MICROFILARIAE SPECIFIC MECHANISMS OF IMMUNOMODULATION IN A MOUSE MODEL OF FILARIASIS

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Declaration

The studies described in this thesis were carried out in the Department of Veterinary Parasitology at the University of Glasgow Veterinary School between October 1997 and March 2001. The author was responsible for all the experiments carried out in this thesis except where it is stated otherwise. 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 filariasis is a long term chronic infection characterised by a Th2 dominated immune response and suppressed Ag-specific proliferation. This immunological hyporesponsiveness is most profound amongst individuals with circulating microfilariae (mf) suggesting an important role for mf in generating proliferative suppression. The use of single life cycle stage infections in murine models of filariasis has facilitated the study of stage specific mechanisms of immunomodulation. Intravenous infection of BALB/c mice with *B. pahangi* mf or L3 (the third stage larvae) leads to development of differentially polarised immune responses. At 12 d.p.i. splenocytes from L3 infected animals produce Ag-specific IL-4, IL-5 and IL-10 and show strong Ag-driven proliferative responses. In contrast splenocytes from mf-infected animals show a cytokine profile dominated by IFN-γ and suppression of Ag-specific proliferation. After 96 hrs of Ag-stimulated culture splenocytes from mf-infected animals proliferate at levels below background indicating that an active form of suppression is operable *in vitro*.

A lack of IL-2 does not account for the defective proliferative response as addition of recombinant IL-2 failed to restore Ag-specific proliferation. Splenocytes from mf-infected animals produce high levels of NO in Ag-stimulated culture which correlates inversely with their proliferative responses. No such accumulation of nitrite is seen in cultures of cells from L3 infected animals. The proliferative defect is dependent upon inducible nitric oxide synthase (iNOS) activity as inhibition of iNOS activity with either L-NMMA or AMG restored Ag-specific proliferation. Experiments in IFN-γR-/- mice demonstrated that signalling via the IFN-γ receptor is essential to the induction of NO production and subsequent proliferative suppression. Significantly elevated levels of IFN-γ were produced upon iNOS inhibition suggesting an NO mediated negative feedback mechanism limits IFN-γ production and proliferation in Ag-stimulated culture. Restimulation of splenocytes from mf-infected animals with an extract of *A. viteae,* a related filarial worm which lacks endosymbiotic bacteria, also resulted in NO production and proliferative suppression, demonstrating that lipopolysaccharide of bacterial origin is not essential to the induction of iNOS activity *in vitro*.

To assess the ability of mf to modulate a pre-established Th2 response, L3 infected mice were subsequently infected with mf at 7 days post infection and *in vitro* responses assessed 12 days later. At 19 days post initial infection splenocytes from L3 infected

animals displayed a mixed response with production of Ag-specific IFN-γ as well as IL-4, IL-5. Intriguingly secondary infection with mf significantly reduced *in vitro* IFN-γ production whilst production of IL-4 and IL-5 remained unaltered. A similar pattern of responsiveness was seen following secondary infection with L3. Although the mechanisms underlying this phenomenon remain undefined such results suggest that *in vivo* restimulation selectively down-regulates IFN-γ production.

CFSE staining allowed identification of the cellular population subject to NO mediated proliferative suppression. The majority of the proliferation in Ag-stimulated culture occurred within the $CD4^+$ population. However, $CD4^+$ T cells from mf-infected animals only display evidence of proliferation upon iNOS inhibition. In the absence of high levels of NO, expansion of a subpopulation of brightly staining $CD4^{hi}$ lymphocytes could clearly be identified. The majority of these cells had divided in Ag-stimulated culture and their division accounted almost entirely for the proliferative response within the $CD4^+$ population.

Significantly elevated levels of apoptosis were observed amongst $CD4^+$ cells from mf-infected animals in Ag-stimulated culture associated with decreased numbers of $CD4^+$ cells in the G0/G1 phase of the cell cycle. Inhibition of iNOS activity significantly reduced levels of apoptosis and led to a corresponding increase in the number of cells in G0/G1 suggesting that the IFN-γ induced NO mediated apoptosis of Ag-reactive T cells limits proliferation. Depletion experiments identified $CD4^+$ T cells from mf-infected animals as the major source of IFN-γ production in Ag-stimulated culture. These results suggest that IFN-γ producing $CD4^+$ T cells from mf-infected animals indirectly induce their own NO mediated apoptosis upon *in vitro* restimulation. Such a mechanism may serve to limit the development of potentially damaging pro-inflammatory responses by down-regulating both IFN-γ production and proliferative responses.

LIST OF ABBREVIATIONS

Ag antigen

AMG aminoguanidine

APC allophycocyanin

APC antigen presenting cell

BSA bovine serum albumin

CFA circulating filarial antigen

CFSE carboxyfluorescein diacetate succinimidyl ester

Ci Curie

CO_2 carbon dioxide

Con A concanavalin A

cpm counts per minute

ddH_20 double distilled water

d.p.i. days post infection

ELISA enzyme linked immunoabsorbent assay

EN endemic normal

FACS fluorescence activated cell sorting

FITC fluorescein isothiocyanate

FCS fetal calf serum

g gram

hr hour

HCL hydrochloric acid

iNOS inducible nitric oxide synthase

i.v. intravenous

i.p. intra-peritoneal

KCL potassium chloride

KH_2PO_4 potassium dihydrogen phosphate

l litre

L3 infective larvae

L.F. lymphatic filariasis

L-NMMA N-g-monomethyl-L-arginine

M molar

MAb monoclonal antibody

μCi micro Curie

μg microgram

mg milligram

μl microlitre

ml millilitre

μM micromolar

mM millimolar

mRNA messenger RNA

mf microfilariae

mf Ag microfilarial antigen

MF+ micfofilaraemic

MF- amicrofilaraemic

min minute

MO macrophage

NaCL sodium chloride

$NaHCO_3$ sodium bicarbonate

Na_2HPO_4 di-sodium hydrogen phosphate

NaOH sodium hydroxide

NA_4CL ammonium chloride

NO nitric oxide

NO_2^- nitrite

°C degrees centigrade

OD 620nm optical density at wavelength 620 nm

PBS phosphate buffered saline

PCR polymerase chain reaction

PEC peritoneal exudate cell

pg picogram

PI propidium iodide

rpm revolutions per minute

r recombinant

s.c. subcutaneously

SD standard deviation

SEB staphylococcal enterotoxin B

SNAP S-nitroso-amino-penacillamine

Th T helper

U unit

w/v weight per volume

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CHAPTER 1.

Introduction

Lymphatic filariasis (L.F.) is a major tropical disease affecting an estimated 128 million individuals world-wide (Micheals, 1997). With approximately 751 million people living in endemic areas, this major cause of clinical morbidity continues to present a serious impediment to socio-economic development. The causative agents of lymphatic filariasis are long-lived nematode worms of the genera *Wuchereria* and *Brugia.* Infection with *Wuchereria bancrofti* is responsible for around 91% of cases, occurring throughout the tropics and subtropics, with the exceptions of the Middle East (where infection is restricted to Egypt) and the western parts of South America. The remaining 9% of infections are caused by *Brugia malayi* and *Brugia timori* (W.H.O., 1992). Brugian filariasis is restricted to South and South East Asia, including Southern China. Infection with *B. timori* occurs in only around 40, 000 people in several islands in Eastern Indonesia (Scott, 2000).

1.1. The parasite life cycle

Filarial worms were first identified as the cause of lymphatic filariasis in the latter half of the nineteenth century. The Scottish physician Sir Patrick Manson, whilst stationed in China, first attributed the profound deforming swelling of the limbs (elephantiasis – a characteristic pathology of L.F.) to infestation with filarial parasites. All filarial worms require a period of larval development (L1 to L3) in a blood feeding insect (Scott, 2000) and Manson's observation that parasites were ingested by mosquitoes along with the blood meal represents the first evidence that arthropods act as vectors of parasitic organisms (Rajan, 2000).

In the human host the adult male and female worms inhabit the lumen of dilated lymphatics. Following insemination, the female releases many thousands of fully formed, sheathed microfilariae (mf, the first larval stage) into the lymphatic circulation. From the lymph the microfilariae find their way into the peripheral circulation. The numbers of mf circulating in the peripheral blood can fluctuate dramatically over a 24 hr period. In most *W. bancrofti* endemic areas mf are nocturnally periodic, disappearing from the circulation during the day and being at maximum numbers in the bloodstream between 10 pm and 2 am. During the day the mf are concentrated in the microvasculature of the deep tissues predominantly in the lungs (Spencer, 1973). It appears that physiological signals such as O_2 tension in the blood and body temperature provide the cues for this periodicity. These fluctuations occur in such a way as to ensure that mf are available in the peripheral blood in maximum numbers during the time that the major vector species are actively feeding. Microfilariae are ingested along with the blood meal when a mosquito feeds upon an infected individual.

The principal mosquito species responsible for transmitting lymphatic filariasis of humans are found within the genera *Anopheles* (*W. bancrofti, B. malayi*), *Aedes* (*W. bancrofti, B. malayi*), *Culex* (*W. bancrofti*), and *Mansonia* (*W. bancrofti, B. malayi*) (Scott, 2000). While over seventy different species and subspecies of mosquito have now been identified as vectors of disease, differences in vector / parasite compatibility and vector distribution partly account for the wider distribution of *W. bancrofti.* Within hours of ingestion the mf penetrate the midgut wall and migrate to the flight muscles. Exsheathment occurs at some stage on this migration. Once within the thoracic muscles the mf begin the first in a series of moults. The L1-L2 moult takes place between 3 and 4

days post infection (d.p.i.), and the L2-L3 moult some 8-9 d.p.i. (Schacher, 1962). The L3, the infective third stage larvae, migrate to the head and become associated with the feeding structures. When an infected insect takes a subsequent blood meal L3 are deposited onto the surface of the skin from where they actively penetrate the feeding wound and enter the lymphatic system. The L3 to L4 moult takes place early post infection (varying between 7 – 12 d.p.i. dependent upon species) and the newly formed L4 undergo a period of dramatic growth to become sexually mature adults over the following 6 – 12 months (Ash, 1970).

1.2. Clinical manifestations of lymphatic filariasis

In endemic areas the vast majority of infected individuals have few overt clinical manifestations of filariasis despite the presence of large numbers of circulating mf (Kumaraswami, 2000). However, increasing evidence suggests that although they may be clinically asymptomatic, virtually all persons with *W. bancrofti* or *B. malayi* microfilaremia have some degree of sub-clinical pathology. Many microfilareamic individuals (~40%) have some degree of heamaturia and / or proteinuria reflecting low grade renal damage. These pathologies appear to be related to the presence of mf in the blood as clearing mf from the circulation results in complete reversal of renal abnormalities (Dreyer, 1994). Lymphoscintography in asymptomatic microfilaraemic (MF+) individuals has demonstrated that they have markedly dilated lymphatics and abnormal patterns of lymphatic flow (Suresh, 1997). The asymptomatic MF+ state is, therefore, not as clinically benign as was once thought, though many individuals may remain in this state for years without developing either acute or chronic pathologies. Indeed some infected individuals have been described as remaining asymptomatic for decades without "progressing" to other forms of clinical expression (Kumaraswami, 2000).

1.2.1. Acute manifestations of L.F.

The acute clinical manifestations of filariasis are characterised by repeated bouts of fever associated with inflammation of the lymph nodes (adenitis) and / or lymph vessels (lymphangitis) termed adenolymphangitis (ADL). Recurrent attacks of fever associated with lymphadenitis are more commonly seen in brugian than bancroftian filariasis (Partono, 1987). In brugian filariasis the affected lymph nodes are mostly found in the inguinal and axillary regions and acute attacks of ADL may involve the limb, breast or male external genitalia. An acute attack may last from several days to 4 – 6 weeks and can result in a prolonged inability to work (Sabesan, 1992). Microfilaremia is generally absent in patients with ADL (Ottesen, 1992).

1.2.2. Chronic manifestations of L.F.

Chronic signs of filariasis rarely develop before 15 years of age and after that are only seen in small proportion of the infected population. The chronic pathologies of L.F. resulting from abnormal lymphatic function may be manifest as hydrocoele, chyluria or lymphoedema. Not all the chronic pathological consequences of filarial infection can be directly attributed to filarial worms. Indeed it is now considered that bacterial infections are the most common cause of elephantiasis in native residents of endemic areas (Dreyer, 2000). Lymphoscintography has shown that adult filarial worms functionally damage parasitised lymphatics in virtually all infected individuals to a greater or lesser extent (Dreyer, 1994; Witte, 1993). Impaired lymphatic drainage greatly predisposes infected individuals to secondary bacterial infections (Jamal, 1990). However, some residents of endemic areas develop lymphoedema over a period of years without any evidence of bacterial infections, and in these cases it is most likely that lymphatic dysfunction caused

by filarial worms themselves is the primary trigger for lymphoedema (Dreyer, 2000). Lymphoedema may be initially transient and reversible but later becomes permanent (elephantiasis). In brugian filariasis the leg below the knee is characteristically affected and sometimes the arm below the elbow. Microfilariae are usually absent in patients with chronic pathology and the condition persists even in the absence of active infection with adult worms (Ottesen, 1992).

1.2.3. Tropical Pulmonary Eosinophilia

This clinical manifestation is much less common than those previously mentioned and appears completely unrelated to lymphatic damage (Ottesen, 1992). TPE presents itself as a nocturnal cough, nodular or diffuse pulmonary lesions, peripheral blood eosinophilia (>3000/mm^3), high levels of IgE and very high levels of filarial specific antibodies (Ottesen, 1992). Mf are very rarely found in the blood of patients displaying TPE. Dramatic clinical improvement can be expected upon anti-filarial therapy.

1.2.4. The endemic normal (E.N.)

The term "endemic normal" in lymphatic filariasis refers to residents of endemic areas who, whilst being continually exposed to infective larvae, display no clinical signs or symptoms of infection. These individuals have been postulated to be putatively immune (King, 1991) but other authors have proposed that immunity displayed by these individuals is more likely concomitant than "perfect" in nature and, in areas of intense transmission, 100% of the population may be infected (Day, 1991). The difficulties of detecting adult worms in human filariasis have complicated the definition of an individual's infection status. In the advent of ever-more sensitive methods of detection however, the criteria determining whether an individual can truly be classed as infection-free have become

increasingly stringent. Tests for circulating filarial Ag (CFA) for example, can provide evidence of active *W. bancrofti* infection in the absence of detectable circulating mf (Weil, 1996). Elevated levels of anti-filarial IgG4 have also been shown to indicate the presence of an active infection in Mf- individuals (Kwan-Lim, 1990). Microfilaremia alone is clearly not satisfactory as the sole indicator of infection status as many individuals may be Mf- but infected with adult worms. E.N. are more likely to represent a heterologous population ranging from those who are truly infection-free to those who harbour single sex infections, few adult worms or occult infection (Day, 1991; Ottesen, 1992).

1.3. Immunology of human lymphatic filariasis.

Lymphatic filariasis may be considered a spectral disease in accordance with the variety of its clinical manifestations (Ottesen, 1992). Taking this into account most studies have sought to separate study populations into three major groups: microfilaremics, chronic pathology patients and E.N. Whilst differences in responsiveness have been reported amongst clinical groups, certain features dominate the immune response in lymphatic filariasis: high level IL-4 production (King, 1992; Mahanty, 1991; Mahanty, 1993; Yazdanbaksh, 1993), Ag-specific proliferative suppression (Ottesen, 1992; Piessens, 1980b; Sartono, 1995a; Yazdanbaksh, 1993) and down-regulation of IFN-γ production (King, 1992; Ravichandran, 1997).

1.3.1. IL-4 producing cells are expanded in human L. F.

Th2 responses, of which IL-4 is the signature cytokine, are classically associated with helminth infections and filariasis is no exception. Infected individuals have a much greater frequency of T cells capable of producing IL-4 and IL-5 than uninfected individuals (King, 1992; King, 1993) and these cells are preferentially stimulated by parasite Ag (Mahanty, 1993). In vitro restimulation with parasite Ag invokes a strong IL-4

response by PBMC from infected individuals (King, 1992; Mahanty, 1993; Yazdanbaksh, 1993) and similarly elevated levels of IL-4 have been observed when measured as protein (Mahanty, 1996) or at the level of mRNA expression (Dimock, 1996). It is interesting to note that whilst the frequency of IL-4 producing T cells is similar across clinical groups, the frequency of IFN-γ secreting cells is significantly lower amongst microfilaremics (King, 1992). In accordance with these findings no relationship has yet been demonstrated between IL-4 production and microfilaremia (Maizels, 1995) or fluctuations in mf density (Sartono, 1999). It is of note that PBMC from Mf+, Mf- CFA+ or CFA- individuals did not differ in their levels of IL-4 mRNA expression upon *in vitro* restimulation with Ag (Dimock, 1996). Individuals who are CFA- may be considered putatively immune and it has been suggested that incoming L3 are the target of this immunity (Day, 1991). In the absence of active infection it is possible that continued exposure to incoming L3 (known to be a potent stimulus for IL-4 production in laboratory mice (Lawrence, 1994; Osborne, 1997b) may contribute to the maintenance of an Ag-specific population of IL-4 producing cells. Interestingly it has recently been reported that production of IL-4 and IL-5 are differentially regulated in human filariasis. Sartono *et al* (1997) studied Ag-specific cytokine production as a function of age in an area endemic for *B. malayi* infection. While both asymptomatic Mf- and Mf+ subjects produced equivalent amounts of IL-4, asymptomatic Mf- subjects produced significantly higher levels of IL-5. Furthermore when considered as a function of age, IL-5 expression was found to segregate distinctly from that of IL-4. Although Mf+ individuals showed increased production of IL-4 over time no such changes were observed in the levels of Ag-driven IL-5 or IFN-γ. Such findings suggest that responses to filarial infection may not fall neatly into line with the Th1/Th2 paradigm.

1.3.2. Proliferative suppression in human L.F.

Ag-specific proliferative suppression is a well documented feature of lymphatic filariasis (Ottesen,1977;Piessens,1980b;Ravichandran,1997;Sartono,1995a;Yazdanbaksh, 1993). Early studies reported the inability of PBMC from infected individuals to proliferate in response to parasite Ag whilst polyclonal, mitogen driven responses remain intact (Ottesen, 1977; Piessens, 1980b). The most profound proliferative suppression was seen amongst asymptomatic microfilaremics leading them to be classified as "hyporesponsive" with regard to filarial Ag (Ottesen, 1977; Piessens, 1980b; Ravichandran, 1997; Yazdanbaksh, 1993). More recently, observations from a larger study population demonstrated that a percentage of individuals in all clinical groups have suppressed Ag-specific proliferative responses (Yazdanbaksh, 1993). Suppression was most profound amongst microfilaremics but was also observed in amicrofilaremics and elephantiasis patients. This study had the great advantage of being able to compare both T and B cell responses within the same individuals and proliferative responses were shown to correlate inversely with IgG4 production. As IgG4 production has been shown to indicate the presence of active infection (Kwan-Lim, 1990), these results suggest that all infected individuals are subject to some form of suppression, the degree of which may vary in line with worm burden (Yazdanbaksh, 1993). It is of interest to note, however, that filarial-specific IgG4 is most highly elevated in microfilaremics compared to other clinical groups (Hussain, 1987; Ottesen, 1992). These findings infer an association between the presence of circulating mf, high level IgG4 production and the down-regulation of Ag-specific proliferative responses.

1.3.3. Mechanisms of proliferative suppression in the infected human

Cells from Mf+ individuals are sensitised by *in vivo* exposure to filarial Ag (as demonstrated by their capacity for Ag-specific cytokine production), but are unable to

proliferate in response to Ag *in vitro*. Although the nature of this hyporesponsiveness is incompletely characterised a number of mechanisms maybe involved, including clonal deletion, induction of parasite-specific anergy, an imbalance in cytokine production, clonal exhaustion, or the development of regulatory cell populations (for review see Maizels, 1995). Various studies have attempted to elucidate the cause of proliferative suppression and whilst there has been some success in partially restoring responsiveness, no single factor has been universally effective (King, 1992; Mahanty, 1997; Piessens, 1980a; Sartono, 1995a). Similar findings have been reported in the mouse model (see below), wherein it appears that proliferative suppression is likely to be the net result of various and diverse mechanisms.

Sartono *et al* (1995a) attempted to restore Ag-specific proliferation *in vitro* using a battery of immunomodulatory reagents. Some restorative effect was seen using recombinant IL-2 but this was inconsistent reversing unresponsiveness in only 12-28% of patients and no factor consistently improved proliferation. TCR ligation in the absence of efficient co-stimulation can result in T cells undergoing apoptosis (Liu, 1990) or entering an unresponsive state (Scwartz, 1990). To address the possibility that inefficient co-stimulation may be involved in generating hyporesponsiveness, Ag stimulated cultures were supplemented with antibodies capable of enhancing co-stimulation via cross-linking of CD2, CD26,CD27 or CD28 (Sartono, 1995a). None of these treatments were able to restore proliferative responses, suggesting that defective co-stimulation alone does not account for impaired T cell responses.

1.3.4. IL-10 and proliferative suppression

There is conflicting evidence surrounding a role for IL-10 in down-regulating proliferative responses in filariasis (King, 1992; Mahanty, 1996; Ravichandran, 1997;

Sartono, 1995a). IL-10 down-regulates production of IL-2 and IFN-γ by Th1 clones (Sher, 1992), and following murine infection with *Nippostrongylus braziliensis* and *Schistosoma mansoni* (Sher, 1992). IL-10 also mediates proliferative suppression in a murine model of trypanosomiasis (Uzonna, 1998).

Cells from microfilaremic individuals produce IL-10 both spontaneously (King, 1992) and in response to Ag (Ravichandran, 1997). At the level of mRNA expression Mf+ individuals produce higher levels of IL-10 than patients in other clinical groups and IL-10 production correlates inversely with proliferation (Ravichandran, 1997). Whilst *in vitro* neutralisation of IL-10 or TGF-β enhanced Ag-specific proliferation of PBMC from *W. bancrofti* infected individuals (King, 1992), neutralisation of IL-10 did not restore proliferative responses of PBMC from individuals with brugian filariasis (Sartono, 1995a). These differences, however, may relate to the treatment of cells prior to *in vitro* assay as Sartono *et al* used cryopreserved cells.

There is good circumstantial evidence to suggest that the increased IL-10 production seen amongst microfilaraemics is APC derived. In an early study by Piessens *et al* it was found that removal of adherent cells significantly improved proliferative responses from "non-reactors" (Piessens, 1980b). Analysis of the adherent cell population led to speculation that monocytes were responsible for suppression, and, as human monocytes have been identified as a major source of IL-10 (de waal Malefyt, 1991), it is tempting to speculate that monocyte derived IL-10 may be a contributory factor. Furthermore serum from "non-reactors" was shown to suppress proliferative responses of PBMC from "reactors" and, whilst the serum factor responsible for suppression was not identified, IL-10 is a potential candidate. It is interesting to note that conditions which improved responses of "non-reactors" also enhanced responses of "reactors" (Piessens,

1980b), again suggesting that all infected individuals are subject to some form of suppression.

1.3.5. T regulatory cells

Several lines of evidence suggest a role for regulatory T cells in modulating responses in lymphatic filariasis. King *et al* (1992) found that that cultures with fewer lymphocytes often showed higher levels of Ag-specific proliferation, suggesting interacting populations of $CD4^+$ cells may modulate this response. As proliferative and pro-inflammatory responses are selectively suppressed, regulatory cytokine production again seems a likely mechanism.

Recently new T cell subsets (termed Th 3 / Tr 1) which differ from Th 0, Th1 or Th2, have been described and characterised at the level of T cell clones (O'Garra, 1998). A common feature of these subsets is their production of high levels of TGF-β and / or IL-10 and only low to undetectable levels of IL-2. It has been suggested that Tr 1 cells may also drive APC to produce IL-10 leading to suppression of responses to third party Ag (Cobbold, 1998). In a recent study on Ag-specific hypo-responsiveness in humans infected with *Onchocerca volvulus* (a related filarial worm and the causative agent of river blindness), IL-10 and TGF-β were shown to effect suppression of proliferative responses (Doetze, 2000). Parasite-specific T cells were cloned from hyporesponsive individuals and shown to have a cytokine production profile similar to that of Th 3/Tr 1 cells (no IL-2, high IL-10 and / or TGF-β). This led to the proposal that hypo-responsiveness in *O. volvulus* infection is mediated by Th 3 / Tr 1 type cytokines rather than a Th1 to Th2 shift. It is not known whether a similar population of regulatory T cells is expanded in hypo-responsive individuals in lymphatic filariasis but the early studies of King and Nutman

(1993) which showed that neutralisation of IL-10 or TGF-β restored proliferative responses suggest that this may indeed be the case.

1.3.6. *In utero* exposure to filarial-Ag

Suppression of proliferative responses in L.F. may be considered as a form of parasite-specific tolerance and it has been proposed that *in utero* exposure to filarial Ag may contribute to its development. Several studies have shown that children born to Mf+ mothers are more likely to become microfilaremic themselves (Hightower, 1993; Lammie, 1991). The findings of Steel *et al* (1994) that the offspring of Mf+ mothers remain unresponsive to Mf Ag throughout their lives suggested clonal deletion of Ag-reactive lymphocytes may occur as a result of *in utero* exposure to parasite Ag. However several findings argue against clonal deletion being the sole mechanism of down-regulation, most notably the widely reported reversal of hyporesponsiveness seen post-chemotherapy (Lammie, 1992; Piessens, 1980b; Sartono, 1995b; Yazdanbaksh, 1993). Treatment with diethyl carbamazine (DEC), a filaricidal drug leads to improved proliferative responses amongst microfilaremics and elephantiasis patients (Lammie, 1992). Treatment with ivermectin, which clears mf without affecting the adult worms, has also been shown to enhance T cell reactivity, suggesting a direct role for mf in generating suppression (Lammie, 1992).

1.3.7. IFN-γ responses are down-regulated, particularly amongst microfilaremic individuals

Down-regulation of IFN-γ production is another prominent feature of filarial infection which is most pronounced amongst Mf+ individuals (King, 1992; Mahanty, 1996; Ravichandran, 1997). While the precursor frequency of Ag-specific IL-4 secreting

cells is comparable across clinical groups, Mf+ individuals have significantly fewer IFN-γ producing cells than CP individuals indicating a more profound suppression of Th1 responses (King, 1992;1993). In several ways suppression of IFN-γ production parallels proliferative suppression as *in vitro* both Ag-specific proliferation and IFN-γ responses are lowest amongst Mf+ individuals (Ottesen, 1977; Piessens, 1980b; Ravichandran, 1997). Furthermore factors which restored proliferation, such as neutralisation of IL-10 or TGF-β (King, 1992; Mahanty, 1997), or drug treatment (Piessens, 1980a; Sartono, 1995b), also enhanced IFN-γ production. Several lines of evidence, (including reversal post-chemotherapy), suggest that down-regulation of IFN-γ production is dependant upon active infection. Putatively immune individuals (Mf-, CFA-) in an area endemic for bancroftian filariasis have higher proliferative and IFN-γ responses than infected (CFA+ Mf- and Mf+) individuals (Dimock, 1996). Similar results have been reported in infection with *Onchocerca volvulus,* where both proliferative and IFN-γ responses are significantly greater amongst putatively immune individuals than those with active infections (Elson, 1995).

That worm burden may influence the degree of suppression is suggested by evidence that there is an inverse relationship between the presence of circulating microfilariae and IFN-γ production by PBMC (Sartono, 1999). Over the course of this study a small number of individuals (6 of 39) had detectable mf at one or two time points and were negative at others. These individuals were termed "converters". IFN-γ production was most affected by changes in mf density in a manner generally consistent with the presence of mf being associated with low IFN-γ release, and the absence of mf with high IFN-γ production. These results, demonstrating lower IFN-γ production amongst Mf+, complement those of Dimock *et al* (96) which demonstrated that anti-filarial IgG2

production was associated with the absence of mf but not the absence of circulating Ag. This suggests a direct role for mf, as distinct to infection with adult worms, in suppressing IgG2 production, most probably via suppression of IFN-γ production. Whilst not recognised as a switching factor for human IgG2 production, IFN-γ can, itself, enhance IgG2 and acts synergistically in this respect with IL-6 (Kawano, 1994).

1.3.8. A role for mf Ag in eliciting proliferative suppression

The origin of the Ag used for *in vitro* restimulation was also demonstrated to affect both the proliferative and cytokine responses of PBMC from infected individuals (Mahanty, 1996). Proliferative responses to both mf Ag and mixed sex adult Ag were markedly impaired amongst Mf+ as compared to CP. In contrast to this, proliferative responses to adult male Ag were not significantly different between groups (being greater in Mf+ individuals than those elicited by mixed sex or female Ag). This was reflected in the observed pattern of cytokine secretion as lower levels of IL-2 and IFN-γ were produced by cells from Mf+ in response to mf Ag and mixed adult Ag than to adult male Ag (Mahanty, 1996). These results indirectly suggest a role for microfilarial Ag in down-regulating proliferation as unlike male Ag, a proportion of mixed sex and female Ag will be composed of mf Ag due to the presence of mf within female worms.

1.3.9. Hyporesponsiveness and microfilaremia

Whilst hyporesponsiveness is not solely restricted to microfilaremic individuals there is a clear association between the presence of circulating mf and immunosuppression. Ag–specific proliferative responses are lowest amongst microfilaremics (Ottesen, 1977; Ravichandran, 1997) and most difficult to restore both *in vitro* and post-chemotherapy in this group (Sartono, 1995b). Mf+ individuals have a lower frequency of Ag-specific lymphocytes and significantly lower numbers of IFN-γ producing

cells (King, 1992;1993). IFN-γ production has been reported to correlate inversely with mf density (Sartono, 1999). PBMC from Mf+ individuals produce the lowest levels of Ag-specific IFN-γ and IL-2 and high levels of both spontaneous and Ag-driven IL-10 upon *in vitro* restimulation (Mahanty, 1996). IL-10 production is highest whilst IL-2 / IFN-γ production and proliferative responses are lowest upon restimulation with Ag preparations containing mf Ag, suggesting a role for mf Ag in generating suppression (Mahanty, 1996).

Work in the mouse model of filariasis has revealed that different life cycle stages of the parasite elicit differentially polarised immune responses, and possess distinct mechanisms for modulating host responses (see below). During the course of long term human infection, individuals are exposed to all life cycle stages, adult worms, circulating mf, incoming L3 and developing larvae, and are subject to their combined immunomodulatory effects. This builds a complex picture of infection, wherein the observed responses are likely to be the net result of multi-factorial interactions between host and parasite. Whilst active infection, or continued exposure to incoming L3, may elicit and maintain the development of an expanded population of IL-4 producing cells, the presence of circulating mf is more particularly associated with distinct suppressive effects. In this sense it maybe considered that circulating mf contribute to the stability of Th2 polarisation by actively down-regulating Ag-specific proliferative and IFN-γ responses, leading to a more profound suppression of pro-inflammatory responses.

1.4. The mouse model of lymphatic filariasis

The study of immune responses in human lymphatic filariasis is complicated by several factors, including levels of worm burden and infection status, prior and current exposure to infective larvae and the presence of concomitant infections. The use of animal

models allows many of these variables to be controlled, such that it is possible to assess responses to infection of known dose, at specific sites, over a given period of time. Unfortunately no suitable animal model has been found to support the development of *W. bancrofti* so most laboratory studies have used models of infection with *Brugia* spp. *Brugia pahangi,* a cat filarid closely related to *B. malayi,* will develop in several species of laboratory rodents, producing patent infections in the Lewis rat (Fox, 1976; Vickery, 1983), the golden hamster (Malone, 1975) and the mongolian jird, *Meriones unguiculatus* (Ash, 1970). The jird is the most susceptible of these hosts and is used successfully to maintain the life cycle of both *B. malayi* and *B. pahangi* for laboratory studies. In the jird model, suppression of both Ag-specific and mitogen driven proliferative responses occurs during infection (Lammie, 1983a/b; Lammie, 1984b; Partaro, 1976; Prier, 1988). Most interestingly, the onset of proliferative suppression has been shown to correlate with the appearance of microfilariae in the blood (Lammie, 1983a/b;1984a). However, the lack of a well characterised immune system, inbred strains or immunological reagents have limited the usefulness of this model.

To overcome these problems much work has been done using infection of inbred laboratory mice. While immunocompetent mice will not support the complete development of the parasite (Ahmed, 1967; Laing, 1961; Vickery, 1983), they have allowed study of the mechanisms of resistance to primary infection and of protective immunity to challenge infections. A great deal of information on the immunomodulatory capacity of filarial worms has also been obtained from studies using single life cycle stage infections. The advent of genetically manipulated mice, lacking highly specific components of the immune response has also allowed the importance of precise parameters to be assessed in terms of protective immunity, parasite survival and host pathology (Rev Lawrence, 1996). Cumulatively these studies build a picture of an

immunologicaly complex infection, with a parasite able to modulate the host response by an array of mechanisms, associated with distinct life cycle stages and cellular interactions.

1.4.1. Resistance and protective immunity in mouse models of filariasis

(i) Immunity to L3

That T cells are necessary for the development of resistance to *B. pahangi* was suggested by the observation that congenitally athymic "nude" mice (lacking functional T lymphocytes) are susceptible to infection, whilst normal mice are resistant (Suswillo, 1980; Vincent, 1980). The importance of T cells was shown by the demonstration that resistance, comparable to that seen in normal mice, could be achieved in nudes by reconstituting T cell function either by thymus grafting or adoptive transfer of splenocytes from infection primed heterozygotes (which are phenotypically normal) (Vickery, 1984). In the absence of transferred cells transferred serum containing anti-parasite antibodies was shown to be non-protective (Vickery, 1983). These results demonstrated that resistance to *B. pahangi* in mice is achieved by a thymus dependant mechanism of which cells are an essential component.

Further evidence of the importance of specific immunity in resistance came from studies using SCID mice. Severely compromised immunodeficient (SCID) mice, are homozygous for a recessive mutation at the locus that influences assembly of intact Ig and TCR genes, and therefore cannot generate functional B or T lymphocytes. Nelson *et al* (1991) demonstrated that subcutaneous injection of *B. malayi* L3 led to the development of patent infections in SCIDs. As SCID mice have normal NK cell and macrophage activity these results further suggested that immunity is dependant upon specific T/B cell activity.

Whilst being refractory to full infection, the potential of the well characterised BALB/c mouse in allowing the dissection of responses to individual life cycle stages was

first utilised by Hayashi *et al* (1984). Having determined that vaccination with irradiated L3 led to a dramatic reduction of survival upon challenge infection they sought to define the mechanisms of this immunity. Using passive transfer of spleen cells from immunised animals they were able to confer resistance upon naïve recipients. As previously described elsewhere (Vickery 83) transfer of serum in the absence of cells did not confer resistance.

Subsequent studies sought to determine the specific components of adaptive immunity involved in generating resistance to primary infections with L3. Having established that cells of the specific adaptive immune system are necessary to achieve resistance (Nelson, 1991), Rajan *et al* failed to demonstrate a role for $CD4^+$ T cells, $CD8^+$ T cells or B cells in generating resistance to L3 of *B. malayi*. CD4 null mice (lacking $CD4^+$ T cells), β2-microglobulin knock-out mice (deficient in $CD8^+$ T cells and NK cells), and μMT-/μMT- mice (lacking functional B cells) were all found to be resistant to infection (Rajan, 1992;1994;1995). The authors concluded that B cells were unnecessary to resistance, and that in the absence of $CD4^+$ or $CD8^+$ T cells, double negative, (CD4-/CD8-) T cells, or the interactions of either $CD4^+$ or $CD8^+$ T cells with cells of the innate immune system may be sufficient to mediate resistance. It is of note, however, that in the above mentioned experiments with β2m-/- and CD4 null mice no comparison was made to worm recoveries in the equivalent "wild type" counterparts and in those using μMT-/μMT- mice, a total of three wild type C57B/6 mice were used in two experiments. However, recent results from the same laboratory have led them to re-evaluate the importance of B cells in generating resistance to *B. malayi* L3. Comparing responsiveness of mice lacking T cells, B cells or both cell types (on a uniform genetic background, C57BL/6) suggested that B cell activity may contribute to resistance at early stages post infection (Babu, 1999).

In contrast to the findings of Rajan *et al* evidence for an important role for $CD4^+$ T cells has been reported in protective immunity to challenge infection of immunised BALB/c mice. Mice immunised with irradiated *B. pahangi* L3 become strongly immune to challenge infection (Bancroft, 1993). Upon *in vitro* restimulation with Ag / ConA, splenocytes from immunised animals produce large amounts of IL-5/IL-9, moderate levels of IL-4 and negligible IFN-γ, consistent with a preferential expansion of Th2 cells. The effects of *in vivo* antibody-mediated depletion of $CD4^+$ or $CD8^+$ T cells upon immunity in immunised mice were assessed. Anti-CD4 treatment resulted in a significant increase in worm recovery and a much reduced capacity for IL-4, IL-5 and IL-9 production *in vitro* and lower levels of parasite-specific IgG and IgE *in vivo*. In contrast depletion of $CD8^+$ T cells had negligible effects on immunity to challenge infection (Bancroft, 1994). This study supports a critical role for $CD4^+$ T cells in protective immunity to *B. pahangi* in BALB/c mice and suggests some component of the Th2 response plays an important role.

There is strong evidence supporting a role for Th2 responses in murine resistance to filarial infection from several closely related model systems. In particular those studies using *Onchocerca* sp., which have also suggested that distinct mechanisms effect immunity to different life cycle stages (Hogarth, 1998). *Litmosoides sigmondontis* is a member of the Onchocercidae family which displays fundamental biological similarities to human filariae (Allen, 2000; Martin, 2000) and is the only filarial worm to undergo full development in inbred laboratory mice. The potential of this important model of filarial infection is now being realised in terms of identifying effector mechanisms of immunity (Le Goff, 1999; Martin, 2000; Petit, 1992; Pfaff, 2000a/b) and providing a model for vaccine development (Allen, 2000). During murine infection, development of *L. sigmondontis* is controlled by $CD4^+$ T cells, and worm loads correlate negatively with Th2 cyokine production, eosinophilia and levels of parasite-specific serum IgE (Al-Qaoud,

1997). Mice with impaired development of Th2 responses display enhanced susceptibility to *L. sigmondontis* (Al-Quoad, 1998), while those with enhanced Th2 responses are highly resistant (Martin, 2000). The B1 cell deficiency of BALB/c Xid mice led to increased susceptibility to infection, which was associated with a lower level of B cell derived IL-10 and parasite driven Th2 cytokines (Al-Quoad, 1998). While these results suggest that B cell derived IL-10 is important in driving the development of Th2 responses during filarial infection, it is important to acknowledge that btk deficiency in Xid mice also affects other cell types in addition to B1 cells.

(ii) The role of IL-5 and eosinophils in protective immunity

A range of gene-knock-out mice and *in vivo* antibody depletion studies have helped identify effector mechanisms, operable post-immunisation, against challenge infection with L3 and mf of *Onchocerca* sp. Following immunisation with irradiated L3 of *O. volvulus* and challenge with L3 contained within diffusion chambers, both IFN-γ-/- and wild type C57BL/6 mice display high levels of larval killing (Johnson, 1998). Immunity was associated with elevated levels of IL-4 and eosinophil recruitment. Unimpaired resistance in IFN-γ-/- mice argues against a role for Th1 responses in the killing of L3. In contrast, immunised IL-4-/- mice were unable to kill challenge larvae showing an absolute requirement for IL-4 in protective immunity to L3 (Johnson, 1998). Interestingly while levels of IL-5 were elevated in all three groups of immunised mice, IL-4-/- mice had significantly fewer eosinophils in their diffusion chambers suggesting IL-4 may play an important role in enabling the transmigration of eosinophils through the vasculature and to the target site.

In contrast to the situation with L3, IL-4-/- mice are able to clear a primary infection with *O. lienalis* mf, and display unimpaired resistance to secondary infection (Hogarth, 1995). However *in vivo* neutralisation of IL-5 in IL-4-/- mice enhanced survival

of primary infections, and ablated infection induced eosinophilia and resistance to challenge infections (Hogarth, 1998). Furthermore infection of μMT mice demonstrated IL-5 does not mediate its protective effects via Ig isotype switching. These results suggest that unlike immunity to L3, IL-5 dependant immunity to mf is mediated by eosinophils independently of IL-4. Indeed the cytokine response during the expression of protective immunity to *Onchcerca* mf is dominated by IL-5 production which correlates with blood and tissue eosinopihlia (Hogarth, 1999). In an interesting parallel to observations in human filariasis (Mahanty, 1996), splenocytes from mf-infected animals showed vigorous proliferative responses to restimulation with adult worm Ag yet failed to proliferate in response to mf Ag (Hogarth, 1999).

The studies of Hogarth and Johnson show a dissociation of IL-4 and IL-5 production, and it has been proposed that IL-5 production may be regulated independently of IL-4 during filarial infection. Indeed the work of Sartono *et al* (1997) demonstrated that levels of IL-5 fluctuate independently of IL-4 in human, infection with *B. malayi*. Most interestingly the studies reviewed above demonstrate that IL-5 dependant protective immunity to mf of *Onchocerca* sp. is IL-4 independent and as such is distinct to that against L3, which requires both IL-4 and IL-5 (Johnson, 1998; Lange, 1994). However, protective immunity to *L. sigmodontis* L3 has been shown to be IL-5 dependent whilst resistance to primary infection is not, illustrating differences in the mechanism of resistance to primary infection and immunity to challenge (Le Goff, 2000).

Elimination of both *B. malayi* mf and L3 is enhanced by prior sensitisation with parasite lysates (Kazura, 1982) and following immunisation, resistance to mf correlates with the development of local Th2 responses (Pearlman, 1993a). Elimination of *B. malayi* mf in multiply immunised BALB/c mice is associated with local eosinophilia and elevated levels of serum IgE. $CD4^+$ T cells from the site of inoculation (the peritoneal cavity)

produce IL-4 and IL-5 but not IFN-γ. Following multiple immunisation, and subsequent infection, differences in cytokine responses were observed between cells from the site of infection and other secondary lymphoid organs. While peritoneal exudate cells (PEC) produce exclusively Th2 cytokines, splenocytes and lymph node cells give a more mixed response producing Ag-specific IL-5 and IFN-γ but no IL-4 (Pearlman, 1993b). These results led to the suggestion that Ag-specific Th1 cells may enter the peritoneal cavity but IFN-γ production maybe down-regulated under the influence of IL-10. Neutralisation of IL-10 in Ag-stimulated culture of cells from multiply immunised, or chronically infected, mice enhanced IFN-γ production further supporting this theory (Pearlman, 1993b).

Collectively these studies are suggestive of an important role for IL-5 and eosinophils in murine resistance to filarial infections. Recently the development of IL-5 transgenic (IL-5 Tg) mice, which have life long eosinophilia, has allowed further analysis of the role of IL-5 in various models of infection. IL-5 Tg mice display enhanced resistance to *Nippostrongylous brasiliensis* (Dent, 1999) and *L. sigmondontis* (Martin, 2000). Infection with *N. brasiliensis* is so short lived that any contribution from the humoral arm of the adaptive response to enhanced resistance was considered unlikely (Dent, 1999). Following infection with L3 of *L. sigmondonis*, IL-5 Tg mice displayed resistance equivalent to that seen in immunised BALB/c mice which also show high levels of IL-5 production and eosinophilia (Martin, 2000). Developing larvae recovered from IL-5 Tg mice were found to be surrounded by an aggregate of eosinophils and macrophages, further suggesting an important role for eosinophils in effecting resistance.

L. sigmondontis infection, of resistant and susceptible strains of inbred mice, has also been used as an approach to highlight potentially important effector mechanisms. Following infection with mf, resistant C57BL/6 mice show enhanced production of nitric

oxide (NO) compared to susceptible BALB/c mice (Pfaff, 2000b). NO, produced by activated macrophages, has been shown to act as an effector molecule in immunity to a diverse array of pathogens (James, 1995). *In vitro* studies have also demonstrated cytostatic and cytotoxic effects of NO upon filarial worms (Taylor, 1995; Thomas, 1997). However, although *L. sigmondontis* mf are susceptible to NO *in vitro*, iNOS-/- mice (lacking the gene for inducible nitric oxide synthase, the enzyme responsible for high level NO production) on the C57BL/6 background, were found to be as resistant as their wild type counterparts (Pfaff, 2000b). Such results suggest NO is not an important factor in the elimination of *L. sigmondontis* mf *in vivo*.

These studies, in several different host / parasite systems, serve to illustrate features of importance in resistance to primary and challenge infection with filariae. Unlike observations in human endemic normals (Dimock, 1996) there is little support from murine models for a role of Th1 responses in resistance to L3. In contrast Th2 responses often correlate with the development of protective immunity to both mf and L3 (Al-Quoad, 1998; Bancroft, 1993; Hogarth, 1998/1999; Johnson, 1998; Lange, 1994; Pearlman, 1993a). Furthermore rather than falling neatly in line with the classic Th1 / Th2 paradigm, production of IL-5 is dissociated from that of IL-4 and maybe produced even in the absence of IL-4 or along with IFN-γ (Hogarth, 1998; Pearlman, 1993b). It is also of note that IFN-γ, IL-4, IL-5 and IL-10 are not uniquely produced by T helper cells and cytokine production by non-T cells such as eosinophils, mast cells and activated MO may also influence the immune response. Protective immunity to different life cycle stages may also be dependant upon different cytokines (Hogarth, 1998; Lange, 1994), may be site specific, and dependant upon route of infection (Pearlman, 1993a). Most mouse models of filariasis are limited in terms of assessing protective adaptive immunity over the course of infection, due to incomplete parasite development in murine hosts. In spite of this, such

studies provide information of obvious importance for vaccine design, in terms of the type of responses that are most effective against the desired target stage of the parasite at a specific site.

1.4.2. Immunomodulation in mouse models of filariasis

(i) Different life cycle stages elicit development of differentially polarised responses

While *Brugia* spp. will not complete their developmental cycle in immunocompetent mice, short term, single life cycle stage infections have been used to great effect in assessing stage-specific responses. Lawrence *et al* (94) made the intriguing discovery that infection with different life cycle stages leads to the development of contrasting cytokine and Ig isotype response in BALB/c mice (Lawrence, 1994). Intraperitoneal infection with adult worms led to the production of IgG1 and IgE *in vivo.* High levels of Ag-specific IL-4 and negligible levels of IFN-γ were produced upon *in vitro* restimulation of splenocytes, indicative of a Th2 type response. L3 given i.p. or s.c. (Lawrence, 1994; Osborne, 1996)also induce strong Th2 responses. In contrast, infection with mf leads to development of a Th1-like response: splenocytes from mf-implanted animals release large amounts of IFN-γ upon *in vitro* restimulation, generate Ag-specific serum antibodies of all IgG subclasses and produce little serum IgE (Lawrence, 1994). This stage-specific skewing of the immune responses is consistent across a variety of mouse strains tested including BALB/c, 129SvxC57B/6 and CBA/Ca mice (Lawrence, 1996).

Although adult female worms continue to produce mf following implantation, splenocytes from these animals still give Th2 recall responses *in vitro,* suggesting that females exert a sufficiently strong influence to override mf-induced IFN-γ production. It is interesting to note that infection with adult females provided a much more potent stimulus

for IL-4 production than infection with adult males (Lawrence, 1994). It is not known whether this is a reflection of the significantly greater biomass of female worms, or if the response is directed towards antigens, possibly of uterine origin, associated solely with female worms.

The importance of IL-4 in down-regulating mf-induced IFN-γ production was further demonstrated following infection of IL-4 KO (IL-4-/-) mice. A dramatic change in polarisation of the response to adult worms was observed in KO mice with a significant reduction in IgG1 and a corresponding increase in IgG2a, IgG2b, IgG3 and IFN-γ release. This "switch" was most marked in recipients of adult female worms which continually produce mf, demonstrating that in the absence of IL-4, down-regulation of mf-induced IFN-γ production does not occur (Lawrence, 1995). Alternatively the absence of IL-4 during priming may allow development of a female induced IFN-γ response. These *in vitro* cytokine responses suggest that under the influence of IL-4, IFN-γ secreting cells may not be primed during infection with life cycle stages driving Th2 responses. However, it has now been shown that Ag-specific IFN-γ producing cells are primed by infection with L3 but their activity *in vitro* is down-regulated by IL-10, whilst polyclonal IFN-γ production is down-regulated under the influence of both IL-4 and IL-10 (Osborne, 1996/1999). The existence of "primed but silenced" Th1 responses provides an interesting parallel to human infection with *W. bancrofti* where Ag-specific IFN-γ production is down-regulated by IL-10 and TGF-β (see above, King, 1992).

(ii) Mf-induced responses in murine infection

At early time-points (12-14 days) following intra-peritoneal infection with mf recall responses *in vitro* are Th1-like, with Ag-specific cytokine responses dominated by IFN-γ (Lawrence, 1994; Pearlman, 1993b). Following chronic infection or repeated

immunisations with mf-extract however, IFN-γ responses are suppressed and a more Th2 like response is seen *in vitro* (Lawrence, 1994; Pearlman, 1993b). Subcutaneous infection with mf also induces development of a Th1 like cytokine profile and the suppression of Ag-specific proliferative responses (Osborne, 1996).

Pearlman *et al* (1993b) investigated the ability of *B. malayi* mf and soluble mf extract to elicit and maintain T cell responses in BALB/c mice. At 14 days post-infection with mf, or following immunisation with mf extract, splenic lymphoid cells produce IFN-γ but little or no IL-5. At later time points, however, Ag-specific IL-5 production was shown to increase coincident with a decrease in IFN-γ production. CD 4^+ T cells were shown to be the major source of Ag-specific cytokine production upon *in vitro* restimulation. Injection with soluble mf extract also induced production of IFN-γ in the absence of IL-5, while repeated immunisation (x3), led to development of a more Th2 polarised response with increased IL-5 and decreased IFN-γ.

As prolonged infection (Lawrence, 1994) or repeated immunisation (Pearlman, 1993b) are required for the generation of mf-induced Th2 responses it may be that increased levels of restimulation are necessary for Th2 development. In this way Th2 responses may be related to the chronicity of infection. Alternatively the development of Th2 responses following mf infection may be due to exposure to somatic antigens only released upon death of the parasite.

Following intravenous infection of BALB/c mice with *B. malayi* mf, high levels of IgE production have been reported even in the presence of IFN-γ and it has been shown that high numbers of mf ($2.5x10^5$) or repeated immunisations with mf extract increases mf-induced IgE production (Lawrence, 2000). After infection with high doses of mf, IFN-γ

production was also shown to decrease over extended periods of time (up to 80 days) supporting the theory that chronicity of exposure to Ag is related to the development of mf-induced Th2 responses. In an interesting parallel to studies on human L.F. (Mahanty, 1996), development of mf-induced Th2 responses was accompanied by the onset of IL-10-mediated down-regulation of IFN-γ production. In vitro treatment with an anti-IL-10 MAb led to increased IFN-γ production by splenocytes from chronically infected (35 dpi), or multiply immunised mice but did not affect IFN-γ production at 14 d.p.i. or following a single immunisation. As seen in studies on brugian filariasis in humans however (Sartono, 1995a), anti-IL-10 treatment failed to enhance proliferative responses (Pearlman, 1993b).

Mf-induced suppression of Ag-specific proliferative responses was recently reported following s.c. infection of BALB/c mice with *B. pahangi* (Osborne, 1996). While splenocytes from L3 infected mice gave strong Ag-specific proliferative responses, cells from mf-infected animals were unable to proliferate under identical conditions. At 12 d.p.i. after 72 hrs of Ag-stimulated culture, cells from mf-infected animals proliferated at only background levels equivalent to those of cells from uninfected control mice. Cells from mf-infected animals were clearly activated, as demonstrated by their production of high levels of Ag-specific IFN-γ. In a situation analogous to that seen in human infection, proliferative suppression was Ag-specific in nature as polyclonal, mitogen driven responses were intact in mf-infected animals. It is of note that proliferative suppression in the *B. pahangi* /BALB/c mouse model occurs at an early time-point post mf-infection, when the response is Th1 like in nature, and precedes the previously reported down-regulation of IFN-γ responses (Pearlman, 1993b) suggesting it is mediated by distinct mechanisms. That suppression is mf-specific is also particularly intriguing considering the close association between the presence of circulating mf and hyporesponsiveness in human

infection (see above 1.3.7-9). The observation of mf-induced Ag-specific proliferative suppression forms the basis of this study which is aimed at identification of the immunomodulatory effector mechanisms involved.

(iii) Mechanisms of immunomodulation in the mouse model of filariasis

Studies in murine models have revealed a multitude of ways in which filarial parasites may regulate host responses. These range from interactions of filarial products with the innate system (Whelan, 2000), via the generation of a suppressive APC population (Allen, 1996; MacDonald, 1998) which may favour Th2 development (Loke, 2000), by production of cytokines such as IL-4, IL-10 and TGF-β capable of down-regulating Th1 responses (Loke, 2000; Osborne, 1999) or via direct effects of parasite products in suppressing proliferation of T and B cells (Harnett, 1998/1993). It is also apparent that different life cycle stages possess different means of effecting suppression (Allen, 1996), clearly illustrating the complex and multi-factorial nature of responses to filarial infection.

The role of Th2 cytokines in the generation of a suppressive APC population following filarial infection has been extensively studied by Allen *et al.* Intra-peritoneal infection with adult *B. malayi* leads to the IL-4 dependant development of a population of alternatively activated $F480^+$ macrophages, which suppress proliferation of the conalbumin specific T-cell clone D10.G4 whilst cytokine production remains intact (Allen, 1996; Loke, 2000). The fact that Ag-specific cytokine responses are not suppressed demonstrates that reduced proliferation is not a result of ineffective Ag-processing or presentation. The observation that D10.G4 T cells are unable to divide even in the presence of excess control APC, further suggests suppression is an active process, which cannot be accounted for by an absence of, or defect in, co-stimulation. Infection with adult worms or L3 leads to the development of a profound suppression suggesting some component of the Th2 response

is important in driving development of suppression. Indeed, studies using gene-knock-out mice have demonstrated an essential role for IL-4 but not IL-10 in development of a suppressive APC population (MacDonald, 1998). *In vitro* neutralisation of IL-4 or IL-10 demonstrated neither cytokine had direct suppressive effects, but rather act via the APC (MacDonald, 1998). While the mechanism whereby infection-derived PEC suppress proliferative responses has not been identified, there are qualitative differences in the suppressive effects generated by infection with different life cycle stages. Proliferative suppression following infection with adult worms, L3 or mf, is independent of H_2O_2 or prostaglandin production. Neutralisation of IFN-γ or TGF-β, both of which have been shown to have cytostatic properties (Gajewski, 1988), also failed to restore proliferative responses. Interestingly, whilst suppression following infection with adults or L3 was not mediated by nitric oxide (NO) (which has been identified as a key mediator of proliferative suppression in several models of infectious disease), inhibition of NO partially reversed the suppression by PEC from mf-implanted mice. However even following mf-infection only background levels of proliferation could be achieved following NO inhibition and Ag-specific proliferation was not restored. These results further demonstrate different life cycle stages possess distinct mechanisms to modulate the host immune response.

