REGULATION OF IMMUNE RESPONSES BY THE L3 OF *Brugia pahangi*

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DECLARATION

The author was responsible for all the experiments carried out in this thesis, except where it is otherwise stated. No part of this work has been submitted for any other degree but has been reproduced in part in the following publication:

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SUMMARY

Lymphatic filarial worms are mosquito borne parasites which cause chronic disease in humans. Infection is characterized by proliferative suppression of T cells in the host and a skewing of the immune response, away from an IFN-γ producing Th1 response, towards a Th2 or regulatory phenotype, with production of IL-4 and IL-10. In this study, a mouse model was used to investigate the role of the infective form of the parasite (the third stage larvae, L3) in regulating immune responses. BALB/c mice were infected by the subcutaneous route to mimic the natural transmission of the parasite, and immune responses generated by the L3 were analysed.

Experiments carried out in the intact BALB/c mouse showed that infection with L3 results in increased IL-4, IL-10 and IL-5 with no IL-2 or IFN-γ, a similar situation to that observed in infected humans. In order to investigate the key components in this skewing of the immune response, experiments were carried out using IL-4-/- mice. IL-4 was shown to have a role in down-regulating Th1 responses in wild type mice, as knock-out animals produced elevated levels of IL-2 and IFN-γ. However these mice still had the capacity to produce IL-5, IL-13 and IL-10, suggesting that although IL-4 is an important Th2 cytokine, it may be dispensable in the initiation of such a response. In addition, reduced levels of proliferation of $CD4^+$ and $B220^+$ cells were observed in infected IL-4-/- mice. Despite elevated levels of IFN-γ, this reduction in proliferation was not associated with increased production of NO, and neutralizing IFN-γ itself did not restore proliferative responses. Addition of rIL-4 to cultures of splenocytes from these mice had a mild effect but did not result in a significant increase in proliferation.

Treatment of splenocytes from intact mice with anti-IL-10 MAb *in vitro* resulted in increased levels of IL-2 and IFN-γ, but no significant effect on Ag-specific proliferation was observed. However, when mice were treated with an anti-IL-10R MAb the opposite effect was seen, with a significant increase in levels of proliferation and no alteration in cytokines. When examined by FACS it was shown that $CD4^+$ cells were the major population which expanded during IL-10R blockade. In addition, $CD4^+$ cells were found to be the major source of IL-10 post-infection with L3, suggesting these cells may

modulate their own proliferation, or perhaps these cells were of a regulatory nature. Experiments were also carried out to determine the effect of natural transmission on host immune responses. L3 were administered via syringe inoculation or via mosquito transmission. Splenocytes from mice infected via syringe inoculation had an increased capacity to produce cytokines and displayed higher levels of proliferation. Further experiments demonstrated that cytokine and proliferative responses were not dose-dependant therefore there may be factors within mosquito saliva which down-regulate immune responses in the mouse model.

To further characterize the cellular source of IL-10 produced in response to infection with L3, RT-PCR was carried out. These experiments demonstrated that $CD4^+$ cells produce not only IL-10 but another regulatory cytokine TGF-β. Further phenotypic analysis of the $CD4^+$ population in L3 infected mice identified a small percentage of $CD4^+CD25^+CTLA\text{-}4^+$ cells which were not present in MF infected or control mice. These cells were IL-4 independent, as was expression of IL-10 and TGF-β mRNA. Limiting the expansion of these cells *in vivo* with the administration of an anti-CD25 MAb, resulted in elevated levels of Ag-specific and spontaneous cytokine and proliferative responses, indicating that these cells are essential for regulating immune responses to infection with L3.

The aim of the final part of this study considered the role of B cells in L3 infection. It was shown by CFSE staining that B cells were the major population which proliferate in response to restimulation with parasite Ag, and B cells were also shown to be a source of IL-10, suggesting an important function for these cells. Experiments carried out in μMT mice confirmed results from depletion experiments as levels of Ag-specific proliferation were decreased in these mice. However, B cell depletion experiments showed that levels of IFN-γ were significantly increased post-depletion, with decreased levels of Th2 cytokines and IL-10, suggesting that B cells are important in maintaining Th2 responses at least in the BALB/c mouse. This profile of cytokine responses differed from that in μMT mice. Further experiments demonstrated that B cells act as APC in this system, as most $B7\text{-}1^+$ and $B7\text{-}2^+$ cells in splenocyte cultures from L3 infected mice were also $B220^+$. B7-1 and B7-2 expression was shown to be regulated by IL-10 as neutralizing IL-10 *in vitro* or analyzing splenocytes from IL-10-/- mice resulted in

significantly increased expression of these costimulatory molecules. As B cells have been shown to be a source of IL-10, this suggests another mechanism of negative feedback, where production of IL-10 leads to down-regulation of costimulatory molecules on the surface, thus attenuating the efficiency of these cells to present Ag to $CD4^+$ cells.

LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
AMG	aminoguanidine
APC	allophycocyanin
APC	antigen presenting cell
ATP	annual transmission potential
BCR	B cell receptor
BSA	bovine serum albumin
CFA	circulating filarial antigen
CFSE	carboxyyfluuorescein diacetate succinimidyl ester
Ci	Curie
CO_2	carbon dioxide
ConA	concanavalin A
CP	chronic pathology
cpm	counts per minute
CTLA-4	cytotoxic T lymphocyte-associated antigen
DC	dendritic cell
ddH_2O	double distilled water
DEC	diethylcarazamine
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate

d.p.i.	days post infection
EAE	experimental autoimmune encephalitis
EDTA	ethylenediamine tetra acetic acid
ELISA	enzyme linked immunoabsorbent assay
EN	endemic normal
ES	excretory secretory
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
FCS	fetal calf serum
g	gram
GEO	generalised onchocerciasis
GITR	glucocorticoid-induced TNF receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hanks balanced salt solution
HCL	hydrochloric acid
ICT	immunochromatographic test
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
i.p.	intra-peritoneal
JAK	janus activating kinase
KCL	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
l	litre

L3	infective larvae
LAP	latency associated peptide
LF	lymphatic filariasis
LN	lymph node
LPS	lipopolysaccharide
M	molar
MAb	monoclonal antibody
MHC	major histocompatability complex
µCi	micro Curie
µg	microgram
µl	microlitre
ml	millilitre
µM	micromolar
mM	millimolar
mRNA	messenger RNA
MF	microfilaria
MF+	microfilaraemic
MF-	amicrofilaraemic
min	minute
NaCl	sodim cholride
$NaHCO_2$	sodium bicarbonate
Na_2HPO_4	di-sodium hydrogen phosphate
NaOH	sodium hydroxide
Na_4Cl	ammonium chloride
NK	natural killer

NO	nitric oxide
°C	degrees centigrade
OD	optical density
OVA	ovalbumin
PBS	phosphate buffered saline
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcriptase-polymerase chain reaction
PBMC	peripheral blood mononuclear cells
PC	phosphocholine
PEC	peritoneal exudate cells
pg	picogram
PGE_2	prostaglandin E2
PHA	phytohaemagglutin
PMA	phorbol myristate acetate
PNG	Papua New Guinea
PS	phosphatidylserine
pTh	precursor Th cells
r	recombinant
R	receptor
rpm	revolutions per minute
RT	room temperature
s.c.	subcutaneously
SD	standard deviation
SEB	staphylococcal enterotoxin B

STAT	signal transducer and activator of transcription
TCR	T cell receptor
Tg	transgenic
TGF-β	transforming growth factor-β
Th	T helper
TNF-α	tumour necrosis factor-α
Treg	T regulatory cell
TT	tetanus toxoid
U	unit

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Chapter 4

Chapter 1. Introduction

Lymphatic filariasis (LF) is a debilitating disease of humans which is responsible for considerable pathology and morbidity in tropical regions of the world. There are approximately 751 million individuals living in endemic areas where transmission is known to occur and of these individuals, an estimated 128 million people are infected (Michael *et al.*, 1996). In a World Health Organisation (WHO) report in 1995, LF was named as the second leading cause of permanent and long-term disability worldwide. The mosquito transmitted, long-lived nematodes of the genera *Wuchereria* and *Brugia* are the causative agents of LF with *Wuchereria bancrofti* accounting for 91% of infections and *Brugia malayi* and *Brugia timori* responsible for the remaining 9%. Infection with *B. malayi* occurs in South and South East Asia, whereas *B. timori* has only been detected in approximately 40,000 individuals on the Indonesian islands of Timor, Flores, Rote and Alor (Scott, 2000). Bancroftian filariasis occurs throughout the tropics and subtropics, with the exception of the Middle East where infection is only prevalent in Egypt. The widespread occurrence of these filarial infections is predominantly associated with the geographical distribution and parasite compatibility of the vector mosquito. For example, *W. bancrofti* can be transmitted by many different species of mosquito, however its main vectors are the domesticated, night-feeding urban mosquito *Culex quinquefasciatus,* with *Anopheles* spp. and *Aedes* spp. being more common in rural areas. It is the ability of this parasite to be transmitted by a number of different vectors that results in bancroftian filariasis being so widespread. Conversely, the definitive host of *W. bancrofti* is very specific, the only occurrence of natural infection is in humans. Mosquitoes of the genus *Anopheles*, *Aedes* and *Mansonia*, transmit *Brugian* filariasis. *B. malayi* is predominantly a parasite of man although it is also a zoonosis, with monkey, cat and dog reservoirs and this animal reservoir is very

important in terms of infection control. *B. timori* is transmitted via *Anopheles barbirtostris* and natural infection is limited to humans. *B. pahangi* is a natural parasite of wild and domestic animals in South East Asia and has proved to be a useful experimental species, as infections in laboratory animals such as the jird, *Meriones unguiculatus*, will develop to patency (Ash and Riley, 1970).

1.1. Parasite Life Cycle

There are no free-living forms of these parasites and the full developmental cycle takes place in the mosquito (intermediate) host where larval development occurs and in the human (definitive) host where larval and adult development takes place. Like other nematodes, the lymphatic filariae have five developmental stages in their life cycle. Infection of the definitive host is initiated by the bite of a mosquito which harbours mature, infective third stage larvae (L3) (approximately 1.5 x 0.3 mm in size), which are present in the head and mouthparts of the mosquito. The L3 is released from the mosquito's proboscis onto the skin and the larvae enter the body via the puncture wound (Denham and McGreevy, 1977). Once the dermis has been penetrated the L3 enters the lymphatic system of the human host. The moult from the L3 to the fourth stage larvae (L4) occurs between days nine and fourteen post infection (p.i.). In a process, which can take 3 - 12 months (depending on species), L4 develop into sexually mature adults which dwell primarily in the afferent lymphatics. The adult parasites are white, unsegmented and thread-like and the females are approximately double the size of the males (e.g. *B. pahangi* male adult measures 22 x 0.1 mm whereas the female measures 48 x 1.5 mm). After mating the females constantly produce sheathed microfilarae (MF/L1) that circulate in the bloodstream, and are available for ingestion by a mosquito upon taking a blood meal. An interesting aspect of the microfilaraemic

stage of infection is the dramatic fluctuation in numbers of MF in the blood over the course of 24 hours. In most *W. bancrofti* endemic areas MF are nocturnally periodic, in that they are at maximum numbers in the blood at night and virtually disappear from the circulation during the day when they are concentrated in the lungs (Spencer, 1973). This phenomenon ensures that MF are available in the peripheral blood at high concentration when vector species are feeding.

Once ingested by the mosquito the MF (measuring only 210 x 6 μm) migrate from the blood meal and penetrate the mid-gut where they pass through the haemocoel before migrating to the thoracic muscles where development is initiated (Agudelo-Silva and Spielman, 1985). The development of the parasite into the L2, then subsequently the L3 is temperature dependent (optimal 28°C and 80% humidity) and by the eighth day the majority of the parasites are L3 or in the process of moulting. Within two days the mature L3 will migrate from the flight muscles and will associate with the feeding structures in the head of the mosquito which facilitates their transmission to the definitive host.

1.2. The Clinical Spectrum of Disease

There are many different clinical manifestations of LF ranging from asymptomatic microfilaraemia to gross pathology. In addition a proportion of exposed individuals remain uninfected. These people are termed endemic normals or asymptomatic amicrofilaraemics. The clinical manifestations vary from one endemic area to another and also differ depending on the parasite involved. The wide spectrum of the disease is summarised below.

1.2.1. The microfilaraemic state

The vast majority of infected individuals living in an endemic area display few overt clinical manifestations of filariasis, despite the presence of high levels of MF in the peripheral blood (Kumaraswami, 2000). However, although these individuals appear to be clinically asymptomatic, recent evidence suggests that virtually all these individuals suffer from some form of sub-clinical disease. For example approximately 40% of MF+ individuals have low-grade renal damage which is associated with the presence of MF, not adult parasites (Dreyer *et al.*, 1992) and many patients present with abnormal patterns of lymphatic flow (Freedman *et al.*, 1994; Suresh *et al.*, 1997). Therefore the clinical aspects of the microfilaraemic state are more complex than was first thought. However, despite these distinct signs of pathology associated with microfilaraemia, the majority of individuals remain in this state for decades without developing either chronic or acute pathologies (Kumaraswami, 2000).

1.2.2. Acute manifestation of Lymphatic Filariasis

Adenolymphangitis (ADL) is the term used to characterise recurrent attacks of fever associated with inflammation of the lymph nodes and/or lymph vessels. Recurrent attacks of fever associated with lymphadenitis are more commonly seen in *Brugian* filariasis than with bancroftian filariasis. In *Brugian* filariasis the affected lymph nodes are mostly situated in the inguinal and axillary regions with inflammation along the course of the distal lymphatic vessels (Partono, 1987). These acute attacks may last up to 6 weeks, therefore resulting in prolonged periods of inability to work. These episodes are characterised by warmth, tenderness and local pain of the limb, breast or male genitalia. ADL is generally associated with individuals who are amicrofilaraemic (Ottesen and Nutman, 1992).

1.2.3. Chronic manifestations of Lymphatic Filariasis

Chronic pathology (CP) only develops in a small proportion of infected individuals and rarely occurs before the age of 15 (Partono, 1987). Individuals presenting with pathology are generally amicrofilaraemic (Ottesen and Nutman, 1992). Pathology associated with filariasis manifests as hydrocoele, chyluria, lymphoedema and elephantiasis. The symptoms of pathology are primarily caused by living or degenerating worms resident in the lymphatic vessels (Dreyer and Piessens, 2000), but these conditions are often exacerbated by the presence of fungal or bacterial infections. The infected individuals are predisposed to secondary infections due to impaired lymphatic drainage (Jamal and Pani, 1990), although some individuals do develop lymphoedema without the presence of secondary infections. In these cases it is thought that the worms themselves are the primary cause of pathology (Dreyer and Piessens, 2000). Swelling of the limbs is the most common presentation of pathology. In bancroftian filariasis the entire limb is normally affected, but in *Brugia*n filariasis the leg below the knee and the arm below the elbow are typically affected.

1.2.4. Tropical Pulmonary Eosinophilia

TPE is a rare clinical manifestation associated with *W. bancrofti* infection. It is thought to reflect an immunological hyper-responsiveness to the parasite (particularly the MF stage) which presents as eosinophil counts greater than 3000/ml, increased levels of IgE and anti-filarial Abs (Ottesen and Nutman, 1992). MF are typically absent from the blood of these individuals, although dead or dying MF have been identified in lung biopsies. Symptoms include cough, dyspnoea and wheezing (Spry and Kumaraswami, 1982). This condition has been shown to respond well to treatment with the anti-filarial drug diethylcabazamine (DEC).

1.2.5. Endemic Normals

In most areas where filariasis is endemic, a proportion of individuals do not show any clinical manifestations of the disease and are amicrofilaraemic despite having been exposed to infective larvae. With the advent of more sensitive diagnostic techniques, i.e. circulating Ag tests, the number of endemic normals has decreased, as many individuals test positive for filarial Ag despite appearing to be MF free (Moore and Copeman, 1994). Therefore the absence of MF alone is not satisfactory for determining whether an individual is infection free. In addition ultrasound examinations can now determine whether an individual is an amicrofilaraemic adult worm carrier or a normal, uninfected individual (Amaral *et al.*, 1994).

1.3. Diagnosis and Treatment

Diagnosis of LF can be carried out in two ways. Firstly, it is possible to detect the parasite itself or to detect parasite products by circulating Ag tests; secondly, serology can be carried out to identify an immune response to the parasite.

1.3.1 Parasitological diagnosis

Detection of MF is the most common form of parasitological diagnosis. In most parts of the world MF are periodic (Manson, 1883), in that they appear at their highest load at night. Therefore it is imperative that blood be taken for diagnostic purposes when MF reach peak levels (between 10 PM and 2 AM). Traditionally this form of diagnosis is made by staining blood films for the presence of MF. However patients with low levels of MF may not be detected using this method (20 MF / ml of blood required for reliable diagnosis). Alternatives to this method have been developed and have proved more

sensitive, such as membrane filtration. This technique involves taking 1 ml of blood and passing it through a polycarbonate membrane with a 3 to 5 μm pore size. MF, which have been retained on the membrane, can be stained with Giemsa. This method is twice as sensitive as carrying out blood films but is more expensive and requires venous puncture. Both of these methods have other disadvantages, such as collection of samples at night and the inability to detect occult infection, when MF are not present in the circulation (Eberhard and Lammie, 1991).

Circulating Ag detecting tests for *Wuchereria* infection can be carried out at any time therefore eradicating the requirement for nocturnal sampling and this form of diagnosis is now recognized as the method of choice for *W. bancrofti* infection. Previously, monoclonal Abs which recognise phosphocholine (PC), a secretory-excretory product of filarial parasites, were used in ELISA to determine whether an individual has circulating filarial Ag (CFA) in their blood (Weil *et al.*, 1997). However this method was difficult to perform in the field, therefore a rapid-format filarial Ag test using an ICT (immunochromatographic test) card was designed by ICT Diagnostics (Balgowlah, New South Wales, Australia). Human serum or plasma is added to a pink sample pad that contains dried polyclonal antifilarial antibodies coupled to colloidal gold. Antigen-antibody complexes form and the gold-labelled conjugate concentrates to form a pink line visible after 5 minutes. This assay has a reported sensitivity of 96-100% and a specificity of 100% (Weil *et al.*, 1997). Since the advent of this technique, many individuals thought previously to be endemic normals have proved to harbour infection.

1.3.2 Serodiagnosis

Serodiagnosis is useful for the detection of Brugian infections, as circulating Ag detecting tests are not available for Brugian infection. Serological detection is generally carried out using the ELISA format. However, such techniques have proved to be problematic due to poor specificity, as extensive cross-reactivity is found in the sera of individuals infected with closely related parasites (Voller and De Savigny, 1981); (Maizels *et al.*, 1985). In addition, serodiagnosis does not distinguish between past and current infections. However, greater specificity can be achieved by detecting *Brugia*-specific antibodies of the IgG4 subclass. IgG4 levels are greatly up-regulated in actively infected individuals, but not in those who have had previous infections. In addition this assay can be useful in assessing the effectiveness of treatment, as levels of IgG4 fall after chemotherapy (Terhell *et al.*, 1996); (Atmadja *et al.*, 1995). However this form of diagnosis is not as specific as parasitological diagnosis.

1.3.3. Treatment and control of Lymphatic Filariasis

In 1998, a Global Programme to Eliminate LF was initiated and is active in 18 of the 80 endemic countries worldwide. The two principle goals of the Programme are to interrupt transmission of the infection and to treat and prevent the disability caused by the disease. However, it is of great importance to ensure that these goals are reached in a cost effective and socially acceptable manner. To eliminate, or more realistically reduce the level of transmission, all at-risk individuals are treated with a yearly, single dose of a two-drug regime to reduce levels of MF in the circulation below those required to sustain transmission, and to kill adult worms. The drugs of choice are: albendazole (400 mg) plus diethylcabamazine (DEC) (6 mg/kg), or albendazole (400 mg) plus ivermectin (200 μg/kg) in sub-Saharan Africa. This regimen will require to be administered for

several years to correspond with the reproductive life span of adult worms. Alternatively DEC can be added to cooking/table salt to ensure daily consumption of the drug over a 6-12 month period (Ottesen, 2000). In addition, transmission of the parasite can be affected by reducing the number of mosquitoes in an endemic area by spraying breeding grounds with biocides, or by reducing the biting activity of the mosquitoes using insecticide impregnated bednets.

In terms of relieving physical pain and suffering associated with the disease, the main strategy involves treatment of secondary opportunistic bacterial and fungal infections, which are the primary cause of the worsening of lymphoedema. The basic components of managing lymphoedema are meticulous hygiene, prevention and cure of entry lesions, exercise, elevation and wearing proper shoes. Treatment of the affected limb with antibiotics and antiseptics will alleviate bacterial infections. Proper management of lymphoedema should result in increased quality of life for the patient, therefore educating the community on these basic measures should make a significant difference towards the control of filariasis (Dreyer *et al.*, 2002).

1.4. Immunology of Lymphatic Filariasis

Filarial parasites cause chronic infections which evoke host responses and immuno-pathological changes. The wide spectrum of disease associated with filariasis reflects an equally wide range of host responses to infection. Most studies have involved comparing the immune response of microfilaraemics, chronic pathology patients and endemic normals. In general it has been shown that peripheral blood mononuclear cells (PBMC) from individuals with patent microfilaraemia are unable to generate cellular immune responses to filarial antigens, thus distinguishing their immune response from

that of non-infected individuals in an endemic area. In contrast, PBMC from patients with chronic pathology (who are typically MF negative) display increased cellular reactivity to filarial Ag (Ottesen *et al.*, 1977; Piessens *et al.*, 1980a; King *et al.*, 1992). However, despite these differences in responsiveness between clinical groups, there are certain features of the immune response which each group have in common, for example, a certain degree of Ag-specific proliferative suppression (Piessens *et al.*, 1980a; Ottesen, 1992; Yazdanbakhsh *et al.*, 1993a; Sartono *et al.*, 1995a), high levels of IL-4 (King *et al.*, 1992; Mahanty *et al.*, 1991; Mahanty *et al.*, 1993; Yazdanbakhsh *et al.*, 1993b), and down-regulated levels of IFN-γ (King *et al.*, 1992; Ravichandran *et al.*, 1997).

1.4.1. Proliferative suppression

T cell hyporesponsiveness is a characteristic of human filarial infection but many other infectious diseases show a similar pattern of down-regulated immune responses. For example in visceral leishmaniasis, lepromatous leprosy, tuberculosis and schistosomiasis, PBMC show poor Ag-specific proliferation. In general this unresponsiveness is attenuated upon treatment (Maizels *et al.*, 2000). In terms of LF, this defect is most profoundly observed in individuals who are microfilaraemic (Ottesen *et al.*, 1977; Piessens *et al.*, 1980a; Yazdanbakhsh *et al.*, 1993a). PBMC taken from microfilaraemic individuals fail to proliferate to filarial Ag whereas in most studies, mitogen stimulated responses remain intact (Ottesen *et al.*, 1977). These depressed levels of proliferation may be regarded as a form of immunological tolerance. In contrast, PBMC taken from EN and chronic pathology patients will proliferate to varying degrees (Maizels *et al.*, 1995), although all clinical groups display a certain degree of suppression (Yazdanbakhsh *et al.*, 1993a). However in a study by King *et al*

(2001), T cells from individuals who lived in a high intensity transmission village in Papua New Guinea (PNG) exhibited depressed levels of proliferation in response to non-filarial Ag and phytohaemagglutin (PHA) compared to individuals who lived in a low transmission area. These results suggest a role for the incoming L3 in mediating unresponsiveness as the subjects were matched for levels of infection. In addition, a series of studies carried out by Cooper *et al* (1998, 1999) demonstrated that infection with *Onchocerca volvulus* effects the efficacy of vaccines. Notably, post-vaccination with tetanus toxoid (TT), proliferative, antibody and IFN-γ levels were significantly greater in individuals who were not infected. Infected individuals did however produce TT specific IL-10 (while non-infected individuals did not), suggesting that IL-10 has a role in down-regulating non-filarial Ag responses.

The precise mechanisms underlying the proliferative suppression are not yet fully elucidated but may include clonal deletion, induction of anergy, an imbalance in cytokine production, clonal exhaustion or the development of regulatory cell populations (Maizels *et al.*, 2000). The induction of this "tolerance" is however, likely to be only partial as some studies have shown that it can be reversed with chemotherapy (Piessens *et al.*, 1981; Sartono *et al.*, 1995b).

1.4.2 *In utero* exposure to filarial Ag

As referred to above one possible explanation of the proliferative defect in LF is a form of immunological tolerance. In support of this hypothesis, Haitian children born to mothers infected with *W. bancrofti* were observed to be more susceptible to filarial infection than those born to uninfected mothers (Lammie *et al.*, 1991). In an additional study carried out in the Cook Islands, the offspring of mothers who were MF+ remained

unresponsive to MF Ag throughout their lives, suggesting that clonal deletion of Ag-reactive T cells may occur due to *in utero* exposure to parasite Ag (Steel *et al.*, 1994). In a recent study in an area of Kenya where *W. bancrofti* is endemic, children of infected mothers had a three to four-fold increased risk of filarial infection as determined by CFA, compared to children born to uninfected mothers. In addition, children of infected mothers exhibited elevated levels of IL-5 and IL-10, and depressed levels of proliferation in response to MF Ag compared to children of uninfected mothers. However, the response to adult Ag was markedly different as there was no difference in cytokine production or proliferation between the groups. These results support the concept that *in utero* exposure to filarial Ag increases the offspring's susceptibility to *W. bancrofti* infection and polarises the immune response towards Th2 (Malhotra *et al.*, 2003). However some studies have shown that treatment with DEC can result in the recovery of Ag-specific proliferative responses, at least in the short term, thus arguing against clonal deletion (Lammie *et al.*, 1992; Sartono *et al.*, 1995a). However this is a controversial area as other studies have shown no recovery of proliferative responses post-chemotherapy (Steel and Ottesen, 2001), indicating that clearance of infection is not sufficient to restore immune responsiveness, at least to a comparable level with EN individuals. Therefore infection with filarial parasites appears to induce long-term defects in the ability to respond to parasite Ag. These findings underline the fact that there are many different pathways which may result in Ag-specific unresponsiveness in LF.

1.4.3. IL-4 in Lymphatic Filariasis

Although PBMC from microfilaraemics do not proliferate in response to parasite Ag they have the ability to produce cytokines. T cells from these individuals, along with

EN and chronic pathology patients secrete significant amounts of Ag-specific IL-4, with a resultant down-regulation in IFN-γ responses (Nutman *et al.*, 1987; Mahanty *et al.*, 1996b). This has been demonstrated at the protein level (Mahanty *et al.*, 1996b) and at the mRNA level (Dimock *et al.*, 1996). In the latter study it was shown that levels of IL-4 mRNA did not differ between PBMC from MF+, MF-, CFA+ or CFA- individuals upon *in vitro* stimulation with parasite Ag. As PBMC from putatively immune CFA- individuals also express elevated levels of IL-4 in the absence of infection, the L3 to which all individuals are exposed may be the stimulus for IL-4 production in this group.

Levels of IL-4 correlate inversely with levels of IFN-γ in filarial infected individuals, however when the cells that produce IL-4 and IFN-γ were enumerated and compared between patients with chronic pathology and those with an active infection, it was observed that the ratio of IFN-γ:IL-4 producing cells was higher in patients with CP and lower in MF+ individuals (King *et al.*, 1993). Therefore it has been suggested that despite elevated levels of IL-4 in individuals from all clinical groups, it is the down-regulation of IFN-γ producing cells in microfilaraemic individuals that may be the important factor in maintaining parasite infection (Maizels *et al.*, 1995; Sartono *et al.*, 1997). The presence of the parasite is clearly associated with down-regulation of IFN-γ production as MF+ individuals who are given chemotherapy can produce IFN-γ (Sartono *et al.*, 1995a).

Interestingly, although IL-4 responses are elevated in active infection, IL-5 is not co-ordinately up-regulated (Sartono *et al.*, 1997). When Ag-specific IL-5 responses were examined as a function of clinical status and age, it was shown that MF- individuals had higher levels of IL-5 than MF+ and that IL-5 decreased significantly with increasing age

in both MF+ and MF- individuals. This is quite different to the situation with IL-4 where levels of IL-4 were not different between MF+ and MF- individuals. In addition, IL-4 levels increase with age in MF+ individuals and decrease in MF- individuals. Therefore the cytokine disregulation observed in LF is observed in both Th1 and Th2 subsets suggesting that immune responses to filarial parasites do not fall neatly into the Th1/Th2 paradigm. This theory was discussed in a review by Allen and Maizels (1997), which notes that even though the Th2 bias in the immune response to filarial infection makes the Th1/Th2 model attractive, it does not account for the mechanisms of hyporesponsiveness observed in filarial patients.

1.4.4. IL-10 in Lymphatic Filariasis

As discussed above, the exact nature of Ag-specific hyporesponsiveness has yet to be elucidated. The role of IL-10 has been of interest in LF due to its capacity to block activation of cytokine synthesis and several accessory macrophage functions. These functions render IL-10 a potent suppressor of the effector functions of macrophages, T cells and NK cells, at least in murine models (Fiorentino *et al.*, 1989). Initial studies into the role of IL-10 in LF were carried out by neutralizing IL-10 in cultures of PBMC from *W. bancrofti* infected MF+ individuals. The characteristic T cell hyporesponsiveness was reversed when IL-10 was neutralized. In addition a similar but less pronounced effect was observed when TGF-β was neutralized (King *et al.*, 1993). In another study, it was found that PBMC from MF+ individuals secreted large amounts of spontaneous IL-10. Levels of IL-10 increased when cells were re-stimulated with mixed sex adult or microfilarial Ag but not adult male Ag, suggesting that IL-10 production is regulated by MF Ag, but also may suggest a role for adult female Ag. IL-10 levels were inversely

correlated with levels of Th1 cytokines, indicating a role for IL-10 in down-regulating Th1 responses in these individuals (Mahanty *et al.*, 1996b).

In a more recent study, Mahanty *et al* investigated the regulation of antigen specific immune responses by IL-10 and IL-12. These two cytokines have opposing functions as IL-12 has a critical role in the stimulation of IFN-γ from NK cells and T cells (D'Andrea *et al.*, 1993). Confirming the previous findings of King *et al* (1993), neutralizing anti-IL-10 was shown to enhance proliferation in response to restimulation with *B. malayi* adult antigen in 78% of MF+ infected individuals (Mahanty *et al.*, 1997). These studies indicate that IL-10 plays a critical role in the hyporesponsiveness to antigen that is associated with MF+. As a result of IL-10 blockade, levels of IFN-γ increased two-fold in 73% of MF+ subjects studied, in keeping with the hypothesis that IL-10 inhibits IFN-γ secretion in response to antigen (Mahanty *et al.*, 1997). However these results also demonstrate that MF+ individuals have antigen-specific T cells capable of mounting a Th1 response under the appropriate conditions. In the same study, when IL-12 was neutralized, levels of IL-5 increased in Ag stimulated cultures while levels of IFN-γ decreased. Augmentation of Type 2 responses by anti-IL-12 suggests that there are significant amounts of IL-12 in the Ag-driven cultures but that the inhibitory effects of endogenously secreted IL-10 on Type 1 responses to Ag are not effectively attenuated by IL-12.

To gain understanding of the molecular basis of the Th1 hyporesponsiveness, Ravichandran *et al* (1997), performed RT-PCR to determine the levels of cytokine mRNA in PBMC from *W. bancrofti* infected MF+, CP and endemic normal individuals. Unstimulated PBMC from EN individuals had a purely Th1 pattern with no IL-4 or IL-5

expression. In PBMC from MF+ there was a marked decrease in the expression of Th1 cytokines (IFN-γ and IL-2), paralleled by increased expression of IL-10. Comparatively little IL-10 expression was observed in CP patients and a ten-fold increase in levels of IFN-γ and IL-2 was observed between CP and MF+ individuals. PBMC and adherent cells in MF+ patients stimulated with Ag or medium only were also shown to express greater levels of IL-10 mRNA in comparison to individuals with elephantiasis. When IL-10 expression in response to parasite antigen was examined it was found that only MF+ individuals expressed IL-10 and that there was a significant negative correlation between IL-10 mRNA expression and PBMC proliferation in the MF+ individuals in comparison with CP and EN patients (Ravichandran *et al.*, 1997). This study lends further support to the hypothesis that IL-10 is involved in the down-regulation of the Th1 response. However when studying individuals infected with *Brugia malayi* a similar phenomenon was not observed. PBMC from MF+, CP and EN individuals were cultured with *B. malayi* antigen and a range of different reagents were added in an attempt to reverse the proliferative defect. These included IL-2, IL-7, anti-IL4, anti-IL-10, anti-CD2, anti-CD27, anti-CD28, indomethacin, phorbol myristate acetate (PMA) or calcium ionophore. Co-culture with IL-2, IL-7 and PMA were the only conditions which resulted in enhanced proliferation to parasite antigen, but only in a few cases. Neutralizing antibodies to IL-10 did not restore responsiveness to antigen (Sartono *et al.*, 1995b). Furthermore, higher levels of IL-10 mRNA expression were observed in CP patients infected with *W. bancrofti* than in MF+ patients using PBMC re-stimulated with fractionated *B. pahangi* Ag (Dimock *et al.*, 1994).

These contrasting findings may be due to a number of different factors, e.g. different species of parasite studied (*W. bancrofti* or *Brugia* species), different geographical

strains of parasites i.e. studies by Dimock *et al*, King *et al* and Sartono *et al* were carried out in different endemic regions (Haiti, Madras, India and Indonesia, respectively). In addition, the use of different antigens for restimulation (*B. malayi, B. pahangi*), possible differences in past chemotherapy, differences in transmission rate or the use of cryopreserved cells (Sartono *et al.*, 1997) may all contribute to the conflicting results described above.

IL-10 has also been postulated to play an important role in the regulation of $CD4^+$ T cell responses induced by the parasitic trematode *Schistosoma*. In a recent study, the contribution of IL-10 and IFN-γ to the regulation of Th1 and Th2 cytokine responses was investigated in individuals with differing clinical forms of *S. mansoni*. It was demonstrated, by the use of cytokine neutralization procedures, that early disease is associated with a significant IFN-γ response and that IL-10 contributes to the suppression of that response during both early and chronic infection. This is due to the measurable cross- regulation between IL-10 and IFN-γ, with IL-10 neutralization up regulating IFN-γ production and vice versa (Montenegro *et al.*, 1999). Broadly similar results were obtained in a study in which responses to adult worm Ag (AWA) were investigated using fresh or cryopreserved PBMC from individuals chronically infected with *Schistosoma haematobium* (Grogan *et al.*, 1998). Neutralizing IL-10 alone resulted in increased background levels of proliferation of PBMC, but did not increase levels of AWA-specific proliferation in freshly isolated cells or cryopreserved cells. Also, IFN-γ levels were increased when IL-10 was neutralized, however this increase was 4-fold greater in freshly isolated cells compared to cryopreserved cells. IL-4 was unaffected by anti-IL10 MAb. IL-10, therefore has a down-regulatory effect on Th1 responses in *S. haematobium* infection but plays no role in modulating IL-4 responses. This study was

also informative in that it demonstrated a difference in the use of cryopreserved and freshly isolated cells (Grogan *et al.*, 1998).

1.4.5. The role of Antigen Presenting Cells in Lymphatic Filariasis

One of the major ways in which IL-10 modulates T cell responses is via an indirect effect on antigen presenting cells (APC). Evidence from several studies has indicated that defects in antigen presentation are critical for development of peripheral tolerance in LF (Ottesen, 1979; MacDonald *et al.*, 1999). For example, an important study indicated that IL-10 from infected individuals may be produced by adherent cells (Mahanty *et al.*, 1996b). The role of impaired APC function in LF was investigated by examining the expression of the costimulatory ligands B7-1 (CD80) and B7-2 (CD86), which are present on the surface of professional APC. The importance of the "second signal" in the activation of responder T cells which initiate Ag-specific responses is outlined in Section 1.6. Ravichandran *et al* addressed the possibility that T cell hyporesponsiveness in individuals infected with *W. bancrofti* could result from the absence of costimulatory signals delivered by APC. mRNA levels of B7-1 were compared in PBMC from EN, CP and MF+ individuals. Levels of B7-1 expression on unstimulated PBMC from MF+ individuals were very low compared to the basal level observed in CP individuals. However, most significant was the baseline expression of B7-1 in EN, in which cells from every individual expressed spontaneous B7-1. In response to parasite Ag, there was a general up-regulation of B7-1 in both MF+ and CP individuals but the level of B7-1 expression in EN individuals did not increase. It was suggested that high Ag-load down-regulates the expression of spontaneous B7-1 in MF+ individuals. Interestingly, it was shown that IL-10 levels do not correlate with B7-1 expression. B7-2 was not examined and this molecule may be of major interest in

future studies on the role of costimulatory molecules in filariasis (Ravichandran *et al.*, 1997). In individuals infected with *Loa loa* levels of B7 were found to be similar between MF+ and MF- groups, however this study failed to discriminate between B7-1 and B7-2 (Baize, 1997). In a study using experimental animals (Giambartolomei *et al.*, 2001), monkeys infected with *B. malayi* were divided into two groups depending on the ability of their PBMC to divide in response to BmAg – responders and non-responders. All of the responders were found to express significantly higher levels of B7-1, whereas half of the non-responders showed no increase in B7-1 and the other half only showed a marginal increase. High levels of B7-2 were observed in all monkeys, regardless of stimulation. Therefore this study demonstrates an association between BmAg induced proliferative responses and expression of B7-1 but not B7-2. The general consensus from these experiments appears to be that proliferative capacity is associated with an up-regulation of B7-1 expression, although not all the studies reviewed above examined the role of B7-1 and B7-2 as separate ligands.

As described above, many studies have attempted to discover the underlying cause of proliferative suppression but none have found a single factor which is universally successful at restoring proliferation. Recently, the impact of transmission intensity on the host immune response in two villages in PNG where the transmission intensity of *W. bancrofti* differed by 63-fold was investigated (King, 2001). Residents of the high transmission village had impaired lymphocyte proliferation and IFN-γ production in response to parasite Ag, to the non-parasite Ag streptolysin-O and to the APC-dependent mitogen phytohaemagluttanin. By contrast, lymphocyte proliferation and IFN-γ production in response to the mitogen PMA and ionomycin (which directly activates protein kinase C and facilitates influx of Ca^{+} into the cell and does not require

APC help) was similar between individuals from both areas. In addition, $CD4^+$ cells which had been purified from individuals living in the area of high transmission could be activated with immobilised anti-CD3 (that cross-links the T cell receptor) and anti-CD28 (which provides an important costimulatory signal). This study provides further evidence that impaired APC function modulates the ability of PBMC to proliferate in response to filarial Ag.

Other studies have shown that Ag-specific hyporesponsiveness is mediated, in part, by diminished APC function and is most obvious when PBMC are stimulated with MF Ag (Semnani *et al.*, 2001; Semnani *et al.*, 2003). DC exposed to live MF up-regulate the expression of ICAM-1 and undergo increased levels of apoptosis compared to unexposed DC. While MF exposed DC up-regulate IL-8, RANTES, IL-1α, TNF-α and IL-β, levels of IL-12 and IL-10 were down-regulated. Soluble excretory secretory (ES) products from MF also resulted in this down-regulation of cytokine production but to a lesser degree. In addition, exposed DC were less able to stimulate $CD4^+$ cells to produce IFNγ and IL-5 (Semnani *et al.*, 2001; Semnani *et al.*, 2003). The consensus from the studies reviewed above is that infection with filarial parasites has a marked effect on the ability of professional APC to present Ag to $CD4^+$ cells effectively, thus inhibiting the ability of immune cells to mount appropriate responses.

1.4.6. T regulatory cells in Lymphatic Filariasis

T regulatory cells (Treg) are a family of $CD4^+$ cells that are anti-inflammatory and profoundly suppressive. Three populations of Treg cells have been identified to date: Th3 (Weiner, 2001), Tr1 (Roncarolo *et al.*, 2001b) and, perhaps the best characterised, $CD4^+CD25^+$ cells, which typically co-express CTLA-4 and OX-40 (Read and Powrie,

2001). These regulatory T cells have the ability to inhibit potentially harmful immune responses via cell-to-cell contact dependent mechanism or by the secretion of cytokines such as TGF-β and IL-10. Most studies to date investigating the role of Treg cells in filariasis have been carried out in humans and indeed it has been proposed that many of the features of LF could be accounted for by the induction of Treg cells, rather than a Th2 bias (Maizels and Yazdanbakhsh, 2003). One such study investigated the role of CTLA-4 in mediating diminished levels of Ag-specific T cell responsiveness that is characteristically observed in filariasis patients. It was observed that individuals who inhabited endemic areas had a higher frequency of $CD4^+CTLA\text{-}4^+$ cells than expatriate infected or uninfected individuals. In addition MF+ patients had a higher frequency of $CD4^+CTLA\text{-}4^+$ cells than MF- individuals. These studies suggest that length of exposure to the L3 and patency of infection are factors associated with the expression of CTLA-4. In addition the greatest intensity of CTLA-4 expression occurred on $CD4^+CD25^+$ cells. Neutralizing CTLA-4 *in vitro* resulted in increased levels of IL-5 and decreased levels of IFN-γ, indicating a role for this molecule in regulating host responses to filarial infections (Steel and Nutman, 2003).

Further studies have examined the role of regulatory T cells in chronic infection with the related filarial nematode *O. volvulus*. Individuals with a patent infection i.e. generalised onchocerciasis (GEO), display an Ag-specific proliferative hyporesponsiveness similar to that described above for LF. It was shown that IL-10 and TGF-β mediate this response, as neutralizing these cytokines resulted in the reversal of the proliferative defect. *O. volvulus*-specific T cells cloned from GEO PBMC produced no IL-2 and high IL-10 and TGF-β, similar to Th3/Tr1 cells, indicating that these cells may be involved in maintaining *O. volvulus* hyporesponsiveness (Doetze *et al.*, 2000).

In an additional study, it was found that these Treg cells display elevated levels of CTLA-4 after stimulation and are able to inhibit proliferation of other T cells in co-culture (Satoguina *et al.*, 2002). More recently it has been found that *S. mansoni* specific lyso-phosphatidylserine (PS) activated the Toll-like receptor 2 and affected DC, such that mature DC gained the ability to induce the development of IL-10-producing Treg cells (van der Kleij *et al.*, 2002), Taken together, the evidence outlined above suggests a role for Treg cells in the maintenance of hyporesponsiveness in helminth infection.

1.4.7. The importance of the L3 in Lymphatic Filariasis

Defining the role of individual life cycle stages in polarising the immune response in human infection can be very difficult as individuals living in an endemic area are exposed to multiple different life cycle stages simultaneously. As the L3 is the first life cycle stage to which the host is exposed, the immune response elicited by the L3 is likely to influence both the establishment of the parasite and the development of immunity.

Evidence in humans that an immune response is directed against the L3 comes from the observation that a percentage of individuals inhabiting an endemic area will remain infection-free (endemic normals) despite exposure to L3. The presence of EN has given rise to the theory of concomitant immunity (whereby individuals are resistant to incoming L3 but cannot clear their adult parasites) (Day *et al.*, 1991). Despite a lack of knowledge regarding the parasitological state of endemic normals, it was previously thought that these individuals were resistant/immune to infection with the L3, whereas microfilaraemic individuals were susceptible. However with the advent of more

sensitive diagnostic procedures it appears that the endemic normal is a very rare individual, at least in areas of high transmission (Day, 1991; Freedman *et al.*, 1989; Lalitha *et al.*, 1998).

Population dynamic studies (Day, 1991; Vanamail *et al.*, 1989) have argued that the concept of the endemic normal should be replaced by the hypothesis that concomitant immunity exists in the majority of the adult population in filariasis-endemic areas. For example, the rate of gain and loss of *W. bancrofti* infection was estimated during a control programme in Pondicherry, South India. The results suggested that the age distribution of bancroftian filariasis is primarily determined by age-dependency in the rate of acquisition of infection. The rate of gain of infection, based on levels of MF, was shown to peak in the 16-20 year age group and then decline in adulthood. This was proposed as evidence for acquired resistance to new infection (Vanamail *et al.*, 1989). A longitudinal study of the age-specific dynamics of *W. bancrofti* infection in a community of East Sepik Province, PNG was carried out. MF density and levels of PC-containing Ag in individuals were used as measures of adult worm burden. Over a 12 month period significant increases in the levels of circulating PC-Ag was noted in subjects less than or equal to 20 years of age, but not in subjects greater than 20 years of age (Day *et al.*, 1991). These data are therefore consistent with the acquisition of resistance to superinfection with age. Further analysis indicated that resistance was directed at the L3 as, when the age-specific dynamics of the Ab response to the L3 surface was examined in this population, a correlation was established between age and prevalence of Ab. Adults, but not children, had detectable anti-L3 surface Ab irrespective of adult worm burden (Day *et al*, 1991). In conclusion, it appears that children living in this endemic area continued to accumulate adult worms, whereas

worm burden was stable in adults. These studies support the notion of concomitant immunity, directed at restricting incoming larval parasites but ineffective at eliminating existing adult parasites.

Clear evidence of acquired resistance to trickle infection of L3 in *B. pahangi* infected cats also supports the hypothesis of concomitant immunity. Cats trickle-infected with L3 become resistant to re-infection and this resistance is directed at the L3, with the majority being destroyed within 24 hours of infection. Adult worms in these animals can be long-lived with a life expectancy of two years and arise from early infections when the animals are naïve (Denham *et al.*, 1983). More recent studies involving the relationship between the levels of transmission of the L3 and the development of infection and disease in bancroftian filariasis have been carried out in two endemic villages in East Africa. One village had an ATP (annual transmission frequency, determined by the number of L3 to which an individual is theoretically exposed to per year), nine times higher than that of the other (Michael *et al.*, 2001). A particular focus of this work was to understand the contribution of host immunity in regulating infection and in the development of chronic disease, and the role of exposure rate in generating such immunity. In the high ATP village, prevalence and mean intensity of MF and incidence of pathology (hydrocele and lymphoedema) were significantly higher. In addition, the geometric mean MF count was also significantly higher (almost 20 times) in the higher transmission community. These data were interpreted to suggest that while immunity against infection reflects past experience of adult worms acting against the establishment of L3, the immunopathological component of lymphoedema is apparently more related to cumulative experience of larvae rather than adult worms. However this interpretation requires further investigation.

The importance of the L3 in modulating the immune responses of individuals living in an area of high transmission compared to responses of individuals living in a low transmission area was investigated study populations from villages in PNG (King, 2001). High levels of L3 transmission were shown to affect Th2 responses with levels of IL-5 being significantly elevated compared to that of the low transmission area. However, levels of IL-4 and IL-10 secreted from PBMC were not significantly different between groups. It was notable that in this study, levels of IL-10 and TGF-β did not correlate with depressed levels of proliferation or IFN-γ levels, a result in contrast to a previous report from India (King *et al.*, 1993). One of the other interesting results from the PNG study was the identification of elevated plasma IL-4 in individuals from the high transmission village (almost two-fold higher). However, it was notable that this increase in IL-4 was not observed in PBMC culture. It was suggested that the IL-4 is likely to derive from mast cells and basophils in the skin, as these cells are abundant in dermal tissues where the L3 are inoculated. These cells may therefore play a critical role in host defence, particularly across epithelial barriers such as the skin. Together, the studies summarised above strongly indicate that the L3 evokes an early immune response in the human host. Further information on the role of the L3 in generating a response will be discussed with reference to work carried out using the mouse model of infection.

1.5. Mouse model of Brugian filariasis

Immunological studies of human filariasis are complicated by many factors, including exposure to multiple life cycle stages, differences in worm burdens and infection status, prior and current exposure to L3, use of chemotherapy and the presence of concomitant infections. The use of laboratory animals allows some of these factors to be controlled

and has provided invaluable information on the immune response to filarial parasites. The ideal animal model would be inbred, would harbour the full developmental cycle of the parasite, display similar pathology to that seen in humans and mimic the immunological characteristics of the human infection. A model with all these traits is not available, therefore most studies have been carried out in mice. A surprising amount has been learnt from the mouse model. Although not fully permissive to filarial infection (with the exception of *Litomosoides sigmodontis*), mice can support single life cycle stages of *Brugia sp* for a limited time, allowing investigation of the immune response to each stage. Unfortunately a suitable animal model for *W. bancrofti* has not been found, as it will not survive in intact or immunocompromised mice.

There are several features of the immune response which are similar in the human and mouse infections, such as the characteristic proliferative defect and skewing of the immune system towards a predominantly Th2 type response. Each life cycle stage of the parasite can elicit differing immune responses and thus the ability to implant the various stages of development into the mouse model allows the responses to be interpreted independently of each other. It is notable that different life cycle stages elicit different immune responses in the murine host, suggesting that natural infection with filarial parasites induces a complex array of immunological responses in the infected individual.

1.5.1. Murine infection with MF

Experiments carried out by Pearlman *et al* (1993b) examined cytokine production in BALB/c mice inoculated i.p. with live MF of *B. malayi*. At early time points (12-14 days) splenocytes from these animals produced an Ag-specific Th1-like response with

elevated levels of IFN-γ and little IL-5. However at later time points levels of IL-5 increased, with a resultant decrease in levels of IFN-γ. Peritoneal exudate cells (PEC) from mice which had been immunized three times with soluble microfilarial Ag were recovered four days after MF challenge and were cultured with MFAg. These cells produced elevated levels of IL-4 and IL-5 with no increase in Ag driven IL-2 or IFN-γ. Depletion experiments demonstrated that $CD4^+$ cells were the main source of IL-4 and IL-5. In addition IL-10 was shown to have a role in controlling IFN-γ production in splenic and lymph node cells from mice chronically exposed to live MF or immunized multiple times with soluble Ag. Addition of neutralizing IL-10 MAb resulted in increased levels of IFN-γ production in splenic and lymph node cells from these animals (Pearlman *et al.*, 1993a). In similar experiments from a different laboratory in which BALB/c mice were infected with MF i.v, IFN-γ was detected at early time points with a gradual increase in IL-4. It was also found that the IFN-γ response to MF was independent of the dose of MF administered, in the range of 10^2 to 10^6 (Lawrence *et al.*, 1994). Furthermore, popliteal lymph node cells from BALB/c mice infected via the footpad with MF of *B. pahangi* express IFN-γ in the absence of IL-10 or IL-4 at day four post infection (Osborne and Devaney, 1998). These results indicate that an early IFN-γ response to MF is accompanied by a later more modest Th2 response.

Interestingly, recent data identified a MF-specific protein (Bm-SPN-2, a secreted serpin) which is a prominent T cell Ag. It was found that mice infected with MF of *B. malayi* mounted a strong Bm-SPN-2 specific Th1 response which was short-lived. By day 35 responsiveness to Bm-SPN-2 was lost. These findings are important as Bm-SPN-2 was recognised by mouse and human T and B cells and is the first example of a MF-specific secreted molecule that acts on T cells (Zang *et al.*, 2000). Studies in the

mouse model demonstrated that the proliferation of splenocytes from MF infected BALB/c mice was suppressed in an Ag-specific manner, in contrast to splenocytes from L3 infected mice, which proliferated well under identical conditions (Osborne *et al.*, 1996). Cells from MF infected animals had been activated as demonstrated by elevated levels of IFN-γ in Ag-stimulated cultures. Similar to the situation observed in human infection, levels of mitogen stimulated proliferation were not suppressed. Further studies carried out in MF infected mice demonstrated that the proliferative defect was associated with elevated production of NO, induced by IFN-γ (O'Connor *et al.* 2000). Inhibition of iNOS or infection of IFN-γ receptor knock-out mice resulted in normal levels of Ag-specific proliferation. Moreover it was shown that elevated levels of NO in these cultures induced apoptosis of $CD4^+$ T cells (Jenson *et al.* 2002). It was hypothesised that the apoptosis of activated T cells represents a negative feedback system, which limits the expansion of potentially harmful pro-inflammatory cells (O'Connor and Devaney, 2002).

1.5.2. Murine infection with adult parasites

Although immunocompetent mice are resistant to natural infection with *B. malayi* or *B. pahangi*, adult worms can be implanted and survive for up to 90 days. In contrast to the situation observed in mice infected with MF, mice implanted with the adult stage of *Brugia* develop a profound Th2 response with elevated levels of IL-4 and good Ag-specific proliferative responses (Lawrence *et al.*, 1994). Mice implanted with female worms displayed a stronger immune response than those implanted with adult males, although the response of both groups of animals followed a similar pattern. Levels of IL-2 and IFN-γ remained low in these animals. This is in stark contrast to responses observed in MF infected animals, where levels of IFN-γ remained high until 28 days

post-infection, and although an IL-4 response was observed at this time point, levels of this cytokine remained less than 10% of that observed in response to adult female worms. Furthermore $CD4^+$ cells were identified as the source of the IL-4 produced in response to adult worms (Lawrence *et al.*, 1994). These results are interesting as they demonstrated that the IL-4 (Th2) response is dominant despite the continual release of IFN-γ inducing MF from the adult females.

Studies which have attempted to unravel the ways in which adult parasites modulate the immune system have demonstrated a multitude of mechanisms, ranging from suppressive ES products, the generation of a suppressive APC population and the induction of suppressor cytokines IL-10 and TGF-β. Using the filarial parasite *Acanthocheilonema viteae* an ES product (ES62) was identified which was immunomodulatory and down-regulated immune responses. ES62 is a phosphoryl-choline (PC)-containing glycoprotein to which PC is covalently attached via an N-type glycan. PC is found on components of the ES of all human filarial nematodes examined to date. (reviewed by Harnett *et al.*, 1999; Harnett and Harnett, 1999). It was further shown that ES62 interacts directly with APC, signalling dendritic cells to acquire a phenotype which drives the development of Th2 cells (Whelan *et al.*, 2000). In these studies the responses of GM-CSF matured DC exposed to LPS or ES62 were compared. It was shown that exposure of DC to LPS results in a phenotypic Th1 response compared to the Th2 response generated by ES62. ES62 also appears to inhibit B cell stimulation and proliferation (Harnett and Harnett, 1993). These results indicate that the production of ES62 by adult parasites modulates the immune response towards a Th2 phenotype and can reduce levels of proliferation of the B cell population.

The role of the adult parasite in eliciting a population of suppressive APC has been extensively studied by the Allen group. Intraperitoneal implantation of adult worms or L3, but not MF, leads to the generation of PEC which suppress the proliferation of T cell clones without blocking Ag-specific cytokine production *in vitro* (Allen *et al.*, 1996). It was demonstrated that IL-4 was essential for the induction of this non-specific suppressor cell population but that IL-10 was not a key component. IL-4-/- mice were unable to generate a suppressive PEC population while mice given anti-IL-4 at the time of infection still developed Th2 responses but were unable to generate the suppressive PEC population. Therefore it appears that early IL-4 production, and not necessarily Th2 establishment, is the essential factor in the development of the proliferative block. In these experiments, infection of IL-10 -/- mice generated fully suppressive PEC. A partial role for IL-10 was suggested as compared to the wild type mice, PEC from IL-10-/- mice induced less profound suppression of hybridomas. These IL-4 dependent suppressor cells were later described as F4/80+ alternatively activated macrophages and their suppressive function was found to operate via a cell-cell contact dependent mechanism. Interestingly, these cells were found to suppress proliferation of various transformed cell lines, including many human tumour cell lines, thus indicating that the suppressive mechanism was neither species nor cell type specific (Loke *et al.*, 2000a).

1.5.3. Murine infection with L3

Studies from many groups have highlighted the importance of the L3 in modulating host immune responses in mouse models. Infection of mice with the L3 of *Brugia malayi* (Lawrence *et al.*, 1995) or *B. pahangi* (Osborne and Devaney, 1998) results in a Th2 type response with elevated production of IL-4 and low IFN-γ and IL-2, similar to adult parasites. To further investigate the role of this Th2 response, experiments were

carried out in IL-4-/- mice. C57BL/6 IL-4 deficient mice or their corresponding WT counterparts were infected with 100 L3 of *B. malayi* i.p. Cytokine responses from these animals showed slightly decreased levels of IL-5 compared to WT mice, but no elevation of IFN-γ levels. These data were interpreted to suggest that Th1 cells were not primed in response to infection with L3 (Lawrence *et al.*, 1995). However in experiments in which IL-10 was neutralized *in vitro*, splenocytes from L3 infected BALB/c mice produced IFN-γ and IL-2 without altering levels of IL-4 or IL-5, suggesting that Th1 cells were primed by L3, but suppressed by IL-10 (Osborne and Devaney, 1999).

Further studies on the role of the adherent APC population in mice infected with *B. pahangi,* demonstrated that replacement of the resident APC population with APC from control mice reversed the inhibition of Th1 responses (Osborne and Devaney, 1999). The precise phenotype of the suppressive APC in this system was not identified. However it was notable that the irradiation of resident APC had the same effect, suggesting that the suppressive population may be B cells, as these cells are more susceptible to γ irradiation that other APC (Sopori *et al.*, 1985). Although these experiments also showed that neutralizing IL-10 resulted in elevated levels of IFN-γ and IL-2, no link between the role of IL-10 and the suppressive APC population was sought (Osborne and Devaney, 1999). Therefore the data presented above demonstrate that Th1 and Th2 cells are primed in response to the L3 but Th1 cells are suppressed by IL-10 and the APC population.

The cytokine environment at the point of T cell priming is very important in determining the differentiation of precursor (p) Th cells into Th1 or Th2 cells (see

section 1.7). In light of this, experiments were carried out to investigate the pattern of cytokine mRNA expression within the draining lymph node shortly after infection with L3. Mice were infected via the footpad and at 24 hours p.i. the popliteal lymph nodes were removed and cytokine gene expression was measured by semi-quantitative RT-PCR. These experiments showed that the L3 elicited a dramatic burst of IL-4 transcription within 24 hours of infection, defining the L3 as a potent stimulator of the Th2 response. Cell separation experiments demonstrated that an $\alpha\beta$ $^{+}CD4^{-}CD8^{-}$ (a double negative) T cell population was responsible for the production of early IL-4 (Osborne and Devaney, 1998). Further characterisation of these cells indicated that this population may be $NK1.1^{+}$ T cells (Balmer and Devaney, 2002). It is not known what components of the L3 stimulate the early IL-4, but the surface of the L3 contains both lipid and carbohydrate moieties and the components of the surface are known to turn over within 24 hours of entry into the mammalian host (Carlow *et al.*, 1987). Therefore one of these moieties may be involved in priming NK cells for IL-4 production.

Experiments carried out by the Rajan group have concentrated on factors which contribute to the clearance of a primary infection when L3 of *Brugia* are administered i.p. Primed B cells from mice immunized with the L3 of *B. pahangi* adoptively transferred into athymic recipients protected these animals from challenge infection (Paciorkowski *et al.*, 2003). Further investigation showed that B cells were required for the early production of Th2 cytokines. These results were interpreted to suggest that primed peritoneal B cells were sufficient to transfer protection against *B. pahangi* infection in mice. IgE has also been shown to have a role in determining the susceptibility of the host strain. IgE-/- mice were more permissive to primary infection with L3 of *B. malayi* than WT mice. Infected IgE-/- mice also displayed defective IgG1

production suggesting that there may be other contributing factors to the permissiveness observed in the KO animals (Spencer *et al.*, 2003b).

