GENETIC CONTROL OF MURINE SCHISTOSOMIASIS: IN SEARCH OF GENES

A dissertation

submitted by

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ABSTRACT

Infection with the trematode parasite *Schistosoma mansoni* results in a distinct heterogeneity of disease severity, both in humans and in an experimental mouse model. Severe disease is characterized by pronounced hepatic egg-induced granulomatous inflammation in a proinflammatory cytokine environment, while mild disease corresponds with reduced hepatic inflammation in a Th2 skewed cytokine environment. This marked heterogeneity indicates that differences in the host's genetic background significantly impact the clinical outcome of schistosomiasis, yet little is known about the genetic basis of dissimilar immunopathology and particularly the specific gene(s) that contribute to disease severity. To investigate the role of genetic susceptibility in murine schistosomiasis, we performed a QTL analysis on an F_2 progeny derived from SJL/J and C57BL/6 mice, which develop severe and mild pathology, respectively. QTL analysis identified several genetic intervals controlling immunopathology as well as IL-17 and IFN-γ production, including two loci, *D4Mit203* and *D17Mit82*, which were highly significantly linked to granuloma formation. Furthermore, A significant reduction of hepatic granulomatous inflammation and IL-17 production in interval-specific congenic mice demonstrated that these loci have a decisive effect on the development of immunopathology in murine schistosomiasis. *D4Mit203* was also identified as controlling severe disease in a second genetic analysis between BL/6 and high pathology BL/10 mice. Subsequent studies in these mice combining microarray analysis with an *in vitro* BMDC-CD4 T cell coculture system, demonstrated that enhanced immunopathology in BL/10 mice was likely due to a defect in the alternative activation pathway of DCs. Further analysis of candidate genes located within *D4Mit203* provided strong evidence that G-CSFR is the underlying causal gene. Finally, we investigated the schistosome infection in wild-derived mouse strains, which possess a diverse gene pool likely to reveal novel phenotypes of immune regulation. We now show that following infection, wild-derived MOLF mice develop exacerbated immunopathology with high levels of IL-17 is controlled by a locus in chr. 6,

designated Why1, in which *Irak2* mediates severe disease in a CD4 T cell specific manner by enhancing IL-1b stimulation of Th17 cell development. The use of wild-derived mice thus unravels IRAK-2 as a novel regulator of IL-1-induced pathogenic Th17 cells in schistosomiasis. In sum, we identified several loci that control both immunopathology and cytokine production during schistosome infection, and provide strong evidence for the role of two genes, *Csf3r* and *Irak2*, that regulate the development of severe disease.

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ABBREVIATIONS

AAM	Alternatively Activated Macrophages
Ag	Antigen
AHR	Aryl Hydrocarbon Receptor
APC	Antigen Presenting Cell
BALB/c	BALB/cJ
BATF	B cell Activating Transcription Factor
BL/6	C57BL/6J
BL/10	C57BL/10J
BMDC	Bone-Marrow derived Dendritic Cell
C3H	C3Heb/FeJ
CBA	CBA/J
CCL	Chemokine Ligand
CCR	Chemokine Receptor
CIA	Collagen Induced Arthritis
DC	Dendritic Cell
EAE	Experimental Autoimmune Encephalomyelitis
FACS	Flourescence-Activated Cell Sorting
Foxp3	Forkhead Box p3
GC	Granuloma Cell
G-CSF	Granulocyte Colony Stimulating Factor
G-CSFR	Granulocyte Colony Stimulating Factor Receptor
IBD	Inflammatory Bowel Disease
IBD	Inherited By Decent
ICOS	Inducible Costimulatory Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRAK	Interleukin-1-Receptor Associated Kinase
IRF	Interferon Regulatory Factor
LACK	*Leishmania* Analogue of the receptors of activated C Kinase
LM	Littermate
LOD	Logarithm of Odds
LPS	Lipopolysaccharide
mAB	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MLN	Mesenteric Lymph Node
MLNC	Mesenteric Lymph Node Cell
PZQ	Praziquantel
QTG	Quantitative Trait Gene
QTL	Quantitative Trait Loci
QTN	Quantitative Trait Nucleotide
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
ROR	RAR-related orphan receptor
SEA	Schistosome Egg Antigens
SEA/CFA	Schistosome Egg Antigens in Complete Freund's Adjuvant
SJL	SJL/J

SLE	Systemic Lupus Erythematosus
STAT	Signal Transducers and Activators of Transcription
TCR	T Cell Receptor
Tg	Transgenic
TGF	Transforming Growth Factor
Th	T helper
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Treg	T regulatory
Why1	Wild-derived Hypersensitivity locus 1
WT	Wild Type

SECTION I: INTRODUCTION

Chapter 1

The Schistosomes

Schistosomiasis is a major tropical parasitic disease ranking second only to malaria among parasitic diseases in terms of a cause of morbidity. The World Health Organization reports that approximately 600 million people are at risk of contracting schistosomiasis, with 200 million reported infections globally, resulting in an estimated 200,000 deaths per year (Chitsulo, Loverde et al. 2004). The major etiological agents of schistosomiasis are blood dwelling flukes called schistosomes, of which there are four major species that afflict humans. *Schistosoma mansoni, S. japonicum and S. mekongi* primarily cause disease of the liver and intestine, while infection with *S.* haematobium results in urinary tract disease (Despommier D. 2005). Each species of schistosome inhabits a distinct region of the world, *S. mansoni* is endemic throughout sub-Saharan Africa, Egypt and the Sudan, as well as parts of South America, and the Caribbean, *S. japonicum* and *S. mekongi* are found mainly in southeast Asia, including China, Malaysia and the Philippines, while *S. haematobium* primarily affects Africa and the Middle East (Chitsulo, Loverde et al. 2004) **(Figure 1).** This distinct geographic distribution among schistosome species is due to their dependency on specific aquatic snails, which serve as intermediate hosts for the parasite. *S. mansoni* is transmitted by snails of the genus *Biomphalaria*, *S. japonicum* and *S. mekongi* require snails of the genus *Oncomelania*, and *S. haematobium* requires snails of the genus *Bulinus (Hokke and Deelder 2001)*.

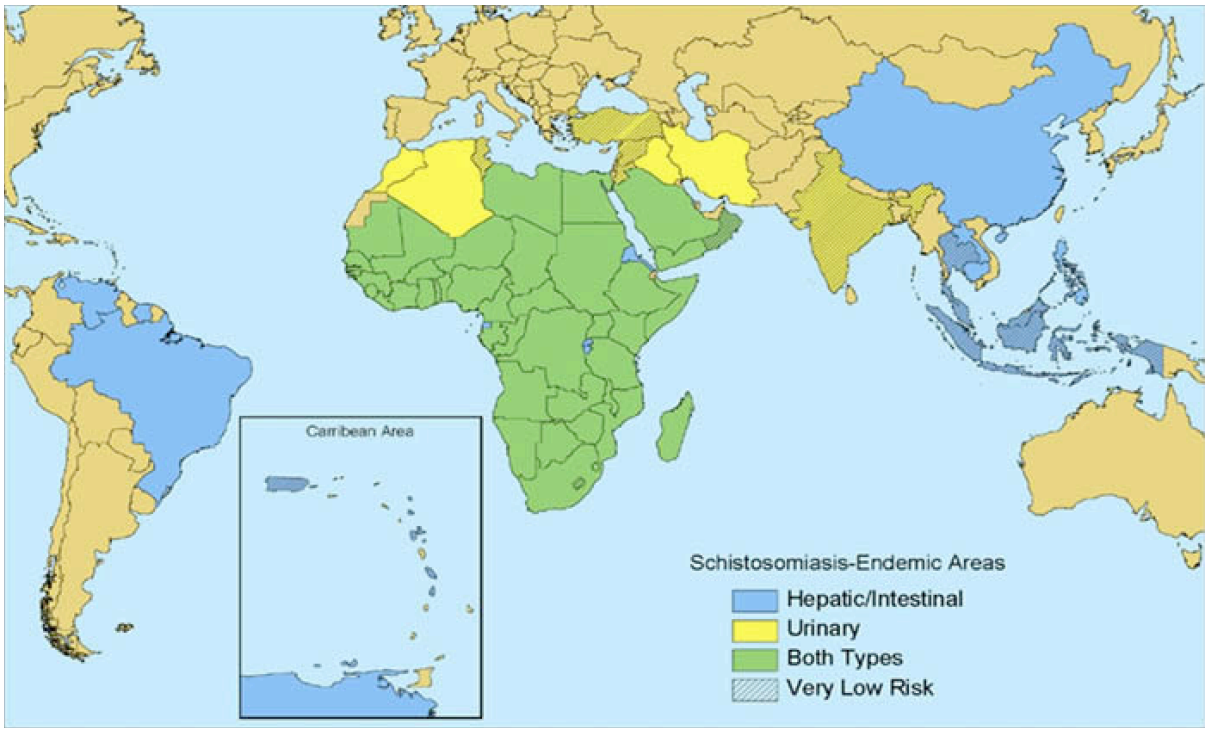


Figure 2: Global distribution of schistosomiasis caused by *S. mansoni, S. haematobium, S. japonicum and S. mekongi.* Adapted from CDC website.

The lack of suitable intermediated host snails, along with improved sanitation, is a major reason that natural infections do not occur in the United States and other industrialized nations. The remainder of this thesis will concentrate on the parasite *S. mansoni*, which is the main focus of our lab.

1.1 *Schistosoma mansoni* and Schistosomiasis

Schistosomiasis, also known as Bilharzia, is an ancient disease, dating back some three thousand years when ancient Egyptians believed that the presence of blood in the urine signaled the advent of manhood (Kloos 2002). However, it was not until the mid 19th century that Theodor Bilharz identified the causative agent of this disease when he described the first accounts of *S. haematobium* infection in humans and identified eggs with a terminal spine that were secreted in the urine of patients. In 1902, Patrick Manson described a new case of schistosomiasis, in which eggs were found in the stool, but not in the urine, and contained a lateral spine as opposed to the terminal spine previously identified by Bilharz. In 1907 Louis Sambon termed this new species of schistosome, *Schistosoma mansoni*, in honor of his mentor (Despommier D. 2005). One year later Piraja de Silva identified the first cases of *S. mansoni* in South America (Katz 2008). Since its discovery researchers have extensively studied this parasite and the disease it causes leading to a better understanding of the parasite itself, the pathogenesis of disease and hopefully better treatments for patients diagnosed with schistosomiasis.

S. mansoni is a digenic parasite, meaning it has a complex life cycle that requires multiple hosts. Specifically, the life cycle of *S. mansoni* has two hosts, an intermediate snail host and definitive mammalian host **(Figure 2)**. Humans become at risk for infection when they come into contact with bodies of fresh water that contain snails infected with the parasite. It is within these snails that asexual reproduction of the parasite occurs, which in turn results in the production of cercariae, the infectious stage to mammals. Cercariae are phototropic, and following exit from the snail they accumulate at the surface of the water, where they use their bifurcated tail to migrate through the water following temperature and chemical attractants, such as linoleic acid, emitted by their human hosts. Once in contact with their definitive hosts, cercariae penetrate the skin, typically through a hair follicle or abrasion (Gordon and Griffiths 1951; Stadecker 2001; Despommier D. 2005; He, Salafsky et al. 2005). Direct penetration of the host by cercariae is one of several unique features of the schistosome life cycle as the majority of digenean parasites infect their hosts as a result of being eaten (Pearce and MacDonald 2002). Following entry into the skin, cercariae shed their tail and transform into schistosomula and migrate into the blood stream. Subsequently, a brief developmental stage occurs in the lungs before the developing parasites travel to their final habitat, the mesenteric venous plexus (Stadecker 2001; Despommier D. 2005). It is here that another unique development occurs, unlike most trematodes, which are hermaphroditic;

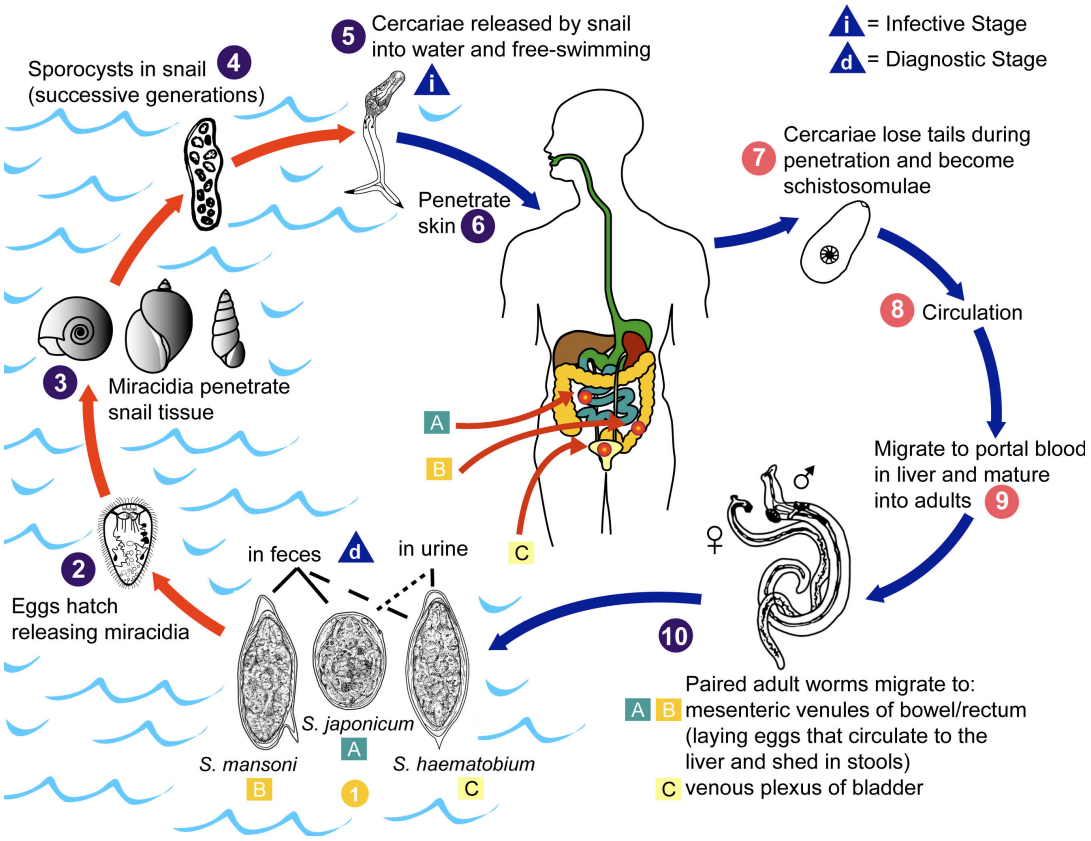


Figure 2. Life cycle of *S. mansoni, S. japonicum and S. haematobium.* Figure adapted from CDC webpage (http://www.dpd.cdc.gov/dpdx/HTML/Schistosomiasis.htm). (1) Schistosome eggs are excreted from humans in the feces or urine. (2) Upon reaching fresh water, the schistosome eggs hatch, releasing miracidia. (3) The free-swimming miracidia invade and infect the species-specific intermediate host snail. (4) Inside the snail the parasite undergoes asexual reproduction forming (5) cercariae. (6) Upon sensing physical or chemical cues emitted from humans, the cercariae leave the snail and penetrate the hosts' skin, typically through a hair follicle. (7) In the skin, the schistosomes shed their bifurcated tail and progress to schistosomula. (8,9) The schistosomula migrate through several tissues and stages to take up residence in the venous system. (10) Adult worms reside in the mesenteric venules in locations specific for each species. (A) *S. japonicum* resides in the superior mesenteric veins draining the small intestine, (B) while *S. mansoni* parasitize the mesenteric veins draining the large intestine. (C) *S. haematobium* resides within the venous plexus of the bladder. Once mature, the male-female worm pairs produce eggs that migrate into the veinous portal system and eventually gain entry into the intestinal (*S. mansoni* and *S. japonicum*) or the bladder (*S. haematobium)* and are eliminated in the feces and urine, respectively (1).

schistosomes have physically separate and morphologically distinct male and female counterparts (Pearce and MacDonald 2002; McManus and Loukas 2008). The female worm is longer and thinner than the male worm and also darker in color, which is caused by the presence of hemozoin in its digestive tract (Oliveira, d'Avila et al. 2000). Once the male and female worms have reached the mesenteric vasculature they sexually mature and form a worm pair, in which the female resides in the gynecophoric canal of the male and is capable of producing approximately 300 eggs per day. At this juncture the schistosome eggs have one of two fates, the first is beneficial to both the host and the parasite, whereas the second is detrimental to both.

In the first scenario, *S. mansoni* eggs enter into the circulation and eventually become trapped in the intestinal wall where they cause a granulomatous inflammatory reaction. This inflammatory response damages the intestinal epithelium and allows the eggs to cross into the lumen where they are excreted from the host in the feces. As a result, the eggs are reintroduced into the environment and upon contact with fresh water hatch, releasing free-swimming miracidia that seek out and infect *Biomphalaria* snails, perpetuating the life cycle (Despommier D. 2005). In the second scenario, the schistosome eggs are carried by the circulatory system upstream through the hepatic portal vein where they embolize in the liver. This leads to an intense granulomatous and fibrosing reaction. The resultant granulomas are comprised of a variety of cells including lymphocytes, macrophages, neutrophils and eosinophils. The accumulation of these granulomas and additional fibrotic lesions results in restriction of blood flow in the liver leading to portal hypertension, ascites, hepatosplenomegally and portal-systemic shunting, which ultimately results in gastrointestinal bleeding, hemorrhage and death

(Stadecker 2001; Pearce and MacDonald 2002). The pathology associated with this second scenario is caused by cells of the host's own immune system, making it a true adaptive immunopathology similar to several autoimmune and inflammatory diseases. Therefore it is of critical importance that we understand the underlying immune mechanisms responsible for this disease.

1.2 Control and treatment of schistosomiasis

Schistosomiasis is the most important human helminth parasitic disease in terms of morbidity and mortality, resulting in a large socioeconomic burden in developing countries whose true impact is greatly underappreciated (King, Dickman et al. 2005; Bergquist, Utzinger et al. 2008; McManus and Loukas 2008). Given the complex nature of the schistosome early intervention attempts focused on targeting the intermediate snail vector with molluscacides, however this proved ineffective as even a few surviving snails were sufficient to re-establish the population. There also were developing concerns about potential contamination of drinking water (Jordan 1977). Additionally attempts focused on providing more sanitation and access to clean uncontaminated water, however long standing cultural habits mixed with economic issues, that led inhabitants of endemic areas to have daily contact with contaminated water derailed these efforts (Despommier D. 2005).

Currently, the most effective chemotherapeutic treatment for individuals with schistosomiasis is praziquantel (PZQ), an acylated quinoline-pyrazine whose exact

mechanism of function is not known (Cioli and Pica-Mattoccia 2003). PZQ functions by reducing the worm burden, and several groups have shown that PZQ acts by compromising the integrity of the tegument of adult male worms. This causes an increased calcium ion flux, which results in worm paralysis and increased surface antigen exposure, leading to enhanced recognition by the immune system (Pax, Bennett et al. 1978; Harnett and Kusel 1986). While PZQ acts as a major anti-schistosomal drug that effectively reduces the number of excreted eggs in endemic areas, schistosomiasis continues to spread into new areas and several significant treatment issues remain. First and perhaps most important from a socioeconomic impact is that mass treatment with PZQ does not provide protective immunity or prevent re-infection as reports from highly endemic regions show that prevalence of infection returns to baseline levels within 6-8 months (McManus and Loukas 2008). As such, up to 80% of children living in high transmission areas can develop recurrent aggressive inflammation termed 'rebound morbidity' (Hotez, Bethony et al.). Second, although currently there is not clear-cut evidence of PZQ resistant schistosome strains there is increasing evidence that treatment with PZQ is becoming less effective (Doenhoff and Pica-Mattoccia 2006; Melman, Steinauer et al. 2009). This has led to concerns of developing parasite resistance and the requirement for additional treatments, with most promising focusing on anti-schistosome vaccines (Gryseels, Polman et al. 2006).

Schistosome vaccines that could confer protective immunity while not requiring monthly treatments like PZQ are necessary and provide hope for long-term control and possible elimination of schistosomiasis. To date only one vaccine has reached the clinical trial phase. This vaccine is directed against a recombinant 28kDA Glutathione S-

transferase (Sh28GST) clone of *S. haematobium (McManus and Loukas 2008)*. Other putative vaccine targets against *S. mansoni* antigens that will soon be ready for clinical trials include a fatty acid binding protein (Sm14) (Moser, Tendler et al. 1991), which elicits a protective response in several animal models (Tendler and Simpson 2008), and Sm-p80, which provides protection in baboons similar to irradiated cercariae (Zhang, Ahmad et al. 2010). Several other vaccines against membrane spanning antigens present on the worm tegument are also close to the clinical trial phase (Hotez, Bethony et al. 2010). These developmental vaccines, together with the recently completed sequencing of the *S. mansoni* genome provide serious hope for the future despite the poor success rates of previously tested anti-schistosome vaccines (Berriman, Haas et al. 2009). Given the critical role of the host immune system in mediating the pathology observed in schistosomiasis, additional studies that focus on controlling or modulating pathogenic immune responses provide another effective therapeutic approach.

1.3 Immunobiology of *S. mansoni* infection

Schistosomes require a significant portion of their life cycle and maturation to adult worms to occur in humans, and have done so successfully for thousands of years (Cox 2002). As such, they have developed a number of clever mechanisms with which to continually evade detection by our immune system (Pearce and Sher 1987). Starting immediately following entry into the skin, schistosomula incorporate host serum proteins into their tegument that act to confuse host immune cells in their attempt to recognize the parasite (McLaren, Clegg et al. 1975; Goldring, Clegg et al. 1976; Sher, Hall et al. 1978).

The parasite also produces factors such as prostaglandin D_2, which prevents local Langerhans cells from migrating to the draining lymph nodes (Angeli, Faveeuw et al. 2001; Herve, Angeli et al. 2003). In addition, the parasite can also avoid immune detection by altering the expression of life cycle stage-specific antigens (Levy-Holtzman and Schechter 1995) and further still by expressing a β_2-microglobulin-like molecule on the service of mature worms that further confuses attempts by innate immune cells, particularly macrophages, to recognize it as foreign and mount a sufficient immune response (Despommier D. 2005). It is not until the worms migrate to the mesenteric venous plexus that the host immune system first recognizes the presence of a pathogen and it is not until the worms begin producing eggs that the parasite can cause severe disease.

Murine models have shown that infection with parasitic worms classically elicits the development of an anti-inflammatory, CD4 T helper type 2 (Th2) response directed against the parasite (Maizels and Yazdanbakhsh 2003; Anthony, Rutitzky et al. 2007). Despite the best efforts of *S. mansoni* to circumvent the immune system similar studies have shown that during the initial phase of infection from approximately 3-5 weeks, the host mounts a mild Th1-biased proinflammatory response characterized by enhanced IFN-γ production against migrating immature parasites (Pearce and MacDonald 2002). As the parasites mature and the females begin producing eggs (~ weeks 5-6) there is a dramatic shift in the immune response starting with the down-modulation of the initial proinflammatory response, coinciding with the rise of a more potent Th2 response, characterized by the production of the cytokines interleukin (IL)-4, IL-5, IL-10 and IL-13, as well as by increased immunoglobulin (Ig) E production and enhanced eosinophilia

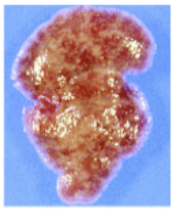
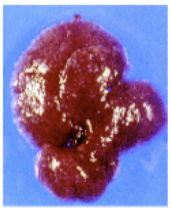
(Pearce, C et al. 2004; Stadecker, Asahi et al. 2004). The development of a full Th2 response requires a number of costimulatory pathways including, B7-CD28 (Hernandez, Sharpe et al. 1999), CD40-CD154 (MacDonald, Patton et al. 2002) and ICOS-B7RP-1 (Rutitzky, Ozkaynak et al. 2003), as well a requirement for B cells, likely because of the dependency on interactions between B7 and CD28 (MacDonald, Patton et al. 2002). A failure to switch from the initial Th1 to Th2 mediated response can result in severe hepatic inflammation and hepatocellular injury resulting in increased morbidity and mortality during the acute phase of the disease beginning at week 7 post infection.

A classic feature of schistosome infection is the distinct heterogeneity of disease severity that develops in humans. Following infection with *S. mansoni* the majority of afflicted individuals develop a relatively mild and chronic infection termed, "intestinal schistosomiasis." However 5-10% of infected individuals develop the more severe and life-threatening form of the disease termed, "hepatosplenic schistosomiasis". Severe disease is characterized by advanced fibrosis of the liver, which can lead to the development of portal hypertension, portal-systemic venous shunting, ascites, gastrointestinal bleeding and which, if left untreated, ultimately can result in death (Pearce and MacDonald 2002; Stadecker, Asahi et al. 2004). Association studies aimed at identifying the basis for the development of dissimilar disease in humans have resulted in the identification of several HLA alleles linked to severe disease (Assaad-Khalil, Helmy et al. 1993; Secor, del Corral et al. 1996; May, Kremsner et al. 1998; McManus, Ross et al. 2001). However these studies have been greatly complicated by several factors, most commonly, individuals who become infected likely receive very different parasite loads and often suffer from various concomitant infections that can affect analyses. Fortunately

the identification of mouse models that closely mimic the development of both forms of disease (Warren and Dewitt 1958; Cameron and Bhattacharyya 1965) have led to a greater understanding and characterization of the molecular mechanisms responsible for the observed heterogeneity.

In the murine model of acute schistosomiasis the dichotomy of mild and severe disease is best represented by the C57BL/6 (BL/6) and CBA mouse strains, respectively **(Figure 3)**. Following infection both strains develop an initial proinflammatory response, however unlike BL/6, the developing Th2 response in CBA mice fails to down modulate the initial proinflammatory response. The result

Liver Pathology

	Severe (CBA)	Mild (BL/6)
		
	• Larger, poorly circumscribed granulomas • Enhanced parenchymal inflammation • Pronounced splenomegaly	• Smaller, well circumscribed granulomas • Mild parenchymal inflammation • Moderate splenomegaly

CD4 T cells

Frequency in Liver:	**Higher**	**Lower**
Response to:		
SEA	**Proinflammatory** **Persistent Th1/Th17 response alongside Th2 response**	**Anti-inflammatory** **Initial Th1 response, replaced by long-lasting Th2 response**
Sm-p40 Ag	**Proinflammatory**	**Weak-No Response**

Figure 3. Mouse model of severe and mild forms of schistosomiasis

is that BL/6 mice develop small well-circumscribed granulomas with little parenchymal inflammation and splenomegaly, while CBA mice develop much larger poorly circumscribed liver granulomas with pronounced parenchymal inflammation and severe hepatosplenomegally, often leading to gastrointestinal hemorrhage and death (Pearce and MacDonald 2002; Stadecker, Asahi et al. 2004). In BL/6 mice, the immune response elicited by schistosome eggs antigens (SEA) is characterized by the production of the Th2 and anti-inflammatory associated cytokines IL-4, IL-5 and IL-10 with virtually no detectable IL-17 or IFN-γ (Rutitzky, Hernandez et al. 2001). Additional studies have identified several other mechanisms by which low-pathology prone mice control disease, including the development of protective alternatively activated macrophages (AAMϕ) (Herbert, Holscher et al. 2004; Noel, Raes et al. 2004) and a higher frequency of apoptosis in immunopathogenic CD4 T cells (Rutitzky, Mirkin et al. 2003). However, during the chronic phase of infection, the initially protective Th2 response becomes detrimental to the host, and IL-13 was identified as responsible for mediating the development of severe egg-induced liver fibrosis (Chiaramonte, Donaldson et al. 1999; Wynn 2004).

In contrast, the immune response in CBA mice is mediated by the production of the proinflammatory cytokines IL-17 and IFN-γ in response to SEA (Rutitzky, Hernandez et al. 2005; Rutitzky, Bazzone et al. 2008). CBA mice also lack several of the protective mechanisms identified in BL/6 mice, including reduced expression of markers associated with AAMϕ and a decrease in frequency of apoptosis in immunopathogenic CD4 T cells. Further analysis of the CD4 T cell response identified another potentially important mechanism in the development of high pathology. As mentioned earlier

immunopathology is critically dependent on CD4 T cells sensitized to schistosome egg antigens. The most abundant of these antigens is *Schistosoma mansoni*-p40 (Sm-p40), which was originally described by Nene *et al* and subsequently described independently by our laboratory and several others. Sm-p40 is a small heat shock protein comprised of 354 amino acids with a molecular weight of 40 kDa. Stimulation with Sm-p40 elicits a strong Th1 biased proliferative response in high pathology mouse strains and using a panel of Sm-p40 specific T cell hybridomas our lab demonstrated that the CD4 T cell response to Sm-p40 is directed against the immunodominant peptide 234-246 (Sm-p40$_{234-246}$) (Asahi, Hernandez et al. 1999; Hernandez and Stadecker 1999; Stadecker 2001). Further analysis of the variable gene usage in these hybridomas revealed that almost all hybridomas specific for Sm-p40$_{234-246}$ utilized the TCR gene rearrangement Vα11.3Vβ8, indicating a potentially important role in the development of high pathology (Finger, Brodeur et al. 2005).

Immunodominance in disease is not a new phenomenon, however it is fairly remarkable that it is exerted by a peptide derived from the eggs of an organism as large and complex as a helminth. A classic example of immundominance stems from studies using infection with the protozoan parasite *Leishmania major* as a model and have shown that development of a lethal Th2 response in BALB/c mice is directed against a specific peptide derived from the LACK antigen and that removal of this peptide or the entire LACK antigen results in the development of protective immunity (Launois, Maillard et al. 1997). While immunodominance is well characterized in the *Leishmania* model the role of immunodominance *in vivo* in schistosomiasis remains unclear. *In vivo* studies have shown that CBA mice have a strong I-A^k restricted T cell response against Sm-

$p40_{234-246}$ and that this T cell population expresses the previously identified Vα11.3Vβ8 TCR gene rearrangement and is expanded following infection (Finger, Brodeur et al. 2005). However further experiments will be needed to clarify the exact nature and specific contribution of this T cell population to the development of severe pathology and will be the focus of chapter 1 of the results section of this thesis.

1.4 Genetics analyses of schistosome infection

Infection with schistosome parasites results in a great disparity of disease outcomes, ranging from mild intestinal disease to the severe hepatosplenic form among human patients. These differences make analysis of the schistosome infection ripe for genetic interrogation, and as such has given rise to a number of studies of human populations in endemic regions to assess the genetic basis of these observed differences. Initially, it had been reported that intensity of infection in a Brazilian pedigree was under the influence of a major gene (Abel, Demenais et al. 1991), providing further evidence that host genetic factors are significantly involved in determining disease outcome. This led Alain Dessein's group to the seminal discovery of the first major locus controlling intensity of schistosome infection (Marquet, Abel et al. 1996). In this analysis, the authors performed a genome wide association study using fecal egg counts to determine the infection level in patients and identified the locus *SM1* as responsible for the phenotype (Marquet, Abel et al. 1996). *SM1* mapped to a region on human chromosome 5, 5q31-q33, which contains a number of candidate genes including the cytokine

grouping of IL-4, IL-5, IL-12 and IL-13. This locus has also been associated with regulating IgE levels, which is known to be important for control of human schistosome infection (Marsh, Neely et al. 1994; Meyers, Postma et al. 1994; Dessein, Marquet et al. 1999). These findings were later independently confirmed in a Senegalese population in which intensity of infection also was significantly linked to 5q31-q33 (Muller-Myhsok, Stelma et al. 1997). Taken together these studies highlighted the importance of the *SM1* locus during schistosome infection and represent the first successful example of a genome-wide screen for a human infectious disease. Importantly they show the utility of genome-wide analysis to help understand the regulation of complex human infectious diseases, which undoubtedly paved the way for numerous other analyses of this type.

Although it was originally thought that disease progression was dependent on a patient's worm load (Cheever 1968; Cook, Baker et al. 1974), more recent evidence has shown that severe fibrosis only correlates with high infection in children (Homeida, Abdel-Gadir et al. 1988; Doehring-Schwerdtfeger, Abdel-Rahim et al. 1990; Domingues, Lima et al. 1993) and that hepatosplenomegally is not necessarily associated with high levels of fibrosis (Homeida, Abdel-Gadir et al. 1988; Doehring-Schwerdtfeger, Kaiser et al. 1992; Richter, Monteiro Eda et al. 1992). Therefore, infection level, although important, is not the only critical factor that determines disease progression in different individuals, particularly in the development of hepatic fibrosis. To address this, Dessein and colleagues used portable ultrasound machines to measure hepatic fibrosis in a Sudanese population whom had not been previously treated for disease. Multipoint linkage analysis identified a locus close to the *IFNγR1* gene located at 6q22-q23, that was significantly linked to hepatic fibrosis and was designated as *SM2 (Dessein, Hillaire et*

al. 1999). Furthermore, this locus was shown to be independent of *SM1* (Dessein, Hillaire et al. 1999), although it was not determined if an interaction occurred between these two loci. Given that intensity of infection is not necessarily a major factor in disease development, these data indicate that anti-infection and anti-disease immunity are under distinct major gene control, which may have a significant clinical impact in the future, particularly as the risk of praziquantel resistant schistosomes increases.

Despite these elegant studies, genetic analysis in humans remains incredibly complex and it is particularly difficult to identify polymorphic genes associated with disease. In contrast, the mouse represents a simpler, and importantly, a more testable model in which to analyze genetic contributions to disease, especially in the case of schistosomiasis, where the murine model so closely mimics the human disease. Our lab previously performed a genetic analysis of an F_2 cohort between low pathology BL/6 and high pathology CBA mice. Two genetic intervals were identified controlling IFN-γ production on chromosome 1 and 5, while a single locus was identified as linked to granuloma formation on chromosome 13 (Rutitzky, Hernandez et al. 2005). However, this analysis was done using a small cohort of mice and therefore only suggestive linkages to the phenotype were identified. Furthermore this study was completed before the emergence of IL-17 as a critical mediator of severe immunopathology. Additional studies are needed to precisely identify the genetic intervals and their candidate genes that significantly affect the outcome and severity of disease. There is great potential for discovery and enhancement of our knowledge of the schistosome infection by employing genetic analyses in mice, and strategies regarding this and the analysis of complex traits in general will be discussed later in the introduction.

Chapter 2

Th17 cells: Discovery, Differentiation, Disease and Schistosomiasis

2.1 Discovery and Differentiation of Th17 cells

The assertion that CD4 T cells can differentiate into various effector populations following antigen stimulation was originally suggested in 1986 when two seminal papers by Coffman and Mossman demonstrated that effector T cells could be categorized into two distinct populations, Th1 and Th2, and defined by their cytokine profiles (Coffman and Carty 1986; Mosmann, Cherwinski et al. 1986). Th1 cells are characterized by the production of large quantities of IFN-γ and enhance cell-mediated responses against viral infection and intracellular pathogens, while Th2 cells produce IL-4, IL-5 and IL-13 and are important for humoral immunity and the regulation of helminthic infections. The Th1, Th2 paradigm has been around for more than 20 years and as such the specific mechanisms required for the differentiation of these cells have been elucidated **(Figure 4)**. IL-12 is the canonical cytokine associated with induction of naïve T cells to differentiate into Th1 and the transcription factor T-bet was later identified as the master regulator of Th1 cell differentiation, Similarly, IL-4 and the transcription factor Gata-3 are required to coordinate the genetic program of differentiating Th2 cells (Zheng and Flavell 1997; Szabo, Kim et al. 2000; Szabo, Sullivan et al. 2002).

Regulation of effector T cell differentiation is critical as uncontrolled and persistent T cells responses can result in the development of autoimmunity. In particular, Th1-cell-mediated responses play a pivotal role in the development of several autoimmune and

inflammatory diseases, such as experimental autoimmune encephalomyelitits (EAE) (Krakowski and Owens 1996; Tran, Prince et al. 2000). However, later studies of CD4 T cell mediated autoimmunity revealed that in some instances loss of IFN-γ signaling failed to provide protective immunity and in some cases mice lacking IFN-γ or the IFN-γ receptor became even more susceptible to autoimmune disease, indicating the possibility of another effector cell population. The majority of *in vivo* data dissecting the role of Th1 cells in autoimmune pathogenesis was based on studies using mice that lacked the IL-12p40 subunit and therefore could not produce IL-12, resulting in the loss of Th1 cell development (Gran, Zhang et al. 2002; Zhang, Gran et al. 2003). In 2000, a dramatic paradigm shift occurred with the discovery of IL-23. IL-23 consists of the pairing of a unique p19 subunit with the p40 subunit also shared by IL-12, and studies by Cua *et. Al.* (Cua, Sherlock et al. 2003) demonstrated that in fact IL-23 primed Th17 cells were much more pathogenic than IL-12 primed Th1 cells in EAE models and ultimately along with work discussed below demonstrated that Th17 cells represented a unique CD4 T cell population.

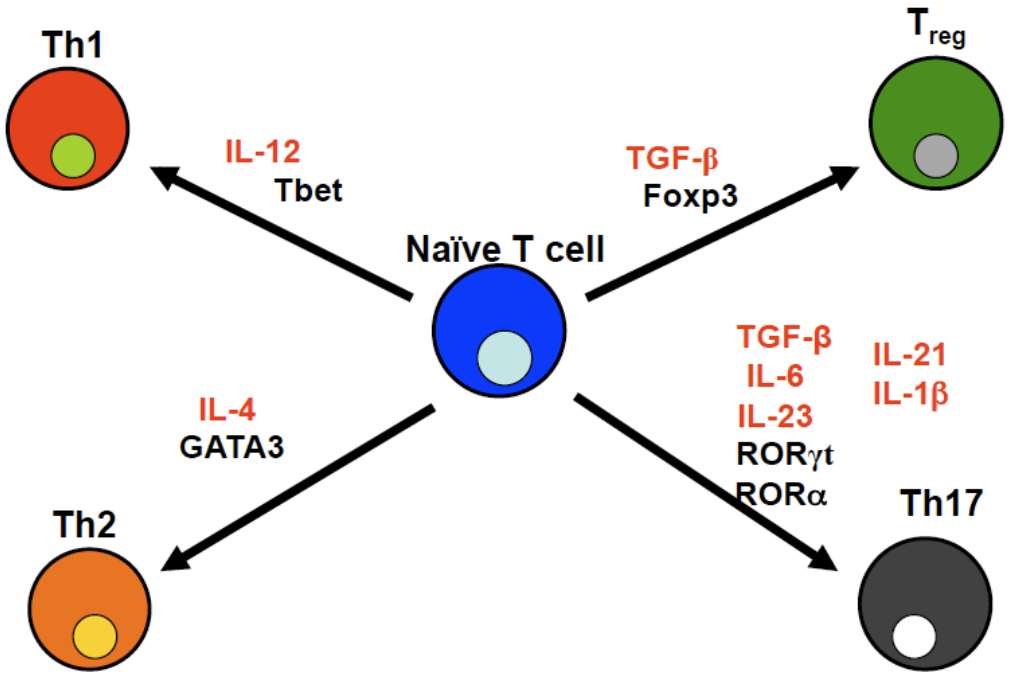


Figure 4. CD4 T cell effector subsets and their respective cytokines and transcription factors required for differentiation.

Since these pioneering experiments the cytokines and transcription factors necessary for the differentiation of Th17 cells have been extensively studied **(Figure 4)**. Three different research groups simultaneously demonstrated that IL-6 and TGF-β were required for the differentiation of naïve CD4 T cells into Th17 cells , while IL-23 is critical for the maintenance and expansion of this population (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006), while more recent evidence has shown that IL-1β alone is capable of driving Th17 cell differentiation (Chung, Chang et al. 2009). If Th17 cells are to represent a novel population than it is likely that their differentiation is governed by a unique set of transcription factors as well. Ivanov and colleagues discovered a splice variant of the gene *Rorc*, RORγt, as the unique transcription factor required for Th17 cell differentiation (Ivanov, McKenzie et al. 2006). It was also shown that IL-6 and TGF-β synergize to enhance transcription of RORγt and thus induce Th17 cell differentiation and providing further evidence for RORγt as the "master regulator" of Th17 cells (Acosta-Rodriguez, Napolitani et al. 2007; Kattah, Wong et al. 2008). Subsequently, RORα and interferon regulatory factor 4 (IRF-4) were also identified as Th17 cell inducing transcription factors either in combination with RORγt or in the case of IRF-4 on its own (Brustle, Heink et al. 2007; Sundrud and Rao 2008; Yang, Pappu et al. 2008). Similar to Th1 and Th2 cells, Th17 cells produce a specific cytokine profile, including its namesake cytokine IL-17A, as well as IL-17F, IL-22, IL-6, TNF-α as well as the neutrophil chemoattractants, CXCL1 (gro-α), CXCL2 (gro-β) and CCL2 (MCP-1) (Korn, Bettelli et al. 2009). Importantly, because Th17 cells are such potent proinflammatory mediators a number of immunological mechanisms are in place to limit their development and expansion (McGeachy and Cua 2008).

Much as there is cross-regulation between Th1 and Th2 cells, these subsets also can regulate Th17 cell differentiation. Addition of the Th1 and Th2 polarizing cytokines IL-12, IFN-γ and IL-4 to cultures inhibited the Th17 cell inducing capabilities of IL-6, TGF-β and IL-23 (Murphy, Langrish et al. 2003; Harrington, Hatton et al. 2005; Park, Li et al. 2005). Furthermore, T-bet knockout mice, which do not produce IFN-γ, have greater numbers of Th17 cells, and our lab has shown that this leads to a dramatic increase Th17-mediated immunopathology in a model of severe schistosomiasis (Rutitzky, Smith et al. 2009). However this cross-regulation is more complex than originally realized and in several models Th17 cells where shown to co-produce IFN-γ, indicating that it may not always be capable of downregulating Th17 cell differentiation and may, in some instances, contribute to Th17 cell-mediated pathology (Chen, Langrish et al. 2006; Acosta-Rodriguez, Napolitani et al. 2007; Annunziato, Cosmi et al. 2007).