Infection with live worms is necessary for the development of suppression which may be in part due to the immunomodulatory properties of filarial excretory/secretory (E/S) products. Besides implantation of live adults or L3, daily injection of adult worm E/S products has been shown to be sufficient to drive development of the suppressive cell population (Allen, 1998). Furthermore injection of E/S from two other nematodes *Nippostrongylous braziliensis* and *Toxocara canis* also leads to reduced T cell proliferation, suggesting that, as a mechanism of down-regulating host responses, this may be a feature common to parasitic nematodes. In light of this, it is interesting that *N.*

brasiliensis E/S products have recently been shown to be capable of stimulating Th2 responses to a soluble bystander Ag, co-administered at the same site, which may have implications regarding responses to concomitant infection (Holland, 2000).

A common feature of E/S of filarial parasites is that they contain phosphoryl choline (PC) (Lal, 1990). ES-62 is a major PC containing E/S product of the rodent filarial parasite *Acanthoceilonema viteae* (Harnett, 1993) and has been shown to have various immunomodulatory properties. It has recently been demonstrated that ES 62 interacts directly with APC, signalling dendritic cells to acquire a phenotype which drives development of Th2 responses (Whelan, 2000). Dendritic cells are specialised APCs required for the priming and activation of $CD4^+$ T cells, and as such can potentially direct the subsequent differentiation of T cell function (Banchereau, 1998). Whelan *et al* (Whelan, 2000) demonstrated that culture of GM-CSF matured bone marrow derived dendritic cells with LPS or ES62 leads to development of DC phenotypes promoting the differentiation of naïve $CD4^+$ T cells (OVA specific DO11.10 T cells) towards either a Th1 or Th2 phenotype respectively. These results, and those previously discussed (Allen, 1998; Holland, 2000), suggest that signals dictating the phenotype of the resultant adaptive immune response may be intrinsic to nematode products and mediated via interactions with the innate immune system. The ability of E/S products to drive dendritic cells to promote Th2 responses following infection may be an important factor in driving IL-4 production, inducing development of alternatively activated macrophages, further cementing Th2 polarisation whilst down-regulating proliferative responses. ES62 can also mediate direct suppressive effects on both T and B cells and can inhibit murine B cell proliferative responses (Harnett, 1993). Pre-exposure of Jurkat T cells to ES62 renders them anergic to intracellular signalling via the TCR (Harnett, 1998). PC containing

somatic extract of *B. malayi* has also been shown to suppress phytohaemagglutinin induced T-cell proliferation *in vitro* (Lal, 1990).

(iv) Down-regulation of IFN-γ production in the mouse model

Ag-specific cytokine responses in murine models of filariasis, like those in human infection, are generally characterised by production of IL-4 with low or absent IFN-γ responses. As reported in human bancroftian filariasis (King, 1993; Mahanty, 1996) there is evidence that cytokines such as IL-10 may play an important role in down-regulating IFN-γ responses following murine infection.

Mitogen driven IFN-γ production is significantly reduced *in vitro* following s.c. infection of BALB/c mice with L3 of *B. pahangi* and splenocytes from these animals do not produce Ag-specific IFN-γ. While anti-IL-4 treatment restored mitogen driven IFN-γ responses, only anti-IL-10 treatment allowed production of Ag-specific IFN-γ (Osborne, 1996; 1999). Anti-IL-10 treatment also enhances Ag-specific IFN-γ production following chronic infection with mf or repeated immunisation with mf-extract (Pearlman, 1993b). As seen in human filariasis there is also evidence of a role for TGF-β in suppressing IFN-γ production. Alternatively activated macrophages, derived from i.p. infection with *B. malayi,* preferentially induce Th2 differentiation of naïve $CD4^+$ T cells from pigeon cytochrome-c-specific TCR transgenic mice. In contrast, stimulation with control PEC leads to development of Th1 cells, producing high levels of IFN-γ and little IL-4 (Loke, 2000). Infection derived PEC down-regulate IFN-γ production via TGF-β independently of IL-10, anti-TGF-β but not anti-IL-10 treatment enhancing IFN-γ production to levels equivalent to those in control PEC primed cultures. In contrast to previous reports in human infection, however (King, 1993), anti-TGF-β was unable to restore proliferative responses. The fact that anti-TGF-β treatment reversed inhibition of IFN-γ production but

not defective proliferation could suggest that there is no direct relationship between these two phenomenon in this form of suppression. Alternatively it may be that proliferative responses are more profoundly suppressed than IFN-γ production. In this respect it is of note that splenocytes from mf-infected BALB/c mice fail to show Ag-specific proliferative responses whilst retaining the ability to produce Ag-specific IFN-γ (see above, Osborne, 1996). These results clearly demonstrate that, dependant upon the life cycle stage, and route of infection, the same end (down-regulation of IFN-γ production), may be achieved by a variety of means.

Whilst some down-regulatory effects may be more profound than others, it is conceivable that during the course of human infection, some, or all of these mechanisms may be operable. Furthermore distinct mechanisms, acting independently, or in concert, may assume greater or lesser significance at different times and even locations over the course of infection. In this way, rather than employing a single "shell" as defence against the elements of the immune system, it seems likely that the parasite has developed multiple mechanisms which may contribute to suppression, effectively forming a "layering system". While no single factor has been shown to account for proliferative suppression, or down-regulation of IFN-γ responses, in human or murine infection, several contributory factors have been identified. The development of various mechanisms to achieve apparently similar goals is indicative of their importance to the parasite. This is further supported by the observations that proliferative and IFN-γ responses are strongest amongst putatively immune E.N. (Dimock, 1996), and may be recovered post-drug-treatment of infected individuals (Lammie, 1992; Piessens, 1980b; Sartono, 1995b). The induction of hyporesponsiveness may then, represent a parasite survival strategy, allowing continued survival and maximising chances of transmission. In this sense it becomes important to

assess the contribution of various mechanisms, and the life cycle stages driving them, to the security of this system. The close association between the presence of circulating mf and the most profound suppression of proliferative responses and IFN-γ production, suggest that mf-specific responses make an important contribution to hyporesponsiveness in human filariasis. The recent observations of mf-induced Ag-specific proliferative suppression (Osborne, 1996), and down-regulation of IFN-γ responses during chronic mf-infection (Pearlman, 1993b), suggest the mouse model provides a powerful system in which to analyse the nature of mf-specific mechanisms of immune modulation.

1.5. Nitric Oxide

Nitric oxide (NO), one of the smallest known biologically active metabolites, displays a fascinating diversity of biological functions. Over the last 15 years renewed interest in the biology of NO has revealed its involvement in a number of fundamental processes, including important roles in both innate and acquired immunity. NO is now known to affect a range of biological functions, including, vascular and muscle relaxation, platelet aggregation, neuronal cell function, apoptosis and host defence, as well as being implicated in various immuno-pathologies (reviewed Liew, 1995). Research in immuno-parasitology has provided a considerable amount of information regarding the role of NO, both as an effector mechanism in host defence and in immunomodulation (James, 1995; Liew, 1995; MacMicking, 1997). The wealth of information gained from these systems probably reflects the intimate and delicate nature of the host parasite relationship. Such studies have also illustrated the immunologically diverse effects of NO, which, besides direct anti-microbial effects, can suppress potentially harmful or beneficial immune responses and may also contribute to pathology. It seems clear then, that production of

such an important bio-active molecule must be under stringent control, and, with NO, this is indeed the case.

NO is derived from L-arginine and molecular oxygen in a reaction catalysed by nitric oxide synthase (NOS). To date three major mammalian isoforms of NOS have been identified, and all are thought to catalyse production of NO via the same pathway, involving two sequential mono-oxygenase reactions (Michel, 1996). One molecule of L-arginine is oxidised at its terminal guadino nitrogen to produce *N*w-OH-L-arginine which is further oxidised to yield one molecule each of NO and L-citrulline (reviewed – MacMicking, 1997). The three isoforms of mammalian NOS are named after the cell type from which they were first isolated and the main characteristic of their regulation (Bredt, 1991; Lyons, 1992). The two constitutive forms (cNOS) were first identified in neuronal (NOS1/ncNOS) and endothelial cells (NOS3/ecNOS). These constitutively expressed isoforms are dependant upon elevated levels of intracellular Ca^{2+} for activity (cNOS) (Bredt, 1990). NOS 2 or macrophage NOS (Lyons, 1992) however, is inducibly expressed and functions independently of elevated levels of Ca^{2+} leading it to be termed iNOS. All three isoforms require calmodulin binding for activity (Bredt, 1990; Dawson, 1992). NOS 1/3 can only bind calmodulin (CaM) when intracellular Ca^{2+} is elevated, while iNOS contains tightly bound CaM and therefore has a much reduced requirement for intracellular Ca^{2+} (Cho, 1992). The differential requirement for intracellular Ca^{2+} is thought to form the biochemical basis for NO production by NOS 1/3 being relatively "low output" while that of NOS 2 is considered "high output". It has generally been considered that high output NO production is involved in cell mediated cytotoxicity and host immunity, while low output constitutively expressed NOS are concerned with homeostatic "housekeeping" functions. However the discovery that cNOS may have roles in leukocyte adhesion (Kubes, 1991) and the induction of apoptosis during thymic maturation (Aiello, 2000;

Williams, 1998) suggest they may also be of relevance in immunology. However, it is clearly the high output NO pathway, under the direction of iNOS, that has central importance with regard to models of parasitic disease and which is reviewed below.

1.5.1. Induction of NO

iNOS is absent in strictly resting cells but is strongly induced by cytokines and other immunological stimuli. Expression is both transcriptionaly and post-transcriptionaly regulated, and involves a number of signal transduction pathways and molecules, including: Jak1/Stat1α/IRF-1; IκB/NFκB; MAPK; PKC and others (reviewed Bogdan, 2000a). The induction of iNOS expression has received considerable attention over recent years. It is clear that expression can be up or down-regulated by a multitude of factors, and that the outcome depends upon the net effect of all factors present, as well as the sequence of their encounter. Generally, pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-1β, associated with Th1 responses, and microbial products such as LPS, promote iNOS induction whilst cytokines such as IL-4, IL-10 and TGF-β inhibit NO synthesis.

Ding *et al* (1988) investigated the induction of NO production in murine peritoneal macrophages (MO), using a battery of recombinant cytokines. When tested as a sole agent only IFN-γ could independently induce NO production, while LPS acted in synergy with IFN-γ to augment NO production. Although TNF-α/β or IFN-α/β could not independently induce iNOS activity, either IFN-γ and TNF-α/β or IFN-α/β/γ and LPS can interact synergistically to enhance NO production. These results reflect the findings of a study on NO production during BCG infection. Splenic and peritoneal macrophages from BCG infected mice produce NO *in vitro,* and rIFN-γ which enhanced NO production also acted synergistically in this respect with either LPS or heat killed BCG (Kamijo, 1994). The iNOS promoter region confers inducibility by IFN-γ and synergistic inducibility by LPS

and IFN-γ, and contains multiple interferon regulatory factor response elements (Xei, 1993). Interferon regulatory factor-1 (IRF-1) is essential to the induction of iNOS activation in murine macrophages, and infection with *Mycobacteria bovis* (BCG) is more severe in IRF-1-/- mice than their wild type counterparts (Kamijo, 1994). These findings demonstrate that whilst IFN-γ appears of primary importance in priming MO, with LPS acting as a "triggering" factor, other cytokines can also enhance NO production.

The sequence of exposure to these signals is, however, essential in determining the outcome. Simultaneous or sequential exposure to IFN-γ and LPS being required to induce NO synthesis, while pre-exposure to LPS has an antagonistic effect upon NO production (Lorsbach, 1992; Severn, 1993). TNF-α production is induced endogenously in macrophages by LPS and is thought to provide the major physiological secondary signal for IFN-γ primed macrophages to produce NO (Drapier, 1988; Oswald, 1992b). The demonstration that *Leishmania major* amastigotes initiate NO production in IFN-γ primed macrophages via the induction of TNF-α suggests that products of parasite origin may also directly induce TNFα production (Green, 1990c). The glycosylphosphatidylinositol toxin of *P. falciparum* has also been shown to promote MO activation and NO production providing further evidence of the ability of parasite products to interact directly with MO (Tochado, 1996).

Like LPS, IFN-α/β can have dual effects upon iNOS expression dependent upon the sequence of exposure. While unable to act alone to induce iNOS expression in murine MO, IFN-α/β can act in synergy with LPS or *L. major* for iNOS induction. Indeed IFN-α/β have been identified as principal inducers of iNOS activity at very early time points following infection with *L. major* (1 day) in a study which demonstrated the importance of iNOS in the innate immune response (Diefenbach, 1998). However, IFN-α/β have an

inhibitory effect when added to MO prior to receipt of the primary stimulus (IFN-γ/LPS or *L. major*) whereupon IFN-α/β inhibit the activation of NFκB a critical transcription factor for iNOS activation (Lopez, 1998).

1.5.2. Factors down-regulating NO production

A variety of factors have been identified which can down-regulate NO production, most notably cytokines associated with Th2 responses, IL-4, IL-10 and TGF-β. As the induction of NO production is initiated by IFN-γ, the prototypic Th1-type cytokine, regulation of an essentially Th1 function by Th2 cytokines represents another level upon which these oppositely polarised subsets, of great importance in parasitic disease, can interact.

IFNγ up-regulates PKC activity in murine MO (Hamilton, 1985) and IFN-γ induced up-regulation of PKC is essential to the induction of iNOS activity (Severn, 1992). Conversely IL-4 inhibits PKC activity and reduces iNOS expression, suggesting that blocking the PKC activation pathway may represent one mechanism whereby IL-4 can inhibit NO production. Exposure to IL-4 prior to activation by IFN-γ is necessary for IL-4 to display such inhibitory effects (Liew, 1991). Intriguingly treatment of pre-activated MO with IL-4 has also been shown to enhance NO production (Stenger, 1991). Such findings indicate further levels of complexity in the interactions of IL-4 and IFN-γ in influencing NO synthesis, and suggest that factors besides PKC activation are probably involved.

IL-10 has been shown to inhibit the IFN-γ induced NO dependant killing of both intra-cellular and extra-cellular parasites in a dose dependant fashion (Gazzinelli, 1992). Unlike IL-4 or TGF-β, IL-10 inhibits IFN-γ induced NO production via the inhibition of endogenous TNF-α production by MO. Recombinant IL-10 potently suppresses both LPS

and IFN-γ induced TNF-α production in murine MO, demonstrating that IL-10 can suppress MO microbicidal function by a pathway distinct from that of other down-regulatory cytokines (Bogdan, 1991; Oswald, 1992b).

TGF-β1, β2 and β3 have also been shown to inhibit NO production when present during the induction phase or prior to activation with IFN-γ (Ding, 1990). Using both anti-sense oligonucleotides, complementary to TGF-β mRNA, and anti-TGF-β antibodies, it has been shown that inhibition of endogenously produced TGF-β during MO activation, significantly enhances NO production (Jun, 1995). Such results suggest that TGF-β production may provide an initial self-regulatory mechanism controlling NO production.

As seen amongst factors promoting NO production, IL-4, IL-10 and TGF-β display synergy in down-regulating NO production, suggesting that, for NO susceptible organisms, induction of these regulatory cytokines may represent a parasite survival strategy (Oswald, 1992a). IL-4, IL-10 and TGF-β are also only able to inhibit IFN-γ induced NO production when present prior to, or simultaneously with, exposure to IFN-γ (Ding, 1990; Liew, 1990b). These results further demonstrate the importance of the sequence of exposure to NO production and emphasise the importance of early events in the immune response.

Asseury *et al* demonstrated that exposure to NO generated by chemical donors inhibited NO synthase activity in both the murine MO cell line J774 and in a cell free system. These findings demonstrate that, in a manner characteristic of important biological systems, NO can down-regulate its own production via a negative feedback loop (Assreuy, 1993). Besides this direct effect, in certain circumstances IFN-γ may also act indirectly to down-regulate NO production. IFN-γ up-regulates expression of the A_{2B} adenosine receptor on the surface of MO (Xaus, 1999). Adenosine modulates different functional

activities in activated MO, including NO production and stimulation of the A_{2B} receptor by adenosine inhibits IFN-γ induced iNOS expression (Hon, 1997), thus representing another potential mechanism of MO deactivation.

The multiple factors inducing and suppressing NO production, the sequential nature of their effects and the presence of feed-back mechanisms to limit over-production, illustrate the delicate balance, and highly context-dependent control, involved in the regulation of NO production.

1.5.3. Microbicidal activity of NO

NO has now been shown to play an important effector function in a great variety of infectious diseases including bacterial, viral, and parasitic infections (Bogdan, 2000b; James, 1995; Liew, 1993). While the study of such infections has provided much information regarding factors affecting NO production during infection, they are only now beginning to precisely identify the anti-microbial targets of NO.

The biochemical basis of the cytostatic action of NO is thought to be primarily due to the inactivation of enzymes involved in critical metabolic pathways (Woods, 1994). NO can inactivate several key metabolic enzymes with [4Fe-4S] prosthetic groups at their catalytic sites, via the formation of iron-dinitrosyl-dithiolate complexes (Woods, 1994). Nitrosylation of Fe-S centres is associated with inactivation of the aconitase enzyme of the Krebs cycle (Drapier, 1986; Stamler, 1992), several enzymes involved in the electron transport chain (Drapier, 1986; Granger, 1982; Stamler, 1992), and ribonucleotide reductase, which is involved in DNA synthesis and cellular proliferation (Kwon, 1991). In addition to reactions with metals, thiols and amines, NO can also react with superoxide

anions to form peroxynitrite anions which decay rapidly to form highly reactive hydroxyl radicals (Beckman, 1990).

Studies on schistosomes have suggested that the principal targets of NO are enzymes containing a catalytically active Fe-S group at their active site (James, 1995). MO derived NO is effective in killing larval schistosomes (schistosomula) *in vitro* (James, 1989). Ultra-structural studies on skin stage schistosomula co-cultured with activated MO recorded progressive disintegration of parasite internal structures (McLaren, 1985). Subsequent studies demonstrated that inhibition of the schistosome achonitase enzyme or of electron transport is similarly toxic to schistosomula, in terms of pathology, suggesting that inhibition of larval metabolism is involved in NO mediated toxicity (Fouad, 1994).

Recently the first direct evidence of the ability of NO to display effector function via *S*-nitrosylation of a critical enzyme has been provided in a model of viral infection. Coxackie virus B3 (CVB3) induces iNOS expression in MO which in turn limits viral replication. NO inactivates the coxsackie virus protease 3C via *S*-nitrosylation of the cysteine residue at the active site, inhibiting protease activity and viral replication (Saura, 1999). As cysteine proteases are critical for virulence or replication in many viruses, bacteria and parasites, it was proposed that *S*-nitrosylation of pathogen cysteine proteases may represent a general anti-microbial mechanism. This is supported by the recent demonstration that NO also inhibits cruzipain the major papain-like cysteine protease of *T. cruzi*. Cruzipain is expressed on all life cycle stages of the parasite and has important roles in parasite nutrition, penetration of host cells and immune evasion. NO mediated *S*-nitrosylation inhibits the activity of both cruzipain and falcipain (the cruzipain homologous cysteine protease of *P. falciparum*), further suggesting inhibition of cysteine proteases maybe a widespread mechanism of anti-microbial activity (Venturini, 2000).

1.5.5. NO in mouse models of leishmaniasis

As a model of disease wherein resistance or susceptibility correlates with the development of differentially polarised immune responses, *Leishmania* provides a prime example of the Th1/Th2 paradigm. Protective responses in *L. major* infection are characterised by the induction of IFN-γ producing $CD4^+$ T cells while susceptibility is associated with the development of a Th2 response (Heinzel, 1989). The availability of genetically resistant (C57BL/6) and susceptible (BALB/c) mice, which develop Th1 and Th2 responses respectively, provides an ideal experimental situation in which to address factors influencing the polarisation of responses, and their associated effector mechanisms (Scott, 1989).

As NO is an effector mechanism associated with Th1 responses and resistance to *Leishmania*, it is perhaps unsurprising that this model system has provided some of the most illuminating results regarding the induction of iNOS activity and its anti-parasitic effects. Green *et al* (1990a) first demonstrated that IFN-γ activated MO destroyed intracellular *L. major* by an L-arginine dependant mechanism. Killing of *L. major, in vitro,* could be inhibited by either, NG-NMMA (a competitive inhibitor of iNOS activity) or by addition of arginase (an enzyme which converts L-arginine to L-ornithine and urea, thus depleting available substrate (Green, 1990b)). Liew *et al* further demonstrated that NO dependant killing is operative *in vivo.* Injection of the iNOS inhibitor L-NMMA into the lesions of resistant CBA mice, infected with *L. major,* led to an exacerbation of disease and a 10^4-fold increase in the number of parasites recoverable from lesions (Liew, 1990a). More recently the observation that iNOS-/- mice on a genetically resistant background are susceptible to *L. major* infection clearly demonstrates the importance of NO in controlling infection (Wei, 1995). This study also recorded no significant difference in lesion size

between iNOS-/- mice and their wild type counterparts up to 5 weeks post-infection, leading to speculation that innate responses are unlikely to be associated with NO-mediated effector mechanisms (Wei, 1995). However, more recently, it has been shown that genetic deletion or functional inactivation of iNOS also abolishes IFN-γ and NK responses at very early time-points (1 dpi.). This effect is associated with increased expression of TGF-β and enhanced parasite dissemination. The induction of iNOS activity at this early stage of infection is dependant upon IFN-α/β. Neutralisation of IFN-α/β mimics the phenotype of iNOS-/-mice, demonstrating that IFN-α/β can act as critical regulators of the innate response to *L. major* (Diefenbach, 1998).

These results of the studies outlined above have allowed the relevance of factors implicated in the induction or inhibition of iNOS activity to be assessed in a model of parasitic infection. IFN-γ and TNF-α have been shown to promote iNOS activity while down-regulatory effects of IL-4, IL-10 and TGF-β have all been reported in the leishmania model. Using recombinant cytokines, Liew *et al* demonstrated that IFN-γ and TNF-α were active *in vivo* in promoting NO production and parasite killing, and did so in a synergistic fashion when administered at individually sub-optimal levels (Liew, 1990b). It was further shown that a combination of IFN-γ and *L. major* amastigotes induced high levels of NO production when administered together but were individually ineffective. The "triggering" effect of amastigotes was shown to be mediated via the induction of endogenous TNF-α production which was further enhanced by IFN-γ (Green, 1990c).

1.5.6. Factors inhibiting iNOS induction: TGF-β, IL-4 and IL-10

The inhibitory effects of TGF-β upon NO production are well documented in models of leishmaniasis wherein early production of TGF-β is a factor associated with

susceptibility. Using immunocytochemistry, Stenger *et al* demonstrated that iNOS is produced earlier and in significantly greater amounts in resistant as compared to susceptible strains of mice (Stenger, 1994). Conversely cells staining positively for TGF-β were more abundant in susceptible (BALB/c) than resistant (C57BL/6) mice. Such findings suggest that the relative lack of iNOS expression in susceptible mice may be a result of MO deactivation by TGF-β and reduced responsiveness to IFN-γ. This has been supported by work showing that TGF-β treatment prior to IFN-γ activation abolished the intracellular killing activity of infected MO (Nelson, 1990). Barral netto *et al* also demonstrated TGF-β production in the footpads of infected BALB/c mice (Barral-Netto, 1992). Furthermore it was shown that anti-TGF-β treatment of BALB/ mice led to development of a healer phenotype, associated with enhanced Th1 responses.

Both IL-4 and IL-10 have also been shown to be effective in inhibiting the lieshmanicidal activities of MO when present prior to IFN-γ activation (Liew, 1991; Vieth, 1994). Down-regulation of NO production by IL-4 may represent one way in which disease promoting Th2 cells counteract the host protective effects of Th1 cells in murine models of leishmaniasis. Neutralisation of endogenously produced IL-10, using either neutralising antibody or an anti-sense approach, also led to an enhancement of IFN-γ induced leishmanicidal activity, suggesting that, like TGF-β, IL-10 may act as an autocrine regulatory factor in MO activation (Vieth, 1994).

1.5.7. The role of IL-12 in promoting and maintaining Th1 responses and NO mediated effector function

That development of Th1 responses is necessary for the induction of NO dependant killing has been further illustrated by studies of factors affecting the polarisation of

responses. IL-12 and IL-4 are known to be involved in the generation of Th1 and Th2 responses respectively (Hsieh, 1993; Mattner, 1997; Schmitt, 1994; Swain, 1990). The IL-12 p70 heterodimer is a potent inducer of IFN-γ production by T cells and NK cells and promotes differentiation of naïve T cells towards the Th1 phenotype (Trinchieri, 1995). Mice susceptible to infection with *L. major* can be cured by administration of rIL-12 or anti-IL-4 antibody early in infection (Corraliza, 1995; Heinzel, 1993; Heinzel, 1995; Mattner, 1997; Sypeck, 1993). Furthermore genetically resistant C57BL/6x129Sv mice with homologous disruption of the gene encoding the p35 or p40 subunit of IL-12 are rendered susceptible to infection (Mattner, 1996). These results convincingly demonstrate the importance of IL-12 in generating an appropriate immune response that allows the resolution of infection.

Interestingly it has recently been shown that dendritic cells, professional APC of primary importance in the induction of immune responses, contain membrane bound stores of bio-active IL-12. This novel source of IL-12 is mobilised and released within minutes of contact with *L. donovani* (Quinones, 2000). Intriguingly it has also been shown that *L. major* infected Langerhans cells (skin dendritic cells) from BALB/c mice are not impaired in their production of IL-12, and can be used successfully to vaccinate syngeneic mice (von Stebut, 2000). These results suggest that defective Th1 development in BALB/c mice may result from enhanced production of Th2 cytokines, or failure to maintain responsiveness to IL-12, rather than an initial deficiency in IL-12 production. In support of this it has recently been shown that continued production of endogenous IL-12 is necessary for the maintenance of IFN-γ dependant resistance to *Toxoplasma gondii* (Yap, 2000). Following anti-IL-12 treatment, *L. major* infected, genetically resistant, CH3 mice, develop a Th2 response similar to that of susceptible BALB/c mice. However upon

withdrawal of anti-IL-12 treatment CH3 mice spontaneously switch to a Th1 response and resolve their lesions (Hondowitz, 1997). Furthermore the differential ability of $CD4^+$ cells from BALB/c or CH3 mice to maintain responsiveness to IL-12 is of central importance in the switch to a Th1 response (Hondowitz, 2000). The findings reported in these studies suggest that the beneficial effects of IL-12 may be more far-reaching than previously supposed, and are not confined to polarisation at the initial stages of the immune response.

1.5.8. LPS and molecules of parasite origin can inhibit development of leishmanicidal activity

Studies using *Leishmania* have also demonstrated that exposure of MO to factors of bacterial and parasitic origin can affect subsequent NO production. Pre-exposure of the murine macrophage cell line J774 to LPS inhibits development of IFN-γ/LPS induced NO production and leishmanicidal activity (Severn, 1993). Inhibition of iNOS induction by prior exposure to LPS is considered to be a manifestation of endotoxin tolerance and a means of regulating NO production which may represent a survival mechanism for intra-cellular parasites.

Leishmania may also affect MO activation directly via the interaction of parasite surface molecules with MO. Glycoinositolphospholipids (GIPL) are the predominant surface glycolipids in both developmental stages of *Leishmania sp.* Purified GIPL can inhibit the IFN-γ/LPS induced production of NO by J774 MO in a dose-dependant fashion. Consequently prior exposure of MO to GIPL enhances survival of *L. major* in activated MO. The inhibitory activity of GIPL is contained within the lipid fraction and functions independently if TNF-α production (Proudfoot, 1995). Such findings demonstrate one way in which molecules of parasite origin may regulate MO function.

1.5.9. NO in models of schistosomiasis

The observation of James and Glaven, that activated MO killed schistosomula of *S. mansoni* by an NO dependant mechanism, represents one of the earliest demonstrations of the microbicidal effector functions of NO (James, 1989). As a model of helminth infection wherein NO clearly displays effector function, studies in mouse models of schistosomiasis have further indicated the diversity of roles NO may play at different stages of infection.

In a murine vaccination model, immunisation with irradiated cercariae of *S. mansoni,* leads to a 60 – 80% reduction in worm survival following challenge infection (Minard, 1978). Resistance to challenge infection is dependant upon $CD4^+$ T cells and the development of Th1 type responses (Sher, 1990; Vignali, 1989). The majority of challenge larvae are thought to be killed traversing the lungs of immunised mice. IFN-γ and iNOS mRNA expression have been found in the pulmonary inflammatory foci surrounding migrating larvae, suggesting iNOS is involved in generating resistance *in vivo* (Wynn, 1994). *In vivo* treatment of mice with aminoguanidine (AMG, a selective inhibitor of iNOS activity), or genetic deletion of the iNOS gene, leads to a significant reduction of resistance to challenge infection (James, 1998; Wynn, 1994). MO taken from iNOS-/- mice also fail to develop larvicidal activity when activated with IFN-γ / LPS *in vitro* (James, 1998). These findings are consistent with a role for NO as an effector mechanism of the protective immune response induced by vaccination with radiation attenuated cercariae.

Studies using iNOS-/- mice, or *in vivo* inhibition of iNOS activity, have shown that NO may affect various parameters of immune responsiveness, including cytokine production and subsequent regulation of isotype switching, as well as an involvement in

inflammatory responses. James *et al* (98) demonstrated that besides acting as an effector molecule, NO plays important roles in regulating the Th1 vs Th2-type cytokine balance following vaccination with irradiated cercariae. Expression of Th1-type cytokines (IFN-γ and TNF-α) was found to be significantly enhanced, and levels of Th2 cytokines (IL-4 and IL-5) lower, in iNOS-/- mice than their wild type counterparts. Th1 associated humoral responses were enhanced in iNOS-/- mice, seen as elevated levels of IgG2a, and decreased levels of IgE production. Vaccinated iNOS-/- mice also developed smaller inflammatory foci around schistosomula in the lungs. Changes in the size and eosinophil content of inflammatory foci correlated well with reduced IL-5 production in iNOS-/- mice (James, 1998). These studies demonstrate that NO can affect the production of both Th1 and Th2 type cytokines, and that the altered cytokine production profiles may be responsible for phenotypic changes observed upon iNOS inhibition.

The immune response to schistosome eggs that become lodged in host tissues is responsible for the pathology of schistosomiasis (Boros, 1989). Several studies have now implicated NO in modulating the development of pathology during infection. It has recently been demonstrated that PBMC from infected humans can produce NO *in vitro*, and that inhibition of NO exacerbates *in vitro* granuloma formation (Oliveira, 1998). These results suggest that NO production may be relevant to the course of human infection, and its influence may go beyond its role as a direct effector mechanism. Furthermore it has been demonstrated that immune complexes from the serum of patients with chronic schistosomiasis are able to reduce the granulomatous reaction developed in an *in vitro* model system. The reduction in granuloma size could be reversed by the inhibition of NO production which resulted in decreased production of prostaglandin E_2

(Neves, 1999). These results suggest that NO may regulate the bio-synthesis of PGE_2, which may further modulate granulomatous hypersensitivity against *S. mansoni* eggs.

Recent studies have also demonstrated that the functions of NO are not static, and that at different stages of infection NO may mediate dramatically different effects. Following the onset of egg deposition in the liver, in murine infection with *S. mansoni,* NO is required to limit the development of pathology. Prolonged production of NO however (as seen in susceptible IL-4-/- mice), is highly deleterious and associated with severe hepatotoxicity. IL-4-/- mice fail to reduce production of NO as infection progresses, suggesting that egg-induced Th2 responses are required to regulate NO production, and limit NO mediated tissue damage. Brunet *et al* (99) reported that the development of hepatosplenomegaly was severely reduced upon iNOS inhibition. Similar observations have recently been reported following iNOS inhibition *in vivo* during murine infection with a highly attenuated strain of salmonella (SL3235). iNOS inhibition by AMG reduced splenomegaly, associated with a decrease in the influx of neutrophils and MO to the spleen (Shearer MacFarlane, 1999). These findings suggest that either NO is directly chemotactic, or that it may regulate expression of chemokines involved in cell trafficking towards sites of inflammation.

An intriguing feature of these studies is that iNOS-/- mice and *in vivo* inhibition of iNOS activity have provided somewhat contrary results regarding the involvement of iNOS in inflammatory responses in murine schistosomiasis. Studies using iNOS-/- mice and *in vivo* inhibition both reported quantitative reductions in inflammatory responses in the absence of NO production, smaller inflammatory foci in the lungs, and hepatic granuloma formation respectively (Brunet, 1999; James, 1998). However only in iNOS-/- mice were qualitative differences in responsiveness reported. James *et al* reported that in

iNOS-/- mice enhanced Th1 responsiveness was associated with formation of smaller inflammatory foci in the lungs which contained significantly fewer eosinophils (James, 1998). In contrast, while Brunet *et al* found iNOS inhibition *in vivo* led to development of smaller hepatic granulomas, they displayed no alteration in cellular make up. Most interestingly Hesse *et al* (2000) found that schistosome egg/IL-12 sensitized iNOS-/- mice, which, like AMG treated IL-4-/- mice are reported to display enhanced Th1 responsiveness in the absence of NO production, did not display many of the pathological changes reported by Brunet *et al*. Furthermore while iNOS inhibition led to development of smaller hepatic granulomas iNOS-/- mice developed significantly larger granulomas than their wild type counterparts. Such differences are difficult to explain, it is possible that AMG incompletely inhibited iNOS activity or alternatively that AMG treatment during infection may generate undesirable toxic side effects.

1.5.10. NO in filariasis

Several studies have presented evidence suggestive of an anti-parasitic role for NO in filarial infection. However while inferring potential effector function, there are conflicting results from studies using iNOS inhibition and those using iNOS-/- mice. Taylor *et al* (1995) investigated the susceptibility of microfilariae of *O. lienalis* and *B. malayi* to H_2O_2 and NO *in vitro*. Whilst *B. malayi* mf proved to be relatively resistant to H_2O_2, exposure to NO reduced microfilarial motility, and prolonged exposure proved toxic. Exposure of mf to NO generated by a chemical donor (SNAP, 32.7+/-0.8 μM NO_2^-) caused a permanent reduction in microfilarial viability. IFN-γ activated MO produced lower levels of NO (7-10 μM NO_2^-) which also significantly reduced mobility. The toxicity of IFN-γ activated MO to mf could be inhibited by L-NMMA, or anti-TNF-α antibodies. Thomas *et al* (1997) obtained similar results and further demonstrated that *B.*

malayi mf are more susceptible than adult worms to the NO mediated anti-parasitic effects of activated MO. The toxic effects of activated MO could be mimicked by exposure to chemical donors of NO, but far higher levels of NO exceeding physiological levels (130μM vs 27μM NO_2^-) were required, suggesting that peroxynitrite or its by products may be more potently damaging to mf than NO itself. Ultrastructural examination of NO damaged worms revealed that hypodermal mitochondria were highly vacuolated, reminiscent of morphological changes observed during the NO mediated killing of schistosomula (James, 1989).

There is also evidence that *B. malayi* L3 are susceptible to NO mediated killing. Treatment of immunocompetent, non-permissive BALB/c mice with AMG was shown to abrogate resistance to L3. AMG treated mice displayed significantly higher worm burdens than their untreated counterparts when examined five weeks post i.p. infection with L3. In complementary experiments it was shown that treatment of susceptible C.B-17*scid* mice with the NO releasing compound DEA/NO led to a significant reduction in worm burden at the same time-point post infection. It is of note however, that contrary to these findings, more recent studies from this same laboratory found iNOS-/- mice no more susceptible to *B. malayi* infection than their wild type counterparts (L. Schultz Pers. comm.).

The results of the *in vitro* studies outlined above demonstrate clearly that *B. malayi* mf are susceptible to NO mediated killing by activated MO. However, results of a recent study on the role of NO in innate resistance to *L. sigmodontis* mf, urge caution in extrapolating such results to the *in vivo* situation. *L. sigmodontis* mf were shown to be similarly sensitive to NO, produced by activated MO (9.7+/-0.75 μM NO_2^-), or chemical donors (SNAP, producing ~30-130μM NO_2^-). Ag stimulated splenocytes from infected animals also produced significantly higher levels of NO *in vitro* as compared to uninfected controls. While such circumstantial evidence appears suggestive of a role for NO in

generating resistance, neither *in vivo* inhibition of NO nor genetic deletion of iNOS abrogated resistance to circulating mf (Pfaff, 2000b).

There is some evidence to suggest that NO maybe involved in the clearance of mf post-drug treatment in human filarial infection. In animals models the *in vitro* cytotoxic effects of ivermectin upon mf of *A. viteae* and *L. carinii* have been shown to require the presence of cells, indicating that killing probably results from the action of host defence mechanisms (Zahner, 1994). Furthermore it has been demonstrated that the ivermectin-induced killing of *L. carinii* mf by neutrophils *in vitro* is mediated by NO and may be inhibited by L-NMMA (Zahner, 1997). This is of particular interest in light of a recent clinical study which demonstrated highly elevated levels of serum nitrite and nitrate during drug-induced mf clearance in human filariasis. Drug treatment of *Loa loa* or *O. volvulus* infected individuals led to increased levels of serum NO derivatives which peaked sharply at two to five days post-treatment and remained elevated for up to six months (Winkler, 1998). It should be noted however, that this was a very small study group of only four individuals and there is no evidence to demonstrate a cause and effect relationship. Although cytokine production profiles were not analysed in this study, it is possible that increased production of NO may reflect a restoration of Th1 responsiveness post-drug treatment rather than an effector mechanism. Studies carried out in an area endemic for *B. malayi* infection have demonstrated that adverse reactions to DEC treatment are most severe amongst Mf+ individuals (Haarbrink, 1999a) and are associated with increased production of pro-inflammatory molecules such as IL-6 and LPS binding protein (LBP) an acute phase protein (Haarbrink, 1999b). Such studies have suggested that adverse reactions post DEC treatment may be the result of an exaggerated host inflammatory response stimulated by the high antigenic load released from killed or degenerating mf or possibly by LPS released by the endosymbiotic *Wolbachia* (Taylor, 1998).

The studies outlined above demonstrate the extent of the influence of NO, affecting a range of dynamic factors during the course of an immune response. They also illustrate the need for caution in attributing any phenotypic results from studies in iNOS-/- mice, or using iNOS inhibition, to a direct action of NO as multiple factors are likely to be altered down-stream of iNOS induction, the effects of which may be seen in both *in vivo* and *in vitro* studies.

Besides its well documented role as an anti-microbial effector mechanism NO has emerged as a dynamic modulator of immune responses. Whilst low level NO production may promote the expansion of IFN-γ producing T cells, high levels of NO can suppress both mitogen-driven and Ag-specific proliferative responses and down-regulate production of cytokines such as IFN-γ and IL-2. Thus NO, a molecule potentially effecting immunity, may also limit the development of protective immune responses. In this way NO production may be of benefit to either host or parasite dependant upon the prevailing circumstances.

1.5.11. NO as a mediator of proliferative suppression

Albina *et al* (1991) first demonstrated that MO derived NO could suppress T cell proliferative responses. It was shown that the addition of excess macrophages inhibited the mitogen-driven proliferation of murine T cells. This effect correlated with an accumulation of NO_2^- in the culture medium and could be reversed by the addition of an iNOS inhibitor. Furthermore neutralisation of IFN-γ in splenocyte / MO co-cultures prevented production of NO and ablated the anti-proliferative effects of MO. These results demonstrate the importance of IFN-γ in inducing NO production by macrophages ultimately leading to proliferative suppression.

The role of NO in the development of immunosuppression has been investigated in various models of infection, and it has been shown to exert suppressive effects both *in vitro* and *in vivo*. Gregory *et al* (1993) reported that levels of serum nitrite and nitrate (indicative of NO production) were elevated during primary infection with *Listeria monocytogenes* in C57BL/6 mice. Hepatocytes from infected mice also produced significantly greater levels of NO_2^- upon *in vitro* stimulation with LPS/TNF-α/IFN-γ than did cells from uninfected animals. However *in vitro* inhibition experiments showed that the anti-listerial activity of hepatocytes was independent of NO production. Stimulation of splenocytes from infected mice with *Listeria* Ag led to the production of high levels of NO and significantly reduced proliferative responses. Inhibition of iNOS activity in these cultures led to reversal of proliferative suppression, demonstrating the role of NO and suggesting that this may be of relevance *in vivo*. Indeed mice treated with L-NMMA *in vivo* exhibited a 10 to 100 fold reduction in the numbers of *Listeria* in their livers on day 3 and 7 pi. respectively. These findings suggest that elevated production of NO during primary infection with *Listeria* limits the effectiveness of host defence mechanisms by suppressing proliferative responses.

NO has also been shown to mediate proliferative suppression in various models of parasitic infection including trypanosomiasis, toxoplasmosis and infection with *Echinococcus multilocularis* (Candolfi, 1994; Dai, 1999; Mabbot, 1995; Schleiffer, 1993). Candolfi *et al* demonstrated that both mitogen driven and Ag-specific proliferative responses are suppressed during the acute phase of murine infection with *Toxoplasma gondii*. Suppression was again associated with elevated levels of nitrite in splenocyte cultures and was reversible upon iNOS inhibition. Furthermore replacement of the resident adherent cell population with adherent cells prepared from uninfected animals reduced levels of NO production and restored proliferative responses. Transfer of adherent cells

from infected mice also suppressed the mitogen driven proliferative responses of splenocytes from uninfected animals in an NO dependant fashion. These results suggest that proliferative suppression is mediated by NO produced by MO activated during acute infection with *T. gondii*. Interestingly it has recently been shown that the NO mediated suppression of proliferative responses during acute infection is not systemic. While responses in the spleen are suppressed, cells from the mesenteric lymph nodes (MLN) maintain proliferative responsiveness. MLN cells and splenocytes produced equivalent amounts of IFN-γ in response to Con-A, but levels of NO_2^- were significantly lower in cultures of MLN cells. Differences in NO production were thought to reflect the presence of significantly greater numbers of MO amongst spleen cells as compared to MLN cells (Neyer, 1998). Such results demonstrate that NO may mediate a localised, organ specific, suppression of proliferative responses.

Further evidence of the anti-proliferative effects of NO, has been found in murine models of trypanosomiasis. Human and murine infection with African trypanosomes (*T. brucei spp.*) is associated with a severe immunosuppression. Peritoneal and splenic MO from infected mice suppress mitogen and Ag-driven proliferative responses in an NO dependant fashion (Sternberg, 1992). Mabbott *et al* (1995) demonstrated that adoptive transfer of suppressive MO to syngeneic recipients inhibited the Con A driven proliferative responses of splenocytes 3 – 4 days post transfer. Inhibition of NO production either *in vivo* or *in vitro* abrogated this suppressive effect, demonstrating that NO produced by donor MO mediates proliferative suppression. Furthermore drug cure of infected animals led to recovery of proliferative responses which was associated with reduced production of NO, both *in vivo* and *in vitro*. More recently studies in both IFN-γR-/- mice (which are unable to respond to infection with MO activation and NO production) and iNOS-/- mice, have further defined the anti-proliferative effect of NO in *T. brucei*

infection. Following infection of IFN-γR-/- mice suppression of T cell proliferative responses was reduced, whilst levels of parasitaemia were elevated and survival time reduced, compared to wild type mice (Mabbot, 1998). Infection of iNOS-/- mice also linked NO production to a depression of cell function. Splenocytes from infected iNOS-/- mice displayed increased proliferative and IFN-γ responses *in vitro* compared to wild type mice. Furthermore spleens from infected iNOS-/- mice contained higher numbers of total $CD4^+$ T cells and activated (IL-2R expressing) $CD4^+$ T cells. Such results suggest that NO affects T cell activation and proliferation *in vivo* as well as *in vitro* (Millar, 1999).

Murine models of malaria have also provided evidence of how NO can impair T cell function. It has been shown that malaria-specific Th1 but not Th2 T cell clones can produce NO upon *in vitro* restimulation at levels which affects their biological function. Th1 cells stimulated with a lysate of parasite-infected RBC expressed iNOS mRNA and produced NO which suppressed both their proliferative response and their ability to produce IFN-γ and IL-2 (Taylor-Robinson, 1994). Both cytokine production and proliferation could be restored upon inhibition of iNOS activity. Furthermore, splenocytes from mice treated with L-NMMA *in vivo,* and infected with *P. chabaudi,* produced elevated levels of IFN-γ and IL-2 *in vitro*, suggesting that NO also modifies cytokine production *in vivo.* NO is known to be involved in resistance against malaria infection and *in vivo* treatment of mice with L-NMMA (but not the inactive D isomer D-NMMA) significantly increased the primary peak of parasitaemia (Taylor-Robinson, 1994). The results outlined above demonstrate that, unlike infection with *Listeria*, during infection with malaria NO may be active in both anti-microbial and immunomodulatory capacities. Whilst being active as an effector mechanism of primary importance, NO may also down-regulate the proliferative and cytokine responses of Th1 cells in a specific fashion. In this

way inhibiting the biological activity of cells responsible for its production may represent one means whereby NO can limit the development of potentially pathological pro-inflammatory responses.

The dual biological roles of NO, on the one hand mediating host defence, whilst also having the potentially adverse effect of immunosuppression, may appear paradoxical, and at times incompatible. However, it has recently been shown that NO may function simultaneously in both capacities. Infection with SL3235, a highly attenuated strain of *Salmonella typhimurium* profoundly suppresses the ability of splenocytes from infected mice to mount an *in vitro* plaque forming response to sheep RBC, and to proliferate in response to Con-A. Inhibition of iNOS activity *in vitro* using L-NMMA alleviates suppression of both these responses, indicating suppression is NO mediated. Interestingly then, in light of this, *in vivo* treatment with AMG prior to infection leads to development of more severe disease. AMG treatment ablated the splenomegaly associated with SL3235 infection, blocking the influx of neutrophils and macrophages into the spleen, further suggesting that NO may be directly chemotactic or may influence production of chemokines involved in cell trafficking. Most unexpectedly AMG treatment of mice infected with this highly attenuated vaccine strain led to a 90% mortality rate, associated with an inability to clear organisms from the spleen and persistent bacteremia (Shearer MacFarlane, 1999). These results suggest that NO may be directly microbicidal in this system or may act via the recruitment of inflammatory cells essential to limit the spread of infection. It appears then, that following murine infection with SL3235, NO is responsible for the development of immunosuppression, whilst at the same time being of vital importance in host defence.

As it has been shown that NO can exert anti-proliferative effects and down-regulate production of IFN-γ and IL-2, it is perhaps unsurprising, that in the absence of

iNOS activity Th1 responsiveness may be enhanced (Wei, 1995). The mechanisms whereby NO affects development of Th1 responses are now being elucidated and modulation of IL-12 production has emerged as an important factor. Huang *et al* (Huang, 1998b) have shown that peritoneal MO from iNOS-/- mice produce significantly higher levels of IL-12 than their wild type counterparts when infected with *L. major in vivo* or stimulated with IFN-γ or LPS *in vitro*. IL-12 production by J774 cells upon stimulation with LPS, or LPS and IFN-γ was also enhanced upon iNOS inhibition, and was inhibited in the presence of the NO generating compound SNAP (Huang, 1998b). IL-12 is a major inducer of Th1 cells, promoting development of IFN-γ producing T cells which can further enhance IL-12 production. In light of the results described above, it was proposed that NO can inhibit this feedback loop via inhibition of IL-12 synthesis, preventing excessive amplification of Th1 responses.

The capacity of IL-12 to promote IFN-γ production leading to NO production and consequent proliferative suppression has been revealed in studies upon the adjuvant effects of recombinant murine IL-12 (rIL-12). As a potent inducer of Th1 $CD4^+$ T cell differentiation, $CD8^+$ T cell cytotoxicity and NK cell activation, IL-12 may appear an attractive means of promoting development of a selectively polarised immune response. However it has been demonstrated that use of rIL-12, during vaccination with genetically modified tumor cells, is associated with a dose dependant suppression of the immune response which was accompanied by suppressed *in vitro* responses to Con-A (Kurzawa Koblish, 1998). Suppression *in vitro* could be reversed by the addition of L-NMMA, and failed to develop in iNOS-/- and IFN-γ-/- mice. Such findings support the theory that high levels of IFN-γ, induced by rmIL-12, result in production of NO, which in turn mediates proliferative suppression (Kurzawa Koblish, 1998). These results further illustrate the

complex regulation involved in the generation of immune responses, and demonstrate how the inadvertent induction of negative feedback mechanisms may override potentially beneficial effects.

In contrast to the majority of data demonstrating the suppressive effects of NO, it has recently been demonstrated that low levels of NO may selectively enhance the development of Th1 cells. Stimulation of naïve, transgenic, OVA specific (DO11.10) T cells, in the presence of low doses of NO and IL-12 significantly enhanced production of IFN-γ as compared to Ag and IL-12 alone. Low level NO did not affect Th2 differentiation when DO11.10 cells were similarly stimulated in the presence of IL-4. NO acted synergistically with IL-12 in enhancing Th1 development and mediated its effects directly upon T cells. This was shown by enhanced IFN-γ production, in the presence of low levels of NO, upon direct stimulation with immobilised anti-CD3 MAb in the absence of APC. Interestingly the presence of low level NO had little, or no effect upon IFN-γ production by established cloned Th1 cell lines, and in all cases, exposure to high levels of NO resulted in suppression of IFN-γ production (Niedbala, 1999). These results show that low levels of NO present during initial T cell activation may selectively enhance the induction of Th1 differentiation in synergy with IL-12.

The studies outlined above illustrate the diversity of regulatory effects mediated by NO during the development of an immune response. Low level NO present during the induction phase may enhance Th1 development, at higher levels however, NO can limit Th1 expansion via suppression of IL-12 production by MO, and IFN-γ and IL-2 production by T cells. Furthermore while NO may act as an anti-microbial effector mechanism, it may also suppress the development of host protective immune responses. As a consequence of this apparent dichotomy of function NO is often referred to as a

"double-edged sword." In the immunological arsenal it is clearly a weapon which must be deployed with care, and yet its effects can be felt from the induction of Th1 responses through to their ultimate down-regulation.

1.6. Apoptosis

Programmed cell death (PCD) or apoptosis is a genetically determined process whereby organisms can safely dispose of unwanted cells. The death program is implemented by proteins encoded in the host genome via mechanisms which are highly evolutionarily conserved. PCD plays important roles in development, homeostasis, defence and ageing (reviewed Vaux, 1996). Apoptosis is involved in various fundamental immunological processes including negative selection in the thymus (Surh, 1994) clonal downsizing and the development of peripheral tolerance (Singer, 1994; Janssen ,2000).

Apoptosis refers to the morphological changes characteristically exhibited by actively dying cells. Such changes include cell shrinkage, cleavage of nuclear DNA chromatin condensation and membrane blebbing (Cohen, 1993). Apoptotic cell death can result from the developmentally controlled activation of endogenous execution programmes or the transduction of death signals triggered by a wide variety of external stimuli. Homeostasis in the immune system is maintained by a balance between the generation of new lymphocytes, Ag-induced proliferation and differentiation and apoptotic cell death. In terms of maintaining this balance and regulation of T cell responses, apoptosis induced via death receptors is thought to be of primary importance.

Whilst stimulation of mature peripheral T cells via the TCR generally results in T cell activation and proliferation, repeated TCR triggering or TCR stimulation in the absence of co-stimulatory signals can lead to T cell apoptosis. Apoptosis induced in this manner is termed activation induced cell death (AICD) (Liu, 1990; Wesselborg, 1993) and

represents a mechanism for controlling development of T cell responses (Alderson, 1995). Studies on the kinetics of responses to superantigens first indicated the importance of apoptosis in regulating responses. Injection of staphylococcal enterotoxin B (SEB, a bacterial super-Ag which reacts predominantly with $V\beta8^+$ T cells) results in an initial increase in the percentage of $V\beta8^+$ T cells, followed by a dramatic decline in their number. This decrease occurs as a result of massive peripheral deletion, achieved via the apoptosis of responding cells (Scott, 1993). Such findings suggest an important regulatory role for apoptosis in T cell responses.

AICD is triggered by signalling via "death receptors" upon TCR engagement (Alderson, 1995; Renno, 1996). Death receptors are members of a growing subfamily of the TNF-R superfamily, which can induce apoptosis upon receptor triggering: Of these the signalling pathway initiated by ligation of the cell surface receptor Fas (CD95 / APO-1) by its ligand Fas-L is the best characterised (reviewed Peter, 1998). Upon triggering of CD95, the adapter molecule FADD (Fas associated death domain containing protein/Mort-1) and pro-caspase 8 are recruited to the receptor forming the death inducing signalling complex (DISC). Cysteine proteases of the IL-1β converting enzyme (ICE) family (caspases), are critical executioners of apoptosis in mammalian cells. Caspases are synthesised as inactive precursors requiring proteolytic conversion to become active proteases. The association of pro-caspase 8 into the DISC results in its auto-proteolytic activation, upon which the active enzyme is released into the cytosol. The resultant protease cascade results in the activation of caspase 3 which leads eventually to apoptosis (Nagata, 1997). Activation of caspases results in the cleavage of various cellular proteins resulting in apoptosis. Targets of caspase activity include structural proteins, proteins involved in DNA repair and cell cycle regulation (reviewed Denis, 1998)

Many studies have now confirmed the importance of Fas mediated apoptosis in AICD and regulation of T cell responses. It has been shown that T cell apoptosis initiated by repeated TCR stimulation is mediated via Fas / Fas-L interactions (Brunner, 1995; Dhein, 1995; Ju, 1995). Peripheral deletion of expanded $V\beta8^+$ T cells following SEB injection was also shown to be impaired in both *lpr* and *gld* mice (deficient in expression of Fas and Fas-L respectively), (Renno, 1996; Scott, 1993). Renno *et al* also demonstrated that dying SEB reactive T cells expressed enhanced levels of Fas and Fas-L. It has also been shown that stimulation of previously activated human T cells results in the expression of Fas-L mRNA and sensitivity to Fas mediated apoptosis, and that the AICD of T cell clones and lines can be inhibited by the action of Fas-L antagonists (Alderson, 1995).

