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The interplay of the parasite and the host immune response during schistosome development in a mammalian host

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The Interplay of the Parasite and the Host Immune Response during Schistosome Development in a Mammalian Host

by

Rebecca B. Blank

DISSERTATION

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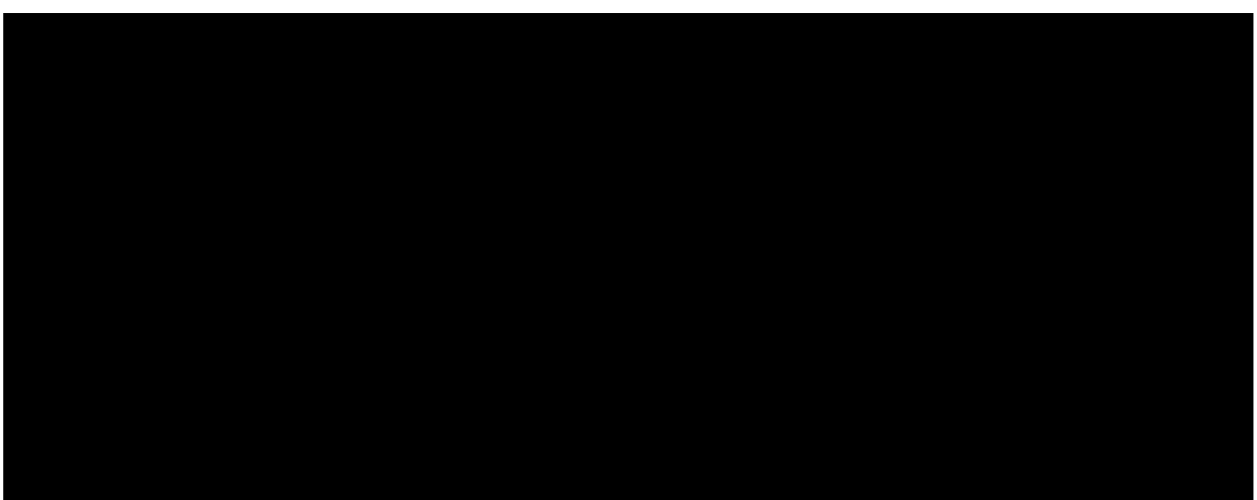

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I would like to dedicate this dissertation in memory of my mother,

Sharon Donna Cosloy, Ph.D.

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The Interplay of the Parasite and the Host Immune Response during Schistosome Development in a Mammalian Host

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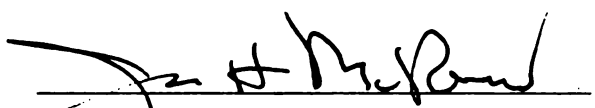

James H. McKerrow, M.D., Ph.D.

Abstract

As a complex, multicellular, and relatively large pathogen, *Schistosoma mansoni* has co-evolved with its vertebrate host to maximize its longevity and transmission while evading the host immune response. We have previously observed that parasite development is severely impaired in recombination activating gene (RAG)-$1^{-/-}$ mouse hosts, which lack an adaptive immune response, Schistosomes grew slower, produced fewer eggs, and did not pair as well as those in wild-type hosts. To elucidate the role of the adaptive immune response in promoting parasite development, we undertook a number of cell reconstitution and cell depletion studies. By reconstituting RAG-$1^{-/-}$ mice with B

cells, $CD8^+$ T cells, or $CD4^+$ T cells prior to infection, we determined that only a $CD4^+$ T cell population was able to rescue worm development. These observations were corroborated by data showing that depletion of $CD4^+$ T cells in wild-type mice and subsequent infection produced an impaired growth phenotype, similar to that observed in RAG-$1^{-/-}$ mice. These observations suggested that immature schistosomes must sense the host environment, in particular the immune fitness of its host, in order to initiate a period of rapid growth and sexual maturation. Specifically, we found that $CD4^+$ $\alpha\beta$ T cells were crucial in promoting parasite development.

A clear understanding of how $CD4^+$ $\alpha\beta$ T cells respond to prepatent schistosome infection is essential to elucidating the mechanism by which $CD4^+$ T cells act to regulate parasite development. To determine the complex interactions between the schistosome and host, three lines of inquiry were undertaken. First, intracellular cytokine production by hepatic $CD4^+$ T cells throughout early infection was analyzed. Second, T cell cytokines that may be required for parasite development were surveyed by infecting mice that possess targeted deletions in specific cytokine signaling pathways. Third, microarray experiments with both *S. mansoni* cDNA and oligonucleotide-based arrays were performed to analyze global gene expression patterns in developing parasites from either wild-type or immunodeficient hosts.

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Chapter One

Introduction

Overview

Infection by the blood fluke *Schistosoma mansoni* is remarkable in that it is both ancient, having been identified in Egyptian mummies, and long lived in the human host; individual worms can live up to thirty years (1). As a complex, multicellular, and relatively large pathogen, *S. mansoni* has co-evolved with its vertebrate host to maximize its longevity and transmission while evading the host immune response. Currently, schistosomiasis is a global health problem for over 250 million people worldwide, particularly for those living in sub-Saharan Africa, Asia, and parts of South America. Infection with *S. mansoni* can vary in intensity and clinical spectrum and may produce fever, diarrhea, hepatomegaly, and splenomegaly. Heavy parasite load and recurrent infection can lead to death from portal hypertension, but even mild infections result in disability. Praziquantel is an effective drug for treating infection, but its value is limited in endemic areas where residents are at risk for reinfection due to their interactions with infested water in work and play.

Evolution and Life Cycle

Schistosoma mansoni is a trematode that evolved from free-living turbellarian or rhabdocoel flatworms. Its free-living ancestors became adapted as parasites of marine mollusks in the early Paleozoic era, hundreds of millions of years ago. Ensuing coevolution of the parasite and molluscan host, in parallel

with the evolution of vertebrates, resulted in a digenetic (two-host) life cycle. Vertebrate predators were passively infected with molluscan trematodes by incidental ingestion of infected mollusks and, eventually, molluscan parasites adapted to the new vertebrate environment and evolved to thrive in that environment. For these reasons, most modern adult trematodes live within the intestines of their vertebrate hosts (2). Genus *Schistosoma* is unique in respect to its niche in the vertebrate host; members of this genus that infect humans, reside in either the hepatic portal venous system (in the case of *S. mansoni* and *S. japonicum*) or in the vasculature surrounding the bladder (*S. haematobium*) (1).

Schistosoma mansoni has a complex two-host life cycle in which humans are its secondary and definitive hosts. The snail, *Biomphalaria glabrata,* is the intermediate host in which large numbers of developing larval flukes (called sporocysts) develop. Primary sporocysts give rise to daughter sporocysts by budding and these daughter sporocysts migrate to the digestive glands (hepatopancreas) of the snail and, eventually, differentiate into numerous infective larvae, called cercariae. Mature cercariae escape through a birth pore in the daughter sporocyst and are shed from the snail into the surrounding fresh water (2). Cercariae home to and penetrate their final mammalian host by sensing motion, temperature gradients, changes in light (2), and skin secretions (3, 4). Upon contact with the host, cercariae penetrate the epidermal layer of the host skin and immediately transform into schistosomula. Schistosomula continue

to invade the dermal layer until they reach and enter venules or small lymphatics in the dermis by day 2. Once they enter the circulatory system, they pass through the right side of the heart and enter the lungs, residing in the lung vasculature from days 4 and onward, peaking at day 6 and then exiting by day 11 to migrate to the liver over the next two weeks. By day 21, all viable immature schistosomula have migrated to the portal veins within the liver, at which point growth and sexual maturation rapidly ensues (5, 6). Pairing of mature male and female schistosomes begins around day 35 whereupon they migrate to the mesenteric veins as a mating pair (7, 8). *S. mansoni* females can lay up to 300 eggs a day; approximately half of them pass through the large intestine to be excreted in the feces. The rest are carried back into the liver and other organs via the flow of blood in the veins in which the worms reside (1). Wayward eggs that lodge in host tissues induce a granulomatous immune response that protects the host from a diffusible parasite toxin in the egg, while at the same time producing the pathology of chronic schistosomiasis. Eggs passed through the feces can hatch in fresh water; the resulting miracidia are then able to infect the primary molluscan host in order to perpetuate the life cycle (2).

Genetics

S. mansoni is a diploid organism, which has 8 pairs of chromosomes. The haploid genome contains 2.7×10^8 base pairs, approximately 10% of that found in mammals. The genome is 65% AT-rich (9). 60% of the genome is composed

of repetitive sequences (10), and ribosomal RNA genes are repeated 500 to 1000 times (9). It has been postulated that the genome contains approximately 15,000 to 20,000 expressed genes (11), compared to the 26,383 genes in the human genome conservatively predicted by Venter and colleagues (12). Efforts are currently underway to sequence the entire *S. mansoni* genome (The Institute for Genomic Research, www.tigr.org), with current coverage at 4-fold the haploid genome. In addition, over 180,000 *S. mansoni* established sequence tags (EST) (13) and over 44,000 *S. japonicum* ESTs (14) have been deposited into GenBank. Yet few sequences have been well characterized. Of those that have been characterized, some have homology to hormone, immune and growth factor receptors (15-25), suggesting that the parasite may respond to host immune or endocrine products.

Immune Response and Pathology

The prevailing paradigm in schistosome research has been that pathology is due mainly to the granulomatous immune reaction to the schistosome egg, and that there is little, if any, host reaction to the worms themselves. Egg production begins at approximately 35 days post-infection (dpi), and numerous studies support the conclusion that the T helper (Th) response to parasite eggs is dominated by the production of type-2 cytokines (such as IL-4, IL-5, and IL-13 cytokines classically defined as immunomodulatory cytokines) (7, 26-29). A Th2 response is central to both circumoval granuloma formation (30) and to limitation

of egg-induced pathology (31). Aside from vaccination studies (27, 32), any immune response earlier in infection has, until recently, been largely undocumented and therefore not characterized for specific T cell and cytokine responses before full egg production has begun. The limited published data suggest that, in contrast to anti-egg responses, Th responses to worms are type 1-like (inflammatory cytokines such as IFN-γ and TNF-α are induced). Analysis of splenocytes from prepatently (before egg production has begun) infected mice revealed a predominantly Th1-like response to parasites during early infection (27). Further, analysis of single-sex infections, where egg production did not occur, demonstrated that schistosome infection evokes a type-1 response in the absence of anti-egg Th2 responses (8, 26). However, due to the longevity of the parasite in the host (up to 30 years), any observed immune response to the worms does not appear to be detrimental to the parasite. Some studies have proposed that schistosomes actively evade the host immune response by either shedding their tegumental surface proteins (33) or by incorporating host proteins into their tegument, effectively masking themselves from detection (34-36).

Environmental Role in Parasite Development

The idea that the host environment may play a role in regulating development and fecundity of *S. mansoni* is not a new concept. Studies on the

role of host nutritional deficits impairing parasite growth and egg production go back to the 1940's. Krakower and colleagues made the interesting observation that vitamin C deficiency in guinea pig hosts led to parasite production of deficient eggshells and disintegrated eggs (37). In a mouse model, animals were fed a diet deficient in selenium, vitamin E, and cysteine, resulting in dietary necrotic degeneration of the heart, liver, muscle and kidneys. Infections in these nutritionally deficient mice produced worms that failed to attain normal size; the majority of which did not reach sexual maturity (38, 39). Interestingly, worm burden was higher in the nutritionally deficient mice than in normal controls, indicating a reduction in host resistance in parallel with host nutrition. In another study, male worms were smaller than normal, had aberrant reproductive systems, and egg production was reduced in mice fed a low-protein diet as compared with normally nourished controls (40).

Of greater import to our current studies are the observations on the effects of host immune and hormonal balance on parasite development. The earliest studies demonstrated that immunosuppressive treatment with corticosteroids impaired parasite establishment (41). In studies of schistosome-infected baboons, dexamethasone treatment, which is an immunosuppressive corticosteroid, reduced egg output, indicating a defect in fecundity or maturation (42). Follow up studies using other immunosuppressants and/or depleting hosts of T lymphocytes demonstrated that host immune deficiency led to delayed worm maturation and delayed onset of oviposition in *S. mansoni*-infected mice (43, 44).

Knopf (1981) reviewed the role of host hormones in the outcome of *S. mansoni* development. Mice that had been made hypothyroid harbored schistosomes that were smaller and sexually matured more slowly as compared to mice rendered hyperthyroid, whose infections produced worms that were larger and sexually matured more rapidly (45). Additionally, rats, which are not normally permissive hosts of schistosomes, can be made to host the parasite after thyroidectomy (46). It is important to note that thyroidectomy leads to a reduction in immune responsiveness (47) and could be a factor in permissiveness of infection in normally nonpermissive rats. More recently, when transgenic and knockout mice became available, studies involving infections of severe combined immunodeficiency (scid) mice, which are homozygous for the *prkdc*^{scid} allele and as a consequence have severely reduced levels of T and B cells, demonstrated that parasite egg production was impaired when the host adaptive immune response was reduced (48). Treatment with exogenous TNF-α, rescued fecundity in this immunodeficient host (48). Similarly, infections in IL-7-deficient hosts, which are severely lymphopenic due to the role of IL-7 in lymphocyte development (49), resulted in worms with attenuated development. Yet, their growth could be rescued by administration of exogenous IL-7 (50, 51).

These previous studies of attenuated schistosome development in lymphopenic hosts did not determine the effects of a complete absence of an adaptive immune response. Our lab determined that in recombination activating gene (RAG)-1$^{-/-}$ mouse hosts, which lack T or B lymphocytes, parasite

development was severely impaired. Worms grew slower, produced fewer eggs, and did not pair as well as those in wild-type hosts (52). These observations were made in both *S. mansoni* and *S. japonicum* infections, indicating that attenuated development occurred across *Schistosoma* species. To elucidate the role of the adaptive immune response in promoting parasite development, we undertook a number of cell reconstitution and cell depletion studies. By reconstituting RAG-1$^{-/-}$ mice with B cells, CD8^{+} T cells, or CD4^{+} T cells and subsequent schistosome infection, we determined that only a CD4^{+} T cell population was able to rescue worm development. These observations were corroborated by data showing that depletion of CD4^{+} T cells in wild-type mice and subsequent infection produced an impaired growth phenotype, similar to that observed in RAG-1$^{-/-}$ mice. Further characterization of the responsible CD4^{+} T cell population determined that it expressed T cell receptor (TCR) α and β chains but did not express NK1.1 and so was not considered to be an NKT cell. Additionally, histological analysis of developing worms in liver venules showed lymphocytes, including CD4^{+} cells, in close proximity with the tegument of the worms (52). To our initial surprise, infections in doubly deficient β2-microglobulin (β2-m)$^{-/-}$/major histocompatibility complex (MHC) class II$^{-/-}$ mice (53), which do not express MHC class I or MHC class II and therefore do not allow development of conventional T cells, were completely normal. Further analysis revealed that an unconventional CD4^{+} T cell population in the liver of these mice, that develop in the absence of MHC class I or MHC class II interactions with TCR, was able to

recapitulate worm development in these immunodeficient hosts (52). Taken together, these observations suggest that immature schistosomes must sense the host environment, in particular the immune fitness of its host, in order to initiate a period of rapid growth and sexual maturation. Specifically, $CD4^+$ $\alpha\beta$ T cells are crucial in promoting parasite development.

Advantage of an Alternative Developmental Program for Long-Lived Parasites

The observation that schistosomes can enter a reversible developmental arrest is consistent with the presumed complex relationship between a long-lived parasite and its host. The working model suggests that the parasite regulates its development depending on the overall health and fitness of the host; parasite development is attenuated in a host that lacks an adaptive immune response (52). In the case of *S. mansoni*, $CD4^+$ T cells are essential both for parasite growth and maturation (52), as well as granuloma formation (43). Paradoxically, granuloma formation is the main cause of pathology; yet, it is essential for both host survival and parasite transmission (31, 43, 48). Doenhoff *et al.* and Amiri *et al.* showed that in the absence of granuloma formation eggs were not found in the feces of mice. Doenhoff proposed that the granuloma shepherds eggs through the bowel wall by virtue of its hydrolytic enzyme activity. The

developmental response of *S. mansoni* that we described may independently serve to increase the likelihood of parasite survival in an immunocompromised host or a host compromised by malnutrition or other concurrent infections (54). Interestingly, analysis of humans coinfected with *S. mansoni* and HIV have found that schistosome fecal egg excretion is reduced in patients with HIV coinfection and that reduced egg excretion correlates with lower $CD4^+$ lymphocyte counts (55, 56). These observations suggest that the phenomenon observed in experimental mouse infections may be of biological relevance and important in understanding the pathogenesis of schistosomiasis in humans.

