Immune modulation by the parasitic nematode *Brugia malayi*

A thesis submitted for the degree of Doctor of Philosophy of the University of Edinburgh

By

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Abstract

The establishment of parasitic helminth infection is characteristically accompanied by a Th2-dominated host immune response. This is the case in human filariasis, in which long term infection with this lymphatic-dwelling nematode is accompanied by parasite-specific immunosuppression. This thesis seeks to address the mechanisms that underlie the development of the profound cellular hyporesponsiveness in filariasis, and the relationship of this to the development of Th2-type responses.

These questions were addressed using a murine model of infection with *Brugia malayi*, one of several species of parasitic nematode that are responsible for filarial disease. In this model, mice develop strong Th2 responses. In addition, adherent PEC from mice implanted with adult parasites are profoundly anti-proliferative but do not prevent antigen-specific cytokine production by T cells.

The findings in this thesis relate to both parasite and host factors involved in the development of immune suppression. Adult and infective larval parasites, but not microfilaria, generated down-regulatory host cells. The excretory/secretory products of the adult parasite were sufficient to induce the generation of PEC that block proliferation. Suppressor cell generation correlated directly with systemic IL-4 production by the host. We were able to demonstrate that there was a direct link between host production of IL-4 and the generation of suppressive PEC, as IL-4-deficient mice failed to induce proliferative block. However, implanted IL-10-deficient mice resulted in T cell suppression, indicating that IL-10 is not essential for the induction of hyporesponsiveness. Experiments using transwell membranes or formaldehyde-fixing of PEC indicated that suppression was not effected by a soluble mediator, and required cell-cell contact.

In order to investigate the identity of the suppressive cell type, we assessed the dynamics of cell recruitment to the site of infection. This showed the rapid influx of inflammatory cells to the site of infection, with marked eosinophilia and macrophage recruitment, coincident with a significant reduction in mast cell numbers. The down-regulatory cell type was identified as MAC-1^+, F4/80^-, and experiments using IL-5-deficient mice indicated that eosinophils were not responsible for suppression.

These data suggest that the mechanism of suppression exhibited by down-regulatory cells from filarial-infected mice appear to be unlike those previously described in other infectious disease systems, being dependent on host IL-4 production, and effected through a cell-contact-mediated mechanism.

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Abbreviations

APC	antigen presenting cell
BmA	adult *Brugia malayi* antigen
ConA	concanavalin A (not conalbumin)
D-NMMA	N^G-monomethyl-D-arginine
ES	excretory/secretory material
FACS	fluorescence activated cell sorter
L3	infective larvae
L-NMMA	N^G-monomethyl-L-arginine
Mf	microfilariae
NO	nitric oxide
PEC	peritoneal exudate cells

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Chapter 1: Introduction

Parasitic nematodes, trematodes and cestodes (helminths) are macroparasites with elaborate life cycles that typically induce chronic infections in their mammalian hosts. Helminth parasites have at their disposal an extensive range of specialised and ingenious mechanisms to facilitate long-term survival within the immunocompetent host (Maizels et al., 1993). Survival strategies include physical adaptations, such as the growth of a thick extracellular cuticle, or shedding of a protective surface coat, or can be more specialised, including secretion of proteases, molecular mimicry, and uptake of host antigens. Perhaps the most complex of avoidance mechanisms are those involved in direct modulation of the host immune system.

The establishment of parasitic helminth infection is characteristically accompanied by a host response dominated by the activation of Th2 cells and the emergence of parasite-specific immunosuppression (Allen and Maizels, 1996; Behnke et al., 1992; Finkelman et al., 1991; Maizels et al., 1993). However, the importance of the emergence of such a response in host resistance to infection, development of pathology, and parasite survival remains uncertain. Further, the mechanism(s) by which these long-lived parasites are able to so strikingly influence the host immune response are not wholly understood.

Lymphatic filariasis is a parasitic disease of humans, mainly caused by two species of helminth, the filarial nematodes *Brugia malayi* and *Wuchereria bancrofti*. Filariasis is mosquito transmitted and is a widespread condition throughout the tropics, infecting up to 120 million people (Ottesen et al., 1997). Infection typically results in high morbidity in chronically infected individuals and can cause a range of pathology, the most severe being the disfiguring condition of elephantiasis. Although dwelling at the 'heart' of the immune system in the lymphatic vessels, these parasites can survive for many years (Kazura et al., 1993). The mechanisms that are responsible for long-term survival of the parasites within the immuno-competent host, and those that cause disease and pathology, are poorly understood.

1.1 Life cycle, morphology and epidemiology (Schmidt and Roberts, 1996; Zaman and Keong, 1990)

The complex life cycles of *B. malayi* and *W. bancrofti* are essentially the same (Figure 1). Infective third stage larvae (L3) enter host tissues via the bite wound at the site of the mosquito blood meal. These larvae then enter the blood stream and migrate to the afferent lymphatics, where they moult and mature to become dioeceous adults. Infection is chronic in nature and adults can live for over 10 years in the lymphatics, apparently unaffected by the immune system of the host. Around three months after infection female worms start producing large numbers of sheathed first larval stage microfilariae (Mf) into the peripheral blood stream. When the circulating Mf are ingested by an appropriate species of mosquito, they penetrate the thoracic musculature where they undergo two successive moults to become infective stage larvae. These then emerge from the mouthparts during feeding to enter the mammalian host.

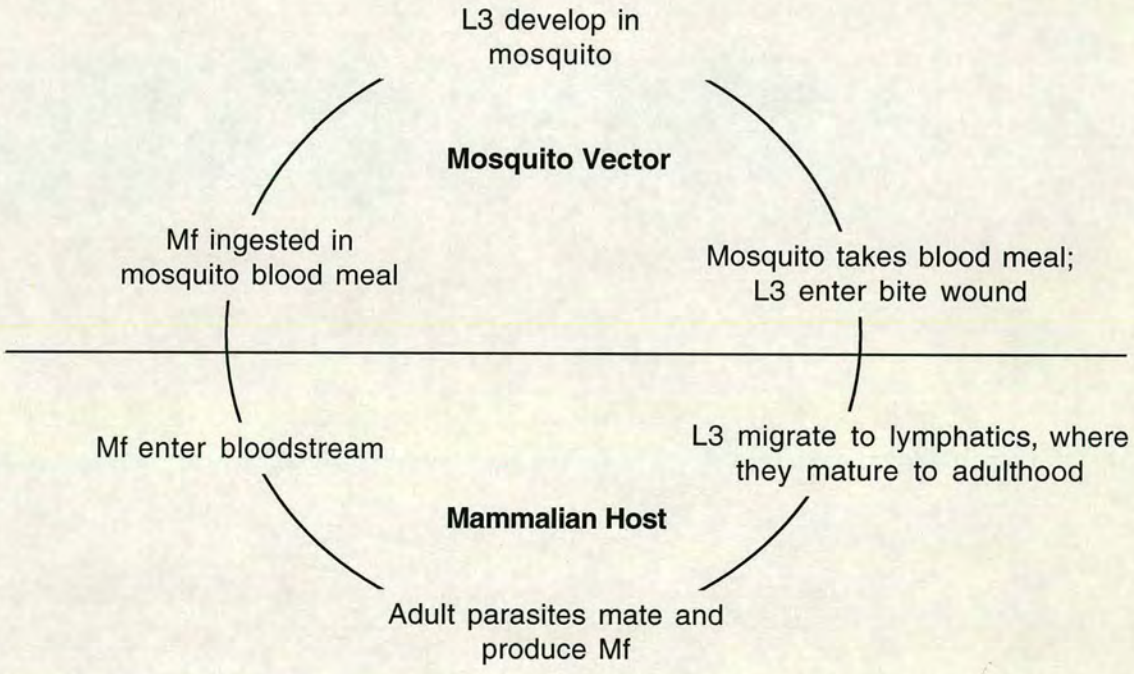


Figure 1. Life cycle of *B. malayi* and *W. bancrofti*. Abbreviations used; infective larvae (L3) and microfilariae (Mf). See body of text for life cycle details.

Adult worms are elongated and slender. In *B. malayi*, the male measures 10 to 20 mm long and 70 to 80 μm wide. The females are 30 to 50 mm long and 100 μm wide. *W. bancrofti* is similar to *B. malayi* but somewhat larger. Species of filarids are generally differentiated by morphological differences between the microfilariae, particularly tail shape and arrangement of nuclei in the tail. The presence or absence of microfilariae in the peripheral blood of the mammalian host varies depending on the feeding times of the species of mosquito vector in a given geographical area.

Many species of mosquito can serve as intermediate hosts for filarial nematodes, including the genera *Anopheles*, *Aedes*, *Culex*, and *Mansonia*. *W. bancrofti* is the most widely distributed filarial infection, occurring in the Far East, major portions of Africa, and South and Central America. *B. malayi* is less widespread being restricted to the eastern regions of India and to Southeast Asia. *W. bancrofti* has no known animal reservoir, whereas *B. malayi* can parasitise a more diverse range of animals, including dogs, cats and monkeys.

1.2 Diagnosis, pathology and treatment (Schmidt and Roberts, 1996; Zaman and Keong, 1990)

The most common method of confirming filarial infection is to find the microfilariae in the peripheral blood, for which there are several techniques available. Blood for analysis is taken at the appropriate time of day or night depending on the periodicity of the infection. More recently, the presence of circulating IgG4 antibody to filaria has been suggested as a marker of active infection (Kurniawan et al., 1993).

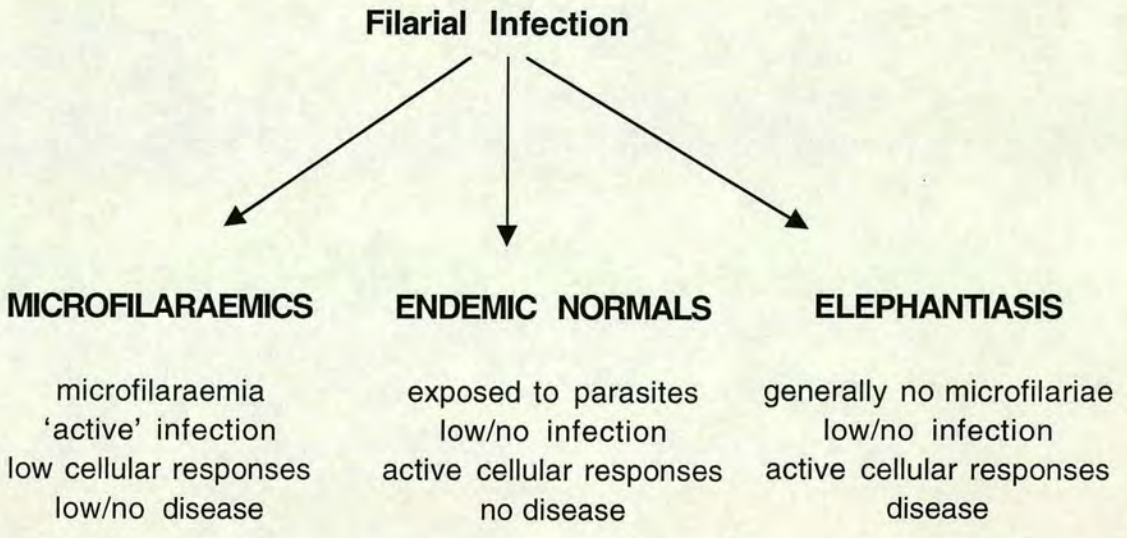
Pathogenesis can be separated into three clinical phases: the incubation stage, the acute stage, and the chronic stage. The incubation stage is generally symptomless, and represents the time between infection and the appearance of microfilariae in the blood. There may be mild fever with transient lymphatic inflammation. The acute stage of the disease is related to the development of the worms in the lymphatics and the first release of microfilariae. Infected individuals may have inflammation of the lymphatic vessels (lymphangitis) and of a lymph node or lymph nodes (lymphadenitis). Lymphangitis can

sometimes be severe and result in high fever and headache, occasionally requiring hospitalisation. Inflammation of the testis and epididymis may also occur at this stage. These symptoms usually subside after a few days, but may recur.

It is the chronic stage of infection that causes the greatest amount of pathology. It may take several years from the time of infection for chronic disease to be manifest. This phase is marked by dilation of the lymph vessels (varices), oedema and lymph in the urine (chyluria) and can ultimately lead to the disfiguring condition known as elephantiasis. There is commonly a decline in microfilaraemia associated with this stage, and often individuals with extreme chronic pathology have a total absence of circulating larvae.

Classically, people in an endemic area are separated into three distinct groups based upon clinical condition and level of active infection (Figure 2): (1) Microfilaraemics; (2) those with elephantiasis; and (3) 'endemic normals' (Ottesen, 1989; Yazdanbakhsh et al., 1993). Microfilaraemics typically have high parasite burdens, down-regulated cellular responses and minor pathology. Individuals with elephantiasis (and other chronic pathology related to infection such as oedema) generally harbour few parasites and have active cellular responses, with vigorous specific immune reponses. 'Endemic normals' are those individuals that are exposed to infection in an endemic area but display no outward sign of infection, also sometimes termed 'asymptomatic amicrofilaemics'. Generally, these individuals also display active cellular responses to the parasite.

Figure 2. The spectral nature of filarial disease.



Classification of individuals in an endemic area into these three groups is an oversimplification of what is actually a complex range of infection states without obvious boundaries of definition (Maizels et al., 1995). This simplification may neglect to include individuals such as those that harbour low level parasitemia, or those that have recently cleared infection. Indeed, the use of new techniques such as ultrasound to identify adult worms in the absence of detectable circulating microfilaria (Dreyer et al., 1996), shows that amicrofilaramic individuals may harbour active infection. Such information reinforces the hypothesis that the perception of the endemic normal as a resistant, infection-clear individual does not adequately reflect the true situation (Day, 1991).

Programmes to reduce the transmission of filarial disease combine vector control and drug treatment strategies (Ottesen and Ramachandran, 1995). Diethylcarbamazine citrate (DEC) is a highly effective human treatment, killing both adult and microfilarial life cycle stages of the parasite, although adverse side effects commonly result from this treatment, which may be due to release of high levels of antigen from dying parasites (Maizels and Denham, 1992). Single or co-administration of DEC with the microfilaricide ivermectin, or with albendazole, has proved highly effective at reducing parasitaemia (Ottesen et al., 1997). Based on this, the WHO has recently targeted filarial infection as a priority for eradication within the near future. However, recent examples of emerging drug resistance to other helminth diseases (such as praziquantel in schistosomiasis (Fallon et al., 1996)), have provided a blunt warning that DEC treatment, although effective for the moment, may not remain so indefinitely.

For many years researchers have been questing for a vaccine to this highly prevalent disease, spurred on by the established efficacy of vaccination with radiation attenuated infective larvae in permissive animal models (Oothuman et al., 1979; Wong et al., 1969), and the establishment of protective immunity through 'trickle' infection with infective larvae (Denham et al., 1992). As yet, no suitably effective vaccine has emerged from such studies (Selkirk et al., 1992).

1.3 Immunology of human filarial infection

A striking feature of active filarial infection (as indicated by the presence of circulating microfilariae) is the induction of antigen specific hypo-responsiveness in the proliferative reaction of host T cells to the parasite (Maizels et al., 1995). This has been consistently demonstrated in human studies (Ottesen et al., 1977; Piessens et al., 1980; Yazdanbakhsh et al., 1993), where peripheral blood mononuclear cells (PBMC) taken from microfilaraemic individuals fail to proliferate to filarial antigen *in vitro* but are still able to respond in an antigen specific manner as measured by cytokine production (Figure 3). Conversely, individuals with chronic lymphatic pathology, who rarely exhibit blood microfilaraemia, generally mount stronger filarial-specific cellular proliferative responses.

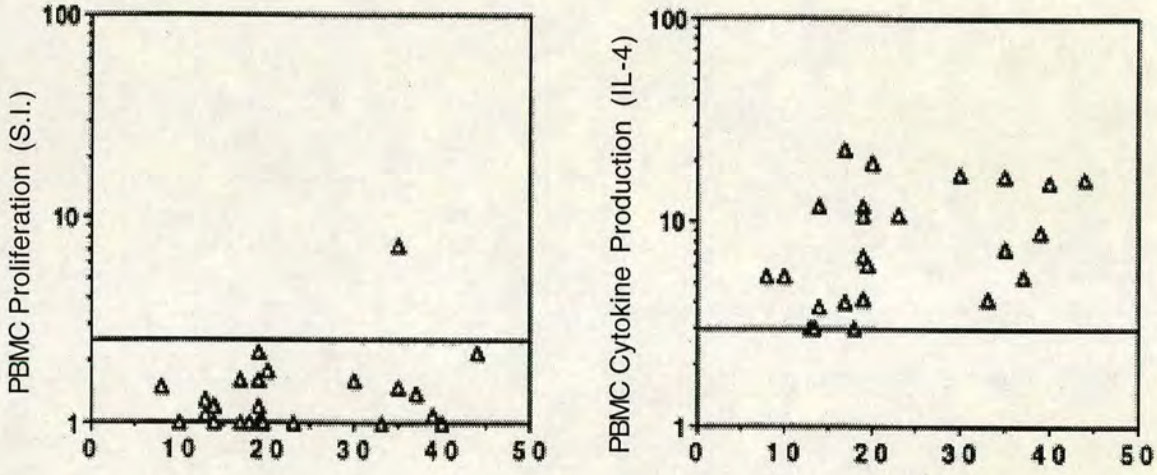


Figure 3. Key immunological features of active filarial infection. PBMC from microfilaraemic patients fail to proliferate to in response to filarial antigen *in vitro*, but still produce antigen-specific cytokine. Reproduced with permission from Sartono *et al.*, Leiden University.

The specific identity of the cell types that are involved, and the underlying mechanisms behind down-regulation of cellular proliferative responses in filarial infection, remain unknown, although previous human studies have correlated increased numbers of CD8+ T cells (Piessens et al., 1982) with hyporesponsiveness, as well as suggesting that adherent monocytes/macrophages may play a role (Piessens et al., 1980).

Epidemiological studies have indicated that hypo-responsiveness caused by filarial infection affects only antigen-specific responses, as proliferative responses to both

mitogens (such as PHA) or non-parasite antigens (such as PPD) have been shown to be similar in individuals from any of the filarial clinical groups (Yazdanbakhsh et al., 1993). However, cellular responses in patients infected with the related filarial nematode *Onchocerca volvulus* can be down regulated to both parasite and non-parasite antigens (Greene et al., 1983).

The most conspicuous antibody isotypes found in filarial infection are those that are promoted by the Th2 cytokine IL-4, specifically IgG4 and IgE (Hussain et al., 1981; Ottesen et al., 1985). The role that these infection-dominant antibodies play is still unclear. IgG4 levels, normally comprising approximately 5% of total serum IgG, can rise to up to 95% of specific antibody in microfilaraemic individuals (Kwan-Lim et al., 1990; Ottesen et al., 1985). Further, asymptomatic microfilaraemics have much higher ratios of filarial specific IgG4 to IgE than chronically infected elephantiasis patients, where the most significant antibody isotype appears to be IgE (Kurniawan et al., 1993). Indeed, high levels of anti-filarial IgE have been related to chronic pathology, particularly tropical pulmonary eosinophilia (Hussain et al., 1981). IgG4 has been shown to have anti-allergic 'blocking' activity in human filariasis (Hussain et al., 1992). Thus, chronic pathology in elephantiasis patients may be due to an increased IgE-mediated hypersensitivity response in the presence of insufficient blocking antibody, similar to the situation that has been described in human schistosomiasis (Hagan, 1992). IgG1, 2 and 3 present a different profile, in that these isotypes are most prominent in individuals with chronic pathology, and not significantly enhanced in microfilaraemics (Kurniawan et al., 1993).

1.4 The cytokine influence

The cytokine environment in which a T cell is first presented antigen by an APC may have a number of effects on the outcome of the type of response induced, and indeed the fate of that particular cell. The most elegant example of this is the division of $CD4^+$ T cells into subsets based on their cytokine profiles (Mosmann et al., 1986). The general acceptance is that Th1 cells are involved in inflammatory responses, secreting IL-2 and IFN-γ under the influence of IL-12, and that Th2 cells are more involved in humoral

responses, secreting cytokines such as IL-4, IL-5 and IL-10 (Abbas et al., 1996). Although this basic framework has proved invaluable in classification of the immune response to infection, it is crucial to avoid over generalisation and to stress that polarisation of an immune response to purely Th1 or Th2 rarely, if ever, occurs *in vivo* (Allen and Maizels, 1997). Rather, each subset represents an extreme, and a 'normal' response will incorporate elements from both. Apart from influencing the development of T cells certain cytokines, such as IL-10 and TGF-β, may exert a direct down-regulatory effect.

Interpretation of cytokine production in response to filarial infection is hampered by the complex range of infection states that are apparent in human filariasis. Analysis of cytokine secretion in response to infection generally supports the trend observed in antibody isotype profiles, with active infection being associated with down-regulation of Th1-type cytokines. In keeping with this, actively infected microfilaraemics show reduced IFN-γ and IL-2 production (King and Nutman, 1991; Sartono et al., 1997) in comparison to other clinical groups, whereas IL-4 secretion in response to parasite antigen is not diminished in actively infected individuals (Mahanty et al., 1993; Yazdanbakhsh et al., 1993). However, even though filarial infection is associated with generation of IL-4 and IL-5 producing T cells (Mahanty et al., 1993), levels of the Th2-type cytokine IL-5 are actually reduced in microfilaraemic individuals (Sartono et al., 1997). This suggests that both Th1 and Th2 responses are to some extent down-regulated during active infection.

Assessment of cellular responsiveness of patients after treatment with DEC or ivermectin has shown that clearance of adult or Mf parasites results in an increase in proliferative responses (Lammie et al., 1992; Piessens et al., 1981; Sartono et al., 1995), and decreased levels of anti-filarial IgG4 (Maizels et al., 1995). Treatment with DEC results not only in elevated proliferative responses, but also enhanced IFN-γ production in individuals from all clinical groups, whereas IL-4 production does not change significantly following therapy (Sartono et al., 1995).

Spontaneous and antigen-specific IL-10 production appear to be elevated in peripheral blood cells from infected individuals (Baize et al., 1997; Mahanty and Nutman, 1995), although the involvement of this down-regulatory cytokine in the induction of hypo-responsiveness by filarial infection remains an area of debate. Some studies support a role for IL-10 in down-regulation of Th1-type cytokine production and proliferative responses to filarial antigen (King et al., 1993; Mahanty et al., 1997), having shown that the addition of neutralising antibody to IL-10 *in vitro* can reverse proliferative block and elevate IFN-γ production. However, other studies have failed to show a role for IL-10 in down-regulation of proliferation or cytokine production by addition of netralising antibody *in vitro* (Baize et al., 1997; Sartono et al., 1995).

The presence of a pronounced blood and tissue eosinophilia has long been recognised as a characteristic feature of the immune response to helminth parasites (Brown, 1898; Finkelman et al., 1991). In human filarial infection, where blood and tissue eosinophilia can be quite dramatic (Mackenzie, 1980), pathology related to this eosinophilia is not common. Eosinophil-related pathology is seen only in rare cases, where eosinophilia directed against Mf in the lungs of infected individuals can result in severe asthmatic symptoms and the condition known as tropical pulmonary eosinophilia (TPE) (Ottesen, 1992). However, in general, high level eosinophilia appears to occur in conjunction with active infection, and in the absence of overt symptoms. Thus the role of helminth-driven eosinophilia in disease remains a topic of much controversy.

IL-5 is a Th2-related cytokine that has several roles, including regulation of B cell development, regulation of antibody production, and mobilisation and maturation of eosinophils (Kopf et al., 1996; Takatsu, 1992). In human infection, IL-5 appears to show a quite different pattern of expression than IL-4, being produced at lower levels by microfilaraemics than by asymptomatic amicrofilaraemics (Sartono et al., 1997). This is consistent with evidence for lower eosinophilia rates in microfilaria-positive as compared to microfilaria-negative patients (Wong et al., 1969). There is currently no human evidence supporting a pivotal protective role for IL-5 or eosinophils in filariasis.

1.5 Immune Tolerance

The hypo-responsiveness that is evident in filarial infection has been proposed as a form of immune tolerance towards the parasite (Maizels and Lawrence, 1991). How this tolerance is induced *in vivo* is not known, but several mechanisms could lead to such a state.

Immune responses to some self-antigens are prevented by clonal deletion of T cells in the thymus, while excessive T cell responses to other self-antigens and external antigens may be controlled by the generation of peripheral tolerance (Mondino et al., 1996). The ability to develop immune tolerance is essential for the mammalian immune system to refrain from responding inappropriately, and potentially destructively, towards self-antigens. As a mechanism by which the immune response can be 'toned down', exploitation of routes towards tolerance might provide a window of opportunity for parasitic organisms.

Activation-induced apoptosis mediated by Fas (CD95) and Fas-ligand is thought to be involved in deletion of potentially autoreactive T cells either in the thymus or the periphery (Brunner et al., 1995). Little is yet known about the regulation of Fas and Fas-ligand during filarial infection. However, it is unlikely that the hypo-responsiveness seen in filarial infection is caused by clonal deletion of parasite-specific T cells for several reasons. Primarily, although parasite-specific proliferative responses are down-regulated in infected individuals, cytokine production remains intact. Further, cellular responses of infected individuals are regained after DEC treatment (Lammie et al., 1992; Piessens et al., 1981), and there is evidence that microfilaraemics, although having predominantly suppressed parasite-specific proliferative responses, still possess very low levels of T cells that can proliferate to filarial antigen (King et al., 1992).

It is more likely that down-regulated responses to filarial infection might be the result of the development of tolerance by non-deletional means. The ever-expanding range of tolerance mechanisms that occur outwith the thymic selection process are often grouped together under the term peripheral tolerance (Cobbold and Waldmann, 1998).

Inadequate or aberrant costimulation, and the action of altered peptide ligands are two examples of the many non-deletional mechanisms that can lead to peripheral tolerance.

Complete activation of T lymphocytes requires two distinct signals, one through the TCR complex and the second in the form of a costimulatory signal (Schwartz, 1990). This signal may be provided by a soluble molecule or receptor-ligand interaction. If the appropriate signals are not provided the T cell will not respond appropriately to a given antigen, and may instead lose the ability to respond to subsequent stimulation by that antigen (Harding et al., 1992). The CD28 receptor on the T cell, and B7 molecules (CD80 and CD86) on the APC are the predominant molecules involved in costimulation, although other molecules can also be involved in this process (Freeman et al., 1993; Linsley et al., 1991). It remains to be seen if parasitic helminths have the ability to interfere with costimulatory events during infection.

A state of lymphocyte unresponsiveness can also be induced by the presence of altered peptide ligands (APL) in the T cell receptor/MHC complex. Understanding of this has stemmed from the discovery that subtle changes in T cell peptide determinants could have dramatic consequences on the subsequent activation state of T cells so stimulated, being able to render those cells anergic (Sloan-Lancaster et al., 1993). Evidence exists that APL's occur naturally, both from self-antigens and from pathogens, and at certain levels can antagonistically affect peripheral T cell responses (Vidal et al., 1996).

1.6 Immune suppression

An alternative 'tolerising' mechanism that would be applicable to self- or non-self-antigen would be the ability to directly suppress potentially reactive cells.

T cell suppression has been a controversial area of immunology for many years, and the existence of $CD8^+$ 'suppressor' cells as a specialised subset of T cells remains a matter of debate. Scepticism in the face of many examples of such suppression in a wide variety of systems has stemmed from the inability to show definitive evidence for $CD8^+$ T cell immunoregulation *in vivo*, to clone suppressor cells, or to define their mechanism

of action. However, several recent discoveries have revived flagging interest in the suppressive ability of T lymphocytes (Chen et al., 1994; Groux et al., 1997). Chen *et al.* described the generation by oral administration of antigen of TGF-β, IL-10 and IL-4 secreting $CD4^+$ T cells, that could suppress the development of experimental autoimmune encephalomyeitis (EAE) *in vivo*. In a separate system, Groux *et al.* defined a $CD4^+$ T cell subset (termed 'regulatory T cells' or Tr1) that acts through secretion of IL-10 and TGF-β to suppress antigen-specific T cell proliferation *in vitro*, and inflammatory bowel disease *in vivo*. Perhaps in fear of provoking the scepticism that has been directed towards $CD8^+$ T cells, the term 'suppressor' T cell has been superseded by 'regulatory' T cell.

For many years it has been known that macrophages, in addition to their well-known immunostimulatory abilities, are also able to down-regulate cellular responses. The presence of 'suppressor macrophages' has been demonstrated in a variety of experimental systems, including tumour-bearing animals (Alleva et al., 1994), bacterial (Saha et al., 1994) and protozoan (Schleifer and Mansfield, 1993; Sternberg and Mabbott, 1996) infections. Additionally, suppressor macrophage activity has been described in the placenta (Chang et al., 1993; Mues et al., 1989), where monocytes/macrophages are suggested to protect the developing embryo, and in the lung, where alveolar monocytes/macrophages may be involved in regulation of inflammatory disease (Herscowitz, 1985; Holt, 1986). The mode of action of these down-regulatory cells, where known, has generally been attributed to inflammatory-related mechanisms under the influence of IFN-γ. Evidence has been presented that implicates hydrogen peroxide (H_2O_2), prostaglandins, TNF-α, and particularly nitric oxide (NO), in mediating the down-regulatory capacity of 'suppressor macrophages' (Albina et al., 1991; Alleva et al., 1994; Metzger et al., 1980; Schleifer and Mansfield, 1993; Sternberg and Mabbott, 1996). In addition to monocytes/macrophages, certain non-classical antigen presenting cells, such as human keratinocytes, have also been shown to be able to inhibit T cell proliferation, by secretion of soluble factors including prostaglandins and TGFβ (Laning et al., 1997).

1.7 Animal studies of filarial infection

The ideal animal model for study of lymphatic filariasis would be one that faithfully mimics human infection, allowing the development of the full life cycle of the parasite and displaying similar pathology with an analogous immune response to that seen in human infection.

Many of the features of human filarial infection can be seen in *B. pahangi* infection of cats (Denham et al., 1992) or dogs (Snowden and Hammerberg, 1989). Such models have been particularly valuable in investigating pathogenesis of disease and development of resistance. However, lack of reagents, as well as the ethical and monetary issues of using large mammals for research purposes, have imposed limitations on the study if the immune response to filarial infection in cats and dogs.

In the absence of an ideal animal model, alternative systems have been developed to allow more detailed investigation of the immunology of filarial infection. Fortunately, it is relatively easy to maintain a supply of *B. malayi* by maintenance of infection in the laboratory in inbred *Meriones unguiculatus*, the mongolian jird (gerbil). The jird provides a permissive rodent host for filarial infection (Philipp et al., 1984), possibly due to a defect in the ability of these outbred rodents to produce NO in response to filarial infection (Nasarre et al., 1998). *W. bancrofti* is less suitable for study due to the inability of this species to survive outside of its primate human host.

Observations using the jird model have noted the ability of *Brugia* spp. to down-regulate both antigen-specific, and non-specific, proliferative responses (Lammie and Katz, 1983; Lammie and Katz, 1984(a); Lammie and Katz, 1984(b); Portaro et al., 1976). Studies in this system have demonstrated that an adherent population of cells is integral to blocking antigen-specific and non-specific proliferative responses (Lammie and Katz, 1983; Portaro et al., 1976; Prier and Lammie, 1988). The mechanism of suppression in this system is unknown but, given the defective ability of macrophages from *Brugia*-infected jirds to produce NO (Nasarre et al., 1998), it is unlikely that this mediator is responsible for down-regulation of proliferation in the jird infection system.

Interestingly, maintenance of down-regulated cellular responses is not dependent on Mf (Bosshardt et al., 1995), despite the fact that suppressed responses are generally not seen until after the onset of patency (Lammie and Katz, 1983; Soboslay et al., 1991).

Studies in the jird have also helped characterise the nature of the cellular response to filarial infection, with the emphasis being on analysis of granulomas that are formed in the lymphatics after subcutaneous infection (Rao et al., 1996). More detailed investigation of the specific cytokines and cell types involved in filarial down-regulation of host proliferative responses in jirds has been limited by a lack of immunological reagents, and the fact that these rodents are only available outbred.

Although immunocompetent mice do not support the full developmental cycle of *Brugia* spp. (Howells et al., 1983), single parasite stages can survive for many weeks when implanted into the peritoneal cavity. Adult male and female *Brugia* implanted into the peritoneal cavity of BALB/c or CBA/Ca mice can survive for at least three months (Suswillo et al., 1980). Mf alone can survive for at least 4 weeks after injection into the murine peritoneal cavity (Rajasekariah et al., 1988). The L3 stage of *B. malayi* does not appear to readily survive in the murine peritoneal cavity, where it appears to die at around ten days post-implant (Carlow and Philipp, 1987).

The use of murine infection models has provided a wealth of information about many areas of the immunology of filariasis and in doing so has helped provide clarity and new direction for human studies (Lawrence, 1996). In common with many helminth species, and consistent with observations from human studies, *Brugia* infection of mice has been shown to induce a strong Th2-type response characterised by elevated production of the archetypal Th2-type cytokine IL-4, marked eosinophilia, and high levels of IgE (Bancroft et al., 1993; Lawrence et al., 1995; Lawrence et al., 1994). Moreover, infection of mice with single life cycle stages has dissected the immune response to filariae further and revealed that adult and L3 parasites are the driving force for Th2-polarisation through induction of IL-4 (Lawrence et al., 1995; Osborne et al., 1996). In contrast, live Mf infection in the absence of adults or L3, initially induces a Th1-type response (Lawrence et al., 1994). This Th1 response is only evident in the unnatural

situation of infection with Mf alone, being normally eclipsed by the dominant Th2-type response to the other life cycle stages. Although the response to Mf alone is initially Th1-type in nature, a Th2-type response may eventually develop as a result of chronic exposure (Lawrence et al., 1994; Pearlman et al., 1993). As in human infection, it is not clear the degree to which Th2-skewing of the immune response caused by filarial infection might affect host cellular responsiveness.

Although a definitive pattern of antibody isotype production has been shown for adult, L3 and Mf life cycle stages in mice, the role of antibody in murine protection against filarial infection remains ambiguous. Antibody is apparently not essential for murine resistance to infection, as B cell deficient mice are able to effectively clear L3 parasites (Rajan et al., 1995). However, several studies have implicated an important role for host production of antibody, in particular IgM (Lawrence and Denham, 1992; Thompson et al., 1981) and IgG2a (Kazura and Davis, 1982), in clearance of Mf. A critical role for T cells in murine resistance to filarial infection has been demonstrated by the fact that infection of athymic (Suswillo et al., 1980) or severe combined immunodeficiency (SCID) (Nelson et al., 1991) mice increases parasite survival and allows L3 to development into patent adult parasites.

Although the antibody and cytokine response to filarial infection has been investigated in murine models, the jird is the only rodent that has been used to assess down-regulation of cellular responses in filarial infection. However, filarial nematodes are not the only parasitic helminth species that have been shown to be able to down-regulate host cellular responsiveness. Excretory-secretory antigen from the trematode *Fasciola hepatica* directly inhibits the mitogenic proliferation of rat splenocytes (Cervi and Masih, 1997). Similarly, secreted products of the cestode *Taenia multiceps* have been shown to be able to generate an adherent population of cells capable of inhibiting lymphocyte proliferation (Rakha et al., 1991). It is not known whether these helminths share common mechanisms of immune modulation.

In addition to experiments that have concentrated on the ability of live parasite infection to generate down-regulatory host cells, several studies have concentrated on the ability

of extracts or secreted products of helminth parasites to directly inhibit lymphocyte proliferation *in vitro*. This seems an attractive mechanism by which the parasite could actively down-regulate responses in the local environment, and examples of secreted parasite molecules have been identified in sera from infected individuals (Lal and Ottesen, 1989).

One candidate to emerge from these studies is phosphorylcholine (PC), a molecule that is found in abundant quantities in the circulating antigen profiles of filarial patients. PC has been shown to be able to directly and non-specifically inhibit lymphocyte function in a number of different *in vitro* studies (Harnett and Harnett, 1993). This seems to be an inherent property of the PC molecule itself, as substitution of PC bound to BSA for parasite-derived PC had an equivalent down-regulatory effect (Lal et al., 1990). More recently, a filarial-derived antigen with homology to cystatin C, a cysteine protease inhibitor that belongs to the cystatin superfamily, was found to be able to non-specifically down-regulate T cell proliferation (Hartmann et al., 1997). Perhaps more interestingly, this effect coincided with enhanced IL-10 production. Homologous antigens have been found in *B. malayi* (Gregory et al., 1997) and *O. volvulus* (Lustigman et al., 1992).

Animal models have staunchly reinforced the clinical observations of human helminthiases, with a wide range of systems illustrating the potent ability of these diverse parasites to induce eosinophilia (Finkelman et al., 1991). Development of transgenic mice has revealed that promotion of expression of IL-5 results in the development of persistent eosinophilia (Dent et al., 1990; Tominaga et al., 1991), whereas mice deficient in production of IL-5 fail to appropriately develop blood and tissue eosinophilia under stimulating conditions (Kopf et al., 1996). There is circumstantial evidence for a role for eosinophils in larval and Mf killing of several species of helminth *in vitro* (Mackenzie, 1980). Use of IL-5-deficient mice, IL-5 transgenic mice, and neutralising antibody, has generally failed to provide convincing evidence that eosinophils play a defensive role against helminth infection *in vivo* (Kopf et al., 1996; Sher et al., 1990; Takamoto et al., 1997). However, two exceptions to this have been reported. In a murine model of infection with *Strongyloides venezuelensis*,

neutralisation of IL-5 had no effect on faecal egg output or intestinal worm burden, although the eosinophil count in peripheral blood was reduced. However, after re-infection, treated mice did not expel worms as effectively as non-treated mice, implicating a role for IL-5 (and therefore eosinophils) in generation of host protective immunity to this nematode (Korenaga et al., 1991). Similarly, a murine model of infection with the filarial nematode *Onchocerca lienalis* has shown higher levels of parasite recovery from animals treated with neutralising antibody to IL-5 (Hogarth et al., 1998). Thus, based on current evidence, the protective role of eosinophils in helminth infection is unclear and requires further investigation.

With no defined role in parasite killing, the fact that there is such a pronounced relationship between helminth infection and eosinophilia suggests that these cells must play some other role in infection. It has become apparent that eosinophils might exercise a more subtle effect on shaping the attending immune response, rather that through direct killing mechanisms. A potential immunoregulatory role for eosinophils is supported by the expanding repertoire of cytokines that both human and murine eosinophils have been shown to be able to be produce, in addition to their ability to function as antigen presenting cells, having the capacity to phagocytose and express adhesion molecules and MHC class II (Wardlaw et al., 1995; Weller, 1994). In helminth infection, class II MHC expression has been shown on eosinophils isolated from the peritoneal cavity of mice infected with the cestode *Mesocestoides corti* (Del Pozo et al., 1992), or with the nematode *B. malayi* (Mawhorter et al., 1993). Additionally, eosinophils have been implicated as a source of early production of IL-4 in response to the trematode *Schistosoma mansoni* (Sabin and Pearce, 1995).

1.8 A murine model for investigation of filarial hyporesponsiveness

The work detailed in this thesis is based on a murine model of filarial infection that was developed in order to try to identify the processes that could lead to the profound form of immune modulation evident during filarial infection (Allen et al., 1996). The premise behind development of this model was to investigate the possibility that filariae down-regulate host immune responses by interfering with effective antigen presenting

cell function. Initially, the approach used to address these questions was to co-culture live parasites *in vitro* with a source of APC and a T cell responder population, but the parasites did not directly inhibit proliferation of T cells, nor did they appear to alter the ability of APC to function effectively. It was therefore decided to take an *in vivo* approach to investigate how filariae modulate host antigen presentation processes.

Mice were implanted in the peritoneal cavity with adult *B. malayi* and the ability of adherent peritoneal cells to present antigen *ex-vivo* was evaluated. In order to delineate the functional role of parasite-exposed APC, the response to a defined model antigen was evaluated, rather than assessment of parasite-specific responses. It was found that when live but not dead parasites were used, the ability of the D10.G4 Th2 cell clone to proliferate in response to its cognate antigen (conalbumin) was completely blocked. However, levels of antigen-specific cytokine production were intact, and even elevated, relative to control PEC indicating that there was no defect in antigen processing. When low numbers of infection-derived PEC were mixed with an excess of normal irradiated splenocytes profound proliferative suppression was still observed. This demonstrated that suppression was an active process and not due to an absence of co-stimulatory molecules or reduced numbers of effective APC. However, differences were seen between the ability of adults or Mf to generate down-regulatory host cells, consistent with the contrasting cytokine responses induced by these life cycle stages. Adult parasites, which induce elevated production of IL-4, generated potent suppressive host cells whereas Mf, which induce IFN-γ, generated only partially suppressive PEC. Further, Mf-related suppression was significantly reversed on the addition of NO inhibitors into culture, whereas adult parasite-mediated suppression was not. This is consistent with the known role of IFN-γ in promotion, and of IL-4 in inhibition, of NO production (Al-Ramadi et al., 1992; Ding et al., 1988). Moreover, while suppression caused by adult parasites could apparently be transferred with supernatants from cultured PEC, this was not due to soluble factors typically associated with 'suppressor macrophages' such as prostaglandins or nitric oxide. Additionally, the anti-proliferative effect was shown not to be antigen-specifc, and to be able to affect a range of cell types, including B cell hybridomas and a colon carcinoma cell line, in addition to T cells. The

observations made in this study, although not identical to what is seen in human filariasis, were sufficiently provocative to warrant further investigation.

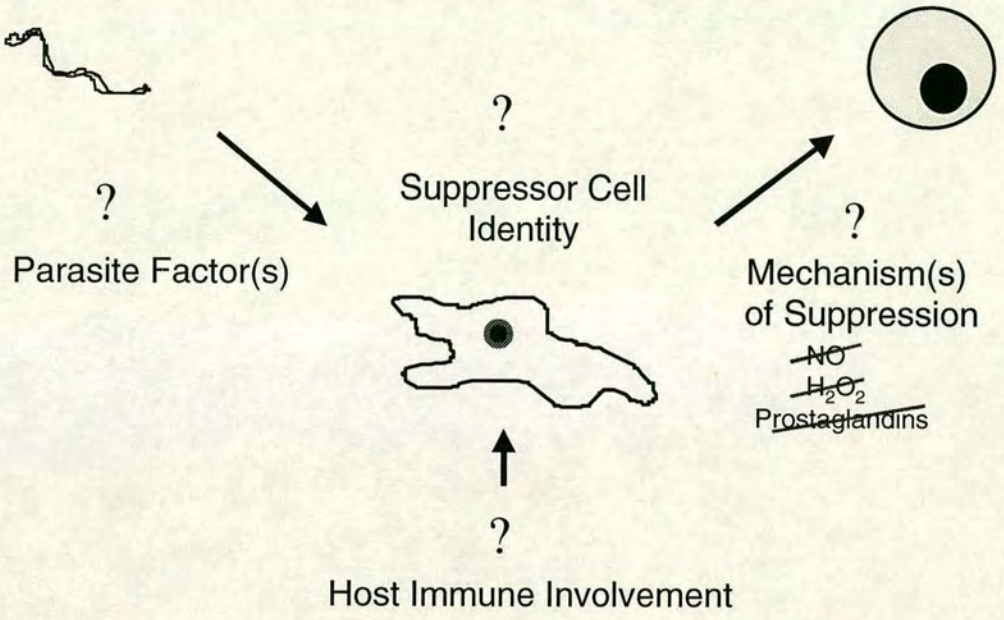


Figure 4. Key areas to be investigated. The initial study by Allen *et al.* raised a number of important unanswered questions about the factors involved in generation of suppressive PEC, and the mechanism by which they effect proliferative block.

1.9 Thesis aims

The aim of the work detailed in this thesis was to address a number of important unanswered questions about the mechanisms involved in filarial down-regulation of cellular proliferative responses (Figure 4). The approach used was to investigate antigen presenting cell function and modulation of T cell responses after implantation of parasite stages into inbred mice. Using this model and the available reagents, the aim of this thesis was to answer these questions:

- What is the role of IL-4 and the Th2 response in infection and in generation of suppressor cells?
- What are the parasitic factors that drive recruitment and/or generation of suppressor cells?

- What is the identity of the suppressive cell?
- What is the mechanism by which suppression occurs?

A more detailed understanding of these areas could then be related to human infection, to help further our understanding of an exciting and as yet poorly understood area of immunology.

Chapter 2: Materials and Methods

2.1 Parasite Isolation

B. malayi adults and microfilariae (Mf) were obtained from infected jirds purchased from TRS laboratories (Athens, GA). Adult worms were removed from the peritoneal cavity of jirds that had been euthanised by cardiac puncture under anaesthesia, and washed in RPMI 1640 (Gibco-BRL, UK) supplemented with 50 μg/ml gentamicin. Mf were isolated from this lavage by passage through PD10 Sephadex® G-25 M columns (Pharmacia Biotech AB, Uppsala, Sweden). Infective larvae (L3) (supplied by W. Gregory and J. Murray, University of Edinburgh) were obtained from *Aedes aegypti* mosquitos that had been fed on blood containing $1.6x10^4$ Mf/ml 12 days previously. After this time, mosquitos were gently crushed between a glass plate and a test tube, following which larvae were separated from mosquito debris using a Baermann apparatus with Grace's insect tissue culture medium (Gibco-BRL, UK).

2.2 Preparation of excretory/secretory material

Adult *B. malayi* excretory/secretory material (ES) was obtained by culture of 1 adult worm per ml at 37°C in 50 ml RPMI 1640 (Gibco-BRL, UK) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 1% glucose. 40 ml of supernatant was harvested daily and replaced with the same volume of fresh supplemented media. Harvested supernatant was passed through a 0.2 μm filter and stored at -70°C prior to use. Adult *N. brasiliensis* ES (supplied by Y. Harcus, University of Edinburgh) was obtained by culture of 125 worms/ml in RPMI supplemented with 2% glucose, 2 mM glutamine, 100 units/ml penicilin and 100 μg/ml streptomycin at 37°C. Supernatants were collected daily from day 2 of culture, passed through a 0.2 μm filter and stored at -70°C. *T. canis* larval ES (supplied by K. Tetteh, University of Edinburgh) was obtained by culture of 2,500 infective larvae/ml at 37°C in RPMI supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml Fungizone (Gibco-BRL, UK), 15 μg/ml gentamicin (Gibco-BRL, UK) and 1% glucose. Supernatants were removed weekly and filtered through a 0.2 μm filter prior to storage

at -70°C. Adult *B. malayi* antigen (BmA) was prepared by homogenisation of mixed sex worms in PBS on ice followed by centrifugation at 10,000 x *g* for 20 mins. The resultant supernatant was passed through a 0.2 μm filter prior to protein concentration determination by the Coomassie Plus protein assay (Pierce, Rockford, Illinois).

2.3 Preparation of parasite antigen

Adult *B. malayi* antigen (BmA) was prepared by homogenization of mixed sex worms in PBS on ice followed by centrifugation at 10,000 x *g* for 20 mins. The resultant supernatant was passed through a 0.2 μm filter prior to protein concentration determination by the Coomassie Plus protein assay (Pierce, Rockford, Illinois). This material was used in T cell assays and for ELISA at the concentration indicated in the text.

2.4 Mouse infection model

2.4.1 Mice

For all experiments, mice used were 6-8 week old males unless otherwise stated. Experimental mice were generally purchased from Harlan-UK (Bicester, UK), or obtained from source (Ann Walker House, Edinburgh University). C57BL/6 IL-4 deficient (IL-$4^{-/-}$)(Kuhn et al., 1991) and C57BL/6 IL-10 deficient (IL-$10^{-/-}$) (Kuhn et al., 1993) and wild-type C57BL/6 mice were purchased from B&K Universal Ltd (North Humberside, UK) with the permission of the Institute of Genetics, University of Cologne. C57BL/6 IL-5 deficient (IL-$5^{-/-}$) mice were the kind gift of Professor Manfred Kopf (Basel, Switzerland). BALB/c nude mice and wild-type controls were obtained from Harlan-UK (Bicester, UK).

2.4.2 Implantation/infection

Mice were surgically implanted intra-peritoneally (i.p.) under anaesthesia with adult *B. malayi* parasites. Mice were generally implanted with mixed-sex infections, using 6 adult female and 2 adult male parasites, unless otherwise stated. For Mf and L3

infections, mice were injected i.p. with $2x10^5$ Mf, or 10 to 400 L3, using a 21 guage needle. Implants were left for 3 to 4 weeks unless otherwise stated. At this point, mice were euthanized by cardiac puncture under anaesthesia, and peritoneal exudate cells (PEC) were harvested by thorough washing of the peritoneal cavity with 15 ml of RPMI containing 50 μg/ml gentamicin. Blood obtained from cardiac puncture was allowed to clot at room temperature, spun at 13,000 r.p.m. for 30 mins at 4°C, supernatants removed and sera stored at -20°C 1:1 with glycerol for subsequent analysis.

2.4.3 Administration of ES

For investigation of the ability of parasite ES to generate suppressive PEC, mice were injected daily for two weeks with 1 ml of *B. malayi*, *N. brasiliensis*, or *T. canis* ES, or with RPMI alone. After this time peritoneal cells were harvested as described above.

2.4.5 Treatment with neutralising antibody

For treatment of animals with neutralising antibody to IL-4 *in vivo*, mice were injected i.p. with 2 mg of anti-IL-4 mAb (11B11), or isotype matched anti-IFN-γ mAb (R4-6A2; ATCC HB170) at the time of parasite implant. For *in vivo* use, 11B11 was kindly provided by F.W. Falkenberg (Ruhr University, Bochum, Germany), and for an isotype matched control, the miniPERM™ monoclonal antibody production system (Heraeus) was used to produce neutralising mAb to IFN-γ, purified using a protein G column (Pharmacia Biotech).

2.5 Cultures and cell lines

Unless otherwise stated, all *in vitro* cultures were carried out in RPMI 1640 medium (Gibco-BRL, UK) supplemented with 2 mM glutamine, 0.25 units/ml penicillin, 100 μg/ml of streptomycin, 5 μM 2-mercaptoethanol and 10% FCS (complete medium), at 37°C in 5% CO_2. The Th2 cell clone, D10.G4 (Kaye et al., 1983), the B cell hybridoma HB32 (ATCC:Rockville, MD) and the murine lymphoma EL-4 (ATCC:Rockville, MD) were maintained in culture for use. The D10.G4 clone was maintained by incubation of cells with 100 μg/ml conalbumin in the presence of syngeneic (CBA/Ca) irradiated

splenocytes for 2-3 days, after which cells were diluted 1:10 in complete medium with 10% (v/v) conditioned medium from a 48 h culture of concanavalin A (Con A)-stimulated mouse lymphocytes. The D10.G4 cells were used 5-7 days after this rest period. HB32 and EL-4 cells were maintained by growth in complete medium. For experiments using C57BL/6 and BALB/c mice, primary ovalbumin-sensitised T cell lines were established by subcutaneous immunisation of mice with 50 μg/footpad ovalbumin in complete Freund's adjuvant. Two weeks later, popliteal lymph nodes were removed and liberated cells cultured in complete medium with 25 μg/ml ovalbumin. After 3-4 days cultures were supplemented with 10% (v/v) conditioned medium from a 48 h culture of Con A-stimulated mouse lymphocytes. Syngeneic splenocytes inactivated by irradiation with 2000 rads were added at the same time as feeders. T cells were used 5-7 days after this rest period. In some experiments, ovalbumin-sensitised T cells were purified from popliteal lymph node suspensions using nylon wool columns, and were then used directly.

2.6 Proliferation assays

For investigation of proliferative suppression caused by parasite-exposed PEC, 100 μl PEC at $1x10^6$/ml were adhered to a flat-bottomed 96 well plate (Nunclon) at 37°C for 2-3 hours, after which non-adherent cells and Mf were removed by washing with complete medium. Plates with adherent PEC were then used for co-culture with $5x10^4$ D10.G4 or HB32 cells, or with primary T cell lines, to a final volume of 200 μl/well. Conalbumin (Sigma Chemical Co.) and ovalbumin (Sigma Chemical Co.) were used at the concentrations indicated in the text. After incubation for 48 hr at 37°C, 100 μl of supernatant was removed from each well of the D10.G4 or primary lymphocyte assays for cytokine analysis; 1 μCi [^{3}H] thymidine in 10 μl complete medium was then added to each well, and plates were incubated for 16-18 hr at 37°C prior to harvesting and counting using a Top Count Microplate Scintillation Counter (Canberra Packard).

For analysis of lymphocyte responses in the spleens of infected animals, splenocytes were obtained by teasing spleens apart, then lysing erythrocytes using red blood cell lysis buffer (Sigma Chemical Co.). Viable cells were then counted by trypan blue

exclusion. Whole spleen cells were incubated at $5x10^5$ cells/well with BmA at a final concentration of 5 μg/ml. After incubation for 65 hr at 37°C, supernatants were taken for subsequent cytokine analysis.