1.6.1. The role of co-stimulation in AICD

Many factors have now been implicated in determining sensitivity of T cells to Fas mediated apoptosis, including, the activation status of the cell (Wesselborg, 1993) and the presence or absence of co-stimulation (Liu, 1990) as well as the action of certain cytokines including IL-2 and IFN-γ (Leonardo, 1991; Liu, 1990). Harding *et al* (1992) demonstrated that while TCR stimulation in the absence of co-stimulation leads to development of clonal anergy, provision of co-stimulation via cross-linking of CD28 blocked anergy induction and resulted in vigorous proliferation. Apoptosis of murine splenic T cells induced by re-cross-linking the TCR during a primary immune response could also be prevented by cross-linking CD28 (Radvanyi, 1996). Inhibition of apoptosis by CD28 stimulation was found to be associated with increased expression of IL-2 (Boise, 1995) and bcl-X_L, an anti-apoptotic member of the bcl-2 gene family (Radvanyi, 1996). Furthermore, using transfection Boise *et al* demonstrated that expression of bcl-X_L was sufficient to prevent T cell apoptosis in response to Fas ligation and IL-2 withdrawal as

well as via TCR stimulation. These results suggest that an important role of co-stimulation is to augment T cell survival during the process of activation.

Van Parjis *et al* (1996.) showed that naïve T cells cultured in the absence of antigen or co-stimulation, or in the presence of Ag without co-stimulation, undergo Fas independent apoptosis. Apoptosis of naïve T cells induced under these conditions was termed programmed cell death in order to differentiate it from the Fas dependent AICD seen upon restimulation of activated T cells. It was further shown that the AICD of previously activated T cells could not be prevented by co-stimulation via CD 28. As co-stimulation has been shown to prevent the apoptosis of naïve T cells upon TCR engagement, differences in responsiveness may reflect the differential activation status of responding cells. The pathways of apoptosis induction also differ, as in this study, TCR induced apoptosis of naïve T cells was shown to be Fas independent whilst AICD of activated T cells was Fas mediated (Van Parijs, 1996).

Differences in the sensitivity of resting and activated T cells to AICD have also been reported. Wesselborg *et al* (1993.) demonstrated that while freshly isolated human peripheral blood T cells are largely resistant to AICD, sensitivity to apoptosis induced by TCR cross-linking increased upon activation and during *in vitro* culture. These results suggest that stimuli which activate T cells may also render them susceptible to AICD. More recently it has been shown that naïve and memory T cells differ in their response to Fas cross-linking. While naïve T cells stimulated with anti-CD3 Ab undergo apoptosis upon Fas ligation, memory T cells, under the same conditions, are co-stimulated to proliferate by Fas ligation. Most interestingly, besides provision of co-stimulation via CD28, the presence of Th1 or Th2 differentiation inducing cytokines (IL-12 and IL-4 respectively) also rescued naïve T cells from AICD and rendered them receptive to Fas mediated co-stimulation (Desbarats, 1999). These results illustrate two important points,

firstly, that the outcome of Fas engagement in $CD4^+$ T cells may be determined by the antigenic history of the cell, and secondly, that the cytokines present during T cell priming may influence not only the subsequent polarisation of responses but also cell survival.

1.6.2. Th1 Vs Th2 : Sensitivity to AICD

While there is considerable evidence that Th1 cells are more susceptible to apoptosis than Th2 cells, the mechanisms underlying this differential sensitivity have been a controversial issue. Liu and Janeway (1990) investigated the propensity of T cell clones to undergo AICD and demonstrated high levels of AICD in all five Th1, but none of the Th2 clones tested. Most importantly they demonstrated that apoptosis induced by TCR ligation in the absence of co-stimulation had a critical requirement for IFN-γ. Novelli *et al* (1997) also demonstrated an important role for IFN-γ in the AICD of human Th1 clones. Once again Th1 but not Th2 clones underwent rapid AICD in the absence of co-stimulation, and the apoptotic response could be blocked by neutralisation of IFN-γ. Most interestingly the addition of IFN-γ to Th2 clones triggered their apoptosis under the same circumstances. It was also shown that IFN-γ increased susceptibility to AICD via the upregulation of Fas-L expression, further illustrating the role of IFN-γ in AICD. Ramsdell *et al* (1994) also found that AICD was readily observed amongst Th1 but not Th2 clones and this was reported to reflect the expression of functional Fas-L, which was found only on Th1 clones (possibly under the direction of IFN-γ?). However Zhang *et al* (1997) found that while Th1 but not Th2 effector cells underwent rapid Fas mediated AICD, Th1 and Th2 clones expressed equivalent levels of Fas and Fas-L. Such results suggested that differences in sensitivity to AICD were unlikely to be accounted for by differential expression of Fas or Fas-L. Th2 effectors however, also expressed high levels of FAP-1, a

Fas-associated phosphatase which inhibits Fas signalling and which may increase resistance to Fas mediated AICD.

That differential sensitivity to AICD may have functional significance has been suggested by studies on the development of peripheral tolerance. Following the induction of peripheral tolerance a small population of Ag-specific T cells, apparently resistant to apoptosis, persists. On investigation the critical difference between apoptosis resistant and sensitive cells was found to lie in the ability of resistant cells to secrete high levels of the Th2 cytokines IL-4 and IL-10 (Zhang, 1996). It may be that increased expression of FAP-1 or lower expression of Fas-L facilitates the preferential survival of Th2 cells, providing a mechanism which may, under certain circumstances, favour the outgrowth of Th2 responses.

IL-4 itself has also been shown to protect chronic leukaemic B cells from apoptosis via the upregulation of bcl-2 expression (Dancescu, 1992), and to maintain expression of bcl-2 and bcl-X_L in resting T cells, supporting their *in vitro* survival (Vella, 1997). Somewhat surprisingly then, and in contrast to IL-4, IL-2, a T cell growth factor associated with Th1 responses has been shown to promote T cell apoptosis. Leonardo (1991) first reported that prior exposure to IL-2 predisposed T cells to undergo apoptosis upon subsequent TCR engagement. More recently exposure to IL–2 has been shown to increase transcription and surface expression of Fas-L and to suppress expression of FLIP (FLICE-like inhibitor protein,) which shuts off Fas signalling (Refaeli, 1998). It seems likely that this dual action of IL-2 accounts for its ability to potentiate T cell apoptosis.

1.6.3. Apoptosis during infection

While inhibition of host cell apoptosis as a viral survival strategy provided the first evidence of apoptosis modulation by infectious organisms, there is now increasing

evidence of a role for apoptosis in the immune response to various bacterial and parasitic infections.

Blocking apoptosis via the inhibition of caspase activity has been shown to increase the level of HIV replication (Chinnaniyan, 1997) and a number of viruses have now been found to encode proteins capable of directly interfering with the death program. CrmA from cowpox virus binds to and inactivates caspase 1 (Ray, 1992) while several other viruses encode proteins which resemble bcl-2 (Reviewed Vaux, 1996). There is now increasing evidence of a role for apoptosis in the immune response to certain parasitic infections. Lopes *et al* (1995) first reported elevated levels of AICD amongst $CD4^+$ T cells from *Trypanosoma cruzi* infected mice *in vitro*. Levels of T cell apoptosis were also shown to be elevated during infection *in vivo*. $CD4^+$ but not $CD8^+$ T cells from infected mice were susceptible to AICD and the development of proliferative unresponsiveness was shown to correlate with the loss of T cell viability (Lopes, 1996). These results suggested that the AICD of $CD4^+$ T cells could contribute to the development of the immunosuppression associated with infection. More recently it has been shown that the Fas mediated AICD of IFN-γ producing T cells enhanced parasite replication *in vitro* (Nunes, 1998). The mechanisms of apoptosis during *T. cruzi* infection have now been further characterised. During infection IFN-γ has been shown to enhance expression of both Fas and Fas-L and to induce production of NO. Cells from infected animals undergo both Fas-dependent and Fas-independent, NO mediated apoptosis during *in vitro* culture (Martins, 1999). These findings illustrate that while IFN-γ may be of importance in mediating resistance to *T. cruzi* it may also affect the development of the immune response via modulation of apoptosis.

A link between IFN-γ production and host cell apoptosis has also been reported during infection with *Mycobacterium avium* and *Toxoplasma gondii*. Following infection of C57BL/10 mice with *M. avium* T cell apoptosis, measured immediately *ex-vivo,* was shown to increase over the course of infection. A close correlation was found to exist between the apoptosis of $CD4^+$ T cells and the level of IFN-γ production by splenocytes (Gilbertson, 1999). IFN-γ has also been shown to increase Fas expression and enhance levels of apoptosis amongst $CD4^+$ and $CD8^+$ α/β T cells in the Peyers patches following peroral infection of mice with *T. gondii* (Liesenfeld, 1997). These studies in murine models demonstrate that IFN-γ may modulate both Fas/Fas-L expression and apoptosis during the course of infection with a variety of pathogens.

Evidence of elevated levels of apoptosis during parasitic infection of humans however, is comparably scarce. It has been shown that PBMC from *P. falciparum* infected individuals undergo higher levels of spontaneous apoptosis in *in vitro* culture than those of uninfected individuals. Furthermore exposure to parasite-Ag was shown to significantly increase apoptosis (Toure-Balde, 1996). Evidence of increased lymphocyte apoptosis has also been found amongst individuals with chronic helminth infection (Kalinkovich, 1998). It is of note that the subjects in this study group were Ethiopian immigrants infected with a variety of helminths. Interestingly amongst a group of older immigrants who had been in a western environment for over 5 years, levels of lymphocyte apoptosis fell within the normal range, further suggesting an association with active infection. Such results suggest that apoptosis is also likely to be of importance during human helminth infection.

1.6.4. NO and apoptosis

As with many aspects of its biology, the role of NO in regulating apoptosis may appear contradictory. Both pro- and anti-apoptotic functions have been attributed to NO.

Once again the outcome of exposure appears to depend largely upon both the level of NO production and the tissue type involved. While exposure to high levels of NO, as produced by iNOS, appears to promote apoptosis, the continuous production of low levels of NO, by constitutive NOS, is more often associated with anti-apoptotic effects (reviewed Dimmler, 1997).

(i) Pro-apoptotic effects of NO

It has now been shown that NO can induce apoptosis in a variety of cell types, including murine thymocytes, splenocytes, peritoneal MO and tumor cells (Albina, 1993; Fehsel, 1995; Okuda, 1996; Sarih, 1993). Sarih *et al* demonstrated that stimuli leading to MO activation and the development of cytotoxicity to tumor cells (LPS and IFN-γ), also led to MO death by apoptosis. Addition of L-NMMA inhibited both NO production and apoptosis. Albina *et al* (1993) further demonstrated that besides activation by LPS and IFN-γ exposure to authentic NO gas induced the apoptosis of murine peritoneal MO. Both murine thymocytes and splenic T lymphocytes have also been shown to undergo apoptosis upon exposure to chemical donors of NO (Fehsel, 1995; Okuda, 1996). Thymocytes co-cultured with activated endothelial cells also underwent NO mediated apoptosis, demonstrating that physiological levels of NO are sufficient for its induction. The ability of NO to induce apoptosis of both APC and T cells suggest that this may represent another mechanism whereby NO can directly or indirectly modulate T cell responses.

Although the precise mechanism whereby NO mediates apoptosis is not known, several potentially contributing factors have now been identified. During the NO mediated apoptosis of thymocytes, Fehsel *et al* (1995) demonstrated the NO induced expression of p53, a tumor suppressor protein which promotes apoptosis. More recently it has been shown that there is an accumulation of p53 resulting from an inhibition of proteosome activity in RAW 264.7 MO undergoing NO mediated apoptosis (Glockzin, 1999). The

pro-apoptotic protein Bax, normally degraded by the proteosome, also accumulates during NO mediated apoptosis, which may be a result of either p53 accumulation or proteosome inhibition. As the proteosome plays an essential role in Ag presentation, inhibition of proteosome activity may represent another mechanism whereby NO can mediate proliferative suppression.

Several lines of evidence suggest that NO can induce apoptosis via modulation of Fas or Fas-L expression. NO induces Fas expression on pancreatic β cells and in this fashion is thought to contribute to β cell damage during insulin dependent diabetes mellitus (Stassi, 1997). More recently it has been shown that human T cell blasts produce NO upon TCR ligation which increases Fas-L expression and sensitivity to Fas mediated apoptosis (Williams, 1998). Human leukaemic T cells have also been shown to upregulate expression of Fas-L upon exposure to NO. Most interestingly however, some lymphoid cells resistant to Fas mediated apoptosis (BL 60, a Burkitt lymphoma cell line) were sensitive to NO mediated apoptosis (Chlichlia, 1998). In this respect NO appears unique in its ability to induce both Fas dependent and independent apoptosis. Regardless of the involvement of the Fas signalling pathway NO mediated apoptosis has been shown to require activation of caspases. Using inhibitors of caspase activity it was shown that activation of caspase 1 but not caspase 3 was essential to the NO mediated apoptosis of thymocytes. Caspase 1 derived from the cytosol of NO treated thymocytes has been shown to be capable of cleaving inhibitor of caspase-activated deoxyribonuclease (I-CAD) leading to DNA fragmentation and apoptosis (Zhou, 2000). There are clearly several mechanisms whereby NO can induce apoptosis which may impact the development of immune responses in a variety of ways. It is possible that NO mediated apoptosis can affect T cells either directly (during thymic maturation or in the periphery), or indirectly

via affecting APC function through induction of apoptosis or inhibition of proteosome activity.

(ii) Anti-apoptotic effects of NO

Various studies have reported that NO can also show anti-apoptotic activity. The protective effects of NO are generally mediated by low levels of NO produced by constitutive NOS, and have been demonstrated with regard to both Fas mediated and TNF-α mediated apoptosis. Mannick *et al* (1997) demonstrated that basal levels of NO production inhibited the Fas mediated apoptosis of human leukocytes. Inhibition of NOS activity by L-NMMA was shown to significantly increase levels of Fas mediated apoptosis in both B and T cell lines. It was also demonstrated that NO inhibited the caspase mediated cleavage of Poly-(ADP-ribose) polymerase (PARP) (Mannick, 1997). NO has been shown to modulate caspase activity via *S*-nitrosylation of active site thiols, and it has been suggested that this may be the mechanism whereby NO mediates its protective effects (Melino, 1997). Kim *et al* (1997) demonstrated that NO protects hepatocytes from apoptosis via effects upon caspase activity. NO was shown to protect hepatocytes from apoptosis induced by withdrawal of growth factors, exposure to TNF-α, or Fas ligation. Induction of apoptosis via these stimuli results in an increase in caspase 3 like activity. Exposure to NO prevented this increase and furthermore, exposure of purified recombinant caspase 3 to NO inhibited its proteolytic activity via *S*-nitrosylation. NO has also been shown to mediate *S*-nitrosylation of cysteine protease (CPP)-32-like cysteine protease (CPP-32) and thus inhibit the TNF-α induced apoptosis of human umbilical venous cells (Dimmeler, 1997). Constitutive expression of endothelial NOS has also been demonstrated to protect human NK cells from AICD via down-regulation of TNF-α expression (Furuke, 1999). Exposure to NO can clearly display both pro- and anti-

apoptotic effects, dependant upon the amount of NO present, and the cell type involved. As an important modulator of cell survival, NO can be added to the list of factors such as previous experience of Ag, co-stimulation and both prior and current exposure to cytokines, which can influence the outcome of specific cellular activation.

CHAPTER 2 Materials and Methods

2.1 The Parasite

2.1.1. Maintenance of the mosquito life cycle

Aedes aegypti (Refm) mosquitoes 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 removed and stored dry until required. Eggs were hatched by submerging egg papers in plastic trays of tap water containing yeast tablets to feed the developing larvae. Pupae were picked daily and transferred to cages. Adult mosquitoes were fed on sucrose and starved for 24 hrs prior to receiving a blood-meal.

2.1.2. Maintenance of the parasite life cycle

Adult parasites and microfilariae were obtained from the peritoneal cavity of infected jirds (*Meriones unguiculatus*) which had been infected for at least three months with 250 L3 of *Brugia pahangi*. Jirds were killed by CO_2 inhalation and exsanguinated by cardiac puncture. Worms were obtained by extensive peritoneal lavage with Hanks Balanced Salt Solution (HBSS, Gibco) at 37°C. Adult worms were washed in HBSS and frozen in liquid nitrogen as a source of material for the preparation of parasite extracts. The mf were washed in HBSS and resuspended in heparinized rabbits blood at a concentration of approximately 400/20 µl. Mosquitoes were fed infected blood using the artificial membrane feeder.

2.1.3. Recovery of infective stage larvae and microfilariae

L3 were harvested from infected mosquitoes at 9 days post infection (p.i.) using standard methods (Devaney, 1991), washed extensively in sterile HBSS and counted. Microfilariae were obtained by extensive peritoneal lavage of infected jirds, adult worms were mechanically removed and any erythrocytes present were lysed using sterile ddH2O. Mf were washed in HBSS (37°C) and separated from host cells by repeated centrifugation over Histpaque-1077 (Sigma) at 1200 rpm for 15 mins without brake. Mf were washed twice in HBSS and counted.

2.1.4. Preparation of antigen extracts

Soluble extracts of *B. pahangi* adult worms, for use in cell culture were prepared by homogenisation of frozen mixed sex or single sex adult worms in RPMI-1640 (Dutch Modification, containing 5mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, all Gibco ($RPMI^+$)) on ice. The homogenate was incubated on ice for 1 hr with occasional agitation. The suspension was then centrifuged at 10,000 g for 30 mins at 4°C. The supernatant was sterilised by centrifugation through a 0.45 µm Spin-X filter unit (Co-star) and assayed for protein concentration using a BioRad dye reagent based on the Bradford method. 50µl aliquots were stored at –70°C until use. Mf Ag was prepared by sonicating mf for 3 minutes at 6µm in $RPMI^+$ on ice and then processing in the same way as adult Ag.

2.2 Animals and infection protocols

6 week old male BALB/c mice were purchased from Harlan-Olac (Bicester, UK) and were maintained in filter topped cages. Groups of 5 mice were injected i.v. via the tail vein with either $1x10^5$ mf, 50x L3 *B. pahangi* or an equivalent volume of HBSS. In certain

experiments, where stated mice were infected s.c. in "the scruff of the neck". Heat killed mf were prepared by plunging into boiling water for 3 mins. At 12 days post infection (d.p.i.) mice were killed by CO_2 inhalation. Spleens were removed aseptically and serum collected by cardiac puncture. In additional experiments NOS-2 deficient mice on the 129/Sv background (obtained from Professor F. Y. Liew, University of Glasgow), IFN-γR-/- mice also on the 129/Sv background (obtained from Dr. Alan Mowat, University of Glasgow) and IL-4 deficient mice on the BALB/c background (from Dr. Eileen Devaney, University of Glasgow) were infected using the same protocol. Equivalent strain and age-matched wild type counterparts for all these animals were purchased from Harlan-Olac.

2.3. Preparation of spleen cells

Spleens were removed aseptically. Single cell suspensions were prepared in RPMI by passage of the spleens through Nytex mesh (Cadisch and Sons, London UK) and debris separated by further passage through Nytex. Erythrocytes were lysed in 0.83% NH_4Cl (pH 7.2), the remaining cells were washed twice in RPMI and numbers of viable lymphocytes assessed by trypan blue exclusion. Cells were resuspended at $1x10^7$/ ml (for proliferations) or $2x10^7$/ ml for cytokines in $RPMI^+$ containing 20% heat inactivated FCS (Gibco), to give a final concentration of 10% FCS.

2.3.1. Proliferation assay

Proliferation was measured by incorporation of ^3H thymidine. Triplicate 100 µl cultures ($5x10^5$ cells / well) in 96 well half area plates (Co-star) were incubated in the presence or absence of 1 µg/ml Con A (Sigma) or 10 µg/ml adult Ag. These concentrations of Con A and adult Ag were determined 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 ^3H thymidine/well (Amersham) during the last 16 hrs of culture. The cells were harvested and radioactivity measured in a "TopCount" microplate scintillation counter (Canberra Packard Instrument Company).

2.3.2. *In vitro* treatments

In some experiments the IFN-γ neutralising MAb XMG1.2 or isotype matched control Ab ((R3-34) both PharMingen) were added to medium only and Ag-stimulated culture at a concentration of 100 μg/ml. After such treatment the proliferation and cytokine response of the cells were assessed as previously described. IFN-γ was assayed by cytokine ELISA which demonstrated that this cytokine had been successfully neutralised.

In certain experiments the following supplements were added to both antigen-stimulated and medium only cultures. All concentrations given are the final concentration active in culture: Recombinant IL-2 (rIL-2, Sigma) at 50 U/ml. The iNOS inhibitor N-g-monomethyl-L-arginine (L-NMMA, Sigma) at 250 μg/ml; Aminoguanidine (AMG, Sigma) a more selective inhibitor of iNOS activity at 500 mM; Polymixin B an inhibitor of the pro-inflammatory activity of LPS 2.5 μg/ml (Sigma); S-nitroso-amino-penacillamine (SNAP) an NO donor (Sigma) at various concentrations and times as noted. The proliferative responses of these cells were compared to those in unsupplemented cultures.

2.3.3. Magnetic Separations

In some experiments splenocytes from mf-infected animals were depleted of either $CD4^+$ or $CD8^+$ cells, by magnetic separation, prior to *in vitro* culture. Splenocytes were prepared as described previously and pooled from 3-5 mf-infected animals. Unseparated cells were plated out prior to depletion. At this point $5x10^6$ cells were removed for staining with an isotype control antibody (KLH/G2a-1-1 PharMingen). Remaining cells were washed in PBS and then stained with either an anti-CD4 or anti-CD8 FITC conjugated MAb (L3T4 or S3-6.7 both PharMingen) in 500 µl PBS on ice for 20 mins. Both antibodies were used at 2 µl/$2x10^6$ cells. Stained cells were washed twice in PBS and $5x10^6$ cells were removed for FACS analysis. Remaining cells were resuspended in degassed separation buffer (90 µl/10^7 cells) containing anti-FITC beads (10µl/10^7 cells) on ice for 15 mins. Cells were resuspended in 500 µl of separation buffer and loaded onto LS positive selection columns (Miltenyl Biotec) pre-wet with 3mls of separation buffer. Cells were washed through the column with 3x3ml of separation buffer and collected on exit. Cells were then washed in PBS and $5x10^6$ cells removed for FACS analysis to allow the efficiency of depletion to be assessed. Remaining cells were washed in $RPMI^+$ and plated out as normal.

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 (Co-star) in medium only or in the presence of Con A (5 µg/ml) or Ag at 10 µg/ml and the supernatants harvested after 48 hrs culture (unless otherwise stated). Cell free supernatants were stored at -20°C. Levels of IL-2, IL-4, IL-5, IL-10 and IFN-γ were determined by two-site ELISA using antibody pairs purchased from PharMingen. The optimal concentrations for the capture and detecting antibodies were determined in

preliminary experiments. Results are expressed as U/ml or pg/ml in reference to commercially available standards (IL-2, IL-4 and IL-10 from PharMingen, IL-4 and IFN-γ from R and D systems). The sensitivity of the assay was determined as the mean + 3 SD of 16 wells containing medium (RPMI 10% FCS) only.

The cytokine ELISA was performed as follows. ELISA plates (Corning Easy-Wash) were coated with 50 µl/well capture antibody in PBS, over night at 4°C and then blocked with 150 µl/well PBS/0.5% Tween 20, 10% FCS, for 45 mins 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. Plates were washed twice with PBS/0.05% Tween 20 (PBS/T.20) quickly and twice for 3 mins. Samples, standards and RPMI 10% FCS were transferred to plates at 50 µl/well for two hours at R.T. Plates were washed as before and biotinylated secondary antibody diluted in 1 % BSA PBS/T.20 was added at 50µl/well for 1 hr at R.T. Plates were washed as before. Streptavidin peroxidase (Serotec) 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) was added at 100 µl/well, for 10 mins. Plates were read at 620nm in a Dynatech MR5000 automated ELISA reader.

2.3.5. Measurement of nitrite in culture supernatants

The Greiss reaction was used to determine levels of nitrite in culture supernatants. All samples were tested in duplicate. Equal volumes of sample (cell free supernatants) and freshly prepared Griess reagent (0.05% alpha-naphthyl-amine, 0.5% sulfanilamide in 2.5% phosphoric acid) were mixed in 96 well flat bottomed-plates and allowed to react for 10 minutes at room temperature before absorbance was determined at 560 nm. NO_2^-

concentration was calculated from a $NaNO_2$ standard curve and sensitivity calculated as for cytokine ELISAs. Greiss reagent was prepared freshly every time by mixing equal volumes of 0.1% alpha-naphthyl-amine and 1% sulfanilamide, 5% phosphoric acid.

2.4. FACS staining

2.4.1. CFSE labelling

Cells were labelled with CFSE *ex-vivo*, and then cultured, with or without stimulation, prior to cell surface staining and FACS analysis. After counting the number of viable splenocytes, $5x10^7$ cells / mouse were washed twice in 5 ml sterile PBS (1000 rpm/5 min). Cells were then incubated in 10 μM CFSE (Molecular Probes) at $5x10^7$ cells/ml for 8 min at R.T. The reaction was stopped by the addition of 5 mls $RPMI^+$ 20% FCS, and all samples were washed twice in $RPMI^+$ 10% FCS. Stained cells were plated out in 1ml cultures as previously described, with or without stimulation, for 96 hr prior to cell surface staining and FACS analysis. In certain experiments CFSE labelled cells were cultured in the presence or absence of AMG at a final concentration of 500 mM.

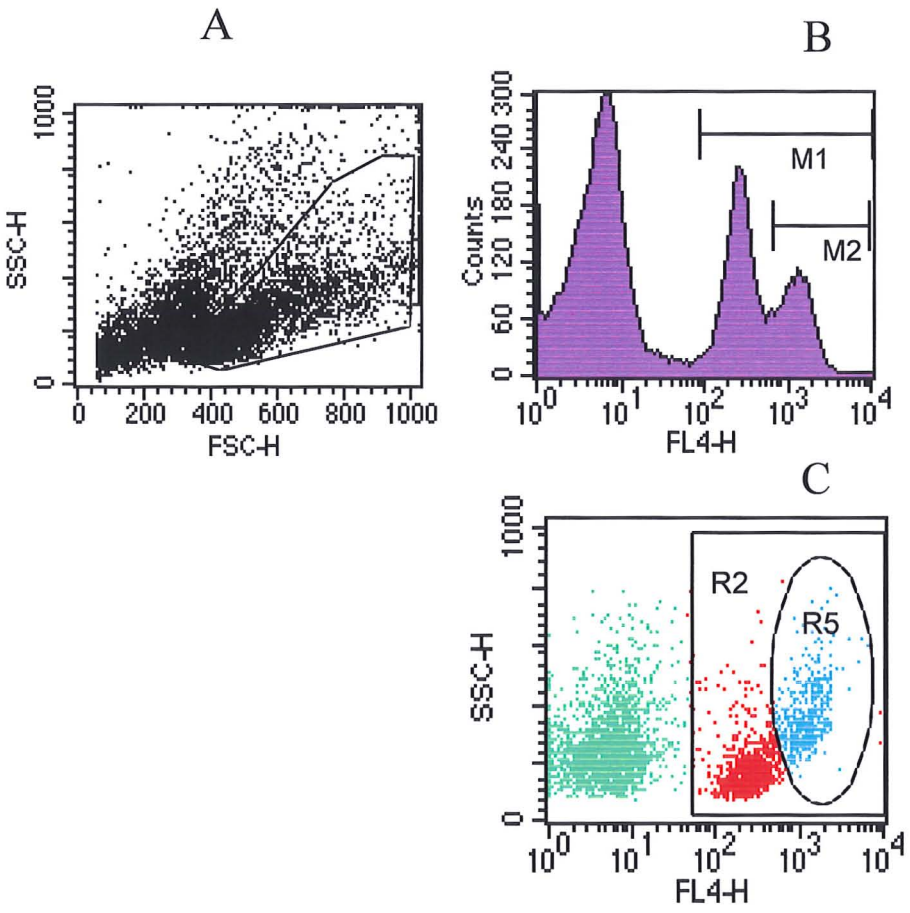
CFSE labelled cells were harvested (around $1x10^6$ cells per test), and surface stained with either an anti-mouse CD4 APC conjugated MAb (L3-T4), or isotype matched control ((R35-95) both PharMingen), as described below, prior to FACS analysis. Samples were gated on both total lymphocytes, as determined by forward and side scatter and $CD4^+$ lymphocytes for analysis of CFSE staining profiles. Controls in each experiment comprised unstained cells, cells stained with Anti-CD4 or isotype matched control only, and cells stained with CFSE only.

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 (1000 rpm /5 min). Cells were then stained with 2 µg/test of flourochrome 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 if not to be analysed immediately. Stained samples were stored in the dark at 4°C. Samples were gated on lymphocytes, as determined by forward and side scatter.

2.4.3. Propidium Iodide Staining

Cells to be stained were removed from culture, transferred to FACS tubes (Falcon), and washed twice in 200 µl staining buffer (1000 rpm /5 min). Cells were then stained with 100 µl/sample of either an APC conjugated anti-mouse CD4 MAb, or isotype matched control MAb (both PharMingen) in staining buffer, for 20 min on ice. Cells were washed in 200 µl staining buffer and incubated for 15 min at R.T. in 200 µl fixation buffer. Cells were washed as before and incubated for a further 15 min at R.T. in permeabilisation buffer. Cells were then washed and resuspended in 300 µl PI/RNase A (5 µg/ml PI, 200 ng/ml RNase A, PharMingen), for 30 min in the dark at R.T. If not to be analysed immediately, cells were washed, resuspended in 300 µl fixation buffer and stored in the dark at 4°C. Samples were analysed on a Becton Dickinson FACScalibur (department of Immunology, University of Glasgow). Gates were set around the lymphocyte population and subsequently upon $CD4^+$ lymphocytes, the PI staining profile of these cells was assessed and those cells with a subdiploid DNA content considered apoptotic. All samples were analysed using Becton Dickinsons CellQuest software.



Histogram Statistics

File: ROCCFSE24.3.038
Sample ID:
Tube:
Acquisition Date: 24-Mar-0
Gated Events: 79045
X Parameter: FL4-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G1
Total Events: 169683

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	79045	100.00	46.58	241.63	26.83	198.77	9.06	1
M1	95, 9910	30257	38.28	17.83	619.78	429.19	98.25	324.88	248
M2	667, 9910	9987	12.63	5.89	1309.64	1217.92	47.22	1197.09	1165

2.4.4. FACS gating

Lymphocytes were originally gated by means of their physical properties in terms of forward and side scatter (A). The number of positively staining lymphocytes were determined using markers set on histograms as in (B), in this case M1=$CD4^{+}$ and M2=$CD4^{hi}$, the corresponding figures are shown in the above table. Dot plots of side scatter vs CD4 were used for selection to allow further analysis, as shown in (C) wherein $CD4^{+}$ events appear red and $CD4^{hi}$ blue.

2.5. 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.

2.6. Buffers / Reagents

2.6.1. Lysis buffer: 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. Sterilised by autoclaving and stored at 4°C.

2.6.2. PBS: 137 mM NaCl, 8.1 mM Na_2HPO_4 , 2.7 mM KCL, 1.47 mM KH_2PO_4 in ddH_2O. Adjusted to pH 7.0, sterilised by autoclaving and stored at RT.

2.6.3. ELISA Buffers

PBS/0.05% Tween 20 (Sigma): 500µl Tween 20 was added per litre of PBS.

2.6.4. Separation buffer

50ml PBS, 200mg BSA, 200µl 0.5M EDTA

2.6.5. FACS Buffers

(i) Staining Buffer

PBS/2% FCS, 0.2% sodium azide, pH 7.4-7.6, filter sterilsed and stored at 4°C.

(ii) Permeabilization Buffer

PBS/2% FCS, 0.2% sodium azide, 0.1% saponin (Sigma), pH 7.4 – 7.6, filter sterilised and stored at 4°C.

(iii) Fixation Buffer

4% formaldehyde: 14ml 10x PBS, 10.8 ml 37.5% formaldehyde, 75.2ml H_2O, filter sterilised and stored at 4°C.

CHAPTER 3. NO suppresses the Ag-specific proliferation of splenocytes from mf-infected animals at 12 d.p.i.

3.1 Introduction

The immune response in human filariasis is characterised by a Th2 bias and defective Ag-specific proliferation (Ottesen, 1977; Piessens, 1980b; Yazdanbaksh, 1993). While suppression of proliferative responses was first reported only amongst Mf+ individuals it is now known to extend to other clinical groups (Yazdanbaksh, 1993). The presence of circulating mf, however, remains associated with the most profound manifestations of proliferative suppression. Ag-specific proliferative responses are lowest amongst microfilaremics (Ottesen, 1977; Ravichandran, 1997) and most difficult to restore post chemotherapy in this group (Sartono, 1995b). In contrast, T cells from patients displaying chronic pathology, who are generally Mf-, have relatively strong parasite-specific proliferative responses (Maizels, 1991). Mf+ individuals have also been shown to have lower frequencies of Ag-specific lymphocytes and fewer IFN-γ producing cells (King, 1992). Furthermore, treatment with ivermectin (a microfilaricidal drug) enhances T cell reactivity, suggesting a direct role for mf in generating proliferative suppression (Lammie, 1992). Studies in the jird/ *B. pahangi* model of infection, wherein loss of proliferative responsiveness accompanies the onset of mf production, also imply a link between microfilaremia and proliferative suppression (Leiva, 1989).

Although a number of mechanisms have been proposed which may account for this hyporesponsiveness (see 1.3. and Maizels, 1995), the exact nature of the proliferative suppression is still not fully understood. Attempts to reverse defective proliferative responses of T cells from *B. malayi* infected individuals, using a variety of immunomodulators or neutralising antibodies, have been largely unsuccessful, although some effect was noted with recombinant IL-2 (rIL-2) (Sartono, 1995a). In *W. bancrofti*

infection several reports have provided evidence of a role for regulatory cytokines such as IL-10 (King, 1993; Mahanty, 1997) and TGF-β (King, 1993) in suppressing proliferative responses. More recently it has also been shown that the source of the Ag used for *in vitro* restimulation is an important determinant of proliferative unresponsiveness. Restimulation of PBMC from *W. bancrofti* individuals with Ag prepared from mixed sex adult worms, or mf, led to down-regulation of proliferative responses whilst adult male-Ag did not (Mahanty, 1996). Such results suggest that the presence of Ag of microfilarial origin may induce hyporesponsiveness.

The use of murine models has greatly facilitated the study of the immunology of filariasis, and has revealed a number of ways in which filarial worms can modulate host responses. It has been shown that infection with different life cycle stages elicits development of differentially polarised immune responses, illustrating the potential for stage-specific immunomodulation (Lawrence, 1994). Infection with L3 (by either the i.p. or s.c. route), leads to development of Th2 responses characterised *in vitro* by production of high levels of IL-4 and negligible levels of IFN-γ (Lawrence, 1994; Osborne, 1996). In contrast, infection with mf leads to the development of Th1-like responses, characterised by high levels of Ag-specific IFN-γ (Lawrence, 1994; Pearlman, 1993b). Most interestingly splenocytes from mf-infected and L3-infected animals have recently been shown to differ in their to ability to mount Ag-specific proliferative responses. Whilst splenocytes from BALB/c mice infected subcutaneously with *B. pahangi* L3 displayed strong Ag-driven proliferative responses, cells from mf-infected animals failed to proliferate under the same conditions (Osborne, 1996). Suppression of proliferative responses was observed after 72 hrs of Ag-stimulated culture, 12 days post-infection. The production of high levels of Ag-stimulated IFN-γ by cells from mf-infected animals

demonstrates that they are activated, yet unable to proliferate, in response to Ag. It is interesting to note that while Ag-driven proliferative responses were down-regulated, the polyclonal (ConA-driven) responses of cells from mf-infected animals were intact. In this respect the Ag-specific nature of suppression parallels that seen in human filariasis, wherein mitogen driven responses are generally unaffected (Ottesen, 1977; Piessens, 1980b).

Down-regulation of proliferative responses is a hallmark of several different parasitic infections (Candolfi, 1994; Dai, 1999; Schleiffer, 1993) and presumably reflects a means of promoting parasite survival. The mechanisms underlying suppression vary from organism to organism, but various mediators such as iNOS (Dai, 1999; Mabbot, 1995), pro- and anti-inflammatory cytokines (Martins, 1999; Osborne, 1999; Uzonna, 1998), and T cell apoptosis (Lopes, 1996; Lopes, 1995) have been implicated in mediating suppression. The observation of mf-specific proliferative suppression, suggests that the BALB/c / *B. pahangi* model provides a suitable system in which to analyse the mechanisms involved in its generation.

The aim of the experiments presented in this chapter was to identify the mechanism(s) underlying the suppression of Ag-specific proliferative responses seen following mf-infection. While previous experiments in this laboratory had been carried out using s.c. infection, intravenous infections were used throughout, in order to place mf directly into the circulatory system. Ag-stimulated proliferative and cytokine responses were assessed in response to stimulation in the presence or absence of various immunomodulators.

3.2. RESULTS

3.2.1. Polyclonal and antigen stimulated immune responses of splenocytes from mf-infected, L3-infected and uninfected control mice at 12 d.p.i.

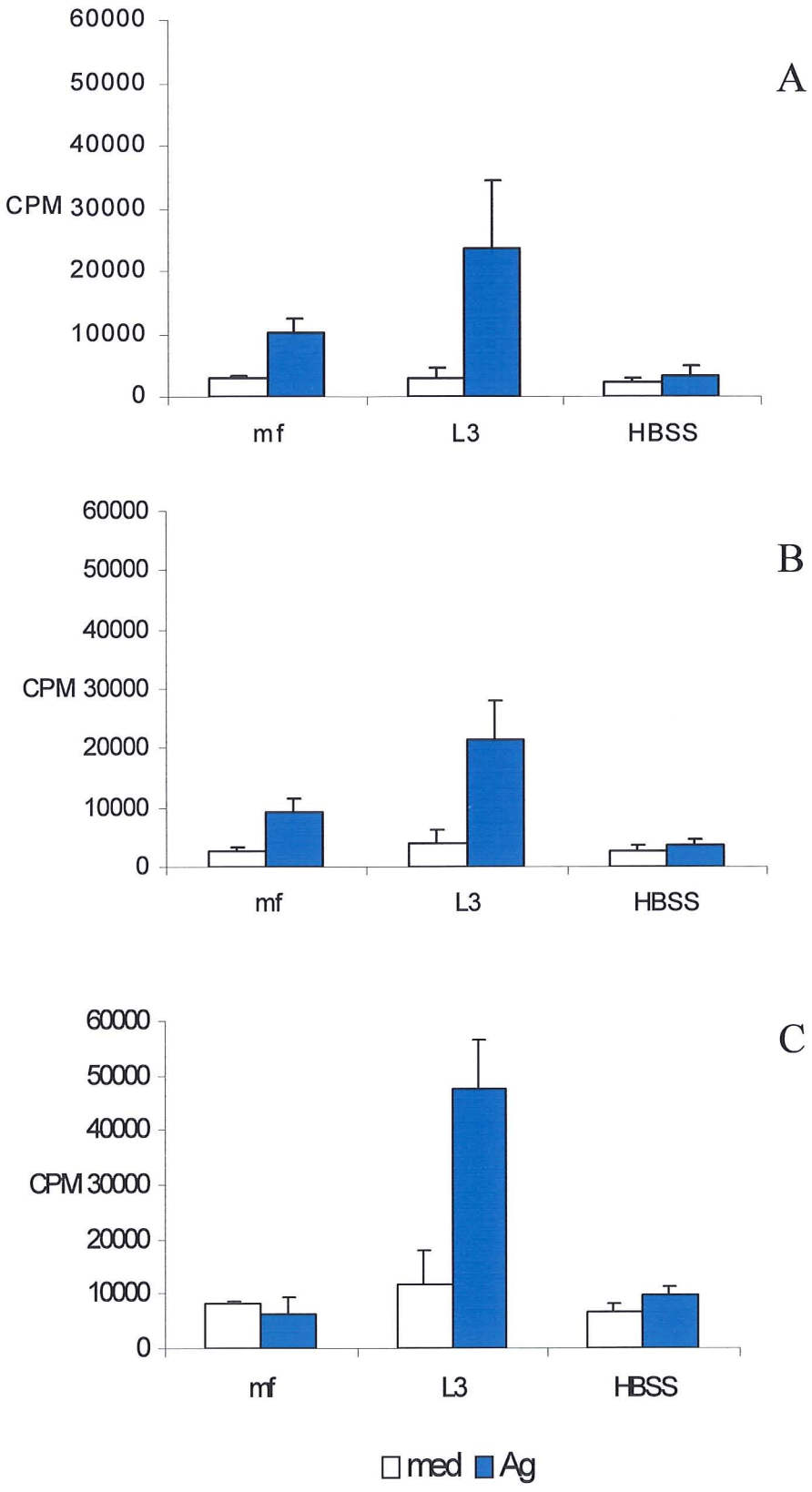
In these experiments groups of five BALB/c mice were injected intravenously via the tail-vein with $1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. spleens were removed for *in vitro* analysis.

(i) Ag-specific proliferative responses

Proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control animals were measured over a time-course of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. Figure 3.1 shows Ag-driven proliferative responses after (A) 48, (B) 72 and (C) 96 hrs *in vitro* culture. At 48 hrs splenocytes from both groups of infected mice show Ag-specific proliferation Figure 3.1 (A), cells from L3-infected animals consistently showing higher levels of Ag-driven proliferation than those from mf-infected animals. After 96 hrs of culture however, Ag-stimulated cells from mf-infected animals routinely incorporated fewer cpm than did cells in culture medium only Figure 3.1 (C). In contrast cells from L3 infected animals displayed strong Ag-specific proliferative responses throughout the period observed. Splenocytes from uninfected control animals did not proliferate in response to Ag. These results are highly comparable to those previously reported following s.c. infection with *B. pahangi* mf and L3 (Osborne, 1996). By virtue of their more robust responsiveness, cells from L3-infected animals were included in many subsequent experiments as positive controls for Ag-driven proliferation. This experiment has been repeated on multiple occasions with equivalent results.

FIGURE 3.1. Proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control mice to *B. pahangi* adult antigen at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen were measured by ^{3}H thymidine incorporation at (A) 48 hrs, (B) 72 hrs and (C) 96 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group.


A
60000
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40000
CPM 30000
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mf
L3
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CPM 30000
20000
10000
0
mf
L3
HBSS
C
60000
50000
40000
CPM 30000
20000
10000
0
mf
L3
HBSS
med
Ag

(ii) Ag-driven cytokine responses at 12 d.p.i.

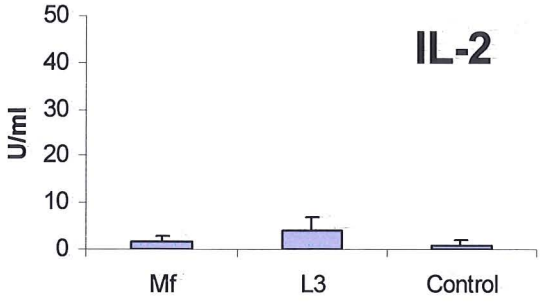
Infection with different life cycle stages has previously been reported to lead to the development of differentially polarised responses. To confirm that this is also the case following i.v. infection cytokine production by splenocytes from mf-infected, L3-infected and uninfected control mice was measured after 48 hrs *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag (Figure 3.2). The cytokine production profiles of i.v. infected mice proved very similar to those previously reported following infection by the subcutaneous (Osborne, 1999) or the intraperitoneal (Lawrence, 1994) route. The only Ag-specific cytokine produced by splenocytes from mf-infected mice was IFN-γ, while cells from L3-infected animals produced IL-4, IL-5 and IL-10 but no IFN-γ. This experiment has been repeated on multiple occasions with equivalent results.

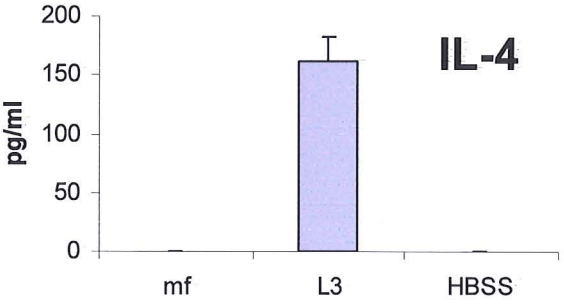
(iii) Polyclonal proliferative responses

Proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control animals were measured after 48 hrs *in vitro* stimulation with ConA, anti-CD3 MAb, or LPS all at a final concentration of 1 μg/ml. The concentrations and culture time used were determined to be optimal in preliminary experiments (data not shown). As shown in Figure. 3.3, proliferative responses to these stimuli were equivalent across all groups. In the case of ConA stimulation these results differ from those previously reported following s.c. infection wherein splenocytes from L3-infected mice showed significantly reduced proliferation (Osborne, 1996). This experiment has been repeated on multiple occasions with equivalent results.

FIGURE 3.2. Ag-stimulated cytokine production by splenocytes from mf-infected, L3-infected and uninfected control mice at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Cytokine levels in supernatants from 48 hr cultures were measured by 2-site ELISA. All values represent the mean and standard deviation of five animals per group.


IL-2
U/ml
50
40
30
20
10
0
Mf
L3
Control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50
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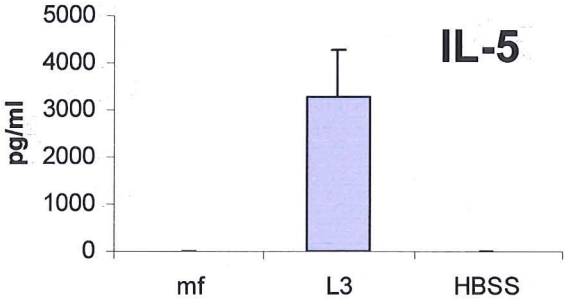
mf
L3
HBSS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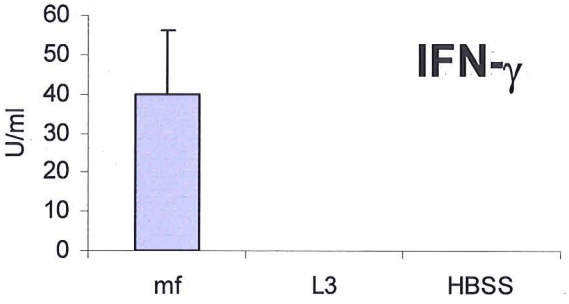
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U/ml
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30
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10
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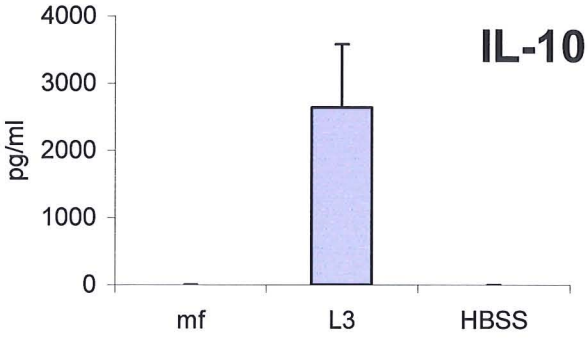
mf
L3
HBSS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pg/ml
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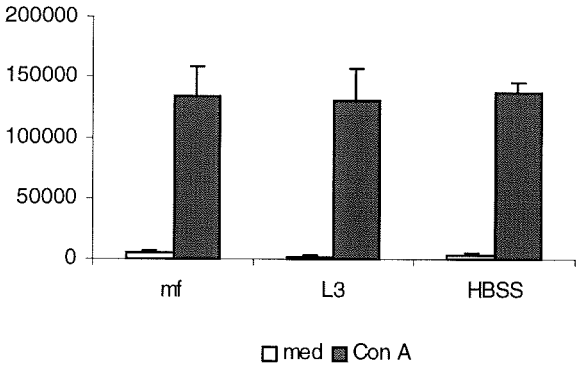
3000
2000
1000
0
mf
L3
HBSS

FIGURE 3.3. Polyclonal proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control mice at 12d.p.i.

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes ($5x10^6$ cells/ml) to (A) 1 μg/ml ConA, (B) 1 μg/ml anti-CD3 MAb and (C) 1 μg/ml LPS were measured by ^3H thymidine incorporation after 48 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group.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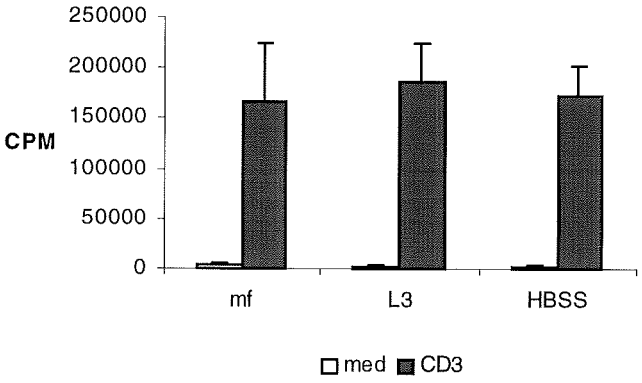
mf
L3
HBSS
med
Con A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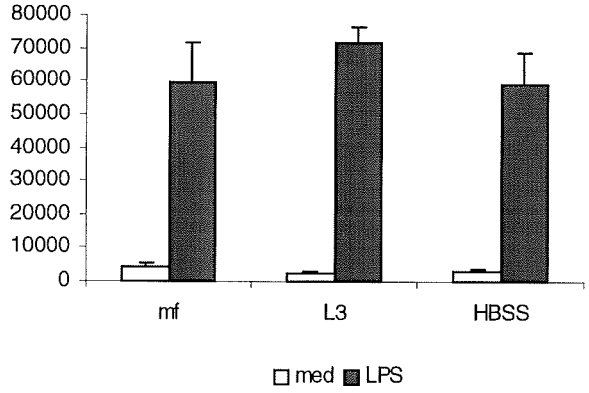
HBSS
med
CD3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60000
50000
40000
30000
20000
10000
0
mf
L3
HBSS
med
LPS

3.2.2. rIL-2 fails to restore Ag–specific proliferation of splenocytes from mf-infected mice

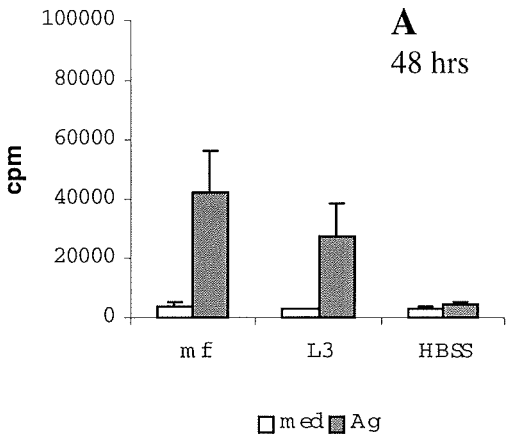
IL-2 is known to be an important factor supporting the survival of activated T cells in *in vitro* culture (reviewed Marrack P, 1998). Furthermore recombinant IL-2 (rIL-2) has been shown reverse the *in vitro* Ag-specific proliferative unresponsiveness in some, but not all, individuals with brugian filariasis (Sartono, 1995a). As Ag-stimulated splenocytes from mf-infected BALB/c mice produce only low or undetectable levels of IL-2 *in vitro,* the possibility that a lack of IL-2 may act as a limiting factor upon the *in vitro* proliferation of these cells was investigated. Ag-stimulated cells from infected and uninfected control animals were cultured in the presence or absence of 50 U/ml rIL-2.

Figure 3.4 shows the Ag-stimulated proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control mice cultured in the presence or absence of rIL-2. Addition of rIL-2 caused a significant increase in the cpm of Ag-stimulated cells from mf-infected but not L3-infected animals at 48 hrs (p=0.012 and 0.09 respectively). However, rIL-2 also significantly increased the cpm incorporated in medium only wells, such that when calculated as stimulation indices (SI = cpm of Ag-stimulated wells/ cpm of unstimulated wells), rIL-2 caused a significant reduction in the SI of cells from infected animals. Ag-stimulated cells from mf–infected animals displayed a mean SI of 10.8 +/-1.7 at 48 hrs while upon addition of rIL-2 this was reduced to 5.8 +/-0.7 (p=0.0004). This demonstrates that while rIL-2 increased proliferation it did not do so in an Ag-specific fashion. Furthermore regardless of the presence or absence of rIL-2, the cpm of Ag-stimulated cells from mf-infected animals were not significantly greater than background levels (p=>0.05) after 96 hrs culture. Whether considered as cpm or SI, the presence of rIL-2 did not significantly alter the Ag-stimulated proliferative responses of cells from mf-

infected or L3-infected animals at 96 hrs. rIL-2 was active as demonstrated by a significant increase (p=0.012) in proliferation in rIL-2 supplemented versus medium-only wells. This experiment was repeated twice with equivalent results.