In addition to the cross-regulation among Th1, Th2 and Th17 cells, CD4 T regulatory cells (Treg), not discussed in detail here, are major anti-inflammatory mediators that also regulate Th17 cell differentiation. Tregs mainly inhibit Th17 cell differentiation through the production of TGF-β, which also highlights the importance of the cytokine milieu in T cell differentiation, as TGF-β alone inhibits Th17 cell differentiation; however, TGF-β in combination with IL-6 enhances it (Chen, Jin et al. 2003; Bettelli, Carrier et al. 2006). Additional regulatory mechanisms include IL-27, another member of the IL-12 cytokine family, which negatively regulates Th17 cell development mainly by inducing the Th1 cell associated transcription factors T-bet and STAT1 (Kastelein, Hunter et al. 2007), and retinoic acid, which induces Foxp3

expression, thereby inhibiting Rorγt and Th17 cell differentiation (Mucida, Park et al. 2007; Elias, Laurence et al. 2008).

2.2 Th17 cells and Disease

It has become increasingly accepted that Th17 cells are responsible for mediating disease in a number of animals models previously thought to be Th1 cell mediated. As mentioned previously Th17 cells were identified as a distinct effector T cell population in models of EAE when mice deficient IL-23p19, which have normal Th1 responses, did not develop EAE due to a lack of encaphalitogenic IL-17 producing CD4 T cells (Langrish, Chen et al. 2005). Abrogation of IL-17 signaling via knockout or administration of *in vivo* antibodies has identified it as the critical cytokine in a number of other autoimmune models, including collagen induced arthritis (CIA), uveitis, psoriasis and inflammatory bowel disease (IBD) (Nakae, Nambu et al. 2003; Kobayashi, Liu et al. 2005; Zhang, Zheng et al. 2006; Ouyang, Kolls et al. 2008; Miossec 2009). More recent reports have shown that Th17 cells gain entry into the central nervous system via expression of the IL-23R or CCR6 where they are able to mediated inflammatory immunopathogenic processes (Awasthi, Riol-Blanco et al. 2009; Reboldi, Coisne et al. 2009).

While Th17 cells have mainly been studied in the pathogenesis of autoimmunity, it is unlikely they would have been evolutionarily selected if they did not also have a beneficial role to the host. As such, subsequent studies have shown that the production of IL-17 is critical for host defense against infection with intracellular pathogens

(McGeachy and Cua 2008). One of the first documented activities of IL-17 was an increase in granulopoiesis and in neutrophil develoment and recruitment. Studies using IL-17 knockout mice revealed that the lack of these processes led to increased susceptibility to *K. pneumoniae* infection, caused by decreased production of granulocyte-colony stimulating factor (G-CSF) and CXCL1, which led to impaired neutrophil recruitment in the lung (Ye, Rodriguez et al. 2001; Happel, Dubin et al. 2005). IL-17 deficient mice were also highly susceptible to infection with *Bacteroides fragilis*, *Toxoplasmosis gondii* and *Candida albicans* (Huang, Na et al. 2004; Kelly, Kolls et al. 2005). Interestingly, these mice were not susceptible to infection with *Mycobacterium tuberculosis* or *Listeria monocytogenes* (Khader, Bell et al. 2007), indicating that IL-17 may not be critical for defense against intracellular pathogens. Thus, these findings indicate that depending on the model, the IL-23/IL-17 inflammatory axis may be active in both host defense and the development of immunopathology.

2.3 Th17 cells in Schistosomiasis

As mentioned previously, laboratory mice infected with *S. mansoni* develop a distinct heterogeneity of disease severity. Initially the development of severe disease was considered to be Th1-cell-mediated, however similar to models of EAE, IL-17 was later identified as a potentially more important mediator of severe immunopathology. IL-12 consists of two subunits, IL-12p40 and IL-12p35, In studies by Rutitzky *et. al* it was shown that mice deficient in IL-12p40 developed mild disease, however mice deficient in IL-12p35 still developed severe pathology despite producing little to no IFN-γ (Rutitzky,

Lopes da Rosa et al. 2005). This indicated that another inflammatory cytokine was likely responsible for the development of severe disease and using mice deficient for IL-23p19 our lab identified this as IL-17 (Rutitzky, Bazzone et al. 2008). Subsequent studies revealed that IL-17 production correlated with increased hepatic immunopathology in an F_2 population and *in vivo* neutralization of IL-17 reduced severe pathology in CBA mice (Rutitzky, Lopes da Rosa et al. 2005; Smith, Shainheit et al. 2009). Furthermore, co-cultures of dendritic cells and naïve CD4 T cells from high pathology CBA mice produced significantly more IL-17 than those from BL/6 mice in response to schistosome eggs. Production of IL-17 in co-cultures from CBA mice was dependent on IL-23 and IL-1β and importantly, no IFN-γ was produced indicating that dendritic cells from high pathology prone mice responding to schistosome eggs drive naïve CD4 T cells towards a Th17, and not a Th1 cell phenotype (Shainheit, Smith et al. 2008). Taken together these data show that IL-17 producing CD4 T cells are critical for the development of severe schistosomiasis and a more likely marker of severe disease than Th1 cells.

Chapter 3

Genetic Analysis of Complex traits

The genetic analysis of complex traits has been critical to our understanding of the molecular mechanisms that underlie disease processes. Most common diseases are considered complex traits whose phenotypes vary widely in a population and are influenced by multiple genes as well as by gene-gene interactions and environmental factors. Much of the genetic variation that leads to disease susceptibility is governed by loci that have quantitative effects on the disease phenotype and the identification and analysis of these loci is an important measure in understanding the molecular basis of complex traits. Currently, there is increased focus on understanding the pathogenesis of complex diseases, including many autoimmune diseases. Murine schistosomiasis is an extensively characterized model of T cell mediated tissue damage and inflammation, and shares many mechanistic features with other T cell mediated inflammatory and autoimmune diseases. The marked phenotypic heterogeneity that develops in our model indicates that genetic differences play a significant role and is therefore ripe for genetic interrogation. In this section I will discuss some of the strategies used to analyze complex traits with the overall goal being the identification of genes responsible for variation in disease susceptibility and resistance.

3.1 Forward vs Reverse Genetics

Genetic studies can be broadly classified into two categories: reverse genetics and forward genetics. Reverse genetics is a gene driven approach and relies on prior knowledge about the gene and some of its functions. It is commonly used to investigate the full range of functions of a known gene, typically by either knocking the gene out, adding back the gene after manipulation or by over-expression of the gene. In fact, the use of knockout mice has been instrumental in identifying novel functions for known genes and in advancing our knowledge of a number of immune related processes (Akira and Takeda 2004). However, reverse genetics can have drawbacks when studying complex diseases in that it requires prior knowledge of a gene and is limited to the study of a single gene. In most cases the genes that underlie disease susceptibility are not known and furthermore deletion and study of a single gene is not an ideal model for analysis of non-Mendelian fashion, as these are likely conferred by multiple genes, making it difficult to study the contributions of a single known gene.

In contrast, forward genetics relies on naturally occurring or experimentally induced genetic variation in mice or other species to identify genes and represents a phenotype driven approach to gene discovery. The fundamental principle of this approach is to identify phenotypic differences in a model and then use this to identify allelic variation in the genome responsible for these differences. Forward genetics is particularly useful to the study of complex traits because of its inclusive nature as it allows for the potential identification of all contributors into a phenotype. While a major strength of this approach is that it often identifies completely new unthought-of phenomena, it is also a weakness in that these discoveries can be completely “out of the

blue" and therefore difficult to interpret. Nevertheless, no other technique has matched the proven ability of forward genetics to identify new genes and novel functions for previously identified genes (Beutler, Du et al. 2007).

3.2 Quantitative Trait Loci

The major goal of forward genetics analysis is to identify the genes responsible for variation of a heritable phenotype. In the case of complex diseases, much of the genetic variation that leads to disease susceptibility is governed by loci that have quantitative effects on the disease phenotype, termed quantitative trait loci (QTL) (Abiola, Angel et al. 2003). Practically speaking a QTL is a genetic locus whose alleles cause variation in the phenotype of interest. Classically inbred mouse strains have been traditionally used for mapping of QTL because much phenotypic variation exists between inbred strains and because the homogeneity of their genetic backgrounds simplifies the process of mapping. Genetic mapping of QTL is a powerful approach that is used to assess if a linkage exists between individual genetic loci and an experimentally determined phenotype. The goal is to identify genetic intervals that are shared between individuals with the same phenotype and differ between affected and unaffected individuals, i.e. "linked" to disease onset.

QTL mapping starts with the choice of parental inbred mouse strains; typically, mouse strains are chosen that are as distinct as possible, both genetically and phenotypically, as this will increase the likelihood of identifying QTL. The ability of forward genetics to identify of QTL relies on meiotic recombination events. To generate a panel of mice with genetic variation, inbred mouse strains are crossed to produce F_1 mice. These F_1 mice will have one copy of every chromosome from each parent.

However, homologous recombination induces random crossover events that result in a single chromosome with genetic information from both parents. Much information can be gleaned from F_1 mice, including whether or not one parental strain has a dominant effect or if inheritance of the trait is influenced by both parents. F_1 mice are then intercrossed to produce F_2 mice or backcrossed to a parental strain to generate N_2 mice depending on the type of mapping experiment. The resultant offspring will have a random genetic assortment of loci from each parent, which can be used to identify QTL. The offspring are next phenotyped and their genotypes can be determined using microsatellite markers or SNPs. A genetic interval is considered to be "linked" to the phenotype if the observed phenotype in progeny mice matches the genetically expected phenotype based on the phenotypes of the parental strains at ratio greater than would be expected to occur by chance (Lander and Kruglyak 1995).

QTL mapping has proven very successful in the identification of loci, which are responsible for the original phenotypic variation seen in the parental strains. However, QTL mapping does have two major disadvantages, both relating to time and resources. The first disadvantage deals with identification of QTL itself. The average "effect" size of a QTL, defined as the percentage of total variance in phenotype a given QTL controls, is approximately 5%. To put this in perspective it is estimated that approximately 300 mice would be require to identify a QTL with an effect size of 5% in a standard F_2 cross (Flint, Valdar et al. 2005). Given that complex diseases likely contain many QTL, each with a small effect size, 300 mice would probably only reveal the ones with the largest effect. The second and more important disadvantage deals with identification of the causal genes that govern these QTL. Assuming a traditional mapping strategy using

markers that are spaced roughly every 20 centimorgans (cM) across the genome a QTL with a 5% effect size can be mapped with a 95% confidence interval (CI) of about 40cM (Flint, Valdar et al. 2005). In the mouse 1 cM corresponds to about 2 megabases and while gene density varies throughout the genome, 1 megabase typically contains about 10 genes. Therefore, while the identification of QTL using the strategy outlined above can be completely in anywhere from 6 months to a year, depending on the phenotype, the researcher may still be left with greater than 500 genes potentially responsible for the QTL. Thus, moving forward it will be critical to test new strategies for the identification of quantitative trait genes (QTG)s (Peters, Robledo et al. 2007).

3.3 Strategies for Narrowing QTL

QTL analysis is a powerful tool for identifying loci that underlie complex traits, however identifying the causal genes that represent each QTL has been very challenging (Flint, Valdar et al. 2005). While there have been many successes in identifying identify QTGs there remain multiple times more that have yet to be identified. In this section I will detail several experimental and bioinformatics strategies that have been proposed for narrowing QTL that are relative to this thesis, with the identification of QTGs being the ultimate goal.

Interval-specific congenic mice are still considered the mainstay of fine mapping QTL previously identified in an initial F_2 intercross (Bolivar, Cook et al. 2001). Once a QTL has been identified, F_1 mice are then backcrossed for several generations (at least 5) to one of the parental strains, selecting for the QTL of interest by using genetic markers at each successive backcross. Resultant animals are then intercrossed to yield mice homozygous for the recombinant haplotype. Interval-specific congenic mice provide

several advantages, by backcrossing over several generations other interfering QTLs are removed which may impede the analysis and allows for confirmation of the QTL. These congenic mice can then be used for further fine mapping by producing subcongenic mice. Subcongenic mice have a shorter differential segment then their parental congenics and can be used to subdivide the critical region into several segments, which can then be individually tested for the QTL. This will allow to significantly reduce the confidence interval and eventually lead to the identification of candidate genes that affect the phenotype (Fehr, Shirley et al. 2002). This method does have risks, as it is possible that the QTL in question may require interactions with other genomic segments to exert its effect. Therefore, if backcrossing based on genotype, it is important to continually test each successive backcross for the presence of the desired phenotype.

While the use of congenic mice is an important and useful tool for narrowing QTL, it is very time consuming and can still leave the researcher with a number of candidate genes that can not be feasibly tested, therefore new methods are needed for narrowing QTL. One such technique, proposed in 2002 by Wayne and McIntyre, is to combine QTL mapping with microarray analysis (Wayne and McIntyre 2002). Several studies have shown that regulatory variation in genes is important in a variety of complex traits and that quantitative expression studies, such as microarray analysis, can reveal these variations (Mackay 2001). Gene expression profiling of known QTLs is typically done comparing congenic mice with inbred strains to remove unwanted background signals, however it can be just as effective when comparing parental strains and has been used to identify a number of candidate genes associated with QTLs including *CD36*, which affects insulin-fatty acid metabolism, complement factor 5, which influences

susceptibility in a model of asthma, and *Ifi202*, which increases susceptibility to systemic lupus, to name a few (Karp, Grupe et al. 2000; Rozzo, Allard et al. 2001). There are potential limitations to this approach as nucleotide variants that alter protein structure and function may not alter expression levels, however a highly regulated gene that is located within a QTL is likely to be a very strong candidate gene.

To this point I have discussed QTL loci and how they are identified and a few strategies with which to shrink the number of candidate genes to a testable level. It is important to note that, as mentioned above, candidate genes usually contain variances in their DNA sequences, termed quantitiative trait nucleotides (QTN)s which in the majority of cases are often used to define and identify candidate genes. These DNA sequence polymorphisms that affect expression or function of a protein are considered the molecular basis for QTL, thus making it very important to identify sequence variants between strains that could lead to expression differences that may identify the causal gene(Abiola, Angel et al. 2003; DiPetrillo, Wang et al. 2005). Since the original sequencing of the BL/6 genome a number of other mouse genomes have been completed making it possible to search for sequence polymorphism between increasingly more strains *in silico*. If the genomes of original parental strains have been sequenced then it is possible to use this approach to narrow the number of candidate genes within a QTL with proven success (Burgess-Herbert, Cox et al. 2008; Stylianou, Affourtit et al. 2008). A blueprint was developed by Marshal *et al.* to identify non-synomous sequence polymorphisms *in silico,* and using this approach they were able to narrow 121 genes within an 8-Mb region to only six candidate genes (Marshall, Godden et al. 2002). This approach has limitations, specifically when searching for QTNs in regulatory or non-

coding regions, however, one can imagine that combining QTL and expression data as discussed above with sequence comparisons could be a very powerful approach.

The continued sequencing or more and more mouse genomes and an increase in the number and quality of public sequence, expression and genotype databases has resulted in a number of bioinformatics techniques that have been increasingly used to narrow QTL (Peters, Robledo et al. 2007). QTLs researchers are able to use these databases as an *in silico* approach for candidate gene identification, particularly for analysis of gene expression differences and to identify nucleotide sequence variants. Two other methods have resulted from the increase in bioinformatics information; the first is the ability to combine data from multiple crosses of inbred mice and the second is the ability to perform haplotype analysis across several mouse strains in order to increase the resolution of quantitative trait loci mapping (Cuppen 2005; Li, Lyons et al. 2005). Combined cross analysis relies on the fact that most of the genetic variation between inbred mouse strains is due to ancestral variation (Frazer, Wade et al. 2004) and therefore if the same QTL is detected in multiple different inbred crosses it likely represents the same ancestral polymorphic loci and thus the same causal gene. This allows data from existing QTL crosses to be combined to narrow the QTL interval without performing additional experiments. Combining crosses increases the total number of mice tested as well as the density of the map generated, altogether leading to a smaller QTL interval with increased resolution. Importantly, by increasing the power and resolution of the QTL, combined cross analysis increases the ability to detect QTLs with a smaller effect size and is also able to resolve closely linked QTLs that might otherwise present as a single peak (Li, Lyons et al. 2005). This method has been used to successfully narrow

QTL in a number of models (Park, Clifford et al. 2003; DiPetrillo, Tsaih et al. 2004; Ishimori, Li et al. 2006).

Haplotype analysis is a completely *in silico* approach to narrowing QTL intervals based on strain distribution patterns of haplotypes in inbred mice (Grupe, Germer et al. 2001). The inbred mouse genome is known to have originated from a limited number of founders and is made up of a mosaic structure primarily consisting of the two major ancestral subspecies *Mus musculus musculus* and *Mus musculus domesticus*, with only a minor contribution from the third major subspecies *Mus musculus castaneous (Wade, Kulbokas et al. 2002)*. It is also known that within a given species discrete patterns of contiguous polymorphic alleles are observed and that these shared polymorphic alleles stem from ancestral meiotic crossovers. Within a given population the number of discernable haplotypes is directly related to the number of ancestral lineages represented in that group (Burgess-Herbert, Cox et al. 2008). This is especially true in inbred mouse strains whose lineage has only been established for ~75 years and whom were derived from a limited set of founders (Yang, Bell et al. 2007). This results in laboratory mouse strains having large regions of the genome that are inherited by descent (IBD), many of which will be shared between inbred mouse strains, and is the basis for haplotype mapping using single nucleotide polymorphisms (SNPs). Because 97% of the genetic variation between inbred mouse strains is ancestral, it is unlikely that shared regions of the genome, which are inherited by descent, will contain the causal polymorphism underlying the QTL (Wiltshire, Pletcher et al. 2003). To put this in practical terms, if a QTL is identified in crosses between mouse strains A and B, and A and C, but does not exist in a separate cross between mouse B and C then the causal polymorphism will be

contained in a region in which the sequences from B and C are the same but different from A.

The techniques discussed here and others for narrowing QTL, are extensively discussed in more detailed reviews (Mackay 2001; DiPetrillo, Wang et al. 2005; Flint, Valdar et al. 2005). However one thing is clear that as more and more bioinformatics information becomes available it will only aid in the search for quantitative trait genes. Dipetrillo *et al.* recently published a systematic method to narrowing QTL that have been previously identified using some of the traits discussed here and others known as the bioinformatics toolbox (DiPetrillo, Wang et al. 2005; Hillebrandt, Wasmuth et al. 2005). This approach outlines a strategy using methods such as combined cross and haplotype analysis to narrow a QTL to a manageable number of genes, from which candidate genes can then be further tested by expression and sequence analysis. Still, some of these methods, particularly those that are *in silico,* are still controversial as to how likely they are to identify causal genes, because they are often not based on experimental evidence and can potentially eliminate causal genes from the analysis. Regardless, when used in concert with experimental methods, these bioinformatic approaches will provide investigators with more tools for identifying strong candidate genes, which can then be tested *in vivo* using knockout or transgenic mice.

3.4 Wild-Derived Mice as a Genetic Tool

Thus far, all of the methods for genetic analysis discussed have been performed using classically inbred mouse strains. Classically inbred mice have been instrumental in the genetic analysis of innate and adaptive immunity in a number of disease models. Observations of the immune response on a molecular level in laboratory mice are highly

reproducible and often recapitulate parallel human processes (Waterston, Lindblad-Toh et al. 2002; Mestas and Hughes 2004). Particularly inbred mice have been used to identify a number of genetic loci that affect disease processes. However, a caveat to genetic analysis in classically inbred mice is that these strains are derived from a relatively restricted number of founders predominantly within the *Mus. mus domesticus* subspecies **(figure 5 and table I)** and therefore do not reach the level of diversity observed in humans, thus limiting their utility for phenotypic screening (Frazer, Eskin et al. 2007; Yang, Bell et al. 2007). Therefore given the polygenic nature of most human diseases it is not surprising that other methods, such as ENU mutagenesis, have been increasingly used to study the genetics of the immune response (Beutler, Jiang et al. 2006; Beutler, Eidenschenk et al. 2007). Wild-derived mice, which diverged from a common ancestor with classical strains more than one million years ago, provide a genetically diverse model more suitable for studies of host-pathogen interactions. As a result of this early divergence, many of the wild-derived strains have large genomic regions originating from the subspecies *M.m. musculus* and *M. m. castaneus* resulting in greater genetic diversity and thus increasing the likelihood of phenotypic variation in these strains **(figure 5 and table I)** (Frazer, Wade et al. 2004; Frazer, Eskin et al. 2007; Yang, Bell et al. 2007)**.** Furthermore novel phenotypes that are identified are likely to have increased biological relevance given that they have arisen in an evolutionarily driven context. Importantly, comparison of wild-derived mice with classical inbred strains may prove ideal for studying complex humans diseases by revealing differences in even simple gene networks. Thus, wild-derived mice represent an important new resource in the analysis of complex phenotypes and immune networks.

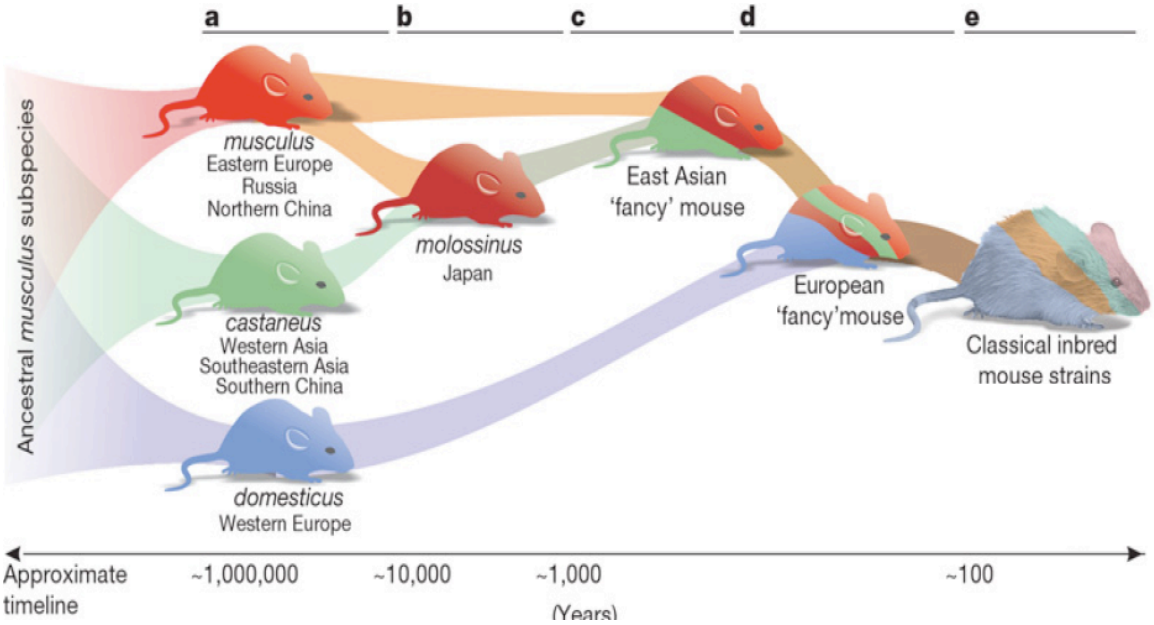


Figure 5. The origin of the classically inbred mouse. (A) Classically inbred mice are derived from predominantly three main subspecies, *Mus musculus domesticus, Mus musculus castaneus and Mus musculus musculus.* (B) Approximately 10,000 years ago the *molossinus* subspecies formed from a convergence of *mus musculus and castaneus. (C) The molossinus and musculus* subspecies were then inter-bred by Asian "fanciers" to produce mice with a variety of coat colors and behavioral characteristics as pets. (D) Soon after "fancy" mice from Asia were imported to England and bred with local mice. (E) In the early 1900s, a limited number of "fancy" mice were brought to the US and used as breeders to give rise to the majority of common classical strains used today. Figure adapted from Frazer et al (Frazer, Eskin et al. 2007)

Table I. Relative contribution of the three main subspecific lineage to the genomes of laboratory mice[1]

	B6	DBA/2	A	BALB/C	C3H	AKR	129S1	NZW	FVB	NOD	BTBR	KK	MOLF
M. m. domesticus	0.92	0.91	0.94	0.95	0.92	0.94	0.91	0.87	0.96	0.93	0.92	0.86	0.11
M. m. musculus	0.07	0.07	0.05	0.04	0.07	0.05	0.08	0.11	0.03	0.06	0.06	0.12	0.74
M. m. castaneus	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.02	0.02	0.15

[1] Table adapted from Yang et. al. (Yang, Bell et al. 2007)

Chapter 4

Summary and Goals of Thesis

Schistosomiasis is a complex human parasitic infection with a major socioeconomic impact throughout parts of South America, Africa and Asia. While treatment with PZQ is effective, schistosomiasis remains a significant public health risk as it claims 200,000 victims per year. For this reason, a greater understanding of its mechanisms of pathogenesis is important for disease prevention. Furthermore, because immunopathology is the result of a CD4 T cell hypersensitivity reaction and as such shares mechanistic features with many T cell mediated autoimmune diseases, it represents an excellent model to study T cell based diseases.

The goal of the work presented here is to understand the genetic mechanisms through which inbred mice control strain specific differences in immunopathology during schistosome infection, with the intention of identifying genes that regulate severe disease. To investigate this, we performed several genetic analyses, including QTL, and using congenic mice we identified several loci that control severe immunopathology. Then using prior knowledge of *in vitro* analyses of wild-derived mouse strains, we characterized the wild-derived MOLF mouse as a novel model of high pathology and using these mice, identified IRAK-2 as a novel mediator of Th17 cell development and severe schistosome infection.

SECTION II: MATERIALS AND METHODS

Results chapter 1

Mice and infection

Five to six week old female CBA/J, BALB/c and BALB/k mice were purchased from The Jackson Laboratory (Bar Harbor, ME). CBA/d congenic mice, as described below, were bred in our colony at Tufts University School of Medicine. All mice were maintained at the Animal Facility at Tufts University School of Medicine in accordance with the American Association for the Assessment and Accreditation of Laboratory Animal Care Guidelines. All mice were age-matched and infected between 6-10 weeks of age i.p. injection with 85 cercariae of *S. mansoni* (Puerto Rico strain). Cercariae were obtained from infected *Biomphalaria glabrata* snails, provided to us by Dr. Fred Lewis, the Biomedical Research Institute, through National Institutes of Health/National Institute of Allergy and Infectious Diseases Contract NO1-AI-55270. All mice were studied after seven weeks of infection. **This infection protocol is standard in our laboratory and is the same for each chapter of the thesis.**

Standard cell preparations, cell cultures and cytokine determinants

The following represents standard preparations for analysis of bulk mesenteric lymph node cells (MLNC), granuloma cells (GC) and purified CD4 T cells used throughout the results section of this thesis unless otherwise noted. Livers and mesenteric lymph nodes (MLN) were removed aseptically from 7-week infected mice. Single cell suspensions from MLN were prepared by teasing the tissues in complete

RPMI-1640 medium (cRPMI) supplemented with 10% fetal calf serum (Atlanta Biologicals, Atlanta, GA), 4 mM L-glutamine, 80 U/ml penicillin, 80 μg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES, 1X NEAA (all from BioWhittaker, Walkersville, MA) and 0.1% 2-mercaptoethanol. Erythrocytes were lysed by exposure to Tris ammonium chloride buffer pH: 7.2 (Sigma) for 15 min on ice. Cells were washed and live cells that excluded trypan blue were counted and resuspended at the desired concentrations in cRPMI. For purification of CD4 T cells, MLNC were negatively selected on CD4 MACS columns (Miltenyi Biotec, Auburn, CA) following the manufacturer's instructions. The resulting cell preparations were > 94% CD4+ cells as determined by flow cytometry. Granuloma cells (GC) were obtained by homogenization of the livers in a Waring blender, isolation of granulomas by 1 g sedimentation, extensive washing and enzymatic digestion with 1 mg/ml of collagenase type H, from *Clostridium histolyticum* (Sigma Chemicals) as previously described (Rutitzky, Hernandez et al. 2001).

For the cell transfer experiments, CBA or BALB/k recipient mice were sublethally irradiated (~500 rad) 3 days prior to infection, allowed to recover for a period of 12 hours and subsequently injected i.v. with 10 x 10^6 naïve splenic CD4 T cells from CBA or BALB/k donor mice, purified by negative selection as described above..

Bulk MLNC and GC suspensions (5 x 10^6 cells/ml) or purified CD4 T cells from MLN (1 x 10^6 cells/ml) plus normal irradiated syngeneic splenic APC (4 x 10^6 cells/ml), were incubated in the presence or absence of 15 mg/ml of SEA. After 48 hours, the culture supernatants were removed, filtered and stored at -36°C until analysis by ELISA. For IL-4, IL-5 and IL-10 Ab, standard cytokines and protocols were obtained from BD-

PharMingen (San Diego, CA), and for IFN-g, IL-17, IL-6 and TNF-a from R&D Systems, Inc. (Minneapolis, MN).

Histopathology assessment by morphometric analysis

Histopathology was assessed by standard procedure used in each chapter of the results section. Liver samples from all mice were fixed in 10% buffered formalin and processed for routine histopathological analysis; 5-μm sections were stained with hematoxylin and eosin. The extent of hepatic granulomatous inflammation around schistosome eggs was measured by computer-assisted morphometric analysis using Image-Pro Plus software (Media Cybernetics) as described previously (Rutitzky, Hernandez et al. 2001). The lesions were assessed blindly by an observer unaware of the experimental parameters. To accurately reflect the true magnitude of the granulomatous inflammation only those granulomas with a single visible central egg were counted. A minimum of 20 granulomas were counted per section with more than one section counted per liver. Mean granuloma size was measured in square micrometers ± SEM.

Congenic strain development

To develop CBA/d congenic mice, BALB/c and CBA/J mice were first bred to produce F_1 mice. These mice were then backcrossed to CBA/J mice to produce N_2 animals.

Selection of breeders at this stage and subsequent backcrosses was based on genotyping at the microsatellite marker D17Mit34 (position at 34 Mb) located within the MHC locus on chromosome 17. Mice were backcrossed for 7 additional generations with selection for this markers followed by intercrossing of N_8 animals to isolate donor homozygotes. The resultant mice were selected for BALB/c homozygotes, yielding a CBA mouse with a haplotype that is H-2^d (CBA/d), and constituted the finished congenic strain. To ensure proper generation of the CBA/d mouse, the background was verified by PCR analysis at several randomly selected chromosomes as well as in the H-2 locus, while MHC expression was verified by FACS analysis. For FACS analysis, we obtained two antibodies from BD pharmingen, one of which was specific for the MHC class I alloantigen H-$2K^k$ and the other for H-$2K^d$. These antibodies are representative of the entire H-2 locus.

Real time quantitative RT-PCR

Total RNA was isolated from the MLNs of infected mice using Trizol as per manufacturer's instructions (Invitrogen). RNA (0.5-2μg) was subjected to DNASE I treatment (Roche Molecular Biochemicals) and reverse transcribed using the high capacity cDNA reverse transcription kit from Applied Biosystems. Real-time quantitative RT-PCR was performed on 10ng of cDNA from each sample either SYBR green or Taqman analysis using an ABI 7300 instrument per manufacturere's instructions. GAPDH levels were used to normalize the data. All Taqman probes were obtained from Applied Biosystems. Using the average mean cycle threshold (Ct) values for GAPDH and the gene of interest for each sample, the equation 1.8 e (Ct GAPDH – Ct gene of

interest) x 10^4 was used to obtain normalized values (Chen, Langrish et al. 2006). For SYBR green analysis of TCR rearrangement, primers were as previously described (Finger, Brodeur et al. 2005): Vα11.3F – caatttttctatcgccacaacaa, Vα11.3R – aggctgccctgggatt, J36R – caaggttgacctgaagtcttgt. The amount of Vα11.3J36 transcripts was expressed as a ratio of Vα11.3J36 over total Vα11.3 transcripts.

Statistical analyses

ANOVA and Student's *t* tests were used to determine the statistical significance of the differences between groups and were calculated with GraphPad Prism.

Results Chapter 2

Mice and infection

BL/6, SJL/J and B6SJLF1/J (F_1) mice, 5-6 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME). 150 male and female BL/6 x SJL/J F_2 mice were bred in house by F_1 brother x sister mating. The interval specific congenic mice, as described below, were also bred in our colony at Tufts University School of Medicine. All mice were maintained at the Animal Facility at Tufts University School of Medicine in accordance with the American Association for the Assessment and Accreditation of Laboratory Animal Care Guidelines. Infection protocol is as described in chapter 1 of the materials and methods.

Histopathology assessment by morphometric analysis

Histopathology was completed as described above in chapter 1 of the materials and methods

Cell preparations and cytokine determinations

All bulk MLNC cultures and cytokine determinants were performed as described in chapter 1 of the materials and methods.

Genetic mapping

DNA was extracted for genotyping from tail biopsies using DNAeasy tissue kit (Qiagen) or DirectPCR (tail) (Viagen) according to manufacturer's instructions. Single strand conformation polymorphic loci were selected from available polymorphic microsatellite markers (Mouse genome informatics (MGI) or www.cidr.jhmi.edu); primers were obtained from Integrated DNA Technologies (www.idtdna.com). Microsatellite marker positions were obtained from the Jackson Laboratory Mouse Genome Database (www.informatics.jax.org). A panel of 100 primer sets that readily distinguish BL/6 and SJL alleles was used for genotyping the F_2 mice. Together, the markers spanned all 19 autosomes and the X chromosome with an average intermarker distance of approximately 20 Mb (~10 cM). Standard PCR was performed at an annealing temperature of 55°C for each primer pair. Amplified products were electrophoresed in 3-4% agarose gels and visualized by ethidium bromide staining with UV transillumination. Data were analyzed by Mapmaker QTL and R/QTL (Broman) using the J/QTL interface (Jackson Labs). Mapmaker is a quantitative trait loci mapping program that tests whether markers show

evidence of linkage to the tested phenotypes. J/QTL is a graphical user interface for R/QTL, which is a powerful statistical software program used for mapping QTL in experimental crosses. Mapmaker and R/QTL programs were also used to determine epistatic interactions between loci. Both programs returned similar results. The linkage was considered highly significant if the logarithm of the odds (LOD) favoring linkage score exceeded 3.3 according to the system established by Lander and Kruglyak (Lander and Kruglyak 1995) in the context of a genome search using an intercross study.

Congenic strain development

B6SJLF1/J mice were backcrossed to SJL/J mice to produce N_2 animals. Selection of breeders at this stage and subsequent backcrosses was based on genotyping at three markers within and flanking the QTL on chromosome 4 (between *D4Mit308* at 123.8Mb and *D4Mit256* at 154Mb, including peak marker *D4Mit203*) and chromosome 17 (between *D17Mit133* at 25Mb and *D17Mit180* at 57Mb, including peak marker *D17Mit82*). Mice were backcrossed for 8 additional generations with selection for these markers followed by intercrossing of N_9 animals to isolate donor homozygotes. The resultant mice constituted the finished congenic strains, SJL.B6-*D4Mit203*, SJL.B6-*D17Mit82* and double congenic SJL.B6-*D4Mit203*:*D17Mit82*, representing mice that were homozygous for a donor (BL/6) segment from the QTL identified by peak marker *D4Mit203*, *D17Mit82*, or both, respectively.

Statistical Analysis

One-way ANOVA was used to determine statistically significant differences between groups of mice. $P < 0.05$ was considered significant and statistical analysis was performed with GraphPad Prism (GraphPad Software).

Results Chapter 3

Mice and infection

BL/6 and BL/10 mice, aged 5-6 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME). F_1 mice were bred in house by crossing BL/6 females with BL/10 males. 35 female BL/6 x BL/10 F_2 mice were bred in house by F_1 brother x sister mating. The B6.B10 interval specific congenic mice were provided to us as part of a collaboration with Derry Roopenian and Tom Sproule at the Jackson Labs (http://research.jax.org/faculty/roopenian/roop_strains.html). All mice were maintained at the Animal Facility at Tufts University School of Medicine in accordance with the American Association for the Assessment and Accreditation of Laboratory Animal Care Guidelines. Infection protocol is as described in chapter 1 of the materials and methods.

Histopathology assessment by morphometric analysis

Histopathology was completed as described above in chapter 1 of the materials and methods

Cell preparations and cytokine determinations

All bulk MLNC cultures, CD4 T cell cultures and cytokine determinants were performed as described in chapter 1 of the materials and methods.

Genetic mapping

Genetic mapping was performed as described in chapter 2. A panel of ~ 40 primer sets that readily distinguish BL/6 and BL/10 alleles was used for genotyping the F_2 mice. Due to the extreme genetic similarity of BL/6 and BL/10 mice it was difficult to provide equal coverage throughout the genome. However, at least one marker was identified for all 19 autosomes, although not the X chromosome. Some markers were provided to us by the Roopenian Lab and others were identified by standard PCR of microsatellite markers (Appendix I). Data were analyzed by Mapmaker QTL and R/QTL (Broman) using the J/QTL interface (Jackson Labs). Mapmaker and R/QTL programs were also used to determine epistatic interactions between loci. Both programs returned similar results. The linkage was considered highly significant if the logarithm of the odds (LOD) favoring linkage score exceeded 3.3 according to the system established by Lander and Kruglyak (Lander and Kruglyak 1995) in the context of a genome search using an intercross study.

Microarray using Affymetrix Mouse Gene 1.0 ST Array

MLNs were aseptically removed from uninfected or 7-wk infected BL/6 and BL/10 mice, placed in 1.5ml, Dnase, Rnase free eppendorf tubes containing RNA*later* and flash frozen using liquid nitrogen. A total of 2 samples were used for each condition. Total RNA was isolated using Trizol reagent as per manufacture's instructions (Invitrogen). Next RNA was subjected to a series of purification, amplification and labeling steps and then hybridized to the Affymetrix Mouse Gene 1.0 ST Array. All reagents and protocols were obtained from Affymetrix, Santa Clara, CA. The Mouse Gene 1.0 ST chip analyzes expression of approximately 28,000 well-annotated genes based on the mouse genome sequence NCBI build 36 (UCSC mm8, NCBI build 36) and has thorough coverage of RefSeq, putative complete CDS GenBank transcripts, all Ensembl transcript classes and syntenically mapped full-length mRNAs and RefSeq NMs from human and rat, as well as complete coverage of NM sequences present in the RefSeq database release as of April 2007. The instrumentation required for analysis of the Mouse Gene 1.0 ST chip included the GeneChip Hybridization over 640, GeneChip Fluidics Station 450 and the GeneChip Scanner 30007G. Expression Console 1.1 software, provided by Affymetrix, was used to confirm significant, comparable gene-level signals from each chip and generate annotated NetAffx CSV file for analysis with Microsoft Excel (Redmond, WA). Data obtained from a single chip was representative of the gene expression profiles of uninfected or infected MLNs from individual BL/6 or BL/10 mice. Two chips were run for each permutation for a total of 8 chips, with similar results.

Bone marrow-derived dendritic cells (BMDCs)

Femurs and tibias were removed from uninfected mice and bone marrow cells were flushed into 10cm petri dishes with cold PBS. Isolated bone marrow cells were cultured at a concentration of 5 x 10^5 cells/ml in 10 ml of complete RPMI medium containing 10% fetal calf serum (Aleken Biologicals, Nash, TX) and granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF containing supernatant isolated from the B cell hybridoma J558L (provided by Dr. Nir Hacohen, Whitehead Institute for Biomedical Research, Cambridge, MA) was added to bone marrow cells at an optimized concentration of 1:10. On days 4 and 7 an additional 10ml of GM-CSF containing medium was added to the cultures. Non-adherent cells, which were > 75% $CD11c^+$ as determined by FACS analysis were harvested on day 9 and used for experiments.

Schistosome egg isolation

Schistosome eggs were isolated under sterile conditions from 7 to 8 week-infected swiss webster mice. To isolate eggs, livers were blended and eggs were sifted from the tissue using a series of sieves and washes with saline solution (Shainheit, Smith et al. 2008).

Dendritic cell-egg co-cultures

BMDCs (1 x 10^6 cells/ml) were cultured in the presence of 250 or 500 live eggs or LPS. After 24 hr, culture supernatants were collected, sterile filtered and assayed by ELISA for IL-1β, IL-12p40, IL-6 and TGF-β (Reagents and protocols from BD-Pharmingen or R&D systems). Potential contamination of schistosome-containing cultures with LPS was ruled out as previously described (Shainheit, Smith et al. 2008).

BMDC-CD4 T cell-Egg co-cultures

Purified CD4 T cells (1 x 10^6) were isolated from uninfected spleens, cultured with syngeneic BMDCs (2.5 x 10^5) and stimulated with 100 or 250 eggs plus ant-CD3/CD28 coated beads (3 x 10^5, Dynal/Invitrogen, Carlsbad, CA). After 4 days, culture supernatants were removed, sterile filtered and assayed by ELISA for IL-17, IL-6, IL-23 and IFN-γ using mAB, standards and protocols from R&D systems. For IL-5 ELISA, mAB, standards and protocols were obtained from eBioscience (San Diego, CA). As a control, some cultures were stimulated with a cytomix consisting of IL-12p40, IL-6 and TGF-β to induce optimal Th17 cell differentiation (Shainheit, Smith et al. 2008).