The *Caenorhabditis elegans* Model

The reversible developmental arrest that occurs in schistosome-infected immunodeficient hosts is reminiscent of the hypobiotic developmental arrest that occurs in the dauer stage of *Caenorhabditis elegans*. Dauer in *C. elegans* is an alternative third-stage larva specialized for survival and dispersal (57). Progression to the dauer stage depends on various environmental cues, such as temperature, food availability, and population density as indicated by the concentration of constitutively secreted dauer pheremone (58, 59). Nematodes can sense these environmental cues at the first and second (L1 and L2) larval stages and then proceed to either the third larval stage (L3) or the dauer. When food availability increases or overcrowding subsides, nematodes can then

proceed from dauer to normal adult development. Therefore, dauer is reversible when environmental conditions improve.

C. elegans' dauer pathway involves several TGFβ signaling molecules including TGFβ receptor and Smads, cofactors involved in transducing a signal from the receptor to the nucleus (60). The TGFβ superfamily is conserved throughout metazoans and regulates cell growth and differentiation in both invertebrates and vertebrates (reviewed in (61)). In fact, a homolog of TGFβ receptor I, named SmRK-1 (17), and homologs of multiple Smads involved in SmRK-1 signaling (19, 25, 62) have been identified in *S. mansoni*, making platyhelminths the most primitive organisms in which TGFβ signaling has been identified. TGFβ signaling pathways are conserved throughout evolution suggesting to Beall *et al.* (63) that host-produced human TGFβ might be able to signal through SmRK-1. Indeed, they found that human TGFβ could signal via SmRK-1 *in vitro*. Similarly, previous studies had determined that a TGFβ receptor-1 homolog of *C. elegans* was able to bind human bone morphogenetic protein-2 and bone morphogenetic protein-4 when expressed in mammalian cells *in vitro* (64), indicating a conservation of this superfamily among diverse organisms. Using the dauer and TGFβ pathway as examples, crosstalk between the human host and parasite can be imagined, leading one to hypothesize that schistosomes can sense and transduce signal from certain host growth factors in the environment of the hepatic portal veins that determine their progression towards sexual maturity.

Parallels in Other Helminth Infections

Like the nonparasitic nematode *C. elegans,* there are a number of parasitic helminths that can reversibly arrest their development. Many of these parasites are similar to *C. elegans*, being members of Nematoda and entering diapause in the L3 stage (65, 66). Some of the genes that are involved in *C. elegans* dauer formation can also be identified in the genomes of parasitic nematodes (67-70). Certain parasites can arrest in response to strong immunity from the host, allowing for resumption of development during suppression of immunity (71). Others, like *Toxocara canis*, arrest in tissues of older dogs; presumably older dogs have a higher degree of immunity. The larvae reactivate when the host becomes pregnant, allowing transmission to nonimmune pups (72, 73). *Strongyloides ratti* females can sense the environment of the small intestine in which they reside, producing free-living male and female offspring or infectious female L3 larvae that are homologous to *C. elegans* dauer (74). Arrested development has also been observed in platyhelminthes (75-77).

Aims

A clear understanding of how $CD4^+$ $\alpha\beta$ T cells respond to prepatent (period before egg production has begun) schistosome infection is essential to

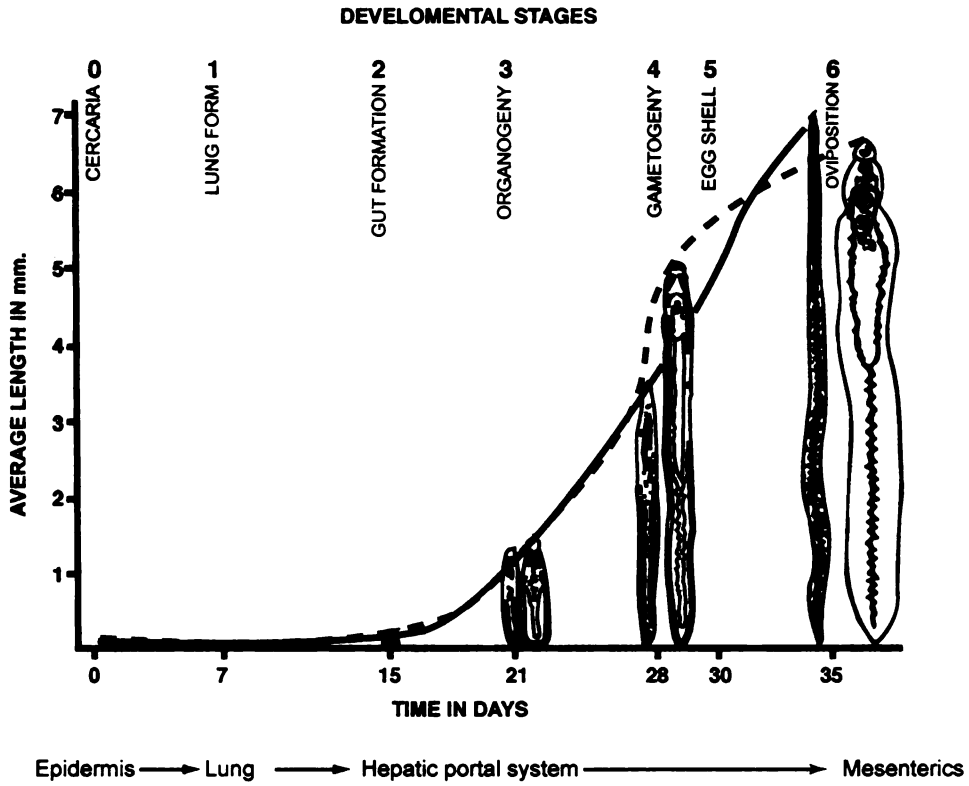
elucidating the mechanism by which $CD4^+$ T cells act to regulate parasite development. To determine the complex interactions between the schistosome and host, I undertook three lines of inquiry. First, in order to gain insights into how $CD4^+$ T cells might affect the outcome of schistosome development, I analyzed intracellular cytokine production by hepatic $CD4^+$ T cells throughout early infection. Second, I examined which T cell cytokines may be required for parasite development by infecting mice that possess targeted deletions in specific cytokine signaling pathways. Third, I performed a number of microarray experiments with both *S. mansoni* cDNA and oligonucleotide-based arrays to analyze global gene expression patterns in developing parasites from either wild-type or immunodeficient hosts.

Chapter Two

Details of Parasite Maturation in an *in vitro* Culture System

Introduction

A reproducible method for maintaining schistosome worms in culture so that their developmental program can be observed and manipulated would be of immense value for experiments designed to elucidate host factors that influence parasite growth and maturation. However, attempts at culturing *S. mansoni in vitro* have been met with varying degrees of success. The earliest attempts at cultivating parasites *in vitro* were reported by Lee and Chu (1935) (78) who were able to culture adult worms for periods of up to five months. Later attempts at setting up *in vitro* culture systems to study growth and development can be found in seminal papers by Cheever and Weller (1958) and Clegg (1965) (79, 80). Cheever and Weller's studies focused on comparing formulations of various nutrient media and the effects of pH on worm maintenance and growth. Yet even with the optimization of the culture media, and the ability to culture both male and female worms for months at a time, worms never reached full sexual maturity, leading to the hypothesis that for complete sexual development, other factors specific to the host environment were necessary. Clegg made very careful and detailed observations that defined six stages of schistosome development throughout the course of infection in the mouse (Fig. 1) (80). From his detailed descriptions of the developmental stages and studies of mitotic cell divisions, he determined that cell division in immature schistosomula only begins once they have reached the portal areas of the liver; they remain in a mitotic state of arrest



Adapted from *Clegg, J.A. 1965. Experimental Parasitology 16, 133-147*

Figure 1. *Schematic detailing schistosome development in a mammalian host.* Rapid growth and development only begins once the immature schistosomula reach the portal veins of the liver beginning around 15 days post infection.

from initial skin penetration through migration past the heart and lung. Additionally, he attempted to reproduce his *in vivo* observations on schistosome development *in vitro*. *In vitro* development of schistosomula isolated from lungs of infected mice occurred rapidly to stage 2 but slowed from stages 2 to 4, so that worms reached the developmental criteria of stage 4 approximately 1 week later than that observed in the mouse. Schistosomula were able to reach stage 5 within 6 weeks, but in all experiments the parasites were smaller in size than their stage 5 counterparts *in vivo*. Adjustments to culture media and apparatus did not support development to stage 6. Therefore, Clegg was not successful in producing worms at full sexual maturity *in vitro* (80). However, in 1981, Basch and colleagues documented *in vitro* development from cercariae to pairing adults (81). This was an important breakthrough, yet even so, the few worms that were able to pair were only able to produce infertile eggs (82). Eggs produced *in vitro* were about half the size of normal eggs, had a thin, poorly formed shell and a blunt lateral spine. In addition, the sexual organs of the *in vitro* pairs were relatively poorly developed. It was hypothesized that worms growing entirely *in vitro* did not obtain a required stimulus that the mammalian host provided (83). Other attempts at maintaining the lifecycle from cercariae through miracidia *in vitro* have thus far been fruitless.

Using previous studies as a model, I designed an *in vitro* co-culture system that allowed our lab to assay the effect of the addition of host cell populations and/or factors to the culture system with or without physical contact

with the developing parasites (Fig. 2). Briefly, parasites were isolated from mice 21 dpi (at the start of the normal period of rapid growth) via perfusion through the hepatic portal vein. They were subsequently washed and transferred to the upper chamber of a transwell culture dish, which is separated from the bottom by a 0.4 μm filter. Host liver lymphocytes isolated from infected wild-type mice were placed in the well below and both were immersed in culture media. Mouse red blood cells (RBC) were added to the upper chamber with the parasites as an additional food source. In this way, both parasite excretory/secretory antigens and host factors secreted by lymphocytes were able to diffuse through the filter. Culture media could be saved for future analysis of secreted proteins. Development was assayed by hematin production and by measuring the worms at the endpoint of culture experiments. Hematin is a waste product produced by metabolism of RBC hemoglobin, and therefore its quantification reflects the digestive capacity and metabolic rate of developing worms. It is insoluble and visible to the eye in culture media, but it can be dissolved in sodium hydroxide and its optical density can be measured using a spectrophotometer (84). Here we discuss initial observations and analysis of methods used to determine development with thoughts on how to improve this system in the future.

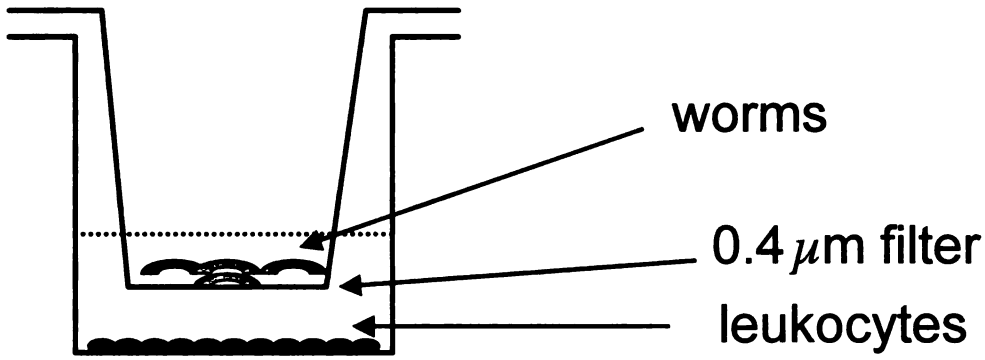


Figure 2. *Diagram of in vitro co-culture assay.* Use of a transwell filter allowed culturing of parasites and host cells in individual compartments, but in shared media. Secreted products from host cells and parasites were able to pass freely through the filter that barred the parasites from physical interaction with host cells.

Results and Discussion

Initial experiments consisted of culturing immature schistosomula (Fig. 2), isolated from either RAG-1$^{-/-}$ mice or wild-type mice with whole liver leukocyte populations from infected wild-type mice at 21, 28, 35, or 42 dpi. It had been previously established that worms from RAG-1$^{-/-}$ mice display an attenuated developmental phenotype, whereas worms mature normally in wild-type mice (52). Therefore, our aim was to determine which host factors from wild-type liver leukocytes could rescue development. Schistosomula from wild-type mice were used as a control. Cultures were terminated between 9 and 11 days after establishment. Media was changed every fourth day. Observation under a dissecting scope throughout the course of experiments revealed a qualitative assessment that schistosomes from either RAG-1$^{-/-}$ mice or wild-type mice, when cultured with liver leukocytes from any stage of infection, appeared healthier and larger than control worms that were cultured in media alone. Quantitative analysis indicated that worms cultured with leukocytes had a significant developmental advantage, but the level of effect varied. In one representative experiment, hematin production by worms isolated from RAG-1$^{-/-}$ mice cultured in media alone was lower than that produced by worms cultured with either 28, 35, or 42 dpi wild-type liver leukocytes, indicating that worms cultured in the presence of host leukocytes were more metabolically active than their controls (Fig. 3 A). In the same experiment, worms were photographed at the termination

of the experiment and measured by using NIH Image J software (http://rsb.info.nih.gov-image/). Measurements of the average area of worms from RAG-1$^{-/-}$ mice indicated accelerated growth in the presence of wild-type liver leukocytes compared to those cultured in media alone (Fig. 3 B); yet, in the same group, measurement of worm length did not illustrate such significant differences (Fig. 3 C). Due to the variations I observed in quantitative assessments, *in vitro* culture experiments were not pursued further as a means of determining individual effector molecules that might be involved in promoting parasite development.

One of the likely causes for the experimental variations I observed is the natural heteromorphic size of the parasites at the start of the culture experiments. Experiments were performed on schistosomula isolated from RAG-1$^{-/-}$ mice at 21 dpi, when most, but not all worms are uniformly small in size. Already at that stage, variability in size can be seen with the naked eye. Therefore at the termination of the experiment, it is quite difficult to quantitatively assess changes in growth in a population that is heteromorphic at the start. Another caveat is that accurate assessment of hematin production is difficult to assess. Hematin deposits on the transwell filter throughout the course of the experiment, but is difficult to physically remove from the filter at the experiment's end. Therefore any loss of hematin in the transfer from the filter to the culture tube for analysis will affect the results.

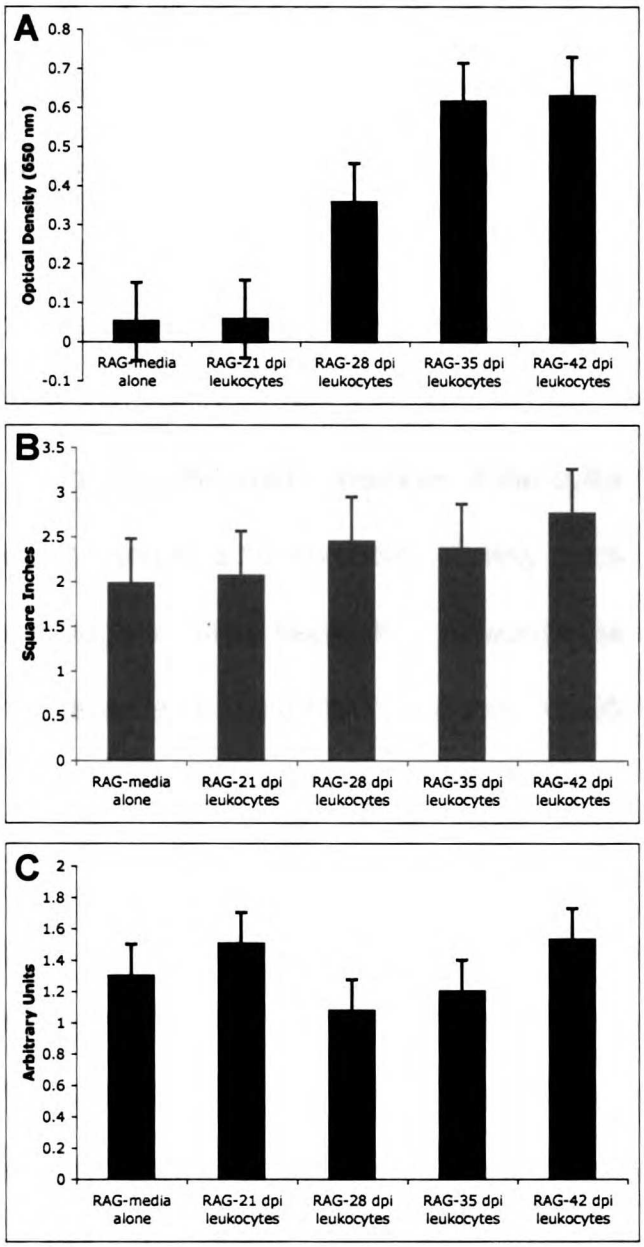


Figure 3. *Schistosome development in vitro.* Parasites isolated from RAG-1$^{-/-}$ mice were cultured in the presence of liver leukocytes from infected wild-type mice and analyzed for growth and hematin production. (A) Parasites cultured with liver leukocytes produce more hematin than those cultured in media alone. (B) Parasites cultured in the presence of liver leukocytes tend to have a larger surface area than do parasites cultured in media alone, the difference is not significant. (C) Measurement of parasite length was variable in control parasites (cultured with media alone) and parasites cultured with wild-type liver leukocytes. Black bars from left to right represent length in arbitrary pixel units of immature worms from RAG-1$^{-/-}$ mice cultured in media alone, liver leukocytes from wild-type mice 21 dpi, 28 dpi, 35 dpi, and 42 dpi. Results are representative of 2 independent experiments.