The data presented above indicate that the host-parasite interaction is complex and highly evolved. However it remains unclear whether the Th2 response to the parasite protects the host from pro-inflammatory pathology or whether the parasite induces Th2 responses to maintain its survival. Whatever the case, analysis of the responses to different life cycle stages has led to the conclusion that there are many different ways in which the parasite can modulate host responses and that each life cycle stage exploits a different mechanism of modulation.

1.6. Immunoregulation

1.6.1. T cell phenotype and effector function

In a seminal study, Th1 and Th2 cells were first distinguished as two different subsets (Mosmann *et al.*, 1986). A panel of mouse helper T cell clones were characterized into two groups according to their lymphokine production. Type 1 helper cells (Th1) produced IL-2, IFN-γ, GM-CSF and IL-3 in response to Ag and APC or to ConA. T helper 2 cells (Th2) produced IL-3, BSF1, and two other activities unique to the Th2 subset, a mast cell growth factor distinct from IL-3 (now known to be IL-5) and a T cell growth factor distinct from IL-2 (now known to be IL-4). A subsequent study showed that Th2 clones also have the ability to synthesise IL-4 and IL-5 (Cherwinski *et al.*, 1987). Th1 and Th2 cells are derived from Th0 cells which have the capacity to produce all these cytokines. Th0 cells are thought to be derived from naïve T cells (pTh) as they differentiate towards the Th1 or Th2 pathway. This new paradigm changed the way in which the immunology of infectious disease was studied, with particular regard to

Leishmania infection in mice, which was instrumental in establishing the functional relevance of disease models in understanding these pathways. However, it has become increasingly clear that the concept of Th1/Th2 cannot account for every aspect of the immune response. In this section, I review the mechanisms by which T cells are activated to effector status or tolerized to result in suppression of immune responses, the role of APC and cytokines in determining the phenotype of differentiated T cells and finally, the role of Treg cells.

1.6.2. T cell activation and tolerance

Lymphocytes have to distinguish self from non-self while at the same time directing immune responses. To avoid damage to self, these responses must be tightly regulated. The 'two signal' concept of lymphocyte activation was proposed to explain discrimination of self from non-self (Bretscher and Cohn, 1970; revised Bretscher, 1999) and has been a useful model of activation and tolerance ever since. Naïve precursor Th (pTh) cells are activated after interaction of the T cell receptor (TCR) with the peptide antigen major histocompatability complex (MHC) (signal 1). Signal 2 is generated via an interaction between costimulatory molecules on the antigen presenting cell (APC) and counter receptors on the T cell, such as the B7-CD28 interaction. In the absence of signal 2, the generation of signal 1 alone results in a state of inactivation and the pTh cell is rendered anergic. The 2 signal model oversimplifies the various ways in which a T cell reacts to each signal; in some cases T cell activation can occur in the absence of signal 2, if the TCR signal is very strong. In addition, positive and negative signals can be received by the T cell via the costimulatory receptors expressed on their surface (e.g. CTLA-4-B7 interaction). Also, the 2 signal model was designed with the activation of naïve pTh cells in mind, however the immune response to foreign Ag is a

diverse process in which costimulatory signals can be delivered to Ag-experienced T cells. It has become clear in recent years that the balance of protective immunity and tolerance relies on a tightly regulated regime of stimulation and inhibition signals (Sharpe and Freeman, 2002).

There are a growing number of T-cell costimulatory molecules, but the best characterized system is the B7-1/B7-2-CD28/CTLA-4 pathway (see Figure 1), discussed in detail below (Karandikar *et al.*, 1998; Oosterwegel *et al.*, 1999; Sansom, 2000; Salomon and Bluestone, 2001; Chambers *et al.*, 2001). B7-1/2 and CD28/CTLA-4 are now known to be part of a much larger family of molecules as at least two new pathways have been discovered in recent years. The first involves the interaction of ICOS (a member of the CD28 family, inducible T cell co-stimulator) with ICOSL (ICOS ligand) (Hutloff *et al.*, 1999). The second involves the PD-1 receptor which interacts with two new B7 family members, PD-L1 and PD-L2 (Ishida *et al.*, 1992; Freeman *et al.*, 2000). ICOS interaction with ICOSL enhances all basic T cell responses to a foreign Ag, namely proliferation, secretion of lymphokines, up-regulation of molecules that mediate cell-cell interaction and effective help for Ab secretion by B cells. Unlike CD28, ICOS is not constitutively expressed on the surface of T cells but has to be induced, and does not up-regulate the production of IL-2 but induces the synthesis of IL-10 (Hutloff *et al.*, 1999).

The PD-1 pathway is thought to be critical for the induction and/or maintenance of peripheral T cell tolerance to self-Ag. Expression of PDL is induced by IFN- γ and recent data suggests that this might mediate some negative regulatory effects of IFN-γ (Latchman *et al.*, 2001). Interaction of PD-1 with PD-L1 or PD-L2 results in inhibition

of TCR-mediated proliferation and cytokine production, although levels of IL-4 and IL-13 were not easily down-regulated, suggesting that Th2 cytokine expression may be less sensitive to the effects of the PD-1 pathway (Latchman *et al.*, 2001).The properties of the best characterised system, B7 interaction with CD28 or CTLA-4, are discussed below.

1.6.3. B7-1/B7-2-CD28/CTLA-4 pathway

CD28 and CTLA-4 (CD152) are transmembrane proteins, both of which are found on the surface of T cells. CD28 is abundantly expressed on the majority of resting T cells whereas CTLA-4 appears predominantly after T cell activation. These molecules share 30% homology and bind to the B7 family members with high affinity (Alegre *et al.*, 1996). The complexity of the CD28/CTLA-4 receptor interactions results from the fact that there are two natural ligands for these receptors, namely B7-1 (CD80) and B7-2 (CD86). Both of these molecules can interact with either receptor, however as they share only 25% homology, it has been postulated that these molecules have differing functions. B7-1 and B7-2 are found on the surface of APC such as DC, monocytes and activated B cells (Razi-Wolf *et al.*, 1992; Azuma *et al.*, 1993; Inaba *et al.*, 1994). B7-1 is induced slowly after activation, whereas B7-2 is abundant on the surface of APC and its expression is rapidly increased after stimulation. However, experiments in B7-1/B7-2 -/- mice, indicated that B7-1 is more potent in terms of activation, as naïve cells respond less well to B7-2 and this is perhaps associated with B7-2 binding to CD28 for a shorter time than B7-1. However, primed cells respond to both molecules equally (Fields *et al.*, 1998; Schweitzer and Sharpe, 1998).

1.6.4. CD28 and CTLA-4 – opposing functions

CD28 receives signals from both B7-1 and B7-2, which ultimately results in the co-stimulation which is required for efficient T cell activation. In the absence of this signal, the cells will become unresponsive and tolerant to Ag. This theory was substantiated when it was shown that APC, which were highly impaired in Ag presentation, were unable to activate T cells to respond to the same Ag (Jenkins *et al.*, 1987; Jenkins and Schwartz, 1987). Along with other experiments, which described the rescue of T cell anergy by the addition of a costimulatory signal, these studies are consistent with the notion that CD28-B7-1 interactions are critical for T cell activation (Gimmi *et al.*, 1991; Linsley *et al.*, 1991). Recently results have come to light that suggest that CD28 may play a role in the development and homeostasis of Treg cells. $CD4^{+}CD25^{+}$ cells, which control autoimmune diabetes, are profoundly decreased in CD28-/- and B7-1/B7-2-/- NOD (nonobese diabetic) mice. Adoptive transfer of this regulatory population into CD28 deficient animals can prevent or delay diabetes (Salomon *et al.*, 2000). Therefore a substantial amount of data suggests that CD28 is a critical molecule in T cell activation and regulation.

Several studies have shown that blocking CTLA-4 results in increased T cell proliferation (Krummel and Allison, 1995). The most convincing data set, however, comes from experiments carried out in CTLA-4-/- mice, which develop lympho-proliferative disease suggesting a key role for CTLA-4 in maintaining self-tolerance (Tivol *et al.*, 1995; Waterhouse *et al.*, 1995). However despite the undeniable importance of CTLA-4, its inhibitory pathway is still not fully understood. Interestingly, within 24 hours of activation (when surface levels of CTLA-4 are very low) CTLA-4 has been found to block T cell function, preventing the up-regulation of activation

markers, entry into the cell cycle and the generation of IL-2. CTLA-4 is detected post-activation on activated T cells at higher levels, however these are not typically the cells upon which it acts, suggesting that there may be an alternative function for CTLA-4 expression on the surface of activated T cells (Krummel and Allison, 1996; Walunas *et al.*, 1996). While it has been shown that CTLA-4 is crucial in maintaining a balance in T cell activation, matters are somewhat complicated by the fact that CD28 and CTLA-4 have opposite functions but share the identical ligands, B7-1 and B7-2.

Thus, it is of interest to decipher how T cells "decide" whether to use CTLA-4 or CD28. Various mechanisms have been described in different model systems, none of which are mutually exclusive. Firstly, the idea of ligand competition has been proposed in many studies. This model suggests that CTLA-4 acts as a competitive inhibitor for the ligands B7-1 and B7-2, so as to limit T cell activation. This concept is more likely to occur during secondary stimulation of T cells as resting T cells express undetectable amounts of CTLA-4. The ligand competition model of CTLA-4 regulation would require that CTLA-4 be expressed in sufficient levels to sequester ligands away from CD28, thus preventing a costimulatory signal being received. This model does not require a signalling component as it would rely on the higher affinity of CTLA-4 for B7-1 and B7-2 than CD28 (Chambers *et al.*, 1998; Chambers *et al.*, 1999).

Alternatively CTLA-4 signalling is a possible mode of action, whereby CTLA-4 is proposed to interfere with TCR signalling events (Marengere *et al.*, 1996; Brunner *et al.*, 1999). This hypothesis is supported by the observation that inhibition via CTLA-4 can occur in the absence of CD28 (Fallarino *et al.*, 1998). Finally it has been shown that a population of Treg cells express CTLA-4 on their surface (Takahashi *et al.*, 2000), so it is possible that these cells regulate T cell activation, a concept which is supported by

the development of spontaneous autoimmune disease when these cells are removed (Sakaguchi *et al.*, 1995; Itoh *et al.*, 1999) (these cells will be discussed in more detail in Section 1.9). For a schematic representation of the interactions of CTLA-4 and CD28 with their ligands refer to Figure 1.1.

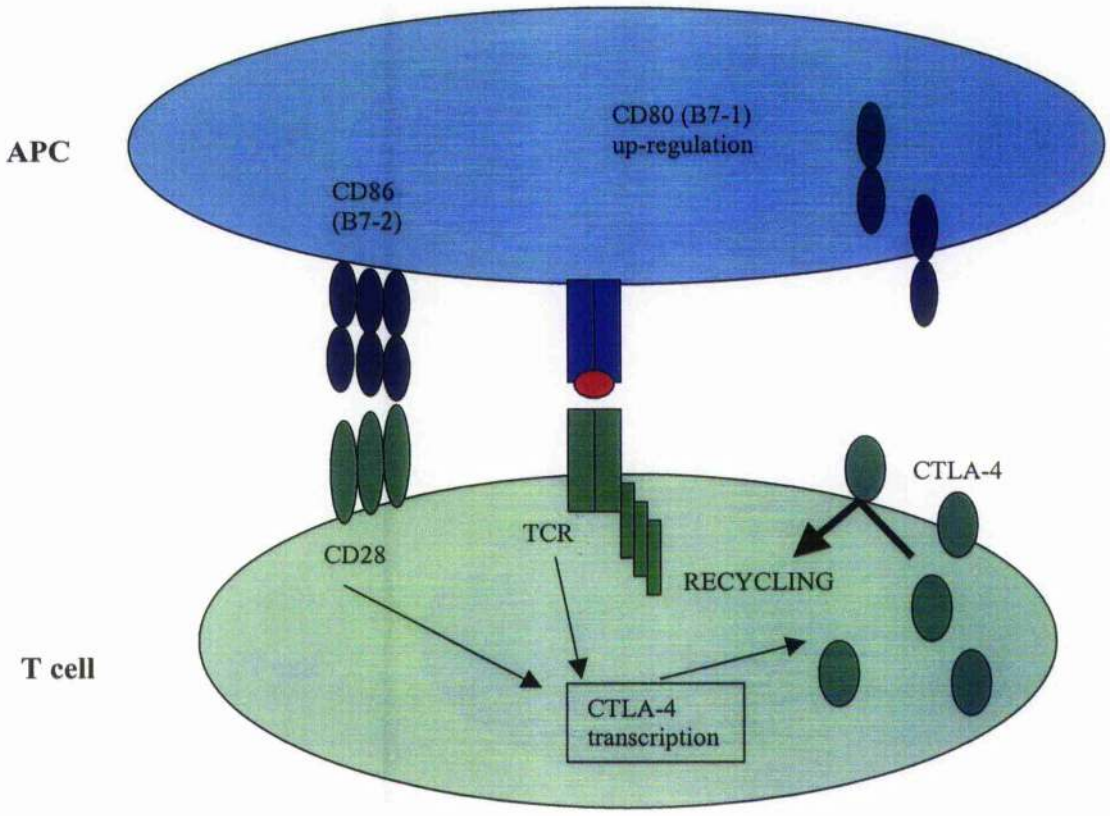


Figure 1.1. Diagram of CD28 and CTLA-4 interactions with their ligands

B7-2 is typically expressed at higher levels on the surface of APC and is found more widely than B7-1. B7-1 is induced on the surface of APC via a number of stimuli and is expressed at lower levels. B7-2 is therefore the primary ligand for CD28. CD28 expression is constitutive on the surface of T cells whereas CTLA-4 is not expressed on resting T cells. Interaction of B7 with CD28 and T cell receptor stimulation result in the up-regulation of CTLA-4, however CD28 stimulation is not absolutely required for up-regulation of CTLA-4. CTLA-4 expression is thought to be transient at the cell surface and rapidly re-internalised by a clathrin pit mechanism. It should be noted that both ligands can interact with both receptors.

1.7. Factors influencing the pathway of T cell differentiation

1.7.1. The role of the APC

Gajewski *et al* (1991) examined the ability of distinct APC populations to stimulate proliferation of OVA- specific Th1 and Th2 clones. It was shown that both subsets proliferated well in response to OVA in the presence of whole spleen cells, however, purified B cells stimulated optimal proliferation of Th2 clones. Optimal proliferation of Th1 clones was observed when adherent cells were used as APC. These results indicated that co-factors produced by professional APC influenced the differentiation of Th1 and Th2 cells, with B cells preferentially inducing a Th2 response whereas adherent cells induce a more Th1-like response. Further studies investigated the roles of various APC in driving a Th1 or Th2 response. In a study using the SCID mouse and the nematode parasite *Nippostrongylus brasiliensis*, it was shown that DC had the ability to prime both Th1 and Th2 cells (Ronchese *et al.*, 1994). However a subsequent study disputed this and demonstrated that DC predominantly produce IL-12, an important cytokine in driving the Th1 response. Furthermore, in this study it was shown that DC could induce the development of Th1 cells from Ag-specific naïve LECAM-1 (leukocyte endothelial adhesion molecule, CD62L) bright $CD4^+$ T cells obtained from αβ–TCR Tg mice, indicating that DC play a direct role in the development of IFN-γ producing Th1 cells (Macatonia *et al.*, 1995). Other studies have examined the role of macrophages in stimulating a biased response. Although macrophages are dedicated APC *in vitro*, they exert this activity only after activation with IFN-γ. However macrophages are an important source of IL-12 and may therefore favour the development of Th1 cells. Indeed, depletion of macrophages *in vivo* supports a Th2

response in BALB/c mice sensitized with bovine serum albumin (BSA) or ovalbumin OVA (Brewer *et al.*, 1994).

Since these discoveries, research into the role of APC has become much more advanced with the discovery of the "alternatively activated" APC (APC2). In parallel to the Th1/Th2 dichotomy, APC may be subdivided into pro-inflammatory, classically activated APC such as mature dendritic cells or IFN-γ activated effector macrophages and anti-inflammatory, alternatively activated APC such as IL-10 activated immature dendritic cells and IL-4 induced suppressor macrophages. These APC2 have now been shown to mediate an indispensable pathway of APC function. It is believed that classically and alternatively activated APC secure the balance between pro-inflammatory and anti-inflammatory immune reactions.

Classically activated macrophages are activated by exposure to IFNγ, TNF-α, or LPS whereas exposure to IL-4, IL-10 or PGE_2 elicits a population of alternatively activated macrophages. These cells are known to be important in the inhibition of T cell proliferation. One study examined the role of IL-4-induced alternatively activated macrophages in a human co-culture system *in vitro*. It was found that alternatively activated macrophages had the ability to inhibit mitogen-mediated proliferation of $CD4^+$ T cells independently of IL-10. The expression of costimulatory molecules, NO or prostaglandin synthesis was also down-regulated. Inhibition of phenotypic differentiation of T cells by alternatively activated macrophages is paralleled by a lack of maturation of T cells (Schebesch *et al.*, 1997). Alternatively activated macrophages have also been identified in helminth infection. Chronic infection of mice with *Taenia crassiceps* led to the generation of a macrophage population which could suppress the

proliferation of T cells and drive T cells to produce IL-4 and not IFN-γ (Rodriguez-Sosa *et al.*, 2002). These data are similar to those where PEC, recruited in response to *B. malayi*, were used to stimulate naïve Tg T cells. Upon primary stimulation, proliferation of these cells was inhibited, but upon secondary stimulation the cells proliferated normally and produced IL-4. In these studies the PEC were found to be dependent on IL-4 as they did not develop in IL-4-/- mice. Further characterisation of these cells identified them as alternatively activated macrophages (MacDonald *et al.*, 1998; Loke *et al.*, 2000a).

The DC1 and DC2 dichotomy was originally proposed to describe the capacity of monocyte-derived myeloid DC to stimulate allogenic T cells to produce IFN-γ and plasmacytoid DC to prime T cells to secrete IL-4 (Rissoan *et al.*, 1999). However, subsequent reports have shown that the induction of a distinct Th phenotype is not dependent on the particular DC lineage but instead depends on the state of activation and maturation of the DC (Cella *et al.*, 2000; Manickasingham *et al.*, 2003). Interestingly, recent data has suggested that, not only is there a role for DC in the differentiation of Th1 and Th2 cells, but that DC also have an important role in controlling the differentiation of Treg cells (see Figure 1.2) (Jonuleit *et al.*, 2000; Roncarolo *et al.*, 2001a; Mahnke *et al.*, 2002; van der Kleij *et al.*, 2002).T cells primed by tolerogenic DC (such as immature or IL-10 treated DC) can develop into Tregs. In general, however, the functional properties of DC are dependent on their maturational state. This was demonstrated in a seminal study, in which immature $CD83^-$ and mature $CD83^+$ human DC were used for stimulation of naïve, allogenic $CD4^+$ cells (Jonuleit *et al.*, 2000). Repetitive stimulation with mature DC resulted in the expansion of the $CD4^+$ cells with the production of Th1-type cytokines. After stimulation with the immature

DC the T cells displayed an inability to proliferate which could not be reversed by the addition of rIL-2, restimulation with PBMC or with mature dendritic cells. These cells displayed an up-regulation of CTLA-4 and despite losing their ability to produce IL-2, IL-4 and IFN-γ, could produce IL-10. In addition, in co-culture these cells had the ability to suppress Ag-driven proliferation of Th1 cells in a cell-contact dependent mechanism. These data strongly indicate that DC (depending on their state of activation) can drive the differentiation of Treg cells. In another important study it was shown that DC matured in the presence of a lipid derived from *S. mansoni* eggs (lyso-phosphatidylserine), results in the induction of IL-10-producing Treg cells via an interaction with TLR2 (Toll-like receptor). These data therefore suggest a role for tolerized/immature DC in inducing a Treg population in helminth infection (van der Kleij *et al.*, 2002).

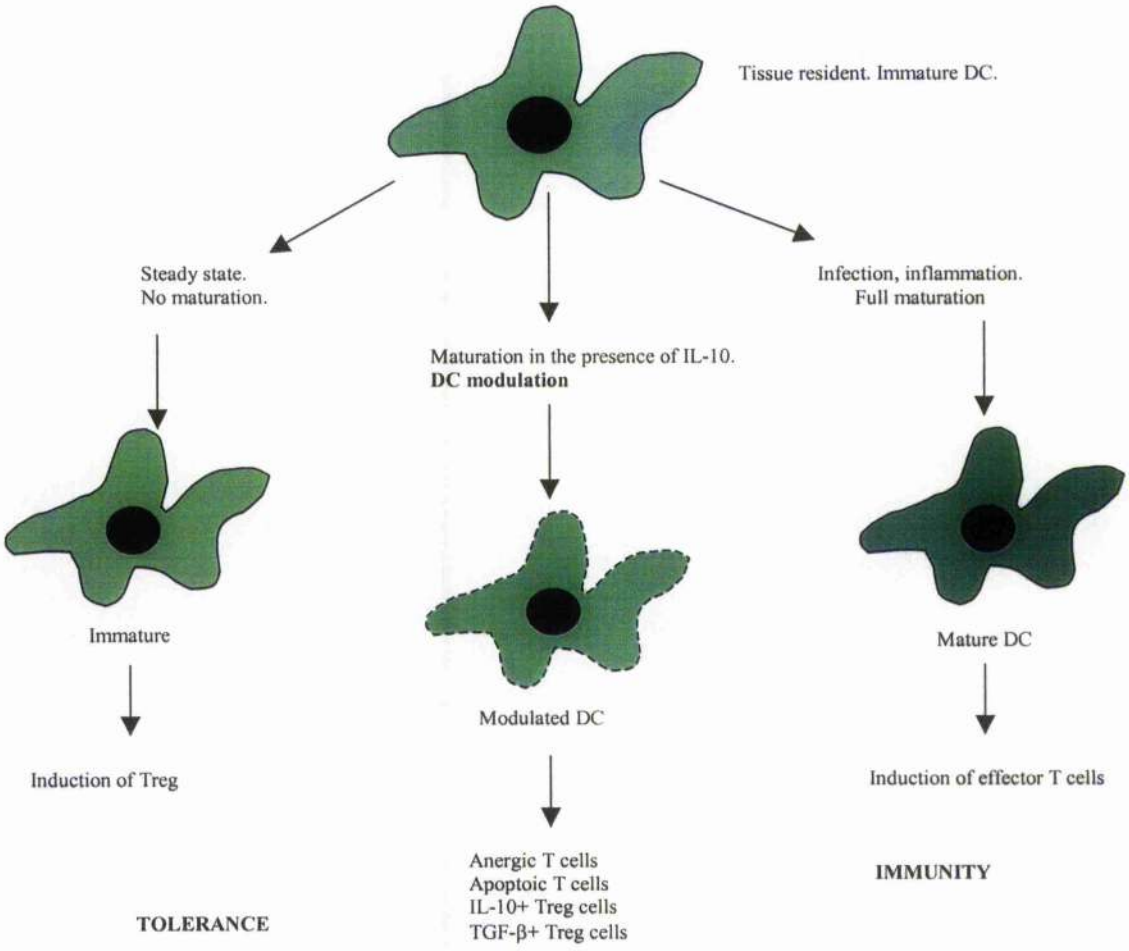


Figure 1.2. DC Maturation
Immature DC capture Ag from dying cells (self matter) and foreign Ag. In the steady state, DC remain immature but enter lymph nodes and prime resting T cells to become Treg cells. Post-infection with a microbe, pro-inflammatory cytokines stimulate the differentiation of DC and these cells mature. Mature DC efficiently prime resting T cells to develop into T effector cells. However the function of the mature DC can be significantly altered if matured in the presence of IL-10 or TGF-β. This results in the conversion of immunostimulatory DC into tolerizing DC (adapted from Mahnke *et al.*, 2002).

1.7.2. B7-1 and B7-2 can bias towards the Th1 or Th2 phenotype

In an initial study in the mouse model (Freeman *et al.*, 1995), it was shown that B7-1 and B7-2 do not deliver identical costimulatory signals. B7-1 and B7-2 co stimulation induces equivalent levels of IL-2 and IFN-γ, but B7-2 induces significantly more IL-4 production than B7-1, with the greatest difference observed in naïve T cells. Repeated stimulation of $CD4^+CD45RA^+$ T cells with B7-2 resulted in moderate levels of IL-4 and IL-2, but repeated stimulation with B7-1 resulted in IL-2 with minimal levels of IL-4. Therefore, this study concluded that B7-2 provides an initial signal to induce naïve T cells to become Th2, IL-4 producing cells, whereas B7-1 is a more neutral differentiative signal. These data were further substantiated by experiments carried out using anti-B7 MAb *in vivo* in a mouse model of experimental allergic encephalitis (EAE). Anti-B7-1 reduced the incidence of disease, whereas anti-B7-2 increased disease severity. Administration of anti-B7-1 at immunization resulted in the generation of Th2 clones, which, when adoptively transferred prevented the induction of EAE and cleared the established disease. Therefore B7-1 and B7-2 interactions with CD28 or CTLA-4 results in different clinical outcomes by influencing the differentiation of pTh cells towards a Th1 or Th2 phenotype (Kuchroo *et al.*, 1995).

The role of B7-1 and B7-2 in mice sensitized with *S. mansoni* eggs was investigated to determine whether either ligand effects expression of Th2 cytokines and the development of Th2-mediated pathologic reactions, or induction of pulmonary granuloma by i.v. injection of eggs. Anti-B7-2 treatment resulted in decreased levels of IL-4, IL-5 and IL-13 but had no effect on IFN-γ in animals that were inoculated with *S. mansoni* ova, *B. malayi* MF, or circulating filarial Ag (CFA). In contrast, when B7-1

was neutralized, IFN-γ was inhibited in mice sensitized with *S. mansoni* ova. In addition, anti-B7-2 treatment inhibited pulmonary granuloma formation by 74% and levels of IL-5 and IL-13 transcripts were decreased by 20- and 5-fold respectively compared to mice given Ig control only. Anti-B7-1 had no such effect (Subramanian *et al.*, 1997). These data therefore support the hypothesis that B7-1 tends to promote the differentiation of Th1 responses whilst B7-2 interactions promote Th2 differentiation.

1.7.3. The role of cytokines

The transmission between innate and adaptive immunity is a tightly regulated process, which involves both the secretion of soluble mediators and cell to cell contact. In particular the cytokine milieu that is established during the innate response to pathogens creates a suitable environment for the migration of Ag-specific T cells to lymph nodes where they meet APC. As described above Th1, Th2 and Treg differentiation is associated with the phenotype of the APC that is involved in presentation of Ag to the T cell. However T cell responses are also determined by the pattern of cytokines that are present during the clonal expansion of T cells (Mosmann and Coffman, 1989) as discussed below.

1.7.3.1. The IL-12 family of Th1 inducing cytokines

1.7.3.1.1. IL-12

IL-12 is a pro-inflammatory cytokine that favours Th1 differentiation and forms a link between innate and adaptive immunity (refer to Figure 1.3A). The main producers of IL-12 are phagocytes (i.e. monocytes, macrophages and neutrophils) and DC in response to pathogens (i.e. bacteria, fungi, intracellular parasites and viruses). IL-12

induces T cells and NK cells to produce several cytokines – for example GM-CSF and TNF-α, however it is extremely efficient at inducing the production of IFN-γ. It has also been shown that IL-12 has a strong synergistic effect with CD28/B7 interactions in inducing proliferation and cytokine production (Kobayashi *et al.*, 1989; Chan *et al.*, 1991; Kubin *et al.*, 1994; Murphy, 1994). The role of IL-10 in regulating IL-12 production has been the focus of many studies, showing that IL-10 inhibits IL-12 production and B7 expression on the surface of monocytes. These two effects are largely responsible for the ability of IL-10 to down-regulate IFN-γ production by lymphocytes, because anti-CD28 Ab and IL-12 can reverse the inhibitory effect of IL-10 on IFN-γ production (Kubin *et al.*, 1994).

IL-12 is expressed as a heterodimer formed by a 35-kDa light chain known as p35 and a 40-kDa heavy chain known as p40 (Kobayashi *et al.*, 1989). Recently it was discovered that IL-12 p40 associates not only with IL-12 p35 but also with another molecule p19, to form a new heterodimeric cytokine, IL-23 (Oppmann *et al.*, 2000). Another newly discovered cytokine is IL-27, which consists of Epstein Barr virus-induced gene 3 (EBI3), a p40 related protein, and p28, a newly discovered IL-12 p35 – related polypeptide (Pflanz *et al.*, 2002). Because of these recently discovered cytokines, it is now thought that the role of IL-12 has been over-interpreted. Many *in vivo* experiments carried out in the past used IL-12 p40-/- mice to investigate the role of IL-12. However when experiments were carried out in IL-12 p35 mice many discrepancies were found between the p40 and p35 -/- mice (Becher *et al.*, 2002). It now seems probable that at least some of these discrepancies are due to the fact that IL-23 also utilises the IL-12 p40 protein subunit.

1.7.3.1.2. IL-23 and IL-27

IL-23 is secreted by mouse and human DC, however the biological activities of IL-23 differ from that of IL-12, at least in the murine model. For example, unlike IL-12, murine IL-23 does not induce IFN-γ. IL-23 is also reported to induce strong proliferation of memory T cells but not of naïve T cells, whereas IL-12 has no effect on memory T cells (Oppmann *et al.*, 2000). In addition, it has been recently shown that murine IL-23 can induce the production of the pro-inflammatory cytokine IL-17 from memory T cells. However, in humans the function of IL-23 is less distinct from IL-12 (Oppmann *et al.*, 2000). Data from p19-deficient mice are clearly required to fully elucidate the role of IL-23 in the murine model. Studies on EAE (experimental autoimmune encephalitis) in p40-/- and p35-/- mice give contrasting results and suggest that IL-12 may not be as important for the establishment of disease as was once thought. Instead IL-23 fulfils this role. Recently, transgenic p19 (IL-23 deficient) mice have been shown to develop multi-organ inflammation, infertility and premature death. These mice display lymphocyte and macrophage infiltrates in organs and increased numbers of circulating neutrophils (Wiekowski *et al.*, 2001). These data clearly indicate a role for IL-23 in IL-12 related responses.

IL-27 is produced early by activated APC and has been shown to be functionally very similar to IL-12 in that it is able to induce clonal proliferation of naïve T cells and synergises with IL-12 in IFN-γ production by naïve T cells (Pflanz *et al.*, 2002). Interestingly IL-27 binds to a newly discovered receptor expressed in large amounts on the surface of naïve T cells, named WSX-1 (Yoshida *et al.*, 2001). This receptor does not associate with IL-12 (Chen *et al.*, 2000; Pflanz *et al.*, 2002) and has been shown to

be required in the early initiation, but not the maintenance, of the Th1 response (Yoshida *et al.*, 2001). This indicates that IL-27 may interact with naïve T cells before IL-12, driving Th1 development, but IL-12 is then required in the maintenance of this response (Yoshida *et al.*, 2001; Pflanz *et al.*, 2002).

In summary the expression of the receptors on T cells for the IL-12 family of cytokines is an important factor in the differentiation of Th1 cells. Expression of the receptor for IL-27 on naïve T cells enables the initiation of Th1 responses (Chen *et al.*, 2000; Pflanz *et al.*, 2002) and subsequent up-regulation of IL-12Rβ2 (Szabo *et al.*, 1997) allows IL-12 to become active in the induction and maintenance of effector Th1 responses (Park *et al.*, 2000; Yap *et al.*, 2000). However, activation of Th1 memory cells appears to be restricted to IL-23 (Oppmann *et al.*, 2000). IL-18 then synergizes with IL-12 after IL-12 has induced the IL-18R (Afkarian *et al.*, 2002) and this cascade of events will eventually lead to the production of IFN-γ and the resultant activation of macrophages and DTH responses. For a diagrammatic representation of the role of IL-12, IL-23, IL27 and IL-18 in the differentiation of Th1 type response, see Figure 1.3A.

1.7.3.2. IL-4 and IL-13

IL-4 was identified as a cytokine that was instrumental in the generation of the Th2 responses when it was shown that IL-4 and IL-2 are required for the *in vitro* generation of IL-4 producing T cells (see Figure 1.3B) (Le Gros *et al.*, 1990). When stimulated with either Con A or Ag in the presence of IL-4, $CD4^+$ cells become primed to follow the Th2 differentiation pathway and after 48 hours these cells switch off IFN-γ gene transcription (Nakamura *et al.*, 1997). These data indicated that IL-4 was important in directing pTh cells down the pathway of Th2 differentiation. Th2-driven responses are

important in disease processes including allergies, asthma and helminth infection. Although IL-4 is a key cytokine in the development of Th2 responses, recent studies of IL-4 receptor (IL-4R)-mediated signalling pathways have implied the presence of alternative routes for Th2 induction (Kaplan *et al.*, 1996; Shimoda *et al.*, 1996; Takeda *et al.*, 1996; Barner *et al.*, 1998). One other way in which Th2 development can be affected is via IL-13, posing the question of how these cytokines can compensate for one another (McKenzie, 1998).

IL-13 shares ~30% homology with IL-4 and appears to have overlapping biological activities. This is due, in part, to these two cytokines utilising the common IL-4Rα chain as a component of their receptor complexes and signalling through a shared signal transducer and activator of transcription (STAT) 6-dependent pathway (Zurawski *et al.*, 1993). IL-4 and IL-13 are produced mainly by Th2-like cells and mast cells (Burd *et al.*, 1995) and it has been demonstrated that they share many biological functions. They both have the ability to suppress the production of IL-1 and TNF-α from monocytes and macrophages (de Waal Malefyt *et al.*, 1993a) and can induce B cells to undergo Ig isotype switching (Punnonen *et al.*, 1993). There are however differences in their molecular function, notably the ability of IL-4 to induce cellular proliferation. The functions of IL-13 and IL-4 are best described when examining their role in infection with *L. major* and *T. muris* (discussed in Section 1.8). For a diagrammatic representation of the pathway of Th2 development, see Figure 3B.

1.7.3.3. IL-10 and TGF-β

IL-10 was originally identified as a molecule secreted by Th2 cells that could inhibit the synthesis of Th1 cytokines. IL-10 is produced by most haemopoitic cells and its

expression is regulated both transcriptionally and at the level of mRNA stability. The functional receptor for IL-10 consists of a heterodimer between IL-10R1 and IL-10R2, (reviewed Moore *et al.*, 2001). The expression of IL-10R determines whether cells will respond to the effects of IL-10. For example, T cells can down-regulate expression of IL-10R1 to become resistant to the suppressive effects of IL-10, whereas activated monocytes can up-regulate IL-10R1 and become susceptible to a negative feedback pathway (Liu *et al.*, 1994). Like other cytokines from this family, IL-10 mediates its effects via the activation of the Jak/STAT pathway (Moore *et al.*, 2001). The principal role of IL-10 is to down-regulate overwhelming and dangerous inflammatory immune reactions. To do this IL-10 has the ability to suppress virtually all pro-inflammatory cytokines and chemokines, while at the same time inhibiting the expression of B7-1 and B7-2 on the surface of APC (Moore *et al.*, 2001).

TGF-β belongs to a large family of proteins, which are highly conserved throughout evolution. In mammals, three members of this family, TGF-β1 - β3, have an important role in regulating immune responses. All three molecules signal through the same receptor complex which consists of TGF-βR1 and R2 (Letterio and Roberts, 1998). The effect of TGF-β is mediated by activation of the SMAD family of transcription factors (Attisano and Wrana, 2002). TGF-β is synthesised as a precursor protein and is released in an inactive form as either a small or large latent complex (Gleizes *et al.*, 1997). In the small complex, a homodimer of active TGF-β is associated with a protein, known as latency associated protein (LAP), which consists of a homodimer of inactive TGF-β. In the large complex, LAP is additionally associated with the latent TGF-β binding protein (LTBP), which then allows the complex to associate with the extracellular matrix. Activation of TGF-β *in vitro* can be achieved by disruption of the non-covalent

interaction between TGF-β and LAP by heating or acidification. *In vivo* activation occurs extracellularly and likely involves the cleavage or conformational modification of LAP (Gleizes *et al.*, 1997). TGF-β has anti-proliferative effects on $CD4^+$ T cells as it has the ability to block production of IL-2 and to up-regulate cell cycle inhibitors (Gorelik and Flavell, 2002). This suppressive cytokine also has the ability to block the differentiation of Th1 and Th2 cells via effects on the transcription factors required for expression of IFN-γ (T-bet) (Gorelik *et al.*, 2002) and IL-4 (GATA-3) (Heath *et al.*, 2000). In terms of APC, TGF-β can inhibit the activation of macrophages and their ability to produce pro-inflammatory cytokines (Gorelik and Flavell, 2002).

TGF-β and IL-10 were shown to work synergistically with one another in down-regulating immune responses. In studies in mice, IL-10 has been shown to regulate the production of TGF-β and vice versa and it was initially shown that exogenous IL-10 can stimulate TGF-β *in vitro* (Seder *et al.*, 1998). This observation was further substantiated by experiments examining intestinal epithelial cells transgenic for IL-10 (De Winter *et al.*, 2002), which display increased levels of TGF-β. Furthermore, IL-10-/- mice produce less TGF-β than wild-type mice (De Winter *et al.*, 2002). In a reciprocal fashion TGF-β can also enhance levels of IL-10. For example, treatment of colitis by administration of a plasmid encoding TGF-β provides a beneficial effect via elevated levels of IL-10 production (Kitani *et al.*, 2000). These studies indicate that blocking one of these suppressive cytokines has a corresponding effect on the other.

T cell anergy is described as a continuous state of unresponsiveness to rechallenge with fully competent APC. There are two ways in which a T cell can be rendered anergic. The first way has been discussed previously (section 1.6.2) and involves the absence of

co-stimulation ("signal 2"). Alternatively T cell anergy can be induced when T cells are activated in the presence of IL-10 or are stimulated with APC which have been pre-treated with IL-10 (Groux *et al.*, 1996; Steinbrink *et al.*, 2002). These results show that not only can IL-10 effect T cell function via the APC, but IL-10 can also have a direct effect on T cells themselves. After cells have become anergized by the presence of IL-10, they acquire regulatory properties which are cytokine independent (Steinbrink *et al.*, 2002). In addition this level of unresponsiveness cannot be reversed by the addition of rIL-2 (Groux *et al.*, 1996; Perrin *et al.*, 1999). IL-10 anergized cells are unable to produce cytokines, but when "forced" to proliferate with high concentrations of anti-CD3, a population of T cells which has the ability to produce cytokines arises. These cells produce IL-10 and TGF-β and have been termed Tr1 cells (as discussed in Section 1.9) (Groux *et al.*, 1997). Therefore it appears that these regulatory cells go through two stages of differentiation to become Tr1 cells. It has been suggested that these are the most powerful regulatory cells, because of their direct effect on other T cells, and their ability to affect proliferation via APC by the production of IL-10.

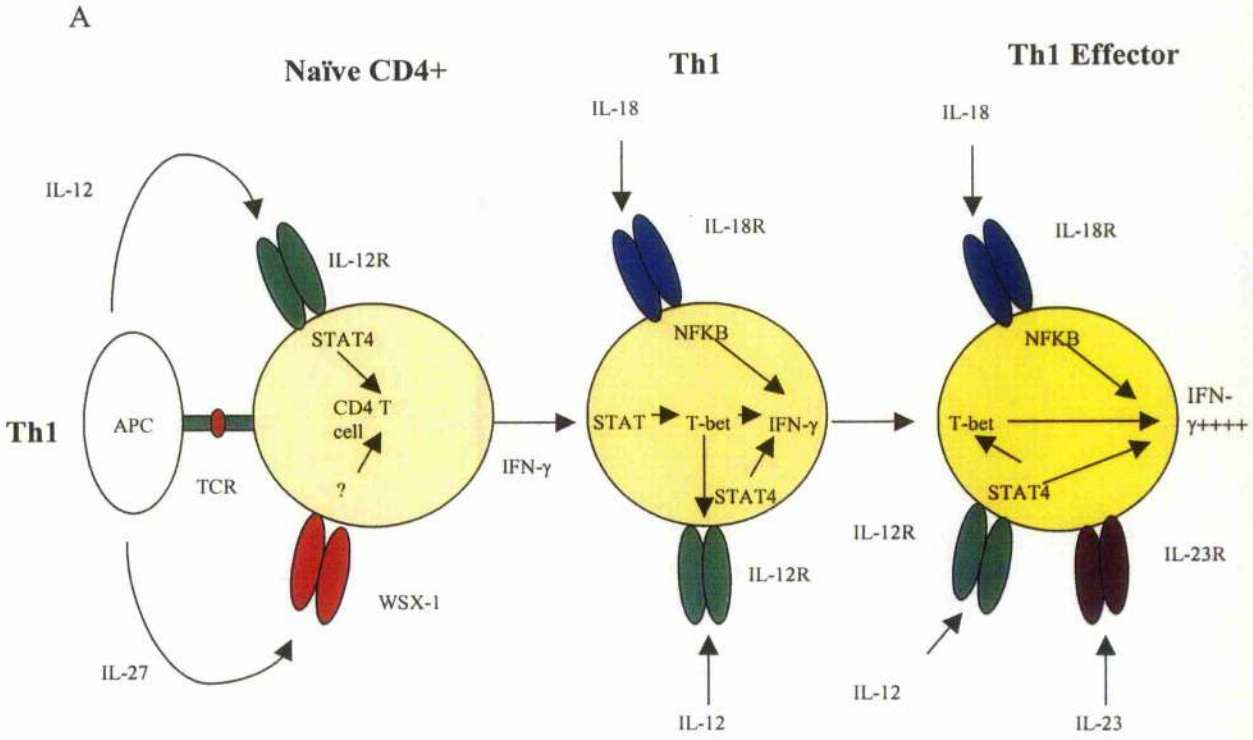
In the mouse, IL-10 alone is not sufficient to induce a state of hyporesponsiveness equivalent to that seen in human cells. Only mouse T cells primed with IL-10 and TGF-β are hyporeponsive *in vivo* (Zeller *et al.*, 1999). However, studies into human T cell anergy reported that IL-10 was fully capable of inducing anergy without TGF-β . In addition, rTGF-β1 or 2 added to IL-10 did not increase the level of anergy induced by IL-10, nor synergize with IL-10 to promote the differentiation of Tr1 cells (Levings *et al.*, 2001). The reason for the differing role of TGF-β in the mouse and in the human remains to be elucidated. In conclusion it appears that both IL-10 and TGF-β are required for the differentiation of Tr1 cells in the mouse model, whereas IL-10 has the

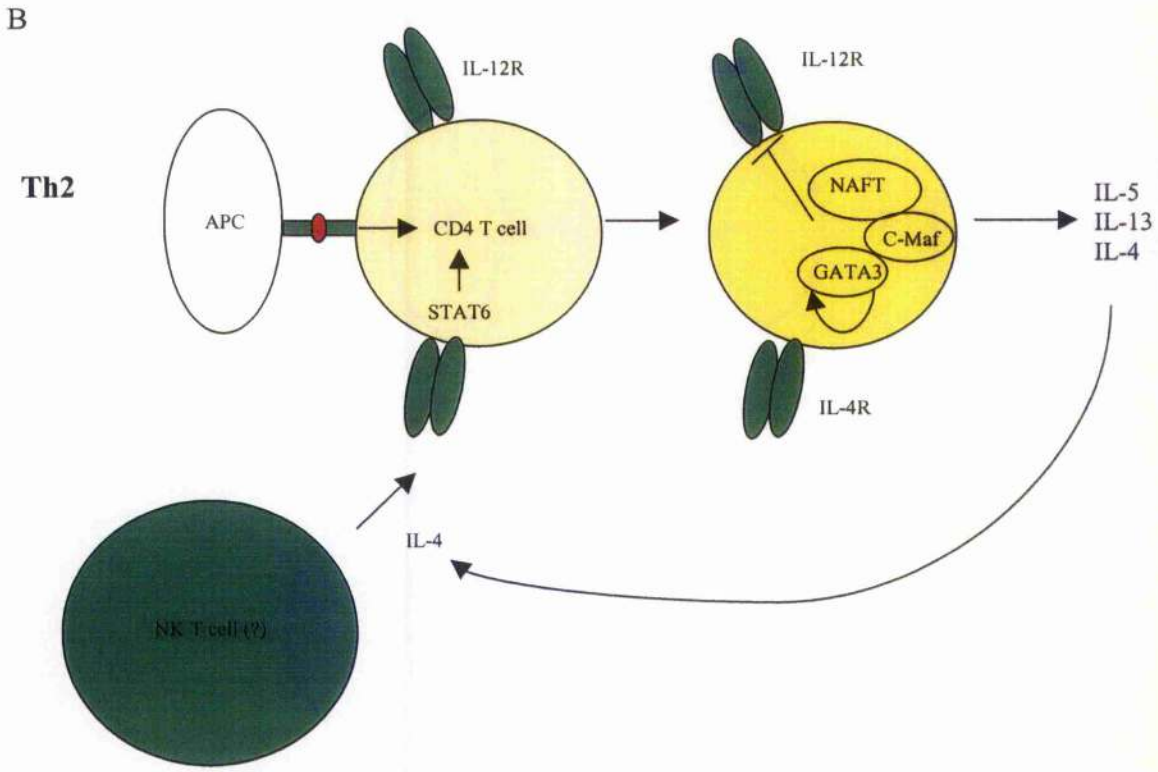
ability to function independent of TGF-β in the generation of these cells in the human system.

Figure 1.3. Differentiation processes of Th1 and Th2 cells

A. The delivery of Ag to the TCR via the APC, results in an up-regulation of IL-12R. APC derived IL-12 and IL-27 results in the priming of naïve T cells toward a Th1 phenotype. IL-27 binds to WSX-1 and IL-12 signals via STAT4 to initiate the up-regulation of IFN-γ. IFN-γ activates STAT1,which induces the transcription factor T-bet. IL-12 induces the IL-18 receptor, which allows binding of IL-18 which results in IL-18 synergizing with IL-12 to increase IFN-γ. IL-18 can activate NFκB which can also amplify the IFN-γ response. Memory T cells respond to IL-23 which enhances proliferation of effector cells and increases IFN-γ responses (adapted from Robinson and O'Garra, 2002).

B. The mechanisms involved in Th2 differentiation are less well understood. IL-4 is the key cytokine involved in inducing Th2 differentiation, however the early source of IL-4 varies in different models. NK cells, mast cells or differentiating T cells themselves have been proposed to produce early IL-4. Early IL-4 activates STAT6, which induces GATA3. GATA3 has many important effects including involvement in chromatin remodelling of the IL-4/IL-13 locus. Also, c-Maf and NFAT transcription factors are important in regulating production of IL-4, IL-5 and IL-13 (adapted from, Agnello *et al.*, 2003).


A
Naïve CD4+
Th1
Th1 Effector
IL-18
IL-18
IL-12
IL-12R
IL-18R
IL-18R
STAT4
NFKB
NFKB
Th1
APC
CD4 T cell
STAT
T-bet
IFN-γ
T-bet
IFN-γ++++
TCR
?
IFN-γ
STAT4
STAT4
IL-12R
IL-12R
IL-23R
WSX-1
IL-27
IL-12
IL-12
IL-23


B
IL-12R
IL-12R
Th2
APC
CD4 T cell
NAFT
IL-5
IL-13
IL-4
C-Maf
GATA3
STAT6
IL-4R
IL-4
NK T cell (?)

1.8. Parasitic models of Th1/Th2 development

In terms of parasite immunology, the first demonstration of the importance of the Th1 /Th2 balance in the regulation of disease outcome *in vivo* came from studies in the mouse model of *Leishmania major*. Infection of inbred strains of mice with *L. major* demonstrated that the differential development of Th1 or Th2 cells during experimental cutaneous leishmaniasis determines the outcome of infection (Reiner and Locksley, 1995). Infection of resistant mice (C57BL/6, CBA and C3H/He) with *L. major* results in the onset of an IL-12 dependent Th1 response, which results in elevated levels of IFN-γ and healing. BALB/c mice, on the other hand, are susceptible to infection and develop a parasite specific Th2 response, with elevated levels of IL-4, progressive lesions and eventually die. It has been shown that early production of IL-4 results in Th2 polarisation and therefore the suppression of the Th1 IFN-γ response which is responsible for activating infected macrophages for parasite killing (Green *et al.*, 1990; Liew *et al.*, 1990). Early IL-4 is produced by a population of $CD4^+$ cells with a Vβ4Vα8 T cell receptor, as Vβ4-deficient BALB/c mice mount a stronger Th1 response than wild-type mice and appear to control lesion development (Launois *et al.*, 1997).

However the role of IL-4 in disease progression is controversial as demonstrated by experiments in IL-4Rα mice (Mohrs *et al.*, 1999; Noben-Trauth *et al.*, 1999), STAT6, and BCL6 deficient mice (Dent *et al.*, 1999). The complex interplay of IL-4 and IL-13 was dissected using IL-13 transgenic mice and IL-13 -/- mice (Matthews *et al.*, 2000). In this study it was shown that over expression of IL-13 in transgenic mice makes the normally resistant C57BL/6 mouse strain susceptible to *L. major* infection even in the absence of IL-4. In addition IL-13 -/- BALB/c mice are resistant to infection. This

resistance is independent of IL-4, however when IL-4 was deleted along with IL-13 there was an additive effect (Matthews *et al.*, 2000). The healing response of *L. major* infection is characterised by an early Th1 response with elevated levels of IFN-γ and IL-12 (Nacy *et al.*, 1991; Swihart *et al.*, 1995). Interestingly a recent study has reported a role for WSX-1 in the initiation of Th1 responses and resistance to *L. major* infection. WSX-1-/- mice were remarkably susceptible to *L. major* infection, showing impaired IFN-γ production in early phases of infection. However, IFN-γ production during the later stages of infection was not impaired in the WSX-/- mice. These data therefore indicate that WSX-1 is essential for the initiation of Th1 responses but dispensable for their maintenance (Yoshida *et al.*, 2001). The role of IL-10 in promoting disease progression in leishmaniasis was investigated by examining lesion development following *L. major* infection of susceptible BALB/c mice deficient in IL-10. Where normal BALB/c mice develop unhealing progressive lesions containing many parasites, IL-10-/- mice controlled disease progression and relatively small numbers of parasites were recovered.

The importance of the Th1 response in clearing infection with *L. major* has been widely demonstrated whereas IL-4 and IL-13 have been shown to be important in maintaining infection. In contrast, resistance to the gastrointestinal nematode *T. muris* is dependent on the Th2 pathway. When IL-4 was blocked in resistant mice, a Th1 response was generated with the development of chronic disease. In addition, administering IL-4 to strains of mice which are susceptible to infection with *T. muris* resulted in the expulsion of worms (Else *et al.*, 1994). These results were later confirmed in IL-4-/- mice infected with *T. muris*. These animals failed to mount a sufficient Th2 response and therefore could not expel the parasite (Bancroft *et al.*, 1998). In further experiments it was found

that the background strain of mouse was important in whether worms were expelled or not. For example, C57BL/6 mice deficient in IL-4 develop chronic unresolving infection, while BALB/C mice deficient in IL-4 clear infection suggesting an IL-4 independent pathway of immunity, at least in BALB/c mice (Bancroft *et al.*, 1998; Artis *et al.*, 1999; Bancroft *et al.*, 2000). This led to studies which investigated the role of IL-13 in *T. muris* infection. As discussed in Section 1.7.3.2, IL-4 and IL-13 have overlapping biological activities, therefore it was important to understand the function of this cytokine in regulating Th2 responses. IL-13 was neutralized *in vivo* in IL-4-/- C57BL/6 mice and it was shown that these mice could expel their parasites, thus indicating that IL-13 is an important cytokine in regulating resistance to *T. muris*.

In contrast, mouse strains that are susceptible to *T. muris* respond to infection by producing IFN-γ and IL-12 and develop chronic infection (Else *et al.*, 1992). The importance of IFN-γ in the maintenance of infection in susceptible strains of mouse (e.g. B10.BR, AKR) was demonstrated by neutralization of this cytokine *in vivo*, resulting in the expansion of Th2 cells and clearance of infection (Else *et al.*, 1994). In addition, when rIL-12 was administered to a resistant strain of mice (BALB/K), these mice developed elevated levels of IFN-γ and were unable to clear their parasites (Bancroft *et al.*, 1997). IL-18 has been shown to work in synergy with IL-12 in promoting Th1 priming (Okamura *et al.*, 1995) and has a role in the regulation of responses to *T. muris*. In susceptible animals infected with *T. muris*, IL-12, IL-18 and IFN-γ mRNA were detected in sequence. IL-18-/- mice expelled their worms faster than IL-12-/- mice. IL-18-/- mice did not produce significantly different amounts of IFN-γ compared to their wild type counterparts but they did produce significantly more IL-13. Interestingly, when resistant mice were given rIL-18, the mice developed a chronic infection. Similar

results were obtained in IFN-γ -/- mice indicating an important role for IL-18 in regulating immune responses to *T. muris* (Helmby *et al.*, 2001).

The role of IL-10 in regulating immune responses to *T. muris* has also been investigated. It was found that normally resistant mice (C57BL/6) mice were rendered susceptible to infection with *T. muris* when IL-10 was knocked out. This susceptibility correlated with an increase in levels of Th1 cytokines (Schopf *et al.*, 2002). Taken together, these data demonstrate the importance of a Th1 response for establishment of infection, whereas the production of Th2 cytokines plays a vital role in the expulsion of this parasite. Interestingly, additional experiments have identified a role for TNF-α in the regulation of IL-13 mediated expulsion of *T. muris* (Artis *et al.*, 1999). Using mice deficient in TNF-αR signalling or anti-TNF-α MAb treatment has demonstrated a role for TNF-α in regulating Th2 cytokine-mediated responses. *In vivo* blockade of TNF-α in normally resistant mice did not alter IL-4, IL-5 or IL-13 production in the draining lymph node, but levels of worm expulsion were significantly delayed. TNFR -/- mice failed to expel *T. muris*, producing high levels of Th1 cytokines suggesting that the absence of TNF-α at the onset of infection alters the phenotype of the response elicited.

1.9. Treg cells

Although evidence for polarized $CD4^+$ T cell cytokine secretion profiles that define Th1 and Th2 subsets is indisputable, these extremely polarized responses are usually observed under highly contrived experimental conditions. The real challenge to the Th1-Th2 paradigm comes when attempts are made to apply this simplistic dichotomy in explaining immune system-dependent disease conditions. These and other issues have recently caused a spate of interest in a recently re-discovered group of cells, namely the

Treg cells. These cells have a suppressive function and are distinguishable by their cytokine profile (IL-10 and TGF-β) and mechanism of suppression. So far at least three separate T regulatory cell types have been characterised. Th3 cells were first discovered during studies into oral tolerance, where low dose administration of Ag was known to result in active suppression of immune responses. These cells are IL-4 independent and CD86 dependent, requiring interaction with APC. Th3 differentiation is enhanced by culture with TGF-β, IL-4, IL-10 and anti-IL-12, and mature Th3 cells predominantly secrete TGF-β and varying amounts of IL-10 and IL-4. In addition, these cells express CTLA-4 on their surface (Weiner, 2001). Tr1 cells were initially discovered in studies of $CD4^+$ cells which were activated in the presence of IL-10 and rendered anergic. Analysis of this population at the clonal level showed a subset of T cell clones with a unique cytokine profile, secreting high levels of IL-10 and TGF- β, moderate IFN-γ and IL-5 with little or no IL-2 or IL-4. These cells have been shown to be involved in the down-regulation of immune responses *in vivo* and *in vitro* via the production of IL-10 and TGF-β (Roncarolo *et al.*, 2001a) Lastly, the third group of Treg cells are the naturally occurring $CD4^+CD25^+$ cells which have potent suppressor functions *in vivo* and *in vitro*. Removal of these cells from normal rodents is associated with the development of various autoimmune diseases. Although it has been suggested that suppression by these cells is cytokine independent, they are known to produce IL-10, TGF- β and low levels of IL-4 or IL-2. Consequently the role of these cytokines is controversial. One study suggested that the production of IL-10 from $CD4^+CD25^+$ cells may lead to the induction of $CD4^+CD25^-$ cells which suppress immune responses via the production of IL-10 (i.e. $CD4^+CD25^-$ cells induce Tr1 like cells) (Dieckmann *et al.*, 2002). $CD4^+CD25^+$ cell suppression is thought to be linked to their ability to inhibit IL-2 production but also requires cell to cell contact to induce suppression which may be

associated with surface bound TGF-β, LAP, CTLA-4 or GITR. In addition these cells differ from Th3 and Tr1 cells in that $CD25^+$ cells exist in the thymus as suppressor cells, while Th3/Tr1 cells can arise from any naïve peripheral $CD4^+$ cell (Read and Powrie, 2001). Until recently very little was known about the molecular mechanism of the development of $CD25^+$ regulatory cells, but in a study by Hori *et al* (Hori *et al.*, 2003) the gene *Foxp3* was shown to encode a transcription factor which is specifically expressed in naturally arising Treg cells (see Figure 1.4A).

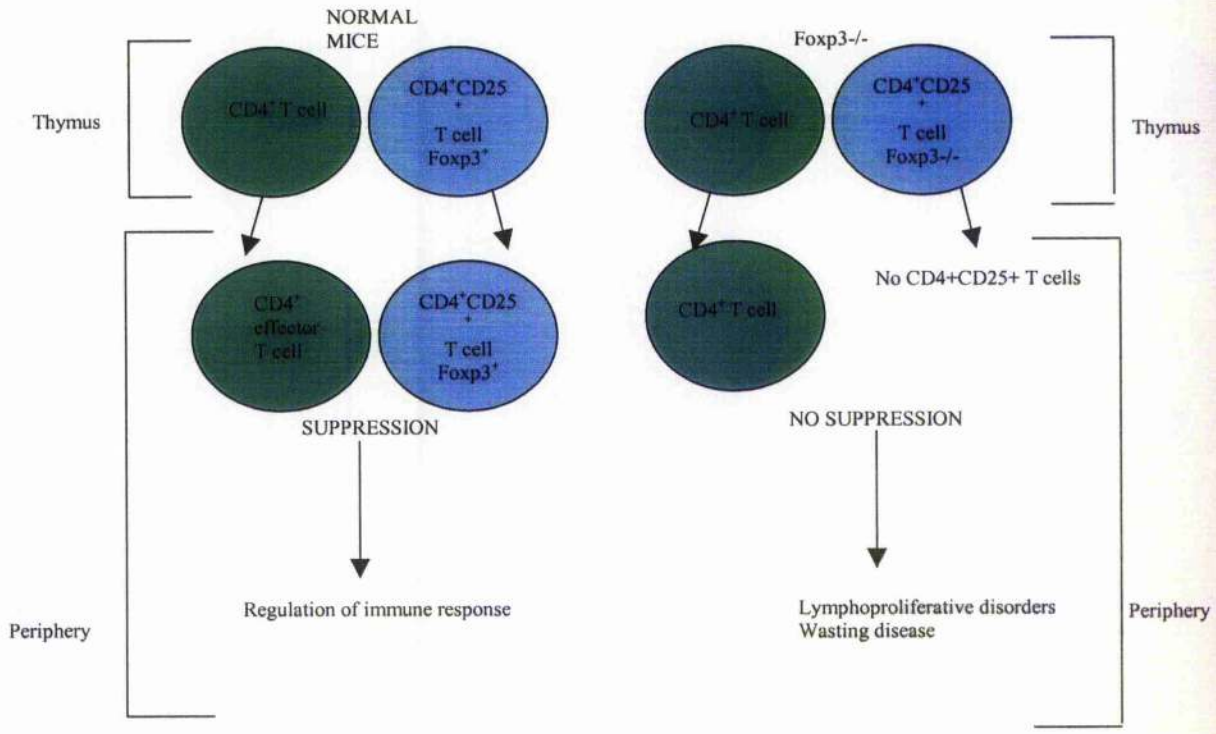
Regulatory cells have the capacity to inhibit potentially harmful immune responses and have been described in various experimental systems. Best characterised are the $CD4^+CD25^+$ cells which have been shown to prevent inflammatory bowel disease (Read *et al.*, 2000) and inhibit autoimmune diabetes in rats (Stephens and Mason, 2000). Treatment with monoclonal Ab specific for CD25 has been shown to suppress a variety of tumours in mice (Onizuka *et al.*, 1999). In addition, several studies have indicated the role of $CD4^+CD25^+$ cells in transplant tolerance, by preventing autoimmunity and enabling the development of tolerance to organ, tissue and cell transplants (Wood and Sakaguchi, 2003). Regulatory T cells also function in immune responses to various infectious agents, whether it be fungal, viral, bacterial or parasitic infections and these studies are reviewed in Chapter 5. Figure 1.4 illustrates the pathways leading to the development of Treg cells.