FIGURE 3.4. Ag-stimulated proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control mice in the presence or absence of rIL-2

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen in the presence (B/D) or absence (A/C) of 50 U/ml rIL-2 were measured by ^{3}H thymidine incorporation. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group.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100000
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60000
40000
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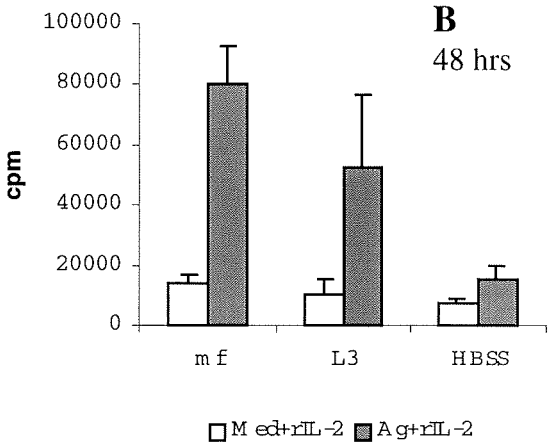
0
cpm
mf
L3
HBSS
med
Ag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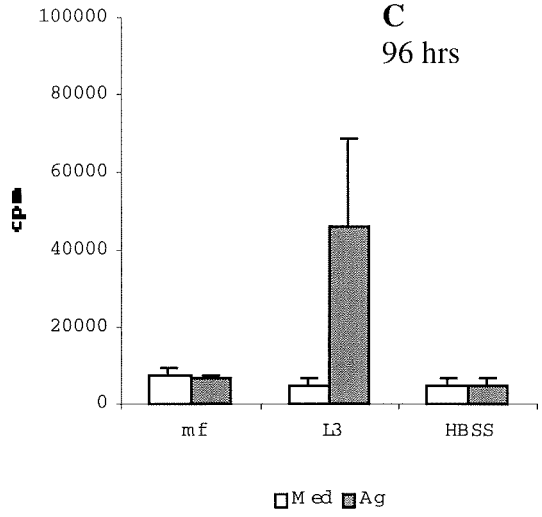
0
cpm
mf
L3
HBSS
Med+rIL-2
Ag+rIL-2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80000
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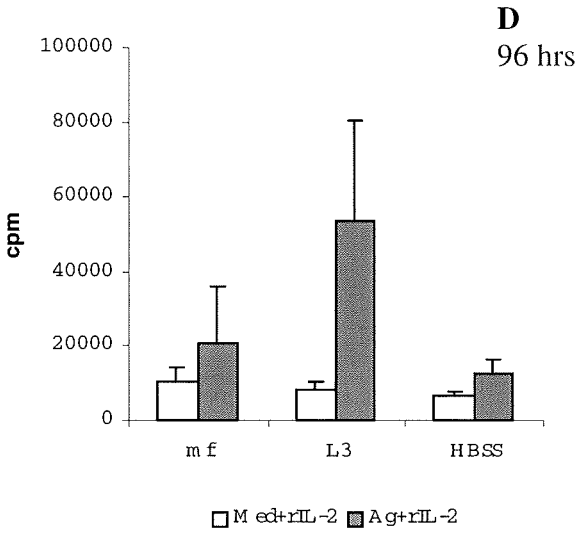
40000
20000
0
mf
L3
HBSS
Med
Ag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96 hrs
100000
80000
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40000
20000
0
cpm
mf
L3
HBSS
Med+rIL-2
Ag+rIL-2

3.2.3. Ag-stimulated splenocytes from mf-infected but not L3-infected animals produce NO

The production of NO by activated macrophages has been identified as a factor mediating proliferative suppression in several models of parasitic infection (Candolfi, 1994; Dai, 1999; Mabbot, 1995; Schleiffer, 1993). In order to assess *in vitro* production of NO, the Greiss reaction was used to determine levels of nitrite, a stable end product of NO breakdown, in culture supernatants of Ag-stimulated splenocytes from infected and uninfected control animals.

Figure 3.5 illustrates levels of nitrite production after 48 hrs. At this time-point significant levels of nitrite were detected only in cultures of cells from mf-infected animals whilst cells from L3-infected and uninfected control animals produced only background levels below the sensitivity of the assay (5μM). Extension of these observations over a time course of Ag-stimulated culture showed that splenocytes from mf-infected animals continued to produce nitrite and that levels in these cultures increased in a manner displaying a strong negative correlation with Ag specific proliferative responses ($r=-0.866$, $P=<0.001$) as shown in Figure 3.6. No such accumulation of nitrite was seen in Ag-stimulated cultures of cells from L3-infected mice.

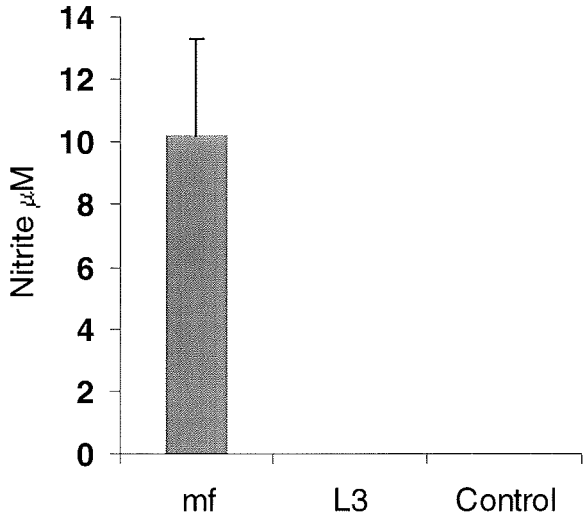


FIGURE 3.5. Nitrite production by Ag-stimulated splenocytes from mf-infected, L3-infected and uninfected control mice at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Nitrite levels in supernatants from 48 hr cultures were determined using the Greiss reaction. All values represent the mean and standard deviation of five animals per group.

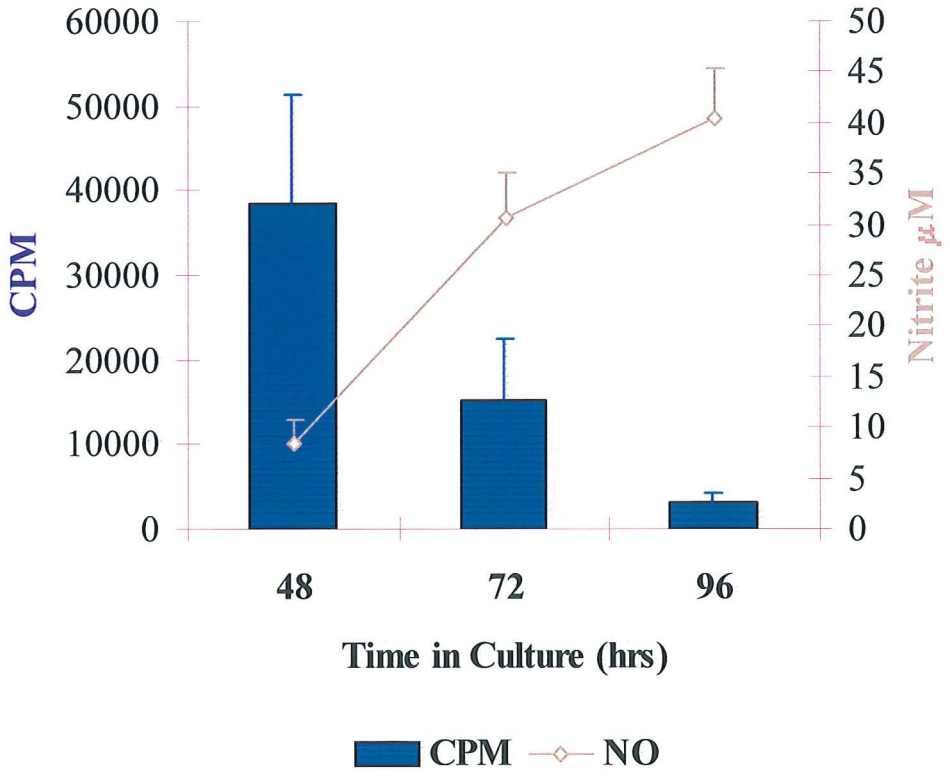


FIGURE 3.6 Proliferative suppression correlates with NO production by splenocytes from mf-infected mice

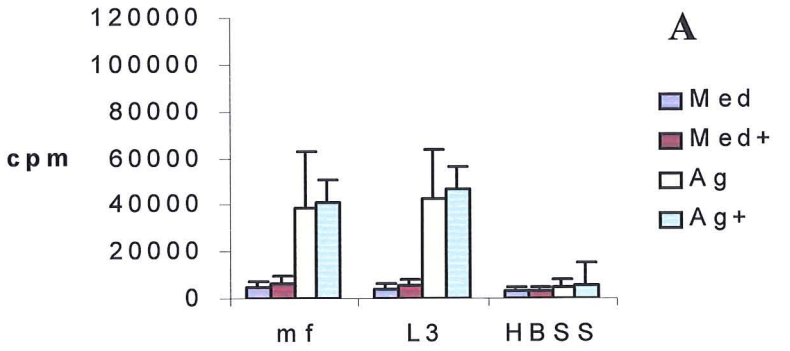
Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 µg/ml *B. pahangi* adult antigen were measured by ^{3}H thymidine incorporation over a time course of *in vitro* culture. Results are expressed as mean cpm incorporated in triplicate wells. Nitrite levels in the supernatants of Ag stimulated cultures were determined using the Greiss reaction. All values represent the mean and standard deviation of five animals per group. Nitrite levels in culture (right hand axis) display a strong negative correlation with proliferative responses (left hand axis) (r = - 0.866, p = <0.001).

3.2.4. Inhibition of NO production restores defective Ag-specific proliferative responses of cells from mf-infected animals

iNOS inhibitors were used to determine whether or not the observed correlation between NO production and proliferative suppression represented a cause and effect relationship. Ag-stimulated cultures of splenocytes from infected and uninfected animals were supplemented with either 250 μg/ml L-NMMA or 500 μM aminoguanidine (AMG), both inhibitors of iNOS activity (Corbett, 1992). Figure 3.7 shows the Ag-stimulated proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control animals in the presence or absence of L-NMMA over a time-course of *in vitro* culture. Figure 3.8 shows the equivalent results from an experiment using AMG. Inhibition of iNOS activity had no effect upon proliferative responses in any group at 48 hrs, and caused only a slight and insignificant improvement in the proliferation of cells from mf-infected animals after 72 hrs culture. After 96hrs culture, however, the addition of either L-NMMA or AMG significantly increased Ag specific proliferation of cells from mf-infected animals (p=0.012 and 0.011 respectively). Levels of nitrite in these culture supernatants were significantly reduced at this time point, L-NMMA and AMG respectively achieving 58% (P=0.012) and 50% (P=0.012) reduction (Figure 3.9). At no time did the presence of either inhibitor affect proliferative responses of cells from L3-infected or uninfected control animals in any way. These experiments have been repeated on multiple occasions with equivalent results. As AMG is known to be a more specific inhibitor of iNOS activity than L-NMMA (Corbett, 1992) it was the inhibitor of choice for all further experiments.

FIGURE 3.7 Ag-stimulated proliferation of splenocytes from mf-infected, L3-infected and uninfected control mice in the presence or absence of L-NMMA

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen, in the presence or absence of 250 μg /ml L-NMMA, were measured by ^{3}H thymidine incorporation incorporation at (A) 48 hrs, (B) 72 hrs and (C) 96 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group. *, significantly different ($P = <0.05$) from unsupplemented cultures.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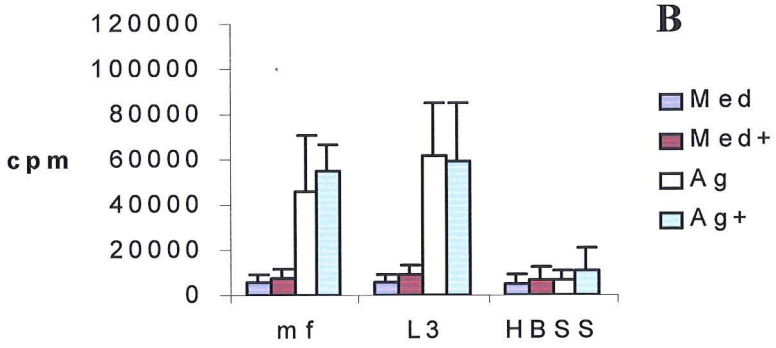
mf
L3
HBSS
A
Med
Med+
Ag
Ag+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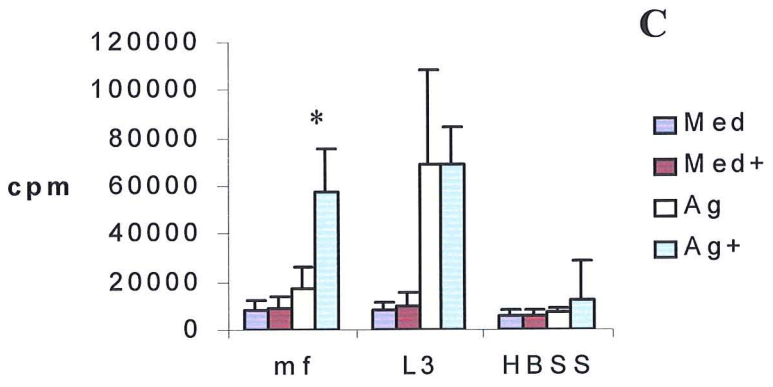
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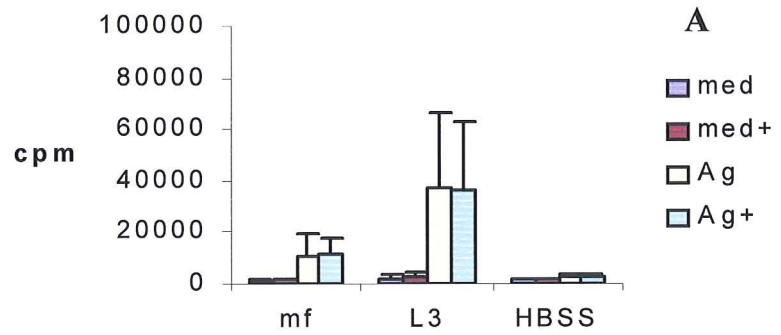
FIGURE 3.8. Ag-stimulated proliferation of splenocytes from mf-infected, L3-infected and uninfected control mice in the presence or absence of aminoguanidine (AMG)

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen, in the presence or absence of 500 μM AMG, were measured by ^{3}H thymidine incorporation incorporation at (A) 48 hrs, (B) 72 hrs and (C) 96 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from unsupplemented cultures.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L3
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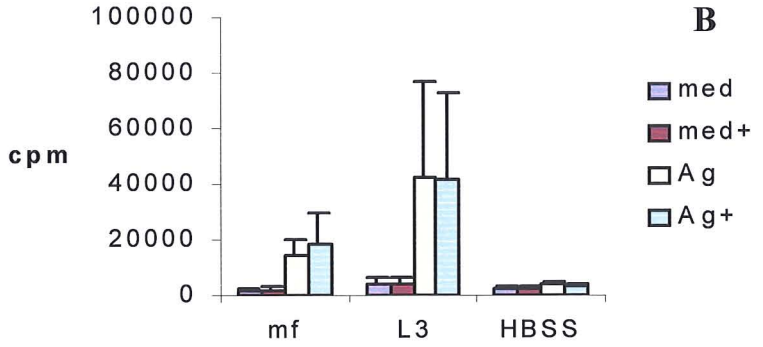
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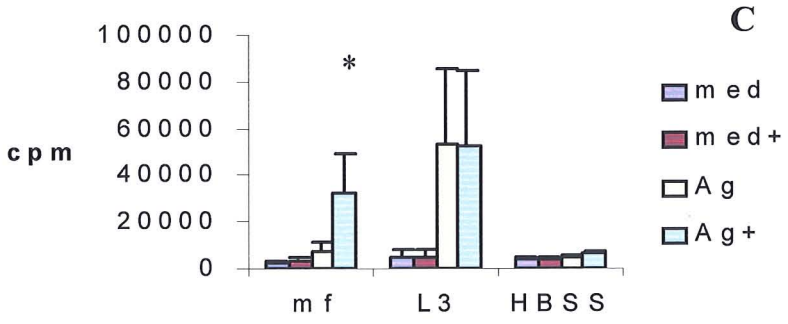
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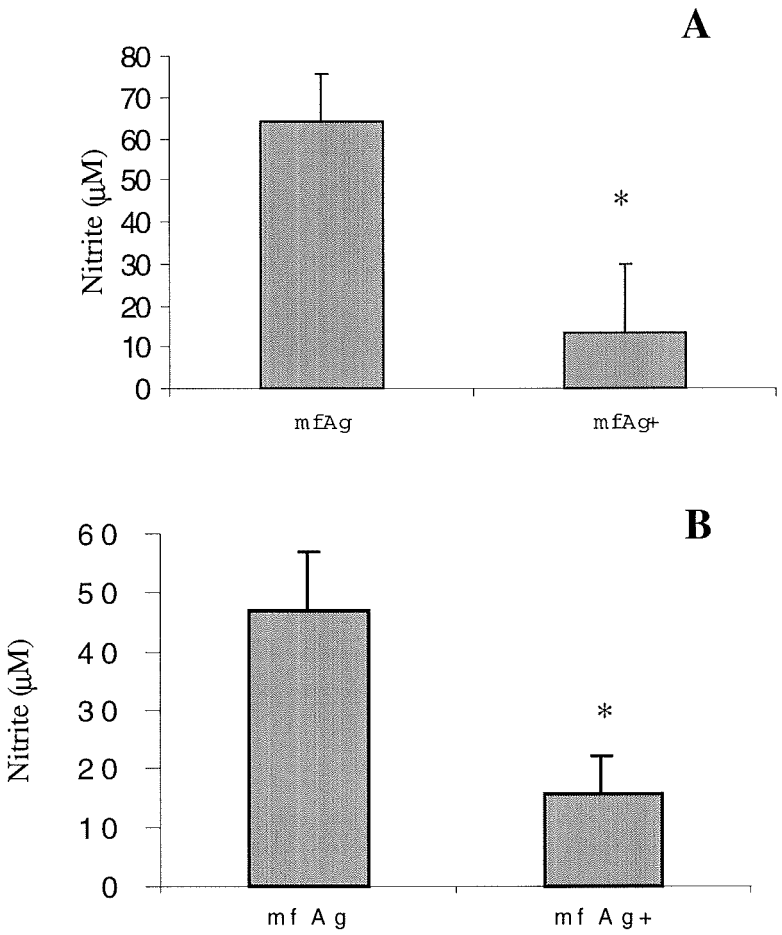


FIGURE 3.9. Effects of iNOS inhibition upon nitrite production in Ag stimulated culture

Mice were injected intravenously with 10^5 mf *B. pahangi*. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 µg/ml *B. pahangi* adult Ag in the presence or absence of (A) 500 µM AMG or (B) 250 µg/ml L-NMMA. Nitrite levels in supernatants from 96 hr cultures were determined using the Greiss reaction. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from unsupplemented cultures.

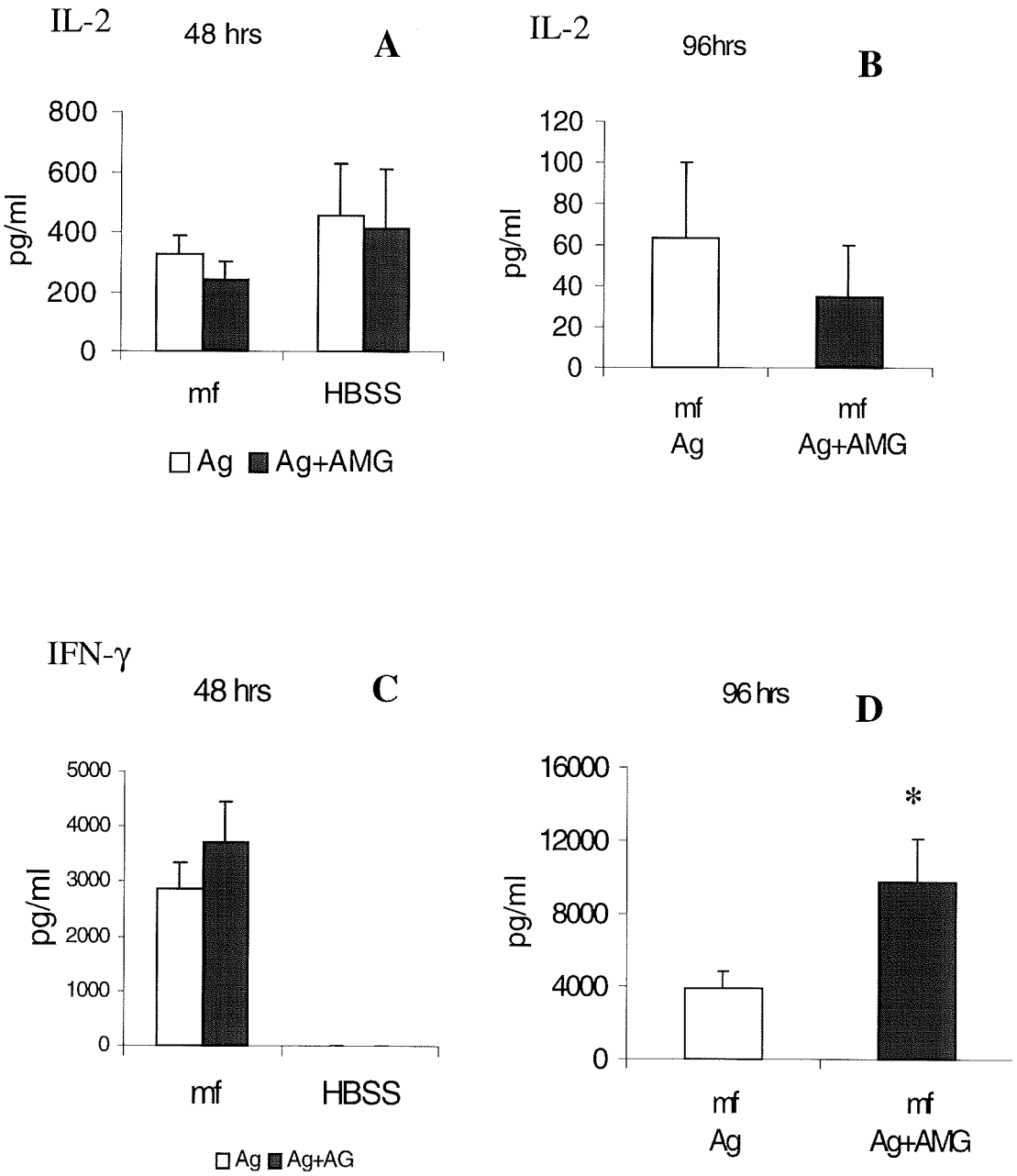
3.2.5. Inhibition of iNOS activity significantly enhances production of IFN-γ but not IL-2 by splenocytes from mf-infected animals after 96 hrs of Ag-stimulated culture

There is evidence that NO can modulate T cell function in a variety of ways; in particular it has been shown to down-regulate production of IFN-γ and IL-2 both *in vitro* and *in vivo* (Taylor-Robinson, 1994). Furthermore it has been suggested that NO selectively inhibits production of these Th1 cytokines as it has been shown to enhance production of IL-4 by Th2 clones (Chang, 1997). To assess the effect of NO upon cytokine production in Ag-stimulated culture, splenocytes from infected animals were cultured in the presence or absence of AMG.

Figure 3.10 illustrates antigen stimulated IL-2 and IFN-γ production by splenocytes from mf-infected animals after 48 and 96 hrs culture in the presence or absence of AMG. At 48 hrs equivalent levels of Ag-stimulated IFN-γ and IL-2 were found regardless of the presence of AMG (it may be of note to recall that proliferative responses of cells from mf-infected animals are not affected by iNOS inhibition at this time-point). After 96 hrs culture however while levels of IL-2 were not improved by iNOS inhibition, levels of IFN-γ were significantly increased compared to those of unsupplemented cultures (p=0.02). No Ag-specific IL-4 or IL-5 were detected in Ag-stimulated cultures of cells from mf-infected animals regardless of iNOS inhibition. This experiment has been repeated three times with equivalent results.

FIGURE 3.10 Effect of iNOS inhibition upon production of IL-2 and IFN-γ in Ag-stimulated culture

Mice were injected intravenously with 10^5 mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg /ml *B. pahangi* adult Ag in the presence or absence of 500 μM AMG. Cytokine levels in supernatants from 48 hr (A/C) and 96 hr (B/D) cultures were measured by 2-site ELISA. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from unsupplemented cultures.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pg/ml
mf
HBSS
Ag
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IL-2
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100
80
60
40
20
0
pg/ml
mf
Ag
mf
Ag+AMG
IFN-γ
48 hrs
C
5000
4000
3000
2000
1000
0
pg/ml
mf
HBSS
Ag
Ag+AG
96 hrs
D
16000
12000
8000
4000
0
pg/ml
*
mf
Ag
mf
Ag+AMG

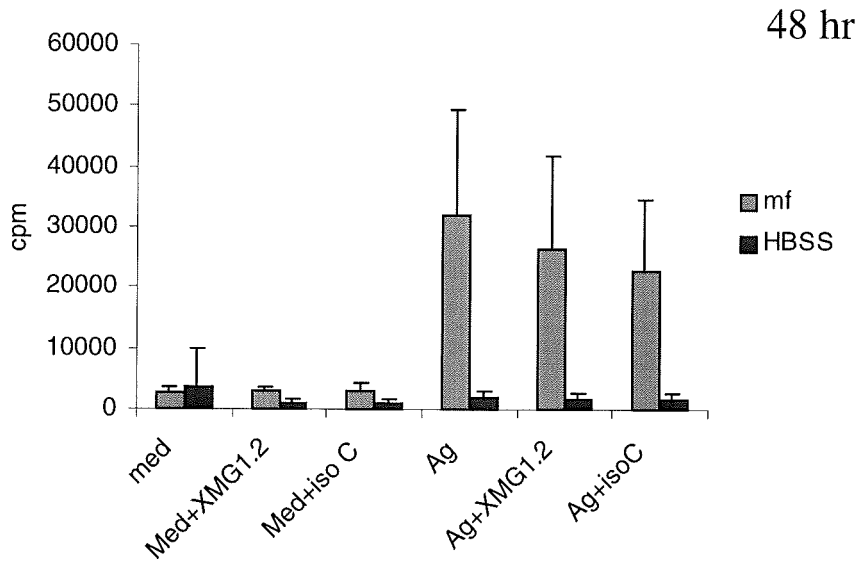
3.2.6. Blocking IFN-γ activity *in vitro* fails to reduce NO production or restore Ag specific proliferation.

IFN-γ is a potent inducer of NO production and is the only cytokine capable of acting alone in this respect (Ding, 1988). Furthermore neutralisation of IFN-γ activity *in vitro* has been shown to prevent NO production and restore suppressed proliferative responses in a murine model of toxoplasmosis (Neyer, 1998). To test whether a similar pathway is operative *in vitro* following mf-infection, Ag-stimulated cultures of cells from infected and uninfected control animals were supplemented with the anti IFN-γ antibody XMG1.2 or an isotype matched control Ab.

Figure 3.11 shows the proliferative response of splenocytes from mf-infected and uninfected control animals over a time-course of Ag-stimulated culture, supplemented where indicated with either 100 μg/ml XMG1.2, or isotype matched control MAb. The addition of XMG1.2 or control MAb had no significant effect upon the proliferative responses of cells from mf-infected animals. As shown in Figure 3.12.A no Ag-stimulated IFN-γ could be detected in cultures containing XMG1.2, indicating that IFN-γ was effectively neutralised at the concentration of MAb used, within the sensitivity of the assay (39 pg/ml). In line with the results of the proliferation assay no significant differences were found in Ag-stimulated nitrite production in the presence of either MAb (Figure 3.12.B). This experiment was repeated three times with equivalent results.

FIGURE 3.11. Proliferative responses of splenocytes from mf-infected and uninfected control animals to *B. pahangi* adult Ag in the presence or absence of anti-IFN-γ MAb

Mice were injected intravenously with 10^5 mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen, alone or in the presence of 100 μg/ml XMG1.2 or an inactive isotype matched control MAb, were measured by ^{3}H thymidine incorporation. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group. *, significantly different ($P = <0.05$) from unsupplemented cultures.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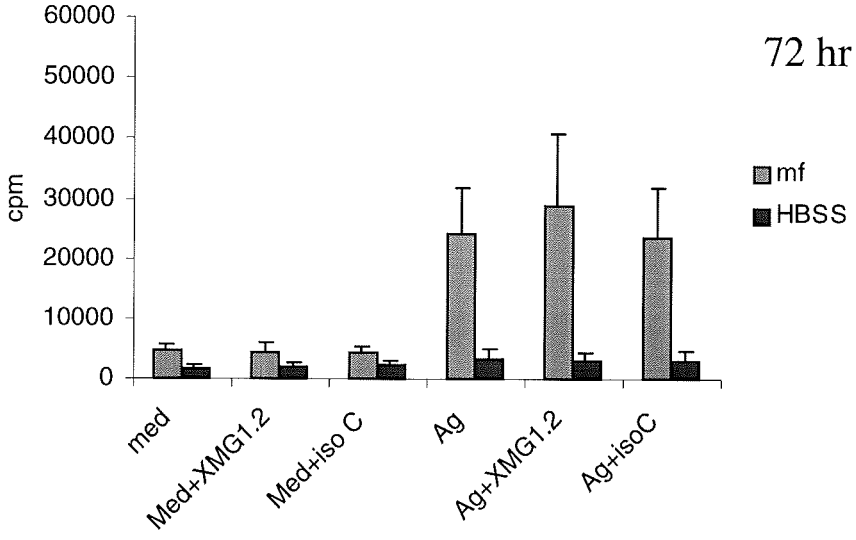
Med+iso C
Ag
Ag+XMG1.2
Ag+isoC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50000
40000
30000
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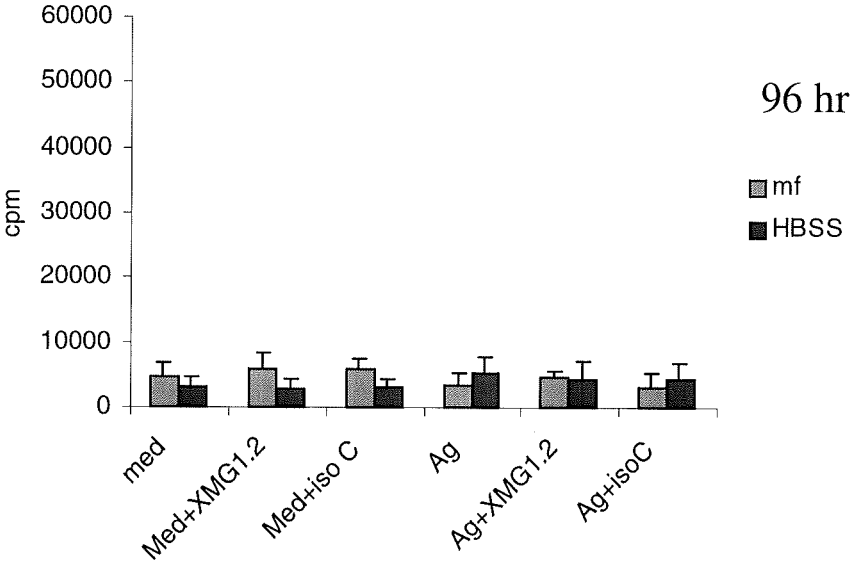
cpm
mf
HBSS
med
Med+XMG1.2
Med+iso C
Ag
Ag+XMG1.2
Ag+isoC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cpm
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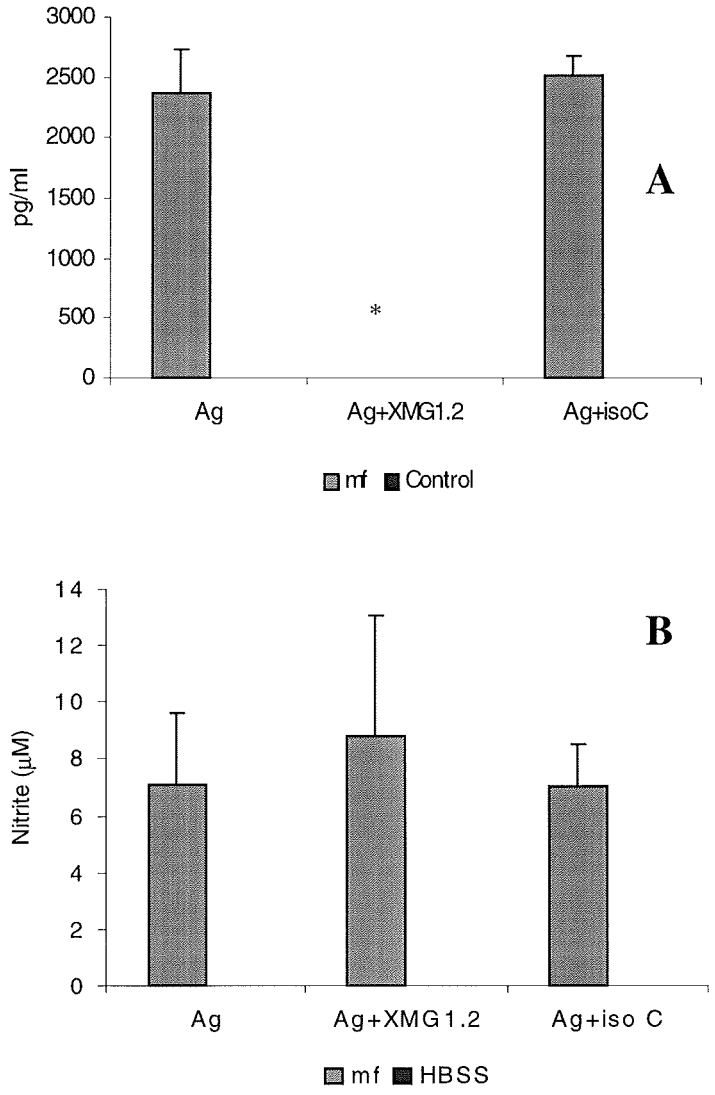


FIGURE 3.12. Upon addition of XMG1.2 IFN-γ is undetectable in Ag-stimulated cultures of splenocytes from mf-infected animals while nitrite production is unaltered

Mice were injected intravenously with 10^5 mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes (1 x 10^7 cells/ml) were restimulated with 10 μg/ml *B. pahangi* adult antigen, alone or in the presence of 100 μg/ml XMG1.2 or an inactive isotype matched control MAb. IFN-γ (A) and nitrite (B) production in supernatants from 48 hr cultures were measured using 2-site ELISA and the Greiss reaction respectively. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from unsupplemented cultures.

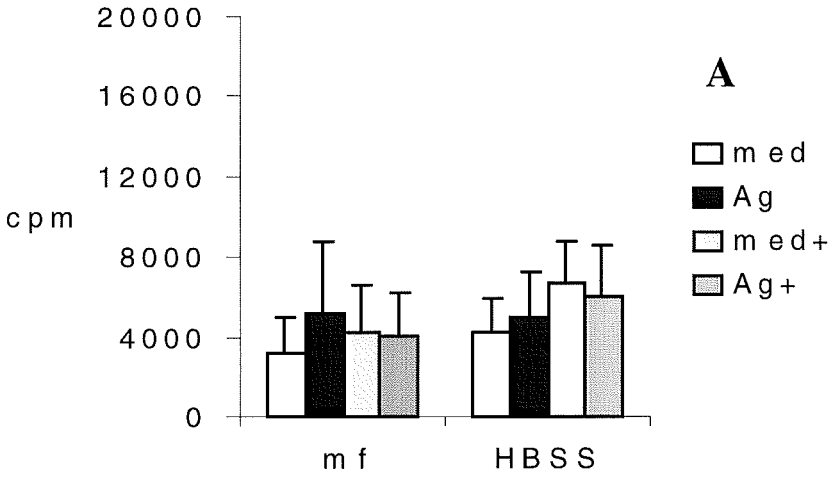
3.2.6. Polymixin-B fails to inhibit nitrite production or proliferative suppression in Ag-stimulated culture

The presence of gram negative micro organisms in filarial worms was first reported by McLaren *et al* in (1975) and there is currently a resurgence of scientific interest directed toward these intracellular symbionts. The potential of these *Wolbachia* as targets for chemotherapy, mediators of pathology and also in terms of possible immunomodulatory effects provides the focus of these studies (recently reviewed Taylor, 1999). Although identified as a consideration for future studies by Kozek in (1977) the effect of products of bacterial origin present in the crude Ag preparations used in *in vitro* studies has not been addressed until recently. IFN-γ and LPS have been shown to display synergism as potent stimulators of NO production (Ding, 1988) and it has been demonstrated that sequential exposure to IFN-γ followed by LPS is efficient at stimulating NO production by murine macrophages (Lorsbach, 1992). To investigate the role of bacterial LPS in driving Ag-stimulated NO production *in vitro,* cells from mf-infected and uninfected control animals were cultured in the presence of polymixin B (PxB) an inhibitor of the biological activity of LPS.

Figure 3.13 shows Ag-driven proliferation in the presence or absence of 2.5 μg/ml PxB at 96 hrs (A) and nitrite production under the same conditions over a time-course of *in vitro* culture (B). The concentration of PxB used was determined to be optimal in preliminary experiments. Addition of polymixin B had no effect upon the Ag-stimulated production of NO_2^- by cells from mf-infected animals or upon Ag-specific proliferative responses, suggesting that LPS is not involved in generating the *in vitro* proliferative suppression seen in this model. This experiment was repeated three times with similar results.

FIGURE 3.13. Ag-stimulated proliferative responses and nitrite production by splenocytes from mf-infected and uninfected control animals in the presence or absence of polymixin B

Mice were injected intravenously with 10^5 mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen, in the presence or absence of 2.5 μg/ml polymixin B (PxB), were measured by ^{3}H thymidine incorporation over a time course of *in vitro* culture. Proliferative responses after 96 hrs are shown (A). Results are expressed as mean cpm incorporated in triplicate wells. Nitrite levels in the supernatants from 48, 72 and 96 hr cultures (1x10^7 cells/ml) were determined using the Greiss reaction (B). All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from unsupplemented cultures.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cpm
8000
4000
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mf
HBSS
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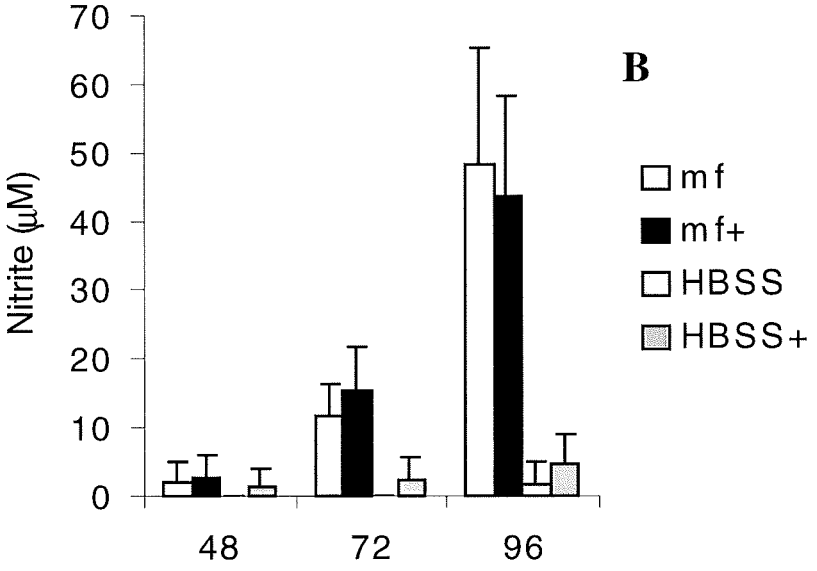
Ag
med+
Ag+


70
60
50
Nitrite (μM)
40
30
20
10
0
48
72
96
B
mf
mf+
HBSS
HBSS+

3.2.8. Splenocytes from infected animals display similar patterns of responsiveness when restimulated *in vitro* with *B. pahangi* or *A. viteae* Ag

To further assess any potential contribution of the *Wolbachia* bacteria to responses seen during *in vitro* assays, cells from infected and uninfected control animals were restimulated with an extract of *Acanthocheilonema viteae,* a related filarial parasite lacking endosymbionts.

Figure 3.14 shows cytokine responses of cells from mf-infected, L3-infected and uninfected control animals restimulated *in vitro* with 10 μg/ml *B. pahangi* or *A. viteae* Ag. The same polarisation of responses was observed regardless of the antigen used: IFN-γ dominating the cytokine profile of cells from mf-infected animals while splenocytes from L3–infected animals produced high levels of IL-4 and IL-5.

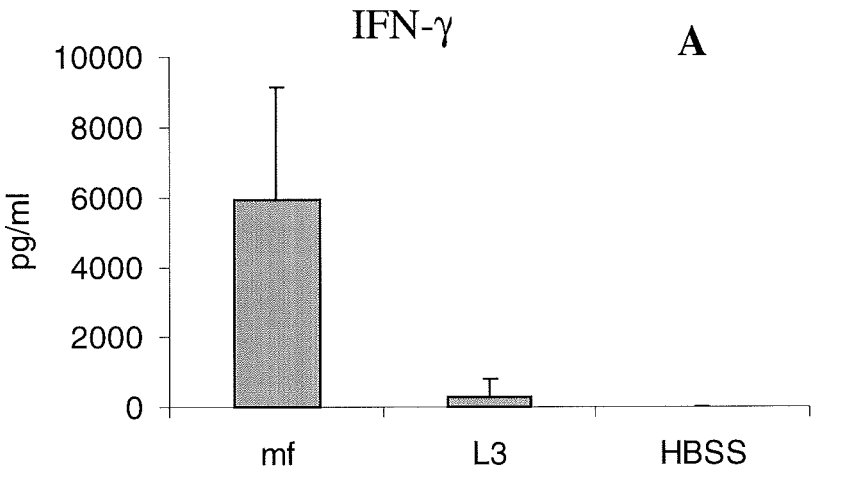
Cells from both mf-infected and L3-infected animals proliferated in response to restimulation with *A. viteae* Ag at 48 hrs (Figure. 3.15A). After 96 hrs however, proliferation of cells from mf-infected animals was significantly lower than background (p=0.012) while cells from L3-infected animals continued to proliferate strongly (Figure 3.15B). Again these results are highly comparable to those of *B. pahangi* stimulated splenocytes (Figure 3.1). These experiments were repeated three times with similar results.

To confirm that the proliferative suppression seen following restimulation with *A. viteae* and *B. pahangi* Ag are mechanistically related, NO production was measured in the culture supernatants. As shown in Figure 3.16A, splenocytes from mf-infected but not uninfected control animals produced high levels of NO upon restimulation with both antigens, confirming NO production *in vitro* does not require the presence of LPS of bacterial origin. Furthermore while not affecting the responses of cells from L3-infected or uninfected control animals, addition of AMG significantly improved the proliferation of

cells from mf-infected animals after 96 hrs of *A. viteae* Ag-stimulated culture (p=0.034) Figure 3.16 (B).

FIGURE 3.14. *A. viteae* Ag-stimulated cytokine production by splenocytes from mf-infected, L3-infected and uninfected control mice at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal of volume HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *A. viteae* adult Ag. Levels of (A) IFN-γ, (B) IL-4 and (C) IL-5 in supernatants from 48 hr cultures were measured by 2-site ELISA. All values represent the mean and standard deviation of five animals per group.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4000
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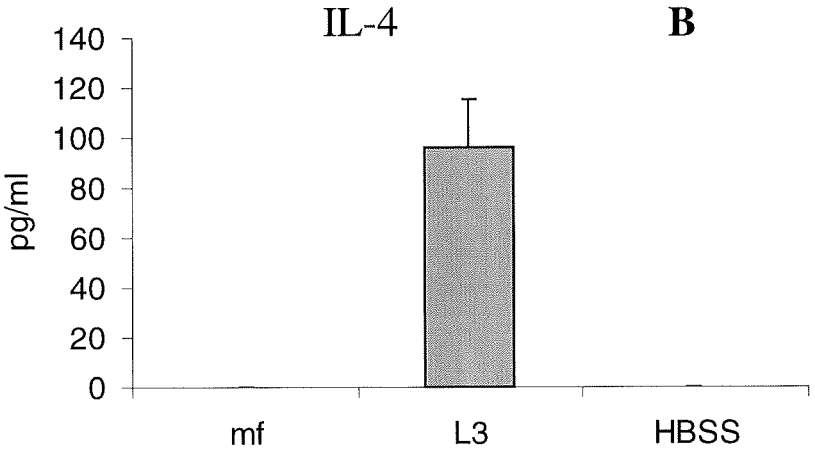
pg/ml
mf
L3
HBSS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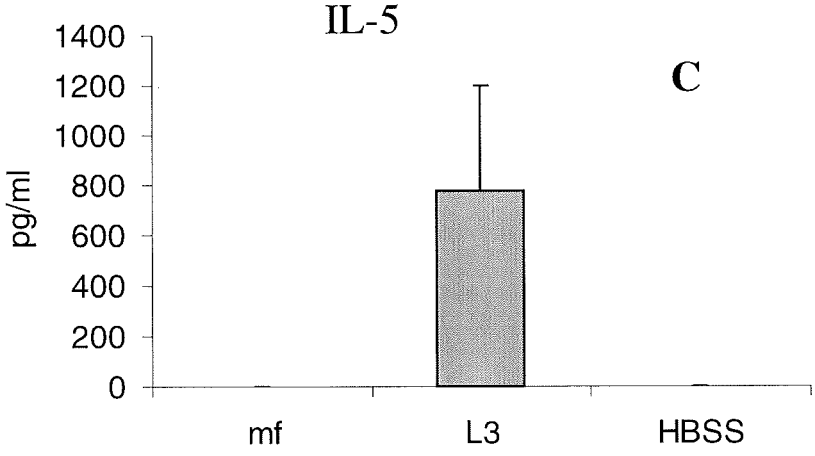
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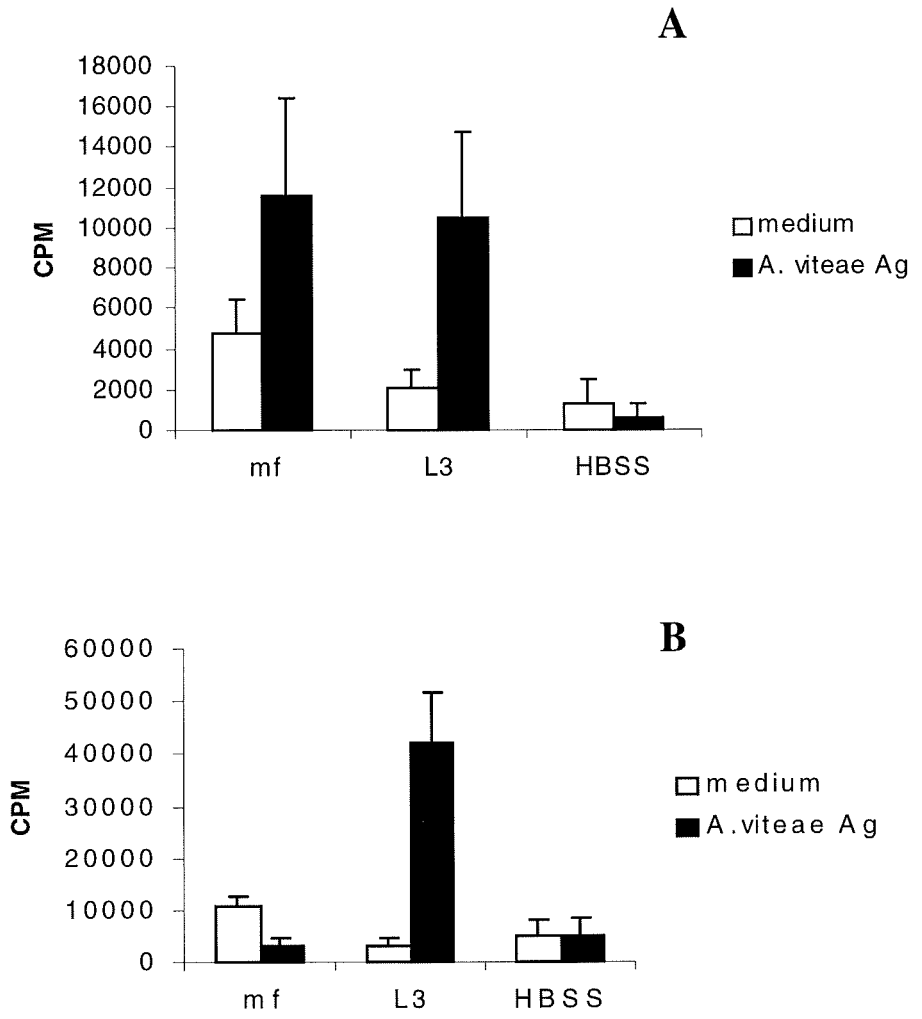


FIGURE 3.15. Proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control mice to *A. viteae* adult antigen at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 µg/ml *A. viteae* adult antigen were measured by ^{3}H thymidine incorporation at (A) 48 hrs and (B) 96 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group.

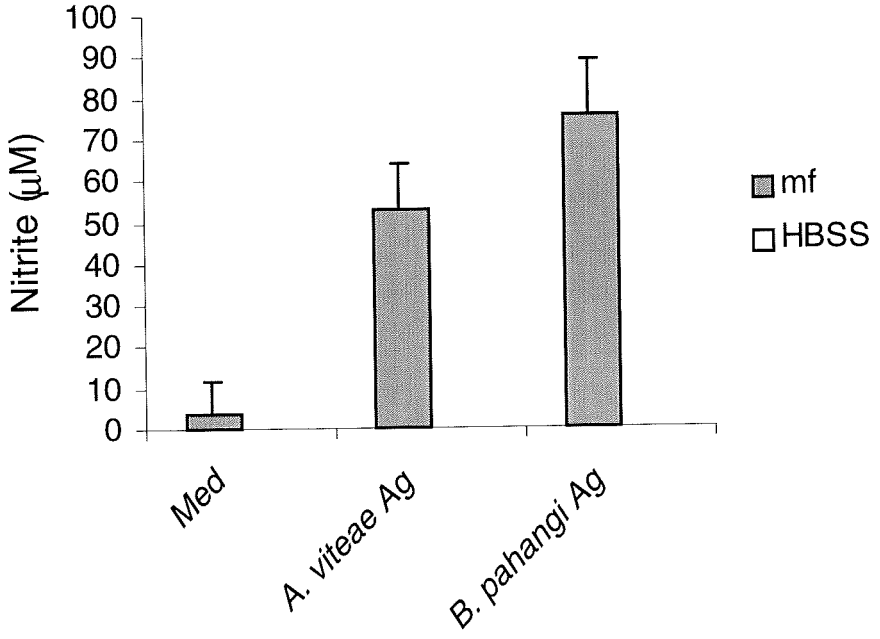


FIGURE 3.16. Nitrite production by *A. viteae* / *B. pahangi* Ag stimulated splenocytes from mf infected and uninfected control mice at 12 d.p.i.

Mice were injected intravenously with 10^5 mf *B. pahangi* or an equal volume HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *A. viteae* or *B. pahangi* adult Ag. Nitrite levels in supernatants from 96 hr cultures were determined using the Greiss reaction. All values represent the mean and standard deviation of five animals per group.

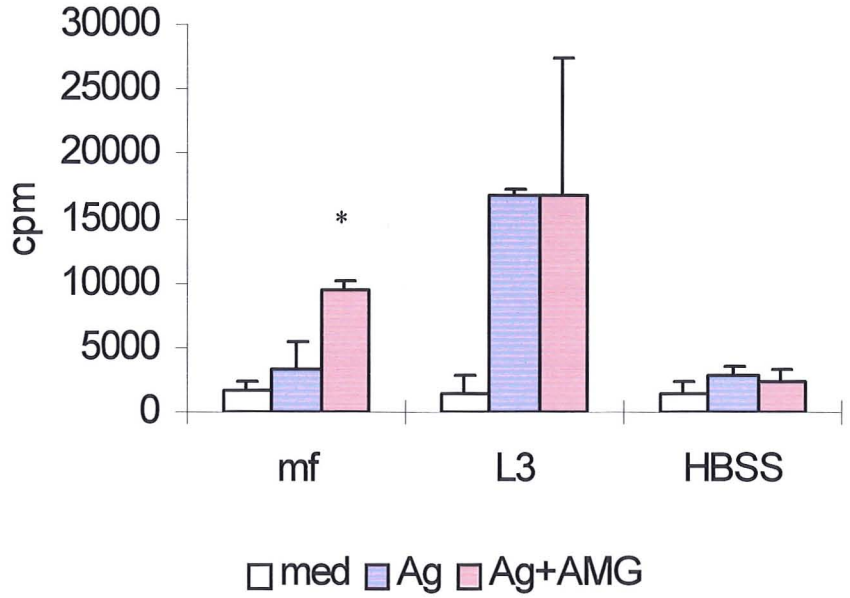


FIGURE 3.17. *A. viteae* Ag-stimulated proliferation of splenocytes from mf-infected, L3-infected and uninfected control mice in the presence or absence of aminoguanidine

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *A. viteae* adult antigen, in the presence or absence of 500 μM aminoguanidine (AMG), were measured by ^{3}H thymidine incorporation. Proliferative responses at 96 hrs culture are shown. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from unsupplemented cultures.

3.2.9. Ag-stimulated proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control mice in the presence of NO

It has been suggested that Th1 cells are more susceptible to the effects of NO in terms of proliferation and cytokine production than Th2 cells (Chang, 1997; Taylor-Robinson, 1994). In the light of this it became of interest to determine whether Ag-stimulated cells from mf-infected and L3-infected animals were equally sensitive to NO. To this end Ag-stimulated cultures of splenocytes from infected and uninfected animals were supplemented with S-nitroso-*N*-acetylpenicillamine (SNAP) an NO donor, after 48 hrs culture to a final concentration of 200 μM. SNAP was added in a 10 μl volume of RPMI, and all wells not receiving SNAP had 10 μl of RPMI added to compensate for any dilution effects. Figure 3.18 shows Ag-specific proliferative responses in the presence or absence of NO. The addition of SNAP caused a significant reduction in the Ag-driven proliferation of cells from mf-infected animals after 72 hrs culture (p=0.03). Cells from L3-infected animals displayed an apparent, yet less marked and statistically insignificant, reduction in proliferation in the presence of SNAP at both 72 and 96 hrs. At 96 hrs there was no difference in the proliferative responses of cells from mf-infected animals in the presence or absence of SNAP.

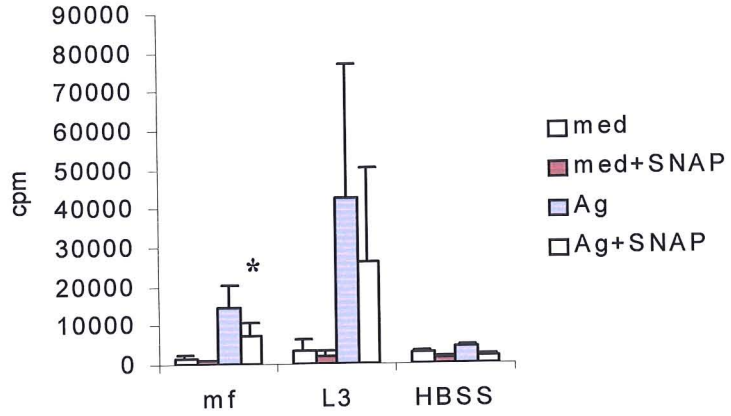
The levels of nitrite generated by 200 μM SNAP were similar to those produced by cells from mf-infected animals in Ag-stimulated culture, as shown in Figure. 3.19. These results demonstrate that cells from mf-infected animals are more acutely sensitive to the anti-proliferative effects of NO derived from chemical donors than are cells from L3-infected animals. However the marked depression of proliferative responses at 72 and 96 hrs in the presence of SNAP suggests that cells from L3-infected animals may also be sensitive to NO. One factor to consider in this respect is that cells from mf-infected animals are continually producing NO in Ag-stimulated culture, which, in the presence of

SNAP results in exposure to slightly higher levels of NO than would be derived from SNAP alone in other groups. It is likely however that the time-scale of NO production is more critical. In preliminary experiments it was determined that the vast majority of SNAP derived NO is evolved over the first 24 hrs while NO production by cells from mf-infected animals is far more gradual and sustained, so its effects would be felt for longer. This experiment has been repeated twice with equivalent results.

