ALTERNATIVELY ACTIVATED MACROPHAGES RECRUITED BY *BRUGIA MALAYI*

by

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DECLARATION

I hereby declare that this thesis was composed by myself and that the work described herein is my own, unless otherwise stated.

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October, 2000.

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I dedicate this thesis to my parents, who gave me all the opportunities to follow my interests and ambitions and have supported and inspired me every step of the way. I still vaguely remember witnessing my mum's thesis writing days, with the childish intentions of following suit one day in the future (although crocodiles were my main interests then)!

One of the joys of science is the intellectual interaction, exchange of ideas and collaborations. I think this is reflected in my thesis, which is in many ways a joint effort, with many experiments having been done with help from other people in the lab. It is not surprising that I have a lot of people to thank!

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ABSTRACT

The adult stage of the parasitic nematode *Brugia malayi* can live in the host lymphatics for many years and must have an extremely effective immuno-suppressive mechanism that prevents rejection. We have discovered that this parasite can induce alternatively activated IL-4 dependent macrophages that can block proliferation of other cells via a receptor-mediated mechanism. The proliferative block is reversible and is not a result of apoptosis. Furthermore, these suppressive cells can reduce the proliferation of a wide range of human cancer cell lines. These data demonstrates that *B. malayi* can lead to the activation of a novel mechanism of proliferative suppression, via IL-4 dependent macrophages. These macrophages may have important roles in altering host immune responses during parasitic infection and may even have the potential to reduce tumour cell growth.

Another feature of *B. malayi* infection is a bias towards a type 2 immune response. To investigate the role that the *B.malayi* recruited antigen presenting cells have on naïve T cells, the suppressive macrophages recruited by *B. malayi* was used to stimulate naïve T cells from TCR transgenic mice. Although the proliferation of the naïve T cells was inhibited by parasite-induced macrophages during primary stimulation, they proliferated normally upon secondary stimulation and were not rendered anergic. However, naïve T cells primed by suppressive macrophages differentiated into IL-4 producing Th2 cells upon secondary stimulation instead of IFN-γ producing Th1 cells, as has been previously described. Th2 differentiation was associated with the inhibition of (or failure to stimulate) IFN-γ producing T cells during primary stimulation. Interestingly, blocking antibodies against TGF-β (but not IL-10) restored the differentiation of IFN-γ producing Th1 cells. These data indicate that T cells exposed to parasite induced alternatively activated macrophages are driven towards Th2 differentiation. This may be an important factor in the Th2 bias that accompanies helminth infection.

In order to identify genes that are highly expressed in suppressive macrophages, which could be involved in proliferative suppression and Th2 induction,

a cDNA library was constructed from these alternatively activated macrophages and an expressed sequence tag (EST) strategy was used to characterise the abundantly expressed genes in these cells. The gene YM1/ECF-L, also recently characterised as a novel eosinophil chemotactic factor (ECF-L), was massively upregulated in this library, representing 8.5% of the EST clones. The recruitment of eosinophils is another key feature of nematode infection and indeed many other type 2 immune responses such as allergic responses. Interestingly, we found that *in vivo* administration of the *Brugia malayi* homologue of the cytokine MIF (BmMIF-1) also induced YM1/ECF-L up-regulation. A single amino acid mutation that abolishes enzyme activity of BmMIF-1 (called BmMIF-1G) also abolishes induction of YM1/ECF-L in $F4/80^{+}$ macrophages. BmMIF-1 injected mice had much higher levels of eosinophilia than BmMIF-1G injected mice, suggesting that eosinophil recruitment is linked to the enzyme activity of BmMIF-1. Thus, parasite derived MIF could activate macrophages to produce YM1/ECF-L, which in turn recruits eosinophils to the local site of inflammation.

In order to identify IL-4 dependent genes in alternatively activated macrophages and also potentially discover novel genes involved in the mechanism of proliferative suppression, we took a number of molecular approaches. Firstly, we constructed a cDNA library from non-suppressive macrophages that are recruited in parasite implanted IL-4 deficient mice and again used an expressed sequence tag (EST) strategy to characterise the abundantly expressed genes in these cells. Comparison of the $IL\text{-}4^{-/-}$ macrophage EST dataset with the suppressive macrophage EST dataset revealed a number of potentially differentially expressed genes. Secondly, we have made a subtractive library by using 'driver' RNA from non-suppressive IL-4 deficient PEC to subtract away 'tester' RNA from suppressive macrophages, yielding a population of 20 000 recombinants enriched in putative suppression generating transcripts. A preliminary assay of 176 randomly selected clones from this library has revealed that between 10-20% of the recombinants are differentially expressed between the 2 macrophage populations.

Thus, we have found that alternatively activated macrophages could play a crucial role in: 1) immune suppression, 2) inducing Th2 differentiation of naïve T cells

and 3) recruiting eosinophils to the site of inflammation. We have also made the first steps towards defining the molecular basis of IL-4 dependent genes in alternatively activated macrophages, which could lead to the identification of a novel mechanism that inhibits proliferation.

GLOSSARY

AAMΦ	Alternatively activated macrophages
APC	Antigen presenting cell
BALF	Bronchoalveolar lavage fluid
CAMΦ	Classically activated macrophages
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CTL	Cytotoxic T lymphocytes
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tags
FITC	Fluorescein isothiocyanate
i.p.	Intraperitoneal
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
Mf	Microfilaria
MΦ	Macrophages
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
NO	Nitric Oxide
PE	Phycoerythrin
PEC	Peritoneal exudate cells
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
WT	Wild type

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Chapter 1

INTRODUCTION

One of the most interesting areas of investigative immunology revolves around the mechanisms involved in maintaining peripheral tolerance. It is becoming increasingly clear that the link between the innate immune system and the adaptive immune system plays a crucial role in peripheral tolerance. During the evolutionary arms race between hosts and parasites, it is likely that parasites have evolved strategies that exploit these mechanisms to prevent rejection by the host immune response. Thus, by studying the interaction between parasites and host responses, we might gain important insights into the process of maintaining peripheral tolerance.

The filarial parasites are remarkable organisms because they can live in the very heart of the immune system (i.e. the lymphatic system) without being rejected for many years. As a foreign organism living within the host, they are behaving in some ways like a successful xeno-transplant. The many mechanisms that filarial parasites must utilise in order to prevent rejection remain unclear. Our laboratory has been particularly interested in the effects that these parasites have on the antigen presenting cells of the immune system.

This introduction will begin by reviewing the mechanisms involved in regulating and maintaining peripheral tolerance. I will then describe the biology and immunological responses to filarial parasites and discuss how the parasites could be exploiting the mechanisms involved in peripheral tolerance. Finally, I will describe the work that has been done previously in the lab with respect to the antigen presenting cells that are recruited by these parasites.

Peripheral tolerance

T cell activation

To understand T cell tolerance, one must first understand T cell activation, since the decision between activation, death or not responding is the basis of most immune mechanisms. T cell activation requires a number of different signals, all of which can also serve as checkpoints in determining whether activation is appropriate.

First of all, T cells need to receive signal one through recognition of a specific antigen in order to undergo clonal expansion and generate extended progeny that are specific to this antigen (Burnet, 1959). Zinkernagel and Doherty showed that the major histocompatitibility complex (MHC) plays a crucial role in delivering signal one to T cells (Zinkernagel and Doherty, 1974), whereas Townsend completed the signal one story by showing that it was peptides presented on MHC molecules that delivered the specific signals to T cells (Townsend et al., 1986).

By then, it was already clear that T cells required a second signal for activation, which was particularly true for "naïve" T cells that have never before encountered antigen. In the mid-1970s, Lafferty and Cunningham had already proposed that signal one from the antigen specific receptor was not enough to activate T cells, and a second signal from an antigen presenting cell was necessary for full activation (Lafferty and Cunningham, 1975). This second signal is also known as co-stimulation. In the 1980s, Schwartz and Jenkins expanded and modified this idea by arguing that in the absence of a second signal, T cells receiving signal one would become "anergic" or non-responsive (Jenkins and Schwartz, 1987). Although there is no disagreement about the fact that signal two clearly enhances activation of T cells, arguments that signal one in the absence of signal 2 leads to deletion (Matzinger, 1994) or tolerance through anergy (Schwartz, 1996) is more controversial (Zinkernagel, 2000).

From these models it was suggested that antigen presenting cells (APC) themselves have to become activated in order to deliver signal 2, the absence of which

could result in tolerance. This implied an instructive role played by the antigen presenting cell, which has the predicted power to determine whether T cells are activated or tolerised. Indeed the recent advances in immunology have shifted away from understanding T cell activation from the T cell point of view towards an emphasis on the antigen presenting cell.

Medzhitov and Janeway (Medzhitov and Janeway, 1997) have proposed that antigen presenting cells are activated by having pattern recognition receptors (PRR), which bind and recognise pathogen associated molecular patterns (PAMPS). In contrast, Matzinger has proposed that antigen presenting cells recognise "danger", which is an endogenous signal from stressed or damaged host cells, instead of "foreign" exogenous factors (Gallucci et al., 1999; Matzinger, 1998). Although this debate continues, both models acknowledge the crucial role played by antigen presenting cells and the innate immune response in instructing T cell (and indeed the adaptive immune response). This clear role for APCs in T cell activation should translate to an equally important role in maintaining or inducing T cell tolerance, although this role for APCs remains a relatively unexplored area of immunology.

Thus, T cell activation (or tolerance) can be regulated via 1) signal one from TCR-peptide-MHC engagament, 2) signal 2 via costimulation and 3) activation of APCs.

Signal one: TCR engagement of peptide-MHC complex

Unlike antibodies, which recognise an unlimited range of molecular structures, the T cell receptor (TCR) recognises antigens as a peptide presented on a major histocompatibility complex (MHC) class I or II molecule. Interestingly, there is a new awareness of how cross reactive T cell receptors are in their recognition of peptides in the context of MHC. This is due to a number of observations including the emerging awareness of 'altered peptide ligands' (APL). These are peptides that differ slightly in sequence from the original peptide recognised by a TCR, and causes either partial activation (weak or partial agonists) or down regulation of the T cell (antagonist) when engaged by the TCR (reviewed by Kersh and Allen 1996 (Kersh and Allen, 1996)).

The resolution of the crystal structure of a TCR-peptide-MHC complex has also shown that there is a considerable amount of space in the region between the TCR and the peptide-MHC complex (i.e. They do not fit together as tightly as previously thought) (Garboczi et al., 1996; Garcia et al., 1996). Slight variations in peptide sequence (e.g. in APLs) are more likely to lead to a difference in the kinetics of molecular interactions, rather than the lack of structural specificity.

Signal two: Costimulation

Costimulation (or signal 2) plays a crucial role in enabling the sensitivity and specificity of T cell activation by enhancing appropriate T cell activation and preventing non-specific T cell activation. CD28 was the molecule that was originally recognised as being a prime positive mediator of costimulation (Hara et al., 1985). The subsequent discovery of CTLA-4 (Linsley et al., 1991), which shares 75% nucleotide sequence homology with CD28, has eventually led to the concept of an opposing role for these 2 costimulatory molecules (Allison and Krummel, 1995). It is now generally accepted that costimulation via CTLA-4 inhibits T cell activation (Thompson and Allison, 1997), while costimulation via CD28 enhances T cell activation (Lenschow et al., 1996). The family of ligands expressed on the surface of APC that bind CD28 and CTLA-4 on T cells are called B7 (Lenschow et al., 1996). The first 2 members of this family (B7-1 & B7-2) will bind to either CD28 or CTLA-4, although CTLA-4 has a much higher affinity for both B7-1 & B7-2 than CD28 (van der Merwe et al., 1997). CD28 is constitutively expressed on T cells and can be down regulated by T cell activation (Linsley et al., 1991), while CTLA-4 is only detectable after T cell activation (Krummel and Allison, 1995) and is mostly sequestered inside the cell (Zhang and Allison, 1997). The distinct expression pattern and function of CD28 and CTLA-4 suggests that the kinetic interplay between these 2 costimulatory molecules and their ligands is extremely complex.

The recent discovery of more costimulatory molecules (ie. ICOS and B7H) has added extra layers of complexity to the costimulation story (Abbas and Sharpe, 1999).

It is important to note that B7-H does not bind CTLA-4 and CD28, but binds to ICOS instead (Ling et al., 2000; Mages et al., 2000; Yoshinaga et al., 1999). In contrast to CD28, ICOS delivers a positive signal to activated T cells, instead of resting or naïve T cells. In particular, ICOS has been implicated in the differentiation of Th2 cells (Kopf et al., 2000), as well as potentially stimulating T regulatory cells, since ICOS signalling induces IL-10 production (Dong et al., 1999).

The combination of TCR and positive costimulatory molecule engagement initiates multiple signal transduction cascades that lead to the expression of genes necessary for T cell responses. However, it is still uncertain whether positive costimulation transduces independent signals that are necessary for T cell activation, or whether costimulation simply amplifies signals delivered via TCR engagement. This is partly due to difficulties in identifying a genuine independent signal. Apart from the ability of CD28 to induce bcl-xl (Boise et al., 1995), there have not been many other examples of changes in gene expression, which is induced by costimulation and not simply an amplified response to TCR engagement. It is possible that such genes do not exist and that costimulation is simply a process that influences TCR induced gene expression. Apart from the difficulties in identifying independent costimulatory signals, the signal amplification theory of costimulation has been boosted by recent studies showing that costimulation leads to cytoskeletal changes (Wulfing and Davis, 1998), reorganization of lipid membrane microdomains (Viola et al., 1999) and contributes towards the formation of the immunological synapse (Grakoui et al., 1999). This could lead to T cell activation at a much lower level of TCR triggering, by enhancing and stabilising early phosphorylation events (Grakoui et al., 1999; Lanzavecchia et al., 1999).

Cytokines: Th1/Th2 paradigm

Lanzavecchia has proposed that the maturation of a naive T cell into a fully activated effector T cell requires a combination of 3 signals (Lanzavecchia, 1999). Apart from signal one and two (as discussed above), he refers to the presence of

polarizing cytokines as signal 3 and argues that it promotes the differentiation of T effector cells. Although the cytokine environment of an immune response is usually attributed to the Th subsets of $CD4^+$ T cells, their differentiation is also subject to the cytokine microenvironment that they are in, which means that the systems works via a positive feedback loop mechanism. Ever since Mosmann et.al. first delineated T-cell clones into Th1 and Th2 subsets (Mosmann et al., 1986), these T helper subsets have provided us with an important framework to understand the regulation of the immune response. The basic concept is that; Th1 cells are involved in cell mediated inflammatory functions and secrete cytokines such as IFN-γ and TNF-α, whereas Th2 cells encourages antibody production, enhances eosinophil response, and is characterised by the cytokines IL-4, IL-5, IL-6 and IL-13. However, the concept of these Th subsets has since become so influential that it is frequently oversimplified and has become almost a 'dangerous dogma' (Allen and Maizels, 1997).

Although the cytokine environment is widely considered to be the most important factor influencing the differentiation of naïve T cells (Abbas et al., 1996; O'garra, 1998), with interleukin-12 (IL-12) as the major Th1 polarizing factor and IL-4 as the major Th2 driving cytokine, many other factors can influence the differentiation of naïve T cells (e.g. altered peptide ligands, antigen dose and costimulatory molecules (Constant and Bottomly, 1997)). It has also been proposed that the type of APC and its previous environmental exposure could also play an important role in influencing T cell differentiation (Goerdt and Orfanos, 1999; Kalinski et al., 1999; Rissoan et al., 1999). This is probably due to the different cytokines (e.g IL-12) that are produced by APC that have been activated under different conditions (Kalinski et al., 1998).

Antigen presenting cells

An important source of cytokines is the antigen presenting cells (APC) that are delivering signals one and two to the T cells. The term APC is generally used to describe cells that express MHC class II, which restricts the term to B cells, macrophages and dendritic cells (Professional APC). Although most cells express MHC class I and can be destroyed by activated cytotoxic T lymphocytes (CTL), only

APC that express MHC class II can stimulate/activate $CD4^+$ T cells. Although most cells that express class I can be surveyed and recognised by CTL, it is thought that only professional APC (probably dendritic cells) can activate naïve $CD8^+$ T cells to differentiate/mature into activated CTL (Lanzavecchia, 1998; Ridge et al., 1998). From studies with naïve T cells from TCR transgenic mice, B cells, macrophages and dendritic cells could all prime and activate naïve T cells *in vitro* (Swain, 1994), although physiologically, it is probably dendritic cells that play this role *in vivo*.

B cells need to present antigen to $CD4^+$ cells in order to receive their equivalent of "signal 2" (Bretscher and Cohn, 1970), which allows them to undergo expansion and somatic hypermutation. Macrophages also need to restimulate activated $CD4^+$ T cells in order to receive their own activation signals to either clear intracellular parasites via oxygen radicals (MacMicking et al., 1997) or perhaps to play an immuno-regulatory role by entering an alternative activation pathway (Goerdt and Orfanos, 1999). It remains a controversy whether naïve T cells are solely activated by dendritic cells *in vivo*, or whether B cells and macrophages can also perform this role in a real infection. Nonetheless, dendritic cells are probably the most important APC in the role of kick starting the immune response, but the role for macrophages in priming the immune response is still unclear.

T cell tolerance

T cell tolerance is essentially a two stage process known as 'central tolerance' and 'peripheral tolerance'. A few decades ago, tolerance was thought to involve mainly the process of clonal deletion or survival. This is the basis of our understanding of "central tolerance", which is the process of positive and negative selection in the thymus (Bevan et al., 1994; Goldrath and Bevan, 1999). Positive selection is the process that restricts the TCR repertoire to peptides that can bind MHC complexes. T cells that bear TCR with no binding affinity to self MHC complexes are deleted by apoptosis, presumably T cells that can't recognise MHC-peptide complexes at all are a waste of space. However, T cells which have TCR that reacts with very high affinity to self MHC complexes in the thymus also undergo apoptosis in a process known as

negative selection. This would in theory delete all auto-reactive T cells that encounter self antigens in the thymus.

Whereas central tolerance depends on signal one, peripheral tolerance depends on all the T cell activation signals discussed above. Although thymic selection (i.e. central tolerance) is crucial for successful tolerance, many self antigens would be expressed in peripheral tissues that never get into the thymus and become involved in the process of selection and deletion. It is now clear that an extremely complex multilayered set of checkpoints are involved in maintaining the fine balance between an effective immune response and an auto-immune reaction in the periphery. This change in perception and emphasis is largely due to advances in technology, especially in our ability to generate T cell receptor (TCR) transgenic mice (Stockinger, 1999). These mice clearly demonstrated that T cells exist with TCR that recognise self antigens, but have not been deleted in the process of thymic selection, as negative selection is not efficient in deleting T cells which react to peripheral tissue specific antigens. The auto-reactive T cells that have escaped negative selection remain unresponsive, but can be stimulated to proliferate in response to auto-antigens. Why are these auto-reactive T cells not activated when their TCR recognises MHC with self peptides? The melange of mechanisms that has emerged to explain these observations is lumped together as the process of "peripheral tolerance".

Peripheral tolerance

As discussed earlier, the understanding of peripheral tolerance requires not only an understanding of the specificity of T cells, but also an understanding of the activation and interaction of T cells with antigen presenting cells (APC). I believe that the decision for a T cell to respond to an antigen depends more on the kinetics of TCR-peptide-MHC engagement, accompanied by the correct molecular context of it's APC, then it does on the specificity of the TCR to the peptide-MHC. The presence of appropriate accessory signalling molecules and adhesion molecules that modify cytoskeletal organisation and stabilises TCR engagement and signalling is probably as important as TCR specificity itself. Hence, peripheral tolerance could be more important than central tolerance in the prevention of auto-immunity. Although this

interpretation of immunological data is consistent with some aspects of Matzinger's "danger hypothesis", the fact that central tolerance occurs at all is clear evidence that there is a self-non-self element to the process of tolerance. Furthermore, it is also consistent with Medzhitov and Janeways interpretation of innate/acquired immunity.

Peripheral tolerance involves a number of mechanisms, all of which probably act in concert to prevent auto-immunity. These mechanisms are: 1) physical barriers to lymphocyte migration, 2) immune ignorance, 3) peripheral deletion/apoptosis, 4) anergy and 5) suppression. Apart from the first mechanism, all the other mechanisms involve the molecular context of T cell activation. Immune privileged sites such as the testes, the anterior chamber of the eye and the brain, which can accept allogeneic or xenogeneic tissue grafts, have improved our understanding of the tolerance mechanisms that underly their protection.

Physical barriers

The simplest mechanism for the prevention of T cell responses to self antigens in the periphery, is that *in vivo*, potentially autoreactive T cells never encounter their cognate antigen in a physiological relevant situation. This may be the situation in immune privileged sites such as the brain and the anterior chamber of the eye, which lack immune surveillance (Vaux, 1995). For example, it is possible that T cells which can react specifically to antigens expressed only in the brain are present, but are never activated because they never cross the blood/brain barrier. This passive mechanism of peripheral tolerance is perhaps less well studied that it should be.

Ignorance

The term immunologically ignorant was first used by Ohashi et.al. (1991). In a classic study, transgenic mice were generated to express a viral glycoprotein (LCMV-GP) in β islet cells of the pancreas, and crossed with mice transgenic for TCR specific to LCMV-GP (Ohashi et al., 1991). Some of the T cells from these double transgenics were neither deleted nor were they anergized, because they could be functionally activated *in vitro*. Ohashi et.al. called these 'ignorant' T cells. Interestingly, if these mice were infected with LCMV, this state of tolerance is reversed and the mice

develop diabetes. Since then, many other examples of ignorance have emerged from similar double transgenic animals, of T cells which can be functionally activated *in vitro* (i.e. not anergised), not deleted, but also non-responsive *in-vivo*.

Another example of ignorance is the observation that immunisation with a self-peptide from cytochrome c (81-104) could elicit a strong autoimmune response (Mamula, 1993). However, the autoreactive T cells which recognise this self-peptide do not react to the entire cytochrome c protein when stimulated *in-vitro*. This example of an ignorant T cell is probably a result of the lack of processing and presentation of this cryptic epitope *in vivo*.

The term 'ignorance' has essentially been used to describe any situation in which there are tolerant auto-reactive T cells which are neither deleted nor anergised. These observations have generated interest because the emergence of autoimmune disease could be a result of the activation of these 'ignorant' T cells (Elson et al., 1995).

Deletion

The best examples of T cell deletion in the periphery are the active process of Fas-ligand induced apoptosis in immune privileged sites of the testis (Bellgrau et al., 1995) and the eye (Griffith et al., 1995). Bellgrau et. al (1995) showed that grafts of mouse testes taken from mice that lack Fas ligand (gld mice), are rejected whereas normal mouse testes which express high levels of Fas-ligand in the testes survive. Although this strongly suggests that Fas-ligand on the testis induces apoptosis in activated T cells which responds to the graft, it was not formally demonstrated. It should also be noted that these experiments could not be replicated by a separate group and remain controversial (Allison et al., 1997; Vaux, 1995).

Griffith et.al (1995) showed that Fas mediated apoptosis was indeed responsible for the death of inflammatory cells infiltrating the eye after the injection of antigen into the eye. This appears to explain the previous observations that injection of antigens into the eye can lead to tolerance to delayed type hypersensitivity response.

However, expression of Fas-ligand alone is not sufficient to prevent rejection since the transfer of pancreata from transgenic mice expressing Fas-ligand in their β-islet cells could not prevent host rejection (Allison et al., 1997).

There are surprisingly few reports of peripheral deletion of other auto-reactive T cells. The best example is work by Rocha and von Boehmer (1991). They showed that the adoptive transfer of T cells from TCR transgenic mice specific for the male antigen HY into male mice resulted in an expansion of these T cells in the first 5 days, which surprisingly did not cause any auto-reactive pathology. The majority of these T cells disappeared from the peripheral lymphoid organs between 5-9 days, leaving behind a smaller 'ignorant' population with down regulated receptors (Rocha and von Boehmer, 1991). This observation appears similar to the expansion and deletion of T cells after response to a foreign antigen.

Anergy

Anergy is a condition for lymphocytes whereby they cannot be fully activated to perform their normal functions although they have received all the appropriate signals (Schwartz, 1996; Schwartz, 1997). The anergic T cell lacks the ability to produce or respond to signals for proliferation. There are many routes to anergy and these include: 1) TCR engagement without co-stimulation, 2) TCR engagement by altered peptide ligands (APL), 3) the cytokine environment in which TCR engagement occurs, 4) an excess of antigen.

The earliest models for anergy revolved around the absence of co-stimulation during TCR engagement. This model was attractive because it provided a mode of peripheral tolerance whereby an APC could induce T cell unresponsiveness by providing signal one without signal two. However, when more ways of anergising cells emerged, this model became inadequate. Jenkins (1992) then argued that anergy occurs when a T cell receptor is engaged but the T cell is prevented from proliferating (Jenkins, 1992), for example when T cell activation occurs in the presence of anti-IL2 and anti-IL2-receptor antibodies. Inhibition by IL-10 during human $CD4^+$ T cell activation has also been reported to result in anergy (Schwartz, 1996). Interestingly,

there have been no reports of anergy as a result of T cell suppression by TGF-β. Although this model can account for some mechanisms which lead to anergy, it is not consistent with induction of anergy by APLs.

As a result of recent signal transduction data, Schwartz has proposed a more mechanistic model for anergy (1996, 1997). He argues that anergy occurs due to a block in IL-2 gene transcription by; 1) a failure to activate $p21^{ras}$ or 2) a repressor molecule such as Nil-2-a. A block to $p21^{ras}$ probably leads to the decreased phosphorylation and activity of ERK and JNK which has been reported in anergic cells (Desilva et al., 1996). This leads to a failure in activating AP-1, a transcription factor that induces IL-2 gene transcription (Schwartz 1996).

It is interesting that although CTLA-4 provides an inhibitory signal towards T cell activation, there are no reports that it induces anergy. Especially since, early reports of signal transduction pathways suggest that CTLA-4 signalling might intersect with the $p21^{ras}$ signalling pathway (Chambers and Allison, 1997).

T suppressor/regulatory cells

Recently, the experimental evidence supporting the existence of $CD4^+$ regulatory T cells has been mounting (Sakaguchi, 2000). This idea is being received with much greater enthusiasm due to the cloning of $CD4^+$ T cells that suppress the proliferation of other T cells (Chen et al., 1994; Groux et al., 1997). Naive T cells cultured in the presence of IL-10 differentiate into a Th subset which secretes high levels of IL-10, low levels of IL-2 and no IL-4, and has been termed Tr1 cells (Groux et al., 1997). Tr1 cells proliferate very slowly, and can suppress antigen specific T cell proliferation via IL-10 and TGF-β dependent pathways. Hence the properties and function of T regulatory cells probably overlaps with phenomenon that had previously been attributed to anergic T cells (Chai et al., 1999; Frasca et al., 1997; Lombardi et al., 1994; Taams et al., 1998). Adoptive transfer of Tr1 cells can prevent the development of inflammatory bowel disease *in vivo*.

In a different system, Chen et.al (Chen et al., 1994) described the generation of $CD4^+$ T cells that secreted TGF-β, IL-4 and IL-10, in response to orally administered antigen. These cells could also suppress the development of experimental autoimmune encephalomyelitis (EAE) *in vivo*. However, there has been no evidence that exposure of naive T cells to either of these sets of regulatory T cells would lead to anergy. Indeed, these regulatory T cells might explain the phenomenon of 'ignorance' instead of anergy.

There is also data emerging from the transplantation field of 'infectious suppression' and 'linked suppression', from the adoptive transfer of $CD4^+$ cells (Cobbold and Waldmann, 1998). Treatment with antibodies to CD4 and CD8 at the time of organ grafts leads to acceptance and tolerance to the graft (Qin et al., 1993). $CD4^+$ cells from mice which have been tolerised in this way can then be adoptively transferred to a naive animal. Interestingly, the naive animal will then be able to accept the same organ graft even though it has not been treated with antibodies. In fact, if $CD4^+$ cells are taken from a naive animal and transferred into a tolerized animal, these $CD4^+$ cells from the naive animal are then converted into suppressor cells, which can prevent the rejection of the organ graft when transferred into a different naive animal (Cobbold and Waldmann, 1998).

More recently, a clearer phenotype of these cells have emerged. It is now thought that suppressive T cells express CD4, CD25 and are $CD45RB^{low}$ (Shevach, 2000). It has been suggested that engagement of CTLA-4 could play an important role in the differentiation of these suppressor cells or be involved in their biological function (Read et al., 2000; Takahashi et al., 2000) (eg. by triggering release of TGF-β (Chen et al., 1998b)). Indeed, it is possible that the lympho-proliferative disease associated with $CTLA\text{-}4^{-/-}$ mice could be due to the lack of T regulatory cells, as well as the inability to downmodulate activated T cells. On the other hand, there is also been evidence that the CD28/B7 interaction is necessary for the development or differentiation of $CD4^+/CD25^+$ suppressor cells (Salomon et al., 2000). The number of these $CD4^+/CD25^+$ cells are reduced in NOD $CD28^{-/-}$ mice as well as in NOD

B7.1/B7.2$^{-/-}$ mice, and can be decreased by treatment with CTLA-4-Ig (Salomon et al., 2000). Shevach and Sakaguchi have recently provided a more detailed review of these suppressive or regulatory T cells (Sakaguchi, 2000; Shevach, 2000).

Alternatively activated or suppressive antigen presenting cells

Although the importance of suppressor T cells in the immune response is becoming more established, immuno-suppresive cells are not restricted to T cells, and there has always been considerable evidence for the presence of suppressive antigen presenting cells, especially macrophages. There are examples of antigen specific (Kirschmann et al., 1994) as well as antigen independent suppression against tumours (Taramelli et al., 1981). There have also been early suggestions of macrophages inducing the activity of regulatory T cells, before the scepticism of suppressor T cells set in (Ptak et al., 1978). Suppressor macrophages have been described to play a role in protecting the immune privileged embryo (Chang et al., 1993) and the alveoli of the lungs (Poulter et al., 1994). *In vitro* studies have shown that macrophages that phagocytose or interact with apoptotic cells will secrete TGF-β, which could suppress production of pro-inflammatory cytokines (Fadok et al., 1998). More recently, there have also been reports that suppressive T cells might act via the antigen presenting cell (Taams et al., 1998), which might act as a bridges that brings suppressive T cells together with naïve T cells.

More significantly, there is a growing view that IFN-γ activated macrophages have different suppressive capabilities from IL-4 activated macrophages (Goerdt and Orfanos, 1999). Whereas IFN-γ activated macrophages, or classically activated macrophages suppress proliferation vie nitric oxide (NO) mediated mechanisms (MacMicking et al., 1997), IL-4 activated macrophages could suppress via novel mechanisms (Schebesch et al., 1997). For example, human macrophages activated *in vitro* by IL-4 and glucocorticoids suppress proliferation independently of IL-10, NO or prostaglandins (Schebesch et al., 1997).

In terms of parasitic infections, *Trypanosoma brucei* can induce suppressive macrophages in mice that act via NO (Mabbott et al., 1995; Schleifer and Mansfield, 1993b; Sternberg and Mabbott, 1996a), whereas Th2 driving nematode infections could induce suppressive macrophages that are activated by IL-4. Macrophages that suppress a pro-inflammatory response via IL-10 have been described in response to schistosome eggs (Stadecker, 1999).

By discussing the various mechanisms of peripheral tolerance, it becomes clear that it is difficult to distinguish between 'tolerance' (i.e. the lack of an immune response) and an anti-inflammatory response, which in turn suppresses proliferation of T cells and cytokines and prevents rejection. It seems to me likely that this distinction can never be clearly made experimentally and thus general theories about 'tolerance' are useful in guiding our thinking, but dangerous in affecting the interpretation of complex empirical data common to biology.

Lymphatic filariasis

Lymphatic filariasis is a serious, debilitating and widespread tropical disease. The WHO estimates the 120 million people are infected with the parasitic filarial nematodes that cause this disease. *Wuchereria bancrofti* causes 90% of the infections and the remainder is mostly caused by *Brugia malayi*. It is estimated that 44 million people suffer from pathology caused by lymphatic filariasis (e.g. lymphoedema, genital pathology (especially hydrocoeles) and elephantiasis), with a further 76 million people having hidden, asymptomatic infections. Due to the global nature of this disease, it is the second leading cause of permanent and long-term disability world wide. Recently (1998), a collaboration between pharmaceutical companies and the WHO has raised hopes that this disease could potentially be eradicated. SmithKline Beecham has agreed to provide albendazole free-of-charge for as long as necessary to ensure success of the elimination programme, while Merck & Co will expand it's onchocerciasis ivermectin donation program to cover the treatment of lymphatic filariasis. Notable successes in countries like China and Japan suggests that a well organised effort can radically reduce the consequences of this tropical disease. However, drug resistance as well as logistical difficulties in continuing treatment for a minimum of 4-6 years in order to successfully interrupt transmission makes global eradication a formidable task.