Figure 1.4. Diagrammatic representation of the development of Treg cells

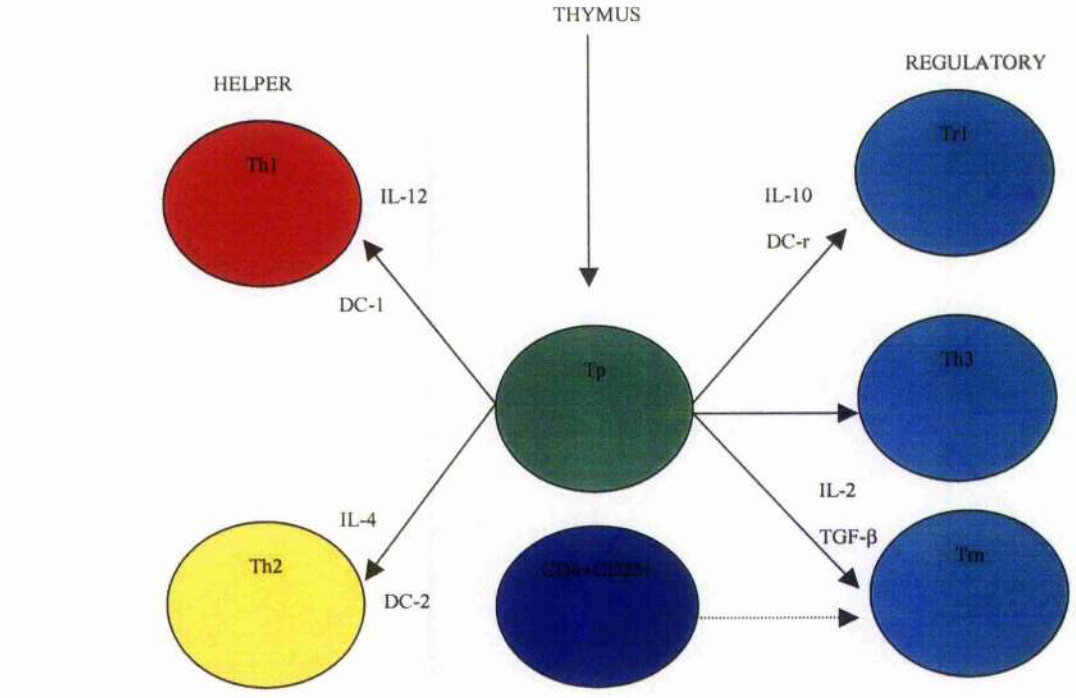
A. Treg cells which express Foxp3 are produced in the thymus, however CD4+ cells which are found in the periphery may also acquire regulatory properties. Loss of function mutations of Foxp3 results in wasting disease and lymphoproliferative disorders. Treg cells inhibit activation of CD4+ effector cells, therefore have an important role in the prevention of these disorders (adapted from O'Garra and Vieira, 2003).

B. Naïve T cells differentiate in the thymus and cells which are selected include low-affinity TCR T cells and high affinity TCR natural $CD4^{+}CD25^{+}$ Treg cells (Trn). Conventional T cells and Tregs are exported to the periphery where they respond to Ag. Conventional CD4+ cells become either T helper or Treg cells depending on the affinity of their TCR to Ag, the strength of co-stimulatory signals and the cytokine environment. Stimulation of naïve T cells with regulatory DC can result in the generation of a population of Treg cells (Tr1 cells), in addition IL-10 can also induce this population. TGF-β and IL-2 can cause Thp cells to differentiate towards a Th3 phenotype. These two Treg cells produce IL-10 and TGF-β and can suppress T cell prolferation in a cytokine dependent manner. $CD4^{+}CD25^{+}$ cells (Trn) suppress T cell proliferation in a cytokine independent, contact dependent manner *in vitro* (adapted from, Horwitz *et al.*, 2003).

A


NORMAL MICE
Thymus
CD4+ T cell
CD4+CD25+ T cell Foxp3+
CD4+ effector T cell
CD4+CD25+ T cell Foxp3+
SUPPRESSION
Regulation of immune response
Periphery
Foxp3-/-
CD4+ T cell
CD4+CD25+ T cell Foxp3-/-
Thymus
No CD4+CD25+ T cells
CD4+ T cell
NO SUPPRESSION
Lymphoproliferative disorders
Wasting disease
Periphery

B


THYMUS
HELPER
REGULATORY
Th1
IL-12
DC-1
Tp
IL-10
DC-r
Tr1
Th3
IL-2
TGF-β
Tm
IL-4
Th2
DC-2
CD4+CD25+

1.10 Aims of thesis

The infective third stage larvae of filarial worms have potent immuno-modulatory effects on the host immune system. The aims of this thesis were to define how L3 modulate cytokine and cellular immune responses in a well-characterised mouse model of infection.

The specific aims are detailed below.

- IL-4 is a prototypic Th2 cytokine and is secreted by PBMC of all individuals in an endemic area. Ag-specific IL-4 is produced by splenocytes and lymph node cells from L3 infected BALB/c mice, therefore it was of interest to investigate whether IL-4 had a role in worm survival and to analyze immune responses in IL-4-/- mice.

- IL-10 is known to be important in maintaining hyporesponsiveness in MF+ individuals and is up-regulated in L3 infected mice. However, as the cellular sources of IL-10 have not been defined, I set out to investigate the role of IL-10 in L3 infection and to identify the major source of IL-10 in the mouse model.

- Recent data suggests the presence of Treg cells in chronic infectious disease. Although the L3 of *Brugia* is the infective form, the production of elevated IL-10 suggests that the L3 may induce a suppressor cell population. Therefore experiments were carried out to determine whether infection with L3 elicits Treg cells.

- There are many immune cells which are known to present Ag to T cells and the capacity of B cells to carry out this function, along with their ability to produce modulatory cytokines was investigated in the L3 BALB/c model.

Chapter 2. Materials and Methods

2.1. The Parasite

2.1.1. Mosquito life cycle

Mosquitoes (*Aedes aegypti*) were kept in mesh cages in a purpose built insectary maintained at a temperature of 28°C and relative humidity of 75-80%. To maintain stocks, mosquitoes were fed twice weekly with heparinized rabbit blood using an artificial membrane feeder. Eggs were collected on moist Whatman 3 mm filter papers which were placed in cages. The papers were then removed and dried and stored until required. Eggs were hatched by placing the egg papers in plastic trays containing tap water with yeast tablets to feed the developing larvae. Pupae were picked daily and placed in cages. Adult mosquitoes were fed on a diet of sucrose before being starved 24 hours prior to receiving a blood meal.

2.1.2. Parasite life cycle

Adult parasites and microfilaria were obtained from the peritoneal cavity of infected jirds (*Meriones unguiculatus*), which had been infected intraperitoneally with 250 L3 of *B. pahangi* for at least three months. Jirds were killed by CO_2 inhalation. Worms were obtained by repeated peritoneal lavage with Hanks Balanced Salt Solution (HBSS, Invitrogen) at 37°C. Adult worms were washed in HBSS and transferred to liquid nitrogen until they were required for the preparation of parasite extracts. The MF contained in the peritoneal washings were given a further wash in HBSS before being resuspended in heparinized rabbits blood at a concentration of 400/20 µl. The infected blood was then fed to mosquitoes using an artificial membrane feeder.

2.1.3. Recovery of infective larvae and microfilariae

Microfilariae were obtained from the peritoneal cavity of infected jirds by extensive washing with HBSS. 1ml ddH_2O was added to lyse any erythrocytes present. MF were then washed in HBSS at 37°C and separated from host cells by centrifugation twice over Histopaque-1077 (Sigma, Poole, Dorset) at 1200 rpm for 15 minutes with no brake. MF were washed twice, resuspended in HBSS and counted. L3 were extracted from infected mosquitoes at day 9 p.i. using standard methods (Devaney and Jecock, 1991).

2.1.4. Preparation of adult antigen extracts

Soluble extract of *B. pahangi* adult worms, for use in cell culture, was prepared by extensive homogenisation on ice of frozen mixed sex adult worms in RPMI-1640 (Dutch Modification, containing 5 mM glutamine, 5 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin, all Invitrogen, UK). The homogenate was incubated on ice for a further 60 minutes with occasional agitation. The suspension was transferred to an Eppendorf tube and centrifuged at 4°C at 10,000g for 30 minutes. The supernatant was removed and sterilized by centrifugation through a 0.45 µm Spin-X filter unit (Co-star, UK) and assayed for protein concentration using the BioRad method. Aliquots were then stored at –70°C.

2.2. Animals and infection protocols

2.2.1. Standard infections

6-week-old male BALB/c mice were purchased from Harlan-Olac (Bicester, U.K.) and were maintained in filter-topped cages. Unless stated otherwise, groups of 5 BALB/c

mice were infected sub-cutaneously (s.c.) with either 50 L3 or 1 x 10^5 MF or an equivalent volume of HBSS in the scruff of the neck. Syringes used for inoculation of L3 were flushed with HBSS after use and the remaining L3 counted. If ten or more L3 remained, these were re-injected. Mice were typically culled on day 12 post-infection (unless otherwise stated) by CO_2 inhalation and spleens were removed.

2.2.2. Mosquito infections with L3

In some experiments, mice were infected by allowing mosquitoes to feed directly. Mosquitoes were infected as described above and blood fed females were removed to a separate cage. On day 9 p.i., 20 mosquitoes were dissected and the numbers of L3 counted to give an average number of L3 per mosquito. The appropriate number of infected mosquitoes were then allowed to feed on the shaved abdomen of an individual mouse, so that each mouse would receive a minimum of 50 L3. The fed female mosquitoes were dissected after feeding to confirm that most L3 had been transmitted. Controls in these experiments were mice infected by syringe inoculation or given HBSS alone in the scruff of the neck.

2.2.3. Adult implants

Groups of 5 BALB/c mice were also infected with adult worms by implanting 10 adult female worms into the peritoneal cavity. Adult worms were obtained from the peritoneal cavity of infected jirds as described previously. Mice were anaesthetized using Halothane and 10 female worms were introduced into the peritoneal cavity through a small incision, using a sterile glass probe. The incision was clipped and mice allowed to recover in warmed boxes. These animals were housed individually for the duration of the experiment. Mice were killed by CO_2 inhalation and spleens were

removed. In addition the peritoneal cavity of these mice was washed extensively with HBSS to assess adult worm and MF recovery.

2.2.4. Other mouse strains

In additional experiments, 6-week-old IL-4 deficient mice on the BALB/c background were infected with MF, L3 or adult worms using the same protocols as described above. Groups of 10-week-old male B cell deficient (IgM transmembrane domain) μMT or IL-10-/- mice on the C57BL/6 background were infected with L3 by the s.c. route. IL-10-/- mice were obtained from Dr. R. Lawrence at the University of Manchester and μMT mice were obtained from either Dr. J Allen at the University of Edinburgh or Dr. R. Lawrence. Appropriate controls were used in all experiments.

2.3. Preparation of spleen cells

Spleens were removed aseptically and single cell suspensions were prepared in RPMI by homogenising the tissue through Nytex mesh using sterile syringe barrels (Cadish and Sons, London U.K.). Remaining tissue debris was removed by passage of the suspension through Nytex mesh. Erythrocytes were lysed using 0.83% NH_4Cl (pH 7.2) following which the single cell suspension was washed twice in RPMI. The number of viable lymphocytes was assessed by the Trypan Blue exclusion method. The cells were resuspended at 1 x 10^7 / ml (for proliferation assays) or 2 x 10^7 / ml (for cytokine assays) in RPMI containing 20% heat inactivated FCS (Invitrogen) to give a final concentration of 10%.

2.3.1. Proliferation assay

Proliferation of splenocytes was measured by the incorporation of ^{3}H thymidine. Triplicate 100 µl cultures (5×10^{5} cells / well) in 96-well flat-bottomed half-area plates (Costar) were incubated in the presence or absence of 10 µg/ml adult antigen. This concentration of Ag was shown to be optimal in previous experiments (Osborne, 1997a). Cells were cultured at 37°C in an atmosphere of 5% CO_2 and pulsed with 0.5 µCi of ^{3}H thymidine/well (Amersham) during the last 16 hours of incubation. The cells were harvested and radioactivity measured in a "Topcount" Microplate Scintillation Counter (Canberra Packard Instrument Company).

2.3.2. *In vitro* treatments

In certain experiments rat anti-mouse IL-10 MAb (JESS-5, Pharmingen, UK) or IgG1 isotype matched control MAb (R59-40, Pharmingen) were added to Ag-stimulated and medium only culture at a final concentration of 1 µg/ml (anti-IL-10). After such treatment, the proliferation and cytokine responses of the cells were assessed. IL-10 was measured by cytokine ELISA assay, which demonstrated that this cytokine had been successfully neutralized in JESS-5. In addition, in all experiments purified anti-mouse CD80 (B7-1, 16-10A1) and purified anti-mouse CD86 (B7-2, GL1) were added to Ag-stimulated culture at a final concentration of 1µg/ml. When these Ab were added to culture simultaneously the final concentration was 2 µg/ml. For experiments in IL-4-/- mice, IFN-γ neutralizing MAb (XMG1.2) or isotype matched control (R3-34, both Pharmingen) were added to medium only and Ag-stimulated culture at a concentration of 100 µg/ml. After such treatment the proliferative responses of the cells were assessed as described in 2.3.1.

In other experiments the following supplements were added to both antigen-stimulated and medium only cultures. All concentrations given refer to final concentrations active in culture: Recombinant IL-4 (rIL-4, Sigma) at 500 pg/ml and 1 ng/ml; Aminoguanidine (AMG, Sigma) at 500 mM. The proliferative and cytokine responses of these cells were compared to those in unsupplemented cultures.

2.3.3. *In vivo* treatments

In some experiments mice were injected intraperitoneally with neutralizing IL-10R MAb (1B1.3a, Pharmingen) or matched isotype control (GL113) at a concentration of 10 μg on day 0 and 4 and 7 post infection. In other experiments, mice were injected intraperitoneally with neutralizing CD25 MAb (PC61) or isotype matched control (GL113) at a concentration of 0.5 mg/ml on day -4 and -2 prior to infection. Hybridomas were cultured in RPMI 1640 + 10% FCS, 1% glutamine, 1% penicillin/streptomycin, 50 μM 2-mercaptoethanol, 2% petone and glucose solution, at 37°C with 5% CO_2. PC61 and GL113 Ab were prepared by ammonium sulphate precipitation of hybridoma supernatant and subsequent dialysis against PBS (Maizels *et al* 1991). The Ab was sterilized using a syringe filter and protein concentration was determined by carrying out a 1:10 dilution and taking an OD_{280} reading (Perkin Elmer U.V. Spectrophotometer). Ab was stored at 4° C. Proliferation assays, cytokine assays and FACS analysis were carried out on the splenocytes from each group of animals as described in 2.3.1, 2.3.4 and 2.4 respectively.

2.3.4. Analysis of cytokine production by ELISA

Spleen cells were incubated at $1x10^7$ cells/ml in 1ml cultures in 24-well flat-bottomed plates (Costar) in the presence of Ag (10 μg/ml) or medium only. Supernatants were

harvested at 48 hours, unless otherwise stated. After centrifugation for 5 mins at 13,000 rpm the cell-free supernatants were stored in aliquots at -20°C. Levels of IL-2, IL-4, IL-5, IL-10 and IFN-γ were determined by two-site ELISA using Ab pairs purchased from Pharmingen. The optimal concentrations for the capture and detecting antibodies were determined in preliminary experiments. Results are expressed as pg/ml in reference to commercially available standards (IL-12, IL-4, IL-5 and IL-10 from Pharmingen and IFN-γ from R and D Systems, UK). The sensitivity of the assay was determined as the mean + 3 SD of the 16 wells containing medium only (RPMI 10% FCS). For measurement of IL-13, the Murine Quantikine kit (R and D Systems) was used according to the manufacturers guidelines. For the measurement IL-2, IL-4, IL-5, IL-10 and IFN-γ, ELISA plates (Corning Easy Wash) were coated with 50 µl/well capture Ab in PBS, over-night at 4°C and then blocked with 150 µl/well PBS, 0.5% Tween 20 (PBS.T20), 10% FCS for 45 minutes at 37°C. During this incubation, samples were defrosted and doubling dilutions of the recombinant cytokine standards were prepared in RPMI/10% FCS on a separate plate. ELISA plates were then washed twice with PBS/T.20 quickly and twice for 3 minutes. Samples, standards and RPMI/10% FCS were transferred to plates at 50 µl/well for 2 hours at R.T. Plates were washed as described above and biotinylated secondary antibody diluted in 1% BSA PBS/T.20 was added at 50 µl/well for 1 hr at R.T. Plates then were washed as before. Streptavidin peroxidase (Serotec, UK) diluted 1/1000 in 1% BSA PBS/T.20 was added at 75 µl/well for 1 hr at R.T. Plates were washed as before and TMB peroxidase substrate (KPL, UK) was added at 100 µl/well for 10 minutes. Plates were read at 620 nm in a Dynatech MR5000 automated ELISA reader.

2.3.5. Analysis of nitrite by the Greiss reaction

The Greiss reaction was used to measure nitrite levels in culture supernatant, using the following protocol. 50 µl of cell culture supernatant and 50µl of Greiss reagent (see 2.7.8) were mixed in 96 well flat bottomed plates and incubated at room temperature for 10 minutes before absorbance was determined at 560 nm (all samples were tested in duplicate). NO_2^- concentration was calculated from a standard curve using two-fold dilutions of a stock control solution of sodium nitrite at 10 µg/ml. Sensitivity was determined as the mean + 3 SD of the 16 wells containing medium only (RPMI 10% FCS).

2.4. FACS staining

2.4.1. Cell surface staining

Cells to be stained were removed from culture, transferred to FACS tubes (Falcon), and washed twice in 200 µl of staining buffer (see 2.7.6), by centrifugation at 1000 rpm for 5 minutes. 2 x 10^6 cells were stained with 2 µg/test of flurochrome-conjugated antibody in staining buffer or staining buffer only (100 µl/sample) on ice for 20 min. Cells were washed twice in staining buffer as before and resuspended in 300 µl of fixation buffer (see 2.7.6) if not to be analysed immediately. Stained samples were stored in the dark at 4 °C. Samples were gated on lymphocytes, as determined by size and granularity (forward and side scatter). Monoclonal antibodies used were as follows, anti-mouse CD4 (L3T4), anti-mouse CD45R/B220 MAb (RA3-6B2), anti-mouse CD8 (Ly-2), anti-mouse CD152 (CTLA-4, UC10-4F10-11), anti-mouse CD25 (PC61), anti-mouse CD80 (B7-1, 16-10A1) and anti-mouse CD86 (B7-2, GL1) (all Pharmingen). In experiments examining the expression of CTLA-4, B7-1 and B7-2, Fc block (Pharmingen) was used

at a concentration of 1 μg/test to reduce non-specific binding. Fc block was added to samples along with the antibody of choice. Isotype controls used were as follows, Rat IgG2a-FITC (KLH/G2a-1-1, Southern Biotechnology Associates), rat IgG2a-APC (R35-95), rat IgG1-PE (both Pharmingen).

All samples were analysed on a Becton Dickinson FACScalibur (department of Immunology, University of Glasgow). Data analysis was carried out by using Becton Dickinson's CellQuest software.

2.4.2. CFSE staining

Splenocytes were harvested from mice as described in 2.3. Cells were labelled with CFSE *ex-vivo* and then cultured with or without stimulation, prior to cell surface staining and flow cytometric analysis. After counting the number of viable splenocytes, 5 x 10^7 cells/sample were washed twice in 5 ml sterile PBS by centrifugation at 1000 rpm for 5 minutes. Cells were then incubated in 10 μM CFSE in PBS (Molecular Probes, Netherlands) at 5 x 10^7 cells/sample for 8 min at R.T. The reaction was stopped by the addition of 5 ml of RPMI containing 20% FCS. All samples were washed twice in RPMI 10% FCS. Stained cells were plated out in 1 ml cultures as described previously, with or without stimulation, for 96 hr prior to cell surface staining and FACS analysis. CFSE labelled cells were harvested and surface stained with either an anti-mouse CD4 APC conjugated MAb (L3-T7), anti-mouse B220 APC conjugated MAb (RA3-6B2), anti-mouse CD8 (Ly-2) or isotype matched control (R35-95) (all Pharmingen), as described above, prior to FACS analysis. Samples were gated on total lymphocytes, as determined by forward and side scatter and $CD4^+$, $B220^+$ or $CD8^+$ lymphocytes for analysis of CFSE staining profiles. Controls in each experiment

comprised unstained cells, cells stained with anti-CD4 or anti-B220 or isotype matched control only and cells stained with CFSE only cultured in medium alone.

2.4.3. FACS gating

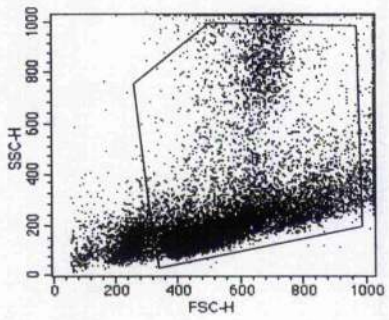
Lymphocytes were initially gated by means of their physical properties in terms of size and granularity (forward and side scatter) (Figure 2.1A). The number of positively stained lymphocytes [in this example $CD4^+$ cells were stained with FITC (FL-1)] were determined using gates set with the isotype control for the particular fluorochrome, shown in B. Positively stained lymphocytes are shown in panel C. Histogram statistics are also shown, indicating that $CD4^+$ cells make up 19.8% of gated lymphocytes.

For three colour staining, lymphocytes were gated as shown in Figure 2.1D, $CD4^+$ cells were stained with FITC and the gate was set on positive cells to create region 2 (R2)(D). The dot plot shown in E is gated on R2 and an isotype control (for PE and APC) was analysed to plot a quadrant, allowing analysis of $CD4^+$ (FITC), $CD25^+$ (APC stained (FL-4)) and CTLA-4+ (PE stained (FL-2) cells as shown in F. Quadrant statistics are also shown demonstrating that 5.98% of $CD4^+$ cells also express CD25 and CTLA4.

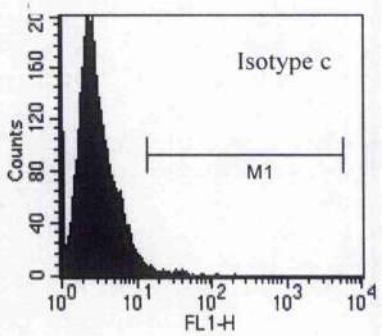
CFSE labelling was used to track the proliferation of specific cell types. Cellular division can be visualised by the serial halving of FL-1 fluorescence, therefore if CSFE labelling is coupled with cell surface staining (APC, FL-4), the proliferation of specific cell types can be analysed by flow cytometry. The gates were set using FL-4 isotype control stained cells (G). The example given in H depicts the division of $CD4^+$ cells.

Histogram statistics are shown and it can be seen that 6.05% of total lymphocytes are dividing $CD4^{+}$ cells.

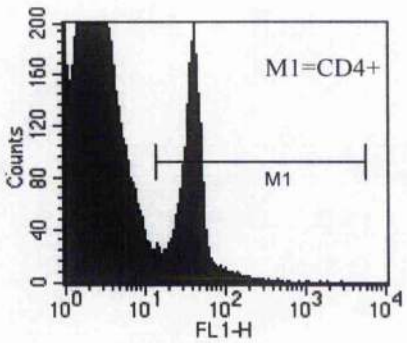
A



B



C



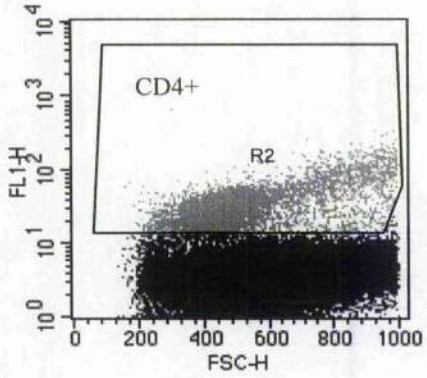
Histogram Statistics

File: vic18.2.03.021
Sample ID:
Tube:
Acquisition Date: 18-Feb-3
Gated Events: 55223
X Parameter: FL1-H (Log)

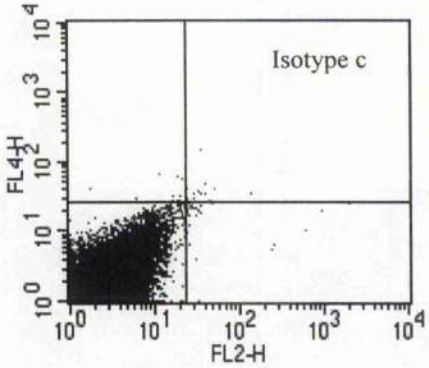
Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 55223

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	55223	100.00	100.00	10.58	4.14	236.69	2.74	1
M1	14, 5882	10936	19.80	19.80	41.68	36.95	105.85	37.18	39

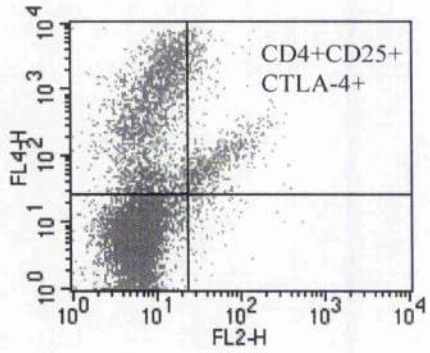
D



E



F

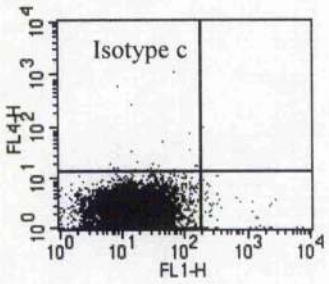


Quadrant Statistics

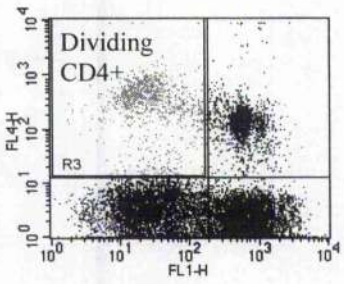
File: vic18.2.03.019
Log Data Units: Linear Values
Sample ID:
Patient ID:
Tube:
Panel:
Acquisition Date: 18-Feb-3
Gate: G2
Gated Events: 9629
Total Events: 66806
X Parameter: FL2-H (Log)
Y Parameter: FL4-H (Log)
Quad Location: 24, 25

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean
UL	1902	19.75	2.85	10.03	8.49	1102.00
UR	576	5.98	0.86	64.25	51.62	856.29
LL	7067	73.39	10.58	6.37	5.91	6.87
LR	84	0.87	0.13	55.30	42.88	13.71

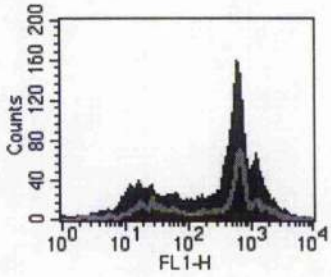
G



H



I



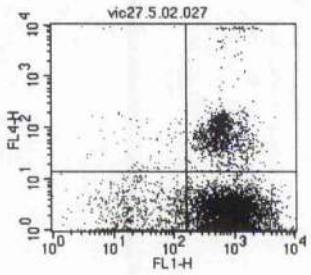
Quadrant Statistics

File: vic27.5.02.019
Sample ID:
Tube:
Acquisition Date: 27-May-2
Gated Events: 18171
X Parameter: FL1-H (Log)
Quad Location: 183, 13

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G1
Total Events: 29733
Y Parameter: FL4-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	1099	6.05	3.70	39.21	26.95	422.22	250.99
UR	2787	15.34	9.37	633.84	588.78	211.50	134.94
LL	5113	28.14	17.20	43.31	27.71	3.37	2.67
LR	9172	50.48	30.85	836.86	681.27	1.91	1.58

J



Quadrant Statistics

File: vic27.5.02.027
Sample ID:
Tube:
Acquisition Date: 27-May-2
Gated Events: 9038
X Parameter: FL1-H (Log)
Quad Location: 93, 15

Log Data Units: Linear Value
Patient ID:
Panel:
Gate: G4
Total Events: 17490
Y Parameter: FL4-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mear	Y Mean	Y Geo Mear
UL	60	0.66	0.34	34.28	24.52	353.48	53.09
UR	1283	14.20	7.34	722.70	621.33	351.32	92.94
LL	591	6.54	3.38	28.37	19.96	2.81	2.08
LR	7104	78.60	40.62	1158.46	851.29	1.91	1.56

2.5. Magnetic Separations

2.5.1. Cell depletion

In experiments to determine the cellular source of IL-10, splenocytes from L3 infected animals were depleted of either $CD4^+$ or $B220^+$ cells by magnetic separation, prior to *in vitro* culture. Splenocytes were prepared as described previously and pooled from five L3 infected mice. Unseparated cells were plated out prior to depletion for proliferation and cytokine assays as described in 2.3.1 and 2.3.4 respectively. At this point 5 x 10^6 cells were removed for FACS analysis and stained with an isotype control antibody (KLH/G2a-1-1, Pharmingen) for use in setting gates for FACS. The remaining pooled cells were washed in PBS and then stained with either an anti-CD4 or anti-B220 FITC conjugated MAb (L3T4 or RM4-5, both Pharmingen) in 500 µl of PBS on ice for 20 minutes. Both Abs were used at 2 µl/2 x 10^6 cells. Cells were then washed twice in PBS and 5 x 10^6 cells were taken for FACS analysis to determine the percentage of $CD4^+$ and B cells in total splenocyte preparations. Cells were then resuspended at 90 µl of buffer/10^7 cells in degassed separation buffer (see 2.7.6) prepared to MACS specifications, with anti-FITC beads (10 µl of beads/10^7 cells) and incubated on ice for 15 minutes. Cells were washed in separation buffer and resuspended in 500 µl of separation buffer. Prior to passing cells over the positive selection LS column (Miltenyl Biotec), 3 ml of separation buffer was passed over the column. Cells were loaded onto the column followed by 9 ml of buffer and collected upon exit from the column. Cells were washed in PBS and 5 x 10^7 cells were removed for FACS analysis to determine the efficiency of the depletion. Cells were then resuspended in RPMI containing FCS 20% and plated out as described previously for proliferation and cytokine assays.

2.5.2. Cell purification

For RNA extraction $CD4^+$ and $B220^+$ cells were purified from splenocytes of L3 infected animals by magnetic separation. Splenocytes were prepared as described previously and pooled from five L3 infected mice. At this point 5 x 10^7 cells were placed in a cryotube and frozen in liquid nitrogen for RNA extraction from whole splenocytes. 5 x 10^6 cells were removed for FACS analysis and stained with an isotype control antibody (KLH/G2a-1-1, Pharmingen) for use in setting gates for FACS. The remaining pooled cells were washed in PBS and then stained with either an anti-CD4 or anti-B220 FITC conjugated MAb (L3T4 or RM4-5) both Pharmingen) in 500 µl of PBS on ice for 20 minutes. Both Abs were used at 2µl / 2 x 10^6 cells. Cells were then washed twice in PBS and 5 x 10^6 cells were taken for FACS analysis to determine the percentage of $CD4^+$ and B cells in total splenocyte preparations. Cells were then resuspended in degassed separation buffer (90 µl of buffer/10^7 cells) with anti-FITC beads (10 µl of beads/10^7 cells) and incubated on ice for 15 minutes. Cells were washed and resuspended in 500 µl of buffer. 3 ml of separation buffer was passed over the positive selection LS column (Miltenyl Biotec) and cells were then loaded onto the column followed by 9 ml of buffer and collected upon exit from the column. To collect purified $CD4^+$ or $B220^+$ cells, the column was removed from the magnet and placed on a new collection tube. 5 ml of buffer was applied to the reservoir of the column and the cells were firmly flushed out using the plunger supplied with the column. 5 x 10^6 cells were then taken for FACS analysis to determine the efficiency of the purification. The purified $CD4^+$ and $B220^-$ cells were placed in a cryotube and stored in liquid Nitrogen.

2.6. Analysis of cytokine mRNA expression following infection with L3

2.6.1. RNA extraction

Cells from the purification experiments described above were removed from liquid nitrogen and total RNA was extracted using TRIzol (Invitrogen), according to the manufacturer's instructions. 1 ml of TRIzol was added to the cryotube containing cells and heated to 60°C for 10 minutes. The samples were then vortexed. At this point 3 µg of glycogen (Invitrogen) was added to each sample to act as an RNA carrier. 200 µl of cholorform was added and the samples were shaken for 15 seconds. After centrifugation at 4 °C for 10 minutes at 12,000 rpm the aqueous layer containing RNA was pipetted off into a fresh Eppendorf tube. 0.5 ml of isopropanol was added to each sample to precipitate the RNA (0.5 ml isopropanol for every ml of TRIzol initially used). Samples were then incubated at room temperature before further centrifugation at 4 °C for 10 minutes at 12,000 rpm. A small pellet of RNA was then formed and the supernatant was removed using a Pasteur pipette attached to a suction pump. To enhance subsequent solubility, 1 ml of 75% ethanol was added to each tube (1 ml of ethanol for every ml of TRIzol initially used) and the sample was vortexed. Samples were then centrifuged at 4°C for 5 minutes at 7500 rpm. The pellet formed was dissolved in an appropriate amount of diethyl pyrocarbonate (DEPC) H_2O (10 µl - 30µl) and incubated at 55 - 60 °C for 10 minutes to ensure the RNA was dissolved.

The RNA was then quantified by spectrophotometry at OD_{260} and checked by ethidium bromide staining on 1% agarose mini-gels. Before the gel was poured, the mini-gel tank and combs were washed in 0.25M HCl, and three times in DEPC treated H_2O. For a 50 ml mini-gel, 0.5 g agarose was melted in a solution containing 5 ml 10X MOPS buffer

and 39.5 ml of DEPC H_2O by boiling in the microwave. The solution was cooled to 55 °C. In a fumehood, 8.5 ml of 17% formaldehyde was added, mixed and the gel was poured. 1 µl of 500 ng/ml ethidium bromide, 1 µl loading buffer and 1 µl of formamide was added to 1-5 µg of each RNA sample. Prior to electrophoresis, the RNA samples were denatured by heating to 65 °C for 10 minutes. When the gel was set, the RNA samples were loaded and the gel was run in 1X MOPS buffer at 45V for 30-40 mins. RNA was visualised on the UV transilluminator. RNA was aliquoted and stored in DEPC-treated H_2O at –70°C until used.

2.6.2. RT-PCR detection of IL-10 and TGF–β

The reverse transcription reaction was carried out in a total volume of 20 µl. 2 µl of random primers (Promega) were added to 10 µl of total RNA (2 µg) and the mix was heated to 70 °C for 10 mins and then cooled on ice for 10 mins. 4 µl of first strand buffer (Invitrogen), 2 µl DTT (Invitrogen) and 1 µl 10 mM dNTP mix were added and incubated at 42 °C. 1 µl of Superscript (reverse transcriptase) was added to the mix which was heated to 42°C for 50 mins with a final extension of 70 °C. 1 µl of RNase was added and the reaction was incubated at 37 °C for 20 mins. The reaction was then chilled on ice and stored at –20 °C.

Real time quantitative reverse-transcriptase-PCR was carried out using the TaqMan method with gene specific primers and internal probes for IL-10 and TGF-β and HPRT (Cruachem) designed to specifically amplify the target cDNA as these primers were designed to span an intron/exon boundary and thus anneal specifically to cDNA (primer and probe sequences shown in Table 2.1). HPRT was used as the constitutively

expressed gene. All primers and probes were HPLC purified and lyophilised. For the standard TaqMan protocol, primers were used at a concentration of 10 μM and probes at 5 μM. Each reaction of 25 μl volume contained 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μl probe, 0.75 μl each primer and 9 μl H_2O. Sufficient mastermix was prepared to analyse each sample in triplicate including a non-template control (water alone). 24 μl of mastermix was then aliquoted into the appropriate wells of a MicroAmp Optical 96 well reaction plate (Applied Biosystems). 1 μl of previously prepared cDNA (or H_2O in the case of the non-template controls) was then added to the wells. Once the samples were added the plate was sealed with Optical Caps (Applied Biosystems). Plates were then spun at 1400 rpm for 1 min to ensure that there were no bubbles in the samples, as this interferes with the laser beam. The plate was then placed in the ABI/PRISM 7700 detection system which was set to the following cycling conditions; 50 °C for 2 mins, 95 °C for 10 mins, 95 °C for 15 secs and 60 °C for 1 min. At the end of the run the software generates a series of amplification plots relating to the samples. The graph shows the cycle numbers plotted against fluorescence. Data from this software was exported into EXCEL where a series of Ct values (the number of cycles at which the amplification plot for the particular sample crosses the threshold line) was generated. When these data were pasted into the previously prepared analysis template the calculations normalise the data to the HPRT gene. The percentage values for each of the samples (IL-10 or TGF-β) were plotted to determine the relative expression levels for the gene of interest. Therefore the normalized value for IL-10 or TGF-β mRNA expression in each sample was calculated as the relative quantity of either IL-10 or TGF-β divided by the relative quantity of HPRT (x100). A diagram of the TaqMan principle shown in Figure 2.2.

Figure 2.1. FACS gating

A-C
2 x 10^6 cells from splenocyte cultures of L3 infected mice were stained with FITC labelled anti-mouse CD4 MAb or the isotype matched control. Panel A shows typical gating of lymphocytes in terms of size and granularity. Panels B and C are gated on this region therefore display events taking place within this area. Panel B shows FL1 fluorescence of the isotype control from which the M1 region can be set. M1 is the region in which all $CD4^+$ cells will fall when stained with anti-CD4 MAb (panel C).

D-F
2 x 10^6 cells from splenocyte cultures of L3 infected mice were stained with FITC labelled anti-mouse CD4, PE labelled anti-mouse CTLA-4 and APC labelled anti-mouse CD25. Panel D shows gating on all $CD4^+$ events which lie within the total lymphocyte population (see panel A). Panel E shows events collected when cells were stained with isotype controls for FITC, PE and APC, thus allowing the quadrant to be set which displays $CD4^+$ cells which co-express CTLA-4 and CD25 (panel F).

G-J
2 x 10^6 CFSE stained and non-CFSE stained cells from splenocyte cultures of L3 infected mice were stained with APC labelled anti-mouse CD4 or the isotype matched control. Panel G shows cells which were not stained with CFSE and stained with FITC and APC isotype control. Panel H shows cells stained with CFSE and anti-CD4. As CFSE loses fluorescence as cells divide, dividing $CD4^+$ cells are shown in the upper left hand part of the quadrant. Panel I is a histogram which shows the distinct peaks which represent each cellular division undergone by the total lymphocyte population. The overlay represents $CD4^+$ cell division. Panel J shows cells which have been cultured with medium only and the statistics below represent the percentage of dividing cells. It can be seen here that the percentage of dividing lymphocytes is much lower in cells from medium only cultures compared to Ag stimulated cultures.

HPRT	Forward 5'-GCA GTA CAG CCC CAA AAT GG-3' Reverse 5'-AAC AAA GTC TGG CCT GTA TCC AA-3' Probe 5'-FAM-TAA GGT TGC AAG CTT GCT GGT GAA AAG GA-TAMRA-3'
IL-10	Forward 5'-ACA ACA TAC TGC TAA CCG ACT CCT T-3' Reverse 5'-AGG TAA AAC TGG ATC ATT TCC GAT A-3' Probe 5'-FAM-TGG CAA CCC AAG TAA CCC TTA AAG TCC TG-TAMRA-3'
TGF-β	Forward 5'–CCC CAC TGA TAC GCC TGA GT – 3' Reverse 5' – ACA AGA GCA GTG AGC GCT GA – 3' Probe 5' – FAM-TGA ACC AAG GAG ACG GA-TAMRA – 3'

Table 2.1. TaqMan primer and probe sequences

Primer and probe sequences were selected by the using the Primer Express software (ABI/Perkin Elmer). Probe sequences were designed according to the following criteria. The melting temperature (T_m) was between 65 and 67 °C, the 5' end of the probe was not a guanosine residue, runs of an identical residues were avoided and the polymorphic site was near the centre of the probe. Primer sequences were designed according to the following guidelines. The G-C content was within the 20 to 80 % range, runs of an identical neucleotide were avoided, the T_m was between 58 and 60 °C, the five neucleotides at the 3' end had no more than two G and/or C bases and the forward and reverse primers were placed as close as possible to the probe without overlapping the probe.

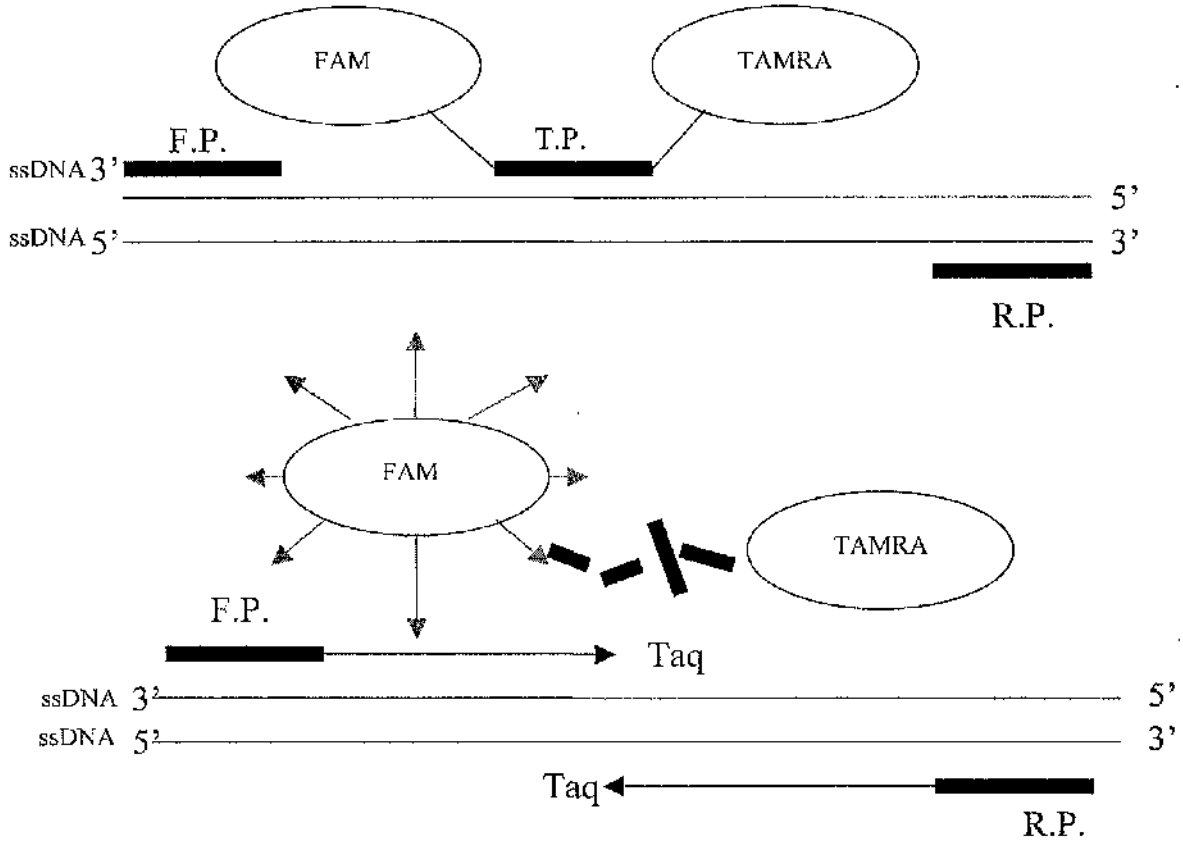


Figure 2.2. The TaqMan principle

During PCR annealing, primers and the fluorogenic probe anneal to denatured single-stranded DNA template. As long as the probe is intact, TAMRA (the 3' quencher dye) absorbs the fluorescent emissions of FAM (5' reporter dye). During extension, the probe is cleaved by the 5' nuclease activity of the Taq, separating the reporter from the quencher signal which results in increased fluorescence of the reporter dye which is quantitative for the initial amount of template. F.P. - forward primer; R.P. - reverse primer; T.P. - flurogenic probe; Taq - Taq Polymerase.

2.6. Statistical analysis

The Mann-Whitney *U*-test was used to determine the statistical significance of differences between groups. $p<0.05$ was considered to be a significant difference.

2.7. Buffers/Solutions

2.7.1. Cell culture

Ammonium chloride solution: Mix 9 vols of 0.83% w/v NH_4Cl in ddH_2O with 1 vol of 2.06% w/v Tris-HCl pH 7.65. pH adjusted to 7.2. Sterilize by autoclaving and store at 4°C.

2.7.2. General purpose

PBS: 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.47 mM KH_2PO_4 in ddH_2O. pH adjusted to 7.2. Sterilize by autoclaving and store at R.T.

2.7.3. ELISA buffers

0.06 M Carbonate Buffer, pH 9.6: To 45.3 ml of 1 M $NaHCO_3$ add 18.2 ml of 1M Na_2CO_3 and make solution to 1 litre with ddH_2O.

PBS/0.05% Tween 20: 500 µl of Tween 20 was added per litre of sterile PBS.

2.7.4. RNA preparation

DEPC (Diethylpyrocarbonate) – treated H_2O: add 900 µl of DEPC to 900 ml of ddH_2O and dissolve by autoclaving. Store at R.T.

Ethidium Bromide for RNA Work: 500 ng/ml stock solution dilute in DEPC-treated H_2O. Store in the dark at R.T.

Loading Buffer (5x): 10% Ficoll, 0.5 % SDS, 0.06% bromophenol blue in ddH_2O. Store at R.T.

MOPS (MOPS (3-[N-Morpholino]propanesulphonic acid) buffer (10x): 0.05 M Na acetate, pH 7, 0.01 M EDTA, 0.2 M MOPS in dd H_2O. Store at R.T. Dilute to 1x for working concentration.

2.7.5. DNA preparation

Ethidium Bromide for DNA Work: 10 mg /ml stock in dd H_2O. Store in the dark at R.T. Working concentration 100 μg/ml diluted in dd H_2O.

TAE buffer (50x): 2 M Tris-HCl, 5.17% glacial acetic acid, 0.05M EDTA pH 8.2 in dd H_2O. Store at R.T. Dilute to 1x for working concentration.

2.7.6. FACS buffers

Staining Buffer: PBS/2 % FCS, 0.2 % sodium azide, pH 7.4-7.6, filter sterilize and store at 4°C.

Permeabilization Buffer: PBS/2 % FCS, 0.2 % sodium azide, 0.1 % saponin (Sigma) pH 7.4-7.6, filter sterilize and store at 4°C.

Fixation Buffer: 14 ml 10x PBS, 10.8 ml 37.5% formaldehyde, 75.2 ml H_2O, filter sterilize and store at 4°C.

2.7.7. MACS buffer

50 ml PBS, 250 mg BSA, 200 µl EDTA. Degass.

2.7.8. Greiss reagent

Mix equal volumes of 0.1% α-naptyl-amine and 1% sulphanilamide, 5% phosphoric acid. Store at 4 °C

Chapter 3. Infection of IL-4-/- mice with L3 of *B. pahangi* results in increased microfilaraemia and a skewed immune response

3.1. Introduction

IL-4 is a central Th2 cytokine because of its ability to drive naïve T cells towards the Th2 pathway while diverting Th cells from differentiation down the Th1 pathway. In recent years the use of mice in which the IL-4 gene has been deleted has provided an important tool in analysing the role of IL-4 in parasitic infection. As reviewed in the general Introduction, studies into murine infection with *Leishmania major* and *Trichuris muris* have highlighted the role of IL-4 in driving Th2 responses and the role of the Th1 vs Th2 balance in host immunity or susceptibility to infection. In this chapter I examined the role of IL-4 in murine infection with the filarial nematode *B. pahangi*.

The rodent filarial nematode, *Litmosoides sigmodontis* is a useful model for examining host immune responses to filarial parasites over the course of infection, as L3 develop into MF producing adults in some inbred strains of mouse, such as the BALB/c. Experiments carried out in IL-4-/- mice on the BALB/c background, have shown that MF levels are up to 100-fold greater and that microfilaraemia is significantly prolonged in KO mice compared to WT BALB/c mice. The difference in MF levels is thought to relate to the fecundity of the adult worms because there is no difference in the recovery of MF when injected directly into mice (Volkmann *et al.*, 2001). The importance of the genetic background of knockout mice was highlighted in a study examining susceptibility of *L. sigmodontis* BALB/c mice and IL-4-/- mice on the C57BL/6 background. WT BALB/c mice are susceptible to infection with *L. sigmodontis*, while WT C57BL/6 mice are resistant. However infection of IL-4-/- C57BL/6 mice results in a susceptible phenotype with worm recovery comparable to that of WT BALB/c mice.

However despite similar levels of susceptibility, the immune responses of these animals are remarkably different with WT BALB/c mice exhibiting a strong Th2 response and IL-4-/- C57BL/6 mice exhibiting a Th1 response. These data suggest that parasite clearance in a resistant host is Th2 dependent but a susceptible host can allow parasite development despite a Th2 response (Le Goff *et al.*, 2002).

IL-4 is also thought to play a role in susceptibility to *Brugia* infection. Again, the studies carried out to date emphasize the importance of the background strain of mouse used. The original study reported no difference in the recovery of L3, MF or adult worms from IL-4-/- mice on the C57BL/6 background, despite profound alterations in the immune response in these animals (Th2 to Th1 switch in the case of adult and L3 infection) (Lawrence *et al.*, 1995). However C57BL/6 mice are not susceptible to *Brugia* infection and one of the aims of the work of the work described in this chapter was to investigate the role of IL-4 in the susceptible BALB/c mouse. Infection of BALB/c mice with L3 of *B. pahangi* results in an early burst of IL-4 transcription from a population of $CD4^{-}CD8^{-}\alpha\beta$ T cells in the draining popliteal lymph nodes and this IL-4 functions primarily to promote Th2 responses (Osborne and Devaney, 1998). However Spencer *et al* showed that in the absence of IL-4, IL-4R or Stat6, BALB/c mice were significantly more susceptible to infection with either *B. malayi* or *B. pahangi*. Somewhat surprisingly the transfer of T cells from IL-4-/-, IL-4R-/- and Stat6-/- mice into SCID recipients induced host protection comparable to that mediated by T cells from a WT origin. These data suggest that while T cells are required for host protection, they need not respond to or produce IL-4 (Spencer *et al.*, 2003a).

There are many unanswered questions regarding the role of IL-4 in lymphatic filariasis. The L3 has been shown to direct an early burst of IL-4 post-infection, but the importance of this remains unclear. Is IL-4 correlated with resistance or susceptibility? If so, which life cycle stages are affected by IL-4 mediated responses. In this chapter, I set out to determine whether IL-4 had any affect on the development of *Brugia* L3 in the mouse model and to investigate whether Ag-specific immune responses varied significantly between L3 infected IL-4-/- and WT BALB/c mice.

3.2. Results

3.2.1. Investigating the role of IL-4 *in vivo*

(i) Worm recoveries from IL-4-/- mice

The worm recovery experiments were carried out in collaboration with Prof. E. Devaney. For these experiments, mice were infected with 50 L3 of *B .pahangi* by the i.p. route, so that worms could be recovered easily. At the following time points the numbers of larvae were recovered and counted: day 6 (moulting L3–L4), day 8 (L4), day 15 (L4), day 27 (L4 to adults) or days 71-77 (patent adults). As shown in Table 3.1, there was no significant difference in recovery of the larvae from the IL-4-/- mice compared to WT mice at any of the time points. The recovery of worms from both strains remained very steady at 30-40% until after the final moult (post-day 27). At day 70 plus, more adult worms were recovered from the IL-4-/- mice than the wild-type mice but this difference was not statistically significant ($p > 0.05$).

(ii) MF recovery from IL-4-/- and WT mice

Mice were infected i.p. with 50 L3 of *B. pahangi* and at day 71 or day 77 post-infection the levels of MF produced by adult worms in each strain of mouse were determined. The results from the experiment carried out on day 77 post-infection are presented. As shown in Table 3.2, seven of ten infected BALB/c mice contained adult worms, while five of ten contained MF. In contrast, six out of six IL-4-/- mice contained adult worms and MF. MF recoveries were significantly different between the strains of mice ($p = 0.0318$) with IL-4 -/- mice containing ~8 fold more MF than WT mice. It can be seen from Figure 3.1 that there was a wide range in the numbers of MF recovered from

individual animals. This was correlated with the numbers of adult females in IL-4-/- mice ($r = 0.993$, $p=0.001$), but not in wild type mice. When WT mice contained the same numbers of adult worms as IL-4-/- mice, significantly fewer MF were recovered from WT mice by comparison. By expressing the data as mean MF per adult female worm it can be concluded that the worms from IL-4-/- mice are more fecund than those from WT mice (3329 vs 722 MF per adult female, respectively, Table 3.2). In addition, dissection of individual female worms from IL-4-/- mice showed that these worms contain many more MF at all stages of development than did adult female worms from WT mice. Even in WT mice that had MF in the peritoneal cavity, the adult female worms contained very few developing stages or mature MF. Similar results were obtained from an experiment analysed on day 71 post-infection.

(iii) Recovery of MF after implantation of adult worms

The results from the experiment described above could relate to an increased rate of MF killing or reduced fecundity of female worms in WT BALB/c mice. In order to distinguish between the two possibilities, ten adult female *B. pahangi* were implanted into the peritoneal cavity of five BALB/c mice and five IL-4-/- mice. The results of this experiment are presented in Table 3.3. Adult worms and MF were recovered 2 weeks post-infection. At this time point IL-4-/- mice contained more adult female worms than WT mice and this difference just reached statistical significance (worm recovery, 7.8 ± 0.8 in IL-4-/- mice and 6.2 ± 0.8 in WT mice, $p < 0.05$). However levels of MF were not significantly different between the two strains of mice. When these results are expressed as MF per adult female worm it can be seen that there was no difference in MF levels, suggesting that IL-4 does not affect MF survival or the ability of sexually

mature female worms to continue producing MF, but rather acts to the fecundity of developing worms.

3.2.2. Cellular recruitment into the peritoneal cavity of IL-4-/- mice

Mice were infected i.p. with 50 L3 of *B. pahangi* and at day 27 post-infection the peritoneal washings were analysed for cell counts and cell phenotype. The peritoneal washings from BALB/c mice contained significantly more cells than those from IL-4-/- mice (Figure 3.2A). The recruitment of individual cell types was analysed by staining of cytospin preparations (carried out in the Haematology Lab, Veterinary Pathology, University of Glasgow). Infected WT mice contained more eosinophils and macrophages, compared to IL-4-/- mice, but there was very little difference in the numbers of lymphocytes and neutrophils, while mast cells accounted for a very small proportion of the total cells found in the peritoneum. At day 71 post-infection, the total cell number was greatly reduced compared to day 27 p.i. but the wild type mice still contained more eosinophils and macrophages than the IL-4-/- mice (see Figure 3.2B).

3.2.3. Ag-specific immune responses of splenocytes from L3 infected and IL-4-/- BALB/c mice

In order to investigate whether Ag-specific immune responses in L3 infected IL-4-/- mice were significantly different to these of WT mice, groups of five IL-4-/- mice and five WT mice were infected s.c. with 50 L3 of *B. pahangi*. In these experiments the mice were infected s.c. to better mimic the natural route of infection with L3. At day 12 p.i. spleens were removed from these animals and *in vitro* analysis was carried out.

(i) Ag-specific cytokine responses

Cytokine responses of splenocytes from L3 infected IL-4-/-, BALB/c wild type mice and control uninfected mice were measured after 72 hours of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. These experiments were carried out on several occasions and similar results observed in each. The results of a representative experiment showing levels of Ag-driven cytokines are presented in Figure 3.3. Splenocytes from L3 infected IL-4-/- mice generated an altered cytokine profile compared to those of their wild type counterparts, with a significant increase in Ag-specific IL-2 and IFN-γ, cytokines which were only ever seen at very low levels in L3 infected wild-type mice. In addition a reduction in IL-5 and IL-10 was observed in IL-4-/- mice. IL-5 was reduced to significant levels in all experiments while the reduction in IL-10 reached significant levels in only one of five experiments. In all experiments, levels of IL-13 were not significantly different between IL-4-/- and wild-type mice, although there was a tendency for IL-13 levels to be higher in IL-4-/- mice.

(ii) Ag-specific proliferative responses

Proliferative responses of splenocytes from L3 infected IL-4-/- and wild type BALB/c mice were measured over a time course of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. Control mice received HBSS alone. Several experiments were carried out each of which gave similar results. In the representative experiment shown in Figure 3.4, levels of Ag-specific proliferation were significantly lower in IL-4-/- mice compared to that of their wild-type counterparts after 72 and 96 hours incubation with Ag. The time-course from 48 to 96 hours indicates that the differences in proliferative responses are unlikely to reflect a difference in the kinetics of the Ag-specific

proliferative response in IL-4-/- mice. There are a number of possible explanations for this apparent decrease in proliferation. One possibility is that the cells which proliferate in response to the L3 may use IL-4 as a growth factor. Alternatively, the increase in IFN-γ in these cultures (as shown in Figure 3.3) may be cytostatic or may lead to an increase in nitric oxide resulting in the suppression of Ag-specific proliferative responses.

(iii) ConA driven proliferative responses

Proliferative responses of splenocytes from L3 infected IL-4-/- and wild type BALB/c mice and control uninfected mice were measured at 48 hours of *in vitro* restimulation with 1 μg/ml of ConA. In the representative experiment shown in Figure 3.5, levels of ConA driven proliferation were not significantly different between IL-4-/- mice and wild-type mice, suggesting that the cells from IL-4-/- mice have the capacity to proliferate and that the defect in proliferation described is Ag-specific in nature.