2.6.1 Inhibition/neutralisation of potential suppressive factors in vitro

Neutralising antibody to mouse IL-10 was purchased from Genzyme Diagnostics (Cambridge, UK) and used at 2 μg/ml. A mAb that neutralises transforming growth factor (TGF)β-1, TGFβ-2 and TGFβ-3 (1D11.16) was kindly provided by the Celtrix Corporation (Santa Clara, CA), and was used at a concentration of 50 μg/ml. The neutralising antibody to IL-4 (11B11) was obtained from ascites for *in vitro* use, and used at 2.5 μl/ml, the optimal concentration for neutralization as determined by titration. The NO inhibitor L-N^G-monomethyl-arginine (L-NMMA) and the control inhibitor D-NMMA (Wellcome Foundation, Beckenham, Kent, UK) were used where indicated at a final concentration of 250 μg/ml.

2.7 Cytokine assays

2.7.1 Interleukin-2 and Interleukin-4

The IL-2/IL-4 responsive NK cell line (Swain et al., 1981) was used to measure cytokine production by D10.G4 cells, primary T cells and splenocytes, as previously described (Allen et al., 1996; Lawrence et al., 1994). Proliferation of the NK cells at 10^5/ml, 100 μl/well, was measured in the presence of 10 μl culture supernatant, with the addition of anti-IL-2 (S4B6)(ATCC:Rockville, MD) neutralising antibody for IL-4 measurement, and anti-IL-4 (11B11)(ATCC:Rockville, MD) neutralising antibody for IL-2 measurement. S4B6 and 11B11 were obtained from ascites for *in vitro* use, and used at 2.5 μl/ml, the optimal concentration for neutralisation as determined by titration. After incubation at 37°C for 24 hours, 1 μCi/well of [^{3}H]TdR (Amersham) was added in 10 μl RPMI complete medium prior to incubation for a further 16 to 18 h. Plates were then harvested and counted using a Top Count microplate scintillation counter (Packard

Instrument Co. Meriden, CT). Standard curves using recombinant IL-2 (Sigma Chemical Co.) and IL-4 (Sigma Chemical Co.) were performed to determine cytokine levels in supernatants.

2.7.2 Interleukin-5

IL-5 was measured by capture ELISA. Plates were coated with 100 µl/well purified rat-anti-mouse IL-5 Ab (TRFK-5)(Pharmingen, San Diego, CA) at 1 µg/ml in 0.06M carbonate buffer (pH 9.6). After overnight incubation at 4°C, plates were blocked by adding 100 µl/well 1% BSA in TBS, incubated for 2 h at 37°C. After washing three times with TBST, 25 µl of samples to be tested were added, and a recombinant IL-5 standard curve was performed starting at 100 ng/ml, to give a final volume of 50 µl/well. Plates were then incubated overnight at 4°C, washed three times with TBST, and 100 µl/well biotinylated anti-mouse IL-5 (Pharmingen, San Diego, CA) at 1µg/ml was added to each well, followed by incubation at room temperature for 2 h. Plates were washed three times with TBST, then streptavidin-alkaline phosphatase was added, 100 µl/well at 1µg/ml, and incubated at room temperature for 1 h. Finally, plates were washed six times with TBST, and 100 µl/well *p*-nitrophenyl phosphate substrate (Sigma Chemical Co.) was added. Once colour had developed, the plates were read on an Anthos 2001 ELISA reader (Anthos Labtec Instruments) at 405nm. The detection limit of this assay was ≥0.1ng/ml, as determined from the standard curve.

2.7.3 Interferon-gamma

IFNγ was measured by capture ELISA. Plates were coated at 3 µg/ml with rat-anti-mouse IFNγ mAb (R46A2)(ATCC, Rockville, MD), 50 µl per well, diluted in 0.06M carbonate buffer (pH 9.6). After overnight incubation at 4°C, plates were blocked by addition of 100 µl per well 1% BSA in carbonate buffer, incubated for one hour at 37°C. 25 µl of sample to be tested, and a recombinant IFNγ (Sigma Chemical Co., I-5517) standard curve, were added to give a final volume of 50 µl/well in TBST. Plates were then incubated overnight at 4°C. After washing four times with TBST, 50 µl per well biotinylated rat-anti-mouse-IFNγ monoclonal Ab (XMG1.2) (Pharmingen, San Diego, CA 18112D) was added at 1 µg/ml in TBST, and incubated for 1 hour at 37°C. Plates

were again washed four times with TBST, followed by addition of 50 µl/well streptavidin-alkaline phosphatase (Sigma Chemical Co.) at 1 µg/ml in TBST, which was incubated for 45 minutes at 37°C. Finally, after washing four times with TBST and rinsing once with deionised water, *p*-nitrophenyl phosphate substrate (Sigma Chemical Co.) was added at 50 µl/well. The plates were developed in the dark for 30 minutes to several hours, with regular checking for colour change. Once a colour change was observed, plates were read at 405 nm in an ELISA reader (Anthos Labtec Instruments). Detection limits using this protocol were ≥0.125 Units/ml IFNγ, as determined from the standard curve.

2.8 Analysis of antibody production in sera by ELISA

2.8.1 Antigen-specific IgG Isotypes

ELISA plates (NUNC-Immunoplate MaxiSorp™ NUNC, Denmark) were coated with 50 µl/well BmA at 5 µg/ml, diluted in 0.06M carbonate buffer (pH 9.6), and incubated overnight at 4°C. They were then blocked for 1 h with 100 µl/ well 5% BSA in PBS at 37°C. After washing four times with PBST, serum samples diluted 1:100 in PBST were added at 50 µl/well, then incubated for 4 h at 37°C. Plates were washed four times with PBST, then 50 µl/well horseradish peroxidase-conjugated isotype-specific mAbs diluted in PBST were added: anti-IgG1 (Southern Biotechnology Associates Inc. Birmingham, AL), diluted 1:6000; anti-IgG2a (SBA Inc.), diluted 1:4000; anti-IgG2b (SBA Inc.), diluted 1:4000; and anti-IgG3 (SBA Inc.). In the case of C57BL/6 mice an alternative anti-IgG2a antibody (R19-15, Pharmingen, San Diego, CA) had to be employed, since the IgG2a isotype produced by this strain of mouse differs significantly from those produced by other strains (Martin and Lew, 1998), and is not recognised by the Southern Biotechnology mAb. Following a 1 h incubation at 37°C and four washes with PBST, ABTS substrate (KPL Biotechnology, Gaithersburg MD) was added at 50 µl/well, and plates read at 405nm on an ELISA reader and comparative optical densities (O.D.) plotted.

2.8.2 Total IgE

A capture ELISA was used to measure the total serum IgE concentration. Plates were coated at 2 μg/ml, 50 μl/well, with monoclonal anti-mouse IgE R35-72 (PharMingen, San Diego, CA) diluted in TBS. Following overnight incubation at 4°C, plates were blocked with 100 μl/well 5% BSA in TBS incubated at room temperature for 1 h. A standard curve was performed using purified mouse IgEκ isotype (Pharmingen, San Diego, CA), serially diluted from 20 μg/ml in TBST, 100 μl/well. Sera were diluted 1:10 in TBST with 100 μl/well added to the plate prior to overnight incubation at 4°C. Plates were washed 3 times with TBST before addition of 100 μl/well biotinylated rat-anti-mouse-IgE (R35-118) (Pharmingen, San Diego, CA), at 2 μg/ml, then incubated for 1 h at room temperature. After three washes with TBST, 100 μl/well streptavidin-alkaline phosphatase (Sigma Chemical Co.) was added at 1 μg/ml, followed by incubation at 37°C for 45 minutes. Plates were washed four times with TBST and once with deionised water, and 100 μl/well Sigma*FAST*™ *p*-nitrophenyl phosphate (Sigma Chemical Co.) substrate was added. Plates were developed in the dark for approximately 1 hour and read at 405 nm on an ELISA reader, when the colour had developed sufficiently.

2.9 Flow cytometric analysis of PEC

Harvested PEC were washed and adjusted to a concentration of $1x10^7$/ml in FACS PBS (PBS containing 1% BSA and 0.1% sodium azide). Antibodies to be used had previously been titrated to determine the optimum amount required for staining of $1x10^6$ cells. The appropriate amount of antibody was then added to cell samples to be stained, either in a 15 ml centrifuge tube or in a round bottomed microtitre plate. Samples were incubated at 4°C for 30 minutes, washed three times with FACS PBS, then transferred to FACS tubes (Becton Dickinson) and either analysed directly, or fixed with a few drops of 10% formalin and stored in the dark prior to analysis, using a FACScan with CELLQuest software (Becton Dickinson). Antibodies used for flow cytometry were; R-phycoerythrin-conjugated anti-CD4 (Caltag), FITC-conjugated anti-CD8 (Caltag),

FITC-conjugated anti γδTCR (Pharmingen), FITC-conjugated anti-CD80(B7-1) (Caltag), R-phycoerythrin-conjugated anti CD86(B7-2) (Caltag),Tri-color-conjugated anti-macrophage (clone F4/80)(Caltag), and FITC-conjugated anti-macrophage (Mac-1)(Caltag).

2.10 Cell sorting using flow cytometry

PEC isolated from mice were washed and adjusted to $1x10^7$/ml in sterile phosphate-buffered saline (PBS) containing 0.02% EDTA. Cells were then incubated at 4°C for 30 min with the appropriate concentration of anti-macrophage (Clone F4/80, R-phycoerythrin-conjugated, Caltag Laboratories, San Francisco, CA) monoclonal antibody, as determined by titration. F4/80 -positive and -negative PEC were sorted using a FACStar (Becton Dickinson), with logarithmic amplification of fluorescence detection and side scatter, and linear amplification of forward scatter. Lysis 2 software was used for acquisition and analysis. Sorted cells were then washed and resuspended in complete RPMI medium for use in the D10.G4 assay.

2.11 Cell sorting using magnetic beads

The MACS magnetic cell sorting system (Milteyni Biotec, Germany) was used for positive selection and depletion of peritoneal subpopulations.

PEC obtained by peritoneal lavage of mice were washed and adjusted to $1x10^7$ per ml in PBS pH 7.2 supplemented with 2 mM EDTA (labelling buffer). Cells were stained with the appropriate amount of the chosen antibody (previously titrated for FACS) for 30 min at 4°C, washed three times, and then resuspended in 90 µl of buffer per $1x10^7$ cells prior to addition of 10 µl magnetic beads and incubation at 4°C for 15 min. Cells were then washed carefully and resuspended in 500 µl PBS pH 7.2 supplemented with 0.5% BSA and 2mM EDTA (separation buffer). MS+ separation columns (Miltenyi Biotec, Germany) were used for all magnetic sorting. Columns were prepared by application of 500 µl separation buffer. Cell samples were then added, and the column was then washed with 3 x 500 µl separation buffer, with the total effluent collected as the

depleted fraction. Positively selected cells were then obtained after removal of the column from the magnet, by washing of the column with 1 ml of separation buffer. To avoid binding of adherent cells to the column matrix, all buffers were stored at 4°C or on ice, and columns were incubated at 4°C prior to use.

PEC were enriched for MAC-1$^+$ cells by magnetic cell sorting using anti-FITC microbeads in conjunction with FITC-conjugated anti-MAC-1 mAb (Caltag). Streptavidin microbeads were used in conjunction with biotin-conjugated anti-F4/80 mAb (Caltag) for purification of macrophages.

2.12 Cytocentrifuge preparations

Harvested PEC were washed and adjusted to $1x10^6$/ml in complete medium. 100 µl of cells ($1x10^5$) plus 100 µl of foetal calf serum (Gibco-BRL, UK) were combined, and cytocentrifuge preparations were made using a Shandon Cytospin (Shandon, PA). Cytocentrifuge preparations of PEC were air-dried and fixed for 2 min in methanol prior to staining with Diff-Quik (Dade, Germany). The proportion of macrophages, monocytes/blasting cells, lymphocytes, mast cells, neutrophils and eosinophils was determined by morphological examination (see Appendix 1 for examples) of at least 300 cells in randomly selected fields using a Nikon Microphot-FX microscope. Photomicrographs were taken using the same equipment.

2.13 Membrane separation of PEC and responder cells

Transwell cell culture chambers (Costar, UK) were used to separate PEC from responder cells to determine if suppression was effected via a soluble mediator. 24 well cluster plates, with 0.4 µm pore size, were used. D10.G4 cells ($5x10^5$), or HB32 cells ($6x10^5$), were added to the lower compartment in 600 µl complete medium. PEC at from *Brugia*-implanted or unimplanted mice (100 µl at $1x10^7$/ml) were then added to the upper compartment. In control wells (with no Transwell), the same numbers of cells were added to individual wells of the 24 well plate. For D10.G4 cultures, $2.5x10^5$ syngeneic irradiated splenocytes were added to culture wells (lower compartment of

Transwell), to provide a source of APC. Conalbumin was also added to a final concentration of 50 μg/ml. Cultures were incubated at 37°C for 24 hours. After this time 5 μCi [^{3}H]thymidine in 50 μl complete medium was added to each well (lower compartment of Transwell), prior to incubation for a further 16-18 h at 37°C. D10.G4 and HB32 cells were then transferred to 96 well plates prior to harvesting and counting using a Top Count Microplate Scintillation Counter (Canberra Packard).

2.14 Antigen presentation by fixed adherent PEC

Using flat-bottomed 96-well microtitre plates 1 x 10^5 peritoneal exudate cells were added to each well in 200 μl RPMI complete medium. Plates were incubated at 37°C for 2 h with media alone or, for D10.G4 cell cultures, PEC were pulsed with 50 μg/ml conalbumin prior to incubation in order to pre-load APC with antigen prior to fixation. After this time, non-adherent cells were removed from the wells and replaced with 50 μl of incomplete RPMI. 50 μl of 2% paraformaldehyde in PBS, or 50 μl of incomplete RPMI was then added to each well. Plates were then incubated for 15 min at room temperature. After this time, the fixative solution was removed and replaced with 120 μl of incomplete RPMI, which was then removed and replaced with 120 μl of lysine wash solution. Plates were then incubated at room temperature for 30 min prior to washing four times with RPMI and replacing with 100 μl of complete RPMI. After incubation, 5 x 10^4 D10.G4, or HB32, or EL-4 cells were added to each well to give a final volume of 200 μl/well. Conalbumin was added to D10.G4 cultures at a final concentration of 50 μg/ml. After incubation for 48 h at 37°C, 100 μl of supernatant was removed from each well of the D10.G4 assay for cytokine analysis. After supernatant removal, 1 μCi of [^{3}H] thymidine in 10 μl complete medium was added to each well, and plates were incubated at 37°C for 16-18 h prior to harvesting and counting using a Top Count Microplate Scintillation Counter (Packard Instrument Co., Meriden, CT).

2.15 Statistical analysis

The Student's t-test and paired t-test were used to determine the statistical significance of differences between and within groups. $P < 0.05$ was considered to be a significant difference.

Chapter 3: Induction of Suppression

(Published in part in Journal of Immunology and Parasite Immunology. See Appendix 2)

3.1 Introduction

Our initial hypothesis was that the loss of T cell responsiveness seen in human filarial infection might be due to the parasite directly interfering with host antigen processing or presentation, or lymphocyte function. We reasoned that this strategy might have evolved to prevent the escalation of a potentially damaging inflammatory immune response directed towards the parasite. Preliminary experiments designed to address this possibility, in which adult and Mf stages of *Brugia* were co-cultured with a source of antigen presenting cells and responding T cells *in vitro*, failed to show a direct down-regulatory effect of the parasite on antigen-specific proliferation and cytokine production (Allen et al., 1996). This indicated that *Brugia* was not directly interfering with APC or responder cell function. More interestingly, it suggested that additional components of the host immune system, lacking in the defined *in vitro* model, might be required for the parasite to inhibit lymphocyte responsiveness.

The inability of live parasites to exert a direct inhibitory effect on lymphocyte proliferation or APC function *in vitro* led to the development of an *in vivo* model, to provide a system that was more comparable to that involved during a natural infection. This approach revealed that antigen presenting cells that were exposed to the parasite *in vivo* were functionally altered. Peritoneal exudate cells (PEC) taken from mice implanted in the peritoneal cavity with live adult *B. malayi* parasites were able to inhibit proliferation, but did not prevent antigen-specific cytokine production, of responding lymphocytes (Allen et al., 1996). Thus, adult filarial parasites can indirectly interfere with lymphocyte function through activation or recruitment of a host cell type which is then able to dramatically block normal proliferation. It is around this core finding that this thesis is based.

Mf were also found to be able to generate a moderate suppressive effect by i.p. implant, but proliferative block by these PEC appeared to operate via a different mechanism to

that of adult parasites, since any suppression caused by this life cycle stage was found to be reversible with the addition of NO inhibitors.

This chapter details an investigation of the parasite factors responsible for the induction of a profoundly suppressive host cell population by asking a series of important questions about the respective roles of such factors.

Firstly, did the inability of the parasite to emulate *in vivo* generation of a host suppressive cell type *in vitro* indicate a necessity to collaborate with additional components of the host immune system, or did it simply reflect insufficient incubation times *in vitro*? The capacity of *B. malayi* to generate suppressive PEC *in vitro* was therefore examined in further detail, primarily by extending the pre-incubation times of parasites with APC prior to the addition of responder lymphocytes to see if this would alter subsequent APC and lymphocyte function.

Secondly, we wanted to determine the time required for generation of suppressive PEC *in vivo* to provide information on whether host cells could be rapidly activated to down-regulatory function by the presence of parasites, or required the development of a host response. By assessing the down-regulatory abilities of host cells exposed to parasites over increasing time *in vivo*, we would also find out how long PEC remained suppressive after the time of parasite exposure, even after the death and clearance of live parasites from the site of infection.

We also wanted to learn if the infective larval stage could generate suppressive PEC, and if so, if the mechanisms underlying the action of such down-regulatory cells bore similarities to either those of adult- or Mf-derived suppressive PEC (the former being NO-independent and the latter NO dependent). Additionally, we wanted to see if the ability of filariae to interfere with host APC function was restricted to either male or female adult parasites. Such questions might help clarify whether, as has been proposed, Mf are the key life cycle stage in causing the loss of peripheral blood T cell responsiveness seen in individuals with filarial infection.

Finally, we wanted to establish the mechanism(s) by which the parasites induce the activation and/or recruitment of host down-regulatory cells. As one of the main interfaces between parasite and host, the excretory/secretory (ES) products of nematode parasites is a likely candidate to play some role in parasite survival through modulating host immune responsiveness in the local infection environment. We wanted to know if exposing host cells to the ES products of *Brugia* could duplicate the ability of live parasites to generate suppressive PEC *in vivo*.

3.2 Results

3.2.1 Co-culture of Adult or Mf stages of *B. malayi* with APC *in vitro* does not block T cell proliferation

Previous work from this lab had shown that direct incubation of the D10.G4 cell clone and mitomycin C treated splenocytes with adult or Mf parasites did not prevent antigen-specific proliferation of the T cell clone (Allen et al., 1996). The same study showed that pre-incubation of the splenocytes for 13 h with adult parasites did not affect their ability to normally stimulate D10.G4 cells. To further investigate the ability of the parasite to generate or activate APC to suppressive function *in vitro*, live Mf or adult parasites were pre-incubated with CBA/Ca splenocytes for longer time periods than the 13 h initially tested, prior to the addition of D10.G4 cells. Pre-incubation of splenocytes with either Mf (Figure 1A), or adult parasites (Figure 2) for up to 96 hours before the addition of D10.G4 cells had no detrimental effect on the ability of these APC to normally stimulate proliferation of the T cell clone.

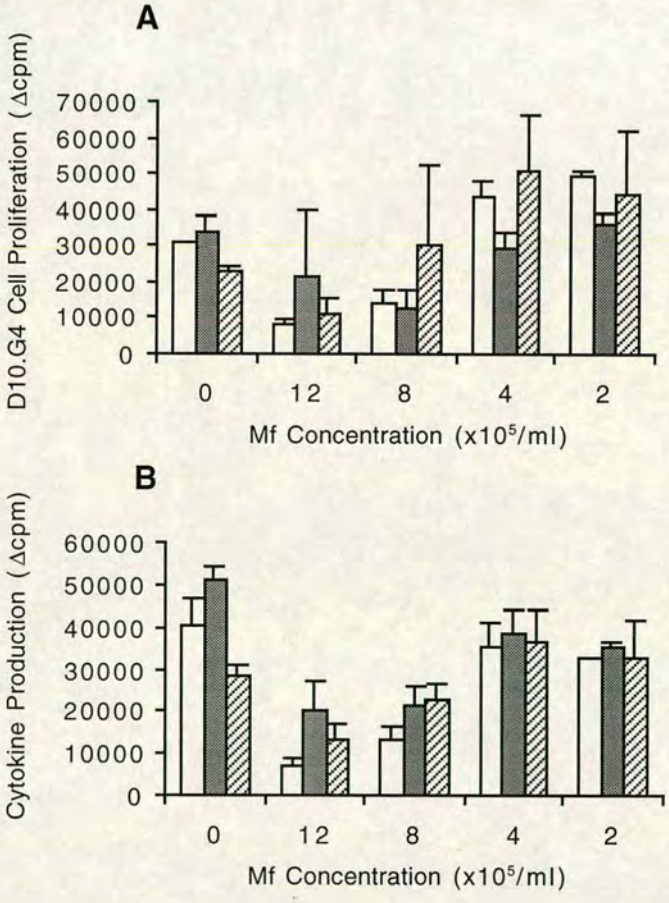


Figure 1. Co-culture of *B. malayi* Mf with APC *in vitro*. (**A**) Antigen-specific proliferation or (**B**) cytokine production of D10.G4 cells in the presence of CBA/Ca splenocytes which had been pre-incubated with the given number of Mf for 48 hr (open bars), 72 hr (grey bars), or 96 hr (hatched bars), prior to the addition of the D10.G4 cells plus 50 µg/ml conalbumin. Proliferation was measured by [^{3}H]TdR incorporation and is shown as delta cpm. Cytokine production was measured by [^{3}H]TdR incorporation by the IL-2/IL-4 indicator NK cell line in response to culture supernatant. Data presented are mean ± SD of three to four mice separately assayed.

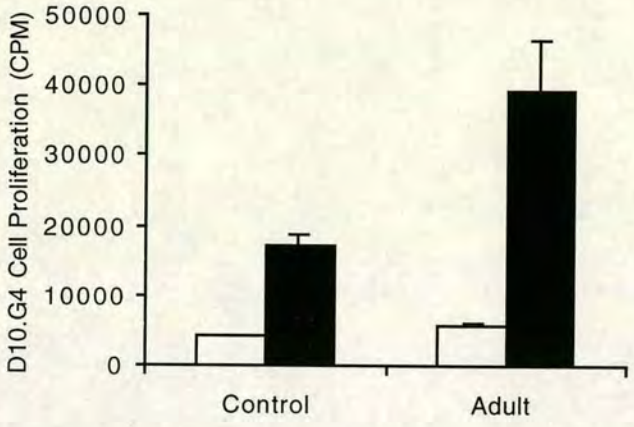


Figure 2. Co-culture of *B. malayi* adults with APC *in vitro*. Proliferation of D10.G4 cells in the presence of CBA/Ca splenocytes which had been pre-incubated with 1 adult female parasite for 96 hr, prior to the addition of the D10.G4 cells plus media (open bars) or 50 μg/ml conalbumin (solid bars). Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute. Data presented are mean ± SD of four wells separately assayed.

Reduced proliferation of the D10.G4 cells was seen only at the highest Mf numbers ($8x10^5$/ml or $1.2x10^6$/ml), but this reduction in proliferation coincided with a reduction in cytokine produced by these cells (Figure 1B). Suppression in this system is typically accompanied by intact, or even enhanced, cytokine production. Thus, this suggested that the high numbers of live parasites in these wells might be depriving the cultures of necessary nutrients, rather than inducing a proliferative block. The presence of adult parasites, or lower numbers of Mf, actually enhanced the proliferation of the T cell clone in comparison to controls. This could be due, as has previously been suggested (Allen et al., 1996), to the high motility of these parasites causing a gentle mixing effect, thereby increasing APC-T cell interaction.

3.2.2 Time course of suppression by *in vivo* implant of adult *B. malayi*

It has been demonstrated that, 3 weeks following adult *B. malayi* implantation into mice, a suppressive cell population can be recovered from the peritoneal cavity (Allen et al., 1996). To provide more information about the dynamics of parasite induction of down-regulatory PEC, a time course experiment was conducted to establish a shorter timeframe. Following implantation of adult *B. malayi*, proliferation of (Figure 3A), and cytokine production by (Figure 3B), the murine T cell clone D10.G4, cultured with adherent peritoneal cells from infected mice, was measured at several time points over a three week period. Seven days of host exposure to the parasite was required to achieve significant proliferative suppression ($P < 0.05$). This suggests that the development of a

host immune response and/or recruitment of particular cells is required, consistent with the inability of the parasites to directly induce proliferative suppression *in vitro*. Although D10.G4 cell proliferation was inhibited in the presence of PEC that had been exposed to adult parasites for from one to three weeks (Figure 3A), cytokine production remained intact (Figure 3B). This refutes the possibility that the reduced proliferation of the D10.G4 cells was due to ineffective antigen presentation, reduced numbers of APC, or heightened cell death.

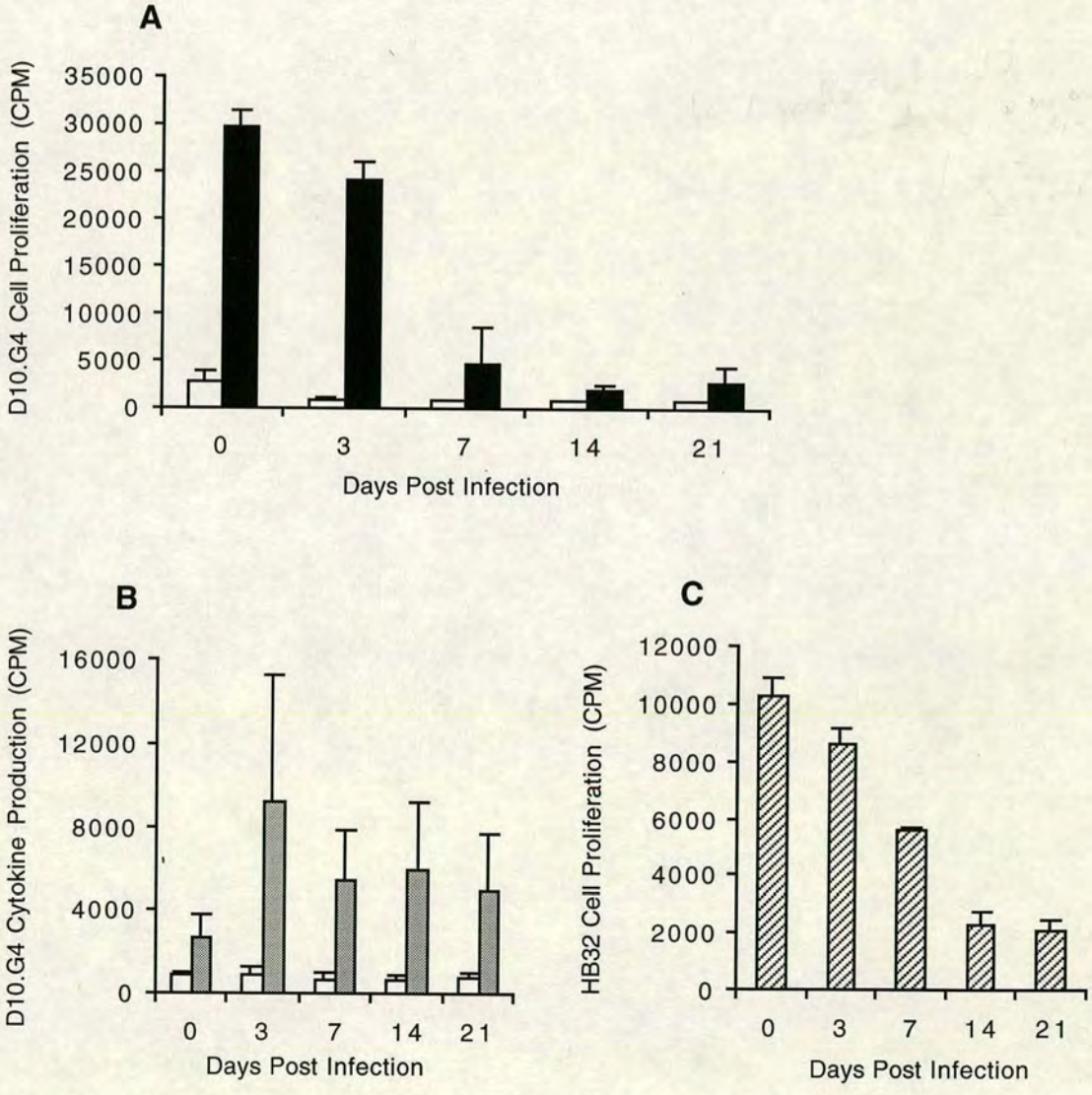


Figure 3. Time course of suppression. (**A**) Proliferation or (**B**) cytokine production of D10.G4 cells with media (open bars) or 50 µg/ml conalbumin (solid bars), or (**C**) proliferation of HB32 cells with media (hatched bars), in the presence of PEC from control (time point 0) or adult parasite-implanted CBA/Ca mice over a three week time course. Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute. Cytokine production was measured by [^{3}H]TdR incorporation by the IL-2/IL-4 indicator NK cell line in response to culture supernatant. Data presented are mean ± SD of three to four individual mice separately assayed.

PEC from parasitised mice also block the proliferation of a range of B cell hybridomas and an adenocarcinoma cell line (Allen et al., 1996). Proliferation of the B cell hybridoma HB32 was assessed in the presence of adherent PEC from adult parasite-implanted animals (Figure 3C). In this case, suppression of proliferation was apparent with cells taken 7 days post-exposure to adult parasites, but was maximal with day 14 cells.

To determine if suppressor cells were a transient feature of parasite implant to the peritoneal cavity requiring the presence of live parasites, implants were left for a long period of time prior to peritoneal cell harvest. Suppressive PEC could be obtained from *Brugia*-implanted CBA/Ca mice as long as 16 weeks after parasite implant, even though no live parasites were recovered from these long-term-exposed mice (Figure 4).

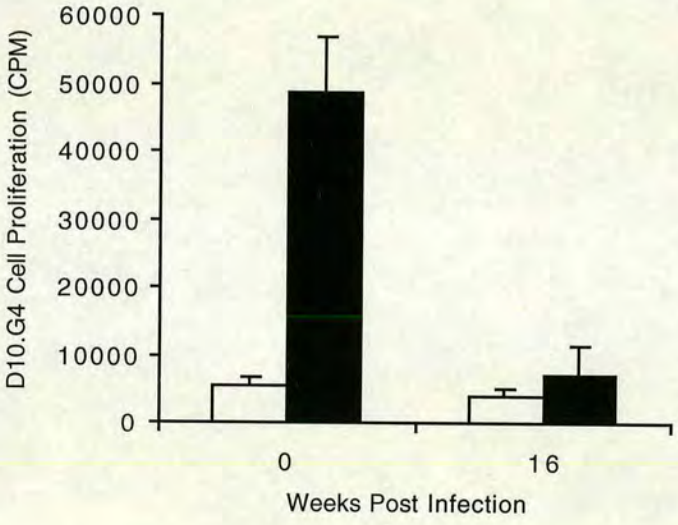


Figure 4. Long term implant. Proliferation of D10.G4 cells with media (open bars) or 50 µg/ml conalbumin (solid bars), in the presence of PEC from control (time point 0) or adult parasite-implanted CBA/Ca mice 16 wk after parasite exposure. Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute. Data presented are mean ± SD of four mice separately assayed.

3.2.3 Infective stage larvae and adult parasites induce similar profiles of T cell suppression in infected animals

Although the ability of adult and Mf *B. malayi* to generate suppressive PEC in mice implanted with either developmental stage had been established, the same had not been shown for the infective larval stage of the parasite. To investigate the ability of L3 parasites to generate suppressive PEC, we infected mice with a range of parasite numbers. CBA/Ca mice were implanted i.p. with 10, 40, 100 or 400 L3 stage parasites,

or with 6 adult female parasites, and the ability of these stages to generate suppression was then compared after two weeks, since L3 parasites do not survive for 3 weeks in the murine host (Carlow and Philipp, 1987). Adherent peritoneal cells from both L3 and adult implanted mice prevented antigen-specific proliferation of the D10.G4 clone (Figure 5A). At high numbers of implanted L3 (400) suppression was greater than that seen with adult implants ($P < 0.05$), despite the lower biomass of 400 L3's as compared to 6 adult parasites. Suppression was not reversed at any parasite dose by the NO inhibitor L-NMMA (Figure 5B), as is seen with adult-implant-derived PEC.

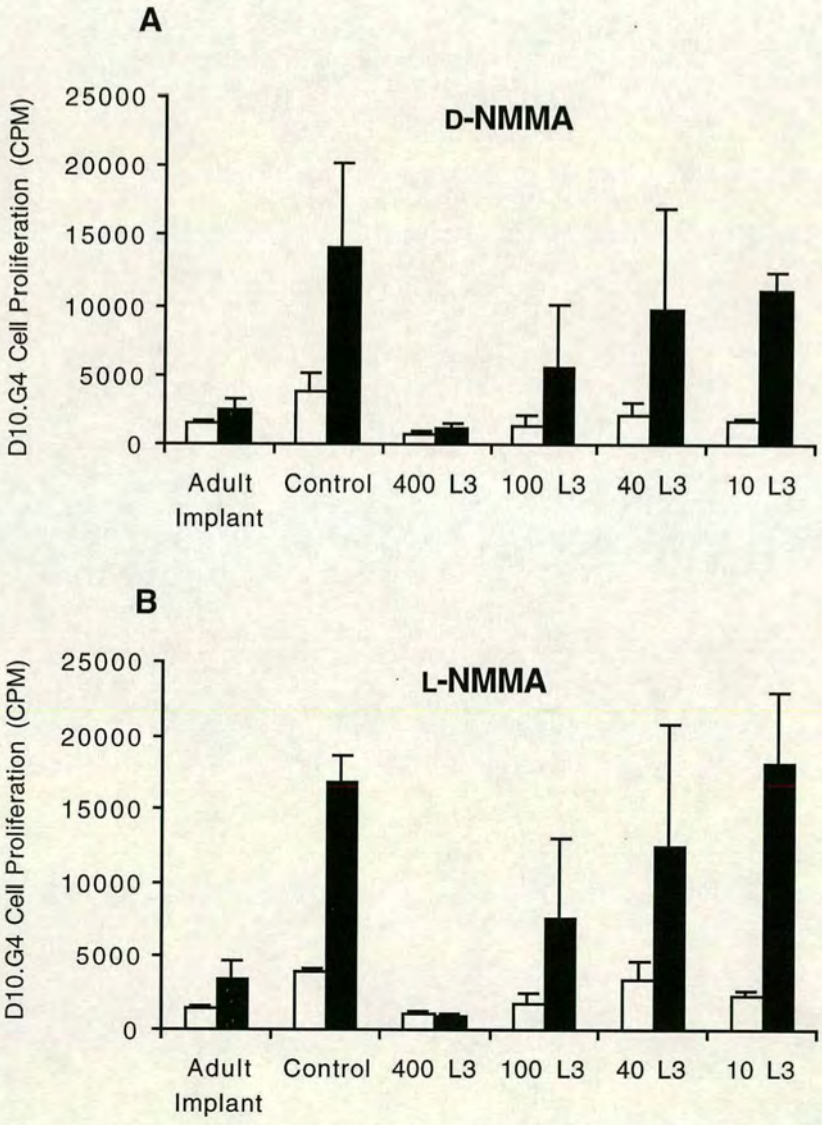


Figure 5. L3 induction of suppression. Proliferation of D10.G4 cells with media (open bars) or 50 µg/ml conalbumin (solid bars), in the presence of PEC from control, adult, or L3-implanted CBA/Ca mice, with the addition of 250 µg/ml (**A**) D-NMMA, or (**B**), L-NMMA. PEC were harvested 2 wk post-implant. Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute. Data presented are mean ± SD of three to four mice separately assayed. Significant proliferative suppression of the D10.G4 clone was achieved when co-cultured with PEC from adult or 400L3 implanted animals in both treatment groups ($P < 0.05$).

Analysis of antigen-specific IL-4 production by D10.G4 cells showed that, although cellular proliferation was ablated in the presence of both adult and L3 implant-derived PEC, cytokine production was enhanced (Figure 6). This demonstrated that, similar to adult parasites, the reduced proliferation of the D10.G4 cells caused by PEC that had been exposed to L3 parasites was not due to ineffective antigen presentation, reduced numbers of APC, or heightened cell death.

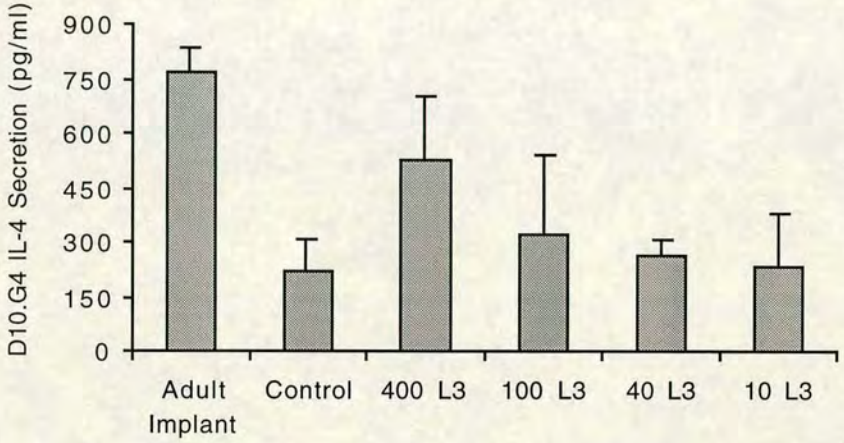


Figure 6. Cytokine production by D10.G4 cells cultured with L3-exposed PEC. Conalbumin-specific IL-4 production, as measured by NK bioassay. Proliferation was measured by [^{3}H]TdR incorporation. Data presented are mean ± SD of three to four individual mice separately assayed.

In addition to T cells, proliferation of the B cell hybridoma HB32 was assessed in the presence of adherent PEC from L3-implanted animals. A significant reduction in hybridoma cell proliferation was seen with administration of as few as 10 L3 parasites (Figure 7) ($P < 0.02$).

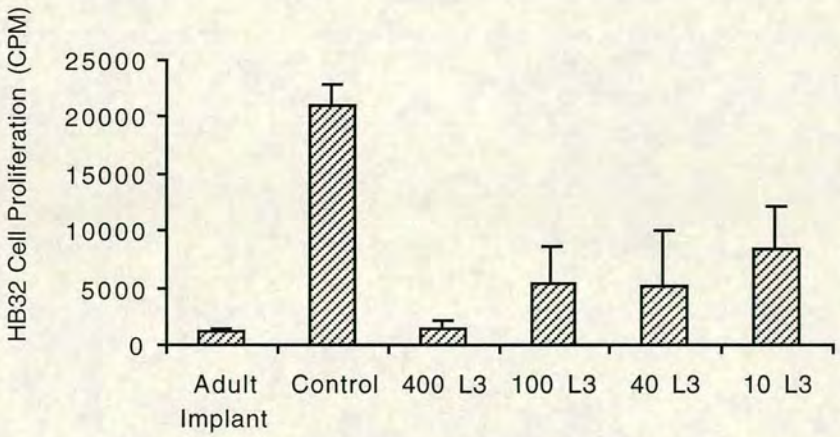


Figure 7. L3 induction of suppression. Proliferation of HB32 cells in the presence of PEC from control CBA/Ca mice, or mice that had been implanted for 2 wk with adult or L3 parasites. Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute. Data presented are mean ± SD of three to four individual mice separately assayed.

3.2.4 Single-sex infections are capable of generating suppressive PEC

Previously, it had been shown that i.p. implant of mixed male and female adult *Brugia* parasites could induce suppressive PEC (Allen et al., 1996). Additionally, several studies have shown an association between the onset of patency of adult female parasites and the emergence of down-regulated immune responsiveness (Lammie and Katz, 1983; Soboslay et al., 1991), thus suggesting an important role for Mf. To resolve if male or female parasites displayed differences in their ability to induce suppressive PEC, mice were implanted i.p. with adult female alone, adult male alone, or a combination of adult female and adult male parasites. Mice implanted exclusively with either adult male or female parasites displayed equivalent ability to generate PEC able to block proliferation of the D10.G4 cell clone, and as effective as PEC taken from mice implanted with a mixture of male and female parasites (Figure 8). Although proliferation of the D10.G4 clone was blocked by parasite-exposed PEC, cytokine production remained equivalent to, or elevated over, control levels (data not shown).

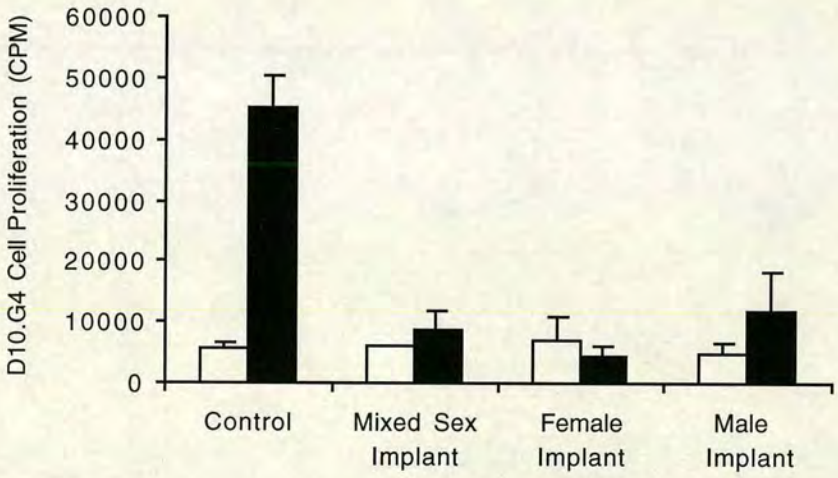


Figure 8. Comparison of induction of suppressive PEC by single or mixed sex *Brugia* infection. Proliferation of D10.G4 cells with media (open bars) or 50 µg/ml conalbumin (solid bars), in the presence of PEC from control CBA/Ca mice, or mice implanted with mixed sex worms (6 adult female and 2 adult male parasites), 6 adult female, or 7 adult male parasites. PEC were harvested 3 wk after implant. Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute Data presented are mean ± SD of four individual mice separately assayed.

To determine the minimum number of live parasites required to generate suppressive cells, CBA/Ca mice were implanted with either a single adult female worm or six adult

female worms, and the ability of PEC from these mice to block proliferation in the D10.G4 system was compared. Not surprisingly, given that suppression had been observed with very low numbers of L3, PEC capable of causing significant proliferative suppression could be generated with the implant of only one adult female parasite (Figure 9)($P < 0.02$).

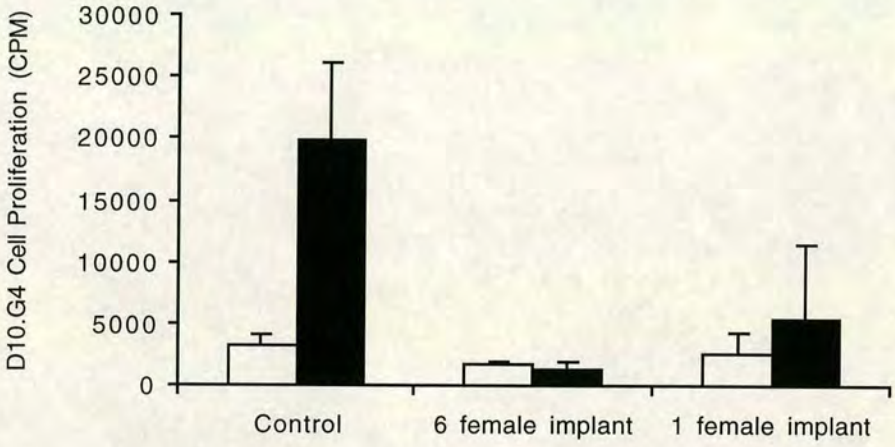


Figure 9. Single parasite implant. Proliferation of the D10.G4 clone with media (open bars) or 50 μg/ml conalbumin (solid bars), co-cultured with PEC from control CBA/Ca mice, or mice implanted with multiple (6) or a single adult female. PEC were harvested 3 wk after implant. Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute Data presented are mean ± SD of five individual mice separately assayed.

3.2.5 Induction of suppression by *B.malayi* ES

Having established that suppressive PEC could be generated by i.p. implant with only one adult female parasite, and given that filarial suppression of proliferation ensues from live, but not dead, adult implants (Allen et al., 1996), we chose to investigate the suppressive potential of the secreted products of the adult stage of the parasite. Adult *Brugia malayi* culture supernatants (ES) contain very low levels of protein (Kwan-Lim et al., 1989), and it was not possible to quantify the concentration of ES produced by standard assay. Instead, *B. malayi* adults were cultured at 1 worm per ml of culture medium, and supernatants collected every 24 hours. Thus, administration of 1ml/mouse/day equated to the amount of ES produced by one worm in 24hr. Daily administration of this amount of ES i.p. for 2 weeks resulted in the generation of a suppressive PEC population (Figure 10A) which blocked proliferation, but not antigen-

specific cytokine production (Figure 10C), in the D10.G4 system ($P < 0.01$). Suppression was not significantly altered on the addition of the NO inhibitor L-NMMA (Figure 10B). Thus, administration of adult ES exactly paralleled the result of implantation of live adults themselves. Interestingly, administration of adult ES every second day did not induce suppressive PEC (data not shown). This may reflect the low concentration of the ES used in these experiments, or may suggest that development of suppression requires constant exposure of host cells to parasite antigen.

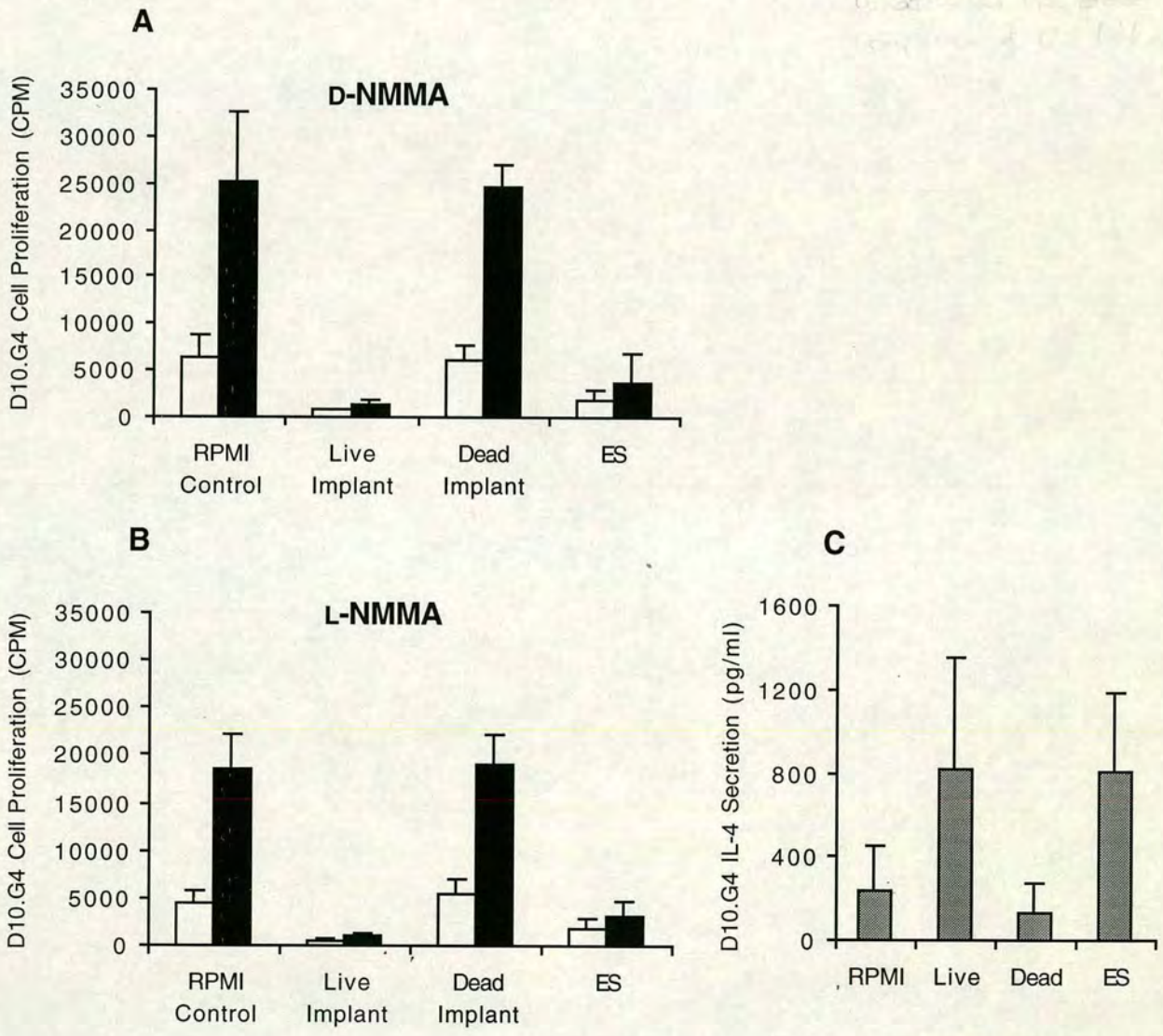


Figure 10. Induction of suppression by *in vivo* administration of *B. malayi* ES. Proliferation of D10.G4 cells (**A**) with media (open bars) or 50 µg/ml conalbumin (solid bars) plus 250 µg/ml D-NMMA or (**B**) with media (open bars) or 50 µg/ml conalbumin (solid bars) plus 250 µg/ml L-NMMA, in the presence of PEC from CBA/Ca mice implanted with 6-8 live adult parasites, 10 dead adult parasites, or injected daily with 1 ml RPMI or 1 ml adult *Brugia* ES. (**C**), Conalbumin-specific D10.G4 cell IL-4 production (from D-NMMA cultures) as measured by NK bioassay. PEC were harvested 3 wk after implant. Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute Data presented are mean ± SD of three to five individual mice separately assayed.

ES products from the rodent filarial parasite *Acanthocheilonema viteae* have been shown to directly inhibit mitogen-stimulated B cell proliferation *in vitro* (Harnett and Harnett, 1993). We therefore decided to investigate the ability of *Brugia* ES products to interfere with proliferation *in vitro*. The very effective suppression generated by adult ES *in vivo* could not be duplicated by the direct addition of the same ES to cultured T cells *in vitro* (Table 1). Although these experiments may have used insufficient quantities of ES, the potency of the same material *in vivo* argues that the comparison is valid. This result is also consistent with our observations that live adult parasites do not have a direct suppressive effect *in vitro* (Allen et al., 1996) (and this chapter).

Table 1. *Addition of Adult ES in vitro*

A. D10.G4 Proliferation (cpm)			
Media	Conalbumin	Media + ES	Conalbumin +ES
7756 ± 746	32358 ± 106	7743 ± 567	36117 ± 2025
B. HB32 Proliferation (cpm)			
Media		Media + ES	
70886 ± 715		77148 ± 2418	

Proliferation of (**A**), D10.G4 cells with media or 50 µg/ml conalbumin, ± 50 µl/well adult *Brugia* ES, or (**B**), HB32 cells with media ± 50 µl/well adult *Brugia* ES, in the presence of PEC from unimplanted CBA/Ca mice. Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute. Data are means ± SD of three individual mice separately assayed.

3.2.6 Suppression can be induced by ES of several nematode species

Having established that *B. malayi* ES has the capacity to generate suppressive PEC *in vivo*, we decided to test the ability of ES from two other nematode species available in the lab, adult *Nippostrongylus brasiliensis* and larval *Toxocara canis*, to generate such cells. We administered 1 ml of ES to each animal i.p every day for two weeks, then removed the PEC for use in the D10.G4 system. Decreased proliferation of the D10.G4

clone was seen when co-cultured with PEC from mice that had been exposed to ES material from all three nematode species tested, although significant suppression was only achieved using PEC from *B. malayi* or *T. canis* ES injected animals (Figure 11)($P < 0.01$ and $P < 0.05$).