FIGURE 3.18. Ag-stimulated proliferation of splenocytes from mf-infected, L3-infected and uninfected control mice in the presence or absence of SNAP

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen were measured by ^{3}H thymidine incorporation at (A) 72 and (B) 96 hrs culture. Where indicated 200 μM SNAP was added after 48 hrs. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from unsupplemented cultures.

A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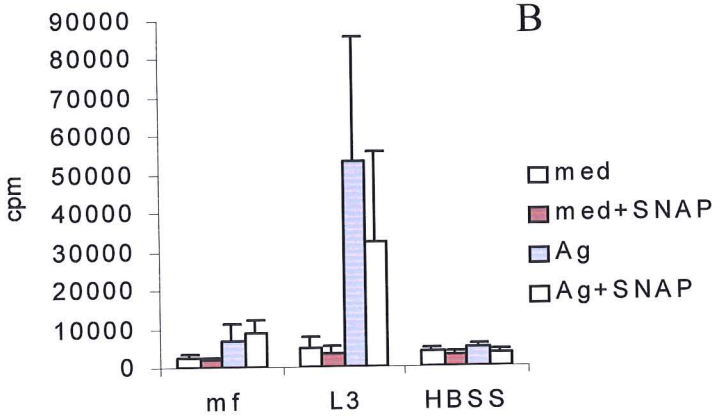
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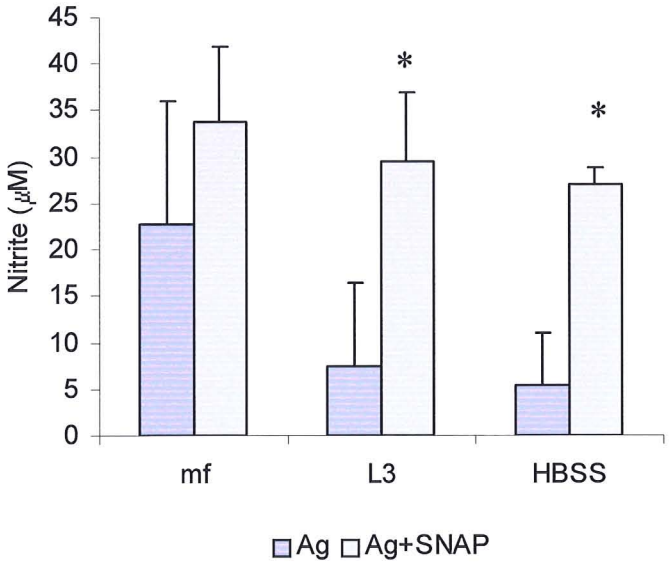


FIGURE 3.19. Nitrite production by Ag-stimulated splenocytes from mf-infected, L3-infected and uninfected control mice in the presence of 200 μM SNAP at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Where indicated 200 μM SNAP was added after 48 hrs. Nitrite levels in supernatants from 96 hr cultures were determined using the Greiss reaction. All values represent the mean and standard deviation of five animals per group. *, significantly different ($P = <0.05$) from unsupplemented cultures.

3.3. Discussion

The results presented in this chapter demonstrate that i.v. infection of BALB/c mice with mf or L3 *B. pahangi* results in development of differentially polarised immune responses. In line with previous studies of infection via the intraperitoneal (Lawrence, 1994), or subcutaneous route (Osborne, 1996), infection with L3 elicits development of a Th2 response, characterised by production of Ag-specific IL-4, IL-5 and IL-10 in the absence of IFN-γ. In contrast to this cells from mf-infected animals produce Ag-specific IFN-γ in the absence of Th2 cytokines. Furthermore while cells from L3-infected animals proliferate well in Ag-stimulated culture, splenocytes from mf-infected animals show lower levels of proliferation which is down-regulated under the influence of NO, independently of IL-2.

Cells from L3-infected animals proliferated well in response to Ag over a time-course of *in vitro* culture whereas cells from mf-infected animals proliferated at levels equivalent to, or lower than, background after 96 hrs culture. The observation in multiple experiments of proliferation significantly below background levels suggests that an active form of suppression is induced by re-exposure to Ag *in vitro*. Although it has previously been reported that following s.c. infection splenocytes from mf-infected animals are unable to proliferate in response to Ag, this observation was made at a single time-point, which may have missed any earlier responses (Osborne, 1996). Following i.v. infection cells from mf-infected animals do display Ag-specific proliferation at earlier time-points which is subsequently suppressed. The observations of early proliferative responses and Ag-specific cytokine production argue against any defects in Ag-processing and presentation, or co-stimulation, in cells from mf-infected animals taken *ex-vivo*.

Although Ag-specific proliferative responses are impaired the polyclonal responses of cells from mf-infected animals (as measured by stimulation with ConA, anti-CD3 MAb

or LPS) remain intact. This reflects the situation in human infection wherein suppression is Ag-specific (Ottesen, 1977; Piessens, 1980b). Interestingly cells from animals infected with L3 via the i.v. route showed normal mitogenic responses, in contrast to observations previously made following s.c. infection wherein ConA driven proliferation was down-regulated under the influence of IL-4 (Osborne, 1996). All other factors being equal this suggests that L3 induced suppression of polyclonal responses is dependent upon the route of infection, as previously suggested by the absence of suppression following i.p. infection (Osborne, 1999).

Ag-stimulated splenocytes from mf-infected animals produce high levels of IFN-γ and only low to undetectable levels of IL-2. The survival of activated T cells *in vitro* is supported by cytokines such as IL-2 and IL-4 and whilst T cells may be exposed to high levels of these growth factors during the initial stages of their response to Ag, their concentration decreases as the response progresses. Thus T cells may die as a result of reduced levels of such growth factors (Reviewed (Marrack, 1998)). Previous studies in the jird/*B. pahangi* model showed that splenocytes from microfilaremic jirds were unable to proliferate or produce significant levels of IL-2 in response to parasite Ag (Leiva, 1989; Prier, 1988). The inability of rIL-2 to prolong the proliferative response of cells from mf-infected animals demonstrates that lack of IL-2 is not the underlying cause of proliferative suppression. Furthermore the enhanced levels of proliferation seen in medium only wells supplemented with rIL-2 illustrates both the activity of rIL-2 in culture, and that the IL-2 responsiveness of cells from mf-infected animals is intact.

Splenocytes from mf-infected animals were shown to produce significantly higher levels of nitrite in Ag-stimulated culture than cells from L3-infected or uninfected control animals. Extension of these observations demonstrated an accumulation of nitrite which showed a strong inverse correlation with proliferative responses. No such accumulation of

nitrite was seen in cultures of cells from L3-infected animals. Furthermore inhibition of iNOS activity by either L-NMMA or AMG reversed proliferative suppression providing evidence of a causative relationship. iNOS inhibition had no effect upon proliferative responses at 48 or 72 hrs suggesting that either a threshold level of NO production needs to be surpassed, or prolonged exposure to NO is necessary for its anti-proliferative effects to become apparent. Neither inhibitor had any effect upon the proliferative responses of cells from other groups.

The production of high levels of NO by cells from mf-infected but not L3-infected animals is perhaps unsurprising when considering their respective cytokine production. Whilst MO activation and NO production are induced by pro-inflammatory stimuli such as IFN-γ and LPS (Ding, 1988), IL-4 and IL-10 are known to down-regulate NO production. Pre-treatment with IL-4 inhibits IFN-γ induced NO production and the development of leishmanicidal activity in murine peritoneal MO (Liew, 1991). IL-10 has also been shown to inhibit the microbicidal activity of activated MO towards both intracellular and extra-cellular parasites (Gazzinelli, 1992). Furthermore IL-4 and IL-10 have been shown to act synergistically to promote MO deactivation at individually sub-optimal levels (Oswald, 1992a). Recently it has been demonstrated that IL-4 and IL-10 promote an alternative pathway of arginine metabolism, via arginase rather than iNOS, in murine MO and dendritic cells (Corraliza, 1995). There are both constitutive and inducibly expressed forms of arginase, both of which convert arginine to L-ornithine and urea. IL-4 and IL-10 trigger expresssion of the inducible form of arginase and display a potent synergy in this respect (Corraliza, 1995; Munder, 1998). While co-culture of Th1 clones with MO led to the exclusive induction of iNOS activity, co-cultures with Th2 cells upregulated arginase activity without inducing iNOS (Munder, 1998). As with many factors affecting MO activation the sequence of exposure is of importance in determining the subsequent

response. Simultaneous exposure of MO to IFN-γ and IL-4/IL-10 led to a reduction in both iNOS and arginase activity. Pretreatment with IFN-γ prevented subsequent induction of arginase activity by IL-4/IL-10 and likewise prior exposure to IL-4/IL-10 prevented subsequent production of NO in response to LPS (Modolell, 1995). It has been proposed that arginase may participate in regulation of iNOS activity via substrate competition (Modolell, 1995). The demonstration that a specific inhibitor of arginase activity enhanced NO production by LPS activated MO suggest that such a regulatory mechanism may be of importance when the extracellular supply of L-arginine is limited (Chang, 1998).

It has also recently been shown than expression of arginase down-regulates NO production and prevents NO mediated apoptosis of IFN-γ/LPS activated RAW264.7 macrophages. Expression of arginase did not inhibit SNAP induced apoptosis, indicating that its effects may be mediated via depletion of intra-cellular arginase (Gotoh, 1999). Such results suggest that the iNOS/arginase balance in MO is competitively regulated by Th1/Th2 cytokines and that specific high level induction of arginase maybe one mechanism whereby Th2 cells can regulate pro-inflammatory responses. Although there is no direct evidence of a role for arginase in suppressing NO production during infection, the ability of Th2 cytokines to down-regulate MO activation is known to be associated with the susceptibility to *L. major* infection in murine models.

Interestingly pre-exposure of J774 MO to crude extract or E/S products of *Anisakis simplex* leads to a dose dependant inhibition of LPS-stimulated NO production (Cuellar, 1998). Such results suggest that products of parasite origin may interact directly with MO to suppress subsequent activation. MO activation is also known to be down-regulated during chronic *B. pahangi* infection in jirds (Nasarre, 1998). Recently it has been shown that arginase expression is upregulated in the profoundly anti-proliferative alternatively activated MO induced by i.p. infection with *B. malayi* adults or L3 (J. Allen, pers. comm).

Both these life cycle stages drive strong Th2 responses (Lawrence, 1994) and IL-4 is known to be essential to the development of the suppressive cell population (MacDonald, 1998). In this respect, it is of interest to note that the suppressive MO population induced by i.p. infection with L3 or adult worms is distinct to that induced by mf-infection. Following i.p. infection PEC from mf-infected animals were unable to support proliferation of a conalbumin specific T cell clone. Whilst inhibition of iNOS activity allowed restoration of background levels of proliferation, Ag-specific responsiveness was not recovered. iNOS inhibition did not affect the suppressive MO induced by infection with L3 or adult worms. These results illustrate that following i.p. infection, mechanisms besides NO production are involved in generating the suppressive effects of infection derived PEC, and further highlight the ability of different life cycle stages to regulate proliferative responses via distinct mechanisms. It is of note that following i.p. infection development of a suppressive APC population is restricted to the peritoneal cavity, while responses in the spleen are not suppressed. Such results illustrate a site specific effect, and provide one possible mechanism whereby filarial worms may modulate localised immune responses within their immediate environment.

NO production can modulate T cell function either directly by inducing the apoptosis of T cells or APC (Albina, 1993; Fehsel, 1995; Okuda, 1996; Sarih, 1993), or indirectly via the modulation of cytokine responses (Taylor-Robinson, 1994; Wei, 1995). Measurement of cytokine production by splenocytes from mf-infected animals in the presence of high levels of NO revealed that NO caused a significant reduction in Ag-driven IFN-γ production, whilst having no effect on IL-2. It is of note that the NO mediated suppression of IFN-γ production coincides with the suppression of proliferative responses, suggesting that NO may limit the expansion of Ag-specific IFN-γ producing T-cells. It has previously been described that NO limits the production of IL-12 by MO thus

indirectly controlling the expansion of Th1 responses (Huang, 1998b). Several studies have now shown that the maintenance of sustained Th1 responsiveness has a requirement for IL-12 production (Stobie, 2000; Yap, 2000). Although not measured in this study it is possible that suppression of IL-12 production by NO may also influence T cell proliferative responses in Ag-stimulated culture.

IFN-γ is known to be a potent inducer of MO activation and NO production (Ding, 1988). Neutralisation of IFN-γ activity abrogated the iNOS inducing activity of ConA stimulated supernatants and relieved subsequent proliferative suppression (Albina, 1991). Unexpectedly neutralisation of IFN-γ in Ag-stimulated culture failed to restore proliferative responsiveness or reduce NO production by cells from mf-infected animals. It is possible that *in vivo* exposure to IFN-γ is sufficient to prime MO for NO production in Ag-stimulated culture with other proinflammatory cytokines, such as TNF-α, providing a secondary stimulus (Ding, 1988; Green, 1990c). It is also possible that any residual IFN-γ activity, below the sensitivity of the ELISA, was efficient in inducing iNOS activity.

Bacteria of the *Wolbachia* sp. family have now been found in many filarial species, including *B. malayi, W. bancrofti* and *O. volvulus* (reviewed Taylor, 1999). Kozek *et al* (77) first identified the potential contribution of products of bacterial origin, present in filarial Ag preparations, as a consideration for *in vitro* studies. It is only recently however, that the immunomodulatory potential of *Wolbachia* has come under scrutiny. As LPS is known to act as an important triggering agent for NO production by IFN-γ activated MO, the presence of bacterial LPS in Ag preparations used for *in vitro* restimulation may assume biological significance. Taylor *et al* (2000) showed that *B. malayi* extracts induced production of proinflammatory cytokines IL-1β and TNF-α and NO by murine MO in a dose-dependent manner. These responses were attributed to the action of LPS of bacterial

origin, as they could be inhibited by polymixin B and could not be induced in MO from LPS unresponsive C3H/HeN mice. Furthermore extracts of *A. viteae,* which lacks endosymbionts, failed to stimulate any pro-inflammatory responses. However, in this study following mf-infection, experiments using polymixin B failed to demonstrate an essential role for LPS in stimulating NO production and proliferative suppression. Restimulation with *A. viteae* Ag also led to high levels of NO production which, in the absence of *Wolbachia,* is assumed to be independent of LPS. In interpreting these apparently contrasting results it may be of importance to consider that cells from infected animals were primed by *B. pahangi,* which bear *Wolbachia* in all life cycle stages, and therefore may already have been activated by exposure to pro-inflammatory cytokines *in vivo*. In the light of this, observations made following *in vitro* restimulation cannot discern any effects of bacterial products which may be mediated during the initiation of an *in vivo* response. However, the *in vitro* responses observed following stimulation with *A. viteae* Ag do demonstrate that filarial antigens cross-reactive between species can stimulate proliferation and production of either Th1 or Th2 cytokines, in a fashion determined by the previous experience of the responding cells.

NO has been shown to affect the production of various cytokines including IL-2, IFN-γ, IL-12 and IL-4 besides regulating proliferative responses (Bauer, 1997; Huang, 1998b; Taylor-Robinson, 1994). Whether or not NO preferentially affects the responses of Th1 vs Th2 cells remains an area of considerable controversy (rev Bogdan, 2000b). Taylor Robinson *et al* (94), demonstrated that Th1 but not Th2 T cell clones may be activated to produce NO which in turn limits their proliferation. NO inhibited production of IL-2 and IFN-γ by Th1 clones but not IL-4 production by Th2 clones. More recently it has been shown that exposure to SNAP-derived NO increased IL-4 production by Th2 clones. Interestingly in EL4 T lymphoma cells, which produce both IL-2 and IL-4, NO inhibited

ConA stimulated IL-2 production whilst enhancing production of IL-4 (Chang, 1997). In contrast to these results it has been reported that when stimulated by pre-activated MO both Th1 and Th2 T cell clones are subject to similar anti-proliferative effects although no down-regulation of cytokine production was observed (van der Veen, 2000). Furthermore NO has been shown to inhibit production of IFN-γ, IL-2, IL-4 IL-5 and IL-10 by anti-CD3 stimulated human T cells and no preferential inhibition of Th1 cytokines was observed (Bauer, 1997). As IL-2 and IL-4 are important T cell growth factors, any differential effects of NO upon their production may manifest itself at the level of proliferation. In order to assess the susceptibility of cells from mf-infected vs L3-infected animals to the anti-proliferative effects of NO, Ag-stimulated cultures were supplemented with SNAP. Addition of exogenous NO significantly suppressed the proliferation of cells from mf-infected animals at 72 hrs, whilst causing a marked, but statistically insignificant, reduction in the response of cells from L3-infected animals. Levels of SNAP-derived NO were within the range of that generated in Ag-stimulated cultures of cells from mf-infected animals and after 96 hrs the response of cells from mf-infected animals were similarly suppressed in the presence of endogenous or exogenous NO. Cells from L3-infected animals continued to display an insignificant reduction in proliferation. These results suggest that cells from L3-infected animals may-be affected by exogenous NO, but are not as acutely sensitive as cells from mf-infected animals. Although not measured in this study, it would be of interest to observe the effects of NO upon Ag-stimulated cytokine production by cells from L3-infected animals, to further analyse what may be the basis of any differential effects. The results presented in this chapter build on observations previously made that infection with mf and L3 of *B. pahangi* leads to development of differentially polarised immune responses, and illustrate one way in which these differences can exert their effects at the level of proliferation. Subsequent experiments

were directed towards further defining the immunomodulatory effects of NO, IFN-γ and IL-4 using a variety of knock-out mice.

CHAPTER 4. Immune responses of iNOS-/-, IFN-γR-/- and IL-4-/- mice

4.1 Introduction

The increasing availability of genetically modified gene knock-out (KO) mice has facilitated research in many areas of immunology. Deletion of specific genes has allowed direct assessment of the contribution of various cytokines and effector molecules to both host-protective and immunopathological responses. In situations associated with strongly polarized immune responses (such as helminth infection), or where the polarisation of responses is critical in determining the outcome of infection (e.g. Leishmaniasis), cytokine knock-out mice have been particularly informative. In terms of both anti-microbial activity and immunomodulation, the use of iNOS-/- mice has now largely superceded the use of iNOS inhibitors in determining the effects of NO *in vivo* (reviewed Bogdan, 2000).

While gross phenotypic changes in knock-out animals can reliably indicate a critical role for the missing gene product, the absence of effect may not necessarily denote a lack of involvement. Several factors may influence the phenotypic outcome of infection in cytokine knock-out mice, including compensation by other cytokines, or the ability of the targeted cytokine to affect factors other than its own production. The background strain on which the knock-out animals have been generated may also influence the observed responses. The importance of such considerations was illustrated in a recent study on the role of IL-4 in the expulsion of the gastrointestinal nematode *Trichuris muris*. IL-4 and IL-13 have overlapping anti-inflammatory functions and the IL-4Rα chain is a critical component of both the IL-4 and the IL-13 receptors (Mohrs, 1999). IL-4-/- mice on the C57Bl/6 background are susceptible to *T. muris* infection whilst wild type C57Bl/6 mice are not, suggesting a critical role for IL-4 in generating resistance. However, IL-4-/- BALB/c mice are resistant to infection. IL-4-/- mice on the BALB/c, but not the C57Bl/6, background produce significant quantities of IL-13 in response to infection. The *in vivo*

neutralisation of IL-13 in IL-4-/- BALB/c mice demonstrated that, in the absence of IL-4, it plays a critical role in the expulsion of *T. muris* (Bancroft, 2000). Such results illustrate both the importance of compensatory mechanisms and the influence of strain-specific effects in the interpretation of results obtained using knock-out mice.

As the results presented in Chapter 3 established that NO mediates the Ag-specific proliferative suppression of cells from mf-infected animals *in vitro,* iNOS-/- mice were used in attempt to further dissect any immunomodulatory action of NO. Genetic deletion of the iNOS gene has been shown to enhance Th1 responsiveness, in terms of both IFN-γ production and proliferation in response to a variety of infectious agents, including *T. brucei* and *L. major* (Millar, 1999; Wei, 1995). Although *in vitro* neutralisation of IFN-γ activity failed to restore proliferative responsiveness, IFN-γR-/- mice were used to further assess the response to infection, free of the influence of IFN-γ. The use of IFN-γR-/- mice provides a more complete assessment of the role of IFN-γ than *in vitro* neutralisation, as the deficiency extends to cover the *in vivo* development of responses.

As previously described, infection with L3 led to Th2 cytokine production and robust Ag-specific proliferative responses (Lawrence, 1994; Osborne, 1996). The recent demonstration that infection of BALB/c mice with *B. pahangi* L3 induces an early burst of IL-4 transcription in the draining lymph node within 24 hrs of infection suggests IL-4 may be of importance in skewing the developing response in a Th2 direction (Osborne, 1997b). Neutralising IL-4 *in vitro* partially restored defective ConA driven proliferation seen following s.c. infection with *B. pahangi* L3. ConA stimulated production of IL-2 and IFN-γ was also enhanced in the absence of IL-4 activity. Although anti-IL-4 treatment failed to enhance Ag-driven IFN-γ production, infection with *B. pahangi* L3 does prime Ag-specific IFN-γ producing cells, which are silenced *in vitro* under the influence of IL-10 (Osborne,

1999). Previously it has been show that Ag-driven IFN-γ production by splenocytes from IL-4-/-129/Sv x C57Bl/6 mice, infected with *B. malayi* L3, was not significantly higher than that of their wild type counterparts. Interestingly however, infection of IL-4-/- mice with adult worms led to a switch to a more Th1-like response, which was most marked in recipients of female worms (Lawrence, 1995). As adult female worms continually release mf, one possible explanation for these results is that IL-4 has the capacity to down-regulate mf-induced IFN-γ production. In the light of these observations, it was of interest to determine the influence of IL-4 upon the polarisation of responses following infection and how this may affect proliferative responses. To address this question IL-4-/- mice (on the BALB/c background) were infected with *B. pahangi* mf or L3 and cytokine production and proliferative responses analysed *in vitro*.

4.2 RESULTS

4.2.1 Immune responses of iNOS-/- and wild type 129/Sv mice at 12 d.p.i.

i) Proliferative responses

iNOS-/- and wild type 129/Sv mice were injected with $1x10^5$ mf *B. pahangi* or HBSS only i.v. and at 12 d.p.i. proliferative responses were measured over a time-course of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. As shown in Figure. 4.1 cells from mf-infected iNOS-/- mice showed significantly greater levels of Ag-stimulated proliferation at both 48 and 72 hrs culture than their wild type counterparts (p=0.013 and 0.025 respectively). These results are consistent with those from experiments using iNOS inhibitors, being indicative of NO mediated proliferative suppression. After 96 hrs of Ag-stimulated culture however, there was no significant difference between the proliferative responses of cells from infected iNOS-/- mice and their wild type counterparts (p=0.093). At this time-point Ag-stimulated proliferation of cells from mf-infected iNOS-/- and wild type129Sv mice was not significantly greater than that seen in medium only wells. This experiment was repeated three times with similar results in each case.

ii) Cytokine production

Figure. 4.2 shows Ag-stimulated IFN-γ production by splenocytes from mf-infected and uninfected iNOS-/- 129S/v mice and their equivalent wild type counterparts. At both 48 and 96 hrs cells from mf-infected iNOS-/- and wild type mice produced equivalent levels of IFN-γ. This result is contrary to those from experiments using iNOS inhibitors which demonstrated that NO suppresses Ag-stimulated IFN-γ production by cells from mf-infected animals. As expected no IL-4 or IL-5 was detected in any group. However, in one of three experiments 3 of 5 mf-infected iNOS-/- mice produced IL-5

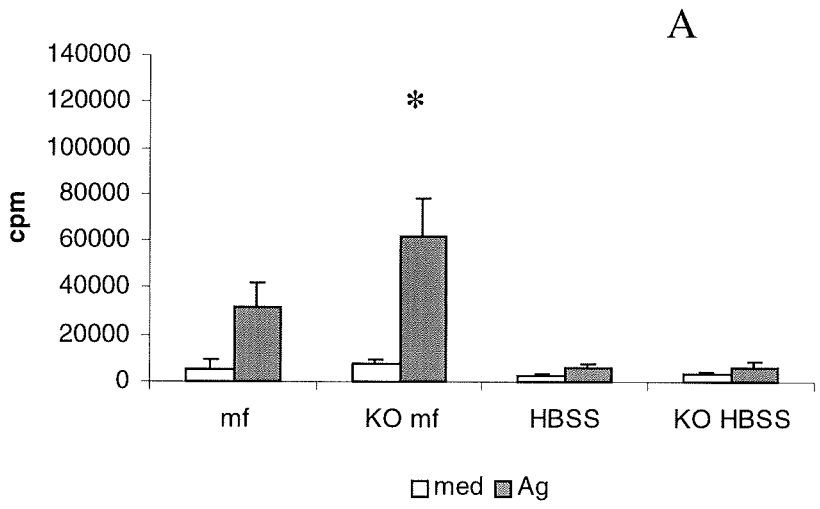
(data not shown). These results show that infection of wild type 129/Sv mice with *B. pahangi* mf leads to development of a Th1-like response at 12 d.p.i., as seen in BALB/c mice, with production of Ag-stimulated IFN-γ and development of proliferative suppression.

iii) Nitrite production

Nitrite production by splenocytes from mf-infected and uninfected mice was measured at 48 and 96 hrs culture. As shown in Figure 4.3, mf-infected iNOS-/- mice produced significantly lower amounts of nitrite than their wild type counterparts at 48 hrs (p=0.039). Unexpectedly however, after 96 hrs of Ag-stimulated culture cells from both groups of mf-infected animals produced high levels of nitrite and there was no significant difference between these groups. Equivalent results were obtained in each of three repeat experiments. The demonstration that cells from mf-infected iNOS-/- mice are capable of producing NO at levels sufficient to suppress proliferative responses (53 +/-5.1 μM), clearly illustrates that these animals are unsuitable for functional studies on NO production at late time-points. Delayed production of high levels of NO can also explain the previously described results, in terms of proliferative suppression and unaltered cytokine production in iNOS-/- mice.

FIGURE 4.1 iNOS-/- vs wild type 129Sv: Ag-stimulated proliferative responses of splenocytes from mf-infected and uninfected control mice at 12 d.p.i.

Wild type 129Sv and iNOS-/- mice were injected intravenously with 10^5 mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen were measured by ^{3}H thymidine incorporation at (A) 48, (B) 72 and (C) 96 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from wild type counterparts.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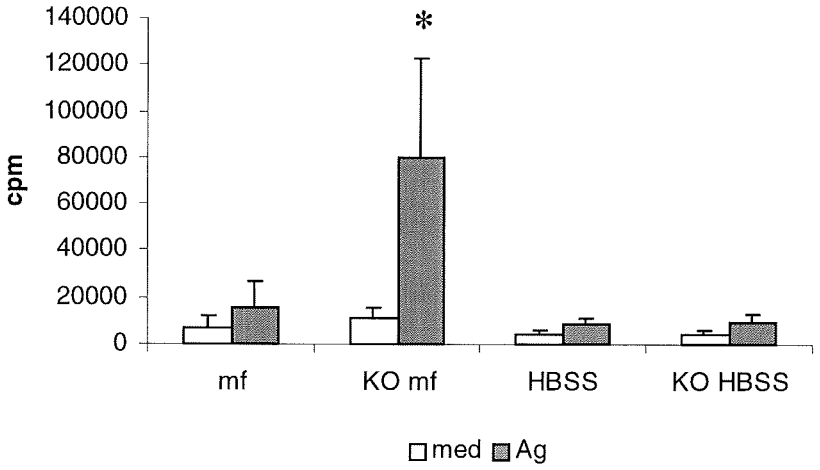
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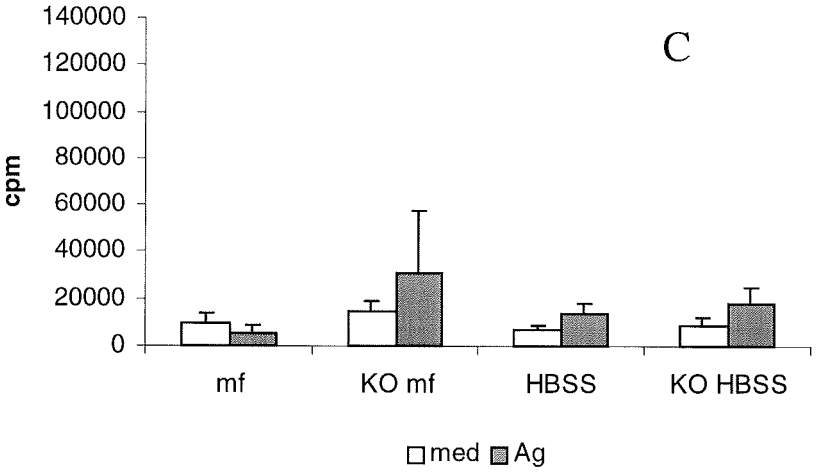
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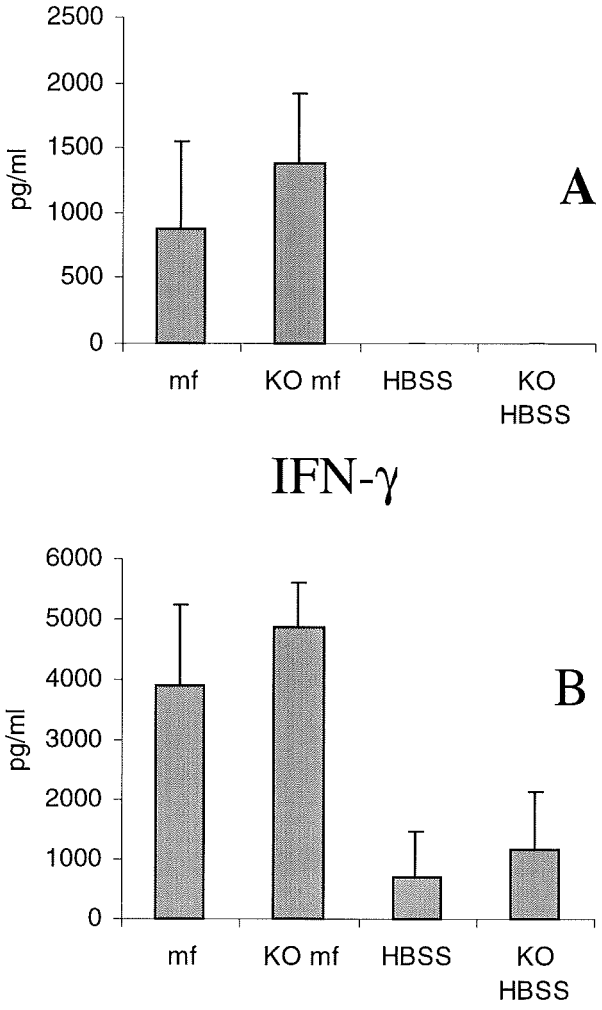


FIGURE 4.2. iNOS-/- vs wild type 129/Sv mice: Ag-stimulated IFN-γ production by splenocytes from mf-infected and uninfected control mice at 12 d.p.i.

Wild type 129/Sv and iNOS-/- mice were injected intravenously with 10^5 mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Levels of IFN-γ in supernatants from (A) 48 and (B) 96 hr cultures were measured by 2-site ELISA. All values represent the mean and standard deviation of five animals per group.

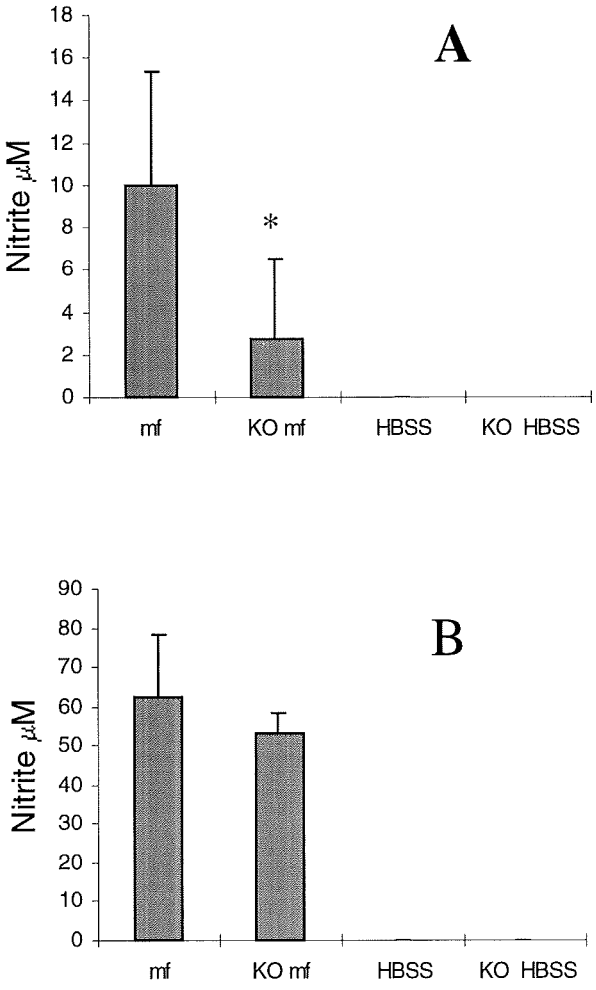


FIGURE 4.3. iNOS-/- vs wild type 129/Sv mice: Nitrite production by Ag-stimulated splenocytes from mf infected and uninfected control mice at 12 d.p.i.

Wild type 129/Sv and iNOS-/- mice were injected intravenously with 10^5 mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Nitrite levels in supernatants from (A) 48 and (B) 96 hr cultures were determined using the Greiss reaction. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from wild type counterparts.

4.2.2 Immune responses of IFN-γR-/- and wild type 129/Sv mice at 12d.p.i.

i) Proliferative responses

As IFN-γ is essential to the induction of NO production in various murine models of infection, proliferative responses of splenocytes from mf-infected and uninfected control IFN-γR-/- and wild type129/Sv mice were measured over a time-course of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. As shown in Figure 4.4, cells from mf-infected animals proliferated in response to Ag at 48 hrs and there was no significant difference between groups (p=0.72). At both 72 and 96 hrs, however, cells from mf-infected IFN-γR-/- mice proliferated at significantly higher levels than those of their wild type counterparts (p=0.0093 and 0.0027 respectively). At 72 and 96 hrs only cells from mf-infected KO animals proliferated at levels significantly higher than background. Equivalent results were achieved in three repeat experiments.

ii) Cytokine responses

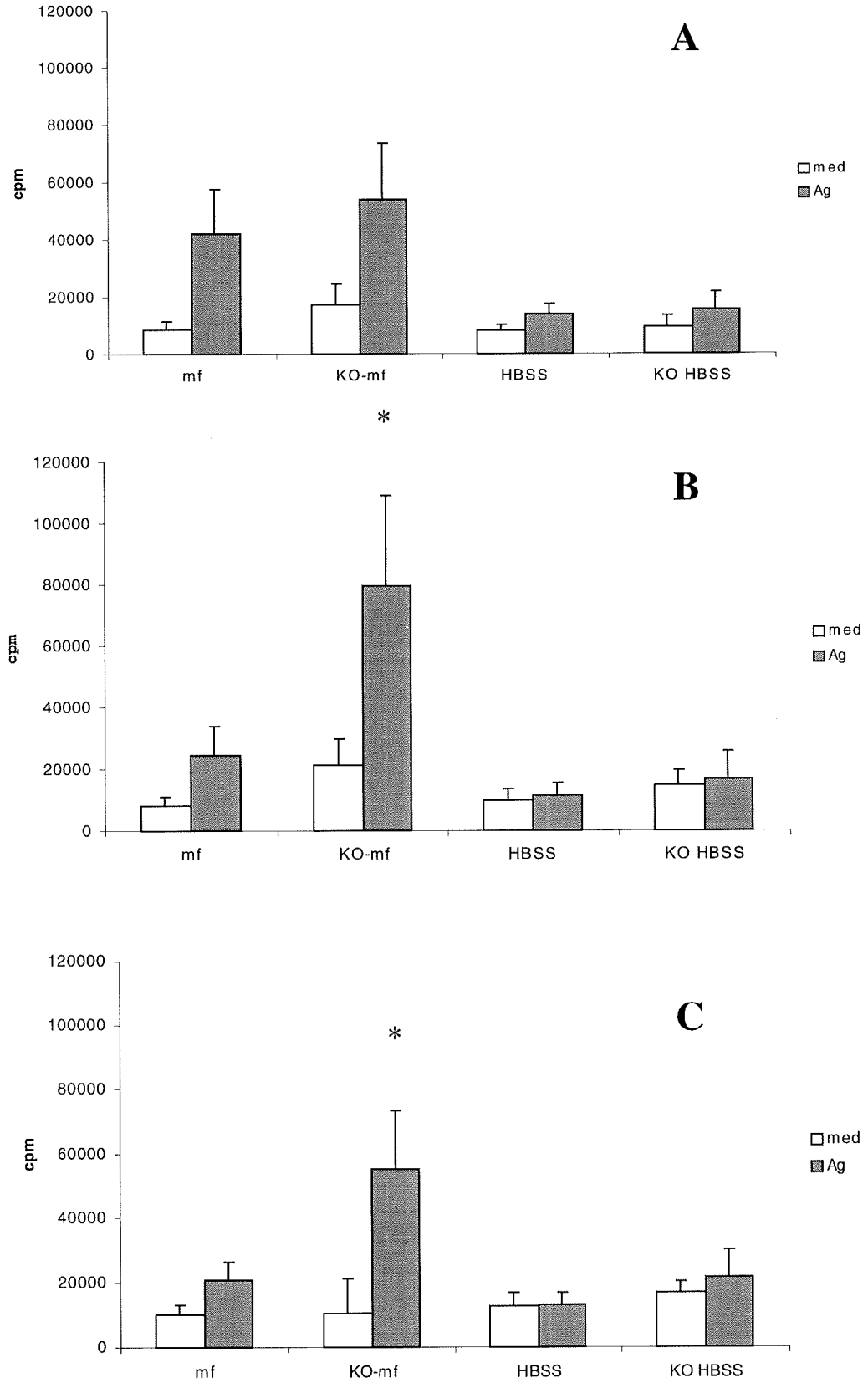
Figure 4.6 shows cytokine production of cells from mf-infected and uninfected control IFN-γR-/- and wild type 129/Sv mice in Ag-stimulated culture. Cells from both KO and wild type mf-infected animals produced low levels of IL-2 which were not significantly higher than those produced by cells from uninfected control animals (data not shown). The observation that cells from infected IFN-γR-/- mice do not produce higher levels of IL-2 than their wild type counterparts suggests that, as described in previous experiments, a lack of IL-2 does not account for the suppression of proliferative responses. No production of IL-4 or IL-5 was detected in any group. Cells from both groups of mf-infected animals produced IFN-γ and at 48 hrs there was no significant difference between these groups.

iii) Nitrite production

Figure 4.6 shows nitrite production by cells from IFN-γR-/- and wild type 129/Sv mice after 48 and 96 hrs of Ag-stimulated culture. Splenocytes from mf-infected IFN-γR-/- mice produced significantly less nitrite than their wild type counterparts indicating that, as previously reported (Mabbot, 1998), they are unable to respond to IFN-γ with MO activation and NO production.

FIGURE 4.4 IFN-γR-/- vs wild type 129Sv: Ag-stimulated proliferative responses of splenocytes at 12 d.p.i.

Wild type 129Sv and IFN-γR-/- mice were injected intravenously with 10^5 mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen were measured by ^{3}H thymidine incorporation at (A) 48 and (B) 72 and (C) 96 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from wild type counterparts.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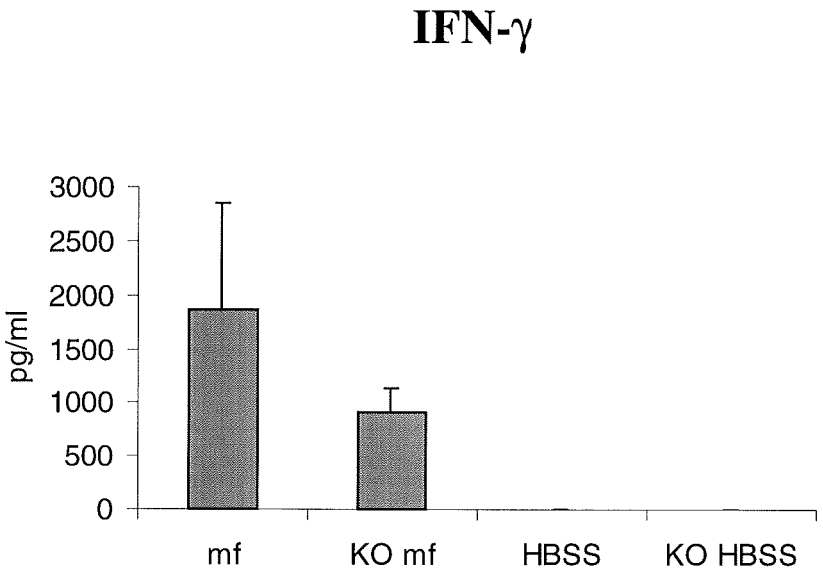


FIGURE 4.5 IFN-γR-/- vs wild type 129/Sv mice: Ag-stimulated IFN-γ production by splenocytes from mf infected and uninfected control mice at 12 d.p.i.

Wild type 129/Sv and IFN-γR-/- mice were injected intravenously with 10^5 mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Levels of IFN-γ in supernatants from 48 hr cultures were measured by 2-site ELISA. All values represent the mean and standard deviation of five animals per group.

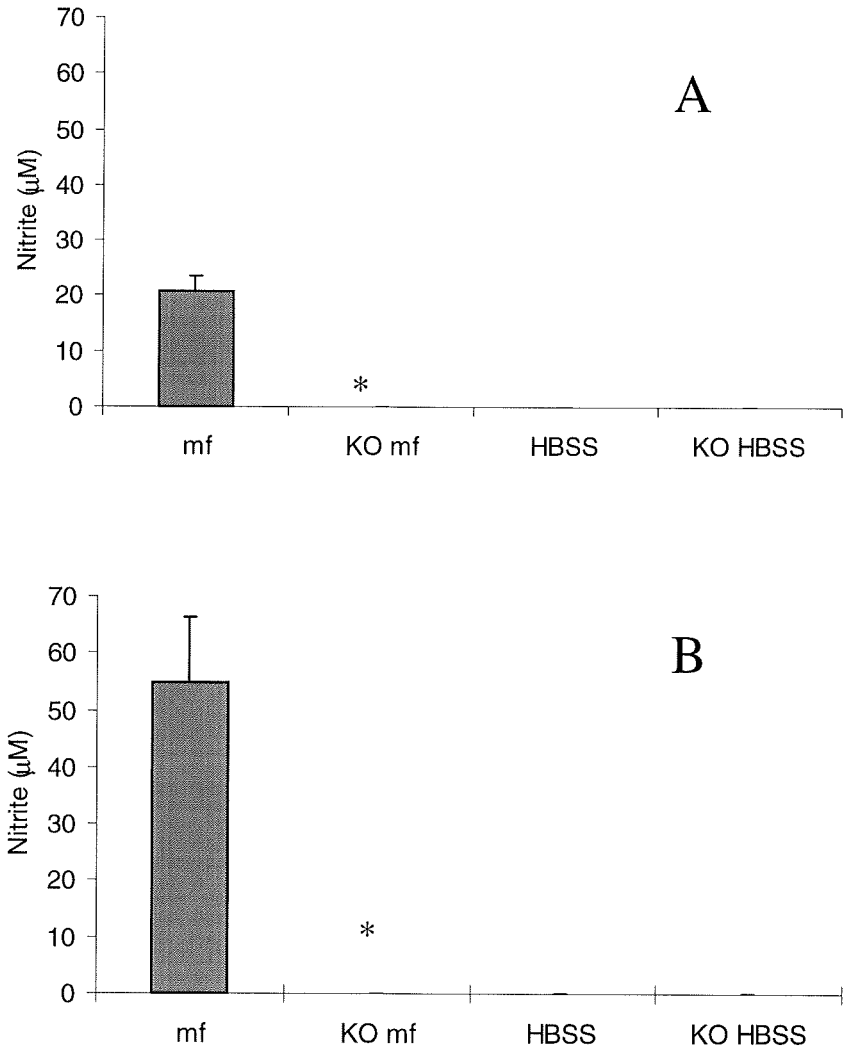


FIGURE 4.6 IFN-γR-/- vs wild type 129/Sv mice: Nitrite production by Ag stimulated splenocytes from mf-infected and uninfected control mice at 12 d.p.i. Mice were injected intravenously with 10^5 mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg /ml *B. pahangi* adult Ag. Nitrite levels in supernatants from (A) 48 and (B)96 hr cultures were determined using the Greiss reaction. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from wild type counterparts.

4.2.3 Immune responses of IL-4-/- and wild type BALB/c mice at 12d.p.i.

i) Proliferative responses

Proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control IL-4-/- and wild type BALB/c mice were measured over time-course of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag (Figure. 4.7). Cells from mf-infected IL-4 KO mice displayed the same pattern of proliferative responses as previously described following infection of BALB/c mice, showing early proliferation before the response is down-regulated. This may be expected as infection with mf does not lead to production of large amounts of IL-4 at 12 d.p.i.. However following infection with L3 proliferative responses of IL-4-/- mice were lower than those of their wild type counterparts after 72 hrs culture (p=0.03). Proliferative responses at 96 hrs culture were not measured in this experiment. In one other experiment in IL-4-/- mice after 96 hrs culture, Ag-stimulated splenocytes from 2 of 5 L3-infected animals proliferated at levels below background.

ii) Cytokine production

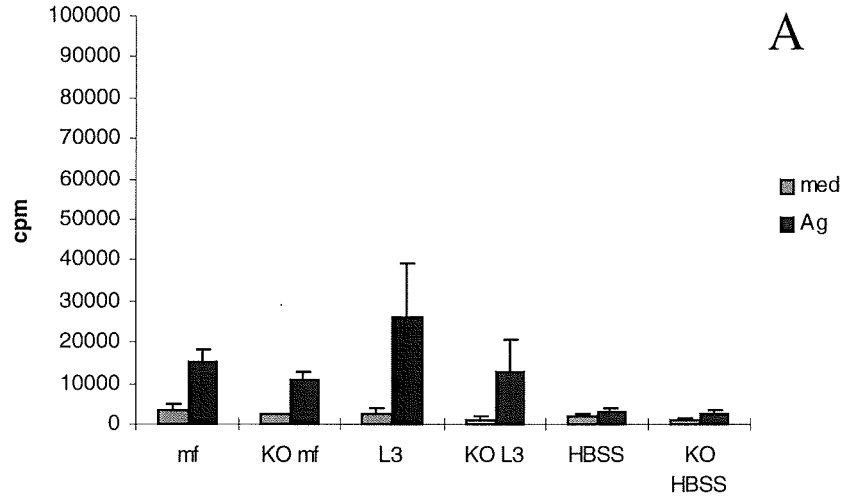
Analysis of cytokine production confirmed that IL-4-/- mice did not produce IL-4 under any circumstances tested (both Ag-stimulated and ConA-stimulated culture). Cytokine production after 72 hrs of Ag-stimulated culture is shown in Figure 4.8. Production of IFN-γ was greater in both groups of infected IL-4-/- mice than their wild type counterparts (p=0.03 for mf-infected and p=0.012 for L3-infected animals). L3-infected BALB/c mice produced only background levels of IFN-γ in Ag-stimulated culture. Interestingly IL-4-/- and wild type BALB/c mice produced equivalent levels of IL-5.

iii) Nitrite production

Figure 4.9 shows nitrite production by cells from IL-4-/- and wild type BALB/c mice in Ag-stimulated culture. Consistent with their increased production of IFN-γ splenocytes from L3-infected IL-4-/- mice produced significantly greater amounts of nitrite than their wild type counterparts. Cells from mf-infected KO animals produced similar levels of NO to their wild type counterparts.

FIGURE 4.7 IL-4-/- vs wild type BALB/c mice: Ag stimulated proliferation of splenocytes from mf-infected, L3-infected and uninfected control animals at 12 d.p.i.

Wild type BALB/c and IL-4-/- mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen were measured by ^{3}H thymidine incorporation at (A) 48 and (B) 72 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from wild type counterparts.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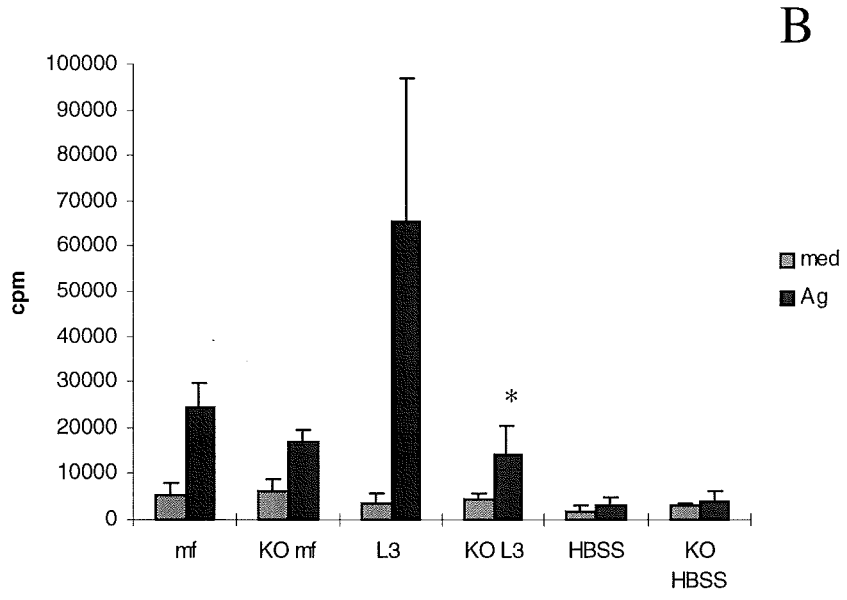
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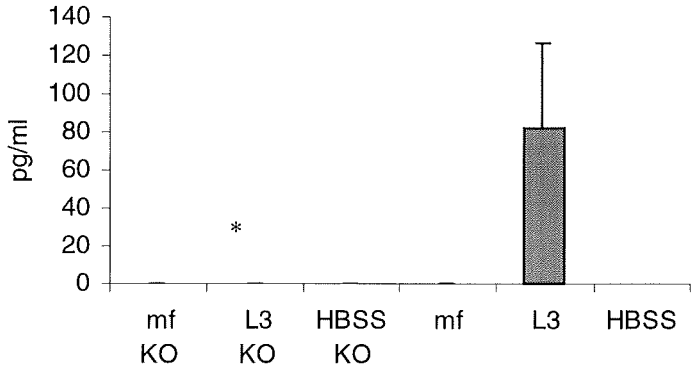
FIGURE 4.8. IL-4-/- vs wild type BALB/c mice: Ag stimulated cytokine production by splenocytes from mf infected, L3 infected and uninfected control mice at 12 d.p.i.

Wild type BALB/c and IL-4-/- mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal of volume HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Levels of (A) IL-4, (B) IL-5 and (C) IFN-γ in supernatants from 72 hr cultures were measured by 2-site ELISA. All values represent the mean and standard deviation of five animals per group. *, significantly different ($P = <0.05$) from wild type counterparts.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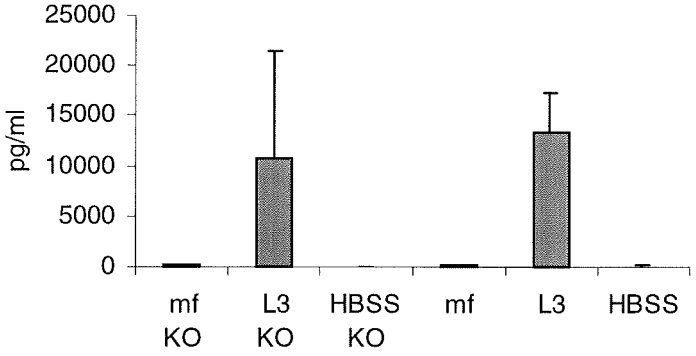
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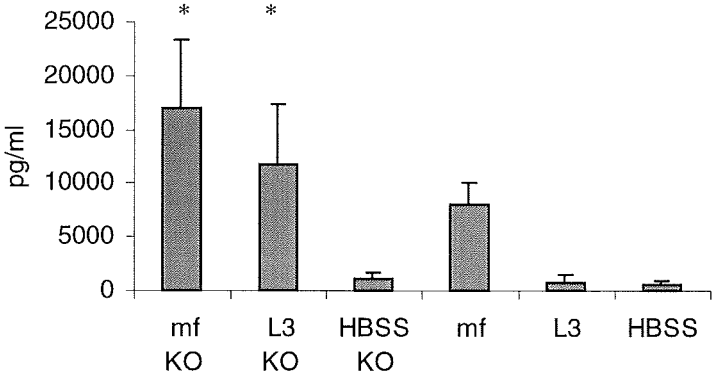
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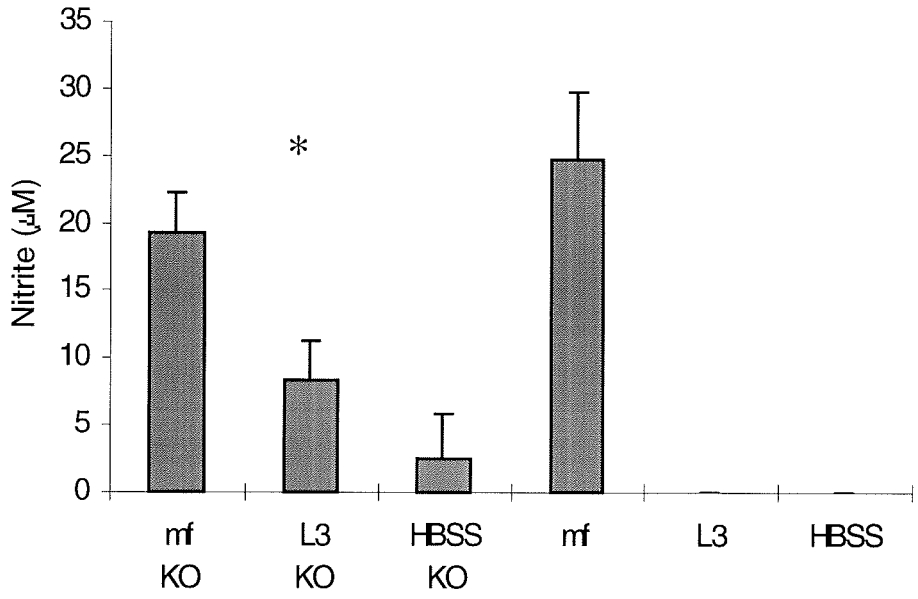


FIGURE 4.9 IL-4-/- vs wild type BALB/c mice: Nitrite production by Ag-stimulated splenocytes from mf-infected, L3-infected and uninfected control mice at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Nitrite levels in supernatants from 72 hr cultures were determined using the Greiss reaction. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) from wild type counterparts.