3.2.4. Investigating the proliferative defect observed in IL-4-/- mice

In order to investigate the mechanisms by which Ag-specific proliferative responses were decreased in L3 infected IL-4-/- mice compared to wild-type mice, a number of experiments were carried out as described below.

(i) CFSE staining of cells from L3 infected IL-4-/- and wild-type mice in Ag stimulated culture

CFSE staining is a sensitive technique used for tracking the proliferation of specific cell types (see Materials and Methods 2.4.2), and was used in this experiment to investigate

the phenotype of proliferating cells in IL-4-/- and wild-type mice. CFSE staining was carried out prior to *in vitro* stimulation of splenocytes with 10 μg/ml *B. pahangi* adult Ag. At 96 hours cells were harvested and labelled with anti-CD4 MAb, anti-CD8 MAb or B220 MAb. The samples were then analyzed by flow cytometry. The data shown in Figure 3.6 and 3.7 are from a representative experiment, with Figure 3.6 showing cells from individual mice, with means of five mice shown in Figure 3.7. The first point apparent from Figure 3.6 is that the major cell type proliferating in L3 infected mice in response to Ag restimulation were B cells with ~ 16 % of $B220^+$ cells proliferating in response to Ag in wild-type mice compared to ~ 5 % of $CD4^+$ cells. As can be seen in Figure 3.7, proliferating B cells account for ~ 17% of total lymphocytes in wild-type mice while proliferating $CD4^+$ cells account for approximately ~ 5% of total lymphocytes in these mice. In the KO mice, proliferating B cells account for only ~ 7 % of total lymphocytes while proliferating $CD4^+$ cells account for ~ 3 % of total lymphocytes. When examining data as mean percentages it can be seen that the percentage of B cells and $CD4^+$ cells that proliferated in response to Ag were significantly reduced in L3 infected IL-4-/- mice compared to that of their wild-type counterparts. No significant difference in the proliferation of $CD8^+$ cells was observed during the course of these experiments.

(ii) *Ex vivo* staining of splenocytes from L3 infected and control IL-4-/- and wild-type mice

One possible explanation for decreased levels of $CD4^+$ and $B220^+$ proliferation observed in the splenocyte cultures from L3 infected IL-4-/- mice may be due to diminished numbers of these cell populations *ex vivo*. To investigate whether there were differences in the cellular make up of the spleen in wild-type and IL-4-/- mice,

splenocytes were phenotyped *ex vivo* using anti-CD4 MAb, anti-B220 MAb and anti-CD8 MAb. However, as can be seen from Figure 3.8, the percentage of $CD4^+$, $B220^+$ and $CD8^+$ cells were constant between both WT and IL-4-/- mice and control uninfected and L3 infected mice. Therefore it can be concluded that the decreased levels of proliferation observed in L3 infected IL-4-/- mice compared to that of L3 infected wild-type mice is not a reflection of a different cellular make-up in the spleens of the respective strains of mouse.

(iii) Production of NO

IFN-γ is known to be a potent inducer of iNOS. The production of NO by activated macrophages has been identified as a factor mediating suppression in several models of parasitic infection (Candolfi, 1994; Dai, 1999, O'Connor 2000). Therefore to assess the production of NO *in vitro*, the Greiss reaction was carried out on supernatants of Ag-stimulated splenocytes from L3-infected IL-4-/- and wild type mice. Figure 3.9 shows the results of one such assay. This analysis demonstrated that there were elevated levels of NO in cultures from L3 infected IL-4-/- mice compared to cultures from infected wild-type mice, however this difference did not reach statistical significance in any one of three experiments.

(iv) Inhibition of iNOS with AMG

Aminoguanidine (AMG) is an iNOS inhibitor and was used to determine whether the increased levels of IFN-γ produced by cells from L3 infected IL-4-/- mice might suppress proliferative responses by induction of iNOS. Ag-stimulated cultures of splenocytes from L3 infected IL-4-/- and wild-type mice and control uninfected mice were supplemented with 500 μM AMG. As shown in Figure 3.10 the inhibition of NO

production did not result in increased proliferation in cultures of Ag-stimulated splenocytes from L3 infected IL-4-/- animals. Nitrite levels were efficiently reduced by AMG in these experiments (data not shown). Therefore, despite the high levels of IFN-γ in cultures of splenocytes from L3-infected IL-4-/- mice, there was a minimal increase in levels of NO and blocking the production of NO had no effect on proliferation.

(v) Neutralization of IFN-γ

An IFN-γ neutralizing MAb was used to determine whether the increased levels of IFN-γ produced by cells from L3 infected IL-4-/- mice might suppress proliferative responses, as IFN-γ is known to have cytostatic effects on T cells as well as inducing NO from macrophages. Ag-stimulated cultures of splenocytes from L3 infected IL-4-/- and wild-type mice and control uninfected mice were supplemented with 100 μg/ml IFN-γ neutralizing MAb. Figure 3.11 shows the Ag-stimulated proliferative responses in the presence and absence of anti-IFN-γ MAb over a time-course of *in vitro* culture. It can be seen that neutralization of IFN-γ did not result in increased proliferation in these cultures. Therefore, despite high levels of IFN-γ being produced in cultures of splenocytes from L3 infected IL-4-/- mice, blocking the production of IFN-γ had no significant effect on proliferation. Measurement of cytokine levels demonstrated that IFN-γ was effectively neutralized using this concentration of MAb. A range of additional cytokines were also measured but there were no differences between the +/- MAb groups (data not shown).

(vi) Addition of recombinant-IL-4 to cultures of Ag-stimulated splenocytes from L3 infected IL-4-/- and wild-type mice

In an attempt to reverse the defect in Ag-specific proliferation, recombinant (r)IL-4 was added to splenocytes from L3 infected IL-4-/- mice at concentrations ranging from 100 pg/ml to 1000 pg/ml. Proliferative responses were then measured over a time course of *in vitro* restimulation with 10 μg/ml *B. pahangi* Ag. Three experiments were carried out giving similar results. The results of a representative experiment are presented in Figure 3.12 showing only those cultures supplemented with the highest dose of rIL-4 (1000 pg/ml). In these experiments there was a trend towards increased levels of Ag-specific proliferation when rIL-4 was added to cultures of splenocytes from IL-4-/- mice. However this difference did not reach statistical significance at any time point, with any concentration of rIL-4. These data suggest that the lack of IL-4 *in vivo* has a profound effect on the proliferation of cells *in vitro* and although proliferation can be rescued to a certain degree by rIL-4, this never reached significance. In addition levels of Ag-specific cytokines were not altered when rIL-4 was added to cultures at any concentration (data not shown).

Time post infection	BALB/cIL-4-/-	BALB/c WT
Day 6	36.8 ± 22.6% (n=6)	36.2 ± 13.9% (n=7)
Day 8	28.8 ± 11.1% (n=5)	47.3 ±23.9%(n=4)
Day 15	28.9 ± 21.2% (n=6)	38.5 ± 14.5% (n=8)
Day 27	36.7 ± 11.6% (n = 6)	34.2 ± 14.2% (n = 6)
Day 71	14.1 ± 11.4% (n = 13)	7.4 ± 7.7 %(n =12)
Day 77	20.5 ± 19.0 % (n = 6)	10.9 ± 7.7% (n = 10)

Table 3.1. The recovery of worms from IL-4-/- or WT BALB/c mice is not significantly different at any time point post-infection

IL-4-/- and wild-type (BALB/c) mice were infected intra-peritoneally with 50 L3 of *B. pahangi*. Figures show the mean percentage recovery of worms ± SD from the peritoneal cavity of infected mice at different time points post infection. Numbers in parenthesis denote the number of animals per group. $p > 0.05$ at all time points.

	IL-4-/-	BALB/c
No. of mice with adult worms	6/6	7/10
Total no. of males:females	38:28	23:30
No. mice with MF	6/6	5/10*
Mean no. MF per mouse with MF	23952 ± 30485	3023 ± 2139
Mean MF per adult female with MF	3329 ± 2794	722 ± 510**

Table 3.2. Adult worm and MF recoveries at day 77 p.i. demonstrate that IL-4-/- mice contain significantly more MF than WT mice

IL-4-/- and BALB/c wild-type mice were infected intra-peritoneally with 50 L3 of *B. pahangi*. The table shows the numbers of adult worms recovered at day 77 p.i. from the two groups of mice, the total numbers of male and female worms recovered from the respective group, the numbers of mice with MF, the mean (±SD) numbers of MF recovered and the mean numbers of MF per adult female worm

* Two mice with adult females contained no adult males hence no MF
** Statistical significance between MF recoveries from IL-4-/- mice and WT mice (p= 0.0318)

	IL-4-/-	WT BALB/c
No. of adult females recovered	**7.4 ± 0.8**	**6.2 ± 0.8 ***
No. of MF recovered	**$1.61 \pm 0.233 \times 10^5$**	**$1.4 \pm 0.18 \times 10^5$**
No. of MF per adult female	**2.2×10^4**	**2.2×10^4**

Table 3.3. Implantation of adult worms into the peritoneal cavity of IL-4-/- or WT mice results in equivalent production of MF

Ten female *B. pahangi* were implanted in to the peritoneal cavity of 5 BALB/c WT mice or 5 IL-4-/- mice. Adult worms and MF were recovered 2 weeks post-infection. The data above shows the number of adult females recovered from infected mice and the total number of MF in the peritoneal lavage of the mice. MF are also represented as the number of MF per adult female recovered.

* Recovery of adult female worms between two strains was significantly different ($p < 0.05$).

Figure 3.1. Recovery of MF in IL-4-/- mice correlates with the number of adult female worms

IL-4-/- mice were infected intra-peritoneally with 50 L3 of *B. pahangi*. At 77 d.p.i. MF and adult worms were recovered. The graph shows the number of MF in the peritoneal lavage of five IL-4-/- mice plotted against the number of female worms recovered from individual mice. MF levels show positive correlation with adult female worm burden (r= 0.993, p= 0.001).

Figure 3.2. Cell recruitment into the peritoneal cavity is reduced in infected IL-4-/- mice compared to that of WT mice

IL-4-/- and wild-type (WTL3) mice were infected intra-peritoneally with 50 L3 of *B. pahangi*. The graph shows the number of cells per ml in the peritoneal lavage of BALB/c WT (■) mice or IL-4-/- (□) at 27 days p.i. (A) or 71 days p.i. (B). Total cells (1) were enumerated by Coulter counting while numbers of eosinophils (2), neutrophils (3), lymphocytes (4), macrophages (5) or mast cells (6) were counted by differential staining of slides prepared by cytospin. The results are presented as means plus SD of six mice per group.

* significant difference between IL-4-/- and WT mice.

WT vs IL-4-/- (day 27 p.i.)
Total cells $p = 0.007$
Eosinophils $p = 0.007$
Macrophages $p = 0.011$

WT vs IL-4-/- (day 71 p.i.)
Total cells $p = 0.004$
Eosinophils $p = 0.0001$
Macrophages $p = 0.02$

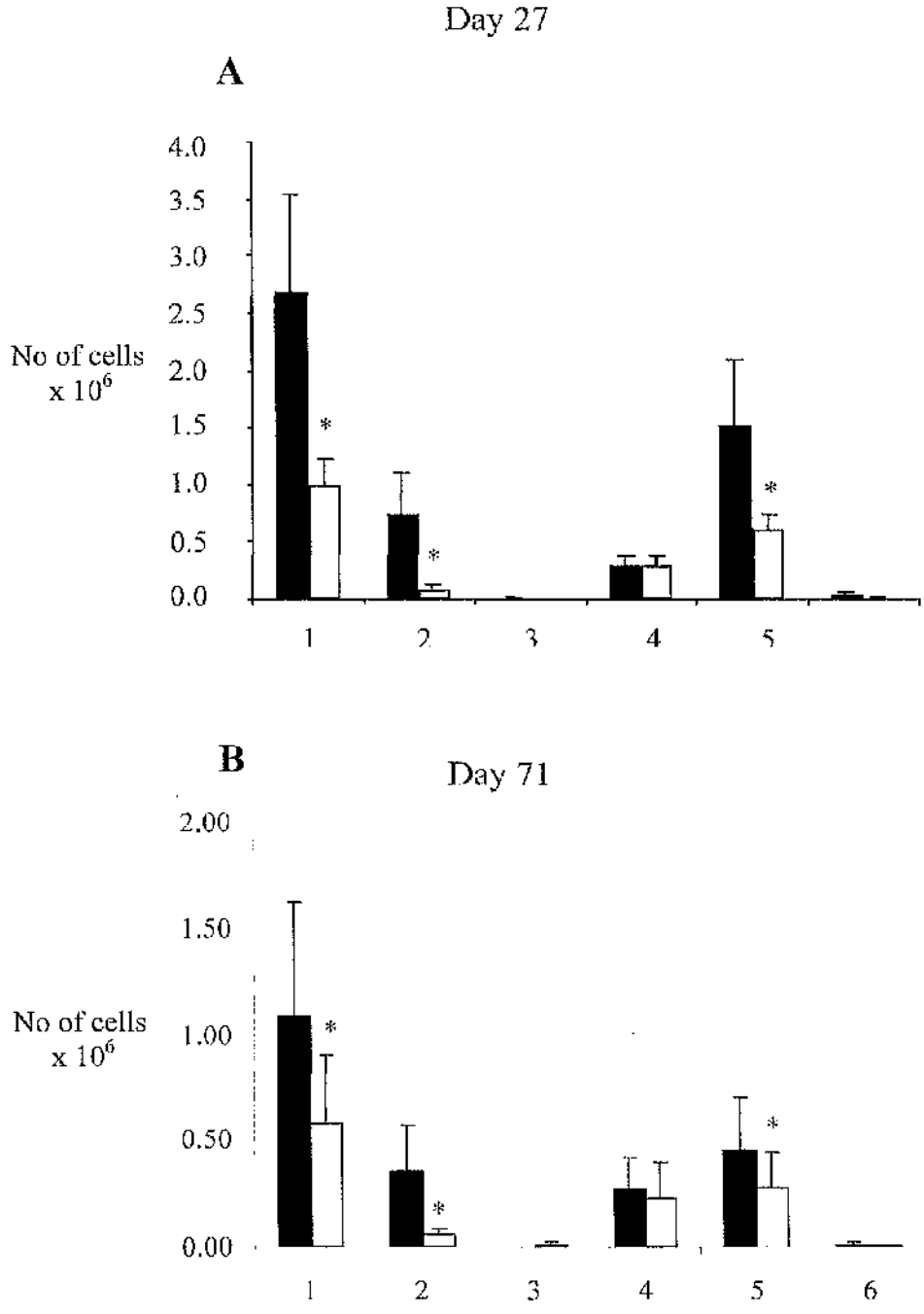
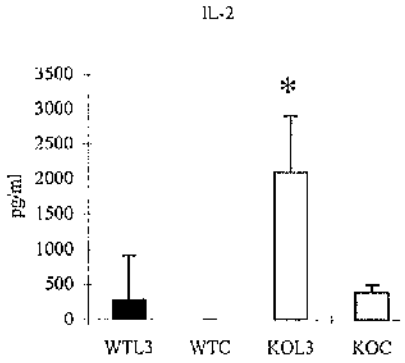
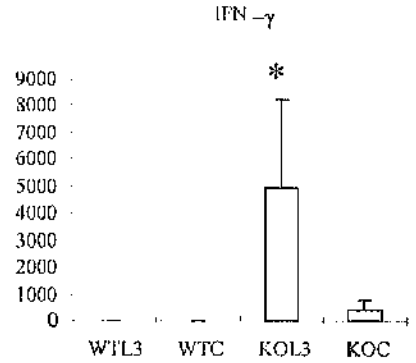

Day 27
A
No of cells x 10^6
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
B
Day 71
No of cells x 10^6
2.00
1.50
1.00
0.50
0.00
1
2
3
4
5
6

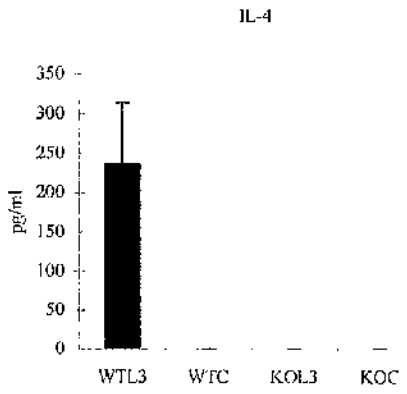
Figure 3.3. Splenocytes from L3 infected IL-4-/- mice display an altered Ag-specific cytokine profile compared to that of their wild-type counterparts

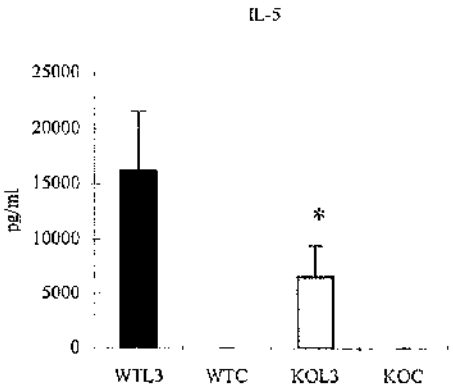
IL-4-/- (KOL3) and wild-type (WTL3) mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i., splenocytes (1 x 10^7/ml) were re-stimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Ag-specific cytokine responses were measured at 72 hours. Results are expressed as pg/ml and all values represent the mean and standard deviation of five mice per group.

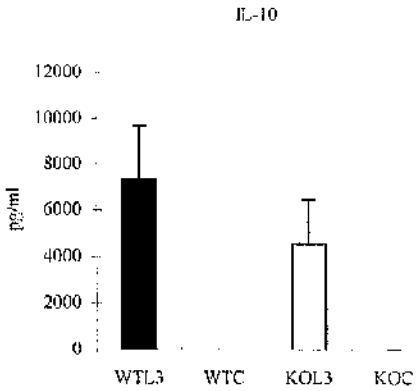
* significantly different ($p = < 0.05$) WT mice vs KO mice
IL-10, WTL3 vs KOL3 $p=0.102$


IL-2
pg/ml
3500
3000
2500
2000
1500
1000
500
0
*
WTL3
WTC
KOL3
KOC


IFN –γ
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
*
WTL3
WTC
KOL3
KOC


IL-4
pg/ml
350
300
250
200
150
100
50
0
WTL3
WTC
KOL3
KOC


IL-5
pg/ml
25000
20000
15000
10000
5000
0
*
WTL3
WTC
KOL3
KOC


IL-10
pg/ml
12000
10000
8000
6000
4000
2000
0
WTL3
WTC
KOL3
KOC

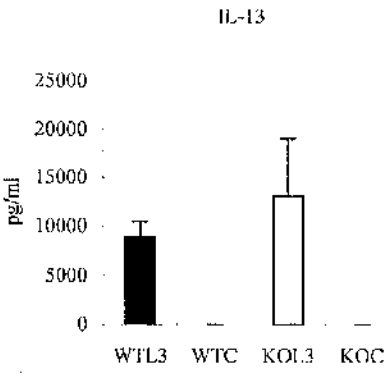
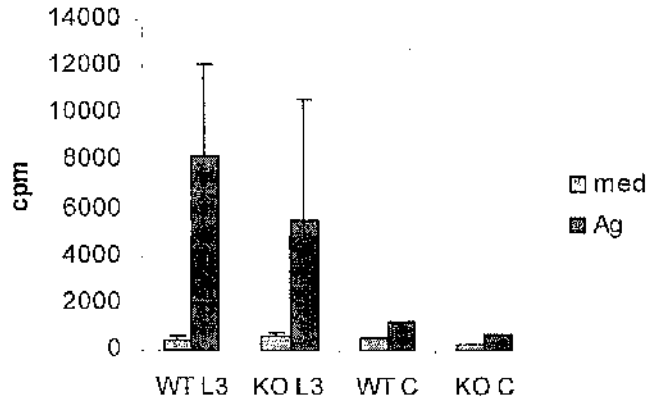

IL-13
pg/ml
25000
20000
15000
10000
5000
0
WTL3
WTC
KOL3
KOC

Figure 3.4. Splenocytes from L3 infected IL-4-/- mice display significantly lower Ag-specific proliferation compared to that of their wild-type counterparts

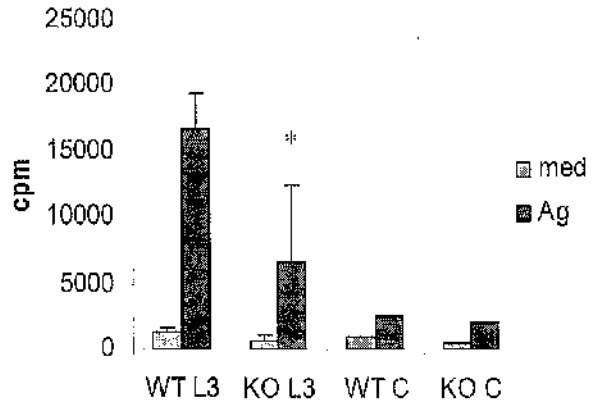
IL-4-/- (KOL3) and wild-type (WTL3) mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i., splenocytes from individual mice from each group were re-stimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Ag-specific proliferative responses from whole splenocytes were measured at 48 (A), 72 (B) and 96 (C) hours. Results are expressed as cpm.

48 hours p=0.3913 (KOL3 vs WTL3)
72 hours p=0.0373 " "
96 hours p=0.0304 " "

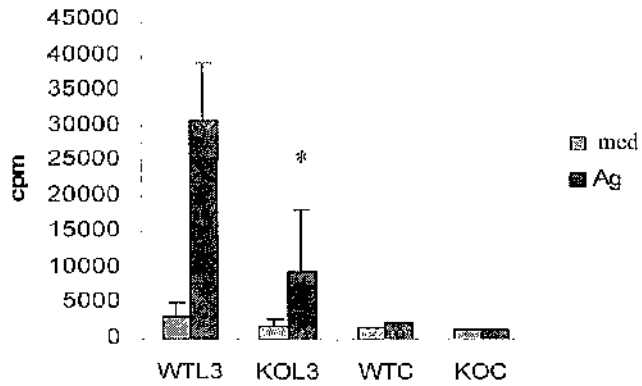
A


14000
12000
10000
8000
6000
4000
2000
0
cpm
WT L3
KO L3
WT C
KO C
med
Ag

B


25000
20000
15000
10000
5000
0
cpm
*
WT L3
KO L3
WT C
KO C
med
Ag

C


45000
40000
35000
30000
25000
20000
15000
10000
5000
0
cpm
*
WTL3
KOL3
WTC
KOC
med
Ag

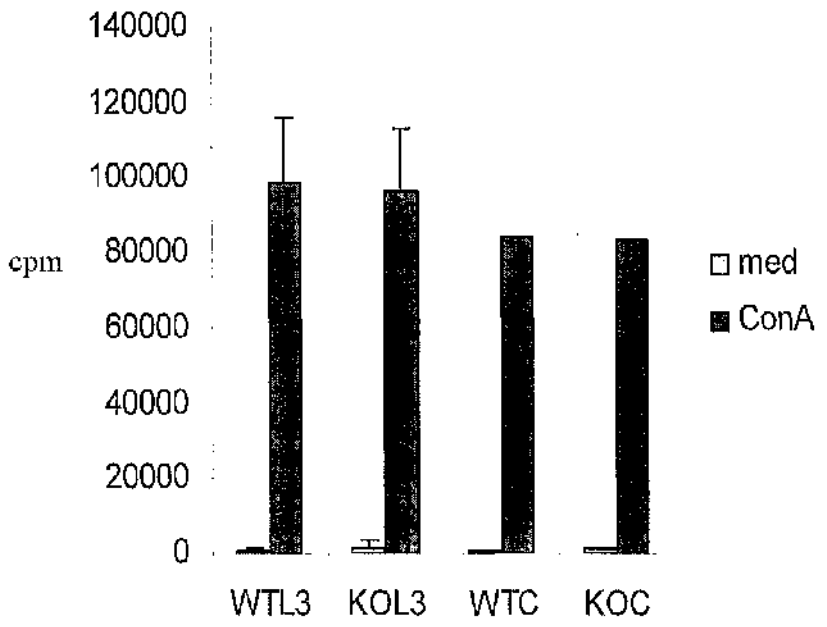


Figure 3.5. Splenocytes from L3 infected wild-type and IL-4-/- mice display similar levels of mitogen-driven proliferation

IL-4-/- (KOL3) and wild-type (WTL3) mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i. splenocytes from individual L3 infected wild-type or IL-4-/- mice or pooled cells from control mice were restimultated *in vitro* with 1 μg/ml ConA. Mitogen-driven proliferative responses from whole splenocytes were measured at 48 hours. Results are expressed as cpm.
WT vs KO p=0.8102

Fig 3.6. $CD4^+$ and $B220^+$ cells proliferate less well in IL-4-/- mice compared to wild-type mice

Splenocytes from wild-type (A and B) and IL-4-/- (C and D) mice given 50 L3 of *B. pahangi* or an equal volume of HBSS were labelled with CFSE and cultured with 10 μg/ml *B. pahangi* adult Ag for 96 hours. Cells from control animals did not proliferate and, for simplicity, are not shown. Cells were then harvested, stained with anti-CD4 MAb (A and C) or anti-B220 MAb (B and D) and analysed by flow cytometry. Each panel shows the staining profile of $CD4^+$ or $B220^+$ splenocytes in an individual mouse. The numbers at the top left hand corner of each panel indicate the percentage of $CD4^+$ or $B220^+$ cells displaying reduced fluorescence intensity in FL-1, indicating they have divided in Ag-stimulated culture. These figures are representative of the responses of five animals per group.

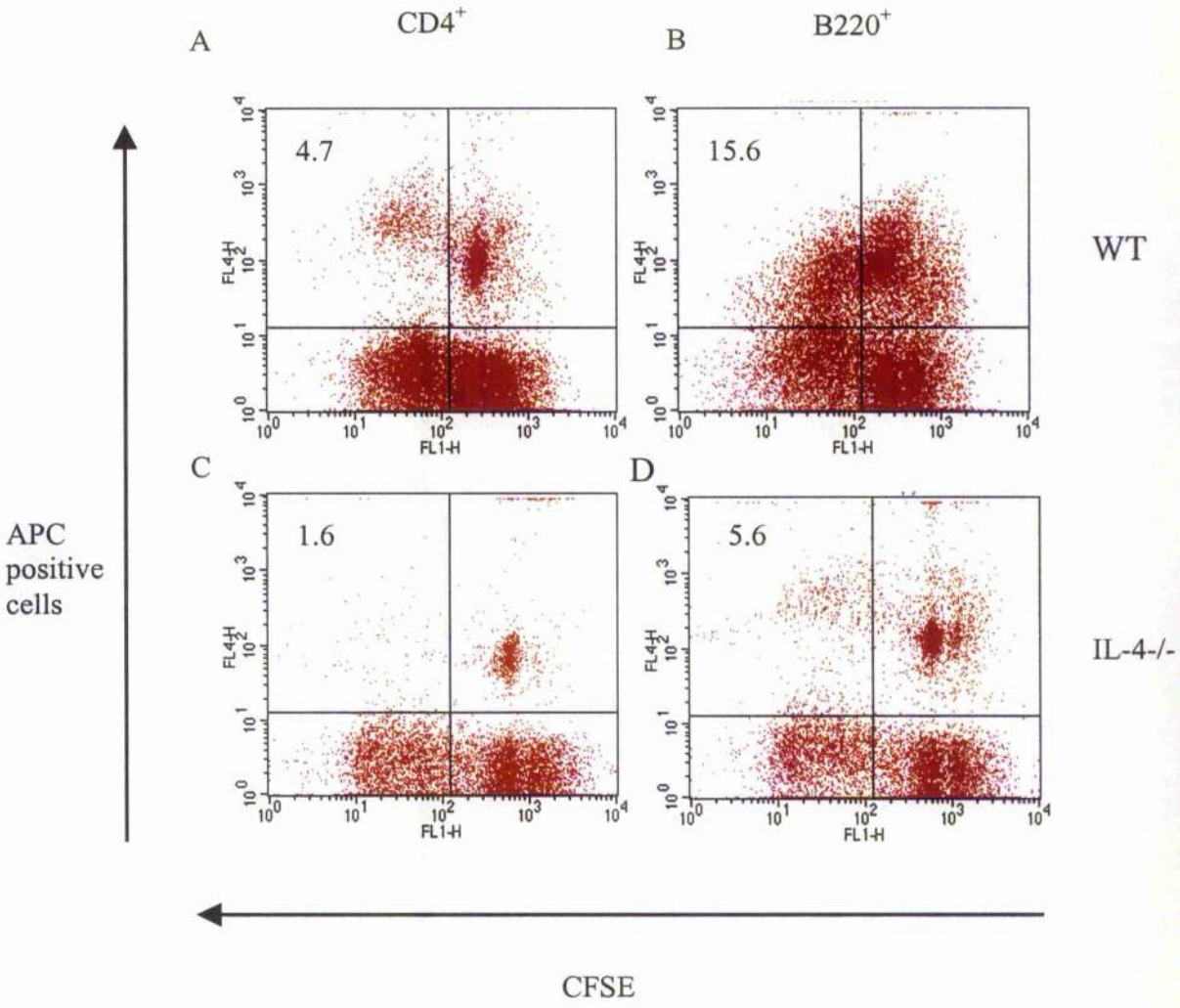
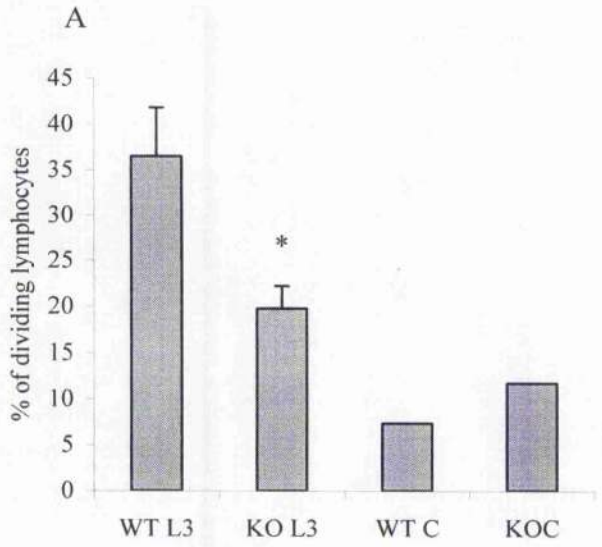
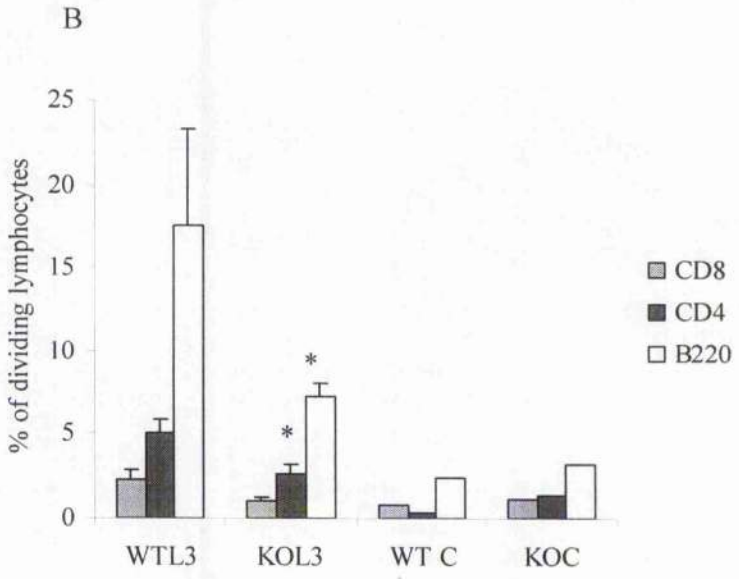

A
CD4+
B
B220+
4.7
15.6
WT
C
D
1.6
5.6
IL-4-/-
APC positive cells
FL4-H
FL1-H
CFSE

Figure 3.7. Proliferation of $CD4^+$ and $B220^+$ cells is significantly reduced in IL-4-/- mice compared to WT controls

Splenocytes from IL-4-/- (KOL3) and wild-type (WTL3) mice given 50 L3 of *B. pahangi* or an equal volume of HBSS (C), were labeled with CFSE and cultured with 10 μg/ml *B. pahangi* adult Ag for 96 hours. Cells were then harvested, stained with anti-CD4 MAb or anti-B220 MAb and analysed by flow cytometry. Data shown in A and B are mean results from five animals per group (excluding control animals where cells were pooled) and results are expressed as a percentage of total dividing lymphocytes. Graph A illustrates the mean percentage of total lymphocytes which have divided from groups of five mice (WTL3 vs KOL3, p=0.02). Graph B shows division of individual cell types as a percentage of dividing lymphocytes ($CD4^+$ division WTL3 vs KOL3 p=0.02; $B220^+$ division WTL3 vs KOL3 p=0.02).


A
45
40
35
30
25
20
15
10
5
0
% of dividing lymphocytes
*
WT L3
KO L3
WT C
KOC


B
25
20
15
10
5
0
% of dividing lymphocytes
*
*
WTL3
KOL3
WT C
KOC
CD8
CD4
B220

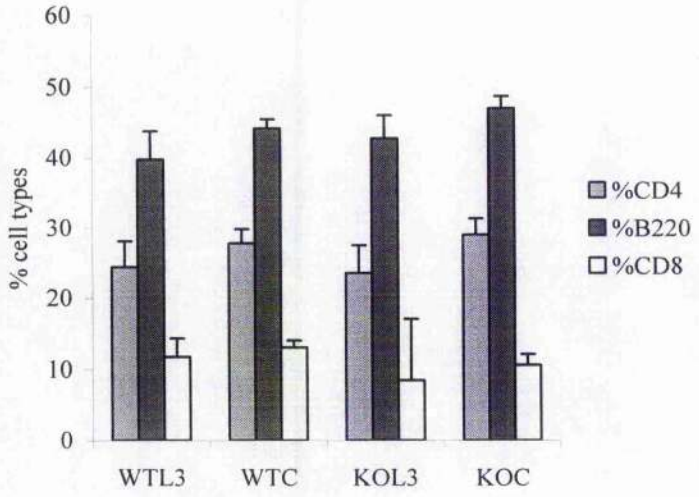


Figure 3.8. ***Ex vivo*** **staining of splenocytes from L3 infected and control IL-4-/- and wild-type BALB/c mice showed no significant differences between groups**

Splenocytes from IL-4-/- (KOL3) and wild-type (WTL3) mice given 50 L3 of *B. pahangi* or an equal volume of HBSS only (C) were surface stained *ex vivo* 12 d.p.i. with anti-CD4 MAb, anti-B220 MAb and anti-CD8 MAb and analyzed by flow cytometry. All results represent the mean +/- standard deviation of five animals per group.

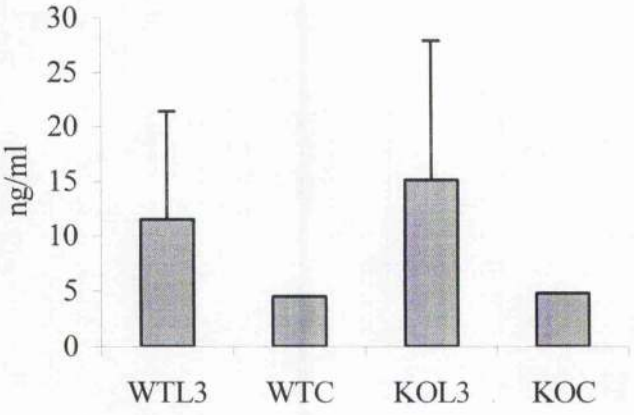


Figure 3.9. Levels of nitrite from 96 hour cultures are not significantly different between IL-4-/- mice and their wild type counterparts

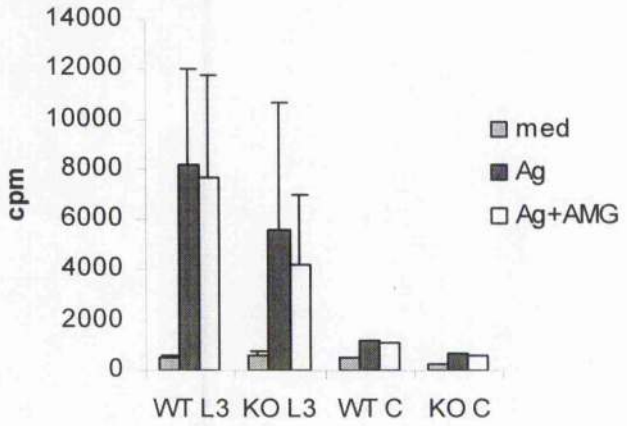
IL-4-/- (KO) and wild-type (WT) mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i. splenocytes from individual mice from each group were re-stimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Nitrite levels from whole splenocytes were measured in supernatants collected at 96 hours. Results are expressed as ng/ml. (WTL3 vs KOL3 p= 0.7133).

Figure 3.10. Addition of AMG to splenocytes from L3 infected BALB/c and IL-4-/- mice had no significant effect on Ag-specific proliferation of either group

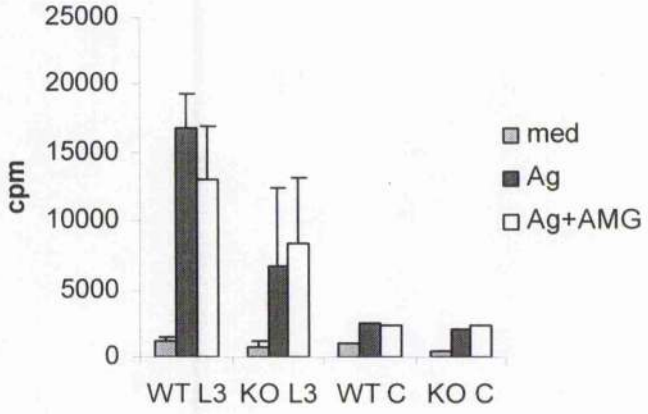
IL-4-/- (KOL3) and wild-type (WTL3) mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i., splenocytes from individual mice from each group were restimultated *in vitro* with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of 500 μM AMG. Ag-specific proliferative responses from whole splenocytes were measured at 48 (A), 72 (B) and 96 (C) hours. Results are expressed as cpm.

WTL3 VS KOL3	KOL3+Ag vs KOL3+Ag+AMG
48 hours p=0.39	48 hours p=1
72 hours p=0.0373	72 hours p=0.665
96 hours p=0.0367	96 hours p=0.4034

A


14000
12000
10000
8000
6000
4000
2000
0
cpm
med
Ag
Ag+AMG
WT L3
KO L3
WT C
KO C

B


25000
20000
15000
10000
5000
0
cpm
med
Ag
Ag+AMG
WT L3
KO L3
WT C
KO C

C

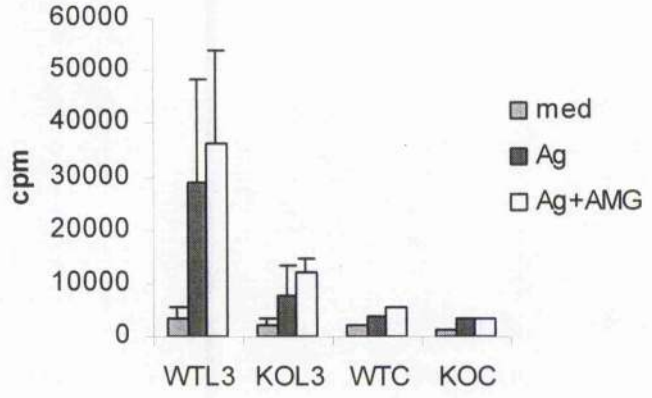
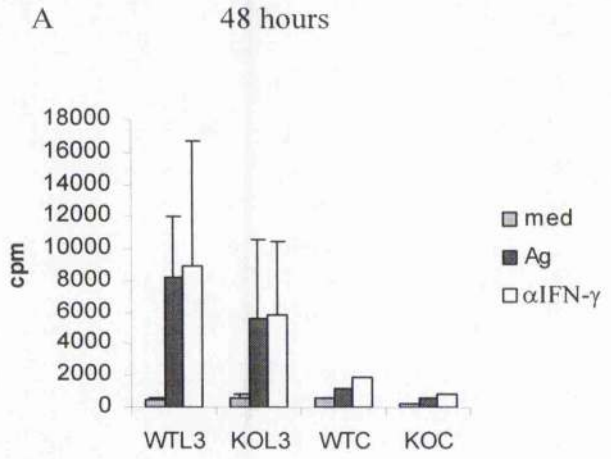

60000
50000
40000
30000
20000
10000
0
cpm
med
Ag
Ag+AMG
WTL3
KOL3
WTC
KOC

Figure 3.11. Addition of IFN-γ neutralizing MAb to splenocytes from L3 infected BALB/c and IL-4-/- mice had no significant effect on Ag-specific proliferation of either group

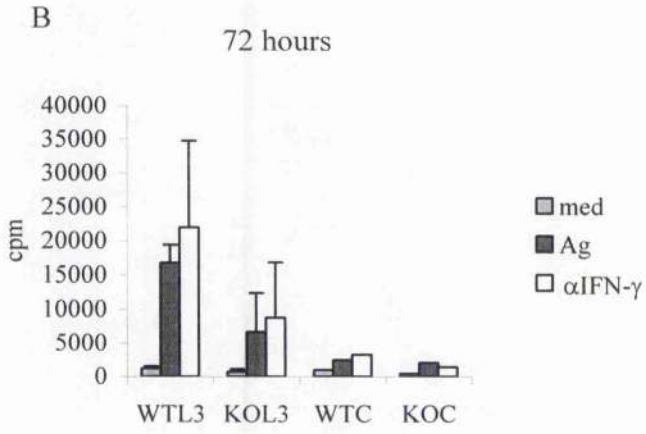
IL-4-/- (KOL3) and wild-type (WTL3) mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i. splenocytes from individual mice from each group were restimultated *in vitro* with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of 1 μg/ml neutralizing IFN-γ MAb. Ag-specific proliferative responses from whole splenocytes were measured at 48 (A), 72 (B) and 96 (C) hours. Results are expressed as cpm.

WTL3 VS KOL3	KOL3+Ag vs KOL3+Ag+αIFN-γ
48 hours p=0.39	48 hours p=0.8852
72 hours p=0.0373	72 hours p=0.665
96 hours p=0.0367	96 hours p=1

A


48 hours
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
cpm
WTL3
KOL3
WTC
KOC
med
Ag
αIFN-γ

B


72 hours
40000
35000
30000
25000
20000
15000
10000
5000
0
cpm
WTL3
KOL3
WTC
KOC
med
Ag
αIFN-γ

C

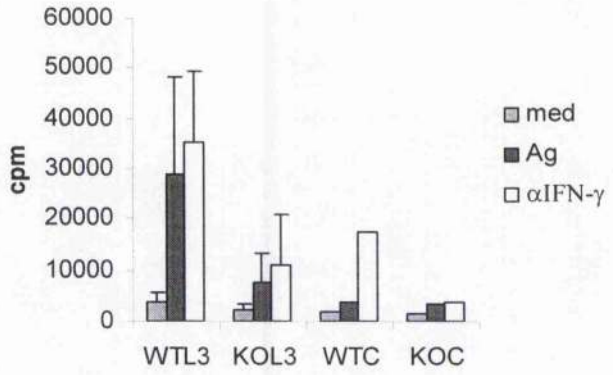
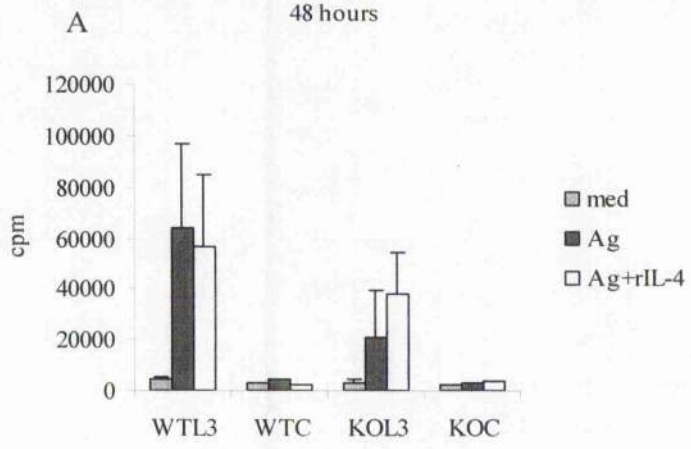
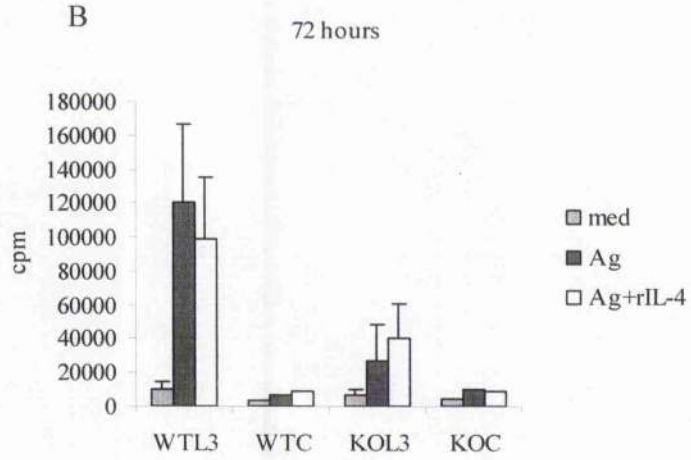

96 hours
60000
50000
40000
30000
20000
10000
0
cpm
WTL3
KOL3
WTC
KOC
med
Ag
αIFN-γ

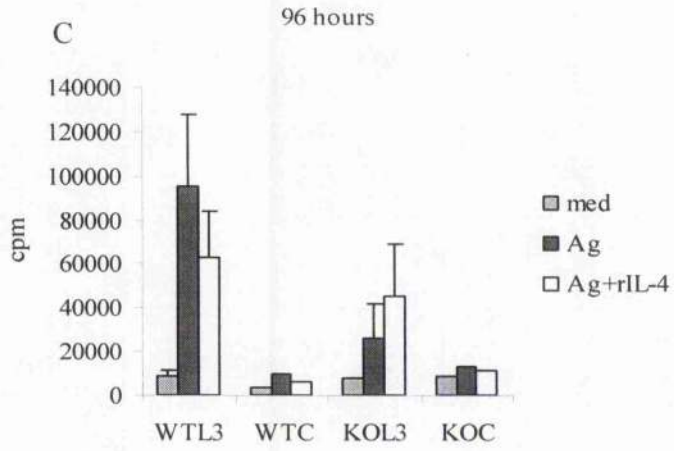
Figure 3.12. Addition of recombinant IL-4 to splenocytes from L3 infected BALB/c and IL-4-/- mice had no significant effect on Ag-specific proliferation of either group

IL-4-/- (KOL3) and wild-type (WTL3) mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i. splenocytes from individual mice from each group were restimultated *in vitro* with 10 μg/ml *B. pahangi* adult Ag and recombinant IL-4 was added at 1000 pg/ml to each individual culture. Ag-specific proliferative responses from whole splenocytes were measured at 48 (A), 72 (B) and 96 (C) hours. Results are expressed as cpm.

WTL3 VS KOL3	KOL3vsKOL3+1000 pg rIL-4
48 hours p=0.0662	48 hours p=0.1939
72 hours p=0.0304	72 hours p=0.3123
96 hours p=0.0304	96 hours p=0.3123


A
48 hours
120000
100000
80000
60000
40000
20000
0
cpm
WTL3
WTC
KOL3
KOC
med
Ag
Ag+rIL-4


B
72 hours
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
cpm
WTL3
WTC
KOL3
KOC
med
Ag
Ag+rIL-4


C
96 hours
140000
120000
100000
80000
60000
40000
20000
0
cpm
WTL3
WTC
KOL3
KOC
med
Ag
Ag+rIL-4

3.3. Discussion

The results presented in this chapter used IL-4-/- mice on the BALB/c background to study the role of IL-4 in parasite survival and immune modulation. The first experiments showed that IL-4 had little effect on worm recoveries from mice infected intraperitoneally with *B. pahangi* L3 as there was no significant difference in worm recoveries at any time point between the groups. However, IL-4 appeared to have a down-regulatory effect on the numbers of MF produced by adult female worms as IL-4-/- animals contained vastly more MF compared to WT mice. As adult female worms from IL-4-/- mice contained more MF at all stages of development compared to worms from WT mice, it was hypothesised that IL-4 may limit worm fecundity. Implantation of mature, fecund female worms into IL-4-/- and WT mice confirmed this theory, as the number of MF recovered was no different between worms from IL-4-/- animals and WT animals, thus ruling out the possibility of increased MF killing in WT mice. Therefore, although IL-4 does not appear to affect L3 to adult development, it clearly has a role in modulating the fecundity of worms. Alternatively, rather than a direct effect on female worms, IL-4 may also affect adult male worms. It was shown previously that male worms were more susceptible to the effects of irradiation than female worms in gerbils infected with irradiated L3 of *B. pahangi* (Devaney *et al.*, 1993) indicating that males may be less resistant to oxidative or immunological stress. However in the present study male worms were recovered, although the ratio of males : females was reduced in WT mice compared to IL-4-/- mice. These results are similar to experiments carried out in the *L. sigmodontis* model, where MF in the peritoneum of IL-4-/- BALB/c mice were increased 100-fold compared to WT mice post-infection with L3 (Volkmann *et al.*, 2001). However, whether IL-4 effects adult female fecundity directly or indirectly is not known. Infection of IL-5-/- mice with the nematode *Strongyloides ratti* resulted in

increased egg production (Ovington *et al.*, 1998). IL-5 was shown to affect worm survival and fecundity as IL-5-/- mice harboured 60% more worms and these worms were more fecund that those taken from WT mice. The results presented in this Chapter demonstrate that levels of IL-5 are significantly greater in WT mice than in IL-4-/- mice. Thus IL-5 may have a similar effect on adult *B. pahangi* to that described for *S. ratti*. No direct link between IL-5 and worm fecundity could be made during the course of these experiments, but it would be interesting to implant adult *Brugia* into the peritoneum of IL-5-/- mice and directly investigate the role of IL-5 in MF production and fecundity of adult worms.

It was notable that despite the fact that worm recoveries, following i.p. infection with L3 were very similar between mouse strains, the immunological responses were very different. Following s.c. infection with L3, splenocytes from WT mice produced Th2 cytokines with no IL-2 or IFN-γ production. However in IL-4-/- mice elevated levels of IL-2 and IFN-γ and significantly reduced levels of IL-5 were observed. IL-10 levels had a tendency to decrease in IL-4-/- mice but this difference only reached statistical significance in one out of five experiments. These data demonstrate that the L3 can prime Th1 cells in response to infection but in WT mice these are suppressed by a mechanism involving IL-4. The ability of IL-4-/- mice to produce typical Th2 cytokines (IL-5, IL-10 and IL-13) is interesting. An obvious candidate for Th2 induction in the absence of IL-4 is IL-13, although, experiments in *L. major* infected mice have shown that Th2 priming can occur via an alternative pathway, independent of both IL-4 and IL-13 (as judged by the stable Th2 marker ST2L) (Kropf *et al.*, 1999). In the experiments described above, the source of Th2 cytokines was not determined and it is possible that cell types other than $CD4^+$ cells may account for IL-5, IL-10 and IL-13. Therefore,

further experiments would be required to unequivocally demonstrate that Th2 cells are expanded in IL-4-/- mice infected with *B. pahangi*.

In addition, there were major differences between IL-4-/- and BALB/c mice in the cellular make up of the peritoneal exudates in infected animals. Numbers of eosinophils were significantly higher in WT animals, results similar to those from the *L. sigmodontis* model, where levels of eosinophilia were greatly reduced in IL-4-/- mice (Volkmann *et al.*, 2001). This result was not unexpected as levels of IL-5, a growth factor for eosinophils are significantly lower in IL-4-/- animals, and, in addition IL-4 is known to be chemotactic for eosinophils (Moser *et al.*, 1993; Johnson *et al.*, 1998). Eosinophils have been shown to be involved in the development of protective immunity to the L3 of *L. sigmodontis*. Worm recoveries were similar during a primary infection of IL-5-/- mice or WT mice but during challenge infection, IL-5-/- mice were unable to generate protective immunity, whereas intact mice cleared their parasites. Further analysis indicated that eosinophils had a role in worm killing (Le Goff *et al.*, 2000). Based on the results of Le Goff *et al*, it would be interesting to study worm recovery in secondary infection in IL-4-/- mice. Macrophage numbers were also reduced in IL-4-/- mice. Macrophages have been implicated in worm killing in various experimental systems, such as the jird or the mouse infected with *B. pahangi*, where adherent cells are required for MF killing (Karavodin and Ash, 1982; Oxenham *et al.*, 1984). Furthermore, macrophages have long been suspected to modulate immune responses to filarial infection. For example, in 1980 it was shown that removal of adherent cells from PBMC of filarial infected humans reversed the proliferative defect, suggesting that macrophages play a role in maintaining hyporesponsiveness in human infection (Piessens *et al.*, 1980b). More recent studies in the mouse model suggested adherent

cells may produce IL-10 and thus down-regulate immune responses (Osborne and Devaney, 1999). These data clearly indicate that IL-4 is important for recruitment of specific cell types into the peritoneal cavity of infected mice. However, despite high cellular recruitment in WT mice, worm recovery was not affected. The importance of cellular infiltration in pathology in relation to IL-4 has been highlighted in studies on *O. volvulus* where IL-4-/- BALB/c mice develop exacerbated pathology due to increased neutrophil infiltration of the corneal stroma (Pearlman *et al.*, 1996; Hall *et al.*, 2002).

In terms of Ag-specific immune responses of IL-4-/- and WT mice, one of the most interesting results was the reduced levels of proliferation observed in the IL-4-/- animals. In order to determine if this defect was Ag-specific, splenocytes from WT and L3 infected mice were also stimulated with the mitogen ConA. No differences were observed in response to ConA, indicating that the defect in proliferation observed was Ag-specific. It was demonstrated by CFSE labelling that the percentage of $CD4^+$ and $B220^+$ proliferation was significantly decreased in L3 infected IL-4-/- mice, suggesting that, in response to Ag, IL-4 may be acting as a growth factor for these cell types. This hypothesis was tested by supplementing the medium with concentrations of rIL-4 up to 1000 pg/ml. However, although there was a slight increase in proliferative responses to Ag in splenocytes from IL-4-/- mice, this difference never reached significance. Phenotypic analysis of the cellular population in the spleen of L3 infected IL-4-/- or WT mice *ex vivo* revealed no significant difference, ruling out the possibility that differences in proliferation relate to diminished numbers of specific cell populations *ex vivo.*

Splenocytes from L3 infected IL-4-/- mice secrete high levels of IFN-γ, which can induce NO production from classically activated macrophages and result in a

consequent reduction in proliferation (Candolfi *et al.*, 1994; Dai and Gottstein, 1999; O'Connor and Devaney, 2002). To assess the production of NO *in vitro* in my experiments, the Greiss reaction was carried out on supernatants of Ag-stimulated splenocytes from L3 infected IL-4-/- and wild type mice. These results demonstrated, somewhat surprisingly, that there was no significant difference in levels of NO in cultures from IL-4-/- mice compared to wild type mice, despite significantly increased levels of IFN-γ. However, to rule out possible effects of low-level NO, further experiments were undertaken. Aminoguanidine (AMG), an iNOS inhibitor, was used to determine whether the increased levels of IFN-γ produced by cells from L3 infected IL-4-/- mice might suppress proliferation responses by induction of iNOS. Inhibition of NO production did not result in increased proliferation in cultures of Ag-stimulated splenocytes from L3 infected IL-4-/- animals. Finally to ensure that elevated levels of IFN-γ observed in cells from IL-4-/- mice were not cytostatic, IFN-γ was neutralized *in vitro*. However, anti-IFN-γ had no effect on the proliferation of cells from L3 infected IL-4-/- mice. These results in IL-4-/- mice contrast with those reported from *S. mansoni* infection. In that model, WT mice develop chronic disease but survive, whereas IL-4-/- animals suffer fatal disease. Disease severity was seen to correlate with increased levels of NO and enhanced IFN-γ production. Splenocytes from IL-4-/- mice displayed a similar defect in proliferation to that described in this Chapter, and it was shown that in the *S. mansoni* model, NO was largely responsible for impaired T cell function (Patton *et al.*, 2002). In contrast to the results presented here, neutralization of IFN-γ in splenocyte cultures from *S. mansoni* infected mice resulted in the rescue of proliferation. In addition, when administered *in vivo* improvements in the gross appearance of the liver and reduction in granuloma size resulted. (La Flamme *et al.*, 2001). It is notable that the cytokine milieu is very complex in cultures from L3 infected

IL-4-/- mice compared to WT mice with IL-2 and IFN-γ production as well as IL-10 and IL-13 and some IL-5. Therefore, these typically Th2 type cytokines (or in the case of IL-10, Treg) may have a role in down-regulating the production of NO from classically activated macrophages, despite high levels of IFN-γ.

The results presented in this Chapter show that Ag-specific proliferation in IL-4-/- animals was significantly reduced but this could not be attributed to NO or IFN-γ production as has been observed in other model systems. These results present an interesting conundrum, in that splenocytes from L3 infected IL-4-/- mice secrete substantial levels of IFN-γ yet this does not appear to induce significant amounts of NO. This situation contrasts with that observed when mice were infected with MF of *B. pahangi*, where high levels of IFN-γ (in the same order as observed in L3 infected IL-4-/- mice) resulted in NO production and a consequent proliferative defect (O'Connor *et al.*, 2000; Jenson *et al.*, 2002; O'Connor and Devaney, 2002). As L3 of *Brugia* are reported to direct the activation of alternatively activated macrophages in the peritoneal cavity of C57BL/6 mice (Allen and Loke, 2001), it is interesting to speculate whether a similar cell may be induced in this system. Alternatively activated macrophages have been shown to suppress the proliferation of T cell clones in a non-specific manner, but are reportedly IL-4 dependent. Whether a similar population may be elicited in L3 infected IL-4-/- BALB/ c mice remains unknown. It is pertinent to note that these cells can be activated by IL-13 (Doyle *et al.*, 1994). IL-13 and IL-4 share certain overlapping biological activities, as both cytokines utilise the IL-4Rα chain as a component of their receptor complexes and signal though a shared transducer (Zurawski *et al.*, 1993). IL-13 is found in large amounts in splenocyte culture from L3 infected IL-4-/- BALB/ c mice and perhaps would be sufficient to prime these alternatively activated macrophages. By

investigating levels of arginase or urea (indicative of alternatively activated macrophages) in these cultures in relation to levels of NO (indicative of classically activated macrophages), it would be possible to determine whether alternatively activated macrophages are present. One possibility for reduced levels of proliferation observed in splenocytes from IL-4-/- mice may be due to a reduced capacity of APC from KO animals to efficiently present Ag (i.e. via an effect on B7-1, B7-2 or MHC class II molecules). One way in which this could be investigated would be to analyze cells by FACS or to perform "mixing" experiments, whereby purified $CD4^+$ cells from naïve animals are cultured with APC from WT or IL-4-/- mice. These experiments would indicate whether APC function is effected in IL-4-/- mice.

