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***Schistosoma mansoni* modulation of
allergic responses**

submitted to
Trinity College Dublin

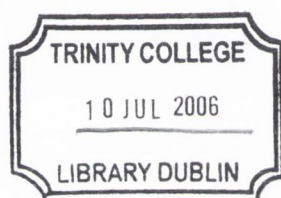
for the degree of
Doctor of Philosophy

by
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October 2005

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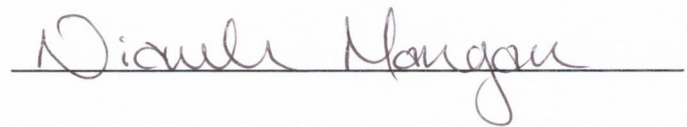
Abstract

It has been proposed that in humans infection with certain parasitic helminths may reduce the propensity to develop allergies in infected populations. *Schistosoma mansoni* is a parasitic trematode that is implicated as a reducer of allergic responses in humans. The association between helminths and allergies is intrinsic to the as yet inadequately tested Hygiene Hypothesis. In this PhD I have used mouse models of allergies to formally experimentally test the role of *Schistosoma mansoni* in the Hygiene Hypothesis. I demonstrate that while mice with conventional *S. mansoni* egg-producing infections are partially resistant to anaphylaxis, they are predisposed to developing exacerbated spontaneous and allergen-induced pulmonary inflammation including airway hyper-responsiveness. In contrast, I now demonstrate that mice infected with only schistosome worms are refractory to both anaphylaxis and allergen-induced airway hyper-responsiveness. I have dissected the underlying immunological mechanisms that mediate the resistance of worm-infected mice to both allergic insults. I propose a new paradigm whereby schistosome worms are resistant to allergic pulmonary inflammation via what I have termed a “helminth-modified pulmonary type 2 response”. In this paradigm schistosome worms suppress allergen-induced interleukin-5 which leads to lower pulmonary eosinophilia and normal lung function. Additionally, I have shown that the regulatory cytokine IL-10 is essential to mediate resistance of worm-infected mice to allergic disease. At the cellular level I show a new role for B cells and CD1d⁺ cells in resistance to anaphylaxis and AHR.

These data are the first experimental demonstration of a human parasitic worm preventing airways inflammation. In this thesis I have not only tested the Hygiene Hypothesis but have developed new theories for the rational development of new therapies for the treatment of allergies in humans.

Declaration of Authorship

This thesis is submitted by the undersigned to the University of Dublin for the examination of a Doctorate in Philosophy. The work herein is entirely my own and has not been submitted as an exercise for a degree to any other university. The library at Trinity College Dublin has my permission to lend or copy this thesis upon request.

A handwritten signature in dark ink, reading "Niamh Mangan", is written over a horizontal line.

Niamh Mangan

Acknowledgements

I would like to sincerely thank my supervisor Dr. Padraic Fallon for helping, guiding, cajoling and most of all encouraging me through my PhD. Padraic is inspirational in his ideas, determination and unrelenting high standards. Padraic has exposed me to science in all its' glory both good and bad from the human to the mouse to the cell and from the tip-box to the Gilson to the computer to the grant proposal to the politics.

I would also like to thank Rosie for her endless patience and training at the very beginning to get me on my way. Thanks to Phil for all his help with the creepy crawlies.

The biggest of thanks to Caitriona who with a personality larger than life, definitely requires her own paragraph here. You are a great friend and have been brilliant to share all the ups and downs with both in and out of the lab and especially with the write up. I must add though that someday we will find that off-switch!!

Next there is the posse that have been there since the start: Joanne, Sarah, Miriam, Michelle, PB, Graham, Brian (BOG and Keogh), Peter and Padraig. Then there is of course Andrew (and his jumpers), Ed, and Oonagh. The 'newbies' in the post-grad room: Caroline, Corinna, Claire and Kevin. I would especially like to mention Neil and Aileen: Neil for the Friday night pints – you can always be counted on; and Aileen for the good times!!

Then there are Geraldine and Jules who kept me sane! Fiona the best friend a person could ask for. Olive, thanks for being there, for listening and encouraging me.

Finally I must thank my family for putting up with me. Thanks to Mam and Dad for supporting and encouraging me through all my years in college and my Ph. D. And thanks to Aileen and Fiona – I couldn't ask for better sisters.

List of Abbreviations

Ab	Antibody
AHR	Airway hyper-responsiveness
Ana	Anaphylaxis
APC	Antigen presenting cell
ASA	Active systemic anaphylaxis
AW	Adult worm antigen
BMMo	Bone marrow derived macrophages
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
Cdyn	Compliance dynamics
CONA	Conalbumin
CTLA	Cytotoxic T lymphocyte Antigen
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DTH	Delayed type hypersensitivity
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
EPO	Eosinophil peroxidase
FCS	Foetal calf serum
FO	Follicular
GL	Lung Resistance

GM-CSF	Granulocyte macrophage colony stimulating factor
IFN	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ION	Ionomycin
IP	intra peritoneal
IR	intra rectal
IV	intra venous
LPS	Lippopolysaccharide
Mch	Methacholine
MHC	Major histocompatibility Complex
MPO	Myeloperoxidase
MZ	Marginal zone
NK	Natural Killer Cell
OD	Optical Density
OVA	Ovalbumin
PAF	Platelet Activating Factor
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD1	Protein Disulphide isomerase
PEC	Peritoneal Exudate cells
Pen V	Penicillin V

Penh	Enhanced pause
PMA	Phorbol Myristate Acetate
PSA	Passive systemic anaphylaxis
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial institute
Sc	Subcutaneous
SEA	Soluble Egg antigen
TCR	T cell receptor
Tg	Transgenic
Th	T helper
TLR	Toll-like Receptor
UV	Ultraviolet

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Chapter 1

Introduction

1.1 Schistosome parasites.

Parasitic helminth infections present some of the most universal health problems in the world and in addition to death can be the cause of marked morbidity. Schistosomes are trematode parasites of man, infecting over two hundred and fifty million people mainly in developing countries. Schistosomiasis, also known as Bilharzia, is a primarily tropical disease caused by worms of one or more of the schistosome helminth family. The name Bilharzia comes from Theodor Bilharz, a German pathologist, who identified the worms in 1851. Schistosomes are prevalent in rural and outlying city areas of 74 countries in Africa, Asia, and Latin America. In Central China and Egypt, the disease poses a major health risk.

The thirteen genera that comprise the family Schistosomatidae are distinct from other digenian parasites in four ways: 1, They have two rather than three hosts: 2, they are dioecious, i.e. sexually-defined male and female individuals, rather than being hermaphrodite: 3, they penetrate the hosts body surface as opposed to being eaten and, fourth, they reside in the vasculature of the definitive host. Schistosomatidae parasitize birds and mammals using various species of snail as an intermediate host. Only the genus *Schistosoma* contains species that infect man. Based on the geographical prevalence and impact on human health there are three major species of schistosome that infect man: *S. mansoni*, *S. haematobium* and *S. japonicum*. Other species, which are not as widely distributed, also infect man; these include *S. intercalatum* and *S. mekongi*. Despite important differences between the three major human species (see Table 1), there are general morphological and biological features of the life-cycle stages that define the *Schistosoma* species.

Feature	<i>S. mansoni</i>	<i>S. japonicum</i>	<i>S. haematobium</i>
Location of adult worm in host	Mesenteric veins	Mesenteric veins	Vesical plexus
Eggs passed in	Faeces	Faeces	Urine
Egg shape (size μm)	Ovoid (61x140)	Round (60x40)	Ovoid (62x150)
Egg spine	Lateral (prominent)	Lateral (reduced)	Terminal (prominent)
Number of eggs/female/day	100-300	3000-4000	20-300
Intermediate host snail species	Biomphalaria	Oncomelania	Bulinus
Geographical distribution	Africa: mainly sub-Saharan countries. S. America: mainly in Brazil	China, Philippines, Indonesia	Africa: countries north and south of the Sahara Middle East.

Table 1. Characteristics of human *Schistosoma* species

1.1.1 *Schistosoma* parasite life-cycle.

The snail host of *Schistosoma* lives in freshwater; thus, the increasingly frequent construction of dams for hydroelectricity or irrigation in many developing countries leads to the spread of schistosomiasis. Infection is initiated when the cercariae reaches their host and penetrates the skin (**Fig 1.1**). Cercariae are adapted for life in water; though rather than snails they are infective to humans. They consist of a tegument bound body and a tail, which splits at the end to form a Y shape, which known as the furca. The overall

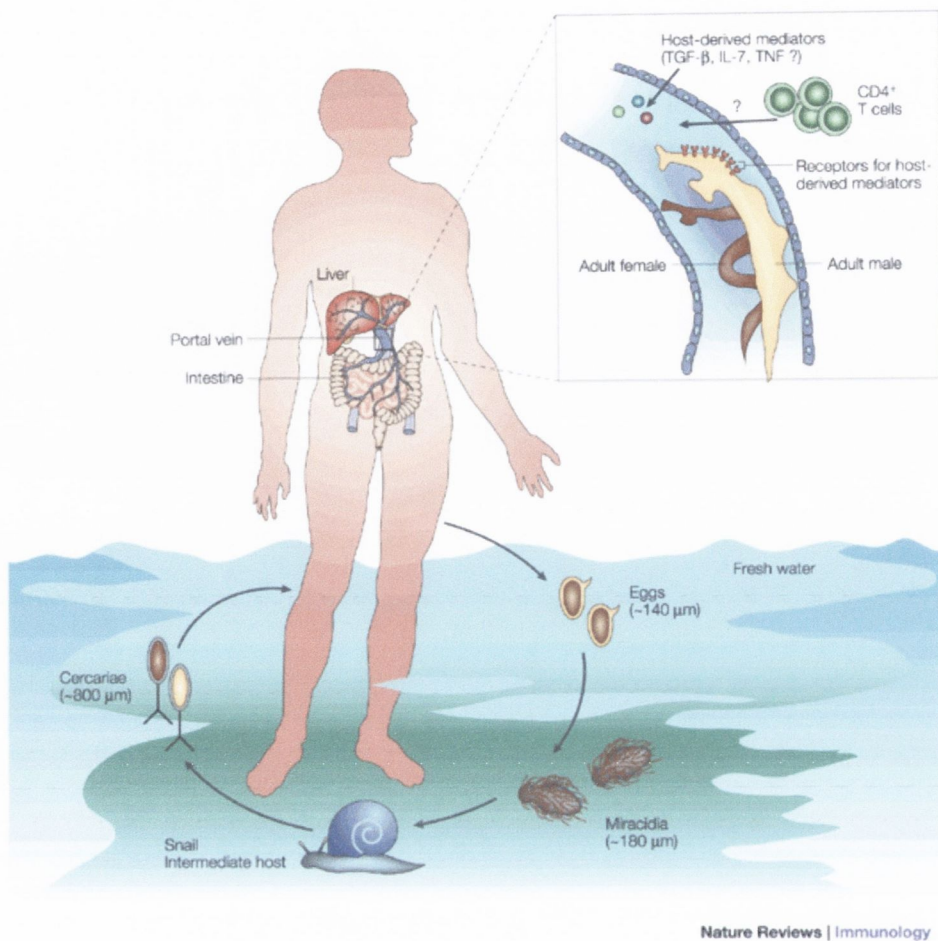


Figure 1.1. Diagrammatic representation of the life cycle of *Schistosoma mansoni* in humans (Pearce *et al.*, 2002).

length is about 1 mm. On contact with a host the cercariae adheres to the skin using its oral sucker and then releases the enzymic contents of the penetration gland, this combined with vigorous tail activity facilitates penetration into the skin. Due to the dramatic change in environment the cercariae undergo profound morphological and physiological changes that result in the transformation to the next life-cycle stage, the schistosomula.

Schistosomula resemble the adult stages of schistosomes having oral and ventral suckers, elongated worm-like body, and a visible gut. After transformation in the dermal tissue schistosomula penetrate the walls of a nearby vein or lymphatic vessel. After 10 to 24 hours, they locate a blood vessel and enter the vasculature. They then migrate passively in the blood stream to the right heart and through the capillary beds of the lungs, and then back to the left heart and finally to the blood vessels surrounding the intestine/liver (*S. mansoni* and *S. japonicum*) or bladder (*S. haematobium*). Only when the schistosomula reach the site of sexual reproduction do they develop into mature male and female worms, which pair up and start producing eggs. The whole process from infection to egg-laying takes approximately twenty-eight to thirty days.

Schistosome male worms are 10-20 mm long and dorsoventrally flattened. The lateral edges are rolled inwards ventrally to form the gynaecophoric canal that embraces the longer (15-30 mm) female, which has a cylindrical, filiform body. Both sexes have a weak oral sucker containing the mouth and a more muscular ventral sucker that is used to attach the worm to the endothelial wall of the host. The body is covered by a tegument capable of taking up nutrients and oxygen direct from the host's blood; additional nutritional requirements are obtained from host blood ingested through the mouth. Proteolytic enzymes secreted by the gut breakdown serum globins and haemoglobin from red blood

cells into tyrosine. A by-product of this process, a black pigment called haematin, can clearly be seen in the caecum, which is eventually regurgitated from the blind-ending gut.

The adult male and female remain in close proximity to each other, indeed the male worm is essential for full maturation of the female worm. Females can produce several hundred to several thousand eggs per day depending on the species (see Table 1). The eggs are non-operculate and characterized by a pointed expansion of the shell known as the spine, located either in a lateral or terminal position. The size and shape of eggs vary between species and thus are an important diagnostic tool. The egg contains an embryo, which matures over six days into the next larval stage, the miracidium. Passage of the eggs through the vein wall and tissues to the lumen of the gut or bladder is also facilitated by a number of mechanisms including blood pressure, mechanical attachment via the spine and the hosts immune response to the egg. Also lytic enzymes secreted by the miracidium pass through the micropores of the egg shell and assist the egg in penetration of the epithelium and basement membrane of the vein, whereby the egg is able to traverse the intervening tissue, basement membrane and epithelium of the intestine (*S. mansoni* and *S. japonicum*) or bladder (*S. haematobium*) en route to the exterior. The eggs are passed in the faeces or urine and under certain environmental cues hatch to release the free-swimming miracidium. Optimal hatching conditions include light, a temperature of 25-30° C and an osmotic pressure close to that of water. The miracidium is between 150 and 170 µm long and approximately 70 µm wide, it propels itself through the water via numerous cilia on its surface.

Miracidiae locate an appropriate snail host or microenvironment where snails maybe found, such as the surfaces of aquatic plants, using a combination of chemical, ionic

and physical cues. Upon contact with the soft tissue of a snail miracidial secretions and mechanical boring facilitates the penetration of the miracidia. During penetration and the following forty-eight hours, structures concerned with swimming such as the ciliated cells and muscle layers are lost and the remaining cells reorganize themselves into a primary sporocyst containing many germinal cells. The sporocyst matures over eight to ten days eventually giving rise to numerous secondary sporocysts that actively migrate to the hepatopancreas and gonads. The second round of asexual reproduction within the sporocyst results in the development of the infective larval stage, cercariae. The cercariae breakout through the sporocyst wall and migrate to the exposed soft tissues where they are shed from the snail. The major stimulus for shedding is light after a period of dark. After 4-7 weeks, thousands of cercariae are produced which shed from the snail tissues into the water under the optimum conditions of temperature and light, the cycle continues if a cercaria penetrates the unbroken skin of a primary host as described previously.

Aspects of *S. mansoni* life-cycle such as the relative ease in maintenance of the *Biomphalaria glabrata* snails in the laboratory and the fact that mice are able to harbour a stable infection, comparable to that of humans, has provided researchers with an excellent experimental model. As a result the majority of research, especially that involving immunobiology of host/parasite interactions are concerned with *S. mansoni*. In this thesis I describe studies on *S. mansoni* infection in mice.

1.1.2 Human infection with *S. mansoni*.

It is currently estimated that one hundred and thirty thousand people die each year from *S. mansoni* infection in sub-Saharan Africa, with a further 13.6 million people

suffering morbidity (van der Werf *et al.*, 2003). However, in endemic areas only a small proportion of infected individuals at any given time manifest severe disease (Jordan *et al.*, 1993). Disease is primarily due to eggs being deposited in host tissue, mainly the liver and the gut, which induces inflammatory and pro-fibrotic responses. *S. mansoni* infection can be sub-divided into two main clinical conditions, acute schistosomiasis and chronic schistosomiasis.

1.1.2.1 Acute schistosomiasis.

Many individuals develop acute disease, also known as Katayama fever, 3-6 weeks after their first exposure to the parasite. Symptoms include fever, chills, weakness, weight loss, headache, anorexia, nausea, vomiting, diarrhoea, hepatosplenomegaly and marked eosinophilia, all of which are hallmarks of a strong hypersensitivity reaction (Gazzinelli *et al.*, 1985). Levels of pro-inflammatory cytokines, such as TNF- α , IL-6 and IL-1, detected in the plasma and supernatants from peripheral blood mononuclear cells (PBMC) are all elevated in patients with acute disease when compared to patients with chronic disease (de Jesus *et al.*, 2002). Strikingly, cytokine production by PBMC after stimulation with parasite antigen reveals a dominant Th1 response in acute disease, rather than the Th2 response to SEA in chronically-infected individuals (de Jesus *et al.*, 2002). Although acute disease can appear before the appearance of eggs in the stool, disease severity generally coincides with maximal egg-laying between six and eight weeks after infection (Rabello, 1995). It is thought that in the progression of the disease, the developing egg-antigen induced Th2 response down-regulates the production and effector functions of the pro-inflammatory mediators. The production of IL-10 during this acute stage might have a

crucial role in the process (Montenegro *et al.*, 1999).

The severity of acute schistosomiasis is related to the intensity of infection, estimated from the number of eggs in the faeces, but lightly infected patients are also sometimes very ill. Acute toxæmic infections are associated with large granulomas around the egg. The granulomatous response peaks around weeks 8-9 and is then gradually down modulated as the infection progresses to the chronic stages. However, acute schistosomiasis is rarely detected in people born in endemic areas and thus tends to be a disease more commonly associated with previously uninfected people moving into endemic areas, or tourists. This suggests that sensitization either as an infant or *in utero* may decrease the pro-inflammatory response upon subsequent contact with the parasite (King *et al.*, 1998).

1.1.2.2 Chronic schistosomiasis.

After the acute symptoms recede infection progresses to the chronic phase, which is graded according to severity. Chronic diseases can be divided into two syndromes: intestinal and hepatosplenic forms. Intestinal schistosomiasis is characterized by irregular bowel movements, diarrhoea, bloody faeces and abdominal pain. The more serious form is the life-threatening hepatosplenic schistosomiasis which is characterized by an enlarged liver and spleen accompanied by increased portal hypertension and portosystematic shunting of venous blood (Dunne and Pearce, 1999). The symptoms are thought to be a consequence of continual inflammatory responses to tissue migrating eggs and subsequent fibrosis. Fibrosis is particularly evident (by ultra-sound) in the portal tracts of the liver and is known as Symmer's pipe-stem fibrosis. Elevated levels of pro-inflammatory cytokines

such as TNF- α and IFN- γ are correlated with hepatosplenomegaly suggesting that these cytokines play a role in the development of severe disease (Mwatha *et al.*, 1998). Infection intensity is one factor that can affect the incidence and severity of chronic schistosomiasis (Mohamed-Ali *et al.*, 1999). However, the prevalence of hepatosplenic disease within communities in Kenya vary despite having similar infection intensities (Fulford *et al.*, 1991), suggesting that other factors such as genetic predisposition to the disease are also important. Indeed a gene polymorphism, IFG, has been shown to be associated with fibrosis in schistosomiasis patients in the Sudan (Dessein *et al.*, 1999). A confounding factor when investigating prevalence and degree of hepatosplenic-associated morbidity is the high incidence of other infections in schistosome endemic areas. Indeed, individuals that harbor both schistosome and malarial infections have been shown to have exacerbated hepatosplenic disease (Booth *et al.*, 2004).

1.1.3 Control measures.

The basis of any control measure is to disrupt the life-cycle of the parasite at one or more life stages. Thus, control measures may involve killing the adult worms by chemotherapy, preventing eggs reaching snail-infested water by providing proper sanitation, removing or killing snails or preventing contact with cercariae by the provision of 'safe' water. In richer countries with stable governments, installing adequate water supply, sewage disposal and primary healthcare programs have proved to be effective in controlling schistosomiasis. Using these strategies countries such as Brazil, Morocco, Egypt, and China have all had successful control programs (Hagan *et al.*, 2004). In Japan complete eradication of *S. japonicum* has been achieved primarily through the use of

molluscicides, backed up with environmental modification of snail habitat, health education and improved water sanitation and agriculture management. However, due to the lack of financial support for control programs and unstable governments many sub-Saharan countries are unable to control schistosomiasis and thus it remains a major public health problem (Chitsulo *et al.*, 2000). Indeed due to water management schemes in certain regions the incidence of schistosomiasis has actually increased (Talla *et al.*, 1990).

1.1.4 *Drug treatment.*

Currently control of infection and disease treatment, especially in sub-Saharan Africa, depends largely on the use of the relatively cheap drug praziquantel (Hagan *et al.*, 2004). Treatment of adult worms with praziquantel induces paralytic muscle contraction and tegumental disruption both of which are thought to be due to changes in calcium flux (Cioli and Pica-Mattoccia, 2003). The disruption of the tegument reveals previously hidden antigens which provide new targets for immune attrition (Harnett and Kusel, 1986) thus efficacy of praziquantel can depend on the immune status of the host (Fallon *et al.*, 1992). This may have implications where praziquantel treatment programs are implemented in areas with high prevalence of HIV infection (Karanja *et al.*, 1997). However, when individuals with concurrent schistosome and HIV infection were treated with praziquantel there was no difference in efficacy between individuals with low CD4⁺ T-cell counts and persons with higher CD4⁺ cell counts (Karanja *et al.*, 1998).

In the past 10 years there have been reports of praziquantel treatment failures (Ismail *et al.*, 1999; Picquet *et al.*, 1998), these have been attributed to either the stage-specific efficacy of the drug, or the development of drug resistance. Stage-specific efficacy

refers to the fact that after penetration by the cercariae, praziquantel is only active against the schistosomula for two days, after which time they lose their susceptibility for approximately four weeks until they become mature worms and are once again susceptible to the drug (Sabah *et al.*, 1986). Thus at the time of treatment all mature worms are killed, however, migrating schistosomula remain unaffected and mature to become egg-producing adult worms giving the appearance of treatment failure (Picquet *et al.*, 1998). Reported treatment failures may also be due to drug resistance, which has been demonstrated both in the laboratory (Fallon and Doenhoff, 1994), and in the field (Fallon *et al.*, 1995; Ismail *et al.*, 1999). Drug treatment-alone, although reducing morbidity, can only have a limited effect on disease transmission as recently cured patients will soon become re-infected the next time they go to snail infested waters to play, wash or collect drinking water. Thus effective control measures need to include environmental management, education, sanitation and drug treatment.

1.1.5 Vaccines.

The fact that mammalian hosts can develop protective immunity to schistosome infection raises the possibility that an effective vaccine can, in theory, be developed. Considering the limitations and failings of drug treatment outlined above the development of a vaccine remains an attractive alternative for controlling schistosomiasis. There are currently numerous vaccine candidates that have either proved successful in experimental animals or have proven links with human protective immunity. They include glutathione S-transferase, paramyosin, triose-phosphate isomerase, Sm23, Sm14, Sm22.6, Sm37GAPDH, Sm20.8 and Sm10-DLC 9 (Capron *et al.*, 2001). The candidates are at

varying stages of development with probably *S. haematobium* GST being the most advanced having successfully completed phase I trails and currently in phase II (Capron *et al.*, 2001; Capron *et al.*, 2005). Vaccines that have been tested in experimental animals have never given 100% protection, however, it is generally considered that partial immunity is better than no immunity (Pearce, 2003). The potency of a partially protective vaccine could be increased with the use of an appropriate adjuvant, for example, when IL-12 is used as an adjuvant with radiation-attenuated cercariae, mice are almost completely protected (Wynn *et al.*, 1996). However, other studies have demonstrated the potentially fatal effects of modulating vaccine-induced immune responses (Rutitzky *et al.*, 2001). A proposed alternative vaccine strategy is to protect against parasite induced pathology rather than the parasite it self. Again IL-12 has been shown to be able to reduce egg-associated pathology (Wynn, 1996; Wynn and Sher, 1995), but others have reported that this strategy may exacerbate pathology (Rutitzky *et al.*, 2001).

1.2 The immune response in *S. mansoni* infection.

With the exception of parasites that require the host to be eaten for transmission to occur, a successful parasite can be defined as one that infects and replicates within its host without causing excessive pathology or death. Thus the host acts as long-term reservoir for transmission. In this respect human helminth parasites can be considered successful as they are characteristically long-lived and, depending on environmental and genetic factors, result largely in asymptomatic infections (Maizels and Yazdanbakhsh, 2003). However, rather than being inert entities that go unrecognized by the hosts immune system helminth parasites have co-evolved with humans to form a complex, delicately balanced relationship with the immune system. Underpinning this relationship is the parasites ability to modulate the immune response.

Typical of helminth parasites, schistosomes are long-lived with worm pairs surviving for up to ten years (Fulford *et al.*, 1995). They also generally result in asymptomatic infections in endemic areas with clinical manifestations becoming apparent only after recurrent exposure and re-infection or with long-term egg deposition without drug treatment. Such long-lived asymptomatic infections are a result of effective immune modulation by the parasite. In an attempt to gain understanding of the mechanisms behind schistosome immune modulation researchers have relied heavily on the murine experimental model of schistosomiasis. Mouse infection with *S. mansoni* has many parallels with human infection in that infection occurs via the skin, the adult worms reside in the venous system around the gut and liver and parasite eggs are either trapped in the liver or translocate through the gut wall. As shown in Figure 1.2, *S. mansoni* infection in

the mammalian host involves interactions within multiple organs. In Figure 1.2, I have divided the phases in mice into the larval-worm stages and the egg-stages. While extrapolating directly from mouse to human can be problematic (Fallon, 2000), much of what is known on how *S. mansoni* and the immune system interacts has been deemed from the murine model.

1.2.1 Protective immunity

Although there is no evidence of immune-mediated clearance of established *S. mansoni* adult worms (Agnew *et al.*, 1993), protective immunity towards infection has been demonstrated in humans and mice. Human studies in endemic areas of Africa where populations are cleared of *S. mansoni* infection by drug treatment have shown that children are more susceptible to re-infection when compared to adults. The low levels of re-infection seen amongst adults cannot be explained entirely by decreased water contact and may be due to the development of protective immunity (Butterworth and Richardson, 1985). Subsequent studies demonstrated that IgE responses to specific parasite antigens correlated with resistance to re-infection (Dunne *et al.*, 1997). Indeed an association between IgE and resistance was first shown in *S. haemtobium*-infected patients (Hagan *et al.*, 1991). The role of IgE in protective immunity is supported by *in vitro* studies where parasite-specific IgE and eosinophils have been shown to participate in schistosomula killing (Capron *et al.*, 1994). IgE is associated with a type 2 response as is the cytokine IL-5. When human peripheral blood mononuclear cells were stimulated with worm antigens, increased levels of IL-5 correlated with older and more resistant age groups (Roberts *et al.*, 1993), suggesting that a type 2 response directed towards the schistsomule/worm stage may

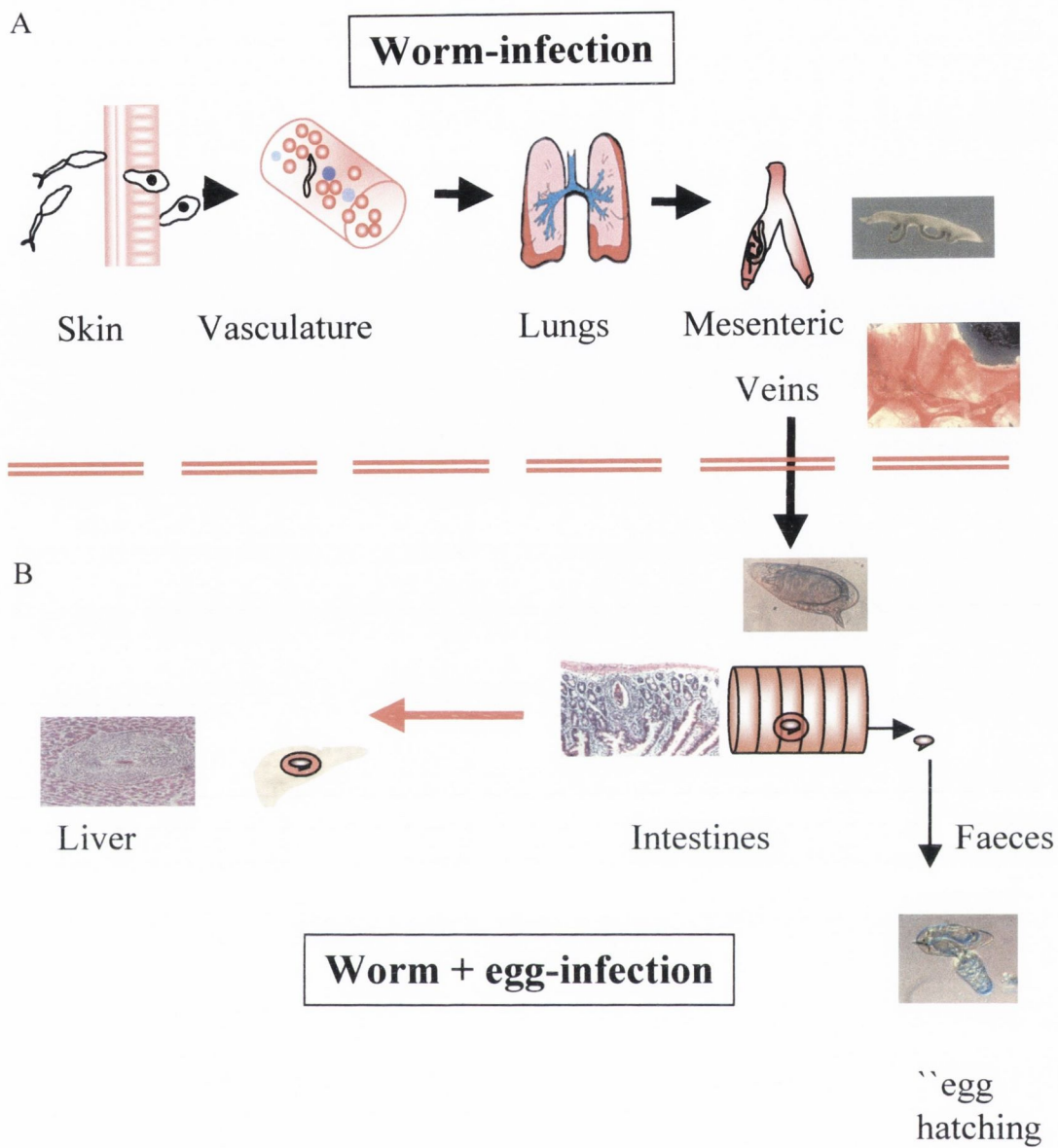


Figure 1.2. Experimental *Schistosoma mansoni* infection in mice.
 A, stages in worm-only infection, above line.
 A + B, conventional worm + egg infection.

confer resistance to re-infection after treatment with praziquantel (Joseph *et al.*, 2004).

An interesting facet of the human-schistosome relationship is the phenomenon of concomitant immunity. Humans are repeatedly reinfected with schistosomes, with new infections requiring migration of larvae through the lung, if this larval-induced pulmonary pathology is not suppressed the host may die. Therefore the adult worm induces an anti-larval immunity or concomitant immune response. This is characterized by the ability of the host to mount an effective defence against larval stages of infection whilst being unable to clear a persistent burden of adult worms (Smithers and Terry, 1969). Smithers & Terry demonstrated this phenomenon when adult worms were transferred from *S. mansoni* infected monkeys to uninfected monkeys. The recipient monkeys were almost totally resistant to cercarial challenge (Smithers and Terry, 1967).

In mice, the irradiated vaccine model provides evidence for protective immunity, whereby infection with attenuated cercariae results in partial immunity to challenge infections with viable parasites. This immunity is reduced after CD4⁺ T-cell or IFN- γ depletion and is thought to be directed against the schistosomula stage (Kelly and Colley, 1988; Smythies *et al.*, 1992). Although the mechanisms involved in human and mouse immunity appear to be different they are both schistosomula-specific. In addition to this transgenic mice that are IgE deficient have increased worm burdens following immunization with irradiated cercariae (King *et al.*, 1997) suggesting that IgE may also play an important protective role in the animal model. Furthermore experiments with rat cells and antibodies *in vitro* have demonstrated the capacity of IgE and IgG2a together with eosinophils to mediate killing of schistosomula (Capron *et al.*, 1994).

1.2.2 Host-pathogen interaction- schistosome innate immune response.

As schistosomes firstly enter through the skin and encounter the innate immune system, the initial priming of the immune response must also be addressed. The strategy of the innate immune system is the early detection of evolutionary conserved microbial structures or pathogen-derived molecules called pathogen-associated molecular patterns (PAMPs) via receptors called pattern-recognition receptors (PRRs). Many immunomodulators activate cells of the innate immune system including macrophages and dendritic cells by binding to PRRs. The PRR include toll-like receptors (TLR), C-type lectins, integrins and complement receptors. These innate receptor-functions include opsonisation, activation of complement cascades, phagocytosis, activation of proinflammatory signalling pathways and induction of apoptosis (Dunne and O'Neill, 2005; Medzhitov, 2001).

1.2.2.1 Toll-like receptors.

Toll-like receptors (TLRs) are the principle sensors (PRRs) of pathogens and have an essential role in host defense, whereby they provide a link between the innate and the adaptive immune responses. TLRs were initially discovered due to sequence similarity of a receptor termed Toll, shown to be essential in host defense against fungal infection in *Drosophila* (Lemaitre *et al.*, 1996), with the intracellular domain of the mammalian cytokine receptor, type I interleukin- (IL-) 1 receptor (IL-1RI; (Gay and Keith, 1991). This conserved intracellular domain is called the Toll/IL-1R/Resistance (TIR) domain. The IL-1 receptor and Toll had very distinct ectodomains, comprised of immunoglobulin folds and leucine-rich repeats respectively. The intracellular signaling pathways of both IL-1RI and

toll were shown to both lead to activation of NF- κ B.

Subsequently, mammalian receptors have been identified that are more Toll-like than IL-1R-like in that as well as the intracellular TIR domain, they also have extracellular leucine-rich repeats. The first mammalian TLR to be discovered was TLR4 (Medzhitov *et al.*, 1997) and since then there have been thirteen TLRs identified. Consistent with their function as PRRs, TLRs are mainly expressed in innate effector cells such as dendritic cells, macrophages, neutrophils, epithelial cells of the mucosa and dermis as well as on T and B lymphocytes (Applequist *et al.*, 2002). TLRs 1-9 are common to mouse and human, TLR10 is only functional in humans, and TLRs 11-13 have been found only in mice (Dunne and O'Neill, 2003; Takeda and Akira, 2005; Zhang *et al.*, 2004). TLRs can recognize several structurally unrelated ligands (Akira and Takeda, 2004) although it is unclear how TLRs recognize their ligands. TLR2 has been shown to be essential in recognizing a multitude of PAMPs including bacterial lipoproteins, gram positive bacterial cell wall components such as peptidoglycan and GPI anchors from *Trypanosoma cruzi* (Campos *et al.*, 2001). This unusually broad range of ligands recognized by TLR2 is explained in part by cooperation between TLR2 and at least two other TLRs ie. TLR1 or TLR6 heterodimerisation. TLR3 recognises polyinosine-polycytidilic acid, poly(I:C), and double stranded RNA (dsRNA) from viruses and also from the eggs of *S. mansoni* (Aksoy *et al.*, 2005). The ligands for TLR4 include lipopolysaccharide (LPS) from gram-negative bacteria. Macrophages and B cells from TLR4 knockout mice have been shown to be hyporesponsive to LPS (Hoshino *et al.*, 1999). Flagellin is a ligand of TLR5 (Hayashi *et al.*, 2001). Single-stranded RNA derived from viruses and synthetic compounds including imidazoquinolines have been found as ligands for TLR7 and TLR8 (Jurk *et al.*, 2002; Lund

et al., 2004). The recognition of CpG motifs requires TLR9 (Hemmi *et al.*, 2000). Also, the malaria pigment, hemozoin, is a non-DNA ligand for TLR9 (Coban *et al.*, 2005). The physiological role of hemozoin-induced, TLR9-mediated innate immune activation and the host responses against it is currently under investigation. The ligand for TLR10 is currently unknown. TLR11 is involved in protection against uropathogenic bacteria (Zhang *et al.*, 2004) and the ligand is thought to be similar to the TLR5 ligand flagellin due to similarities between the two TLRs.

Activation of almost all TLRs culminates in signaling cascades that utilize Toll/IL-R1 receptor domain-containing adapter molecule myeloid differentiation factor 88 (MyD88)-dependent pathways which culminate in the activation of transcription factor NF- κ B and mitogen-activated protein kinases (Akira and Takeda, 2004; Takeda and Akira, 2005). However, there are also MyD88-independent pathways for recognition of pathogens. In the course of analyzing MyD88-independent activation of LPS signalling, a novel adaptor molecule named MyD88-adaptor-like (Mal) / TIR domain-containing adaptor protein (TIRAP) was identified (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001). Mal/TIRAP also involves NF- κ B activation and it is involved in TLR-4 signal transduction.

1.2.2.2 Innate immune recognition in schistosome infection.

The dendritic cells (DC) is an essential factor in innate immunity and in priming of CD4⁺ T cells. The maturation of DC represents a key regulatory step in T cell activation. PAMPs are among the principal DC maturation factors. Antigen uptake by DC leads to DC maturation and migration to the lymph node. At this site DC are then able to present antigen on MHC class II molecules and along with co-stimulation thus activate naïve CD4⁺

T cells. Classical activation in response to bacterial products is characterized by the release of IL-12 and up-regulation of co-stimulatory surface markers. However, mouse bone-marrow DC stimulated by helminths show limited upregulation of CD80, CD86 and MHC class II expression in comparison to expression induced by bacterial stimulants (Jankovic *et al.*, 2001; MacDonald *et al.*, 2001).

Carbohydrates from schistosome eggs are recognized as the main inducers of the Th2 immune response that favours granuloma formation. The role of carbohydrates that are important for the induction of the immune response during schistosomiasis leads to the possibility that PRRs that identify schistosome PAMPs might have a crucial role in the induction of the Th2 response. In the murine schistosome model, the most well understood structures of egg-derived glycoconjugates are the lactosamine carbohydrates lacto-*N*-fucopentaose III (LNFPIII) and lacto-*N*-neotetraose (LNnt). LNFPIII has been shown to be a potent inducer of a Th2 response and can also act as an adjuvant by inducing antibody to coupled protein antigens (Okano *et al.*, 1999). The oligosaccharide determinant is expressed on schistosome glycoproteins and glycolipids and appears to polarize the immune response by activating cells of the innate immune system (Atochina *et al.*, 2001). Helminth antigens fail to induce the classical production of IL-12 by DC. DC pulsed with SEA *in vitro* have been shown to promote Th2 cell responses *in vivo* (MacDonald *et al.*, 2001).

It has been shown that DC-SIGN, a receptor for SEA present on DC and macrophages, recognizes the glycan antigen Lewis^x which occurs in several stages of the parasite life-cycle including cercariae, eggs, SEA and adult worms (van Die *et al.*, 2003). Relevant to these findings, recent data suggests that schistosomes glycans might affect Th2

response development by influencing the way in which DC and macrophages become activated via TLR4 (Pearce and MacDonald, 2002; Thomas *et al.*, 2003), although there are inconsistencies in these data. Glycosylated components of schistosome eggs have also been shown to have effects on the production of cytokines by DC involved in schistosome protection against the onset of diabetes in these mice (Zacone *et al.*, 2003). Therefore in addition to various activation of adaptive immunity it is evident that *Schistosoma mansoni* can also stimulate innate responses via host recognition of schistosome-derived PAMPs.

1.2.2.3 Helminth modulation of macrophages.

Suppressive adherent cells have been shown in infected individuals with suppression mediated by IL-10 (Flores Villanueva *et al.*, 1994a; Osborne and Devaney, 1999). However, there are also nematode induced-IL-10-independent pathways of macrophage suppression (Allen and Loke, 2001) (MacDonald *et al.*, 1998). The production of anti-inflammatory mediators such as IL-10, the induction of Th2 cytokine secretion and a mechanism of suppression of T-cell proliferation reflects an alternatively activated phenotype for LNFPIII- and LNnt –primed macrophages (Noel *et al.*, 2004). Recent studies have showed that activated macrophages are capable of expressing two cytokine inducible enzymes, inducible nitric oxide synthase (iNOS) and arginase, which share L-arginine as a substrate. Modolell and coworkers demonstrated that different combinations of type-1 and type-2 cytokines induced iNOS and arginase (Arg-1) expression (Modolell *et al.*, 1995). The Th1 cytokines, IFN- γ and TNF- α , induced iNOS in macrophages that were designated classically activated, whereas the Th2 cytokines IL-4 and IL-13 induced Arg-1 in cells macrophages described as alternatively activated (aaMØs) (Gordon, 2003).

Classically-activated macrophages are the best-characterized. Stimulation of macrophages with pro-inflammatory microbial molecules such as LPS results in the production of various cytokines such as TNF- α , IL-1, IL-6 and IL-10 and inflammatory effector substances such as prostaglandins, leukotrienes and nitric oxide (NO). These classically activated macrophages have an enhanced antigen-presenting capability and exert anti-proliferative and cytotoxic anti-microbial and anti-tumour effects. aaMØs in contrast to classically activated macrophages are generated in a Th2 environment and do not generate NO from L-arginine. Recent studies have identified Ym 1 and Fizz 1 as the most abundantly expressed genes in murine-elicited alternatively activated macrophages (Loke *et al.*, 2000; Nair *et al.*, 2003). As aaMØs are antagonistically regulated by Th1 cytokines, it could be hypothesized that these macrophages, by secreting anti-inflammatory cytokines such as IL-10, down-regulate the inflammatory processes initiated by classically activated macrophages (Noel *et al.*, 2004). Thus evaluating the iNOS-arginase balance provides evidence for the presence of aaMØs as does the presence of Arg 1, Ym 1 and Fizz 1 that are strongly induced by aaMØs.

Several studies have investigated whether the iNOS-arginase balance is tilted during schistosome infection (Noel *et al.*, 2004). It has been found that the induction of arginase activity depends on the Th2 immune response and correlates with maximal egg-induced pathology. Contrastingly, high iNOS activity is associated with a reduction in the number of eggs and pathology in *Schistosoma*-infected mice (Pearce and MacDonald, 2002). When mice are sensitized to SEA in the presence of IL-12 before infection, the normal Th2 egg-specific response is converted to a dominant Th1 immune response. Consequently, arginase-positive aaMØs and the associated Th2 liver pathology decrease (Noel *et al.*,

2004). These data indicate that aaMØs contribute to the granuloma pathology during *Schistosoma* infection. In another study Herbert *et al.*, demonstrated that macrophages deficient in the IL-4R α are extremely susceptible to acute morbidity after *S. mansoni* infection (Herbert *et al.*, 2004). This morbidity occurred with concomitant elevation in type 1 immunity thus further indicating a role for aaMØs in schistosome infection and their potential for inhibiting inflammatory responses.

1.2.3 Host-pathogen interaction- schistosome adaptive immune responses.

In the course of an infection, the immune response progresses through at least three phases. In the first 3-4 weeks of *S. mansoni* infection, prior to egg-laying, the initial infective stages of schistosomes (cercariae) actually stimulate a type 1 response in the mouse model with associated expansion in the frequency of CD8⁺ Tc1 and CD4⁺ Th1 cells. Th1 cells are characterized by the secretion of IFN- γ , tumour necrosis factor (TNF) and IL-2 and are associated with cell-mediated responses, such as activation of cytotoxic and phagocytic functions in effector cells and delayed-type hypersensitivity (**Fig. 1.3**). As the schistosome infection progresses and parasites mature, mate and begin to produce eggs at weeks 4-5, the response alters markedly with a switch towards a strong type 2 response (Sabin and Pearce, 1995) with an increase in the numbers of Th2 cells and a dramatic reduction in the frequency of Tc1 cells (Fallon *et al.*, 1998). IL-4 can skew the development to production of Th2 cells (**Fig. 1.3**). The source of IL-4 early in the immune response is not conclusive. The candidates include memory and possibly naïve MHC class II restricted CD4⁺ T cells (Bradley *et al.*, 1991), the NK1⁺ subset of CD4⁺ T cells (Bendelac *et al.*, 1997) and non T cell sources e.g. mast cells, eosinophils and basophils (Paul *et al.*,

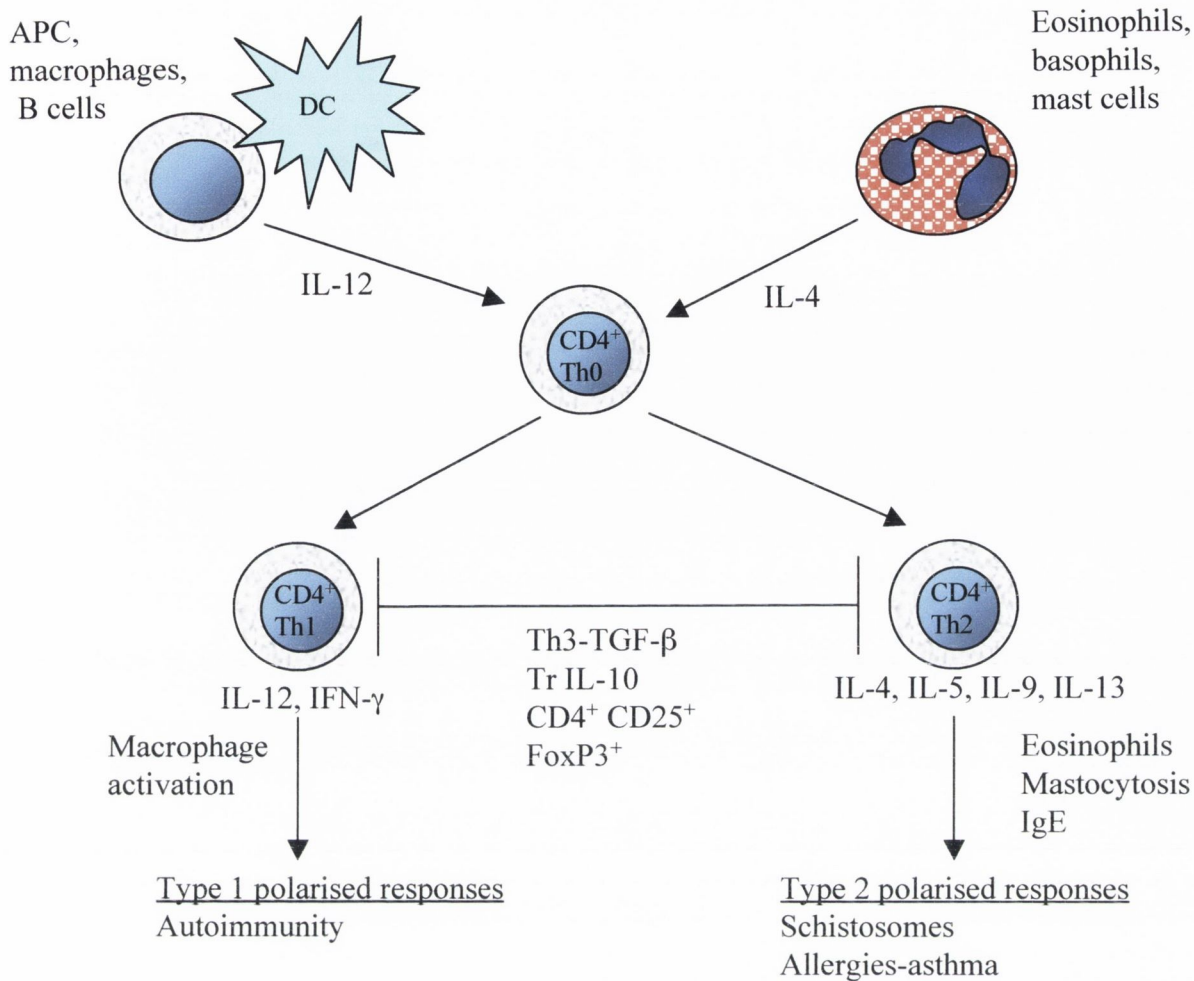


Figure 1.3. Schematic of the Th1 and Th2 cell response.

1993) and Gr1⁺ cells (Jordan *et al.*, 2004). The characteristic cytokines produced by Th2 cells include IL-4, IL-5, IL-10 and IL-13, (Mosmann *et al.*, 1986).

Exogenous antigens are initially processed by antigen presenting cells (APC), which include professional APC such as dendritic cells along with macrophages and B cells, prior to presentation to T cells. Antigen presenting cells play a pivotal role in determining the outcome of an infection due to their unique ability to prime naïve CD4⁺ T cells. The docking of T cells and APCs depends upon strong mutual interactions between complementary molecular pairs on their surface: MHC II/CD4, MHC I/CD8, VCAM-1/VLA-4, ICAM-1/LFA-1, LFA-3/CD2, B7/CD28 (or CTLA-4) respectively. Activation of T cells requires two signals as one alone produces unresponsiveness (anergy). Signal 1, the interaction of the TCR with the (MHC) molecule containing the pathogen derived antigenic peptides, indicates the molecular identity of the pathogen. The pathogenic potential of the infectious agent is translated by signal 2, supplied through the interaction of co-stimulatory molecules on the surface of both cells (Kalinski *et al.*, 1999).

Downstream activation of transcription factors have been shown to play critical roles in the initiation of either Th1 or Th2 responses. Induction of the T-box transcription factor (T-bet) is involved in the differentiation of T cells (Th0) to a Th1 phenotype by driving IFN- γ production. Mice with the T-bet gene knocked out lack Th1 cells and have elevated numbers of Th2 cells making them thus susceptible to Th2 disorders e.g. allergic asthma. GATA binding protein 3 (GATA-3) induction is important in some Th2 responses including the activation of the Th2 gene cluster, which includes IL-4, -5 and -13 (Abbas *et al.*, 1996), and in maintaining Th2 memory through the inhibition of IFN γ . GATA-3 transcription is activated by signal transduction and activator of transcription (STAT) -6

(Ray and Cohn, 1999). STAT-6, induced by IL-4, plays a central role in Th2 cytokine-induced pathology. Epithelial cells devoid of STAT-6 are not affected by IL-13 induced airway hyper-responsiveness and mucus production (Kuperman *et al.*, 2002). Also, decreasing STAT-6 transcription inhibits IL-4 induced cell proliferation (Wang *et al.*, 2000). STAT-6 defective mice are severely impaired in granuloma formation and fibrosis while STAT-4-defective mice show only minor changes in pathology confirming the critical role of type-2 cytokines in disease progression on schistosomiasis (Kaplan *et al.*, 1998).

Type 2 responses in schistosome infection are also characterized by elevated serum IgE levels, as well as eosinophilia in blood and tissues, factors that are controlled by IL-4 (Fallon *et al.*, 2002; Finkelman *et al.*, 1986) and IL-5 (Coffman *et al.*, 1989). *S. mansoni* eggs are the major stimulus responsible for the production of Th2 cytokines during infection (Grzych *et al.*, 1991). The developing schistosome-egg antigen-induced Th2 responses down-regulates the production and effector functions of the earlier Th1 pro-inflammatory mediators; the production of IL-10 during this period is thought to have a crucial role in this process. Although the Th2 response might have limited effects on parasite burden, it exerts an enormous influence on the survival of the host as the granulomatous response around the egg is maximal at this stage of infection (Grzych *et al.*, 1991). Th2 cytokines as described modulate excessive inflammatory reactions preventing a runaway pathology.

1.2.4. Immunopathology of schistosome infection.

Most pathology in schistosome-infected mice is attributed to the host's reaction to

the eggs which is maximal by the 8th week of infection. The granuloma formed around a schistosome egg is primarily a T-lymphocyte-mediated host response with diminished granulomas in T cell-depleted and severe combined immunodeficiency mice (SCID) mice (Amiri *et al.*, 1992; Doenhoff *et al.*, 1981; Fallon *et al.*, 2000). B cells have also been implicated in playing a regulatory role in schistosome granuloma formation (Jankovic *et al.*, 1998). Granulomas that form in response to antigenic stimulation represent a form of delayed-type hypersensitivity. Initially, macrophages surround the antigen and fuse to form characteristic epitheloid and giant cells. These then recruit T cells, which in turn recruit myeloid effector cells such as eosinophils, neutrophils and activated macrophages (Sandor *et al.*, 2003). This initial development of a granuloma is thought to be governed, to a degree by TNF- α (Joseph and Boros, 1993) and the chemotactic activity of various chemokines (Qiu *et al.*, 2001), and through secretion from the egg of a chemokine-binding protein (Smith *et al.*, 2005) (in press).

The cellular components and structural organisation of granulomas are highly regulated by an array of cytokines, chemokines and neurokines. Schistosome eggs induce a type 2 granuloma characterized by a high eosinophilic component (approximately 40-50%) (Weinstock *et al.*, 1999) and Th2 cells (Boros and Whitfield, 1999). During schistosome infection in mice the formation of the granuloma occurs in two distinct immune events. The acute stage (6-12 weeks post infection) is characterized by reaction to soluble egg antigens, which induces a switch from a type 1 to a type 2 response and is accompanied by maximal granulomatous inflammatory responses (Lukacs and Boros, 1992). Following peak granuloma formation inflammatory responses progress towards a chronic phase characterized by spontaneous down-modulation of the inflammatory response with

diminished Th2 cytokine production and decrease in granuloma formation.

IL-13 has been distinguished as a key cytokine in the development of fibrosis in schistosome egg-induced liver pathology (Fallon *et al.*, 2000). Indeed IL-13R α 2-deficient mice infected with *S. mansoni* have increased IL-13-dependent fibrosis and this decoy receptor for IL-13 has been shown to be important in down-modulation of the immune response in infection and hence host survival (Chiaramonte *et al.*, 2003; Mentink-Kane *et al.*, 2004). Studies in IL-10^{-/-}, IL-12^{-/-} and IFN- γ ^{-/-} mice demonstrate this further as these mice develop elevated IL-13 responses and consequently have elevated hepatic fibrosis (Hoffmann *et al.*, 2000). Furthermore, increased mortalities occur in IL-10^{-/-} mice with *S. mansoni* infection, with these mice developing egg Ag-specific Th1 responses (Wynn *et al.*, 1998). While prolonged Th2 responses contribute to the development of hepatic fibrosis and chronic morbidity, the initial Th2 response in the acute stage of infection protects the host from the potential pathogenic damage caused by unrestricted Th1 cell-mediated inflammation (Pearce and MacDonald, 2002). So paradoxically, granulomas may have an essential host-protective role. In mice that are tolerised against *S. mansoni* egg antigen, granuloma formation is severely limited. Mice have elevated type 1 cytokine responses and diminished type 2 responses with severe hepatotoxic liver damage (Fallon and Dunne, 1999).

These observations also indicate the importance of a balanced immune response in infection to prevent overt or runaway pathology. Thus the formation of the granuloma is controlled by both type 1 and type 2 cytokines. From work in the mouse, there now seems to be a correlation between the inability to form granulomas, or the development and persistence of a highly pro-inflammatory Th1-like response beyond the acute phase, and the

development of severe hepatic fibrosis (Rutitzky *et al.*, 2001). By contrast, Th2 cell-mediated granulomas seem to protect hepatocytes, but allow the development of fibrosis as cumulative damage occurs as older lesions involute to leave fibrous scars. Th2 responses are also strongly implicated in naturally acquired resistance to reinfection with schistosomes. The prominence of IL-10 as a crucial mediator of regulation in parasite infections has long been recognised, particularly for its role in attenuating pathogenesis.

To date, murine studies have focused primarily on CD4⁺ cells as the T cell source of cytokines that determine the type 2 outcome in murine schistosome infections and regulate the granulomatous response to parasite eggs. However, other cell types such as eosinophils, FcεR⁺ non-B non-T cells or B1 cells have also been implicated as additional cell sources of type 2 cytokines during murine infection (Kullberg *et al.*, 1996; Palanivel and Harn, 1994; Rumbley *et al.*, 1999).

During the chronic phase of infection (> week 14 in mice), the Th2 response is modulated and granulomas that form around newly deposited eggs are smaller than at earlier times during infection. It is during this chronic infection stage that there is an acceptable balance between the host's immune system and the disease state. This Th2 modulation, is undergone by the host to down-regulate inflammatory reactions to invading schistosomes and ultimately prevents the death of the host from massive widespread inflammation. One study demonstrated that IL-10^{-/-} mice infected with *S. mansoni* fail to enter this late down-modulating phase (Sadler *et al.*, 2003). Although the switch to a Th2 response is beneficial to the host, it is also beneficial to the worm and leads to long-term infections. Evidence indicates chronic exposure to *S. mansoni* down regulates the type 1 immune response and prevents the onset of Th1-mediated diseases such as multiple

sclerosis, diabetes mellitus and Crohn's disease (Cooke *et al.*, 1999; Moreels *et al.*, 2004; Sewell *et al.*, 2003).

1.2.5 *T cell regulation in schistosome infection.*

During infection, levels of the Th1 type cytokine- IFN- γ fluctuate (Smith *et al.*, 2001), and it is the dominant cytokine during the first four weeks of infection. This pro-inflammatory response during the first 3-5 weeks of infection is stimulated by the cercariae, but decreases by the eighth week of infection with the reciprocal increase in Th2 type cytokines. This switch is thought to involve regulation by IL-10, and suggests a central role for various types of T regulatory cells.

It is thought that immune responses are controlled by a broad range of T cell populations with putative regulatory activity. Some 5-10% of T cells are so-called T-regulatory cells. These include regulatory T cells, Th3 cells and CD4⁺CD25⁺ cells which all can suppress unwanted adaptive immune responses, whether they be Th1 or Th2 (Shevach, 2002). Like other T cells, T reg cells also express the $\alpha\beta$ T-cell receptor for antigen (TCR) and can only be activated if it binds to the peptide class II MHC molecule for which it is specific and the cells also receive co-stimulation from B7 molecules CD80 and CD86 on the APC. However if activated they begin to secrete large amounts of the immunosuppressive cytokines IL-10 and in some cases TGF- β . IL-10 has been shown to down-regulate Th1 immune responses and also Th2 skewed response to agents such as allergens and certain parasites demonstrating an inhibition of IL-4 and IL-5 production, antibody secretion and eosinophilic inflammation. It has been shown that numerous cell types secrete IL-10 including, but not limited to; mast cells, macrophages, eosinophils,

microglia, langerhans cells, fibroblasts, and T cells (Moore *et al.*, 2001; O'Garra *et al.*, 1997).

Sakaguchi and others illustrated active *in vivo* T cell suppression as a mechanism for immunological tolerance against autoimmunity (Sakaguchi *et al.*, 1995). More recently Shevach and co-workers developed an *in vitro* system, that is now widely used, showing that CD4⁺ CD25⁺ T cells do not proliferate in response to either anti-CD3 or antigenic stimulation and furthermore can inhibit the proliferative response of CD4⁺ CD25⁻ T cells (Shevach, 2002). The mechanism of suppression by T reg subsets remains controversial; different *in vivo* and *in vitro* studies have investigated roles for the immunosuppressive cytokines IL-10 and TGF- β , the negative co-stimulatory molecules cytotoxic T-lymphocyte-associated antigen (CTLA)-4 and programmed death 1 (PD-1) and the glucocorticoid-induced TNF receptor (GITR).

Recently it has been shown that CD4⁺ CD25⁺ T cells, with a role for IL-10, are elevated in worm + egg infection (Hesse *et al.*, 2004), which may suppress Th2 responses (McKee and Pearce, 2004). Indeed the phenomenon of T cell suppression during the conventional schistosome worm + egg infection is well documented (Flores Villanueva *et al.*, 1994b; Stadecker, 1999). Jankovic *et al* have shown that conditioning a DC for Th2 differentiation by SEA correlates with down-regulation of activation signals in the APC, which leads to a temporary delay in T cell cycling (Jankovic *et al.*, 2004). Also a schistosome egg glycan has been shown to induce a Gr1⁺/F4/80⁺ macrophage population that suppresses CD4⁺ T cell proliferation (Atochina *et al.*, 2001). Recently we have shown that *S. mansoni* infection induces two forms of T cell anergy during the acute stages of infection, including a new model involving up-regulation of the co-stimulatory molecule

PDL1 on the surface of macrophages (Smith *et al.*, 2004).

An important advance in the understanding of the biology of regulation by CD4⁺ CD25⁺ T cells occurred in the demonstration of the dependence of their suppressive phenotype on expression of the transcription factor FoxP3 (Chen *et al.*, 2003; Marie *et al.*, 2005). Gene deletion of *foxp3* abrogated suppression by CD4⁺CD25⁺ T cells whereas ectopic gene expression in CD25⁺ T cells rendered these cells suppressive. It has hence been suggested that FoxP3 acts as a master switch for the regulatory phenotype in much the same way as do GATA-3 and T-bet in Th2 and Th1 cell phenotypes respectively (Cohn *et al.*, 1999). Although FoxP3 mRNA is found in isolated CD4⁺CD25⁺ cells from schistosome-infected mice (McKee and Pearce, 2004), we have shown it is selectively expressed on creation CD4⁺CD25⁺ cells (Walsh, unpublished). In addition, FoxP3 gene transfer in livers of *S. mansoni*-infected mice has been shown to strongly suppress full granuloma development (Singh *et al.*, 2005).

1.3 Allergies.

Atopy is a generic term that covers a range of aberrant responses to environmental targets termed allergens. Atopic individuals may develop a range of allergic diseases including asthma, anaphylaxis, allergic rhinoconjunctivitis, atopic dermatitis and food allergies. Clinically these conditions range from relatively mild discomfort in allergic rhinitis to death due to anaphylaxis. The two most important prerequisites for the development of atopic disorders such as asthma, hay fever, eczema or allergic rhinitis are allergen-exposure followed by the generation of allergen specific CD4⁺ Th2 cells. Thus mechanistically, atopy neatly fits into the Th1/Th2 cytokine paradigm of immunological

responses with a type 2 cytokine response as Th2 cells secrete IL-4, IL-5 and IL-13 which in turn induce the production of allergen-specific IgE by B cells, the development and recruitment of eosinophils and mast cells (via IgE cross-linking) thus leading to most clinical manifestations of allergic disease (Girolomoni *et al.*, 2001).