Lifecycle

The related lymphatic filariae have similar lifecycles that depends on larval development in the intermediate hosts (i.e. mosquitoe) as well as development in humans, which are considered the definitive hosts. Like all nematodes, filarial worms develop through a moulting process, which distinguishes the 5 developmental stages (L1-L5) that occur during their life cycle (Figure 1). We have been particularly interested in the adult (or L5) stage of this parasite, because this is the stage that resides in the lumen of the lymphatics for long periods of time. The female adults are considerably longer than the males averaging about 4.8cm in length in comparison to an average of about 2.2cm for male adults. After mating, the female adults can live for 5-10 years in the host lymphatics, during which time they are constantly producing thousands of microfilariae (Mf), which circulate in the bloodstream. The microfilariae are ingested by mosquitoes, where they migrate into the thoracic flight muscles and moult twice (from L1-L3 stage) in a period of about 10 days. The infective L3 larvae migrate throughout the body cavities of the mosquito, including the head, and can then be transmitted to a mammalian host when the mosquito feeds. After entering the mammalian host through the lesion created by the feeding mosquito the larvae migrate rapidly to the afferent lymphatics where they moult 2 more times (L3-L5) and remain for the rest of their lives.

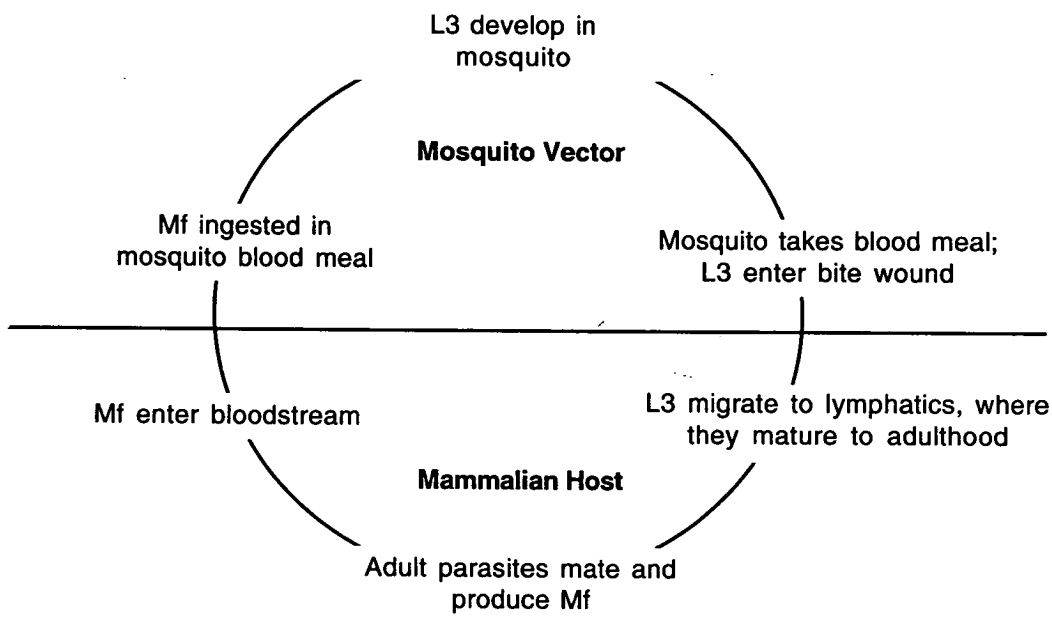


Figure 1: The life cycle of *Brugia malayi*.

Animal models

It is interesting to note that most filarial worms have a narrow host specificity, which could be an important clue to their remarkable abilities of immune evasion. However, this has been a major hindrance towards a better understanding of this infection since suitable laboratory animal models have not been readily available. Whereas *W. bancrofti* cannot be maintained in any non primate laboratory animals, at least *B. malayi* can develop to maturity in jirds, rats, hamsters, ferrets, dogs and cats. The *Brugia*-Jird model via intraperitoneal infection results in the best recovery of sexually mature adult parasite, and is routinely used in the maintenance of this parasitic life cycle.

Unfortunately, inbred strains of laboratory mice are resistant to the full developmental lifecycle of *B.malayi*, which deprives us of many immunological tools that could significantly improve our understanding of the immune responses that are necessary for resistance and protection against this parasite. However, the individual stages of *B.malayi* can survive in mice for various periods of time, and the analysis of genetically deficient mice have been useful in developing our understanding for the cytokine responses that are associated with these Th2 inducing nematode parasites (Lawrence, 1996). At present, the most promising rodent model is the recent discovery that the rodent filarial parasite *Litomosoides sigmodontis* can undergo full development in inbred strains of laboratory mice (Maréchal et al., 1997; Maréchal et al., 1996). In our laboratory, we are beginning to switch over from the *Brugia*-mouse model to the *Litomosoides*-mouse model in order to ask questions about natural and acquired immunity, as well as to verify whether our findings with the *Brugia*-mouse model are relevant to a natural host-parasite interaction.

Unfortunately, none of the animal models can reproduce the extremely wide spectrum of clinical presentation in an endemic region, which is one of the most interesting and important aspects of filariasis (Ottesen, 1992). It has been reported that infection of SCID or athymic nude mice with *Brugian* parasites can result in an elephantiasis-like disease with dilated lymphatics, but without inflammation (Nelson

et al., 1991; Vincent et al., 1984). However, the *Brugia pahangi*-infected cat model (a natural host-parasite pair) is probably the best mimic of the natural course of disease development.

Human lymphatic filariasis

In order to study the disease in an endemic area, researchers have divided the population into 3 categories; 1) endemic normals, 2) asymptomatic microfilaraemics, and 3) individuals with clinical manifestations. This classification system has been useful in defining the immune parameters that could be associated with various disease states, but as always, is an oversimplification of the situation.

Endemic normals

The term endemic normals is used to describe individuals who are constantly exposed to the filarial parasites, but have no clinical manifestations or signs of infection. They seem to be truly immune to the parasites and have high levels of anti-filaria antibodies as well as active cellular responses (Maizels et al., 1995). However, because detection of microfilarae in the blood is a relative insensitive indicator of current infection, some of these individuals might harbour undetected infections.

Asymptomatic microfilaraemic

Asymptomatic microfilaraemic individuals represent a large proportion of the people infected with filarial parasites, who although have circulating Mf, do not show obvious signs of clinical disease. It is estimated that 76 million people fall into this category of the minimum of 120 million infected people. Contrary to earlier models of a clear dichotomy between asymptomatic microfilaraemics and the clinically affected patients, modern lymphoscintigraphic analysis has revealed that asymptomatic microfilaraemics often suffer from significant damage to the lymphatic vessels (Freedman et al., 1994), although this seems to be sustained without clinical manifestations.

Clinical filariasis

Individuals with clinical manifestations had previously been grouped into a category termed "chronic pathology" and thought to be amicrofilaraemic. More recent studies have demonstrated that 10-40% of individuals with chronic diseases remain microfilaraemic. Furthermore, the wide spectrum of clinical manifestations (not all of which are a result of chronic infection), has led to the term "clinical filariasis" being proposed as an alternative term of classification (Freedman, 1998). An important question revolves around the reasons for the development of clinical manifestations, since almost all infected individuals (including asymptomatic microfilaraemics) suffer from some form of lymphatic damage.

Clinical pathogenesis can be separated into the acute and chronic stages (Kumaraswami, 2000). The acute stage is characterised by recurrent fever attack associated with inflammation of lymph nodes and lymphatic vessels. More recent evidence suggests that secondary infections could play an important role in triggering the episodes of fever and inflammation. These attacks have serious impacts on the ability of patients to work productively. The chronic stages of disease manifestation are usually associated with obstruction of lymphatics, which result in elephantiasis of the limbs or hydrocoeles (male genital disease). There is some epidemiological evidence which suggests that many individuals in an endemic area will progress from asymptomatic microfilaraemics, to suffering from acute filarial disease and eventually develop signs of chronic pathology (Bundy et al., 1991). However, this hypothesis is controversial and conversion is certainly not inevitable since some infected individuals will never develop clinical filariasis (Ottesen, 1992).

A distinct group of infected individual suffer from an extreme manifestation immmunological hyperesponsiveness known as tropical pulmonary eosinophilia (Ottesen and Nutman, 1992). They are almost always amicrofilaraemic, but remnants of microfilaria surrounded by eosinophils are often found in tissue biopsies. These patients suffer from symptoms similar to asthma, including severe obstructive lung function abnormalities, probably as a result of eosinophil infiltration into the lungs.

However, treatment with antifilarial drugs is usually accompanied by rapid and dramatic clinical improvement, which is surprising considering the fact that these patients are usually amicrofilariaemic.

Immunology of filarial infection

The immunological features of filarial infection share remarkable similarities with other helminth (defined as multicellular macroparasites that do not undergo asexual expansion in their hosts) infections. They have 1) high levels of type 2 responses, 2) immune suppression or downmodulation, 3) ineffective immune responses 4) resulting in chronic infections due to long lived parasites

Th1/Th2 paradigm

Since Mosmann et. al. distinguished Th1 from Th2 clones (Mosmann et al., 1986), the Th1/Th2 paradigm has been a major guiding principle in most of the immunological studies in infectious diseases. The work on filarial immunology is no exception in relying heavily upon the concepts behind this paradigm. Like other nematodes, many studies have suggested that infected individuals show a bias towards a Th2 response. However, a more significant concept in filarial immunology was the perspective that differential Th1/Th2 responses is associated with different levels of clinical disease (Ottesen, 1992).

Consistent with the concept that a Th2 response is associated with reduced pathology, the predominant serum antibody that is specific for parasites in asymptomatic Mf+ patients is IgG4, which is often also accompanied by high levels of IgE. These antibody isotypes are known to be promoted by the Th2 cytokine IL-4. In contrast, patients that are suffering from the chronic stage of the disease often have higher IgG 1-3 antibodies, which are not significantly enhanced in Mf+ individuals (Kurniawan et al., 1993).

In terms of T cell responses, many studies have consistently shown that both asymptomatic individuals as well as those in the chronic stages of disease have

comparable levels of IL-4 production. However, asymptomatic individuals have suppressed/reduced IFN-γ production, whereas clinically affected patients often have elevated IFN-γ production. Although these data suggests that the Th1 response is associated with clinical pathology, it should be noted that IgE antibodies are also elevated in elephantiasis patients as well as individuals suffering from TPE.

Thus, clinical disease can be associated with both a hyperresponsive Th1 or Th2 response. Because there is not a suitable animal model for human disease, as well as the fact that human *in vitro* studies can only be relied upon to provide associations and correlation, whether pathology is 1) immune mediated or 2) associated with the switch from a Th2 to a Th1 response, remains difficult to answer.

Immunological tolerance to filarial infections

Because one of the most consistent findings of human studies is the antigen specific hyporesponsiveness of T cells in asymptomatic Mf+ individuals, it has long been suggested that filarial nematodes induce a state of tolerance in the host to the parasite burden (Maizels and Lawrence, 1991). However, more recent evidence indicates that the term tolerance oversimplifies a complex interaction between the host and the parasite (Maizels et al., 1999a). For example, it seems that T cell unresponsiveness is restricted to Th1 responses, proliferation and IL-5 production, whereas IL-4 production and antibody responses remain intact (Maizels et al., 1999a). Thus, as was discussed in the previous section, it is often difficult to distinguish between a state of peripheral tolerance and anti-inflammatory responses that prevent rejection.

Parasites can prevent host rejection by either evading the immune system (a strategy directed towards itself), or by actively down modulating the immune response (a strategy directed towards the host). With regard to helminth parasites, there are numerous examples of the first strategy (Maizels et al., 1993) but considerably less knowledge about the latter mechanism. However, there is a clear recognition that this is likely to be a key strategy for parasites that do not replicate in the host and do not undergo antigenic variation. Further understanding of strategies directed towards the

host provides the opportunity for us to learn potentially novel means by which to regulate the mammalian immune response. Although, it is unlikely that any single mechanism is responsible for this balance of responsiveness/unresponsiveness, by dissecting a piece of the puzzle at a time, we hope to understand this complex host-parasite interaction.

Which of the mechanisms of peripheral tolerance discussed in the earlier section on peripheral tolerance could be involved in parasite specific hypo-responsiveness?

Since filarial nematodes live in the lymphatic system of the host, there are no **physical barriers** involved in preventing T cells from being exposed to parasite antigen. There have been no reports of immune **ignorant** T cells in filarial patients in the literature. The existence of inactivated T cells that are parasite specific, but are neither anergised nor deleted remains an interesting prospect. Although there have been no reports of genes homologous to Fas-ligand found in parasites, it is possible that structural homologies, or molecular mimicry of Fas-ligand could lead to the **deletion** of parasite activated T cells. This remains an intriguing possibility for a mechanism of peripheral tolerance to parasites. Our current knowledge of peripheral tolerance to parasites is restricted to reports of parasite induced **anergy** and **suppression.**

T cell anergy in lymphatic filariasis

As mentioned before, a consistent finding of human studies is the observation that T cells isolated from parasite infected individuals do not proliferate in response to stimulation *in vitro*. This lack of proliferation is accompanied by the lack of IFN-γ, and IL-5 secretion (Maizels et al., 1999a). However, antigen specific IL-4 production remains high even in the absence of proliferation. These observations have led to the suggestion that parasite induce tolerance by inducing anergy in parasite specific T cells (Nutman et al., 1987). However, there is still no direct evidence of a parasite mechanism for inducing anergy, especially since there are several possible mechanisms that can lead to anergy. It is important to note that hyporesponsiveness

observed in infected individuals is antigen specific and Th1 response to mycobacterial antigens remains the same (Sartono et al., 1996).

There are reports that high antigen doses can lead to anergy in human T cell clones (O'Hehir et al., 1991). Since it is common for individuals to have chronic high level infections in filiriasis, high antigen load causing anergy is an attractive explanation (Maizels et al., 1999a). Another model is the suggestion that parasites could prevent the expression of co-stimulatory molecules on APC (Maizels et al., 1999a). This would cause T cells that have parasite specific TCR to become anergic when TCR engagement occurs in the absence of costimulation. Finally, the prevention of T proliferation by active suppression in the presence of antigen might also lead to anergy.

Suppression in lymphatic filariasis

T cell responses can either be 1) actively suppressed by the parasite itself or 2) actively suppressed by a cell type which is induced/recruited by the presence of the parasite. Piessens et.al. have reported both forms of suppression (Wadee et al., 1987: Piessens, 1980). A high molecular weight fraction from microfilarial extracts of *Brugia* appears to suppress lymphocyte proliferation in response to conA (Wadee et al., 1987). Maizels et.al has also postulated that *B.malayi* might express homologues of the cytokine TGF-β that can down regulate T cell responses (Gomez-Escobar et al., 1998). Piessens et.al. also reported an adherent cell type isolated from blood of patients, which suppresses antigen specific responses (1980).

So far, there have been few descriptions of suppressor T cells being involved in immune-suppression by filarial parasites. However, a recent study by Doetze et.al. found that hypo-responsive T cells in individuals infected with Onchocerca volvulus is associated with the cytokines IL-10 and TGF-β (Doetze et al., 2000). More significantly, they were able to clone 27 *O. volvulus* specific T cell clones from an

infected individual, and found that 3 of these T cell clones produced IL-10 only, 11 of these clones produced TGF-β and 4 clones produced both IL-10 and TGF-β.

APC in a murine model for filarial infection

Our group has been interested in the possibility that filarial parasites interfere with the function of antigen presenting cells and hence down modulate the immune response and potentially induce the antigen specific tolerance that is observed in Mf^+ patients. Initial studies involved *in vitro* co-culture studies whereby live parasites were cultured with APC and T cells. There was no a direct effect of co-culturing live parasites *in vitro*, on the proliferation of T cells or the function of APCs and so this approach was abandoned.

We subsequently decided to take an *in vivo* approach and developed a mouse model to investigate the interaction of filarial parasites with APC *in vivo*. Adult *B. malayi* parasites that are implanted in the peritoneal cavity of mice can survive for many weeks, although the full lifecycle of the parasite cannot be reproduced in mice. A result of this implantation is the generation of a population of antigen presenting cells (APCs) that can induce antigen specific cytokine production, but block cellular proliferation of a Th2 cell clone D10.G4 (Allen et al., 1996). The suppression of proliferation is an active process that cannot be reversed by mixing an excess of irradiated splenocytes with the D10s and suppressor cells. The generation of these suppressor cells only occurs when live but not dead parasites were used, and is dependent on IL-4 but not IL-10 (Allen et al., 1996; MacDonald et al., 1998). Furthermore, suppressor cells also block proliferation of a wide range of cell types including B cell hybridomas and a colon carcinoma (Allen et al., 1996). Depletion of T cells suggests that suppressive T cells are not involved. This raises the possibility that we are studying a novel suppressor cell type acting through a novel mechanism and represents the starting basis for this thesis.

MATERIALS AND METHOD

Immunological techniques

Parasite isolation and infection model

B. malayi adult parasites were obtained from infected jirds purchased from TRS laboratories (Athens, GA) or maintained in house courtesy of Bill Gregory, Janice Murray and Yvonne Harcus. Adult worms were removed from the peritoneal cavity and washed in RPMI. For all experiments, mice used were 6-8 week old males. Mice were surgically implanted intra-peritoneally (i.p.) with 6 live adult *B. malayi* females and 3-6 weeks later mice were euthanized by cardiac puncture and PEC were harvested by thorough washing of the peritoneal cavity with 15 ml of RPMI wash.

Mouse strains

Apart from experiments comparing C57BL/6 wild type mice (WT) with genetically deficient mice, 6-8 week old CBA/Ca males were used. 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-$4^{-/-}$) breeding pairs were purchased from B&K Universal Ltd. (North Humberside, UK) with permission of the Institute of Genetics, University of Cologne. C57BL/6 IL-5 deficient (IL-$5^{-/-}$) mice were the kind gift of Prof. Manfred Kopf (Basel, Switzerland). Naïve $CD4^+$ lymphocytes were obtained from splenocytes of mice expressing transgenic TCR for PCC peptide 88-104/I-E^K (B10.A X B10 F1).

Cultures and cell lines

All *in vitro* cultures were carried out in RPMI 1640 medium supplemented with 2 mM glutamine, 0.25 units/ml penicillin, 100 µg/ml of streptomycin, 5 µM 2-mercaptoethanol and 10% FCS (RPMI complete). The Th2 cell clone, D10.G4 was maintained by incubation of cells with 100 µg/ml conalbumin in the presence of syngeneic 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 concanavilin A (Con A)-stimulated mouse lymphocytes. The D10.G4 cells were used 5-7 days after this rest period. The murine T cell lymphoma EL4 and the B cell hybridoma HB32 were maintained by growth in complete RPMI. The tumour cell lines used were acclimatized to RPMI media for at least 2 weeks prior to exposure to PEC. HB32 and EL-4 cells were maintained by growth in complete medium. The tumour cell lines were provided courtesy of Scott Bader and Jim Ross.

Cell sorting by magnetic beads

$CD4^+$ lymphocytes (>95% pure) were isolated with directly conjugated L3T4 beads and either MS^+ or VS^+ columns in conjunction with MiniMacs or VarioMacs magnet (Miltenyi Biotec). Before magnetic bead cell purification, PEC were passed through a 70μm cell strainer and purified by centrifugation over Histopaque (Sigma) in order to remove any microfilariae. PEC were then sorted with MS^+ or VS^+ columns according to the manufacturers instructions (Miltenyi Biotec). $F4/80^+$ cells were purified with biotin conjugated F4/80 (rat IgG2b;Caltag) and Streptavidin Microbeads (Miltenyi Biotec).

Antigen presenting cell suppression assays

For investigation into the effect of suppressive PEC on apoptosis, recovery and the cell cycle, 1ml of PEC from parasite implanted or control mice, adjusted to $1x10^6$/ml, were adhered on 24 well plates for 2-3 hours at 37°C. Non-adherent cells and microfilariae were then removed by washing with complete medium and the adherent PEC were co-cultured with D10.G4, EL4 or HB32 cells to a final volume of 2ml/well. D10.G4 (1ml at $1x\ 10^6$/ml) were added to each well, with conalbumin at a final concentration of 50μg/ml. EL4 and HB32 cells were added in 1ml of complete RPMI (at 5×10^5/ml). In all these experiments, suppression was also confirmed by $[^3H]$ thymidine incorporation assays in 96 well plates. 100 μl PEC at $1x10^6$/ml were adhered to flat–βottom 96 well plates as described above and co-cultured with different cell lines, at the same concentration as above, to a final volume of 200μl/well. After incubation for 48 hr at 37°C, 1 μCi $[^3H]$ 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 Microplate Scintillation Counter (Wallac). The same protocol was used to measure the suppression of human cancer cell lines, which were adjusted to $1x10^5$ cells/ml before adding 100µl to the adherent PEC in 96 well flat bottom plates. To determine if suppressed cells recovered ability to proliferate after removal from suppressive PEC: EL4, HB32 and D10.G4 cells (with 50µg/ml conalbumin) were co-cultured in 24 well plates with PEC for 48 hours at the same concentration as described above. The non-adherent HB32, EL4 and D10.G4 cells were then aspirated from the adherent PEC and further purified using nylon wool columns to remove all the adherent PEC. The suppressed HB32 and EL4 cells were then cultured in 96 well plates at the same starting concentration ($5x10^4$ cells per well), and their proliferation was assayed by [^{3}H] thymidine incorporation 48 hours later. $1x10^4$ nylon-wool purified D10.G4 cells (per well) were restimulated with $5x10^4$ syngeneic irradiated splenocytes and 50µg/ml conalbumin in each well, and proliferation was also assayed 48 hours later.

T cell stimulation assays

For primary stimulation studies, $1x10^5$ PEC from control or parasite-implanted mice were plated out on flat–βottom 96 well plates for 2-3 hours at 37°C. The non-adherent cells and microfilariae were then removed by washing with complete medium. The adherent PEC were used to stimulate $1x\ 10^5$ purified naïve $CD4^+$ T cells, with 10µg/ml PCC protein. Supernatants were harvested after 48 hours for cytokine assays (see below) and proliferation was measured by [^{3}H] thymidine incorporation. For two stage assays, $1x\ 10^6$ PEC were adhered to 24 well plates, washed and used to stimulate $1x\ 10^6$ naïve T cells (at 10µg/ml PCC protein). After 48 hours co-culture, non adherent T cells were aspirated from adherent PEC and allowed to expand/rest for 72 hours in complete media before being repurified for $CD4^+$ cells as above. In secondary cultures, $1x10^5$ repurified T cells were stimulated with $5x10^5$ irradiated splenocytes and 10µg/ml PCC protein. After 48 hours, proliferation and cytokine production were assayed as described for primary stimulation. Neutralizing antibodies against TGF-β (25µg/ml, clone 1D11.16, Celtrix Corporation, Santa Clara, CA) and

IL-10 (5μg/ml, clone JES5-2A5, Pharmingen, San Diego, CA) were added to the primary stimulation cultures at the recommended concentrations.

Cytokine assays

IL-2 and IL-4 production were measured with the IL-2/IL-4 responsive NK cell line, as described previously (Allen et al., 1996). Briefly, $1x10^4$ NK cells were added to 10μl of cell culture supernatant in the presence of saturating anti-IL-2 (S4B6) or anti-IL-4 (11B11) antibody to distinguish between the two cytokines responsible for proliferation. Proliferation data was converted to concentrations with standard curves of recombinant IL-4 and IL-2 (Sigma). IFN-γ and IL-10 was measured by capture ELISA, with reference to standard curves of known amounts of rIFN-γ (Sigma) and rIL-10 (Genzyme). IFN-γ was measured using R46A2 (ATCC) as capture Ab and biotinylated rat anti-mouse IFN-γ monoclonal XMG1.2 (Pharmingen). IL-10 was measured using JES5-2A5 (Pharmingen) as capture Ab and biotinylated rat anti-mouse IL-10 clone SXC-1 (Pharmingen). Avidin-alkaline phosphatase (Sigma) was used for detection.

CFSE and intracellular cytokine staining

A modification of Lyons and Parish's technique for CFSE staining was used (Bird et al., 1998; Lyons and Parish, 1994). Briefly, 1 x 10^7 cells/ml were incubated with 10μM CFSE (Molecular Probes) in PBS for 8 minutes at room temperature. Staining was stopped with an equal volume of FCS, and the cells were washed 3 times with complete RPMI. Stained naïve T cells were primed by PEC (as described above) for 72 hours before being harvested and stained for intracellular cytokines using the Cytofix/Cytoperm Plus™ kit (Pharmingen) according to the manufacturer's instructions. Briefly, GolgiStop was added to the culture at 1:1500 dilution together with 50ng/ml PMA and 500ng/ml Ionomycin and incubated for 4 hours at 37°C. Non-adherent cells were harvested and fixed in Cytofix/Cytoperm solution before being stained with anti-IL-4 (11B11, Pharmingen), anti-IFN-γ XMG1.2, Pharmingen_ or control Ig-PE conjugates (Pharmingen) in Perm/Wash solution. After staining, cells were analyzed by flow cytometry using a Becton-Dickinson FACSCAN and Cell

Quest Software. All data plots shown represent activated lymphocytes, gated by forward and side light scatter.

Cell cycle analysis

To investigate changes in the cell cycle as a result of suppression, EL4 and D10.G4 cells were stained with propidium iodide according to the protocol of Darynkiewicz (Darynkiewicz, 1993). Briefly, after 24 or 48 hours culture with PEC, suppressed cells were fixed with 0.5% formaldehyde in HBSS, permeabilised with 0.1% Triton X-100 and 1% BSA in HBSS, and resuspended in HBSS solution containing 5μg/ml or propidium iodide and 100 units of Rnase A. The cells were then analyzed by a FACScan (Becton Dickinson). FACS data was analyzed using the cell cycle analysis software Modfit (Verity Software House), which calculates the percentage of cells in the different phases of the cell cycle using a Gaussian model for G0/G1 and G2/M cells, and estimates S-phase cells as a trapezoid component. Suppressed cells were also double stained, by incubating with FITC conjugated Ki-67 (Boehringer Mannheim) prior to incubation with propidium iodide containing HBSS solution (Darynkiewicz, 1993). Cell division of suppressed EL4 cells was investigated with the proliferation dyes PKH26 (Sigma) and carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) with identical results. PKH26 staining was carried out according to manufacturer instructions (SIGMA). PKH26 and CFSE labeled cells were then co-cultured with control and implanted PEC as described above. A TUNEL kit from Boehringer Mannheim was used to assay the level of apoptosis after 48 hours co-culture with PEC according to the manufacturer's instructions.

Cytology and immunohistochemistry

Cytocentrifuge preparations of the adherent 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 morphological examination of at least 300 cells in randomly selected fields using an Olympus BH2 microscope.

Molecular Biology Techniques

Construction of cDNA libraries

$F4/80^+$ macrophages from the PEC of *B. malayi* implanted IL-5 and IL-4 deficient mice were purified as described above. 30µg of total RNA, which was extracted using RNAStat60 (Biogenesis), was used to synthesize cDNA and unidirectionally cloned into the pCMV-Script plasmid vector, using the cDNA library construction kit from Stratagene (CA). Briefly, first strand cDNA was synthesised with a poly(dT) primer containing a XhoI restriction site protected by GAGA sequences and an 18 base poly(dT) sequence. Methylated dCTP was used in the first strand reaction to protect the cDNA from restriction enzymes in subsequent cloning. The second strand was synthesised with RNase H and DNA polymerase I, and blunt ended with cloned Pfu DNA polymerase before being ligated with EcoR I adapters. The subsequent Xho I digest releases the EcoR I adapter from the 3' end of the cDNA to enable unidirectional cloning. The cDNA was then size separated on a cDNA size separation column (Gibco). In order to reduce inherent bias for smaller fragments, the different sized fractions were ligated and transformed separately to produce 10 different sub-libraries. About half of each sub-library was stored as primary recombinants by freezing down immediately in 15% glycerol at –80°C, while the other half was amplified by plating out on large petri dishes and incubated at room temperature for 48 hours. The unamplified IL-5-/- library contained 5 X 10^5 primary recombinants, whereas the unamplified IL-4-/- library contained 1 X 10^5 primary recombinants.

Construction of subtractive cDNA library

Total RNA was extracted from $F4/80^+$ purified macrophages from PEC of either WT implanted mice or $IL4^{-/-}$ mice. 1µg of total RNA was converted into 1^{st} strand cDNA using the Clontech SMART system (according to manufacturers instructions). After synthesis of first strand cDNA from WT macrophage, 2 rounds of subtractive hybridisation were undertaken with 20 times the amount of $IL\text{-}4^{-/-}$ macrophage RNA (20µg) as was used in the 1^{st} strand synthesis reaction of WT macrophage RNA using the Invitrogen 'Subtractor kit'. After 2 rounds of subtractive

hybridisation and phenol-choloroform extraction, the remaining single stranded cDNA was PCR amplified for 25 cycles. The PCR products were then size separated on a sepharose cDNA size selection column (Gibco) and the fractions were cloned separately in order to reduce cloning bias of small DNA fragments. The subtracted and then Taq amplified cDNA products with 3'-A overhangs were cloned into the eukaryotic expression vector (pcDNA3.1/V5/His-TOPO), which has a single 3' thymidine (T) overhang, and Topoisomerase I, for TA cloning (*In vitro*gen). The ligated plasmids were transformed into XL-10 gold ultracompetent cells (Stratagene). Using this combination of kits, we generated a combined subtractive cDNA library with between 10-20,000 primary recombinants. Since cDNA were cloned bidirectionally into plasmid vectors, a special sequencing primer, called SMART seq primer 5'-AAC GCA GAG TAC GCG GG (AGCT)-3', was designed to sequence unidirectionally from the 5' end of the transcripts.

Express Sequence Tag analysis of cDNA libraries

Single clones from the unamplified library were randomly picked and the cDNA inserts were amplified using vector primers T3 (AATTAACCCTCACTAAAGGG) and T7 (CGGGATATCACTCAGCATAATG). Aliquots of the amplified PCR products were checked by agarose gel and clones containing inserts greater than 200 bp were selected for sequencing. PCR products were prepared for sequencing using shrimp alkaline phosphatase and exonuclease I (Amersham Pharmacia Biotech Ltd, Sweden). Inserts were sequenced using the 5' vector primer SAC (GGGAACAAAAGCTGGAG) and ABI Big DYE terminators (Perkin Elmer Corporation, CT). Sequencing reactions were analysed using an ABI 377 automated sequencer. The sequences were edited manually and poor 3' sequence removed. The edited sequences were sent to NCBI for Blastn, blastx and blastn against dbest analysis. The search results were downloaded in html format and assembled into a web table that can be viewed using an internet browser (i.e. Internet Explorer or Netscape), which links the blast search results to other NCBI based information.

RT-PCR

For reverse transcription (RT)-PCR, first strand cDNA was made with oligo-dT primers from total RNA using GeneAmp RT-PCR kits according to manufacturer's instructions (Perkin Elmer Corporation, CT). Gene specific primers were used to PCR amplify from the first strand cDNA. The conditions used for PCR were; an initial denaturation at 94°C for 3 min, then 35 cycles of 20 sec 94°C, 30 sec 55 °C, 90 sec at 72 °C, resulting in a 1506 bp amplicon for Ym1/ECF-L and 348 bp for β-actin. Gel images were captured and analysed with GelDoc2000 (BioRad) software.

RT-PCR primers

Ym1/ECF-L-For: 5'-TGG GGG ATC CGT ACC AGC TGA TGT GCT ACT-3'

Ym1/ECF-L-Rev: 5'-GTA AAG GAT CCT CAA TAA GGG CCC TTG CA-3'

β-actin-For: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'

β-actin-Rev: 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'

PNG-1-For: 5'-GGT CCC AGT GCA TAT GGA TGA GAC CAT AGA-3'

PNG-1-Rev: 5'-CAC CTC TTC ACT CGA GGG ACA GTT GGC AGC-3'

IL-1β-For: 5'-GCA AGT GTC TGA AGC AGC TAT GG-3'

IL-1β-Rev: 5'-GGG TGT GCC GTC TTT CAT TAC AC-3'

TNF-α-For: 5'-GGA AAT AGC TCC CAG AAA AGC AAG-3'

TNF-α-Rev: 5'-TAG CAA ATC GGC TGA CGG TGT G-3'

Arginase I-For: 5'-CAG AAG AAT GGA AGA GTC AG-3'

Arginase I-Rev: 5'-CAG ATA TGC AGG GAG TCA CC-3'

Arginase II-For: 5'-TGA TTG GCA AAA GGC AGA GG-3'

Arginase II-Rev: 5'-CTA GGA GTA GGA AGG TGG TC-3'

DNA blots

Single clones from the subtractive library were randomly picked and the cDNA inserts were amplified with T7-For (5'-TAA TAC GAC TCA CTA TAG GG-3') and pcDNA3.1-Rev (5'-TAG AAG GCA CAG TCG AGG-3'). Aliquots of the amplified PCR products were checked by agarose gel and clones containing inserts greater than 500 bp were selected for differential expression analysis. Aliquots of the PCR products were immobilised onto nitrocellulose membranes using a Bio-Dot microfiltration apparatus (BioRad) according to manufacturers instructions Briefly, nitrocellulose membrane was pre-wet in 6X SSC, before being clamped in place into the microfiltration unit. The PCR products were denatured at a final concentration of 0.4M NaOH, 10mM EDTA and incubation at 100°C for 10 minutes. After neutralisation with an equal volume of 2M ammonium acetate, the samples were loaded onto the microfiltration apparatus and pulled through to the nitrocellulose membrane by a gentle vacum. The membrane was washed in 2X SSC and air dried, before being baked for 2 hours at 80°C.

