly, by interacting with other cells to make the liver a more permissive environment for parasite development. Our microarray data reveal that the presence of naïve $CD4^+$ T cells is sufficient to alter global gene expression in the liver, with many of the differentially regulated genes we identified having roles in the acute phase response – a component of the innate immune system that performs functions analogous to the humoral arm of the adaptive immune system. These data suggest that $CD4^+$ T cells facilitate schistosome development through priming of the innate immune system and possibly of the acute phase response. We show

that $CD4^+$ T cells are required for priming of the modest acute phase response to schistosome worms but that treatment of immunodeficient mice with potent acute phase stimuli, such as LPS, circumvents this requirement. Finally, we show that induction of an acute phase response in the absence of $CD4^+$ T cells is associated with a significant increase in *S. mansoni* growth and development in $RAG^{-/-}$ mice, demonstrating that the requirement for host $CD4^+$ T cells in schistosome development can be bypassed by direct stimulation of the innate immune system. These data suggest both that it is acute phase responses or other innate inflammatory processes that are required for normal schistosome development and that the previously described role of $CD4^+$ T cells in schistosome development operates via the innate immune system.

To further evaluate the role of adaptive-innate interactions in schistosome development, we have begun to analyze *S. mansoni* infections in $\gamma_C/RAG^{-/-}$ mice (148). $\gamma_C/RAG^{-/-}$ mice are completely deficient in B and T lymphocytes and NK cells and are defective in IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 signaling. We find a striking deficiency in *S. mansoni* growth and development in these animals, which is significantly more severe than in $RAG^{-/-}$ mice (Fig. 20), further suggesting that adaptive ($CD4^+$ T cell) and innate (NK cell) components of the host immune system have synergistic effects on schistosome development. These interactions are currently being explored further.

Figure 20: *S. mansoni* development is severely impaired in γ_C/RAG-1$^{-/-}$ mice.

Wild type, RAG-1$^{-/-}$, and γ_C/RAG-1$^{-/-}$ mice were infected with *S. mansoni* cercariae and evaluated 42 days post-infection. (A) Worm length (B) percent pairing and (C) egg production are shown, as described in Figure 12.


A
P<0.001
P<0.001
P<0.001
Worm length (mm)
0
1
2
3
4
5
6
Wild type
RAG-2-/-
γC/RAG-2-/-
B
P<0.01
P>0.05
P>0.05
Paired females (%)
0
25
50
75
100
Wild type
RAG-2-/-
γC/RAG-2-/-
C
P<0.001
P<0.01
P>0.05
Eggs per pair
0
2500
5000
7500
10000
12500
Wild type
RAG-2-/-
γC/RAG-2-/-

Models of Schistosome Development

We propose three possible models to explain how $CD4^+$ T cells mediate effects on schistosome development (Fig. 21). First, $CD4^+$ T cells may prime, by mechanisms that do not require responses to antigen, one or more intermediate innate immune components, such as monocytes, macrophages and dendritic cells, which in turn prime other components of the innate immune system such as the acute phase response. In support of this model, the microarray data presented in Chapter 4 reveal several macrophage and dendritic cell specific genes that are differentially regulated in the presence of naïve T cells. Also, severely impaired schistosome development in $\gamma_C/RAG^{-/-}$ mice suggests that both adaptive and innate components play a role in schistosome development. The role of innate immune components will be further evaluated in appropriate animal models. Since IL-1 and IL-6, produced by macrophages and monocytes, are key mediators of the acute phase response, the ability of these molecules to mediate *S. mansoni* development can be evaluated by treating $RAG^{-/-}$ mice with exogenous IL-1 and IL-6. The role of IL-1 and IL-6 can be further evaluated by studying *S. mansoni* infections in $IL\text{-}1R^{-/-}$ (149) and $IL\text{-}6^{-/-}$ (150) mice. Additionally, schistosome development can be evaluated following treatment of wild type mice with blocking antibodies directed against IL-1β or IL-6. Alternatively, parasite development may be examined in mice which overproduce the acute phase stimulators TNFα and IL1β. $AUF\text{-}1^{-/-}$ mice have dysregulated control of TNFα and IL1β production in response to inflammatory stimuli and

overproduce acute phase mediators (151). Augmentation of *S. mansoni* development in AUF-$1^{-/-}$ mice would indicate an important role for acute phase proteins in schistosome development. Finally, the effect on schistosome development of inhibition of specific acute phase proteins, such as complement component C3, could be examined (152, 153).

In the second model, primed innate immune cells stimulate production of acute phase proteins in addition to providing the schistosome growth-enhancing signals. Thus, the acute phase response serves only as a marker for processes that facilitate schistosome development, though it is not directly involved. To test this model, production of acute phase proteins could be arrested at key points in schistosome development through D-galactosamine treatment, which specifically blocks RNA and protein synthesis in the liver (154).