Although it was not possible to identify the basis of the proliferative defect observed in L3 infected IL-4-/- mice, one possibility yet to be investigated is that splenocytes from IL-4-/- mice undergo increased apoptosis when re-exposed to Ag *in vitro*. Data presented in this Chapter show that levels of Ag-specific proliferation in IL-4-/- mice are significantly different to that of WT mice only after 72 and 96 hours. The defect is not observed at 48 hours suggesting that the cells start to proliferate, but cannot maintain cellular divisions. It would be worthwhile investigating apoptosis in these cultures using Annexin V staining at various time points to determine whether differences exist between L3 infected WT and IL-4-/- cells. It was notable that, when carrying out FACS analysis on CFSE stained splenocytes from IL-4-/- mice, fewer events were collectable when gating on live lymphocytes compared to WT mice. This may provide indirect evidence that these cells are undergoing apoptosis in culture.

The results presented in this Chapter demonstrated that the presence or absence of IL-4 did not significantly influence the recovery of the parasite from the peritoneal cavity of

BALB/c mice, despite major differences in cytokine profile and cellular infiltration between groups. While these results indicate that IL-4 or IL-4-/- mediated responses are not required for killing a primary infection of L3 of *B. pahangi*, their involvement in protective immunity to challenge infection has yet to be demonstrated. Perhaps the most significant effect of IL-4 demonstrated by these experiments was its effect on worm fecundity, as female worms from IL-4-/- mice were significantly more fertile than worms from WT mice. Exactly how IL-4 affects worm fecundity remains unclear and will require further investigation.

Chapter 4. IL-10 down-regulates Th1 cytokine production and $CD4^+$ cell proliferation in L3 infection

4.1. Introduction

IL-10 was first described as cytokine synthesis inhibitory factor (CSIF), a product of mouse Th2 cells that was shown to inhibit activation and cytokine production by Th1 cells (Fiorentino *et al.*, 1989). IL-10 inhibits cytokine production via an indirect effect on accessory cells, such as monocytes and macrophages. However in the absence of APC, IL-10 can affect T cells directly by suppressing production of IL-2 (de Waal Malefyt *et al.*, 1993b; Taga *et al.*, 1993). IL-10 was found to profoundly down-regulate the synthesis of monokines, the production of NO and the expression of class II MHC and costimulatory molecules on activated monocytes and macrophages. *In vivo* and *in vitro* studies have also demonstrated a role for IL-10 in modulating B,T, NK, dendritic and mast cell function. IL-10 can also inhibit the production of IL-12, which in turn suppresses the stimulation of NK cells, the activation of macrophages, DC and IFN-γ Th1 responses (Moore *et al.*, 2001). These studies therefore indicate the diverse effects of this cytokine in dampening and, ultimately, terminating inflammatory responses.

In terms of infectious disease, the challenge faced by the immune system is to respond to such an extent that infection is eliminated while minimizing the non-specific injury to host tissue. Most of what has been learned about IL-10 in infectious disease derives from studies carried out in mice, where levels of IL-10 have been manipulated *in vivo* or *in vitro*. In murine studies of schistosomiasis, rIL-10 inhibited the proliferation of schistosome-specific T cell clones (Flores Villanueva *et al.*, 1993) and down-regulated

expression of B7 costimulatory molecules on APC (Flores Villanueva *et al.*, 1994). The administration of an IL-10/Fc fusion protein (with a prolonged half-life *in vivo*) caused a decrease in Th1 responses in infected mice which resulted in a reduction in granuloma size. These results suggest that regulation of Th1 responses reduces the immunopathologic damage associated with schistosomiasis (Flores-Villanueva *et al.*, 1996). However, there is conflicting evidence as to whether IL-10 is required for the immune down-modulation, which defines the transmission from the acute to the chronic disease state in *S. mansoni* infection in the mouse model (Wynn *et al.*, 1998); (Sadler *et al.*, 2003).

As the Th1/Th2 balance is essential in determining the outcome of *L. major* infection, experiments have also been carried out to examine the role of IL-10 in this model system. One particular study gave a clear demonstration that IL-10 contributes to susceptibility to *L. major* in BALB/c mice. It was shown that normal BALB/c mice develop progressive lesions, whereas IL-10-/- BALB/c mice control disease progression and have 1000-fold fewer parasites by week 5 post-infection (Kane and Mosser, 2001). IL-10 has also been shown to be a critical anti-inflammatory molecule in other disease models in mice e.g. IL-10-/- mice develop inflammatory bowel disease and other exaggerated inflammatory responses (Kuhn *et al.*, 1993). In human inflammatory disease, the therapeutic potential of IL-10 has been tested in specific patient populations such as those with Crohn's disease (van Deventer *et al.*, 1997), rheumatoid arthritis (RA) (Keystone *et al.*, 1998), psoriasis (Asadullah *et al.*, 1998) and hepatitis C (Nelson *et al.*, 2000) – all chronic inflammatory conditions. In both Crohn's disease and RA, IL-10 had a trend towards efficacy and a good safety profile and in cases of psoriasis, after a seven week treatment, there was a significant decrease in the psoriatic area in 9/10

patients (van Deventer *et al.*, 1997; Asadullah *et al.*, 1998). Conversely when IL-10 is neutralized in PBMC from leprosy patients, proliferative responses are enhanced (Sieling *et al.*, 1993).

The role of IL-10 in lymphatic filariasis has been the subject of several studies by the Nutman laboratory. In the original study (King *et al.*, 1993) it was shown that neutralizing IL-10 with a monoclonal antibody restored proliferative responses in cultures of PBMC from *W. bancrofti* infected individuals. Interestingly, similar results were obtained with neutralizing antibody to TGF-β. In a subsequent study by Mahanty *et al*, T cell proliferative responses were studied in asymptomatic microfilaraemics (MF+) and it was shown that MF+ individuals were preferentially hyporesponsive to microfilarial derived antigens via induction of IL-10 (Mahanty *et al.*, 1996a). The concurrent reduction in IFN-γ and IL-2 observed in this study is in agreement with previously reported results of down-regulated Th1 cytokine secretion in MF+ individuals (King *et al.*, 1993; Yazdanbakhsh *et al.*, 1993a) and is consistent with the notion that parasite antigen-specific Th1 responses are inhibited in MF+ individuals. These studies were notable in that they illustrated that PBMC from MF+ individuals secrete more spontaneous IL-10 (without stimulation) than CP individuals. Spontaneous IL-10 was found to be secreted by plastic adherent cells (Mahanty *et al.*, 1996b), suggesting that cells, other than T lymphocytes, are producing IL-10 and that APC (adherent cells) have a role in suppressing the immune response in active infection. All in all, these data suggest that MF+ individuals preferentially produce IL-10 in response to MF-derived Ag, in an inverse pattern to that observed with Th1 cytokines. The L3 stage of *B. pahangi* is also known to induce high levels of IL-10 in antigen-stimulated spleen cell cultures from BALB/c mice. Neutralization of IL-10 resulted in the secretion

of IFN-γ and IL-2 without affecting levels of Ag-specific IL-4 or IL-5 (Osborne and Devaney, 1999). In addition, APC were also shown to contribute to the suppression of mitogen-driven Th1 responses in spleen cells from L3-infected animals, although a direct role for IL-10 in modulating APC function was not shown.

The aims of the experiments in this chapter were three-fold. Firstly, as described above, IL-10 is known to effect *in vitro* cytokine responses in mice infected with L3, but nothing is known of the role of IL-10 *in vivo* in this model system. Secondly, IL-10 is produced by a variety of cell types including T cells, B cells activated mast cell lines, activated macrophages and keratinocytes (Fiorentino *et al.*, 1989) and T regulatory cells (Roncarolo *et al.*, 2001b). Consequently, it was of interest to determine the source of IL-10 in mice infected with L3, as a first step to determine whether this life cycle stage can elicit a population of Treg cells. Thirdly, studies in *L. major* infected mice have shown that sand fly transmission enhances lesion size and parasite burden within the lesion (Titus and Ribeiro, 1988). This effect is thought to be associated with a vasodilatory peptide which reportedly up-regulates levels of IL-10 (Bozza *et al.*, 1998). Therefore, it was of interest to examine the role of mosquito saliva on L3 transmission using natural feeding methods.

4.2. Results

4.2.1. Ag-specific immune responses of splenocytes from L3 infected mice +/- anti-IL-10 MAb

In these experiments groups of five BALB/c mice were injected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. spleens were removed for *in vitro* analysis. 10 μg/ml of neutralizing anti-IL-10 (JESS-25A) or isotype matched control (R59-40) was added to Ag-stimulated cultures from L3 infected and control mice. In addition, Ag only cultures were set up.

(i) Ag-specific cytokine responses

Cytokine responses of splenocytes from L3 infected and control animals in the presence and absence of neutralizing anti-IL-10 were measured after 72 hours of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. Figure 4.1 shows levels of Ag-driven cytokines in supernatants produced by spleen cells after 72 hours of *in vitro* culture. Cells incubated in medium only did not produce any of the cytokines measured. These results confirm that IL-10 is important in regulating IL-2 and IFN-γ production, in that neutralizing IL-10 significantly up-regulates the production of these Th1 cytokines. However, although there was a trend for increased levels of IL-4 and IL-5 when IL-10 was neutralized, these differences did not reach statistical significance. These results confirm those previously reported following *in vitro* neutralization of IL-10 (Osborne and Devaney, 1999)

(ii) Ag-specific proliferative responses

Proliferative responses of splenocytes from L3 infected and control mice in the presence and absence of neutralizing anti-IL-10 were measured over a time course of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. As shown in Figure 4.2, at no time point (48 or 72 hour) did anti-IL-10 have an effect on Ag-stimulated proliferation, as previously reported (Osborne, 1999).

4.2.2. Investigating the cellular source of IL-10

Defining the phenotype of the cells responsible for IL-10 production is important in terms of understanding how the L3 polarizes the immune response. For example, do high levels of IL-10 merely reflect Th2 polarization or is IL-10 produced by cells other than Th2 cells?

4.2.2.1. *In vitro* depletion experiments

In an attempt to characterize the cellular source of IL-10, selected populations were depleted from splenocytes from L3 infected BALB/c mice. In these experiments the appropriate cellular population was depleted from pooled splenocytes using magnetic beads and the relevant cell surface Ab. Depletions were monitored by FACS analysis and in all experiments were greater than or equal to 95% efficient. Cytokine responses were compared in Ag-stimulated cultures of depleted and non-depleted populations at 48 hours. Post depletion, cells were cultured at 1 x 10^7 / ml per sample, thus cell numbers in cultures from depleted splencytes did not contain fewer cells than whole splenocyte cultures.

(i) Depletion of $CD4^+$ cells

In three separate experiments, depletion of $CD4^+$ cells resulted in a dramatic decrease in levels of IL-10 relative to unseparated splenocytes from the same animals, although levels of IL-10 were never reduced to background despite the removal of almost all $CD4^+$ cells. The results of a representative experiment are shown in Figure 4.3, where levels of IL-10 were decreased by 60% (mean value of three experiments 71% ± 12.3 reduction in IL-10). In addition levels of IL-4 were reduced by 40% and IL-5 by 60% (mean values of three experiments 45% ±12 and 73.5 %± 20.5 respectively). No Th1 cytokines were detected in supernatants from $CD4^+$ depleted cultures or whole splenocyte cultures.

(ii) Depletion of B cells

In four separate experiments, depletion of $B220^+$ cells resulted in a more modest effect on IL-10 production relative to unseparated splenocytes from the same animals. The results of a representative experiment are shown in Figure 4.3, where levels of IL-10 were reduced by 40% (mean data for four experiments 53.5% ± 14.85).

4.2.2.2. Semi-quantitative real time RT-PCR

The results of the depletion experiments suggested that $CD4^+$ cells were the major producers of IL-10 with B cells contributing to a lesser extent. However using this method it was not possible to rule out indirect effects of depletion, e.g. the depletion of one cell type could affect the ability of another cell to produce IL-10. Therefore a more direct method of determining the cellular source of IL-10 was required. To address this point IL-10 mRNA levels from purified $CD4^+$ cells and B cells were quantified by real-

time PCR using the ABI/PRISM 7700 sequence detection system. Analyses were performed using primers and an internal fluorescent TaqMan probe specific to IL-10 and HPRT. All samples were run in triplicate.

(i) IL-10 mRNA expression in $CD4^+$ and $B220^+$ cells

At 12 d.p.i. $CD4^+$ or B cells were purified from the spleens of ten L3 infected mice using magnetic separation. Depletions were monitored by FACS and in all experiments were greater than or equal to 95% efficient (data not shown). Following RNA extraction, real time RT-PCR was carried out on the selected populations. Figure 4.4 shows IL-10 mRNA levels relative to HPRT and demonstrates that the $CD4^+$ population express approximately two-fold more IL-10 mRNA compared to B cells. This experiment was carried out three times and each data set showed a similar profile. Thus the results on mRNA expression in different cellular populations equate reasonably well with those from the depletion experiments, measuring levels of IL-10 protein.

4.2.3. Investigating the role of IL-10 *in vivo*

The *in vitro* experiments described above demonstrate that IL-10 can suppress the production of Th1 cytokines in response to the L3 and indicate that Th1 cells are primed, but are suppressed by IL-10. To investigate the role of IL-10 *in vivo*, an antibody which neutralizes the IL-10 receptor was administered to mice intraperitoneally at days 0, 4 and 7 post-infection at a dose of 10 μg each injection. The use of this Ab gave further insight into the role of IL-10 in the immune response elicited by the L3.

(i) Ag-specific proliferative responses of splenocytes from L3 infected mice in which IL-10R has been blocked

Data from previous experiments demonstrated that neutralizing IL-10 *in vitro* had no effect on levels of Ag-specific proliferation of splenocytes from L3 infected mice, despite a significant increase in levels of Th1 cytokines. However, as shown in Fig. 4.5, after mice have been infected with L3 and then treated with IL-10R neutralizing Ab, levels of Ag-specific proliferation were significantly increased compared to mice given the isotype control, suggesting a role for IL-10 in down-regulating proliferation *in vivo*. This increase in proliferation was shown to be Ag-specific in nature as when cpm from medium only cultures were subtracted from cpm in Ag-stimulated cultures, there was still a significant difference in the levels of proliferation between mice given IL-10R MAb and mice given the isotype control (p= 0.0122).

(ii) Ag-specific cytokine responses of splenocytes from L3 infected mice in which IL-10R has been blocked

It was shown previously that neutralising IL-10 *in vitro* resulted in increased levels of IL-2 and IFN-γ. However, results shown in Fig. 4.6 demonstrate that neutralising IL-10R *in vivo* did not result in any changes in cytokine profile, with levels of IL-4, IL-5 and IL-10 remaining consistent between groups and no Th1 cytokines observed.

(iii) CFSE staining of cells from L3 infected mice in which IL-10R has been blocked

In an attempt to define the phenotype of the cells that proliferate when the IL-10R is blocked, CFSE staining (see Materials and Methods 2.4.2) was carried out prior to *in vitro* stimulation with 10 μg/ml *B. pahangi* adult Ag. At 96 hours, cells were harvested and labelled with anti-CD4 MAb or B220 MAb and the samples were analyzed by flow cytometry. The data shown in Figure 4.7 and 4.8 are from a representative experiment in which the proliferation of $CD4^+$ and B cells were followed using CFSE. The FACS plots in Figure 4.7 show cells from individual mice, which are representative of a group of five, with the means of five mice shown in Figure 4.8. These results demonstrate that levels of $B220^+$ proliferation are unaffected by the neutralization of IL-10R, with ~15% of B cells proliferating in mice given the neutralizing Ab, or given the isotype control (Figure 4.8). However, there is a difference in the levels of $CD4^+$ proliferation between these groups of mice. In splenocytes culture from isotype control treated mice, ~5% of $CD4^+$ cells proliferate compared to ~13.5% in cultures from mice given the neutralizing Ab (Figure 4.8). However the difference in proliferation of $CD4^+$ cells between the groups did not reach statistical significance (p=0.0662). In Figure 4.9, histogram plots generated by the flow cytometer are shown to illustrate the division of $CD4^+$ and $B220^+$ cells. The plots represent two individual animals from a group of five infected with 50 L3 of *B. pahangi* and given either neutralizing IL-10R MAb (panel A) or isotype matched control (panel B). It can be seen from these plots that the division of $B220^+$ cells (green overlay) and $CD4^+$ cells (pink overlay) accounts for the majority of dividing cells in these cultures. The initial peak which is shown at approximately 10^3 on the x axis reflects undivided cells while the peak to the left represents cells which have

divided. It can be seen that there are fewer $CD4^+$ cells dividing in panel B, which is from the mouse which received GL113.

(iv) *Ex vivo* staining of splenocytes from L3 infected mice in which IL-10R has been blocked

Ex vivo staining of splenocytes from L3 infected mice given IBL3a or GL113 was carried out using anti-CD4 MAb, anti-B220 MAb and anti-CD8 MAb, to investigate whether there were differences in the cellular make up of the spleen after the administration of IBL3a. One possible explanation for the increased levels of $CD4^+$ cell proliferation observed in the splenocytes from L3 infected mice given IBL3a compared to L3 infected mice given GL113, may be due to differences in numbers of these cell populations *ex vivo*. However, it can be seen from Figure 4.10 that this is not the case, as the percentage of $CD4^+$, $B220^+$ and $CD8^+$ cells are not significantly different between groups. Therefore it can be concluded that neutralizing the IL-10R directly affects proliferation of $CD4^+$ cells, following Ag-restimulation.

4.2.4. The role of mosquito transmission on immune responses

In all the afore mentioned experiments, mice were infected with L3 by syringe inoculation. In the sand fly-leishmania system there is now evidence that sand fly saliva inoculated along with the parasite, has a major effect on the immune response (Ribeiro, 1987). In particular, the principle vasodilator in sand fly saliva, a peptide termed maxadilan (MAX) (Lerner *et al.*, 1991) has been shown to have the ability to decrease levels of IFN-γ mainly by up-regulating levels of IL-10 (Bozza *et al.*, 1998). It is not clear at present whether this effect is specific to the sand fly or might be shared with other *Diptera*. Experiments carried out to investigate the role of mosquito saliva in

transmission of L3, included analysis of cytokine production in the spleen and peripheral lymph nodes following syringe inoculation or natural transmission via mosquito bite. The number of L3 administered to the mice by the mosquitoes was estimated by counting L3 per mosquito prior to and after feeding (as described in Materials and Methods 2.2.2). The relevant controls included mice infected sub-cutaneously with 50 L3 and mice given HBBS alone. At 12 d.p.i. spleens were removed and cytokine and proliferation assays were carried out.

(i) Cytokine responses of splenocytes and lymph nodes of mice infected with L3 via syringe or natural transmission

Cytokine responses of splenocytes and lymph nodes from syringe infected and naturally infected and control animals were measured after 72 hours of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. Figure 4.11 shows levels of Ag-driven cytokines in supernatants produced by spleen cells and lymph node cells after 72 hours of *in vitro* culture. These results show that there are significantly higher levels of IL-4, IL-5 and IL-10 produced by splenocytes from syringe inoculated mice compared to those from mice exposed to infective mosquitoes. In addition levels of IL-4 and IL-10 were significantly higher in lymph node cells from syringe inoculated mice compared to mosquito exposed mice.

(ii) Ag-specific proliferative responses of splenocytes and lymph node cells of mice infected with L3 via syringe or natural transmission

Proliferative responses of splenocytes and lymph node cells from syringe infected and naturally infected and control animals were measured after 48, 72 and 96 hours of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. Figure 4.12 shows that although

there are slight differences in the levels of proliferation in splenocytes from syringe inoculated mice compared to naturally infected animals, this did not reach significance at any time point. However at 96 hours, levels of proliferation were significantly higher in lymph node cells from syringe inoculated animals compared to levels in lymph node cells from mosquito exposed animals. However levels of proliferation are extremely low and indeed were lower than the proliferation of splenocytes from control animals. Lymph nodes from control animals were not used in these experiments as they were too small.

4.2.5. Infection with different doses of L3

As there were significant differences in the levels of IL-4, IL-5 and IL-10 between splenocytes from syringe inoculated and mosquito exposed mice, it was important to ascertain whether this was a dose dependent effect (i.e. there was a possibility that mosquito exposed animals were not receiving as many L3). In order to investigate this possibility, groups of five mice were infected with either 50, 25 or 10 L3 and control animals were given HBSS. At12 d.p.i. spleens were removed and cytokine assays were carried out.

(i) Cytokine responses of splenocytes of mice infected with 50, 25 or 10 L3

Cytokine responses of splenocytes from all groups were measured after 72 hours of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. Figure 4.13 shows that there were no significant differences in the levels of IL-4, IL-5 and IL-10 between the groups. Therefore there was no dose dependent effect on cytokine production.

(ii) Ag-specific proliferative responses of splenocytes of mice infected with 50, 25 or 10 L3

Ag specific proliferative responses of splenocytes from all groups were measured after 48, 72 and 96 hours of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. Figure 4.14 shows that despite being infected with different numbers of L3, there was no significant difference in levels of proliferation at any time point between any group.

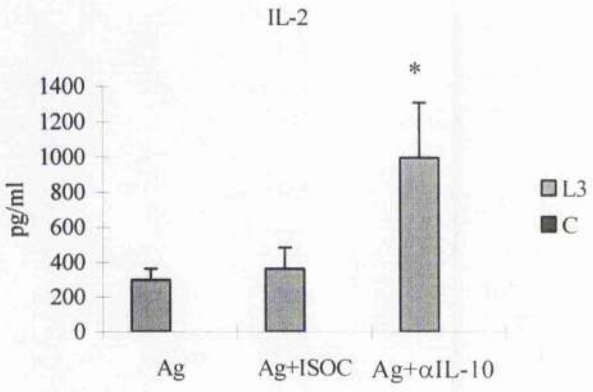
Figure 4.1. Neutralizing IL-10 in Ag-stimulated cultures of splenocytes from L3 infected mice resulted in significantly increased levels of Th1 cytokines

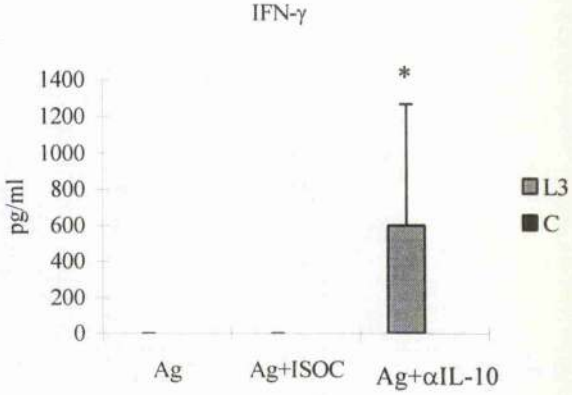
Mice were infected s.c. with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i., splenocytes (1 x 10^7/ml) were re-stimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag (Ag). Ag-specific cytokine responses were measured at 72 hours in the presence of 1μg/ml neutralising IL-10 Ab (αIL-10) or 1μg/ml isotype matched control (isoc). Results are expressed as pg/ml and all values represent the mean and standard deviation of five mice per group.

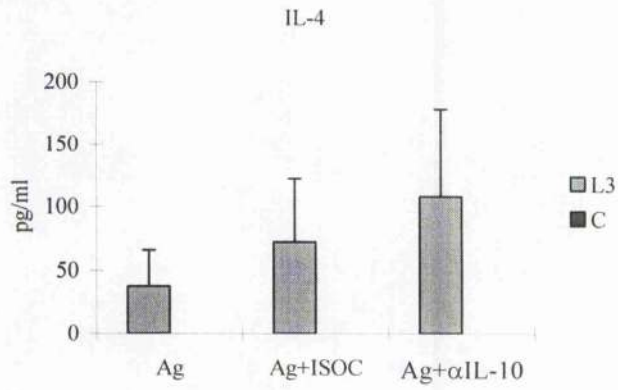
IL-2 – Ag vs αIL-10 p= 0.0122
isoc vs αIL-10 p= 0.0122

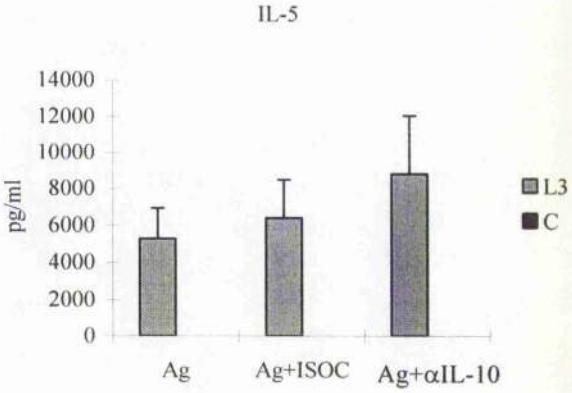
IL-4 - Ag vs nαIL-10 p= 0.0947
isoc vs αIL-10 p= 0.5309

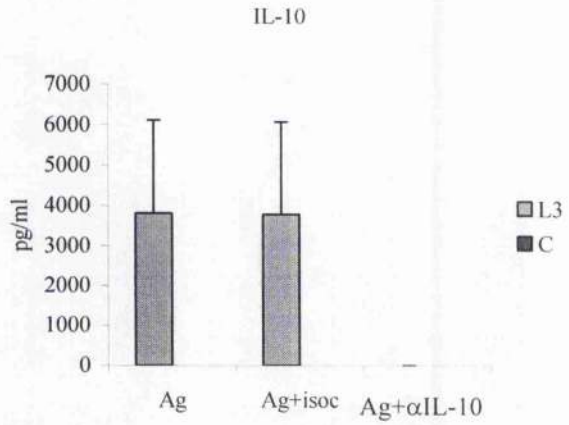
IL-5 - Ag vs αIL-10 p= 0.0947
isoc vs αIL-10 p= 0.2101


IL-2
pg/ml
1400
1200
1000
800
600
400
200
0
*
Ag
Ag+ISOC
Ag+αIL-10
L3
C


IFN-γ
pg/ml
1400
1200
1000
800
600
400
200
0
*
Ag
Ag+ISOC
Ag+αIL-10
L3
C


IL-4
pg/ml
200
150
100
50
0
Ag
Ag+ISOC
Ag+αIL-10
L3
C


IL-5
pg/ml
14000
12000
10000
8000
6000
4000
2000
0
Ag
Ag+ISOC
Ag+αIL-10
L3
C


IL-10
pg/ml
7000
6000
5000
4000
3000
2000
1000
0
Ag
Ag+isoc
Ag+αIL-10
L3
C

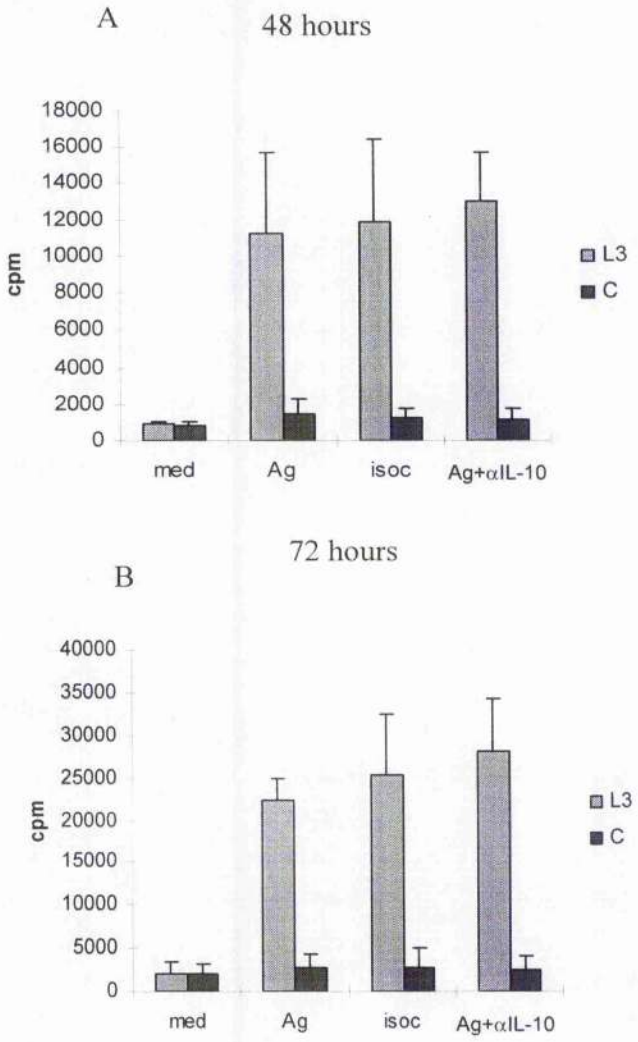


Figure 4.2. Neutralizing IL-10 in Ag-stimulated cultures of splenocytes from L3 infected mice did not alter levels of Ag-specific proliferation

Mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i., splenocytes (5 x 10^6/ml) were re-stimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag (Ag) in the presence or absence of 1μg/ml neutralizing IL-10 Ab (αIL-10) or 1μg/ml isotype matched control (isoc). Ag-specific proliferative responses were measured by uptake of ^{3}H thymidine at 48 hours (A) and 72 hours (B). Results are expressed as mean cpm incorporated in triplicate wells and all values represent the mean and standard deviation of five mice per group.

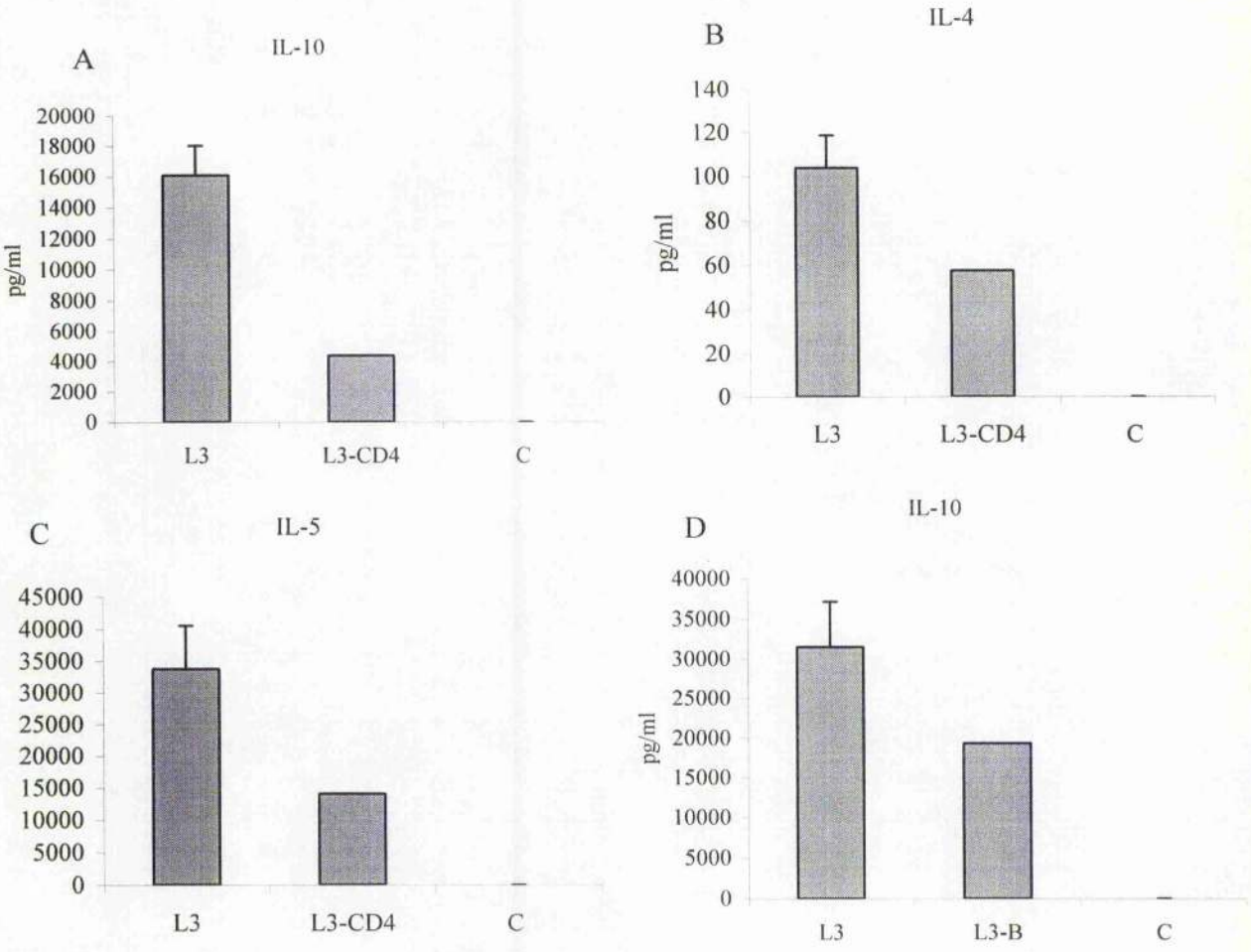
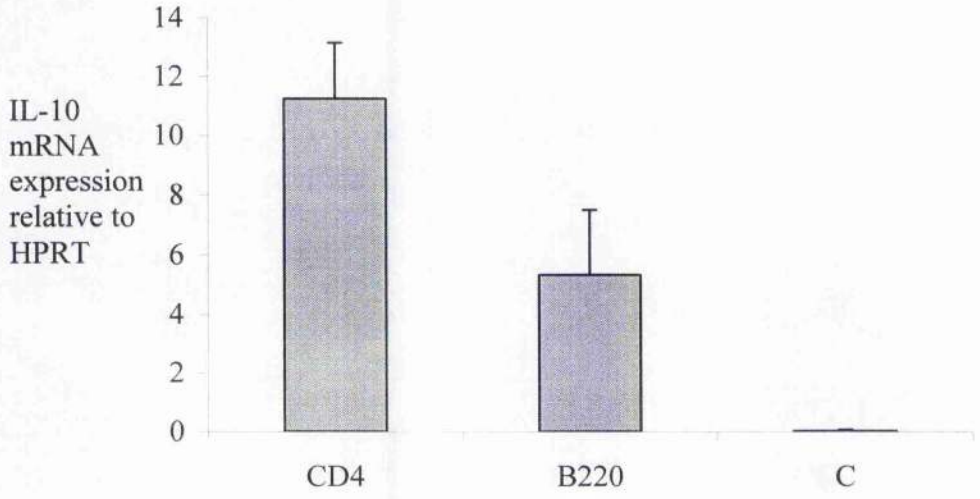


Figure 4.3. Depletion of $CD4^+$ or B cells from spleens of L3 infected mice resulted in decreased levels of IL-10

Mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i. splenocytes from L3 infected mice were then pooled and $CD4^+$ or B cells were depleted from culture using MACS anti-FITC beads and passing the suspension through MACS magnetic columns. The negative fraction was then restimultated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Ag-specific cytokine responses from whole splenocytes (L3) and splenocytes depleted of $CD4^+$ (L3-CD4) were measured at 72 hours (A-C). Panel D represents IL-10 levels from whole splenocytes and splenocytes depleted of B cells. Results are expressed as pg/ml.



	HPRT	IL-10
CD4	100	11
B220	100	5
Control	100	0.02

Figure 4.4. Semi-quantitative RT-PCR carried out on *ex vivo* splenocytes from L3 infected mice shows that CD4+ cells express more IL-10 mRNA relative to HPRT than do $B220^+$ cells

Mice were infected s.c. with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i. splenocytes from L3 infected mice were pooled and magnetically separated into purified $CD4^+$ and $B220^+$ cells. Semi-quantitative RT-PCR was carried out on these cells (using the TaqMan method) and levels of IL-10 mRNA are expressed relative to the constitutive gene HPRT.

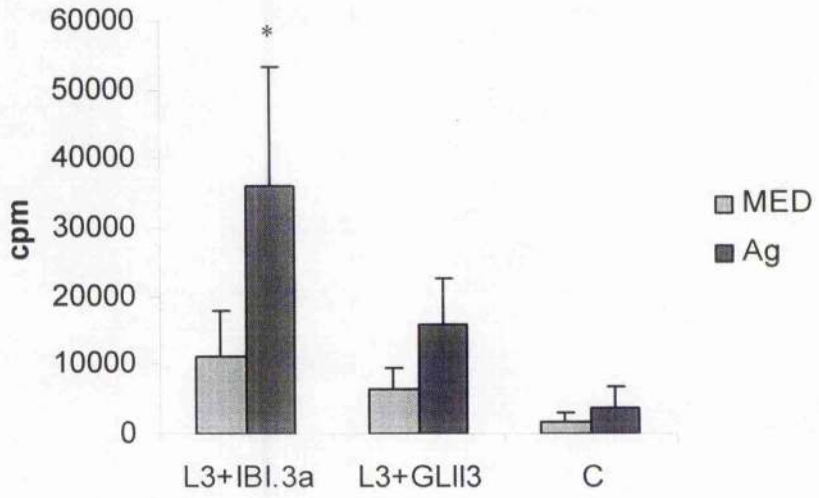


Figure 4.5. ***In vivo*** **neutralization of IL-10R resulted in increased levels of Ag-specific proliferation in splenocytes from L3 infected mice**

Two groups of BALB/c mice were infected sub-cutaneously with 50 L3 of *B. pahangi* and a third group were given an equal volume of HBSS only (C). One of the infected groups was injected intraperitoneally with 10 μg neutralizing IL-10R MAb (IBI.3a) and the other group received the same concentration of isotype control (GL113) at day 0, 4 and 7 post-infection. At 12 d.p.i., splenocytes (5 x 10^6/ml) were re-stimulated *in vitro* with 10 μg/ml of *B. pahangi* adult Ag and Ag-specific proliferative responses were measured by uptake of ^{3}H thymidine at 72 hours. Results are expressed as mean cpm incorporated in triplicate wells and all values represent the mean and standard deviation of five mice per group.
Data show an increase in levels of proliferation when IL-10R is blocked.
(L3+IBI.3a Ag vs L3+GL113 Ag p=0.0216).

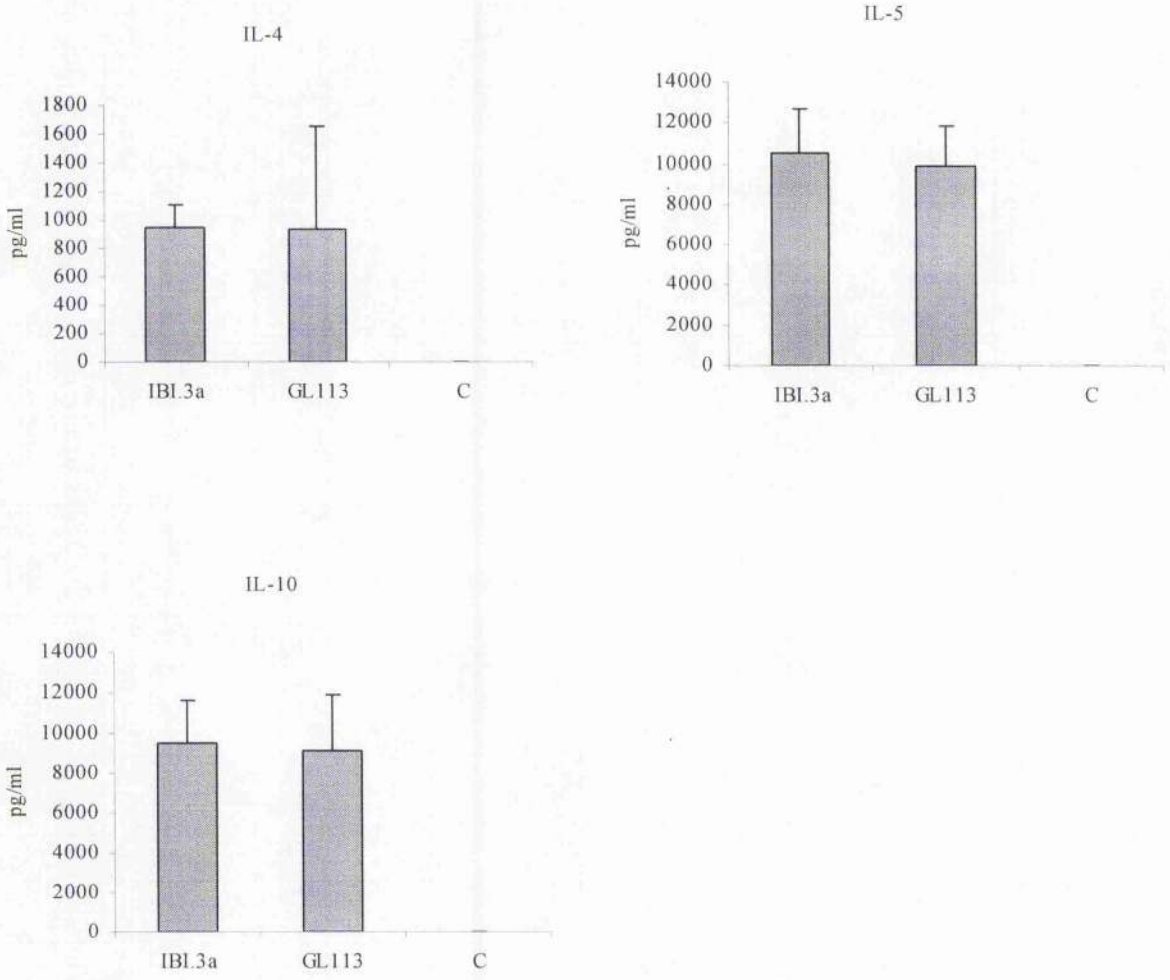


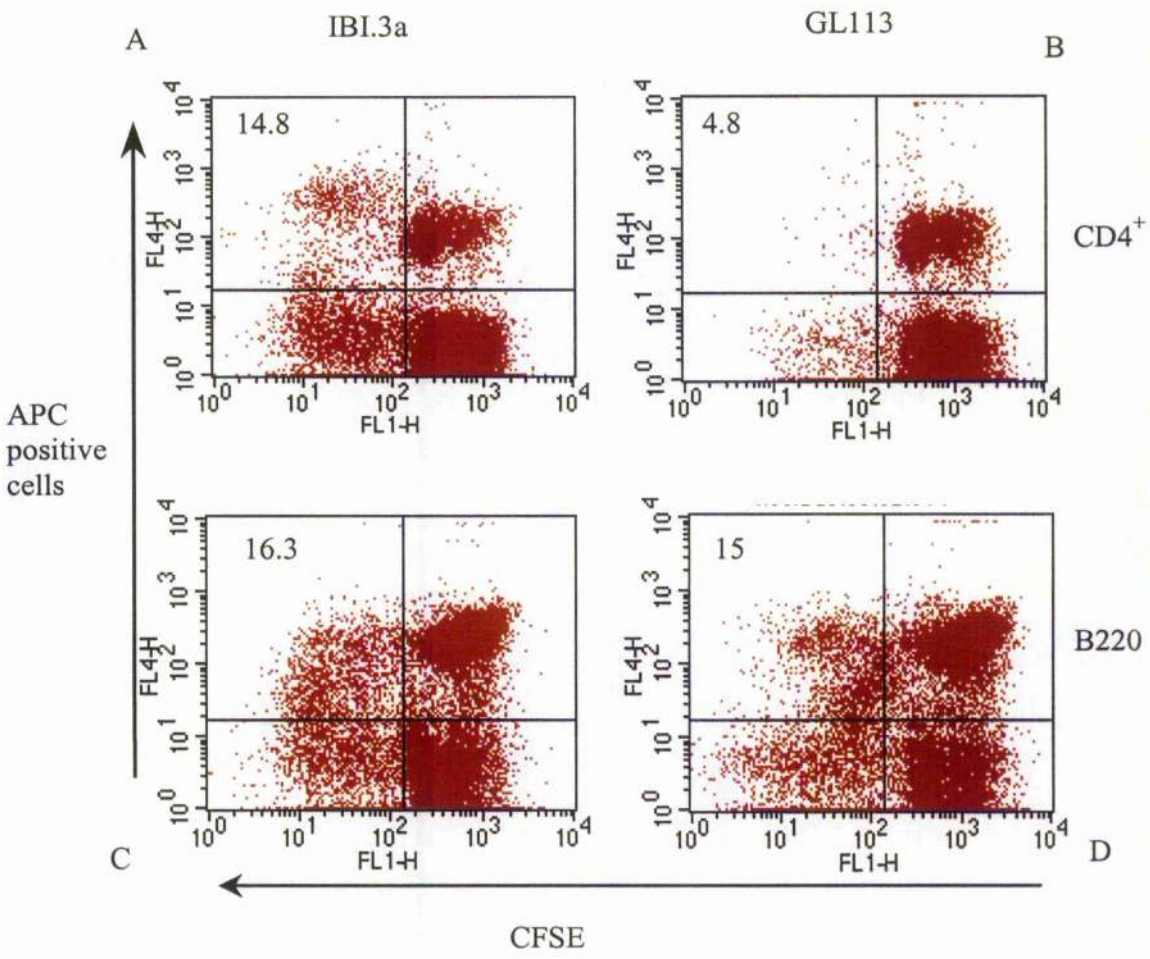
Figure 4.6. *In vivo* neutralization of IL-10R resulted in no differences in cytokine production

Two groups of BALB/c mice were infected sub-cutaneously with 50 L3 of *B. pahangi* and a third group were given an equal volume of HBSS only (C). One of the infected groups was injected intraperitoneally with 10 μg neutralizing IL-10R MAb (IBI.3a) and the other group received the same concentration of isotype control (GL113) at day 0, 4 and 7 post-infection. At 12 d.p.i., splenocytes (1 x 10^7/ml) were re-stimulated *in vitro* with 10 μg/ml of *B. pahangi* adult Ag and Ag-specific cytokine responses were measured by ELISA. Results are expressed as pg/ml and all values represent the mean and standard deviation of five mice per group.

IL-4 – IBI.3a vs GL113 $p = 1$
IL-5 - IBI.3a vs GL113 $p = 0.7728$
IL-10 - IBI.3a vs GL113 $p = 0.6650$

Figure 4.7. CFSE labelling of splenocytes from L3 infected mice shows that the proliferation of $CD4^+$ cells is increased when IL-10R is blocked

Two groups of BALB/c mice were infected sub-cutaneously with 50 L3 of *B. pahangi*. One of the infected groups was injected intraperitoneally with 10 μg neutralising IL-10R MAb (IBI.3a) (A and C) and the other group received the same concentration of isotype control MAb (GL113) (B and D) at day 0, 4 and 7 post infection. Splenocytes from these mice were labelled with CFSE and cultured with 10 μg/ml *B. pahangi* adult Ag. Cells were harvested at 96 hours, surface stained with anti-$CD4^+$ MAb (A and B) or anti-B220 MAb (C and D) and analyzed by flow cytometry. Each panel shows the CFSE staining profile of $CD4^+$ or $B220^+$ splenocytes from an individual mouse. The numbers at the top left hand corner of each panel indicate the percentage of $CD4^+$ or $B220^+$ cells displaying reduced fluorescence intensity in FL-1, indicating they have divided in Ag-stimulated culture. These figures are representative of the responses of five animals per group.


A
IBI.3a
GL113
B
14.8
4.8
CD4+
APC positive cells
16.3
15
B220
C
D
FL4-H
FL1-H
CFSE

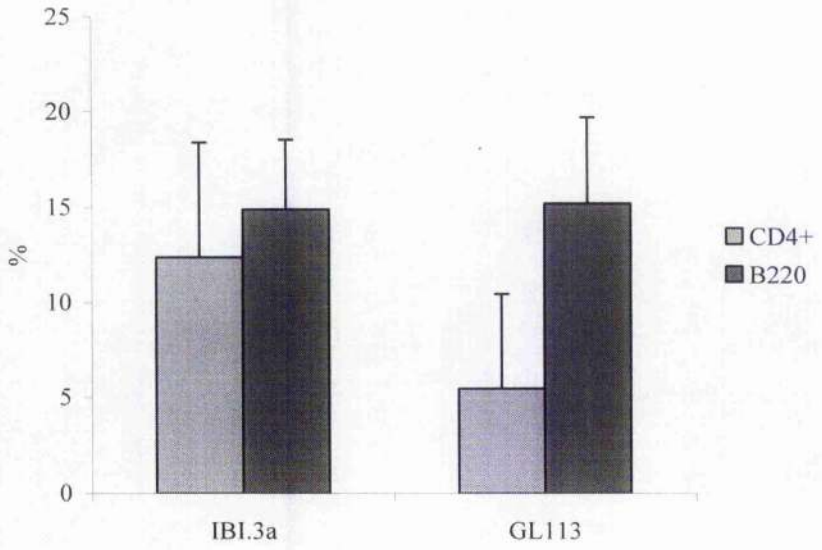


Figure 4.8. CFSE staining demonstrates that mean levels of proliferation of $CD4^+$ cells from L3 infected mice are increased when IL-10R is blocked

Two groups of BALB/c mice were infected sub-cutaneously with 50 L3 of *B. pahangi.* One of the infected groups was injected intraperitoneally with 10 μg neutralizing IL-10R MAb (IBI.3a) and the other group received the same concentration of isotype control MAb (GL113) at day 0, 4 and 7 post-infection. Splenocytes from these mice were labelled with CFSE and cultured with 10 μg/ml *B. pahangi* adult Ag. Cells were harvested at 96 hours, surface stained with anti-CD4 MAb or anti-B220 MAb and analyzed by flow cytometry. Graphs show the mean levels of proliferation of $CD4^+$ or B cells calculated from CFSE labelling at 96 hours of 5 mice per group. No difference in B cell proliferation was observed while $CD4^+$ proliferation showed a tendency to increase when IL-10R was blocked but did not reach significance p=0.0662.

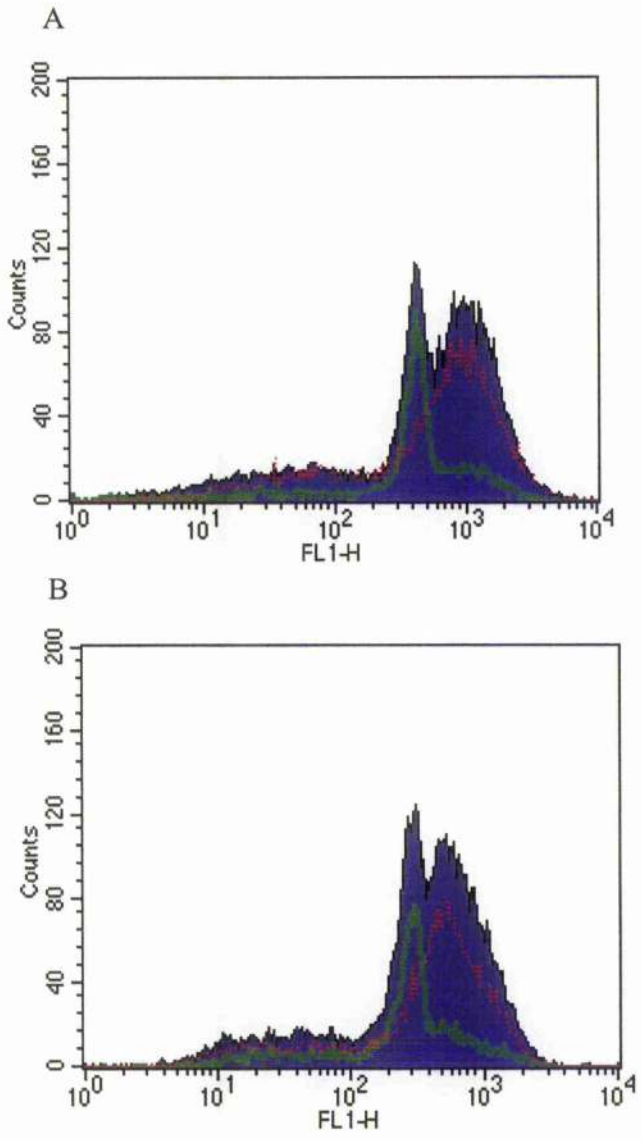


Figure 4.9. $CD4^+$ expansion is increased in L3 infected mice given blocking IL-10R Ab

Two groups of BALB/c mice were infected sub-cutaneously with 50 L3 of *B. pahangi*. One of the infected groups was injected intraperitoneally with 10 μg neutralizing IL-10R MAb (IBI.3a) (A) and the other group received the same concentration of isotype control MAb (GL113) (B) at day 0, 4 and 7 post-infection. Splenocytes from mice given 50 L3 of *B. pahangi* s.c. were labelled with CFSE and cultured with 10 μg/ml *B. pahangi* Ag. After 96 hours the cells were harvested and labelled with anti-CD4 MAb or anti-B220 MAb and analysed by flow cytometry. The purple fill shows the CFSE staining profile of total splenocytes. The green overlay represents the CFSE staining profile of $CD4^+$ cells and the pink overlay shows the CFSE staining profile of $B220^+$ cells.

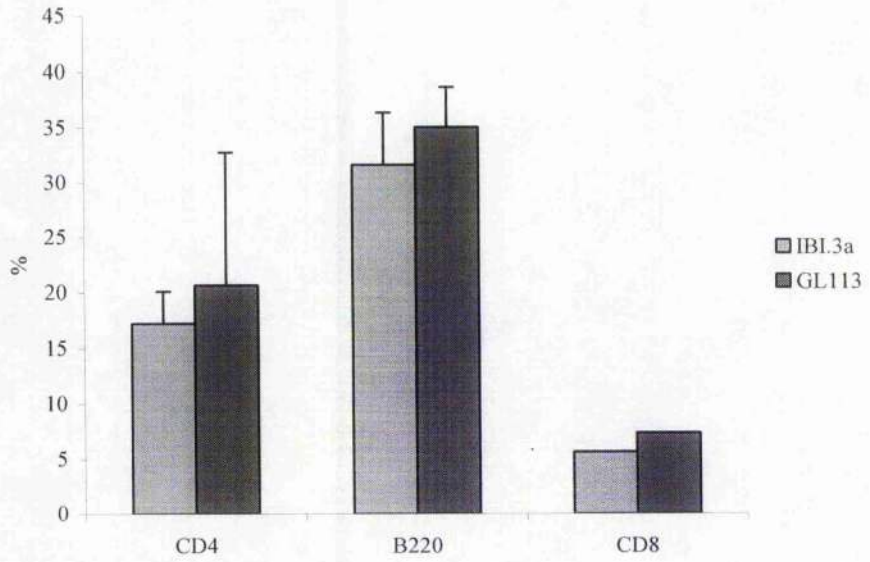


Figure 4.10. ***Ex vivo*** **staining of splenocytes from L3 infected mice shows no difference in levels of cellular populations in the spleens of mice in which IL-10R was blocked**

Two groups of BALB/c mice were infected sub-cutaneously with 50 L3 of *B. pahangi*. One of the infected groups was injected intraperitoneally with 10 μg neutralizing IL-10R MAb (IBI.3a) and the other group received the same concentration of isotype control MAb (GL113) at day 0, 4 and 7 post infection. Splenocytes from these mice were surface stained *ex vivo* 12 d.p.i. with anti-CD4 MAb, anti-B220+ MAb and anti-CD8+ MAb and analyzed by flow cytometry. All results represent the mean +/- standard deviation of 5 animals per group.

Figure 4.11. Cytokine responses to L3 are exaggerated in mice infected via syringe compared to mice infected by mosquito bite

One group of mice were infected sub-cutaneously with 50 L3 of *B. pahangi* via syringe inoculation. A second group of mice were infected via mosquito transmission, so that each animal was estimated to receive approximately 50 L3. A third control group were given HBSS. At 12 d.p.i., spleens and lymph nodes were removed. Ag-specific cytokine responses were measured at 72 hours. Results are expressed as pg/ml and all values represent the mean and standard deviation of five mice per group.

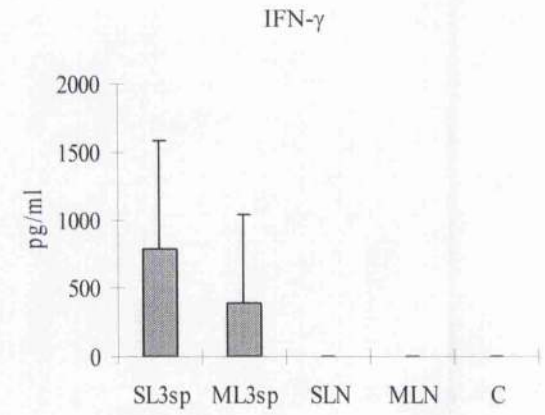
SL3sp – syringe inoculation, spleen
ML3sp – mosquito transmission, spleen
SLN – syringe inoculation, lymph node
MLN – mosquito transmission, lymph node

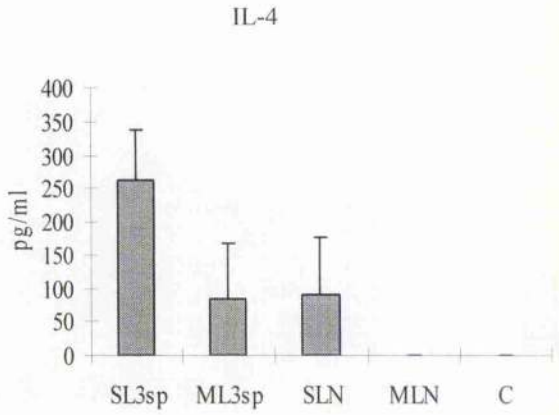
IFN-γ – SL3sp vs ML3sp $p = 0.2506$
SLN vs MLN $p = 1$

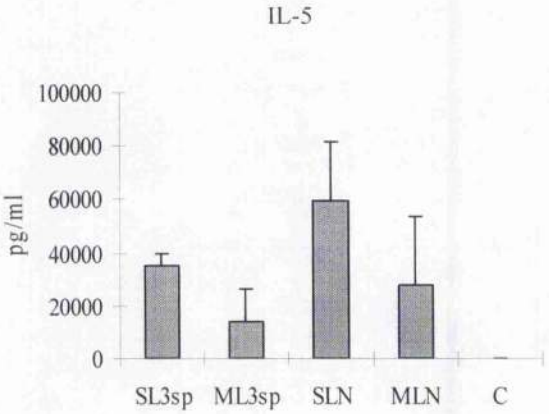
IL-4 – SL3sp vs ML3sp $p = 0.0367$
SLN vs MLN $p < 0.05$

IL-5 - SL3sp vs ML3sp $p = 0.0122$
SLN vs MLN $p = 0.0947$

IL-10 - SL3sp vs ML3sp $p = 0.0367$
SLN vs MLN $p = 0.0122$


IFN-γ
2000
1500
1000
500
0
pg/ml
SL3sp
ML3sp
SLN
MLN
C


IL-4
400
350
300
250
200
150
100
50
0
pg/ml
SL3sp
ML3sp
SLN
MLN
C


IL-5
100000
80000
60000
40000
20000
0
pg/ml
SL3sp
ML3sp
SLN
MLN
C

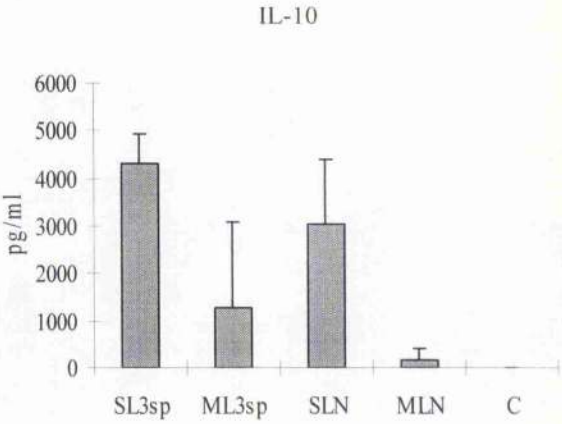

IL-10
6000
5000
4000
3000
2000
1000
0
pg/ml
SL3sp
ML3sp
SLN
MLN
C

Figure 4.12. Proliferative responses in spleens and lymph nodes of mice infected with L3 via syringe inoculation are exaggerated compared to mice exposed to infective mosquitoes

One group of mice were infected sub-cutaneously with 50 L3 of *B. pahangi* via syringe inoculation. A second group of mice were infected via mosquito transmission, so that each animal was estimated to receive approximately 50 L3. A third control group were given HBSS. At 12 d.p.i., spleens and lymph nodes were removed. Ag-specific proliferative responses were measured at 48, 72 and 96 hours. Results are expressed as cpm and all values represent the mean and standard deviation of five mice per group.