To minimize the heteromorphogeneity of the initial worm population, parasites could be isolated from hosts at an earlier stage, such as at the lung stage, where it has been shown previously that worms are mitotically inactive, quite small and similar in shape and size (80). We did not originally use worms from this stage of infection due to very low recovery of worms from the lung. Another possibility would be to separate heteromorphic worms into groups of similar size before adding to the upper chamber of the culture wells by means of a discontinuous bovine serum albumin (BSA) density gradient. A BSA gradient should be gentle enough to allow separation of worms based on size without being toxic to the parasite. For hematin analysis, careful transfer from the transwell filter is most likely the best way to optimize the assay. Hematin is composed of iron, therefore use of a magnet to concentrate hematin on the filter may help to diminish loss at the end of the experiment. Use of a magnet in hematin recovery has not been attempted in our lab. Future attempts at *in vitro* culture of parasites should take these observations into account in designing experiments. Presently, another graduate student in the lab is following up on these suggestions and optimizing the assay by exploring different readouts for worm maturation. Her preliminary experiments have validated my observation that co-culture with host leukocytes supports development and sexual maturation. She has also tentatively ruled out direct effects of TGFβ, TNF-α, IL-2, IL-7, and epidermal growth factor on worm development.

Chapter Three

$CD4^+$ T Cell Responses to Developing Worms in the Liver: Clues to Host Cell and Cytokine Requirements

Introduction

T helper (Th) cell responses to schistosomes have received considerable attention, although previous studies have focused primarily on the response to parasite eggs during patent infection. Numerous studies support the conclusion that the Th response to parasite eggs is dominated by production of type-2 cytokines (26) and is central to circumoval granuloma formation (30) and to limitation of egg-induced pathology (31). In contrast, Th responses to the parasites themselves have received relatively little attention. The limited published data indicate that, in contrast to anti-egg responses, Th responses to worms are type 1-like. Analysis of splenocytes from prepatently infected animals revealed a predominantly Th1-like response to parasites during early infection (27). Further, analysis of single-sex infections, where egg production does not occur, demonstrated that schistosome infection evoked a type-1 response in the absence of an anti-egg Th2 response (8, 26). However, there is no information on $CD4^+$ T cell responses to developing parasites in the liver, the anatomic site of parasite development, during prepatent infection. Therefore, it was of great interest to analyze liver specific $CD4^+$ T cell responses.

To gain insights into how $CD4^+$ T cells might affect the outcome of schistosome development, we analyzed intracellular cytokine production by hepatic $CD4^+$ T cells throughout the early course of infection. We determined that $CD4^+$ T cells in infected wild-type mice express proinflammatory cytokines in the

liver during prepatency, coincident with the arrival of schistosomula in the portal system and subsequent parasite growth and development at this anatomic site. To determine the role of these T cell effector molecules in parasite development, infections were examined in mice that possess specific defects in proinflammatory cytokine signaling pathways. *S. mansoni* displayed a normal developmental phenotype in all animals, with the exception of strains that possessed targeted mutations in interleukin- (IL-) 2 signaling. Early egg production, an indicator of worm maturation, was dramatically reduced in IL-2-deficient mice. A similar phenotype was observed in IL-2 receptor alpha (IL-2Rα)-deficient mice, indicating that the effect of IL-2 on the parasite is indirect. A severely attenuated developmental phenotype was observed in common gamma (γ_c) chain-deficient mice, presumably due to the loss of both IL-2 and IL-7 signaling, as we show here that parasite growth and development is also significantly impaired in IL-7 receptor-deficient mice. The effects of T cell growth factor deficiency on schistosome infection underscore the importance of T cells in determining the outcome of *S. mansoni* development.

Results

To characterize the $CD4^+$ T cell response to developing schistosomes in the liver, we examined cytokine production by hepatic $CD4^+$ T cells in prepatently-infected wild-type mice using intracellular cytokine antibody staining. Throughout the course of infection the absolute number of hepatic $CD4^+$ T cells remained constant so that changes in cytokine production by hepatic $CD4^+$ T cells could be compared accurately. To determine cytokine production, liver leukocytes, isolated from uninfected and infected mice, were stimulated with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin A *in vitro*; cell walls were then permeabilized, cells were incubated with antibodies to Th type 1 and 2 cytokines and analyzed by flow cytometry. Early schistosome infection induced expression of IFN-γ and TNF-α by hepatic $CD4^+$ T lymphocytes (Fig. 4 A, C). In contrast, schistosome infection did not induce detectable expression of IL-4, IL-5, IL-10, or IL-12 by hepatic $CD4^+$ T cells (data not shown), indicating that Th responses in the liver during prepatent infection are predominantly type 1-like, as has been reported for other organs (26, 27). In uninfected mice, approximately 8% of hepatic $CD4^+$ T cells produced IFN-γ. The number of IFN-γ-producing T cells increased coincident with the arrival of immature schistosomes in the liver at about 14 dpi (5), and reached 30% at the peak of schistosome development in the venules of the liver at 28 dpi (Fig. 4 A). The percentage of IFN-γ-producing hepatic $CD4^+$ T cells remained high until egg

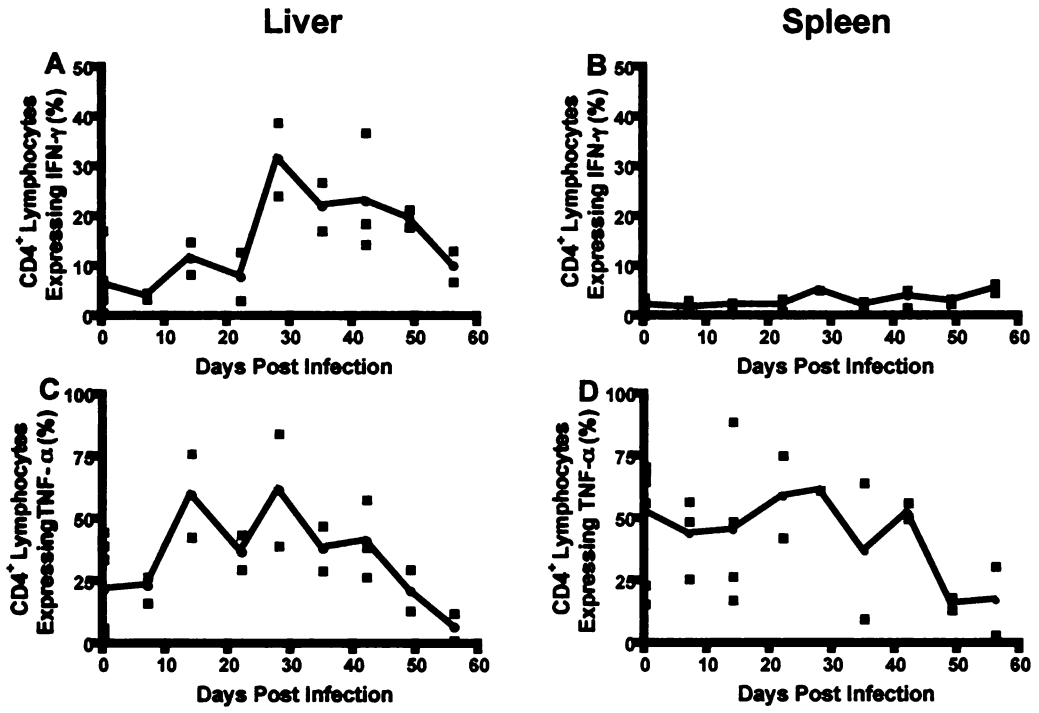


Figure 4. *Cytokine production by CD4$^+$ lymphocytes in the liver and spleen during prepatent infection of wild-type hosts.* Percentages of cytokine producing CD4$^+$ lymphocytes were determined throughout the course of infection by flow cytometric analysis. (A, B) Percentage of IFN-γ-producing CD4$^+$ lymphocytes in the liver (A) and spleen (B) throughout infection. (C, D) Percentage of TNF-α-producing CD4$^+$ lymphocytes in the liver (C) and spleen (D) throughout infection. Each data point represents the percentage of cytokine producing lymphocytes in an independent experiment. The line represents the mean of the percentages of cytokine producing cells at each time point.

production began at around 42 dpi, at which point IFN-γ-producing T cells diminished sharply (Fig. 4 A), consistent with previously published data for other organs (26, 27). In contrast, in the spleen, the percentage of IFN-γ-producing $CD4^+$ T cells remained low throughout the early course of infection, not increasing above 10% at any time point (Fig. 4 B).

The percentage of TNF-α-producing $CD4^+$ T cells was high in both liver and spleen in uninfected mice and throughout early infection (Fig. 4 C, D). The percentage of TNF-α-producing hepatic $CD4^+$ T cells in uninfected mice was 25% after *in vitro* stimulation with PMA and ionomycin, increasing to approximately 50% once immature schistosomes began arriving in the liver between 7-14 dpi (Fig. 4 C). As with hepatic IFN-γ-producing T cells, the percentage of TNF-α-producing T cells in the liver quickly diminished once egg production began at about 42 dpi (Fig. 4 C). In the spleen, TNF-α production by $CD4^+$ T cells was high in uninfected mice and during the early course of infection. Approximately 50% of splenic $CD4^+$ T cells expressed TNF-α at days 0-42 (Fig. 4 D). However, as observed in the liver, TNF-α-producing $CD4^+$ T cells rapidly diminished to below 25% once egg production began at about 42 dpi. That the hepatic T helper response to developing parasites is predominantly type 1-like was corroborated by results of stimulating hepatic leukocyte preparations *in vitro* with schistosome antigens and analyzing cytokine accumulation in culture supernatants by a cytometric bead array (data not shown) and subsequent flow cytometry. Beads in the cytometric bead array are designed to have discrete fluorescence intensities

to simultaneously detect multiple soluble proteins, such as cytokines. Bead capture of soluble cytokines in culture supernatants can then be accurately quantified by flow cytometry.

To determine whether IFN-γ or TNF-α is required for parasite development, we examined schistosome infections in TNF-$\alpha^{-/-}$ and IFN-$\gamma^{-/-}$ mice. Parasite development was assessed by analysis of (i) worm pairing, (ii) early egg production and (iii) worm length. We determined that the percentage of female worms found in pairs in TNF-$\alpha^{-/-}$ infected animals was not significantly different from that found in pairs in wild-type controls (Fig. 5 A). Analysis of egg production per worm pair at 42 dpi is a sensitive and readily quantified indicator of worm development during prepatency (52). We found that the amount of eggs produced in infected TNF-$\alpha^{-/-}$ mice was not significantly different from that produced in wild-type controls (Fig. 5 B). We also determined that worms recovered from infected mice at 42 dpi were similar in length from both TNF-$\alpha^{-/-}$ mice and their wild-type controls. Male worms isolated from both animal groups had a mean length of just over 3 mm (Fig. 5 C). Similarly, results from IFN-$\gamma^{-/-}$ mouse infections showed that the number of female worms in pairs, eggs produced during prepatency and parasite length were not significantly different from wild-type controls (data not shown).

We next examined the effects of IL-2 on schistosome development. We proposed IL-2 as a candidate for modulating parasite development because previous studies have suggested that Th1/proinflammatory responses are

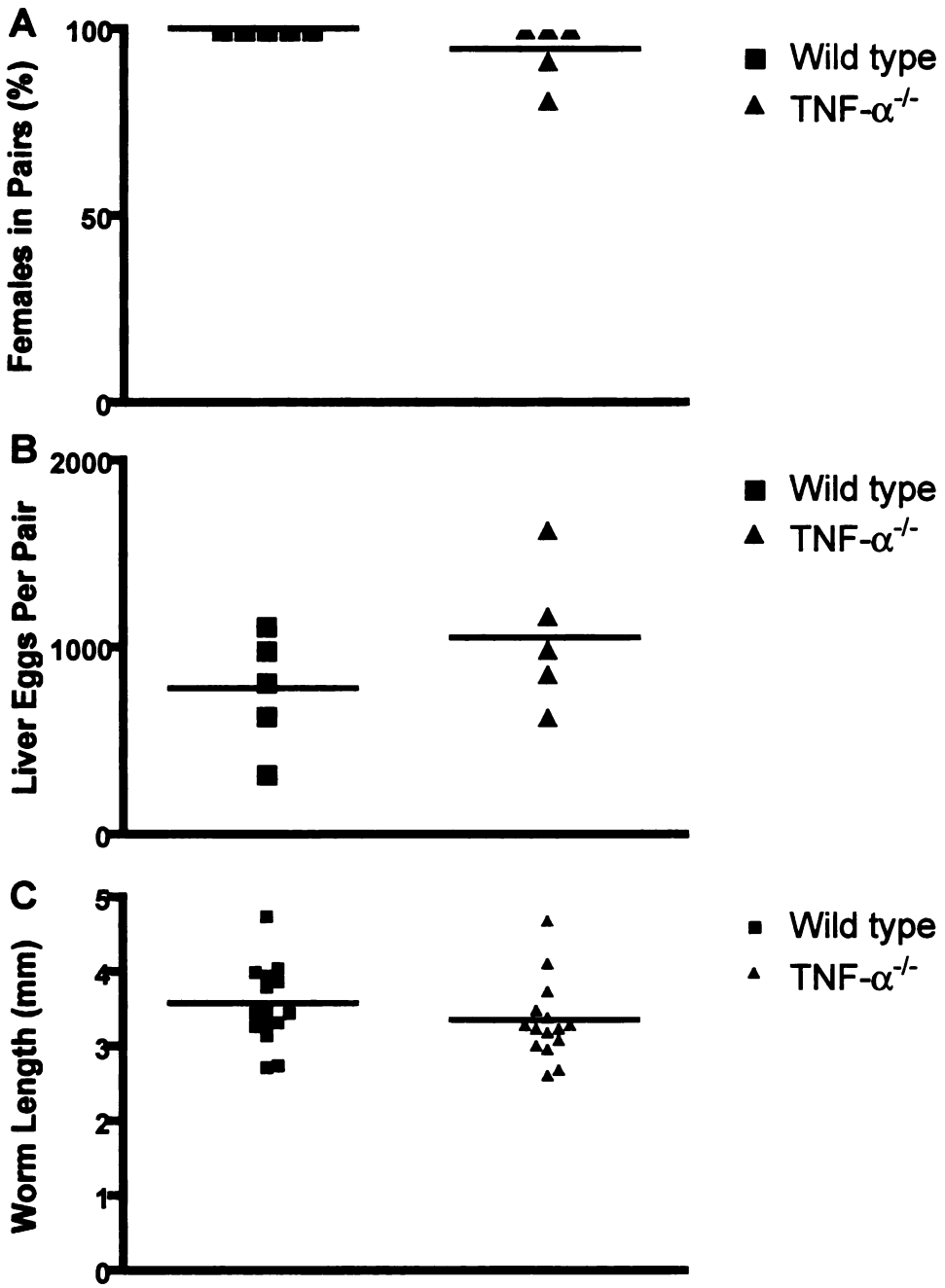


Figure 5. *Parasite development in TNF-α$^{-/-}$ mice.* Wild-type and TNF-α$^{-/-}$ mice were infected on day 0 and sacrificed 42 days later. (A) Percentage of female worms found in pairs after recovery from the portal vein. (B) Egg burdens in the liver were determined by counting eggs in a weighed liver tissue sample. Egg burden is expressed as liver eggs per worm pair. (C) Worm length of recovered parasites. Lengths of a random sample of single males were measured from digital images of fixed parasites. Results are representative of at least 2 independent experiments.

reduced in mice that have a specific defect in IL-2 signaling (85) and that IL-2 is produced during schistosome infection (86, 87). Furthermore, although not as striking as the observed increases in IFN-γ- and TNF-α-producing T cells during early infection, a modest, but consistent increase in the percentage of IL-2-producing $CD4^+$ T cells was observed in both the liver and spleen during infection (data not shown) by intracellular cytokine staining. Mean egg production by worm pairs in IL-$2^{-/-}$ mice was significantly lower than egg production in wild-type controls ($p = 0.001$); worm pairs in IL-$2^{-/-}$ mice produced only 60% of the number of eggs produced in wild-type mice (Fig. 6 B). Fig. 6 C shows a representative experiment confirming that worms recovered from IL-$2^{-/-}$ mice were small, having a mean length of just over 2.5 mm, whereas worms from wild-type mice measured almost 1.5 times longer at approximately 4 mm ($p = 0.003$). However, in one of three experiments, although the same trend was observed, a statistically significant difference in length was not observed. Over 90% of all female worms recovered were paired in both IL-$2^{-/-}$ and wild-type mice (Fig. 6 A), indicating that while fecundity was affected, there was no defect in worm pairing in IL-2-deficient animals. We infected young IL-$2^{-/-}$ mice and examined them at necropsy for signs of immunopathology (88) in various tissues, including the intestine. Experimental mice showed no signs of autoimmune disease, and we therefore conclude that this did not contribute to the altered parasite phenotype in these mice.