Probing the DNA arrays

Total RNA was extracted from $F4/80^+$ purified macrophages from PEC of either WT implanted mice or $IL4^{-/-}$ mice. Total RNA was treated with DNAse to remove contaminating genomic DNA with DNA-free™ (Ambion) according to manufacturers instructions. 5µg of total RNA was converted into radioactivelly labelled cDNA probes in an RT reaction with oligo-dT primers with 35µCi of [α-^{32}P] dATP. The DNA blots described above were pre-hybridized with pre-warmed Rabid-hyb buffer (Amersham Pharmacia) for 15 minutes before addition of the radioactive probes. After overnight hybridixation, blots were washed 1X in 2X SSC, 1%SDS and 4X with 0.1X SSC, 0.5%SDS, or until background counts were low. hybridized blots were visualized using a phosphorimager (Molecular Dynamics) as well as with x-ray film (Kodak).

Statistical Analysis

The Students t-test or the non-parametric Mann-Whitney test (for smaller samples) was used to determine if the differences between groups of animals were statistically significant. In order to determine if the representation of clones between the two different EST datasets were significantly different, the Fishers exact test was used. All statistical analysis were performed using Prism Version 2.0 (Graphpad, San Diego)

Chapter 3

WHAT IS THE SUPPRESSOR CELL?

(Some of the experiments described in this chapter have been published in part in the *European Journal of Immunology* and were done with help from Andrew S. MacDonald and Amy O. Robb)

Summary

Previous work has shown that *Brugia malayi* induced suppressive cells in the peritoneal cavity of mice after surgical implantation. Here, we describe the identification of these suppressive cells as IL-4 dependent F4/80$^+$ macrophages. The adherent population of peritoneal exudate cells (PEC) is highly enriched for macrophages, but still contains eosinophils and lymphocytes. Purification of CD11c$^+$ cells from the PEC population demonstrated that dendritic cells were not the suppressive cells. Purification of Mac-1 (CD11b$^+$) cells from IL-4 deficient as well as IL-5 deficient mice strongly suggested that the suppressive cells were IL-4 dependent macrophages (MΦ) and not eosinophils. Purification of F4/80$^+$ cells from suppressive PEC confirmed that MΦ were the suppressive cell type. Using purified MΦ as antigen presenting cells to stimulate a Th2 clone (D10.G4) showed that these MΦ retained antigen processing and presentation capabilities and could stimulate antigen specific cytokine production while suppressing proliferation. Pro-inflammatory factors such as IFN-γ, LPS and anti-CD40 antibodies, did not reverse the suppressive phenotype of these cells.

Introduction

There is a fine balance between an effective immune response that can eliminate infectious organisms and a pathological autoimmune response that induces the process of self-destruction. A prominent feature of an activated immune response is the massive proliferation of immune cells necessary for the effective clearance of parasites. Understanding mechanisms that regulate proliferation is therefore a key issue in understanding immune-modulation. This could provide novel approaches to either boosting a more effective immune response against pathogens, or down-modulating an overblown autoimmune response.

Filarial parasites are adept at preventing rejection by the host immune response (Maizels et al., 1993; Maizels et al., 1999b), perhaps due to their ability to induce hypo-responsive T cells (Piessens et al., 1980; Yazdanbakhsh et al., 1993). It was previously found that *Brugia malayi*, when implanted into the peritoneal cavity of mice, generates host cells that inhibit proliferation of immune cells, while retaining the capacity to stimulate antigen specific cytokine production by Th2 cells (Allen et al., 1996; MacDonald et al., 1998). A key objective of my studies was to first establish the identity of the suppressor cell, in order to further characterise at a cellular, molecular or biochemical level, which molecules are involved in the mechanism of proliferative inhibition.

Previous work had provided some clues towards the identification of the suppressive cell type (MacDonald, 1998; MacDonald et al., 1998). Whereas implantation of live adult stage parasites and high doses (400) of L3 stage larvae induces recruitment of suppressive cells, dead adult parasites and microfilariae do not induce suppression (MacDonald et al., 1998). Because the suppressive cells can be induced by the daily injection of excretory secreted (ES) products from the adult parasites (Allen and MacDonald, 1998), instead of implantation, but cannot be generated by the implantation of dead parasites (MacDonald, 1998), it is clear that live adult parasites secrete factors that can generate the suppressive cells. Interestingly,

even a single adult parasite, irregardless of sex, was sufficient to drive recruitment of suppressive cells (MacDonald, 1998).

A time course experiment had showed that suppressive cells are generated 1 week after parasite implantation and remained almost indefinitely for many months (MacDonald, 1998). The emergence of suppressive cells is paralleled by the emergence of eosinophils into the peritoneal cavity (MacDonald, 1998). Significantly, the recruitment or induction of these adherent suppressive cells is dependent on host production of the Th2 cytokine IL-4, as they are not found in mice genetically deficient in IL-4 (MacDonald et al., 1998). This finding is consistent with the induction of type 2 responses by *B. malayi*, a characteristic feature of infection with nematode parasites (Maizels et al., 1993). Since infected IL-4 deficient mice also had reduced numbers of eosinophils (MacDonald, 1998), this suggested that eosinophils might be the suppressor cell. Experiments involving purified macrophages (MΦ) based on sorting $F4/80^+$ cells by flow cytometry suggested that the suppressive cells were not MΦ (MacDonald et al., 1998), further encouraging the hypothesis that eosinophils were the suppressive cell. However, when parasites were implanted into IL-5 deficient mice, that fail to recruit any eosinophils into the peritoneal cavity, the adherent peritoneal cells from implanted mice remained highly suppressive (MacDonald, 1998). The complete absence of eosinophils in parasite implanted $IL5^{-/-}$ mice unequivocally showed that eosinophils were not the suppressive cells (MacDonald, 1998). These confusing results led us to re-evaluate the identity of the suppressive cell.

In this chapter, we establish that $F4/80^+$ macrophages are in fact the suppressive population, and exclude a suppressive role for dendritic cells. The requirement for the type-2 cytokine IL-4 places these macrophages in the recently defined category of 'alternatively activated' macrophages (AAMΦ), which have been suggested to play an important role as anti-inflammatory cells (Goerdt and Orfanos, 1999).

Results

The cellular composition of adherent peritoneal cells recruited by *Brugia malayi*

Previous work had described an almost 10 fold increase in total cell numbers ($3.1 \pm 0.8 \times 10^7$) recovered from the peritoneal cavity of parasite-implanted mice, in comparison to control mice ($3.8 \pm 1.1 \times 10^6$) (MacDonald, 1998). The peritoneal exudate cells (PEC) recruited by *B. malayi* consist of a mixed population consisting mainly of macrophages ($74.3 \pm 3.5\%$), eosinophils ($11.7 \pm 0.5\%$) and lymphocytes ($8.6 \pm 4.7\%$) (MacDonald et al., 1998) (Figure 1B). In comparison to control mice, parasite-implanted mice had a marked reduction in masts cells and an increase in both macrophages and eosinophils (MacDonald et al., 1998). In this study, we first established that the adherent component of the PEC populations show a similar profile (Figure 1A). The adherent cells are slightly enriched for macrophages ($78.2 \pm 2.8\%$) and slightly depleted of eosinophils ($6.1 \pm 0.43\%$) in comparison to total cells, while lymphocyte numbers remained similar ($10.3 \pm 1.8\%$).

The suppressive cell is not a $CD11c^+$ dendritic cell

Although there are extremely few $CD11c^+$ dendritic cells in the peritoneal cavity, both in control mice as well as parasite implanted mice (Figure 2A), we decided to ensure that a small population of $CD11c^+$ dendritic cells did not mediate suppression. We used indirect magnetic sorting to purify $CD11c^+$ cells from PEC of parasite implanted mice (Figure 2A), and found that $CD11c^+$ cells were not suppressive when co-cultured with the T cell lymphoma EL4 (Figure 2C), or the B cell hybridoma HB32 for 48 hours (Figure 2B). The CD11c- fraction that had been almost entirely depleted of $CD11c^+$ dendritic cells (Figure 2A) blocked proliferation as effectively as the total PEC population (Figure 2B & C). This data provided strong evidence that the suppressive cells are not $CD11c^+$ dendritic cells.

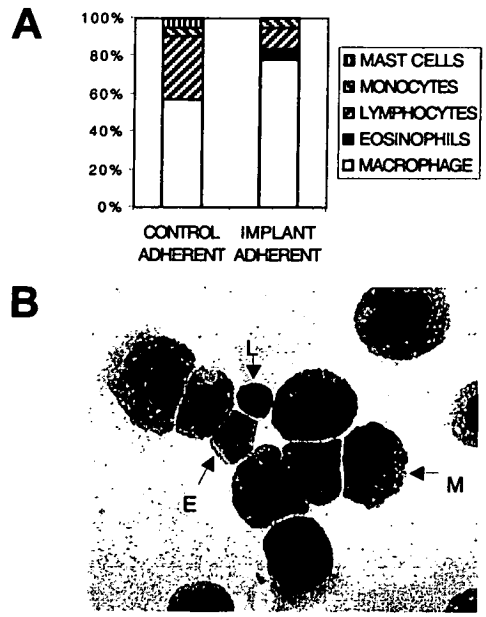


Figure 1. Adherent peritoneal cell populations in control and parasite implanted mice. Adherent cells were removed non-enzymatically from tissue culture wells with a cell scraper, after the non-adherent cells had been washed away with medium. The cell composition of adherent PEC was determined by microscopy of cyto-centrifuge preparations stained with Diff Quik (A). Data shown are mean percentage of mast cells, eosinophils, lymphocytes, macrophages and unidentified monocytes, from 3 cyto-centrifuge preparations per sample. 500 cells were counted from randomly selected fields per cyto-centrifuge preparation. (B) An example of PEC recruited by *B. malayi.* L; lymphocyte, E; eosinophil, M; macrophage.

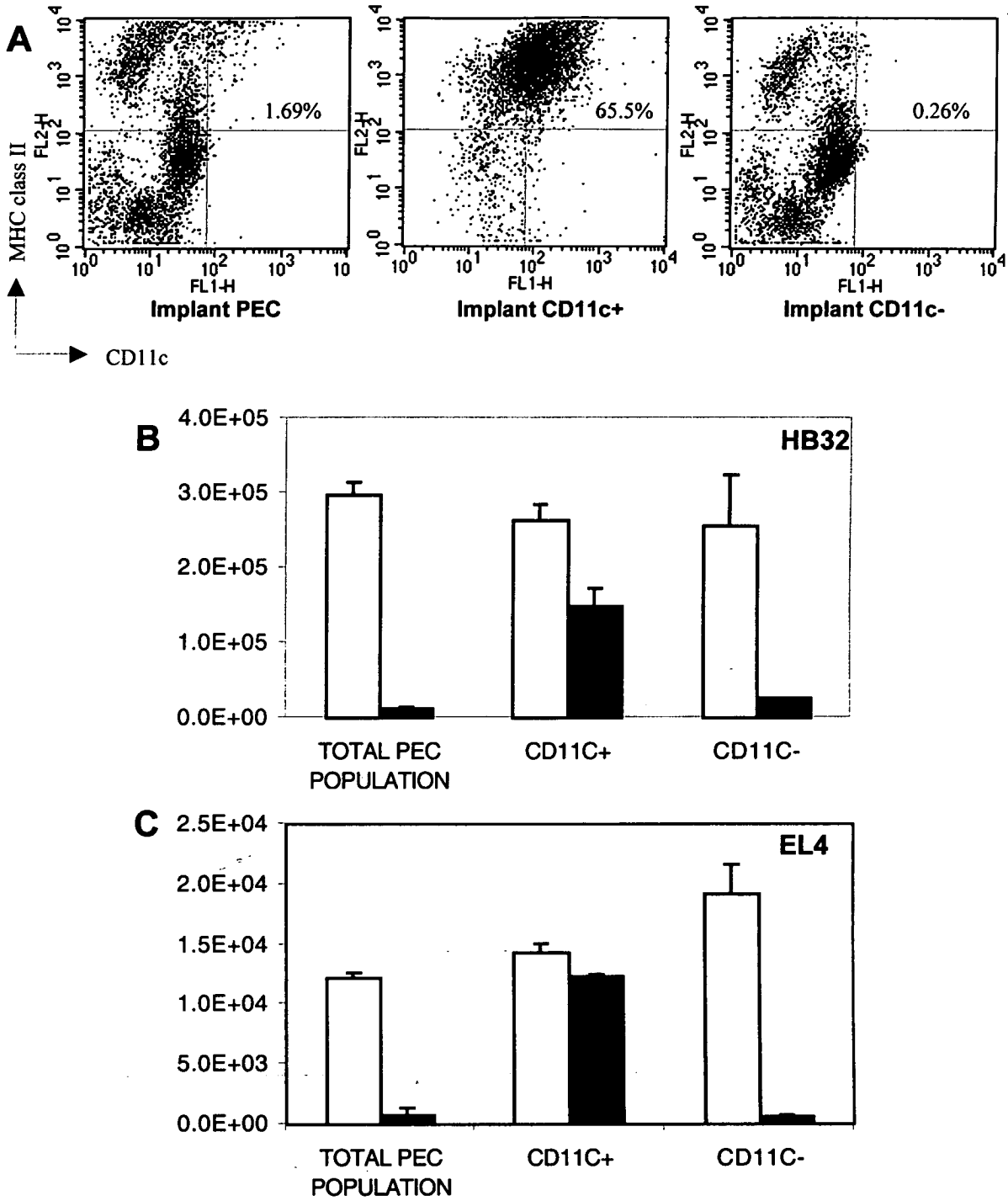


Figure 2: Suppressive cells are not CD11c+ dendritic cells. (A) CD11c+ dendritic cells were purified from PEC of parasite implanted mice (as shown) as well as control mice (not shown) using FITC conjugated α-CD11c antibodies and anti-FITC conjugated magnetic beads. Total as well as purified cells were stained with FITC conjugated α-CD11c (FL1) and PE conjugated α-IE^k (FL2) and analysed by FACS (B) $1x10^5$ cells from the CD11c+ and CD11c- fractions from control (□) or implanted mice (■) were co-cultured with $5x10^4$ HB32 and EL4 cells and after 48 hours, proliferation was measured by [^{3}H]TdR incorporation. Data shown are mean ± SD of quadruplicate wells.

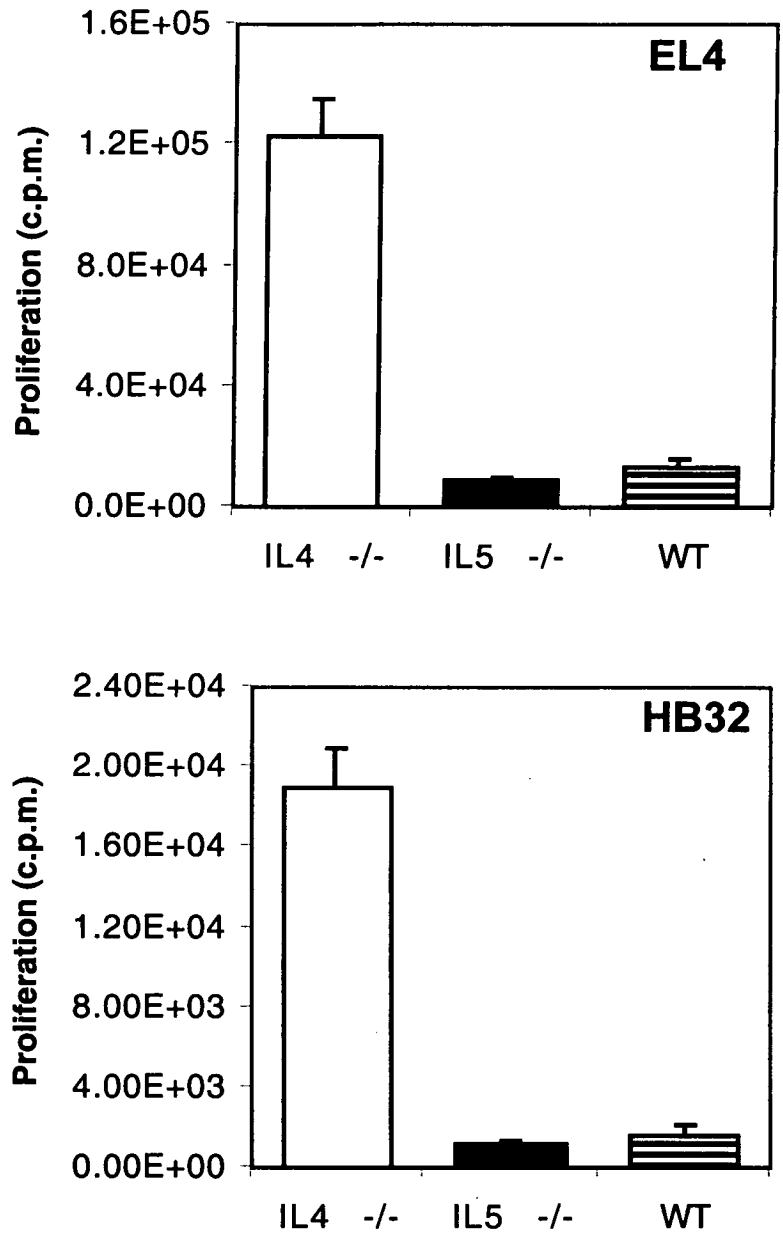


Figure 3: Mac1+ cells from IL-5 -/- mice suppress proliferation. Mac1+ cells were purified from parasite implanted IL-4 -/-, IL-5 -/- and WT mice using FITC conjugated α-Mac1 antibodies and α-FITC conjugated magnetic beads. $1x10^5$ Mac1+ cells were co-cultured with $5x10^4$ EL4 or HB32 cells and proliferation was measured with [^{3}H]TdR after 48 hours. Data shown are mean ± SD of quadruplicate wells.

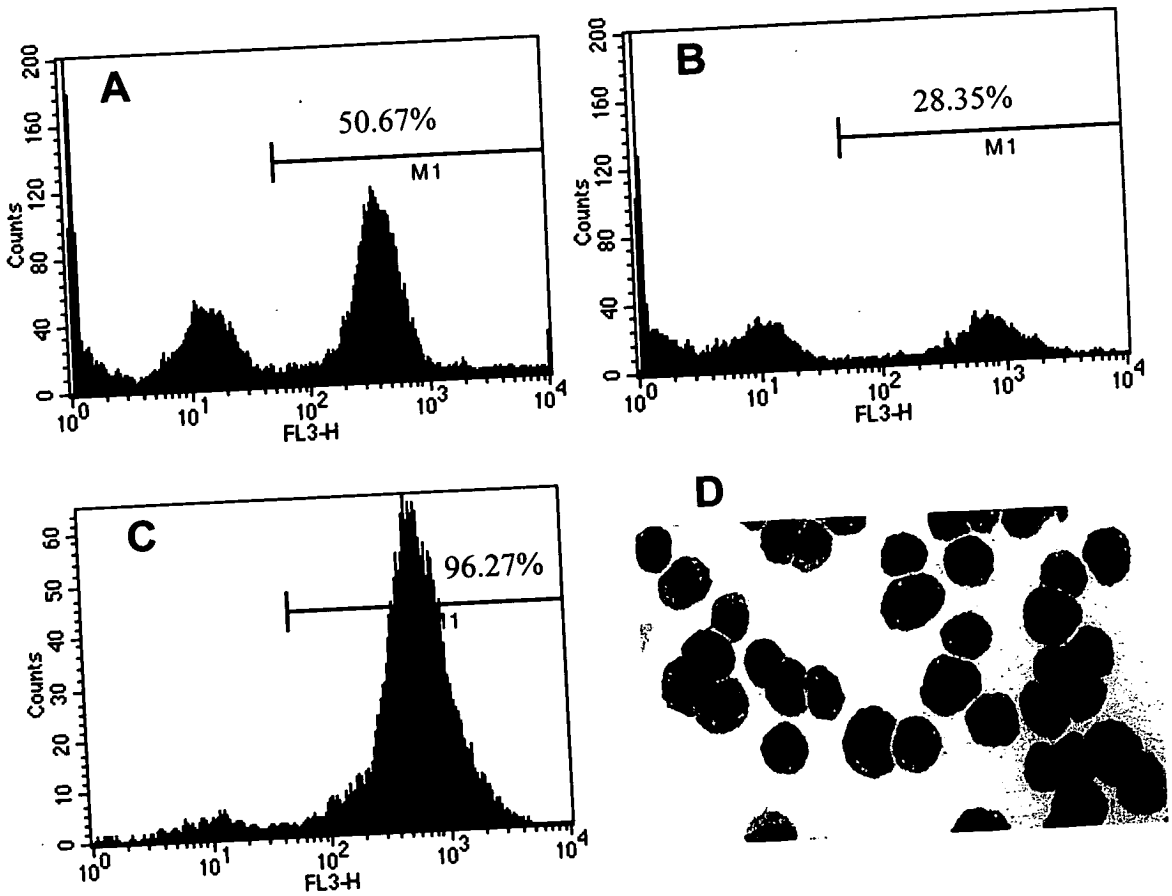


Figure 4: A typical example of F4/80 purification of macrophages from PEC of parasite implanted mice. (A) Histogram showing the number of F4/80+ cells in the PEC of parasite implanted mice before magnetic bead purification. PEC were stained with F4/80-Tricolor. (B) Histogram showing the negative fraction of PEC after magnetic bead purification with F4/80-biotin and streptavidin conjugated microbeads. It was difficult to obtain negative fractions of greater purity even after two rounds of passage through purification columns. (C) Histogram showing the F4/80 positive fraction of PEC after magnetic bead purification. There are fewer cells in this sample because only 5000 data points were collected. Purity of F4/80+ cells are usually within the range of 90-95% F4/80+. (D) Cyto-centrifuge preparations of F4/80+ purified cells stained with DiffQuik.

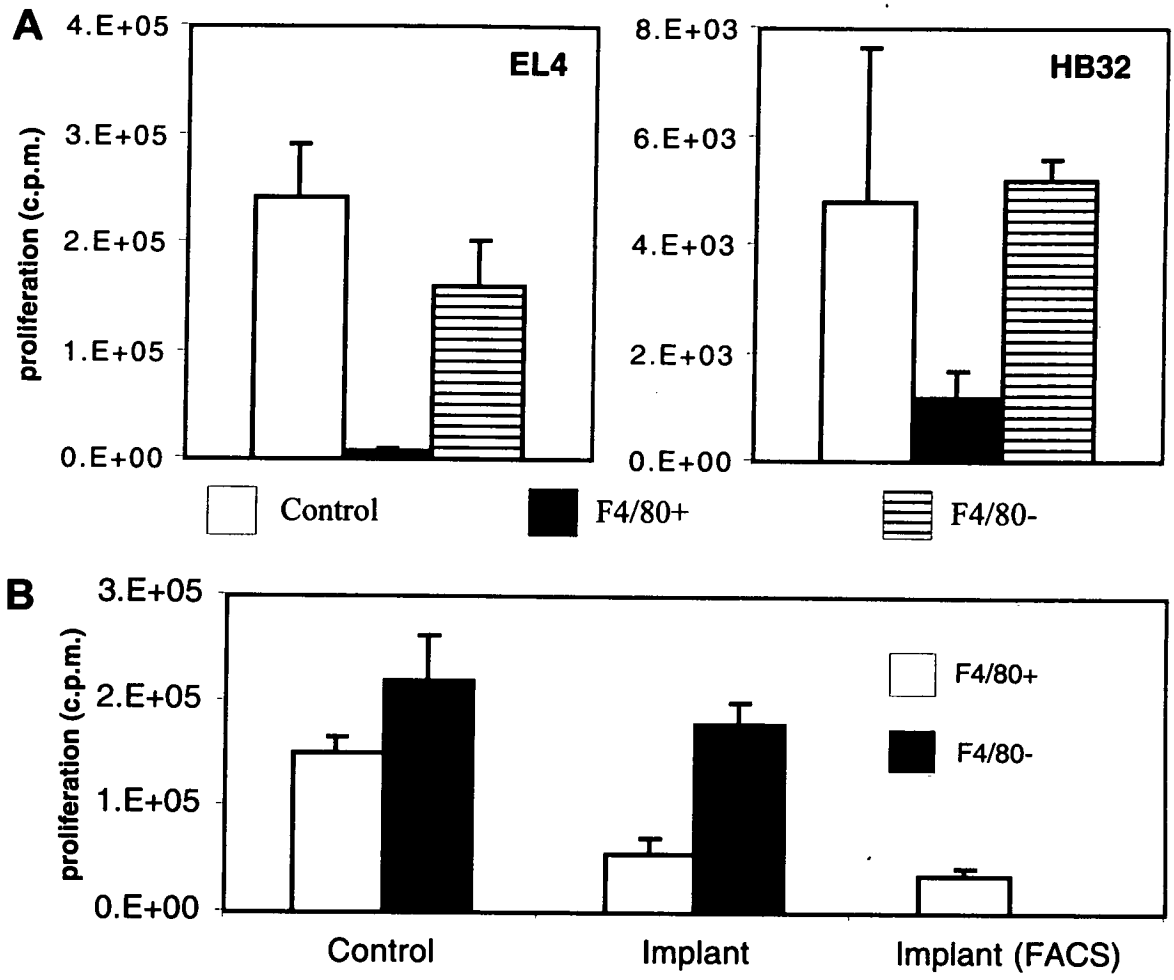


Figure 5: Suppressive cells are F4/80+ macrophages (MΦ) (A) F4/80+ MΦ were purified from PEC of parasite implanted mice using F4/80-biotin and streptavidin conjugated microbeads. $1x10^5$ cells from resident peritoneal cells of control mice, and the F4/80+ and F4/80- fractions of parasite implanted mice were co-cultured with $5x10^4$ HB32 and EL4 cells and after 48 hours, proliferation was measured by [^{3}H]TdR incorporation. (B) F4/80+ and F4/80- fractions from control mice and parasite implanted mice were separated using magnetic beads. F4/80+ cells were also purified from parasite implanted PEC using a FACS cell sorter. $1x10^5$ cells from these populations were co-cultured with $5x10^4$ HB32 and EL4 cells and after 48 hours, proliferation was measured by [^{3}H]TdR incorporation. Data shown are mean ± SD of quadruplicate wells.

We found that F4/80$^+$ macrophages purified from *B. malayi* implanted mice suppress proliferation of EL4 and HB32 after 48 hours of co-culture (Figure 6B), in comparison to F4/80- cells and also resident peritoneal cells from control mice (Figure 5A). To confirm that these results were not an artifact of magnetic bead separation, we used flow cytometry to sort for F4/80$^+$ cells, which produced identical results (Figure 5B). Additionally, co-culture with different concentrations of EL4 and HB32 cells with purified F4/80$^+$ cells gave identical results as adherent PEC (Figure 6A). This demonstrated that proliferative suppression is directly mediated by F4/80$^+$ macrophages.

Furthermore, we found that F4/80 purified macrophages from implanted mice can stimulate antigen specific cytokine production by D10.G4 cells, while inhibiting proliferation (figure 6B) repeating our original observations using unsorted adherent peritoneal exudate cells. The lack of proliferation of D10.G4 cells is not a result of defective antigen processing and presentation capabilities of the macrophages, but is instead due to an active mechanism. This confirmed that parasite-exposed macrophages alone can directly mediate proliferative suppression while presenting antigen and stimulating cytokine production.

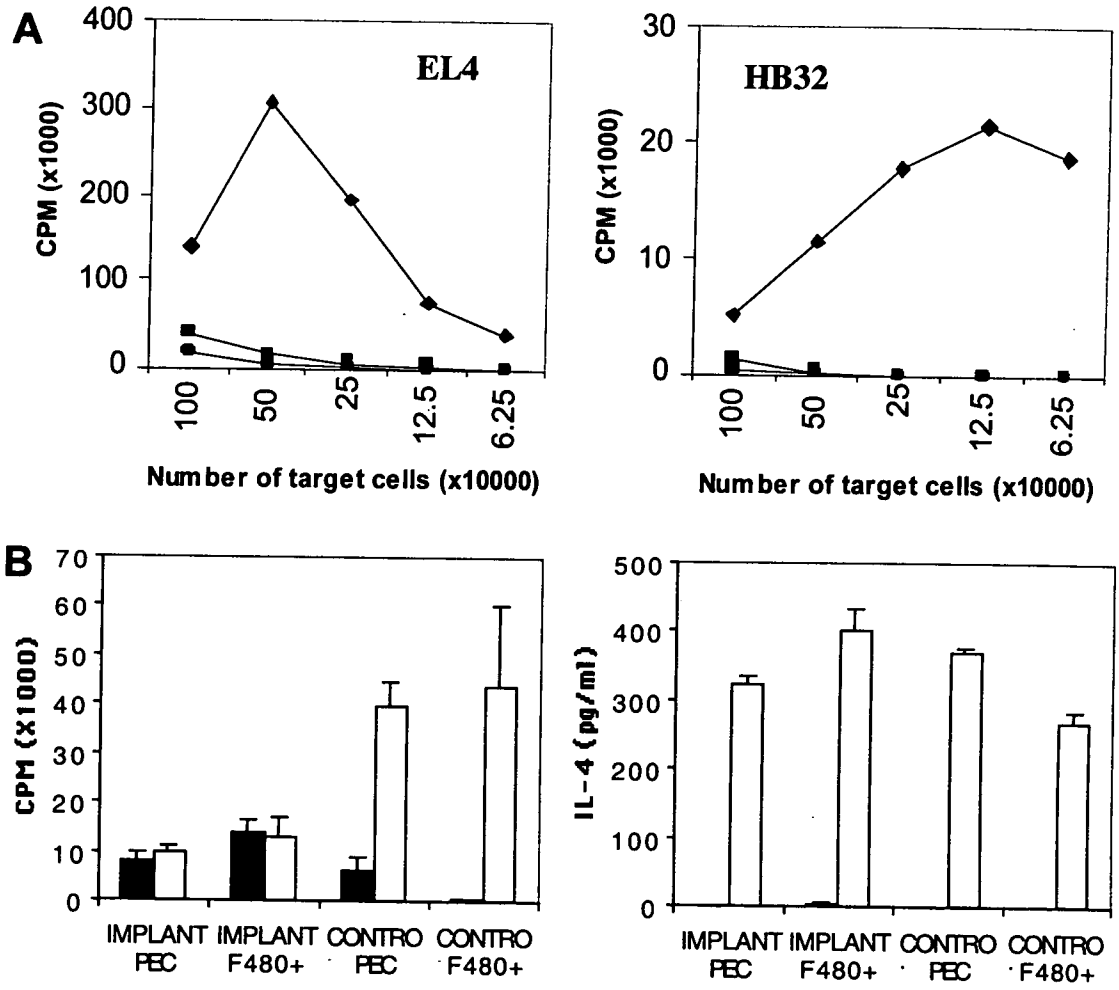


Figure 6: F4/80+ macrophages suppress proliferation but stimulate cytokine production. (A) EL4 and HB32 cells were cultured at different cell concentrations with fixed numbers ($1x10^5$ cells per well) of control PEC (♦), implant PEC ( ) and $F4/80^+$ purified cells from implant PEC (■). After 48 hours, proliferation was measured by [^{3}H] TdR incorporation. (B) D10 cells were co-cultured with control and implant PEC, as well as $F480^+$ cells purified from these PEC populations. After 48 hours of co-culture with (□) or without antigen (■), IL-4 production was assayed by NK bioassay, and proliferation was measured by [^{3}H]TdR incorporation. Data shown are mean ± SD of quadruplicate wells.