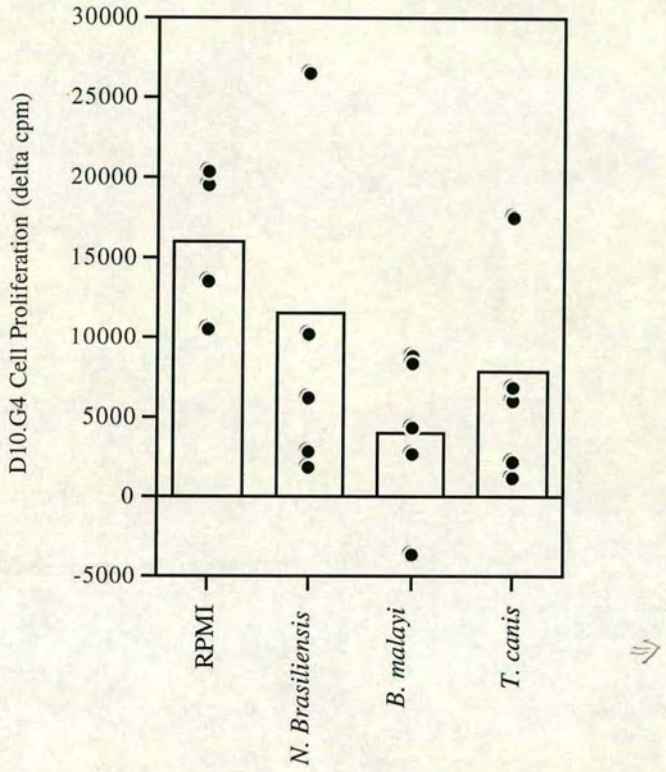


Figure 11. ES-induced suppression by other nematode species. Proliferation of the D10.G4 clone when co-cultured with PEC from control (unimplanted) CBA/Ca mice, or mice injected daily for 2 wk with 1 ml RPMI, or 1 ml adult *Brugia* ES, adult *N. brasiliensis* ES, or L3 *T. canis* ES. Proliferation was measured by [^{3}H]TdR incorporation and is shown as delta cpm. Data represent individual mice within each group (points) and mean (bars) of five individual mice in each group, separately assayed. Proliferation of D10.G4 cells was significantly suppressed after co-culture with PEC from mice that had been injected with *Brugia* ES ($P < 0.01$) or *T. canis* ES ($P < 0.05$).

3.3 Discussion

The inability of *Brugia* to alter APC function *in vitro*, even after pre-culture of live adult or Mf parasites with APC for up to 96 hours prior to the addition of responder cells, reinforces the idea that additional input is required from the host immune system to generate down-regulatory mechanisms. This hypothesis is further strengthened by the fact that at least one week of host exposure to the parasite is required before generation of suppressive PEC *in vivo*.

L3 stage parasites need to evade immune defences on penetration of the host, on migration to the lymphatic vessels, and during growth and development towards adulthood. The observation that the infective larval stage can induce pronounced suppression of lymphocyte proliferation is intriguing given the relatively short life-span of this developmental stage and its initial migratory nature (Schacher, 1962). In particular, it indicates that cellular suppression is initiated extremely early in the course of this long-term infection. In other animal models, filarial modulation of proliferative responses is generally not observed until after patency (Lammie and Katz, 1983; Soboslay et al., 1991), and thus is frequently attributed to Mf release. The results presented here suggest that immune down-modulation is a characteristic of all mammalian stages, even if it is not apparent at a systemic level until later in infection. This hypothesis is supported by the ability of single-sex implant of male parasites to generate suppressive PEC, and is consistent with work involving *Brugia* spp. in gerbils which has failed to show a definitive relationship between Mf and lymphocyte suppression (Bosshardt et al., 1995; Rao et al., 1996). Furthermore, within the peritoneal environment, Mf do not induce the profound suppression seen with adult and L3 stages, but may have other as yet undefined roles in the induction of antigen-specific immune suppression.

Interestingly, we have found that adult excretory-secretory (ES) material injected daily into mice can induce the PEC-mediated proliferative block, even given the very low levels of protein present in the material that is collected from the parasites *in vitro*. This proliferative block, like that caused by implant of live adult or L3 parasites, is not

reversed by the addition of NO inhibitors. These data, along with the inability of dead parasites to generate down-regulatory PEC, strongly suggest that the parasite produces a soluble factor(s) responsible for the subsequent recruitment and/or development of a suppressive cell type. Adult parasites are ideally placed within the afferent lymphatics to 'bathe' the cells within the lymph nodes with down-regulatory ES. Generation of profound localized proliferative suppression by this mechanism could explain the absence of local inflammation at the site of infection in human filariasis (Amaral et al., 1994; Jungmann et al., 1991; Ottesen, 1980), and in animal models (Denham and McGreevy, 1977; Miller et al., 1991). The profundity of the suppressive mechanism(s) at play in this system is demonstrated by the fact that, thus far, we have been able to block proliferation of fully differentiated T cell clones, hybridomas, or primary cell lines. This discovery that the suppressive ability of *Brugia* may be carried in ES provides an ideal tool to allow identification of the parasite factor(s) contained within the ES mix that are critical for generation of down-regulatory cells by the host.

Other helminth studies have shown direct anti-proliferative effects of concentrated ES, or purified or recombinant components of ES *in vitro* (Cervi and Masih, 1997; Harnett and Harnett, 1993; Hartmann et al., 1997). We have not been able to show any down-regulatory *in vitro* effect using either live parasites or unconcentrated ES. This inability of *B. malayi* ES to prevent cellular proliferation *in vitro* may reflect the low concentration of the ES used, although the potency of the same material *in vivo* argues that the concentrations used in our study are physiologically relevant. Alternatively, it could suggest that parasite-derived factors must act in concert with host immune system components to generate suppression. This is consistent with the requirement for at least one week of host exposure to the parasite to allow development of PEC capable of causing proliferative block. Although filariae may posess alternative mechanisms that can *directly* suppress lymphocyte proliferation, the generation of a host cell population that is anti-proliferative may allow amplification of such a negative signal, thus resulting in longer lasting, more profound inhibition of the host response.

Studies using cestode ES have demonstrated a similar ability for these parasites to generate suppressive host cells (Rakha et al., 1991; Sciutto et al., 1995). This led us to

question if the development of a non-specific down-regulatory host cell might be a general feature of helminth infection. To further investigate this possibility, we examined the ability of ES from two other species of nematode, *Toxocara canis* and *Nippostrongylus brasiliensis*, to reproduce the *in vivo* suppressive effect of *Brugia* ES. Daily injection of ES from adult stage *N. brasiliensis* or L3 *T. canis* led to the generation or recruitment of PEC capable of downregulating the proliferation of the D10.G4 cell clone. Unlike *Brugia*, the adult stages of both of these nematodes localise to the intestinal tract, but both species have a larval stage that must survive through a tissue migratory phase. These results suggest that the immuno-modulatory capacity of *Brugia* ES may be a feature common to nematode infection.

In summary, this chapter has detailed the advances that we have made in our understanding of the parasitic factors involved in the generation of down-regulatory PEC. In particular, we have learned a great deal more about the differential ability of the life cycle stages (adult and L3 versus Mf) to generate suppressive PEC. We have shown that the parasite needs to collaborate with its host's immune system to allow immunosuppression to occur, but that surprisingly few parasites need to be present to generate a profoundly suppressive host cell population. Further, we have defined the source of adult stage-mediated suppression as a parasite-derived soluble factor, and have raised the interesting suggestion that this immunomodulatory mechanism may be utilised by a diverse range of organisms.

Chapter 4: Host Immune Involvement

(Published in part in Journal of Immunology. See Appendix 2)

4.1 Introduction

Human filarial infection is associated with an immune response that is skewed towards a Th2-type response, with infected individuals producing high levels of IL-4, IgE and IgG4, and showing marked eosinophilia (Maizels et al., 1995; Ottesen, 1992). There are many examples that illustrate the dominance of Th2 responses in animal models of helminth infection (Allen and Maizels, 1996; Maizels et al., 1993), with one key immunological feature being the production of IL-4 by infected animals in response to parasite antigen (Lawrence et al., 1994; Pearce et al., 1991). Although characteristic of helminth infection, the benefits conferred on host or parasite by the emergence of an immune response dominated by Th2-type antibody and cytokine production are still poorly understood. It is often generally, and perhaps mistakenly, accepted that Th2 responses are 'protective responses' in helminth infection. Although this is apparently the case with gastrointestinal nematodes (Else et al., 1994), the degree of protection conferred by a Th2 response on individuals infected with helminths inhabiting extra-intestinal locations is certainly not as clearly defined (Allen and Maizels, 1997).

Lawrence *et al* previously demonstrated that contrasting cytokine profiles are induced in mice by different life cycle stages of *B. malayi*, with intra-peritoneal implantation of adult or L3 parasites inducing high levels of IL-4 with a matching 'Th2' antibody isotype profile, while microfilariae induce IFN-γ and a 'Th1' antibody profile (Lawrence et al., 1995; Lawrence et al., 1994). This Mf-specific Th1 response is normally eclipsed by the stronger Th2 response to the adult stage of the parasite, and can only be seen when mice are infected with Mf in the absence of adults, a situation which would not occur during a natural infection. In addition, contrasting what is seen with the adult stage of the parasite, peritoneal implant of the blood-circulating microfilariae generates a less profound form of suppression that is reversible by addition of NO inhibitors *in vitro* (Allen et al., 1996). This would be consistent with the cytokine profile typical of

infection with Mf, as one role of IFNγ is as a stimulant of nitric oxide production (Ding et al., 1988).

We wanted to examine the relationship of the host Th2 response, and particularly production of the key Th2 cytokine IL-4, in the generation of down-regulatory cells after exposure to *B malayi* parasites. We postulated that modification of the host response by downregulating the expansion of immune effector cells might be one function of the pronounced Th2 skewing associated with helminth infection. Given our observation that adults and L3 generate suppressive PEC, and that both stages are potent inducers of systemic IL-4, and given the well-documented general relationship between IL-4 production and filarial infection in both animal models and human studies, this primary Th2 type cytokine was the initial candidate for investigation.

The importance of IL-4 production in the development of a host immune response that could generate suppressive PEC was investigated by *in vivo* administration of neutralising antibody. This work was corroborated with the use of IL-4-deficient mice. Since this approach proved successful in extending our understanding of the role of IL-4 in the generation of down-regulatory PEC, we applied the same method to further investigate IL-10. This potent down-regulatory cytokine is associated with IL-4, can act to dampen macrophage inflammatory responses through inhibition of IFN-γ, and may also be critical in regulation of Th1 and Th2 polarisation (Fiorentino et al., 1991; Mosmann and Moore, 1991).

Since we had shown that one week of host exposure to *Brugia* was required to develop suppressive PEC (see Chapter 3), we wished to assess whether the adaptive immune response was required to generate suppression. Thus, in addition to targeting specific cytokines, we assessed the involvement of T cells in the generation of suppressive PEC. To see if T cells were a requisite source for the production of cytokines required for the generation of suppressive PEC, we implanted nude mice (deficient in T cells) and RAG knock-out mice (deficient in both T and B cells) with *B. malayi* to determine if down-regulatory cells could be generated in the absence of T cells, or both T and B cells.

4.2 Results

4.2.1 Systemic IL-4 production correlates with PEC suppression

Mice infected with *Brugia* adults or L3 stimulate high levels of production of the Th2 cytokine IL-4 (Bancroft et al., 1993; Lawrence et al., 1995; Lawrence et al., 1994). Consistent with this previously published data, splenocytes taken from adult or L3-implanted animals produced elevated IL-4 levels (Figure 1A) in response to parasite antigen. Increasing levels of splenocyte *Brugia*-specific IL-4 production correlated with increasing size of L3 inoculum. Further, as levels of systemic IL-4 increased, so did the level of the observed suppression (Chapter 3, Figure 5). IFN-γ levels were also elevated in adult and L3 implanted animals, although there was no correlation between cytokine levels detected and L3 inoculum dose (Figure 1B).

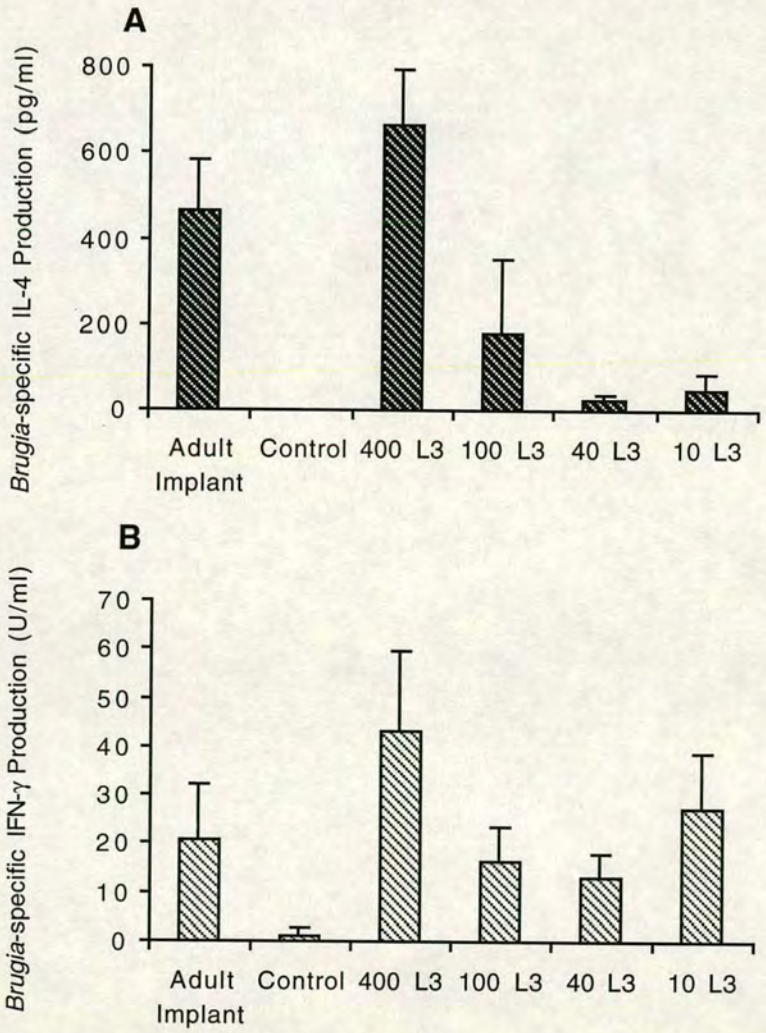


Figure 1. L3-induced splenocyte cytokine production. *Brugia*-specific (**A**) IL-4 or (**B**) IFN-γ production by splenocytes from control or L3-implanted CBA/Ca mice stimulated with BmA, as measured by NK bioassay and ELISA, respectively. Background levels of cytokine production without the addition of BmA were in all cases below detectable level for IL-4, and < 10 U/ml for IFN-γ

4.2.2 Treatment of parasite implanted mice with neutralising antibody to IL-4

Because of the striking correlation between splenic IL-4 production and the generation of down-regulatory PEC, we chose to investigate the hypothesis that IL-4 plays a role in the development of the suppressive PEC by the administration of neutralising antibody to IL-4 (11B11) at the time of parasite implant. Adult parasite-implanted mice that received 2 mg of anti-IL-4 at the time of implant had impaired ability to generate strongly suppressive PEC, with cells from only 2 out of 4 animals showing significant suppressive ability. In contrast, PEC from control implanted mice, or implanted mice injected with isotype-matched anti-IFN-γ (R46A2), significantly blocked cellular proliferation (Figure 2A)($P < 0.01$). This effect was achieved under conditions in which anti-IL-4 did not block the subsequent development of IL-4-producing T cells in the spleens of implanted mice (Figure 2B). Thus, a reduction in IL-4 levels at the time of initial exposure to the parasite was sufficient to reduce the ability of PEC to suppress proliferation.

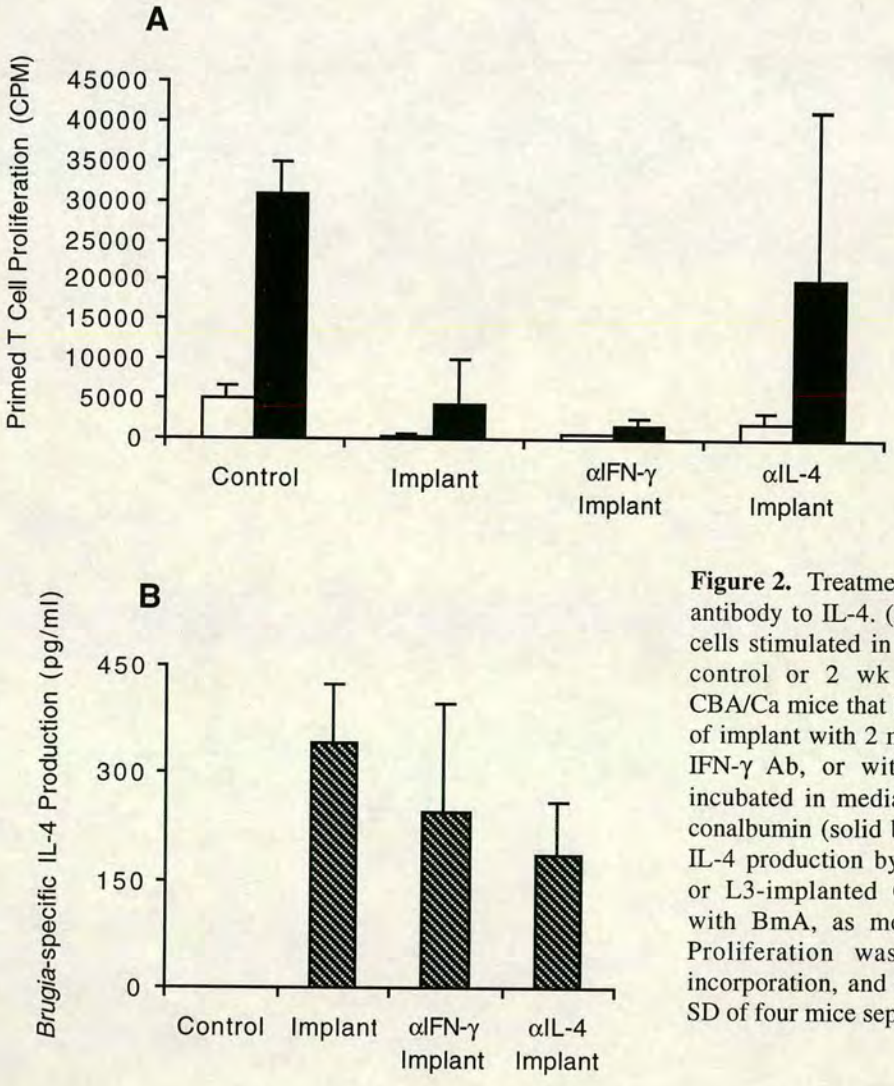


Figure 2. Treatment of mice with neutralising antibody to IL-4. (**A**) Proliferation of D10.G4 cells stimulated in the presence of PEC from control or 2 wk adult parasite-implanted CBA/Ca mice that had been treated at the time of implant with 2 mg/mouse anti-IL-4 or anti-IFN-γ Ab, or with no Ab. Cultures were incubated in media (open bars) or 50 μg/ml conalbumin (solid bars). (**B**) Antigen-specific IL-4 production by splenocytes from control or L3-implanted CBA/Ca mice stimulated with BmA, as measured by NK bioassay. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of four mice separately assayed.

4.2.3 Induction of proliferative suppression requires IL-4 production by the host

In order to more firmly establish the role of IL-4 in the generation of suppressive PEC, given the equivocal result gained from the neutralising antibody experiment, mice genetically deficient for the production of IL-4 (IL-$4^{-/-}$) on the C57BL/6 background were implanted with adult *B. malayi* parasites. In these experiments use of the D10.G4 cell clone was precluded as IL-4-deficient mice were not available on an H-2k background. Nylon wool purified lymphocytes from ovalbumin-primed syngeneic mice were therefore used as an alternative T cell source (see materials and methods). The ability to stimulate antigen-specific proliferation was compared in PEC taken from gene-deficient and wild-type C57BL/6 animals implanted with *B. malayi.* Cellular proliferation as measured by tritiated thymidine incorporation showed that, in contrast to wild-type mice, IL-$4^{-/-}$ mice implanted i.p. with adult *B. malayi* parasites failed to generate adherent PEC that blocked the proliferation of either ovalbumin-specific T cells (Figure 3A) or the B cell hybridoma, HB32 (Figure 3B).

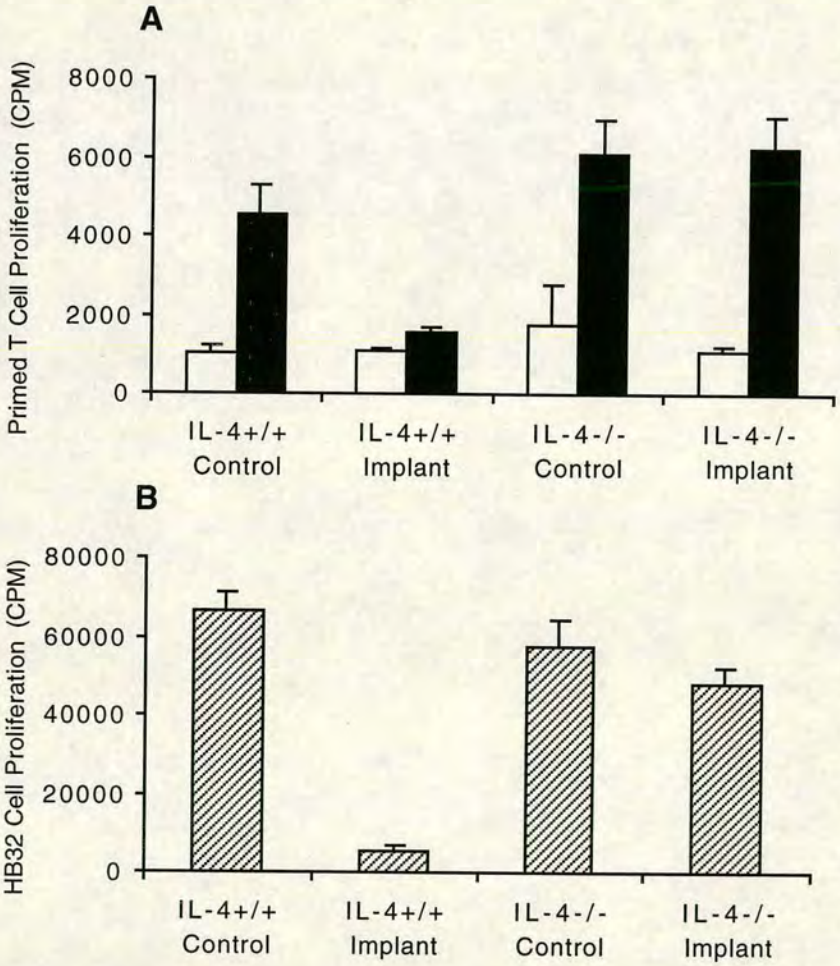


Figure 3. *Brugia* implant of IL-$4^{-/-}$ mice. Proliferation of (**A**) syngeneic ovalbumin-sensitised primary T cells or (**B**) the B cell hybridoma HB32, with PEC from IL-$4^{+/+}$ or IL-$4^{-/-}$ control or adult parasite-implanted C57BL/6 mice. T cells were incubated with PEC media (open bars), or 25 µg/ml ovalbumin (solid bars), and HB32 cells were incubated with PEC plus media (hatched bars). Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of four mice separately assayed.

Assessment of splenocyte cytokine responses showed that adult parasite implanted IL-$4^{+/+}$ mice produced elevated levels of parasite-specific IL-4 relative to uninfected controls. As expected, no IL-4 was produced by IL-$4^{-/-}$ mice (Figure 4).

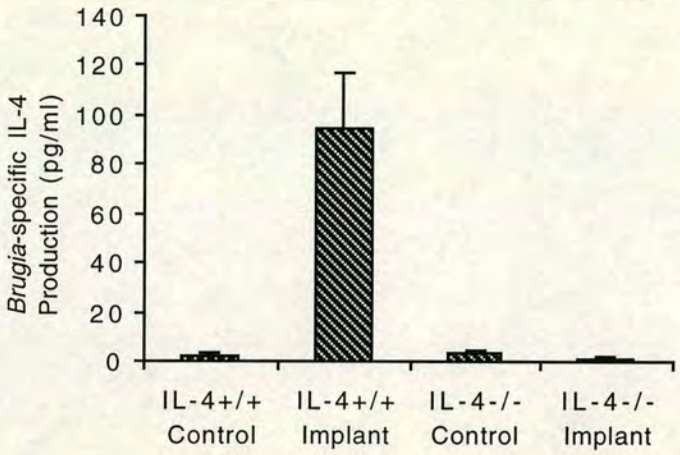


Figure 4. IL-$4^{-/-}$ splenocyte cytokine production. Antigen-specific IL-4 production by splenocytes from IL-$4^{+/+}$ or IL-$4^{-/-}$ control or adult parasite-implanted C57BL/6 mice stimulated with BmA, as measured by NK bioassay. Data are shown as mean ± SD of four mice separately assayed.

4.2.4 IL-10 production by the host is not essential for the induction of proliferative suppression

Having established that *in vivo* production of IL-4 is necessary for the induction of competent suppressor cells by filarial parasite implant, and given the importance of IL-10 as a down regulatory cytokine influenced by IL-4, we decided to investigate the role of IL-10 in the induction phase of suppression. Ovalbumin-primed lymph node cells were used as a responder lymphocye population as IL-10 mice were also not available on an H-2k background. C57BL/6 mice deficient in the production of IL-10 (IL-$10^{-/-}$) were implanted with adult *B. malayi* parasites. Adherent peritoneal exudate cells taken from these mice were capable of inducing proliferative suppression of ovalbumin-specific T cells as effectively as wild type mice (Figure 5A), thus IL-10 is not required for the induction of T cell hypo-responsiveness. However, suppression of proliferation of the B cell hybridoma HB32 was not as complete as that seen in similarly infected IL-$10^{+/+}$ mice (Figure 5B), suggesting that the suppressive PEC population may encompass a minor IL-10-dependent pathway effective on B cells. Similar levels of IL-4 were produced by splenocytes from IL-$10^{+/+}$ and IL-$10^{-/-}$ infected mice, with both groups

producing greater levels of cytokine than uninfected animals in response to parasite antigen challenge *in vitro* (data not shown).

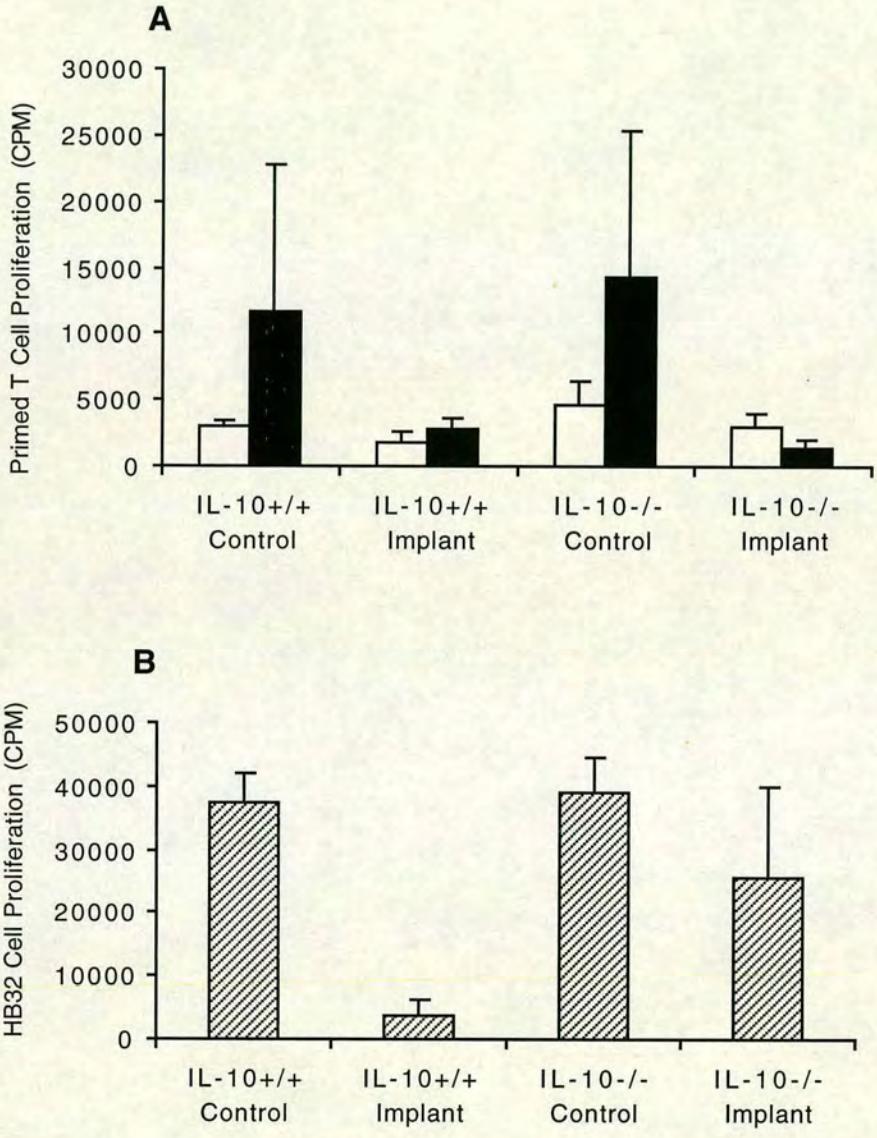


Figure 5. *Brugia* implant of IL-$10^{-/-}$ mice. Proliferation of (**A**) syngeneic ovalbumin-sensitised primary T cells or (**B**) the B cell hybridoma HB32, with PEC from IL-$10^{+/+}$ or IL-$10^{-/-}$ control or adult parasite-implanted C57BL/6 mice. T cells were incubated with PEC media (open bars), or 25 µg/ml ovalbumin (solid bars), and HB32 cells were incubated with PEC plus media (hatched bars). Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of five to six mice separately assayed.

4.2.5 Infection of nude mice with *B. malayi*

To investigate the involvement of host T cells at the induction phase of suppression, nude mice were implanted in the peritoneal cavity with adult *Brugia* parasites, and their ability to generate suppressive PEC assessed. As with the gene-deficient mice, ovalbumin-specific cells were used as responders in these experiments. Although these

experiments were carefully repeated several times, the results obtained were equivocal (Figure 6A). This was apparently due to the high levels of NO being produced in cultures containing ovalbumin-sensitised primary T cells in all of these experiments, as removal of these background levels of NO by addition of the NO inhibitor L-NMMA revealed a pattern of suppression. PEC from nude parasite-implanted mice displayed no significant suppressive ability, compared to the significant suppression exerted by PEC from parasite-exposed wild-type mice ($P < 0.04$). A second experiment not shown here gave a similar pattern, but parasite-implant-derived PEC did display a small but significant level of suppression of ovalbumin-primed cells. The two experiments together suggest that parasite-exposed PEC from nude mice are not as capable of generating suppressive host cells as their wild-type counterparts (Figure 6B).

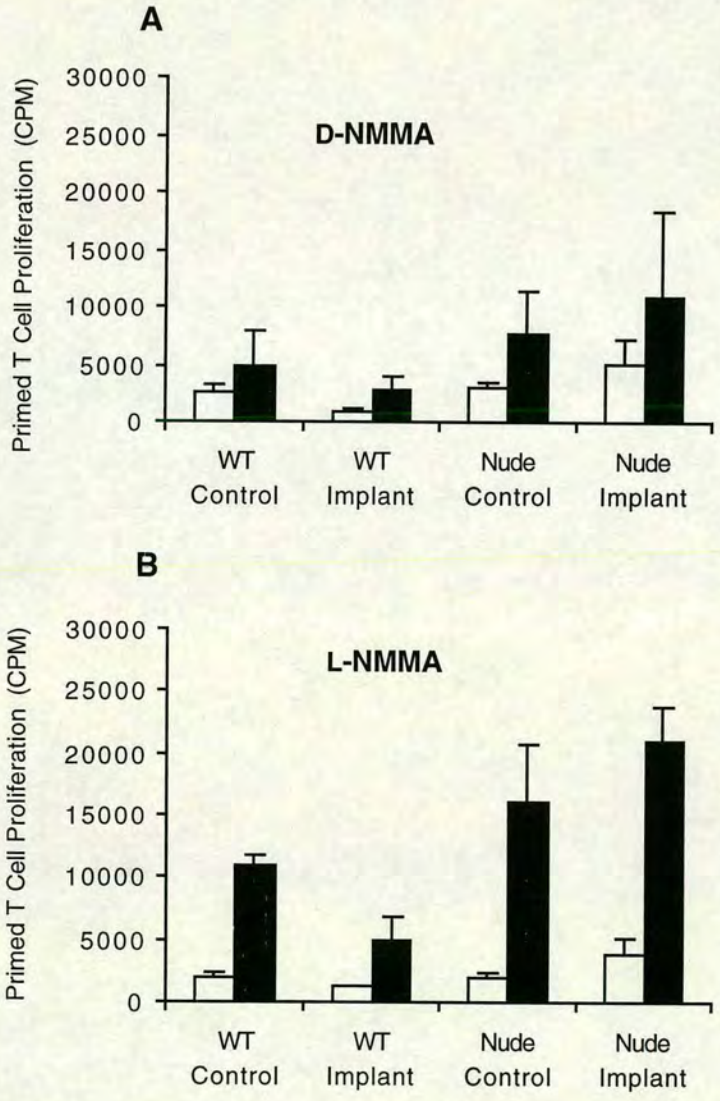


Figure 6. Adult parasite implant of nude mice (i). Proliferation of syngeneic ovalbumin-sensitised primary T cells with PEC from wild-type or nude control or adult *Brugia*-implanted BALB/c mice in the presence of (**A**) 250 µg/ml D-NMMA or (**B**) 250 µg/ml L-NMMA. T cells were incubated with media (open bars) or with 25 µg/ml ovalbumin (solid bars). Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of three to five mice separately assayed.

Additionally, proliferation of the B cell hybridoma HB32 was significantly suppressed by PEC from parasite-exposed wild-type mice ($P < 0.01$), but not by PEC from parasite-exposed nude mice (Figure 7).

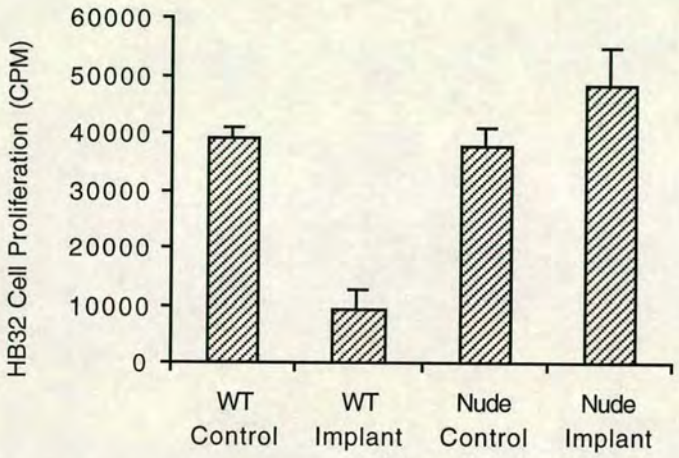


Figure 7. Adult parasite implant of nude mice (ii). Proliferation of HB32 cells plus media in the presence of PEC from wild-type or nude control or adult *Brugia*-implanted BALB/c mice. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of three to five mice separately assayed.

4.2.6 Infection of RAG knock-out mice with *B. malayi*

Having demonstrated a potential role for T cells in the generation of down-regulatory host cells, we chose to test RAG$^{-/-}$ mice, deficient in both B and T cells, for their ability to generate suppressive PEC. PEC from RAG$^{-/-}$ mice implanted with adult stage parasites failed to significantly block proliferation of either the HB32 hybridoma (Figure 8A), or the EL-4 lymphoma (Figure 8B).

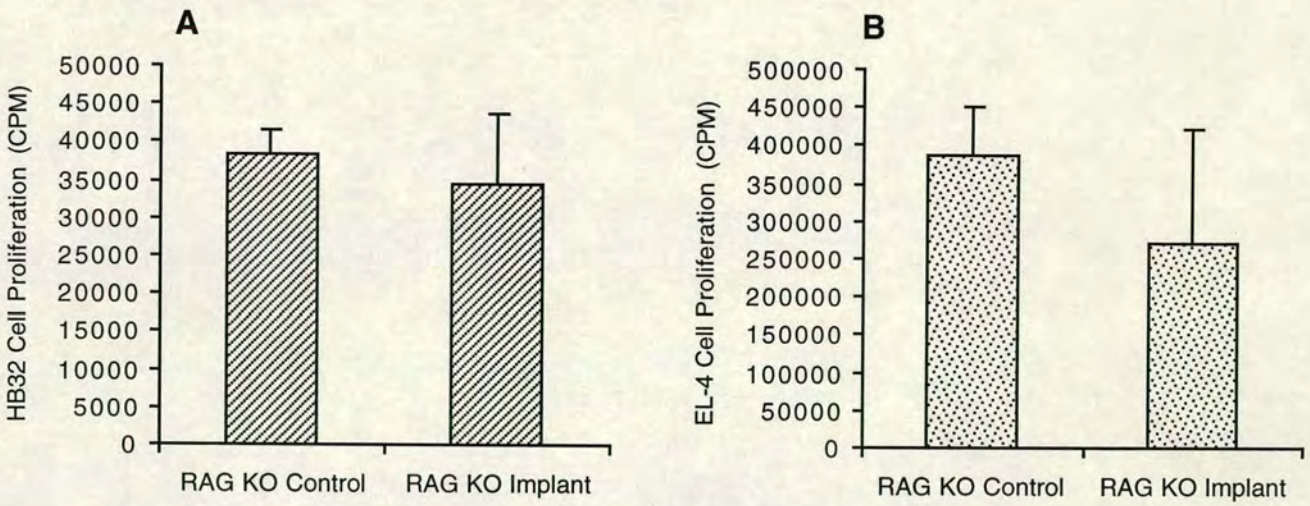


Figure 8. Infection of RAG$^{-/-}$ mice with *B. malayi*. Proliferation of (**A**) HB32 or (**B**) EL-4 cells with PEC from control or adult parasite implanted RAG$^{-/-}$ mice. Proliferation was measured by [^{3}H]TdR incorporation and data shown are mean ± SD of five mice in each group, separately assayed.

4.2.7 Parasite survival in genetically deficient mice

To determine if the emergence of down-regulatory host cells in the peritoneal cavity correlated with increased or decreased parasite survival, the presence of live adult parasites, and recovered numbers of live Mf was assessed at the time that peritoneal washes were carried out. IL-4-deficient mice showed no significant difference to wild-type mice regarding parasite recovery (Table 1), consistent with previously published data (Lawrence et al., 1995).

Table 1. *Parasite recovery from gene-deficient mice*

Mouse Type	% Recovery of adult parasites	Mf recovered from adult-implanted animals (Mf/μl)
IL-4+/+	100	9.4 ± 8.0
IL-4-/-	100	4.4 ± 1.2
IL-10+/+	67	6.0 ± 3.8
IL-10-/-	60	3.6 ± 4.3

Table 1. Recovery of live parasites from the peritoneal cavity of wild-type and gene-deficient C57BL/6 mice. Data shown represent (a) the percentage of mice in each experimental group from which live adult parasites were recovered, and (b) the numbers of live Mf recovered from adult parasite-implanted mice. Recoveries are shown as Mf/μl from a total volume of 10 ml, and differences were not statistically significant. Numbers of Mf recovered are mean ± SD of counts performed on four to six individual mice in each group. Counts were made three weeks after parasite implant.

Although IL-10-deficient mice also showed no significant reduction in recovery of live parasites from the peritoneal cavity (Table 1), recovery of low numbers of Mf correlated with enhanced systemic production of IFN-γ in individual mice (Figure 9). This correlation was not observed in any other experimental group (data not shown).

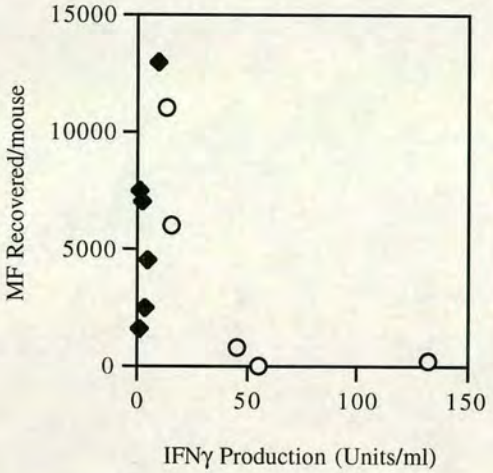


Figure 9. Correlation of IFN-γ production with low Mf recovery in IL-$10^{+/+}$ (diamonds) and IL-$10^{-/-}$ (circles) mice. Total numbers of Mf recovered (y-axis), plotted against BmA-specific splenocyte IFN-γ production (x-axis) in IL-$10^{-/-}$ mice which had been implanted with adult parasites 3 wk earlier. Each data point represents one individual animal.

4.3 Discussion

We have shown that live *B. malayi* parasites, or physiological concentrations of *B. malayi* ES, are unable to directly affect cell proliferation *in vitro*, and that at least one week of host exposure is required before suppressive cells can be recovered from infected mice ((Allen et al., 1996)and Chapter 3). Thus, it seemed probable that the generation of down-regulatory cells required additional input from components of the host immune system.

IL-4 production is likely to be one of the first events to occur following exposure to helminth parasites (Sabin et al., 1996). Production of IL-4 is greatly expanded in *Brugia* infected mice (Bancroft et al., 1993; Lawrence et al., 1995; Lawrence et al., 1994), and in this study we see elevated levels of IL-4 produced by mice exposed to adult *Brugia* parasites, and a direct correlation between parasite-specific IL-4 production in the spleen and PEC-mediated suppression of lymphocyte proliferation at each dose of L3.

IL-4$^{-/-}$ mice were unable to generate a suppressive PEC population, formally demonstrating that IL-4 is required. We cannot yet determine whether IL-4 alone is sufficient for suppressive PEC development, or if a mature Th2 response must first emerge. However, mice given anti-IL-4 at the time of implantation still develop 'Th2' responses but are unable to generate full suppression. This suggests that it is *early* IL-4 production, and not necessarily Th2 establishment, that may be the essential factor in the development of a proliferative block. The results obtained from the nude mouse experiments suggest that T cells may be involved in activation or recruitment of suppressive PEC, perhaps acting through amplification of the IL-4 signal. However, since it is possible to generate some down-regulatory activity in the absence of a T cell contribution, there may be a degree of redundancy in this system, with alternative cellular sources of IL-4. The data obtained from implant of RAG knock-out mice deficient in T or B cells also support this hypothesis, where an absence of lymphocytes, and therefore a greatly reduced cytokine environment, resulted in a failure to reliably recruit or activate suppressive PEC. Further investigation of *Brugia*-exposed RAG$^{-/-}$

mice, for example adoptive transfer of defined lymphocyte populations prior to implant of parasites, should help delineate the respective roles of T and B cells in generation of down-regulatory cells.

IL-10 has been implicated as a mediator of filarial non-proliferative responses in human patients in some (Mahanty and Nutman, 1995; Mahanty et al., 1997) but not all (Baize et al., 1997; Sartono et al., 1995) studies. Surprisingly, IL-$10^{-/-}$ mice generated PEC able to fully suppress the D10.G4 clone. However, a partial role for this potent down-regulatory cytokine is suggested by the fact that, in contrast to the wild-type, IL-$10^{-/-}$ mouse implant-derived PEC induce less profound suppression of the HB32 hybridoma. Intriguingly, a role for IL-10 in promoting parasite survival was implicated by reduced recovery of Mf and adults from the peritoneal cavity of infected IL-$10^{-/-}$ mice. The *in vivo* relevance of this finding is not clear, as the reduced parasite survival observed in IL-10-deficient mice may be due to artificially high levels of IFNγ produced by these animals. Elevated IFN-γ production in IL-$10^{-/-}$ mice would be consistent with the proposed role for IL-10 involvement in regulation of IFN-γ (Fiorentino et al., 1991). Irrespective of the precise mechanism and the role of IL-10, the fact that reduced numbers of live parasites are recovered from animals producing high levels of the Th1 cytokine IFNγ, and not the Th2 cytokine IL-4, adds strength to the argument that the Th2 response seen in filarial infection may not mediate parasite killing.

We have established that IL-4, but not IL-10, is critical for the *in vivo* induction of suppressor cells, but we still do not know the mechanism that underlies this relationship. It is possible that IL-4 or IL-4-driven cytokines may act alone or in concert with parasite-derived factors to directly activate recruited or resident cells to suppressor function. It has recently been shown that 'alternative' activation of macrophages with IL-4 can cause inhibition of mitogen-mediated proliferation of T cells, apparently independently of IL-10 production (Schebesch et al., 1997). The strong Th2 response that results from filarial infection may provide a suitable environment for the generation of such alternatively activated macrophages. Additionally, our results indicate that host lymphocytes are important in the generation of down-regulatory cells, perhaps through providing an appropriate cytokine environment for development or recruitment.

However, we have seen that suppressive PEC may in some cases be generated in the absence of T cells, suggesting that there is a degree of redundancy in the system leading to generation of suppresser cells. In some cases production of IL-4 by alternative cell types may be able to compensate for the absence of T cells. Difficulty in interpretation of the results obtained in the nude mouse experiments may be a consequence of the complexity of the balance existing between alternative inductive mechanisms. Finally, we have revealed a potential link between parasite killing and host production of the Th1-type cytokine IFNγ, a finding that is somewhat surprising given that infection, and host protection, is often more generally associated with production of the Th2-type cytokine IL-4.

Chapter 5: Suppressor cell identity

(Published in part in Journal of Immunology. See Appendix 2)

5.1 Introduction

A key objective of this work was identification of the cell population(s) responsible for the development of the anti-proliferative effect. One of the attractions of this murine model of *B. malayi* infection was that it allowed us the opportunity both to carry out a detailed analysis of the phenotype of the cells that were recruited to the site of parasite exposure (the peritoneal cavity), and to test these cells for suppressive function.

Helminth infection, as well as being associated with the development of a pronounced Th2-type immune response, is also associated with the generation of a distinctive pattern of cell infiltration to the site of infection. This cellular infiltrate, best studied in granuloma formation in schistosomiasis (Brunet et al., 1998), is characteristically composed of a high proportion of eosinophils, in addition to activated macrophages and other inflammatory cell types. In *Brugia* infection, work in animal models has shown that, although there is a recruitment of inflammatory cells to the site of infection, there is generally an absence of inflammatory pathology at these sites (Denham and McGreevy, 1977; Miller et al., 1991).

The recruitment of macrophages to the site of filarial infection is not surprising given the important role this cell type plays in co-ordinating host immune responses. The macrophage has long been recognised as having both immunostimulatory and immunosuppressive ability, with the mantle assumed being determined by the circumstances involved. The study of 'suppressor macrophages', highly fashionable during the '80's, revealed a multitude of instances where macrophages could assume an immunosuppressive role.

It is the dramatic eosinophilia that accompanies filarial and other helminth infections that provides somewhat of an enigma. Although being a dominant feature of helminth infection, the actual function of eosinophils is still not wholly understood. The accepted

dogma, that these granulocytic cells are integral in the response that eventually leads to killing and clearance of parasites, is based on somewhat questionable *in vitro* killing assays (Hamann et al., 1990), or elaborate diffusion chamber experiments (Lange et al., 1993; Rotman et al., 1996). Additionally, attempts to define eosinophil function in filarial infection have essentially concentrated on the killing of Mf, and not adult parasites. In fact, adult and Mf life cycle stages of the parasite appear to be able to survive for a substantial time in the presence of eosinophils, both *in vitro* and *in vivo* (Lawrence, 1996).

This chapter details the investigation of the possible phenotype of the cell type(s) recruited or activated to suppressive function by intra-peritoneal implant of mice with *B. malayi*. The identification of suppressive cell(s) is a necessary prerequisite to fully understand the down-regulatory mechanisms employed by such a cell type. Apart from helping to identify the cell types involved in the generation of suppressive PEC, this approach would also yield important information regarding the dynamics of cell recruitment to the site of infection.

As a prerequisite to identification of the suppressive cell type, we sought to characterise the differences in cell recruitment between control and parasite-implanted mice, the premise being that any obvious differences might provide immediate clues for the identification of the suppressive cell type. Cytospins of peritoneal washes were made to allow morphological examination of cell types present at the site of infection, and the proportions of different cell subpopulations were compared between control and parasite-implanted animals.

In addition to cytospin analysis, flow cytometry was used to allow a comparison of expression of a range of cell surface markers, including CD4, CD8, B7-1 and B7-2, on peritoneal exudate cells taken from parasite-implanted or control mice. The data from this work was used to identify candidate suppressor cells. These were then tested for suppressive ability after enrichment using flow cytometry or magnetic bead sorting.

5.2 Results

5.2.1 Peritoneal cell recruitment in *B. malayi* implanted mice

To investigate cell recruitment initiated by *Brugia* implant, the proportions of different peritoneal cell sub-populations were determined from cytocentrifuge preparations (cytospins) taken from unimplanted mice, mice implanted with *Brugia* adults or L3, or mice implanted with dead adult parasites, live adult parasites, Mf, or injected with the inflammatory agent thioglycolate (For examples see Appendix 1).

Adult and L3 implanted mice showed recovery of similar populations of cells from the peritoneal cavity, with implant of both stages resulting in a marked reduction in proportions of mast cells, and an increase in proportions of both macrophages and eosinophils, in comparison to unimplanted control mice (Figure 1A). During the course of these investigations, eosinophils consistently comprised 10-20% of total cells recovered from the peritoneal cavity of adult parasite implanted mice, and in some cases reached up to 50% of cells. Significant recruitment of eosinophils to the peritoneal cavity was seen in mice implanted with as few as 10 L3 parasites.

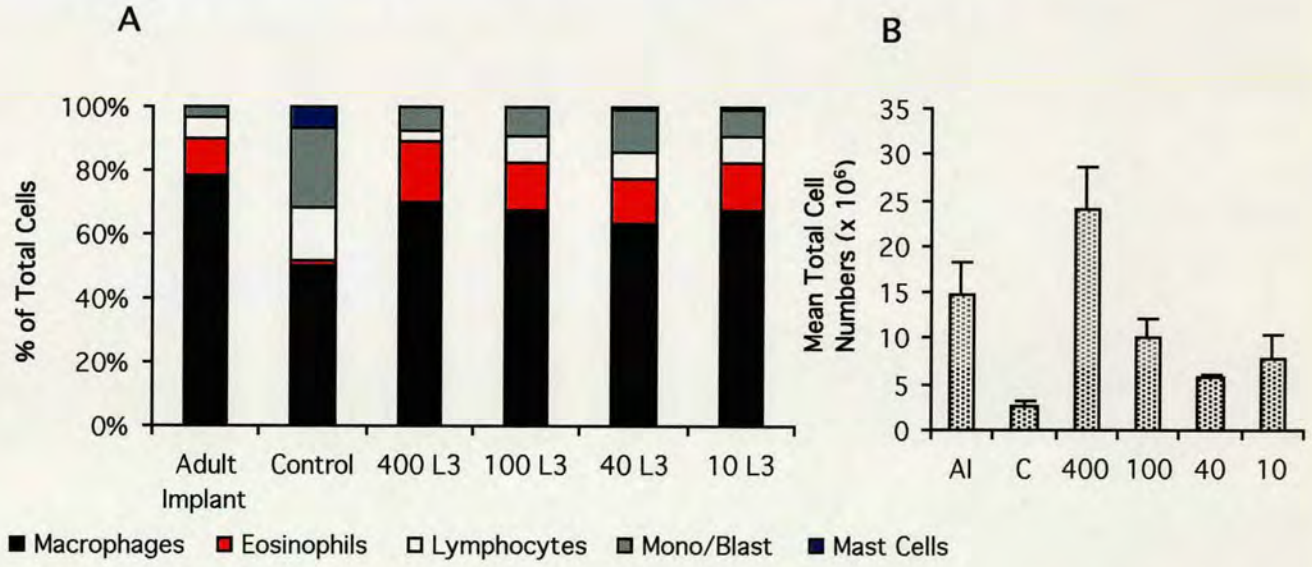


Figure 1. Peritoneal cell populations in control, and adult- and L3-implanted CBA/Ca mice. (**A**) Mean percentage of total cells that were identified as mast cells (blue bars), monocytes/blasting cells (grey bars), lymphocytes (open bars), eosinophils (red bars), and macrophages (black bars). The cell composition of PEC from control and implanted mice was determined from cytospins by microscopy. Data shown are mean of three to four individual mice separately analysed. (**B**) mean total cell numbers recovered from the peritoneal cavity of control or implanted mice. Data presented are mean ± SD of three to four individual mice. See Chapter 3, Figure 5 for proliferation data from this experiment.

Implant with either the adult or infective larval stage of *Brugia* into mice provoked a dramatic increase in total cell numbers recruited to the peritoneal cavity in comparison to unimplanted control mice, with three to five times as many cells being routinely recovered from implanted animals (Figure 1B). Thus, increased ratios of macrophages and eosinophils reflected not only a proportional increase, but also a dramatic increase in total cell numbers of each type (Table 1). Notably, despite the increased number of recruited cells in adult and L3 implanted mice, both the percentage and total number of mast cells was significantly decreased ($P < 0.05$).