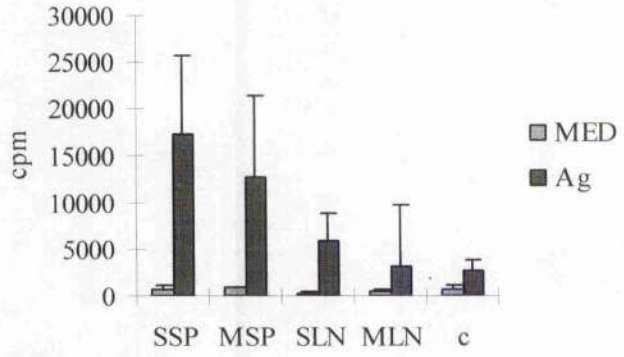
SSP – syringe inoculation, spleen
MSP – mosquito transmission, spleen
SLN – syringe inoculation, lymph node
MLN – mosquito transmission, lymph node

48 hours – SSP vs MSP $p = 0.4034$
SLN vs MLN $p = 0.5403$

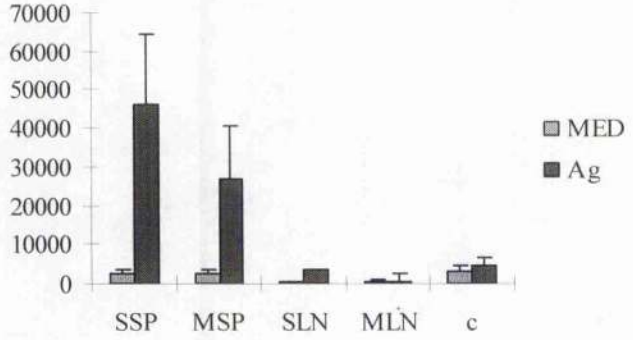
72 hours – SSP vs MSP $p = 0.1437$
SLN vs MLN $p = 0.373$

96 hours – SSP vs MSP $p = 0.067$
SLN vs MLN $p = 0.0200$

48 hours



72 hours



96 hours

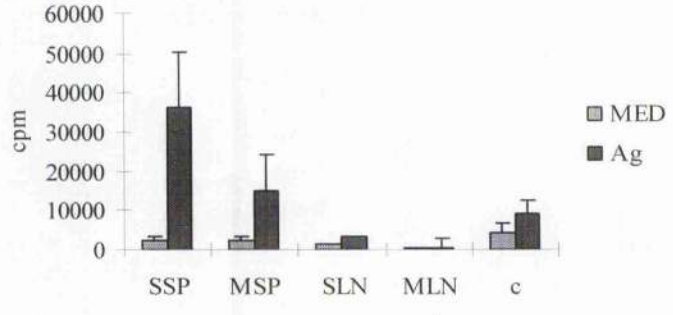


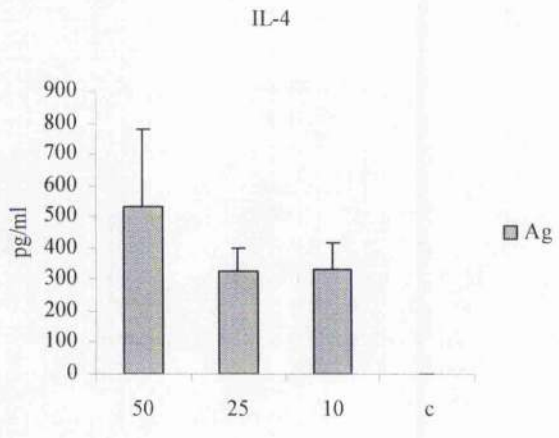
Figure 4.13. Cytokine responses of mice given 50, 25 or 10 L3 are not significantly different

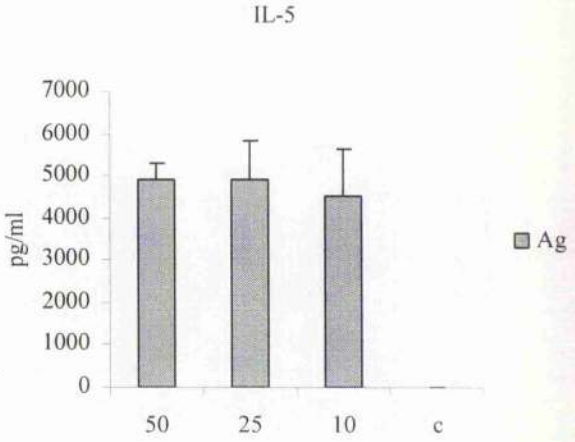
One group of five mice were infected sub-cutaneously with 50 L3 *B. pahangi*. A second group were given 25 L3 and a third group given 10 L3. A fourth group of control mice were given HBSS. At 12 d.p.i., spleens were removed from these mice. Ag-specific cytokine responses were measured at 72 hours. Results are expressed as pg/ml and all values represent the mean and standard deviation of five mice per group.

IL-4 – 50 vs 25 $p = 0.4034$
50 vs 10 $p = 0.2963$
25 vs 10 $p = 1$

IL-5 – 50 vs 25 $p = 0.5309$
50 vs 10 $p = 0.8345$
25 vs 10 $p = 0.7540$

IL-10 – 50 vs 25 $p = 0.8345$
50 vs 10 $p = 0.5309$
25 vs 10 $p = 0.4034$


IL-4
900
800
700
600
500
400
300
200
100
0
pg/ml
50
25
10
c
Ag


IL-5
7000
6000
5000
4000
3000
2000
1000
0
pg/ml
50
25
10
c
Ag

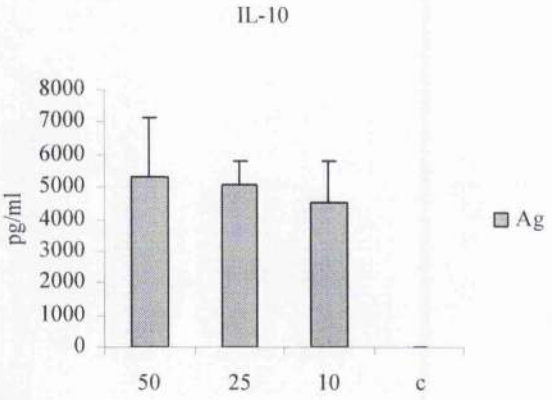

IL-10
8000
7000
6000
5000
4000
3000
2000
1000
0
pg/ml
50
25
10
c
Ag

Figure 4.14. Ag-specific proliferative responses of mice given 50, 25 or 10 L3 are not significantly different at any time point

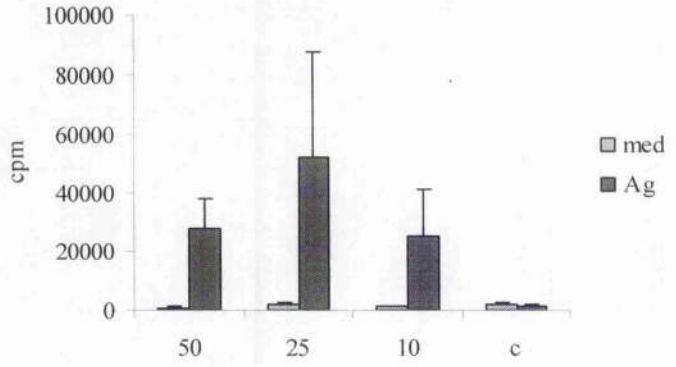
One group of five mice were infected sub-cutaneously with 50 L3 *B. pahangi*. A second group were given 25 L3 and a third group given 10 L3. A fourth group of control mice were given HBSS. At 12 d.p.i., spleens were removed from these mice. Ag-specific proliferative responses were measured at 48, 72 and 96 hours. Results are expressed as cpm and all values represent the mean and standard deviation of five mice per group.

48 hours – 50 vs 25 $p = 0.2101$
50 vs 10 $p = 0.6761$
25 vs 10 $p = 0.2101$

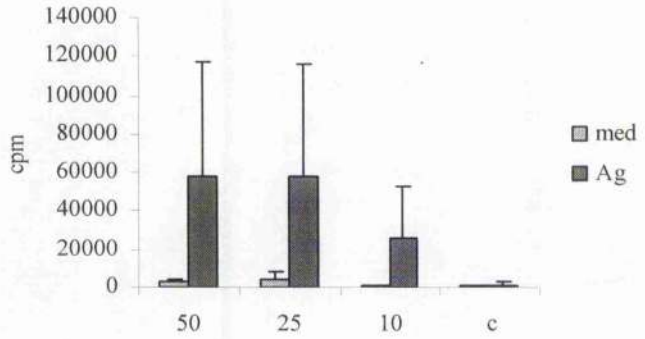
72 hours – 50 vs 25 $p = 0.8345$
50 vs 10 $p = 0.0510$
25 vs 10 $p = 0.1437$

96 hours – 50 vs 25 $p = 0.5309$
50 vs 10 $p = 0.6761$
25 vs 10 $p = 0.4034$

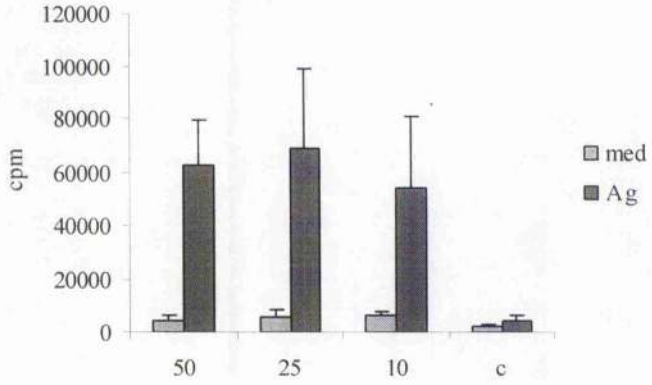
48 hours



72 hours



96 hours



4.3. Discussion

The results presented in this Chapter highlight the importance of IL-10 in the regulation of immune responses in L3 infected BALB/c mice. Initial experiments carried out using a neutralizing anti-IL-10 MAb *in vitro* confirmed that IL-10 has an important role in suppressing the production of Th1 cytokines, IL-2 and IFN-γ. The increase in IFN-γ may derive from Th1 cells or alternatively could derive from activated macrophages. When IL-10 is neutralized, macrophages may become activated and, in turn produce IFN-γ. This involves a mechanism whereby IL-10 can inhibit IFN-γ gene transcription within monocytes by controlling aspects of the IFN-γ signalling pathway (Ito *et al.*, 1999). In terms of Th2 cytokine production, IL-4 and IL-5 also had a tendency to increase in the absence of IL-10, however these differences did not reach statistical significance in any experiment. These results suggest that IL-10 is suppressing cytokine production by Th1 cells and possibly Th2 cells, to a lesser extent. In this respect, it might be predicted that neutralizing IL-10 *in vitro* would result in increased levels of proliferation. Previously, IL-10 has been shown to down-regulate proliferation of T cells via an affect on APC (Ding *et al.*, 1993), but IL-10 has also been shown to have a direct effect on T cells in the absence of APC by inhibiting IL-2 production (de Waal Malefyt *et al.*, 1993a). However, in numerous experiments in this study, levels of proliferation were never significantly different between cultures where IL-10 was neutralized or not. It is possible that neutralizing IL-10 *in vitro* has an effect on the ability of Th1 cells to produce cytokines without affecting their proliferation. Other studies have described a similar scenario where proliferation and cytokine production are differentially regulated (Evavold and Allen, 1991; Allen *et al.*, 1996). Alternatively, as alluded to above, cell types other than $CD4^+$ cells may produce IFN-γ but not proliferate. This could be investigated using intra-cellular cytokine staining to identify

the cellular source of IFN-γ. Finally, the concurrent production of Th1 and Th2 cytokines may be cross-regulatory, so that no increase in Th1 or Th2 proliferation is observed *in vitro*.

As *in vitro* assays had determined that IL-10 had a role in cytokine regulation but not down-regulation of proliferation in cultures from L3 infected mice, it was of interest to investigate the role of IL-10 *in vivo*. An obvious way to examine the role of IL-10 in the mouse model of *Brugia* would be to use IL-10-/- mice. Unfortunately, the conditions in the Animal Unit at the University of Glasgow are not compatible with the survival of IL-10-/- mice as they are prone to pro-inflammatory pathologies such as inflammatory bowel disease. An alternative approach is to neutralise the effects of IL-10 *in vivo* using a blocking IL-10R Ab (O'Farrell *et al.*, 1998; Belkaid *et al.*, 2001). Using this Ab had the added benefit of ensuring that the background strain of mouse was the same as used in previous experiments. In contrast to the *in vitro* situation, blockade of IL-10R *in vivo* showed very little difference in cytokine production. However, there was a significant increase in levels of proliferation in splenocytes from mice given the neutralizing Ab compared to those receiving the isotype control. These results are intriguing, as the L3 generates good proliferative responses in the intact mouse in the presence of IL-10. However after *in vivo* administration of this Ab, levels of proliferation were seen to almost double. It was therefore of interest to identify the cellular population inhibited by IL-10. FACS analysis of CFSE stained cells demonstrated that $CD4^+$ cells were the major population which expanded during IL-10R blockade. As IL-10R is expressed on the surface of APC and on T cells (Asadullah *et al.*, 2003), blockade of IL-10R will result in altered signals being delivered to $CD4^-$ cells from APC and/or direct effects upon T cells both of which could result in increased levels of proliferation. Ligation of

the IL-10 receptor results in signalling via the Jak/Stat pathway and has multiple downstream effects. For example, IL-10 suppresses inflammatory responses via its effect on pro-inflammatory cytokines, chemokines, APC and costimulatory molecules (Moore *et al.*, 2001). IL-10 promotes an anti-inflammatory Th2 response by inhibiting the production of IL-12 from APC, thus suppressing the production of IFN-γ from T cells. Macrophages are particularly susceptible to these effects, with IL-10 down-regulating the production of TNF-α, IL-1, IL-6, IL-12 and the expression of MHC class II and B7-1 and B7-2 on the cell surface. Altogether, the ability of macrophages to present Ag efficiently to T cells is attenuated by IL-10 (Fiorentino *et al.*, 1989; de Waal Malefyt *et al.*, 1993b). Besides the dominating indirect impact via the APC, IL-10 can also exert some direct effect on T cells. Naive T cells are known to be more sensitive to the effects of IL-10 due to the down-regulation of the IL-10R on activated cells (Liu *et al.*, 1994). IL-10 was shown to inhibit T cell proliferation in the presence of CD28 co-stimulation (Akdis *et al.*, 2000). In addition, the presence of IL-10 during the activation of naïve T cells results in these cells developing a regulatory phenotype, characterised by weak proliferation and absence of IL-2 after repeated stimulation (Groux *et al.*, 1997). Thus, increased levels of $CD4^+$ proliferation observed when IL-10R is blocked, may be attributed to one or more of the suppressive mechanisms of IL-10 mentioned above.

Neutralizing IL-10 *in vitro* resulted in production of Th1 cytokines but had no effect on proliferation. In contrast blockade of the IL-10R *in vivo* produced the opposite effect, no difference in cytokines but an increase in proliferation. Interestingly when the IL-10R Ab was used *in vitro,* the results were identical to neutralizing IL-10 (increased IL-2 and IFN-γ). How can these differences be reconciled? Perhaps in the *in vivo* situation IL-

10R is blocked very quickly after the administration of L3, allowing little time for the down-regulatory effects of IL-10 to be manifest, resulting in more efficient Ag presentation to $CD4^+$ cells with a consequent increase in proliferation. However it is difficult to account for the fact that no increase in Th1 cytokines is observed. This may reflect a difference in sensitivity to receptor blockade in different cells. In the case of the *in vitro* system where IL-10 is present during T cell priming *in vivo*, IL-10 may inhibit the ability of Th1 cells to divide, but after neutralization of IL-10 *in vitro* they have the ability to release cytokines. For example, cells which have been continuously exposed to IL-10 can be rendered anergic and these cells cannot recover from this state even with the addition of IL-2. Therefore, it may be the case that cells primed in the presence of IL-10 are unable to proliferate even when IL-10 is neutralized *in vitro*. An interesting experiment in this respect would be to examine proliferation by CFSE using cell surface markers for Th1 and Th2 cells, and to determine whether the $CD4^+$ cells that expand following blockade of the receptor are $CD25^-$, as it has been shown in previous *in vitro* studies, that $CD4^+CD25^+$ cells have the ability to suppress proliferation of co-cultured $CD4^+CD25^-$ T cells (Takahashi *et al.*, 1998).

Defining the phenotype of the cells responsible for the production of IL-10 in response to L3 infection is important in terms of understanding how the L3 polarizes the immune response e.g. does IL-10 derive from a Th2 or perhaps Treg phenotype or from an APC (B cells or macrophages). Initial attempts to identify the source of IL-10 using intra-cellular cytokine staining were unsuccessful (data not shown), therefore, to define the cellular source of IL-10, depletions of selective populations were carried out on splenocytes of L3 infected mice. These experiments showed that $CD4^+$ cells were the major producers of IL-10 with B cells contributing to a lesser extent. In addition,

depletion of $CD4^+$ cells had marked effects on levels of Ag-specific IL-4 and particularly IL-5. While it is clear from these experiments that depletion of both $CD4^+$ and B cells has a dramatic effect on cytokine production, a more direct method of defining the cellular source of IL-10 was required. Quantitative RT-PCR on selected cellular populations confirmed the depletion experiments, in that $CD4^+$ cells were demonstrated to produce the majority of IL-10 with B cells having a lesser role. As there is an additional sub-set of $CD4^+$ cells which have regulatory properties, either by their ability to produce IL-10 (Tr1 and Th3) or via cell to cell contact dependant mechanisms ($CD4^+CD25^+$ cells), the finding that $CD4^+$ cells are the major source of IL-10 clearly required further investigation (see results presented in Chapter 5).

Finally, the remaining experiments in this Chapter were carried out to determine whether mosquito saliva had an effect on the transmission of L3 in the mouse model, and if there was an effect, whether IL-10 played a role. These experiments were relevant, as it has been reported previously that sand fly saliva has potent immunomodulatory functions. When *L. major* is administered to mice along with sand fly salivary gland lysate, disease is exacerbated and the parasite burden is greatly enhanced (Titus and Ribeiro, 1988). One important vasodilatory peptide in sand fly saliva, termed maxadilan (MAX), has been shown to induce elevated levels of IL-10 (Bozza *et al.*, 1998), thus down-regulating levels of IFN-γ and NO, which in this system is beneficial to the parasite (Titus *et al.*, 1994). Salivary gland lysate also has a profound ability to suppress T cell proliferation in a non-specific manner (Titus, 1998). Therefore, to determine whether the saliva of mosquitoes has any such affect mice were either exposed to infective mosquitoes, which were estimated to transmit ~50 L3 per mouse, or to syringe inoculation (the method of infection used during the course of all

experiments up to and beyond this point). When examining cytokine responses from both groups of animals it appeared that the group which received L3 via natural transmission, had a reduced capacity to produce cytokines (it is notable that splenocytes in this experiment were producing low levels of IFN-γ, a rare occurrence in most experiments). This contrasts to the sand fly system, where elevated levels of IL-10 are observed. When levels of proliferation were measured in splenocytes and lymph node cells, a general reduction in proliferation was observed in cells (lymph node cells and splenocytes) from the animals which received L3 via mosquito transmission.

While these differences could be due to an inhibitory effect of mosquito saliva during the course of transmission, they may also be accounted for by fewer L3 being administered by mosquito transmission than by syringe inoculation. To clarify this point, additional experiments were carried out in which varying numbers of L3 were administered to mice. Somewhat surprisingly, cytokine analysis of supernatants from cultures of splenocytes from mice infected with 50, 25 or 10 L3 showed very little difference in levels of IL-4, IL-5 and IL-10. In addition, levels of Ag-specific proliferation between the groups was not significantly different at any time point. These results confirm that the L3 is an extremely potent modulator of the immune response, in that as few as 10 L3 polarised the immune response towards the Th2 phenotype. In addition, it casts doubt on the possibility that differences between syringe and mosquito administered L3 can be solely accounted for by variation in levels of transmission. Whether there is an immunosuppressive component in saliva injected simultaneously with L3 when a blood meal is taken or whether natural transmission of L3 is immunologically "silent" requires further investigation. For example, L3 could be administered via syringe along with salivary gland lysate from mosquitoes.

Interestingly, previous studies have shown that mosquito saliva contains factors which modulate cytokine expression (Gillespie *et al.*, 2000). Saliva from the malaria vector *Anopheles stephensi* has a strong neutrophil chemotactic activity (Owhashi *et al.*, 2001) while *Aedes aegypti* (the species used in this study) salivary gland extracts were reported to stimulate TNF-α release from mast cells (Bissonnette *et al.*, 1993). During mosquito transmission, L3 are deposited in the skin where they presumably encounter different populations of APC compared to those administered via syringe inoculation. In addition, they may migrate to the lymphatics with different kinetics compared to those administered by s.c. inoculation. Either factor could influence the subsequent immune response.

In conclusion it is apparent that IL-10 has an important role in controlling immune responses in the mouse model post-infection with the L3 of *B. pahangi*. However the way in which IL-10 modulates these responses remains inconclusive as there are many different ways in which this potent anti-inflammatory cytokine can operate. Neutralization of IL-10 *in vitro* resulted in the production of Th1 cytokines, suggesting that IL-10 can suppress Th1 responses, however it is not clear whether IFN-γ is being produced by macrophages or T cells. When IL-10R was neutralized *in vivo,* a different situation was observed where cytokine levels were unchanged but Ag-specific proliferation was enhanced. Experiments to examine the cellular source of IL-10 demonstrated that a population of $CD4^+$ cells are the main producers of IL-10 with B cells contributing to a lesser extent. Further experiments to characterise the phenotype of these cells are presented in Chapter 5. Finally, there appeared to be no obvious role for IL-10 in the natural transmission of L3, in contrast to the situation observed in the sand fly/*Leishmania* model. There may however be immunomodulatory properties in

mosquito saliva which down-regulate levels of Th2 cytokines but this does not appear to be associated with elevated IL-10. Alternatively, natural infection with L3 may be a less immuno-stimulatory route than s.c. inoculation.

Chapter 5. The L3 of *B. pahangi* elicits a population of $CD4^+CD25^+CTLA\text{-}4^+$ cells which regulate proliferation and cytokine production

5.1. Introduction

Over 40 years ago, experiments demonstrated that thymectomy of mice three days after birth leads to systemic autoimmunity, indicating the importance of T cells in the regulation of tolerance. Recently there has been a re-emergence of interest in the theory of regulatory T cells. The modern view of these cells is based on research by Sakaguchi, who demonstrated that the adoptive transfer of T cells depleted of $CD25^+$ cells resulted in multi-organ autoimmunity in recipient animals (Sakaguchi *et al.*, 1995; Asano *et al.*, 1996). Since these seminal studies, an enormous amount has been learnt about Treg cells. These cells were demonstrated to induce active suppression of T cell function, which is essential for the induction of peripheral tolerance to both foreign and self antigens (Roncarolo *et al.*, 2001b). The different subsets and properties of these cells have been outlined in the main Introduction, but it is also important to understand the role of Treg cells in relation to infectious disease. Studies carried out on *Candida albicans* have shown that $CD4^+CD25^+$ regulatory T cells are essential for memory-protective immunity to this fungal infection in mice (Montagnoli *et al.*, 2002). Iwashiro *et al* further demonstrated a mechanism of virus-induced immunosuppression by means of the generation or expansion of $CD4^+CD25^+$ cells in mice persistently infected with the Friend retrovirus (Iwashiro *et al.*, 2001a; Iwashiro *et al.*, 2001b), while $CD4^+CD25^+$ cells were shown to have an essential role in the control of *Pneumocystis carinii* pneumonia in mice (Hori *et al.*, 2002). In terms of parasitic infections, $CD4^+CD25^+$ cells have been studied in mice infected with *L. major*. In this system, they regulate progressive disease by controlling production of early IL-4, an essential cytokine

determining whether an infected animal will progress towards a resistant (Th1) or a susceptible phenotype (Th2). Depletion of $CD4^+CD25^-$ cells from susceptible BALB/c mice resulted in increased levels of IL-4 and exacerbated disease (Aseffa *et al.*, 2002). In an additional study, resistant C57BL/6 mice were infected with *L. major* and it was demonstrated that a population of parasite specific $CD4^+CD25^+$ cells accumulate at the site of infection in the dermis, where they suppress the ability of effector T cells to eliminate the parasite from the skin (Belkaid *et al.*, 2002). This equilibrium between T effector and Treg function enables the host to develop resistance to further infection whilst benefiting the parasite by enabling transmission by its vector to continue. Removal of the $CD4^+CD25^+$ population resulted in total eradication of the parasite from the site of infection, resulting in the loss of concomitant immunity. Thus it appears that elimination of $CD4^+CD25^+$ cells in *L. major* infection can make resistant mouse strains more resistant and susceptible mouse strains more susceptible. The Belkaid study also demonstrated a role for IL-10 in the survival of parasites within C57BL/6 mice, in that IL-10-/- mice or mice given IL-10R Ab, eradicated the parasites resulting in a loss of concomitant immunity. IL-10 produced by $CD4^+CD25^+$ cells may suppress parasite killing by macrophages, but this does not rule out other possible mechanisms of suppression, such as cell-contact via CTLA-4 or GITR binding. $CD4^+CD25^+$ cells have also been shown to have a role in modulating responses in mice infected with *Plasmodium berghei* NK65. Following infection and administration of an anti-CD25 MAb, the proportion of $CD4^+CD25^+$ cells within the $CD4^+$ population decreased despite a general increase in the number of $CD4^+$ cells. Depletion of $CD25^+$ cells resulted in a delayed growth of parasitaemia during challenge infection, particularly in immunised mice. These findings are interesting in that they demonstrate that $CD4^+CD25^+$ cells are able to influence protective immunity to *P. berghei* NK65 (Long *et al.*, 2003).

In terms of filariasis most studies to date have considered the function of Treg cells in human disease. For example the role of Treg cells in onchocerciasis was examined by Doetze *et al* who demonstrated that hypo-responsiveness observed in *O. volvulus* infected individuals was associated with the presence of these cells (Doetze *et al.*, 2000). More recently, analysis of T cells from individuals with lymphatic filariasis showed that a population of CTLA-4^{+} cells are expanded in hyporesponsive individuals. Addition of neutralizing Ab to CTLA-4 to PBMC culture, resulted in increased levels of IL-5 and decreased levels of IFN-γ, indicating that CTLA-4 plays a role in maintaining the Th1/Th2 balance (Steel and Nutman, 2003). Finally, it has been found that *S. mansoni* specific lyso-phosphatidylserine (PS) activated the Toll-like receptor 2 and affected dendritic cells such that mature dendritic cells gained the ability to induce the development of IL-10-producing Treg cells (van der Kleij *et al.*, 2002).

The experiments in this chapter set out to investigate whether Treg cells were induced following infection of BALB/c mice with the L3 of *B. pahangi.* While it is generally considered that L3 induce a strong Th2 response in infected mice, many previous observations are consistent with the induction of Tregs. These cells were characterised in terms of cell surface markers, cytokine production and their ability to down-regulate proliferative responses.

5.2. Results

5.2.1. T cell characterization

(i) IL-10 and TGF-β mRNA expression in CD4+ cells from L3 infected mice

T regulatory cells frequently co-express another suppressor cytokine, TGF-β, along with IL-10. Therefore the first experiments investigated the possibility that $CD4^+$ cells from L3 infected mice might also produce TGF- β. To address this point, quantitative RT-PCR was carried out using primers and probes for TGF-β, IL-10 and HPRT on RNA isolated from $CD4^+$ and B cells purified from the spleens of ten L3 infected mice at 12 d.p.i. $CD4^+$ and B cells were purified using magnetic beads (as described in Material and Methods 2.6.1). In all experiments the efficiency of the purifications was greater than or equal to 95% (data not shown). The results of these experiments confirmed that $CD4^-$ cells from L3 infected mice produced TGF-β (Figure 5.1) along with IL-10. The $B220^+$ population expressed very low levels of TGF-β in the experiment shown. However, in a subsequent experiment TGF-β was not detected in the $B220^+$ population. It is notable that although the absolute amount of IL-10 expression in this experiment is lower than that described previously (Figure 4.5), the relative levels of IL-10 mRNA expressed by $CD4^+$ and $B220^+$ cells is similar. This finding confirms that the L3 of *B. pahangi* elicit a population of $CD4^+$ T cells that express both IL-10 and TGF-β in the BALB/c mouse model.

(ii) Three-colour FACS analysis of $CD4^+$ cells from L3 infected mice

An alternative way to identify T regulatory cells is to analyse cell surface markers which are associated with this population. Such markers include co-expression of CD4

with one or more of CD25, CTLA-4 (CD152), GITR, OX40 (CD134), CD103 or Ly6 (McHugh *et al*, 2002). Therefore three-colour FACS analysis was carried on splenocytes from L3 infected, MF infected and control mice using anti-CD4 MAb (FITC), anti-CD25 MAb (APC) and anti-CTLA-4 (PE). Mice infected with MF were included in these experiments as splenocytes from these animals display a Th1-type cytokine profile, and it was therefore of interest to compare the expression of these cell surface markers in L3 and MF infected animals. In these experiments, cells were cultured with Ag for 72 hours prior to labelling and flow cytometry. The FACS plots in Figure 5.2 show results from individual mice, representative of the group of five animals, while Figure 5.3 shows the mean percentage of $CD4^+$ cells which co-express $CD25^+$ and CTLA-4^+ for each group of five animals. These data showed that $CD4^+CD25^+$CTLA-4^+ cells were expanded only in cultures from L3 infected mice where they comprised ~7 % of total $CD4^+$ cells. In control or MF infected mice, this population accounted for ~ 2% of total $CD4^+$ cells.

The results of this same experiment are presented in Table 5.1, and show the percentage of $CD4^+$ cells that also express each individual marker. As would be expected, there was no significant difference in the percentage of lymphocytes expressing $CD4^+$ in either group (L3 $CD4^+$ vs MF $CD4^+$, p= 0.0518; L3 $CD4^+$ vs C $CD4^+$, p= 0.0518). Approximately 25% of $CD4^+$ cells from L3 infected mice expressed the activation marker CD25, while control uninfected mice and MF infected mice express only low levels of CD25 (~6-7%). This is somewhat surprising, as it might be expected that infection with MF would cause activation of $CD4^+$ cells. As with CD25, CTLA-4 was expressed only on a significant percentage of cells from L3 infected mice. Although CD25 is also a marker of T cell activation, the co-expression of CTLA-4 on a

proportion of the $CD25^+$ population indicates that L3, but not MF, elicit a population of T cells with some characteristics of T regulatory cells.

5.2.2. L3 induced $CD4^+CD25^+CTLA4^+$ cells are IL-4 independent

One population of T regulatory cells, the Th3 cells have been identified in IL-4-/- animals which suggests that these cells do not require the Th2 pathway to undergo their process of differentiation (Powrie *et al*, 1996). It was therefore of interest to carry out experiments in IL-4-/- mice and compare the results to those previously described from L3 infected wild-type mice.

(i) IL-10 and TGF-β expression is IL-4 independent

In the first analysis, the expression of IL-10 and TGF-β mRNA was quantified from purified $CD4^+$ or $B220^+$ cells from IL-4-/- or WT mice, as described previously. Following RNA extraction and cDNA synthesis (as described in Materials and Methods 2.6), real-time RT-PCR was carried out on the selected populations. Figure 5.4 shows IL-10 and TGF-β mRNA expression relative to HPRT in $CD4^+$ and $B220^+$ cells from L3 infected wild-type and IL-4-/- mice. Although levels of IL-10 and TGF-β mRNA were slightly lower in KO mice vs WT mice, the difference was not significant, suggesting that the expression of these cytokines are not IL-4 dependent in this model.

(ii) Three-colour FACS analysis of $CD4^+$ cells from L3 infected wild-type and IL-4-/- mice

Three-colour FACS analysis was carried on splenocytes from L3 infected wild-type and IL-4-/- mice following *in vitro* restimulation with Ag, as described previously. The

FACS plots in Figure 5.5 show data from individual mice, representative of the group, while Figure 5.6 depicts the mean percentage of $CD4^+$ cells which co-express CD25 and CTLA-4 in splenocytes from five L3 infected wild-type or IL-4-/- mice. This analysis demonstrates that there was no difference in the expression of CD25 or CTLA-4 in $CD4^+$ cells from WT or IL-4-/- mice. Both groups of infected animals showed a large increase in this population compared to their uninfected counterparts (see Figure 5.6).

5.2.3. Investigating the regulatory role of $CD25^+$ cells *in vivo*

The experiments described above demonstrate that a population of T cells with some of the characteristics of T regulatory cells was expanded *in vitro,* only in culture from L3 infected mice. In order to investigate whether these cells were present *in vivo* and to investigate their function, further experiments were required. To this end, a neutralizing Ab (PC61) was used to deplete $CD25^+$ cells *in vivo*, prior to L3 infection.

(i) Three-colour FACS analysis of $CD4^+$ cells from L3 infected mice given neutralizing $CD25^+$ Ab (PC61)

In these experiments, mice were treated with 0.5 mg (total protein) of PC61 (anti-CD25 MAb) or 0.5 mg of GL113 (isotype matched control) on days – 4 and –2 prior to s.c. infection with 50 L3 of *B. pahangi.* Administration of the MAb prior to infection is important to ensure that T cells activated by infection are not targeted (Onizuka et al, 1999). On day 12 p.i., spleens were harvested and splenocytes cultured *in vitro* for 72 hours with 10 μg/ml *B. pahangi* Ag. Three-colour FACS analysis was then carried on splenocytes from L3 infected mice given either PC61 or GL113, using the combination of anti-CD4 MAb, anti-CD25 MAb and anti-CTLA-4. The FACS plots in Figure 5.7 show the results from individual mice, with the mean percentages of $CD4^+$ cells co-

expressing CD25 and CTLA-4 in PC61 treated mice and GL113 treated mice shown in Figure 5.8. In mice given PC61 MAb, there was a significant reduction in the percentage of $CD4^+CD25^+CTLA\text{-}4^+$ cells compared to those given the isotype control, indicating that the Ab was effectively depleting these cells. However, at the dose given, the PC61 Ab did not completely ablate the $CD25^+CTLA\text{-}4^+$ population. In most experiments, ~50% reduction in the numbers of these cells was observed.

(ii) Ag–specific and spontaneous proliferation of splenocytes from L3 infected mice given PC61 and GL113

To determine whether the removal of a significant proportion of the $CD25^+$ $CTLA\text{-}4^+$ population had any effect on immune responses, proliferation and cytokine assays were carried out. Proliferative responses of splenocytes from L3 infected mice given either PC61 or GL113 were measured over a time course of *in vitro* culture with medium only or 10 μg/ml *B. pahangi* adult Ag. Control mice received HBSS alone. Three experiments were carried out, each of which gave similar results (see Figure 5.9). Two points are immediately obvious from this experiment, firstly, levels of Ag-specific proliferation were significantly higher in splenocytes from mice given PC61 compared to mice given GL113 after 72 and 96 hours incubation. Secondly, it was notable that levels of spontaneous proliferation (medium only) were significantly increased in cultures from PC61 treated mice at all time points. The increase in the levels of Ag-specific and spontaneous proliferation in cultures from mice depleted of $CD25^+$ cells *in vivo*, indicates a regulatory function for this population

(iii) Ag–specific and spontaneous cytokine production of splenocytes from L3 infected mice given PC61 or GL113

Cytokine responses of splenocytes from L3 infected mice given either PC61 or GL113 MAb treatment and control uninfected mice were measured after 72 hours of *in vitro* culture with medium only or 10 μg/ml *B. pahangi* adult Ag. The results of a representative experiment are presented in Figure 5.10. Mice depleted of $CD25^+$ cells *in vivo*, produced significantly more IL-10 and IL-4 than animals given the isotype control MAb. There was also a trend for levels of IL-5 to increase but this did not reach statistical significance. In the experiment shown, splenocytes from mice given the isotype control Ab secreted low levels of IFN-γ, which were not significantly altered in mice given PC61. Interestingly, cells from PC61 treated mice also produced large amounts of spontaneous cytokines in medium only cultures. As is shown in Figure 5.11, spontaneous IL-2 was detected in medium only culture whereas this cytokine is rarely seen in Ag-stimulated cultures. Table 5.2 shows the results of this experiment as the net levels of Ag-stimulated and spontaneously produced cytokines from mice depleted of $CD25^+$ cells prior L3 infection.

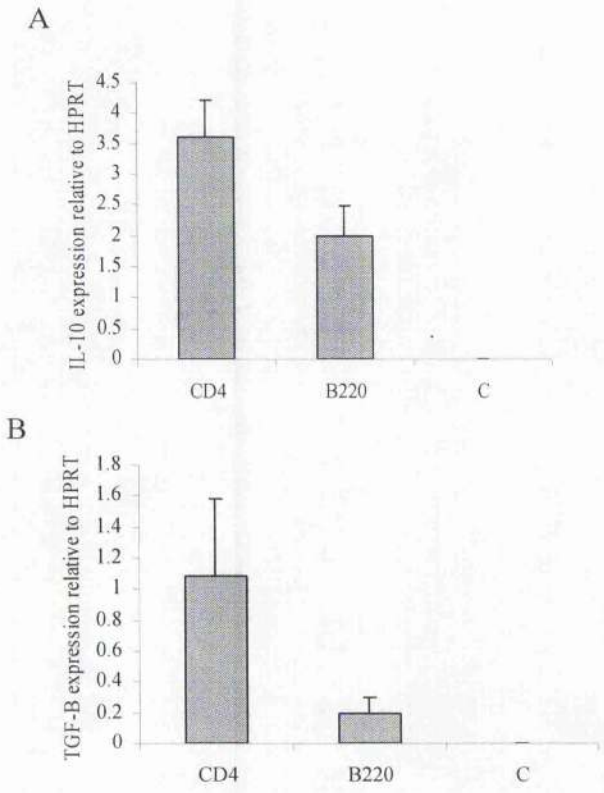


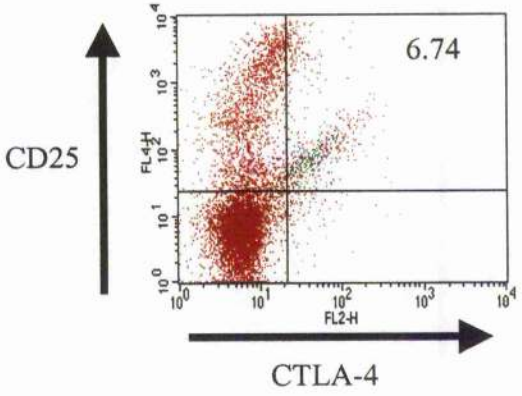
Figure 5.1. $CD4^+$ cells analysed *ex vivo* from L3 infected mice express more IL-10 and TGF–β mRNA relative to HPRT mRNA than do B cells

Mice were infected s.c. with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At day 12 p.i., splenocytes from L3 infected mice were pooled and magnetically separated into purified $CD4^+$ and $B220^+$ cells. Quantitative RT-PCR was carried out on these cells and levels of IL-10 and TGF–β mRNA were expressed relative to HPRT. Quantitative RT-PCR was carried out using the TaqMan method (as described in Materials and Methods 2.6.2).

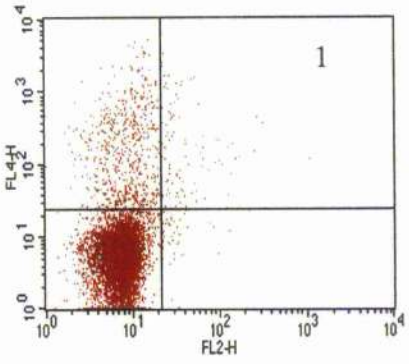
Figure 5.2. Three colour FACS analysis demonstrates that a population of $CD4^+$ cells which co-express CD25 and CTLA-4 are expanded only in L3 infected mice

Mice were infected s.c. with 50 L3 or 1 X 10^5 MF of *B. pahangi,* or an equal volume of HBSS only. At day 12 p.i., splenocytes from L3 infected (A), MF infected (B) and HBSS control mice (C) were cultured with 10 μg/ml *B. pahangi* adult Ag for 72 hours. Cells were harvested and stained with anti-mouse CD4 MAb, anti-mouse CD25 MAb and anti-mouse CTLA-4 MAb, each bound to a different flurochrome (CD4 – FITC, CD25-PE, CTLA-4-APC). Cells were analysed by flow cytometry using gates set with isotype matched control MAb. Each panel shows the staining profile of an individual mouse from each group. The numbers at the top right hand corner of each panel indicate the percentage of $CD4^+$ cells which are $CD25^+$ and $CTLA\text{-}4^+$. These figures are representative of the responses of five animals per group.

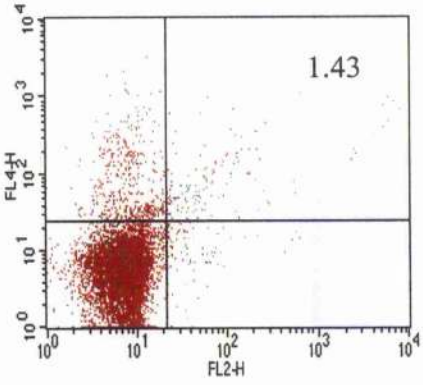
A L3



B MF



C Control



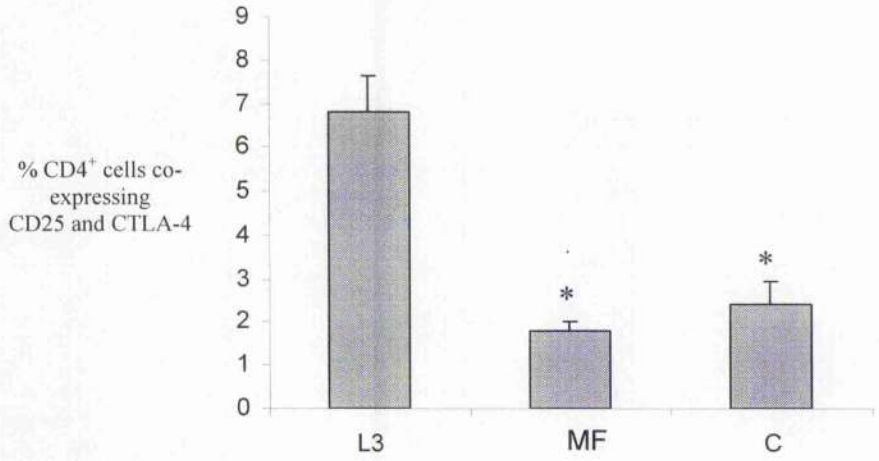


Figure 5.3. L3 infected mice have a higher percentage of $CD4^+$ cells which co-express CD25 and CTLA-4 than MF infected or control animals

Mice were infected s.c. with 50 L3 or 1 X 10^5 MF of *B. pahangi,* or an equal volume of HBSS only. At 12 d.p.i. splenocytes from L3 infected (L3), MF infected (MF) and HBSS control mice (C) were cultured with 10 μg/ml *B. pahangi* adult Ag for 72 hours. Cells were harvested and stained with anti-CD4 MAb, CD25 MAb and CTLA-4 MAb, each bound to a different flurochrome (CD4 – FITC, CD25-PE, CTLA-4-APC). Graph depicts significant differences in the expression of $CD4^+CD25^+CTLA\text{-}4^+$ cells between groups. Statistical analysis was carried out on results from 5 mice per group. Results are expressed as mean percentage of $CD4^+$ cells expressing CD25 and CTLA-4.

L3 VS MF, p=0.034
L3 vs C, p=0.034

	% CD4	% CD4 co-expressing CD25	% CD4 co-expressing CTLA-4	% CD4 co-expressing CD25 CTLA-4
L3	17.7 ± 1.8	24.66 ± 1.17	7.59 ± 0.59	6.9 ± 0.44
MF	23 ± 2.7	7.93 ± 0.65	1.82 ± 0.19	1.32 ± 0.195
C	20.37 ± 0.93	6.77±0.34	3.34 ± 0.48	2.13±0.23

Table 5.1. Expression of cell surface markers on the surface of $CD4^+$ cells from splenocytes of L3 and MF infected mice

Mice were infected s.c. with 50 L3 or 1 X 10^5 MF of *B. pahangi,* or an equal volume of HBSS only. At 12 d.p.i. splenocytes from L3 infected (L3), MF infected (MF) and HBSS control mice (C) were cultured with 10 μg/ml *B .pahangi* adult Ag for 72 hours. Cells were harvested and stained with anti-CD4 MAb and CD25 MAb and CTLA-4 MAb, each bound to a different flurochrome (CD4 – FITC, CD25-PE, CTLA-4-APC). Figures shown in Table 5.1 represent the % of splenocytes which express $CD4^+$, the percentage of $CD4^+$ cells which express $CD25^+$ alone, CTLA-4 alone and the percentage of $CD4^+$ cells which co-express both of these cell surface markers.

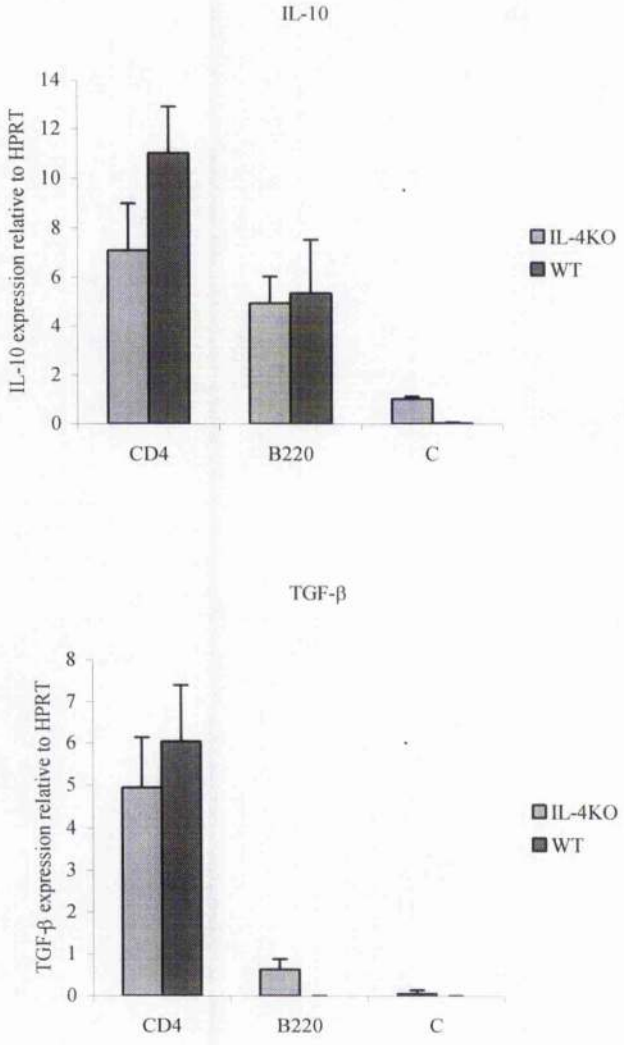


Figure 5.4. $CD4^+$ cells analyzed *ex vivo* from L3 infected IL-4-/- mice express equivalent levels of IL-10 and TGF-β mRNA to WT mice

IL-4-/- (IL-4 KO) and wild-type (WT) mice were infected s.c. with 50 L3 of *B. pahangi* or an equal volume of HBSS only. At day 12 p.i. splenocytes from L3 infected mice were pooled and magnetically separated into purified $CD4^+$ and $B220^+$ cells. Efficiency of the purification was analysed by FACS at > 95%. RT-PCR was carried out on these cells and levels of IL-10 and TGF-β mRNA were expressed relative to HPRT. Quantitative RT-PCR was carried out using the TaqMan method (as described in Materials and Methods 2.6.2).

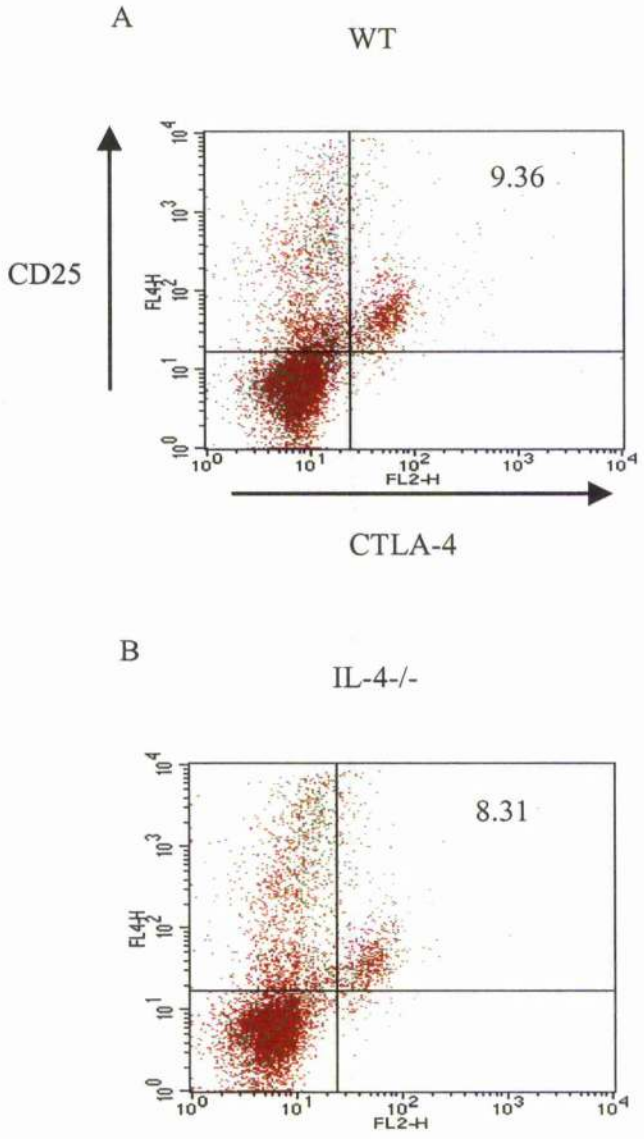


Figure 5.5. Three colour FACS labelling shows that a population of $CD4^+$ cells which co-express CD25 and CTLA-4 is also expanded in splenocyte cultures from L3 infected IL-4-/- mice

Splenocytes from L3 infected wild type (WT) (A) and IL-4-/- mice (B) were cultured with 10 μg/ml *B. pahangi* adult Ag for 72 hours. Cells were harvested and stained with anti-mouse CD4 MAb, anti-mouse CD25 MAb and anti-mouse CTLA-4 MAb, each bound to a different flurochrome (CD4 – FITC, CD25-PE, CTLA-4-APC) and analysed by flow cytometry. Gates were set on $CD4^+$ cells and these cells were then analysed for the expression of CD25 and CTLA-4 Each panel shows the staining profile of an individual mouse from each group. The numbers at the top right hand corner of each panel indicate the percentage of $CD4^+$ cells which are $CD25^+$ and $CTLA\text{-}4^+$. These figures are representative of the responses of five animals per group.

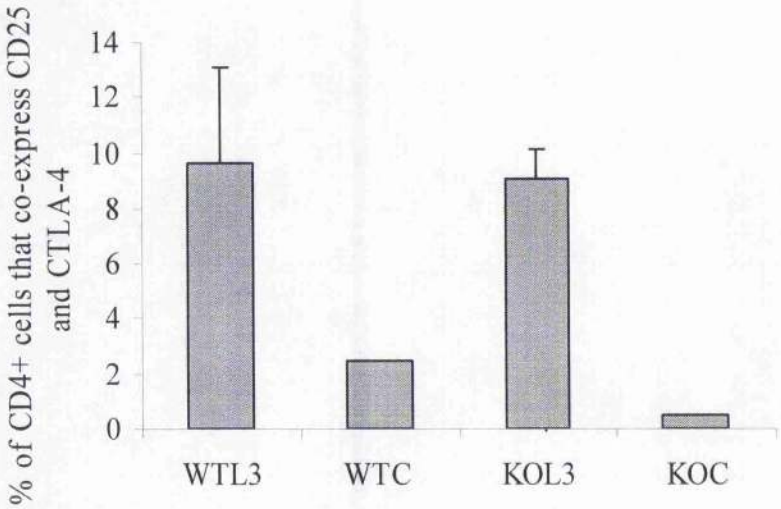


Figure 5.6. Mean percentage of $CD4^+$ cells which co-express CD25 and CTLA-4 from L3 infected and control IL-4-/- mice and wild type mice

Splenocytes from L3 infected and control (C) wild type (WT) and IL-4-/- mice (KO) were cultured with 10 μg/ml *B. pahangi* adult Ag for 72 hours. Cells were harvested and stained with anti-CD4 MAb and CD25 MAb and CTLA-4 MAb, each bound to a different flurochrome (CD4 – FITC, CD25-PE, CTLA-4-APC) and analysed by flow cytometry. Analysis was carried out on results from 5 mice per group for L3 infected animals and results are expressed as mean percentage. Cells pooled from five animals for control animals.

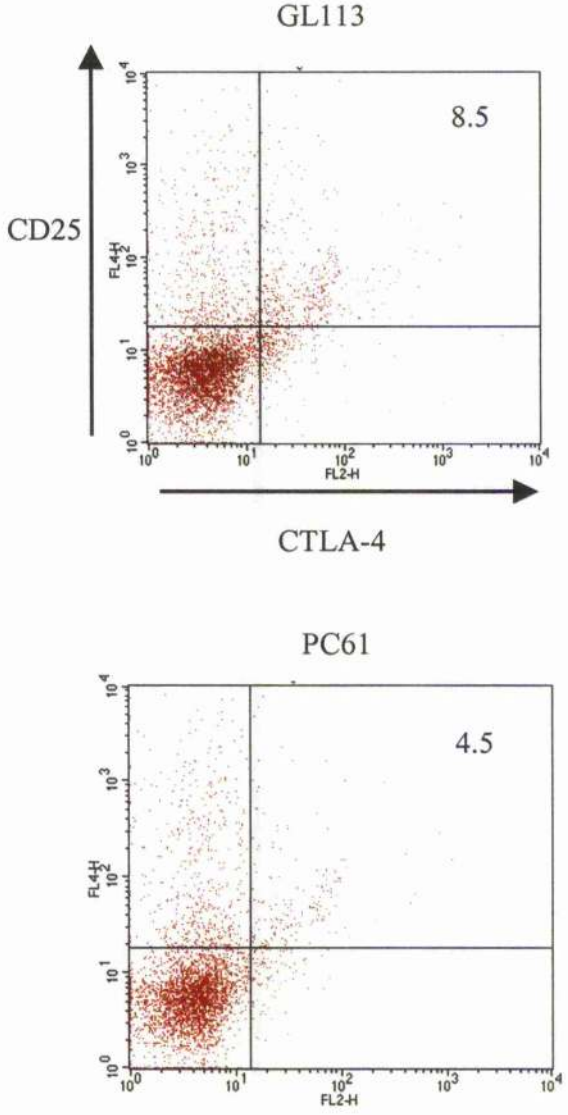


Figure 5.7. Three colour FACS analysis of cells from L3 infected mice given neutralizing CD25 MAb show reduced levels of $CD4^+$ cells which co-express CD25 and CTLA-4

Splenocytes from two groups of mice given GL113 (A) or PC61 (B) prior to infection with 50 L3 of *B. pahangi,* were cultured with 10 μg/ml *B. pahangi* adult Ag for 72 hours. Cells were harvested and stained with anti-mouse CD4 MAb, anti-mouse CD25 MAb and anti-mouse CTLA-4 MAb, each bound to a different flurochrome (CD4 – FITC, CD25-PE, CTLA-4-APC) and analysed by flow cytometry. Each panel shows the staining profile of an individual mouse from each group. The numbers at the top right hand corner of each panel indicate the percentage of $CD4^+$ cells which are $CD25^+$ and $CTLA\text{-}4^+$. These figures are representative of the responses of five animals per group.

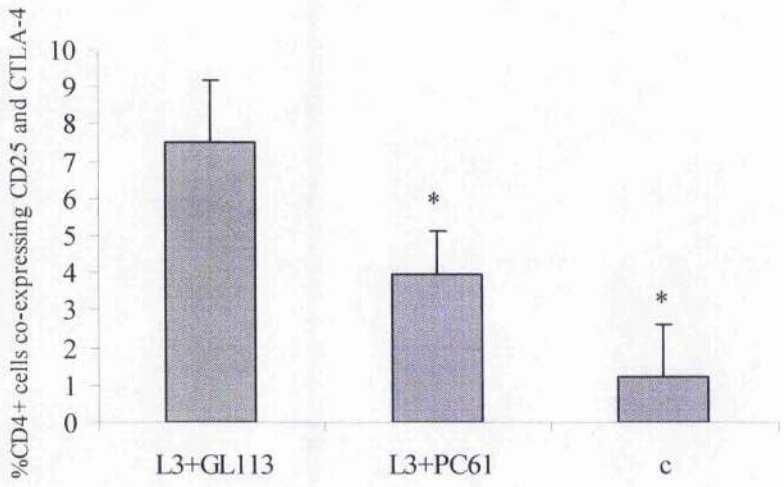


Figure 5.8. Three colour FACS analysis of cells demonstrates that administration of CD25 blocking MAb results in a significant decrease in the percentage of $CD4^+$ cells co-expressing CD25 and CTLA-4

Splenocytes from two groups of mice given GL113 or PC61 prior to infection with 50 L3 of *B. pahangi,* were cultured with 10 μg/ml *B. pahangi* adult Ag for 72 hours. C represents responses in control animals. Cells were harvested and stained with anti-mouse CD4 MAb and anti-mouse CD25 MAb and anti-mouse CTLA-4 MAb, each bound to a different flurochrome (CD4 – FITC, CD25-PE, CTLA-4-APC) and analysed by flow cytometry. Graph displays mean data from a group of five mice.

L3 + PC61 VS L3+ GL113, p=0.0122

L3 + PC61 VS C p = 0.0369

L3 + GL113 VS C p = 0.02

Figure 5.9. Levels of Ag-specific and spontaneous proliferation of splenocytes from L3 infected mice given neutralizing CD25 MAb are significantly increased compared to those given isotype matched control MAb

Two groups of mice were given either PC61 (L3+PC61) or GL113 (L3+GL113) prior to s.c. infection with 50 L3 of *B. pahangi*. At day 12 p.i., splenocytes from these groups of mice and control mice (C) were cultured with medium only and 10 μg/ml *B. pahangi* adult Ag for 48 (A),72 (B) and 96 (C) hours and proliferative responses were measured. Results are expressed as cpm.