Quantitative Real-time RT-PCR.

Total RNA was isolated from individual samples using TriZol reagent (Invitrogen) as per manufacturers instructions. RNA (1-5μg) was subjected to DNASE I treatment (Roche) and reverse-transcribed using the high capacity cDNA reverse synthesis kit (Applied Biosystems). Real-time quantitative RT-PCR was performed by Taqman analysis using an ABI 7300 instrument. GAPDH levels were used to normalize the data. Taqman real-time probes for IL-4, Ym1, Fizz1 and Arg1 were obtained from Applied Biosystems. Using the average mean cycle threshold (Ct) value for GAPDH and the gene of interest for each sample, the equation 1.8 e (Ct (GAPDH) – Ct (GOI)) x 10^4 was used to obtain normalized values (Chen, Langrish et al. 2006)

Statistical analyses.

One-way ANOVA or Student's *t* tests were used to determine the statistical significance of differences between samples and were calculated with GraphPad Prism Software.

Results Chapter 4

Mice, infection and immunization.

C57BL/6J, CBA/J and MOLF/Ei (MOLF) mice, 5-8wk old, were purchased from the Jackson Laboratory. Why1 mice were produced as previously described(Conner, Smirnova et al. 2009). These congenic mice are homozygous for the MOLF allele selected by marker *D6Mit328* (chromosome 6 at 112.7 Mb) on a BL/6 background. IRAK-2-/- mice were obtained from Dr. Shizuo Akira (Research Institute for Microbial

Diseases, Osaka, Japan). Maintenance of mice and infection protocols are as described in chapter 1 of the materials and methods. For some experiments, IRAK-2-/-, IRAK-2+/- and BL/6 mice were immunized s.q. with 50 mg of SEA/CFA before and after infection, as previously described (Rutitzky, Hernandez et al. 2001). Treatment of mice with SEA/CFA causes marked exacerbation of hepatic egg-induced immunopathology; either SEA or CFA by themselves are ineffective. SEA was prepared as previously described (Boros and Warren 1970)

Assessment of histopathology.

Histopathology was assessed as described in chapter 1 of the materials and methods

Cell preparations, cell cultures and cytokine determinations.

All standard cell preparations, cell cultures and cytokine determinants were performed as described in chapter 1 of the materials and methods section unless otherwise noted.

Why1-BL/6 cell cocultures and in vivo CD4 T cell transfers.

1 x 10^6 purified MLN CD4 T cells from 7 week-infected BL/6 and Why1 mice were cultured *ex vivo* with 4 x 10^6 irradiated naïve splenic APCs from BL/6 or Why1 mice for 48 hours in the presence or absence of 15mg/ml of SEA. IL-17 levels in cell supernatants were measured by ELISA. For the cell transfer experiments, BL/6 recipient mice were sublethally irradiated (500 rad) 3 days prior to infection and subsequently injected i.v. with 8 x 10^6 naïve splenic CD4 T cells from BL/6 or Why1 donor mice, purified by

negative selection as described above. After 7 weeks of infection, IL-17 production by SEA-stimulated bulk MLNC, and by purified MLN CD4 T cells was measured by ELISA. ELISAs were performed as described above.

Quantitative Real-time RT-PCR.

Total RNA was isolated from individual samples using TriZol reagent (Invitrogen) as per manufacturers instructions. RNA (1-5μg) was subjected to DNASE I treatment (Roche) and reverse-transcribed using the high capacity cDNA reverse synthesis kit (Applied Biosystems). Real-time quantitative RT-PCR was performed by SYBR green or Taqman analysis using an ABI 7300 instrument. GAPDH levels were used to normalize the data. Taqman real-time probes for IL-17, IFN-γ, IL-4, IL-12p40, IL-12p35, IL-23p19, IL-22, IL-1β and *batf* were obtained from Applied Biosystems. Primers for SYBR green analysis of *ror*γt were described previously (Ivanov, McKenzie et al. 2006).

Western Blot analysis.

1 x 10^6 cells CD4 T cells were stimulated with IL-1β (4ng/ml, R&D Systems) for 0, 5, 10, 20 and 30 minutes followed by lysis on ice in a cytoplasmic lysis buffer (50mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM NaVanadate and 10 mM NaF) supplemented with Halt protease inhibitor cocktail (Thermo Fisher Scientific) for 10 min. Lysates were then centrifuged at 13,000 rpm at 4°C for 10 min. Cleared lysates were resolved on a 4-12% gradient Bis-Tris SDS gel (NuPAGE; Invitrogen) and

transferred to a nitrocellulose membrane. Rabbit polyclonal antibodies to phosphorylated ERK and p105 were obtained from Cell Signaling Technology. After incubation with specific Abs, chemiluminescence was detected using ECL substrate (Thermo Fisher Scientific).

Lentiviral transduction.

To down-regulate the expression of IRAK-2 in mouse T cells, we used infection with lentiviral particles expressing IRAK-2-targeting shRNA. This procedure was performed as previously described (Conner, Smirnova et al. 2009). Lentiviral particles were produced by transfecting (Fugene, Roche) 293-T cells with a plasmid encoding IRAK-2-specific shRNA in the pLKO.1 vector (Open Biosystems clone ID TRCN000022502) together with two other plasmids, pSPAX2 and pMD2.G (Addgene), encoding packaging components of the lentivirus. Supernatants from 293-T cells were harvested on days 2 and 3 after transfection and passed through a 0.45mm filter. Naïve CD4 T cells were purified from mouse spleens using the Easysep kit (StemCells). Cells were resuspended to a density of 2 x 10^6 cells/ml followed by mixing in a 1:1 ratio with the viral supernatant and infected for 18 hours. Subsequently, the T cells were washed and allowed to recover for 96 hrs with no selection before assays were performed. For activation, the T cells were seeded in 96-well plates.

Stimulation of naïve CD4 T cells.

Naïve CD4 T cells were incubated in either 96 well plates (3.5×10^5 cells/ml) for ELISA detection, or 48 well plates (1×10^6 cells/ml) for real-time analysis in triplicates, and stimulated with anti-CD3/CD28 coated beads (3×10^5, Dynal) together with rIL-1β, at indicated concentrations, and rIL-23 (20ng/ml). For ELISA, cell culture supernatants were collected after 4 days and analyzed for IL-17 as described above. For real-time PCR, cells were collected at 0, 2, 24, 48 and 96 hrs in Trizol reagent and assayed as described in the results section.

Statistical analyses.

ANOVA and Student's *t* tests were used to determine the statistical significance of the differences between groups and were calculated with GraphPad Prism.

SECTION III: RESULTS

Chapter 1

The Role of MHC vs. background genes in Severe Schistosomiasis

1.1 Premise and Rationale

As mentioned above, previous work in our lab has shown that CBA mice mount a strong I-A^k restricted T cell response against the immunodominant peptide 234-246 (Sm-$p40_{234-246}$) of the major Sm-p40 schistosome egg antigen (Hernandez and Stadecker 1999). This T cell population expresses a restricted Vα11.3β8 T cell receptor (TCR) that expands in CBA, but not BALB/c mice (Finger, Brodeur et al. 2005). The activation and expansion of an oligo-clonal T cell population against an immunodominant antigen specific antigen may be responsible for the persistent pro-inflammatory environment that is conducive to high pathology in schistosomiasis. *In essence, these results suggest that MHC and MHC related genes play a critical role in the development of high pathology in schistosomiasis.*

The development of a persistent Th1 polarized T cell response in high pathology mice was initially thought to be driven by IL-12 induced production of IFN-γ (Stadecker, Asahi et al. 2004). However, recently it has been reported from our lab that IL-17, and not IFN-γ, is the main pathology-inducing cytokine in schistosomiasis (Rutitzky, Lopes da Rosa et al. 2005). IL-17 is produced by a novel CD4 T cell subset, Th17 that arises from a lineage distinct from T helper type 1 and 2 cells (Harrington, Hatton et al. 2005). Th17 cells express an activated memory phenotype (Rutitzky, Lopes da Rosa et al. 2005) and have been shown to mediate chronic inflammation in experimental autoimmune encephalomyelitis (Langrish, Chen et al. 2005) and collagen induced arthritis (Murphy,

Langrish et al. 2003). *The contribution of a pro-inflammatory environment caused by IL-17 production thus suggests that factors other than MHC restricted peptide presentation can also determine the degree of immunopathology in schistosomiasis.* Given the differences of these factors contributing to disease severity, it is necessary to determine the relative importance of MHC versus background genes in the development of high pathology.

To dissect the relative importance of MHC and background genes to the development of severe disease we used recombinant congenic mice. Recombinant congenic mouse models are a commonly used research tool that allow for the genetic dissection of genes involved in complex traits. Congenic mice are assumed to only differ at a single gene or region of the genome from their inbred partner strains. These mice have great importance in evaluating the effect that a single locus may have on phenotypic characteristics and they have been used for many years to help determine the functions of multiple genes associated with single and multigenic diseases (Mullerova and Hozak 2004). For example, MHC congenic mice provide a useful model to study the importance of the T cell repertoire in response to an antigenic challenge. MHC genes are responsible for producing peptides derived from antigenic factors and then presenting them to a specific TCR. The composition of the peptide that is presented can have a dramatic effect on the T cell response against the antigen. MHC congenic mice could be produced by choosing a marker in the H-2 region of chromosome 17 in mice and then selecting those genes from 1 mouse strain and crossing it onto another mouse of the desired background. As mentioned previously high pathology (CBA), but not low pathology (BALB/c) mice, exhibit expansion of a restricted TCR CD4 T cell subset. Congenic mice,

in which cells from a low pathology (BL/6, BALB/c) background would express the haplotype of a high pathology (CBA H-2^k) mouse and vice versa will be very useful in determining the relative contributions of MHC and background genes to the development of severe disease.

1.2 Results

1.2.A Generation of MHC congenic mice

CBA (H-2^k) and BL/6 (H-2^b) mice are the typical examples of high and low pathology studied in our lab, respectively. Therefore the first choice for MHC congenic mice was to produce CBA mice that express the H-2^b haplotype and BL/6 mice that express H-2^k. Unfortunately neither of these mice exists. Another mouse on a black background, the B10.Br mouse is H-2^k, however, upon further study it was found that this mouse has intermediate pathology and thus could not be used as a model for low pathology in schistosomiasis. Another well-characterized low pathology mouse strain studied in our lab is the BALB/c mouse, which is H-2^d. Importantly there already exists a mouse on the BALB background that expresses the H-2^k haplotype, the BALB/k mouse. Because BALB/c mice express an H-2^d hapotype the equivalent congenic mouse would be a CBA mouse expressing the H-2^d haplotype.

In order to develop a CBA mouse expressing the H-2^d haplotype, CBA and BALB/c mice were crossed to produce F_1 progeny. The F_1 mice were then selected for those that were heterozygous for both the H-2^k and H-2^d haplotypes. Genetic selection

was determined by PCR amplification of a microsatellite (D17Mit34) located in the H-2 locus on chromosome 17 (at ~ 35Mb). This ensures that all of the MHC genes in this region are selected for and carried on through the backcrosses. The heterozygous F_1 mice were then backcrossed to normal CBA mice. The backcrossing was repeated for 7 generations eliminating approximately 99.6 percent of the BALB/c genome while maintaining the H-2^d haplotype. After 7 backcrosses, heterozygous mice were selected and then intercrossed. The resultant mice were selected for homozygosity of the H-2^d haplotype, which was verified by PCR and FACS analysis **(Figure 6)**. CBA/d mice also did not increase expression of the schistosome egg specific TCR gene rearrangement Vα11.3J36 following subcutaneous immunization with SEA in complete freund's adjuvant (CFA), similar to BALB/c mice, while CBA and BALB/k mice, both of which express the k haplotype, had higher levels of the schistosome specific TCR **(Figure 7)**. Taken together these results show that CBA/d congenic mice are positive for the H-2^d haplotype and do not have increased expression of the schistosome specific Vα11.3J36 TCR gene rearrangement despite being mainly derived from the CBA background. These mice, along with CBA, BALB/c and BALB/k, were then used to determine what role MHC may play in the development of severe pathology.

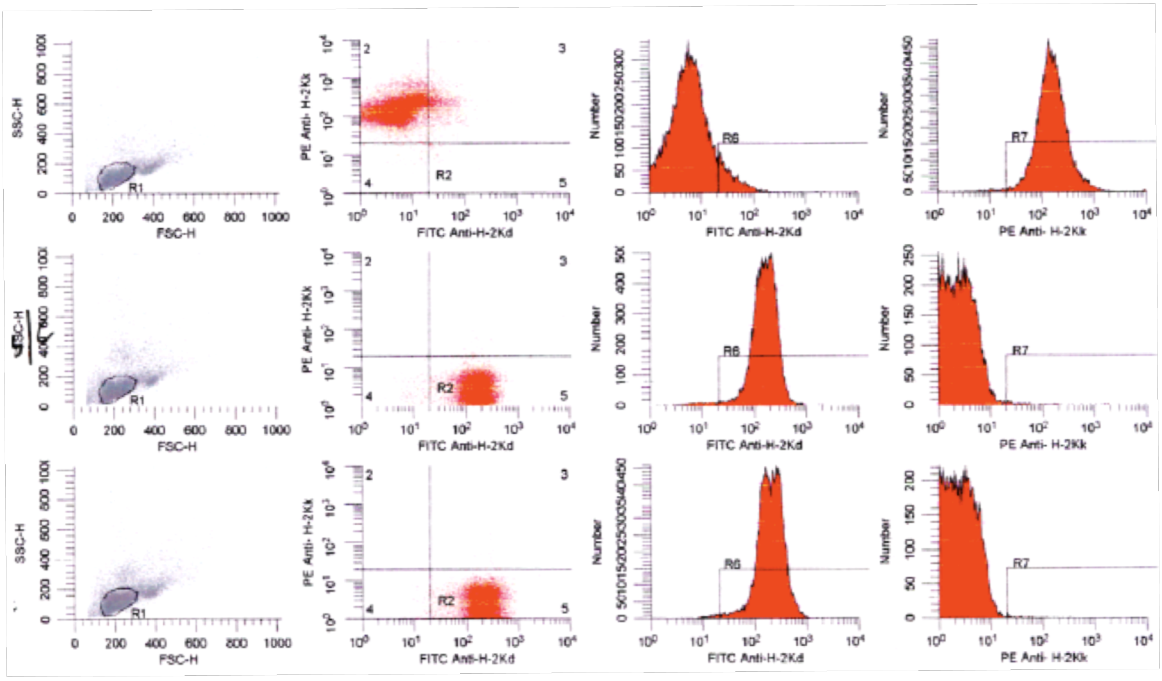


Figure 6. FACS verification of congenic CBA/d mice. Rows 1 and 2 show FACS analysis for the parental mouse strains CBA (H-2^k) and BALB/c (H-2^d), which act as positive controls. Lanes 3 verifies that CBA/d mice are > 99% H-2^d positive. The antibodies selected for staining specifically recognize the K and D haplotypes in the K region of the H-2 locus. No cross reactivity has been observed between these antibodies. Bulk splenocytes were gated for live lymphocytes in region R1. Unstained, Isotype and single positive staining for each antibody were used as controls (not shown here). Data are representative of 5 individual mice.

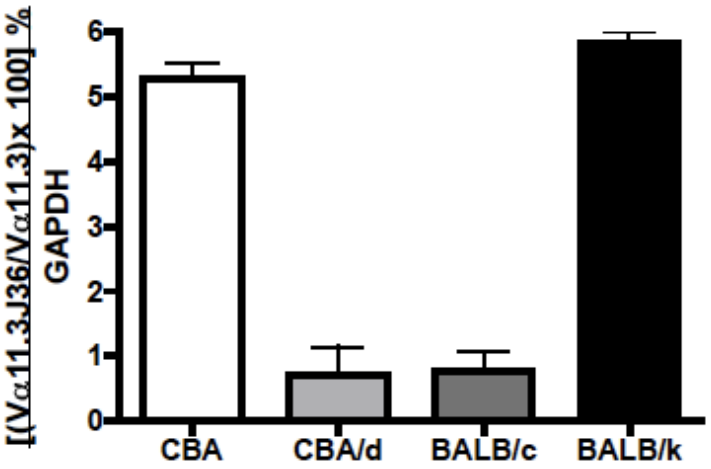


Figure 7. Expression of Vα11.3J36 TCR gene rearrangement. Mice were subcutaneously immunized with and emulsion of 50μg SEA in CFA and splenic RNA was used to measure expression of the Vα11.3J36 TCR gene rearrangement. Following immunization, CBA and BALB/k mice had increased expression of the Vα11.3J36 TCR gene rearrangement, while CBA/d and BALB/c did not. Expression of Vα11.3J36 rearrangement as a percentage of total Vα11.3 expressing T cells as measured by taqman real-time PCR. Results are from triplicate determinants and were normalized against GAPDH. Results are representative of two independent experiments.

1.2.B MHC genes alter IFN-γ production but do not affect the development of severe disease.

To determine the effect of the schistosome specific TCR gene rearrangement on the development of severe immunopathology mice were infected with *S. mansoni* and the extent of hepatic granuloma formation was determined. 7 weeks after infection with 85 cercariae CBA/d mice exhibited significantly enhanced granulomatous inflammation compared with BL/6 mice and similar to CBA control mice, while BALB/k mice developed granulomas similar to those seen in BL/6 mice **(Figure 8).** Analysis of SEA-specific cytokine production by bulk mesenteric lymph node cells (MLNCs) revealed a similar pattern to granuloma size. CBA/d mice produced high levels of the proinflammatory cytokines IL-17, IFN-γ, TNF-α and IL-6, similar to CBA mice and significantly higher than BALB/c and BALB/k mice **(Figure 9 A-D)**. MHC genes did also not affect the production of anti-inflammatory cytokines as all mice produced similar levels of IL-5 and IL-10 **(Figure 9 E, F).**

Production of IL-17 and IFN-γ, the two cytokines most associated with the development of severe pathology(Rutitzky, Hernandez et al. 2005; Smith, Shainheit et al. 2009), was also measured from SEA stimulated CD4 T cells in congenic mice, because this is the most likely affected cell population. As before, CD4 T cells mixed with irradiated APCs from CBA/d and CBA mice produced similar amounts of IL-17, which was significantly higher than BALB/c and BALB/k mice (**Figure 10 A)**. Interestingly, CBA/d CD4 T cells had significantly reduced IFN-γ production when compared with CBA CD4 T cells, while BALB/k CD4 T

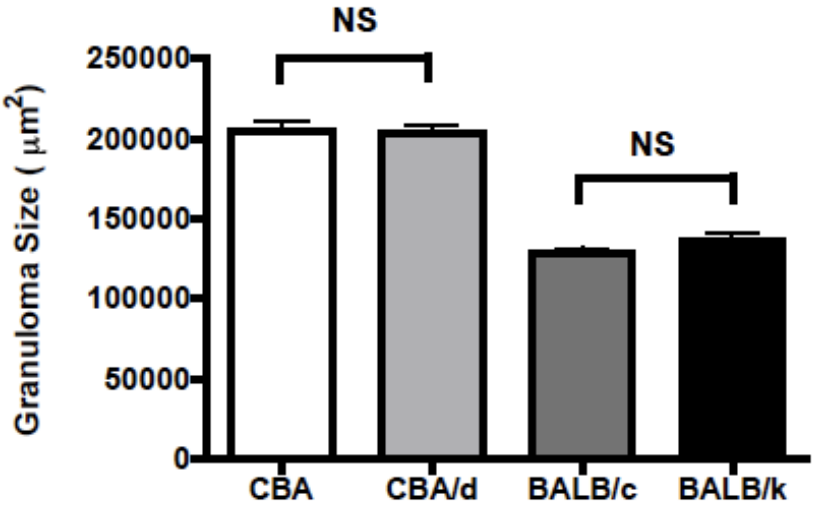


Figure 8. Granuloma size in CBA, CBA/d, BALB/c and BALB/k mice. Granuloma size was measured in CBA, CBA/d, BALB/c and BALB/k mice. CBA and CBA/d mice developed significantly larger liver granulomas than BALB/c and BALB/k mice ($p < 0.05$). There was no difference between CBA and CBA/d mice. Groups consisted of at least 4 mice per strain and greater than 50 granulomas were measured per mouse strain by morphometric analysis. Results are representative of 4 individual experiments and standard deviation represents variance in individual granulomas. One-way ANOVA of statistical significance was performed by Prism GraphPad software.

cells produced significantly more IFN-γ than BALB/c CD4 T cells **(Figure 10 B)**. This also held true in cells directly isolated from hepatic lesions, where CBA/d and CBA mice produced similar amounts of IL-17, but different amounts of IFN-γ **(Figure 10 C, D).**

Previously our lab has shown that high pathology mouse strains increase expression of the schistosome specific Vα11.3J36 TCR gene rearrangement, while low pathology strains do not, and this is restricted to the H-2^k haplotype(Finger, Brodeur et al. 2005). Following infection with *S. mansoni* we sorted $V\beta^+$ CD4 T cells from MLNCs in order to enrich for expression of the schistosome specific TCR. CBA and BALB/k mice, both H-2^k positive, had enhanced expression of the Vα11.3J36 TCR gene rearrangement compared with CBA/d and BALB/c mice. Importantly, as a control $V\beta^-$ CD4 T cells showed little to no expression of this gene rearrangement, which is associated with only $V\beta^+$ CD4 T cells **(Figure 11).** Taken together these results imply that MHC genes, and in particular the absence or presence of a schistosome specific TCR, do not play a significant role in the development of severe disease in this model.

1.2.C CD4 T cells and background genes differentially contribute to the development of high pathology schistosomiasis

Although MHC genes did not appear to greatly affect the development of pathology, indicating that the presence of a schistosome specific TCR does not critically effect pathology, the relative contribution of CD4 T cells and non-MHC/T

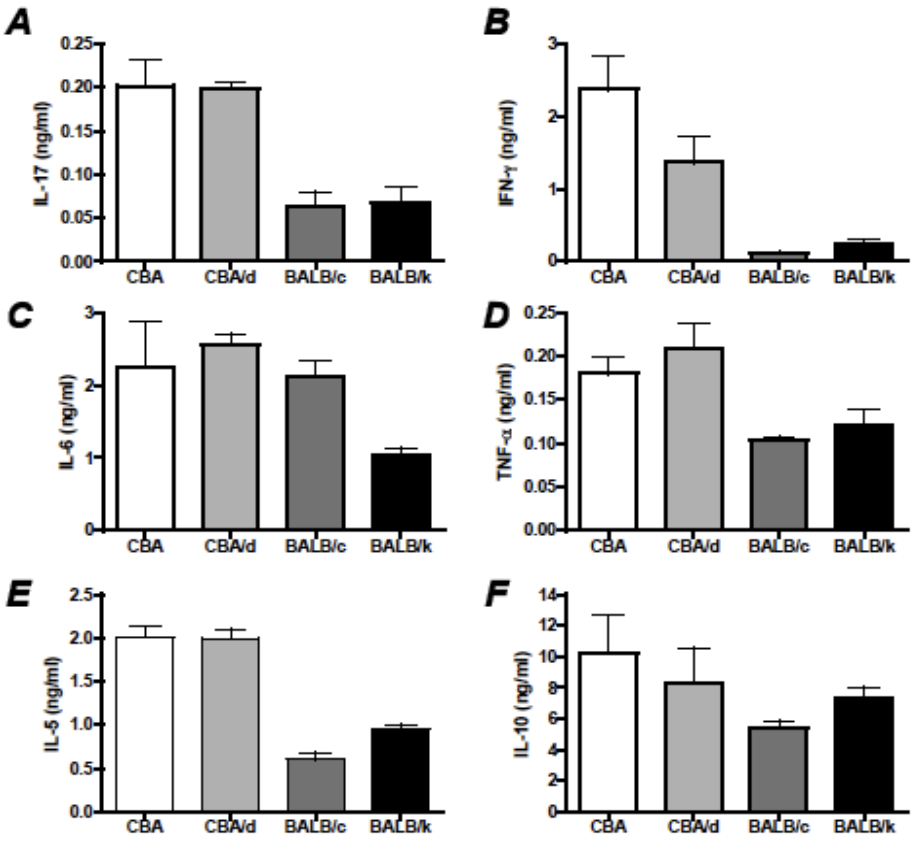


Figure 9. Cytokine profile of infected MLNs from CBA, CBA/d, BALB/c and BALB/k mice. SEA-specific IL-17 (A), IFN-γ (B), TNF-α (C), IL-6 (D), IL-5 (E) and IL-10 (F) production was measured from MLN cells by ELISA. CBA/d and BALB/k mice produced similar amounts of cytokines compared to their control mice, CBA and BALB/c, respectively. Cytokine levels are expressed as means of triplicate ELISA determinations. Results are representative of 4 individual experiments. No statistically significant difference was identified between CBA vs CBA/d or BALB/c vs BALB/k groups.

cell genes to the development of immunopathology is not known. The generation of MHC congenic mice afforded us the opportunity to further dissect this relationship. To accomplish this, naïve splenic CD4 T cells were isolated from CBA and BALB/k mice and subsequently transferred by i.v. injection into recipient mice prior to infection. Following infection, BALB/k mice that received CBA CD4 T cells developed larger granuloma size, and had increased production of the proinflammatory cytokines IFN-γ, IL-17 as well as increased expression of IL-23 **(Figure 12)**. Alternatively, CBA mice that received BALB/k CD4 T cells exhibited a decrease in granuloma size and proinflammatory cytokine production **(Figure 12).** However, a closer analysis revealed that while transfer of T cells did alter immunopathology in recipient mice, both IL-17 production and IL-23 expression appeared to be more dependent on the host background, while IFN-γ production was dependent on CD4 T cells **(Table II)**. Taken together, these data indicate that both the innate and adaptive genes differentially contribute to the development of severe immunopathology.

1.3 Discussion

Immunodominance plays a critical role in the development of the immune response, as numerous reports have shown prominent T cell responses directed against immunogenic epitopes ranging from self antigens involved in autoimmune disease, to viruses, bacteria and fungi (Yewdell and Bennink 1999; Sant, Chaves et al. 2007; Rodrigues, Alencar et al. 2009; Stenger, Poelen et al. 2009; Streeck, Jolin et al. 2009; Weaver and Sant 2009). Previously our lab

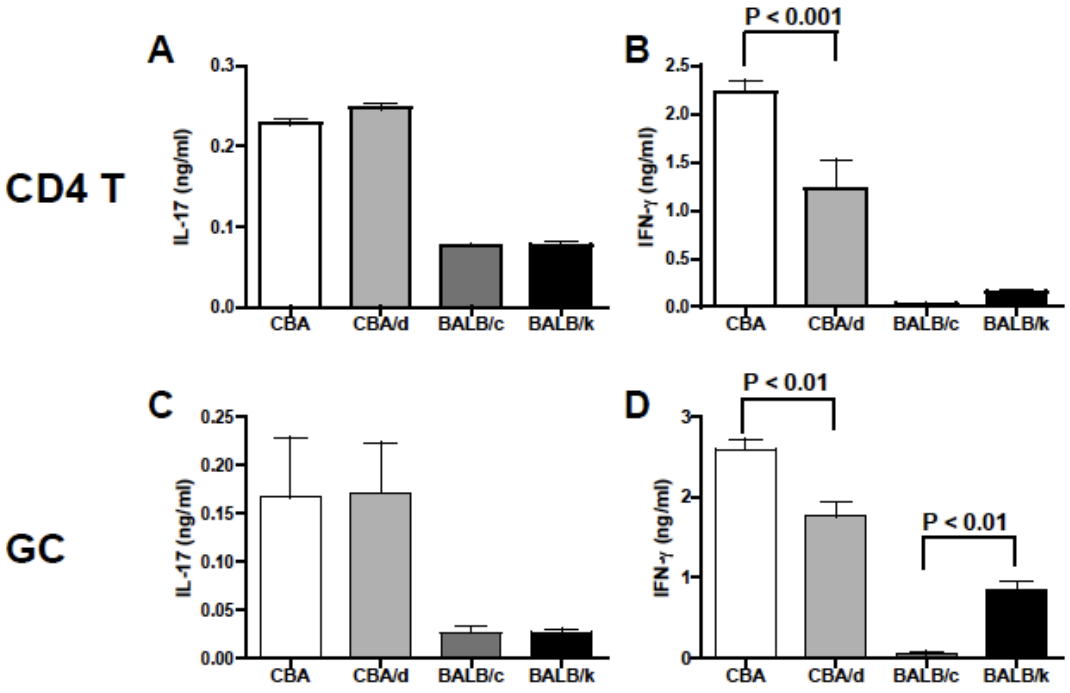


Figure 10. IL-17 and IFN-γ production from CD4 T cells and GC in MHC congenic mice. SEA specific cytokine production from CD4 T cells isolated from the MLNs of infected mice and GC isolated from hepatic lesions. CBA/d and BALB/k congenic mice produced similar amounts IL-17 in the (A) MLN and GC (D) compared with CBA and BALB/c mice, respectively. CBA/d mice produced significantly less IFN-γ in the MLN (B) and GC (D) than CBA mice, while BALB/K mice produced significantly more IFN-γ in GC than BALB/c mice. Cytokine levels are expressed as means of triplicate ELISA determinations. Results are representative of 2 individual experiments. One-way ANOVA was used to determine the statistical significance using GraphPad Prism software.

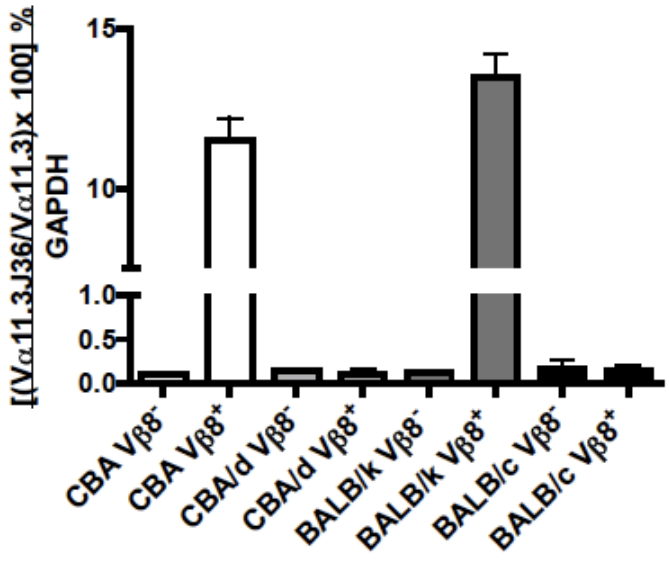


Figure 11. Expression of TCR Vα11.3J36 gene rearrangement in infected mice. Expression of the restricted TCR Vα11.3J36 gene rearrangement was measured by real time quantitative PCR in CD4 T cells FACS sorted for Vβ8 expression. CBA and BALB/k mice increase expression of the restricted TCR in Vβ8 positive CD4 T cells compared with CBA/d and BALB/c mice. Vβ8 negative CD4 T cells express only background levels of the restricted TCR. Expression is shown as the percentage of the total population of CD4 T cells that express Vα11.3J36 and normalized relative to GAPDH.

identified the first example of immunodominance during helminth infection (Hernandez, Edson et al. 1998; Hernandez and Stadecker 1999; Murphy, Langrish et al. 2003; Finger, Brodeur et al. 2005). CD4 T cells from high pathology CBA and C3H mice displayed a strong Th1 type T cell response against SEA, of which a substantial portion was directed against the major Sm-p40 antigen (Hernandez, Edson et al. 1998). It was later shown that these T cells used a restricted TCR structure composed of Vα11.3 in association with Vβ8 directed against a single parasite epitope (Sm-p40$_{234-246}$), which were expanded after infection in high, but not low pathology mice (Finger, Brodeur et al. 2005). Although these data suggest a prominent a role for these immunodominant T cells in schistosomiasis, the true biological significance is best appreciated by the impact of the involved T cells directly on the outcome of the disease, therefore a causal relationship has not yet been established in our model.

In order to assess the role of the H-2^k restricted, Vα11.3Vβ8 schistosome specific T cells, we made MHC congenic mice, which enabled the expression of these immunodominant T cells on an otherwise low pathology background, as well as removing them from their natural high pathology environment. Interestingly, despite a strong correlation of expression in high pathology mice and a strong Th1 type T cell response, the presence or absence of immunodominant T cells did not affect severe immunopathology in congenic mice. This is contrary to other models in which immunodominance has been shown to play a critical role, most notably during infection with the protozoan parasite *Leishmania major*, the classic paradigm of Th1 vs Th2 responses involving parasitic infection (Sacks and Noben-Trauth 2002). This is likely due to the complexity of the disease course as well as the schistosome itself.

Immunodominance in schistosomiasis is directed against a specific peptide derived from the major Sm-p40 antigen, which is the major antigen driving Th1 mediated responses in our model. The majority of other known schistosome egg antigens have mainly been shown to drive a Th2-mediated response, such as IPSE/alpha 1 and omega 1(Schramm, Gronow et al. 2006; Schramm, Mohrs et al. 2007; Everts, Perona-Wright et al. 2009; Steinfelder, Andersen et al. 2009). However, it is likely that in the absence of Sm-p40 other antigens are capable of inducing the Th1/Th17 response critical for the development of severe disease. Sm-PEPCK and Sm-Tpx-1 are eggs antigens that were identified as inducing a higher response in BL/6 mice than CBA with a more Th1/Th2 balanced cytokine profile (Stadecker, Asahi et al. 2004), however in the absence of an immunodominant response it is possible that the response to these antigens may become stronger in high pathology mouse strains.

A more likely scenario is that other untested or unidentified schistosome antigens are responsible for driving the proinflammatory response in the absence of Sm-p40 or Sm-p40 specific T cells, that is necessary for severe disease. A recent report describing a complete proteomic analysis of *S. mansoni* eggs secretions identified 188 proteins, including 32 that were previously uncharacterized (Cass, Johnson et al. 2007). This analysis identified several heat shock proteins as well enolase, both of which have been implicated in a number of autoimmune models that share similar mechanistic features with schistosome infection (Pancholi 2001; Mayer and Bukau 2005). This may have increased relevance in our model as previous work from our lab has shown that antigens necessary to drive CD4 T cells into Th1 or Th17 cells are likely secretory proteins (unpublished observations). Furthermore it is known that non-antigen specific T cells

play a major role in enhancing tissue inflammation in a number of models, including schistosomiasis (Hogan, Wang et al. 2002).

Perhaps the most salient finding of this study was the role of immunodominance relating to IFN-γ production. BALB/k and CBA/d congenic mice consistently had altered IFN-γ responses compared with control mice and transfer of CD4 T cells revealed that IFN-γ production was CD4 T cell-mediated while innate cells controlled IL-23 and IL-17 production. IFN-y plays a critical role in host defense in a number of infection and disease models (Farrar and Schreiber 1993). However, more recently, IL-17 was shown to be the critical factor in a number of autoimmune models originally described as mediated by IFN-γ (Korn, Bettelli et al. 2009). Taken together, these findings suggest that the immunodominant T cell response directed against Sm-p40 controls differentiation of Th1 cells but not other T cell subsets, such as Th17 cells which are the more likely mediators of severe disease, and thus does not have an effect on the overall outcome of disease severity. Based on these results we decided to take a more genome-wide based genetic approach in an attempt to identify factors that control disease severity, which will be the focus of the next two sections.

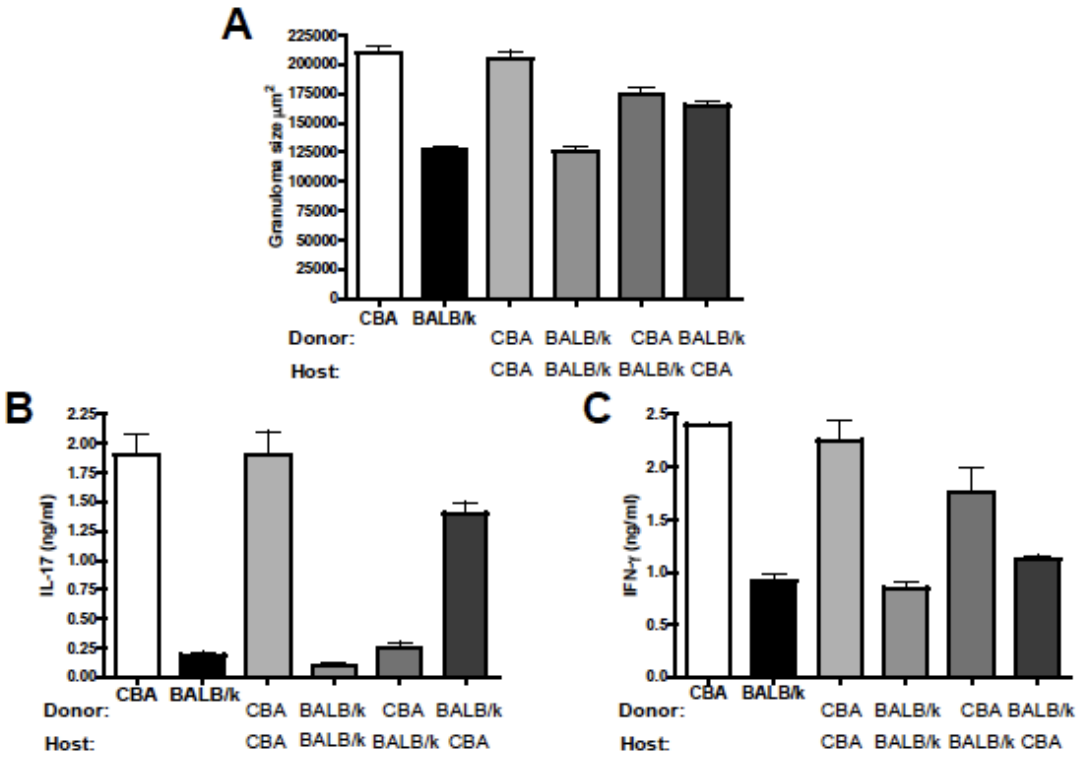


Figure 12. Liver pathology and cytokine production from CD4 T cell transfer experiment. Donor purified naïve CD4 T cells (~ 10 x 10^6) were transferred i.v. as indicated in the graphs into sublethally irradiated recipient mice, which were subsequently infected with *S. mansoni*; CBA and BALB/k mice are shown for comparison. (A) Granuloma size was significantly reduced in CBA mice that received BALB/k CD4 T cells compared with control mice ($p < 0.05$); likewise BALB/k mice that received CBA CD4 T cells had significantly increased granuloma size compared with control mice ($p < 0.01$). (B and C) CD4 T cell transfer also significantly affected both IL-17 and IFN-γ in CBA ($p < 0.01$) and BALB/k ($p < 0.05$) recipient mice. Standard error is based on analysis of 4-5 individual mice per group. Results are representative of 2 independent experiments. One-way ANOVA was used to determine statistical significance between groups.

IFN-γ	BALB/k donor T cells	CBA donor T cells
BALB/k host	0.9	1.8
CBA host	1.0	2.4

IL-17	BALB/k donor T cells	CBA donor T cells
BALB/k host	0.10	0.25
CBA host	1.40	1.90

IL-23p19	BALB/k donor T cells	CBA donor T cells
BALB/k host	12	15.5
CBA host	30	40

Granuloma size	BALB/k donor T cells	CBA donor T cells
BALB/k host	12.5	17.5
CBA host	16.5	20.5

Table II. CD4 T cells and background genes differentially control cytokine production and granuloma size. Data are taken from Figure 11 and reformatted into table II. Cytokine values are in ng/ml for IL-17 and IFN-γ, expression of IL-23p19 is displayed as relative units as measured by quantitative realtime PCR and granuloma size x 10^4 μm^2. The columns represent contributions from donor T cells (highlighted in red) and the rows represent contributions from the host background genes (highlighted in blue). The data were grouped based on similar production of cytokines or granuloma size. IFN-γ production appears to be controlled more by the donor T cells (red), while IL-17 and IL-23p19 appear to be controlled more by host background genes (blue). Granuloma size is controlled by contributions from both.

Chapter 2

Genome-Wide Screen for loci controlling Immunopathology

2.1 Premise and Rationale

The outcome of the schistosome infection depends on complex innate and adaptive immune responses, including DC and T cell interactions and regulatory mechanisms such as those afforded by alternatively activated macrophages and regulatory T cells. Based on the polygenic nature of the immune response and the striking heterogeneity in the development of immunopathology, it has long been thought that disease severity is under genetic control, prompting the search for host genetic factors that account for these differences. Human studies have revealed the association of several HLA alleles with disease intensity and identified two genetic loci, designated *Sm1* and *Sm2*, that control disease susceptibility and severe hepatic fibrosis, respectively. However as mentioned previously human studies remain extremely challenging, especially when trying to identify loci, and particularly genes, that affect complex traits such as schistosomiasis. However, the advent of genetic mapping in mice using microsatellite or SNP markers along with advances in analytical approaches has greatly simplified the analysis of complex traits and made gene discovery a realistic goal as discussed above.

A previous QTL analysis from our lab demonstrated a correlation between granuloma size and IFN-γ production and identified several suggestive loci responsible for controlling the magnitude of granulomatous inflammation and IFN-γ. However, no statistically significant linkages were identified and furthermore this analysis was performed before IL-17 was identified as the most important mediator of severe disease. This afforded us with the opportunity to perform a more comprehensive QTL analysis in

which we would be able to modify our approach slightly. As mentioned above, a larger phenotypic difference among parental strains provides a greater resolution in F_2 mice. Studies by Murray and Bottomly on ligand density and TCR affinity have shown that the number of peptides selected by a give MHC class II haplotype and the affinity of these peptides to a specific TCR influences CD4 T cell polarization. The lower number of epitopes for a given antigen that can be presented by an MHC class II molecule and the higher their affinity for a given TCR leads to a Th1 CD4 T cell polarization, whereas an increased number of possible epitopes with a lower affinity will yield a Th2 type polarization. The completion of crystal structures for several MHC class II haplotypes, combined with computer software programs, has allowed for the study of peptide presentation from a given antigen.