1.3.1 *Development of an allergic response.*

In response to exposure to an allergen the immune response is stimulated to produce an early Th1 response that is succeeded by a predominantly late-phase Th2 response. The Th2 cytokines IL-4, -5, -9 and -13 have been strongly implicated, by both generic and experimental studies of asthmatics, in generating and perpetuating allergic inflammation through multiple mechanisms including the stimulation of mucus production, isotype switching of immunoglobulin (Ig) E, expression of adhesion molecules and chemokines that attract eosinophils and other inflammatory cells to an inflammatory focus, and the suppression of the production of IL-12, a cytokine that inhibits allergic inflammation. In addition, IL-4 stimulates mast cell growth and activation and the differentiation and/or growth of T cells that produce pro-allergic type 2 cytokines including recruitment of activated eosinophils and neutrophils into airways, airways hyper-responsiveness, and airflow obstruction generated by goblet cell hyperplasia causing an infiltrate of mucus in the airways. The critical requirement for IL-4, in the initial priming of Th2 effector cells, was demonstrated in studies using IL-4^{-/-} mice whereby defective priming of Th2 cells in the absence of IL-4 resulted in a failure to generate allergic inflammatory responses after subsequent airway challenge (Corry *et al.*, 1996; Coyle *et al.*, 1995; Kips *et al.*, 1995).

DC play an important role in sensitization to inhaled allergens (van Rijt *et al.*,

2005). Airway DC act as antigen presenting cells in the bronchial epithelium where they capture inhaled antigens and migrate to the draining lymph nodes in the lung. Here the DC present antigen to circulating naïve CD4⁺ and CD8⁺ T cells (Brimnes *et al.*, 2003; Lambrecht *et al.*, 2000b). Antigen presentation by airway DC is necessary in Th2 sensitization in allergies (Stumbles *et al.*, 1998). Molecules expressed on the surface of these cells are involved in production of Th2-dependent airway eosinophilia (Lambrecht *et al.*, 2000a). Lung DC have been found to be important proinflammatory cells that are necessary for Th2 cell stimulation during airway inflammation

Akbari *et al* (2003) demonstrated an essential role for natural killer T (NKT) cells in the development of asthma and Th2 biased respiratory immunity against allergens. IL-10 is one of the main cytokines that is implicated in regulatory T cell function and, with respect to asthma, has also been shown to suppress immune and inflammatory responses including airway inflammation (Zuany-Amorim *et al.*, 1995).

Genetic analysis has also demonstrated evidence of genetic predispositions to developing allergies. The Th2 cluster on human chromosome 5q23-35, containing the loci for the Th2 cytokines IL-4, IL-5, IL-9, IL-13 and other potentially relevant genes, has been shown by various studies to have a significant linkage with total IgE levels (Marsh *et al.*, 1994). As discussed earlier TLR4 and TLR9 have been differentially associated with a regulatory role in inducing Th2 responses. Genetic analysis has shown no association between TLR4 and asthma susceptibility (Raby *et al.*, 2002), whereas polymorphism in TLR9 may be associated with asthma in certain populations (Lazarus *et al.*, 2003).

1.3.2 Asthma.

Asthma affects approximately 10-15% of the population in the Western world and its reported incidence has increased dramatically in many developed nations (Gergen and Weiss, 1990). Asthma is defined as a chronic inflammatory lung disease that is characterized by airway hyper-reactivity, subepithelial fibrosis, collagen deposition, goblet cell hyperplasia and mucus hyper-secretion that results in intermittent airway obstruction (Busse and Neaville, 2001; Cookson, 1999; Marsh *et al.*, 1994; Umetsu *et al.*, 2002).

Although most asthma is associated with atopy, the patho-physiological basis of the clinical features remains poorly defined. However, it has been suggested that acute inflammation is ordinarily responsible for episodic bronchoconstriction, while chronic inflammation and airway remodelling contribute to airway hyper-reactivity and to the fixed airflow obstruction that develops in chronic asthmatics. The asthma response is characterized in two parts, firstly the early phase in which smooth muscle of the airways is attacked, followed by constriction of the lumen of the bronchioalveolar. In the late-phase response, inflammatory cells infiltrate the lungs and it is in this phase that lungs can become damaged especially after repeated attacks (Bousquet *et al.*, 2000).

Allergic asthma is initially driven by a response to a specific allergen, resulting in allergen-induced activation of mast cells in the lower airways triggering them to secrete inflammatory mediators e.g. histamine which causes increased vascular permeability and smooth muscle contraction (Williams and Galli, 2000). But the subsequent chronic airway inflammation can be continued in the absence to further exposure of allergen. For example, the airways of asthmatics characteristically show airway hyper-responsiveness (AHR) to environmental chemical irritants such as cigarette smoke. These environmental factors are

bronchoconstrictors. Studies of bronchoalveolar lavage (BAL) and asthmatic airways have shown an increase in activated T lymphocytes, expressing a Th2 like cytokine profile of IL-4, IL-5, IL-9 and IL-13 (Fallon and Dunne, 1999; Robinson *et al.*, 1993). Th2 cells, in particular, their cytokines IL-4 and IL-5, have been implicated in the development of AHR (Lee *et al.*, 1997).

DC at the site of allergen entry-under the epithelial surface, migrate to the mediastinal lymph nodes and activate allergen-specific Th2 cells. Activated Th2 cells secrete cytokines that augment eosinophil activation and degranulation. IL-5 is particularly important in asthma as it is directly implicated in the increased production of eosinophilia in bone marrow causing increased numbers in circulation but not in tissues. Entry of eosinophils into tissues is regulated by the chemokine eotaxin (Collins *et al.*, 1995). Activated eosinophils produce eotaxin, and at the site of inflammation eosinophils release leukotrienes C4, D4 and E4 which causes smooth muscle contraction, increased vascular permeability and increased mucus secretion. Alterations in the IL-5-eotaxin-eosinophil axis may result in chronic inflammation of the airways and AHR.

1.3.3 Anaphylaxis.

Anaphylaxis is an acute, life-threatening, allergic hypersensitivity reaction that can cause fatalities following exposure to allergenic antigens in atopic individuals. Allergic reactions, as described above, are characterized by an over-production of Th2 cytokines contributing to elevated levels of IgE and eosinophilia. The initiation of the response requires the binding of IgE to its high affinity receptor Fc receptor, FcR1, expressed on mast cells and basophils (Ando *et al.*, 1993). Cross-linking of receptor bound IgE by

specific antigen initiates an intracellular signal transduction cascade that culminates in the degranulation of these cells resulting in release of inflammatory mediators (for example histamine and platelet activating factor (PAF)), enzymes and cytokines that mediate the clinical manifestations of atopy which include acute bronchoconstriction, tachycardia, hypotension and vascular leakage (Strait *et al.*, 2002)

The central role of Fc ϵ RI in mediating the response was demonstrated by observations that mice deficient in this receptor fail to undergo IgE-dependent, passive cutaneous (Takai *et al.*, 1994) and passive systemic anaphylaxis (Dombrowicz *et al.*, 1993). However IgE does not account for all anaphylactic pathways as earlier observations indicated that the low-affinity Fc receptors for IgG (Fc γ RIIB and Fc γ RIII) on mouse mast cells, macrophages, and the rat mucosal type mast cell RBL-2H3 can bind IgE immune complexes in vitro and the engagement of Fc γ RIIB/III with the IgE immune complexes triggers C57.1 mast cells to release serotonin suggesting a greater potential complexity to the IgE-mediated anaphylactic response (Oettgen *et al.*, 1994; Ujike *et al.*, 1999). Also the low-affinity IgE receptor Fc ϵ RII (CD23), is expressed by several immune cells including B cells and dendritic cells. IgE that is bound to CD23 facilitates allergen uptake by B cells, enhancing presentation to T cells and augmenting secondary immune responses.

Mast cell degranulation is also essential for rapid expulsion of some gastrointestinal nematode parasites, such as *Trichinella spiralis*, during a second infection; however, IgE is not absolutely essential for this process. In contrast, IL-4 enhancement of target cell sensitivity to mediators appears to be critical for host protection against this parasite. Interestingly, the cross-linking of IgE bound to Fc ϵ R1 on basophils leads to the rapid secretion of large quantities of IL-4 and IL-13 that could increase the sensitivity of

intestinal cells to the effects of mediators released by mast cells or macrophages. Furthermore the basophil cytokine response requires considerably less IgE cross-linking than the induction of mast cell mediator release, allowing targeted cells to develop maximal sensitivity to mediators by the time they are produced and released (Strait *et al.*, 2002).

1.4 The Hygiene Hypothesis.

Th2 responses are specifically associated with Helminth parasitic diseases as described in **Fig. 1.4**. However, in modern societies the most widespread Th2-mediated diseases are the spectrum of atopic disorders. One hypothesis for the increasing prevalence of Th2-associated allergic disease, the Hygiene Hypothesis, is that western lifestyles reduce contact with environmental microorganisms. Such microorganisms are thought to modify antigen presenting cells (in part via TLRs) so that they induce either immune deviation in favour of Th1 responses or the development of T reg responses to allergens. Indeed the frequency and severity of atopic disorders are steadily increasing, particularly in developing countries where approximately 20-30% of the population are affected (Erb *et al.*, 1998).

The basic principle of this hypothesis is that the concurrent improvement in hygiene in industrialized societies, which includes improved public health and the use of antibiotics and vaccines, obesity, diet and reduction in childhood infections has led to the speculation that early childhood infections may reduce the risk of developing allergy (Strachan, 1989; Umetsu *et al.*, 2002). More direct scientific evidence comes from epidemiological studies that show an inverse association between infection with a variety of pathogens and atopy. These pathogens include bacteria, such as *Mycobacterium tuberculosis* (Shirakawa *et al.*,

Developing Countries

Large family size
Rural homes
Livestock
Intestinal microflora, variable
No antibiotics/vaccines
Poor sanitation
High orofaecal burden
Active lifestyle

High helminth burden

Regulated immunity

Non-allergic

Westernised Countries

Small family size
Urban homes
Intestinal microflora -stable
High antibiotic use
Vaccines used routine
Good sanitation
Low orofaecal burden
Diet
Obesity
Sedentary lifestyle
Affluence

Low / absent helminth burden

Dysregulated immunity

Allergic disorders
*asthma, anaphylaxis,
eczema & rhinitis*

Figure 1.4. The allergic balance in the Hygiene Hypothesis.

1997) and *Helicobacter pylori* (Stroffolini *et al.*, 1998), viruses, such as measles and Hepatitis A, (Matricardi *et al.*, 1997), parasitic protozoan *Toxoplasma gondii* (Matricardi *et al.*, 2000) and parasitic helminths (van den Biggelaar *et al.*, 2000). The traditional explanation given for infections decreasing the risk of atopy have largely been explained by the infection shifting the immune system towards type 1 dominated response upon early exposure to infections. This shift towards inducing type 1 immunity to allergens protects against allergic diseases by reducing the expression of Th2 cytokines generally associated with allergy.

Arguably the most compelling supportive evidence of the hygiene hypothesis is the potential negative association between mycobacteria and allergies. An immunological study of a cohort of 6,000 Japanese children demonstrated a strong inverse relationship between delayed hypersensitivity to *M. tuberculosis* and atopic responses (Shirakawa *et al.*, 1997). These early positive tuberculin responses were associated with lower serum IgE levels and cytokine profiles skewed towards a Th1 bias. Positive tuberculin responses in infants were associated with lower incidence of asthma, rhinoconjunctivitis and eczema in later childhood (Shirakawa *et al.*, 1997). This view is further supported by the demonstration of a significant inverse correlation between the prevalence of asthma and reported rates of tuberculosis (von Mutius *et al.*, 2000). In mouse studies, pulmonary exposure of mice to live Bacillus Calmette-Guerin prevented allergen-induced airway eosinophilia and the development of airway hyper reactivity (Erb *et al.*, 1998; Herz *et al.*, 1998). As bacterial (Shirakawa *et al.*, 1997; Stroffolini *et al.*, 1998) and viral (Matricardi *et al.*, 2000) infections shift the immune system towards type 1 dominated

responses upon early exposure to infection, it is argued that this shift towards type 1 immunity protects against allergic diseases by reducing the expression of Th2 cytokines generally evoked by allergens. Paradoxically, in the context of this model of the so-called Hygiene Hypothesis, human populations with high rates of parasitic helminth infections, which induce an immunological shift towards Th2 responses, have less prevalence of allergic disorders (Yazdanbakhsh *et al.*, 2002)(**Fig. 1.4**).

A range of parasitic helminths have been implicated in the hygiene hypothesis, including gastro-intestinal nematodes, e.g. *Ascaris lumbricoides*, and the vascular trematodes *Schistosoma mansoni* and *S. haematobium* (Yazdanbakhsh *et al.*, 2002). As host morbidity is closely related to parasite burden most natural helminth infections of humans are likely to have co-evolved with their host mechanisms to maintain infections but control parasite numbers. Several mechanisms have been postulated to underlie the low incidence of respiratory allergy and asthma individuals with certain worm infections. These hypotheses include 1) effects of worm burden, 2) induction of immunosuppressive IL-10 or 3) competition between helminth-induced polyclonal IgE and aeroallergen-specific IgE for mast cells IgE receptors, 4) production of antibodies against allergens in the IgG4 subclass, a Th2-dependent Ig isotype that is not associated with clinical allergic manifestations.

1.5 *Schistosomes and the Hygiene Hypothesis.*

Schistosoma parasites have been postulated to ameliorate atopic disorders in humans (Yazdanbakhsh *et al.*, 2002). Schistosomes are characteristically associated with being potent inducers of Th2 cytokine responses including eosinophilia and IgE responses as described in Section 1.2 (Fallon, 2000). Despite type 2 responses being considered

necessary for the development of allergies, it is argued that the presence of schistosome infections in humans reduces the propensity to develop allergies in infected populations (Yazdanbakhsh *et al.*, 2001). This inverse relationship between schistosome infections and allergies was recently formally demonstrated by van den Biggelaar and colleagues; *S. haematobium*-infected school children in The Gabon had a lower prevalence of skin reactivity to house dust mite than those free of this infection (van den Biggelaar *et al.*, 2001). Therefore the chronic downregulation of the immune system during helminth infections evokes a regulatory environment that may impart protection from allergies (Maizels and Yazdanbakhsh, 2003).

It is important to note that a study by Araujo found no significant difference in total and *Dermatophagoides pteronyssinus* antigen (Derp) -1-specific production of IgE between *S. mansoni*-infected patients with low and those with high parasite loads (Araujo *et al.*, 1996). However, in another study, this group found an inverse association between the skin prick test reactivity and infection with *S. mansoni* and showed that the severity of asthma is reduced in subjects living in an endemic area of schistosome infection and this helminth-induced protection is probably IL-10-mediated (Araujo *et al.*, 2000).

1.6 Thesis objectives

There is contradictory data to support or disprove the role of schistosomes in the Hygiene Hypothesis. Therefore, the objective of this thesis was to examine, using mouse models, the role of the helminth parasite *Schistosoma mansoni* in the Hygiene Hypothesis with respect to allergies. As part of this objective I intended to dissect the underlying immunological mechanism of schistosome modulation.

I had 3 AIMS to address this overall objective:

- 1 Evaluate the role of 2 types of *Schistosoma mansoni* infection in a severe form of allergic hypersensitivity reaction, i.e. in anaphylaxis.
- 2 Extend studies in AIM 1 to address the role of *S. mansoni* in a less severe allergic reaction in airways hyper-responsiveness.
- 3 As B cells have an influence in *S. mansoni* protection in 1 and 2 above, the protective role of B cells was assessed.

Chapter 2

Materials and Methods

2.1 Mice.

BALB/c or C57BL/6 mice were initially purchased from Harlan, (Bicester, U.K.), and were bred in-house. Outbred male or female CD1 strain mice (Bioresources TCD) were used for egg and worm production. CBA/*xid* and wild-type (CBA/Ca) mice were obtained from Harlan (Horst, The Netherlands) and bred in-house; age-matched male mice were used in experiments. IL-13 transgenic (Tg) (Emson *et al.*, 1998) IL-4 (Kuhn *et al.*, 1991) IL-5 (Fallon *et al.*, 2002), IL-9 (Townsend *et al.*, 2000) and IL-13 (McKenzie *et al.*, 1998) deficient ($^{-/-}$) mice and combined IL-4, -5, -9 and -13 $^{-/-}$ mice (Fallon *et al.*, 2002) were bred in-house. IL-10 $^{-/-}$ breeding pairs (Makela *et al.*, 2000), on a C57BL/6 background, and CD1d $^{-/-}$ (Nieuwenhuis *et al.*, 2002) mice on a BALB/c background were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) and bred in-house. 4GET mice, that express IL-4 linked with enhanced green fluorescent protein (Mohrs *et al.*, 2001), on a BALB/c background were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) and bred in-house.

Mice were maintained on an irradiated and OVA-free diet (Harlan, UK) with food and water supplied *ad libitum*. Sentinel mice were routinely screened (Harlan, UK; Surrey Diagnostics Ltd, UK) to ensure the Specific Pathogen-Free status of the unit where breeding of mice occurred, and also the unit where mice were housed while undergoing experimental procedures. Experimental mice were housed in individually ventilated and filtered cages under positive pressure (Techniplast, UK).

2.2 Parasitology.

2.2.1 *Schistosoma mansoni* infection.

The *Schistosoma mansoni* life-cycle is routinely maintained in the Department of Biochemistry (Trinity College, Dublin). A Puerto Rican strain of *S. mansoni* was maintained by passage in CD1 mice and albino *Biomphalaria glabrata* snails.

2.2.2 *Snail maintenance.*

Snails were kept in plastic containers filled with artificial aquarium water (Lepple water; see Appendix I). The snails were kept in incubators with an ambient temperature of 28°C with a 12 hour day/night cycle. Snail tanks were aerated using air pumps (RENA, France). The snails were fed daily with commercially available fish food (TetraPond). The water in the plastic containers was changed weekly. For this the snails were collected in a sieve, the water was removed from the containers, the containers were scrubbed and cleaned with tap water and rinsed in Lepple water before return of the snails. Polystyrene rafts were placed in breeding tanks and the hermaphroditic snails laid their eggs on the underside of rafts. Rafts with the eggs attached could then be transferred to separate tanks for hatching.

2.2.3 *Parasite infection.*

To prepare miracidia for infection of snails, the liver of a *S. mansoni*-infected mouse was removed 49 days post infection. The tissue surrounding the egg was gently disrupted by homogenizing the liver in a blender (Philips) in double-strength saline (1.8% NaCl; 2x-saline). The use of 2x-saline stops the eggs from hatching. Lepple water was

added to the resulting solution to start egg hatching. The solution was placed in a conical flask wrapped in aluminium foil with the neck exposed to a light source. Following hatching of the eggs the phototropic miracidia swim towards the light source. After 30 minutes a sample of miracidia was collected from the top of the solution using a glass pipette and the number of miracidia per ml was calculated. The snails were infected in individual wells of a 24 well plate in 1 ml Lepple water with 4-6 miracidium for a mixed male and female (bisexual) infections. The snails were infected for approx. 6 hours or overnight. Infected snails were placed in fresh plastic containers in a dark incubator to allow asexual multiplication and development of the cercariae. After 28 days the snails were transferred into the individual wells of a 24 well plate with 1 ml Lepple water and placed under a lamp for 90 minutes. Wells were examined under a microscope to ascertain those snails that were infected and shedding cercariae. Cercariae were stained for counting using Lugol's iodine (Appendix I) to kill the cercariae and for visualisation under the microscope. Any snails not shedding cercariae, i.e. uninfected, were removed and killed.

2.2.3.1 Unisexual snail infection.

To prepare unisexual (male-only) snail infections individual snails were exposed to a single miracidium. After 28 days snails were checked for infection as described above. The shell of infected snails were colour-marked with nail varnish for identification and cercariae from each individual snail were collected. The sex of cercariae was determined as described previously (Smith *et al.*, 2004). In brief, genomic DNA was isolated from cercariae (Section 2.10.1) and PCR was performed (Section 2.10.3) to establish the presence or absence of two female specific sequences, named W1 and W2 (Grevelding, 1999). Sequence from a gene, Protein disulphide isomerase (PDI), present in both male

and female cercariae was also amplified as a control. Cercariae were determined to be male by the absence of the W1 and W2 gene sequences (**Fig. 2.1**). Mice were then infected with cercariae obtained from individual snails shedding only these pre-determined male cercariae.

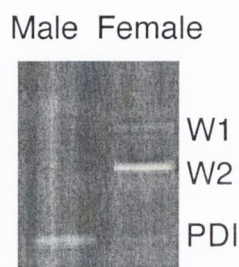


Fig. 2.1. *Sexing of cercariae by PCR.* Snails infected by a single miracidium were shed, and cercariae DNA isolated. Sequences from female-specific W1 and W2 sequences were amplified by PCR, with PDI as an internal control.

2.2.4 *Schistosoma mansoni* infection of mice.

2.2.4.1 Experimental infection of mice.

Mice were infected percutaneously (pc) with *S. mansoni* cercariae. Six to eight week-old mice were anaesthetized with sodium pentobarbitone (Sagatal; 6 mg/kg). Their abdomen was shaved and moistened with Lepple water. The mice were secured on a specially adapted infection board and a metal ring was secured with tape over their shaved abdomen. For experimental infections mice were infected pc with 40 male (as tested by PCR) cercariae to establish unisexual (worm-only) infections and 40 male and female cercariae for worm + egg infections. The mice were kept on the infection board for 25 minutes after which time they were placed on a heated mat and monitored until they

recovered. Upon full recovery all laboratory animals were returned to their cages. Mice were killed after 8 weeks for acute infection and worm + egg-infected mice were killed at 12-16 weeks in chronic infections. In experimental worm + egg infection liver and guts were frozen for tissue egg counts (Section 2.2.5) at -20°C.

In some experiments worm-infected mice were treated, five weeks post infection, with the schistosomicidal drug Praziquantel (Sigma; 100 mg/kg orally for 5 consecutive days) to kill the worms. Mice were checked by portal perfusion (described below in Section 2.2.4.2) at 8 weeks later for the presence of worms.

2.2.4.2. Infection of mice for life-cycle maintenance and antigen production.

Male or female outbred CD1 strain mice were infected with 350 cercariae pc as described above to establish worm + egg infections for antigen production and schistosome life-cycle maintenance. Mice were injected subcutaneously (sc) with hydrocortisone (see Appendix I) 35 days post infection; this suppresses the hosts' immune response to the parasite eggs and reduces inflammation around the egg granuloma which aids egg extraction from tissue. Also mice were placed on a fibre-free diet (Special Diet Services, Essex, UK) on day 47 to reduce intestinal detritus to aid egg extraction from the gut tissue. The animals were killed 49 days post-infection by an overdose of sodium pentobarbitone injected intraperitoneal (ip). The thoracic and peritoneal cavities were exposed and the hepatic portal vein was cut, and mice were portally-perfused (Smithers and Terry, 1965) through the left heart with 20 ml perfusion media (see Appendix I) and worms were collected in a glass beaker. The liver and the intestines were removed for isolation of *S. mansoni* eggs.

2.2.5 *Schistosoma* antigen production.

2.2.5.1 Isolation of eggs.

Isolation of eggs from tissue was as described (Fallon *et al.*, 1998). The liver and guts were processed separately and placed in 2x-saline solution. Trypsin (Sigma) was added at a concentration of 0.05 g per liver or gut. The liver egg suspension was homogenized (five second bursts at low speed), using a blender to mechanically break up the tissue surrounding the egg. The homogenate was incubated at 37°C for 3-4 hours on a rotating tray and then filtered through a series of sieves (500 µm-150 µm pore sizes) to recover the eggs. The suspension was spun at 2000 rpm for 2 minutes at 4°C and the egg pellet was resuspended in 2X-saline and spun up to 1500 rpm for 1 minute. The eggs were washed through two smaller sieves and eggs were caught in the 32 µm sieve. Liver eggs were used for egg antigen preparations (Section 2.2.4.2) and for *in vivo* studies and gut eggs were used for proteomics and ELISA assays. Eggs were stored in liquid nitrogen before use. The eggs were washed several times in endotoxin-free Dulbecco's PBS (DPBS; Sigma) prior to use. Endotoxin levels in all preparations were tested before use as described in Section 2.11. SEA batches had <1EU per mg of protein (Chromogenic LAL, Biowhittaker, MD).

2.2.5.2 Preparation of soluble egg antigen.

Soluble egg antigen (SEA) was prepared from liver eggs isolated as described above. SEA was prepared in ice-cold PBS using eggs which were disrupted under liquid nitrogen with a percussion mortar that had also been pre-chilled with liquid nitrogen. Samples were examined periodically under a microscope until there were no intact eggs left. 5 ml DPBS was added to the resulting egg homogenate/paste that was then sonicated

for three 1 min bursts and centrifuged at 10,000 rpm for 1 hr at 4°C in a Sorval centrifuge. The supernatant was further centrifuged for 15 min at 10,000 rpm at 4°C in a microcentrifuge. The supernatant was repeatedly clarified using a microcentrifuge at 4°C and filtered through a 0.45 and 0.22 micron syringe filter. SEA batches were tested for endotoxin contamination (Section 2.11) and confirmed to have <1EU per mg of protein (Chromogenic LAL, Biowhittaker, MD). The protein content was determined by a BCA protein estimation kit (Section 2.12). SEA was aliquoted and stored at -20°C.

2.2.5.3 Preparation of adult worm antigens.

Worms were obtained by portal perfusion (Smithers and Terry, 1965), as described in Section 2.2.4.2, and were washed and frozen in liquid nitrogen in pellet form. A mortar and pestle was cooled with liquid nitrogen. Frozen worm pellets were then crushed under liquid nitrogen until a very fine paste was formed. The homogenized worms were then allowed to thaw. The worm homogenate was then transferred to a Sorval tube rinsing the mortar and pestle with PBS. Worms were then spun for an hour 10,000rpm in a Sorval centrifuge. Supernatant was removed, transferred to Falcon tubes and respun. This cleaning process was repeated until no further pellets were visible. Supernatant containing soluble worm antigen (AW) was then filtered through a 0.45 and 0.22 micron filters and frozen in aliquots at -20°C. All batches of worm antigens were tested for endotoxin contamination (Section 2.11) and confirmed to have <1EU per mg of protein (Chromogenic LAL, Biowhittaker, MD).

2.2.6 Tissue egg counts.

Frozen livers and guts, which had been stored at -20°C, from worm + egg-infected animals (Section 2.2.3.1) were thawed. Tissue was digested overnight at 37°C with continuous shaking in 20 ml 4% potassium hydroxide solution. Three 50 µl aliquots per sample were assayed microscopically and egg counts were calculated as the average number of eggs over the three. If variation between the aliquots was more than 10%, egg numbers from a fourth, or more, aliquot were counted. Tissue egg counts were expressed as total eggs per tissue.

2.2.7 Histology.

Livers and intestines from mice were taken and fixed in formal saline (Appendix I) for histology. Samples were sent to Cambridge University, UK, or Dept. Physiology, TCD, where they were paraffin-embedded for sectioning. Tissue sections were stained with hematoxylin and eosin (H+E), eosinophil infiltration was counted on Giemsa-stained sections, and Martius Scarlet Blue for collagen. Pulmonary collagen was quantified by differential staining, and is expressed as mg collagen per mg lung protein, as described (Fallon and Dunne, 1999). Goblet cells were counted on Periodic Acid-Schiff (PAS)-stained lung sections using an arbitrary scoring system (Grunig *et al.*, 1998; Townsend *et al.*, 2000). PAS-stained goblet cells in airway epithelium were measured double-blind using a numerical scoring system (0: <5 % goblet cells; 1: 5 to 25 %; 2: 25 to 50 %; 3: 50 to 75 %; 4: >75 %). The sum of airway scores from each lung was divided by the number of airways examined, 20-50 airways per mouse, and expressed as mucus cell score in arbitrary units.

2.3 mAbs and reagents.

See appendices II and III for a list of antibodies and reagent used in flow cytometry and cell activation/stimulation studies.

2.3.1 *Depletion studies antibodies and reagents.*

Depleting mAbs against IL-10 receptor (clone 1B1.3a), CD25 (clone PC61 5.3), TGF- β (clone 1D11.16.8), anti-B220 (clone TB146) and F4/80 (clone F4/80) were purchased from American Type Culture Collection (ATCC). The anti-CD4 (clone YTS 191), anti-CD8 (YTS 169) and anti-mouse Thy-1 depleting antibody (YTS 148.3.2) hybridomas were kindly provided by Prof. Anne Cooke (University of Cambridge) under a Materials Transfer Agreement (MTA) from Prof. Herman Waldman (University of Oxford). Murine IgE (clone IGELa2) and murine IgG1 (clone B7.11) were raised against 2,4,6-trinitrophenyl (TNP) were also from ATCC. Anti-CD1d (clone 1B1) was a kind gift from Prof. Mitch Kronenberg (under an MTA from La Jolla Institute for Allergy and Immunology, San Diego, USA). Anti-mouse IgM was obtained from Jackson Laboratories. Control rat IgG was from Sigma. In all *in vivo* depletion experiments the efficacy of treatment was initially tested, with doses of 0.1 - 0.5 mg of mAb administered per mouse. In all experiment where depleting mAb were used the efficacy of depletion was confirmed.

Macrophages were depleted by the treatment of mice with liposomes containing dichloromethylene bisphosphonate (clodronate-liposomes), prepared as described (Van Rooijen and Sanders, 1994). Clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany. Macrophages were depleted by two injections of clodronate- or control PBS-liposomes; 24 hours before challenge each mouse received 0.2 mls ip and an additional 0.1 mls was injected iv 4-6 hours before elicitation of anaphylaxis. Efficacy of

macrophage depletion was confirmed by flow cytometry analysis of F4/80⁺Mac-1⁺ spleen populations.

2.3.2 Hybridoma culturing and mAb production.

The above hybridoma cell lines were cultured in standard conditions in RPMI-1640 5-10% FCS with routine passages, when confluent, every 3-5 days. Cells were maintained in T-175 cell culture flasks (Greiner) or roller-bottles (Costar). Supernatants were harvested and stored frozen at -20°C.

To purify mAbs, supernatants were first precipitated in 50% ammonium sulfate (313 g/litre supernatant; Sigma) while stirring overnight at 4°C. The ammonium sulfate supernatant suspensions were centrifuged at 7000 rpm for 30 min in a Sorval centrifuge at 4°C. The pellets were re-suspended in a 10X volume of DPBS (Sigma; pH 7.2; Endotoxin-free). The suspension was placed in dialysis tubing (molecular weight cut-off at 12,400 kDa; Sigma). Samples were extensively dialysed at 4°C in 5 litre volumes of DPBS with three changes over 48 hours. Dialysed suspensions were applied to a Protein G separation column (Sigma) and absorbance monitored using a BioLogic LP (Bio Rad). Bound antibody was eluted with a solution of 100 mM glycine (pH 2.5) and solution was neutralized (pH 7) by addition of Tris base (Sigma). Eluted mAb was extensively dialysed against DPBS using dialysis cassettes with a molecular weight cut-off at 10,000 kDa (Pierce). mAbs were tested for correct sized bands on an SDS-PAGE gel, functional binding to appropriate target cells (flow cytometry) or efficacy in mediating specific *in vivo* depletion. mAb protein content was quantified before use using a BCA assay (Pierce; Section 2.12). All purified antibodies were tested for endotoxin levels by LAL assay (Section 2.11) and confirmed to have < 0.5 EU/ml.

2.4 Induction of anaphylaxis.

2.4.1 Preparation of Penicillin V-protein conjugates.

Penicillin V- (Pen V-) OVA and Pen V-BSA conjugates were prepared as described (Park *et al.*, 1997). In brief, 20 mg of BSA or OVA (both from Sigma) were prepared in 5 ml of 50 mM Veronal buffer (Appendix I; pH 8.5). These BSA or OVA suspensions were added to 5 ml of Veronal buffer containing 100 mg of Pen V. The suspension was stirred overnight at 37°C. The reaction mixture was centrifuged and the supernatant was dialysed, using dialysis cassettes with a molecular weight cut-off at 10,000 kDa (Pierce). The supernatant was extensively dialysed for 7 days against 2 litres of DPBS (pH 7.2) with daily changes of DPBS. All batches of antigen were tested for endotoxin contamination and were confirmed to have <1EU per mg of protein (Chromogenic LAL, Biowhittaker, MD), see Section 2.11. Protein was measured using the BCA assay (Section 2.12) and aliquots of the dialysed supernatant were stored at -20°C.

2.4.2 Induction of active systemic anaphylaxis.

For the induction of Pen V-induced active systemic anaphylaxis, mice were first primed by ip injection of 500 µg of Pen V-OVA (Section 2.4.1) plus 2×10^9 *B. pertussis* (Evans, Liverpool, UK; Wako Pure Chemical Industries Ltd, Japan) and 1 mg of Imject alum (Pierce). Anaphylaxis was elicited 14 days later by iv challenge with 100 µg Pen V-BSA (Section 2.4.1) (Choi *et al.*, 1998). The rectal temperatures of mice were measured with a Digital Thermocouple Thermometer linked to a murine rectal probe (size: 3/4" long, 0.028" diameter, tip diameter 0.065"; Physitemp Instruments, Clifton, NJ). Temperatures were measured just before challenge, then every 10 min for the next 60 min. Activity

levels were assessed at the same time the rectal temperatures were obtained. Clinical signs of anaphylaxis, adapted from Park *et al.*, 1997, were assessed with a 0-4 scoring system as outlined below (Table 2.1). Mice were sacrificed after 60 minutes by terminal anaesthesia and blood and plasma were obtained for further analysis.

2.4.3 Induction of PAF-mediated anaphylaxis.

Commercial platelet-activating factor (PAF; Calbiochem) was used to induce systemic fatal anaphylaxis (Haribabu *et al.*, 2000). A dose of 200 µg/kg PAF is fatal within 10 minutes following injection of uninfected mice. Mice received an iv injection of 100 or 200 µg/kg body weight of PAF in 0.25% BSA/DPBS. Core body temperature was recorded every 5 minutes.

Score	Symptoms of anaphylaxis
0	Normal activity
1	Mild shock including slow movement, dyspnea, ruffling of fur
2	Moderate shock including prostration, sluggish gait and slight activity after prodding
3	Severe shock including complete paresis, no activity following prodding
4	Death

Table 2.1. *Clinical scores of anaphylaxis.* Mice were scored according to the above parameters on susceptibility to the induction of anaphylaxis in response to treatment. Adapted from Park *et al.*, 1997.

2.4.4 Induction of passive systemic anaphylaxis (PSA).

2.4.4.1 PSA induced using sera from Pen V-immunised mice.

PSA with immune mouse sera was induced as described previously (Park *et al.*, 1997). Uninfected or worm-infected BALB/c mice were immunized with 500 µg of Pen V-OVA (Section 2.4.1) plus 2×10^9 *Bordetella pertussis* and 1.0 mg of alum. 14 days later blood was recovered from immunized mice and the anti-Pen V serum extracted and pooled (n=10). To induce PSA, naïve mice were injected ip with 0.4 ml of anti-Pen V serum, from uninfected or worm-infected mice, three times at 10 minute intervals. After 24 hours, anaphylaxis was induced by iv challenge with 100 µg Pen V-BSA (Section 2.4.1).

2.4.4.2 mAb-induced PSA.

Passive IgE or IgG1 mAb-mediated systemic anaphylaxis was induced as described previously (Ujike *et al.*, 1999). Mouse anti-TNP IgG1 (200 µg) or anti-TNP IgE (20 µg) mAbs were administered through iv tail vein injections in volumes of 200 µl/mouse. 30 min after injection of anti-TNP IgG1 or 24 h after injection of anti-TNP-IgE, mice were injected with 1.0 mg iv TNP₄-OVA (Biosearch Technologies) in PBS. Control mice received OVA in PBS. The concentration of IgG1 and IgE mAbs used for passive sensitization and the amount of TNP-OVA used for challenge was determined based on preliminary dose-response experiments. Rectal temperatures were recorded following challenge, as described in Section 2.2.

2.5 Airways hyper-responsiveness (AHR).

2.5.1 Preparation of OVA.

Mice were sensitized with OVA and airways challenged with OVA to induce

pulmonary allergic inflammation and AHR. Ovalbumin (OVA fraction V: Sigma) that is available commercially has ~1.5 - 3 EU per mg protein. Endotoxin itself has previously been shown to alter the development AHR (Delayre-Orthez *et al.*, 2004) and would thus modify Penh values and immunological responses in mice under investigation in these studies. However, despite published claims to the contrary, we have so far found that it is impossible to rigorously completely remove endotoxin from OVA preparations, with even the use of polymyxin B, to inactivate lipopolysaccharide (LPS) in preparations, being inappropriate. My observations on OVA and endotoxin contamination have recently being substantiated by others (Watanabe *et al.*, 2003). Furthermore, polymyxin B itself has been shown to have direct effects on cells as demonstrated in a study on human dendritic cells (DC) whereby it induced partial maturation of the DC (Valentinis *et al.*, 2005).

Therefore every effort was made to maintain sterile procedures and use “clean-up” techniques to minimize the contamination in all OVA preparations. OVA was prepared using endotoxin-free reagents and all glassware and equipment were endotoxin decontaminated (NaOH), see Section 2.11. Endotoxin was partially removed with Detoxi-gel endotoxin removing gel columns (Pierce) used according to the manufacturer’s instructions (Section 2.11). Following endotoxin removal, OVA was extensively dialysed against sterile Dulbecco’s PBS (Sigma) before endotoxin measurement and BCA protein estimation (see Section 2.12). After endotoxin removal, samples were screened for endotoxin as described in Section 2.11. Endotoxin levels were measured according to the manufacturer’s instructions and expressed as EU/mg protein. OVA for use in experiments had < 0.5 (range 0.186 - 0.447) Endotoxin units/mg. Samples with endotoxin levels greater than 1EU/mg were not used in experiments and were discarded.

2.5.2 Antigen sensitisation and challenge.

Mice were sensitized with ovalbumin (OVA; prepared as described in 2.5.1) as described by Hamelmann *et al* (1997). On days 1 and 14 mice received an intraperitoneal injection of 100 μ l of a sensitization solution containing 20 μ g OVA and 2 mg Imject™ Alum (Pierce). Mice received a 20 minute aerosol challenge with 1% OVA in DPBS from days 28-30 (**Fig. 2.2**). Control mice received an aerosol of PBS. On day 32, airway responsiveness of mice was assessed by barometric WBP (see Section 2.5.3) in response to Methacholine challenge. 24 hours later mice were sacrificed by terminal anaesthesia and samples taken for further immunological analysis.

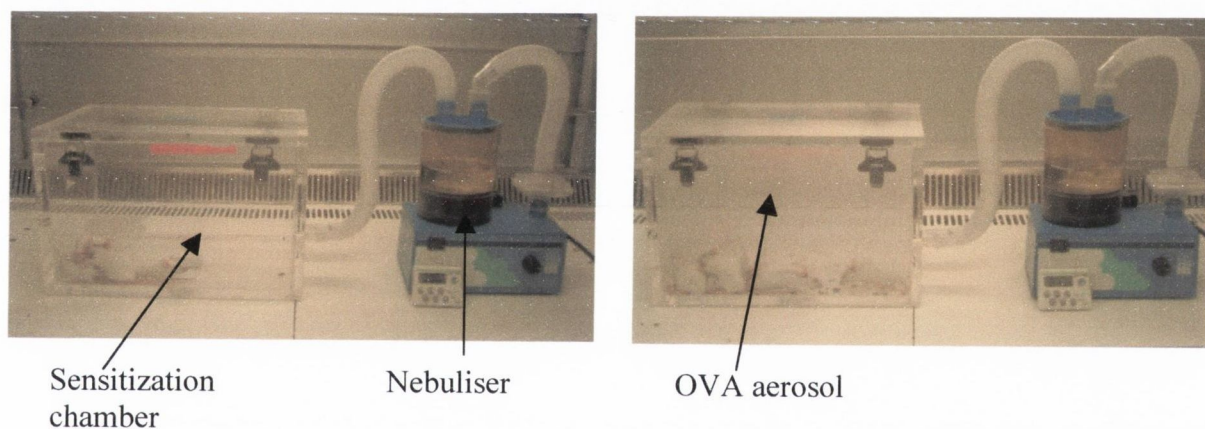


Figure 2.2. OVA sensitisation of mice. Mice are placed in the sensitization chamber (left photograph) and then challenged via the airways for 20 mins with 1% OVA (right photograph).

2.5.3 Whole-body Plethysmograph (WBP).

WBP (**Fig. 2.4**) is a functional *in vivo* measurement that is used to analyse airway reactivity (breathing) of unrestrained and conscious mice. In this model the extent of

airway reactivity of individual mice is quantified as enhanced pause (Penh; (Hamelmann *et al.*, 1997)). Penh is an empirical parameter that reflects changes in box flow waveform from both inspiration and expiration and combines it with the comparison of early and late expiratory box flow (Pause). Spontaneously breathing and non-anaesthetised mice were placed in the plethysmograph chamber (**Fig. 2.3A**) and left to adjust to the environment for 8 minutes (**Fig. 2.3B**). Baseline readings were recorded for 3 minutes before initiation to obtain baseline values, $Penh_{base}$. PBS aerosol was administered prior to Methacholine (β -acetyl methycholine chloride: Mch; Sigma) challenge and recordings were taken for 3 minutes after every aerosol ($Penh_{PBS}$). Methacholine aerosol was administered (**Fig. 2.3C**) in increasing, serial 2 fold concentrations from 3.125 - 50 mg/ml ($Penh_{Mch}$). Data are presented as the average Penh for each reading period. Penh is calculated as the folds increase of $Penh_{Mch}$ compared to $Penh_{PBS}$ for each Mch concentration. All data presented are representative of at least 2 separate experiments (n=8-10).

2.5.4 Airway conductance and dynamic compliance studies.

Pulmonary conductance (GL) and compliance (Cdyn) in response to Mch were measured as described previously (Martin *et al.*, 1988). In brief, mice were assessed on day 31 after OVA sensitization and challenge using the same protocol as outlined above (Section 2.5.2). Mice were anaesthetized with 105 mg/kg pentobarbitone (Sagatal; Rhone Merieux Ltd, Essex). Mice were tracheostomized and then mechanically ventilated (EMKA Technologies, France) for airway assessment. A two-way connector was attached to the tracheal cannulation tube to allow inspiration and expiration via the ventilator. Breaths were stabilized at a rate of 60 breaths per min and the tidal volume was fixed at 250 μ l. Increasing doses of Mch (3.125 - 25 mg/kg) were administered ip and readings were taken

continuously for 4 min after injection. Maximum pulmonary resistance (GL is the inverse of resistance) and compliance (Cdyn) values were taken after each dose for statistical analysis to express changes in these functional parameters.

2.5.5 Lung immunology.

2.5.5.1 Bronchoalveolar lavage and cytopins.

Bronchoalveolar lavage (BAL) fluids were collected on ice after lavaging lungs twice with 0.8 ml of ice-cold PBS via tracheal cannula. Supernatant was removed for measurement of cytokines by ELISA (Section 2.9). BAL cells were pelleted for immunofluorescence staining for surface marker expression (van Rijt *et al.*, 2004) or cytopins. Cytopins were prepared with 5×10^4 bronchoalveolar cells using a cytocentrifuge (Shandon, Pittsburgh, PA). Cytopins were fixed and stained using Diff-Quik Wright-Geimsa Stain kit (Thermo Shandon). Differential cell counts were determined by counting a minimum of 100 cells/slide using morphologic criteria.

2.5.5.2 Lung processing.

Whole lungs were removed from the mice and either snap-frozen, placed in formal saline for histology or digested for FACS analysis. Lungs were digested in 1mg/ml collagenase D (Roche Diagnostics GmbH) in RPMI for 30 mins at 37°C with shaking. Lung digests were then filtered through 100 µm Falcon Cell Strainers (Beckton Dickinson) followed by three washes and further filtering through 40 µm Falcon Cell Strainer. In some experiments the left lobes of the lungs were removed for assessment of tissue cytokines and chemokines (IL-4, -5, -13, -10, IFN-γ and eotaxin; Section 2.9).

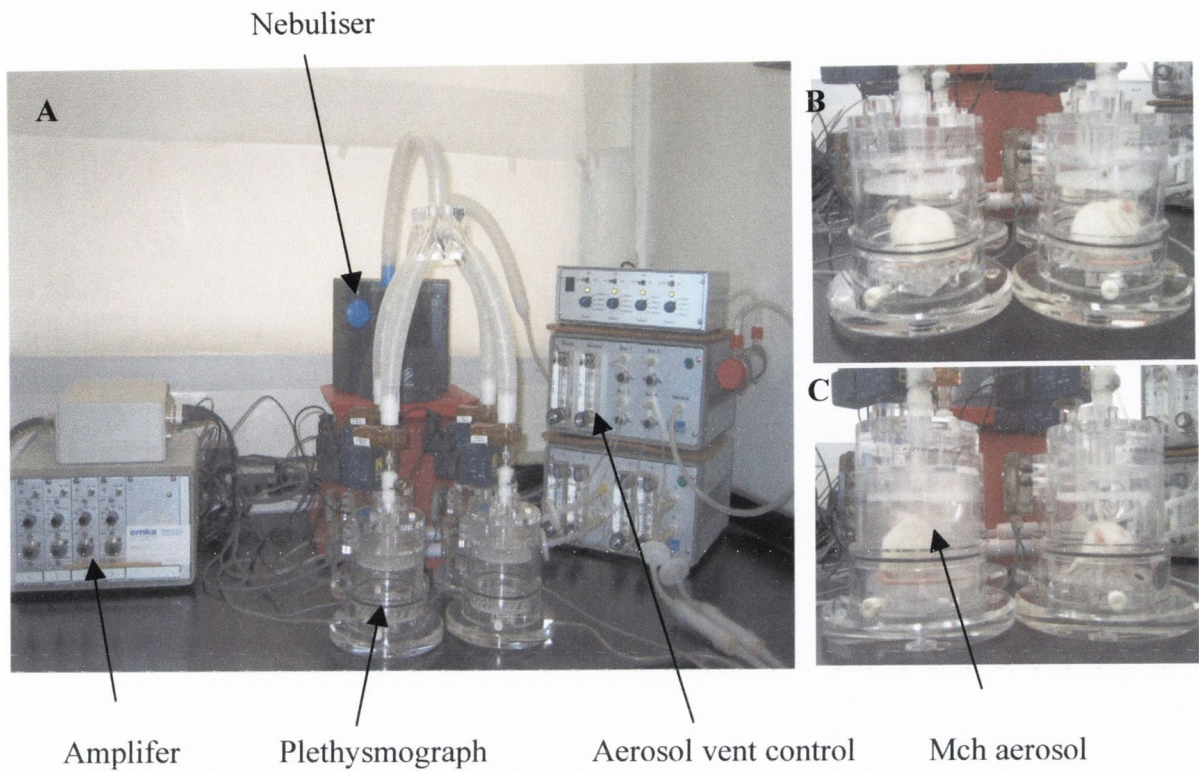
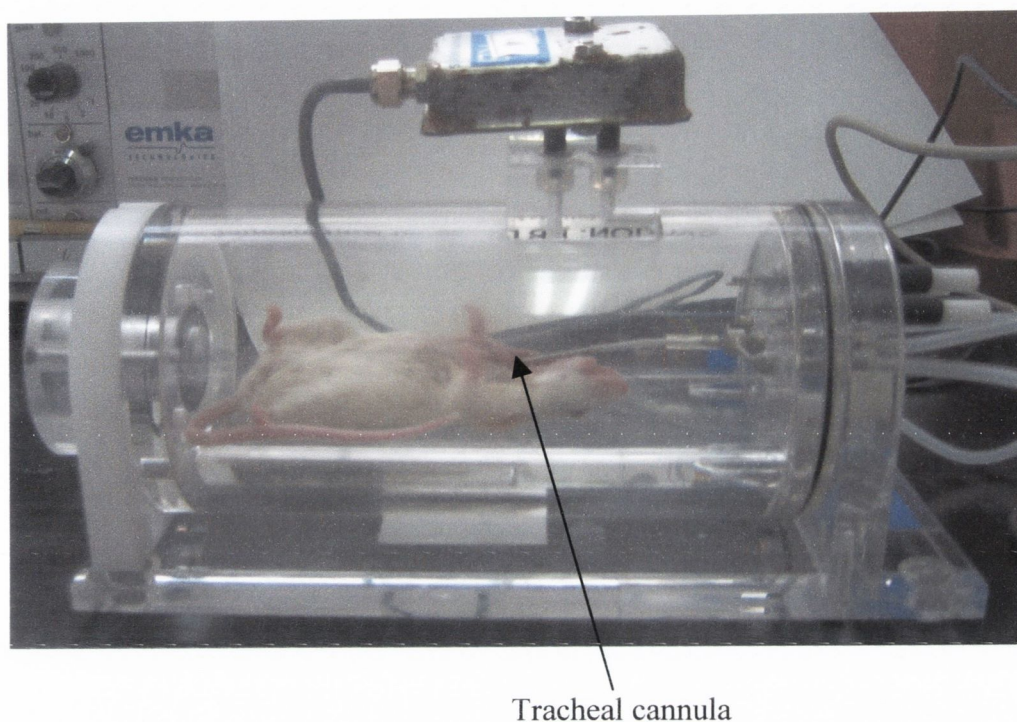


Figure 2.3. *Sample setup for unrestrained whole body plethysmograph.* Mice are placed (B) in the plethysmograph chambers and challenged with PBS or methacholine (Mch) via aerosol (C) as described in 2.5.3. The results are collected and amplified (A) for analysis by computer using IOX software. All hardware and software described for WBP was purchased from EMKA Technologies, Paris, France.



Tracheal cannula

Figure 2.4. *Sample set-up for resistance and compliance dynamic studies.* Mice are anaesthetised and a tracheal cannula inserted (arrow) to maintain breathing via a mechanical ventilator. The mouse is then placed in the plethysmograph chamber and challenged with PBS or methacholine (Mch) as described in 2.5.4. The results are collected and amplified as described for WBP for analysis by computer using IOX software. All hardware and software was purchased from EMKA Technologies, Paris, France.

2.6 Cell culture and immunological analysis.

2.6.1 *Sample processing.*

Mice were killed at the indicated times. Spleen, lymph nodes or tissue were aseptically removed. Single cell suspensions were prepared from spleens or lymph nodes by passing through 70 μm sieves (Falcon). Erythrocytes from cell suspensions were depleted by lysis with 0.85% ammonium chloride solution. Viability of the isolated cells was determined by dye exclusion using trypan blue (Sigma) staining to be >95%. For *in vitro* experiments, cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco BRL; endotoxin levels were <0.04 EU/mg protein) supplemented with 10% (v/v) heat-inactivated (56°C for 30 min) foetal calf serum (FCS) (Labtech, UK; endotoxin levels were <0.1 EU/mg protein), 100 mM L-glutamine, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin (Sigma).

2.6.2 *Isolation of blood granulocytes.*

For isolation of blood cells, mice were exsanguinated by cardiac puncture. Blood was collected on ice over citrate-phosphate-dextrose solution (Sigma) and centrifuged immediately at 2000 g for 5 min at 4°C. Plasma was removed and red blood cells were lysed twice in ammonium chloride solution. Cells were then counted and resuspended in FACS buffer for cytometric analysis (Section 2.8).

2.6.3 *Isolation of peritoneal macrophages/cells.*

Mice were killed by CO₂ or cervical dislocation and the peritoneal cavities of the mice exposed. The peritoneum was lifted up with a forceps and a 19G needle was carefully inserted into the cavity and 10 mls of ice-cold RPMI (1 % FCS) injected. Keeping the

needle in place with the forceps, the peritoneal cavity was gently massaged to allow the cavity contents to be flushed and the media now containing the cells was removed gently. Peritoneal exudates cells (PEC) were centrifuged and washed twice in complete media before being counted. PEC were plated at 2×10^6 cells in 1 ml complete media on a 12 well flat-bottomed plate and allowed to adhere for 2 hours at 37°C . The non-adherent cells were gently removed by pipetting and cells were counted before adding fresh media containing various antigens or mitogens for cell restimulation (Section 2.6.6).

2.6.4 *Isolation of bone marrow macrophages (BMM ϕ).*

Mice were killed and hind legs removed for isolation of bone marrow. Excess muscle was removed from the legs holding the end of the bone with forceps and using a scissors. The femurs and tibia were severed at the joint and the bone cavity flushed through with 5 ml wash media using a syringe with attached 27G needle. The bone appears white upon removal of the marrow. The cells were flushed through a $70\ \mu\text{m}$ cell strainer and then centrifuged for 10 min at 500 g, room temperature. The pellet was resuspended in 5 ml of wash media. 5 ml of Histopaque (Sigma) was placed in a 15 ml Falcon tube and 5 ml of bone marrow was overlaid. The cells were centrifuged for 20 min at 500 g with the brake off. The mononuclear cells were removed at the interface and washed twice for 10 min at 500 g, 4°C . The cells were then resuspended at 5×10^6 cells/ml in complete media and aliquoted at 3×10^7 cells per T-25 flask. The volume was made up to 10ml total volume with recombinant mouse Granulocyte macrophage - colony stimulating factor (GM-CSF; R&D Systems) added at 10 ng/ml. Cells were then incubated at 37°C , 5% CO_2 humidified incubator for 24 hr. The non-adherent cells were then transferred to a T-75 flask and 10 ml complete media was added supplemented with 10 ng/ml GM-CSF. The cells were

incubated for another 4 days and then a further 10 ml complete media (plus 10 ng/ml GM-CSF) was added. The cells were incubated at 37°C for a further 3 days after which the macrophages were harvested.

The culture supernatant in the T-75 was discarded and the remaining adherent cells were washed with 15 ml of PBS. 1 mg/ml dispase (5 ml/flask) was prewarmed and filter sterilized and then incubated in the flask for 5 mins at 37°C. The side of the flask was gently tapped to loosen adherent cells and the remaining attached cells were removed by uni-directional scraping with a rubber scraper. 10mls of complete media was added to the flask to harvest detached cells. Cells were then washed twice before counting for further assaying and analysis. The general FACS profile of these putative BMM ϕ is >95% positive for the general macrophage marker F4/80 and of these F4/80⁺ cells there are 98% Mac-1⁺. There is < 1% dendritic cell contamination as assessed by CD11c staining.

2.6.5 In vitro worm culture.

Adult worms were cultured as described (Mangan *et al.*, 2004; Smith *et al.*, 2004). Adult schistosome worms were obtained 8 weeks post-infection by portal perfusion as described (Smithers and Terry, 1965) (Section 2.2.4.2). Male and female worms were separated by anatomical examination using a binocular microscope. Any motionless, inert worms were removed using a Pasteur pipette and discarded. Mature, healthy male worms were segregated and washed three times in medium without FCS and three times in medium with FCS. For transwell experiments, spleen cells were plated at a density of 1×10^7 per well in a 12-well TranswellTM plate (Costar; pore size 3 μ m) in 2 ml RPMI with Hepes (25 mM). In the upper filter portion of each TranswellTM 15 male worms were placed. Plates were incubated for 48 hrs at 37°C, after which time the worms were

removed and cells were washed several times. The viability of cells was checked via dye exclusion. Worm-modulated spleen cells were used for isolation of B cells (Section 2.6.7.1) or immunofluorescence staining (Section 2.8).

2.6.6 *In vitro stimulated cell cytokine production.*

Peritoneal exudate cells (PEC), BMM ϕ , spleen or draining lymph node cells were plated at 5×10^6 /ml for *in vitro* activation in a 24 well plate at 37°C for 24 and 72 hours. Cells were unstimulated (media) or stimulated with soluble anti-CD3 ϵ (BD PharMingen; 0.5 μ g/ml), plate-bound anti-CD3 ϵ mAb with anti-CD28 (4 μ g/ml), IL-4 (RnD; 20 ng/ml) LPS (*E coli* 0128:B12 or *Salmonella minnesota*: Sigma; 100 μ g/ml), OVA (Sigma; 50 - 500 μ g/ml), OVA peptide (Cambridge Research Biochem UK; 0.1-100 ng/ml) or worm antigens (Section 2.2.4; 20 μ g/ml). Plates were pre-coated with anti-CD3 mAb at 10 μ g/ml for 2 hours at 37°C and then washed twice in sterile DPBS before addition of cells. Supernatants were harvested after 24 and 72 hours and cytokine levels (IL-2, IL-4, IL-5, IL-6, IL-13, IFN- γ , IL-10 and TGF- β) were measured by ELISA (see Section 2.9.1).

2.6.7 *Isolation of B cells for in vivo and ex vivo studies.*

Magnetic sorting of B cells.

B cells were purified from splenic populations by magnetic sorting using an autoMACS system (Miltenyi Biotec, Germany). Splenic populations were incubated with anti-CD43⁺ magnetic microbeads followed by capture and negative depletion of resting B cells with autoMACS separation columns. Single cell suspensions of splenocytes were prepared as described in Section 2.6.1. Cells were washed and re-suspended in DPBS with 0.5% FCS (DPBS/FCS) at a volume of 90 μ l containing 10^7 total cells. 10 μ l of anti-

CD43⁺ microbeads was added per 10^7 total cells and mixed gently before incubation at 6°C for 15 minutes. This mixture of cells and beads was then washed in 20 ml DPBS/FCS and centrifuged at 300 g for 10 minutes. The pellet was resuspended in 500 μ l DPBS/FCS per 10^8 total cells followed by capture with autoMACS separation columns. The conditions for separation were optimized before use and the programme separating fractions with highest B cell purity was used. The POSSELS programme separates a highly pure population of untouched B cells from the CD43⁺ splenic populations by negative depletion with consequent elution as the non-labelled negative fraction. The resultant B cell populations were routinely >95% pure (**Fig. 2.5**), as assessed by FACS staining (Section 2.8.1) for CD19 expression, and > 99% viable. For transfer of purified cells, 0.5 - 1.0 $\times 10^7$ purified B cells were injected ip to individual mice 24 hours prior to elicitation of anaphylaxis.

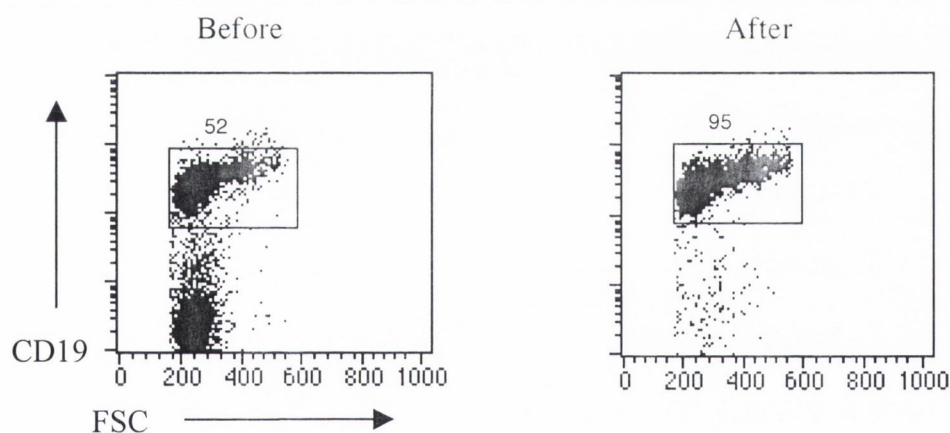


Figure 2.5. Purity of CD19⁺ B cell population after Automacs isolation. CD19⁺ B cells were purified by CD43⁺ Microbead separation on Automacs cell separator and cells were found to be 95% pure as determined by FACS analysis. Representative images are shown, the gate was set on an isotype control mAb.

2.7 *In vitro* proliferation assays.

Single cell suspensions of spleen cells, draining lymph nodes or purified B cell populations were cultured in 96 well round-bottomed microtiter plates (Greiner) at 2×10^5 cells per well in RPMI with various stimuli for 72 hours at 37°C. Cells were activated with 0.5 µg/ml anti-CD3 mAb, IL-2 (20 ng/ml), CD28 (4 µg/ml), LPS (1 µg/ml) AW (20 µg/ml) or SEA (20 µg/ml). All samples were prepared in triplicate. The cells were then co-incubated for 72 hours and pulsed with 1 µCi/well [3 H] thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) for the last 16 hours of proliferation. Samples were collected using a cell harvester (TOMTEC) and radioactivity measured with a Microbeta liquid scintillation system (1450 Microbeta plus liquid scintillator counter; Wallac).

2.8 Flow cytometric analysis of T and B cell populations.

Surface marker expression and intracellular cytokine phenotyping was assessed by immunofluorescent staining of T and B cell populations as described (Fallon *et al.*, 1998; Mauri *et al.*, 2003). Staining was carried out using the Tri-, FITC- or PE-conjugated mAbs (Pharmingen/Caltag) (See Appendix II for list of antibodies). Data were analysed using CellQuestTM software (version 3.13; Immunocytometry systems). Quadrants and gates were drawn based on isotype-control Ig staining and were plotted on logarithmic scales.

2.8.1 *Surface staining.*

Surface marker expression and intracellular cytokine phenotyping of cells was assessed by flow cytometry using a FACScanTM flow cytometer (Becton-Dickinson, San Jose, CA) using a previously described method (Fallon *et al.*, 1998). Cells to be analysed

were resuspended and washed in ice-cold FACS buffer (PBS, 2% FCS and 0.05% sodium azide) and plated on a 96 well plate at $2-5 \times 10^6$. Cells were stained with surface antibodies for 30 min on ice at recommended concentrations and then washed three times in FACS buffer. The gates were set up according to unstained cells and using appropriate fluorochrome (single stained cells) and isotype control mAbs. The appropriate compensation was determined and gates were set using forward and side scatter gates to exclude non-viable cells. See Appendix II for a full list of fluorochrome-conjugated antibodies used for surface staining.

For immunofluorescence staining in the lung the various cell populations in digests were identified as described (van Rijt *et al.*, 2004). Lungs cells were first gated on CD19, CD4 and CD8 vs forward side scatter (FSC). Lymphocytes were identified as FSC^{lo} , SSC^{lo} $CD19^+$, $CD4^+$, $CD8^+$. Eosinophils distinguished as SSC^{hi} , $CD19^-$, $CD4^-$, $CD8^-$, $CCR3^+$ non-autofluorescent granulocytes that stained positive for CCR3. Dendritic cells were characterized as SSC^{lo} , $CD11c^+$. Alveolar macrophages were characterized as $CD19^-$, $CD4^-$, $CD8^-$ mononuclear cells that were highly autofluorescent, and confirmed to be $F4/80^+$.

PE-conjugated $CD1d^+$ -tetramers loaded with α -galactosylceramide (GalCer) (Matsuda *et al.*, 2000) were used for evaluating $CD1d^+$ cells in lung digests. Cells were firstly blocked with Fc-block (CD16/32 at 1/100 dilution) and neutravidin (Molecular Probes, US; $0.4 \mu g / 10^6$ cells), for 15 mins at room temperature to block non-specific binding. Cells were then incubated with PE-loaded or unloaded tetramers (1/300 dilution) for 30 min on ice. Following staining, cells were washed and surface stained with CD3, DX5, CD19 or CD11c as described above. Cells stained with unloaded tetramers were used to set appropriate gates for $CD1d$ -tetramer $^+$ cells.