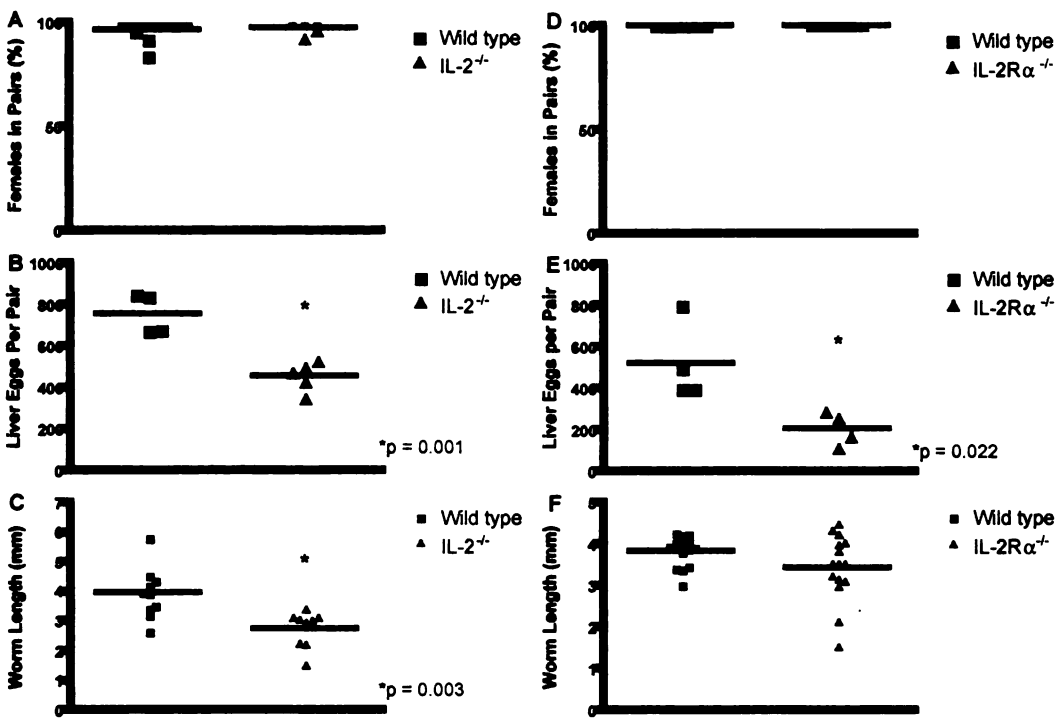


Figure 6. *Parasite development in the absence of IL-2 and IL-2 signaling.* Parasite infections and analysis of parasitological parameters were performed as described in Fig. 2. (A) Percentage of female worms found in pairs in IL-$2^{-/-}$ mice. (B) Worm pairs in IL-$2^{-/-}$ mice produce significantly fewer eggs than do worm pairs in wild-type mice. Differences between gene-deficient and wild-type groups in this experiment (and in Figs. 4-6) were analyzed by student's t test. In (B) * represents p = 0.001. (C) Worm length of recovered parasites is significantly smaller in IL-$2^{-/-}$ mice infections as compared to wild-type infections. *, p = 0.003. (D) Percentage of female worms found in pairs in IL-2R$\alpha^{-/-}$ mice. (E) Worm pairs in IL-2R$\alpha^{-/-}$ mice produce significantly fewer eggs than do worm pairs in wild-type mice. *, p = 0.022. (F) Mean worm length in both wild-type and IL-2R$\alpha^{-/-}$ mice was comparable, although the trend of measurements is smaller for worms recovered from IL-2R$\alpha^{-/-}$ mice. Results are representative of at least 2 independent experiments.

To determine whether IL-2 acted directly on the parasite or whether IL-2 signaling via its receptor was required to promote schistosome development, parasite development was examined in IL-2Rα$^{-/-}$ mice, in which IL-2 signaling is impaired. Similar to results from IL-2$^{-/-}$ mice, egg production was significantly reduced in IL-2Rα$^{-/-}$ mice compared to wild-type mice ($p = 0.022$) (Fig. 6 E). Egg production by worm pairs in IL-2Rα$^{-/-}$ mice was 39% of that produced by worm pairs in wild-type mice. Worms recovered from IL-2Rα$^{-/-}$ mice were slightly smaller than worms from wild-type controls (Fig. 6 F), but this trend was not statistically significant. Similar to IL-2$^{-/-}$ mice, worm pairing was not impaired in IL-2Rα$^{-/-}$ mice (Fig. 6 D). Autoimmune disease in IL-2Rα$^{-/-}$ mice (89) could be a possible confounding factor, therefore precautions were taken similar to those taken for IL-2$^{-/-}$ mice. No evidence of autoimmune disease was observed.

RAG-1$^{-/-}$ mice adoptively transferred with either whole spleen preparations from wild-type or IL-2$^{-/-}$ mice were infected with schistosomes to further rule out possible adverse effects that autoimmunity in the IL-2$^{-/-}$ host could have played in limiting worm development. In all three experiments performed, the amount of CD4^{+} T cells found at 42 dpi in spleens of RAG-1$^{-/-}$ mice adoptively transferred with IL-2$^{-/-}$ lymphocytes was either equal to or slightly greater than that found in RAG-1$^{-/-}$ mice adoptively transferred with wild-type cells (Fig. 7 A). Although the number of recovered IL-2$^{-/-}$ CD4^{+} T cells was similar to that of wild-type CD4^{+} T cells at 42 dpi, egg production by worms in IL-2$^{-/-}$ cell reconstituted hosts was just 33% of that produced by worms in wild-type reconstituted hosts, and was as low

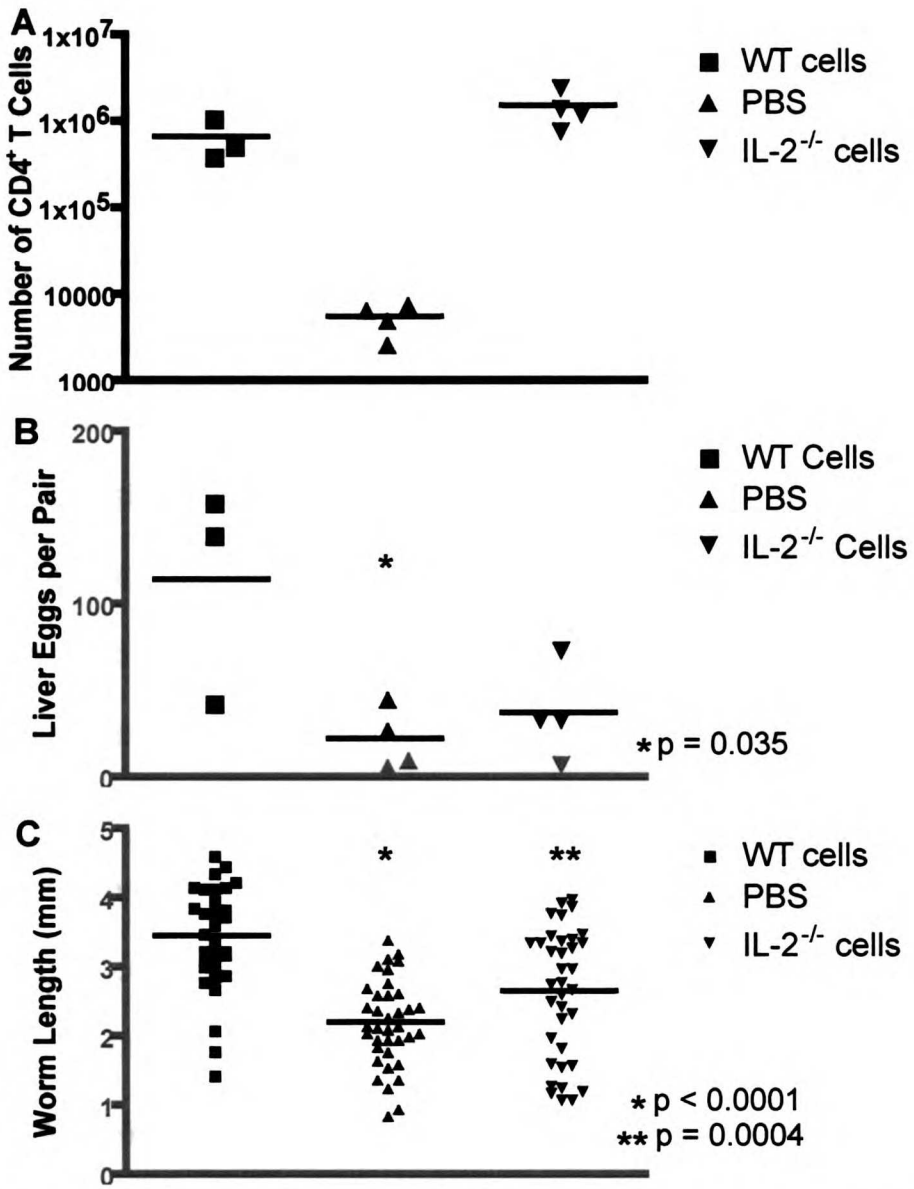


Figure 7. *Parasite development in RAG-1$^{-/-}$ mice reconstituted with IL-2$^{-/-}$ splenocytes.* IL-2$^{-/-}$ or wild-type splenocytes or PBS were intravenously injected into RAG-1$^{-/-}$ mice 1 day prior to schistosome infection. Parasite infections and analysis of parasitological parameters were then performed as described in Fig. 2. (A) Absolute numbers of CD4^{+} T cells recovered from spleens of reconstituted hosts were determined by counting total splenocytes. Splenocytes were then labeled with fluorochrome-conjugated antibodies to CD4, TCRb, and NK1.1 and analyzed by flow cytometry to determine percentage of CD4^{+} T cells per spleen. (B) Egg production by worm pairs in PBS and IL-2$^{-/-}$ cell reconstituted RAG-1$^{-/-}$ mice was similarly lower than egg production by worm pairs in wild-type cell reconstituted hosts. Egg production by worms in PBS injected hosts was significantly lower than that in wild-type cell reconstituted hosts. *, p = 0.035. (C) Worm length of recovered parasites is significantly smaller in both PBS and IL-2$^{-/-}$ cell reconstituted host infections as compared to wild-type cell reconstitution infections. *, p < 0.0001; **, p = 0.0004. Results are representative of at least 2 independent experiments.

as egg production by worms in PBS injected RAG-1$^{-/-}$ control mice (Fig. 7 B). In addition, worms isolated from IL-2$^{-/-}$ cell reconstituted RAG-1$^{-/-}$ mice at 42 dpi were more similar in length to their PBS injected RAG-1$^{-/-}$ controls, measuring an average of 2.66 mm and 2.2 mm, respectively, as compared to worms isolated from wild-type reconstituted mice which measured an average 3.44 mm (Fig. 7 C).

Since IL-2 signaling is not entirely abrogated in IL-2R$\alpha^{-/-}$ mice due to the presence of an intermediate affinity receptor comprised of the IL-2Rβ and γ_c chains, we infected $\gamma_c^{-/-}$ mice to examine the effect of a complete absence of IL-2 signaling on schistosome development. A profoundly altered developmental phenotype was observed in parasites isolated from $\gamma_c^{-/-}$ mice. The percentage of female worms in pairs was significantly reduced in $\gamma_c^{-/-}$ mice (43%) as compared to their wild-type controls (100%) ($p = 0.027$) (Fig. 8 A). Egg production in $\gamma_c^{-/-}$ mice was drastically reduced, producing approximately 5% the amount of eggs produced in wild-type mice ($p = 0.002$) (Fig. 8 B). Furthermore, a representative experiment shows that worms recovered at 42dpi from $\gamma_c^{-/-}$ hosts were significantly smaller, with a mean length of 1.9 mm, than those recovered from wild-type controls that had a mean length of 3.6 mm. ($p < 0.0001$) (Fig. 8 C-E).

Common gamma chain is a component of the receptors for IL-4 (90, 91), IL-7 (92), IL-9 (93, 94), and IL-15 (95). There is no evidence to suggest that schistosome development is impaired in IL-4- (31, 96), IL-9-, or IL-15-deficient (unpublished observations) hosts. Yet it has previously been shown that parasite

development is impaired in IL-7$^{-/-}$ mice (50), and it was proposed that IL-7 may act directly on the parasite to stimulate its development (51). To determine whether loss of IL-7 signaling contributed to the altered worm development observed in $\gamma_c^{-/-}$ mice and to discriminate between the possible direct and indirect effects of IL-7 on the developing worm, we examined infections in IL-7R$^{-/-}$ mice. Again, a profoundly altered developmental phenotype was observed in IL-7R$^{-/-}$ mice similar to that seen in $\gamma_c^{-/-}$ mice. The percentage of females in pairs (42%) in IL-7R$^{-/-}$ mice was significantly less than the percentage of females in pairs in wild-type mice (97%) ($p < 0.0001$) (Fig. 9 A). Egg production was drastically reduced in IL-7R$^{-/-}$ mice compared to wild-type mice; worm pairs from IL-7R$^{-/-}$ mice produced approximately 2% of eggs that worms in wild-type mice produced ($p < 0.0001$) (Fig. 9 B). Further, worms recovered from IL-7R$^{-/-}$ mice were significantly smaller than worms recovered from wild-type controls (Fig. 9 C-E). Fig. 9 C shows a representative experiment in which worms recovered from IL-7R$^{-/-}$ mice had a mean length of 2.2 mm, whereas worms recovered from wild-type mice had a mean length of 3.6 mm ($p < 0.0001$).

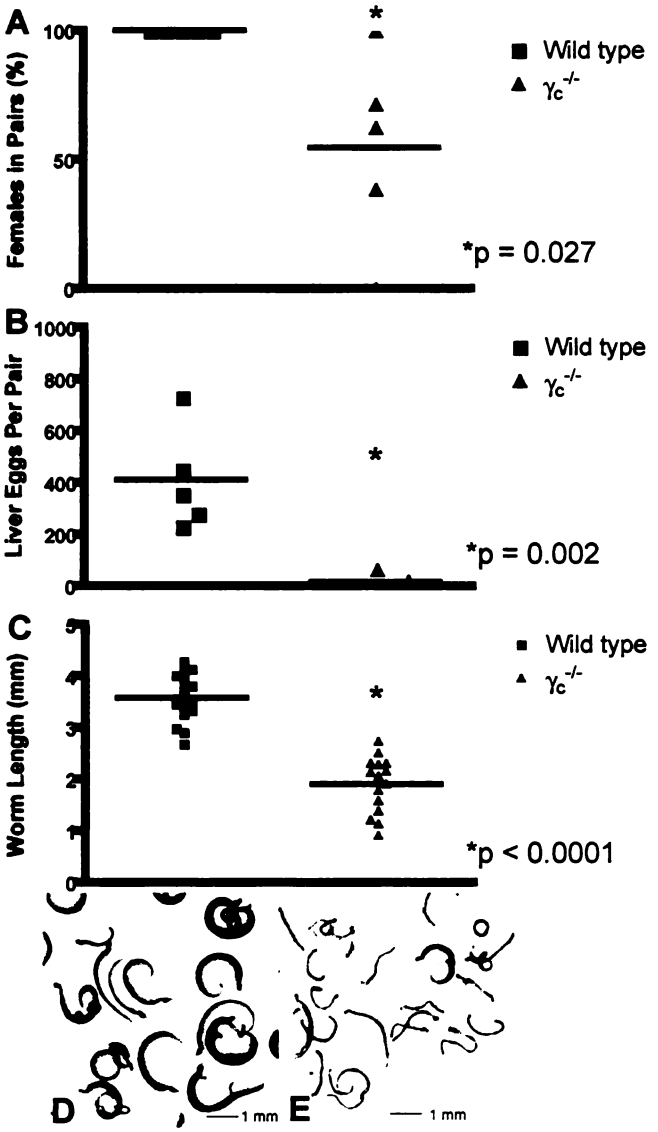


Figure 8. *Parasite development in the absence of* γ_C. Parasite infections and analysis of parasitological parameters were performed as described in Fig. 2. (A) Percentage of female worms found in pairs is significantly lower in $\gamma_C^{-/-}$ mice compared to wild-type mice. *, p = 0.027. (B) Worm pairs in $\gamma_C^{-/-}$ mice produce significantly fewer eggs than do worm pairs in wild-type mice. *, p = 0.002. (C) Worm length of recovered parasites is severely attenuated in $\gamma_C^{-/-}$ mice as compared to wild-type mice. *, p < 0.0001. (D, E) Micrographs of fixed parasites perfused from wild-type (D) or $\gamma_C^{-/-}$ (E) mice at 42 dpi. Results are representative of at least 2 independent experiments.

