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**Modulation of cellular immunity by
Schistosoma mansoni in mice**

submitted to
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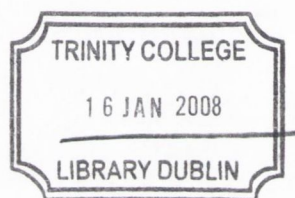
for the degree of
Doctor of Philosophy

by
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October 2006

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Abstract

Schistosoma mansoni is a trematode parasite that elicits a potent Th2 response in its mammalian host. Schistosomes are powerful stimulators and suppressors of the immune system, with these alterations in immune function permitting the parasite to live for up to 40 years in humans. In this thesis T cell responses during acute and chronic murine *S. mansoni* infection were addressed. A novel mechanism of immune modulation in a worm-only infection was identified, with cells from worm-infected mice having impaired T cell proliferation, that was restored by addition of IL-2. F4/80⁺ splenic macrophages from worm-infected mice also induced anergy in naïve CD4⁺ and CD8⁺ T cells. The inhibitory co-stimulatory molecule PD-L1 was shown to mediate this anergy. Levels of PD-L1 declined during *S. mansoni* worm infection, and this correlated with a reduction in anergy induced by the worm infection. This is the first demonstration that PD-L1 has been employed by a pathogen to undermine the immune system.

I showed that levels of PD-L1, its related molecule PD-L2, and their receptor PD-1 are markedly up-regulated during worm-only and conventional worm-and-egg-infection. The role of these molecules was addressed in *S. mansoni* infection using blocking antibody studies. Blocking of PD-L1 and studies in PD-L1 ko mice suggest a potential role for PD-L1 in regulating the type 1/type 2 balance in schistosome infection.

The role of regulatory T cells in infection was also examined in this thesis. I demonstrated that the negative co-stimulatory marker CTLA-4, but not CD25, was involved in the regulation of Th2 responses during *S. mansoni* infection.

These data provide novel findings on the role of regulatory cells and inhibitory T cell co-stimulatory pathways during schistosome infection, and deepen our understanding of the immunomodulatory mechanisms at play in schistosome infection.

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, except where stated 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.

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List of Abbreviations

Ab	Antibody
APC	Antigen presenting cell
AW	Adult worm antigen
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CARD	Caspase-recruitment domain
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLR	C-type lectin receptors
Con A	Concanavalin A
CRD	Carbohydrate recognition domain
CTLA-4	Cytotoxic T lymphocyte-associated protein-4
DALY	Disability-adjusted life year
DC	Dendritic Cell
DC-SIGN	Dendritic cell-SIGN
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum
FO	Follicular
GITR	Glucocorticoid-induced tumour necrosis factor receptor-related proten

GM-CSF	Granulocyte macrophage colony stimulating factor
GPI	Glycosylphosphatidylinositol
IFN	Interferon
Ig	Immunoglobulin
IKK	Inhibitor of kappaB kinase
IL	Interleukin
ip	intra peritoneal
IRF	Interferon regulatory factor
iv	intra venous
LLR	leucine-rich repeats
LPS	Lipopolysaccharide
mDAP	D-glutamyl-mese-DAP
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NF- κ B	Nuclear factor κ B
NK cell	Natural killer cell
NLR	NOD-like receptor
NOD	Nucleotide oligomerisation domain
OVA	Ovalbumin
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDI	Protein disulphide isomerase
PGN	Peptidoglycan
PPF	Periportal fibrosis
PRR	Pathogen recognition receptors
PYD	Pyrin domain
RA	Radiation-attenuated
RIG-1	Retinoic acid inducible gene-1
RLR	RIG-1like receptor
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial institute
sc	Subcutaneous
SEA	Soluble egg antigen
SIGN	Specific ICAM-3-grabbing non-integrin
SIGNR	SIGN-related
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Tg	Transgenic
TGF	Transforming growth factor
Th	T helper
ThFH	T helper follicular
TIR	Toll-IL-1R
TLR	Toll-like receptor
TNF	Tumour necrosis factor

TNFR	TNF-receptor
TSP	Tetraspanin
UV	Ultraviolet
WHO	World Health Organisation

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Chapter 1

Introduction

1. Introduction

1.1 Function of the immune system

The immune system is a versatile defense organization, composed of many interdependent cell types that have evolved to collectively protect animals from invading pathogens. An immune system functions in two main ways: recognition and response. Immune recognition is highly specific, and the body's immune system can distinguish between self and non-self molecules and cells. Once an invading organism has been recognized, the immune system then recruits various cells to mount an effector response, to eliminate or neutralize the organism. Subsequent exposure to the same invader induces a memory response, with a faster and more intense immune reaction (Goldsby *et al.*, 2002).

1.1.1 Development of the immune response: innate and adaptive immunity

There are two main arms of the immune system, the innate and the adaptive. The innate system recognizes non-self molecules and operates a wide range of immune mechanisms that do not improve upon repeated exposure to infection. The innate response is necessary to control pathogens at the early stage of infection while the adaptive immune system is being primed. Adaptive immunity is an acquired immunity, which is highly specific in recognizing antigens. It is controlled by both T and B lymphocytes. Memory to antigens is developed which means that the acquired secondary response is faster and greater.

The physical barriers of the skin and mucosa provide the first form of innate defense against pathogens. When these barriers are breached, innate effector cells are mobilized to assist in pathogen clearance and mediate initiation of the acquired immune response. Physiological barriers such as host temperature and pH also form part of the innate defenses, and can restrict the growth of microorganisms. Other principal components of the innate immune system include complement, interferons α and β , phagocytic cells and natural killer

(NK) cells. Phagocytes such as neutrophils and macrophages can engulf microbes and induce killing by reactive oxygen or reactive nitrogen species, or by antimicrobial factors.

Pathogen-derived molecules, or pathogen associated molecular patterns (PAMPs) are recognized by innate cells such as macrophages and dendritic cells (DCs) through a series of pattern recognition receptors (PRR). These PRR include the Toll-like receptors (TLRs), C-type lectins such as the mannose receptor, dendritic cell specific ICAM-3-grabbing non-integrin (DC-SIGN) and the SIGN-related (SIGNR) molecules, nucleotide oligomerisation domain (NOD)-like receptors (NLRs), Retinoic acid inducible gene-1(RIG-1)-like proteins (RIG-like receptors, RLRs), integrins and complement receptors. Each recognizes and binds PAMPs to facilitate differential recognition of pathogens and microbial products. Stimulation of these receptors causes an intracellular signaling cascade and an immediate defensive response, which includes the production of an array of antimicrobial peptides and cytokines.

These innate cells can function as antigen presenting cells (APCs) to the resting cells of the adaptive immune response. Exogenous antigens are engulfed and processed, before being complexed with class I or class II major histocompatibility complex (MHC) molecules on the surface of the APC. They are then presented to T cells. T lymphocyte cells have specialized subsets, as defined by differences in the cluster of differentiation (CD) markers on their surfaces. CD4 is a marker of T cell populations that promote activation and maturation of B cells and cytotoxic T cells, and control antigen-specific chronic inflammatory reactions through stimulation of macrophages. CD4 molecules, with the T cell receptor (TCR), associate with the antigen-MHC II complex on the APC. CD8⁺ T cells are cytotoxic lymphocytes that associate with MHC I on APCs. The interaction of the TCR with MHC I or II containing the processed antigen peptides indicates the molecular identity of the pathogen. However complete T cell activation requires two signals, as one signal alone produces

unresponsiveness (anergy). The interaction of co-stimulatory molecules on the surface of T cells and APCs provides the second signal.

1.2 Schistosomiasis

In Egypt in 1851 a young pathologist named Theodor Bilharz described a parasitic worm infection (bilharzia) that was schistosomiasis. After malaria, schistosomiasis is the world's most prevalent serious parasitic disease, estimated to affect 200 million people in 74 countries in the tropical regions of Africa, Asia and South America (Chitsulo *et al.*, 2000). Of this, 120 million people have symptoms, and 20 million develop severe illness (WHO, 2002). The World Health Organisation (WHO) reported that deaths due to this disease could be as high as 200,000 a year, and that up to 600 million people live in areas at risk.

There are 13 genera in the schistosomidae family and of these, 5 species of *Schistosoma* are known to infect man. The family is one of digenian trematode parasites, that have two hosts; snails as intermediary hosts and birds and mammals as definitive hosts. They have defined male and female individuals, i.e. they are dioecious. They infect the host by surface penetration rather than by being ingested, as is the case in other digenian parasites, and they reside in the vasculature of the definitive host.

Schistosoma intercalatum and *S. mekongi* are two of the 5 schistosoma species that infect humans. However *S. mansoni*, *S. haematobium* and *S. japonicum* are the main parasitic species as they are the most widespread geographically and have the greatest impact on human health. The differences between these three species are outlined in **Table 1**. The life-cycle of these major species were described in the first few decades of the last century, with Leiper describing the life cycle of *S. mansoni* in 1915.

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.

1.2.1 Life cycle of the schistosome

As mentioned above, the schistosome life cycle occurs in two stages; the intermediate host, which is a freshwater snail, and the mammalian (definitive) host. The life cycle of *S. mansoni*, the parasite used in this study, is outlined in **Figure 1.1**. Eggs are laid in the blood vessels of the mammalian host. They contain a developing larva, a miracidium. A female worm will lay a few hundred to several thousand eggs per day, depending on the species (see **Table 1**). Schistosome eggs have a characteristic pointed expansion of the shell in a lateral or terminal position, termed the spine. The eggs lodge against the vessel walls, aided by their spines and adhesive exudates. Peristalsis and the host's immune response to the egg also facilitate passage of the eggs to the lumen of the intestine or bladder. The miracidium secretes

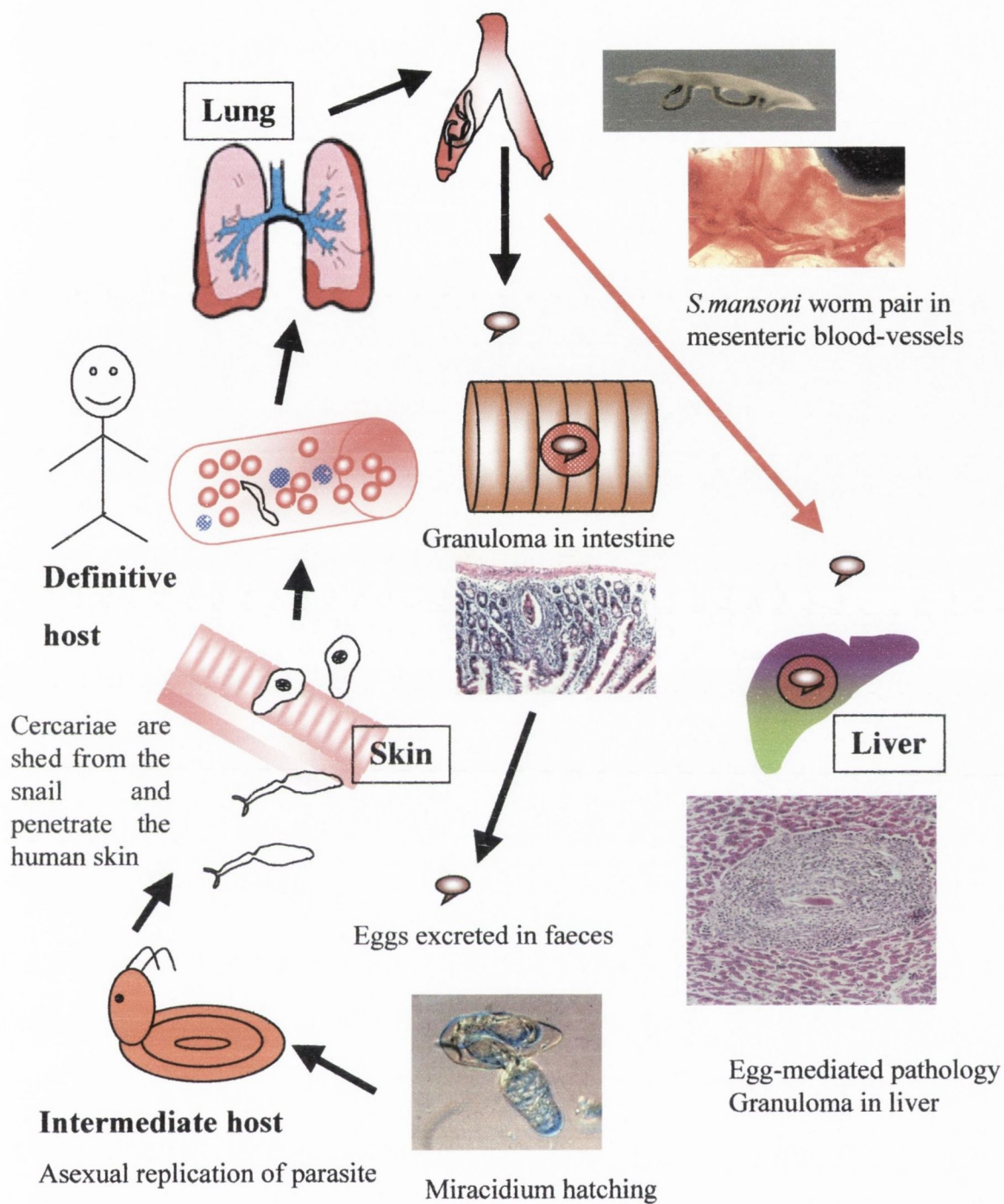


Figure 1.1. Life cycle of *Schistosoma mansoni*.

lytic enzymes, which diffuse through the micropores of the eggs shell. These enzymes help the egg penetrate the epithelium and basement membrane of the vein, and pass into the intervening tissue, basement membrane and epithelial of the intestine (*S. mansoni* and *S. japonicum*), or bladder (*S. haematobium*). The eggs can then make their way out of the host. *S. mansoni* and *S. japonicum* eggs are released in the faeces, and *S. haematobium* in the urine. However many of the eggs laid by female *S. mansoni* worms are swept back with the blood-flow to the liver where they get trapped in the sinusoids. The trapped eggs evoke an immune response, which induces the formation of a granuloma around them.

On contact with fresh water, and under certain environmental conditions, the excreted eggs can hatch and release the free-swimming miracidia. Light, a temperature between 25-30°C, and an osmotic pressure close to that of water are required for optimal hatching conditions. To develop further into a primary sporocyst, the miracidium needs to penetrate the soft tissue of a compatible freshwater snail. The miracidium is approx 70 µm wide, and between 70-150 µm long, with numerous cilia that aid its movement through water. Aquatic pulmonate snails of the genus *Biomphalaria* are the intermediate hosts of *S. mansoni*, while *Oncomelania* and *Bulinus* snail species are the hosts for *S. japonicum* and *S. haematobium*, respectively. Miracidial secretions and mechanical boring facilitate the penetration of the miracidia into the soft tissue of the snail. In the next 48 hours it develops into a primary sporocyst, which matures over 8-10 days to produce a number of secondary sporocysts which then migrate to the hepatopancreas and gonads of the snail. The sporocysts asexually replicate and after 4-7 weeks thousands of cercariae are produced. Under the most favorable conditions of light and temperature, the cercariae are shed from the snail tissues into the water. Light

after a period of dark is the optimal period for shedding. The schistosome life-cycle continues if an infective cercaria penetrates the unbroken skin of the primary host.

Cercariae are approx. 1 mm long, with a tegument body and a tail that is Y-shaped at the end, known as a furca. They burrow into the skin using their oral sucker to adhere, and also through use of digestive enzymes and strong tail activity. Once in the host, cercariae lose their tail and transform into schistosomula, before entering into a nearby vein or lymphatic vessel. Soon after they enter the blood stream and migrate passively through the right side of the heart, pulmonary capillaries, back through the left side of the heart and eventually to the hepatic portal system (*S. mansoni* and *S. haematobium*), or the blood vessels surrounding the bladder (*S. japonicum*). It is here where maturation into adult male and female worms occurs. The male schistosome worm is essential for the female to fully mature, and they remain in close proximity to each other. The male clasps the female in a longitudinal 'schist' or gynaecophoric canal, and the female begins to lay eggs. This takes 28-30 days from the point of original infection.

1.2.2 Human *S. mansoni* infection

In a study in sub-Saharan Africa, it was estimated that *S. mansoni* caused 130,000 deaths a year in the area, with 13.6 million people suffering morbidity (van der Werf *et al.*, 2003). As mentioned earlier, in endemic areas, only a small portion of infected people may display severe symptoms (Jordan *et al.*, 1993). The estimated average life span of a schistosome is 5-10 years, although adult worms can live for decades (Fulford *et al.*, 1995). Indeed it was shown that immigrants coming from areas where schistosomes are endemic can remain infected for 30 to 40 years (Whitty *et al.*, 2000). The range of morbidities caused by schistosome infection is influenced by the nature of the induced immune response and its effects on egg granuloma formation and associated pathologies in target organs. Severity of

the disease varies greatly between individuals, with host genetics, infection intensity, *in utero* sensitization to schistosome antigen and co-infection status all contributing to development of the immune response. So while some infected individuals develop granulomatous disease and organ failure, leading to death due to hepatosplenic schistosomiasis, others display no clinical symptoms, and host and parasite live in harmony together for many years. *S. mansoni* infection of man can be sub-divided into two main clinical conditions, acute schistosomiasis and chronic schistosomiasis.

1.2.3 Acute schistosomiasis

Acute schistosomiasis is also known as Katayama fever. Patients usually present with symptoms 2-12 weeks after contact with contaminated water. Granulomas form around the eggs that are deposited in tissue such as the liver and intestine, and it is often the immune response to egg deposition that induces the symptoms of acute schistosomiasis. Disease severity generally coincides with maximal egg-laying, between 6-8 weeks post-infection (Rabello, 1995). Symptoms include fever, headache, general malaise, right-upper-quadrant pain, bloody diarrhea and tender hepatomegaly. Up to 70% of *S. mansoni* patients develop respiratory symptoms, and all have marked eosinophilia. Splenomegaly is present in one third of cases (Ross *et al.*, 2002).

Patients with acute disease have elevated levels of the pro-inflammatory cytokines tumour necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) and IL-1 in plasma and supernatant from peripheral blood mononuclear cells (PBMCs) compared to patients with chronic disease (de Jesus *et al.*, 2002). Stimulation of PBMCs with the parasite antigen, soluble egg antigen (SEA), induced a dominant T helper (Th) 1, rather than a Th2 response in acutely-infected individuals, whereas a Th2 response was elicited in chronically-infected patients (de Jesus *et al.*, 2002). As the disease progresses, the Th2 response that develops in

response to the egg antigens may down-regulate the production and dampen the effects of the pro-inflammatory cytokines, and it is believed that this process could be mediated in part by the regulatory cytokine IL-10 (Montenegro *et al.*, 1999).

Acute schistosomiasis is a problem for travelers to areas endemic for the disease, and for previously uninfected individuals. It is rare for people born in areas endemic for the disease to develop acute schistosomiasis. One reason for this may be that *in utero* sensitization or sensitization as an infant may render individuals less likely to develop such a severe pro-inflammatory response upon first infection with schistosomes (King *et al.*, 1998; Malhotra *et al.*, 1997).

1.2.4 Chronic schistosomiasis

Schistosome infection progresses into the chronic phase after the acute symptoms recede. Chronic schistosomiasis is graded according to severity, and can be divided into intestinal and hepatosplenic forms of the disease. Irregular bowel movements, diarrhoea, bloody faeces and abdominal pain are characteristics of the intestinal form of schistosomiasis (Zhou *et al.*, 1998).

The hepatosplenic form of the disease is more serious, and can be life-threatening. It is characterized by an over-sized liver and spleen, with severe hepatic and periportal fibrosis (PPF), portal hypertension and porto-systemic shunting of venous blood being common features of the disease. The intensity and duration of infection determine the amount of egg antigen released and the severity of chronic fibro-obstructive disease that accompanies egg deposition. Although granulomas destroy the ova, they result in fibrotic depositions in the host tissues. As mentioned earlier, most granulomas occur where egg deposition is maximal, i.e. in the liver and intestine, however periovular granulomas have been found in such tissues as the skin, lung, brain and skeletal muscle (King, 2001). Severe hepatosplenomegaly is also

associated with the production of high levels of the Th1 cytokines TNF- α and interferon- γ (IFN- γ), and elevated levels of soluble TNF receptors (Mwatha *et al.*, 1998). Individuals who had less severe disease, but a similarly intense infection, had Th2 responses and low plasma levels of soluble TNFR (King, 2001).

Genetic predisposition may play a role in the outcome of disease among individuals. Dessein and co-workers showed that advanced hepatic fibrosis appeared to be closely linked to a polymorphism in the IFN- γ receptor gene (Dessein *et al.*, 1999). In a study of a population living in an endemic area for *S. mansoni* in the Sudan, two polymorphisms on the IFN- γ gene were shown to be associated with periportal fibrosis (Chevallard *et al.*, 2003). IFN- γ +2109 A/G polymorphism is associated with a higher risk for developing PPF, while the IFN- γ +3810 G/A polymorphism is associated with less PPF. Resistance to re-infection with *S. mansoni* is associated with another locus (SMI) and is located on chromosome 5q31-q33 (Rodrigues *et al.*, 1999), with the gene product of this locus involved in regulation of the development of Th2 cells. Within this locus subjects with an IL-13-1055T/T genotype were on average much less infected with *S. haematobium* than individuals with other genotypes (Kouriba *et al.*, 2005), while IL-13-1055C/T and IL-13-591A/G polymorphisms were associated with *S. haematobium* infections (Kouriba *et al.*, 2005).

Individuals living in areas endemic for schistosomes are often also at risk of infection from other parasites. Co-infection with other parasites can cause difficulties when trying to assess the prevalence and degree of morbidity due to chronic *S. mansoni* infection. Co-infection with hepatitis B (HBV) or hepatitis C virus (HCV) and *S. mansoni* is associated with more rapid deterioration of liver function (Aquino *et al.*, 2000). Exacerbated hepatosplenic disease is also found in patients with both schistosome and malaria infections (Booth *et al.*,

2004). However an association of *S. haematobium* infection with protection against acute *Plasmodium falciparum* malaria was found in young (aged 4-8) Malian children (Lyke *et al.*, 2005).

1.2.5 Treatment and control measures

1.2.5.1 Treatment

The schistosomidal drug, praziquantel was discovered in the mid-1970's (Thomas and Gonnert, 1977). It has been used extensively to treat and control the disease, especially in sub-Saharan Africa; it is relatively cheap, and is also safe and efficacious (Hagan *et al.*, 2004). It is absorbed well by the patient, but undergoes extensive first-pass hepatic clearance. The drug functions by inducing paralytic muscle contraction and tegumental disruption in the adult worm, these are believed to be due to changes in calcium flux (Cioli and Pica-Mattoccia, 2003). The worms detach from the wall of the vein and die. New worm targets for immune attack by the host are revealed upon the tegumental disruption (Harnett and Kusel, 1986), and the treatment of disease has been shown to be immune-dependent (Fallon *et al.*, 1992). In mice, the presence of host antibodies is crucial for the drug to work (Brindley and Sher, 1987). Although this may highlight concerns for the treatment of individuals that are co-infected with HIV, Karanja and co-workers found no difference in the effect of praziquantel in patients who were co-infected with HIV-1, regardless of whether such persons had low CD4⁺ T cell counts (Karanja *et al.*, 1998). However, a more recent study in Uganda has shown that treatment of *S. mansoni* infection leads to an increase in helminth-specific type 2 cytokine responses, and a subsequent increase in HIV-1 viral load in co-infected individuals (Brown *et al.*, 2005), making the use of this drug undesirable in HIV-infected patients.

After nearly 20 years of intensive use, treatment failure with praziquantel has been demonstrated in studies in Egypt and Kenya (Ismail *et al.*, 1999; William *et al.*, 2001). This is

due to drug resistance, or the stage-specific efficacy of the drug. Schistosomes are susceptible to praziquantel only in the first 2 days after infection, at the schistosomula stage, and then again 4 weeks later when the adult worms have matured (Sabah *et al.*, 1986). So administration of praziquantel does not kill the migrating schistosomula, and they continue their journey through the vasculature, where they mature and the females begin to lay eggs (Fallon *et al.*, 1996). Drug resistance is also a factor, with various groups demonstrating this, both in the field and in the laboratory (Fallon and Doenhoff, 1994; Fallon *et al.*, 1995; Ismail *et al.*, 1999). However a lack of drug efficacy in the geographical areas where reported 'resistance' was observed has also been suggested for the treatment failure. Oxamniquine is another drug that can be used to treat *S. mansoni*, but it has limited availability. It may be necessary to increase access to this drug if widespread resistance to praziquantel develops.

The use of drug treatment to prevent schistosomiasis is another way to control for the disease. However praziquantel is inefficient in this manner, as it has a half-life of only 1 to 1.5 hours, and because of its stage-specific efficacy, as discussed above. The anti-malarial drug Artemether, can kill schistosomula during the first 21 days in the body, and fortnightly treatment with the drug was successful in reducing the number of new cases of *S. japonicum* in a study group in southern China (Xiao *et al.*, 2000). Although the prospects for the use of this drug as a chemoprophylactic are good, it is unlikely that this drug will be used in areas endemic for malaria, as it could inadvertently result in the selection of artemether-resistant *Plasmodium falciparum* (Ross *et al.*, 2002).

1.2.5.2 Control measures

Although treatment of schistosomiasis with drugs such as praziquantel is efficient in reducing the morbidity associated with schistosomiasis, the fact that it is only prevalent in certain areas of the world indicate that environmental factors play a major role in the spread of

the disease. Proper control measures need to include environmental management, sanitation and education, in addition to drug treatment. Control of schistosomiasis can involve disruption of the life-cycle of the parasite at one or more of its life stages, outlined below.

As discussed in the previous section, drug treatment kills the adult worms in the definitive host to prevent any further egg-laying. Adequate water sanitation systems can prevent the eggs in the faeces or urine from reaching snail-infested waters. The removal or killing of the intermediate host, the snail, from waters that humans use, or providing 'safe water' to prevent contact with cercariae are all important control measures. Governments in emerging '2nd world' countries such as Brazil, China, Egypt and Morocco have adopted measures such as installing proper water supplies and sewage treatment, and initiating primary healthcare and education programs. These have proved to be successful in the control of schistosomiasis (Hagan *et al.*, 2004). In Egypt, the widespread campaign to treat schistosomiasis using intravenous tartar emetic, which ceased in the 1980's, has had the unfortunate consequence of creating a high prevalence of hepatitis C virus-infected individuals, due to the re-use of contaminated needles. HCV has replaced schistosomiasis as the principal cause of chronic liver disease in Egypt (Frank *et al.*, 2000; Strickland, 2006).

Japan has successfully eradicated *S. japonicum* infections by initiating the measures mentioned above, but mainly through the eradication of the intermediate snail host using molluscides (Hagan *et al.*, 2004). Natural molluscides are also under investigation. The endod (*Phytolacca dodecandra*) is an extensively studied plant molluscide (Webbe and Lambert, 1983); it is potent but with no major toxic properties for other animals and plants. A study in Ethiopia from 1994 to 1999 investigated the locally available endod as a suitable molluscide for the control of the *Biomphalaria pfeifferi* population. Endod berry powder soap was given to locals every week, and snail-infested waters were drip-fed or sprayed with the

molluscicide every 3 months. Results showed that endod-spray could obtain 100% snail mortality 24 h after application. In the stream where the endod berry soap was used for laundry, 59% of sentinel mice exposed to stream water before the soap was used became infected with *S. mansoni*, but only 11% of mice exposed to the water after the use of endod soap became infected, suggesting that endod is a suitable schistosomiasis control agent in Africa (Abebe *et al.*, 2005). Laboratory studies on the use of the Ghanaian strain of the snail *Lanistes varicus* to control the population of *Biomphalaria pfeifferi* also suggest that this could have potential as a biological control measure against schistosomiasis (Anto *et al.*, 2005).

Disease control measures are not being implemented in all areas endemic for the disease, with a lack of financial support for control programs and unstable governments in some sub-Saharan countries (Chitsulo *et al.*, 2000). In fact, schistosomiasis continues to spread to new geographic locations, even with the major advances in disease control. The construction of the Diama Dam on the Senegal River caused the introduction of *S. mansoni* into Mauritania and Senegal, also the Aswan Dam in Egypt led to the virtual elimination of *S. haematobium* from the Nile Delta, it resulted in the establishment of *S. mansoni* in upper Egypt. Population displacement and refugee movement has introduced *S. mansoni* into Somalia and Djibouti (Patz *et al.*, 2000; Ross *et al.*, 2001).

The recent re-evaluation of the disease prevalence and its impact according to disability weighting (disability-adjusted life year) DALY, show that schistosome infection remains a serious health problem (King *et al.*, 2005). As discussed earlier, although the control measures are crucial to limit the spread of disease, they are often costly and difficult to sustain, particularly in poor financial and political climates. This, and the limitations of drug treatment mean that the development of a vaccine, or a reliable prophylactic, is an attractive alternative to efficiently control the disease in the long term.

1.2.6 Vaccines

The WHO commissioned an independent trial of the six most promising vaccine candidates against *S. mansoni* 15 years ago. The results were disappointing as none gave greater than a 40% protection in mice, and immune responses to the candidates in human studies did not indicate that any had great potential as a successful vaccine (Bergquist *et al.*, 2002). Despite the limited efficacy in mice, paramyosin; triose phosphatase isomerase and Sm14, which binds fatty acids, are under further development as vaccine candidates (Hewitson *et al.*, 2005). Glutathione-S-transferase (Bilhvax) from *S. haematobium* is probably the most advanced of vaccine candidates, with Phase I/Phase II clinical trials having been performed (Capron *et al.*, 2005). A number of other vaccine candidates have been identified since and may yet hold long-term promise.

Although animal studies have never shown 100% protection for schistosome vaccines, the general consensus is that some protection is better than no protection at all (Pearce, 2003). Adjuvants have been used to increase the potency of partially protective vaccines. The use of Th1 (IL-12 or IL-2), but not Th2 (GM-CSF or IL-4), enhancer cytokines, was found to increase protective immune response induced by the *S. mansoni* vaccine candidate Sm-p80 (Siddiqui *et al.*, 2005). The vaccine was administered to mice in the form of a DNA vaccine formulation containing Sm-p80 (the large subunit of calpain) and one of the Th1 or Th2 enhancer cytokines. Almost complete protection of mice was also demonstrated when IL-12 was used as an adjuvant with radiation-attenuated cercariae (Wynn *et al.*, 1996). However others have shown that modulating the schistosome-induced immune response by addition of Th1 adjuvants could have potentially fatal effects (Rutitzky *et al.*, 2001).

The search for a vaccine has been helped by the recent work on the schistosome genome sequencing project (Verjovski-Almeida *et al.*, 2003). Instead of isolating molecules

from the parasite to use as vaccines, many studies have focused on developing 2nd (recombinant) and 3rd (DNA) generation vaccines, and the genome project has aided the identification of a number of vaccine candidates (Mountford, 2005).

There has been some recent success with regard to recombinant schistosome vaccines. Tran and co-workers targeted schistosome proteins that were expressed on the outer membrane of the tegument, at the host-parasite interface. Signal sequence trapping was used to identify cDNAs that had membrane-targeting signals and that could putatively be expressed in the tegument. Two cDNAs of interest were identified that encoded tetraspanin (TSP) proteins. The major ligand-binding domain of the protein was expressed and purified as a soluble fusion protein with *E. coli* thioredoxin. Murine studies using recombinant TSP-2 demonstrated 57% and 64% reduction in adult worm and liver egg burdens respectively upon subsequent infection with *S. mansoni* compared to mice given thioredoxin alone. That recombinant TSP-2 is strongly recognized by IgG1 and IgG3 from naturally resistant individuals, but not unexposed or chronically infected individuals is also promising for the potential use of this vaccine in human schistosomiasis (Tran *et al.*, 2006).

Until the recent studies on recombinant tetraspanin vaccine antigens, the vaccine model that provided the highest protection against schistosomes was the radiation-attenuated vaccine (RA), whereby infective cercariae are attenuated by ionizing radiation (gamma, X-rays, or UV). To date 2nd and 3rd generation molecule-specific vaccines have never equaled it in terms of efficacy. The RA vaccine against *S. mansoni* has been shown to protect in mice and rats (Anderson *et al.*, 1998; Ford *et al.*, 1984), and also against infection in non-human primates such as baboons and chimpanzees (Eberl *et al.*, 2001; Soisson *et al.*, 1993; Yole *et al.*, 1996). The method by which the RA vaccine induces protection against *S. mansoni* is complex, involving Th1-associated cell mediated immune responses, and Th2 or Th1-

associated humoral responses. The genetic background of the host, and the vaccine course of therapy all play a role in the generation of an immune response to the vaccine (Hewitson *et al.*, 2005). The RA vaccine does not give sterile immunity, and it is hypothesized that administration of the vaccine also induces a self-limiting regulatory mechanism. IL-10 may play a role in this regulation, as IL-10^{-/-} mice have increased levels of protection (Hoffmann *et al.*, 1999). TGF- β has also been implicated (Williams *et al.*, 1995), and works on the identification of other molecules and cell types involved in this regulatory mechanism are ongoing (Hewitson *et al.*, 2005).

The success of the RA vaccine in non-human primates is welcoming, as the vaccine regime is more physiologically relevant to humans. The vaccine also works on previously infected animals, which is important, as that would most likely be the case if one were to administer the vaccine to humans in areas endemic for the disease. However some 20 years ago, the use of attenuated cercariae in humans was not considered ethically acceptable (Von Lichtenberg, 1985), and this has not changed significantly today (Hewitson *et al.*, 2005). The intimate studies on immune response to RA vaccines will enable greater understanding of the immune mechanisms involved, and ultimately may lead to the generation of more efficient 2nd and 3rd generation vaccines.

1.2.7 Murine *S. mansoni* studies as a model for investigations into schistosomiasis

Mice can harbour a stable infection of *S. mansoni*, and have become a standard experimental model, with studies on experimental infections of mice being published since 1915. In addition, the intermediate host of *S. mansoni*, *Biomphalaria glabrata*, is relatively easy to maintain in the laboratory. There are many discrepancies between experimental infection of mice and natural infection of humans (Fallon, 2000), and there has long been a call for parallel human studies to complement murine experiments (Warren, 1964). However

the mouse model of *S. mansoni* infection has lead to greatly increased understanding of the immunological basis of the disease, especially since the advent of new transgenic mouse technology. The access to cells from multiple tissues, other than just peripheral blood, as is the case in human studies, also enables us to examine immune events in more detail in the murine system. It has become a very useful model for the study of many aspects of granulomatous inflammation and fibrosis, and also in the study of the regulation of the Th1 versus Th2 response. I describe studies on *S. mansoni* infection of mice in this thesis.

1.3 Innate Immunity

The anatomical and physiological barriers that form part of innate immunity have been outlined above (section 1.1.1). The importance of the TLR family of pathogen recognition receptors in host defense is now well established. The function of C-type lectins as PRRs has re-ignited interest into this diverse group of molecules in recent years, as have significant advances in the understanding of other pathogen sensor families, the NLRs and RLR. They all provide important links between innate and adaptive immunity. Future studies may address what interplay occurs between these families of innate immune receptors, and further our understanding of the complexities of innate immunity. These pathogen sensor families are further described in the following sections.

1.3.1 Toll-like receptors

Toll-like receptors are type 1 transmembrane receptors, with Toll being first described in *Drosophila* as a critical molecule for embryonic patterning, and subsequently also in anti-fungal immunity (Lemaitre *et al.*, 1996). TLRs recognize PAMPs through the leucine-rich repeats (LRRs) in their extracellular domains. These LRRs have been implicated in auto-regulation and ligand binding. They contain a Toll-IL-1R (TIR) domain in the cytoplasmic

portion of the molecule, however the extracellular domain of IL-1R is considerably different to the TLRs (Akira *et al.*, 2001). TLRs are evolutionarily conserved from insects to humans. To date, eleven TLRs have been identified in humans and mouse genome searches have identified thirteen, with TLRs 1-9 conserved between both species (Takeda and Akira, 2005).

Lipids, carbohydrates, nucleic acids and various proteins are among the PAMPs that are recognized by TLRs. The TLRs signal through their TIR domain, and share some downstream signaling molecules with members of the IL-1R family. TLR signaling can lead to activation of several transcription factors such as nuclear factor- κ B (NF- κ B) and interferon regulatory factors (IRFs), which can induce expression of a number of immune response genes. Different adaptor molecules can associate with the intracytoplasmic region of TLRs, these adaptors include MyD88, Mal (TIRAP), TRIF and TRAM. An overview of TLR ligand recognition and signaling through TLR4 as an example is outlined in **Figure 1.2**. Some TLRs are associated with the plasma membrane of the cell, while others are localized in intracellular compartments, such as the endosome (Dunne and O'Neill, 2005; Takeda and Akira, 2005). TLR 1, 2, 4, 5 and 6 reside mainly in the plasma membrane, while those TLRs that recognise nucleic acid derivatives (TLR 3, 7, 8 and 9) are localized to the endosome (**Fig. 1.2**). They require endosomal maturation in order to recognise their ligands.

TLRs have two primary functions; induction of inflammation and the establishment of adaptive immunity. TLRs can activate the complement cascade and induce anti-microbial proteins and peptides. TLR signaling induces production of cytokines such as IL-1 β , IL-6 and TNF- α , which stimulates chemokines and recruits inflammatory cells to the infected site. They also enhance phagocytosis and killing of microbes by phagocytes such as macrophages and neutrophils. Type 1 interferons that are induced by TLRs are critical for anti-viral

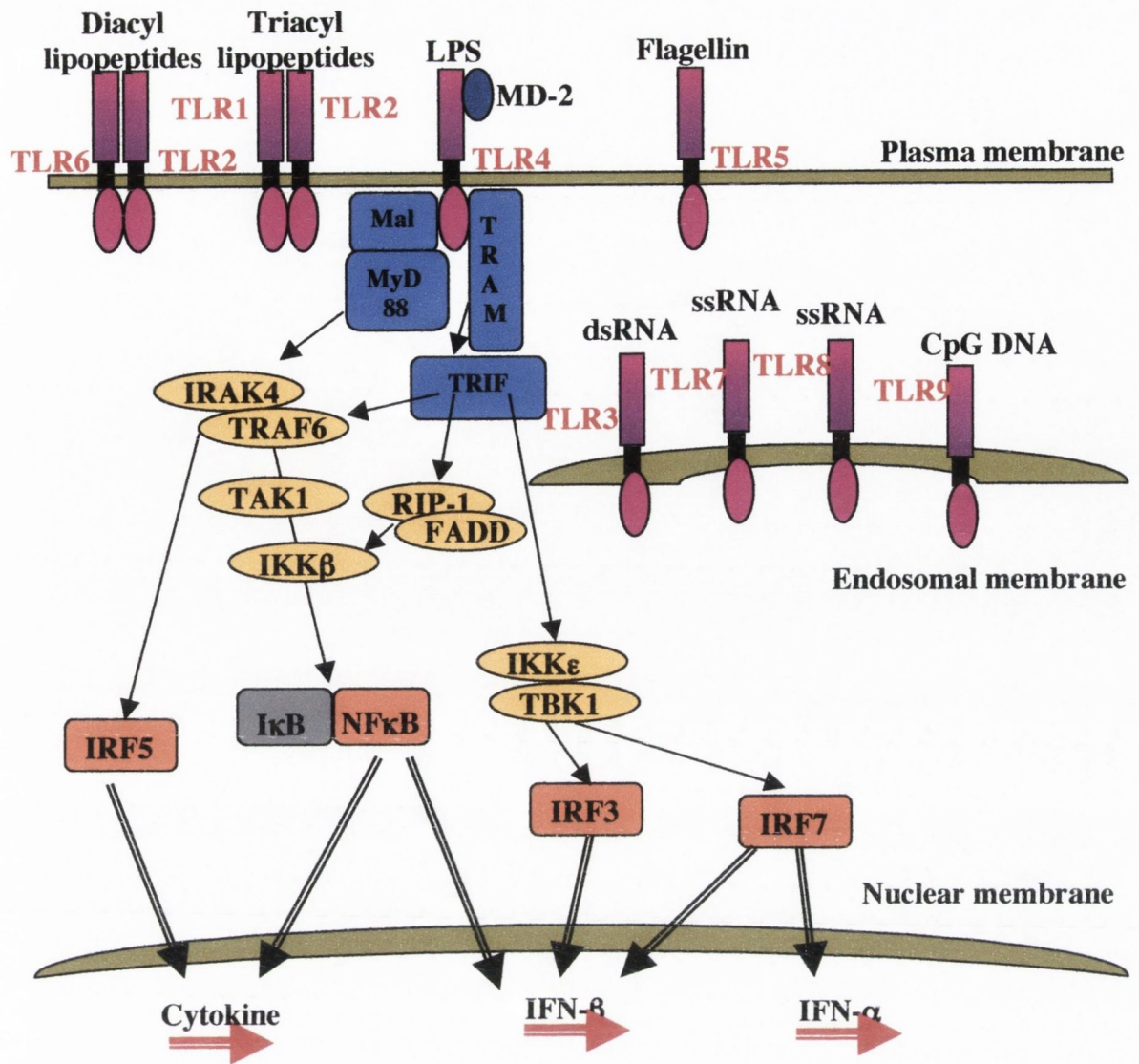


Figure 1.2. Toll-like receptors and their ligands. A simplistic overview of signaling through the TLR4 receptor: TLR4 detects LPS, which is specific to Gram-negative species. A TIR domain-containing adaptor, MyD88, associates with the cytoplasmic TIR domain of TLRs, and is also dependent on Mal (TIRAP). IRAK is recruited to the receptor upon ligand binding, which then activates TRAF6. This activates the I κ B kinase complex, which phosphorylates I κ B and leads to the nuclear translocation of NF κ B which induces transcription of genes for inflammatory cytokines. Activation of IRF-3 and induction of IFN- β occurs via the MyD88-independent pathway. The adaptor TRIF is essential for this pathway, and the adaptor TRAM is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway. TLRs are in purple, adaptor proteins are shown in blue, and transcription factors in orange. Modified from (Dunne and O'Neill, 2005; Takeda and Akira, 2005; Kaisho and Akira, 2006).

immunity. TLRs also form a link with adaptive immunity. Engagement of TLRs on DCs activates these cells and leads to upregulation of MHC and co-stimulatory molecules. The DC migrates to lymph nodes where it then interacts with and presents antigen to naïve T cells, thus stimulating an adaptive immune response (Kaisho and Akira, 2006; Pasare and Medzhitov, 2004).

1.3.2 NOD-like receptors

The nucleotide oligomerisation domain (NOD)-like receptor family (NLR) is a family of proteins that are involved in the intracellular recognition of bacteria. Like the TLRs, the C-terminus of the protein contains leucine rich repeats, which is implicated in ligand binding and auto-regulation (Tschopp *et al.*, 2003). The N terminus contains an effector domain, usually a Pyrin domain (PYD) or a caspase-recruitment domain (CARD). A role for some of the NLR proteins in pro-inflammatory caspase regulation and activation has been identified (Ting and Davis, 2005). NOD1 and NOD2 are perhaps the best studied of the NLR family. They bind to subcomponents of bacterial peptidoglycan (PGN); D-glutamyl-meso-DAP (mDAP) and muramyl dipeptide (MDP) respectively (Girardin *et al.*, 2003a; Girardin *et al.*, 2003b). Upon recognition of PGN motifs by NOD1 and NOD2, they can oligomerise and recruit Rip2/RICK, which is a serine/threonine kinase. This recruits the IKK complex and allows NF- κ B activation. Mutations in the NOD2 gene have been implicated in the inflammatory bowel disorder, Crohn's disease, and also with Blau syndrome, which is a disease characterized by granulomatous arthritis, uveitis and skin rash (Miceli-Richard *et al.*, 2001; Ogura *et al.*, 2001). Various NLRs have also been implicated in caspase-1 activating complexes, termed inflammasomes, which then leads to IL-1 β processing, which is essential for host defense and inflammation. An integrated response from the TLRs and NLRs is required to induce IL-1 β

production, as TLR priming is essential in addition to inflammasome activation (Creagh and O'Neill L, 2006; Kanneganti *et al.*, 2006).

1.3.3 RIG-1-like receptors (RLRs)

Retinoic acid inducible gene-1 (RIG-1) and melanoma differentiation-associated gene (MDA)-5 are two members of the RLR family of proteins that function as viral sensors (Kang *et al.*, 2002; Yoneyama *et al.*, 2004). They are similar to the anti-viral TLRs in that their signaling pathway also activates NF- κ B and IRF3, and that they induce transcription of similar genes (type 1 interferons). They also contain the CARD domains present in NLR family members. RLRs are widely expressed and are upregulated upon IFN- α and β activation. While the anti-viral activity of TLRs is primarily limited to detection by plasmacytoid DCs, RLRs can protect all virus-infected cells. Some viruses have evolved to evade the RLR mechanism of anti-viral immunity, and produce proteins that inhibit RLR signaling (Meylan *et al.*, 2005).

1.3.4 C-type lectin receptors

C-type lectins receptors (CLRs) are expressed on APCs and are involved in pathogen capture, processing and antigen presentation, which activate immune responses against these pathogens. Some members of this family are more involved in immune regulation, as pathogens hijack the function of the CLR to evade immune surveillance. These receptors also interact with a range of endogenous ligands during the immune response, implicating roles for these molecules not only in pathogen recognition but also in cell adhesion or homeostasis (Geijtenbeek *et al.*, 2004). CLRs are principally type II transmembrane receptors that recognize their ligands through the structurally related Ca^{2+} -dependent carbohydrate-recognition domain (CRD). Such CLRs include dendritic cell specific ICAM-3-grabbing non-

integrin (DC-SIGN), the related murine receptor family SIGN-related SIGNR(1-4), dectin-2, langerin, and blood DC antigen-2 (BCDA-2). Type I CLR has multiple lectin-like domains, although not all are capable of acting as functional CRDs. These include the mannose receptor and DEC-205. Although dectin-1 is a type II CLR, it does not have a standard Ca^{2+} -dependent CRD (McGreal *et al.*, 2005).

Ca^{2+} -dependent CLR can be broadly divided into two main categories; those that recognise mannose-type ligands and those that recognise galactose-type ligands. Although there appears to be overlapping ligand specificity among the range of CLR, distinct functions for each receptor are observed with differences in temporal and locational expression of the CLR, differing degrees of cell-surface multimerisation, and also differing contexts in which the ligand is recognised. The CLR DC-SIGN interacts with a wide range of pathogen-derived and endogenous ligands. It binds through high mannose glycans to viruses such as HIV and dengue virus, to mycobacterium tuberculosis and the Lewis^x antigen in *S. mansoni* egg antigens and in the LPS of *Helicobacter pylori*. It also binds the cellular ligands ICAM-2 and ICAM-3. DC-SIGN can mediate internalization of ligands for antigen presentation (Engering *et al.*, 2002), and HIV has exploited this function to gain access to CD4^+ T cells (Geijtenbeek *et al.*, 2000).

Murine SIGNR1 also binds various high mannose structures, and is essential for the clearance of *Streptococcus pneumoniae* by marginal zone macrophages (Lanoue *et al.*, 2004). A further role for SIGNR1 in innate immunity has recently been demonstrated, whereby SIGNR1 was critically implicated in the activation of a dominant complement fixation pathway for pneumococcal polysaccharides (Kang *et al.*, 2006). SIGNR1 can directly bind the complement C1 subcomponent C1q to allow assembly of a C3 convertase without the traditional requirement for either antibody or factor B.

Further cross-talk between components of the innate immune system has been suggested, with speculation that CLRs can act in synergy with members of the TLR family. Both the mannose receptor and DC-SIGN can bind to *Candida albicans*, while TLR4^{-/-} mice show enhanced infection rate of *Candida*, suggesting that the CLRs are required for antigen uptake and processing, while the TLR is required for complete activation of the downstream immune response. In contrast, MR^{-/-} mice show poor clearing of serum proteins but do not suffer from systemic *Candida* infections (Lee *et al.*, 2002; Lee *et al.*, 2003). Mannosylated lipoarabinomannan (ManLAM) is a mannose-capped glycolipid on the cell wall of *M. tuberculosis*. DC-SIGN can bind this to mediate capture and internalization of the pathogen, and it has been shown that in doing so, ManLAM inhibits IL-12 and stimulates IL-10 production in DC that have been activated by TLR stimuli (Geijtenbeek *et al.*, 2003). The expression of co-stimulatory molecules is also down-modulated. This is a potential mechanism by which the simultaneous activation of a CLR and TLRs can result in immune escape for the pathogen, by shifting the balance of the immune system from a protective inflammatory response towards a tolerogenic Th2 response.

1.3.5 Innate response to schistosome infection

As mentioned earlier, the first form of innate defense against pathogens is often in the form of the physical barriers of the skin or mucosa. *Schistosoma mansoni* cercariae gain entry to the host through the skin, and migration through the skin layers induces inflammatory reactions (Mountford and Trottein, 2004). In the mouse model of *S. mansoni* infection, over 90% of larvae have successfully exited the skin within the first 5-7 days of infection (Mountford *et al.*, 1988). Macrophage inflammatory protein (MIP)-1 α and MIP-1 β are released immediately upon the presence of schistosome larvae in the skin. Expression of pro-

inflammatory cytokines IL-1 β and IL-6 rapidly ensues, with other cytokines such as IL-12 and IL-18 induced as the response progresses. Infiltration of innate immune cells occurs, and these cells are activated. Once activated, innate immune cells with antigen-presenting capacity such as macrophages and DCs can then emigrate from the skin to the skin-draining lymph nodes, to stimulate the adaptive immune response.

Studies using TLR4^{-/-} and MyD88^{-/-} mice demonstrated that schistosome larvae can stimulate macrophage cytokine production through both TLR4-dependent and independent pathways, although MyD88 was critical for both IL-12p40 and IL-6 production (Jenkins *et al.*, 2005). The activation of TLR pathways by schistosome larvae was mediated by the glycan components present, as sodium meta-periodate treatment almost ablated the production of cytokines (Jenkins *et al.*, 2005). DCs activated by such schistosome larvae products also expressed some IL-12p40 and IL-6, and drove Th2 polarisation of CD4⁺ T cells (Jenkins and Mountford, 2005). However ligation of the tumor necrosis factor receptor (TNFR) family member CD40 on DCs altered the immune polarisation profile of the schistosome larvae-activated DCs to induce a more Th1-type response (Jenkins and Mountford, 2005).

Many pattern recognition receptors involved in the innate response have been shown to interact with schistosome antigens. Certain schistosome worm lipids (lysophosphatidylserine) can modulate APCs (human DCs) to induce regulatory T cells via TLR2 (van der Kleij *et al.*, 2002). In addition it has been shown that a schistosome carbohydrate (lacto-N-fucopentaose III) functions as an innate Th2 promoter, by inducing maturation of a dendritic cell type 2 (DC2) phenotype via a TLR4-dependent mechanism (Thomas *et al.*, 2003). However Jankovic and co-workers have shown that conditioning of DC for Th2 differentiation by SEA (soluble egg antigen) was MyD88 independent (Jankovic *et al.*, 2004). This is in agreement with previous studies that demonstrate normal development of Th2

responses to SEA in MyD88^{-/-} mice (Jankovic *et al.*, 2002). Although TLR4 can also signal through pathways other than the MyD88 pathway, Jancovic and co-workers also showed that SEA-induced Th2 differentiation could also occur in TLR4^{-/-} DC. In a study of *S. mansoni*-infected MyD88^{-/-} mice, Layland and co-workers reported a lack of antigen-specific Th1 responses and subsequent alterations in granuloma formation and composition (Layland *et al.*, 2005). The dendritic cell-specific C-type lectin DC-SIGN has also been shown to bind SEA, with the glycan antigen Lewis^x being critically involved in the binding (van Die *et al.*, 2003).

1.4 Adaptive Immunity

1.4.1 Macrophages: classically-activated versus alternatively-activated

Macrophages are essential for host defense. They are involved primarily in innate, but also in adaptive immunity, and display a wide variety of biological functions, including phagocytosis, killing and pro- and/or anti-inflammatory activities. Macrophages can also function as antigen presenting cells, and signal adaptive responses through co-stimulatory molecules and antigen presentation, in addition to cytokine secretion. Microbial antigens and effector T cells, and their secretory products, influence the activation state of macrophages. The concept of two major macrophage classes has developed in the past few years, with Stein and co-workers describing ‘alternatively’ activated macrophages (aaMφ) as being phenotypically distinct from the traditional ‘classically’ activated macrophage (caMφ) (Stein *et al.*, 1992).

‘Classical’ immune activation was demonstrated in the 1960s, with the observation that infection of mice with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) or *Lysteria monocytogenes* enhanced the antimicrobial activities of macrophages in a stimulus-dependent,

but antigen-non-specific, manner (Gordon, 2003). Activation of these classical macrophages requires priming by the Th1 cytokine IFN- γ , secreted by Th1 lymphocytes, and a cytokine network involving IL-12 and IL-18, produced by APCs. When the primed macrophage encounters a pro-inflammatory microbial product, such as LPS, it becomes classically activated. They display a Th1-type phenotype, promoting inflammation, extracellular matrix (ECM) destruction, and apoptosis. caM ϕ characteristically produce nitric oxide (NO), and have enhanced antigen-presenting capability, with augmented expression of MHC II and co-stimulatory molecule B7-1. They are critical in the defense against intracellular pathogens, displaying increased oxidative burst and NO release. The ability to produce NO, in addition to pro-inflammatory cytokines such as TNF- α , IL-1 and IL-6, confers the anti-proliferative and cytotoxic activities to the macrophage. While the activities of the caM ϕ are essential for host defense and direction of the adaptive immune system, persistence of its pro-inflammatory activities often elicits severe tissue damage if unchecked. The immune system faces a challenge to develop anti-inflammatory activities to maintain host survival.

Alternatively activated macrophages (aaM ϕ) arise in a Th2 environment, with the development of caM ϕ being inhibited by Th2 cytokines. They are activated by Th2 cytokines IL-4 and/or IL-13, and require no further priming in order to differentiate (Gordon, 2003; Stein *et al.*, 1992). Early studies have included the regulatory cytokine IL-10, with IL-4 and IL-13, as having deactivating effects on macrophages. However IL-4 and IL-13 stimulate phenotypic changes to the macrophage that are distinct from the changes induced by IFN- γ in classical activation of macrophages, and indeed the deactivation of inflammatory cytokine production induced by IL-10. aaM ϕ differ from classically activated macrophages in many ways, and as a result, exert different effects on the host. aaM ϕ promote ECM construction,

angiogenesis, and tend to resolve inflammation and facilitate wound healing through the molecules it secretes.

One of the defining features of the aaM ϕ is its failure to generate NO from L-arginine, and so it cannot readily limit the growth of intracellular pathogens. aaM ϕ have increased levels of arginase, which converts L-arginine to ureum and L-ornithine. Indeed the iNOS-arginase balance correlates with the balance between Th1- and Th2-type activities (Munder *et al.*, 1998), with IFN- γ inhibiting arginase and IL-4/IL-13 mediating iNOS inhibition. Recent studies have expanded our knowledge of aaM ϕ , and a number of genes have been identified as markers of alternative macrophage activation. Raes and co-workers used suppression subtractive hybridization to show induction of Fizz 1 (found in inflammatory zone 1) and Ym 1 in aaM ϕ *in vivo* and *in vitro* (Raes *et al.*, 2002). Expression of these markers was controlled by IL-4/IFN- γ in an antagonistic manner. Loke and co-workers also showed up-regulation of these genes in IL-4-dependent aaM ϕ elicited *in vivo* following implantation of the nematode *Brugia malayi* in the peritoneum of mice (Loke *et al.*, 2002). Fizz 1/RELM- α (resistin-like molecule- α) is a resistin-like secreted protein, associated with pulmonary inflammation and originally discovered in a murine model of asthma (Holcomb *et al.*, 2000). It is also expressed in white adipose tissue, as well as mammary tissue and tongue. Ym 1/ECF-L is a chitinase-like secretory lectin that forms crystals in alveolar spaces and in hyperactive lung macrophages. It functions as an eosinophil chemotactic factor, and can be induced in macrophages by the secretion of a nematode homologue of macrophage migration inhibitory factor (MIF) (Falcone *et al.*, 2001). This may explain why the recruitment of helminth-induced aaM ϕ is accompanied by an infiltration of eosinophils to the site of parasite residence. The intelectin family of proteins have joined the arginase family, resistin-like molecules, and

the chitinase-like mammalian proteins, as effector molecules in type 2 inflammation (Nair *et al.*, 2006). The intelectin family is composed of two calcium-dependent galactose-binding lectins, intelectin-1 and intelectin-2. Although relatively little is known about the regulation of their expression and function, intelectin expression is a feature of helminth infection in the murine gastro-intestinal tract, with dramatic up-regulation of intelectin-2 in intestinal epithelial in *Trichinella spiralis* infection (Pemberton *et al.*, 2004). Maximal expression was associated with expression of type 2 cytokines and worm expulsion.

As discussed earlier, the classically activated macrophage is induced with *Mycobacterium* infection. A potent Th1 response is crucial for the control of *Mycobacterium tuberculosis*, and indeed alternative activation of macrophages prevents such cells from enabling an efficient control mechanism to limit the growth of mycobacteria *in vivo* (Kahnert *et al.*, 2006).

1.4.2 Alternatively-activated macrophages during parasite infection

The parasitic nematode *Brugia malayi* was first used to characterize aaM ϕ . IL-4-dependent aaM ϕ were elicited upon implantation of *Brugia malayi* into the peritoneal cavity of mice. They were shown to actively suppress lymphocyte proliferation *in vitro*, and in a cell-contact dependent manner (Loke *et al.*, 2000b). The mechanism of suppression via soluble factors associated with suppressive macrophages such as NO, prostaglandins, IL-10 and TGF- β , had previously been discounted (Allen *et al.*, 1996; MacDonald *et al.*, 1998). The F4/80⁺ peritoneal aaM ϕ also inhibited proliferation of various transformed cell lines. Suppression of proliferation was reversible and not due to apoptosis of responder cells. Although the regulatory cytokine TGF- β was not involved in induction of cell suppression,

parasite-induced aaM ϕ were shown to prime a Th2 response by preventing Th1 differentiation by the inhibition of early IFN- γ production via TGF- β (Loke *et al.*, 2000a).

Murine infection with the cestode *Taenia crassiceps* has been shown to induce a population of peritoneal macrophages with suppressive activity (Terrazas *et al.*, 2005). These cells had high levels of alternatively-activated macrophage (aaM ϕ) markers, in addition to elevated PD-L1 and PD-L2 expression. The suppressive activity was mediated by the PD-1:PD-L pathway.

Murine studies using the filarial nematode *Litomosoides sigmodontis* have demonstrated a role for F4/80⁺ aaM ϕ s in both pre-patent and patent infection (Taylor *et al.*, 2006b). Infection induced a suppressive population of aaM ϕ s at the site of infection. Suppression of CD4⁺ Ag-specific proliferative responses was partially dependent on TGF- β , but not on IL-10. As the infection progressed into the patent stage, the suppressive activity of the F4/80⁺ aaM ϕ was also observed at peripheral sites such as the draining lymph nodes.

A recent publication has shed some light on the mechanism of type 2 effectors leading to host protection against helminthic parasites (Anthony *et al.*, 2006). Using the mouse gastrointestinal parasite *H. polygyrus*, the group demonstrated an infiltration of memory IL-4-producing Th2 cells, that resulted in the induction of aaM ϕ that were IL-4R^{hi} and CD206⁺. The macrophages functioned in an arginase-dependent manner to impair larval mobility, and contributed to an eventual expulsion of adult worms (Anthony *et al.*, 2006).

The role for aaM ϕ during schistosomiasis has been demonstrated using macrophage/neutrophil specific IL-4R α -deficient (lysM^{Cre}, IL-4R α ^{-flox}) mice (Herbert *et al.*, 2004). aaM ϕ activation was shown to be essential for survival during schistosomiasis, with 100% mortalities during acute *S. mansoni* infection. This mortality was not neutrophil-

dependent. aaM ϕ are critical during infection to downmodulate Th1 responses and egg-induced inflammation, as death was associated with increased Th1 cytokines and iNOS activity, impaired egg expulsion, sepsis and hepatic and intestinal histopathology.

The production of arginase by aaM ϕ provides a potential explanation for the association of Th2-type inflammation with enhanced fibrosis and is implicated in granuloma formation. Arginase levels correlate with maximal egg-induced pathology. In an SEA-induced granuloma model, Hesse and colleagues showed that type 2 cytokine-stimulated macrophages produced proline, which is under the control of arginase (Hesse *et al.*, 2001). Proline is a precursor to collagen, and it has been proposed that aaM ϕ increase collagen deposition and fibrosis in the liver with their high arginase activity. Blocking arginase activity in aaM ϕ impairs the production of proline. Arginase activity in aaM ϕ also leads to increased synthesis of polyamines (Abdallahi *et al.*, 2001), which may be important as helminth parasites are believed to be dependent on their hosts for the uptake and interconversion of polyamines. Hesse and co-workers also sensitized mice to SEA in the presence of IL-12, and observed a dominant Th1 reaction in contrast to the normal Th2-egg-specific response. This was associated with high iNOS activity, and a strong decrease in arginase-positive aaM ϕ , with a dramatic reduction in liver pathology (Hesse *et al.*, 2001).

1.4.3 T helper cell effector lineages

APCs play a pivotal role in determining the outcome of an infection due to their unique ability to prime naïve CD4⁺ T cells. A mature DC can polarize a T cell towards a certain phenotype by translating ‘messages’ received from the pathogen to the cell. Naïve CD4⁺ T helper (Th0) cells are maintained in a pluripotent state and they conventionally respond to stimulation by peptide-MHC complexes on APCs via proliferation and cytokine secretion.

They differentiate into various effector lineages depending on various intra- and extracellular stimuli. The best characterized lineages are T helper 1 (Th1) or T helper 2 (Th2) CD4⁺ cells, however additional subsets of effector T cells have recently been described, including IL-17-producing T helper (Th17) cells and T helper follicular (ThFH) cells (Reinhardt *et al.*, 2006). An overview of the T helper effector lineages is outlined in **Figure 1.3**.

The Th1/Th2 division was initially typified by the distinct cytokines that are produced (Mosmann *et al.*, 1986). Th1 cells secrete IL-2, IFN- γ , and TNF- α , while Th2 cells secrete IL-4, -5, -9, and -13. IL-12 is a major APC factor that acts via transcription factors Stat4 and T-bet to drive IFN- γ -producing Th1 cells. Th1 cells are predominately involved with cell-mediated immunity, such as activation of cytotoxic and phagocytic functions in effector cells and delayed-type hypersensitivity. They are crucial for cellular immunity against intracellular pathogens (Abbas *et al.*, 1996; Murphy and Reiner, 2002). IL-4 acts via Stat6 and GATA-3 to drive the differentiation of Th2 cells. Th2 cells are mainly associated with humoral immunity, by assisting the progression of B cells to antibody secretion, and also in anti-helminth immunity. CD8⁺ T cells (cytotoxic T cells; Tc) may also be subdivided into Tc1 or Tc2 subsets, depending on their cytokine secretion pattern. The type 0 response is characterized by the simultaneous release of type 1 and type 2 cytokines, with these cells capable of differentiating into either Th1 or Th2 cells. A Th1 or Th2 phenotype will ultimately dominate a response by both amplification of a particular Th cell subset and downregulation of the opposing cell response (Th1 via IL-2 and IFN- γ , Th2 via IL-4) (Abbas *et al.*, 1996; Fallon, 2000). The newer members of the T helper lineages are described below, but as seen in **Figure 1.3**, there is dynamic interaction and overlap between the various T helper subsets.

ThFH cells are T cells found in germinal centres that may be broadly defined as CXCR5⁺ and display a cytokine profile distinct from Th1 or Th2 cells. Upon activation, they migrate to B cell follicles in a CXCR5⁺-dependent manner, where they interact with B cells to ensure efficient B cell activation and antibody production (Chtanova *et al.*, 2004; Hardtke *et al.*, 2005). Co-stimulatory molecules such as ICOS have been implicated in enhancing CXCR5 expression and a ThFH fate (Akiba *et al.*, 2005). ThFH cells were recently implicated as possible regulators of autoantibody production when a study showed that mice with elevated levels of ICOS had higher ThFH levels and eventually developed autoimmune disease (Akiba *et al.*, 2005). It is currently uncertain whether ThFH cells are a separate distinct Th lineage, or whether they are a terminally differentiated state of either Th1 or Th2 cells.

The cytokines IL-17 and IL-23 were originally believed to belong to the Th1 lineage, however this view has been reassessed in recent years, resulting in a separate and distinct Th lineage termed Th17. Studies of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and adjuvant-induced arthritis initially highlighted a role for this IL-17-producing sub-set (Chabaud *et al.*, 1999; Langrish *et al.*, 2005; Zhang *et al.*, 2003a). The cytokine secretion profile of Th17 cells includes IL-17A, IL-17F, IL-6 and TNF- α . They induce inflammation by production of chemokines and also G-CSF and GM-CSF, which act to recruit and activate neutrophils. Macrophage mediation of Th17-induced inflammation has also been described (Park *et al.*, 2005). That Th17 cells are their own lineage, distinct from Th1 or Th2, is increasingly well-supported amongst the literature. It has been shown that transcription factors associated with Th1 and Th2 lineages respectively, T-bet and GATA-3, are not required for Th17 induction *in vivo* (Veldhoen *et al.*, 2006). IL-23 is implicated in the

survival and maintenance of Th17 cells, however it was the regulatory cytokine, TGF- β , that has been shown to induce Th17 differentiation in the context of an inflammatory cytokine milieu (Veldhoen *et al.*, 2006). Indeed two recent papers published in Nature also support this view that it is the regulatory cytokine TGF- β that induces development of the Th17 lineage (Bettelli *et al.*, 2006; Mangan *et al.*, 2006b). TGF- β is known to induce expression of the regulatory transcription factor Foxp3 (Chen *et al.*, 2003), however Bettelli *et al.* demonstrate that the presence of IL-6 inhibits this generation of CD4⁺ Foxp3⁺ T regulatory (T_{reg}) cells, and instead acts with TGF- β to induce pro-inflammatory Th17 cells. They hypothesise that this reciprocal development pathway for the pathogenic Th17 and protective T_{reg} subsets may have evolved to induce or regulate tissue inflammation, and it certainly has helped to increase our understanding of these emergent T cell lineages.

Another member of the IL-17 family is the Th2-like cytokine IL-25 (IL-17E). Recent studies have highlighted a role for IL-25 in host defense and controlling inflammation. The role for IL-25 in anti-helminth immunity using IL-25 deficient mice was demonstrated in two separate studies (Fallon *et al.*, 2006; Owyang *et al.*, 2006). IL-25 was shown to increase IL-4 production in order to augment Th2-mediated immunity. Fallon *et al.* describe a non-lymphoid c-kit⁺ population that is induced by IL-25 to produce IL-4 during *Nippostrongylus brasiliensis* infection. This then leads to enhanced Th2 differentiation by CD4⁺ cells. Owyang *et al.* also show a role for IL-25 in anti-helminth Th2 responses using the parasitic helminth *Trichuris muris*. In addition, the group has identified an anti-inflammatory function for IL-25 in dampening Th1 and Th17 responses. Whether IL-25-secreting cells are a unique effector T cell lineage (Th25), or merely a subset of Th2 cells remains to be discovered, and no doubt will be the subject of much future work.

1.4.4 Development of immune response during schistosome infection

Helminths are the main examples of Th2 cell inducers in both humans and experimental models, with high-level tissue eosinophilia and elevated serum IgE serving as immunological hallmarks of infection with parasitic helminths (Pearce and MacDonald, 2002.; Sher and Coffman, 1992). However the initial infective stages of schistosomes (cercariae) stimulate a dominant pro-inflammatory Th1 response in the mouse model. The Th1 response is observed throughout the first 3-5 weeks of infection, as the cercariae transform into schistosomula upon penetration of the host's skin, and migrate into the blood stream, travel through the lungs and arrive in the hepatic portal system.

Only as the infection progresses and the mature parasites mate and the female begins to lay eggs does the response switch to a strong Th2 response (Pearce *et al.*, 1991). This is characterized by elevated levels of IL-4, -5, -9 and -13 (Sabin and Pearce, 1995). This developing schistosome-egg antigen induced Th2 response downregulates the production and effector functions of the earlier Th1 pro-inflammatory mediators (Pearce *et al.*, 1991), with the production of IL-10 during this time thought to play a crucial role in this process.

Although it is widely acknowledged that egg deposition is the major stimulus for the production of Th2 cytokines in murine schistosomiasis (Grzych *et al.*, 1991), there is evidence to suggest that the adult worm stage of the parasite may initiate the Th2 response, to prime the host for the arrival of the eggs, and so limit egg-induced pathology (Hayashi *et al.*, 1999). Hepatic lymphocytes from *S. mansoni* infected mice during the prepatent period (before arrival of eggs) already produced a higher amount of IL-4 and lesser amount of IFN- γ than those from uninfected mice (Hayashi *et al.*, 1999). Immune priming by the adult worm, with production of pro-inflammatory cytokine TNF- α , has also been shown to be necessary for

granuloma formation around the egg (Leptak and J.H., 1997). Murine studies on worm-only infection of mice demonstrated worm-modulation of macrophages (Smith *et al.*, 2004), and a helminth-modified type 2 response in the lung (Mangan *et al.*, 2006a), highlighting a role for the worm stage of the parasite for immune modulation.

Most pathology in schistosome-infected mice is attributed to the host's reaction to the egg, which is maximal by the 8th week of infection. Th2 cytokines modulate excessive inflammatory reactions preventing a runaway pathology (Hoffmann *et al.*, 2002). In *S. mansoni* infection, many eggs laid by the female are carried by the blood flow to the liver, the sinusoids of which are often too small for the eggs to traverse. They remain trapped in the liver, and eventually die within the tissue. A granuloma forms around eggs in the liver, and also in the intestinal wall, where traversing eggs often get trapped en route to the exterior. The granuloma formed around a schistosome egg is primarily a T-lymphocyte-mediated host response with diminished granulomas in T cell-depleted and SCID mice (Amiri *et al.*, 1992; Doenhoff *et al.*, 1981; Fallon *et al.*, 2000b). B cells have also been implicated in playing a regulatory role in schistosome granuloma formation (Fallon *et al.*, 1998; Jankovic *et al.*, 1998). Type 1 and type 2 cytokines are associated with the granuloma, although studies in STAT 6 deficient mice demonstrate the essential role of type 2 cytokines in granuloma formation (Kaplan *et al.*, 1998). The granuloma is composed of collagen fibres and cells, including macrophages, eosinophils and CD4⁺ T cells (Dunne and Pearce, 1999). The egg eventually dies and the granuloma resolves, leaving fibrotic plaques. IL-13 is the principal cytokine responsible for hepatic fibrosis during schistosome infection (Chiaramonte *et al.*, 1999; Fallon *et al.*, 2000a). Although the Th2 response might have limited effects on parasite burden, it exerts an enormous influence on the survival of the host. 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 were tolerized against *S. mansoni* egg antigen, granuloma formation was severely limited. Mice had elevated type 1 cytokine responses and diminished type 2 responses, with severe hepatotoxic liver damage (Fallon and Dunne, 1999).

High pathology schistosomiasis linked with a Th1 response is observed in certain inbred mouse strains, such as C3H and CBA, but not in other strains such as C57BL/6 (Cheever *et al.*, 1987; Rutitzky *et al.*, 2005a). In a vaccine model for prevention of fibrosis, it was shown that immunization with *S. mansoni* eggs and IL-12 partially prevented granuloma formation and reduced fibrosis induced by a natural infection of *S. mansoni* (Wynn *et al.*, 1995). However it has also been shown that Th1-polarising immunization with egg antigens in low pathology strains such as C57BL6 can induce a marked exacerbation of the disease (Rutitzky *et al.*, 2001). Such severe immunopathology is dependent on IL-12p40 and also correlates with high levels of IL-17, with neutralization of IL-17 with mAbs greatly inhibiting hepatic granulomatous inflammation (Rutitzky *et al.*, 2005b).

To date murine studies have focused primarily on CD4⁺ T cells as the cellular source of cytokines that determine the type 2 outcome in murine schistosome infection and regulate the granulomatous response to parasite eggs. However other cells types such as eosinophils, FcεR⁺ non B non T cells or B1 cells have also been implicated as additional sources of type 2 cytokines during murine infection (Kullberg *et al.*, 1996; Palanivel *et al.*, 1996; Rumbley *et al.*, 1999). Th2 responses are also strongly implicated in naturally acquired resistance to reinfection with schistosomes.

The role of eosinophils in *S. mansoni* infection has recently been investigated using eosinophil lineage-ablated mice (Swartz *et al.*, 2006). While *S. mansoni* egg-induced early IL-4 production was found to be dependent on IL-5 and eosinophils (Sabin *et al.*, 1996), lack of eosinophils in *S. mansoni*-infected eosinophil lineage-ablated mice had no impact on worm burden, egg deposition or on granuloma formation other than the eosinophil depletion itself (Swartz *et al.*, 2006).

The prominence of IL-10 as a crucial mediator of regulation in parasite infections has long been recognized, particularly for its role in attenuating pathogenesis (Sher *et al.*, 1992). Increased mortalities in IL-10 deficient mice are observed with *S. mansoni* infection (Sadler *et al.*, 2003; Wynn *et al.*, 1998), with these mice developing egg Ag-specific Th1 responses. Unregulated IL-12 production is the cause of this, as mice lacking both IL-10 and IL-12 fail to polarize in this fashion and excessively skewed Th2 responses develop (Hoffmann *et al.*, 2000).

As the infection moves into its chronic phase (> week 12), there is a modulation of the Th2 response. Granulomas that form around newly deposited eggs are smaller than at earlier times during infection (Boros *et al.*, 1975). Another example of Th2 modulation is induction of expression of the decoy receptor IL-13Ra2, which blocks IL-13-dependent fibrotic reactions (Chiaramonte *et al.*, 2003). It is during the chronic infection stage that there is an acceptable balance between the host's immune system and the disease state. There is a decline in parasite-specific T cell responses, and a more regulated environment prevails. In contrast to previous studies (Wynn *et al.*, 1998), one study showed that IL-10 deficient mice infected with *S. mansoni* fail to enter this late downmodulating phase (Sadler *et al.*, 2003).

The phenomenon of T cell suppression during the conventional schistosome egg-and-worm infection is well documented (Flores Villanueva *et al.*, 1994; Stadecker, 1999). T cell

unresponsiveness is often mediated by APCs such as macrophages or DCs, or can be induced by a population of T regulatory cells. Jankovic and co-workers have shown that conditioning a DC for Th2 differentiation by SEA correlates with down-regulation of activation signals in the APC, which lead to a temporary delay in initial T cell cycling (Jankovic *et al.*, 2004). A schistosome egg glycan has also been shown to induce a Gr1⁺/F4/80⁺ macrophage population that suppresses CD4⁺ T cell proliferation (Atochina *et al.*, 2001). Mitogen un-responsiveness in worm-only infected mice has also been reported (Attallah *et al.*, 1979; Diab *et al.*, 1989), and more recently worm modulation of macrophages was shown to induce T cell suppression (Smith *et al.*, 2004).

As mentioned earlier a schistosome worm lipid activated TLR2 on human DCs, which conferred on these cells the ability to induce IL-10 producing T regulatory cells (van der Kleij *et al.*, 2002). Hesse and co-workers have shown that the pathogenesis of schistosomiasis is controlled by co-operating IL-10-producing innate effector and regulatory T cells (Hesse *et al.*, 2004). The role of regulatory T cells in schistosome infection is discussed in section 1.4.6.

1.4.5 Regulatory T cells

It was in the early 1970's when the idea of suppressor or regulatory T (T_{reg}) cells first emerged. Three-day-old mice that were thymectomized developed organ-specific autoimmunity, however this was not the case in mice thymectomized at day 0 or day 7, suggesting that a suppressor population of T cells was induced after day 3 of life to regulate self-reactive T cells in the thymus. The field of T regulatory cells really took off in 1995 when Sakaguchi and co-workers identified T cells expressing the IL-2 receptor α -chain (CD25) as being critical in maintaining self-tolerance by down-regulating immune responses to both self and non-self antigens (Sakaguchi *et al.*, 1995).

Regulatory T cells are capable of suppressing naïve CD4⁺ and CD8⁺ T cell proliferation *in vitro* via IL-2 inhibition (Thornton and Shevach, 1998), and this is generally used as a distinguishing factor when identifying a T_{reg} population. A prime feature of T_{reg} cells is their ability to suppress both innate and adaptive immune responses. New evidence implicates T_{reg} cells in regulation of other immune cell subsets, such as dendritic cells and B cells. Lewkowich *et al.* showed that when anti-CD25 mAb was used to deplete CD4⁺CD25⁺ T_{reg} cells *in vivo* in A/J mice, the mice displayed increased levels of pulmonary myeloid DCs, with these cells having a greater propensity to induce Th2 cytokines and proliferation in T cells (Lewkowich *et al.*, 2005). Lim and co-workers have also shown direct suppression of B cells by CD4⁺CD25⁺ cells (Lim *et al.*, 2005). Although a T_{reg} cell's primary function is to limit damage and down-modulate an excessive immune response, a consequence of this in terms of disease and infection may be enhanced pathogen survival, and long-term persistence, and they may also be detrimental in tumour biology, as is discussed later.

Various subsets of regulatory CD4⁺ T cells that functionally limit the immune response and have a maintenance role in preventing autoimmune disease have been described. CD4⁺ T cells with regulatory activity may be broadly divided into T regulatory cells that occur naturally (natural T_{reg}) and cells that are induced by certain stimuli (termed adaptive T_{reg} or inducible T_{reg}) (Belkaid and Rouse, 2005; Bluestone and Abbas, 2003; O'Garra and Vieira, 2004). The regulatory T cell subsets are outlined in **Figure 1.4**. Inducible T_{reg} cells include Tr1 cells, which are IL-10-dependent regulatory cells (Groux *et al.*, 1996), and also Th3 cells, that are TGF- β -secreting cells (Chen *et al.*, 1994). Tr1 cells were induced *in vitro* and, upon antigen stimulation, were able to inhibit both Th1 and Th2 responses via IL-10 production (Cottrez *et al.*, 2000; Groux *et al.*, 1997). Recently Foxp3⁺, IL-10-producing, antigen peptide-induced regulatory cells were shown to be independent of CD25⁺ T_{reg} cells for their growth,

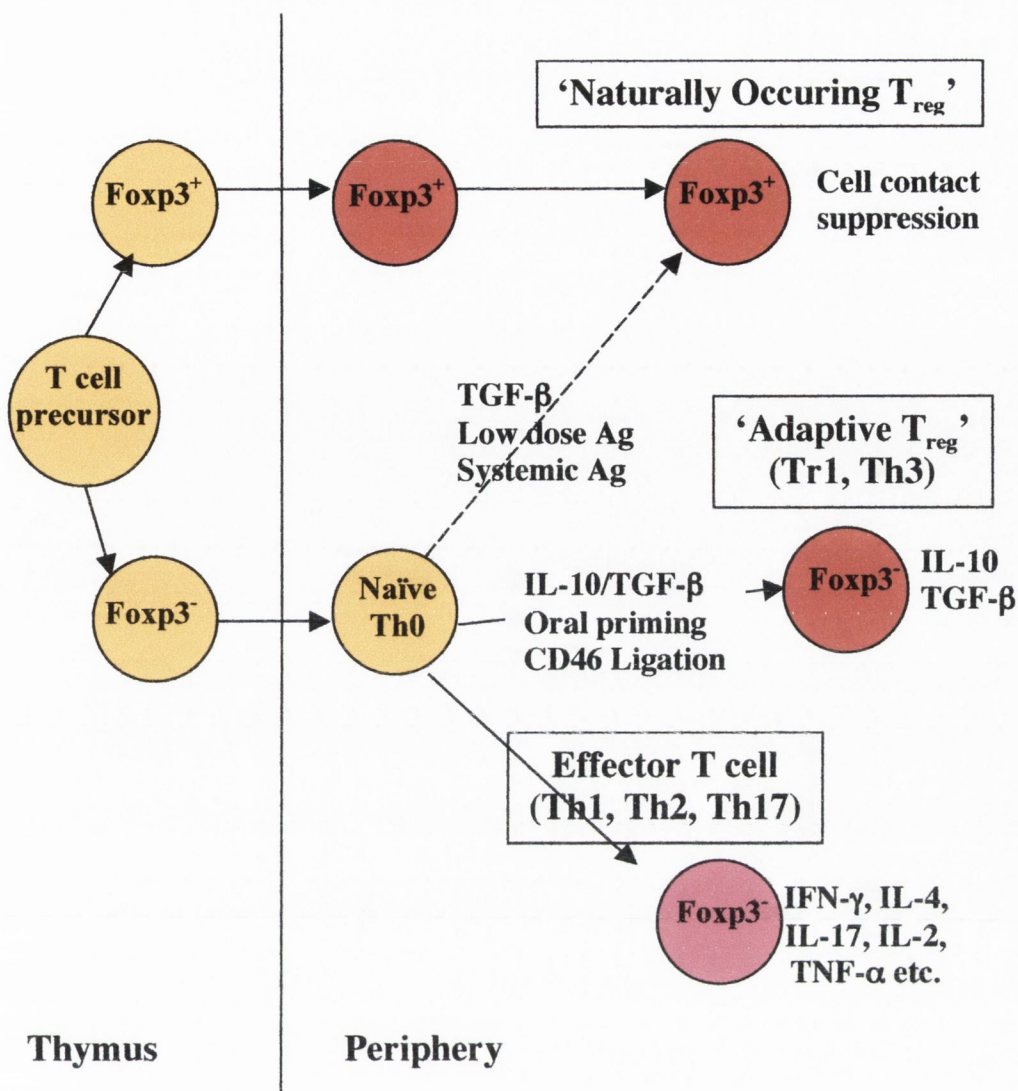


Figure 1.4. Development of regulatory T cell subsets. Naïve Th0 and Foxp3⁺ T_{regs} originate in the thymus. The naturally occurring T_{reg} mediates its suppression via a contact-dependent mechanism. Th0 cells differentiate into Th1, Th2, and Th17 lineages under different immunogenic stimuli. Stimulation of Th0 cells under certain *in vitro* conditions or via an oral route can induce adaptive T_{regs} that are Foxp3⁻ and primarily dependent on IL-10 or TGF-β to mediate suppression. Stimulation of Th0 cells with TGF-β or low dose antigen *in vivo* can induce a Foxp3⁺ T_{reg} population that is indistinguishable from the naturally-occurring T_{reg} that developed in the thymus. Taken from (Wing *et al.*, 2006).

differentiation and function (Nicolson *et al.*, 2006). Th3 cells were shown to be induced upon oral administration of antigen, and have been implicated in limiting autoimmune pathology in models of colitis, EAE and diabetes (Chen *et al.*, 1994; Faria and Weiner, 2005). Inducible T_{reg} cells are generated from mature T-cell populations under certain antigenic stimulation conditions, and they function in a cytokine-dependent manner *in vivo*. However natural T_{reg} cells mature in the thymus and survive in the periphery as natural T_{reg} cells.

Foxp3 as a marker for T regulatory cells

Natural T_{reg} cells were initially characterized by surface expression of CD25, however this may also be present on activated CD4⁺ cells, as mentioned above. A hallmark of T_{reg} cells is the expression of forkhead family transcription factor Foxp3 (Hori *et al.*, 2003). Foxp3 was shown to be predominately expressed in naturally arising CD4⁺CD25⁺ regulatory cells, and retroviral gene transfer of Foxp3 can confer a regulatory phenotype upon naïve T cells (Hori *et al.*, 2003). Foxp3 deficient mice (*scurfy* mutant mice) develop severe lymphoproliferative disease and die within 3 weeks (Godfrey *et al.*, 1991). Khattri *et al.* showed that CD4⁺CD25⁺ T cells from Foxp3 deficient mice lack regulatory T cell activity, and that overexpression of Foxp3 in mice produced more T_{reg} cells (Khattri *et al.*, 2003). Two human isoforms of Foxp3 exist, and while overexpression of both Foxp3 isoforms together markedly increased expression of T_{reg} markers from autologous CD4⁺ T cells, suppressor activity was only slightly increased (Allan *et al.*, 2005). Expression of the IL-7R, CD127, has been shown to inversely correlate with Foxp3 expression and the suppressive function of human CD4⁺ T_{reg} cells (Liu *et al.*, 2006a).

As mentioned earlier, the regulatory cytokine TGF- β is known to induce Foxp3 expression (Chen *et al.*, 2003), and leads to the conversion of murine peripheral CD4⁺CD25⁻ naïve T cells to CD4⁺CD25⁺ T_{reg} cells. Peripheral Foxp3⁺ T_{reg} cells were also induced in a

TGF- β -dependent manner in murine transplant tolerization models (Cobbold *et al.*, 2004). Marie *et al.* demonstrated that while TGF- β was required to maintain Foxp3 expression and regulatory cell function in the periphery, it was not required for their development in the thymus (Marie *et al.*, 2005). Recently Fontenot and co-workers proposed that a dedicated T regulatory cell lineage could be specified by Foxp3 (Fontenot and Rudensky, 2005). Using Foxp3-GFP reporter mice they showed that suppressor activity was demonstrated in Foxp3⁺ T cells irrespective of CD25 expression. It was also demonstrated that Foxp3 has no cell-intrinsic role in effector T cell function, and that pathogen-driven immune responses do not induce Foxp3 in non-regulatory cells (Fontenot *et al.*, 2005). That there is no *de novo* generation of Foxp3⁺ T_{reg} cells in the periphery is conflicting with some previous studies, as outlined above (Chen *et al.*, 2003). However as these studies were performed on bulk CD4⁺ T cells, Fontenot and co-workers postulate that it is unclear whether increased Foxp3 expression is from expansion of a small population of CD25⁺Foxp3⁺ T_{reg} cells or conversion of non-regulatory cells (Fontenot *et al.*, 2005). Together with the striking lymphoproliferative autoimmune phenotype displayed in Foxp3 deficient mice, this data argues strongly for the case that Foxp3 acts as a regulatory T cell lineage specification marker, and that such T_{reg} cells are the critical mediators of dominant tolerance. However more recently, conversion of truly naïve T cells (Foxp3 protein expression was below the limit of detection) into Foxp3⁺ suppressor cells by targeting of peptide-agonist ligands to dendritic cells was demonstrated (Kretschmer *et al.*, 2005), adding further support to the theory that Foxp3⁺ regulatory cells may develop extrathymically as well as intrathymically.