Previous work from our lab analyzed the number of peptides that could be presented and their binding affinity from the Sm-p40 antigen of SEA with different MHC class II haplotypes and predicted that the I-A^s haplotype would present only 1 peptide with a very high binding affinity and suggests that mice expressing this haplotype would generate a massive ligand density leading to a dramatic Th1 CD4 T cell polarization, even greater than those expressing the I-A^k haplotype, which is classically associated with severe disease. Therefore we hypothesized that mice expressing the I-A^s haplotype would develop severe immunopathology and represent the perfect complement parental strain to the BL/6 mouse in order to increase resolution in F_2 mice.

2.2 Results

2.2A SJL mice develop significantly enhanced immunopathology and produce higher levels of IL-17 than BL/6 mice and in an F_2 progeny, enhanced pathology correlates with an increase in proinflammatory cytokines

Seven weeks after infection with 85 cercariae, SJL mice developed large liver granulomas (mean size, 221 ± 19.8 μm^2 x 10^3), while in BL/6 mice they were significantly smaller (mean size, 137 ± 13.5 μm^2 x 10^3) (**Fig 13 A)**. These results confirmed our hypothesis in that SJL mice develop the most severe liver pathology of any strain previously examined by us and others (Cheever, Duvall et al. 1987). F_1 mice developed small granulomas, close to those in BL/6 mice, indicating that low pathology was dominant. To further elucidate the genetic basis of granuloma formation, F_2 mice were studied in a similar manner. The F_2 progeny displayed a wide range in granuloma size with some reaching that displayed by either the SJL or BL/6 parental strains **(Fig 13 A)**.

Since proinflammatory cytokines, particularly IL-17, have been shown to be of pathogenic significance in schistosomiasis (Rutitzky, Lopes da Rosa et al. 2005; Rutitzky, Bazzone et al. 2008), we analyzed SEA-induced cytokine production by MLN cells from SJL, BL/6, F_1 and F_2 mice. SJL mice produced significantly higher levels of IL-17 than BL/6 mice, while F_1 mice displayed a more intermediate phenotype for IL-17 production (**Fig 13 B)**. Cytokine analysis in F_2 mice again revealed a wide variation in IL-17 production and a significant correlation of the proinflammatory cytokines IL-17, IFN-γ and TNF-α with granuloma size (**Fig 14 A, B and C**). In contrast, the Th2

cytokines IL-4 and IL-5, as well as the anti-inflammatory cytokine IL-10, exhibited no significant correlation to granuloma size **(Fig 14 D, E and F)**. IL-13 production was not determined because it plays a more significant role during the chronic and not the acute phase of infection. Since granuloma size and cytokine values did not follow a normal distribution, data were reanalyzed after log transformation and yielded similar results (data not shown).

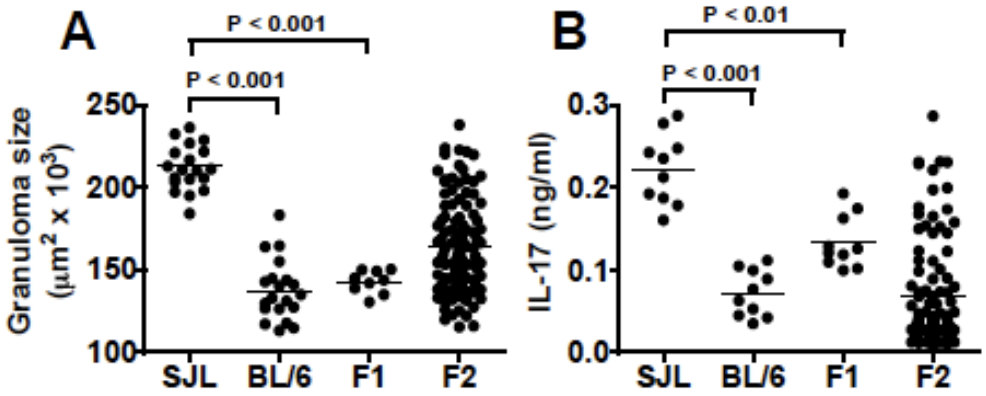


Figure 13. Granuloma size and IL-17 production by SEA-stimulated MLN cells from SJL, BL/6, F_1 and F_2 mice. (A) Granulomas were measured in liver sections obtained from 7-wk-infected SJL (n = 19), BL/6 (n = 20), F_1 (n = 10) and F_2 mice (n = 150), as described in materials and methods. A minimum of 20 granulomas were measured per mouse. Each dot represents an individual mouse. (B) IL-17 production by SEA-stimulated MLN cells from these mice was measured in 48 h culture supernatants by ELISA, as described in materials and methods. Each dot represents the mean cytokine level of triplicate determinations per mouse. Black horizontal lines represent the mean of the group.

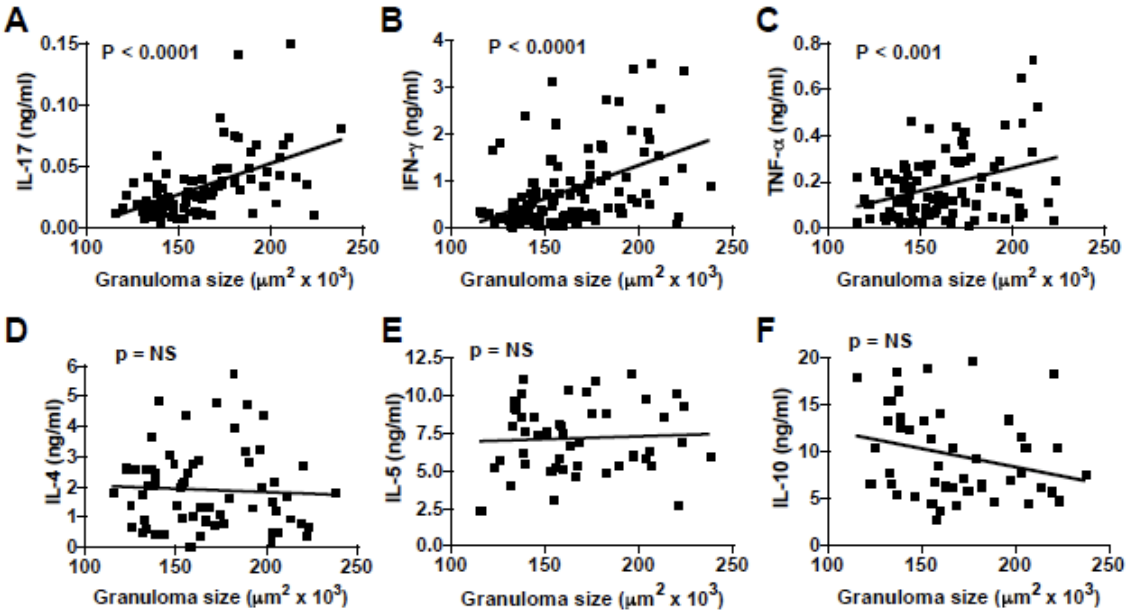


Figure 14. Linear regression analysis of mean granuloma size vs cytokine production for individual F_2 mice. (A) IL-17, (B) IFN-γ and (C) TNF-α production from MLN cells significantly correlate with granuloma size in F_2 mice. There was no correlation between (D) IL-4, (E) IL-5 and (F) IL-10 production and granuloma size in these mice. Each dot represents an individual F_2 mouse. NS = not significant.

2.2B QTL analysis of immunopathology identified significant linkage to granuloma formation

We next performed a QTL analysis of F_2 mice to try and identify loci linked to the onset of severe disease. Linkage analysis of granuloma size identified a statistically significant linkage to two loci, *D4Mit203* on chromosome 4 (peak position, 129.2 Mb, LOD 3.4, $p < 0.001$, 95% CI 115-142Mb) and *D17Mit82* on chromosome 17 (peak position, 33.9 Mb, LOD 6.0, $p < 0.0001$, 95% CI 21-40Mb). An additional locus was also identified on chromosome 9 with a suggestive linkage to granuloma size (peak position, 95.2 Mb, LOD 2.5, $p < 0.05$, 95% CI 63-105 Mb) (**Fig 15**). F_2 mice that were homozygous for the SJL (SS) alleles at *D4Mit203* and *D17Mit82* developed significantly larger liver granulomas than F_2 mice homozygous for the BL/6 (BB) allele at both loci, while heterozygous F_2 mice (SB) developed small granulomas equal to those homozygous for BL/6, indicating that both loci were inherited in a BL/6 dominant manner (**Fig 16 A and B)**. We further analyzed the extent of control these loci exert on immunopathology by their effect on cytokine production and found that IL-17 and IFN-γ responses were significantly higher in SS versus BB mice, each of which was also inherited in a BL/6 dominant manner. There was no significant difference in TNF-α, IL-4, IL-5 and IL-10 production between homozygous and heterozygous mice at either locus, suggesting that they had no effect on the production of these cytokines (**Fig 16 A and B**).

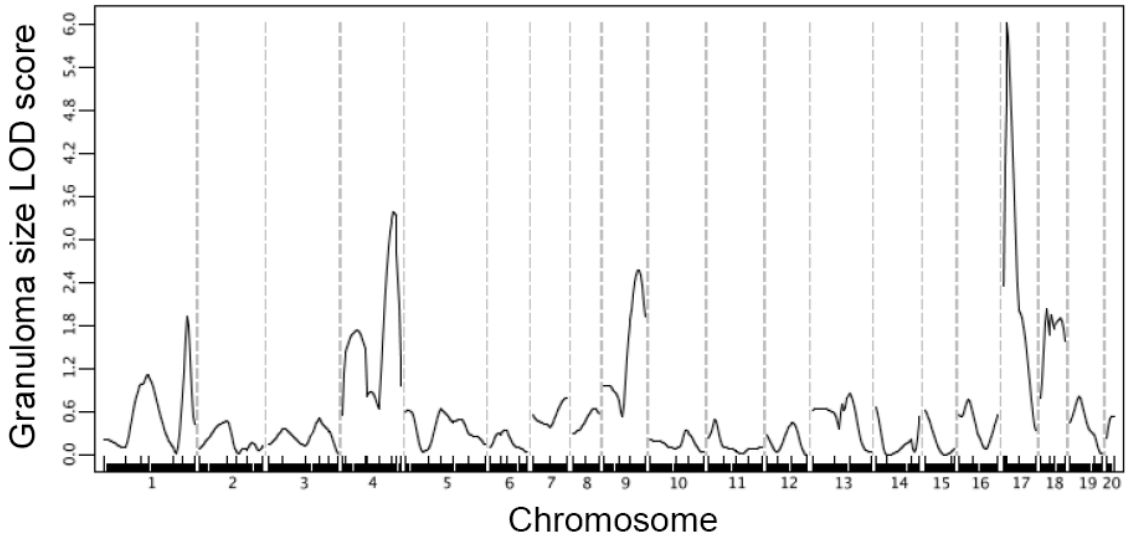


Figure 15. Interval map for granuloma size in *S. mansoni* infected F_2 mice. The markers *D4Mit203* (LOD of 3.4) and *D17Mit82* (LOD of 6.0) were highly significantly linked to granuloma size. LOD scores are represented on the Y-axis and chromosomal positions on the X-axis. Vertical dashed lines represent chromosomal breaks. Results are based on analysis performed by J/QTL (5000 permutations).

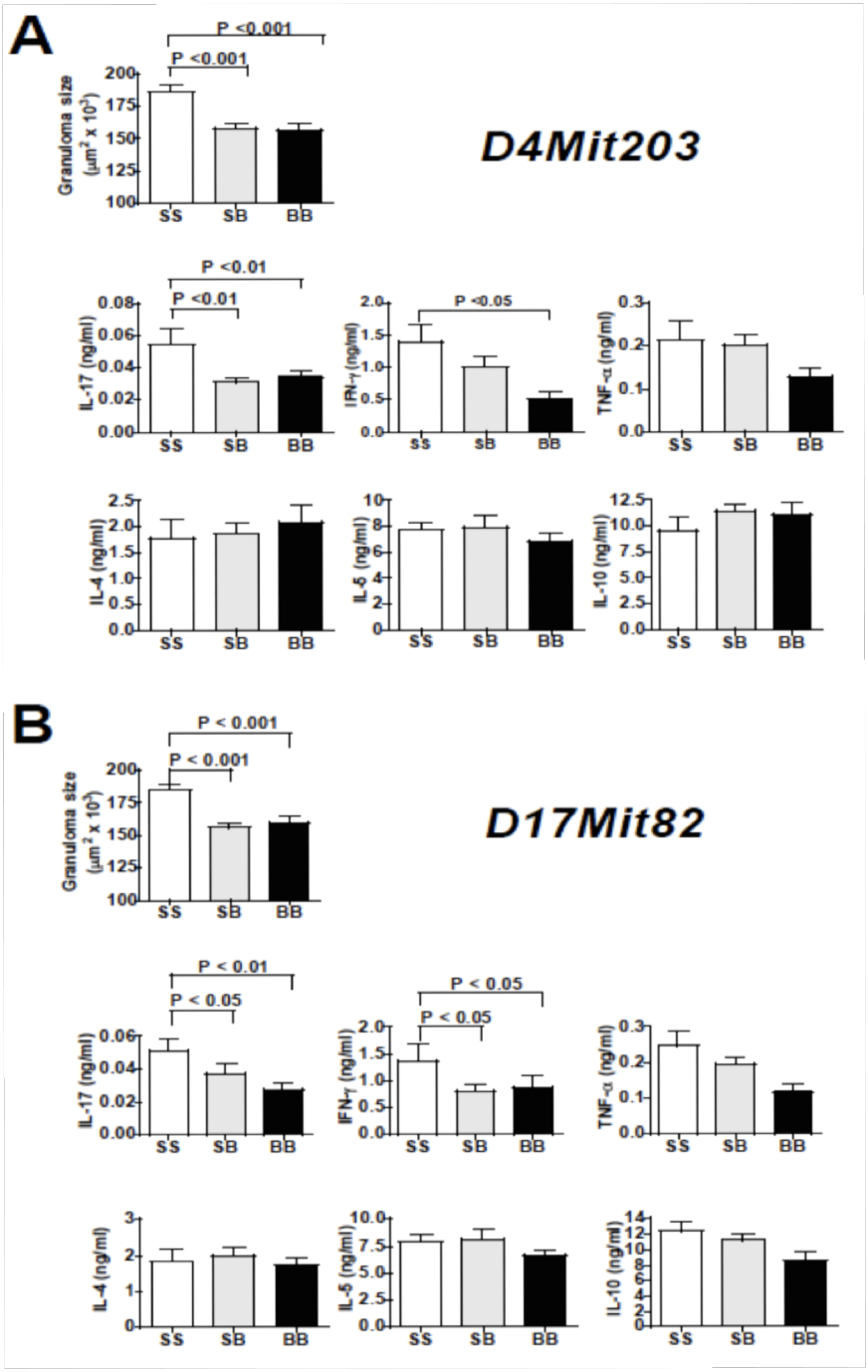


Figure 16. Segregation of granuloma size and cytokine production linked to peak markers (A) *D4Mit203* and (B) *D17Mit82* in F_2 mice. Average granuloma size was calculated for individual mice within the indicated genotypes before grouping. For the cytokines, each bar represents mean values ± SEM of mice of each genotype. Statistically significant differences between groups are as indicated in graphs. S = SJL, B = BL/6. For *D4Mit203*: SS (n = 38), SB (n = 70), BB (n = 42), and for *D17Mit82*: SS (n = 36), SB (n = 71), BB (n = 41).

Further analysis of F_2 mice disclosed that those that were homozygous for the SJL allele at both *D4Mit203* and *D17Mit82* had significantly increased pathology compared with those that were homozygous for BL/6 at each locus. Interestingly, while F_2 mice that expressed the BL/6 allele only at *D4Mit203* developed small granulomas close to mice that expressed BL/6 alleles at both *D4Mit203* and *D17Mit82*, mice that expressed the BL/6 allele only at *D17Mit82* still developed larger granulomas similar to mice that expressed SJL alleles at both loci, suggesting that *D4Mit203* plays a more significant role in pathology (**Fig 17**).

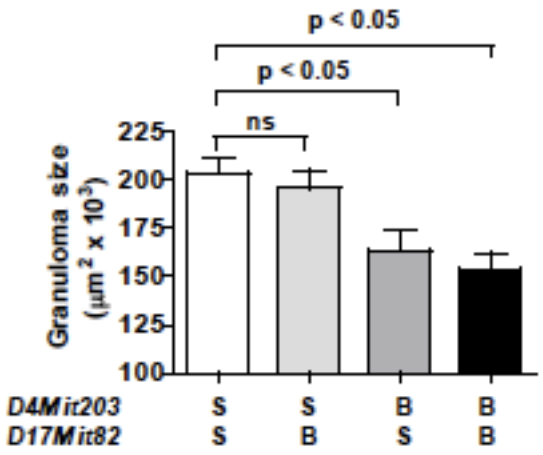


Figure 17. Segregation of granuloma size in F_2 mice homozygous for the SJL or BL/6 allele or both at *D4Mit203* and *D17Mit82*. Each bar represents mean granuloma size of mice per group. At least 20 granulomas were measured per mouse. NS = not significant.

2.2C QTL analysis of cytokine production reveals significant linkage to IL-17 production

The computer software used to perform our analyses treats each phenotype as its own individual parameter. Therefore, we were also able to analyze the production of different cytokines as its own individual phenotype, unrelated to granuloma size. QTL analysis of cytokine production from *in vitro* SEA stimulated MLN cells identified one major and several minor loci linked to increased IL-17 production (**Figure 18)**. *D4Mit211* on chromosome 4 (peak position, 24 Mb, LOD 3.4, 95% CI 10-33 Mb) was significantly linked to IL-17 production ($p < 0.01$) and analysis of F_2 mice showed that this locus was inherited in a BL/6 dominant manner (**Table III**). Suggestive linkage to IL-17 production was also detected at the previously described loci *D4Mit203* (LOD 1.8) and *D17Mit82* (LOD 2.2) as well as at *D3Mit191* (peak position, 101.5 Mb, LOD 2.3, 95% CI 75-120 Mb) and *D9Mit269* (peak position, 87.8 Mb LOD 2.1, 95% CI 61-104 Mb) (**Table III**). Statistical analysis using J/QTL and mapmaker programs revealed an epistatic interaction between *D4Mit211* and two markers, *D3Mit191* ($p < 0.01$) and *D9Mit269* ($p < 0.001$), but not between *D9Mit269* and *D3Mit191*. Increased IFN-γ production showed minor linkage to two loci, *D5Mit233* (peak position, 53 Mb, LOD 2.2, 95% CI 35-71 Mb) and *D10Mit148* (peak position, 44.7 Mb, LOD 2.5, 95% CI 21-59 Mb). The locus containing *D5Mit233* was inherited in a BL/6 dominant manner, whereas the locus containing *D10Mit148* was inherited in an intermediate fashion (**Table III**). No linkage was detected for TNF-α, IL-4, IL-5 or IL-10 production (data not shown).

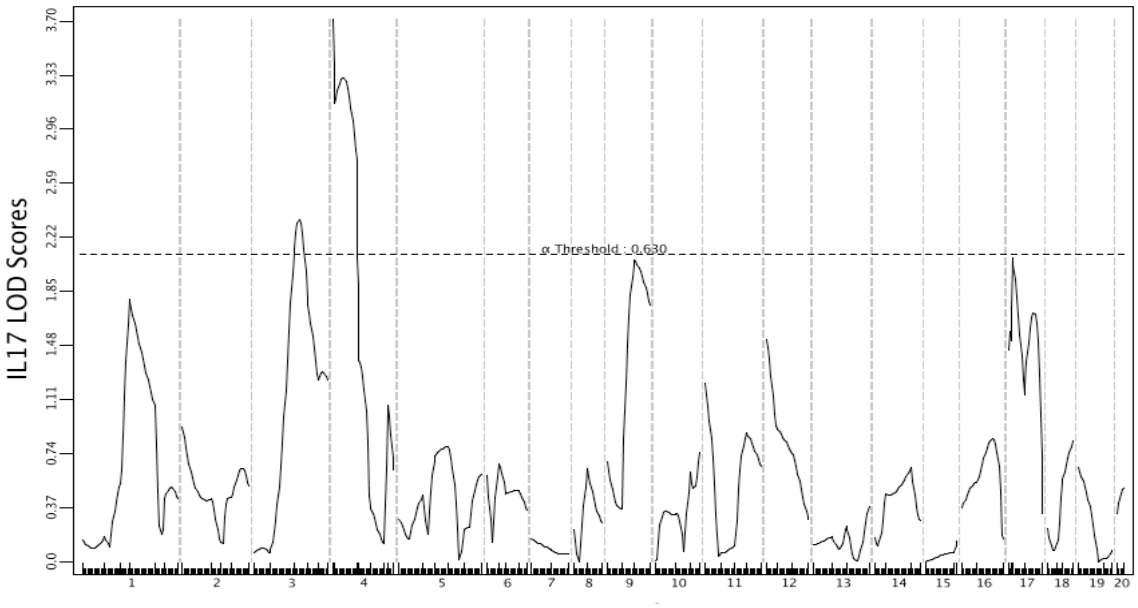


Figure 18. Interval map for IL-17 production in *S. mansoni* infected F_2 mice. The marker *D4Mit211* (LOD of 3.4) was highly significantly linked to granuloma size. LOD scores are represented on the Y-axis and chromosomal positions on the X-axis. Vertical dashed lines represent chromosomal breaks. Results are based on analysis performed by J/QTL (5000 permutations).

Table III Loci controlling cytokine production in *S. mansoni*-infected F_2 mice

Phenotype[1]	Locus peak marker	Genotype			LOD score[2]	p-value[3]
		SS	SB	BB		
IL-17 production	*D4Mit211*	1.120±0.130 (n=33)	0.366±0.288 (n=77)	0.303±0.194 (n=40)	3.4	< 0.0001
	D3Mit191	0.632±0.187 (n=31)	0.393±0.214 (n=81)	0.351±0.186 (n=38)	2.3	< 0.001
	D9Mit269	0.726±0.331 (n=34)	0.435±0.126 (n=75)	0.315±0.252 (n=39)	2.0	< 0.001
IFN-γ production	*D5Mit233*	1.704±1.039 (n=37)	0.442±0.262 (n=71)	0.577±0.233 (n=42)	2.2	<0.001
	D10Mit148	1.600±0.899 (n=39)	0.714±0.217 (n=72)	0.465±0.172 (n=37)	2.5	<0.01

1. Cytokine levels (ng/ml) are based on segregation according to genotype of F_2 mice at the specified peak marker.
2. LOD scores were calculated by the statistical program R/QTL using the graphical interface J/QTL.
3. P-values represent statistical significance for mode of inheritance between SS and SB groups.

2.2D SJL.B6-D4Mit203 and SJL.B6-D17Mit82 congenic mice exhibit significantly reduced granuloma size and IL-17 production in comparison with parental high pathology SJL mice

We were most interested in the loci identified for granuloma size, *D4Mit203* and *D17Mit82*, because they effect the overall disease development as opposed to production of a specific cytokine. To assess the effect of these loci directly on immunopathology we produced interval-specific congenic mice that were homozygous for the BL/6 allele at these loci on an otherwise SJL background. For this purpose F_1 mice were backcrossed to the SJL strain and mice were selected at each generation for heterozygosity at *D4Mit203* and *D17Mit82*. After 9 generations these mice were intercrossed and selected for homozygosity at each locus, resulting in the production of the interval specific congenic mice SJL.B6-*D4Mit203* and SJL.B6-*D17Mit82*.

Following schistosome infection, both SJL.B6-*D4Mit203* and SJL.B6-*D17Mit82* mice exhibited a significant reduction in granuloma size when compared with SJL mice and littermate controls (**Fig 19 A**), with an even greater reduction observed in mice that were congenic for both loci, suggesting that there may be an additive effect. Both congenic mouse strains also had significantly lower production of IL-17 by SEA stimulated MLN cells **(Fig 19 B)**, again with a greater effect seen in the double congenic mice, however, neither locus significantly affected IFN-γ production **(Fig 19 C)**. There was no effect on IL-4, IL-5 or IL-10 production in either congenic mouse (data not shown).

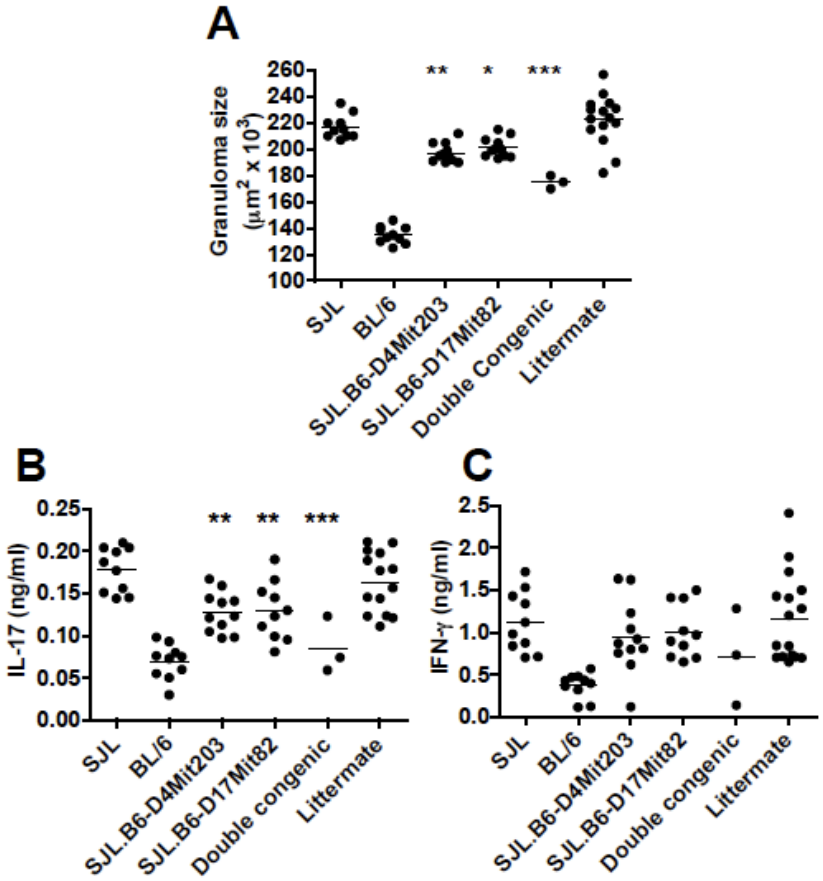


Figure 19. Granuloma size and cytokine production in SJL.B6-*D4Mit203* and SJL.B6-*D17Mit82* congenic mice. Congenic mice were produced as described in materials and methods. SJL.B6-*D4Mit203* and SJL.B6-*D17Mit82* congenic mice, or mice congenic for both intervals, had significantly reduced (A) granuloma size and (B) IL-17 production compared with SJL and littermate controls; however, these loci did not affect (C) IFN-γ production. Granulomas were counted from individual mice and at least 20 granulomas were counted per mouse. Each dot represents an individual mouse. For cytokine values each dot represents the average of triplicate determinations of individual mice. Black horizontal lines represent the mean of the group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.3 Discussion

Given that in chapter 1 non-MHC-related genes were shown as the more likely mediators of severe immunopathology, we were next interested in identifying genetic regions that control disease development. The availability of a well-established mouse model of human infectious disease with strain specific differences in immunopathology provided a unique opportunity to investigate the genetic basis of a complex disease phenotype. It is clear that the course of infection with *S. mansoni* is profoundly affected by the host genome and identification of genes responsible for the variability in immunopathology among infected individuals will provide a greater understanding of its mechanisms of pathogenesis. The results of our analysis of a progeny from a cross between high (SJL) and low (BL/6) pathology mouse strains showed that F_1 mice developed low pathology indicating that the BL/6 background is dominant. Further analysis of an F_2 progeny revealed a significant correlation between granuloma formation and SEA-induced levels of proinflammatory, but not anti-inflammatory, cytokines. Our observations confirm that severe immunopathology occurs in a proinflammatory cytokine environment dominated mainly by Th17 and Th1 cell subsets, but not Th2, suggesting that IL-17 and IFN-γ are an important part of a larger and more complex series of biological events that result in severe disease.

Severe hepatic granulomatous inflammation was highly significantly linked to two loci, *D4Mit203* and *D17Mit82*, which have also demonstrated linkage to other autoimmune and infectious disease-related conditions. The locus on chromosome 4 contains overlapping QTL for the autoimmune diseases experimental autoimmune

encephalomyelitis (EAE), insulin dependent diabetes (IDD), systemic lupus erythematosus (SLE) and psoriasis (Morel, Tian et al. 1999; Reifsnyder, Li et al. 2005; Kess, Lindqvist et al. 2006; Teuscher, Doerge et al. 2006) and has several candidate genes implicated in immune signaling and regulation including the signaling proteins LCK and map3k6 as well as CSF3R. Perhaps the most intriguing candidate gene is *IL-22ra1*, which pairs with IL-10R2 to form the IL-22R and is specific for IL-22. IL-22 is a major cytokine produced by Th17 cells and because the IL-22R is expressed on epithelial and endothelial cells, Th17 cells use IL-22 to communicate directly and specifically with tissues and not other immune cells. IL-22 has been shown to play a role in several autoimmune conditions, however more recent evidence has also identified a protective function for IL-22, particularly in inflammation of the liver by promoting survival of hepatocytes (Lafdil, Miller et al. 2007; Zenewicz, Yancopoulos et al. 2007; Korn, Bettelli et al. 2009). Whether IL-22 helps to mediate inflammation or plays a protective role in the liver during schistosome infection is not known, however it is clear that a defect in the IL-22R could have important consequences on disease outcome. This locus corresponds to a region on human chromosome 1, near the *D1S252* marker that demonstrated linkage with infection levels of *S. mansoni* in humans (Zinn-Justin, Marquet et al. 2001), providing a potential link to human disease.

The locus identified on chromosome 17 has also been linked with EAE and SLE, and contains overlapping QTL for response to trypanosome infection (*Tir1*) and resistance to malaria (*Char3*) and leishmaniasis (*Lmr1*) (Kemp, Iraqi et al. 1997; Roberts, Baldwin et al. 1997; Fortin, Stevenson et al. 2002) (**Table IV**). The most immunologically relevant candidate genes in this interval are *TNFα,* which is prominent

in acute inflammation, and *Notch3,* which has been shown to affect autoimmune disease and T cell development (Jurynczyk, Jurewicz et al. 2008). This locus corresponds to a region on human chromosome 5, near the *D5S410* marker, which was used to identify the *Sm1* locus that controls intensity of infection in humans (Marquet, Abel et al. 1996). Another likely linkage is to MHC genes located near this region, as they have been suggested to play a role in both human and murine schistosome infection (Assaad-Khalil, Helmy et al. 1993; Secor, del Corral et al. 1996; May, Kremsner et al. 1998; McManus, Ross et al. 2001; Finger, Brodeur et al. 2005).

We produced congenic mice specific for the intervals containing *D4Mit203* and *D17Mit82*. These intervals were chosen for closer analysis because they affect granuloma formation, which is a better measure of overall disease severity than individual cytokines that are a smaller part of a more complex response. Both congenic mice displayed significantly reduced granuloma size in comparison with SJL parental controls, with a greater reduction seen in SJL.B6-*D4Mit203* congenic mice compared with SJL.B6-*D17Mit82*. These mice also produced significantly lower amounts of IL-17, but not IFN-γ, IL-4, IL-5 or IL-10, thus demonstrating that the BL/6 allele at each of these loci regulates pathology and IL-17 production. Interestingly, the locus containing *D4Mit203* has a greater effect than *D17Mit82* in the congenic mice. This is in agreement with the F_2 progeny in that mice that contain the BL/6 allele at *D4Mit203* have reduced pathology, whereas mice that have the BL/6 allele at *D17Mit82* still develop higher pathology. Importantly, the smaller genetic differences in congenic mice reduces the intervals underlying these QTL, making it easier for future identification of candidate genes.

Genetic analysis of cytokine production identified one locus, *D4Mit211*, with a highly significant linkage to IL-17 production and 4 additional loci, *D3Mit191*, *D4Mit203*, *D9Mit269* and *D17Mit82*, which were merely suggestive (Lander and Kruglyak 1995). Since, it is known that IL-17 correlates with the development of large granulomas and *D4Mit203* and *D17Mit82* were linked to granuloma size and regulated IL-17 production in congenic mice, it is not surprising that there is a suggestive linkage to these loci. Statistical analysis of the loci affecting cytokine production revealed epistatic interactions between *D4Mit211* and *D3Mit191,* and between *D4Mit211* and *D9Mit269*. *D3Mit191* and *D9Mit269* contain candidate genes encoding for the nuclear orphan receptor family members *Rorc* and *Rora*, respectively (**Table IV**), which are critical for Th17 cell differentiation. The *Rorc* gene, which encodes for the transcription factor RORγt, was first identified as the transcription factor responsible for Th17 cell differentiation from naïve cells (Ivanov, McKenzie et al. 2006), similar to *Tbet* for Th1 cells and *Gata3* for Th2 cells (Glimcher and Murphy 2000). Since then it has been shown that in the absence of RORγt a second transcription factor, RORα was able to induce naïve CD4 T cells to differentiate to Th17 cells (Yang, Pappu et al. 2008). *D4Mit211* was the only locus that was significantly linked to IL-17 production in our analysis and based on the candidate genes contained in the loci for *D3Mit191* and *D9Mit269* it is possible that this locus contains a potential unidentified transcription factor or other gene that is important for IL-17 production *in vivo*. One potential candidate is *Runx1t1*. Originally identified in humans as part of a fusion translocation protein and associated with acute myeloid leukemia, the mouse homologue *Runx1tl* (Niwa-Kawakita, Miyoshi et al. 1995) is a member of the runt related family of transcription factors, one of which, *Runx1*, plays

a significant role in Th17 differentiation by interacting with RORγt (Zhang, Meng et al. 2008). These loci also contain overlapping QTL from the autoimmune diseases EAE, IDD, SLE and collagen induced arthritis (CIA) as well as infectious diseases such as *Salmonella typhimurium* susceptibility, *Borrelia burgdorferi*-associated arthritis and resistance to malaria (Sundvall, Jirholt et al. 1995; Rothe, Jenkins et al. 1997; Butterfield, Sudweeks et al. 1998; Jirholt, Cook et al. 1998; Haywood, Hogarth et al. 2000; Roper, Weis et al. 2001; Fortin, Stevenson et al. 2002; de Souza, Morel et al. 2004; Caron, Loredo-Osti et al. 2005; Bernstein-Hanley, Balsara et al. 2006). All these QTL may be of interest since the schistosome infection shares mechanistic features with many autoimmune and infectious diseases.

Table IV Candidate genes for loci controlling immunopathology and cytokines and colocalization with related QTL[1]

Chromosome	Marker	Position[2]	Phenotype	Candidate gene/s	Colocalization with QTL
4	*D4Mit203*	129.2	Granuloma formation	*Lck, Csf3r* (granulocytes) *IL22ra1, Map3K6*	*Eae40* (*Experimental autoimmunce encephalomyelitis 40*) *Idd9, Idd25* (*Insulin dependent diabetes 9,25*) *Sles2* (*Systemic lupus erythematosous 2*)
	D4Mit211	24	IL-17 production	*Runx1t1, Map3K7* (TGF-β activating kinase 1)	*Ses8* (*Salmonella enteriditis susceptibility 8*) *Lxw1* (Susceptibility to lupus)
17	*D17Mit82*	33.9	Granuloma formation	*TNFa, Notch3, Traf7*	*Char3* (Resistance to malaria) *Tir1* (*Trypanosome infection response 2*) *Lmr1* (*Leishmania major resistance 1*)
3	*D3Mit191*	101.5	IL-17 production	*Rorc, Notch2*	*Ctrq2* (*C. trachomatis resistance 2*) *Eae3* (*Experimental autoimmune encephalomyelitis 3*)
5	*D5Mit233*	53.5	IFN-γ production	*TLR6*	*Lmr3* (*Leishmania major* resistance *3*) *Listr1* (Resistance to *Listeria*) *Bbaa3* (*B. burgdorferi-associated arthritis 3*)
9	*D9Mit269*	87.8	IL-17 production	*Rora, Irak1bp1*	*Char1* (Resistance to malaria) *Eae9* (*Experimental autoimmune encephalomyelitis 9*)
10	*D10Mit148*	44.7	IFN-γ production	*?*	*Eae15* (*Experimental autoimmune encephalomyelitis 15*)

1. The upper section of the table lists loci that are highly significantly linked to a particular phenotype and the lower section lists loci that have only a suggestive linkage.
2. Position in megabases (Mb)

Of the two loci linked with IFN-γ levels, the locus on chromosome 5 proved to be the most interesting. This locus has been associated with control of IFN-γ levels in a previous independent QTL analysis of *S. mansoni* infection (Rutitzky, Hernandez et al. 2005), as well as in a model of *Leishmania major* infection (Havelkova, Badalova et al. 2006). This interval has also been implicated in Lyme arthritis (Roper, Weis et al. 2001) and susceptibility to *Listeria* infection (Boyartchuk, Broman et al. 2001). The fact that this locus has been identified in multiple mouse strains and in different disease models highly supports the notion that it contains a candidate gene likely to control IFN-γ production during an immune response in general and not jut specific to a particular disease model. One potential candidate gene is *TLR6*, which interacts with TLR2 as part of the innate immune response against a number of pathogens and in response to autoimmune diseases (Palsson-McDermott and O'Neill 2007). The majority of loci controlling cytokine response in our model did not significantly affect granuloma formation; one possible reason for this is that immunopathology in schistosomiasis is a very complex trait and cannot be determined by genes regulating a single cytokine.

In humans, variation in the severity of schistosomiasis has been linked to a variety of HLA haplotypes as well as two major loci outside the MHC designated *Sm1* and *Sm2* (Assaad-Khalil, Helmy et al. 1993; Secor, del Corral et al. 1996; May, Kremsner et al. 1998; Dessein, Hillaire et al. 1999; McManus, Ross et al. 2001). *Sm1* and *Sm2* are linked with a number of cytokine and cytokine receptor genes that influence the outcome of immune responses against pathogens, including *IFNγR1* (*Sm2*). CD4 T cell clones from individuals homozygous for the susceptibility allele of *Sm1* tended to be of the Th1 type whereas those homozygous for the resistance allele tended to be of the Th2 type

(Rodrigues, Piper et al. 1999). Furthermore, field studies demonstrated that severe clinical presentations of acute schistosomiasis correlated with a proinflammatory cytokine environment similar to the murine model (Zwingenberger, Irschick et al. 1990; King, Medhat et al. 1996; Malaquias, Falcao et al. 1997; Mwatha, Kimani et al. 1998; de Jesus, Silva et al. 2002; Booth, Mwatha et al. 2004). It is likely that humans, like mice, carry different subsets of susceptibility and resistance genes that predispose them to more severe disease. In humans, however, it is difficult to control for a number of variables, such as concomitant infections, intensity of infection and other environmental factors, which hampers the identification of candidate genes. Many of these problems do not exist in mouse models making it possible to map these genes in mice and then identify their human homologues, which can then be tested for their role in infection.

Taken together, our genetic analysis has identified the first two genomic intervals that directly and significantly control the development of severe immunopathology in murine schistosomiasis. These loci correspond to regions of the human genome that have shown association to factors relating to severe disease in human studies. Therefore it is possible that genes identified using our model will have relevance to human disease. These loci have also been linked to a variety of other infectious and autoimmune disorders with similar pathogenic mechanisms, suggesting that they may contain genes that play important roles in the host immune response. However, each locus contains over a hundred genes and even if these are narrowed based on immunological parameters the number of candidate genes will still far exceed that which can be tested in our current model. Therefore despite the success of our analysis the subsequent chapters of this thesis will focus on other models with which to identify individual genes that control pathology.

Chapter 3

C57BL/6 and C57BL/10: Similar but different

3.1 Premise and Rationale

In the previous genetic study we analyzed BL/6 and SJL mice because they are genetically distinct and displayed the greatest disparity in disease phenotype. However, these differences also likely increase the number of contributing loci to the development of severe immunopathology, making it difficult to identify causal genes that affect disease development. In this chapter we analyzed mouse strains that are more closely related genetically yet still develop dissimilar pathology, thereby decreasing the number of loci that contribute to the disease phenotype.

BL/6 and BL/10 mice share a close ancestral relationship and as such, they share the same haplotype and differ in only approximately 5% of their genome. Not including individual point mutations, these differences were mapped to ~ 26 sections with different ancestral origins that make up greater than 99% of the diversity between these two strains **(Fig 20 and personal correspondence Dr. Derry Roopenian).** Despite this very strong genetic similarity these mice can elicit very different immune responses.

In a model of lupus, BL/10 mice exhibit enhanced nephritis and autoantibody production compared to BL/6 mice and this difference was mapped to a locus on mid chromosome 13 (Rozzo, Vyse et al. 2000). The responses of BL/10 mice to *S. mansoni* infection are markedly different from BL/6 mice. BL/6 mice develop very low pathology

and produce low levels of pro-inflammatory cytokines such as IL-17, IFN-γ and TNF-α and are the major strain used to study low pathology. Conversely, BL/10 mice develop significantly higher pathology and produce larger amounts of these pro-inflammatory cytokines (unpublished observations). Given this significant difference in immunopathology, despite the similarity in their genomes, we were interested in performing a genetic analysis of BL/6 vs BL/10 mice.