The suppressive property of F4/80$^+$ AAMΦ is not reversed by classical activation factors

Since the F4/80$^+$ macrophages are dependent on the Th2 cytokine IL-4 for their suppressive phenotype, this suggests that they belong to the recently defined category of 'alternatively activated' macrophages (AAMΦ) (Goerdt and Orfanos, 1999). We therefore asked if treatment with inflammatory factors that are known to activate macrophages, as well as mature dendritic cells, would be able to reverse the suppressive phenotype of these cells. Overnight pre-incubation of adherent PEC from parasite implanted mice with LPS, IFN-γ and anti-CD40 antibodies, did not reverse the ability of these cells to block proliferation of HB32 cells (Figure 7). Overnight treatment of resident peritoneal cells from control mice with IFN-γ resulted in the suppression of co-cultured HB32 cells (Figure 7), most probably as a result of inducing nitric oxide production (although this was not measured). This data suggests that the activation status of these suppressive anti-inflammatory macrophages cannot be simply reversed by pro-inflammatory factors.

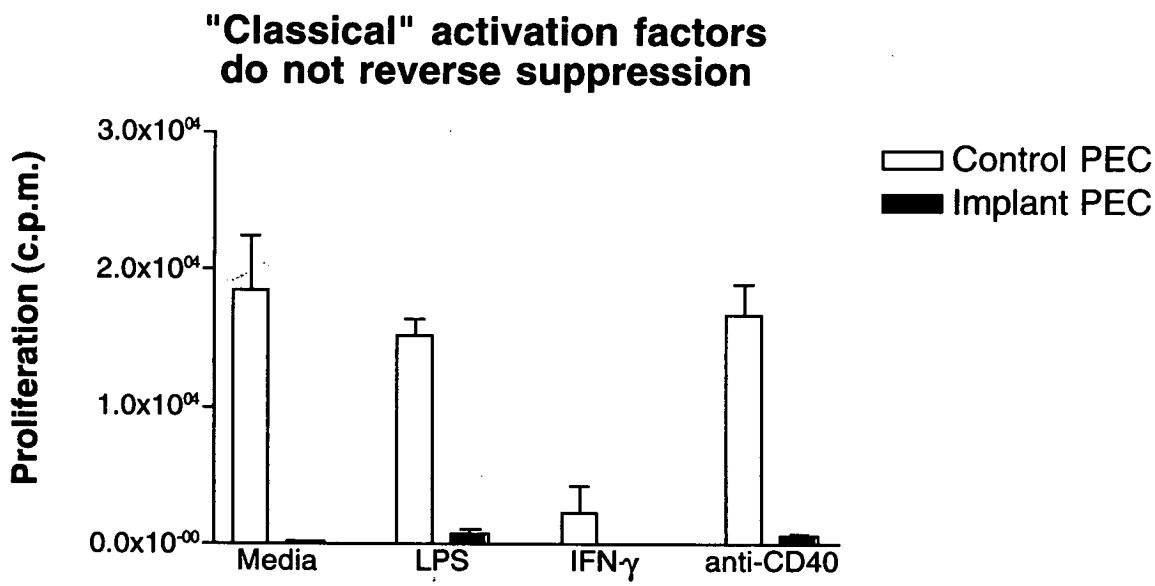


Figure 7: Classical activation factors do not reverse suppressive property of AAMΦ. Adherent PEC from control or parasite implanted mice were cultured overnight with LPS, IFN-g and anti-CD40, before being co-cultured with HB32 cells. Proliferation of HB32 cells was measured after 48 hours by [3H] Tdr incorporation. Data shown are mean ± SD of triplicate wells.

Discussion

We had previously found that filarial nematodes induce the recruitment and/or activation of a suppressor cell type in the peritoneal cavity of mice, which profoundly inhibits the proliferation of immune cells. The recruitment of these anti-proliferative cells is induced by the excretory/secretory (ES) products of the worms (Allen and MacDonald, 1998), and is dependent on IL-4 production by the host (MacDonald et al., 1998). In this study, we have identified this suppressive cell type as an IL-4 dependent $F4/80^+$ macrophage. These data suggest that filarial nematodes can induce the activation of a macrophage population that inhibits proliferation of cells in the local vicinity, while retaining their ability to process/present antigen and stimulate antigen specific cytokine production.

Protozoan parasites (*Trypanosoma brucei* and *Toxoplasma gondii*) induce IFN-γ dependent macrophages that suppress splenocyte proliferation via nitric oxide (Channon and Kasper, 1996; Mabbott et al., 1995; Schleifer and Mansfield, 1993a; Sternberg and McGuigan, 1992). Unlike these classically-activated macrophages, suppressive macrophages that do not rely on IFN-γ for activation have been described as 'alternatively activated' (reviewed recently by Goerdt and Orfanos (Goerdt and Orfanos, 1999)). Alternative activation is associated with type 2 immune response mediators such as IL-4, glucocorticoids, IL-13 and TGF-β. Thus, it is appropriate to categorize the macrophages that are recruited by the Th2 driving nematode *Brugia malayi* as 'alternatively activated'. Furthermore, by performing a small EST project from a cDNA library constructed from these nematode induced suppressive macrophages, we have found that they highly express many genes that are associated with alternative activation and are induced by Th2 cytokines, including type I hepatic arginase and the chemokine C10 (described in the EST study in chapter 7).

More interestingly, we have previously found that these macrophages do not exert their suppressive effects via well-known mediators such as PGE_2, IL-10 and TGF-β (Allen et al., 1996; MacDonald et al., 1998). Whether suppressive macrophages are recruited by the parasite during a natural filarial infection to

modulate the immune response, or they perform some role in preventing immunopathology, remains to be established. The possibility that these cells act to induce regulatory T cells in response to infection is another exciting prospect that we are currently testing. Recently, we have found that these suppressive macrophages can drive Th2 differentiation when used as APC to prime naive T cells from TCR transgenic mice (described in chapter 6). The parasite-induced macrophages prevent Th1 differentiation by the inhibition of early IFN-γ production via TGF-β. Interestingly, while TGF-β is involved in Th2 differentiation, it is not involved in the proliferative suppression. The biased induction of Th2 cells by these alternatively activated macrophages, as well as their anti-proliferative effects, is consistent with their potential role as important anti-inflammatory cells.

The identification of the suppressive cells as IL-4 dependent macrophages forms the basis of further molecular analysis of this cell type. (as described in chapters 7-9). Most studies describing the properties and biological effects of macrophages generated under Th2 conditions (AAMΦ) arise from *in vitro* studies (Doyle et al., 1994; King et al., 1998; McWilliam et al., 1992; Munder et al., 1998; Schebesch et al., 1997; Stein et al., 1992), although other *in vivo* studies have also been described (Lee et al., 1999). Our ability to purify relatively large numbers of an AAMΦ population that has been generated by a Th2 driving nematode parasite *in vivo*, puts us in a ideal situation to play a leading role in characterising the molecular basis of AAMΦ.

Chapter 4

WHAT IS THE MECHANISM OF SUPPRESSION?

(Some of the experiments described in this chapter have been published in part in the *European Journal of Immunology* and were done with help from Andrew S. MacDonald)

Summary

We have previously found that the suppressive mechanism is not mediated by the action of soluble factors such as prostaglandins, nitric oxide, IL-10 and TGF-β (Allen et al., 1996; MacDonald et al., 1998), but instead is mediated by a contact dependent mechanism suggesting a receptor mediated interaction (MacDonald, 1998). Here, we show that the proliferative block is reversible and is not a result of apoptosis. Suppressed cells accumulate in the G1 and G2/M phase of the cell cycle. Interestingly, the G1 and G2/M block correlates with increased levels of Ki-67 protein, suggesting a mechanism that effects degradation of cell cycle proteins. We also show that, in addition to lymphocyte cell lines of murine origin, these suppressive cells can inhibit proliferation of a wide range of transformed human carcinoma lines. Apart from a potential role in altering host immune responses during parasitic infection, these macrophages and may even have the potential to reduce tumour cell growth.

Introduction

Understanding mechanisms that regulate cellular proliferation is a fundamental goal of cell biology, developmental biology, oncology, as well as immunology. In immunological systems, antigen-presenting cells and T cells can both adopt an important role in regulating or suppressing the proliferation of immune cells and thereby modulate immune response expansion and development. Prominent $CD4^+$ regulatory T subsets (Groux et al., 1997; Read et al., 1998; Thornton and Shevach, 1998) have been shown to inhibit the proliferation of other T cells via cytokines such as IL-10 and TGF-β. Most recently, a direct suppressive mechanism dependent on cell to cell contact between regulatory and responder T cells has been proposed (Read et al., 1998; Thornton and Shevach, 1998; Thornton and Shevach, 2000).

Among APC, the most well characterised suppressive (or regulatory) cells are macrophages (MΦ) 'classically activated' by Th1 cytokines (such as IFN-γ). By the release of nitric oxide (Abrahamsohn and Coffman, 1995; Channon and Kasper, 1996; Sternberg and Mabbott, 1996b) these cells suppress cellular proliferation by multiple mechanisms including the inhibition of DNA synthesis and its rate-limiting enzyme, ribonucleotide reductase (MacMicking et al., 1997). In addition to activation by pro-inflammatory cytokines, MΦ can be alternatively activated by type 2 cytokines such as IL-4 and IL-13 (Doyle et al., 1994; Stein et al., 1992). These cells have been less well-characterized than classically activated MΦ but appear to have anti-inflammatory roles and suppress T cell proliferation via cytokines such as TGF-β and IL-10 (Goerdt and Orfanos, 1999).

We have previously found that the suppressive peritoneal cells are induced by the implantation of live adult *Brugia malayi* (Allen et al., 1996; MacDonald et al., 1998). As described in the previous chapter, we have now identified these suppressive cells as IL-4 dependent 'alternatively activated' MΦ. Although alternatively activated MΦ have been suggested to suppress proliferation via TGF-β and IL-10 (Goerdt and

reverse the proliferative inhibition exerted by *B. malayi* recruited cells (Allen et al., 1996; MacDonald et al., 1998). Inhibitors of other mediators known to inhibit proliferation, such as NO, H_2O_2, prostaglandins and IFN-γ did not reverse the proliferative block (Allen et al., 1996). More recently, we found that instead of soluble cytokines, proliferative suppression occurred via cell-cell contact, suggesting a receptor mediated mechanism (Figure 1). This conclusion was made since the anti-proliferative activity of parasite-recruited PEC could be reversed by transwell separation, while fixed PEC from implanted mice fully retained their ability to block proliferation (MacDonald, 1998) (Figure 1).

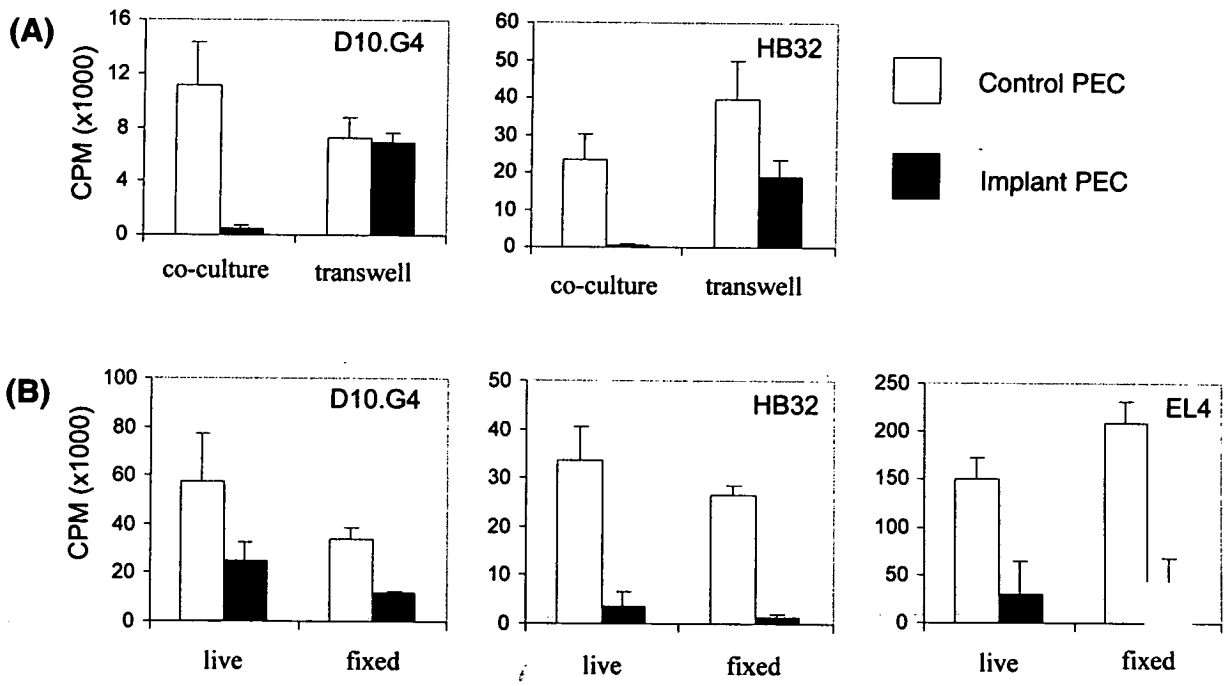


Figure 1 (courtesy of A.S. MacDonald): Proliferative inhibition is mediated by cell to cell contact. (A) D10.G4 and HB32 cells were either directly co-cultured with PEC or placed in separate transwell chambers separated by a 0.4 μm membrane. To obtain sufficient cell numbers, PEC from separate animals were combined, and data are shown as mean ± SD for six wells. Data shown are representative of three experiments. Although proliferation of separated HB32 cells was slightly reduced in some experiments (as shown), there were no significant differences in other experiments. (B) Control or implanted PEC were fixed with 1% paraformaldehyde before being co-cultured with D10.G4, EL4 and HB32 cells. Proliferation was measured by [^{3}H]TdR incorporation.

In this chapter, I describe some other unusual features regarding the suppressive mechanism. The data presented here, in combination with previous observations, suggest that alternatively activated macrophages induced by a Th2 driving infection can regulate proliferation of a broad range of cell types via a novel receptor-mediated mechanism.

Results

Cell cycle arrest does not lead to apoptosis.

The proliferative block of affected responder cells could be a result of apoptosis induced by suppressive peritoneal exudate cells (PEC) induced by *B. malayi*. We therefore assessed apoptosis in suppressed D10.G4 cells and EL4 cells by TUNEL staining. Apoptosis of PEC alone was also assessed as a control for background levels of apoptosis. These experiments revealed that some of the parasite-recruited PEC undergo apoptosis after 48 hours in culture (Figure 2A). However, apoptosis of D10.G4 cells and EL4 cells was not significantly increased by suppression (Figure 2B and 2C) when compared to the background levels of parasite recruited PEC apoptosis (Figure 2A). Apoptosis is therefore not the explanation for the reduction of proliferation.

Cell cycle arrest is reversible

Since the non-proliferating responder cells were not undergoing apoptosis when exposed to suppressive PEC, we then asked if they could recover their ability to proliferate after being removed from the suppressive cells. Experiments with D10.G4 and EL4 cells, which were removed and purified from co-culture with suppressive PEC after 48 hours, demonstrated that suppressed responder cells could fully recover their ability to proliferate (Figure 3). Indeed, thymidine incorporation of recovered cells was actually elevated (Figure 3B), perhaps because their cell cycles had been synchronized after release from the cell cycle block.

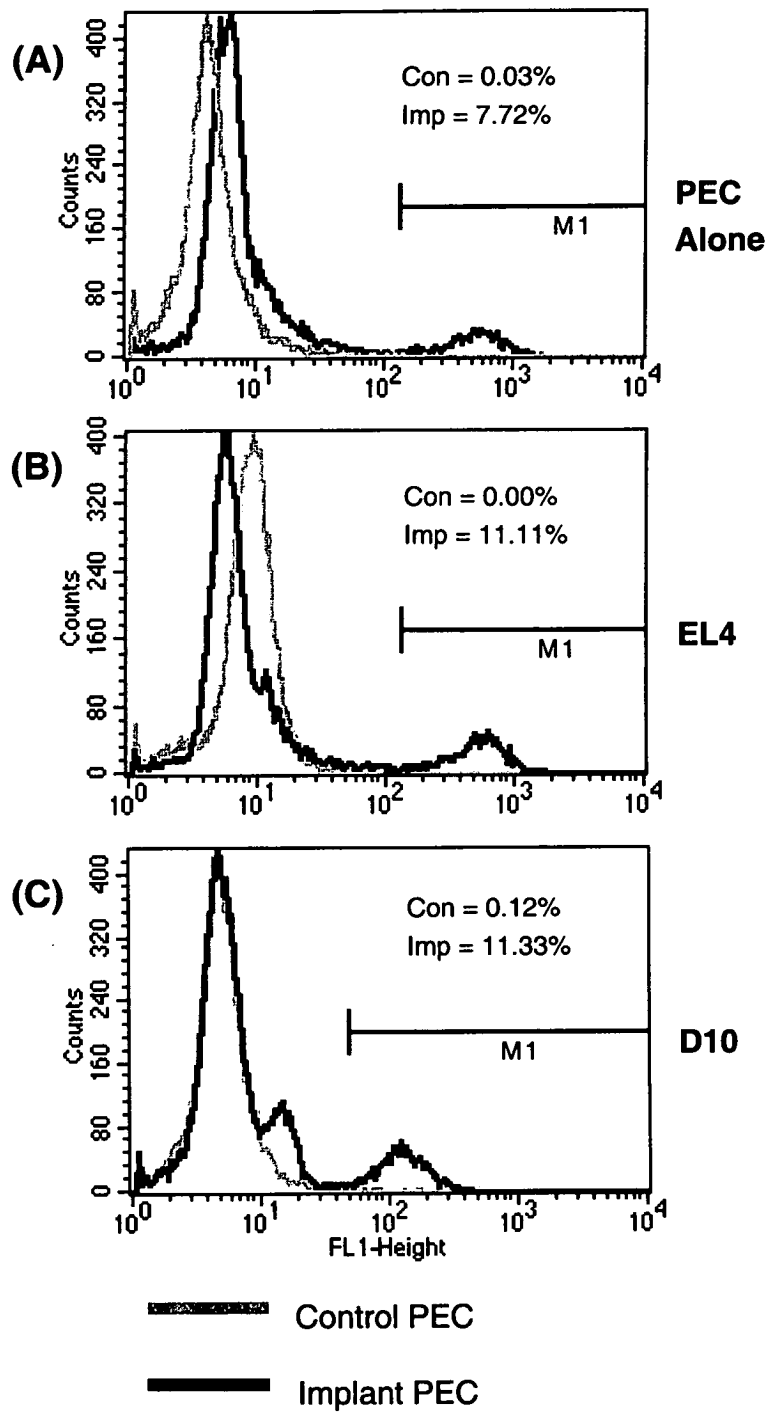


Figure 2. Proliferative inhibition does not lead to apoptosis. Cells were TUNEL stained for apoptotic cells 48 hours after stimulation/co-culture with control PEC (black line) or implant PEC (gray line). M1 represents apoptotic cells (A) Implant PEC alone undergo some apoptosis, whereas control PEC do not. (B) EL4 and (C) D10.G4 cells had slightly elevated levels of apoptosis in comparison to implant PEC alone. Data shown are representative of two experiments.

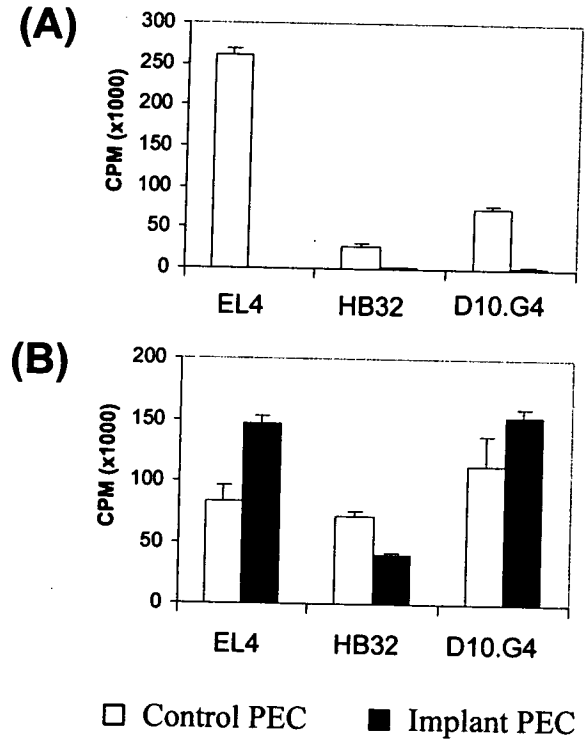


Figure 3. Proliferative suppression is reversible. (A) Proliferation of EL4, HB32 and D10.G4 (with 50μg/ml conalbumin) cells was measured by [^{3}H]TdR incorporation after 48 hours co-culture with control and implant PEC. (B) After 48 hour exposure to PEC, non-adherent HB32, EL4 and D10 cells were purified through nylon wool columns. Recounted cells were cultured for 48 hours and proliferation measured. D10 cells were restimulated with fresh APC and antigen. PEC of individual animals were combined for this experiment and results shown are mean ± SD of quadruplicate wells.

Proliferation of various transformed cell lines are inhibited

We had previously observed that proliferative suppression is very non-specific, acting not only on mitogen activated T cells, but also affecting transformed lymphocyte lines. We wanted to address how wide-ranging this anti-proliferative effect was by exposing a broad panel of human cancer cells to the suppressive PEC. Strikingly, the proliferation of many human cancer cell lines was considerably reduced when co-cultured with suppressive PEC, in comparison to control PEC (Table 1). This indicated that the suppressive mechanism was neither species specific nor cell type specific. However, the sensitivity of the different types of carcinoma to suppression by PEC did vary quite considerably. Colon carcinomas appeared to be more sensitive to suppression than pancreatic carcinomas, while the neuroblastoma line NGP was completely unaffected (Table 1).

Effect of suppressive PEC on the cell cycle.

In previous experiments, proliferation of cells co-cultured with adherent PEC was measured by [^{3}H] thymidine incorporation after 48 hours (Allen et al., 1996; MacDonald et al., 1998). In order to obtain a more complete picture, we performed experiments to assess the effect of suppressive PEC on the cell cycle of the responder cells.

Firstly, in order to determine whether the proliferative block occurred immediately after exposure to suppressive PEC, EL4 cells were labelled with proliferation marker dyes PKH26 or CFSE before co-culture with PEC. These dyes lose intensity as cells divide and provided the means to track cell divisions that occured after exposure to suppressive PEC. In these experiments, EL4 cells cultured with suppressive PEC completed one round of cell division after 24 hours, but failed to undergo a second round of cell division after 48 hours (Figure 4B). This was also observed when CFSE labelled D10.G4 cells were stimulated with either control or parasite implanted PEC. Whereas control PEC stimulated D10.G4 cells had undergone up to 3 rounds of cell division after 48 hours, suppressed D10.G4 cells had only completed one round of cell division (Figure 4C).

Cell line	Control PEC (c.p.m.)	Implant PEC (c.p.m.)	Suppression (%)	Cancer type
H358	115888	15111	87	Non-small-cell lung adenocarcinoma
HCT116	140116	19996	85.7	Colon carcinoma
HT29	55420	12832	76.8	Colon carcinoma
LoVo	180491	54441	69.8	Colon carcinoma
Colo320	251051	86639	65.4	Colon carcinoma
H2122	148989	55024	63	Non-small cell lung carcinoma
Capan-1	50261	25675	48.9	Pancreatic carcinoma
COR L24	2522	1312	48	Small cell lung carcinoma
H524	69167	42403	38.7	Small cell lung carcinoma
Panc-1	86934	54109	37.7	Pancreatic carcinoma
MiaPaCa-2	172272	135719	21.2	Pancreatic carcinoma
NGP	112649	119571	-6.1	Neuroblastoma

Table1: Effect of suppressive PEC on human tumor cell lines.
$1x10^4$ cancer cells were cultured with adherent PEC ($1x10^5$) from control or implanted animals in 96 well flat bottom plates. Proliferation by [^{3}H] thymidine incorporation was measured at 48h and results are shown as c.p.m. Numbers shown are the mean of quadruplicate wells and representative of at least 2 experiments with each cell line.

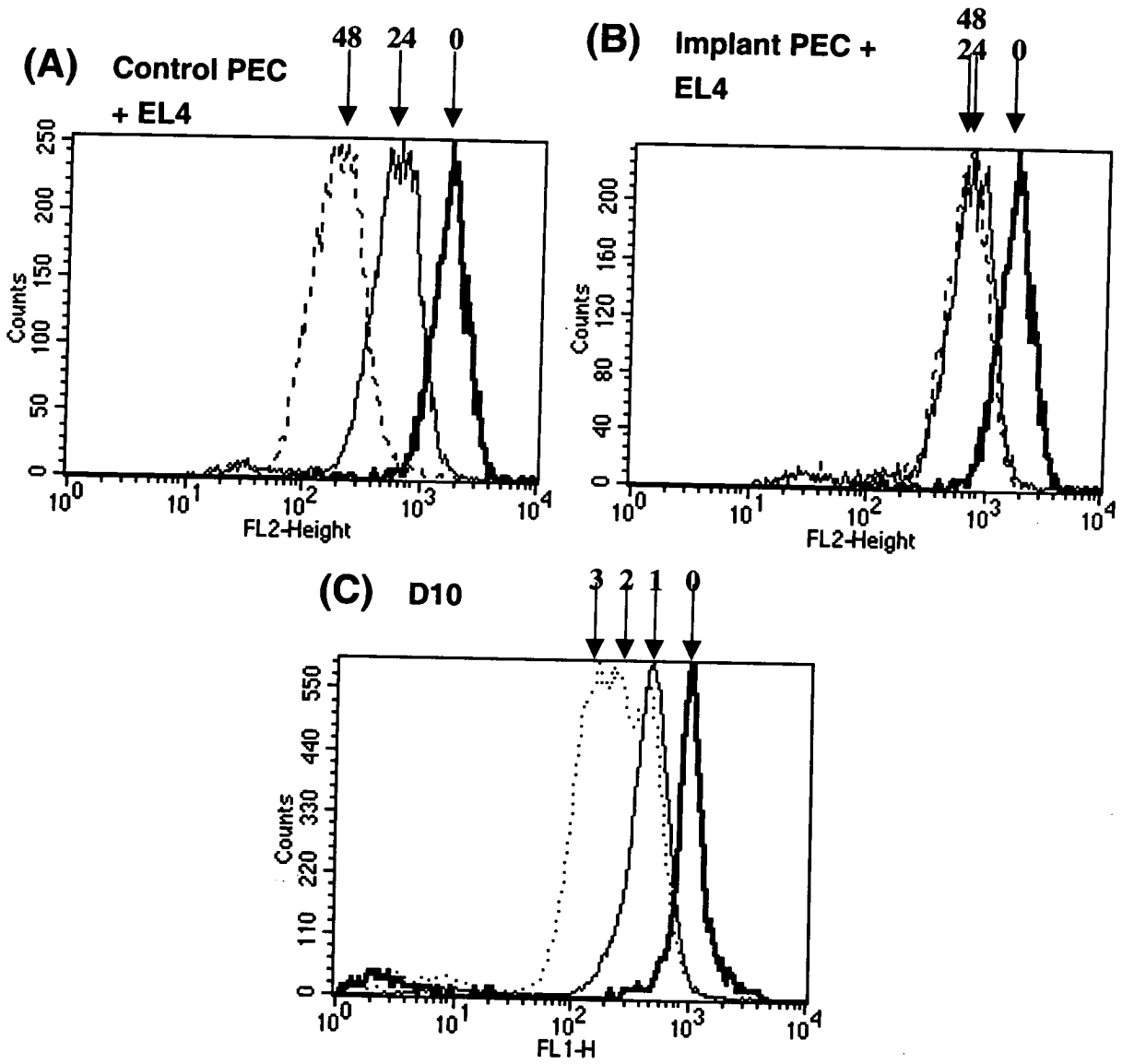


Figure 4. Proliferation is blocked after one cell division. EL4 cells stained with PKH26 were co-cultured with control PEC (A) or implant PEC (B) for 24 and 48 hours before harvest and analysis by FACS. With each cell division fluorescence intensity halves. The arrow marked 0 indicates the original intensity of cells that were fixed immediately after staining. The arrows marked 24 and 48 indicate fluorescent intensity of cells harvested after 24 or 48 hours culture with PEC. This experiment was repeated with CFSE staining with identical results. (C) D10.G4 cells stained with CFSE were stimulated (50μg/ml conalbumin) with implant PEC (thin line) or control PEC (broken line) for 3 days. The marked arrows indicate the number of cell divisions and the thick line represents the original intensity of the D10 cells.

Thymidine incorporation is associated with cells cycling through S phase of the cell cycle. To obtain additional information on the cell cycle, D10.G4 cells and EL4 cells were stained with propidium iodide after co-culture with PEC for 48 hours. Whereas the Th2 clone D10.G4 proliferates only in response to antigen (conalbumin) specific stimulation, the T cell lymphoma EL4 proliferates continuously and does not require antigen stimulation. The effect of co-culture with suppressive PEC on both cell lines (in comparison with control PEC) was to increase the percentage of cells in G0/G1 and G2/M, and to reduce the number of cells in S phase (Figure 5). The decrease in the percentage of S phase cells is consistent with the inhibition of thymidine incorporation in standard proliferation assays. The accumulation of suppressed cells at both G0/G1 as well G2/M phases suggested that there were two blocks on the cell cycle.

To further distinguish between cells in G0 and G1, cells were double stained with antibody against Ki-67 as well as propidium iodide. Ki-67 is expressed only in proliferating cells and not in quiescent G0 phase cells. Surprisingly, suppressed D10.G4 cells and EL4 cells had an increased level of Ki-67 protein in comparison to proliferating cells cultured with control PEC (Figure 5). The accumulation of Ki-67 was evident in G1 cells (2N) as well as G2/M cells (4N). Proliferative inhibition is therefore not a result of entering a quiescent G0 phase, but might instead be associated with accumulation of cell cycle proteins.

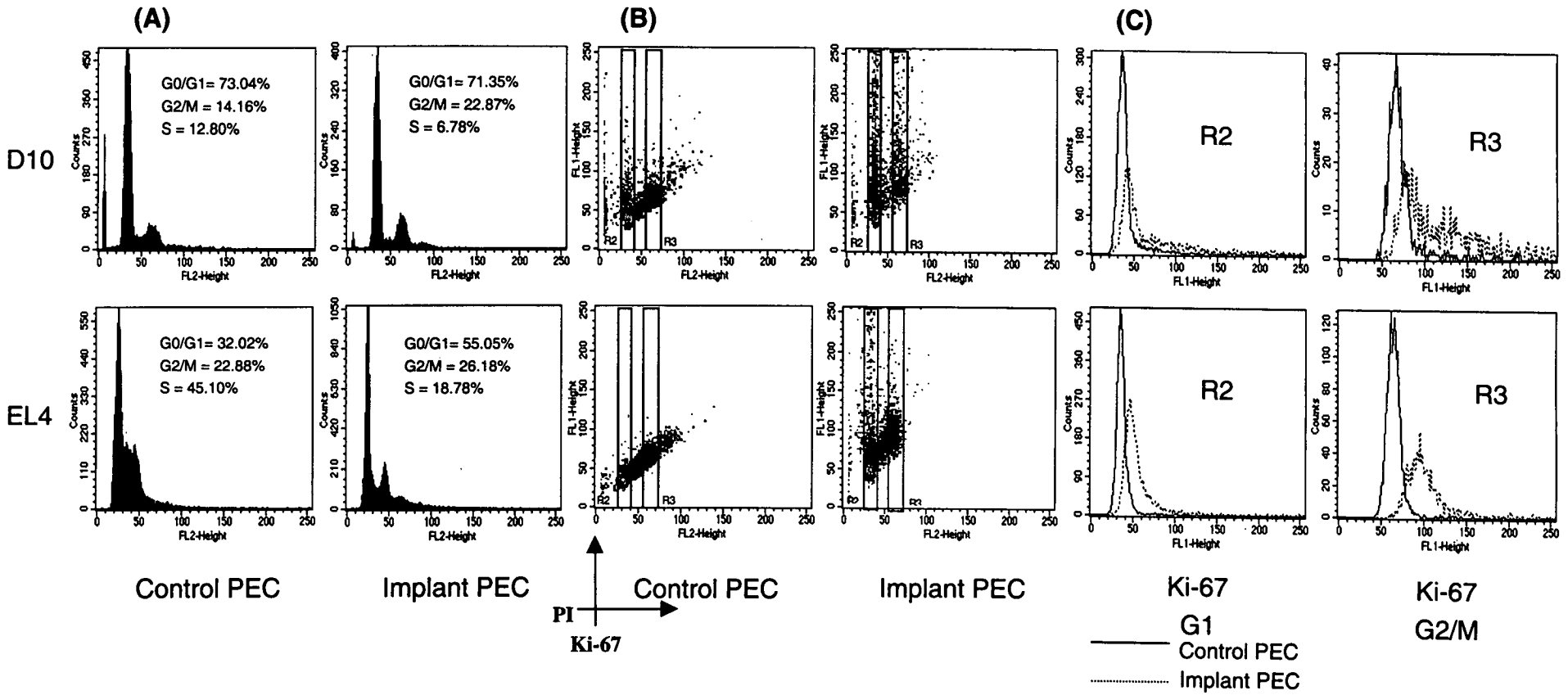


Figure 6. Cell cycle analysis of PEC exposed cells. (A) Histograms of D10.G4 and EL4 cells stained with propidium ioidide (PI) 48 hours after co-culture with either control or implant PEC. In both cell lines, implant PEC exposed cells have a decreased percentage of cells in S phase, and an accumulation of cells in G1 and G2/M. Results shown are representative of 3 independent experiments. (B) FACS analysis of cells double stained with Ki-67 as well as PI after 48 hours co-culture with PEC. Ki-67 expression is shown on the Y-axis (FL-1) and PI staining is shown on X-axis (FL-2). The R2 gate represents 2N cells in G1. The R3 gate represents 4N cells in G2/M. (C) Histogram analysis of Ki-67 expression in G1 (R2) and G2/M (R3) cells, using gates shown in B. Implant PEC exposed cells (dotted lines) have increased expression of Ki-67 in both these phases of the cell cycle, compared to control (unbroken line).