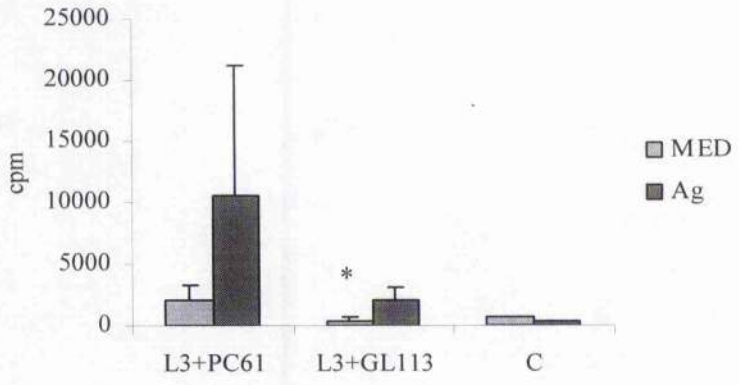
Spontaneous proliferation (medium culture)

48 hours p=0.0216 (L3+PC61 vs L3+GL113)
72 hours p=0.0122 " "
96 hours p=0.0122 " "

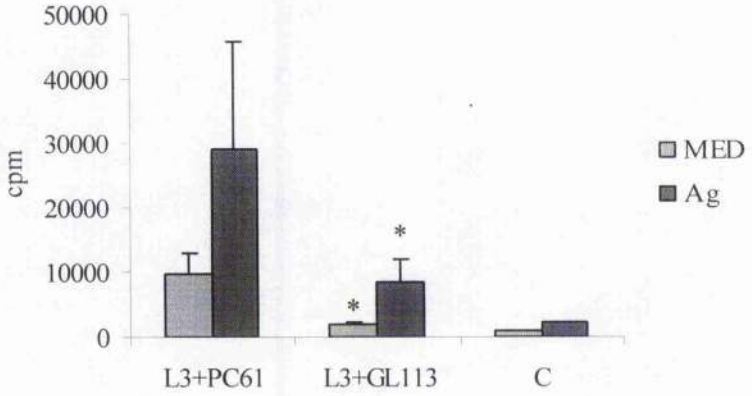
Ag-specific proliferation

48 hours p=0.0947 (L3+PC61 vsL3+GL113)
72 hours p=0.0122 " "
96 hours p=0.0367 " "

A


25000
20000
15000
10000
5000
0
cpm
MED
Ag
*
L3+PC61
L3+GL113
C

B


50000
40000
30000
20000
10000
0
cpm
MED
Ag
*
*
L3+PC61
L3+GL113
C

C

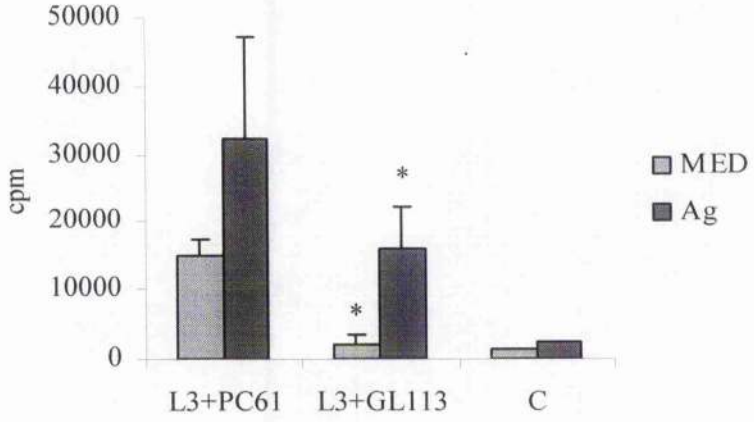

50000
40000
30000
20000
10000
0
cpm
MED
Ag
*
*
L3+PC61
L3+GL113
C

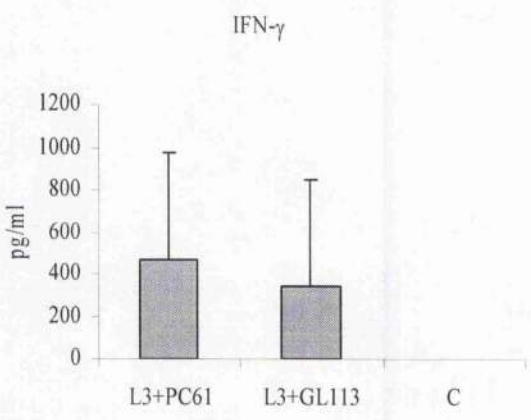
Figure 5.10. Ag-stimulated cytokine responses of splenocytes of L3 infected mice given neutralising CD25 MAb are significantly increased compared to those given isotype matched control

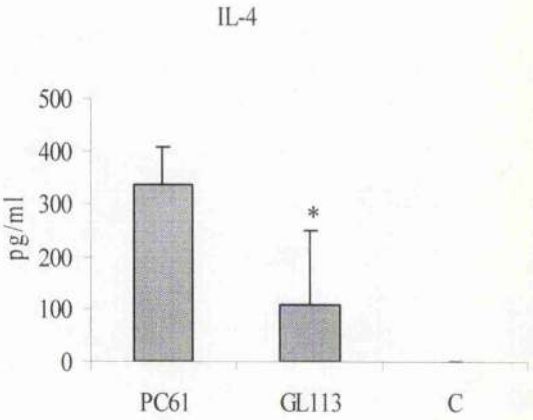
Two groups of mice were given PC61 (L3+PC61) or GL113 (L3+GL113) prior to s.c. infection with 50 L3 of *B. pahangi*. At 12 d.p.i. splenocytes from these groups of mice and control mice (C) were cultured with 10 μg/ml *B. pahangi* adult Ag. Ag-specific cytokine responses were measured at 72 hours. Results are expressed as pg/ml and all values represent the mean and standard deviation of five mice per group.

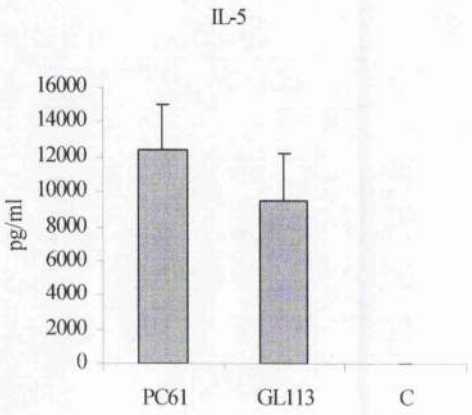
L3+PC61 vs L3+GL113

IL-5, p=0.1437
IL-4, p=0.0369
IL-10, p=0.0216
IFN-γ, p=0.8354

* significantly different.


IFN-γ
pg/ml
1200
1000
800
600
400
200
0
L3+PC61
L3+GL113
C


IL-4
pg/ml
500
400
300
200
100
0
*
PC61
GL113
C


IL-5
pg/ml
16000
14000
12000
10000
8000
6000
4000
2000
0
PC61
GL113
C

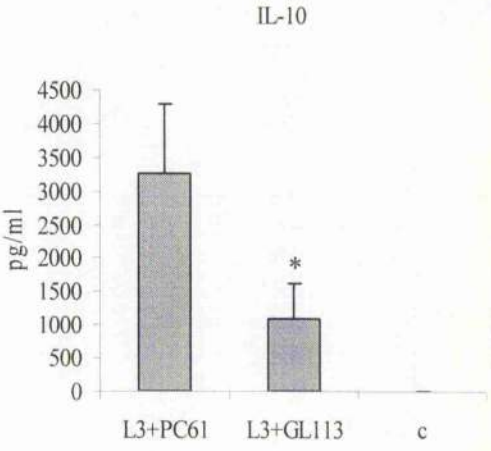
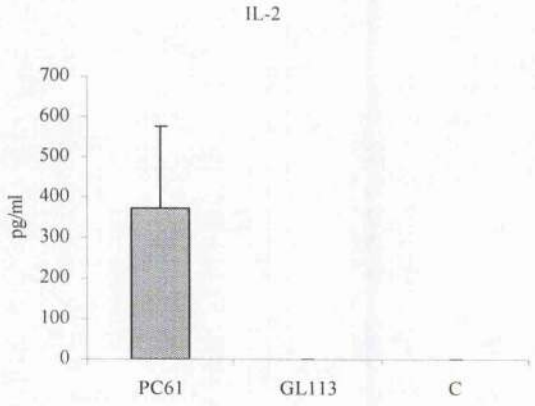
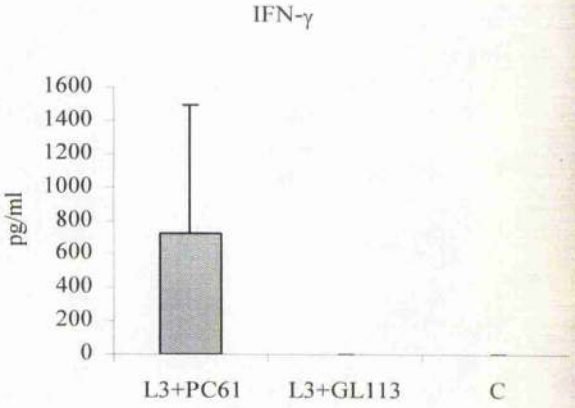
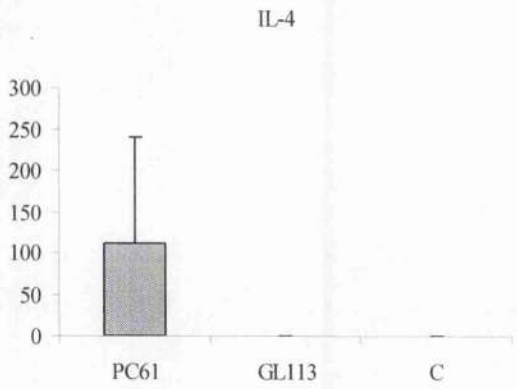

IL-10
pg/ml
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
L3+PC61
L3+GL113
c

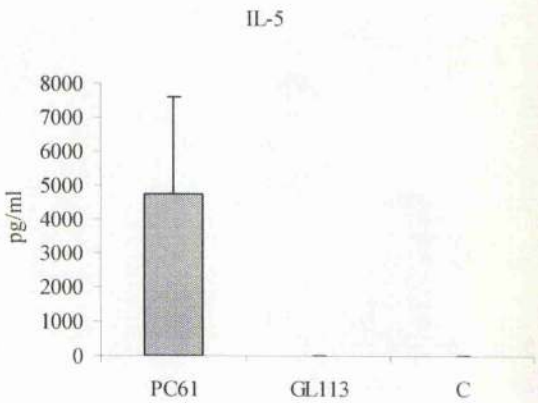
Figure 5.11. Spontaneous cytokine responses of splenocytes from L3 infected mice given neutralising CD25 MAb are significantly increased compared to those given isotype matched control

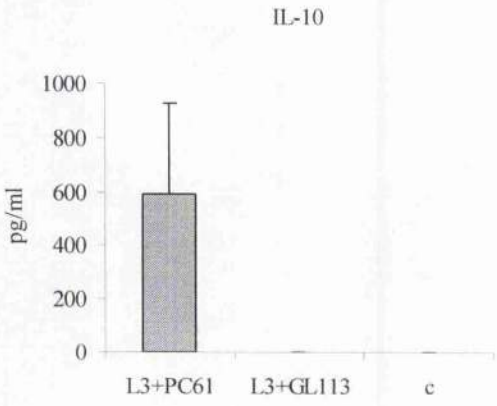
Two groups of mice were given PC61 (L3+PC61) or GL113 (L3+GL113) prior to s.c. infection with 50 L3 of *B. pahangi*. At 12 d.p.i. splenocytes from these groups of mice and control mice (C) were cultured with medium only. Spontaneous cytokine responses were measured at 72 hours. Results are expressed as pg/ml and all values represent the mean and standard deviation of five mice per group.


IL-2
pg/ml
700
600
500
400
300
200
100
0
PC61
GL113
C


IFN-γ
pg/ml
1600
1400
1200
1000
800
600
400
200
0
L3+PC61
L3+GL113
C


IL-4
300
250
200
150
100
50
0
PC61
GL113
C


IL-5
pg/ml
8000
7000
6000
5000
4000
3000
2000
1000
0
PC61
GL113
C


IL-10
pg/ml
1000
800
600
400
200
0
L3+PC61
L3+GL113
c

	Ag-stimulated cytokine levels (pg/ml)	Spontaneous cytokine levels (pg/ml)	Net amount of Ag-specific cytokine production (pg/ml)
IL-2	0	370.06	-370.06
IFN-γ	465.455	724.425	-258.97
IL-4	305.56	110.945	194.055
IL-5	12405.18	4752.275	7652.905
IL-10	3269.1	589.05	2680.05

Table 5.2. Net levels of Ag-specific cytokine production from splenocytes of mice depleted of $CD25^+$ cells prior to infection with L3

A group of mice were given PC61 prior to s.c. infection with 50 L3 of *B. pahangi*. At day 12 p.i. splenocytes from these groups of mice were cultured with medium only (RPMI+) or with 10 μg/ml *B. pahangi* adult Ag. Ag-stimulated and spontaneous cytokine responses were measured at 72 hours. To calculate the net amount of Ag-specific cytokine production, levels of spontaneous cytokines were subtracted from levels of Ag-stimulated cytokines. Results are expressed as pg/ml.

5.3. Discussion

Experiments carried out in Chapter 4 indicated that a population of $CD4^+$ cells produced IL-10 in response to L3 infection, and when IL-10R was blocked *in vivo,* increased levels of Ag-specific proliferation resulted. With recent studies reporting that T cells cloned from *O. volvulus* infected individuals bear the characteristics of Tr1 cells (produce elevated levels of IL-10, TGF-β and express CTLA-4) (Doetze *et al.*, 2000; Satoguina *et al.*, 2002), it was of interest to further characterize the cellular source of IL-10 in the *B. pahangi* mouse model. Therefore the experiments outlined in this Chapter were carried out to investigate if L3 infection resulted in the expansion of Treg cells and also to define the role of Tregs in L3 infection.

Initial experiments confirmed that $CD4^+$ T cells are the main producers of IL-10, and also demonstrated that this population express elevated levels of TGF-β *ex vivo.* Characteristically, these two cytokines are associated with the presence of a regulatory population of T cells. Tr1 cells have been shown to produce high levels of IL-10 and TGF-β (Weiner, 2001), and Th3 cells produce high levels of TGF-β with varying amounts of IL-10 (Roncarolo *et al.*, 2001b). The role of cytokines in the suppressive mechanisms of $CD4^+CD25^-$ cells is controversial. Although these cells do produce IL-10, it is thought that cell-cell contact is their mode of suppression, with surface bound TGF-β being implicated (Nakamura *et al.*, 2001; Nakamura *et al.*, 2004). However, although my results show that the $CD4^+$ population express both cytokines, they do not determine whether an individual cell co-expresses IL-10 and TGF-β. To confirm if an individual cell was producing both cytokines, intra-cellular cytokine staining, ELISPOT or single cell RT-PCR would be required.

Further characterization of the $CD4^+$ population was carried out by examining the cell surface expression of markers commonly associated with a regulatory phenotype. There are many markers which have been reported to be associated with Treg cells (GITR, OX40, LAP) (Read and Powrie, 2001; Nakamura *et al.* 2004). However, the markers I chose to use in the course of my experiments were CD4, CD25 and CTLA-4. CTLA-4 was selected, as this molecule had been identified previously to be expressed on the Treg cells identified in the *O. volvulus* studies (Satoguina *et al.*, 2002). In addition, a significant role for CTLA-4 was recently identified in mediating the diminished parasite Ag-specific hyporesponsiveness observed in *W. bancrofti* infected individuals. In that study, the greatest intensity of CTLA-4 expression occurred on $CD4^+CD25^+$ cells (Steel and Nutman, 2003). In splenocyte culture from L3 infected mice there was a significant expansion of $CD4^+$ cells which co-expressed CD25 and CTLA-4, compared to cells from MF infected or control animals. This was, therefore, a strong indication that the L3 of *B. pahangi* elicited a population of Treg-like cells in the mouse model. It was also observed that the percentage of activated cells ($CD25^-$) was much higher in L3 infected animals compared to MF infected animals, as was the proportion of cells expressing CTLA-4. This is not surprising, as cells which have been activated express elevated levels of CTLA-4 after stimulation. However when CD4 cells co-express both molecules, it suggests that these cells may be regulatory in function. These results are intriguing, in that these cells are not expanded in MF infected mice, whereas the most significant down-regulation in proliferation seen in human studies is in MF+ patients. Also, CD25 is a marker of activation, yet in MF infected mice, the expression of this marker was not up-regulated. However, previous studies have shown that $CD4^+$ cells are clearly activated in MF infected mice, as demonstrated by their ability to produce Ag-specific IFN-γ (Jenson *et al.*, 2002) and also to express high levels of an alternative

marker of activation, CD44 (O'Connor and Devaney, 2002).These results suggest that the L3 and MF stages of the parasite use different mechanisms to down-regulate immune responses, at least in the mouse. However the situation is bound to be more complex in infected humans, who are exposed to multiple life cycle stages simultaneously.

To further characterize these cells, parallel experiments were carried out in IL-4-/- mice. Previous studies showed that IL-4 was not required for the development of a population of Treg cells which down-regulated the effects of Th1-mediated colitis in the mouse. This effect was dependent on TGF-β, but these cells did not require IL-4 (Powrie *et al.*, 1996). Therefore RT-PCR was carried out on purified $CD4^+$ and $B220^+$ cells from WT and IL-4-/- mice infected with L3 to detect levels of IL-10 and TGF-β mRNA expression. These experiments showed that there was no significant difference in mRNA levels for either cytokine in the $CD4^+$ population, supporting the hypothesis that these cytokines are being produced by T cells other than Th2 cells. However, Th2 cells can expand via an IL-4 independent pathway (Huang *et al.*, 1997; Bird *et al.*, 1998; Kropf *et al.*, 1999) therefore, further experiments would be required to formally prove that IL-10 and TGF-β are expressed by Treg cells. For example, it would be possible to distinguish between Th2 and Treg cells by using RT-PCR to detect Foxp3, the transcription factor associated with Treg cells (O'Garra and Vieira, 2003; Sakaguchi, 2003) or ST2L which is a marker for Th2 cells (Kropf *et al.*, 1999). However, further experiments carried out in IL-4-/- mice demonstrated that there was similar expansion of $CD4^+CD25^+$CTLA-4^+ cells in splenocyte cultures from these animals compared to infected WT mice, lending further support to the hypothesis that these cells are a regulatory population, that may express IL-10 and TGF-β.

In order to investigate the function of these cells in L3 infected mice, depletion experiments were carried out using an Ab (PC61), which eliminates $CD25^+$ cells. It was important to administer this Ab prior to infection, as administration post-infection would lead to the depletion of activated cells which express CD25 (the α chain of the high affinity IL-2R) upon activation, whereas administration before infection targets Treg cells (Sakaguchi *et al.*, 1995). It is notable that ~ 25% of $CD4^+$ cells express CD25 post-infection with L3 but only ~ 7% of this sub-population co-express CTLA-4. Therefore, cells co-expressing all three markers are a minor population within the splenocyte cultures from these animals. However results from these experiments demonstrated that this small population was vital in regulating immune responses to infection with L3. FACS analysis of splenocyte cultures from animals given PC61, revealed a significant reduction in the expansion $CD4^+CD25^+CTLA4^+$ cells *in vitro*. In all experiments there was approximately 50% reduction in the numbers of Treg cells following administration of PC61. This may relate to the fact that the MAb was not affinity purified prior to administration. In future experiments, the Ab could be purified using Protein G columns, which would allow a higher concentration of pure Ab to be administered and may further reduce expansion of Treg cells. Another interesting point from these experiments was that the percentage of $CD4^+CD25^-$ cells did not decrease in splenocyte cultures from mice given PC61 (data not shown), indicating that activated cells were not being deleted. This provides additional confirmation that the depleted population are regulatory cells, as opposed to activated cells.

Proliferation and cytokine assays were carried out on splenocytes from PC61 treated animals to investigate whether these cells had a regulatory function. Depletion of Tregs had a profound effect on immune Ag-specific responses but also affected spontaneous

responses (medium only). For example, Ag-specific proliferation was significantly enhanced in PC61 treated mice. Along with the ability to down-regulate Th1 responses, Treg cells can also suppress Th2 responses (although to a lesser extent) (Cosmi *et al.*, 2003), therefore, increased levels of proliferation observed in PC61 treated mice may reflect modulation of Th2 responses by Treg cells as proposed by Maziels and Yazdanbakhsh (2003). In addition, elevated levels of spontaneous proliferation were observed demonstrating an extreme dysregulation in the absence of Treg cells. Similar results were observed with Ag-specific cytokine responses, where levels of IL-4 and IL-10 were significantly higher in cultures of splenocytes from mice given PC61. Increased levels of IL-5 were observed in each experiment but this did not reach statistical significance, while no effect was observed on levels of IFN-γ. In addition, the potent immunomodulatory effect of these cells was demonstrated by spontaneous cytokine production in these cultures. In intact mice, spontaneous cytokine production is never observed. However in mice given PC61, there were elevated levels of each cytokine (IL-2, IFN-γ, IL-4, IL-5, IL-10). Again this illustrates that removal of these regulatory cells appears to have resulted in major dysregulation of responses. Interestingly, in the absence of these cells, IL-10 was elevated, suggesting that Treg cells may regulate IL-10 production by other cell types, such as Th2 cells. These results suggest a complex network of regulation in which Th1 responses are limited by Th2 cells elicited by helminth infection, with Tregs then preventing an overwhelming Th2 response, which can also be damaging to the host.

Other parasitic models which have utilized the PC61 Ab to eliminate Treg cells have shown that the balance between T effector and Treg mechanisms is important for parasite clearance (Long *et al.*, 2003; Hisaeda *et al.*, 2004). In addition, in the *L. major*

system, elimination of $CD4^+CD25^+$ cells resulted in a sterile cure with loss of concomitant immunity. This is a very different model system to that described in this thesis, as the Belkaid studies deal with a chronic infection where up to 50% of $CD4^+$ cells in the dermis are $CD25^+$ cells. In addition, IL-10 produced by these $CD4^+CD25^+$ cells has been shown to have an important role in parasite persistence. Transfer of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from IL-10-/- mice into RAG-/- mice resulted in complete clearance of parasites. In contrast, when cells from WT mice were implanted, this resulted in non-healing lesions at the dermal site and maintenance of parasitaemia. These data indicate that IL-10 may modulate APC function, directly inhibiting IFN-γ or rendering macrophages refractory to activation. Therefore, in this system, IL-10 has a role in the establishment of persistent infection, which in the long term results in concomitant immunity, with benefits to both host and parasite (Belkaid *et al.*, 2002).

These studies and those presented in this Chapter address the question as to whether immune suppression elicited by Tregs protects the parasite or the host. This is a difficult question to answer as maintenance of parasitaemia is required for resistance in the *L. major* and *Plasmodium* studies and also for the transmission of the parasite. From these studies it appears that the presence of Tregs is involved in maintenance of low-level infection. In the context of lymphatic filariasis, there are many outstanding questions. Do L3 elicit Treg cells in humans? Do these cells regulate immune responses that might otherwise kill adults and MF? How does protective immunity develop in this situation? Clearly, to answer these questions, further investigation into the role of Treg cells in filarial infection is required.

Investigations into the role of Treg cells in infectious disease are only just beginning (see 5.1) as previously most studies were carried out examining their properties in autoimmune disease and tumour immunity. Now these cells have been shown to be involved in parasitic, bacterial and fungal infection. However despite recent advances there are still many unanswered questions relating to Treg cells. For example, three different subsets of Treg have been defined, $CD4^{+}CD25^{+}$, Tr1 and Th3, but there are many similarities between these groups and further study is required to define their origins. In terms of helminth infection, the work of van der Kleij and colleagues (2002) is very important as they identified IL-10 producing Treg cells (which were clearly in a separate lineage from Th2 cells), the APC driving this population and the molecule responsible for this immune response. Whether a similar cell type is involved in initiation of responses that give rise to a Treg population in the L3 infected BALB/c mouse remains to be seen. Further characterization of these cells and the L3-specific molecules which induce their development are required.

Chapter 6. B cells modulate immune responses in mice infected with L3

6.1. Introduction

The development of appropriate and effective immune responses requires T cells to come into contact with antigen-bearing cells of many sorts, such as DC, macrophages and B cells. Various sub-groups of APC are essential in initiating and directing T cell responses (as reviewed in the Introduction). APC provide ligands for signalling via the TCR by processing and presenting Ag in the form of peptides associated with MHC class I or II molecules, or lipid molecules bound to CD1 proteins. These cells also deliver a costimulatory signal to the T cell via B7-1 and B7-2 ligation with CD28 on the surface of the T cell. In addition, DC can influence the pathway of differentiation by the secretion of cytokines (Banchereau and Steinman, 1998). However, $CD4^+$ cells must also physically associate with Ag-bearing, Ag-receptor activated B cells (Cyster, 1999; Jenkins *et al.*, 2001). B cells are a major component of the adaptive mammalian immune system as they have the unique ability to secrete immunoglobulins of various isotypes. Alone, this property ensures that B cells are essential to immunity, but activated B cells can also produce a variety of chemokines and cytokines and act as APC. For example, the role of B cells in Ag-presentation has been described in the NOD mouse model of autoimmune type 1 diabetes. B cells were shown to contribute to the disease as they have the ability to preferentially trigger pathogenic $CD4^+$ cells (Silveira *et al.*, 2002), thus initiating insulitis and sialitis (Noorchashm *et al.*, 1997). B cells can act as potent APC, in the main part due to the ability of the Ig receptor to mediate rapid and specific Ag-uptake (Chesnut and Grey, 1981). Early experiments in B cell-deficient mice supported the hypothesis that B cells play a significant role in Ag-presentation required for T cell activation (Hayglass *et al.*, 1986; Kurt-Jones *et al.*,

1988). Ron *et al* (1987) described a major role for B cells in T cell priming in LN of mice. Irradiated mice injected i.v. with purified T cells manifested poor T cell priming in the LN after s.c. injection with Ag. It was reasoned that as B cells are highly radio-sensitive, the lack of B cells resulted in decreased levels of T cell priming. To confirm this, i.v. injection of B cells into irradiated recipients of T cells led to high priming of T cells in the LN after s.c. injection of Ag (Ron and Sprent, 1987).

Initital studies into the expression of B7 on the surface of B cells described up-regulation of this molecule (B7-1 was the only molecule known at this time) on the surface of activated B cells and its ligation with the CD28 receptor on the surface of naïve T cells. The costimulatory effect of this interaction was also demonstrated as stimulation of T cells with $B7^+$ transfected cells resulted in increased proliferation and production of IL-2 (Linsley *et al.*, 1991). Further studies identified an additional ligand for CD28 which was constitutively expressed on the surface of DC and expressed on activated B cells. This molecule was named B7-2 and was shown to co-stimulate naïve T cells upon exposure to alloantigen. Interestingly, B7-1 was shown to be expressed on the surface of only a subset of B cells after 48 hours, whereas B7-2 was shown to be expressed on the surface of the majority of LPS-activated B cells within 6-12 hours post-stimulation (Lenschow *et al.*, 1993). In a subsequent study it was shown that naïve B cells do not express B7-1 and B7-2, but that engagement of the B cell receptor (BCR) resulted in the rapid up-regulation of B7-2 on the B cell surface. These data help explain why Ag-pulsed B cells are such potent APC (Lenschow *et al.*, 1994).

I chose to investigate the properties of B cells in relation to infection with L3 due to a number of key factors. Firstly, it has been shown previously that B cells preferentially induce a Th2 type response in OVA-specific clones from lymph node cells, compared to

adherent cells which induce Th1 type responses (Gajewski *et al.*, 1991). Therefore, there was a possibility that B cells were acting as APC post-infection with L3 in the mouse model, as splenocytes from infected animals produce high levels of Th2 cytokines. Secondly, previous data from this model system has shown that infection of BALB/c mice with the L3 of *B. pahangi* resulted in inhibited Th1 responses. However, when APC from control mice were added to cultures in place of resident APC, this inhibition was reversed. Although the phenotype of the suppressive APC was not identified, it was notable that irradiation of the resident APC resulted in reversal of Th1 inhibition. As mentioned previously, B cells are very sensitive to radiation, suggesting that B cells may be the suppressive APC in this model system (Osborne and Devaney, 1999). In addition, recent work carried out by Wilson and colleagues, demonstrated that ES-62, a major ES product of filarial nematodes, has a potent immuno-modulatory effect on B cells, rendering them hyporesponsive to Ag-stimulation. In addition, a separate lineage of B cells, B1 cells, has been shown to produce IL-10 in response to exposure to ES-62 (Wilson *et al.*, 2003a; Wilson *et al.*, 2003b) and taken together, these results suggest that the secreted products of helminth parasites can modulate B cell responses.

Therefore, the work presented in this chapter aimed to further clarify the role of B cells in L3 infection, in terms of cytokine production, capacity to proliferate in response to Ag, and Ag presentation.

6.2. Results

6.2.1. The role of B cells in L3 infection

(i) B cell depletion and μMT experiments

To define the role of B cells in L3 infection, experiments were carried out with groups of five BALB/c mice injected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS. At 12 d.p.i., spleens were removed and B cells were depleted from single cell suspensions using magnetic beads and a FITC anti-mouse B220 MAb (RA3-6B2) (see Materials and Methods 2.5.1). The efficiency of the depletion was analysed by FACS and was greater than, or equal to 95% in all experiments. Proliferation and cytokine assays were carried out on the remaining depleted populations. In addition, similar experiments were carried out with groups of five L3 infected B-cell deficient (μMT) mutant mice on the C57BL/6 background. At 12 d.p.i. spleens were removed from these animals and the appropriate controls and *in vitro* analysis was carried out.

(ii) Ag-specific proliferative responses

Proliferative responses of splenocytes from L3 infected mice, pre- and post-depletion of B cells were measured after 72 hours of *in vitro* re-stimulation with 10 μg/ml *B. pahangi* adult Ag. As shown in the representative experiment in Figure 6.1, depletion of the B cell population resulted in a decrease in levels of Ag-specific proliferation (62% decrease in the experiment shown). In three separate experiments, the mean percentage reduction was 49%± 15.17 compared with whole splenocytes culture (p=0.0809). As *in vitro* depletion of B cells had a major effect on Ag-specific proliferation, the role of B cells in L3 infection was further examined using μMT mice. As illustrated in Figure 6.2,

levels of Ag-specific proliferation were significantly lower (85% reduction) in splenocytes from L3 infected μMT mice, than in wild type mice (p=0.013). Despite the fact that the μMT mice are on the C57BL/6 background the results were similar to those obtained by depletion of B cells from BALB/c mice. These data suggest that either B cells may be proliferating in response to re-stimulation with Ag, or that B cells may act as APC for $CD4^+$ T cell proliferation.

(iii) CFSE labelling of splenocytes from L3 infected mice

In order to define the precise role of B cells in L3 infected BALB/c mice, CFSE staining was carried out prior to *in vitro* stimulation of cells with 10 μg/ml *B. pahangi* adult Ag. At 96 hours cells were harvested and labelled with anti-CD4 MAb, anti-B220 MAb or CD8 MAb. The samples were then analyzed by flow cytometry. Figures 6.3 and 6.4 show data from a representative experiment (medium only controls not shown) in which the proliferation of $CD4^+$, $B220^+$ and $CD8^-$ cells were tracked using CFSE. The FACS plots in Figure 6.3 are from individual mice, whereas Figures 6.4A and B depict the mean results from five mice. It can be seen from these Figures that the major cell type proliferating in response to Ag is in fact the B cell population with proliferating $B220^+$ cells accounting for 17% of the total lymphocyte population. Proliferating $CD4^-$ cells accounted for ~ 5% and proliferating $CD8^+$ cells only ~ 2.2% of the total lymphocyte population (Figure 6.4A). When the results are expressed as a percentage of dividing cells, it is clear that B cells account for the majority of the dividing cells in L3 infected mice (Figure 6.4B). Therefore it can be concluded that B cells have the ability to proliferate in response to L3 infection in an Ag-specific manner. These data confirm findings outlined in 3.2.4, where it was shown that B cells proliferate in response to Ag, in both WT and IL-4-/- mice.

(iv) Ag-specific cytokine responses after *in vitro* depletion of B cells

In four separate experiments, the depletion of B cells from splenocytes of L3 infected mice resulted in decreased levels of Ag-specific cytokines. The results of a representative experiment are shown in Figure 6.5. As shown previously, levels of IL-10 were reduced by 40%, results which are similar to those from mRNA expression, where B cells were shown to account for ~ 40% of IL-10 message. However of all cytokines measured, levels of IL-5 were most reduced (75% in the experiment shown), while little difference was recorded in IL-4 levels (22% reduction in the experiment shown). Levels of IFN-γ were increased in two out of four experiments (75% increase in the experiment shown), while in the remaining two experiments, there was no increase following B cell depletion. No IL-2 was detected in either group. The mean percentage decrease/increase in cytokines post-depletion of B cells in several experiments is shown in Table 6.1 and illustrates a consistent decrease in Th2 cytokines following B cells depletion.

(v) Ag-specific cytokine responses of L3 infected μMT mice

Cytokine responses of splenocytes from L3 infected and control μMT mice were examined at 72 hours. As shown in Figure 6.6, levels of IL-10 were reduced by 60% in the KO mice compared to levels in their wild type counterparts. However, IL-4 and IL-5 remained unchanged between groups. No IL-2 or IFN-γ was detected in either group. The reduction in levels of IL-10 in μMT mice was similar to that described previously following depletion of B cells from BALB/c mice. However in contrast to the depletion experiments, there was no difference in levels of IL-5 or IL-4. It is notable that levels of these cytokines were considerably lower in both μMT mice and their WT counterparts

compared to BALB/c mice. This may be due to the fact that μMT mice are on the C57BL/6 background while the depletion experiments were carried out in BALB/c mice (the parasite induces a stronger Th2 response in the BALB/c mice than in C57BL/6 mice).

6.2.2. Investigating a role for B7 cell surface molecules expressed on B cells

As B cells generate a Th2 like response with model Ag such as OVA, it is possible that these cells act as APC in mice infected with *B. pahangi*. An important way in which APC modulate T cell responses is by the expression of their costimulatory ligands B7-1 and B7-2. Therefore the following experiments examined the role of these molecules in L3 infection. B7-1 and B7-2 are known to play an essential role in the proliferative capacity of T cells via interaction with the costimulatory molecule CD28, so it was of interest to investigate the possibility of increased expression of these molecules on splenic cells which had been primed by the L3 compared to that of control animals. MF infected animals were also included in some of these experiments as these mice display a different, more Th1-like profile.

(i) B7-1 and B7-2 expression on the surface of $B220^+$ cells

Splenocytes from L3 infected, MF infected and control BALB/c mice were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag for 72 hours. Cells were then harvested and two-colour FACS analysis was carried out to determine the percentage of $B220^+$ cells which expressed B7-1 or B7-2. Figure 6.7 shows FACS plots from individual mice, representative of a group of five. In control animals ~7.8% of total lymphocytes co-express B220 and B7-1 while ~ 3.6% co-express B220 and B7-2. Following infection with L3 the expression of both B7-1 and B7-2 on B cells is up-regulated with ~14.8% of

all lymphocytes expressing B7-1 and B220 and 21.7% expressing B7-2 and B220. Infection with MF results in a similar up-regulation of B7-1 on the surface of $B220^+$ cells (~15.9%) whereas approximately 10.2% of total lymphocytes from these animals co-express B220 and B7-2. These results indicate that infection with L3 and MF induces the up-regulation of both B7-1 and B7-2 on the surface of B cells. However the L3 has the ability to up-regulate B7-2 with approximately double the efficiency of MF. These data also show that the vast majority of cells which express B7-1 or B7-2 are in fact B cells, with ~90% of $B7\text{-}2^+$ cells co-expressing B220 and 70% of $B7\text{-}1^-$ cells co-expressing B220 (Figure 6.8). However, further analysis of these data indicated that not all B cells express B7-1 or B7-2 (see Figure 6.7).

(ii) Effect of IL-10 on the expression of B7-1 and B7-2 on the surface of $B220^+$ cells

Previous experiments described in this thesis have shown that neutralizing IL-10 *in vitro* has a significant effect on cytokine production in splenocytes culture from L3 infected mice. In addition when IL-10R was blocked *in vivo* levels of Ag-specific proliferation were increased significantly in splenocytes from infected mice. One mechanism by which IL-10 can affect T cell responses is via its effect on APC, where it is reported to down-regulate expression of B7-1 and B7-2. To assess the effect of IL-10 on B7-1 and B7-2 in L3 infected mice, experiments were carried out using IL-10-/- mice and *in vitro* neutralization of IL-10.

(iii) Effect of *in vitro* neutralization of IL-10 on B7-1 and B7-2 expression

Splenocytes from L3 infected mice were re-stimulated *in vitro* for 72 hours with 10 μg/ml *B. pahangi* adult Ag, Ag plus 1μg/ml neutralizing IL-10 MAb or Ag plus 1μg/ml

isotype matched control. Cells were then harvested and two colour FACS analysis was performed as described previously. The results of these experiments are shown in Figure 6.9 demonstrate that neutralizing IL-10 results in a small, but significant increase in the percentage of B cells that express B7-1 and B7-2. Notably and confirming previous results, in all these experiments more B cells from L3 infected mice express B7-2 than B7-1. The percentage of lymphocytes which co-express B7-1 or B7-2 and B220 was increased in cultures where IL-10 was neutralized compared to Ag only or Ag plus isotype control cultures. Figure 6.10 shows mean results of five mice per group as a percentage of total lymphocytes which co-express B7-1 or B7-2 and B220.

(iv) B7-1 and B7-2 expression in IL-10-/- mice

During the course of this study, there was an opportunity to infect IL-10-/- mice on the C57BL/6 background at the University of Manchester. Splenocytes from L3 infected IL-10-/- mice and their wild type counterparts were re-stimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag for 72 hours. Cells were then harvested and two-colour FACS analysis performed as described above. Figure 6.11 shows FACS plots from an individual WT and IL-10-/- mouse. It can be seen that the expression of both B7-1 and B7-2 on B cells is elevated in IL-10-/- mice. In addition, B7-2 and in part B7-1 are also expressed on a significantly higher percentage of non-B cells in IL-10-/- mice. Figure 6.12 shows the mean percentage of total lymphocytes which co-express B220 and B7-1 or B7-2 and demonstrates a number of differences between the groups. L3 infected IL-10-/- mice express significantly higher levels of both B7-1 and B7-2 compared to L3 infected WT mice, consistent with the role of IL-10 in regulating expression of these costimulatory molecules. Included in Figure 6.12 is data from control animals, which shows that levels of B7-1 are significantly up-regulated in L3 infected KO animals

compared to control uninfected IL-10-/- mice, whereas this result did not reach significance in WT animals. However, when examining expression of B7-2 on B cells from both KO and wild type mice, levels were significantly increased between L3 infected and control animals from both groups of mice.

(v) Effect of neutralizing B7-1 and B7-2 on Ag-specific proliferation

The following experiments were carried out to address the significance of B7-1 and B7-2 in the regulation of proliferation. As infection with L3 results in a significant up-regulation of these molecules on the surface of B cells, it was proposed that blocking the B7-1/2 interaction with CD28 might influence T cell proliferation.

Proliferative responses of splenocytes from L3 infected mice were measured over a time course from 48 to 96 hours of *in vitro* re-stimulation with 10 μg/ml *B. pahangi* adult Ag in culture containing 1 μg/ml neutralizing B7-1, B7-2 or both MAb or the appropriate isotype control. Figure 6.13 shows data from 48 and 72 hour cultures. At 48 hours, levels of Ag-specific proliferation were significantly reduced when either B7-1, B7-2 or both molecules were neutralized. However, at 72 hours levels of Ag-specific proliferation were only reduced significantly when B7-2 was neutralized. Neutralization of both molecules together resulted in reduced levels of proliferation but this failed to reach statistical significance. By 96 hours, no differences were observed (data not shown).

(vi) Effect of neutralizing B7-1 and B7-2 on Ag-specific cytokine production

Ag-specific cytokine responses of splenocytes from L3 infected mice were measured after 72 hours of *in vitro* re-stimulation with 10 μg/ml *B. pahangi* adult Ag, Ag plus

1μg/ml anti-B7-1 MAb or Ag plus 1μg/ml anti-B7-2 MAb or Ag plus both Abs. As shown in the representative experiment in Figure 6.14, levels of Ag-specific IL-5 were significantly reduced when B7-2 or both molecules were neutralized together. Neutralization of B7-1 also reduced levels of IL-5, but this did not reach statistical significance. Levels of both IL-4 and IL-10 were significantly reduced when B7-1 and B7-2 were neutralized simultaneously but no significant difference was observed when either B7-1 or B7-2 was neutralized alone. Interestingly, despite the significant decrease in Th2 cytokines observed in these experiments, there was no consequent increase in Ag-specific IL-2 or IFN-γ.

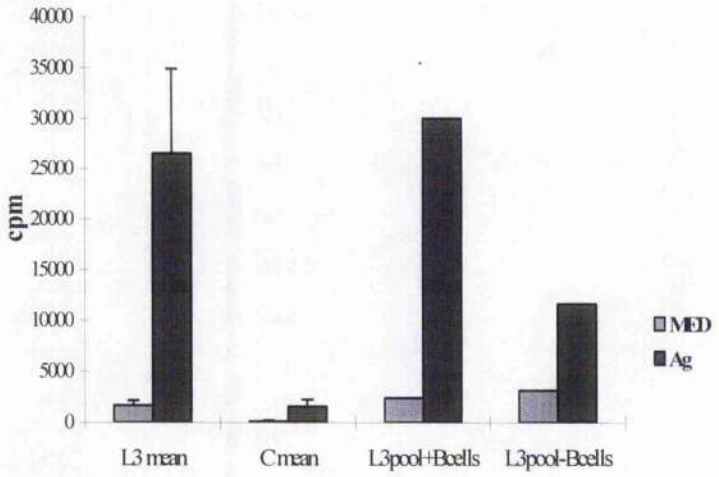


Figure 6.1. Depletion of B cells from splenocytes of L3 infected mice results in reduced levels of Ag-specific proliferation

Mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i. splenocytes from L3 infected mice were pooled and B cells were depleted using MACS anti-FITC beads and magnetic columns. The negative fraction was then re-stimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Ag-specific proliferative responses from whole splenocytes from five animals (L3 mean), from pooled cells prior to depletion (L3pool+Bcells) and from splenocytes depleted of B cells (L3pool-Bcells) were measured at 72 hours. Results are expressed as cpm.

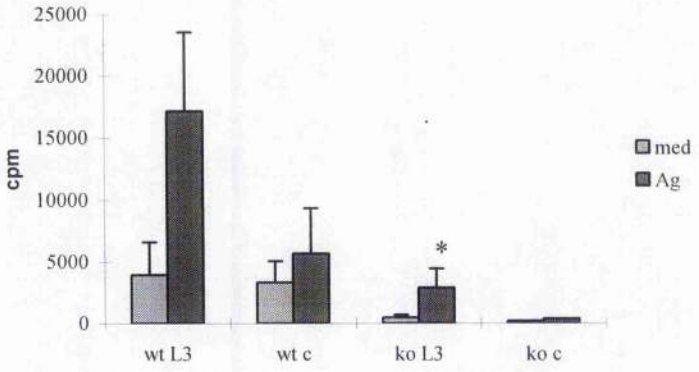


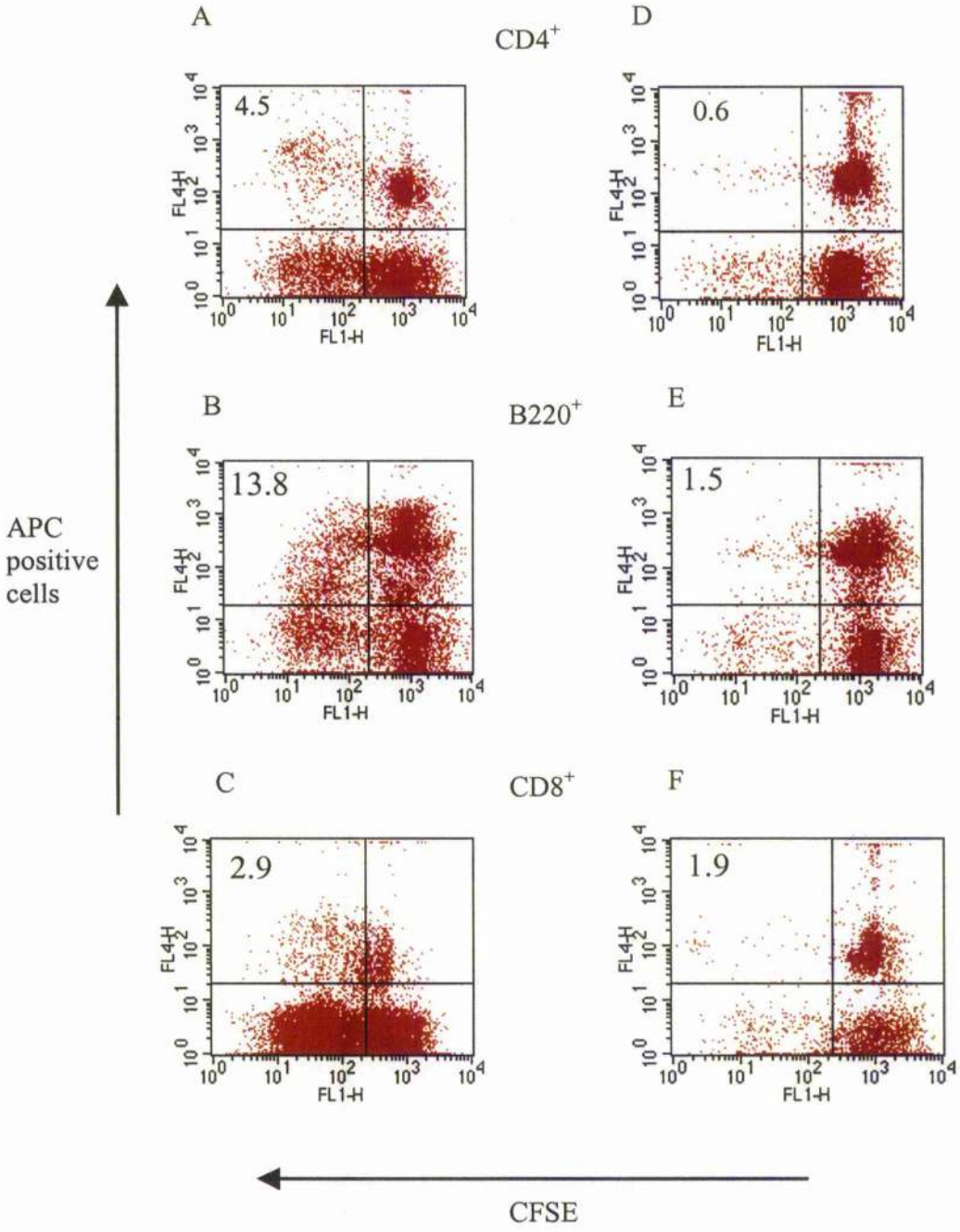
Figure 6.2. Splenocytes from L3 infected μMT mice display significantly lower Ag-specific proliferation compared to their wild-type counterparts

μMT mice on the C57BL/6 background (KO) and wild-type mice (WT) were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes from individual mice were re-stimulated *in vitro* with 10μg/ml *B. pahangi* adult Ag. Ag-specific proliferative responses from whole splenocytes were measured at 72 hours. Results show means and standard deviations of five mice per group and are expressed as cpm.

WTL3+Ag vs KOL3+Ag – p=0.013

Figure 6.3. CFSE labelling of cells from L3 infected mice in Ag-stimulated culture shows that B cells proliferate in response to Ag

Splenocytes from BALB/c mice given 50 L3 of *B. pahangi* (A - C) or an equal volume of HBSS (D - F), were labelled with CFSE and cultured with 10μg/ml *B. pahangi* adult Ag. Cells were harvested at 96 hours, surface stained with anti-CD4 MAb (A and D) or anti-B220 MAb (B and E) or anti-CD8 (C and F) and analyzed by flow cytometry. Each panel shows the CFSE staining profile of $CD4^+$, $B220^+$ or $CD8^+$ splenocytes in an individual mouse. The numbers at the top left hand corner of each panel indicate the percentage of $CD4^+$, $B220^+$ or $CD8^+$ cells displaying reduced fluorescence intensity in FL-1, indicating they have divided in Ag-stimulated culture. These figures are representative of the responses of five animals per group.


A
CD4+
D
4.5
0.6
B
B220+
E
13.8
1.5
C
CD8+
F
2.9
1.9
APC positive cells
FL4-H
FL1-H
CFSE

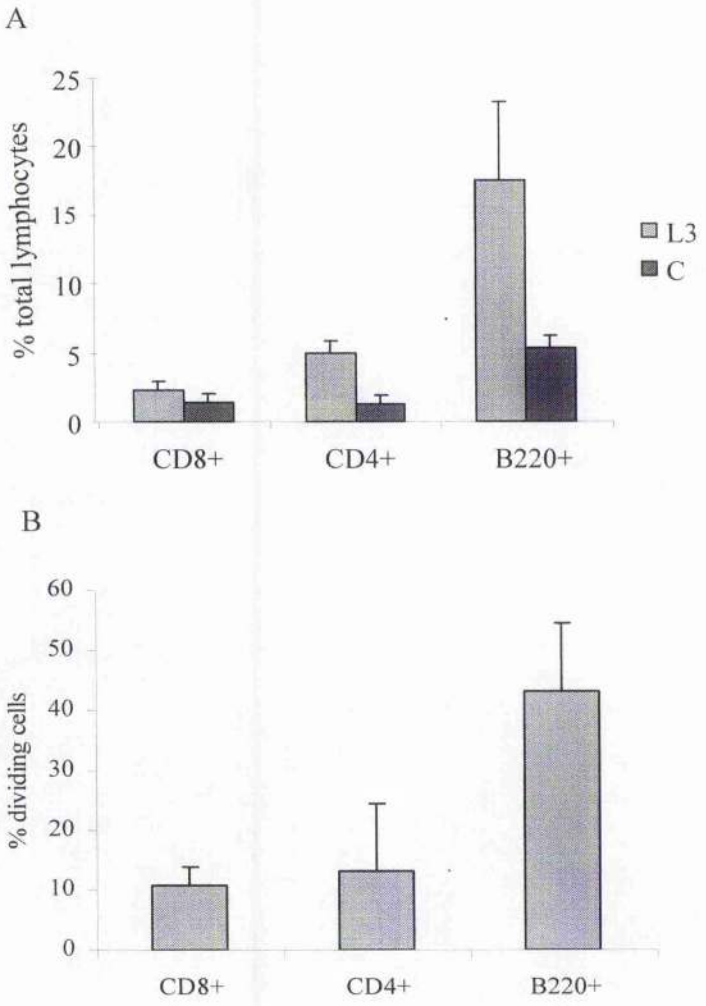


Figure 6.4. B cells proliferate in response to L3 infection

Splenocytes from mice given 50 L3 of *B. pahangi* or an equal volume of HBSS (C), were labelled with CFSE and cultured with 10 μg/ml *B. pahangi* adult Ag for 96 hours. Cells were harvested, stained with anti-CD4 MAb, anti-CD8 MAb or anti B220 MAb and analysed by flow cytometry. Graph A depicts the mean results of five animals per group, expressed as division of individual cell types as a percentage of total lymphocytes. Graph B depicts the mean results of five animals per group expressed as division of individual cell types as a percentage of dividing cells.

Graph A stats

L3 infected

$CD8^+$ division vs $CD4^+$ $p = 0.0122$
$CD8^+$ division vs $B220^+$ $p = 0.0122$
$CD4^+$ division vs $B220^+$ $p = 0.0122$

C vs L3 infected

$CD8^+$ $p = 0.0864$
$CD4^+$ $p = 0.020$
$B220^+$ $p = 0.020$

Graph B stats

CD4 vs B220 $p = 0.0122$
CD4 VS CD8 $p = 0.147$
B220 VS CD8 $p = 0.0216$

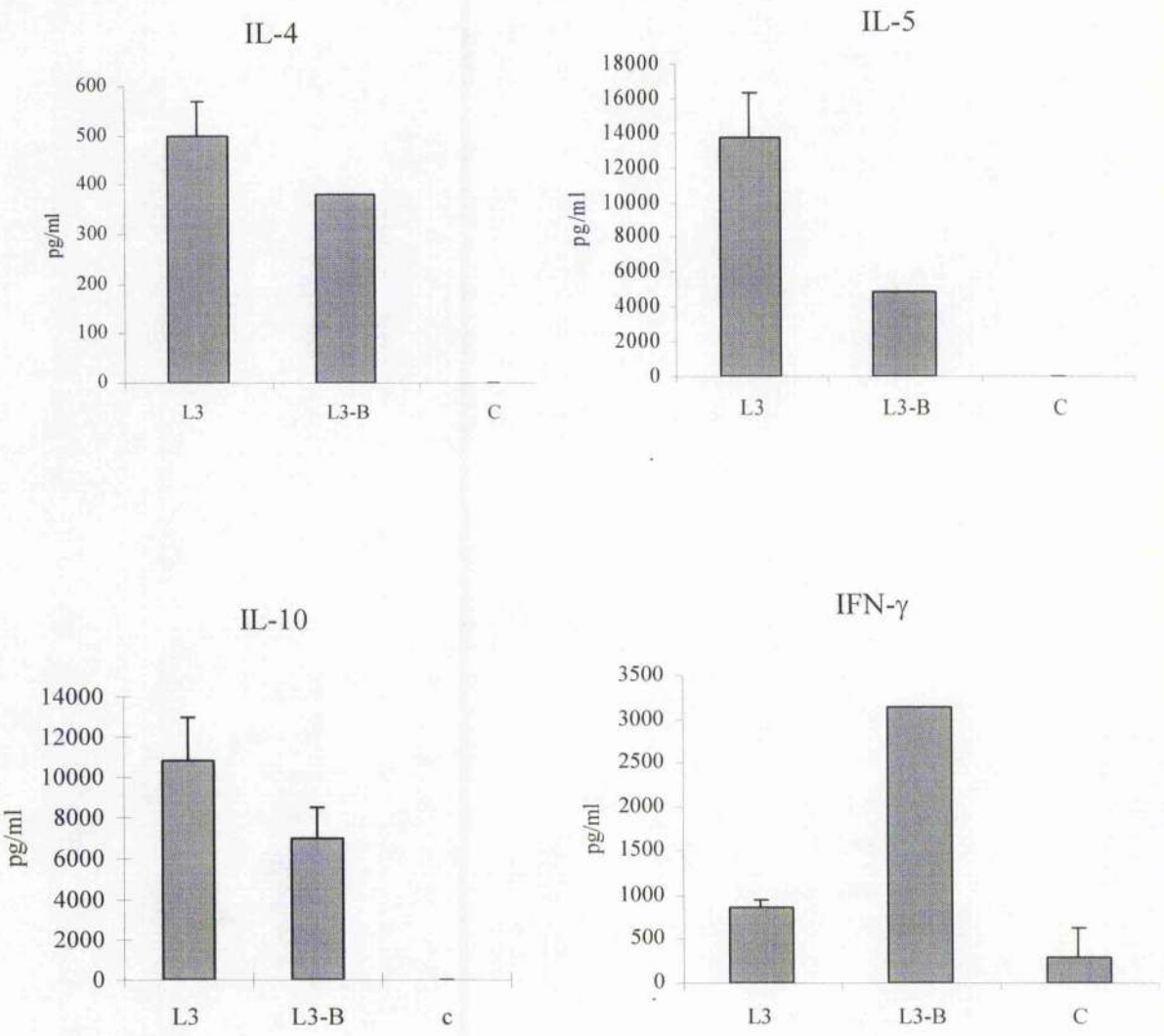


Figure 6.5. Depletion of B cells population from spleens of L3 infected mice results in decreased levels of Ag-specific IL-10 and IL-5

Mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i. splenocytes from L3 infected mice were pooled and B cells were depleted from culture using MACS anti-FITC beads and magnetic columns. The negative fraction was then re-stimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Ag-specific cytokine responses from whole splenocytes (L3) and splenocytes depleted of B cells (L3-B) were measured at 72 hours. Results are expressed as pg/ml.

	IL-4	IL-5	IL-10	IFN-γ
% change in cytokine levels post–depletion of B cells	32 ± 11.3 DECREASE (n=4)	80.5 ± 27.5 DECREASE (n=4)	53.5 ± 14.85 DECREASE (n=4)	59.5 ± 40.4 INCREASE (n=2)

Table 6.1. Changes in cytokine levels post-depletion of B cells

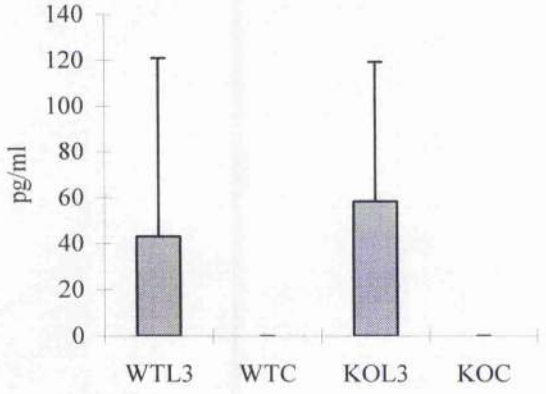
Mice were infected sub-cutaneously with 50 L3 of *B. pahangi* or an equal volume of HBSS only (C). At 12 d.p.i. splenocytes from L3 infected mice were pooled and B cells were depleted from culture using MACS anti-FITC beads and magnetic columns. The negative fraction was then re-stimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Ag-specific cytokine responses from whole splenocytes (L3) and splenocytes depleted of B cells (L3-B) were measured at 72 hours. Results are expressed a mean percentage decrease or increase in cytokine levels post-depletion of B cells in four separate experiments, except for IFN-γ which is the mean of two experiments.

Figure 6.6. Splenocytes from L3 infected μMT mice produce less IL-10 compared to those from L3 infected WT mice

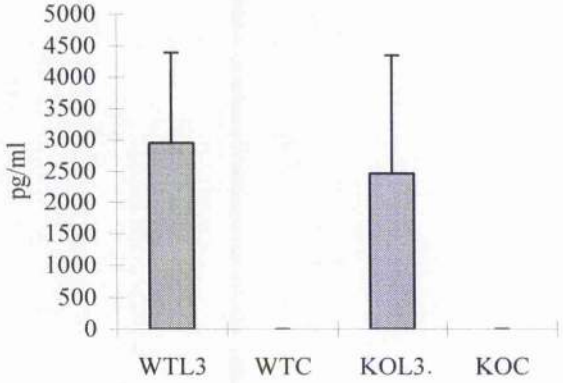
Five μMT mice (KO) (C57BL/6 background) and five WT mice (C57BL/6 background) were infected sub-cutaneously with 50 of L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i., splenocytes from individual mice from each group were restimultated *in vitro* with 10μg/ml *B. pahangi* adult Ag. Ag-specific cytokine responses from whole splenocytes were measured at 72 hours. Results show means and standard deviations of five mice per group and are expressed as pg/ml.