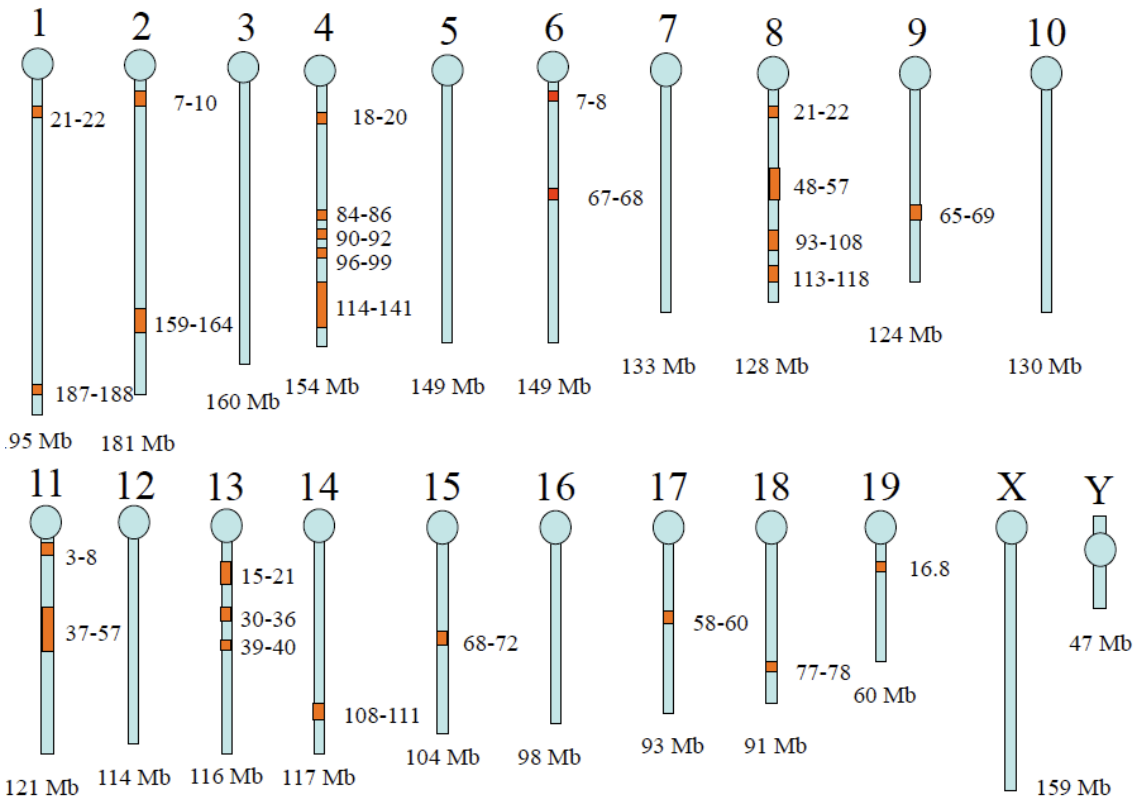


Figure 20. BL/6 and BL/10 mice have different ancestral origins for approximately 26 sections, on 13 chromosomes, totaling ~5% of the genome.

3.2 Results

3.2A C57BL/10 mice develop significantly enhanced immunopathology and produce higher levels of IL-17 and IFN-γ than BL/6 mice, and in an F_2 progeny, enhanced pathology correlates with an increase in proinflammatory cytokines

Similar to the analysis between SJL and BL/6 mice, seven weeks after infection, BL/10 mice developed large liver granulomas (mean size, 205 ± 22.7 μm^2 x 10^3), while in BL/6 mice they were significantly smaller (mean size, 128 ± 17.5 μm^2 x 10^3) (**Fig 21 A)**. F_1 mice developed small granulomas, close to those in BL/6 mice, indicating that low pathology was dominant. To further elucidate the genetic basis of granuloma formation, F_2 mice were studied in a similar manner. The F_2 progeny displayed a wide range in granuloma size ranging between those displayed by either the BL/10 or BL/6 parental strains **(Fig 21 A)**.

We next analyzed cytokine production and found that BL/10 mice produced significantly higher levels of IL-17 and IFN-γ than BL/6 mice, while in F_1 mice IL-17 and IFN-γ levels were closer to those of BL/6 (**Fig 21 B and C**). Cytokine analysis in the F_2 progeny again revealed a wide variation in both IL-17 and IFN-γ production and there was a significant correlation of these cytokines with granuloma size (**Fig 22 A and B**). In contrast, the Th2 cytokine IL-4 and the anti-inflammatory cytokine IL-10 exhibited no significant correlation to granuloma size **(Fig 22 C and D)**. Taken together these results show that similar to SJL mice, BL/10 mice act as a model of high pathology in which granuloma size correlates with increased proinflammatory cytokine production in F_2 mice.

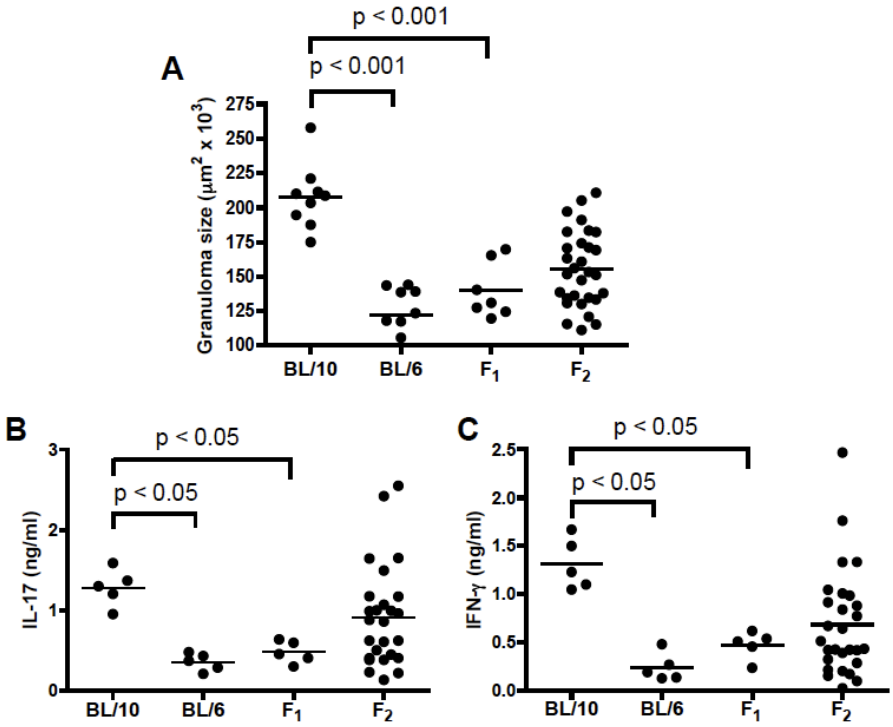


Figure 21. Granuloma size and IL-17 and IFN-γ production by SEA-stimulated MLN cells from BL/10, BL/6, F_1 and F_2 mice. (A) Granulomas were measured in liver sections obtained from 7-wk-infected BL/10 (n = 5), BL/6 (n = 5), F_1 (n = 5) and F_2 mice (n = 35), as described in materials and methods. A minimum of 20 granulomas were measured per mouse. Each dot represents an individual mouse. (B) IL-17 and (C) IFN-γ production by SEA-stimulated MLN cells from these mice were measured in 48 h culture supernatants by ELISA, as described in materials and methods. Each dot represents the mean cytokine level of triplicate determinations per mouse. Black horizontal lines represent the mean of the group. Statistical analysis was determined by one-way ANOVA using GraphPad Prism software.

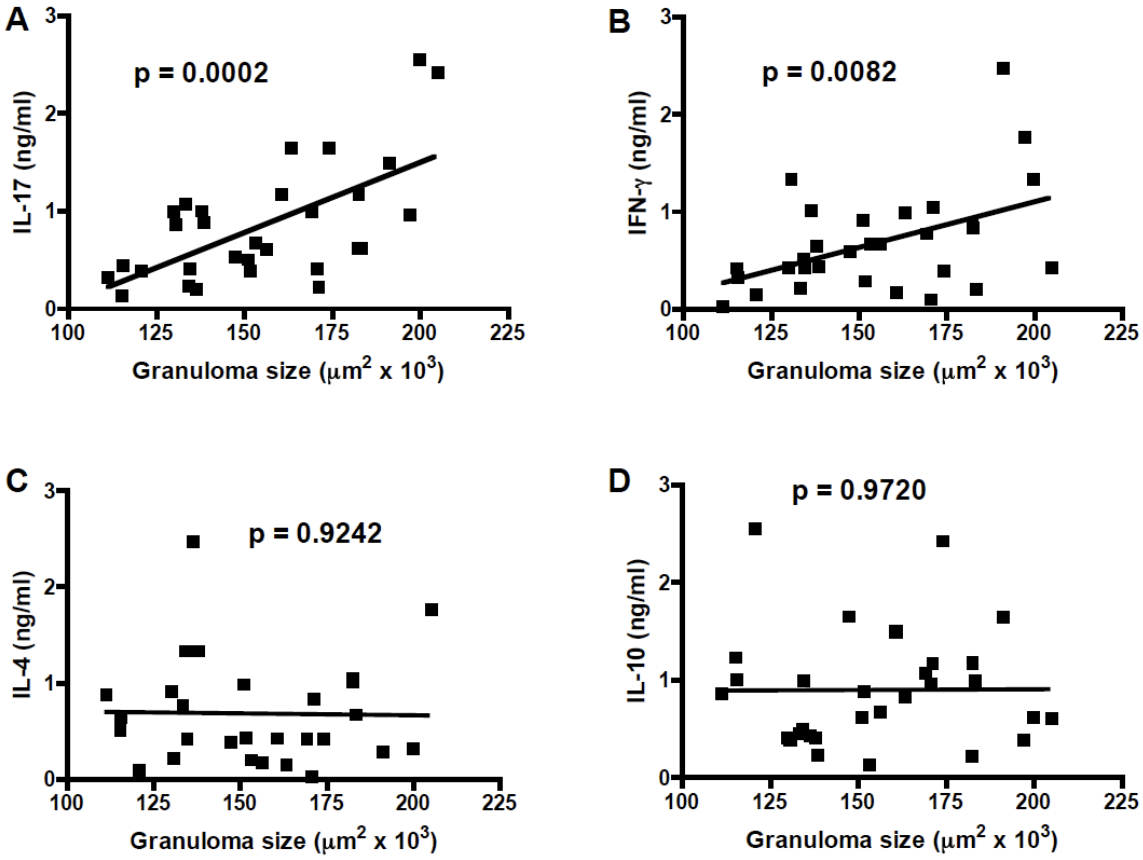


Figure 22. Linear regression analysis of mean granuloma size vs cytokine production for individual F_2 mice. (A) IL-17 and (B) IFN-γ production from MLN cells significantly correlate with granuloma size in F_2 mice. There was no correlation between (C) IL-4 and (D) IL-10 production with granuloma size in these mice. Each dot represents an individual F_2 mouse. Statistical analysis was performed by linear regression using GraphPad Prism software.

3.2B QTL analysis of immunopathology identified one locus with significant linkage to granuloma formation

Given the success of our previous genetic study (Results: chapter 2) and the wide range of pathology in F_2 mice, we performed an initial QTL analysis on a cohort of 35 F_2 mice (F_1(BL/6BL/10) x F_1(BL/6BL/10)). Linkage analysis of granuloma size identified a strongly suggestive linkage to *D1Mit504* on chromosome 1 (peak position 152.12 Mb, LOD 2.97, CI 110-190) and an additional locus with a suggestive linkage on chromosome 8 (peak position 68.27 Mb, LOD 1.91, CI 38-94) **(Fig 23)**. F_2 mice that were homozygous for the BL/10 (AA) allele at *D1Mit504* developed significantly larger granulomas than those homozygous for the BL/6 (BB) allele, while heterozygous F_2 mice (AB) developed smaller granulomas similar to those homozygous for BL/6, indicating that this locus was inherited in a BL/6 dominant manner **(fig 24)**. QTL analysis of cytokine production identified no significant or suggestive linkages to IL-17, IFN-γ, IL-4 or IL-10 production (data not shown). This is possibly due to the small number of F_2 mice in our analysis or more likely due to the limited number of available polymorphic markers between BL/6 and BL/10 mice (Appendix I).

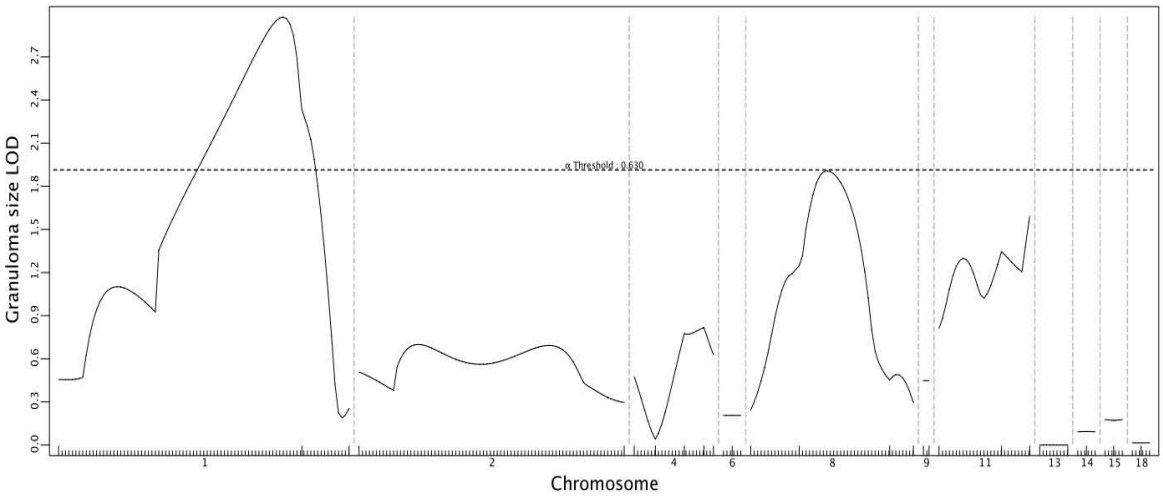


Figure 23. Interval map for granuloma size in *S. mansoni* infected F_2 mice. The marker *D1Mit504* (LOD of 2.97) was highly suggestively linked to granuloma size. An additional locus on chromosome 8 was identified with a merely suggestive linkage. LOD scores are represented on the Y-axis and chromosomal positions on the X-axis. Vertical dashed lines represent chromosomal breaks. Results are based on analysis performed by J/QTL (5000 permutations).

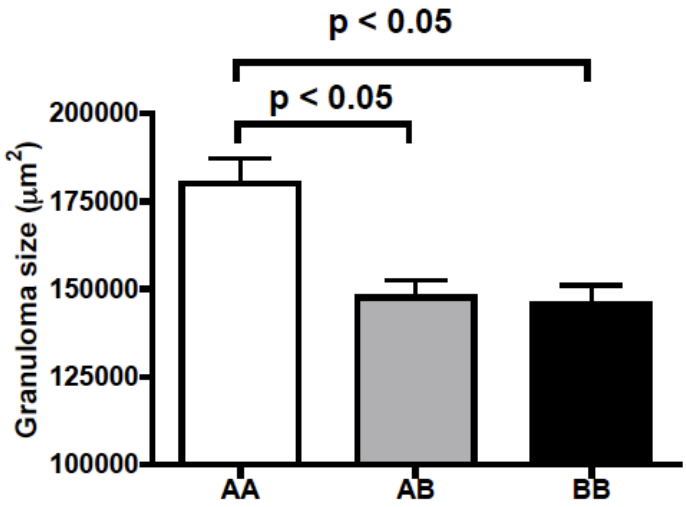


Figure 24. Segregation of granuloma size linked to peak markers *D1Mit504* in F_2 mice. Average granuloma size was calculated for individual mice within the indicated genotypes before grouping. For the cytokines, each bar represents mean values ± SEM of mice of each genotype. Statistically significant differences between groups are as indicated in graphs. A = BL/10, B = BL/6. For *D1Mit504* AA (n = 8), SB (n = 18), BB (n = 9).

3.2C Identification of loci that confer severe immunopathology and cytokine production using congenic mice

QTL analysis identified two peaks linked to granuloma size on chromosomes 1 and 8 and the given confidence intervals for each of these loci contain within them regions of known difference between BL/6 and BL/10 mice. To directly test the contribution of these QTL, and others, to the development of severe disease we examined 15 different congenic mouse lines in which BL/6 mice possessed segments of the BL/10 genome. These congenic mice were provided to us by Derry Roopenian and colleagues at Jackson Laboratories**.**

Following schistosome infection, three congenic mouse strains, B6.B10-4:87-99 (locus on chr. 4, 87-99Mb), B6.B10-4/125-135 (locus on chr. 4, 127-135Mb) and B6.B10-13/20-35 (locus on chr. 13, 20-35Mb) exhibited a significant increase in granuloma size when compared with BL/6 control mice **(Fig 25)**. B6.B10-4/87-99 and B6.B10-4/125-135 also had significantly increased production of IL-17 by SEA-stimulated MLN cells **(Fig 26 A)**, while B6.B10-13/20-35 had reduced IL-17 levels similar to BL/6 controls. Interestingly, only B6.B10-4/87-99 also had significantly increased IFN-γ production **(Fig 26 B)**, while none of these loci affected production of IL-5 and IL-10 **(Fig 26 C and D).** Several other congenic mice that did not develop severe immunopathology produced high amounts of proinflammatory cytokines compared with BL/6, suggesting that the presence of these cytokines alone was not enough to induce hepatic disease. Because BL/10 mice develop severe clinical lupus we measured type I IFN production and found that B6.B10-4/87-99 produced large amounts of both IFNα and IFNβ **(Fig 27 A and B)**.

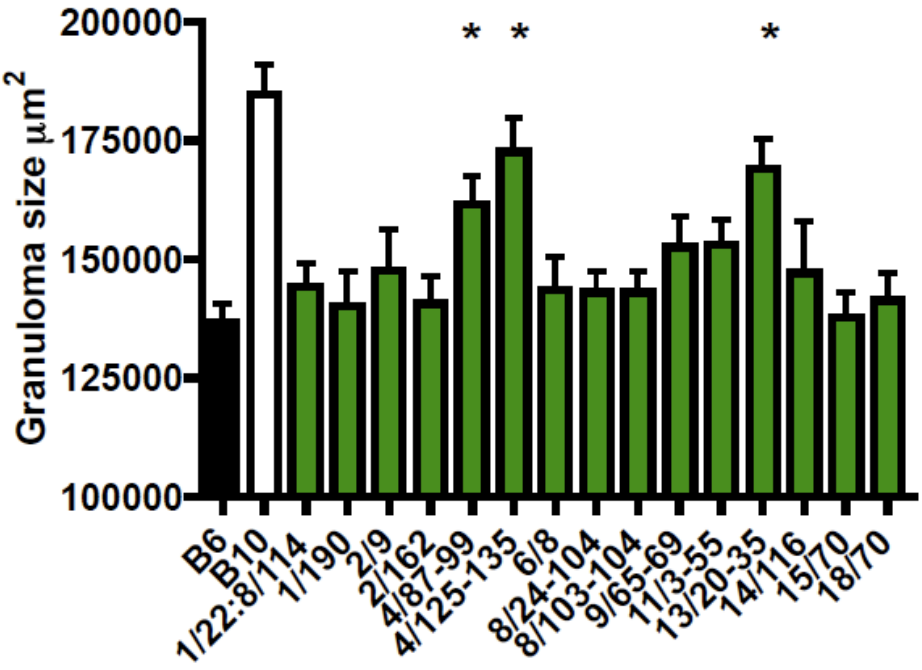


Figure 25. Granuloma size in BL/6, BL/10 and B6.B10 congenic mice. Congenic mice contain segments of the BL/10 genome on an otherwise BL/6 background. Congenic mice designations consist of the chromosome followed by the position of the congenic segment in Mb. Granuloma size was measured by morphometric analysis as described in the materials and methods. At least 10 granulomas were measured per mouse with at least 5 mice per group. Error bars represent standard deviation among individual mice. * = $p < 0.05$. Statistical significance was determined by one-way ANOVA using GraphPad Prism software.

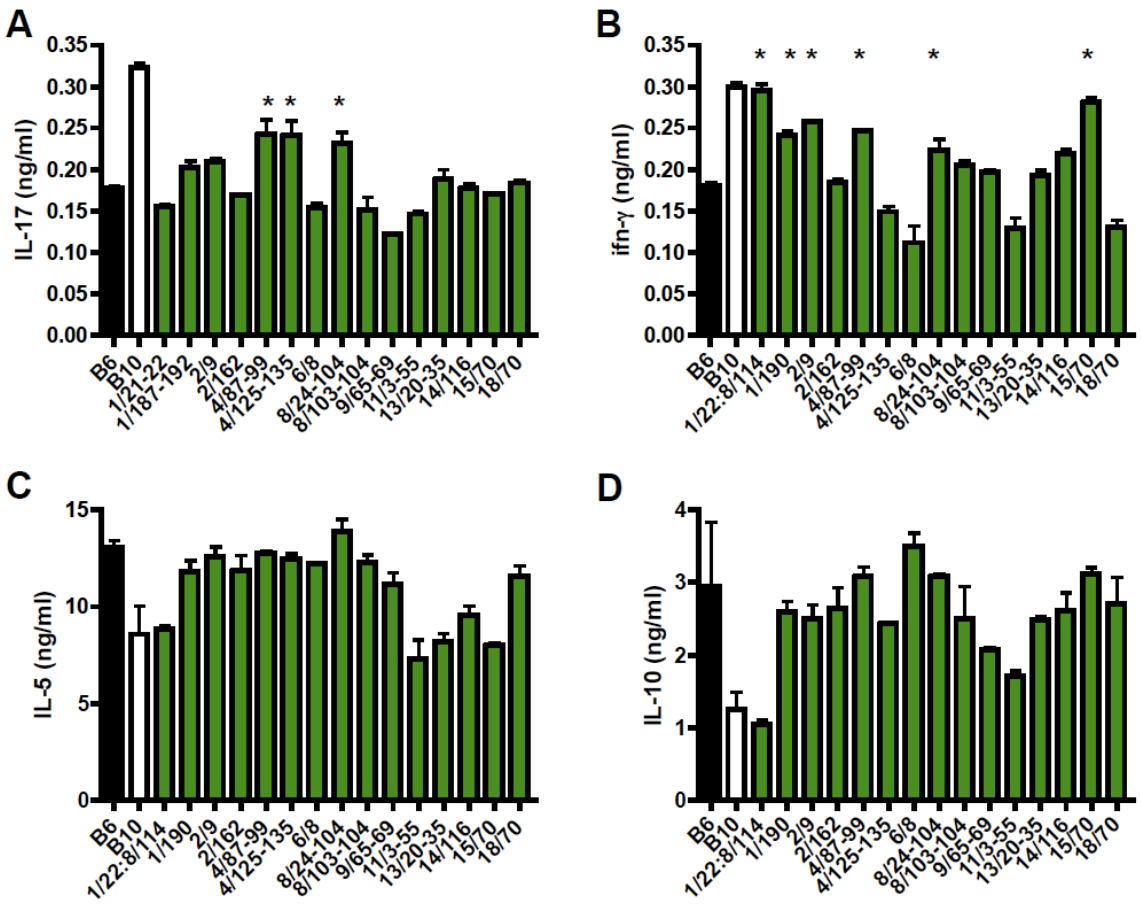


Figure 26. Cytokine profile of infected MLNs from BL/6, BL/10 and B6.B10 congenic mice. SEA-specific IL-17 (A), IFN-γ (B) IL-5 (C) and IL-10 (D) production was measured from MLN cells by ELISA. Cytokine levels are expressed as means of triplicate ELISA determinations. Results are representative of 3 (IL-17 and IFN-γ) or 2 (IL-5 and IL-10) individual experiments. * = $p < 0.05$ of congenic mice compared with BL/6 mice. Statistical significance was determined by one-way ANOVA using GraphPad Prism software.

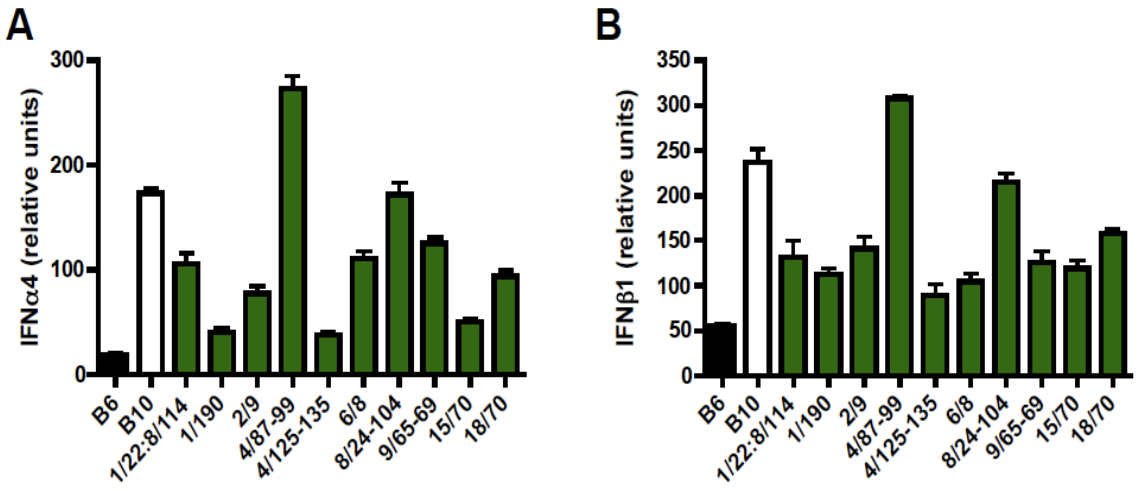


Figure 27. Type I interferon production in BL/6, BL/10 and B6.B10 congenic mice. RNA was isolated from MLNs of infected BL/6, BL/10 and B6.B10 congenic mice and expression of IFN-α (A) and IFN-β (B) was measured by real time quantitative PCR as described in the materials and methods. Standard error represents variance between two individual mice per group. Results are representative of 2 independent experiments.

3.2D Microarray analysis reveals significant differences in markers of alternative activation between infected BL/6 and BL/10 MLNs

Given the large number of candidate genes located within the loci identified as controlling immunopathology, we performed microarray experiments comparing the gene expression profiles of MLN from uninfected and 7-wk infected BL/6 and BL/10 mice. Microarray analysis will narrow our candidate gene list, as well as providing a global picture of the differences between these mice that lead to dissimilar pathologies. Initial analysis of MLN from uninfected BL/6 and BL/10 mice using the standard 2-fold expression difference as a minimum threshold produced a list of only 9 genes whose expression was increased in BL/10 over BL/6 mice **(Table V)** and likewise, only 7 genes had a greater than 2-fold increase in expression in BL/6 over BL/10 mice **(Table VI)**. This is not surprising given the extremely close genetic background of these two strains and indicates that the difference in immunopathology between these mice likely results from a differing ability to respond to infection from the same starting point.

Microarray analysis of MLN from infected BL/10 MLN revealed a broad array of roughly 20 well-annotated genes that were enhanced by a factor of 2-fold or greater compared to infected BL/6 MLN, most of which had known immunological functions **(Table VII)**. Of these genes the most immunologically relevant are *CCR3,* which is the receptor for eotaxin (Amerio, Frezzolini et al. 2003; Pease 2006) and *CXCL13*, which is important for B cell homing as well as eosinophil chemotaxis (Rossi and Zlotnik 2000; Viola and Luster 2008). An additional gene of immunological interest with enhanced expression in BL/10 MLN is the dual specificity phosphatase, *Dusp1*, also known as

MAP kinase phosphatase-1, which plays a critical role in the regulation of the innate immune response (Wang and Liu 2007).

Evaluation of the gene expression pattern in MLN from infected BL/6 mice revealed only 15 genes enhanced by a factor of 2-fold or greater with several of these possessing known immunological function **(table VIII)**. Despite this small number of differentially expressed genes, this provided the most interesting findings. The gene most highly expressed in infected BL/6 MLN compared with BL/10 was *chi3l3* or Ym1, with and increase greater than 7-fold. Ym1 is most commonly known as a marker for alternatively activated macrophages (Raes, De Baetselier et al. 2002), which are critical for survival against schistosome infection (Herbert, Holscher et al. 2004). Interestingly, *retnla* or Fizz1, another marker of AAMs (Raes, De Baetselier et al. 2002) also had enhanced expression in infected BL/6 MLN, although to a lesser extent than Ym1. Taken together these data suggest that the alternative activation pathway may be differentially regulated between these two strains. Also interesting was the small number of genes differentially regulated by greater than 2-fold between both the uninfected and infected samples in these mice, however when the minimum threshold was dropped to 1.5 fold this number jumped to over 100 genes, indicating that while there are few large differences in gene expression there are many more smaller differences which may also be important.

Table V. Genes preferentially expressed[a] in uninfected BL/10 MLNs[b]

Gene Symbol	Chromosome	Gene Accession number	Fold Diff	mRNA description
Rsad2	chr12	NM_021384	1.971699921	radical S-adenosyl methionine domain containing 2
Hsph1	chr5	NM_013559	1.984902304	heat shock 105kDa/110kDa protein 1
Hba-a1	chr11	NM_008218	2.010354605	hemoglobin alpha, adult chain 1 (Hba-a1), mRNA.
Saa3	chr7	NM_011315	2.011794577	serum amyloid A 3 (Saa3), mRNA.
Hba-a2	chr11	NM_001083955	2.097042354	hemoglobin alpha, adult chain 2 (Hba-a2), mRNA.
Glycam1	chr15	NM_008134	2.270281196	glycosylation dependent cell adhesion molecule 1 (Glycam1)
Naaladl2	chr3	XM_910834	2.31431782	N-acetylated alpha-linked acidic dipeptidase-like 2
Hbb-b1	chr7	NM_008220	2.512429301	hemoglobin, beta adult major chain (Hbb-b1), mRNA.
Hbb-b2	chr7	AB364478	2.606778498	HBB2 mRNA for hemoglobin beta chain subunit, complete cds.
LOC629446	chr10	BC057932	6.115057227	Mus musculus gag protein, mRNA

[A] Genes shown are expressed by a factor of ~ 2-fold or greater in uninfected BL/10 MLNs as compared to BL/6 uninfected MLNs

[B] Data obtained from a single microarray chip represents the gene expression profile of an individual mouse. Data shown are from one analysis representative of two with similar results

Table VI. Genes preferentially expressed[a] in uninfected BL/6 MLNs[b]

Gene Symbol	chromosome	Gene Accession Number	Fold Diff	mRna - Description
Trav15-1/dv6-1	chr14	ENSMUST00000103653	11.00543298	TRADV15-1 (Fragment) gene:ENSMUSG00000076841
Trav7d-5	chr14	ENSMUST00000103649	3.626080616	similar to TRAV7D-5 gene:ENSMUSG00000076837
Mpv17 // Gtf3c2	chr5	ENSMUST00000101411	3.358011102	General transcription factor IIIC
Igh	chr12	BC092271	2.514253296	immunoglobulin heavy chain 1a (serum IgG2a)
Mybl1	chr1	NM_008651	2.427597096	myeloblastosis oncogene-like 1 (Mybl1), mRNA.
Rgs13	chr1	NM_153171	2.323771156	regulator of G-protein signaling 13 (Rgs13), mRNA.
Igj	chr5	NM_152839	2.292540149	immunoglobulin joining chain (Igj), mRNA.
Raver2	chr4	NM_183024	2.278137776	ribonucleoprotein, PTB-binding 2 (Raver2), mRNA.
Igk	chr6	BC128281	1.967145949	immunoglobulin kappa chain complex
Olfr604	chr7	NM_147070	1.907329371	Mus musculus olfactory receptor 604 (Olfr604), mRNA.
Cldn10	chr14	NM_021386	1.838051258	Mus musculus claudin 10 (Cldn10), transcript variant 2

[A] Genes shown are expressed by a factor of ~ 2-fold or greater in uninfected BL/6 MLNs as compared to BL/10 uninfected MLNs

[B] Data obtained from a single microarray chip represents the gene expression profile of an individual mouse. Data shown are from one analysis representative of two with similar results

Table VII. Genes preferentially expressed[a] in infected BL/10 MLNs[b]

Gene Symbol	Chromosome	Gene Accession number	Fold Diff	mRna - Description
Dub2a	chr7	NM_001001559	7.471091339	deubiquitinating enzyme 2a (Dub2a), mRNA.
Ccr3	chr9	NM_009914	2.943359984	chemokine (C-C motif) receptor 3 (Ccr3), mRNA.
Igh	chr12	BC092271	2.931077267	immunoglobulin heavy chain 1a (serum IgG2a),
Snord1c	chr11	AJ543402	2.773429398	R38c snoRNA.
Fut11	chr14	AK034234	2.386671486	adult male diencephalon cDNA,
Asns	chr6	NM_012055	2.300843728	asparagine synthetase (Asns), mRNA.
Idi1	chr13	NM_177960	2.279902296	isopentenyl-diphosphate delta isomerase, transcript variant 2
Retnlg	chr16	NM_181596	2.252098613	resistin like gamma (Retnlg), mRNA.
Car4	chr11	NM_007607	2.249635085	carbonic anhydrase 4 (Car4), mRNA.
Ubxn11	chr4	NM_026257	2.217705814	UBX domain protein 11 (Ubxn11), mRNA.
Eif2c4	chr4	NM_153177	2.159788438	eukaryotic translation initiation factor 2C, 4 (Eif2c4), mRNA.
Snhg1	chr19	AK051045	2.12880824	small nucleolar RNA host gene (non-protein coding) 1
Rgs13	chr1	NM_153171	2.121275496	regulator of G-protein signaling 13 (Rgs13), mRNA.
Cxcl13	chr5	NM_018866	2.074841311	chemokine (C-X-C motif) ligand 13 (Cxcl13), mRNA.
Ndufa1	chrX	NM_019443	2.026943676	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1
Lphn2	chr3	NM_001081298	2.026586845	latrophilin 2 (Lphn2), mRNA.
Dusp1	chr17	NM_013642	2.015828634	dual specificity phosphatase 1 (Dusp1), mRNA.
Igk-V28	chr6	ENSMUST00000103302	2.005589016	Igk-V28 protein gene
P2rx1	chr11	NM_008771	1.997867631	purinergic receptor P2X, ligand-gated ion channel, 1
Xlr3b	chrX	NM_001081643	1.979443653	X-linked lymphocyte-regulated 3B
Adamdec1	chr14	NM_021475	1.959735567	ADAM-like, decysin 1 (Adamdec1), mRNA.

[A] Genes shown are expressed by a factor of ~ 2-fold or greater in infected BL/10 MLNs as compared to BL/6 infected MLNs

[B] Data obtained from a single microarray chip represents the gene expression profile of an individual mouse. Data shown are from one analysis representative of two with similar results

Table VIII. Genes preferentially expressed[a] in infected BL/6 MLNs[b]

Gene Symbol	Chromosome	Gene acession number	Fold Diff	mRNA description
Chi3l3 (Ym1)	chr3	NM_009892	7.009740321	chitinase 3-like 3 (Chi3l3), mRNA.
Trav15-1/dv6-1	chr14	ENSMUST00000103653	5.024144645	TRADV15-1 (Fragment) gene:
Trav7d-5	chr14	ENSMUST00000103649	4.338908011	similar to TRAV7D-5 gene
Eps8l1	chr7	NM_026146	3.356956867	EPS8-like 1 (Eps8l1), mRNA.
Olfr767	chr10	NM_146318	3.310488228	olfactory receptor 767 (Olfr767), mRNA.
Retnla (Fizz1)	chr16	NM_020509	3.059462042	resistin like alpha
Mid1	chrX	NM_010797	2.846894439	midline 1 (Mid1), transcript variant 1, mRNA.
Amy2-1	chr3	NM_001042712	2.631032373	amylase 2-1, pancreatic (Amy2-1), mRNA.
Amy2-1	chr3	NM_001042712	2.631032373	amylase 2-1, pancreatic (Amy2-1), mRNA.
Amy2-1	chr3	NM_001042712	2.631032373	amylase 2-1, pancreatic (Amy2-1), mRNA.
Amy2-1	chr3	NM_001042712	2.631032373	amylase 2-1, pancreatic (Amy2-1), mRNA.
Ldlrap1	chr4	NM_145554	2.264166416	low density lipoprotein receptor adaptor protein 1
Amy2	chr3	NM_009669	2.154383372	amylase 2, pancreatic (Amy2), mRNA.
Fcgr4	chr1	NM_144559	2.12647663	Fc receptor, IgG, low affinity IV (Fcgr4), mRNA.
Tgtp	chr11	NM_011579	2.090801356	T-cell specific GTPase (Tgtp), mRNA.

[A] *Genes shown are expressed by a factor of ~ 2-fold or greater in infected BL/6 MLNs as compared to BL/10 infected MLNs*

[B] *Data obtained from a single microarray chip represents the gene expression profile of an individual mouse. Data shown are from one analysis representative of two with similar results*

Based on microarray data, the alternative activation pathway may play a critical role in the differing susceptibility of BL/6 and BL/10 mice. To confirm our findings, RNA was isolated from the MLN of 7-wk-infected BL/6 and BL/10 mice and Ym1 expression was determined. In agreement with our microarray data, *Ym1* transcript levels were approximately 6 times higher in BL/6 MLN compared with BL/10 MLN and interestingly, while the majority of congenic mice had similar expression to BL/6, B6.B10-4/125-135 had reduced Ym1 expression, similar to BL/10 mice **(Fig 28)**. Taken together these results indicate that differences in the alternative activation pathway may be responsible for the differing pathology between BL/6 and BL/10 mice and that this may be controlled by a locus on chromosome 4. Unfortunately, neither Ym1, Fizz1 nor any other differentially regulated genes of interest mapped to the polymorphic regions between BL/6 and BL/10 mice, which were previously identified to control severe immunopathology. Therefore, to further dissect our phenotype, we were interested in identifying the cell types that may affect severe disease development in our model.

3.2E Schistosome eggs induce BL/10, but not BL/6, DCs to produce proinflammatory cytokines

Innate cytokines produced by dendritic cells are critical for the differentiation of Th17 cells and the development of severe schistosomiasis. Our lab has shown that bone marrow derived DCs (BMDC) from high pathology CBA mice, but not low pathology BL/6, produce mediators of Th17 cell development in response to schistosome eggs (Shainheit, Smith et al. 2008). Given the critical role of dendritic cells to induce Th17 cell differentiation and the strong association of Th17 cells with severe immunopathology we were interested if

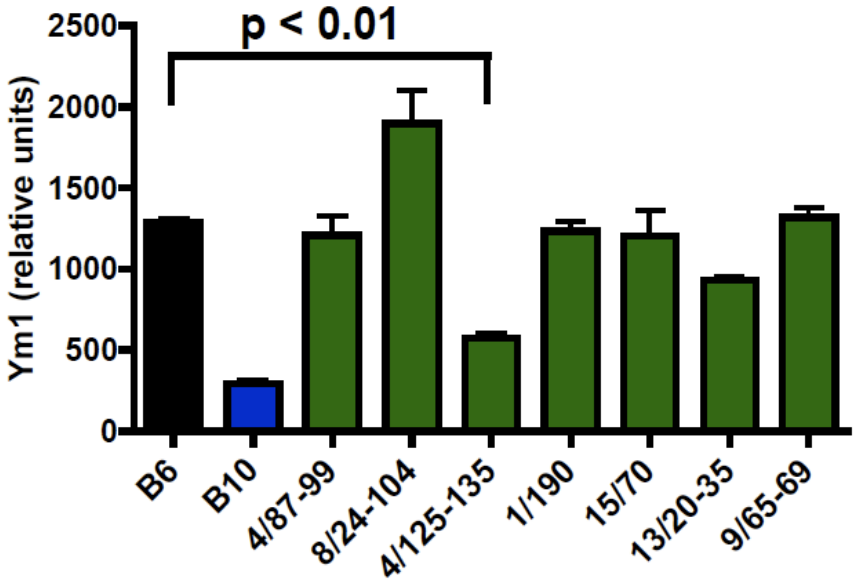


Figure 28. Expression of *chi3l3* (Ym1) is enhanced in infected MLNs from BL/6 mice compared with BL/10. MLNs were isolated from infected BL/6, BL/10 and congenic mice and total RNA was isolated as described in the materials and methods. Taqman RT-PCR was performed using primers and probes for *Chi3l3* from applied biosystems. All data were normalized against *Gapdh* and are presented as relative units. Using the average mean cycle threshold (Ct) value for GAPDH and the gene of interest for each sample, the equation 1.8 e (Ct GAPDH – Ct GOI) x 10^4 was used to obtain normalized values (Chen, Langrish et al. 2006). Each bar represents the mean mRNA level from two individual mice. Statistical significance was determined by one-way ANOVA analysis performed by GraphPad Prism software.

BMDCs from BL/10 mice were also prone to a proinflammatory phenotype. For this, we assessed cytokine production by BMDCs following a 24-h incubation with live schistosome eggs. BMDCs derived from BL/10 mice produced significantly higher amounts of IL-1 and IL-12p40 than those derived from BL/6 mice **(Fig 29 A and B)**. Both strains produced similar amounts of IL-6 and TGF-β and of all 4 cytokines in response to LPS **(Fig 29 C and D).** These results suggest that BMDCs from BL/10 mice possess a similar ability to BMDCs from high pathology CBA mice to react to schistosome eggs with proinflammatory cytokines response.