All gates and quadrants were set on the appropriate isotype control. Each stain had

it's own individual isotype and individual mice also had their own isotype control for each stain. Gates were set on these isotype such that there was less than 1% fluorescent cells in the fluorochrome gate. This gate was then copied to all positive stained samples.

2.8.2 Intracellular cytokine staining.

See Appendix II for a full list of fluorochrome-conjugated antibodies used for intracellular cytokine staining.

For intracellular cytokine staining, splenocytes or purified cells were stimulated for 6 hrs prior to surface staining (Fallon *et al.*, 1998; Mauri *et al.*, 2003). Cells were stimulated with PMA (10 ng/ml; Sigma) and ionomycin (100 ng/ml; Sigma) for 6 hours and Brefeldin A (10 mg/ml; Sigma) was added after 2 hours. Following surface staining, cells were fixed and permeabilised using a Fix & Perm^R Cell Permeabilization Kit (Caltag Laboratories) with cytokine staining antibodies added upon permeabilisation. Upon analysis gates were set on the appropriate isotype control as outlined in 2.8.1.

For Foxp3, intracellular immunofluorescence staining was detected according to manufacturers (eBioscience) protocol. Cells were blocked with Fc block (1/100 dilution) prior to staining. Antibodies for Foxp3 staining are described in Appendix II and were used at 1/100 dilution / 10^6 cells. Fix/Perm buffers were supplied with Foxp3 Staining set (eBioscience).

2.9 Enzyme Linked Immunosorbent Assay.

General ELISA protocol

Checkerboards titrations of coating and detecting antibodies or antigens were performed to determine optimum concentrations. 96 well flat-bottomed microtitre high-

binding plates (Nunc; Biosciences, Ireland) were coated with capture mAbs (0.1-2 µg/ml) in coating buffer (PBS) at 50 µl/well and were left overnight in a wet-box at 4°C. Following coating overnight, the plates were allowed to reach room temperature. They were then washed three times in ELISA wash buffer (PBS/T) and blotted dry. Plates were blocked for 2 hours, 200 µl/well, at room temperature in blocking buffer (1% BSA/PBS). Plates were then washed three times in ELISA wash buffer and blotted dry. The cytokine standards were prepared in incubation buffer (see Appendix I). Standards were added in duplicate in a two-fold serial dilution from the top to the bottom of the plate leaving the last two wells blank (incubation buffer only). Test samples were added to duplicate wells according to predetermined plate format. Plates were then left overnight at 4°C in a wet box. The following day, plates were washed five times in ELISA wash buffer as described and 50 µl of biotinylated anti-cytokine mAb (0.1 - 2 µg/ml) was then added to each well for another 2 hours at room temperature. Plates were then washed five times in ELISA wash buffer. Horseradish peroxidase-conjugated Streptavidin (BD Pharmingen, UK) was added at 1/1000 dilution, 50 µl per well, and plates incubated at room temperature for thirty minutes. ELISA plates were washed three times and blotted dry followed by addition of 50 µl/well of substrate. The reaction development substrate o-Phenylenediamine (OPD; Sigma) was prepared by dissolving a 5 mg OPD tablet in 12.5 ml phosphate-citrate buffer (pH 5.0) with 9 µl of 30% hydrogen peroxide (H₂O₂) in a 50 ml Falcon tube that was kept in the dark. Plates were incubated in the dark for 10 minutes. The reaction was stopped with 25 µl/well of 1M H₂SO₄ and the plates were placed in the microplate spectrophotometer (Versa_{Max} Tunable Microplate Reader, Molecular Devices, CA) and absorbance was read at a wavelength of 492 nm. The levels of cytokines were quantified by interpolation from a standard curve using Soft Max Pro 3.0 software. Data was

transferred to Microsoft Excel 2.11 for analysis.

2.9.1 Cytokine ELISA.

Sandwich cytokine ELISA were performed to quantify levels of specific cytokines (Eotaxin, IL-2, IL-4, IL-5, IL-6, IL-13, IFN- γ , IL-10, TNF- α and TGF- β) in the supernatants from lung tissue homogenates, BAL fluid and *in vitro* cell stimulation cultures. A commercial kit (Promega, UK) was used to measure total TGF- β (acidified samples) according to the manufacturers instructions. Reagents for detection of IL-2, -4, -5, -6 and IL-13 and were from BD Pharmingen, UK. TNF- α , IFN- γ and IL-10 DuoSet ELISA development kits and reagents were purchased from R&D Systems, UK. Eotaxin detection reagents were also purchased from R&D systems.

2.9.2 Total IgE ELISA.

Total IgE was assayed using sandwich ELISA. 96 well plates (medium binding) were coated with monoclonal anti-IgE capture antibody (BD Pharmingen). Diluted serum samples in 0.1% BSA PBS were added and incubated overnight. Bound IgE was detected using monoclonal biotin-labelled anti-IgE detection antibody and strepavidin HRP (R&D Systems). Concentrations were calculated using purified IgE as standards.

2.9.3 Antigen Specific ELISA (IgE, IgG, IgG1, IgG2a).

Schistosome egg antigen specific Ig isotype responses were measured using ELISA by coating 96-well (medium binding; Nunc) plates with SEA (100 μ l) at 2.5 μ g/ml in carbonate buffer (Appendix I) at 4⁰C overnight in a wet box. Plates were washed

three times in PBS Tween (0.05%) and blocked with 200 μ l 1% marvel for 2 hours at room temperature. Diluted serum samples (100 μ l) were added at starting dilutions of 1 in 100 for IgG and IgG1, and 1 in 25 for IgG2a and IgE. Samples were plated as doubling serial dilutions in 0.1% BSA across the plate. The plates were incubated at room temperature for 2 hrs for IgG or overnight at 4⁰C for IgE. Plates were washed three times in PBS Tween (0.05%). Bound Ig was detected using HRP conjugated anti-Ig isotype detection antibodies (Zymed) at 1/1000 dilution and plates were incubated for 2hrs at RT. For IgE detection, 100 μ l of rat anti-mouse IgE biotin at 1/2000 dilution in incubation buffer was added to each well for 2 hours followed by 3 washes. Streptavidin (100 μ l) was then added at 1/1000 dilution for 30 min. All plates were washed three times and OPD substrate (one 10 mg tablet dissolved in 25 ml phosphate citrate buffer with 18 μ l H₂O₂) was then added. The reaction was stopped with 50 μ l of H₂SO₄ and plates read at 490nm.

2.9.4 Antibody and other ELISA.

OVA-specific Abs, were detected by direct ELISA (Fallon and Dunne, 1999). Mouse mast cell protease 1 (mMCP-1) was measured in sera using a commercial kit according to the manufacturers instructions (Moredun Scientific Ltd., Scotland). Histamine was measured in plasma samples using a commercial kit (IBL, Germany).

Pen V-specific serum IgG1 and IgE levels were detected by direct ELISA (Park *et al.*, 1997). In brief, 96 well microtiter plates were coated with 100 μ l of Pen V-BSA (10 μ g/ml in carbonate buffer; pH 9.6) overnight at 4⁰C. A serial dilution of sera was added to plates that were then incubated at 4⁰C overnight. Detection was with peroxidase conjugated IgG1 (1/1000, 2 hours; Zymed) or biotin labeled anti-IgE (2.5 μ g/ml, 2 hours; Pharmingen) followed by peroxidase- conjugated Streptavidin (1/1000, 30 min;

Pharmingen) before substrate development using o-Phenylenediamine.

2.9.5 *Measurement of Eosinophil Peroxidase (EPO) and Myeloperoxidase (MPO).*

Eosinophil peroxidase and myeloperoxidase levels were determined based on a method previously described (Schneider and Issekutz, 1996). Each mouse lung/gut tissue was homogenized using an Ultra-Turrax homogeniser (IRK-WERKE, Germany) in 800 μ l of homogenization buffer (2% FCS, 0.5% hexadecyltrimethylammonium bromide (CTAB; Sigma) with a designated protein probe (CAT. No 361-118). The homogenate was microcentrifuged at 13,000 rpm for 15mins at 4°C. The supernatant was aliquoted and the tissue extract was stored for protein estimation cytokine analysis and EPO and MPO assay. The protein content of tissue extracts was determined by BCA protein estimation kit (see Section 2.15). The tissue extracts were diluted 1 in 10 in EPO buffer, or MPO dilution buffer (See Appendix I). Aliquots of 50 μ l were pipetted in duplicate onto a 96 well medium binding plate. EPO substrate solution and MPO substrate solution were added 50 μ l/well (See Appendix I). The reactions ran for a minimum of 30 s (EPO) or 2 min (MPO) until colour was observed. 50 μ l/well of EPO stop solution and MPO stop solution was added to each well (See Appendix I). The OD_{490nm} (EPO) or OD_{450nm} (MPO) was determined using a microplate reader. Homogenisation buffer was used as a blank for each assay. The enzyme activities of the samples were calculated relative to the amount of protein present in tissue. MPO or EPO activity was expressed as ng per mg of protein.

2.10 Molecular and biochemical techniques.

2.10.1 *Isolation of genomic DNA for genotyping.*

Tail snips (0.1-0.5 cm) from mice to be genotyped or cercariae shed from snails

for sexing were taken to isolate genomic DNA for PCR. DNA was isolated using the Wizard SV Genomic DNA Isolation Kit (Promega) according to manufacturer's protocol for "Isolation of genomic DNA from animal tissue using a microcentrifuge". Pieces of tail were cut in half or approx 200 cercariae were digested overnight at 55°C in digestion mix containing proteinase K (10 ng/ml; Invitrogen). For DNA isolation from tails, undigested tail and hair was spun down and DNA was isolated from the supernatant by elution twice in 100 µl volumes of nuclease free water. 5 µl (150 ng) of tail genomic DNA or 10 ng of cercarial DNA was used per PCR reaction. If the DNA collected was not used straight away, it was stored at -20°C to prevent degradation.

2.10.2 *RNA isolation.*

Total RNA was isolated using TRI Reagent (Sigma). 1 ml TRI Reagent was added to 5-10 X 10⁶ cells, and pipetted up and down several times to form a homogenous lysate. When isolating RNA from tissue, the tissue was homogenized in 1ml TRI Reagent using a homogeniser. Samples were left to stand for 5 minutes at RT. The mixture was centrifuged at 12,000 g for 15 mins at 4°C. The upper aqueous phase (containing RNA) was transferred to fresh centrifuge tube and 0.5 ml isopropanol added. Sample was left to stand for 10 mins at RT, before spinning at 12,000 g for 10 mins at 4°C. The resultant RNA pellet was washed by adding 1 ml of 75% ethanol. Samples were vortexed and then centrifuged at 7,500 g for 5 mins at 4°C. Supernatant was removed and RNA pellets left 10-15 mins to air-dry, before resuspension in 50 µl of RNase-free H₂O (Sigma).

2.10.3 *Complementary strand DNA (cDNA) synthesis.*

RNA was quantified using a spectrophotometer (Eppendorf,

BioPhotometer). cDNA was synthesized from 2 µg RNA using oligo dT-primed reverse transcription. RNA was added to 1 X First-strand Buffer, 0.5 µg oligo dT, 10 U Ribonuclease Inhibitor, 100 U SuperScript™ II reverse transcriptase (all Invitrogen), 1 mM dioxynucleotide triphosphates (dNTP's), 10 mM dTT (both Promega) in RNase-free H₂O) to a total volume of 20 µl in thin walled PCR tubes (Eppendorf). Tubes were incubated in a thermocycler (DYAD™) and cDNA synthesis performed (Table 2.2).

Step	Action	Temp (°C)	Time (minutes)
1	Binding	25	10
2	Extension	42	60
3	Enzyme deactivation	95	5
4	Cooling	4	

Table 2.2. *cDNA synthesis reaction.* The above reaction conditions were applied to synthesis of cDNA from RNA samples.

2.10.4 Polymerase Chain Reaction.

Reverse-transcription PCR (RT-PCR) was used to amplify cDNA. A ‘master-mix’ was prepared with a final concentration of 1X reaction buffer, 0.25 mM dNTPs, 2.5 mM MgCl₂, 1 U taq Polymerase (Invitrogen) and 10 pmol forward and reverse primers. 1 µl of cDNA was placed in a thin-walled PCR tube (Eppendorf) and the master mix added to each tube. RNase-free water (Sigma) was added to a total volume of 25 µl. Tubes were placed in a thermocycler (DYAD™) and PCR cycles started (Table 2.3). Cycle number and

annealing temperature varied depending on primer pairs.

Step	Action	Temp (°C)	Time
	Denaturing	95	5 min
1	Denaturing	95	45 sec
2	Annealing	54-65	45 sec
3	Extension	72	30-60 sec
	Repeat from step 2 for 25 – 35 cycles		
4	Extension	72	10 min
	Cooling	4	

Table 2.3. *PCR reaction.* The general protocol for PCR reactions were as described here.

2.10.5 *Agarose gel electrophoresis.*

A 2% agarose gel was prepared by mixing 1.2 g agarose (Sigma) in Tris-borate EDTA (TBE; see Appendix I) and boiling the mixture for approximately 3 mins to dissolve the agarose. After cooling 5 µl of 20 mg/ml ethidium bromide (EtBr) was added in order to visualise the bands under UV light. The gel was then poured into gel casting cassette (Biorad) and allowed to set for 30 mins. The gel was then placed in a gel box (Bio-Rad) containing 1X TBE buffer.

6X loading dye (Invitrogen) was added to PCR products and DNA ladder

(Size: 100 Bp - 1000 Bp; Invitrogen) and 10 μ l of each sample was run on a gel. Samples were electrophoresed in 1X TBE buffer at 100 volts using a power-pack (Bio-rad) for approximately 40 mins. DNA bands on the gel were visualized under ultraviolet light using Sygene computer software and photographed using a Sony Syngene digital graphic printer.

The images were analysed and densitometry performed with Syngene image analysis software. The relative intensity of β -actin cDNA was used as a comparison and water was used as negative control for each cDNA sample.

2.11 Endotoxin analysis.

In an effort to limit endotoxin contamination all antigens or mAbs were prepared using endotoxin-free reagents and all glassware and equipment were endotoxin decontaminated (NaOH). All glassware to be used was first washed in a 0.5 M NaOH solution for 30 minutes followed by baking at 200°C for 1 hour, and rinsing in endotoxin-free water. All samples from the endotoxin columns were then extensively dialysed, using dialysis cassettes (Pierce), against endotoxin-free DPBS (Sigma) and sterile-filtered (0.45 μ m and 0.22 μ m filters). The levels of LPS present in purified antibodies or antigen preparations were assessed using the *Limulus amoebocyte* lysate (LAL) assay, obtained commercially from Biowhittaker (UK). Endotoxin levels were measured according to the manufacturer's instructions and semi-quantified by a simple equation to give endotoxin amounts in EU/mg protein. Any reagent that had contamination of >1EU/mg was not used.

2.12 Protein quantification using the BCA assay.

Protein estimation of samples was performed using the BCA Protein Assay Reagent Kit (Pierce) according to the manufacturer's instructions. Standards were prepared from a

stock solution of 2 mg/ml BSA in dH₂O and ranged from 125 µg/ml to 2000 µg/ml. Standards and samples were added in triplicate to a 96 well microtiter plate in a volume of 10 µl and 200 µl of the BCA solution was then added to each well. The plate was then incubated at 37°C for 30 min to allow the reaction to develop and the absorbance was measured at 562 nm. The concentration of protein in the supernatants or tissue samples was then calculated from the standard curve generated with the BSA standards.

2.13 Statistical analysis.

GraphPad Prism and GraphPad Instat software was used to analyse the data. Student's t-test and ANOVA were used to determine statistical significance between groups. Differences were considered significant when $P < 0.05$.

Chapter 3

*Helminth infection protects mice from
anaphylaxis via IL-10 producing B cells.*

3.1 Introduction

Anaphylaxis is an acute, life-threatening, allergic hypersensitivity reaction that can cause fatalities following exposure to allergenic antigens in atopic individuals. Allergic reactions, as described in Chapter 1, are characterized by an over-production of Th2 cytokines contributing to elevated levels of IgE and eosinophilia. In mice, the Th2 cytokines IL-4 and IL-13 are critical for the induction of the inflammatory response that protects against nematode parasites and IL-4 and IL-13 also cause much of the immunopathology in mouse models of asthma. IL-4 and IL-13 have also been shown to exacerbate anaphylaxis through a type 2 IL-4R-, Stat-6-dependent mechanism that potentiates the effects of vasoactive mediators (Strait *et al.*, 2003).

Previously Fallon *et al* demonstrated experimentally, using mice over expressing IL-13 (IL-13Tg) in a lymphoid specific manner (Emson *et al.*, 1998), that *S. mansoni* egg antigens induce fatal anaphylaxis of pre-sensitized IL-13Tg mice (Fallon *et al.*, 2001). *S. mansoni* infection of mice stimulates marked Th2 immune responses (Fallon, 2000; Pearce *et al.*, 1991) that can be lethal in certain Th2 polarised transgenic mice, including IL-9Tg mice (Fallon *et al.*, 2000) or IL-10/IL-12-deficient mice (Hoffmann *et al.*, 2000). Therefore as schistosome infection is a potent Th2 inducer and IL-13Tg mice are highly susceptible to egg-induced anaphylaxis it was anticipated that these mice would succumb to anaphylaxis during conventional schistosome infection. Unexpectedly, despite IL-13Tg mice being predisposed to anaphylaxis caused by schistosome eggs (Fallon *et al.*, 2001), these mice had no anaphylactic symptoms during an acute or chronic *S. mansoni* infection.

Therefore, in this chapter I evaluated if schistosome infection renders mice less susceptible to anaphylaxis using experimental murine models of systemic anaphylaxis. I have identified that the worm stage of *S. mansoni* infection modulates mice so they are completely refractory to active or passive systemic anaphylaxis. I demonstrate that *S. mansoni* worms upregulate IL-10 producing B cells that can protect against anaphylaxis.

Chapter objectives:

1. To investigate the influence of *Schistosoma mansoni* infection of mice on susceptibility to anaphylaxis.
2. To investigate the differences of worm + egg versus worm-infection in anaphylaxis.
3. To determine the mechanism of protection by the worm.

3.2 Results

3.2.1 *S. mansoni* infection prevents anaphylaxis.

To dissect the underlying mechanism of anti-allergic protective responses induced by schistosome infection, an anaphylaxis model using Pen V as the allergen was used (Chapter 2; Section 2.4.1). To investigate the relative roles of schistosome worms versus eggs in anaphylaxis mice were infected with an *S. mansoni* conventional mixed male and female infection, where eggs are laid (worm + egg infection), or with a male only-infection where there are no eggs (worm-only infection). In the Pen V anaphylaxis model, intravenous Pen V challenge of pre-sensitized uninfected BALB/c mice leads to approx. 10-20% of animals dying and all mice having a marked drop in core body temperature within 10 minutes of challenge, (**Fig. 3.1A**). The drop in temperature in uninfected mice is associated with immediate clinical signs of anaphylactic shock, as shown on 10 minute severity scores, that subside as the mice recover (**Fig. 3.1B**). Uninfected mice undergoing Pen V-induced anaphylaxis have an attendant mast cell degranulation, as determined by detection of substantial elevations in circulating levels of mast cell protease-1 (mMCP-1; **Fig. 3.2A**) and histamine (**Fig. 3.2B**). In contrast, BALB/c mice that had been infected with *S. mansoni* worm + egg or worm-only infections were both protected from anaphylaxis with no mortalities, reduced severity of symptoms and negligible release of mast cell by-products (**Fig. 3.1, 3.2**). Mice with worm-only infections were completely resistant to anaphylaxis, as determined by all parameters tested. In contrast, although worm + egg-infected mice were protected they did develop some symptoms of anaphylaxis, and had detectable, but limited (45 ± 20 pg/ml), release of histamine. These results indicate that the worm stage of *S. mansoni* infection may elicit protection from Pen V-induced anaphylaxis.

3.2.2. *Schistosome worm-infected mice are not resistant to PAF.*

During anaphylaxis the initial shock symptoms are the result of mast cells and basophils releasing various chemical mediators, including platelet-activating factor (PAF). Injection of PAF directly into mice induces rapid fatal anaphylaxis without the need for allergen-antibody-induced mast cell degranulation (Mencia-Huerta *et al.*, 1983). Uninfected and worm-infected mice were treated with PAF to address if schistosome worm-infected mice were susceptible to direct PAF-induced anaphylaxis. At two different doses of PAF both uninfected and worm-infected BALB/c mice were equally susceptible, with all animals dead within 5- 10 minutes of treatment (**Fig. 3.3**). Worm-infected mice are therefore fully susceptible to direct chemical-induced anaphylaxis induced by PAF.

3.2.3 *Schistosome worm-infected mice are resistant to passive mAb mediated anaphylaxis.*

The protective mechanism by which chronic helminth infection seemingly protects from allergic disease is unknown. The IgE blocking hypothesis was an initial paradigm that suggested the high concentration of helminth-induced polyclonal IgE would block the binding to, and subsequent crosslinking of, FcεR with allergen-specific IgE. I have also found that both *S. mansoni* worm- and worm + egg-infected mice had elevated levels of total IgE compared with uninfected mice (**Fig. 3.4**). Therefore the reduced susceptibility of worm-infected mice to Pen V-induced anaphylaxis could be due to the infection modifying production of anti-Pen V Ab, suppressing antigen specific IgE production or helminth induced non-specific polyclonal IgE saturating mast cells and basophils thus preventing degranulation (Godfrey and Gradidge, 1976; Hagel *et al.*, 1993). However, ELISA for Pen

V-specific Ab responses of sera from Pen V-OVA immunized uninfected and worm-infected mice demonstrated that both groups had comparable titers of Pen V-specific IgE (**Fig. 3.5A**) and IgG1 (**Fig. 3.5B**).

As I had shown that worm-infected mice produce anti-Pen V antibodies (**Fig. 3.5**) I tested if they were functional. Uninfected and worm-infected mice were immunized with Pen V-OVA and the sera from mice were pooled for transfer to naïve animals. Following subsequent Pen V-BSA challenge there were comparable levels of anaphylaxis in both groups that received immune sera (**Fig. 3.6**). Indeed mice passively-transferred with sera from Pen V-immunized worm-infected mice had a greater relative initial drop in core temperature than mice that received sera from uninfected mice (**Fig. 3.6**). Therefore following Pen V immunization worm-infected mice were indeed capable of producing functional antibodies to Pen V that could induce anaphylaxis.

3.2.4 Schistosome worm-infected mice are resistant to TNP-mAb-mediated passive-systemic anaphylaxis.

In order to further confirm that resistance of worm-infected mice to anaphylaxis was not due to alterations in the functionality of Ab, a model of passive systemic anaphylaxis was used. Uninfected and worm-infected mice were passively-transferred monoclonal IgG1 or IgE antibodies, specific for TNP, prior to eliciting anaphylaxis by challenge with TNP-OVA. Uninfected mice treated with TNP-IgE were susceptible to TNP-induced anaphylaxis, which evoked a rapid and severe drop in temperature in uninfected mice within 5 minutes of challenge (**Fig. 3.7A**). In contrast, the temperature of worm-infected mice was not altered by TNP-IgE sensitization. Similarly, worm-infected mice were not affected by anaphylaxis induced by TNP-IgG1 mAb treatment, with a drop in body

temperature following challenge (**Fig. 3.7B**). Therefore, a schistosome worm infection completely protects mice from both IgG1- and IgE-mediated passive systemic anaphylaxis.

To summarise, worm-infected mice are protected from Pen V-induced anaphylaxis (**Diagram 3.1**). However, these mice are fully susceptible to the end-stage mediators of anaphylaxis as these mice die upon treatment with PAF (**Fig. 3.3**). They produce allergen-specific Abs at comparable titres to uninfected mice (**Fig. 3.5**). These Abs are functional as they are capable of inducing anaphylaxis upon transfer to uninfected mice (**Fig. 3.6**). Worm-infection also protects mice from direct IgE or IgG1 mediated anaphylaxis which bypasses the development of a sensitization response (**Fig. 3.7**). Thus worm-infected mice are effectively sensitized to allergen, the cells involved in anaphylaxis are there and the mice are susceptible to the downstream mediators of anaphylaxis. This thus indicates that protection from anaphylaxis in worm-infected mice is an upstream event. This protection may involve upstream direct modulation of the interaction and/or cross-linkage of allergen-specific Abs on the mast cells, basophils or macrophages that mediate elicitation of the anaphylactic response.

3.2.5 *S. mansoni* worm-infected mice have elevated Th2 cytokines and regulatory cytokines in comparison to uninfected mice.

As anaphylaxis is mediated by type 2 cytokines (Strait *et al.*, 2002), the production of cytokines in the spleens of uninfected and worm-infected mice was analysed. IFN- γ and IL-4 production were measured as representative type 1 and type 2 cytokines, respectively (**Fig. 3.8**). None of the splenocytes spontaneously produced either cytokine when incubated with media-alone. T cell (anti-CD3) and APC (LPS) induced production of IFN-

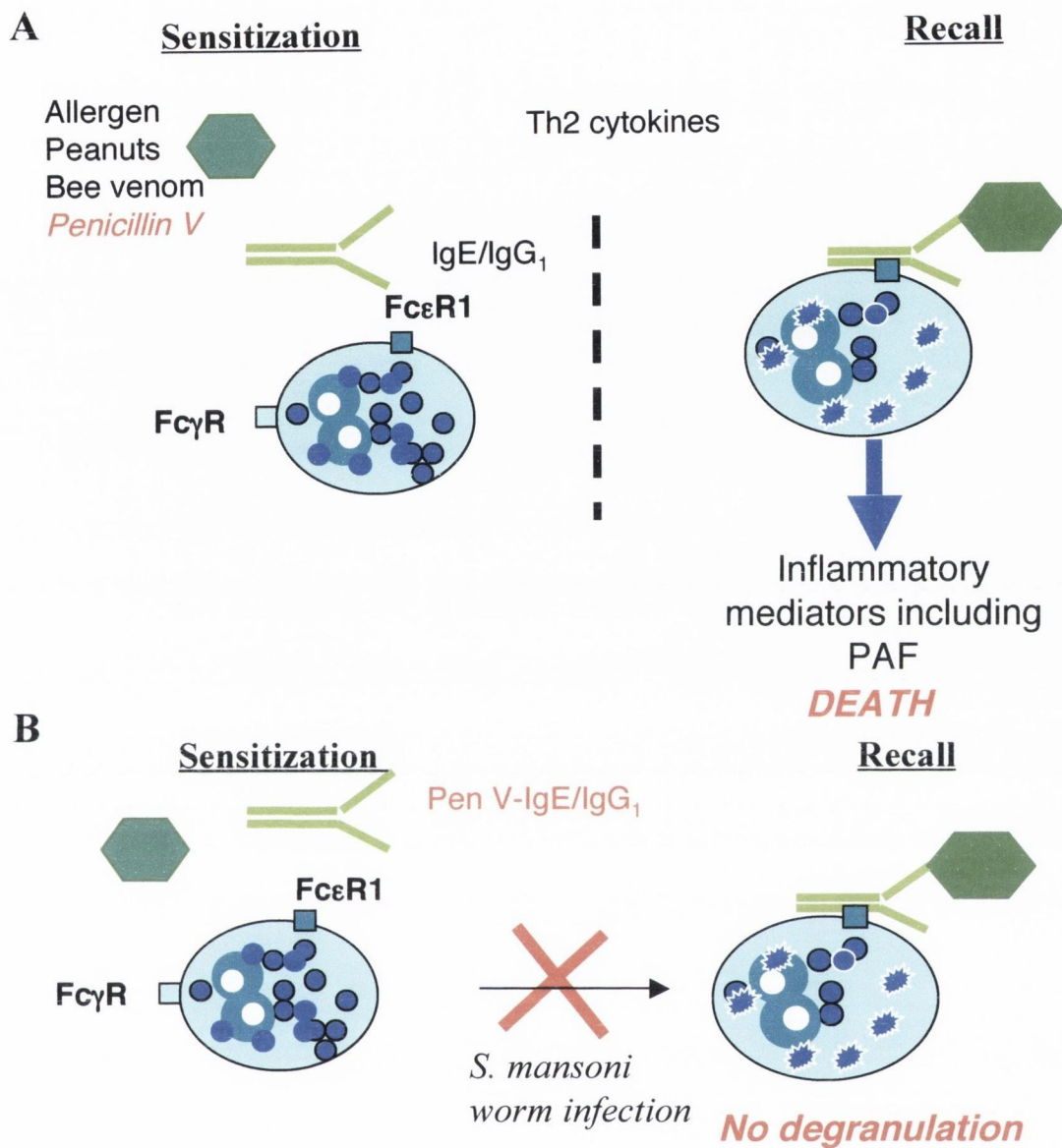


Diagram 3.1. Mechanism of mast cell/basophil degranulation during anaphylaxis. A, conventional response in anaphylaxis. B, response with worm infection.

γ demonstrated no differences in cytokine levels between groups (**Fig. 3.8**). In contrast, following direct T cell activation with anti-CD3 mAb there was marked production of IL-4 by cells from worm-infected mice in comparison to limited IL-4 release by cells from uninfected mice (**Fig. 3.8**), indicating a type 2 cytokine bias.

Infection with helminth parasites also induces an upregulation in the levels of the regulatory cytokines IL-10 and TGF- β (King *et al.*, 1996; Xu *et al.*, 2003). Indeed, cells from schistosome worm-infected mice did have elevated levels of both cytokines compared to cells from uninfected mice (**Fig. 3.8**). The presence of TGF- β in unstimulated (media) cells is due to the presence of FCS in the culture media which stimulates TGF- β production from cells. Although there is TGF- β in media of uninfected spleen cells, the worm-infected spleen cells still have increased spontaneous production compared with uninfected cells. Therefore, in addition to the known increase in IL-4 production by cells from worm-infected mice (Grzych *et al.*, 1991) the levels of the regulatory cytokines IL-10 and TGF- β were also both elevated in worm-infected (**Fig. 3.8**).

3.2.6 Role for IL-10 in schistosome protection from anaphylaxis.

As both IL-10 and TGF- β are implicated in modulation of the immune system by helminth parasites (Maizels and Yazdanbakhsh, 2003), and worm-infected mice had greater production of these cytokines, I investigated the role of these cytokines in the greater resistance of worm-infected mice to anaphylaxis. Pen V-sensitised mice received an intraperitoneal injection of 0.5 mg/mouse of anti-IL-10 receptor (clone 1B1.3a) or anti-TGF- β (clone 1D11.16.8) on two consecutive days prior to anaphylaxis challenge. The dose regimes used were based on published protocols, with the mAbs prepared in-house and also confirmed to block specific cytokine-mediated responses *in vivo*.

Sensitized uninfected BALB/c mice treated with a control mAb developed anaphylaxis with a drop in core body temperature and mortalities (2/7) after 10 minutes (**Fig. 3.9**). Uninfected mice that were treated with anti-TGF- β had a comparable drop in temperature as compared to untreated uninfected mice. As shown earlier, worm-infected mice were protected from Pen V anaphylaxis with no drop in core body temperature over time and also no mortalities (**Fig. 3.9**). Worm-infected mice that had been treated with a depleting TGF- β mAb were also completely protected from anaphylaxis (**Fig. 3.9**), indicating that this cytokine had no role in mediating protection. In contrast, worm-infected mice treated with anti-IL-10 receptor mAb were highly susceptible to anaphylaxis, with a significant drop in core body temperature and greater mortalities of mice (4/8) than seen in uninfected animals (2/7; **Fig. 3.9**). Anti-IL-10R treatment of uninfected sensitized mice did not further alter the susceptibility of these mice to anaphylaxis. These mAb neutralization studies suggested that IL-10, but not TGF- β , has a role in mediating the resistance of *S. mansoni* worm-infected mice to anaphylaxis.

To further formally validate if worm resistance to anaphylaxis was mediated by IL-10 we infected IL-10-deficient mice and their C57BL/6 wild-type controls with schistosome worms and tested their susceptibility to Pen V-induced anaphylaxis. Uninfected C57BL/6 were susceptible to anaphylaxis as determined by a drop of $\sim 3^{\circ}\text{C}$ in body temperature (**Fig. 3.10**). Worm-infected C57BL/6 mice were resistant to anaphylaxis but did have a drop in temperature (**Fig. 3.10**). Clinical scores were not taken as these mice are difficult to accurately assess and compare as they are darker and symptoms are not as obvious. As all data shown previously (**Figures 3.1 - 3.9**) were in BALB/c strain mice it is significant that worm-infection of both BALB/c (considered Th2-biased) and C57BL/6 (considered Th1-biased) strains renders mice less susceptible to anaphylaxis. Worm-

infected IL-10 deficient mice developed anaphylaxis, with a marked drop in core body temperature, of $>5^{\circ}\text{C}$, and also high mortalities (**Fig. 3.10**). These data further support a central role for IL-10 in mediating worm-induced protection against anaphylaxis.

3.2.7 Role of IL-10 producing cells in worm resistance to anaphylaxis.

IL-10 is a cytokine produced by numerous cell types, including activated T cells, B cells and macrophages (Moore *et al.*, 2001). To empirically determine the relative roles of defined IL-10 producing cell populations in worm resistance to anaphylaxis we used mAbs for selective *in vivo* cell depletion. The efficacy of all *in vivo* cell depletion treatments was confirmed by flow cytometry.

Previously it has been shown that regulatory cells including $\text{CD4}^+\text{CD25}^+$ T cells that produce IL-10 have a suppressive role in diseases (Belkaid *et al.*, 2002; Maloy and Powrie, 2001) including allergic diseases such as asthma (Chen *et al.*, 2003). Indeed, the levels of $\text{CD4}^+\text{CD25}^+$ T cells in worm-infected mice (14-16%) are elevated compared to what is observed in naïve uninfected mice (8-11%; **Fig. 3.11A**). However, although worm-infected mice had elevated levels of $\text{CD4}^+\text{CD25}^+$ T cells there were no major differences between uninfected and worm-infected mice in the percentage of $\text{CD4}^+\text{CD25}^+\text{IL-10}^+$, indeed worm-infected mice have more $\text{CD4}^+\text{CD25}^+$ cells that did not produce IL-10 relative to frequencies of IL-10 producers (**Fig. 3.11B**). Interestingly, in the $\text{CD4}^+\text{CD25}^+$ cell populations in the spleens of worm-infected, the expression of the regulatory marker Foxp3, detected by intracellular staining (Marie *et al.*, 2005), was also not increased compared to Foxp3 expression in cells from uninfected mice (**Fig. 3.11C**). These preliminary data suggests that schistosome worm-infection induces an increase in the frequencies of $\text{CD4}^+\text{CD25}^+$ cells that do not produce IL-10 and do not express Foxp3.

CD25⁺ cells were depleted *in vivo* to address the role of these regulatory T cells in worm-infection and anaphylaxis (**Fig. 3.12A**). Depletion of CD25⁺ cells had no effect on the protection afforded by worm infections (**Fig. 3.12B**) or susceptibility of uninfected mice to anaphylaxis. This result indicates that CD25⁺ cells do not mediate the protection against anaphylaxis that is observed in worm-infected mice.

It has previously been shown that macrophages are modulated by schistosome worm infection (Smith *et al.*, 2004). Although, macrophages can release IL-10 (Ajuebor *et al.*, 1999; de Waal Malefyt *et al.*, 1991; Moore *et al.*, 2001), there was no differences in IL-10 production from spleen macrophages of uninfected and worm-infected mice (**Fig. 3.13A**), suggesting that they may not be a major source of IL-10 during worm infection. Nevertheless, as macrophages have been implicated in anaphylaxis (Bieber, 1997; Holt *et al.*, 1989; Strait *et al.*, 2002), we assessed the role of macrophages in worm-mediated protection from anaphylaxis. Macrophages were depleted *in vivo* by administration of clodronate-liposomes (**Fig. 3.13B**). This treatment had no further effect on the susceptibility of Pen V-sensitised uninfected mice to anaphylaxis and importantly depletion of macrophages had no effect on worm-mediated protection against anaphylaxis. These data indicated that macrophages are not an effector cell in the worm-induced protection from Pen V anaphylaxis (**Fig. 3.13C**).

Worm-infected mice have elevated levels of IL-10 production following spleen cell activation in comparison to uninfected mice (**Fig. 3.8**). Using intracellular immunofluorescence staining it was shown that worm infection increased the frequencies of spleen IL-10 producing CD4⁺ T and CD19⁺ B cells (**Fig. 3.14A,B**). To empirically determine the relative roles of these defined IL-10-producing cell populations in worm resistance to anaphylaxis we selectively depleted each cell type *in vivo*. CD4⁺ T cells were

depleted by mAb treatment (**Fig. 3.15A**). *In vivo* depletion of this cell population in uninfected mice did not alter the susceptibility of these mice to Pen V-induced anaphylaxis. Despite worm-infected mice having ~2-fold more IL-10-producing CD4⁺ T cells than uninfected mice (**Fig. 3.14A**), the depletion of CD4⁺ cells had no effect on worm-mediated protection against anaphylaxis (**Fig. 3.15B**). To assess the role of B cells in anaphylaxis an anti-IgM Ab was administered to deplete B cells. Although Ab treatment only caused partial depletion of IgM⁺ B cells (**Fig. 3.16A**), this reduction in B cells was sufficient to render *S. mansoni* worm-infected mice highly susceptible to anaphylaxis, with mortality of all mice after 10 minutes (**Fig. 3.16B**). In contrast, uninfected and untreated worm-infected mice showed similar trends of susceptibility and protection, respectively, to anaphylaxis as was previously noted.

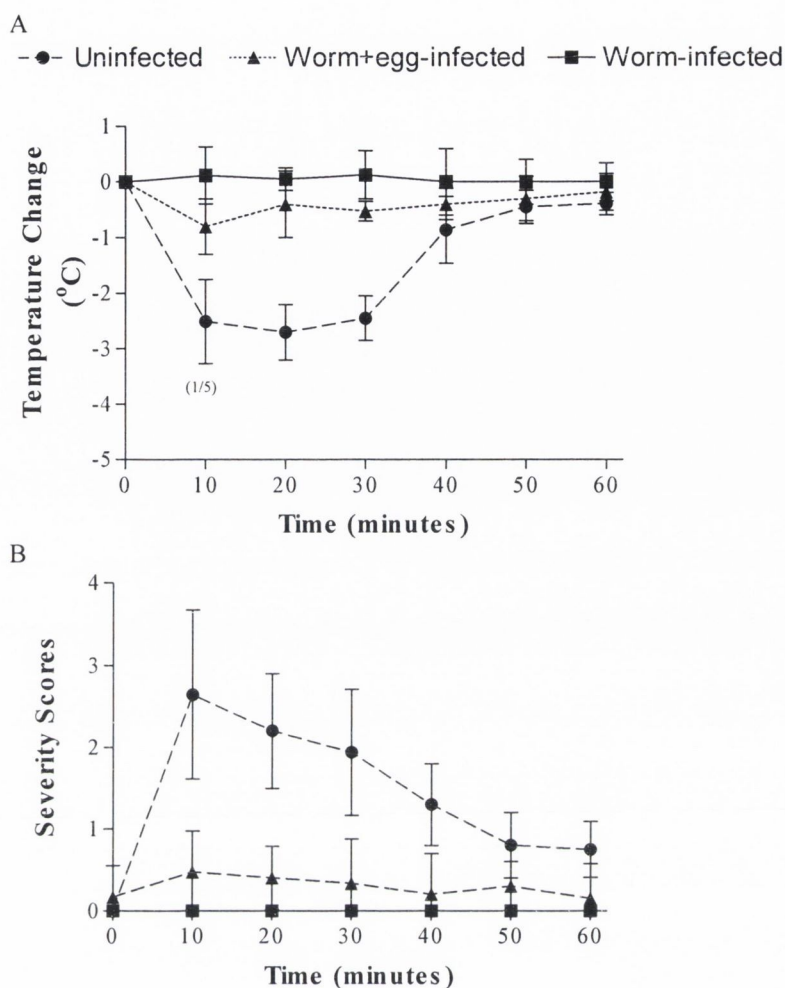


Figure 3.1. *Schistosoma mansoni* infection protects mice from systemic anaphylaxis. Uninfected and schistosome worm- or worm + egg-infected BALB/c mice were sensitized with Pen V-OVA on day 1. Infected mice were sensitized 7 weeks after infection. Pen V-BSA was injected intravenously 14 days later to elicit anaphylaxis. Rectal body temperature (A) and anaphylaxis severity scores (B) were recorded prior to Pen V-BSA challenge (time 0) and then every 10 minutes for one hour. Temperature change or severity score are representative of three separate experiments. Data are presented as group mean $\pm$ SEM (n=5-7). Number of mice dying per group are shown in brackets. Data are representative of three separate experiments.

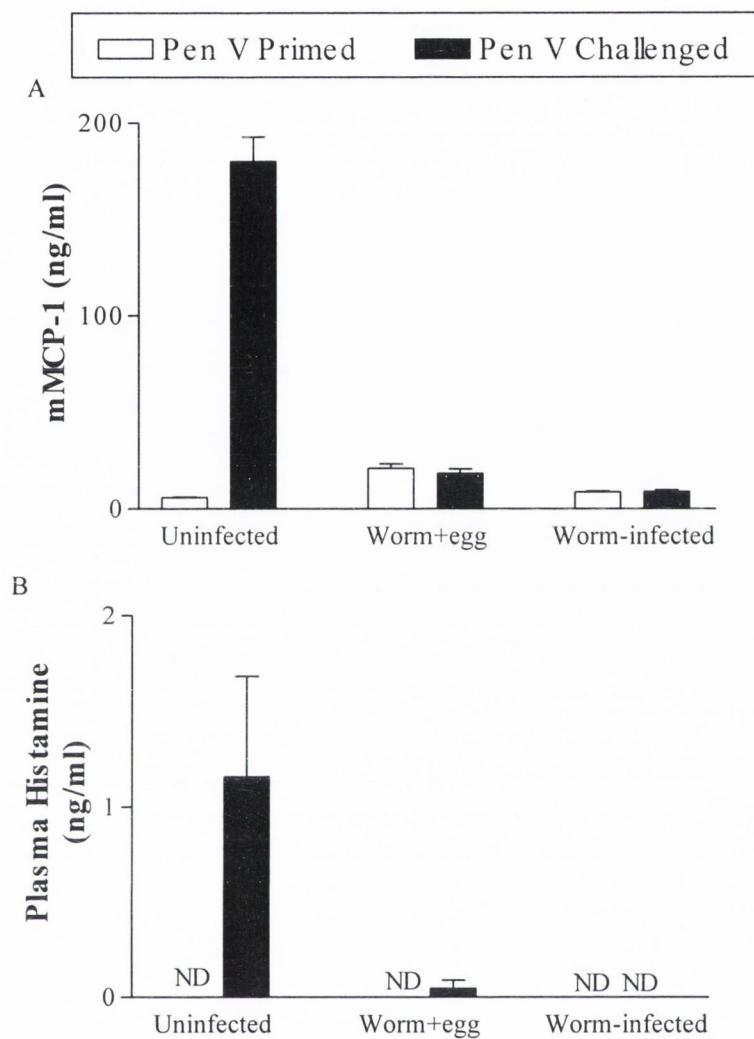


Figure 3.2. *Lack of inflammatory mediators in worm-infected and worm + egg-infected mice following anaphylaxis challenge.* Serum was harvested 60 min post challenge and mMCP-1 (A) and plasma histamine (B) levels in uninfected, worm + egg-infected and worm-infected (open bars) or Pen V-challenged (closed bars) mice were measured by ELISA (ND; not detected). Data are presented as group mean + SEM (n=5-7) and are representative of two separate experiments.

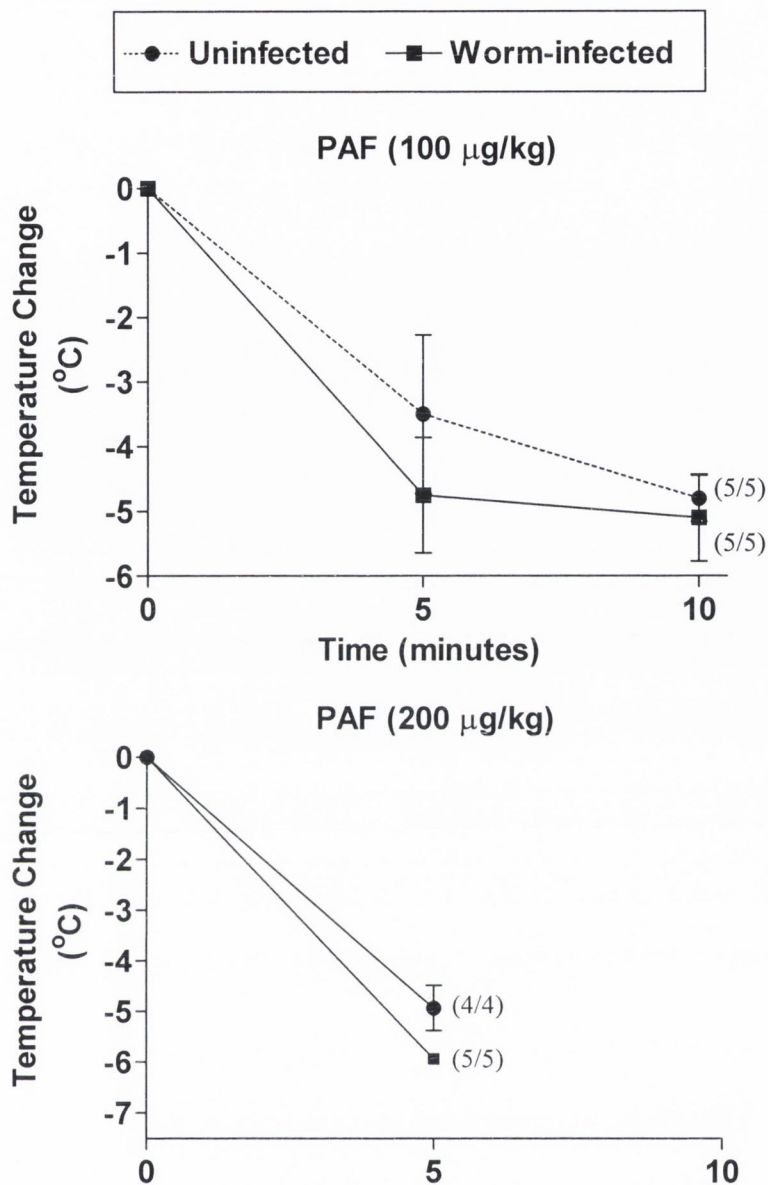


Figure 3.3. *S. mansoni*-infected mice are equally susceptible to PAF-mediated fatal anaphylaxis as uninfected mice. Uninfected or worm-infected BALB/c mice were injected with 100 or 200 µg/kg body weight of PAF ip. Rectal temperature was recorded at 0, 5 and 10 minutes after PAF administration. There was 100% mortality of all animals after 10 minutes following treatment. Results represent group mean $\pm$ SEM (n=4-5) change in temperature and are representative of two separate experiments.

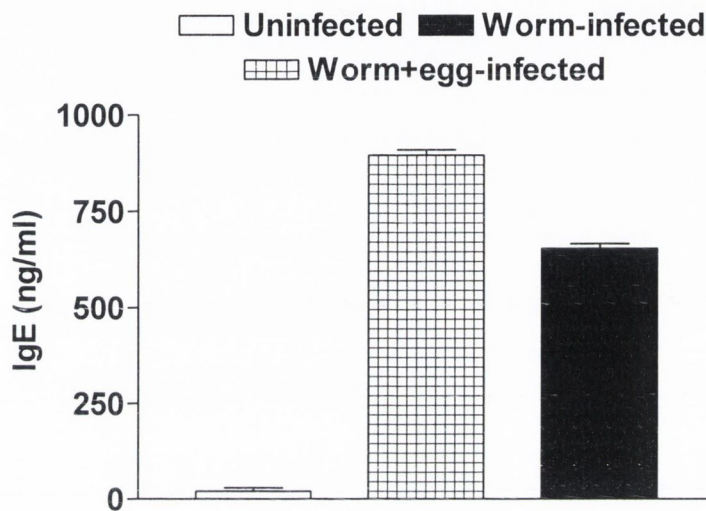


Figure 3.4. *S. mansoni*-induced production of total IgE. Serum levels of total IgE in uninfected, worm-infected and worm + egg-infected mice (week 8 post infection) was detected by capture ELISA. Results presented as group mean + SEM (n=5-10) and are representative of 3 separate repeat experiments.

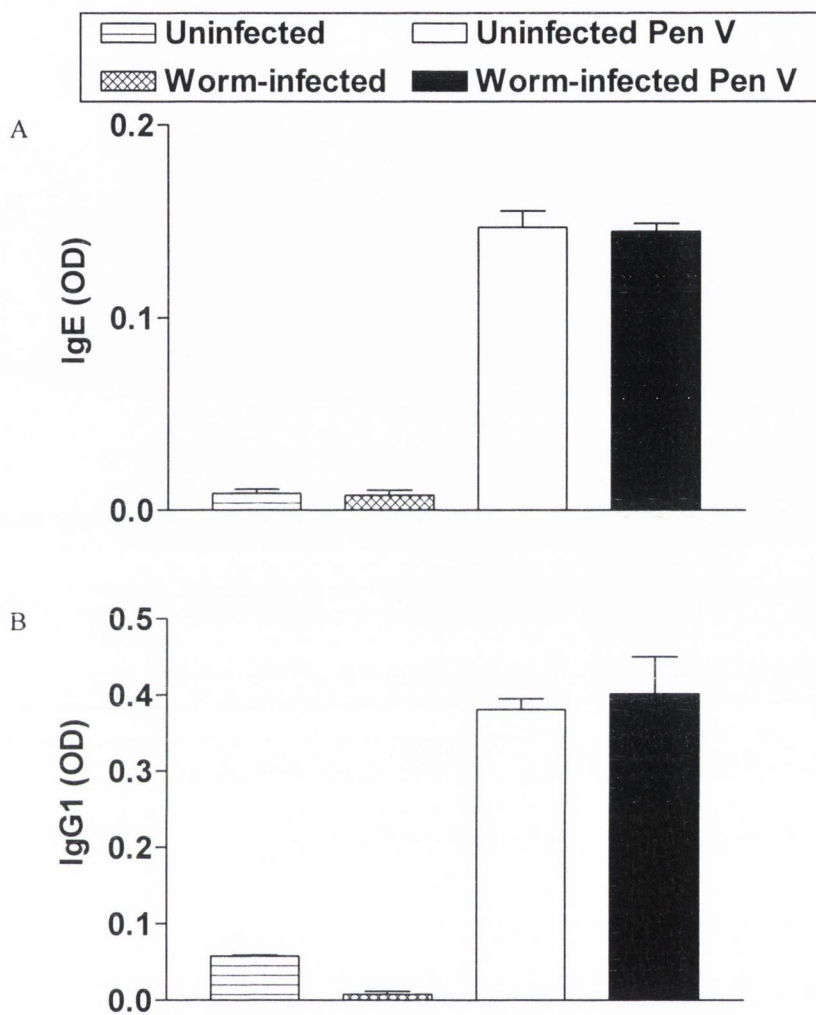


Figure 3.5. *S. mansoni*-induced production of *Pen V*-specific antibodies. Serum levels of *Pen V*-specific IgE (A) and IgG1 (B) were measured in unsensitized uninfected and worm-infected mice and in *Pen V*-sensitized uninfected and worm-infected mice by direct ELISA. Data are from 2 separate experiments and are presented as group mean + SEM (n=5-7).

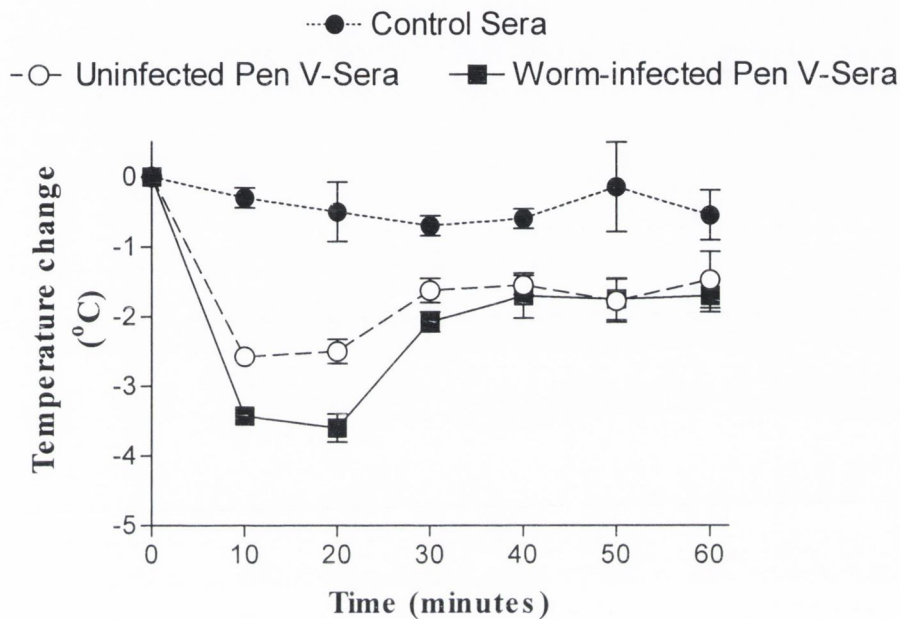


Figure 3.6. *S. mansoni* worm-infected mice produce Pen V-specific Abs that are capable of transferring passive anaphylaxis. Passive transfer of systemic anaphylaxis was induced by transfer of 0.4 ml of sera (ip) from 14 day Pen V-OVA sensitized uninfected or worm-infected mice to naïve recipient mice. Mice were treated with sera three times at 10 minute intervals and challenged after 24 hours with 100 µg Pen V-BSA iv. Control mice received normal mouse sera. Rectal body temperature was recorded prior to Pen V-BSA challenge (time 0) and every 10 minutes for one hour. Temperature changes are representative of two separate experiments. Results represented as group mean $\pm$ SEM (n=5-10).

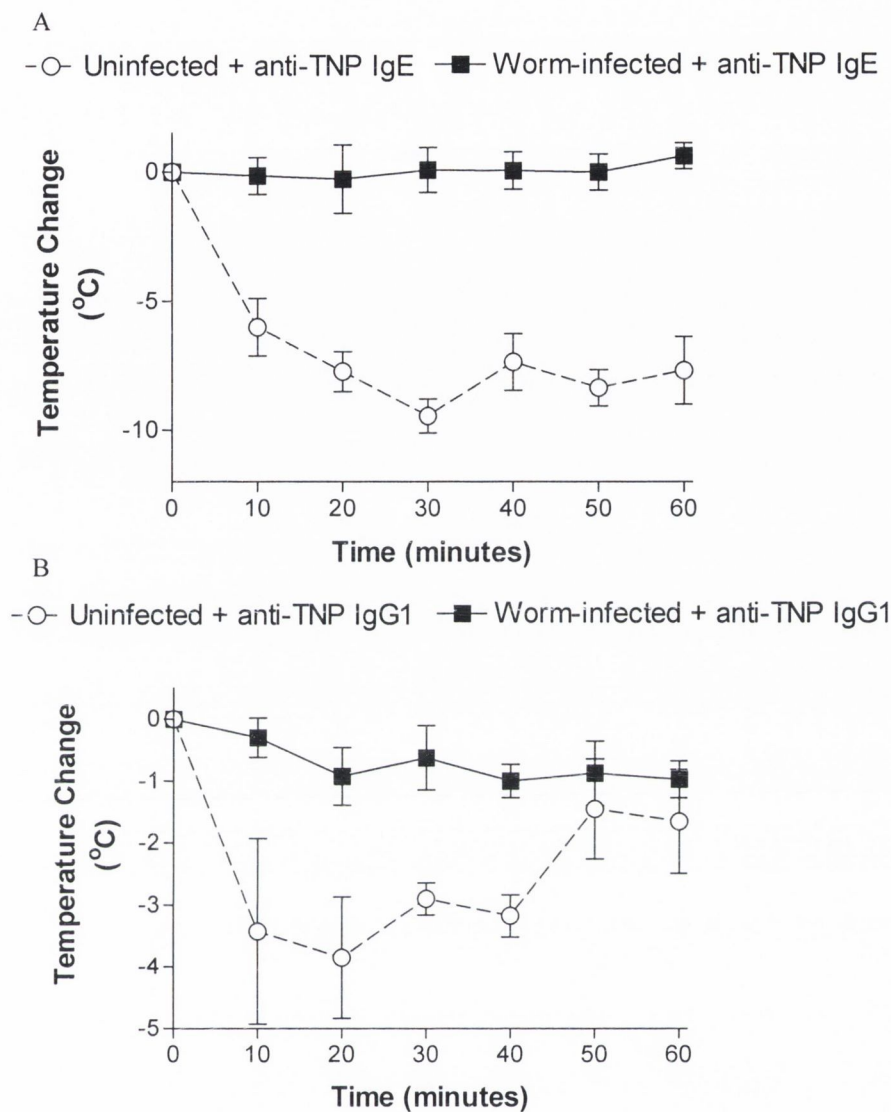


Figure 3.7. *S. mansoni* worm-infected mice are themselves protected from direct IgE- and IgG1-passive systemic anaphylaxis. Direct IgE- or IgG1-passive systemic anaphylaxis was induced by iv injection of anti-TNP IgG1 (200 μ g; **A**) or anti-TNP IgE (20 μ g; **B**) in uninfected and worm-infected mice. Thirty minutes after IgG1, or 24 hours after IgE, treatment mice were challenged intravenously with TNP-OVA (1 mg). Rectal body temperature was recorded prior to challenge (time 0) and every 10 minutes for one hour. Temperature changes are representative of two separate experiments. Results represented as group mean $\pm$ SEM (n=4-6).

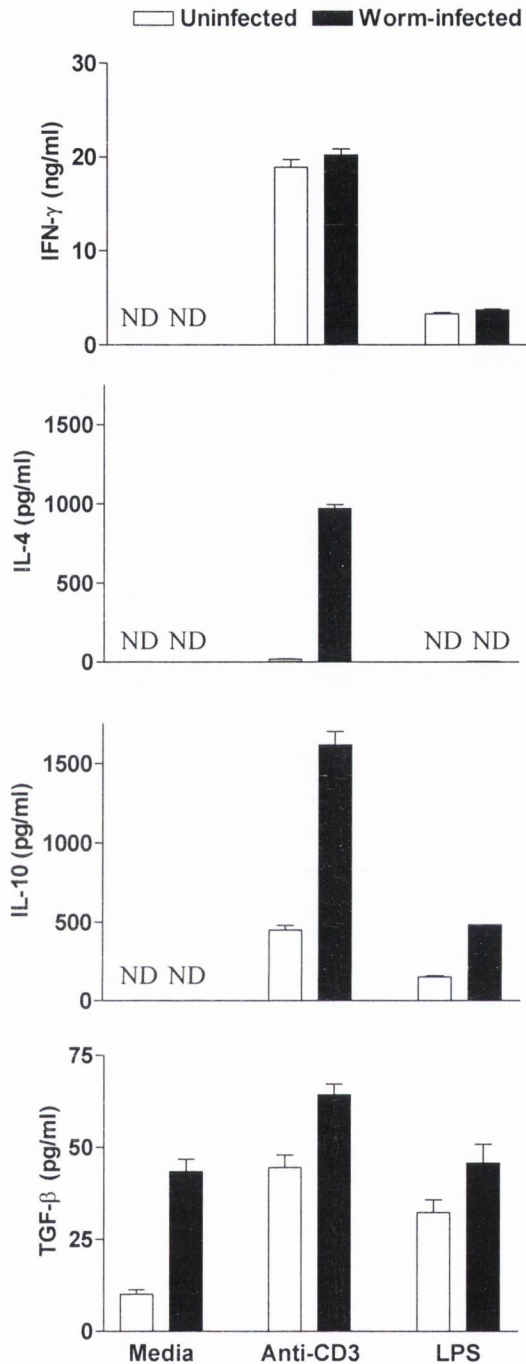


Figure 3.8. *S. mansoni* worm-infection induced alterations in cytokine profiles compared with uninfected mice. Spleen cells were isolated from two to three uninfected and worm-infected mice (8 weeks post infection), pooled and were stimulated with plate-bound anti-CD3 mAb (10 μ g/ml) or LPS (1 μ g/ml) for 72 hours. Levels of IFN- γ , IL-4, IL-10, TGF- β were measured in the culture supernatants by ELISA. Results are presented as the mean + SD from triplicate wells. Data are representative of 2-3 separate experiments. ND, not detected.

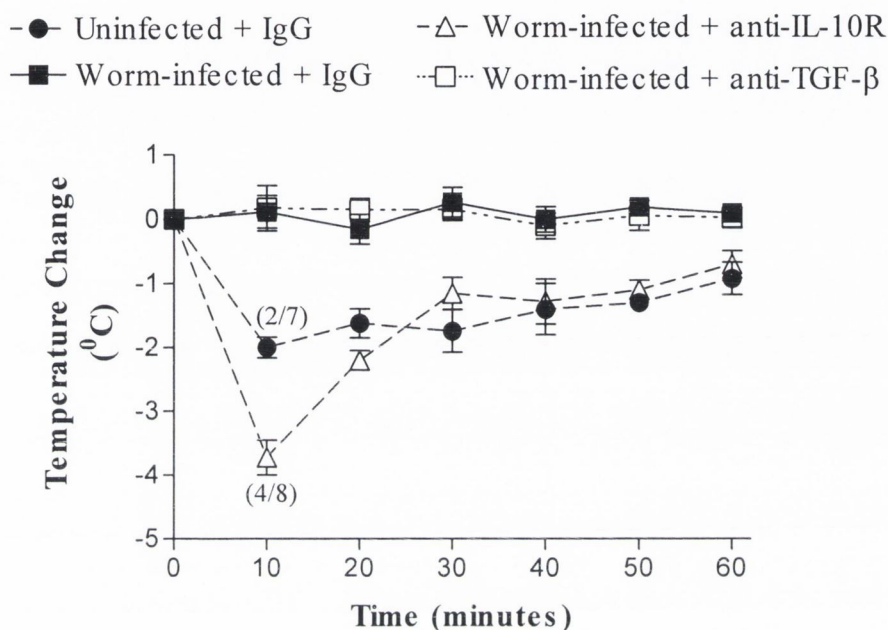


Figure 3.9. *IL-10* has a role in protecting *S. mansoni* worm-infected mice from *Pen V*-induced anaphylaxis. Depleting mAbs against TGF- β (1D11.16.8) or IL-10 receptor (IL-10 R; 1B1.3a) were injected (0.5 mg was injected ip per mouse at each time point) 2 days and 1 day before eliciting anaphylaxis in *Pen V*-OVA sensitized mice. Groups of uninfected and worm-infected (8 weeks post infection) mice also received control IgG1. Rectal body temperature was recorded prior to *Pen V*-BSA challenge (time 0) and every 10 minutes for one hour. Temperature changes are representative of three separate experiments. Number of mice dying/group are shown in brackets. Results represented as group mean $\pm$ SEM (n=7-8).