Table 1. *Adult and L3 recruited peritoneal cell types*

	Total Number of Cells/animal ($x10^6$)					
	Adult Implant	Control	400 L3	100 L3	40 L3	10 L3
Macrophages	11.3 ± 0.67	1.4 ± 0.21	16.6 ± 1.8	6.6 ± 0.67	3.5 ± 0.58	5.2 ± 1.1
Eosinophils	1.7 ± 0.8	0.04 ± 0.02	4.5 ± 1.5	1.5 ± 0.6	0.77 ± 0.3	1.2 ± 0.4
Lymphocytes	0.95 ± 0.23	0.45 ± 0.08	0.75 ± 0.37	0.8 ± 0.4	0.45 ± 0.08	0.61 ± 0.2
Monocytes/ Blasting cells	0.39 ± 0.05	0.67 ± 0.15	1.8 ± 0.38	0.9 ± 0.31	0.74 ± 0.15	0.66 ± 0.1
Mast Cells	0.04 ± 0.03	0.19 ± 0.04	0.01 ± 0.02	ND	0.03 ± 0.01	0.05 ± 0.07

Data represent the mean total number ($x\ 10^6$) of each cell sub-population recovered in peritoneal washes. The cell composition of PEC from control and implanted CBA/Ca mice was determined from cytospins by microscopy. Data shown are mean ± SD of three to four individual mice separately assayed. N.D. = not detectable.

Consistent with differences in their ability to generate suppressive PEC (Figure 2) adults and Mf showed markedly different cell recruitment profiles.

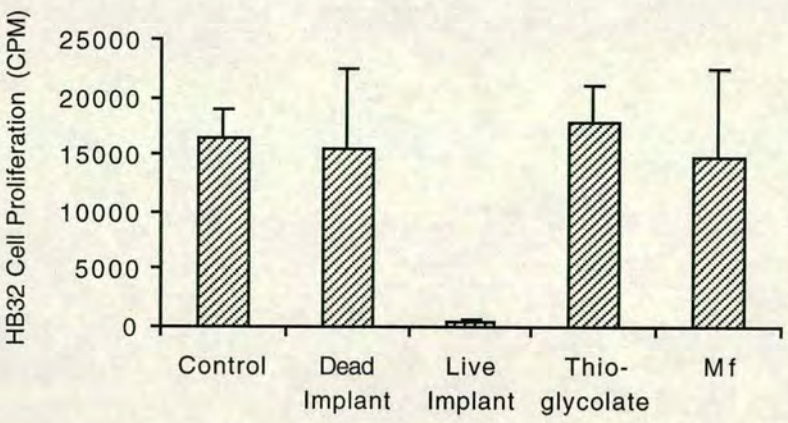


Figure 2. Comparison of suppressive ability of differentially induced PEC. Proliferation of HB32 cells in the presence of PEC from control, dead adult, live adult, thioglycolate injected, or Mf-implanted CBA/Ca mice. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of three to five mice separately assayed.

Mf-implanted mice showed a minor increase in the proportion of lymphocytes, a decrease in the proportion of macrophages, and only a minor eosinophilia in comparison to adult implants (Figure 3A). Additionally, the large increase in total cell numbers that was apparent in L3- and adult- implanted mice (Figure 1B) was not seen with Mf-injected animals (Figure 3B). However, Mf-implanted mice did mount an immune response against the parasite, as a high level of antigen-specific lymphocyte reactivity was seen in response to Mf implant (data not shown).

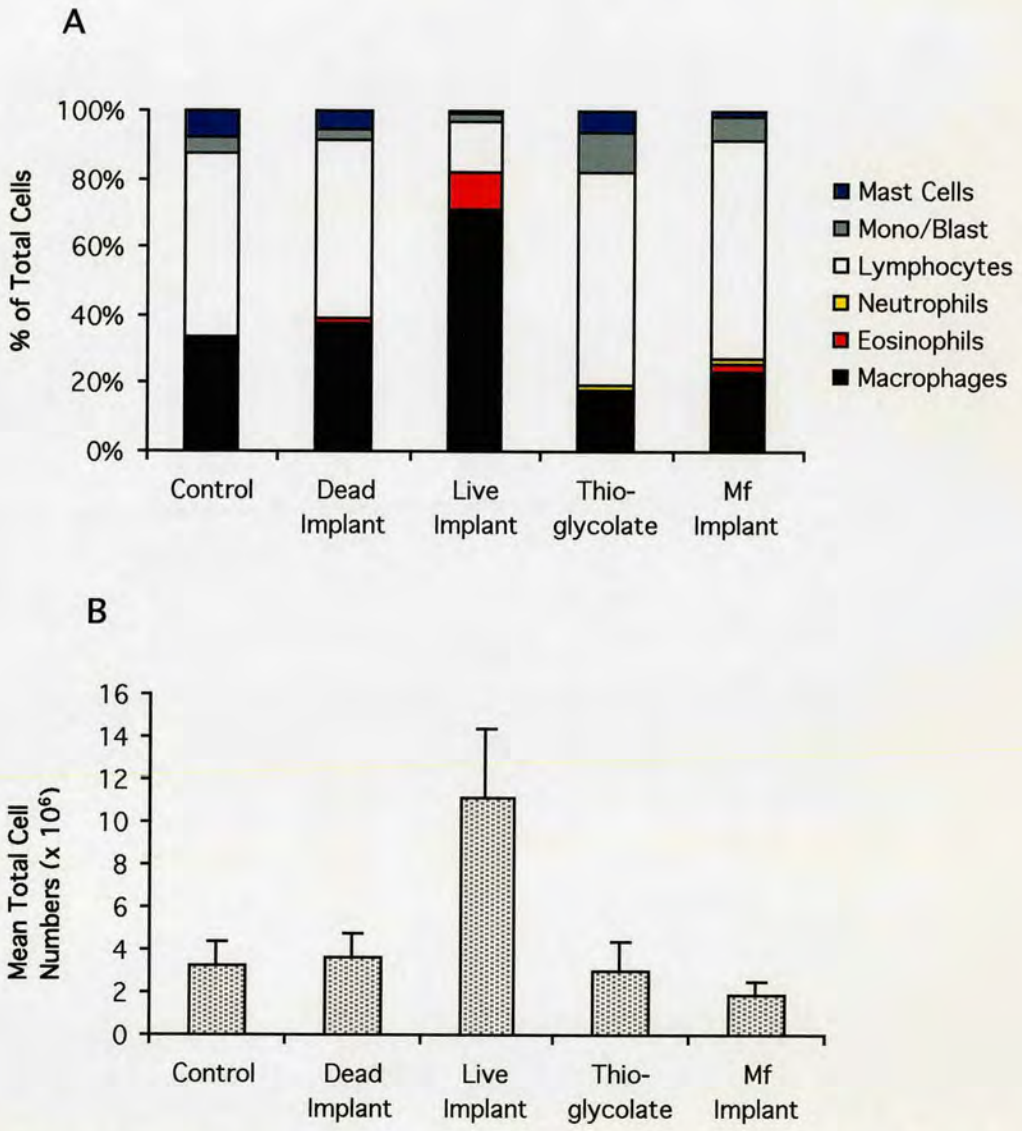


Figure 3. Peritoneal cell populations in control and parasite implanted CBA/Ca mice. **A**) mean percentage of total cells that were identified as mast cells (blue bars), monocytes/blasting cells (grey bars), lymphocytes (open bars), neutrophils (yellow bars), eosinophils (red bars), and macrophages (black bars). The cell composition of PEC from control and implanted mice was determined from cytospins by microscopy. Data shown are mean of three to five individual mice separately analysed. (**B**) mean total cell numbers recovered from the peritoneal cavity of control or implanted mice. Data presented are mean ± SD of three to five individual mice.

Mf did not provoke a significant increase in total numbers of any of the cell sub-types, with most cell types showing equivalent total numbers to controls, and a significant decrease in total numbers of macrophages in comparison to controls. However Mf, like the adult and L3 stages, also showed a significant decrease in total mast cell numbers recovered from the peritoneal cavity in comparison to controls (Table 2).

Table 2. *Recruited and resident peritoneal cell types*

	Total Number of Cells/animal ($x10^6$)				
	Control	Dead Adult Implant	Live Adult Implant	Thiogly-colate	Live Mf Implant
Macrophages	1.12 ± 0.5	1.47 ± 1.0	7.85 ± 2.28	0.51 ± 0.2	0.42 ± 0.16
Eosinophils	0.01 ± 0.01	0.07 ± 0.1	1.23 ± 0.53	0.02 ± 0.01	0.04 ± 0.06
Neutrophils	ND	ND	0.04 ± 0.03	0.03 ± 0.02	0.03 ± 0.04
Lymphocytes	1.76 ± 0.55	1.78 ± 0.44	1.56 ± 0.45	1.96 ± 1.14	1.22 ± 0.5
Monocytes/ Blasting cells	0.17 ± 0.07	0.14 ± 0.03	0.29 ± 0.05	0.32 ± 0.11	0.14 ± 0.06
Mast Cells	0.25 ± 0.09	0.19 ± 0.08	0.06 ± 0.04	0.15 ± 0.04	0.02 ± 0.02

Data represent the mean total number (x 10^6) of each cell sub-population recovered in peritoneal washes. The cell composition of PEC from control and implanted CBA/Ca mice was determined from cytospins by microscopy. Data shown are mean ± SD of three to five individual mice separately assayed. N.D. = not detectable.

In addition to recruitment of the cell sub-populations described above, examination of cytospins made of PEC from adult parasite implanted mice revealed the presence of a significant number of large, multi-nucleate macrophages (Figure 4).

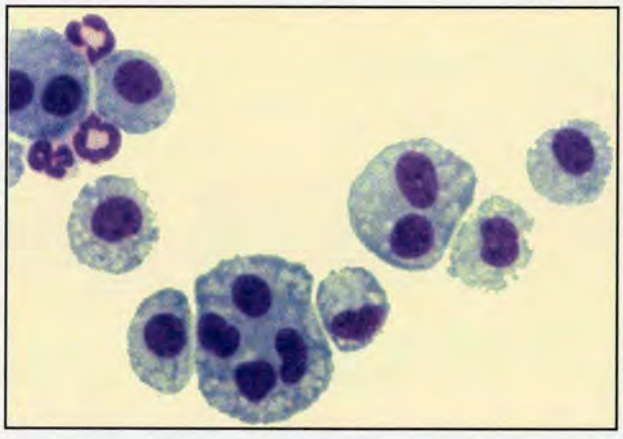


Figure 4. Adult parasite implant induces formation of multi-nucleate macrophages. Cytocentrifuge preparation of PEC from adult *Brugia*-implanted mice, stained with Diff-Quick, and examined at 400x magnification. Cells with ring-shaped nuclei and red staining granules are eosinophils, and the remainder are single or multi-nucleate macrophages.

5.2.2 Time course of cell recruitment into the peritoneal cavity

Suppressive PEC are not recovered from the peritoneal cavity of adult-implanted mice until at least one week after initial host exposure (see chapter 3). To assess the dynamics of cell recruitment during the period that suppressor cells are presumably recruited and/or activated, the percentage of different cell subpopulations was determined by examination of cytocentrifuge preparations of PEC taken from mice that had been exposed to adult parasites for an increasing amount of time (Figure 5A).

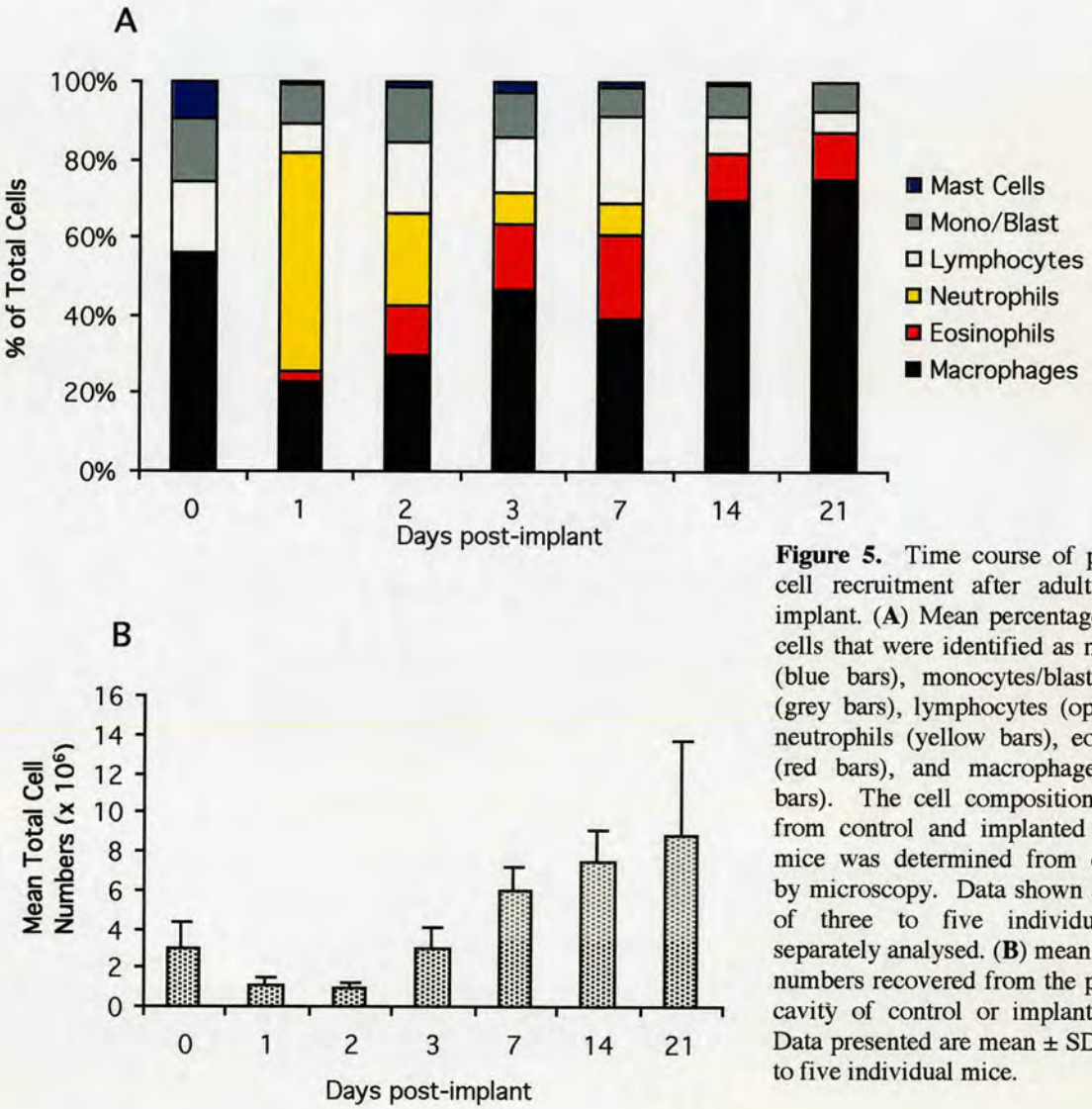


Figure 5. Time course of peritoneal cell recruitment after adult parasite implant. (**A**) Mean percentage of total cells that were identified as mast cells (blue bars), monocytes/blasting cells (grey bars), lymphocytes (open bars), neutrophils (yellow bars), eosinophils (red bars), and macrophages (black bars). The cell composition of PEC from control and implanted CBA/Ca mice was determined from cytospins by microscopy. Data shown are mean of three to five individual mice separately analysed. (**B**) mean total cell numbers recovered from the peritoneal cavity of control or implanted mice. Data presented are mean ± SD of three to five individual mice.

The proportion of total cells recovered that comprise those of the macrophage phenotype increased steadily from 2 days post-implant to reach a maximum after 2 to 3 weeks. The proportion of lymphocytes decreased after parasite exposure although,

when the increase in total numbers of cells recovered from the peritoneal cavity of these implanted mice is taken into account (Figure 5B), real numbers of this cell type are actually elevated in implanted mice (Table 3). Eosinophil infiltration could be seen as early as 1 day post-implant, and rose to comprise approximately 20% of total PEC after 1 week. Initial high neutrophilia evident from day 1 reduced steadily until little or no neutrophils could be detected by day 14, consistent with known dynamics of rapid neutrophil recruitment to, and clearance from, sites of inflammation. Mast cells were absent in peritoneal washes from adult parasite implanted mice from the earliest time point examined, and could only be found at very low levels throughout infection, even as rapidly as day 1 post-implant.

Table 3. *Time course of peritoneal cell recruitment*

	Total Number of Cells/animal ($x10^6$)						
	Day 0	Day 1	Day 2	Day 3	Day 7	Day 14	Day 21
Macrophages	1.67 ± 0.81	0.21 ± 0.06	0.9 ± 0.22	1.42 ± 0.53	2.29 ± 1.27	5.12 ± 0.86	6.57 ± 3.54
Eosinophils	ND	0.03 ± 0.01	0.14 ± 0.06	0.55 ± 0.34	1.24 ± 0.39	0.93 ± 0.4	1.07 ± 0.6
Neutrophils	ND	0.5 ± 0.03	0.25 ± 0.1	0.27 ± 0.19	0.44 ± 0.15	0.01 ± 0.02	ND
Lymphocytes	0.52 ± 0.17	0.06 ± 0.02	0.2 ± 0.03	0.44 ± 0.17	1.12 ± 0.26	0.71 ± 0.42	0.46 ± 0.24
Monocytes/ Blasting cells	0.52 ± 0.25	0.09 ± 0.01	0.15 ± 0.04	0.37 ± 0.18	0.39 ± 0.13	0.61 ± 0.26	0.73 ± 0.49
Mast Cells	0.27 ± 0.16	0.01 ± 0.01	0.02 ± 0.01	0.06 ± 0.02	0.06 ± 0.01	0.05 ± 0.03	0.01 ± 0.02

Data represent the mean total number (x 10^6) of each cell sub-population recovered in peritoneal washes. The cell composition of PEC from control and implanted CBA/Ca mice was determined from cytospins by microscopy. Data shown are mean ± SD of three to five individual mice separately assayed. N.D. = not detectable.

5.2.3 Flow cytometric analysis of parasite-exposed PEC populations

A second approach used to investigate cell recruitment was to use flow cytometry to analyse PEC from mice implanted with Mf, L3, or adult parasites and use PEC from control, non-implanted mice as a comparison. Simple examination of forward scatter (size) and side scatter (internal complexity) profiles of PEC from control, adult, L3, or Mf infected mice showed a significant increase in the percentage of large, granular cells recovered from adult and L3 implanted mice (Figure 6).

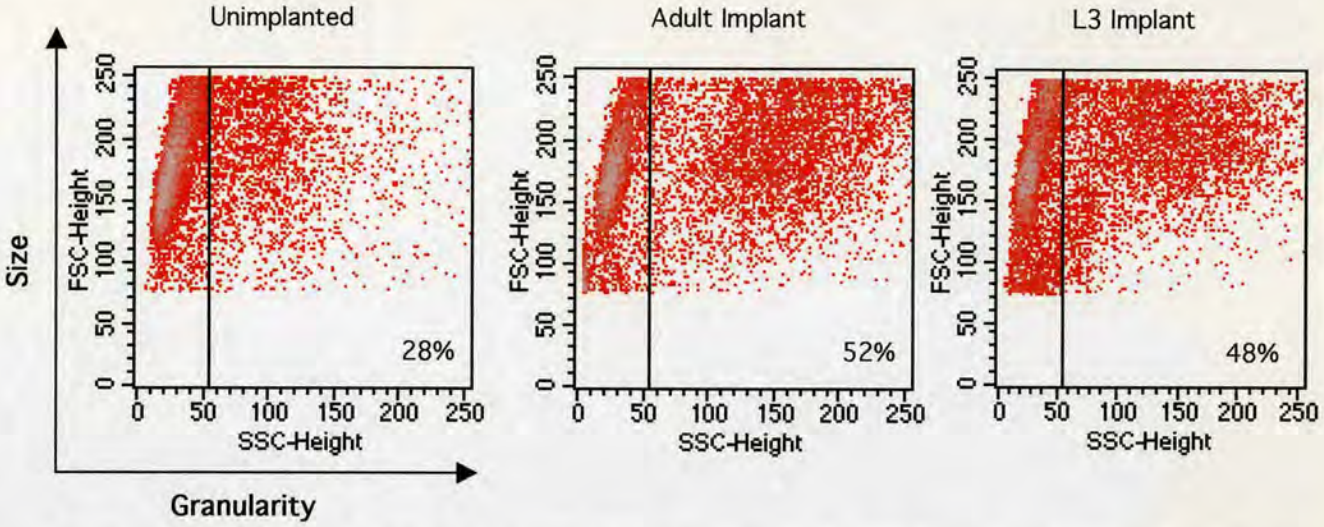


Figure 6. Scatter profiles of PEC from adult- and L3-implanted mice. Forward scatter (FSC) and side scatter (SSC) profiles of PEC from control (unimplanted), adult-, or L3-implanted CBA/Ca mice. Control PEC were combined cells from 5 unimplanted mice, whereas adult- and L3-implant-derived PEC were typical results obtained from individual mice. Figures refer to the percentage of total cells sampled that fall within the granulocyte/macrophage gate. Implant with either life cycle stage provoked the dramatic recruitment of large, granular cells.

The profile for Mf implant derived PEC was strikingly different to that of both the control and the adult implant derived PEC. The large granular cells recruited to the peritoneal cavity by adult or L3 parasites were not present in the Mf exposed samples (Figure 7), again consistent with the data obtained from cytospin analysis.

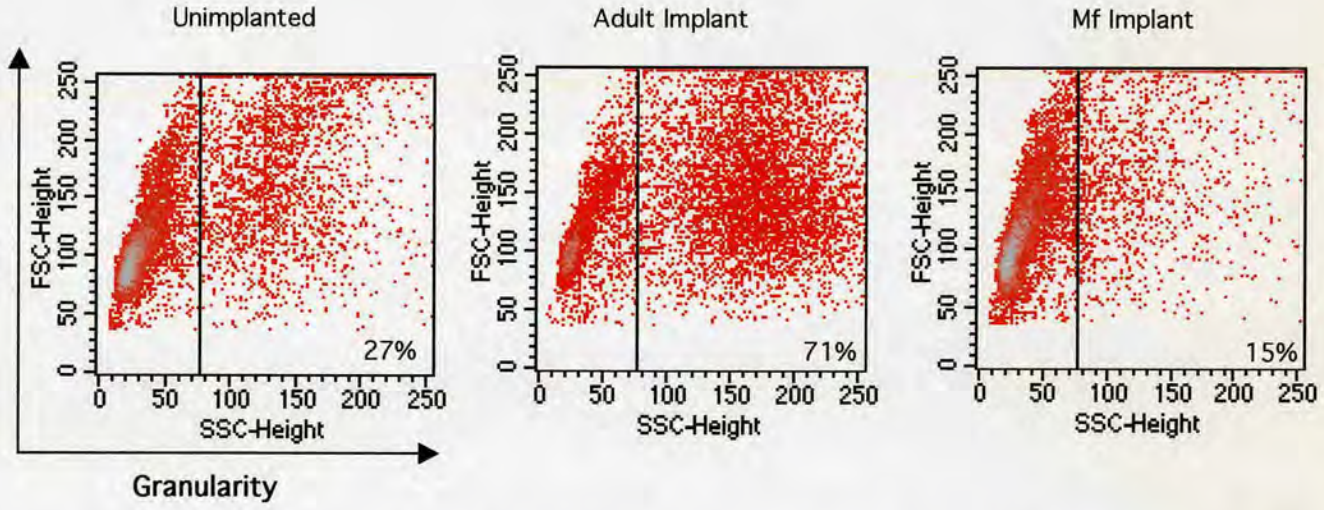


Figure 7. Scatter profiles of PEC from adult- and Mf-implanted mice. Forward scatter (FSC) and side scatter (SSC) profiles of PEC from control (unimplanted), adult-, or Mf-implanted CBA/Ca mice. Control PEC were combined cells from 4 unimplanted mice, and Mf PEC were combined cells from 4 implanted mice, whereas adult-implant-derived PEC were typical results obtained from one implanted mouse. Figures refer to the percentage of total cells sampled that fall within the granulocyte/macrophage gate. Implant with Mf did not provoke the dramatic recruitment of large, granular cells.

PEC resulting from daily immunisation with adult *Brugia* ES, as well as being suppressive, were also phenotypically similar to those induced by infection with live adult parasites. Flow cytometric analysis showed that PEC recovered from ES injected animals included large granular cells characteristic of adult or L3 implanted mice (Data not shown). Hence, PEC from adult ES injected animals are also phenotypically quite distinct from those recovered from Mf-implanted animals.

Three-colour staining was used to carry out a more detailed assessment of the phenotype of resident or recruited PEC from unimplanted mice, or mice that had been implanted with live adult *Brugia*, dead adults, or Mf.

Cytospins had shown that adult parasites did not induce an overall increase the percentage or total numbers of lymphocytes recovered from the peritoneal cavity, but could not identify if there was an increase or decrease in defined lymphocyte types. The various treatment groups were compared for the percentage of cells within the lymphocyte gate that expressed the surface markers CD4 or CD8 (Figure 8). Adult-implanted mice showed a marked increase in the proportion of those lymphocytes found to be $CD4^+$ or $CD8^+$, in comparison to unimplanted, dead-adult-implanted, or Mf-implanted mice. Mf alone caused little change over controls in terms of the percentage of gated lymphocytes that were $CD4^+$ or $CD8^+$.

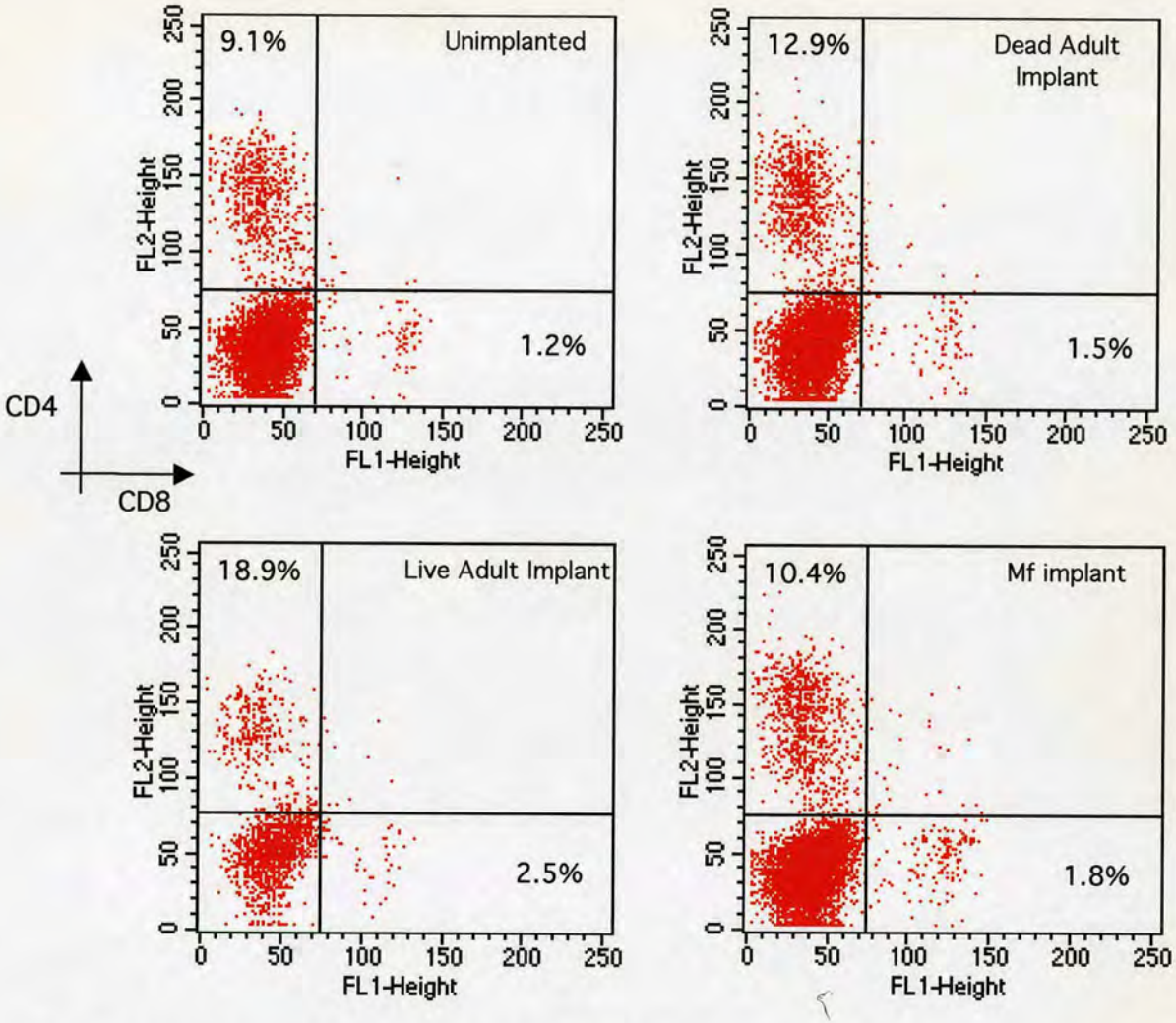


Figure 8. Lymphocyte expression of CD4 and CD8. CD4 (FL2) and CD8 (FL1) expression was assessed on gated lymphocytes of PEC from control (unimplanted), live adult-, dead adult-, or Mf-implanted CBA/Ca mice. Control PEC were combined cells from 4 unimplanted mice, dead implant PEC were combined cells from 5 mice each implanted with 10 dead adult parasites, Mf PEC were combined cells from 4 mice each injected with $2x10^5$ Mf i.p., and adult-implant-derived PEC were typical results obtained from one mouse implanted with live parasites. Lymphocytes were gated on the basis of their forward and side scatter profiles. Quadrants were set on unstained samples from each group. Figures refer to the percentage of gated lymphocytes staining positive for either CD4 (vertical axis) or CD8 (horizontal axis).

In order to determine whether downregulation of cellular proliferative responses might be due to a reduction in the ability of parasite-exposed host cells to effectively costimulate, PEC were analysed by flow cytometry for expression of the two key costimulatory molecules B7-1 and B7-2. Adult parasite-implanted animals actually showed an increased percentage of granulocytes/macrophages expressing B7-2, in

comparison to PEC from unexposed mice, with levels of B7-1 in both groups being low (Figure 9).

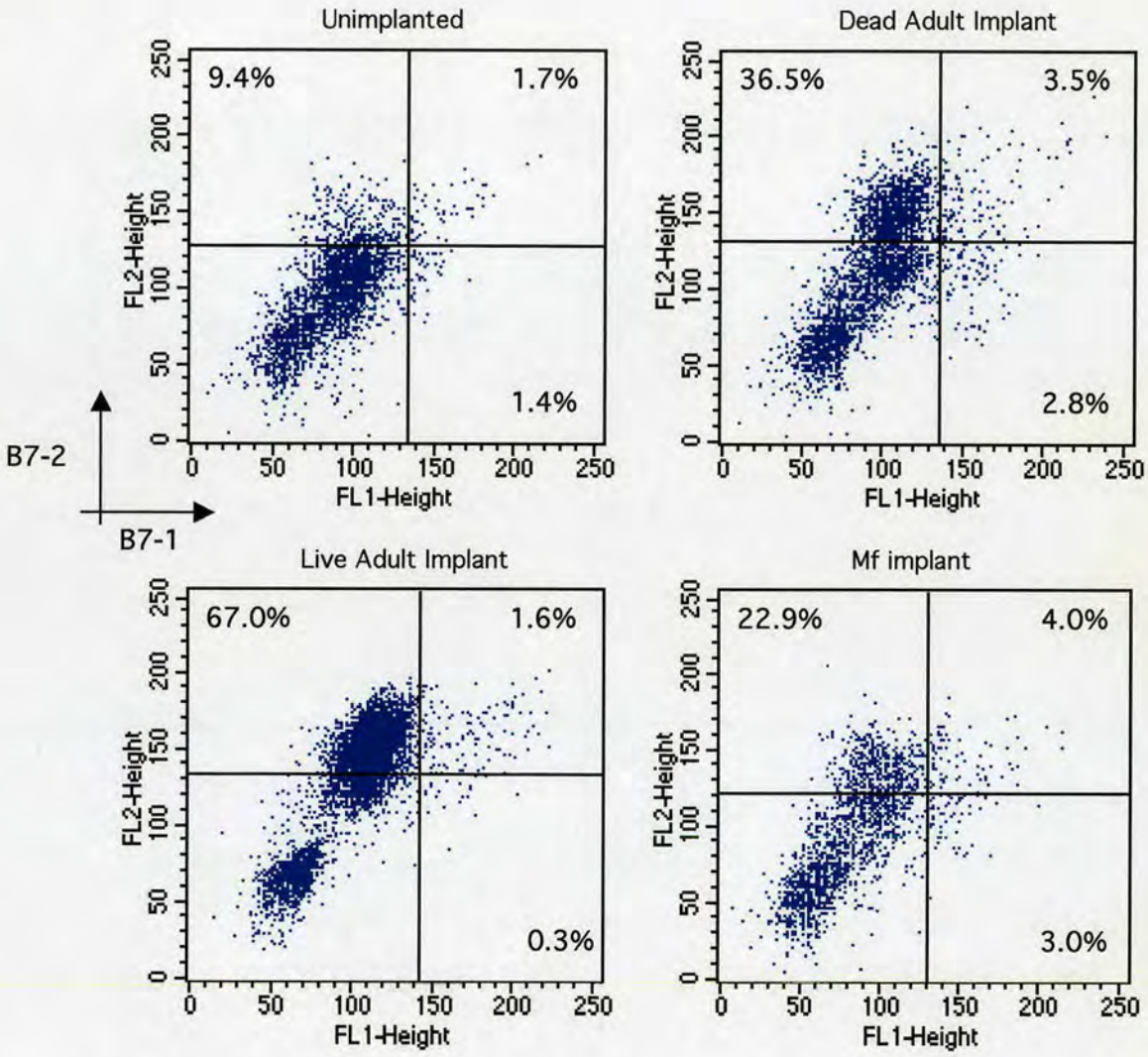


Figure 9. Granulocyte/macrophage expression of B7-1 and B7-2. B7-1 (FL1) and B7-2 (FL2) expression was assessed on PEC from control (unimplanted), live adult-, dead adult-, or Mf-implanted CBA/Ca mice. Control PEC were combined cells from 4 unimplanted mice, dead implant PEC were combined cells from 5 mice each implanted with 10 dead adult parasites, Mf PEC were combined cells from 4 mice each injected with $2x10^5$ Mf i.p., and adult-implant-derived PEC were typical results obtained from one mouse implanted with live parasites. Granulocytes/macrophages were gated on the basis of their forward and side scatter profiles. Quadrants were set on unstained samples from each group. Figures refer to the percentage of gated granulocytes/macrophages staining positive for either B7-1 (horizontal axis) or B7-2 (vertical axis).

Antibodies against CD5 and IgM were used in combination to determine if there was an expansion of $CD5^+$ B1 cells in PEC samples from *Brugia*-implanted mice. No such

population was observed (data not shown). Similarly, no increase in cells staining positive with antibody against γδ TCR was observed (data not shown).

Additionally, Mf implant-, but not adult implant-, derived PEC were found to contain a defined sub-population of T cells that were low expressers of the CD4 surface marker ($CD4^{low}$ cells)(Figure 10). Although a less distinct population of $CD4^{low}$ lymphocytes was also present in adult implants, it is interesting that PEC populations resulting from exposure to the two parasite life cycle stages appear to be so dramatically different in phenotype. These findings, combined with the data obtained from cytospin analysis, further support current evidence for differential immune responses to the Adult and Mf stages of *Brugia*.

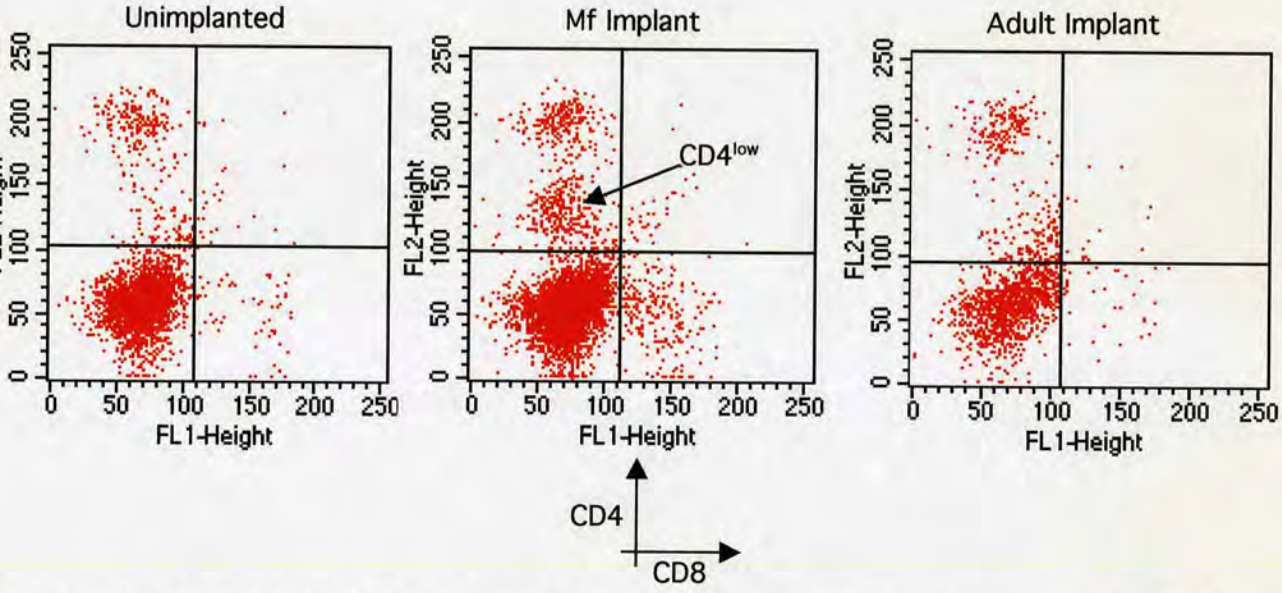


Figure 10. Mf induction of $CD4^{low}$ lymphocytes. CD4 (FL2) and CD8 (FL1) expression was assessed on PEC from control (unimplanted), live adult-, or Mf-implanted CBA/Ca mice. Control PEC were combined cells from 5 unimplanted animals, Mf PEC were combined cells from 4 implanted animals, and adult-implant-derived PEC were typical results obtained from one implanted animal. Lymphocytes were gated on the basis of their forward and side scatter profiles. Quadrants were set on unstained samples from each group. The presence of a defined population of $CD4^{low}$ cells in Mf implant-derived PEC is indicated by an arrow.

Another intriguing observation that resulted from FACS staining of PEC populations was the presence of a substantial population of granulocytes/macrophages, found only in adult parasite-implant PEC, which co-expressed both the T cell marker CD4 and the non-T cell marker F4/80 (Figure 11). These '$CD4^{+}$ macrophages' did not co-express F4/80 with either CD8 or CD3 (data not shown), suggesting that the double-staining seen with CD4 was not an experimental artifact.

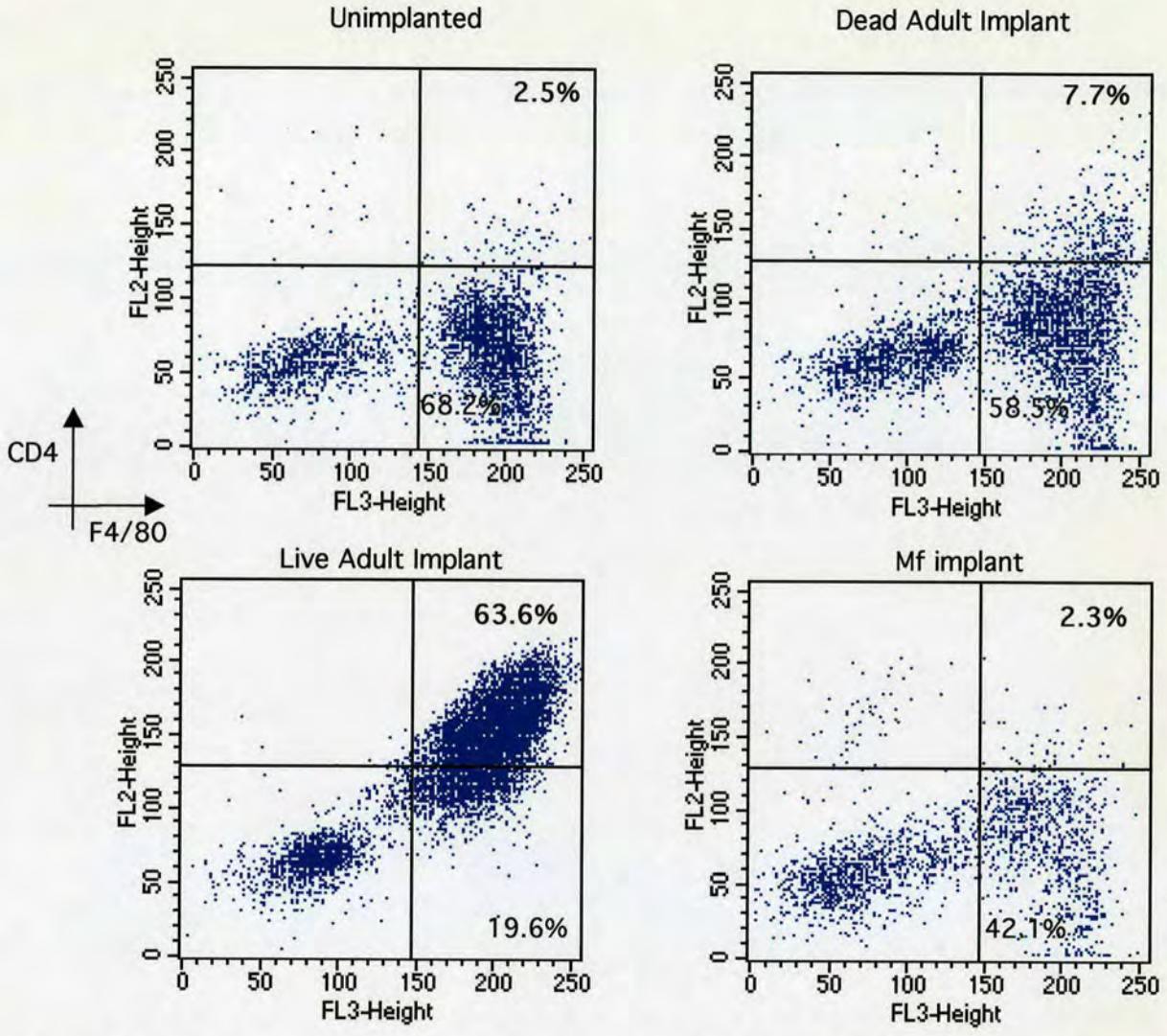


Figure 11. Adult *Brugia*-induction of $CD4^+$ macrophages. CD-4 (FL2) and F4/80 (FL3) expression was assessed on gated granulocyte/macrophage PEC from control (unimplanted), live adult-, dead adult-, or Mf-implanted CBA/Ca mice. Control PEC were combined cells from 4 unimplanted mice, dead implant PEC were combined cells from 5 mice each implanted with 10 dead adult parasites, Mf PEC were combined cells from 4 mice each injected with $2x10^5$ Mf i.p., and adult-implant-derived PEC were typical results obtained from one mouse implanted with live parasites. Granulocytes/macrophages were gated on the basis of their forward and side scatter profiles. Quadrants were set on unstained samples from each group. Figures refer to the percentage of gated granulocytes/macrophages staining positive for either CD4 (vertical axis) or F4/80 (horizontal axis), with the percentage of double-positive cells highlighted in bold.

5.2.4 Comparison of FACS sorted cell populations

Based on the observation that adult implant-derived PEC contain an unusually large cell population, we wanted to test if this population might be responsible for suppression. PEC taken from adult *B.malayi* implanted mice were sorted on size basis (using FSC)

into 'small' and 'large' cell populations which were then used as APC in the D10.G4 system (Figure 12). Anti-proliferative ability was evident in the small cell population, which was 95% pure. A much lower level of suppression of D10.G4 cells was effected by large PEC. However, these large cells were contaminated with approximately 30% small PEC, which may have been responsible for the suppression observed. However, lack of proliferation was not due to lack of adequate antigen presentation, as cytokine production was intact in suppressed cultures (data not shown).

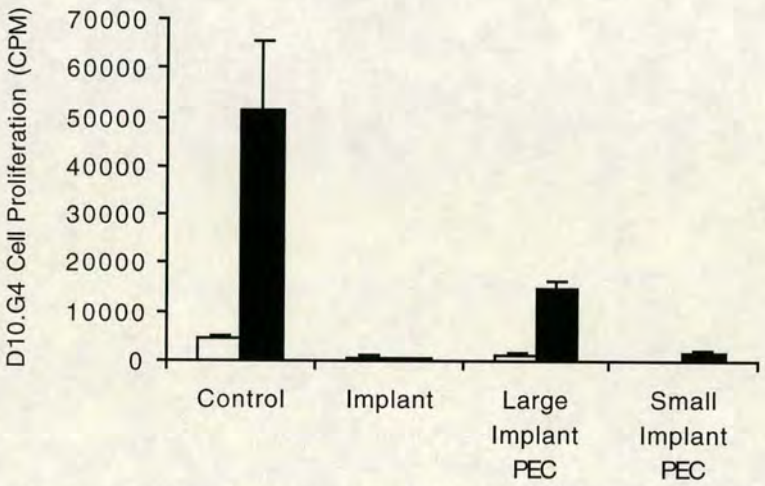


Figure 12. Differential ability of large or small PEC to suppress. Proliferation of D10.G4 cells stimulated in the presence of PEC from control or adult parasite-implanted CBA/Ca mice incubated in media (open bars) or 50 µg/ml conalbumin (solid bars). Control PEC were combined cells from 4 unimplanted mice and implant PEC were combined cells from 3 implanted mice. Cells were sorted on the basis of their forward and side scatter profiles, achieving 95% purity for large cells, and 70% purity for small cells. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of quadruplicate wells.

MAC-1 (CD11b) is a leukocyte adhesion molecule that can be found on the surface of monocytes/macrophages, granulocytes, NK cells and CD5$^+$ B cells (B1 cells) (Lai et al., 1998). To further characterise the suppressor cell population, PEC from adult parasite-implanted mice were sorted by FACS into large MAC-1 positive (92% pure), small MAC-1 positive cells (93% pure), and small MAC-1 negative cells (93% pure). FSC was used to determine size. These sorted peritoneal cells were then used as APC in the D10 assay (Figure 13). Once again, the results indicated that the large cell population was not suppressive, since large MAC-1$^+$ PEC did not potently prevent the proliferation of D10.G4 cells. Reduced proliferation was also seen in MAC-1$^-$ cultures but, unlike the other suppressed cultures, this was accompanied by a lack of cytokine production

(data not shown), suggesting that the proliferative block in these cultures was due to inadequate APC function rather than direct suppression.

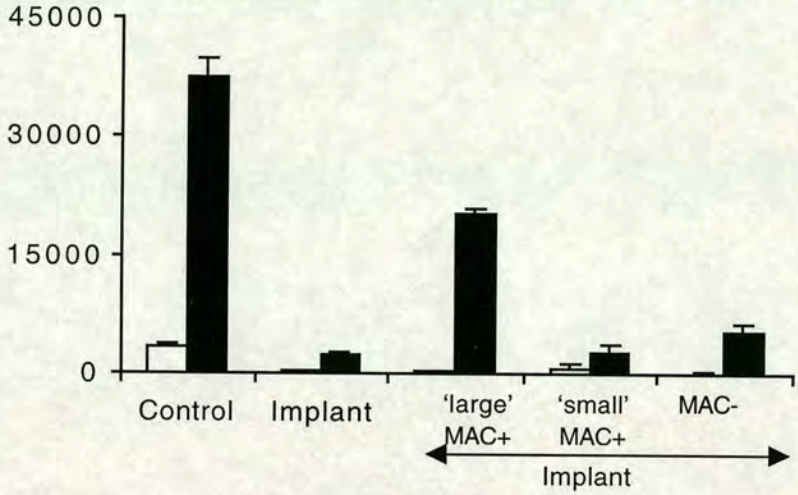


Figure 13. Large MAC-1$^+$ cells do not suppress. Proliferation of D10.G4 cells stimulated in the presence of PEC from control or adult parasite-implanted CBA/Ca mice incubated in media (open bars) or 50 µg/ml conalbumin (solid bars). Control PEC were combined cells from 5 unimplanted mice and implant PEC were combined cells from 2 implanted mice. Cells were first sorted into MAC-1$^+$ and MAC-1$^-$, and then by size on the basis of their forward and side scatter profiles. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of quadruplicate wells.

5.2.5 The suppressive cell type is MAC-1 positive

To further examine the ability of MAC-1 bearing cells to effect a proliferative block, PEC were sorted into MAC-1$^+$ and MAC-1$^-$ populations using magnetic beads that bind to FITC, in combination with FITC conjugated antibody to MAC-1. Using this approach, PEC were sorted into high purity MAC-1$^+$ and MAC-1$^-$ fractions (data not shown). When used as APC in the D10.G4 system, MAC-1$^+$ cells, but not MAC-1$^-$, suppressed the proliferation of the D10.G4 T cell clone (Figure 14).

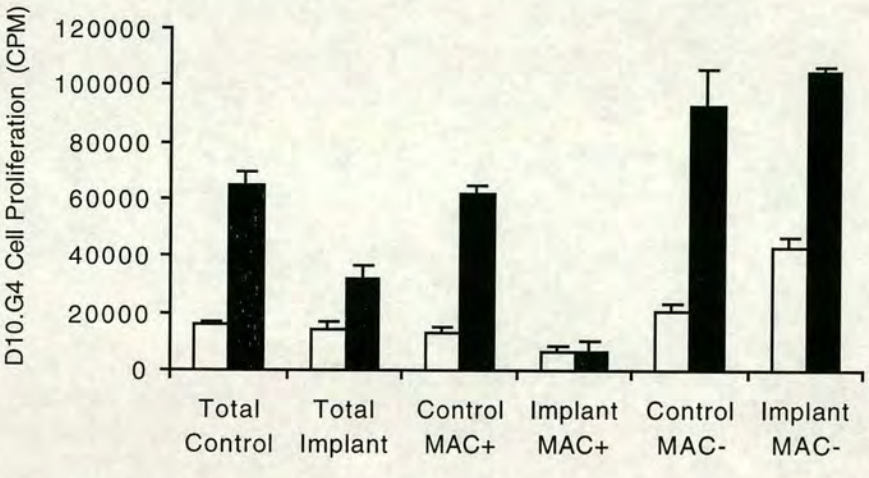


Figure 14. Suppressive PEC are MAC-1$^+$. Proliferation of D10.G4 cells stimulated in the presence of magnetic bead sorted PEC from control or adult parasite-implanted CBA/Ca mice incubated in media (open bars) or 50 µg/ml conalbumin (solid bars). Control PEC were combined cells from 6 unimplanted animals and data are shown as mean ± SD of quadruplicate wells. Implant PEC were not combined, and data shown are mean ± SD of two individual mice separately sorted and assayed. Proliferation was measured by [^{3}H]TdR incorporation, and is shown as cpm.

5.2.6 The suppressive cell type is not an F4/80$^+$ macrophage

Given the well documented role of macrophages in cellular suppression (Alleva et al., 1994; Schleifer and Mansfield, 1993), the expansion of macrophage numbers in the peritoneal cavity of parasite-implanted mice (see cytospin data in this chapter), the adherent properties of the suppressive cell, and the fact that it expressed the MAC-1 marker, we investigated the possibility that macrophages were responsible for proliferative block. The F4/80 monoclonal antibody has been extensiveley used to identify macrophages and some dendritic cell populations in murine studies (Haidl and Jefferies, 1996). Peritoneal cells from 10 control or adult parasite-implanted mice were combined and sorted by FACS into macrophage positive or negative populations using a monoclonal antibody specific for the macrophage surface marker F4/80. Greater than 97% purity was achieved for each group (data not shown). Proliferation of the T cell clone D10.G4 was then measured in the presence of each cell population (Figure 15). D10.G4 cells proliferated well when co-cultured with the macrophage-enriched population, while F4/80$^-$ cells prevented cellular proliferation. The absence of proliferation in the F4/80$^-$ cultures was not due to a lack of effective APC, as high levels of antigen-specific IL-4 were produced in these cultures, and similar results were obtained with the addition of irradiated syngeneic splenocytes as a source of APC (data not shown).