IL-10 – WTL3 vs KOL3 = p=0.5403
IL-4 – WTL3 vs KOL3 = p=0.4647
IL-5 – WTL3 vs KOL3 = p=0.3711

IL-4


140
120
100
80
60
40
20
0
pg/ml
WTL3
WTC
KOL3
KOC

IL-5


5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
pg/ml
WTL3
WTC
KOL3.
KOC

IL-10

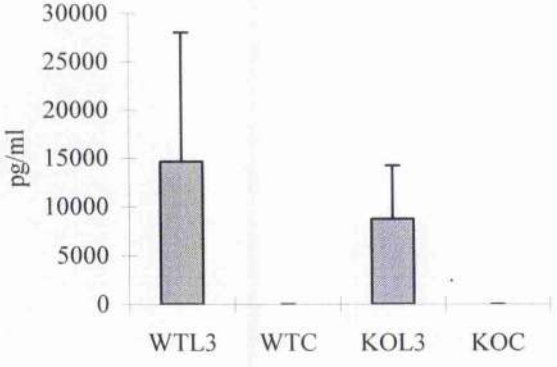
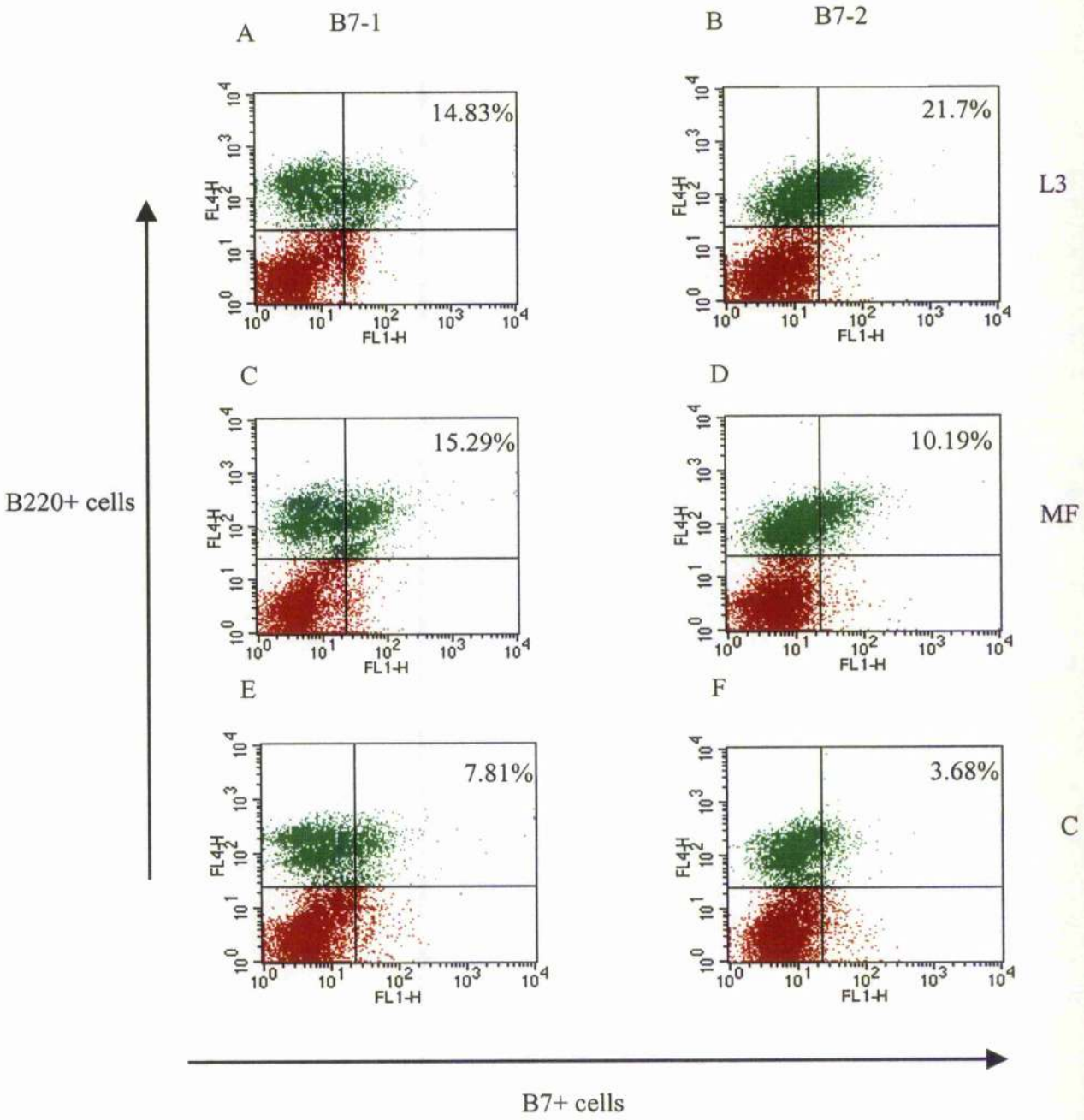

30000
25000
20000
15000
10000
5000
0
pg/ml
WTL3
WTC
KOL3
KOC

Figure 6.7. Two colour FACS labelling shows more B220$^+$ cells express B7-1 and B7-2 in L3 and MF infected mice compared to control mice

Mice were infected s.c. with 50 L3 of *B. pahangi*, or 1 X 10^5 MF or an equal volume of HBSS only. At day 12 p.i. splenocytes from L3 infected (A and B), MF infected (C and D) and HBSS control mice (E and F) were cultured with 10 μg/ml *B. pahangi* adult Ag for 72 hours. Cells were harvested and stained with anti-mouse B220 and either anti-mouse B7-1 MAb anti-mouse or B7-2 MAb (B220 - APC, B7-1/B7-2 - FITC). Cells were analysed by flow cytometry. Each panel shows the staining profile of an individual mouse from each group. The numbers at the top right hand corner of each panel indicate the percentage of splenocytes which are B220$^+$ and either B7-1$^+$ or B7-2$^+$. These figures are representative of the responses of five animals per group.


A
B7-1
B
B7-2
14.83%
21.7%
L3
C
D
15.29%
10.19%
MF
B220+ cells
E
F
7.81%
3.68%
C
FL4-H
FL1-H
B7+ cells

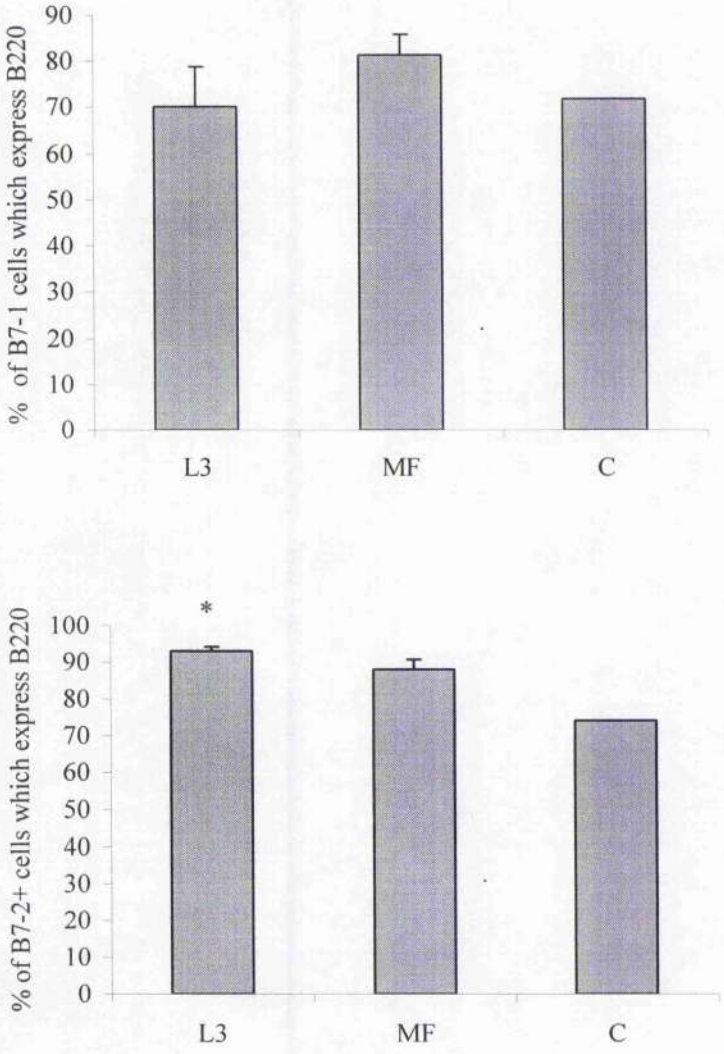


Figure 6.8. The majority of cells expressing B7-1 or B7-2 co-express B220

Mice were infected s.c. with 50 L3 of *B. pahangi*, or 1 X 10^5 MF or an equal volume of HBSS only. At day 12 p.i. splenocytes from L3 infected (A and B), MF infected (C and D) and HBSS control mice (E and F) were cultured with 10 μg/ml *B. pahangi* adult Ag for 72 hours. Cells were harvested and stained with anti-mouse B220 and either anti-mouse B7-1 MAb anti-mouse or B7-2 MAb (B220 – APC, B7-1/B7-2 - FITC). Cells were analysed by flow cytometry. Graphs depict levels of B7-1 or B7-2 expressing cells from L3 and MF infected and control animals which co-express B220.

B7-1^+ cells which co-express B220 L3 VS MF $p = 0.0601$
B7-2^+ cells which co-express B220 L3 VS MF $p = 0.0373$

Figure 6.9. The percentage of B cells which express B7-1 and B7-2 is elevated when IL-10 is neutralized

Mice were infected s.c. with 50 L3 of *B. pahangi*. At 12 d.p.i., splenocytes (1 x 10^7/ml) were re-stimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag (A and D) in the presence of 1μg/ml neutralizing IL-10 Ab (B and E) or 1μg/ml isotype matched control (C and F) for 72 hours . Cells were harvested and stained with anti-mouse B220 and either anti-mouse B7-1 MAb (A-C) or anti-mouse B7-2 MAb (D-F) (B220–APC, B7-1/B7-2 - FITC). Cells were analysed by flow cytometry. Each panel shows the staining profile of an individual mouse from each group. The numbers at the top right hand corner of each panel indicate the percentage of splenocytes which are $B220^+$ and either $B7\text{-}1^+$ or $B7\text{-}2^+$. These figures are representative of the responses of five animals per group.

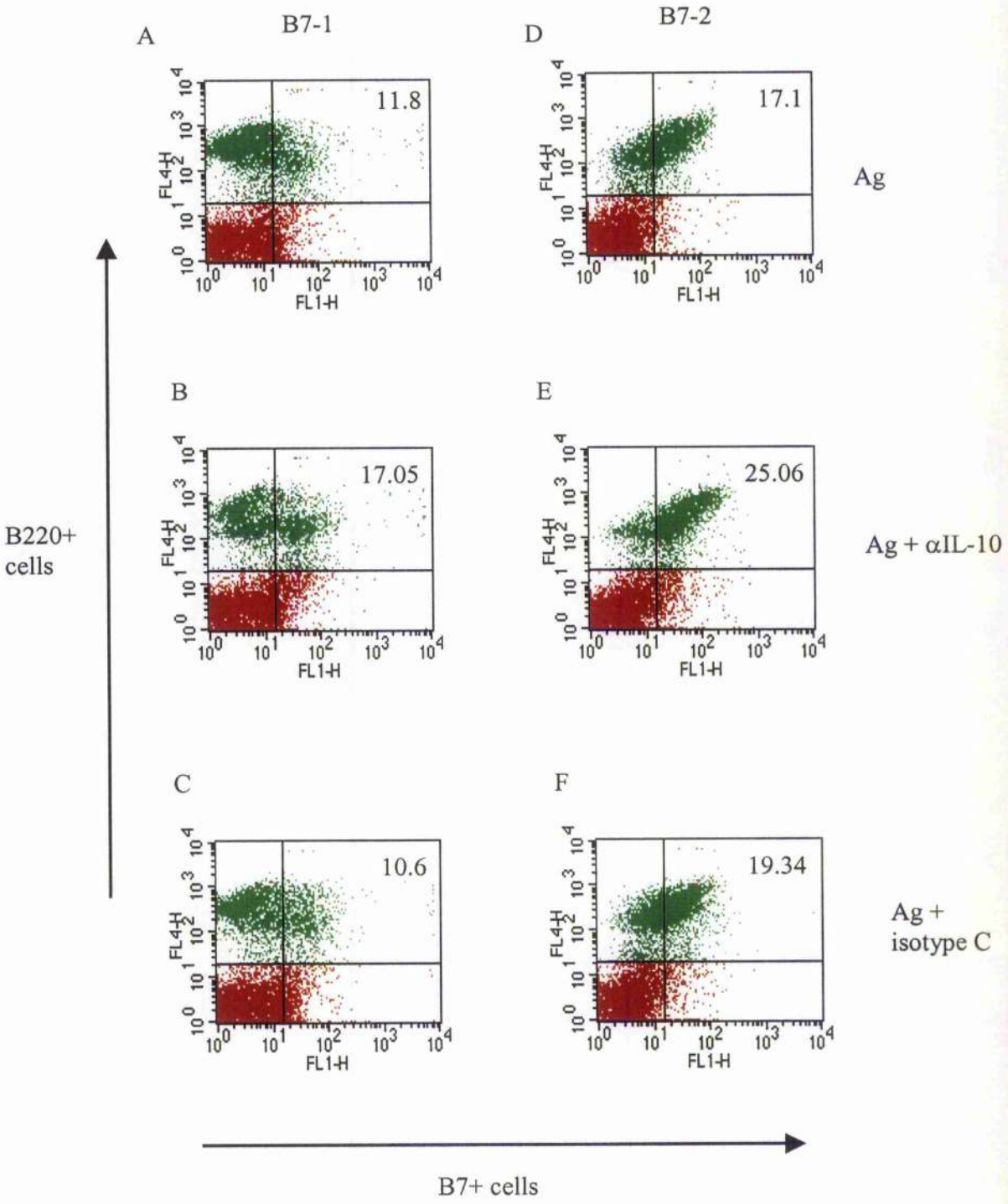

A
B7-1
D
B7-2
11.8
17.1
Ag
B
E
17.05
25.06
Ag + αIL-10
B220+ cells
C
F
10.6
19.34
Ag + isotype C
FL4-H
FL1-H
B7+ cells

Figure 6.10. Neutralizing IL-10 results in increased levels of B7-1 and B7-2 expression on splenocytes

Mice were infected s.c. with 50 L3 of *B. pahangi*. At 12 d.p.i., splenocytes (1 x 10^7/ml) were re-stimulated *in vitro* with 10μg/ml *B. pahangi* adult Ag in the presence of 1μg/ml neutralizing IL-10 Ab (αIL-10) or 1μg/ml isotype matched control for 72 hours. Cells were harvested and stained with anti-mouse B220 and either anti-mouse B7-1 Mab (A) or anti-mouse B7-2 Mab (B) (B220 – APC, B7-1/B7-2 - FITC). Cells were analysed by flow cytometry. Graphs depict the mean percentages of splenocytes which are B7-1/2 expressing $B220^+$ cells from 5 mice per group.

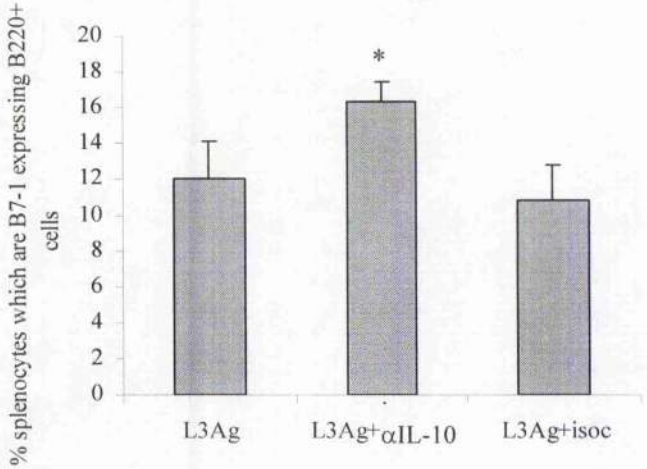
<u>B7-1 expression</u>

L3Ag vs L3AIL-10 = 0.0122
L3isoc vs L3AIL-10 = 0.0122

<u>B7-2 expression</u>

L3Ag vs L3AIL-10 = 0.0122
L3isoc vs L3AIL-10 = 0.0601

A


% splenocytes which are B7-1 expressing B220+ cells
20
18
16
14
12
10
8
6
4
2
0
*
L3Ag
L3Ag+αIL-10
L3Ag+isoc

B

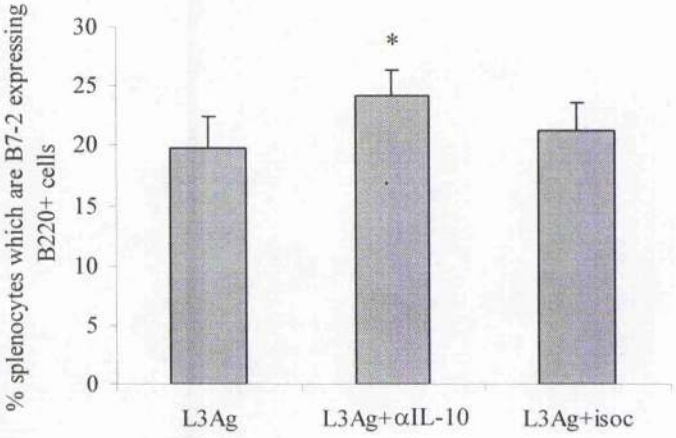

% splenocytes which are B7-2 expressing B220+ cells
30
25
20
15
10
5
0
*
L3Ag
L3Ag+αIL-10
L3Ag+isoc

Figure 6.11. The percentage of B cells which express B7-1 and B7-2 is higher in IL-10-/- mice

Wild-type (WT) and IL-10-/- mice were infected s.c. with 50 L3 of *B. pahangi*. At 12 d.p.i. splenocytes from WT (A and C) and IL-10-/- mice (B and D) were cultured with 10 μg/ml *B. pahangi* adult Ag for 72 hours. Cells were harvested and stained with anti-mouse B220 and either anti-mouse B7-1 MAb or anti-mouse B7-2 MAb (B220 – APC, B7-1/B7-2 - FITC). Cells were analysed by flow cytometry. Each panel shows the staining profile of an individual mouse from each group. The numbers at the top right hand corner of each panel indicate the percentage of splenocytes which are $B220^+$ and either $B7\text{-}1^+$ or $B7\text{-}2^+$. These figures are representative of the responses of five animals per group.

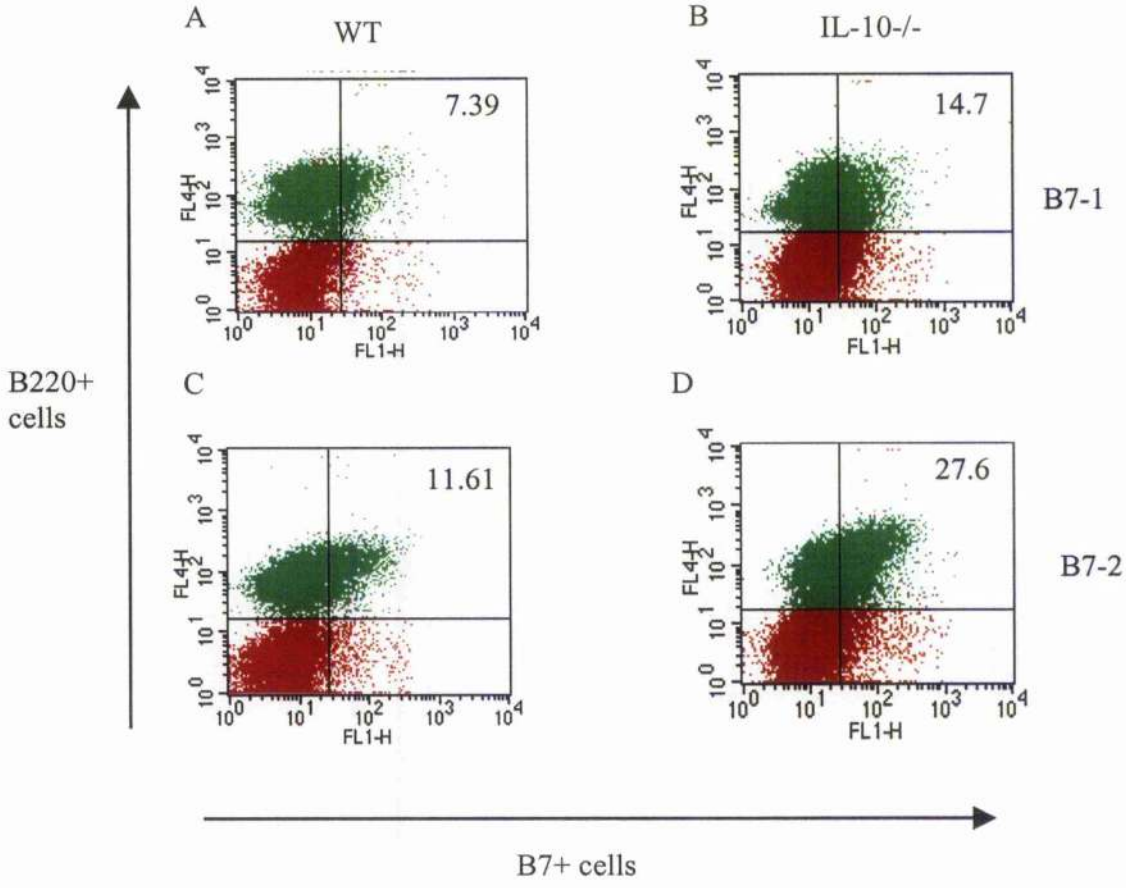

A
WT
7.39
B
IL-10-/-
14.7
B7-1
B220+
cells
C
11.61
D
27.6
B7-2
FL4-H
FL1-H
B7+ cells

Figure 6.12. B7-1 and B7-2 are up-regulated on B cells from IL-10-/- mice

Wild-type (WT) and IL-10-/- (KO) mice were infected s.c. with 50 L3 of *B. pahangi*. At day 12 p.i. splenocytes from WT and IL-10-/- mice were cultured with 10 μg/ml *B. pahangi* adult Ag for 72 hours. Cells were harvested and stained with anti-mouse B220 and either anti-mouse B7-1 MAb (A) or anti-mouse B7-2 MAb (B) (B220 – APC, B7-1/B7-2 - FITC). Cells were analysed by flow cytometry. Graphs depict the mean percentages of splenocytes which are B7-1/2 expressing $B220^+$ cells from 5 mice per group.

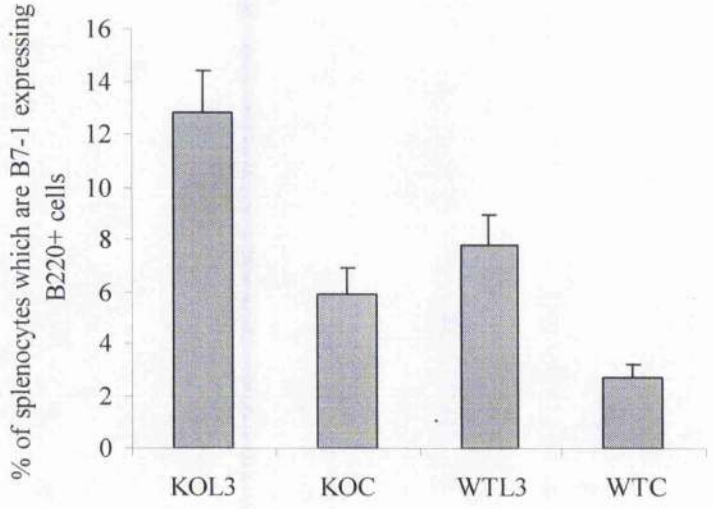
B7-1 expression

KOL3 vs WTL3 = 0.0122
KOL3 vs KOC = 0.02
WTL3 vs WTC = 0.0814

B7-2 expression

KOL3 vs WTL3 = 0.0122
KOL3 vs KOC = 0.0369
WTL3 vs WTC = 0.02

A


% of splenocytes which are B7-1 expressing B220+ cells
16
14
12
10
8
6
4
2
0
KOL3
KOC
WTL3
WTC

B

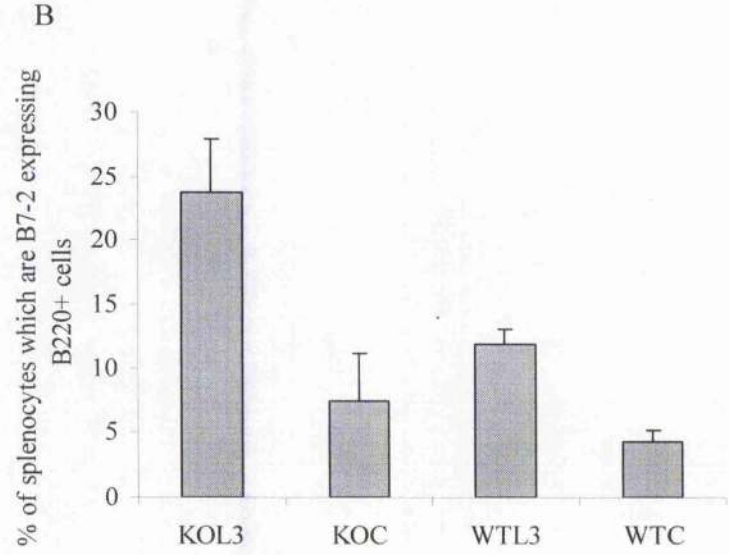
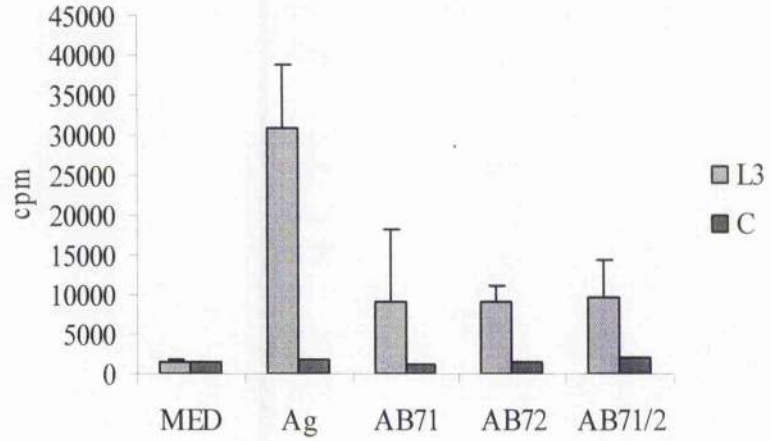

% of splenocytes which are B7-2 expressing B220+ cells
30
25
20
15
10
5
0
KOL3
KOC
WTL3
WTC

Figure 6.13. Neutralizing B7-1 and B7-2 in cultures of splenocytes from L3 infected mice results in significantly reduced levels of proliferation at 48 and 72 hours

Mice were infected sub-cutaneously with 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i., splenocytes were re-stimulated *in vitro* with 10μg/ml *B. pahangi* adult Ag. Ag-specific proliferative responses were measured at 48 (A) and 72 (B) hours in the presence or absence of 1μg/ml neutralizing B7-1 (AB71) or/and B7-2 (AB72). Results are expressed as cpm and all values represent the mean and standard deviation of five mice per group.

48 hours	72 hours
Ag vs AB7-1 = 0.0304	Ag vs AB7-1 = 0.67
Ag vs AB7-2 = 0.0304	Ag vs AB7-2 = 0.0217
Ag vs B71/B72 = 0.0304	Ag vs B71/B72 = 0.0601

A


48 hours
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
cpm
L3
C
MED
Ag
AB71
AB72
AB71/2

B

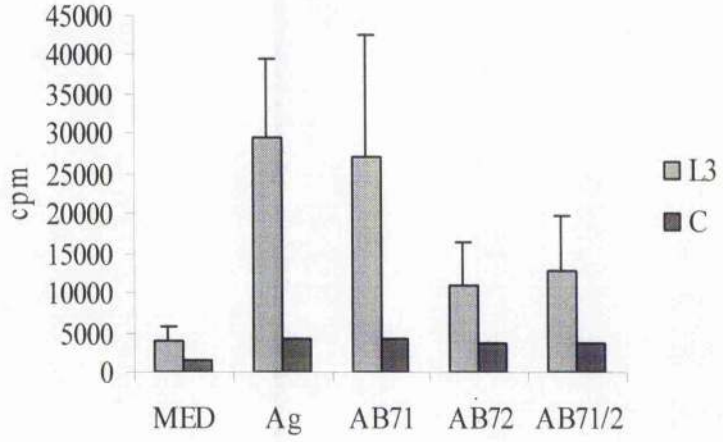

72 hours
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
cpm
L3
C
MED
Ag
AB71
AB72
AB71/2

Figure 6.14. Neutralizing B7-1 and B7-2 in cultures of splenocytes from L3 infected mice results in significantly reduced levels of specific cytokines

Mice were infected sub-cutaneously with 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i., splenocytes (1 x 10^7/ml) were re-stimulated *in vitro* with 10μg/ml *B. pahangi* adult Ag. Ag-specific cytokine responses were measured at 72 hours in the presence or absence of 1μg/ml neutralizing B7-1 (AB71) or/and B7-2 (AB72). Results are expressed as pg/ml and all values represent the mean and standard deviation of five mice per group.

IL-5

Ag vs B7-1 = 0.0606
Ag vs B7-2 = 0.0304
Ag vs B7-1/2 = 0.0302

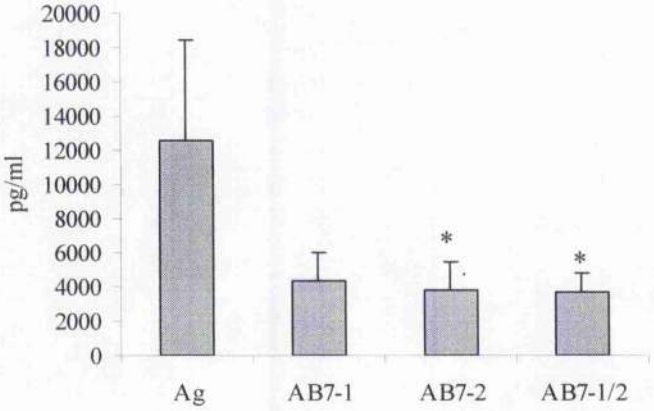
IL-4

Ag vs B7-2 = 0.2101
Ag vs B7-1 = 0.4
Ag vs B7-1/2 = 0.02

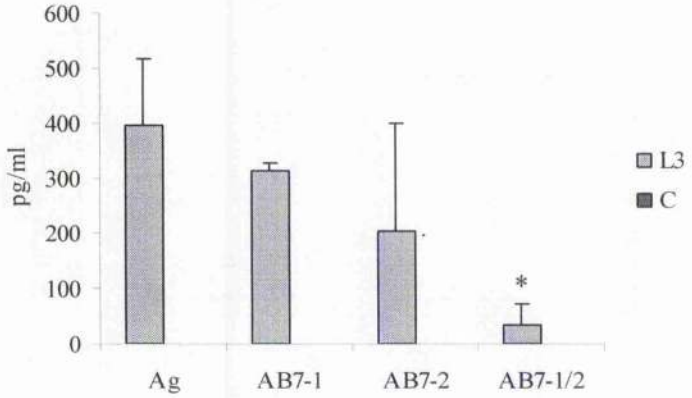
IL-10

Ag vs B7-1 = 0.2101
Ag vs B7-2 = 0.2101
Ag vs B7-1/2 = 0.0373

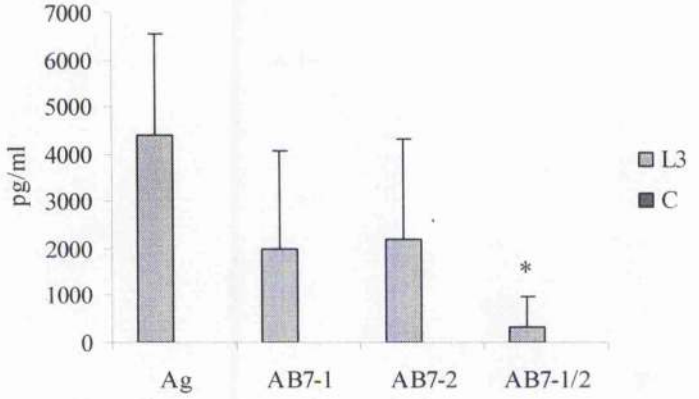
IL-5


20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
pg/ml
*
*
Ag
AB7-1
AB7-2
AB7-1/2

IL-4


600
500
400
300
200
100
0
pg/ml
*
L3
C
Ag
AB7-1
AB7-2
AB7-1/2

IL-10


7000
6000
5000
4000
3000
2000
1000
0
pg/ml
*
L3
C
Ag
AB7-1
AB7-2
AB7-1/2

6.3. Discussion

The results presented in this chapter indicate that B cells play an important role in the development of immune responses in mice infected with L3 of *B. pahangi* via the ability to proliferate, to act as APC and to produce cytokines. Infection with *Brugia* L3 results in the up-regulation of Th2 cytokines and good proliferative responses at day 12-post infection. The contribution of B cells to this response was investigated by either depleting B cells from culture or by carrying out experiments in B cell deficient mice (μMT mice on the C57BL/6 background). The results of depletion experiments indicated that B cells were important in the development of good proliferative responses in the spleen of infected animals, as there was a 62% reduction in proliferation post-depletion. *In vivo* experiments in μMT mice gave similar results, in that the proliferative capacity of splenocytes from L3 infected μMT mice was significantly lower than that of WT mice. However, these experiments do not identify the precise role of B cells, as removal of one population of cells may have dramatic effects on the ability of another population to expand. For example, B cells may proliferate in response to the L3 but may also act as APC for T cells, as described in other model systems (Noorchashm *et al.*, 1997; Silveira *et al.*, 2002). Therefore, cells from L3 infected and control animals were labelled with CFSE and cultured for 96 hours to track proliferation of dividing cells. Data from these experiments revealed that B cells were in fact the major splenocyte population dividing in response to Ag, confirming previous experiments in IL-4-/- mice (Chapter 3). Therefore, one explanation for the decrease in proliferation following depletion or in μMT mice is the lack of B cell expansion.

Similar to this situation, studies on the role of B cells in *S. mansoni* infected mice have shown that splenic $B220^{+}$, $CD4^{-}$, $CD8^{-}$ (B cells) proliferate in response to a sugar found

on soluble egg antigen (SEA) named lacto-N-fucopentaose (LNFPIII) which contains the trisaccharide Lewis^x Ag. In addition, LNFPIII induced splenic B cells to produce IL-10 (Velupillai and Harn, 1994). Whether the L3 of *B. pahangi* contain similar molecules that induce B cells to proliferate and produce cytokines is not known. Further experiments in murine *S. mansoni* infection examined the role of B-1 cells ($CD5^+B220^+$) from the peritoneum of infected animals. These cells are autoreactive, have been shown to produce large amounts of IL-10 and are typically found in the peritoneal and plural cavities but not in the spleen (O'Garra *et al.*, 1992). Exposure to SEA or LNFPIII resulted in elevated numbers of B-1 cells in susceptible hosts (CBA/J, C3H/HeJ and BALB/c). These studies also identified a role for IL-12, IL-10 and IFN-γ in regulating the expansion of B-1 cells (Velupillai *et al.*, 1996). Although the focus of these studies was peritoneal B-1 cells, it was also observed that coincident with the decline in B-1 expansion observed 5 to 6 weeks post-infection, splenic $CD23^+B220^+$ cells increased from 11% to 30% of total splenocytes. These results indicate that the expansion of B-1 cells at 2 to 3 weeks post infection may initiate the down-regulation of Th1 responses and that the continued release of IL-10 by splenic B cells during the acute phase of infection may continue to polarize the immune response towards a Th2 phenotype (Velupillai *et al.*, 1997). Therefore, in that model system B cells are expanding and also producing Th2 enhancing cytokines.

These results are similar to data presented in this Chapter, in that B cells proliferate in response to infection with the L3 of *B. pahangi* and also contribute to the production of cytokines such as IL-4, IL-5 and IL-10. In the absence of the splenic B cell population, levels of IL-5 and IL-10 were dramatically reduced, while a more modest effect on IL-4 levels was observed. One interpretation of these results is that B cells are affecting the

ability of T cells to produce Th2 cytokines, an observation supported by the production of IFN-γ in the absence of these suppressive Th2 cytokines. These results indicate the importance of B cells in maintaining a Th2 type environment in WT mice. An alternative explanation is that B cells themselves are a source of Th2 cytokines, as demonstrated for IL-10 in Chapter 4. Additional studies will be required to address this point using either TaqMan RT-PCR or ELISPOT. In this study, an interesting correlation was observed between the strength of the IL-10 response and the production of IFN-γ post-depletion of B cells. For example, in the experiment shown in Figure 6.5 levels of IL-10 were reduced to approximately 6400 pg/ml post-depletion of B cells. In the other experiment where IFN-γ was detected, levels of IL-10 were reduced to 4000 pg/ml following B cell depletion. However, in the experiments where IFN-γ was not detected following the removal of B cells, IL-10 levels were much higher, (approximately 20,000 pg/ml post depletion). Thus, it is possible that the intensity of the IL-10 response determines whether IFN-γ is produced or not. The differences in IL-10 levels between experiments may relate to the viability of L3 used or the differences in the batch of Ag used to re-stimulate the splenocytes.

Interestingly, in L3 infected μMT mice there were no significant differences in cytokine production compared to WT animals. Presumably *in vivo* in μMT mice, other cell types compensate for the lack of B cells by acting as APC for $CD4^+$ cells. It is notable that levels of IL-10 and IL-5 were lower in L3 infected μMT mice than in WT mice, although these differences did not reach statistical significance. In addition, no IFN-γ was detected in the KO animals as was observed in some B cell depletion experiments. However, these results are complicated by the fact that the μMT mice available were on the C57BL/6 background while all other experiments used BALB/c

mice. Ideally, further experiments would be carried out using KO mice on the BALB/c background. Although the removal of B cells results in a decrease in Ag-specific Th2 responses, L3 are still capable of inducing a Th2 response in infected μMT mice, reinforcing the concept that this life cycle stage is important in driving the T helper bias.

The results of this present study are in contrast to the situation observed with *T. muris* infection of μMT mice (Blackwell and Else, 2001). These animals were permissive to infection, producing significantly less IL-4 and IL-5 compared to resistant WT mice. In addition *T. muris* infected μMT mice produced increased levels of IFN-γ compared to WT mice indicating that these mice mount a Th1 response in the absence of B cells, thus conferring susceptibility. The importance of B cells in this model system was further confirmed when it was shown that reconstitution with B cells from WT mice restores resistance to *T. muris,* with elevated levels of IL-4 and IL-5.

B cells can modulate the host response to parasitic infection, particularly via their capacity to produce IL-10. Additionally, B cells may also enhance the production of IL-10 and Th2 cytokines from other cell types while down-regulating potentially harmful Th1 responses. The role of B cells has been investigated in other parasitic infections using Xid mice, which are deficient in B-1 cells. Lack of B-1 cells and B-cell derived IL-10 in mice carrying the Xid immunodeficiency was associated with reduced Th2-induced granuloma formation in *S. mansoni* infection (Velupillai *et al.*, 1997) and lowered susceptibility to *L. major* (Hoerauf *et al.*, 1994). The Xid mouse has also been used in studies on *L. sigmodontis*. Worm development in susceptible BALB/c mice is controlled by $CD4^+$ cells and worm loads correlate negatively with Th2 cytokine production. BALB/c.Xid mice were shown to be more susceptible than WT mice to L3

of *L. sigmodontis* with a lower Th2 response, which was attributed to the decrease in IL-10 from B cells (Al-Qaoud *et al.*, 1998). These studies further support the hypothesis that B cells are a source of IL-10 in many parasite systems.

One way in which IL-10 modulates the immune response is via the regulation of B7-1 and B7-2 on the surface of APC. The up-regulation of these ligands on the surface of B cells post-infection with L3 implies that B cells act as APC in this model. As the ligand for CD28 and CTLA-4, the level of expression of B7-1 and B7-2 on APC is an important determinant of T cell activation. FACS analysis of splenic B cells illustrated that infection with L3 resulted in a significant up-regulation in levels of both B7-2 and B7-1 compared to control animals. In addition there was a significant increase in the percentage of $B220^+$ cells that express B7-2 in L3 infected mice compared to that of MF infected mice. Up-regulation of B7-2 is consistent with the observation that L3 elicit a strong proliferative response and induce Th2 like cytokines in the mouse model. However in MF infection reduced capacity to up-regulate B7-2 may partially account for reduced levels of proliferation observed in splenocytes from MF infected animals. However, expression of B7-1 on the surface of $B220^+$ cells from MF infected mice was equal to that of L3 infected mice. Co-stimulation via B7-1 has been shown to be associated with up-regulation of Th1 responses (Freeman *et al.*, 1995; Kuchroo *et al.*, 1995), therefore this may be a contributing factor to the production of IFN-γ in cultures from MF infected mice. Co-stimulation via B7-2 in L3 infected mice may be a strong enough stimulus to promote a Th2 over a Th1 response.

The role of IL-10 in modulating expression of B7-1 and B7-1 on the surface of B cells from L3 infected mice was first examined using a neutralizing IL-10 MAb *in vitro*. It

was found that neutralizing IL-10 resulted in significant up-regulation of both B7-1 and B7-2. These results were further substantiated in a single experiment in which IL-10-/- mice on the C57BL/6 background were infected with L3 of *B. pahangi*. B cells from IL-10-/- animals exhibited elevated levels of both B7-1 and B7-2 compared to those of their WT counterparts. These data confirm previous findings that IL-10 regulates the ability of cells to proliferate via its effect on APC. Therefore it is possible that B cells act in a negative feedback mechanism to restrict Th2 expansion in mice infected with L3 of *Brugia*. B cells produce IL-10, which in turn modifies the expression of surface costimulatory molecules, thus attenuating their own efficiency in presenting Ag to T cells. This may be a further important mechanism of regulating Th2 responses in *Brugia* infection.

To further clarify the role of B7-1 and B7-2 in L3 infection, these molecules were neutralized *in vitro* and cytokine and proliferation assays were carried out. When B7-1 and B7-2 were neutralized together there was a significant reduction in proliferation of splenocytes from L3 infected mice at 48 and 72 hours. The effects of neutralizing B7-1 and B7-2 individually also gave interesting results. When B7-1 was neutralized alone, proliferation was only significantly reduced at 48 hours and levels recovered after this time point. However, when B7-2 was neutralized levels of proliferation were significantly inhibited at 48 and 72 hours. It has been reported previously that B7-1 and B7-2 deliver different costimulatory signals. Stimulation of naïve T cells with B7-2 results in a more Th2 like response with the production of IL-4, by comparison to stimulation with B7-1 (Freeman *et al.*, 1995). With this in mind it is probable that neutralizing B7-2 will have a greater effect on levels of proliferation in response to the L3 as this life cycle stage induces Th2 cells. However the results of these experiments

suggest that B7-1 does play an important role in the co-stimulation of T cells, at least at 48 hours of culture, as a reduction in proliferation was observed at this time point. Cytokine responses in these cultures were also examined and levels of IL-5, IL-4 and IL-10 were significantly reduced when B7-1 and B7-2 were neutralized simultaneously. Only IL-5 was reduced significantly when B7-2 was neutralized alone, whilst neutralizing B7-1 had no significant effect on any of the cytokines measured (although levels of IL-5 were reduced, this did not reach statistical significance). As there was no concurrent increase in the levels of Th1 cytokines, it is likely that the reduction in cytokine levels reflects lower levels of proliferation in these cultures.

The results presented in this chapter highlight the importance of B cells in filarial infection, in terms of the expression of costimulatory molecule (i.e. acting as APC), producing anti-inflammatory cytokines and proliferating in response to infection. While results of the depletion experiments indicate that B cells provide help for Th2 cells, μMT mice also generate a Th2 response. These results indicate that B cells are not essential for Th2 generation and provide further evidence for the strong Th2 priming nature of the L3.

Chapter 7. General Discussion

Lymphatic filarial worms have an extraordinary ability to modulate host immune responses. These long-lived helminth parasites down-regulate pro-inflammatory immune responses (Maizels *et al.*, 2000), in a manner which protects the parasite from being eliminated, but in turn, minimizes severe pathology in the host. PBMC from infected individuals display a skewing of the immune response, secreting IL-4 and IL-10 in favour of inflammatory Th1 cytokines. IL-4 is the prototype Th2 cytokine and IL-10, although produced by many cells types, has recently been shown to be produced by Treg cells (Groux *et al.*, 1997; Foussat *et al.*, 2003; Battaglia and Roncarolo, 2004). In fact, in some studies, neutralizing IL-10 or TGF-β in cultures of PBMC from infected individuals resulted in elevated levels of proliferation (King *et al.*, 1993). Therefore, what was once thought to be a skewing of the immune response towards a Th2 phenotype, is also likely to involve the expansion of regulatory cells; together these mechanisms will result in the down-regulation Th1 responses and suppression of Ag-specific proliferation in infected individuals. However Treg cells suppress both Th1 and Th2 responses and it is equally likely that Treg cells in lymphatic filariasis also function to suppress over-expansion of Th2 cells, which can also be pathogenic. In endemic areas some degree of proliferative suppression occurs in each clinical group, even EN and those who have cleared their parasites (Yazdanbakhsh *et al.*, 1993a). This suggests that the L3 may be the cause of this suppression, as all groups are constantly exposed to incoming infective larvae. In addition, proliferative suppression has been linked to intensity of L3 transmission, lending further support to the theory that the L3 is a highly immunoregulatory life cycle stage (King, 2001).

Results described in this thesis confirm that the L3 of *B. pahangi* is a potent modulator of immune responses in the mouse model. Previous experiments have shown that IL-4 mRNA transcripts can be detected in draining lymph nodes of mice at 24 hour post-infection with L3 (Osborne and Devaney, 1998). In the current study, it was demonstrated that IL-4 was essential for down-regulating Th1 responses, as elevated levels of IL-2 and IFN-γ were detected in splenocytes from L3 infected IL-4-/- mice. In addition, levels of Ag-specific proliferation were significantly reduced, with B cell and $CD4^+$ expansion inhibited in the absence of IL-4. These results demonstrate that in WT mice, L3-driven IL-4 has a major effect on Th1 cells. Extrapolation of these results to humans would suggest that infection with L3 may lead to the development of IL-4 producing cells prior to the establishment of patent adults. Interestingly, in areas of high transmission of *W. bancrofti*, plasma IL-4 increases but PBMC derived IL-4 does not, indicating that IL-4 may be produced by cells other than T cells. In fact, it was shown that basophils preferentially respond to L3 extracts with IL-4 secretion (King *et al.*, 2001). As these cells are present in the dermis, events in the skin immediately following infection may be an important determinant of subsequent responses. The importance of early events in the skin was well documented in a study by Hogg *et al*, who demonstrated that elevated levels of IL-12 were detected in skin-draining lymph nodes following immunization with irradiated cercariae of *S. mansoni*. In that study, IL-10 was implicated in the development of immunity, as challenge infection with irradiated parasites resulted in lower levels of IL-10. IL-10 was also shown to have an important role in controlling inflammation at the infection site as IL-10-/- mice displayed a thickening of the dermis with elevated cellular infiltration to the site. Thus, production of early IL-10 at the site of infection controls Th1 events which lead to immunity (Hogg *et al.*, 2003). In this study, immune responses were compared in the draining lymph

node and spleen of mice infected by mosquito transmission or syringe inoculation of L3. No major effect was observed, although it was notable that lymphocytes from mice that had been infected via mosquito transmission were generally less responsive to Ag restimulation. However, whether mosquito transmission of L3 is immunologically "silent" or whether mosquito saliva contains immunomodulatory factors, has yet to be determined. In light of work carried out in the schistosome model, future studies should attempt to investigate cytokines and cell types which are present at the site of infection.

Although IL-4-/- mice showed a dramatic change in the immune response to the L3, there was no difference in worm recovery compared to WT mice . However, there was a significant difference in the levels of MF produced by adult female worms in IL-4-/- mice. Therefore, IL-4, or IL-4 driven responses, appears to affect the fecundity of the adult worm. It is interesting to consider whether IL-4 may have a similar effect in humans. Although there are no studies that have indicated a direct link between MF levels and IL-4, a seasonal study of infected individuals showed that MF density decreased in April to July and increased in July to November. IFN-γ levels appeared to be associated with low parasite density, but a small increase in IL-4 levels was noted between July and November in MF+ subjects. These results are difficult to interpret as the numbers of individuals studied was very small and there are known differences in mosquito biting rates (and presumably transmission of L3) in wet and dry areas (Sartono *et al.*, 1999).

Along with IL-4, IL-10 has been implicated in the control of Th1 responses (Moore *et al.*, 2001). As infection of mice with L3 of *Brugia* leads to elevated levels of IL-10 with diminished Th1 responses, experiments were carried out to determine the role of this

inhibitory cytokine. Results from these experiments indicated an important role for IL-10 in down-regulating Th1 responses and in addition, when IL-10R was blocked *in vivo*, levels of proliferation were significantly enhanced. This increase in proliferation was found to be $CD4^+$ specific, as levels of B cell proliferation were unaffected. These results are similar to those observed using PBMC from infected humans where spontaneous and Ag-driven levels of IL-10 correlate negatively with levels of T cell proliferation (Mahanty *et al.*, 1996b). Additionally, neutralizing IL-10 resulted in a significant elevation in levels of lymphocyte proliferation to filarial Ag in MF infected humans (King *et al.*, 1993). In the infected human, MF Ag has been implicated in the production of IL-10, but the data presented in this thesis suggest that the L3 is also an important stage for the induction of an IL-10 response. Although infected humans (who harbour all life cycle stages) have been shown to express high levels of both spontaneous and Ag-specific IL-10, it is very difficult to study the human disease in terms of stage-specific responses and this is where the laboratory animal is extremely useful. While long-lived adult parasites or MF probably have a role in maintaining the production of IL-10, which ultimately suppresses damaging Th1 responses and T cell proliferation, the initial life cycle stage which induces IL-10 may be the L3.

In the past, immune responses in lymphatic filariasis used the Th1/Th2 dichotomy to explain the down-regulation of IFN-γ and proliferation associated with active infection. However, further studies indicated that lymphatic filariasis did not fit neatly into the Th1/Th2 cross-regulation observed in inbred mouse models of parasite infection (Allen and Maizels, 1997). For example, while IFN- γ is clearly suppressed, so is IL-5, suggesting that both Th1 and Th2 responses are modulated over the course of infection (Sartono *et al.*, 1997). Thus, additional mechanisms of suppression have been the focus

of recent studies. IL-10 has long been known to be produced by a variety of cell types (Fiorentino *et al.*, 1989). However the concept of Treg cells has been the focus of many recent studies. The suppressive activity of at least two of the Treg subsets (Th3 and Tr1 cells) is associated with IL-10 and TGF-β, while the role of cytokines is controversial in the mode of action of the $CD4^+CD25^+$subset. These cells are thought to function via a cell-cell contact dependant mechanism, which may involve surface bound TGF-β or CTLA-4. The notion that Treg cells may be elicited in filarial infection is not surprising as the generation of a suppressive cell type could explain immune responses in infected individuals. For example, the induction of IL-10 and TGF-β, proliferative hyporesponsiveness, down-regulation of IFN-γ and IL-5 responses and constant exposure of T cells to parasite Ag all support the hypothesis that filarial worms may induce Treg cells. The first formal proof of a Treg population in filarial infection came with the cloning of T cells from PBMC of *O. volvulus* infected individuals. These T cell clones had a unique cytokine profile consistent with Tr1 cells (no IL-2 but high IL-10 and/or TGF-β production) (Doetze *et al.*, 2000). In addition, $CD4^+CD25^+CTLA\text{-}4^+$ cells have been shown to be important in maintaining the Th1/Th2 balance in *W. bancrofti* individuals (Steel and Nutman, 2003). As these are chronic infections of humans it might be expected that the long-lived adult worms would induce such a population. Therefore, it was somewhat surprising during the course of these experiments in L3 infected mice, to identify a population of $CD4^+$ cells which co-express $CD25^+$ and $CTLA\text{-}4^+$, suggesting that regulatory T cells are being generated in response to the L3. These cells were not observed in MF infected animals or control uninfected animals suggesting that, at least in the mouse, this is an L3-specific effect. In additional experiments, adult worms were implanted into the peritoneal cavity of mice, but this did not result in the development of these cells in the spleen (data not shown).

This observation may be due to the fact that peritoneal implantation does not result in vigorous immune responses in the spleen. However, $CD4^+CD25^+CTLA4^+$ cells were elicited in L3 infected IL-4-/- mice and these mice also had the ability to express IL-10 and TGF-β, demonstrating that these cytokines are being produced via an IL-4 independent pathway. Although the development of Treg cells may be associated with chronic infection and long-term exposure to Ag, these experiments demonstrate just how potent the effects of infection with the L3 can be. The profound immunomodulatory effects of the L3 are also evident in infected humans as demonstrated in studies in PNG by King *et al* (2001), who compared immune responses of individuals from villages with different levels of L3 transmission. Immune responses were suppressed in individuals from the high transmission village, even when MF and CFA levels were equivalent, indicating an L3–specific effect. Levels of proliferation of PBMC from individuals from the high transmission village were 11-fold less than that of those from the low transmission village. A degree of "spill over" was also observed, as PBMC from individuals in the area of high transmission also had a reduced capacity to proliferate in response to non-parasite Ag and PHA.

Experiments described in this thesis attempted to demonstrate the regulatory function of the $CD4^+CD25^+$ CTLA-4^+ cells by depletion with an anti-CD25 MAb (PC61). PC61 treatment resulted in a 50% reduction in the expansion of $CD4^+CD25^+CTLA\text{-}4^+$ T cells from L3 infected mice and Ag-specific and spontaneous cytokine and proliferative responses were increased in these cultures. Further studies are required to phenotype the cells that expand in the absence of Tregs cells, but these are likely to be T cells (Sakaguchi *et al.*, 1995). Interestingly, when the Treg population was reduced, elevated levels of IL-10 were observed. However the depletion of these cells appeared to

override any of the effects of IL-10 described previously, i.e. down-regulating Th1 responses and proliferation. These data therefore implicate a Treg population in the control of immune responses to the L3 and gives rise to an interesting question. Are these T cells naturally occurring and non-specific or is there a population of Treg cells which can develop into Ag-specific T cells after exposure to L3? Other studies on the role of Treg cells in bacterial (McGuirk and Mills, 2002) and protozoan infection (Belkaid *et al.*, 2002) have also addressed this point, with Belkaid *at al* defining a population of *L. major* Ag-specific $CD4^{+}CD25^{+}$ Treg cells at the site of infection (Belkaid *et al.*, 2002). In addition, parasites may have the ability to mediate the conversion of naïve T cells to Treg-like cells by mechanisms, such as that described by van der Kleij *et al.* In that study DC were shown to induce IL-10 producing Treg cells when matured in the presence of a lipid fraction from *S. mansoni* eggs (van der Kleij *et al.*, 2002). Further analysis is clearly required of parasite molecules, which drive this response, along with more precise characterization of Treg cells.

Other experiments in this thesis aimed to examine to role of B cells in filarial infection, an area which has been neglected until recently. Both macrophages and DC have been implicated in driving the characteristic Th2 response elicited by helminth infection. For example work by the Allen lab has described the development of alternatively activated macrophages in the peritoneum of C57BL/6 mice infected with L3 or adult worms of *B. malayi*. These cells were shown to be IL-10 independent and IL-4 dependent, but have not yet been identified in the spleen or in the BALB/c mouse (MacDonald *et al.*, 1998; Allen and Loke, 2001). Alternatively activated macrophages suppress the proliferation of T cell clones and non-lymphoid cells by a cell-contact dependent mechanism (Loke *et al.*, 2000b). DC have also been shown to be important in the generation of a Th2

response, as demonstrated by studies using the PC bearing ES62 secreted by *A. vitae* (Goodridge *et al.*, 2001). However, as B cells have been shown to be a source IL-10 preferentially diverting naïve T cells towards the Th2 pathway (Gajewski *et al.*, 1991), experiments were carried out to examine the role of these cells in L3 infection. B cells were shown to be the major proliferating population in splenocyte cultures from L3 infected mice and were shown to be an important source of IL-10. Depletion of B cells also resulted in diminished levels of IL-4 and IL-5, and interestingly in two out of four experiments, levels of IFN-γ were increased, suggesting that B cells preferentially inhibit Th1 responses by affecting Th2 expansion, perhaps through the production of IL-10 or via effects on T cells during Ag presentation. To investigate the latter, the expression of costimulatory molecules B7-1 and B7-2 were measured on the B cell surface. Firstly it was shown that levels of B7-2 were significantly up-regulated on the surface of B cells from L3 infected animals compared to MF infected or control mice. This observation is in keeping with other studies which have demonstrated that B7-2 is important in the generation of a Th2 response. Another interesting finding in these experiments was that in L3 infected IL-10-/- mice, or when IL-10 was neutralized in cultures from L3 infected WT mice, a significant increase in B7-1 and B7-2 was observed. These results suggest that IL-10 has no preference for either molecule and demonstrates a mechanism by which IL-10 might be involved in modulating of proliferative responses in this model system. The production of IL-10 by B cells and consequent down-regulation in B7-1/2 expression, is proposed to represent a negative feedback mechanism to control $CD4^+$ responses in the mouse.

One important factor which is missing from these studies is an understanding of the component/s of the L3 which cause the characteristic skewing of the immune response.

Molecules associated with immunomodulation have been identified in *B. malayi* from the EST project (Maizels *et al.*, 2001). For example a cystatin which interferes with the MHC class II pathway, blocking Ag-presentation has been characterized (Manoury *et al.*, 2001). A similar molecule is secreted by O. *volvulus* (onchocystatin) (Schonemeyer *et al.*, 2001). *B. malayi* MF have also been shown to synthesize and release Prostaglandin E_2, and this molecule may have effects on macrophages and DC (Liu *et al.*, 1992). Therefore it would be of considerable interest to investigate whether molecules secreted by the L3 of *B. pahangi*, are involved in eliciting a population of Treg-like cells, or inducing the production of high levels of IL-4, TGF-β and IL-10.

The results presented in this thesis have identified novel mechanisms by which the L3 modulate immune responses, particularly with respect to B cell expansion and the development of a Treg–like population. These data confirm that the L3 use a multiplicity of mechanisms to influence the host immune response. However it is important to remember that the L3 can also induce protective immunity in both mice and man, suggesting that under the appropriate conditions the immune response can override the modulatory effects of the L3. With the identification of novel molecules which stimulate protective immunity (Gregory *et al.*, 2000), the challenge for the future will be to dissect the mechanistic basis by which the host–parasite interaction results in a balance between regulatory and effector functions.

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