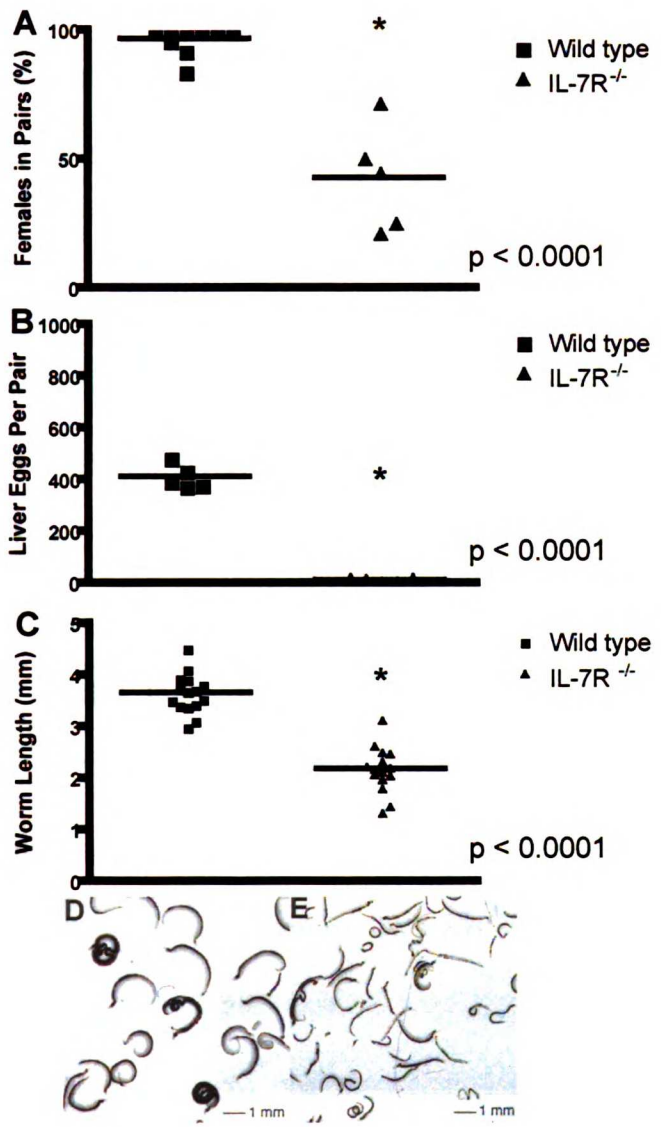


Figure 9. *Severely attenuated parasite development in IL-7Rα$^{-/-}$ mice*. Parasite infections and analysis of parasitological parameters were performed as described in Fig. 2. (A) Percentage of female worms found in pairs is significantly lower in IL-7Rα$^{-/-}$ mice than in wild-type mice. *, $p < 0.0001$. (B) Worm pairs in IL-7Rα$^{-/-}$ mice produce significantly fewer eggs than do worm pairs in wild-type mice. *, $p < 0.0001$. (C) Worm length of recovered parasites is severely attenuated in IL-7Rα$^{-/-}$ mice as compared to wild-type mice. *, $p < 0.0001$. (D, E) Micrographs of fixed parasites perfused from wild-type (D) or IL-7Rα$^{-/-}$ (E) mice at 42 dpi. Results are representative of at least 2 independent experiments.

Discussion

We have previously shown that signals from the host's adaptive immune system are essential for normal schistosome development and that $CD4^+$ $\alpha\beta$ T cells play a central role in supplying the required host signals (52). To gain insights into the molecular identity of the signals that affect parasite development, it is essential to characterize $CD4^+$ T cell responses to developing parasites where they reside in the host. Whereas previous studies have analyzed splenic immune responses to early schistosome infection as an indication of a systemic immune response during prepatency (26, 27), we focused our analysis on immune responses in the liver, the anatomic site of parasite growth and maturation. We observed an increase in IFN-γ-producing $CD4^+$ T cells in the liver coincident with the arrival of immature schistosomula in the portal system. This Th1 response was sustained throughout prepatent infection. However, the percentage of IFN-γ-producing cells quickly decreased once egg production began (Fig. 4 A), consistent with well-documented observations that egg production elicits an anti-inflammatory Th2 response (26, 30, 31). In addition, the percentage of inflammatory TNF-α-producing $CD4^+$ T cells was relatively high both in the liver and spleen during early infection (Fig. 4 C, D) but, like IFN-γ, was downregulated once egg production began. Taken together, our results indicate that the Th1-like inflammatory response that we and others have observed extends to the liver and is more profound at this site than in the spleen.

Whereas TNF-α and IFN-γ are prominent components of the hepatic Th response to developing parasites, *S. mansoni* developed normally in IFN-$\gamma^{-/-}$ and TNF-$\alpha^{-/-}$ mice (Fig. 5 and data not shown), indicating that TNF-α and IFN-γ do not play a role in development of the parasite. Previous studies have shown that TNF-α is required for egg laying (48), but our data suggest that although this cytokine may be involved in modulating parasite fecundity later in infection, it is not required for normal parasite development. However, we cannot exclude the possibility that TNF-α and IFN-γmay play a redundant role in inducing development of schistosomula or that another factor may be upregulated in IFN-$\gamma^{-/-}$ and TNF-$\alpha^{-/-}$ mice.

We also examined the role of IL-2 in promoting schistosome development because this cytokine was elevated in the spleen and liver during infection, and because it plays an important role in the proliferation and function of Th1 cells. Significant alterations in worm development were observed upon infection of IL-$2^{-/-}$ mice. Specifically, egg production per worm pair early in infection of IL-$2^{-/-}$ mice was significantly lower than in control mice (Fig. 6 B) and in most experiments worms recovered from IL-$2^{-/-}$ mice at 42 dpi were significantly smaller than worms from control mice (Fig. 6 C). The observed impaired developmental phenotype was not due to a diminution in the population of T cells present in the livers of IL-$2^{-/-}$ mice because flow cytometric analysis of spleens and livers from IL-$2^{-/-}$ mice revealed just as many, if not more $CD4^+$ $TCR\beta^+$ T cells in both organs compared to wild-type mice (data not shown).

Two possible conclusions can be drawn from these results; (i) that IL-2 acts directly on the parasite or (ii) that IL-2 promotes schistosome development indirectly via signaling through the host IL-2 receptor. To discriminate between these possibilities, we analyzed worm development in mice that do not express IL-2Rα because these animals are able to produce IL-2, but are impaired in their ability to respond to it. The developmental phenotype of schistosomes in IL-2Rα$^{-/-}$ mice was similar to that observed in IL-2$^{-/-}$ mice with the exception that although worms recovered from IL-2Rα$^{-/-}$ mice were smaller than those from wild-type controls, the difference in length was not significant. Taken together, these data indicate that IL-2 signaling via its receptor is required to promote worm development presumably because of its essential role in Th1 cell function. To prevent spontaneously occurring autoimmune pathology from interfering with our experiments, we infected young mice and examined them at necropsy for signs of immunopathology (88, 89). Mice used in experimental infections showed no signs of autoimmune disease and we conclude that this did not contribute to the altered parasite phenotype in IL-2$^{-/-}$ and IL-2Rα$^{-/-}$ mice.

We endeavored to determine whether worm development could be rescued in a mouse whose CD4^{+} T cells could not make IL-2, yet could respond to it through their intact IL-2 receptor. To do this, we transferred IL-2$^{-/-}$ splenocytes into RAG-1$^{-/-}$ hosts prior to infection. In this model, innate immune cells, such as dendritic cells (97, 98), from the RAG-1$^{-/-}$ host could produce the proliferative cytokine IL-2 that in turn could act on IL-2$^{-/-}$ CD4^{+} T cells. In fact,

schistosome development is impaired in this model as well, indicating that IL-2 may act in an autocrine manner in $CD4^+$ T cells to promote parasite development.

Interestingly, the parasite developmental phenotype in IL-2R$\alpha^{-/-}$ mice was not as profound as that in IL-$2^{-/-}$ mice. This might be explained by the fact that the β and γ_c chains of the IL-2 receptor complex are still able to function as a low affinity receptor through which residual IL-2 signaling may occur (99). To examine the effect of a complete block in IL-2 signaling on parasite development, we examined parasite development in γ_c-deficient mice. A severely impaired developmental phenotype was observed in $\gamma_c^{-/-}$ mice which was even more severe than that seen in either IL-$2^{-/-}$ or IL2R$\alpha^{-/-}$ mouse infections. Further, the percentage of females found in pairs, egg production per worm pair, and worms recovered from $\gamma_c^{-/-}$ mice were reminiscent of the altered parasite phenotype observed in RAG-$1^{-/-}$ mice (52). Yet, the severely impaired developmental phenotype may also be due to a T cell deficiency that is characteristic of $\gamma_c^{-/-}$ mice (100).

Because γ_c is a component of the receptors for IL-4, IL-7, IL-9, and IL-15, it is possible that the severe parasite developmental phenotype observed in $\gamma_c^{-/-}$ mice is due to a combined loss of signaling by other cytokines. There is currently no evidence to suggest that schistosome development is altered in IL-$4^{-/-}$ (31, 96), IL-$9^{-/-}$, or IL-$15^{-/-}$ mice (unpublished observations). However, it has previously been shown that schistosome development is impaired in IL-$7^{-/-}$ mice (50) and

that IL-7 may act directly on the parasite to increase parasite responsiveness to host endocrine factors involved in stimulating worm development (51). Because IL-7 plays a critical role in lymphocyte development, an alternative explanation for these observations might be that parasite development is impaired in IL-$7^{-/-}$ mice due to the lymphopenic phenotype of these animals.

To determine whether loss of IL-7 signaling contributes to the parasite developmental phenotype observed in $\gamma_c^{-/-}$ mice and to discriminate between the possible direct and indirect effects of IL-7 on parasite development, we examined schistosome infections in IL-$7R^{-/-}$ mice. Infections of IL-$7R^{-/-}$ mice produced a profound developmental impairment similar to that observed in $\gamma_c^{-/-}$ mice. Like infections in $\gamma_c^{-/-}$ mice, the percentage of females in pairs (Fig. 9 A), the amount of eggs produced per pair (Fig. 9 B), and worm length (Fig. 9 C) was significantly reduced compared to wild-type controls. Because IL-$7R^{-/-}$ mice can synthesize IL-7 but are unable to respond to it, our data indicate that it is host IL-7 signaling and not IL-7 acting directly upon the worm that promotes worm growth and development. From these data, we conclude that the impairment of parasite development previously reported in IL-$7^{-/-}$ mice (50) is likely due to the lymphopenic phenotype of these animals and the resulting deficit in $CD4^+$ T cells, which we have shown to be critical in allowing parasite development to proceed normally (52).

Recent studies have determined that Bim, a proapoptotic Bcl-2 family member, plays a role in apoptosis of immature and mature T cells in IL-7- and

IL7R-deficient mice. Generation of doubly-deficient $Bim^{-/-}$/IL-$7R\alpha^{-/-}$ mice restored mature T cells to near normal numbers (101). To discriminate between the possibility that the lack of T cells in IL-$7^{-/-}$ and IL-$7R\alpha^{-/-}$ mice is clouding our assessment that IL-7 signaling specifically induces attenuated development in schistosomes, further analysis of development in $Bim^{-/-}$/IL-$7R\alpha^{-/-}$ mice should be pursued. Analysis of schistosome development in mice that lack IL-7 signaling, but retain near normal numbers of mature T cells, will allow us to determine if the absence of IL-7 signaling has a real effect on schistosome development.

The biological significance of developmental responses in schistosomes to host T cell factors remains unclear. In other helminth parasites alternative developmental programs function to increase the likelihood of transmission and life cycle propagation in the face of adverse conditions (54). For *S. mansoni*, two aspects of the immune response may be relevant in development and propagation. First, $CD4^+$ T cells are essential for granuloma formation, which in turn is essential for host survival and the establishment of a chronic infection (31). In an immunodeficient host, granuloma formation would be impaired, leading to decreased host survival and potential termination of schistosome transmission. Second, schistosomes may live for over a decade in the mammalian host so an alternative developmental program might afford an opportunity for worms to "hibernate" when a host is compromised by malnutrition until optimal host conditions return. The developmental response of *S. mansoni* that we have described may, therefore, serve to increase the likelihood of

parasite survival (54) and transmission in an immunocompromised host or a host compromised by malnutrition or other concurrent infections.

Chapter Four

Characterization of $CD4^+$ $\alpha\beta$ T cell populations in $\beta 2\text{-}m^{-/-}$ /MHC $II^{-/-}$ mice

Introduction

In the absence of normal $CD4^+$ T helper and $CD8^+$ cytotoxic T cells, a unique population of $CD4^+$ $\alpha\beta$ T cells, found in $\beta 2\text{-}m^{-/-}$/MHC $II^{-/-}$ mice and not restricted by either MHC class I or MHC class II, are able to promote worm development (52). Studying their development, function, and secreted products would help to elucidate the minimal requirements for supporting parasite development.

$\beta 2\text{-}m^{-/-}$/MHC $II^{-/-}$ mice were generated by Grusby and colleagues (53). These mice do not maintain normal surface expression of either MHC class I or MHC class II. Immature $CD4^+$ and $CD8^+$ T cells express unique TCRs that bind to MHC molecules expressed on thymic stromal epithelium. T cells can mature and exit the thymus only if they can sufficiently recognize MHC molecules. Therefore, lack of expression of MHC molecules leads to a deficiency in mature T cells in the periphery. Yet these mice appear healthy when housed under sterile conditions, even though they are immunocompromised due to T cell deficiency. Upon closer inspection we detected a $CD4^+$ $\alpha\beta$ T cell population in these mice lacking expression of MHC class I and MHC class II. Its presence allowed parasite development to proceed. We characterized this cell population in various compartments in the host and provide insight into its probable source of development.

Results

There is a discrete CD4$^+$ $\alpha\beta$ T cell population in the livers of β2-m$^{-/-}$/MHC II$^{-/-}$ mice that is larger than that observed in the spleens of these animals (52). Other organs have not heretofore been analyzed for the presence or absence of this unique T lymphocyte population. Therefore, we were interested in determining the origin and tissue distribution of this unusual T cell population. By isolating various compartments of gut associated lymphoid tissue (GALT) in uninfected animals and subsequently staining with antibodies to CD4, TCRβ, and NK1.1, we determined that CD4$^+$ $\alpha\beta$ T cells were also present in lamina propria lymphocytes (LPL), Peyer's patches (PP), intraepithelial lymphocytes (IEL), and mesenteric lymph nodes (MLN). We determined that β2-m$^{-/-}$/MHC II$^{-/-}$ CD4$^+$ $\alpha\beta$ T cells were most prevalent in GALT, comprising 18% of LPL, 10% of IEL, 5% of PP, 5% of MLN and 10% of liver lymphocytes (Fig. 10). It is noteworthy that immature worms pass first through the vasculature of the intestine in order to reach the liver and initiate development. Therefore, these gut-associated cells may have been exposed to parasite antigens prior to schistosome residence in the portal venous system. Additionally, MLN is the draining lymph node of the liver and intestine; therefore, parasite antigens will be transported there during early infection.

CD4$^+$ $\alpha\beta$ T cells found in the GALT and the liver are phenotypically similar in their cell surface marker expression, suggesting that they are the same

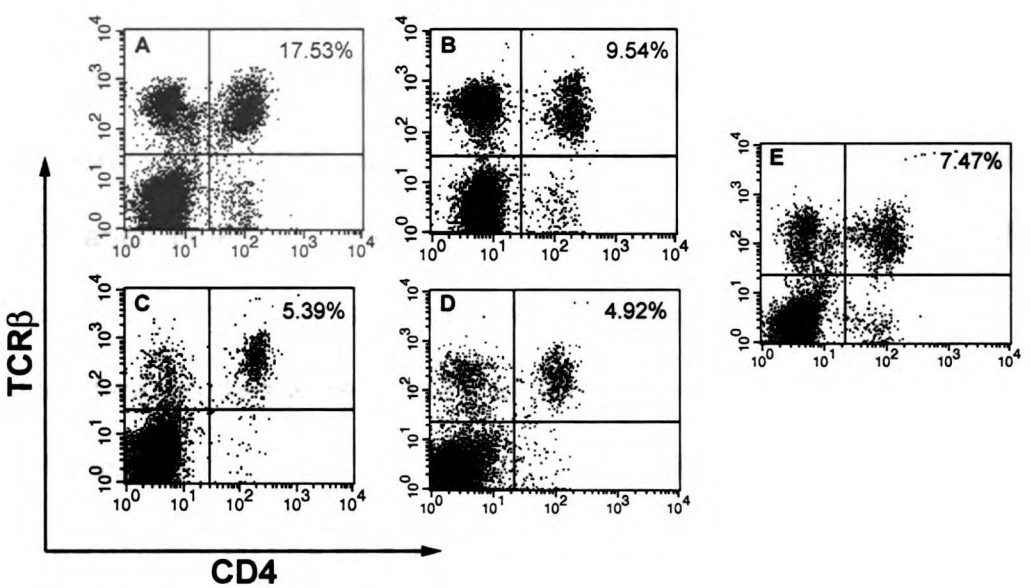


Figure 10. *$CD4^+$ αβ T cells in GALT of uninfected β2-$m^{-/-}$/MHC $II^{-/-}$ mice.* GALT and liver lymphocytes were stained with anti-CD4 and anti-TCRβ antibodies and analyzed by flow cytometry to determine percentage $CD4^+$ αβ T cells in each compartment. (A) Lamina propria lymphocytes. (B) Intraepithelial lymphocytes. (C) Peyer's patches. (D) Mesenteric lymph node. (E) Liver lymphocytes. Results are representative of at least 2 independent experiments.

population of cells and/or arise from the same precursors. Surface expression of molecular markers was analyzed by antibody staining and subsequent flow cytometric analysis. We determined that $CD4^+$ $\alpha\beta$ T cells from any compartment in uninfected $\beta2\text{-}m^{-/-}$/MHC $II^{-/-}$ animals expressed moderate levels of CD69, little to no CD62L and moderate levels of CD44. These $CD4^+$ $\alpha\beta$ T cells are not likely to be T regulatory (T reg) cells because they do not express CD25, a marker of T regulatory cells in uninfected mice. Nor did they express NK1.1, a cell surface marker expressed on NK and NK T cells in C57BL/6 mice (Table 1).