Discussion

B. malayi induced macrophages showed several novel properties not previously described for anti-inflammatory macrophages. Instead of soluble cytokines, proliferative suppression occurred via cell-cell contact, suggesting a receptor mediated mechanism. Furthermore, suppressed cells were not simply blocked at the G1/G0 stage of the cell cycle but also at the G2/M stage. Thus, the suppressive mechanism has a profound but reversible effects on the G1 and G2/M phase of the cell cycle. Finally, the proliferation of a wide range of transformed human cell lines was also inhibited by these macrophages, showing that the suppressive effect could transcend the species barrier.

The identity of the cell surface molecule(s) involved in inhibiting proliferation remains mysterious and efforts are under way to determine it's identity (described further in chapter 7). The observation that the suppressive macrophages affect such a wide range of human tumour cells, as well transformed mouse cells, strongly suggests the engagement of a highly conserved receptor. Based on our findings, it is tempting to suggest that parasitic nematode infection could have a beneficial effect against tumour expansion as a by-product of recruiting macrophages that suppress proliferation. However, the effect of these cells is probably extremely limited because of the local nature of receptor mediated mechanisms. Furthermore, the reversibility of the proliferative block would allow any dividing cells that migrate away from the vicinity of the suppressor cells, to continue proliferating. Indeed, *in vivo* experiments described in chapter 6 indicate that EL4 cells that have been injected into the peritoneal cavity of *B.malayi* implanted mice can eventually evade suppression. Nonetheless, it would be interesting to investigate if lymphomas are less prevalent in regions endemic for filariasis.

It is unusual for growth regulatory mechanisms to induce a cell cycle block in G2/M as well as G1. The cell cycle machinery usually responds to external stimuli (such as mitogenic or anti-mitogenic factors) only during a defined window of time, from the beginning of G1 to the restriction point (R) (Pardee, 1989; Planas-Silva and Weinberg, 1997; Zetterberg et al., 1995). After *R*, cells are relatively refractory to most

external stimuli, the remaining phases of the cell cycle being executed in a quasi-automatic fashion (Planas-Silva and Weinberg, 1997). Thus, the majority of anti-proliferative signals would lead to a G1 arrest of the cell cycle, or entry into the quiescent G0 state. A G2/M arrest usually occurs only as a result of interference to DNA replication (Elledge, 1996; Nurse, 1997). The effect on two phases of the cell cycle raises the possibility that more than one mechanism could be acting together to inhibit proliferation. Alternatively, the suppressive macrophages may be slowing down the cell cycle by delaying the passage through the checkpoint controls at G1 and G2/M, while having no effect on progression through S phase.

It was interesting that the accumulation of cells at G1 and G2/M was associated with an increased level of Ki67 protein. Although the monoclonal antibody (mAb) Ki-67 has been used to identify proliferating cells since 1983 (Gerdes et al., 1983), the function of the protein recognized by this mAb is still uncertain. Anti-sense oligonucleotide treatment or microinjection of antibodies into the nucleus will delay cell cycle progression, suggesting that Ki-67 antigen is essential for cell cycle progression (Schluter et al., 1993; Starborg et al., 1996). However, there are no reports that increased expression also leads to cell cycle arrest. Interestingly, one of the few factors which can lead to a G2/M as well as a G1 block is inhibition of the Proteasome (Machiels et al., 1997), which leads to an accumulation of ubiquitinated proteins, including the various cyclins necessary for cell cycle progression (King et al., 1996; Machiels et al., 1997). Perhaps the anti-proliferative macrophages engage receptors that lead to changes in protein degradation of the suppressed cells, leading to accumulation of cell cycle proteins such as Ki-67. We need to establish whether Ki-67 accumulation in G1 and G2/M cells is directly involved in the mechanism of suppression or merely an indirect consequence of cell cycle arrest. We are also investigating the effect of suppression on the levels of cyclins in the non-proliferating cells.

In conclusion, we report the observation of a contact dependent mechanism of inhibiting proliferation that has profound effects on the G1 and G2/M phases of the

cell cycle. This novel mechanism of suppression by filarial-induced macrophages (which are dependent on host IL-4) can also significantly reduce the proliferation of a wide range of human tumour cell. Thus, the identification of the molecular players involved will be of interest to tumour biologists as well immunologists and parasitologists.

Chapter 5

WHAT IS THE EFFECT OF AAMΦ ON NAÏVE T CELLS?

(The experiments described in this chapter have been published in the *European Journal of Immunology*)

Summary

A key feature of nematode infection is a bias towards a type 2 immune response. To investigate the role that antigen presenting cells (APC) may play in promoting this bias, we used adherent peritoneal exudate cells (PEC) recruited in response to the filarial nematode *Brugia malayi*, to stimulate naïve T cells from pigeon cytochrome c (PCC) specific TCR transgenic (PCC-tg) mice. Although the proliferation of PCC-tg T cells was inhibited by parasite-induced PEC during primary stimulation, they proliferated normally upon secondary stimulation and were not rendered anergic. However, PCC-tg T cells primed by suppressive APC differentiated into IL-4 producing Th2 cells upon secondary stimulation instead of IFN-γ producing Th1 cells, as has been previously described. Studies with CFSE labeled cells indicated that Th2 differentiation was associated with the inhibition of (or failure to stimulate) IFN-γ production during primary stimulation. Interestingly, blocking antibodies against TGF-β (but not IL-10) restored the differentiation of IFN-γ producing Th1 cells. Identical results with CFSE labeled cells were obtained using purified F4/80$^+$ macrophages. These data indicate that T cells exposed to parasite-induced alternatively-activated macrophages are driven towards Th2 differentiation. This may be an important factor in the Th2 bias that accompanies nematode infection.

Introduction

Understanding why helminth infection induces type 2 cytokines has been a major goal for parasite immunologists. Since the cytokine environment is considered the most important factor influencing the differentiation of naïve T cells (Abbas et al., 1996; O'garra, 1998), the source of early IL-4 production during helminth infections has been of particular interest (Osborne and Devaney, 1998; Sabin et al., 1996). However, many other factors can influence the differentiation of naïve T cells, including altered peptide ligands, antigen dose and costimulatory molecules (reviewed by Constant and Bottomly (Constant and Bottomly, 1997)). Most recently, it has been proposed that the type of APC and its previous environmental exposure could also play an important role in influencing T cell differentiation (Goerdt and Orfanos, 1999; Rissoan et al., 1999). In this chapter, we show that macrophages recruited by *Brugia malayi* can induce Th2 differentiation in the absence of detectable levels of IL-4, via a mechanism that involves TGF-β.

Apart from a type 2 cytokine profile, another key feature of filariasis is the presence of hyporesponsive, or anergic, T cells in the infected host (Piessens et al., 1980; Yazdanbakhsh et al., 1993). In the previous chapters, we have described the recruitment of suppressive macrophages by *B. malayi*, which can process and present antigen and stimulate antigen-specific IL-4 production by the Th2 clone D10.G4 while actively inhibiting proliferation via a contact dependent mechanism. Based on the model of anergy induction proposed by Schwartz and Jenkins, linking cell division with clonal anergy (Beverly et al., 1992; Jenkins, 1992), we hypothesized that stimulation of naïve T cells by these anti-proliferative APC could lead to the development of anergic, or hypo-responsive cells. Additionally, to explore the possibility that these suppressive APC affect T cell differentiation, we investigated their impact on the differentiation of a clonal population of naïve $CD4^+$ lymphocytes from pigeon cytochrome C (PCC)-specific TCR transgenic (PCC-tg) mice.

Results

Suppressive APC inhibit proliferation of naïve $CD4^+$ T cells but do not induce anergy

To investigate whether stimulation of naïve T cells with anti-proliferative APC could induce anergy, $CD4^+$ T cells were purified from mice expressing a transgenic TCR specific for the PCC peptide (88-104) with $I\text{-}E^k$. These naïve $CD4^+$ cells were stimulated with either adherent PEC taken from control mice or from mice implanted with *B. malayi*. As expected $CD4^+$ T cells proliferated strongly in response to PCC protein (10 μg/ml) when presented on control PEC, but proliferated poorly in response to stimulation by implanted PEC (Figure 1A). IL–2 production was also severely abrogated on primary stimulation (Figure 1B) with suppressive PEC. In a parallel experiment, suppressed $CD4^+$ T cells were removed after 48 hours and rested for 72 hours before repurification and stimulation. These cells proliferated normally in response to secondary stimulation with PCC and irradiated splenocytes (Figure 1A). IL–2 production was also fully restored (Figure 1B).

Suppressive APC induce Th2 differentiation of naïve $CD4^+$ cells

It has been reported in several systems (Seder et al., 1992; Stockinger et al., 1996; Takeuchi et al., 1998) that upon secondary stimulation, $CD4^+$ T cells derived from TCR transgenic mice will develop into Th1 cells and produce high levels of IFN-γ and little IL-4, if primed in the absence of any exogenous cytokine. The addition of IL-4 during primary stimulation stimulates Th2 differentiation leading to the production of high levels of IL-4 and little IFN-γ (Seder et al., 1992). We observed an identical pattern of T cell differentiation, when irradiated splenocytes were used as APC to stimulate the PCC-tg T cells (Figure 2A). However, PCC-tg T cells primed by parasite-derived APC (in the absence of exogenous cytokines) also produced high levels of IL-4 upon secondary stimulation. This was in contrast to $CD4^+$ T cells primed with control PEC, which as expected produced high levels of IFN-γ (Figure 2B). The mechanism by which Th2 differentiation is induced in this system did not appear to be mediated via IL-4 secretion, since IL-4 produced by PEC was generally undetectable

(<5 pg/ml) in both control and implanted mice. Indeed, when very low levels of IL-4 were detected, control PEC produced equal if not greater levels of IL-4 than implant PEC (e.g. control PEC = 27.8 ± 11.7 pg/ml, implant PEC = 16.1 ± 10.4 pg/ml).

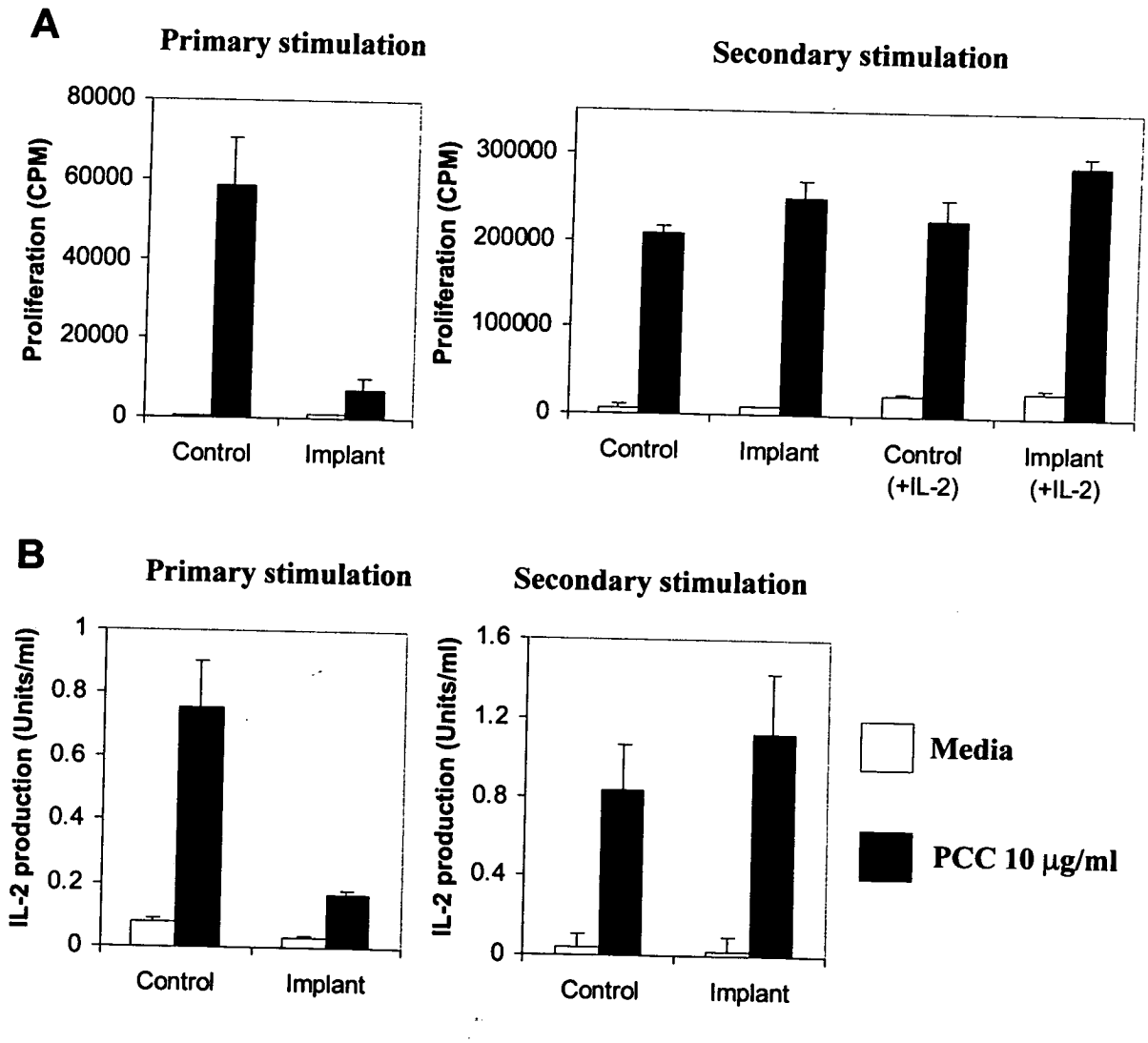


Figure 1. Suppressive APC block proliferation but do not induce anergy. Purified CD4+ cells from splenocytes of PCC specific TCR transgenic mice were stimulated with adherent PEC from control or parasite-implanted animals and 10 μg/ml PCC protein. Proliferation (**A**) was measured by [^{3}H] thymidine incorporation and IL-2 production (**B**) was measured with the NK bioassay after 48 hours. Stimulated cells were also rested for 72 hours, repurified with anti-CD4 magnetic beads and restimulated with PCC and irradiated normal splenocytes for another 48 hours (secondary stimulation). The addition of exogenous IL-2 (10 Units/ml) had no effect on proliferation of restimulated cells (**A**).

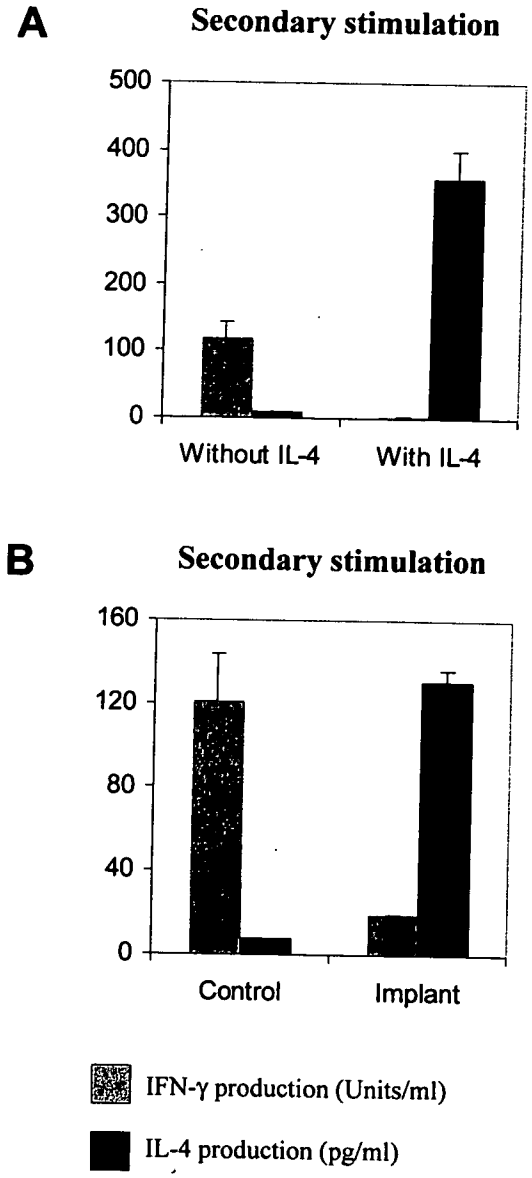


Figure 2. Suppressive APC induce Th2 differentiation. (**A**) PCC-specific TCR transgenic CD4+ cells were stimulated with 10 μg/ml PCC protein and irradiated splenocytes in the absence or presence of IL-4 (200 pg/ml). After 5 days, primed cells were washed and restimulated with fresh irradiated splenocytes. 48 hours later, supernatants were harvested and tested for IL-4 and IFN-γ production. (**B**) Naïve T cells were primed with control or parasite implanted PEC without additional cytokines (as described in Fig. 1). 5 days later, primed cells were restimulated with PCC and fresh irradiated normal splenocytes. 48 hours after secondary stimulation, supernatants were harvested and tested for IL-4 and IFN-γ production.

Relationship between cytokine production and proliferation

An interesting relationship between cellular proliferation and cytokine production has recently been demonstrated (Bird et al., 1998; Gett and Hodgkin, 1998). Whereas IFN-γ production can be stimulated without cell division, IL-4 production can only be detected after at least 3 rounds of cell division (Bird et al., 1998). We therefore investigated whether parasite-derived APC could stimulate PCC-tg T cells to produce IL-4 in the absence of proliferation (during primary stimulation), by staining the T cells with the proliferation marker CFSE before primary stimulation with antigen and suppressive APC. IL-4 and IFN-γ production were assessed by intracellular cytokine staining.

We found that naïve T cells primed with control PEC underwent up to 9 rounds of cell division (in 72 hours) (Figure 3A) and produced high levels of IFN-γ as they divided (Figure 3B). In contrast, suppressive PEC-primed T cells went through very few rounds of cell division in the same period of time (Figure 3A). In some experiments, they failed to divide altogether (data not shown). Furthermore, there were no IFN-γ producing cells among the T cells that have undergone 3-4 cell divisions (Figure 3B) so that suppression of IFN-γ appears more profound than proliferative suppression. No IL-4 producing cells were observed from T cells stimulated by either control or implant PEC (Figure 3B). These data suggest that the shift to Th2 differentiation we observe is more likely due to the inhibition of IFN-γ rather than the induction of IL-4 producing T cells during primary stimulation.

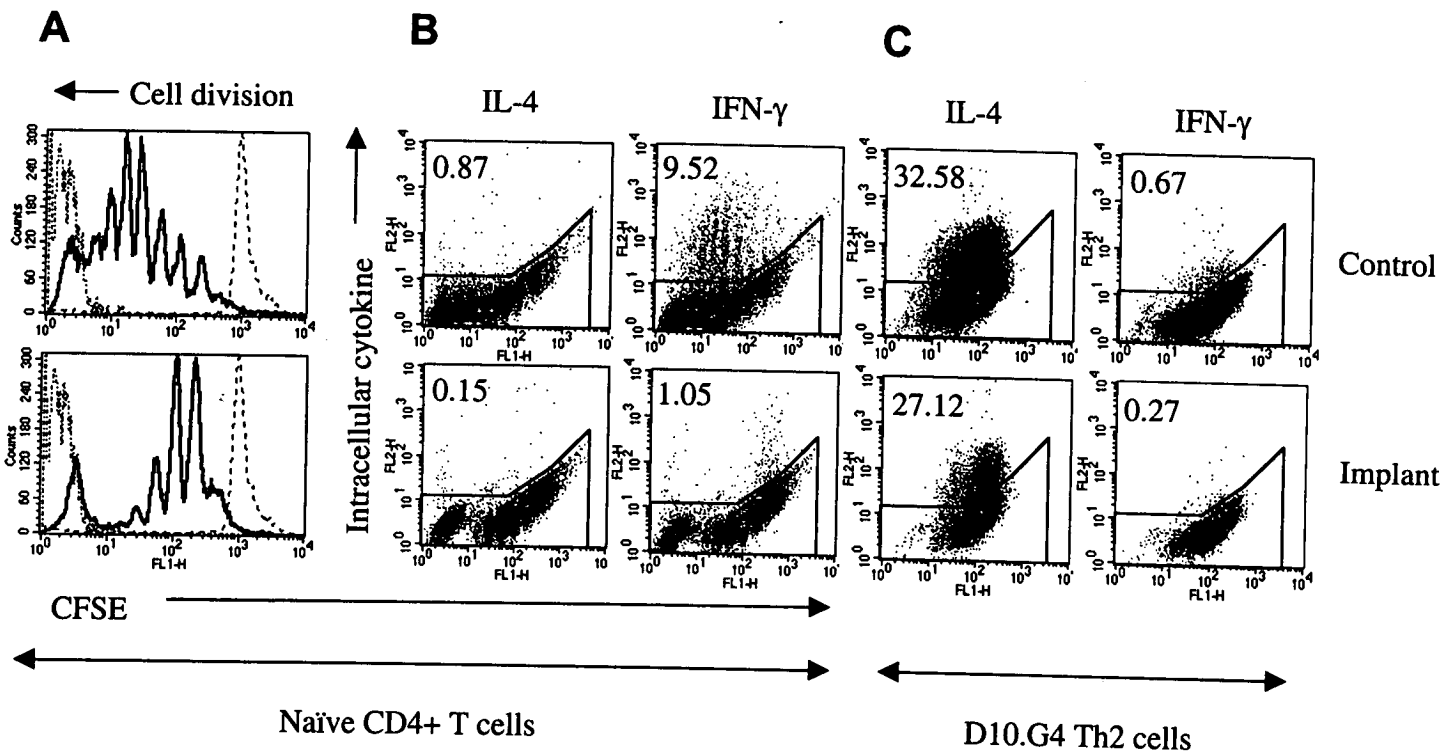


Figure 3. Analysis of cell division and cytokine production during primary stimulation. Naïve CD4+ T cells and the Th2 clone D10.G4 were labeled with CFSE before stimulation with antigen and adherent PEC from control or parasite implanted mice. After 72 hours, the non-adherent T cells were harvested and stained for intracellular cytokine (IL-4 and IFN-γ). The numbers in (**B**) and (**C**) represent the percentage of cytokine positive cells. (**A**) Cell division of naïve T cells stimulated with PCC and control PEC or implant-derived PEC. (**B**) Intracellular IL-4 and IFN-γ staining of dividing CD4+ transgenic T cells. (**C**) As a positive control for IL-4 staining, the Th2 clone D10.G4 was also stimulated with conalbumin and control or implant PEC. Intracellular IL-4 and IFN-γ was assessed.

TGF-β is involved in inhibition of IFN-γ producing T cells

IL-10 and TGF-β are cytokines commonly associated with the capacity to down regulate IFN-γ as well as inducing a Th2 differentiation bias (Iwasaki and Kelsall, 1999; King et al., 1998; Stumbles et al., 1998). In order to determine whether these molecules were involved in the inhibition of IFN-γ production or proliferative suppression of naïve T cells, we used blocking antibodies to IL-10 and TGF-β to see if the observed phenomena could be reversed (Figure 4). Consistent with the fact that IL-10 was undetectable in primary stimulation assays (<50pg/ml), we found that blocking antibodies against IL-10 did not have any significant effect on naïve T cells primed by parasite induced APC (Figure 4C). However, blocking antibodies against TGF-β doubled the number of IFN-γ positive cells stimulated by the suppressive PEC (Figure 4D), reaching numbers which were indistinguishable from control PEC stimulated T cells. Upon secondary stimulation, IFN-γ production by T cells primed with suppressive PEC and anti-TGF-β was also indistinguishable from those primed with control PEC (Figure 5A), and was much enhanced compared to priming with suppressive PEC alone. IL-4 production was also significantly reduced as a result of primary stimulation with anti-TGF-β antibodies (Figure 5B). This suggests that TGF-β plays a role in the induction of IL-4 production in naïve T cells stimulated by suppressive PEC, as well as inhibiting their production of IFN-γ.

Although these experiments suggested that TGF-β was involved in Th2 induction by suppressive PEC, blocking antibodies against TGF-β did not reverse the proliferative suppression of naïve T cells (Figure 4D). The observation that antibodies against TGF-β and IL-10 do not reverse proliferative suppression is consistent with our previous work using Th2 clones and transformed cell lines (Allen et al., 1996; MacDonald et al., 1998). The observation that antibodies against TGF-β could reverse IFN-γ inhibition, but not proliferative suppression, also suggests that there is no direct relationship between these two phenomena.

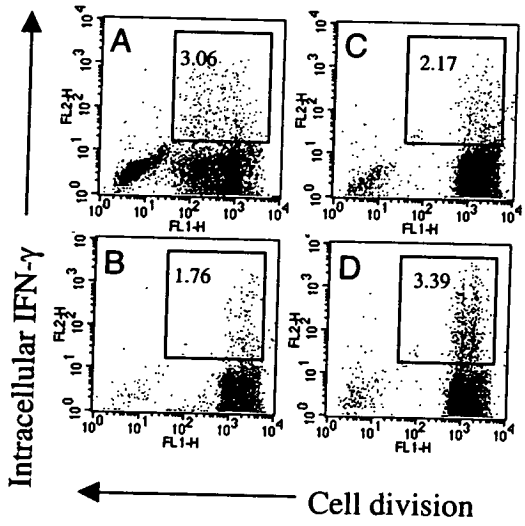


Figure 4. TGF-β is involved in inhibition of IFN-γ by implant PEC, but not inhibition of proliferation. Naïve CD4+ T cells were labeled with CFSE before stimulation with antigen and PEC from control mice (A) or parasite implanted mice (B, C & D). Media (A, B), neutralizing anti-IL-10 (C) or neutralizing anti-TGF-β (D) were added to the in vitro cultures. Blocking antibodies had no effect on T cells stimulated by control PEC (data not shown). After 72 hours, the naïve T cells were harvested and stained for intracellular IL-4 (undetectable, data not shown) and IFN-γ. Numbers within gates represent the percentage of IFN-γ positive cells.

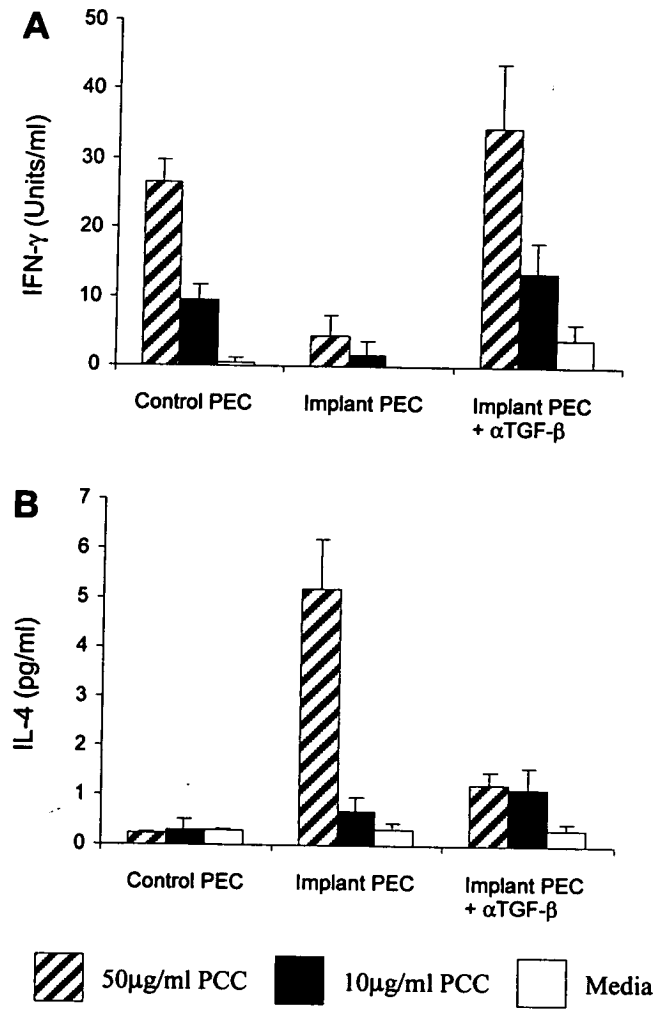


Figure 5. TGF-β is involved in inducing Th2 differentiation. Naïve T cells were primed (as described in Fig. 1 and 2) with control PEC, parasite implanted PEC alone and parasite implanted PEC with blocking antibodies against TGF-β. 5 days later, primed cells were restimulated with 10 or 50 μg/ml PCC and fresh irradiated splenocytes. 72 hours after secondary stimulation, supernatants were harvested and tested for IL-4 and IFN-γ production.

Effect of parasite recruited macrophages on naïve T cells

In chapter 3 we described how F4/80$^+$ macrophages have been recently identified as the PEC cell type directly responsible for proliferative suppression in implanted mice. To examine the possibility that macrophages are responsible for the observed effect on naïve T cells, F4/80$^+$ cells were purified from the PEC population of control and parasite implanted mice, and used to stimulate CFSE stained naïve T cells (Figure 6). Whereas F4/80$^+$ cells from control animals stimulated cell division and IFN-γ production by naïve T cells, parasite recruited F4/80$^+$ cells suppressed proliferation and did not stimulate IFN-γ producing cells (Figure 6). This data demonstrates that it is the macrophage population that is responsible for the observed effects on IFN-γ production. This suggests that alternatively-activated macrophages influence naïve T cell differentiation by failing to stimulate, or inhibiting, IFN-γ production, perhaps permitting a Th2 default pathway of differentiation.

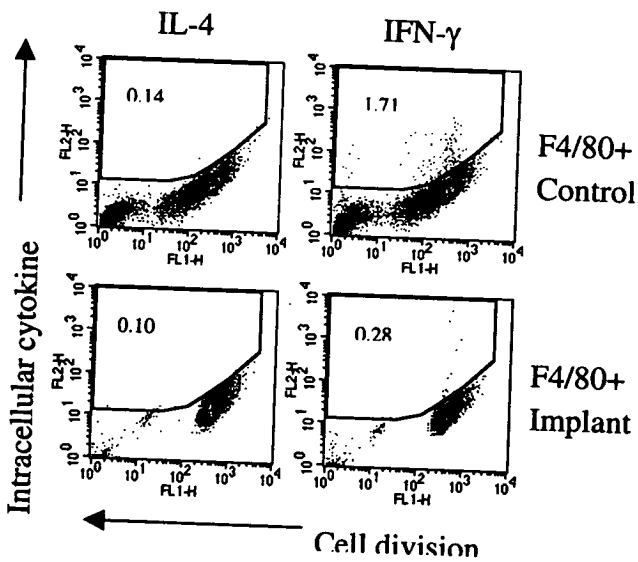


Figure 6. F4/80+ macrophages from the implant PEC population inhibit IFN-γ. Naïve CD4+ T cells were labeled with CFSE before stimulation with antigen and F4/80+ purified cells from control or parasite implanted mice. After 72 hours, the naïve T cells were harvested and stained for intracellular IL-4 and IFN-γ. Numbers in gate represents percentage of IFN-γ positive cells.

Discussion

In addition to the ability to inhibit cellular proliferation, we have found that the macrophages recruited by *B.malayi* appear to drive Th2 differentiation of naïve T cells *in vitro*. The Th2 differentiation is linked with the absence of IFN-γ producing cells during the primary encounter of antigen and this effect appears to be mediated via TGF-β. Future efforts will be made to determine if these APC can drive Th2 responses *in vivo* as well.