4.3 Discussion

The results presented in this chapter further define the contribution of NO, IFN-γ and IL-4 to *in vivo* and *in vitro* responses following infection. Experiments in IFN-γR-/- mice demonstrated an essential role for IFN-γ in the induction of NO-mediated proliferative suppression. The functional significance of the oppositely polarised responses elicited by infection with mf or L3 is illustrated by the results from IL-4-/- mice. The increased production of IFN-γ by both mf and L3-infected mice suggests that IL-4 may act to down-regulate IFN-γ production and thereby maintain proliferative responsiveness.

Previous experiments using iNOS inhibitors clearly demonstrated that the suppression of Ag-specific proliferative responses seen *in vitro* following mf-infection of BALB/c mice is NO dependent. In keeping with these observations splenocytes from mf-infected iNOS-/- mice, on the 129/Sv background, showed significantly greater levels of Ag-driven proliferation at 48 and 72 hrs culture than did their wild type counterparts. Unexpectedly however, proliferation at 96 hrs was not significantly greater than background levels in medium only wells. Inhibition of iNOS activity was previously shown to enhance IFN-γ production (Figure. 3.10) but in contrast to these findings, cells from iNOS-/- and wild type mf-infected mice produced equivalent levels of IFN-γ at both 48 and 96 hrs. This was also surprising as in previous studies using the same KO mice, infected with *L. major* or *T. brucei,* enhanced Th1 cytokine responses were reported (Millar, 1999; Wei, 1995). Analysis of NO_2^- production in Ag-stimulated culture demonstrated that cells from iNOS-/- mice produced significantly less NO at 48 hrs than wild type mice. However after 96 hrs culture, cells from both groups of infected mice produced similarly high levels of NO. It was originally reported that IFN-γ/LPS activated cells from this strain of iNOS-/- mice produced low levels of NO_2^- which was attributed to

the action of constitutive NOS (Wei, 1995). It has now been demonstrated that the intended deletion of exon 1 to 5 of the NOS2 gene had not occurred in these mice and that an alternative mRNA transcript of NOS2 is expressed, clearly capable of producing functional levels of NO (Niedbala, 1999). This urges extreme caution in the interpretation of results gained from these mice and necessitates a re-evaluation of results from previous studies. Whilst clearly not entirely functional as iNOS-/- mice, it is nevertheless interesting to speculate on another unexpected aspect of their *in vitro* response. Inhibitors of iNOS activity only significantly enhance proliferative responses at later time points in culture, however cells from iNOS-/- mice showed enhanced responsiveness at 48 hrs when only relatively low levels of NO (~10 μM) are produced by wild type mice. In murine infection with *Mycobacterium tuberculosis* it has been shown that IFN-γ production by $CD4^+$ T cells declines after the third or fourth week of infection, (Orme, 1993). Both Ag-specific and mitogen-driven proliferative responses are also suppressed, under the influence of NO. Most interestingly it has recently been shown that the frequency of mycobacterial purified protein derivative (PPD)-specific $CD4^+$ T cells also declines during infection (Nabeshima, 1999). This led to speculation that the NO-mediated elimination of Ag-specific T cells by activated macrophages may reduce the T cell response and the number of PPD-specific $CD4^+$ T cells *in vivo*. Although not quantified in this study, the presence of increased numbers of Ag-specific $CD4^+$ T cells in the spleens of iNOS mice (possibly as a result of enhanced survival *in vivo*), could provide a possible explanation for the greater magnitude of the proliferative response at 48 hrs when NO mediated suppression does not appear to be operative *in vitro*.

Infection of IFN-γR-/- demonstrated that signalling via the IFN-γR is essential to the induction of high level NO production and subsequent proliferative suppression. This

finding appears to contradict the results of previous experiments using anti-IFN-γ MAb in *in vitro* culture. One possible explanation for this discrepancy is that in IFN-γR-/- mice the influence of IFN-γ is effectively removed both *in vivo* and *in vitro* whereas neutralisation was only effective *in vitro.*

There are several mechanisms whereby IFN-γ could potentially suppress proliferative responses. Besides promoting MO activation and NO production, it has previously been shown that IFN-γ plays a critical role in AICD of effector T cells (Liu, 1990). IFN-γ also induces the Fas-dependent apoptosis of both $CD4^+$ and $CD8^+$ T cells following peroral infection with *T. gondii* (Liesenfeld, 1997). Furthermore, where the effects of NO in mediating proliferative suppression have been investigated, it appears that the direct effects of NO and those mediated via IFN-γ may synergise to promote apoptosis. In murine infection with *T. cruzi* it has been shown that IFN-γ may modulate lymphocyte apoptosis by both NO-dependent and independent mechanisms. IFN-γ promoted NO production and expression of both Fas and Fas-L by splenocytes from infected animals. Inhibition of iNOS activity partially reduced levels of lymphocyte apoptosis whilst not affecting Fas expression. As levels of apoptosis in the absence of NO were still higher than those found amongst splenocytes from uninfected mice this suggested that apoptosis may be induced by more than one mechanism (Martins, 1999). In this study use of iNOS inhibitors and IFN-γR-/- mice made it possible to differentiate between the effects of IFN-γ and NO. Upon inhibition of iNOS activity, splenocytes from mf-infected animals proliferated well in the presence of very high levels of IFN-γ, suggesting that the primary role of IFN-γ in mediating proliferative suppression is via the induction of iNOS. However it remains a possibility that exposure to NO, besides directly inducing apoptosis, may

increase the susceptibility of cells to IFN-γ mediated apoptosis, as has recently been demonstrated using human T cells (Allione, 1999).

The Ag-stimulated cytokine production profiles of cell from mf-infected IFN-γR-/- and wild type mice were highly comparable. IFN-γ production dominated responses in both groups at 48 hrs and there was no detectable production of IL-4. This suggests that signalling via the IFN-γR does not down-regulate production of Th2 cytokines and demonstrates the same polarisation of responses as seen following infection of BALB/c mice. Although not measured, it would be of interest to determine levels of IFN-γ production after 96 hrs culture, when NO-mediated suppression of IFN-γ production becomes apparent in cells from BALB/c mice (see section 3.2.4.). Levels of IL-2 production were similarly low in all infected animals, suggesting again, that a lack of IL-2 does not limit proliferative responsiveness and confirming previous results from experiments using rIL-2 (see section 3.2.2.).

Infection of IL-4-/- BALB/c mice demonstrated the importance of IL-4 in driving development of L3 induced Th2 responses and showed that production of IL-5 is regulated independently of IL-4. Splenocytes from L3 infected IL-4-/- mice displayed lower levels of Ag-stimulated proliferation than did those of wild type mice at 72 hrs whilst producing elevated levels of IFN-γ and NO. These results are suggestive of a role for IL-4 in down-regulating proinflammatory responses and thus allowing sustained proliferative responses.

L3 infected IL-4-/- BALB/c mice produced significantly greater levels of IFN-γ than their wild type counterparts. This result is in contrast to findings previously reported following infection of IL-4-/- mice on the C57/Bl/6 x 129/Sv background with L3 of *B. malayi* a result which probably reflects the influence of background strain (Lawrence, 1994). It has been shown that following s.c. infection with L3 Ag-specific IFN-γ producing

cells are primed, but silenced under the influence of IL-10 (Osborne, 1999). Although anti-IL-4 treatment was unable to restore Ag-specific IFN-γ production *in vitro* in that study, the results described above clearly illustrate a role for IL-4 in down-regulating IFN-γ production *in vivo*. In a situation comparable to that previously proposed for IFN-γ, the *in vivo* action of IL-4 may be critical in driving development of Th2 responses, promoting IL-10 production, which in turn subsequently suppresses IFN-γ production. Early IL-4 production following infection with *L. major* has been shown to promote the development of Th2 responses and susceptibility in BALB/c mice, demonstrating its functional importance (Launois, 1997). It seems likely that the early burst of IL-4 production reported following footpad infection with L3 functions in a similar directional capacity (Osborne, 1997b). The results presented here following i.v. infection clearly indicate that, as may be expected, IL-4 is the critical cytokine driving development of Th2 responses. Interestingly cells from both groups of L3-infected animals generated large amounts of IL-5 in Ag-stimulated culture demonstrating that IL-5 production is independent of IL-4. This result is in accordance with observations from L3 infected IL-4-/- C57Bl/6 x 129/Sv mice, wherein IL-5 production was not significantly lower than that of their wild-type counterparts (Lawrence, 1995). In BALB/c IL-4-/- mice, however, IL-5, generally considered a Th2 type cytokine, is produced along with IFN-γ. Elevated levels of IL-5 have previously been reported in the absence of IL-4 following infection of C57BL/6 mice with *O. volvulus* L3 (Johnson, 1998). It has been proposed that in this case IL-5 production may be regulated by cytokines other than IL-4 in particular IL-2 (Steel, 1993). While earlier studies on human helminth infection reported parallel regulation of IL-4 and IL-5 (Mahanty, 1993), more recent studies have shown that production of IL-4 and IL-5 are independently regulated in human filariasis (Sartono, 1997). Such findings suggest that responses to

filarial infection do not fall strictly in line with the Th1/Th2 paradigm and that the rigid application of such a scheme may be an over-simplification of events. It is also of note however, that IL-5 may be produced by cells other than $CD4^+$ T cells including $CD8^+$ T and non-T cells such as mast cells and eosinophils which may allow for continued production of IL-5 when Th2 responses are down-regulated. Recent studies showing that IL-5 plays an important role in protective responses to infection with *Onchocerca* sp. independently of IL-4 (Hogarth, 1998) and that IL-5-/- deficient mice are less resistant to challenge infection with *L. sigmodontis* L3 than their wild-type counterparts (Le Goff, 2000) suggest the role of IL-5 may be of greater significance than previously credited. In this respect it is of interest to note that IL-5 was recently shown to be required for the optimal generation of cytotoxic T lymphocytes (CTL) in response to immunisation with the modified tumor Ag oxidised mannan MUC1 fusion protein (Apostolopoulos, 2000).

Ag-stimulated proliferative responses of cells from L3 infected IL-4-/- mice were lower than those of wild type animals after 72 hrs culture, this was associated with enhanced production of both IFN-γ and NO. Whilst responses at 96 hrs were unfortunately not recorded in the experiment shown, in one similar experiment proliferation of cells from 2 of 5 L3-infected IL-4-/- mice fell below background levels at 96 hrs. This suggests that in the absence of IL-4, a response which is more Th1 like develops and is associated with suppression of proliferative responses. Subsequent experiments performed in this laboratory have shown that this is indeed the case (Jenson pers. com.). It would be of interest to record the proliferative responses over a fuller time-course in the presence or absence of AMG to determine whether reduced proliferative responses were directly related to enhanced NO production. It is also possible however that lower levels of proliferation are more directly related to a lack of IL-4 in terms of its action as an important T cell growth factor. Unexpectedly, Ag-stimulated cells from mf-infected IL-4

-/- mice displayed lower levels of proliferation than their wild type counterparts after 72 hrs culture. This is intriguing as levels of NO production were not significantly different between groups at this time-point. Cells from mf-infected IL-4-/- mice were shown to produced enhanced levels of IFN-γ however, it has been shown previously that cells from mf-infected animals can proliferate in the presence of high levels of IFN-γ, suggesting that reduced proliferation is unlikely to be a direct effect of IFN-γ. It is possible that a greater proportion of Ag-reactive T cells from mf-infected IL-4-/- mice are IFN-γ producers and as such may be more susceptible to the suppressive effects of NO which may manifest itself at the level of proliferation. As NO appears to selectively suppress IFN-γ production and proliferative responses, it is interesting to speculate on the possibility that the increased susceptibility of Th1 cells to NO may lead to a depletion of IFN-γ producing cells which may in turn allow the outgrowth of relatively resistant Th2 cells. This is a particularly intriguing possibility considering the previously reported instability of the mf-induced Th1 response (Lawrence, 1994; Pearlman, 1993b). The experiments presented in the following chapter were aimed at determining the stability of proliferative suppression and polarisation of the immune responses in longer term infections, and whether or not suppression is restricted to the spleen or extends to other secondary lymphoid organs.

CHAPTER 5. Further characterisation of factors influencing proliferative suppression

5.1 Introduction

In murine models it is now well established that infection with mf elicits development of a Th1 polarised response at relatively early time-points (12-14 d.p.i.). This has been shown to be independent of the route of infection (whether i.p., s.c., or i.v.), and mf numbers (in the range of 10^2 to 10^6) (Lawrence, 1994; Osborne, 1996; Pearlman, 1993b). However several studies have indicated that the polarisation of mf-induced responses is not inherently stable over longer term infections. Lawrence *et al* (94) showed that whilst at 14 d.p.i. splenocytes from mf-infected animals produced high levels of IFN-γ and little IL-4, a more mixed response was observed at 28 d.p.i. with production of IL-4 and IL-5 as well as IFN-γ. Pearlman *et al* (93) described a similar situation wherein levels of Ag-stimulated IL-4 and IL-5 production increased with duration of infection, coincident with a decline in IFN-γ production, suggesting a switch to a more Th2 like response. As IFN-γ production is essentially a Th1 function, and is central to the induction of NO production, any changes in cytokine production over the course of infection which result in decreased IFN-γ production, or enhanced production of Th2 cytokines, may impact upon NO production and proliferative responses. In order to assess the stability of the Th1 response initiated by i.v. infection with *B. pahangi* mf, and to determine how any changes in cytokine/nitrite production may affect Ag-specific proliferation, *in vitro* responses were analysed at 30 d.p.i.

Several studies have shown that the information gained from *in vitro* studies can differ dependent upon which lymphoid organ is used for analysis. For example, during the acute stage of infection with *T. gondii* the mitogen-driven proliferation of splenic T cells is

suppressed under the influence of NO (Candolfi, 1994), while the response of mesenteric lymph node T cells remains intact (Neyer, 1998). Thus the suppressive effects of NO may be localised rather than systemic and restricted to the major lymphoid organ of the spleen.

Compartmentalisation of Ag-specific Th1 and Th2 like responses has been observed following infection with *Trichinella spiralis,* such that splenocytes produce predominantly IFN-γ while MLN cells produce mainly IL-5 (Becky Kelly, 1991). There is also evidence of site-specific effects in murine models of filariasis. Following i.p. infection of multiply immunised mice with *B. malayi* mf, $CD4^+$ T cells from the peritoneal cavity produced exclusively Th2 cytokines (IL-4 and IL-5), while splenocytes and lymph node cells produced both IL-5 and IFN-γ (Pearlman, 1993a). More recently splenocytes and tracheo-broncheal lymph node (TBLN) cells from mf-infected mice were shown to display differences in Ag-specific cytokine production. Production of IL-4 by TBLN cells increased over time while the IL-4 response of splenocytes varied little over the course of infection. Furthermore while splenocytes showed high levels of IFN-γ production at early time-points post-infection TBLN cells only produced IFN-γ at 80 d.p.i.. Interestingly a dose response effect was noted in respect to IFN-γ production, in that TBLN cells from mice given a low dose of mf (2.5×10^4) produced IFN-γ at 80 d.p.i. while those from mice given a higher dose (2.5×10^5 mf) did not. Splenocytes from animals given a high dose of mf also showed a marked decrease in IFN-γ production over time (Lawrence, 2000).

As different life cycle stages occupy distinct locations within the mammalian host, L3 in the skin, developing larvae and adult worms in the lymphatics and mf in the circulatory system, it is possible that any stage-specific mechanisms of immunomodulation may also be to some degree site specific. To determine whether the development of mf-induced Th1 responses and associated proliferative suppression is restricted to the spleen

the proliferative and cytokine responses of splenocytes and peripheral lymph node cells (LN cells) were assessed *in vitro*.

Aside from duration of infection and site specific effects there is also evidence to suggest that the origin of the Ag-used for *in vitro* restimulation can influence both the proliferation and cytokine production of Ag-reactive cells. It has been shown that whilst the proliferative responses of PBMC from Mf+ individuals to mf-Ag and mixed sex adult Ag were lower than those of individuals displaying chronic pathology, responses to adult male Ag were comparable across groups (Mahanty, 1996). Such observations are suggestive of a role for mf-Ag in suppressing proliferation of PBMC from Mf+ individuals. To determine whether the type of Ag-used for *in vitro* restimulation affects proliferative responses, splenocytes from infected animals were restimulated *in vitro* with mixed sex adult, adult female, adult male or mf-Ag.

L3 and adult *Brugia* are known to be potent inducers of Th2 responses and the ability of both IL-4 and IL-10 to down-regulate IFN-γ production has been demonstrated in murine models of filariasis. Experiments previously described (see section 4.2.3) showed that, following infection with *B. pahangi* L3, IFN-γ production is enhanced in the absence of IL-4. Evidence from another study using IL-4 KO mice suggested that adult induced IL-4 is capable of suppressing the development of mf-induced IFN-γ production (Lawrence, 1995). IL-10 has also been shown to suppress IFN-γ production following chronic infection with mf (Pearlman, 1993b) and to "silence" IFN-γ producing cells primed by s.c. infection with L3 (Osborne, 1999). As in human infection exposure to L3, developing larvae and adult worms precedes exposure to mf, the ability of mf to modulate a pre-established Th2 response is a subject of considerable interest. In order to further assess the immunomodulatory potential of mf, L3 infected animals were super-infected with mf and proliferative and cytokine responses assessed *in vitro*.

The experiments presented in this chapter were aimed at addressing some of the issues raised above, in particular the stability of mf-induced Th1 responses, whether the responses in the spleen reflect those in other secondary lymphoid organs, how restimulation with different Ag-preparations affects proliferative responses, and the effects of mf on a pre-existing Th2 response.

5.2 RESULTS

5.2.1 Ag-stimulated immune responses of splenocytes from mf-infected, L3-infected and uninfected control mice at 30 d.p.i.

In these experiments groups of five BALB/c mice were injected intravenously via the tail vein with $1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 30 d.p.i. spleens were removed for *in vitro* analysis.

(i) Proliferative responses

Proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control animals were measured over a time-course of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. Figure 5.1 shows Ag-driven proliferative responses after (A) 48, (B) 72 and (C) 96 hrs of *in vitro* culture. At 48 hrs splenocytes from both mf-infected and L3-infected mice show Ag specific proliferation (Figure 5.1A). As at 12 d.p.i. the proliferative response of cells from mf-infected animals is short lived and falls to background levels by 72 hrs. However, unlike the situation previously described at 12 d.p.i. splenocytes from L3 infected animals at 30d.p.i. did not show sustained proliferation throughout the time-course observed. After 96 hrs Ag-stimulated cells from both groups of infected animals failed to proliferate at levels significantly greater than those of unstimulated cultures (Figure. 5.1C). This experiment has been repeated twice with similar results.

(ii) Cytokine production

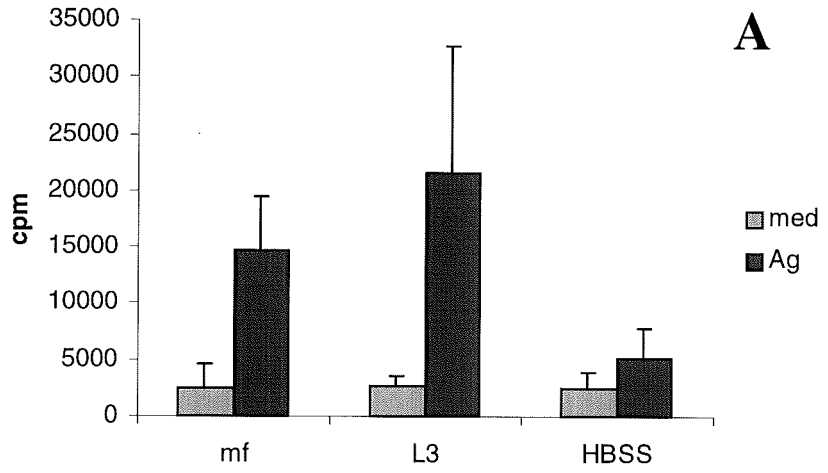
To investigate the stability of the differentially polarised responses observed at 12 d.p.i. Ag-stimulated cytokine production by splenocytes from mf-infected, L3-infected and uninfected control mice was measured at 30 d.p.i.. After 48 hrs of *in vitro* restimulation, cells from mf-infected animals displayed no evidence of switching to a more Th2 like response, producing only IL-2 and IFN-γ. Unexpectedly however, cells from L3 infected animals displayed a much more mixed response than at 12 d.p.i. producing IL-2 and IFN-γ alongside IL-4, IL-5 and IL-10. This experiment has been repeated twice with similar results.

(iii) Nitrite production

Having established that NO mediates the Ag-specific proliferative suppression of splenocytes from mf-infected mice at 12 d.p.i., the Greiss reaction was used to determine the levels of NO_2^- in Ag-stimulated culture at 30 d.p.i. At 48 hrs, only cells from mf-infected animals produced significant levels of nitrite whilst cells from L3 –infected and uninfected control animals produced only background levels below the sensitivity of the assay (2.5 μM). After 96 hrs of culture, however, cells from both mf-infected and L3-infected animals produced high levels of NO (39+/-7.6 and 42 +/-7.6 μM respectively (Figure. 5.3B)). This observation demonstrates that, while the onset of NO production may occur later in these cultures, cells from L3-infected animals can produce NO at levels sufficient to down-regulate proliferative responses.

FIGURE 5.1. Ag stimulated proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control mice at 30 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 x L3 *B. pahangi* or an equal volume of HBSS only. At 30 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen were measured by ^{3}H thymidine incorporation at (A) 48, (B) 72 and (C) 96 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group.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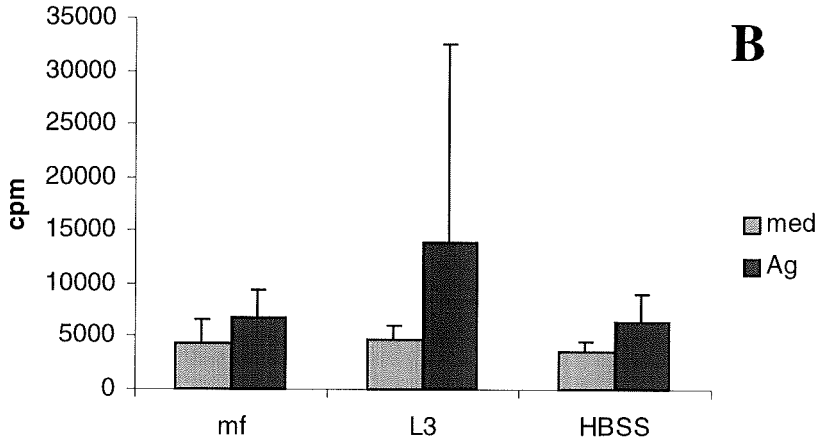
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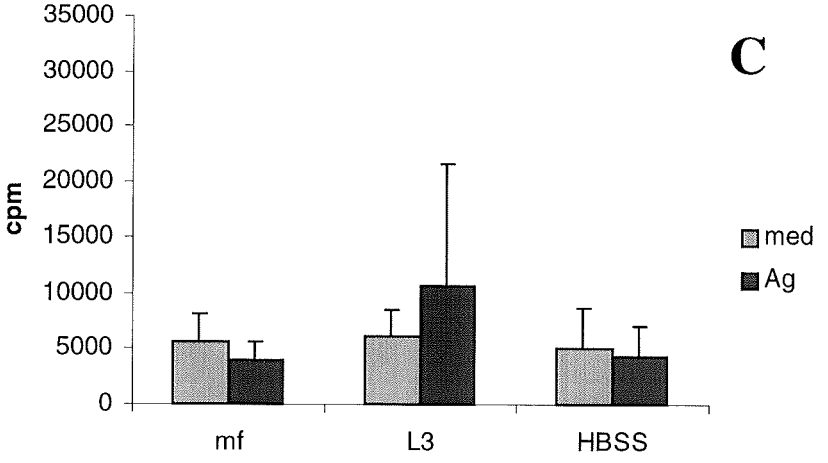
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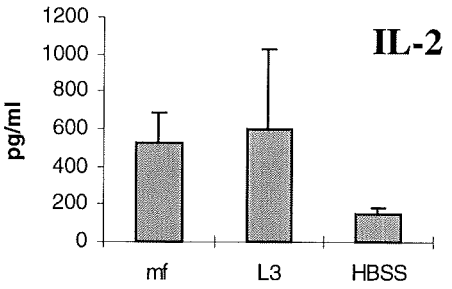
FIGURE 5.2 Ag-stimulated cytokine production by splenocytes from mf-infected, L3-infected and uninfected control mice at 30 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 x L3 *B. pahangi* or an equal volume of HBSS only. At 30 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Levels of IL-2, IFN-γ, IL-4, IL-5 and IL-10 in supernatants from 48 hr cultures were measured by 2-site ELISA. All values represent the mean and standard deviation of five animals per group.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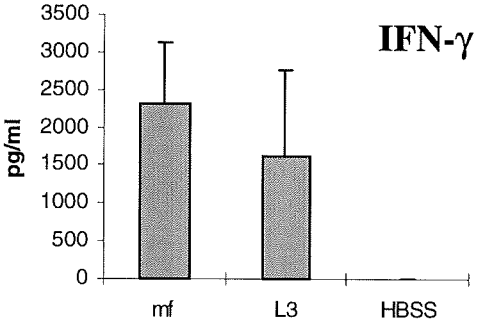
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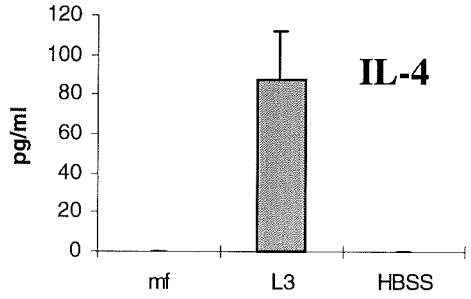
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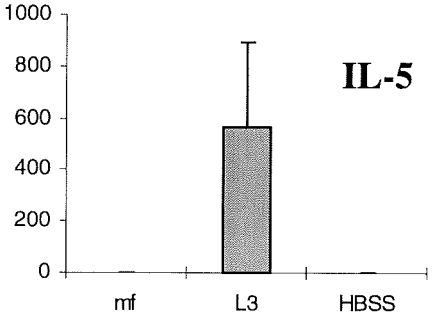
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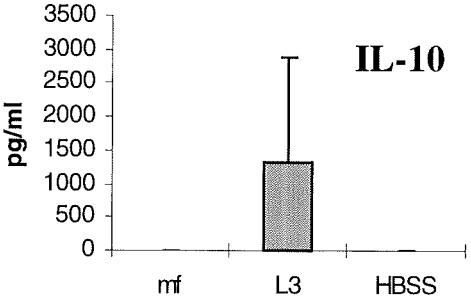
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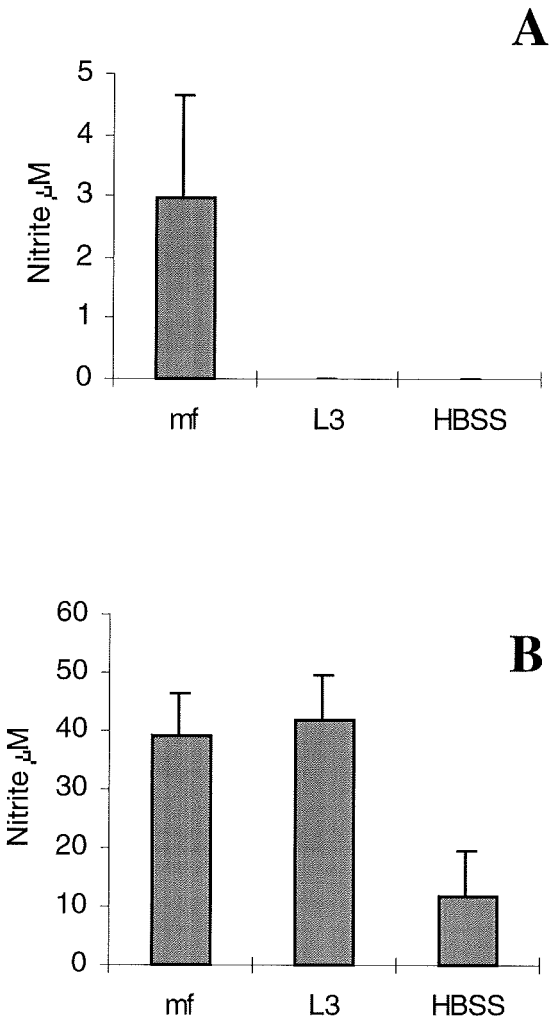


FIGURE 5.3. Nitrite production by Ag-stimulated splenocytes from mf-infected, L3-infected and uninfected control mice at 30 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 30 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 µg/ml *B. pahangi* adult Ag. Nitrite levels in supernatants from (A) 48 and (B) 96 hr cultures were determined using the Greiss reaction. All values represent the mean and standard deviation of five animals per group.

5.2.2 Ag-stimulated immune responses of splenocytes and lymph node cells from mf-infected, L3-infected and uninfected control mice at 12 d.p.i.

To determine whether or not the Ag-specific proliferative suppression seen following mf infection is restricted to the spleen, the Ag-stimulated responses of peripheral lymph node cells were assessed. In these experiments groups of six BALB/c mice were injected intravenously via the tail-vein with $1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. spleens and peripheral lymph nodes (inguinal, axillary and brachial) were removed for *in vitro* analysis. Due to low cellular recoveries lymph node cells from individual animals were pooled prior to *in vitro* culture.

(i) Proliferative responses

Proliferative responses of splenocytes and peripheral lymph node cells (LN cells) from mf-infected, L3-infected and uninfected control animals were measured over a time-course of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. Figure 5.4 shows Ag-driven proliferative responses after 48 (A/B), 72 (C/D) and 96 (E/F) hrs of *in vitro* culture. Splenocytes from infected and uninfected animals displayed a typical pattern of responsiveness as previously described (Figure. 5.4.A/C/E). The proliferative responses of LN cells from infected animals generally reflected that of splenocytes (Figure. 5.4 B/D/F). LN cells from mf-infected animals showed low levels of Ag-specific proliferation even at 48 hrs, while LN cells from L3-infected animals proliferated well in response to Ag throughout the period observed. LN cells showed lower levels of both background (medium only), and Ag-stimulated proliferation than did splenocytes at all time-points. This experiment was repeated twice with equivalent results.

(ii) Cytokine production

LN cells from mf-infected, L3-infected and uninfected animals displayed equivalent Ag-stimulated cytokine production profiles to splenocytes from the same animals. At 48 hrs LN cells from mf-infected animals produced Ag-specific IFN-γ in the absence of Th2 cytokines, while LN cells from L3-infected animals produced IL-4 and IL-5 in the absence of IFN-γ (Figure. 5.5). LN cells produced lower levels of all cytokines tested than did splenocytes (data not shown).

(iii) Nitrite production

Whilst only splenocytes from mf-infected animals produced high levels of NO_2^- in Ag-stimulated culture (40.5 +/-4.7 μM at 96 hrs), no nitrite was detected in Ag-stimulated cultures of LN cells from any group, at any time-point (data not shown).

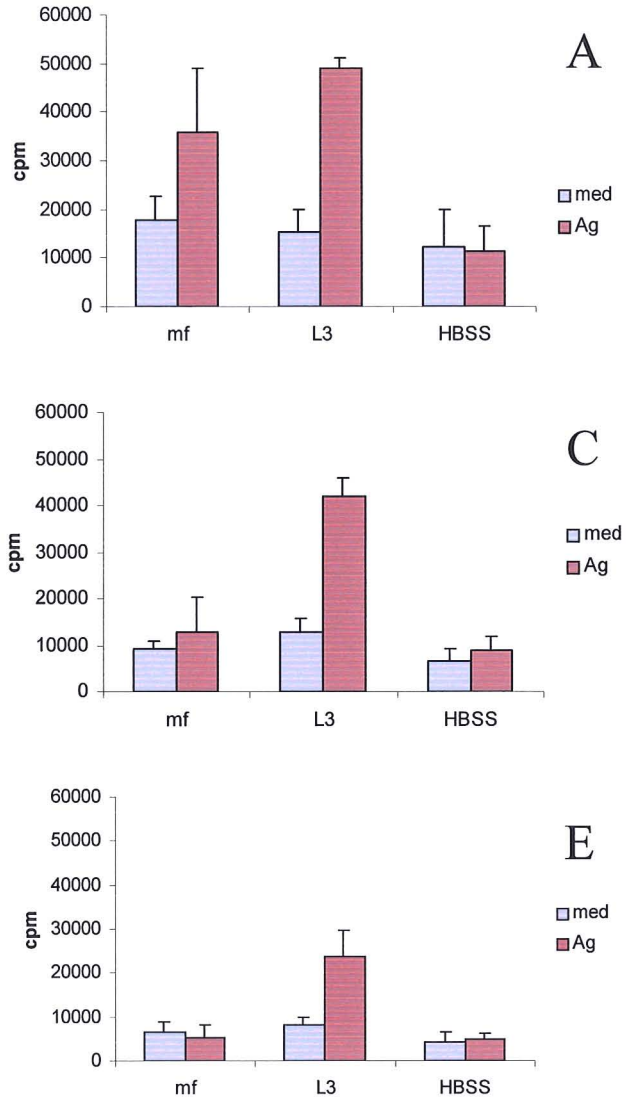
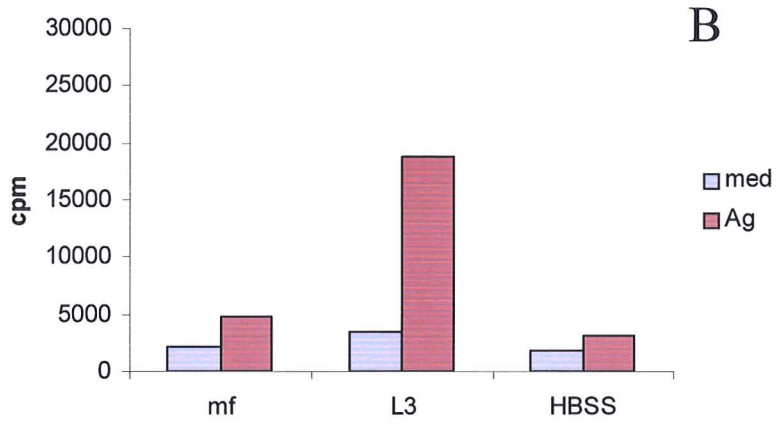
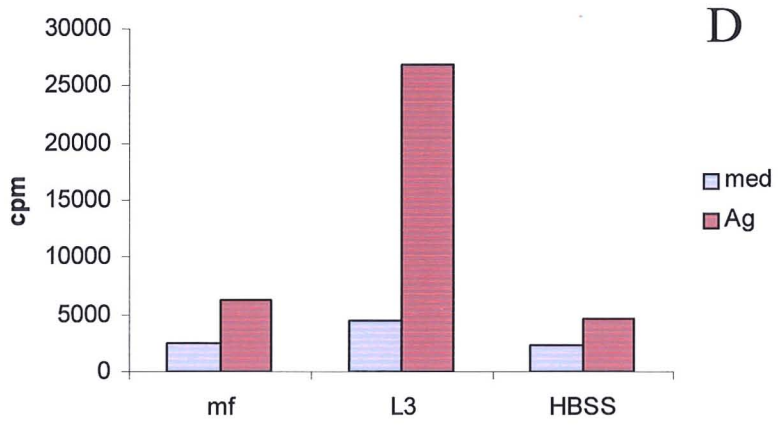


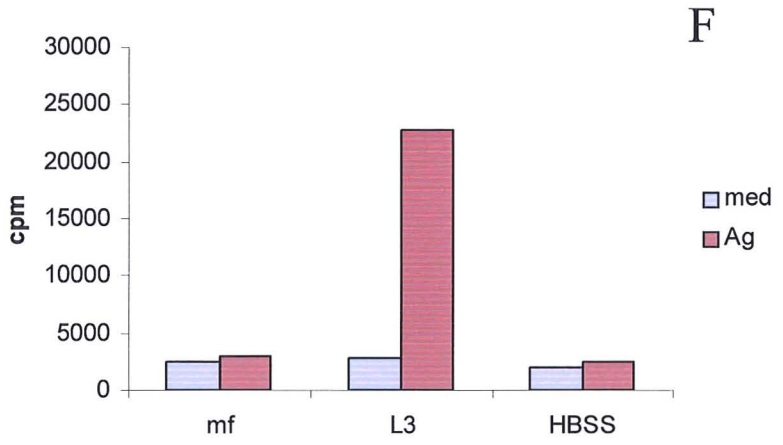
FIGURE 5.4 Ag-stimulated proliferative responses of splenocytes and LN cells from mf-infected, L3-infected and uninfected control mice at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 x L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes and peripheral LN cells (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen were measured by ^{3}H thymidine incorporation at 48 (A/B), 72 (C/D) and 96 (E/F) hrs culture. LN cells from 6 individual animals per group were pooled prior to *in vitro* restimulation. Results are expressed as mean cpm incorporated in triplicate wells. All values for splenocytes represent the mean and standard deviation of six animals per group.

Lymph node cells







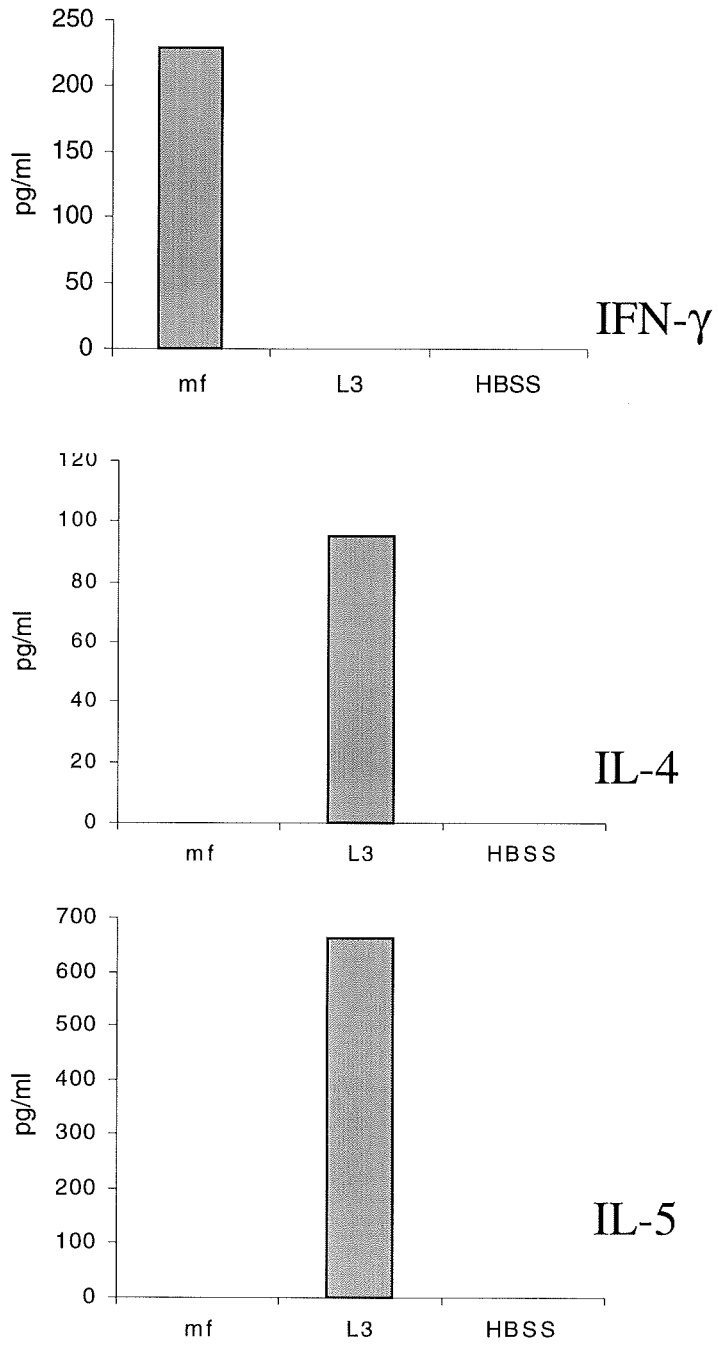


FIGURE 5.5. Ag-stimulated cytokine production by LN cells rom mf-infected, L3-infected and uninfected control mice at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 x L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. peripheral lymph node cells ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult Ag. Levels of IFN-γ, IL-4 and IL-5 in supernatants from 48 hr cultures were measured by 2-site ELISA. LN cells from 6 individual animals per group were pooled prior to *in vitro* restimulation.

5.2.3. Live vs heat-killed mf

In these experiments groups of five BALB/c mice were injected intravenously via the tail-vein with $1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. spleens were removed for *in vitro* analysis.

(i) Proliferative responses

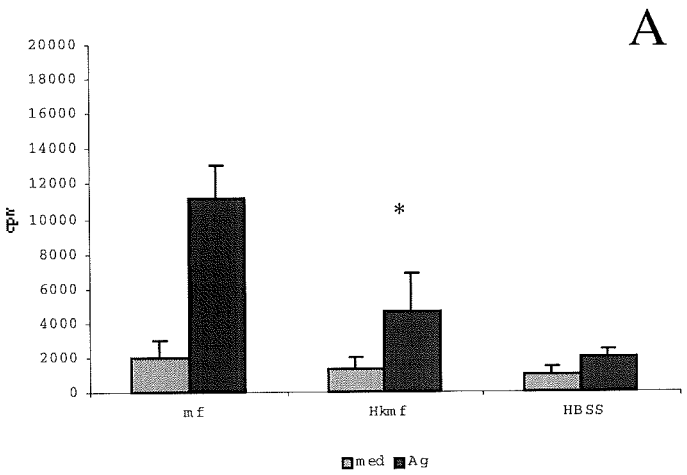
Proliferative responses of splenocytes from mice given $1x10^5$ live or heat-killed mf of *B. pahangi* or an equal volume of HBSS only i.v. were measured over a time-course of *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag. Figure 5.6 shows Ag-driven proliferative responses after (A) 48, (B) 72 and (C) 96 hrs *in vitro* culture. Cells from animals infected with live mf displayed significantly greater levels of Ag-driven proliferation at both 48 and 72 hrs than those given heat-killed mf (p= 0.037 and 0.004 respectively). Only cells from mice given live mf proliferated at levels significantly below background (p=0.013). This experiment has been repeated twice with similar results.

(ii) Cytokine and nitrite production

As shown in Figure 5.7 (C), cells from mice given live or heat killed mf produced IFN-γ upon restimulation with Ag. Whilst cells from recipients of live mf produced markedly more IFN-γ, there was no significant difference between these groups. No IL-4 or IL-5 production was detected in Ag-stimulated culture of cells from any group of mice. Although splenocytes from mf-infected animals produced higher levels of nitrite at 48 hrs, after 96 hrs culture cells from animals given live or heat-killed mf produced similarly elevated levels of NO_2^-. Such results indicate that although injection of heat-killed mf leads to lower proliferative responses upon *in vitro* restimulation than live mf, levels of Ag-stimulated IFN-γ are sufficient to induce NO production at levels capable of suppressing proliferative responses.

FIGURE 5.6 Ag-stimulated proliferative responses of splenocytes from mice given 1x10^5 live vs heat killed mf *B. pahangi*

Mice were injected intravenously with 10^5 live or heat killed mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen were measured by ^{3}H thymidine incorporation at (A) 48, (B) 72 and (C) 96 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group. *, significantly different (P = <0.05) response to live *B. pahangi* mf.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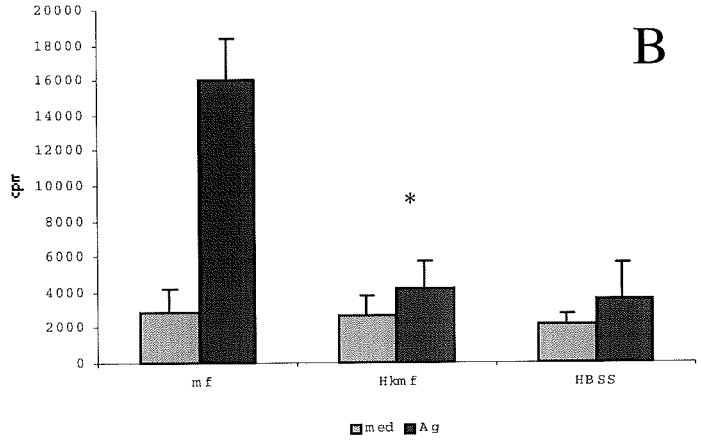
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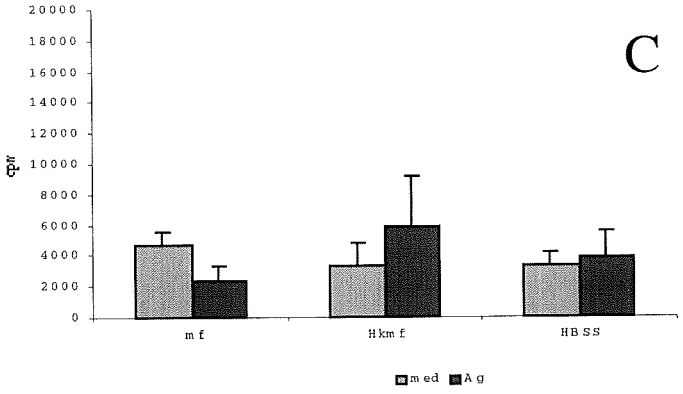
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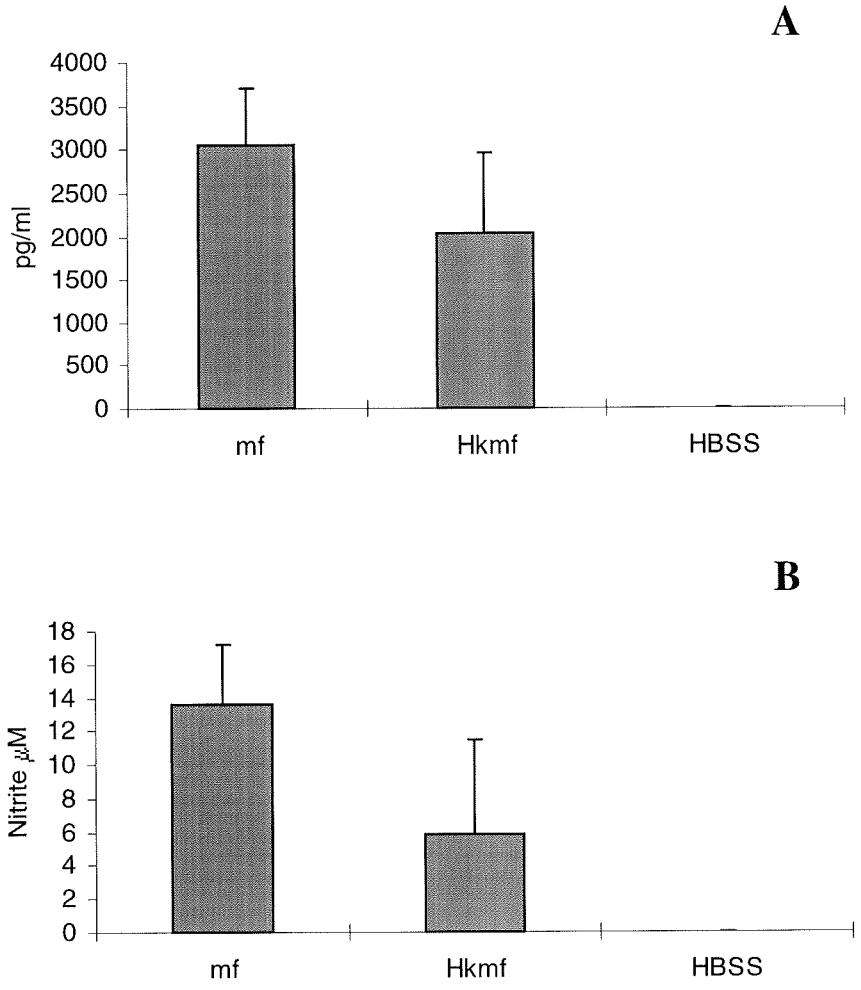

FIGURE 5.7 Live vs heat-killed mf: IFN-γ and nitrite production in Ag-stimulated culture

Mice were injected intravenously with 10^5 live or heat killed mf *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes (1 x 10^7 cells/ml) were restimulated with 10 μg/ml *B. pahangi* adult antigen. IFN-γ (A) and Nitrite (B) production in supernatants from 48 hr cultures were measured using 2-site ELISA and the Greiss reaction respectively. All values represent the mean and standard deviation of five animals per group.

5.2.4 Restimulation with Ag-derived from distinct life cycle stages at 12 d.p.i.

(i) Proliferative responses

To test whether the proliferative suppression seen following mf-infection is influenced by the origin of the Ag used for *in vitro* restimulation, cells from mf-infected, L3-infected and uninfected control animals were cultured with 10 μg/ml mixed sex adult, adult female or adult male Ag. As shown in Figures 5.8 and 5.9 restimulation with mixed sex or adult female Ag stimulated highly comparable patterns of proliferative responsiveness in both groups of infected animals. Responses to male Ag however were inconsistent across these two experiments. In the first experiment (Figure 5.8B) splenocytes from mf-infected animals proliferated at significantly higher levels in response to male Ag than to mixed sex or female Ag at 72 hrs (P=0.0127 and 0.032 respectively). The reverse situation was seen with cells from L3 infected animals which proliferated at significantly lower levels in response to male Ag than mixed sex or female Ag at the same time-point (p=0.0045 and 0.037 respectively). In this same experiment there were no significant differences in proliferative responses to different Ag preparations at either 48 or 96 hrs culture. In one subsequent experiment however no differences were observed in the proliferative responses to mixed sex adult female or adult male Ag at any time-point (Figure 5.9). It may be of consequence to note that different batches of male Ag were used in these experiments.

Figure 5.9 also illustrates the proliferative responses of cells from infected and uninfected animals to restimulation with 10 μg/ml mf Ag. As shown, mf Ag stimulates a similar profile of proliferative responses to that described for adult Ag with cells from mf-infected animals showing suppressed proliferation at 96 hrs while cells from L3 infected animals proliferated well at all time-points. Several experiments were performed using mf Ag with equivalent results.

(ii) Adult vs mf Ag: cytokine production

Cytokine production by cells from mf-infected, L3-infected and uninfected control animals in response to restimulation with adult and mf Ag are shown in Figure 5.10, (corresponding proliferative responses are shown in Figure 5.9). Both Ag-preparations elicited similar responses after 48 hrs of culture. Cells from mf-infected animals produced only IFN-γ, whilst cells from L3-infected animals produced IL-4 and IL-5 levels of which were unaffected by the origin of the Ag used.

FIGURE 5.8. Proliferative responses of splenocytes from mf-infected, L3-infected and uninfected control mice to mixed vs single sex *B. pahangi* adult Ag at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 x L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* mixed sex adult, adult female or adult male antigen, were measured by ^{3}H thymidine incorporation at (A) 48, (B) 72 and (C) 96 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group. *, significantly different ($P = <0.05$) response to *B. pahangi* mixed sex adult Ag.

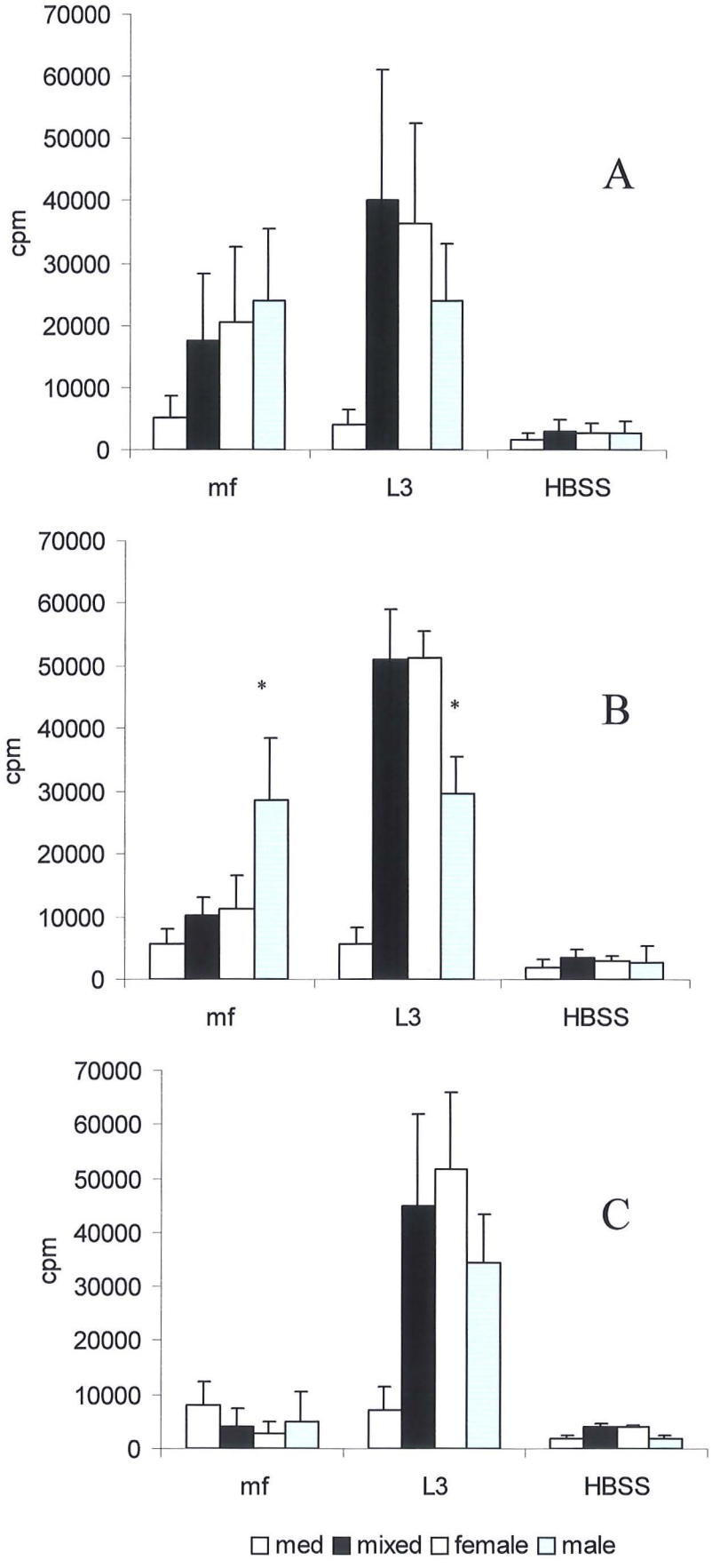
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FIGURE 5.9. Proliferative responses of splenocytes from infected and uninfected animals to mixed sex, single sex *B. pahangi* adult Ag and mf Ag at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 x L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* mixed sex adult, adult female, adult male and mf Ag, were measured by ^{3}H thymidine incorporation at (A) 48, (B) 72 and (C) 96 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group.