To assess if congenic mice also were capable of responding to schistosome eggs in a proinflammatory manner we derived BMDCs from B6.B10-4/125-135 congenic mice. This strain was chosen because it exhibited enhanced immunopathology and IL-17 production, as well as decreased Ym1 expression, *in vivo*. Furthermore, this locus was also identified as controlling severe immunopathology in our previous cross (BL/6 v. SJL). Following incubation with live eggs, BMDCs derived from B6.B10-4/125-135 produced significantly more IL-1 and IL-12p40 than BL/6 BMDCs and similar to that of BL/10 BMDCs **(Fig 30 A and B)**, while there were no difference seen in any of the strains between IL-6 and TGF-β **(Fig 30 C and D)**. BMDCs derived from a control congenic strain that did not develop severe immunopathology produced protein levels similar to that of BL/6. These results suggest that BMDCs derived from B6.B10-4/125-135 mice retain the ability of BL/10 BMDCs to initiate a proinflammatory cytokine response following stimulation with schistosome eggs.

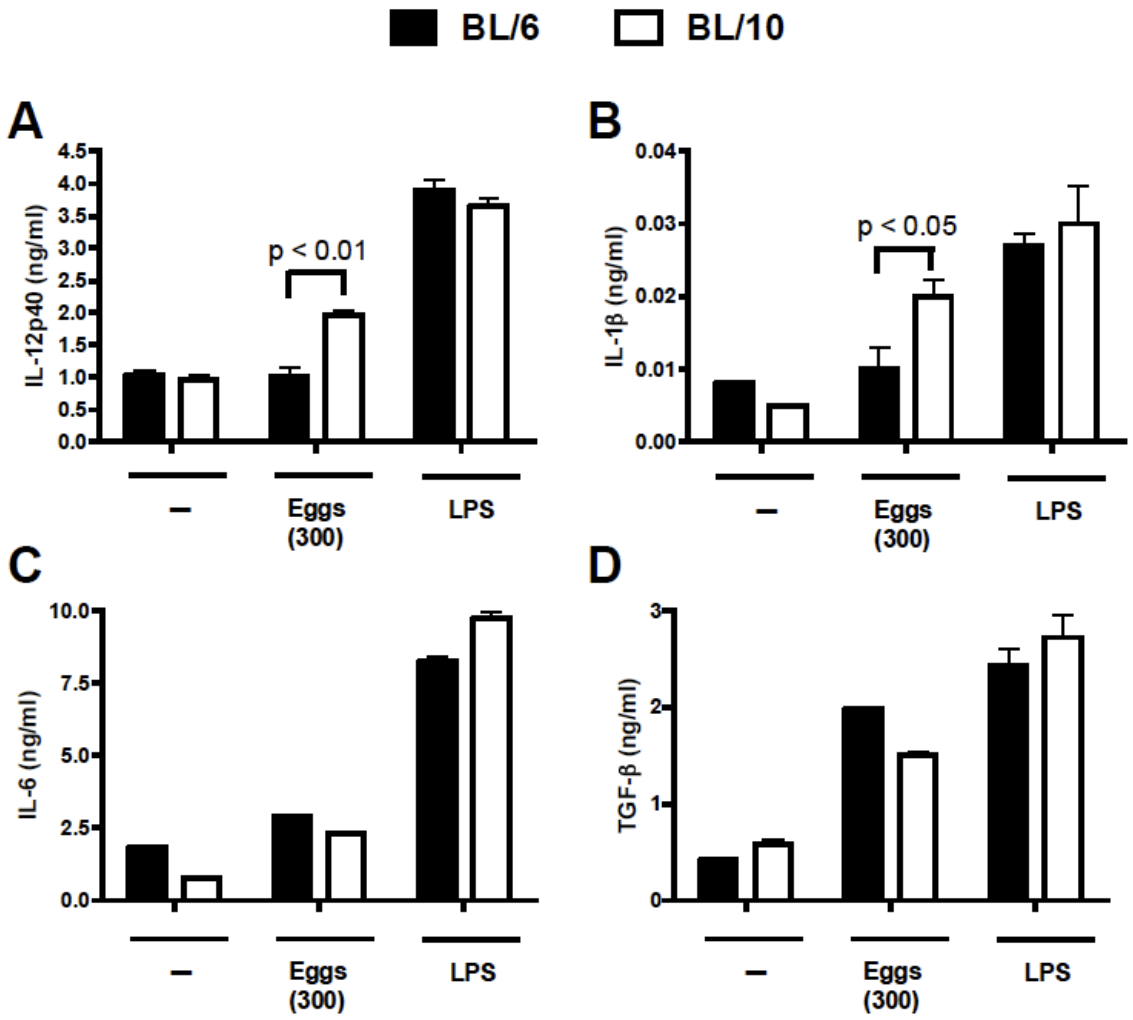


Figure 29. Cytokine production induced by live schistosome eggs from BMDCs derived from BL/6 and BL/10 mice. (A-D) BMDCs were co-cultured with the indicated number of live eggs as described in detail in the materials and methods. (A) IL-12p40, (B) IL-1β, (C) IL-6 and (D) TGF-β cytokine levels in 24-h supernatants were measured by ELISA. Induction with LPS was used as a positive control for cytokine production. Cytokine levels are expressed as means of triplicate ELISA determinations. Results shown are from one of three independent experiments with similar results. Student's *t* test performed by GraphPad Prism software was used to determine statistical significance among groups.

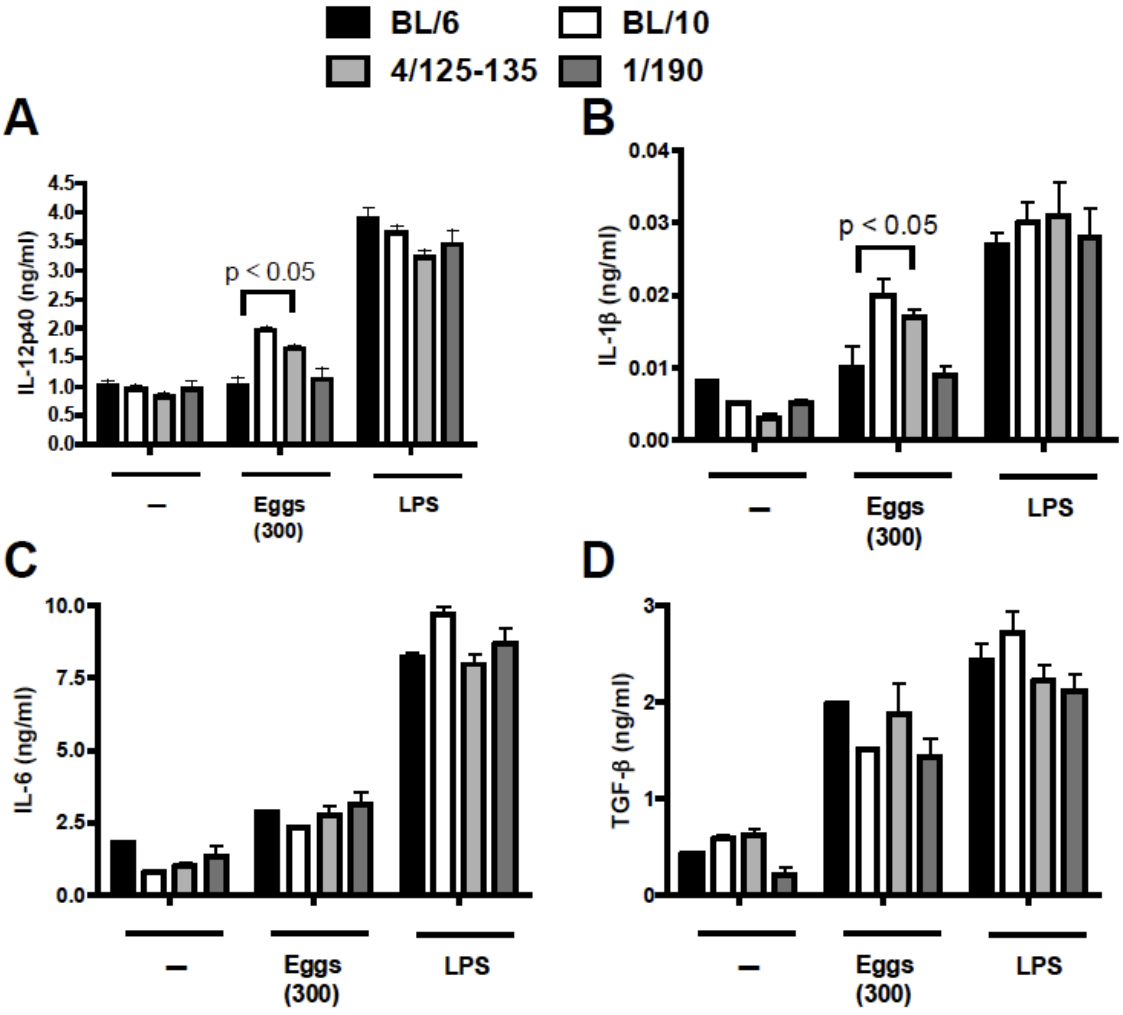


Figure 30. B6.B10-4/125-135 BMDC cytokine production induced by live schistosome eggs. (A-D) BMDCs were co-cultured with the indicated number of live eggs as described in detail in the materials and methods. (A) IL-12p40, (B) IL-1β, (C) IL-6 and (D) TGF-β cytokine levels in 24-h supernatants were measured by ELISA. Induction with LPS was used as a positive control for cytokine production. Cytokine levels are expressed as means of triplicate ELISA determinations. Results shown are from one of three independent experiments with similar results. One-way ANOVA performed by GraphPad Prism software was used to determine statistical significance among groups.

3.2F BL/10, but not BL/6, BMDCs induce Th17 cell differentiation, which is controlled by a locus on chromosome 4.

Previously our lab has shown, using a well-established model, that BMDCs from CBA but not BL/6 mice can induce Th17 cell differentiation. To examine the ability of egg-stimulated BL/10 derived BMDCs to instruct CD4 T cell differentiation, BMDCs were incubated together with syngeneic naïve CD4 T cells in the presence of live schistosome eggs and anti-CD3/CD28 beads and cytokine production was assessed after 4 days of culture. BL/10 BMDC-CD4 T cell cocultures produced significantly larger amounts of IL-1 and IL-23 than BL/6 cocultures in response to schistosome eggs **(Fig 31 A and B).** Consistent with the prevailing notion that IL-1 and IL-23 are critical for the development of pathogenic Th17 cells, there was a robust IL-17 response in egg-stimulated BL/10 cocultures, which was negligible in their BL/6 counterparts **(Fig 31 C)**. In contrast, BL/6 cocultures produced high amounts of IL-5 consistent with their Th2 biased response *in vivo* **(Fig 31 D)**. As has been reported schistosome eggs did not induce IFN-γ production from any coculture (data not shown) (Shainheit, Smith et al. 2008). Interestingly, cocultures of BMDCs and naïve CD4 T cells derived from B6.B10-4/125-135 congenic mice also elicited a significantly greater IL-17 response than BL/6 cocultures following egg stimulation **(Fig 31 C)** and this corresponded with increased production of IL-1 and IL-23 in the cocultures derived from B6.B10-4/125-135 congenic mice **(Fig 31 A and B)**. Cocultures from control congenic mice produced cytokine levels similar to BL/6 cultures **(Fig 31 A-D)**. These findings indicate that BMDCs from BL/10 mice are capable of driving Th17 cell differentiation in response to schistosome eggs and that this is controlled by a locus on chromosome 4.

3.2G BMDCs derived from BL/6 mice significantly increase expression of alternative activation markers following stimulation with schistosome eggs, compared with BL/10 and B6.B10-4/125-135 congenic BMDCs

Alternatively activated macrophages are critical for protection against excessive inflammation during *S. mansoni* infection and their absence results in death(Herbert, Holscher et al. 2004). More recent evidence has shown that dendritic cells also are capable of expressing markers of alternative activation and that this results in a more regulatory DC phenotype (Arora, Chen et al. 2006). To assess if this pathway is affected in our *in vitro* system we measured the expression of Ym1 and Fizz1 by BMDCs cultured alone or with 500 schistosome eggs for 24-h. While BMDCs derived from BL/6 mice expressed about twice the levels of Fizz1 compared with BMDCs from BL/10 mice **(Fig 32 A)**, they expressed substantially higher levels of the lectin Ym1 **(Fig 32 B).** BMDCs derived from B6.B10-4/125-135 congenic mice expressed similar amounts of Fizz1 to BL/10 BMDCs and while they did increase expression of Ym1 over BL/10 BMDCs it was still significantly lower than BL/6 BMDCs and not egg-specific **(Fig 32 A and B)**. Consistently, control BMDCs expressed levels of all markers equal to BL/6. Taken together, our results imply that a defect in the alternative activation pathway of APCs may be responsible for increased pathology in BL/10 and congenic mice.

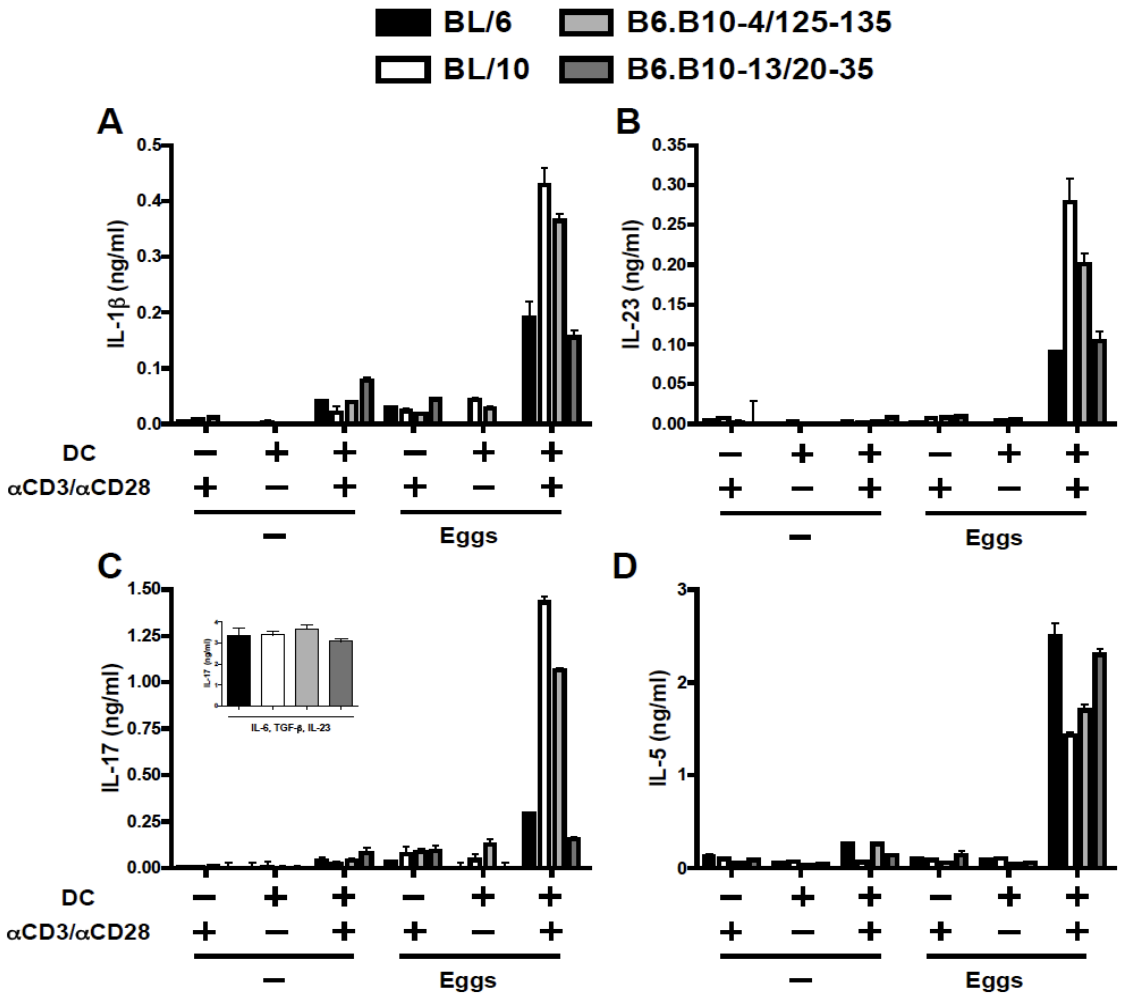


Figure 31. Cytokine production in DC-T cell cocultures stimulated with live schistosome eggs. (A-D) Naïve CD4 T cells from BL/6, BL/10, B6.B10-4/125-135 and B6.B10-13/20-35 mice were cocultures with syngeneic BMDCs in the presence or absence of αCD3/CD28 coated beads, eggs or rIL-6, TGF-β and IL-23 as described in the materials and methods. Cytokine levels in 4-day supernatants were measured by ELISA. Stimulation with 200 eggs induced markedly higher levels of IL-1 (A), IL-23 (B) and IL-17 (C) in BL/10 and B6.B10-4/125-135 cocultures as compared with BL/6 cocultures (all $p < 0.01$). In contrast, BL/6 cocultures produced significantly more IL-5 than both BL/10 and B6.B10-4/125-135 cocultures ($p < 0.05$). p values compare egg-stimulated cocultures in the presence of αCD3/CD28 coated beads. (inset) All cocultures produced equal amounts of IL-17 following stimulation with recombinant cytokines. Cytokine levels are expressed as means of triplicate ELISA determinations. Results shown are from one experiment representative of 2 (IL-1, IL-23, IL-5) or 3 (IL-17) independent experiments. Students *t* test performed by GraphPad Prism software was used to determine statistical significance.

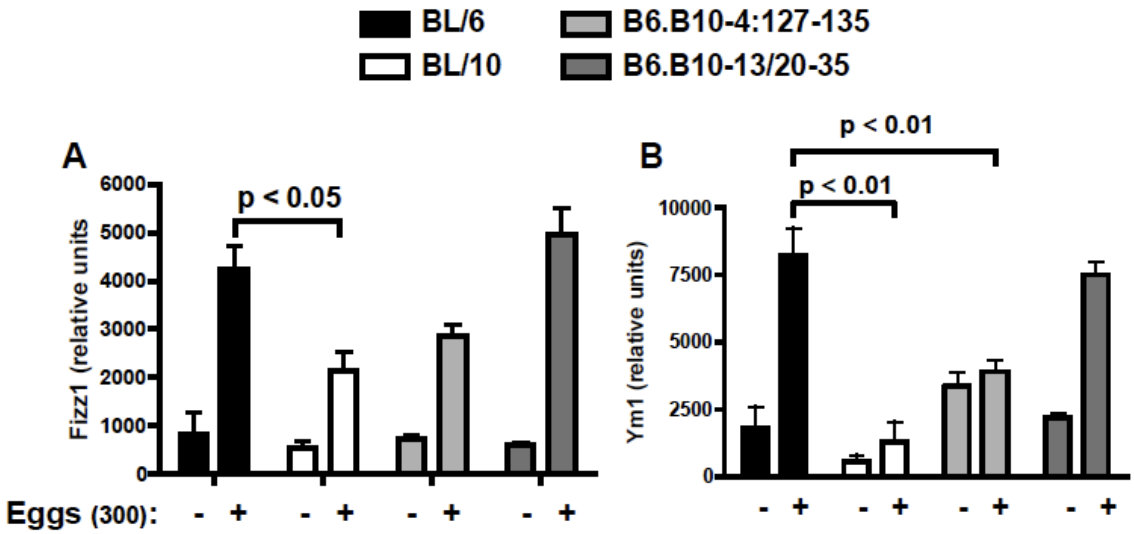


Figure 32. BMDC expression of Ym1 and Fizz1 following stimulation with live schistosome eggs. BMDCs were cocultured with or without live schistosome eggs as described in the materials and methods. (A and B) mRNA expression was measured by real-time quantitative RT-PCR as described in the materials and methods. All data were normalized against *Gapdh* and are presented as relative units. Using the average mean cycle threshold (Ct) value for GAPDH and the gene of interest for each sample, the equation 1.8 e (Ct GAPDH – Ct GOI) x 10^4 was used to obtain normalized values (Chen, Langrish et al. 2006). Each bar represents the mean mRNA level between two independent experiments. Statistical significance was determined by one-way ANOVA analysis performed by GraphPad Prism software.

3.3 Discussion

Schistosomiasis, despite effective treatment, remains a significant public health risk, in which patients develop drastically different pathologies that can result in death in about 5-10% of affected individuals. Schistosomiasis is a genetically heterogeneous disease inherited in a polygenic nature, and despite ample evidence that the course and degree of infection are profoundly affected by the host genome, genetic factors that control this process have been difficult to identify. We hypothesized that this may be due in part to the large number of contributing loci in a complex disease phenotype such as schistome infection. In our mouse model of schistosomiasis, BL/6 and BL/10 mice develop significantly different immunopathology, which afforded us a unique opportunity to interrogate the genetic basis of this complex trait using ancestrally very closely related mouse strains, and thus reducing the overall number of contributing loci. The results of our analysis of a progeny from a cross between BL/6 and BL/10 mice revealed that F_1 mice develop low pathology, indicating that the BL/6 background is dominant. Further analysis of an F_2 progeny demonstrated a significant correlation between granuloma size and SEA-induced IL-17 and IFN-γ production, but not IL-4 and IL-10. These observations confirm that severe immunopathology occurs in a proinflammatory cytokine environment, dominated by Th1 and Th17 cell subsets and importantly confirm that BL/10 mice represent an effective new model to study high pathology.

QTL analysis identified one locus, *D1Mit504,* with a strongly suggestive linkage to the magnitude of hepatic granulomatous inflammation, and one additional locus, *D8Mit205*, that was merely suggestive, both of which have previously demonstrated linkage to other immune-mediated conditions. The locus on chromosome 1 contains

overlapping QTL for the autoimmune diseases systemic lupus erythematosus (*Lxw3*) and the development of spontaneous colitis in mice (*Gpdc2*) (Kono, Park et al. 2003; Borm, He et al. 2005), as well as response to trypanosome infection (*Tir3b*) and immunity to *Salmonella typhimurium* (*Ity3*) (Sebastiani, Olien et al. 1998; Iraqi, Clapcott et al. 2000). The most immunologically relevant candidate gene in this interval is CXCR4, which is the chemokine receptor for CXCL12. While most well known for its role in HIV infection, CXCR4 is also important for the recruitment of infiltrating cells at the site of pathology in rheumatoid arthritis as well as several cancer models (Viola and Luster 2008). This locus closely corresponds to a region on human chromosome 1 that demonstrated linkage with infection levels of *S. mansoni* in humans (Zinn-Justin, Marquet et al. 2001). The locus on chromosome 8 has been linked with regulation of antibody responses, including susceptibility to infectious parasites (Puel, Mevel et al. 1998; de Souza, Morel et al. 2004), and has several immunologically relevant candidate genes, including TLR3, which is expressed on almost all liver cells and plays a role in hepatic inflammation and autoimmune disease (Yin and Gao 2010), Caspase 3, which plays a major role in apoptosis (Budihardjo, Oliver et al. 1999), and the IL-12Rb1, which is the common chain of the IL-12 receptor and is critical for IL-12 and IL-23 signaling (Hunter 2005).

Interestingly, congenic mice that contained segments of these loci did not develop severe immunopathology following schistosome infection, however the mouse strain congenic for chromosome 8 consistently produced high levels of IL-17. QTL analysis identified a strong epistatic interaction between these loci and it is possible that these loci are required to act in concert to promote severe disease. It would be interesting to

speculate that while the locus on chromosome 8 solely promotes IL-17 production, it requires interaction with a specific gene/s or regulatory factors on chromosome 1 to exert downstream effects that translate cytokine production into severe immunopathology.

Our analysis did identify three independent congenic mouse strains that developed significantly worse immunopathology compared with BL/6 mice. The first locus, B6.B10-4/87-99, induced increased granuloma size and enhanced production of several proinflammatory cytokines, including both IL-17 and IFN-γ. The majority of this locus is inhabited by the cluster of type I interferon genes, and given that BL/10 mice have enhanced susceptibility to SLE and the role of type I IFNs in the development of this disease, these are obvious candidate genes. In support of this, SEA-stimulated MLNC from B6.B10-4/87-99 congenic mice produced very large quantities of both IFNα and IFNβ. The second locus, B6.B10-13/20-35, also had significantly larger granulomas than BL/6 mice. This locus was identified in a previous genetic analysis performed by our lab using BL/6 and CBA mice as having suggestive linkage to granuloma formation It contains several potential candidate genes, most notably interferon regulator factor 4 (Irf4), which regulates Th17 cell differentiation. Interestingly however, was that B6.B10-13/20-35 congenic mice did not have enhanced proinflammatory cytokine production, particularly IL-17 and IFN-γ, yet still developed large granulomas. Therefore it is possible that an as yet unidentified cytokine may be responsible for the development of severe liver pathology or that severe hepatic granulomas may develop without the need for a proinflammatory cytokine environment in these mice. Thus these mice may represent an interesting model in which to study the development of severe disease without the need for IL-17 and IFN-γ.

Of the three loci identified that controlled severe immunopathology we were most interested in the third locus, B6.B10-4/125-135. These mice developed significantly larger liver granulomas and increased IL-17, but not IFN-γ, production compared with BL/6 mice, indicating that this effect may be specific to IL-17, which is the more reliable marker of severe disease. This locus is also contained within the interval previously identified as affecting severe disease in a cross between BL/6 and SJL mice. The locus identified here is considerably smaller than the one identified in chapter 2, and therefore contains a smaller number of candidate genes. However, although this region is fairly small, it still contains greater than 200 candidate genes, many of which have known immune functions. Given that expression differences in genes that lead to functional regulatory variation are important for a variety of complex traits, and that sequence variation leading to expression differences underlie the large majority of QTLs, combining QTL and expression studies can be a powerful tool in decreasing the number of candidate genes (Mackay 2001; Wayne and McIntyre 2002).

The first notable observation from our microarray analysis was that there were very few differences in expression, less than 15 genes in total with greater than 2-fold difference in MLNs from uninfected BL/6 and BL/10 mice. Of interest in BL/6 MLNs was the upregulation of three immunoglobulin genes, *Igh*, *Igj* and *Igk*, compared with BL/10 MLNs. The precise role of B cells in schistosomiasis is not clear at present, however several reports have shown that B cells are important for the induction of a Th2 response during infection (Hernandez, Sharpe et al. 1999; MacDonald, Patton et al. 2002) and that in the absence of B cells mice develop very large granulomas and increased morbidity (Jankovic, Cheever et al. 1998). These results are concordant with murine

studies in which BL/6 mice develop a protective Th2-mediated response following infection with *S. mansoni* that is critical for down-modulation of severe disease (Stadecker 2001; Stadecker, Asahi et al. 2004), and suggests that BL/10 mice may have a defect in this process.

As anticipated, more genes displayed enhanced expression by a factor of 2-fold in either BL/6 or BL/10 MLNs isolated from infected mice compared with those from uninfected mice. Of those genes overexpressed in BL/10 infected MLN, the most relevant for our model was *Ccr3*, which is more commonly known as the eotaxin receptor. Helminth infections typically elicit a protective Th2 mediated response by the host and CCR3 was originally thought to mediate chemotaxis of various cell types, including Th2 cells, eosinophils and mast cells, into the affected tissues. However subsequent studies have revealed that while CCR3 is expressed on all eosinophils, it is only expressed on a small number of Th2 type cells (Viola and Luster 2008). Furthermore, circulating eosinophils play an important role in the inflammation process of acute schistosomiasis and expression of CCR3 promotes chemotaxis of infiltrating eosinophils into hepatic granulomas following infection (Chiu and Chensue 2002). Taken together it is possible that upregulation of CCR3 leads to increased accumulation of eosinophils, but not Th2 cells, in the granulomas of BL/10 mice and that these, in combination with other infiltrating proinflammatory cell types (neutrophils, Th1, Th17 cells) create larger granulomas that results in increased morbidity and mortality in BL/10 mice.

Among the genes preferentially expressed by BL/6 infected MLNs, we were most interested in *Chi3l3* (Ym1) due to its association with anti-inflammatory processes. Ym1 has been extensively studied in the context of alternatively activated macrophages

(Gordon 2003). AAMs are induced by production of IL-4 and IL-13, leading to an increased expression of several proteins, including Ym1, which serves as an identifying marker (Gordon 2003). Due to their association with Th2 responses, it is not surprising that AAMs are critical for protection against helminths, and are specifically required for survival during schistosome infection. (Gordon 2003; Herbert, Holscher et al. 2004). However, more recent reports have identified Ym1 as an inducer of regulatory dendritic cells as well (Arora, Chen et al. 2006; Cai, Kumar et al. 2009). Enhanced expression of Ym1 on dendritic cells resulted in the development of Th2 effector cells (Arora, Chen et al. 2006), and more specifically, when Ym1 expressing DCs were co-cultured with CD4 T cells they induced production of the Th2 cytokines IL-4, IL-5 and IL-13 (Cai, Kumar et al. 2009). Importantly, our lab has shown a role for DCs both in the induction of these cytokines in low pathology BL/6 mice, and in the induction of Th17 cell differentiation in high pathology CBA mice, indicating a potentially critical role for DCs in modulating severe schistosomiasis (Shainheit, Smith et al. 2008).

To this effect, we made use of an *in vitro* model previously established in our lab (Shainheit, Smith et al. 2008) to further investigate whether the alternative activation pathway in dendritic cells is deficient in BL/10 mice. Here we show that cocultures of BMDCs and CD4 T cells from BL/10 mice produced significantly more IL-1 and IL-23 than BL/6 co-cultures and that this leads to a substantial increase in Th17 cell differentiation. There were no detectable differences in IL-6 and TGF-β production under any conditions. These finding are consistent with the idea that IL-23 and IL-1 in combination, and not IL-6 and TGF-β, are the critical factors for promoting pathogenic Th17 cell differentiation (Langrish, Chen et al. 2005; Kastelein, Hunter et al. 2007;

Chung, Chang et al. 2009; Dinarello 2009; Ghoreschi, Laurence et al. 2010). Furthermore, BL/6 BMDC-CD4 T cell co-cultures responded to egg stimulation with an increase in IL-5 secretion, suggesting the induction of Th2 responses. This is the result of differing capabilities of BL/6 and BL/10 BMDCs to respond to schistosome eggs, where following stimulation, BL/10 derived BMDCs secreted significantly more IL-12p40 and IL-1 than BL/10 BMDCs. Interestingly, and in support of our previous findings, BL/6 BMDCs displayed a more regulatory phenotype and had significantly upregulated expression of the alternative activation markers Ym1 and Fizz1 compared with BL/10 BMDCs. To understand the mechanism through which this may occur we analyzed BMDC and CD4 T cell co-cultures derived from B6.B10-4/125-135 congenic mice and found that they behaved similarly to BL/10 co-cultures. Following egg stimulation, B6.B10-4/125-135 co-cultures produced increased amounts of IL-17 and Th17 cell associated factors compared with BL/6 co-cultures, and again similar to BL/10 co-cultures, B6.B10-4/125-135 co-cultures had significantly reduced expression of Ym1 and Fizz1. Taken together our data support the hypothesis that BL/10 mice develop severe pathology due to a defect in the alternative activation pathway, which is controlled by a locus on chromosome 4.

The goal of this chapter was to understand how the small genetic variation between BL/6 and BL/10 mice could result in drastic differences in immunopathology. Interestingly, in all the genetic analyses performed throughout this thesis the BL/6 genetic background is dominant over various high pathology strains in both F_1 and F_2 mice. Therefore it appears that BL/10 mice develop high pathology, not because of the addition of proinflammatory mediators, but because of the subtraction of potential

regulatory mechanisms. In agreement with this, our data indicate that a significant portion of these phenotypic differences is the result of a defect in the alternative activation of dendritic cells to a regulatory phenotype, which is controlled by a locus on chromosome 4. Analysis of the genes located within the locus identified one particularly strong candidate gene, *Csf3r* (CD114, G-CSFR).

Granulocyte colony stimulating factor receptor, or G-CSFR, was originally identified in 1990 as the receptor for murine granulocyte colony-stimulating factor (G-CSF) (Fukunaga, Ishizaka-Ikeda et al. 1990; Fukunaga, Ishizaka-Ikeda et al. 1990). G-CSFR is expressed mainly on myeloid and endothelial cell types and is the only receptor for G-CSF and as such is critical its function (Demetri and Griffin 1991). G-CSF is involved in a variety of immune functions, including acting as the main granulocyte inducing cytokine from a common progenitor cell with macrophages (Rieger, Hoppe et al. 2009) and as a growth and activation factor for neutrophils (Demetri and Griffin 1991). More recently, G-CSF has been identified as a key regulator of both dendritic cell and T cell function (Rutella, Zavala et al. 2005). Several studies have shown that treatment of human DCs and DC progenitors or murine myeloid progenitors with G-CSF resulted in mobilization to a tolerogenic DC phenotype, which, in turn induced naïve CD4 T cells to differentiate into both Th2 and regulatory T cell subsets (Arpinati, Green et al. 2000; Pulendran, Banchereau et al. 2000; Fagnoni, Oliviero et al. 2004; Rutella, Bonanno et al. 2004; MacDonald, Rowe et al. 2005; Rutella, Zavala et al. 2005). The authors of these studies did not determine the expression of alternative activation markers in these cell populations, however G-CSF demonstrates a clear ability to induce regulatory phenotypes in both APCs and CD4 T cells. Administration of G-CSF has also

been shown to significantly ameliorate disease severity in EAE and type I autoimmune diabetes, both T cell-dependent murine models, similar to schistosomiasis (Rutella, Zavala et al. 2005).

Our microarray analysis revealed that expression of G-CSFR was slightly decreased in BL/10 MLNs compared with BL/6 although not to a significant extent. This could be due in part to a diluting effect because of the abundance of more highly expressed mRNAs and the wide variety of different cell types in the MLNs. We measured expression of G-CSFR in our BMDC-Egg co-cultures and found that both BL/10 and B6.B10-4/125-135 BMDCs had a significant defect in G-CSFR expression following egg stimulation **(Fig 33)**. These data, combined with the known role of G-CSF to induce tolerogenic APCs, identify *Csf3r* as a strong candidate gene in the induction of alternatively activated dendritic cells and protection against severe disease in BL/10 mice.

In parallel with these studies, we performed an independent bioinformatics analysis on BL/6 and BL/10 mice, which further strengthened the candidacy of G-CSFR as our QTG. As described in the introduction, bioinformatics when combined with experimental laboratory work can be a useful way to narrow QTL intervals and identify candidate genes. The genome of classically inbred laboratory mice consists of a mosaic of segments derived from three primary founder sources (Wade, Kulbokas et al. 2002) and approximately 97% of the genetic variation between inbred strains is ancestral (Frazer, Eskin et al. 2007). Therefore, causal genes or genetic polymorphisms that underlie QTL will likely reside in regions that are not similarly inherited by descent (IBD) between two strains, but in a region with alleles derived from a difference ancestral

source. We were able to employed several bioinformatics strategies, discussed in detail in the introduction (DiPetrillo, Wang et al. 2005), that narrowed the number of candidate genes in our QTL to a testable level **(Fig 34)**. First, because our QTL was identified in two separate crosses it is likely that the causal gene will be contained within both QTL. This narrowed our locus by about 11Mb and 200 genes. We then employed a combined cross analysis, which takes data from two separate crosses and combines them into one larger cross. This increases the statistical power of the QTL analysis and results in a smaller 95% confidence interval. Combined cross analysis further decreased the size of the QTL by 1 Mb. Next, we performed an interval-specific haplotype analysis using publicly available SNP databases as described briefly in the introduction and in more detail here and found that many regions within our QTL are inherited by descent, thus making them unlikely to contain the causal gene. Interval-specific haplotype analysis significantly reduced our QTL by another 16 Mb and reduced the number of candidate genes to 60, which when analyzed for immune function was further reduced to 30 **(Fig 34)**. Interestingly, an approximately 3Mb region within our QTL that contains the *Csf3r* gene is not IBD, therefore bioinformatics analysis also identified *Csf3r* as a strong candidate gene. The region containing *Csf3r,* not only had allelic differences between BL/6 and BL/10 mice but between BL/6 and SJL mice as well. Bioinformatics analyses assume that the majority of QTL identified are based on ancestral variation and therefore if the same locus is identified in two separate crosses, it implies that the same gene may underlie each QTL. Further experiments will be necessary to determine if SJL mice have a defect in the alternative activation pathway or in G-CSFR expression.

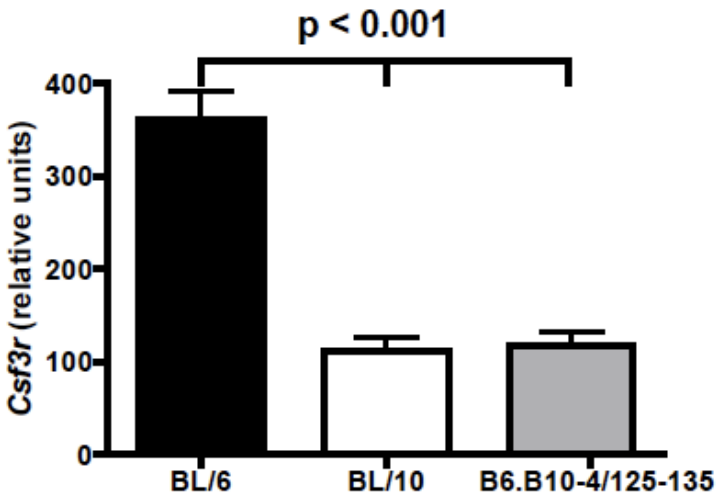


Figure 33. Expression of *Csf3r* in BMDCs stimulated with live schistosome eggs. BMDCs were cocultured with or without live schistosome eggs as described in the materials and methods. mRNA expression was measured by real-time quantitative RT-PCR as described in the materials and methods. All data were normalized against *Gapdh* and are presented as relative units. Using the average mean cycle threshold (Ct) value for GAPDH and the gene of interest for each sample, the equation 1.8 e (Ct GAPDH –I) x 10^4 was used to obtain normalized values (Chen, Langrish et al. 2006). Each bar represents the mean mRNA level between three coculture experiments. Statistical significance was determined by one-way ANOVA analysis performed by GraphPad Prism software.

In Sum, our initial genetic analysis identified three loci that significantly contribute to the development of severe immunopathology in BL/10 mice. To identify potential causal genes we performed a microarray analysis, which revealed that these differences in immunopathology were likely the result of a defect in the alternative activation pathway in BL/10 mice, a known regulatory mechanism of Helminth infections (Anthony, Rutitzky et al. 2007). Then using an *in vitro* model we showed that in response to schistosome eggs BL/6 BMDCs develop a regulatory phenotype and upregulate expression of the alternative activation markers Ym1 and Fizz1, which directs the differentiation of naïve CD4 T cells into a Th2 phenotype. In contrast BL/10 BMDCs do not develop a regulatory phenotype and instead produce high amounts of IL-23 and IL-1, which results in the development of pathogenic Th17 cells. Using congenic mice we were then able to show that this defect in the development of tolerogenic BMDCs maps to a locus on chromosome 4 and identified *Csf3r* as a strong potential candidate gene. It is likely that this is not a total defect in the alternative activation pathway as previous studies have shown that schistosome infection is fatal in mice deficient in IL-4, due to a lack of AAMs (Herbert, Holscher et al. 2004). It would be interesting to determine if this defect is specific to DCs or if BL/10 mice also have a decrease in AAMs. These and other potential experiments will be discussed in the future directions section.

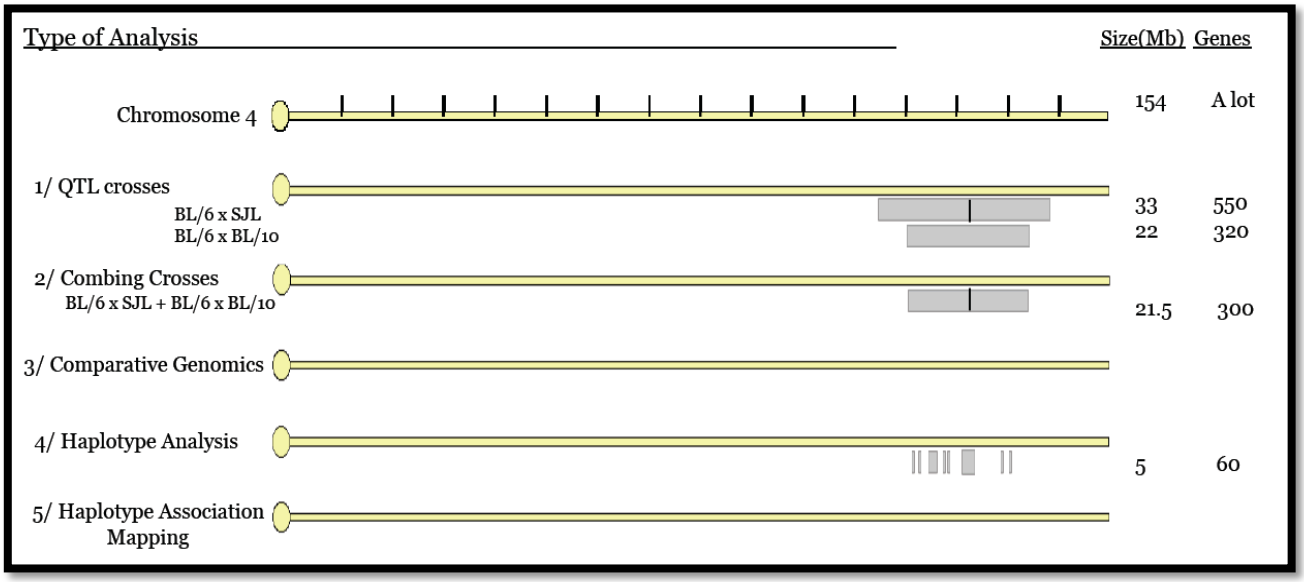


Figure 34. Application of the Bioinformatics Toolbox. We performed several bioinformatics analyses, including overlapping QTL crosses, combined cross and interval specific haplotype analysis to significantly reduce the number of candidate genes within the QTL identified on chromosome 4. These techniques are described in the introduction. The rectangular gray bars represent the relative sizes of the locus throughout each analysis. The vertical black line represents peak marker *D4Mit203* identified in the QTL analysis between BL/6 and SJL mice. These analysis were able to narrow our candidate genes from over 500 to less than 60, only 30 of which have known immune functions. Among the 30 remaining genes is *Csf3r*, which was identified as the likely candidate gene through our experimental analyses.