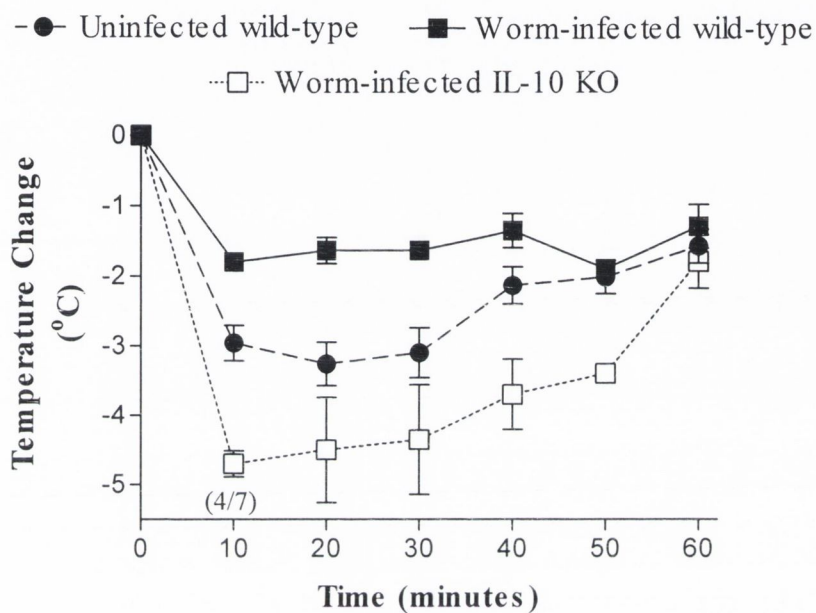


Figure 3.10. *Worm-infected IL-10^{-/-} mice are susceptible to anaphyaxis.* IL-10^{-/-} (IL-10 KO) and wild-type control C57BL/6 mice were infected with schistosome worms and Pen V-induced anaphylaxis was performed, 8 weeks post infection, as described in section 2.4. Results are group mean $\pm$ SEM (n=6-7) change in body temperature. Data presented are representative of two separate experiments. Number of mice dying/group are shown in brackets.

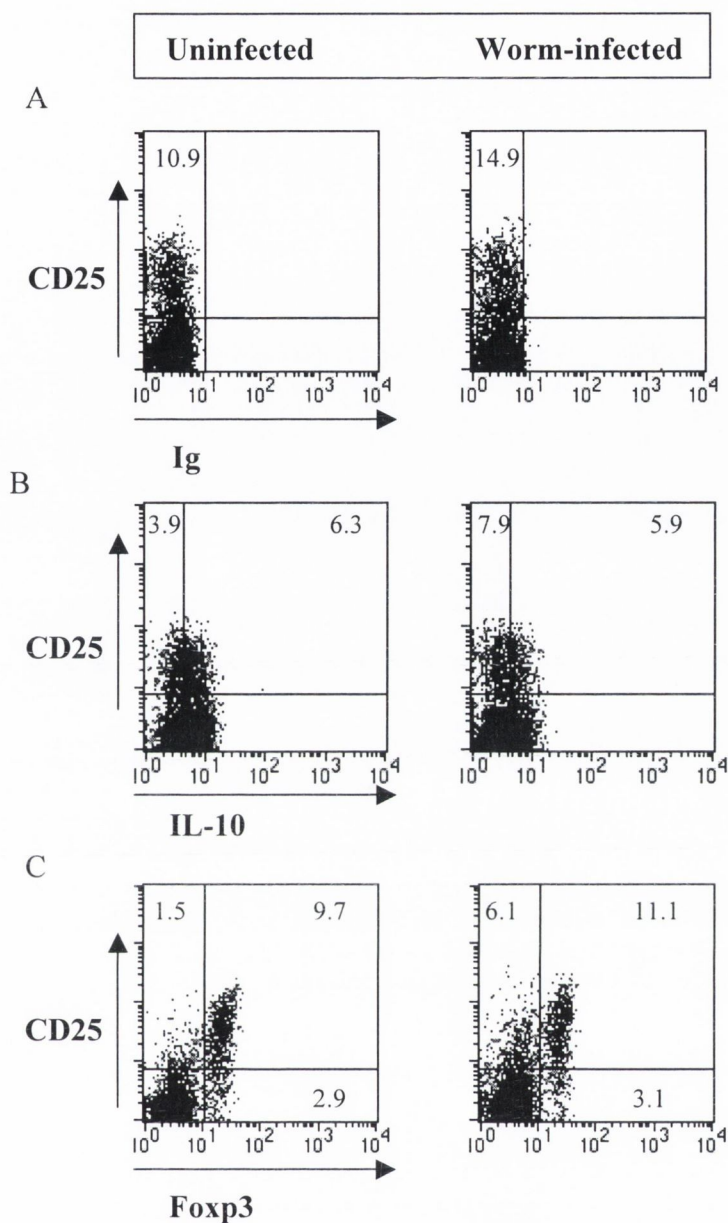


Figure 3.11. Alterations in the levels of regulatory T cells populations in worm-infected and uninfected mice. Spleen cells from worm-infected (8 weeks post infection) and uninfected mice were isolated and stained with the relevant surface and intracellular labels for detection of cell populations by flow cytometry. (A) Frequencies of $CD4^+CD25^+$ in worm-infected and uninfected mice. (B) Intracellular immunofluorescence cytokine staining was used to detect the frequency of IL-10 producing $CD4^+CD25^+$ T cells in spleens of uninfected and worm-infected mice. (C) Intracellular staining for Foxp3 in $CD4^+CD25^+$ cells was as described in section 2.8.2. Cells were analyzed by flow cytometry. Gates were set on individual isotype controls. Data are representative of 3 replicates each.

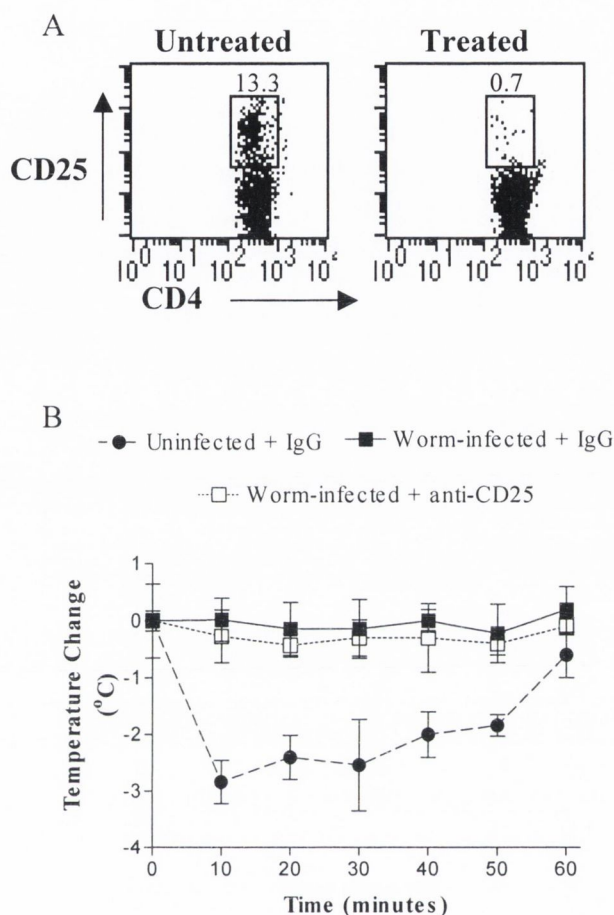


Figure 3.12. *CD25⁺ T cell populations are not involved in worm-mediated protection against anaphylaxis.* (A) CD25⁺ cells were depleted *in vivo* with anti-CD25 mAb (PC61; 0.5 mg ip per mouse) by injection 2 days and 1 day before eliciting anaphylaxis in Pen V-sensitized worm-infected mice. As a control mice received IgG1. Gates for analysis were set on individual isotype controls. (B) Temperature changes in Pen V-challenged uninfected and worm-infected (8 weeks post infection) mice treated with IgG or anti-CD25 mAb. Results are group mean $\pm$ SEM (n=7-8) change in body temperature. Data presented are representative of two separate experiments.

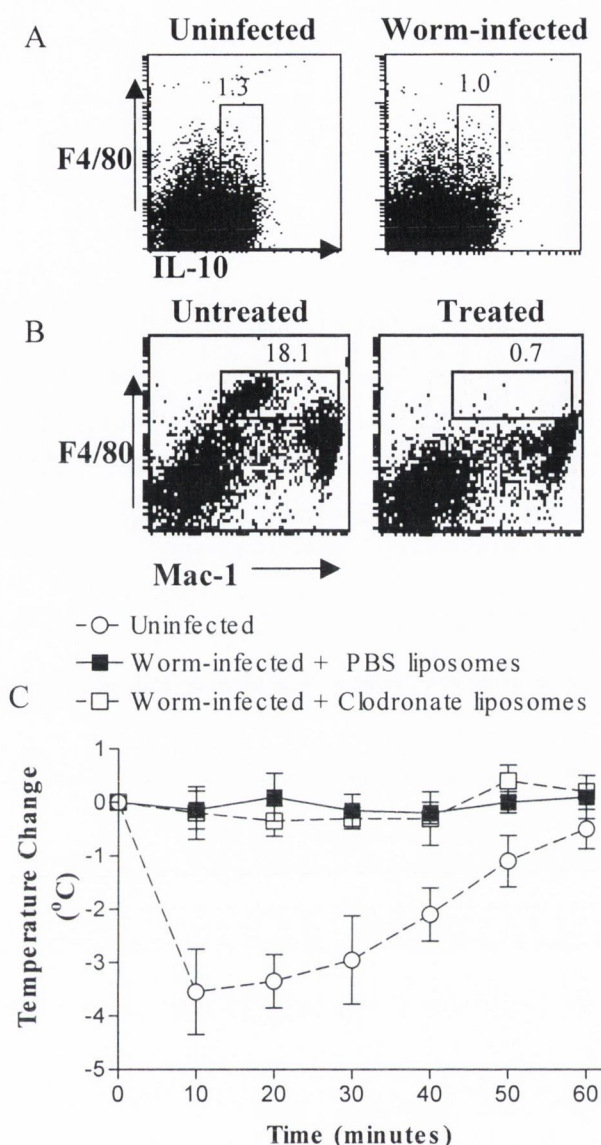


Figure 3.13. *Macrophages are not involved in worm-mediated protection against anaphylaxis.* (A) Intracellular cytokine immunofluorescence staining for the levels of IL-10 production by spleen F4/80⁺ macrophages. Gates were set on individual isotype controls. (B) Efficacy of *in vivo* depletion of spleen macrophages. Percentages of relevant cell type in spleens of untreated or treated worm-infected mice are shown. (C) Depletion of F4/80⁺ Mac-1⁺ macrophage populations does not alter the resistance to anaphylaxis of worm-infected mice. Macrophages were depleted in Pen V-sensitized mice by 2 injections of clodronate- or control PBS-liposomes; 24 hours before challenge, each mouse received 0.2 ml ip and an additional 0.1 ml was injected iv 4-6 hours before elicitation of anaphylaxis. Results are group mean $\pm$ SEM (n=5-7) change in body temperature. Data presented are representative of two separate experiments.

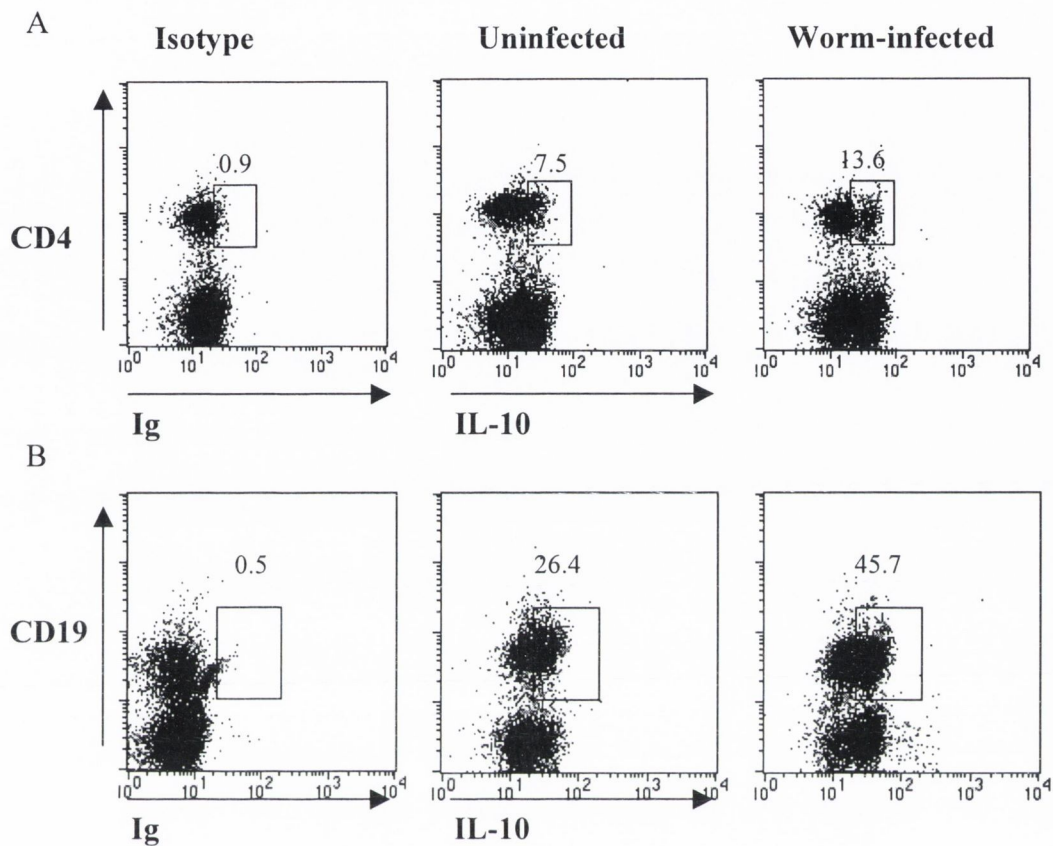


Figure 3.14. *IL-10-producing cell population in the spleen.* Spleen cells from worm-infected and uninfected mice were isolated and stained with the relevant surface and intracellular labels for detection of cell populations by flow cytometry. Cells were unstimulated and treated for 4 hours with Brefeldin A (10 μ g/ml). Levels of IL-10 in (A) CD4⁺ T cells and (B) CD19⁺ B cells were assessed after this time. Gates were set on individual isotype controls. Data are representative of 3-4 separate experiments (n=8-10).

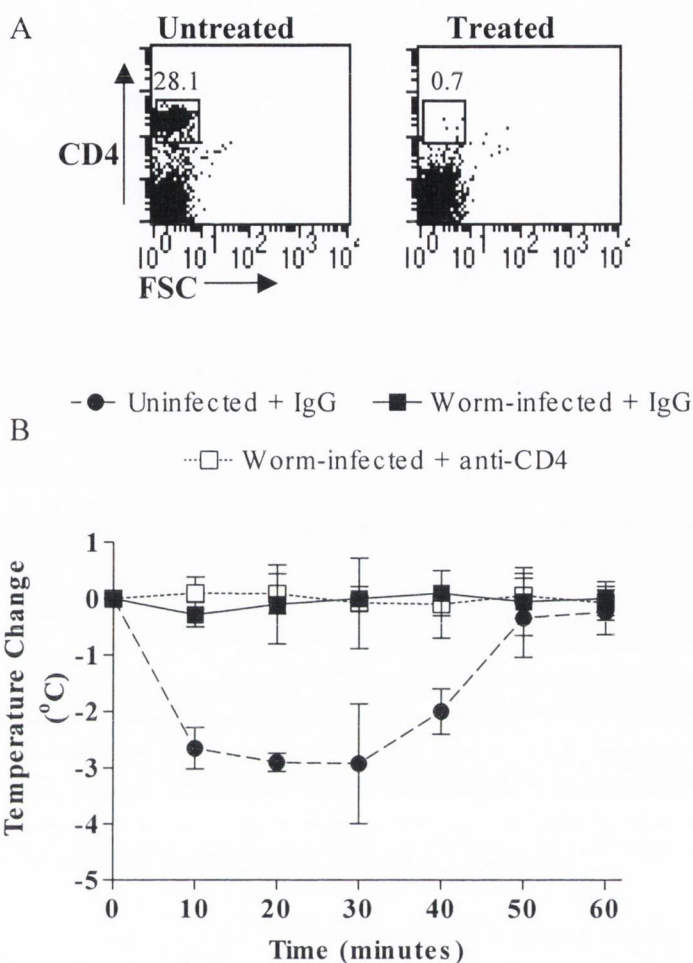


Figure 3.15. Depletion of $CD4^+$ T cells does not alter worm-induced protection against anaphylaxis. Depleting mAbs against CD4 (YTS 191) were injected (0.5 mg was injected ip per mouse at each time point) 2 days and 1 day before eliciting anaphylaxis in Pen V-sensitized mice. Uninfected and worm-infected (8 weeks post infection) mice also received control IgG. (A) *In vivo* depletion of $CD4^+$ T cells. Percentages of $CD4^+$ T cells in spleens of untreated or treated mice are shown. (B) Anaphylaxis in CD4-depleted worm-infected mice. Uninfected and worm-infected mice received YTS 191 or control IgG1. Data presented are representative of three separate experiments. Results are group mean $\pm$ SEM ($n=7-8$) change in body temperature.

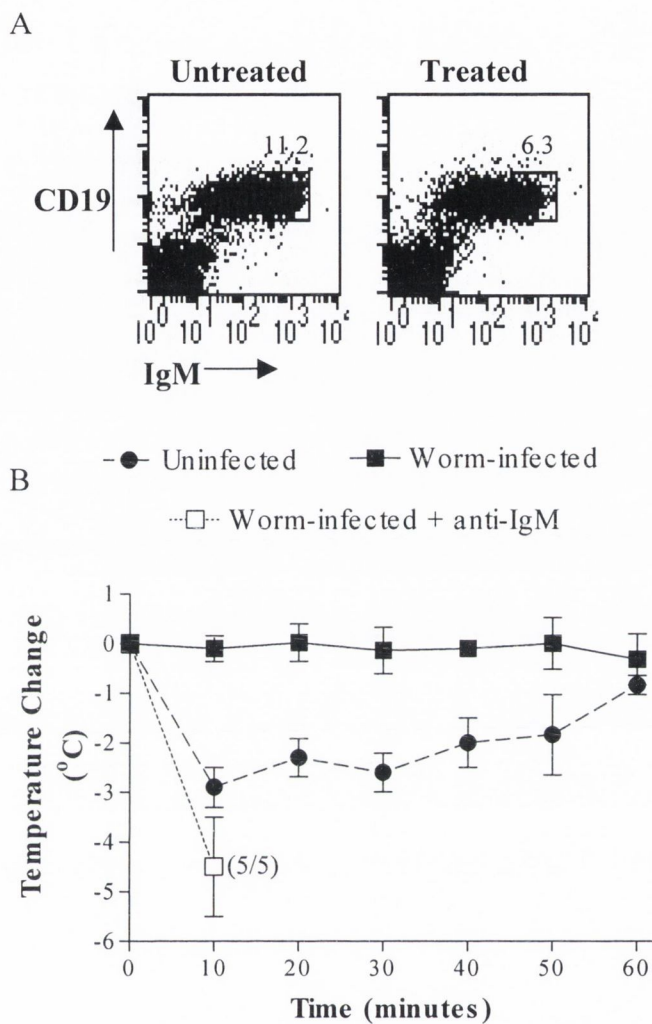


Figure 3.16. Depletion of B cells obliterates worm-induced protection against anaphylaxis. B cells were partially depleted in anaphylaxis in Pen V-sensitized mice by anti-IgM treatment (0.5 mg was injected ip per mouse at each time point) 2 days and 1 day before eliciting anaphylaxis in Pen V-sensitized mice. (A) Flow cytometry showing efficacy of *in vivo* depletion of spleen B cells. Percentages of B cells in spleens of untreated or treated mice are shown. (B) *S. mansoni* worm-infected mice (8 weeks post infection) develop anaphylaxis after depletion of IgM⁺ cells. Number of mice dying/group are shown in brackets and results are group mean $\pm$ SEM (n=5-7). Data are representative of 2-3 separate experiments.

3.3 Discussion

In this study I demonstrate that mice infected with *S. mansoni* are refractory to experimental systemic anaphylaxis. I assessed the role of infection with schistosome worms compared with a worm + egg infection in immunoregulation during anaphylaxis, using a murine model of Penicillin V-induced anaphylaxis. It was established that it is the worm stage of infection that elicits the protective phenotype in this model, with worm-infected mice completely protected against anaphylaxis whereas worm + egg-infected mice were only partially protected. I have identified that schistosome worm infection of mice prevents anaphylaxis via a B cell and IL-10 dependent mechanism.

Using different anaphylaxis models it is evident that worm infection does not alter sensitization to the allergen but protects the mice from anaphylaxis when re-challenged with the allergen. Therefore resistance of schistosome-infected mice to anaphylaxis was not due to the known propensity of helminth infections to alter responses to vaccination (Sabin *et al.*, 1996) or the stimulation of polyclonal IgE, that can saturate Fc ϵ receptors and thereby prevent mast cell degranulation (Hagel *et al.*, 1993). This is in agreement with studies by Jarrett *et al* showing that helminth-induced non-specific IgE does not inhibit hypersensitivity reactions *in vivo* (Jarrett *et al.*, 1980). Protection from anaphylaxis is therefore primarily mediated when the sensitized mice are re-challenged with appropriate allergen. This indicates that alterations in immune responses in *S. mansoni* worm infection potentially induce a blocking effect in susceptibility of these mice to allergen recall responses.

In the context of the Hygiene Hypothesis, it has previously been proposed that schistosome infection can ameliorate allergic disorders in humans (Maizels and

Yazdanbakhsh, 2003). In support for this argument, schistosome-infected Gabonese schoolchildren have lower allergic responses and elevated IL-10 levels (van den Biggelaar *et al.*, 2000). Furthermore, there is an increase in atopic responses in Gabonese schoolchildren after chemotherapy to eliminate gastro-intestinal helminth infections (Van Den Biggelaar *et al.*, 2004). It is well documented that schistosome worm + egg-infections induce elevated IL-10 production (Flores Villanueva *et al.*, 1994; Sher *et al.*, 1991; Wynn *et al.*, 1997). IL-10 production during such infections has an essential function in suppressing egg-induced immuno-pathology (Boros and Whitfield, 1998; Hesse *et al.*, 2004; Hoffmann *et al.*, 2000; Wynn *et al.*, 1998). In this study spleen cells from schistosome worm-infected mice also have elevated IL-10 production, with resistance of these mice to anaphylaxis being IL-10 dependent. Resistance to anaphylaxis was found to be IL-10 dependent in both worm-infected BALB/c and C57BL/6 strains of mice although C57BL/6 mice demonstrate different symptoms of anaphylaxis to BALB/c mice. IL-10 is one of the main cytokines that is implicated in certain regulatory T cell types (Shevach, 2002) and, with respect to asthma, has also been shown to suppress immune and inflammatory responses including allergic airway inflammation (Zuany-Amorim *et al.*, 1995). In murine schistosome worm + egg infections, a recent study has addressed for the first time the role of IL-10-producing CD4⁺CD25⁺ T cell in ameliorating morbidity (Hesse *et al.*, 2004). However, in my studies although natural CD4⁺CD25⁺ T cells and IL-10-producing CD4⁺ T cells are both elevated in worm-infected mice, depletion studies demonstrated that CD4⁺ or CD25⁺ cells had no role in worm-mediated protection against anaphylaxis. The data here indicates that T cells are not the only source of IL-10 during a schistosome worm infection, an observation also demonstrated during *S.mansoni* worm + egg-infections by Hesse and colleagues (Hesse *et al.*, 2004). I show here that B cells from

schistosome worm-infected mice have a 2-fold elevation in the levels of IL-10-producing B cells. Previously, it has indeed been shown that B cells play an important regulatory role during conventional *S. mansoni* worm + egg infection (Cheever *et al.*, 1985), with high mortalities of infected B cell-deficient mice (Hernandez *et al.*, 1997), (Ferru *et al.*, 1998; Jankovic *et al.*, 1998). As schistosome infection of mice stimulates a number of cells to produce IL-10 it highlights the known significant role for this cytokine in immunomodulation of the host to suppress parasite-induced pathology.

The central role of B cells in protecting worm-infected mice from anaphylaxis was illustrated by depletion of B cells causing these animals to be fully susceptible to anaphylaxis. B cells are difficult to deplete *in vivo* but even partial depletion of B cells as seen in this experiment was sufficient to demonstrate a profound effect. B cells possess a variety of immune functions, including production of immunoglobulins, the presentation of antigens, and the production of distinct cytokines (Harris *et al.*, 2000). B cells have also been shown to participate in the induction of immune tolerance and suppression of disease giving rise to the suggestion of IL-10-producing regulatory B cells (Fillatreau *et al.*, 2002; Korganow *et al.*, 1999; Mizoguchi *et al.*, 2002).

It is of note that the pathways for some autoimmune diseases (Th1) and allergies (Th2) are very similar and involve crossover of receptors and cells involved in both diseases. Molecules involved in disease can potentially modulate the onset of other diseases as seen in the blockade of histamine and PAF, the main mediators in inducing anaphylaxis, significantly decreasing murine experimental autoimmune encephalomyelitis severity (Pedotti *et al.*, 2003). However, an unexpected development in the quest for peptide-based tolerance therapies for the treatment of autoimmune disease is the occurrence of anaphylaxis to potential therapeutic peptides (Smith *et al.*, 2005). Therefore it is

imperative that there be a full and thorough understanding of the pathways involved in the development of anaphylaxis to ensure the effective design of any future peptide therapeutics for disease. My studies here present some further insight into the mechanisms and complexities in the induction of murine allergen-induced systemic anaphylaxis whilst under the added immunomodulatory influence of a parasite infection.

It is noteworthy that when a protective factor, i.e. B cells or IL-10 was inhibited the worm-infected mice had a heightened propensity to develop fatal anaphylactic reactions upon antigen challenge. This highlights that in the absence of regulation by IL-10 or B cells schistosome-infected mice are in fact highly predisposed to type 2 allergic responses. Thus, these data demonstrate the sensitive balance of *S. mansoni* modulation of host immunity to achieve host protective versus detrimental immune responses. Hence IL-10-production and B cells are necessary in maintaining a fine balance of regulation in the allergic predisposed environment of schistosome infection. Others have demonstrated directly that it is IL-10-producing B cells that have this effect. As I have not formally demonstrated that the worm protective effect is mediated specifically by IL-10-producing B cells I have referred to protection involving both IL-10 and B cells.

Collectively, these experimental data demonstrate that schistosome worms evoke IL-10-producing B cells during infection with these cells protecting against anaphylaxis. I have shown the IL-10-producing B cells are induced during schistosome infection as part of the parasite's regulation of host immunity, with this modulation also influencing unrelated allergic (anaphylaxis) responses. These data demonstrate for the first time conclusive experimental evidence that *S. mansoni* can suppress allergic responses via a novel regulatory cell mechanism.

Chapter 4

*Helminth modified pulmonary type 2
response protects mice from allergen-induced
airway hyperresponsiveness.*

4.1 Introduction

I have previously demonstrated in Chapter 3 that *Schistosoma mansoni* infection protects mice from an allergic response through a regulatory mechanism induced by the worm (Mangan *et al.*, 2004). These studies in Chapter 3 were on anaphylaxis, a severe and immediate form of allergic reaction. Therefore in order to further address the role and development of worm-protection in allergies, I have assessed the role of *S. mansoni* infection in a less severe form of allergic reaction, namely airway hyper-responsiveness (AHR). The OVA-induced AHR protocols that I have used in this chapter are widely used as model of human pulmonary inflammation (Boyce and Austen, 2005); see methods section 2.5. I have identified that the worm stage of *S. mansoni* infection modulates mice so they are refractory to AHR. This protection involves the *S. mansoni*-induced modulation of the pulmonary response in AHR. This is the first formal demonstration of a mechanism that human parasitic worms employ to suppress allergen-induced airway inflammation.

Chapter objectives:

1. To investigate the role of *Schistosoma mansoni* in a less severe form of allergic sensitivity then was described in Chapter 3.
2. To look at differences in worm + egg and worm-only infections in a murine asthma model of OVA-induced airway hyper-responsiveness.
3. To investigate the mechanism of action of *S. mansoni* worms in protection against AHR.

4.2. Results

4.2.1 *Schistosoma mansoni* worm + egg infection of mice exacerbates AHR.

To experimentally investigate if schistosome infection modulates immune responses following exposure to an allergen schistosome infected mice were immunized with OVA-alum, a model type 2 cytokine-inducing allergen, and OVA-specific immunity analysed. Mice were exposed to a conventional male and female worm (worm + egg) *S. mansoni* infection and were initially sensitized with OVA systemically, and then in the lungs during the acute (between 7-11 weeks of infection) and chronic (between 12-16 weeks of infection) stages of a worm + egg-infection (**Fig. 4.1**). Spleen cells from worm + egg-infected mice immunized with OVA during acute or chronic infection both had elevated *in vitro* production of allergen-specific type 2 cytokines (IL-4, IL-5 and IL-13) and also elevated IL-10 compared to production of these cytokine by cells from uninfected OVA-immunized mice (**Fig 4.2A**). The increased type 2 cytokine response in worm + egg-infected mice was also associated with greater levels of OVA-specific IgE compared to uninfected OVA-immunized mice (**Fig. 4.2B**).

In the context of the Hygiene Hypothesis schistosome worm + egg-infected mice did not have suppressed allergic immune responses, on the contrary they have elevated type 2 cytokine responses to a model allergen. To analyse if the altered allergen-induced immune response in worm + egg-infected mice altered pulmonary inflammation we used whole-body plethysmography on conscious, unrestrained mice to determine airway function, with enhanced pause (Penh) calculated to determine pulmonary AHR (Hamelmann *et al.*, 1997). In this model uninfected mice sensitized with OVA develop AHR, demonstrated by a dose-dependent elevation in Penh in response to Mch challenge when compared to Penh response of uninfected and PBS-treated mice (**Fig. 4.3A**). Unexpectedly, both PBS- and OVA-sensitized worm + egg-infected mice developed severe respiratory distress following exposure to the lowest

dose of Mch (3.125 mg/ml) aerosol (**Fig. 4.3A**). This distress was manifested by significant elevated Penh values at all doses of Mch when compared to uninfected OVA mice ($P < 0.05$), with all worm + egg-infected mice dying in the plethysmograph chamber at doses of 12.5 - 25 mg/ml Mch (**Fig. 4.3A**). There was no difference in the rate of mortalities between acute versus chronically worm + egg-infected mice, but in both groups sensitization with OVA accelerated the death of mice, with all OVA-sensitized animals dead after exposure to 12.5 mg/ml Mch (**Fig. 4.3A, B**). Therefore following allergen challenge worm + egg-infected mice develop elevated type 2 cytokine responses and, independent of allergen sensitization, are also predisposed to exacerbated and fatal AHR.

As the accuracy of Penh as a measure of murine lung function has recently been questioned (Adler *et al.*, 2004), we also tested pulmonary function by analysis of pulmonary compliance (Cdyn) and resistance (GL) in unconscious and restrained mice (Martin *et al.*, 1988). Initial Penh results were corroborated by Cdyn and GL studies (**Fig. 4.4A, B**), which confirmed that worm + egg-infected mice are indeed predisposed to developing AHR, independent of OVA-sensitization. In homogenates of lungs of OVA-treated worm + egg-infected there was elevated levels of IL-5 ($P < 0.05$) compared with uninfected animals, with no differences in IFN- γ levels. There was also a significant increase in the levels of the regulatory cytokine IL-10 in lungs of OVA-treated worm + egg infection (**Fig. 4.5**; $P < 0.05$). I did not measure the other Th2 cytokines IL-4 and IL-13 as there was a finite amount of supernatant from the lung homogenates.

It was unexpected that worm + egg-infected mice that were not immunized with OVA spontaneously develop respiratory distress and die when exposed to Mch. An obvious potential explanation is that during a murine schistosome worm + egg infection eggs that are laid by the female worms are swept to various organs, in this case the

lungs, where they evoke granulomatous inflammation. When schistosome eggs are injected intravenously into naïve mice they are trapped in the lung and indeed stimulate a potent pulmonary inflammation (Fallon *et al.*, 2002). Therefore an initial explanation for the increased susceptibility of worm + egg-infected mice to AHR was the presence of eggs in the lungs inducing pathology. However, we have not detected any eggs present in digests of lungs from acute or chronically worm + egg-infected mice, nor have we seen eggs/egg granulomas in histology sections of lungs from worm + egg-infected mice. In the literature there have been no reports of eggs in the lungs of schistosome-infected mice however, with the exception of CD154^{-/-} mice (MacDonald *et al.*, 2002). Intriguingly, I have detected eggs in the mediastinal lymph nodes, which may have profound effects on the cytokine milieu in the node.

Histology sections of lungs from uninfected and worm + egg-infected mice demonstrated comparable appearance (**Fig. 4.6A**), with no overt increase in peribronchial inflammation or cellular infiltration associated with elevated AHR. There was substantial subepithelial and parenchymal collagen deposition in worm + egg-infected mice as seen on Martius Scarlet Blue staining of lung sections (**Fig. 4.6B**). Quantification of pulmonary collagen indeed showed worm + egg-infection of mice induced a significant, $P < 0.001$ versus uninfected mice, 2-3 fold elevation in collagen (**Fig. 4.7A**). Previously, it has been shown that schistosome worm + egg-infected IL-13^{-/-} mice do not develop the elevated levels of hepatic collagen seen in comparably infected wild-type mice (Fallon *et al.*, 2000a), which is consistent with the role of IL-13 in fibrosis (Wynn, 2003). I show here for the first time that worm + egg schistosome infected IL-13^{-/-} mice do not develop pulmonary fibrosis (**Fig. 4.7A**), as was reported previously in the livers of worm + egg-infected IL-13^{-/-} mice (Fallon *et al.*, 2001). To address if the pulmonary fibrosis in worm + egg-infected mice influences AHR we infected IL-13^{-/-} mice and tested their lung function during acute infection. Worm +

egg-infected IL-13^{-/-} mice did not develop AHR and had comparable pulmonary function as observed in uninfected mice (**Fig. 4.7B**). Therefore worm + egg-infected mice develop elevated, IL-13-dependent, pulmonary fibrosis that contributes to the exacerbated lung pathology in these mice.

4.2.2 *Schistosome worm-infected mice are resistant to OVA-induced AHR.*

Previously I have shown that mice infected with schistosome male worms are completely refractory to anaphylaxis, whereas worm + egg-infected mice were only partially resistant (Chapter 3). Mice with a male-only worm infection have elevated basal type 2 cytokine production but this response is not as marked as seen in a worm + egg infection probably due to the absence of eggs that are laid by female worms (Smith *et al.*, 2004). Spleen cells from OVA-immunized worm-infected mice had greater allergen stimulated type 2 cytokine (IL-4, IL-5 and IL-13) production (**Fig. 4.8A**). Interestingly, there was greater relative allergen-specific IL-10 released by spleen cells from OVA-sensitized worm-infected mice compared to IL-10 production from spleen cells from comparable treated worm + egg-infected mice (**Fig. 4.2A, Fig. 4.8A**), which is consistent with previous studies (chapter 3; (Smith *et al.*, 2004)).

OVA-treated worm-infected mice had markedly elevated ($P < 0.05$) sera levels of OVA-specific IgE (**Fig. 4.8B**), compared to OVA-immunized uninfected mice. Worm-infected mice also had elevated serum levels of total anti-OVA IgG, with the type 2-biased antibody isotype response reflected in elevated OVA-specific IgG1 in these mice compared to OVA-treated uninfected mice, whereas there was no difference in the limited IgG2a produced (**Fig. 4.8C**).

Mice with a worm infection, but not exposed to OVA, had no spontaneous AHR (**Fig. 4.9**), which is not what was observed in worm + egg-infected animals (**Fig. 4.3**). Furthermore, OVA-sensitized worm-infected mice did not develop AHR, with no

alterations in Penh following Mch challenge, whereas OVA-sensitized uninfected mice had dose-dependent increases in Penh (**Fig. 4.9**). In addition to quantification of Penh, alterations in pulmonary function were validated by analysis of pulmonary resistance (GL) and compliance (Cdyn) in unconscious and restrained animals (Martin *et al.*, 1988). Similar to the Penh data, mice with worm-infections had reduced Cdyn and GL responses to methacholine challenge compared to resistance/compliance responses of sensitized uninfected mice (**Fig. 4.10A, B**).

During schistosome infection there is a larval migration phase that passes through the lung (Pearce and MacDonald, 2002), with any tissue damage potentially altering pulmonary function. However, worm-infected mice that were treated with the schistosomicidal drug Praziquantel 5-6 weeks post-infection, thus allowing larval migration and any associated lung pathology to occur, were fully susceptible to OVA-induced AHR (**Fig. 4.11**). This data demonstrates that mice infected with *S. mansoni* worms do not develop OVA-induced AHR independent of any infection-induced lung pathology. The removal of worms was assessed by portal perfusion of worm-infected-treated mice after 8 weeks. Effective clearance of schistosome male worms requires both Praziquantel and Oxamniquine treatment (Fallon *et al.*, 1995) and as I had only used Praziquantel treatment, there was incomplete removal of all worms, with 0-3 stunted male worms remaining, as determined at week 8. However, there was sufficient reduction in worm burden to affect the threshold of protection afforded by the experimental worm-infection and thus the removal of worms rendered mice susceptible to AHR.

4.2.3 Modified pulmonary type 2 response in OVA-sensitized worm-infected mice.

I have addressed lung-specific responses in worm-infected mice, as in the OVA pulmonary challenge model used, the elevated AHR in sensitized mice is associated

with elevations in pulmonary type 2 cytokines, eosinophil infiltration and goblet cell hyperplasia (Wills-Karp, 1999). Uninfected OVA-sensitized mice had a type 2 cytokine profile in the lungs with production of IL-4, -5, and -13 (**Fig. 4.12**). These mice also produced the type 1 cytokine IFN- γ and demonstrated low-level production of IL-10 in both the BAL fluid and homogenates of lung (**Fig. 4.12**). OVA-sensitized worm-infected mice had significantly elevated levels of both IL-4 and IL-13 in BAL fluid and lung homogenates ($P < 0.05$) compared to OVA-sensitized uninfected mice, with no differences in IFN- γ levels (**Fig. 4.12**). Therefore the absence of AHR in OVA-sensitized worm-infected mice was surprising as the lungs of these animals had more allergic cytokines, IL-4 and IL-13, than the OVA-sensitized uninfected mice that had AHR. In contrast, however, IL-5 levels in BAL and lung homogenates from worm-infected mice were lower than uninfected mice, with BAL IL-5 significantly reduced ($P < 0.05$; **Fig. 4.12A,B**). Despite certain pulmonary Th2 cytokines levels being significantly elevated in worm-infected mice, they had dramatically reduced IL-5, indicating worm-infected mice stimulated a modified type 2 cytokine response in the lungs. Additionally, sensitized worm-infected mice had a striking >3 fold increase in total IL-10 in both homogenates of lungs and BAL fluid, which was significantly elevated above IL-10 levels detected in lungs of sensitized but uninfected mice ($P < 0.01-0.001$; **Fig. 4.12A,B**).

OVA-specific cytokine production by the lung mediastinal lymph node cells also demonstrated the modified type 2 response; with elevated IL-4 and IL-13 but reduced IL-5, in sensitized worm-infected mice compared to sensitized uninfected mice, with an associated pronounced increase in OVA-induced IL-10 (**Fig. 4.13A**). Another potential mechanism of protection by the worm at the site of inflammation may be to induce a suppression of T cell responsiveness in the lung thus dampening the inflammatory allergic response to OVA. However, there were no differences in OVA-

specific proliferation of mediastinal lymph node cells from OVA sensitized uninfected and worm-infected mice, indicating normal functional antigen-induced T cell responsiveness (**Fig. 4.13B**).

Histology sections of lungs of OVA-sensitized uninfected mice showed the characteristic allergen-induced airway inflammation and peribronchial infiltrating eosinophils seen in this model (**Fig. 4.14A**). Enumeration of cells in the BAL showed that worm-infected mice did not have the normal increase in eosinophils that occurred in OVA-sensitized uninfected mice, with significantly ($P < 0.005$) less eosinophils recovered in BAL from infected versus uninfected mice (**Fig. 4.14B**). The reduced pulmonary eosinophilia in worm-infected mice was also confirmed by flow cytometry of lung digests, with significantly less ($P < 0.01$) lung eosinophils (FSC/SSC^{hi}, CD19⁻CD4⁻CD8⁻CCR3⁺ cells) in worm-infected mice (**Fig. 4.15**). In accordance with the absence of eosinophils in the lungs of worm-infected mice the levels of eotaxin, the chemokine attractant for eosinophilia, were significantly lower in lungs recovered from sensitized worm-infected mice relative to uninfected mice ($P < 0.01$; **Fig. 4.16A**). Concordant with reduced eosinophil activity there were also reduced levels of eosinophil peroxidase (EPO) enzymatic activity detected in the lungs of sensitized worm-infected mice compared with the eosinophil-rich lungs of comparable treated uninfected mice (**Fig. 4.16B**; $P < 0.01$). In agreement with these data worm-infected mice had also reduced numbers of neutrophils in their lungs as was determined by levels of myeloperoxidase enzymatic activity (**Fig. 4.16C**; $P < 0.05$). Worm-infected mice also had limited goblet cell hyperplasia, with significantly reduced mucus scores compared to uninfected sensitized mice (**Fig. 4.17A,B**; $P < 0.01$).

4.2.4 Neutralisation of TGF- β does not reverse worm-infection suppression of AHR.

Regulatory T cells have previously been shown to suppress allergen responsiveness in target organs such as the lung. Production of the regulatory cytokines TGF- β (McMillan and Lloyd, 2004) and IL-10 (Akbari *et al.*, 2002) have been strongly implicated in suppression of allergic disease. It has been postulated that TGF- β is involved in airway remodeling due to its increased expression in the airways. TGF- β is upregulated in asthma, especially by eosinophils and airway epithelium and its potential role in tissue repair and modulation of other inflammatory cytokines have previously been investigated (Hansen *et al.*, 2000). Indeed, *S. mansoni* infection upregulates TGF- β production in infected mice (Smith *et al.*, 2004) and here, in this study, it was also shown that worm-infection of OVA-sensitized mice induces increases in TGF- β production to OVA in the lungs of sensitized mice as detected in the mediastinal lymph nodes (**Fig. 4.18A**). Despite this increase, TGF- β was not a critical component in worm protection in AHR; treatment of worm-infected (**Fig. 4.18B**) and uninfected mice with anti-TGF- β did not significantly influence protection or susceptibility noted previously (**Fig. 4.9**). It is noteworthy that there is a minor increase in Penh in both sensitized uninfected and worm-infected anti-TGF- β -treated mice at higher doses of methacholine (25-50 mg/ml). This change in Penh is proportional in both groups and may be as a direct influence of TGF- β on Mch-induced bronchoconstriction, as reported previously (McMillan and Lloyd, 2004), and not a result of anti-TGF- β mAb altering infection-induced protection. Therefore although the protective pulmonary response with infection is lessened in the absence of TGF- β (**Fig. 4.18B**), it is not reduced such that the protective effect to OVA-induced AHR noted in worm-infection is reversed.

4.2.5 *IL-10 mediates resistance of worm-infected mice to AHR.*

I have previously demonstrated that worm-infected mice are resistant to anaphylaxis through an IL-10-dependent mechanism (Chapter 3). In view of the elevated pulmonary IL-10 in OVA-sensitized worm-infected mice we used an anti-IL-10R mAb to block IL-10 activity and analysed lung function. OVA-sensitized worm-infected mice were fully susceptible to AHR when IL-10 was blocked, with significantly greater Penh values in these animals than OVA-sensitized uninfected mice at doses >12.5 mg/ml Mch ($P<0.01-0.001$; **Fig. 4.19**). Blocking IL-10 in uninfected mice did not alter the susceptibility of these mice to AHR. The increase in AHR in infected mice with IL-10 blocked was associated with a restoration of OVA-induced eosinophilia in the BAL (**Fig. 4.20A**). In addition OVA-sensitized worm-infected mice treated with anti-IL-10R mAb had significant elevations ($P<0.01$) in lung IL-5 levels versus OVA-sensitized worm-infected mice treated with a control Ig (**Fig. 4.20B**). This data demonstrates that schistosome worm infection prevents AHR in a mouse model of allergen-induced pulmonary inflammation via IL-10 dependent suppression of pulmonary eosinophilia.

4.2.6 *Cells infiltrating the lungs of worm-infected versus uninfected sensitized mice.*

In mice with worm + egg or worm infections a number of IL-10 producing cells have been identified, including CD4⁺ cells, CD4⁺CD25⁺ regulatory cells, macrophages and B cells (Hesse *et al.*, 2004) (Mangan *et al.*, 2004). The airway inflammatory response in asthma is associated with mucosal infiltration by a number of different cell types including eosinophils, basophils, mast cells and CD4⁺ lymphocytes (Bradley *et al.*, 1991). I have previously addressed the reduction in eosinophil numbers infiltrating the lungs of worm-infected mice (**Fig. 4.14-16**). As I was addressing a pulmonary allergic phenotype I characterized by flow cytometry which of the different potential

cellular sources of IL-10 preferentially infiltrated the lungs of worm-infected mice (**Fig. 4.21**). I found no major differences in the percentages of CD4⁺ cells, CD25⁺ cells or macrophages infiltrating the lungs of sensitized worm-infected versus sensitized uninfected mice (**Fig. 4.21A, 22A, 23A**).

4.2.7 *Resistance of worm-infected mice to AHR is independent of CD4⁺, CD25⁺ cells and macrophages.*

The absence of a role for CD4⁺ and CD25⁺ cells in the resistance of worm-infected mice to AHR was further corroborated by *in vivo* depletion studies whereby depletion of either CD4⁺ (**Fig. 4.21A**) or CD25⁺ (**Fig. 4.22A**) cells in worm-infected mice demonstrated that each cell population has no role in affording protection from OVA-induced AHR (**Fig. 4.21B, 4.22B**) or in altering the susceptibility of uninfected mice to AHR. Furthermore, depletion of pulmonary macrophages had no effect on the worm-infection mediated airway protection, with OVA-sensitized worm-infected mice having lower Penh values compared with uninfected mice that developed AHR (**Fig. 4.23**). Efficacy of all depletions was assessed as described in chapter 3.

4.2.8 *Resistance of worm-infected mice to AHR is dependent on B cells.*

Previously, I have shown that worm-infected mice are resistant to anaphylaxis via an uncharacterized schistosome-induced spleen B cell subpopulation (Chapter 3). Significantly, there was a ~30 % increase in the number of infiltrating B cells in the lungs of sensitized worm-infected mice when compared with sensitized uninfected mice (**Fig. 4.24**). Following anti-IgM treatment, OVA-sensitized worm-infected mice were fully susceptible to AHR (**Fig. 4.25A**). Thus, even partial depletion of B cells was sufficient to mitigate the protection afforded by worm-infection. These data suggest that a B cell population is intrinsic in the mechanism of resistance of worm-infected

mice to AHR. In fact, the airway reactivity of these mice was even more enhanced than in uninfected mice indicating the significance of B cells in the protection from pulmonary inflammation. The susceptibility of worm-infected mice to AHR after B cell depletion was associated with a restoration of eosinophil infiltration of the lungs (**Fig. 4.25B**).

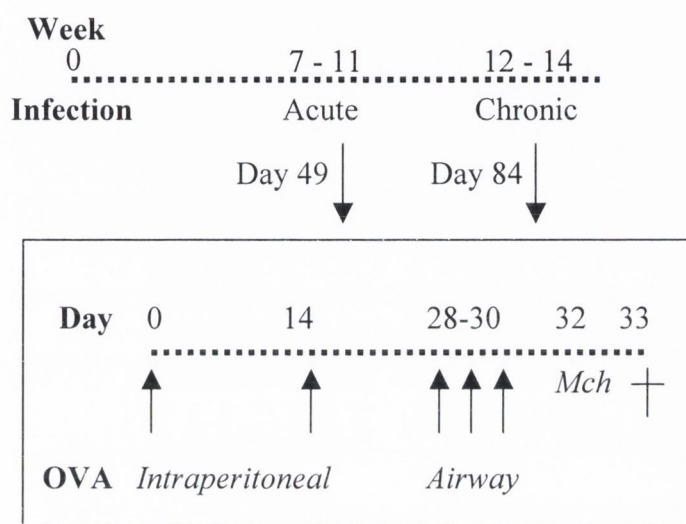


Figure 4.1. Protocol for OVA sensitization and challenge in mice for induction of AHR. Infected mice were sensitized in the acute, starting week 7, or chronic, starting week 12, stages of infection. Mice were systemically sensitized with an ip injection of 20 μ g OVA (plus 2 mg alum) on Day 0 and 14. Mice were challenged via the airways from days 28-30 with an aerosol of OVA (1% OVA for 20 min). On day 32 lung function in response to methacholine (Mch) was tested using either restrained or unrestrained plethysmography, as described in Section 2.5. Depletion regimes were performed on days 27 and 30. Mice were killed on day 33 (cross).

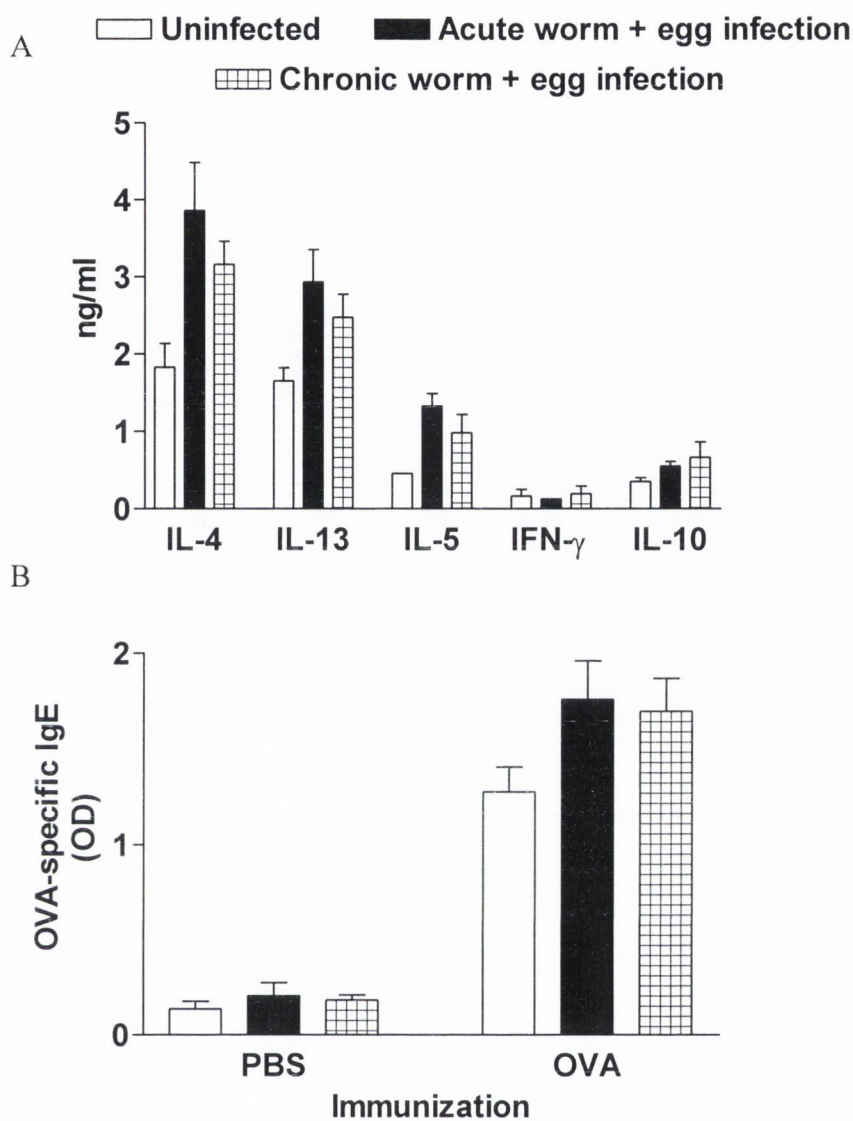
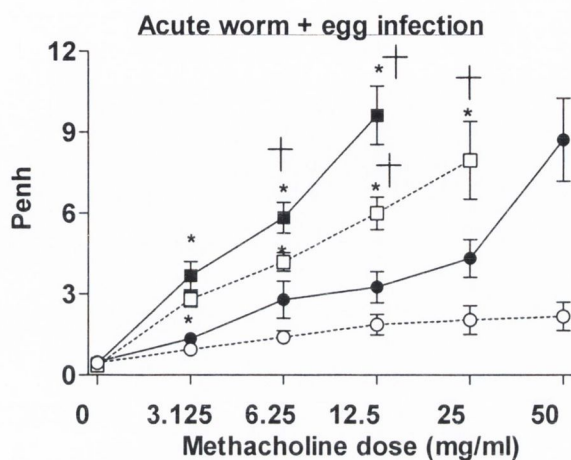


Figure 4.2. *Schistosoma mansoni* worm + egg-infected mice produce elevated type 2 responses to ovalbumin sensitization. OVA was administered to uninfected mice and to animals during the acute or chronic stages of a worm + egg-infection, as described in Fig. 4.1. (A) Detection of *in vitro* production of cytokines from spleen cells stimulated with OVA (200 μ g/ml). Spleens from 3 mice were pooled. (B) Levels of OVA-specific IgE. All data are mean + SD. Data are representative of 2-3 separate experiments.

A

○ Uninfected-PBS □ Worm + egg-infected-PBS
 ● Uninfected-OVA ■ Worm + egg-infected-OVA



B

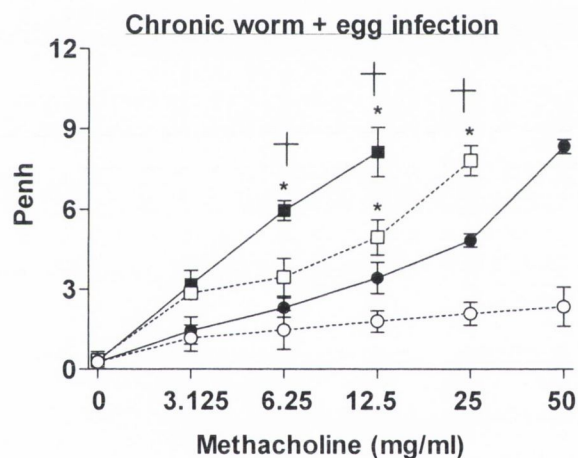


Figure 4.3. *Schistosoma mansoni* worm + egg-infected mice have increased susceptibility to AHR. Uninfected and worm + egg-infected (acute and chronic) mice were unsensitized (PBS) or sensitized with OVA, as described in Fig. 4.1. Penh responses of (A) acute and (B) chronically worm + egg-infected mice. Data represents the mean change from baseline PBS values for each group. Penh is presented as mean $\pm$ SEM from 3 separate experiments ($n = 12-15$ mice). Statistical differences between infected mice and uninfected OVA-sensitized mice was tested by Student's t-test; * = $P < 0.05$. Deaths are denoted with crosses.

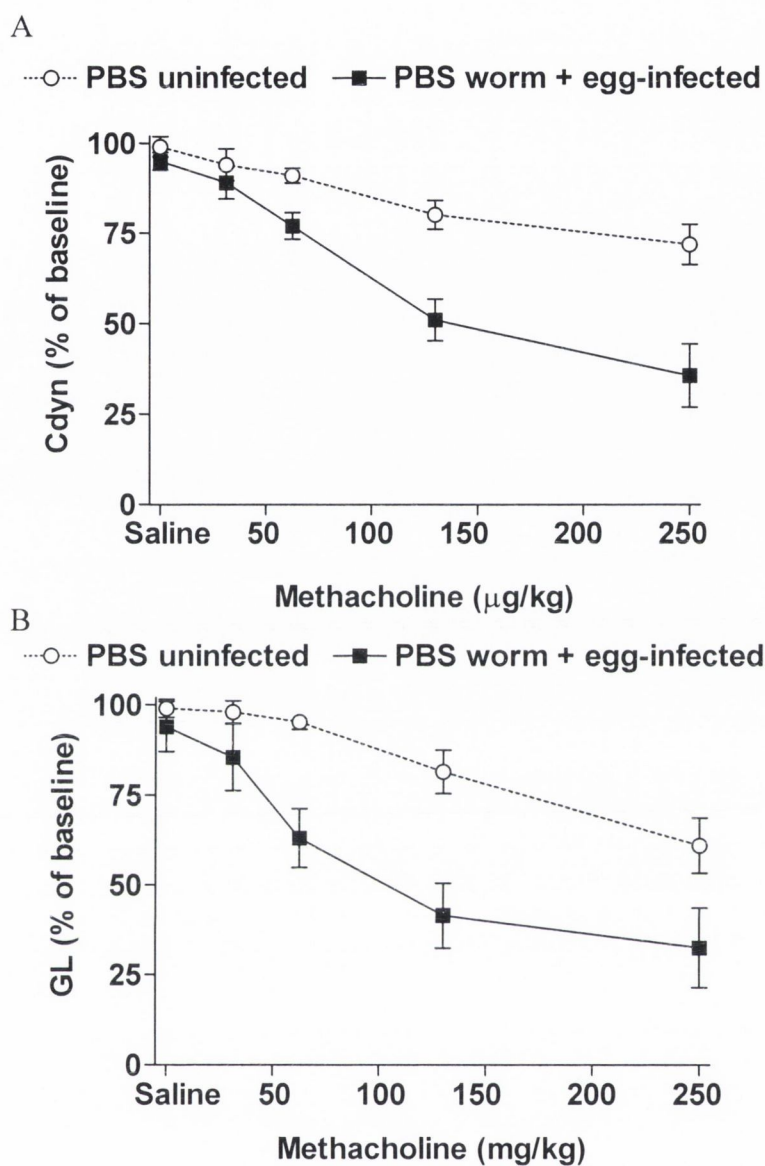


Figure 4.4. *Schistosoma mansoni* worm + egg-infected mice have increased susceptibility to AHR as determined by compliance and resistance studies. Uninfected and worm + egg-infected mice were unsensitized (PBS) or sensitized with OVA, as described in Fig. 4.1. Compliance dynamics (Cdyn; A) and Resistance (GL; B) were measured in restrained and anaesthetised mice for increasing doses of methacholine administered ip. Results are mean $\pm$ SEM with 5-6 mice per group and are representative of 2 separate experiments.

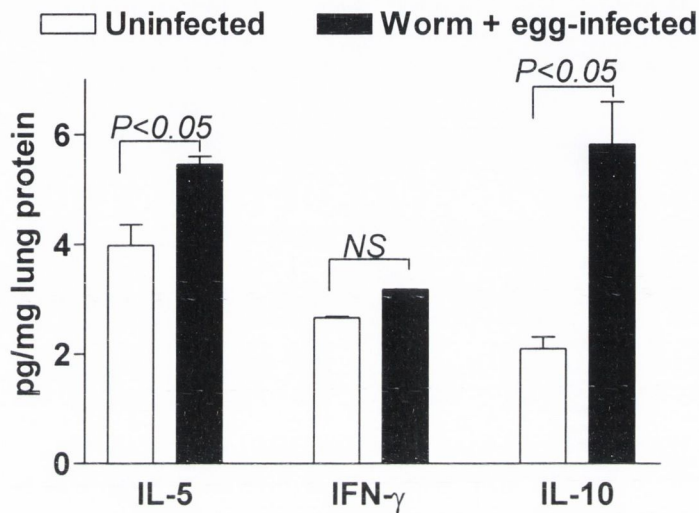


Figure 4.5. *Schistosoma mansoni* worm + egg-infected mice produce an elevated type 2 responses following ovalbumin sensitization in the lung. OVA was administered to uninfected mice and to animals during a worm + egg-infection, as described in Fig. 4.1. Cytokines from lung homogenates of treated mice were measured. Lungs from individual mice were assessed (n=3-5 per group). Statistical analysis was performed by Student's t-test. NS = not significant.

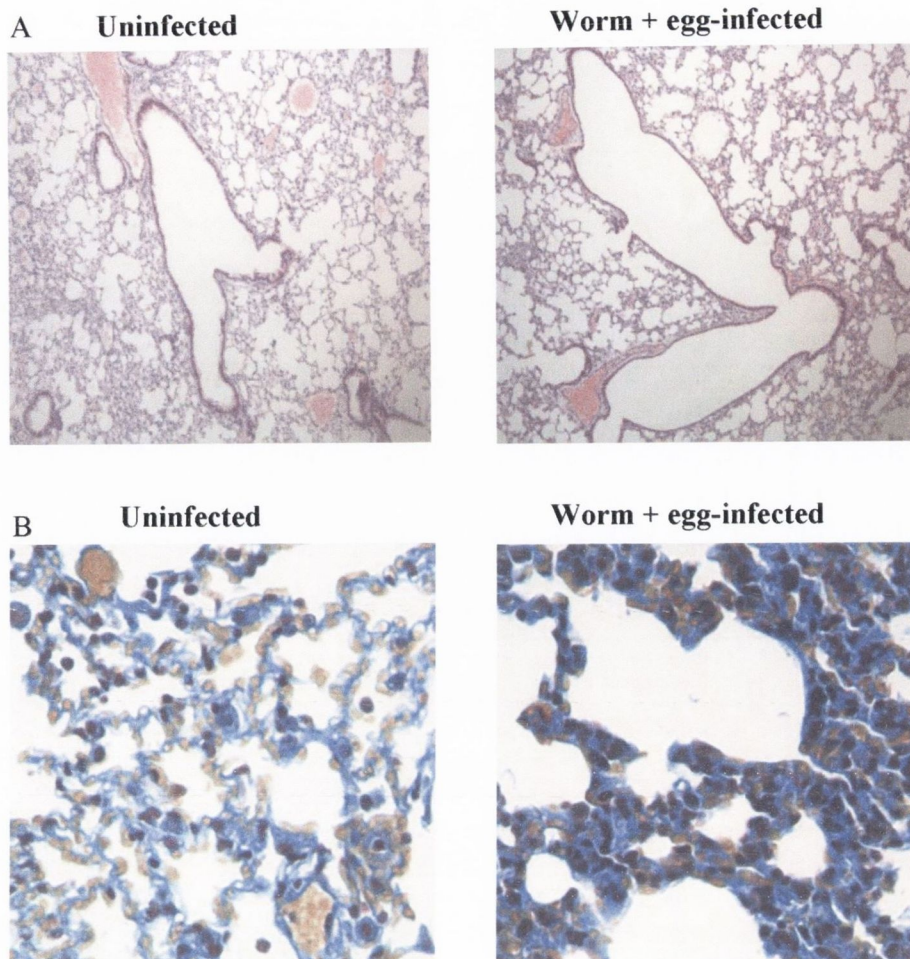


Figure 4.6. *S. mansoni* worm + egg-infected mice have increased pulmonary fibrosis. (A) Representative hematoxylin and eosin-stained sections (10X magnification) from lungs from uninfected and worm + egg-infected mice indicating the absence of inflammation in lungs of a 10 week worm + egg-infected mouse following Mch challenge. (B) Increased pulmonary fibrosis (blue stain; 40X magnification) in worm + egg-infected mice relative to uninfected mice (Martius Scarlett Blue stain) following Mch challenge.