Other T_{reg} markers

In addition to CD25, and the now hallmark Foxp3, other putative surface markers for T_{reg} cells include glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR), and the integrin CD103 (McHugh *et al.*, 2002). GITR is predominately expressed on $CD4^+CD25^+$ T_{reg} cells in naïve mice (Shimizu *et al.*, 2002), and antibody stimulation of GITR removed T_{reg} -mediated suppression. Tone *et al.* identified the GITR ligand, and showed that it was indeed co-stimulatory for T cells, with GITR-GITR-L interaction reversing suppression by $CD4^+CD25^+$ cells (Tone *et al.*, 2003). $GITR^{high}$, $Foxp3^+$ T_{reg} cells that were $CD25^+$ or $CD25^-$ were demonstrated to be essential in the control of autoimmune myocarditis and multiorgan inflammation (Ono *et al.*, 2006). CD103 is the α_E chain of the integrin $\alpha_E\beta_7$, and is believed to play an important role in the retention of natural T_{reg} cells to the site of *Leishmania major* infection (Suffia *et al.*, 2005), with T_{reg} cells from $CD103^{-/-}$ mice being impaired in their ability to be retained at the site of infection. Its expression is positively regulated by the cytokine TGF- β (Robinson *et al.*, 2001), and CD103 is expressed at high levels on T_{reg} cells in the infected site (Suffia *et al.*, 2005). BALB/c mice are susceptible to *L. major* infection, but $CD103^{-/-}$ mice on a BALB/c background were rendered refractory to infection (Suffia *et al.*, 2005). CD103 may also be expressed on other cell types, including subsets of $CD8^+$ cells, DCs and mast cells (Cepek *et al.*, 1994; Kilshaw, 1993; Smith *et al.*, 1994). Indeed it has recently been shown that $CD103^+$ DCs in the gut are important for the generation of gut-tropic $CD8^+$ effector cells (Johansson-Lindbom *et al.*, 2005).

Although expressed on activated T cells, the co-stimulatory molecule cytotoxic T lymphocyte-associated protein 4 (CTLA-4) is also associated with regulatory activity (Chikuma and Bluestone, 2003). CTLA-4 deficient mice develop massive

lymphoproliferation and early death (Tivol *et al.*, 1995; Waterhouse *et al.*, 1995), and CTLA-4 also plays a vital role in Th2 cell differentiation (Khattri *et al.*, 1999; Oosterwegel *et al.*, 1999). Neuron interaction with T cells has recently been shown to convert encephalitogenic T cells to CD25⁺TGF- β 1⁺CTLA-4⁺Foxp3⁺ T_{reg} cells. These cells were capable of suppressing encephalitogenic T cells and inhibiting experimental autoimmune encephalomyelitis (EAE) in a CTLA-4-dependent manner (Liu *et al.*, 2006b). The role of CTLA-4 in immunity is discussed further in section 1.5.

T regulatory cells in disease

Regulatory T cells have also been implicated in tumour immunity, particularly in the suppression of CTLs and effective anti-tumour immunity. Onizuka *et al.* showed that depletion of CD4⁺CD25⁺ cells using the anti-CD25 mAb PC61 caused tumour regression in mice in 6 out of 8 tumour models used (Onizuka *et al.*, 1999). Another group showed that transfer of CD25-depleted CD4⁺ BALB/c spleen cells to BALB/c athymic nude mice given RL1 leukaemia cells lead to tumour regression and long-term host survival (Shimizu *et al.*, 1999). This is in direct contrast to mice that received whole CD4⁺ spleen cells (including the CD25⁺ sub-population), where 100% mortality was seen by day 40. Many studies have focused on determining how to induce an effective immune response against advanced tumours by limiting T_{reg}-mediated suppression and in conjunction, stimulating tumour-reactive T cells. One group recently demonstrated that administration of an agonistic anti-GITR mAb to tumour-bearing mice caused tumour regression that was mediated by tumour-infiltrating IFN- γ -secreting CD4⁺ and CD8⁺ cells (Ko *et al.*, 2005). When coupled with TCR stimulation, this GITR mAb has been shown to abrogate CD4⁺CD25⁺-mediated suppression and in fact induce T_{reg} cell proliferation (McHugh *et al.*, 2002; Shimizu *et al.*, 2002). Ko *et al.* found

ameliorated tumour regression and eradication when blocking antibody against CTLA-4 was administered along with the anti-GITR mAb. However tumour eradication was less effective when a CD25-depleting mAb was given with anti-GITR (Ko *et al.*, 2005). This was due to depletion of activated CD25⁺ T cells as well as naturally occurring CD4⁺CD25⁺ T_{reg}. In control mice, the dominant infiltrating population of T cells into growing tumours were CD4⁺CD25⁺Foxp3⁺ T_{reg} cells, reaffirming the central role they play in suppression of anti-tumour immunity.

5-10% of peripheral CD4⁺ T cells also express CD25 in naïve mice, however levels of such natural CD4⁺CD25⁺ T_{reg} cells, have been shown to be expanded by various pathogens and are implicated in limiting immunopathology during pathogen infection (Belkaid and Rouse, 2005).

The involvement of T_{reg} cells in IBD, colitis or Crohn's Disease has been extensively studied (Asseman *et al.*, 2000). In mouse models, gut inflammation is induced by transfer of naïve CD4⁺CD45RB^{high} T cells into T-cell deficient mice (SCID mice). However this effect is suppressed when CD4⁺CD25⁺CD45RB^{lo} T cells are co-transferred (Powrie *et al.*, 1993). IL-10-dependent Tr1 regulatory cells are also involved in protection from colitis (Groux *et al.*, 1997), as are the TGF- β -dependent Th3 cells (Chen *et al.*, 1994). Indeed the suppressive effect of the CD45RB^{lo} T_{reg} cells in the SCID model of colitis is mediated by TGF- β (Powrie *et al.*, 1996), and also by IL-10 (Asseman *et al.*, 1999; Hagenbaugh *et al.*, 1997). CTLA-4 expression on T_{reg} cells also plays a central role in suppression of colitis as blockade of CTLA-4 on CD4⁺CD25⁺ T_{reg} cells eliminates their protective role in colitis (Read *et al.*, 2000). It has recently been shown that Foxp3⁺ T_{reg} cells with suppressive activity can be cultured from the

colonic mucosa of Crohn's Disease patients, which could be an option for adoptive immunotherapy to treat the disease (Kelsen *et al.*, 2005).

Natural T_{reg} cells are central to the preservation of host homeostasis, and it has been postulated that one of their principal functions is to respond to, and limit, tissue damage in the host. In addition to the colitis studies described above, evidence for T_{reg} control of gastrointestinal bacteria exists, with natural T_{reg} cells being implicated in control of *Helicobacter pylori* infection in humans (Lundgren *et al.*, 2003). The pulmonary pathogen *Pneumocystis carinii* (PS) causes lethal pneumonitis in infected RAG-2 mice upon CD4⁺CD25⁻ cell transfer, however co-transfer of CD4⁺CD25⁺ cells conferred protection to the infected mice (Hori *et al.*, 2002). Natural CD4⁺CD25⁺ T_{reg} cells are also central to the control of *Leishmania major* persistence and immunity (Belkaid *et al.*, 2002). In susceptible BALB/c mice, T_{reg} cells control the early IL-4 response and the maturation of Th2 cells in *L. major*-infected mice (Aseffa *et al.*, 2002). More recently it has been shown that in C57 mice *L. major* superinfection can cause reactivation of the disease at the primary site, in a T_{reg}-dependent manner. Depletion of T_{reg} cells from the primary site using anti-CD25 mAb prevented such disease reactivation at the site of persistent infection upon secondary challenge (Mendez *et al.*, 2004). However although natural T_{reg} cells can promote pathogen persistence, subsequent removal of such suppressor cells leads only to a 'sterile cure', and is not sufficient to maintain long-term immunity (Belkaid *et al.*, 2002; Belkaid and Rouse, 2005).

The control of viral infections by natural T_{reg} cells has also been demonstrated, with these cells being implicated limiting liver damage in hepatitis C patients (Cabrera *et al.*, 2004), and also in preventing blinding keratitis which can be caused by herpes virus (Suvas *et al.*, 2004). A role for T_{reg} cells in immunity to human immunodeficiency virus (HIV) infection has also been postulated, with anti-HIV CD4⁺ T cell responses being increased upon removal

of T_{reg} cells from PBMCs (Aandahl *et al.*, 2004). HIV was shown to induce CD4⁺CD25⁺ T_{reg} cells which suppress HIV-specific CD4⁺ T cell responses in HIV-infected individuals (Weiss *et al.*, 2004). Indeed there is evidence that both natural and Foxp3-transfected human T_{reg} cells can be infected with HIV (Oswald-Richter *et al.*, 2004). In the murine model of AIDS, removal of regulatory T cells prevented disease progression (Beilharz *et al.*, 2004).

1.4.6 T regulatory cells in helminth infection

There are numerous studies on the role of T_{reg} cells in parasitic disease, in particular in the role they play in helminth modulation of the immune system. As discussed earlier, APCs, in particular aaMφs, are associated with immune suppression during filarial nematode infection (section 1.4.3). However the long-lived survival of filarial worms is also associated with suppression of protective immune responses via regulatory T cells. T cells with regulatory activity have been isolated from individuals infected with *Onchocerca volvulus*, *Loa loa* and *Wuchereria bancrofti* (Satoguina *et al.*, 2002; Steel and Nutman, 2003).

In murine models, T_{reg} cells are important in promoting filarial survival during *Litomosoides sigmondontis* infection (Taylor *et al.*, 2005). Infection induced upregulation of the regulatory markers CD25, GITR and CTLA-4 in the thoracic cavity. GITR and CTLA-4 expression was also increased in distal sites such as the thoracic lymph nodes and the spleen. Ablation of T_{reg} cells using the anti-CD25 depleting mAb PC61 and an agonistic mAb against GITR resulted in parasite clearance *in vivo*, however when these mAbs were used alone there was no effect on worm burden (Taylor *et al.*, 2005). A recent study on patients infected with the parasite *Brugia malayi* showed that regulatory networks down-regulated both Th1 and Th2 pathways during patent infection (Babu *et al.*, 2006). Expression of the Th1/Th2 regulators T-bet and GATA-3 was down-modulated in PBMCs in response to live microfilaria stimulation, but Foxp3 expression was higher in infected individuals compared to uninfected. In addition,

regulatory markers CTLA-4, TGF- β and the negative co-stimulation marker PD-1 were all increased upon stimulation with live microfilaria (Babu *et al.*, 2006).

The role of macrophages in the control of T cell responses during schistosome infection has been discussed earlier. The importance of CD4⁺ T cells for schistosome egg granuloma formation has also been addressed. Studies in recent years have begun to investigate what role, if any, that regulatory T cells play in schistosome infection. Hesse and co-workers showed that IL-10-producing CD4⁺CD25⁺ regulatory cells, in addition to IL-10 derived from a non-T cell source, were important for the development of type 2 immunity and host survival (Hesse *et al.*, 2004). CD4⁺CD25⁺ T_{reg} cells were also shown to play a role in suppressing the Th1 response and promoting Th2 cell polarization during *S. mansoni* infection (McKee and Pearce, 2004). More recent publications by the same groups describe IL-10-independent functions for naturally occurring T_{reg} cells (Baumgart *et al.*, 2006; Taylor *et al.*, 2006a). The T_{reg} cell response induced by schistosome egg injection suppressed the Th1 response, and also limited the magnitude of the Th2 response. Such T_{reg}-mediated suppression of Th responses was IL-10 independent (Taylor *et al.*, 2006a). Baumgart and co-workers also show IL-10 independent functions for T_{reg} cells in regulation of egg-induced cytokine production, and showed that depletion of CD25⁺ T_{reg} cells lead to increased frequency of IL-4-producing T cells in egg-induced inflammation (Baumgart *et al.*, 2006).

1.4.7 Regulatory CD8⁺ T cells

Two types of CD8⁺ T cells were described in the 1970's: cytotoxic and suppressor. The suppressor, or regulatory cells, inhibited lectin-induced T cell proliferation, B cell immunoglobulin synthesis and T cell proliferation in mixed lymphocyte cultures (Abdou *et al.*, 1976). The existence of CD8⁺ regulatory cells has not been the subject of much study, with most of the focus on the biology of regulatory T cells pertaining to the CD4⁺ T cell

subset. However interest in this cell population has returned in the past few years, with groups generating CD8⁺ T_{reg} cells by stimulation of immature or plasmacytoid DCs (Chang *et al.*, 2002; Gilliet and Liu, 2002). TGF- β is also used to generate CD8⁺ T_{reg} cells, and these cells were capable of suppressing a stimulatory graft-versus-host disease in mice (Zheng *et al.*, 2004). The Qa-1 protein in mice (HLA-E in humans) has been linked to CD8⁺ T cell-dependent suppression, and studies on Qa-1^{-/-} mice showed that suppressive CD8⁺ T cells are important in prevention of secondary autoimmune responses (Hu *et al.*, 2004). It was demonstrated that peptide-specific CD8⁺ T_{reg} cells inhibit peptide-specific CD4⁺ T cell proliferation by using IFN- γ to elaborate TGF- β -based suppression of the CD4⁺ T cells (Myers *et al.*, 2005). CD8⁺ regulatory cells have been found to express the marker CD122 (IL-2 receptor β chain) (Rifa'i *et al.*, 2004). IL-10 was crucial for the inhibition of CD8⁺CD122⁻ cell proliferation and IFN- γ production *in vitro*, but other regulatory mechanisms may compensate for IL-10 *in vivo*, as CD8⁺CD122⁺ T_{reg} cells that were deficient in IL-10 still showed some inhibitory effect on wild-type CD8⁺CD122⁻ cells when they were co-transferred into RAG-deficient mice (Endharti *et al.*, 2005).

A recent paper by Noble and co-workers has demonstrated that cytokine-induced IL-10 secreting CD8⁺ T cells represent a phenotypically distinct suppressor T cell lineage (Noble *et al.*, 2006). They found that CD8⁺ T_{reg} cells are generated in the presence of IL-4 and IL-12, and could inhibit the generation of Th1/Tc1 and Th2/Tc2 antigen-specific effector responses. The mechanism of suppression was contact-dependent, but the T_{reg} cells also produced high levels of IL-10 and IFN- γ . They also suppressed IgG/IgE antibody responses and graft-versus-host disease *in vivo*. The presence of glucocorticoids also increased the development of

the CD8⁺ T_{reg} cells. These cells exhibited a unique cell surface phenotype, with both naïve and activation cell markers being co-expressed on the cells.

1.5 T cell activation and co-stimulation

As mentioned earlier, T lymphocyte activation requires two signals, antigen recognition and co-stimulation. Signal 1 is antigen-specific, and involves the TCR complex recognizing antigenic peptides presented on the MHC molecules of the APC. This is critical in maintaining the specificity of the immune response. The second, co-stimulation signal is antigen-independent, and adds an additional layer of regulation to lymphocyte activation. Positive co-stimulation is necessary for proper activation of T and B cells, i.e. sustained cell proliferation, effector/memory cell generation and prevention of anergy or apoptosis. However inhibitory co-stimulation molecules expressed on APCs and peripheral tissues can regulate immune responses by sending negative costimulatory signals to the T cell.

1.5.1 The B7:CD28 pathway

One of the best-studied co-stimulatory pathways is the CD28/CTLA-4-CD80/CD86 pathway. CD28 is constitutively expressed on T cells. Its ligands are CD80 (B7-1) and CD86 (B7-2), which are induced by microbial products and inflammation and expressed on the APC. Interaction between CD28 and its ligands is crucial for activation of naïve T cells. It results in induction of IL-2, expression of CD25 and entry into the cell cycle. This is outlined in **Figure 1.5**, with the CD28 receptor in green inducing a 'GO' signal, with IL-2 production and T cell proliferation.

Only in exceptional circumstances is TCR/CD3 complex engagement alone sufficient to induce proliferation, such as stimulation with CD3-specific Abs at high surface density, or in the physiological response to high avidity antigen from the non-cytolytic lymphocytic

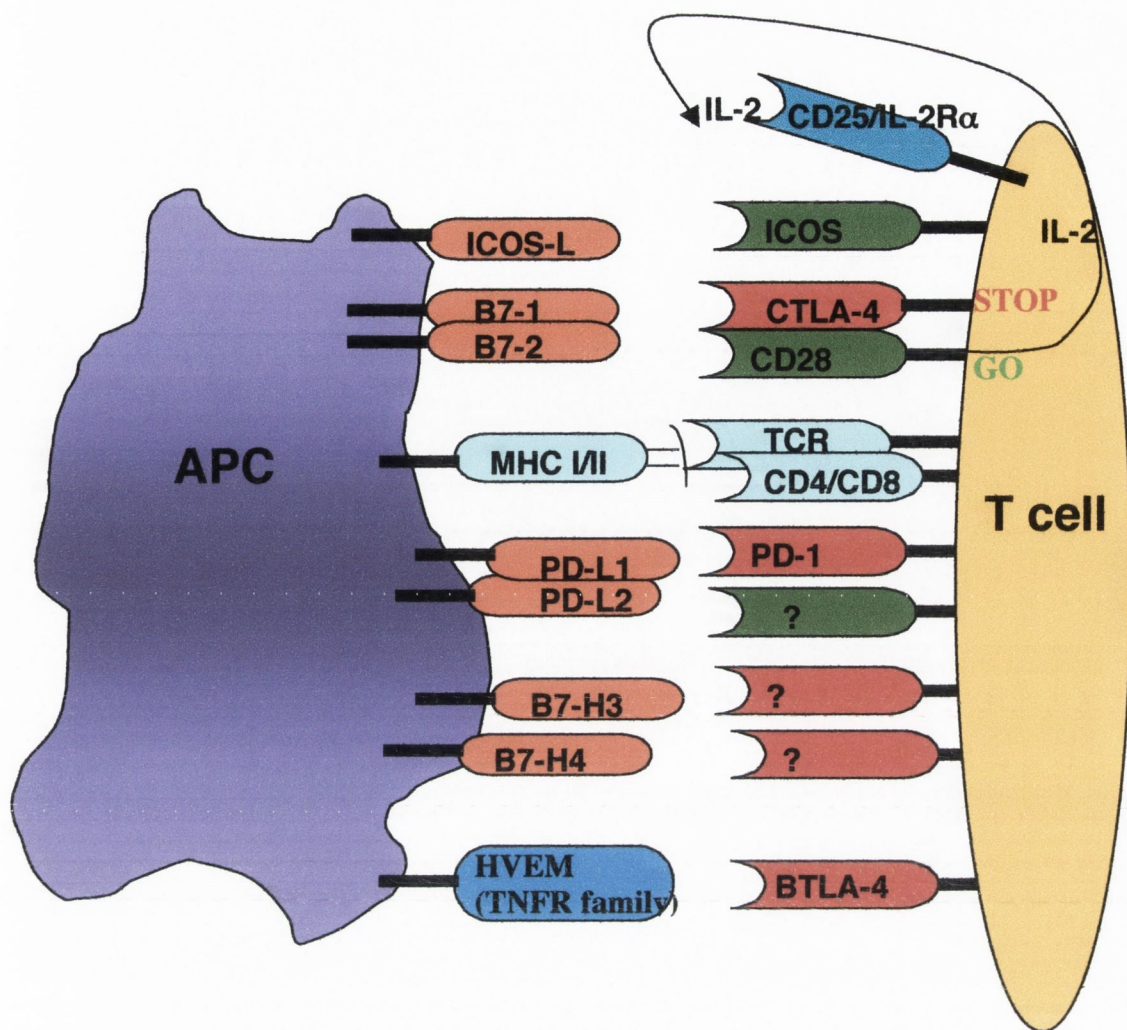


Figure 1.5. B7 family members and their receptors. B7 family members are shown in orange. CD28 family receptors are shown in green (co-stimulators) or red (co-inhibitors). ‘GO’, in green signals T cell activation, with IL-2 production and proliferation. ‘STOP’, in red, signals T cell inhibition. HVEM, the ligand for BTLA is a TNFR family member (blue).

choriomeningitis virus (Christensen *et al.*, 2002; Viola *et al.*, 1999). At the other end of the spectrum, CD28 engagement without TCR activation has been shown to induce proliferation, using CD28-specific ‘superagonist’ Abs (Luhder *et al.*, 2003; Tacke *et al.*, 1997). CD28 is a homodimer, but there have been no crystal structures of this to date. However a complex of a CD28 superagonist Ab Fab’ with the human CD28 monomer has recently been described (Evans *et al.*, 2005). Modelling based on this structure and previous biacore studies (Collins *et al.*, 2002), indicate that CD28 is functionally monovalent. This monovalency of CD28 is essential for physiological co-stimulation, and it acts as a safeguard to prevent T cell activation in the absence of TCR ligation (Dennehy *et al.*, 2006).

However the pathway is more complex, as the B7 ligands B7-1 and B7-2 also bind to an inhibitory receptor, CTLA-4 (cytotoxic T lymphocyte-associated protein 4). Inhibitory co-stimulators serve as a self-limiting mechanism to regulate T cell tolerance, and attenuate the immune response. Unlike CD28, CTLA-4 is expressed only on activated T cells, or regulatory cells. It binds B7-1 and B7-2, but with a much greater affinity than CD28, and subsequently its inhibitory interaction eventually prevails, terminating T cell activation (Walunas *et al.*, 1994). CTLA-4 is of great importance in immune regulation as mice deficient in this molecule develop massive lymphoproliferation and early death (Tivol *et al.*, 1995; Waterhouse *et al.*, 1995). In addition, CTLA-4 gene polymorphisms in humans have been associated with autoimmune disease (Ueda *et al.*, 2003). The role of CTLA-4 in immunity was discussed in section 1.4.5 and is also reviewed in greater detail below (section 1.5.4).

1.5.2 The B7 Family of co-stimulators

Other CD28:B7 members include the PD-1:PD-L pathway, the ICOS:ICOS-L pathway, unknown receptor:B7H3, and unknown receptor:B7H4, with BTLA being a new CD28 family member. The B7 family members and receptors are outlined in **Figure 1.5**, with the

expression profile of the B7 ligands listed on **Table 2**. Members of the receptor family (CD28, PD-1, BTLA) are type 1 transmembrane proteins with a single IgV extracellular domain. The 7 known members of the B7 family [B7-1, B7-2, PD-L1 (B7-H1), PD-L2 (B7-DC), ICOS-L, B7-H3 and B7-H4 (B7x)] are type 1 transmembrane or glycosylphosphatidylinositol (GPI)-linked proteins with IgV and IgC extracellular domains (Collins *et al.*, 2005). B7-H4 is GPI-linked to the cell membrane while all of the others have the transmembrane domain. The intracellular domains contain serine and threonine residues, which may act as phosphorylation sites for signaling pathways, but no function for this domain has as yet been identified. B7-1 and B7-2 genes are closely linked on mouse chromosome 16 and human chromosome 3q21. PD-L1 and PD-L2, which both bind the receptor PD-1, are also closely linked, but the genes encoding the other family members are unlinked.

<i>Ligand/Receptor</i>	<i>Expression</i>	<i>Positive co-stimulatory Receptor</i>	<i>Inhibitory co-stimulatory Receptor</i>	<i>Expression</i>
B7-1 (CD80)	Activated APC	CD28	CTLA-4	T and NK cells
B7-2 (CD86)				Activated T cells
B7h (LICOS)	APC	ICOS		T _{reg} cells
B7-H1 (PD-L1)	DCs, M ϕ , T and B cells, some non-lymphoid tissues and some tumour cells	?	PD-1	Activated T cells
B7-DC (PD-L2)	DCs, macrophages, some tumour cells			B cells, monocytes
B7-H3	DCs, monocytes, some non-lymphoid tissue, tumour cell lines		?	
B7-H4 (B7x)	DCs, peritoneal M ϕ , T and B cells, some non-lymphoid tissues and tumour cell lines		? (HVEM, a TNFR family member)	

Table 2. Co-stimulatory markers of the B7 family.

B7-1 and B7-2

B7-1 and B7-2 have only about 25% amino acid homology, but both have their receptor-binding domains in the amino-terminal IgV domain. Although the amino acid sequence within the receptor-binding domain is not highly conserved, structural studies of the two molecules demonstrate that each has a shallow cavity in its front face that facilitates binding of CD28 and CTLA-4 receptors through their highly conserved MYPPPY motif (Zhang *et al.*, 2003b). B7-1 binds to CTLA-4 with a much greater affinity than B7-2, but it is

predicted that B7-2 binds CD28 more efficiently than B7-1 (Collins *et al.*, 2002). B7-1 and B7-2 expression is limited to lymphoid cells, with B7-2 being constitutively expressed at low levels in B cells, DCs and monocytes, and some resting T cells. It is rapidly upregulated upon activation. B7-1 expression is inducibly expressed at a later stage than B7-2 in T and B cells, monocytes and DCs (Greenwald *et al.*, 2005; van der Merwe and Davis, 2003). The role of CD28:B7 interactions in initial T cell activation is well recognized, with ligation of B7-1 or B7-2 to CD28 leading to T cell activation, expansion, differentiation and survival. This in turn promotes efficient antibody-mediated and cellular immune responses, as is apparent in B7-1 and B7-2 deficient mice, where these responses are impaired (Borriello *et al.*, 1997). As mentioned earlier, interactions of these ligands with the negative co-stimulation receptor CTLA-4 balances this activation signal. The differences in temporal expression profile and the differential binding abilities of B7-1 and B7-2 to CTLA-4 or CD28 propose a model whereby B7-2:CD28 interactions induce a dominant positive signal initially, which is followed by B7-1:CTLA-4 negative signals, that eventually terminate the T cell response (Bluestone *et al.*, 2006).

Additional functions for these B7 molecules have been described in recent years. CD28:B7 interactions are necessary for effective CD4⁺CD25⁺ regulatory cell development and maintenance (Lohr *et al.*, 2003; Tang *et al.*, 2003). Using non-obese diabetic (NOD) mice that are deficient in B7-1 and B7-2 or CD28, it was also demonstrated that CD28:B7 co-stimulation was required for the maintenance of the T_{reg} cells that control autoimmune diabetes (Salomon *et al.*, 2000).

B7 expression on T cells may contribute to down-modulation of immune responses. It was demonstrated that T cell:T cell interactions involving B7 and CTLA-4 lead to downregulation of alloresponses (Taylor *et al.*, 2004). In addition it was shown that T_{reg} cells

interacted with B7 on effector T cells to suppress proliferation, but not necessarily in a CTLA-4-dependent manner. $CD4^+CD25^+$ T_{reg} cells failed to suppress effector ($CD4^+CD25^-$) cells from B7-1/B7-2^{-/-} mice compared to wildtype $CD4^+CD25^-$ cells. Addition of B7-1 or B7-2 mutants that were co-stimulation competent but lacked the transmembrane and intracellular domain were unable to restore suppression, but addition of full-length protein resulted in establishment of T_{reg} -induced suppression (Paust *et al.*, 2004).

‘Reverse signaling’ into B7-expressing APCs has also been demonstrated. Ligation of B7-1 and B7-2 with a soluble CTLA-4Ig activates the immunosuppressive pathway of tryptophan catabolism in DC. Ligand interaction induces a signaling pathway that leads to IFN- γ production, which then acts to induce indolamine 2,3-dioxygenase (IDO) production. This is an enzyme that is involved in tryptophan catabolism, the products of which lead to inhibition of T cell proliferation (Grohmann *et al.*, 2002). Ligation of B7-1 and B7-2 on APCs with CD28Ig induces IL-6 production and co-stimulatory signals (Orabona *et al.*, 2004). In summary, it is clear that these B7 molecules play an important role in regulation of T cell activation and tolerance.

ICOS-L (B7-H2)

Inducible co-stimulator (ICOS) is a co-stimulatory receptor homologous to CD28 and CTLA-4. It is expressed only on activated T cells, so interactions with its ligand, (ICOS-L), enhances the activation and survival of effector/memory, and not naïve, T cells. *In vitro*, it stimulates the production of IL-4 and IL-10, but not IL-2, via a CD28-independent mode of action (Hutloff *et al.*, 1999). ICOS^{-/-} mice have impaired ability to produce IL-4, IL-10, and IL-13, but not IFN- γ following immunization, supporting a role for the pathway in regulation of Th2 effector responses (Liang and Sha, 2002). The ICOS ligand (ICOS-L, LICOS, B7h,

B7-H2) is expressed constitutively on B cells, splenic and peritoneal macrophages, DCs, and peripheral blood-derived mononuclear cells (PBMCs) (Swallow *et al.*, 1999). This costimulation pathway has been shown to be essential for both T helper and B cell functions in T cell-dependent B cell responses (Mak *et al.*, 2003). Studies on ICOS-L^{-/-} mice reinforced the theory that the ICOS:ICOS pathway was important for efficient activation of a Th2 immune response. Effector T cells that were restimulated with APCs from ICOS-L^{-/-} mice, had a reduction in Th2 cytokine production (Nurieva *et al.*, 2003). Another group that generated ICOS-L^{-/-} mice described impaired germinal centre formation and recall T cell-dependent immune responses in these mice (Wong *et al.*, 2003).

B7-H3

B7-H3 is an orphan ligand with an as yet unidentified receptor. mRNA for this molecule is broadly expressed in both mice and humans on lymphoid and non-lymphoid organs. It was originally described to induce T cell proliferation (Chapoval *et al.*, 2001), although generation of mice deficient for this molecule suggests that it functions as an inhibitory co-stimulator, and preferentially down-modulates Th1 immune responses (Suh *et al.*, 2003). Message RNA for B7-H3 was induced in murine DCs by IFN- γ , but was suppressed by IL-4. The demonstration of co-stimulatory and co-inhibitory functions for B7-H3 could indicate that there are two opposing receptors for the molecule, similar to CTLA-4 and CD28. B7-H3 binds to activated, not resting CD4⁺ and CD8⁺ T cells (Sun *et al.*, 2002). A positive function for this immune co-stimulatory molecule in bone formation has also been proposed (Suh *et al.*, 2004). B7-H3 is highly expressed in osteoblasts during bone formation. Disruption of B7-H3 inhibited *in vitro* bone formation by osteoblastic cells, and B7-H3^{-/-} mice had lower bone mineral density in cortical bones compared to WT mice (Suh *et al.*, 2004).

B7-H4

B7-H4 (B7x) is another inhibitory B7-ligand, expressed on immune cells, and some nonlymphoid tissues and tumour cell lines (Zang *et al.*, 2003), which has no known receptor. It can be induced on human T cells, B cells, DCs and monocytes after *in vitro* stimulation. Murine B7-H4 is found constitutively on B220⁺ B cells, and can also be induced on macrophages and DCs. B7-H4 differs from the other B7-members in that it is a GPI-linked protein. Studies using B7-H4Ig demonstrated that it binds to a receptor on activated T cells. It was initially believed that BTLA (B and T lymphocyte attenuator) was its probable receptor, as B7H4Ig was shown to bind wild-type, but not BTLA^{-/-} cells (Watanabe *et al.*, 2003). However it has since been shown that BTLA is not the receptor for B7-H4, and that in fact BTLA interacts with herpesvirus entry mediator (HVEM) (Sedy *et al.*, 2005). B7-H4 acts to inhibit TCR-mediated CD4⁺ and CD8⁺ T cell proliferation and IL-2 production (Sica *et al.*, 2003; Zang *et al.*, 2003). It has recently been implicated in T_{reg}-mediated suppression of APCs (Kryczek *et al.*, 2006). T_{reg} cells were shown to stimulate IL-10 production in APCs, which induced B7-H4 expression, and it is this that is partially responsible for the immunosuppressive properties of T_{reg}-conditioned APCs. B7-H4 is expressed on some tumours, suggesting a possible role for this molecule in prevention of anti-tumour immunity (Choi *et al.*, 2003). A study on renal cell carcinoma (RCC) patients showed an association with B7-H4 expression and tumour progression and survival (Krambeck *et al.*, 2006). Patients with tumours that expressed B7-H4 were 3 times as likely to die from RCC than those who had B7-H4⁻ tumours. B7-H4 expression was also found on endothelial cells in the tumour vasculature in over 80% of samples studied, while only 6% of normal renal tissue vessels had endothelial expression of B7-H4. Patients with tumours that expressed both PD-L1 and B7-H4 were at an even greater risk of death from RCC.

BTLA receptor

B and T lymphocyte attenuator (BTLA) is a member of the CD28 family of receptors for B7 members. It is an inhibitory receptor that functions similarly to PD-1 and CTLA-4, and is expressed on T and B cells, splenic macrophages and bone-marrow derived DCs (Carreno and Collins, 2003). It is induced upon naïve T cells activation, and while expression is down-modulated on polarized Th2 cells, it remains on Th1 cells (Watanabe *et al.*, 2003). BTLA^{-/-} T cells proliferate more in response to antigen presented by DCs than wild-type mice, and they also display an increased susceptibility to peptide-antigen induced EAE (Watanabe *et al.*, 2003). Deppong and co-workers have shown that BTLA, and also PD-1, are necessary for the resolution and termination of acute allergic airway inflammation (Deppong *et al.*, 2006). Loss-of-function studies using mice deficient in BTLA or PD-1 showed persistent inflammation up to day 15 following a single inhaled allergen challenge, while wild-type mice had resolved inflammation by day 10. As mentioned above, the receptor for BTLA is the herpesvirus entry mediator (HVEM) (Sedy *et al.*, 2005). Gonzalez and co-workers also identify this ligand as a receptor for BTLA, and show that the two interact with high affinity and can form a trimeric complex with the TNF ligands LIGHT or lymphotoxin α (LT α) (Gonzalez *et al.*, 2005). HVEM is a member of the tumour necrosis factor receptor (TNFR) family of co-stimulatory molecules, and binding of this molecule to BTLA represents a direct interaction between the CD28 family and the TNFR family to mediate T cell inhibition.

Positive co-stimulation is required to augment T and B cell activation, however inhibitory co-stimulation can just as firmly attenuate these responses. An effective immune response is dependent upon the delicate balance between these positive and negative signals.

1.5.3 The TNFR Family of co-stimulatory molecules

In addition to B7 co-stimulation, the TNFR (tumour necrosis factor receptor) family of receptors are also involved in T cell activation. They can function after initial T cell activation to sustain T cell responses, and TNFR family members are emerging as key mediators of survival signaling in T cells following the initial effects of CD28:B7 interactions. A summary of receptors and their ligands is detailed in **Table 3**. Interactions of the TNFR/TNF family can influence innate immunity and inflammation, lymphoid organization, or activation of APCs, and they can also act to provide direct signals to T cells (Watts, 2005). The future use of TNFR/TNF agonists or antagonists in therapeutic applications to human disease is also a possibility.

Ligand/Receptor	Expression	Receptor	Expression
CD30L (CD153)	Neutrophils, activated B and T cells	CD30	Activated T, NK and B cells
CD40	APC, T subset, endothelium, cardiac myocytes, fibroblasts	CD40L	Activated T cells
CD70	Activated B cells	CD27	T cells, B subset, NK
4-1BBL	Activated B, DC, peritoneal cells	4-1BB (CD137)	Activated T cells
OX-40L	Activated B cells, cardiac myocytes	OX-40	Activated T cells
AITRL (GITRL)	APC	AITR (GITR)	Treg, T (upregulated)
LIGHT (HVEM-L)	Activated T cells, monocytes, NK cells, immature DC	HVEM	Resting T cells, monocytes, immature DC
B7-H4 (a B7 family member)			

Table 3. The TNFR family of co-stimulatory receptors and their ligands.

1.5.4 CTLA-4: Negative regulator of T cell activation

As mentioned earlier, CTLA-4 binds to the B7-members B7-1 and B7-2. It functions as a negative regulator of T cell activation, as exemplified by studies in CTLA-4 deficient mice, who die from massive lymphoproliferation within 3-4 weeks of birth (Tivol *et al.*, 1995). Consistent with the autoimmune phenotype observed in CTLA-4 deficient mice, there is a correlation between some CTLA-4 gene polymorphisms in humans and the susceptibility to autoimmune disorders (Kristiansen *et al.*, 2000).

Structure and expression

CTLA-4, like CD28, is a type 1 transmembrane glycoprotein of the immunoglobulin superfamily, and it forms a covalent homodimer of 41-43 kDa (Brunet *et al.*, 1987; Linsley *et al.*, 1995). It shares approximately 30% homology with CD28 at the protein level. The extracellular region is composed of an IgV-like domain that contains the B7-1/B7-2 binding site. The cytoplasmic domain is 36 aa long, and the presence of a lysine-rich motif, two tyrosine residues and a proline-rich region in this portion are associated with a signaling role for the molecule (Lee *et al.*, 1998; Teft *et al.*, 2006). The gene encoding CTLA-4 is on chromosome 1 in mice and chromosome 2 in humans. Alternative splicing of the transcript can occur in the mouse and human. In humans, three forms of CTLA-4 can be detected; the full length protein with all 4 exons, a soluble form that is missing exon 3, which codes for the transmembrane domain, and a form that contains only the leader peptide sequence (exon 1) and the cytoplasmic tail (exon 4). Murine CTLA-4 can also have another form, ligand-independent CTLA-4 (liCTLA-4), which is missing exon 2, the ligand-binding domain (Teft *et al.*, 2006). In addition to humans and mice, CTLA-4 is expressed in primates, dogs, cats, cows, sheep and rats (Teft *et al.*, 2006), and there are putative CTLA-4-like molecules in rainbow trout and chickens (NCBI accession numbers XM_4211960.1 and AY789436.1).

As mentioned earlier, CTLA-4 is principally expressed on activated T cells, but it is also constitutively present on CD4⁺CD25⁺ T_{reg} cells (Lindsten *et al.*, 1993; Takahashi *et al.*, 2000). It is commonly found intracellularly, and is quickly expressed on the cell surface upon activation (Jago *et al.*, 2004). CTLA-4 gene expression has been discovered in non-T cells, such as B cells, monocytes, granulocytes and embryonic cells, but the exact function of CTLA-4 in non-T cells has not yet been fully elucidated (Ling *et al.*, 1998; Pistillo *et al.*, 2003).

CTLA-4 binds its ligands B7-1 and B7-2 (dissociation constant K_d 0.2 and 2.6 μM, respectively) with a much greater affinity than CD28 (K_d 4 and 20 μM respectively). The structure of the CTLA-4 homodimer allows for bivalent B7 binding, whereas CD28 is monovalent (see section 1.5.1 above). The majority of the functions of CTLA-4 are mediated through these ligands. Indeed a triple knock-out mouse, deficient in B7-1 and B7-2 in addition to CTLA-4 did not display the lymphoproliferative phenotype observed in mice deficient in CTLA-4 alone (Mandelbrot *et al.*, 1999). However the presence of the murine ligand-independent isoform of CTLA-4, and recent studies outlining B7-independent inhibition of T cells by CTLA-4 point to B7-independent functions of CTLA-4, and also the possibility of additional ligands (Chikuma *et al.*, 2005; Vijayakrishnan *et al.*, 2004). The liCTLA-4 molecule was associated with increased risk for disease in the NOD mouse model of type 1 diabetes (Vijayakrishnan *et al.*, 2004). LiCTLA-4 inhibits both T cell production and cytokine production with dephosphorylation of the TCR ζ chain, indicating that the cytoplasmic chain, independent of B7 binding, can induce a negative signal (Vijayakrishnan *et al.*, 2004). Chikuma and co-workers used a ligand non-binding mutant of CTLA-4 in otherwise CTLA-4^{-/-} cells to demonstrate B7-independent inhibition of T cell proliferation,

and CTLA-4^{-/-} mice that were bred with the mutant transgene delayed the lethal autoimmune phenotype observed in CTLA-4^{-/-} mice (Chikuma *et al.*, 2005).

Mechanism of inactivation and signaling

CTLA-4 employs two mechanisms of T cell inactivation, antagonism of CD28-dependent co-stimulation and direct negative signaling through the cytoplasmic tail. APC:T cell interactions lead to the formation of an immunological synapse (IS), which contains lipid rafts on the surface of both cells. CD28 engagement by B7-1 or B7-2 leads to the relocation of intracellular CTLA-4 closer to the site of TCR engagement. Its surface expression is increased and clustering occurs within the lipid rafts at the IS. Once at the IS, CTLA-4 may now compete with CD28 for the binding of B7 molecules (Linsley *et al.*, 1995). Antibody cross-linking of CTLA-4 and TCR even in the presence of non-limiting CD28 can also inhibit IL-2 production and cause cell cycle arrest, suggesting the second mechanism of operation for CTLA-4 (Krummel and Allison, 1996). This mechanism does not require high surface expression of CTLA-4, but is dependent on the presence of the cytoplasmic tail (Carreno *et al.*, 2000).

There are three models in which CTLA-4 may deliver its negative signal, inhibition of the early events of TCR signaling, inhibition of the downstream events of TCR and CD28 signaling and direct inhibition of CD28-dependent signaling (Rudd and Schneider, 2003). Cytokine production is down-modulated through the inhibition of transcription factors AP-1, NF- κ B and NFAT in the nucleus of activated T cells. It exerts its anti-proliferative effect via inhibition of cyclin D3, cyclin-dependent kinases 4 and 6 and degradation of cell-cycle inhibitor p27^{kip1} (Brunner *et al.*, 1999).

The exact CTLA-4 signaling pathway has not been fully elucidated, but the cytoplasmic tail does not have enzymatic activity and it lacks the immune receptor tyrosine-

based inhibitory motif (ITIM) found in other inhibitory receptors. However many other protein:protein interaction motifs are present. An overview of the mechanism of T cell inactivation by CTLA-4 is outlined in **Figure 1.6**, taken from (Teft *et al.*, 2006). The current theory is that CTLA-4 signaling is principally regulated by the serine/threonine phosphatase PP2A, acting downstream of early TCR and CD28 signaling, either by direct competition with PI3K or by inhibition of the PKB/Akt pathway.

CTLA-4 in Th2 regulation

CTLA-4 plays a crucial role in control of Th2 responses, in particular in regulation of Th2 differentiation. *In vitro* TCR stimulation of T cells from CTLA-4 deficient mice induces secretion of high levels of the Th2 cytokines IL-4 and IL-5. T cells from CTLA-4^{-/-} mice treated with a murine CTLA-4Ig do not exhibit this Th2 polarisation (Khatttri *et al.*, 1999). Studies using wild-type or CTLA-4^{-/-} OVA-TCR transgenic mice demonstrate that CD28 is necessary for promotion of Th2 differentiation, while CTLA-4 is critical for its limitation (Oosterwegel *et al.*, 1999). CTLA-4 surface expression has been shown to be induced at high levels in Th2 cells compared to Th1 cells (Pandiyani *et al.*, 2004). CTLA-4 regulated activation induced cell death (AICD) in these cells via suppression of the Fas system by targeting PI3K (Pandiyani *et al.*, 2004). As mentioned earlier (section 1.4.3), T-bet and GATA-3 are the principal Th1/Th2 differentiation markers in T cells respectively. CTLA-4 inhibits mRNA expression of the Th2 transcription factor GATA-3 in naïve CD4⁺ T cells upon neutral or Th2-polarising conditions (Nasta *et al.*, 2006). Engagement of CTLA-4 had little effect on T-bet mRNA expression during helper cell differentiation (Nasta *et al.*, 2006).

Biological function for CTLA-4

A role for CTLA-4 has been purported in the control of Th2-mediated diseases, such as nematode infection. Blockade of CTLA-4 induced protective immunity to mice infected

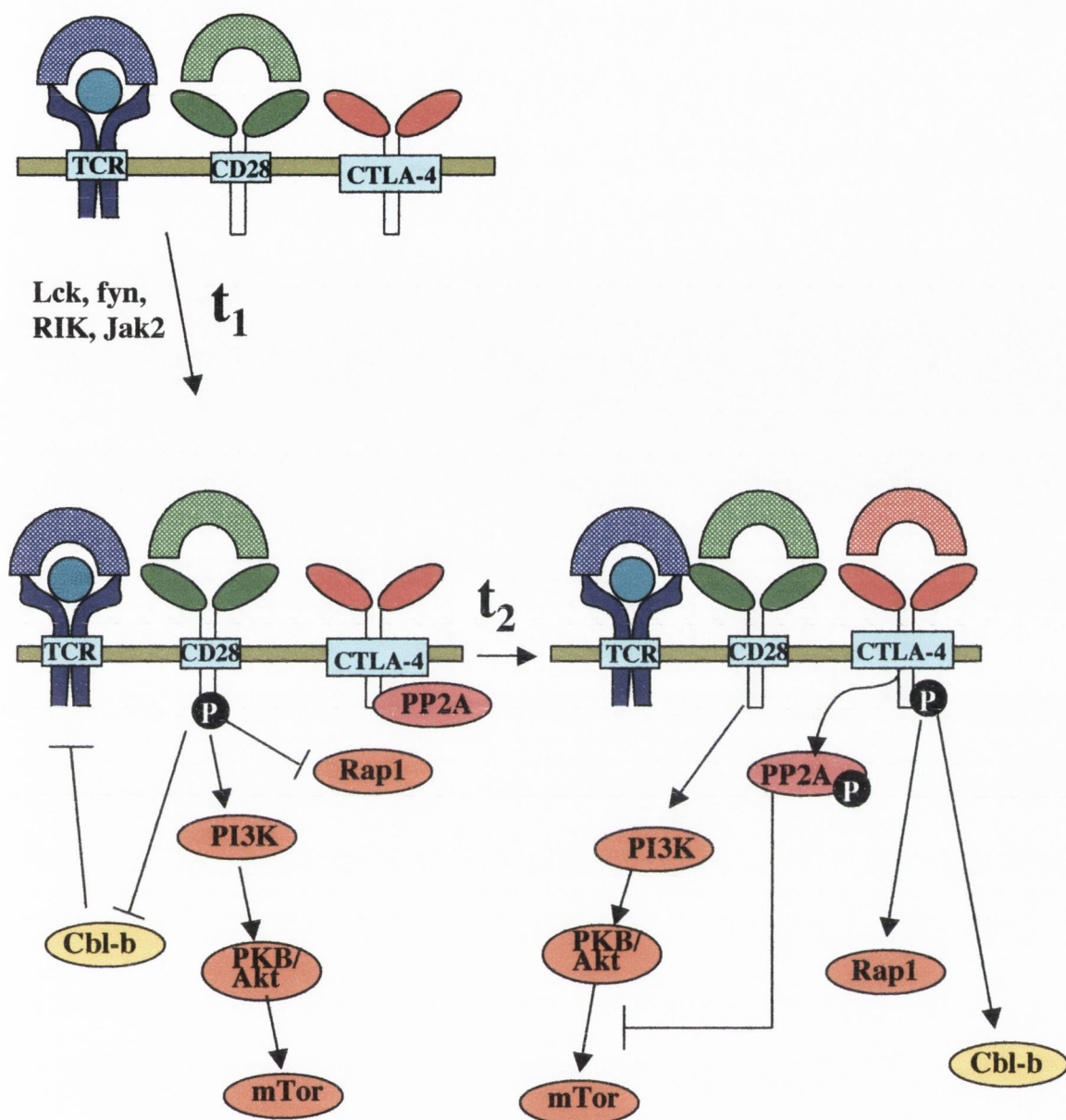


Figure 1.6. Mechanisms of CTLA-4-mediated T cell inactivation. CD28 and CTLA-4 are sequentially (t_1 and t_2) phosphorylated by different kinases in a Lck/ZAP-70-dependent manner following T cell activation. CD28, when phosphorylated, activates PKB and also down-regulates Rap1 (G protein) and Cbl-b (E3 ubiquitin ligase). B7-ligation of CTLA-4 is associated with phosphorylation-dependent dissociation of PP2A. This may cause inhibition of the PKB pathway, or enhance the activity of Rap1 and Cbl-b, which may lead to inhibition of downstream signaling events. Taken from Teft *et al.*, 2006.

with *Nippostrongylus brasiliensis* (McCoy *et al.*, 1997), and it has been implicated in the T cell hyporesponsiveness observed in filaria-infected patients (Steel and Nutman, 2003). In addition, blocking of CTLA-4 was shown to enhance allergic sensitization and eosinophilic airway inflammation in BALB/c mice, with a significant reduction in lung levels of the regulatory cytokine, TGF- β (Hellings *et al.*, 2002). However while another group also found that blockade of CTLA-4 confounded airway inflammation, in a chronic model it failed to prevent inhalation tolerance (Alenmyr *et al.*, 2005). Using a murine peanut allergy model, it was shown that while CTLA-4 was involved in regulating the intensity of hypersensitivity responses, it was not decisive in preventing the induction of sensitization (van Wijk *et al.*, 2005). In the Th1 malaria model *Plasmodium berghei*, CTLA-4 is important for the prevention of T-cell induced liver pathology at the erythrocyte stage, with increased IFN- γ -producing liver T cells and liver damage in mice treated with a blocking antibody against CTLA-4 (Jacobs *et al.*, 2004).

CTLA-4 and T_{reg} cells

Regulation of cell activation by CTLA-4 has previously been discussed in the context of T_{reg} cells and in other disease models such as tumours and colitis (section 1.4.5). The suppressive properties of human CD4⁺CD25⁺ T_{reg} cells are dependent on CTLA-4 expression (Birebent *et al.*, 2004). A murine study showed that T cell suppression by wild-type T_{reg} is CTLA-4 dependent, but T_{reg}-mediated suppression was still observed in T_{reg} from CTLA-4^{-/-} mice, and functioned in a partially TGF- β -dependent manner (Tang *et al.*, 2004). T_{reg} cells are important in peripheral allograft tolerance, and it was recently demonstrated that tolerance could be established by blocking of co-stimulation using anti-CD40L and anti-CD86 mAbs, where CD4⁺CD25⁺ T_{reg}-mediated mechanisms dominated (Coenen *et al.*, 2006). CTLA-4 was

required for this anti-CD40L/anti-CD86-mediated inhibition of the allogeneic response, as were CD4⁺CD25⁺ T_{reg} cells, however CTLA-4 engagement was not required for the activation or function of the T_{reg} cells (Coenen *et al.*, 2006). T_{reg} cells may also function to inhibit T cell responses indirectly, with T_{reg}-conditioned DCs undergoing rapid down-modulation of B7-1 and B7-2, and inducing poor T cell proliferative responses. This down-modulation was CTLA-4 dependent (Oderup *et al.*, 2006).

IDO

As mentioned earlier, CTLA-4Ig was used to reverse signal through B7-1 and B7-2 on DCs to induce IDO expression and tryptophan catabolism (Grohmann *et al.*, 2002). CTLA-4-bound B7 molecules on DCs also lead to IDO production in human studies (Munn *et al.*, 2004). The functional association between CTLA-4 and IDO is gaining stature through studies in allograft rejection, the control of autoimmune conditions, and increased anti-tumour responses upon blocking CTLA-4 (Phan *et al.*, 2003; Salomon and Bluestone, 2001). Boasso and co-workers recently demonstrated that CTLA-4 can induce IDO expression in human CD4⁺ T cells as well as in DCs, and that engagement of B7 molecules with CTLA-4Ig inhibited T cell activation in an IDO-dependent mechanism (Boasso *et al.*, 2005).

Cbl-b

CD28 and CTLA-4 may also have an additional role in controlling the threshold of T cell activation by regulating protein expression of Casitas B-cell lymphoma (Cbl-b) (Li *et al.*, 2004), also shown in **Figure 1.6**. Cbl-b is an E3 ubiquitin ligase that plays a crucial role in regulating the threshold of signaling in T cells, and Cbl-b deficient mice have an increased susceptibility to autoimmune disease (Bachmaier *et al.*, 2000; Chiang *et al.*, 2000). Cbl-b regulates the activity of Vav, Vav-1 is activated by CD28 co-stimulation, which uses Vav-1 to enhance TCR signaling and NF-AT activation (Michel *et al.*, 2000). The function of CD28

and CTLA-4 may be dependent on Cbl-b, as their co-stimulation and co-inhibition effects are uncoupled in Cbl-b^{-/-} T cells. CD28 costimulation results in the downregulation of Cbl-b, while engagement of CTLA-4 on leads to re-expression on Cbl-b. In addition, CTLA-4^{-/-} T cells have significantly lower levels of Cbl-b (Li *et al.*, 2004). In a study of healthy Israelis and persistently helminth-infected individuals (Ethopian migrants to Israel), increased TGF- β , Cbl-b and CTLA-4 levels were detected in association with immunosuppression and this correlated with chronic immune activation (Leng *et al.*, 2006).

It is clear that CTLA-4 is a potent inhibitor of CD28-mediated T cell activation, and that there is great potential to exploit its functional suppressive ability in the treatment of autoimmune disorders. Orencia, from Bristol-Myers-Squibb, is a CTLA-4Ig that is the first-in-class antagonist of CD28 costimulation. It was licensed for use by the FDA in December 2005 for the treatment of rheumatoid arthritis, and may be a promising therapeutic for the treatment of other diseases in the future (Bluestone *et al.*, 2006).

1.5.5 The PD-1:PD-L pathway

The PD-1:PD-L pathway is a relatively recently described inhibitory co-stimulatory pathway. Engagement of programmed death-1 (PD-1) with its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC), was reported to inhibit T and B cell proliferation and cytokine production (Freeman *et al.*, 2000; Latchman *et al.*, 2001; Okazaki *et al.*, 2001). The PD-1 receptor was originally thought to be involved in the programmed death pathway, hence its name. It is an Ig superfamily member that is related to CD28 and CTLA-4, but it does not contain the membrane proximal cysteine residue that enables homodimerisation, and so is monomeric (Freeman *et al.*, 2000; Zhang *et al.*, 2004). It shares 23% homology with CTLA-4, but lacks the MYPPPY motif in its extracellular domain, which is necessary for B7-1 and B7-2 binding.

There are two tyrosine residues in its cytoplasmic domain, one is located within an ITIM, which is typically associated with inhibitory signals (Ravetch and Lanier, 2000). However the second tyrosine residue is located within an ITSM (immunoreceptor tyrosine-based switch motif), and it is this motif that is responsible for the inhibitory activity of PD-1 (Latchman *et al.*, 2001; Okazaki *et al.*, 2001). Upon interaction of PD-1 with its ligands (PD-L1 or PD-L2), the N-terminal phosphorylated tyrosine on the ITSM motif recruits src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2). SHP-2 then dephosphorylates signal transducers of the TCR or BCR pathways (Latchman *et al.*, 2001; Okazaki *et al.*, 2001), although its exact molecular targets remain unclear. SHP-1 is also recruited through the PD-1 ITSM motif in T cells, however only SHP-2 is recruited in B cells (Chemnitz *et al.*, 2004). PD-1 is similar to CTLA-4 in that only very low levels of the molecule are required to induce potent suppression of T cell activation (Chemnitz *et al.*, 2004).

PD-1-mediated inhibition requires co-localisation of PD-1 with TCR/CD28. CTLA-4 and PD-1 both limit glucose metabolism and Akt activation, albeit by different mechanisms, PD-1 via blocking of CD28-mediated activation of PI3K, and CTLA-4 via protein phosphatase 2a (PP2A). PD-1 also inhibits expression of the cell survival gene *bcl-x_L* (Carter *et al.*, 2002). PD-1 is expressed on activated CD4⁺ and CD8⁺ T cells, B cells and myeloid cells (Agata *et al.*, 1996). It is also expressed on CD4⁺CD8⁻ cells and double negative $\gamma\delta$ thymocytes during thymic development (Nishimura *et al.*, 2000). Studies in PD-1 deficient mice have highlighted the crucial role for PD-1 in immune regulation. Increased T and B cell proliferation and propensity to develop autoimmune disease were observed in PD-1 knock-out (ko) mice (Nishimura *et al.*, 1998). C57BL/6 PD-1 ko mice develop lupus-like autoimmune proliferative arthritis and glomerulonephritis (Nishimura *et al.*, 1999), and PD-1 ko BALB/c mice develop severe autoimmune dilated cardiomyopathy followed by death due to congestive

heart failure (Nishimura *et al.*, 2001). Human studies have also suggested a potential regulatory role for PD-1. An association between an intronic single nucleotide polymorphism (SNP) in the PD-1 gene and development of systemic lupus erythematosus (SLE) has been shown in a recent study on SLE patients (Prokunina *et al.*, 2002). The same SNP was also associated with increased risk of disease in a study on type 1 diabetes patients (Nielsen *et al.*, 2003).

PD-1 ligands

The ligands for PD-1 are PD-L1 and PD-L2, both members of the B7 family of co-stimulatory ligands. PD-L1 shares 40% homology with PD-L2, and both genes are mapped on the same chromosome in both humans and mice (9p24.2, and between 19C2 and 19C3 respectively). PD-L1 is constitutively expressed on T cells, B cells, macrophages and DCs in the spleen (Liang *et al.*, 2003). It is also expressed on a variety of non-lymphoid organs, such as the heart, lung, placenta and pancreas (Liang *et al.*, 2003). PD-L2 is predominantly expressed on DCs, but also on macrophages and B cells (Latchman *et al.*, 2001; Yamazaki *et al.*, 2002). The expression of PD-L1 on peripheral tissues may be important in providing inhibitory co-stimulation to previously-activated T cells, to suppress aggressive inflammatory responses and self reactivity at the effector site. Since PD-1 may also be expressed on B cells and macrophages, this suggests potential bi-directional interactions between PD-L1 and PD-1. Expression of PD-L1 on tumour cell lines (Dong and Chen, 2003) suggests that tumour cells have usurped the natural function of this ligand, to escape from immune attrition.

Interaction of PD-L with PD-1 causes cell cycle arrest in G0/G1, however cell death is not induced (Latchman *et al.*, 2001). The inhibition of T cell proliferation is mediated by inhibition of IL-2, and affects both CD4⁺ and CD8⁺ T cells (Carter *et al.*, 2002). CD8⁺ cells may be more susceptible because of their inherent inability to produce significant levels of IL-

2. Co-stimulation with soluble anti-CD28 stimulates IL-2 production, which can overcome PD-1-mediated inhibition. However the PD-1 pathway continues to inhibit IL-2 production even with positive co-stimulation, and the inhibitory pathway eventually prevails. The anergised T cells do not lose IL-2 responsiveness as addition of exogenous IL-2 overcomes the PD-1 mediated inhibition and restores T cell proliferation (Carter *et al.*, 2002). Antigen-specific T cell proliferation was also augmented upon blocking of the PD-1 pathway in response to immunization *in vivo* (Salama *et al.*, 2003). PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells (Loke and Allison, 2003). PD-L1 was shown to be highly expressed on inflammatory macrophages, with further up-regulation upon stimulation by LPS and IFN- γ . It was demonstrated that PD-L2 could be induced on APCs by alternative activation via IL-4, and it was not expressed on inflammatory macrophages. This suggests that PD-L1 and PD-L2 may have distinct roles in the regulation of Th1 and Th2 responses (Liang *et al.*, 2003; Loke and Allison, 2003). Although PD-1^{-/-} mice exhibit marked predisposition to autoimmune disease, and show increased T and B cell proliferation (Nishimura *et al.*, 1998), PD-L2 deficient mice show no abnormal phenotype up to one year of age, with no indication of autoimmunity or immunopathology (Shin *et al.*, 2003). Recent studies using anti-PD-L1 mAbs *in vivo* suggest a role for PD-L1 in regulating autoimmune diseases (Ansari *et al.*, 2003; Salama *et al.*, 2003).

Dong and co-workers recently showed that PD-L1 determines accumulation and deletion of intrahepatic CD8⁺ T lymphocytes, as PD-L1^{-/-} mice had spontaneous accumulation of CD8⁺ cells in the liver, with normal CD4⁺ levels (Dong *et al.*, 2004). Normal numbers and ratios of T cells in the spleen, thymus and lymph nodes were found, and PD-L1 ko mice up to 14 months old showed no obvious pathological changes, with a life-span comparable to aged-match wild-type (wt) mice. However experimental autoimmune hepatitis is accelerated in ko

mice compared to wt, with 40% of ko mice dying upon induction of the disease compared to 10% of wt animals. More recently, another group have also prepared PD-L1^{-/-} mice and demonstrated that PD-L1 on T cells, APCs and host tissues negatively regulates T cells, and plays a critical role in T cell tolerance (Latchman *et al.*, 2004). They showed that CD4⁺ and CD8⁺ responses of ko mice were significantly enhanced *in vivo* and *in vitro*. An experimental model of autoimmune encephalomyelitis showed that PD-L1 on T cells and host tissues limits responses of self-reactive CD4⁺ cells *in vivo*. A PD:PD-L pathway overview is outlined in **Figure 1.7**.

The PD-L pathway in disease

The PD-1:PD-L pathway of co-inhibition has been implicated in many diseases, such as autoimmunity, asthma, infectious disease including viral infection, transplantation and tumour immunity. As mentioned earlier, a role for the pathway in autoimmunity and immune tolerance was initially suggested from studies on PD-1^{-/-} mice. The use of blocking mAbs against PD-1 and PD-L1 in pre-diabetic female NOD mice or male NOD mice caused rapid disease development, which correlated with increased IFN- γ -producing GAD-reactive splenocytes. However treatment with blocking antibodies against PD-L2 had no effect on the onset of disease (Ansari *et al.*, 2003). PD-L1 is expressed on infiltrating cells in the islets, and is markedly upregulated on islet cells in pre-diabetic mice, implicating it in the control of self-reactive T cell responses in the pancreatic islets (Liang *et al.*, 2003). In contrast to this, another study showed that transgenic C57/BL6 mice that have constitutive expression of PD-L1 on pancreatic islet β cells are more susceptible to spontaneous development of autoimmune diabetes (Subudhi *et al.*, 2004). Keir and co-workers have now shown that PD-L1 expression on parenchymal cells rather than haematopoietic cells is involved in protection against

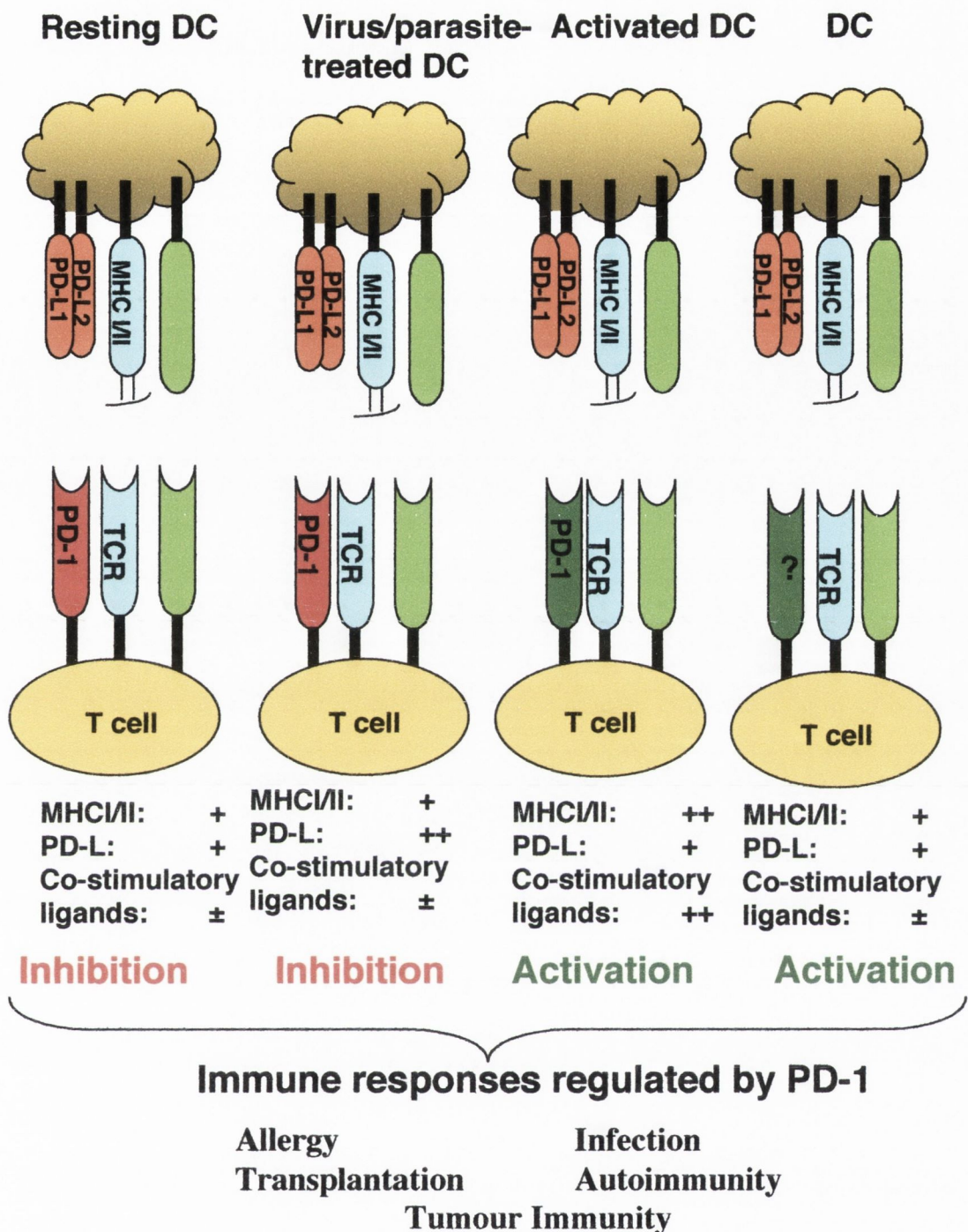


Figure 1.7. The PD-1:PD-L Pathway. Resting DCs and virus- or parasite-treated DCs express higher levels of PD-L than other co-stimulatory ligands (lime green) and MHC class I/II (blue), leading to PD-PD-L induced T cell anergy. Activated DCs express lower levels of PD-L than other co-stimulatory ligands and MHC class I/II, and activating stimuli overcome the negative effect of PD-1, leading to T cell activation. Another possibility is that PD-L can bind to a positive co-stimulatory receptor that induces T cell activation. Modified from Okazaki *et al.*, 2006.

autoimmune diabetes (Keir *et al.*, 2006). Bone-marrow chimeras that only express PD-L1 and PD-L2 on cells of nonlymphoid hematopoietic origin demonstrated that expression of PD-L1 and PD-L2 on APCs alone was not sufficient to protect against the early onset diabetes associated with PD-L1/PD-L2^{-/-} mice (Keir *et al.*, 2006).

Autoimmune disease

The PD-1 pathway also plays an important role in the regulation of experimental autoimmune encephalomyelitis (EAE). This has been highlighted in both PD-L1 mAb blocking studies (Salama *et al.*, 2003) and studies using PD-L1^{-/-} mice (Latchman *et al.*, 2004), where disease was exacerbated in mice lacking functional PD-L1. PD-L1 has also been implicated in a mouse model of inflammatory bowel disease. In the CD45RB^{hi} T cell adoptive transfer model, blockade of PD-L1, but not PD-L2 suppressed the development of intestinal inflammation. IFN- γ , IL-2 and TNF- α production were reduced, but IL-4 and IL-10 levels remained the same (Kanai *et al.*, 2003).

Rheumatoid arthritis (RA) may also be regulated in some ways by the PD-1 pathway. PD-1 is expressed at higher levels on T cells in the synovial fluid of patients with RA (Hatachi *et al.*, 2003). Autoantibodies against PD-L1 have also been detected in patients with RA, they were detected in 29% of patients versus 4% in healthy donors (Dong *et al.*, 2003). The pathway is also implicated in Sjogren's syndrome (SS), with enhanced expression of PD-1 and PD-L1 in salivary gland lymphocytes of patients with the syndrome (Kobayashi *et al.*, 2005).

Transplantation

Cardiac and islet transplantation studies have demonstrated a role for members of the PD-1 pathway. It has been shown that PD-1, PD-L1, and PD-L2 are upregulated in cardiac allografts that are undergoing rejection (Ozkaynak *et al.*, 2002). In islet cell transplantation, expression of PD-L1 promoted organ-specific autoimmunity, suggesting that here it

functioned to activate T cell responses, rather than suppress them (Subudhi *et al.*, 2004). Mycophenolic acid (MPA) is a non-competitive, reversible inhibitor of the inosine 5'-monophosphate dehydrogenase, which plays a major role in the *de novo* synthesis of guanosine nucleotides, and so is important for immunosuppression. MPA was shown to upregulate PD-L2 expression on DCs, and this was associated with the impaired allostimulatory capacity of the DCs (Geng *et al.*, 2006).

PD-L in tumour immunity

Both PD-L1 and PD-L2 are expressed on various tumour cells (Greenwald *et al.*, 2005). PD-L1 on tumour cells can suppress the cytolytic ability of CD8⁺ T cells (Hirano *et al.*, 2005; Iwai *et al.*, 2002). Antibody blockade of PD-L1 improved myeloid DC-mediated anti-tumour immunity (Curiel *et al.*, 2003). The use of gene therapy to block PD-1 interactions also improved the anti-tumour effect of secondary lymphoid tissue chemokine (He *et al.*, 2004). An association between tumour cell expression of PD-L1 or PD-L2 and poor prognosis has been demonstrated in humans. Patients with renal cell carcinoma that had enhanced lymphocyte and/or tumour cell expression of PD-L1 were 4.5 times more likely to die from their illness than patients with low levels of PD-L1 (Thompson *et al.*, 2004). Patients with eosophageal cancer that have PD-L1⁺ and PD-L2⁺ tumour cells have significantly reduced survival rate than patients with PD-L⁻ tumour cells, with only 50% survival after 1 year compared to 100% in PD-L⁻ patients, and no survivors past 3 years, compared to 70% survival in patients with PD-L⁻ tumours (Ohigashi *et al.*, 2005). It is clear that blockade of the PD-1 pathway to reverse the immunosuppressed host could have major implications in future cancer therapies.

Allergies and asthma

The use of a murine hapten-induced contact hypersensitivity model showed a role for PD-1:PD-L1, but not PD-L2 in the regulation of allergic inflammation (Tsushima *et al.*, 2003). mAb blocking of PD-1, and PD-L1, but not PD-L2 resulted in increased contact hypersensitivity responses. In murine models of asthma, allergen challenge with OVA increased PD-1 expression on T cells in the lung and draining lymph node (DLN). PD-L1 was upregulated on macrophages, DCs and B cells in the lungs, while PD-L2 expression was significantly elevated in DCs and slightly increased in macrophages in the lung and DLN (Matsumoto *et al.*, 2004). Treatment with blocking antibodies to PD-1 or PD-L1 at the time of allergen challenge had no effect on airway hyperresponsiveness (AHR). However treatment with anti-PD-L2 mAb lead to increased AHR, associated with increased eosinophilia and elevated IL-5 and IL-13, while IFN- γ production in the lungs and DLN was reduced (Matsumoto *et al.*, 2004). This highlights a PD-1 independent function for PD-L2. IFN- γ depletion showed that the effects were in part mediated by an IFN- γ -dependent mechanism (Matsumoto *et al.*, 2004). Another study showed that while administration of a PD-L2 fusion protein (PD-L2-Fc) was capable of inhibiting proliferation of pre-activated Th0, and also Th2-polarised cells *in vitro*, administration of PD-L2-Fc *in vivo* in an OVA AHR model resulted in increased eosinophil and lymphocyte infiltration into the bronchial lavage fluid, with an associated increase in Th2 cytokines IL-5 and IL-13 from the DLN (Oflazoglu *et al.*, 2004). This appears to contradict the work of Matsumoto and colleagues, however the treatment regime was different, with administration of the fusion protein before sensitization as well as at the time of allergen challenge. However both studies seem to implicate a PD-1 independent role for PD-L2.

Role in Th1/Th2 responses?

The induction of PD-L1 and PD-L2 by different stimuli has been discussed earlier (Loke and Allison, 2003), suggesting that PD-L1 may preferentially regulate Th1 responses. PD-L1^{-/-} and PD-L2^{-/-} mice were used to dissect what role, if any, these two ligands play in the regulation of immunity to cutaneous leishmaniasis (Liang *et al.*, 2006). Wild-type 129Sv mice were susceptible to infection with *L. mexicana*, but PD-L1^{-/-} mice were more resistant to infection, and displayed reduced parasite burden and growth of cutaneous lesions. However PD-L2^{-/-} mice had increased parasite burden and elevated disease pathology. In contrast to other studies on PD-L1^{-/-} mice (Dong *et al.*, 2004; Latchman *et al.*, 2004), the IFN- γ Th1 response was not elevated in *L. mexicana*-infected PD-L1^{-/-} mice. There was however a significant reduction in production of the Th2 cytokine IL-4, which could indicate a defect in polarizing cells towards the Th2 phenotype, or a skewing towards a Th1 response. There was no alteration in the Th1/Th2 response in PD-L2^{-/-} infected mice, but there were elevated levels of *L. mexicana* antigen-specific IgM and IgG2a, suggesting a role for this molecule in regulation of B cell responses.

Role in viral immunity

Perhaps the most exciting discovery relating to the PD-1:PD-L pathway in recent times is its involvement in the generation of exhausted T cells during chronic viral infection (Barber *et al.*, 2006). Initial studies showing that PD-1:PD-L1 interactions may be important for controlling anti-viral responses involved adenovirus infection of PD-1^{-/-} mice, with these mice displaying augmented effector T cell proliferation in the liver, and improved virus clearance (Iwai *et al.*, 2003). The demonstration of expanded CD8⁺ T cell populations in the liver of PD-L1^{-/-} mice also implied a role for the PD-1:PD-L1 pathway in antiviral immunity (Dong *et al.*, 2004). This pathway is involved in induction of the T cell anergy that occurs upon

infection with the human rhinovirus (HRV) (Kirchberger *et al.*, 2005). Treatment of DCs with HRV lead to increased PD-L1 and sialoadhesion expression, and HRV-treated DCs were unable to stimulate T cell proliferation. Blocking of PD-L1 and sialoadhesion resulted in restoration of DC-mediated T cell proliferation.

Chronic viral infection or progressive tumours in mice and humans can lead to impairment of effector functions of the responding CD8⁺ T cells. Such T cells are said to become exhausted (Appay *et al.*, 2000; Zajac *et al.*, 1998). These exhausted T cells usually die soon after, and until recently it was unknown whether this exhausted stage could be reversed. Barber and co-workers have shown that blockade of PD-1:PD-L1 interaction in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) was sufficient to enhance T cell responses and restore function to the exhausted CD8⁺ T cells (Barber *et al.*, 2006). Blocking PD-1:PD-L1 interactions during acute infection with Armstrong-LCMV does not affect the number of LCMV-specific CD8⁺ T cells. PD-1 was highly expressed on exhausted T cells in the LCMV clone 13, used in the chronic infection model, but was only transiently expressed on CD8⁺ T cells after infection with Armstrong LCMV. Clone 13 LCMV infects DCs, but the Armstrong LCMV does not, implicating a role for PD-L1-expressing DCs in induction of these exhausted T cells. Various mechanisms on how PD-1 blockade may restore T cell function have been suggested. That blockade of the PD-1:PD-L1 pathway can have a beneficial effect on anti-viral immunity is an important discovery in the field, and will surely play a role in future clinical research into anti-viral therapies.

Positive co-stimulatory function of the PD-1:PD-L pathway

Although a large body of evidence reports that the PD-1 pathway mediates inhibitory responses through its receptors PD-L1 and PD-L2, a number of laboratories have also reported positive co-stimulatory activities for PD-L1 and PD-L2 (Dong *et al.*, 1999; Tseng *et al.*,

2001). It has recently been shown that blockade of PD-L1 on macrophages induces CD4⁺ T cell suppression. Such suppression was principally mediated by increasing IFN- γ -induced nitric oxide production (Yamazaki *et al.*, 2005). This raises the possibility that signaling through PD-1 can mediate positive as well as negative co-stimulatory signals. The ITSM motif is involved in PD-1 mediated activities, as opposed to the ITIM motif more generally associated with co-stimulator molecule inhibition. The ITSM motif on another molecule, CD150, has been shown to bind alternative downstream targets to affect either positive or negative signaling (Sharpe and Freeman, 2002; Shlapatska *et al.*, 2001). PD-1 could also function in this way, which would explain the discrepancies between the positive and negative effects of the pathway (Khoury and Sayegh, 2004). Another mechanism for how PD-L expression on activated DCs induces T cell activation has also been postulated. PD-L expression is lower than other co-stimulatory ligands and MHC class I and II, and the activating stimuli overcome the negative effect of PD-1 (Okazaki and Honjo, 2006).

B7-1 and B7-2 bind to both positive (CD28) and negative (CTLA-4) co-stimulatory receptors, and by analogy to this, an as yet unidentified second receptor for PD-L2 and PD-L2 has also been suggested (Dong *et al.*, 2002). A number of PD-1-independent, co-stimulatory, functions for PD-L1 and PD-L2 have been shown. PD-L2 on DCs can positively co-stimulate PD-1^{-/-} T cells *in vitro*, and synergises with B7-1 and B7-2 to promote T cell proliferation and cytokine production (Shin *et al.*, 2003). Liu and co-workers also suggested a positive role for PD-L2 *in vivo*, by showing that it promoted tumour immunity via a PD-1-independent mechanism (Liu *et al.*, 2003). Studies on PD-L2^{-/-} mice imply an *in vivo* co-stimulatory role for this molecule in selectively enhancing Th1 and cytotoxic T lymphocyte responses (Shin *et al.*, 2005). CD4⁺ T cell IFN- γ production was impaired, as were antigen specific CD8⁺ cells

responses and CTL activity. In other studies, PD-L1 and PD-L2 mutants, which had lost the ability to bind PD-1, could co-stimulate proliferation and cytokine production of T cell from wt and PD-1^{-/-} mice (Wang *et al.*, 2003). So while the inhibitory functions of the PD-1:PD-L pathway appear to act via PD-1, the positive co-stimulatory properties of the PD-L ligands could be mediated via an unknown receptor. Identification of this receptor will further elucidate the mechanisms behind this B7 pathway, and benefit our understanding of the intricacies of immune regulation. An overview of the PD:PD-L pathway, including potential mechanisms for its dual co-stimulatory/inhibitory activity, is outlined in **Figure 1.7**.

1.5.6 T cell activation and co-stimulation in *S. mansoni* infection

The role of T cell activation and co-stimulation during schistosome infection has been addressed in relatively few studies. Hernandez and co-workers demonstrated exacerbated pathology during *S. mansoni* infection of mice deficient in both B7-1 and B7-2 (Hernandez *et al.*, 1999). CD4⁺ Th cells from B71/2^{-/-} mice had markedly reduced proliferation to egg antigen compared to wildtype infected mice, with secretion of only IFN- γ , and not IL-10 or IL-4. Similarly, exacerbated pathology was also observed in mice that had disrupted ICOS-ICOS-L interactions by mAb treatment, with enhanced hepatic immunopathology and elevated levels of CD4⁺ T cell-derived IFN- γ production (Rutitzky *et al.*, 2003). CD40-L (CD154)^{-/-} mice infected with *S. mansoni* have impaired Th2 development and increased morbidity and mortality (MacDonald *et al.*, 2002a), and CD40 has been shown to be essential for induction of the SEA-driven Th2 response in DCs (MacDonald *et al.*, 2002b). Using *S. mansoni* or *Toxoplasma gondii*-infected CD154^{-/-} mice, the same group also demonstrated that CD40/CD40-L interaction is critical for maintaining activation of DC, regardless of whether the outcome is a Th1 or Th2 response (Straw *et al.*, 2003). The inhibitory co-stimulator PD-

L2 was also shown to be upregulated on splenic DCs from *S. mansoni*-infected mice (Straw *et al.*, 2003). Infection of CBA/J mice with *S. mansoni* also lead to increased expression of PD-L2 on DCs, with PD-1 expression on CD4⁺ T cells also being increased (Colley *et al.*, 2005). Colley and co-workers found a correlation with PD-L2⁺ DCs and PD-1⁺ CD4⁺ T cells and morbidity in chronic schistosomiasis.

1.6 Thesis objectives

The introduction highlighted the importance of effector T cells and macrophages in *S. mansoni* infection, and showed that schistosome infection can exert immunomodulatory effects on the host. Regulatory T cells, and the co-stimulatory molecules CTLA-4 and PD-1, with its ligands PD-L1 and PD-L2, have also been highlighted as crucial regulators of immunity. In this thesis I aimed to examine the underlying mechanism of T cell activation during *S. mansoni* infection of mice. Another objective as part of this was to assess the role of regulatory T cells and the CTLA-4 and PD-1 co-stimulatory pathways during *S. mansoni* infection. I had three aims to address these overall objectives.

1. Examine the role of regulatory T cells during *S. mansoni* infection, by assessing regulatory cell markers and function, and using mAb depletion or blocking studies against regulatory cell markers during acute and chronic infection.
2. Investigate the mechanism of T cell activation during schistosome infection, and extend this to examine the levels of expression of co-stimulatory pathway molecules during worm-only and worm-and-egg-infection.
3. Evaluate the role of the PD-1:PD-L1/PD-L2 pathway during *S. mansoni* infection, using mAb blocking studies and also a transgenic (PD-L1 ko) mouse model.

Chapter 2

Materials and Methods

2.1 Mice

Female and male C57/BL6 and BALB/c mice were purchased from Harlan, U.K., and bred in-house. Outbred female CD1 mice from TCD were obtained for egg and worm production. DO.11.10 OVA₃₂₃₋₃₃₉ specific TCR transgenic mice (Murphy *et al.*, 1990), and bicistronic 4get IL-4 reporter mice (4get) (Mohrs *et al.*, 2001), both on a BALB/c genetic background, were purchased from The Jackson Laboratories (USA), and bred in-house. PD-L1^{-/-} mice, on a C57/BL6 background, were obtained from Professor Lieping Chen, (Department of Biology, The John Hopkins University, Baltimore, Maryland, US)(Zhang *et al.*, 2004), and bred in-house. Mice were housed in individually ventilated and filtered cages under positive pressure (Techniplast, UK). Irradiated food and water were supplied. Sentinel mice were screened (Surrey Diagnostics Ltd, UK) to ensure Specific Pathogen Free status.

I am grateful for the assistance of lab members Dr. Padraic Fallon, Dr. Phil Smith and Dr. Niamh Mangan in the procedures involving the requirement for an animal licence.

2.1.1 Genotyping

Tail snips (0.5 - 1 cm) were taken to isolate genomic DNA, and PCR was performed to genotype the mice. DNA was isolated using the Wizard SV Genomic DNA Isolation Kit (Promega). Pieces of tail were cut in half and digested overnight at 55°C in digestion mix containing proteinase K. Undigested tail and hair was spun down and DNA isolated from the supernatant. 5 µl of DNA was used per PCR reaction. Primers and protocols for PCR genotyping of tails are detailed in appendix II.

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). *Biomphalaria glabrata* snails, an albino strain from Puerto Rico, served as intermediate hosts. Female and male BALB/c or CD1 mice, 6-8 weeks, are used as the mammalian hosts.

2.2.1 Snail maintenance and infection

2.2.1.1 Snail Maintenance and breeding

Snails were maintained in plastic containers containing aerated artificial aquarium water (Lepple water; see appendix I). The containers were stored in incubators with a constant temperature of 28°C, and a 12 hour day/night cycle. The snails were fed daily with commercial fish food (Tetrapond). For the purposes of breeding, polystyrene rafts were placed in breeding tanks where the eggs laid by the hermaphrodite snail attach to the underside of the rafts. These rafts were then removed to separate tanks for hatching.

2.2.1.2 Snail Infection

The liver of an infected mouse was removed approximately 49 days post infection, and homogenized in a Waring blender in double strength (2x) saline. This gently disrupts the tissue surrounding the egg, while the use of 2x-saline serves to prevent the eggs from hatching. Lepple water was added to the resulting solution to start eggs hatching into miracidia. The solution was transferred to a conical flask wrapped in aluminium foil. A lamp was shone onto the neck of the flask for 30 minutes. The miracidia that hatch from the eggs are phototropic and swim to the top towards the light source. The number of miracidia was

assessed by collecting a sample from the top of the solution and counting them under the microscope. For snail infections, individual snails were placed in 1 ml Lepple water in a 24-well plate and were exposed to 4-6 miracidia for 6 hours. They were then placed back into fresh containers in a dark incubator for 21 days, to allow asexual multiplication and development of the redia. Snails were checked for infection after 28 days, where individuals were transferred into the wells of a 24 well plate in 1ml Lepple water and placed under a lamp for 90 minutes. Infected snails shedding cercariae were visualized under a microscope. Cercariae were counted by obtaining ten 10 μ l samples per well, and staining with Lugol's iodine, which killed the cercariae and allowed easier visualization under the microscope.

2.2.1.3 *Unisexual (male only) infection*

Snails were exposed to a single miracidium for a unisexual snail infection. After 28 days snails were checked for infection as described above. Snails shedding only male cercariae were required. As there are no visual differences to distinguish between male and female cercariae, a PCR-based technique modified from a method by Grevelding was implemented (Grevelding, 1999). Infected snails were colour-coded for identification and cercariae from each individual snail were collected. Cercariae genomic DNA was isolated using the Wizard SV isolation kit (Promega), and PCR was carried out to determine the presence or absence of female specific sequences, termed W1 and W2. Primers for protein disulphide isomerase (PDI), present in both males and females, were used as an internal control (see appendix for primer sequences). Figure 2.1 is a gel showing the PCR products. Snails shedding male-only cercariae were then used to establish a male worm-only mouse infection.