Chapter 4

IRAK-2 regulates IL-1-mediated pathogenic Th17 cell development and severe schistosomiasis

4.1 Premise and Rationale

As mentioned in the introduction, the majority of diseases are complex in nature, whose phenotypes vary widely within a population and are influenced by multiple gene-gene interactions. The genetic analysis of these complex traits has been critical to our understanding of the molecular mechanisms that underlie disease processes. Allelic variation in QTL is responsible for the majority of genetic diversity in both human and mouse disease susceptibility and severity. Our genetic analyses thus far have included inbred mouse strains that ranged from very distinct genetically (BL/6 vs. SJL), to those with greater than %95 homology (BL/6 vs. BL/10) and to date we have identified several QTL that confer severe immunopathology (Rutitzky, Hernandez et al. 2005; Smith, Shainheit et al. 2009), however, genes that underlie these loci remain elusive. One possible reason for this is the relatively limited genetic diversity among classically inbred strains. These mice are derived from a restricted number of founder animals predominantly within the *Mus mus domesticus* subspecies and therefore do not reach the level of diversity observed in humans (Frazer, Eskin et al. 2007; Yang, Bell et al. 2007). We reasoned that this limited diversity was a significant problem that has made it difficult to identify genes that underlie even well defined traits, such as schistosomiasis.

We accounted for this by examining the schistosome infection in wild-derived mouse strains, which are evolutionarily divergent from classical inbred strains and thus are likely to contain novel phenotypes of immune regulation. Wild-derived mice have proved useful as genetic models in identifying novel phenotypic variants in studies exploring host responses to infection with pathogens, such as *Salmonella typhimurium (Sancho-Shimizu and Malo 2006),* as well as identifying several loci that confer resistance to TNF-α induced toxic shock (Staelens, Wielockx et al. 2002). In *in vitro* models wild-derived mice are skewed towards a pro-inflammatory cytokine response as evidenced by their significantly enhanced transcription of pro-inflammatory mediators, particularly IL-6, when compared with BL/6 mice. We have shown that dendritic cells from several high pathology prone mouse strains produce significantly more IL-6, as well as two other innate cytokines IL-1 and IL-23, in response to schistosome eggs than those derived from low pathology strains, and these cytokines are known to be critical for the development of Th17 cells. Taken together these results suggest that wild-derived mice may represent a new model of severe immunopathology. In an attempt to identify novel mechanisms that govern severe disease in schistosomiasis we infected the wild-derived mouse strains MOLF and MSM with *S. mansoni* and assessed their immunopathology and cytokine production during the acute phase of the infection.

4.2 RESULTS

4.2A MOLF mice develop severe immunopathology with high levels of IL-17 following schistosome infection.

To determine if the *in vitro* bias of wild-derived mice towards a proinflamatory response also occurs in an *in vivo* model of infection, we infected MOLF and MSM mice with *S. mansoni*. Seven weeks after infection with 85 cercariae, MOLF and MSM mice exhibited significantly enhanced hepatic egg-induced immunopathology when compared with BL/6 mice, with lesions in some instances larger than those seen in the high pathology control CBA mice **(Fig 35 A)**. Individual granulomas from MOLF and MSM mice consisted of significantly larger perioval aggregates of macrophages/histiocytes, as well as lymphocytes, eosinophils and neutrophils, in a more collagenized matrix than in BL/6 mice **(Fig 35 B)**. Analysis of cytokine production by schistosome egg antigen (SEA)-stimulated draining mesenteric lymph node (MLN) cells (MLNC) from infected mice revealed that wild-derived mice produced strikingly high amounts of IL-17, approximately 5 times high than BL/6 and more than twice that produced by the high pathology control CBA mice **(Fig 35 C)**. There also was a significantly higher, but less pronounced, increase in IFN-γ and IL-6 **(Fig 35 D and E).**

Cytokine production in MLNC correlates very well with that produced in the affected liver. In order to confirm this in the wild-derived mouse strains, we isolated granuloma cells and analyzed their specific response to SEA. Similar to the MLNC, MOLF and MSM granuloma cells (GC) produced very high amounts of IL-17 compared with both BL/6 and CBA mice **(Fig 35 F)**, whereas IFN-γ and IL-6 production were not significantly different between MOLF and BL/6 mice **(Fig 35 G and H)**, suggesting that

increased IL-17 expression plays a more prominent role in the granulomas of wild-derived mice. Analysis of cytokines involved in the development of Th17 cells revealed that IL-1b, as well as IL-23p19 and IL-12p40, the subunits that make up IL-23, were expressed at much higher levels in the livers of MOLF and MSM mice than BL/6 and CBA mice **(Fig 35 I, J and K)**. These mice also expressed increased levels of the IL-12-specific subunit IL-12p35 in comparison with BL/6 mice **(Fig 35 L).** These results demonstrate that wild-derived mice produce unusually high levels of Th17-related cytokines, suggesting a potentially novel mechanism of severe immunopathology.

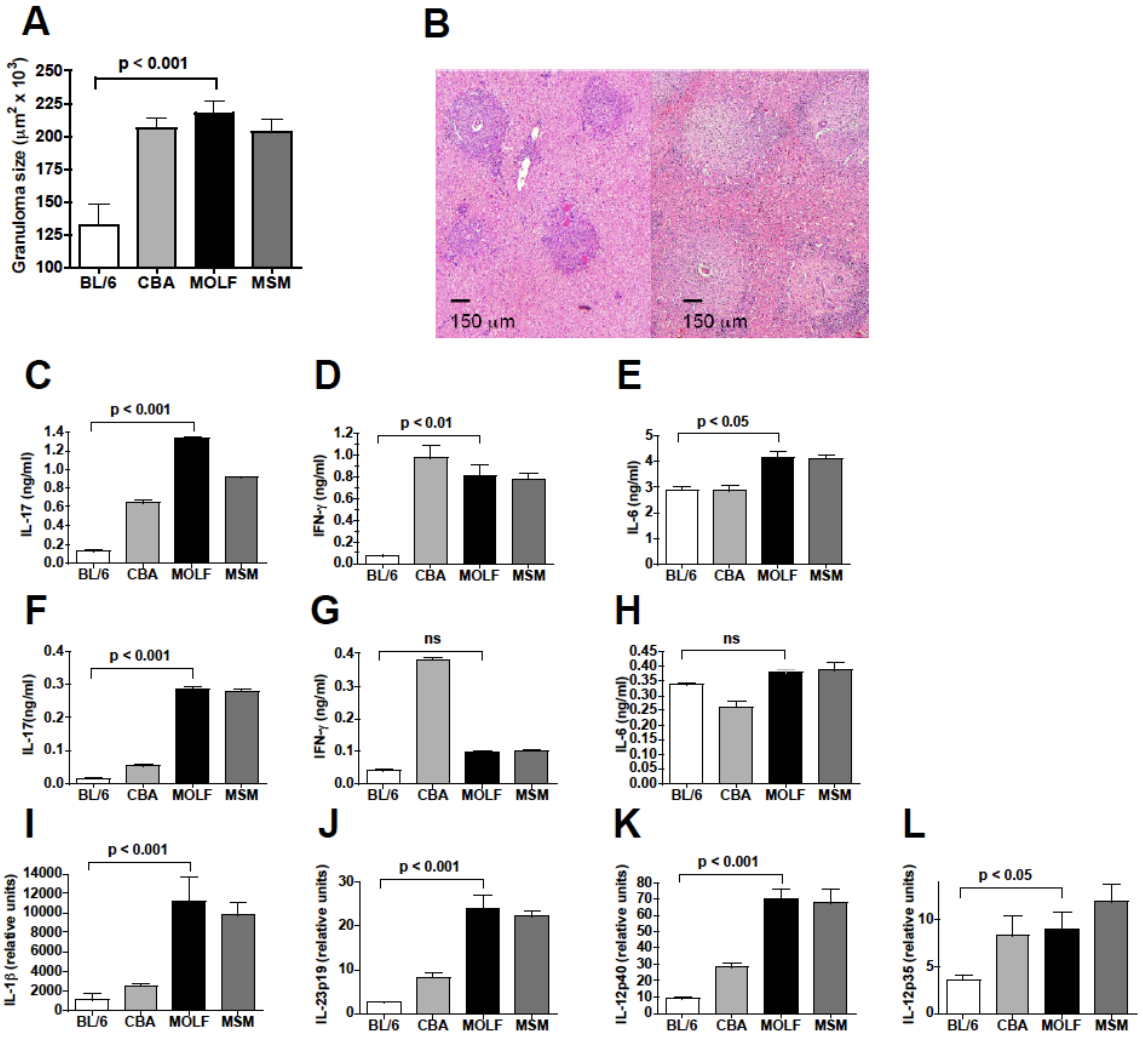


Figure 35. Schistosome-infected wild-derived MOLF and MSM mice develop severe egg-induced immunopathology and a proinflammatory cytokine profile. After a 7-week infection, mice were analyzed for hepatic immunopathology and cytokine expression. **(A)** Granuloma size was measured by morphometric analysis in BL/6, CBA, MOLF and MSM mice. **(B)** H & E stain of hepatic egg granulomas from BL/6 (left panel) and MOLF mice (right panel). IL-17, IFN- γ and IL-6 production by **(C-E)** MLNC and **(F-H)** GC stimulated with 15 mg/ml of SEA for 48 hours, measured by ELISA. **(I-L)** Expression of IL-1β, IL-23p19, IL-12p40 and IL-12p35 measured by real-time quantitative RT-PCR. RNA was isolated from the livers of infected mice and data were normalized to GAPDH. Error bars represent means of triplicate determinations +/- SD. Results are representative of at least 4 independent experiments with at least 5 mice per group. P values were determined by one-way ANOVA. ns = not significant.

4.2B Severe immunopathology and increased IL-17 production are controlled by the MOLF allele of the *Why1* locus.

The Poltorak lab previously mapped the IL-6 hyperresponsiveness of MOLF macrophages following TLR stimulation to a dominant locus on chromosome 6, designated *Why1 (Conner, Smirnova et al. 2008)*. Since MOLF mice reacted to schistosome infection with an overwhelmingly proinflammatory response, we postulated that the *Why1* locus may also play a role in this phenotype in the context of live infection. To assess the effect of the *Why1* locus directly, we used congenic mice (Why1 mice), which contain the MOLF allele of the *Why1* locus on a BL/6 background. Following schistosome infection, Why1 mice displayed significantly increased liver granuloma size compared with BL/6 mice **(Fig 36 A)**. IL-17 production by SEA-stimulated MLNC from Why1 mice was significantly greater than BL/6 mice, equal to that of high pathology CBA mice **(Fig 36 B, C and D).** IFN-γ and IL-6 production were also elevated although not to the same extent of IL-17. Why1 mice also expressed higher levels of IL-1β and IL-23p19 **(Fig 36 E and F)**, but not of IL-12p40 or IL-12p35 (**Fig 36 G and H)**. These findings demonstrate that Why1 mice largely recapitulate the pathology and IL-17 secretion seen in MOLF mice, and identify Why1 as a key locus responsible for controlling this phenotype.

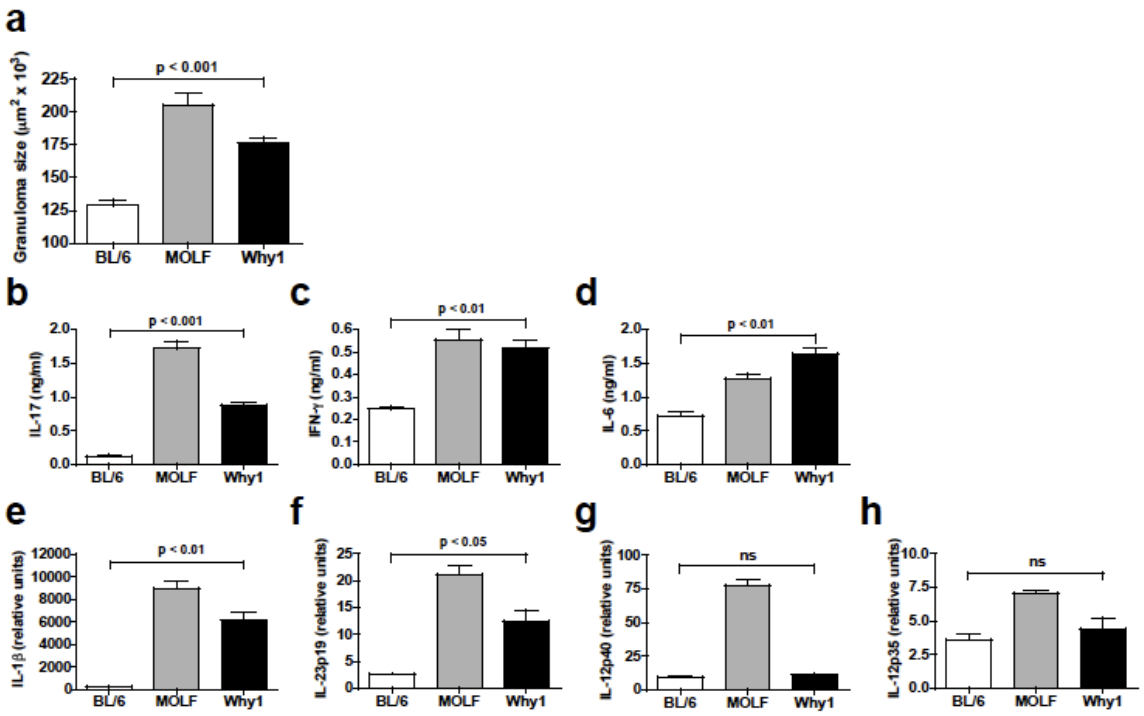


Figure 36. Severe egg-induced immunopathology and proinflammatory cytokine production are controlled by the Why1 locus. After a 7-week infection, mice were analyzed for hepatic immunopathology and cytokine expression. **(a)** Granuloma size was measured by morphometric analysis in BL/6, MOLF and Why1 mice. **(b-d)** IL-17, IFN-γ and IL-6 production by MLNC stimulated with SEA for 48 hours, measured by ELISA. **(e-h)** Expression of IL-1β, IL-23p19, IL-12p40 and IL-12p35 measured by real-time quantitative RT-PCR. RNA was isolated from the livers of infected mice and data were normalized to GAPDH. Error bars represent means of triplicate determinations +/- SD. Results are representative of at least 3 independent experiments with at least 5 mice per group. P values were determined by one-way ANOVA. ns = not significant.

4.2C Severe immunopathology and increased IL-17 production controlled by the *Why1* locus are mediated by CD4 T cells.

The *Why1* locus contains >200 possible causal genes that could underlie pathology in a complex trait such as the response to schistosome infection. We therefore sought to reduce the number of possible candidate genes by further defining the phenotype of Why1 mice. Based on previous mapping of the *Why1* locus in macrophages (Conner, Smirnova et al. 2008; Conner, Smirnova et al. 2009), we hypothesized that severe disease and increased IL-17 production was mediated by innate immune cells. To this effect, in order to avoid bias towards any one particular APC type, we used an *in vitro* system involving bulk naïve splenic APC together with CD4 T cells isolated from MLN of infected mice. SEA-stimulated Why1 APC-CD4 T cell cocultures produced markedly higher levels of IL-17 than BL/6 controls **(Fig 37 A)**. However, surprisingly, Why1 T cells in combination with BL/6 APC resulted in higher IL-17 production than Why1 APC in combination with BL/6 T cells, which did not significantly differ from the IL-17 produced by the all-BL/6 coculture **(Fig 37 A)**. These findings clearly indicated that CD4 T cells play a more prominent role than APCs in CD4 T cell IL-17 production.

To confirm this observation *in vivo*, we adapted an adoptive transfer model of high pathology. Adoptively transferred naïve splenic CD4 T cells from uninfected Why1 mice caused a sharp increase in granulomatous inflammation in infected BL/6 recipients, whereas a similar transfer of BL/6 T cells had no effect **(Fig 37 B)**. Furthermore, bulk MLNC **(Fig 37 C)**, or MLN CD4 T cells **(Fig 37 D)** from BL/6 recipients of Why1 T cells produced high levels of IL-17, similar to those from infected Why1 mice themselves. Adoptive transfer of Splenic APCs from uninfected Why1 mice did not

affect granulomatous inflammation in BL/6 recipient mice. These results demonstrate that the *Why1* locus controls severe immunopathology and IL-17 production via a CD4 T cell-mediated mechanism.

4.2D IRAK-2 regulates IL-17 production by CD4 T cells.

Why1 CD4 T cells confer enhanced immunopathology and IL-17 production to infected recipient BL/6 mice, however, it is not known if naïve CD4 T cells from Why1 mice are inherently programmed to be proinflammatory and mature differently upon activation compared to CD4 T cells from BL/6 mice. To determine if an inherent proinflammatory bias exists, Why1 and BL/6 CD4 T cells were isolated by negative selection from the spleens of naïve uninfected mice and stimulated with anti-CD3/CD28. Stimulated Why1 T cells expressed significantly higher IL-17 and IFN-g than BL/6 T cells **(Fig 38 A and B).** In contrast, BL/6 T cells expressed higher levels of IL-4 than Why1 T cells **(Fig 38 C).** These results suggest that Why1 CD4 T cells are predisposed to differentiate into a Th17/Th1 proinflammatory phenotype following stimulation.

Using shRNA knockdown technology, the Poltorak lab previously demonstrated that *Irak2* is the gene responsible for the enhanced proinflammatory response of Why1-derived macrophages following TLR stimulation (Conner, Smirnova et al. 2009). This observation supported the notion that *Irak2* is primarily involved in innate immune response signaling. However, we now show that lentiviral knockdown of IRAK-2 in CD4 T cells significantly decreased IL-17 expression by both BL/6 and Why1 T cells stimulated with anti-CD3/CD28 Abs **(Fig 38 D)**, whereas this treatment had no

significant effect on IFN-γ or IL-4 expression **(Fig 38 E and F)**. These unexpected results uncover a novel role for IRAK-2 in directing Th17 cell polarization.

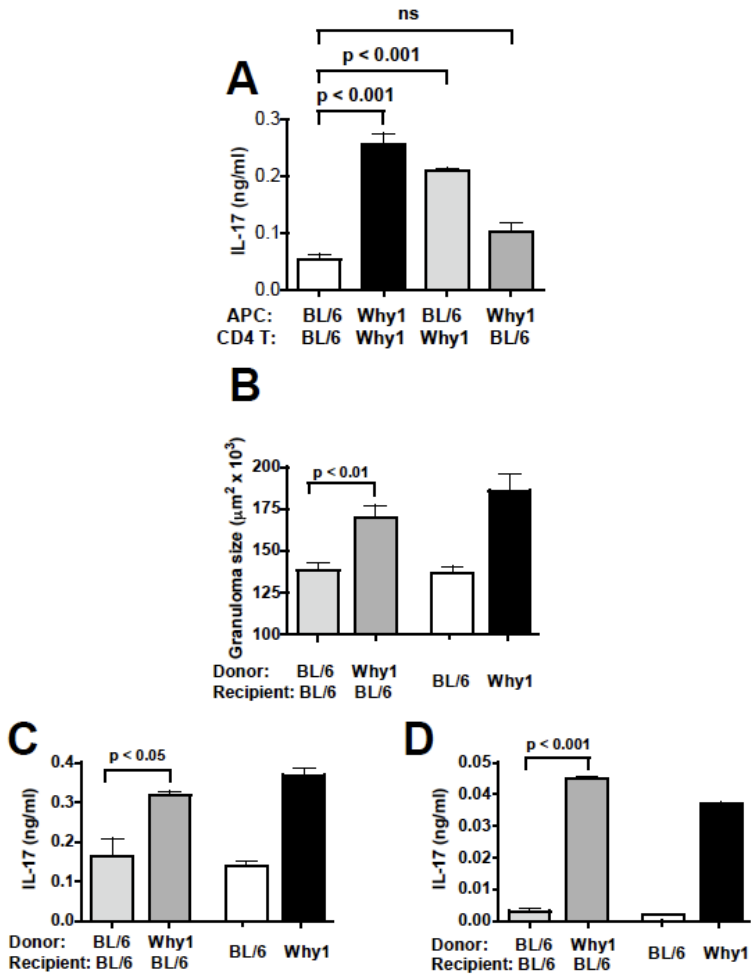


Figure 37. Why1-dependent severe egg-induced immunopathology is mediated by CD4 T cells. (A) CD4 T cells from 7 week-infected mice plus normal irradiated splenic APC were stimulated with SEA for 48 hours, and IL-17 production was measured by ELISA. **(B-D)** Purified naïve splenic CD4 T cells were transferred i.v. as indicated and recipient mice were subsequently infected with *S. mansoni*; BL/6 and Why1 mice are shown for comparison. **(B)** Granuloma size in recipient mice was measured by morphometric analysis. IL-17 production by **(C)** bulk MLNC and **(D)** purified MLN CD4 T cells plus irradiated splenic APC stimulated with SEA for 48 hours and measured by ELISA. Error bars represent means of triplicate determinations +/- SD. Results are representative of two **(A)** or three **(B-D)** independent experiments with at least 4 mice per group. P values were determined by one-way ANOVA. ns = not significant.

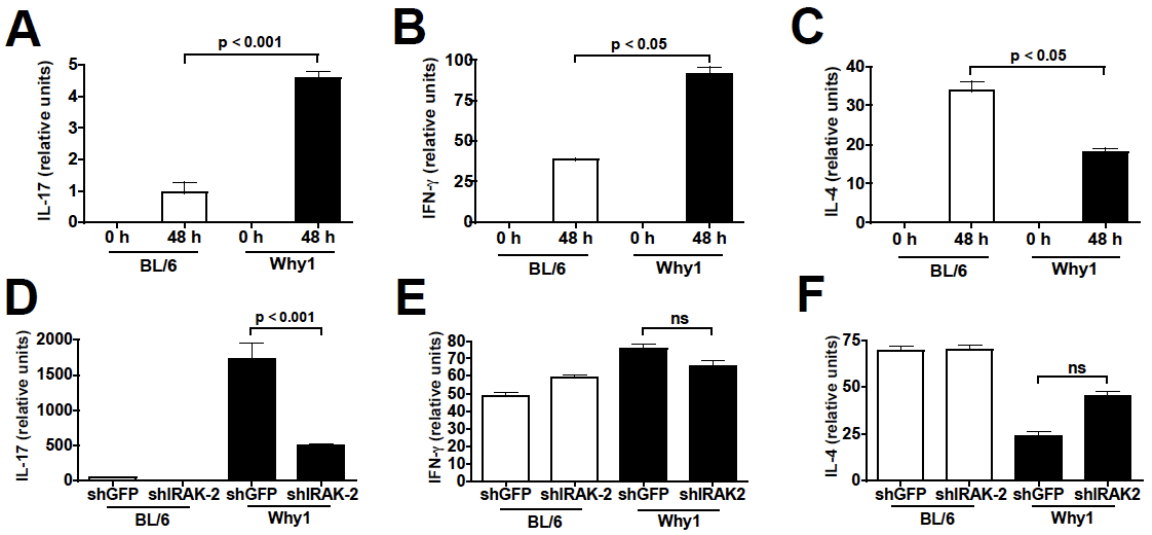


Figure 38. IRAK-2 is necessary for CD4 T cell-specific IL-17 production. (A-C) Naïve CD4 T cells from BL/6 and Why1 mice were stimulated with anti-CD3/CD28 for 48 hours and IL-17, IFN-g and IL-4 mRNA expression was measured by quantitative real-time PCR. **(D-F)** BL/6 and Why1 CD4 T cells were first treated with shRNA against IRAK-2 or control GFP and then stimulated with anti-CD3/CD28 for 48 hours. IL-17, IFN-g and IL-4 mRNA expression was measured by quantitative real-time PCR. Error bars represent means of triplicate determinations +/- SD. from one of three independent experiments with similar results. P values were determined by one-way ANOVA. ns = not significant.

4.2E IRAK-2 determines the severity of schistosome infection *in vivo*

To directly assess the effect of IRAK-2 on the schistosome infection *in vivo,* we examined the immunopathology and cytokine profile in IRAK-2-deficient (IRAK-2-/-) mice. BL/6 and IRAK-2-/- mice were first immunized with an emulsion of SEA and complete freund's adjuvant (CFA) and then infected with *S. mansoni*. Immunization with SEA/CFA induces severe pathology and high IL-17 production in mice that are typically resistant to infection (Rutitzky, Hernandez et al. 2001). After 7 weeks of infection, the SEA/CFA-immunized IRAK-2-/- mice exhibited significantly reduced granulomatous inflammation in comparison with similarly treated WT BL/6 mice and IRAK-2+/- littermate controls **(Fig 39 A)**. SEA-stimulated MLN CD4 T cells from infected, SEA/CFA-immunized IRAK2-/- mice also produced significantly less IL-17 than BL/6 and IRAK-2+/- controls, which was negligibly higher than their unimmunized counterparts **(Fig 39 B)**. Interestingly, IRAK-2 did not influence the levels of IFN-γ production **(Fig 39 C)**. These results demonstrate that IRAK-2 mediates the development of severe immunopathology and enhanced IL-17 production in schistosomiasis.

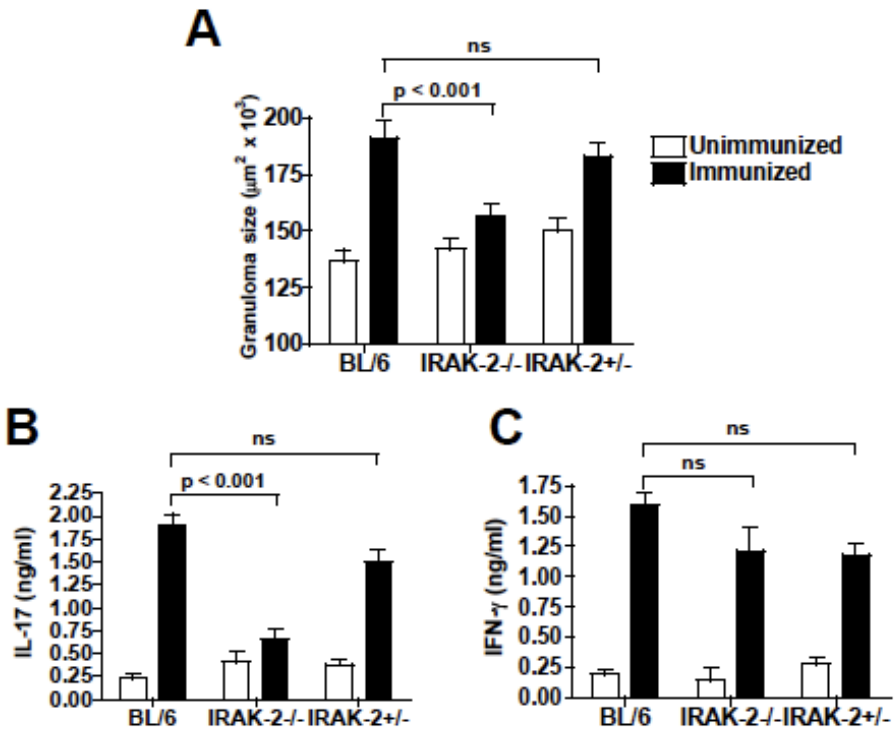


Figure 39. IRAK-2 mediates severe egg-induced immunopathology and CD4 T cell IL-17 production. IRAK-2-/-, littermate IRAK-2-/+ and BL/6 mice were immunized s.c., or not, with 50 mg of SEA/CFA prior and following infection as previously described. **(A)** Granuloma size was measured by morphometric analysis after 7 weeks of infection. At least 30 granulomas were measured per group consisting of 5-7 individual mice. **(B)** IL-17 and **(C)** IFN-γ production by CD4 MLN T cells plus irradiated splenic APC stimulated with SEA for 48 hours, measured by ELISA. Error bars represent means of triplicate determinations +/- SD. from 5-7 individual mice per group. P values were determined by one-way ANOVA. ns = not significant.

4.2F IRAK-2 enhances IL-1-induced Th17 cell differentiation.

Although a number of cytokines have been demonstrated to play key roles in Th17 cell development (Dong 2008), we have observed that production of IL-1β and IL-23 by egg-stimulated dendritic cells is essential for Th17 cell differentiation in high-pathology CBA mice (Shainheit, Smith et al. 2008). As shown herein, IL-1β and IL-23 expression was also markedly and consistently higher in MOLF and Why1 mice than in BL/6 mice **(Fig 35 I and J and 36 E and F).** To address the molecular basis by which wild-derived IRAK-2 leads to enhanced IL-17 production, we stimulated naïve CD4 T cells with each of these two cytokines, or in combination, and found that IL-1 *per se* induced IL-17 production only in CD4 T cells from Why1, but not from BL/6 or IRAK-2-/- mice (**Fig 40 A)**. IL-23 alone was ineffective but synergized with IL-1 for a significantly greater increase in IL-17 production by Why1 than either BL/6 of IRAK-2-/- cells (**Fig 40 A)**. In CD4 T cells additionally stimulated non-specifically with anti-CD3/CD28, IL-1 again elicited significantly more IL-17 production in Why1 than in BL/6 cells, whereas there was a significant defect in IRAK-2-/- mice **(Fig 40 B)**. Importantly when stimulated with anti-CD3/CD28 and IL-23 alone there was no difference in IL-17 production between any of the groups, indicating that the effect of IRAK-2 is specific to IL-1 mediated IL-17 production. Under more optimal Th17 cell differentiating conditions, IL-23 markedly enhanced IL-1-induced IL-17 production by both Why1 and BL/6 T cells, but significantly less so in IRAK-2-/- T cells **(Fig 40 C)**. These results demonstrate that IL-1-induced IL-17 production by T cells is highly dependent on IRAK-2; they also indicate that IL-23 by itself is unable to stimulate IL-17 production, but significantly potentiates IL-1 in carrying out this function.

As further evidence of the role of IRAK-2 in boosting Th17 differentiation, we found a significantly higher expression of the Th-17-cell associated cytokine IL-22 in IL-1-stimulated CD4 T cells from Why1 than from BL/6 or IRAK-2-/- mice **(Fig 40 D)**. Following stimulation with antiCD3/CD28 and IL-1, Why1 T cells also displayed significantly increased expression of the IL-23R compared with BL/6 mice, while level remained close to background in IRAK-2-/- mice **(Fig 40 G)**. Additionally, in Why1 T cells there was significantly higher expression of the Th17 cell lineage-associated transcription factors RORγt **(Fig 40 E)** and AP-1 B-cell activating transcription factor (BATF) **(Fig 40 F)** (Ivanov, McKenzie et al. 2006; Schraml, Hildner et al. 2009). Time course analysis revealed an earlier and short-lived induction of Rorγt in comparison with BATF, but at all times both expression levels were higher in Why1 than in BL/6 cells, and were profoundly down-regulated in IRAK-2-/- cells. Interferon regulatory factor 4 (IRF4) and the aryl hydrocarbon receptor (AHR) also play important roles in Th17 cell biology (Brustle, Heink et al. 2007; Quintana, Basso et al. 2008; Veldhoen, Hirota et al. 2008), however, there were no significant differences in their expression among the various cell populations (data not shown). Likewise, no significant differences were observed in the induction of the Th1 and Th2 cell-associated transcription factors T-bet and Gata-3 (data not shown).

For effective induction of responsive genes following TLR/IL-1R stimulation, IRAK family kinases are known to activate a series of downstream signaling events, including NF-κB and certain MAPK family members (Kawagoe, Sato et al. 2008; Wan, Xiao et al. 2009). To assess the effect of IRAK-2 on these molecular mediators, CD4 T cells were stimulated with IL-1 and the activation of two pathways downstream of the

IRAK signaling complex, NF-κB and MAP kinases, were compared. Western blot analysis of phosphorylation levels revealed that Why1 T cells had significantly enhanced activation of the IκB kinase (IKK) family member p105 in comparison with BL/6 controls, suggesting increased activation of the NF-κB axis via IL-1 receptor signaling. At the same time, changes in Erk phosphorylation were insignificant, indicating that the MAPK pathway is less affected by the pro-inflammatory IRAK-2 isoform **(Fig 40 H)**. Taken together, these results demonstrate that IL-1-induced Th17 cell polarization via IRAK-2 is associated with increased expression of the transcription factors RORγt and BATF, likely through enhancement of NF-κB activity.

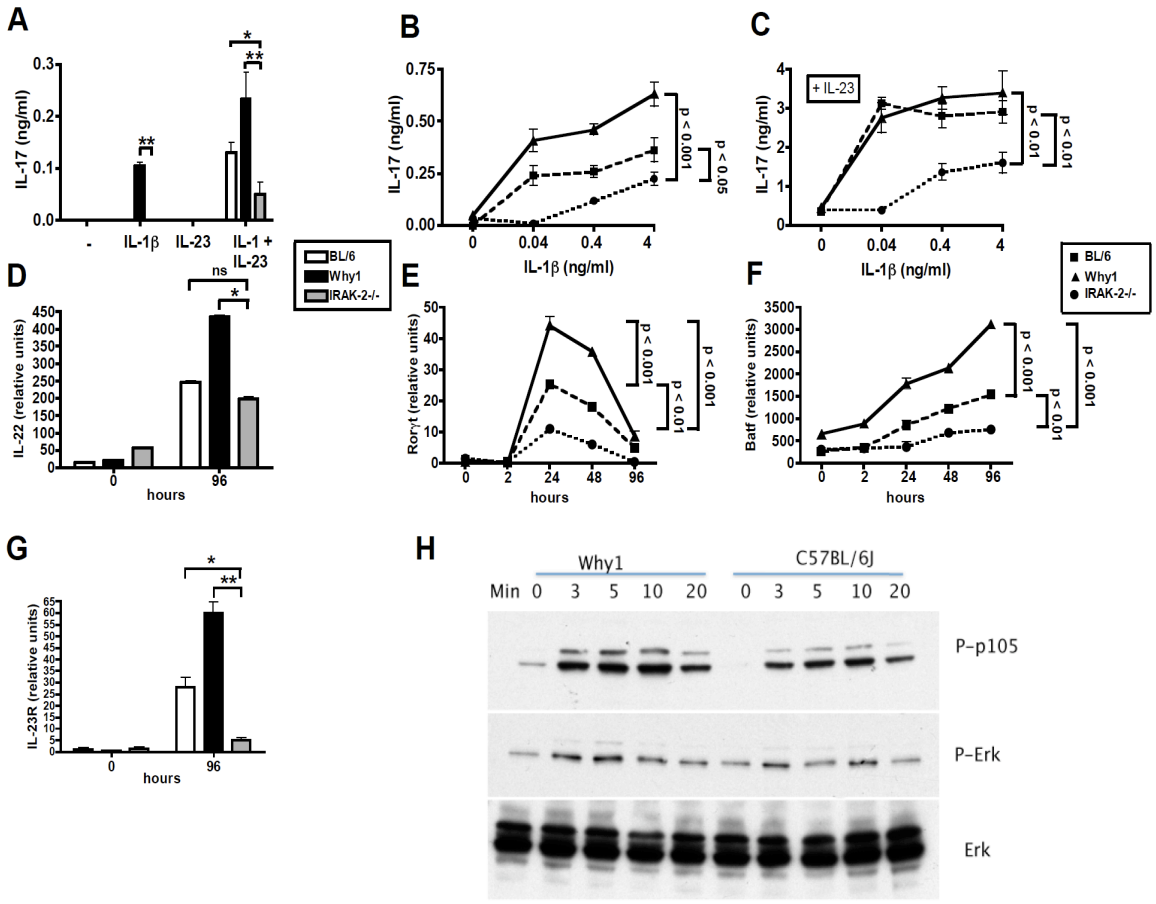


Figure 40. IRAK-2 promotes CD4 T cell IL-17 production, Th17 lineage commitment and activation. (A) Naïve splenic CD4 T cells from BL/6, Why1 and IRAK-2-/- mice were stimulated with 4 ng/ml of IL-1β, or 20 ng/ml of IL-23, or both, for 96 hours, and IL-17 production was measured by ELISA. Naïve splenic CD4 T cells were stimulated with anti-CD3/CD28 plus IL-1β, at indicated concentrations, **(B)** either alone or **(C)** together with 20ng/ml of IL-23 for 96 hours, and IL-17 production was measured by ELISA. Naïve splenic CD4 T cells were stimulated with anti-CD3/CD28 plus 0.4 ng/ml of IL-1β, and **(D)** IL-22, **(E)** Rorγt and **(F)** BATF expression were measured by quantitative real-time PCR analysis at the indicated time points. Data were normalized to GAPDH. Error bars represent means of triplicate determinations +/- SD. **(G)** BL/6 and Why1 CD4 T cells were stimulated with 4 ng/ml of IL-1β for the indicated times and cytoplasmic protein lysates were analyzed by Western blot for MAPK (P-Erk) and NF-κB (P-p105) pathway activity. Unphosphorylated Erk kinase (ERK) was used as a loading control. Data are representative of 3 **(A-E)** or 2 **(F, G)** independent experiments. P values were determined by one-way ANOVA and student's *t* test using GraphPad Prism software (* = $p < 0.05$, ** = $p < 0.001$).

4.3 Discussion

Murine schistosomiasis is a well-established experimental model of a major human infectious disease. Humans as well as mice develop marked differences in disease severity and it is clear that immunopathology is profoundly affected by the host genome. Thus, a greater understanding of its pathogenic mechanisms and underlying genes has widespread implications. In this chapter, a novel role for IRAK-2 in CD4 T cell signaling and the development of severe disease in schistosomiasis was identified. IRAK-2 exerted its influence by enhancing IL-1-mediated differentiation of pathogenic Th17 cells through increased expression of the transcription factors RORγt and BATF. The effect of IRAK-2 was apparent in both wild-derived (MOLF) and classically inbred (BL/6) mouse strains, indicating that its function is not strain-specific and therefore will likely have wide-ranging implications for other disease phenotypes. Our study also demonstrates the utility of an unbiased phenotypic approach in an evolutionarily diverse subspecies of mice to reveal novel gene networks and refine our understanding of immune signaling pathways.

Previously, our laboratory has identified several genetic intervals that are associated with severe disease in mice (Rutitzky, Hernandez et al. 2005; Smith, Shainheit et al. 2009), of which some correspond to regions in the human genome that contain the loci *Schistosoma mansoni 1* (*Sm1)* and *Sm2*, which have been associated with intensity of infection and hepatic fibrosis during human infection, respectively (Marquet, Abel et al. 1996; Dessein, Hillaire et al. 1999; Zinn-Justin, Marquet et al. 2001). Despite these efforts, and the many similarities between murine and human pathology, specific genes

that control severe disease have not been identified. One reason for this is the genetic redundancy of classical inbred mouse strains, which facilitates the analysis of "simple" monogenic and fully penetrant traits. However, greater genetic diversity may be required when investigating traits that are conferred by multiple loci that impart a quantitative contribution to the phenotype. Hence, the limited diversity of classically used strains can make it particularly difficult to identify genes that underlie complex traits, such as those involved in the host response to schistosome infection.

Using the more genetically diverse wild-derived mice as a model, we provide evidence of how genetic mapping of complex traits can be dissected with prior knowledge of the loci or genes identified in relatively simple screens. Previously, the Poltorak lab positionally cloned a mutation in the promoter of IRAK-2C that limits the expression of the inhibitory isoform of IRAK-2 in MOLF mice. The outcome of this differential expression is a higher ratio of proinflammatory IRAK-2A relative to the inhibitory isoform IRAK-2C, which in turn leads to an enhanced proinflammatory response in MOLF macrophages (Hardy and O'Neill 2004; Conner, Smirnova et al. 2009). Extending these findings to a physiological setting *in vivo*, we now show that addition of the MOLF *Why1* interval, which contains *Irak2*, markedly increases expansion of pathogenic Th17 cells and the severity of egg-induced hepatic immunopathology in schistosome-infected BL/6 mice. Using a reciprocal approach, we also observed that deletion of *Irak2* leads to a significant defect in pathogenic Th17 cell development and a marked reduction of immunopathology, thus identifying *Irak2* as the causal gene behind this *in vivo* phenotype. The effect of *Irak2* on immunopathology is especially striking since susceptibility to *S. mansoni* infection is likely conferred by many

genes, which have been elusive in previous genetic screens measuring immunopathology as a direct phenotypic read-out. The effect of the *Why1* locus and *Irak2* on pathology thus sets up an important precedent for how results of a genetic screen *in vitro* can be used for identification of genes influencing complex traits *in vivo*.