We also investigated where these cells may arise and mature. Due to their lack of expression of MHC class I and class II, it was hypothesized that they either escaped deletion in the thymus or arose extrathymically. Extrathymic development of T cells has been observed in animals that lack a thymus (102, 103); therefore, studies were initiated to determine whether the observed $CD4^+$ $\alpha\beta$ T cell population in $\beta2\text{-}m^{-/-}$/MHC $II^{-/-}$ mice were similarly of extrathymic origin. Initially, we focused on LPL as a source of extrathymic lymphopoeisis because of the abundance of $CD4^+$ $\alpha\beta$ T cells found there. We found no evidence for extrathymic lymphopoeisis in the LPL, but initial analysis suggested MLN could be a source due to the observation of triple negative cells that also expressed CD25 in the MLN (Fig. 11). Following a similar antibody staining protocol to Guy-Grand *et al.* (103), and subsequent flow cytometric analysis, we observed lymphocyte populations in the thymi of both wild-type and $\beta2\text{-}m^{-/-}$/MHC $II^{-/-}$ animals that were $CD3^-$ $CD4^-$ $CD8^-$ (triple-negative (TN)), $CD19^-$ $CD25^H$ $Thy\text{-}1.1^H$

	LPL	IEL	PP	MLN	LIVER
NK1.1	-	-	-	-	-
CD25	-	-	-	-	-
CD44	+	++	++	+	+/++
CD62L	-	-	-	+	-
CD69	+	+ one expt - one expt	+	+	+

Table 1. Extracellular lymphocyte marker expression in GALT of uninfected $\beta 2\text{-}m^{-/-}$/MHC $II^{-/-}$ mice.

corresponding to pro-pre T cells. We also observed a population of pro-pre T cells in the MLN of β2-m$^{-/-}$/MHC II$^{-/-}$ mice that was significantly larger than that observed in wild-type mice ($p = 0.0374$), indicating the MLN as a source of extrathymic lymphopoeisis in MHC-deficient mice (Fig. 11 A). Analysis of these pro-pre T cells (immature T cells at the double-negative (DN) DN2 (TN and CD25^{H} CD44^{+}) stage and DN3 (TN and CD25^{H} CD44^{-}) stage of thymic development), suggested that precursor T cells could develop in both the thymus and MLN of β2-m$^{-/-}$/MHC II$^{-/-}$ mice as compared to only thymic development in wild-type hosts. One caveat between our analysis and that of Guy-Grand *et al.* was that Guy-Grand *et al.* were able to further differentiate triple-negative cells by analyzing expression of the fusion RAG-green fluorescent protein gene that had been engineered in their experimental mice. Although we observed cells in MLN of β2-m$^{-/-}$/MHC II$^{-/-}$ mice that appeared to correspond to pro-pre T cells, RAG gene expression in these cells was not analyzed. Additionally, analysis of lymphocytes at the double-positive stage of T cell development, when precursor T cells express both CD4 and CD8 on their surface, revealed that lymphocytes express both CD4 and CD8 in the thymus but not in the MLN (Fig. 11 B) of either wild-type or β2-m$^{-/-}$/MHC II$^{-/-}$ mice, suggesting that development of CD4^{+} T cells occurs primarily in the thymus, even though classically, T cells should be deleted due to the absence of MHC class I and class II molecules.

We next determined whether the CD4^{+} αβ T cells found in β2-m$^{-/-}$/MHC II$^{-/-}$ mice were functional, as determined by their ability to produce effector cytokines.

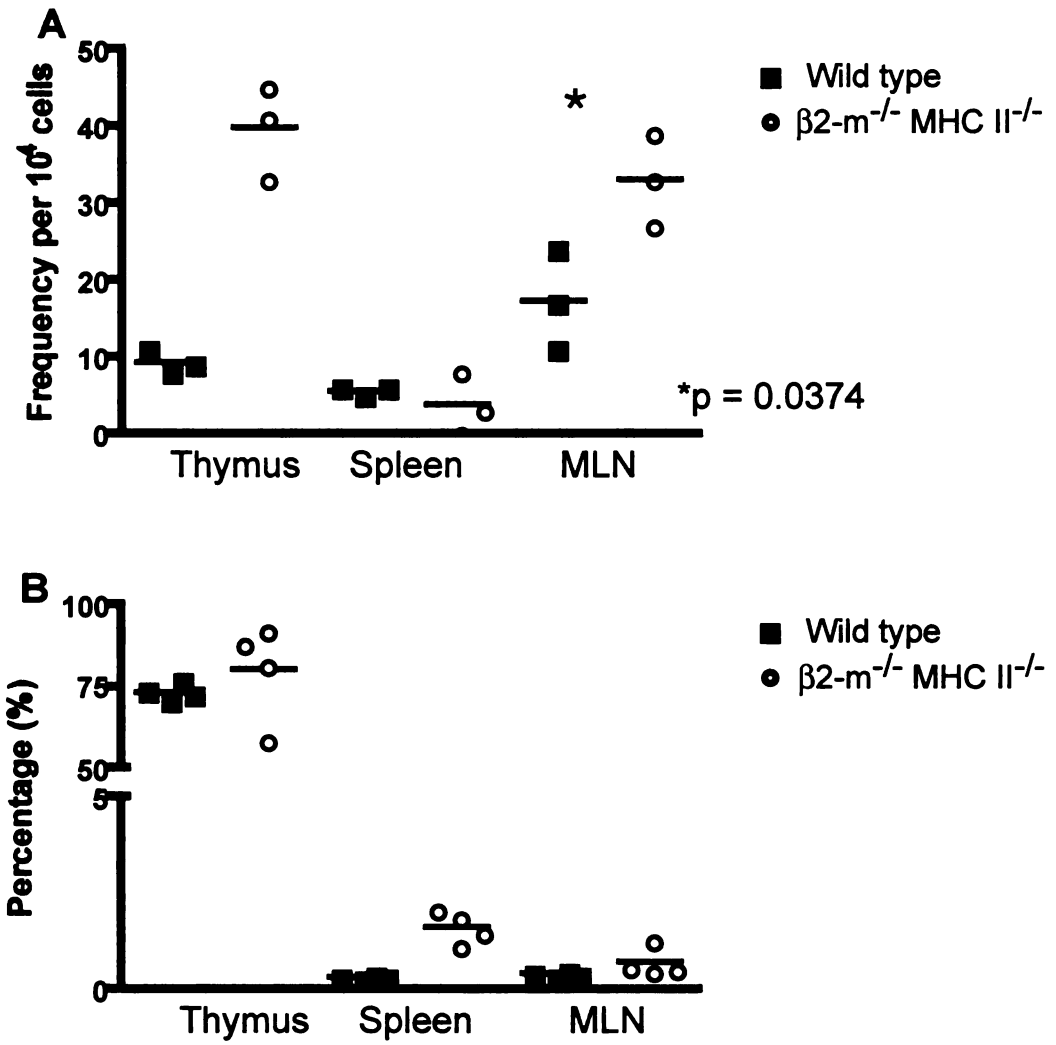


Figure 11. *Mesenteric lymph node is not a site of extrathymic lymphopoeisis in β2-$m^{-/-}$/MHC $II^{-/-}$ mice.* (A) Frequency of pro-pre-T (triple negative) cells in individual β2-$m^{-/-}$/MHC $II^{-/-}$ (open circles) and wild-type (filled squares) mice. Lymphocytes were gated on the $CD3^-$, $CD4^-$, $CD8\alpha^-$, $CD19^-$ population and $CD25^+$ Thy 1.2^+ were considered to be pro-pre-T cells. There are significantly more pro-pre-T cells in MLN of β2-$m^{-/-}$/MHC $II^{-/-}$ than wild-type mice. *, $p = 0.0374$. (B) Percentage of double-positive (DP) T cells in individual β2-$m^{-/-}$/MHC $II^{-/-}$ (open circles) and wild-type (filled squares) mice. Double positive T cells are defined here as $TCR\beta^+$ staining lymphocytes that positively stain for both CD4 and CD8. Results are representative of 2 independent experiments.

To this end, we stimulated $CD4^+$ αβ T cells, isolated by flow cytometric cell sorting from β2-$m^{-/-}$/MHC $II^{-/-}$ mice, with either PMA and ionomycin or anti-CD3 and anti-CD28 antibodies *in vitro* and analyzed the culture supernatants for the presence of cytokines after 72 hours by using a cytometric bead array. We determined that these lymphocytes were able to produce a full range of type 1 cytokines, including TNF-α and IFN-γ, and type 2 cytokines, including IL-2, IL-4, and IL-5 (Fig. 12). The concentrations of cytokine proteins, found in the supernatants of $CD4^+$ αβ T cells from β2-$m^{-/-}$/MHC $II^{-/-}$ mice that were stimulated with PMA and Ionomycin, were comparable to that of stimulated splenocytes from wild-type mice (data not shown). Analyses were not performed on splenocytes from wild-type mice that had been stimulated with anti-CD3 and anti-CD28 antibodies. Additionally, flow cytometric analysis of intracellular cytokine staining of $CD4^+$ αβ T cells in uninfected and 22 dpi β2-$m^{-/-}$/MHC $II^{-/-}$ livers showed a similar rise in TNF-α, IFN-γ, and IL-2 production, as was observed in wild-type infections (Fig. 13 and Fig. 4). The percentage of IFN-γ–producing $CD4^+$ αβ T cells increased almost two-fold by 22 dpi (Fig. 13 B, E), and TNF-α- and IL-2-producing T cells increased over 8- fold by 22 dpi (Fig. 13 C-G).

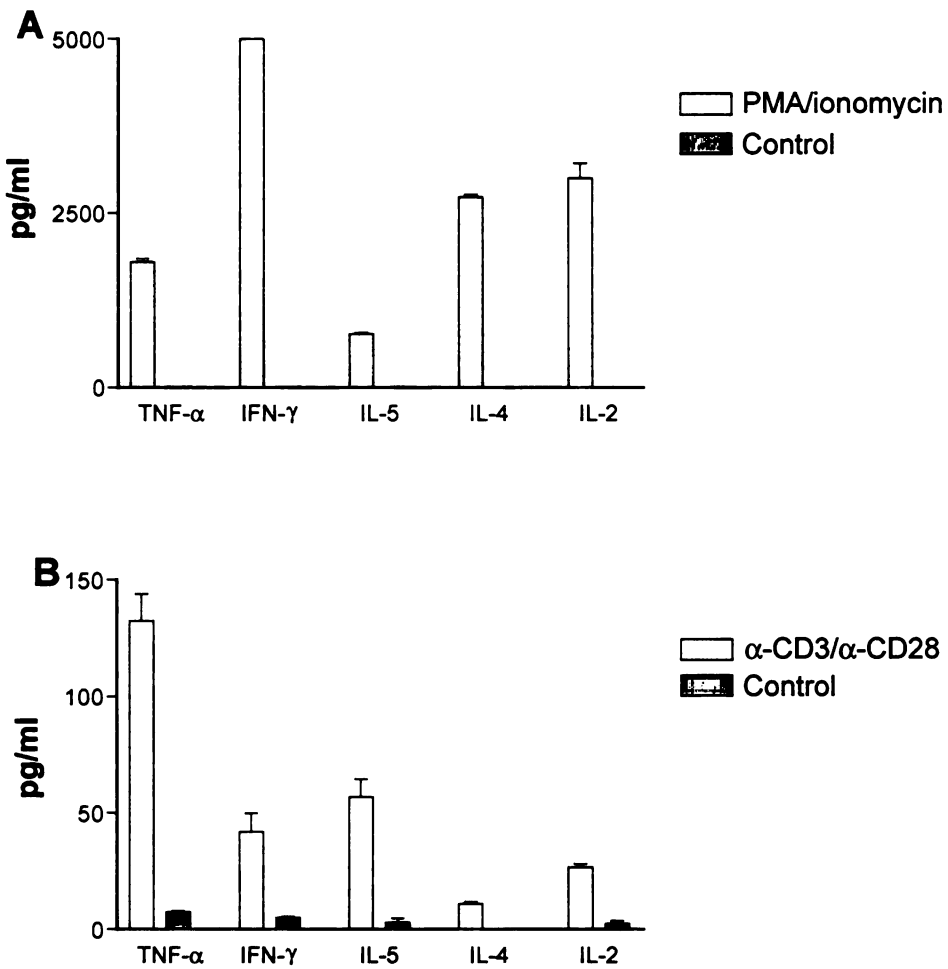


Figure 12. *Cytokine production by β2-m$^{-/-}$/MHC II$^{-/-}$ CD4^{+} αβ T cells stimulated in vitro.* CD4^{+} αβ T cells were sorted from uninfected β2-m$^{-/-}$/MHC II$^{-/-}$ mice and stimulated by PMA and Ionomycin in (A) and anti-CD3 and anti-CD28 antibodies in (B). Vertical bars indicate amount of cytokine found in the supernatant after 72 hours. Results are representative of at least 2 independent experiments.

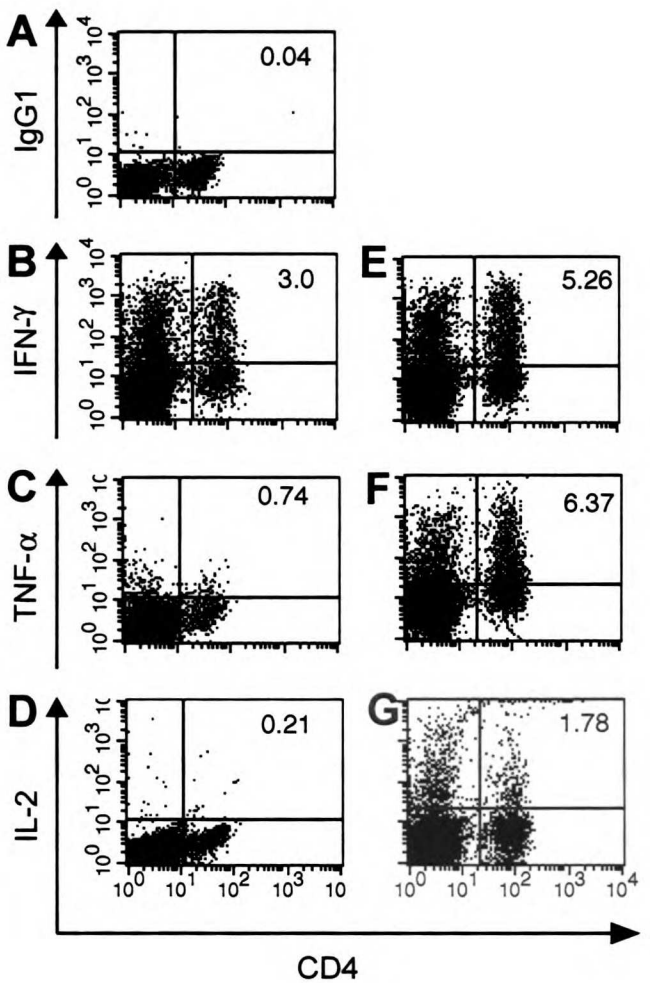


Figure 13. *Cytokine production by CD4$^+$ lymphocytes in the liver during prepatent infection of β2-m$^{-/-}$/MHC II$^{-/-}$ mice.* Percentages of cytokine producing CD4$^+$ lymphocytes from livers of β2-m$^{-/-}$/MHC II$^{-/-}$ mice were determined at 0 dpi (B-D) and 22 dpi (E-G) by flow cytometric analysis. (A) Percentage of lymphocytes that bound IgG1 isotype antibody control. (B, E) Percentage of IFN-γ-producing CD4$^+$ lymphocytes at 0 dpi (B) and 22 dpi (E). (C, F) Percentage of TNF-α-producing CD4$^+$ lymphocytes at 0 dpi (C) and 22 dpi (F). (D, G) Percentage of IL-2-producing CD4$^+$ lymphocytes at 0 dpi (D) and 22 dpi (G). Results are representative of at least 2 independent experiments.