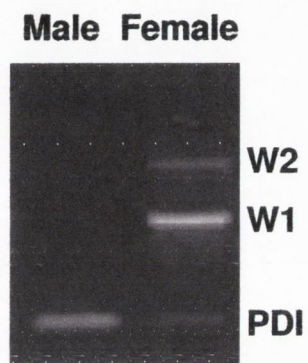


Figure 2.1. *Sexing of cercariae by PCR.* Snails infected by a single miracidium were shed, and cercariae DNA isolated. Sequences from female-specific W1 and W2 were amplified by PCR, with PDI as an internal control.

2.2.2 Infection of mice

2.2.2.1 *Experimental mouse infection*

Mice were infected percutaneously (pc) with cercariae, 250-350 for egg and worm infections and 35-100 for experimental unisexual (male worm only) or bisexual (worm and egg) infections. 6-8 week old mice were anaesthetized with sodium pentobarbitone (Sagatal; 6 mg/kg). The abdomen was shaved and swabbed with cotton wool moistened with Lepple water. Mice were laid on a special mat, a metal ring was placed on the abdomen, and each was secured down with tape. Cercariae, in 400µl lepple water, were pipetted onto the ring using a cut tip, and mice were left for 25 minutes. Mice were then allowed recover on a heating mat before being returned to their cage.

2.2.2.2 *Infection of mice for antigen production and maintenance of life cycle*

Female outbred CD1 mice were infected with 250-350 cercariae pc. A hydrocortisone mixture (see appendix I) was administered subcutaneously to the mice 35 days post-infection. This serves to suppress the host's immune response to the parasite egg and reduce inflammation in the granuloma. At 49 days post-infection the mice were sacrificed by ip injection of 200 µl sodium pentobarbitone (60 mg/ml) with heparin (10U/ml) added for perfusion of worms. The hepatic portal vein was cut and worms were collected upon portal perfusion of the mice with perfusion media (see appendix I). Eggs were harvested from the liver and intestines of the infected animal.

2.2.3 *Schistosoma mansoni* antigen production

2.2.3.1 Isolation of eggs and soluble egg antigen (SEA) production

Eggs were isolated from tissue as described (Fallon *et al.*, 1998). Infected livers and guts were taken and placed into separate containers of 2x-saline. 0.05 g trypsin (Sigma) was added per liver or gut, before homogenization in a blender. The homogenate was incubated at 37°C on a shaker for 4 hours. Eggs were then recovered from the homogenate by filtering through a series of sieves (pore sizes 500 µm- 150 µm). They were then pelleted at 2000 rpm for 2 minutes at 4°C. This was resuspended at in 2x-saline and spun up to 1500 rpm. Eggs were further washed through two smaller sieves (45 µm and 32 µm sizes). The mature/live eggs were caught in the 32 µm sieve. Eggs were frozen in liquid nitrogen until use.

Liver eggs were used for preparation of SEA. They were washed several times in endotoxin-free Dulbecco's PBS (DPBS, Sigma), before being homogenized with ice cold PBS using a glass homogenizer with a Teflon plunger. The eggs and the glass homogenizer were kept on ice at all times. Samples were examined periodically under a microscope until there were no intact eggs left. The homogenized eggs were then spun at 10,000g for 60 minutes at 4°C. The supernatant was harvested and re-spun for 15 minutes at 4°C. The resulting supernatant was filtered through a 0.45 and 0.22 micron syringe filter before being stored at -20°C. Protein content was determined using the BCA assay (section 2.10.5). Endotoxin levels were tested (section 2.11) and SEA was only used if it contained < 1 EU per mg of protein.

2.2.3.2 Adult worm (AW) production

Worms collected from an infected animal were washed and pelleted before freezing in liquid nitrogen. The frozen pellets were crushed under liquid nitrogen using a pre-cooled mortar and

pestle. When a fine paste had formed, it was left to thaw. The mortar and pestle were rinsed with DPBS and the worm paste and washes were spun at 10,000 rpm for 60 minutes. The supernatant was collected and was spun again several times until no further pellets remained. The soluble adult worm antigen contained in the supernatant was filtered through a 0.45 and 0.22 micron filter and frozen at -20°C . Protein content was determined using the BCA assay (section 2.10.5). Endotoxin levels were tested (section 2.11) and AW was only used if it contained < 1 EU per mg of protein.

2.2.4 Tissue Egg Counts and parasite fecundity

Portal perfusion for worm recovery, liver, intestinal and fecal egg counts were as described (Fallon and Dunne, 1999). Frozen liver and guts from infected animals were thawed and livers were weighed. Tissue was digested overnight at 37°C on a shaker in 20 mls 4% potassium hydroxide. Three 50 μl aliquots from each sample were taken. They were placed on a slide and covered with a coverslip. Eggs were viewed microscopically and counted. An average of the three aliquots was taken. If variation between aliquots was more than 10%, egg numbers from a fourth sample were counted. Tissue egg counts were expressed as total eggs per gut or per liver. Parasite fecundity was determined by dividing total tissue (liver and intestine) egg counts by the number of worm pairs present.

2.2.5 Histology and sectioning

Livers and intestine from mice were taken and fixed in formal saline (see appendix I) for histology. Samples were sent to Cambridge University, UK, or Dept. Physiology, TCD, where they were paraffin-embedded for sectioning. Sections of liver were stained with hematoxylin and eosin for egg granuloma diameter measurements, Giemsa-stained for

eosinophil quantification and Martius Scarlet Blue stained for visualization of collagen deposition. Hepatic fibrosis was measured by quantification of hydroxyproline (Bergman and Locksley, 1963). Hepatic hydroxyproline was measured in weighed samples of livers from acutely- or chronically-infected mice and also from uninfected age- and sex-matched mice. Hepatic collagen is expressed as the increase in infected mice above levels in age-sex matched uninfected mice adjusted to μg collagen per 10,000 liver eggs, as described (Kaviratne *et al.*, 2004). Collagen quantification was performed by Dr. Philip Smith.

2.2.6 AST and ALT quantification

To quantify the extent of liver damage, plasma was recovered for analysis of aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) levels, as described (Fallon and Dunne, 1999; Fallon *et al.*, 2001). This was performed by Dr. Philip Smith.

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 Hybridoma culturing and antibody production

Anti-CD25 (clone PC61 5.3), anti-CTLA-4 (clone UC10-4F10-11), anti-B220 (clone TB146), murine IgG1 (clone B7.11) raised against 2,4,6-trinitrophenyl (TNP), anti-F4/80 (clone F4/80), and hamster IgG (clone UC8-1B9) hybridomas were purchased from American Type Culture Collection (ATCC). The anti-CD4 (YTS191), anti-CD8 (YTS169) and anti-Thy-1 (YTS148.3.2) hybridomas, originally from Prof. Herman Waldmann (University of Oxford,

UK), was provided by Prof. Anne Cooke (University of Cambridge, UK). Control Rat IgG was from Sigma.

Hybridoma cell lines were cultured in RPMI-1640 containing 10% FCS/pen-strep/l-glut, with routine passages, when confluent, every 3-5 days. Cells were maintained in T-175 flasks (Greiner) or roller bottles (Costar). Supernatants were harvested and frozen at -20°C until further purification. Thawed supernatant was precipitated in 50% ammonium sulphate (Sigma), with constant stirring overnight at 4°C. The resulting mixture was centrifuged at 7000 rpm for 30 minutes at 4°C in a Sorvall centrifuge. The pellets were re-suspended in sterile endotoxin-free Dulbecco's PBS (pH 7.2; Sigma), 100 mls per litre supernatant. The suspension was dialysed at 4°C over 48 hours with three changes of 20 volumes of DPBS (Dialysis tubing with molecular weight cut-off at 12,400 kDa; Sigma). The suspension was then added to a protein G column (Sigma). Absorbance was monitored using a Biologic LP (Bio Rad). Antibody was eluted with 100 mM glycine (pH 2.5), and solution was neutralized to pH 7 by addition of Tris base (Sigma) before further dialysis against DPBS using dialysis cassettes (Pierce). Protein was quantified using a BCA assay (section 2.10.5). Endotoxin levels were tested by LAL assay (section 2.11) and confirmed to have levels of <0.5 EU/mg (Chromogenic LAL, Biowhittaker, MD). Antibodies were run down an SDS-PAGE gel (section 2.10.6) to test for purity. Functionality of the antibody was tested by flow cytometry or by efficacy in mediating specific *in vivo* depletion. 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.

2.3.2 *In vivo* blocking and depletions

BALB/c mice were infected with 35 (chronic)-100 (acute) mixed cercs pc to obtain an *S. mansoni* infection. 4 or 12 weeks post-infection, mice were administered with 100-250 µg of

blocking or depleting antibody, or a control antibody. Dose was optimized from published papers and initial efficacy studies in the lab. This was repeated three times weekly for the following four weeks, and mice were weighed each time. The treatment regime for *in vivo* mAb studies is outlined in Figure 2.2. Differences in weight loss or gain were noted and mice were sacrificed 7-8 or 16 weeks post-infection for immunological and histological examination. Efficient blocking or depletion was verified by flow cytometry.

2.4 Immunology

2.4.1 Sample Processing

Mice were sacrificed and spleen, mesenteric lymph nodes (MLN) or tissue were aseptically removed. Spleens and MLN were passed through 70 μm sieves to obtain single cell suspensions. Cells were spun and pellet re-suspended in 0.87% ammonium chloride for 3 minutes at 37°C. This lysed the red blood cells. Fat clumps were removed and cells were washed twice in wash media before counting. Cell viability was determined by staining with trypan blue (Sigma). Cells were resuspended at the appropriate concentrations in complete media: Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco BRL; endotoxin levels were <0.04 EU/ml protein) supplemented with 10% (v/v) heat-inactivated (56°C for 30 minutes) foetal calf serum (FCS) (Labtech, UK; endotoxin levels were <0.1 EU/ml protein), 100 mM L-glutamine, 100U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma).

Throughout the experiments samples were preferentially processed on individual mice, but with logistics and time this was not always feasible, and so pools of mice were sometimes used.

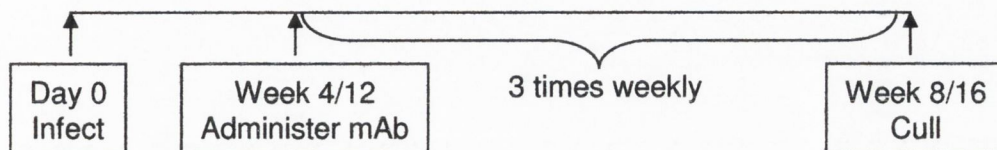


Figure 2.2. *Treatment regime for the administration of control mAb or depleting mAb against CD25, and blocking mAbs against CTLA-4, PD-1, PD-L1 and PD-L2. BALB/c or 4get mice were infected with *S. mansoni* cercariae on day 0, and mAbs were administered three times a week from week 4 until week 8 or week 12 until week 16 post-infection.*

2.4.2 Isolation of sera from blood

For isolation of sera from blood, mice were exsanguinated by cardiac puncture. Blood was allowed to clot at room temperature for a few hours. Samples were placed at 4°C overnight to allow contraction of clots. A small amount (~100 µl) of Sera Sieve (Hughes and Hughes Ltd., Wellington) was added to clotted blood and samples were centrifuged at 5000 rpm for 10 mins at 4°C. Sera was then used immediately for detection of antibodies by ELISA, or frozen at -20°C until further use.

2.4.3 Isolation of liver granuloma cells

Liver granuloma cells were isolated as described previously (Fallon and Dunne, 1999). Briefly, livers from infected mice were perfused with perfusion fluid *in situ*. They were then taken and homogenized briefly using a Waring blender. Livers were washed rigorously in wash media before collagenase digestion (collagenase D, Roche) for 1 hour at 37°C. The homogenate was passed through a 5 ml syringe several times to disrupt the granulomas, then passed over 100 µm and 70 µm sieves (Falcon) to suspend the granuloma cells.

2.4.4 Preparation of accessory cells

Splenic accessory cells (AC) were prepared by depletion of B (B220⁺) and T (Thy-1⁺) cells with two rounds of complement-mediated lysis (Lo-Tox rabbit complement; Cedarlane Laboratories, Hornby, Canada) using anti-Thy-1 mAb (YTS148) and anti-B220 mAb (TB146). Briefly, spleen cells were incubated with mAb (50 µg/ml) on ice for 1 hour, washed and incubated for 1 hour at 37°C with complement. AC preparations were shown to contain

<1% T or B cells by flow cytometry analysis. AC were irradiated at 30 Grays using a gamma irradiator (Gamma Cell 3000, Nordion International Inc.) before use.

2.4.5 Depletion of CD4⁺, CD8⁺, or B220⁺ cells for *in vitro* culture

Spleen cells were depleted of CD4⁺, CD8⁺, or B220⁺ cells using the complement-depletion mediated method outlined above (section 2.4.4). Efficacy of depletion is shown in Figure 2.3.

2.4.6 Isolation of CD4⁺CD25⁺ cells

Splenic CD4⁺CD25⁺ cells were isolated using the CD4⁺CD25⁺ T regulatory cell isolation kit (Miltenyi Biotec, UK), according to the manufacturers instructions. Briefly, spleen cells were incubated with a cocktail of non-T cell biotinylated mAbs, and also with anti-CD25 PE mAb. Anti-biotin microbeads were added and T cells were enriched by depletion using a depletion programme on the autoMACS separator (Miltenyi Biotec). These cells were then incubated with anti-PE micro beads and CD4⁺CD25⁺ cells were purified using a positive selection programme on the autoMACS separator. CD4⁺CD25⁺ cells were shown to be 92-95% pure by flow cytometry (Figure 2.4). For proliferation assays, CD4⁺CD25⁻ responder cells were prepared by taking the negative fraction from the CD25⁺ selection and putting them through a CD4⁺ enrichment column (R&D Systems).

2.4.7 Isolation of F4/80⁺ splenic macrophages

A method for the isolation of F4/80⁺ splenic macrophages (M ϕ) was optimized. Spleens were processed as outlined above, and re-suspended in 1%FCS/HBSS. Cells were incubated with 5 μ g/ml rat anti-mouse F4/80 (Serotec) for 30 minutes at 4°C, rocking gently. After three

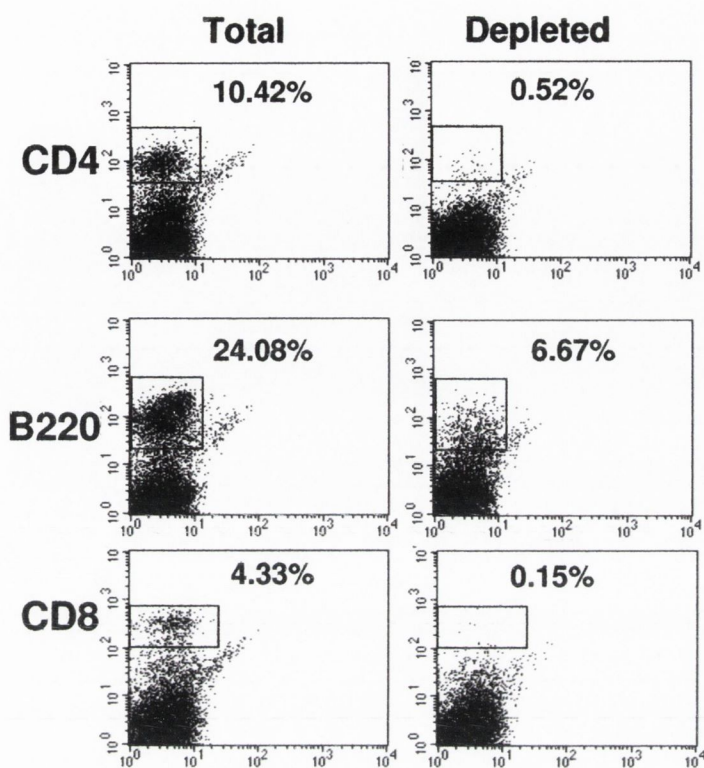
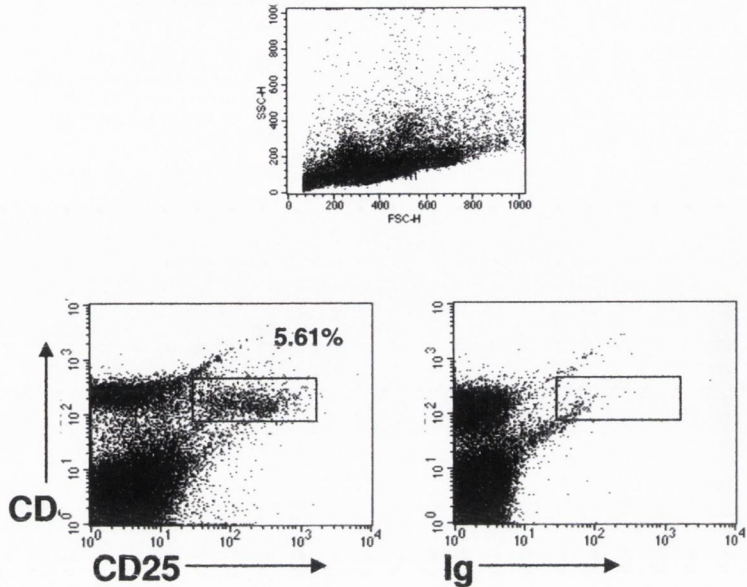


Figure 2.3. *Efficacy of in vitro depletion of CD4⁺, CD8⁺, and B220⁺ spleen cells.* Spleen cells from infected mice were isolated and CD4⁺, CD8⁺, and B220⁺ cells were depleted as described in section 2.4.6. Cells were stained for CD4, CD8, or B220 and graphs plotted on logarithmic scales using Cellquest™ software.

A: Whole spleen cells



B: After AUTOMACS isolation

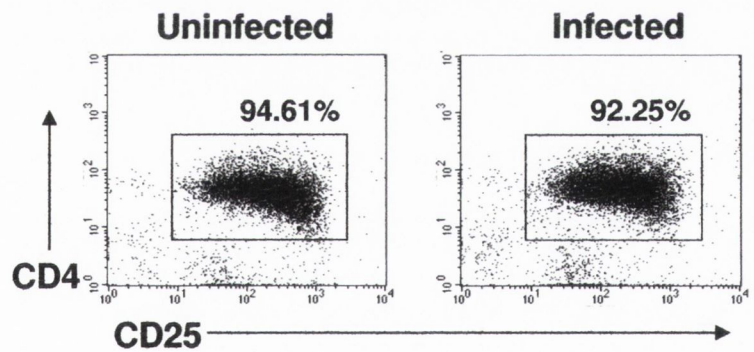


Figure 2.4. Purity of CD4⁺CD25⁺ cell isolation from spleen cells using AUTOMACS. FACS profile of A, whole spleen cells or B, purified CD4⁺CD25⁺ cells from uninfected and *S. mansoni*-infected spleens. Cells were purified using the AUTOMACS CD4⁺CD25⁺ regulatory T cell isolation kit, and stained for CD4.

washes in HBSS, washed sheep anti-rat IgG Dynabeads (DynaL Biotech, Great Neck, NY) were added to the cells (5 μ l per 4×10^7 splenocytes). Beads were incubated with the cells for 20 minutes at 4°C on a rocker. The cells were then placed on a Dynarack (DynaL Biotech, Great Neck, NY), where beads bound magnetically. Multiple rounds of this magnetic separation were performed. Purity was assessed by flow cytometry, with <1% each of contaminating CD11c⁺, CD19⁺, CD4⁺, or CD8⁺ cells. If the F4/80⁺ M ϕ were to be used as accessory cells, they were irradiated.

2.4.8 *In vitro* activation of cells for cytokine production

Spleens and MLNs were processed as outlined above, and re-suspended at 1×10^7 /ml. Cells were seeded in a 24 well plate (Greiner) at 5×10^6 /well, and cultured with various stimulants for 24 and 72 hours. Stimulants were as follows: soluble anti-CD3 mAb (0.5 μ g/ml), AW (20 μ g/ml), and SEA (20 μ g/ml). Supernatants were harvested after 24 or 72 hours and cytokines in supernatants were analysed using conventional sandwich ELISAs (section 2.9).

2.4.9 *In vitro* modulation of cells by live worms

As described in (Smith *et al.*, 2004), live worms were collected upon perfusion of a heavily infected mouse (250-350 cercariae), described earlier (section 2.2). Male worms were separated from the females, and mature, intact males were counted before being washed several times in sterile RPMI (Gibco) containing 1 % penicillin/streptomycin (pen-strep). They were then washed 4 times in complete RPMI (see appendix). Spleen cells (1×10^7 /well) were seeded in a 12-well Transwell culture plate (Costar, Cambridge, MA) in 2 ml complete RPMI with Hepes (25mM). 12 male worms were placed in each transwell insert. The

cells/worms were incubated at 37°C and 5% CO₂ for 48 hours. The worms were still alive at this point, as they were observed to be still moving. Worms were removed, and cells harvested and washed before being used for flow cytometry and proliferation.

2.5 *In vitro* cell proliferation assays

Spleen or MLN (2×10^5 /well) cells were cultured in 96-well U-bottomed plates for 72 h at 37°C. Cells were activated with 0.5 µg/ml anti-CD3 mAb alone or with IL-2 (20 ng/ml) or anti-CD28 mAb (4 µg/ml). Antigen-specific stimuli were AW (20 µg/ml), or SEA (20 µg/ml). In co-culture proliferation assays, accessory cells (AC) or F4/80⁺ Mφ were added to enriched CD4⁺ or CD8⁺ cells at a range of effector/responder ratios. CD4⁺ and CD8⁺ spleen cells were isolated using enrichment columns (R&D Systems), according to manufacturer's instructions.

For CD4⁺CD25⁺ proliferation assays, CD4⁺CD25⁺ cells were added to CD4⁺CD25⁻ responders at a range of effector/responder ratios, and irradiated AC were also added. All samples were set up in triplicate wells. Cultures were pulsed with 1 µCi/well [³H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) for the last 14 hours of culture. Cells were harvested using a cell harvester (TOMTEC) and [³H]thymidine incorporation was determined using a Microbeta liquid scintillation system (1450 Microbeta plus liquid scintillator counter; Wallac).

2.6 *In vitro* proliferation studies: CFSE staining

Spleen cells (1×10^7 /ml) were labeled with 0.5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) and unstimulated or stimulated with 0.5 µg/ml anti-CD3 mAb for 24-72 h. Spleen cells were washed twice in PBS and then stained in the dark at 37°C for

15 mins with pre-warmed 0.5 μ M CFSE in PBS. Cells were washed twice in ice-cold complete medium, and once in room-temperature medium. They were then counted and set up for in vitro culture, either unstimulated (media), or with 0.5 μ g/ml anti-CD3 mAb. CFSE fluorescence staining was detected using flow cytometry. Cells were stained for CD4 or CD8 using TRI-coloured antibodies, propidium iodide (PI) was detected in the FL2 channel, and CFSE was detected in the FL1 channel.

2.7 *In vivo* proliferation studies: Bromodeoxyuridine labeling

Bromodeoxyuridine (BrdU) is a uridine derivative that can be incorporated into DNA in place of thymidine. BrdU (Sigma) can be administered to mice and those cells that are synthesizing DNA (i.e proliferating cells) will incorporate BrdU into the DNA. Anti-BrdU FITC (BD) is used to identify BrdU in single stranded DNA. BrdU staining was performed as described (Humphreys *et al.*, 2003), with several in-house modifications.

Uninfected, worm, and worm and egg-infected OVA-TCR mice were given 1 mg OVA per mouse ip. 36 hours later mice were given 2 mg BrdU ip. Mice were sacrificed 12 hours later and spleens processed and stained with anti-TCR clonotypic mAb KJ126 PE (BD) and anti-BrdU FITC (BD). Firstly, cells were stained with anti-KJ126 PE. After washing, cells were resuspended in 500 μ l ice-cold 0.15M NaCl. 1.2 mls ice-cold 95% ethanol was added dropwise while mixing gently. Cells were incubated in the dark for 30 mins at 4°C, then washed in cold PBS and fixed for 30 mins in 100 μ l fixation buffer (BD) at RT. Cells were spun down before a ten minute incubation in 50 U DNase 1 (Roche) in 4.2 mM MgCl₂, 0.15M NaCl at RT. Cells were centrifuged and incubated in the dark with FITC anti-BrdU (BD) in FACS

buffer for 30 minutes at RT. Cells were washed three times in FACS buffer and analysed on a FACScan™ flow cytometer (Becton-Dickinson, San Jose, CA).

2.8 Flow cytometry analysis

2.8.1 Surface marker staining

Immunofluorescent staining of splenic, peritoneal, and lymph nodes populations was carried out using a variety of TRI-, FITC-, or PE-conjugated mAbs (BD Pharmingen/Caltag), as listed in appendix II. Surface marker expression of cells was assessed by flow cytometry using a FACScan™ flow cytometer (Becton-Dickinson, San Jose, CA) using a previously described method (Fallon *et al.*, 1998). Cells were plated on a 96-well U-bottomed plate (2-10 x 10⁵/well) and washed in FACS buffer (PBS with 2% FCS and 0.05% sodium azide). The cells were then stained with surface antibodies for 30 minutes on ice, followed by three washes in FACS buffer. Appropriate isotype controls were used at each timepoint in all experiments, and titrations of antibodies were initially performed to determine recommended concentrations for use (see Appendix).

2.8.2 Intracellular cytokine detection

Intracellular staining was performed similar to a previously described method (Fallon *et al.*, 1998). Cells (1 x10⁷/ml) were unstimulated or stimulated *in vitro* for 6 hours with Concanavalin A (1 µg/ml; Sigma). Brefeldin A (10 µg/ml; Sigma) was added for the final four hours of culture. Cells were harvested with a transfer pipette and washed 3 times with FACS buffer. Cells were surface stained as described in section 2.8.1 above. After surface staining, the cells were washed three times in FACS buffer and fixed at room temperature for 15 mins using fixation buffer from a Fix and Perm^R Cell Permeabilisation Kit (Caltag

Laboratories). The cells were again washed three times before permeabilisation using the Fix and Perm^R Cell Permeabilisation Kit. Antibodies to the cytokines were added at the permeabilisation step at a 1:10 dilution for 15 mins at room temperature. Cells were washed three times in FACS buffer before being analysed on the flow cytometer. Data were analysed using CellQuestTM software (version 3.13; Immunocytometry Systems). Quadrants were gated according to appropriate isotype controls, as shown in Figure 2.5.

2.8.3 Other intracellular staining

Intracellular staining for CTLA-4 was performed using the Fix and Perm^R Cell Permeabilisation Kit, according to the manufacturers instructions. Foxp3 intracellular staining was performed using a Foxp3 Staining Kit (eBiosciences), according to manufacturers instructions.

2.8.4 4-colour immunofluorescence staining

APC-conjugated and APC-Alexaflour-conjugated mAbs were used in addition to PE- and FITC-conjugated mAbs for 4-colour staining. Analysis was performed in the Conway Institute (University College Dublin, Ireland) using a DAKO Cyan cytometer and SummitTM software. For all flow cytometry work, quadrants and gates were drawn based on appropriate isotype-control Ig staining and were plotted on logarithmic scales.

2.8.5 Annexin V staining

Annexin V and propidium iodide (PI) staining was performed using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen), according to manufacturers instructions. Cells were initially stained for CD4 or CD8 using TRI-conjugated antibodies. Briefly, cells were

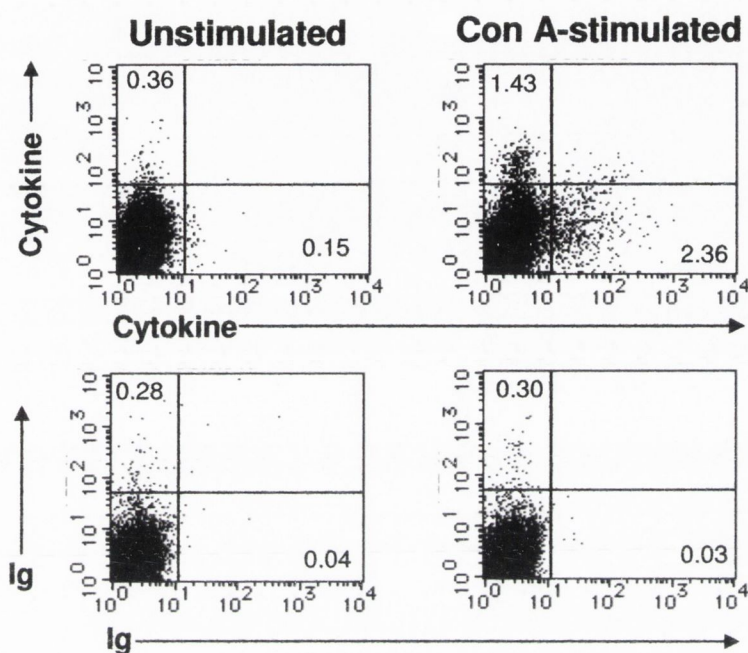


Figure 2.5. *Isotype gating for intracellular staining for cytokine production.* Brefeldin A was added to unstimulated or Con A-stimulated cells for the last 4 h of culture. Cells were stained for appropriate surface marker expression before intracellular (IL-4/IFN- γ) staining was performed. Appropriate isotypes for the intracellular markers were used, and quadrants were gated according to these isotype controls.

washed twice in cold PBS then resuspended in 1X binding buffer. Annexin V-FITC and PI were added and mixed gently before incubation in the dark for 15 mins at room temperature. More binding buffer was added, and cells were analysed immediately by flow cytometry.

2.9 Enzyme Linked Immunosorbent Assay (ELISA)

Cytokine levels in cell culture supernatants were quantified using sandwich ELISAs. Antibodies to mouse IL-2, IL-4, IL-5 and IL-6 were obtained from BD Pharmingen, UK. IL-1 β , IL-13, IFN- γ , IL-10 and TNF- α DuoSet kits were purchased from R&D Systems, UK. Checkerboards of coating and detecting antibodies were performed to determine optimum concentrations. Capture antibodies (0.1-2 μ g/ml) were coated on high-binding 96-well plates (Greiner) in coating buffer (PBS), 50 μ l/well, and left overnight at 4°C in a wet-box. Plates were washed three times in ELISA wash buffer (PBS/T) and blotted dry with tissue paper. Plates were blocked in blocking buffer (1% BSA/PBS), 200 μ l/well for 2 hours at room temperature. Plates were then washed three times in PBS/T and blotted dry before addition of samples, 50 μ l/well, to duplicate wells. A two-fold serial dilution of cytokine standards was added in duplicate to the wells, with the remaining two wells left blank (PBS), on each plate. Plates were left at overnight at 4°C in a wet-box. The plates were washed four times the following morning and blotted dry. Total IgE in sera samples was also measured using this ELISA protocol. An IgE standard was used (BD), and sera samples were diluted 1:25 or 1:50 before being added to the plate. Biotinylated detecting antibodies (0.1-2 μ g/ml) were added in PBS, 50 μ l/well, for two hours at room temperature. After 5 washes horseradish peroxidase-conjugated streptavidin (BD Pharmingen, UK) was added to the wells at 1:1000 dilution, 50 μ l/well for 30 minutes at room temperature. o-phenylenediamine (OPD; Sigma) was used as

substrate to develop the reaction. In a darkened 50 ml Falcon tube a 5 mg tablet of OPD was dissolved in 12.5 mls phosphate citrate buffer, pH 5.0, and 9 μ l of 30% hydrogen peroxide was then added. After strep-HRP step, plates were washed 5 times and 50 μ l substrate/well was added. The plates were incubated in the dark for 10 minutes. The reaction was stopped by adding 1M H_2SO_4 , 25 μ l/well. Absorbance at wavelength 492 nm was read using a microplate reader (Versa_{Max} Tunable Microplate Reader, Molecular Devices, CA). Cytokine levels in the samples were quantified by extrapolation from the standard curve using Soft Max Pro 3.0 software.

2.10 Molecular and Biochemical Techniques

2.10.1 RNA isolation

RNA was isolated using TRI Reagent (Sigma). 1 mL TRI Reagent was added to $5-10 \times 10^6$ 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 (GmbH & Co., Germany). Samples were left to stand for 5 mins at room temperature. 0.2 ml chloroform per mL of TRI Reagent was added. Samples were covered tightly, shaken vigorously for 15 seconds and allowed to stand for 15 mins at room temperature. The mixture was centrifuged at 12,000g for 15 mins at 4°C. The upper aqueous phase (containing RNA) was transferred to a fresh centrifuge tube and 0.5 ml isopropanol added. Sample was left to stand for 10 mins at RT, before spinning at 12,000g for 10 mins at 4°C. The resulting RNA pellet was washed by adding 1 ml of 75% ethanol. Samples were vortexed and then centrifuged at 7,500g for 5 mins at 4°C. Supernatant was removed and RNA pellets left for 10-15 mins to air-dry, before resuspension in 50 μ l of RNase-free H_2O (Sigma).

2.10.2 Co mplementary 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), 1mM dioxynucleotide triphosphates (dNTP's), 10mM 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 under the following conditions:

1. Binding at 25°C 10 minutes
2. Extension at 42°C 60 minutes
3. Enzyme Deactivation at 95°C 5 minutes
4. Cooling at 4°C

2.10.3 Polymerase Chain Reaction (PCR)

Reverse-transcription PCR (RT-PCR) was used to amplify cDNA. A cocktail was prepared with a final concentration of 1 X reaction buffer, 0.25 mM dNTPs, 2.5 mM MgCl₂, 1 U Taq Polymerase (all Promega), and 10 pmol forward and reverse primer. 1 µl of cDNA was placed in a thin-walled PCR tube (Eppendorf) and cocktail added to each tube. RNase-free water was added to a total volume of 25 µl. Tubes were placed in a thermocycler (DYAD™) and PCR cycles started. Cycle number and annealing temperature varied depending on primer pairs, but in general PCR reaction was as follows:

1. Denaturing: 95°C 5 minutes
2. Denaturing: 95°C 45 seconds

cd

3. Annealing: 54°C-67°C 45 seconds
4. Extension: 72°C 45 seconds
5. Go to 2 for 25-35 cycles
6. Extension: 72°C 10 minutes
7. Cooling: 4°C

2.10.4 Agarose gel electrophoresis

6 X loading dye (Promega) was added to PCR products and 10 µl of each was run on a 1.2% agarose gel (1.2 g agarose in Tris-borate-EDTA (TBE), see appendix) containing 5 µl ethidium bromide (EtBr). Samples were electrophoresed in 1 X TBE buffer at 100 volts. DNA bands on the gel were visualized under ultraviolet light and photographed using a Syngene digital graphic printer.

2.10.5 Protein Estimation

Protein concentrations were determined using the BCA Protein Assay Reagent Kit (Pierce) according to manufacturer's instructions. 10 µl samples were added in triplicate to wells of a 96-well plate. Standards of bovine serum albumin (BSA) were prepared ranging from 2000 µg/ml up to 125 µg/ml. 200 µl of working reagent was added to each well and the plate was incubated at 37°C for 30 minutes. Absorbance was measured at 562 nm, and the concentration of protein in supernatants or tissue samples could be determined from the standard curve.

2.10.6 SDS -PAGE and Western Blot

A 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was cast on a BioRad casting cassette. Casting gels were prepared by mixing 4 ml protogel, 2.6 ml

protogel buffer (BioRad), 3.3 ml dH₂O, 100 µl of 10% ammonium persulphate (APS), and 20 µl TEMED (Sigma). A 4% stacking gel was made with 1.3 ml protogel, 2.5 ml protogel stacking buffer, 6.1 ml dH₂O, 20 µl 10% APS, and 5 µl TEMED, and BioRad combs inserted. Samples were diluted 1:2 in loading buffer containing 5% β-mercaptoethanol (see appendix I) and boiled for 5 minutes before being loaded. The cassette was placed in electrophoresis chamber and run at 200 V for 45 minutes in 1 x electrophoresis buffer (see appendix I).

Proteins on the gel were visualized using silver stain, or were transferred to a nitrocellulose membrane (Pall Corporation) for western blot. The gel was transferred to a nitrocellulose membrane in a tank with transfer buffer (see appendix) for 1 hour at 400 mA. Membrane was blocked in 5 % dry milk/PBST for 1 hour at room temperature. Primary antibody was added (1:1000) in 3 % dry milk/PBST for 1 hour at rt, or overnight at 4°C. Membrane was washed 5 x 5 mins in PBS/T. Secondary antibody (peroxidase-conjugated) was added (1:1000) in 3 % dry milk/PBST for 1 h rt, or o/n at 4°C. Membrane was washed 5 x 5 mins in PBS/T. 2 mls of 1:1 ECL reagent (Amersham) was added to the membrane for 1 min before membrane was drained and placed in a Hyper cassette (Amersham). Hyperfilm (Amersham) was placed in the cassette and exposed for varying times (30 sec- 5 mins) and blot developed using a developer machine.

Silver staining of the SDS-PAGE gel was performed as follows:

The gel was pre-fixed in 50% methanol/10% acetic acid (BDH) for 30 mins followed by 5% methanol/7% acetic acid for 30 mins. It was then fixed in 10% glutaraldehyde (Sigma) for 30 mins. The gel was rinsed several times in distilled H₂O for 1 hour. It was soaked in 5 µg/ml DTT (Sigma) for 30 mins, then 30 mins in 0.1% silver nitrate (Sigma). It was rinsed once in distilled H₂O, then twice in developer (0.019% formaldehyde (Sigma) and 3% sodium

carbonate (Sigma)), before soaking in developer for 30 mins. Proteins were visualized on the gel using a light box. Reaction was stopped by addition of acetic acid, and gel was washed in distilled H₂O.

2.11 LPS level determination

The level of lipopolysaccharide (LPS) in purified antibodies or antigen preparations was assessed using the *Limulus ameobocyte* lysate (LAL) assay (Biowhittaker, UK), according to manufacturer's instructions. A simple equation then gave endotoxin amounts in EU/ml. Samples with endotoxin levels > 1EU/mg were not used.

Endotoxin-free reagents were used in all antigen and antibody preparations. Sterile plastics were used wherever possible. Glassware and equipment were decontaminated by washing with 0.5M NaOH for 30 minutes.

2.12 Statistical analysis

GraphPad Prism and GraphPad Instat software was used to analyse the data. Unpaired or paired Student's t-test was used to determine statistical significance between groups. Differences were considered significant when $P < 0.05$. Data analysed was from 3-6 individual mice, or a mixture of individual numbers and numbers from pools of mice from different experiments.

Chapter 3

Role of selected regulatory T cell markers in S. mansoni infection

3.1 Introduction

A central cause of the immunopathology during infection with the helminth parasite *Schistosoma mansoni* is the granulomatous inflammation evoked by the parasite's eggs that become trapped in various tissues (Wynn *et al.*, 2004). An obligate role for T cells in the formation of the schistosome granuloma was initially shown using various mice with T cell deficiencies (Amiri *et al.*, 1992; Byram and von Lichtenberg, 1977; Doenhoff *et al.*, 1979). More recent studies have demonstrated granuloma formation around the schistosome egg is dependent on CD4⁺, and not CD8⁺, T cells (Fallon *et al.*, 2000; Hernandez *et al.*, 1997; Mathew and Boros, 1986; Yap *et al.*, 1997).

There is accumulating evidence for various subsets of CD4⁺ T cells that functionally limit the immune response and have a maintenance role in preventing autoimmune disease. Although CD4⁺ cells are known to be essential to schistosome egg granuloma formation, the role of distinct CD4⁺ regulatory subsets in schistosome infection has not been fully addressed. As discussed in chapter 1, natural T_{reg} cells were initially characterized by surface expression of the IL-2 α receptor, CD25, which is also on activated CD4⁺ cells. Natural CD4⁺CD25⁺ T_{reg} cells have been shown to be expanded by various pathogens and are implicated in limited immunopathology during pathogen infection (Belkaid and Rouse, 2005). A recent study showed that CD4⁺CD25⁺ T cells have an important role in schistosome infection as they are a source for IL-10 (Hesse *et al.*, 2004), which is a significant finding as IL-10 has an important role in prevention of pathology during schistosome infection of mice (Flores Villanueva *et al.*, 1993; Hoffmann *et al.*, 2000; Wynn *et al.*, 1998). In a separate study it was also demonstrated that CD4⁺CD25⁺ regulatory cells from *S. mansoni*-infected mice may contribute to Th2 cell polarization during infection by suppression of the Th1 cell response (McKee and Pearce, 2004). In addition to CD25, other putative surface markers for T_{regs} cells include

glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) and the integrin CD103 (McHugh *et al.*, 2002). A hallmark characteristic of T_{reg} cells is the expression of forkhead family transcription factor Foxp3 (Hori *et al.*, 2003). Although expressed on activated T cells, the co-stimulatory molecule cytotoxic T lymphocyte associated protein 4 (CTLA-4) is also associated with regulatory activity (Chikuma and Bluestone, 2003).

In this chapter I show that although the levels of CD4⁺CD25⁺ cells are upregulated during *S. mansoni* infection, there is no abrogate role for CD25⁺ cells in immune regulation in infection. I therefore evaluated if schistosome infection induced alterations in known regulatory markers on CD4⁺ but CD25⁻ spleen cells. I have identified that schistosome infection expanded a CD4⁺CD25⁻CTLA-4⁺ spleen cell population. A functional role for CTLA-4 in suppression of type 2 cytokine response in schistosome infection was shown when CTLA-4 was blocked with mAb during acute but not chronic infection.

Chapter objectives:

1. To investigate the expression and functionality of T regulatory (CD4⁺CD25⁺) cells during *S. mansoni* infection.
2. To determine the role of T regulatory cells (CD4⁺CD25⁺) during acute and chronic *S. mansoni* infection.
3. To investigate the biological function of CTLA-4 during acute and chronic *S. mansoni* infection.

3.2 Results

3.2.1 Frequency of $CD4^+CD25^+$ T cells are increased in spleens during *S. mansoni* infection.

I first tested if the frequencies of $CD4^+CD25^+$ T cells increased during the peak of inflammation during schistosome infection of mice, i.e. at week 8 post-infection. Infected mice had ~ 50% more $CD4^+CD25^+$ cells in the spleens compared to levels seen in uninfected mice (Fig. 3.1A). As $CD4^+$ cell activity is modulated during acute to chronic stages of infection (Fallon, 2000; Pearce and MacDonald, 2002.), I analysed the frequencies of $CD4^+CD25^+$ cells in the spleens of mice infected with *S. mansoni* for 4, 8, and 16 weeks compared to age-matched uninfected mice (Fig. 3.1B). Interestingly, the frequencies of $CD4^+CD25^+$ cells had already significantly ($P < 0.05$) increased in the spleens of 4 week-infected mice, which is prior to eggs being laid, indicating that the initial larval/worm stages of infection can induce expansion of $CD4^+CD25^+$ cells (Fig 3.1B). The level of $CD4^+CD25^+$ cells increased further by week 8, and maintained these levels in the chronic stage of infection (week 16) (Fig. 3.1B).

The transcription factor Foxp3 is another marker of regulatory cells that is predominantly expressed on $CD4^+CD25^+$ cells (Hori *et al.*, 2003). I investigated expression of Foxp3 on $CD4^+CD25^+$ cells during the acute stages of schistosome infection. Despite the increased frequency of spleen $CD4^+CD25^+$ cells during infection, there was little or no increase in the percentage of $CD4^+CD25^+$ cells that co-expressed Foxp3 at week 4 or 8 post-infection (Fig. 3.2). The increase in $CD4^+CD25^+$ cells at week 4, and the greater increase observed at week 8 of infection was largely confined to a Foxp3⁻ population (Fig. 3.2), with these $CD4^+CD25^+Foxp3^-$ cells possibly representing activated T cells induced by the *S. mansoni* infection.

3.2.2 $CD4^+CD25^+$ T cells from acutely-infected mice are more potent at suppressing T cell proliferation *in vitro* than $CD4^+CD25^+$ T cells from uninfected mice.

I isolated $CD4^+CD25^+$ cells from the spleens of mice infected for 4 and 8 weeks to test if they had the known *in vitro* suppressive activity of $CD4^+CD25^+$ cells isolated from uninfected mice (Thornton and Shevach, 1998); with the proliferation of uninfected $CD4^+CD25^-$ responders suppressed in a dose-dependent manner (Fig. 3.3). It was interesting that $CD4^+CD25^+$ from 4 and 8 week-infected mice were relatively more potent than $CD4^+CD25^+$ cells from uninfected mice, demonstrating a significantly greater degree of suppression ($P < 0.01$ or $P < 0.05$) at a range of effector:target ratios (Fig. 3.3).

I also investigated expression on the regulatory marker Foxp3 in $CD4^+CD25^+$ cells from week 16 chronically-infected mice. Cells from 16 week-infected mice have a small, but significant ($P < 0.01$), increase in $CD4^+CD25^+Foxp3^+$ cells compared to cells from uninfected mice (Fig. 3.4). As also observed in week 8-infected mice (Fig. 3.2), there was an increase in $Foxp3^+CD25^+$ cells compared to uninfected mice. However, despite this it was remarkable to observe that $CD4^+CD25^+$ cells isolated from spleens of chronically infected mice were unable to suppress proliferation of $CD4^+CD25^-$ T cells from uninfected mice (Fig. 3.5). The isolated $CD4^+CD25^+$ cells from chronically-infected mice were not dead, as seen by trypan blue staining. Therefore functionally suppressive $CD4^+CD25^+$ cells are induced in acute *S. mansoni* infection, but despite being elevated in the chronic stage of infection they have lost their *in vitro* suppressive activity.

One concern with these functional studies was the method of isolation of the suppressive $CD4^+CD25^+$ cells. I used the AUTOMACS $CD4^+CD25^+$ regulatory T cell isolation kits for my studies. When initially optimizing the cell isolation procedure I confirmed an approx. 92-95% purity of isolated $CD4^+CD25^+$ cells (section 2.4.6). Despite a

greater than 93% purity from uninfected and week 8-infected spleen cells, purity of CD4⁺CD25⁺ cells isolated from 16-week infected mice was only approx 80%, as assessed by flow cytometry (Fig.3.6). From the forward and side-scatter profiles of the isolated cells, it appears that some of the 20% contamination in chronically infected samples comes from larger, more granular cell populations. The CD4⁺ and CD25⁺ staining profile also shows a contaminating population of CD4⁺CD25⁻ cells. Whether it is this contamination that is responsible for ablation of all suppressive activity of the remaining CD4⁺CD25⁺ cells remains to be seen. However since suppression of naïve CD4 cells by CD4⁺CD25⁺ cells is present even at an effector:target ratio of 1:16 in uninfected mice, an 80% pure CD4⁺CD25⁺ population should be capable of mediating suppression, certainly at lower effector:target ratios.

3.2.3 *Depletion of CD25⁺ cell depletion during acute or chronic S. mansoni infection has no effect on the outcome of infection.*

The expansion of CD4⁺CD25⁺ T cells during the first 4 - 8 weeks of *S. mansoni* infection (Fig. 3.1) is temporally coincident with the essential role for CD4⁺ T cells in the granulomatous response during the acute stages of infection of mice (Fallon *et al.*, 2000b; Mathew *et al.*, 1990). Therefore we used mAb to deplete either CD4⁺ or CD25⁺ cells during the acute 4-8 weeks of infection. Depleting mAbs were administered from week 4 post-infection, i.e. prior to the onset of egg-laying but when CD4⁺CD25⁺ are increased in infected mice (Fig. 3.1), until week 8, which is the peak of granulomatous inflammation. The known essential role of CD4⁺ cells in acute schistosome infection was confirmed by a range of parameters, including the progressive weight loss and deaths of anti-CD4 mAb-treated mice (Fig. 3.7), due to hepato-intestinal pathology, as described previously (Fallon *et al.*, 2000b;

Hernandez *et al.*, 1997; Mathew and Boros, 1986). The CD4 cell depletion data was performed by the Fallon lab in Cambridge, UK, and here in Trinity College Dublin, with comparable results.

Depletion of CD25⁺ cells caused no mortalities, with anti-CD25 mAb-treated animals having comparable weight changes as seen in control mAb-treated mice (Fig. 3.8). Anti-CD25 mAb treatment did not alter egg granuloma size (Fig. 3.9A), eosinophil content (Fig. 3.9B), liver fibrosis (Fig. 3.9C, 3.9D), as well as tissue egg counts (Fig. 3.9E) or egg excretion and parasite fecundity (Fig. 3.9F). There were no gross changes in the appearance of livers of anti-CD25 mAb-treated mice, with these animals having comparable plasma transaminase levels, which are markers for hepatocyte damage, as control infected mice (Fig. 3.9D).

Spleen (Fig. 3.10) and mesenteric lymph node cells (MLN) (Fig. 3.11) from anti-CD25 mAb-treated mice had comparable production of Th2 (IL-4, IL-5), Th1 (IFN- γ) or regulatory (IL-10 and TGF- β) cytokines as control mAb-treated mice when stimulated with anti-CD3 mAb or parasite worm or egg antigens. Production of IL-2, which is critical for T cell proliferation, was also equivalent between groups.

It has been suggested that CD25⁺ cells may preferentially function in the chronic stages of *S. mansoni* infection (Singh *et al.*, 2005). I therefore depleted CD25⁺ cells in mice during the chronic stages of infection, from weeks 12-16. Depletion of CD25⁺ cell in chronic infection caused no weight change compared to infected mice treated with control rat IgG mAb (Fig. 3.12). CD25⁺ cell depletion during chronic infection also had no effect on any parasitological parameter investigated (Table 3.1). Table 3.1 includes data from CD25⁺ depletion during both acute and chronic infection. Down-modulation of the immune response from acute to chronic infection is observed, with reduction in granuloma size and eosinophil

counts in the livers of chronically infected mice from both groups. Cytokine production from stimulated spleen (Fig. 3.13) cells or MLN (Fig. 3.14) cells during the chronic stage of infection were also comparable between groups.

In both acute and chronic infection experiments the efficacy of CD25 cell depletion was checked weekly by flow cytometry. After 4 weeks of anti-CD25 mAb treatment, at the end of acute or chronic infection, flow cytometry confirmed >85% depletion of CD25⁺ cells in the spleens, mesenteric lymph nodes or liver granuloma cells (Fig. 3.15A). So despite the marked increase in CD4⁺CD25⁺ cells in mice during schistosome infection, depletion of the CD25⁺ cells did not alter cytokine responses, granuloma formation and immunopathology during acute or chronic stages of an *S. mansoni* infection. It is relevant to note that CD25⁺ cell depletion did not remove the Foxp3⁺ population of CD4⁺ cells, with only a 30% reduction in total CD4⁺Foxp3⁺ spleen cells in CD25⁺-depleted mice, even though >85% of CD25⁺ cells had been efficiently depleted (Fig. 3.15B).

Collectively, these data show that while CD4⁺ cells are critically involved in schistosome infection, depletion of the CD25⁺ cells has no effect in acute or chronic infection, it is indicative that a CD4⁺ but CD25⁻ cell may have a regulatory role in schistosome infection.

3.2.4 CD25 expression on T cells is not critical for production of the Th2 cytokine IL-4, or the regulatory cytokine IL-10.

Mice deficient in IL-4 succumb to pathology in acute *S. mansoni* infection (Fallon *et al.*, 2000a; Hoffmann *et al.*, 2000; Rosa Brunet *et al.*, 1997), highlighting a protective role for IL-4 in infection. Spleen and mesenteric cells from mice with CD25⁺ cells depleted had intact, or possibly elevated, IL-4 production *in vitro* (Figs. 3.10, 3.11 and 3.13, 3.14), suggesting no deficiency in IL-4. To further assess whether depletion of CD25⁺ cell resulted

in a reduction in Th2 cells I checked if the Th2 cell specific marker T1/ST2, up-regulated on Th2 cells in schistosome infection (Lohning *et al.*, 1998), was expressed on CD25⁺ or CD25⁻ CD4⁺ cells. As shown in Fig. 3.16A, T1/ST2 expression is predominately on the CD25⁻ fraction of CD4⁺ T cells ($P < 0.01$). This may explain why depletion of CD25⁺ cells had a negligible effect on *S. mansoni* infection, as the Th2 cells would not be removed. Indeed when intracellular expression of IL-4 in Con A-stimulated spleen cells from uninfected versus infected mice was examined, IL-4 production was also almost entirely limited to the CD25⁻ fraction of CD4⁺ T cells (Fig. 3.16B; $P < 0.005$).

I also looked at production by CD4⁺CD25⁺ or CD4⁺CD25⁻ cells of the regulatory cytokine IL-10, a cytokine with an essential role in schistosome infection (Flores Villanueva *et al.*, 1993; Hoffmann *et al.*, 2000). In infected mice there is a marked expansion of spleen CD4⁺ cells that produce IL-10, with significantly more CD4⁺ IL-10 producing cells that were CD25⁻ than CD25⁺ (Fig. 3.16C). Thus based on intracellular IL-10 detection, the depletion of CD25⁺ cells would not considerably alter IL-10 levels, which is what was detected in spleen or mesenteric lymph node cells from infected mice treated with anti-CD25 mAb (Figs. 3.10, 3.11 and 3.13, 3.14).

3.2.5 *S. mansoni* infection induces elevation of a CD4⁺CD25⁻CTLA-4⁺ population.

Since CD25 depletion had such a negligible effect on the outcome of *S. mansoni* infection, I then tested for differential expression of putative regulatory cell markers on CD4⁺ cells that were CD25⁺ or CD25⁻ during acute infection. Expression of CD103 was marginally elevated on CD4⁺ spleen cells from infected mice, with CD103 expression largely confined to the CD25⁺ population cells from both uninfected and infected mice (Fig. 3.17A; $P < 0.01$). The expression of the regulatory marker GITR was increased on CD4⁺ cells from infected mice

but, similar to CD103, expression was predominately ($P < 0.01$) on $CD4^+CD25^+$ cells (Fig. 3.17B). Levels of the regulatory cell marker CTLA-4 were also assessed on cells from uninfected and *S. mansoni*-infected mice. Similar to CD103 and GITR there was an increase in CTLA-4 expression on the $CD4^+CD25^+$ population in infected mice compared to uninfected animals (Fig. 3.17C). Interestingly, however, *S. mansoni* infection also induced a 3-4-fold increase in CTLA-4 expression on the $CD4^+$ but $CD25^-$ cell population, with significantly elevated levels compared to those on $CD25^+$ cells in infected mice (Fig. 3.17C).

3.2.6 *Foxp3* and CTLA-4 expression on $CD4^+CD25^+$ during acute *S. mansoni* infection.

It has previously been observed that the phenotype observed in mice deficient in CTLA-4 is similar to that which develops in *Foxp3* deficient (*scurfy*) mice, with both types of deficient mice developing massive lymphoproliferation and early death (Khattari *et al.*, 2003) (Tivol *et al.*, 1995). As *Foxp3* is potentially one of the more robust markers for regulatory cell activity (Fontenot and Rudensky, 2005), we examined *Foxp3* and CTLA-4 co-expression on $CD4^+CD25^+$ and $CD4^+CD25^-$ spleen cells from uninfected mice and animals infected for 4 and 8 weeks. Consistent with *Foxp3* being a marker for $CD4^+CD25^+$ cells, >95% of such cells expressed *Foxp3*, with a slight reduction in *Foxp3*⁺ cells by week 8 of infection (Fig. 3.18). Within the $CD4^+CD25^+$ cell population there was increased expression of CTLA-4 in cells from mice with an 8-week schistosome infection, with almost 40% of $CD4^+CD25^+$ cells from infected mice co-expressing *Foxp3* and CTLA-4. In addition, by week 8 of infection ~5% of $CD4^+CD25^+$ cells also expressed CTLA-4 but were negative for *Foxp3*, compared to <1% of such cells from uninfected or 4 week infected mice (Fig. 3.18). Strikingly, in the $CD4^+CD25^-$ cell population from 8 week-infected mice there was 10-fold expansion of CTLA-4⁺*Foxp3*⁻ cells relative to these cells in uninfected mice (Fig. 3.18). This specific expansion during

acute infection of a CD4⁺CTLA-4⁺ cell population, that was CD25⁻Foxp3⁻, prompted me to examine the role of CTLA-4 during infection.

3.2.7 *Blocking CTLA-4 alters acute schistosome-infection in mice.*

Schistosome-infected mice (35-100 cercariae) were administered a blocking mAb against CTLA-4 or a control hamster mAb from the 4th to 8th week of acute stages of infection. Mice with CTLA-4 blocked had significant ($P<0.05-0.0001$) progressive loss of weight compared to control hamster mAb treated mice, with 15-20 % loss in body weight by week 8 (Fig. 3.19). However, despite the marked weight-loss in anti-CTLA-4 mAb-treated mice these animals did not appear moribund and in 7 experiments there were no deaths. Anti-CTLA-4 mAb-treated mice had comparable schistosome worm burdens and fecundity as untreated mice (Table 3.2). At autopsy there was no overt appearance of exacerbated intestinal pathology in anti-CTLA-4 mAb-treated mice. Consistent with normal infection-associated changes in the intestines, the ability of CTLA-4 mAb-treated mice to excrete eggs was also normal (Table 3.2).

Hematoxylin and eosin-stained sections of the liver showed an absence of parenchymal or peri-oval hepatocyte damage in anti-CTLA4 mAb-treated mice (Fig. 3.20A). The size of the granuloma around schistosome eggs in the liver was larger in anti-CTLA-4-mAb treated mice (Fig. 3.20A), but the increase in size was not statistically significant from control mice (Fig. 3.20B). Additionally, there was also a non-significant increase in liver fibrosis (Fig. 3.20C), with collagen deposition on Martius Scarlet Blue-stained sections indicative that the increase was possibly due to the larger-sized granulomas rather than a generalized increase in parenchymal fibrosis in anti-CTLA-4 mAb-treated mice (Fig. 3.20C).

Most notably, on liver sections there was a significantly greater influx of eosinophils into the granuloma of anti-CTLA-4 mAb-treated mice compared to control mice (Fig. 3.21A; $P < 0.05$). Rather than relying solely on histological counts for eosinophils, liver granuloma cells were isolated and flow cytometry was used to quantify eosinophils ($CD4^+CD8^+CD19^-CCR3^+$). By flow cytometry there was also a significant ($P < 0.01$) increase in eosinophils within the liver granuloma of anti-CTLA-4 mAb-treated mice compared to levels in granuloma cells from control mAb-treated infected mice (Fig. 3.21B). Absolute numbers of granuloma eosinophils were determined from total liver granuloma cells (Fig. 3.21C).

4get mice, which have IL-4 linked to eGFP, were also infected, and the mice were treated with anti-CTLA-4 mAb to quantify the effects of treatment on Th2 ($CD4^+IL4-GFP^+$) cells within the liver granuloma. There were more Th2 cells within the granuloma of anti-CTLA-4 mAb-treated 4get mice compared to untreated mice (Fig. 3.21D). Absolute numbers were also determined and the increase in Th2 cells was found to be significant ($P < 0.05$) (Fig. 3.21E). These data indicate that although blocking anti-CTLA-4 during acute schistosome infections causes marked weight loss but not deaths or overt increased pathology in mice, this treatment did cause significant increases in the eosinophil and Th2 cell content of the granuloma in the liver.

3.2.8 *Blocking CTLA-4 does not alter chronic schistosome-infection in mice.*

Mice with chronic 16-week infections also have greater up-regulation in the expression of CTLA-4 on $CD25^-$ relative to the increase on the $CD25^+ CD4$ cells (Fig. 3.22A). However, when mice were treated with anti-CTLA-4 mAb during the chronic stages (weeks 12-16) of infection there was no difference between groups in body weight or mortalities when compared to infected mice treated with a control hamster mAb (Fig. 3.22B).

The size of liver granulomas and eosinophil content were unaltered between control and anti-CTLA-4 mAb treated groups, respectively, as were egg excretion levels, and parasite fecundity. A complete list of parasitological parameters tested on CTLA-4 mAb-treated mice in both acute and chronic infection is shown in Table 3.2. Spleen cells (Fig. 3.23), and mesenteric lymph node cells (Fig. 3.24), from chronically-infected mice treated with anti-CTLA-4 mAb had comparable cell proliferation and the production of a range of cytokines as mice treated with control mAb. However there was evidence of spontaneous IL-5 production in unstimulated spleen cells from anti-CTLA-4 mAb treated mice. Despite CTLA-4 expression being up-regulated in both acute and chronic stage of schistosome infection, blocking CTLA-4 altered the infection only in acute stages of infection.

3.2.9 *Spontaneous proliferation of cells from acutely-infected mice treated with anti-CTLA-4 mAb.*

To address effects of anti-CTLA-4 mAb treatment in acutely infected mice on cellular responses, we removed the spleens and mesenteric lymph nodes from schistosome-infected control or anti-CTLA-4 mAb-treated mice. Spleen cells from anti-CTLA-4 mAb-treated mice had greater relative cell proliferation to various *in vitro* stimulations when compared to control infected mice, with marked spontaneous proliferation of cells from these mice when cultured in media (Fig. 3.25A). Using CFSE staining of spleen cells from anti-CTLA-4 mAb-treated mice I have not observed spontaneous proliferation in CD4⁺, CD8⁺ or CD19⁺ T cells (Fig. 3.25B).

3.2.10 Elevated IL -5 production in acutely-infected mice treated with anti-CTLA-4-mAb.

The production of a range of cytokines by spleen cells from the control and anti-CTLA-4 mAb-treated groups was also examined (Fig. 3.26). Although there was no difference in production of the Th2 cytokine IL-13 between groups, the levels of IL-4 were elevated in schistosome antigen-stimulated, but not anti-CD3 mAb-treated, cells from the anti-CTLA-4 mAb-treated mice (Fig. 3.26). Strikingly, there was spontaneous production of the Th2 cytokine IL-5 in the media of the CTLA-4 mAb-treated group, similar to what was observed in chronically-infected mice (Fig. 3.23B), but at much higher levels. There was no difference in levels of IL-5 in response to anti-CD3 mAb or antigen-specific stimuli. Levels of the Th1 cytokine IFN- γ were comparable between groups, with slightly less IFN- γ production in the anti-CTLA-4 mAb-treated group upon anti-CD3 stimulation. IL-10 and TGF- β production in response to anti-CD3, and antigen-specific AW and SEA, was equivalent between groups. In contrast, mesenteric lymph node cells from anti-CTLA-4 mAb-treated mice had comparable cell proliferation, with some spontaneous proliferation, and production of all cytokines tested as control mice (Fig. 3.27).

Consistent with the elevated production of IL-4 by cells from anti-CTLA-4 mAb-treated there was a significant increase ($P < 0.05$) in total serum IgE in these mice relative to IgE detected in serum of control or untreated mice (Fig. 3.28). This is another indicator of the enhanced type-2 response observed in mice treated with anti-CTLA-4.

3.2.11 Elevated Th2 cells in the spleens of acutely-infected mice treated with anti-CTLA-4 mAb.

Spleen cells from *S. mansoni*-infected 4get mice that were untreated or treated with anti-CTLA-4 mAb were examined for levels of IL-4-eGFP. *S. mansoni* infection caused a ~10 fold increase in IL-4-eGFP⁺ CD4⁺ T cells in the spleen compared to levels in spleen cells from uninfected mice (Fig. 3.29A). There was a marked elevation in the frequency of IL-4-GFP⁺ CD4⁺ T cells in spleen cells from anti-CTLA-4 mAb-treated 4get mice compared to control infected 4get mice (Fig. 3.29A, 3.29B), which was similar to the increase in Th2 cells in the liver granuloma (Fig. 3.21D). Absolute numbers of IL-4-GFP⁺ CD4⁺ cells in the spleen were determined, with significantly ($P < 0.05$) greater numbers in anti-CTLA-4 mAb-treated infected mice compared to control infected mice (Fig. 3.29B).

3.2.12 IL -5 production by splenocytes from mice with acute schistosome infection treated with anti-CTLA-4 mAb is predominantly from CD4⁺ cells.

In view of the elevated IL-4 and IL-5 in spleen, but not mesenteric, cell cultures (Fig. 3.26, 3.27) and increased IL-4-eGFP⁺ CD4⁺ cells in the spleen of anti-CTLA-4 mAb-treated mice (Fig. 3.29A), I addressed if CD4⁺ cells are the primary source of the IL-4 and IL-5 in these mice. CD4⁺ cells were depleted *in vitro* and cytokine production in response to anti-CD3 or schistosome AW or SEA stimulation was tested. The production of the schistosome-antigen-induced IL-4 was almost completely abrogated when CD4⁺ cells were depleted from spleen cells from untreated and anti-CTLA-4 mAb-treated mice, with a marked reduction in IL-4 induced by anti-CD3 treatment (Fig. 3.30), indicating that in both groups of mice IL-4 is predominately from CD4⁺ cells. IL-5 release from *in vitro* stimulated cells from infected untreated and anti-CTLA-4 mAb-treated mice was also produced primarily from CD4⁺ cells

(Fig. 3.30). In contrast, the spontaneous production of IL-5 in unstimulated (media) cells from spleens of anti-CTLA-4 mAb-treated mice was only slightly diminished when CD4⁺ T cells were depleted (Fig. 3.30). The spontaneous production of IL-5 by spleen cells from anti-CTLA-4 mAb-treated mice was also not reduced when CD8⁺ cells or B (B220⁺) cells were depleted *in vitro* (3.31A and 3.21B). This highlights an alternative, non-T/non-B cellular source for IL-5 production when CTLA-4 is blocked in acute schistosome infection.

IL-5 is produced primarily by T cells, but may also be produced by non-T cell sources, such as eosinophils and mast cells (Castro *et al.*, 1995). Previously, eosinophils from *S. mansoni*-infected mice were shown to produce a major portion of Th2 cytokines, including IL-5, in the granuloma milieu (Rumbley *et al.*, 1999). In view of the increase in granuloma eosinophils in anti-CTLA-4 treated mice it is possible that the spontaneous release of IL-5 is from eosinophils, but using intracellular cytokine staining we have been unable to confirm this. Some IL-5 expression was detected in CD4⁺ T cells, and also from non-CD4⁺ T cells, but expression was comparable between groups (Fig. 3.32).

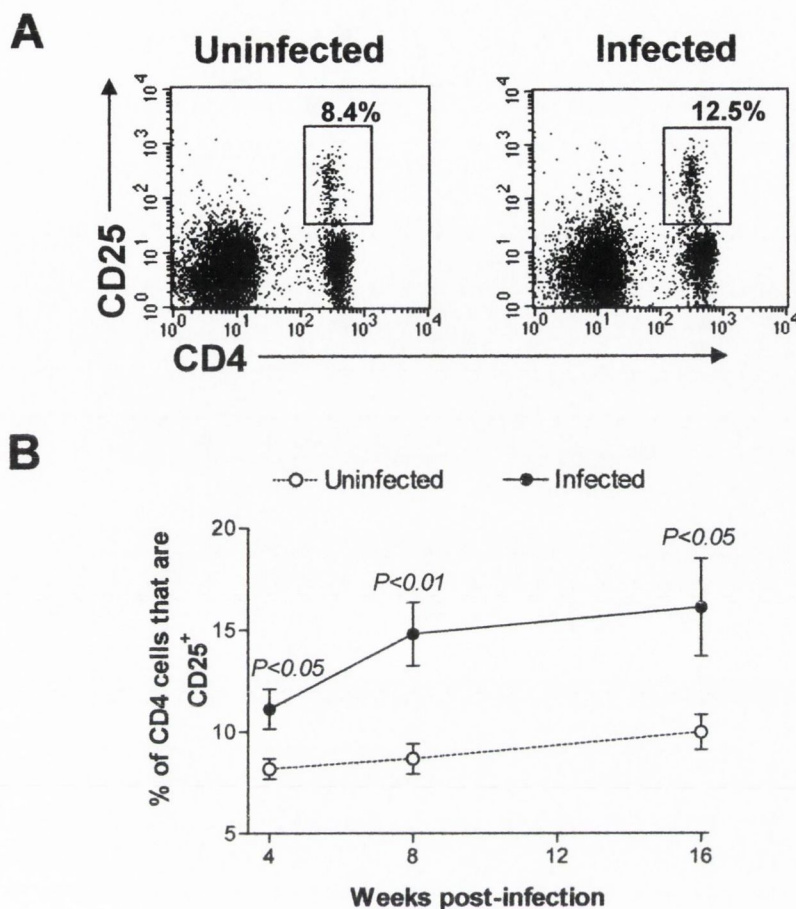


Figure 3.1. $CD4^+CD25^+$ regulatory *T* cells are upregulated in the spleen throughout *Schistosoma mansoni* infection. *A*, expression of CD25 on $CD4^+$ spleen cells from uninfected and 8-week *S. mansoni*-infected mice. *B*, increase in CD25 expression on $CD4^+$ spleen cells from 4, 8, and 16 week *S. mansoni*-infected mice. Data are expressed as a % increase in $CD4^+CD25^+$ levels relative to age-matched uninfected mice. Student's t-test was used to compare differences between uninfected and infected groups at each timepoint. Data shown is mean $\pm$ SD from 3 - 4 individual mice with similar levels seen in at least 2 separate experiments per time-point.

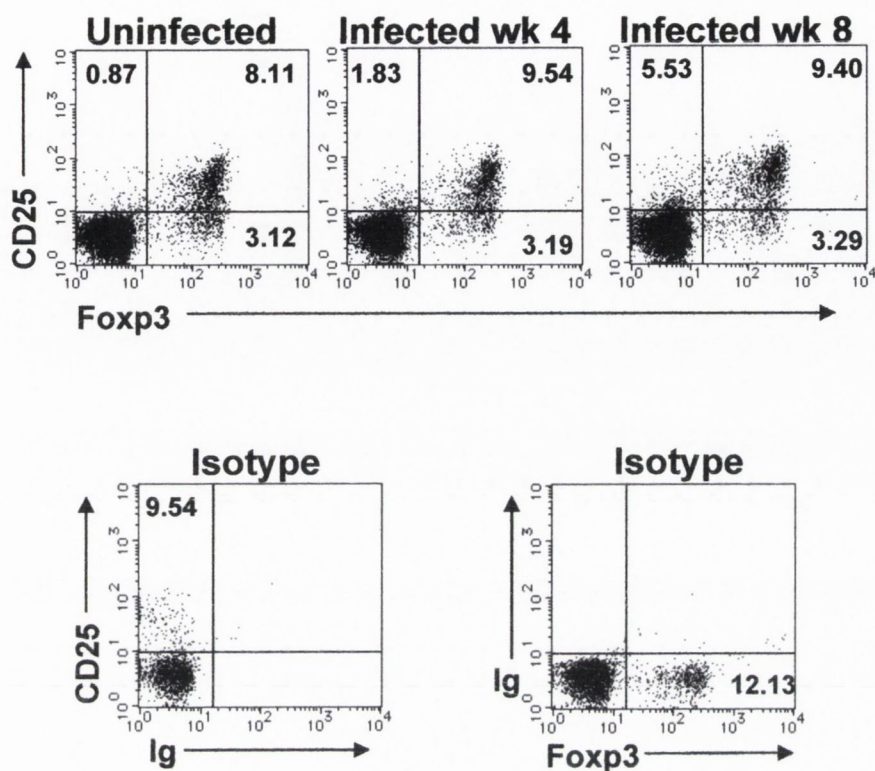


Figure 3.2. *The increase in frequency of CD4⁺CD25⁺ cells during acute infection is predominately confined to a Foxp3⁺ population. CD25 and Foxp3 expression on CD4⁺-gated spleen cells from uninfected and 4 or 8 week-infected mice. Data shown is from individual mice and representative from at least 2 separate experiments. Isotype gates for CD25 and Foxp3 are shown.*

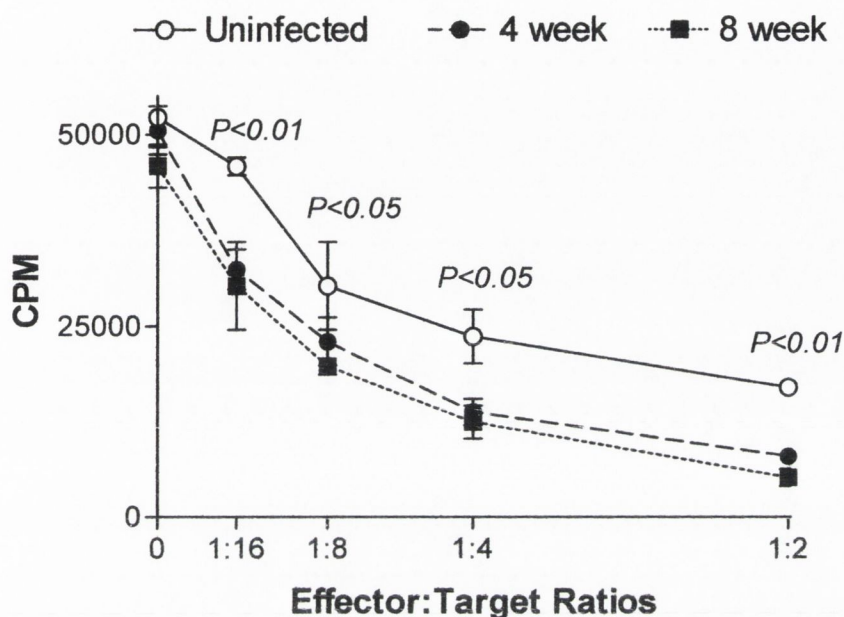


Figure 3.3. $CD4^+CD25^+$ cells isolated from the spleens of infected mice are functionally suppressive in vitro. $CD4^+CD25^-$ spleen cells from uninfected mice were mixed with $CD4^+CD25^+$ cells from uninfected mice or mice infected for 4 or 8 weeks. Cells were mixed in triplicate at different effector:target ratios. Irradiated naïve AC were added and cells were stimulated with 0.5 $\mu\text{g/ml}$ anti-CD3 mAb for 72h. [^3H]thymidine was added for the last 16h and incorporation was measured and expressed as counts per minute, mean $\pm$ SD. Student's t-test was performed to compare uninfected from either week 4 or week 8 infected and data was considered significant if $P < 0.05$. Data are representative of 2-3 separate experiments.

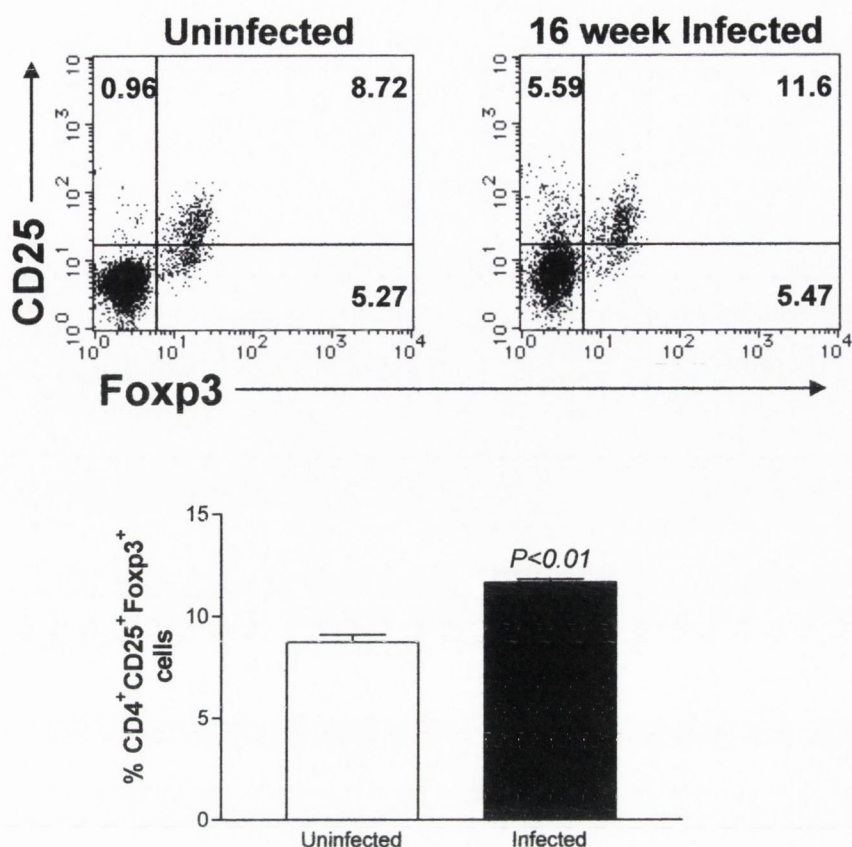


Figure 3.4. Upregulation of $CD4^+CD25^+Foxp3^-$ and $CD4^+CD25^+Foxp3^+$ cells in spleens of chronically infected mice. CD25 and Foxp3 expression on $CD4^+$ -gated spleen cells from uninfected and 16 week-infected mice. Data shown is from individual mice and representative from at least 2 separate experiments. Student's t-test was used to determine differences in $Foxp3^+CD25^+$ cells between groups.

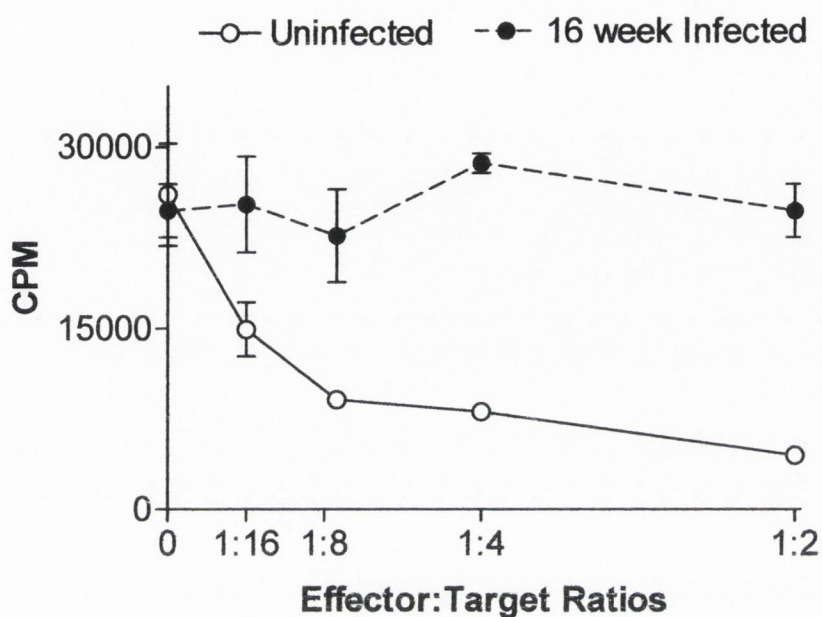


Figure 3.5. *CD4⁺CD25⁺ cells from chronically infected mice do not suppress uninfected T cell proliferation in vitro.* CD4⁺CD25⁺ spleen cells from uninfected mice were mixed with CD4⁺CD25⁺ cells from uninfected mice or mice infected for 16 weeks. Cells were mixed in triplicate at different effector:target ratios. Irradiated naïve AC were added and cells were stimulated with 0.5 µg/ml anti-CD3 mAb for 72h. [³H]thymidine was added for the last 16h and incorporation was measured and expressed as counts per minute. Data are representative of 2 separate experiments.

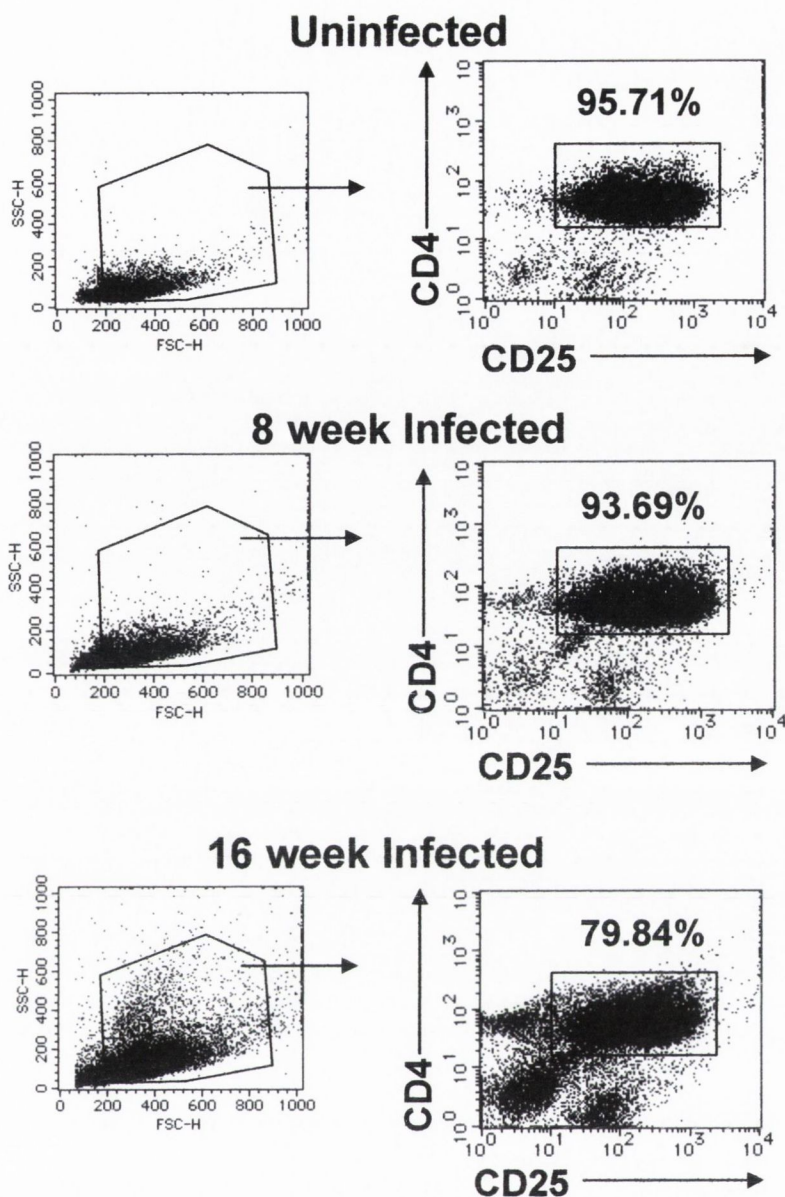


Figure 3.6. *AUTOMACS-isolated CD4⁺CD25⁺ spleen cells from chronically infected mice have increased contaminating cells compared to CD4⁺CD25⁺ cells isolated from uninfected or week 8-infected mice. FACS profile of purified CD4⁺CD25⁺ cells after AUTOMACS isolation. Cells were stained with PE-conjugated anti-CD25 during isolation, then stained with TRI-conjugated anti-CD4.*

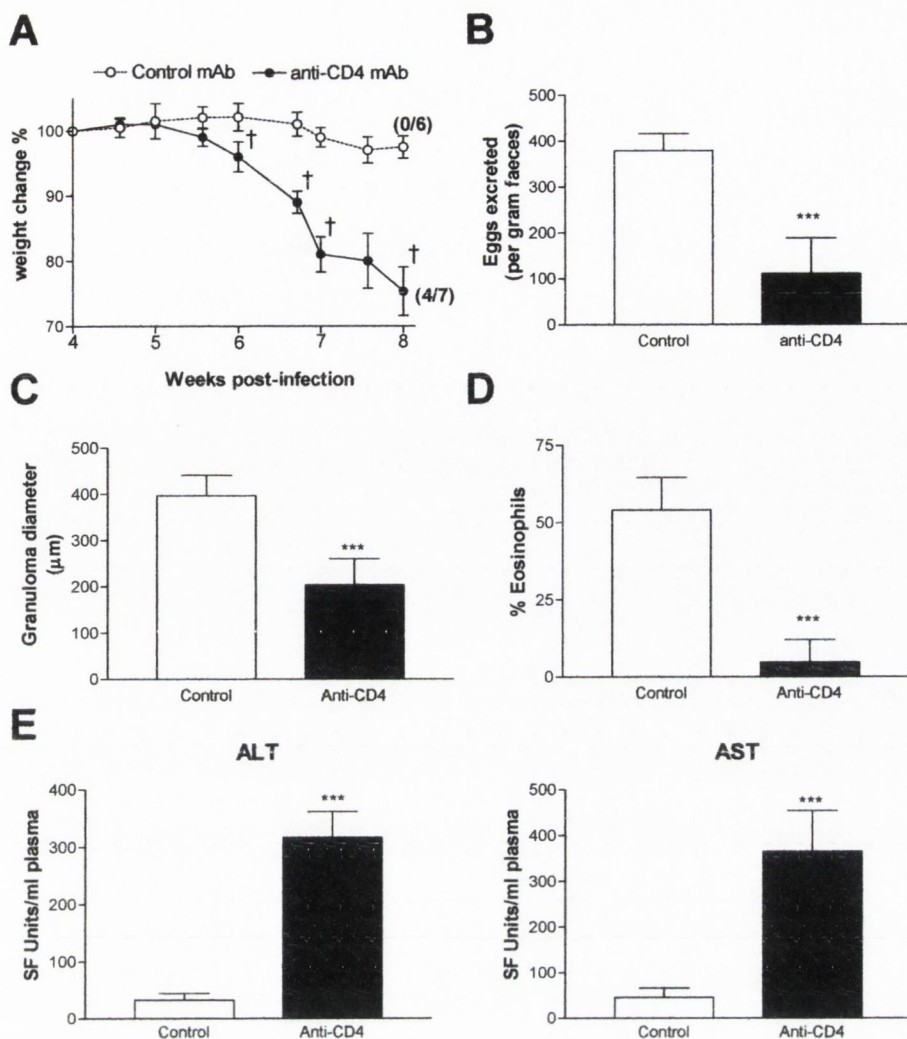


Figure 3.7. *CD4⁺ cells are central in granuloma formation and limiting liver damage during S. mansoni infection.* Infected BALB/c mice were administered with control mAb or anti-CD4 mAb 3 times a week from 4 weeks post-infection. Mice were culled at week 8. *A*, weight loss, shown as % change from weight at week 4. Mouse numbers are shown in brackets, number of deaths/total number in group. *B*, egg excretion expressed as eggs per gram faeces. *C*, liver granuloma size (diameter) as determined from H&E-stained slides. *D*, eosinophils, expressed as a % of total liver granuloma cells. *E*, liver damage as expressed by transaminase levels, AST and ALT. Data shown are mean $\pm$ SD from 5-7 mice per group, and are representative of 3 separate experiments. Student's t-test was used to compare differences between groups. *** = $P < 0.0001$. Experiments were performed by Dr. Phil Smith in Cambridge UK, and by the lab in Trinity College Dublin.