IRAK family kinases are central to TLR signaling and a critical factor in innate immunity (Janssens and Beyaert 2003). Recently, IRAK family kinases have been studied in the adaptive immune response with some discrepancy as to their precise role. IRAK-4 has first been suggested to be an essential factor in TCR induced T cell responses (Suzuki, Suzuki et al. 2006). However, these results have not been confirmed as it was later shown that IRAK-4 is dispensable for normal T cell responses and TCR activity (Kawagoe, Sato et al. 2007; Staschke, Dong et al. 2009). Here we provide evidence that another IRAK family member, IRAK-2, critically affects T cell biology by regulating the ability of IL-1 to promote Th17 differentiation. Thus, stimulation of naïve T cells with either IL-1 alone, or together with anti-CD3/CD28, resulted in a dramatic increase in IL-17 production by Why1 CD4 T cells compared with BL/6, while IL-17 from IRAK-2-/- T cells was minimal. Stimulation of Why1 CD4 T cells with IL-1 alone also led to increased activation of the IκB kinase p105, which promotes the degradation of IκB and allows NF-κB to translocate to the nucleus (Vallabhapurapu and Karin 2009). These observations identify IRAK-2 as a key regulator of Th17 cell biology by enhancing IL-1R signaling through NF-κB activation. Our data also show that stimulation with IL-1 alone is sufficient to induce Th17 cells in Why1 mice, suggesting that their high expression of IRAK-2 is responsible for the increase in Th17 cells. Importantly stimulation with IL-23 together with anti-CD3/CD28 did not induce any strain specific

differences in IL-17 production, indicating that this effect is specific to IL-1 signaling. Altogether, these findings imply that the role of IRAK family members in T cell responses is not limited to an effect on TCR signaling directly, but rather that they can also act via the IL-1R-MyD88 complex.

Among several candidate transcription factors, RORγt has been demonstrated to play a central role in Th17 cell differentiation, as its absence significantly impairs IL-17 production (Brustle, Heink et al. 2007; Stockinger and Veldhoen 2007). However, IRF4, another transcription factor involved in Th17 cell differentiation via IL-21 (Brustle, Heink et al. 2007; Huber, Brustle et al. 2008), has recently been identified as critical for IL-1-induced Th17 cell differentiation and that this may act independently of RORγt. We now show that Why1 CD4 T cells significantly up-regulate RORγt expression following stimulation with IL-1, suggesting that IL-1 *per se* can drive Th17 cell differentiation through an IRAK-2 dependent pathway. Interestingly, while there was an increase in IRF4 expression following stimulation with IL-1 and antiCD3/CD28, there were no strain-specific differences. More recently, BATF was identified as a key transcription factor in Th17 cell differentiation (Schraml, Hildner et al. 2009), as BATF-deficient mice displayed impaired Th17 cell activity and were resistant to EAE despite normal IL-6 signaling. BATF synergized with RORγt to enhance IL-17 production and sustained RORγt expression in Th17 cells, although the exact nature of their interaction remains to be elucidated (Martinez and Dong 2009). Here we show that BATF expression is significantly enhanced in IL-17-producing Why1 CD4 T cells compared with BL/6 T cells and that this function is IRAK-2 dependent. Interestingly, in our model RORγt expression peaks earlier than BATF **(Fig 40 D and E),** suggesting that RORγt up-

regulates BATF during Th17 cell development. Our findings also suggest that BATF functions downstream of the IL-1 receptor thus explaining why BATF-/-mice have a defect in Th17 cell differentiation. Similar to IRF4, we found no significant differences in the expression of AHR, which has a demonstrated regulatory role in Th17 development and function (Veldhoen, Hirota et al. 2008; Veldhoen, Hirota et al. 2009) (data not shown).

As demonstrated in this study, wild-derived IRAK-2 confers on T cells a powerful, TCR-independent hypersensitivity to stimulation with IL-1, which is further amplified in the presence of IL-23. Mechanistically, this is due to a deletion in the IRAK-2C promoter leading to unopposed activation of the main proinflammatory isoform of IRAK-2A. This contrasts with BL/6 mice, in which the inhibitory isoform IRAK-2C is abundantly expressed and up-regulated in response to inflammatory stimuli (Conner, Smirnova et al. 2009). Tissue inflammation induces large amounts of IL-1 and IL-23 and it has been suggested that non-antigen specific Th17 cells responding to these stimuli may aggravate tissue damage (Chung, Chang et al. 2009). In schistosomiasis, IL-1 and IL-23 are highly expressed in MLN and hepatic lesions of high-pathology CBA, but not low-pathology BL/6 mice. Furthermore, dendritic cells derived from the bone marrows of normal CBA mice produce abundant IL-1 and IL-23 in response to stimulation with live schistosome eggs, whereas those from BL/6 mice do not, clearly linking these cytokines with exacerbated disease (Rutitzky, Bazzone et al. 2008; Shainheit, Smith et al. 2008). With respect to the above, we speculate that IRAK-2 may enhance the sensitivity of Th17 cells in a TCR-independent manner and further aggravate tissue damage.

In summary, using wild-derived mice as a model, we illustrate the first example of a gene controlling severe pathology in murine schistosomiasis, setting an example of how analysis of simple monogenic traits *in vitro* can be applied to complex *in vivo* models of infection or autoimmunity. We used this model to uncover a novel role for IRAK-2 in CD4 T cell signaling via the IL-1 receptor and show that IRAK-2 is a key regulator of IL-1-mediated Th17 cell biology, which may have wide-ranging effects on other chronic inflammatory diseases.

SECTION IV: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Schistosomiasis is a serious and potentially fatal tropical parasitic disease caused by trematode helminthes. Infection with one such species, *S. mansoni*, results in the development of strikingly different pathologies among affected individuals, with the majority developing the mild form of the disease, "intestinal schistosomiasis," while approximately 5-10% develop the more severe and potentially fatal "hepatosplenic schistosomiasis" (Bica, Hamer et al. 2000). Taking advantage of a mouse model, which accurately recapitulates these polar forms of schistosomiasis, despite similar environmental and infection conditions, we used forward genetics analyses to dissect the genetic contributions to the development of severe immunopathology in an attempt to identify novel genes and gene networks that influence disease outcome.

Overall summary of findings

The first study examined the differential contributions of MHC and background genes to the development of severe disease. Previously our lab has identified an H-2^k restricted schistosome specific TCR gene rearrangement developed against Sm-p40, the major antigen of SEA. Following infection, several high pathology mouse strains demonstrate increased expansion of this specific TCR compared with low pathology mouse strains, indicating a potentially important role for immunodominance in severe disease. To assess this directly, we used MHC congenic mice and found that either removal from high pathology mice or addition to low pathology mice of the schistosome specific TCR did not affect the development of severe disease. Following infection, CBA/d mice developed large liver granulomas and produced high amounts of IL-17 similar to CBA mice, while BALB/k mice developed smaller liver granulomas and

produced less IL-17, similar to BALB/c mice. Interestingly, expression of the schistosome specific TCR did affect IFN-γ production as CBA/d mice produced significantly less IFN-γ than CBA, while BALB/k mice produced significantly more than BALB/c. Taken together these data suggest that background genes, and not MHC, play a more significant role in the outcome of disease processes.

In the second study, we examined a cohort of 150 F_2 mice and confirmed that severe immunopathology occurs amidst a pro-inflammatory cytokine environment. QTL analysis identified two loci that were significantly linked to granuloma formation, *D4Mit203* and *D17Mit82.* These loci were inherited in a BL/6 dominant manner and also exerted control over pro-inflammatory cytokine production in F_2 mice. Congenic mice, in which the BL/6 alleles of these loci were expressed on an otherwise SJL background, had significantly reduced pathology and IL-17 production, indicating that these loci directly contribute to the development of severe disease. QTL analysis also identified several loci that were linked to cytokine production, including one that was significantly linked to IL-17 production (see table III). These data represent the first QTL identified that are significantly linked to both granuloma formation and cytokine production in murine schistosomiasis.

We next examined the schistosome infection in BL/6 and BL/10 mice, two very closely related strains ancestrally, to potentially limit the number of contributing loci to severe disease. Using congenic mice, in which BL/6 mice contain segments of the BL/10 genome, we identified 3 loci that directly contribute to disease development. Microarray analysis of infected BL/6 and BL/10 MLNs revealed a major phenotypic difference, in which BL/6 mice respond to schistosome infection by upregulating the alternative

activation markers Ym1 and Fizz1, whereas BL/10 did not. *In* vitro experiments traced this differential expression of Ym1 and Fizz1 was to a defect in BL/10 DCs, which led to increased production of DC IL-1 and IL-23 and resulted in increased Th17 cell differentiation in BL/10 mice. Combining these data with a bioinformatics approach we identified Csf3r as a strong candidate gene in this model.

Lastly, we initiated experiments in wild-derived mice in an attempt to identify novel mechanisms that govern severe disease. Wild-derived mice developed significantly increased immunopathology and produced extremely high amounts of IL-17 following schistosome infection. This was controlled by a locus on chromosome 6, designated *Why1*, and, using lentiviral infection and knockout mice, led us to identify IRAK-2 as the gene responsible for the phenotype. IRAK-2 mediates severe disease by enhancing IL-1β-induced Th17 cell differentiation, through increased expression of the Th17 cell transcription factors Rorγt and BATF. Taken together, we used wild-derived mice to identify a novel regulator of Th17 cell differentiation and severe disease in murine schistosomiasis.

General Discussion of Findings

Schistosomiasis is a serious and potentially fatal tropical parasitic disease caused by trematode helminthes. Infection with one such species, *S. mansoni*, results in the development of drastically different pathologies among affected individuals, with the majority developing the mild form of the disease, "intestinal schistosomiasis," while approximately 5-10% develop the more severe and potentially fatal "hepatosplenic schistosomiasis" (Cheever, Duvall et al. 1987; Bica, Hamer et al. 2000). Taking

advantage of a mouse model, which substantially recapitulates these polar forms of disease, despite similar environmental and infection conditions (Warren and Dewitt 1958; Cameron and Bhattacharyya 1965), we used forward genetics analyses to dissect the genetic contributions to the development of severe immunopathology in an attempt to identify novel genes and gene networks that influence disease outcome.

One of the most intriguing observations of *S. mansoni* infection involves the strikingly disparate disease pathologies observed in humans and among mouse strains. In murine schistosomiasis, low pathology-prone BL/6 mice develop mild immunopathology in a Th2-biased immune environment, whereas high pathology-prone mouse strains, such as CBA, SJL and BL/10, develop significantly larger hepatic granulomas amidst a proinflammatory cytokine environment, characterized by robust production of IFN-γ and IL-17 (Pearce, C et al. 2004; Stadecker, Asahi et al. 2004). The outcome of schistosome infection depends on complex innate and adaptive immune responses, including regulatory mechanisms such as those afforded by alternatively activated macrophages. While it is clear that the course of infection with *S. mansoni* is profoundly affected by the host genome, genetic control of the specific immunological mechanisms that lead to the development of mild versus severe disease have not been well characterized.

One reason for this is that genetic analyses in humans can be extremely difficult, especially those involving parasitic infections, and must account for a variety of environmental factors that do not exist in mouse models. One of the most obvious is the likely co-infection of individuals with other parasites, including helminths, which have a dramatic effect on the immune response. Previously our lab has shown that co-infection of mice with intestinal nematodes significantly reduced hepatic egg-induced

immunopathology and proinflammatory cytokine production (Bazzone, Smith et al. 2008). Pre-infection of high pathology mice with *H. polygyrus* promoted a strong protective Th2-polarized cytokine environment associated with an increase in markers of alternative activation in the liver. This prevented the development of Th1 and Th17 cell-mediated granulomatous inflammation and ultimately resulted in the significant amelioration of schistosome-induced immunopathology (Bazzone, Smith et al. 2008). Observations such as these, as well as the observation that helminthic infections can prevent and protect against the development of aberrant adaptive immune responses (Capron, Dombrowicz et al. 2004; Dunne and Cooke 2005; Maizels 2005), provides strong evidence as to why the predominance of human infections result in the onset of the mild form of schistosomiasis. However, despite this, a significant number of patients in regions endemic for helminth infections develop the severe and potentially fatal form of schistosomiasis, indicating a substantial contribution of host genetic factors to the development of severe immunopathology (Bica, Hamer et al. 2000). Based on the polygenic nature of the immune response, the schistosome infection is likely under the control of a relatively large number of contributing loci that are responsible for the striking heterogeneity in egg-induced immunopathology. While the majority of these genetic factors will require interactions with other loci/regulatory regions to have even a relatively small effect, it is likely that QTL analysis will identify loci with a larger, more measurable contribution to the phenotype (Mackay 2001). Thus, using a mouse model that accurately recapitulates the disease dichotomy observed in humans, the overriding goal of this thesis was to discover and characterize loci that contribute to severe

immunopathology, eventually leading to the identification of novel genes that regulate schistosome infection.

To identify novel genes that contribute to the development of severe immunopathology, the present study focused on two distinct, yet similar methods. In the first part of this thesis (Results: chapters 2 and 3) we used a QTL based approach, in which two separate crosses led to the identification of a number of loci linked to severe hepatic granulomatous inflammation and cytokine responses. In the first analysis we crossed SJL and BL/6 mice, which were chosen because they exhibited the largest phenotypic differences of any strain we had previously tested increasing the likelihood that QTL could be detected. We indeed identified two loci, *D4Mit203* (peak position, 129.2 Mb, LOD 3.4, $p < 0.001$, 95% CI 115-142Mb) and *D17Mit82* (peak position, 33.9 Mb, LOD 6.0, $p < 0.0001$, 95% CI 21-40Mb), which were significantly linked to granuloma size. A closer analysis revealed that these loci not only controlled granuloma formation but also proinflammatory cytokine production. F_2 mice that were homozygous for the BL/6 allele at either *D4Mit203* or *D17Mit82* developed significantly smaller liver granulomas and produced much less IL-17 and IFN-γ than mice homozygous for the SJL allele, while mice heterozygous at either locus were phenotypically similar to those carrying only the BL/6 allele. We then used interval-specific congenic mice to assess the direct contribution of these loci to the development of severe disease and found that mice homozygous for the BL/6 allele of either locus on an otherwise SJL background developed significantly smaller liver granulomas and had less IL-17 production. Interestingly, SJL.B6-D4Mit203 congenic mice appeared to have a stronger overall effect on immunopathology than SJL.B6-D17Mit82. This is in agreement with data from F_2

mice, which showed that in mice homozygous for both loci, *D4Mit203* was dominant **(Fig 17).** There was an additive effect in double congenic mice, as immunopathology and IL-17 production was even further reduced, this indicates that these loci likely control different processes that regulate the schistosome infection. Taken together, these data identified the first genomic intervals that significantly and directly control the development of severe murine schistosomiasis.

The next step in this process was to identify the causal genes that underlie these loci. For this, we were most interested in *D4Mit203*, given its dominance over *D17Mit82*, as well as the fact that *D17Mit82* is likely associated with the MHC complex. Unfortunately, this locus was over 25 Mb in size and contained greater than 400 genes, many with known immunological functions, making it difficult to zero in on specific potential candidate genes. Further complicating the matter was the lack of a suitable *in vitro* model to test genes once they were identified as candidates. This led us to perform our second genetic analysis of BL/6 versus BL/10 mice. As mentioned previously, these mice are genetically very similar yet develop dramatically disparate disease pathology. Therefore the number of contributing loci will likely not only be fewer, but also significantly smaller, than in our cross between BL/6 and SJL mice, thus limiting the number of candidate genes. For this analysis we again used congenic mice, in which BL/6 mice contained segments of the BL/10 genome, and discovered several additional loci that directly and significantly controlled immunopathology and cytokine responses. However, the key observation was that mice congenic for B6.B10-4/125-135, which is contained within *D4Mit203,* significantly controlled both granuloma formation and IL-17 production. Interestingly, this locus had no effect on IFN-γ production, similar to

D4Mit203, indicating a strikingly similar phenotype between these two congenic mouse strains and increasing the likelihood that the same causal gene underlies both crosses. Since the locus identified in this cross is less than half the size of *D4Mit203* we continued on with our analysis of BL/6 versus BL/10 mice in an attempt to identify candidate genes. We have now shown that the locus identified on chromosome 4 regulates both immunopathology and cytokine production in two separate crosses, however congenic mice express the BL/6 allele of this locus on an otherwise SJL background developed reduced pathology, while addition of the high pathology BL/10 allele to BL/6 mice increased pathology. Thus it is unclear whether the effect of this locus is of a regulatory or proinflammatory nature, making it difficult to reduce the number of candidate genes.

Recently, combining QTL mapping and microarray analysis was shown to be an efficient, objective and most importantly effective way to narrow candidate gene identification (Wayne and McIntyre 2002; Peters, Robledo et al. 2007; Burgess-Herbert, Cox et al. 2008). Of the QTLs that have been identified in rodents, the majority of these are caused by allelic variations that can result in expression differences (Glazier, Nadeau et al. 2002; Abiola, Angel et al. 2003), and even when this is not the case, microarray analysis would still reveal interesting differences in molecular pathways, which may be altered by the causal gene. As such, while our initial observations of the microarray data did not identify any genes with substantially different expression levels located within any of our QTL, it did reveal striking differences in markers of the alternative activation pathway, particularly Ym1. Ym1 is best characterized as a marker of alternatively activated macrophages, which regulate a number of inflammatory processes and are particularly important during helminth infection (Gordon 2003; Martinez, Helming et al.

2009). AAMs are also critical for survival and are associated with the protective Th2 responses induced by co-infection with intestinal nematodes during schistosome infection (Herbert, Holscher et al. 2004; Bazzone, Smith et al. 2008).

Ym1 is typically associated with protective responses in macrophages, however, using an *in vitro* model (Shainheit, Smith et al. 2008) we identified dendritic cells as the cell type most likely responsible for our phenotype. BMDCs derived from BL/10 mice produced significantly more IL-1 and IL-23 than BMDCs derived from BL/6 mice following stimulation with schistosome eggs. This led to enhanced Th17 cell differentiation in BL/10 co-cultures, while in BL/6 cocultures there was enhanced IL-5 production, indicative of a Th2 response. These data are in agreement with recent evidence that suggests that IL-1 and IL-23, but not IL-6 and TGF-β, are the critical cytokines for the development of pathogenic Th17 cells in a number of disease models, (Cua, Sherlock et al. 2003; Langrish, Chen et al. 2005; McGeachy, Bak-Jensen et al. 2007; Chung, Chang et al. 2009; Ghoreschi, Laurence et al. 2010) including schistosomiasis (Rutitzky, Bazzone et al. 2008; Shainheit, Smith et al. 2008). We next showed that BMDCs from BL/6 mice also had significantly enhanced expression of Ym1 and Fizz1 compared with BL/10 BMDCs, following egg stimulation. Importantly, BMDCs derived from B6.B10-4/125-135 congenic mice also drove increased Th17 cell differentiation and had reduced expression of alternative activation markers similar to BL/10 co-cultures. Taken together, our data indicate that one potential mechanism for the differential response of BL/6 and BL/10 mice to schistosome infection is due to the ability of BL/6 DCs to become alternative activated following contact with schistosome eggs, which results in the development of a protective Th2-biased cytokine environment.

In contrast, production of innate proinflammatory cytokines by BL/10 DCs led to enhanced pathogenic Th17 cell differentiation.

The ability of DCs to drive CD4 T cells to differentiate into both Th2 and regulatory T cell subsets has created the idea of "tolerogenic" DC subsets (Pulendran, Tang et al. 2010). Tolerogenic DCs play important roles in the defense against many inflammatory and autoimmune diseases (Thomson and Robbins 2008; Matta, Castellaneta et al. 2010). These DCs are also critical for driving protective Th2 responses during helminth infections (Mendlovic and Flisser 2010). However, while the induction of Th1 responses by DCs is relatively well documented, very little is known about the mechanism by which DCs induce Th2 responses, particularly the DC receptor/ligand pairs involved in this process (MacDonald and Maizels 2008). Recent evidence has shown that Ym1 may be important in this process as in several models DCs required expression of Ym1 to drive Th2 T cell differentiation (Arora, Chen et al. 2006; Cai, Kumar et al. 2009). The data presented here fit well with this model and reveal a potentially key role for Ym1 expressing tolerogenic DCs in protection against severe schistosome infection. Given this model we were interested in genes that may promote tolerogenic DC function in BL/6 mice, which led us to identify *Csf3r* (G-CSFR) as a strong potential candidate gene.

G-CSFR is located directly within our QTL of interest and is expressed mainly on myeloid cells, including DCs and macrophages (Fukunaga, Ishizaka-Ikeda et al. 1990). G-CSF, the only ligand for G-CSFR, is a key regulator of both DC and T cell function, and DCs treated with G-CSF drive naïve CD4 T cells to differentiate into both Th2 and T regulatory subsets (Arpinati, Green et al. 2000; MacDonald, Rowe et al. 2005; Rutella,

Zavala et al. 2005; Pulendran, Tang et al. 2010). Administration of G-CSF also has protective effects against EAE and type I autoimmune diabetes (Rutella, Zavala et al. 2005). We analyzed expression of G-CSFR in schistosome egg-stimulated DCs and found that it correlated with an increase in Ym1 and Fizz1 expression in BL/6, but not BL/10 DCs. Taken together, these data indicate that G-CSF induces DCs into a "tolerogenic" phenotype and identifies G-CSFR as a strong candidate gene in our model. Further testing will be needed to address the role of G-CSFR during the schistosome infection. These data also do not preclude a role for alternatively activated macrophages in our model and further experiments will be needed to determine the relationship between G-CSF, DCs and macrophages.

Our genetic data also indicate that the QTL that we identified, at least those with larger effect sizes, control regulatory responses as opposed to promoting inflammatory ones. This is in agreement with another interesting observation, throughout all of the genetic analyses performed in this thesis, or previously by the lab, the low pathology background was always dominant. F_1 mice from several crosses developed low pathology and had reduced proinflammatory cytokines production, and F_2 mice heterozygous for an identified quantitative allele also developed mild disease. Applying this to human genetics, if the loci controlling regulatory mechanisms are dominant as in mice, it would likely require a combination of rare mutations to develop severe disease. This may provide another explanation as to why the majority of humans develop mild "intestinal" schistosomiasis.

In the last part of this thesis we used wild-derived mouse strains, which are genetically more diverse than classically inbred strains and therefore likely to contain

novel mechanisms of immune regulation. Furthermore, these phenotypes have been selected throughout the process of subspeciation and therefore are likely to have increased biological relevance (Frazer, Eskin et al. 2007; Yang, Bell et al. 2007). The initial key observation in this study was that along with developing severe immunopathology, wild-derived mice produced strikingly high amounts of IL-17, indicating a potential novel mechanism of Th17 cell regulation. Using prior knowledge of the MOLF hyper-responsive phenotype derived from *in vitro* studies (Conner, Smirnova et al. 2009), we were able to identify IRAK-2 as the underlying causal gene. This was an important finding in that it demonstrated the first successful example of gene identification in murine schistosomiasis and provided a blueprint for how prior knowledge of a phenotype through "simpler" *in vitro* studies can be applied to the analysis of complex traits.

IRAK-2 is centrally located within the TLR signaling cascade it is also downstream of the IL-1R (Janssens and Beyaert 2003), however we have shown that the development of severe disease in MOLF mice is CD4 T cell mediated. IRAK-2 also signals downstream of the IL-1 receptor (Janssens and Beyaert 2003), and given the critical role of IL-1 in the development of Th17 cells, we hypothesized that IRAK-2 functioned downstream of the IL-1R to direct Th17 cell differentiation. This proved to be correct, as T cells expressing proinflammatory IRAK-2 produced excessive amounts of IL-17 while those from IRAK-2 knockout mice had a significant defects in IL-17 production. IRAK-2 also increased synergy between IL-1 and IL-23 to further enhance Th17 cell differentiation. IL-1 and IL-23 being the key cytokines for pathogenic Th17 cell differentiation. Taken together our data identify a novel role for IRAK-2 as critical

for IL-1-mediated pathogenic Th17 cell differentiation. One of the more interesting findings, was that IRAK-2 mediated Th17 cell differentiation was reliant on the transcription factors Rorγt and BATF, but not the other Th17 cell associated TFs, Ror-α, IRF4 or AHR. The nature of the interactions of these TFs during Th17 cell development is not well defined and varies depending on the model (Martinez and Dong 2009; Schraml, Hildner et al. 2009) (McGeachy and Cua 2008; Korn, Bettelli et al. 2009). Our data suggest Rorγt and BATF are the critical TFs required for IL-1-induced pathogenic Th17 cell differentiation. Rorγt and BATF also appeared to have a temporal relationship as the former peaks early after stimulation and the latter peaks during maximal IL-17 production. This indicates that Rorγt may serve as a first wave transcription factor during early Th17 cell differentiation, which then activates BATF transcription to expand and maintain the phenotype. These are the first data generated showing time-specific differences in Th17 cell transcription factor expression involving BATF and more experiments are needed dissect these results.

IRAK-2 directs Th17 cell differentiation by enhancing the sensitivity of naïve T cells to IL-1 signaling. However the intracellular mechanisms by which this occurs are not well understood. IRAK-2 was originally identified as controlling proinflammatory responses in MOLF macrophages due to a mutation in the promoter of inhibitory IRAK-2C, which led to an increase in proinflammatory IRAK-2A expression (Conner, Smirnova et al. 2009). This held true in MOLF and Why1 congenic mice infected with *S. mansoni* as these mice produced large amounts of proinflammatory cytokines and developed severe immunopathology, likely due to decreased expression of the inhibitory form of IRAK-2C. However, the data presented within this thesis show that IRAK-2 also

mediates severe disease in BL/6 mice, which do not have a mutation in IRAK-2C. This implies additional regulation of IRAK-2 following schistosome infection that results in increased IRAK-2A expression. It would be very interesting to determine the internal signaling mechanisms that regulate this process in BL/6 mice, which could have broader clinical applications for the treatment of T cell-mediated inflammatory diseases.

In sum, we identified the first genetic loci that significantly control both immunopathology and cytokine production in murine schistosomiasis. Using a series of genetic analyses we also provide evidence for two separate pathways controlling schistosome infection **(Figure 41)**. The first involves the role of G-CSF to induce alternative activation of DCs, which leads to the development of protective Th2 responses following schistosome infection and the second identified IRAK-2 as a critical mediator of IL-1 induced pathogenic Th17 cell differentiation. These findings highlight the complexity of the schistosome infection, in that every high or low pathology situation may be different, i.e. governed by a different set of genes in different mouse strains. In this sense, individual mouse strains are similar to individual people, in that they each have a different set of genetics, which may result in different mechanisms that cause severe disease. Therefore, each of these pathways are of equal importance in regulating the schistosome infection, and more work must be done to identify the undoubtedly many more mechanism that underlie severe disease. Schistosomiasis is a well-characterized model of CD4 T cell mediated tissue inflammation and as such it shares many mechanistic features with other inflammatory and autoimmune conditions. The data presented here may also have more broad ranging clinical implications for potential therapeutic involvement in inflammatory and autoimmune diseases.

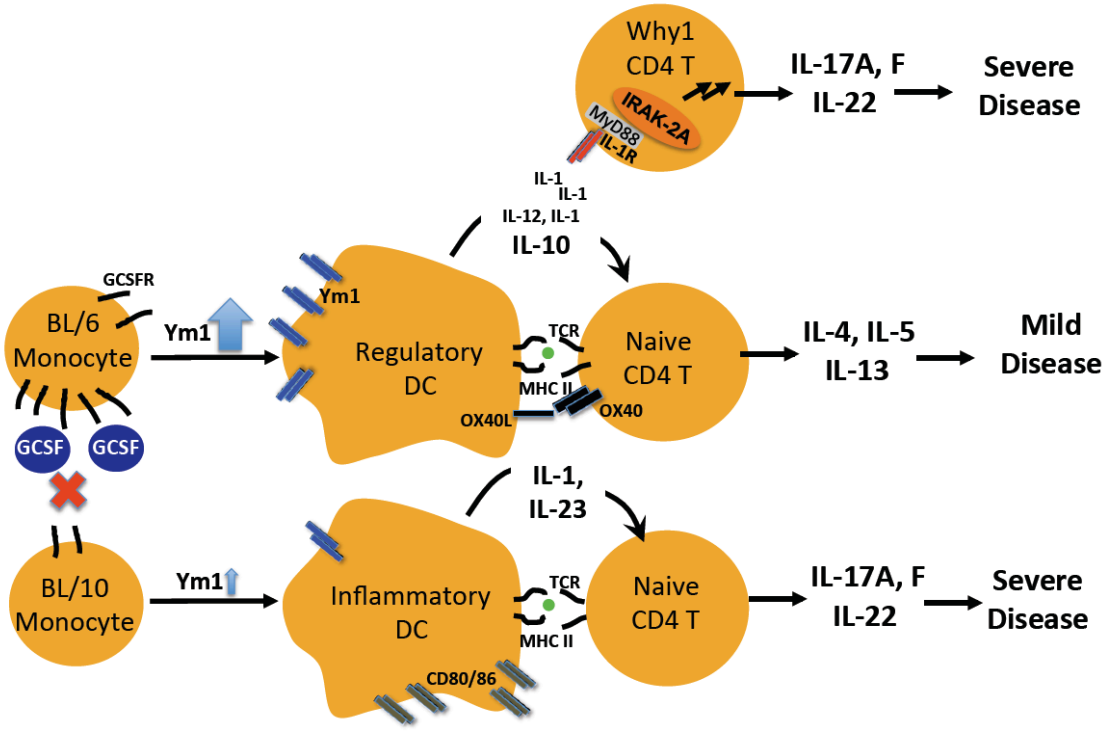


Figure 41. Divergent pathways control immunopathology in murine schistosomiasis. In our model of immunopathology high expression of the G-CSFR in BL/6 monocytes leads to an increase in Ym1 expression and differentiation into a regulatory DC phenotype. BL/6 regulatory DCs produce low levels of the proinflammatory cytokines IL-12 and IL-1 and drive naive CD4 T cells to produce IL-4, IL_5 and IL-13, possibly through secreation of IL-10 and OX40-OX40L interactions. In contrast BL/10 monocytes express low levels of G-CSFR, which results in differentiation into an inflammatory DC phenotype characterized by the production of IL-1 and IL-23. These cytokines then induce naive CD4 T cells to produce IL-17 and Th17 cell associated cytokines, which ultimately results in the development of severe disease. However, in contrast to BL/6 CD4 T cells, naive T cells derived from Why1 mice express high levels of proinflammatory IRAK-2A. Overexpression of IRAK-2A in naive CD4 T cells results in IL-1 hyperresponsiveness and thus the small amount of IL-1 produced by BL/6 DCs induce naive Why1 T cells to differentiate into pathogenic Th17 cells.

Future Directions

The data presented in this thesis provide new insight into the immunological mechanisms that regulate the development of severe forms of schistosomiasis. To date, a number of loci have been identified that affect murine schistosomiasis **(Figure 42),** however many questions still, many questions remain, and I will address future experiments for several here, both specific to this thesis and to broader ranging genetic analyses of schistosome infection. In chapter 3 of this thesis we demonstrated that BL/10 mice develop high pathology, likely due in part to a defect in their alternative activation pathway, however, future experiments must validate the functional nature of this observation. One potential experiment is to isolate $CD11c^{+}$ DCs from the spleen of infected BL/6 and BL/10 mice and look at markers of alternative activation by FACS analysis. FACS will measure actual protein expression as opposed to previous analyses, which only measured RNA levels. If, as expected, BL/6 DCs possess a "tolerogenic" phenotype compared with BL/10 DCs the regulatory capabilities of these cells could then be assessed. A straightforward experiment would be to titrate BL/6 DCs into BL/10 MLN cultures stimulated with SEA and determine if proinflammatory cytokine production is dampened. Another interesting experiment would be to assess if BL/6 macrophages are also alternatively activated and then assess their regulatory capabilities. Regulatory DCs and macrophages are not mutually exclusive and it would be very informative to determine if one, the other, or both are required for protection against severe schistosome pathology.

Csf3r as a candidate gene as well as the role of G-CSF/G-CSFR interactions must also be validated during the schistosome infection. The simplest way to do this would be

to sequence the *Csf3r* gene from BL/6 and BL/10 mice. If a mutation exists that alters protein structure then functional studies could be carried out to assess the role of this gene *in vivo* either by using *Csf3r* knockout mice or by administration of G-CSF antibody into infected mice. Even if a mutation does not exist functional studies could still be carried out because not all QTL are the result of sequence variation and we have already shown expression differences of *Csf3r* between BL/6 and BL/10 mice. Also the existence of an *in vitro* DC-T co-culture model that mimics the *in vivo* model of IL-17 production will provide and excellent and potentially easier model with which to test candidate genes in. It would also be interesting to sequence *Csf3r* and begin functional experiments in SJL mice, such as quantifying Ym1 expression on DCs, because it is likely that the same causal gene underlies both these QTLs.

With regards to our studies in wild-derived mice, we have shown that IRAK-2 is the causal gene underlying the Why1 locus and any additional experiments would focus on further elucidating the regulatory mechanisms that control this process. Why1 mice however did not entirely recapitulate the phenotype of MOLF mice and while I would not recommend further experiments to identify additional contributing loci in these mice I would in MSM. MSM mice developed severe immunopathology and produced extremely high levels of IL-17 similar to MOLF mice. These mice, like BL/6 and BL/10, are also very similar genetically, having been derived from the same ancestral subspecies of *M. mus. molossinus*. Consomic mice for every chromosome are now available between BL/6 and MSM mice. This would be a straightforward and simple way to identify new loci that contribute to severe disease. Consomic mice could then be used to generate smaller subcongenic mice with expected successful recombination obtained relatively easily.

More broadly several bioinformatics techniques may be useful for the genetic analysis of the schistosome infection. Sequence analysis using the mouse phenome database at Jackson lab (www.jax.org/phenome/SNPwizard) identified a SNP mutation between BL/6 and SJL mice that results in a non-synonymous coding mutation in exon 10 of *Csf3r*. Unfortunately, very few SNPs are available for BL/10 mice, however this increases the likelihood that CSF3R may also contribute to high pathology in SJL mice. Recently the Sanger Institute has completed sequencing of several more mouse genomes including the CBA (www.sanger.ac.uk/resources/mouse/genomes). The locus identified on chromosome 13 as controlling granulomas size between BL/6 and BL/10 mice was also identified in a cross between BL/6 and CBA mice (Rutitzky, Hernandez et al. 2005). Therefore it is possible that again the same causal gene is responsible for both QTL and sequence analysis between CBA and BL/6 mice may elucidate this.

Lastly, genome-wide haplotype analysis (GWHA) could provide a useful tool for identifying new QTL. This is based on the same principles as interval-specific haplotype analysis except it does not require *a priori* knowledge of a QTL. As mentioned previously the majority of genetic variation among inbred mouse strains is ancestral. Therefore if the same QTL are identified in different crosses there is a good chance that the same causal gene is responsible for both. GWHA requires the analysis of at least 20 mouse strains for a given phenotype, which are then broken into groups of high responders and low responders. The idea is that a genomic interval that is shared by all the high responders but different from the low responders is likely to contain evolutionarily conserved genes that regulate this response. GWHA also requires that the strains analyzed have their genomes sequences so successful analysis can be performed.

This technique has been successfully used to map several traits (Pletcher, McClurg et al. 2004; Burgess-Herbert, Cox et al. 2008; Stylianou, Affourtit et al. 2008; Sarah L. BurgessHerbert 2009).

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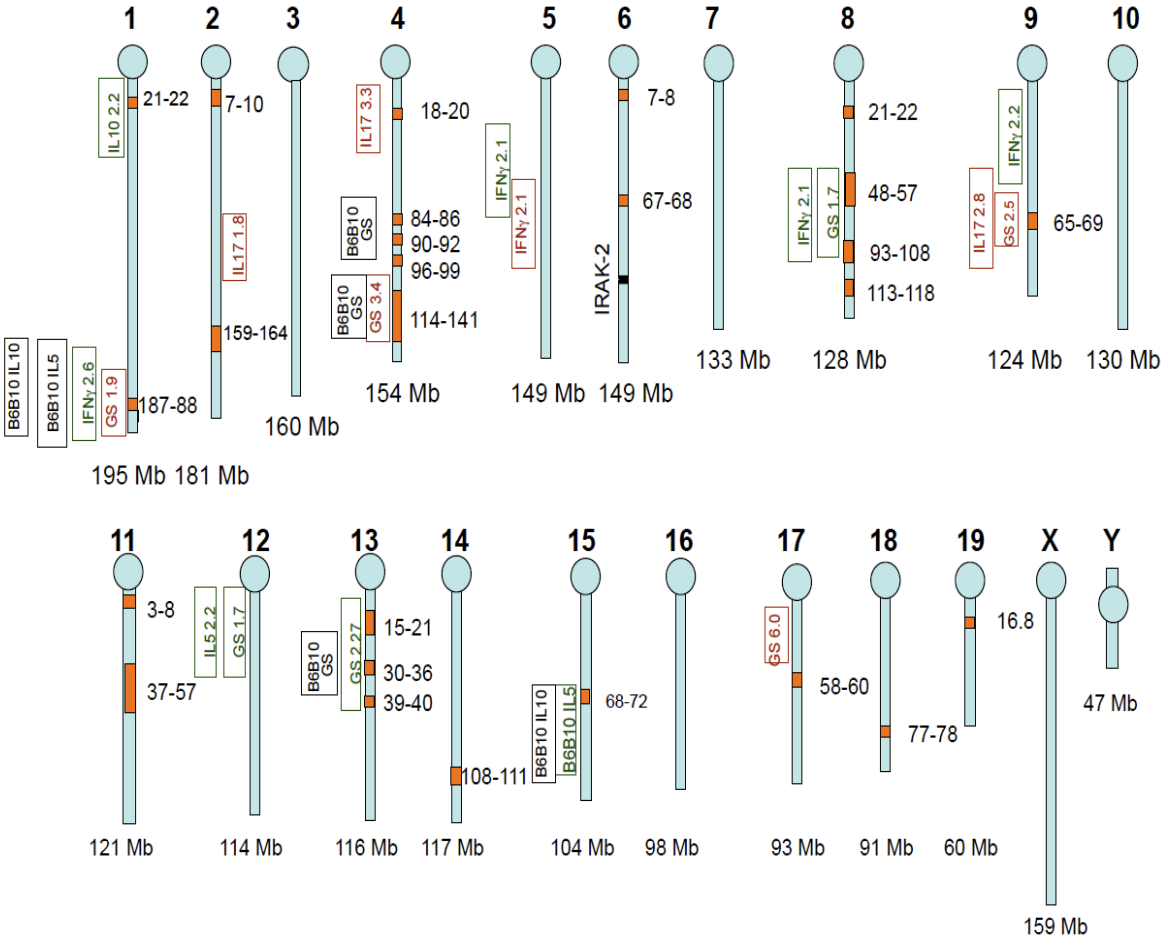

Figure 41. Chromosomal locations of QTL affecting murine schistosomiasis. The solid orange rectangles represent regions of the genome where BL/6 and BL/10 mice have different ancestral origins. Red rectangles denote QTL identified between BL/6 and SJL mice and indicate the phenotype followed by the LOD score. Green rectangles denote QTL identified between BL/6 and CBA mice and indicate the phenotype followed by the LOD scores. Black rectangles denote QTL identified using B6.B10 congenic mice and indicate the phenotype

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Appendix I

Chr	MGI Name	UCSCPosition(bp)	B6 Size (bp)
1	D1Dcr8	chr1:22,123,450-22,123,548	99
1	D1Dcr19	chr1:190,243,207-190,243,304	98
2	D2Dcr45	chr2:9,524,317-9,524,491	175
2	D2Dcr115	chr2:163,020,182-163,020,283	102
4	D4Dcr10	chr4:87,449,311-87,449,412	102
4	D4Dcr14	chr4:99,710,295-99,710,394	100
4	D4Dcr17	chr4:116,468,644-116,468,747	104
4	D4Dcr25	chr4:127,792,499-127,792,594	97
4	D4Dcr28	chr4:133,341,853-133,341,951	99
4	D4Dcr29	chr4:135,917,004-135,917,106	103
6	D6Dcr2	chr6:8,203,036-8,203,139	104
6	D6Dcr3	chr6:8,237,720-8,237,815	96
8	D8Dcr5	chr8:23,912,491-23,912,586	96
8	D8Dcr17	chr8:51,922,715-51,922,812	98
8	D8Dcr20	chr8:55,683,156-55,683,251	96
8	D8Dcr30	chr8:103,157,082-103,157,187	106
8	D8Dcr32	chr8:104,042,219-104,042,331	113
8	D8Dcr33	chr8:104,261,409-104,261,507	99
8	D8Dcr34	chr8:104,344,822-104,344,926	105
8	D8Dcr46	chr8:118,046,431-118,046,526	96
8	D8Dcr47	chr8:118,251,609-118,251,708	100
8	D8Dcr48	chr8:118,268,254-118,268,349	96
9	D9Dcr2	chr9:65,425,664-65,425,779	116
9	D9Dcr3	chr9:65,833,998-65,834,102	105
9	D9Dcr6	chr9:67,377,034-67,377,129	96
9	D9Dcr7	chr9:67,841,551-67,841,659	109
9	D9Dcr8	chr9:68,676,723-68,676,826	104
11	D11Dcr1	chr11:3,215,496-3,215,594	99
11	D11Dcr7	chr11:39,411,812-39,411,915	105
11	D11Dcr12	chr11:55,703,489-55,703,592	104
13	D13Dcr1	chr13:20,523,201-20,523,327	127
13	D13Dcr3	chr13:36,768,179-36,768,274	96
14	D14Dcr7	chr14:117,924,597-117,924,698	102
15	D15Dcr4	chr15:70,887,962-70,888,059	100
18	D18Dcr3	chr18:77,762,753-77,762,852	100