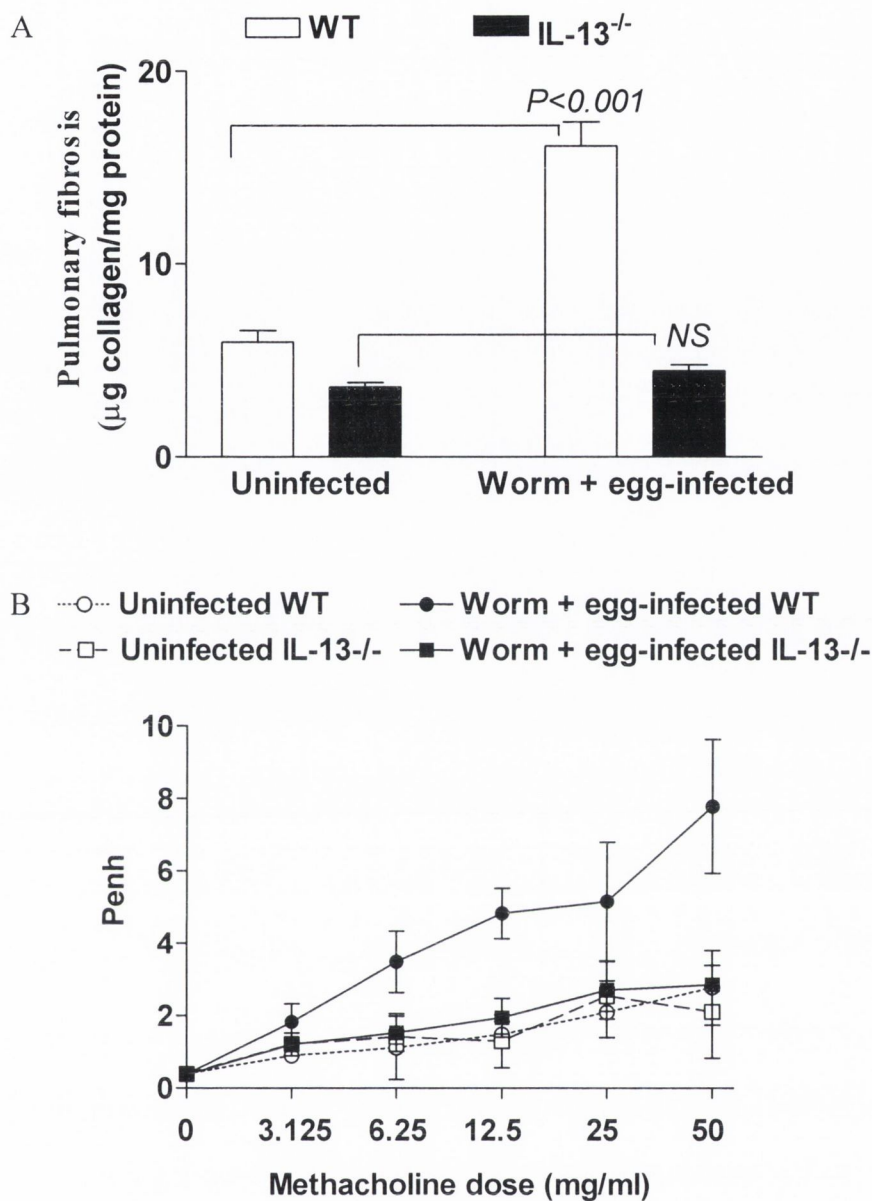


Figure 4.7. Worm + egg-infected IL-13^{-/-} mice are protected from AHR. (A) Quantification of pulmonary collagen levels in worm + egg-infected wild type (WT) and IL-13 deficient (-/-) mice relative to levels in uninfected mice. Data are mean + SEM from 6-8 mice per group. Statistical differences between levels of collagen in uninfected versus infected WT or IL-13^{-/-} mice were tested by Student's t-test, NS = not significant. (B) Worm + egg-infected IL-13^{-/-} do not develop spontaneous AHR, whereas worm + egg-infected WT mice have dose-dependent elevations in Penh. Penh values are mean $\pm$ SEM from 7-12 mice from two separate experiments.

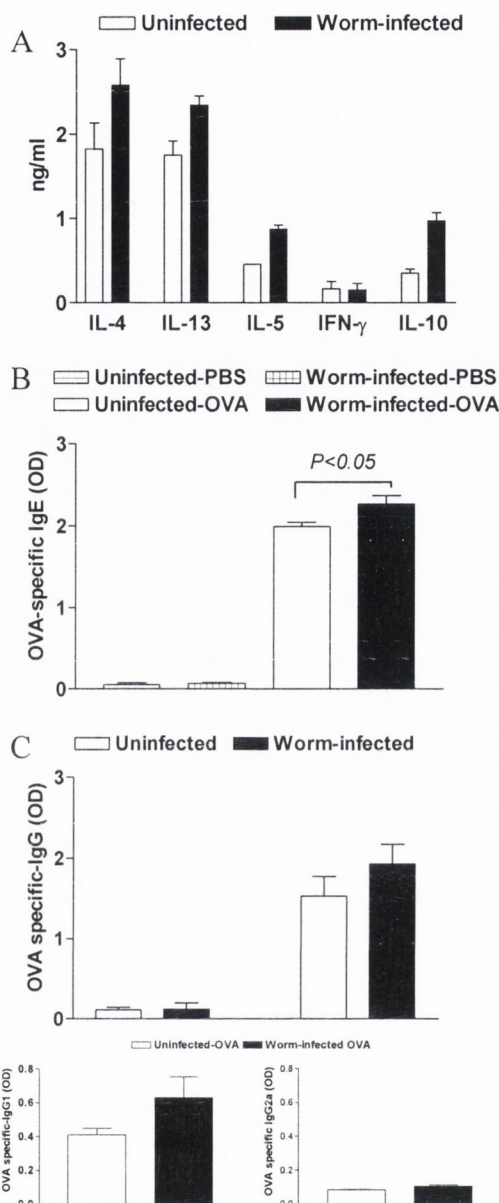


Figure 4.8. Spleen cells from *Schistosoma mansoni* worm-infected mice produce elevated allergen-induced IL-10 and type 2 responses following OVA sensitization. (A) Uninfected and worm-infected (8 weeks post infection) mice were sensitized with OVA as described in Fig. 4.1. Spleen cells were stimulated with OVA (200 μ g/ml) *in vitro* and cytokines in supernatants detected by ELISA. Data are means + SD from pools of spleens from 3 mice, and are representative of three experiments. (B) Serum levels of OVA-IgE were detected by direct ELISA. (C) Total OVA-specific IgG and OVA-IgG1 and IgG2a were also detected in the serum of individual uninfected or worm-infected OVA sensitized mice. Differences were assessed by Student's t-test; n=6-7 mice per group.

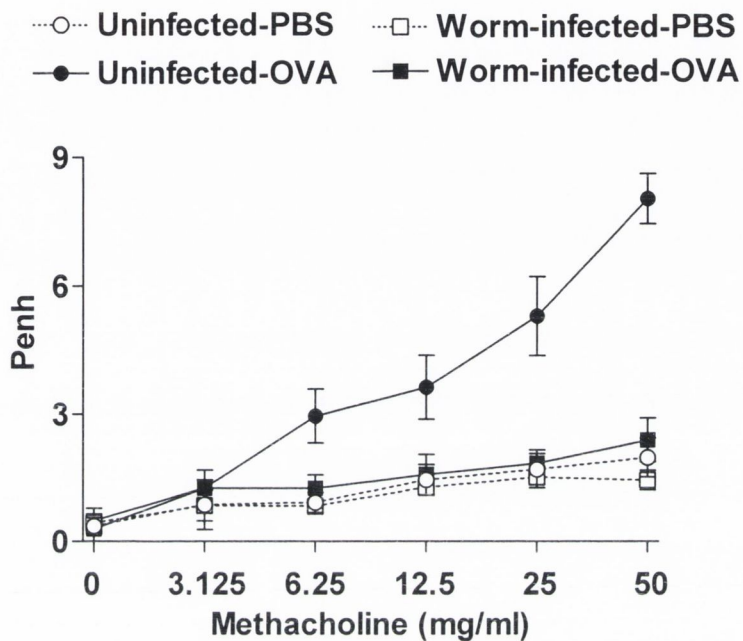


Figure 4.9. *Schistosoma mansoni* worm-worm infected mice are refractory to OVA-induced AHR as determined by Penh. Uninfected and worm-infected mice (8 weeks post infection) were exposed to OVA or PBS and AHR was determined as Penh as described in Fig. 4.1. Data are presented as mean $\pm$ SEM. Penh values are from 2 separate experiments, n = 16-21 mice per group.

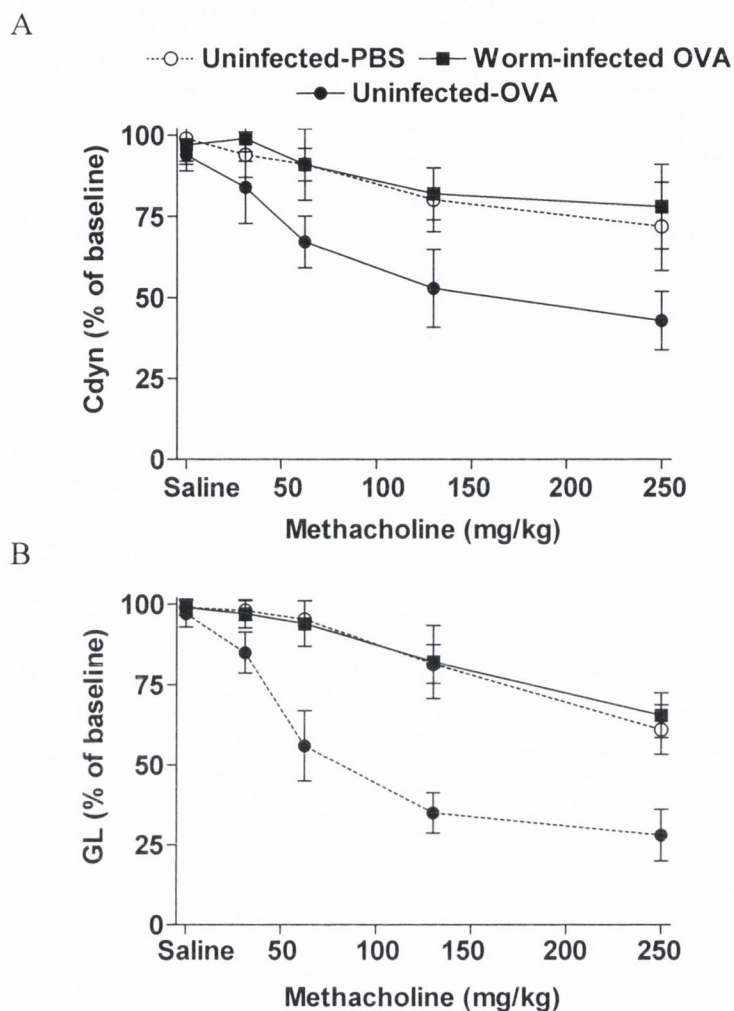


Figure 4.10. *Schistosoma mansoni* worm-infected mice are refractory to OVA-induced AHR as determined by compliance and resistance studies. (A) Compliance (Cdyn) or (B) pulmonary resistance (GL). Uninfected and worm-infected mice (8 weeks post infection) were sensitized with OVA as described in Fig. 4.1. Lung function was assessed on restrained and anaesthetised mice for increasing doses of methacholine administered ip. GL and Cdyn were from 2 separate experiments; n = 8-10 mice per group. Data are presented as mean $\pm$ SEM.

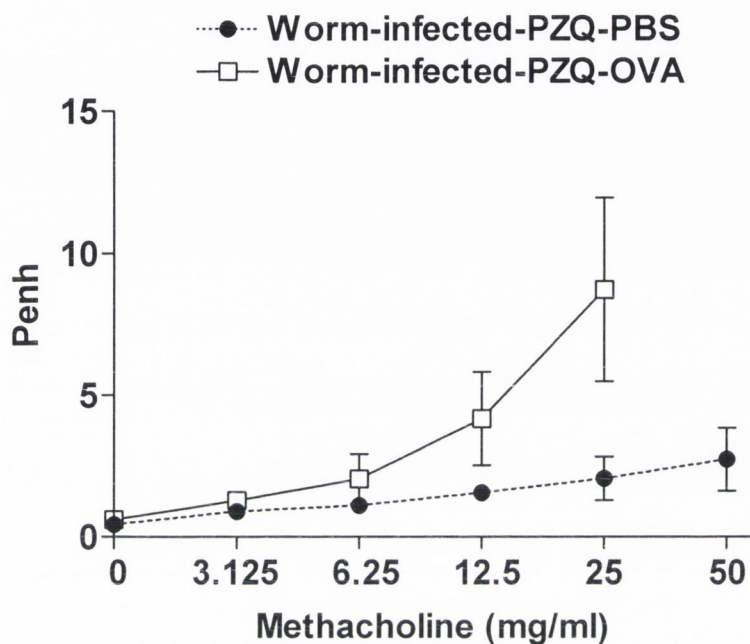


Figure 4.11. *Praziquantel treatment of worm-infected mice.* 5-6 week-worm-infected mice were treated with intra-gastric administration of the schistosomicidal drug praziquantel (PZQ; 100 mg/kg) over five days. The efficacy of treatment was assessed eight weeks later by portal perfusion. OVA was administered to mice as described in Fig. 4.1. Penh responses of treated and untreated PBS or OVA-sensitized worm-infected mice were assessed in response to methacholine challenge. Data represent the mean change from baseline PBS values for each group. Penh is presented as mean $\pm$ SEM, (n=7-9).

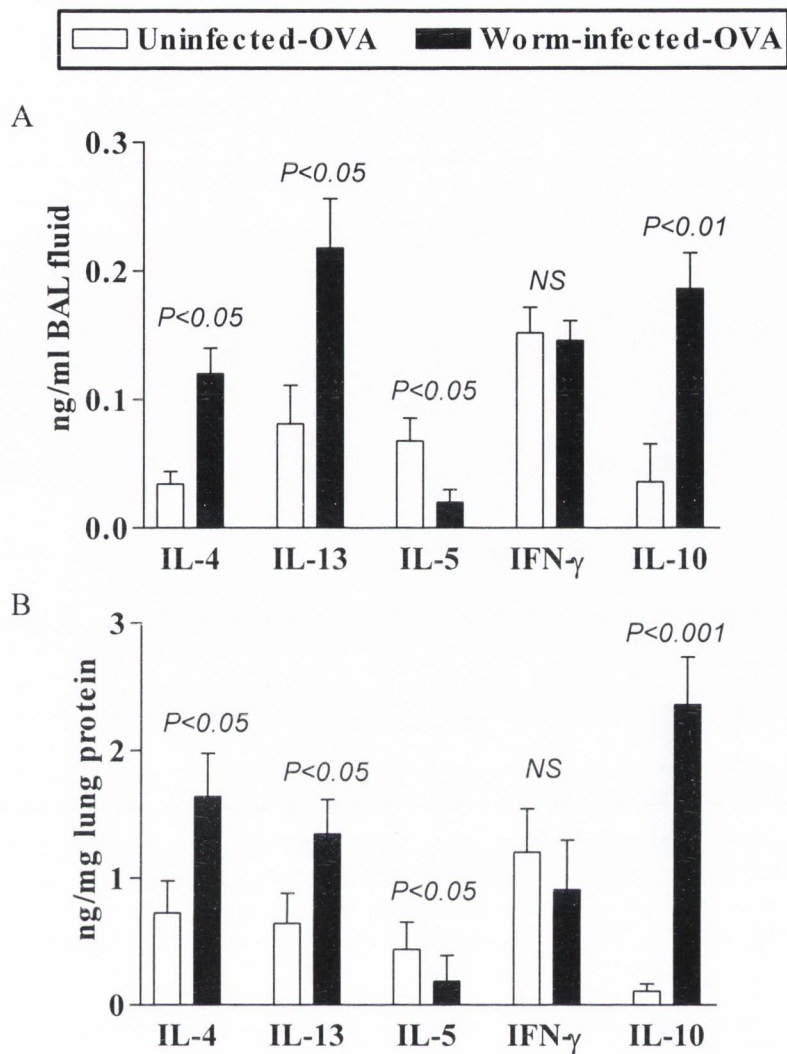


Figure 4.12. *Modified type 2 response and elevated pulmonary IL-10 in OVA-sensitized worm-infected mice.* (A) Levels of cytokines in BAL from OVA-sensitized uninfected and worm-infected mice. Data are mean + SEM from 5-8 individual mice, and are representative from 2 separate experiments. Student's t-test was used to determine differences between uninfected and infected mice. (B) Levels of cytokines in lung homogenates from OVA-sensitized uninfected and worm-infected mice. Cytokine values were adjusted to ng per mg lung protein. Data are mean + SEM from 6-9 individual mice, and are representative from 3 separate experiments. Student's t-test was used to determine difference between uninfected and infected mice: NS = not significant.

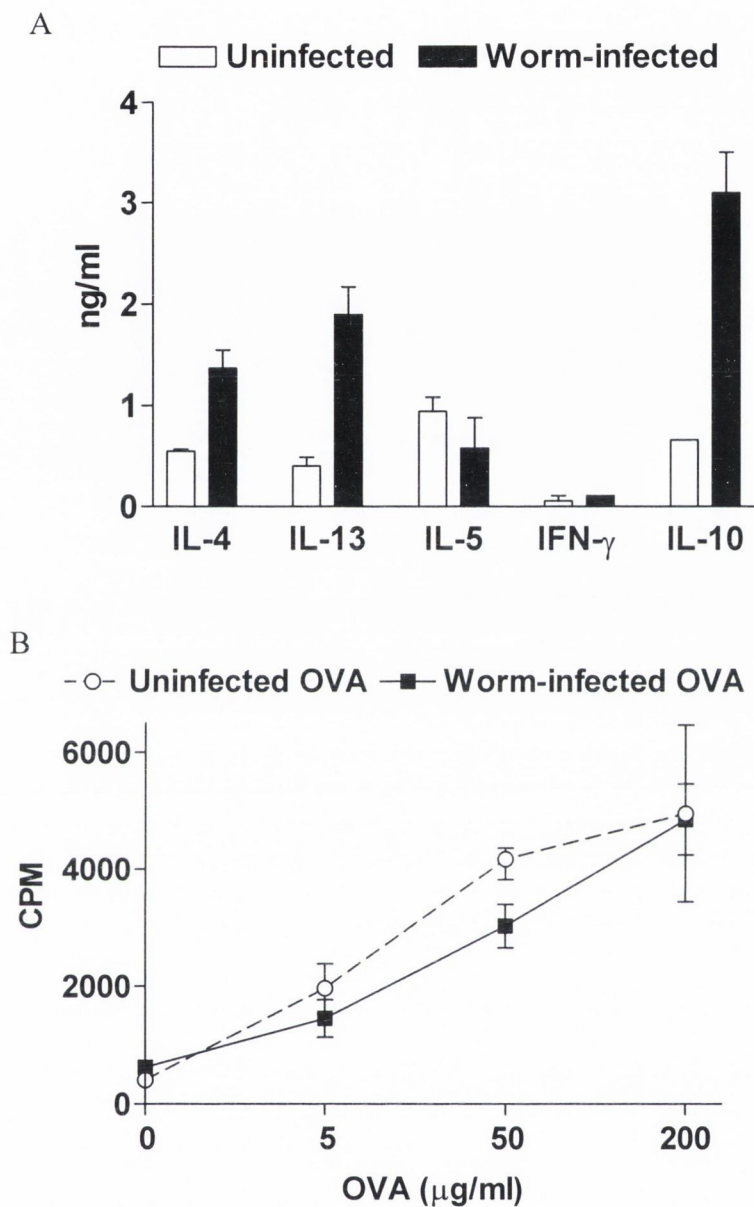


Figure 4.13. *Worm-infected mice had a modified type 2 responses but normal T cell proliferation to OVA.* (A) OVA-specific cytokine responses from mediastinal lymph node cells from OVA-sensitized uninfected and worm-infected mice. Lymph nodes from 4-8 mice were pooled and cells cultured in duplicate or triplicate with OVA (200 μ g/ml). (B) Cell proliferation of mediastinal lymph node cells to different concentrations of OVA was measured by [3 H] incorporation and are expressed as CPM. Data are representative of two separate experiments.

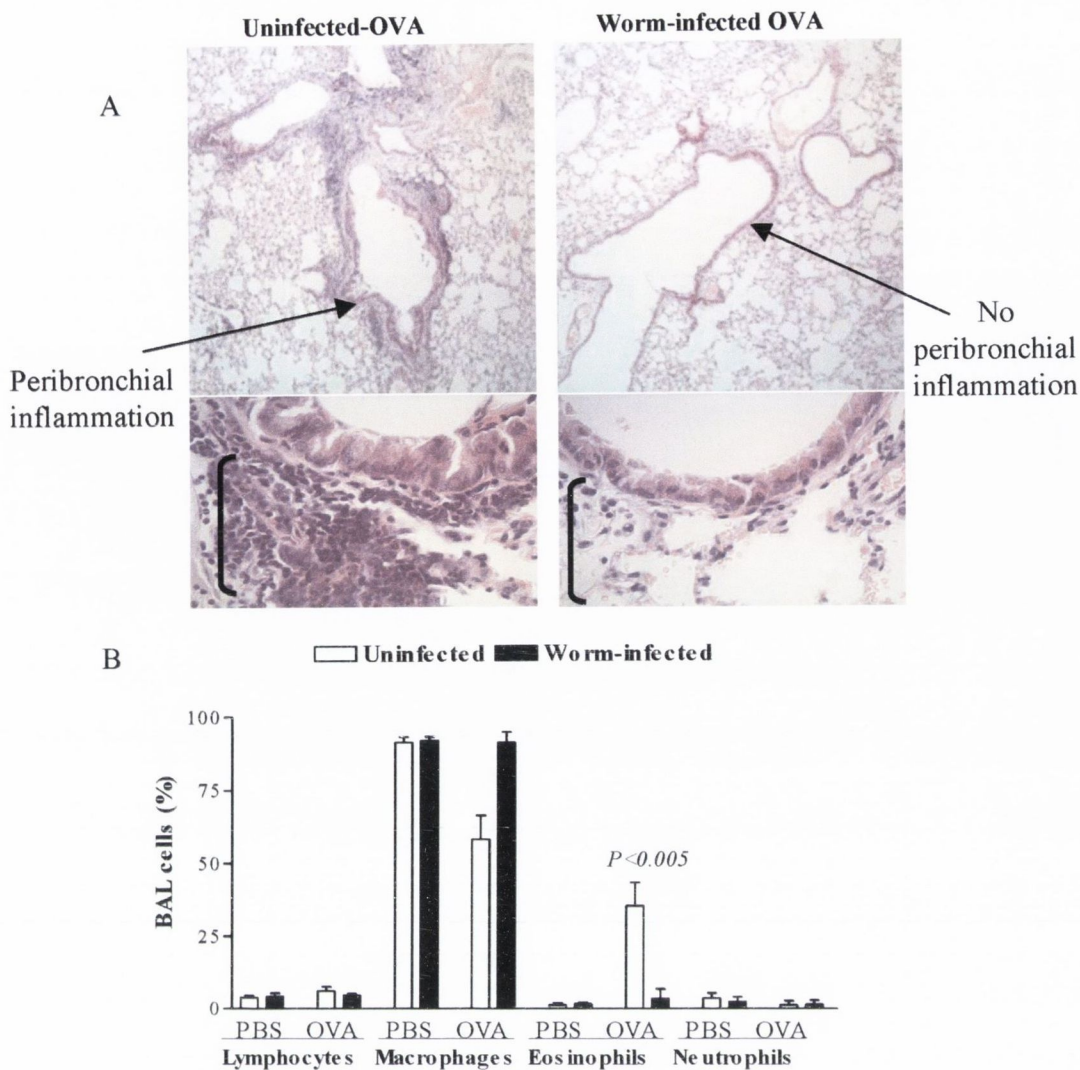


Figure 4.14. *Absence of pulmonary eosinophilia in OVA-sensitized worm-infected mice.* (A) Hematoxylin and eosin-stained sections of lungs from OVA-sensitized uninfected and worm-infected mice at 10X (top panels) and 40X magnification (lower panels). H + E-stained sections of lungs from OVA-sensitized uninfected and worm-infected mice, showing peribronchial eosinophilia in uninfected mice (bracket). (B) Differential cell counts of cytopins of BAL cells from uninfected and worm-infected mice exposed to OVA or PBS. Data are means + SEM from 5-8 individual mice and are representative of two separate experiments. Student's test was used to test for statistical differences in the percentage of BAL eosinophils.

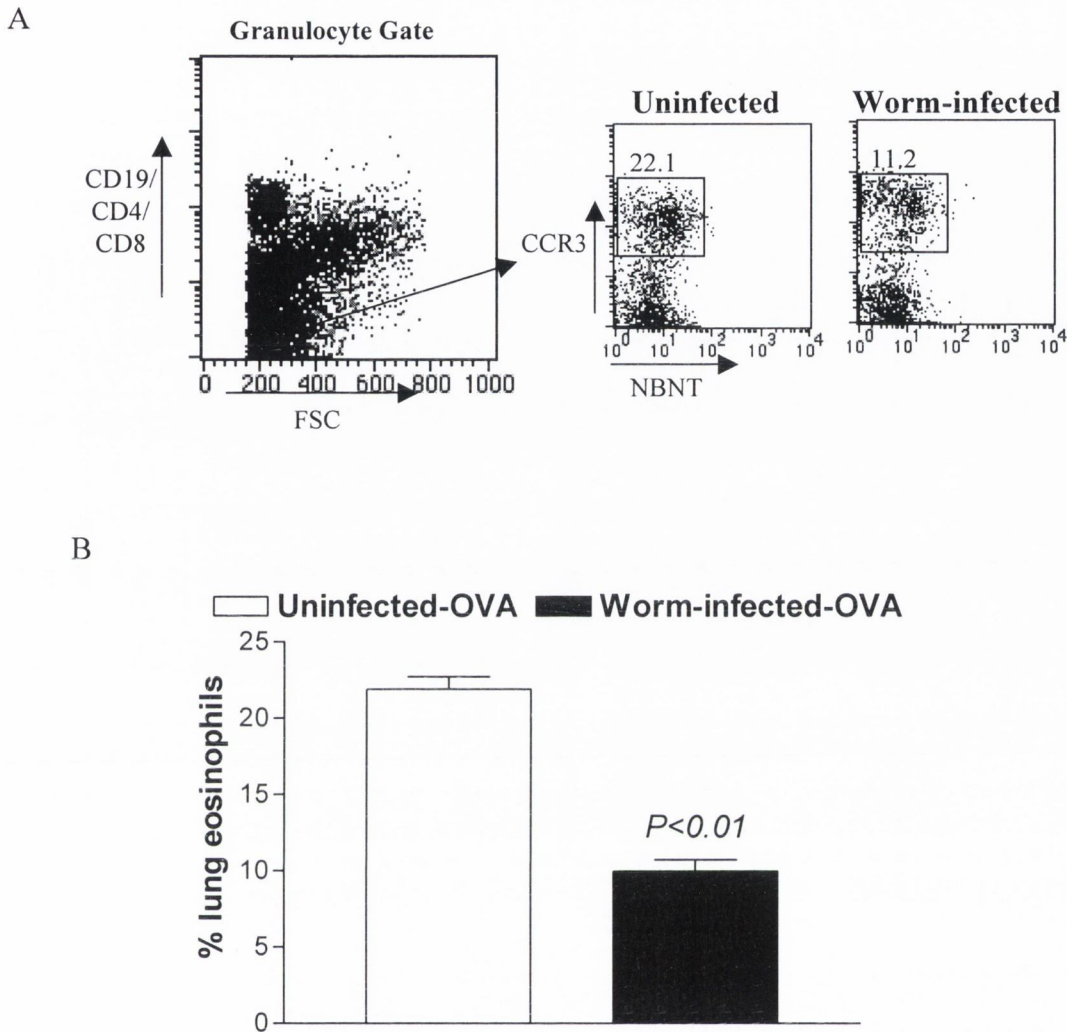


Figure 4.15. *Reduced frequency of eosinophils in the lungs of worm-infected mice.* (A) Representative image of granulocytes that are eosinophils present in the lungs of uninfected and worm-infected (8 weeks post infection) OVA-sensitized mice as determined by immunofluorescence surface staining in lung digests of OVA-sensitized uninfected and worm-infected mice. Eosinophils were phenotyped as SSC^{hi}, CD19⁻, CD4⁻, CD8⁻, CCR3⁺ by flow cytometry. (B) Percentages of eosinophils as assessed by flow cytometry in lung digests of uninfected and worm-infected mice. Statistical analysis was performed by Student's t-test; n=5 mice per group.

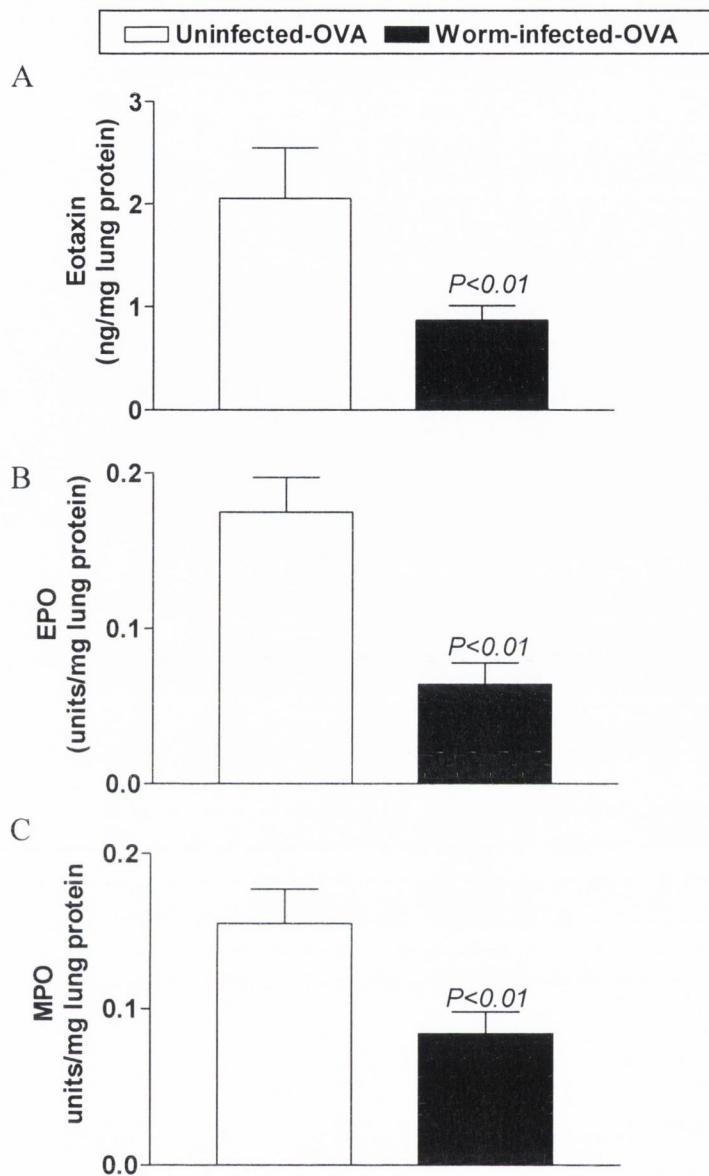


Figure 4.16. *Reduced recruitment of eosinophils to the lungs of worm-infected mice.* Detection of eotaxin (A) and eosinophil peroxidase enzymatic activity (EPO; B) by ELISA in lung homogenates from OVA-sensitized uninfected and worm-infected mice. (C) Measurements of the levels of myeloperoxidase (MPO) enzyme activity from the lung digests of OVA-sensitized uninfected and worm-infected mice. Statistical differences between lungs of infected mice and uninfected OVA-sensitized mice was tested by Student's t-test; $P < 0.01$. All data are mean + SEM from 5-9 individual mice from 3 separate experiments.

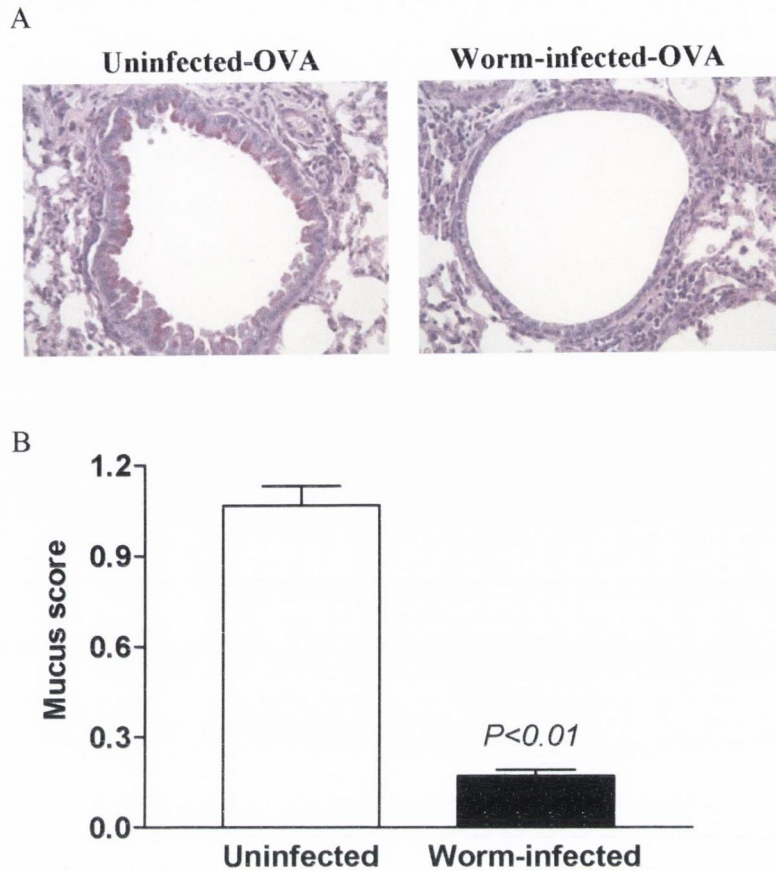


Figure 4.17. *Worm-infected mice have reduced mucus in the lungs.* Worm-infected mice sensitized with OVA, as described in Fig 4.1, demonstrate reduced goblet cell hyperplasia (A) and mucus score (B) compared with sensitized uninfected mice. Lung sections from mice were stained with Periodic Acid-Schiff stain to visualise goblet cells. See section 2.2.7 for mucus scoring system. Statistical differences between lungs (n=6) of worm-infected mice and uninfected OVA-sensitized mice was tested by Student's t-test; $P < 0.01$.

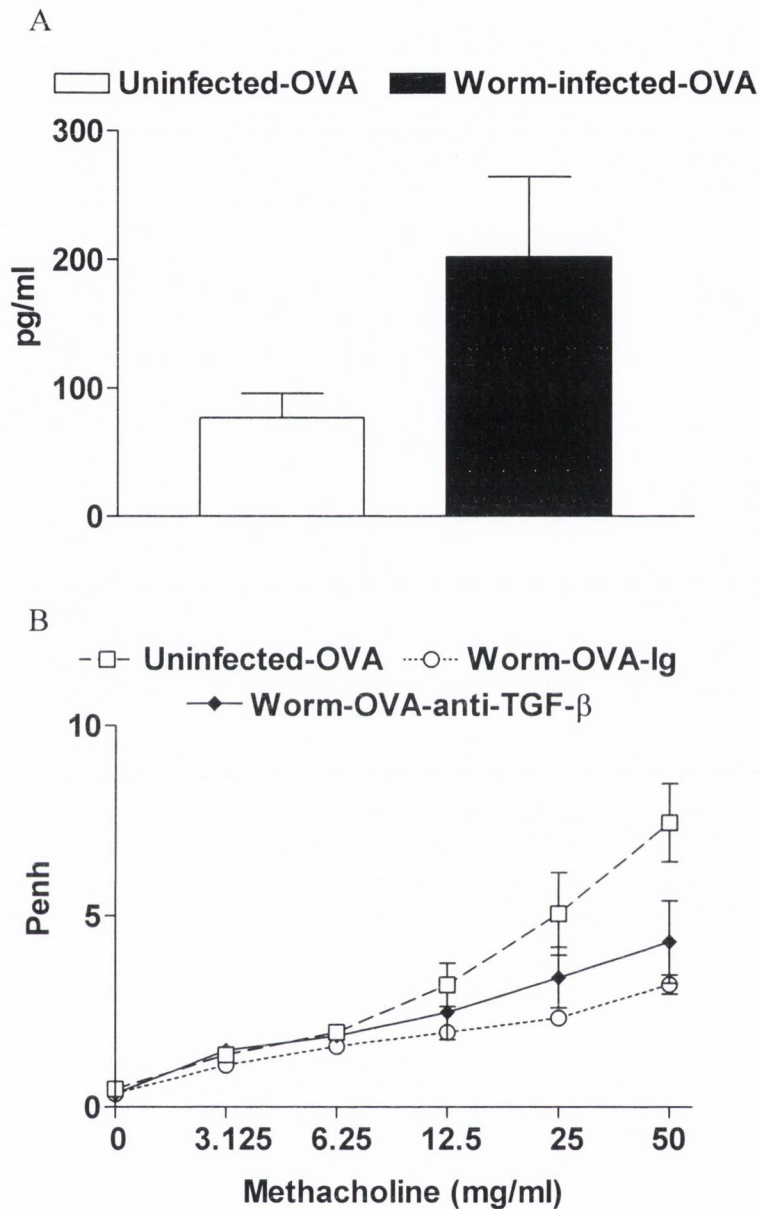


Figure 4.18. *No role for TGF- β in worm-infection protection in AHR.* Uninfected and worm-infected mice were sensitized with OVA as described in Fig. 4.1. (A) Levels of TGF- β in the supernatant from mediastinal lymph node cell cultures of uninfected and worm-infected mice were detected by ELISA. (B) Mice were treated with anti-TGF- β antibody. Data represent the mean change from baseline Penh values for each group. Results represent 2 separate experiments $n=8-10$.

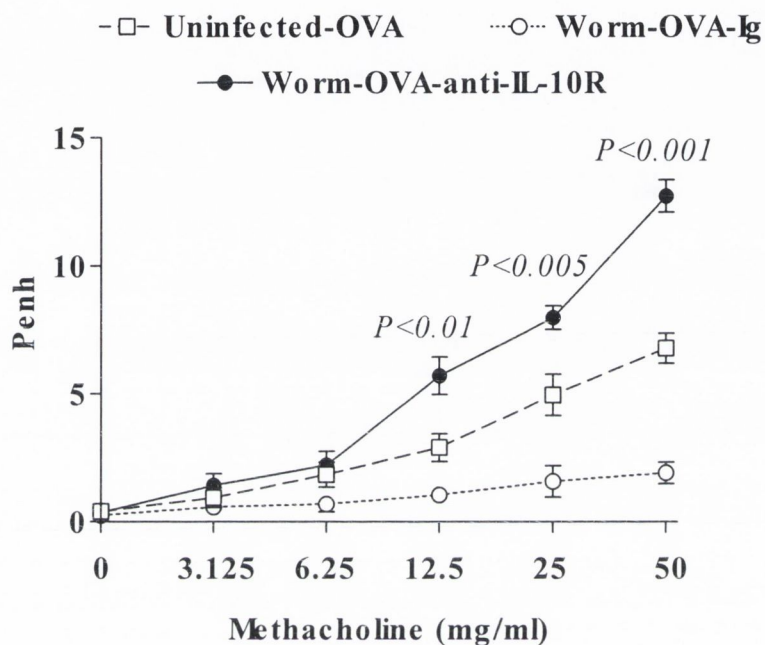


Figure 4.19. Resistance of worm-infected mice from AHR is dependent on IL-10. Penh responses of OVA-sensitized uninfected mice and sensitized-infected mice treated with anti-IL-10R or control IgG. Student's t-test was used to determine difference between uninfected mice and anti-IL-10R treated infected mice. Data are presented as the mean change from baseline Penh values for each group. All data are presented as group mean $\pm$ SEM (n=8-10) and are representative of at least 2 separate experiments. Statistical differences between infected mice and uninfected OVA-sensitized mice were tested by Student's t-test.

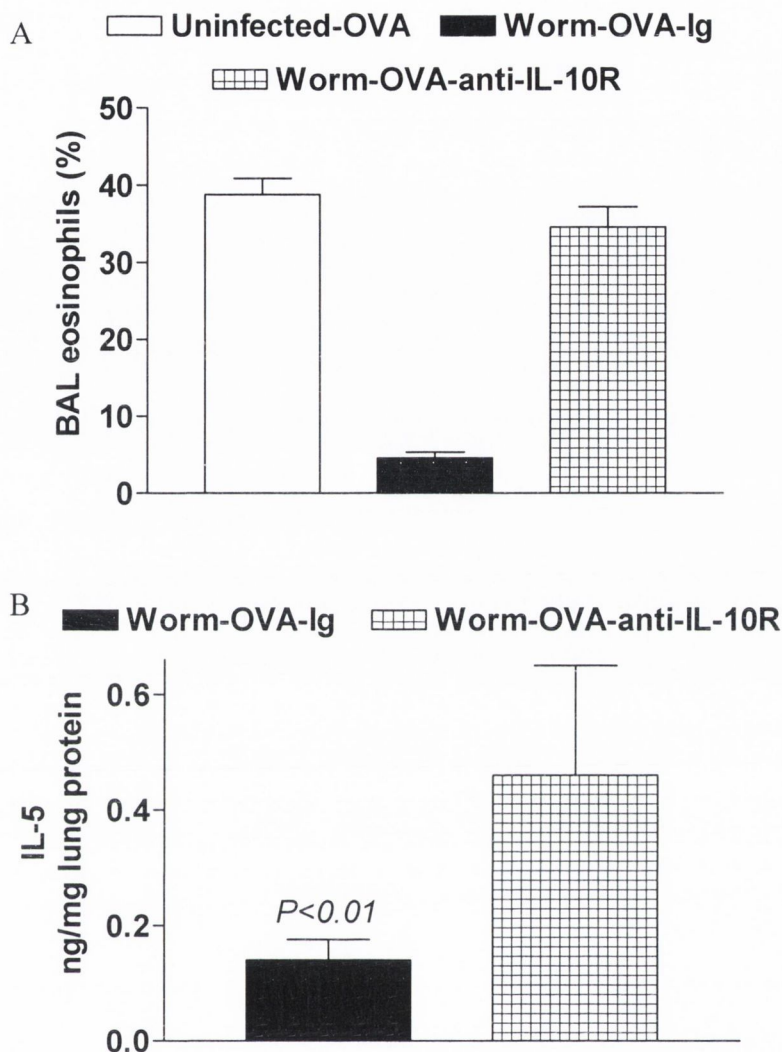


Figure 4.20. Differential cell counts of cytopins of BAL cells from uninfected and worm-infected mice exposed to OVA or PBS. (A) Percentage of eosinophils in the BAL of OVA-sensitized mice. Data are mean + SEM from 5-8 individual mice and are representative of two separate experiments. (B) Levels of IL-5 cytokine in lung homogenates from OVA-sensitized anti-IL-10R-treated and Ig-treated worm-infected mice. Cytokine values were adjusted to ng per mg lung protein. All data are presented as group mean + SEM (n=7-10) and are representative of at least 2 separate experiments. Statistical analysis was performed by Student's t-test.

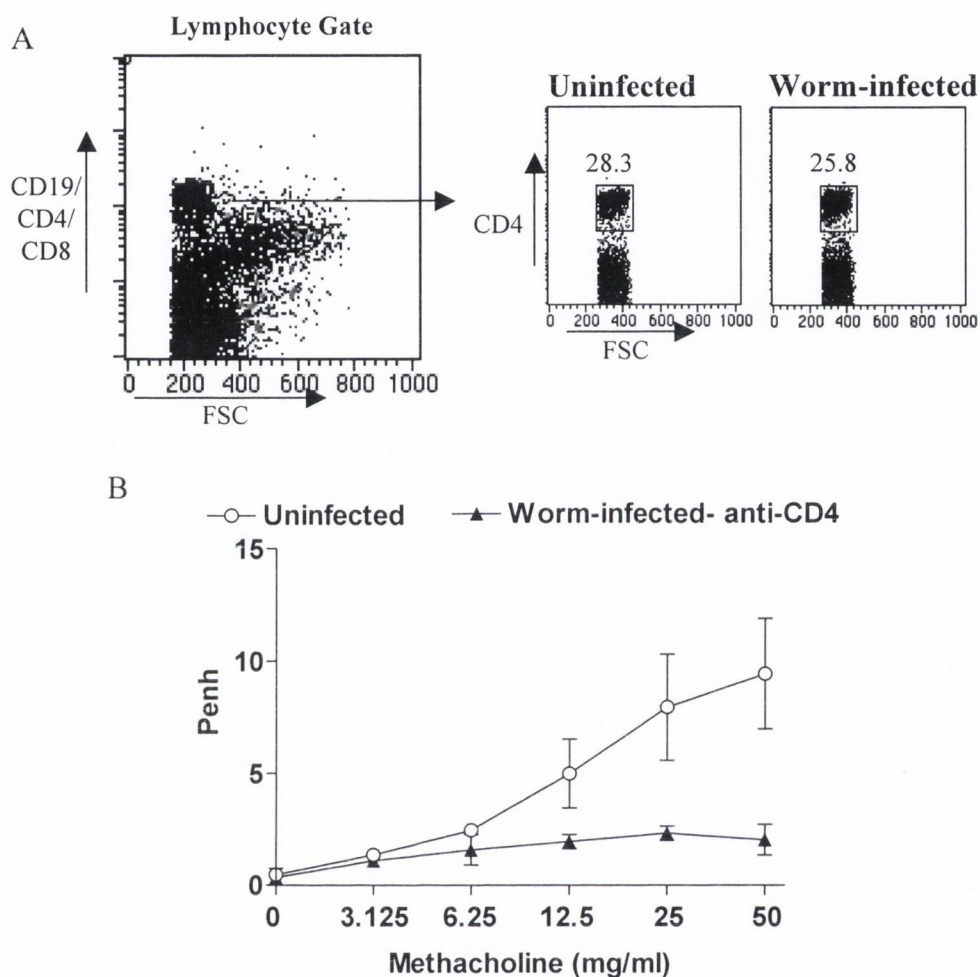


Figure 4.21. *No role for CD4⁺ T cells in worm protection in AHR.* (A) Percentage of CD4⁺ T cells present in the lungs of uninfected and worm-infected mice as determined by immunofluorescence surface staining for lymphocytes in lung digests of OVA-sensitized uninfected and worm-infected mice. (B) OVA sensitized mice were administered anti-CD4 (YTS 191; 0.5 mg/mouse) depletion treatments on days 27 and 30 of OVA sensitization and challenge protocol. All depletions were confirmed to be effective by flow cytometry (Chapter 3). Data represent the mean change from baseline Penh values for each group. Data are group mean $\pm$ SEM from 3 separate experiments; n=10 - 12 mice per group.

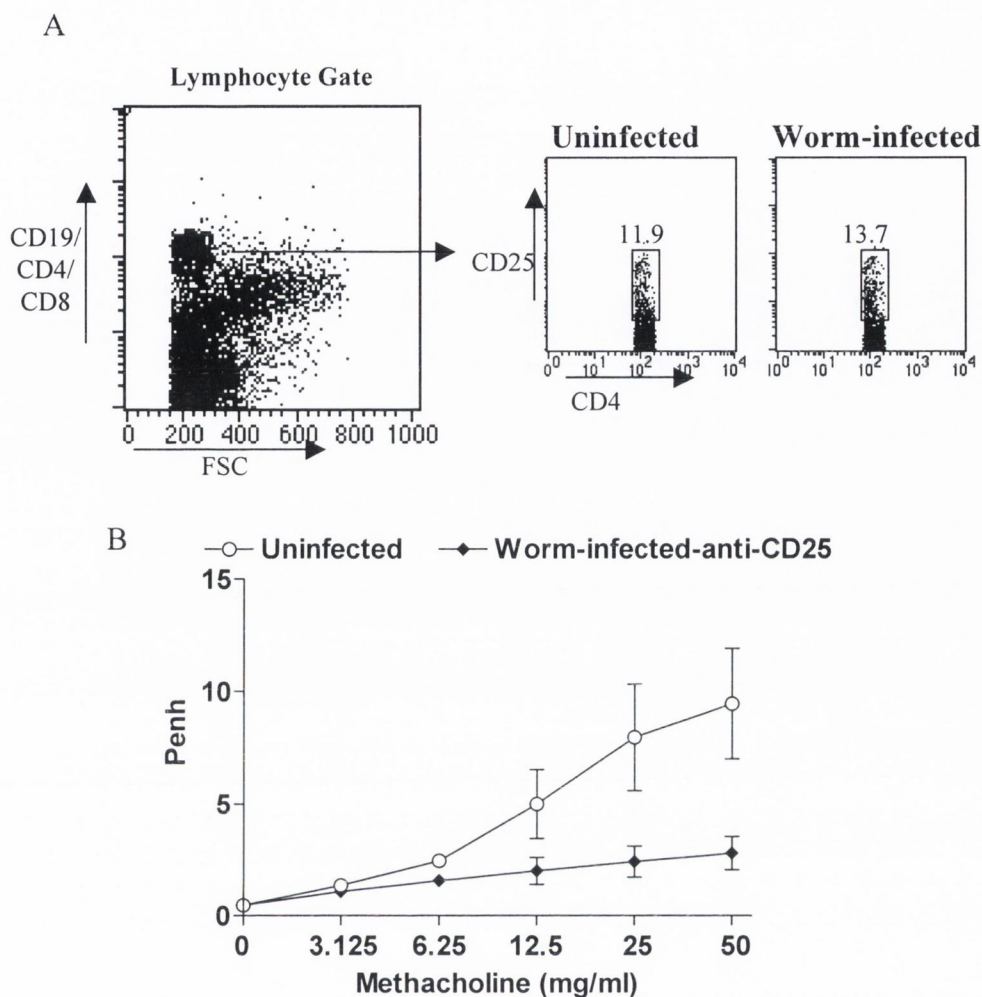


Figure 4.22. No role for $CD25^+$ cells in worm protection in AHR. (A) FACS of lung cell populations. Percentage of $CD4^+ CD25^+$ T cells present in the lungs of uninfected and worm-infected mice as determined by immunofluorescence surface staining for lymphocytes in lung digests of OVA-sensitized uninfected and worm-infected mice. (B) OVA-sensitized mice were administered anti-CD25 (0.25 mg/mouse) depletion treatments on days 27 and 30 of OVA sensitization and challenge protocol. Efficacy of depletion were confirmed by flow cytometry (Chapter 3). Data represent the mean change from baseline Penh values for each group. Data are from 2 separate experiments; $n = 8 - 10$ mice per group.

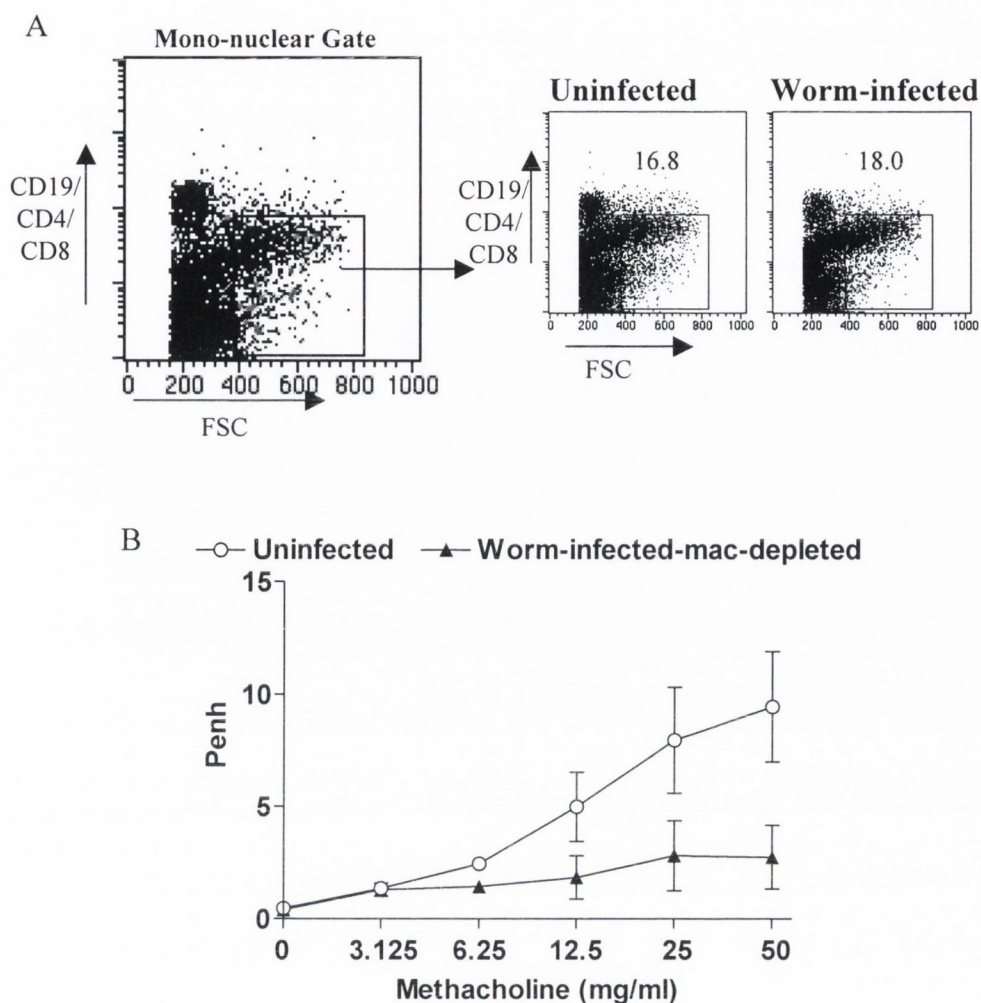


Figure 4.23. *Worm protection in AHR is independent of macrophages.* (A) Percentage of mono-nuclear cells present in the lungs of uninfected and worm-infected mice as determined by immunofluorescence staining of lung digests of OVA-sensitized uninfected and worm-infected mice. These cells were confirmed to be F4/80⁺. (B) OVA sensitized mice were administered clodronate coated liposomes (50 μ l liposomes in on day 27 and 0.1 ml ip on day 30). Depletions were confirmed to be effective by flow cytometry (refer to Chapter 3). Data are from 2 separate experiments; n = 10 - 12 mice per group. Data represent the mean change from baseline Penh values for each group.

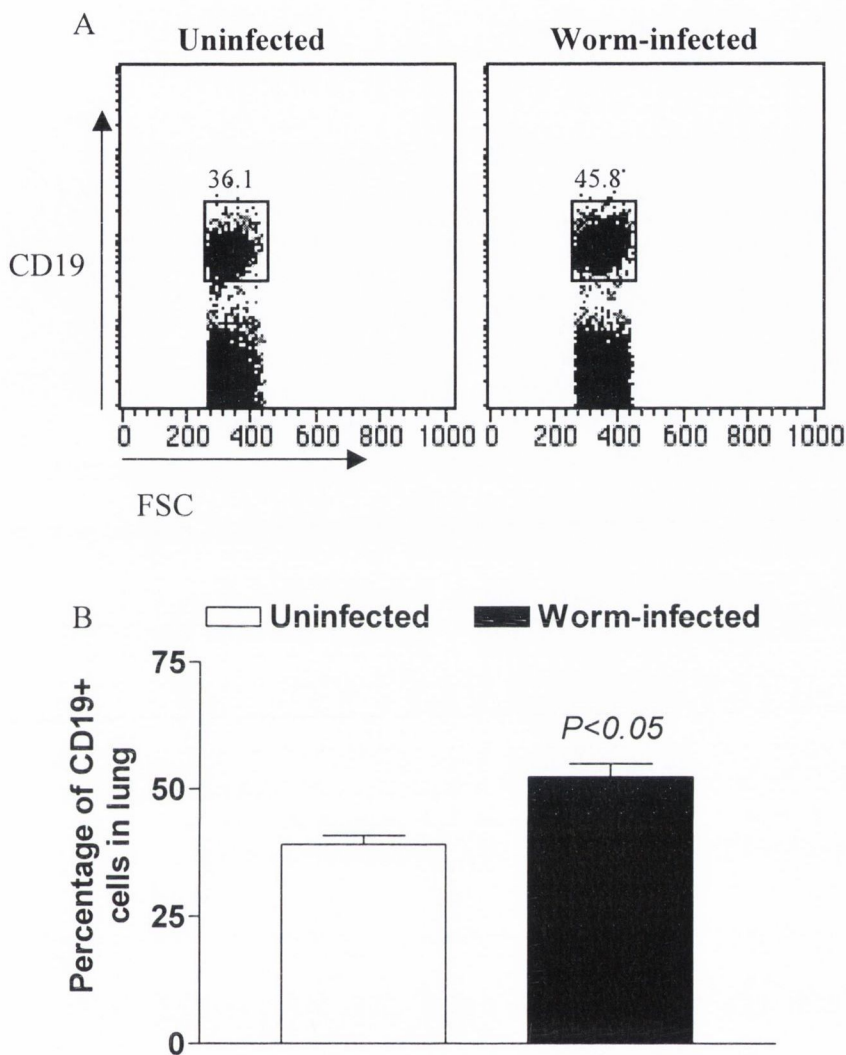


Figure 4.24. *Levels of CD19⁺ cells in the lungs of worm-infected.* (A) Representation of levels of B cells present in the lungs digests of uninfected and worm-infected mice as determined by immunofluorescence surface staining. Cells were characterized on FSC/ SSC characteristics and B cells are gated as CD19⁺ lymphocytes (see Fig 4.21). (B) Percentages of B cells present in the lungs of uninfected and worm-infected mice. Statistical analysis was performed using Student's t-test, (n=7).

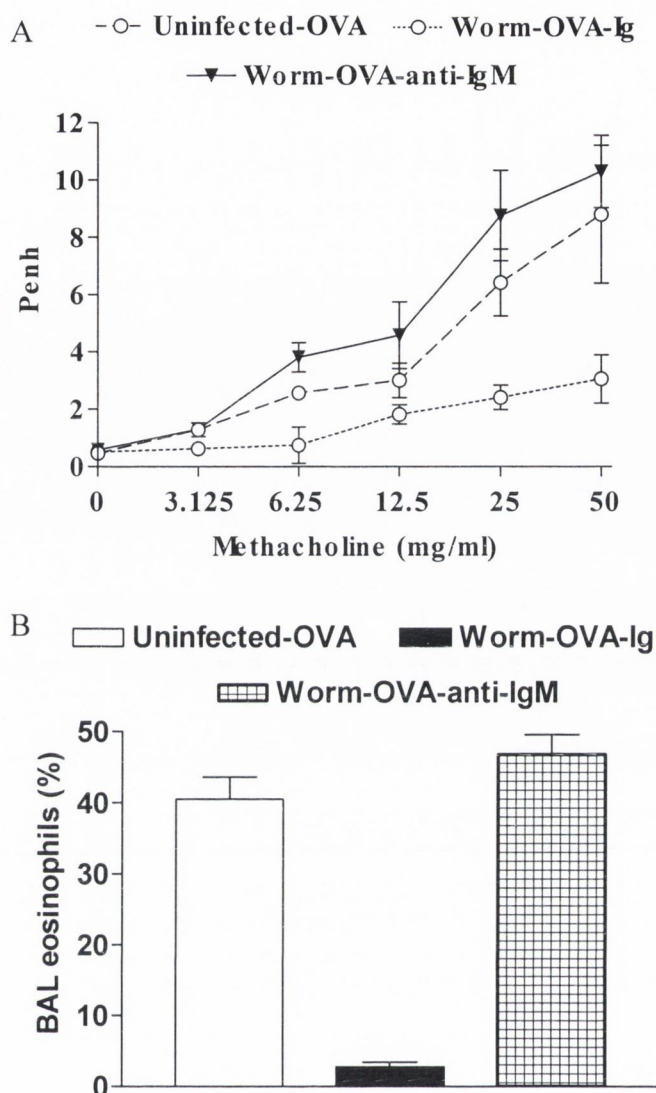


Figure 4.25. *B* cells have an integral role in *Schistosoma mansoni* worm-dependent protection of mice from AHR. (A) Increased Penh responses in worm-infected mice with B cells depleted. Mice were sensitized against OVA and treated with anti-IgM (μ -chain specific; 0.5 mg/mouse) prior to OVA aerosol challenge. Data represent the mean change from baseline Penh values for each group. (B) BAL eosinophil counts in OVA-sensitized mice and sensitized-infected mice treated with anti-IgM. Data are presented as group mean $\pm$ SEM ($n=8-10$) and are representative of 2 separate experiments.

4.3. Discussion

In this chapter I have experimentally investigated the role of a helminth that infects humans in modulation of allergies and in particular the role of schistosome infection on allergen-induced pulmonary airway hyper-reactivity. I have demonstrated both the protective and the exacerbating roles of schistosome infections in a murine model of allergen-induced pulmonary inflammation. Schistosome worms stimulated a modified pulmonary type 2 response that prevents OVA-induced AHR, with protection involving B cell and IL-10 dependent suppression of pulmonary eosinophil infiltration.

The failure of schistosome worm-infected mice to develop OVA-induced AHR was not associated with an inability of infected mice to respond to the allergen. On the contrary, cells from infected mice had normal *in vitro* cell proliferation to OVA and their spleen and mediastinal lymph nodes cells produced greater levels of both IL-4 and IL-13 than uninfected mice. Strikingly, in the lungs of worm-infected mice there was reduced levels of total and OVA-specific IL-5 compared to uninfected animals, suggesting a unique selective alteration in type 2 cytokines in the lungs. Recently, Platts-Mills and colleagues have suggested that a “modified Th2 response” to cat allergens may explain the reduced levels of asthma in children exposed to cats (Platts-Mills *et al.*, 2004). I now describe that schistosome worms induce what I have termed “a helminth modified pulmonary type 2 response” to OVA in mice, that is characterized by elevated pulmonary allergen-specific IL-4, IL-13, but reduced IL-5 and elevated IL-10, that renders mice refractory to allergen-induced AHR (**Diagram 4.1**).