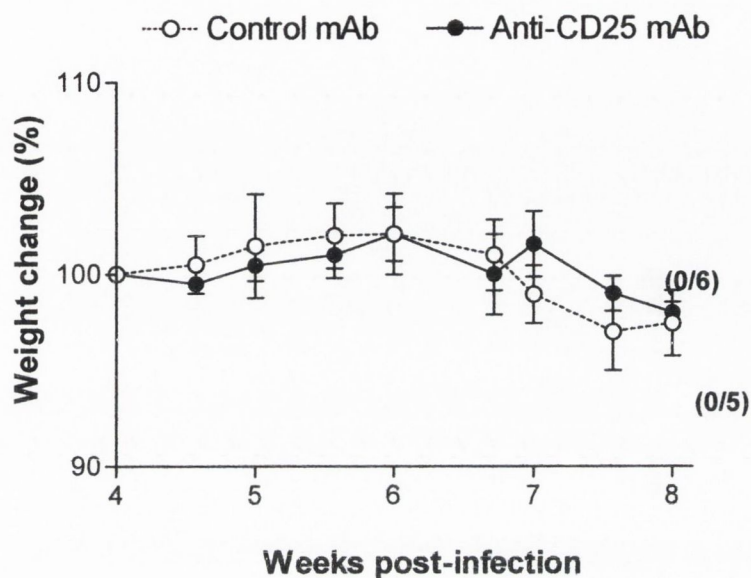


Figure 3.8. Mice treated with anti-CD25 mAb during acute *S. mansoni* infection have comparable weight change to infected mice treated with control IgG Ab. BALB/c mice were infected with *S. mansoni* and control mAb or mAb against CD25 (PC61) were administered 3 times weekly from 4 weeks post-infection. Weights were measured routinely up to 7 weeks post-infection and are shown as % weight change from day 28. Mouse numbers are shown in brackets, number of deaths/total number in group. Data shown are mean $\pm$ SEM from 5-7 mice per group, and are representative of 3 separate experiments.

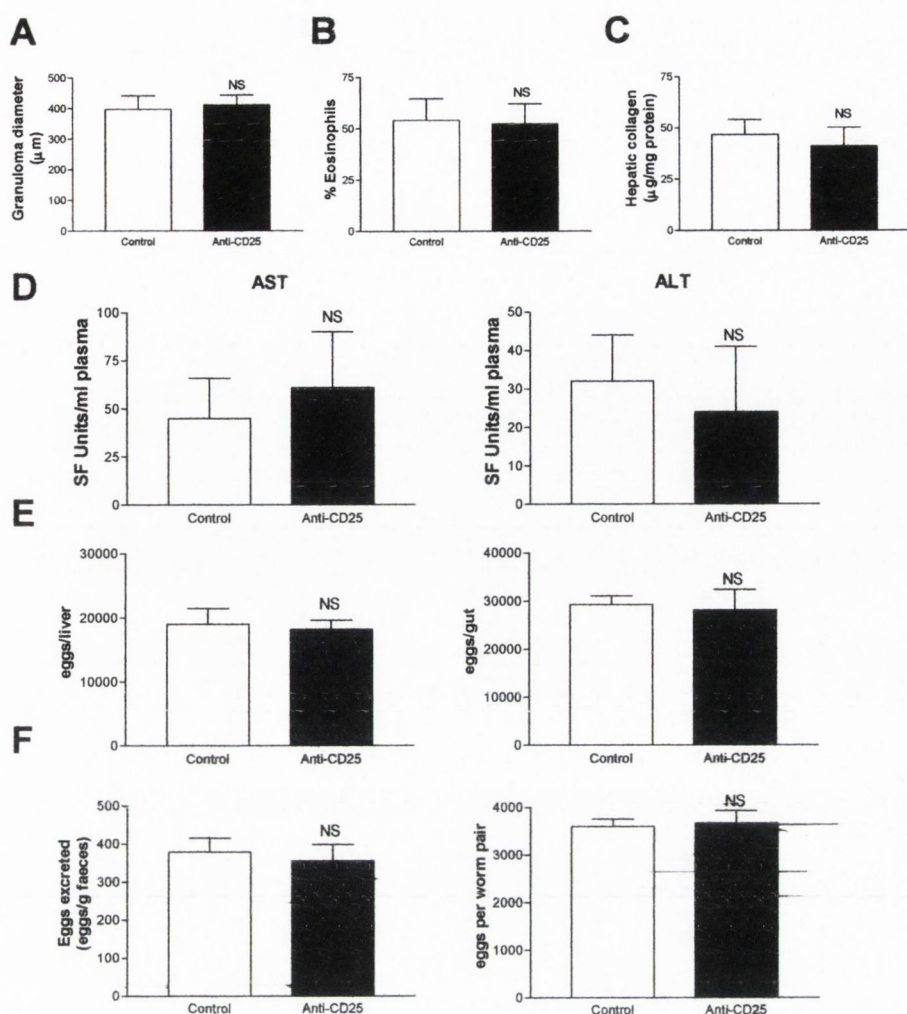


Figure 3.9. *CD25 cell depletion does not alter schistosome infection.*

Infected mice were treated with control mAb, or anti-CD25 mAb from days 28 to 56 of infection. Mice were culled on day 56. Data shown represents mean $\pm$ SD/SEM from 4-7 mice and are representative of 2-3 separate experiments. *A*, diameter of granuloma around eggs in the liver. *B*, percentage of eosinophils within the granuloma. *C*, hepatic fibrosis, expressed as μg collagen per mg protein. *D*, AST and ALT levels in plasma expressed as SF Units/ml. *E*, eggs in the liver and gut, expressed as eggs per liver/gut. *F*, eggs excreted in the faeces, expressed as eggs per gram faeces. *G*, fecundity of worms, expressed as eggs per worm pair. Students' t-test was performed to compare differences between the groups. *NS* = not significant.

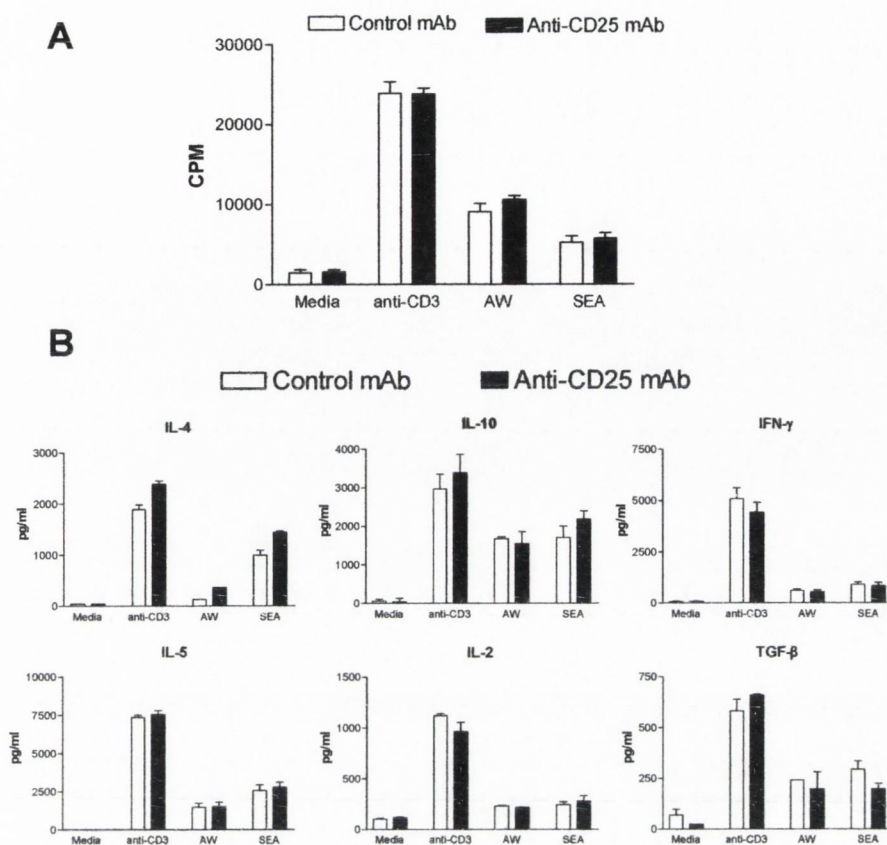


Figure 3.10. *Unaltered proliferation and cytokine production in spleens of infected mice treated with anti-CD25 compared to control mAb-treated infected mice. A, proliferation of spleen cells from control mAb-treated or anti-CD25 mAb-treated infected mice. Cells were unstimulated (media) or stimulated with 0.5 μ g/ml anti-CD3 mAb, 20 μ g/ml AW (adult worm antigen) or 20 μ g/ml SEA (soluble egg antigen). [3 H]thymidine was added for the last 16h and incorporation was measured and expressed as counts per minute (CPM). B, spleen cells were unstimulated (media), or stimulated with anti-CD3 mAb, AW or SEA. Cytokine levels were measured in supernatants after 24 (IL-2) or 72 hours (IL-4, IL-5, IL-10, IFN- γ and TGF- β) by sandwich ELISA, and expressed as picograms per ml supernatant. Data is mean $\pm$ SD from a pool of 3-5 mice and is representative of at least 3 separate experiments.*

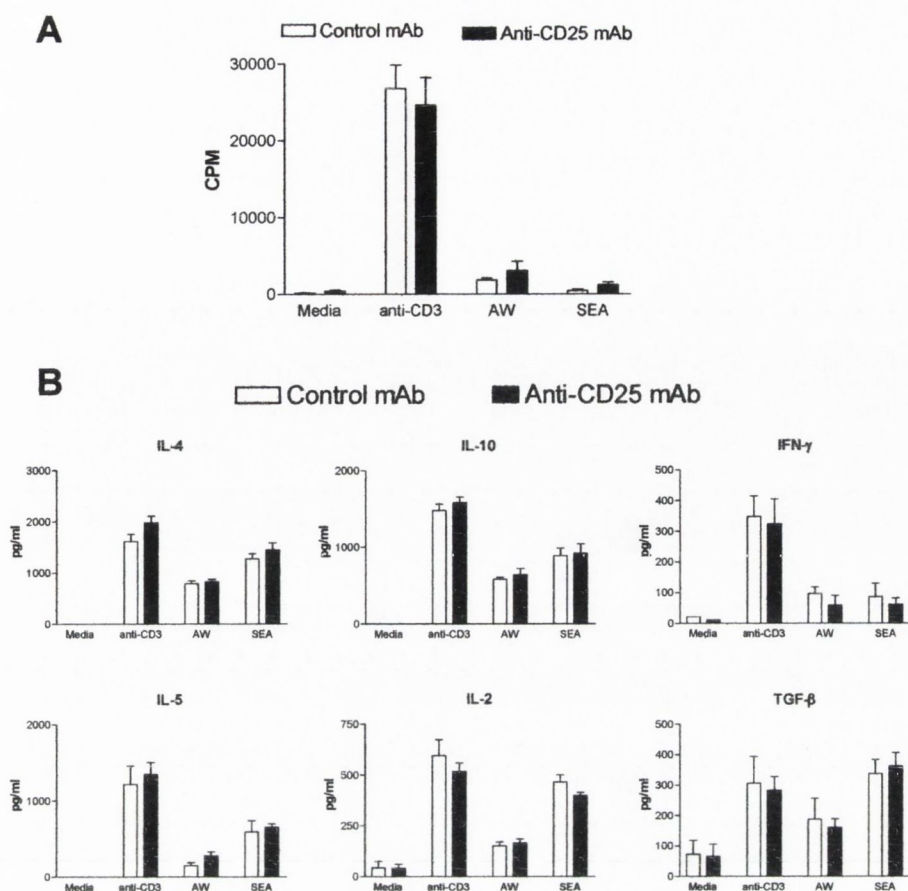


Figure 3.11. *Unaltered proliferation and cytokine production in MLN of infected mice treated with anti-CD25 compared to control infected mice.* *A*, proliferation of spleen cells from infected or anti-CD25 mAb-treated infected mice. Cells were stimulated with media, 0.5 $\mu\text{g/ml}$ anti-CD3 mAb, or 20 $\mu\text{g/ml}$ AW or SEA. [^3H]thymidine was added for the last 16h and incorporation was measured and expressed as counts per minute (CPM). *B*, MLN cells were unstimulated (media), or stimulated with anti-CD3 mAb, AW or SEA. Cytokine levels were measured in supernatants after 24 (IL-2) or 72 hours (IL-4, IL-5, IL-10, IFN- γ and TGF- β) by sandwich ELISA, and expressed as picograms per ml supernatant. Data is from a pool of 3-5 mice and is representative of 3 separate experiments.

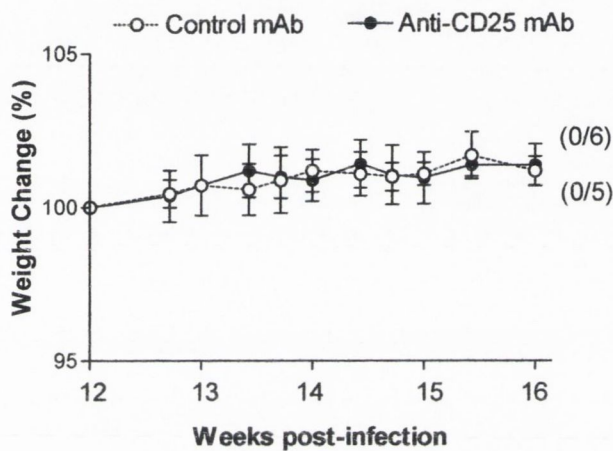


Figure 3.12. *Weight change in CD25-depleted mice during the chronic stage of S. mansoni infection.* BALB/c mice were infected with *S. mansoni* and control mAb or mAb against CD25 (PC61) were administered every 3 days from 12 weeks post-infection. Weights were measured routinely up to 16 weeks post-infection and are shown as % weight change from day 84. Data shown are mean $\pm$ SEM from 5-6 mice per group, and are representative of two separate experiments.

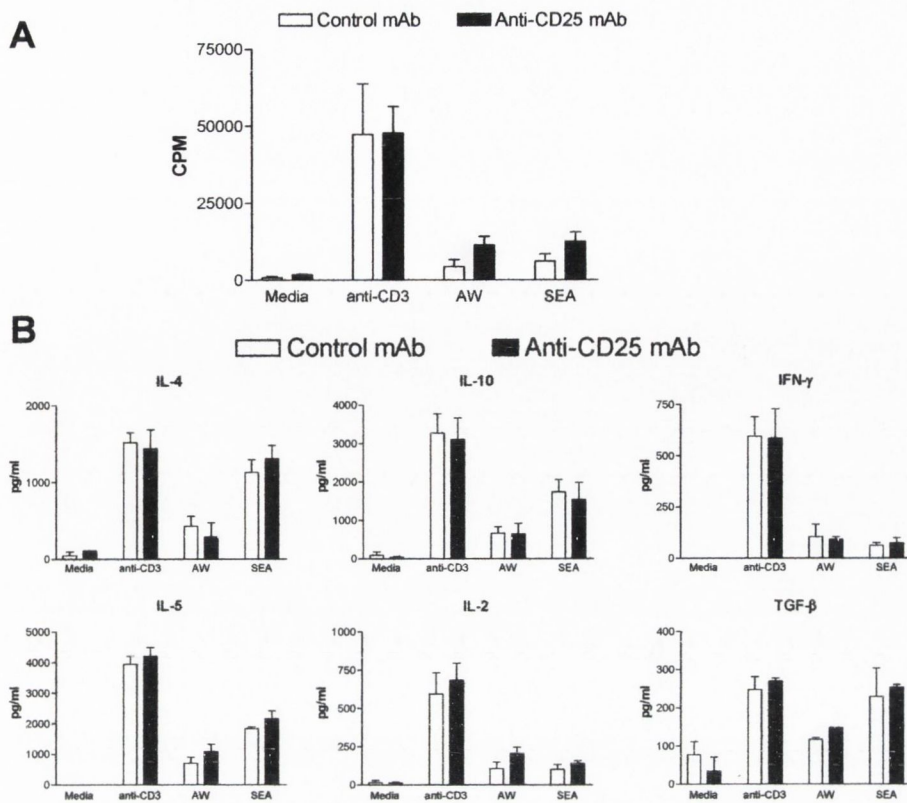


Figure 3.13. *CD25 cell depletion during chronic S. mansoni infection has no effect on proliferation or cytokine production of spleen cells.* *A*, proliferation of spleen cells from infected or anti-CD25 mAb-treated infected mice. Cells were stimulated with media, 0.5 $\mu\text{g/ml}$ anti-CD3 mAb, or 20 $\mu\text{g/ml}$ AW or SEA. [^3H]thymidine was added for the last 16h and incorporation was measured and expressed as counts per minute (CPM). *B*, spleen cells were unstimulated (media), or stimulated with anti-CD3 mAb, AW or SEA. Cytokine levels were measured in supernatants after 24 (IL-2) or 72 hours (IL-4, IL-5, IL-10, IFN- γ and TGF- β) by sandwich ELISA, and expressed as picograms per ml supernatant. Data is mean $\pm$ SEM from of 3-5 individual mice and is representative of 2 separate experiments.

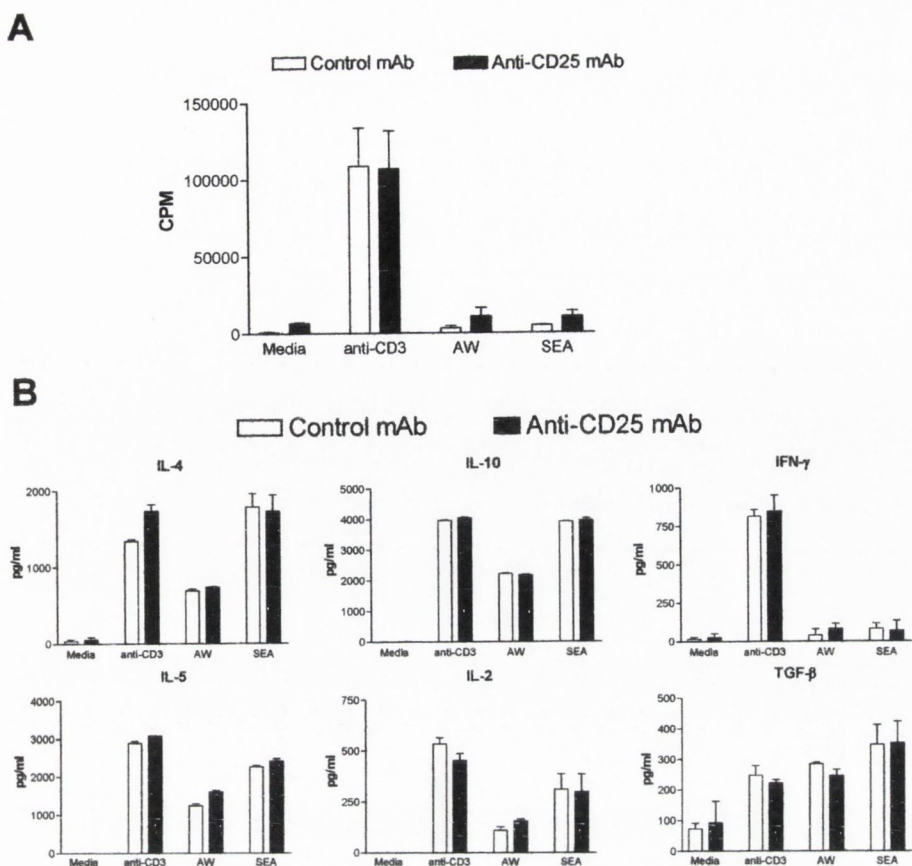


Figure 3.14. *CD25 depletion during chronic *S. mansoni* infection has no effect on proliferation or cytokine production of MLN cells.* *A*, proliferation of spleen cells from infected or anti-CD25 mAb-treated infected mice. Cells were stimulated with media, 0.5 μ g/ml anti-CD3 mAb, or 20 μ g/ml AW or SEA. [3 H]thymidine was added for the last 16h and incorporation was measured and expressed as counts per minute (CPM). *B*, MLN cells were unstimulated (media), or stimulated with anti-CD3 mAb, or 20 μ g/ml AW or SEA. Cytokine levels were measured in supernatants after 24 (IL-2) or 72 hours (IL-4, IL-5, IL-10, IFN- γ and TGF- β) by sandwich ELISA, and expressed as picograms per ml supernatant. Data is from a pool of 3-5 mice and is representative of 2 separate experiments.

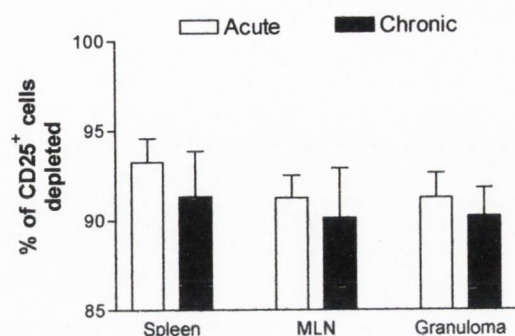
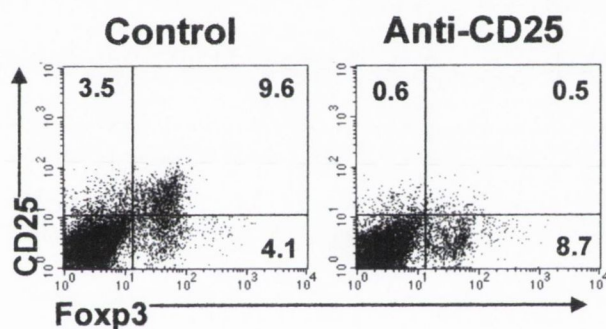
A**B**

Figure 3.15. *Efficient depletion of CD25⁺ cells in acute and chronic infection.* *A*, % depletion of CD25⁺ CD4⁺ T cells in the spleen, MLN and liver granuloma from anti-CD25 mAb-treated mice compared to control mAb-treated mice was determined by flow cytometry. Data shown is from 4 individual mice at both acute and chronic stages of infection. *B*, representative FACS image showing the efficacy of CD25 cell depletion in the spleen of infected mice, and also the alteration in Foxp3 expression following CD25⁺ cell depletion. Cells were stained with TRI-conjugated anti-CD4 mAb and FITC-conjugated anti-CD25 mAb (clone 7D4), and PE-conjugated anti-Foxp3.

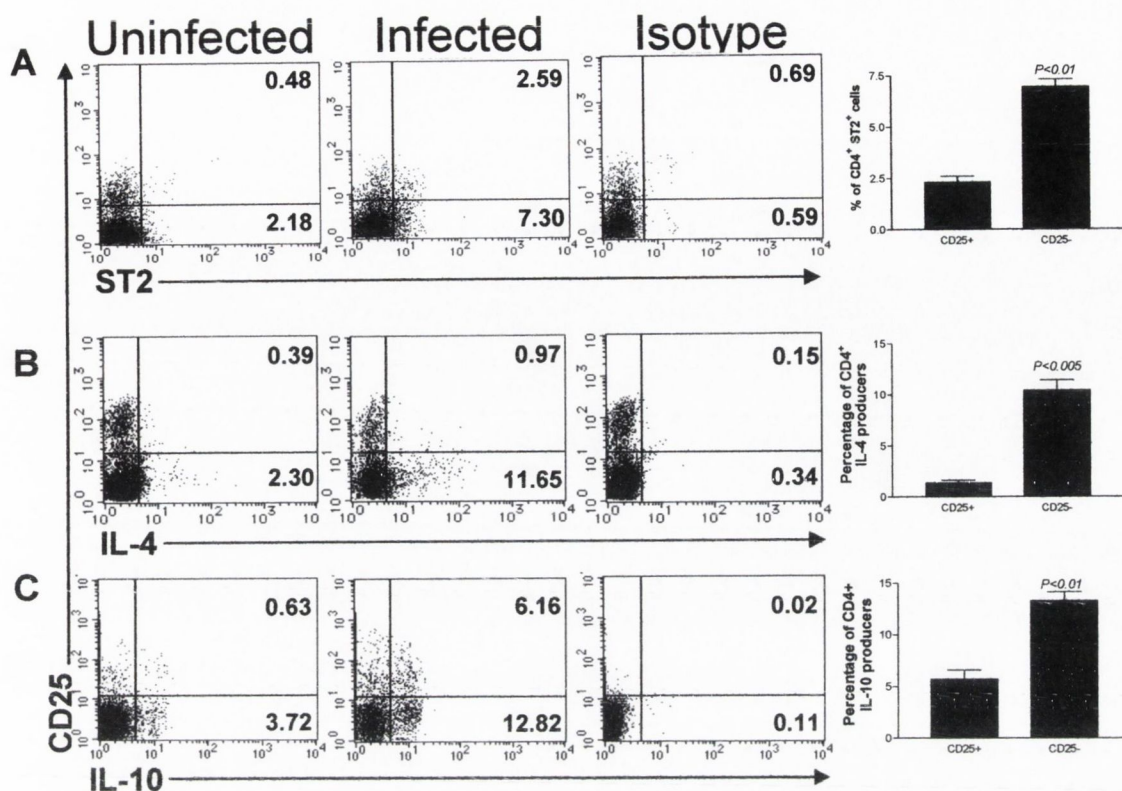


Figure 3.16. Increase in Th2 marker and IL-4 and IL-10 with acute infection is largely associated with the CD25⁻ population of CD4⁺ cells. Spleen cells from uninfected and *S. mansoni* infected BALB/c mice were incubated with TRI-conjugated anti-CD4 mAb, PE-conjugated anti-CD25 mAb and FITC-conjugated anti-ST2, FITC-conjugated anti-IL-10 or FITC-conjugated anti-IL-4 mAb. CD4⁺ cells were gated and FACS graphs drawn plotting CD25 against A, ST2, B, intracellular IL-4 or C, intracellular IL-10. Quadrants were drawn based on isotype controls. Data shown is from individual mice and is representative of at least 6 individual mice from 2-3 separate experiments. Bar graphs represent mean $\pm$ SEM of infected individual mice from all experiments. Student's t-test was performed to compare differences between CD25⁺ and CD25⁻ populations in the infected group.

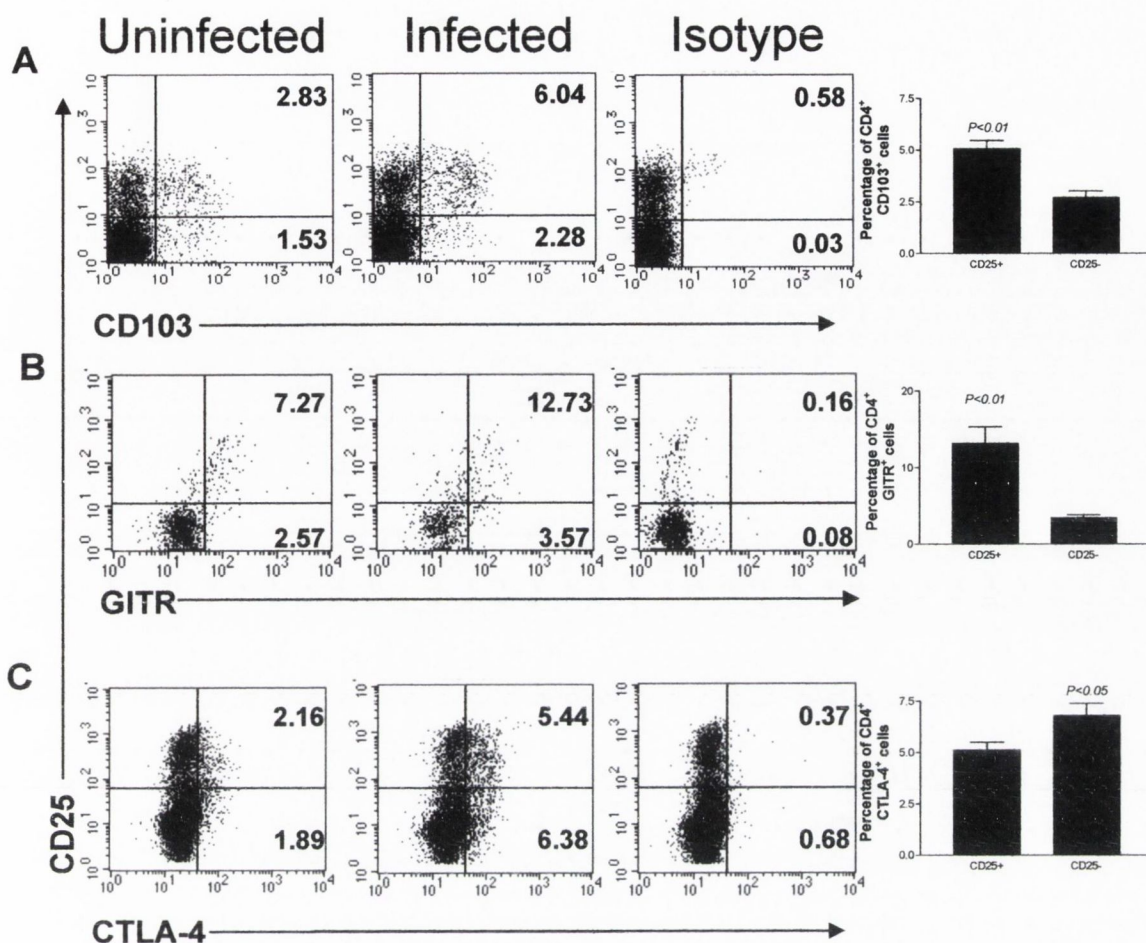


Figure 3.17. *CD103, GITR and CTLA-4 expression on CD4⁺CD25⁺ and CD4⁺CD25⁻ spleen cells during S.mansoni-infection.* Spleen cells were isolated from uninfected or 7-8 week infected BALB/c mice. The expression of A, GITR B, CD103 and C, CTLA-4, on CD4⁺CD25⁺ and CD4⁺CD25⁻ cell populations was analysed. Data shown are representative of an individual mouse from 3-4 analysed in 2-4 separate experiments. All quadrants were set on appropriate mAb isotype controls. Bar graphs represent mean $\pm$ SEM of infected individual mice from all experiments. Student's t-test was performed to compare differences between CD25⁺ and CD25⁻ populations in the infected group.

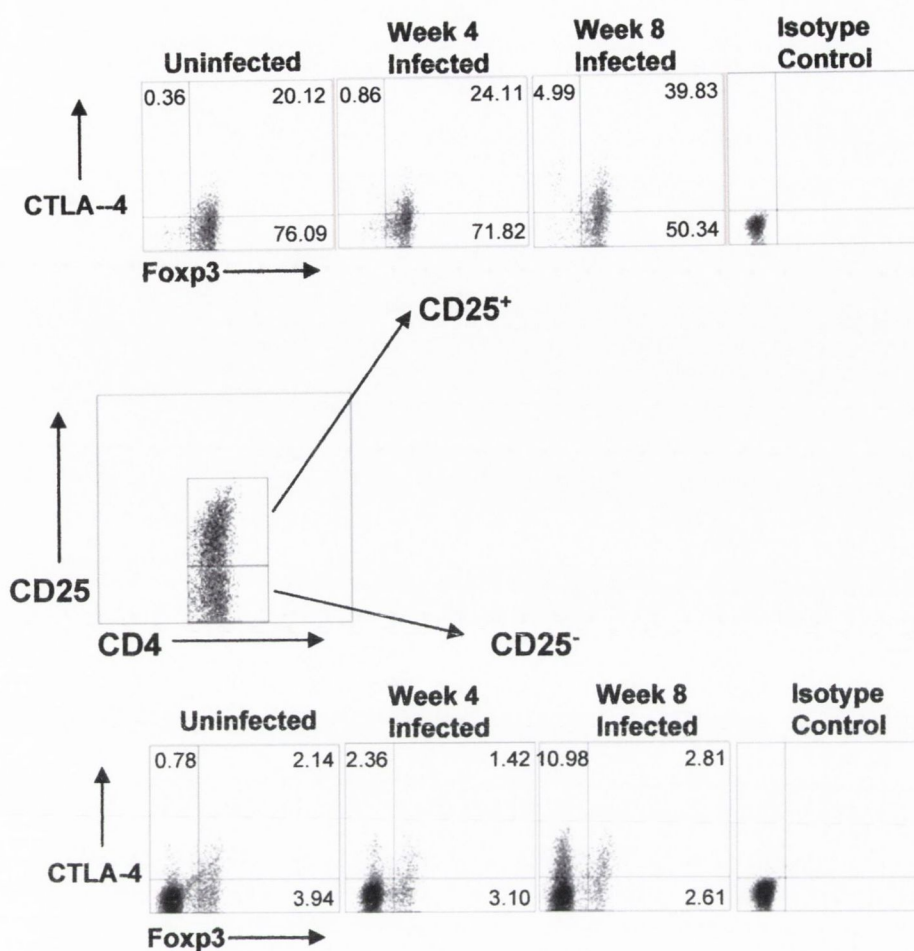


Figure 3.18. Infection induced alterations in CTLA-4 and Foxp3 expression on CD25⁺ or CD25⁻ CD4⁺ spleen cells. Cells were gated as CD4⁺CD25⁺ or CD4⁺CD25⁻ and expression of CTLA-4 and Foxp3 on each population was assessed. CTLA-4 and Foxp3 mAb isotypes are shown. Data are representative of 2 separate experiments. Data was analysed using Summit software.

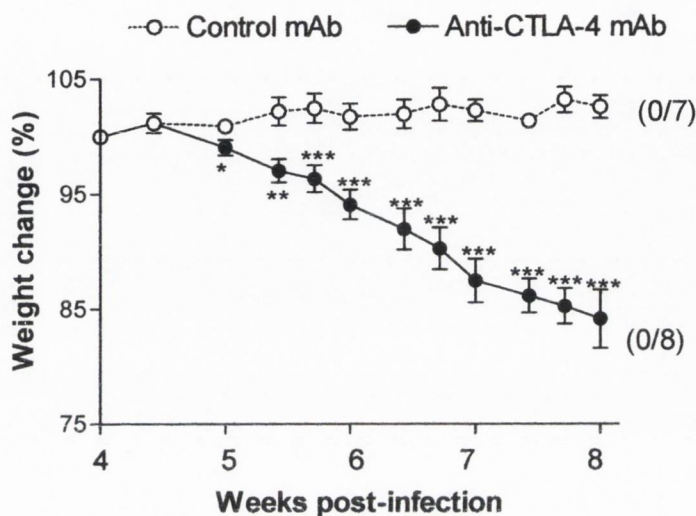


Figure 3.19. *Weight change in control mAb or anti-CTLA-4 mAb treated S. mansoni-infected mice.* 100 cercariae-infected BALB/c mice were treated with control hamster mAb (clone UC8-1B9) or anti-CTLA-4 mAb (UC10-4F10-11) from 4 weeks post-infection, and mice were weighed periodically until 8 weeks post-infection. Data are mean values from 6-8 infected mice per group, and are representative of at least 4 separate experiments. Student's t-test was used to test for differences in weight loss between groups (*, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.0001$).

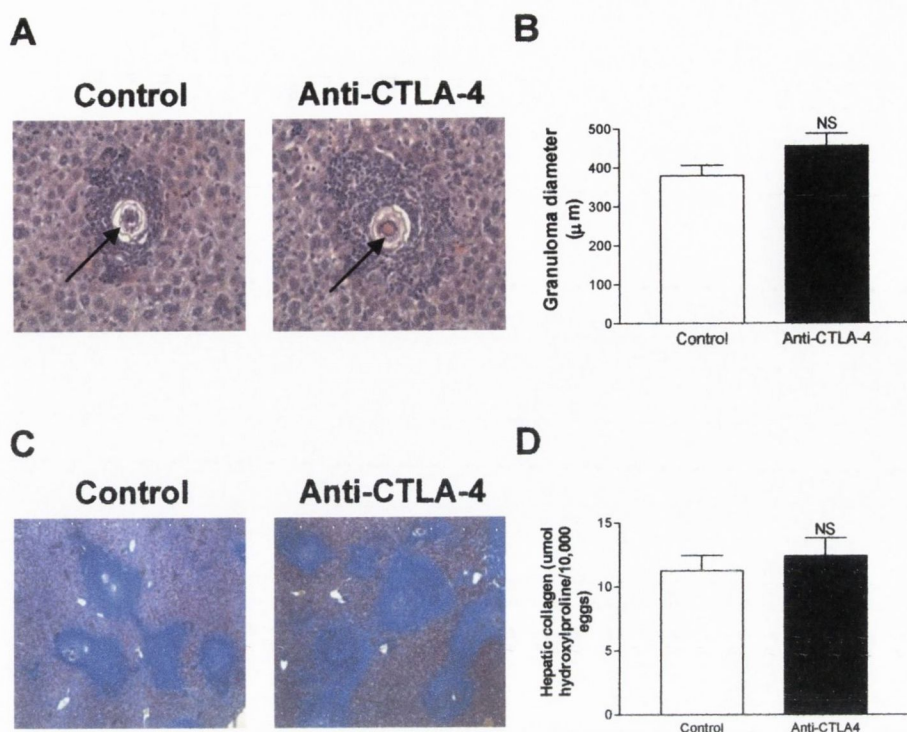


Figure 3.20. *Liver granuloma size and fibrosis in control mAb and anti-CTLA-4 mAb-treated mice during acute infection.* *A*, Representative image of granuloma around egg in the liver of untreated or anti-CTLA-4 mAb-treated mouse. Arrows are pointing to the egg. Sections were stained with hematoxytin and eosin. *B*, Liver granuloma diameter. *C*, representative image of Martius Scarlet Blue-stained sections of liver granuloma. Fibrosis is shown in blue. *D*, liver fibrosis, as shown by hepatic collagen, expressed as μmol hydroxyproline/10,000 eggs. Mice were culled on day 56 and data are mean $\pm$ SD from 5-8 mice per group. Student's t-test was used to compare differences between groups. All experiments were repeated at least twice.

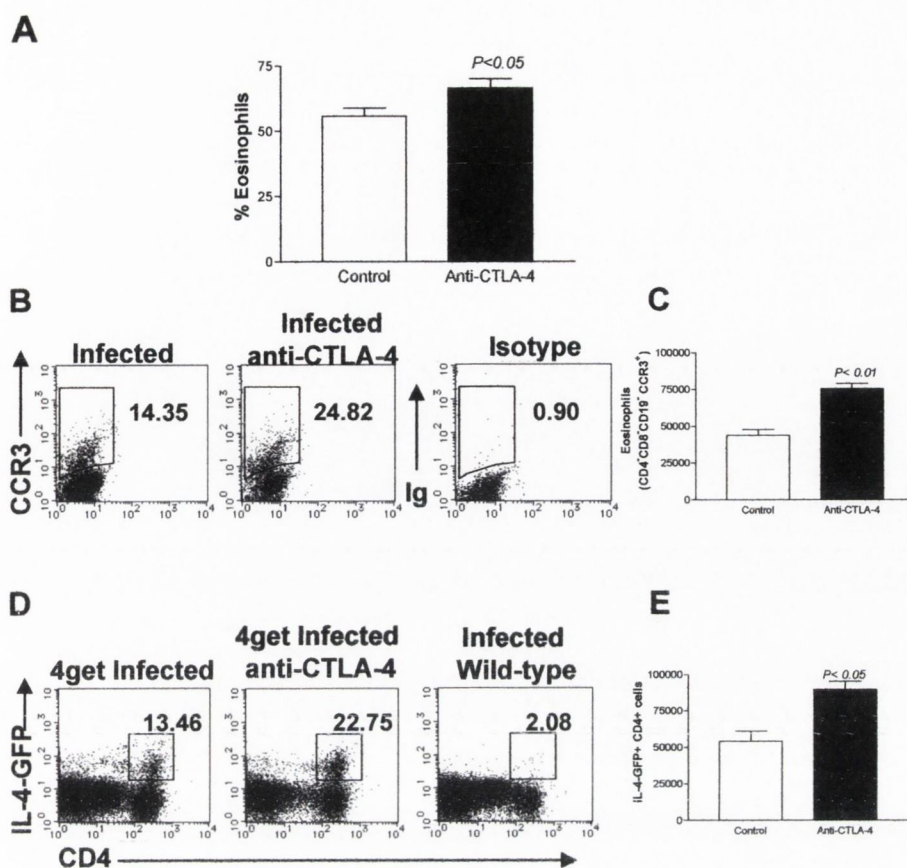


Figure 3.21. Increased eosinophils and Th2 cells in liver granuloma of anti-CTLA-4 mAb-treated infected mice. *A*, numbers of eosinophils within the granuloma on histology slides. *B*, representative image of granuloma eosinophils by flow cytometry. Values are percentage of non-B, non-T (CD4⁺CD8⁺CD19⁺) cells that were CCR3⁺ from pools of livers from 3 infected mice. *C*, bar graph with mean $\pm$ SEM from 2-3 pools from 2-3 different experiments. *D*, frequencies of Th2 cells, CD4⁺ IL-4-GFP⁺, in granulomas from pools of livers from control or anti-CTLA-4 mAb-treated *S. mansoni*-infected 4get mice. An infected wild-type mouse is shown for gating as a GFP⁻ control. *E*, bar graph with mean $\pm$ from 2-3 pools from 2-3 different experiments. Mice were culled on day 56 and data are mean $\pm$ SD from 5-8 mice per group. All experiments were repeated at least twice. Student's t-test was used to compare differences between groups.

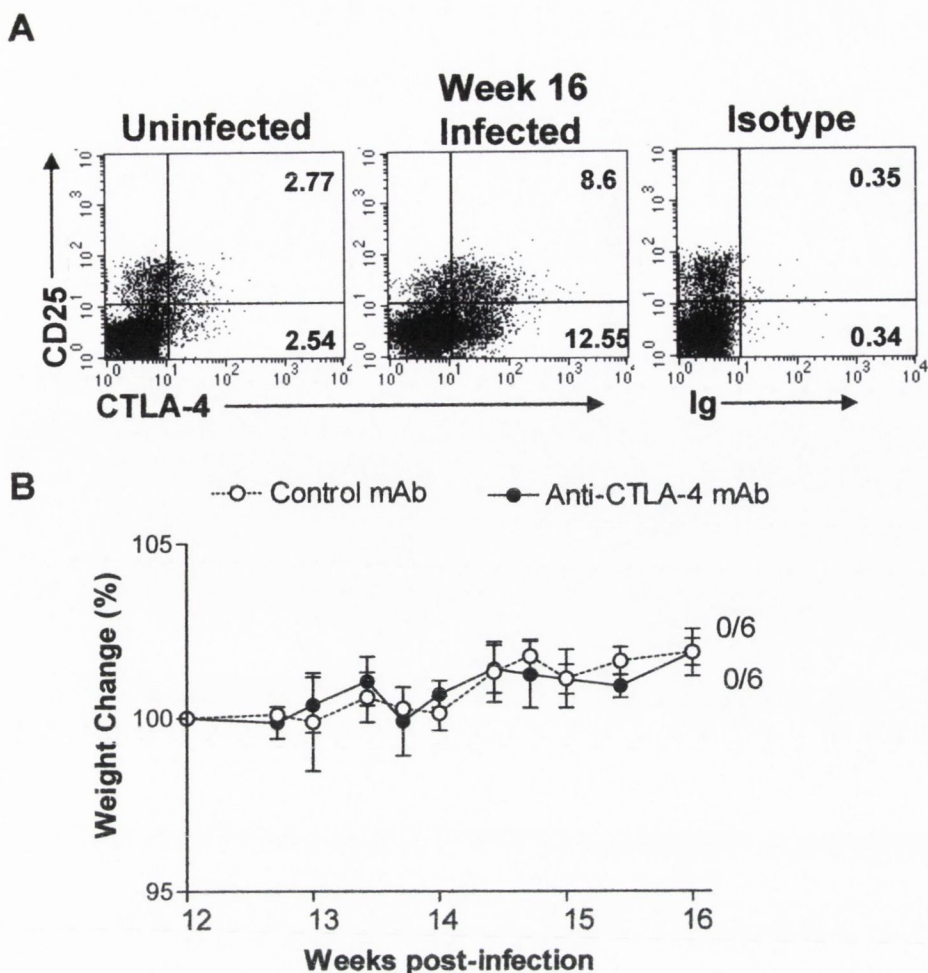


Figure 3.22. Elevated CTLA-4 expression in chronically infected mice but blockade of CTLA-4 in the chronic stage of infection does not induce weight change between groups. *A*, spleen cells from uninfected or 16-week infected BALB/c mice were assessed for expression of CTLA-4 on CD4⁺CD25⁺ and CD4⁺CD25⁻ cell populations. Data shown are representative of an individual mouse from 3-4 analysed in 2-3 separate experiments. Quadrants were set on appropriate mAb isotype controls. *B*, BALB/c mice were treated with control hamster IgG (clone UC8-1B9) or anti-CTLA-4 mAb (UC10-4F10-11) from 12 weeks post-infection, and mice were weighed periodically until 16 weeks post-infection. Data are mean values from 6 infected mice per group, and are representative of 3 separate experiments.

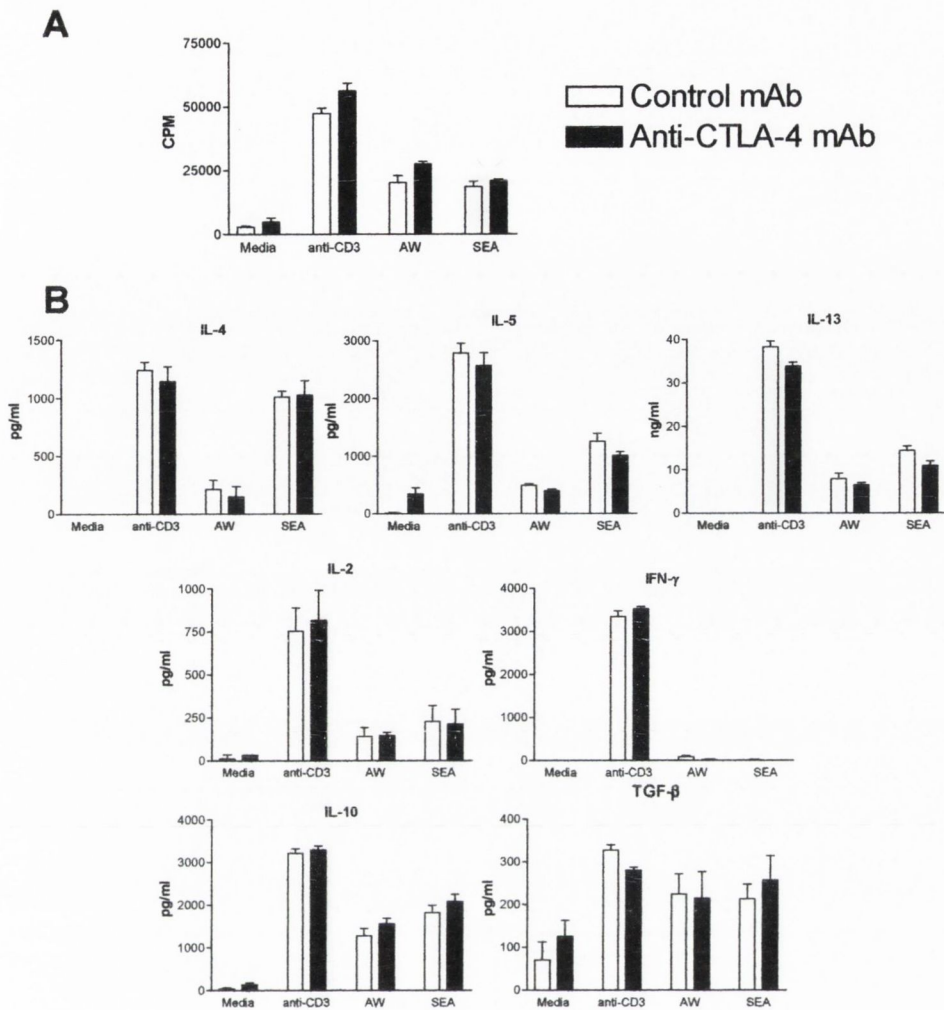


Figure 3.23. *Blocking CTLA-4 during the chronic stage of infection induced little alteration in cytokine profile, but induced some spontaneous IL-5 production from spleen cells. A, proliferation of unstimulated or stimulated (anti-CD3 mAb, AW, SEA) spleen cells, expressed as CPM. B, cytokine production from unstimulated (media) or stimulated (anti-CD3 mAb, AW, SEA) spleen cell supernatants. Cytokines were measured by ELISA (IL-2 after 24h, IL-4, IL-5, IL-13, IL-10, IFN- γ , TGF- β after 72h). Data shown is from pools of spleen cells from three individual mice, and are representative of at least 3 separate experiments.*

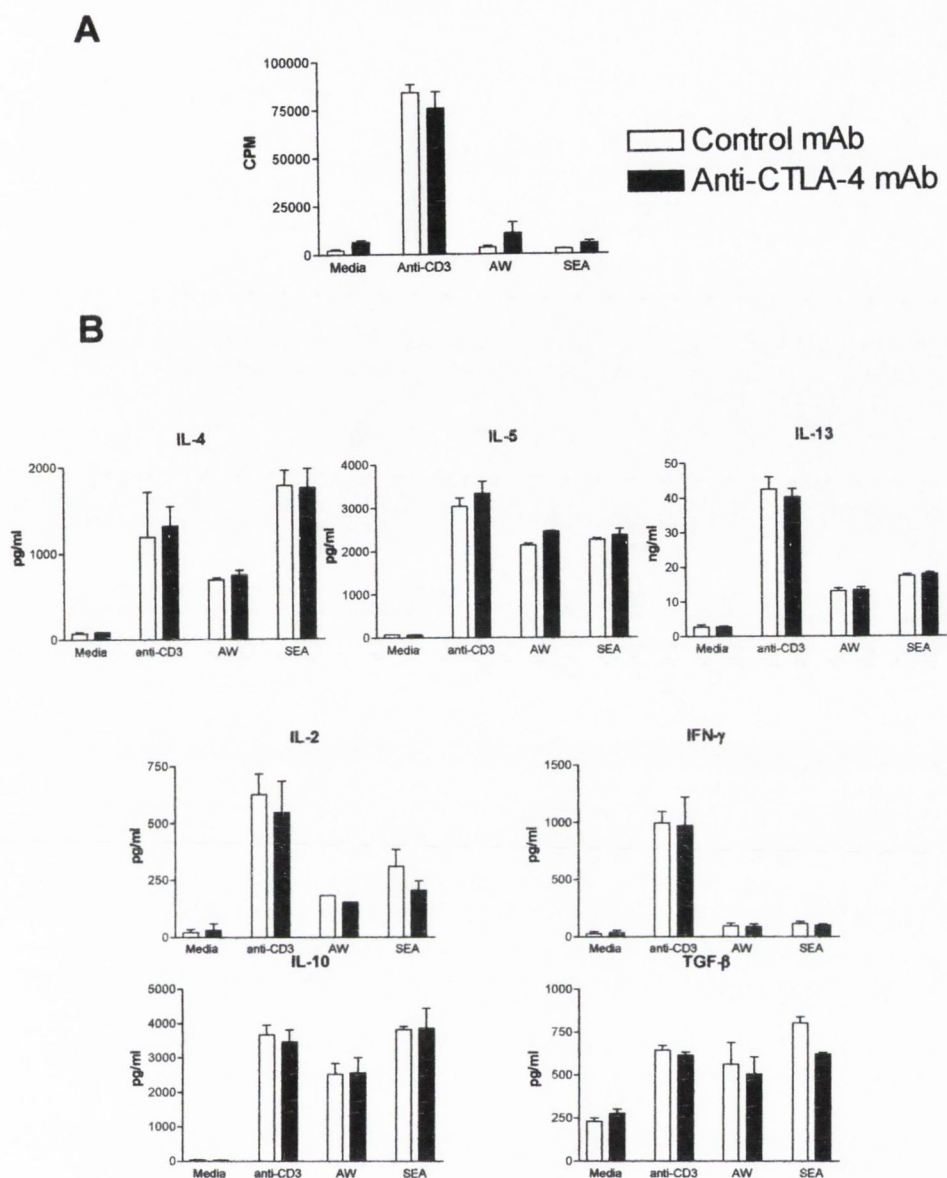


Figure 3.24. *CTLA-4 blockade induces no alteration in MLN cell responses in chronic *S. mansoni* infection.* A, proliferation of unstimulated or stimulated MLN, analysed as described in Figure 3.23. B, cytokine production from MLN, analysed as described in Figure 3.23. Data is from pools of MLN from 3 individual mice, and is representative of 3 separate experiments

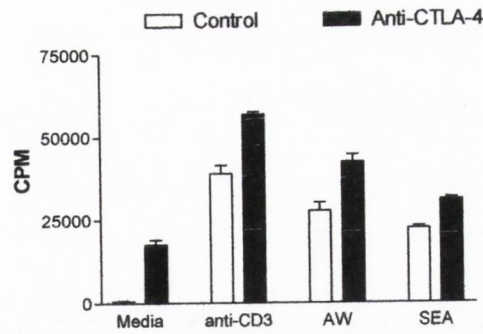
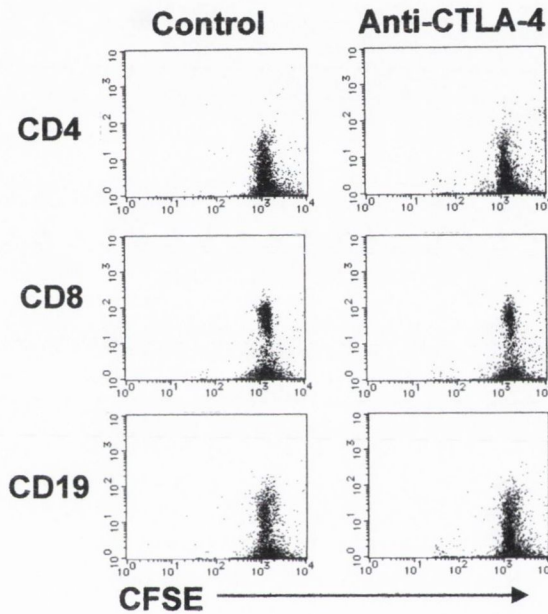
A**B**

Figure 3.25. *CTLA-4 blockade during acute *S. mansoni* infection caused some spontaneous proliferation of unstimulated spleen cells.* *A*, proliferation of spleen cells from infected control or anti-CTLA-4 mAb-treated infected mice. Cells were stimulated with media, anti-CD3 mAb, AW or SEA. [³H]thymidine incorporation is expressed as counts per minute (CPM). *B*, spleen cells from both groups were stained with 0.5 μ M CFSE and cultured *in vitro* for up to 72h. Cells were stained with antibodies for CD4, CD8, and CD19, and analysed by flow cytometry. Data is from pooled spleens from at least 3 separate experiments.

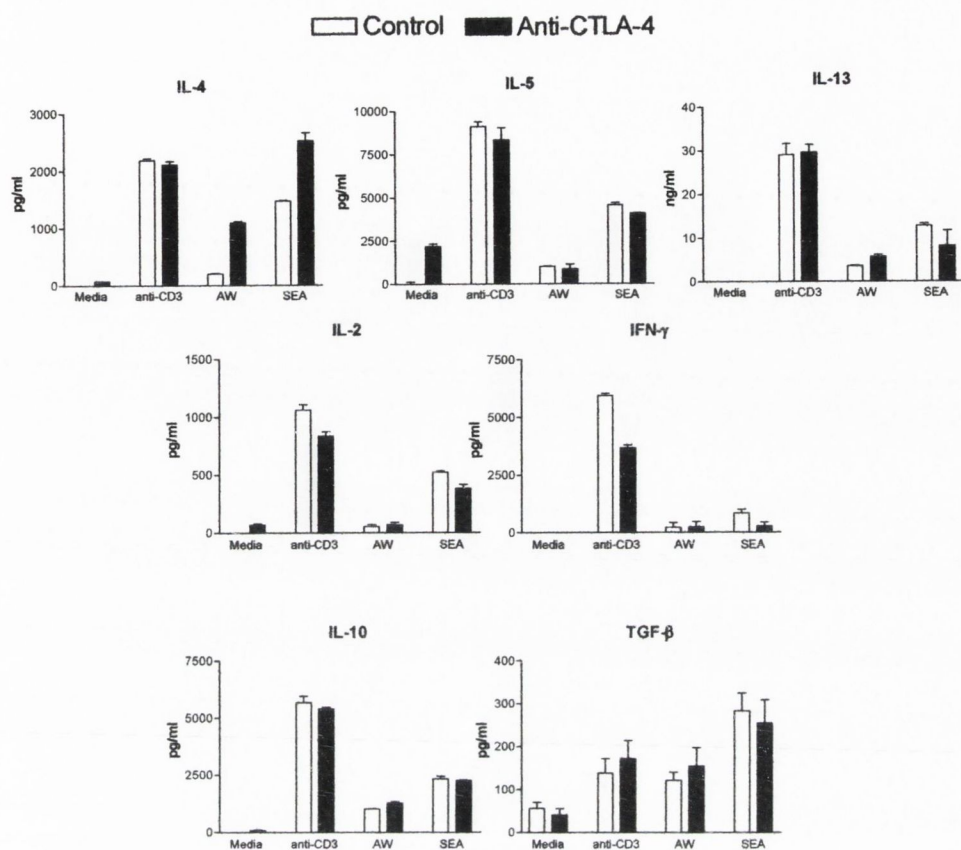


Figure 3.26. *Spontaneous production of IL-5 by spleen cells from infected mice treated with anti-CTLA-4 mAb.* Cytokine production from stimulated spleen cells from infected control or anti-CTLA-4 mAb-treated infected mice. Data shown is from pools of spleen cells from three individual mice, and are is representative of at least 4 separate experiments.

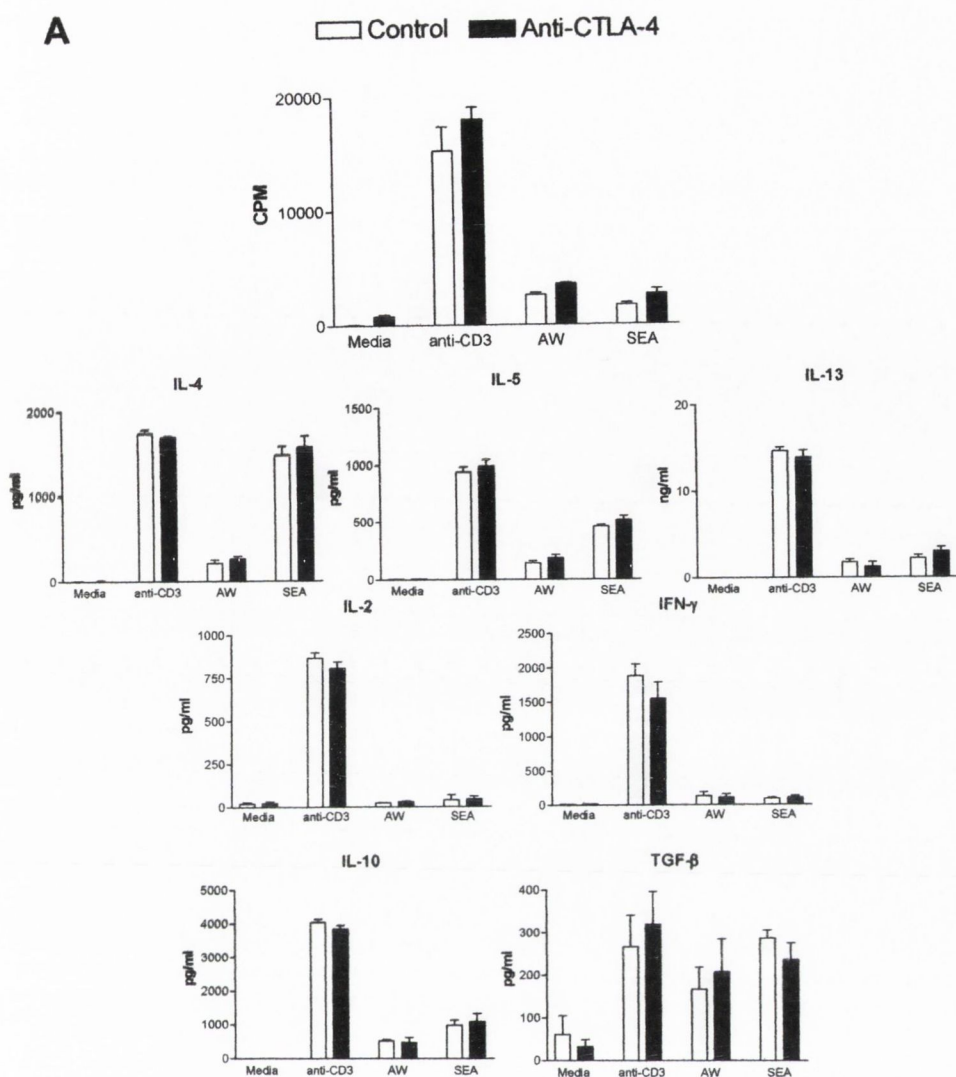


Figure 3.27. Normal proliferation and cytokine profile in stimulated MLN from acutely infected mice treated with anti-CTLA-4 mAb. *A*, proliferation of MLN cells from infected control or anti-CTLA-4 mAb-treated infected mice. Cells were stimulated with media, anti-CD3 mAb, AW or SEA. [³H]thymidine incorporation is expressed as counts per minute (CPM). *B*, cytokine production from stimulated MLN cells from infected control or anti-CTLA-4 mAb-treated infected mice. Data shown is from pools of spleen cells from three individual mice, and are representative of at least 3 separate experiments.

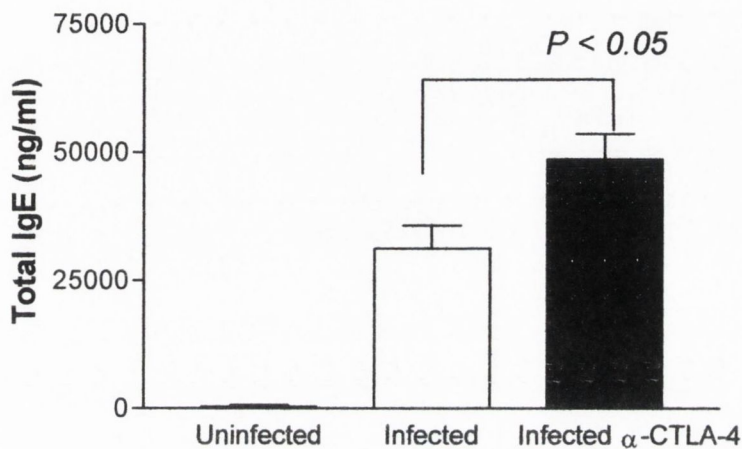
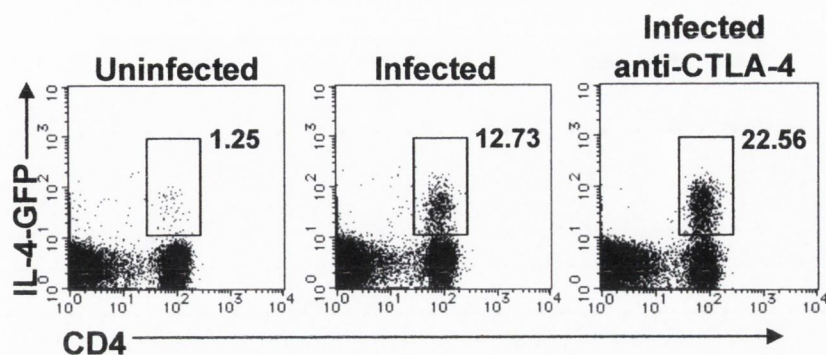


Figure 3.28. *Increased levels of total serum IgE in anti-CTLA-4 mAb treated infected mice compared to control infected mice. Total IgE in sera from infected or anti-CTLA-4 mAb-treated infected mice was measured by direct ELISA, and expressed as ng/ml. Student's t-test of 10 mice per group was performed to test differences between infected groups.*

A



B

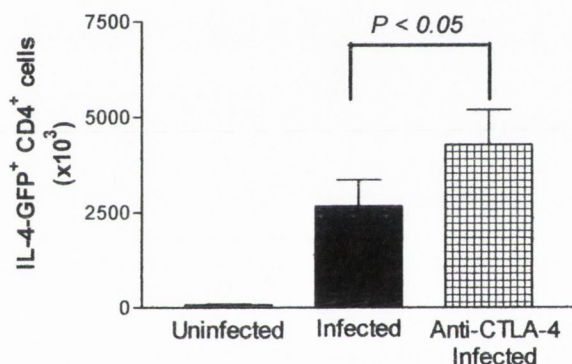


Figure 3.29. Increased Th2 cells in the spleens of infected anti-CTLA-4 mAb treated mice, as shown by IL-4-GFP expression. *A*, Representative image of frequencies of Th2 cells (CD4⁺ IL-4-GFP⁺) in spleen cells from anti-CTLA-4 mAb-treated infected 4get mice relative to levels in infected mice treated with control mAb. *B*, Absolute numbers were determined based on whole spleen cell counts, and data represents mean $\pm$ SD of 4 individual mice. Student's t-test was used to determine differences between infected groups.

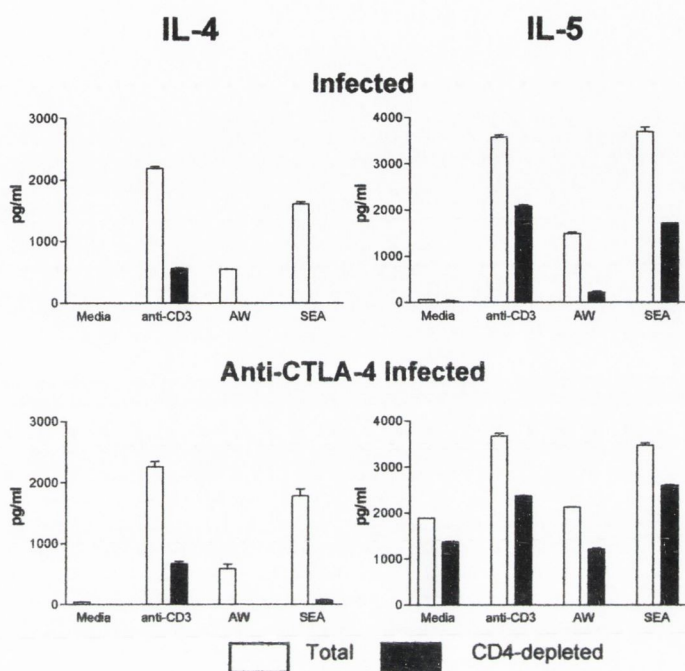


Figure 3.30. *IL-5* from *anti-CTLA-4* mAb-treated mice is predominately derived from non- $CD4^+$ cells. *IL-4* and *IL-5* production by whole spleen cells (Total) or $CD4^+$ cell-depleted spleen cells from control or *anti-CTLA-4* mAb-treated *S. mansoni* infected mice. $CD4^+$ cells were depleted by incubating cells with anti- $CD4^+$ (YTS191) followed by complement depletion and cells were stimulated for 72 h with media, anti- $CD3$ mAb, AW or SEA. Levels of the cytokine *IL-4* and *IL-5* were detected by ELISA. Data are pooled cells from three spleens and are representative of 3 separate experiments.

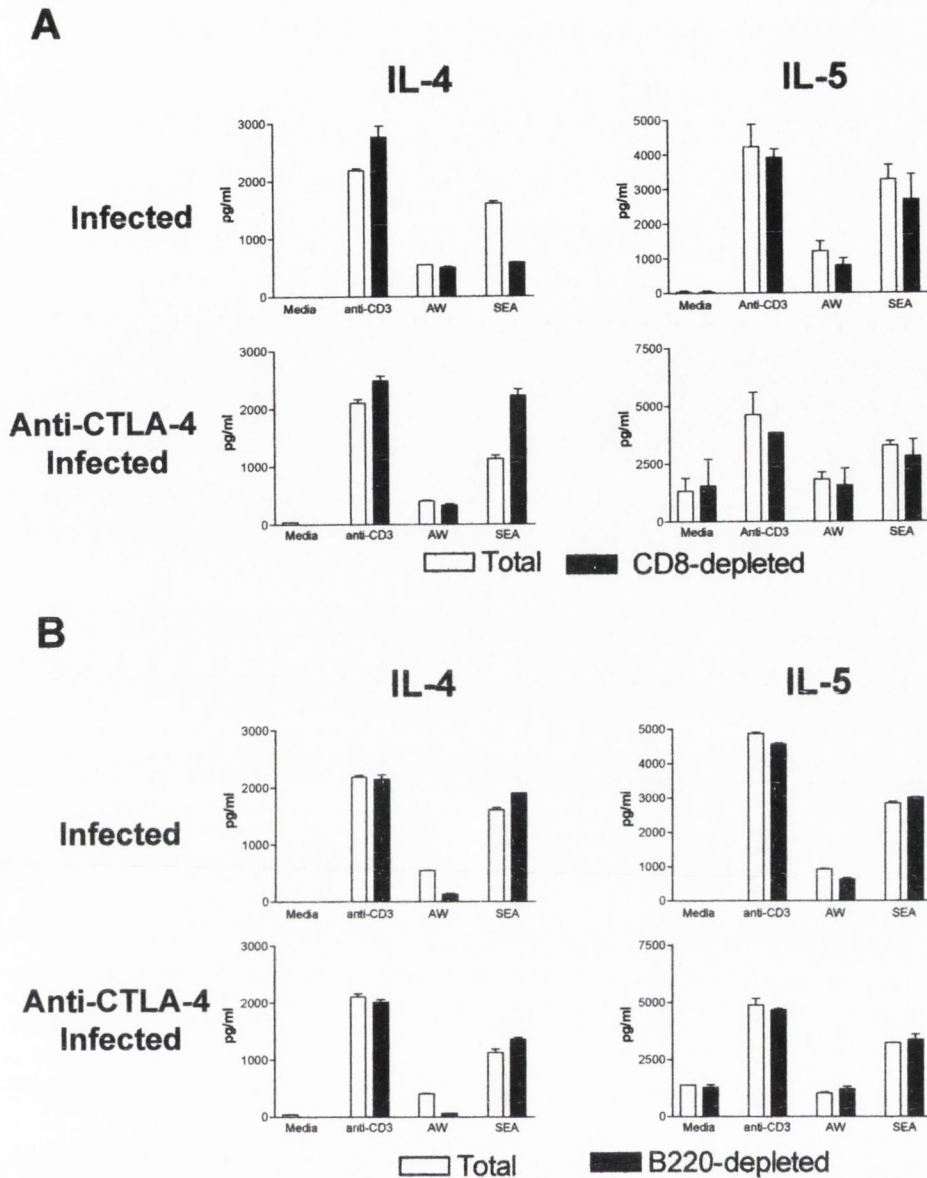


Figure 3.31. *IL-5 is mainly produced by a non-B, non-T cell source.* Splenic CD8⁺ cells (A) and B cells (B) were depleted *in vitro* using antibody-mediated complement depletion. (YTS169 to deplete CD8, TB146 to deplete B cells). Total cells and *in vitro* depleted cells were stimulated for 72 h with media, anti-CD3 ϵ , AW or SEA. Levels of the cytokine IL-4 and IL-5 were detected by ELISA. Spleens were removed 56 days post-infection. Data are pooled cells from three spleens and are representative of 3 separate experiments.

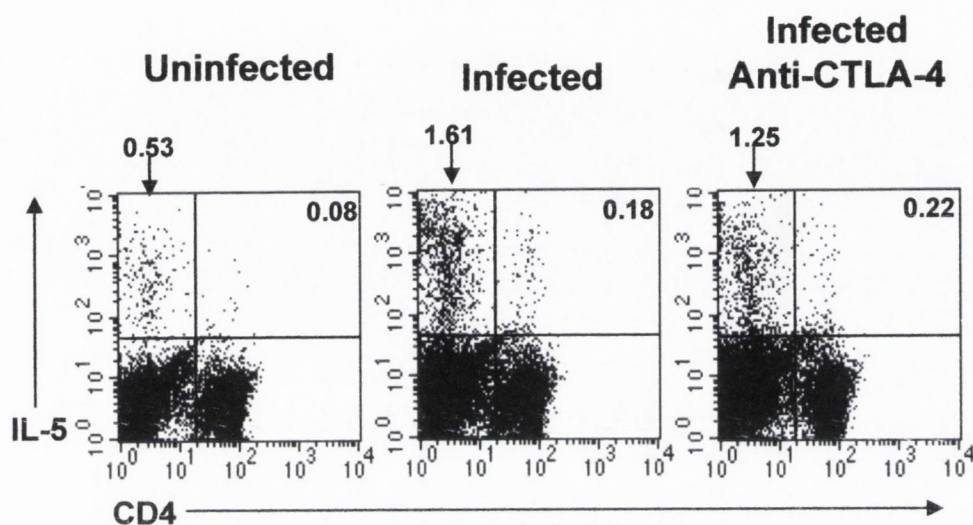


Figure 3.32. *IL-5-production of unstimulated spleen cells using intracellular cytokine staining.* Spleen cells were cultured in media for 6 h @ 37°C, with Brefeldin A (10 µg/ml) added for the final 4 hours of culture. Cells were stained for surface CD4 and intracellular IL-5. Quadrants were drawn based on appropriate isotype controls. Data is from pools of spleen cells from 3 mice and is representative of 2 separate experiments.

3.3 Discussion

This chapter demonstrates that during *S. mansoni* infection of mice there is an expansion of CD4⁺ cells that co-express CD25, a putative marker for natural T_{reg} cells. These CD4⁺CD25⁺ cells were isolated from the spleen of 4 and 8 week infected mice and were shown to potently suppress proliferation of naïve CD4⁺CD25⁻ cells *in vitro*. However, despite the expansion of these CD4⁺CD25⁺ cells in acute through chronic stages of infection, the *in vivo* depletion of CD25⁺ cells from weeks 4-8 or 12-16 of infection had no effect on schistosome infection or parasite-induced changes in immunity. These data indicate no function for CD25-expressing cells during *S. mansoni* infection of mice. As an essential role for CD4⁺ cells in infection has been confirmed, it indicates that a CD4⁺, but CD25⁻ cell population may regulate immunopathology in infection.

As mentioned above, CD4⁺CD25⁺ cells from 4- and 8-week infected mice were more potent at suppressing naïve CD4⁺CD25⁻ cell proliferation *in vitro* than uninfected mice (Fig. 3.3). CD25 and Foxp3 expression was sustained throughout infection, with slight increase in Foxp3 observed at week 16 (Fig. 3.2, 3.4). It was somewhat surprising, therefore, for the suppressive activity of CD4⁺CD25⁺ cells from chronically infected mice to be almost completely ablated (Fig. 3.5). This may be attributed to contamination of approx. 20% in the AUTOMACS-isolated CD4⁺CD25⁺ cells from 16-week infected mice (Fig. 3.6). Spleen cells from infected mice are more activated than uninfected mice, with expanded populations of B cells and also large, granular cells that may be autofluorescent. This is exacerbated with chronic infection, where spleens often have up to 3 times as many cells as uninfected mice. In addition, there are also a greater number of T cells undergoing activation-induced cell death (AICD) in spleens and granulomas of infected mice (Estaquier *et al.*, 1997; Fallon *et al.*, 1998;

Lundy *et al.*, 2001). However even with the reduced purity, the range of effector:target ratios used would have been expected to demonstrate some suppression of naïve T cell proliferation at the higher ratios. Another possibility is that the contaminating cells (appear to be larger and more granular from forward-side-scatter) are somehow inhibiting the function of the $CD4^+CD25^+$ cells, or are themselves stimulating proliferation of the naïve T cells. There is also contamination from a $CD4^+$ but $CD25^-$ population. Indeed it has recently been shown that IL-4 can induce protection of $CD4^+CD25^-$ cells from $CD4^+CD25^+$ T_{reg} -mediated suppression (Pace *et al.*, 2006). The contaminants present in $CD4^+CD25^+$ cells isolated from chronically infected mice may be a source of IL-4, and thus could be inhibiting the T_{reg} -mediated suppression. The recent access to cell-sorting facilities in TCD ($CD4^+CD25^+$ cells could potentially be sorted at 98% purity in both groups) could help solve the dilemma and determine whether the data is reliable, or merely due to reduced purity from chronically-infected mice compared to $CD4^+CD25^+$ cells from uninfected mice.

Recent studies have already highlighted a potential role for $CD4^+CD25^+$ T cells and IL-10 producing T cells in schistosome infection (Hesse *et al.*, 2004; McKee and Pearce, 2004; Singh *et al.*, 2005). Hesse and co-workers first demonstrated a central role for both IL-10-producing innate effectors and regulatory T cells in reduction of morbidity and mortality in chronic schistosomiasis (Hesse *et al.*, 2004). They demonstrated that within liver $CD4^+$ granuloma cells of chronically-infected mice (>12 weeks), the main producer of IL-10 was the $CD4^+CD25^+$ subset, with these cells having a significant role in transferring protection from morbidity during infection (Hesse *et al.*, 2004). I studied the expression of $CD25^+$ on spleen $CD4^+$ cells before arrival of eggs (week 4) and at peak (week 8) and chronic (week 16) stages of infection. Despite showing elevation of $CD4^+CD25^+$ cells from week 4 of infection, $CD25$ cell depletion had no influence on acute or chronic stages of schistosome infection (Fig. 3.8,

3.12). CD25⁺ cell-depleted mice had comparable worm burdens/fecundity, degree of weight loss, tissue egg counts and granuloma size/composition and immune responses as control infected mice. Since CD4⁺CD25⁺ T cells are elevated by week 4 of infection, which is prior to egg-laying, there could be a role for these cells in infectivity of the schistosome parasite, but I have not addressed this in this chapter.

As CD4⁺ cells are protective in acute infection it had been postulated that CD25⁺ cells would be implicated in regulation of immunopathology in infection. It was therefore unexpected that CD25⁺ cell depletion did not alter acute or chronic infection. One potential concern with the data could have been that the CD25⁺ cells were not adequately depleted. However this was not the case as I obtained >85 % reduction in the spleen, mesenteric lymph nodes and liver granuloma (Fig. 3.15). Furthermore, the levels of CD25 cell depletion obtained using the anti-CD25 mAb PC61 over the 4 week regime used in this study are similar to a number of other systems where *in vivo* depletion of > 80% have successfully demonstrated a biological function for CD25 cells. PC61 depletion of CD25⁺ cells has been used in studies of, for example, experimental allergic/autoimmune encephalomyelitis (McGeachy *et al.*, 2005; Stephens *et al.*, 2005; Zhang *et al.*, 2006), tumors (Onizuka *et al.*, 1999; Shimizu *et al.*, 1999), *Leishmania major* infection (Aseffa *et al.*, 2002; Mendez *et al.*, 2004), and viral keratitis (Suvas *et al.*, 2004). It is significant that a recent study has issued questions on interpretation of *in vivo* depletion of CD25⁺ cells using PC61 (Kohm *et al.*, 2006). Kohm *et al.* have found that administration of anti-CD25 mAb (clones PC61 or 7D4) to naïve mice does not alter spleen or mesenteric lymph node Foxp3 protein expression (Kohm *et al.*, 2006), however another group reported almost complete removal of CD25⁺Foxp3⁺ cells in lymph nodes one week after PC61 administration (Taylor *et al.*, 2006).

I have not addressed if there was functional inactivation of CD25 in mice treated with anti-CD25 mAb (PC61). It is relevant that in this study, despite an overall 30% reduction in Foxp3 expression on CD4⁺ cells following anti-CD25 mAb treatment, there was an increase in Foxp3⁺ CD25⁻ cells (Fig. 3.15), but we do not know if such cells were functional *in vivo* and compensated for the absence of CD25⁺ cells. The advent of Foxp3-red fluorescent protein reporter mice (Wan and Flavell, 2005), which are now being bred in-house, will enable such studies to be performed in the future.

Potentially, the essential role for CD4⁺ cells in acute infection could be due to their production of the Th2 cytokine IL-4, which has an important host protective role (Fallon *et al.*, 2000a; Hoffmann *et al.*, 2000; Rosa Brunet *et al.*, 1997) or IL-10, which also potently prevents morbidity and mortalities in acute infection (Flores Villanueva *et al.*, 1993; Hoffmann *et al.*, 2000; Wynn *et al.*, 1998). However, spleen and mesenteric cells from CD25⁺ cell-depleted mice had normal production of IL-4 and IL-10 *ex vivo* (Fig. 3.10, 3.11). Furthermore, CD25⁺ cell-depletion may have not dramatically altered IL-4 and IL-10 production in infected mice as there was a substantial population of IL-10- and IL-4-producing CD4⁺ cells in addition to expression of the Th2 marker T1/ST2 on CD25⁻ populations (Fig. 3.16). A recent paper has also shown that both IL-4 and IL-10 is preferentially produced by CD25⁻ CD4⁺ cells in schistosome infection (Baumgart *et al.*, 2006).

Others have highlighted that using CD25 as a target for selective depletion of T_{reg} cells is confounded by the removal of CD25⁺-activated effector T cells as well as CD4⁺CD25⁺ naturally occurring T_{reg} cells (Ko *et al.*, 2005). Our data shows that in schistosome infection CD25 expression on CD4⁺ cells is possibly more a marker for cell activation than putative naturally occurring T_{reg} cell activity. Foxp3 is considered to be a better marker than CD25, with some groups preferring to use it as an exclusive marker for T regulatory cells (Fontenot

and Rudensky, 2005). However, despite the increase in spleen $CD4^+CD25^+$ cells following schistosome infection, we observed no increase in intracellular Foxp3⁺-expressing cells at week 4 or 8 post-infection, but with a significant small increase by week 16 (Fig. 3.2, 3.4). Recently, Singh and colleagues also have found that mRNA expression for Foxp3 in the spleens of 8 week-infected mice was comparable to mRNA levels in uninfected spleens, with almost a 4-fold increase in Foxp3 mRNA in spleens of chronic (week 16) infected mice (Singh *et al.*, 2005). As I used intracellular detection, I could discriminate the $CD4^+$ subpopulation that expressed Foxp3. The increase in Foxp3-expressing $CD4^+CD25^+$ cells observed in this study was not of the same magnitude as that reported by Singh and co-workers (Fig. 3.4). There was no increase in Foxp3 expression on $CD4^+CD25^+$ cells from acute 4 and 8 week-infected mice versus levels in cells from uninfected mice, similar to what was seen in mRNA detection of Foxp3 (McKee and Pearce, 2004).

In a range of infectious pathogens that, like schistosomes, stimulate an expansion of $CD4^+CD25^+$ cells with infection, depletion of $CD25^+$ cells has a profound effect on the outcome of infection (Belkaid and Rouse, 2005). However, that $CD25$ appeared to play no role in any aspect of acute or chronic schistosome infection prompted me to examine expression of other regulatory cell markers during infection. While it was shown that the expression of other regulatory markers $CD103$ and $GITR$ were predominately associated with the $CD25^+$ sub-population of $CD4^+$ cells, infection also induced a significant population of $CTLA-4^+$ cells that were $CD25^-$ (Fig. 3.17). $CTLA-4$ binds $B7-1/B7-2$ on APCs and is known to have a role in limiting Th2 differentiation (Oosterwegel *et al.*, 1999), as discussed in section 1.5.

The use of $CTLA-4$ deficient mice for *in vivo* studies is limited due to the potent autoimmune and lymphoproliferative phenotype that develops, and mice do not survive past 3

weeks of age (Tivol *et al.*, 1995). Therefore to address the function of this regulatory marker in *S. mansoni* infection, a blocking antibody against CTLA-4 was administered in acute and chronic stages of infection. In acute infection anti-CTLA-4 mAb-treated mice suffered significant weight loss with no mortalities (Fig. 3.19). In contrast, despite having up-regulation in CTLA-4 expression on CD4⁺ cells in chronic infection, blocking mAb treatment had no effects on any parameter tested (Fig. 3.22). Weight loss in acute infection in CTLA-4 mAb treated mice was associated with selective effects on type 2 responses, with elevated IL-4 and IL-5 production, and significant increases in eosinophils and Th2 cells in liver granulomas (Fig. 3.21). In other studies it has been shown that schistosome infection of IL-10/IL-12 double-deficient mice, or IL-9 transgenic mice, develop an extreme type 2 polarized response leading to exacerbated pathology and mortalities (Fallon *et al.*, 2000b; Hoffmann *et al.*, 1999). However, in this study the magnitude of increase of production of IL-4 and IL-5 was not as high, which might explain the weight loss without overt severe pathology or mortalities. The exacerbated Th2 response demonstrated in this chapter led to increases in eosinophils in the liver granuloma, but as mentioned earlier, this caused no severe pathology. This is consistent with a recent paper, which demonstrated no role for eosinophils in schistosome infection using eosinophil-lineage ablated mice (Swartz *et al.*, 2006).

IL-5 is important for induction of B cell growth, differentiation and immunoglobulin production (Takatsu *et al.*, 1994). It is also critical in regulating the growth, activation and survival of eosinophils, which are important effector cells in helminth infections and allergies. In allergic airway models, eosinophils are shown to produce IL-5 once they enter the airways, thus establishing a positive feedback loop (Dubucquoi *et al.*, 1994). IL-5 mediates its effects through signalling via different pathways than those of the other Th2 cytokines IL-4 and IL-13 (Adachi and Alam, 1998). A recent study using IL-5 deficient mice showed that regulation of

IL-13 activity by IL-5 increased the progression of liver fibrosis in *S. mansoni* infection (Reiman *et al.*, 2006). IL-5^{-/-} mice had smaller granulomas, with less fibrosis, and only very few eosinophils present. The group postulate that IL-13 production by eosinophils may contribute to hepatic fibrosis in infected mice.