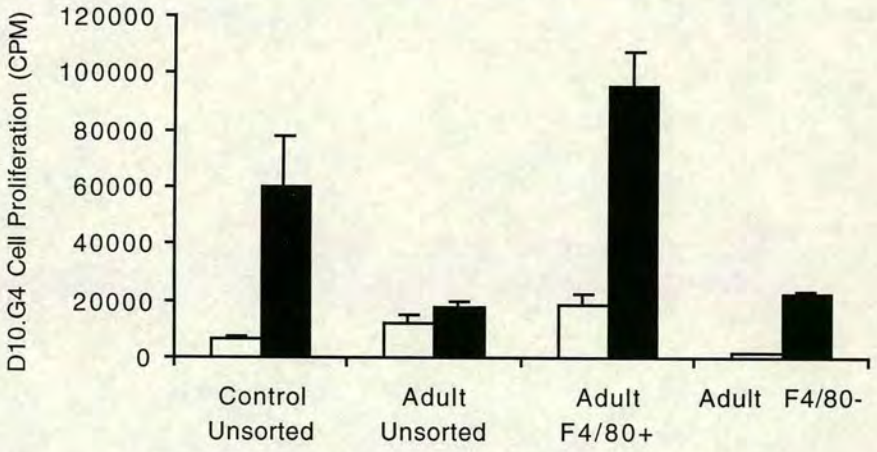


Figure 15. Suppressive PEC are not F4/80$^+$. Proliferation of D10.G4 cells with media (open bars) or 50 µg/ml conalbumin (solid bars), stimulated in the presence of unsorted PEC from control or adult parasite-implanted CBA/Ca mice, or macrophage-enriched (F4/80$^+$) or depleted (F4/80$^-$) PEC from adult parasite implanted mice. PEC were combined cells from 10 control or implanted animals prior to sorting. Proliferation was measured by [^{3}H]TdR incorporation, and is shown as cpm. Data presented are mean ± SD of quadruplicate wells.

5.2.7 MAC-1$^+$, F4/80$^-$ granulocytes are recruited by adult *Brugia* implant

Having shown that the suppressive cell type was MAC-1$^+$ and F4/80$^-$, we then examined PEC from adult parasite-implanted mice by flow cytometry for expression of these surface markers. This revealed the presence of a distinct population of MAC-1$^+$, F4/80$^-$ granulocytes in PEC from adult parasite implanted mice that was not present in control mice (Figure 16).

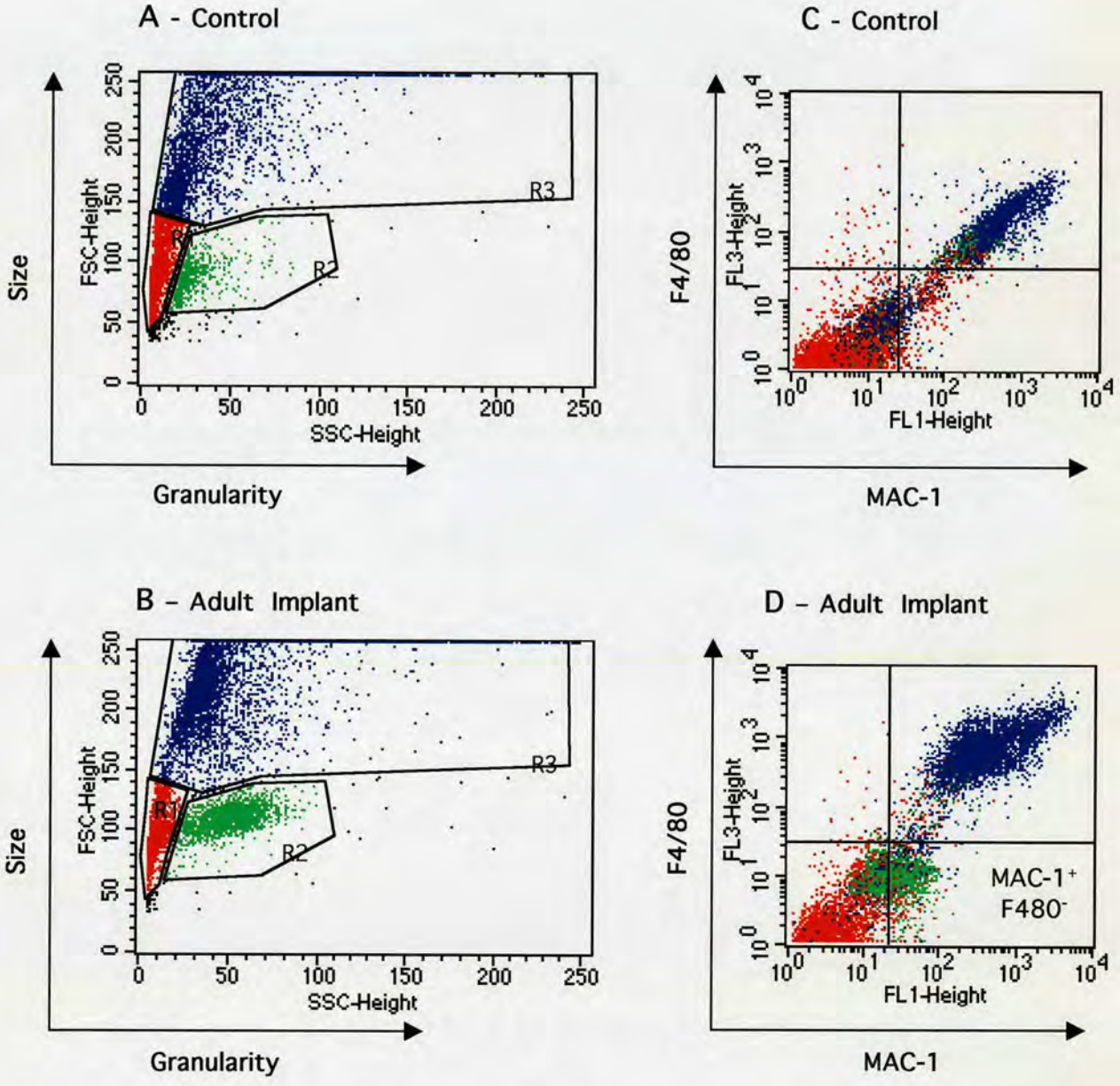


Figure 16. Adult *Brugia* recruitment of MAC-1$^+$, F4/80$^-$ granulocytes. PEC from control (**A**), or adult implanted (**B**), CBA/Ca mice were gated on the basis of their forward and side scatter profiles into lymphocytes (R1/red), granulocytes (R2/green) or macrophages (R3/blue). The same samples were then assessed for expression of MAC-1 (FL1) and F4/80 (FL3), (**C**) and (**D**). Control PEC were combined cells from 5 unimplanted mice and parasite-exposed PEC were typical results obtained from one implanted mouse. Quadrants were set on unstained samples from each group. MAC-1$^+$, F4/80$^-$ cells were present in PEC from implanted (**D**), but not control (**C**) mice, and color gating showed that these cells originated from cells in the granulocyte gate (R2/green).

5.2.8 Cell recruitment in the absence of host IL-4 production

To help further identify the phenotype of the down-regulatory cell, we next chose to look in more detail at the cell types recruited to the site of infection of IL-4-deficient mice, which fail to generate suppressive PEC (Chapter 4), in comparison to wild-type mice. These experiments had the further benefit of assessing the role of IL-4 in recruitment of cells to the site of infection. Cytocentrifuge preparations were made of peritoneal cells taken from IL-4-deficient mice implanted with adult stage *Brugia*. Observation of these cytospins showed that, in comparison to wild-type control mice, IL-4-deficient mice failed to generate a marked eosinophilia in response to adult parasite implant, but did show a decreased recovery of mast cells (Figure 17)(Table 4).

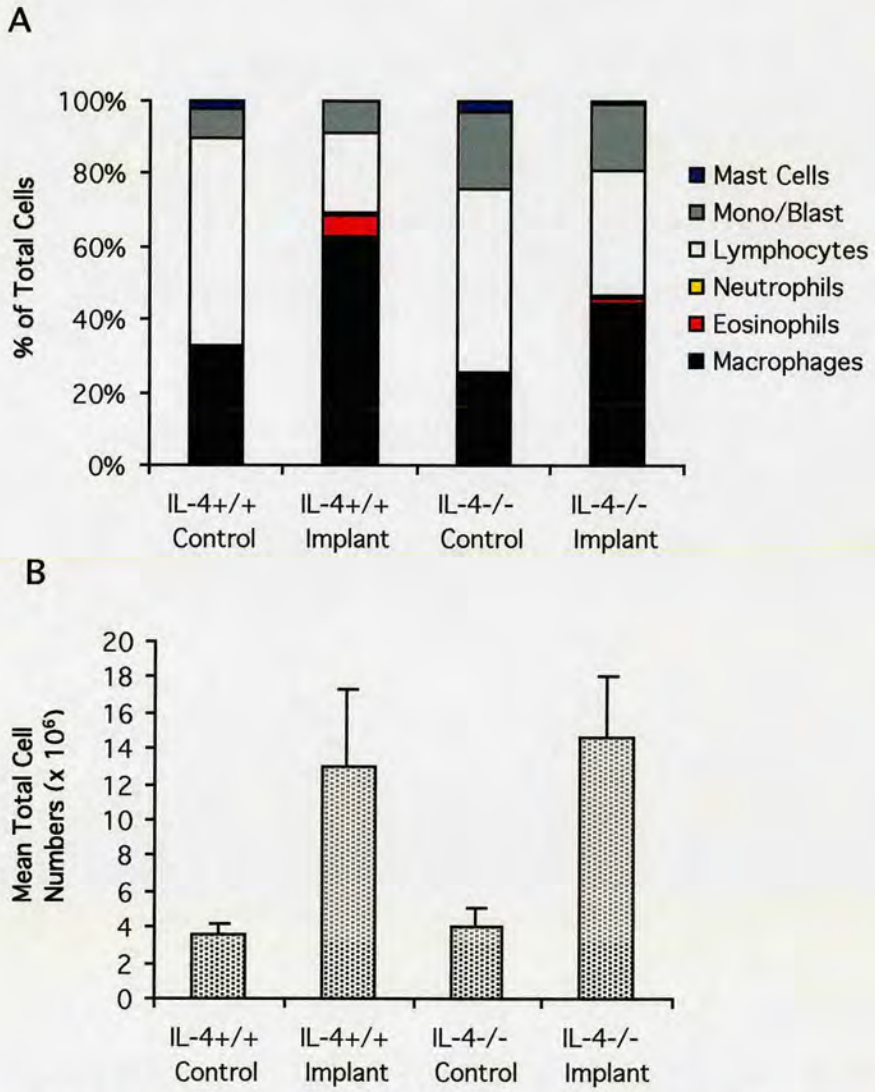


Figure 17. Peritoneal cell populations in control and adult-implanted wild-type and IL-4$^{-/-}$ C57BL/6 mice. (**A**) Mean percentage of total cells that were identified as mast cells (blue bars), monocytes/blasting cells (grey bars), lymphocytes (open bars), neutrophils (yellow bars), eosinophils (red bars), and macrophages (black bars). The cell composition of PEC from control and implanted mice was determined from cytospins by microscopy. Data shown are mean of three to four individual mice separately analysed. (**B**) mean total cell numbers recovered from the peritoneal cavity of control or implanted mice. Data presented are mean ± SD of three to four individual mice in each group separately assayed.

Table 4. *Peritoneal cell recruitment by IL-4-deficient mice*

	Total Number of Cells/animal ($x10^6$)			
	IL-4+/+ Control	IL-4+/+ Implant	IL-4-/- Control	IL-4-/- Implant
Macrophages	1.15 ± 0.14	8.08 ± 2.65	1.02 ± 0.23	6.28 ± 1.33
Eosinophils	0.03 ± 0.01	0.82 ± 0.42	0.01 ± 0.01	0.2 ± 0.13
Neutrophils	0.01 ± 0.01	0.09 ± 0.13	ND	0.16 ± 0.08
Lymphocytes	2.07 ± 0.24	2.88 ± 1.14	2.09 ± 0.65	5.12 ± 2.24
Monocytes/ Blasting cells	0.29 ± 0.05	1.08 ± 0.41	0.86 ± 0.21	2.79 ± 0.81
Mast Cells	0.10 ± 0.03	ND	0.12 ± 0.02	0.06 ± 0.05

Data represent the mean number (x 10^6) of each cell sub-population recovered in peritoneal washes. The cell composition of PEC from control and implanted C57BL/6 IL-$4^{+/+}$ and IL-$4^{-/-}$ mice was determined from cytospins by microscopy. Data shown are mean ± SD of three to four individual mice separately assayed. N.D. = not detectable.

5.2.9 Suppressive PEC are generated in the absence of eosinophilia

Having shown that the down-regulatory cell type was MAC-1^+ but F4/80^-, and given the recruitment of a MAC-1^+, F4/80^- granulocyte population in adult *Brugia*-implanted mice, we hypothesised that the suppressive cell type might be an eosinophil, since granulocytes express MAC-1, but not F4/80. Additionally, we had observed a dramatic recruitment of eosinophils to the peritoneal cavity following filarial implant, and had shown that only minor eosinophil recruitment was evident in peritoneal washes from non-suppressive IL-4-deficient mice implanted with adult parasites. To test the possibility that eosinophils were down-regulatory, we implanted IL-5-deficient mice with adult *Brugia*. IL-5 deficient mice, although possessing almost normal basal levels of eosinophils in the blood and bone marrow, fail to develop blood and tissue eosinophilia when infected with helminths (Kopf et al., 1996; Sabin et al., 1996; Takamoto et al., 1997). Intra-peritoneal implant of adult *Brugia* into IL-5-deficient mice generated PEC that were equally capable to those of parasite-exposed wild-type mice in their ability to block the proliferation of the B cell hybridoma HB32 (Figure

18A) and the murine lymphoma EL-4 (Figure 18B). This proliferative block occurred independently of NO production (Figure 18).

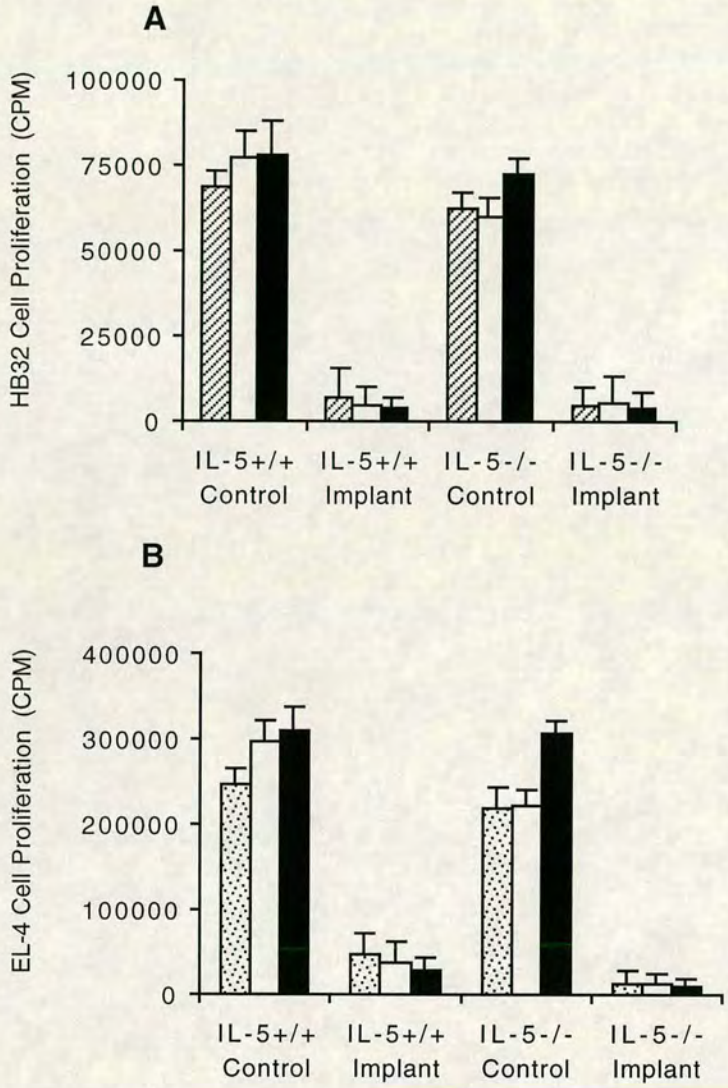


Figure 18. *Brugia* implant of IL-$5^{-/-}$ mice. Proliferation of (**A**) HB32 cells with media (diagonal striped bars), media plus D-NMMA (open bars) or media plus L-NMMA (solid bars) or (**B**) EL-4 cells with media (spotted bars), media plus D-NMMA (open bars), or media plus L-NMMA (solid bars), in the presence of PEC from control or implanted IL-$5^{+/+}$ or IL-$5^{-/-}$ C57BL/6 mice. Data presented are mean ± SD of three to five individual mice separately assayed.

Analysis of cytospins of PEC from parasite-implanted IL-$5^{-/-}$ mice showed that proliferative block occurred in the absence of eosinophilia, although there was a significant increase in total cell numbers recovered from infected mice in comparison to controls (Figure 19)(Table 5).

Equivalent numbers of live Mf were recovered in the peritoneal washes from implanted wild-type and IL-5-deficient mice (Data not shown).

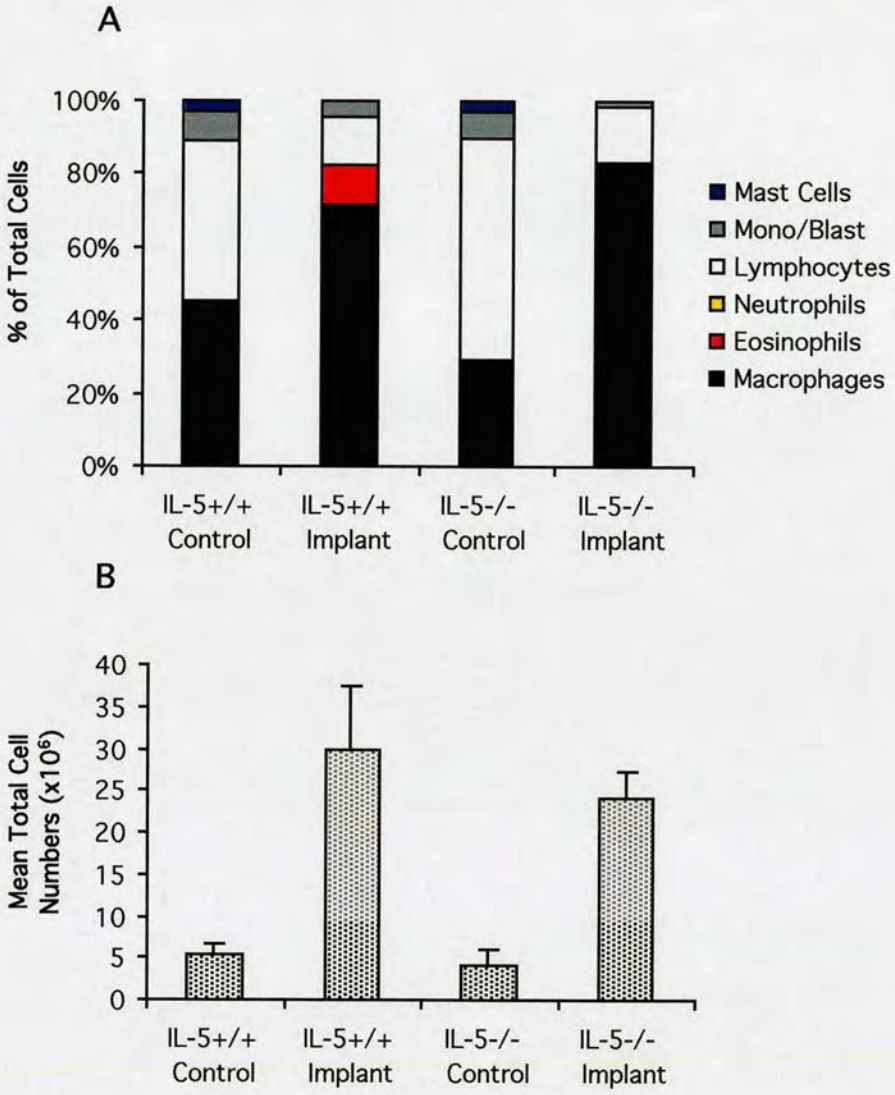


Figure 19. Peritoneal cell populations in control and adult-implanted wild-type and IL-5$^{-/-}$ C57BL/6 mice. (**A**) Mean percentage of total cells that were identified as mast cells (blue bars), monocytes/blasting cells (grey bars), lymphocytes (open bars), neutrophils (yellow bars), eosinophils (red bars), and macrophages (solid bars). The cell composition of PEC from control and implanted mice was determined from cytospins by microscopy. Data shown are mean of three to five individual mice separately analysed. (**B**) Mean total cell numbers recovered from the peritoneal cavity of control or implanted mice. Data presented are mean ± SD of three to five individual mice.

Table 5. *Peritoneal cell recruitment by IL-5-deficient mice*

	Total Number of Cells/animal ($x10^6$)			
	IL-5+/+ Control	IL-5+/+ Implant	IL-5-/- Control	IL-5-/- Implant
Macrophages	2.39 ± 0.31	21.53 ± 5.70	1.19 ± 0.41	19.99 ± 2.16
Eosinophils	0.04 ± 0.02	3.28 ± 1.08	0.01 ± 0.01	0.06 ± 0.06
Neutrophils	ND	0.03 ± 0.05	ND	0.02 ± 0.02
Lymphocytes	2.44 ± 0.66	3.79 ± 0.66	2.55 ± 1.16	3.64 ± 0.90
Monocytes/ Blasting cells	0.45 ± 0.10	1.06 ± 0.14	0.29 ± 0.10	0.41 ± 0.07
Mast Cells	0.16 ± 0.04	0.09 ± 0.05	0.13 ± 0.06	0.02 ± 0.02

Data represent the mean number (x 10^6) of each cell sub-population recovered in peritoneal washes. The cell composition of PEC from control and implanted C57BL/6 IL-$5^{+/+}$ and IL-$5^{-/-}$ mice was determined from cytospins by microscopy. Data shown are mean ± SD of three to four individual mice separately assayed. N.D. = not detectable.

5.2.10 Cell recruitment in the absence of host lymphocytes

To investigate the involvement of host T cells in recruitment of inflammatory cells, nude mice were implanted in the peritoneal cavity with adult *Brugia* parasites, followed by assessment of cell recruitment by analysis of cytocentrifuge preparations. In the absence of host T cells, there was a similar pattern of eosinophil recruitment and loss of mast cells in parasite-implanted mice in comparison to controls (Figure 20), even though PEC from implanted nude mice were not suppressive (Chapter 4). However the proportion of macrophages recruited to the peritoneal cavity of infected nude animals was less elevated than was seen in infected wild-type mice.

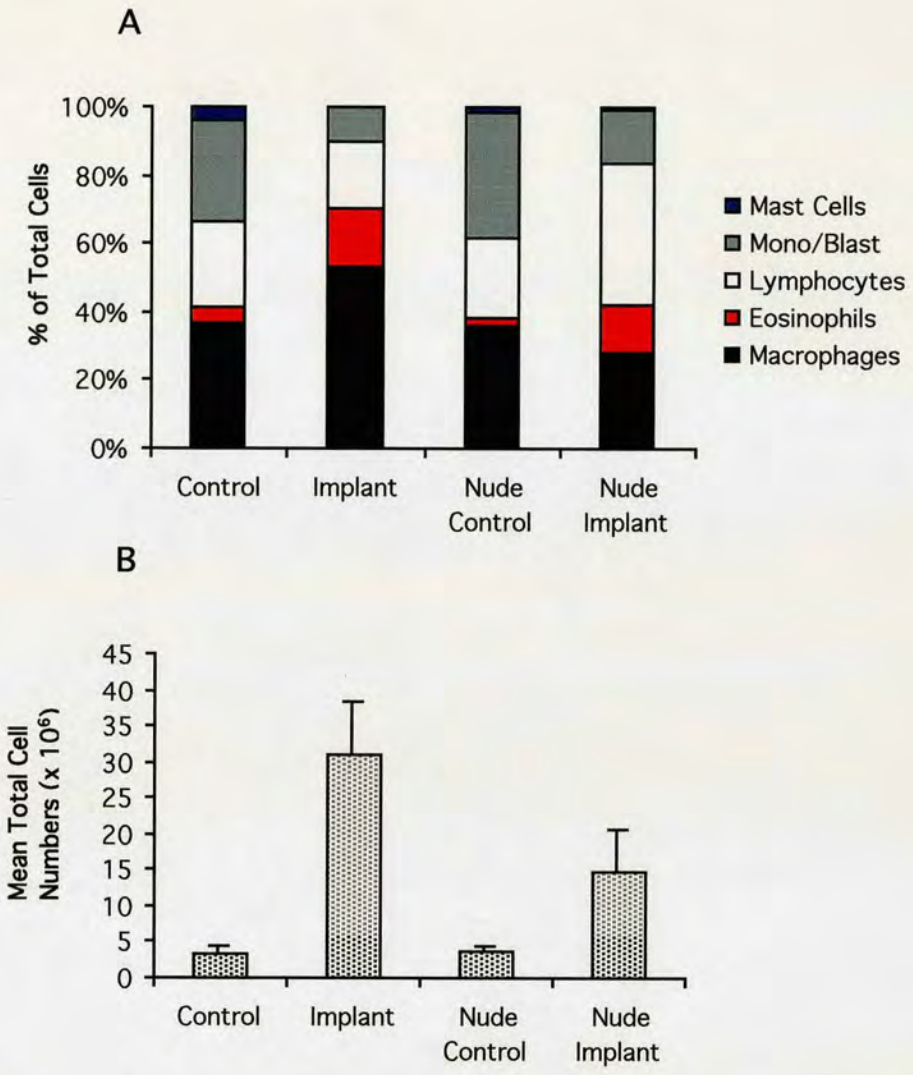


Figure 20. Peritoneal cell populations in control and adult-implanted wild-type and nude BALB/c mice. (**A**) Mean percentage of total cells that were identified as mast cells (blue bars), monocytes/blasting cells (grey bars), lymphocytes (open bars), eosinophils (red bars), and macrophages (solid bars). The cell composition of PEC from control and implanted mice was determined from cytospins by microscopy. Data shown are mean of three to five individual mice separately analysed. (**B**) mean total cell numbers recovered from the peritoneal cavity of control or implanted mice. Data presented are mean ± SD of three to five individual mice.

We also assessed $Rag^{-/-}$ mice, deficient in both B and T cells, for their cell recruitment abilities. These mice fail to generate a suppressive cell population (Chapter 4). Intriguingly, in contrast to wild-type or nude mice, these animals showed a dramatic neutrophilia after 3 weeks exposure to adult *Brugia* parasites, with neutrophils comprising approximately 20% of total cells recovered. Little increase in total cell

numbers was observed, and mast cell proportions were significantly decreased, in peritoneal washes from parasite-implanted RAG knock-out mice (Figure 21).

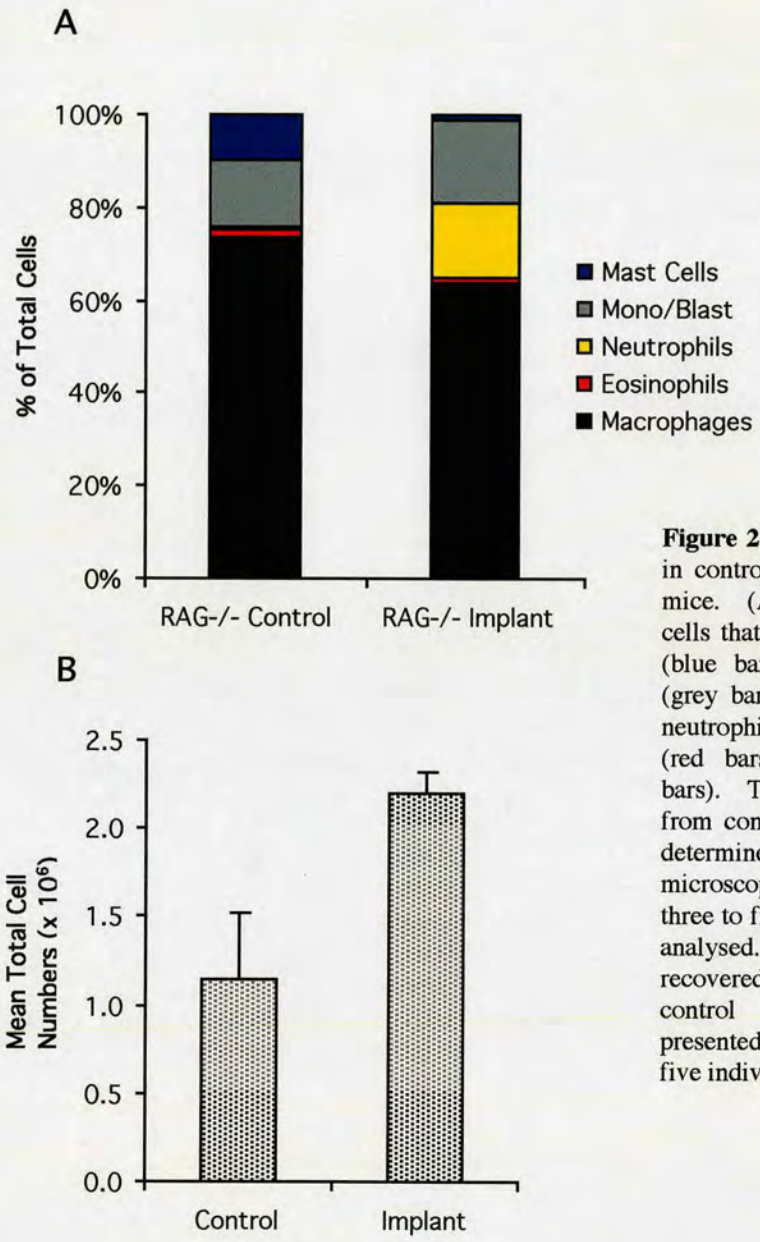


Figure 21. Peritoneal cell populations in control and adult-implanted $RAG^{-/-}$ mice. (**A**) Mean percentage of total cells that were identified as mast cells (blue bars), monocytes/blasting cells (grey bars), lymphocytes (open bars), neutrophils (yellow bars), eosinophils (red bars), and macrophages (solid bars). The cell composition of PEC from control and implanted mice was determined from cytospins by microscopy. Data shown are mean of three to five individual mice separately analysed. (**B**) Mean total cell numbers recovered from the peritoneal cavity of control or implanted mice. Data presented are mean ± SD of three to five individual mice.

5.3 Discussion

Analysis of the cell types recruited to the site of filarial infection has yielded some intriguing information about the interaction of *B. malayi* with the host immune system. The kinetics of cell recruitment to the site of infection have revealed a surprisingly rapid infiltration of eosinophils from as early as day 1 after adult parasite exposure. This eosinophilia persists throughout the course of infection, and can represent up to 50 percent of the total cells recovered from the peritoneal cavity during this time. However, even given this eosinophil-rich environment, adult filarial parasites have been shown to be able to survive for several months, and Mf for at least 1 month in the peritoneal cavity of mice (Lawrence, 1996), and we have recovered live adult parasites as long as six months after implant into the peritoneal cavity. This does not support the proposal that eosinophils are the effector cell type in filarial killing. It is possible that eosinophils do play a role in parasite destruction, but that this role is dependent on the co-operation of a number of controlling factors and is evident only in the resolution phase of infection.

The reduction in the proportions and numbers of mast cells recovered from the peritoneal cavity of *Brugia*-implanted mice suggests that the presence of the parasite has activated this cell type, causing either degranulation, or adherence to the wall of the peritoneal cavity. The activation of mast cells could have many implications on the outcome of infection. Human and murine mast cells are capable of producing IL-4 on stimulation (Schwartz, 1994), as well as playing a key role in the recruitment of inflammatory cells to the site of infection through the release of mediators such as histamine or tryptase (He et al., 1997). Indeed, IL-4 itself has recently been described as an effective inducer of release of the eosinophil chemokine eotaxin by epithelial cells (Mochizuki et al., 1998). It is possible that mast cells play a key role in providing IL-4 in response to the parasite to drive the subsequent Th2 phenotype that is typical of helmith infection, and may be involved in provoking the rapid influx of eosinophils.

It is evident that the Th2-inducing adult and L3 stages of the parasite generate a markedly different population of cells after implant into the murine peritoneal cavity in

comparison to Th1-inducing Mf. This is shown by the striking differences in cell populations seen by cytospin analysis, backed up by the contrasting scatter profiles of PEC from the different implant groups, with adults and L3 inducing a larger, more granular population of cells than are present in Mf implants. The remarkable differences in PEC profiles seen by both cytospin and FACS analysis suggests that the induction of such distinct cell populations reflects the contrary cytokine environments induced by the different life cycle stages.

The appearance of a conspicuous subgroup of lymphocytes expressing low levels of CD4 in Mf-infected mice is of particular interest. $CD4^{low}$ T cells have been implicated in T cell regulation of responses to self-ligands (Vidal et al., 1996), where it has been suggested that alterations in the level of CD4 expression could change the fine specificity of the TCR. Additionally, low expression of this surface molecule has also been reported on $NK1.1^{+}$ T cells (MacDonald, 1995).

Early experiments, where parasite-exposed PEC had been mixed with excess 'normal' (non-parasite-exposed) mitomycin-C-treated splenocytes, had shown that the proliferative block was not overcome by the addition of excess non-suppressive PEC (Allen et al., 1996). This suggested that the defective division was not due to an absence of costimulation or effective antigen processing or presentation. Consistent with these findings, flow cytometric analysis of PEC from parasite-implanted mice showed no impaired expression of the key costimulatory molecules B7-1 or B7-2 in comparison to PEC from control mice. In fact, the high levels of B7-2 provoked by the adult parasites could potentially contribute to the production of IL-4, and thus the development of the Th2 response induced by this life cycle stage (Freeman et al., 1995). However, the role of both B7-1 and B7-2 in differential regulation of Th1 and Th2 cell development remains controversial (Abbas et al., 1996; Thompson, 1995).

IL-4 has been shown to be able to induce cultured monocytes/macrophages to form giant multinucleated cells (McInnes and Rennick, 1988). It is likely that the high levels of IL-4 present in filarial-infected animals are causing the emergence of the large, multinucleate macrophages seen in cytospins from adult and L3 implanted mice.

Alternatively, this phenotype could represent end-stage cells, although multinucleate macrophages isolated from filarial-infected animals have been shown to be functionally active (Mackenzie et al., 1985). Although these large multinucleate cells are a remarkable feature of filarial implant into the peritoneal cavity of mice, they do not appear to be directly involved in downregulation of proliferative responses, as flow cytometric sorting of PEC from infected mice showed that suppression is not caused by these large cells.

The results obtained from the implant of $RAG^{-/-}$ and nude mice implicate both T and B cells as important in generating appropriate cell recruitment to the site of filarial infection. The absence of lymphocytes resulted in reduced total numbers of recruited cells in comparison to wild-type implants. Notably, neither nude nor $RAG^{-/-}$ mice showed a marked increase in macrophage recruitment, a prominent feature in wild-type implants. In addition to this, $RAG^{-/-}$ mice showed a striking neutrophilia 3 weeks after parasite implant, and in marked contrast to nude implants, which showed characteristic eosinophilia. These observations require further investigation, but suggest that both T and B cells are required to allow the development of an appropriate recruitment signal, perhaps by providing sources for increasing the strength of the appropriate cytokine/chemokine signal.

We have not yet determined the identity of the suppressive cell type, but have demonstrated that the PEC responsible for ablating proliferative responses *in vitro* is MAC-1-positive, yet is not an F4/80-positive macrophage. This suggests that the suppressive cell type is not a macrophage, since F4/80 is expressed by virtually all cells of this subtype to some degree depending upon their state of activation or development (Haidl and Jefferies, 1996). However, the suppressive cell type may be an unusual subpopulation of macrophages that do not express F4/80, or that express very low levels of F4/80, and the techniques used for selection of this marker might not have been sensitive enough to enable their detection.

The generation of a population of '$CD4^{+}$ macrophages' by adult parasites may represent the recruitment of dendritic cells to the site of infection, since dendritic cells can express

both CD4 and F4/80. However, the extremely high numbers of these cells recruited make this unlikely. It is also conceivable that the presence of $CD4^+/F480^+$ cells may be the result of fusion of T cells with macrophages, or consumption of T cells by macrophages. However, if this were the case, double staining of F4/80 with other T cell markers such as CD3 or CD8 might be expected, but was not observed. The function of these '$CD4^+$ macrophages' remains unknown but, since we have shown that the suppressive cell type is F4/80-negative, it would appear that these unusual macrophages do not play a down-regulatory role. An unusual population of $CD4^+$, $CD3^-$ cells has previously been observed in a murine infection model with the parasitic cestode *Mesocestoides corti* (Estes et al., 1993). The authors postulated that these cells might play a role in infection, but could not define a function. Notably, they did not look for expression of non-T cell markers, raising the interesting possibility that these $CD4^+$, $CD3^-$ cells might be the same as the $CD4^+$, $CD3^-$, but $F4/80^+$ cells that we have observed in filarial infected mice.

The rapid recruitment of eosinophils to the peritoneal cavity following parasite implant raised the interesting suggestion that these granulocytes, or a subset of these cells, might be the suppressive cell type. Other encouraging evidence supporting a suppressive role for eosinophils was the marked reduction in recruitment of these cells displayed by non-suppressive IL-4-deficient animals, and the reduction seen in proportions of eosinophils recovered from the peritoneal cavity of essentially non-suppressive nude and RAG knock-out mice. The possibility that this somewhat mysterious cell type might possess a novel immunomodulatory role was attractive given their unknown role in helminth infection, and the impressive repertoire of abilities that have been attributed to these granulocytes, such as expression of class II (Mawhorter et al., 1993), and secretion of a range of important mediators including IL-4 (Moqbel et al., 1995), IL-5 (Dubucquoi et al., 1994), IL-12 (Grewe et al., 1998) and TGFβ (Elovic et al., 1998). Additionally, eosinophils have been implicated in IL-4 dependent regression of some tumor types (Tepper et al., 1992).

IL-5 is a Th2-type cytokine, production of which is elevated in response to helminth infection, coincident with the recruitment and activation of eosinophils (Mahanty et al.,

1993; Takamoto et al., 1997). However, *Brugia*-implanted IL-5-deficient mice, which fail to develop eosinophilia, were able to competently generate a population of down-regulatory cells. This suggests that the marked eosinophilia and elevated production of IL-5 that accompanies lymphatic filarial infection are not related to the development of proliferative suppression. This indicates either that eosinophils are not the suppressive cell type, or that there is a redundancy in the suppressive system caused by *Brugia* implant, and that the suppression seen in IL-$5^{-/-}$ animals is effected by an alternative source. Although several studies have implicated that host clearance of tissue dwelling nematodes may be due to the action of eosinophils, dependent upon IL-5 (Hogarth et al., 1998; Rotman et al., 1996), we could see no evidence for decreased parasite survival in IL-5-deficient mice. At this stage, the precise role of eosinophils in lymphatic filariasis remains unknown.

The identity of the suppressive cell type generated by adult or L3 *B. malayi* is still undefined. We do know that this cell type is MAC-1^{+}, and F4/80^{-}. This data, taken together with that obtained from implant of IL-5-deficient animals, suggests that neither macrophages nor eosinophils are the down-regulatory cell. Further work needs to be carried out to reinforce these findings, to ensure that a minor subset of F4/80^{-} macrophages is not responsible, or that eosinophils from competent mice cannot suppress. The fact that we see a marked recruitment of macrophages to the site of infection, and the adherent nature of the suppressive cell type, promotes an unusual macrophage subpopulation as the most attractive candidate. However, we remain open to the possibility that multiple mechanisms may be operating concurrently to effect suppression. The lack of suppressive ability of PEC taken from nude or $RAG^{-/-}$ mice raises the interesting possibility that proliferative down-regulation may involve a regulatory T cell, although the MAC-1^{+} phenotype suggests that this would not be the dominant suppressive cell type.

Chapter 6: Mechanism of suppression

6.1 Introduction

Determination of the mechanism(s) by which host cells exposed to filarial parasites profoundly block the proliferation of such a wide range of responder cell types was one of the prime objectives of this study.

The initial approach to try and identify the mechanism by which the division of responder cells was being blocked was to use a panel of inhibitors or neutralising antibodies *in vitro* in culture wells containing parasite-exposed peritoneal cells. The candidates to be inhibited or blocked were chosen on the basis of their known regulatory properties and on whether or not they had been associated with filarial infection in other animal models or in human studies.

Using this approach, addition of inhibitors of classical macrophage-derived suppressive mediators such as nitric oxide, hydrogen peroxide or prostaglandin, or of antibodies to IFN-γ or TGF-β, had been shown to have no effect on the proliferative block (Allen et al., 1996). This chapter expands upon these initial findings by analysing the effect of *in vitro* neutralisation of IL-4, IL-10, and IL-10 and TGF-β together, on the proliferative block.

Initial experiments had also suggested that the mechanism by which suppression was effected *in vitro* was at least in part due to release of a soluble factor, since suppression could be partially transferred in culture supernatants taken from parasite-exposed PEC (Allen et al., 1996). In addition to the ability of suppressor cells to act on such a wide range of cell types, this finding was the basis for the pursuit of a soluble suppressive factor released by down-regulatory cells.

We decided to confirm if a soluble mediator was involved, firstly using Transwell membranes to separate responder from suppressor cells, and secondly by formaldehyde fixing of adherent PEC prior to addition of responder cells.

We also wanted to investigate the functional state of lymphocytes that had been exposed to suppressive PEC. To determine if lymphocytes that had been exposed to down-regulatory cells were irreversibly anergised, or had been temporarily prevented to divide, we investigated the ability of suppressed lymphocytes to recover from their state of proliferative block by isolation of non-adherent lymphocytes after exposure to adherent suppressor cells.

Finally, we investigated the involvement of membrane-bound signals in the mechanism(s) leading to proliferative block by stimulation of T cells downstream of any membrane bound activation or inhibitory signal by using PMA and ionomycin.

6.2 Results

6.2.1 Neither IL-4 nor IL-10 appear to be the *in vitro* suppressive factor

Prior evaluation of the ability of gene deficient mice to generate suppressive PEC suggested a critical role for IL-4 (see chapter 4). The D10.G4 Th2 clone itself secretes high levels of IL-4 during normal activation and proliferation (Kaye et al., 1983), but can be suppressed by parasite-exposed PEC. This suggested that, although important in the generation of suppressive PEC, IL-4 was unlikely to be the *in vitro* effector molecule responsible for blocking proliferation. This hypothesis was confirmed by the inability of antibody to IL-4 (11B11) to reverse the proliferative block *in vitro* of either the D10.G4 clone or the HB32 hybridoma (Table 1).

Table 1. *Neutralisation of IL-4 in vitro*

	A. HB32 Proliferation (cpm)			
Source of PEC	Medium		Medium + anti-IL-4	
Control	13,459 ± 5,244		13,178 ± 5,037	
Adult Implant	2,636 ± 1,214		2,294 ± 472	
	B. D10.G4 Proliferation (cpm)			
Source of PEC	Medium	Conalbumin	Medium + anti-IL-4	Conalbumin + anti-IL-4
Control	9,583 ± 490	45,685 ± 2,398	6,219 ± 258	10,255 ± 216
Adult Implant	1,106 ± 484	13,917 ± 5,271	731 ± 106	1,496 ± 399

Proliferation of (**A**) HB32 cells with media ± anti-IL-4 mAb or (**B**) D10.G4 cells with medium or 50 µg/ml conalbumin ± anti-IL-4 mAb, in the presence of PEC from control or adult implanted CBA/Ca mice. Removal of IL-4 from culture wells reduced D10.G4 proliferation in all cases because IL-4 is an autocrine growth factor for this T cell clone. Proliferation was measured by [^{3}H]TdR incorporation, and data shown are mean ± SD of three to five individual mice separately assayed.

IL-10 has been shown to exert major down-regulatory effects *in vitro* in parasitic helminth infection (Sher et al., 1991), and in the development of tumour-derived suppression (Maeda and Shiraishi, 1996), and has also been implicated in filarial infection (Mahanty and Nutman, 1995). Addition of neutralising antibody to IL-10 did not restore proliferative responses of T cells exposed to infection derived PEC (Table 2). The removal of IL-10 did enhance responses with control derived PEC, with increased proliferation of D10.G4 cells seen in these cultures. Further, we have shown

that, unlike IL-4-deficient mice, PEC from IL-10-deficient mice are capable of causing proliferative block (see Chapter 4). Thus, IL-10 is not required for either the induction or effector phases of suppression.

Table 2. *Neutralisation of IL-10 in vitro*

	A. HB32 Proliferation (cpm)			
Source of PEC	Medium		Medium + anti-IL-10	
Control	10,250 ± 630		10,101 ± 523	
Adult Implant	2,151 ± 344		2,551 ± 588	
	B. D10.G4 Proliferation (cpm)			
Source of PEC	Medium	Conalbumin	Medium + anti-IL-10	Conalbumin + anti-IL-10
Control	2,692 ± 554	29,598 ± 1,750	4,434 ± 449	52,286 ± 4,829
Adult Implant	697 ± 57	2,885 ± 1,579	781 ± 223	2,028 ± 984

Proliferation of (**A**) HB32 cells with media ± anti-IL-10 mAb or (**B**) D10.G4 cells with medium or 50 µg/ml conalbumin ± anti-IL-10 mAb, in the presence of PEC from control or adult implanted CBA/Ca mice. Proliferation was measured by [^{3}H]TdR incorporation, and data shown are mean ± SD of three to five individual mice separately assayed.

TGF-β has the ability to inhibit cellular proliferation. However, it has been shown that addition of several different anti-TGF-β antibodies to *in vitro* cultures containing PEC from *Brugia*-implanted mice does not restore proliferative responses (Allen et al., 1996). Since it has been suggested that in some circumstances IL-10 and TGF-β can have a synergistic relationship (Maeda and Shiraishi, 1996), we added neutralising antibody to both of these potent down-regulatory cytokines at the same time to *in vitro* cultures of the D10.G4 cell clone with parasite-exposed PEC. Concurrent neutralisation of both IL10 and TGF-β *in vitro* did not reverse suppression (Figure 1).

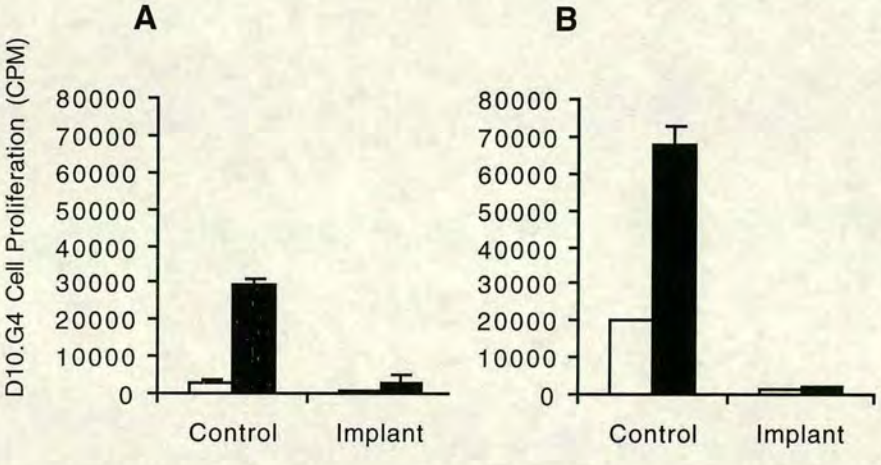


Figure 1. Simultaneous neutralisation of IL-10 and TGFβ does not reverse proliferative suppression. Proliferation of D10.G4 cells stimulated in the presence of PEC from control or adult parasite-implanted CBA/Ca mice incubated in media (open bars) or 50 µg/ml conalbumin (solid bars) (**A**), with no Ab added or (**B**), with 2 µg/ml anti-IL-10 and 50 µg/ml anti-TGF-β Ab added. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of three to four mice individually assayed.

6.2.2 Dilution of suppressive PEC negates the down-regulatory effect

The *in vitro* experiments referred to throughout this thesis standardly use $1x10^5$ PEC/well. Previously published work had shown that the suppressive effect of *Brugia*-exposed PEC could be overcome by addition of 8-fold more non-suppressive APC (Allen et al., 1996). We wanted to determine if suppressive function would be lost before APC function if the proportion of parasite-exposed PEC to responder lymphocytes was reduced. This would give an indication of the proportion of adherent PEC from parasite-implanted mice that possessed suppressive function. PEC from parasite-implanted mice were serially diluted prior to the addition of the D10.G4 T cell clone to see at what point suppressive ability would be lost in the absence of excess non-suppressive APC. Suppressive ability was rapidly lost in the cultures containing PEC that had been diluted (Figure 2). Indeed, at low numbers of parasite-exposed PEC ($2.5x10^4$/well and $1.3x10^4$/well) the antigen-specific proliferation of the D10.G4 cell clone was enhanced over that of the T cells grown in culture with the same numbers of control PEC.

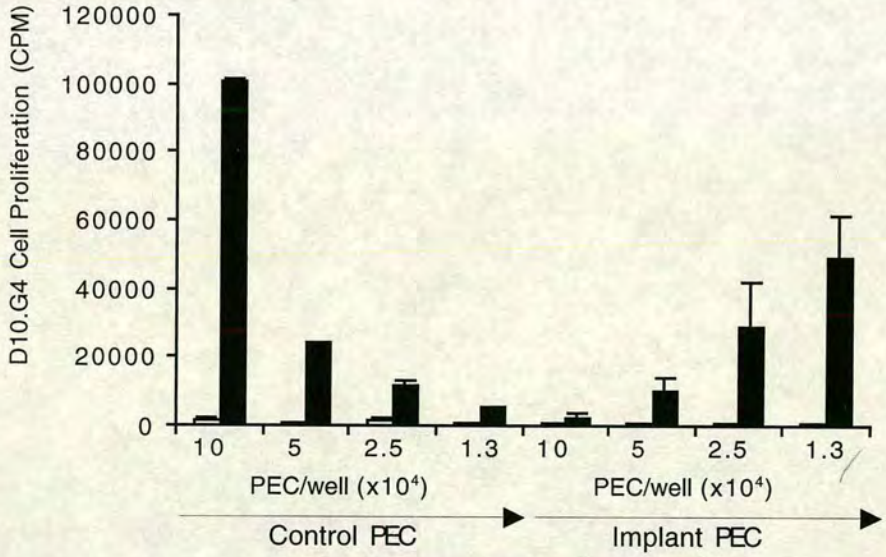


Figure 2. Dilution of suppressive PEC negates the proliferative block. Proliferation of D10.G4 cells stimulated in the presence of PEC from control or adult parasite-implanted CBA/Ca mice incubated in media (open bars) or 50 µg/ml conalbumin (solid bars) with decreasing numbers of PEC/well. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of (i) quadruplicate wells for controls, which were combined cells from four unimplanted mice or (ii) three implanted mice individually assayed.

6.2.3 A membrane barrier inhibits proliferative suppression

Preliminary experiments had shown that the down-regulation of proliferative responses could be partially transferred in the supernatants of PEC from adult parasite infected mice (Allen et al., 1996). To confirm that proliferative suppression was due to a soluble factor(s) released by the parasite-exposed peritoneal cells, PEC and responder cells (the D10.G4 clone or the HB32 hybridoma) were separated using the Transwell membrane system. Surprisingly, separation of suppressive PEC from responder cells using this technique prevented the downregulation of proliferation evident without the transwell barrier (Figure 3A), although in some cases a low level of suppression was seen with the HB32 hybridoma (Figure 3B).