An important question is why would schistosomes selectively suppress pulmonary immunity? The answer may relate to the biology of schistosome infection. Humans are repeatedly reinfected with schistosomes, with new infections requiring migration of larvae through the lung. In fact already established schistosomes induce an immune response against any invading new schistosome larvae, with new invading

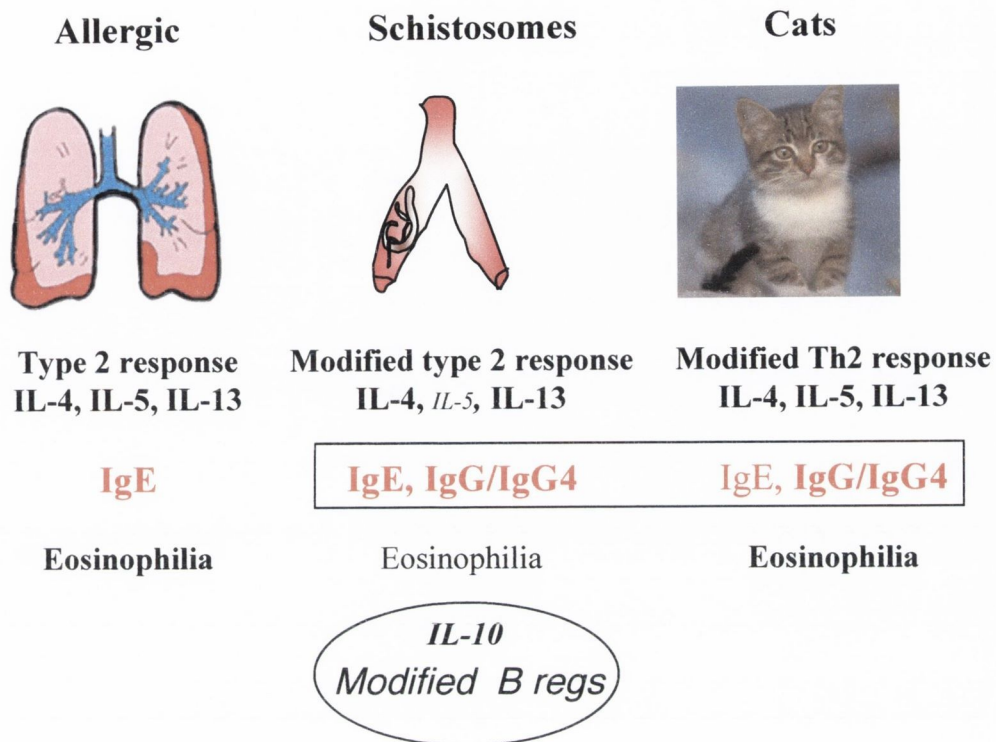


Diagram 4.1. Overlap between the helminth modified pulmonary type 2 response and the modified Th2 response to cats.

larvae being killed in the lungs. This phenomenon, called ‘concomitant immunity’ is thus characterized by the ability of the host to mount an effective defense against larval stages of infection whilst being unable to clear a persistent burden of adult worms (Smithers and Terry, 1969) (**Diagram 4.2**). Smithers & Terry classically demonstrated this phenomenon when adult worms were transferred from *S. mansoni* infected monkeys to uninfected monkeys. The recipient monkeys were almost totally resistant to cercarial challenge (Smithers and Terry, 1967). Interestingly this anti-larval immunity is thought to involve blocking IgG Ab (Butterworth *et al.*, 1987), which is similar to what is proposed in the modified Th2 response to cat allergens (Platts-Mills *et al.*, 2004). Therefore in our murine model, the schistosome worm may induce a modified pulmonary type 2 response to suppress inflammation in the lung induced by new infections.

Suggested reasons for the diminished prevalence of allergic diseases in helminth-infected populations, despite highly raised concentrations of IgE and Th2 cytokines, have included certain features of these infections such as raised polyclonal IgE and a high heightened capacity to produce IL-10 (Jarrett and Bazin, 1974; King *et al.*, 1996). However, another possibility involves the production of blocking antibodies. In humans it has been found that the production of antigen-specific IgG4 can play a predominant role in blocking IgE-mediated allergic responses to helminth parasite antigens (Hussain *et al.*, 1992). As discussed, established schistosome worms can induce suppression in immunity to prevent further reinfection by cercariae, but they also induce protection against the development of allergies (**Diagram 4.2**). Platts-Mills ‘modified Th2 response’ mentioned previously, involves the presence of cat-specific IgG/IgG4 leading to a modified T cell response with production of IL-10. This modified response induced tolerance in these patients with a high exposure to cat allergens in early childhood that prevents delayed hypersensitivity upon later exposure

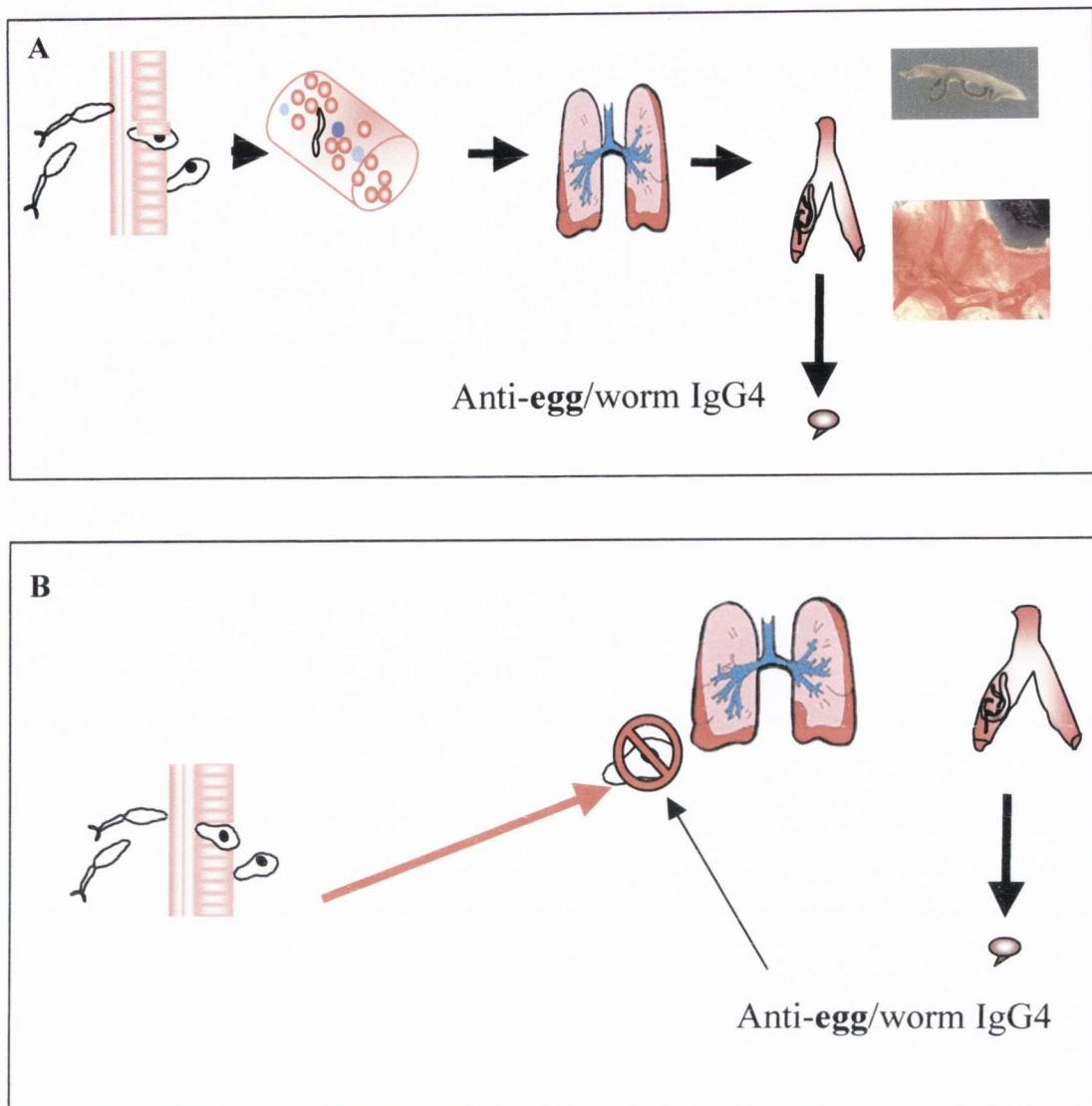


Diagram 4.2. Induction of concomitant immunity to reinfection by *S. mansoni*.

- A. Primary infection leads to generation of IgG/IgG4 to egg and worm antigens.
- B. New infections are killed in the lungs as the anti-egg IgG “blocking antibodies” cross-react with larval surface antigens.

to cat allergens (Platts-Mills *et al.*, 2001; Platts-Mills *et al.*, 2004). In contrast, house dust-mite exposure showed no such protective effect, with allergic dust-mite specific IgE, and not potentially protective IgG being produced (Platts-Mills *et al.*, 2004). Therefore in this chapter, the presence of the persistent antigen challenge from schistosome worms that have been shown to induce a modulated lung phenotype to control parasite burden and prevent reinfection may also indirectly protect against allergies by inducing a non-allergic or modified pulmonary phenotype. In worm-infected mice in addition to elevated IgE there is also elevated anti-OVA IgG. However, the mouse is a limited model for studies on blocking human antibodies, as mice do not have a functional homologue of human IgG4, thus the protective mechanism will require further dissection.

In mice after a primary schistosome challenge, the invading larvae migrate through the lungs and, in doing so, will cause some tissue damage in the lungs. Such pathology may have influenced the resistance of worm-infected mice to pulmonary OVA-sensitization and/or subsequent methacholine response while undergoing plethysmography. This is unlikely as worm + egg-infected mice would also have had larval migration are fully susceptible to AHR. Nevertheless, to formally validate my proposal that worm-infected mice are resistant to lung inflammation via the worm eliciting a modified pulmonary type 2 response, and not because of migratory larvae-induced lung pathology, I treated worm-infected mice with the schistosomicidal drug Praziquantel (PZQ) 5-6 weeks after infection, i.e. after the larvae have migrated through the lungs. However, treatment with PZQ-alone is not sufficient to completely remove all the male worms, with the drug Oxamniquine being more effective against male worms (Fallon *et al.*, 1995). As Oxamniquine is not commercially available in Ireland it is not easily obtained. However, despite the concerns with efficacy of PZQ, even the incomplete reduction in worm burden was sufficient, with 0-3 stunted worms remaining

per mouse after PZQ versus 15-25 worms in untreated mice, to render worm-infected mice susceptible to AHR. I was unable to determine the immune response in these mice following treatment as there were still some worms present after PZQ treatment. It would thus be interesting to repeat the drug treatment experiment but use Oxamniquine instead of PZQ and analyse the immune response.

There is already evidence from field studies in Africa that schistosome infection of humans can indeed reduce allergic responses. *S. haematobium*-infected school children in the Gabon have lower prevalence of skin reactivity to house dust mite than those free of this infection (van den Biggelaar *et al.*, 2001). Strikingly, when worms, primarily *Ascaris* and *Trichuris* and not schistosomes, are removed from patients by chemotherapy there is an increase in atopy, directly establishing a link between the presence of worms and suppression of allergic responses (Van Den Biggelaar *et al.*, 2004). In this experimental study presented here, when adult schistosome worms were killed by drug treatment the previously resistant mice became susceptible to OVA-induced AHR. There is therefore a requirement for the continual presence of the worm during infection to sustain a helminth modified pulmonary response to suppress allergic inflammation. Therefore these experimental data support the argument that the downregulation of the immune system during helminth infections evokes a regulatory environment, called here a helminth modified pulmonary type 2 response, that may impart protection from allergies.

In *S. haematobium*-infected schoolchildren the reduced mite-specific allergic response was statistically associated with production of parasite-specific IL-10 (van Den Biggelaar *et al.*, 2000.). I have shown that worm-infected mice are resistant to allergen-induced AHR via suppression of pulmonary eosinophilia via IL-10. IL-10 is a potent regulatory cytokine suppressing a range of immune-mediated responses (Moore *et al.*, 2001). In mouse AHR models there is various data showing a role for IL-10 in

suppressing airway inflammation and AHR (Hernandez *et al.*, 2005; Makela *et al.*, 2002; Nakagome and Dohi, 2005; Stampfli *et al.*, 1999). One of these recent studies showed that when IL-10 was administered *in vivo* by gene delivery it suppresses OVA-induced AHR and airway eosinophilia (Nakagome and Dohi, 2005). Similarly, this data presented here shows worm-infection-induced IL-10 also blocks AHR and airway eosinophilia. However, unlike in the gene delivery of IL-10, worm-infected OVA-sensitized mice have elevated, not reduced, allergen-specific IL-4 and IL-13 cytokines and IgE and there were no alterations in the cellular response to OVA and lung APC function. In my studies, IL-10 may be a regulatory component of the helminth modified pulmonary type 2 response we describe, as pulmonary IL-5 levels are restored in worm-infected mice with IL-10 blocked *in vivo*.

The production of IL-10 in worm + egg-infected mice has been shown to have a central role in preventing infection induced-pathology (Flores-Villanueva *et al.*, 1996) (Hoffmann *et al.*, 2000) (Hesse *et al.*, 2004). However, despite this elevated IL-10, worm + egg-infected mice were highly susceptible to AHR, with worm + egg-infected mice dying, even without allergen sensitization, when exposed to Mch-induced bronchoconstriction. The predisposition of worm + egg-infected mice to AHR was evident in both the acute stages of infection, which is the peak of Th2 cytokine induction, and also during the chronic stages, when the parasite has down-modulated host immunity. Although worm-infected mice produce relatively more IL-10 than comparably infected worm + egg-infected mice (Chapter 3) (Smith *et al.*, 2004), the discrepancy between worm infection causing mice to be resistant to allergen-induced inflammation whereas a worm + egg-infection exacerbated AHR, is unlikely to be solely due to IL-10 levels. In this respect I have previously highlighted that the balance between levels of protective IL-10 and detrimental IL-4 may determine the susceptibility of schistosome-infected mice in induction of anaphylaxis (Chapter 5).

Worm + egg-infected mice also have the modified Th2 response in the lungs that is observed in worm-infected mice (**Fig. 4.5**). However, the presence of marked IL-13 dependent fibrosis in the lungs (**Fig. 4.6**) of worm + egg-infected mice, and not in mice infected with worms (data not shown), is relevant due to the effects of IL-13-induced fibrosis on lung inflammation (Wynn, 2003). Therefore a simple association of certain parasite worms inducing IL-10 as part of their immunomodulatory strategy that will, as a bystander effect, suppress allergic inflammation is too simplistic and does not highlight the complexity of the host-parasite immune interaction.

It would be of interest to assess mechanistically how the worm induces IL-10. It remains to be determined what worm antigens or molecules interact with the host innate and adaptive immune responses to induce a sustained modulatory environment. With respect to IL-10, Van der Kleij *et al.* have demonstrated that schistosome-specific phosphatidylserine activates TLR2 on human dendritic cells (DC) to mature DC to induce the development of IL-10 producing T cells (van der Kleij *et al.*, 2002). Thus one avenue for future work lies in addressing the interplay of TLR2 in worm-infection protection against AHR; to this end I have infected TLR2^{-/-} mice with schistosome worms and will address immune responses, in particular IL-10 production, and AHR in the absence of TLR2.

In chapter 3 I had infected IL-10^{-/-} mice on a C57/BL6 background with a worm-infection to assess the importance of *S. mansoni* worm-induced IL-10 in anaphylaxis. I have not used IL-10^{-/-} mice to in worm modulation of AHR for several reasons. Firstly, IL-10 deficient mice have been shown to have an already aberrant *in vivo* AHR response, with this discrepancy generating conflicting roles for IL-10 in AHR depending on variations in timing and methods of allergen sensitization (Makela *et al.*, 2000; Zuany-Amorim *et al.*, 1995). Secondly, IL-10^{-/-} mice were not available on a BALB/c background. As all other mice used in this study were of BALB/c strain,

switching to mice on a C57/BL6 background would introduce the added variable of strain differences to OVA (Shinagawa and Kojima, 2003). I did back-cross IL-10^{-/-} C57BL/6 mice to BALB/c, but after 3 backcrosses (~9 months) the F4 heterozygous studs were lost due to a leaky water bottle and I have not since re-started the back-cross.

Animal studies in short-term high-level exposure models of bronchopulmonary inflammation suggest an important role for Th2 cytokines in inflammation. Although worm-infection enhances the type 2 environment of the lung in infected mice as noted by Th2 cytokines, OVA-IgE and eosinophils, these mice do not develop AHR. The regulatory effect of the worm-infection seems to be IL-10-driven dampening of IL-5 and hence eosinophil recruitment. However, there are many unanswered questions in allergies and even the importance of the role of eosinophilia in mouse AHR has been extensively investigated (Gleich *et al.*, 1993; Humbles *et al.*, 2004). Also I have not addressed the role of IL-9 in this model. IL-9 has been demonstrated to have a role in the parasite immune responses in the lung (Townsend *et al.*, 2000), and schistosome worm + egg infection (Fallon *et al.*, 2000b), and to be involved in the development of eosinophilic airway inflammation (McLane *et al.*, 1998; Temann *et al.*, 1998).

Previous experimental studies have shown that infection with various worms causes reduced allergic responses (Bashir *et al.*, 2002; Negrao-Correa *et al.*, 2003; Wang *et al.*, 2001; Wohlleben and Erb, 2004). Using the rodent gastro-intestinal nematode *Nippostrongylus brasiliensis* it was shown that infection suppressed allergen-induced airway eosinophilia via IL-10 from an unidentified cell (Wohlleben and Erb, 2004). It has been argued that CD4⁺ cells, either Th2 or regulatory, are the potential source of the worm-induced IL-10 (Wohlleben and Erb, 2004). As CD4⁺ IL-10-producing cells generated by pathogens, or genetically prepared, suppress airway inflammation (Oh *et al.*, 2002; Zuany-Amorim *et al.*, 2002) they are an attractive possible source for helminth-induced IL-10. Indeed we have previously shown that

worm-infections induce elevated frequencies of natural CD4⁺CD25⁺ regulatory cell and IL-10 producing CD4⁺ cells (Mangan *et al.*, 2004). However, depletion of CD4⁺ or CD25⁺ cells did not alter the resistance of worm-infected mice to OVA-induced AHR. Worm-infected mice do not have elevated levels of CD4⁺CD25⁺FoxP3⁺ cells, which have also been highlighted as important regulatory cells which further negates a role for T regulatory cells in worm-pulmonary protection. Furthermore, TGF- β is directly involved in FoxP3⁺ T regulatory maintenance *in vivo* (Chen *et al.*, 2003; Marie *et al.*, 2005). Therefore the lack of alteration in CD4⁺ CD25⁺ FoxP3⁺ cells in worm-infected mice further supports that worm modulation in AHR is dependent on IL-10 and that this IL-10 is from a non-T cell source. As discussed in chapter 3 worm-infection of mice induces novel modified T regulatory cells.

Depletion of alveolar macrophages, which also produce IL-10, did not alter the worm-induced protection against AHR. Previously I have shown B cell-IL-10 levels were significantly enhanced in worm-infected mice (Chapters 3 and 5), with B cells having a crucial role in schistosome worm infection mediated resistance to anaphylaxis (Chapter 3). B cells have been shown to participate in the induction of immune tolerance and suppression of disease giving rise to the suggestion of B regulatory cells or IL-10 producing B cells (Chapter 3; Fillatreau *et al.*, 2002; Korganow *et al.*, 1999; Mizoguchi *et al.*, 2002). B cells and the regulatory cytokine IL-10 play a crucial role in this *S. mansoni* worm-induced protection unlike in the conventional worm + egg-infection where there were mortalities in mice. This is indicative of the important differences in parasite-induced immunity during the establishment of these infections in mice and the implications of this polarized immunity on the susceptibility of the host to other immune insults. The *S. mansoni* worm infection is an experimental refinement of the induction of the life cycle in the host unlike the conventional egg-producing worm + egg infection therefore the anti-helminthic immune response is limited in worm-infected

mice to worm and not egg antigens. These results parallel the conflict that exists in human studies defining the role of helminth infection in the development of allergies (Yazdanbakhsh *et al.*, 2002) (Lynch *et al.*, 1997). There is however potential for use of worms in the treatment of asthma and allergic diseases. One study is attempting to investigate the immune suppressive properties of hookworms (*Necator americanus*) in human trials as a possible treatment in allergic diseases and asthma (Falcone and Pritchard, 2005). This highlights the varying roles of specific worm infections with relation to altered immune responses in diseases.

Therefore the results in this chapter demonstrate both the protective and antagonistic roles of schistosome worm-modulation of mice in an experimental test of the Hygiene Hypothesis. In a schistosome worm + egg infection the extreme immune conditions afforded and enforced by continual infection prove to be detrimental to the survival of the host upon exposure to a further Th2 immune insult i.e. pulmonary allergic Th2 inflammation. In marked contrast, *S. mansoni* worm-only worm-infection of mice diminishes the disease effects in a model of OVA-induced AHR via induction of a protective modified type 2 response. The worm is able to modulate the pulmonary response of the host by evoking a carefully regulated environment that dampens further putative exacerbatory allergic reactions. In this way the worm is able to control the host and thus prevent its expulsion through overactive immune responses which would be detrimental to its survival. Similarly in a conventional infection, this worm-modified immune response may serve to control the immune response to the egg which is immunogenic. As discussed, this is also a protective mechanism to prevent against re-infection.

In conclusion, *S. mansoni* infection of mice has an important influence in the host's susceptibility to allergic disease. Parasite-modulation of the host's immune system here is such that there is protection against both severe (anaphylaxis; Chapter 3)

and moderate (AHR) forms of immune activation. Overall, these data highlight the complex role of the helminth parasite *Schistosoma mansoni* in regulation of the immune response. Importantly, this is the first formal demonstration of protection by a human pathogen in a mouse model of allergic pulmonary disease.

Chapter 5

B cells in S. mansoni infection

5.1 Introduction

In chapters 3 and 4, I have shown that in mice schistosome-mediated protection from allergic models was associated with B cells. This B cell-mediated protection was associated with an elevation in IL-10 production. As B cells are important in humoral immunity and in schistosome infection, I characterised the B cell sub-populations present in mice with *S. mansoni* infection. B cells were surface phenotyped as described in Table 5.1. In this chapter I demonstrate a protective role for CD1d⁺ cells in worm-induced anaphylaxis and airway hyper-responsiveness in mice.

B cells have been shown to participate in the induction of immune tolerance and maintenance of a protective cytokine environment in the gut (Gonnella *et al.*, 2001; Ozaki *et al.*, 1999). They can also drive the development of several autoimmune diseases through the production of pathogenic Igs (Fagarasan *et al.*, 2000; Korganow *et al.*, 1999; Myers *et al.*, 1992). In contrast, B cells have been associated with suppression of experimental autoimmune encephalomyelitis as depletion of B cells promoted severe disease (Fillatreau *et al.*, 2002; Wolf *et al.*, 1996). Furthermore, in a rheumatoid arthritis mouse model, in which Igs are required for the development of the disease, the presence of B cells has been proposed to control disease progression in an IL-10 dependent manner (Korganow *et al.*, 1999; Mauri *et al.*, 2003). Therefore, B cells are gaining support as important immunoregulatory cells. Indeed previously, I have shown a role for helminth-induced B cell regulation in modulating allergic responses (Chapters 3 and 4).

There are several categories of mature B cell subtypes, each characterized by distinct anatomic location, lifespan, phenotype and function. B1 cells reside primarily in the pleural and peritoneal cavities and share many functional and phenotypic characteristics of marginal zone (MZ) B cells (Anzelon *et al.*, 2003). Marginal zone B cells are a sessile

population of cells positioned at the junction between the red and white pulp and provide the initial response to particulate antigens entering by means of the blood. B2 cells are the main B cell sub-population in the spleen and are similar in function and phenotype to follicular (FO) B cells. FO B cells are mature recirculating cells that traverse between the follicles of the secondary lymphoid organs and represent the majority of spleen B cells. They participate in T-cell-dependent and T cell-independent antibody responses and in the former case can further differentiate in the germinal centre to give rise to high-affinity, isotype-switched plasma and memory B cells (Achtman *et al.*, 2003; Loder *et al.*, 1999; Otero *et al.*, 2003).

The particular mechanism of regulation and interaction of B cells with other cells of the innate and adaptive immune response is still not fully understood. It is likely to incorporate many different cells and pathways. Similar to the functional divisions of CD4⁺ T cells into Th1 and Th2 it is also suggested that B cell regulation involves B cell effector subsets; termed B effector (Be) 1 and Be 2 cells. These B cell subsets are divided by secretion of different cytokines in a similar way to Th1 and Th2 cells (Harris *et al.*, 2000). B cells secrete IL-10 (O'Garra *et al.*, 1992), which may be relevant to their role in immunoregulation. However, the importance of particular IL-10 producing B cell populations is also still very much unknown.

Type 2 cytokine responses in host-parasite immunity have been well characterized in schistosome infection (Chapter 1). As discussed in chapters 3 and 4, schistosome infection protects mice from allergic disorders with protection involving B cells. In this chapter I have further addressed the role of B cells in the schistosome-modulated immune response to allergic models. Furthermore I have defined a subset of schistosome worm-induced B cells that via IL-10 regulate allergic disorders.

Chapter objectives:

The objective of this chapter was to investigate and characterise the influence of schistosome-induced B cell regulation on the anti-allergy phenomena described in Chapters 3 and 4.

- 1) To assess alterations in the temporal B cell profile in the spleen during the course of *S. mansoni* worm + egg and worm-only infection of mice.
- 2) To address the role of B cells in *S. mansoni* protection in anaphylaxis and airway hyper-responsiveness.
- 3) To identify the B cell sub-population induced by the *S. mansoni* worm that elicits the protective phenotype described in 2.

5.2 Results

5.2.1 *Worm-infection protection from anaphylaxis is not via B1 cells.*

B cells can be divided into B1 and B2 B cell sub populations depending on their phenotypic characteristics (Table 5.1). B1 cells in the peritoneal cavity produce a large amount of IL-10 in response to different stimuli (Babai *et al.*, 1999; O'Garra *et al.*, 1992) including worm + egg-infection (Velupillai and Harn, 1994). I now show for the first time that worm-infected mice have a 50% increase in the frequencies of peritoneal B1 cells that produce IL-10 (**Fig. 5.1A**). This increase in peritoneal B1 cells that produce IL-10 is relevant to results in Chapter 3 and 4. Therefore to specifically investigate the relative roles of B1 versus B2 cell sub-populations in allergic models I used mice expressing the X-linked immunodeficiency (*xid*) mutation that are deficient in peritoneal B1 cells (Berning *et al.*, 1980).

S. mansoni worm-infected CBA/Ca mice were resistant to Pen V-induced anaphylaxis (described in Chapter 3) unlike uninfected CBA/Ca mice (**Fig 5.1B**). Thus, BALB/c (H-2d), C57BL/6 (H-2b), and CBA/Ca (H-2k) mouse strains are all protected from anaphylaxis by schistosome infection. This highlights the range of protection afforded by the worm across a wide range of different mouse MHC haplotypes.

Worm-infected CBA/*xid*, which are devoid of B1 cells, were also protected from Pen-V anaphylaxis compared to the anaphylaxis observed in uninfected CBA/*xid* and wildtype CBA/Ca mice (**Fig. 5.1B**). Also in an OVA-induced model of airway hyper responsiveness (AHR; Chapter 4), worm-infected CBA/*xid* and wildtype CBA/Ca mice did not develop the enhanced Penh responses noted in uninfected mice and were still resistant to AHR (**Fig. 5.2**). These data indicate that despite worm infection increasing peritoneal

IL-10-producing B1 cells, these cells have no role in the B cell-dependent protection from anaphylaxis (Chapter 3) and AHR (Chapter 4). Therefore it was necessary to address the specific B2 cell sub-population that was evoking the IL-10-mediated protection from allergic insult in worm-infected mice.

5.2.2 *Phenotyping of spleen B cells in uninfected, worm-infected and worm + egg-infected mice.*

I have shown that worm-infected mice were protected from anaphylaxis (Chapter 3) and AHR (Chapter 4) but worm + egg-infected mice were only partially protected from anaphylaxis and were fully susceptible to AHR. As worm-infected and not worm + egg-infected mice were protected from AHR and anaphylaxis, I hypothesised that as this protection was B cell dependent, a distinct B cell sub-population would be up- or down-regulated in worm-infected mice. Thus I compared B cell surface phenotype (Table 5.1) of infected mice to investigate differences in B cells between a conventional egg-producing worm-infection and the experimental worm-only non-egg-producing infection. Having already excluded a role for peritoneal B1 cells (Section 5.2.1) in schistosome protection, the various other B cell populations present in the spleens of worm-infected mice versus worm + egg-infected and uninfected mice were characterized by surface immunofluorescence analysis (**Fig. 5.3-5.8**). I assessed differences in B cell populations through the course of schistosome infection of mice from weeks 4, before egg production, through the acute phase of infection to a chronic infection at week 16.

<i>B cell sub-population</i>	<i>Spleen surface marker phenotype</i>
B1	CD19 ⁺ , IgD ^{lo} , IgM ^{hi}
B2	CD19 ⁺ , IgM ^{lo} , IgD ^{hi}
B1a	B220 ^{lo} , CD5 ^{hi}
B1b	B220 ^{hi} , CD5 ^{neg}
T2	CD19 ⁺ , IgM ^{hi} , IgD ^{hi}
FO	CD19 ⁺ , CD21 ^{int} , CD23 ^{hi}
MZ	CD19 ⁺ , CD21 ^{hi} , CD23 ^{lo}

Table 5.1. *Surface phenotype for characterisation of B cell sub-populations in the spleen of uninfected and worm-infected mice.* Cells were first analysed on forward and side scatter characteristics and then gated for on the lymphocyte population. Gates were set on appropriate isotype controls.

By the acute stage of infection (8 weeks) worm-infected and worm + egg-infected mice have approximately 20% more total CD19⁺ B cells in the spleen than uninfected mice (**Fig. 5.3A**). Over the course of both a worm + egg and a worm infection the percentages of B cells increases from weeks 4 to 8 post-infection with numbers of spleen B cells maximal at week 8 in both infection groups (**Fig. 5.3B**). After week 8, the spleen B cell levels decline in worm-infected mice but remain elevated until weeks 12-16 in worm + egg-infected mice. I first characterized spleen B cells into B1 and B2 cells (Table 5.1) (Lam and Rajewsky, 1999). B1 cells are identified as IgD^{lo} and IgM^{hi} and represent immature transitional (T1) B cells recently migrated from the bone-marrow, they are similar in phenotype to marginal zone (MZ) B cells. These cells then enter the T2 stage (IgM^{hi},

IgD^{hi}). Finally, a maturation to follicular, recirculating B2 cells takes place when cells can be identified as IgM^{lo}, IgD^{hi}. Percentages of B1 and B2 cells in the spleen are comparable between uninfected and worm-infected mice (**Fig. 5.4A**). However, worm + egg-infected mice have lower numbers of both B1 and B2 cells with higher numbers of T2 cells (**Fig. 5.4A**). B1 cells can be further subdivided into B1a (CD5^{hi}B220^{lo}) and B1b (CD5^{neg}B220^{hi}) cells (Table 1; **Fig. 5.4B**). Uninfected mice, worm + egg-infected and worm-infected mice have similar numbers of B1a cells but both worm + egg-infected and worm-infected mice have higher numbers of B1b cells (18% and 8% greater respectively; **Fig. 5.4B**). In schistosome infection spleen B1 cell levels are comparable in worm-infected to worm + egg-infected mice over the course of infection (**Fig. 5.5A**). The frequencies of B2 cells in infected mice remain high in the spleens of both infection groups, up to week 8 when the immune response in infection is most prominent. Following this point there is a reduction in B2 cells numbers in worm-infected mice up to week 16 at which point the levels of B2 cells return to normal frequencies (Week 0). Worm + egg-infected mice have similar levels of B2 cell numbers to worm-infected mice up to week 8 after which point the frequencies in the spleen remains constantly elevated (**Fig. 5.5B**).

To further identify spleen B cell sub-populations I used the following categorization: follicular (FO) B cells are defined as CD21^{int} and CD23^{hi}, while marginal zone (MZ) B cells are defined as CD21^{hi} and CD23^{lo} (Achtman *et al.*, 2003). Using these criteria (Table 5.1), worm + egg-infected and uninfected mice have similar levels of FO B cells at week 8, whereas worm-infected mice have approximately 10% more FO B cells than the other 2 groups (**Fig. 5.6A**). The FO B cells constitute the most abundant number of cells in the spleen and are similar to B2 cells. Over the course of infection, numbers of FO cells remained similar in both infection groups (**Fig. 5.6B**).

Worm-infected mice have less MZ B cells compared with uninfected mice. Worm + egg-infected mice have almost 50% less MZ B cells compared to uninfected (**Fig. 5.7A**). Percentages of MZ B cells were comparable from 4-8 after which point (week 12-16) the frequency of MZ B cells continues to decrease in worm-infected groups but remain relatively constant in worm-infected mice (**Fig. 5.7B**).

Therefore the outcome of analysis over the course of a 16 week infection is that *S. mansoni* infection increases the percentages of spleen B cells in mice. The most heightened B cell response is at week 8 post infection in both worm-infected and worm + egg-infected mice. The B cell frequency dampens from weeks 8-16 until it is comparable with uninfected mice. All the spleen B cell sub-populations assessed are elevated in infected mice compared with uninfected mice. Unfortunately, the objective of the screen was unsuccessful. There were no distinct alterations between worm- and worm + egg-infection in most B cell sub-populations assessed.

5.2.3 *B cell proliferation and cytokine responses in infection.*

Proliferation of spleen cells from infected groups following B cell-specific stimulation (CD40 + IgM or LPS) over the course of infection was assessed (**Fig. 5.8**). Direct B cell stimulation with CD40 + IgM evoked a stronger cell proliferation response at all time points compared with LPS stimulation. Both worm-infected and worm + egg-infected mice have similar levels of spleen B cell responsiveness to stimulation at week 4 of infection, ie prior to eggs in worm + egg-infected mice. Worm-infected mice demonstrated a reduced proliferation at week 8 to CD40 + IgM as the B cell responsiveness of worm + egg-infected mice increased. However, at week 12 there is a return to comparable and normalised spleen cell responsiveness to B cell stimulants versus

uninfected mice. By week 16 post-infection, the worm + egg proliferation is completely reduced and is approx. equal to that of worm-infected mice (**Fig. 5.8**). Uninfected mice have barely detectable proliferative responses to LPS over the time course. On the other hand worm + egg-infected mice demonstrate LPS responsiveness which increases from weeks 8-12 (**Fig. 5.8**). The limited response of worm-infected mice to LPS compared with worm + egg-infected mice may be attributable to the presence of eggs in the intestine, and release of LPS systemically, in worm + egg infected mice after weeks 5-6 (Fallon *et al.*, 2002).

5.2.4 *Passive transfer of in vivo modulated spleen B cells.*

As IgM depletion renders worm-infected mice susceptible to anaphylaxis and AHR and B cells from these mice produce IL-10 (Chapters 3 and 4) it was suggestive that B cells mediate allergic protection. As I was unable to identify a distinct B cell sub-population uniquely elevated in worm-infection, I therefore investigated whether *in vivo* worm-mediated protection in allergic disorders could be transferred to naïve mice by transfer of spleen B cells from worm-infected mice. To assess the potential of transferred worm-modulated B cells to transfer protection I used the Pen V anaphylaxis model described in Chapter 3 rather than the OVA-induced AHR model. I used the anaphylaxis model because the duration of the transfer of cell protection is shorter, 2-3 days, and the procedure has a single readout for assessment.

B cells were purified from the spleens of worm-infected or uninfected mice and transferred to uninfected mice. Spleen B cells were separated by autoMACS and were typically >95% pure (Chapter 2, Section 2.6.7.1). Pen V-sensitized BALB/c mice were injected ip with 0.5×10^7 purified B cells on each of the 2 days prior to intravenous Pen V

challenge and anaphylaxis was induced as per the standard protocol. B cells isolated from the spleen of uninfected mice did not alter susceptibility to anaphylaxis (**Fig. 5.9**). Unexpectedly, transfer of B cells from worm-infected mice not only did not protect mice from anaphylaxis, but these animals had a greater drop in body temperature, increased mortalities and prolonged symptoms of anaphylaxis, compared to mice receiving no cells or recipients of B cells from uninfected mice (**Fig. 5.9**). The unanticipated finding that transfer of spleen B cells from worm-infected mice exacerbates anaphylaxis does not support my initial observation that worm infection induces B cells that mediate protection.

5.2.5 *Passive transfer of in vitro modulated B cells.*

To further determine if B cells are involved in protection from anaphylaxis rather than allowing 8 weeks of *in vivo* infection to modulate the cells an *in vitro* modulation system was used. We have developed a model of short-term *in vitro* culture of distinct cell populations with live worms (Smith *et al.*, 2004). Spleen cells from a naïve mouse were cultured with 15 live worms in Transwells for 48 hours at 37°C. *In vitro* exposure of spleen cells to worms significantly ($P<0.01$) elevated the frequency of B cells expressing IL-10 (**Fig. 5.10A, B**). Following *in vitro* culture of spleen cells in the presence of worms, B cells were purified by autoMACs selection as described (Chapter 2, section 2.6.7.1). Transfer of *in vitro* worm-modulated B cells made recipient mice more susceptible to anaphylaxis (**Fig. 5.10C**), similar to what occurred with transfer of B cells modulated *in vivo* by worm-infection (**Fig. 5.9**). It is noteworthy that I consistently observed that mice receiving *in vivo* modulated B cells (**Fig. 5.9**) had a sustained anaphylaxis, with reduced temperature until 50 minutes after challenge, but animals that were transferred *in vitro* worm-modulated cells (**Fig. 5.10**) had increased anaphylaxis for 10-20 minutes and then rapidly recovered from

anaphylaxis within 30 minutes. This alteration in recovery (**Fig. 5.11**), despite both having approx. two-fold increase in IL-10-producing cells, suggests a difference in *in vivo* compared to *in vitro* worm-modulation of B cells.

5.2.6 *Worm-modulated B cells from IL-10^{-/-} mice do not protect against anaphylaxis.*

B cells have previously been shown to participate in the induction of immune tolerance and suppression of disease giving rise to the suggestion of B regulatory cells or IL-10 producing B cells (Fillatreau *et al.*, 2002; Korganow *et al.*, 1999; Mizoguchi *et al.*, 2002). The frequency of B cells that produced IL-10 is enhanced in worm-infected mice as was previously described in Chapter 3.

Despite the unexpected observations that *in vivo* or *in vitro* worm-modulated B cells exacerbated anaphylaxis, both B cell populations have approx. 2 fold greater basal levels of IL-10 than uninfected (Chapter 3) or unmodulated B cells (**Fig. 5.10A**). In Chapter 3 I demonstrated a role for IL-10 in worm-mediated protection in anaphylaxis as worm-infected IL-10^{-/-} mice were fully susceptible to Pen V-induced anaphylaxis. Therefore, to confirm IL-10-producing B cells have a role in this worm-mediated protection, B cells from IL-10^{-/-} and wildtype C57BL/6 mice were modulated *in vitro* and transferred to Pen V-OVA immunized mice prior to eliciting anaphylaxis. All mice were susceptible to anaphylaxis (**Fig. 5.12**). However, mice that received IL-10^{-/-} worm-modulated B cells had markedly more severe temperature drop and mortalities than mice receiving B cells from wild-type animals. These data support a protective role for IL-10 producing B cell in the resistance of schistosome worm-infected mice to anaphylaxis.

5.2.7 *Elevated IL-4 in B cells from infected and uninfected mice.*

Our initial data from depletion studies suggested that schistosome worm infection induces B cells producing elevated levels of IL-10, that render the mice refractory to experimental anaphylaxis. Unexpectedly, transfer of B cells from worm-infected mice, or *in vitro* worm-modulated cells, to naïve mice not only did not mediate protection from Pen V-induced anaphylaxis but significantly exacerbated disease (**Figs. 5.9-5.12**). It has already been shown that worm-infection induces elevation in IL-4 production from CD4⁺ T cells (Smith *et al.*, 2004), which I now confirm here using 4GET mice, which express IL-4 in a GFP dependent manner (**Fig. 5.13A**). I have previously demonstrated in Chapter 3 that worm-infected mice have elevated IL-10-producing B cells, which I also saw in worm-infected 4GET mice (**Fig 5.13B**), and again following *in vitro* worm-modulation of B cells (**Fig. 5.14B**). However, using 4GET mice, immunofluorescence detection of GFP staining on CD19⁺ B cells illustrated that worm-infected mice have a two-fold elevation in the frequencies of IL-4-producing B cells compared with uninfected mice (**Fig. 5.13B**). It is important to note that <1% of B cells had IL-4 detected by intracellular cytokine staining in GFP mice compared with the detection of IL-4 by intracellular cytokine staining in wild-type mice (**Fig. 5.14A**). The elevation in both IL-10 and IL-4 in B cells was further confirmed by RT-PCR of purified B cells isolated from both worm-infected mice compared against uninfected mice (**Fig. 5.13C**). Therefore in experiments described in Figures 5.10 and 5.11, we were not only injecting mice with IL-10-producing B cells, but we were also transferring ~ 1% of B cells that produced IL-4.

5.2.8 *Exogenous IL-10 or IL-4 alters susceptibility of mice to anaphylaxis.*

As IL-4 is known to exacerbate experimental anaphylaxis (Strait *et al.*, 2003), the increased anaphylaxis in mice receiving B cells from worm-infected mice, or *in vitro* worm-modulated B cells, could be due to co-transfer of these IL-4-producing cells (**Fig. 5.15**). To address this scenario, I firstly examined the relative roles of both IL-4 and IL-10 in the Pen V-mediated anaphylaxis model. Sensitized mice were administered recombinant (r)IL-4 +/- rIL-10 prior to Pen V-BSA challenge. Analysis of the serum levels of IL-4 and IL-10 in these mice (**Fig. 5.15A**) demonstrated that there were artificially elevated levels of each cytokine in mice during challenge compared to control mice. Administration of rIL-4 exacerbated anaphylaxis in recipient mice with these mice showing a 2°C greater drop in body temperature compared with control mice and a prolonged lapse in recovery (**Fig. 5.15B**). In contrast, mice that received rIL-10 were protected mice from anaphylaxis with only a slight drop (~1°C) in body temperature at 10 minutes followed by a rapid recovery (**Fig. 5.15C**). Interestingly, mice that were treated with both rIL-4 and rIL-10 showed a similar trend to mice that received rIL-10. These *in vivo* data demonstrate that IL-10 can reduce the deleterious effects of IL-4 in anaphylaxis.

5.2.9 *Transfer of in vitro worm-modulated Th2 cytokine deficient B cells.*

To exclude a role for IL-4 and other Th2 cytokines (IL-5, -9, -13) known to induce allergic responses, a B cell transfer experiment was performed with cells from a multi-knockout (mKO) mice that have a combined deficiency in IL-4, -5, -9, and -13 (Fallon *et al.*, 2002). B cells from mKO and wild-type mice were modulated with worms *in vitro* and transferred to Pen V-OVA immunized mice prior to Pen V-BSA challenge and induction of anaphylaxis. *In vitro* modulation of mKO splenocytes with worms increased the frequency

of IL-10-producing B cells by 32% (Mangan *et al.*, 2004). Mice that received unmodulated B cells from mKO, or cells from wild-type animals both developed anaphylaxis (**Fig. 5.16A**). Similar to earlier data (**Fig. 5.9**) mice that received worm-modulated B cells from wild-type mice had a more severe initial (first 10 minutes) drop in temperature after which they recovered rapidly (**Fig. 5.9**). However, passive transfer of worm-modulated B cells from mKO mice completely protected animals from anaphylaxis (**Fig. 5.16**).

5.2.10 *Individual Th2 cytokine depleted mice.*

To formally determine the role of the individual Th2 cytokines I compared IL-4-, IL-5-, IL-9- or IL-13-deficient mice. Spleen cells from these cytokine-deficient mice were modulated by worms *in vitro*, B cells were isolated and transferred to Pen V-sensitized mice. Worm-modulated B cells deficient in IL-4 completely protected mice from anaphylaxis (**Fig. 5.17D**). In contrast, animals receiving modulated B cells from mice deficient in IL-5, IL-9 or IL-13 developed a comparable drop in temperature as seen in animals administered wild-type cells (**Figs. 5.17A-C**). Therefore the presence of IL-4 in transferred B cells may have been a contributing factor to the development of anaphylaxis in recipient mice. Indeed, worm-infected mice have elevated frequencies of IL-4-producing B cells following *in vitro* worm-modulation compared with uninfected mice (**Fig. 5.15A**). Most relevant is that these results further corroborate a protective role for schistosome worm-modulated B cells in preventing anaphylaxis in the absence of IL-4.

5.2.11 *Identification of an IL-10-producing spleen B cell population.*

My earlier studies did not find alterations in a distinct B cell sub-populations in worm-infected mice. However, recent studies have shown a role for IL-10-producing B

cells that were CD1d⁺ in suppressing intestinal inflammation (Mizoguchi *et al.*, 2002). In an investigation of B cell sub-populations I found that worm-infected mice had non-significantly elevated (~20%) CD19⁺ CD1d⁺ B cells in the spleen compared with uninfected mice (**Fig. 5.18A, B**). Furthermore upon OVA sensitisation, the levels of CD19⁺ CD1d⁺ B cells in the spleen of OVA-sensitised worm-infected mice significantly increased by 47% ($P<0.001$) compared with sensitised uninfected mice that only had a non-significant increase in this B cell population of 15% (**Fig. 5.18B**). Not only was there an increase in numbers of CD19⁺ CD1d⁺ cells but, upon further analysis, I found that the frequency of IL-10-producing CD19⁺ CD1d⁺ B cells in the spleen was significantly increased greater ($P<0.01$) in worm-infected mice compared with uninfected mice (**Fig 5.19**).

5.2.12 *Role for CD1d in worm-protection against anaphylaxis.*

To ascertain an *in vivo* function of CD1d⁺ cells induced by *S. mansoni*, worm-infected mice were treated with an anti-CD1d (IBI) mAb and challenged with the Pen V anaphylaxis model described previously (**Fig. 5.20**). Confirmation of depletion was assessed by immunofluorescence staining for CD1d in the spleen of uninfected mAb treated mice (**Fig. 5.20A**). Treatment with anti-CD1d mAb in worm-infected mice completely abrogated the protective effects of worm-infection seen previously, with worm-infected mice now rendered fully susceptible to systemic anaphylaxis unlike the control Ig-treated worm-infected (**Fig. 5.20B**).

5.2.13 *Detection of CD1d⁺ cell populations in the lungs of worm-infected mice.*

As I had shown a role for CD1d⁺ cells in systemic protection of worm-infected mice against anaphylaxis (**Fig 5.20**), I investigated the local expression of CD1d on various cell

types in the lung including B cells. In anaphylaxis worm-infected mice had elevated levels of spleen $CD19^+CD1d^+$ cells and upon anti-CD1d mAb treatment worm-infected mice were rendered fully susceptible to anaphylaxis. Therefore I assessed the frequencies of $CD1d^+$ cells in the lungs of worm-infected mice that were protected from pulmonary allergic responses. I did not find alterations in frequencies of $CD1d^+ CD3^+$ (**Fig. 5.21A**) or $CD1d^+ DX-5^+$ (**Fig. 5.21B**) cells which have both previously been used as potential markers for regulatory NKT cells. I found that $CD19^+ CD1d^+$ expression was elevated in the lungs of OVA-sensitised worm-infected mice (**Fig. 5.21C**). In fact this was the only cell sub-population that was significantly (69 %) elevated in the lung in comparison to all other cell types in infected mice.

5.2.14 *CD1d-tetramer analysis of cells in the lungs of worm-infected mice.*

CD1d has been shown to bind glycolipids including as α -galactosylceramide (α -GalCer) (Naidenko *et al.*, 1999). Therefore I was interested to further evaluate $CD1d^+$ populations in the lungs of worm-infected mice using CD1d-loaded α -GalCer tetramers which are a powerful tool for evaluating $CD1d^+$ cell numbers and function (Matsuda *et al.*, 2000). Lung digests from uninfected or worm-infected mice that were either unsensitised or OVA-sensitised were stained for immunofluorescence detection with unloaded-PE-CD1d or α -GalCer-loaded tetramers. I firstly used $\alpha\beta$ TCR to co-stain $CD1d$ -tetramer $^+$ T cells, however I did not find this mAb stained cells effectively when used in conjunction with tetramers and I could not find tetramer-staining, therefore I used CD3 to assess $CD1d^+$ T cells. I did not find any differences in the levels of $CD3^+ CD1d$ -tetramer $^+$ cells in the lungs of any of the groups when treated with OVA (**Fig. 5.22**), similar to findings using surface staining in Figure 5.21A.

Equally, I did not find differences in levels of CD19⁺ tetramer⁺ cells in worm-infected mice with and without OVA-sensitisation (**Fig. 5.23A, B**). However there were significant differences (5 fold increase) in the levels of CD19⁺ tetramer⁺ cells in the lung digest of uninfected mice that had been sensitised with OVA compared with unsensitised (PBS) mice. As there was no OVA-induced increase in CD19⁺ tetramer levels in worm-infected mice unlike uninfected OVA-treated mice, these data were suggestive of the important role of CD19⁺ CD1d⁺ in worm-infection mediated protection in the lungs (**Fig. 5.24**); i.e. in worm-infected mice the CD19⁺ CD1d⁺ population in the lung is not expanded with OVA.

As dendritic cells (DC) have important functions in the innate response and have been widely evaluated in pulmonary responses in AHR, I assessed the DC-CD1d interaction also (**Fig. 5.24**)(Nishimura *et al.*, 2000). Firstly there were similar percentages of CD11c⁺ cells in the lungs of both uninfected and worm-infected mice. The activation and maturation state of the DC present in the lungs of mice was not fully assessed and this may be of importance in the role of antigen presentation in the lungs in infection. However, upon analysis of CD1d⁺ tetramer staining on CD11c⁺ pulmonary DC, I found that worm-infected mice did not have positive α -GalCer co-staining (**Fig. 5.24**). This is similar to results found for CD3⁺ CD1d α -GalCer staining. Therefore initial studies on pulmonary DC in worm-infection do not indicate an important role in pulmonary responses compared with uninfected mice but more detailed studies are underway to confirm this.

5.2.15 *Depletion of CD1d abrogates worm-infection mediated protection in AHR.*

Therefore considering the important role of CD1d⁺ cells in innate responses and its' potentially significant role in the lung I investigated the putative protective role of CD1d⁺

cells in worm-modulation of AHR. OVA-sensitised uninfected and worm-infected mice were treated with anti-CD1d depleting mAb to assess the role of CD1d⁺ cells in AHR. Uninfected anti-CD1d treated mice sensitised to OVA developed exacerbated AHR compared with Ig-treated OVA-sensitised mice that developed the normal OVA-induced elevations in Penh (**Fig. 5.25**). When worm-infected mice were treated with anti-CD1d⁺ depleting mAb and subjected to OVA-induced AHR these mice also developed AHR (**Fig. 5.25**). Indeed OVA-sensitised worm-infected mAb treated mice were so distressed upon increasing methacholine challenge, as demonstrated by their increased Penh, such that methacholine administration was stopped after 12.5 mg/ml. As the lack of worm-mediated protection upon CD1d depletion is similar to that in anaphylaxis (**Fig. 5.20**), this was indicative of a potentially similar mechanism of action in both models of allergy.

5.2.16 *CD1d^{-/-} S. mansoni-infected mice are not protected from AHR.*

To definitively assess a role for CD1d in AHR we infected CD1d deficient mice with an *S. mansoni* worm-infection. When CD1d^{-/-} mice were infected with *S. mansoni* and challenged with OVA they developed AHR (**Fig. 5.26**). This was in complete contrast to the protection with worm infection (**Fig. 5.26**). In fact the lack of CD1d in these worm-infected mice exacerbated the pulmonary insult of OVA challenge.

5.2.17 *Role of CD1d in worm immunity in AHR*

As CD1d has a role in protection of worm-infected mice from AHR, I then looked at alterations in pulmonary immunity in OVA-sensitised wildtype (WT) and CD1d^{-/-} mice (**Fig. 5.27**). I did not find any differences in cell proliferation responses of mediastinal lymph node cells to three different concentrations of OVA (**Fig. 5.27A**). Similarly upon

restimulation of draining lymph node cells, worm-infected mice lacking CD1d were able to induce an effective Th2 response to OVA as seen by production of IL-4, -5, and IL-13 (**Fig. 5.27B**). The production of IL-5 from mediastinal lymph node cells was actually markedly enhanced in these worm-infected CD1d^{-/-} mice. Also, there were elevations in the production of IL-13 in the draining lymph nodes of worm-infected CD1d^{-/-} mice. The production of IL-13, which is an important type 2 cytokine involved in the production of mucus in the airway inflammatory response, was not elevated in worm-infected WT mice which did not develop AHR (**Fig. 5.27A**). In correlation with these data I found that IL-5 was also significantly elevated in the BAL fluid (**Fig. 5.28A**) and lung homogenates (**Fig. 5.28B**) of CD1d^{-/-} worm-infected mice. Interestingly the levels of IL-10 were reduced in the lungs of worm-infected CD1d^{-/-} mice (**Fig. 5.28A**), but not in OVA-stimulated mediastinal lymph nodes. In chapter 4, I showed that worm-mediated protection from OVA-induced lung inflammation correlated with a defect in lung eosinophils. I confirmed this with worm-infected mice having less lung EPO enzymatic activity and less eosinophil counts in the BAL (**Fig. 5.29**). Significantly worm-infected CD1d^{-/-} mice that had AHR also elevated eosinophilia (**Fig. 5.29**).

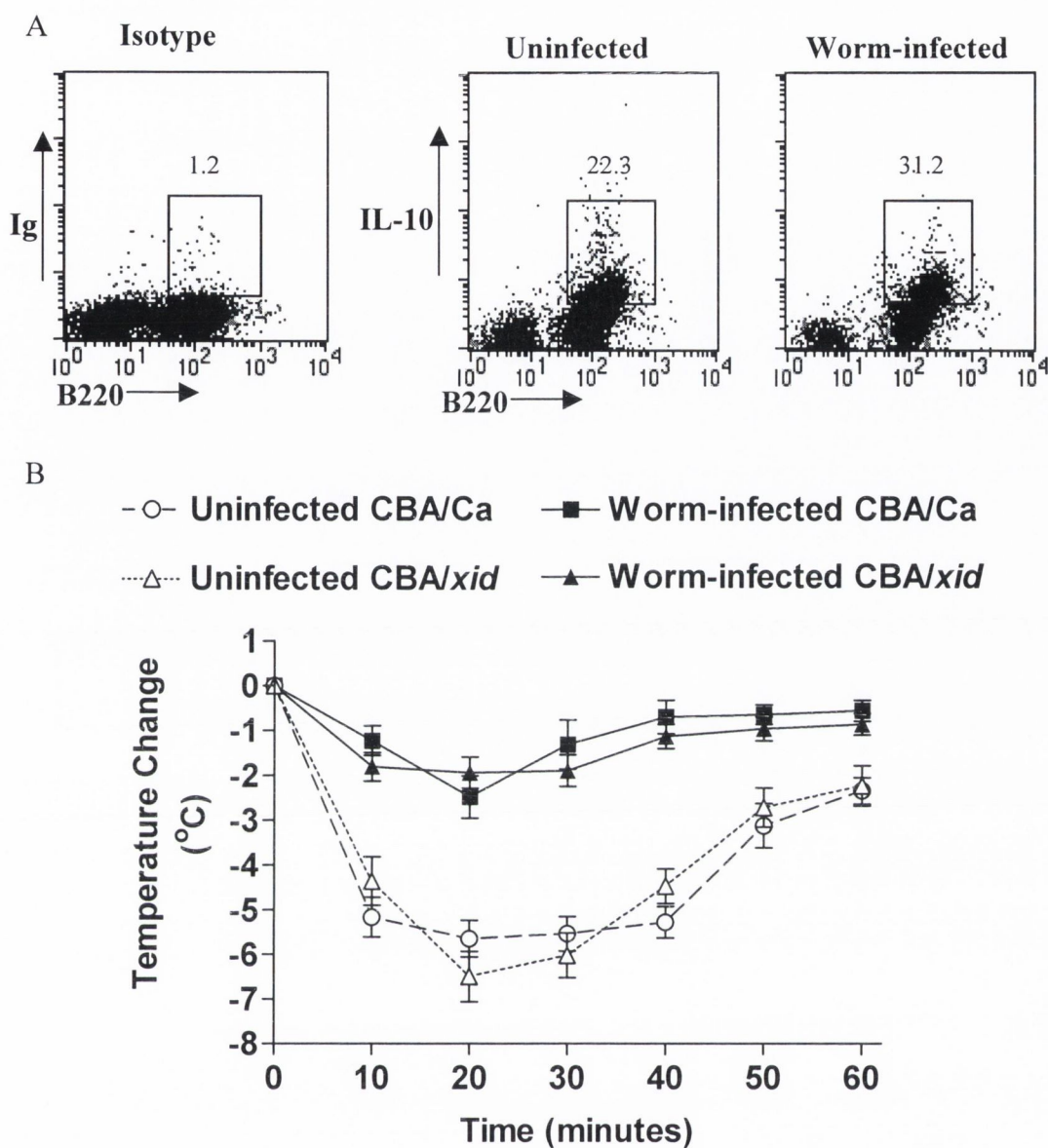


Figure 5.1. No role for B1 cells in worm-protection from anaphylaxis. (A) Frequencies of IL-10 producing B1 (B220⁺) cells in the peritoneal cavities of uninfected and worm-infected mice. (B) *S. mansoni* worm-infected B1 cell deficient *xid* mice are protected from anaphylaxis. Rectal body temperature was recorded prior to Pen V-BSA challenge (time 0) and every 10 minutes after that for one hour. Results are group mean $\pm$ SEM (n=5-6) and are presented as change in body temperature. Data presented are representative of two separate experiments.

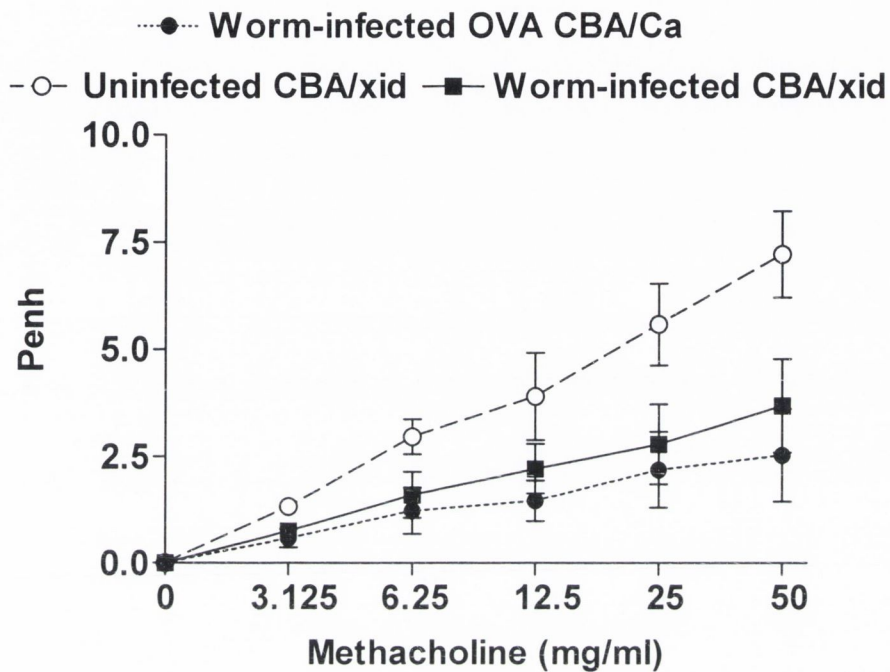


Figure 5.2. No role for B1 cells in resistance of worm-infected mice to AHR. *S. mansoni* worm-infected B1 cell deficient CBA/*xid* mice are protected from AHR. Mice were sensitized to OVA as described in Chapter 2. Penh responses of OVA-sensitized uninfected mice and sensitized-infected mice are presented. Data represent the mean change from baseline Penh values for each group (n=6-7) and represent 2 individual experiments.

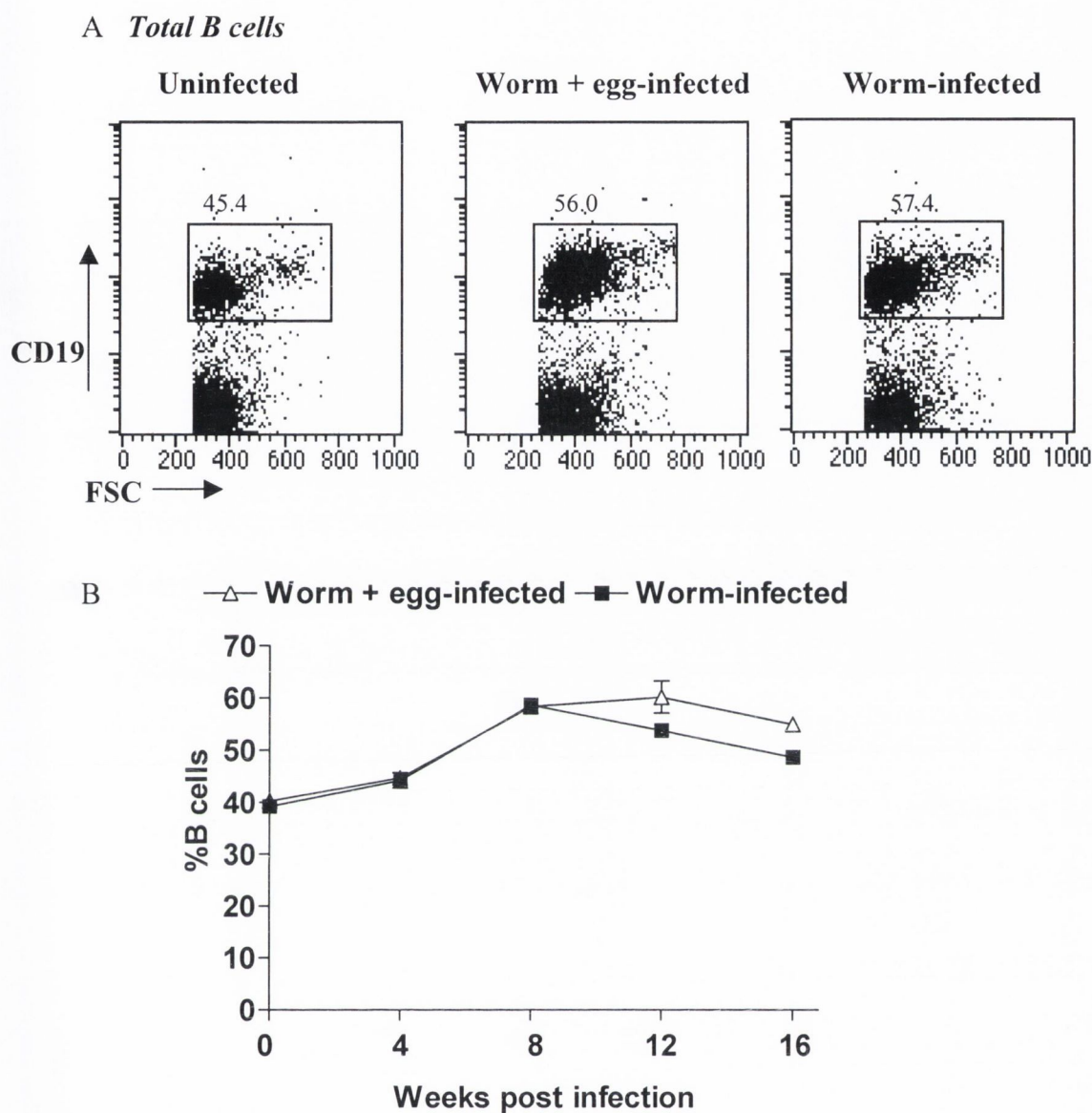


Figure 5.3. Alterations in total spleen B cell numbers in *S. mansoni* infection. Representative flow cytometry images of the percentages of total CD19⁺ B cells populations in uninfected, and 8 week *S. mansoni* worm + egg- or worm-infected mice. (A). The levels of B cells were assessed from weeks 4-16 over the course of murine *S. mansoni* infection. The relative increase in B cells in worm + egg- and worm-infected mice is presented (B). Lymphocytes were first gated on forward (FSC) and side scatter characteristics. Cells were stained with mAbs and gates were set on appropriate isotype control mAbs.