In this chapter the observation of increased IL-4 and IL-5 when CTLA-4 is blocked in mice with acute schistosome infections is comparable to earlier studies in mice infected with the gastro-intestinal nematode *Nippostrongylus brasiliensis* (McCoy *et al.*, 1997). Anti-CTLA-4 mAb treatment of *N. brasiliensis*-infected mice caused a dramatic reduction in worm burden, with enhanced production of Th2 cytokines IL-4 and IL-5 in the mesenteric lymph nodes that drain the site of infection (McCoy *et al.*, 1997). In this study there was also an increase in IL-4 production in anti-CTLA-4 mAb-treated mice, involving an expansion of spleen and granuloma Th2 cells and a consequential elevation in serum IgE. Surprisingly, the marked spontaneous production of IL-5 in the media of cultured spleen cells from anti-CTLA-4 mAb treated mice was not from CD4⁺, or from CD8⁺ or B cells (Fig. 3.30, 3.31). It had been previously reported that mixing of spleen cells from infected mice with granuloma eosinophils resulted in spontaneous IL-5, but not IL-4, production from spleen cells, in a manner that was partially dependent on spleen cell-derived IL-2 (Metwali *et al.*, 1993). IL-5 may also be produced by non-T cell sources, such as eosinophils and mast cells (Castro *et al.*, 1995). Previously, eosinophils from *S. mansoni*-infected mice were shown to produce a major portion of Th2 cytokines, including IL-5, in the granuloma milieu (Rumbley *et al.*, 1999). In view of the increase in granuloma eosinophils in anti-CTLA-4 treated mice it is possible that the spontaneous release of IL-5 is from eosinophils. Using intracellular cytokine staining I detected very low levels of IL-5 in CD4⁺ and non-CD4⁺ T cells, but there was no increase in production in the anti-CTLA-4 mAb-treated group (Fig. 3.32). It is interesting to note that

another group have also reported to have had difficulty in detecting IL-5 production using flow cytometry intracellular antibodies (Rumbley *et al.*, 1999). The recent use of anti-Siglec-F mAb to isolate eosinophils from liver granulomas from *S.mansoni*-infected mice for detection of IL-13 mRNA (Reiman *et al.*, 2006), is one avenue that may be worth pursuing. Indeed Reiman and co-workers noted that significant amounts of type 2 cytokines (including IL-5) were detected in unstimulated purified eosinophils. The generation of a transgenic mouse with GFP linked to the IL-5 reporter sequence, similar to the 4get mice described in this chapter, would also be of immense benefit for the study of IL-5-producing populations.

Brugia malayi worm-infected patients have elevated CTLA-4⁺ expression on CD4⁺ cells compared to uninfected individuals, with CTLA-4 expression associated with suppression of T cell reactivity (Steel and Nutman, 2003). Steel *et al.* showed that CTLA-4 was expressed on the CD4⁺CD25⁺ population of PBMCs at a greater intensity than on the CD4⁺CD25⁻ population. This is of interest as it has recently been shown that the suppressive properties of human CD4⁺CD25⁺ cells are dependent on CTLA-4 expression (Birebent *et al.*, 2004). Steel and co-workers found that *in vitro* blocking of CTLA-4 in PBMC of infected patients resulted in a substantial increase in IL-5 expression and a decrease in IFN- γ production in response to microfilarial antigen (Steel and Nutman, 2003), in contrast when PBMCs were exposed to live parasites blocking CTLA-4 restored both Th1 and Th2 cytokine genes (Babu *et al.*, 2006). In the murine studies with schistosome infection described here, there was also an increase in CTLA-4 expression on CD4⁺ cells and we also observed an increase in IL-5 production when CTLA-4 was blocked *in vivo*. This is suggestive that a common mechanism in the type 2 cytokine biased responses evoked by helminth parasites may involve the up-regulation of CTLA-4 expression on CD4⁺ cells to suppress the magnitude

of Th2 cell activity, as shown in *N. braziliensis* (McCoy *et al.*, 1997), *B. malayi* (Steel and Nutman, 2003) and *S. mansoni* (this study).

In a pulmonary type 2 allergen-sensitization model blocking CTLA-4, using the same mAb and BALB/c strain mice as this study, induced elevated IL-4 and IL-5 production, increased IgE as well as elevated lung eosinophilia (Hellings *et al.*, 2002), which I observed in mice with CTLA-4 blocked during acute schistosome infection. Similarly, blocking CTLA-4 in a peanut allergen oral-sensitization model also up-regulated polyclonal Th2 cytokine responses and total serum IgE (van Wijk *et al.*, 2005). However, whereas in the allergen pulmonary sensitization model (Hellings *et al.*, 2002) TGF- β was reduced, in this chapter I did not see a reduction in TGF- β production in cells from mice with CTLA-4 blocked, which may indicate that TGF- β is not involved in the CTLA-4-mediated mechanism of suppression in schistosome infection. Treatment of acutely-infected mice with anti-TGF- β mAb does not alter CD4 cellular response (data not shown), but the interplay between CTLA-4 and TGF- β in acute infection warrants further address. This data in schistosome infection using CTLA-4 mAb blocking experiments indicates that the increased expression of CTLA-4 on CD4⁺ cells may function to block excessive IL-4, and in particular IL-5.

It is surprising that in the chronic stages of infection, when CTLA-4 expression on CD4⁺ cells remains up-regulated, blocking mAb treatment had no effects. This could be due to CTLA-4 functioning to dampen the magnitude of the potent initial Th2 response evoked in the acute stages of infection, whereas in the down-modulated stages of chronic infection the Th2 responses is reduced by CTLA-4-independent mechanisms. Although IL-5 production was observed in unstimulated spleen cells, it was of a lesser magnitude than in spleen cells from acutely infected mice. In support of this concept, it has been shown that blocking

CTLA-4 mAb treatment altered the initial induction of tolerance to allergen in mice, but once inhalation tolerance was established anti-CTLA-4 mAb had no effect (Alenmyr *et al.*, 2005). So while CTLA-4 may be required for the initial regulation of T cell activation, other co-stimulatory pathways may come into play, that add to the complex layers of regulation on down-modulated cells, for example the ICOS pathway is known to regulate Th2 cells, and the PD-1 pathway is another B7 co-stimulatory pathway that mediates T cell inhibition (section 1.5).

In summary, I have shown that although there is induction of $CD4^+CD25^+$ T_{reg} cells in schistosome infection of mice, these cells do not have a critical role in acute or chronic infection. The increase in $CD25^+CD4^+$ cells in schistosome infection may be more indicative of cell activation than a regulatory activity. However, *S. mansoni* infection also induces a marked CTLA-4⁺ cell population on $CD4^+$ cells that were $CD25^-$. Blocking mAb treatment suggests that CTLA-4 plays an important role in regulating the Th2 response, and limit type 2-associated responses such as eosinophilia in acute infection but not chronic stages of infection. This study illustrates the complexity of regulation of T cells in schistosome infection and highlights a role for CTLA-4⁺, but not $CD25^+$ cells regulation of Th2 responses in helminth infection.

Chapter 4

Investigations into T cell activation during schistosome infection

4.1 Introduction

Infectious pathogens have evolved a range of strategies that can positively or negatively modulate immune activation and thereby alter immune function to the pathogens benefit. The trematode parasite *Schistosoma mansoni* is one such pathogen that is not only a potent activator of T cells, but also regulates lymphocyte function *in vivo* by suppressing T cell activation, as described in section 1.4.

In chapter 3 the role of regulatory T cells in schistosome infection was explored. Another cell type capable of inducing T cell suppression and so modulate parasite infections are macrophages, as discussed in section 1.4.2. Schistosome egg granuloma macrophages have been shown to induce T cell unresponsiveness (Stadecker *et al.*, 1990). IL-4 dependent alternatively-activated M ϕ (aaM ϕ) are induced by nematode infection and inhibit T cell proliferation via a cell-contact dependent mechanism (Loke *et al.*, 2000). Schistosome eggs have been shown to induce expression of the aaM ϕ marker Arginase 1 (Hesse *et al.*, 2001), and the use of macrophage/neutrophil-specific IL-4 receptor α -deficient mice demonstrated that aaM ϕ are essential for survival during schistosome worm-and-egg-infection (Herbert *et al.*, 2004). In addition it has been demonstrated that a schistosome egg glycan induces a peritoneal Gr1⁺ macrophage population that is also capable of suppressing naïve T cell proliferation, in a nitric oxide-dependent mechanism (Atochina *et al.*, 2001).

This chapter examines T cell responses during acute and chronic stages of *S. mansoni* infection in mice. 2 different mechanisms of T cell suppression are identified that are temporally distinct and are induced by different parasite stages. I describe a new mechanism whereby schistosome adult worms induce anergy of both CD4⁺ and CD8⁺ T cells during acute infection. I also aim to examine the expression of another of the B7 family co-

stimulatory pathways, namely the PD-1:PD-L1/PD-L2 pathway, during schistosome infection. Some of the work described in this chapter was performed with Dr. Philip Smith.

Chapter objectives:

1. To investigate the mechanism of suppression of T cells in schistosome-infected mice at acute and chronic stages of infection.
2. To examine the expression of the PD-1 and its ligands PD-L1 and PD-L2 during schistosome infection.
3. To determine the role of PD-L1 in T cell anergy during schistosome worm infection.

4.2 Results

4.2.1 Two distinct mechanisms of suppression of T cell activation are induced by different *S. mansoni* infections.

As discussed in chapter 1, in addition to activating Th2 cell-biased responses, infection with schistosomes also regulates lymphocyte function *in vivo* by suppressing T cell activation (King *et al.*, 1996; Maizels and Yazdanbakhsh, 2003). This study aimed to further examine *S. mansoni* induced T cell suppression in mice. BALB/c mice were given a light (35 cercariae) *S. mansoni* infection, either all male cercariae, for a worm-only infection, or male and female cercariae, for a worm-and-egg-infection. Spleen cells from 8-week *S. mansoni* worm only or worm-and-egg-infected BALB/c mice had a marked defect in anti-CD3 mAb-stimulated cell proliferation (Fig. 4.1). Cell proliferation after anti-CD3 mAb stimulation was consistently 40-60% lower in cells from both worm-only-infected and worm-and-egg-infected mice than in splenocytes from uninfected mice. This was not due to activation-induced cell death (AICD) of T cells during *in vitro* culture of infected mice, as Trypan blue and annexin V staining showed comparable *in vitro* cell death between uninfected and worm-infected groups, with slightly elevated levels of cell death in the worm-and-egg-infected group (data not shown).

Certain forms of T cell suppression can be restored by IL-2 treatment, (Schwartz, 2003). Such IL-2-responsive T cells are said to be anergised. To determine if cells from schistosome-infected mice were anergic, exogenous IL-2, or anti-CD28 mAb, which stimulates endogenous IL-2 production, was added to cultures. Addition of IL-2 or anti-CD28 mAb partially restored anti-CD3 mAb-stimulated proliferation in worm-only infected mice (Fig. 4.2). It can therefore be said that *S. mansoni* worm-infection of mice induces T cell anergy, which is restored by IL-2 treatment. However, there was no restoration of proliferation in worm-and-egg-infected mice (Fig. 4.2). So this data shows that splenic T cells

from both worm-only and worm-and-egg-infected mice are unresponsive to anti-CD3 mAb activation, and that the mechanism of T cell unresponsiveness differs between the two types of infection.

4.2.2 Temporal induction of *S. mansoni*-induced T cell anergy.

In order to further investigate these differences in T cell suppression between infected groups, mice were given a chronic worm-only or worm-and-egg *S. mansoni* infection, and T cell proliferation was assessed at 4 week intervals, up to 16 weeks. As mentioned in section 1.4 and also in chapter 3, week 4 post-infection is before the arrival of the egg, and so immune responses at this timepoint are traditionally thought to be more Th1-biased. Week 8 is the timepoint when immune responses are at their strongest, and these immune responses gradually decline as the infection moves into its chronic phase, with diminished responses by week 16. It was observed that after 4 weeks of infection, before the production of eggs in the worm-and-egg-infected group, both groups had the same T cell anergy, restored by addition of IL-2. This anergy remained apparent in the worm-only-infected group at 8 and 12 weeks. However the levels of anergy were markedly reduced after this timepoint (Fig. 4.3). In contrast, the T cell suppression seen in the worm-and-egg-infected group continued throughout the course of the infection. T cell anergy was only seen at week 4 of *S. mansoni* worm-and-egg infection, with the suppression of T cell activation at the other timepoints (week 8-16; after arrival of eggs) not being restored by IL-2 (Fig. 4.3).

4.2.3 Role of cytokines in worm-induced anergy.

The fundamental difference between worm-only and worm-and-egg infections are the eggs produced by the females in the worm-and-egg infected mouse. *S. mansoni* eggs evoke a

powerful Th2 response (Pearce *et al.*, 1991; Pearce and MacDonald, 2002.). It was pertinent, therefore, to examine differences in cytokine responses between the two types of infection. Splenocytes were stimulated with anti-CD3 mAb or with adult worm antigen (AW). Cytokine responses from both infected groups were Th2 biased, with elevated production of IL-4 and IL-13 in anti-CD3 mAb-stimulated cells compared to levels detected in stimulated cells from the uninfected group (Fig. 4.4). Levels of IFN- γ in infected groups were lower than in the uninfected group. Cells from infected mice that were stimulated with AW induced Th2 cytokines, but not IFN- γ , which is consistent with previous studies (Grzych *et al.*, 1991; Pearce *et al.*, 1991). These cells also had markedly limited IL-2 production compared with cells from uninfected mice. This correlates with the reduction in anti-CD3 mAb stimulated proliferation in infected groups (Fig. 4.1).

It is known that IL-10 has a regulatory role in acute murine schistosomiasis (Flores Villanueva *et al.*, 1993; Wynn *et al.*, 1998), and consistent with this the levels of this cytokine are noticeably elevated in the worm-and-egg-infected group. Production of IL-10 is also shown in the worm-infected group, at levels marginally higher than that of the worm-and-egg-infected group. The regulatory cytokine, TGF- β , is also present in elevated levels in infected groups compared to uninfected cells. Similar to IL-10 there is greater release of TGF- β from cells in worm-infected mice than cell from worm-and-egg-infected mice.

Consistent with earlier studies (Grzych *et al.*, 1991), worm-infected mice had a Th2 biased cytokine response, which is less marked than the Th2 response of the worm-and-egg infected mice. However, worm-infected mice had greater relative production of both IL-10 and TGF- β compared to worm-and-egg-infected mice (Fig. 4.4). Although IL-10 and TGF- β are regulatory cytokines that can suppress T cell proliferation (Shevach, 2002), it has been

shown that the addition of neutralizing anti-IL-10 mAb and anti-TGF- β has no effect on the T_E cell activation defect (Smith *et al.*, 2004).

Another factor previously shown to suppress T cell activation is nitric oxide (NO). NO has been implicated in suppression of T cells by filarial worms (O'Connor *et al.*, 2000), and also by a schistosome egg glycan (Terrazas *et al.*, 2001). Indeed inhibition of NO partially restored anti-CD3 mAb-stimulated proliferation in spleen cells from worm-and-egg-infected mice (Smith *et al.*, 2004). However, the reduction in anti-CD3 mAb-stimulated T cell proliferation observed in splenocytes from worm-infected mice was independent of NO, with no restoration of proliferation upon inhibition of NO (Smith *et al.*, 2004).

The worm-induced T cell suppression is mechanistically distinct from that of the worm-and-egg suppression. It is NO independent, and the suppression is a form of T cell anergy, as proliferation is restored upon treatment with IL-2. The question then remained as to what was the cell population(s) that were mediating this worm-induced anergy?

4.2.4 *Worm infections mediate T cell anergy via splenic F4/80⁺ macrophages.*

As discussed in detail in the previous chapter, it is known that CD4⁺CD25⁺ T cells mediate T cell suppression (Schwartz, 2003; Shevach, 2002). I have already shown that the frequency of CD4⁺CD25⁺ cells are elevated in the spleens of schistosome-infected mice (chapter 3), so it was relevant to investigate whether the worm-induced anergy was mediated by CD4⁺ cells. CD4⁺ T cells and accessory cells (AC) from uninfected and worm-only infected spleens were isolated, and it was determined that AC were mediating the T cell suppression in worm-infected mice (Smith *et al.*, 2004). By separating the CD4⁺ T cells and AC by transwells it was demonstrated that the anergy was mediated in a cell-contact dependent manner (Smith *et al.*, 2004).

Adherent cells, the majority of which are macrophages (M ϕ), in the granuloma surrounding eggs in the liver of worm-and-egg-infected mice have been shown to induce T cell suppression (Stadecker *et al.*, 1990). Therefore to investigate whether the splenic AC that induced T cell suppression during worm infections were also M ϕ , I developed an isolation method which used the mouse M ϕ -specific marker F4/80⁺ to magnetically isolate M ϕ from spleens from uninfected and worm-infected mice (section 2.4.7). Splenic F4/80⁺ M ϕ from each group were co-cultured with CD4⁺ and CD8⁺ T cells from the spleens of uninfected mice (Fig. 4.5). F4/80⁺ M ϕ from worm-infected spleens induced anergy of both uninfected CD4⁺ and CD8⁺ T cells, while anti-CD3 mAb-stimulated proliferation was not impaired by F4/80⁺ M ϕ from uninfected mice. Addition of IL-2 to T cells from uninfected mice mixed with M ϕ from worm-infected mice completely restored T cell proliferation (Fig. 4.5), which is consistent with the anergy induced by whole spleen cells from worm-infected mice (Fig. 4.2).

4.2.5 *Worm-only schistosome infection does not induce alternatively-activated or Gr1⁺ macrophages compared to uninfected mice.*

IL-4-dependent alternatively-activated M ϕ (aaM ϕ), elicited upon i.p. injection of *Brugia malayi* worms, were shown to induce a cell contact-dependent suppression of naïve CD4⁺ T cells (Loke *et al.*, 2000; MacDonald *et al.*, 1998). To address if schistosome worm-induced anergy was also due to aaM ϕ , levels of markers of alternative activation, Arginase 1, Ym-1, and Fizz 1 (Loke *et al.*, 2002) were measured in uninfected, worm-infected and worm-and-egg-infected F4/80⁺ macrophages using RT-PCR. aaM ϕ markers were comparable in uninfected and worm-infected F4/80⁺ macrophages (Fig. 4.6A). However levels of these

markers were elevated in M ϕ from worm-and-egg-infected spleens, with transcripts for Arginase 1 being detected only in this group (Fig. 4.6A).

It has also been shown that T cell suppression is induced by a schistosome egg glycan via induction of novel F4/80⁺Gr1⁺ cells (Atochina *et al.*, 2001; Terrazas *et al.*, 2001). Gr1 expression on F4/80⁺ splenocytes was assessed in uninfected and infected mice, and it was shown that levels of Gr1 from schistosome worm-infected mice are not up-regulated, in fact levels are marginally lower than on uninfected mice (Fig. 4.6B). In contrast, spleen cells from worm-and-egg-infected mice have elevated Gr1 expression on F4/80⁺ cells relative to uninfected or worm-infected mice (Fig. 4.6B).

4.2.6 *In vitro modulation by schistosome worms induces anergy of T cells.*

Schistosome worm-infected mice induce T cell anergy after 4 weeks of infection, as shown in Fig 4.2 above, however it remained unclear if it was the antigens secreted by the worms that mediated the anergy. Live male worms were used *ex vivo* as a source of worm antigens. 12 male worms were exposed to uninfected splenocytes on a Transwell for 48 hours, and this was shown to induce T cell anergy upon subsequent anti-CD3 mAb stimulation (Fig. 4.7). Again, proliferation was restored by addition of IL-2, confirming that the cells were not dead. The number of worms added for in vitro culture was optimized and 10-12 worms produced the optimal level of T cell anergy without cell death or worm death.

4.2.7 *Schistosome worms selectively up-regulate PD-L1 expression on M ϕ .*

Co-stimulation with anti-CD28 mAb, or addition of IL-2 restores the cell contact-dependent, schistosome worm-induced T cell anergy, suggesting that the anergy may be caused by the dysregulation of co-stimulatory molecules on the surface of the M ϕ . Expression

levels of a range of B7 co-stimulatory ligands on F4/80⁺ M ϕ were assessed on spleens from uninfected or 8-week worm-infected mice, when the immune response to the infection is at its peak (Fig. 4.8A). MHC class I and II expression levels were distinctly up-regulated in worm-infected M ϕ compared to uninfected M ϕ . There was a marginal increase in M ϕ expression of the co-stimulatory molecule CD80 (B7-1) in infected spleens, and CD86 (B7-2) and ICOS-L (B7-H2) levels were slightly reduced. Expression of PD-L2 (B7-DC) was slightly higher in infected M ϕ , however there was a marked up-regulation of its related molecule, PD-L1 (B7-H1) (MFI of 189 on M ϕ from uninfected mice compared to MFI of 341 on M ϕ from worm-infected mice).

Surface expression of co-stimulatory molecules was also examined on *in vitro* worm-modulated M ϕ . Levels of CD80, CD86 and PD-L2 were either the same or slightly reduced on worm-modulated M ϕ compared to un-modulated M ϕ (Fig. 4.8B). However, the expression of PD-L1 was up-regulated on worm-treated cells (MFI of 168 compared to 311). So PD-L1 was selectively up-regulated on both *in vivo* and *in vitro* worm-modulated M ϕ , representing a potential function for this molecule in the worm-induced T cell anergy.

4.2.8 PD-L1 mediates the schistosome worm-induced T cell anergy.

In vitro mAb blocking studies were performed to investigate the role of PD-L1 in the worm-induced anergy. Blocking of PD-L1 by the mAb was first confirmed by addition of fluoro-chrome-conjugated anti-PD-L1 mAb to cells that had been treated with 0.5 μ g/ml of the blocking mAb. There was a dramatic reduction in binding of the fluorochrome mAb to the pre-treated cells (Fig. 4.9). The blocking was specific to PD-L1 as anti-PD-L2 mAb stained the same cells.

F4/80⁺ Mφ isolated from uninfected and worm-infected mice were pre-treated with blocking mAbs against PD-L1, PD-L2, or isotype control rat IgG2a mAb. Uninfected CD4⁺ cells were added and stimulated with anti-CD3 mAb. Blocking of PD-L1 on worm-infected Mφ completely restored proliferation of CD4⁺ cells at two different effector:target ratios (Fig. 4.10). However, blocking of PD-L2 had no effect on the worm-infected Mφ-induced anergy, with proliferation levels being similar to the control mAb-treated group. CD8⁺ cell proliferation was also reinstated using anti-PD-L1 mAb (Fig. 4.11), with anti-PD-L2 mAb treatment having no effect on CD8⁺ T cell suppression.

4.2.9 *PD-L1 expression on F4/80⁺ splenic Mφ is reduced during the chronic stages of schistosome worm infection.*

The worm-induced T cell anergy was not constant throughout the course of an infection, with levels peaking at week 4-12, and having largely declined by week 16 (Fig. 4.3). Since PD-L1 was shown to mediate this anergy (Fig. 4.10, 4.11), it was pertinent to examine the levels of PD-L1 expression on F4/80⁺ Mφ at these timepoints. Expression was consistently higher in worm-infected mice than in uninfected mice, with levels peaking from weeks 4-12, and then declining by week 16 (Fig. 4.12). This mirrors the pattern of T cell anergy throughout the infection. So the worm-induced T cell anergy observed from weeks 4-12 of infection corresponds to an increase in PD-L1 levels on F4/80⁺ spleen cells, while at week 16 post-infection both a reduction in anergy and in PD-L1 expression levels were observed.

4.2.10 *Despite displaying antigen non-specific T cell anergy in vitro, S. mansoni-infected DO.11.10 mice have comparable antigen-specific responses in vivo.*

DO.11.10 mice have a transgenic T cell receptor (TCR) that reacts to an ovalbumin (OVA) peptide antigen. The peptide corresponds to amino acids 323-339 of the OVA protein. These mice therefore provide a robust model for investigation of T cell responses without antigen priming. It has been shown in Fig 4.1 that both worm-infected and worm-and-egg-infected mice exhibit T cell suppression *in vitro*. While this anti-CD3 mAb proliferation defect is also present in spleen cells from *S. mansoni*-infected OVA-TCR (Ovalbumin T-cell receptor) mice, their *in vitro* OVA-specific proliferative responses were intact (Smith, Walsh *et al.*, unpublished). To date all cell proliferation studies performed were using cells *in vitro*, with *in vivo* suppression of T cell activation not been demonstrated. It was relevant to examine *in vivo* proliferative responses, as they provide a more real insight into the immune response of mice, and any defect in proliferation would show a biological relevance to the earlier *in vitro* data. Administration of anti-CD3 mAb *in vivo* is not practicable, as it can induce apoptosis in T cells (Tang *et al.*, 2003). OVA-TCR transgenic mice were used as a model to investigate OVA-specific responses of *S. mansoni* infected mice using bromodeoxyuridine (BrdU). I optimized various methods to determine optimal incorporation of BrdU and a method modified from (Humphreys *et al.*, 2003) was used. 1 mg OVA per mouse was administered ip. 36 hours later mice were each given 2 mg (BrdU) ip. Mice were sacrificed 12 hours later, and splenocytes stained with the anti-TCR clonotypic mAb KJ126 and with an anti-BrdU mAb (chapter 2, section 2.7). The percent of transgenic TCR⁺ cells that had incorporated BrdU into their DNA (i.e., that had proliferated) was comparable among groups (Fig. 4.13). Transgenic TCR⁺ T cells from uninfected and infected groups all proliferated in response to OVA, with 13.11% of uninfected TCR transgenic cells taking up

BrdU, compared to 14.30% and 13.2% for worm-infected and worm-and-egg-infected mice respectively (Fig. 4.13). So it appears that worm-only and worm-and-egg-infected mice respond normally to OVA both *in vitro* and *in vivo*, suggesting that the anergised cells observed *in vitro* are still functional and have intact OVA-specific responses. Spleen cells from worm-infected mice also proliferate in response to adult worm antigen (Smith, Walsh *et al.*, unpublished).

4.2.11 Temporal changes in PD-L1 and PD-L2 expression on F4/80⁺ splenocytes during infection.

It has been shown above that levels of PD-L1 on F4/80⁺ splenocytes decline during a *S. mansoni* worm-only infection (Fig. 4.12). This correlated with a decline in T cell anergy. Since the T cell anergy was not observed in egg-and-worm-infected mice after week 4 of infection, PD-L1 levels on F4/80⁺ splenic M ϕ on this group of infected mice were also examined. Both infected groups had comparable levels of PD-L1 expression at week 4, prior to egg deposition, (Fig 4.14), displaying a significant increase over uninfected PD-L1 levels. However in contrast to worm-infected mice, levels of PD-L1 on worm-and-egg-infected mice had declined by week 8. This expression level was sustained throughout the infection, dropping only slightly by week 16. Levels of PD-L1 were comparable between infected groups by week 16 (Fig. 4.14).

PD-L2, a related molecule to PD-L1, was moderately increased in worm-infected F4/80⁺ splenocytes (Fig. 4.8). However, the inhibition studies showed it was not involved in the worm-induced T cell anergy (Fig. 4.10, 4.11). Levels of PD-L2 on uninfected, worm-infected and worm-and-egg-infected mice were examined throughout the course of infection (Fig. 4.15). Expression levels in week 4 infected mice were similar to uninfected PD-L2

levels. However by week 8 of infection, expression had significantly increased in both infected groups, with an approximately 24% increase over uninfected mice. Levels of PD-L2 had further increased in worm-infected mice by week 12, however expression levels in worm-and-egg-infected mice were slightly reduced. There was an increase in expression by week 16 in worm-infected mice, while expression levels in worm-and-egg-infected mice was similar to the 12 week timepoint (Fig. 4.15).

4.2.12 Increase in PD-1 expression on CD4⁺ and CD8⁺ cells during *S. mansoni* infection.

As discussed in chapter 1, PD-L1 and PD-L2 act through their receptor PD-1, to induce a T cell anergy that is restorable by IL-2 (Carter *et al.*, 2002), although PD-1-independent activities for PD-L1 and PD-L2 have also been observed (Liu *et al.*, 2003; Shin *et al.*, 2003; Wang *et al.*, 2003). Given the increase in PD-L1 and PD-L2 levels on both worm-only and worm-and-egg-infected Mφ (Fig. 4.14 and 4.15), it was therefore relevant to examine the levels of PD-1 on T cells during infection. PD-1 levels on CD4⁺ cells increased in both infected groups throughout the infection (Fig. 4.16). Expression levels were comparable between infected groups at week 4, with an approximately 60% increase in expression over uninfected mice. Expression levels were also significantly increased by week 8. Levels in the worm-only infected group had declined by week 16, but the worm-and-egg-infected group still exhibited greater expression levels than the worm-infected group (Fig. 4.16).

PD-1 levels on CD8⁺ spleen cells are also increased during *S. mansoni* infection (Fig. 4.17). Again, levels are comparable between infected groups at week 4 of infection. There is a significant increase in expression in both groups by week 8. The levels of CD8⁺ PD-1⁺ cells continue to increase slightly in both groups through to the chronic stage of infection. So at week 8 of infection, where *S. mansoni* infection is most acute, and T cell responses are at their

strongest, there is three-fold increase in PD-1 levels on CD4⁺ cells from worm-infected mice, with a lesser increase in CD8⁺ cells. Worm-and-egg-infected mice exhibit a greater than three-fold increase of PD-1 on CD4⁺ cells and a three-fold increase on CD8⁺ cells.

4.2.13 *PD-1⁺ CD4⁺ and CD8⁺ cells do not produce IL-2, and frequencies of IL-2 producing CD4⁺ and CD8⁺ cells are reduced in infected groups.*

Cytokine data shown in Fig. 4.4 shows a marked reduction in IL-2 production in infected mice when stimulated with anti-CD3 mAb. As PD-1 is upregulated on T cells of *S. mansoni*-infected mice (Fig. 4.16 and 4.17), it was also of interest to examine whether PD-1⁺ T cells were producing any IL-2. Spleen cells from each group were stimulated with Concanavalin A for 6 hours, with Brefeldin A added for the last 4 hours of culture. Cells were stained for surface markers for CD4 or CD8, and also for PD-1. They were stained intracellularly for IL-2. PD-1 was upregulated on CD4⁺ and CD8⁺ cells from worm-infected and worm-and-egg-infected mice (Fig. 4.18), this is consistent with data shown in Fig. 4.16 and 4.17. Levels of IL-2 are markedly reduced in worm-infected mice compared to uninfected mice, with 8.31% of CD4⁺ cells from uninfected mice producing IL-2 compared to 3.68% on CD4⁺ T cells from worm-infected mice. 2.31% of CD8⁺ cells from uninfected mice produce IL-2 compared to 1.18% on CD8⁺ cells from worm-infected mice (Fig. 4.18). There is also a reduction in IL-2-producing CD4⁺ cells from worm-and-egg-infected mice, comparable to that observed in worm-infected mice. A two-fold decrease in CD8⁺ IL-2 producing cells is also observed in worm-and-egg-infected groups compared to the uninfected group. From Fig. 4.18 it is clear that there are few PD-1⁺IL-2⁺ double positive cells in all groups, and most PD-1⁺ cells do not produce IL-2. This is consistent with its role as an inhibitory co-stimulator (Carter *et al.*, 2002; Latchman *et al.*, 2001).

4.2.14 *Worm-infected mice on a C57BL/6 background do not exhibit the same T cell anergy as in BALB/c mice, thereby hindering studies on PD-L1 deficient mice.*

The differential expression of PD-L1 during worm-only and worm-and-egg schistosome infection, coupled with the mAb treatment data in worm-induced anergy implied a role for PD-L1 in schistosome infection. PD-L1 deficient (PD-L1 ko) mice were generated by two groups, with publications in 2004 (Dong *et al.*, 2004; Latchman *et al.*, 2004). Latchman and co-workers demonstrated that the phenotype of the PD-L1 ko mouse highlighted the negative regulatory role of PD-L1 *in vivo*, and Dong and co-workers showed an accumulation of CD8⁺ T cells in the livers of PD-L1 ko mice. PD-L1 ko were kindly provided to the laboratory by Professor Lieping Chen (John Hopkins University, Maryland, US).

Since the PD-L1 deficient mouse was on a C57BL/6 background, and all previous studies were on a BALB/c background, I first examined C57BL/6 mice for PD-L1 expression on worm-only infected F4/80⁺ Mφ compared to uninfected F4/80⁺ Mφ. Expression of PD-L1 was increased in worm-infected mice compared to uninfected mice (Fig. 4.19A), however the magnitude of the increase was significantly less than that observed in BALB/c mice (Fig. 4.19B). Stimulated splenocytes from uninfected or worm-infected C57BL/6 (wt) or PD-L1 ko (ko) mice produced a comparable cytokine profile between wt and ko (Fig. 4.20). However although worm-infected cells from both groups produced IL-10 in response to adult worm antigen, only minimal IL-4 was produced, with some mice producing none of this Th2 cytokine at all (Fig. 4.20). This is in stark contrast to the cytokine profile observed earlier in worm-infected BALB/c mice, where there was greater production of IL-4 in response to AW and anti-CD3 mAb, and also reduced production of the Th1 cytokine IFN-γ (Fig. 4.4).

To determine whether lack of PD-L1 expression would alter the worm-induced T cell suppression previously described (Fig. 4.2), splenocytes were taken from uninfected and worm-only infected wt and PD-L1 ko mice and stimulated with anti-CD3 mAb with or without addition of IL-2. The previously observed reduction in anti-CD3 mAb-stimulated proliferation in worm-infected mice was not apparent in the C57BL/6 background (Fig. 4.21), with uninfected splenocytes (white) having comparable proliferation to worm-infected splenocytes (black). PD-L1 ko cells from uninfected and infected mice responded similarly to the wild-type groups, with perhaps slightly less proliferation in response to anti-CD3 mAb alone (Fig. 4.21), however as the original anergy phenotype was not present, this data is difficult to interpret.

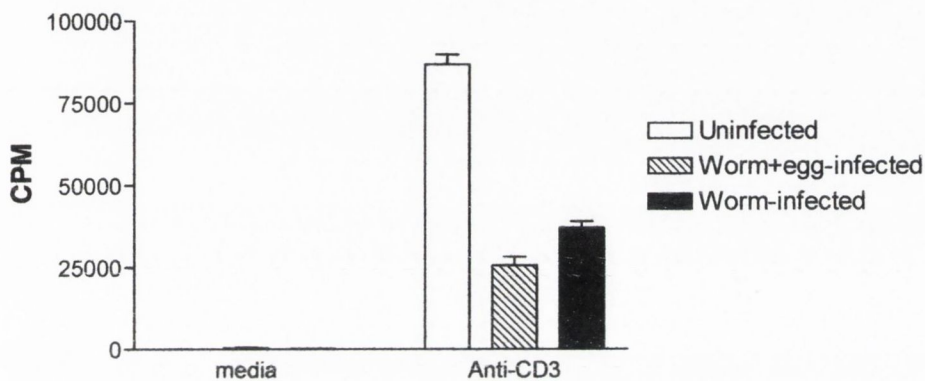


Figure 4.1. *Two types of S. mansoni infection induces suppression of T cell activation.* Proliferation of spleen cells from uninfected mice or from mice infected for 8 weeks with a worm-and-egg or worm-only schistosome infection. Cells were activated with soluble anti-CD3 mAb (0.5 μ g/ml). Proliferation was measured by [3 H] incorporation and expressed as counts per minute (CPM). Data shown is mean $\pm$ SD of triplicate wells from pools of 3-4 mice, and is representative of at least 4 separate experiments.

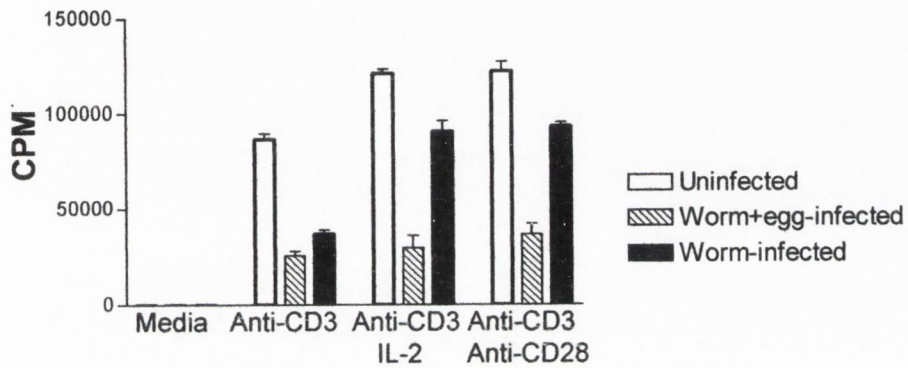


Figure 4.2. *S. mansoni* worm infection induces *T* cell anergy. Proliferation of spleen cells from uninfected mice or from mice infected for 8 weeks with a worm-and-egg or worm-only schistosome infection. Cells were activated with soluble anti-CD3 mAb (0.5 μ g/ml), or anti-CD3 mAb with IL-2 (20 ng/ml), or anti-CD28 mAb (4 μ g/ml). Proliferation was measured by [3 H] incorporation and expressed as CPM. Data shown is mean $\pm$ SD of triplicate wells, and is representative of at least three separate experiments.

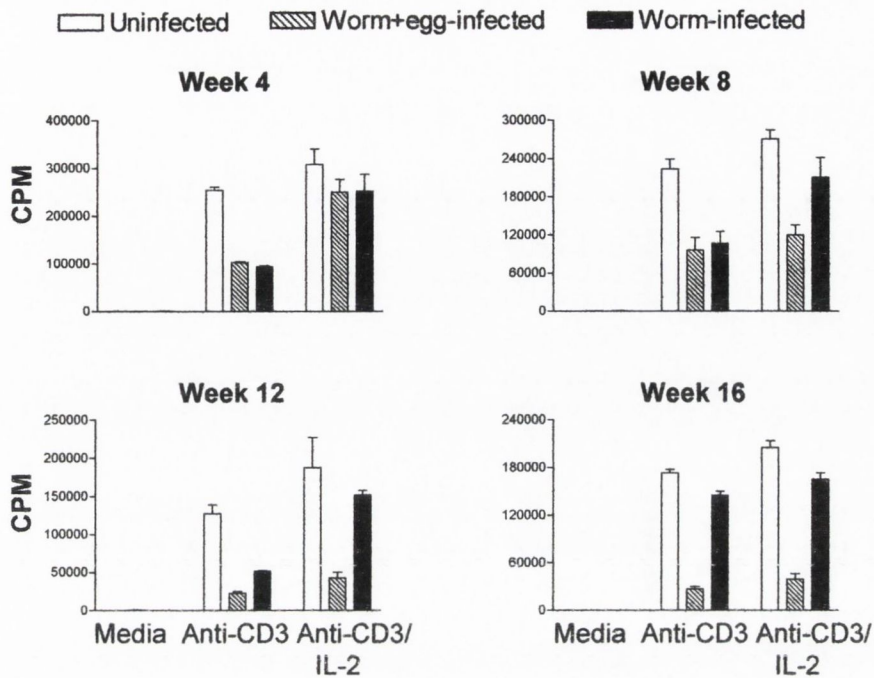


Figure 4.3. *Proliferation of spleen cells from uninfected, worm-and-egg infected, and worm-only infected mice at weeks 4, 8, 12 and 16 of infection. Cells were activated with soluble anti-CD3 mAb (0.5 μ g/ml), or anti-CD3 mAb with IL-2 (20 ng/ml). Proliferation was measured by [3 H] incorporation and expressed as CPM. Data shown is mean $\pm$ SD of triplicate wells, and is representative of two (week 12 and 16) to four (week 4 and 8) separate experiments.*

□ Uninfected ▨ Worm+egg-infected ■ Worm-infected

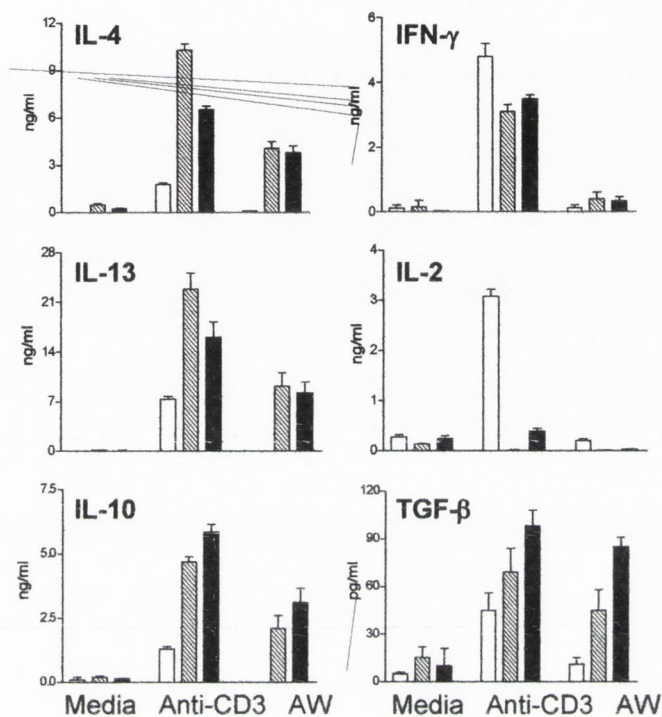


Figure 4.4. *Th2-biased cytokine production by cells from worm- and-egg-infected and worm-infected mice.* Spleen cells from uninfected or 8-week infected mice were activated with anti-CD3 mAb (0.5 μ g/ml) or AW (20 μ g/ml). Supernatants were collected after 24 h (IL-2) and 72 h (IL-4, IL-10, IL-13, IFN- γ , TGF- β) for cytokine ELISA. Data are from pools of three individual mice and are representative of at least three separate experiments.

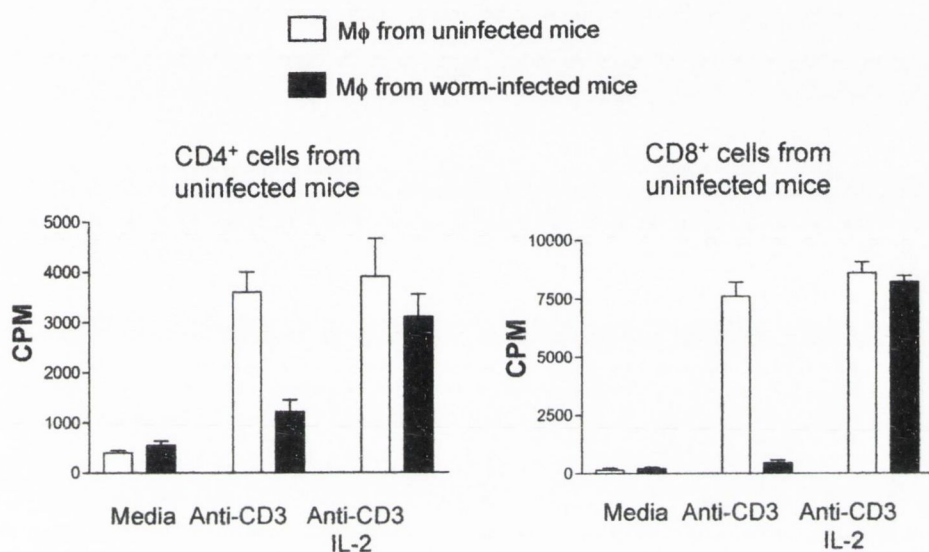
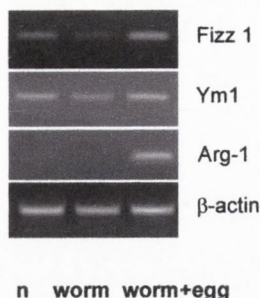


Figure 4.5. *T cell anergy is mediated by splenic F4/80⁺ Mφ.* F4/80⁺ Mφ from the spleens of uninfected or worm-infected mice were added to naïve CD4⁺ and CD8⁺ cells. Cells were activated with anti-CD3 mAb with or without IL-2. Data is presented at the optimal effector:responder ratio of 1:5. Data shown is mean $\pm$ SD of triplicate wells, and is representative of at least three separate experiments.

A



B

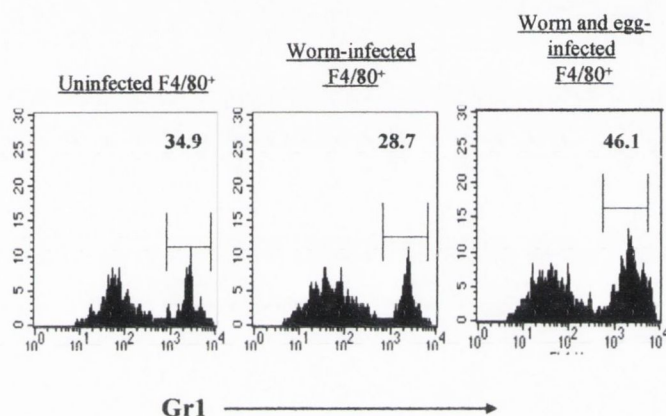


Figure 4.6. *Schistosome worm+egg infection, not worm-only infection, induced alternatively-activated M ϕ markers in spleen M ϕ , and also expands Gr1⁺ M ϕ s. A, worm-infected M ϕ do not have elevated levels of alternative-activation markers. RT-PCR of AAM ϕ markers Ym1, Fizz 1 and Arginase 1 (Arg-1) from uninfected (n), worm-infected (worm) and worm+egg-infected (worm+egg) F4/80⁺ splenic M ϕ , with β -actin as control. B, Gr1 expression on F4/80⁺-gated spleen cells from uninfected, worm-infected, or worm+egg-infected mice. The mean percentage of Gr1⁺ cells from three individual mice per group is shown. Results shown are representative of at least three separate experiments.*

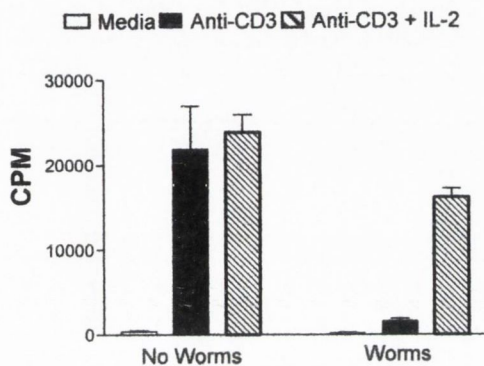


Figure 4.7. *In vitro modulation of spleen cells by schistosome worms induces anergy of T cells.* 12 live male schistosome worms were incubated, using Transwell inserts, over uninfected spleen cells for 48 hours. Cells were washed and stimulated with medium alone, anti-CD3 mAb, or anti-CD3 mAb with IL-2 for an additional 48 hours. Proliferation was measured by [^3H] incorporation and expressed as CPM. Data shown is representative of 3 separate experiments.

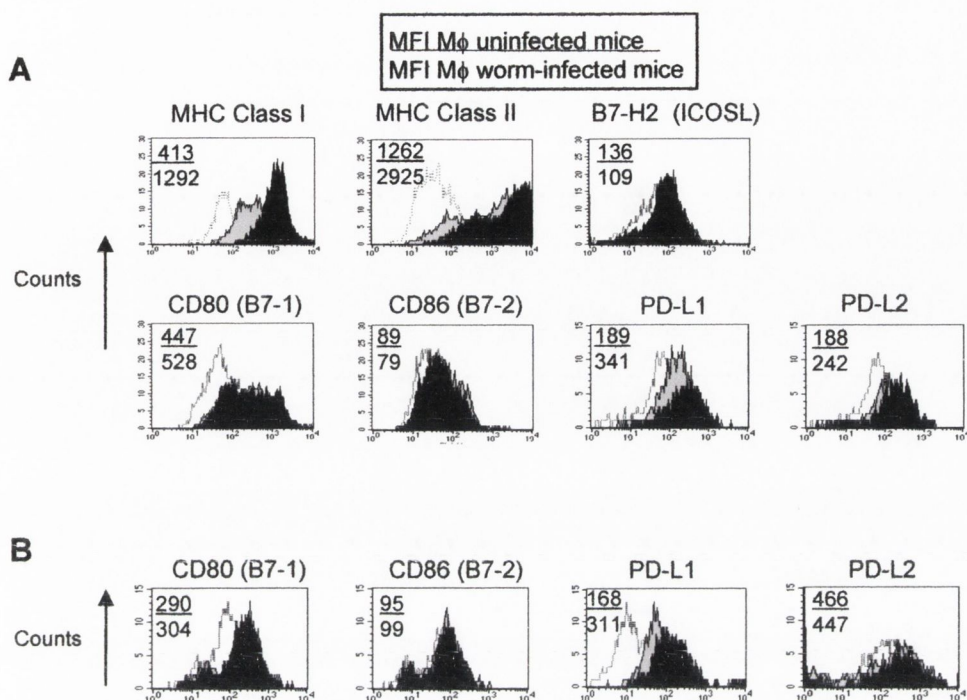


Figure 4.8. Selective alterations in expression of surface markers on *in vivo* or *in vitro* worm-modulated F4/80⁺ M ϕ . *A*, expression of markers on F4/80⁺-gated cells from uninfected (light shading) or worm-infected (dark-shading) mice. Isotype control Ab is indicated (hatched line, no shading). *B*, expression of B7 family members on F4/80⁺-gated *in vitro* worm-modulated spleen cells. Medium-treated cells (light grey shading), worm-treated cells (dark shading), and control Ab-treated cells (no shading) are shown. All data represented are the geometric mean of mean fluorescence intensities (MFI) from pooled or individual mice. Data are representative of at least three or four separate experiments.

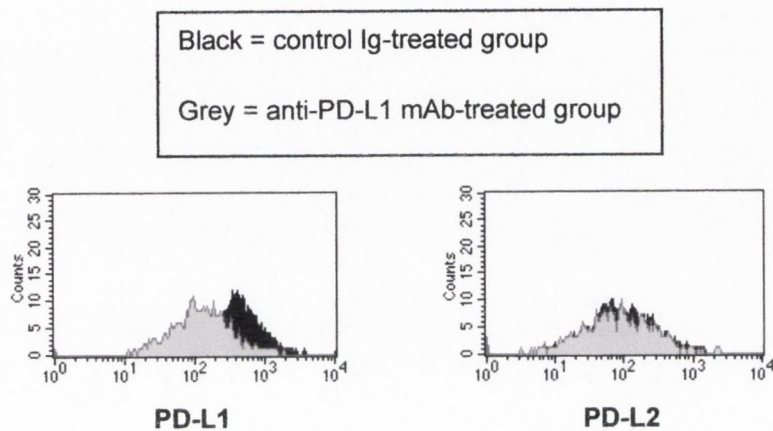


Figure 4.9. *Confirmation of blocking of PD-L1 by the blocking mAb on F4/80⁺ cells.* Flurochrome-conjugated antibodies to PD-L1 and PD-L2 were added to splenocytes that had been treated with 0.5 μ g/ml of control IgG2a mAb or anti-PD-L1 blocking mAb. Cells were gated on the F4/80⁺ population. Black shading is control Ab treated group, light grey shading is anti-PD-L1 treated group.

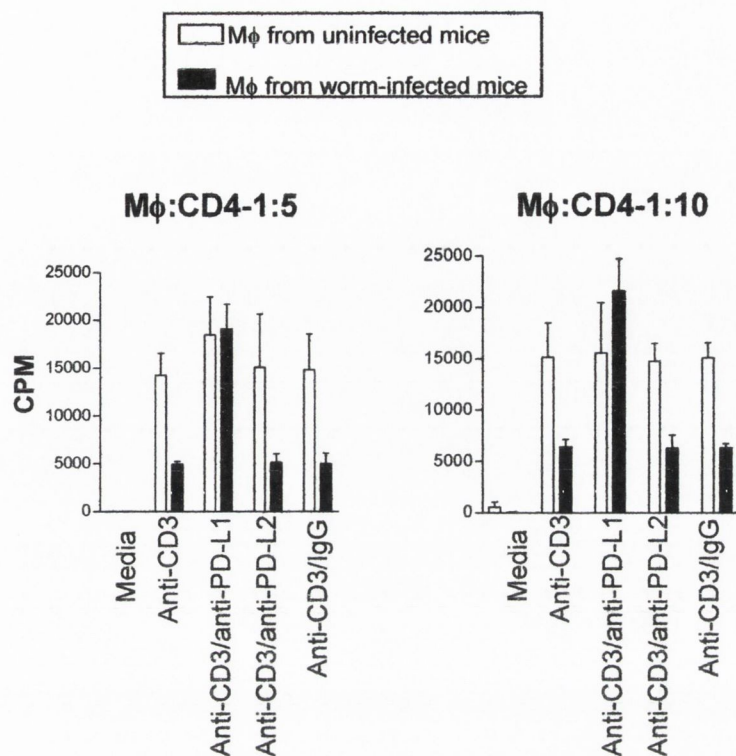


Figure 4.10. Blocking PD-L1 prevents $F4/80^+$ Mφ from schistosome worm-infected mice from inducing $CD4^+$ T cell anergy. $F4/80^+$ Mφ were isolated from the spleens of uninfected or worm-infected mice and incubated with blocking anti-PD-L1 (rat IgG2a, λ), anti-PD-L2 (rat IgG2a, κ), or control (rat IgG2a, κ) mAb (all at 0.5 μ g/ml). RatIgG2a, λ isotype control Ab was also used as a negative control. $CD4^+$ cells (1:5 or 1:10) were added to $F4/80^+$ Mφ, and cells were treated with anti-CD3 ϵ mAb. Data shown are the mean $\pm$ SD of triplicate wells, and representative of three separate experiments.

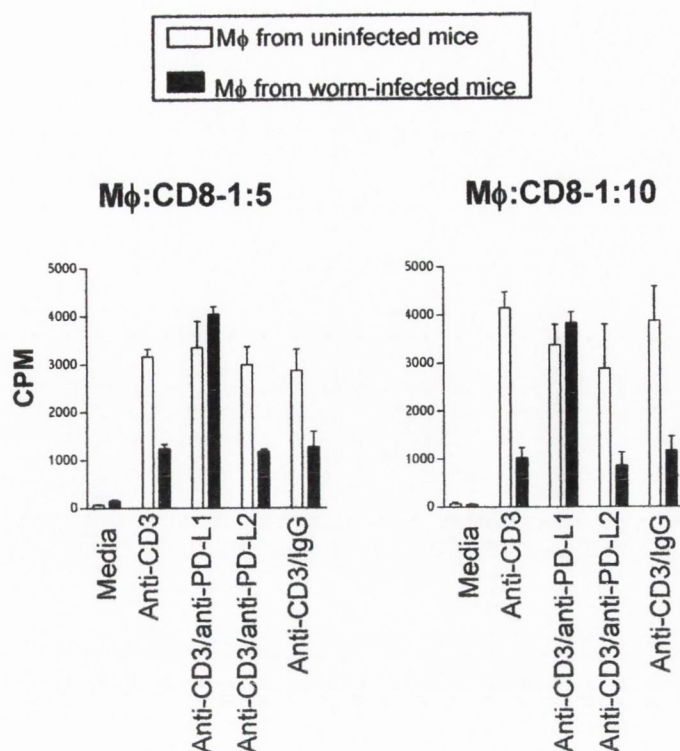


Figure 4.11. *Blocking PD-L1 also prevents F4/80⁺ Mφ from schistosome worm-infected mice from inducing CD8⁺ T cell anergy.* F4/80⁺ Mφ were isolated from the spleens of uninfected or worm-infected mice and incubated with blocking anti-PD-L1 (rat IgG2a, λ), anti-PD-L2 (rat IgG2a, κ), or control (rat IgG2a, κ) mAb (all at 0.5 mg/ml). Rat IgG2a, λ isotype control Ab was also used as a negative control. CD8⁺ cells (1:5 or 1:10) were added to F4/80⁺ Mφ, and cells were treated with anti-CD3ε mAb. Data shown are the mean ± SD of triplicate wells, and representative of three separate experiments.

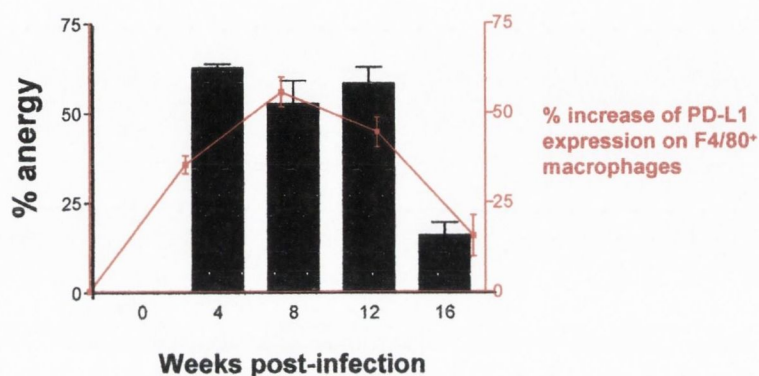


Figure 4.12. *Temporal changes in anergy and PD-L1 expression during acute and chronic schistosome worm infection. Bar graph, Levels of anergy in worm-infected mice 4, 8, 12, and 16 weeks after infection. Data are represented as the mean percent increase in anergy in infected mice relative to that in uninfected mice. Line graph, red, Increase in PD-L1 expression on gated F4/80⁺ spleen cells from worm-infected mice relative to their expression on cells from age-matched uninfected mice. At each timepoint flow cytometry was performed on individuals or pools of spleen cells from two to four mice per group. Data are representative of four (week 4 and 8) or two (week 12 and 16) separate experiments.*

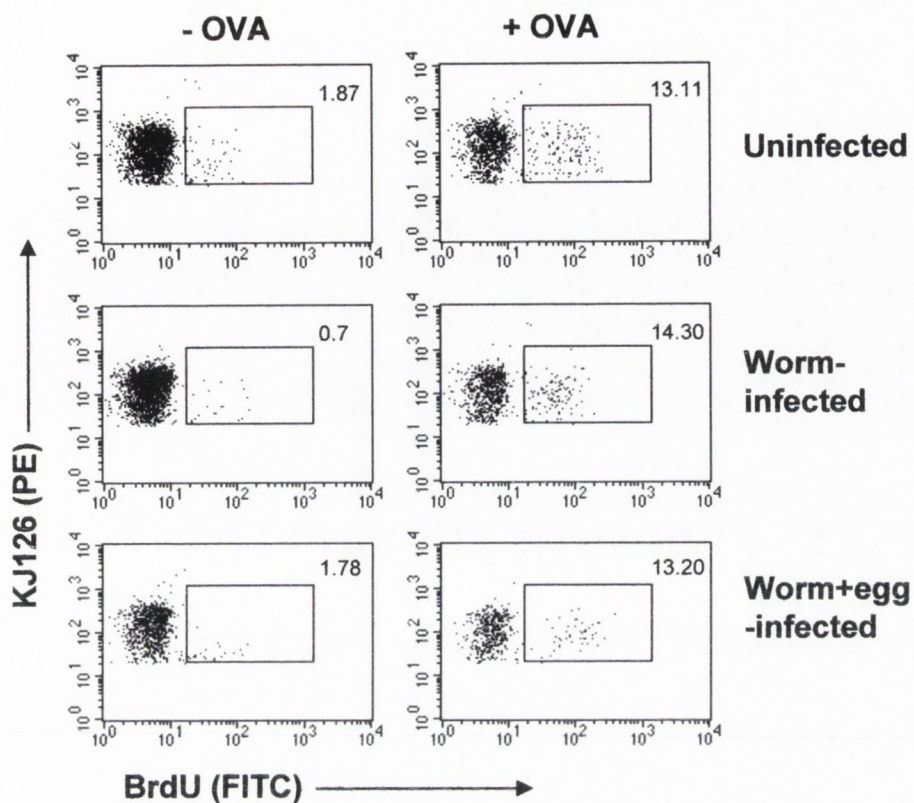


Figure 4.13. *Infected mice have similar OVA-specific responses to uninfected OVA-TCR mice.* Mice were administered with 1 mg OVA ip., and 2 mg BrdU ip. 36 h later. Spleens were taken after 48 h and stained with anti-TCR clonotypic mAb KJ126 and anti-BrdU. Numbers represent the percent of BrdU⁺ OVA-specific TCR⁺ cells. Staining was performed on 3 individual mice and data shown is representative of 2 separate experiments.

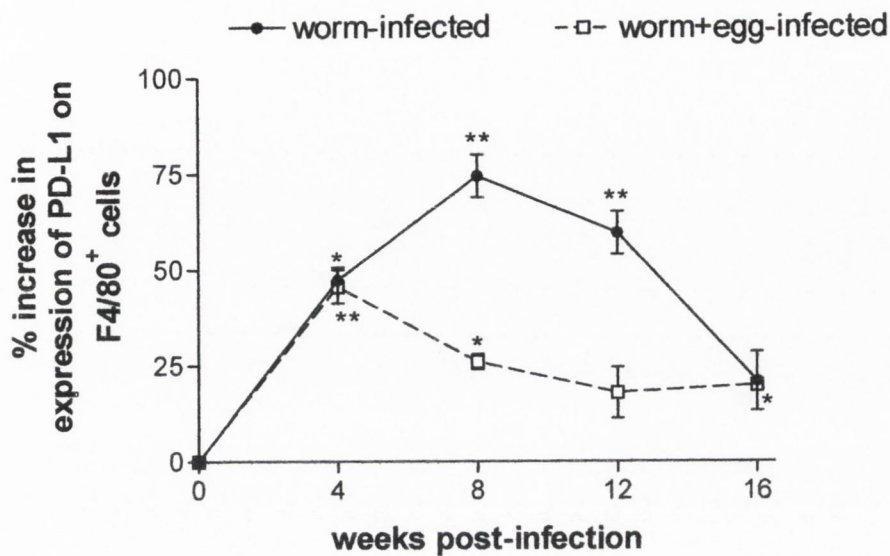


Figure 4.14. Increase in PD-L1 expression on F4/80⁺ M ϕ in *S. mansoni* infection. Increase in PD-L1 expression on gated F4/80⁺ spleen cells from worm-infected and worm-and-egg-infected mice relative to their expression on cells from age-matched uninfected mice. At each timepoint flow cytometry was performed on 3 individual mice or pools of spleen cells from two to four mice per group. Data are representative of four (week 4 and 8) or two (week 12 and 16) separate experiments. Student's t-test was used to compare differences between uninfected and infected groups. (*= $P<0.05$, **= $P<0.01$, ***= $P<0.001$)

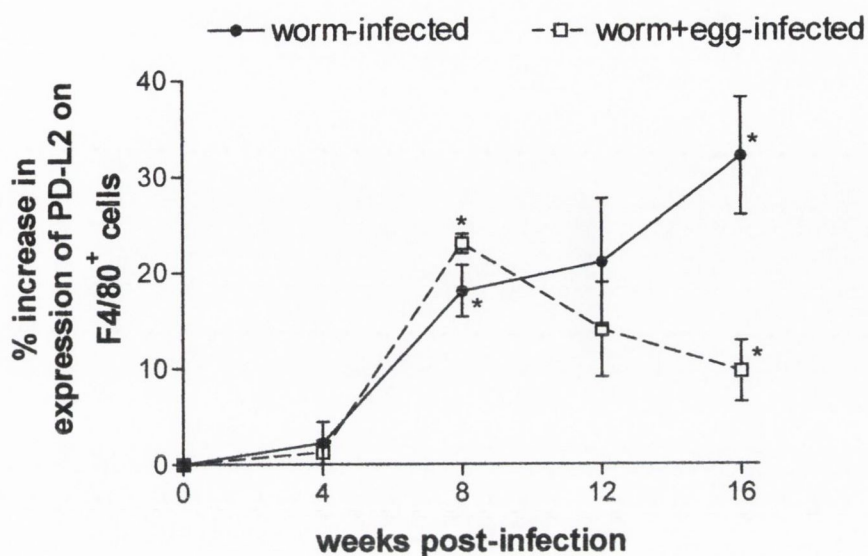


Figure 4.15. Increase in PD-L2 expression on F4/80⁺ M ϕ in *S. mansoni* infection. Increase in PD-L2 expression on gated F4/80⁺ spleen cells from worm-infected and worm-and-egg-infected mice relative to their expression on cells from age-matched uninfected mice. At each timepoint flow cytometry was performed on 3 individuals or pools of spleen cells from two to four mice per group. Data are representative of four (week 4 and 8) or two (week 12 and 16) separate experiments. Student's t-test was used to compare differences between uninfected and infected groups.

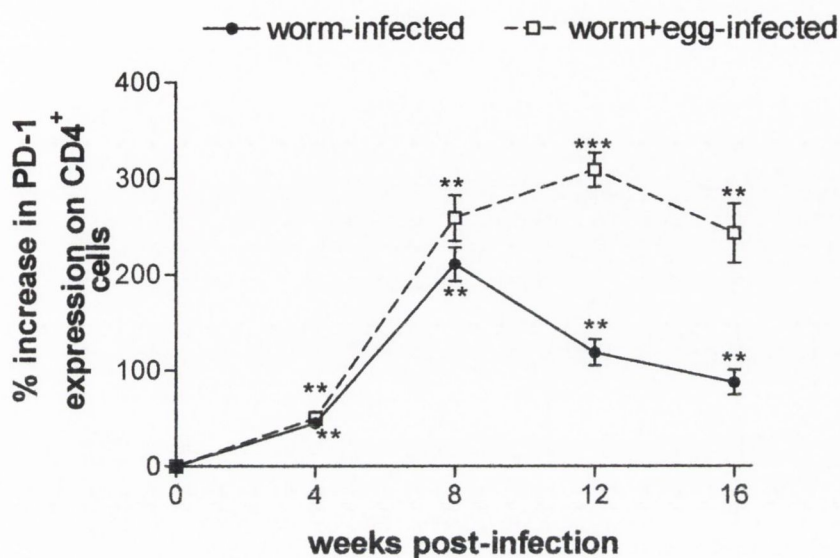


Figure 4.16. Increase in PD-1 expression on CD4⁺ cells during *S. mansoni* infection. Increase in expression on gated CD4⁺ spleen cells from worm- infected and worm-and-egg-infected mice relative to their expression on spleen cells from aged-matched uninfected mice. At each timepoint flow cytometry was performed on 3 individuals or pools of spleen cells from two to four mice per group. Data are representative of four (week 4 and 8) or two (week 12 and 16) separate experiments. Student's t-test was used to compare differences between uninfected and infected groups.

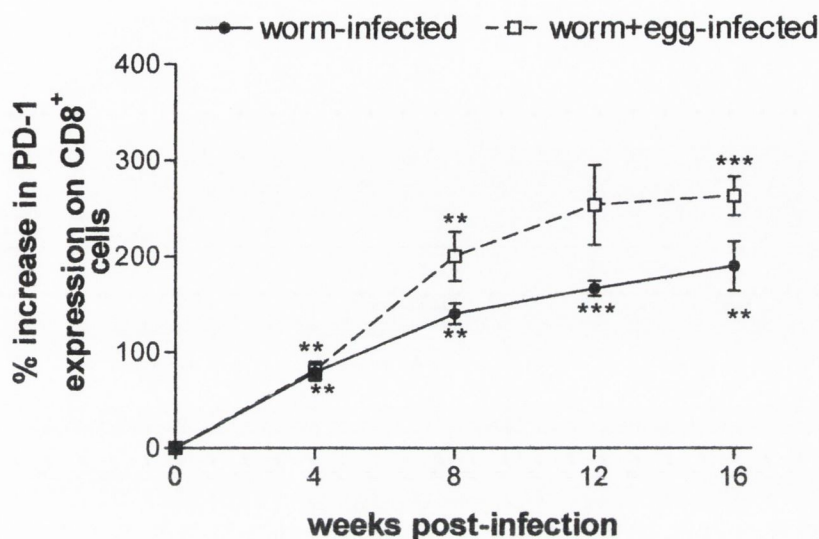


Figure 4.17. *Increase in PD-1 expression on CD8⁺ cells during S. mansoni infection.* Increase in expression on gated CD8⁺ spleen cells from worm- infected and worm-and-egg-infected mice relative to their expression on spleen cells from aged-matched uninfected mice. At each timepoint flow cytometry was performed on 3 individuals or pools of spleen cells from two to four mice per group. Data are representative of four (week 4 and 8) or two (week 12 and 16) separate experiments. Student's t-test was used to compare differences between uninfected and infected groups.

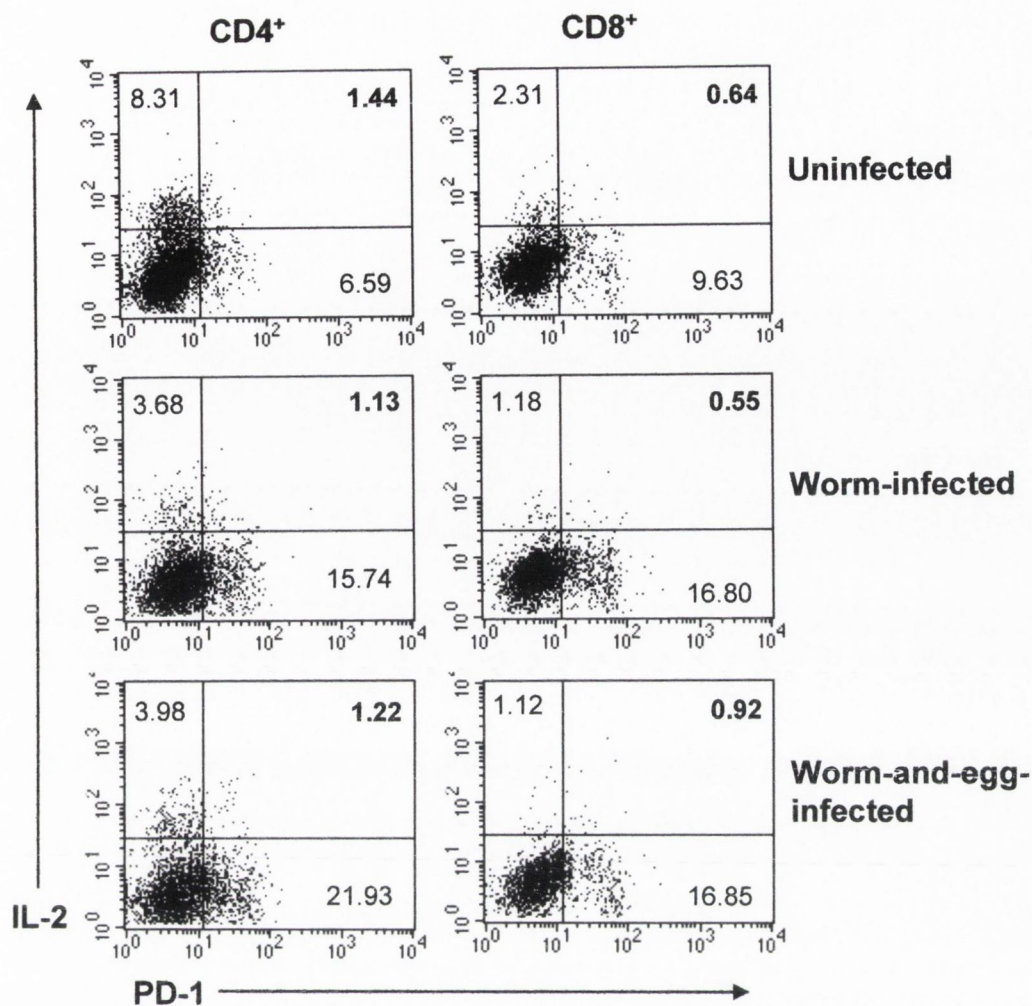
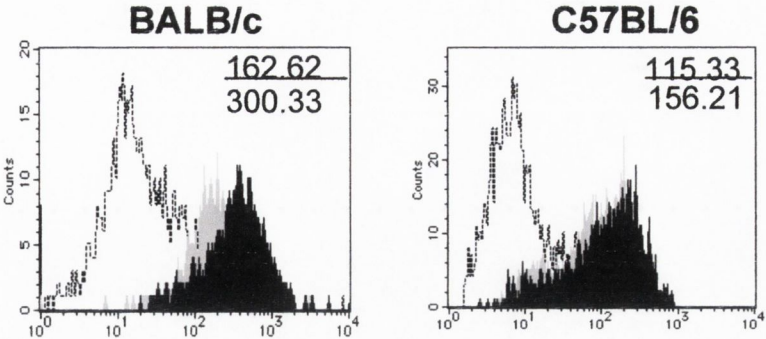


Figure 4.18. *PD-1⁺ CD4⁺/CD8⁺ T cells do not co-express IL-2.* Spleen cells from uninfected or 8-week infected mice were taken and stimulated with Con A (2 μ g/ml) for 6 h, with Brefeldin A added for final 4 hours (10 μ g/ml). Cells were stained for CD4 or CD8 and also for PD-1. Cells were fixed and permeabilised and stained for IL-2. Samples were from 3 individuals or pools of 3-5 mice from 2 separate experiments.

A



B

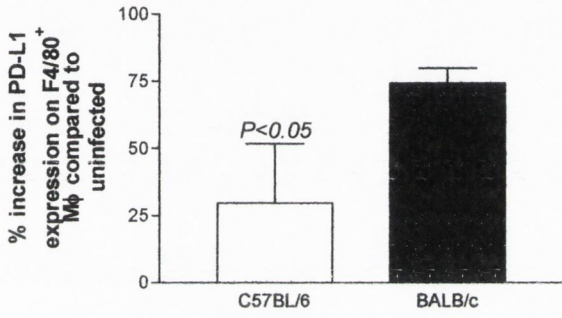


Figure 4.19. *PD-L1 is also upregulated in worm-infected mice on a C57BL/6 background, although not to the same extent as in BALB/c infected mice. A, Expression of PD-L1 on F4/80⁺-gated cells from uninfected (light shading) or worm-infected (dark-shading) C57BL/6 or BALB/c mice. Staining of cells from a PD-L1 knock-out mouse was used as a negative control in the C57BL/6 histogram, with an isotype control used in the BALB/c histogram (hatched line, no shading). Data represented are the geometric mean of mean fluorescence intensities (MFI) from 3 individual mice. B, Bar graph showing % increase in PD-L1 expression on F4/80⁺ macrophages on worm-infected C57BL/6 and BALB/c mice compared to uninfected C57BL/6 or BALB/c mice. Student's t-test was used to determine differences between groups.*

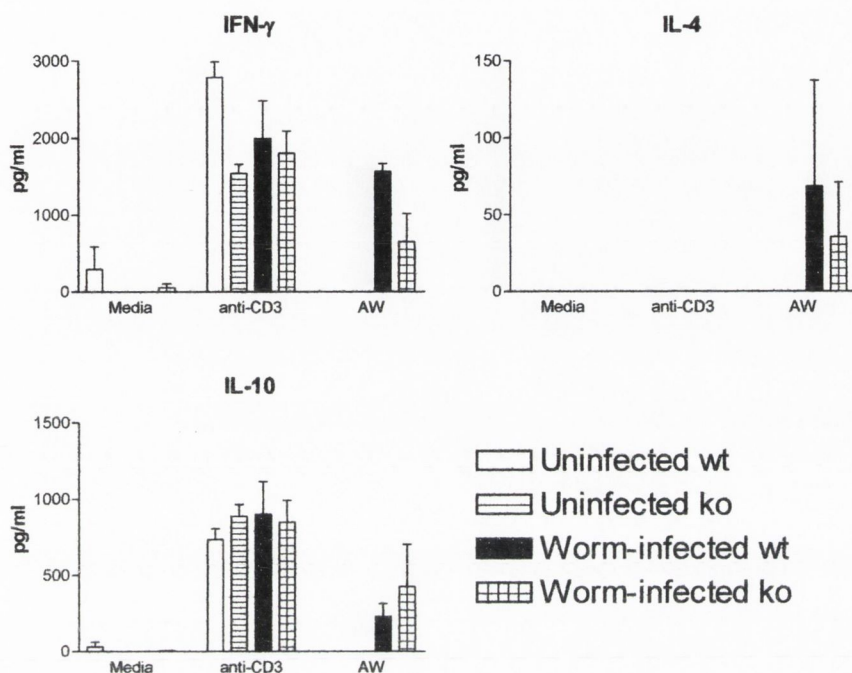


Figure 4.20. *S. mansoni* worm-only infection in C57BL/6 mice induces similar cytokine profiles as PD-L1 ko infected mice. Spleen cells from uninfected or 8-week infected mice were activated with anti-CD3 mAb (0.5 μ g/ml) or adult worm antigen (AW, 20 μ g/ml). Supernatants were collected after 72 h (IL-4, IL-10, IFN- γ) for cytokine ELISAs. Data is mean $\pm$ SEM from 3 individual mice.

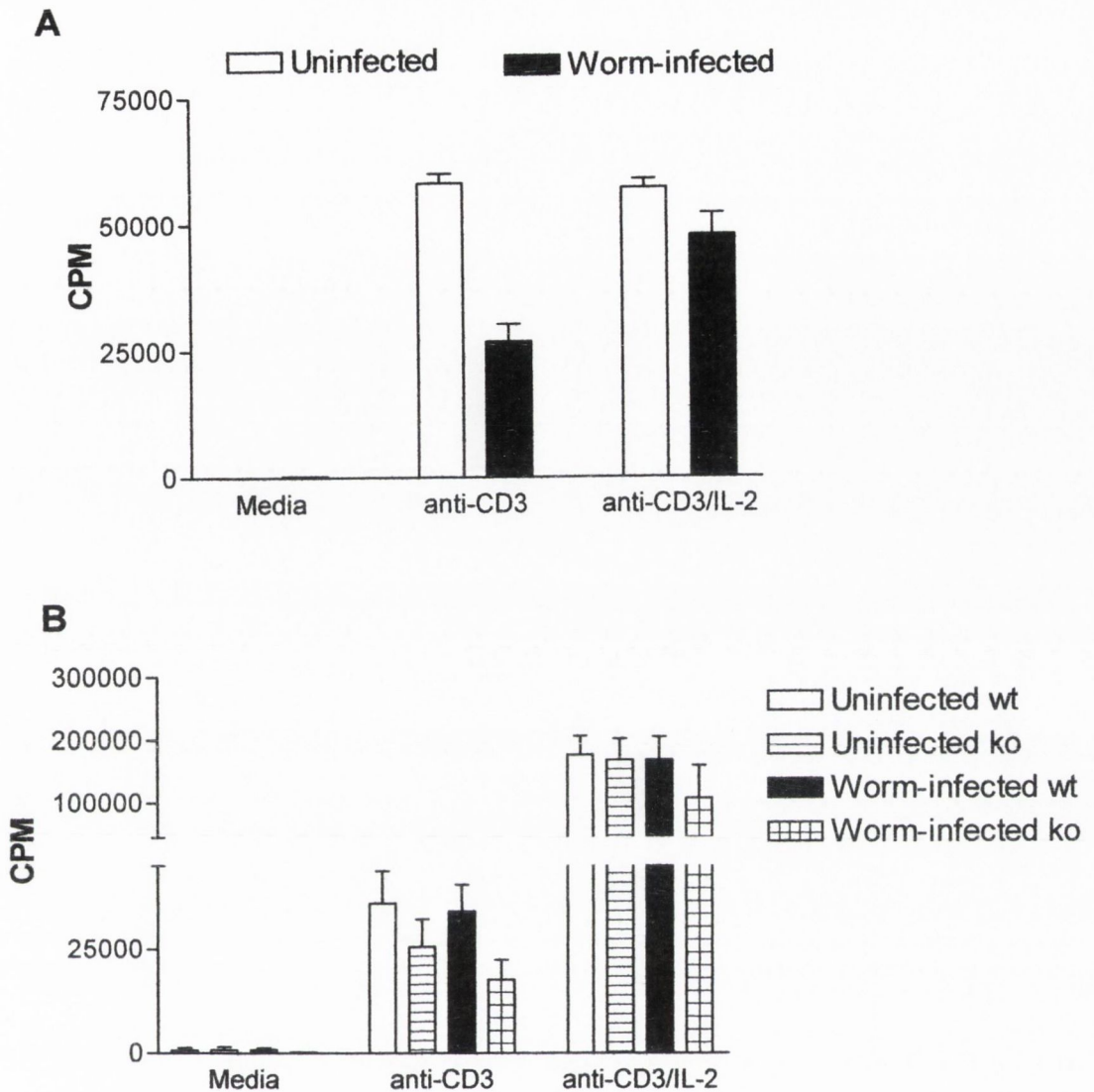


Figure 4.21. *C57BL/6 mice with S. mansoni worm-infection do not induce the same T cell suppression observed in BALB/c mice.* *A*, proliferation of spleen cells from uninfected or worm-infected BALB/c mice. *B*, proliferation of spleen cells from uninfected C57BL/6 or PD-L1 ko mice or from wt or ko mice infected for 8 weeks with a worm-only schistosome infection. Cells were activated with soluble anti-CD3 mAb (0.5 μ g/ml). Proliferation was measured by [3 H] incorporation and expressed as counts per minute (CPM). Data shown is mean $\pm$ SD of 3 individual mice.

4.3 Discussion

It is widely known that schistosome infections bias T cell immune responses to a type 2 response (Pearce and MacDonald, 2002.). However T cell hyporesponsiveness and its mechanism in schistosome infection is less well studied. This chapter focused on the underlying mechanisms of such T cell hyporesponsiveness. Two forms of T cell hyporesponsiveness were demonstrated, that were induced by infection with different stages of the parasite life cycle, and that were active at different times during infection. Earlier studies have shown T cell suppression during the conventional egg-and-worm infection (Flores Villanueva *et al.*, 1994; Stadecker, 1999), and this is similar to the T cell suppression observed in this study. Although mitogen un-responsiveness in worm-only infected mice has been reported (Attallah *et al.*, 1979; Diab *et al.*, 1989), relatively little is known about the T cell suppression induced by worm infection. This chapter describes a novel mechanism of worm-induced T cell anergy.

Both APC and T cells play a role in the schistosome down-modulation of T cell responses (Maizels and Yazdanbakhsh, 2003), with multiple mechanisms being involved. Previous studies have identified M ϕ as mediators of T cell suppression in conventional worm-and-egg *S. mansoni* infections (Flores Villanueva *et al.*, 1994; Stadecker *et al.*, 1990), with granuloma M ϕ surrounding the egg inducing unresponsiveness in specific cloned Th-1 lymphocytes *in vitro* and down-regulating schistosomal granulomatous disease *in vivo*. Marshall *et al.* identified a non-T-lymphocyte suppressor population, which acted via a soluble factor to inhibit virus-specific CTL induction (Marshall *et al.*, 2001). The population involved was isolated from the spleen, and was non-adherent and F4/80^{dull}. This is distinct from an F4/80^{bright}, adherent, and contact-dependent suppressor M ϕ described by Harn and co-

workers (Atochina *et al.*, 2001). They show that injection of a schistosome egg glycan into the peritoneum of mice elicits an F4/80⁺/Gr1⁺ T cell suppressor population. In this study I observe an increase of F4/80⁺/Gr1⁺ Mφ in the spleens of worm-and-egg infected mice. However the F4/80⁺ Mφ from spleens of worm-infected mice described here do not have elevated Gr1 expression.

As discussed in section 1.4.2, alternatively-activated macrophages play a role in host survival during murine schistosomiasis (Herbert *et al.*, 2004). I show that while aaMφ markers are upregulated in splenic F4/80⁺ Mφ from worm-and-egg-infected mice, levels are comparable between uninfected and worm-infected mice (Fig. 4.6). This implies that it is the egg-stage of the parasite, rather than the worm itself, that leads to induction of this suppressive macrophage population. Independent analysis of expression of aaMφ-specific markers by real-time PCR on macrophage populations from worm-infected and worm-and-egg-infected mice have recently confirmed my initial findings (Smith, Walsh *et al.*, submitted).

Dysregulation of co-stimulation has also been shown to be involved in regulation of T cell activation during schistosome infection, with exacerbated pathology during *S. mansoni* infection of mice deficient in B71/2 (Hernandez *et al.*, 1999). Similarly, exacerbated pathology was also observed in mice that had disrupted ICOS-LICOS interactions by mAb treatment (Rutitzky *et al.*, 2003). In this study I describe a new mechanism by which schistosome worms regulate T cell activation during acute stages of infection via selective up-regulation of the co-stimulatory molecule PD-L1 on Mφ. This study is the first demonstration that a pathogen has evolved with the ability to selectively up-regulate PD-L1 on macrophages to subvert the immune system. PD-L1 is expressed on a range of cells, including T cells, B cells, Mφ and dendritic cells. Expression of PD-L1 and PD-L2 on APCs have been implicated as inducers of negative signals in T cells (Brown *et al.*, 2003; Dong *et al.*, 2002; Latchman *et*

al., 2001). The PD-1:PD-L pathway affects both CD4⁺ and CD8⁺ T cells and is overcome by IL-2 (Carter *et al.*, 2002), similar to the anergy found in this study. Enhanced expression of PD-1 on T cells and PD-L1 and PD-L2 on macrophages in both worm-only and worm-and-egg-infected mice compared to uninfected mice was observed. PD-1-independent activities for PD-L1 and PD-L2 have also been shown (Liu *et al.*, 2003; Shin *et al.*, 2003; Wang *et al.*, 2003), pointing to the possibility of additional unknown T cell inhibitory receptors. It would be interesting to block PD-1 on the uninfected CD4⁺ and CD8⁺ cells before adding worm-modulated Mφ, and then assess the levels of anti-CD3 mAb stimulated proliferation. This would help determine if the PD-L1 on Mφ was working solely through PD-1 to induce T cell anergy, or if another receptor was involved.