We expected the naïve T cells to be anergized by the first encounter of antigen in the presence of suppressive APC. The recovery of proliferative ability was surprising given that Schwartz and Jenkins have proposed that anergy is induced when signal 1 (TCR engagement of the peptide-MHC complex) is encountered in the absence of proliferation and IL-2 production (Beverly et al., 1992; Jenkins, 1992). Based on this model, Powell et al. showed that inhibiting proliferation of T cells with rapamycin during stimulation also leads to anergy even in the presence of co-stimulation (Powell et al., 1999). Our data show no induction of anergy despite profound inhibition of cell division and IL-2 production in naïve T cells during primary stimulation. Since anergy studies have primarily used established T cell clones (Beverly et al., 1992), our results support the observations of Hayashi et al., who proposed that naïve T cells are resistant to anergy induction (Hayashi et al., 1998).

It is significant that Th2 differentiation appeared to occur as a result of inhibiting differentiation of IFN-γ producing T cells, instead of driving early differentiation of IL-4 producing cells. This supports the hypothesis that Th2 responses might be a default pathway that occurs in the absence of a Th1 driving factor. This is consistent with observations by Cua and Stohlmans (Cua and Stohlman, 1997), which suggest that reduced IL–12 production by a subpopulation of macrophages, could be associated with type 2 responses (Cua and Stohlman, 1997). IL-12 production by monocyte/macrophages can be down regulated by a number of

factors including engagement of CD47 (Armant et al., 1999) and ligation of the Fc gamma, complement, or scavenger receptors (Sutterwala et al., 1997). Alternatively, the suppressive APC may be producing down regulatory cytokines that directly inhibit IFN-γ production (Nagelkerken et al., 1993; Tripp et al., 1995).

Although TGF-β clearly plays an important role in the Th2 driving effect of the AAMΦ, there remain many unanswered questions about the role of TGF-β in our system, especially since its role in naïve T cell differentiation is extremely complex with conflicting data from different experimental systems (Letterio and Roberts, 1998). While our observations are consistent with reports that TGF-β is associated with the inhibition of IFN-γ and Th2 development (Gorham et al., 1998; Schmitt et al., 1994), it is in contrast with reports suggesting that TGF-β inhibits Th2 differentiation (Nagelkerken et al., 1993; Sad and Mosmann, 1994; Swain et al., 1991). Thus, the effects of TGF-β varies depending on a variety of factors (eg. cytokine concentration (Lingnau et al., 1998) and genetic background (Hoehn et al., 1995)), and cannot be generalized beyond a particular system. Furthermore, it is uncertain in our system whether TGF-β acts directly on naïve T cells or does it have an indirect effect? TGF-β production by parasite derived APC could be directly influencing IFN-γ production by inhibiting the IL-12 responsiveness of the naïve T cells as a result of downregulating IL-12R expression (Gorham et al., 1998). However, TGF-β has also been shown to affect T cell differentiation by modulating the phenotype of APC rather than acting directly on T cells (Takeuchi et al., 1998; Takeuchi et al., 1997). PEC pulsed with TGF-β can induce IL-4 production by DO11.TCR transgenic naïve T cells even during primary stimulation (Takeuchi et al., 1998; Takeuchi et al., 1997). The stimulation of IL-4 appeared to be a result of CD40 up-regulation, as well as inhibition of IL-12 production (Takeuchi et al., 1998). It would be interesting to establish whether TGF-β is acting directly or indirectly in our system.

Recent reports have described specific subsets of dendritic cells that differ in their capacity to stimulate the differentiation of naïve T cells (Iwasaki and Kelsall, 1999; Rissoan et al., 1999; Stumbles et al., 1998). However, we are unaware of any reports suggesting that macrophages can also differ in their capacity to stimulate naïve T cell differentiation, although this has been proposed by Goerdt et.al. (Goerdt and Orfanos, 1999). Indeed, macrophages are expected to stimulate a Th1 type response (Desmedt et al., 1998). Our observations imply that macrophages, like dendritic cells, might also differ in their capacity to stimulate T cell differentiation.

Chapter 6

CAN THE AAMΦ BLOCK TUMOUR PROLIFERATION AND PREVENT MHC MEDIATED REJECTION *IN VIVO*?

Summary

The ability to design new therapies to prevent the expansion of tumour cells and to prevent graft rejection is one of the ultimate aims of applied immunology. The properties of AAMΦ that have been discussed in the previous chapters raised possibilities that nematode parasites might induce novel immune mechanisms that could be of medical significance. We found that when EL4 cells were injected (i.p.) into mice implanted with parasites, the proliferation of these cells was severely curtailed ex vivo, after 1 week of *in vivo* expansion in the peritoneal cavity. As expected, parasite implanted IL-4 deficient mice did not suppress the ex vivo proliferation of injected EL4 cells. However, using CFSE labelled EL4 cells to track proliferation *in vivo*, we found that the recruitment of suppressive AAMΦ into the peritoneal cavity of parasite implanted mice could not prevent the *in vivo* proliferation of EL4 cells (i.p.). This suggests that EL4 cells can migrate away from suppressive AAMΦ in the peritoneal cavity *in vivo* and escape proliferative suppression, which they cannot do when co-cultured *in vitro*. We also found that CFSE labelled suppressive AAMΦ could not prevent MHC restricted host rejection when transferred *in vivo* (i.p.) into allogeneic mice. However, due to a mistake in the choice of mouse strains, we have not been able to determine if AAMΦ can prevent minor histocompatibility antigen mediated rejection.

Introduction

In the previous chapters, we have shown that suppressive macrophages can 1) block proliferation of tumour cells, and 2) drive differentiation of Th2 cells from naïve cells. These results suggested that suppressive macrophages might be able to prevent expansion of tumours and downmodulate the immune response. In this chapter, we describe experiments aimed at testing if the effects we have so far observed *in vitro*, could translate to effects *in vivo*. The T cell lymphoma EL4 is a very aggressive cell line that has been widely used in many tumour rejection studies (Stremmel et al., 1999; Townsend et al., 1994). It's susceptibility to being suppressed by the AAMΦ *in vitro*, as well as it's aggressive and well characterised features *in vivo* (Stremmel et al., 1999; Townsend et al., 1994), encouraged us that it would make a good candidate for testing *in vivo* suppression.

In the previous chapter, we have already described using the intracellular fluorescent dye 5,6-carboxyfluorescein diacetate succinimyl ester (CFSE) to investigate the *in vitro* division of naïve T cells. More recently, this technique has been exploited to investigate *in vivo* proliferation in the study of T cell homeostasis (Ernst et al., 1999). When analysed by flow cytometry *ex vivo*, each division of CFSE labelled cells *in vivo* causes a 2 fold decrease in fluorescence. We therefore decided to use this technique to investigate the proliferation of CFSE labelled EL4 cells when injected into the peritoneal cavity of mice, which have been implanted with *B. malayi*.

The ability to drive Th2 differentiation made us speculate that the suppressive macrophages might function as antigen presenting cells that induce the differentiation of T regulatory cells. T regulatory cells have been proposed to be involved in "infectious tolerance" and play a role in preventing graft rejection (Cobbold and Waldmann, 1998; Waldmann and Cobbald, 1998). In order to test the hypothesis that suppressive macrophages could induce T regulatory cells and prevent host rejection, we decided to transfer these macrophages into allogeneic mice and investigate if they can prevent themselves from being rejected by the host immune response. If the macrophages could prevent themselves from being rejected from allogeneic hosts, we

could then test if their presence would also prevent skin graft rejection on the allogeneic hosts that have received suppressive macrophages.

Unfortunately, the experiments described in this chapter do not show a clear indication that the recruitment of suppressive macrophages into the peritoneal cavity would prevent the *in vivo* proliferation of EL4 cells. There was also no indication that suppressive macrophages can prevent themselves from being rejected when trasnferred into MHC mismatched hosts.

Results

EL4 cells are suppressed *ex vivo* after a week of expansion *in vivo*

Since macrophages recruited into the peritoneal cavity of mice by *B. malayi* can suppress the proliferation of tumour cells when co-cultured *in vitro*, we decided to investigate if implantation of the parasite would prevent tumour proliferation *in vivo*. We injected EL4 lymphoma cells into the peritoneal cavity of mice that have been implanted with parasites for 3 weeks. To control for cytotoxic effects of inflammatory macrophages, we injected mice with thioglycollate for 3 days before injection of EL4 cells. After allowing the EL4 cells to expand *in vivo* for 7 days, we removed the PEC from the EL4 injected mice as well as control mice, and assessed the proliferation of the total PEC population *ex vivo* (Figure 1A). PEC from EL4 injected mice were found to proliferate in thioglycollate injected mice but not parasite implanted mice. PEC of control mice (thioglycollate injected as well as parasite implanted) not injected with EL4 cells, showed no signs of proliferation. (Figure 1A)

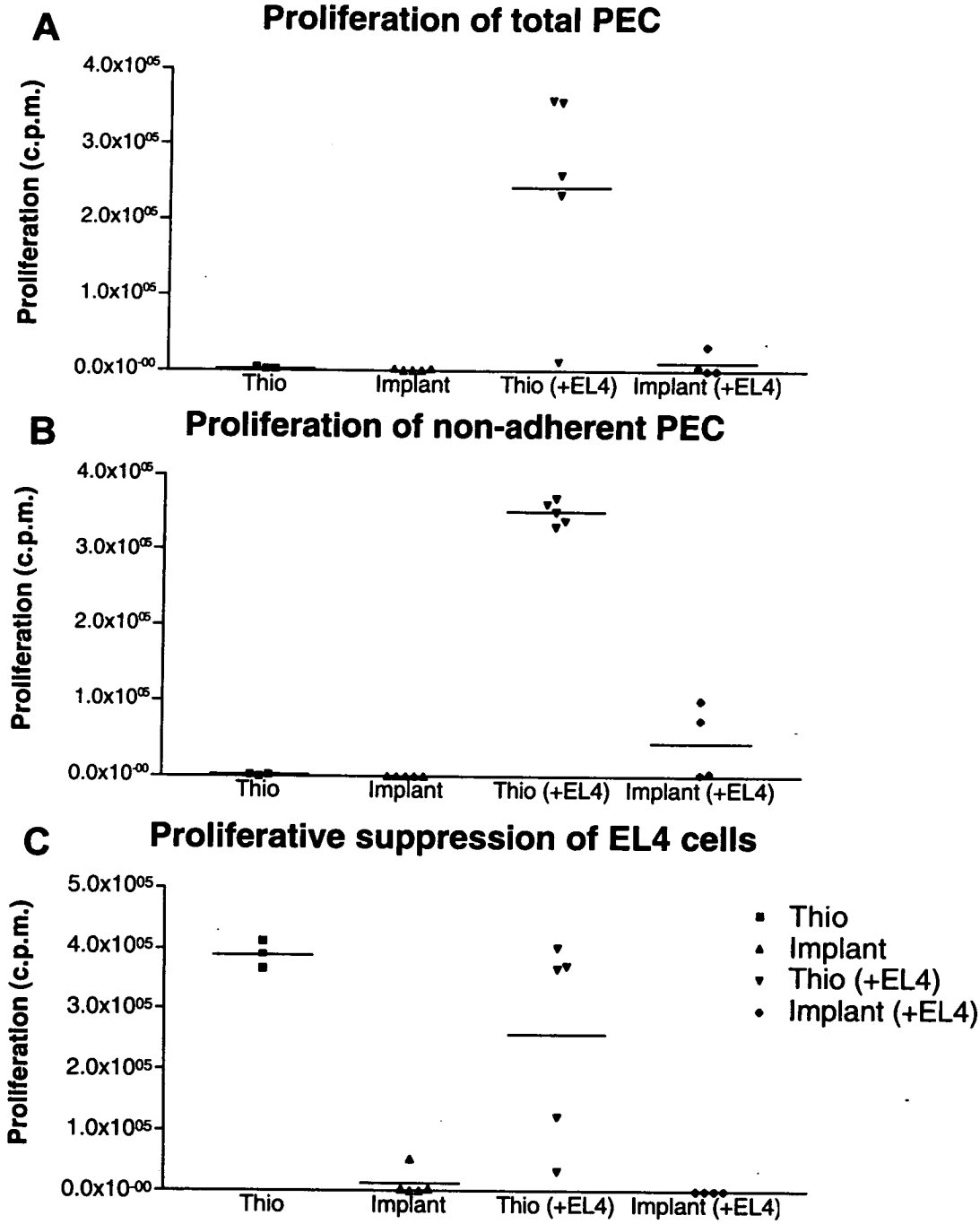


Figure 1: EL4 cells are suppressed *ex vivo*. Mice were either implanted with parasites for 3 weeks or injected (i.p.) with thioglycollate for 3 days, before being injected (i.p.) with $2x10^6$ EL4 cells. After 7 days, PEC were removed from EL4 injected mice (+EL4) as well as control mice. **(A)** $1X10^5$ total PEC were cultured in 96 well flat bottom plates for 48 hours and proliferation was measured by [^{3}H]TdR incorporation. **(B)** In the same experiment, $1X10^5$ total PEC were adhered to 96 well flat bottom plates for 2 hours, before removing the non-adherent cells (containing EL4 cells but not suppressive AAMΦ) to separate plates and after 48 hours, proliferation was measured by [^{3}H]TdR incorporation. **(C)** In the same experiment, the adherent PEC from **B** were co-cultured with $5x10^4$ fresh EL4 cells and after 48 hours, proliferation was measured by [^{3}H]TdR incorporation. Data shown are the mean of quadruplicate wells for individual animals. Horizontal bar indicates the mean of the separate experimental groups.

Because the total PEC population would have both suppressive macrophages as well as EL4 cells, we decided to ask if the non-adherent EL4 cells would proliferate if separated from the adherent suppressive macrophages. In the same experiment, we adhered the total PEC population to flat bottom plates for 2 hours, before removing and assessing the proliferation of the non-adherent population of cells (Figure 1B). The non-adherent fraction containing EL4 cells from thioglycollate injected mice proliferated much more strongly and consistently than the non-adherent cells from PEC of implanted mice (Figure 1B), and also more consistently between animals in the same group in comparison to total PEC proliferation (Figure 1A). However, in 2 of the parasite implanted mice, there was some indication of EL4 proliferation when separated from the suppressive adherent cells (Figure 1B).

Finally, we wanted to know if the adherent suppressive macrophages were influenced by the presence of *in vivo* expanding EL4 cells. By adding fresh EL4 cells (that were growing *in vitro*) to the adherent PEC population of the different experimental groups, we asked if the parasite implanted cells retained their suppressive activity in comparison to thioglycollate injected mice (Figure 1C). As expected, the thioglycollate injected mice that have not been injected with EL4 cells did not suppress proliferation of EL4 cells, whereas adherent PEC from parasite implanted mice (EL4) suppressed proliferation (Figure 1C). Implanted mice that had been injected with EL4 cells *in vivo* retained the presence of suppressive cells, whereas the majority of thioglycollate treated and EL4 injected mice did not suppress proliferation of fresh EL4 cells (Figure 1C).

Since IL-4 deficient ($^{-/-}$) mice failed to generate suppressive macrophages, we decided to repeat the experiment by injecting EL4 cells (i.p.) into parasite implanted IL-4$^{-/-}$ mice as well as implanted wild type (WT) mice and thioglycollate injected mice (Figure 2). In this experiment, we also labelled the EL4 cells with CFSE so that we could track their proliferation *in vivo* as well as *ex vivo* (as described in the next section). This experiment again showed that injected EL4 cells that have expanded for 7 days *in vivo* failed to proliferate *ex vivo* in parasite implanted mice (Figure 2). This was not the case for IL-4 deficient mice, whereby proliferating EL4 cells were observed *ex vivo* (Figure 2).

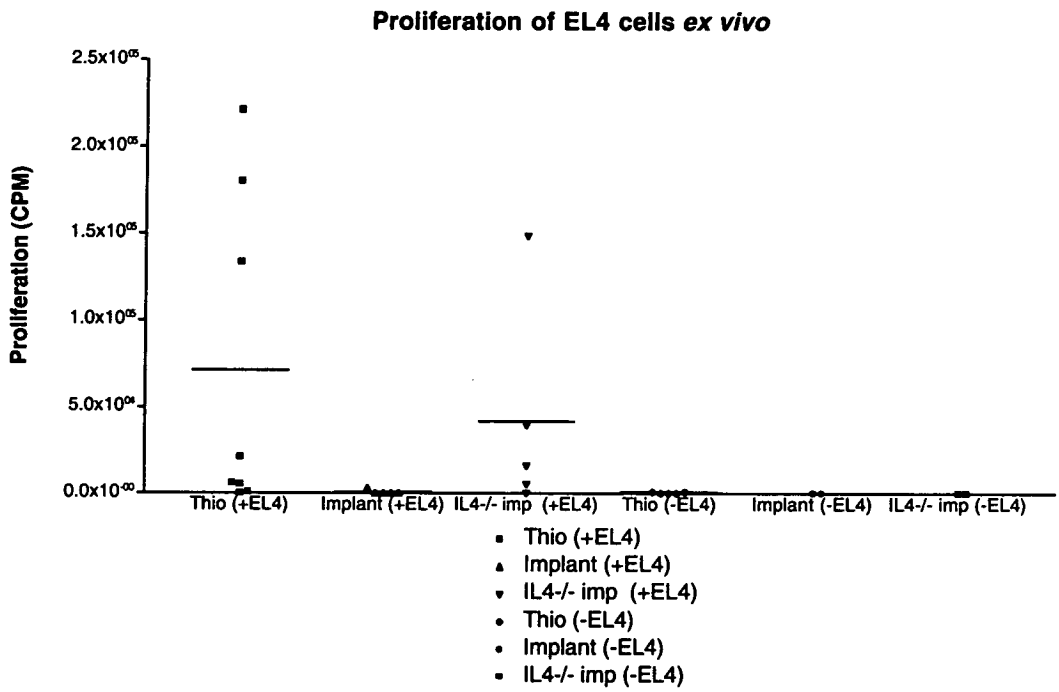


Figure 2: EL4 cells are suppressed *ex vivo* by parasite implanted WT PEC but not parasite implanted IL-4 deficient PEC. IL-4 deficient and C57/BL6 mice were implanted with parasites for 3 weeks, and C57/BL6 mice were injected (i.p.) with thioglycollate for 3 days, before being injected (i.p.) with $2x10^6$ CFSE labelled EL4 cells. After 7 days, PEC were removed from EL4 injected mice (+EL4) as well as control mice (-EL4). $1x10^5$ total PEC were cultured in 96 well flat bottom plates for 48 hours and proliferation was measured by [^{3}H]TdR incorporation. Data shown are the mean of quadruplicate wells for individual animals. Horizontal bar indicates the mean of the separate experimental groups.

These experiments were encouraging because they suggested that parasite implantation could have reduced the proliferation of EL4 cells *in vivo* to such an extent that they failed to proliferate *ex vivo* after 7 days. However, because the proliferation assay was still carried out *in vitro*, we could not be sure that suppression was really occurring *in vivo* as well. Furthermore, we found that the thioglycollate injected mice did not have as severe an inflammatory peritonitis (total PEC of between 5-8 x 10^6 cell) as parasite implanted mice (1-2 x 10^7). Thus, when we plated out the same number of PEC for proliferation assays (Figure 1 & 2), there would be proportionally fewer injected EL4 cells in the PEC population of thioglycollate injected mice relative to the other PEC. However, this would not account for the differences observed between WT implanted mice and IL-4 deficient mice, which have similar number of total PEC (Figure 2).

CFSE labelled EL4 cells allow *in vivo* tracking of proliferation

To assess the proliferation of EL4 cells *in vivo*, we decided to label these cells with the fluorescent dye CFSE before injecting them into the peritoneal cavity of experimental mice. CFSE stained cells allow us to assess proliferation *in vivo* in 2 ways. First of all, we can distinguish between injected fluorescent EL4 cells with the rest of the peritoneal cell population. This would enable us calculate the number of EL4 cells recovered from individual mice. Secondly, the loss of fluorescence in CFSE labelled dividing cells is directly proportional to cell division. By measuring the mean fluorescence of recovered EL4 cells we can compare the number of cell divisions undergone by these cells *in vivo*.

As described in the previous section, we injected CFSE labelled EL4 cells into parasite implanted IL4 $^{-/-}$ mice as well as parasite implanted WT mice and thioglycollate injected mice. 7 days later the total PEC population were recovered from these mice and assessed by flow cytometry (FACS) (Figure 3). This showed that the CFSE labelled EL4 cells (Figure 3, M1) could still be distinguished from the unlabelled peritoneal cells of the control mice (Figure 3A). The number of EL4 cells recovered from each individual mouse could be calculated (Figure 4B) by multiplying the percentage of cells in M1 with the total number of PEC recovered from the mouse.

The mean fluorescence of cells in M1 also gave an indication of cell division (Figure 4A).

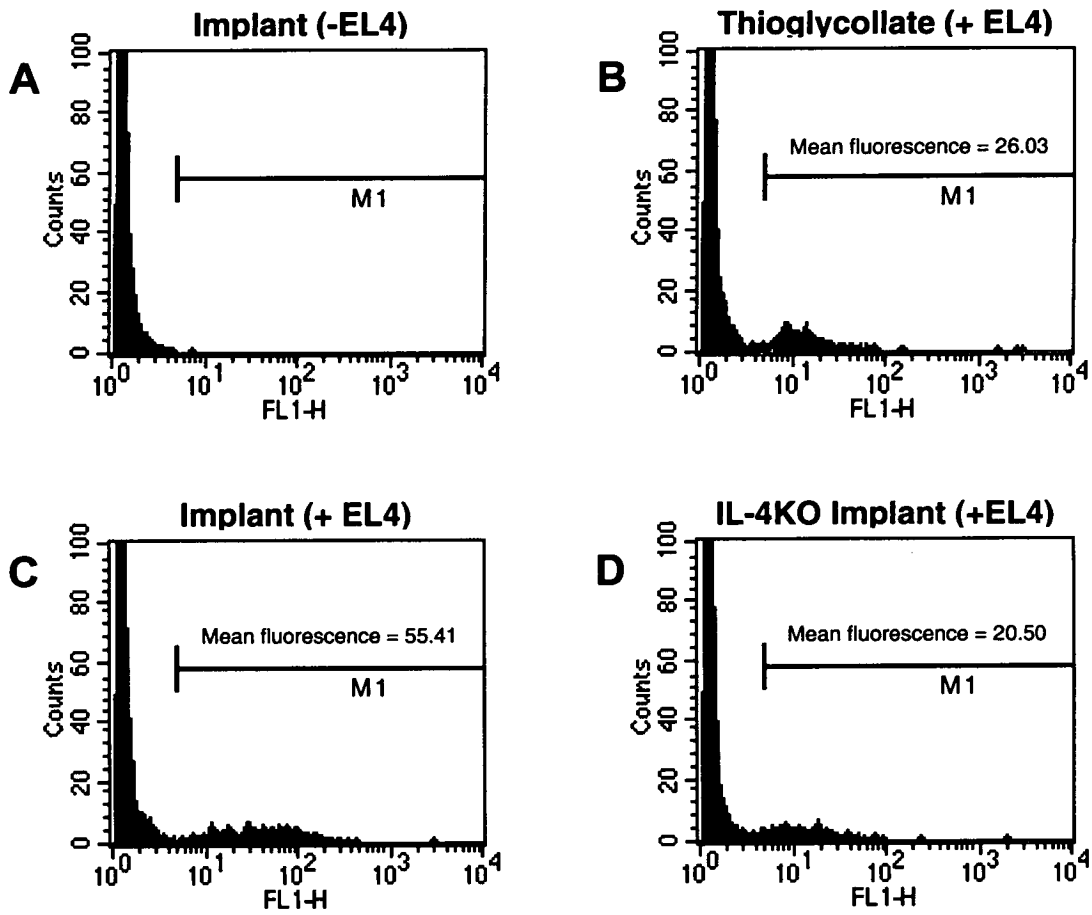


Figure 3: CFSE labelled EL4 cells can be detected by FACS. IL-4 deficient and C57/BL6 mice were implanted with parasites for 3 weeks, and C57/BL6 mice were injected (i.p.) with thioglycollate for 3 days, before being injected (i.p.) with $2x10^6$ CFSE labelled EL4 cells. After 7 days, PEC were removed from EL4 injected mice (+EL4) as well as control mice (-EL4) and analysed by FACS. FACS histograms shown are from an individual animal (representative of 4-5 animals per group), gated by FSC and SSC to enrich for EL4 cells. M1 indicates the CFSE+ EL4 cells and provides the statistical basis for the analysis presented in Figure 4. Mean fluorescence gives an indication towards the number of cell divisions undergone since the CFSE labelled EL4 cells lose half their fluorescence each time they divide (Figure 4A). The percentage of cells in M1 (of the ungated population) also allowed us to calculate the number of EL4 cells recovered from each individual mice (Figure 4B).

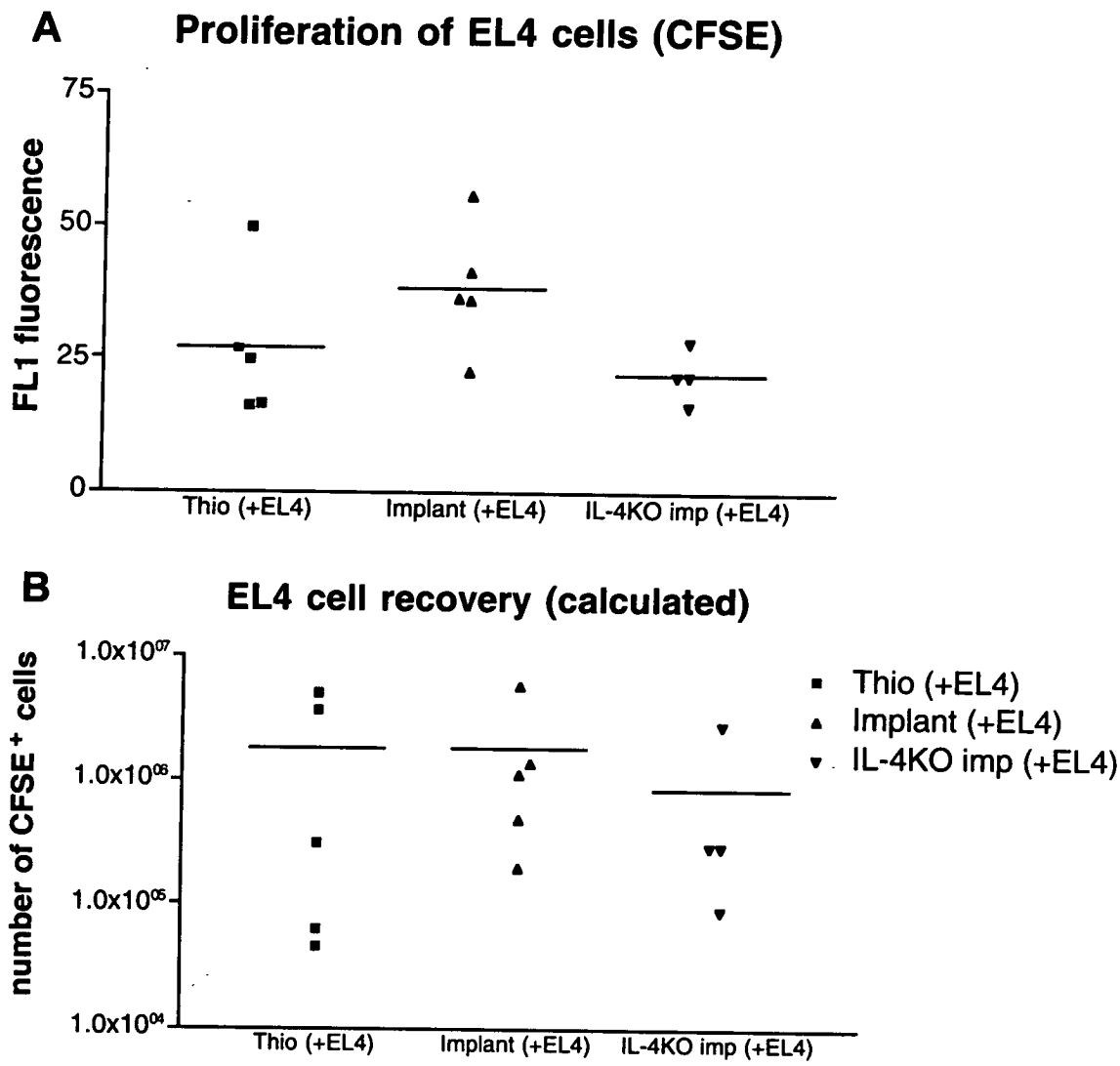


Figure 4: EL4 cells are not significantly suppressed *in vivo*. Statistical analysis of CFSE labelled EL4 detected by FACS, as described in Figure 3. IL-4 deficient and C57/BL6 mice were implanted with parasites for 3 weeks, and C57/BL6 mice were injected (i.p.) with thioglycollate for 3 days, before being injected (i.p.) with $2x10^6$ CFSE labelled EL4 cells. After 7 days, PEC were removed from EL4 injected mice (+EL4) as well as control mice (-EL4) and analysed by FACS. The numerical values for the CFSE+ EL4 cells that fall in M1 (Figure 3) are shown here. **(A)** Mean fluorescence gives an indication towards the number of cell divisions undergone since the CFSE labelled EL4 cells lose half their fluorescence each time they divide. EL4 cells in WT implanted mice appear to have undergone slightly less cell division. Although this is significantly different from IL-4 -/- implanted mice ($p = 0.0373$), it is not signifcantly different from thioglycollate injected mice ($p = 0.1895$). **(B)** The percentage of cells in M1 (of the ungated population) also allowed us to calculate the number of EL4 cells recovered, by taking into account the total number of PEC from each individual mouse. There were no significant differences between the 3 groups. Horizontal bar indicates the mean of the separate experimental groups.

Proliferation of EL4 cells is not suppressed *in vivo*.

There was some indication that EL4 cells in parasite implanted WT mice had undergone fewer rounds of cell division, since the mean fluorescence of these cells was slightly higher (Figure 3C & 4A). Although this difference was statistically significant in comparison to implanted IL-4 $^{-/-}$ mice ($p=0.0373$), it fell short of being significant in comparison to thioglycollate injected mice ($p=0.1895$). Furthermore, the differences in mean fluorescence was very small and would represent a difference in only one round of cell division. These results indicated that parasite recruited cells might slightly reduce the number of cell divisions of injected EL4 cells, but this was a qualitative reduction rather than an effective inhibition. Consequently, the number of EL4 cells recovered remained similar (Figure 4B)

Data analysis of CFSE labelled EL4 cells showed that there were no significant differences in the number of EL4 cells recovered from WT implanted mice in comparison to implanted IL-4 $^{-/-}$ mice and thioglycollate injected mice (Figure 4B). In order to determine if the reduction in cell division was real, a time course experiment would provide more information about the kinetic changes that occur in the peritoneal cavity. We carried out a time course experiment by injecting CFSE labelled EL4 cells into parasite implanted WT mice and thioglycollate injected mice and assayed over a period of 5 days. PEC for the first time point was collected 2 hours (Figure 5, Day 0) after injection of labelled EL4 cells to assess the recovery and fluorescence of undivided EL4 cells. PEC were collected subsequently 1, 3, 4 and 5 days after injection. FACS data was collected in 3 ways. Data for total PEC were collected without any gates, as well as gating for EL4 cells by FSC and SSC. We also adhered PEC for 2 hours and only FACS analysed the non-adherent fraction to enrich for data collection of EL4 cells. The two latter strategies enriched significantly for collection of EL4 datapoints and resulted in mean fluorescence data (as shown in Figure 5 and Figure 6A) that was consistent between individual mice as well as between the two different strategies of FACS analysis.