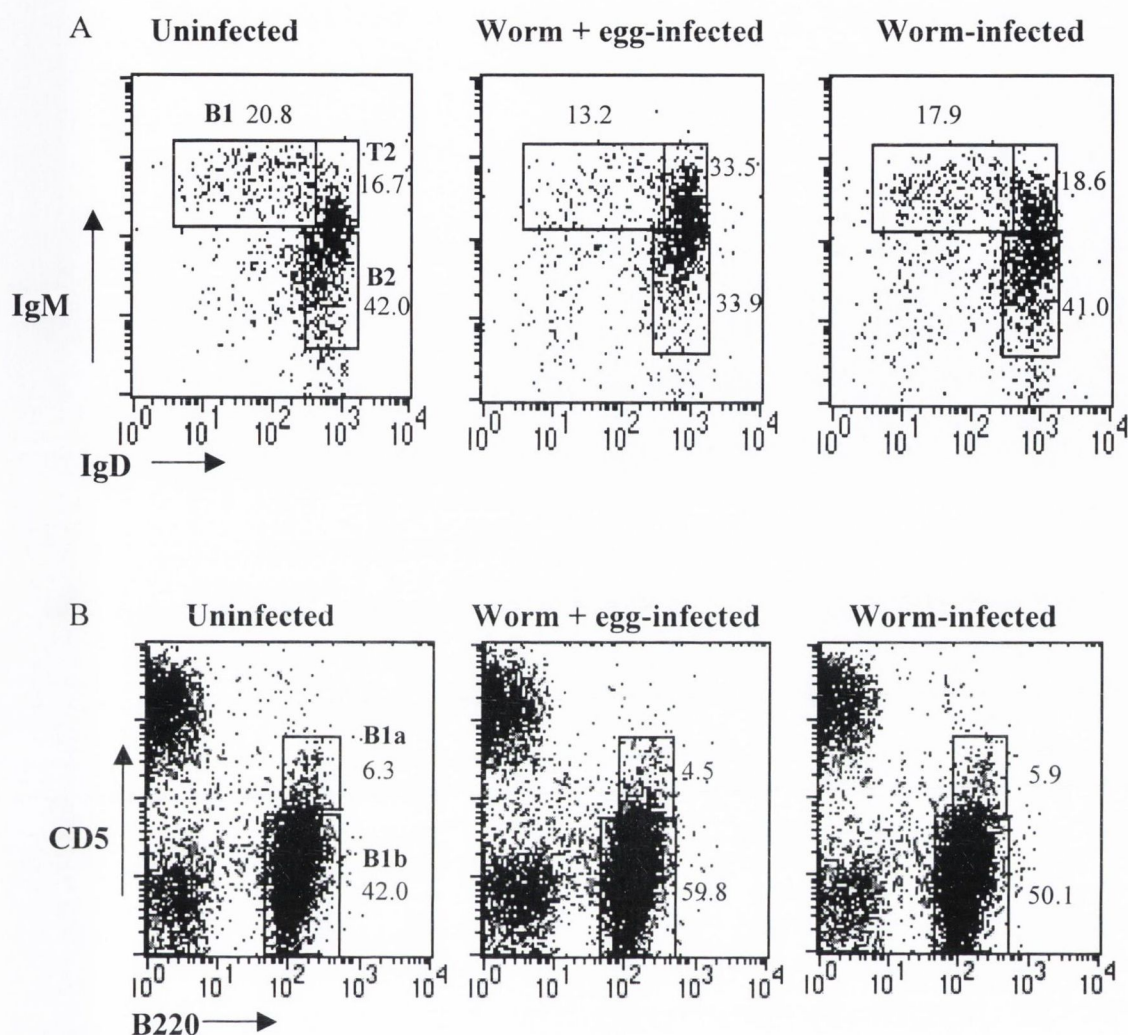


Figure 5.4. Alterations in B1 cell populations in the spleen of uninfected, worm + egg-infected or worm-infected mice. Cells were stained with mAbs against IgM, IgD, CD5 and CD19 to characterize the different B cell populations in the spleens of uninfected mice and infected mice (8 weeks post infection). Lymphocytes were first gated on forward and side-scatter characteristics. Cells were stained with mAbs and gates set on appropriate isotype control mAbs. Representative images of the levels of mature recirculating B2 cells were identified as IgM^{lo} IgD^{hi} cells, T2 B cells as (IgM^{hi} IgD^{hi}), whereas immature B1 cells that had recently migrated from bone marrow were IgD^{lo} IgM^{hi} (A). The B1 cell populations were further subdivided into B1a (CD5^{neg} B220^{hi}) and B1b (CD5^{hi} B220^{lo}) cells (B). Data are representative of flow cytometry analysis of at least 10 individual mice per group from 2-3 separate experiments.

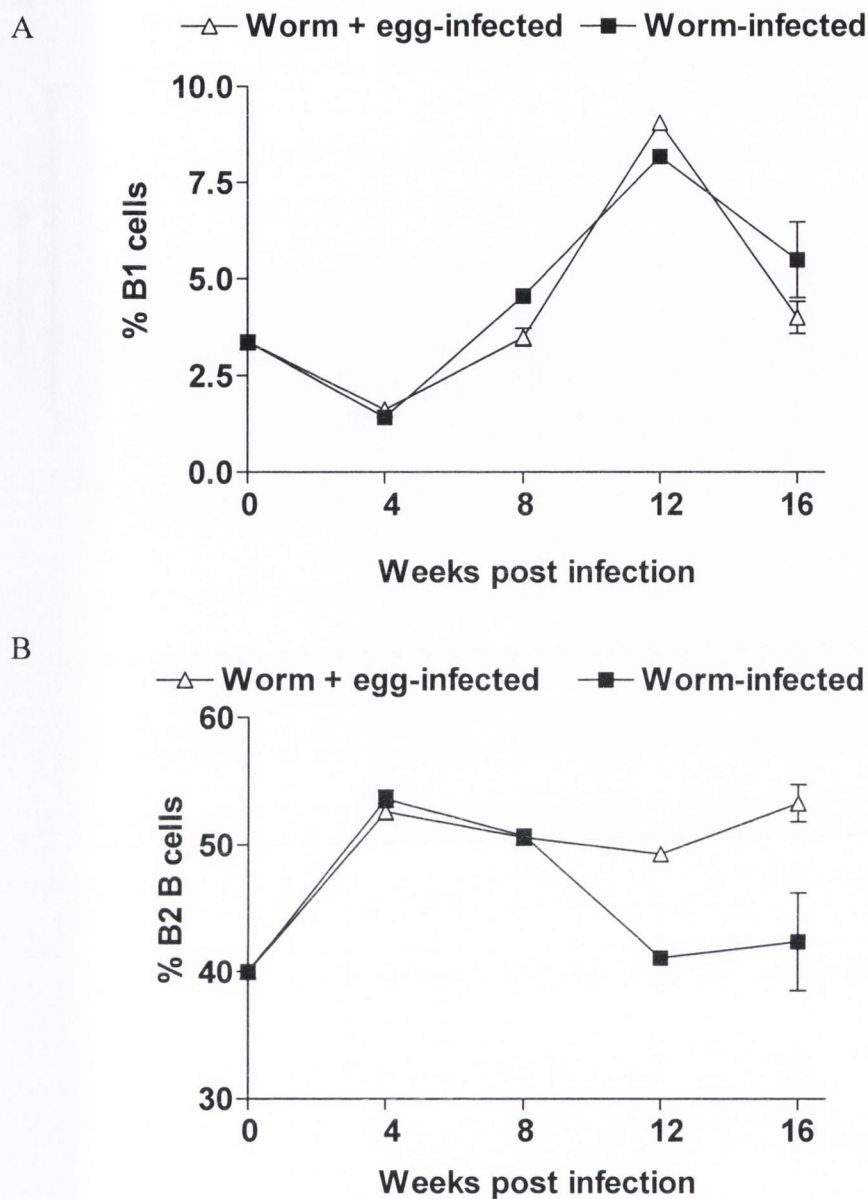


Figure 5.5. Alterations in B1 and B2 cell populations in the spleen of uninfected, worm + egg-infected or worm-infected mice. Cells were stained with mAbs against IgM, IgD, CD5 and CD19 to characterize the different B cell populations in the spleen as described in Fig. 5.6. The levels of B1 (A) and B2 (B) cells were assessed from weeks 4-16 over the course of murine *S. mansoni* infection. Data are expressed as relative change in each B cell population. Data are from 2-3 separate experiments and are representative of flow cytometry analysis of at least 10 individual mice per group.

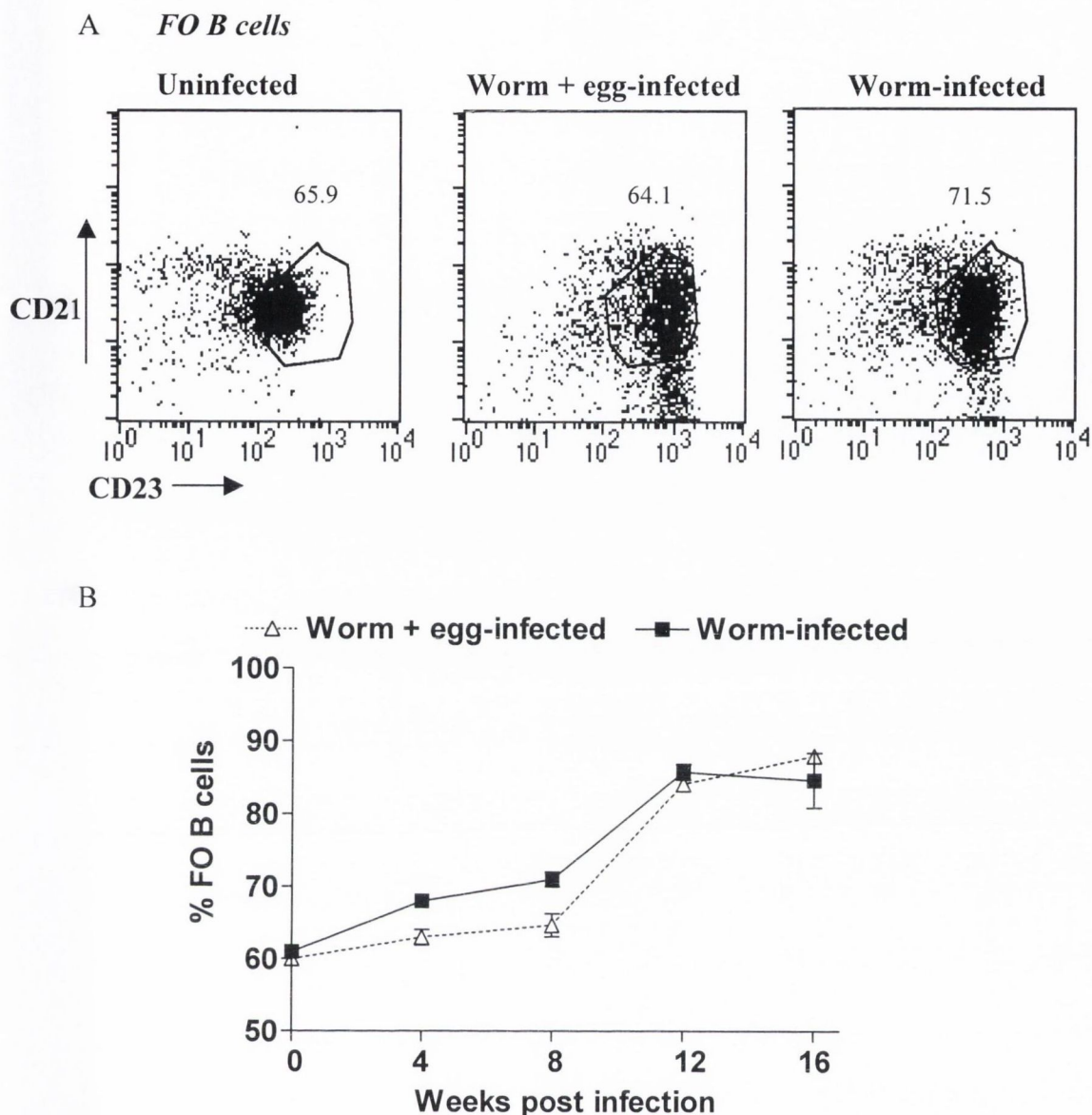


Figure 5.6. Alterations in follicular B cell populations in the spleen of uninfected, worm + egg-infected or worm-infected mice. Follicular (FO) recirculating B cells were identified as CD21^{int}, CD23^{hi} (A). The levels of FO B cells were assessed from weeks 4-16 over the course of murine *S. mansoni* infection. (B) Lymphocytes were first gated on forward (FSC) and side scatter characteristics and then gated on CD19. Cells were stained with mAbs and gates were set on appropriate isotype control mAbs. Data are representative of flow cytometry analysis on at least 10 individual mice per group (2-3 separate experiments).

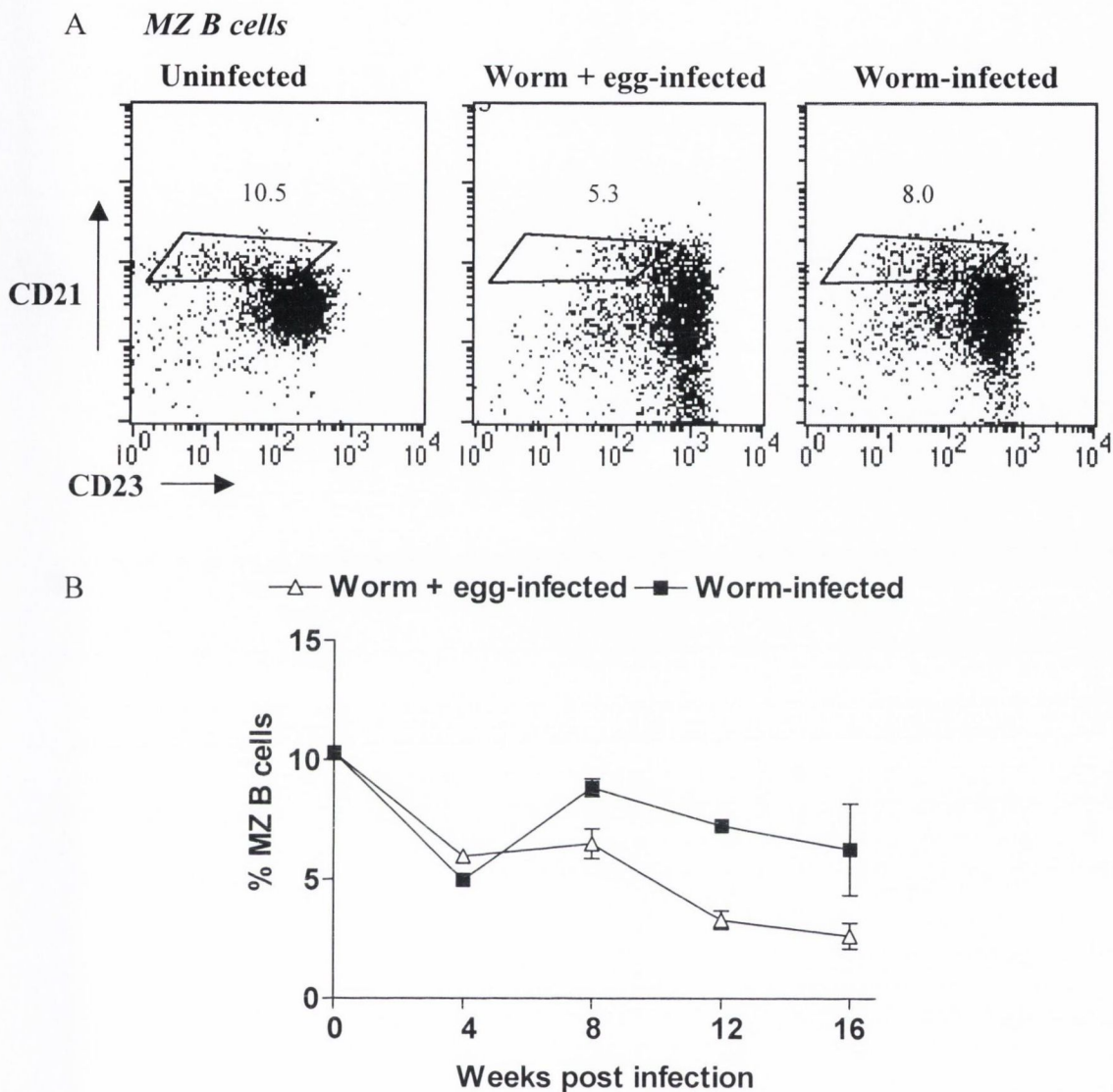


Figure 5.7. Alterations in MZ B cell populations in the spleen of uninfected, worm + egg-infected or worm-infected BALB/c mice. Marginal zone (MZ) B cells were identified as CD21^{hi}, CD23^{lo} (A). The levels of MZ B cells were assessed from weeks 4-16 over the course of murine *S. mansoni* infection and are presented (B). Lymphocytes were first gated on forward and side-scatter characteristics. Cells were stained with mAbs and gates were set on appropriate isotype control mAbs. Data are representative of flow cytometry analysis on at least 10 individual mice per group per time point from 2-3 separate experiments.

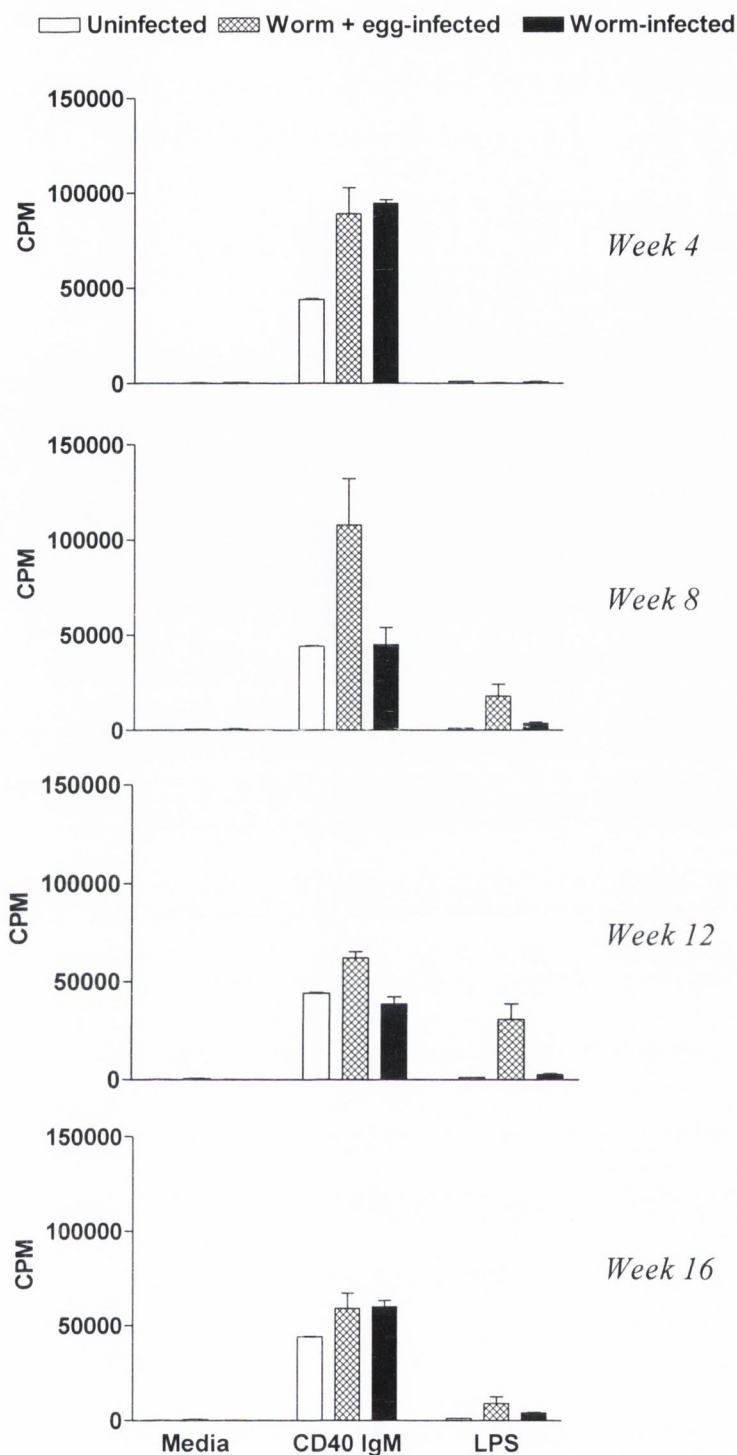


Figure 5.8. *B cell proliferation in S. mansoni infection.* Mice were infected with worm + egg or worm-infections for 4-16 weeks. Spleen cells were then isolated from the different groups at the various time points post infection. Cells were stimulated with anti-CD40+IgM or LPS as described in section 2.7. Proliferations was assessed by $[H]^3$ thymidine incorporation for the last 16 hours of a 72 hour stimulation. All groups were assessed in triplicate, results are mean + SEM and are representative of 2 separate experiments.

—△— Control —●— Uninfected B cells ...■... Worm-infected B cells

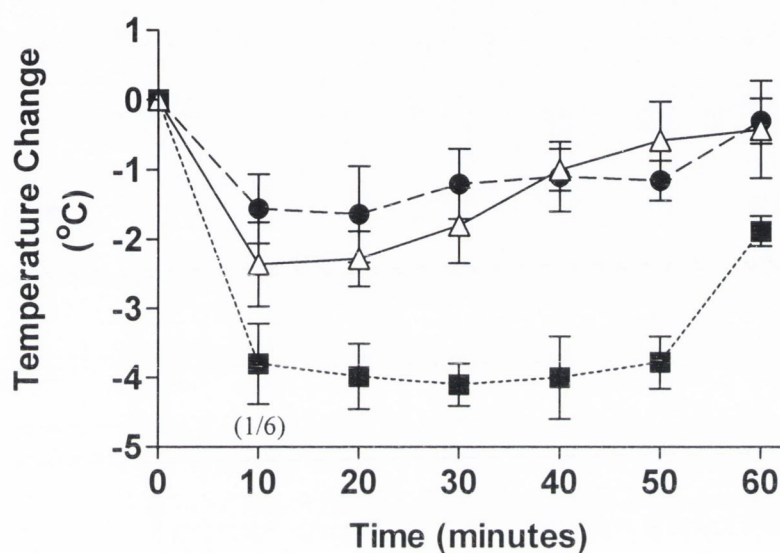


Figure 5.9. *Transfer of B cells from worm-infected mice does not prevent anaphylaxis in recipient mice.* CD19⁺ B cells were isolated from spleen cells from uninfected and worm-infected mice and injected (2 X (1 X 10⁷) cells) into Pen-V-sensitized BALB/c mice 2 and 1 day before inducing anaphylaxis. Data are presented as group mean $\pm$ SEM (n=5-7) temperature change and are representative of three separate experiments.

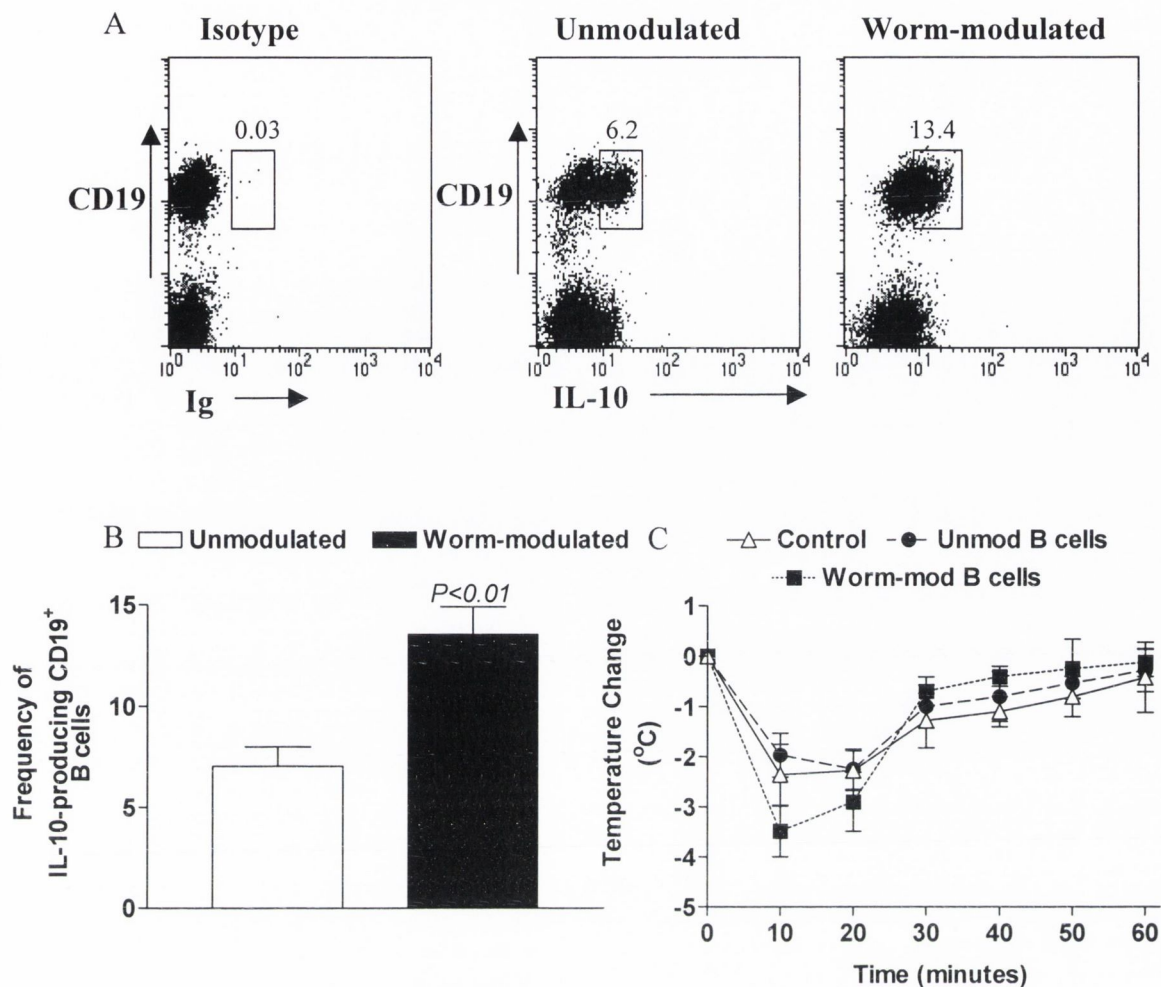


Figure 5.10. Transfer of B cells modulated *in vitro* with worms does not prevent anaphylaxis in recipient mice. Spleen cells from BALB/c mice were modulated by worms *in vitro* (mod) or unmodulated (unmod). (A) Representative image of worm-modulation which increases the frequency of IL-10-producing spleen B cells compared with unmodulated cells. IL-10 levels were detected in CD19⁺ spleen cells by intracellular cytokine staining. (B) Frequency of IL-10-producing B cells following worm modulation. Statistical analysis was performed with Student's t-test; representative of 3-4 separate experiments. (C) B cells were isolated from *in vitro* mod and unmod spleen cells and transferred to Pen V-sensitized BALB/c mice on days 12 and 13 following PenV-OVA sensitisation. Mice were then challenged with Pen V-BSA on Day 14. Data are presented as group mean $\pm$ SEM (n=5-8) temperature change and are representative of two separate experiments. Significant differences between groups was determined by Student's t-test.

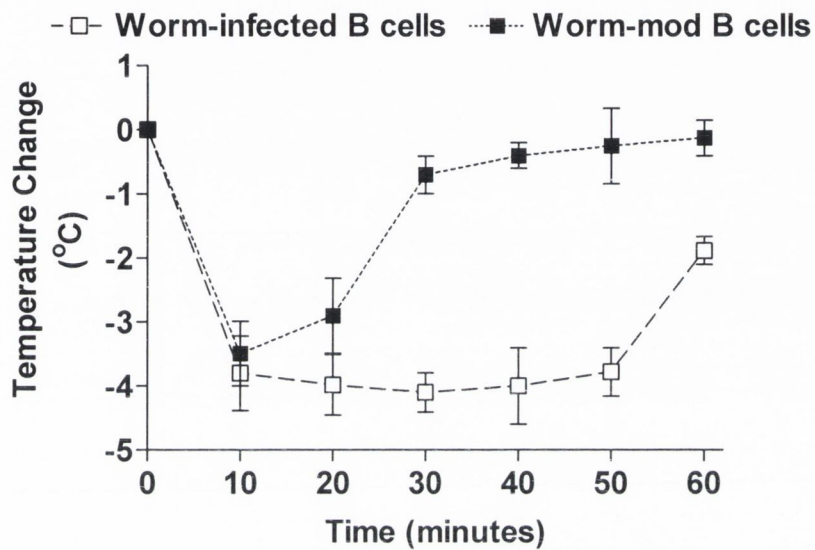


Figure 5.11. Differences in recovery of mice that were transferred B cells modulated either *in vivo* or *in vitro* with worms. Data presented here to demonstrate the altered susceptibility to Pen V anaphylaxis upon B cell transfer are from Figures 5.10 (*in vivo* B cell modulation) and 5.11 (*in vitro* B cell modulation).

—○— Unmod wild-type B cells ···●··· Worm-mod wild-type B cells
 ···□··· Unmod IL-10 KO B cells ···■··· Worm-mod IL-10 KO B cells

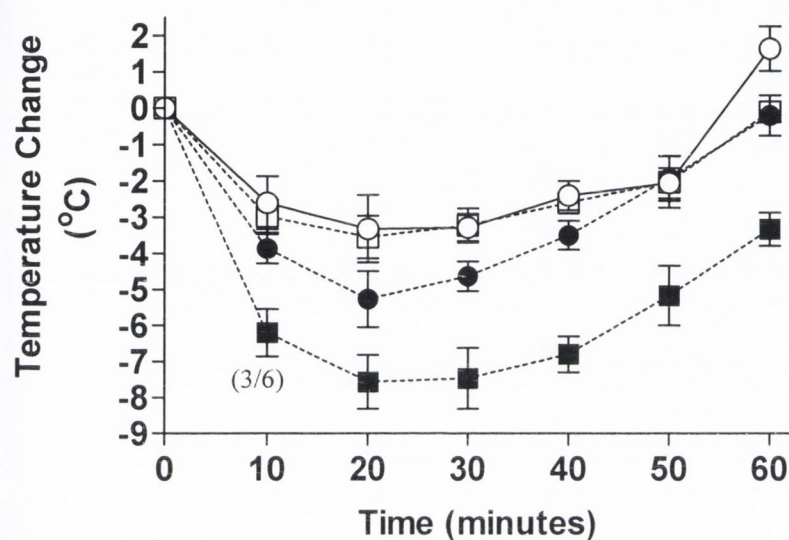


Figure 5.12. *Transfer of B cells modulated in vitro with worms does not prevent anaphylaxis in recipient mice and this effect is exacerbated in the absence of IL-10.* Spleen cells from IL-10 deficient mice (IL-10 KO) and wild-type C57BL/6 mice were worm-modulated (mod) or unmodulated (unmod) *in vitro*. B cells were isolated and transferred to Pen V-sensitized C57BL/6 mice. Data are presented as group mean $\pm$ SEM (n=5-6). Results are changes in rectal temperature over 60 mins with recordings every 10 mins and are representative of two separate experiments.

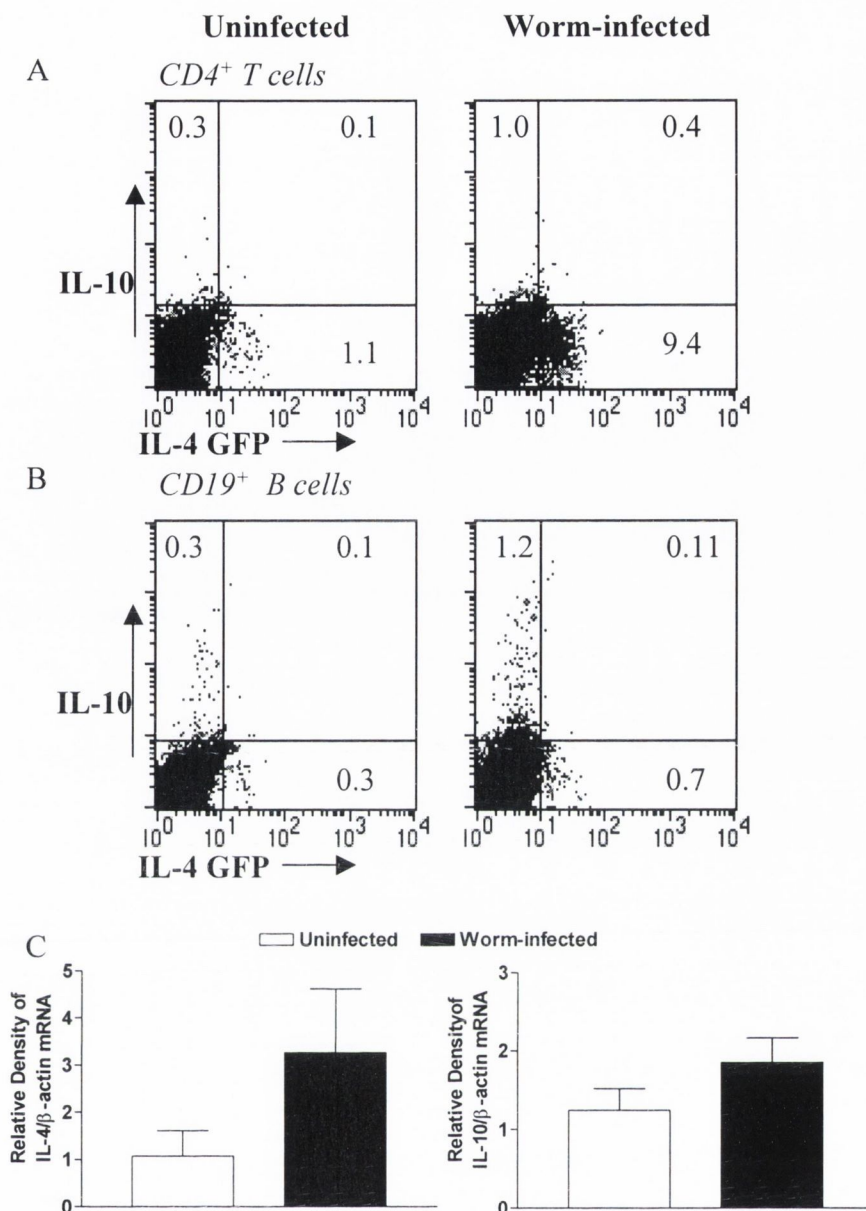


Figure 5.13. *Worm-infected mice have elevated levels of B cell IL-10 and IL-4 compared to uninfected mice.* Intracellular cytokine staining to detect the percentages of IL-10-producing CD4⁺ T (A) and CD19⁺ B (B) cells in spleen cells from uninfected and worm-infected 4GET (IL-4 GFP) mice. Quadrants were assigned from isotype controls. 3 spleens were pooled and data representative of two separate experiments. (C) Ratio of IL-4 and IL-10 mRNA expression to β -actin in purified B cells isolated from spleens from uninfected and worm-infected BALB/c mice. Data are from three separate autoMACS isolations of B cells.

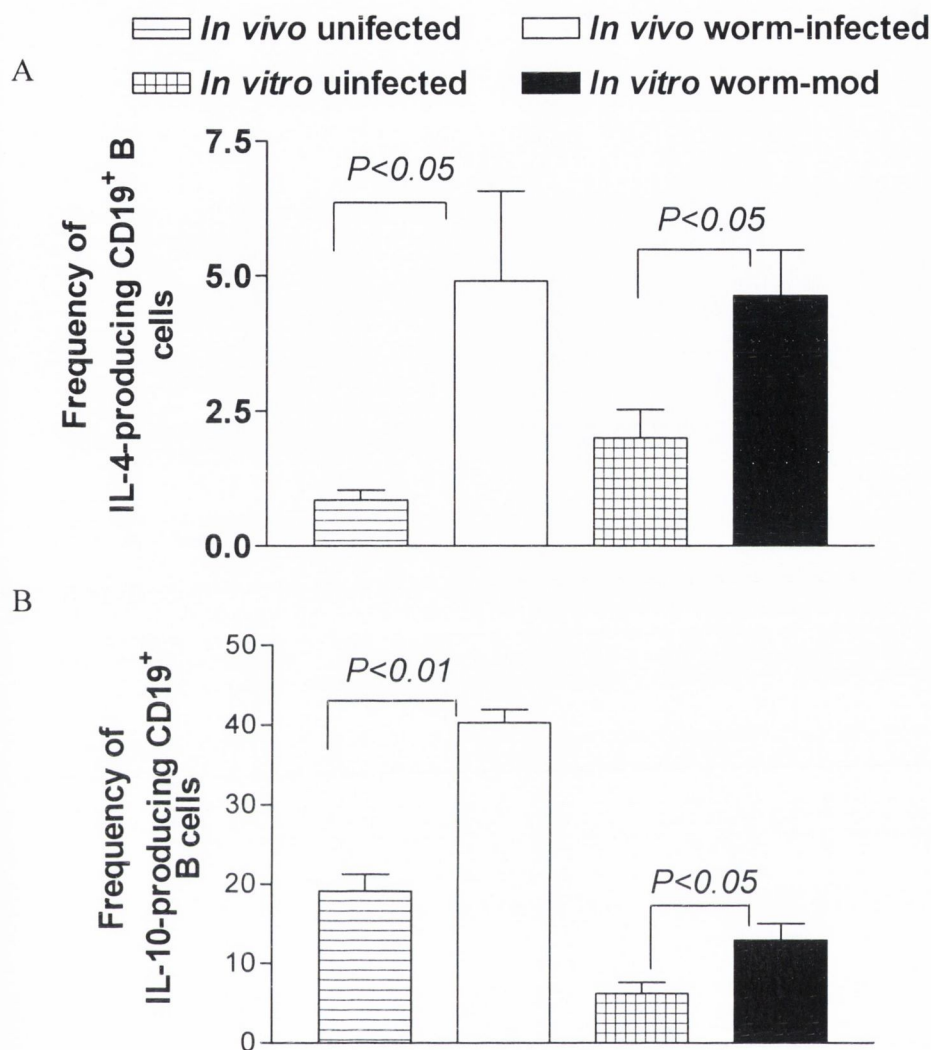


Figure 5.14. Elevated IL-4 and IL-10 producing B cells following worm-modulation both *in vivo* and *in vitro*. Frequency of (A) IL-4 and (B) IL-10-producing CD19⁺ B cells in the spleen from uninfected or *S. mansoni* worm-or worm + egg-infected mice or in cells modulated *in vitro* with worms. Spleen cells were isolated from 2-3 uninfected and worm-infected mice and pooled. Cells were gated on CD19 expression and quadrants for IL-4 or IL-10 were set with appropriate isotype controls. Data presented are mean + SE of cytokine-producing cells from 4-6 experiments. Student's t-test was used to determine differences between groups.

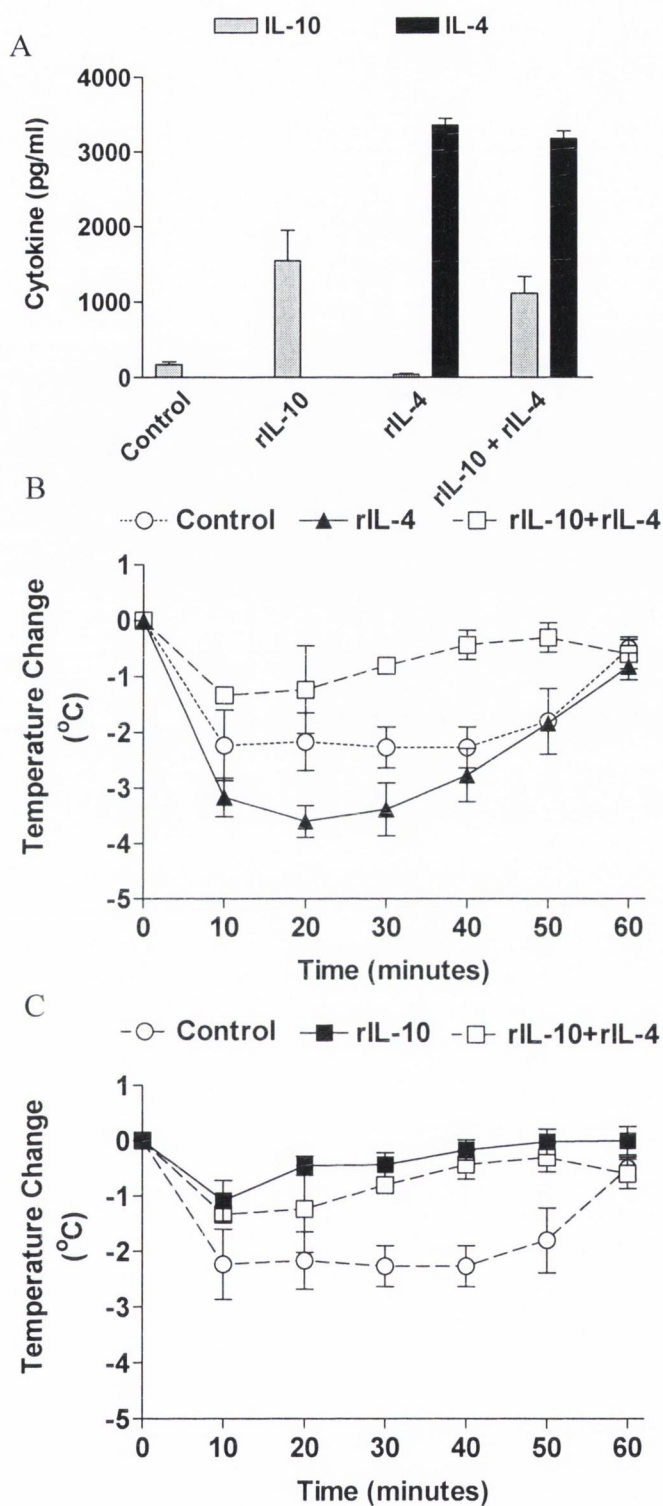


Figure 5.15. *IL-10 diminishes IL-4-mediated exacerbation of Pen V-mediated anaphylaxis in vivo.* (A) Serum levels of IL-4 and IL-10 in mice injected with PBS (Control) or recombinant IL-4 $\pm$ IL-10. (B, C) Drop in body temperature in IL-4- $\pm$ IL-10-treated Pen-V sensitized mice. Data are presented as group mean $\pm$ SEM (n=5-7) temperature change and are representative of two separate experiments.

—○— Unmod wild-type B cells ···●··· Worm-mod wild-type B cells
 —□— Unmod mKO B cells ···■··· Worm-mod mKO B cells

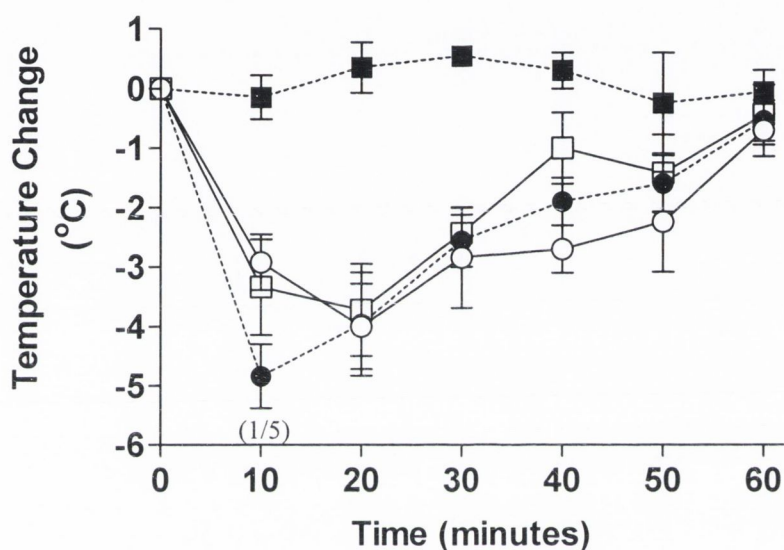


Figure 5.16. *Transfer of Th2 cytokine deficient B cells modulated by S. mansoni worms in vitro prevents anaphylaxis.* Spleen cells from IL-4, -5, -9, -13 (mKO) mice were modulated with worms *in vitro*. B cells were isolated by autoMACs and transferred to Pen V-sensitized mice. *In vitro* modulation and cell transfer are as described in Chapter 2. Data are presented as group mean $\pm$ SEM (n=5-6) temperature change and are representative of two separate experiments.

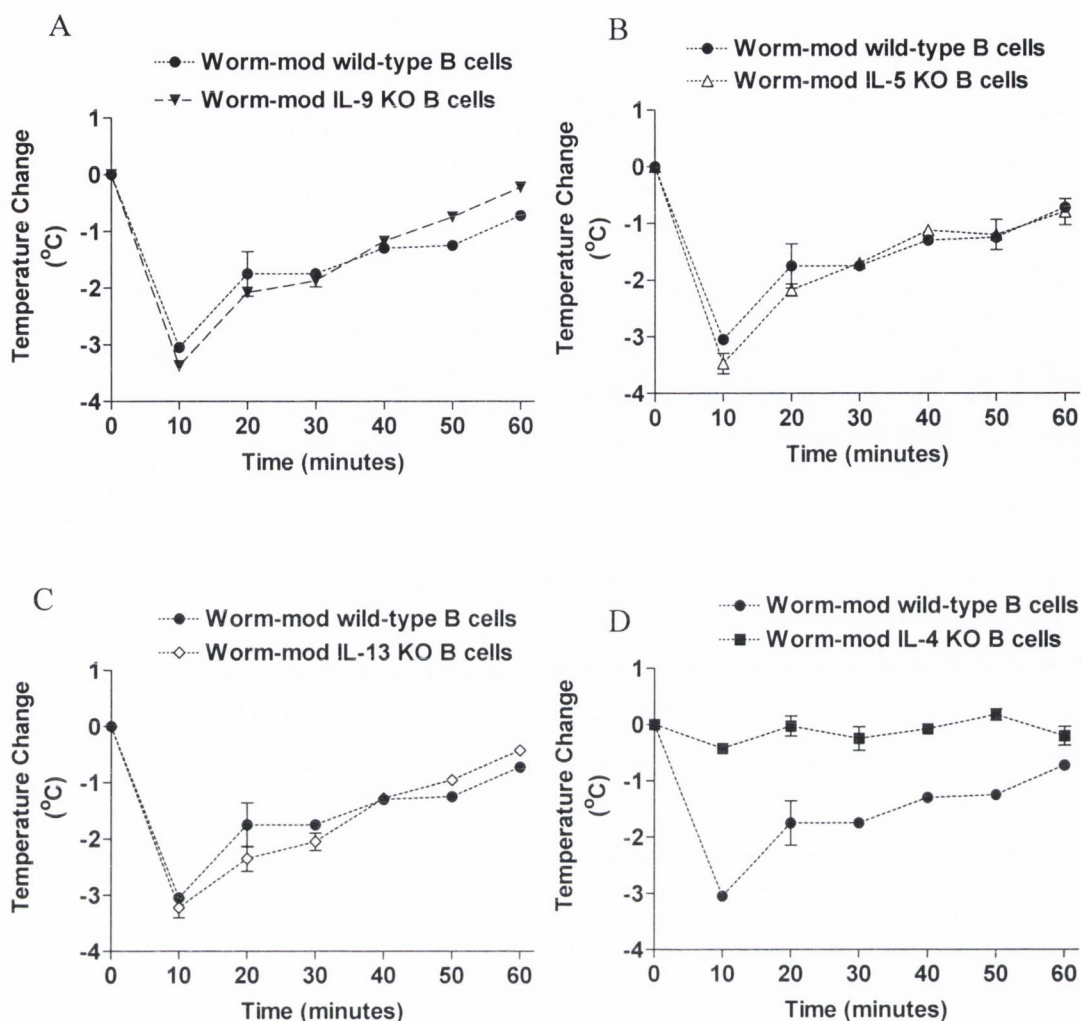


Figure 5.17. *Transfer of IL-4 deficient B cells modulated by worms in vitro prevents anaphylaxis.* (A-D) Worm-modulated B cells from IL-4^{-/-} (IL-4 KO) mice, but not modulated cells from IL-5 KO, IL-9 KO and IL-13 KO, prevent anaphylaxis when passively transferred to Pen V-sensitized mice. *In vitro* modulation and cell transfer are as described in Chapter 2. Rectal body temperatures were recorded prior to Pen V-BSA challenge (time 0) and then every 10 minutes for 60 minutes. Data are presented as group mean $\pm$ SEM (n=6-8) and are representative of two separate experiments.

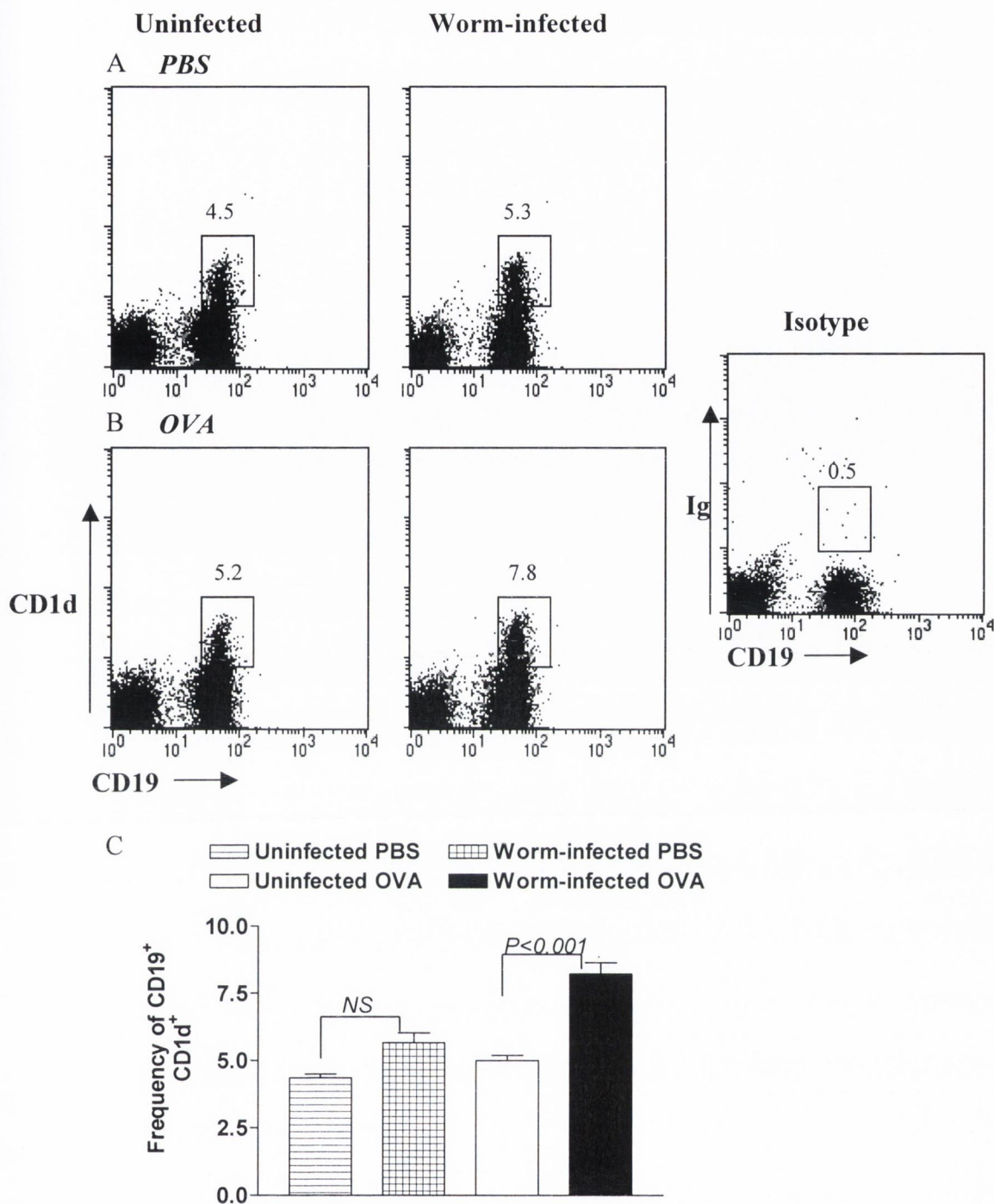
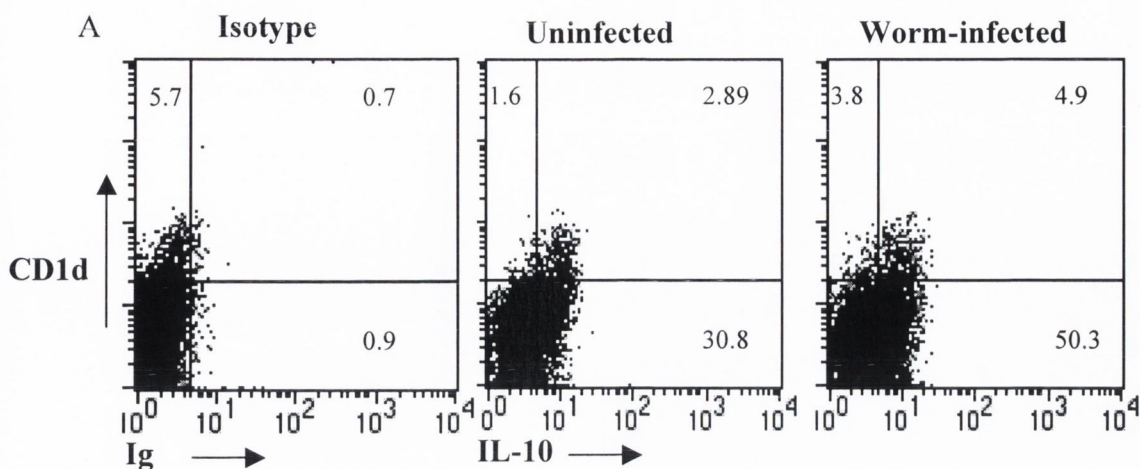


Figure 5.18. Following OVA sensitization *S. mansoni*-infected mice have elevated frequencies of spleen CD19⁺ B cells compared with uninfected mice. Representative images of levels of CD19⁺ CD1d⁺ cells in the spleens of uninfected and worm-infected PBS (A) and OVA-sensitized (B) mice analysed by flow cytometry. (C) The frequency of CD19⁺ CD1d⁺ cells in the spleen was assessed. Cells were stained with mAbs and gates were set on appropriate individual isotype control mAbs. Statistical analysis was performed using Student's t-test. Data are from representative of 2 repeat experiments (n=5).



□ Uninfected-OVA ■ Worm-infected-OVA

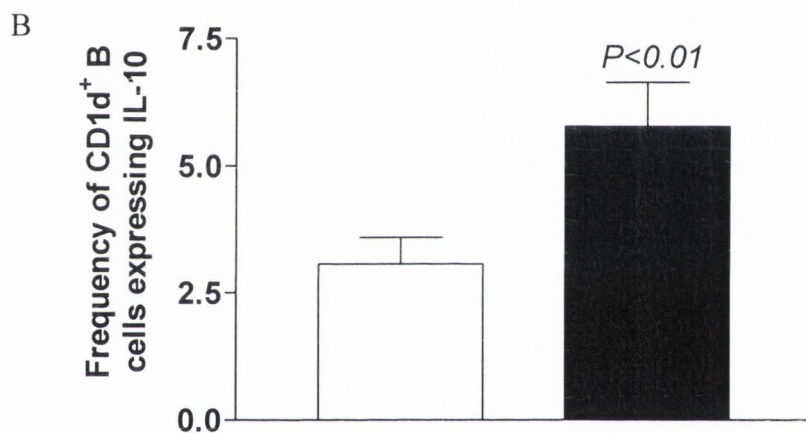


Figure 5.19. *OVA-sensitized S. mansoni-infected mice have elevated frequencies of spleen CD1d⁺ B cells expressing that produce IL-10 compared with sensitized uninfected mice.* (A) CD19⁺ CD1d⁺ spleen cells in uninfected and worm-infected OVA sensitized mice were characterised to ascertain IL-10 levels by intracellular cytokine staining. (B) The frequency IL-10 producing-CD19⁺ CD1d⁺ in the spleens of uninfected compared with worm-infected mice. Spleens were pooled from 3 mice per group and data are representative of 3 experiments. Cells were stained with mAbs and gates were set on appropriate isotype control mAbs. The isotype control shown is spleen cells from a worm-infected mouse.

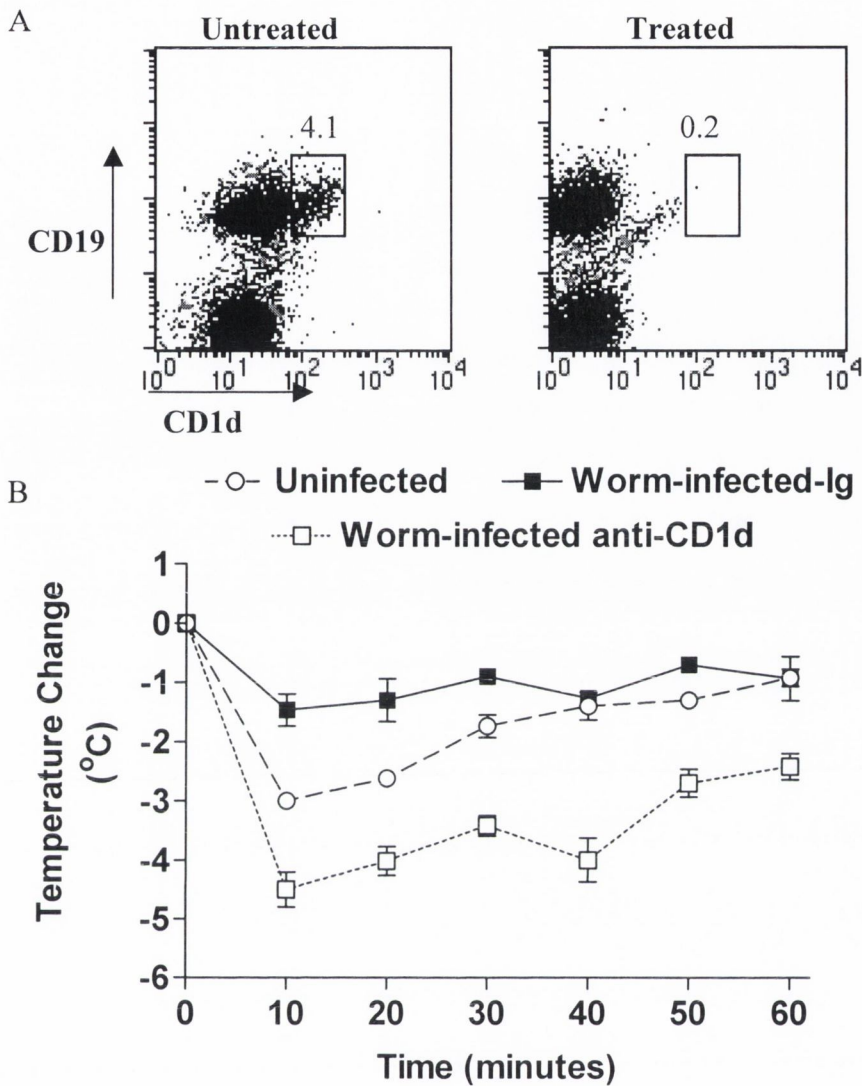


Figure 5.20. *S. mansoni* worm-infected *CD1d*-depleted mice are protected from anaphylaxis. (A) *In vivo* depletion of antibody (mAb) targeted cell populations. Mice received two antibody depleting ip injections (0.5 mg/mouse) of anti-*CD1d* (1B1). Depletion of the cell populations was determined by FACS analysis for the surface expression of the Ab-targeted markers. (B) Pen-V anaphylaxis was induced in anti-*CD1d*-treated and untreated worm-infected and uninfected mice as described in Chapter 2. Rectal body temperature was recorded prior to Pen V-BSA challenge (time 0) and every 10 minutes for one hour. Results are group mean $\pm$ SEM ($n=6-8$) change in body temperature. Data presented are representative of two separate experiments.