In this study T cell anergy was observed in both infected groups at week 4 of infection, and this continued in the worm-infected group throughout the chronic phase of infection, before declining after week 12. The decline in anergy induced by worm-infected mice correlates with a decline in PD-L1 expression on Mφ (Fig. 4.12). This decline in anergy in the chronic phase of infection, coupled with the fact that the anergy was present in worm-and-egg-infected mice at week 4, prior to egg deposition, suggests that the worm has evolved to modulate Mφ to suppress the potent initial T cell activation that is induced by the egg. Previously, worm infections of mice have been shown to drive liver lymphocytes in the prepatent period toward a Th2 phenotype (Hayashi *et al.*, 1999), thereby priming the liver for the onset of egg deposition and the generation of a Th2-mediated granuloma (Cheever *et al.*, 1997; Leptak and J.H., 1997). This would serve to limit inflammation before the emergence of the egg-induced T cell suppression. PD-L1 upregulation by schistosome worms could be involved in suppressing T cells to prime the host against the initial potent immune response of the egg.

While the *in vitro* blocking of PD-L1 demonstrated the importance of this molecule in the worm-induced T cell anergy (Fig. 4.10, 4.11), the use of knock-out mice would provide an alternative approach to further elucidate the role of this molecule in the immune response. Knock-out mouse studies can be critical to dissect the functions of a particular molecule *in vivo*. Antibody depletion studies are important in studying the role of a molecule, particularly where knock-out studies result in a non-viable or early death phenotype, as was the case with CTLA-4 in chapter 3. However, there are some limitations with the use of antibody inhibition studies to determine a function for a molecule, including non-specific blocking at other sites, and the accumulation of antibodies leading to anti-antibody responses and the formation of immune complexes. Also many antibodies only block the molecule of interest and do not deplete the cell type involved, as is the case with anti-PD-L1 mAb treatment.

As the PD-L1 deficient mice were on a C57BL/6 background, I first examined the expression of PD-L1 on F4/80⁺ M ϕ on uninfected C57BL/6 compared to worm-infected mice. While there was increased expression compared to uninfected mice, the magnitude of the increase was significantly less than the increase between uninfected and infected BALB/c mice (Fig. 4.19). In addition the cytokine profile of worm-infected mice was biased towards a more Th1 response compared to that observed in BALB/c infected mice, with little or no induction of IL-4 (Fig. 4.20). Strain differences between C57BL/6 and BALB/c mice have been examined in relation to infection with *Leishmania major*, with C57BL/6 mice resolving infection but BALB/c mice being unable to do so (Heinzel *et al.*, 1989). The resolution of murine leishmaniasis has been shown to be associated with IFN- γ production, while IL-4 production with low IFN- γ is related to disease progression (Heinzel *et al.*, 1989). In *S. mansoni* infection, different in-bred strains of mice develop different degrees of pathology, with C3H and CBA mice acquiring high pathology with larger egg granulomas than the low

pathology observed in C57BL/6 mice (Cheever *et al.*, 1987). Some investigation into the strain differences between C57BL/6 and BALB/c mice in *S. mansoni* infection has been performed in this lab, but this has not as yet been published.

When splenocytes from uninfected and worm-infected C57BL/6 mice were stimulated with anti-CD3 mAb *in vitro*, the previously observed worm-induced suppression was not present (Fig. 4.21). This made any comparisons between wild-type and PD-L1 knock-out very difficult to interpret. The mice are currently being back-crossed 6 generations to a BALB/c background in-house in TCD, however this will take quite some time to complete. More intimate *in vitro* proliferation studies can be performed on the BALB/c backcross in the future, and this will give us a more definitive answer to the questions on the role of PD-L1 in worm-induced anergy.

In summary, in this chapter a new mechanism of pathogen modulation of T cell activation was described. Schistosome worms selectively upregulate the inhibitory co-stimulatory molecule PD-L1 to induce T cell anergy during the acute stages of infection. So PD-L1 may play an important role in pathogen immune evasion strategies, with the pathogen usurping the natural function of this molecule to limit host morbidity and so facilitate its own survival.

Chapter 5

*Role of the PD-1:PD-L1/
PD-L2 co-stimulatory
pathway during S. mansoni
infection*

5.1 Introduction

In the previous chapter, I examined T cell unresponsiveness in schistosome worm infection. It was demonstrated that the co-stimulatory molecule PD-L1, present on worm-modulated macrophages, was responsible for the worm-induced anergy of T cells. In addition it was shown that PD-L1, PD-L2, and their receptor PD-1, had altered expression during both schistosome worm and worm-and-egg infection. In this chapter I therefore addressed the role of the PD-1:PD-L co-stimulation pathway in infection using mAb blocking and also PD-L1 ko mouse studies.

The PD-1:PD-L pathway has been implicated in many immune-mediated diseases, as discussed in chapter 1 (section 1.5.5). Alternate roles for PD-L1 and PD-L2 have been demonstrated in *Leishmania mexicana* infection of mice, with PD-L1 ko mice being more resistant to infection, while PD-L2 ko mice developed more severe pathology than wild-type 129Sv mice (Liang *et al.*, 2006). As PD-1, PD-L1, and PD-L2 are up-regulated in schistosome worm-and-egg infection (chapter 4), it would be interesting to see what effect, if any, that an absence of PD-L1, PD-L2 or their receptor PD-1 would have on *S. mansoni* infection.

Studies on PD-L1 deficient mice have shown a selective accumulation of CD8⁺ cells in the liver (Dong *et al.*, 2004). It is known that CD8⁺ cells in the livers of schistosome-infected mice undergo activation-induced-cell-death (AICD) (Estaquier *et al.*, 1997). It would therefore be of interest to examine what effect an absence of PD-L1 would have on liver granuloma formation during infection.

In this chapter I first examined the role of PD-1, PD-L1 and PD-L2 during *S. mansoni* infection using blocking mAb. I observed an alteration in the type 1/type 2

cytokine response in cells from infected anti-PD-L1 treated mice. PD-L1 ko mice were also used to examine the role of this molecule during acute and chronic *S. mansoni* infection. Some alterations in T cell proliferation, type 1/type 2 cytokine responses, and apoptosis levels were observed. This is the first study using gene-deleted mice to examine the role of the PD:PD-L pathway during parasitic helminth infection.

Chapter objectives:

1. To investigate the role of PD-1, PD-L1 and PD-L2 during *S. mansoni* worm- and-egg-infection using blocking mAbs.
2. To examine the role of PD-L1 during acute and chronic *S. mansoni* infection using PD-L1 ko mice.
3. To determine whether the alterations in CD8⁺ T cell accumulation in the livers of PD-L1 ko mice have any effect on *S. mansoni* granuloma formation and composition during infection.

5.2 Results

5.2.1 Effect of *in vivo* blocking of PD-L1, PD-L2, and PD-1 on *S. mansoni* infection.

Chapter 4 demonstrated that *S. mansoni* worms selectively up-regulate PD-L1 on M ϕ to induce T cell anergy. Levels of PD-L1 and its related molecule, PD-L2 were shown to be increased on M ϕ from both worm-infected and worm-and-egg-infected mice, and their receptor PD-1, was also dramatically increased on T cells from infected mice compared to uninfected mice (Fig. 4.14-4.17). In each case, expression levels between worm-only and worm-and-egg-infected groups were comparable at 4 weeks post-infection. As mentioned earlier, eggs have not yet been deposited by the female worm at this timepoint, and so the immune response observed in the worm-and-egg-infected group is elicited by the worm. Therefore it was of interest to block these co-stimulatory molecules in worm-and-egg-infected groups at this timepoint, and see what effect, if any, it would have on the subsequent egg-induced immune response. BALB/c mice were given an acute (100 cercs) mixed-sex cercariae infection (worm-and-egg-infection). Four weeks post-infection, mice were administered with 100-250 μ g/ml blocking antibody against PD-1, PD-L1, PD-L2, or with a control rat IgG antibody (Kanai *et al.*, 2003; Tsushima *et al.*, 2003). Antibody was given every three days, and mice were also weighed at these intervals. Mice were sacrificed 7 weeks post-infection and tissue taken for immunological and histological analysis.

As the infection progressed, mice from all groups lost weight to a comparable degree, (Fig. 5.1). There were comparable egg counts in the liver or intestine between the control mAb-treated group and the anti-PD-L1 or anti-PD-L2 mAb-treated groups (Fig. 5.2A, 5.2B), with a non-significant reduction in intestine egg counts in the anti-PD-1 group (Fig. 5.2B).

This suggests that the blocking of members of the PD-1:PD-L1/PD-L2 pathway does not affect the level or mechanism of egg deposition during infection. Analysis of liver granuloma size from histology slides also showed no overt differences in granuloma size or eosinophil content between groups (Fig. 5.2C, 5.2D).

Spleen cells from each group were cultured *in vitro* for 72 h with various stimulations. Some cell proliferation was observed in unstimulated cells in the anti-PD-1 and anti-PD-L1 groups, which shows non-specific proliferation (Fig. 5.3A). The anti-CD3 mAb stimulated proliferation defect observed in egg-and-worm-infected mice in chapter 4 (Fig. 4.1) was also observed here, with the infected control antibody group showing markedly less anti-CD3 mAb responses than the uninfected group. The anti-PD-L1 and anti-PD-L2 groups have comparable anti-CD3 mAb responses to the control antibody infected group, however the anti-PD-1 group responses are higher than the other mAb treated infected groups. As in chapter 4 (Fig. 4.2), the proliferative defect observed in worm-and-egg-infected mice is not restored by IL-2, with anti-PD-L1 and anti-PD-L2 group responses being similar to the control IgG. Again, the anti-PD-1 responses are higher than the control IgG group. Cells from all infected groups also proliferated in response to AW and SEA antigens, with the anti-PD-1 and anti-PD-L1 groups displaying increased responses compared to the control IgG and anti-PD-L2 infected groups (Fig. 5.3A). To ensure *in vitro* blocking of cells, each blocking antibody was added to anti-CD3-stimulated spleen cells from each group (Fig. 5.3B). This had no effect on the anti-CD3 mAb responses of infected groups, with anti-PD-L1 and anti-PD-L2-treated groups having comparable proliferation to the control mAb-treated group, and the anti-PD-1-treated group proliferating more than the control group.

5.2.2 Cytokine responses in worm-and-egg-infected mice upon in vivo blocking of PD-1, PD-L1 and PD-L2.

It is well documented that cells from worm-and-egg-infected mice display a Th2 cytokine response upon stimulation (Pearce *et al.*, 1991) and indeed this bias towards Th2 cytokine responses was demonstrated in chapters 3 and 4 (Fig. 4.4). It was interesting to examine whether blocking PD-1, PD-L1, or PD-L2 had any effect on worm-and-egg-infected cytokine responses. Splenocytes were stimulated with soluble anti-CD3 mAb or with AW or SEA antigens. Cytokine responses from infected groups were Th2 biased, with elevated production of IL-4 in anti-CD3 mAb-stimulated cells compared to stimulated cells from the uninfected group (Fig. 5.4). IL-4 was produced in infected groups in response to AW and SEA. Levels of this cytokine were comparable among blocking antibody groups, with the control Ig group displaying slightly elevated levels in response to SEA. Levels of IFN- γ in response to anti-CD3 mAb were comparable among all groups (Fig. 5.4). Interestingly, IFN- γ was present in the media of anti-PD-L1 and to a lesser extent, anti-PD-L2 infected groups. Some of this cytokine was also produced in response to AW and SEA. As in the previous chapter (Fig. 4.4), levels of the regulatory cytokine IL-10 were elevated in infected groups compared to uninfected upon stimulation with anti-CD3 mAb. IL-10 is also produced in response to AW and SEA. Antibody-blocked groups are similar to that of the control Ig group. Cells from infected mice had markedly limited IL-2 production compared with uninfected mice, as also demonstrated in the previous chapter (Fig. 4.4). Interestingly, blocking of PD-1, PD-L1 or PD-L2 had no effect on IL-2 production in response to anti-CD3 mAb stimulation. Production of the pro-inflammatory cytokine TNF- α in response to anti-

CD3 mAb stimulation was also markedly reduced in infected groups. Again blocking of PD-1, PD-L1, or PD-L2 had little effect on production of this cytokine (Fig. 5.4).

In the previous chapter I demonstrated that PD-L1 was upregulated on macrophages from worm-infected mice to induce T cells anergy. *In vitro* blocking of PD-L1 using anti-PD-L1 mAb showed that PD-L1 was the mediator of this anergy, as blockade of PD-L1 on macrophages from worm-infected mice restored proliferation of CD4⁺ and CD8⁺ T cells from uninfected mice. The role of *in vivo* blocking of PD-L1 during schistosome worm-and-egg-infection was therefore examined more closely.

5.2.3 Confirmation of *in vivo* blocking of PD-L1 with mAb.

Flow cytometry staining was performed to verify that the anti-PD-L1 antibody used to block PD-L1 *in vivo* was functional, and could bind effectively to PD-L1. Spleen cells from 7-week infected mice that had been given control Ig or anti-PD-L1 mAb since week 4 of infection were taken and stained with a fluorescently-conjugated anti-rat IgG antibody. This would stain any rat antibody bound to the splenocytes. Cells were then stained for F4/80, CD4, CD8, and CD19 (a B cell marker). Cells from each gated population stained for the rat antibody in the anti-PD-L1 group, but not the control Ig group (Fig. 5.5). This showed that there was still bound antibody on the cells in the anti-PD-L1 group.

5.2.4 Infected mice treated with blocking anti-PD-L1 mAb have elevated levels of Th1/Tc1 cells compared to controls.

Spleen cells from uninfected mice or *S. mansoni* worm-and-egg-infected mice given control antibody, or blocking antibody against PD-L1, were stimulated *in vitro* with

Concanavalin A and surface-stained for CD4 or CD8 and intracellular IL-4 and IFN- γ . As expected, CD4⁺ cells in infected mice have a more Th2 phenotype than uninfected mice, with 5.66% IL-4⁺ CD4⁺ cells compared to 1.90% in the uninfected (Fig. 5.6). The levels of Th2 cells in the anti-PD-L1 group are comparable to the control mAb infected group. However there is an almost two-fold increase in levels of the Th1 cytokine, IFN- γ in the anti-PD-L1 mAb-treated group compared to the control mAb-treated group (3.87% versus 2.01% respectively).

There was also a striking difference in the Tc1 cell population between the two infected groups. The levels of IFN- γ producing CD8⁺ cells (Tc1 cells) were markedly elevated in the anti-PD-L1 treated group compared to control (Fig. 5.6).

5.2.5. Use of PD-L1 deficient mice (PD-L1 ko) to investigate the role of PD-L1 in *S. mansoni* infection.

The mAb data generated in the last few figures, particularly the skewing towards a Tc1 response (Fig. 5.6), prompted me to consider further investigations into the role of PD-L1 in *S. mansoni* infection. Blocking mAb against PD-L1 was a kind gift from Professor Azuma, Tokyo, Japan, but as the hybridoma was not available in-house, there was limited supply of mAb for use. In addition, there were the potential problems with mAb, as discussed earlier (chapter 4), such as development of anti-antibody responses with prolonged use. The lab began the generation of PD-L1 deficient (ko) mice, PD-L2 ko, or a double PD-L1/PD-L2 ko mouse, with our collaborators in Cambridge, UK. This gene inactivation model would aid our examination of the physiological relevance of PD-L1 and PD-L2.

During preparation of these mice, two papers involving the use of PD-L1 ko mice were published (Dong *et al.*, 2004; Latchman *et al.*, 2004). The observation by Dong and co-workers that CD8⁺ cells selectively accumulate in the liver of PD-L1 ko mice was especially interesting in light of my Tc1 data, and also as the liver is an important organ in terms of the pathology observed during *S. mansoni* infection. PD-L1 ko mice, on a C57BL/6 background, were kindly provided by Professor Lieping Chen (John Hopkins University, Maryland, US). The mice were then bred in-house to generate enough numbers for experiments.

Wildtype (wt) C57BL/6 or PD-L1 ko mice were given a light (35 cercariae) mixed sex *S. mansoni* infection. Weight was monitored from week 4 (pre-egg deposition) to week 8 of infection, where the infection is most acute (Fig. 5.7). There were no overt differences in infection between wt and ko with respect to weight change, with both groups maintaining a constant body weight throughout infection. The distinct weight loss observed earlier during infection (Fig. 5.1) was not present here, due to the lesser intensity of infection, and also the strain differences between mice. Analysis of parasitological parameters showed that there was no significant difference in egg counts in the liver or intestine between the wt or PD-L1 ko group (Fig. 5.8A, 5.8B), as was the case when PD-L1 was blocked in the mAb studies. H&E-stained histology slides of liver sections showed no overt differences in granuloma size or eosinophil counts between groups (Fig. 5.8C, 5.8D). Using flow cytometry analysis of liver granuloma cells showed a non-significant decrease in absolute numbers of eosinophils in the PD-L1 ko (Fig. 5.8E).

5.2.6 Cytokine responses in uninfected and worm-and-egg-infected C57BL/6 and PD-L1 ko mice.

Spleen cells from wt and PD-L1 ko mice were unstimulated or stimulated with anti-CD3 mAb, AW or SEA. Consistent with that observed in a BALB/c infection in chapter 4 (Fig. 4.4), infection induced a marked skewing towards a Th2 response, with greater production of Th2 cytokines IL-4 and IL-5, and reduced IFN- γ (Th1) in infected groups compared to uninfected (Fig. 5.9). The magnitude of the Th2 response in infected C57BL/6 mice was not as high as observed in the BALB/c infection in the previous chapter, in particular in relation to IL-4 production in response to anti-CD3 mAb and *S. mansoni* antigen. This could be due to the lesser intensity of infection, and also mouse strain differences. Low levels of IFN- γ were also produced in response to AW and SEA in infected groups (Fig. 5.9). Production of the regulatory cytokine IL-10 was elevated with infection, with increased IL-10 compared to uninfected groups upon anti-CD3 mAb stimulation, and also production in infected groups with AW and SEA stimulation. This is consistent with that observed previously in the BALB/c model (Fig. 4.4). However the lack of PD-L1 on spleen cells did not appear to have any effect on cytokine production, as there were no alterations between wt and PD-L1 ko groups in either uninfected or infected mice (Fig. 5.9). Minor exceptions were a reduction in IL-5 production in the infected PD-L1 ko group compared to wt in response to AW, and also a slight decrease in IL-10 in infected ko group in response to anti-CD3 mAb stimulation.

5.2.7 Alterations in T cell proliferation in PD-L1 ko mice.

Levels of IL-2, which drives T cell proliferation, were markedly reduced in infected groups compared to uninfected groups (Fig. 5.10A), which is consistent with what was seen in worm-and-egg-infected mice in chapter 4 (Fig. 4.4). However, in uninfected groups, I observed a slight reduction in anti-CD3 mAb-stimulated IL-2 production in the PD-L1 ko group compared to wt. This was less apparent in the infected group, and proliferation in response to SEA in infected groups was comparable between wt and ko (Fig. 5.10A). Examination of spleen cell proliferation also showed the T cell unresponsiveness similar to that previously observed in BALB/c infected mice (Fig. 5.10B). Again the levels of T cell proliferation in cells from infected mice were not restored by addition of exogenous IL-2, while it elevated proliferation in uninfected groups. Similar to what was observed when PD-L1 was blocked using mAb (Fig. 5.3), there was no obvious difference in proliferation in infected wt and ko groups, with equivalent proliferation in response to anti-CD3 mAb, AW and SEA. Uninfected wt and ko groups also had comparable proliferation in response to T cell stimuli, with the ko groups proliferating slightly less in response to anti-CD3 mAb than wt. Although some non-specific proliferation was observed in cells from mice treated with anti-PD-L1 mAb (Fig. 5.3), this was not observed in uninfected or infected PD-L1 ko C57BL/6 mice (Fig. 5.10B).

Although these initial studies showed no alterations in proliferative response in PD-L1 ko mice, since the publication by Dong and co-workers described a selective accumulation of CD8⁺, and not CD4⁺, T cells, it was of interest to examine whether there were any differences in proliferation between wt and ko in these specific T cell subsets. Spleen cells were labeled with CFSE and stimulated with anti-CD3 mAb to examine the *in vitro* proliferative response of CD4⁺ and CD8⁺ T cells respectively. Cells were surface stained for CD4 or CD8, and dead

cells were excluded by propidium iodide (PI) staining. In uninfected mice, a reduction in CD4⁺ T cell proliferation was observed (42% of cells with more than 4 divisions in wt versus 23.5% in ko) (Fig. 5.11A). The T cell unresponsiveness observed in infected mice was also present, with cells from infected mice undergoing less rounds of cell division than uninfected. However there was no difference in CD4⁺ T cell proliferation between wt and ko infected groups (Fig. 5.11A).

Examination of CD8⁺ T cell proliferation in response to anti-CD3 mAb showed that in uninfected groups, cells from PD-L1 ko mice proliferated less than wt, with far less cells undergoing a high number of divisions (78.8% in wt versus 24.7% in ko) (Fig. 5.11B). The reduction in proliferation in ko versus wt was also observed in CD8⁺ T cells responses in infected mice, albeit not to the same degree as that observed in the uninfected groups (Fig. 5.11B).

5.2.8 *PD-1 is markedly increased on CD4⁺ and CD8⁺ cells in PD-L1 ko mice compared to wt C57BL/6 mice.*

As discussed earlier, PD-1 is a known receptor for PD-L1. I have shown in the previous chapter that PD-1⁺ T cells and IL-2-producing T cells are distinct populations (Fig. 4.18). In light of the alterations in CD8⁺ T cell proliferation (Fig. 5.11B), and the reduction in IL-2 production in uninfected spleen cells (Fig. 5.10A), it was of interest to examine PD-1 and IL-2 expression on CD4⁺ and CD8⁺ T cell sub-populations. The known significant reduction in IL-2-producing CD4⁺ T cells from infected mice compared to uninfected mice was observed (Fig. 5.12A, 5.12B). There was a small, non-significant decrease in IL-2 production between wt and PD-L1 ko groups (Fig 5.12B). Similar to that described in chapter 4 (Fig. 4.18), PD-1⁺ CD4⁺ T cells were distinct from the IL-2-producing population in all groups. In

C57BL/6 mice infection induced a significant increase in PD-1⁺ CD4⁺ T cells, which was similar to that observed with BALB/c infection of mice in chapter 4 (Fig. 4.16). However what was most startling was the significant increase in PD-1⁺ CD4⁺ cells in both uninfected and infected PD-L1 ko groups compared to wt (Fig. 5.12A, 5.12C). Levels of PD-1 were elevated in uninfected ko mice, with a non-significant increase being observed on the already higher levels observed in infected ko mice (Fig. 5.12).

Although levels of IL-2 production are dramatically lower in CD8⁺ cells compared to CD4⁺ T cells, infection significantly reduced CD8⁺ IL-2-production (Fig. 5.13A, 5.13C). In uninfected groups, there was significantly less IL-2 production in the PD-L1 ko group compared to the wt group (Fig. 5.13), which is consistent with the ELISA data discussed earlier (Fig. 5.10A). There is little PD-1 expression on wt uninfected or infected CD8⁺ T cells, however CD8⁺ spleen cells from PD-L1 ko mice display significantly higher levels of PD-1 in both uninfected and infected groups (Fig 5.13A, 5.13C). Levels of PD-1 on CD8⁺ T cells from PD-L1 ko mice are comparable between uninfected and infected groups.

5.2.9 Investigations into T regulatory cell marker expression in PD-L1 ko mice.

The elevated PD-1 expression on T cells from PD-L1 ko mice was intriguing, and as PD-1 may sometimes be expressed on CD4⁺CD25⁺ regulatory T cells as well as activated T cells (Lechner *et al.*, 2001), it prompted me to assess the levels of regulatory cell markers on uninfected and infected wt and ko mice. It was also of interest to examine whether PD-1 expression was co-incident with regulatory cell marker expression on T cells.

Spleen cells from uninfected and week 8-infected mice were stained for Foxp3 and PD-1 expression on CD4⁺ T cells. Similar to that observed in BALB/c infected mice in chapter 3 (Fig. 3.2), there were no major differences in Foxp3 expression between uninfected

and acute *S. mansoni* infected C57BL/6 mice (Fig. 5.14A, 5.14B). PD-L1 ko mice also had comparable levels of Foxp3 expression compared to wt. PD-1 was again upregulated on CD4⁺ T cells from PD-L1 ko mice in both uninfected and infected mice. This contributed to an approximate 2-fold increase in the Foxp3⁺PD-1⁺ CD4⁺ T cell population in PD-L1 ko groups, with approx 4% of CD4⁺ T cells co-expressing Foxp3 and PD-1 in uninfected and infected PD-L1 ko mice compared just over 2% in wt mice (Fig. 5.14A).

It is known that the majority of Foxp3 expression is limited to the CD4⁺ T cell population, however some CD8⁺ T cell Foxp3 expression has been reported in mice (Bienvenu *et al.*, 2005). I observed low levels (>1%) of Foxp3 expression on CD8⁺ T cells in all groups (Fig. 5.15A), with a non-significant increase in Foxp3 in the infected PD-L1 ko group compared to the wt infected group (Fig. 5.15B). The previously noted significant increase in CD8⁺ PD-1 expression in the PD-L1 ko groups (Fig. 5.13) was also seen here, again with no alterations between the uninfected and infected group (Fig. 5.15).

The IL-2R β , CD122, is known to be expressed on regulatory CD8⁺ cells (Rifa'i *et al.*, 2004). *S. mansoni* infection induced a highly significant increase in CD122⁺ CD8⁺ T cells compared to uninfected C57BL/6 or PD-L1 ko mice (Fig. 5.16A, 5.16B). There was a significant increase in CD122 expression in splenic CD8⁺ T cells from uninfected PD-L1 ko mice compared to wt, however there was only a non-significant increase between wt and ko in *S. mansoni*-infected groups. The expansion of PD-1⁺ cells in PD-L1 ko groups was associated with a CD122^{lo} population, that appears distinct from the CD122⁺ PD-1⁻ population observed (Fig. 5.16A).

5.2.10 Alterations in IL-4/IFN- γ frequencies during infection in PD-L1 ko mice.

During *S. mansoni* infection of BALB/c mice, blocking PD-L1 using mAb induced an expansion of IFN- γ -producing CD4⁺ and CD8⁺ T cells (Fig. 5.6). Although whole spleen cell stimulation from wt and PD-L1 ko mice produced comparable levels of cytokines between infected groups (Fig. 5.9), I investigated IL-4 and IFN- γ production on CD4⁺ and CD8⁺ T cell subsets using intracellular flow cytometry. In contrast to the mAb blocking study, there was a non-significant increase in Tc1 cells in infected PD-L1 ko mice compared to wt infected mice, and there was no difference in Tc1 levels between uninfected groups (Fig. 5.17).

I previously observed an increase in the level of IFN- γ -producing CD4⁺ spleen cells (Th1 cells) from anti-PD-L1-treated infected mice (Fig. 5.6), but Th1 levels were comparable between wt and PD-L1 ko infected mice, and also between wt and ko uninfected mice (Fig. 5.18A). There was however a significant decrease in IL-4-producing CD4⁺ T cells (Th2 cells) in the infected PD-L1 ko group compared to the wt infected group (Fig. 5.18B). Levels of the Th2 cell surface marker, T1/ST2, were also significantly reduced on CD4⁺ T cells in the infected ko group compared to wt (Fig. 5.18C).

5.2.11 Reduced levels of apoptosis in CD8⁺ spleen cells from PD-L1 ko mice in infection.

The alterations in PD-1 expression on T cells from PD-L1 ko mice prompted me to examine the levels of cell death and apoptosis between wt and ko with and without *S. mansoni* infection. Infection induced an increase in both dead cells (PI⁺) and apoptotic CD4⁺ T cells (AnnexinV⁺ PI⁺) (Fig. 5.19A and 5.19B), which is consistent with previous studies (Fallon *et al.*, 1998). However, I observed a small but significant increase in the levels of apoptotic cells in the uninfected PD-L1 ko group compared to the wt uninfected group (Fig. 5.19C). CD4⁺

spleen cells from infected PD-L1 ko mice did not have this increase over wt infected mice, and indeed there was a non-significant decrease in apoptosis levels in this group (Fig. 5.19C).

Dead (PI⁺) CD8⁺ T cells are significantly increased with infection in both wt and ko groups (Fig. 5.20A, 5.20B). Levels of apoptotic cells are also increased in infected groups compared to uninfected groups (Fig. 5.20A, 5.20C), again consistent with previous studies (Fallon *et al.*, 1998). In uninfected groups, CD8⁺ T cell apoptosis levels as determined by annexin V detection, are comparable between wt and PD-L1 groups. However the increase in apoptotic cells observed with infection is not as great in the PD-L1 ko group, with levels being significantly lower than those of the wt infected group (Fig. 5.20A, 5.20C).

5.2.12 Accumulation of CD8⁺ T cells in the liver of uninfected PD-L1 ko mice, but no alteration in liver or granuloma composition with infection.

Dong and co-workers demonstrated a selective accumulation of CD8⁺ T cells in the liver of PD-L1 ko mice compared to wild-types (Dong *et al.*, 2004). The liver is an important organ in *S. mansoni* infection, often becoming enlarged and it is also the site of many egg granulomas. I investigated the absolute numbers of liver and liver granuloma CD4⁺ and CD8⁺ T cells, and the levels of apoptosis in these populations.

Determination of absolute numbers of liver CD4⁺ T cells showed no difference in levels between uninfected wt and ko groups (Fig. 5.21A). Infection induced an increase in liver CD4 cells, with comparable levels between wt and ko infected groups. Liver CD4⁺ T cells were assessed for levels of dead and apoptotic cells. Both levels of dead (PI⁺) and apoptotic (Annexin V⁺ PI⁺) cells are comparable between uninfected wt and uninfected ko groups (Fig. 5.21B). Infection induces a significant increase in both dead and apoptotic cells (Fig. 5.21B, 5.21C), with comparable levels between wt and ko infected groups.

The accumulation of CD8⁺ T cells in the liver of PD-L1 ko mice reported by Dong and co-workers was also present in this study, with a highly significant increase in absolute numbers in the liver from uninfected PD-L1 ko mice compared to uninfected wt mice (Fig. 5.22A). Infection also induced an increase in liver CD8⁺ T cell number, but there was only a non-significant increase in numbers between infected wt and ko groups. The level of dead cells was comparable between wt and ko groups, with infection inducing a small increase on an already high % of dead cells (Fig. 5.22B) (compared to CD4⁺ T cells, Fig. 5.21). In contrast to the spleen data (Fig. 5.20), levels of apoptotic CD8⁺ T cells were comparable between all groups (Fig. 5.22C).

As observed earlier, liver granuloma size and eosinophil composition was unaltered in PD-L1 ko compared to wt groups (Fig. 5.8), with a non-significant decrease in absolute number of granuloma eosinophils as determined by flow cytometry. CD4⁺ and CD8⁺ T cell liver granuloma numbers are also comparable between ko and wt groups (Fig. 5.23A, 5.23B). Although there is a significant reduction in levels of the Th2 marker T1/ST2 on splenic CD4⁺ T cells from infected PD-L1 ko mice (Fig. 5.18C), there was only a non-significant decrease in this cell population in the liver granuloma (Fig. 5.23C). There are a greater percentage of dead CD8⁺ T cells in the granuloma than CD4⁺ T cells, but this is comparable between wt and ko groups (Fig. 5.24). Levels of apoptotic cells are also comparable between wt and ko groups (Fig. 5.24).

5.2.13 *The down-modulation of the immune response observed during the chronic stage of S. mansoni infection is unaltered in PD-L1 ko mice.*

In this study an absence of PD-L1 in 8-week infected mice has had only marginal effects, with no alterations in weight loss or granuloma formation (Fig. 5.7, 5.8). To examine

if lack of PD-L1 has any effect on chronic *S. mansoni* infection, C57BL/6 wt and PD-L1 ko mice were given a mixed sex cercariae infection for 16 weeks, and their weights were measured routinely from 12 weeks post-infection. There was no overt difference in weight change between groups, with both groups maintaining a fairly constant body weight through to week 16 post-infection (Fig. 5.25A). Liver and intestine egg counts, granuloma size and eosinophil composition were also comparable between groups (Fig. 5.25 B-E).

Spleen cells from chronically-infected wt and ko mice displayed comparable proliferation in response to anti-CD3 mAb, AW or SEA stimulation (Fig. 5.26A). Levels of the Th2 cytokine IL-4 were reduced in chronically infected mice compared to week 8 infected mice (Fig. 5.9), again with no differences between wt and ko groups (Fig. 5.26B). IL-2 levels were also very low in chronically infected mice, but in all cases there was no alterations in ko groups compared to the wt infected group (Fig. 5.26B).

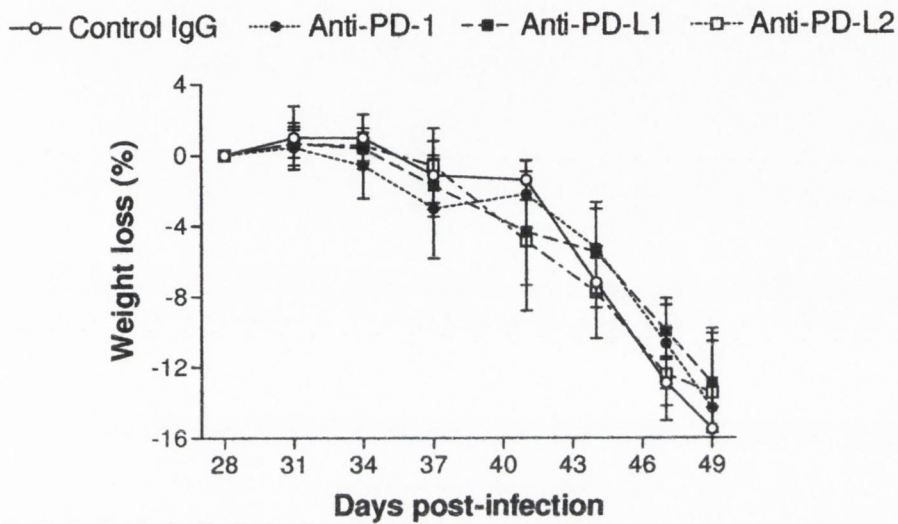


Figure 5.1. Weight change in *S. mansoni*-infected mice treated with control mAb or blocking mAb against PD-1, PD-L1, or PD-L2. BALB/c mice were treated with control or blocking mAbs from 4 weeks post-infection, and mice were weighed periodically until 7 weeks post-infection. Data are mean $\pm$ SEM values from 6-7 infected mice per group, and are representative of 2 separate experiments.

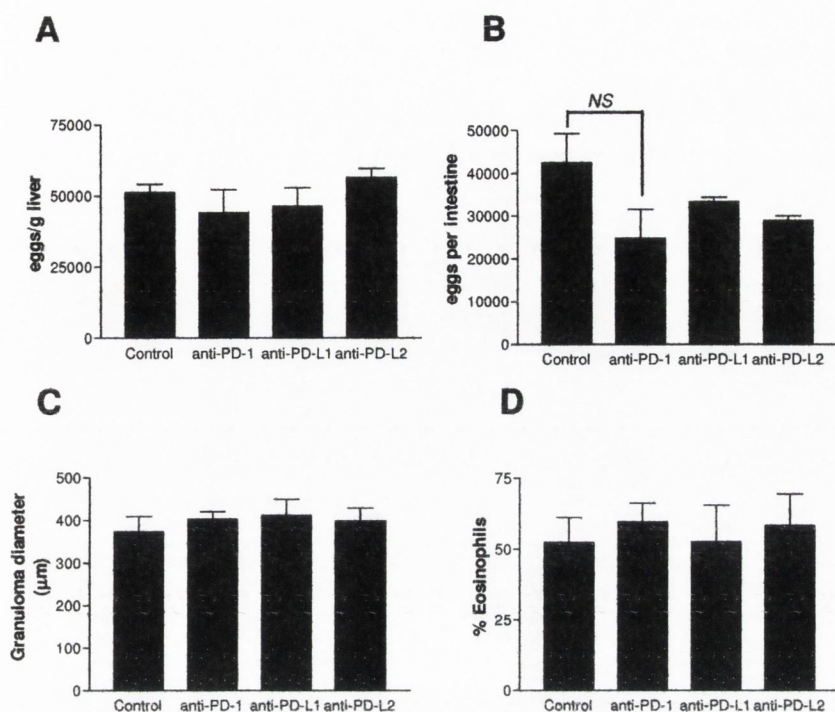


Figure 5.2. *Parasitological parameters from PD-1/PD-L1/PD-L2 treated mice are comparable to those in control mAb-treated infected mice.* Egg counts from (A) liver and (B) intestine of worm-and-egg-infected mice treated with control antibody or blocking antibody to PD-1, PD-L1, or PD-L2. C, liver granuloma diameter, and D, % eosinophils in the liver granuloma. Counts were performed on four individual mice, and error bars represent the SEM.

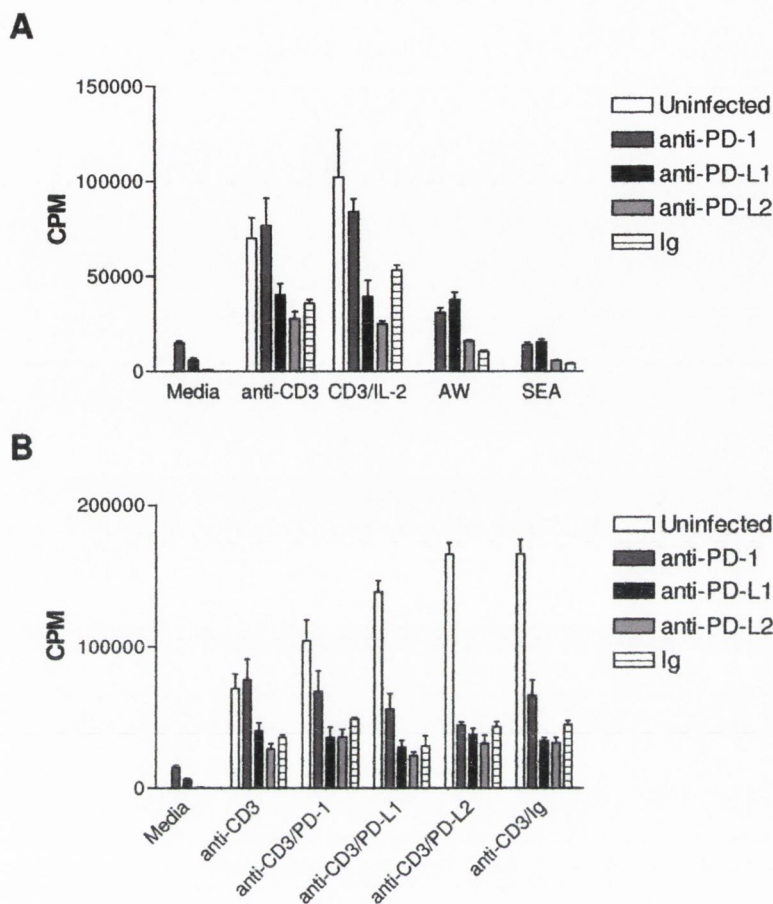


Figure 5.3. Proliferation of spleen cells from uninfected mice or worm-and-egg-infected mice treated with mAbs. Infected mice have been administered an IgG control mAb or anti-PD-1, anti-PD-L1, or anti-PD-L2 mAbs from 4 weeks post-infection. Cells were activated with soluble anti-CD3 mAb (0.5 μ g/ml), with or without IL-2 (20 ng/ml), anti-PD-1, anti-PD-L1, anti-PD-L2, or control Ig (0.5 μ g/ml). Cells were also activated with 20 μ g/ml AW or SEA. Proliferation was measured by [3 H] incorporation and expressed as CPM. Data shown is mean $\pm$ SD of triplicate wells from pools of 3-5 mice per group, and is representative of two separate experiments.

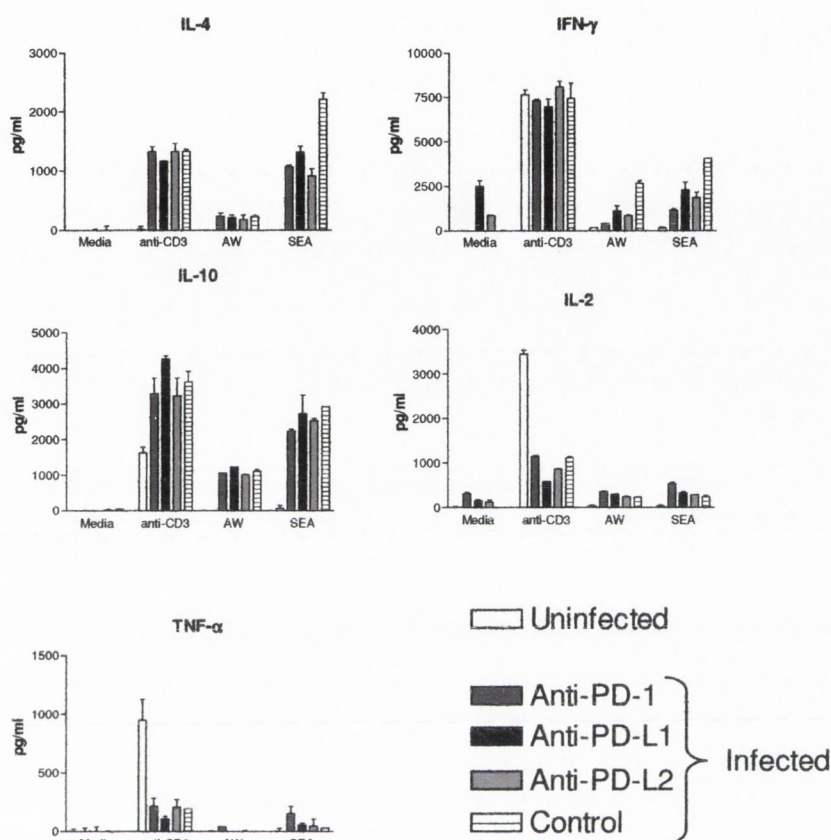


Figure 5.4. Cytokine profiles from uninfected mice and worm- and egg-infected mice treated with control or blocking mAb. Infected mice have been administered an IgG control mAb or anti-PD-1, anti-PD-L1, or anti-PD-L2 mAbs from 4 weeks post-infection. Spleen cells from each group were activated with anti-CD3 mAb (0.5 μ g/ml) or SEA or AW (20 μ g/ml). Supernatants were collected after 24 h (IL-2 and TNF- α) and 72 h (IL-4, IL-10, IFN- γ) for cytokine ELISA. Data are from pools of three or four individual mice and are representative of two separate experiments.

Black =	Rat IgG control (worm-and-egg-infected) treated group
Grey =	Rat anti-mouse PD-L1 (worm-and-egg-infected) treated group

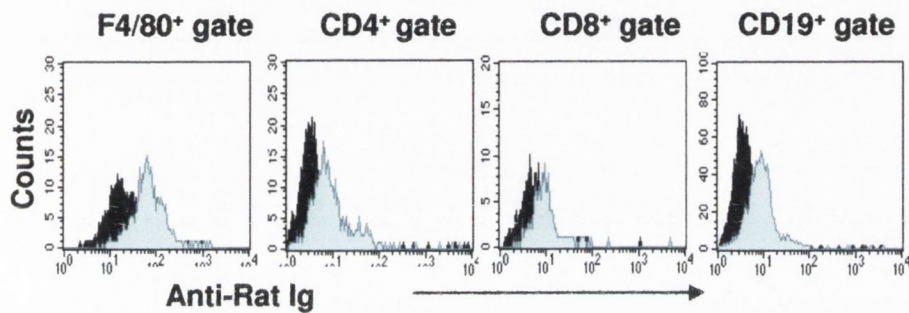


Figure 5.5. *Verification of in vivo blocking of PD-L1 with mAb.* Spleen cells from control IgG and anti-PD-L1 mAb infected mice were stained with anti-rat IgG antibody followed by staining for F4/80, CD4, CD8, and CD19. Histograms show anti-rat Ig fluorescence on various gated populations of cells. Shading: Black = control IgG mAb, Grey = anti-PD-L1 mAb.

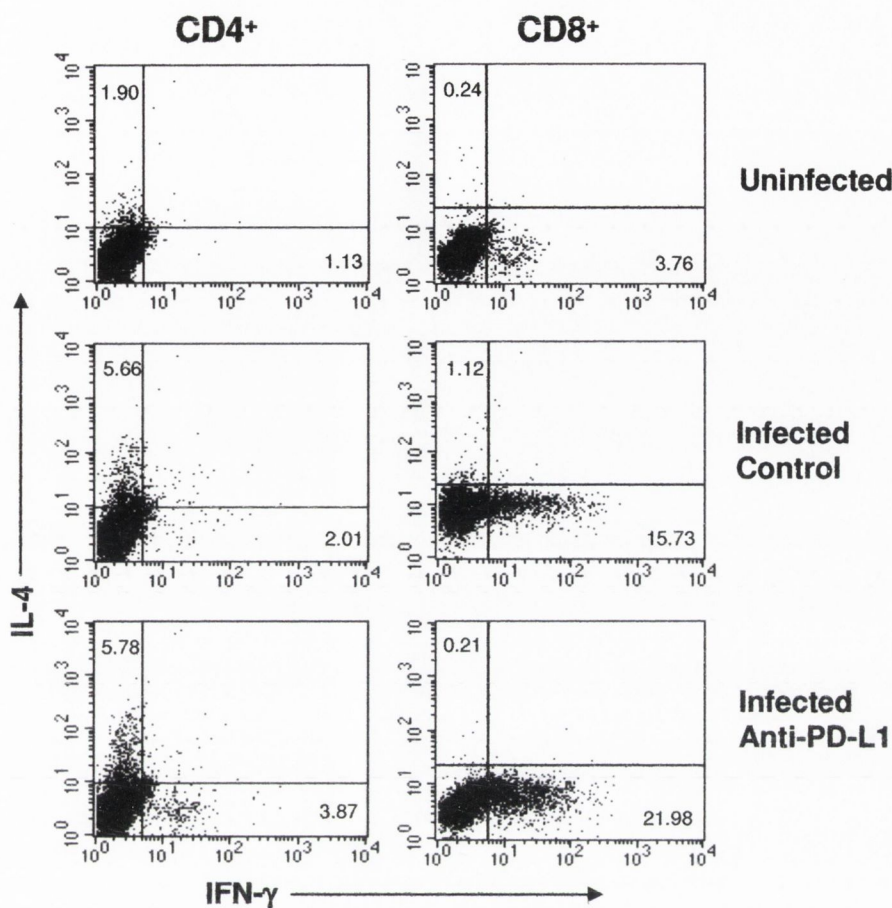


Figure 5.6. *Infected mice treated with blocking anti-PD-L1 mAb have elevated levels of Tc1 cells, and diminished Tc2 cells compared to controls. Spleen cells were taken from uninfected or worm-and-egg-infected mice treated with control mAb or anti-PD-L1 mAb, and were stimulated with Concanavalin A (2 μ g/ml) for 6 hours, Brefeldin A (10 μ g/ml) was added for the final 4 hours. Cells were stained for CD4 or CD8, and fixed and permeabilised to stain for IL-4 and IFN- γ . Numbers indicate percentage of CD4⁺/CD8⁺ cells positive for IL-4 or IFN- γ . Data is representative of 2 separate experiments from pools of 2-4 mice.*

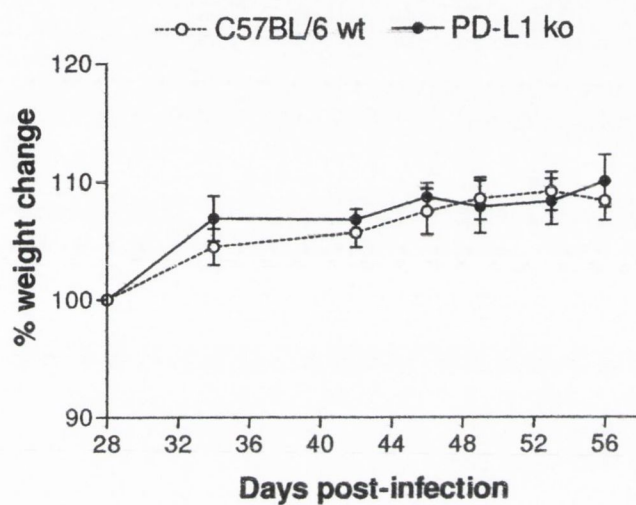


Figure 5.7. Weight change in wild-type *C57BL/6* and *PD-L1 ko* mice with *S. mansoni* infection. *C57BL/6* and *PD-L1 ko* mice were infected with *S. mansoni*. Weights were measured periodically from week 4 post-infection, and mice were sacrificed for immunological and histological analysis at 8 weeks post-infection. Data is mean $\pm$ SEM of 4 individual mice and is representative of three separate experiments.

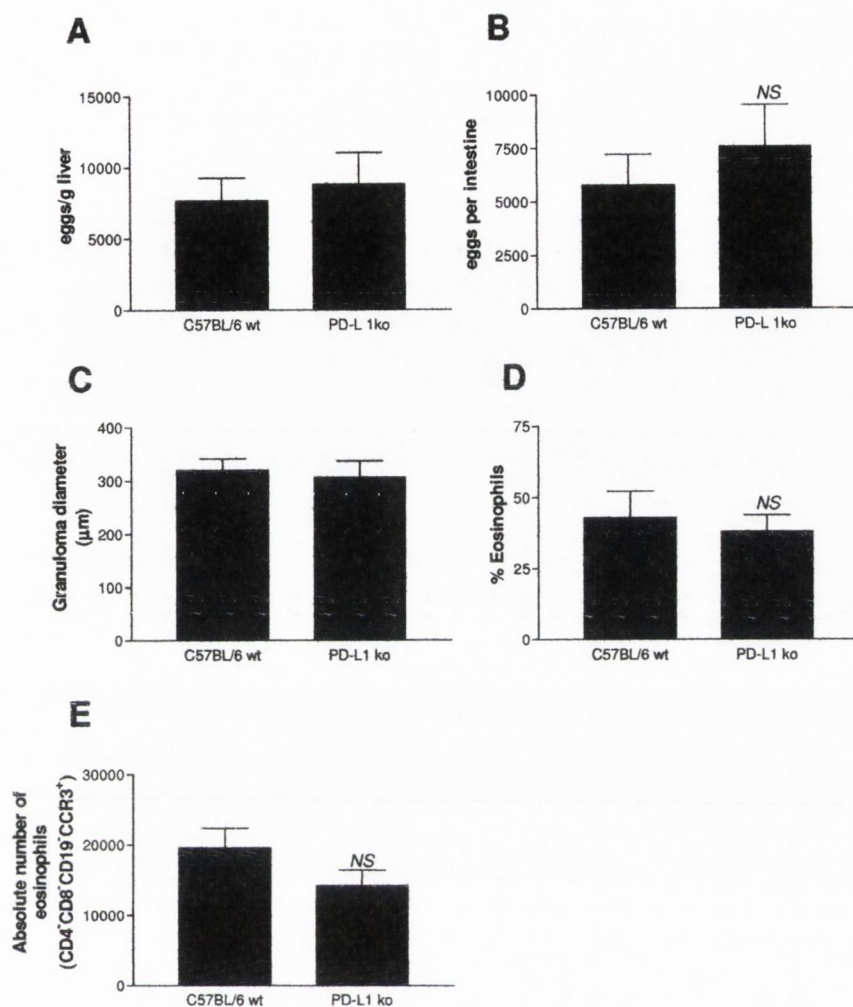


Figure 5.8. Parasitological parameters from PD-L1 ko mice are comparable to those in wild-type C57BL/6 infected mice. Egg counts from A, liver and B, intestine of wt and PD-L1 ko worm-and-egg-infected mice. C, liver granuloma size (diameter) from wt and ko infected mice. D, % eosinophils in liver granuloma. Data is mean $\pm$ SEM from 5-6 mice. E, absolute numbers of granuloma eosinophils, as detected by flow cytometry (CD4⁺CD8⁺CD19⁺CCR3⁺). Data is mean $\pm$ SEM from 3 individual mice and is representative from 2-3 separate experiments.

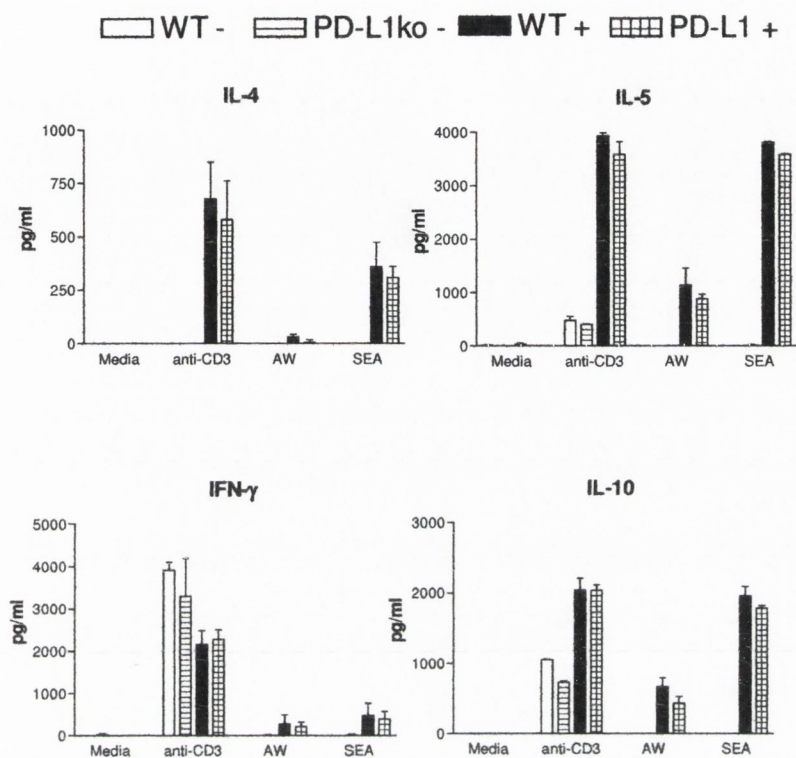
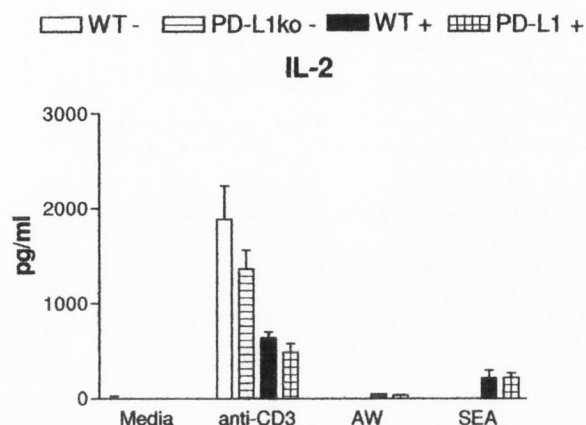


Figure 5.9. Cytokine profiles from uninfected and 8 week worm- and-egg-infected wt and PD-L1 ko mice. Spleen cells from each group were activated with anti-CD3 mAb (0.5 μ g/ml) or SEA or AW (20 μ g/ml). Supernatants were collected after 72 h for cytokine ELISA. Data are mean $\pm$ SEM from three or four individual mice and are representative of two separate experiments. (WT- = uninfected C57BL/6, PD-L1 ko- = uninfected PD-L1 ko, WT + = infected C57BL/6, PD-L1 ko + = infected PD-L1 ko)

A



B

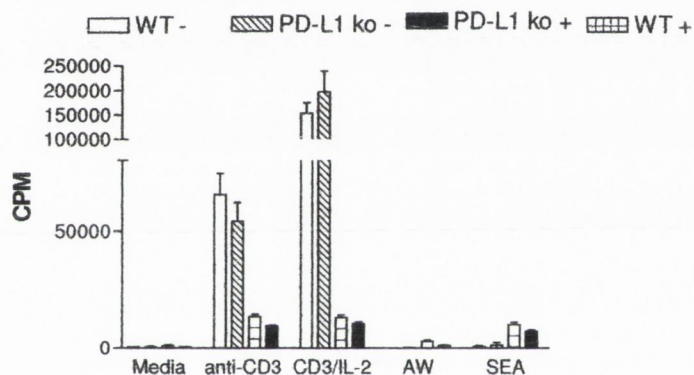


Figure 5.10. *IL-2 production and proliferation in uninfected and infected C57BL/6 wt and PD-L1 ko mice.* A, spleen cells from each group were activated with anti-CD3 mAb (0.5 μ g/ml) or SEA or AW (20 μ g/ml). Supernatants were collected after 24 h for IL-2 cytokine ELISA. B, spleen cells were activated with soluble anti-CD3 mAb (0.5 μ g/ml), with or without IL-2 (20 ng/ml), AW or SEA (20 μ g/ml). Proliferation was measured by [3 H] incorporation and expressed as CPM. Data shown is mean $\pm$ SEM from 3-4 individual mice per group, and is representative of two separate experiments. (WT- =uninfected C57BL/6, PD-L1 ko- = uninfected PD-L1 ko, WT + = infected C57BL/6, PD-L1 ko + = infected PD-L1 ko)

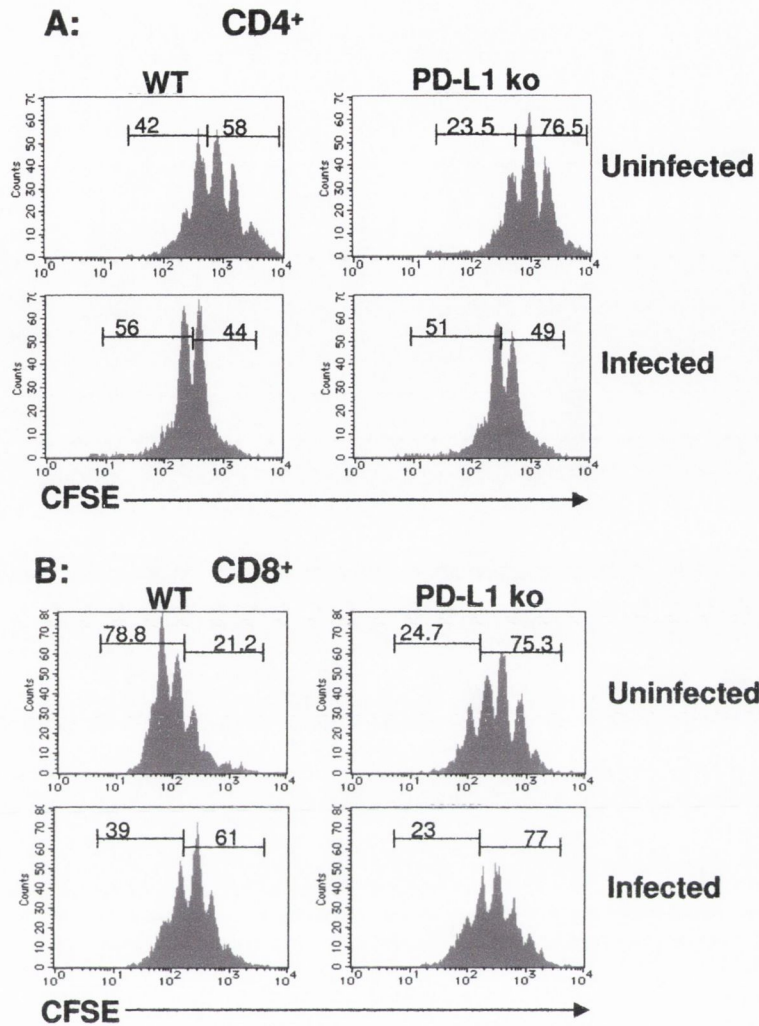


Figure 5.11. Alterations in CD4⁺ and CD8⁺ T cell proliferation in uninfected PD-L1 ko mice compared to wt. Spleen cells were taken from uninfected or week 8 infected wt C57BL/6 or PD-L1 ko mice and stained with 0.5 μ M CFSE. Cells were stimulated with 0.5 ug/ml anti-CD3 mAb for 72 h. Cells were harvested and fluorescently stained for CD4 or CD8. Dead cells were excluded by PI staining and cell divisions of gated CD4⁺ (A) or CD8⁺ (B) T cells was visualised by the fluorescent CFSE. Data is from pools or individual mice and is representative of 2-3 separate experiments.

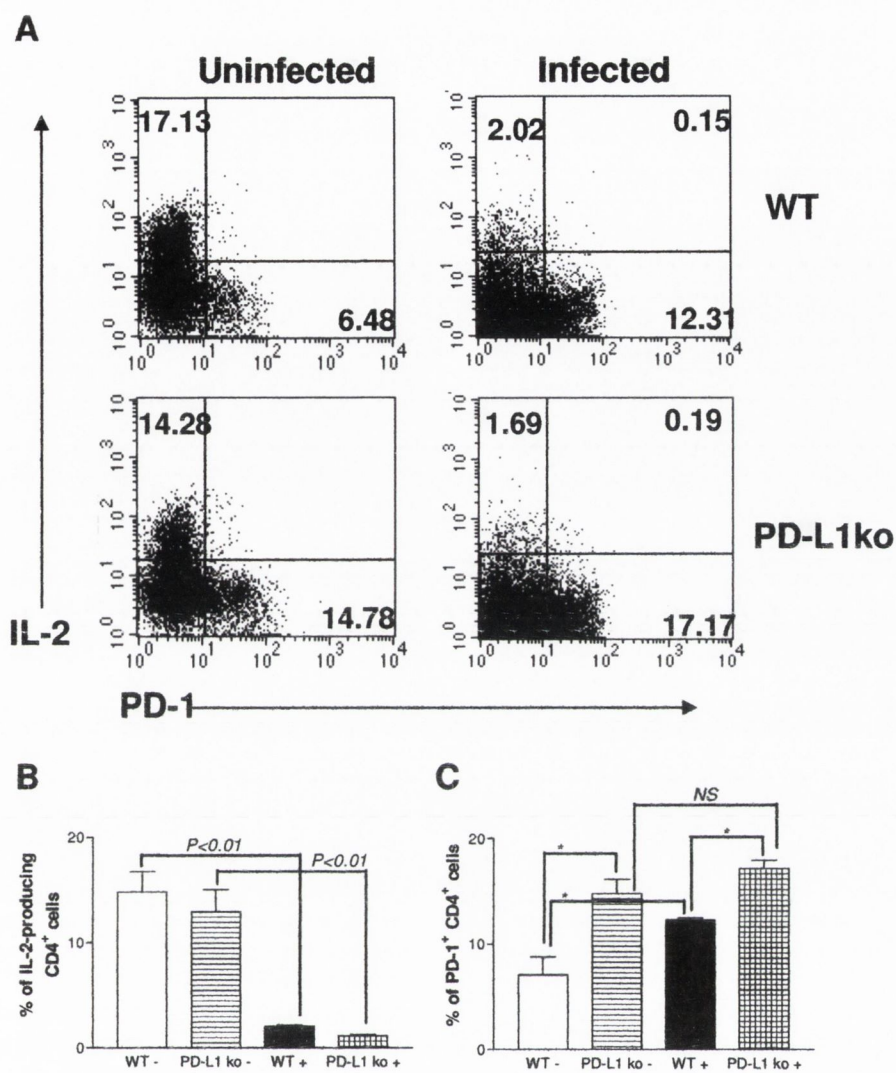


Figure 5.12. *PD-1 is increased in uninfected and infected CD4⁺ T cells from PD-L1 ko mice.* *A*, intracellular IL-2 and surface PD-1 expression on CD4⁺-gated spleen cells from uninfected and *S. mansoni*-infected wt and PD-L1 ko mice. *B*, bar graph showing % of IL-2-producing CD4⁺ T cells. *C*, bar graph representing % of PD-1⁺ CD4⁺ T cells. Student's t-test was used to compare differences between groups. (* = $P < 0.05$). Data are mean $\pm$ SEM of 3-4 individual mice and is representative of 2-3 separate experiments.

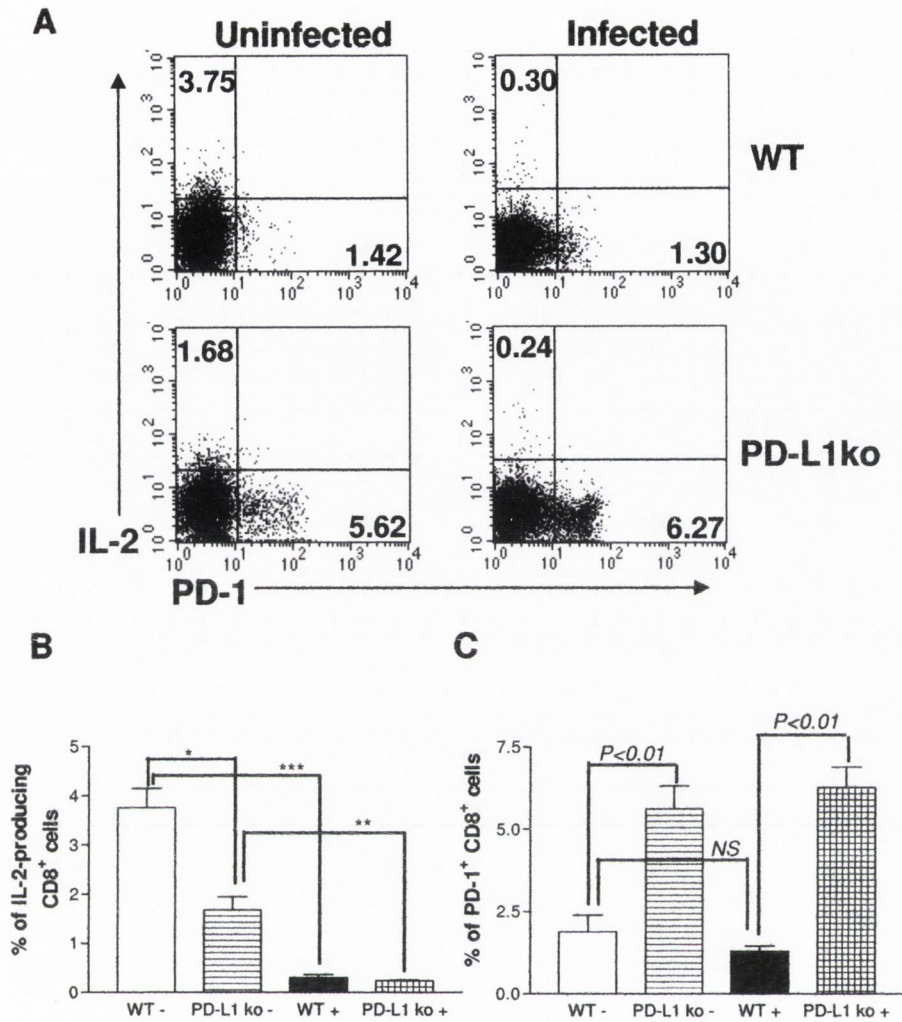


Figure 5.13. *PD-1 is increased in uninfected and infected CD8⁺ T cells from PD-L1 ko mice, and IL-2 is reduced in uninfected PD-L1 ko compared to uninfected wt CD8⁺ T cells. A, intracellular IL-2 and surface PD-1 expression on CD8⁺-gated spleen cells from uninfected and infected wt and PD-L1 ko mice. B, bar graph showing % of IL-2-producing CD8⁺ T cells. C, bar graph representing % of PD-1⁺ CD8⁺ T cells. Student's t-test was used to compare differences between groups. Data are mean $\pm$ SEM of 3-4 individual mice and is representative of 2-3 separate experiments.*

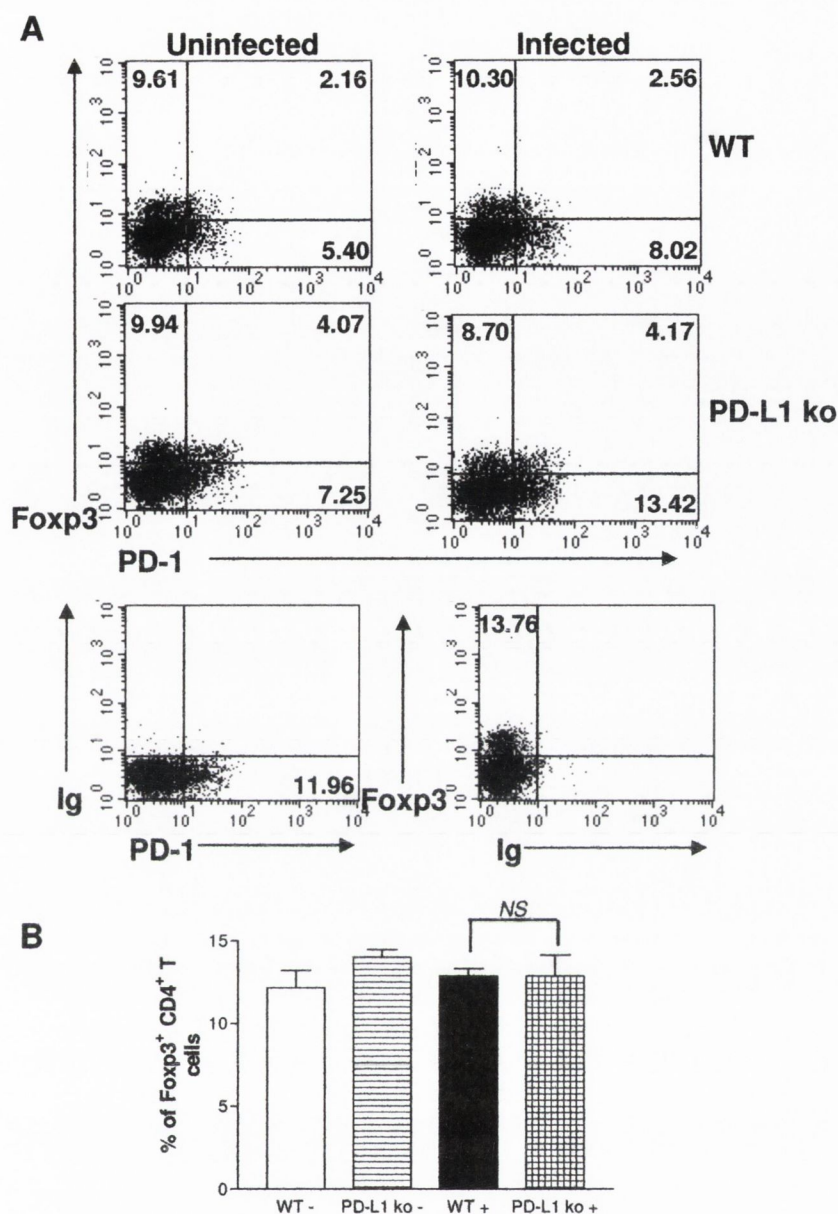


Figure 5.14. CD4⁺ T cell *Foxp3* expression is comparable between uninfected and infected C57BL/6 and PD-L1 ko mice. *A*, intracellular Foxp3 and surface PD-1 expression on CD4⁺-gated spleen cells from uninfected and infected wt and PD-L1 ko mice. *B*, bar graph showing % of Foxp3⁺ CD4⁺ T cells. Student's t-test was used to compare differences between groups. Data are mean $\pm$ SEM of 3-4 individual mice and is representative of 2-3 separate experiments.

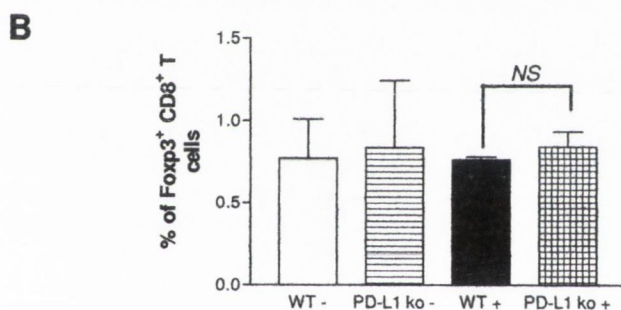
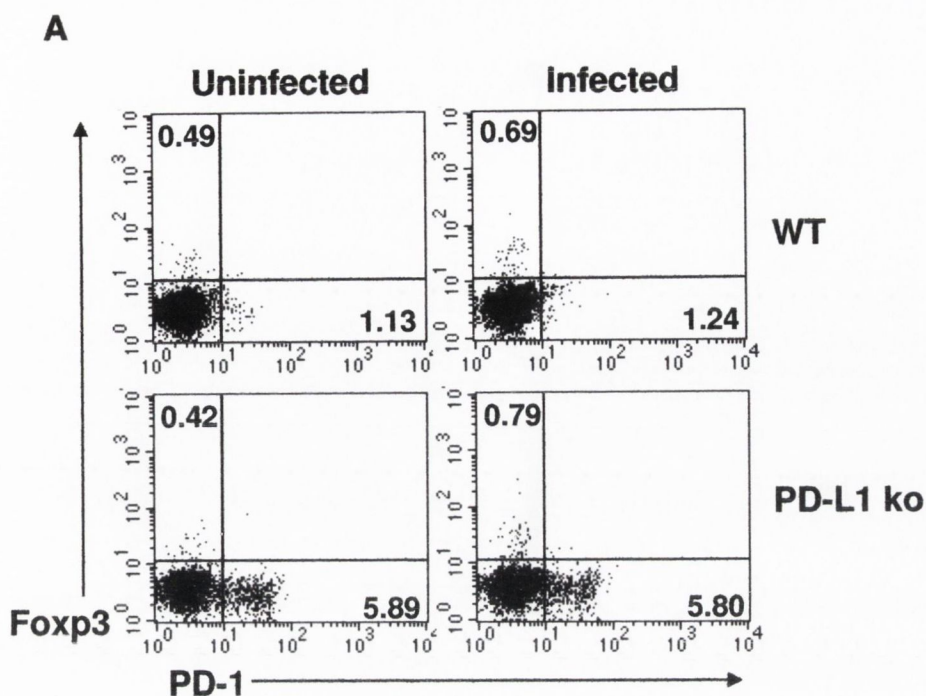


Figure 5.15. CD8⁺ T cell *Foxp3* expression is comparable between uninfected and infected C57BL/6 and PD-L1 ko mice. *A*, intracellular Foxp3 and surface PD-1 expression on CD8⁺-gated spleen cells from uninfected and infected wt and PD-L1 ko mice. *B*, bar graph showing % of Foxp3⁺ CD8⁺ T cells. Student's t-test was used to compare differences between groups. Data are mean $\pm$ SEM of 3-4 individual mice and is representative of 2-3 separate experiments.

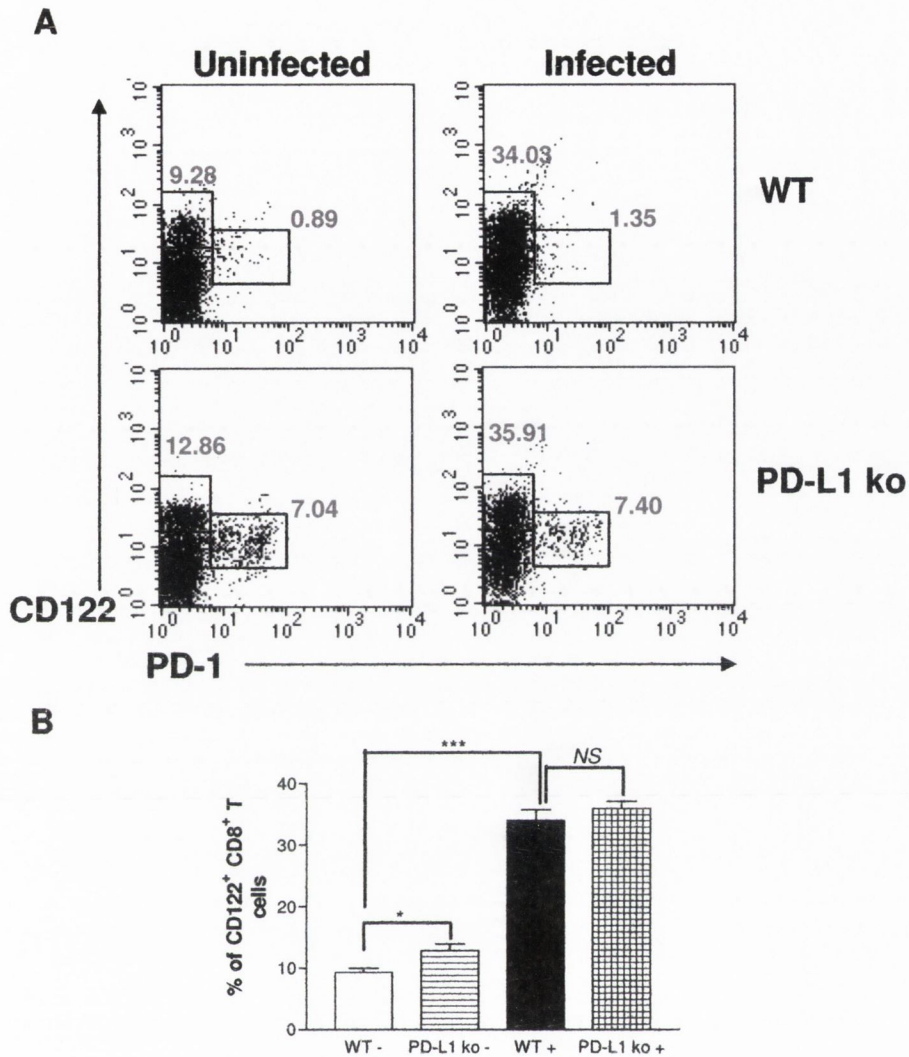


Figure 5.16. *CD122 is upregulated on uninfected PD-L1 ko CD8⁺ T cells compared to wt, and infection also induces an increase in CD122 expression on CD8⁺ T cells. A, surface PD-1 and CD122 expression on CD8⁺-gated spleen cells from uninfected and infected wt and PD-L1 ko mice. B, bar graph showing % of CD122⁺ CD8⁺ T cells. Student's t-test was used to compare differences between groups. Data are mean $\pm$ SEM of 3-4 individual mice and is representative of 3 separate experiments.*

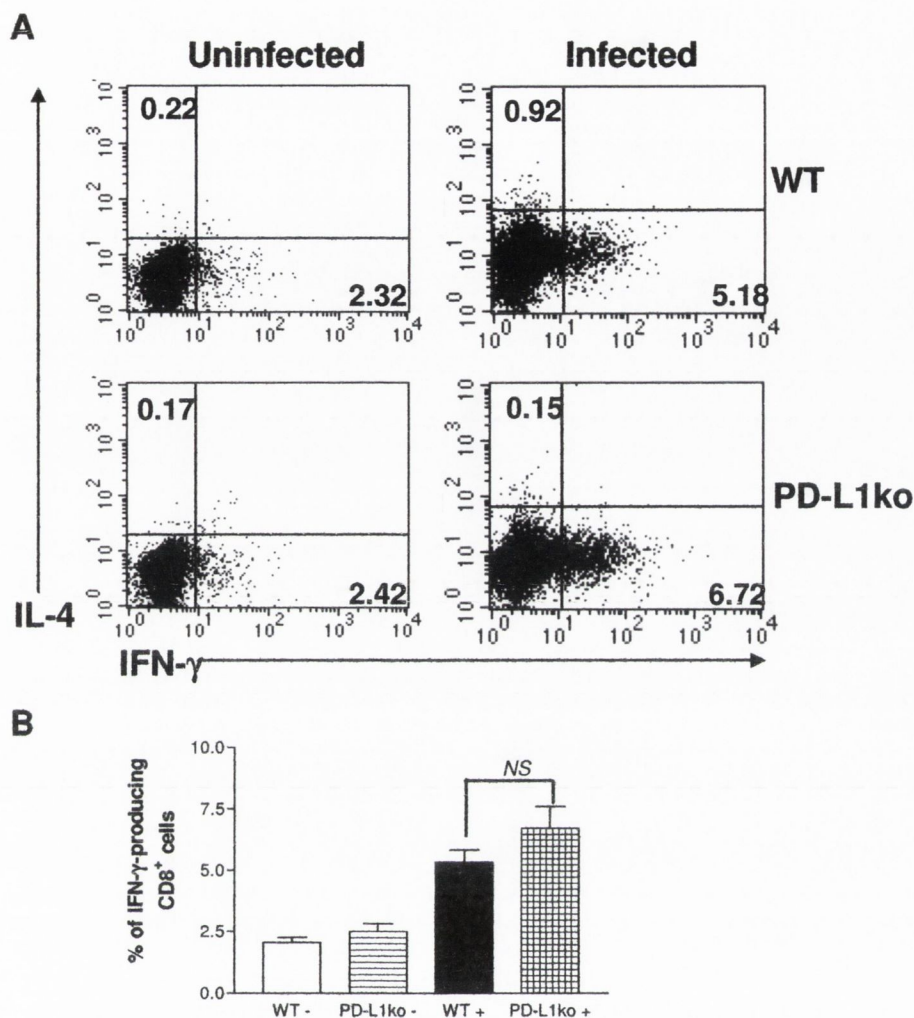


Figure 5.17. *S. mansoni*-infected *PD-L1* ko mice have a non-significant increase in IFN- γ -producing CD8⁺ T cells compared to wt infected mice. **A**, spleen cells from uninfected or infected wt and ko mice were stimulated with Concanavalin A (2 μ g/ml) for 6 hours, Brefeldin A (10 μ g/ml) was added for the final 4 hours. Cells were stained for CD8, and fixed and permeabilised to stain for IL-4 and IFN- γ . **B**, bar graph representing the % of IFN- γ -producing CD8⁺ T cells. Student's t-test was used to compare differences between groups. Data are mean $\pm$ SEM of 3-4 individual mice and is representative of 3 separate experiments.

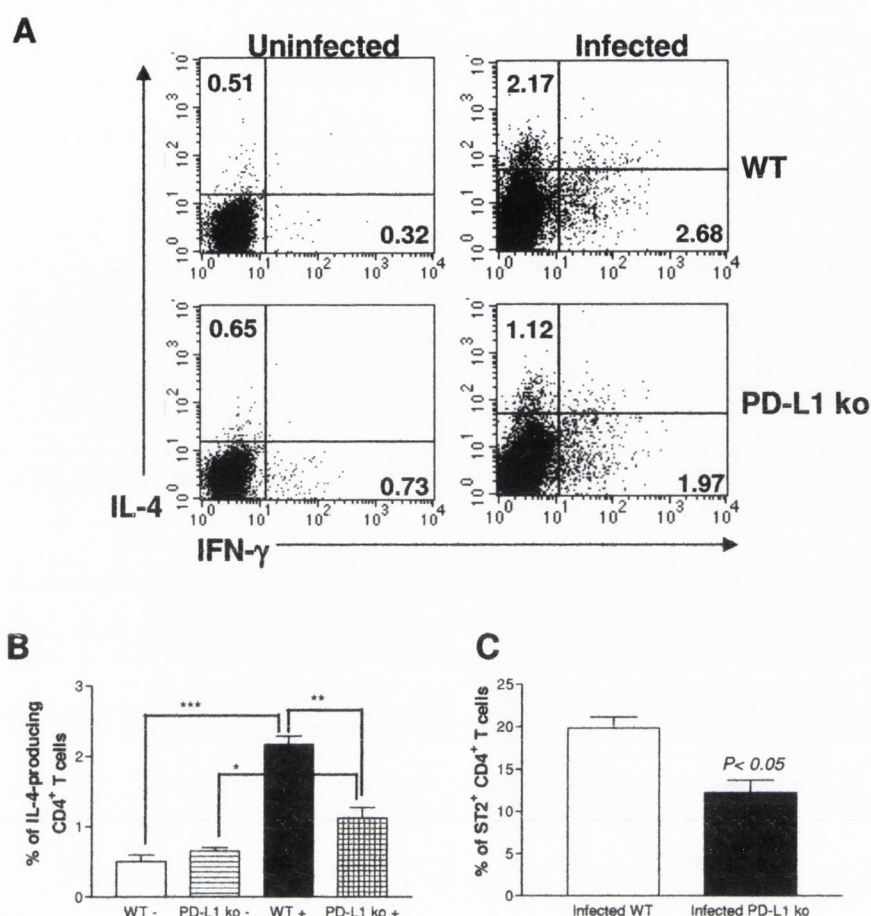


Figure 5.18. *S. mansoni*-infected PD-L1 ko mice have a significant decrease in IL-4-producing and ST2⁺ CD4⁺ T cells compared to wt infected mice. A, spleen cells from uninfected or infected wt and ko mice were stained for CD4, and intracellular IL-4 and IFN- γ . B, bar graph representing the % of IL-4-producing CD4⁺ T cells. C, bar graph representing the % of T1/ST2⁺ CD4⁺ cells in infected groups. Student's t-test was used to compare differences between groups. Data are mean $\pm$ SEM of 3-4 individual mice and is representative of 2-3 separate experiments.