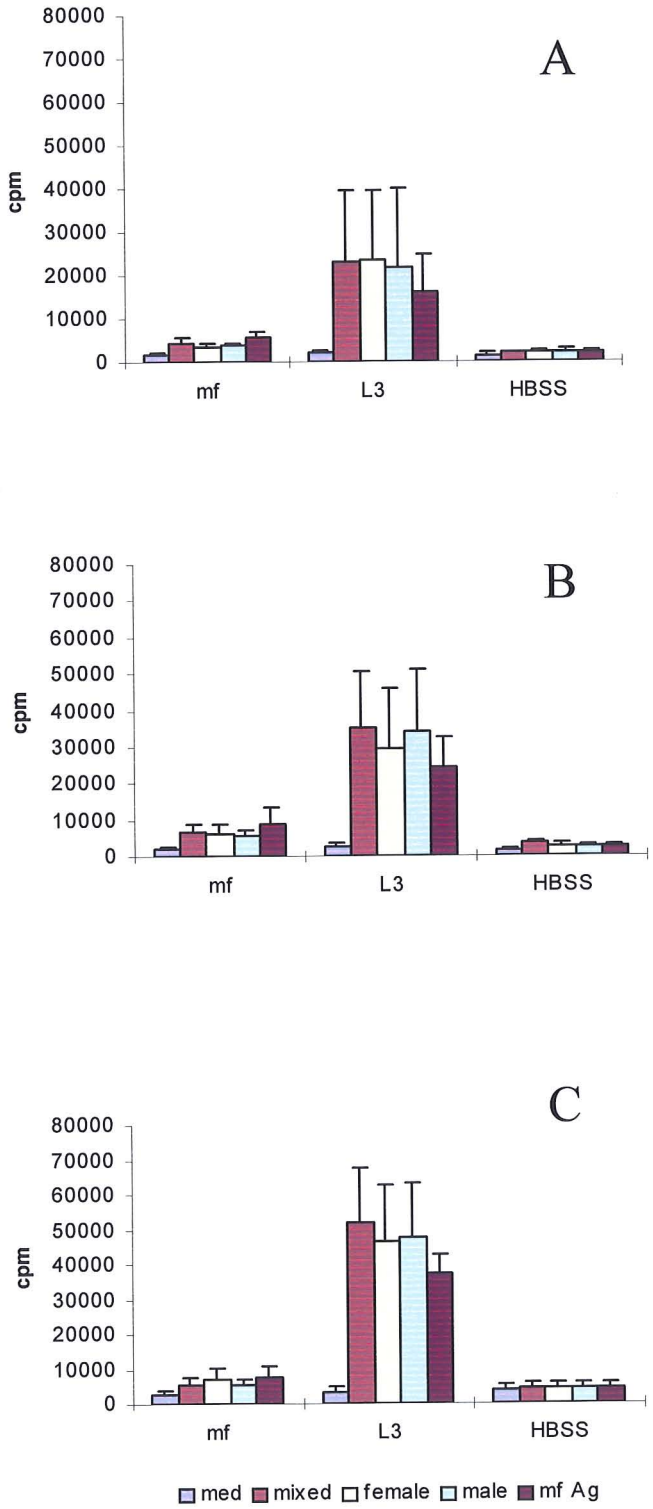
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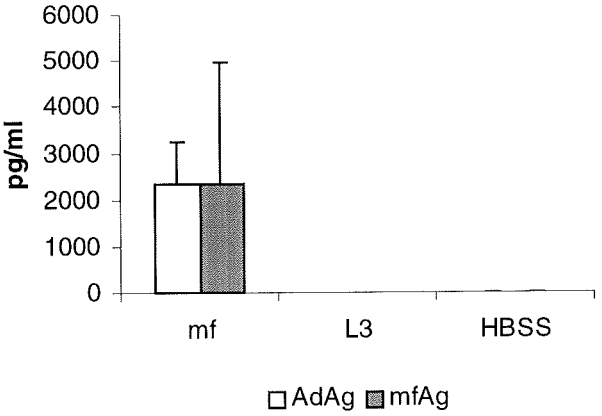
FIGURE 5.10. Adult vs mf Ag-stimulated cytokine production by splenocytes from mf-infected, L3-infected and uninfected control mice at 12 d.p.i.

Mice were injected intravenously with 10^5 mf, 50 x L3 *B. pahangi* or an equal volume of HBSS only. At 12 d.p.i. splenocytes ($1x10^7$/ml) were restimulated *in vitro* with 10 μg/ml *B. pahangi* adult or mf Ag. Levels of IFN-γ, IL-4 and IL-5 in supernatants from 72 hr cultures were measured by 2-site ELISA. All values represent the mean and standard deviation of three animals per group.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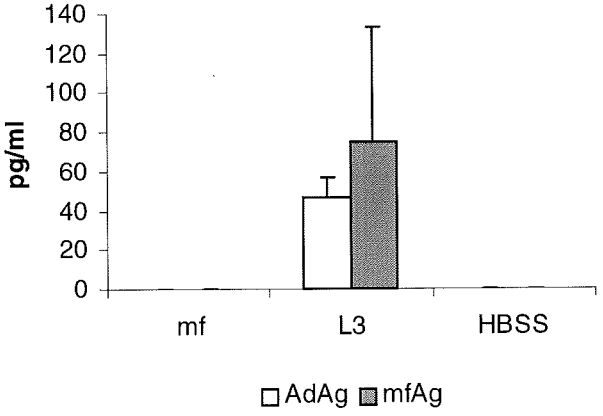
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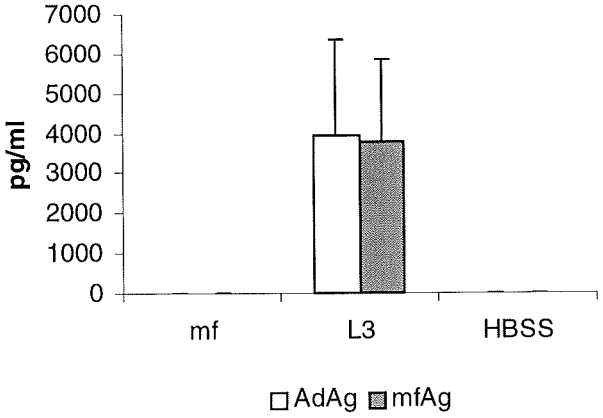
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5.2.5 Immune responses of splenocytes from L3-infected animals super-infected with mf

Infection with *B. pahangi* L3 is known to induce early development of a Th2 response whilst infection with mf elicits development of a Th1 responses at both 12 and 30 d.p.i. As the proliferative suppression following mf-infection is mediated by the IFN-γ dependent induction of NO, essentially a Th1 function, it was of interest to assess how prior exposure to L3, and initiation of a Th2 polarised response, would influence the response to subsequent infection with mf. To address this question groups of 5 BALB/c mice were infected s.c. with 50 *B. pahangi* L3 (this being closer to the natural route of infection for L3). Seven days post initial infection one group of 5 L3-infected and 5 naïve mice were given $1x10^5$ mf *B. pahangi* i.v. whilst control animals received an equal volume of HBSS only. 12 days post infection with mf (19 days post infection with L3), spleens were removed for *in vitro* analysis.

(i) Ag-stimulated proliferative responses

Splenocytes from mice given L3 only, L3 followed by mf (L3/mf), mf or HBSS only were restimulated with 10 μg/ml *B. pahangi* adult Ag over a time-course of *in vitro* culture. Figure 5.11 shows Ag-driven proliferative responses at (A) 48, (B) 72 and (C) 96 hrs culture. At both 48 and 72 hrs splenocytes from L3/mf infected animals showed a significant increase in Ag-driven proliferation over cells from animals given L3 only. After 96 hrs culture however there were no significant differences between these two groups, cells from both L3/mf and L3-infected animals showing strong Ag-specific responses, while cells from mf-infected animals gave only background levels of proliferation.

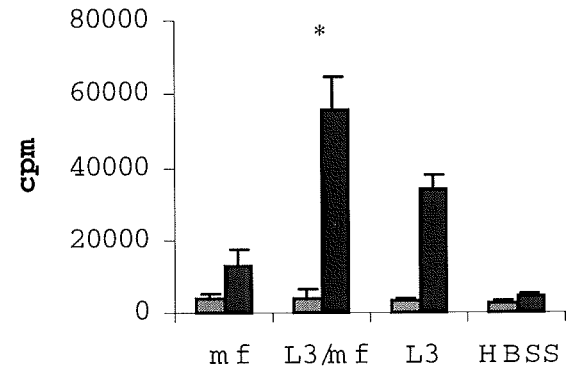
(ii) Ag-stimulated cytokine and nitrite production

Production of IL-4, IL-5 and IFN-γ was measured in the 48 hr supernatants of Ag-stimulated culture (Figure 5.12). As shown, IL-4 and IL-5 were produced by cells from all animals exposed to L3 and their production was not altered following super-infection with mf. Interestingly, however, splenocytes from animals infected with L3 for 19 days produced equivalent levels of IFN-γ to cells from mf-infected animals (at 12 d.p.i.). L3 infected animals super-infected with mf actually produced significantly lower amounts of IFN-γ than mice given L3 only. This observation was made in two subsequent experiments.

At 48 hrs, only cells from mf-infected animals produced significant levels of nitrite in Ag-stimulated culture. After 96 hrs, however, while cells from mf-infected animals produced the highest levels of NO_2^-, cells from L3-infected animals produced significantly more NO_2^- than super-infected animals (Figure 5.13). This experiment has been carried out on two occasions with equivalent results. In one similar experiment wherein an extra group of mice was included which had been infected with L3 at both day 0 and day 7 (L3/L3), it appeared that super-infection with either mf or L3 led to significant reductions in IFN-γ production at 48 hrs and nitrite production at 96 hrs (Figure 5.14).

FIGURE 5.11. Ag-stimulated proliferative responses of splenocytes following primary infection or super-infection with mf

Mice were injected subcutaneously with 50 x L3 *B. pahangi*. Seven days post initial infection one group of L3-infected mice and five naïve mice were given $1x10^5$ mf *B. pahangi* i.v.. Uninfected control mice received an equal volume of HBSS only. 19 days post initial infection with L3, proliferative responses of splenocytes (5 x 10^6 cells/ml) to 10 μg/ml *B. pahangi* adult antigen were measured by ^3H thymidine incorporation at (A) 48, (B) 72 and (C) 96 hrs culture. Results are expressed as mean cpm incorporated in triplicate wells. All values represent the mean and standard deviation of five animals per group. *, significantly different ($P = <0.05$) response to infection with L3 alone.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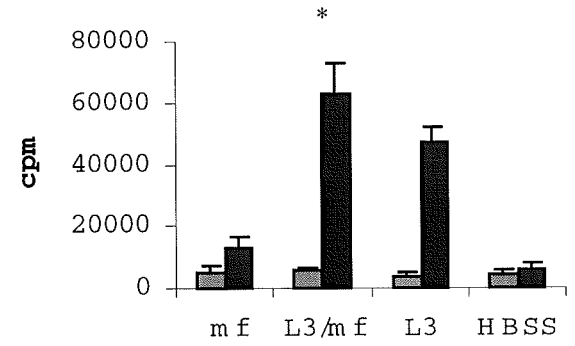
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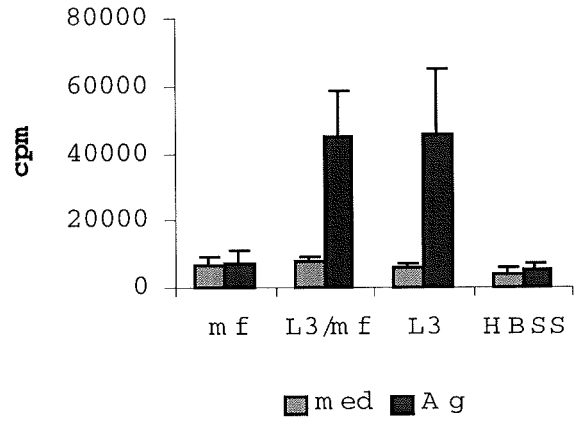
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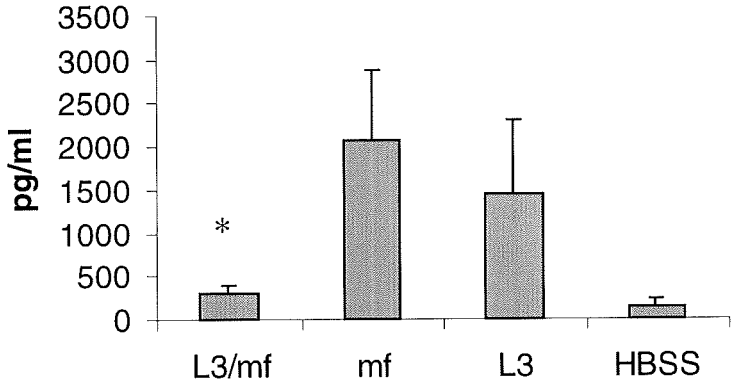
FIGURE 5.12. Cytokine production by Ag-stimulated splenocytes following primary infection and super-infection with mf

Mice were injected subcutaneously with 50 x L3 *B. pahangi*. Seven days post initial infection one group of L3-infected mice and five naïve mice were given $1x10^5$ mf *B. pahangi* i.v. Uninfected control mice received an equal volume of HBSS only. At 19 days post initial infection with L3, splenocytes (1×10^7 cells/ml) were restimulated with 10 μg/ml *B. pahangi* adult antigen. IFN-γ, IL-4 and IL-5 production in supernatants from 48 hr cultures were measured using 2-site ELISA. All values represent the mean and standard deviation of five animals per group. *, significantly different ($P = <0.05$) response to infection with L3 alone.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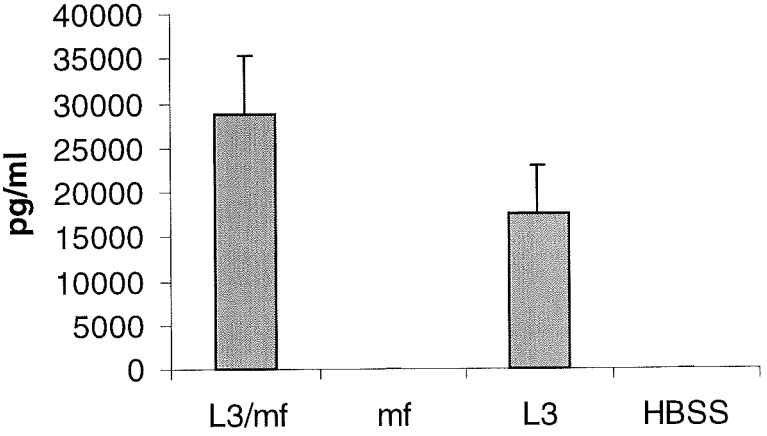
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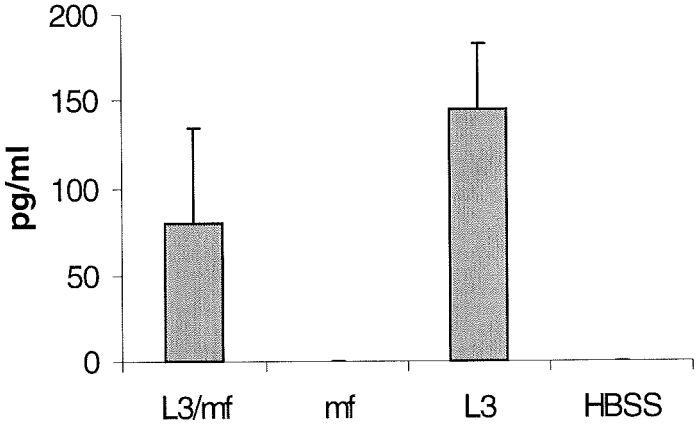
HBSS

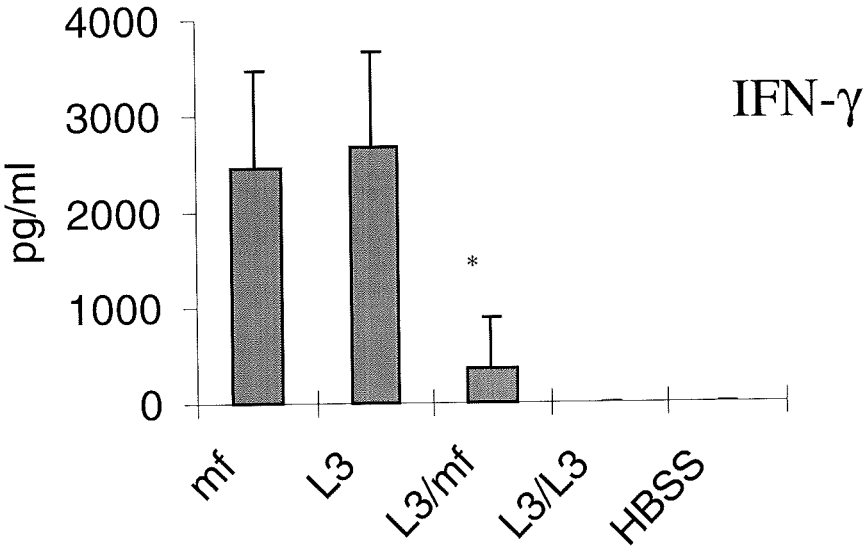
IL-4

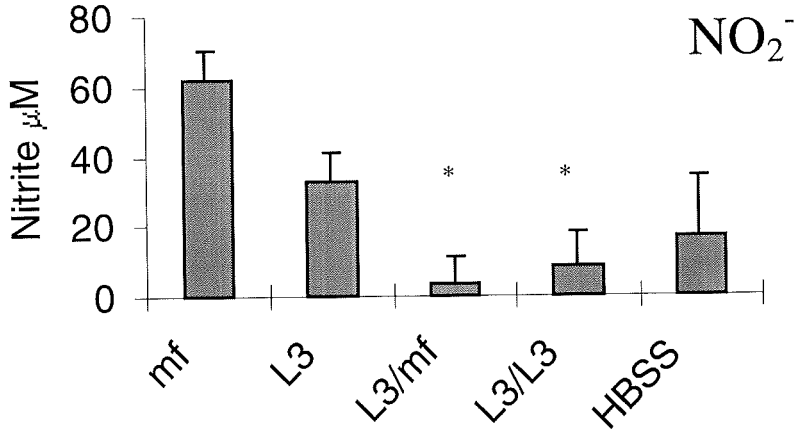

200
150
100
50
0
pg/ml
L3/mf
mf
L3
HBSS

IL-5

FIGURE 5.13 IFN-γ and nitrite production by Ag-stimulated splenocytes following primary infection and super-infection with mf or L3

Mice were injected subcutaneously with 50 x L3 *B. pahangi*. Seven days post initial infection one group of L3-infected mice and five naïve mice were given $1x10^5$ mf *B. pahangi* i.v. whilst another group of L3 infected mice received 50 x L3 *B. pahangi* i.v. Uninfected control mice received an equal volume of HBSS only. At 19 days post initial infection with L3, splenocytes (1×10^7 cells/ml) were restimulated with 10 μg/ml *B. pahangi* adult antigen. IFN-γ and nitrite levels in supernatants from 48 hr and 96 hrs respectively are shown. All values represent the mean and standard deviation of five animals per group. *, significantly different ($P = <0.05$) response to infection with L3 alone.


4000
3000
2000
1000
0
pg/ml
IFN-γ
*
mf
L3
L3/mf
L3/L3
HBSS


80
60
40
20
0
Nitrite μM
NO2-
*
*
mf
L3
L3/mf
L3/L3
HBSS

5.3 Discussion

The results presented in this chapter demonstrate that the Th1 polarisation induced by i.v. infection with *B. pahangi* mf is stable at least up to 30 d.p.i. Splenocytes from mf-infected animals produced high levels of IFN-γ and NO in Ag-stimulated culture and proliferative responses were down-regulated as at 12 d.p.i. This is in contrast to the findings of previous studies using i.p. infection wherein more mixed or Th2 like responses were observed at later time-points (Lawrence, 1994; Pearlman, 1993b). Such differences may be due to the different routes of infection used, or alternatively they may reflect differences in the species of parasite used (*B. malayi* vs *B. pahangi*).

Unexpectedly, cells from L3-infected animals displayed differences in both cytokine production and proliferative responses following long term infection. A more mixed response was seen at 30 d.p.i. with production of high levels of IFN-γ alongside Ag-stimulated IL-4, IL-5 and IL-10. The levels of IFN-γ produced by cells from L3 infected and mf-infected animals were similar (while being slightly higher following mf-infection). Interestingly in the light of this, although only cells from mf-infected animals produced significant levels of NO at 48 hrs, by 96 hrs splenocytes from both groups of infected animals produced NO at levels capable of suppressing proliferative responses. This was reflected in the proliferative response of cells from L3-infected animals which was considerably weaker at 30 d.p.i. than at 12 d.p.i. such that at 96 hrs Ag-stimulated cells proliferated at levels not significantly greater than background. Although not tested in this study it would be of interest to determine whether inhibition of iNOS activity in these cultures was able to enhance proliferative responses of cells from both groups of infected animals.

While several studies have followed responses to mf-infection over longer time-courses, there is little information in regard to temporal changes in L3 induced responses.

While the Th2 response induced by adult worms is known to be stable up to at least 28 d.p.i. (Lawrence, 1994), production of Ag-stimulated IFN-γ has previously been observed at 19 d.p.i. following s.c. infection with *B. pahangi* L3 indicative of a more mixed response (Osborne, 1997). This change in cytokine production and the mechanisms underlying it, whilst outwith the scope of this study, are particularly intriguing given that Th2 responses are generally considered more stable than Th1. Although the fate of L3 following i.v. infection is not known, the L3 is a dynamic stage within the mammalian host, and it is possible that altered cytokine production results from exposure to Ag associated with the L3/L4 or L4/adult moults. As male worms moult from the L4/adult at around 18 d.p.i. and female worms at around 23 d.p.i. it is likely that any parasites surviving up to day 30 are juvenile adults.

It has been suggested that besides chronicity of exposure, the change in cytokine profiles seen following i.p. infection with mf maybe related to mf survival, with development of Th2 responses resulting from to exposure to somatic Ag only released by dead or dying mf (Lawrence, 2000). Splenocytes from mice given heat-killed mf or live mf displayed similar polarisation *in vitro*. Heat-killed mf induced production of lower levels of IFN-γ than live mf but no IL-4 or IL-5 were detected in either group. Live and heat-killed mf induced production of similar levels of NO_2^- in Ag-stimulated culture at both 48 and 96 hrs. However dead mf stimulated only minimal proliferative responses *in vitro*, even at 48 hrs. It is possible that any protein Ag against which proliferative responses are directed may have been denatured by heat killing. Interestingly only splenocytes from mice given live mf proliferated at levels significantly below those in medium only wells at 96 hrs. As a greater proportion of these cells have proliferated in response to restimulation this may suggest that proliferating cells are more acutely susceptible to the suppressive effects of NO.

The demonstration that dead mf can stimulate production of IFN-γ indicates that this response is not induced by E/S products only produced by live mf. Lawrence *et al* (Lawrence, 2000) reported that freeze killed mf given i.p. failed to elicit Ag-specific cytokine production *in vitro* yet induced a qualitatively similar Ab response to live mf (although at much lower levels). The lack of cytokine responses in the spleen following i.p. administration of freeze killed mf compared to i.v. injection of heat killed mf may be a function of the route of injection, or may reflect the different methods of mf killing used. Interestingly while live mf could be readily recovered from the peritoneal cavity at 14 d.p.i. no dead mf were recovered at this time, suggesting that live and dead mf may be processed differently by the immune system with dead mf being cleared more rapidly (Lawrence, 2000). Although recovery of mf was not assessed, it is likely that this is also the case following i.v. infection.

Studies in several murine models of infection, including filariasis, have demonstrated differences between responses in the spleen and other secondary lymphoid organs. Following infection with mf or L3 proliferative and cytokine responses of peripheral lymph node cells were shown to reflect those seen in the spleen at 12 d.p.i. Whilst LN cells from mf-infected animals produced IFN-γ upon restimulation, they failed to display strong proliferative responses even in the absence of high levels of NO, suggesting that an NO independent mechanism maybe operative in these cultures. The absence of high levels of NO in Ag-stimulated cultures of LN cells from mf-infected animals is likely to reflect both the lower levels of IFN-γ produced by these cells and the fact that MO are more abundant in the spleen than peripheral lymph nodes. Although proliferative responses of LN cells were generally lower than those of splenocytes, LN cells from L3-infected animals showed strong Ag-driven proliferative responses throughout the period observed. In comparison to splenic responses LN cells from L3-

infected animals produced similar amounts of IL-4, while LN cells from mf-infected animals produced much lower levels of IFN-γ. This suggests that there are lower numbers of Ag-reactive cells in the peripheral lymph nodes of mf-infected animals. In this respect it is likely that the active migratory nature of the L3, and their tropism for the lymphatics, facilitates induction of a more widespread response than mf infection. This is further supported by the observation that LN from L3 infected animals were considerably larger and gave higher cellular recoveries than those from mf-infected or uninfected control mice (data not shown).

Splenocytes from infected animals generally displayed highly comparable proliferative responses to restimulation with the different Ag preparations tested. In one of two experiments, however, cells from mf-infected animals displayed significantly greater levels of proliferation in response to male Ag, as compared to female or mixed sex adult Ag at 72 hrs. However, responses to all antigens tested were equally suppressed at 96 hrs. Unfortunately, due to a paucity of male Ag, the basis of any alteration in the kinetics of suppression, in terms of cytokine production and generation of NO, were not assessed. This phenomenon was not observed in a repeat experiment, although distinct batches of Ag were used and it is possible they differed in purity. The similarity of proliferative responses induced by all Ag preparations tested suggest that, as described previously in experiments using *A. viteae* Ag, the *in vivo* experience of Ag, in terms of the polarisation of responses, is of primary importance in determining *in vitro* responses. It is of note that the results presented here regarding Ag-stimulated cytokine production differ from those found in human studies. Mahanty *et al* (1996) reported that PBMC from Mf+ individuals produce high levels of both spontaneous and Ag-driven IL-10 which is associated with suppression of IFN-γ production (Mahanty, 1997; 1997). In contrast to this, splenocytes from mf-infected mice do not produce high levels of IL-10 at 12 d.p.i. suggesting that IL-

10 mediated down-regulation of IFN-γ production may be a feature of chronic infection or may be induced by life cycle stages other than mf.

Although super-infection of L3 infected animals did not lead to suppression of proliferative responses it had a striking effect upon cytokine production. At 19 days post-L3 infection a mixed response was seen with production of IFN-γ, IL-4 and IL-5. Intriguingly, rather than enhancing Th1 responsiveness, super-infection with mf reduced IFN-γ production in Ag-stimulated culture. Similar observations were made following secondary infection with L3. These findings demonstrate that restimulation *in vivo* can consolidate Th2 polarisation by down-regulating IFN-γ production.

Heavily polarised responses are generally associated with chronic infections and in filariasis it is likely that long term exposure to adult worms and high levels of circulating Ag serve to strengthen Th2 responses. Whilst T cell lines generated under Th1 or Th2 polarising conditions display great stability in terms of cytokine production, the situation during active infection is likely to be less clear cut.

In a recent study investigating the ability of mf to modulate a pre-existing Th2 response induced by infection with adult male worms it was shown that while mf induced production of IFN-γ this was not reflected in the Ab isotypes elicited (Lawrence, 2000). The results presented here suggest that the response to L3 is less stable than that induced by adult males which may in part account for the different results in terms of IFN-γ production. It is interesting to speculate on the shared ability of mf and L3 to down-regulate L3-induced IFN-γ production. It is possible that, in a situation analogous to that previously described *in vitro* following mf-infection, restimulation of IFN-γ producing cells *in vivo* suppresses their proliferation and IFN-γ production. This is particularly interesting considering the previous reports that repeated immunisations with mf extract

leads to a reduction in IFN-γ production (Pearlman, 1993b). Furthermore in human infection Mf+ individuals have a lower frequency of IFN-γ producing Ag specific T cells (King, 1992; King, 1993) and in bancroftian filariasis Ag negative, and Mf- individuals produce elevated levels of IFN-γ (Dimock, 1996). Most recently it has been demonstrated that changes in mf density were accompanied by fluctuations in IFN-γ production such that the presence of mf was associated with reduced IFN-γ production (Sartono, 1999). It may be that in the presence of high antigen loads, IFN-γ producing cells are preferentially driven to undergo terminal differentiation or AICD.

Interestingly levels of IL-4 and IL-5 production *in vitro* were not affected by alterations in IFN-γ production. Although enhanced production of Th2 cytokines *in vivo* following secondary infection may have suppressed development of IFN-γ producing cells, these findings suggest that IL-4 does not down-regulate IFN-γ production *in vitro*. It is of interest to note that IL-4 production is much less affected by fluctuations in parasite density than is IFN-γ (Sartono, 1999) and varies little between clinical groups (King, 1992; Maizels, 1995). Recently analysis of cytokine production at the single cell level has shown that co-expression of Th1 and Th2 cytokines is a rare event, even in a response which may be characterised as Th0 on the population level (Bucy, 1994). In PBMC from infected humans, which produce both IFN-γ and IL-4 in response to *B. malayi* Ag, IFN-γ production is totally segregated from that of IL-4 (De Boer, 1998). Segregation of IFN-γ and IL-4 production would facilitate their independent regulation as seen following super-infection and in human filariasis. As IL-10 has been shown to down-regulate IFN-γ production following chronic infection with mf, and s.c. infection with L3, it would be of interest to determine levels of IL-10 production following super-infection of L3 infected animals.

Polarised murine Th1 and Th2 cells differ in their ability to respond to IL-12 as strongly polarised Th2 cells lose expression of the IL-12Rβ2 subunit of the IL-12 receptor (Szabo, 1997a). IL-12Rβ2 expression in upregulated following stimulation via the TCR and thereafter expression is influenced by cytokines. IL-4 inhibits expression leading to loss of IL-12 responsiveness and regulating commitment to the Th2 pathway, in contrast IFN-γ maintains IL-12Rβ2 expression (Szabo, 1997b). Th2 cells primed in the absence of IFN-γ can only subsequently respond to IL-12 with IFN-γ production in the absence of IL-4 (Hu-Li, 1997). These findings demonstrate that IL-4 production by Th2 cells is a relatively stable property whilst production of IFN-γ is highly regulated by cytokines, providing another mechanism whereby IFN-γ levels may fluctuate independently of IL-4. Interestingly the Th2 response seen following multiple immunisations with mf extract is also subject to modulation by IL-12 indicating that at least some of these cells retain IL-12 responsiveness (Pearlman, 1995). This suggests, as do responses seen following L3 infection at 30d.p.i., that responses in murine model of filariasis cannot accurately be described by strict application of the Th1/Th2 paradigm.

It has recently been shown that continued production of IL-12 is required to maintain ongoing Th1 responses following infection with *T. gondii* (Yap, 2000). It is possible then, that any reduction in IL-12 during the course of chronic infection could lead to reduced IFN-γ production. It may be that restimulation *in vivo* results in IL-12 production promoting expansion of IFN-γ producing cells and leading to NO production which in turn down-regulates IL-12 via a negative feedback loop. In this fashion restimulation of IFN-γ producing cells may ultimately result in lower levels of IFN-γ production.

CHAPTER 6. Tracking the fate of Ag-reactive lymphocytes in *in vitro* culture

6.1 Introduction

NO has been shown to mediate proliferative suppression in various model of parasitic infection (Candolfi, 1994; Dai, 1999; Mabbot, 1995), but few studies have investigated the means by which NO exerts this effect. As IFN-γ induced NO production is critical in down-regulating the responses of cells from mf-infected animals, the mechanism whereby this effect is manifested is of particular interest. NO could potentially limit proliferation by a variety of means, from effects upon APC function and cytokine production to regulation of apoptosis. The ability to selectively inhibit NO production and alleviate proliferative suppression *in vitro* provides a means of analysing the basis of the suppressive effects of NO.

APC function can be affected by NO on several levels; at the extreme it has been shown that NO can act in an autocrine fashion to induce MO apoptosis (Albina, 1993), effectively reducing APC numbers which may ultimately limit proliferative responses. There are however, more subtle ways in which NO can influence APC activity. It has been shown in various cell types, including human neutrophils and murine peritoneal MO, that phagocytic activity can be negatively regulated by NO (Forslund, 1997; Jun, 1996) which may inhibit Ag-uptake. Ag-presentation may also be affected as activated MO have been shown to inhibit MHC II expression on the surface of alveolar dendritic cells (Holt, 1993) and NO has been shown to down-regulate protein synthesis and MHC II expression in peritoneal MO (Sicher, 1994).

Furthermore, whilst low level NO production can have a direct effect upon T cells, enhancing their capacity for IFN-γ production (Huang, 1998a) high level NO production inhibits IL-12 synthesis by MO consequently limiting the expansion of IFN-γ producing T

cells (Niedbala, 1999). These findings illustrate that there are several ways in which NO can adversely affect APC function, at least one of which (inhibition of IL-12 production), may selectively limit the expansion of Th1 responses.

NO may also affect the development of immune responses by directly affecting T cells. It has been shown that, besides suppressing cytokine production, NO can directly induce the apoptosis of splenic T lymphocytes (Okuda, 1996). Although the high levels of NO generated by chemical donors in many *in vitro* studies exceed physiological levels, it is possible that there may be much higher levels of NO in the direct vicinity of MO than in the culture medium as a whole. In this sense it is not known to what levels of NO T cells may be exposed during their intimate interactions with activated MO.

NO-mediated proliferative suppression has been described during murine infection with *Mycobacterium tuberculosis* and *Trypanosoma cruzi,* and in both cases NO has been implicated in inducing the apoptosis of Ag-reactive T cells (Martins, 1999; Nabeshima, 1999). Where the effects of NO have been investigated it appears that the direct effects of NO and those mediated via IFN-γ may synergise to promote apoptosis. During the acute phase of *T. cruzi* infection both IFN-γ induced upregulation of Fas expression and NO production contribute to splenocyte apoptosis (Martins, 1999). IFN-γ has also been shown to induce the Fas dependent apoptosis of Peyer's patches T cells during peroral infection with *T. gondii* (Liesenfeld, 1997). NO itself has also been shown to modulate Fas expression in a variety of cell types including murine T lymphocytes, wherein iNOS inhibition attenuated TCR induced Fas expression (Williams, 1998).

The fact that Ag-stimulated cells from mf-infected animals frequently display lower levels of proliferation than unstimulated cells after 96 hrs culture suggests that an active form of suppression is induced by restimulation which may involve the AICD of Ag-reactive cells. Furthermore elevated levels of apoptosis amongst splenocytes taken

immediately *ex-vivo* have previously been observed following infection of BALB/c mice with *B. pahangi* mf but not L3. Intriguingly *in situ* analysis of splenocyte apoptosis revealed clusters of apoptotic nuclei in the spleens of mf-infected animals at 12 d.p.i. whilst only single apoptotic nuclei were seen scattered throughout the spleens of L3 infected and uninfected control mice (Osborne, 1997a). Further analysis revealed that the apoptotic bodies are associated with T cells within the germinal centres demonstrating that *B. pahangi* mf can induce lymphocyte apoptosis *in vivo* (Jenson *et al* submitted).

The experiments described in this chapter were aimed at further characterising the mechanism of NO-mediated suppression operative *in vitro* following mf-infection, in particular to identify the cellular population unable to proliferate in the presence of NO and the fate of these cells in Ag-stimulated culture. The tracking dye CFSE was combined with surface staining and FACS analysis to identify proliferating cells. Levels of lymphocyte apoptosis were assessed using propidium iodide staining and depletion experiments allowed identification of the cellular source of IFN-γ which is critical to the induction of iNOS activity and proliferative suppression.

6.2 RESULTS

6.2.1. CFSE staining of cells from mf-infected, L3-infected and uninfected control animals in Ag-stimulated culture in the presence or absence of AMG

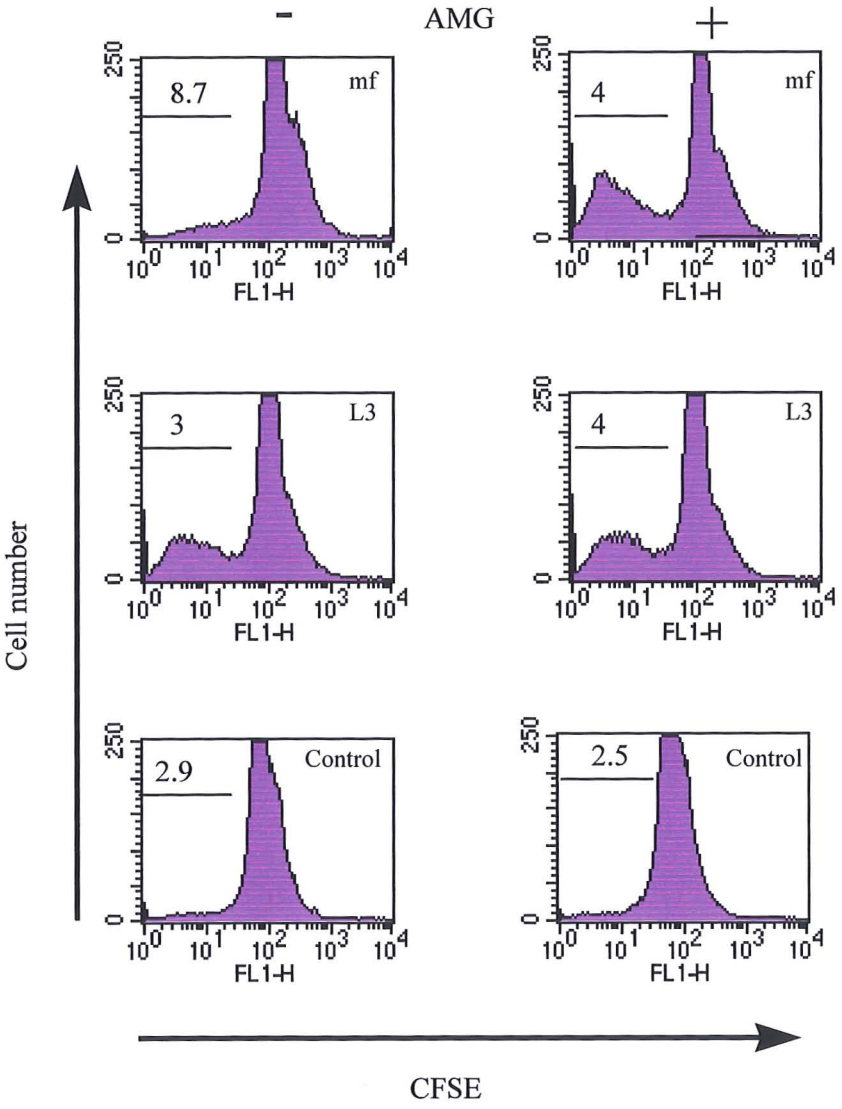
CFSE is a fluorescent dye which labels cells in a non-specific fashion. Upon division the dye is distributed evenly between daughter cells resulting in a decrease in fluorescence intensity and a peak shift to the left. Used in combination with cell surface staining this technique allows division within a phenotypically distinct subpopulation of cells to be tracked.

In these experiments splenocytes from mf-infected, L3-infected and uninfected control mice were labelled with CFSE prior to *in vitro* restimulation with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of the iNOS inhibitor AMG. After 96 hrs cells were harvested and surface stained with an anti-CD4 MAb. Samples were analysed using flow cytometry. Lymphocytes were initially gated on their physical parameters, in terms of size and granularity using forward and side scatter, and the staining profile of $CD4^+$ lymphocytes was analysed using CellQuest software. As shown in Figure 6.1 $CD4^+$ cells from mf-infected animals show little evidence of proliferation in Ag-stimulated culture, consistent with results previously reported using ^3H thymidine incorporation. However, upon inhibition of iNOS activity a secondary peak of CFSE low $CD4^+$ cells which have divided in culture is clearly identifiable. The proliferation of cells from L3 infected animals was unaltered by inhibition of iNOS activity, and cells from uninfected control animals displayed only background levels of proliferation under all conditions. Addition of AMG significantly increased the percentage of $CD4^+$ cells from mf-infected animals showing evidence of Ag-stimulated proliferation (P=0.012 Figure 6.2) whilst not significantly affecting responses within other groups. Comparison of the CFSE staining

profile of total lymphocytes and $CD4^+$ cells demonstrated that the Ag-stimulated proliferative response is contained almost entirely within the $CD4^+$ population (Figure 6.3). These results clearly demonstrate that IFN-γ dependent NO production suppresses the proliferation of Ag-reactive $CD4^+$ T cells from mf-infected mice. This experiment has been carried out on three separate occasions with equivalent results each time.

FIGURE 6.1 CFSE labelling of cells from mf-infected, L3-infecetd and uninfected control mice in Ag-stimulated culture in the presence or absence of aminoguanidine

Splenocytes from mice given $1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only, were labelled with CFSE and cultured with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of 500 μM AMG. After 96 hrs cells were harvested, surface stained with an anti-CD4 MAb and analysed by flow cytometry. Each panel shows the CFSE staining profile of $CD4^+$ splenocytes from an individual mouse in the presence or absence of AMG. The numbers in the top left hand corner of each panel indicate the percentage of $CD4^+$ 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


AMG
-
+
8.7
mf
4
mf
3
L3
4
L3
2.9
Control
2.5
Control
250
0
10^0 10^1 10^2 10^3 10^4
FL1-H
Cell number
CFSE

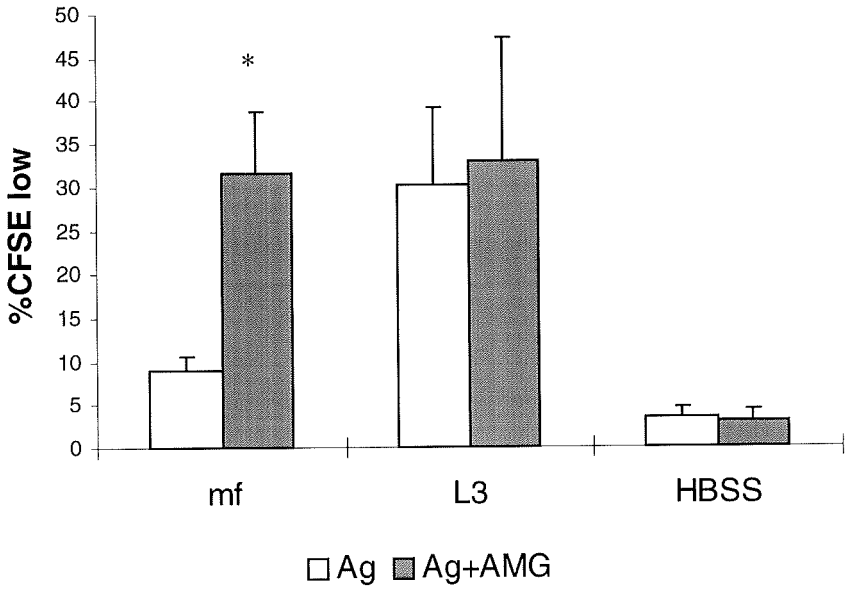
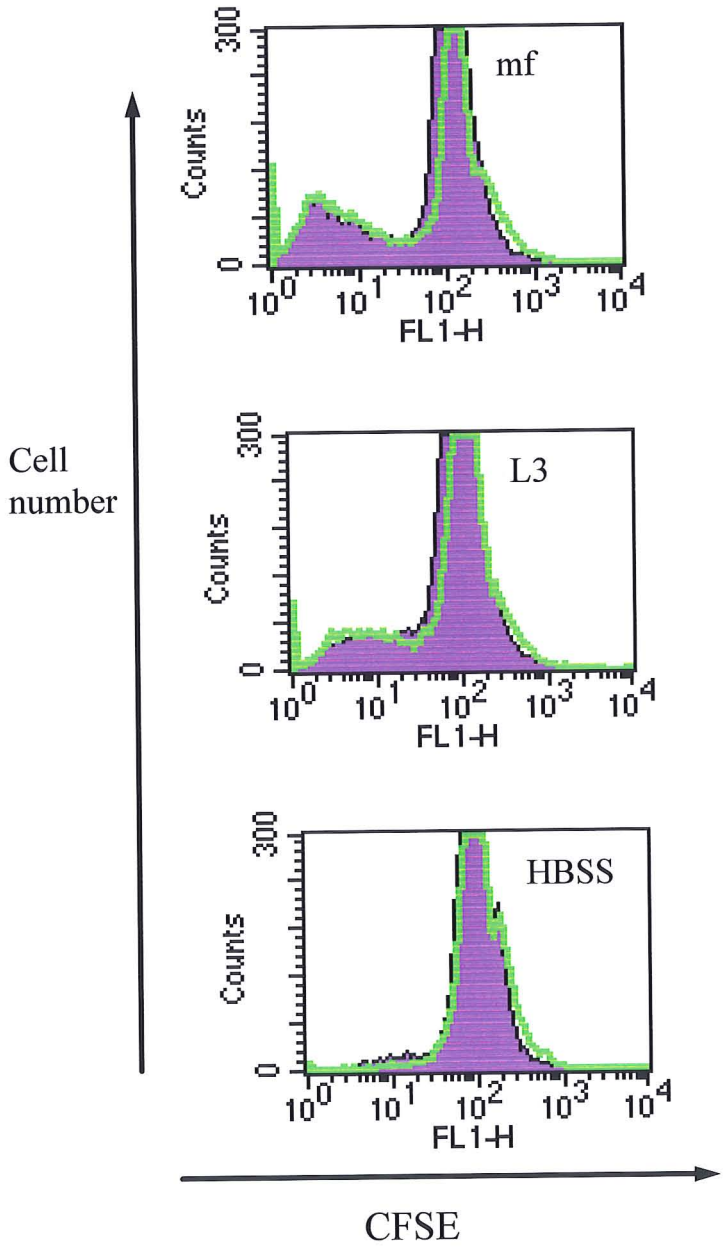


FIGURE 6.2 iNOS inhibition enhances Ag-stimulated proliferation of $CD4^+$ cells from mf-infected animals

Splenocytes from mice given$1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only, were labeled with CFSE and cultured with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of 500 μM AMG. After 96 hrs cells were harvested, surface stained with an anti-CD4 MAb and analysed by flow cytometry. The percentage of $CD4^+$ cells which have divided (i.e. display reduced fluorescence intensity in FL-1 (CFSE low)) are shown. All values represent the mean +/- standard deviation of five animals per group. *, significantly different (p=<0.05) from unsupplemented cultures.

FIGURE 6.3 The majority of dividing cells in Ag-stimulated culture are contained within the $CD4^+$ population

Splenocytes from mice given $1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only, were labelled with CFSE and cultured with 10 μg/ml *B. pahangi* adult Ag in the presence of 500 μM AMG. After 96 hrs cells were harvested, surface stained with an anti-CD4 MAb and analysed by flow cytometry. The purple fill represents the CFSE staining profile of total lymphocytes and the green overlay shows the staining profile of $CD4^+$ cells. The results shown are for individual mice and are representative of five animals per group.


mf
L3
HBSS
Counts
300
0
10^0 10^1 10^2 10^3 10^4
FL1-H
Cell number
CFSE

6.2.2. Inhibition of iNOS activity allows the expansion of a population of $CD4^{hi}$ Ag reactive cells

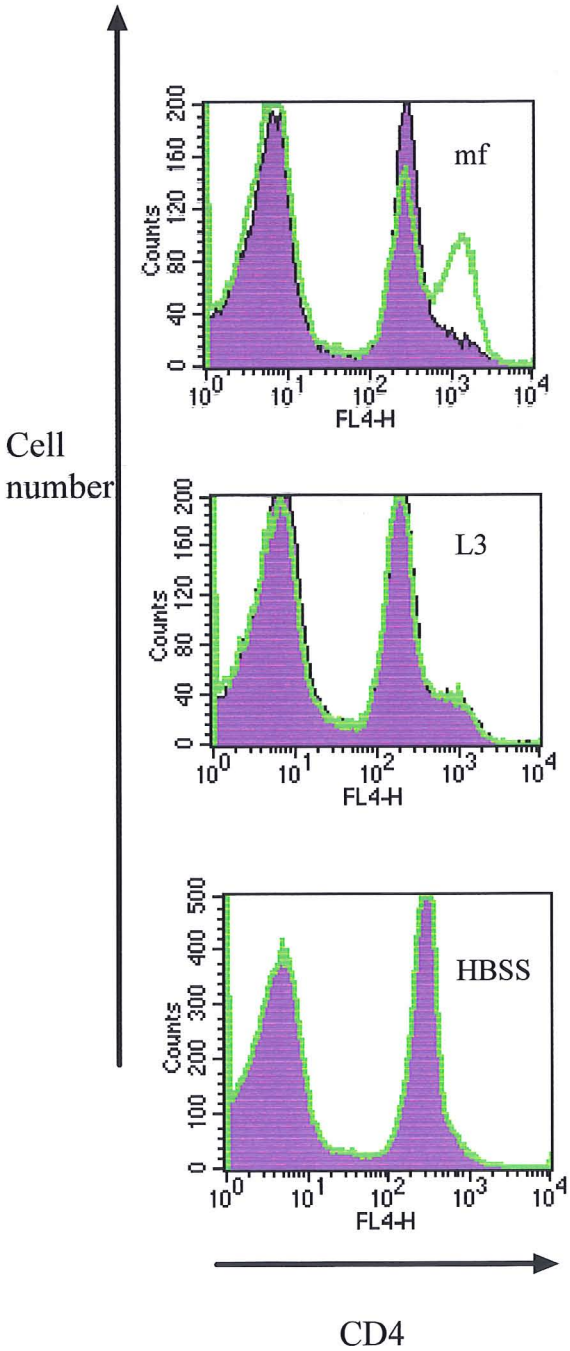
Intriguingly the CD4 staining profile of cells from mf-infected animals in Ag-stimulated culture was clearly altered upon iNOS inhibition. In the presence of high levels of NO, cells from mf-infected animals segregated into two discrete peaks of $CD4^-$ and $CD4^+$ cells. However, upon addition of AMG a tertiary peak of brightly staining $CD4^{hi}$ cells could clearly be identified (Figure 6.4). $CD4^{hi}$ cells were observed in cultures of cells from L3-infected animals regardless of the presence or absence of AMG and were not seen in cultures from uninfected control animals. iNOS inhibition significantly enhanced the number of $CD4^{hi}$ cells in cultures from mf-infected animals (p=0.012) and did not affect responses in other groups (Figure 6.5). Furthermore the $CD4^{hi}$ population showed increased side scatter, indicative of increased granularity and a characteristic of activated lymphocytes (Figure 6.6)

6.2.3. The $CD4^{hi}$ population contains Ag-reactive cells

As $CD4^{hi}$ cells are only detected in cultures of cells from mf-infected animals in the absence of high levels of NO it was of interest to determine whether this population contained dividing cells. Analysis of the CFSE staining profile of $CD4^{hi}$ cells revealed that this is indeed the case. As shown in Figure 6.7 the majority of cells within this population had divided in Ag-stimulated culture and their division accounted almost entirely for division within the $CD4^+$ population.

FIGURE 6.4 $CD4^{hi}$ cells are observed in Ag-stimulated cultures of cells from mf-infected animals only upon iNOS inhibition

Splenocytes from mice given$1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only, were labelled with CFSE and cultured with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of 500μM AMG. After 96 hrs cells were harvested, surface stained with an anti-CD4 MAb and analysed by flow cytometry. The purple fill shows the CD4 staining profile of cells in Ag-stimulated culture and the green overlay represents the CD4 staining profile in the presence of Ag and AMG. The results shown are for cells from individual animals and are representative of five animals per group.


Cell number
mf
L3
HBSS
Counts
FL4-H
CD4

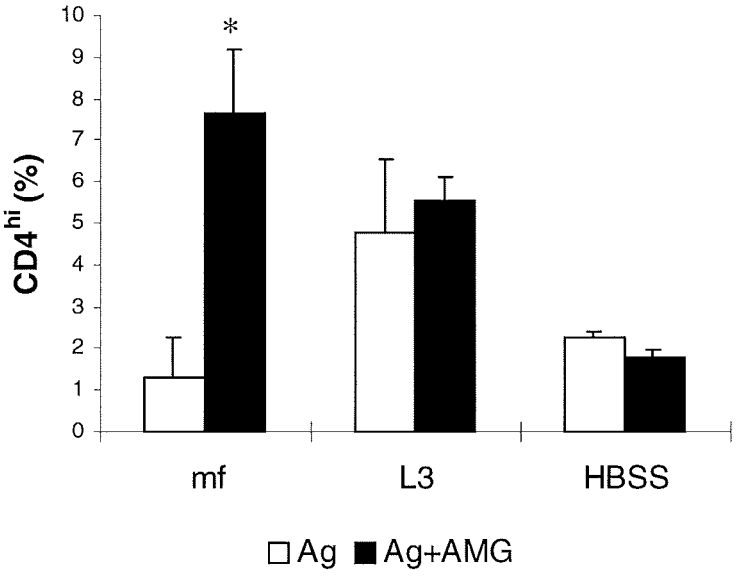


FIGURE 6.5 NO inhibits the expansion of $CD4^{hi}$ cells in Ag-stimulated cultures of cells from mf-infected animals

Splenocytes from mice given $1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only, were cultured with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of 500 μM AMG. After 96 hrs cells were harvested, surface stained with an anti-CD4 MAb and analyzed by flow cytometry. The number of brightly staining $CD4^{hi}$ cells is shown as a percentage of total lymphocytes. All values represent the mean +/- standard deviation of five animals per group. *, significantly different (p=<0.05) from unsupplemented cultures.