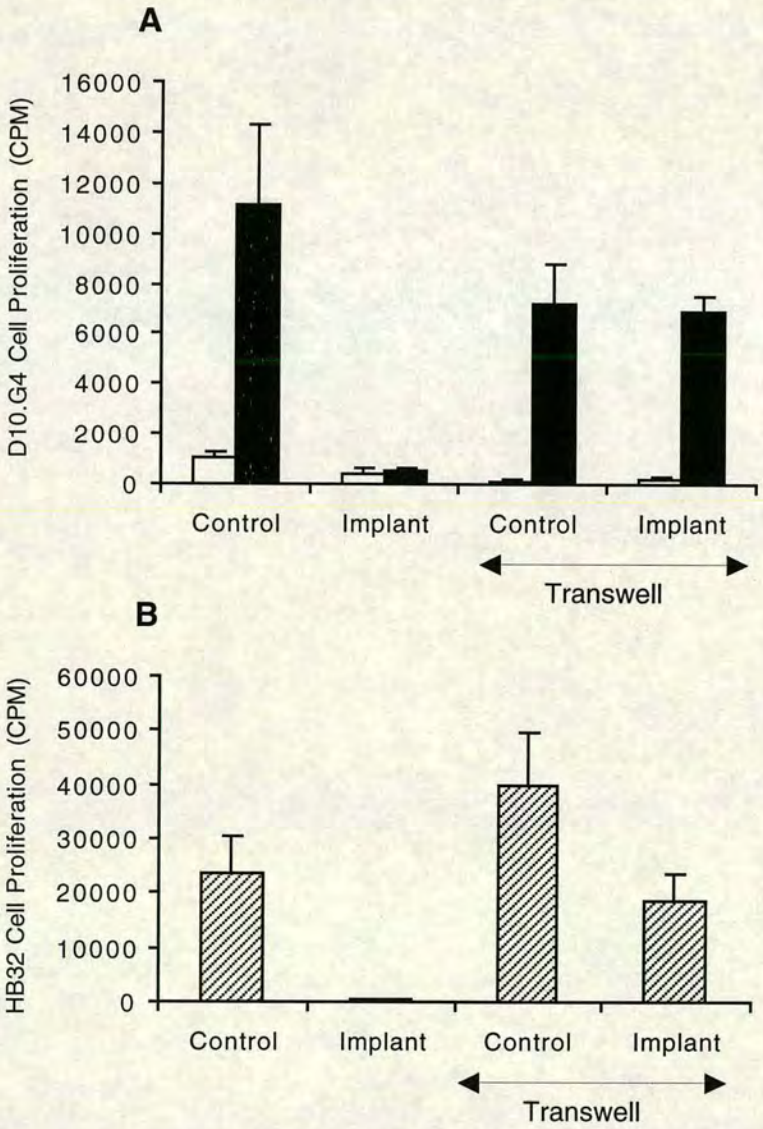


Figure 3. Transwell inhibition of proliferative suppression. Proliferation of (**A**), D10.G4 cells with media (open bars) or 50 µg/ml conalbumin (solid bars)or (**B**), HB32 cells, in the presence of PEC from control or adult parasite-implanted CBA/Ca mice. D10.G4 cells or HB32 cells were either directly co-cultured with PEC, or separated from PEC by a 0.4 µm Transwell membrane. Irradiated syngeneic splenocytes were added to all D10.G4 cell cultures to provide a source of APC. Control PEC were combined cells from 5 unimplanted mice, and implant cells were combined cells from 4 adult implanted mice. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of six wells.

6.2.4 Irradiated cells can effect proliferative block

To determine if growth and division of down-regulatory cells was necessary to allow suppression to occur, parasite-exposed PEC were irradiated with 2000 rads to prevent proliferation while preserving APC function prior to the addition of HB32 or EL-4 cells. Implant-derived PEC so treated were still capable of blocking division of either cell type (Figure 4).

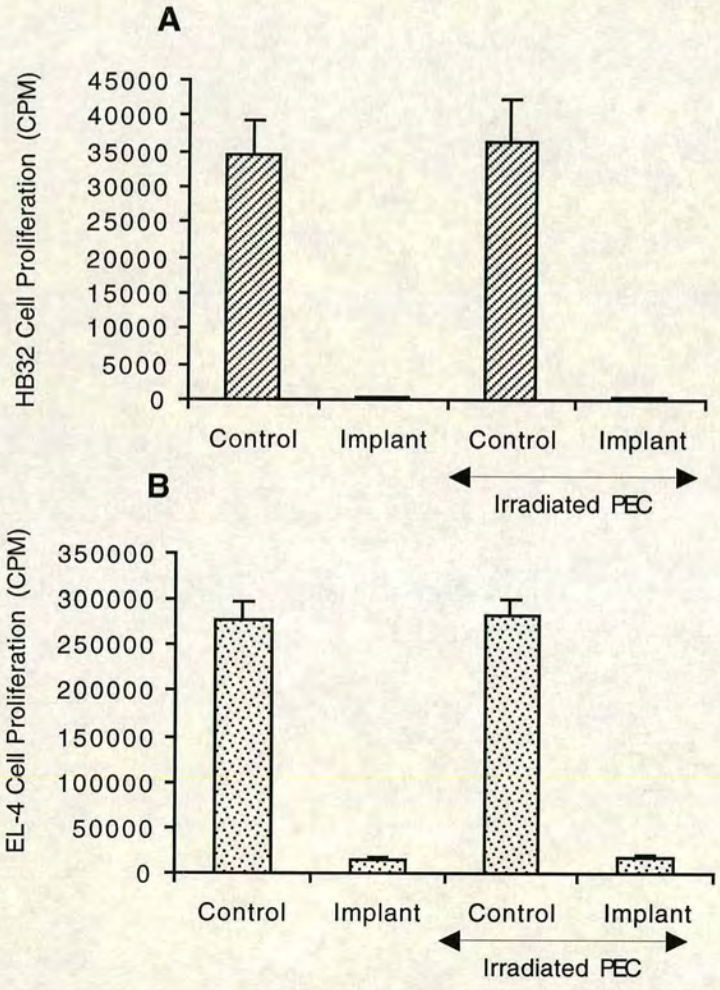


Figure 4. Irradiated PEC retain suppressive ability. Proliferation of (**A**) HB32 cells or (**B**) EL-4 cells, in the presence of PEC from control or adult parasite-implanted CBA/Ca mice. Irradiated PEC were exposed to 2000 rads prior to use. Proliferation was measured by [^{3}H]TdR incorporation, and data shown are mean ± SD of five to six mice individually assayed.

6.2.5 Fixed cells can block proliferation

The experiments detailed above demonstrated that proliferative block required close proximity of suppressor and responder cells, and that suppressive PEC did not need to be able to divide in order to inhibit proliferation. We thus chose to fix parasite-exposed

PEC with formaldehyde to determine if the mechanism of down-regulation involved a cell surface, rather than a secreted molecule. Fixed cells are essentially inert, being unable to process or present antigen, or to synthesise and release any soluble products. Thus fixation of suppressive PEC prior to the addition of responder cells would determine if cell-cell contact was necessary or sufficient to block proliferation. Strikingly, fixed parasite-exposed PEC effected suppression as effectively as non-fixed cells. Significant suppression by formaldehyde-fixed PEC from *Brugia*-implanted mice was seen with the HB32 B cell hybridoma, and the EL-4 lymphoma cell line (Figure 5).

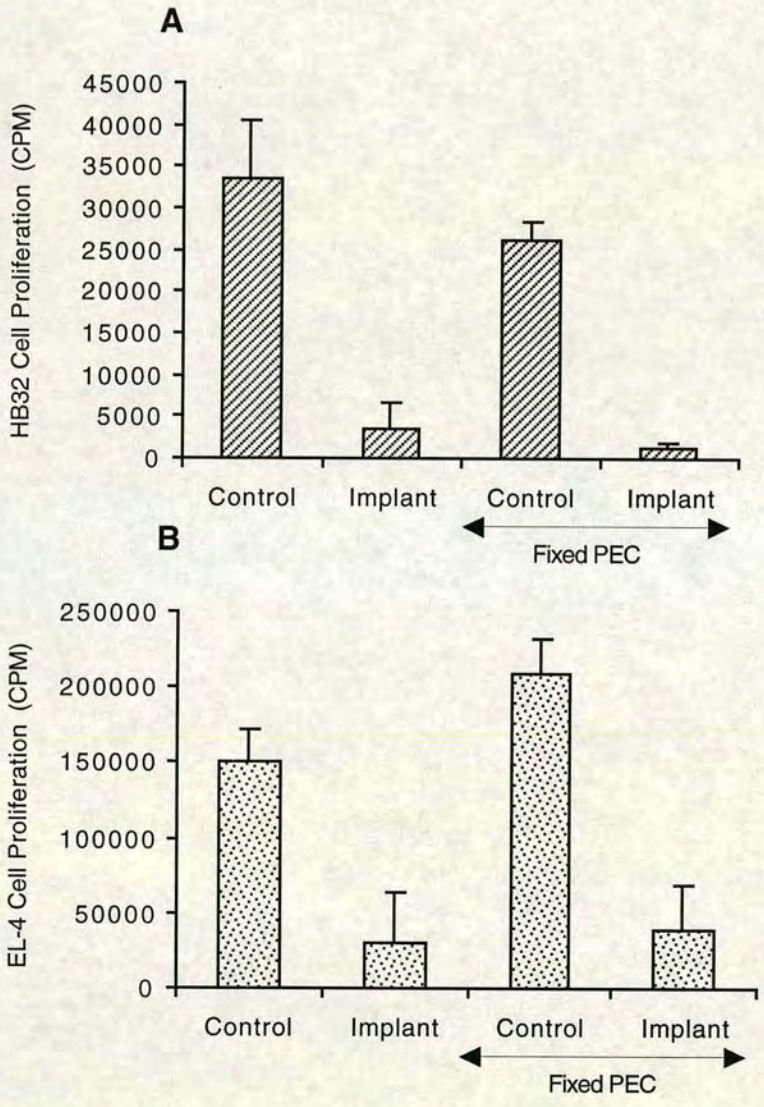


Figure 5. Formaldehyde fixed PEC retain suppressive ability. Proliferation of (**A**) HB32 cells or (**B**) EL-4 cells, in the presence of PEC from control or adult parasite-implanted CBA/Ca mice that were untreated, or had been formaldehyde fixed. Proliferation was measured by [^{3}H]TdR incorporation, and data shown are mean ± SD of five mice individually assayed.

Since the D10.G4 clone requires antigenic stimulation to divide, parasite-exposed PEC were pre-pulsed with conalbumin prior to fixation and the addition of D10.G4 cells. Using this protocol to 'pre-load' APC with antigen, significant suppression of the

D10.G4 cell line could still be seen with fixed parasite-exposed PEC ($P < 0.02$)(Figure 6).

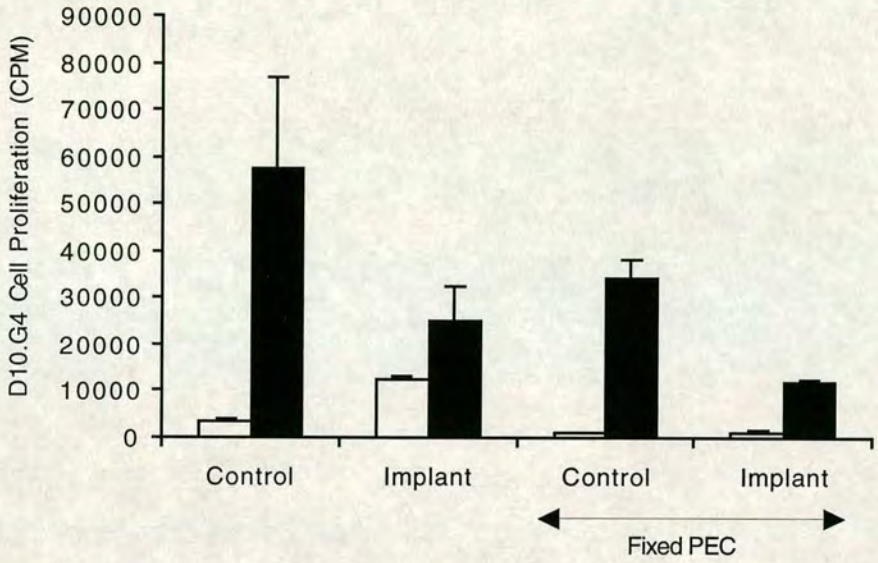


Figure 6. Fixed cells can block proliferation of T cells. Proliferation of D10.G4 cells stimulated in the presence of control or formaldehyde-fixed PEC from control or adult parasite-implanted CBA/Ca mice that had been pre-pulsed for 2 hr at 37°C with media (open bars) or 50 µg/ml conalbumin (solid bars) prior to the addition of the D10.G4 cells. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of three mice individually assayed.

To ensure that the fixing protocol used was effective, splenocytes were taken from control mice and fixed according to the protocol, then stimulated with the mitogen ConA. Fomaldehyde-fixed splenocytes did not divide in response to stimulation, indicating that the fixation protocol had been successful (Figure 7).

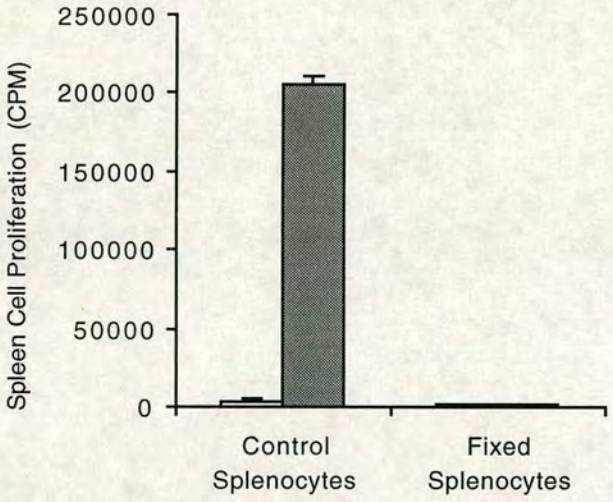


Figure 7. Efficacy of fixation protocol. Proliferation of control or formaldehyde-fixed splenocytes stimulated with media (open bars), or 10 µg/ml ConA (solid bars) Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of quadruplicate wells.

6.2.6 Suppressed lymphocytes are not irreversibly anergised

When directly stimulated with parasite antigen or mitogen *in vitro, Brugia*-specific T cells taken from the peritoneal cavity of parasite-implanted mice do not proliferate, but retain the ability to produce cytokine (Allen et al., 1996). However, it is unclear if this T-cell block represents an irreversible anergic state imposed upon parasite-exposed T cells. To determine if parasite-specific lymphocytes from the peritoneal cavity of implanted mice could recover their ability to divide in response to parasite antigen after withdrawal of suppressive cells, non-adherent cells were purified from PEC of implanted mice using nylon wool columns. The non-adherent fraction, comprising mainly lymphocytes with some granulocytes, proliferated well in response to parasite antigen in comparison to unfractionated PEC (Figure 8), suggesting that the T cells are not irreversibly anergised, but require constant exposure to suppressive PEC to remain non-proliferative.

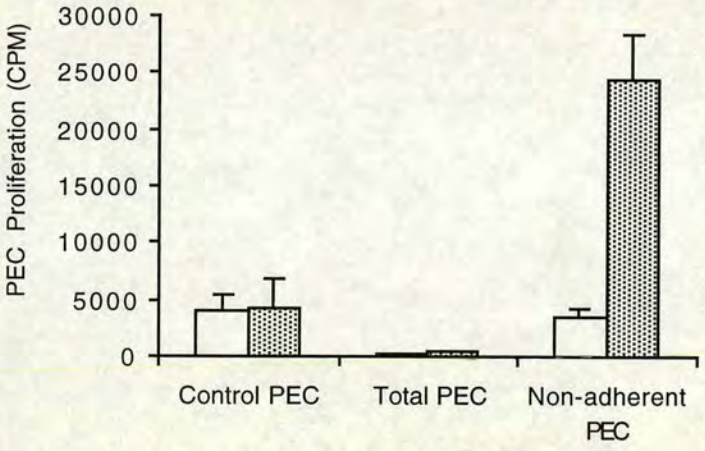


Figure 8. Suppressed lymphocytes are not anergised. Proliferation of total PEC or non-adherent PEC (purified lymphocytes) from control implanted mice stimulated with media (open bars), or 5 μg/ml BmA (hatched bars) Control PEC were combined cells from 5 unimplanted CBA/Ca mice, and implant PEC were combined cells from 3 adult *Brugia* implanted CBA/Ca mice. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of quadruplicate wells.

6.2.7 T Cells stimulated with PMA/Ionomycin are still suppressed

To determine if suppression of T cells required a signal delivered through the T cell receptor, D10.G4 cells were stimulated with PMA and ionomycin in the presence of parasite-exposed PEC. PMA is a phorbol ester that mimics the effect of DAG (diacylglycerol) in cell activation. Thus PMA, through activating PKC (protein kinase C), in conjunction with the calcium influx caused by the calcium ionophore ionomycin,

directly stimulates T cell division in the absence of a TCR-derived proliferative signal. T cells stimulated by this method in the presence of inhibitory PEC were still unable to divide (Figure 9), suggesting that suppression is not due to a block of stimulation via the TCR/MHC, but at a more profound level downstream of any such proliferative signal.

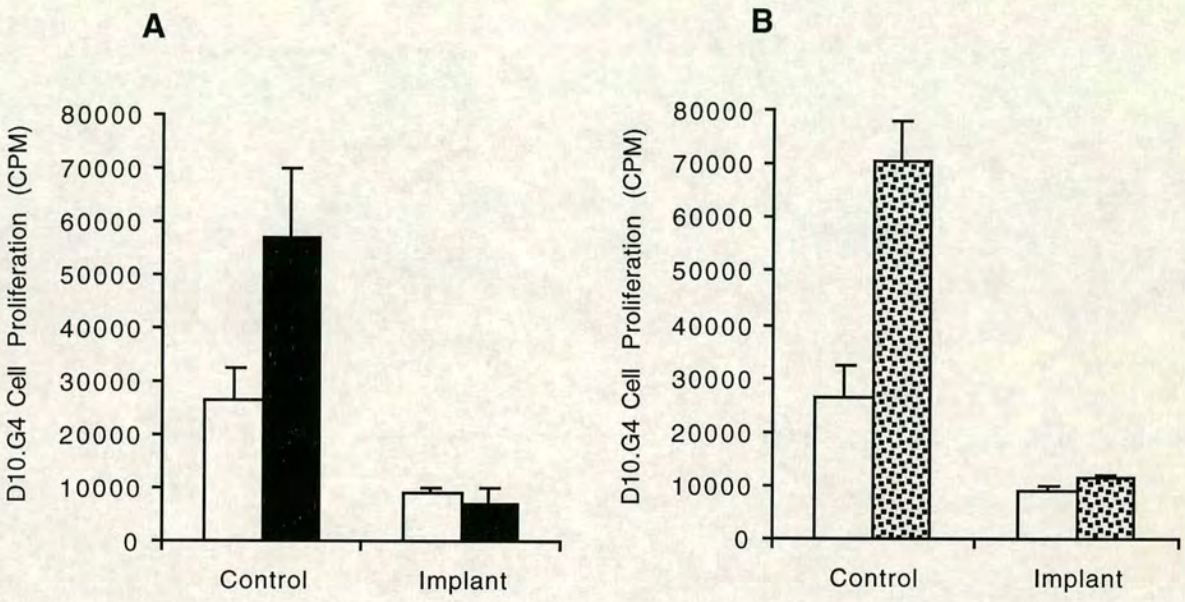


Figure 9. Proliferative block of T cells stimulated with PMA and Ionomycin. Proliferation of D10.G4 cells stimulated in the presence of PEC from control or adult parasite-implanted CBA/Ca mice (**A**), with media (open bars) or 50 µg/ml conalbumin (solid bars) or (**B**), with media (open bars) or 20 ng/ml PMA and 2 ng/ml ionomycin (hatched bars). Control PEC were combined cells from four unimplanted mice, and implant PEC were combined cells from five adult *Brugia* implanted mice. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of triplicate wells.

6.3 Discussion

The profound nature of the suppression seen in this system is demonstrated by the fact that thus far we have been able to block proliferation of fully differentiated T cell clones, primary cell lines, hybridomas, adenocarcinomas and lymphomas.

Proliferative block by *Brugia*-exposed PEC is not due to soluble factor(s) such as IFN-γ, nitric oxide, hydrogen peroxide or prostaglandins (Allen et al., 1996). A direct role *in vitro* for IL-4 has been implicated in L3-induced down-regulation of IL-2 driven T cell mitogenic proliferative responses (Osborne et al., 1996). However, even though IL-4-deficient mice fail to generate down-regulatory PEC (Chapter 4), IL-4 is not the PEC-derived suppressive factor, as proliferative responses are not restored by the addition of neutralising anti-IL-4 antibody *in vitro*, and the Th2 cell clone D10.G4 itself produces copious amounts of IL-4 during its normal proliferative response.

IL-10 has been implicated as a mediator of filarial non-proliferative responses in human patients in some (Mahanty and Nutman, 1995; Mahanty et al., 1997) but not all (Sartono et al., 1995) studies. In our experiments neutralisation of IL-10 *in vitro* did not restore proliferation to treated cultures. Transforming growth factor-β (TGF-β) is another cytokine with down-regulatory ability that has been reported to reduce macrophage anti-parasitic responses during helminth infection (Williams et al., 1995). Moreover TGF-β, together with IL-10, has been shown to have a close relationship in the development of tumour-related immune suppression (Maeda and Shiraishi, 1996). TGF-β appeared to be a likely candidate as we have here and previously shown that at very low concentrations of parasite-exposed cells, enhanced proliferation can be seen, consistent with the reported properties of TGF-β (Massague, 1990). However, addition of two commercially available anti-TGF-β antibodies did not restore proliferation to suppressed cultures *in vitro*, either alone or in combination with antibody to IL-10. Other potential soluble candidates remain to be investigated, including non-peptide soluble mediators such as glycosphingolipids (Persat et al., 1996) .

The experiments referred to above were founded on the premise that the suppressive factor was a soluble mediator based on previous evidence that suppression was at least partially transferable in PEC culture supernatants (Allen et al., 1996). In light of our more recent data, the supernatant transfer of suppression may in fact have been due to the unwitting transfer of suppressive PEC or due to transfer of 'spent' media. The Transwell experiments suggested that if a soluble component was responsible for suppression that this mediator was highly labile. Alternatively, it might suggest that cell-cell contact was required. This question was resolved by experiments in which formaldehyde-fixed PEC were shown to effect proliferative block as well as non-fixed PEC. This intriguing result suggests that the proliferative block seen with fixed PEC from parasite-implanted mice is due to a molecule or molecules already present on the surface of the cells prior to fixation. The identity of this molecule is not yet known, but the fact that *Brugia*-exposed PEC can block division of such a diverse range of cell types suggests that the molecule(s) involved must act through a receptor common to many cell types. Contact-mediated suppression of such a wide range of cell types is an exciting and potentially novel finding, but the supernatant transfer experiments, and the partial suppression of the HB32 hybridoma in the Transwell experiments, suggest that there may also be a soluble component involved in the down-regulation occurring. An additional potential complication is that different cell types may show variable sensitivity to the action of either soluble or membrane-bound effector mechanisms.

The idea that suppression is effected through an active mechanism, and not by simply blocking appropriate activation signals at the cell surface, is supported by the fact that lymphocytes stimulated with PMA and ionomycin are still unable to proliferate in the presence of down-regulatory PEC. This indicates that the mechanism of suppression functions downstream of any initial signal delivered through conventional routes of activation.

Interestingly, one of the few treatments that have had any effect on reversing the proliferative block induced by *Brugia*-implant-derived PEC is the addition of the calcium chelator EDTA at low concentrations in the cell cultures. During the course of flow cytometric analysis, we observed that incubation of suppressive PEC in buffers

containing low concentrations of EDTA caused a small, but significant, reversal of the proliferative block exerted by such cells (data not shown). In the light of the Transwell and fixing experiments, this partial reversal of suppression caused by the presence of EDTA could be due to a reduced ability of cells to contact and bind to their neighbours. Additionally, it could be that the suppressive mechanism acts through a calcium-dependant signalling pathway.

It is possible that peritoneal washes from parasite-implanted mice contain a combination of highly activated conventional APC and down-regulatory cells, and that these conventional APC may be responsible for activating suppressed cells to produce cytokine. The dilution experiments strengthen this hypothesis, as low numbers of PEC from parasite-implanted mice appear to stimulate lymphocytes much more effectively than the same number of control PEC. This dilution of regulatory ability also suggests that suppression may require a certain threshold ratio of down-regulatory PEC to responder cells, and that when this ratio is not achieved responding cells, instead of being blocked, may be stimulated to divide by the activated APC also present in the peritoneal wash. In effect, the fate of the responding T cell may be determined by whether the strength of the negative signal provided by the suppressive PEC is sufficient to overcome any positive signal provided by any activated, non-suppressive APC present.

We are still in the preliminary stages of analysing what happens to responder cells that have been prevented from dividing by suppressive PEC. However, it appears that suppressed cells are not irreversibly 'anergised', but are instead actively prevented from dividing, recovering the ability to proliferate once they have been separated from the down-regulatory cells.

Important concepts to emerge from the work detailed in this chapter is that the mechanism(s) that block cell division may be delivered by a combination of both a membrane-bound and labile soluble mediators, and that the cell type that is effecting proliferative block may not be the same cell type that is stimulating the production of cytokine. Given the profound nature of the suppression observed, and its IL-4

dependence, we are considering the possibility that proliferative block is effected by a suppressive mechanism not previously described, possibly acting in combination with known down-regulatory mechanisms.

Chapter 7: The influence of background genetics on the immune response to *B. malayi*

7.1 Introduction

Although much has been learned in filarial research by using murine models of infection, little is known about the role of background genetics of the murine host on the outcome of the host response to infection. In mouse models of filarial infection, the effect of variation in host genetic background has been investigated in terms of impact on parasite survival. However, research into any effect that the strain of mouse used might have on the immune response elicited in such experiments has to some extent been neglected.

Through the course of the work detailed in this thesis, it has been necessary to use a variety of different inbred strains of mice, primarily to accommodate the use of specific gene-deficient mice only available on a particular genetic background. Murine studies of filarial infection have shown that mice infected with different life cycle stages of *Brugia* stimulate contrasting Th-subsets (Lawrence et al., 1994; Pearlman et al., 1993). Adult and L3 *Brugia* parasites drive strong Th2-type responses, with high levels of IL-4, IgG1 and IgE being produced. Conversely, in the early stages of exposure, Mf induce a strong Th1-type response, characterised by production of elevated levels of IFN-γ accompanied by enhanced IgG2a, IgG2b and IgG3 responses. Chronic exposure to Mf may eventually lead to a Th2-type response. These studies have almost exclusively been carried out in BALB/c mice.

The genetic background of the mouse strain used in experiments may have a profound impact on the resulting response that develops towards a particular pathogen. It has been suggested that particular strains of mice can differ in their propensity to mount either a Th1 or Th2 type immune response. The specifics of the mechanism(s) behind this are poorly understood, but several explanations have been proposed. It has been shown that naïve T cells isolated from mice of different backgrounds exhibit distinct abilities to differentiate into either Th1 or Th2 type cells *in vitro*, independent of the

cytokine environment in which they are stimulated (Hsieh et al., 1995). Strain differences in development of T cell phenotype could be due to many mechanisms, including differences in production of, and responsiveness to, key cytokines such as IL-12 (Guler et al., 1996). This hypothesis is strengthened by the demonstration of a number of functional IL-4 receptor allotypes that differ between mouse strains in their molecular and functional properties (Gorham et al., 1996). Irrespective of the specific mechanism(s), the importance of recognising and addressing any potential immune bias that might have an impact on experimental research, especially that which closely involves Th-subset analysis, cannot be overlooked.

Murine infection studies of the immune response to the intracellular protozoan parasite *Leishmania major* provide a well-characterised example of the influence that the background genetics of the host can exert on the outcome of infection (Reiner and Locksley, 1995). *L. major* disease manifests as cutaneous lesions caused by parasite replication within host lymphoid cells. Development of a Th1-type response is protective resulting in a 'healing' lesion phenotype, whereas a Th2-type response results in progressive disease and a 'non-healing' lesion phenotype. Mice on a BALB background are susceptible 'non-healers', developing an inappropriate Th2-type response to infection, while C57BL/6 mice develop an appropriate Th1-type response and are resistant 'healers'.

Infection with intestinal nematodes shows a different pattern of strain susceptibility, with the best example being provided by studies of expulsion (resistant) versus non-expulsion (susceptible) of *Trichuris muris* in mice, where susceptible strains develop a Th1-type response, compared to the Th2-type response mounted by resistant strains (Else et al., 1994). Some variation in strain susceptibility has been seen with filarial nematodes, where BALB/c mice have generally been less resistant to infection than other strains (Petit et al., 1992). However, the nature of the immune response to filarial infection in these different strains has not been thoroughly investigated.

To determine if there were important strain differences in the immune response to filarial implant we wanted to directly compare the immune response to *B. malayi* in the

three strains of mice that we routinely used in our experiments (CBA/Ca, BALB/c and C57BL/6), by analysis of cytokine and antibody isotype production in response to infection. Additionally, and for the same reasons, we wanted to compare cellular recruitment patterns in the three strains of mice.

7.2 Results

7.2.1 Strain-specific responses to infection with *B. malayi*

7.2.1.1 IgG isotype production

Sera from mice that had been implanted for three weeks was analysed to determine antibody isotypes produced in response to parasite exposure. In all three strains tested, intra-peritoneal implant of adult stage *Brugia* parasites resulted in dominant production of the Th2-type isotype IgG1, consistent with previously published experiments using adult parasite implanted BALB/c mice (Lawrence et al., 1994). BALB/c and CBA/Ca mice showed similar profiles of antibody isotype production in response to adult *B. malayi*, stimulating predominantly IgG1. In contrast, levels of IgG1, IgG3 and, to a lesser extent, IgG2b, were elevated in C57BL/6 mice (Figure 1).

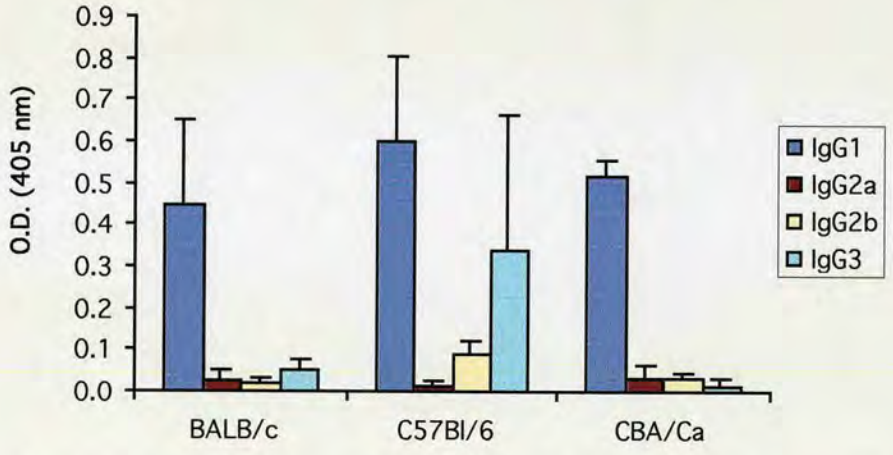


Figure 1. IgG isotype response in mice implanted with adult *B. malayi*. Data represent BmA-specific antibody production as measured by capture ELISA from sera. Data are shown as comparative OD at 405 nm and are mean ± SD of results obtained from sera of five individual mice in each group separately assayed.

Unlike adult parasite implanted mice, and consistent with previously published data, Th2-related IgG1 was not the dominant isotype produced in response to Mf, with all mouse strains producing equivalent or elevated levels of IgG3 relative to IgG1 (Figure 2). Some strain-specific differences were also evident. CBA/Ca and BALB/c strains showed marked differences in the profiles of IgG isotypes produced in response to Mf as compared to their response to adults, whereas C57BL/6 showed a similar response

profile to both life cycle stages. Only CBA/Ca mice showed evidence of elevated production of Th1-related IgG2a. In contrast to previously published data, Mf-implanted BALB/c mice did not produce elevated levels of IgG2a and IgG2b isotypes.

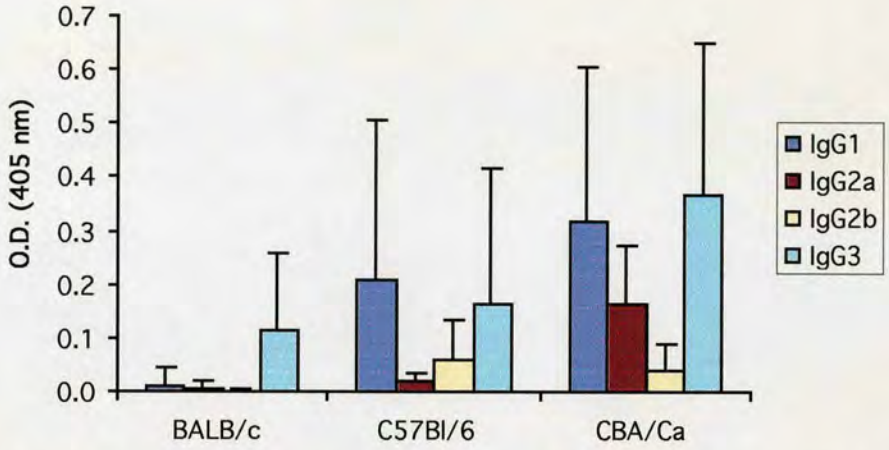


Figure 2. IgG isotype response in mice implanted with *B. malayi* Mf. Data represent BmA-specific antibody production as measured by capture ELISA from sera. Data are shown as comparative OD at 405 nm and are mean ± SD of results obtained from sera of five individual mice in each group separately assayed.

7.2.1.2 Total IgE production

Like other helminth parasites, production of high levels of IgE is a characteristic of filarial infection. All three strains of mice implanted with adult parasites showed an increase in total serum IgE over unimplanted controls, significantly so in C57BL/6 ($P < 0.05$) and CBA/Ca ($P < 0.01$) strain mice (Figure 3). BALB/c mice constitutively produced higher levels of IgE than either C57BL/6 or CBA/Ca mice.

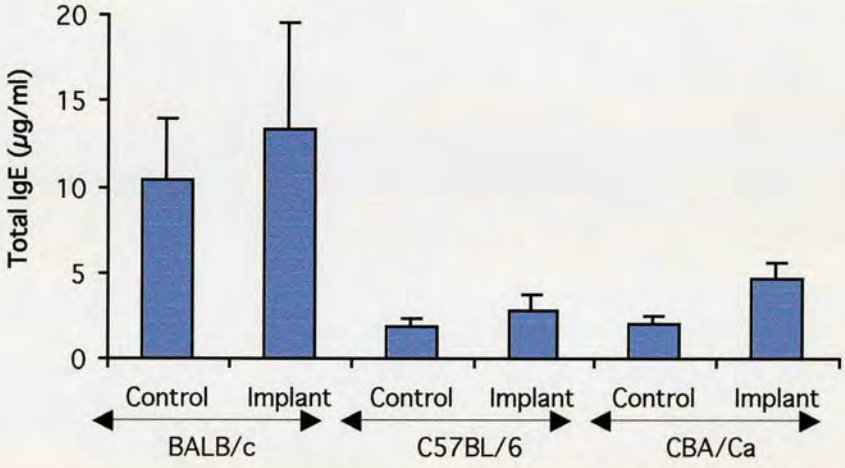


Figure 3. Total IgE in sera of mice implanted with adult *B. malayi*. Data represent total antibody production as measured by capture ELISA from sera. Data shown are mean ± SD of results obtained from sera of five individual mice in each group separately assayed.

In contrast to adult stage parasites, and consistent with their Th1-inducing nature, elevated levels of total IgE were not detected in sera of mice of all three strains implanted with Mf (Figure 4). Once again, BALB/c strain mice constitutively produced higher levels of IgE than either C57BL/6 or CBA/Ca strains.

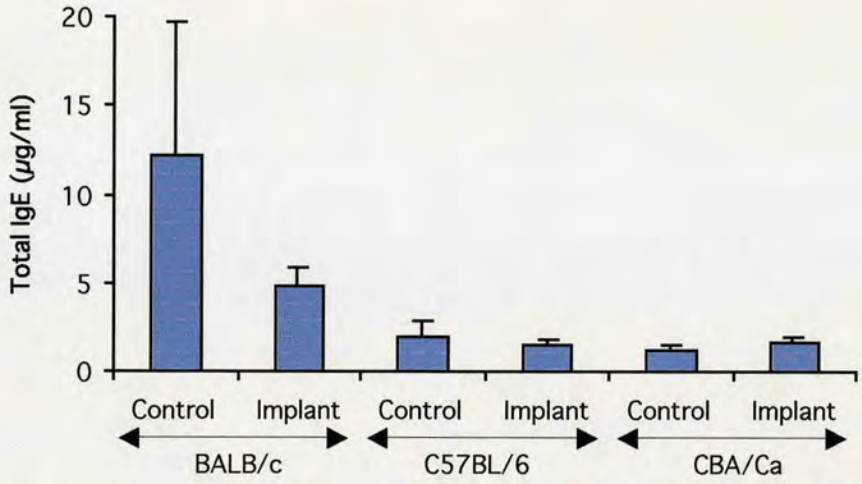


Figure 4. Total IgE in sera of mice implanted with *B. malayi* Mf. Data represent total antibody production as measured by capture ELISA from sera. Data shown are mean ± SD of results obtained from sera of five individual mice in each group separately assayed.

7.2.1.3 Systemic cytokine production by adult *Brugia*-implanted mice

In keeping with published data in BALB/c mice (Lawrence et al., 1994), splenocytes from adult parasite implanted mice of all three strains tested produced elevated levels of Type-2 cytokine on stimulation with BmA *in vitro*, in comparison to unimplanted controls (Figure 5). High levels of IL-4 and IL-5, and low levels of IFN-γ and IL-2 were produced. In general, C57BL/6 mice consistently produced less parasite-specific cytokine (of those tested) than CBA/Ca or BALB/c strains, although this was not significant.

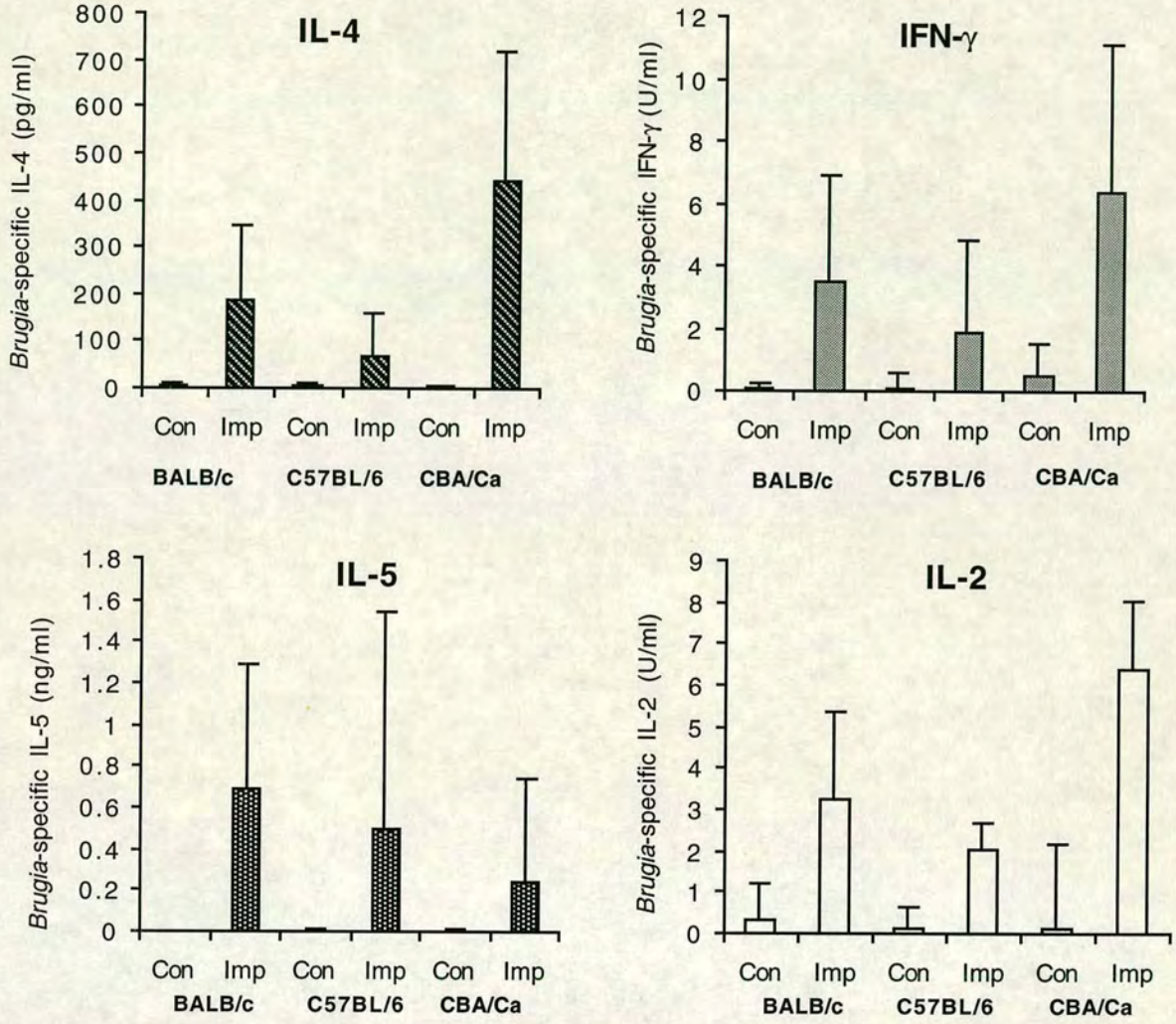


Figure 5. Adult parasite-induced splenocyte cytokine production. IL-4 (diagonal striped bars), IFN-γ (grey bars), IL-5 (spotted bars), or IL-2 (open bars) production by splenocytes from control (unimplanted) or adult implanted BALB/c, C57BL/6 or CBA/Ca mice. Cultures were stimulated with 5 μg/ml BmA, and levels of cytokine produced were measured by NK bioassay (IL-2 and IL-4), or by ELISA (IL-5 and IFN-γ). Data shown are mean ± SD of results obtained from sera of five individual mice in each group separately assayed.

7.2.1.4 Systemic cytokine production by Mf-implanted mice

Splenocytes from Mf implanted mice of all three strains tested produced higher levels of cytokine on stimulation with BmA *in vitro*, than unimplanted controls (Figure 6).

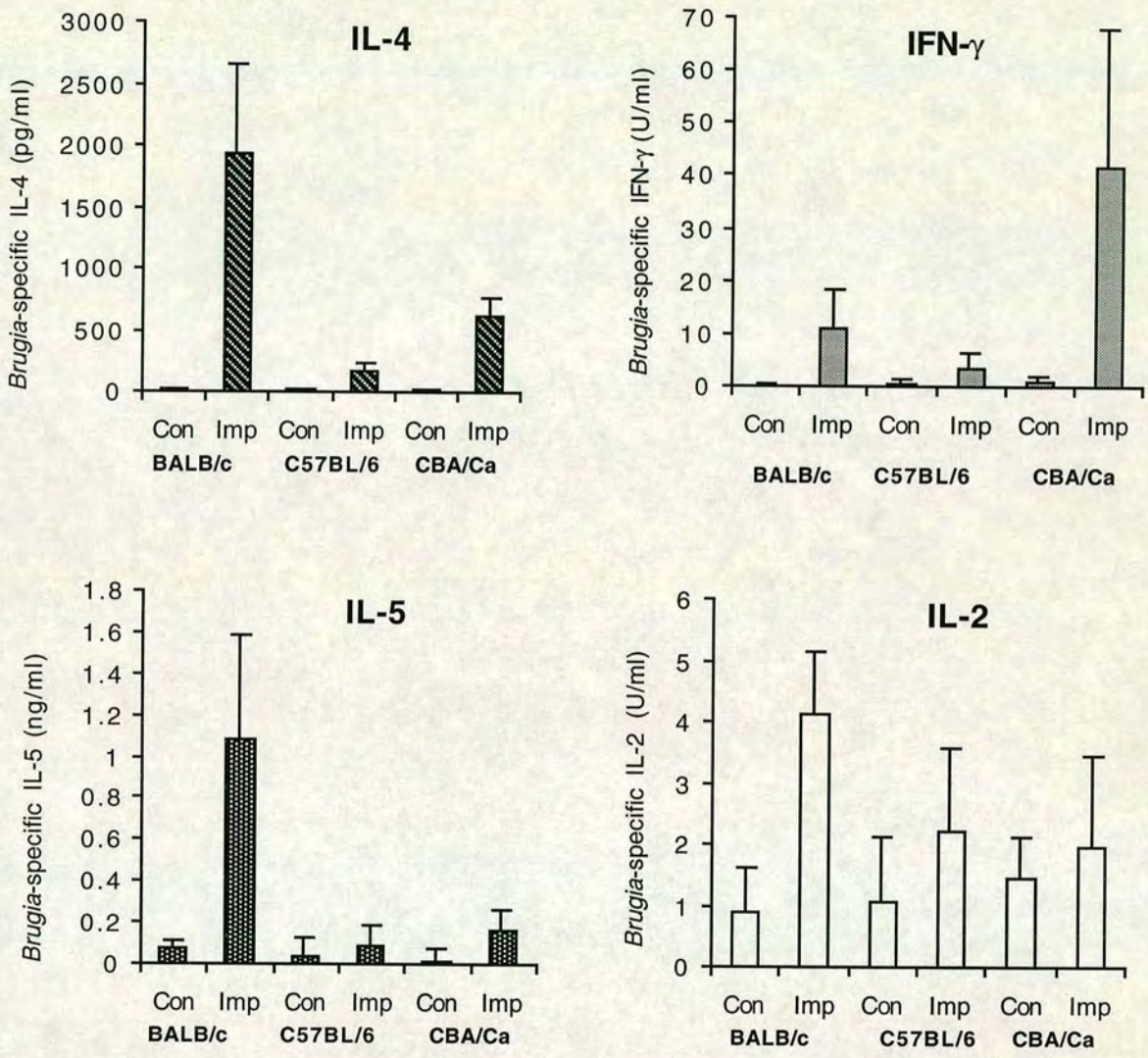


Figure 6. Mf-induced splenocyte cytokine production. IL-4 (diagonal striped bars), IFN-γ (grey bars), IL-5 (spotted bars), or IL-2 (open bars) production by splenocytes from control (unimplanted) or implanted (200,000 Mf) BALB/c, C57BL/6 or CBA/Ca mice. Cultures were stimulated with 5 μg/ml BmA, and levels of cytokine produced were measured by NK bioassay (IL-2 and IL-4), or by ELISA (IL-5 and IFN-γ). Data shown are mean ± SD of results obtained from sera of five individual mice in each group separately assayed.

Surprisingly high levels of IL-4 were produced in response to Mf, particularly by BALB/c mice. However, in keeping with published reports in BALB/c mice (Lawrence et al., 1994), Mf also induced high levels of IFN-γ production in all three strains of mice, particularly CBA/Ca. In addition to producing high levels of IL-4, BALB/c mice also produced high levels of IL-5 in response to Mf stage parasites, whereas CBA/Ca

and C57BL/6 produced lower levels of this cytokine. Implanted C57BL/6 mice produced less IL-4 or IFN-γ than CBA/Ca or BALB/c strains.

7.2.1.5 Cell recruitment

To determine if any differences existed between strains in the cell recruitment dynamics of mice implanted in the peritoneal cavity with *B. malayi*, cytocentrifuge preparations were made of cells recovered in peritoneal washes three weeks after implant with either adult or Mf parasites (Figure 7). The three stains of mice showed similar proportional recruitment of different cell subpopulations to the site of adult parasite implant. However, a marked difference was seen between strains in recruitment dynamics towards Mf, and also in the resident cell populations present prior to parasite exposure.

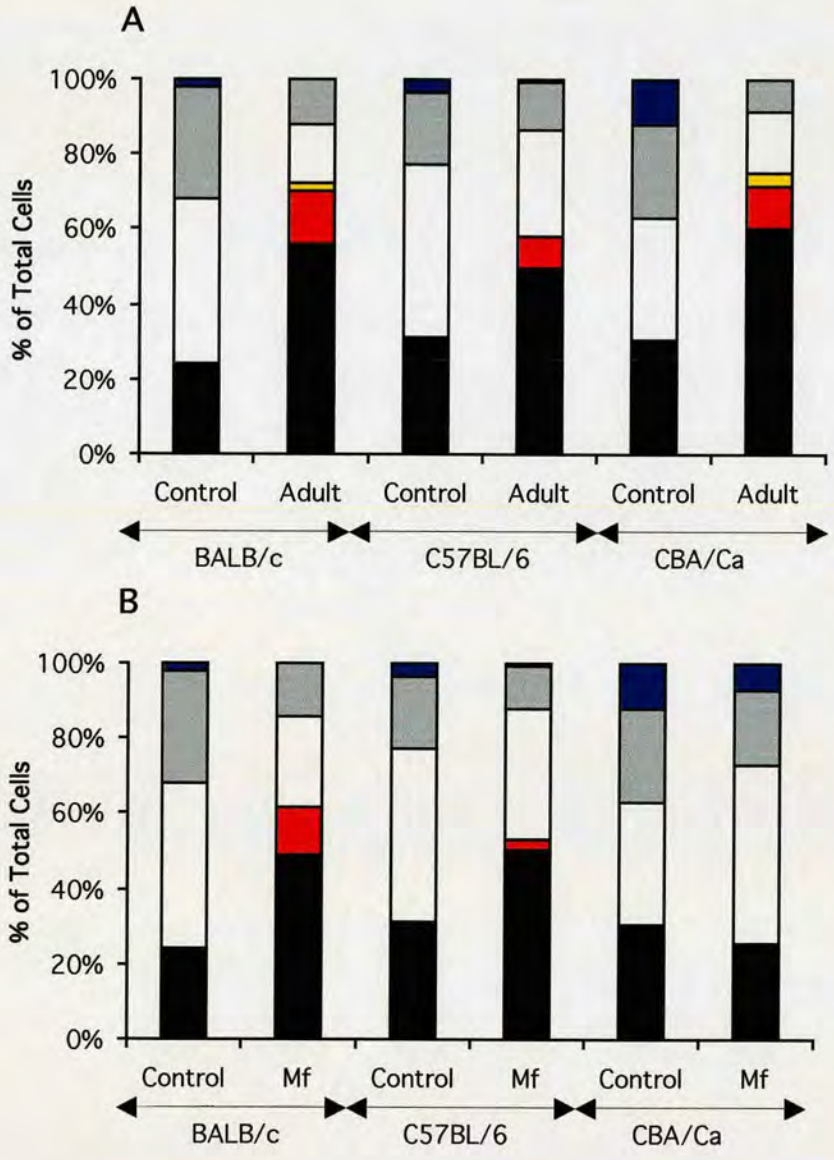


Figure 7. Peritoneal cell populations in control and parasite implanted BALB/c, C57BL/6 and CBA/Ca mice (i). Mean percentage of total cells that were identified as mast cells (blue bars), monocytes/blasting cells (grey bars), lymphocytes (open bars), neutrophils (yellow bars), eosinophils (red bars), and macrophages (solid bars), in control (unimplanted) mice, and mice implanted in the peritoneal cavity with (**A**) adult, or (**B**) Mf parasites. The cell composition of PEC from control and implanted mice was determined from cytospins by microscopy. Data shown are mean of three to six individual mice separately analysed.

BALB/c mice exhibited the lowest proportion of resident mast cells, with CBA/Ca having the highest. BALB/c and CBA/Ca mice showed a proportional increase in neutrophils recruited to the site of adult parasite implant, which was not seen in C57BL/6 mice. BALB/c mice also showed a striking eosinophilia towards Mf that was not seen in CBA/Ca mice, and although evident, was much less marked in the C57BL/6 strain.

All three strains of mice showed significantly increased numbers of cells recovered from the peritoneal cavity in response to adult parasites, but not Mf, in comparison to controls. Numbers of recovered cells from both BALB/c and C57BL/6 mice implanted with adult parasites was significantly greater than CBA/Ca mice ($P < 0.01$ and $P < 0.05$)(Figure 8A). None of the strains showed significant increases in cell numbers recruited in response to implant with Mf stage parasites, in comparison to unimplanted controls (Figure 8B).

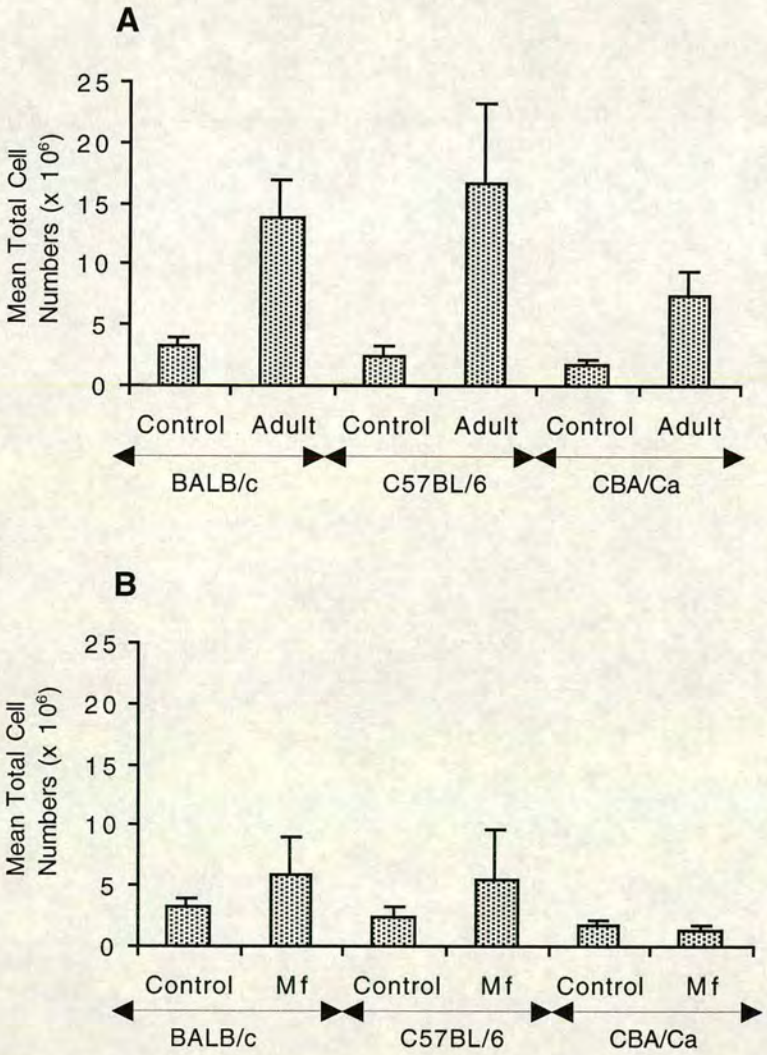


Figure 8. Peritoneal cell populations in control and parasite implanted BALB/c, C57BL/6 and CBA/Ca mice (ii). Mean total cell numbers recovered from the peritoneal cavity of control (unimplanted) mice, and mice implanted in the peritoneal cavity with adult (**A**) or Mf (**B**) parasites. Data presented are mean ± SD of three to six individual mice in each group.