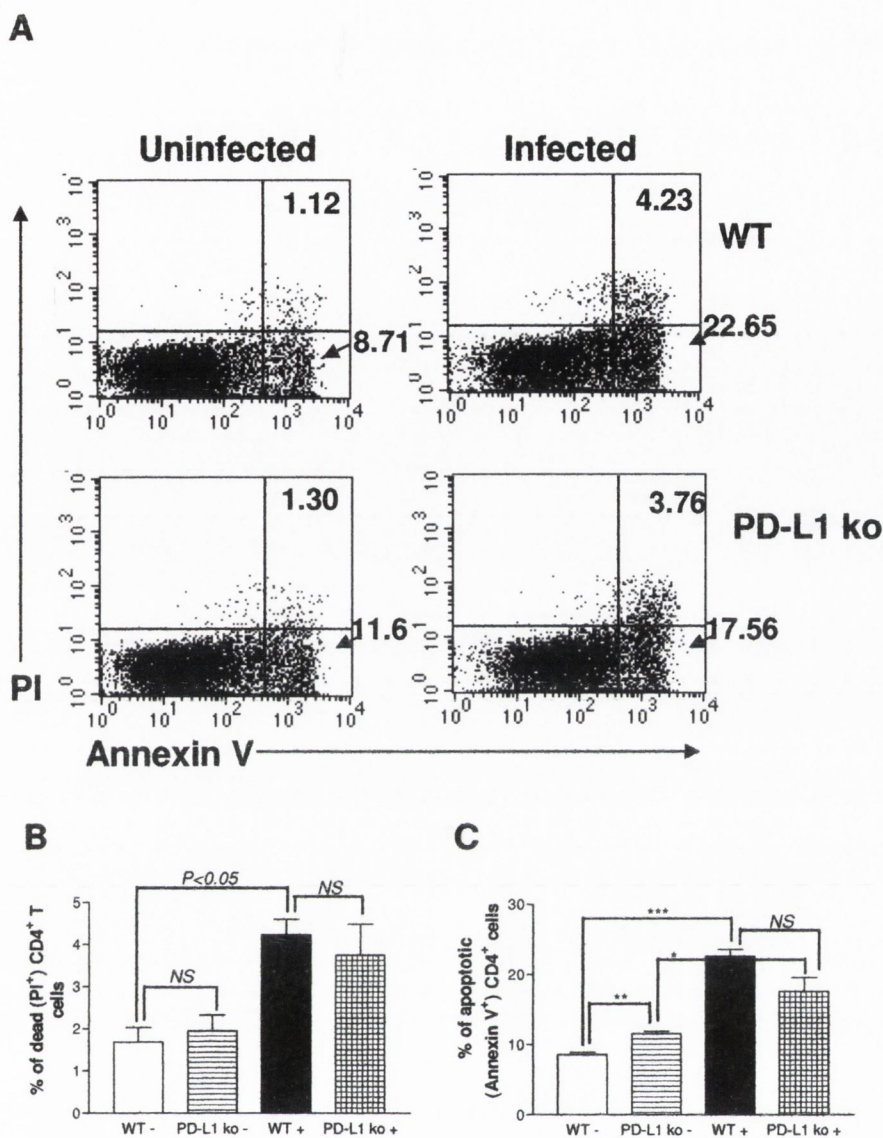


Figure 5.19. Infection increases apoptotic and dead CD4⁺ T cells in both C57BL/6 wt and PD-L1 ko mice, and there is an increase in apoptotic CD4⁺ T cells in uninfected PD-L1 ko mice compared to wt. A, FACS images of CD4⁺-gated spleen cells stained for annexin V and PI. B, bar graph showing % of dead (PI⁺) CD4⁺ T cells. C, bar graph showing % of apoptotic (annexin V⁺ PI⁻) CD4⁺ T cells. Student's t-test was used to compare differences between groups. Data is mean $\pm$ SEM from 3-4 individual mice per group and is representative of 2 separate experiments.

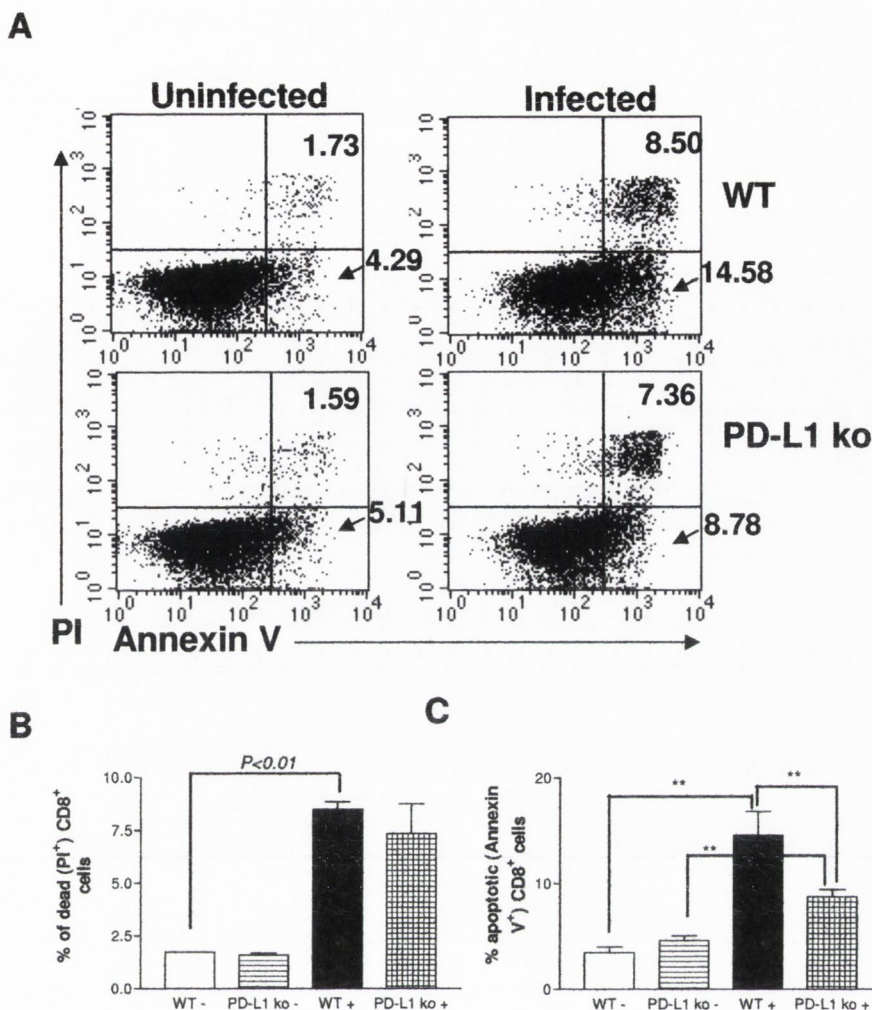


Figure 5.20. Infection increases apoptotic and dead CD8⁺ T cells in both C57BL/6 wt and PD-L1 ko mice, but there is significant decrease in apoptotic CD8⁺ T cells in infected PD-L1 ko mice compared to wt. A, FACS images of CD8⁺-gated spleen cells stained for annexin V and PI. B, bar graph showing % of dead (PI⁺) CD8⁺ T cells. C, bar graph showing % of apoptotic (annexin V⁺ PI⁻) CD8⁺ T cells. Student's t-test was used to compare differences between groups. Data is mean $\pm$ SEM from 3-4 individual mice per group and is representative of 2 separate experiments.

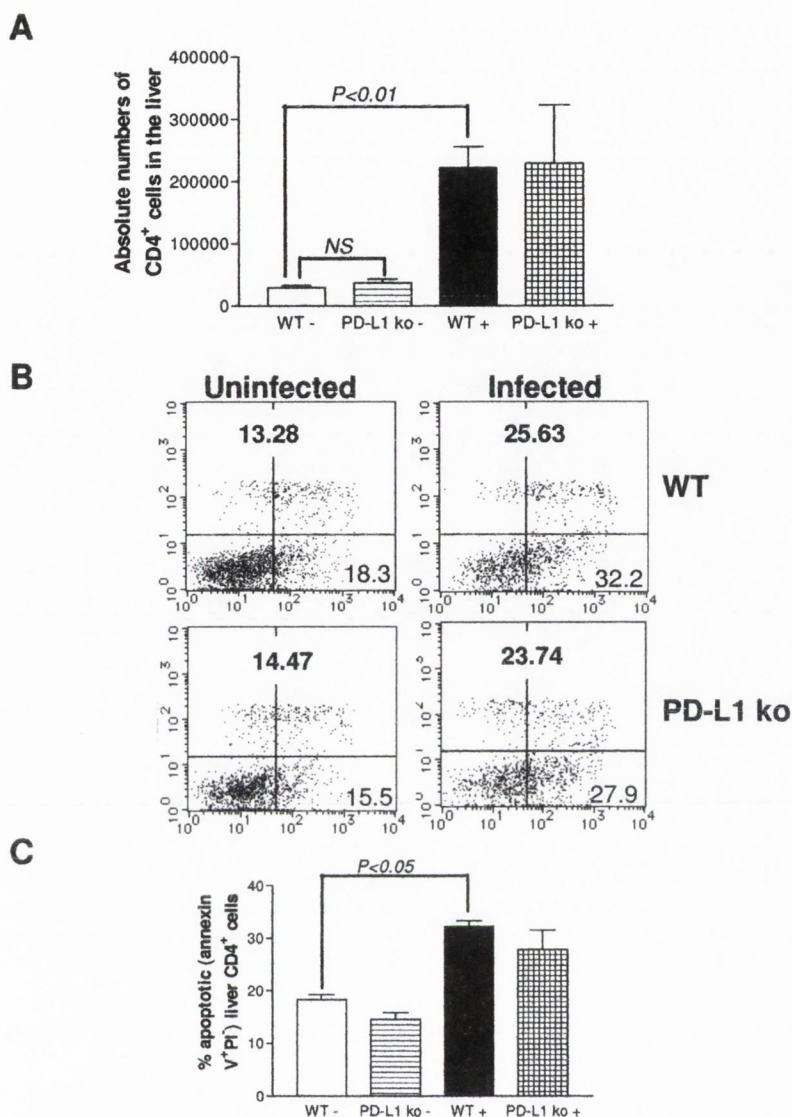


Figure 5.21. Infection increases apoptotic and dead liver CD4⁺ T cells, but levels are comparable between wt and ko groups. **A**, bar graph showing absolute numbers of CD4⁺ T cells in the liver. **B**, FACS images of CD4⁺-gated liver cells stained for annexin V and PI. **C**, bar graph showing % of apoptotic (annexin V⁺ PI⁺) CD4⁺ T cells. Student's t-test was used to compare differences between groups. Data is mean $\pm$ SEM from 3 individual mice per group and is representative of 2 separate experiments.

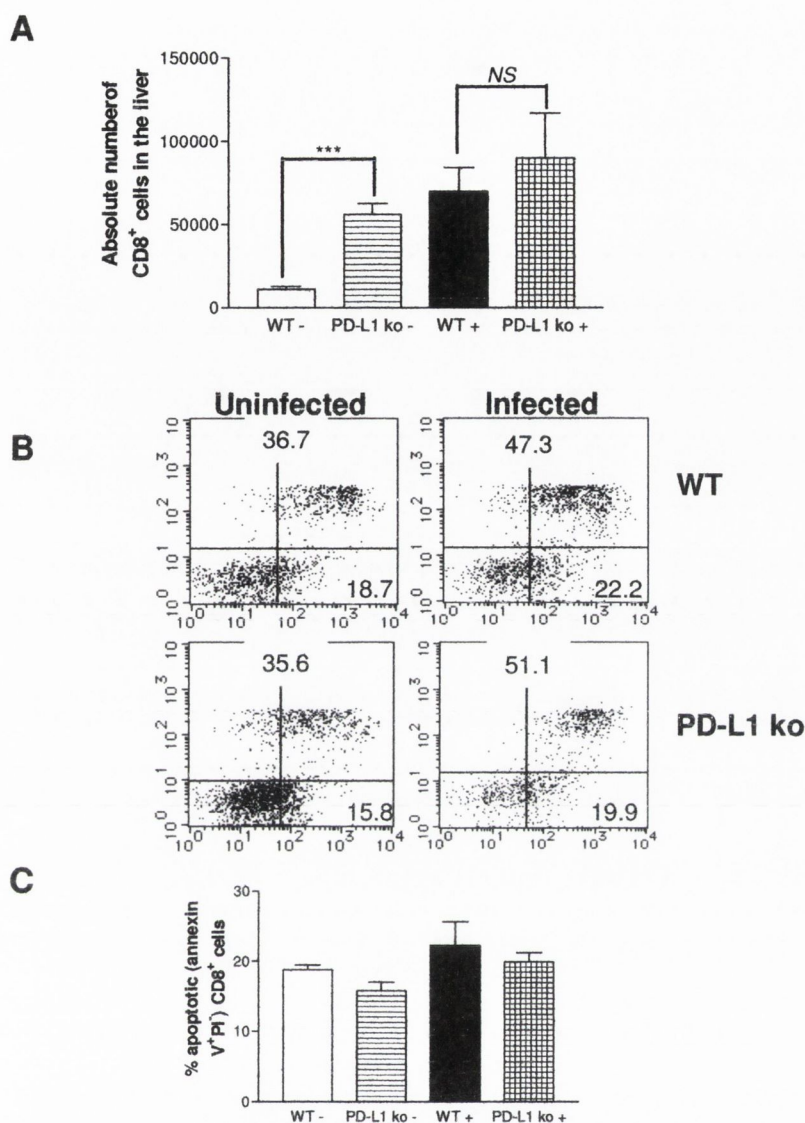


Figure 5.22. Infection increases apoptotic and dead liver CD8⁺ T cells, but levels are comparable between wt and ko groups. *A*, bar graph showing absolute numbers of CD8⁺ T cells in the liver. *B*, FACS images of CD8⁺-gated liver cells stained for annexin V and PI. *C*, bar graph showing % of apoptotic (annexin V⁺ PI⁻) CD8⁺ T cells. Student's t-test was used to compare differences between groups. Data is mean $\pm$ SEM from 3 individual mice per group and is representative of 2 separate experiments.

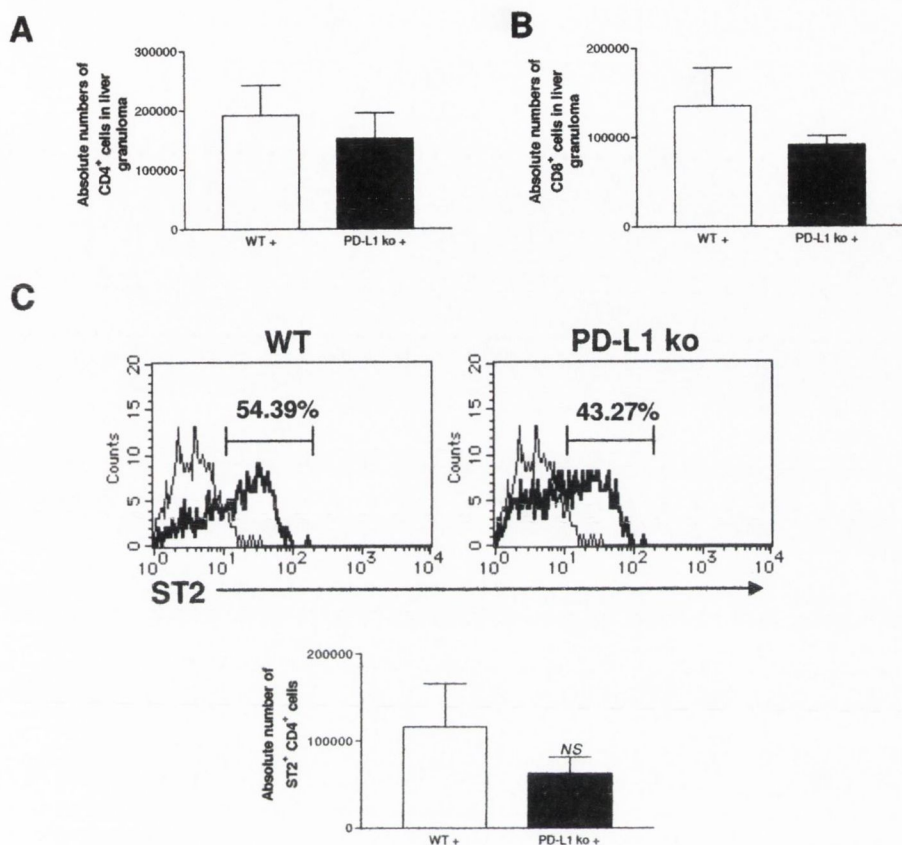


Figure 5.23. Liver granuloma T cells in the liver of wt and ko infected mice are comparable, with a non-significant decrease in T1/ST2⁺ CD4⁺ T cells in the ko group. Bar graph showing absolute numbers of CD4⁺ (A) and CD8⁺ (B) T cells in the liver granuloma. C, %T1/ST2 expression on liver granuloma CD4⁺ T cells, shown as a histogram, thin line represents isotype control, thick line WT or PD-L1 ko group. Student's t-test was used to compare differences between groups. Data is mean $\pm$ SEM from 3 individual mice per group and is representative of 2 separate experiments.

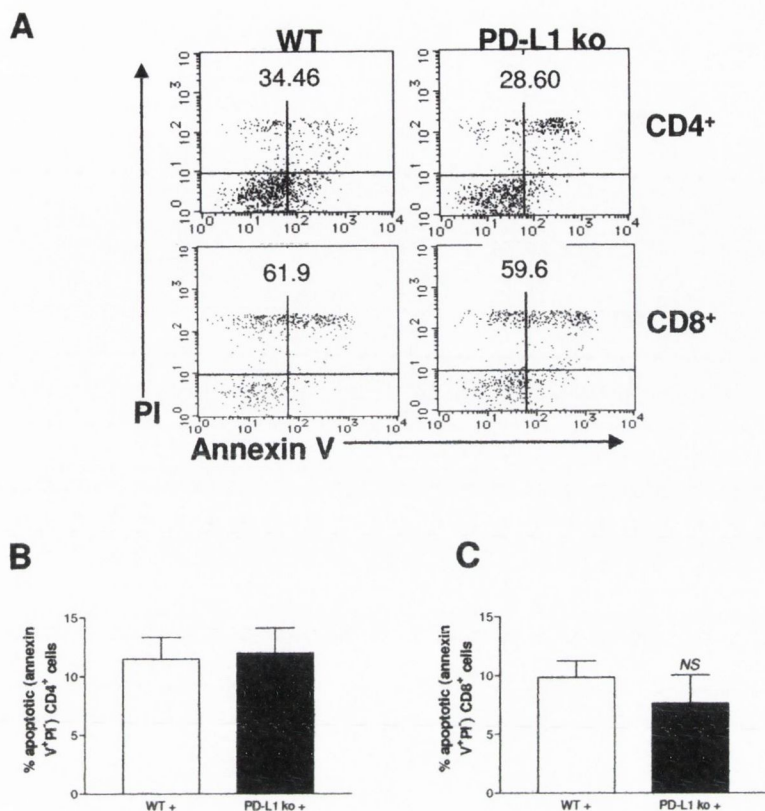


Figure 5.24. Liver granuloma T cells in the liver of wt and ko infected mice have comparable apoptosis and cell death levels between groups. A, FACS images of CD4⁺ and CD8⁺-gated liver granuloma cells stained for annexin V and PI, with bar graph showing % of apoptotic (annexin V+ PI-) CD4⁺ (B) and CD8⁺ (C) T cells. Student's t-test was used to compare differences between groups. Data is mean $\pm$ SEM from 3 individual mice per group and is representative of 2 separate experiments.

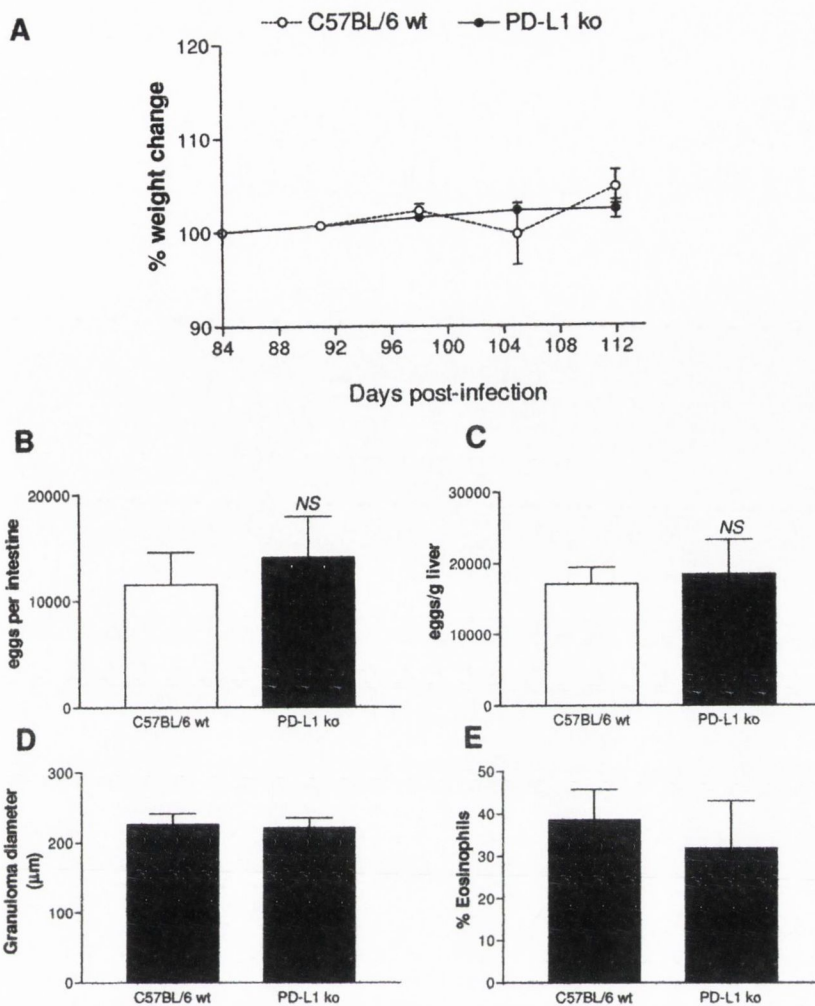


Figure 5.25. Weight change and parasitology in wild-type C57BL/6 and PD-L1 ko mice with chronic *S. mansoni* infection. C57BL/6 and PD-L1 ko mice were infected with *S. mansoni*. A, weights were measured periodically from week 12 post-infection, and mice were sacrificed for immunological and histological analysis at 16 weeks post-infection. Data is mean $\pm$ SEM of 4 individual mice and is representative of two separate experiments. Egg counts from B, liver and C, intestine of wt and PD-L1 ko worm-and-egg-infected mice. D, liver granuloma size (diameter) from wt and ko infected mice. E, % eosinophils in liver granuloma. Data is mean $\pm$ SEM from 5-6 mice. Student's t-test was used to compare differences between groups.

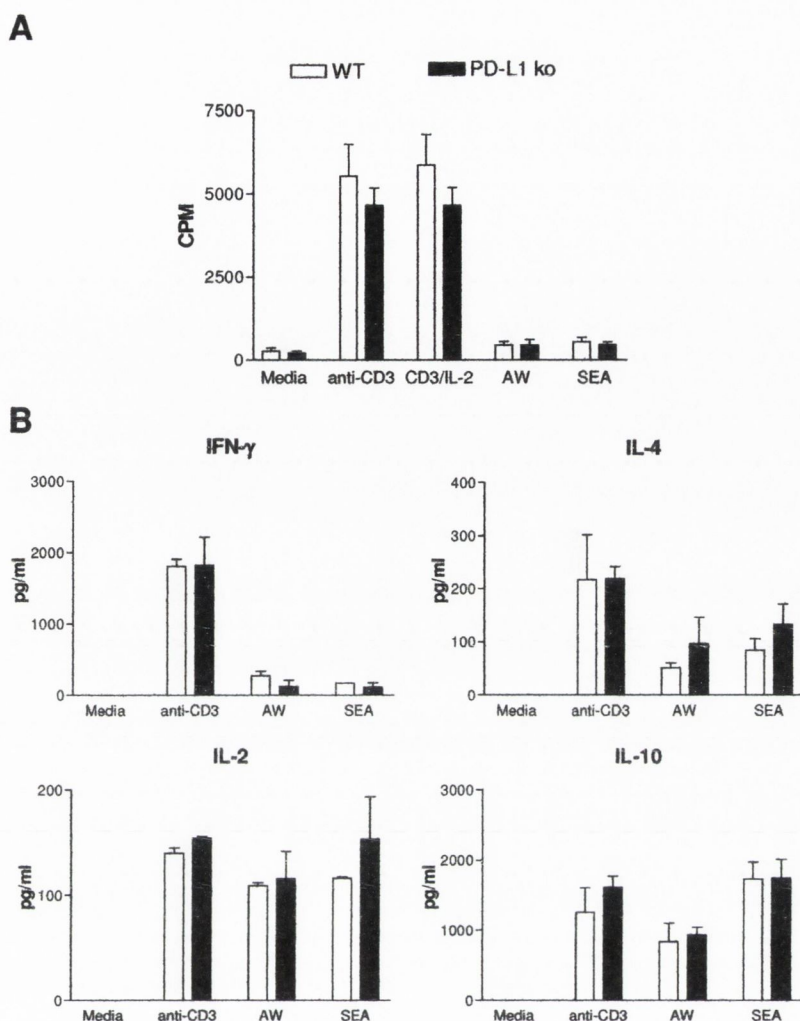


Figure 5.26. *Comparable proliferation and cytokine profiles from chronically infected (16 week) wt and PD-L1 ko mice. A, spleen cells from each group were activated with anti-CD3 mAb (0.5 μ g/ml), anti-CD3 mAb with IL-2 (20 ng/ml), or SEA or AW (20 μ g/ml). Proliferation was measured by [3 H] incorporation and expressed as CPM. B, spleen cells were stimulated with anti-CD3 mAb, AW or SEA. Supernatants were collected after 24h or 72 h for cytokine ELISA. Data are mean $\pm$ SEM from three or four individual mice and are representative of two separate experiments.*

5.3 Discussion

The previous chapter highlighted a member of the B7 family of co-stimulators, PD-L1, as having a role in the induction of T cell anergy in *S. mansoni* worm-infected mice. It also demonstrated that in worm-only and worm-and-egg infection of mice, there is an expansion of macrophage expression of PD-L1 and PD-L2, and also elevation of PD-1 expression on CD4⁺ and CD8⁺ T cells. In this chapter I examined the role of B7 members PD-L1 and PD-L2 and their receptor PD-1 by using monoclonal antibodies to block their function during conventional murine *S. mansoni* infection. This study also describes the use of a gene-deleted mouse (PD-L1 ko) to investigate the role of this molecule in infection.

In an acute infection of mice, blocking members of the PD:PD-L pathway had marginal effects on the outcome of infection, with egg deposition in the liver and intestine being comparable between groups, and no overt changes in weight during infection compared to control mAb-treated mice (Fig. 5.1). Analysis of proliferation showed that spleen cells from infected mice had elevated proliferative responses to anti-CD3 mAb stimulation when the negative co-stimulator, PD-1, was blocked during infection. This is consistent with its role as a negative regulator of T cell activation, as discussed in chapter 1 (section 1.5.5).

Although cytokine production from stimulated cells was equivalent between groups as analysed by ELISA, I observed an alteration in the type 1/type 2 balance in cells from infected mice treated with anti-PD-L1 by intracellular staining (Fig. 5.6). At the peak stage of infection, Th1 and Tc1 populations were elevated in Con A-stimulated cells from mice treated with anti-PD-L1 mAb compared to control mAb-treated mice. It had previously been reported that PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells (Loke and Allison, 2003), PD-L1 was shown to be highly expressed on inflammatory macrophages, with further

up-regulation upon stimulation by LPS and IFN- γ . The observation of increased IFN- γ production by CD4⁺ and CD8⁺ T cells when PD-L1 is blocked suggests that the induction of PD-L1 in an inflammatory setting may act as a negative regulation step, with PD-L1 functioning to inhibit the type 1 response.

Subsequent reports on studies using PD-L1 ko mice were consistent with my initial findings regarding the type 1 response skewing. CD4⁺ T cells from PD-L1 ko mice had enhanced IFN- γ production compared to wt when stimulated with low dose antigen, but this increase was non-significant at higher doses (Latchman *et al.*, 2004). There was also a significant increase in IFN- γ production by stimulated CD8⁺ cells in PD-L1 ko mice compared to wt mice. The group also demonstrated augmented clonal CD8⁺ T cell expansion and CTL activity in PD-L1 ko mice (Latchman *et al.*, 2004).

I did not observe an increase in CD4⁺ IFN- γ production in uninfected or *S. mansoni*-infected PD-L1 ko mice in my studies (Fig. 5.18). Infection induced a small, but non-significant increase in IFN- γ -producing CD8⁺ T cells (Fig. 5.17). However I reported a significant reduction in *S. mansoni*-induced IL-4 production by CD4⁺ T cells, and also significantly reduced ST2 expression on CD4⁺ T cells, demonstrating a role for PD-L1 in regulating Th cell differentiation during infection. This is consistent with another study, which has used the PD-L1 ko mouse to investigate immunity to the parasite *L. mexicana* (Liang *et al.*, 2006). In this study, the Th2 cytokine response to schistosome antigens was intact in PD-L1 ko mice (Fig. 5.9), which may explain why there was no significant alterations in the variety of infection parameters tested (weight loss, granuloma size, egg deposition). Another indicator of a reduced Th2 response was the decrease in liver granuloma eosinophils

and ST2⁺ CD4⁺ cells (Fig. 5.8 and 5.23), but again these were non-significant and would not have any effect on the overall outcome of infection.

Latchman and co-workers demonstrated that PD-L1 on T cells, APCs and host tissues has a role in the negative regulation of T cell responses (Latchman *et al.*, 2004). As mentioned earlier (section 1.5.5), some co-stimulatory functions for PD-L1 have also been reported. While the initial phenotypic studies on PD-L1 ko mice suggest that its principal role is one of down-modulation, they do not preclude the possibility of a costimulatory function for PD-L1. They suggest that the PD-L1 receptor, PD-1, may be the initial dominant negative signal, but that another receptor with positive co-stimulatory properties may also be up-regulated. The group report that they observed no alterations in CD4⁺ or CD8⁺ T cell proliferation between wt and ko groups (Latchman *et al.*, 2004). My initial studies on spleen cell proliferation also showed comparable proliferation between control mAb and anti-PD-L1 mAb treated infected groups (Fig. 5.3), and between wt and PD-L1 ko uninfected and infected mice (Fig. 5.10). However when I assessed the *in vitro* proliferation of the T cell subsets by CFSE labeling the cells, I noted that the CD8⁺ T cells from uninfected PD-L1 ko mice had impaired proliferative responses compared to cells from wt mice (Fig. 5.11). There was also a significant reduction in IL-2 production in uninfected PD-L1 ko CD8⁺ T cells compared to wt uninfected cells (Fig. 5.13). As infection already results in dramatically reduced IL-2 production and T cell proliferation, it was not surprising to observe more marginal alterations in PD-L1 ko CD8⁺ T cell proliferation compared to the changes observed between uninfected PD-L1 ko and wt groups.

These findings of reduced proliferation in the PD-L1 ko seemed to suggest a potential positive costimulatory role for this molecule, which is contrary to the initial results by Latchman and co-workers. Further examination of the CD4⁺ and CD8⁺ T cells from PD-L1 ko

mice demonstrated that expression of the PD-L1 receptor, PD-1, is significantly elevated on both CD4⁺ and CD8⁺ T cells (Fig. 5.12 and 5.13). This is most obvious in the CD8⁺ T cell subset as PD-1 expression on wt cells was negligible to begin with, while a population of CD4⁺ cells constitutively express PD-1. This increased PD-1 expression could explain the reduced proliferation and the reduction in IL-2 production, as PD-1 inhibition of T cell proliferation is mediated by IL-2 (Carter *et al.*, 2002).

I also showed that CD8⁺ T cells from PD-L1 ko mice also have significantly elevated levels of the CD8⁺ regulatory cell marker, CD122 (Fig. 5.16), which again could suggest an explanation for the reduction in CD8⁺ T cell proliferation in PD-L1 ko mice. It is interesting to also observe that *S. mansoni* infection induces a highly significant increase in CD122⁺ CD8⁺ T cells, which could be the subject of future study. However, as CD122 expression is not restricted to regulatory CD8⁺ T cells, and it is also found on NK cells (Phillips *et al.*, 1989), the use of a ko mouse or antibody depletion studies to dissect its role in infection would be of limited value, due to the nature of cellular expression of this molecule. The levels of CD122 expression on CD8⁺ T cells from infected mice are comparable between wt and ko, which is not surprising given the already dramatic increase in expression in infected groups compared to uninfected. In this study I attempted to examine the levels of intracellular IFN- γ and IL-10 in CD8⁺CD122⁺ T cells from infected wt and ko mice, however this was unsuccessful as CD122 surface expression was rapidly down-regulated upon incubation with Brefeldin A. It was interesting to note that to examine levels of cytokine from CD122⁺ cells, other groups describe a method of cell isolation followed by detection of cytokine at the mRNA level using RT-PCR (Endharti *et al.*, 2005).

During this study I observed that the levels of PD-1 expression in uninfected C57BL/6 wt mice were different (lower) than from expression levels noted on BALB/c mice during

infection in the previous chapter (Fig. 4.16 and 4.17). This was particularly apparent in the CD8⁺ T cell subset, where PD-1 infection on uninfected or infected C57BL/6 wt mice was negligible (Fig. 5.13). This could be due to strain differences between the mice. However I have also noted that the flow cytometry antibody used in the study on the C57BL/6 wt and PD-L1 ko mice is a different clone to the one originally provided by the company (eBiosciences). The first flow cytometry antibody to PD-1 had a hamster IgG isotype, but the company no longer produces this and instead provides an antibody to PD-1 with a Rat IgG isotype. The appropriate isotype controls were used in all instances in my studies, and no background staining was observed. Whether the discrepancies in PD-1 expression between C57BL/6 and BALB/c mice is due to strain difference or difference in detection reagents remains to be seen. As mentioned in the discussion of the previous chapter, investigations into strain differences in infection between C57BL/6 and BALB/c mice are underway in the lab.

Another publication investigating the role of PD-L1 using PD-L1 ko mice reported an accumulation of CD8⁺ T cells in the liver of PD-L1 ko mice (Dong *et al.*, 2004). This is of particular interest in light of the recent findings that blockade of the PD-1 pathway using anti-PD-L1 mAb restores function to exhausted CD8⁺ T cells during chronic viral infection (Barber *et al.*, 2006). PD-1 is highly upregulated on exhausted cells, and PD-L1 was also found to be significantly upregulated on virus-infected cells. My study was consistent with the observations by Dong and co-workers, in that I also reported an accumulation of CD8⁺, but not CD4⁺ T cells, in the livers of uninfected PD-L1 ko mice compared to wt mice (Fig. 5.21 and 5.22). Liver T cells were increased in infected mice compared to uninfected, but I found no difference in T cell numbers between wt and ko infected groups. T cell numbers in the liver granulomas were also equivalent between groups (Fig. 5.23).

There is a small but significant increase in the levels of splenic CD4⁺ T cell apoptosis in uninfected PD-L1 ko mice compared to wt mice (Fig. 5.19), but there was no difference in CD8⁺ T cell apoptosis in uninfected groups (Fig. 5.20). Why there is such an increase in CD4⁺ cell apoptosis is unknown, perhaps it could be partially due to the increased PD-1 expression, but this is unlikely as PD-1 is also increased on CD8⁺ T cells, and these have unchanged apoptosis levels. Dong and co-workers observed a slight decrease in apoptotic CD8⁺ cells in the liver of PD-L1 ko mice, but it was not statistically significant (Dong *et al.*, 2004). Infection induced increased apoptosis in CD4⁺ and CD8⁺ T cells, in the liver and spleen (Fig. 5.19-5.22), as is consistent with previous reports (Estaquier *et al.*, 1997; Fallon *et al.*, 1998). In this study there was a significant reduction in the levels of apoptotic CD8⁺ T, but not CD4⁺ T cells in the spleens of infected PD-L1 ko mice (Fig. 5.20), but levels in the liver and liver granuloma were comparable between infected groups (Fig. 5.21-5.23). These data together with the observations by Dong and co-workers infer that PD-L1 has a non-essential role in liver T cell apoptosis induction *in vivo*. However *in vitro* studies have recently shown that PD-L1 expression on human hepatocytes can induce T cell apoptosis, as blockade of PD-L1 significantly reduced T cell apoptosis levels (Muhlbauer *et al.*, 2006). This study used both monoclonal antibody blocking and gene-deleted mice to examine what effect an absence of PD-L1 would have on the outcome of *S. mansoni* infection. Although my initial observations of increased IFN- γ -producing CD4⁺ and CD8⁺ T cells in infected mice treated with anti-PD-L1 mAb were not fully borne out in the ko model, I did observe a slight down-modulation of the Th2 response in infection, as determined by expression of the Th2 marker T1/ST2 and reduced detection of intracellular IL-4 on CD4⁺ T cells.

It is also of interest to note that other groups have observed discrepancies in relation to PD-L1 ko studies versus blocking antibody studies. Barber and co-workers found that

blockade of the PD-1:PD-L1 pathway using anti-PD-L1 mAb enhanced T cell responses and restored exhausted T cells during chronic viral infection (Barber *et al.*, 2006). However the group also noted that when PD-L1 ko mice were infected with a virus clone that induced chronic, but not acute infection, the mice died due to immunopathologic damage. This suggests that PD-1 pathway may function to limit the immune-mediated damage during persistent infection, which results in the establishment of the pathogen in the host.

Contrary to the idea of PD-L1 being a negative regulator of T cell activation, reduced proliferation of CD8⁺ T cells was observed in PD-L1 ko mice. However this could be explained by the dramatic increase in PD-1 expression on T cells from PD-L1 ko mice. Why PD-1 expression is induced so highly on PD-L1 ko mice is unknown, perhaps PD-L1 binding to PD-1 normally results in a negative feedback loop, with down-modulation of PD-1 occurring, or alternatively binding of PD-L2 to PD-1 without competition from PD-L1 could induce PD-1 expression.

In conclusion, I have demonstrated that the members of the PD:PD-L pathway play non-essential roles in *S. mansoni* infection of mice. There was no overt differences in acute or chronic stages of infection between wt and PD-L1 ko mice. Although I observed minor differences in regulation of Th2 cell responses in infected PD-L1 ko mice, these were not of a magnitude great enough to alter the outcome of infection. It would be interesting to examine *S. mansoni* infection in PD-L1 ko mice on a BALB/c background, as cells from infected BALB/c mice more readily produce Th2 responses when stimulated compared to C57BL/6. Some other mechanisms *in vivo* can compensate for the lack of negative regulation by PD-L1 during *S. mansoni* infection.

Chapter 6

General Discussion

6.1 General Discussion

This PhD thesis examined the role of T cell activation during murine *S. mansoni* infection. It is clear that schistosomes are potent stimulators and suppressors of the immune system, with these alterations in immune function permitting the parasite to live for up to 40 years in humans. The challenge is to understand how the immune system deals with such pathogens, and to dissect the mechanism whereby the parasite exerts its regulatory effect. In this thesis, I have addressed the role of two co-stimulatory pathways involved principally in the negative regulation of T cell activation. I demonstrated upregulation of many regulatory markers and negative co-stimulatory markers during infection, and have examined the relative importance of some of these through mAb depletion or blocking studies, and the use of transgenic mice, during acute and chronic murine *S. mansoni* infection. My data demonstrates that while some regulatory markers are of critical importance during infection, others play a non-essential role in the immune response to *S. mansoni* infection, and highlights certain molecules as potential therapeutic immunomodulatory targets for the future.

As discussed in chapter 1, T regulatory cell populations are important in the homeostatic regulation of immune responses to parasite infection. Pathogens may favour T_{reg} survival, to down-modulate the immune response and enable their own survival. I showed that although CD4⁺CD25⁺ T cells were upregulated during infection, depletion of CD25⁺ T cells did not cause any overt changes in acute or chronic infection. This is an important finding in the field of schistosome immunology, and one which may direct the researcher towards the examination of other regulatory pathways in schistosome infection. It is pertinent to note that the studies on CD25⁺ cell depletion during infection were performed in an SPF facility in TCD, and studies were also performed in an SPF unit in Cambridge, UK. It has been previously observed that mice housed in a non-SPF facility have elevated levels of

CD4⁺CD25⁺ T_{reg} cells than those housed in SPF facilities (Fallon, personal communication). In my studies, on all occasions no alteration in intestinal or other organ pathology was observed in anti-CD25 mAb-treated infected mice compared to controls.

The use of the PC61 mAb for depletion of CD25⁺ cells has been the subject of much recent controversy, with Kohm and co-workers demonstrating that mAb injection results in the functional inactivation, and not depletion of T_{reg} cells (Kohm *et al.*, 2006). However, this finding has been questioned by other groups (Stephens and Anderton, 2006; Zelenay and Demengeot, 2006). In this thesis I found that although the levels of CD25 were greatly reduced (>85%) upon anti-CD25 mAb treatment, levels of Foxp3 were not reduced to the same degree. Future studies in the regulation of Foxp3 during infection would add further insight to the role of T_{reg} cells during schistosome infection. Although Foxp3^{-/-} or *scurfy* mice die at an early age (Godfrey *et al.*, 1991), the use of a conditional Foxp3 knock-out mouse, whereby one could inactivate the gene at a later stage, could be a useful tool for the study of T_{reg} cells in *S. mansoni* infection. This would enable examination of the role of T_{reg} cells in the initial infectivity of the parasite, in addition to dissecting their role in acute and chronic infection. Mice with Foxp3 linked to a bicistronic reporter expressing a red fluorescent protein have been generated (Wan and Flavell, 2005), and they are currently being bred in-house to identify Foxp3-expressing cells during *S. mansoni* infection. They have been crossed with 4get (IL-4-GFP) mice, IL-10^{-/-}, IL-4^{-/-}, and PD-L1^{-/-} mice; such resources will aid future work to examine what effect infection may have on the relationship between these cytokines/markers and T_{reg} cells.

This thesis identified the negative regulatory receptor, CTLA-4, as a regulator of the type 2 response to schistosome infection. The elevated Th2 response observed in infected mice treated with anti-CTLA-4 mAb has also been demonstrated in other studies involving

nematodes (McCoy *et al.*, 1997), highlighting a common mechanism in the regulation of Th2 responses. However, it must be noted that blocking CTLA-4, and the subsequent enhancement of the immune response in the study by McCoy and co-workers resulted in the generation of protective immunity to the parasite *Nippostrongylus brasiliensis*, whereas in acute schistosome infection it resulted in excessive and damaging Th2 responses, causing significant weight loss in infected mice. Blocking of CTLA-4 during chronic infection did not cause the immunopathology observed when the molecule was blocked during acute infection, suggesting that it functions primarily at the initial stage of immune regulation, and leads to the subsequent activation of other negative immune regulation pathways, which persist to the chronic stage of infection.

Human helminth studies have also shown that CTLA-4 is associated with immunosuppression and chronic immune activation in persistently helminth-infected individuals (Leng *et al.*, 2006). A fraction of soluble schistosome adult worm antigen, PIII, has been shown to modulate *in vitro* granuloma formation by the upregulation of CTLA-4 and B7-2 in human T cells (Zouain *et al.*, 2004). The function of CTLA-4 as Th2 regulator in filarial infection has been demonstrated, with *in vitro* blocking of CTLA-4 expression in PBMC from filaria-infected individuals resulting in increased IL-5, and decreased IFN- γ responses to filarial antigen stimulation (Steel and Nutman, 2003). The blockade of CTLA-4 during infection in this thesis resulted in the spontaneous production of IL-5 from spleen cells^{ti}. In humans, IL-5 production has been associated with resistance to infection by the helminth *Necator americanus* (Quinnell *et al.*, 2004), and it is also linked to resistance from *S. mansoni* infection (Roberts *et al.*, 1993), which could suggest that CTLA-4 upregulation in helminth infection may be beneficial to the parasite, both by limiting excessive damaging Th2 responses in the host, and also by reducing IL-5 production to regulate resistance to re-

infection. However, murine studies on IL-5 deficient mice have shown no role for this cytokine in host resistance mechanisms to infection by *Schistosoma mansoni* (Brunet *et al.*, 1999).

In this thesis I demonstrated that the B7 molecule, PD-L1, was upregulated on macrophages from worm-infected mice. I investigated the role of this inhibitory co-stimulatory molecule, and the other members of the pathway in *S. mansoni* infection. Infection induced increases in expression of B7 ligands PD-L1 and PD-L2 on macrophages, while their receptor PD-1 was upregulated by infection on CD4⁺ and CD8⁺ T cells. The relative importance of CTLA-4 and PD-1 in immunity is perhaps best described in studies on mice deficient in these receptors. While CTLA-4^{-/-} mice die within 3-4 weeks of birth from massive lymphoproliferation, PD-1^{-/-} mice have less pronounced hyper-immune phenotype, and survive for longer periods than the CTLA-4^{-/-} mice. During the course of this thesis, I noted that in contrast to CTLA-4, blocking the members of this pathway did not have any gross effect on the outcome of infection as determined by weight loss and granuloma formation, indicating that these molecules are non-essential in schistosome infection and that compensatory mechanisms may come into play to limit the effect of blocking one of these molecules during infection. However, the alterations in the type 1/type 2 response observed in cells from infected PD-L1 ko mice or cells from infected mice treated with anti-PD-L1 mAb are interesting, particularly when taken with the recent data supporting this on the role of PD-L1 in *Leishmania* infection and EAE (Latchman *et al.*, 2004; Liang *et al.*, 2006). While CTLA-4 functions to limit the Th2 response, PD-L1 could work in the opposite manner. PD-L1 has been shown previously to be upregulated on inflammatory macrophages (Loke and Allison, 2003). The studies in this thesis and others (Liang *et al.*, 2006) lead me to suggest

that PD-L1 may be induced on inflammatory macrophages as part of a negative feedback loop, and functions to inhibit the Th1 and promote the Th2 response.

My observations of increased Tc1 cells in infected mice treated with anti-PD-L1 mAb, and the data showing less apoptotic CD8⁺ T cells in the spleens of infected PD-L1 ko mice are interesting in light of the recent publication on the role of the PD-1:PD-L1 pathway in the generation of 'exhausted' CD8⁺ T cells during chronic viral infection (Barber *et al.*, 2006). CTLA-4 and PD-1 have been shown to act synergistically in the induction of peripheral CD8⁺ T cell tolerance by resting dendritic cells (Probst *et al.*, 2005). The upregulation of CTLA-4, PD-1 and its ligands PD-L1 and PD-L2 in addition to the CD8⁺ T regulatory cell marker, CD122, during *S. mansoni* infection, warrants further investigation into the role of CD8⁺ T cells during infection.

The proposals of the possibility of a dual role for PD-L1 in positive co-stimulation of naïve T cells and suppression of activated T cells, as discussed in section 1.5.5, could be of interest in the context of schistosome infection. Many groups hypothesise that PD-L1 may play an important immunoregulatory role in the chronicity of inflammatory responses, as recently reviewed (Dong and Chen, 2006). In my studies, worm priming of the immune system resulted in the anergic or T cell suppressive phenotype by week 4 of infection, with PD-L1 functioning as a negative co-stimulator. However, it would be interesting to examine the function of PD-L1 in the initial larval stages of infection, to see if it is involved in the priming of naïve T cells.

The regulatory function of the CTLA-4 molecule as discussed in this thesis may be harnessed for clinical use, when activation or suppression of the immune system by the respective blocking or activation of CTLA-4 may be advantageous to the patient. As mentioned in chapter 1, Orencia (abatecept), from Bristol-Myers-Squibb, is a CTLA-4 agonist

that has been recently licensed for use by the FDA for the clinical treatment of severe rheumatoid arthritis. It is the first-in-class antagonist of CD28 co-stimulation, and the human clinical trial results show the first success of a biologic for the treatment of rheumatoid arthritis that is specifically aimed at T cell inhibition (Bluestone *et al.*, 2006).

The role for PD-1:PD-L1 in chronic viral infection, and the correlation between PD-L1 expression on tumours and poor prognosis in cancer patients (as discussed in section 1.5.5), suggests that PD-1 blockade should also be tested clinically, as it seems that blockade of this pathway can have a beneficial effect on the host's immune responses (Barber *et al.*, 2006). PD-1, rather than PD-L1, blockade would be more useful for the reactivation of immune responses, since PD-L1 is expressed on a variety of non-lymphoid tissues in addition to T and B lymphocytes and APCs. However, the side-effects on the use of any immuno-suppressant or immuno-stimulant (e.g. patients may be at higher risk of bacterial infection due to T cell suppression, and there is a risk of uncontrolled immune activation with immuno-stimulants) must be carefully monitored. The recent outcome of the first human clinical trial using the CD28 agonist from TeGenero, is a stark reminder of the different responses that may occur in humans compared to mice or non-human primates. The identification of host molecules that are specifically targeted by human parasites such as schistosomes, which have been around for millions of years, may provide another avenue for the development of future biologics in the treatment of immune-mediated disease.

Some of the studies in this thesis involved the use of a laboratory model of murine schistosomiasis, a male worm-only infection. I demonstrated that worm-only infected mice have distinct T cell unresponsiveness than that observed in worm-and-egg-infected mice. The worm stage of the infection induces a form of T cell anergy, which can be restored by the addition of IL-2, or co-stimulation via CD28. In a conventional worm-and-egg-infection, the

worm-induced T cell anergy at week 4 (prior to egg-laying) could function to dampen down the subsequent potent immune response elicited by the egg. PD-L1-expressing macrophages were the mediators of this T cell anergy. The *S. mansoni*-induced T cell suppression described in this study has the potential to be harnessed for the future treatment of immune disorders. Indeed worm-modulation of the immune system has already been shown to have a protective role in murine asthma and anaphylaxis models (Mangan *et al.*, 2004; Mangan *et al.*, 2006). Other data from our laboratory implies a role for worm-mediated protection from colitis (Smith, Walsh *et al.*, submitted).

The studies on worm-infected mice were performed on a BALB/c strain of mouse, but C57BL/6 worm-infected mice did not display the same T cell unresponsiveness observed in worm-infected BALB/c mice. The sequencing of the mouse genome has been based on a C57BL/6 strain, and many of the transgenic mice generated in recent years have also been on this background. Developments in transgenic technology have also enabled easier generation of BL6 ES cells rather than 129 ES cells. The PD-L1 ko mice were only obtained in the last 10 months of my PhD, and so we are still currently back-crossing these mice to the BALB/c strain and also to different transgenics.

It may be questioned that since such differences in *S. mansoni* infection exist between mouse strains, what is the relevance of mouse infection data to the human situation? Many discrepancies exist between experimental mouse infection and human schistosomiasis (Fallon, 2000). For example, a single experimental mouse infection usually lasts 8-16 weeks before the mouse is sacrificed, while humans are naturally infected over years and those living in areas endemic with infection are persistently being exposed to repeat infections. Mice are genetically inbred and are housed in specific pathogen free environments. In humans factors such as genetic predisposition and *in utero* sensitization also have effects on schistosome

infection. One worm pair in a mouse corresponds on a body-weight basis to 5000 worm pairs in a human, which is far in excess of the worm burden typically seen in schistosome-infected humans. However, as mentioned in section 1.2.7, mouse studies are of great importance in the study of *S. mansoni* infection, in terms of examination of disease effects and immunological response. Experimental infection of humans with the *S. mansoni* parasite is ethically unacceptable, and cohorts of naturally infected individuals that have not been treated with a schistosomidal drug, or are not co-infected with another parasite, are difficult to find.

The use of biological agents, or biologics, are increasingly popular for the treatment of immune-mediated disease, for example the CTLA-4Ig described earlier. The use of monoclonal antibodies against TNF (TNF-blockers, e.g. Remicade) have also been used successfully in the treatment of arthritis and Crohn's Disease. However caution must be exercised in the use of any immunomodulatory drug in human clinical trials, and patients made aware of the risk of re-occurrence of underlying parasitic or bacterial infections (eg tuberculosis)(Keane *et al.*, 2001). In this thesis, the mechanism of the selective upregulation of PD-L1 by the worm to induce T cell anergy could be exploited as a future therapeutic. However, another alternative is to exploit the worm itself as a target for the development of novel therapeutics. Parasitic worms have evolved over millions of years to modulate our immune system in such a way as to benefit themselves, but also ensuring survival of the host. In the field of Crohn's Disease and Ulcerative Colitis, there have been recent human clinical trials on the porcine whipworm *Trichuris suis* and also the human nematode *Necator americanus*, with varying degrees of success (Croese *et al.*, 2006; Summers *et al.*, 2005). It is currently ethically unacceptable to experimentally infect a human with a male worm-only schistosome infection. The immuno-modulatory mechanisms induced by the worm in a murine schistosome infection are not yet fully characterized. To my knowledge, a human

worm-only schistosome infection has never been documented. There are also safety issues involved with human schistosome infection, as the worms can occasionally become misguided and travel to the CNS, resulting in death (Nascimento-Carvalho and Moreno-Carvalho, 2005).

The identification of worm molecules with immunomodulatory properties represents the best avenue for the development of therapeutics against immune-mediated and inflammatory diseases. There is great potential for the advancement of therapeutics based on pathogen-derived (viral, bacterial, fungal or parasite) immunomodulatory molecules (Fallon and Alcami, 2006). This study highlighted that a number of negative co-stimulatory markers on different cell types are targeted for upregulation by the worm and worm-and-egg-stage of schistosome infection. The *in vitro* modulation of worms is a good model system for the further identification of worm targets on the cell surface. Worm secretions can be harvested and concentrated to produce worm excretion secretions (WES), and from this secretions can be fractionated and the exact worm molecule responsible for the worm-induced anergy may be identified. Parasite-derived immunomodulatory molecules could play a vital role in the future treatment of immunopathological and inflammatory diseases, and they may indeed become 'the drug cabinet of the future'.

6.2 Future work

This thesis addressed the role of schistosome modulation of the immune system in mice by T regulatory cells and also by the negative co-stimulatory pathways of T cell activation. I made several novel findings, including the finding that CTLA-4, but not CD25, was important for the negative regulation of Th2 responses during infection. I also found that macrophages from worm-infected mice induce T cell anergy via upregulation of the co-stimulatory molecule PD-L1. While PD-L1 does not play a critical role in *S. mansoni* worm- and egg-infection in terms of host or parasite survival, it does induce alterations in the type1/type 2 cytokine balance. My studies have opened up several avenues of work that will require further investigation.

1. Use of the Foxp3-reporter (FIR) mice and FIR/IL-10^{-/-} or FIR/4get mice to further study the regulatory pathways in *S. mansoni* infection.
2. Examine the strain differences between BALB/c and C57BL/6-infected mice.
3. Examine the role of CD8⁺ T cells in infection, particularly in relation to PD-1/PD-L1 and CD122 expression.
4. Investigate if worm-modulated human PBMCs have impaired T cell activation upon stimulation, and assess co-stimulatory and regulatory cell expression on these worm-modulated cells.
5. Identify the worm molecule that is responsible for the induction of T cell anergy.

Chapter 7

References

7. References

- Aandahl, E. M., Michaelsson, J., Moretto, W. J., Hecht, F. M., and Nixon, D. F. (2004). Human CD4⁺ CD25⁺ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol* 78, 2454-2459.
- Abbas, A. K., Murphy, K. M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. *Nature* 383, 787-793.
- Abdallahi, O. M., Bensalem, H., Augier, R., Diagana, M., De Reggi, M., and Gharib, B. (2001). Arginase expression in peritoneal macrophages and increase in circulating polyamine levels in mice infected with *Schistosoma mansoni*. *Cell Mol Life Sci* 58, 1350-1357.
- Abdou, N. L., Alavi, J. B., and Abdou, N. I. (1976). Human bone marrow lymphocytes: B and T cell precursors and subpopulations. *Blood* 47, 423-430.
- Abebe, F., Erko, B., Gemetchu, T., and Gundersen, S. G. (2005). Control of *Biomphalaria pfeifferi* population and schistosomiasis transmission in Ethiopia using the soap berry endod (Phytolacca dodecandra), with special emphasis on application methods. *Trans R Soc Trop Med Hyg* 99, 787-794.
- Adachi, T., and Alam, R. (1998). The mechanism of IL-5 signal transduction. *Am J Physiol* 275, C623-633.
- Agata, Y., Kawasaki, A., Nishimura, H., Ishida, Y., Tsubata, T., Yagita, H., and Honjo, T. (1996). Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* 8, 765-772.
- Akiba, H., Takeda, K., Kojima, Y., Usui, Y., Harada, N., Yamazaki, T., Ma, J., Tezuka, K., Yagita, H., and Okumura, K. (2005). The role of ICOS in the CXCR5⁺ follicular B helper T cell maintenance *in vivo*. *J Immunol* 175, 2340-2348.
- Akira, S., Takeda, K., and Kaisho, T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2, 675-680.
- Alenmyr, L., Matheu, V., Uller, L., Greiff, L., Malm-Erfjelt, M., Ljunggren, H. G., Persson, C. G., and Korsgren, M. (2005). Blockade of CTLA-4 promotes airway inflammation in naive mice exposed to aerosolized allergen but fails to prevent inhalation tolerance. *Scand J Immunol* 62, 437-444.

- Allan, S. E., Passerini, L., Bacchetta, R., Crellin, N., Dai, M., Orban, P. C., Ziegler, S. F., Roncarolo, M. G., and Levings, M. K. (2005). The role of 2 FOXP3 isoforms in the generation of human CD4⁺ Tregs. *J Clin Invest* 115, 3276-3284.
- Allen, J. E., Lawrence, R. A., and Maizels, R. M. (1996). APC from mice harbouring the filarial nematode, *Brugia malayi*, prevent cellular proliferation but not cytokine production. *Int Immunol* 8, 143-151.
- Amiri, P., Locksley, R. M., Parslow, T. G., Sadick, M., Rector, E., Ritter, D., and McKerrow, J. H. (1992). Tumour necrosis factor alpha restores granulomas and induces parasite egg-laying in schistosome-infected SCID mice. *Nature* 3, 0-07.
- Anderson, S., Shires, V. L., Wilson, R. A., and Mountford, A. P. (1998). In the absence of IL-12, the induction of Th1-mediated protective immunity by the attenuated schistosome vaccine is impaired, revealing an alternative pathway with Th2-type characteristics. *Eur J Immunol* 28, 2827-2838.
- Ansari, M. J., Salama, A. D., Chitnis, T., Smith, R. N., Yagita, H., Akiba, H., Yamazaki, T., Azuma, M., Iwai, H., Khoury, S. J., *et al.* (2003). The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med* 198, 63-69.
- Anthony, R. M., Urban, J. F., Jr., Alem, F., Hamed, H. A., Roza, C. T., Boucher, J. L., Van Rooijen, N., and Gause, W. C. (2006). Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat Med* 12, 955-960.
- Anto, F., Bosompem, K., Kpikpi, J., Adjuik, M., and Edoh, D. (2005). Experimental control of *Biomphalaria pfeifferi*, the intermediate host of *Schistosoma mansoni*, by the ampullariid snail *Lanistes varicus*. *Ann Trop Med Parasitol* 99, 203-209.
- Appay, V., Nixon, D. F., Donahoe, S. M., Gillespie, G. M., Dong, T., King, A., Ogg, G. S., Spiegel, H. M., Conlon, C., Spina, C. A., *et al.* (2000). HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J Exp Med* 192, 63-75.
- Aquino, R. T., Chieffi, P. P., Catunda, S. M., Araujo, M. F., Ribeiro, M. C., Taddeo, E. F., and Rolim, E. G. (2000). Hepatitis B and C virus markers among patients with hepatosplenic mansonic schistosomiasis. *Rev Inst Med Trop Sao Paulo* 42, 313-320.
- Aseffa, A., Gumy, A., Launois, P., MacDonald, H. R., Louis, J. A., and Tacchini-Cottier, F. (2002). The early IL-4 response to *Leishmania major* and the resulting Th2 cell maturation

- steering progressive disease in BALB/c mice are subject to the control of regulatory CD4⁺CD25⁺ T cells. *J Immunol* 169, 3232-3241.
- Asseman, C., Fowler, S., and Powrie, F. (2000). Control of experimental inflammatory bowel disease by regulatory T cells. *Am J Respir Crit Care Med* 162, S185-189.
- Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L., and Powrie, F. (1999). An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 190, 995-1004.
- Atochina, O., Daly-Engel, T., Piskorska, D., McGuire, E., and Harn, D. A. (2001). A schistosoma-expressed immunomodulatory glycoconjugate expands peritoneal Gr1(+) macrophages that suppress naive CD4(+) T cell proliferation via an IFN-gamma and nitric oxide-dependent mechanism. *J Immunol* 167, 4293-4302.
- Attallah, A. M., Smith, A. H., Murrell, K. D., Fleischer, T., Woody, J., Vannier, W. E., Scher, I., Ahmed, A., and Sell, K. W. (1979). Characterization of the immunosuppressive state during *Schistosoma mansoni* infection. *J Immunol* 122, 1413-1420.
- Babu, S., Blauvelt, C. P., Kumaraswami, V., and Nutman, T. B. (2006). Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. *J Immunol* 176, 3248-3256.
- Bachmaier, K., Krawczyk, C., Kozieradzki, I., Kong, Y. Y., Sasaki, T., Oliveira-dos-Santos, A., Mariathasan, S., Bouchard, D., Wakeham, A., Itie, A., *et al.* (2000). Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* 403, 211-216.
- Barber, D. L., Wherry, E. J., Masopust, D., Zhu, B., Allison, J. P., Sharpe, A. H., Freeman, G. J., and Ahmed, R. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439, 682-687.
- Baumgart, M., Tompkins, F., Leng, J., and Hesse, M. (2006). Naturally occurring CD4⁺Foxp3⁺ regulatory T cells are an essential, IL-10-independent part of the immunoregulatory network in *Schistosoma mansoni* egg-induced inflammation. *J Immunol* 176, 5374-5387.
- Beilharz, M. W., Sammels, L. M., Paun, A., Shaw, K., van Eeden, P., Watson, M. W., and Ashdown, M. L. (2004). Timed ablation of regulatory CD4⁺ T cells can prevent murine AIDS progression. *J Immunol* 172, 4917-4925.

- Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M., and Sacks, D. L. (2002). CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* 420, 502-507.
- Belkaid, Y., and Rouse, B. T. (2005). Natural regulatory T cells in infectious disease. *Nat Immunol* 6, 353-360.
- Bergman, I., and Locksley, R. (1963). Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Anal Chem* 35, 1961.
- Bergquist, R., Al-Sherbiny, M., Barakat, R., and Olds, R. (2002). Blueprint for schistosomiasis vaccine development. *Acta Trop* 82, 183-192.
- Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L., and Kuchroo, V. K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector T(H)17 and regulatory T cells. *Nature*.
- Bienvenu, B., Martin, B., Auffray, C., Cordier, C., Becourt, C., and Lucas, B. (2005). Peripheral CD8+CD25+ T lymphocytes from MHC class II-deficient mice exhibit regulatory activity. *J Immunol* 175, 246-253.
- Birebent, B., Lorho, R., Lechartier, H., de Guibert, S., Alizadeh, M., Vu, N., Beauplet, A., Robillard, N., and Semana, G. (2004). Suppressive properties of human CD4+CD25+ regulatory T cells are dependent on CTLA-4 expression. *Eur J Immunol* 34, 3485-3496.
- Bluestone, J. A., and Abbas, A. K. (2003). Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 3, 253-257.
- Bluestone, J. A., St Clair, E. W., and Turka, L. A. (2006). CTLA4Ig: bridging the basic immunology with clinical application. *Immunity* 24, 233-238.
- Boasso, A., Herbeuval, J. P., Hardy, A. W., Winkler, C., and Shearer, G. M. (2005). Regulation of indoleamine 2,3-dioxygenase and tryptophanyl-tRNA-synthetase by CTLA-4-Fc in human CD4+ T cells. *Blood* 105, 1574-1581.
- Booth, M., Vennervald, B. J., Butterworth, A. E., Kariuki, H. C., Amaganga, C., Kimani, G., Mwatha, J. K., Otedo, A., Ouma, J. H., and Dunne, D. W. (2004). Exposure to malaria affects the regression of hepatosplenomegaly after treatment for *Schistosoma mansoni* infection in Kenyan children. *BMC Med* 2, 36.
- Boros, D. L., Pelley, R. P., and Warren, K. S. (1975). Spontaneous modulation of granulomatous hypersensitivity in schistosomiasis mansoni. *J Immunol* 114, 1437-1441.

- Borriello, F., Sethna, M. P., Boyd, S. D., Schweitzer, A. N., Tivol, E. A., Jacoby, D., Strom, T. B., Simpson, E. M., Freeman, G. J., and Sharpe, A. H. (1997). B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity* 6, 303-313.
- Brindley, P. J., and Sher, A. (1987). The chemotherapeutic effect of praziquantel against *Schistosoma mansoni* is dependent on host antibody response. *J Immunol* 139, 215-220.
- Brown, J. A., Dorfman, D. M., Ma, F. R., Sullivan, E. L., Munoz, O., Wood, C. R., Greenfield, E. A., and Freeman, G. J. (2003). Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J Immunol* 170, 1257-1266.
- Brown, M., Mawa, P. A., Joseph, S., Bukusuba, J., Watera, C., Whitworth, J. A., Dunne, D. W., and Elliott, A. M. (2005). Treatment of *Schistosoma mansoni* infection increases helminth-specific type 2 cytokine responses and HIV-1 loads in coinfecting Ugandan adults. *J Infect Dis* 191, 1648-1657.
- Brunet, J. F., Denizot, F., Luciani, M. F., Roux-Dosseto, M., Suzan, M., Mattei, M. G., and Golstein, P. (1987). A new member of the immunoglobulin superfamily--CTLA-4. *Nature* 328, 267-270.
- Brunet, L. R., Sabin, E. A., Cheever, A. W., Kopf, M. A., and Pearce, E. J. (1999). Interleukin 5 (IL-5) is not required for expression of a Th2 response or host resistance mechanisms during murine schistosomiasis mansoni but does play a role in development of IL-4-producing non-T, non-B cells. *Infect Immun* 67, 3014-3018.
- Brunner, M. C., Chambers, C. A., Chan, F. K., Hanke, J., Winoto, A., and Allison, J. P. (1999). CTLA-4-Mediated inhibition of early events of T cell proliferation. *J Immunol* 162, 5813-5820.
- Byram, J. E., and von Lichtenberg, F. (1977). Altered schistosome granuloma formation in nude mice. *Am J Trop Med Hyg* 26, 944-956.
- Cabrera, R., Tu, Z., Xu, Y., Firpi, R. J., Rosen, H. R., Liu, C., and Nelson, D. R. (2004). An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 40, 1062-1071.
- Capron, A., Riveau, G., Capron, M., and Trottein, F. (2005). Schistosomes: the road from host-parasite interactions to vaccines in clinical trials. *Trends Parasitol* 21, 143-149.

- Carreno, B. M., Bennett, F., Chau, T. A., Ling, V., Luxenberg, D., Jussif, J., Baroja, M. L., and Madrenas, J. (2000). CTLA-4 (CD152) can inhibit T cell activation by two different mechanisms depending on its level of cell surface expression. *J Immunol* 165, 1352-1356.
- Carreno, B. M., and Collins, M. (2003). BTLA: a new inhibitory receptor with a B7-like ligand. *Trends Immunol* 24, 524-527.
- Carter, L., Fouser, L. A., Jussif, J., Fitz, L., Deng, B., Wood, C. R., Collins, M., Honjo, T., Freeman, G. J., and Carreno, B. M. (2002). PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. *Eur J Immunol* 32, 634-643.
- Castro, A. G., Silva, R. A., Minoprio, P., and Appelberg, R. (1995). *In vivo* evidence for a non-T cell origin of interleukin-5. *Scand J Immunol* 41, 288-292.
- Cepek, K. L., Shaw, S. K., Parker, C. M., Russell, G. J., Morrow, J. S., Rimm, D. L., and Brenner, M. B. (1994). Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. *Nature* 372, 190-193.
- Chabaud, M., Durand, J. M., Buchs, N., Fossiez, F., Page, G., Frappart, L., and Miossec, P. (1999). Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum* 42, 963-970.
- Chang, C. C., Ciubotariu, R., Manavalan, J. S., Yuan, J., Colovai, A. I., Piazza, F., Lederman, S., Colonna, M., Cortesini, R., Dalla-Favera, R., and Suciu-Foca, N. (2002). Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. *Nat Immunol* 3, 237-243.
- Chapoval, A. I., Ni, J., Lau, J. S., Wilcox, R. A., Flies, D. B., Liu, D., Dong, H., Sica, G. L., Zhu, G., Tamada, K., and Chen, L. (2001). B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. *Nat Immunol* 2, 269-274.
- Cheever, A. W., Duvall, R. H., Hallack, T. A., Jr., Minker, R. G., Malley, J. D., and Malley, K. G. (1987). Variation of hepatic fibrosis and granuloma size among mouse strains infected with *Schistosoma mansoni*. *Am J Trop Med Hyg* 37, 85-97.
- Cheever, A. W., Lewis, F. A., and Wynn, T. A. (1997). *Schistosoma mansoni*: unisexual infections sensitized mice for granuloma formation around intravenously injected eggs. *Parasitol Res* 83, 57-59.
- Chemnitz, J. M., Parry, R. V., Nichols, K. E., June, C. H., and Riley, J. L. (2004). SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1

- upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* 173, 945-954.
- Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G., and Wahl, S. M. (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198, 1875-1886.
- Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A., and Weiner, H. L. (1994). Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265, 1237-1240.
- Chevillard, C., Moukoko, C. E., Elwali, N. E., Bream, J. H., Kouriba, B., Argiro, L., Rahoud, S., Mergani, A., Henri, S., Gaudart, J., *et al.* (2003). IFN-gamma polymorphisms (IFN-gamma +2109 and IFN-gamma +3810) are associated with severe hepatic fibrosis in human hepatic schistosomiasis (*Schistosoma mansoni*). *J Immunol* 171, 5596-5601.
- Chiang, Y. J., Kole, H. K., Brown, K., Naramura, M., Fukuhara, S., Hu, R. J., Jang, I. K., Gutkind, J. S., Shevach, E., and Gu, H. (2000). Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* 403, 216-220.
- Chiaromonte, M. G., Donaldson, D. D., Cheever, A. W., and Wynn, T. A. (1999). An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *J Clin Invest* 104, 777-785.
- Chiaromonte, M. G., Mentink-Kane, M., Jacobson, B. A., Cheever, A. W., Whitters, M. J., Goad, M. E., Wong, A., Collins, M., Donaldson, D. D., Grusby, M. J., and Wynn, T. A. (2003). Regulation and function of the interleukin 13 receptor alpha 2 during a T helper cell type 2-dominant immune response. *J Exp Med* 197, 687-701.
- Chikuma, S., Abbas, A. K., and Bluestone, J. A. (2005). B7-independent inhibition of T cells by CTLA-4. *J Immunol* 175, 177-181.
- Chikuma, S., and Bluestone, J. A. (2003). CTLA-4 and tolerance: the biochemical point of view. *Immunol Res* 28, 241-253.
- Chitsulo, L., Engels, D., Montresor, A., and Savioli, L. (2000). The global status of schistosomiasis and its control. *Acta Trop* 77, 41-51.
- Choi, I. H., Zhu, G., Sica, G. L., Strome, S. E., Cheville, J. C., Lau, J. S., Zhu, Y., Flies, D. B., Tamada, K., and Chen, L. (2003). Genomic organization and expression analysis of B7-H4, an immune inhibitory molecule of the B7 family. *J Immunol* 171, 4650-4654.

- Christensen, J. E., Christensen, J. P., Kristensen, N. N., Hansen, N. J., Stryhn, A., and Thomsen, A. R. (2002). Role of CD28 co-stimulation in generation and maintenance of virus-specific T cells. *Int Immunol* 14, 701-711.
- Chtanova, T., Tangye, S. G., Newton, R., Frank, N., Hodge, M. R., Rolph, M. S., and Mackay, C. R. (2004). T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol* 173, 68-78.
- Cioli, D., and Pica-Mattoccia, L. (2003). Praziquantel. *Parasitol Res* 90 Supp 1, S3-9.
- Cobbold, S. P., Castejon, R., Adams, E., Zelenika, D., Graca, L., Humm, S., and Waldmann, H. (2004). Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J Immunol* 172, 6003-6010.
- Coenen, J. J., Koenen, H. J., van Rijssen, E., Boon, L., Joosten, I., and Hilbrands, L. B. (2006). CTLA-4 engagement and regulatory CD4+CD25+ T cells independently control CD8+-mediated responses under costimulation blockade. *J Immunol* 176, 5240-5246.
- Colley, D. G., Sasser, L. E., and Reed, A. M. (2005). PD-L2+ dendritic cells and PD-1+ CD4+ T cells in schistosomiasis correlate with morbidity. *Parasite Immunol* 27, 45-53.
- Collins, A. V., Brodie, D. W., Gilbert, R. J., Iaboni, A., Manso-Sancho, R., Walse, B., Stuart, D. I., van der Merwe, P. A., and Davis, S. J. (2002). The interaction properties of costimulatory molecules revisited. *Immunity* 17, 201-210.
- Collins, M., Ling, V., and Carreno, B. M. (2005). The B7 family of immune-regulatory ligands. *Genome Biol* 6, 223.
- Cottrez, F., Hurst, S. D., Coffman, R. L., and Groux, H. (2000). T regulatory cells 1 inhibit a Th2-specific response *in vivo*. *J Immunol* 165, 4848-4853.
- Creagh, E. M., and O'Neill L, A. (2006). TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol*.
- Croese, J., O'Neil, J., Masson, J., Cooke, S., Melrose, W., Pritchard, D., and Speare, R. (2006). A proof of concept study establishing *Nippostrongylus brasiliensis* in Crohn's patients and reservoir donors. *Gut* 55, 136-137.
- Curiel, T. J., Wei, S., Dong, H., Alvarez, X., Cheng, P., Mottram, P., Krzysiek, R., Knutson, K. L., Daniel, B., Zimmermann, M. C., *et al.* (2003). Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med* 9, 562-567.

- de Jesus, A. R., Silva, A., Santana, L. B., Magalhaes, A., de Jesus, A. A., de Almeida, R. P., Rego, M. A., Burattini, M. N., Pearce, E. J., and Carvalho, E. M. (2002). Clinical and immunologic evaluation of 31 patients with acute schistosomiasis mansoni. *J Infect Dis* 185, 98-105.
- Dennehy, K. M., Elias, F., Zeder-Lutz, G., Ding, X., Altschuh, D., Luhder, F., and Hunig, T. (2006). Cutting edge: monovalency of CD28 maintains the antigen dependence of T cell costimulatory responses. *J Immunol* 176, 5725-5729.
- Deppong, C., Juehne, T. I., Hurchla, M., Friend, L. D., Shah, D. D., Rose, C. M., Bricker, T. L., Shornick, L. P., Crouch, E. C., Murphy, T. L., *et al.* (2006). Cutting edge: B and T lymphocyte attenuator and programmed death receptor-1 inhibitory receptors are required for termination of acute allergic airway inflammation. *J Immunol* 176, 3909-3913.
- Dessein, A. J., Hillaire, D., Elwali, N. E., Marquet, S., Mohamed-Ali, Q., Mirghani, A., Henri, S., Abdelhameed, A. A., Saeed, O. K., Magzoub, M. M., and Abel, L. (1999). Severe hepatic fibrosis in *Schistosoma mansoni* infection is controlled by a major locus that is closely linked to the interferon-gamma receptor gene. *Am J Hum Genet* 65, 709-721.
- Diab, M., Kamal, K., Cavender, D., and Higashi, G. (1989). Modulation of cell-mediated immunity in mice with chronic unisexual or bisexual *Schistosoma mansoni* cercarial infection. *Ann Trop Med Parasitol* 83, 25-30.
- Doenhoff, M., Musallam, R., Bain, J., and McGregor, A. (1979). *Schistosoma mansoni* infections in T-cell deprived mice, and the ameliorating effect of administering homologous chronic infection serum. I. Pathogenesis. *Am J Trop Med Hyg* 28, 260-273.
- Doenhoff, M. J., Pearson, S., Dunne, D. W., Bickle, Q., Lucas, S., Bain, J., Musallam, R., and Hassounah, O. (1981). Immunological control of hepatotoxicity and parasite egg excretion in *Schistosoma mansoni* infections: stage specificity of the reactivity of immune serum in T-cell deprived mice. *Trans R Soc Trop Med Hyg* 75, 41-53.
- Dong, H., and Chen, L. (2003). B7-H1 pathway and its role in the evasion of tumor immunity. *J Mol Med* 81, 281-287.
- Dong, H., and Chen, X. (2006). Immunoregulatory role of B7-H1 in chronicity of inflammatory responses. *Cell Mol Immunol* 3, 179-187.

- Dong, H., Strome, S. E., Matteson, E. L., Moder, K. G., Flies, D. B., Zhu, G., Tamura, H., Driscoll, C. L., and Chen, L. (2003). Costimulating aberrant T cell responses by B7-H1 autoantibodies in rheumatoid arthritis. *J Clin Invest* 111, 363-370.
- Dong, H., Strome, S. E., Salomao, D. R., Tamura, H., Hirano, F., Flies, D. B., Roche, P. C., Lu, J., Zhu, G., Tamada, K., *et al.* (2002). Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 8, 793-800.
- Dong, H., Zhu, G., Tamada, K., and Chen, L. (1999). B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 5, 1365-1369.
- Dong, H., Zhu, G., Tamada, K., Flies, D. B., van Deursen, J. M., and Chen, L. (2004). B7-H1 determines accumulation and deletion of intrahepatic CD8(+) T lymphocytes. *Immunity* 20, 327-336.
- Dubucquoi, S., Desreumaux, P., Janin, A., Klein, O., Goldman, M., Tavernier, J., Capron, A., and Capron, M. (1994). Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. *J Exp Med* 179, 703-708.
- Dunne, A., and O'Neill, L. A. (2005). Adaptor usage and Toll-like receptor signaling specificity. *FEBS Lett* 579, 3330-3335.
- Dunne, D. W., and Pearce, E. J. (1999). Immunology of hepatosplenic schistosomiasis *mansoni*: a human perspective. *Microbes Infect* 1, 553-560.
- Eberl, M., Langermans, J. A., Frost, P. A., Vervenne, R. A., van Dam, G. J., Deelder, A. M., Thomas, A. W., Coulson, P. S., and Wilson, R. A. (2001). Cellular and humoral immune responses and protection against schistosomes induced by a radiation-attenuated vaccine in chimpanzees. *Infect Immun* 69, 5352-5362.
- Endharti, A. T., Rifa, I. M. s., Shi, Z., Fukuoka, Y., Nakahara, Y., Kawamoto, Y., Takeda, K., Isobe, K., and Suzuki, H. (2005). Cutting edge: CD8+CD122+ regulatory T cells produce IL-10 to suppress IFN-gamma production and proliferation of CD8+ T cells. *J Immunol* 175, 7093-7097.
- Engering, A., Geijtenbeek, T. B., van Vliet, S. J., Wijers, M., van Liempt, E., Demareux, N., Lanzavecchia, A., Fransen, J., Figdor, C. G., Piguet, V., and van Kooyk, Y. (2002). The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J Immunol* 168, 2118-2126.

- Estaquier, J., Marguerite, M., Sahuc, F., Bessis, N., Auriault, C., and Ameisen, J. C. (1997). Interleukin-10-mediated T cell apoptosis during the T helper type 2 cytokine response in murine *Schistosoma mansoni* parasite infection. *Eur Cytokine Netw* 8, 153-160.
- Evans, E. J., Esnouf, R. M., Manso-Sancho, R., Gilbert, R. J., James, J. R., Yu, C., Fennelly, J. A., Vowles, C., Hanke, T., Walse, B., *et al.* (2005). Crystal structure of a soluble CD28-Fab complex. *Nat Immunol* 6, 271-279.
- Falcone, F. H., Loke, P., Zang, X., MacDonald, A. S., Maizels, R. M., and Allen, J. E. (2001). A *Brugia malayi* homolog of macrophage migration inhibitory factor reveals an important link between macrophages and eosinophil recruitment during nematode infection. *J Immunol* 167, 5348-5354.
- Fallon, P., and Alcamí, A. (2006). Pathogen-derived immunomodulatory molecules: future immunotherapeutics? *Trends in Immunology* 27:IN PRESS.
- Fallon, P. G. (2000). Immunopathology of Schistosomiasis: a cautionary tale of mice and men. *Immunology Today* 21, 29-34.
- Fallon, P. G., Ballantyne, S. J., Mangan, N. E., Barlow, J. L., Dasvarma, A., Hewett, D. R., McIlgorm, A., Jolin, H. E., and McKenzie, A. N. (2006). Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med* 203, 1105-1116.
- Fallon, P. G., Cooper, R. O., Probert, A. J., and Doenhoff, M. J. (1992). Immune-dependent chemotherapy of schistosomiasis. *Parasitology* 105 Suppl, S41-48.
- Fallon, P. G., and Doenhoff, M. J. (1994). Drug-resistant schistosomiasis: resistance to praziquantel and oxamniquine induced in *Schistosoma mansoni* in mice is drug specific. *Am J Trop Med Hyg* 51, 83-88.
- Fallon, P. G., and Dunne, D. W. (1999). Tolerisation of mice to *Schistosoma mansoni* egg antigens causes elevated type 1 and type 2 cytokine responses and increased mortality in acute infection. *J Immunol* 162, 4122-4132.
- Fallon, P. G., Emson, C. L., Smith, P., and McKenzie, A. N. (2001). IL-13 Overexpression Predisposes to Anaphylaxis Following Antigen Sensitization. *J Immunol* 166, 2712-2716.
- Fallon, P. G., Richardson, E. J., McKenzie, G. J., and McKenzie, A. N. (2000a). Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. *J Immunol* 164, 2585-2591.

- Fallon, P. G., Richardson, E. J., Smith, P., and Dunne, D. W. (2000b). Elevated type 1, diminished type 2 cytokines and impaired antibody responses are associated with hepatotoxicity and mortalities during schistosome infection of CD4-depleted mice. *European Journal of Immunology* 30, 470-470.
- Fallon, P. G., Smith, P., and Dunne, D. W. (1998). Type 1 and type 2 cytokine-producing mouse CD4⁺ and CD8⁺ T cells in acute *Schistosoma mansoni* infection. *Eur J Immunol* 28, 1408-1416.
- Fallon, P. G., Smith, P., Richardson, E. J., Jones, F. J., Faulkner, H. C., Van Snick, J., Renaud, J. C., Grencis, R. K., and Dunne, D. W. (2000c). Expression of interleukin-9 leads to Th2 cytokine-dominated responses and fatal enteropathy in mice with chronic *Schistosoma mansoni* infections. *Infect Immun* 68, 6005-6011.
- Fallon, P. G., Sturrock, R. F., Niang, A. C., and Doenhoff, M. J. (1995). Short report: diminished susceptibility to praziquantel in a Senegal isolate of *Schistosoma mansoni*. *Am J Trop Med Hyg* 53, 61-62.
- Fallon, P. G., Tao, L. F., Ismail, M. M., and Bennett, J. L. (1996). Schistosome resistance to praziquantel: Fact or artifact? *Parasitol Today* 12, 316-320.
- Faria, A. M., and Weiner, H. L. (2005). Oral tolerance. *Immunol Rev* 206, 232-259.
- Flores Villanueva, P. O., Chikunguwo, S. M., Harris, T. S., and Stadecker, M. J. (1993). Role of IL-10 on antigen-presenting cell function for schistosomal egg-specific monoclonal T helper cell responses *in vitro* and *in vivo*. *J Immunol* 151, 3192-3198.
- Flores Villanueva, P. O., Harris, T. S., Ricklan, D. E., and Stadecker, M. J. (1994). Macrophages from schistosomal egg granulomas induce unresponsiveness in specific cloned Th-1 lymphocytes *in vitro* and down-regulate schistosomal granulomatous disease *in vivo*. *J Immunol* 152, 1847-1855.
- Fontenot, J. D., Rasmussen, J. P., Williams, L. M., Dooley, J. L., Farr, A. G., and Rudensky, A. Y. (2005). Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22, 329-341.
- Fontenot, J. D., and Rudensky, A. Y. (2005). A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 6, 331-337.

- Ford, M. J., Bickle, Q. D., and Taylor, M. G. (1984). Immunization of rats against *Schistosoma mansoni* using irradiated cercariae, lung schistosomula and liver-stage worms. *Parasitology* 89 (Pt 2), 327-344.
- Frank, C., Mohamed, M. K., Strickland, G. T., Lavanchy, D., Arthur, R. R., Magder, L. S., El Khoby, T., Abdel-Wahab, Y., Aly Ohn, E. S., Anwar, W., and Sallam, I. (2000). The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. *Lancet* 355, 887-891.
- Freeman, G. J., Long, A. J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., Fitz, L. J., Malenkovich, N., Okazaki, T., Byrne, M. C., *et al.* (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 192, 1027-1034.
- Fulford, A. J., Butterworth, A. E., Ouma, J. H., and Sturrock, R. F. (1995). A statistical approach to schistosome population dynamics and estimation of the life-span of *Schistosoma mansoni* in man. *Parasitology* 110 (Pt 3), 307-316.
- Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., *et al.* (2000). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* 100, 587-597.
- Geijtenbeek, T. B., van Vliet, S. J., Engering, A., t Hart, B. A., and van Kooyk, Y. (2004). Self- and nonself-recognition by C-type lectins on dendritic cells. *Annu Rev Immunol* 22, 33-54.
- Geijtenbeek, T. B., Van Vliet, S. J., Koppel, E. A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C. M., Appelmelk, B., and Van Kooyk, Y. (2003). Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med* 197, 7-17.
- Geng, L., Jiang, G., Xie, H., Fang, Y., Dong, S., Chen, Y., Shen, M., and Zheng, S. (2006). Mycophenolic Acid Upregulates B7-DC Expression on Dendritic Cells, Which Is Associated With Impaired Allostimulatory Capacity of Dendritic Cells. *Transplant Proc* 38, 1622-1624.
- Gilliet, M., and Liu, Y. J. (2002). Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J Exp Med* 195, 695-704.