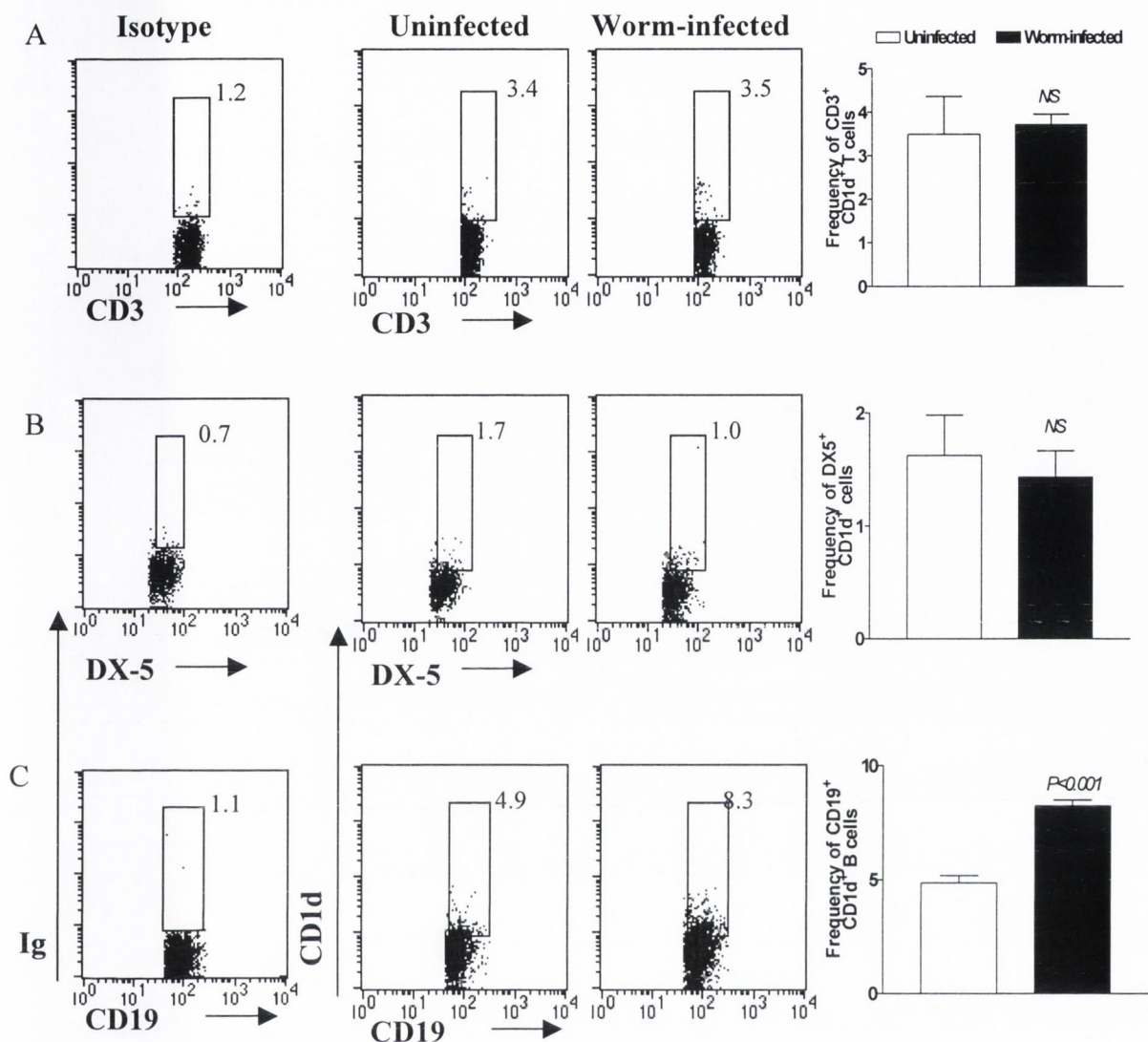


Figure 5.21. *OVA-sensitized S. mansoni-infected mice have elevated levels of B cells expressing CD1d in the lung.* Lung digests from uninfected and worm-infected OVA sensitised mice were analysed for levels of CD1d expression in various cell population in the lung by immunofluorescence staining. Representative images of CD1d⁺ (A) CD4⁺ T cells, (B) DX-5⁺ NK cells, and (C) CD19⁺ B cells are shown. Bar graphs are mean + SD indicating the frequency of CD1d⁺ cells (A-C). Gates were set on individual isotype control mAbs for each surface marker. Student's t-test was used to determine statistical differences between groups n = 4-6. Results presented are representative of 2 different experiments. NS = Not significant.

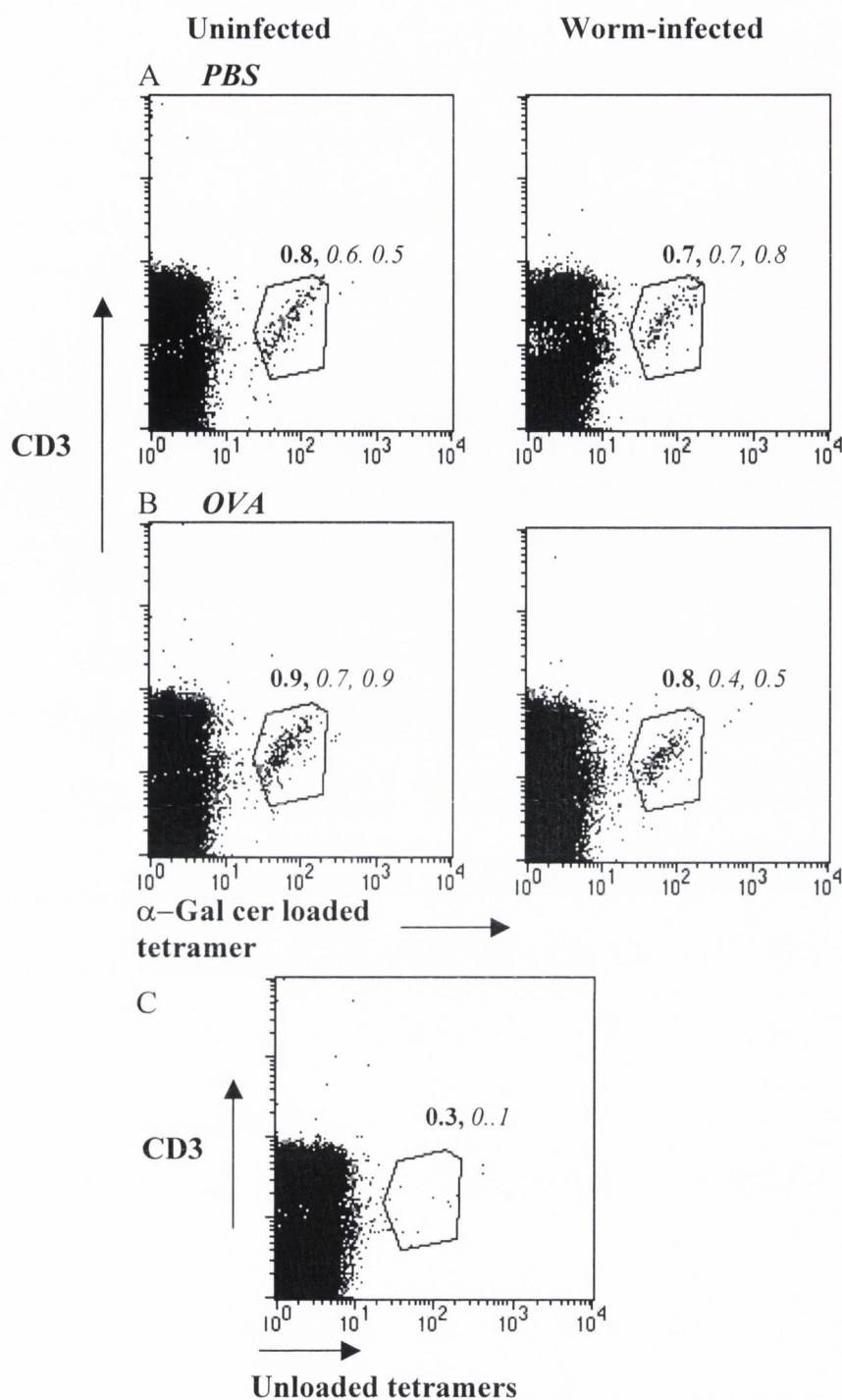


Figure 5.22. Worm-infected mice do not have elevated $CD1d^+$ T cells in the lung by tetramer staining. $CD1d$ loaded α -GalCer tetramers were used to identify $CD3^+$ $CD1d^+$ cells in the lungs of uninfected and worm-infected PBS (A) or OVA-sensitised (B) BALB/c mice. Cells from lung digests were blocked with neutravidin to prevent non-specific binding and were then stained with mAbs and tetramers as described in Chapter 2. Numbers in bold represent values for that individual mouse image, and in italics values from other individual mice. Data are representative of 2 experiments ($n=5$). Gates were set on unloaded tetramers for uninfected OVA lung digests (C) and appropriate isotype control mAbs.

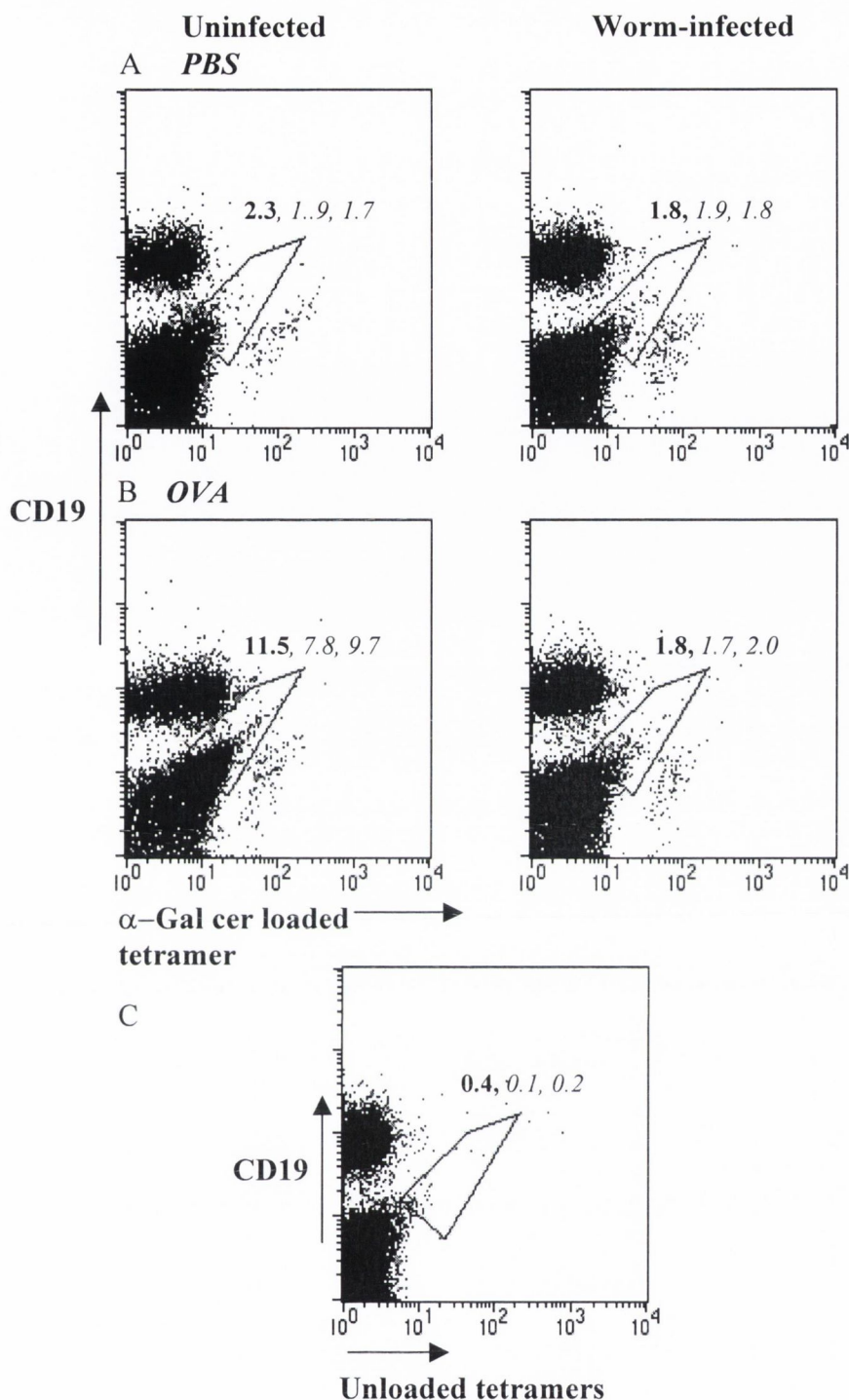


Figure 5.23. Worm-infected mice do not have elevated $CD1d^+$ B cells in the lung by tetramer staining. $CD1d$ loaded α -GalCer tetramers were used to identify $CD19^+$ $CD1d^+$ cells in the lungs of uninfected and worm-infected PBS (A) or OVA-sensitised (B) BALB/c mice. Cells from lung digests were blocked with neutravidin to prevent non-specific binding and were then stained with mAbs and tetramers as described in Chapter 2. Numbers in bold represent that image, repeats in italics. Data are representative of 2 separate experiments $n=5$. Gates were set on uninfected OVA sensitised lung homogenates (C) with unloaded tetramers and appropriate isotype control mAbs.

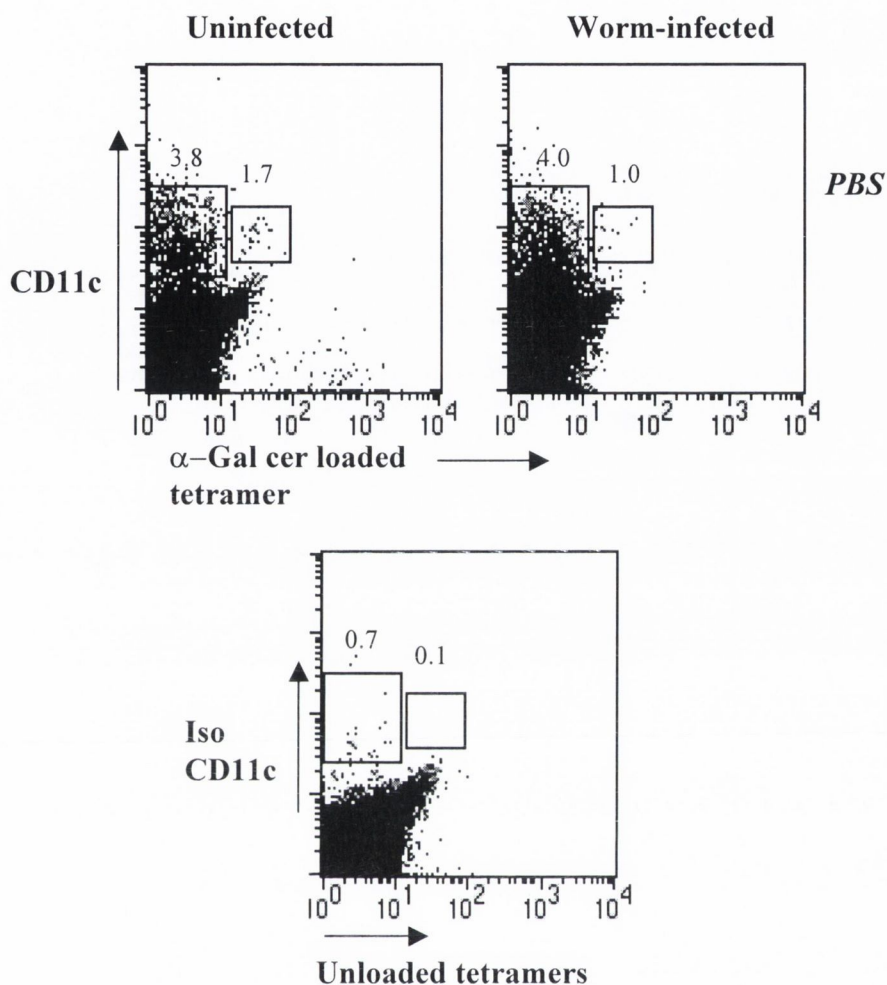


Figure 5.24. *Worm-infected mice do not have elevated $CD11c^+CD1d^+$ in the lung by tetramer staining.* $CD1d$ loaded α -GalCer tetramers were used to identify $CD11c^+ CD1d^+$ cells in the lungs of uninfected and worm-infected BALB/c mice. Cells from lung digests were blocked with neutravidin to prevent non-specific binding and were then stained with mAbs and tetramers as described in Chapter 2. Data are representative of 2 experiments ($n = 4$). Gates were set on unloaded tetramers and appropriate isotype control mAbs.

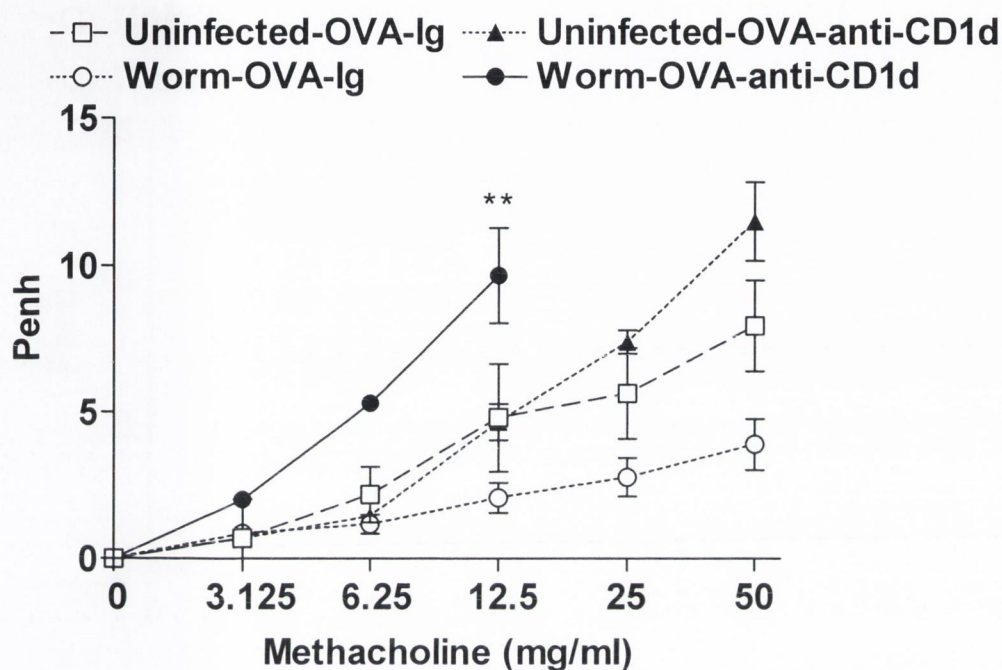


Figure 5.25. Role for CD1d in *S. mansoni* protection against AHR. Penh responses of OVA-sensitized uninfected mice and sensitized-infected mice treated with anti-CD1d (IBI) or control IgG. Student's t-test was used to determine difference between uninfected mice and anti-CD1d-treated infected mice. Data represent the mean change from baseline Penh values for each group. Statistical differences between infected mice and uninfected OVA-sensitized anti-CD1d treated mice was tested by Students t-test; ** $P < 0.01$.

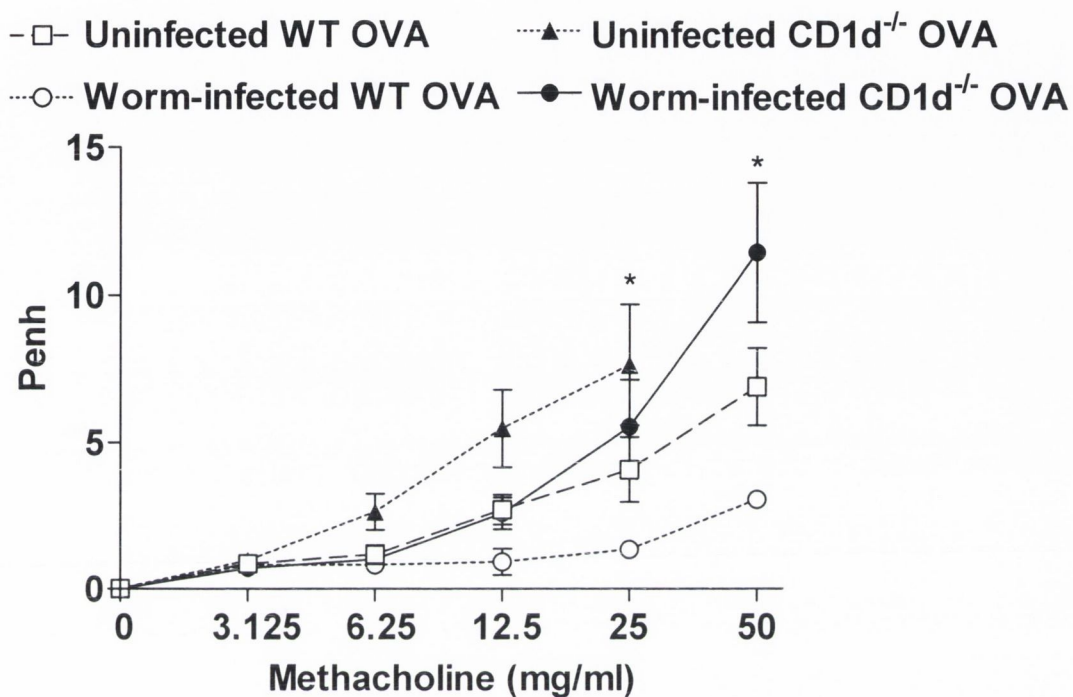


Figure 5.26. *CD1d* deficient worm-infected mice are fully susceptible to AHR. Penh responses of OVA-sensitized uninfected mice and sensitized-infected *CD1d* deficient (-/-) and wildtype (WT) BALB/c mice. Data represent the mean change from baseline Penh values for each group. Results are representative of 2 separate experiments (n=8-10). Statistical differences between infected mice and uninfected (8 weeks post infection) OVA-sensitized mice was tested by Student's t-test; * $P < 0.05$.

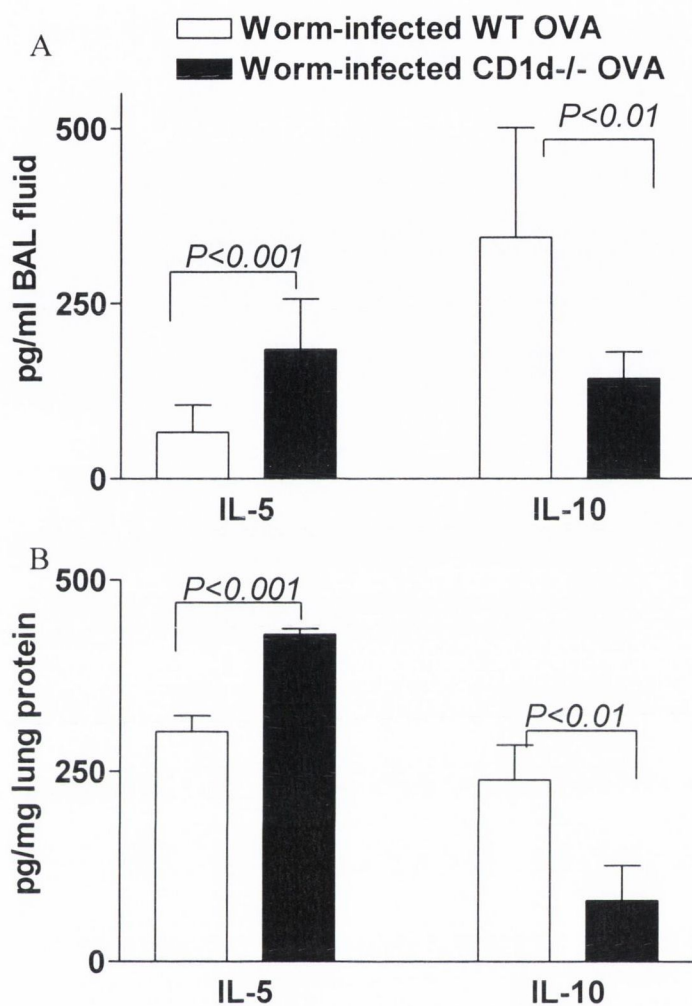


Figure 5.28. *Worm-infected CD1d^{-/-} mice have elevated levels of IL-5 and reduced IL-10 in the lungs.* (A) Levels of IL-5 and IL-10 in the (A) BAL fluid and (B) lung homogenates from OVA-sensitized worm-infected WT and CD1d^{-/-} OVA sensitized mice. Data are from mean + SEM from 4-6 individual mice, and are representative of 2 separate experiments. Cytokine values in lung homogenates were adjusted to ng per mg lung protein. Student's t-test was used to determine differences between groups.

□ Uninfected WT OVA ▨ Uninfected CD1d^{-/-} OVA
 ■ Worm-infected WT OVA ▩ Worm-infected CD1d^{-/-} OVA

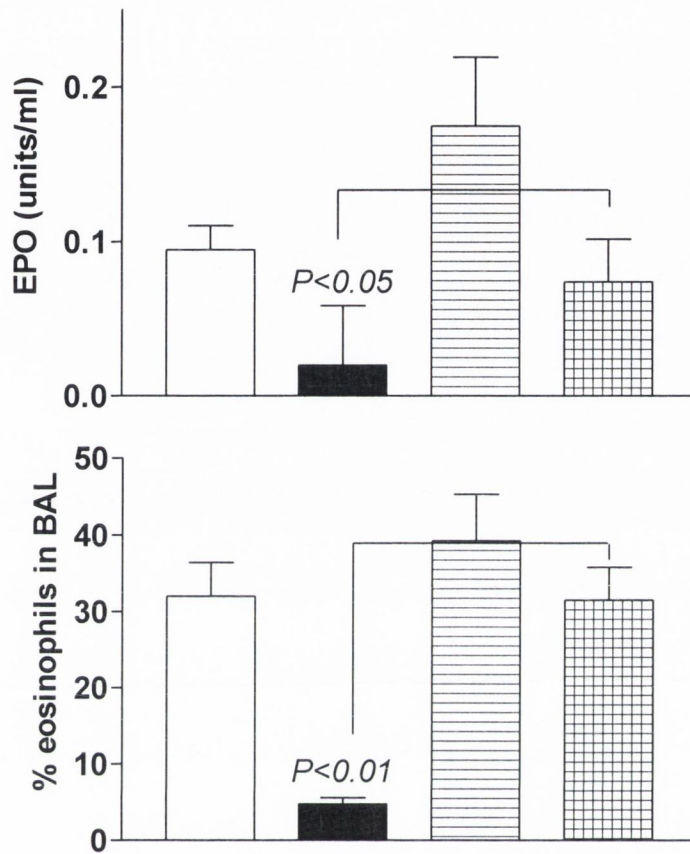


Figure 5.29. *In the absence of CD1d OVA-sensitized worm-infected mice have increased pulmonary eosinophils.* (A) Levels of eosinophil peroxidase enzymatic activity in the lung homogenates from uninfected and worm-infected WT and CD1d^{-/-} mice sensitised with OVA. (B) Percentages of eosinophils in BAL of mice as determined by differential cell counts of cytopins from mice indicated. Data are mean $\pm$ SE, $n = 4-6$. Results are representative of 2 separate experiments. Student's t-test was used to determine differences between groups.

5.3 Discussion

In chapters 3 and 4 I showed that worm mediated protection from anaphylaxis and AHR was dependent on B cells. This lead me to investigate alterations in B cells in schistosome infection. I have now identified a role for CD1d⁺ cells in this B cell worm-modified pulmonary protective response.

Previously, a protective role for IL-10-producing B cells has been demonstrated in experimental encephalomyelitis (EAE) and collagen-induced arthritis (Mauri *et al.*, 2003; Fillatreau *et al.*, 2002). In EAE, IL-10 producing B cells alter recovery of mice from disease through suppression of type 1 autoantigen responses (Fillatreau *et al.*, 2002). Similarly, transfer of *in vitro* anti-CD40 activated B cells from spleen of arthritic DBA/1 mice prevented collagen-induced arthritis in recipients, with B cells from IL-10^{-/-} mice failing to mediate this protective function (Mauri *et al.*, 2003). In this chapter, transfer of B cells from schistosome worm-infected mice (*in vivo* modulated) or *in vitro* worm-modulated B cells not only did not prevent anaphylaxis, as I expected, but induced more severe anaphylaxis in recipients.

During cytokine phenotyping of B cells in infected mice I had observed that splenocytes from schistosome worm-infected mice had 4-5 fold more IL-4 producing B cells than detected in B cells from uninfected mice. As schistosome worm infections are type 2 cytokine-biased (Grzych *et al.*, 1991) it is perhaps not unexpected that B cells from worm-infected mice have a type 2, or B effector 2 (Be2), profile. In the more type 2-biased worm + egg-infected mice there was even more marked increased levels of IL-4 producing B cells (Mangan *et al.*, 2004). It has previously been shown that mesenteric lymph node cells from mice infected with the type 2-inducing gastro-intestinal nematode worm

Heligmosomoides polygyrus have elevated Be2 cells (Harris *et al.*, 2000). Thus upon transferring worm-modulated B cells that had elevated IL-10 to recipient mice we were also transferring IL-4 producing Be2 cells. This contamination with Be2 cells became relevant following Finkelman and colleagues demonstration that an injection of mice with only 65 ng of IL-4 significantly increases the severity of anaphylaxis (Strait *et al.*, 2003). Indeed, earlier findings by Park *et al.* also demonstrated that blockade of IL-4, by anti-IL-4 mAb treatment, prevented fatal anaphylaxis in the same antibiotic model as used in this study (Park *et al.*, 1997). We therefore treated mice with exogenous IL-4 and/or IL-10 and illustrated not only the respective, deleterious or protective effects of these cytokines in Pen V-induced anaphylaxis, but also showed that IL-10 can suppress the pathogenic effects of IL-4. Previously, it has been reported that treatment of mice with 50 µg of IL-10 had no effect on their susceptibility to anaphylaxis (Strait *et al.*, 2003), whereas, I observed that administration of 1 µg of IL-10 protected mice from anaphylaxis. As this discrepancy could be due to the difference in anaphylaxis models used, it would be of interest to evaluate the effects of IL-10 in different models of anaphylaxis. The use of mice deficient in Th2 cytokines permitted confirmation that it was up-regulation of IL-4 in schistosome worm-modulated B cells that was responsible for the exacerbated anaphylaxis in recipient mice transferred worm-modulated B cells. Therefore in experiments described in Fig. 5.11, where transferred B cells exacerbated anaphylaxis we were not only injecting mice with IL-10-producing B cells, but we were also transferring IL-4-producing B cells. Therefore in the B cell transfer studies mice injected with *in vivo* modulated cells had more severe anaphylaxis than mice receiving *in vitro* modulated cells which is consistent with B cells from infected mice having greater relative frequencies of IL-4 producing B cells.

Bashir and co-workers have previously demonstrated a role for experimental helminth infection in protection against the development of allergy in mice (Bashir *et al.*, 2002). They assessed the effect of type 2 responses induced by infection with the gastro-intestinal nematode (*H. polygyrus*) on the development of an allergic response to peanut antigen, an important food allergen. They show protection being mediated, at least in part, by the production of IL-10. Although we have demonstrated a novel mechanism whereby a helminth parasite can prevent anaphylaxis in mice it is possibly too simplistic to envisage a common mechanism whereby different helminth parasites may prevent allergic responses. This can be illustrated by infection of mice with one intestinal nematode (*H. polygyrus*) having been shown to reduce allergic responses (Bashir *et al.*, 2002), whereas in a separate study mice infected with another gastro-intestinal nematode *Trichinella spiralis* had exacerbated anaphylaxis (Strait *et al.*, 2003). There are defined differences on the infectivity and immunity of these two parasites (Finkelman *et al.*, 1997), and also the allergy models used, that could explain this discrepancy. It is relevant that IL-10-producing B cells were first shown in *H. polygyrus* (Harris *et al.*, 2000), and subsequently, infection with this same parasite has been demonstrated by Bashir and co-workers to afford protection against allergic reactions via IL-10.

In *S. mansoni* worm + egg-infection CD1d has also been shown to be important in the generation of an optimal Th2 response and in the subsequent pathology during infection of mice (Faveeuw *et al.*, 2002.). CD1d is capable of binding a wide variety of lipid antigens including α -galactosyl ceramide, β -galactosyl ceramide which is unable to activate natural killer T (NKT) cells (Naidenko *et al.*, 1999) and phospholipids that are present in mammalian cells and considered to be potential self-antigens (Gumperz, 2000). In general glycolipids presented by CD1d rapidly activate NKT cells to produce IFN- γ

and IL-4 (Bendelac *et al.*, 1997). *S. mansoni* eggs and worms produce a variety of glycoconjugates including α -GalCer (Makaaru *et al.*, 1992). Thus the continual chronic presence of the parasite-derived antigens in infection ensures the immune response remains heightened with IL-10 being produced to regulate the allergic Th2 phenotype. The role of CD1d⁺ cells in the immune response in schistosome infection of mice may be to provide a link between the innate and adaptive immunity by binding glycolipids derived from *S. mansoni* worms and eggs and thus inducing a host response.

CD1d⁺ cells have been shown to be upregulated and have a protective role in the lungs of mice infected with *Streptococcus pneumoniae* (Kawakami *et al.*, 2003) or *Pseudomonas aeruginosa* (Nieuwenhuis *et al.*, 2002). An essential role for NKT cells has been shown in the development of asthma and Th2 biased respiratory immunity against allergens. CD1d^{-/-} mice, which lack CD1d on the surface of cells, failed to develop OVA-induced AHR (Akbari *et al.*, 2003). In agreement with this I have found that OVA-sensitised BALB/c mice that develop AHR have elevated levels of CD1d-loaded α -GalCer⁺ cells in their lungs. Also, worm-infected mice that do not develop AHR did not have an increase in the frequency of CD1d-tetramers seen in the lungs of infected mice. In contrast to Akbari however, in these studies here OVA-sensitised CD1d^{-/-} mice developed elevated AHR compared with OVA-sensitised WT mice. Furthermore CD1d^{-/-} mice infected with an *S. mansoni* worm infection also had substantial pulmonary distress as determined by AHR.

It is possible that in CD1d^{-/-} mice, as used in this thesis, the absence of CD1d on all cells may impact on the overall pulmonary inflammatory response. In Akbari *et al.* studies on CD1d^{-/-} mice, they also demonstrated AHR in the absence of CD1d only on NKT cells by using J α 281^{-/-} mice (Akbari *et al.*, 2003). Therefore to assess the function of NKT cells

specifically in worm-protection in allergic disorders, we are currently breeding J α 281^{-/-} mice in-house. J α 281^{-/-} mice, which lack V α 14i NKT cell, will be infected with *S. mansoni* worms and then assessed in the OVA-AHR model to look at the specific role of NKT cells in worm modulation in pulmonary immune responses.

CD1d is expressed by a number of cells but seems to be mostly on effector or antigen presenting cells and appears to function in chronic inflammatory models (Blumberg *et al.*, 1991; Brossay *et al.*, 1997; Saubermann *et al.*, 2000). Ligation of CD1d results in rapid and sustained production of IL-10 from intra-epithelial cells (Colgan *et al.*, 1999). I assessed the alterations in the expression of CD1d on different cell types with infection using CD1d tetramers loaded with α -GalCer. As the NKT cell marker NK1.1 is not expressed on BALB/c mice I used DX5. This is a marker on NK cells which has also been used for assessing NKT cell numbers, although not all NKT cells are DX5⁺. B cells have been shown to express CD1d, with subsets such as MZ B cells (Amano *et al.*, 1998) and T2 B cells (Loder *et al.*, 1999) having a particularly high expression. In fact, both of these B cell subpopulations are increased in worm-infected mice. Ligation of CD1d on B cells by worm-derived molecules or glycolipids may be responsible for the production of IL-10. Alternatively the differentiation of regulatory B cells producing IL-10 may depend on an interaction with CD1d-restricted T cells. However, the exact mechanistic flow of the interaction between the *S. mansoni* worm, CD1d⁺ cells, and B cells that leads to the production of IL-10 which mediates the immune modulation in a protective phenotype has yet to be definitively characterised.

The reduced binding of CD1d loaded α -GalCer tetramers to lung cells in worm-infected mice compared with uninfected mice is interesting. This may be due to the type of cell being assessed by tetramer staining as there are also less well characterised CD1d-

dependent, non- α -GalCer reactive type II NKT cells (Godfrey *et al.*, 2004). However, a more likely scenario is that only recently described by Parekh *et al.*, where activated NKT cells become unresponsive upon secondary immune challenge (Parekh *et al.*, 2005). Parekh's studies involved a 30-day acute model of α -GalCer activation of cells, whereas in *S. mansoni* worm-infection there is a chronic exposure of cells to worm antigens. In the case of *S. mansoni* infection this may translate to worm-induced CD1d activation of NKT cells causing long-term anergy upon subsequent immune challenge with allergens. We have previously shown that *S. mansoni* worm-infection of mice induces T cell anergy *in vivo* (Smith *et al.*, 2004). Therefore this conveys a tolerance mechanism by schistosomes in sustained immune regulation in the presence of chronic antigen stimulation. Further support for this hypothesis is that worm-infected CD1d^{-/-} mice are no longer protected in OVA-AHR and demonstrate in fact elevated pulmonary inflammatory responses compared with similar treated WT mice. Importantly, Parekh *et al* show that α -GalCer-pulsed B cells induce NKT cell unresponsiveness. It would therefore have been interesting to compare α -GalCer-activated B cells against worm-activated B cells for further determination of the role of CD1d⁺ on B cells. However, we could not acquire α -GalCer for these studies from Kirin due to patent reasons.

B cells, CD1d and IL-10 are essential in the immune response as is demonstrated with removal of B cells and more specifically CD1d⁺ cells from worm-infected mice in allergies. Depletion of these cells disrupts the fine balance in regulation and thus exacerbates allergic disorders such as OVA-induced pulmonary inflammation by removal of a potentially critical regulatory cell. Collectively these data further strengthen the case for the involvement of schistosomes in allergies that was discussed in Chapters 3 and 4. In this chapter I have demonstrated how co-operative regulation from the innate to the

adaptive immune response is essential in the sensitive regulation in schistosome-induced immunity. I have illustrated the importance of B cell in this response. Also I have further highlighted a putative regulatory mechanism evoked by helminths to sustain their survival in the face of potentially detrimental immune challenge.

Chapter 6

General Discussion

6.1 General discussion

In the course of this PhD thesis I have addressed a role for schistosomes in the Hygiene Hypothesis. The Hygiene Hypothesis is but a theory as discussed in the introduction. This hypothesis infers a putative link between protection from allergies and infection of individuals infected with parasites (Yazdanbakhsh *et al.*, 2002). I have assessed the role of *Schistosoma mansoni* in susceptibility of mice to severe and mild hypersensitivity reactions i.e. anaphylaxis and airway hyper-responsiveness. I have demonstrated positive protective effects and also a negative exacerbatory effect for schistosomes in allergies. Indeed my data are of huge significance in the context of the Hygiene Hypothesis as schistosomes fit arguments both for, and against, the hypothesis.

In this thesis I have proposed a novel method of immune-modulation by *S. mansoni* worms in allergic responses. Protection was mediated by IL-10 production, which has been widely investigated by others, but is described here as part of a new mechanism of protection in allergies in what I have called a helminth-modified pulmonary type 2 response. Indeed the results I have discussed in Chapters 4 and 5 demonstrate that schistosome infection of mice modulates the pulmonary response to subsequent allergen exposure. This protective response involves a modified type 2 response with decreased IL-5 production and hence reduced pulmonary eosinophil recruitment with associated reductions in airway inflammation. Studies by others have highlighted the central role of IL-5 and eosinophilia in the development and progression of asthma. Furthermore, once airway inflammation has been established, neutralisation of the effects of IL-5 or reducing eosinophilia may not result in inhibition of established AHR in atopic asthma. Therefore further characterization of the mechanism of protection by the worm and investigation of the impact of helminth infections on allergic responses to non-parasite allergens (house

dust-mite) has the potential for revealing other elusive factors that control expression of allergic diseases, leading to the development of novel therapeutics for asthma and other allergic diseases.

There is a need for new effective therapies for atopic disorders. Atopic disorders have a complex and chronic pathogenesis that provides many potential cellular and molecular targets for therapeutic intervention, but may also include redundant pathways that mediate disease. Characterized mechanisms of protection from allergic diseases include decreases in IL-4, IL-5 and IL-13 (decreased Th2 bias), increases in IL-10 (a regulatory mechanism) and an elevated ratio of allergen-specific IgG4 to IgE (blocking). This increase in IgG4, which blocks the effects of IgE, has been proposed to be due to increased IL-10, which directly acts on B cells to promote production of IgG4 relative to IgE. Children with early exposure to allergens who do not develop allergic disease also frequently develop elevated allergen specific IgG4 as discussed in Chapter 4 (Platts-Mills *et al.*, 2001) (**Fig. 6.1**).

Therefore therapies have looked towards modulating the Th2 cytokine balance as an effective treatment in asthma, primarily by mAb against these cytokines. However, only limited benefit was observed for asthma patients treated with inhaled soluble forms of IL-4R α which as discussed previously (Chapter 1) is an important component in IL-4 and IL-13 receptor activation (Cohn *et al.*, 1999). As eosinophils are important in induction of AHR (McKenzie *et al.*, 2001), this has lead to studies on IL-5, which is involved in the production, differentiation, maturation and activation of eosinophils. Several studies have looked at the use of humanized anti-IL-5 mAb treatment in allergies. Treatment effectively depletes eosinophils but has not demonstrated effective therapeutic potential in asthma (Bousquet *et al.*, 2000). As *S. mansoni* worm infection of mice modulates the pulmonary

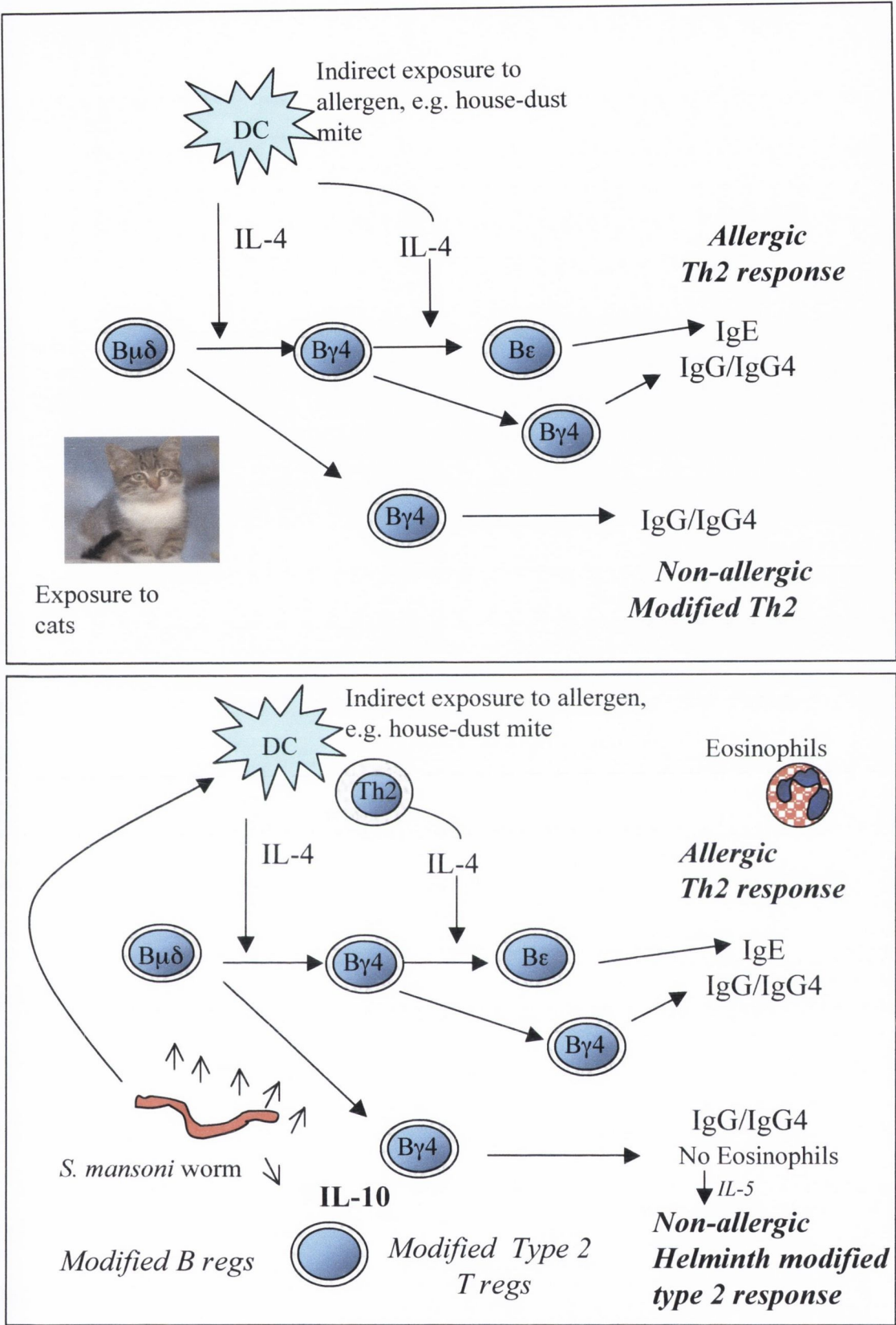


Figure 6.1. Proposed development of modified Th2 response to cats and helminth modified type 2 response.

type 2 response in airway inflammation and this is the ideal of asthma treatments perhaps therein lies a new therapy from these helminth worms.

Exploiting parasite worms for their immunoregulatory properties does have potential and is currently under investigation in trials of hookworms in asthma (Falcone and Pritchard, 2005). I have discussed in chapters 3-5 that it is the worms' presence that affords protection to the host from development of allergies, which suggests the worm may be of benefit in the treatment or prevention of asthma. However, there are many ethical and biological reasonings against this. Most importantly, the immune-modulatory mechanisms of the worm are not fully characterized. It could not be guaranteed that the worm would not elicit side-effects and even death in a patient. Therefore further studies and characterization of the host-parasite immune interaction are necessary before even considering use of the schistosome worm in the treatment of allergies. However, there is currently a 'worm-rush' to put worms into patients in the treatment of allergic disorders.

Perhaps, instead of infecting a patient with a worm, a peptide or molecule secreted from the worm could be harvested for its beneficial regulatory effects? Some therapies for EAE have been based on self-peptide therapies but there is also the potential for a worm-based peptide therapy against allergies. However, aside from the possibility for allergic reactions in peptide therapy, the protective factor produced by the worm indeed may not be efficant as the peptide/lipid may need the worm or other worm factors to ensure it's correct expression e.g. correct exposure of certain glyco-lipids on the surface (in view of the role for CD1d). I have however identified a restricted number of worm antigens that may be therapeutic. The allergy models I have developed in this thesis will be of use to further identify and test worm molecules in anaphylaxis and airway hyper-responsiveness.

The findings of my thesis have important implications for understanding a role for schistosomes in allergies and dissecting immune mechanisms of regulation. However, in the context of the Hygiene Hypothesis, the fundamental theory behind my results in chapters 3, 4 and 5 is flawed. To published knowledge humans do not develop worm-only *S. mansoni* infections. Conventional schistosome infections in the field involve male and female parasite worms residing in a human for a number of years where they produce eggs. Although there is a male worm bias in infections, I have found that worm + egg infections do not protect mice from anaphylaxis or airway hyper-responsiveness. In fact the presence of the egg is actually exacerbates the allergic response in these mice. Also in these mouse studies, the presence of a worm or worm pair does not correlate with worm burden in humans; in the mouse one worm pair can produce 300 eggs per day but this number is out by about 10 fold in humans. Therefore the worm-Ag exposure in the mouse model of infection does not reflect true infection. Also humans are infected over years and are exposed to repeat infections whereas mice infected with *S. mansoni* experimentally are infected for only 8-16 weeks.

The host's immune response over the course of infection changes with acute and chronic stages in infections. This then raises the question of the type of challenge posed by the parasite. The acute phase of the immune response is maximal with the production of eggs by the worms that have now become established in the vasculature of the host. In a conventional infection the presence of the worm in the host preceeds egg production, thereby priming the immune system against the egg. The worm and the egg present antigen to the immune system continuously with the progression of their life-cycle and therefore maintains a constant regulatory environment with infection. I have assessed the protection of the worm + egg infection in airway hyper-responsiveness and even though there is a

regulatory environment at this stage, there is still no protection. Established schistosome worms themselves induce an immune response to prevent reinfection which would introduce crowding via concomitant immunity. Therefore it is this induction of concomitant immunity in infection that I am proposing that results in the protection by worms against allergies. Indeed the fact that there is continual immune challenge from antigens produced both by the worm and the egg has implications for potential in allergy treatments. It is highly unlikely that one particular molecule would be able to recreate the persistent immune stimulation as in a worm infection.

Most mechanistic investigations, including this thesis, have used mouse models of human disease. There are many differences and conundrums between human and mouse immune responses (Fallon, 2000). Mouse models are an excellent way for determining disease effects and assessing the immunology of infections and diseases. However, results from rodent studies can only be extrapolated to a certain point with relevance to the human condition. The main limitation in interpretation of my results using a mouse model of human asthma is that mice do not represent a good model of the IgE/IgG4 interplay in allergies. As discussed in concomitant immunity by the worm (chapter 1) and induction of tolerance to allergens (chapter 4), the blocking Ab hypothesis is potentially the mechanism for reduced allergies in parasite-infected individuals. I have not been able to directly assess this vital factor in the Hygiene Hypothesis as mice do not have a blocking Ig like the human isotype IgG4.

The mouse model is essentially a contrived experimental laboratory system. Mouse strains used for this thesis are inbred strains of mice that are essentially sterile environments. Inbred mice have the same genetic background and are housed in specific pathogen-free environments under strict housing conditions. Everything from temperature

to diet is controlled. Also the regimes of allergen exposure and the age of the mice are selected for in the experimental investigation. Nevertheless, it is worth stressing that the mouse model of AHR I have used in this thesis is the standard model to test new therapies for human asthma. Mice do not reflect the genetic and environmental influences that are argued to be the basis of the Hygiene Hypothesis. Indeed, in the first two years of AHR experiments during my PhD, I have found that alterations in the housing conditions of mice, i.e. food and bedding, are sufficient to alter the allergic response of naïve mice. I have thus not been able to use all that data. Therefore mouse data does not allow for other differences in human populations such as location, age, co-infection, lifestyle, diet, exercise, exposure to environmental allergens.

The association between schistosome infection and allergies, although tantalizing, is limited in its definition. Indeed the conflicting evidence in humans for and against the hypothesis is potentially a statistical war. The list of factors that may influence the development of allergies is growing rapidly and straining the argument for this hypothesis. Everything from lifestyle to diet to vaccinations to whether we played in the dirt as a child has been attempted to be fitted to this hypothesis. There is such an amount of variability that is very difficult to discern whether you can attribute the increase in allergies solely to low helminth burden or decreased early exposure to infection. The life-threatening nature of anaphylaxis and allergies suggests that the biological processes responsible for this disorder would only have evolved if they are host protective when activated in an appropriately limited and focused fashion.

So to conclude – “Will a worm a day keep the doctor away?”

In the case of the experimental worm-infection that has been the focus of my thesis, I concur that *Schistosoma mansoni* infection of mice does impart a protective regulatory

immunity to allergies. Overall I have identified a novel mechanism by worms to modify pulmonary type 2 responses in allergies. My work is very exciting in the field of asthma research and opens up potential areas for future research in the treatment of allergies. In the future, treatments derived from schistosome worms may indeed keep the doctor from the door.

Chapter 7

References

7.1 References

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Appendices

Appendix I

Preparation of media and buffers

All reagents are from Sigma-Aldrich unless otherwise indicated.

Preparation of spleen cells and cell culture.

Culture media

RMPI-1640 Medium (Gibco)

2mM L-Glutamine

10% Fetal Calf Serum (FCS; Labtech, U.K.)

100 U/ml Penicillin

100 µg/ml Streptomycin

Peritoneal lavage fluid

RPMI-1640 medium with 1% FCS

Wash media

RPMI-1640 medium

100 U/ml Penicillin

100 µg/ml Streptomycin

Erythrocyte Lysis Buffer

0.85% ammonium chloride (NH₄Cl) in dH₂O, filter sterilised.

FACS Buffer

PBS with 2% FCS and 0.05% sodium azide.

Phosphate Buffered Saline (PBS; 10X)

85g Sodium Chloride (NaCl)

12.8g Di-sodium hydrogen orthophosphate (Na_2HPO_4)

1.56g Sodium dihydrogen phosphate (NaH_2PO_4)

Add to 1L distilled water (dH_2O) and adjusted to pH 7.6 for a 10X solution.

A 1X solution was prepared by diluting 1L of the 10X PBS in 9L dH_2O and adjusting to pH 7.6.

ELISA Buffers

Coating Buffer: PBS

ELISA Wash Buffer: PBS with 0.05% Tween 20

Blocking Buffer: 1% BSA in PBS

Incubation Buffer: PBS

Phosphate-citrate Buffer

10.19 g Citric acid ($\text{C}_6\text{H}_8\text{O}_7$)

36.9 g Di-sodium hydrogen orthophosphate (Na_2HPO_4)

Dissolve in 1L dH_2O and adjust to pH 5.

Carbonate buffer

0.795 g Na_2CO_3

1.465 g NaHCO_3

Dissolve in 500 ml dH_2O (pH 2.6)

MPO buffers

Dilution Buffer: Phosphate citrate buffer, pH 5.0

Substrate Buffer: TMB, pH.5.5, 120 μM Resorcinol

Stop solution: 1M H_2SO_4

EPO buffers

Dilution Buffer: 50mM Hepes, pH 8.0

Substrate Buffer: 50 mM Hepes, pH 6.5, 6 mM KBr, 3 mM OPD, 8.8 mM H_2O_2

Stop solution: 1M H_2SO_4 , 2 mM Resorcinol

Tris-borate EDTA (TBE, 10X)

107.8 g Tris Base

50 g Boric acid

7.44 g EDTA

Dissolve in 1 L dH_2O and adjust to pH 8.3.

A 1 X solution was prepared by diluting 1 L of the 10X TBE in 9 L dH_2O .

Snail maintenance and *Schistosoma mansoni* infections.*Lepple water (10X concentrated)*

13.90 g	Calcium chloride (CaCl_2)
30.70 g	Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)
1.07 g	Potassium sulphate (K_2SO_4)
10.50 g	Sodium hydrogen carbonate (NaHCO_3)

1.2 ml of a solution containing 2.5 g of ferric chloride ($\text{FeCl}_2 + 6\text{H}_2\text{O}$) in 500 ml H_2O .

Dissolve each chemical in 1L of distilled H_2O , mix together and make up to a final volume of 5 litres.

Use at a 1 in 10 dilution.

Hydrocortisone

625 mg	Hydrocortisone 21-acetate
625 μl	Cremophor EL.
24.36 ml	Distilled water

Sonicate the mixture and store at 4°C .

Perfusion media

13.65 g	Lactalbumin hydrolysate with Earle's salts (Gibco)
2.2 g	Sodium hydrogen carbonate (NaHCO_3)
15 g	Tri-sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)

Lugol's Iodine

1.0 g iodine

2.0 g potassium iodate

100 mls distilled water

Formal saline

270 mls 37% formaldehyde

9 g NaCl

730 mls distilled water

Veronal buffer

50 mM Sodium barbitone in PBS

Appendix II: Antibodies

Flow cytometry antibodies:

Marker	Clone	Isotype	Company	Fluorochrome
<i>Surface</i>				
Rat anti-mouse CD4	CT-CD4	Rat IgG2a	Caltag	TRI
Rat anti-mouse CD8a	5H10	Rat IgG2b	Caltag	TRI
Rat anti-mouse CD45R	RA3-6B2	Rat IgG2a	Caltag	TRI
Rat anti-mouse CD19	6D5	Rat IgG2a	Caltag	TRI
Rat anti-mouse F4/80	F4/80	Rat IgG2b	Caltag	TRI
Rat anti-mouse CD11b (Mac-1)	M1/70	Rat IgG2b	Caltag	TRI
Hamster anti-mouse CD11c	HL3	Armenian Hamster IgG1	BD	PE
Rat anti-mouse Gr1 (Ly-6G)	RB6-8C5	Rat IgG2b	Caltag	FITC
Rat anti-mouse Pan-NK (CD49RB)	DX5	Rat IgM	eBioscience	PE
Rat anti-mouse CD25	PC61 5.3	Rat IgG1	Caltag	PE
Rat anti-mouse I-A/I-E (MHC II)	2G9		BD	FITC
Rat anti-mouse IgD	11-26c.2a	Rat IgG2a	BD	FITC
Goat anti-mouse IgM	μ-chain		Caltag	PE

	specific			
Rat anti-mouse CD21/CD35	7G6	Rat IgG2b	BD	FITC
Mouse CD16/32			Caltag	
Rat anti-mouse CD23	B3B4	Rat IgG2a	BD	PE
Rat anti-mouse CD5	Ly-1	Rat IgG2a	BD	PE
Rat anti-mouse CD1d	1B1	Rat IgG2b	BD	PE/FITC
Rat anti-mouse CCR3	83101	Rat IgG2a	R&D Systems	PE
<i>Intracellular</i>				
Foxp3	FJK-16s	Rat IgG2a, κ	eBioscience	FITC
Rat anti-mouse IL-4	BVD6-24G2	Rat IgG1	BD	PE
Rat anti-mouse IL-10	JES5-2A5	Rat IgG1	BD	PE/FITC
Rat anti-mouse IFN-γ	R4-6A2	Rat IgG1	BD	PE/FITC
<i>Isotypes</i>				
Rat IgG2a	eBR2a		eBiosciences	
Rat IgG2a	R35-95		BD	
Rat IgG1	R3-34		BD	
Rat IgG2b	A95-1		BD	
Hamster IgG1	A19-3		BD	

In vitro antibodies

Marker	Clone	Company
Rat anti-mouse CD3ε	145-2C11	BD
Rat anti-mouse CD28	37.51	BD
Anti-mouse CD40	HM40-3	BD
IgM	μ-chain specific	Southern Biotech

Appendix III

PCR Primers:

β-actin fwd	GGACTCCTATGTGGGTGACGAGG
β-actin rev	TCTTTGCCAATAGTGATGACTTGGC
IL-10 fwd	CTGGACAACATACTGCTAACCGAC
IL-10 rev	TTCATTCATGGCCTTGTAGACACC
IL-4 fwd	GAACGAGGTCACAGGAGAAGG
IL-4 rev	GGA CTCATTCATGGTGCAGCT
W1 fwd	CAACACAGTGAAATTCTTCC
W1 rev	GAATTCACCACTCGACATTC
W2 fwd	TTGCTGATGTGCAGTTTGCC
W2 rev	TCTTCCGAGTATGATGCAGG
PDI fwd	TGAAGCCCTGAAAAAGTTCG
PDI rev	TGCACTGTCAAATAGCACTC

Mouse Genotyping primers

IL-10 KO primer 1	GTGGGTGCAGTTATTGTCTTCCCG
IL-10 KO primer 2	GCCTTCAGTATAAAAGGGGGACC
IL-10 KO primer 3	CCTGCGTGCAATCCATCTTG
IL-4 KO primer 1	CTATCAGGACATAGCGTTGGCTAC
IL-4 KO primer 2	TAGAGAGATCTCTGCCAGCATTGC
IL-4 KO primer 3	CCCTTCTCCTGTGACCTCGTTC
IL-5 KO primer 1 (WT)	CACCAGCTATGCATTGGAG
IL-5 KO primer 2 (WT)	CAGGAACTGCCTCGTCCTCCGTCTCTCCTCGC

IL-5 KO primer 3 (Targeted)	GGCGGATGAGCGGCATTTTCCGTG
IL-5 KO primer 4 (Targeted)	GCCGAAAGGCGCGGTGCCGCTGGC
IL-13 KO primer 1	GGGTGACTGCAGTCCTGGCT
IL-13 KO primer 2	GTTGCTCAGCTCCTCAATAAGC
IL-13 KO primer 3	GGCGGATGAGCGGCATTTTCCGTG
IL-13 KO primer 4	GCCGAAAGGCGCGGTGCCGCTGGC
IL-9 KO primer 1	TGATTGTACCACACCGTGCTACAGGG
IL-9 KO primer 2	CGGACACGTTATGTTCTTTAGGACTTC
IL-13 Tg primer 1	ATATCTCAGGAATGGGATTGTGAC
IL-13 Tg primer 2	CTACTGTATTTAAGTGAAG
Neo CD1d KO primer 1	CTTGGGTGGAGAGGCTATTC
Neo CD1d KO primer 2	AGGTGAGATGACAGGAGATC
CD1d KO primer 1	AATTACACCTTCCGCTGCC
CD1d KO primer 2	CTTCGTGAAGCTGATGGTGG
4GET primer 1 (GFP)	AAGTTCATCTGCACCACCG
4GET primer 2 (GFP)	TCCTTGAAGAAGATGGTGCG
4GET primer 3 (WT)	CCTTATGGCCAAATGAAGTGA
4GET primer 4 (WT)	AAAGACTTCCTGGAAAGCCTA

Publications

Publication to-date

1. **Mangan, N.E.**, Fallon, R.E., Smith, P., Rooijen, N. McKenzie, A.N.J. & Fallon, P.G. (2004). Helminth infection protects mice from anaphylaxis via IL-10 producing B cells. *Journal of Immunology* **173**, 6346-6356.
2. Smith, P., Walsh, C.M, **Mangan, N.E.**, Fallon, R.E., Sayers, J.R., McKenzie, A.N. & Fallon, P.G. (2004). *Schistosoma mansoni* worms induce anergy of T cells via selectively upregulation of Programmed Death Ligand 1 on macrophages. *Journal of Immunology* **173**, 1240-1248.
3. Hewett, D.R., Simons, A.L., **Mangan, N.E.**, Jolin, H.E., Green, S.M., Fallon, P.G. & McKenzie, A.N.J. (2005). Lethal, neonatal ichthyosis with increased proteolytic processing of filaggrin in a mouse model of Netherton syndrome. *Human Molecular Genetics* **14**, 335-346.
4. Smith, P., Fallon, R.E., **Mangan, N.E.**, Walsh, C.M., Saraiva, M., Sayers, J.R., McKenzie, A.N., Alcamí, A. & Fallon P.G. *Schistosoma mansoni* secretes a chemokine binding protein with anti-inflammatory activity. *Journal of Experimental Medicine* **IN PRESS**
5. **Mangan, N.E.**, Rooijen, N. McKenzie, A.N.J. & Fallon, P.G. Helminth modified pulmonary type 2 response protects mice from allergen-induced airway hyper-responsiveness. *Journal of Immunology* **accepted subject to revision**
6. Fallon, P.G., Jolin, H.E., **Mangan, N.E.**, Barlow, J., Smith, P., Hewett, D.R. & McKenzie, A.N.J. IL-25 regulates a mast cell precursor population that provides IL-4, IL-5 and IL-13 at the onset of a type-2 response. *Journal of Experimental Medicine* **under review**

7. Walsh, C.M, **Mangan, N.E.**, Smith, P. & Fallon, P.G. *Schistosoma mansoni* type 2 cytokine responses in acute infection are suppressed by modified T regulatory cells. *Journal of Immunology* **submitted**.