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The proportional differences in peritoneal subpopulations seen in control and parasite-implanted mice was reflected in total numbers of each cell type recovered from the peritoneal cavity of these mice (Table 1). BALB/c and C57BL/6 mice responded similarly to adult stage parasites in terms of total numbers of macrophages, eosinophils and monocytes/blasting cells recruited to the peritoneal cavity. BALB/c and CBA/Ca mice showed similar numbers of recruited neutrophils to adult parasites, which were not present in C57BL/6 strain mice. BALB/c mice also showed a marked eosinophilia in response to Mf that was not seen in either of the other mouse strains. Even given the increases in total cell numbers, BALB/c and CBA/Ca strain mice showed significantly decreased numbers of mast cells recovered from the peritoneal cavity of either adult or Mf implanted mice, in comparison to controls. C57BL/6 mice showed significantly deceased numbers of mast cells only in Mf implanted mice.

Table 1. *Strain comparison of recruited peritoneal cell types*

	Total Number of Cells/animal ($x10^6$)					
	Macrophages	Eosinophils	Neutrophils	Lymphocytes	Monocytes & Blasting cells	Mast Cells
BALB/c Control	0.75 ± 0.13	0.02 ± 0.02	0.02 ± 0.02	1.43 ± 0.35	0.96 ± 0.22	0.07 ± 0.02
C57BL/6 Control	0.73 ± 0.15	0.01 ± 0.01	ND	1.21 ± 0.58	0.49 ± 0.19	0.09 ± 0.03
CBA/Ca Control	0.52 ± 0.26	ND	ND	0.55 ± 0.24	0.41 ± 0.16	0.18 ± 0.02
BALB/c Adult Implant	7.92 ± 2.19	1.87 ± 0.47	0.25 ± 0.06	2.15 ± 0.37	1.70 ± 0.60	0.02 ± 0.02
C57BL/6 Adult Implant	8.42 ± 3.50	1.54 ± 0.92	0.02 ± 0.02	4.56 ± 1.50	2.07 ± 0.01	0.07 ± 0.05
CBA/Ca Adult Implant	4.55 ± 1.43	0.77 ± 0.22	0.24 ± 0.09	1.27 ± 0.40	0.59 ± 0.15	0.01 ± 0.02
BALB/c Mf Implant	2.78 ± 1.50	0.74 ± 0.44	0.03 ± 0.02	1.56 ± 0.96	0.71 ± 0.30	0.01 ± 0.01
C57BL/6 Mf Implant	3.37 ± 3.09	0.12 ± 0.1	ND	1.50 ± 0.73	0.59 ± 0.39	0.02 ± 0.01
CBA/Ca Mf Implant	0.35 ± 0.21	ND	ND	0.62 ± 0.21	0.27 ± 0.16	0.08 ± 0.04

Data represent the mean total number (x 10^6) of each cell sub-population recovered in peritoneal washes. The cell composition of PEC from control (unimplanted), adult and Mf implanted BALB/c, C57BL/6 and CBA/Ca mice was determined from cytospins by microscopy. Data shown are mean ± SD of three to six individual mice separately assessed. N.D. = not detectable.

7.2.1.6 Parasite recovery

Counts were made of live Mf recovered in peritoneal washes three weeks after implant with adult stage parasites. No significant difference was seen in numbers of Mf recovered from BALB/c, C57BL/6 or CBA/Ca strain mice, although the highest numbers were seen in BALB/c mice, and the lowest in C57BL/6 mice.

Table 2. *Strain comparison of parasite recovery*

Mouse Strain	Mf recovered from adult-implanted animals (Mf/µl)
BALB/c	10.6 ± 7.4
C57BL/6	2.0 ± 1.9
CBA/Ca	4.7 ± 2.8

Table 1. Recovery of live parasites from the peritoneal cavity of BALB/c, C57BL/6, and CBA/Ca mice. Data shown represent the numbers of live Mf recovered from adult parasite-implanted mice and are shown as Mf/ml from a total volume of 10ml. Numbers of Mf recovered are mean ± SD of counts performed on four to five individual mice in each group. Counts were made three weeks after parasite implant, and no significant difference was noted between groups.

7.3 Discussion

This chapter details the analysis of antibody profiles, cytokine responses, and cellular recruitment profiles of BALB/c, C57BL/6 and CBA/Ca mice in response to adult and Mf stage *B. malayi*. Despite the variety of mouse strains that were used in this thesis, and have been used in the published literature, little is known about inherent differences that might exist in the immune response of different inbred strains of mice to filarial parasites. Comparative studies in this field have primarily dealt with the survival of parasites within various mouse strains, and have not examined the immune response elicited in any great detail.

We chose to assess cytokine production, antibody profiles, and cellular recruitment three weeks after parasite implant to the peritoneal cavity as this is the timepoint at which we standardly carry out our experiments, and represents a timepoint that should have allowed development of a measurable immune response.

The cytokine and antibody results obtained were generally consistent with previously published work, which had been carried out primarily in BALB/c mice (Lawrence et al., 1994), but revealed several notable differences. Adult parasites provoked activation of a Th2-type response, with high levels of IL-4 promoting production of elevated levels of IgG1 and IgE in all three inbred strains of mouse. In contrast, and again consistent with previously published data, the response to Mf generally resulted in development of a Th1-polarised response, demonstrated by production of high levels of IFN-γ coupled with elevated levels of IgG2b and IgG3 and, in the case of CBA/Ca mice, IgG2a.

Of the three strains tested, CBA/Ca mice responded to filarial exposure in the way that was most expected based on the data previously published on BALB/c mice. Adult parasites induced a strong Th2-type response, with elevated production of IgG1, IgE and parasite-specific IL-4, while Mf induced a strong Th1-type response, with isotype switching to IgG2a, IgG2b and IgG3, reduced IgE, and high levels of production of IFN-γ. CBA/Ca mice also differed from BALB/c and C57BL/6 strains by failing to mount an eosinophilia against implanted Mf.

Implant of BALB/c mice with adult parasites resulted in elevated IgG1, IgE, and parasite-specific IL-4 and IL-5. However, BALB/c mice implanted with Mf failed to completely switch to a Th1-type response. Although failing to produce elevated IgG1, in keeping with the documented propensity of mice of this strain to develop Th2-biased responses to infection, BALB/c mice implanted with Mf produced high levels of IL-4, IL-5, and IgE, as well as IFN-γ. IL-4, IL-5 and IgE are all implicated in development of eosinophilia, and BALB/c mice were found to significantly recruit eosinophils to the site of Mf implant.

In *L. major* infection studies, C57BL/6 mice display the 'healer' phenotype, developing a protective Th1-type response (Sadick et al., 1990), while in murine infection with the intestinal helminth *T. muris*, C57BL/6 mice also develop a resistant phenotype, but this time by mounting a protective Th2-type response (Faulkner et al., 1998). C57BL/6 mice appeared to mount neither a Th1-nor a Th2-polarised response to adult or Mf stage filarial parasites. The profile of antibody production presented by mice of this strain was similar on implant with adult or Mf parasites, with elevated IgG1, IgG2b and IgG3. Low levels of IgE were produced to the adult parasites, and minimal amounts to Mf. Furthermore, in contrast to BALB/c and CBA/Ca strains, parasite-specific production of both type-1 and type-2 cytokines was in most cases not significantly elevated over unimplanted control levels. Although this 'muted' cytokine production was a consistent feature of responses to *Brugia* in this strain of mice, cell recruitment was unimpaired, and generally comparable to that of the elevated cytokine producing BALB/c mice.

Mf exposure led to higher levels of IL-4 production by all three strains of mice than had previously been reported in BALB/c mice at the time point measured (21 days), and was particularly high in BALB/c mice. Late stage production of elevated levels of IL-4 by Mf-exposed BALB/c mice has been noted by Lawrence *et al.*, with 28 days parasite exposure required for the emergence of this response, and it was proposed that a threshold of exposure time to Mf might be required for 'late' IL-4 production (Lawrence et al., 1994) Although we see IL-4 production in response to Mf

approximately one week earlier than the 28 day timepoint, this may be due to experimental differences, for example in mouse age or Mf dosage.

Consistent with the data presented in chapter 5, adult parasites stimulated recruitment of large numbers of inflammatory cells to the peritoneal cavity of BALB/c, C57BL/6 and CBA/Ca mice, whereas Mf did not. All three strains of mice provoked a marked eosinophilia coupled with loss of mast cells in response to adult parasite implant. Conversely, there were notable strain differences in the response to Mf, the most striking of which were the Mf-driven eosinophilia and macrophage recruitment seen in BALB/c and C57BL/6 mice that was not present in CBA/Ca mice. It is possible that the differences in eosinophilia could be a reflection of the cytokine environment that was stimulated by Mf in each strain. BALB/c mice displayed the most Th2-like cytokine production against Mf, CBA/Ca the most Th1-like, and C57BL/6 mice mounting neither a Th1 or Th2-polarised response. As eosinophils may themselves provide an important source of IL-4 during helminth infection, it is possible that the eosinophilia evident in BALB/c mice implanted with Mf might contribute to, as well as being a consequence of, the resultant Th2-type cytokine response that is seen in these mice.

The data presented in this chapter illustrates that, as might have been expected, the genetic background of the murine host can have a distinct influence on the phenotype of the elicited response to adult or Mf *B. malayi*. Differences were not as great as those seen in other experimental infections in which opposing, highly polarised responses are evident in different mouse strains, which can have an impact on parasite survival. This could be due to the fact that mice are not a permissive host for infection with *B. malayi*. However, the strain differences in response to filarial infection detailed here should be borne in mind when comparing studies on filarial parasites that have been carried out using different mouse strains.

Chapter 8: General Discussion

Immune down-regulation is a fascinating feature of helminth infection, and one that is poorly understood in terms of induction and effector mechanisms, and involvement in the outcome of infection. It is possible that down-modulated immune responses can be beneficial to both host and parasite. The study of host-parasite relationships provides the opportunity to learn about fundamental aspects of mammalian immunity. The emphasis of these studies was not to determine the factors involved in the survival of filarial parasites within the immunocompetent host, but rather to unravel the elements involved in development of down-regulation of proliferative responses by *B. malayi.*

Mf have been implicated as the stage of the parasite responsible for effecting suppression, since down-regulation of proliferative responses coincides with the presence of Mf in the bloodstream of infected humans (Ottesen et al., 1977; Piessens et al., 1980; Yazdanbakhsh et al., 1993) and with the onset of patency in several animal models (Lammie and Katz, 1983; Soboslay et al., 1991). Additionally, evidence exists that T cell reactivity can be restored after treatment with the microfilaricidal drug ivermectin (Lammie et al., 1992). However, we have now shown that adult and L3 stage parasites, or adult male parasites alone, are capable of down-regulating proliferative responses, even if in human filariasis it is not apparent systemically until later in infection. Implant of Mf into the murine peritoneal cavity can induce suppression, but of a less profound nature and by a different mechanism to adult and L3 stages. It appears that the parasite employs more that one method to modulate host cellular responses, and generation of a host anti-proliferative cell population by L3 and adults may represent only one of multiple regulatory mechanisms.

B. malayi does not directly block the proliferation of lymphocytes, but instead subverts the host immune system to recruit or activate host cells to suppressive function. The inability of either adult or Mf parasites to directly block lymphocyte proliferation when co-cultured *in vitro*, coupled with the requirement for a substantial amount of *in vivo* exposure time to the parasite prior to development of suppressive PEC provides further evidence for this hypothesis. It appears that the parasite requires the collaboration of

the host in order to down-regulate the very immune response that might otherwise prove effective at combating infection. Further, this recruitment or activation is effected through the release of soluble factor(s) from the parasite, a tactic that may be generally employed by nematodes, and indeed helminths, other than *Brugia* spp. (Maizels et al., 1993)

Helminth infection typically results in a Th2-dominated host response, with elevated production of IL-4. The data presented in this thesis reveals that host IL-4 production is essential for the induction of a non-specific suppressor cell population, but that the major down-regulatory cytokine associated with Th2 responses, IL-10, is not a key component. An important unanswered question is if IL-4 alone is sufficient to generate down-regulatory host cells. It is possible that the signal carried in parasite ES is to 'switch on' IL-4 production by host cells in the local environment, which alone is sufficient to generate down-regulatory cells, or is given in conjunction with another signal in ES that is required to activate host cells to suppressive function. These possibilities remain to be investigated in further detail, through analysis of responses induced by components or fractions of parasite ES in mice, as well as testing the effect of direct inoculation with IL-4.

Eosinophils have been suggested as a potential source for early production of IL-4 in helminth infection (Sabin and Pearce, 1995), and analysis of cell recruitment to the peritoneal cavity of mice implanted with the different life cycle stages of *Brugia* has shown that the Th2-inducing adult and L3 stage parasites induce a rapid and more pronounced eosinophilia than Th1-inducing Mf. Additionally, IL-4-deficient mice implanted with adult parasites show a significantly reduced eosinophilia in comparison to wild-type counterparts. Further investigation is required to test whether these recruited eosinophils provide a source of IL-4, and to determine if mast cells and/or resident lymphocytes may also play a role in providing early IL-4. It is possible that factors in parasite ES initiate an activation cascade that, through release of IL-4 and/or IL-4-driven cytokines, leads to recruitment of eosinophils and lymphocytes to the site of infection. Recruited cells might then amplify production of IL-4 and direct the systemic

immune response towards a Th2-type phenotype, as well as generating down-regulatory host cells.

The identity of filarial-induced suppressor cells remains a mystery. Previous work in other animal models, primarily in jirds, had assumed but not demonstrated that macrophages were the regulatory cell type. As shown in this thesis, the two major cell types recruited by adult and L3 stage parasites are macrophages and eosinophils. We have shown that suppressive PEC do not bear the common macrophage marker F4/80, a finding that encouraged us to consider the possibility of non-macrophage cell types as down-regulatory cells. The multi-faceted abilities of eosinophils, combined with their ambiguous function in helminth infection, promoted them as an attractive candidate cell type. Even though mice unable to mount an eosinophilia were able to generate suppressive PEC, the potential for compensatory mechanisms in gene-deficient mice warrants the further investigation of this intriguing cell type as a potential regulatory cell. Purification of eosinophils recruited to the site of parasite infection, followed by use of these cells as APC and analysis of cytokine production, should help clarify if they can assume a suppressive role. The possibility remains that a minor, as yet unidentified, cell population is responsible for the proliferative suppression seen in this system, and further investigation through systematic analysis of the suppressive ability of recruited cell subpopulations will be required.

The induction of down-regulatory cells by parasitic infection is a well-documented example of one of a variety of immune evasion strategies employed by infectious organisms to aid survival within the immunocompetent host. However, the suppression induced by adult *B. malayi* is not due to classical mechanisms of suppressor macrophage function such as hydrogen peroxide, prostaglandins, or NO (Allen et al., 1996). Indeed, the PEC suppression that we have observed is in striking contrast to any currently defined infection model. For example, a suppressor cell population is generated by infection of mice with African trypanosomes in which nitric oxide and prostaglandins are the mediators (Schleifer and Mansfield, 1993). The influence of IFN-γ production on NO synthesis (Ding et al., 1988), and evidence for a direct involvement of IFN-γ in NO-mediated suppression of *Trypanosoma brucei* infection

(Darji et al., 1996; Sternberg and Mabbott, 1996) suggests that it is IFN-γ, not IL-4, that is necessary for induction of proliferative block by trypanosomes. Similarly, the importance of IL-4 in our system differs from IL-4-independent down-regulation of proliferation in *Toxoplasma gondii* (Roberts et al., 1996), a process that is at least partly attributable to IFN-γ (Channon and Kasper, 1996) and IL-10 (Neyer et al., 1997). The profound nature of filarial-mediated suppression and its IL-4 dependence suggested a potentially novel mechanism of action. Further, the discovery that proliferative block can be effected by a contact-dependent mechanism or by fixed cells that are unable to secrete soluble mediators strongly indicates that this is a down-regulatory mechanism not previously described.

It is difficult to comprehend a surface-bound suppressive molecule that can block the proliferation of such a wide range of cell types, including fully differentiated T cell clones, primary cell lines, hybridomas, lymphomas and adenocarcinomas. Presumably, the mechanism of suppression must act through a surface molecule or receptor that is common to all of these cell types. Further work is necessary to identify the molecules involved. The situation is further complicated by the fact that this is likely to be only one of potentially many immunomodulatory mechanisms employed by the parasite.

Although not identical to what is seen in human filarial infection, the immune down-regulation observed in this system does provide some striking parallels. Through the release of ES products that generate suppressive host cells, adult or L3 parasites could effectively create a local zone of muted responsiveness. Dampening of the host inflammatory response by this mechanism could help explain the absence of inflammation at the site of filarial infection that has been observed in human studies (Amaral et al., 1994; Jungmann et al., 1991; Ottesen, 1980) and in animal models (Denham and McGreevy, 1977; Miller et al., 1991). It may be that this mechanism is restricted to down-regulation of localised cellular responses, preventing inflammatory responses within the lymphatics, and aiding L3 survival during migration and development.

We have found that the proliferative block caused by peritoneal exudate cells from *B. malayi*-infected mice is not antigen-specific. The induction of non-specific host anti-proliferative mechanisms does not currently account for the antigen-specific hypo-responsiveness observed during human filarial infection. Although some evidence for generalised unresponsiveness in human filarial infection is found in onchocerciasis (Greene et al., 1983), studies on lymphatic filariasis generally show that only parasite-specific proliferation is lost (Ottesen et al., 1977; Piessens et al., 1980; Yazdanbakhsh et al., 1993). However, animal studies have demonstrated that both specific and non-specific proliferative suppression can be induced by infection with *Brugia* species (Allen et al., 1996; Lammie and Katz, 1983; Lammie and Katz, 1984(a); Lammie and Katz, 1984(b); Lammie and Katz, 1983). The possibility that a cell population that is profoundly anti-proliferative may lead to the development of antigen-specific suppression requires further investigation. We do not know if the mechanism of proliferative suppression described in this thesis might play a role in the peripheral tolerance evident in human infection. It is difficult to predict how a potent anti-proliferative signal might affect the development of a naïve T cell that first comes into contact with antigen while being exposed to such a negative signal. Unable to proliferate, such cells may be rendered tolerant or anergic to later antigenic challenge in the periphery (Jenkins, 1992). Alternatively, this process could account for the reduced frequency of filarial-specific T cells in the periphery of actively infected individuals (King et al., 1992).

Application of observations made in this study to general filarial infection is somewhat hampered by the artificial nature of the infection system. Similar investigations in a model that allows the full developmental cycle of filarial parasites, such as *Litomosoides* spp. in BALB/c mice, should prove invaluable in assessing the relevance of the observations we have made to filarial infection. Irrespective of the role that these suppressor cells might play in filarial infection, their unusual phenotype and potentially novel mechanism of action make them of fundamental immunological interest.

The mechanism(s) of suppression exhibited by filarial-induced suppressor cells appear to be unlike those previously described in other infectious disease systems. Further

investigation of this model, as well as enhancing our understanding of filarial immunology, should help us to clarify the relationship of IL-4 induction, Th2-development, and the generation of immunosuppression, and may provide insight into potentially novel areas of immune modulation.

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Appendix 1: Photomicrographs of cytocentrifuge preparations

Plate 1	Cytospin of PEC from unimplanted CBA/Ca mouse (x200)
Plate 2	Cytospin of PEC from unimplanted CBA/Ca mouse (x400)
Plate 3	Cytospin of PEC from a CBA/Ca mouse 3 wk after adult *Brugia*-implant (x200)
Plates 4 & 5	Cytospin of PEC from a CBA/Ca mouse 3 wk after adult *Brugia*-implant (x400)
Plate 6	Cytospin of PEC from a CBA/Ca mouse 3 wk after adult *Brugia*-implant (x1000)
Plate 7	Cytospin of PEC from a CBA/Ca mouse 3 wk after adult *Brugia*-implant (x400)

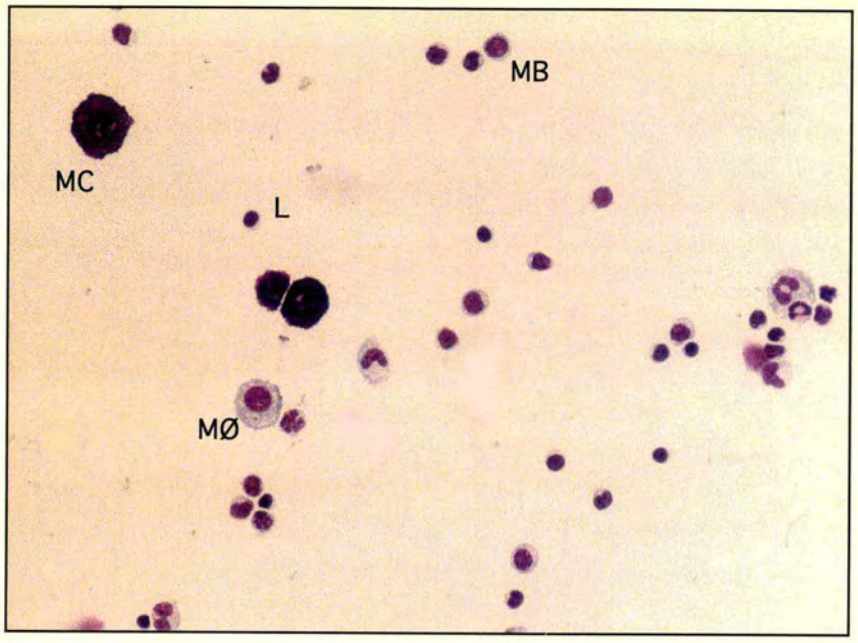

Plate 1. Cytocentrifuge preparation of PEC from an unimplanted CBA/Ca mouse. MØ = macrophage, L = lymphocyte, MC = mast cell, MB = monocyte/blasting cell Photomicrograph taken at x200 using a Nikon Microphot-FX microscope.

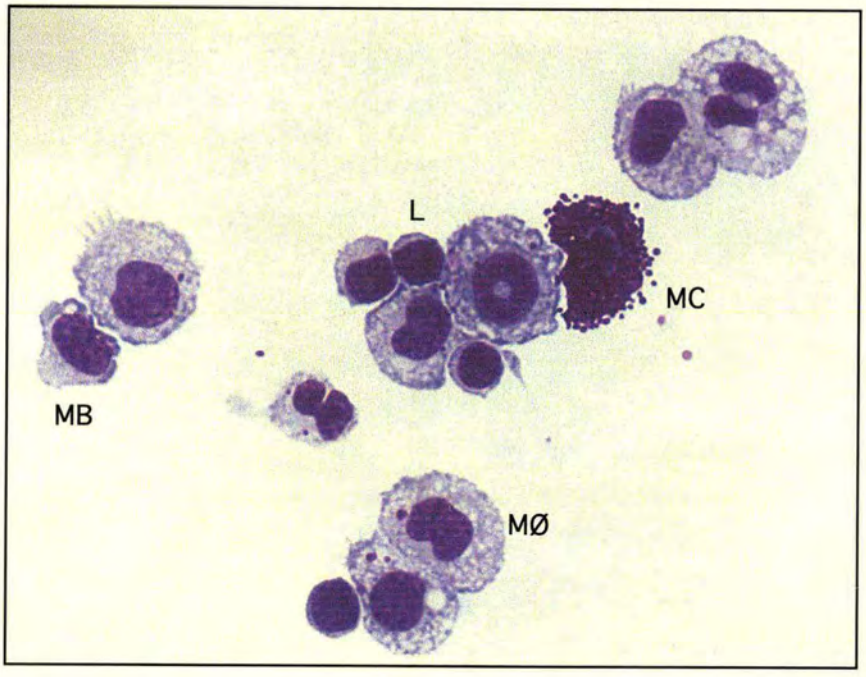

Plate 2. Cytocentrifuge preparation of PEC from an unimplanted CBA/Ca mouse. MØ = macrophage, L = lymphocyte, MC = mast cell, MB = monocyte/blasting cell Photomicrograph taken at x400 using a Nikon Microphot-FX microscope.

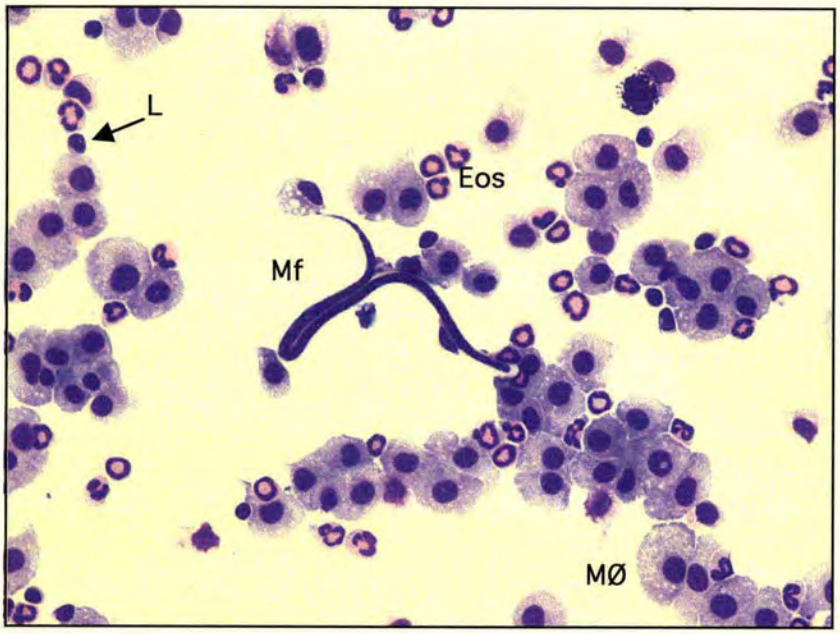

Plate 3. Cytocentrifuge preparation of PEC from a CBA/Ca mouse that had been implanted with adult *B. malayi* parasites 3 weeks previously. Mf = microfilaria, MØ = macrophage, Eos = eosinophil, L = lymphocyte. Eosinophils are distinguished by their ring-shaped nuclei and pink-staining granules. Photomicrograph taken at x200 using a Nikon Microphot-FX microscope.

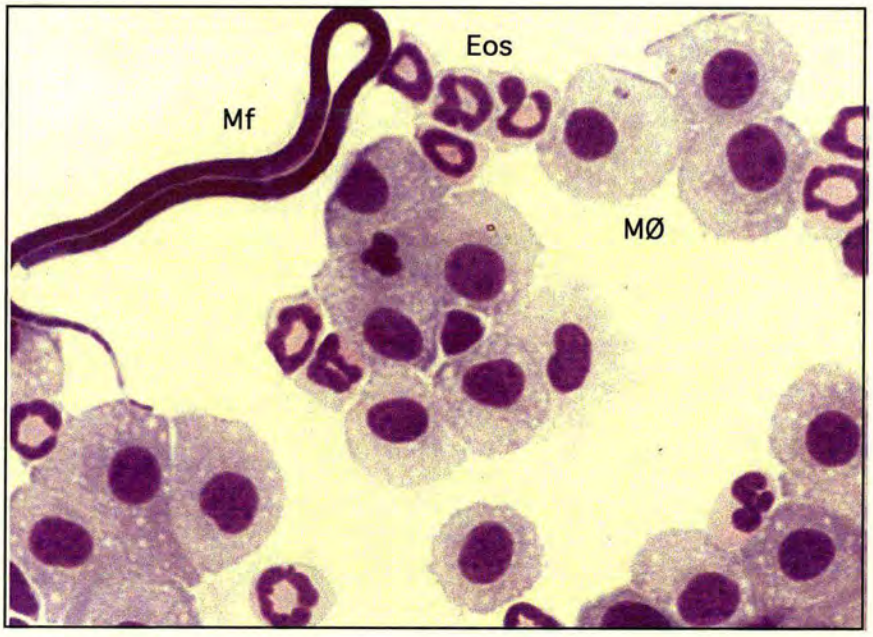

Plate 4. Cytocentrifuge preparation of PEC from a CBA/Ca mouse that had been implanted with adult *B. malayi* parasites 3 weeks previously. Mf = microfilaria, MØ = macrophage, Eos = eosinophil. Eosinophils are distinguished by their ring-shaped nuclei and pink-staining granules. Photomicrograph taken at x400 using a Nikon Microphot-FX microscope.

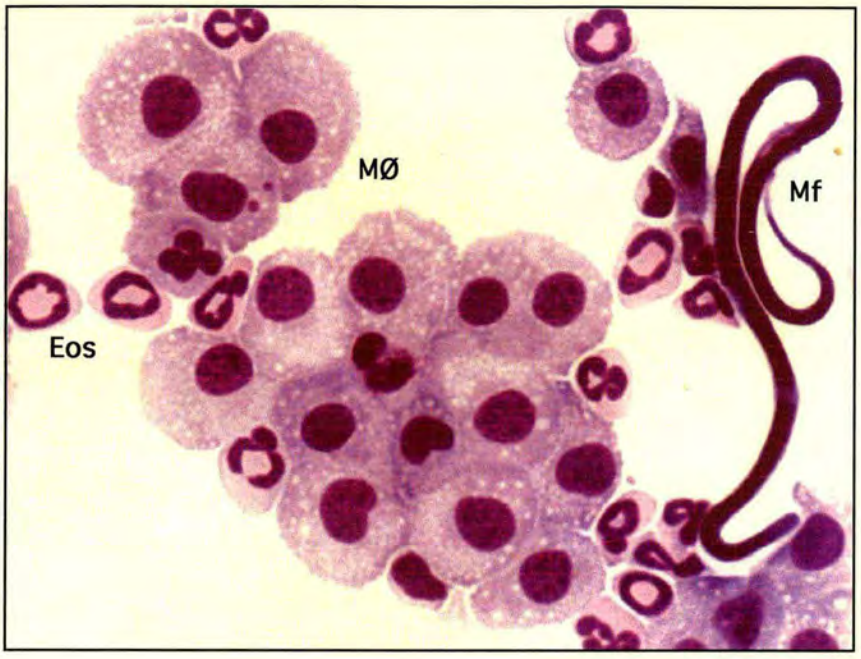

Plate 5. Cytocentrifuge preparation of PEC from a CBA/Ca mouse that had been implanted with adult *B. malayi* parasites 3 weeks previously. Mf = microfilaria, MØ = macrophage, Eos = eosinophil. Eosinophils are distinguished by their ring-shaped nuclei and pink-staining granules. Photomicrograph taken at x400 using a Nikon Microphot-FX microscope.

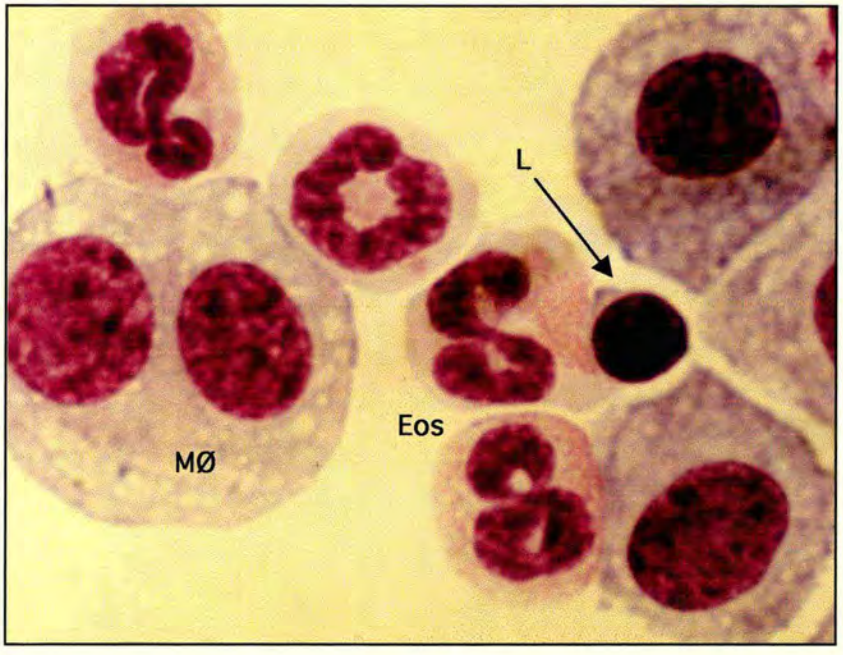

Plate 6. Cytocentrifuge preparation of PEC from a CBA/Ca mouse that had been implanted with adult *B. malayi* parasites 3 weeks previously. MØ = macrophage, L = lymphocyte, Eos = eosinophil. Eosinophils are distinguished by their ring-shaped nuclei and pink-staining granules. Photomicrograph taken at x1000 using a Nikon Microphot-FX microscope.

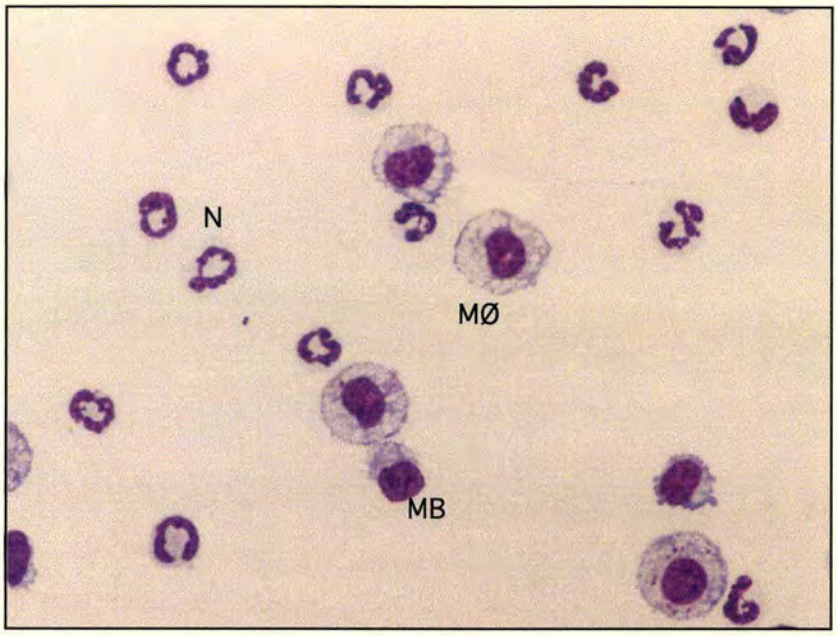

Plate 7. Cytocentrifuge preparation of PEC from a CBA/Ca mouse that had been implanted with adult *B. malayi* parasites 1 day previously. MØ = macrophage, N = neutrophil, MB = monocyte/blasting cell. Neutrophils are distinguished by their 'ragged' multi-lobed nuclei. An additional morphological difference from eosinophils is the lack of pink-staining granules. Photomicrograph taken at x400 using a Nikon Microphot-FX microscope.

Appendix 2: Published Papers

Erratum: In the article printed below, which appeared in J. Immunol. 160:1304, please note the following correction. Due to a printer's error, the word conalbumin was replaced with Con A. The corrected paper is printed in its entirety.

Requirement for In Vivo Production of IL-4, But Not IL-10, in the Induction of Proliferative Suppression by Filarial Parasites[1]

Andrew S. MacDonald,* Rick M. Maizels,* Rachel A. Lawrence,† Ian Dransfield,‡ and Judith E. Allen[2]*

Loss of T lymphocyte proliferation and the emergence of a host response that is dominated by a Th2-type profile are well-established features of human filariasis. We have previously reported that adherent peritoneal exudate cells (PEC) from mice transplanted with adult *Brugia malayi* parasites suppress the proliferation of lymphocytes without blocking Ag-cytokine production in vitro. We now show that infection of mice with the infective larval (L3) stage of *B. malayi* generates a similar population of PEC. Suppressive cells are generated within 7 days of infection and mediate their effects through a nitric oxide-independent pathway. Both L3 and adult infection elicit high levels of host IL-4 whereas the microfilarial stage of the parasite induces IFN-γ production and does not generate a similar form of suppression. Production of host IL-4 was necessary to allow the generation of suppressive PEC, given that IL-4-deficient mice implanted with adult parasites failed to induce proliferative block. However, IL-10-deficient mice implanted with adult parasites resulted in T cell suppression, indicating that IL-10 is not essential for the induction of hyporesponsiveness. Neither IL-4 nor IL-10 were directly responsible for ablating cellular proliferation in vitro, as the addition of neutralizing Ab to either cytokine did not reverse the proliferative block. Thus, IL-4 produced in vivo in response to filarial L3 and adult parasites is essential for the induction of proliferative suppression but is not itself the suppressive factor.
***The Journal of Immunology*, 1998, 160: 4124–4132.**

The establishment of a parasitic helminth infection is characteristically accompanied by a host response dominated by a Th2-type profile and the emergence of parasite-specific immunosuppression (1–4). This is the case in human filariasis, in which long term infection with this lymphatic-dwelling nematode is accompanied by down-regulated host immune responses (5–7). However, the mechanisms that underlie the development of the profound cellular hyporesponsiveness in filariasis and the relationship of this to the development of a Th2-type response are unknown. These questions can be addressed in *Brugia malayi*-infected mice, because Th subset bias and immune suppression, both specific and nonspecific, are hallmarks of infection with this filarial nematode (8–11).

We investigated the possibility that filariae down-regulate host immune responses by interfering with effective APC function. To delineate the role of the APC, we tested their competence in a defined model Ag system. Mice were implanted in the peritoneal cavity with adult *B. malayi*, and the ability of adherent peritoneal cells to present Ag ex vivo was evaluated (11). We found that when live but not dead parasites were used, the ability of the D10.G4 Th2 cell clone to proliferate in response to its cognate Ag (conalbumin) was completely blocked. However, levels of Ag-cytokine production were intact, and even elevated, relative to control peritoneal exudate cells (PEC),[3] indicating that there was no defect in Ag processing. When low numbers of infection-derived PEC were mixed with an excess of normal irradiated splenocytes, profound proliferative suppression was still observed. This demonstrated that suppression was an active process and not due to an absence of costimulatory molecules or reduced numbers of effective APC. Moreover, while suppression could be transferred with supernatants from cultured PEC, this was not due to soluble factors typically associated with "suppressor macrophages" such as prostaglandins or nitric oxide (11).

In contrast to adult *B. malayi*, peritoneal implant of the blood-circulating microfilarial stage (Mf) of the parasite generates a less profound form of suppression that is reversible by addition of NO inhibitors in vitro (11). Previous studies have demonstrated that contrasting cytokine profiles are induced by these two stages of the parasite. Implantation of adult parasites induces high levels of IL-4 with a matching "Th2" Ab isotype profile, while microfilariae induce IFN-γ and a "Th1" Ab profile (10). In this study, we chose first to evaluate the infective larval stage (L3) which, like the adult stage, induces high levels of IL-4 in the infected host (9, 12) and, as the stage of parasite that establishes infection, must also have means to avoid host immune responsiveness. A striking correlation was observed between splenic IL-4 production and the level of proliferative suppression in L3-infected animals. We therefore investigated the role of IL-4 in the in vivo induction of proliferative suppression. This work has led to the identification of a potentially

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[3] Abbreviations used in this paper: PEC, peritoneal exudate cells; ES, excretory/secretory; D-NMMA, N^G-monomethyl-D-arginine; L-NMMA, N^G-monomethyl-L-arginine; L3, infective larvae; Mf, microfilariae; BmA, adult *Brugia malayi* Ag.

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novel form of immunosuppression induced by filarial infection that is dependent on IL-4 but not the down-regulatory cytokine IL-10.

Materials and Methods

Parasite material

B. malayi adults and Mf were obtained from infected jirds purchased from TRS laboratories (Athens, GA). Adult worms were removed from the peritoneal cavity and washed in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 50 μg/ml gentamicin. L3 were obtained from *Aedes aegypti* mosquitoes that had been fed on blood containing Mf 12 days previously. Adult *B. malayi* Ag (BmA) was prepared by homogenization of mixed sex worms in PBS on ice followed by centrifugation at 10,000 × *g* for 20 min. The resultant supernatant was passed through a 0.2-μm pore size filter before protein concentration determination by the Coomassie Plus protein assay (Pierce Chemical Co., Rockford, IL).

Mouse infection model

For all experiments, mice used were 6- to 8-wk-old males unless otherwise stated. CBA/Ca mice plus age-matched control mice were purchased from Harlan-U.K. (Bicester, U.K.). IL-4-deficient (IL-$4^{-/-}$) (13) C57BL/6, IL-10-deficient (IL-$10^{-/-}$) (14) C57BL/6, and wild-type C57BL/6 mice were purchased from B&K Universal Ltd. (North Humberside, U.K.) with the permission of the Institute of Genetics, University of Cologne, Cologne, Germany. Mice were implanted i.p. with either 6 live adult *B. malayi* females or 10 to 400 L3 (as shown in text). Implants were left for 3 wk unless otherwise stated. At this point, mice were euthanized by cardiac puncture, and PEC were harvested by thorough washing of the peritoneal cavity with 15 ml of RPMI containing 50 μg/ml gentamicin.

For treatment of animals with neutralizing Ab to IL-4 in vivo, mice were injected i.p. with 2 mg of anti-IL-4 mAb (11B11), or isotype-matched anti-IFN-γ mAb (R4-6A2; American Type Culture Collection (ATCC) HB170) at the time of parasite implant. For in vivo use, 11B11 was kindly provided by F.W. Falkenberg (Ruhr University, Bochum, Germany), and for an isotype-matched control, the miniPERM mAb production system (Heraeus) was used to produce neutralizing mAb to IFN-γ, purified using a protein G column (Pharmacia Biotech, Uppsala, Sweden).

Cultures and cell lines

All in vitro cultures were conducted in RPMI 1640 medium (Life Technologies) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μM 2-ME, and 10% FCS (complete medium). The Th2 cell clone D10.G4 (15) and the B cell hybridoma HB32 (ATCC, Rockville, MD) were maintained in culture as previously described (11). For experiments with C57BL/6 mice, primary OVA-sensitized T cell lines were established by s.c. immunization of mice with 50 μg/footpad OVA in CFA. Two weeks later, popliteal lymph nodes were removed, and liberated cells were cultured in complete medium with 25 μg/ml OVA. After 3 to 4 days, cultures were supplemented with 10% (v/v) conditioned medium from a 48-h culture of conalbumin-stimulated mouse lymphocytes. Syngeneic splenocytes inactivated by irradiation with 2000 rads were added at the same time as feeders. T cells were used 5 to 7 days after this rest period.

Proliferation and cytokine assays

For investigation of proliferative suppression caused by parasite-exposed PEC, 100 μl of PEC at 1×10^6/ml were adhered to a flat-bottom 96-well plate (Nunclon; Life Technologies) at 37°C for 2 to 3 h, after which nonadherent cells and Mf were removed by washing with complete medium. Plates with adherent PEC were then used for coculture with 5×10^4 D10.G4 or HB32 cells or with primary T cell lines, to a final volume of 200 μl/well. Conalbumin and OVA (both from Sigma Chemical Co., St. Louis, MO) were used at the concentrations indicated in the text. After incubation for 48 h at 37°C, 100 μl of supernatant were removed from each well of the D10.G4 or primary lymphocyte assays for cytokine analysis; 1 μCi of [^{3}H]TdR in 10 μl of complete medium was then added to each well, and plates were incubated for 16 to 18 h at 37°C before harvesting and counting using a Top Count microplate scintillation counter (Packard Instrument Co., Meriden, CT).

For analysis of lymphocyte responses in the spleens of infected animals, splenocytes were obtained by teasing spleens apart and then lysing erythrocytes using red blood cell lysis buffer (Sigma Chemical Co.). Viable cells were then counted by trypan blue exclusion. Whole spleen cells were incubated at 5×10^5 cells/well with BmA at a final concentration of 5 μg/ml. After incubation for 65 h at 37°C, supernatants were taken for subsequent cytokine analysis.

The IL-2/IL-4-responsive NK cell line (16) was used to measure cytokine production by D10.G4 cells, primary T cells and splenocytes, as previously described (10, 11). Briefly, proliferation of the NK cells at 10^4 cells/well was measured in the presence of 20 μl of culture supernatant with the addition of anti-IL-2 (S4B6) neutralizing Ab for IL-4 measurement. S4B6 was obtained from ascites for in vitro use and used at 2.5 μl/ml, the optimal concentration for neutralization as determined by titration. Cells and supernatants were incubated for 24 h at 37°C before addition of 1 μCi of [^{3}H]TdR in 10 μl of complete medium. After a further 12 h of incubation at 37°C, plates were harvested and counted. IFN-γ was measured by ELISA using R46A2 (ATCC) as capture Ab and biotinylated rat anti-mouse IFN-γ monoclonal XMG1.2 Ab (PharMingen, San Diego, CA, 18112D), followed by avidin-alkaline phosphatase (Sigma Chemical Co., A-7294) for detection. Standard curves using mouse recombinant IL-4 (Sigma Chemical Co., I-1020), and IFN-γ (Sigma Chemical Co., I-5517) were performed to determine cytokine levels in supernatants.

Neutralizing Ab to mouse IL-10 was purchased from Genzyme Diagnostics (Cambridge, U.K.) and used at 2 μg/ml. The neutralizing Ab to IL-4 (11B11) was obtained from ascites for in vitro use and used at 2.5 μl/ml, the optimal concentration for neutralization as determined by titration. The NO inhibitor L-N^G-monomethylarginine (L-NMMA) and the control inhibitor D-NMMA (Wellcome Foundation, Beckenham, U.K.) were used where indicated at a final concentration of 250 μg/ml.

Cell sorting and analysis

PEC isolated from mice were washed and adjusted to 1×10^7/ml in PBS containing 0.02% EDTA. Cells were then incubated at 4°C for 30 min with the appropriate concentration of anti-macrophage (Clone F4/80, R-phycoerythrin-conjugated, Caltag Laboratories, San Francisco, CA) mAb, as determined by titration. F4/80-positive and -negative PEC were sorted using a FACStar (Becton Dickinson, San Jose, CA), with logarithmic amplification of fluorescence detection and side scatter and linear amplification of forward scatter. LYSIS 2 software was used for acquisition and analysis. Sorted cells were then washed and resuspended in complete RPMI medium for use in the D10.G4 assay.

Cytocentrifuge preparations of PEC (1×10^5 cells/slide) were air-dried and fixed in methanol before staining with Diff-Quik (Dade, Diagnostics, Unterschleissheim, Germany). The proportion of macrophages, lymphocytes, mast cells, and eosinophils was determined by morphologic examination of at least 300 cells in randomly selected fields using an Olympus BH2 microscope with a ×100 objective.

Statistical analysis

Student's *t* test and the paired *t* test were used to determine the statistical significance of differences between and within groups. $P < 0.05$ was considered to be a significant difference.

Results

Time course of suppression by adult B. malayi

In previous work, we demonstrated that 3 wk after adult *B. malayi* implantation into mice, a suppressive cell population was recovered from the peritoneal cavity. Because L3 stage parasites do not survive for 3 wk in the murine host (17), a time course experiment was conducted to establish a shorter time frame, as a prelude to testing L3 themselves. Following implantation of adult *B. malayi*, proliferation of the murine T cell clone D10.G4 cultured with adherent peritoneal cells from infected mice was measured at several time points during a 3-wk period (Fig. 1*A*). Seven days of host exposure to the parasite were required to achieve significant proliferative suppression ($P < 0.05$). This suggests that the development of a host immune response or recruitment of particular cells is required, consistent with the inability of parasites to directly induce proliferative suppression in vitro (11).

PEC from parasitized mice also induce a proliferative block in a range of B cell hybridomas and a colon carcinoma cell line (11). Proliferation of the B cell hybridoma HB32 was assessed in the presence of adherent PEC from adult parasite-implanted animals (Fig. 1*B*). In this case, suppression of proliferation was apparent with cells taken 7 days postexposure to adult parasites but was maximal with day 14 cells. We thus chose to test cells from L3-infected mice at 2 wk postinfection to ensure that sufficient time

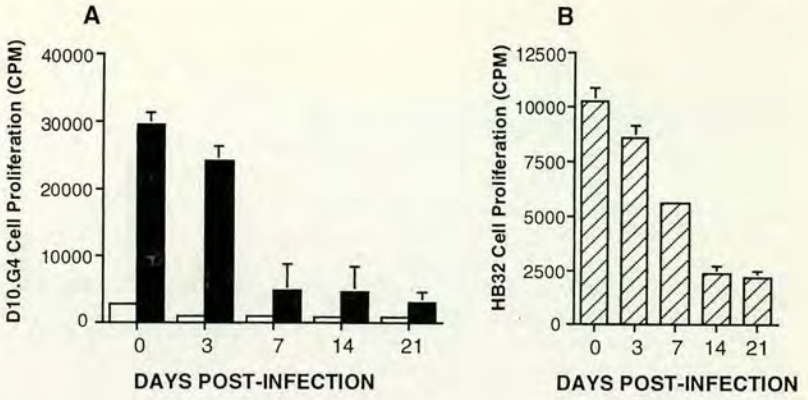

FIGURE 1. Time course of suppression. Proliferation of D10.G4 cells with media (□) or 50 μg/ml conalbumin (■) (*A*), or HB32 cells with media (▨) (*B*), in the presence of PEC from control (time point 0) or adult parasite-implanted CBA/Ca mice over a 3-wk time course. Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute. Data presented are mean ± SD of three to four individual mice separately assayed. Ag-D10.G4 proliferation was significantly less than that of controls after 1 wk of parasite exposure ($P < 0.05$).

had elapsed for the development of a fully suppressive cell population.

Infective stage larvae and adult parasites induce similar profiles of T cell suppression in infected animals

A preliminary experiment showed that 600 L3 parasites implanted i.p. into CBA/Ca mice generated a population of adherent peritoneal cells which induced profound and significant cellular proliferative suppression ($P < 0.04$) in cultured T cells that was not reversible with the NO inhibitor L-NMMA (Table I). We have previously reported similar findings in adult parasite-implanted mice, because even at minimal PEC numbers mixed with a 10-fold higher concentration of control APC, adult parasite-derived suppression cannot be reversed by NO inhibitors (11).

To establish the minimum dose of L3 parasites required to generate suppressive PEC, we infected mice with a range of parasite numbers. CBA/Ca mice were implanted i.p. with 10, 40, 100, or 400 L3 stage parasites or with 6 adult female parasites, and the ability of these stages to generate suppression was then compared after 2 wk. Adherent peritoneal cells from both L3 and adult implanted mice prevented Ag proliferation of the D10.G4 clone (Fig. 2*A*). At high numbers of implanted L3 (400), suppression was greater than that seen with adult implants ($P < 0.05$), despite the lower biomass of 400 L3s compared with 6 adult parasites. Suppression was not reversed at any parasite dose by L-NMMA (data not shown).

Correlation of IL-4 response and suppression

Analysis of Ag-IL-4 production by D10.G4 cells showed that although cellular proliferation was ablated in the presence of both adult and L3 implant-derived PEC, cytokine production was enhanced (Fig. 2*B*). This argues against the possibility that the reduced proliferation of the D10.G4 cells was due to ineffective Ag presentation, reduced numbers of APC, or heightened cell death.

In addition to T cells, proliferation of the B cell hybridoma HB32 was assessed in the presence of adherent PEC from L3-implanted animals. A significant reduction in hybridoma cell proliferation was seen with administration of as few as 10 L3 parasites (Fig. 2*C*) ($P < 0.02$).

Consistent with previously published data, splenocytes taken from L3-implanted animals produced elevated IL-4 levels (Fig. 3*A*) in response to parasite Ag (12). Increasing levels of splenocyte *Brugia*-specific IL-4 production correlated with increasing size of L3 inoculum and inversely correlated with suppression. IFN-γ levels were also elevated in L3-implanted animals, although there was no correlation between cytokine levels detected and inoculum dose (Fig. 3*B*).