- Girardin, S. E., Boneca, I. G., Carneiro, L. A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M. K., Labigne, A., Zahringer, U., *et al.* (2003a). Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300, 1584-1587.
- Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D. J., and Sansonetti, P. J. (2003b). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 278, 8869-8872.
- Godfrey, V. L., Wilkinson, J. E., and Russell, L. B. (1991). X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. *Am J Pathol* 138, 1379-1387.
- Goldsby, R., Kindt, T., Osborne, B., and Kuby, J. (2002). *Immunology*, W.H. Freeman and Company).
- Gonzalez, L. C., Loyet, K. M., Calemine-Fenau, J., Chauhan, V., Wranik, B., Ouyang, W., and Eaton, D. L. (2005). A coreceptor interaction between the CD28 and TNF receptor family members B and T lymphocyte attenuator and herpesvirus entry mediator. *Proc Natl Acad Sci U S A* 102, 1116-1121.
- Gordon, S. (2003). Alternative activation of macrophages. *Nat Rev Immunol* 3, 23-35.
- Greenwald, R. J., Freeman, G. J., and Sharpe, A. H. (2005). The B7 family revisited. *Annu Rev Immunol* 23, 515-548.
- Grevelding, C. G. (1999). Genomic instability in *Schistosoma mansoni*. *Mol Biochem Parasitol* 101, 207-216.
- Grohmann, U., Orabona, C., Fallarino, F., Vacca, C., Calcinaro, F., Falorni, A., Candeloro, P., Belladonna, M. L., Bianchi, R., Fioretti, M. C., and Puccetti, P. (2002). CTLA-4-Ig regulates tryptophan catabolism *in vivo*. *Nat Immunol* 3, 1097-1101.
- Groux, H., Bigler, M., de Vries, J. E., and Roncarolo, M. G. (1996). Interleukin-10 induces a long-term antigen-specific anergic state in human CD4⁺ T cells. *J Exp Med* 184, 19-29.
- Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E., and Roncarolo, M. G. (1997). A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389, 737-742.
- Grzych, J. M., Pearce, E., Cheever, A., Caulada, Z. A., Caspar, P., Heiny, S., Lewis, F., and Sher, A. (1991). Egg deposition is the major stimulus for the production of Th2 cytokines in murine schistosomiasis mansoni. *J Immunol* 146, 1322-1327.

- Hagan, P., Appleton, C. C., Coles, G. C., Kusel, J. R., and Tchuem-Tchuente, L. A. (2004). Schistosomiasis control: keep taking the tablets. *Trends Parasitol* 20, 92-97.
- Hagenbaugh, A., Sharma, S., Dubinett, S. M., Wei, S. H., Aranda, R., Cheroutre, H., Fowell, D. J., Binder, S., Tsao, B., Locksley, R. M., *et al.* (1997). Altered immune responses in interleukin 10 transgenic mice. *J Exp Med* 185, 2101-2110.
- Hardtke, S., Ohl, L., and Forster, R. (2005). Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help. *Blood* 106, 1924-1931.
- Harnett, W., and Kusel, J. R. (1986). Increased exposure of parasite antigens at the surface of adult male *Schistosoma mansoni* exposed to praziquantel *in vitro*. *Parasitology* 93 (Pt 2), 401-405.
- Hatachi, S., Iwai, Y., Kawano, S., Morinobu, S., Kobayashi, M., Koshiba, M., Saura, R., Kurosaka, M., Honjo, T., and Kumagai, S. (2003). CD4+ PD-1+ T cells accumulate as unique anergic cells in rheumatoid arthritis synovial fluid. *J Rheumatol* 30, 1410-1419.
- Hayashi, N., Matsui, K., Tsutsui, H., Osada, Y., Mohamed, R. T., Nakano, H., Kashiwamura, S., Hyodo, Y., Takeda, K., Akira, S., *et al.* (1999). Kupffer cells from *Schistosoma mansoni*-infected mice participate in the prompt type 2 differentiation of hepatic T cells in response to worm antigens. *J Immunol* 163, 6702-6711.
- He, Y. F., Zhang, G. M., Wang, X. H., Zhang, H., Yuan, Y., Li, D., and Feng, Z. H. (2004). Blocking programmed death-1 ligand-PD-1 interactions by local gene therapy results in enhancement of antitumor effect of secondary lymphoid tissue chemokine. *J Immunol* 173, 4919-4928.
- Heinzel, F. P., Sadick, M. D., Holaday, B. J., Coffman, R. L., and Locksley, R. M. (1989). Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J Exp Med* 169, 59-72.
- Hellings, P. W., Vandenberghe, P., Kasran, A., Coorevits, L., Overbergh, L., Mathieu, C., and Ceuppens, J. L. (2002). Blockade of CTLA-4 enhances allergic sensitization and eosinophilic airway inflammation in genetically predisposed mice. *Eur J Immunol* 32, 585-594.
- Herbert, D. R., Holscher, C., Mohrs, M., Arendse, B., Schwegmann, A., Radwanska, M., Leeto, M., Kirsch, R., Hall, P., Mossmann, H., *et al.* (2004). Alternative Macrophage

- Activation Is Essential for Survival during Schistosomiasis and Downmodulates T Helper 1 Responses and Immunopathology. *Immunity* 20, 623-635.
- Hernandez, H. J., Sharpe, A. H., and Stadecker, M. J. (1999). Experimental murine schistosomiasis in the absence of B7 costimulatory molecules: reversal of elicited T cell cytokine profile and partial inhibition of egg granuloma formation. *J Immunol* 162, 2884-2889.
- Hernandez, H. J., Wang, Y., Tzellas, N., and Stadecker, M. J. (1997). Expression of class II, but not class I, major histocompatibility complex molecules is required for granuloma formation in infection with *Schistosoma mansoni*. *Eur J Immunol* 27, 1170-1176.
- Hesse, M., Modolell, M., La Flamme, A. C., Schito, M., Fuentes, J. M., Cheever, A. W., Pearce, E. J., and Wynn, T. A. (2001). Differential Regulation of Nitric Oxide Synthase-2 and Arginase-1 by Type 1/Type 2 Cytokines *In vivo*: Granulomatous Pathology Is Shaped by the Pattern of L-Arginine Metabolism. *J Immunol* 167, 6533-6544.
- Hesse, M., Piccirillo, C. A., Belkaid, Y., Prufer, J., Mentink-Kane, M., Leusink, M., Cheever, A. W., Shevach, E. M., and Wynn, T. A. (2004). The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells. *J Immunol* 172, 3157-3166.
- Hewitson, J. P., Hamblin, P. A., and Mountford, A. P. (2005). Immunity induced by the radiation-attenuated schistosome vaccine. *Parasite Immunol* 27, 271-280.
- Hirano, F., Kaneko, K., Tamura, H., Dong, H., Wang, S., Ichikawa, M., Rietz, C., Flies, D. B., Lau, J. S., Zhu, G., *et al.* (2005). Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res* 65, 1089-1096.
- Hoffmann, K. F., Cheever, A. W., and Wynn, T. A. (2000). IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J Immunol* 164, 6406-6416.
- Hoffmann, K. F., James, S. L., Cheever, A. W., and Wynn, T. A. (1999). Studies with double cytokine-deficient mice reveal that highly polarized Th1- and Th2-type cytokine and antibody responses contribute equally to vaccine-induced immunity to *Schistosoma mansoni*. *J Immunol* 163, 927-938.

- Hoffmann, K. F., Wynn, T. A., and Dunne, D. W. (2002). Cytokine-mediated host responses during schistosome infections; walking the fine line between immunological control and immunopathology. *Adv Parasitol* 52, 265-307.
- Holcomb, I. N., Kabakoff, R. C., Chan, B., Baker, T. W., Gurney, A., Henzel, W., Nelson, C., Lowman, H. B., Wright, B. D., Skelton, N. J., *et al.* (2000). FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. *Embo J* 19, 4046-4055.
- Hori, S., Carvalho, T. L., and Demengeot, J. (2002). CD25+CD4+ regulatory T cells suppress CD4+ T cell-mediated pulmonary hyperinflammation driven by *Pneumocystis carinii* in immunodeficient mice. *Eur J Immunol* 32, 1282-1291.
- Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299, 1057-1061.
- Hu, D., Ikizawa, K., Lu, L., Sanchirico, M. E., Shinohara, M. L., and Cantor, H. (2004). Analysis of regulatory CD8 T cells in Qa-1-deficient mice. *Nat Immunol* 5, 516-523.
- Humphreys, I. R., Walzl, G., Edwards, L., Rae, A., Hill, S., and Hussell, T. (2003). A critical role for OX40 in T cell-mediated immunopathology during lung viral infection. *J Exp Med* 198, 1237-1242.
- Hutloff, A., Dittrich, A. M., Beier, K. C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., and Kroczeck, R. A. (1999). ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397, 263-266.
- Ismail, M., Botros, S., Metwally, A., William, S., Farghally, A., Tao, L. F., Day, T. A., and Bennett, J. L. (1999). Resistance to praziquantel: direct evidence from *Schistosoma mansoni* isolated from Egyptian villagers. *Am J Trop Med Hyg* 60, 932-935.
- Iwai, Y., Ishida, M., Tanaka, Y., Okazaki, T., Honjo, T., and Minato, N. (2002). Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A* 99, 12293-12297.
- Iwai, Y., Terawaki, S., Ikegawa, M., Okazaki, T., and Honjo, T. (2003). PD-1 Inhibits Antiviral Immunity at the Effector Phase in the Liver. *J Exp Med* 198, 39-50.
- Jacobs, T., Plate, T., Gaworski, I., and Fleischer, B. (2004). CTLA-4-dependent mechanisms prevent T cell induced-liver pathology during the erythrocyte stage of *Plasmodium berghei* malaria. *Eur J Immunol* 34, 972-980.

- Jago, C. B., Yates, J., Camara, N. O., Lechler, R. I., and Lombardi, G. (2004). Differential expression of CTLA-4 among T cell subsets. *Clin Exp Immunol* 136, 463-471.
- Jankovic, D., Cheever, A. W., Kullberg, M. C., Wynn, T. A., Yap, G., Caspar, P., Lewis, F. A., Clynes, R., Ravetch, J. V., and Sher, A. (1998). CD4+ T cell-mediated granulomatous pathology in schistosomiasis is downregulated by a B cell-dependent mechanism requiring Fc receptor signaling. *J Exp Med* 187, 619-629.
- Jankovic, D., Kullberg, M. C., Caspar, P., and Sher, A. (2004). Parasite-induced Th2 polarization is associated with down-regulated dendritic cell responsiveness to Th1 stimuli and a transient delay in T lymphocyte cycling. *J Immunol* 173, 2419-2427.
- Jankovic, D., Kullberg, M. C., Hieny, S., Caspar, P., Collazo, C. M., and Sher, A. (2002). In the absence of IL-12, CD4(+) T cell responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in an IL-10(-/-) setting. *Immunity* 16, 429-439.
- Jenkins, S. J., Hewitson, J. P., Ferret-Bernard, S., and Mountford, A. P. (2005). Schistosome larvae stimulate macrophage cytokine production through TLR4-dependent and -independent pathways. *Int Immunol* 17, 1409-1418.
- Jenkins, S. J., and Mountford, A. P. (2005). Dendritic cells activated with products released by schistosome larvae drive Th2-type immune responses, which can be inhibited by manipulation of CD40 costimulation. *Infect Immun* 73, 395-402.
- Johansson-Lindbom, B., Svensson, M., Pabst, O., Palmqvist, C., Marquez, G., Forster, R., and Agace, W. W. (2005). Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J Exp Med* 202, 1063-1073.
- Jordan, P., Webbe, G., and Sturrock, R. F. (1993). *Human Schistosomiasis*, CAB International).
- Kahnert, A., Seiler, P., Stein, M., Bandermann, S., Hahnke, K., Mollenkopf, H., and Kaufmann, S. H. (2006). Alternative activation deprives macrophages of a coordinated defense program to *Mycobacterium tuberculosis*. *Eur J Immunol* 36, 631-647.
- Kaisho, T., and Akira, S. (2006). Toll-like receptor function and signaling. *J Allergy Clin Immunol* 117, 979-987; quiz 988.
- Kanai, T., Totsuka, T., Uraushihara, K., Makita, S., Nakamura, T., Koganei, K., Fukushima, T., Akiba, H., Yagita, H., Okumura, K., *et al.* (2003). Blockade of B7-H1 suppresses the development of chronic intestinal inflammation. *J Immunol* 171, 4156-4163.

- Kang, D. C., Gopalkrishnan, R. V., Wu, Q., Jankowsky, E., Pyle, A. M., and Fisher, P. B. (2002). mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proc Natl Acad Sci U S A* 99, 637-642.
- Kang, Y. S., Do, Y., Lee, H. K., Park, S. H., Cheong, C., Lynch, R. M., Loeffler, J. M., Steinman, R. M., and Park, C. G. (2006). A dominant complement fixation pathway for pneumococcal polysaccharides initiated by SIGN-R1 interacting with C1q. *Cell* 125, 47-58.
- Kanneganti, T. D., Ozoren, N., Body-Malapel, M., Amer, A., Park, J. H., Franchi, L., Whitfield, J., Barchet, W., Colonna, M., Vandenabeele, P., *et al.* (2006). Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 440, 233-236.
- Kaplan, M. H., Whitfield, J. R., Boros, D. L., and Grusby, M. J. (1998). Th2 cells are required for the *Schistosoma mansoni* egg-induced granulomatous response. *J Immunol* 160, 1850-1856.
- Karanja, D. M., Boyer, A. E., Strand, M., Colley, D. G., Nahlen, B. L., Ouma, J. H., and Secor, W. E. (1998). Studies on schistosomiasis in western Kenya: II. Efficacy of praziquantel for treatment of schistosomiasis in persons coinfectd with human immunodeficiency virus-1. *Am J Trop Med Hyg* 59, 307-311.
- Kaviratne, M., Hesse, M., Leusink, M., Cheever, A. W., Davies, S. J., McKerrow, J. H., Wakefield, L. M., Letterio, J. J., and Wynn, T. A. (2004). IL-13 activates a mechanism of tissue fibrosis that is completely TGF-beta independent. *J Immunol* 173, 4020-4029.
- Keane, J., Gershon, S., Wise, R. P., Mirabile-Levens, E., Kasznica, J., Schwiertman, W. D., Siegel, J. N., and Braun, M. M. (2001). Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med* 345, 1098-1104.
- Keir, M. E., Liang, S. C., Guleria, I., Latchman, Y. E., Qipo, A., Albacker, L. A., Koulmanda, M., Freeman, G. J., Sayegh, M. H., and Sharpe, A. H. (2006). Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med* 203, 883-895.
- Kelsen, J., Agnholt, J., Hoffmann, H. J., Romer, J. L., Hvas, C. L., and Dahlerup, J. F. (2005). FoxP3(+)CD4(+)CD25(+) T cells with regulatory properties can be cultured from colonic mucosa of patients with Crohn's disease. *Clin Exp Immunol* 141, 549-557.

- Khatttri, R., Auger, J. A., Griffin, M. D., Sharpe, A. H., and Bluestone, J. A. (1999). Lymphoproliferative disorder in CTLA-4 knockout mice is characterized by CD28-regulated activation of Th2 responses. *J Immunol* 162, 5784-5791.
- Khatttri, R., Cox, T., Yasayko, S. A., and Ramsdell, F. (2003). An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 4, 337-342.
- Khoury, S. J., and Sayegh, M. H. (2004). The roles of the new negative T cell costimulatory pathways in regulating autoimmunity. *Immunity* 20, 529-538.
- Kilshaw, P. J. (1993). Expression of the mucosal T cell integrin alpha M290 beta 7 by a major subpopulation of dendritic cells in mice. *Eur J Immunol* 23, 3365-3368.
- King, C. H., Dickman, K., and Tisch, D. J. (2005). Reassessment of the cost of chronic helminthic infection: a meta-analysis of disability-related outcomes in endemic schistosomiasis. *Lancet* 365, 1561-1569.
- King, C. L. (2001). Initiation and regulation of disease in schistosomiasis. In *Schistosomiasis*, M. AAF, ed. (London, Imperial College Press), pp. 213-264.
- King, C. L., Malhotra, I., Mungai, P., Wamachi, A., Kioko, J., Ouma, J. H., and Kazura, J. W. (1998). B cell sensitization to helminthic infection develops in utero in humans. *J Immunol* 160, 3578-3584.
- King, C. L., Medhat, A., Malhotra, I., Nafeh, M., Helmy, A., Khaudary, J., Ibrahim, S., El-Sherbiny, M., Zaky, S., Stupi, R. J., *et al.* (1996). Cytokine control of parasite-specific anergy in human urinary schistosomiasis. IL-10 modulates lymphocyte reactivity. *J Immunol* 156, 4715-4721.
- Kirchberger, S., Majdic, O., Steinberger, P., Bluml, S., Pfistershammer, K., Zlabinger, G., Deszcz, L., Kuechler, E., Knapp, W., and Stockl, J. (2005). Human rhinoviruses inhibit the accessory function of dendritic cells by inducing sialoadhesin and B7-H1 expression. *J Immunol* 175, 1145-1152.
- Ko, K., Yamazaki, S., Nakamura, K., Nishioka, T., Hirota, K., Yamaguchi, T., Shimizu, J., Nomura, T., Chiba, T., and Sakaguchi, S. (2005). Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3+CD25+CD4+ regulatory T cells. *J Exp Med* 202, 885-891.
- Kobayashi, M., Kawano, S., Hatachi, S., Kurimoto, C., Okazaki, T., Iwai, Y., Honjo, T., Tanaka, Y., Minato, N., Komori, T., *et al.* (2005). Enhanced expression of programmed death-

- 1 (PD-1)/PD-L1 in salivary glands of patients with Sjogren's syndrome. *J Rheumatol* 32, 2156-2163.
- Kohm, A. P., McMahon, J. S., Podojil, J. R., Begolka, W. S., DeGutes, M., Kasprovicz, D. J., Ziegler, S. F., and Miller, S. D. (2006). Cutting Edge: Anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells. *J Immunol* 176, 3301-3305.
- Kouriba, B., Chevillard, C., Bream, J. H., Argiro, L., Dessein, H., Arnaud, V., Sangare, L., Dabo, A., Beavogui, A. H., Arama, C., *et al.* (2005). Analysis of the 5q31-q33 locus shows an association between IL13-1055C/T IL-13-591A/G polymorphisms and *Schistosoma haematobium* infections. *J Immunol* 174, 6274-6281.
- Krambeck, A. E., Thompson, R. H., Dong, H., Lohse, C. M., Park, E. S., Kuntz, S. M., Leibovich, B. C., Blute, M. L., Cheville, J. C., and Kwon, E. D. (2006). B7-H4 expression in renal cell carcinoma and tumor vasculature: Associations with cancer progression and survival. *Proc Natl Acad Sci U S A* 103, 10391-10396.
- Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M. C., and von Boehmer, H. (2005). Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6, 1219-1227.
- Kristiansen, O. P., Larsen, Z. M., and Pociot, F. (2000). CTLA-4 in autoimmune diseases--a general susceptibility gene to autoimmunity? *Genes Immun* 1, 170-184.
- Krummel, M. F., and Allison, J. P. (1996). CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med* 183, 2533-2540.
- Kryczek, I., Wei, S., Zou, L., Zhu, G., Mottram, P., Xu, H., Chen, L., and Zou, W. (2006). Cutting Edge: Induction of B7-H4 on APCs through IL-10: Novel Suppressive Mode for Regulatory T Cells. *J Immunol* 177, 40-44.
- Kullberg, M. C., Berzofsky, J. A., Jankovic, D. L., Barbieri, S., Williams, M. E., Perlmann, P., Sher, A., and Troye-Blomberg, M. (1996). T cell-derived IL-3 induces the production of IL-4 by non-B, non-T cells to amplify the Th2-cytokine response to a non-parasite antigen in *Schistosoma mansoni*-infected mice. *J Immunol* 156, 1482-1489.
- Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A., and Cua, D. J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201, 233-240.

- Lanoue, A., Clatworthy, M. R., Smith, P., Green, S., Townsend, M. J., Jolin, H. E., Smith, K. G., Fallon, P. G., and McKenzie, A. N. (2004). SIGN-R1 contributes to protection against lethal pneumococcal infection in mice. *J Exp Med* 200, 1383-1393.
- Latchman, Y., Wood, C. R., Chernova, T., Chaudhary, D., Borde, M., Chernova, I., Iwai, Y., Long, A. J., Brown, J. A., Nunes, R., *et al.* (2001). PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2, 261-268.
- Latchman, Y. E., Liang, S. C., Wu, Y., Chernova, T., Sobel, R. A., Klemm, M., Kuchroo, V. K., Freeman, G. J., and Sharpe, A. H. (2004). PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. *Proc Natl Acad Sci U S A* 101, 10691-10696.
- Layland, L. E., Wagner, H., and da Costa, C. U. (2005). Lack of antigen-specific Th1 response alters granuloma formation and composition in *Schistosoma mansoni*-infected MyD88^{-/-} mice. *Eur J Immunol* 35, 3248-3257.
- Lechner, O., Lauber, J., Franzke, A., Sarukhan, A., von Boehmer, H., and Buer, J. (2001). Fingerprints of anergic T cells. *Curr Biol* 11, 587-595.
- Lee, K. M., Chuang, E., Griffin, M., Khattri, R., Hong, D. K., Zhang, W., Straus, D., Samelson, L. E., Thompson, C. B., and Bluestone, J. A. (1998). Molecular basis of T cell inactivation by CTLA-4. *Science* 282, 2263-2266.
- Lee, S. J., Evers, S., Roeder, D., Parlow, A. F., Risteli, J., Risteli, L., Lee, Y. C., Feizi, T., Langen, H., and Nussenzweig, M. C. (2002). Mannose receptor-mediated regulation of serum glycoprotein homeostasis. *Science* 295, 1898-1901.
- Lee, S. J., Zheng, N. Y., Clavijo, M., and Nussenzweig, M. C. (2003). Normal host defense during systemic candidiasis in mannose receptor-deficient mice. *Infect Immun* 71, 437-445.
- Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* 86, 973-983.
- Leng, Q., Bentwich, Z., and Borkow, G. (2006). Increased TGF-beta, Cbl-b and CTLA-4 levels and immunosuppression in association with chronic immune activation. *Int Immunol* 18, 637-644.

- Leptak, C. L., and J.H., M. (1997). Schistosome egg granulomas and hepatic expression of TNF- α are dependent on immune priming during parasite maturation. *J Immunol* 158, 301-307.
- Lewkowich, I. P., Herman, N. S., Schleifer, K. W., Dance, M. P., Chen, B. L., Dienger, K. M., Sproles, A. A., Shah, J. S., Kohl, J., Belkaid, Y., and Wills-Karp, M. (2005). CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med* 202, 1549-1561.
- Li, D., Gal, I., Vermes, C., Alegre, M. L., Chong, A. S., Chen, L., Shao, Q., Adarichev, V., Xu, X., Koreny, T., *et al.* (2004). Cutting edge: Cbl-b: one of the key molecules tuning CD28- and CTLA-4-mediated T cell costimulation. *J Immunol* 173, 7135-7139.
- Liang, L., and Sha, W. C. (2002). The right place at the right time: novel B7 family members regulate effector T cell responses. *Curr Opin Immunol* 14, 384-390.
- Liang, S. C., Greenwald, R. J., Latchman, Y. E., Rosas, L., Satoskar, A., Freeman, G. J., and Sharpe, A. H. (2006). PD-L1 and PD-L2 have distinct roles in regulating host immunity to cutaneous leishmaniasis. *Eur J Immunol* 36, 58-64.
- Liang, S. C., Latchman, Y. E., Buhlmann, J. E., Tomczak, M. F., Horwitz, B. H., Freeman, G. J., and Sharpe, A. H. (2003). Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur J Immunol* 33, 2706-2716.
- Lim, H. W., Hillsamer, P., Banham, A. H., and Kim, C. H. (2005). Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J Immunol* 175, 4180-4183.
- Lindsten, T., Lee, K. P., Harris, E. S., Petryniak, B., Craighead, N., Reynolds, P. J., Lombard, D. B., Freeman, G. J., Nadler, L. M., Gray, G. S., and *et al.* (1993). Characterization of CTLA-4 structure and expression on human T cells. *J Immunol* 151, 3489-3499.
- Ling, V., Munroe, R. C., Murphy, E. A., and Gray, G. S. (1998). Embryonic stem cells and embryoid bodies express lymphocyte costimulatory molecules. *Exp Cell Res* 241, 55-65.
- Linsley, P. S., Nadler, S. G., Bajorath, J., Peach, R., Leung, H. T., Rogers, J., Bradshaw, J., Stebbins, M., Leytze, G., Brady, W., and *et al.* (1995). Binding stoichiometry of the cytotoxic T lymphocyte-associated molecule-4 (CTLA-4). A disulfide-linked homodimer binds two CD86 molecules. *J Biol Chem* 270, 15417-15424.

- Liu, W., Putnam, A. L., Xu-Yu, Z., Szot, G. L., Lee, M. R., Zhu, S., Gottlieb, P. A., Kapranov, P., Gingeras, T. R., de St Groth, B. F., *et al.* (2006a). CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. *J Exp Med* 203, 1701-1711.
- Liu, X., Gao, J. X., Wen, J., Yin, L., Li, O., Zuo, T., Gajewski, T. F., Fu, Y. X., Zheng, P., and Liu, Y. (2003). B7DC/PDL2 promotes tumor immunity by a PD-1-independent mechanism. *J Exp Med* 197, 1721-1730.
- Liu, Y., Teige, I., Birnir, B., and Issazadeh-Navikas, S. (2006b). Neuron-mediated generation of regulatory T cells from encephalitogenic T cells suppresses EAE. *Nat Med* 12, 518-525.
- Lohning, M., Stroehmann, A., Coyle, A. J., Grogan, J. L., Lin, S., Gutierrez-Ramos, J. C., Levinson, D., Radbruch, A., and Kamradt, T. (1998). T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc Natl Acad Sci U S A* 95, 6930-6935.
- Lohr, J., Knoechel, B., Jiang, S., Sharpe, A. H., and Abbas, A. K. (2003). The inhibitory function of B7 costimulators in T cell responses to foreign and self-antigens. *Nat Immunol* 4, 664-669.
- Loke, P., and Allison, J. P. (2003). PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proc Natl Acad Sci U S A* 100, 5336-5341.
- Loke, P., MacDonald, A. S., and Allen, J. E. (2000a). Antigen-presenting cells recruited by *Brugia malayi* induce Th2 differentiation of naive CD4(+) T cells. *Eur J Immunol* 30, 1127-1135.
- Loke, P., MacDonald, A. S., Robb, A., Maizels, R. M., and Allen, J. E. (2000b). Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell-to-cell contact. *Eur J Immunol* 30, 2669-2678.
- Loke, P., Nair, M. G., Parkinson, J., Guiliano, D., Blaxter, M., and Allen, J. E. (2002). IL-4 dependent alternatively-activated macrophages have a distinctive *in vivo* gene expression phenotype. *BMC Immunol* 3, 7.
- Luhder, F., Huang, Y., Dennehy, K. M., Guntermann, C., Muller, I., Winkler, E., Kerkau, T., Ikemizu, S., Davis, S. J., Hanke, T., and Hunig, T. (2003). Topological requirements and signaling properties of T cell-activating, anti-CD28 antibody superagonists. *J Exp Med* 197, 955-966.

- Lundgren, A., Suri-Payer, E., Enarsson, K., Svennerholm, A. M., and Lundin, B. S. (2003). *Helicobacter pylori*-specific CD4⁺ CD25^{high} regulatory T cells suppress memory T-cell responses to *H. pylori* in infected individuals. *Infect Immun* 71, 1755-1762.
- Lundy, S. K., Lerman, S. P., and Boros, D. L. (2001). Soluble egg antigen-stimulated T helper lymphocyte apoptosis and evidence for cell death mediated by FasL(+) T and B cells during murine *Schistosoma mansoni* infection. *Infect Immun* 69, 271-280.
- Lyke, K. E., Dicko, A., Dabo, A., Sangare, L., Kone, A., Coulibaly, D., Guindo, A., Traore, K., Daou, M., Diarra, I., *et al.* (2005). Association of *Schistosoma haematobium* infection with protection against acute *Plasmodium falciparum* malaria in Malian children. *Am J Trop Med Hyg* 73, 1124-1130.
- MacDonald, A. S., Maizels, R. M., Lawrence, R. A., Dransfield, I., and Allen, J. E. (1998). Requirement for *in vivo* production of IL-4, but not IL-10, in the induction of proliferative suppression by filarial parasites. *J Immunol* 160, 1304-1312.
- MacDonald, A. S., Patton, E. A., La Flamme, A. C., Araujo, M. I., Huxtable, C. R., Bauman, B., and Pearce, E. J. (2002a). Impaired Th2 development and increased mortality during *Schistosoma mansoni* infection in the absence of CD40/CD154 interaction. *J Immunol* 168, 4643-4649.
- MacDonald, A. S., Straw, A. D., Dalton, N. M., and Pearce, E. J. (2002b). Cutting edge: Th2 response induction by dendritic cells: a role for CD40. *J Immunol* 168, 537-540.
- Maizels, R. M., and Yazdanbakhsh, M. (2003). Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat Rev Immunol* 3, 733-744.
- Mak, T. W., Shahinian, A., Yoshinaga, S. K., Wakeham, A., Boucher, L. M., Pintilie, M., Duncan, G., Gajewska, B. U., Gronski, M., Eriksson, U., *et al.* (2003). Costimulation through the inducible costimulator ligand is essential for both T helper and B cell functions in T cell-dependent B cell responses. *Nat Immunol* 4, 765-772.
- Malhotra, I., Ouma, J., Wamachi, A., Kioko, J., Mungai, P., Omollo, A., Elson, L., Koech, D., Kazura, J. W., and King, C. L. (1997). In utero exposure to helminth and mycobacterial antigens generates cytokine responses similar to that observed in adults. *J Clin Invest* 99, 1759-1766.

- Mandelbrot, D. A., McAdam, A. J., and Sharpe, A. H. (1999). B7-1 or B7-2 is required to produce the lymphoproliferative phenotype in mice lacking cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). *J Exp Med* 189, 435-440.
- Mangan, N. E., Fallon, R. E., Smith, P., van Rooijen, N., McKenzie, A. N., and Fallon, P. G. (2004). Helminth infection protects mice from anaphylaxis via IL-10-producing B cells. *J Immunol* 173, 6346-6356.
- Mangan, N. E., van Rooijen, N., McKenzie, A. N., and Fallon, P. G. (2006a). Helminth-modified pulmonary immune response protects mice from allergen-induced airway hyperresponsiveness. *J Immunol* 176, 138-147.
- Mangan, P. R., Harrington, L. E., O'Quinn D, B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R., and Weaver, C. T. (2006b). Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature*.
- Marie, J. C., Letterio, J. J., Gavin, M., and Rudensky, A. Y. (2005). TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med* 201, 1061-1067.
- Marshall, M. A., Jankovic, D., Maher, V. E., Sher, A., and Berzofsky, J. A. (2001). Mice infected with *Schistosoma mansoni* develop a novel non-T-lymphocyte suppressor population which inhibits virus-specific CTL induction via a soluble factor. *Microbes Infect* 3, 1051-1061.
- Mathew, R. C., and Boros, D. L. (1986). Anti-L3T4 antibody treatment suppresses hepatic granuloma formation and abrogates antigen-induced interleukin-2 production in *Schistosoma mansoni* infection. *Infect Immun* 54, 820-826.
- Mathew, R. C., Ragheb, S., and Boros, D. L. (1990). Recombinant IL-2 therapy reverses diminished granulomatous responsiveness in anti-L3T4-treated, *Schistosoma mansoni*-infected mice. *J Immunol* 144, 4356-4361.
- Matsumoto, K., Inoue, H., Nakano, T., Tsuda, M., Yoshiura, Y., Fukuyama, S., Tsushima, F., Hoshino, T., Aizawa, H., Akiba, H., *et al.* (2004). B7-DC regulates asthmatic response by an IFN-gamma-dependent mechanism. *J Immunol* 172, 2530-2541.
- McCoy, K., Camberis, M., and Gros, G. L. (1997). Protective immunity to nematode infection is induced by CTLA-4 blockade. *J Exp Med* 186, 183-187.

- McGeachy, M. J., Stephens, L. A., and Anderton, S. M. (2005). Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. *J Immunol* 175, 3025-3032.
- McGreal, E. P., Miller, J. L., and Gordon, S. (2005). Ligand recognition by antigen-presenting cell C-type lectin receptors. *Curr Opin Immunol* 17, 18-24.
- McHugh, R. S., Whitters, M. J., Piccirillo, C. A., Young, D. A., Shevach, E. M., Collins, M., and Byrne, M. C. (2002). CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16, 311-323.
- McKee, A. S., and Pearce, E. J. (2004). CD25+CD4+ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development. *J Immunol* 173, 1224-1231.
- Mendez, S., Reckling, S. K., Piccirillo, C. A., Sacks, D., and Belkaid, Y. (2004). Role for CD4(+) CD25(+) regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity. *J Exp Med* 200, 201-210.
- Metwali, A., Elliott, D., Blum, A. M., and Weinstock, J. V. (1993). Granuloma eosinophils enhance IL-5 production by lymphocytes from mice infected with *Schistosoma mansoni*. *J Immunol* 151, 7048-7056.
- Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R., and Tschopp, J. (2005). Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437, 1167-1172.
- Miceli-Richard, C., Lesage, S., Rybojad, M., Prieur, A. M., Manouvrier-Hanu, S., Hafner, R., Chamaillard, M., Zouali, H., Thomas, G., and Hugot, J. P. (2001). CARD15 mutations in Blau syndrome. *Nat Genet* 29, 19-20.
- Michel, F., Mangino, G., Attal-Bonnefoy, G., Tuosto, L., Alcover, A., Roumier, A., Olive, D., and Acuto, O. (2000). CD28 utilizes Vav-1 to enhance TCR-proximal signaling and NF-AT activation. *J Immunol* 165, 3820-3829.
- Mohrs, M., Shinkai, K., Mohrs, K., and Locksley, R. M. (2001). Analysis of type 2 immunity *in vivo* with a bicistronic IL-4 reporter. *Immunity* 15, 303-311.
- Montenegro, S. M., Miranda, P., Mahanty, S., Abath, F. G., Teixeira, K. M., Coutinho, E. M., Brinkman, J., Goncalves, I., Domingues, L. A., Domingues, A. L., *et al.* (1999). Cytokine

- production in acute versus chronic human Schistosomiasis mansoni: the cross-regulatory role of interferon-gamma and interleukin- 10 in the responses of peripheral blood mononuclear cells and splenocytes to parasite antigens. *J Infect Dis* 179, 1502-1514.
- Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136, 2348-2357.
- Mountford, A. P. (2005). Immunological aspects of schistosomiasis. *Parasite Immunol* 27, 243-246.
- Mountford, A. P., Coulson, P. S., and Wilson, R. A. (1988). Antigen localization and the induction of resistance in mice vaccinated with irradiated cercariae of *Schistosoma mansoni*. *Parasitology* 97 (Pt 1), 11-25.
- Mountford, A. P., and Trottein, F. (2004). Schistosomes in the skin: a balance between immune priming and regulation. *Trends Parasitol* 20, 221-226.
- Muhlbauer, M., Fleck, M., Schutz, C., Weiss, T., Froh, M., Blank, C., Scholmerich, J., and Hellerbrand, C. (2006). PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis. *J Hepatol*.
- Munder, M., Eichmann, K., and Modolell, M. (1998). Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. *J Immunol* 160, 5347-5354.
- Munn, D. H., Sharma, M. D., and Mellor, A. L. (2004). Ligation of B7-1/B7-2 by human CD4+ T cells triggers indoleamine 2,3-dioxygenase activity in dendritic cells. *J Immunol* 172, 4100-4110.
- Murphy, K. M., Heimberger, A. B., and Loh, D. Y. (1990). Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes *in vivo*. *Science* 250, 1720-1723.
- Murphy, K. M., and Reiner, S. L. (2002). The lineage decisions of helper T cells. *Nat Rev Immunol* 2, 933-944.
- Mwatha, J. K., Kimani, G., Kamau, T., Mbugua, G. G., Ouma, J. H., Mumo, J., Fulford, A. J., Jones, F. M., Butterworth, A. E., Roberts, M. B., and Dunne, D. W. (1998). High levels of TNF, soluble TNF receptors, soluble ICAM-1, and IFN-gamma, but low levels of IL-5, are associated with hepatosplenic disease in human schistosomiasis mansoni. *J Immunol* 160, 1992-1999.

- Myers, L., Croft, M., Kwon, B. S., Mittler, R. S., and Vella, A. T. (2005). Peptide-specific CD8 T regulatory cells use IFN-gamma to elaborate TGF-beta-based suppression. *J Immunol* 174, 7625-7632.
- Nair, M. G., Guild, K. J., and Artis, D. (2006). Novel effector molecules in type 2 inflammation: lessons drawn from helminth infection and allergy. *J Immunol* 177, 1393-1399.
- Nascimento-Carvalho, C. M., and Moreno-Carvalho, O. A. (2005). Neuroschistosomiasis due to *Schistosoma mansoni*: a review of pathogenesis, clinical syndromes and diagnostic approaches. *Rev Inst Med Trop Sao Paulo* 47, 179-184.
- Nasta, F., Ubaldi, V., Pace, L., Doria, G., and Pioli, C. (2006). Cytotoxic T-lymphocyte antigen-4 inhibits GATA-3 but not T-bet mRNA expression during T helper cell differentiation. *Immunology* 117, 358-367.
- Nicolson, K. S., O'Neill, E. J., Sundstedt, A., Streeter, H. B., Minaee, S., and Wraith, D. C. (2006). Antigen-induced il-10+ regulatory T cells are independent of CD25+ regulatory cells for their growth, differentiation, and function. *J Immunol* 176, 5329-5337.
- Nielsen, C., Hansen, D., Husby, S., Jacobsen, B. B., and Lillevang, S. T. (2003). Association of a putative regulatory polymorphism in the PD-1 gene with susceptibility to type 1 diabetes. *Tissue Antigens* 62, 492-497.
- Nishimura, H., Honjo, T., and Minato, N. (2000). Facilitation of beta selection and modification of positive selection in the thymus of PD-1-deficient mice. *J Exp Med* 191, 891-898.
- Nishimura, H., Minato, N., Nakano, T., and Honjo, T. (1998). Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. *Int Immunol* 10, 1563-1572.
- Nishimura, H., Nose, M., Hiai, H., Minato, N., and Honjo, T. (1999). Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 11, 141-151.
- Nishimura, H., Okazaki, T., Tanaka, Y., Nakatani, K., Hara, M., Matsumori, A., Sasayama, S., Mizoguchi, A., Hiai, H., Minato, N., and Honjo, T. (2001). Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 291, 319-322.
- Noble, A., Giorgini, A., and Leggat, J. A. (2006). Cytokine-induced IL-10-secreting CD8 T cells represent a phenotypically distinct suppressor T-cell lineage. *Blood* 107, 4475-4483.

- Nurieva, R. I., Mai, X. M., Forbush, K., Bevan, M. J., and Dong, C. (2003). B7h is required for T cell activation, differentiation, and effector function. *Proc Natl Acad Sci U S A* 100, 14163-14168.
- O'Connor, R. A., Jenson, J. S., and Devaney, E. (2000). NO contributes to proliferative suppression in a murine model of filariasis. *Infect Immun* 68, 6101-6107.
- O'Garra, A., and Vieira, P. (2004). Regulatory T cells and mechanisms of immune system control. *Nat Med* 10, 801-805.
- Oderup, C., Cederbom, L., Makowska, A., Cilio, C. M., and Ivars, F. (2006). Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology* 118, 240-249.
- Oflazoglu, E., Swart, D. A., Anders-Bartholo, P., Jessup, H. K., Norment, A. M., Lawrence, W. A., Brasel, K., Tocker, J. E., Horan, T., Welcher, A. A., and Fitzpatrick, D. R. (2004). Paradoxical role of programmed death-1 ligand 2 in Th2 immune responses *in vitro* and in a mouse asthma model *in vivo*. *Eur J Immunol* 34, 3326-3336.
- Ogura, Y., Bonen, D. K., Inohara, N., Nicolae, D. L., Chen, F. F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R. H., *et al.* (2001). A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606.
- Ohigashi, Y., Sho, M., Yamada, Y., Tsurui, Y., Hamada, K., Ikeda, N., Mizuno, T., Yoriki, R., Kashizuka, H., Yane, K., *et al.* (2005). Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. *Clin Cancer Res* 11, 2947-2953.
- Okazaki, T., and Honjo, T. (2006). The PD-1-PD-L pathway in immunological tolerance. *Trends Immunol* 27, 195-201.
- Okazaki, T., Maeda, A., Nishimura, H., Kurosaki, T., and Honjo, T. (2001). PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Natl Acad Sci U S A* 98, 13866-13871.
- Onizuka, S., Tawara, I., Shimizu, J., Sakaguchi, S., Fujita, T., and Nakayama, E. (1999). Tumor rejection by *in vivo* administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res* 59, 3128-3133.

- Ono, M., Shimizu, J., Miyachi, Y., and Sakaguchi, S. (2006). Control of autoimmune myocarditis and multiorgan inflammation by glucocorticoid-induced TNF receptor family-related protein(high), Foxp3-expressing CD25+ and CD25- regulatory T cells. *J Immunol* 176, 4748-4756.
- Oosterwegel, M. A., Mandelbrot, D. A., Boyd, S. D., Lorscheid, R. B., Jarrett, D. Y., Abbas, A. K., and Sharpe, A. H. (1999). The role of CTLA-4 in regulating Th2 differentiation. *J Immunol* 163, 2634-2639.
- Orabona, C., Grohmann, U., Belladonna, M. L., Fallarino, F., Vacca, C., Bianchi, R., Bozza, S., Volpi, C., Salomon, B. L., Fioretti, M. C., *et al.* (2004). CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. *Nat Immunol* 5, 1134-1142.
- Oswald-Richter, K., Grill, S. M., Shariat, N., Leelawong, M., Sundrud, M. S., Haas, D. W., and Unutmaz, D. (2004). HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS Biol* 2, E198.
- Owyang, A. M., Zaph, C., Wilson, E. H., Guild, K. J., McClanahan, T., Miller, H. R., Cua, D. J., Goldschmidt, M., Hunter, C. A., Kastelein, R. A., and Artis, D. (2006). Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. *J Exp Med* 203, 843-849.
- Ozkaynak, E., Wang, L., Goodearl, A., McDonald, K., Qin, S., O'Keefe, T., Duong, T., Smith, T., Gutierrez-Ramos, J. C., Rottman, J. B., *et al.* (2002). Programmed death-1 targeting can promote allograft survival. *J Immunol* 169, 6546-6553.
- Pace, L., Rizzo, S., Palombi, C., Brombacher, F., and Doria, G. (2006). Cutting edge: IL-4-induced protection of CD4+CD25- Th cells from CD4+CD25+ regulatory T cell-mediated suppression. *J Immunol* 176, 3900-3904.
- Palanivel, V., Posey, C., Harauf, A. M., Solbach, W., Piessens, W. F., and Harn, D. A. (1996). B-cell outgrowth and ligand-specific production of IL-10 correlate with Th2 dominance in certain parasitic diseases. *Exp Parasitol* 84, 168-177.
- Pandey, P., Gartner, D., Soezner, O., Radbruch, A., Schulze-Osthoff, K., and Brunner-Weinzierl, M. C. (2004). CD152 (CTLA-4) determines the unequal resistance of Th1 and Th2 cells against activation-induced cell death by a mechanism requiring PI3 kinase function. *J Exp Med* 199, 831-842.

- Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., and Dong, C. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6, 1133-1141.
- Pasare, C., and Medzhitov, R. (2004). Toll-like receptors: linking innate and adaptive immunity. *Microbes Infect* 6, 1382-1387.
- Patz, J. A., Graczyk, T. K., Geller, N., and Vittor, A. Y. (2000). Effects of environmental change on emerging parasitic diseases. *Int J Parasitol* 30, 1395-1405.
- Paust, S., Lu, L., McCarty, N., and Cantor, H. (2004). Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc Natl Acad Sci U S A* 101, 10398-10403.
- Pearce, E. J. (2003). Progress towards a vaccine for schistosomiasis. *Acta Trop* 86, 309-313.
- Pearce, E. J., Caspar, P., Grzych, J. M., Lewis, F. A., and Sher, A. (1991). Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. *J Exp Med* 173, 159-166.
- Pearce, E. J., and MacDonald, A. S. (2002.). The immunobiology of Schistosomiasis. *Nature Reviews Immunology* 2, 499-511.
- Pemberton, A. D., Knight, P. A., Wright, S. H., and Miller, H. R. (2004). Proteomic analysis of mouse jejunal epithelium and its response to infection with the intestinal nematode, *Trichinella spiralis*. *Proteomics* 4, 1101-1108.
- Phan, G. Q., Yang, J. C., Sherry, R. M., Hwu, P., Topalian, S. L., Schwartzentruber, D. J., Restifo, N. P., Haworth, L. R., Seipp, C. A., Freezer, L. J., *et al.* (2003). Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A* 100, 8372-8377.
- Phillips, J. H., Takeshita, T., Sugamura, K., and Lanier, L. L. (1989). Activation of natural killer cells via the p75 interleukin 2 receptor. *J Exp Med* 170, 291-296.
- Pistillo, M. P., Tazzari, P. L., Palmisano, G. L., Pierri, I., Bolognesi, A., Ferlito, F., Capanni, P., Polito, L., Ratta, M., Pileri, S., *et al.* (2003). CTLA-4 is not restricted to the lymphoid cell lineage and can function as a target molecule for apoptosis induction of leukemic cells. *Blood* 101, 202-209.

- Powrie, F., Carlino, J., Leach, M. W., Mauze, S., and Coffman, R. L. (1996). A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. *J Exp Med* 183, 2669-2674.
- Powrie, F., Leach, M. W., Mauze, S., Caddle, L. B., and Coffman, R. L. (1993). Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int Immunol* 5, 1461-1471.
- Probst, H. C., McCoy, K., Okazaki, T., Honjo, T., and van den Broek, M. (2005). Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol* 6, 280-286.
- Prokunina, L., Castillejo-Lopez, C., Oberg, F., Gunnarsson, I., Berg, L., Magnusson, V., Brookes, A. J., Tentler, D., Kristjansdottir, H., Grondal, G., *et al.* (2002). A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. *Nat Genet* 32, 666-669.
- Quinnell, R. J., Pritchard, D. I., Raiko, A., Brown, A. P., and Shaw, M. A. (2004). Immune responses in human necatoriasis: association between interleukin-5 responses and resistance to reinfection. *J Infect Dis* 190, 430-438.
- Rabello, A. (1995). Acute human schistosomiasis mansonii. *Mem Inst Oswaldo Cruz* 90, 277-280.
- Raes, G., De Baetselier, P., Noel, W., Beschin, A., Brombacher, F., and Hassanzadeh Gh, G. (2002). Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J Leukoc Biol* 71, 597-602.
- Ravetch, J. V., and Lanier, L. L. (2000). Immune inhibitory receptors. *Science* 290, 84-89.
- Read, S., Malmstrom, V., and Powrie, F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 192, 295-302.
- Reiman, R. M., Thompson, R. W., Feng, C. G., Hari, D., Knight, R., Cheever, A. W., Rosenberg, H. F., and Wynn, T. A. (2006). Interleukin-5 (IL-5) augments the progression of liver fibrosis by regulating IL-13 activity. *Infect Immun* 74, 1471-1479.
- Reinhardt, R. L., Kang, S. J., Liang, H. E., and Locksley, R. M. (2006). T helper cell effector fates--who, how and where? *Curr Opin Immunol* 18, 271-277.

- Rifa'i, M., Kawamoto, Y., Nakashima, I., and Suzuki, H. (2004). Essential roles of CD8+CD122+ regulatory T cells in the maintenance of T cell homeostasis. *J Exp Med* 200, 1123-1134.
- Roberts, M., Butterworth, A. E., Kimani, G., Kamau, T., Fulford, A. J., Dunne, D. W., Ouma, J. H., and Sturrock, R. F. (1993). Immunity after treatment of human schistosomiasis: association between cellular responses and resistance to reinfection. *Infect Immun* 61, 4984-4993.
- Robinson, P. W., Green, S. J., Carter, C., Coadwell, J., and Kilshaw, P. J. (2001). Studies on transcriptional regulation of the mucosal T-cell integrin α E β 7 (CD103). *Immunology* 103, 146-154.
- Rodrigues, V., Jr., Piper, K., Couissinier-Paris, P., Bacelar, O., Dessein, H., and Dessein, A. J. (1999). Genetic control of schistosome infections by the SM1 locus of the 5q31-q33 region is linked to differentiation of type 2 helper T lymphocytes. *Infect Immun* 67, 4689-4692.
- Rosa Brunet, L., Finkelman, F. D., Cheever, A. W., Kopf, M. A., and Pearce, E. J. (1997). IL-4 protects against TNF- α -mediated cachexia and death during acute schistosomiasis. *J Immunol* 159, 777-785.
- Ross, A. G., Bartley, P. B., Sleigh, A. C., Olds, G. R., Li, Y., Williams, G. M., and McManus, D. P. (2002). Schistosomiasis. *N Engl J Med* 346, 1212-1220.
- Ross, A. G., Li, Y., Williams, G. M., Jiang, Z., and McManus, D. P. (2001). Dam worms. *Biologist (London)* 48, 121-124.
- Rudd, C. E., and Schneider, H. (2003). Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. *Nat Rev Immunol* 3, 544-556.
- Rumbley, C. A., Sugaya, H., Zekavat, S. A., El Refaei, M., Perrin, P. J., and Phillips, S. M. (1999). Activated eosinophils are the major source of Th2-associated cytokines in the schistosome granuloma. *J Immunol* 162, 1003-1009.
- Rutitzky, L. I., Hernandez, H. J., and Stadecker, M. J. (2001). Th1-polarizing immunization with egg antigens correlates with severe exacerbation of immunopathology and death in schistosome infection. *Proc Natl Acad Sci U S A* 98, 13243-13248.
- Rutitzky, L. I., Hernandez, H. J., Yim, Y. S., Ricklan, D. E., Finger, E., Mohan, C., Peter, I., Wakeland, E. K., and Stadecker, M. J. (2005a). Enhanced egg-induced immunopathology

- correlates with high IFN-gamma in murine schistosomiasis: identification of two epistatic genetic intervals. *J Immunol* 174, 435-440.
- Rutitzky, L. I., Lopes da Rosa, J. R., and Stadecker, M. J. (2005b). Severe CD4 T cell-mediated immunopathology in murine schistosomiasis is dependent on IL-12p40 and correlates with high levels of IL-17. *J Immunol* 175, 3920-3926.
- Rutitzky, L. I., Ozkaynak, E., Rottman, J. B., and Stadecker, M. J. (2003). Disruption of the ICOS-B7RP-1 Costimulatory Pathway Leads to Enhanced Hepatic Immunopathology and Increased Gamma Interferon Production by CD4 T Cells in Murine Schistosomiasis. *Infect Immun* 71, 4040-4044.
- Sabah, A. A., Fletcher, C., Webbe, G., and Doenhoff, M. J. (1986). *Schistosoma mansoni*: chemotherapy of infections of different ages. *Exp Parasitol* 61, 294-303.
- Sabin, E. A., Kopf, M. A., and Pearce, E. J. (1996). *Schistosoma mansoni* egg-induced early IL-4 production is dependent upon IL-5 and eosinophils. *J Exp Med* 184, 1871-1878.
- Sabin, E. A., and Pearce, E. J. (1995). Early IL-4 production by non-CD4+ cells at the site of antigen deposition predicts the development of a T helper 2 cell response to *Schistosoma mansoni* eggs. *J Immunol* 155, 4844-4853.
- Sadler, C. H., Rutitzky, L. I., Stadecker, M. J., and Wilson, R. A. (2003). IL-10 is crucial for the transition from acute to chronic disease state during infection of mice with *Schistosoma mansoni*. *Eur J Immunol* 33, 880-888.
- Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155, 1151-1164.
- Salama, A. D., Chitnis, T., Imitola, J., Ansari, M. J., Akiba, H., Tushima, F., Azuma, M., Yagita, H., Sayegh, M. H., and Khoury, S. J. (2003). Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J Exp Med* 198, 71-78.
- Salomon, B., and Bluestone, J. A. (2001). Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu Rev Immunol* 19, 225-252.

- Salomon, B., Lenschow, D. J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A., and Bluestone, J. A. (2000). B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12, 431-440.
- Satoguina, J., Mempel, M., Larbi, J., Badusche, M., Loliger, C., Adjei, O., Gachelin, G., Fleischer, B., and Hoerauf, A. (2002). Antigen-specific T regulatory-1 cells are associated with immunosuppression in a chronic helminth infection (onchocerciasis). *Microbes Infect* 4, 1291-1300.
- Schwartz, R. H. (2003). T cell anergy. *Annu Rev Immunol* 21, 305-334.
- Sedy, J. R., Gavrieli, M., Potter, K. G., Hurchla, M. A., Lindsley, R. C., Hildner, K., Scheu, S., Pfeffer, K., Ware, C. F., Murphy, T. L., and Murphy, K. M. (2005). B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. *Nat Immunol* 6, 90-98.
- Sharpe, A. H., and Freeman, G. J. (2002). The B7-CD28 superfamily. *Nat Rev Immunol* 2, 116-126.
- Sher, A., and Coffman, R. L. (1992). Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annu Rev Immunol* 10, 385-409.
- Sher, A., Gazzinelli, R. T., Oswald, I. P., Clerici, M., Kullberg, M., Pearce, E. J., Berzofsky, J. A., Mosmann, T. R., James, S. L., and Morse, H. C. d. (1992). Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. *Immunol Rev* 127, 183-204.
- Shevach, E. M. (2002). CD4+CD25+ Suppressor T cells: more questions than answers. *Nature Reviews: Immunology* 2, 389-400.
- Shimizu, J., Yamazaki, S., and Sakaguchi, S. (1999). Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 163, 5211-5218.
- Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y., and Sakaguchi, S. (2002). Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 3, 135-142.
- Shin, T., Kennedy, G., Gorski, K., Tsuchiya, H., Koseki, H., Azuma, M., Yagita, H., Chen, L., Powell, J., Pardoll, D., and Housseau, F. (2003). Cooperative B7-1/2 (CD80/CD86) and B7-DC Costimulation of CD4+ T Cells Independent of the PD-1 Receptor. *J Exp Med* 198, 31-38.

- Shin, T., Yoshimura, K., Crafton, E. B., Tsuchiya, H., Housseau, F., Koseki, H., Schulick, R. D., Chen, L., and Pardoll, D. M. (2005). *In vivo* costimulatory role of B7-DC in tuning T helper cell 1 and cytotoxic T lymphocyte responses. *J Exp Med* 201, 1531-1541.
- Shlapatska, L. M., Mikhalap, S. V., Berdova, A. G., Zelensky, O. M., Yun, T. J., Nichols, K. E., Clark, E. A., and Sidorenko, S. P. (2001). CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. *J Immunol* 166, 5480-5487.
- Sica, G. L., Choi, I. H., Zhu, G., Tamada, K., Wang, S. D., Tamura, H., Chapoval, A. I., Flies, D. B., Bajorath, J., and Chen, L. (2003). B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity* 18, 849-861.
- Siddiqui, A. A., Pinkston, J. R., Quinlin, M. L., Kavikondala, V., Rewers-Felkins, K. A., Phillips, T., and Pompa, J. (2005). Characterization of protective immunity induced against *Schistosoma mansoni* via DNA priming with the large subunit of calpain (Sm-p80) in the presence of genetic adjuvants. *Parasite* 12, 3-8.
- Singh, K. P., Gerard, H. C., Hudson, A. P., Reddy, T. R., and Boros, D. L. (2005). Retroviral Foxp3 gene transfer ameliorates liver granuloma pathology in *Schistosoma mansoni* infected mice. *Immunology* 114, 410-417.
- Smith, P., Walsh, C. M., Mangan, N. E., Fallon, R. E., Sayers, J. R., McKenzie, A. N., and Fallon, P. G. (2004). *Schistosoma mansoni* worms induce anergy of T cells via selective up-regulation of programmed death ligand 1 on macrophages. *J Immunol* 173, 1240-1248.
- Smith, T. J., Ducharme, L. A., Shaw, S. K., Parker, C. M., Brenner, M. B., Kilshaw, P. J., and Weis, J. H. (1994). Murine M290 integrin expression modulated by mast cell activation. *Immunity* 1, 393-403.
- Soisson, L. A., Reid, G. D., Farah, I. O., Nyindo, M., and Strand, M. (1993). Protective immunity in baboons vaccinated with a recombinant antigen or radiation-attenuated cercariae of *Schistosoma mansoni* is antibody-dependent. *J Immunol* 151, 4782-4789.
- Stadecker, M. J. (1999). The regulatory role of the antigen-presenting cell in the development of hepatic immunopathology during infection with *Schistosoma mansoni*. *Pathobiology* 67, 269-272.

- Stadecker, M. J., Kamisato, J. K., and Chikunguwo, S. M. (1990). Induction of T helper cell unresponsiveness to antigen by macrophages from schistosomal egg granulomas. A basis for immunomodulation in schistosomiasis? *J Immunol* 145, 2697-2700.
- Steel, C., and Nutman, T. B. (2003). CTLA-4 in filarial infections: implications for a role in diminished T cell reactivity. *J Immunol* 170, 1930-1938.
- Stein, M., Keshav, S., Harris, N., and Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 176, 287-292.
- Stephens, L. A., and Anderton, S. M. (2006). Comment on "Cutting Edge: Anti-CD25 Monoclonal Antibody Injection Results in the Functional Inactivation, Not Depletion, of CD4+CD25+ T Regulatory Cells". *J Immunol* 177, 2036.
- Stephens, L. A., Gray, D., and Anderton, S. M. (2005). CD4+CD25+ regulatory T cells limit the risk of autoimmune disease arising from T cell receptor crossreactivity. *Proc Natl Acad Sci U S A* 102, 17418-17423.
- Straw, A. D., MacDonald, A. S., Denkers, E. Y., and Pearce, E. J. (2003). CD154 plays a central role in regulating dendritic cell activation during infections that induce Th1 or Th2 responses. *J Immunol* 170, 727-734.
- Strickland, G. T. (2006). Liver disease in Egypt: hepatitis C superseded schistosomiasis as a result of iatrogenic and biological factors. *Hepatology* 43, 915-922.
- Subudhi, S. K., Zhou, P., Yerian, L. M., Chin, R. K., Lo, J. C., Anders, R. A., Sun, Y., Chen, L., Wang, Y., Alegre, M. L., and Fu, Y. X. (2004). Local expression of B7-H1 promotes organ-specific autoimmunity and transplant rejection. *J Clin Invest* 113, 694-700.
- Suffia, I., Reckling, S. K., Salay, G., and Belkaid, Y. (2005). A role for CD103 in the retention of CD4+CD25+ Treg and control of *Leishmania major* infection. *J Immunol* 174, 5444-5455.
- Suh, W. K., Gajewska, B. U., Okada, H., Gronski, M. A., Bertram, E. M., Dawicki, W., Duncan, G. S., Bukczynski, J., Plyte, S., Elia, A., *et al.* (2003). The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. *Nat Immunol* 4, 899-906.
- Suh, W. K., Wang, S. X., Jheon, A. H., Moreno, L., Yoshinaga, S. K., Ganss, B., Sodek, J., Grynepas, M. D., and Mak, T. W. (2004). The immune regulatory protein B7-H3 promotes

- osteoblast differentiation and bone mineralization. *Proc Natl Acad Sci U S A* 101, 12969-12973.
- Summers, R. W., Elliott, D. E., Urban, J. F., Jr., Thompson, R. A., and Weinstock, J. V. (2005). *Nippostrongylus brasiliensis* therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterology* 128, 825-832.
- Sun, M., Richards, S., Prasad, D. V., Mai, X. M., Rudensky, A., and Dong, C. (2002). Characterization of mouse and human B7-H3 genes. *J Immunol* 168, 6294-6297.
- Suvas, S., Azkur, A. K., Kim, B. S., Kumaraguru, U., and Rouse, B. T. (2004). CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *J Immunol* 172, 4123-4132.
- Swallow, M. M., Wallin, J. J., and Sha, W. C. (1999). B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. *Immunity* 11, 423-432.
- Swartz, J. M., Dyer, K. D., Cheever, A. W., Ramalingam, T., Pesnicak, L., Domachowske, J. B., Lee, J. J., Lee, N. A., Foster, P. S., Wynn, T. A., and Rosenberg, H. F. (2006). *Schistosoma mansoni* infection in eosinophil lineage-ablated mice. *Blood*.
- Tacke, M., Hanke, G., Hanke, T., and Hunig, T. (1997). CD28-mediated induction of proliferation in resting T cells *in vitro* and *in vivo* without engagement of the T cell receptor: evidence for functionally distinct forms of CD28. *Eur J Immunol* 27, 239-247.
- Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W., and Sakaguchi, S. (2000). Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 192, 303-310.
- Takatsu, K., Takaki, S., and Hitoshi, Y. (1994). Interleukin-5 and its receptor system: implications in the immune system and inflammation. *Adv Immunol* 57, 145-190.
- Takeda, K., and Akira, S. (2005). Toll-like receptors in innate immunity. *Int Immunol* 17, 1-14.
- Tang, Q., Boden, E. K., Henriksen, K. J., Bour-Jordan, H., Bi, M., and Bluestone, J. A. (2004). Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. *Eur J Immunol* 34, 2996-3005.

- Tang, Q., Henriksen, K. J., Boden, E. K., Tooley, A. J., Ye, J., Subudhi, S. K., Zheng, X. X., Strom, T. B., and Bluestone, J. A. (2003a). Cutting edge: CD28 controls peripheral homeostasis of CD4⁺CD25⁺ regulatory T cells. *J Immunol* 171, 3348-3352.
- Tang, Q., Smith, J. A., Szot, G. L., Zhou, P., Alegre, M. L., Henriksen, K. J., Thompson, C. B., and Bluestone, J. A. (2003b). CD28/B7 regulation of anti-CD3-mediated immunosuppression *in vivo*. *J Immunol* 170, 1510-1516.
- Tato, C.M., Laurence, A., O' Shea, J.J. (2006). Helper T cell differentiation enters a new era: le roi est mort; vive le roi! *J Exp Med* 203, 809-812.
- Taylor, J. J., Mohrs, M., and Pearce, E. J. (2006a). Regulatory T cell responses develop in parallel to th responses and control the magnitude and phenotype of the th effector populatio. *J Immunol* 176, 5839-5847.
- Taylor, M. D., Harris, A., Nair, M. G., Maizels, R. M., and Allen, J. E. (2006b). F4/80+ alternatively activated macrophages control CD4⁺ T cell hyporesponsiveness at sites peripheral to filarial infection. *J Immunol* 176, 6918-6927.
- Taylor, M. D., LeGoff, L., Harris, A., Malone, E., Allen, J. E., and Maizels, R. M. (2005). Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance *in vivo*. *J Immunol* 174, 4924-4933.
- Taylor, P. A., Lees, C. J., Fournier, S., Allison, J. P., Sharpe, A. H., and Blazar, B. R. (2004). B7 expression on T cells down-regulates immune responses through CTLA-4 ligation via T-T_H interactions [corrections]. *J Immunol* 172, 34-39.
- Teft, W. A., Kirchhof, M. G., and Madrenas, J. (2006). A molecular perspective of CTLA-4 function. *Annu Rev Immunol* 24, 65-97.
- Terrazas, L. I., Montero, D., Terrazas, C. A., Reyes, J. L., and Rodriguez-Sosa, M. (2005). Role of the programmed Death-1 pathway in the suppressive activity of alternatively activated macrophages in experimental cysticercosis. *Int J Parasitol* 35, 1349-1358.
- Terrazas, L. I., Walsh, K. L., Piskorska, D., McGuire, E., and Harn, D. A. J. (2001). The schistosome oligosaccharide lacto-N-neotetraose expands Gr1(+) cells that secrete anti-inflammatory cytokines and inhibit proliferation of naive CD4(+) cells: a potential mechanism for immune polarization in helminth infections. *J Immunol* 167, 5294-5303.
- Thomas, H., and Gonnert, R. (1977). The efficacy of praziquantel against cestodes in animals. *Z Parasitenkd* 52, 117-127.

- Thomas, P. G., Carter, M. R., Atochina, O., Da'Dara, A. A., Piskorska, D., McGuire, E., and Harn, D. A. (2003). Maturation of dendritic cell 2 phenotype by a helminth glycan uses a Toll-like receptor 4-dependent mechanism. *J Immunol* 171, 5837-5841.
- Thompson, R. H., Gillett, M. D., Cheville, J. C., Lohse, C. M., Dong, H., Webster, W. S., Krejci, K. G., Lobo, J. R., Sengupta, S., Chen, L., *et al.* (2004). Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. *Proc Natl Acad Sci U S A* 101, 17174-17179.
- Thornton, A. M., and Shevach, E. M. (1998). CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J Exp Med* 188, 287-296.
- Ting, J. P., and Davis, B. K. (2005). CATERPILLER: a novel gene family important in immunity, cell death, and diseases. *Annu Rev Immunol* 23, 387-414.
- Tivol, E. A., Borriello, F., Schweitzer, A. N., Lynch, W. P., Bluestone, J. A., and Sharpe, A. H. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3, 541-547.
- Tone, M., Tone, Y., Adams, E., Yates, S. F., Frewin, M. R., Cobbold, S. P., and Waldmann, H. (2003). Mouse glucocorticoid-induced tumor necrosis factor receptor ligand is costimulatory for T cells. *Proc Natl Acad Sci U S A* 100, 15059-15064.
- Tran, M. H., Pearson, M. S., Bethony, J. M., Smyth, D. J., Jones, M. K., Duke, M., Don, T. A., McManus, D. P., Correa-Oliveira, R., and Loukas, A. (2006). Tetraspanins on the surface of *Schistosoma mansoni* are protective antigens against schistosomiasis. *Nat Med* 12, 835-840.
- Tschopp, J., Martinon, F., and Burns, K. (2003). NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol* 4, 95-104.
- Tseng, S. Y., Otsuji, M., Gorski, K., Huang, X., Slansky, J. E., Pai, S. I., Shalabi, A., Shin, T., Pardoll, D. M., and Tsuchiya, H. (2001). B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med* 193, 839-846.
- Tsushima, F., Iwai, H., Otsuki, N., Abe, M., Hirose, S., Yamazaki, T., Akiba, H., Yagita, H., Takahashi, Y., Omura, K., *et al.* (2003). Preferential contribution of B7-H1 to programmed death-1-mediated regulation of hapten-specific allergic inflammatory responses. *Eur J Immunol* 33, 2773-2782.

- Ueda, H., Howson, J. M., Esposito, L., Heward, J., Snook, H., Chamberlain, G., Rainbow, D. B., Hunter, K. M., Smith, A. N., Di Genova, G., *et al.* (2003). Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 423, 506-511.
- van der Kleij, D., Latz, E., Brouwers, J. F., Kruize, Y. C., Schmitz, M., Kurt-Jones, E. A., Espevik, T., de Jong, E. C., Kapsenberg, M. L., Golenbock, D. T., *et al.* (2002). A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *J Biol Chem* 277, 48122-48129.
- van der Merwe, P. A., and Davis, S. J. (2003). Molecular interactions mediating T cell antigen recognition. *Annu Rev Immunol* 21, 659-684.
- van der Werf, M. J., de Vlas, S. J., Brooker, S., Looman, C. W., Nagelkerke, N. J., Habbema, J. D., and Engels, D. (2003). Quantification of clinical morbidity associated with schistosome infection in sub-Saharan Africa. *Acta Trop* 86, 125-139.
- van Die, I., van Vliet, S. J., Nyame, A. K., Cummings, R. D., Bank, C. M., Appelmelk, B., Geijtenbeek, T. B., and van Kooyk, Y. (2003). The dendritic cell-specific C-type lectin DC-SIGN is a receptor for *Schistosoma mansoni* egg antigens and recognizes the glycan antigen Lewis x. *Glycobiology* 13, 471-478.
- van Wijk, F., Hoeks, S., Nierkens, S., Koppelman, S. J., van Kooten, P., Boon, L., Knippels, L. M., and Pieters, R. (2005). CTLA-4 signaling regulates the intensity of hypersensitivity responses to food antigens, but is not decisive in the induction of sensitization. *J Immunol* 174, 174-179.
- Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., and Stockinger, B. (2006). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24, 179-189.
- Verjovski-Almeida, S., DeMarco, R., Martins, E. A., Guimaraes, P. E., Ojopi, E. P., Paquola, A. C., Piazza, J. P., Nishiyama, M. Y., Jr., Kitajima, J. P., Adamson, R. E., *et al.* (2003). Transcriptome analysis of the acoelomate human parasite *Schistosoma mansoni*. *Nat Genet* 35, 148-157.
- Vijayakrishnan, L., Slavik, J. M., Illes, Z., Greenwald, R. J., Rainbow, D., Greve, B., Peterson, L. B., Hafler, D. A., Freeman, G. J., Sharpe, A. H., *et al.* (2004). An autoimmune disease-associated CTLA-4 splice variant lacking the B7 binding domain signals negatively in T cells. *Immunity* 20, 563-575.

- Viola, A., Schroeder, S., Sakakibara, Y., and Lanzavecchia, A. (1999). T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* 283, 680-682.
- Von Lichtenberg, F. (1985). Conference on contended issues of immunity to schistosomes. *Am J Trop Med Hyg* 34, 78-85.
- Walunas, T. L., Lenschow, D. J., Bakker, C. Y., Linsley, P. S., Freeman, G. J., Green, J. M., Thompson, C. B., and Bluestone, J. A. (1994). CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1, 405-413.
- Wan, Y. Y., and Flavell, R. A. (2005). Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc Natl Acad Sci U S A* 102, 5126-5131.
- Wang, S., Bajorath, J., Flies, D. B., Dong, H., Honjo, T., and Chen, L. (2003). Molecular Modeling and Functional Mapping of B7-H1 and B7-DC Uncouple Costimulatory Function from PD-1 Interaction. *J Exp Med* 197, 1083-1091.
- Warren, K. S. (1964). Correlation between Experimental and Human Infection with *Schistosoma mansoni*. *Nature* 201, 899-901.
- Watanabe, N., Gavrieli, M., Sedy, J. R., Yang, J., Fallarino, F., Loftin, S. K., Hurchla, M. A., Zimmerman, N., Sim, J., Zang, X., *et al.* (2003). BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol* 4, 670-679.
- Waterhouse, P., Penninger, J. M., Timms, E., Wakeham, A., Shahinian, A., Lee, K. P., Thompson, C. B., Griesser, H., and Mak, T. W. (1995). Lymphoproliferative disorders with early lethality in mice deficient in Ctl4-4. *Science* 270, 985-988.
- Watts, T. H. (2005). TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol* 23, 23-68.
- Webbe, G., and Lambert, J. D. (1983). Plants that kill snails and prospects for disease control. *Nature* 302, 754.
- Weiss, L., Donkova-Petrini, V., Caccavelli, L., Balbo, M., Carbonneil, C., and Levy, Y. (2004). Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood* 104, 3249-3256.
- Whitty, C. J., Mabey, D. C., Armstrong, M., Wright, S. G., and Chiodini, P. L. (2000). Presentation and outcome of 1107 cases of schistosomiasis from Africa diagnosed in a non-endemic country. *Trans R Soc Trop Med Hyg* 94, 531-534.

- WHO (2002). TDR Strategic direction for research. Disease TDR Strategic direction for research. Disease burden and epidemiological trends.
- William, S., Botros, S., Ismail, M., Farghally, A., Day, T. A., and Bennett, J. L. (2001). Praziquantel-induced tegumental damage *in vitro* is diminished in schistosomes derived from praziquantel-resistant infections. *Parasitology* 122 Pt 1, 63-66.
- Williams, M. E., Caspar, P., Oswald, I., Sharma, H. K., Pankewycz, O., Sher, A., and James, S. L. (1995). Vaccination routes that fail to elicit protective immunity against *Schistosoma mansoni* induce the production of TGF-beta, which down-regulates macrophage antiparasitic activity. *J Immunol* 154, 4693-4700.
- Wong, S. C., Oh, E., Ng, C. H., and Lam, K. P. (2003). Impaired germinal center formation and recall T-cell-dependent immune responses in mice lacking the costimulatory ligand B7-H2. *Blood* 102, 1381-1388.
- Wynn, T. A., Cheever, A. W., Jankovic, D., Poindexter, R. W., Caspar, P., Lewis, F. A., and Sher, A. (1995). An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. *Nature* 376, 594-596.
- Wynn, T. A., Cheever, A. W., Williams, M. E., Hieny, S., Caspar, P., Kuhn, R., Muller, W., and Sher, A. (1998). IL-10 regulates liver pathology in acute murine Schistosomiasis mansoni but is not required for immune down-modulation of chronic disease. *J Immunol* 160, 4473-4480.
- Wynn, T. A., Reynolds, A., James, S., Cheever, A. W., Caspar, P., Hieny, S., Jankovic, D., Strand, M., and Sher, A. (1996). IL-12 enhances vaccine-induced immunity to schistosomes by augmenting both humoral and cell-mediated immune responses against the parasite. *J Immunol* 157, 4068-4078.
- Wynn, T. A., Thompson, R. W., Cheever, A. W., and Mentink-Kane, M. M. (2004). Immunopathogenesis of schistosomiasis. *Immunol Rev* 201, 156-167.
- Xiao, S. H., Booth, M., and Tanner, M. (2000). The prophylactic effects of artemether against *Schistosoma japonicum* infections. *Parasitol Today* 16, 122-126.
- Yamazaki, T., Akiba, H., Iwai, H., Matsuda, H., Aoki, M., Tanno, Y., Shin, T., Tsuchiya, H., Pardoll, D. M., Okumura, K., *et al.* (2002). Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol* 169, 5538-5545.

- Yamazaki, T., Akiba, H., Koyanagi, A., Azuma, M., Yagita, H., and Okumura, K. (2005). Blockade of B7-H1 on macrophages suppresses CD4+ T cell proliferation by augmenting IFN-gamma-induced nitric oxide production. *J Immunol* 175, 1586-1592.
- Yap, G., Cheever, A., Caspar, P., Jankovic, D., and Sher, A. (1997). Unimpaired down-modulation of the hepatic granulomatous response in CD8 T-cell- and gamma interferon-deficient mice chronically infected with *Schistosoma mansoni*. *Infect Immun* 65, 2583-2586.
- Yole, D. S., Pemberton, R., Reid, G. D., and Wilson, R. A. (1996). Protective immunity to *Schistosoma mansoni* induced in the olive baboon *Papio anubis* by the irradiated cercaria vaccine. *Parasitology* 112 (Pt 1), 37-46.
- Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 5, 730-737.
- Zajac, A. J., Blattman, J. N., Murali-Krishna, K., Sourdive, D. J., Suresh, M., Altman, J. D., and Ahmed, R. (1998). Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 188, 2205-2213.
- Zang, X., Loke, P., Kim, J., Murphy, K., Waitz, R., and Allison, J. P. (2003). B7x: A widely expressed B7 family member that inhibits T cell activation. *Proc Natl Acad Sci U S A* 100, 10388-10392.
- Zelenay, S., and Demengeot, J. (2006). Comment on "Cutting Edge: Anti-CD25 Monoclonal Antibody Injection Results in the Functional Inactivation, Not Depletion, of CD4+CD25+ T Regulatory Cells". *J Immunol* 177, 2036-2037.
- Zhang, G. X., Gran, B., Yu, S., Li, J., Siglienti, I., Chen, X., Kamoun, M., and Rostami, A. (2003a). Induction of experimental autoimmune encephalomyelitis in IL-12 receptor-beta 2-deficient mice: IL-12 responsiveness is not required in the pathogenesis of inflammatory demyelination in the central nervous system. *J Immunol* 170, 2153-2160.
- Zhang, X., Reddy, J., Ochi, H., Frenkel, D., Kuchroo, V. K., and Weiner, H. L. (2006). Recovery from experimental allergic encephalomyelitis is TGF- β dependent and associated with increases in CD4+LAP+ and CD4+CD25+ T cells. *Int Immunol* 18, 495-503.
- Zhang, X., Schwartz, J. C., Almo, S. C., and Nathenson, S. G. (2003b). Crystal structure of the receptor-binding domain of human B7-2: insights into organization and signaling. *Proc Natl Acad Sci U S A* 100, 2586-2591.

- Zhang, X., Schwartz, J. C., Guo, X., Bhatia, S., Cao, E., Lorenz, M., Cammer, M., Chen, L., Zhang, Z. Y., Edidin, M. A., *et al.* (2004). Structural and functional analysis of the costimulatory receptor programmed death-1. *Immunity* 20, 337-347.
- Zheng, S. G., Wang, J. H., Koss, M. N., Quismorio, F., Jr., Gray, J. D., and Horwitz, D. A. (2004). CD4⁺ and CD8⁺ regulatory T cells generated ex vivo with IL-2 and TGF-beta suppress a stimulatory graft-versus-host disease with a lupus-like syndrome. *J Immunol* 172, 1531-1539.
- Zhou, H., Ross, A. G., Hartel, G. F., Sleight, A. C., Williams, G. M., McManus, D. P., Luo, X. S., He, Y., and Li, Y. S. (1998). Diagnosis of schistosomiasis japonica in Chinese schoolchildren by administration of a questionnaire. *Trans R Soc Trop Med Hyg* 92, 245-250.
- Zouain, C. S., Falcao, P. L., Goes, T. S., Leite, M. F., and Goes, A. M. (2004). *Schistosoma mansoni* PIII antigen modulates *in vitro* granuloma formation by regulating CD28, CTLA-4, and CD86 expression in humans. *Immunol Lett* 91, 113-118.

Appendices

Appendix 1: Buffers and solutions

All reagents are from Sigma-Aldrich, unless otherwise indicated.

Lepple water (10X)

13.9 g Calcium chloride (CaCl_2)

30.7 g Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)

1.07 g Potassium sulphate (K_2SO_4)

10.5 g Sodium hydrogen carbonate (NaHCO_3)

1.2 ml of a solution containing 2.5 g of ferric chloride in 500 ml distilled water (dH_2O)

Each chemical was dissolved in 1 L of dH_2O , mixed together, and made up to a final volume of 25 L with dH_2O . To prepare a 1X solution, 1 L of 10X Lepple water was added to 9 L dH_2O .

Hydrocortisone

625 mg Hydrocortisone 21-acetate

625 μl Cremophor E.L.

24.36 ml dH_2O

The mixture was sonicated and stored 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 ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)

Add dH_2O to 1 L.

Formal Saline

10% formaldehyde in saline (0.9% NaCl)

Culture media (complete media)

Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco)

2mM L-glutamine

10% Foetal Calf Serum (FCS; Labtech, UK)

100 U/ml Penicillin

100 $\mu\text{g/ml}$ Streptomycin

Wash media

RPMI-1640 medium

100 U/ml Penicillin

100 $\mu\text{g/ml}$ Streptomycin

Erythrocyte Lysis Buffer

0.87% ammonium chloride (NH_4Cl) in dH_2O , filter sterilised.

Phosphate Buffered Saline (PBS) (10X)

85 g Sodium Chloride (NaCl)

12.8 g Di -sodium hydrogen orthphosphate (Na_2HPO_4)

1.56 g Sodium di-hydrogen phosphate (NaH_2PO_4)

Add distilled water (dH_2O) to 1 L and adjust to pH 7.2.

To prepare a 1X solution, 1L of 10X PBS was added to 9 L of dH_2O and pH adjusted to 7.2.

Tissue Homogenisation Buffer

PBS with 2% FCS and 0.05% CTAB

(CTAB = hexadecyltrimethylammonium bromide, Sigmaultra)

FACS Buffer

PBS with 2% FCS and 0.05% sodium azide.

ELISA wash buffer

PBS with 0.05% Tween 20

Blocking buffer

1% BSA in PBS

Phosphate-citrate buffer

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

36.9 g Di-sodium hydrogen orthphosphate (Na_2HPO_4)

Dissolve in 1L dH_2O and adjust to pH 5.0.

Tris-borate EDTA (TBE, 10X)

107.8 g Tris Base

50 g Boric acid

7.44 g EDTA

Dissolve in 1 L dH₂O and adjust to pH 8.3.

A 1 X solution was prepared by diluting 1 L of the 10X TBE in 9 L dH₂O.

Appendix II: Antibodies

Flow cytometry:

Marker	Clone	Company	Dilution
<i>Surface</i>			
Rat anti-mouse CD4	CT-CD4	Caltag	1:300
Rat anti-mouse CD8 α	5H10	Caltag	1:250
Rat anti-mouse CD19	6D5	Caltag	1:300
Rat anti-mouse F4/80	F4/80	Caltag	1:100
Hamster anti-mouse CD11c	HL3	BD	1:100
Rat anti-mouse Gr1 (Ly-6G)	RB6-8C5	Caltag	1:100
Rat anti-mouse CD25	PC61 5.3	Caltag	1:100
Rat anti-mouse CD25	7D4	BD	1:100
Rat anti-mouse I-A/I-E (MHC II)	2G9	BD	1:300
Rat anti-mouse H-2k ^d (MHC I)	SFI-1.1	BD	1:200
Hamster anti-mouse CD80 (B7-1)	16-10A1	BD	1:100
Rat anti-mouse CD86	GL2	BD	1:100

(B7-2)			
Rat anti-mouse CD103	M290	BD	1:100
Rat anti-mouse CD122	TM-β1	Caltag	1:200
Rat anti-mouse GITR	108619	RnD Systems	1:10
Rat anti-mouse T1/ST2	DJ8	MD Biosciences	1:100
Rat anti-mouse LICOS	HK5.3	eBiosciences	1:100
(B7-H2)			
Hamster anti-mouse PD-1	J43	eBiosciences	1:100
Rat anti-mouse PD-1	RMPI-30	eBiosciences	1:100
Rat anti-mouse PD-L1	MIH5	eBiosciences	1:100
Rat anti-mouse PD-L2	TY25	eBiosciences	1:100
Anti-Bromodeoxyuridine (BrdU)	B44	BD	1:5
<i>Intracellular</i>			
Hamster anti-mouse CTLA-4	UC10-4F10-11	BD	1:100
Rat anti-mouse Foxp3	FJK-16s	eBioscience	1:100
Rat anti-mouse IL-4	BVD6-24G2	Caltag	1:10
Rat anti-mouse IL-10	JES5-2A5	Caltag	1:10
Rat anti-mouse IFN-γ	R4-6A2	Caltag	1:10
Rat anti-mouse IL-2	JES6-5H4	BD	1:10
Rat anti-mouse IL-5	TRFK5	BD	1:10
<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 cell culture:

Marker	Clone	Company
Rat anti-mouse CD3ε	145-2C11	BD
Rat anti-mouse CD28	37.51	BD
Rat anti-mouse PD-L1 (blocking mAb)	MIH5	eBioscience
Rat anti-mouse PD-L2 (blocking mAb)	TY25	eBioscience
Rat IgG2a κ	eBR2a	eBiosciences
Rat IgG2a λ	B39-4	BD

In vivo blocking/depletions:

Marker	Clone	Supplier
Rat anti-mouse PD-L1	MIH5	Prof. Azuma, Tokyo Medical and Dental University
Rat anti-mouse PD-L2	TY25	Dr. Yagita, Juntendo University School of Medicine, Tokyo
Rat anti-mouse PD-1	RPM1-14	Dr. Yagita, Juntendo University

		School of Medicine, Tokyo
Hamster anti-mouse CTLA-4	UC10-4F10-11	ATCC
Control Hamster IgG	UC8-1B9	ATCC
Rat anti-mouse CD25	PC61.3	ATCC
Control Rat IgG		Sigma

Appendix III

PCR Primers:

β-actin fwd	GGACTCCTATGTGGGTGACGAGG
β-actin rev	TCTTTGCCAATAGTGATGACTTGGC
Ym 1 fwd	TCACAGGTCTGGCAATTCTTCTG
Ym 1 rev	TTGTCCTTAGGAGGGCTTCCTC
Fizz 1 fwd	GGTCCCAGTGCATATGGATGAGACCATAGA
Fizz 1 rev	CACCTCTTCACTCGAGGGACAGTTGGCAGC
Arginase 1 fwd	CAGAAGAATGGAAGAGTCAG
Arginase 1 rev	CAGATATGCAGGGAGTCACC

PCR determination of female/male *S.mansoni* cercariae

W1 fwd	CAACACAGTGAAATTCTTCC
W1 rev	GAATTCACCACTCGACATTC
W2 fwd	TTGCTGATGTGCAGTTTGCC
W2 rev	TCTTCCGAGTATGATGCAGG
PDI fwd	TGAAGCCCTGAAAAAGTTTCG
PDI rev	TGCACTGTCAAATAGCACTC

Genotyping primers

4get: 59°C anneal	WT= primers 1-2 173bp, Knock-in= primers 3-4 173bp
4get.1	CCT TAT GGC CAA ATG AAG TGA
4get.2	AAA GAC TTC CTG GAA AGC CTA
4get.3	AAG TTC ATC TGC ACC ACC G
4get.4	TCC TTG AAG AAG ATG GTG CG
PD-L1: 62°C anneal	WT= primers 1-2 250 bp, KO= primers 2-3 450 bp
PD-L1.1	AGA ACG GGA GCT GGA CCT GCT TGC GTT AG
PD-L1.2	ATT GAC TTT CAG CGT GAT TCG CTT GTA G
PD-L1.3	TTC TAT CGC CTT CTT GAC GAG TTC TTC TG

List of publications to date:

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 selective upregulation of Programmed Death Ligand 1 on macrophages. *Journal of Immunology* **173**: 1240-1248.

Carroll, A.M., Haines, L.R., Pearson, T.W., Fallon, P.G., **Walsh, C.M.**, Brennan, C., Breen, E. & Porter, R.K. (2005). Identification of a Functioning Mitochondrial Uncoupling Protein 1 in Thymus. *Journal of Biological Chemistry* **280**:15534-15543.

Smith, P., Fallon, R.E., Mangan, N.E., **Walsh, C.M.**, Saraiva, M., Sayers, J.R., McKenzie, A.N., Alcamí, A. & Fallon P.G (2005). *Schistosoma mansoni* secretes a chemokine binding protein with anti-inflammatory activity. *Journal of Experimental Medicine* **202**: 1319-1325.

Walsh, C.M., Smith, P., & Fallon P.G. No role for CD25⁺ T cells in *Schistosoma mansoni* infection, whereas CTLA-4 downregulates Th2 responses in acute but not chronic infection. *European Journal of Immunology* **submitted**.

Smith, P., **Walsh, C.M.**, Mangan, N.E., Fallon, R.E., van Rooijen, N., McKenzie, A.N. Azuma, M., & Fallon, P.G., A helminth prevents experimental colitis via a macrophage dependent mechanism. *Journal of Clinical Investigation* **submitted**.