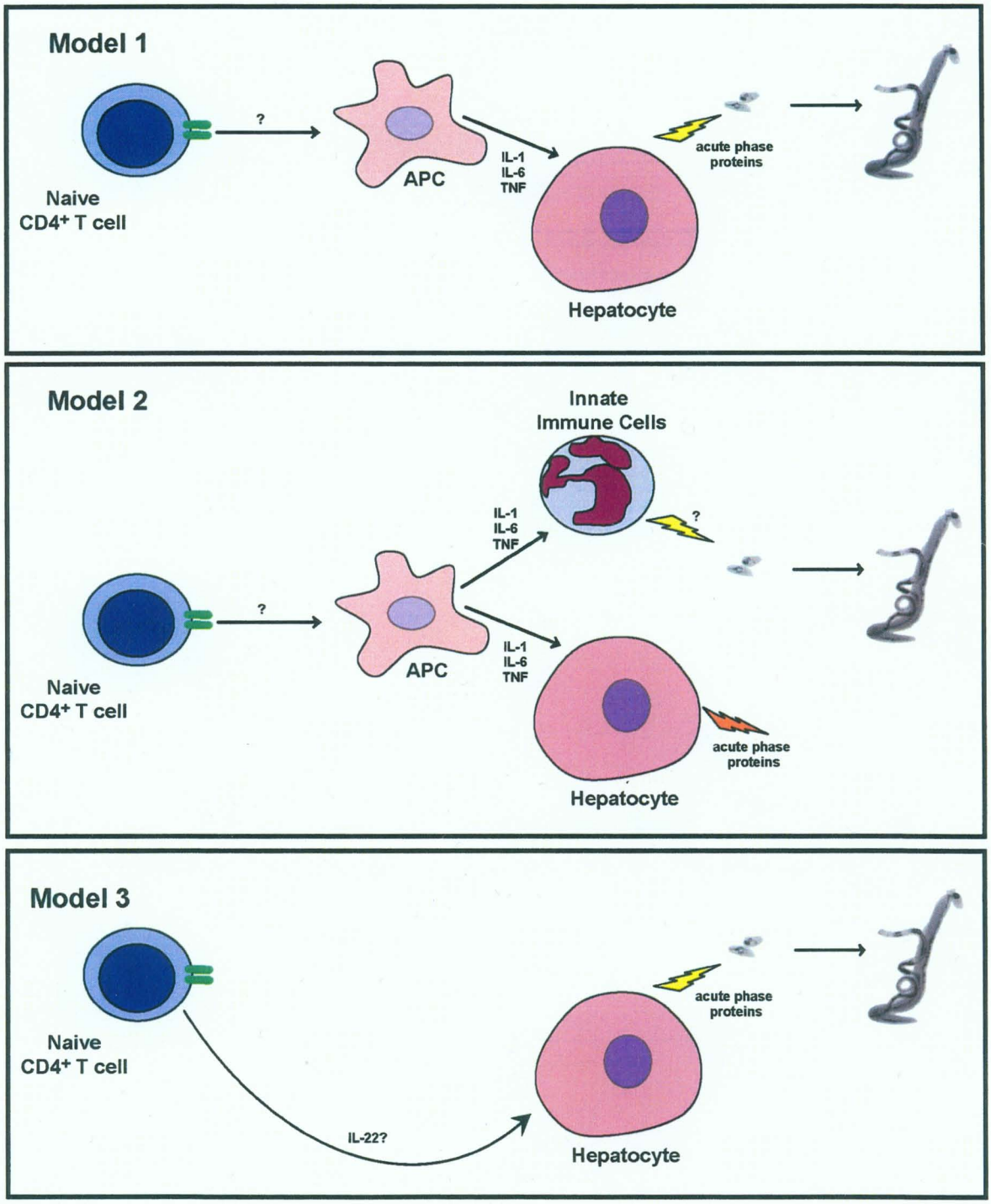
Finally, naïve $CD4^+$ T cells may interact directly with hepatocytes and prime them to produce acute phase proteins or other factors which promote parasite development. Although activated $CD4^+$ T cells have been shown to interact with hepatocytes through IL-22 signaling (155), a similar role has not been identified for naïve T cells. This model will be evaluated through targeted analysis of IL-22 signaling in addition to the continued analyses of both $CD4^+$ T cell and innate immune cell contributions to schistosome development, as described above.

Figure 21: Proposed models for host adaptive-innate immune facilitation of schistosome development.

Model 1: $CD4^+$ T cells prime, through non-antigen specific mechanisms, one or more intermediate innate immune components, which in turn prime other components of the innate immune system such as the acute phase response

Model 2: Primed innate immune cells stimulate production of acute phase proteins in addition to providing the schistosome growth-enhancing signals.

Model 3: Naïve $CD4^+$ T cells interact directly with hepatocytes and prime them to produce acute phase proteins or other factors which promote parasite development.


Model 1
?
Naive
CD4+ T cell
APC
IL-1
IL-6
TNF
acute phase
proteins
Hepatocyte
Model 2
Innate
Immune Cells
IL-1
IL-6
TNF
?
?
Naive
CD4+ T cell
APC
IL-1
IL-6
TNF
acute phase
proteins
Hepatocyte
Model 3
Naive
CD4+ T cell
IL-22?
acute phase
proteins
Hepatocyte

Summary

The relationship between helminth parasites and their hosts is remarkably complex and our understanding of how these parasites exploit host resources to facilitate life cycle completion and transmission is likely still rudimentary. In the case of *Schistosoma* blood flukes, we have shown that these pathogens exploit fundamental interactions between $CD4^+$ T cells and the innate immune system that appear not to require recognition of antigen by the T cell. While initially surprising, these observations are more understandable if one considers that trematodes first acquired vertebrate hosts at a very early stage in vertebrate evolution, before the adaptive immune system had evolved the capacity to form lymph nodes and germinal centers. However, regardless of the evolutionary origins, a detailed understanding of host-*Schistosoma* interactions will hopefully provide a basis for the rational development of new and much needed strategies to treat and prevent schistosome infections.

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