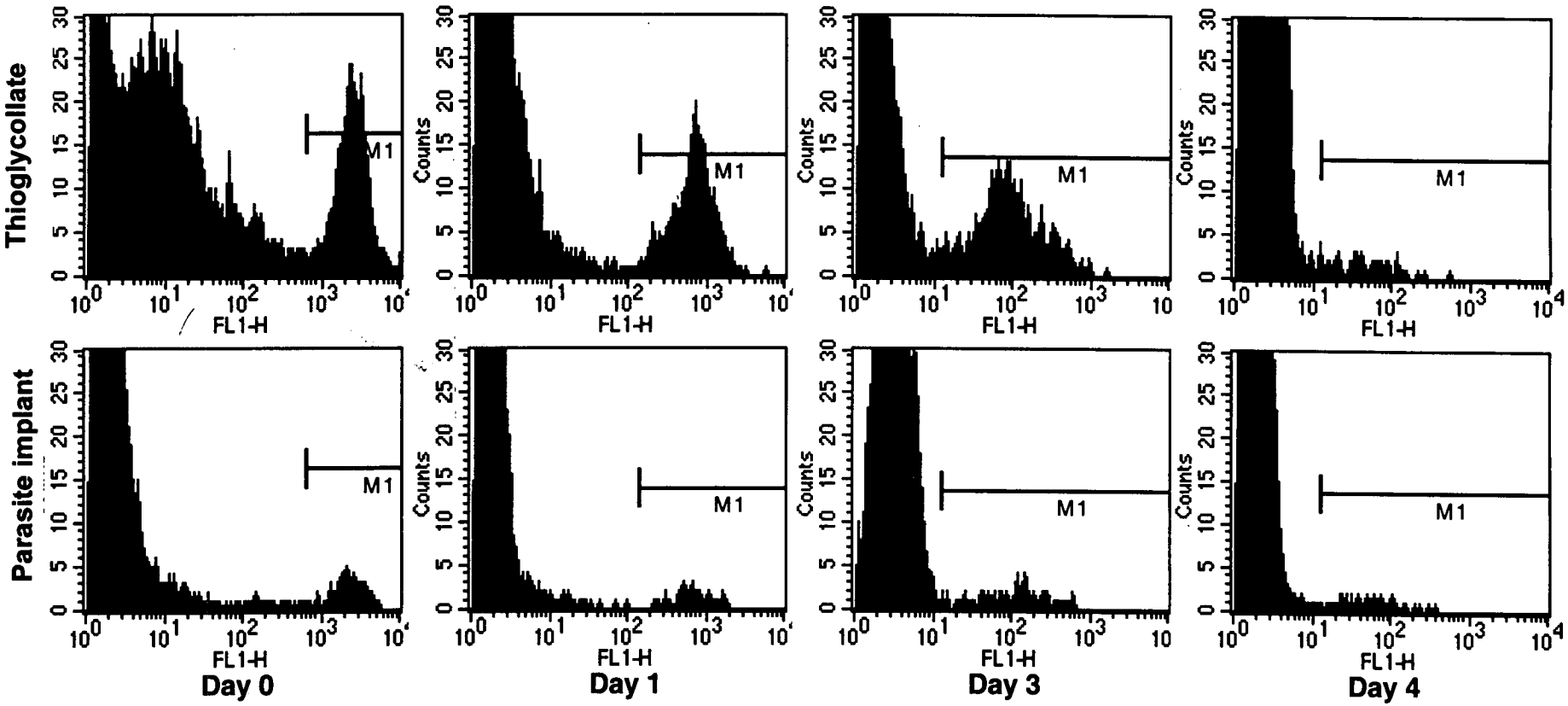


Figure 5: Parasite implantation does not prevent cell division of CFSE labelled EL4 cells in an *in vivo* time course experiment. FACS histograms show CFSE labelled EL4 cells (M1) and the loss of fluorescence as they divide *in vivo* (i.p.). Mice were implanted (i.p.) with parasites for 3 weeks or injected with thioglycollate (i.p.) for 3 days before being injected (i.p.) with $2x10^6$ CFSE labelled EL4 cells. PEC were removed from mice 2 hours later (Day 0), a day later (Day 1) and at Day 3, Day 4 and Day 5 (not shown), and analysed by FACS. Data shown from one of 2 mice per group. Histograms shown are enriched for EL4 cells (gated by FSC and SSC), FACS data also collected for ungated total PEC samples (to calculate EL4 cell recovery) and samples enriched for non-adherent EL4 cells (with similar results to histograms shown). Data analysis for the mean fluorescence (indicating cell division) and the percentage of cells in M1 (indicating cell recovery) is shown in Figure 6.

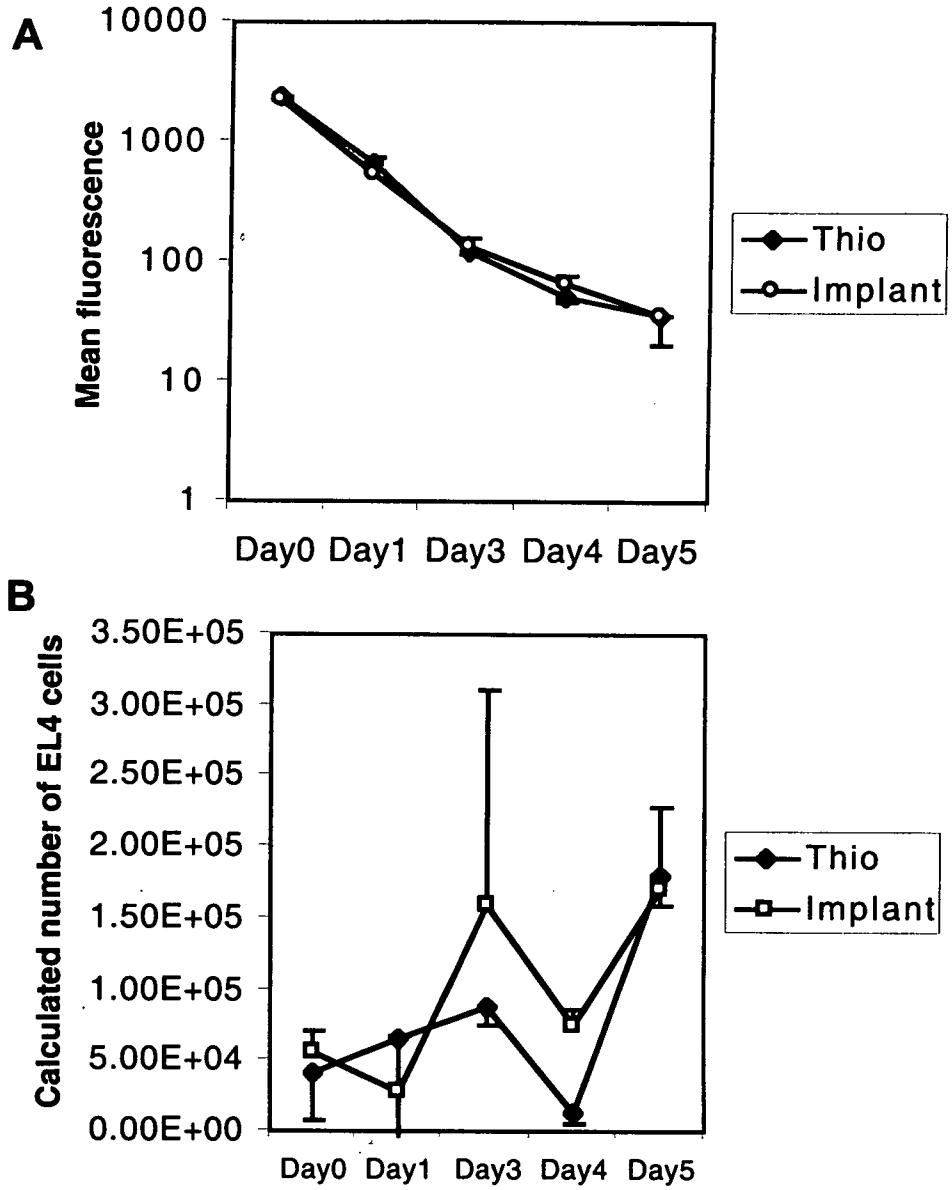


Figure 6: Parasite implantation does not prevent cell division of CFSE labelled EL4 cells in an *in vivo* time course experiment. **(A)** As described in Figure 5, the loss of mean fluorescence of EL4 cells in M1 of the FACS analysis indicates cell division. Data shown are mean ± SD of 2 mice per group, over the different time points. **(B)** The percentage of cells in M1 (of the ungated population), indicates the number of EL4 cells recovered, by taking into account the total number of PEC from each individual mouse. Data shown are mean ± SD of 2 mice per group, over the different time points.

We found that EL4 cells divided at a relatively similar pace over time, in the peritoneal cavity of parasite implanted mice and thioglycollate treated mice, and there were no significant differences in the mean fluorescence of these cells over time (Figure 5 and Figure 6A). Furthermore, there were again no significant differences in the calculated number of EL4 cells recovered from the peritoneal cavity after injection (Figure 6B). The numbers of EL4 cells recovered increases over time as they expand *in vivo*, but quite a few cells might have migrated away from the peritoneal cavity or are destroyed by resident cells (Figure 6B). Considering that we injected $2x10^6$ cells (i.p.), the numbers that we recover from the peritoneal cavity are very small (starting at $5x10^4$).

These results suggest that while suppressive macrophages induced by *B.malayi* can suppress the proliferation of EL4 cells that are co-cultured *in vitro* very effectively, they are unable to prevent the *in vivo* expansion of these cells in the peritoneal cavity.

Suppressive AAMΦ cannot prevent MHC mediated host rejection

Since the suppressive macrophages induced by *B. malayi* exhibit properties that suggest immuno-modulatory function, such as the ability to block proliferation of T cells and induce Th2 differentiation of naïve T cell, we decided to investigate whether the they could prevent MHC mediated rejection. We purified $F4/80^+$ cells from resident peritoneal cells from control CBA/Ca mice, as well as from PEC of parasite implanted CBA/Ca mice. $F4/80^+$ cells were labelled with CFSE and injected into the peritoneal cavity of either allogeneic BALB/C mice or syngeneic CBA/Ca mice. Since the $F4/80^+$ maccrophages do not proliferate, they should retain their CFSE stain for a considerable period of time. 3 weeks later, we removed the peritoneal cavity cells and analysed for the presence of CFSE stained cells by FACS. In the control mice, where the $F4/80^+$ cells from parasite implanted CBA/Ca mice had been transferred back into naïve CBA/Ca mice, we could detect the presence of CFSE labelled cells (Figure 7A). This demonstrated that CFSE stained cells could be detected after 3 weeks and that at least some of the $F4/80^+$ cells remained in the peritoneal cavity and did not migrate away. As expected, resident $F4/80^+$ mice from

control CBA/Ca mice were rejected when transferred into BALB/C mice, since no $CFSE^+$ cells were detected (Figure 7B). Unfortunately, this was also the case for suppressive $F4/80^+$ macrophages from parasite implanted mice (Figure 7C). This experiment clearly showed that the suppressive macrophages did not have the immuno-suppressive capabilities of preventing MHC mediated rejection.

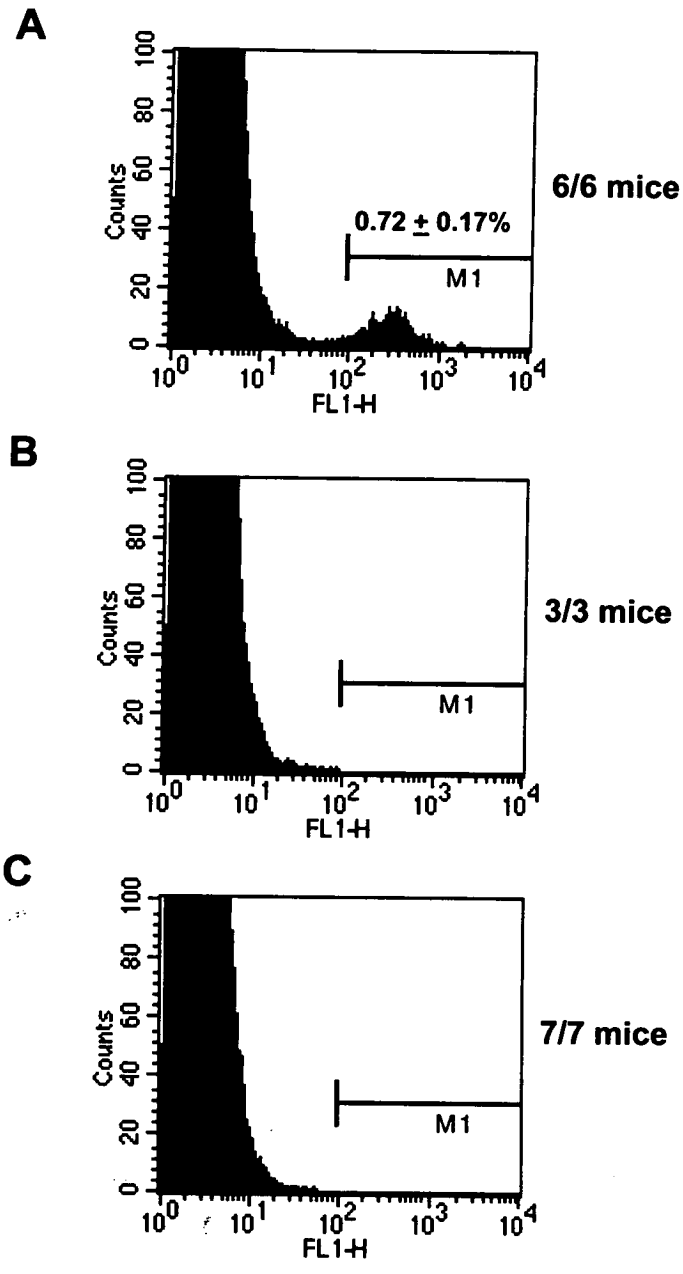
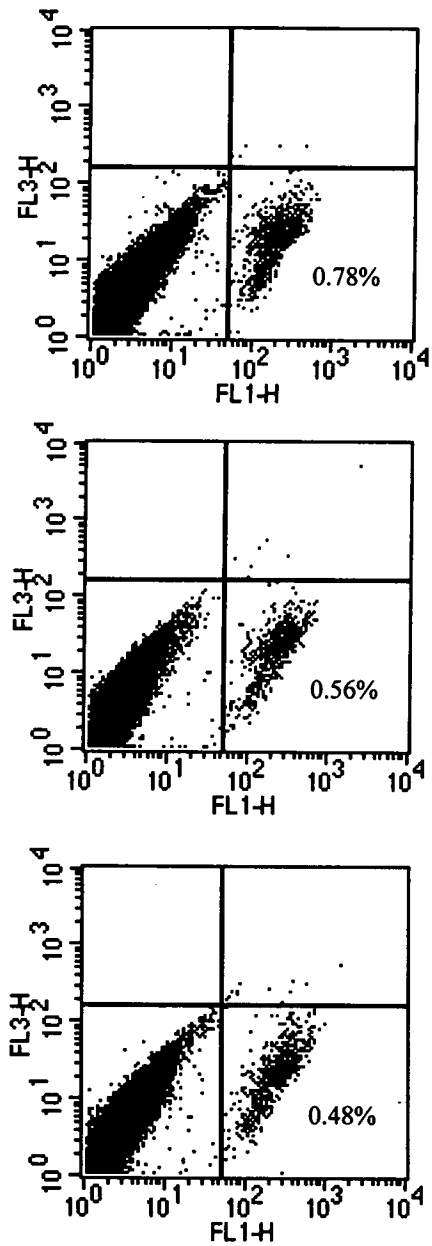


Figure 7:Suppressive macrophages cannot prevent MHC mediated rejection. F4/80+ purified macrophages from PEC of control CBA/Ca mice and parasite implanted CBA/Ca mice were labelled with CFSE and transferred into the peritoneal cavity of naïve BALB/C mice (B & C). $1x10^6$ cells were transferred into each mouse. As a control, F4/80+ purified macrophages from PEC of parasite implanted CBA/Ca mice were transferred into naïve CBA/Ca mice (A). After 3 weeks, total PEC population of the BALB/C (B & C) and CBA/Ca (A) mice were removed and assayed by FACS for the presence of CFSE+ macrophages. Data shown is from one mouse of several in each group, as shown besides the figures, all of which gave identical results.

AAMΦ and H-Y antigen mediated host rejection

Since suppressive macrophages could not prevent MHC mediated rejection, we investigated whether they could prevent rejection as a result of minor histocompatibility antigen differences. To investigate the male HY antigen mediated rejection, we implanted parasites into male and female CBA/Ca mice and purified the $F4/80^+$ cells from these mice 3 weeks later. As a control, we also purified $F4/80^+$ cells from resident peritoneal cells from male mice. All the $F4/80^+$ cells were then labelled with CFSE before being transferred into recipient mice. We injected 1 x 10^6 $F4/80^+$ cells from resident male peritoneal cells into the peritoneal cavity of naïve female mice (n = 3). We then injected 1 x 10^6 $F4/80^+$ cells from parasite implanted female mice into the peritoneal cavity of naïve female mice (n = 5). We also injected 1 x 10^6 $F4/80^+$ cells from PEC of parasite implanted male mice into peritoneal cavity of naïve female mice (n = 5). We also decided to inject the same number of $F4/80^+$ cells from parasite implanted male mice into the footpads of naïve female mice (n = 5), because we hoped that the macrophages would migrate to the popliteal lymph nodes and have a better chance of encountering naïve T cells than in the peritoneal cavity.

Almost 4 weeks later (26 days), we removed either the PEC or the popliteal lymph nodes from the recipient mice and analysed the cells by flow cytometry. Unfortunately, the popliteal lymph nodes of recipient female mice, which had received footpad injections of macrophages from parasite implanted male mice, did not contain any $CFSE^+$ cells at all. The suppressive macrophages had clearly not migrated to the lymph nodes as expected. Furthermore, $CFSE^+$ cells were observed in the control group of female mice (Figure 8a, n = 3/3), which had received resident macrophages from male mice, and had therefore not rejected these macrophages as a result of HY mismatch. Hence, it was no surprise that the suppressive macrophages from parasite implanted mice were also not rejected from recipient female mice (Figure 8c, n = 5/5 male→female, Figure 8b, n=5/5 female→female). Due to the absence of rejection in the control group, the question of whether parasite recruited suppressive macrophages can prevent minor histocompatibility antigen mediated rejection remains uncertain.



Control F4/80+ cells
male → female (i.p.)
3/3 mice

Implant F4/80+ cells
female → female (i.p.)
5/5 mice

Implant F4/80+ cells
male → female (i.p.)
5/5 mice

Figure 8: HY mediated rejection does not occur in CBA/Ca mice. F4/80+ purified macrophages from PEC of control CBA/Ca mice and parasite implanted CBA/Ca mice were labelled with CFSE and transferred into the peritoneal cavity of male or female CBA/Ca mice. $1x10^6$ cells were transferred into each mouse. After 4 weeks, total PEC population of the recipient mice were removed and assayed by FACS for the presence of CFSE+ macrophages. Data shown is from one mouse of several in each group, as shown besides the figures, all of which gave identical results.

Discussion

We have found that the recruitment of suppressive macrophages by implantation of *B. malayi* into the peritoneal cavity of mice does not prevent the proliferation of EL4 cells *in vivo*. However, this is not due to the differentiation of mutant EL4 cells that are no longer susceptible to macrophage suppression, because the injected EL4 cells remain suppressed when they are cultured in the presence of suppressive macrophages *ex vivo* from parasite implanted animals. There is also no indication that the injection of EL4 cells into the peritoneal cavity of parasite implanted mice will result in the loss of suppressive macrophages, since adherent PEC from these mice retain the ability to suppress proliferation of new EL4 cells that are co-cultured *in vitro*. It is possible that EL4 cells are simply too aggressive and the peritoneal cavity is too open a space for sufficient cell-cell contact to occur.

These results suggest that suppressive macrophages can only inhibit the proliferation of cells that are in close contact, as would be expected from a receptor mediated mechanism of proliferative suppression. In the peritoneal cavity, EL4 cells that do not come into contact with suppressive macrophages, or migrate away from suppressive macrophages, would not be preventing from dividing. This is also consistent with the observation that suppressed cells can recover from proliferative inhibition after being removed from contact with suppressive macrophages. These results imply that suppressive macrophages would not be effective in the prevention of tumour proliferation, especially after metastasis. The suppressive effect is restricted to cells that are directly in contact with the macrophages, for example, in the context of T cell/APC interaction.

It was perhaps too optimistic to predict that the suppressive macrophages could prevent allogeneic rejection, because approximately 2-10% of T cells are alloreactive (Beretta et al., 1986; Lindahl and Wilson, 1977a; Lindahl and Wilson, 1977b), whereas T cell frequencies specific for an infectious organisms is between 1 in 10^{-5} -10^{-6}. Interestingly, it is still unclear why there is such a high frequency of alloreactive T cells and hence such a vigorous rejection response. Since the suppressive macrophages could not prevent MHC antigen mediated rejection, we decided to turn

to minor histocompatibility antigens differences, which are analogous to foreign peptide fragments that are presented on host MHC-molecules. The H-Y male transplantation antigen serves as a convenient model for our analysis (Simpson et al., 1997). Unfortunately, due to my ignorance I did not realise that the CBA/Ca strain, like other H-2^k strains, are probably non-responders in terms of H-Y mediated rejection (Simpson et al., 1997). We are currently repeating these experiments using C57/BL6 (H-2^b) mice, which are well characterised in terms of being able to rapidly reject male skin grafts. We will also transfer PEC from IL-$4^{-/-}$ parasite implanted mice to compare rejection rates with suppressive macrophages from WT mice.

Chapter 7

MOLECULAR CHARACTERISATION OF AAMΦ AND THE IDENTIFICATION OF IL-4 DEPENDENT GENES?

Summary

In order to identify the suppressive mechanism, we decided to take a molecular approach towards characterising the suppressive AAMΦ recruited by *B. malayi*. cDNA libraries were constructed from suppressive F4/80^{+} macrophages purified from parasite implanted IL-5$^{-/-}$ mice and non-suppressive macrophages from parasite implanted IL-4$^{-/-}$ mice. An expressed sequence tag (EST) approach was taken to analyse genes expressed by these 2 macrophage populations. 252 clones were sequenced from the suppressive macrophages and 213 clones were sequenced from the non-suppressive macrophages. This analysis highlighted a number of interesting genes that led to further analysis. Furthermore, we constructed a subtractive cDNA library using 2 rounds of subtraction of photo–biotinylated non-suppressive IL-4$^{-/-}$ RNA from the WT suppressive macrophage cDNA. Between 10-20,000 primary recombinant were obtained. 700 clones were picked and screened for inserts. 400 clones had inserts greater than 500bp. 176 clones were screened for differential expression of which 22 clones potentially showed higher expression in the WT macrophages and were sequenced. 5 of these clones represented transcripts of a gene that has very recently been described as FIZZ1, and is an abundantly expressed protein found in the bronchoalveolar lavage fluid (BALF) of ova-induced asthmatic mice, which affects neuronal function. This gene was also identified as being potentially differentially expressed from the EST analysis of IL-5$^{-/-}$ and IL-4$^{-/-}$ macrophages. Our gene expression studies suggest that IL-4 dependent macrophages that have evolved to play a role in expulsion of nematode parasites, could induce changes in the responsiveness of smooth muscle cells, which is an important feature of clinical asthma. These preliminary studies encourage a more detailed genomic characterisation of the 2 different macrophage populations.

Introduction

In the previous chapters I have discussed the IL-4 dependent suppressive mechanism that is exerted by the macrophages induced by *Brugia malayi*. In order to identify this suppressive mechanism, as well as to describe IL-4 dependent genes that are expressed in these suppressive macrophages, I decided to embark on a molecular biology strategy. This is in part due to the close proximity to labs that have expertise in undertaking genomic approaches towards answering biological questions. The revolution in sequencing technology has made such approaches both relatively easy as well as cost effective. In order to be able to compare genes expressed in suppressive macrophages with non-suppressive IL-$4^{-/-}$ macrophages, I constructed 2 different cDNA libraries. I chose to construct a cDNA library of suppressive macrophages from parasite implanted IL-$5^{-/-}$ mice in order to reduce the possibility of contamination with eosinophils. I also constructed a cDNA library from non-suppressive IL-$4^{-/-}$ macrophages.

Since all cells within an organism contain the same genetic information, it is the variation in gene expression that defines the phenotypic characteristics of a particular cell type. This fundamental aspect of biology is a crucial factor in our understanding of developmental biology, oncogenesis as well as immune function. Thus, several techniques have been invented to identify tissue specific gene expression. These are "differential display" (DD), subtractive hybridisation (SD), and more recently, SAGE (or serial analysis of gene expression) and DNA microarrays. However, a direct random sequencing approach can also be very informative.

An expressed sequence tag (EST) strategy is a very rapid and informative technique that is useful for analysing gene expression of specific cells or organisms (Adams et al., 1991). Randomly selected clones from a cDNA library are sequenced once only. By carrying out a non-bias survey sequencing expedition, we can identify abundantly expressed genes at random. This is essentially an indirect survey of a gene expression profile from a particular cell type or developmental stage in an organism. At the NCBI depository of EST sequences, they have set up a web based comparitive tool that is called digital differential display (DDD), which essentially relies on the

Fisher's exact test for a statistical comparison of the representation of genes in different sets of cDNA libraries (http://www.ncbi.nlm.nih.gov/CGAP/info/ddd.cgi). We decided that this was a viable strategy for a rapid comparison of abundantly expressed genes between the 2 different macrophage populations. However, we also decided that one of the more directed approaches towards establishing differential gene expression would be a complementary strategy. Especially since a large number of ESTs are necessary for statistically significant conclusions.

One of the first techniques that became widely used for this purpose was invented by Liang and Pardee (Liang and Pardee, 1992), and is termed "Differential Display" (DD). Briefly, this PCR based technique relies on 2 sets of oligonucleotide primer. The first set is anchored to the poly-A tail of mRNAs and the other set is short (10-13bp) and random in sequence. This combination amplifies a subpopulation of the genes expressed and can be resolved on a DNA sequencing gel. Differences in gene expression would be identified as differing bands resolved on the sequencing gel, which can be PCR amplified further for cloning purposes. The problem with this technique is that it is extremely labour intensive because the differentially displayed bands have to be cloned individually and multiple clones from each band has to sequenced. Furthermore, there is a high percentage of false positive bands. Cloned bands are also often very short (<500bp) and are usually in the non coding region at the 3' end of mRNA, which makes homology searching extremely difficult. Nonetheless, it has been used widely and is referred to in more than 3000 papers.

An alternative to this approach is to use "subtractive hybridization", which is an even older technique and was used to identify some key genes expressed on T cells (Chien et al., 1984; Littman et al., 1985; Maddon et al., 1985), including CD4 and CD8 (Littman et al., 1985; Maddon et al., 1985). As the name suggests, this technique involves using a 'driver' population of RNA or cDNA to subtract away common genes that are also expressed in the 'tester' population by hybridization. The most recent variations of this technique is known as subtractive suppression hybridization (SSH), which is based on PCR and was developed by CLONTECH (Diatchenko et al., 1996). This is becoming more widely used because it is of particular advantage under

circumstances where the amount of RNA isolated can be particularly limiting (e.g. from stem cells (Phillips et al., 2000)).

More recently, a technique known as SAGE, or serial analysis of gene expression, was invented by Velculescu et. al. (1995). This technique works on a similar principle to using EST data to profile gene expression, since the number of times an EST sequence is observed reflects the expression level of a corresponding transcript. Genes that are highly expressed would be highly represented in a cDNA library. Based on the principle that short sequence tags of between 10-14bp are sufficient to identify unique genes, special SAGE cDNA libraries are constructed by ligating these short sequence tags into longer serial molecules that can be cloned and sequenced (Velculescu et al., 1995). Thus, a sequencing reaction that produces a read of 5-600bp would provide information equivalent to about 50 EST sequences instead of just one EST read in a conventional cDNA library. Using this technique, Hashimoto et. al made a comprehensive comparison of the gene expression profiles, by assaying in excess of 50,000 tags from (1) human monocyte, (2) macrophage matured from monocytes with M-CSF, (3) macrophage matured from monocytes with GM-CSF and (4) dendritic cells matured from monocytes with GM-CSF, IL-4 and TNF-α (Hashimoto et al., 1999a; Hashimoto et al., 1999b).

Another recent development to analyse differential gene expression is the development of DNA microarray technology. The concept is simple and based on the hybridisation properties of DNA, but has really become feasible as a result of the advancement in high speed robotics. Thousands of either oligonucleotides pairs (e.g. by Affymetrix) or PCR products (e.g. by Incyte) of known as well as unknown genes (i.e. EST clusters) are spotted onto a glass substrate. RNA populations of interests are then usually labelled with fluorescence during conversion to cDNA, before being hybridised to the DNA microarrays. Specialised imaging equipment that is closely related to a confocal microscope is used to scan for the level of fluorescence on each of the spots on the array. Hence, a single experiment can produce expression data on thousands of known, as well as unknown genes. This is by far the most rapid way of characterising gene expression and has already revolutionised diagnosis of cancer

patients (Golub et al., 1999). However, it depends on several pieces of capital equipment that we do not possess at the moment. Further, the disadvantage of DNA microarrays is that differential expression of genes can only be characterised for known EST clusters. However, DNA microarray technology can be used in combination with clones from subtractive hybridisation libraries. Interestingly, a direct comparison of SAGE with DNA microarray analysis indicated that these 2 methods correlated quite well in terms of describing differentially expressed genes, especially those that are quite abundantly expressed (Ishii et al., 2000).

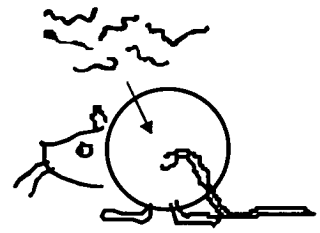
Because we do not have access to the technology necessary for SAGE analysis or DNA microarray analysis, I decided that the most cost and time efficient strategy to identify IL-4 dependent genes would be to use a combination of the 'Clontech' SMART PCR cDNA synthesis kit and the 'Invitrogen' subtractor kit to generate a subtractive cDNA library. This combination relies on a simple hybridization technique using photo-activated biotin and phenol-chloroform extraction, with the SMART system for generating full length, amplified, double stranded cDNA. The PCR amplified cDNA can then be cloned into a mammalian expression vector using the highly efficient new *In vitro*gen TOPO TA cloning system.

Results

EST analysis of suppressive AAMΦ

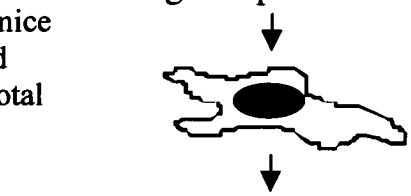
Live adult *B.malayi* parasites were implanted into IL-$5^{-/-}$ mice and the suppressive peritoneal cells were removed 3 weeks later. Total RNA was extracted from the purified F4/80+ macrophages and a unidirectional cDNA library was constructed directly from total RNA using a cDNA library construction kit (Stratagene) with the pCMV-script mammalian expression plasmid (Figure 1). This library contained 5 x 10^5 primary recombinants.

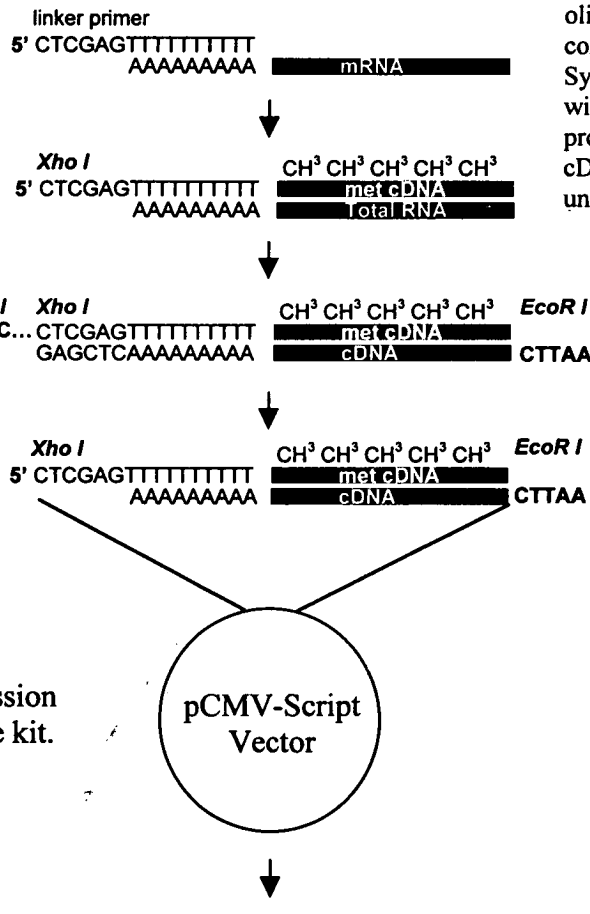
1) Live adult parasites are implanted into the peritoneal cavity of IL-5$^{-/-}$ or IL-4$^{-/-}$ mice for 3 weeks



Brugia implanted mice

2) PEC are removed from mice and F4/80+ cells purified using magnetic beads. Total RNA is extracted from purified macrophages





1st strand cDNA is made from total RNA using an oligo(dT) linker primer containing an XhoI site. Synthesis is performed with 5-methyl dCTP to produce hemimethylated cDNA with an unmethylated 5' end

2nd strand cDNA is made with RNaseH and DNA polymeraseI and ligated with EcoRI adapters. XhoI digestion produces vector ready cDNA for unideractional cloning into pCMV-Script Vector. Hemimethylated cDNA is protected from being digesteded by XhoI .

3) Plasmid unidirectional cDNA libraries are constructed into pCMV-script mammalian expression vector using a Stratagene kit.