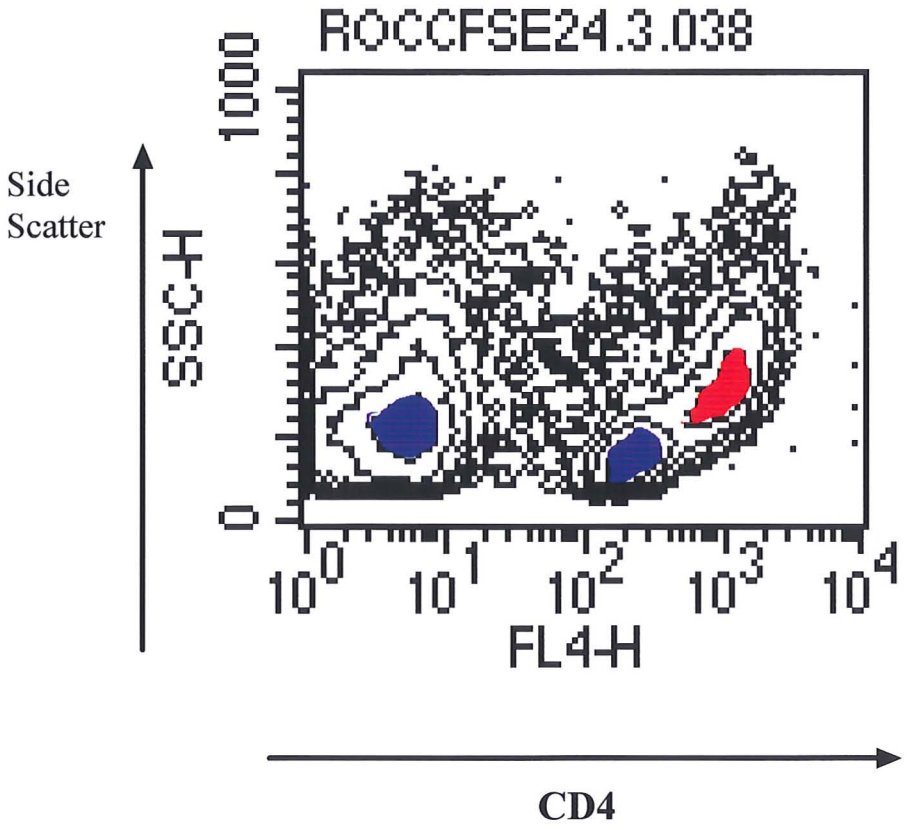
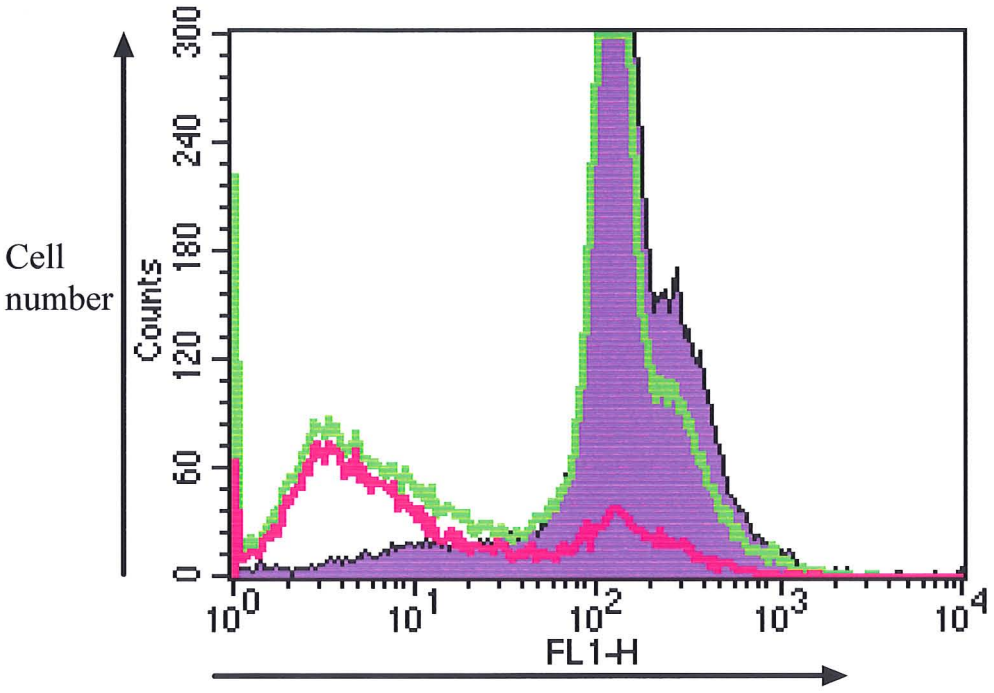


FIGURE 6.6. CD4^{hi} cells show increased side scatter

Splenocytes from mice given 1x10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only, were cultured with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of 500 μM AMG. After 96 hrs cells were harvested, surface stained with an anti-CD4 MAb and analysed by flow cytometry. The contour plot shows side scatter versus CD4 staining and illustrates the staining pattern of splenocytes from a mf-infected animal cultured in the presence of AMG. CD4$^-$, CD4 $^+$ and CD4^{hi} populations can be clearly identified. The highest points of the contours have been filled, and the peak of the CD4^{hi} population is coloured red. The results shown are for cells from an individual animal and are representative of responses seen in five mice per group.

FIGURE 6.7 The $CD4^{hi}$ population contains dividing cells

Splenocytes from mice given $1x10^5$ mf *B. pahangi* were labelled with CFSE and cultured with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of 500 μM AMG. After 96 hrs cells were harvested, surface stained with an anti-CD4 MAb and analysed by flow cytometry. The purple fill shows the CFSE staining profile of $CD4^+$ cells after 96 hrs Ag-stimulated culture. The green overlay represents the CFSE staining profile of total $CD4^+$ cells in the presence of AMG and the pink overlay shows the CFSE staining profile of the $CD4^{hi}$ population seen only upon iNOS inhibition. The results shown are representative of five mice per group.

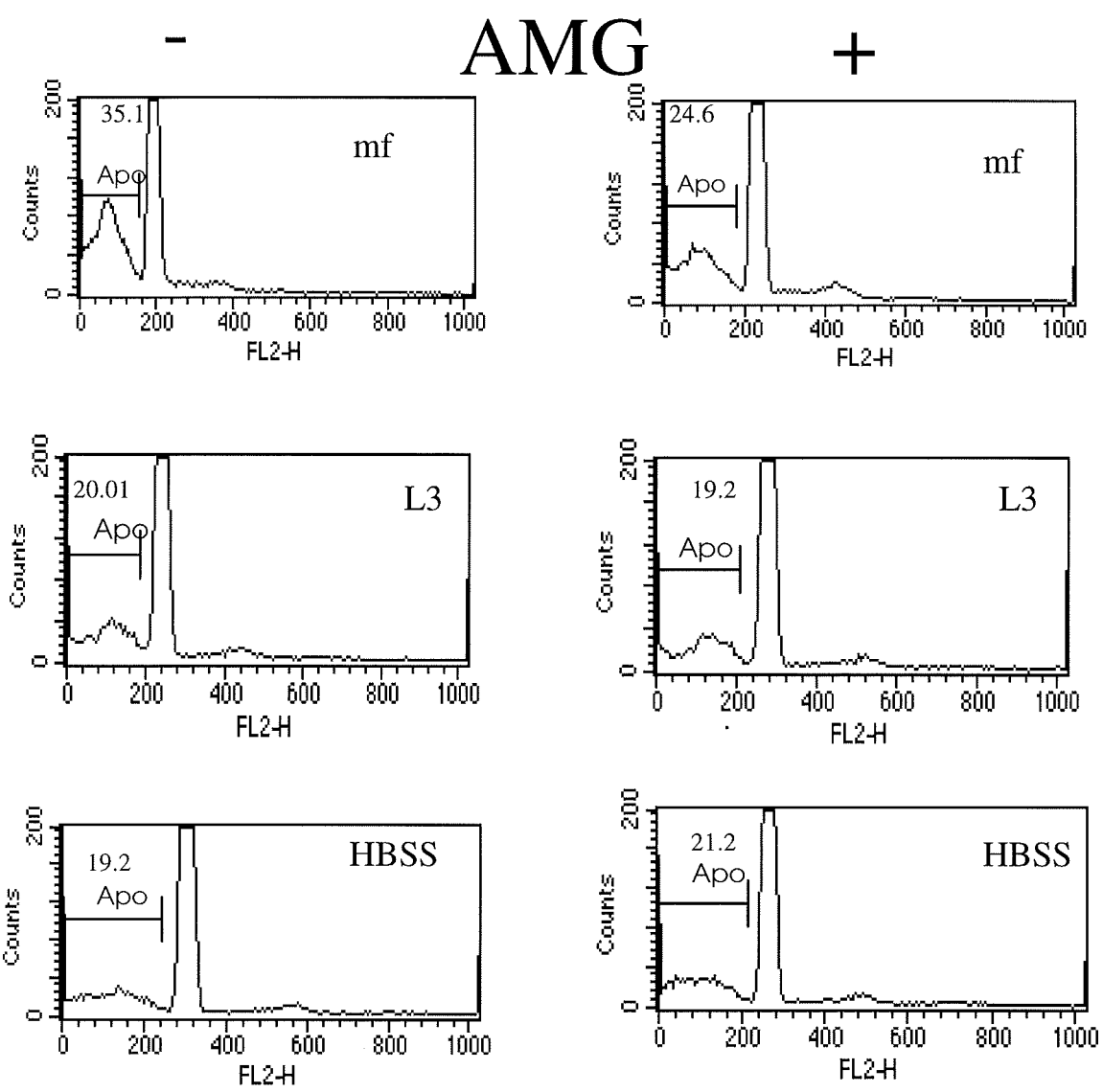

Cell number
Counts
300
240
180
120
60
0
10^0
10^1
10^2
10^3
10^4
FL1-H
CFSE

6.2.4. $CD4^+$ cells from mf-infected animals display elevated levels of apoptosis in Ag-stimulated culture which is reduced following inhibition of iNOS activity

Although cells from mf-infected animals proliferate at early time points, by 96 hrs evidence of Ag-stimulated division is only apparent upon iNOS inhibition. Propidium iodide staining was performed to determine whether enhanced levels of proliferation were associated with reduced levels of apoptosis (as assessed by subdiploid DNA content). As shown in Figures 6.8 and 6.9, $CD4^+$ cells from mf-infected animals showed significantly greater levels of apoptosis in Ag-stimulated culture than cells from L3-infected or uninfected control animals (p=0.03 and 0.03 respectively). The elevated levels of apoptosis in Ag-stimulated cultures of cells from mf-infected animals were associated with a significantly reduced number of cells in G1/GO compared to cells from L3-infected and uninfected control animals (p=0.034 and 0.03 respectively). No significant differences were observed in the proportion of cells in other stages of the cell cycle (data not shown). Inhibition of iNOS activity significantly reduced apoptosis of $CD4^+$ T cells in cultures from mf-infected animals (p=0.0294) but had no significant effect within other groups (Figure 6.9). iNOS inhibition also significantly increased the number of cells from mf-infected animals in G1/GO (p=0.034, Figure 6.10). These results suggest that NO mediates proliferative suppression by inducing the apoptosis of Ag-reactive $CD4^+$ T cells from mf-infected animals. This experiment was repeated three times with equivalent results each time. In parallel experiments in this laboratory highly comparable results were achieved using the TUNEL staining method to demonstrate elevated levels of lymphocyte apoptosis in Ag-stimulated cultures from mf-infected animals. Again, apoptosis occured within the $CD4^+$ population, and was reduced upon iNOS inhibition (Jenson *et al* submitted).

FIGURE 6.8 $CD4^+$ cells from mf-infected animals show elevated levels of apoptosis in Ag-stimulated culture which are reduced upon iNOS inhibition

Splenocytes from mice given$1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only, were labelled with cultured with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of 500 μM AMG. After 48 hrs cells were harvested, stained with propidium iodide and an anti-CD4 MAb and analysed by flow cytometry. The propidium iodide staining profile of $CD4^+$ cells in Ag-stimulated culture in the presence or absence of AMG are shown. Cells with a subdiploid DNA content were considered apoptotic. The results shown are representative of five mice per group.


-
AMG
+
35.1
Apo
mf
24.6
Apo
mf
20.01
Apo
L3
19.2
Apo
L3
19.2
Apo
HBSS
21.2
Apo
HBSS
Counts
FL2-H
0
200
400
600
800
1000

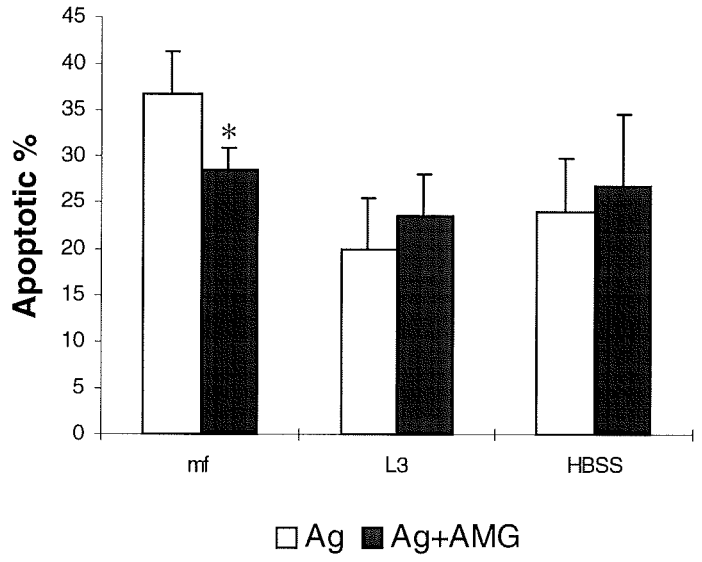


FIGURE 6.9 iNOS inhibition reduces apoptosis of $CD4^+$ cells from mf-infected animals in Ag-stimulated culture

Splenocytes from mice given $1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only, were cultured with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of 500 μM AMG. After 48 hrs cells were harvested, stained with propidium iodide and an anti-CD4 MAb and analyzed by flow cytometry. Cells with a subdiploid DNA content were considered apoptotic. Levels of apoptosis amongst $CD4^+$ cells are shown. All values represent the mean +/- standard deviation of five animals per group. *, significantly different (p=<0.05) from unsupplemented cultures.

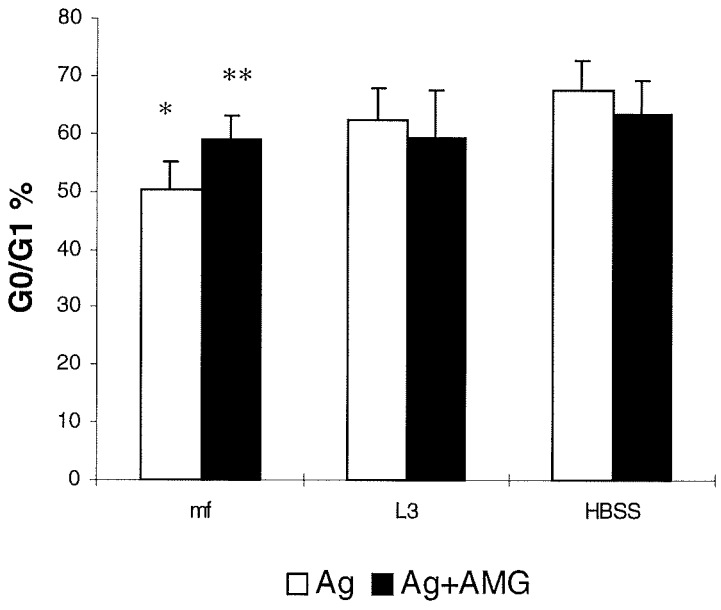


FIGURE 6.10 iNOS inhibition increases the percentage of CD4+ cells from mf-infected animals in G0/G1

Splenocytes from mice given $1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only, were cultured with 10 μg/ml *B. pahangi* adult Ag in the presence or absence of 500 μM AMG. After 48 hrs cells were harvested, stained with propidium iodide and an anti-CD4 MAb and analysed by flow cytometry. The percentage of $CD4^+$ T cells in G0/G1 (diploid cells) is shown. All values represent the mean +/- standard deviation of five animals per group. *, significantly different (p=<0.05) from other groups, **, significantly different from unsupplemented cultures, .

6.2.5. $CD4^{hi}$ cells display enhanced levels of Fas expression independently of IFN-γ and NO production

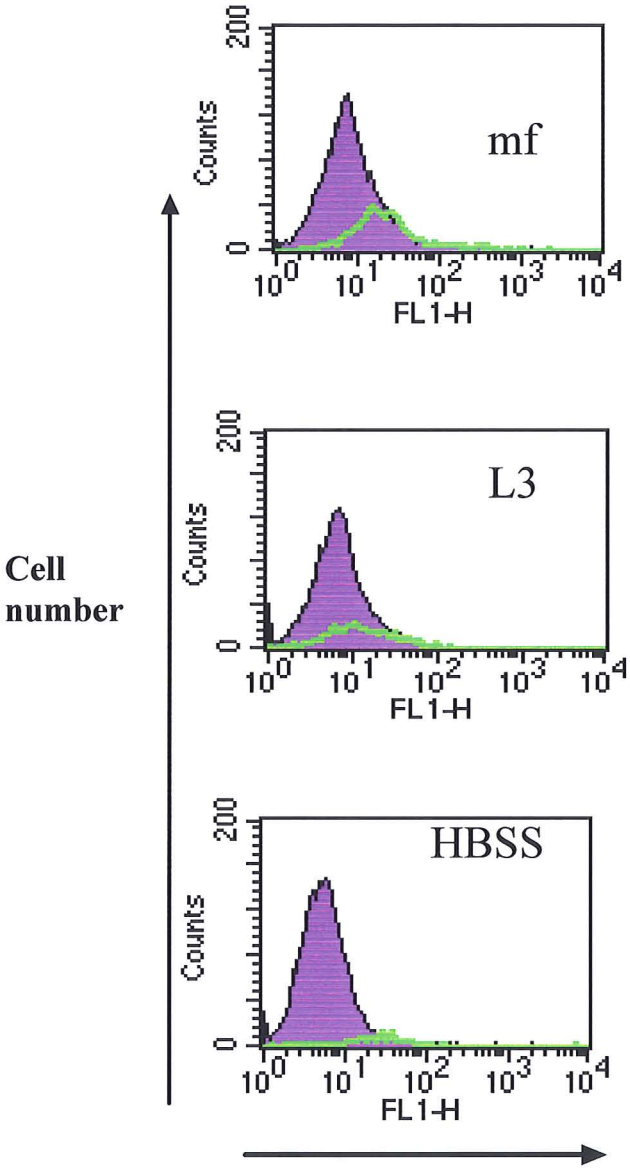
Several studies have suggested that Th1 cells are more susceptible to AICD than Th2 clones (Liu, 1990; Novelli, 1997) and both IFN-γ and NO have been shown to upregulate Fas expression (Martins, 1999; Stassi, 1997). Surface staining was used to assess levels of Fas expression amongst cells from mf-infected, L3-infected and uninfected control animals in Ag-stimulated culture. Although staining with anti-Fas MAb did not clearly identify Fas^+ and Fas^- populations amongst total lymphocytes, when the Fas staining profiles of $CD4^+$ and $CD4^{hi}$ cells were compared (Figure 6.11) it was apparent that $CD4^{hi}$ cells showed higher levels of Fas expression than normal $CD4^+$ cells. This could be seen most clearly when comparing the mean fluorescence intensity of Fas staining in the two populations (Figure 6.12). Although $CD4^{hi}$ cells in all groups were more brightly stained than normal $CD4^+$, cells the number of $CD4^{hi}$ cells in cultures from uninfected control animals was far lower than seen in other groups (as shown previously in Figures 6.5 and 6.6).

In order to assess whether Fas expression on $CD4^{hi}$ cells was influenced by IFN-γ, splenocytes from mf-infected and uninfected control IFN-γR-/- and wild type 129/Sv mice were removed from Ag-stimulated culture after 48 hrs and double stained for Fas and CD4. As shown in Figure 6.13 $CD4^{hi}$ cells from both groups of mf-infected mice showed elevated levels of Fas expression. Interestingly there were greater numbers of $CD4^{hi}$ cells in cultures from infected IFNγR-/- than wild type mice suggesting, as did the results of PI staining, that NO mediated apoptosis of Ag-responsive is acting at this time to limit the

expansion of this population. These results demonstrate that Fas expression is upregulated in $CD4^{hi}$ cells independently of the influence of IFN-γ or NO.

FIGURE 6.11 $CD4^{hi}$ cells show enhanced levels of Fas expression

Splenocytes from mice given $1x10^5$ mf, 50 L3 *B. pahangi* or an equal volume of HBSS only, were cultured with 10 μg/ ml *B. pahangi* adult Ag in the presence of 500 μM AMG. After 96 hrs cells were harvested, surface stained with an anti-CD4 MAb and analysed by flow cytometry. The purple fill represents the Fas staining profile of $CD4^+$ cells and the green overlay shows the Fas staining profile of $CD4^{hi}$ cells. The results shown are representative of five animals per group.


mf
L3
HBSS
Counts
200
0
FL1-H
Cell number
Fas

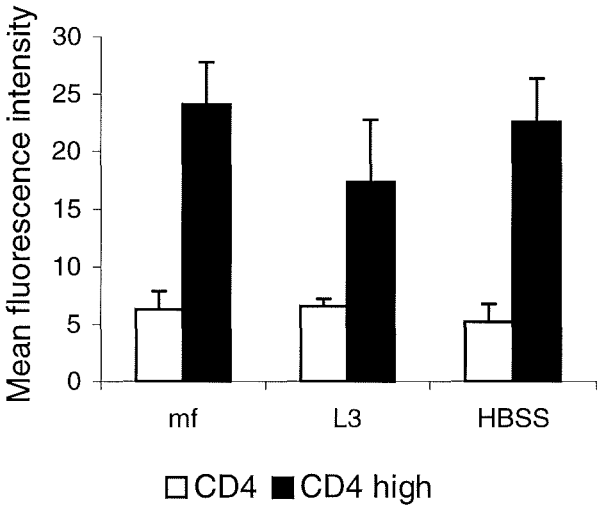
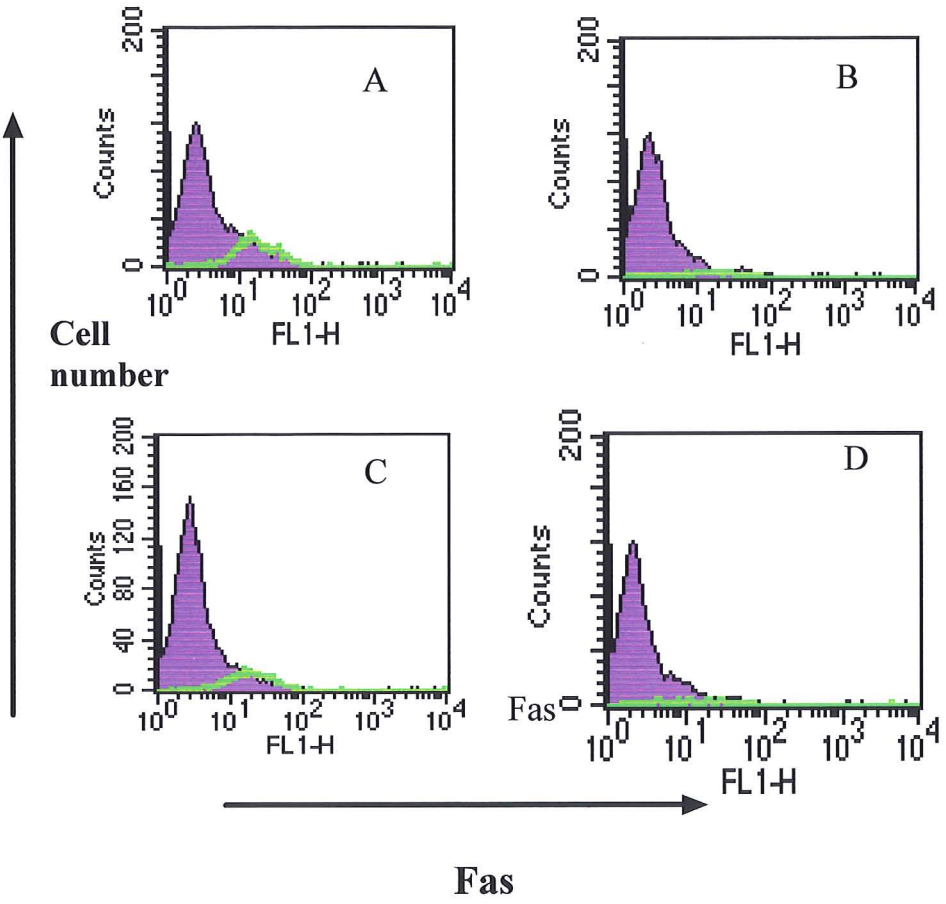


FIGURE 6.12 CD4^{hi} cells display elevated levels of Fas expression

Splenocytes from mice given 1x10^5 mf, 50 L3 *B. pahangi* or an equal volume of HBSS only, were cultured with 10 μg/ml *B. pahangi* adult Ag in the presence of 500 μM AMG. After 96 hrs cells were harvested, double stained with anti-Fas and anti-CD4 MAbs and analyzed by flow cytometry. The geometric mean fluorescence intensity of anti-Fas staining on CD4 normal and CD4^{hi} cells is shown. All results represent the mean +/- standard deviation of three animals per group.

FIGURE 6.13 $CD4^{hi}$ cells from mf-infected IFN-γR-/- and wild type 129/Sv mice show enhanced levels of Fas expression

Splenocytes from IFN-γR-/- and wild type 129/Sv mice given $1x10^5$ mf, or an equal volume of HBSS only, were cultured with 10 μg/ml *B. pahangi* adult Ag. After 48 hrs cells were harvested, surface stained with an anti-Fas and anti-CD4 MAb and analysed by flow cytometry. The panels show representative staining profiles of cells from mf-infected and uninfected IFN-γR-/- mice (A/B) and mf-infected and uninfected wild type 129/Sv mice (C/D). The purple fill represents the Fas staining profile of $CD4^+$ cells and the green overlay shows the Fas staining profile of $CD4^{hi}$ cells. Results shown are representative of three animals per group.



Fas

6.2.6. $CD4^+$ cells are the major source of IFN-γ in Ag-stimulated culture

In order to identify the cellular source of IFN-γ in Ag-stimulated culture, splenocytes from mf-infected animals were depleted of either $CD4^+$ or $CD8^+$ cells, by magnetic cell sorting, prior to *in vitro* restimulation. As shown in Figure 6.14 depletion of $CD4^+$ cells completely abolished IFN-γ production and consequent NO production whilst removal of $CD8^+$ cells only partially reduced these responses. These results clearly demonstrate that $CD4^+$ cells from mf-infected mice are the major source of IFN-γ production in Ag-stimulated culture. This experiment was carried out on three occasions with equivalent results.

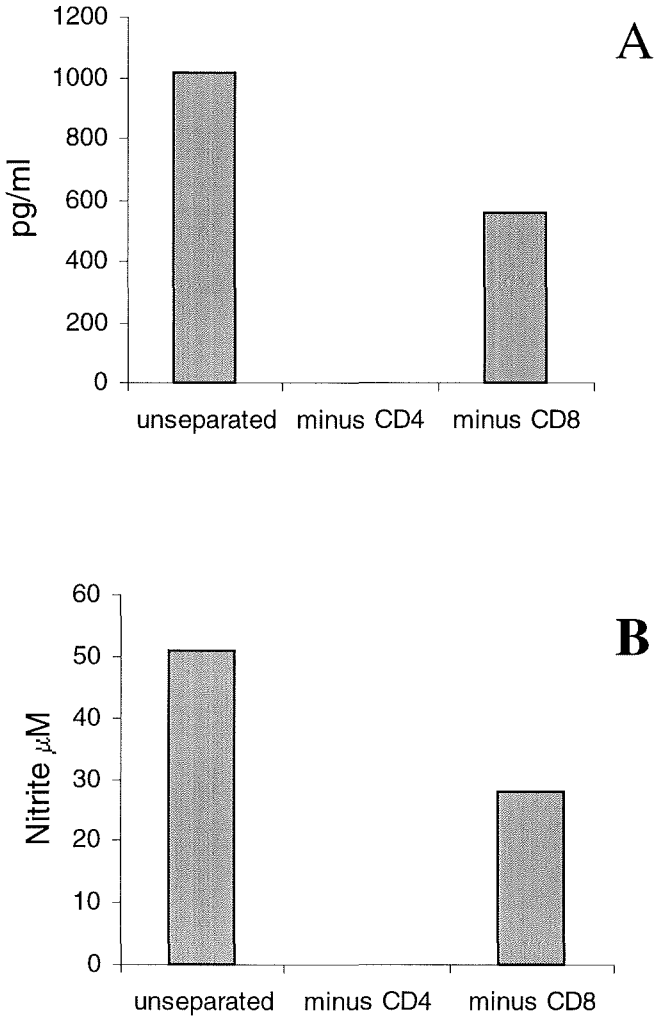


FIGURE 6.14 CD4$^+$ cells are the major source of IFN-γ production in Ag-stimulated culture

Splenocytes from five mf-infected animals were pooled and depleted of either CD4$^+$ or CD8$^+$ cells prior to restimulation *in vitro* with 10 μg/ml *B. pahangi* adult Ag. The efficiency of depletion was checked by FACS and in the experiment shown was 96% for CD4 and 86% for CD8. IFN-γ (a) and nitrite (b) production at 96 hrs of Ag-stimulated culture are shown.

6.3 Discussion

The results presented in this chapter show that NO mediated apoptosis limits the *in vitro* proliferation of Ag-reactive $CD4^+$ T cells from mf-infected animals. Elevated levels of apoptosis were observed amongst $CD4^+$ lymphocytes from mf-infected animals in Ag-stimulated culture. Inhibition of iNOS activity rescued these cells from apoptosis and allowed the continued expansion of a population of $CD4^{hi}$ proliferating cells. Such results demonstrate that cells from mf-infected animals are not irreversibly committed to undergo AICD upon restimulation with Ag and show that these cells can continue to proliferate given the right conditions in culture.

The majority of the proliferative response seen in both groups of infected animals was accounted for by division within the $CD4^+$ population. $CD4^+$ cells from L3-infected animals showed clear evidence of Ag-driven proliferation, irrespective of iNOS inhibition, and cells from uninfected control animals showed only background levels of proliferation. In cultures containing proliferating cells a subpopulation of brightly staining $CD4^{hi}$ cells could be clearly identified, the majority of which had divided in response to filarial Ag. Furthermore the $CD4^{hi}$ population showed increased side scatter a characteristic of activated lymphocytes. Levels of Fas expression were found to be elevated within the $CD4^{hi}$ population in a fashion independent of IFN-γ or NO and likely to reflect their activation status. $CD4^+$ T cells from mf-infected animals were also shown to be the major source of IFN-γ production in Ag-stimulated culture. Taken together these results suggest that IFN-γ production leads to MO activation and production of NO which in turn suppresses proliferation by inducing the apoptosis of Ag-reactive T cells. In this way Ag-specific IFN-γ producing T cells may indirectly limit their own expansion.

It has recently been reported that T cells upregulate cell surface expression of CD4 following encounter with their specific antigen both *in vitro* and *in vivo*. Following *in vitro* restimulation cells from animals immunised with sperm whale myoglobin were shown to upregulate expression of CD4. The resultant $CD4^{hi}$ population also expressed memory and activation markers ($CD45RB^{low}$, $CD62L^{low}$, $CD69^{high}$ and $CD4^{high}$). Separation and restimulation of CD4 normal and $CD4^{hi}$ cells revealed that all the Ag-reactive cells were contained within the $CD4^{hi}$ population (Ridgway, 1998). Analysis of the CFSE staining profile of $CD4^{+}$ and $CD4^{hi}$ T cells from infected animals revealed a similar pattern of responsiveness. Although not tested in this study it would be of interest to determine whether the $CD4^{hi}$ cells which develop in Ag-stimulated culture also display markers of memory and activation and whether cells from mf-infected and L3-infected animals do so at similar levels. Elevated levels of CD4 expression has also been used to select Ag-reactive cells from samples taken immediately *ex-vivo* (gating on the brightest 1% of CD4+ cells). This technique has now been successfully used to isolate auto-reactive T cells from non-obese diabetic mice (Lejon, 1999). Such findings suggest that using $CD4^{hi}$ expression as a marker maybe a useful method of enrichment for Ag-reactive T cells. It would be of interest to systemically inhibit NO production during mf-infection, or to use infection of IFN-γR-/- mice, in attempt to determine whether NO also limits the expansion of Ag-reactive $CD4^{hi}$ T cells *in vivo*. It has been proposed that CD4 itself may act as a co-receptor through association with the TCR leading to enhanced signal amplification (Janeway, 1992); CD4 may also act to increase the avidity of T cell:MHCII interactions (Konig, 1996). In these ways increased expression of CD4 is likely to promote T cell activation. Interestingly it has recently been shown that besides playing an important role during Ag-presentation, CD4 may also regulate the susceptibility of T cells to apoptosis. Cross-linking of CD4 was shown to render resting T cells susceptible to Fas mediated

apoptosis and to induce the expression of Fas-L within the same population (Algeciras, 1998). $CD4^{hi}$ proliferating cells from both mf-infected and L3-infected animals were shown to display enhanced levels of Fas expression. Both IFN-γ and NO have previously been shown to regulate expression of Fas and/or Fas-L in a variety of cell types (Martins, 1999; Stassi, 1997; Williams, 1998). However Fas expression was equally upregulated in $CD4^{hi}$ cells from both mf-infected IFN-γR-/- mice and their wild type counterparts, suggesting expression is not under the influence of either NO or IFN-γ.

Several studies have reported on the differential sensitivity of Th1 and Th2 cells to AICD. Whilst it has been suggested that the increased susceptibility of Th1 cells is due to enhanced levels of Fas-L expression (Ramsdell, 1994) other studies have reported equivalent levels of Fas and Fas-L expression on Th1 and Th2 cells and suggested that enhanced levels of FAP-1 (Fas-Associated-Phosphatase 1) expression in Th 2 cells selectively protects them from Fas-induced apoptosis (Zhang, 1997). Following murine infection with *T. cruzi,* IFN-γ but not NO was shown to upregulate expression of Fas-L. Interestingly *in vivo* treatment of infected mice with AMG decreased levels of apoptosis whilst not affecting Fas/Fas-L expression suggesting that IFN-γ induces NO dependent and independent mechanisms of apoptosis in this model of infection. The ability of iNOS inhibition to rescue $CD4^{+}$ T cells from mf-infected animals from apoptosis in the presence of high levels of IFN-γ suggests that NO mediated apoptosis is of primary importance in suppressing proliferation in Ag-stimulated culture.

NO has been shown to induce apoptosis in a variety of cells types, and whilst the mechanism by which NO induces activation of the apoptotic pathway remains incompletely defined recent studies have identified several possibilities. The NO induced apoptosis of murine T cells is associated with an accumulation of the tumor suppressor

protein p53 (Fehsel, 1995). Expression of p53 can be induced by DNA damage, which may be directly induced by NO (Nguyen, 1992). Furthermore NO mediated inhibition of proteosome activity has been shown to result in elevated levels of p53 and induction of apoptosis in RAW 264.7 MO (Glockzin, 1999). In this fashion it is possible that NO both induces expression of p53 and prevents its degradation by inhibiting proteosome activity. Intriguingly it has previously been demonstrated that dividing cells respond to proteosome inhibitors by undergoing apoptosis whilst in non-dividing cells the same inhibitors displayed anti-apoptotic effects. Such differential effects may form the basis of one means whereby NO can selectively induce the apoptosis of activated dividing cells.

Lissy *et al* (98) recently demonstrated that AICD occurs from a late G1 phase cell cycle checkpoint. Control of cell cycle progression is a highly regulated process involving both positive and negative regulatory proteins that function at specific cell cycle checkpoints. The late G1 checkpoint is a key regulatory point in determining whether a cell will proliferate or undergo apoptosis. Stimulated BrdU-labelled Jurkat T cells were shown to progress from S phase into G2/M and then into G1 before appearing in the apoptotic compartment suggesting that AICD occurs from the late G1 checkpoint (Lissy, 1998). Positive regulators of cell cycle progression include regulatory cyclin subunits which bind and activate cyclin dependent kinase subunits. Cyclins generally display cell cycle dependent expression and progression through the late G1 restriction point is thought to require inactivation of the retinoblastoma tumor suppressor protein pRB via hyperphosphorylation by activated cyclin E:Cdk 2 complexes (Dou, 1993; Ezhevsky, 1997). Most interestingly NO has recently been shown to prevent activation of cyclin-dependent kinase (Cdk)-2 in smooth muscle cells (Guo, 1998). As Cdk-2 is a key regulator of the transition through the late G1 checkpoint it is interesting to speculate on the

possibility that NO may induce apoptosis by preventing the inactivation of pRB. In Ag-stimulated culture a significantly lower percentage of $CD4^+$ T cells from mf-infected mice were found in G1, as compared to cells from L3-infected and uninfected control animals. The lack of cells in G1 was reflected in the increased number of apoptotic cells in these cultures, while the percentage in all other stages of the cell cycle were similar across groups. Inhibition of iNOS activity reduced elevated levels of apoptosis and caused a corresponding increase in the number of cells in G1. Such results suggest that an inability of cells from mf-infected animals to exit G1 may result in their death by apoptosis. The reversibility of this phenomenon demonstrates a critical role for NO which may potentially be realised at the level of cell cycle regulation.

It has recently been demonstrated that NO can suppress the proliferation of human T cells by IFN-γ dependent and IFN-γ independent induction of apoptosis. Both normal and malignant human T cells ceased to proliferate and underwent apoptosis upon exposure to NO. T cells which evaded NO induced apoptosis were shown to overexpress both chains of the IFN-γR rendering them susceptible to apoptosis in the presence of IFN-γ (Allione, 1999). Such findings illustrate another mechanism whereby NO and IFN-γ may synergise to promote apoptosis.

Studies in several murine models of infection have suggested that NO may limit the expansion of activated T cells *in vivo* as well as *in vitro* (Dalton, 2000; Millar, 1999; Nabeshima, 1999). Indeed there is now a growing body of evidence to suggest that NO induced apoptosis eliminates activated T cells in both infectious and autoimmune diseases. It has recently been shown in murine infection with *Mycobacterium bovis* Bacille Calmette-Geurin (BCG) that IFN-γ eliminates responding T cells in an NO dependent manner. During infection there is a large expansion of activated ($CD44^{hi}$) cells in the

spleen which is followed by a rapid contraction of this population associated with increased T cell apoptosis. However, following infection of IFN-γ-/- mice activated $CD44^{hi}$ $CD4^{+}$ T cells failed to undergo apoptosis and accumulated in large numbers. IFN-γ mediated apoptosis could be blocked by removing adherent cells or inhibiting NO production (Dalton, 2000).

Interestingly in two distinct models of autoimmune disease, wherein Th1 responses were previously thought to be solely pathogenic, it has now been shown that the IFN-γ mediated elimination of activated T cells has protective effects. Both IFN-γ-/- and IFN-γR-/- mice develop progressive and fatal experimental autoimmune encephalomyelitis (EAE). Exacerbated disease was associated with an enhanced accumulation of activated autoreactive T cells. EAE is also exacerbated in iNOS-/- mice and it was proposed that IFN-γ induced NO mediated apoptosis of activated T cells is required to down-regulate responses and allow remission of disease (Chu, 2000). Similarly in experimental autoimmune uveitis (EAU) while administration of rIL-12 was expected to increase susceptibility, it was shown to increase resistance through the hyperinduction of IFN-γ, initiating a negative feedback mechanism resulting in the elimination of pathogenic autoreactive T cells (Tarrant, 1999). Interestingly in a study on IFN-γ dependent superantigen induced tolerance it has recently been shown that both NO and reactive oxygen intermediates contribute to the death of activated T cells (Cauley, 2000). Such findings suggest that the negative feedback mechanism previously described may constitute a mechanism of peripheral down-regulation during intense immune responses.

The results presented here demonstrate that a similar mechanism of down-regulation is induced by filarial infection as $CD4^{+}$ T cells from mf-infected mice are the major source of IFN-γ and thus indirectly trigger their own NO mediated apoptosis. It

would be of interest to determine whether a similar mechanism is operative *in vivo*. Circumstantially, enhanced proliferation of cells from mf-infected iNOS-/- mice at early time points in culture (as shown in Figure 4.1) suggests that they may develop or maintain higher frequencies of Ag-reactive T cells than their wild type counterparts. Elevated levels of apoptosis in the spleens of mf-infected mice also suggest that this may be the case, although there is currently no evidence suggest this is linked to NO production (Jenson et al submitted). Most intriguing however are the results of super-infection experiments which showed that restimulation *in vivo* decreased the magnitude of the IFN-γ response (5.2.5.ii). It is also interesting to note that repeated immunisation with mf-extract has also previously been shown to down-regulate Th1 responses (Pearlman, 1993b).

NO mediated down-regulation of pro-inflammatory responses may be of particular importance in the face of high antigenic loads wherein such responses are more likely to be damaging. In this sense mf, which may be present at very high levels in the circulation of infected individuals, represent a considerable challenge. It is known that the presence of mf is associated with suppression of IFN-γ production in human filariasis. Treatment with drugs which are largely microfilaricidal in nature (DEC/Ivermectin) restores both IFN-γ and proliferative responses in some infected individuals (Lammie, 1992). Furthermore IFN-γ production has been shown to correlate inversely with mf-density (Sartono, 1999). Such results suggest that Th1 responses are enhanced upon reduction of the antigenic load. A similar pattern of responsiveness is seen in bancroftian filariasis where Th1 responses dominate amongst antigen-negative individuals (Dimock, 1996). It would be of considerable interest to determine whether or not NO mediated apoptosis of IFN-γ producing cells occurs during human infection, and how, if at all, this may contribute to maintenance of the clinically asymptomatic state seen amongst microfilaraemic individuals.

CHAPTER 7

Final discussion

Lymphatic filariasis is a long term chronic infection characterised by a Th2 dominated immune response and suppressed Ag-specific proliferation. While helminth infections are generally associated with development of Th2 responses it appears increasingly unlikely that strict application of the Th1/Th2 paradigm reflects the vagaries of natural responses (reviewed Allen, 1997). IL-4 producing cells are greatly expanded in L.F. but IFN-γ producing cells are also primed during infection and may be silenced under the influence of IL-10 (King, 1992/93) or other as yet unidentified factors. Ag-specific proliferative responses and IFN-γ production are most profoundly suppressed in individuals with circulating microfilariae. This is reflected in the fact that Mf+ individuals have a lower precursor frequency of Ag-specific T cells, and in particular IFN-γ producing cells than patients displaying chronic pathology (King, 1992). Such observations have long suggested an important role for mf in generating proliferative suppression and limiting development of Th1 responses.

Whilst there is a lack of good longitudinal data, the differential responsiveness seen amongst clinical groups suggest that the host/parasite relationship is highly dynamic and the immune response is likely to reflect this with both qualitative and quantitative changes over the course of infection. Murine models of infection have facilitated laboratory study and identified a variety of mechanisms by which filarial worms may influence the development of host responses. In particular the use of single stage infections has allowed the identification of stage-specific immunomodulatory mechanisms and revealed that infection with different life cycle stages elicits development of differentially polarised responses. Most intriguingly, it has been shown that mf, a life cycle stage

associated with the most profound suppression of IFN-γ production in human infection, uniquely elicit development of a Th1-like response in mice (Lawrence, 1994). The ability of mf to elicit IFN-γ production may appear counter-intuitive given the association between mf and suppression of IFN-γ production in infected humans. However the results presented in this study demonstrate that the IFN-γ response induced by mf is self-limiting *in vitro,* resulting in suppression of both proliferation and IFN-γ production and suggesting one way in which the apparent dichotomy between mf-induced IFN-γ responses in man and mouse may potentially be resolved.

Upon *in vitro* restimulation, $CD4^{+}$T cells from mf-infected animals produce large quantities of IFN-γ leading to production of NO which in turn induces the apoptotic death of Ag-reactive T cells. There is now a growing body of evidence to suggest that the IFN-γ induced elimination of activated T cells represents an important self-regulatory mechanism in both infectious and autoimmune disease (Cauley, 2000; Dalton, 2000; Gilbertson, 1999; Tarrant, 1999). This mechanism of down-regulation is particularly associated with strong IFN-γ responses and/or high antigenic loads. Following infection with *M. avium* loss of IFN-γ production (associated with high levels of T cell apoptosis), does not occur until bacterial numbers are greatly expanded suggesting that a threshold of stimulation may exist beyond which IFN-γ production cannot be sustained (Gilbertson, 1999). While circulating mf clearly present a large antigenic challenge, the ability of mf to induce IFN-γ production within the infected human is questionable. Single stage infection with mf is a uniquely experimental situation, as in natural infections exposure to L3, developing larvae and adult worms precedes exposure to mf, suggesting that Th2 responses are likely to be pre-established. Interestingly however, PBMC from infected humans, and splenocytes from infected BALB/c mice with an established Th2 response, retain the ability to respond

to IL-12 with IFN-γ production (Mahanty, 1997; Pearlman, 1995). Following intra-peritoneal implantation of adult female worms which continually produce mf, IFN-γ production by splenocytes is only observed in IL-4-/- mice (Lawrence, 1995). Interestingly however, following implantation of adult male worms, i.v. infection with mf induces an IFN-γ response (Lawrence, 2000). Whilst the differences in these results may suggest that adult females are more suppressive than adult males they are also likely to reflect the effects of implantation upon APC in different locations. Following implantation of adult females, mf are restricted to the peritoneal cavity wherein it is known that adult worms exert profoundly suppressive effects upon APC function (Allen, 1996). Intravenous administration of mf more closely resembles the natural situation, wherein adults and mf inhabit distinct locations, and these results suggest that mf may induce IFN-γ production in the face of an established anti-filarial Th2 response.

Unlike murine Th2 cells, human Th2 cells retain expression of the IL-12β2 receptor and can respond directly to IL-12 with increased production of both IL-4 and IFN-γ suggesting that there may be a greater degree of plasticity in human as compared to murine responses (Hu, 1999). Although not addressed directly in this study, the IFN-γ response seen following mf infection of both wild type and IFN-γR-/- mice strongly suggest that mf are potent inducers of IL-12. Furthermore using *in situ* immunocytochemistry IL-12 has been detected in the spleen, in the immediate vicinity of mf, shortly after infection (R. Lawrence pers. Comm.). Interestingly IL-12 has been shown to enhance production of IgG4 by PBMC from infected individuals (De Boer, 1997). IgG4 is an indicator of active infection (Kwan-Lim, 1990), production of which correlates inversely with lymphocyte responsiveness (Yazdanbaksh, 1993) and is seen at highest levels amongst Mf+ individuals (Hussain, 1987). Whilst IL-12 is not a recognised

switching factor for IgG4 it may act to enhance production initially elicited by adult induced IL-4. These results suggest that it may be worthwhile investigating IL-12 production amongst Mf+ individuals.

It has recently been shown that high doses of Ag promote development of Th1 responses by upregulating expression of CD40 on T cells and its ligand CD40L on dendritic cells (Ruedl, 2000). Ligation of CD40 induces high level IL-12 production by DC and upregulates their expression of ICAM-1 (Cella, 1996) which further promotes Th1 development via its interaction with LFA-1 (Ruedl, 2000). Whilst IL-12 promotes Th1 development, at high levels it displays immunosuppressive effects, mediated via the IFN-γ induced production of NO (Kurzawa Koblish, 1998; Lasarte, 1999; Tarrant, 1999). It is of interest to note that repeated immunisation with mf extract or infection with high doses of mf is associated with downregulation of IFN-γ production in mouse models (Lawrence, 1994; Pearlman, 1993b). In this sense it appears that suppression of IFN-γ production is dependent upon chronic infection or repeated stimulation. In this study the results of super-infection experiments demonstrated that *in vivo* restimulation following secondary infection, with either mf or L3, significantly reduced *in vitro* IFN-γ responses, further suggesting that repeated stimulation is associated with down-regulation of IFN-γ production. Although the mechanisms underlying this phenomenon are currently unknown, apoptosis of Ag-reactive IFN-γ producing T cells maybe one potential explanation. Interestingly it has been shown that a faster and greater loss of Ag-reactive T cells occurs *in vivo* following repeated administration of SEB than following a single dose (Leonardo 1991). It is known that the decrease in numbers of $V\beta8^+$ T cells seen following injection of SEB is associated with the apoptotic death of Ag-reactive lymphocytes, a response which is enhanced by repeated restimulation. In the light of these results it is

interesting to note that when mf-infected animals are subsequently re-infected with live mf a secondary, more extensive wave of apoptosis is observed in the spleen (J. Jenson pers. comm.). It would be of interest to determine the effects of this on *in vitro* cytokine and proliferative responses. The apoptosis observed in the spleen at 12 d.p.i. (Jenson et al submitted) is clearly not associated with a complete loss of Ag-reactive IFN-γ producing T cells as indicated by responses at 30 d.p.i. However as suggested by super-infection experiments and the *in vitro* results it may be that restimulation is necessary to achieve downregulation of IFN-γ responses. It appears unlikely that downregulation of IFN-γ production is associated with increased numbers of dead mf as it has been shown here and elsewhere (Lawrence, 2000) that dead mf do not inherently induce Th2 responses.

The results presented in this thesis raise several interesting questions regarding *in vivo* responses during murine infection with mf. Particularly in relation to the $CD4^{hi}$ population of proliferating cells, expansion of which is limited *in vitro* by NO. Does a similar population develop following *in vivo* restimulation? If not, is expansion limited in an NO-dependent fashion as seen *in vitro*? Further experiments in IFN-γR-/- mice which are unable to respond to IFN-γ with MO activation and NO production would be necessary to explore these possibilities. Further definition of the properties of these cells in terms of cytokine production and activation status would also help to establish their functional significance. In the light of the data assembled here the fate of Ag-reactive $CD4^+$ T cells following *in vivo* restimulation is of considerable interest. Adoptive transfer of CFSE labelled $CD4^{hi}$ T cells, derived from *in vitro* culture, into infected / uninfected mice potentially offers insight to this intriguing area. It would also be of interest to investigate the effect of *in vivo* restimulation on numbers of activated ($CD44^{hi}$) T cells and to

determine whether NO has any influence upon their expansion (i.e. IFN-γR-/- vs wild type).

Both murine and human studies have shown that in certain circumstances IFN-γ production maybe suppressed under the influence of IL-10 (Mahanty, 1995; Oswald, 1992b; Pearlman, 1993b) suggesting that not all IFN-γ producing cells are lost during infection. Interestingly a study on the induction of peripheral tolerance indicated that apoptosis resistant Ag-specific T cells produced high levels of IL-10 (Zhang, 1996). Furthermore these cells failed to proliferate in response to subsequent restimulation and were capable of suppressing proliferation of other Ag-specific cells, a situation reminiscent of that seen in filariasis (King, 1993). Additionally, high level IL-10 production is a characteristic of the recently described Tr1/Th3 subset of regulatory T cells (Groux, 1997). Intriguingly T regulatory cells have recently been isolated and cloned from an individual with patent *O. volvulus* infection demonstrating their potential importance in filarial infection (Doetze, 2000).

A recent study, following lymphocyte responsiveness amongst *B. malayi* infected individuals over time, demonstrated that IFN-γ production fluctuates most markedly in response to changes in parasite density whilst levels of IL-4 remain relatively stable (Sartono, 1999). This situation reflects the previous reports which have indicated that while levels of IL-4 production are comparable between clinical groups, IFN-γ production is lowest amongst microfilaremics (Dimock, 1996; King, 1992). It is now known that production of IL-4 by $CD4^+$ T cells requires several rounds of cellular division which act in concert with cytokine signalling to relieve epigenetic constraints upon IL-4 expression. In contrast IFN-γ expression increases in frequency with successive cell divisions (Bird, 1998). Furthermore whilst IL-4 production by differentiated $CD4^+$ T cells is cytokine

autonomous, IFN-γ production is highly cytokine regulated under the influences of IL-4, IFN-γ and IL-12 (Hu-Li, 1997). Thus production of IFN-γ may be more susceptible to modulation by such environmental factors than that of IL-4. This may, in part, account for its increased responsiveness to changes in parasite density. An alternative explanation may be that adult worms provide the main stimulus for IL-4 production whilst changes in IFN-γ production are more reflective of the levels of circulating mf and consequently subject to more dynamic fluctuations.

Most data from human infections have been generated using *in vitro* restimulation of PBMC with crude parasite extract and consequently are likely to reflect the influence of various mechanisms of immunomodulation. Whilst there has been no assessment of whether or not the proliferative defect seen in human infection is associated with elevated levels of apoptosis, the data presented here suggest this is an area worthy of investigation. As outlined above it is difficult to ascertain whether mf elicit IFN-γ production in human infection and whether such a response could be down-regulated by elimination of IFN-γ producing T cells as described here *in vitro*. It is conceivable however, that such a mechanism maybe operable in human infection and may serve to compound the Th2 response in the periphery and prevent expansion of a potentially damaging pro-inflammatory response. Consequently it would be of interest to assess levels of lymphocyte apoptosis during human filarial infection both *ex-vivo* and following *in-vitro* restimulation. It has recently been shown that PBMC from *S. mansoni* infected individuals are capable of producing NO which displays anti inflammatory effects upon granuloma formation (Oliveira, 1998). Such findings suggest that NO may influence development of immune responses in human helminth infection. The results presented here also demonstrate a regulatory role for NO in limiting T cell responses. Whether or not the NO

dependent mechanism of apoptosis described here in the mouse model is relevant to human infection, inducing the apoptotic death of Ag-reactive T cells remains an attractive means of achieving peripheral tolerance and its potential role in human infection warrants investigation.

Murine models have clearly demonstrated that there are a multitude of ways in which filarial worms can modulate host immune responses, several of which may be stage and/or site specific. In human infection the immunological situation is far more complex, influenced by various life cycle stages, in distinct locations, in varying numbers, at different times. The responses observed in *in vitro* studies are likely to represent the net result of various mechanisms of immune modulation operative *in vivo*. The onset of patency is a pivotal point in infection beyond which immune evasion strategies must be more widely implemented. Following mf-production the parasite is unlikely to rely on suppression in the immediate environment of the lymphatics to protect its assets in the periphery. Indeed the establishment of a circulating population of mf is the realisation of functional filarial infection and, as may be expected, is associated with the most profound form of immunosuppression. It appears likely that stage-specific effects play distinct yet complementary roles in immunomodulation, as suggested by studies in murine models. In this way L3 may initially induce development of Th2 responses, and adult worms create a profoundly suppressive effect in the immediate environment of the lymphatics, whilst mf additionally suppress proliferative and IFN-γ responses throughout the periphery.

The results presented in this study add the IFN-γ induced elimination of Ag-reactive T cells to the list of immunomodulatory mechanisms which may be employed by filarial worms. Whilst "fighting fire with fire" in this way may appear a convoluted means of downregulating responses, host defence and parasite immune evasion strategies have evolved in intimate association during which any potential weakness may be exploited.

Hijacking mechanisms of suppression, which may be more commonly associated with the down-regulation of autoimmune responses, in order to induce what effectively resembles a state of peripheral tolerance would be an attractive stratagem to facilitate development of long term chronic infection. In this sense the potential ability of mf to employ host mechanisms to eliminate Ag-reactive T cells may represent the implementation of an intimate knowledge of host biology.

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