Discussion

Because immature schistosomula pass through the vasculature of the gut on their way to the hepatic portal venous system and because the gut is home to a number of alternative lymphocytes such as TCR $\gamma\delta^+$ cells and CD8$\alpha\alpha$ T cells (104-111), we turned our attention towards the GALT as a source of $CD4^+$ $\alpha\beta$ T cells found in β2-$m^{-/-}$/MHC $II^{-/-}$ mice. Indeed, β2-$m^{-/-}$/MHC $II^{-/-}$ $CD4^+$ $\alpha\beta$ T cells are present in the GALT and are most abundant in the lamina propria. That these cells are found in the GALT, as well as the liver, suggests that worm antigens may activate lymphocytes in the GALT even before schistosomula reach the liver, or that the $CD4^+$ $\alpha\beta$ T cells found in the liver may originate in the GALT. Additionally, $CD4^+$ $\alpha\beta$ T cells from the various tissues analyzed displayed a similar phenotype, indicating that they were a uniform population. In uninfected β2-$m^{-/-}$/MHC $II^{-/-}$ mice, they displayed some cell surface markers that correspond to activated and/or memory populations; they were $CD44^+$ $CD69^+$ $CD62^L$. Yet they did not express CD25, which is transiently expressed by activated T cells. In that sense, they displayed a mixed activation phenotype, but due to the singularity of these cells, perhaps usual indicators of activation are not applicable in determining their activation status. In fact, others have found that IEL in germ-free β2-$m^{-/-}$ mice maintain a low activation state in the absence of antigenic stimulation and that they fail to express CD25 on their surface (112). As yet, we have not found unique markers on $CD4^+$ $\alpha\beta$ T cells in β2-$m^{-/-}$/MHC $II^{-/-}$ mice, so

we are not able to isolate and differentiate them from normal $CD4^+$ $\alpha\beta$ T cells in wild-type animals.

An important consideration was whether these unusual $CD4^+$ $\alpha\beta$ T cells in $\beta2\text{-}m^{-/-}$/MHC $II^{-/-}$ mice were of thymic origin. Under normal circumstances, $CD4^+$ and $CD8^+$ T cells are selected in the thymus by either MHC class I or MHC class II. Without stromal expression of either of these self molecules, T cells will not complete maturation and should not exit the thymus into the periphery. Therefore, the presence of $CD4^+$ $\alpha\beta$ T cells in $\beta2\text{-}m^{-/-}$/MHC $II^{-/-}$ mice suggested an extrathymic developmental pathway. Indeed, there have been numerous studies debating the presence of extrathymic lymphopoeisis, particularly in GALT of both mice (102, 104, 113-115) and humans (116). Yet, extrathymic development may occur in GALT only in athymic mice; under euthymic conditions, extrathymic development is not significantly detected (103, 117, 118). Additionally, the above analyses of extrathymic lymphopoeisis focused on the unusual IEL populations that expressed $CD8\alpha\alpha$ homodimers and either $\alpha\beta$ or $\gamma\delta$ TCR. Therefore, the previous results may have little bearing on our observed $CD4^+$ $\alpha\beta$ T cell population. We originally focused our studies on the LPL due to the high percentage of $CD4^+$ $\alpha\beta$ T cells found there, but found no indication of extrathymic lymphopoeisis. However we did observe the presence of triple-negative ($CD3^-$ $CD4^-$ $CD8^-$) $CD19^-$ and $CD25^+$ cells in MLN of $\beta2\text{-}m^{-/-}$/MHC $II^{-/-}$ mice, suggesting that the MLN may be a source of lymphopoeisis. Due to our inability to analyze RAG gene expression as in (103) some other cell types that are not considered

immature T cells may have been included in our analysis. Guy-Grand and colleagues had determined that MLN was a source of lymphopoeisis in athymic nude mice (103). Using their studies as a model, we analyzed MLN from β2-$m^{-/-}$ /MHC $II^{-/-}$ mice, but could not detect the presence of precursor T cells there. In fact, the thymus was the only compartment we analyzed that contained precursor T cells, leading us to conclude that $CD4^+$ αβ T cells can develop in the thymus and must somehow escape deletion in order to exit to the periphery. It is also possible that these cells may develop extrathymically in a compartment we have not yet analyzed.

$CD4^+$ αβ T cells from β2-$m^{-/-}$/MHC $II^{-/-}$ mice cannot recognize antigen in the context of classical MHC class I or class II protein; therefore, how they are activated remains a mystery. We have observed that they can be activated by potent general stimulators *in vitro,* such as PMA and ionomycin or anti-CD3 and anti-CD28 antibodies and that they can produce cytokines like classical effector T cells. Additionally, they can be induced to increase production of inflammatory cytokines after infection by *S. mansoni.* Their ability to be stimulated by foreign antigens is unquestioned; it remains to be determined in what molecular context that they recognize foreign antigen in order to respond.

Chapter Five

Analysis of Global Transcription Patterns during Development in Normal and Attenuated Parasites

Introduction

The striking developmental attenuation observed in immunocompromised hosts encouraged us to analyze parasite transcriptional patterns throughout development in both worms from wild-type hosts and worms from RAG-$1^{-/-}$ hosts. The advent of microarray technology for analyzing transcriptional patterns has become a very important tool. Microarrays are very useful in determining transcription patterns in organisms whose genomes have been annotated or whose expressed-sequence tag (EST) databases are rather large and well defined. There are three main types of arrays that are frequently used: arrays that reflect the genes of an organism that has been sequenced (representing all defined open reading frames (ORF) or a subset in the genome), cDNA arrays (made from RNA from an organism at a particular stage in development), and EST arrays (oligonucleotide-based arrays that represent known ESTs in the database). Due to the large size of the *S. mansoni* genome and its high percentage of redundancy, sequencing of the genome has not yet been completed. I created a random genomic shotgun microarray that represented approximately 5% of the genome. Many spots on this array represented non-coding genomic regions, yet the array represented the first completely unbiased *S. mansoni* array in terms of the sequences represented. Preliminary studies were done by using these microarrays and adult stage cDNA microarrays

produced by J. Salter and D. Khiem in our lab, which although yielding few "hits", did validate one gene product of particular interest, Sm13 (discussed below).

In late 2003, a windfall of data was submitted to the *S. mansoni* and *S. japonicum* databases, allowing the ability to generate much more diverse and sensitive microarrays than previously possible. Verjovski-Almeida and colleagues assembled 163,000 *S. mansoni* ESTs from cDNA libraries from 6 different developmental stages (13), and Hu and colleagues assembled 43,707 *S. japonicum* ESTs from adults and eggs (14). With the addition of almost 200,000 ESTs to the database, a shotgun genomic-based microarray approach became obsolete. Hoffmann and colleagues, drawing from this new vast resource of ESTs, recently created an oligo-based array representing 7,500 different, unique messages and kindly supplied me with printed slides with which I analyzed global expressions patterns in parasites isolated from either wild-type or RAG-$1^{-/-}$ mice at 28 or 42 dpi.

Results and Discussion

To identify the differences in gene expression patterns in attenuated and wild-type worms during development in the mammalian host, we analyzed two time points during infection; 28 dpi at which time all immature worms have arrived at the liver and wild-type worms are undergoing a period of rapid growth, and 42 dpi, when wild-type worms are fully sexually mature, females are producing eggs and pairs have begun to mate. We harvested worms from wild-type and RAG-1$^{-/-}$ mice (herein, worms will be called wild-type and RAG-1$^{-/-}$ worms for simplicity) at 28 dpi and 42 dpi three separate times, totaling 12 separate worm pools. It was necessary to gather RNA from three separate infections and to hybridize independent samples in order to ensure high quality analysis. Total RNA was isolated from these pools and was used for subsequent hybridizations to the EST arrays. A reference pool of total RNA was made from the following life cycle stages: male and female adults, eggs, sporocysts (snail stage), and cercariae (invasive larvae), and was used as a reference in hybridizations with each of the 12 sample pools. Therefore, changes in gene expression were compared to the expression patterns of the reference pool and not to the other samples. Due to the difficulty in isolating large amounts of parasite product, sample and reference pool RNA were amplified linearly (119), allowing for the amplification of RNA in such a way as to not distort the ratios of expressed transcripts in the samples (120-122). The reason the ratios of

amplified transcripts are maintained is because RNA polymerase activity is generally not affected by either the concentration of individual templates in a complex mixture or by the sequences of the template molecules that are being transcribed. The reference pool was labeled with Cy3 dye while the samples were labeled with Cy5 dye. Equal microgram quantities of reference pool and sample RNA were then hybridized to the array.

We hypothesized that we would find different patterns in expression between wild-type and RAG-1$^{-/-}$ worms in both 28 dpi and 42 dpi samples, and also that there would be differences in expression across all 4 groups of samples. After hybridizing Cy5 labeled RNA from wild-type and RAG-1$^{-/-}$ worms with Cy3 labeled reference pool RNA in triplicate for both time points, high quality spots were assessed by stringent criteria (Genepix 4.1 software). Only high quality spots were further analyzed in the 12 individual arrays. Spot dye intensity data was transformed to a log scale so that individual arrays could be compared to each other and then clustered by similarities of dye intensities into groups. These clustered groups represented spots (and therefore ESTs) of similar intensities and hypothetically genes that were related transcriptionally. As a control, each oligo representing an EST was spotted twice; therefore, good quality data would produce clusters of spots that represented an EST in duplicate. Indeed, our clustering produced duplicate spots with highly related dye intensities (Fig. 14).

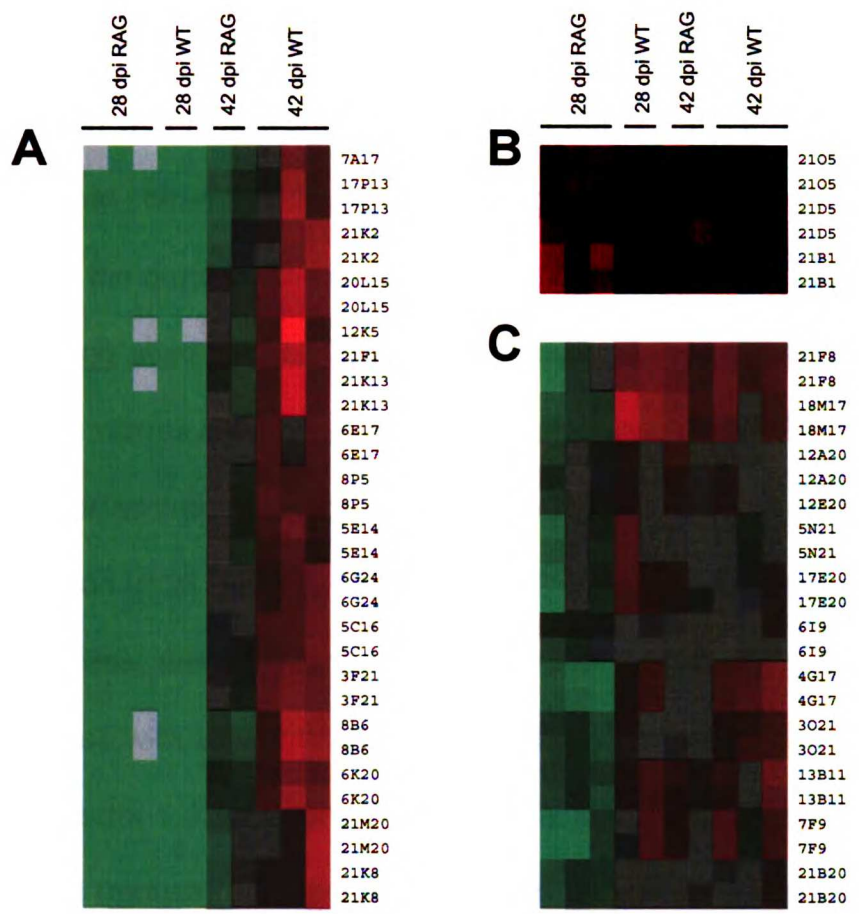


Figure 14. *Microarray analysis of gene expression in wild-type and RAG-1$^{-/-}$ worms during development.* (A-C) Green intensity represents Cy3 labeled *S. mansoni* reference pool. Red intensity represents Cy5 labeled sample pools. Represented here are three independent hybridizations of 28 dpi RAG-1$^{-/-}$ worms vs. pool, two independent hybridizations of 28 dpi wild-type vs. pool, two independent hybridizations of 42 dpi RAG-1$^{-/-}$ vs. pool, and three independent hybridizations of 42 dpi wild-type vs. pool. (A) Cluster represents most differentially regulated genes across wild-type and RAG-1$^{-/-}$ worms over two time points. Clustered genes are highly downregulated compared to reference pool at 28 dpi. Clustered genes are less downregulated in 42 dpi RAG-1$^{-/-}$ worms versus those at 28 dpi, but are still substantially downregulated compared to 42 dpi wild-type worms. Codes on the right indicate the EST represented at that spot. Note that spots duplicated on the array cluster together. (B) Genes that are upregulated in all conditions except 28 dpi wild-type. (C) Genes that are upregulated in all conditions except 28 dpi RAG-1$^{-/-}$.

The most striking changes in expression patterns can be observed in Figure 14 A. Here we show a number of genes in both wild-type and RAG-1$^{-/-}$ worms that are downregulated compared to the reference pool at 28 dpi become upregulated in wild-type worms compared to the reference pool at 42 dpi. It is also clear that these genes are not upregulated in 42 dpi RAG-1$^{-/-}$ worms. Interestingly, the gene cluster contains a number of schistosome female specific genes and egg shell genes. This validates our previous observation that at 42 dpi, wild-type worms are actively paired and mating whereas RAG-1$^{-/-}$ worms are not mature or as frequently paired. Also included in the clusters that showed downregulation in 28 dpi and 42 dpi RAG-1$^{-/-}$ worms and upregulation in 42 dpi wild-type worms, were a number of genes with unknown function as well as parasite genes with similarities to known eukaryotic genes, such as eukaryotic elongation factor-1 kinase and TATA box binding associated factor for RNA polymerase I (mouse). Characterization of those genes with currently unknown function may be key to determining how RAG-1$^{-/-}$ worms are delayed in their development. Future studies on characterizing promising genes will be undertaken by other lab members.

Interestingly, a number of schistosome superoxide dismutases were upregulated in this cluster by 42 dpi wild-type worms. Schistosome-specific superoxide dismutases are known to be expressed at various levels throughout development (123), and have been found to play a role in parasite defense against the host immune system (124, 125). That superoxide dismutases are

down regulated in RAG-1$^{-/-}$ worms at 42 dpi may indicate a role of these genes in development, but more likely, RAG-1$^{-/-}$ worms are not as robust targets for immune attack in their environment as are the rapidly growing and differentiating wild-type worms. Also, there is only a limited immune response in RAG-1$^{-/-}$ hosts that the parasites must defend against.

There were some gene clusters where gene regulation was upregulated in 28 dpi RAG-1$^{-/-}$ worms but not in 28 dpi wild-type worms (fig. 14 B). This cluster contained schistosome heat shock proteins. We hypothesize that in the RAG-1$^{-/-}$ host, immature worms do not receive proper signals to develop along the normal program, therefore, this environmental stress may induce expression of heat shock proteins. It is interesting to note that the same heat shock proteins are also expressed at 42 dpi in both wild-type and RAG-1$^{-/-}$ worms, but was slightly more upregulated in the RAG-1$^{-/-}$ worms.

There were multiple gene clusters in which gene expression was downregulated in 28 dpi RAG-1$^{-/-}$ worms and was upregulated to varying degrees in all other samples, including 42 dpi RAG-1$^{-/-}$ worms. An example of such a cluster is shown in Figure 14 C. A number of cathepsin protease genes, (cathepsins B, C, D, and L1, which are involved in hemoglobin degradation, a major metabolic function in developing and adult worms) were downregulated in 28 dpi RAG-1$^{-/-}$ worms. This is consistent with a model of developmental arrest akin to dauer formation in *C. elegans*, whereby *C. elegans* limit their uptake of nutrients and metabolism in adverse environmental conditions. Yet, by 42 dpi,

these genes had been upregulated in RAG-1$^{-/-}$ worms. Assessment of downregulation of hemoglobinases by microarray confirms our observations in Figure 3 A that RAG-1$^{-/-}$ worms grown in the absence of wild-type lymphocytes *in vitro* produced less hematin, a RBC waste product, than did those cultured with wild-type lymphocytes.