Treatment of parasite-implanted mice with neutralizing Ab to IL-4

Because of the correlation between splenic IL-4 production and the generation of PEC that block proliferation, we chose to investigate the hypothesis that IL-4 plays a role in the development of the suppressive PEC, by the administration of neutralizing Ab to IL-4 (11B11) at the time of parasite implant. Adult parasite-implanted mice that received 2 mg of anti-IL-4 at the time of implant had impaired ability to generate strongly suppressive PEC. In contrast, PEC from control implanted mice, or implanted mice injected with isotype-matched anti-IFN-γ, significantly blocked cellular proliferation (Fig. 4*A*) ($P < 0.01$). This effect was achieved under conditions in which anti-IL-4 did not block the subsequent development of IL-4-producing T cells in the spleens of implanted mice (Fig. 4*B*). Thus, a reduction in IL-4 levels at the time of initial exposure to the parasite was sufficient to reduce the ability of PEC to suppress proliferation.

Table I. *L3 induction of proliferative suppression*[a]

Source of PEC	D10.G4 Proliferation (cpm)			
	Medium + D-NMMA	Conalbumin + D-NMMA	Medium + L-NMMA	Conalbumin + L-NMMA
Control	6,793 ± 5,388	33,719 ± 15,986	9,184 ± 3,745	68,917 ± 4,889
Adult implant	1,331 ± 719	1,634 ± 1,037	1,127 ± 667	1,123 ± 440
L3 implant	1,424 ± 455	3,320 ± 1,212	939 ± 167	2,078 ± 424

[a] Proliferation of D10.G4 cells with medium plus D-NMMA or L-NMMA, or with 50 μg/ml conalbumin plus D-NMMA or L-NMMA in the presence of PEC from control mice or from mice implanted with 6 adult or 600 L3 parasites. Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute. Data are means ± SD of three to four individual mice separately assayed.

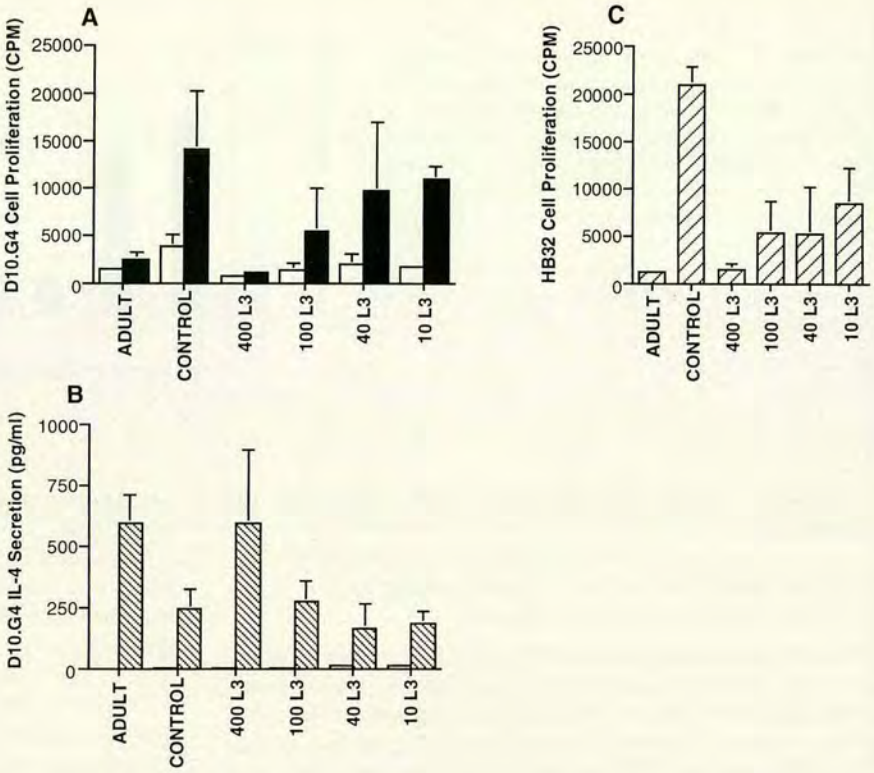

FIGURE 2. L3 induction of suppression. Proliferation of D10.G4 cells with media (□) or 50 μg/ml conalbumin (■) in the presence of PEC from control, adult, or L3 implanted CBA/Ca mice (*A*) or D10.G4 IL-4 production with media (□) or 50 μg/ml conalbumin (▨) measured by NK bioassay (*B*). PEC from implanted mice were taken 2 wk postimplantation. These experiments were conducted in the presence of D-NMMA (shown) or L-NMMA (not shown) with similar results. *C*, Proliferation of HB32 cells in the presence of PEC from control mice or 2-wk adult- or L3-implanted mice. Proliferation was measured by [^{3}H]TdR incorporation. Data presented are mean ± SD of three to four individual mice separately assayed. Significant proliferative suppression of the D10.G4 cell clone was achieved when cocultured with PEC from adult or 400 L3 implanted animals ($P < 0.05$). Hybridoma proliferation was significantly reduced in the presence of PEC from animals implanted with 10 L3 parasites ($P < 0.02$), in comparison to hybridoma proliferation in the presence of control animal PEC.

Induction of proliferative suppression requires IL-4 production by the host

To more firmly establish the role of IL-4 in the induction of PEC suppression, mice genetically deficient for the production of IL-4 (IL-4$^{-/-}$) were implanted with adult *B. malayi* parasites. The ability to stimulate Ag proliferation was compared in PEC taken from knockout and wild-type C57BL/6 animals implanted with *B. malayi*. Cellular proliferation as measured by [^{3}H]TdR incorporation showed that in contrast to wild-type mice, IL-4$^{-/-}$ mice implanted i.p. with adult *B. malayi* parasites failed to generate adherent PEC that blocked the proliferation of either OVA-specific T cells (Fig. 5*A*) or the B cell hybridoma, HB32 (Fig. 5*B*). Assessment of splenocyte cytokine responses showed that adult parasite-implanted IL-4$^{+/+}$ mice produced elevated levels of parasite-specific IL-4 relative to uninfected controls. As expected, no IL-4 was produced by IL-4$^{-/-}$ mice (data not shown).

Neither IL-4 nor IL-10 appear to be the in vitro suppressive factor

Because PEC from IL-4$^{+/+}$ mice suppress proliferation of the IL-4-secreting clone D10.G4, it is unlikely that IL-4 itself can be the effector molecule for blocking proliferation. This was confirmed by the inability of 11B11 to reverse the proliferative block in vitro of either the D10.G4 clone or the HB32 hybridoma (Table II). We also tested IL-10 because it exerts major down-regulatory effects in vitro in parasitic helminth infection (18) and in the development of tumor-derived suppression (19). Addition of neutralizing Ab to IL-10 did not restore proliferative responses of T cells exposed to

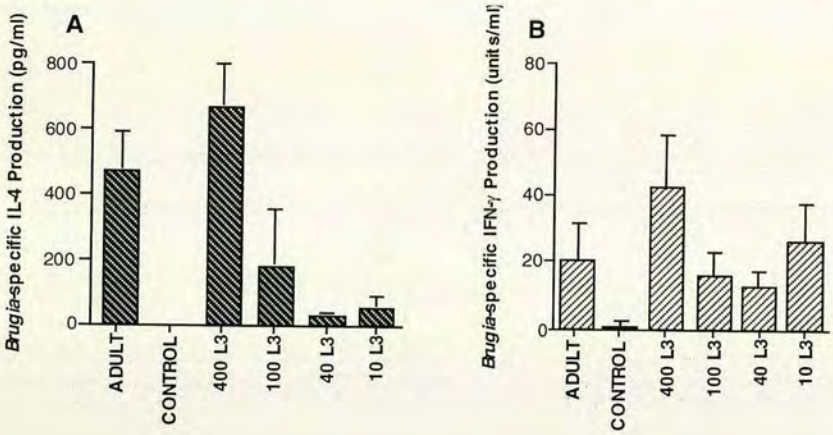

FIGURE 3. L3-induced splenocyte cytokine production. IL-4 production (*A*) or IFN-γ production (*B*) by splenocytes from control or L3 implanted CBA/Ca mice stimulated with BmA as measured by NK bioassay and ELISA, respectively. Background levels of cytokine production without the addition of BmA were in all cases below detectable level for IL-4 and <10 U/ml for IFN-γ.

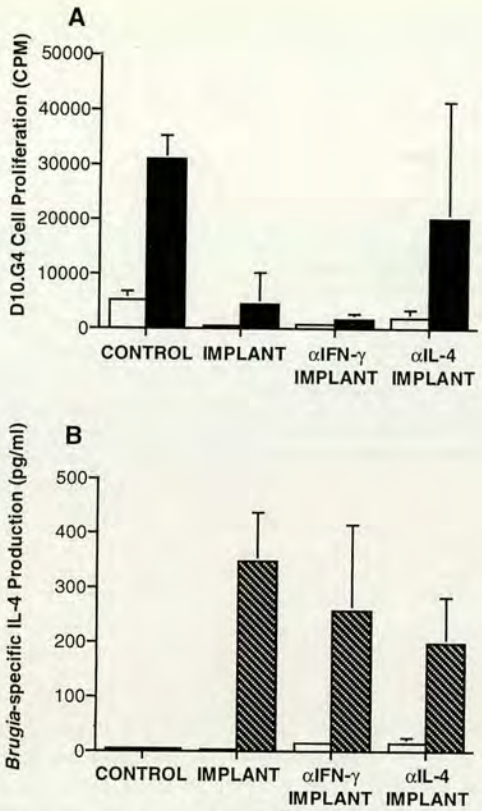

FIGURE 4. Treatment of mice with neutralizing Ab to IL-4. *A*, Proliferation of D10.G4 cells stimulated in the presence of PEC from control or 2-wk parasite-implanted CBA/Ca mice that had been treated at the time of implant with 2 mg/mouse anti-IL-4 or anti-IFN-γ Ab or with no Ab. Cultures were incubated in media (□) or 50 μg/ml conalbumin (■). *B*, Production of IL-4 by splenocytes from control and implanted mice incubated with media (□) or BmA (▨) as measured by NK bioassay. Proliferation was measured by [^{3}H]TdR incorporation and data are shown as mean ± SD of four mice separately assayed.

infection derived PEC (Table II). The removal of IL-10 did enhance responses with control-derived PEC, with increased proliferation of D10.G4 cells seen in these cultures.

IL-10 production by the host is not essential for the induction of proliferative suppression

Having established that in vivo production of IL-4 is necessary for the induction of competent suppressor cells by filarial implant, and given the importance of IL-10 as a down-regulatory cytokine influenced by IL-4, we decided to investigate the role of IL-10 in vivo in the induction phase of suppression. C57BL/6 mice deficient in the production of IL-10 (IL-10$^{-/-}$) were implanted with adult *B. malayi* parasites. Adherent peritoneal exudate cells taken from these mice were capable of inducing proliferative suppression in OVA-specific T cells as effectively as wild-type mice (Fig. 6*A*), thus IL-10 is not required for either the induction or effector phases of T cell hyporesponsiveness. However, suppression of proliferation of the B cell hybridoma HB32 was not as complete as that seen in similarly infected IL-10$^{+/+}$ mice (Fig. 6*B*), suggesting that the suppressive PEC population may encompass a minor IL-10-dependent pathway effective on B cells. Similar levels of IL-4 were produced by splenocytes from IL-10$^{+/+}$ and IL-10$^{-/-}$ infected mice, with both groups producing much greater levels of cytokine than uninfected animals in response to parasite Ag challenge in vitro (data not shown).

Recruitment of cells into the peritoneal cavity

To investigate the dynamics of cell recruitment during infection, the percentage of different peritoneal cell subpopulations was determined from cytocentrifuge preparations taken from control mice or mice implanted with *Brugia* adults or L3. Parasite-implanted mice had a marked reduction in mast cells and an increase in both macrophages and eosinophils, compared with unimplanted control mice (Fig. 7). Recruitment of eosinophils to the peritoneal cavity was seen in mice implanted with as few as 10 L3 parasites. Parasite-implanted mice showed a dramatic increase in total cell numbers recruited to the peritoneal cavity in comparison to unimplanted control mice; three to five times as many cells were routinely recovered from implanted animals. Thus, increased ratios of macrophages and eosinophils reflect not only a proportional increase but also a dramatic increase in total cell numbers of each type. In contrast, despite the increased number of cells in parasite-implanted mice, both the percentage and total number of mast cells were decreased.

The suppressive cell type is not an F4/80-positive macrophage

Given the well-documented role of macrophages in cellular suppression (20, 21), the expansion of macrophage numbers in the peritoneal cavity of parasite-implanted mice (Fig. 7), and the adherent properties of the suppressive cell, we investigated the possibility that macrophages were responsible for proliferative block. Peritoneal cells from 10 control or adult parasite-implanted mice were combined and sorted by FACS into macrophage positive or negative populations using the macrophage-specific mAb F4/80. Greater than 97% purity was achieved for each group (data not shown). Proliferation of the T cell clone D10.G4 was then measured in the presence of each cell population (Fig. 8). D10.G4 cells proliferated well when cocultured with the macrophage-enriched population, while F4/80$^-$ cells prevented cellular proliferation. The absence of proliferation in the F4/80$^-$ cultures was not due to

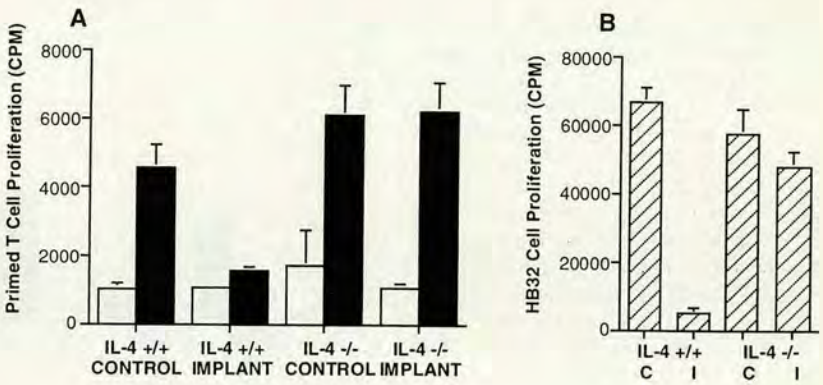

FIGURE 5. Parasite implant of IL-4$^{-/-}$ mice. Proliferation of syngeneic OVA-sensitized primary T cells (*A*) or the B cell hybridoma HB32 (*B*) with PEC from IL-4 +/+ or IL-4−/− control (*C*) or implanted (I) C57BL/6 mice. T cells were incubated with media (□) or 25 μg/ml OVA (■) (*A*) and HB32 cells were incubated with media (▨) (*B*), in the presence of PEC from control or parasite-implanted mice. Proliferation was measured by [^{3}H]TdR incorporation, and data are shown as mean ± SD of four mice separately assayed.

Table II. *Neutralization of IL-4 and IL-10 in vitro*[a]

Source of PEC	A. HB32 Proliferation (cpm)	
	Medium	Medium + anti-IL-4
Control	13,459 ± 5,244	13,178 ± 5,037
Adult implant	2,636 ± 1,214	2,294 ± 472

	B. D10.G4 Proliferation (cpm)			
	Medium	Conalbumin	Medium + anti-IL-4	Conalbumin + anti-IL-4
Control	9,583 ± 490	45,685 ± 2,398	6,129 ± 258	10,255 ± 216
Adult implant	1,106 ± 484	13,917 ± 5,721	731 ± 106	1,496 ± 399

	C. D10.G4 Proliferation (cpm)			
	Medium	Conalbumin	Medium + anti-IL-10	Conalbumin + anti-IL-10
Control	2,692 ± 554	29,598 ± 1,750	4,434 ± 449	52,286 ± 4,829
Adult implant	697 ± 57	2,885 ± 1,579	781 ± 223	2,028 ± 984

[a] Proliferation of HB32 cells with media (A), D10.G4 cells with medium or 50 μg/ml conalbumin, ± anti-IL-4 (B), or D10.G4 cells with medium or 50 μg/ml conalbumin, ± anti-IL-10 Ab (C), in the presence of PEC from control mice or from mice implanted with 6 adult parasites. Removal of IL-4 reduced D10.G4 proliferation in the presence of both control and adult parasite-exposed PEC because IL-4 is an autocrine growth factor for this T cell clone. Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute. Data are means ± SD of three to five individual mice separately assayed.

a lack of effective APC, because high levels of Ag-IL-4 was produced in these cultures (data not shown).

Discussion

Parasite immune evasion strategies are increasingly being characterized in terms of host cytokine networks (1, 22, 23). In the case of the filarial nematode *B. malayi*, as with most other helminth parasites, infection stimulates a dramatic Th2-type response with abundant IL-4 production whether exposure occurs to the L3 or adult stage of the parasite (10, 25). A central question, therefore, is whether the characteristic Th2-type response is a necessary prerequisite for the induction of suppression in filarial infection. The data presented herein demonstrate that host IL-4 production is indeed essential for the induction of a nonspecific suppressor cell population but that the major down-regulatory cytokine associated with Th2 responses, IL-10, is not a key component.

IL-4 production is likely to be one of the first events to occur following exposure to helminth parasites (24). Production of IL-4 is greatly expanded in *Brugia* infected mice (9, 10, 12), and in this study we see a direct correlation between parasite-specific splenocyte IL-4 production and PEC-mediated suppression of lymphocyte proliferation at each dose of L3. IL-4$^{-/-}$ mice were unable to generate a suppressive PEC population, formally demonstrating that IL-4 is required. We cannot yet determine whether IL-4 alone is sufficient for suppressive PEC development or whether a mature Th2 response must first emerge. However, mice given anti-IL-4 at the time of implantation still develop "Th2" responses but are unable to generate full suppression. This suggests that it is early IL-4 production, and not necessarily Th2 establishment, that may be the essential factor in the development of a proliferative block.

The ablation of cellular proliferation in this system is due to soluble factor(s) released from host PEC that does not include IFN-γ, nitric oxide, or prostaglandins (11). A direct role in vitro for IL-4 has been implicated in the L3-induced down-regulation of IL-2-driven T cell mitogenic proliferative responses (25). However, IL-4 is not the PEC-derived suppressive factor, as proliferative responses are not restored by the addition of neutralizing anti-IL-4 Ab in vitro, and the Th2 cell clone D10.G4 itself produces copious amounts of IL-4 during its normal proliferative response.

IL-10 has been implicated as a mediator of filarial nonproliferative responses in human patients in some (26, 27) but not all (28) studies. Surprisingly, in our experiments neutralization of IL-10 in vitro did not restore proliferation to treated cultures, and IL-10$^{-/-}$ mice generated full suppression of the T cell clone D10.G4. However, a partial role for this potent down-regulatory cytokine is suggested by the fact that, in contrast to the wild-type, IL-10$^{-/-}$ mouse implant-derived PEC induce less profound suppression of hybridomas.

TGF-β is another cytokine with down-regulatory ability that has been reported to reduce macrophage antiparasitic responses during

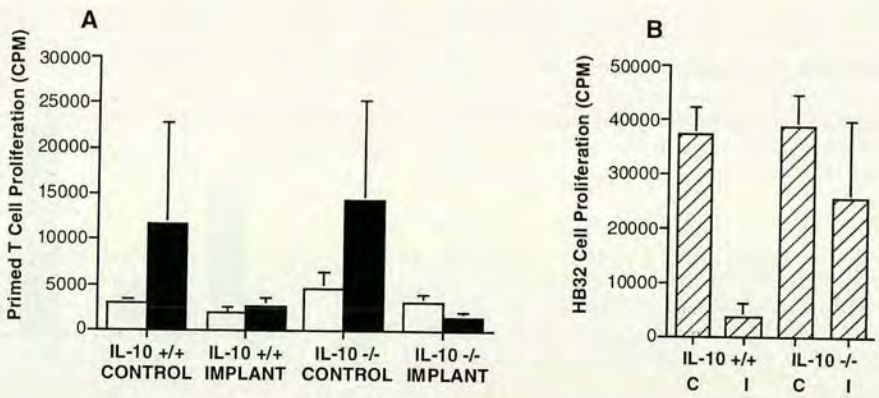

FIGURE 6. Parasite implant of IL-10$^{-/-}$ mice. Proliferation of syngeneic OVA-sensitized primary T cells (*A*) or the B cell hybridoma HB32 (*B*), with PEC from IL-10$^{+/+}$ or IL-10$^{-/-}$ control (*C*) or implanted (I) C57BL/6 mice. T cells were incubated with media (□) or 25 μg/ml OVA (■) (*A*) and HB32 cells/well were incubated with media (▨) (*B*), in the presence of PEC from control or parasite-implanted mice. Proliferation was measured by [^{3}H]TdR incorporation. Data are shown as mean ± SD. Counts per minute from five individual mice separately assayed.

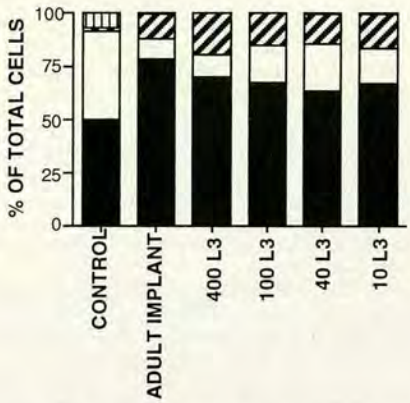

FIGURE 7. Peritoneal cell populations in control and parasite-implanted mice. The cell composition of PEC from control and implanted mice was determined by microscopy. Data shown are mean percentage of mast cells (▥), eosinophils (▨), lymphocytes (□), and macrophages (■). Data shown are mean of three to four individual mice separately analyzed.

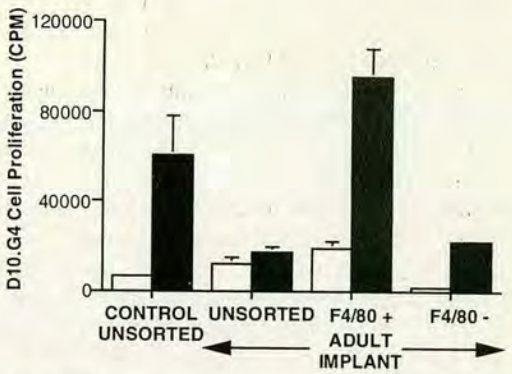

FIGURE 8. FACS sorting of macrophages. Proliferation of D10.G4 cells with media (□) or 50 μg/ml conalbumin (■) in the presence of unsorted PEC from control or adult parasite-implanted mice or macrophage-enriched ($F4/80^+$) or depleted ($F4/80^-$) PEC from adult parasite-implanted mice (97% and 98% purity, respectively). Proliferation was measured by [^{3}H]TdR incorporation and is shown as counts per minute. Data presented are mean ± SD of quadruplicate wells.

helminth infection (29). Moreover, TGF-β, together with IL-10, has a close relationship in the development of tumor-related immunosuppression (19). TGF-β appears to be a likely candidate since we have previously shown that at very low concentrations of parasite-exposed cells, enhanced proliferation can be seen (11), consistent with the reported properties of TGF-β (30). However, addition of two commercially available anti-TGF-β Abs did not restore proliferation to suppressed cultures in vitro, either alone or in combination with Ab to IL-10 (11) (data not shown). Nonetheless, TGF-β remains an attractive possibility that is consistent with the data, and we are still investigating this cytokine as a potential mediator. Other potential candidates remain to be investigated, including such possibilities as glycosphingolipids (31) and other nonpeptide mediators. However, given the profound nature of the suppression and its IL-4 dependence, we are considering the possibility that the proliferative block is effected by a suppressive factor not previously described, possibly acting in combination with known down-regulatory mechanisms.

We have previously shown that *Brugia*-specific T cells generated in the peritoneal cavity fail to proliferate in response to parasite Ag while producing IL-2 and IL-4, but that this proliferative block is not Ag specific given that Con A proliferative responses are also ablated (11). The induction of nonspecific host antiproliferative mechanisms does not currently account for the Ag-specific hyporesponsiveness observed during human filarial infection. Although some evidence for generalized unresponsiveness in human filarial infection is found in onchocerciasis (32), studies on lymphatic filariasis generally show that only parasite-specific proliferation is lost (5, 6, 33). However, animal studies have demonstrated that both specific and nonspecific proliferative suppression can be induced by infection with *Brugia* species (11, 34–37). The possibility that a cell population that is profoundly antiproliferative may lead to the development of Ag-specific cell suppression remains to be explored. For example, if naive T cells first encounter Ag in an environment in which they are unable to proliferate, this may render them tolerant or anergic to later antigenic challenge (38).

Alternatively, the suppression we describe here may be important only at the site of infection, where potentially damaging host inflammatory responses in the local environment are down-regulated. Adult worms live within the lymphatic vessels for many years, apparently impervious to damage by the host immune system (39). We have found that adult excretory-secretory (ES) material injected daily into mice can induce the PEC-mediated proliferative block (unpublished data). Adult parasites are ideally placed within the afferent lymphatics to "bathe" the cells within the lymph nodes with down-regulatory ES. Generation of profound localized proliferative suppression by this mechanism could explain the absence of local inflammation at the site of infection in human filariasis (40–42) and in animal models (43, 44). The profundity of the suppressive mechanism(s) at play in this system is demonstrated by the fact that thus far we have been able to block proliferation of fully differentiated T cell clones, hybridomas, and primary cell lines.

L3 stage parasites must evade immune defenses on penetration of the host, on migration to the lymphatic vessels, and during growth and development toward adulthood. The observation that the infective larval stage can induce pronounced suppression of lymphocyte proliferation is intriguing given the relatively short life span of this developmental stage and its initial migratory nature (45). In particular, it indicates that cellular suppression is initiated extremely early in the course of this long term infection. In other animal models, filarial modulation of proliferative responses is generally not observed until after patency (34, 46) and thus is frequently attributed to Mf release. The results presented here suggest that immune down-modulation is a characteristic of all mammalian stages, even if it is not apparent at a systemic level until later in infection. This is consistent with work involving *Brugia* spp. in gerbils that has failed to show a definitive relationship between Mf and lymphocyte suppression (47, 48). Furthermore, within the peritoneal environment, Mf do not induce the profound suppression seen with adult and L3 stages but may have other as yet undefined roles in the induction of Ag-specific suppression.

The PEC suppression observed in this system is in striking contrast to any currently defined model. For example, a suppressor cell population is generated by infection of mice with African trypanosomes in which nitric oxide (NO) and prostaglandins are the mediators (20). Dependence of inducible NO synthesis on IFN-γ production (49) and evidence for a direct involvement of IFN-γ in NO-mediated suppression of *Trypanosoma brucei* infection (50, 51) suggests that it is IFN-γ, not IL-4, that is necessary for induction of proliferative block by trypanosomes. We show here and have previously shown that neither nitric oxide, nor IFN-γ, nor prostaglandins are mediating proliferative suppression by filaria-exposed PEC in vitro and that IFN-γ production is also not essential in vivo (11). Similarly, the evident importance of IL-4 in our

model differs from IL-4-independent down-regulation of proliferation in *Toxoplasma gondii* (52), a process that is at least partly attributable to IFN-γ (53) and IL-10 (54).

We have yet to determine the identity of the suppressive cell type but have demonstrated that the adherent PEC responsible for ablating proliferative responses in vitro is not an F4/80-positive macrophage, a further distinction from models of suppression that are dependent on suppressor macrophages for proliferative block (20, 49, 55). Additionally, the dramatic increase in eosinophils recruited to the peritoneal cavity following parasite implant raises the interesting suggestion that eosinophils are the suppressive cell type. However, the possibility remains that a minor as yet unidentified cell population is responsible for the proliferative suppression seen in this system. We are now actively investigating these possibilities.

We have established that IL-4 is critical in vivo for the induction of suppressor cells but plays no direct effector role in vitro. Similar patterns of cell recruitment can be seen in adult- and L3-implanted mice, even at low L3 numbers. One possibility is that IL-4 production early in infection may be necessary for recruitment of the appropriate cell type into the peritoneal cavity, perhaps via the production of a secondary cytokine or chemokine such as IL-5 or eotaxin. However, recruitment alone is unlikely to account for such profound suppression. The virtual absence of mast cells in implanted mice may represent mast cell degranulation, which could lead to eosinophil recruitment resulting in enhanced production of IL-4, as has been described in schistosomiasis (24). Thus, IL-4- or IL-4-driven cytokines may act alone or in concert with parasite-derived factors to directly activate recruited or resident cells to suppressor function.

The mechanism(s) of suppression exhibited by filarial-induced suppressor cells from filarial-infected mice appear to be unlike those previously described in other infectious disease systems. Further investigation of this model, as well as enhancing our understanding of filariasis, should help us to clarify the relationship of IL-4 induction to the generation of immunosuppression and may provide insight into potentially novel areas of immune modulation.

Acknowledgments

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Profound suppression of cellular proliferation mediated by the secretions of nematodes

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SUMMARY

Loss of T lymphocyte proliferation and the emergence of a host response that is dominated by a Th2-type profile are well-established features of human filarial infection. Down-regulation and modulation of host T cell responses during lymphatic filariasis has been investigated by implantation of parasite stages into inbred mice. Adherent peritoneal exudate cells (PEC) from mice transplanted with adult or larval Brugia malayi *parasites are profoundly anti-proliferative but do not prevent antigen-specific cytokine production by T cells. We demonstrate here that the excretory/secretory (E/S) products of the adult parasite are sufficient to induce PEC that block proliferation if injected daily into mice. We have previously shown that* in vivo *production of host IL-4 is required for the generation of suppressive cells. Because the induction of host IL-4 is characteristic of infection with nematodes, we asked whether E/S from two other nematode parasites,* Nippostrongylus braziliensis *and* Toxocara canis *were also capable of generating a suppressor cell population. Injection of E/S from these two parasites also led to a reduction in T cell proliferation suggesting that this mechanism of down-regulating host responses is a feature common to nematode parasites.*

Keywords Brugia, *IL-4, tolerance, T-cell, antigen presenting cells, filaria*

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INTRODUCTION

Chronicity, immune suppression and Th2-type immune responses are characteristic features of human infection with multicellular parasites but the relationship between these features remains largely unresolved. Immune suppression and Th2 responses have been attributed to the chronicity of infection with these parasites which include nematodes, trematodes and cestodes. However, long-term parasite survival is almost certainly dependent on immune suppression from the onset of infection while the importance of Th2 cytokines in mediating protection *vs* suppression is an area of sometimes heated debate (Allen & Maizels, 1997).

We have been particularly interested in the relationship of Th2 responses to immune suppression as well as trying to consider what common attributes of multicellular organisms might lead to profound down-regulation of host immune responses. Impaired T cell responses have been described in some detail for individuals infected with the filarial nematodes, *Brugia malayi* and *Wuchereria bancrofti* (Ottesen *et al.* 1977, Piessens *et al.* 1980, Maizels & Lawrence 1991, Yazdanbakhsh *et al.* 1993). Peripheral blood lymphocytes from people with active infection (in the absence of overt disease) do not proliferative in response to parasite antigen but produce antigen-specific IL-4. However, proliferative responses to other antigens are unimpaired.

We set out to investigate whether T cell hypo-responsiveness could be due to a defect in antigen presenting cell function caused by infection with filarial nematodes. To provide a source of *in vivo* parasite-exposed APC, we implanted mice in the peritoneal cavity with live *Brugia malayi* parasites and asked the question: Can APC from infected animals present antigen to a T cell clone (D10.G4) as effectively as cells from control animals? We found that infection-derived peritoneal exudate cells (PEC) are profoundly anti-proliferative (Allen *et al.* 1996). In the presence of these cells, T cells fail to proliferate but produce high levels of cytokine in response to cognate antigen. Antigen processing and presentation are thus fully intact. The

proliferative block is apparently an active process that cannot be accounted for by an absence or defect in co-stimulation as T cells do not divide in response to antigen, even with the addition of excess control APC. Although these observations have striking similarities to human peripheral blood hypo-responsiveness, they differ in that the anti-proliferative effect is non-specific, blocking mitogen responses as well as preventing the proliferation of many different cell types (Allen *et al.* 1996).

During our investigations, we noted a striking correlation between the ability of certain parasite life-cycle stages to induce systemic IL-4 and the induction of profound proliferative suppression. Thus, the infective larval (L3) and adult stages, which induce high levels of parasite-specific IL-4 in the spleen, are both capable of inducing profound PEC-mediated suppression whereas microfilariae (Mf) which induce systemic IFN-γ generate a moderate suppressive effect that can be fully reversed with inhibitors of nitric oxide (Lawrence *et al.* 1994, Allen *et al.* 1996, MacDonald *et al.* 1998). Using both neutralizing antibodies and IL-4 deficient mice, we have demonstrated that although IL-4 plays no direct role *in vitro*, the development of a suppressor cell population induced by filarial infection is critically dependent on IL-4 *in vivo* (MacDonald *et al.* 1998).

To determine the mechanism by which filarial nematodes might generate suppressor APC, we have asked whether excretory/secretory (E/S) products of *B. malayi* could duplicate the effect of implantation with live adult parasites. E/S was found to generate suppressive PEC as effectively as the live parasite itself which has significant implications for our conceptual understanding of the processes leading to suppression *in vivo*. Further, given that IL-4 is required for the generation of this suppressive mechanism (MacDonald *et al.* 1998), we asked whether E/S from two other nematode species known to be strong IL-4 inducers (Finkelman *et al.* 1988; Lawrence *et al.*, 1996); (R. Maizels, pers. comm.) could generate a similar form of suppression and found that this was indeed the case.

MATERIALS AND METHODS

Parasite material

B. malayi adults and microfilariae (Mf) were obtained from infected jirds purchased from TRS laboratories (Athens, GA, USA). Adult worms were removed from the peritoneal cavity and washed in RPMI 1640 (Gibco) supplemented with 50 μg/ml gentamicin. Adult parasite E/S was obtained by culture of one adult worm per ml at 37°C in 50 ml RPMI supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% glucose. 40 ml of supernatant was harvested daily and replaced with the same volume of fresh supplemented media. Harvested supernatant was passed through a 0·2 μm filter and stored at −70°C prior to use. Adult *N. brasiliensis* E/S was obtained by culture of 125 worms/ml in RPMI 1640 supplemented with 2% glucose, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C. Supernatants were collected daily from day 2 of culture, passed through a 0·2 μm filter and stored at −70°C. *T. canis* larval E/S was obtained by culture of 2500 infective larvae/ml at 37°C in RPMI supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml Fungizone (Gibco), 15 μg/ml gentamycin (Gibco) and 1% glucose. Supernatants were removed weekly and filtered through a 0·2 μm filter prior to storage at −70°C. Dead parasites were collected from those that had died after overnight *in vitro* culture.

Generation of suppressive peritoneal exudate cells

Six-to-eight-week old male CBA/Ca mice purchased from Harlan-UK (Bicester, UK) were implanted intra-peritoneally (i.p.) with either the given number of live adult *B. malayi* females, or with ten dead female adults. After three weeks mice were euthanized by cardiac puncture and PEC were harvested by thorough washing of the peritoneal cavity with 15 ml of RPMI containing 50 μg/ml gentamicin. For investigation of the ability of parasite E/S to generate suppressive PEC, mice were injected daily for two weeks with 1 ml of *B. malayi*, *N. brasiliensis*, or *T. canis* E/S, or with RPMI alone. After this time, peritoneal cells were harvested as described above.

Proliferation and cytokine assays

Unless otherwise stated, all *in vitro* cultures were carried out in RPMI 1640 medium (Gibco) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml of streptomycin, 5 μM 2-mercaptoethanol and 10% FCS (complete medium).

For investigation of the ability of parasite- or E/S-exposed PEC to present antigen *in vitro*, 100 μl PEC at 1×10^6/ml were adhered to a flat-bottomed 96 well plate (Nunclon) at 37°C for 2–3 h, after which non-adherent cells and Mf were removed by washing with 100 μl of complete medium/well. Proliferation of the conalbumin-specific Th2 cell clone D10.G4 (Kaye *et al.*, 1983) was determined in the presence of adherent PEC by adding 5×10^4 D10.G4 cells to each well to a final volume of 200 μl/well. Conalbumin (Sigma), the cognate antigen for the D10.G4 clone, was used at a final concentration of 50 μg/ml where indicated. The NO inhibitor L-N^G-monomethyl-arginine (L-NMMA) and the control inhibitor D-NMMA (Wellcome Foundation, Beckenham,

Kent, UK) were used where indicated at a final concentration of 250 μg/ml. After incubation for 48 h at 37°C, 100 μl of supernatant was removed from each well of the D10.G4 assay for cytokine analysis. After supernatant removal, 1 μCi [^{3}H] thymidine in 10 μl complete medium was added to each well, and plates were incubated for 16–18 h at 37°C prior to harvesting and counting using a Top Count Microplate Scintillation Counter (Canberra Packard).

The IL-2/IL-4 responsive NK cell line (Swain *et al.* 1981) was used to measure cytokine levels by D10.G4 culture supernatants, as previously described (Lawrence *et al.* 1994, Allen *et al.* 1996). In brief, proliferation of the NK cells at 10^4 cells/well was measured after the addition of 20 μl culture supernatant with anti IL-2 (S4B6) neutralizing antibody. Standard curves were established using mouse recombinant IL-4 (Sigma, I-1020). Cells and supernatants were incubated for 24 hours at 37°C prior to addition of 1 μCi of [^{3}H] thymidine in 10 μl complete medium. After a further 12 h incubation at 37°C, plates were harvested and counted. S4B6 ascites was used at 2·5 μl/ml, the optimal concentration for neutralization as determined by titration.

Statistical analysis

The Student's *t*-test and paired *t*-test were used to determine the statistical significance of differences between and within groups. $P<0{\cdot}05$ was considered to be a significant difference.

RESULTS

Suppression can be generated by a single adult parasite

We have previously shown that mice implanted i.p. with six *B. malayi* adult parasites generate a population of adherent peritoneal cells capable of blocking the proliferation of a range of lymphocytes (Allen *et al.* 1996). To determine the minimum number of live parasites required to generate proliferative suppression, we investigated the ability of PEC from mice implanted with a single *B. malayi* female to block proliferation of D10.G4 cells, compared to a multiple female implant. We found that significant suppression could be achieved with the implant of only one adult female parasite ($P<0{\cdot}02$) (Figure 1).

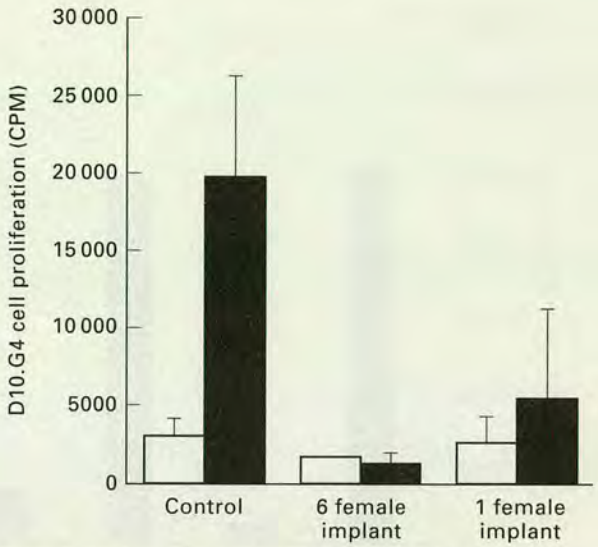

Figure 1 Single parasite implant. Proliferation of the D10.G4 clone with media (open bars) or 50 μg/ml conalbumin (solid bars) co-cultured with PEC from control (unimplanted), single or multiple (six) female adult *B. malayi*-implanted CBA/Ca mice. Proliferation was measured by [^{3}H] thymidine incorporation and is shown as c.p.m. Data are mean ± SD of five individual mice separately assayed.

E/S induction of suppression

Having established that suppressive PEC could be generated by i.p. implant of only one adult female parasite, and given that filarial suppression of proliferation ensues from live, but not dead, adult implants (Allen *et al.* 1996), we investigated the suppressive potential of the secreted products of the adult stage of the parasite. We cultured *B. malayi* adults at 1 worm per ml of culture medium and collected supernatants every 24 h. Thus, administration of 1 ml/animal/day equated to the amount of E/S produced by one worm in 24 h. Daily administration of this amount of E/S i.p. for two weeks resulted in the generation of a suppressive PEC population which ablated proliferation (Figure 2a) ($P<0{\cdot}01$) but not antigen-specific cytokine production (Figure 2c) in the D10.G4 system. Suppression was not significantly altered on the addition of the NO inhibitor L-NMMA (Figure 2b). Thus, administration of adult ES exactly paralleled the result with implantation of live adults parasites. Interestingly, administration of adult E/S every second day did not induce suppressive PEC (data not shown). This may reflect the low concentration of the ES used in these experiments, or may suggest that development of suppression requires constant exposure of host cells to parasite antigen. Additionally, the very effective suppression generated by adult E/S *in vivo* could not be duplicated by the direct addition of the same E/S to cultured T cells *in vitro* (data not shown).

Suppression can be induced by E/S of several nematode species

Having established that *B. malayi* E/S has the capacity to generate suppressive PEC, we decided to test E/S from two other nematode species available in the laboratory. E/S was

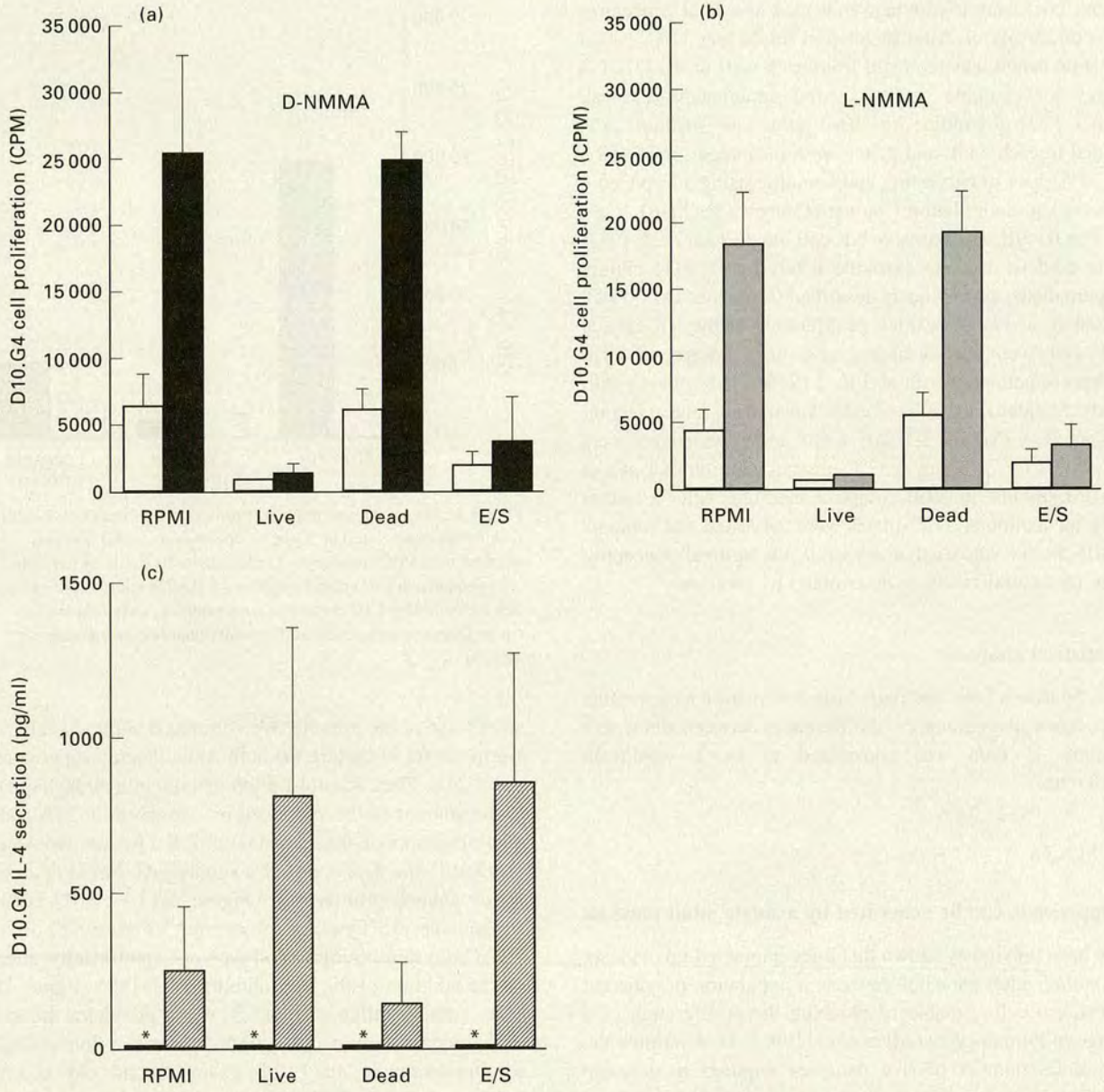

Figure 2 Induction of suppression by *in vivo* administration of *B malayi* E/S. Proliferation of D10.G4 cells (a) with media plus D-NMMA (open bars) or 50 μg/ml conalbumin plus D-NMMA (solid bars), or (b) with media plus L-NMMA (open bars) or 50 μg/ml conalbumin plus L-NMMA (flecked bars) co-cultured with PEC from mice implanted with; six live adult *B. malayi*, ten dead adult parasites, or injected daily woth 1 ml RPMI or 1 ml adult parasite E/S. (c) D10.G4 IL-4 production in media plus D-NMMA (open bars) or 50 μg/ml conalbumin plus D-NMMA (dark hatched bars), as measured by NK bioassay. Asterisks represent cytokine production of < 10 pg/ml. Proliferation was measured by [^{3}H] thymidine incorporation and is shown as c.p.m. Data are mean ± SD of 3–4 individual mice separately assayed. Significant D10.G4 proliferative suppression was seen with daily administration of adult parasite E/S, in comparison to RPMI-injected animals ($P < 0{\cdot}01$)

generated from adult *Nippostrongylus brasiliensis* and larval stage *Toxocara canis*. A similar biomass of parasites was used to generate E/S from these nematodes. We administered one ml of E/S to each animal i.p every day for two weeks, then removed the PEC and tested their ability to stimulate antigen-specific proliferation of the D10.G4 clone. Decreased proliferation of the D10.G4 clone was seen when co-cultured with PEC from mice that had been exposed to E/S material from all three nematode species tested, although significant suppression was only achieved using PEC from *B. malayi* or *T. canis* E/S injected animals ($P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively).

DISCUSSION

The full developmental cycle of the human filarial parasite, *Brugia malayi*, does not occur in the laboratory mouse but individual stages of the parasite can survive for considerable lengths of time when implanted into the peritoneal cavity (Lawrence 1996). We have used this approach to study modulation of the host cytokine responses by this parasite (Lawrence *et al.* 1994, 1995) as well as the response to individual antigens (Allen *et al.* 1995). More recently, we have tried to address the impact of infection on antigen-presenting cell function as a means to understanding the dramatic down-regulation of parasite-specific T cell proliferative responses observed during infection with filarial parasites (Allen *et al.* 1996, MacDonald *et al.* 1998). We found no apparent defect in antigen-specific processing or co-stimulation but rather that peritoneal cells derived from infected animals exert a profound and non-specific anti-proliferative effect on cells with which they are co-cultured *in vitro*.

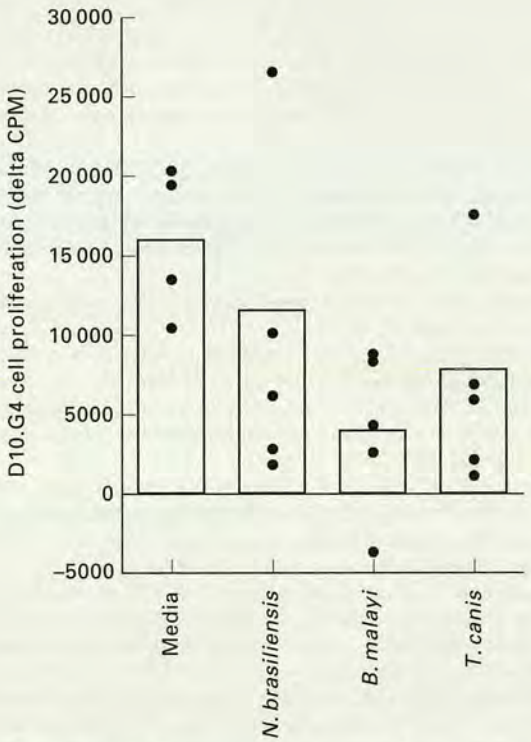

Figure 3 E/S-induced suppression by other nematode species. Proliferation of the D10.G4 clone when co-cultured with PEC from control (unimplanted) CBA/Ca mice, or mice injected daily for two weeks with 1 ml of *N. brasiliensis*, *B. malayi*, or *T. canis* E/S. Proliferation was measured by [^{3}H] thymidine incorporation and is shown as delta cpm. Data are individual mice (spots) and mean (bars) of five mice separately assayed. Significant suppression of the D10.G4 clone was seen on co-culture with PEC that had been exposed to *B. malayi* E/S ($P < 0{\cdot}01$), or *T. canis* E/S ($P < 0{\cdot}05$).

This has led us to ask several questions regarding the *in vivo* relevance of this observation. Firstly, 'How does the parasite generate a suppresive host cell population?' The work described in this manuscript shows that host cells capable of blocking proliferation can be generated by the daily administration of adult *B. malayi* E/S. Notably, this suppression, like that of adult and L3 implants, cannot be reversed on the addition of nitric oxide inhibitors. These data, together with the inability of dead parasites to ablate proliferation, strongly point to the parasite producing a soluble factor(s) responsible for the subsequent development of a suppressive PEC population. Fractionation and chemical manipulation of the *Brugia* E/S will permit a practical approach to the identification of the parasite factor(s) involved in the recruitment or development of a down-regulatory host cell.

Secondly, 'How might a non-specific proliferative block lead to the antigen specific hyporesponsiveness seen in human infection?' Filarial parasites live in the afferent lymphatics where E/S products carried into the adjacent lymph node could directly effect lymphocyte function or maturation. We postulate that naive T cells migrating into the lymph node will first encounter filarial antigen in an environment in which they are unable to undergo normal cellular division. The impact of this is difficult to interpret directly but an active proliferative block may mimic what occurs in the absence of co-stimulation, allowing the accumulation of negative regulators in the cell and leading to an anergic state (Jenkins 1992). These cells, hypo-responsive to filarial antigen, would now enter the periphery.

One prediction of this model would be that at very high levels of infection, when adult parasites can be found at multiple sites, responses to non-parasite antigens would also be suppressed. In human studies of lymphatic filariasis, responses to non-filarial antigens are apparently intact (Yazdanbakhsh *et al.* 1993). However, PPD responses increase following chemotherapy suggesting there is an element of non-specific downregulation during infection (Sartono *et al.* 1995). In onchocerciasis, the evidence that responses to non-filarial antigens decrease with increasing infection is much stronger (Greene 1992 and J. Bradley, pers.comm. and M. Murdoch, unpublished). We are currently testing this model using naive T cells from T cell receptor transgenic mice to directly address the question of whether the proliferative block can lead to a state of anergy. Whether proliferative suppression plays a role in the generation of peripheral tolerance remains to be established, but it may directly facilitate L3 survival through the migratory phase and prevent extensive inflammatory responses to the adult stage within the lymphatic vessel.

Interestingly, other helminth studies have shown direct anti-proliferative effects of purified components of E/S or

concentrated E/S on lymphocytes *in vitro* (Harnett & Harnett 1993, Kadian *et al.* 1996, Cervi & Masih 1997). We have not observed any *in vitro* effect with either live *Brugia malayi* or unconcentrated E/S. The inability of *B. malayi* ES to prevent cellular proliferation *in vitro* may reflect the low concentration of the E/S used, anthough the potency of the same material *in vivo* argues that the concentrations used in our study are physiologically relevant. Alternatively, it could suggest that parasite-derived factors must act in concert with host immune system components to induce suppression. This is consistent with the requirement for IL-4 and our previous observation that one week of host exposure to the parasite is required for development of the proliferative block (MacDonald *et al.* 1998). Although filariae may directly suppress lymphocyte proliferation, the generation of a host cell population that produces an anti-proliferative factor may amplify the negative signal, thus resulting in a longer lasting, more profound inhibition of the host response.

Studies with cestode E/S have demonstrated a similar pattern of proliferative suppression (Rakha *et al.* 1991, Sciutto *et al.* 1995). This led us to question if the generation of a non-specific anti-proliferative host cell is a general phenomenon associated with helminth infection. We chose preliminary to look at two members within the Nematoda phlyum distantly related to filarial parasites. We observed that E/S products from *Toxocara* and *Nippostronglyus* were able to reproduce the *in vivo* suppressive effect of *Brugia*. Unlike *Brugia*, the adult stage of both these nematodes lives primarily in the intestinal tract but like filarial nematodes, a larval stage must survive through a tissue migratory phase. These results suggest that the immuno-modulatory capacity of E/S may be a feature common to nematode infection presumably predicated on the ability of these organisms to induce host IL-4, as we have shown that the ability to generate a cell population capable of blocking proliferation does not occur in the absence of *in vivo* IL–4 (MacDonald *et al.* 1998). These studies will be extended to other helminths to further evaluate the universality of the observed phenomenon. It is possible that innate defence systems in the mammalian host can recognize features common to higher animals leading to a similarity in response to organisms that are phylogenetically quite distinct.

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