One intriguing gene that was downregulated in 28 dpi RAG-1$^{-/-}$ worms was Sm13, a tegumental protein expressed in adult worms not before 21 dpi (126). This observation was of particular importance because Sm13 was the only differentially regulated gene that I observed during my initial work on *S. mansoni* adult cDNA arrays (D. Khiem) or on *S. mansoni* shotgun genomic arrays (R. B. Blank) (data not shown). Sm13 is a tegumental protein and has a transmembrane domain, suggesting that it might function in transducing information from the host environment.

Clustered together with the cathepsins and Sm13 were a number of unknown genes that will be of interest to characterize further. These genes of unknown function may provide additional clues as to how worms are developmentally regulated in wild-type versus immunocompromised hosts.

Chapter Six

Concluding Remarks

The co-evolution of trematode parasites with their vertebrate hosts is an ancient relationship, beginning well before mammals existed. Trematode ancestors of *Schistosoma mansoni* were parasites of marine mollusks. With the evolution of vertebrate predators such as bony fishes, parasites of marine mollusks adapted to parasitize vertebrates (2). These primitive vertebrates represent the earliest known source of lymphocytes and some form of adaptive immunity (127). In fact, it has been hypothesized that adaptive immunity evolved in the gut of ancient jawed fishes (128), the niche where ancient trematodes resided. Therefore, trematodes have long interacted with a host adaptive immune response whose function was to expel or destroy them. That they have evaded the host protective response for so long suggests a highly evolved and complex relationship between the parasite and immune response.

The long evolutionary coexistence of trematodes and the vertebrate immune response is also consistent with the hypothesis that trematode parasites have evolved mechanisms to utilize factors of the host immune response as cues to the condition of the host environment. Since these parasites are long-lived in the host, it would be advantageous if they were able to sense whether host conditions were initially favorable for infection and egg production. In immunocompromised hosts, eggs that are produced by parasites fail to induce granuloma formation, leading to egg toxins damaging the liver and induction of cachexia and death of the host. In infections of immunocompetent hosts, eggs induce granulomas that sequester egg toxins and limit egg-mediated

hepatocellular damage. Hosts that induce granulomas around eggs are able to survive longer and parasites are able to disseminate more eggs to the outside environment. In addition, parasites may use the immune response of its host as a gauge of host robustness, entering a state of developmental arrest until a malnourished condition or co-infection is resolved. That the parasite is able to sense averse conditions and change its developmental pathway is reminiscent of *C. elegans* dauer formation.

We have determined that two $CD4^+$ T cell signaling pathways, IL-2 and IL-7, are involved in promoting schistosome development and our array analysis has provided us with clues as to which parasite genes are differentially regulated during development in both normally developing worms and in worms that display an attenuated phenotype. Although a specific host factor has not been identified as key to determining the developmental alternatives, work with cultured parasites and transgenic mice has ruled out several obvious choices, i.e. TGFβ, TNF-α, IFN-γ, and Th type 2 cytokines. Our data are consistent with two different models of how host $CD4^+$ T cells may affect parasite development (Fig. 15). First, a T cell effector molecule other than those we have studied may affect schistosomes directly. IL-2 and IL-7 signaling pathways may induce expression of unknown downstream T cell factors that are directly sensed by the parasite. Schistosomes have been found to express several molecules that are homologous to components of host signaling pathways (15, 17, 23, 24).

Therefore, it is possible that this parasite may have evolved signaling pathways that can respond to mammalian cytokines.

Alternatively, $CD4^+$ T cells may affect schistosome development indirectly by eliciting a response in another cell type or tissue that is closely associated with the parasite. Since developing worms are intimately associated with the endothelial lining of blood vessels in the liver during their maturation, $CD4^+$ T cells may elicit secretion of factors from endothelial cells that act upon the parasite. $CD4^+$ T cells might also act upon another endocrine site or hepatocytes to induce factors involved in directly stimulating worm maturation. In either case, our observations that two cytokines essential for $CD4^+$ T cell development and function profoundly affect parasite development underscore the complex relationship between host $CD4^+$ T cells and *Schistsoma mansoni* and suggest fruitful research avenues to pursue in the future.

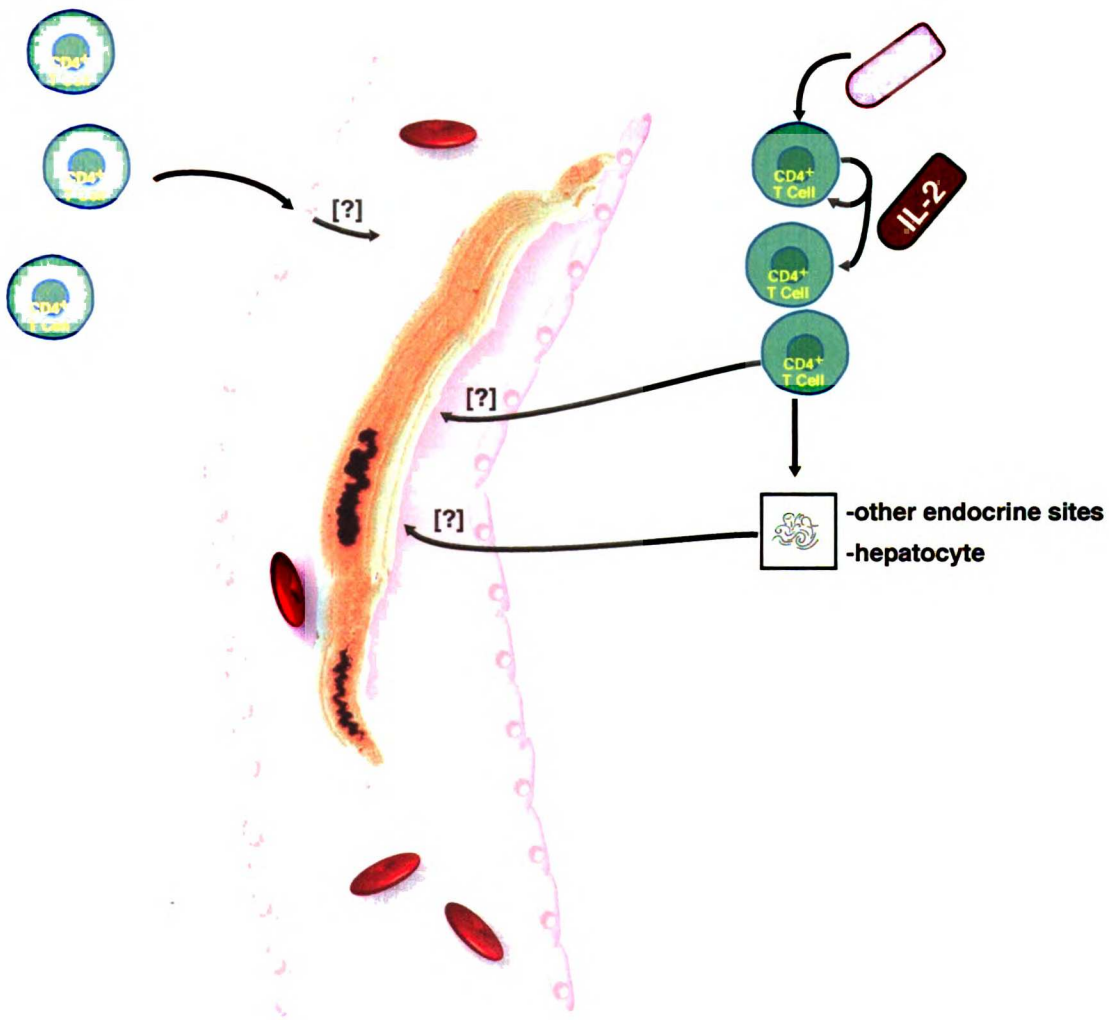


Figure 15. *Schematic diagram of potential roles for CD4$^+$ T cells and IL-2 and IL-7 in parasite development.* On the right, the possibility of a direct role (top) or indirect role (square) of CD4$^+$ T cell cytokines is shown. On the left is an alternative indirect role via vascular endothelial cell interaction.

Chapter Seven

Materials and Methods

Experimental animals. Wild-type, RAG-1$^{-/-}$, IFN-$\gamma^{-/-}$, IL-2$^{-/-}$, IL-2R$\alpha^{-/-}$, IL-7R$^{-/}$, and common gamma chain (γ_c)$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME). TNF-$\alpha^{-/-}$ mice were provided by J. Sedgwick (DNAX Research Inc.) and are described elsewhere (129). β2-m$^{-/-}$/MHC II$^{-/-}$ mice were purchased from Taconic. All wild-type and deficient animals were of the C57BL/6 genetic background. Animals were age- and sex-matched in each experiment. All animals were infected when approximately 8 weeks of age. All IL-2$^{-/-}$ (88) and IL-2R$\alpha^{-/-}$ (89) mice were infected at an early age, before the onset of autoimmune disease and were assessed for gross and histological evidence of autoimmune pathology at necropsy. All animals were infected percutaneously via the tail skin using 150 *S. mansoni* cercariae of (Puerto Rican strain) shed from infected *Biomphalaria glabrata* snails (130). All experiments performed on mice were in accordance with protocols approved by UCSF's Institutional Animal Care and Use Committee.

Cell isolation. For time points prior to 56 dpi and for reconstitution experiments, spleens and livers were removed aseptically from wild-type mice and were dispersed through a 70 μm nylon filter to produce single cell suspensions. For 56 dpi time points, individual livers were homogenized at low speed and granulomas were sedimented by centrifugation. Granulomas were resuspended in 5 mg/ml collagenase type I (Sigma) in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS) and incubated at 37°C for 30 min. Digested granulomas were then dispersed through a 70 μm filter to produce

single cell suspensions. Liver leukocytes were isolated as described by Matsui *et al.* (131). Dispersed liver tissue or granulomas from a single liver were resuspended in a 30% Percoll gradient and centrifuged at 500 x *g* for 20 min at room temperature in order to separate hepatocytes from leukocytes. Hepatic leukocytes and RBC were recovered from the pellet after centrifugation. Both splenocytes and hepatic leukocytes were treated with 0.16 M Ammonium chloride/ 0.17 M Tris pH 7.2 to lyse RBC. Organs from at least 5 mice at each time point were pooled for each experiment.

For isolation of lamina propria, peyer's patches, mesenteric lymph nodes, and intraepithelial lymphocytes, mice were sacrificed and small intestine was removed aseptically. MLN were isolated with tweezers aseptically and pooled. Peyer's patches were removed by pinching off the small intestine with tweezers and pooled. Both were dispersed through 70 μm filter to produce single cell suspensions. IEL and LP isolation techniques were adapted from (132, 133). The remainder of the small intestine was incubated with agitation in 5 mM EDTA. Supernatant contains IEL population. Remaining intestine is incubated with collagenase type VIII (300 U/ml) to isolate lamina propria. Both LP and IEL were concentrated by Percoll gradient centrifugation as described above.

In vitro co-culture and assessment of worm development. Female RAG-1$^{-/-}$ mice were infected with up to 3000 cercariae for 21 days. Immature schistosomula were then harvested and cultured in RPMI-1640 medium with 10% FBS and 100 U/ml Penicillin and 100 μg/ml Streptomycin. Washed RBC

from the mouse were added to the culture wells as a food source. Wild-type liver leukocyte preparations, prepared as above, were added to co-culture apparatus. The media was changed every third day and fresh RBC were added as needed. Parasites were cultured at least 9 days. Parasites were photographed at various time-points during the experiment. Worm length and surface area was quantified using NIH imaging software (http://rsb.info.nih.gov/ij/). At the termination of experiment, hematin was collected from the culture wells, pelleted by using a 40% Percoll gradient and quantified as a measure of RBC digestion. The hematin pellet was dissolved in 0.1 M NAOH and emission spectra at 650nm was calculated to determine concentration. (84).

Assessment of parasite phenotype. All experiments included 4-5 mice per group and were performed at least twice. Worms were perfused from the hepatic portal vein 42 days post infection (dpi) and immediately fixed in 4% neutral buffered formaldehyde solution to prevent paired worms from dissociating. Worms were counted, sexed, and number of pairs determined by using a dissecting microscope. Two different methods were used to determine egg burden in the liver. In some experiments, tissue samples were taken from the left lateral lobe and digested in 0.7% trypsin in a phosphate buffer solution (PBS). Eggs were counted under a dissecting microscope to determine total liver egg burden, assuming uniform distribution of eggs throughout the liver (52). Alternatively, whole livers were digested in 0.7% trypsin solution and eggs were counted from aliquots of liver digest. Identical results were obtained when both

methods were applied to the same mouse (unpublished data). Therefore, we consider both methods comparable and accurate. For analysis of worm size, digital images were acquired by using a Nikon Coolpix 4500 digital camera, and worm length was measured using NIH Image J software (http://rsb.info.nih.gov/ij/). At least 15 male worms were randomly selected and measured in each sample.

Reconstitution experiments. Whole spleens from either wild-type or IL-2$^{-/-}$ mice were prepared as above and resuspended in PBS. 4 X 10^6 cells were transferred into RAG-1$^{-/-}$ mice by intravenous injection 1 day prior to infection with cercariae. Control mice received PBS alone. At 42 dpi, mice were sacrificed and 1 X 10^6 splenocytes from reconstituted RAG-1$^{-/-}$ mice were surface labeled with FITC-conjugated anti-CD4, PE-conjugated anti-NK1.1, and CyChrome-conjugated anti-TCRβantibodies in the presence of unlabeled monoclonal antibodies to CD16 and CD32 ("Fc Block"). All antibodies were purchased from BD Biosciences. Cells were analyzed using a FACS®Calibur flow cytometer and CellQuest software (Becton Dickinson).

Intracellular cytokine staining. Single cell suspensions of hepatic or splenic lymphocytes were resuspended at a concentration of 1 X 10^7 in RPMI 1640 supplemented with 10% FBS and were stimulated with 5 ng/ml PMA and 0.5 μg/ml ionomycin for 4 hours at 37°C in the presence of 1 μg/ml brefeldin A (GolgiPlug, BD Biosciences). 1 X 10^6 cells were surface labeled with FITC-conjugated anti-CD4 and CyChrome-conjugated anti-TCRβantibodies in the

presence of Fc Block.After fixing and permeabilizing with Cytofix/Cytoperm solution (BD Biosciences), cells were stained with PE-conjugated antibodies to IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10, and IL-12. All antibodies were purchased from BD Biosciences. Cells were analyzed using a FACS®Calibur flow cytometer and CellQuest software (Becton Dickinson).

Cytometric bead array assay and in vitro stimulation of CD4⁺ αβ T cells from β2-m$^{-/-}$/MHC II$^{-/-}$ mice. Peripheral lymph nodes and spleens were isolated from mice and lymphocytes were stained with antibodies to CD4, TCRβ, CD19, CD8α, and NK1.1. Cells were sorted by using a MOFLO cell sorter (Cytomation) and only CD4⁺ TCRβ⁺ cells were collected. Single cell suspensions were resuspended at a concentration of 1 X 10^6 in RPMI-1640 medium supplemented with 10% FBS and were stimulated with 1 μg/ml anti-CD3ε and 1μg/ml anti-CD28 antibodies or with 5 ng/ml PMA and 0.5 μg/ml ionomycin or not stimulated for 72 hours at 37°C. To analyze cytokine production by cultured cells, supernatants were collected and cytokine production was determined by using a cytometric bead array kit that can sensitively detect multiple soluble cytokines from one supernatant at physiologically relevant concentrations (pg/ml) (Pharmingen, BD Biosciences).

RNA isolation and amplification. Female wild-type and RAG-1$^{-/-}$ mice were infected with up to 3000 cercariae for 28 or 42 days. Schistosomes were quickly harvested and immediately snap frozen in liquid nitrogen. For other life cycle stages, eggs were harvested from trypsinized livers, sporocysts were harvested

from snail hepatopancreas, and cercariae were harvested from infected snail-primed water and quickly placed in liquid nitrogen. Total RNA was recovered from all life cycle stages by using an RNeasy kit (Qiagen) to obtain very pure RNA from small tissue samples. 1 μg of total RNA from each of the samples were amplified in a linear way by using an Amino Allyl MessageAmp aRNA kit (Ambion) in order to obtain a sufficient quantity of RNA transcripts to use in microarray analyses.

Microarray experiments. Microarray experiments were performed using an oligonucleotide-based *S. mansoni* array. These arrays were generously supplied by Karl Hoffmann of University of Cambridge, UK. An RNA reference pool containing RNA from various lifecycle stages was labeled with Cy3 dye and hybridized with Cy5-labeled sample worm pool (isolated from either wild-type or RAG-$1^{-/-}$ mice) to the array. Hybridized arrays were incubated at 50°C to ensure hybridzation of transcripts with little background hybridization.

Statistical analysis. Student's *t* test was used to determine the statistical significance of differences in (i) percentages of females in pairs, (ii) liver egg burdens and (iii) worm length in wild-type versus deficient animal groups. P values of <0.05 were considered significant.

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