XL10-Gold ultracompetent cells

Figure 1: Protocol for the construction of cDNA libraries from parasite recruited macrophages

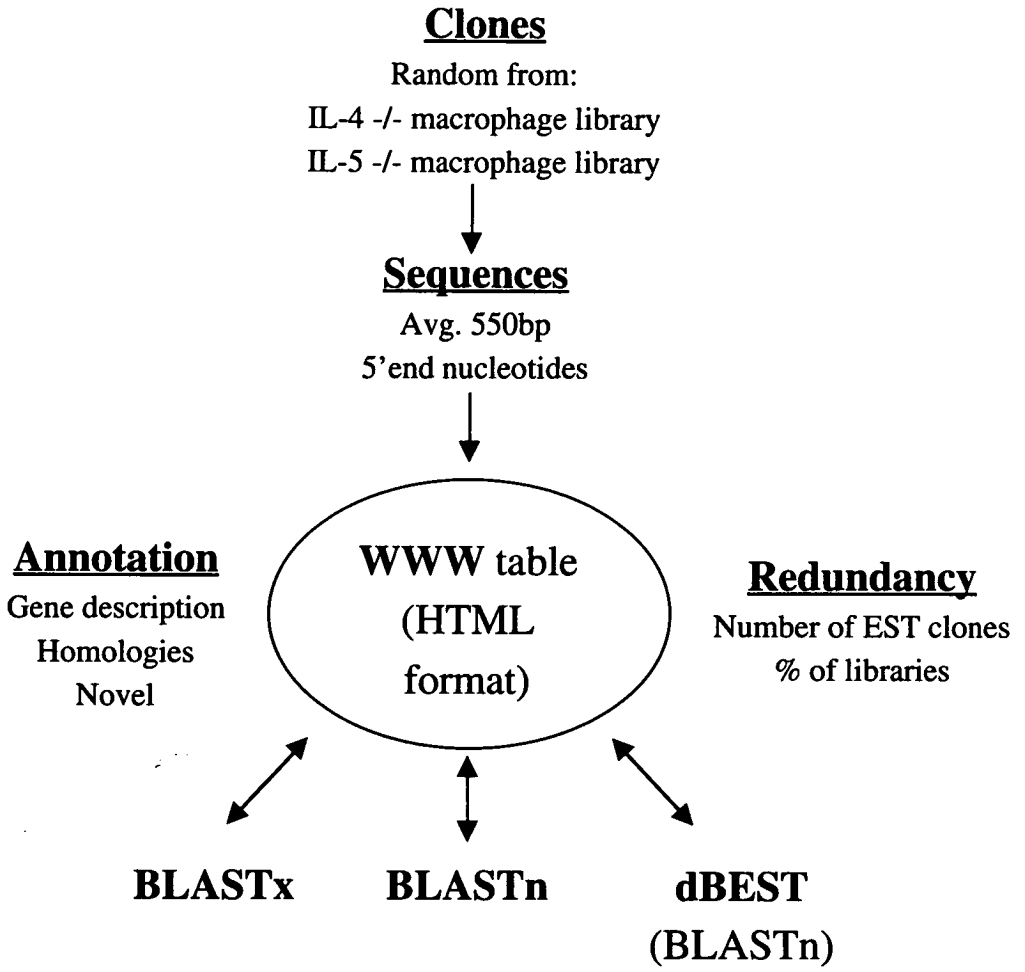


Figure 2: Data analysis of the EST sequences. The sequences from the EST project are sent to NCBI for 3 different blast searches; 1) Blastn against genbank, 2) Blastx for protein homologies and 3)Blastn search against EST databases (dBest). The results from the blast searches are downloaded from NCBI and linked to the sequence information through a WWW table, where all the sequence data and blast search results can be viewed and edited online. Redundant clones were identified by clustering the sequences with 'Assemblyline' software.

Expressed sequence tags (EST) of randomly selected clones provide a snapshot of the abundant genes that are expressed in a particular cell type (Figure 2). A total of 252 clones, from the cDNA library made from suppressive AAMΦ, were successfully sequenced from the 5' end (Table 1). Some of the genes highly represented in the library such as Arginase I and a scavenger receptor (SP-α), have been shown to be induced by Th2 cytokines in macrophages (Goerdt and Orfanos, 1999). We have verified that arginase I is indeed up regulated by parasite implantation (Figure 3). These data supported our classification of *B. malayi* recruited macrophages as AAMΦ.

We also observed an abundantly expressed transcript that has no homology to any known sequences, and has only been submitted to the NCBI databases as EST sequences. We had decided to temporarily call this gene **P**arasite induced **N**ovel **G**ene (*PNG1*). Unfortunately, it has recently been published as FIZZ1 (Holcomb et al., 2000) (Genbank accession number AF205951). Further description of this gene is described in a following section. However, the most striking finding was the extremely high representation of a gene known as ECF-L/Ym1/ECF-L (Jin et al., 1998) in this library, accounting for 8.5 % of the cDNA clones. This gene has subsequently been identified as an eosinophil chemotactic factor (Owhashi et al., 2000). Further characterisation of ECF-L/Ym1 is the subject of the next chapter.

Table 1: Abundantly expressed genes in the suppressive AAMΦ

Gene name	Number of clones	Percentage of library
YM-1	23	8.5
PNG1/FIZZ1	8	2.9
Serum Amyloid A (SAA)	6	2.2
Arginase	5	1.8
Scavenger receptor (SP-alpha)	3	1.2
Beta actin	3	1.2
LFA-1	3	1.2
Ribosomal phosphoprotein (PO)	3	1.2
ATPase 6	2	0.8
Ferritin light chain	2	0.8
Cytochrome c oxidase	2	0.8
Fc gamma receptor	2	0.8
Cysteine Rich Intestinal Protein (CRIP)	2	0.8
EST	2	0.8
Total 252 clones		ribosomal RNA = 6

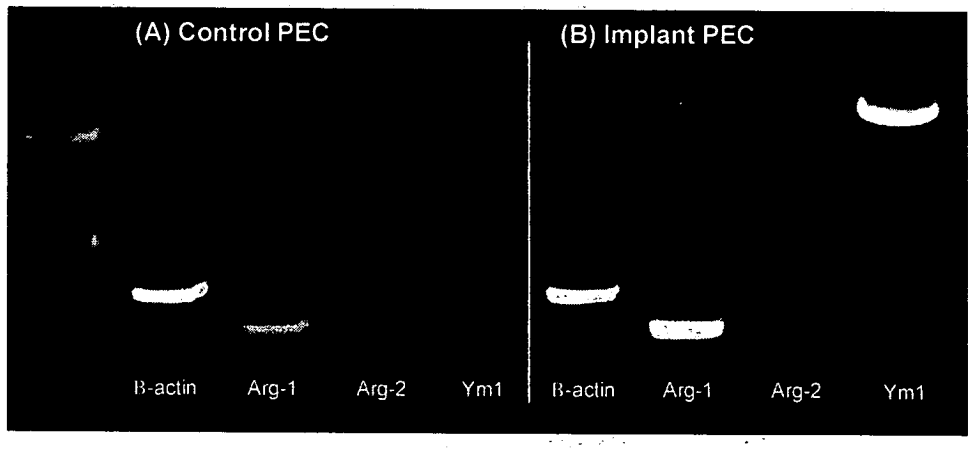


Figure 3: Arginase I is upregulated by parasite implantation.
Total RNA was extracted from PEC of control animals or animals implanted with *B. malayi* and analysed by RT-PCR. After 35 rounds of amplification the PCR reaction was run on a 1% agarose gel for visualisation. While arginase I was upregulated in parasite implanted mice, the PCR product from the arginase II primers were non-specific. Further studies are necessary for the verification of arginase I upregulation in the absence of arginase II.

EST analysis of non-suppressive IL-4 $^{-/-}$ macrophages

As described in the previous section, a cDNA library was constructed from non-suppressive IL-4$^{-/-}$ macrophages. This library was not as good quality as the suppressive macrophage library and only contained 1 X 10^5 primary recombinants. Using an EST approach again, a total of 213 clones were sequenced from this library, which revealed a higher proportion of contaminating ribosomal RNA clones in the library (Table 2). Interestingly, Ym1 was again by far the most abundantly expressed transcript, representing 4.7% of the clones. This indicated that the induction of Ym1 in macrophages recruited by *B. malayi* was not IL-4 dependent, unlike the suppressive mechanism. Interestingly, several abundantly expressed genes in the suppressive macrophage dataset were not equally well represented in this EST project. However, due to the small sample size of the 2 EST projects, it is difficult to determine statistically if these differences are significant. We plan to sequence a much larger number of EST clones from these 2 different cDNA libraries (see Discussion).

Table 2: Abundantly expressed genes in the non-suppressive IL-4$^{-/-}$ macrophages

Gene name	Number of clones	Percentage of library
YM-1	10	4.7
Thymosin beta 4	3	1.4
Translationally regulated transcript (21 kDa (Trt)	3	1.4
C1q C chain	2	0.94
Lactate dehydrogenase 1	2	0.94
DORA cell surface receptor	2	0.94
Zinc finger protein 30	2	0.94
Ribosomal protein L7a	2	0.94
Ribosomal protein L12	2	0.94
Calcium/calmodulin-dependent protein kinase (CAMK1)	2	0.94
Ribosomal protein L35	2	0.94
Ferritin light chain 1	2	0.94

Total 213 clones ribosomal RNA = 20

Construction of subtractive cDNA library (Figure 4)

To construct a subtractive library, total RNA was extracted from F4/80^{+} purified macrophages from PEC of either WT implanted mice or IL4$^{-/-}$ mice. 1μg of total RNA was converted into 1st strand cDNA using the Clontech SMART system. A modified oligo(dT) primer (with a specific 3' PCR primer sequence) primes the synthesis of 1st strand cDNA from the mRNA portion of total RNA. When the RT reaches the 5' end of mRNA, the enzymes terminal transferase activity adds primarily deoxycytidines(C) to the 3' end of the cDNA. The CLONTECH SMART oligo contains an oligo(G) sequence at it's 3' end, which base-pairs with the dCTP stretch and creates an extended template for the RT to continue replicating until it reaches the end of the SMART oligo. This results in full-length cDNA that contain the complete 5' end of the mRNA as well as incorporating sequences that are complemetary to the SMART oligo. These sequences can be used to prime the amplification of full length cDNA together with the additional sequences incorporated into the oligo(dT) primer. Only full length transcripts that have incorporated the SMART oligo sequence will be amplified. This is an ideal system to amplify the rare transcripts that remain after subtractive hybridisation of the 1st strand cDNA made from suppressive WT macrophages with the non-suppressive IL-4 $^{-/-}$ macrophages.

After the synthesis of first strand cDNA from WT macrophage, 2 rounds of subtractive hybridisation were undertaken with 20 times the amount of IL-4$^{-/-}$ macrophage RNA (20μg) as was used in the 1st strand synthesis reaction of WT macrophage RNA. After 2 rounds of subtractive hybridisation and phenol-choloroform extraction, the remaining single stranded cDNA was PCR amplified for 25 cycles. The PCR products were then size separated on a GIBCO sepharose size selection column, which removes DNA fragments of less than 500bp. We cloned the 5 different size selected fractions separately in order to reduce cloning bias of small DNA fragments. Since the subtractive template was PCR amplified with TAQ polymerase, which adds a single deoxyadenosine (A) to the 3' end of the product, the most efficient technique is to use the TOPO cloning system by Invitrogen to clone the subtracted/amplified cDNA. This technology relies on the ligation activity of topoisomeraseI that is attached to the linearized "activated" TA vector, which enables

greater than 95% efficiency of recombinant cloning in 5 minutes. The ligated plasmids were then transformed into XL-10 gold ultracompetent cells from Stratagene. Using this combination of kits, I managed to generate a combined subtractive cDNA library with between 10-20,000 primary recombinants.

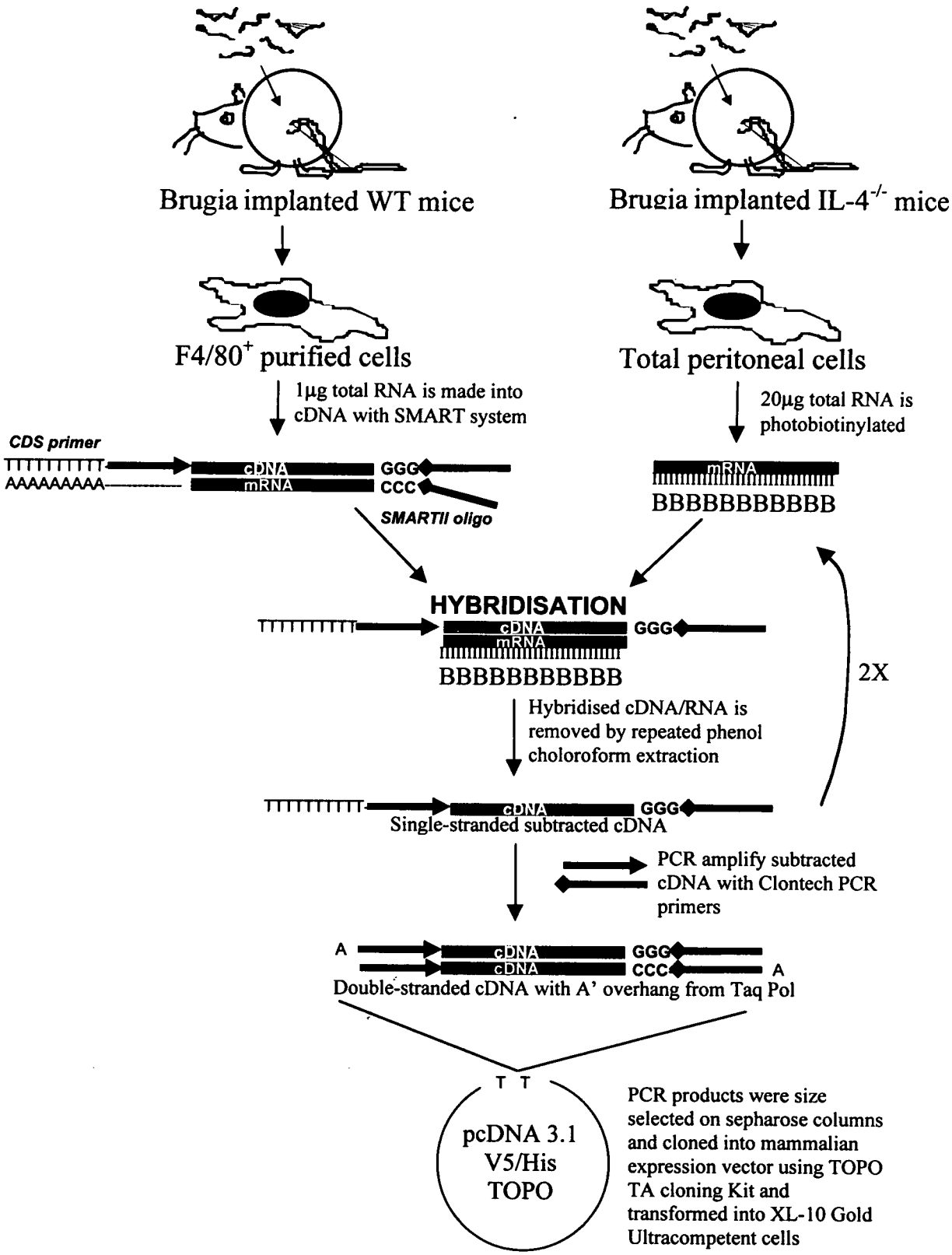


Figure 4: Strategy for the construction of an IL-4 dependent subtractive cDNA library.

Analysis of subtractive cDNA library

We picked 700 clones from the subtractive library and screened for inserts by PCR. Between 60 and 70% of the clones had inserts greater than 500bp and were selected for subsequent analysis. PCR products from approximately 400 clones were blotted onto nitrocellulose membranes to form self-made macro-arrays to analyse differential gene expression and to test the efficiency of the subtraction procedure. Due to the limitations of time, only 2 nitrocellulose membranes were analysed (Figure 5), representing 176 clones. Radioactively labelled 1st strand cDNA was made through a RT reaction with ^{32}P-dCTP, from total RNA of PEC from WT mice and PEC from IL-4$^{-/-}$ mice. RNA from total PEC were used instead of F4/80^{+} purified macrophages due to the limitations of material, especially since this was intended to be a preliminary analysis. The cDNA probes were used to probe 2 duplicate nitrocellulose membranes, one for WT PEC and the other for IL-4$^{-/-}$ PEC. From this preliminary analysis, 22 clones showed potential for being more highly expressed in WT PEC in comparison to IL-4$^{-/-}$ PEC, and the PCR products were selected for sequencing.

It has not been technically possible to sequence PCR products through the poly(A) tail on the 3' end of transcripts. Since the subtractive cDNA library was not cloned unidirectionally into the plasmid vector (unlike the conventional cDNA libraries), we encountered the problem of having 50% of the transcripts cloned in the wrong orientation. In order to circumvent this problem, I designed a special sequencing primer (SMART seq primer) that incorporated the dCTP stretch resulting from the terminal transferase activity of RT when it reaches the end of mRNA, and should be restricted to only the 5' ends of all the transcripts that have been cloned. The 3 additional base pairs of deoxycytidines (C), with an additional degenerate base, was sufficient to prime 5' specific sequencing reactions, thus enabling us to sequence all the PCR products from the 5' end of the transcript using the same primer irregardless of which orientation the transcript had cloned into plasmid vector. This primer (5'-AAC GCA GAG TAC GCG GG (AGCT)-3') will be used to sequence all the differentially expressed clones from the subtractive library.

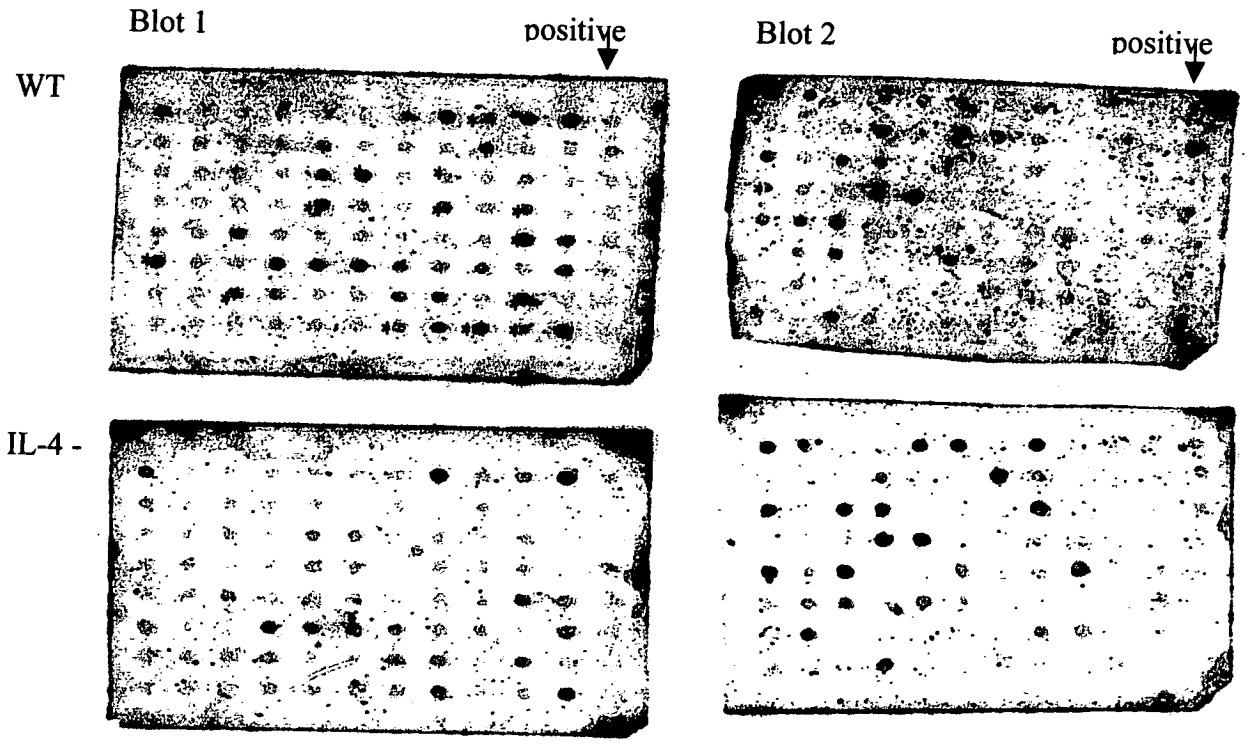


Figure 5: Blots hybridised with first strand cDNA of peritoneal cells from implanted WT and IL-4 $^{-/-}$ *Brugia*-implanted mice and exposed to the phosphoimager. Positive controls included β-actin, YM-1 and β2 microglobulin. * indicates clones selected for sequencing.

Of the 22 clones we sequenced (Table 3), 5 clones represented *PNG1/FIZZ1* and 2 clones represented serum amyloid A3 (SAA3). Both of these genes were highly represented in the suppressive macrophage library (Table 1), but not found in the non-suppressive IL-4 deficient macrophage EST project (Table 2). This result suggested that a combination of EST sequencing and analysis of the subtractive library would produce complementary data. However, from this preliminary screen of the subtractive library, we also picked up clones representing β-actin and β2-microglobulin, which should not be differentially expressed between the 2 different macrophage populations. This could be the result of screening with cDNA isolated from total peritoneal exudate cells instead of purified macrophages. It also suggested that we should refine our differential screening for the future.

Table 3: Potentially differentially expressed clone from the subtractive library

Genes potentially upregulated in AAM	**Frequency**
PNG1/FIZZ1	5
Serum amyloid A3	2
BAC 271B7 chromosome X Gabre gene	1
β-Actin	1
β_2-microglobulin	1
Cyclin ania-6b	1
Cysteine-rich intestinal protein	1
Leukotriene C4 synthase	1
Mitochondrial protein	1
Ribosomal protein S7	1
Ribosomal protein S18	1
Failed Sequences	6

PNG1/FIZZ1

PNG1/FIZZ1 (Figure 6) was highly represented in the suppressive macrophage library (almost 3% of the library) and represented the second most abundantly expressed gene after Ym1/ECF-L. Furthermore, this gene was not found in the EST project on the non-supressive IL-4 deficient macrophages. It was also highly represented in the subtractive cDNA library. All this evidence clearly suggests that this abundantly expressed gene is IL-4 dependent. Preliminary RT-PCR analysis by Meera Nair has confirmed that this gene is indeed upregulated in WT suppressive macrophages, athough it is also expressed at a basal level in resident peritoneal cells as well as non suppressive IL-$4^{-/-}$ macrophages.

Since this protein did not have any clear homologues via the basic BLAST searches, I decided to utilise a number of Web based bioinformatic tools for protein property predictions to analyse the amino acid sequence of this novel EST. The amino acid sequence was submitted for simultaneous prediction by several servers via the "Predict Protein" Website (http://dodo.cpmc.columbia.edu/predictprotein). Using the SignalP WWW server (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen et al., 1997), a clear signal peptide was predicted with the most likely cleavage site being between amino acids at position 23 and 24 (VNT-DE) (Figure 7).

A PROSITE motif search (http://www.expasy.ch/prosite/) revealed potential protein kinase C phosphorylation sites at positions 57 (SVK) and 104 (TAR). The serine at position 57 was also predicted to be phosphorylated by the NetPhos server. Another PROSITE predicted motif is the presence of a N-myristoylation site at position 70 (GMTATG), with the prediction pattern being G[^EDRKHPFYW].{2}[STAGCN] [^P]. Serine residue number 47 is predicted to be O-glycosylated by the NetOglyc WWW server, which produces neural network predictions of mucin type GalNAc O-glycosylation sites in mammalian proteins.

FIZZ1 has also been identified as a major protein band, from SDS-acrylamide gel analysis, of bronchoalveolar lavage fluid (BALF) from mice with experimentally induced asthma (via ovalbumin). Expression of this gene was confirmed to be

upregulated in the bronchial mucosal epithelial cells during pulmonary infammation. It is expressed at a low level in small clusters of epithelial cells in a control lung. The authors suggest that this protein could be involved in modulate neuronal function, because it antagonises the neurotrophic effect of NGF *in vitro* (Holcomb et. al., 2000).

It is important to note that Holcomb et.al. did not find the expression of this protein in the alveolar macrophages of the inflammed lung. Interestingly, Lee et.al. have described lung interstitial F4/80+ macrophages in another experimentally induced asthma model, which share many similar properties to our suppressive macrophages. This is more evidence that AAMΦ recruited in parasitic nematode infection could be very similar to the ones induced under type 2 conditions in pulmonary inflammation.

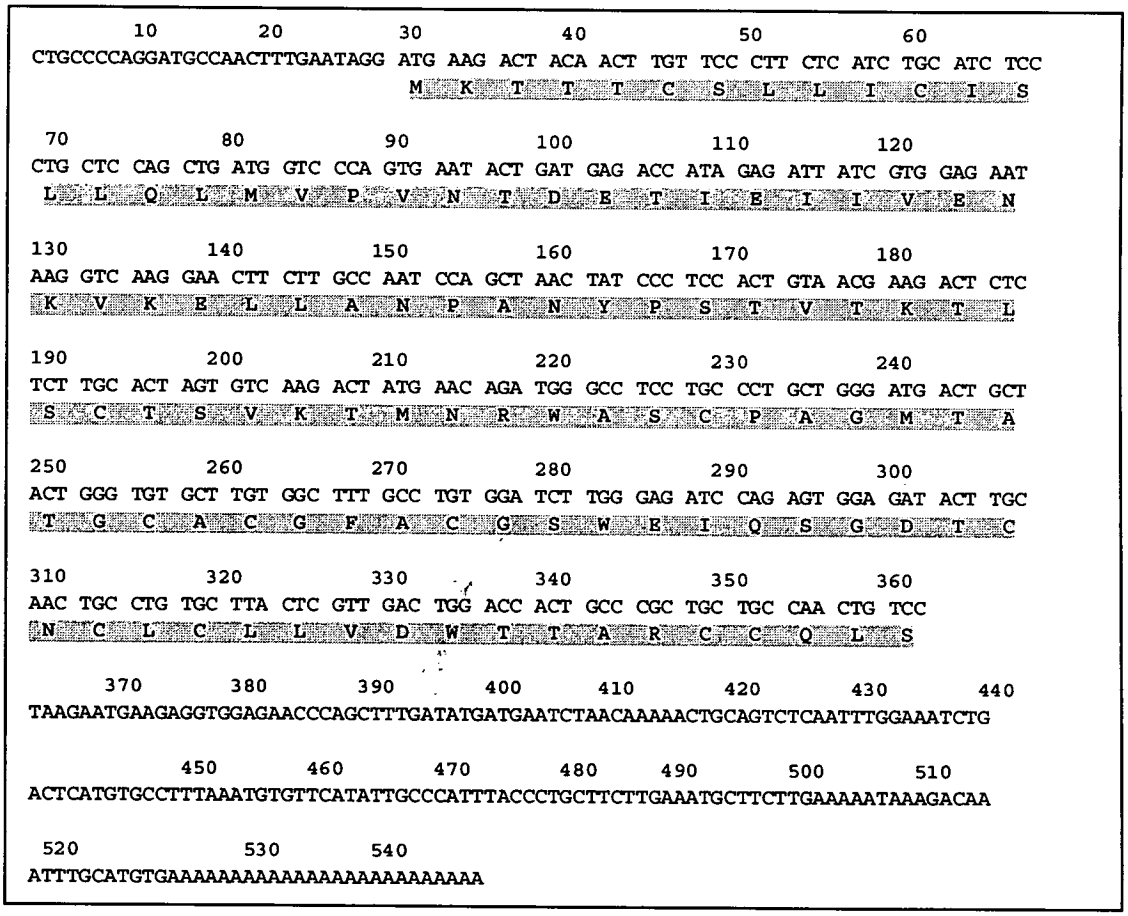


Figure 6:Nucleotide and protein sequence of *PNG1/FIZZ1*. The protein is predicted to have 111 amino acids, which is consistent with protein sequencing data from the identification of FIZZ1 protein (AF205951) as a major protein band in the bronchoalveolar lavage fluid in murine ova-induced asthma. There are 2 other homologous mouse genes and two human genes have been identified (Holcomb et. al., 2000).

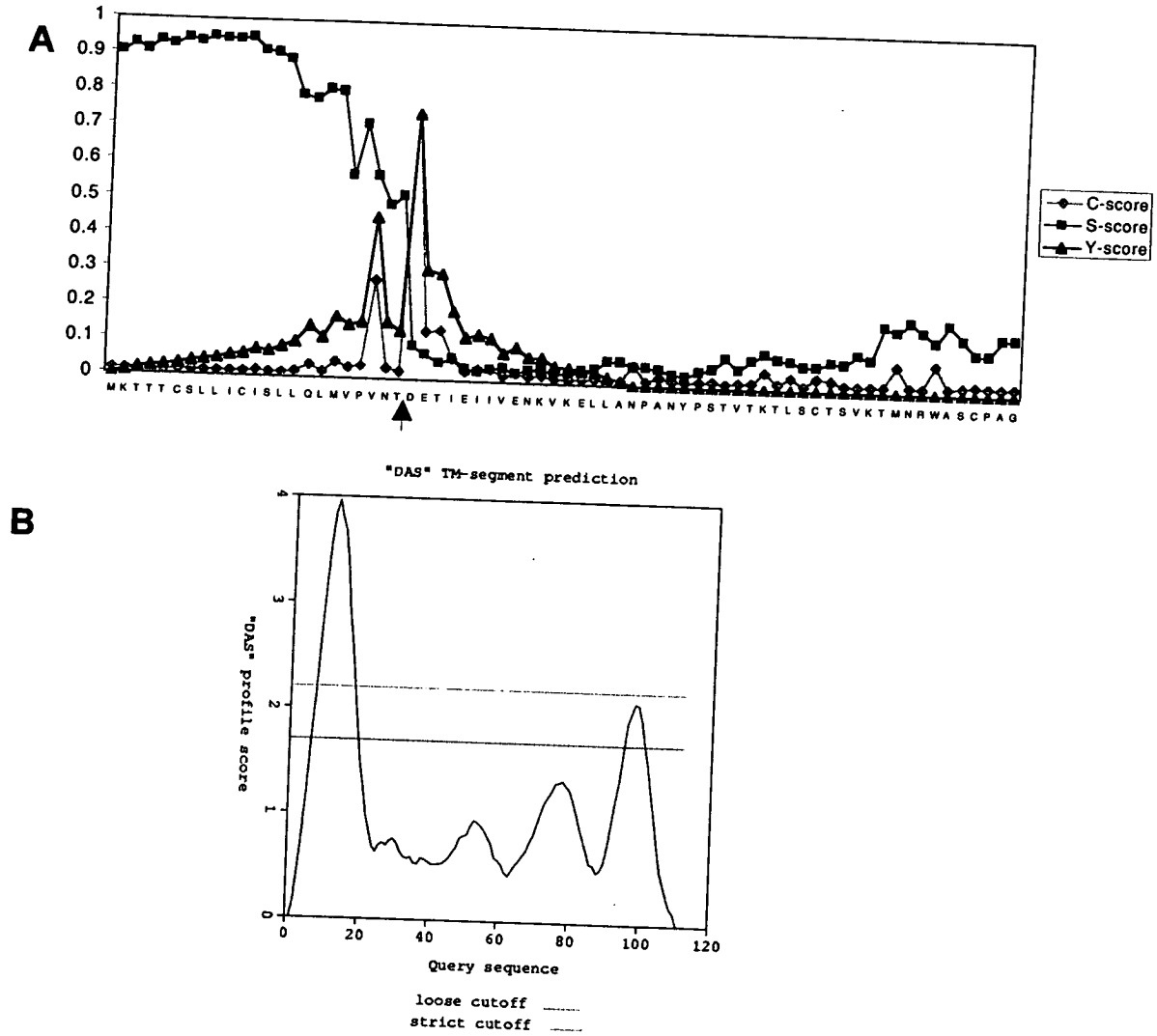


Figure 7: (A) PNG1/FIZZ1 has a clear signal peptide, with the most likely cleavage site at position 23 and 24. This signal peptide was predicted using the SignalP WWW server. The signal peptide prediction is consistent with the prediction of transmembrane domains (B), which was predicted using the "predict protein" webserver.

Discussion

The primary aim of this chapter was to begin characterising the genes that are expressed by AAMΦ recruited by *B.malayi*. Although small, the EST data sets have already provided us with significant information about the biology of these macrophages. More importantly, it has highlighted a number of extremely interesting genes that could play an important role in the biological function of these macrophages. It led to our significant interest in the Ym1/ECF-L gene, although unfortunately, the publication describing the characterization of this gene as a eosinophil chemotactic factor occurred during the midst of our experiments (Owhashi et al., 2000). However, this initiated a whole new set of experiments that are described in the next chapter.

We are also now very interested in the expression and function of Arginase I (Figure 3), which appears to be one of the most robust markers for alternative activation. Munder et.al. found that Th2 cells induced high levels of arginase in macrophages that were used as APCs to present antigen. This was in contrast to Th1 cells, which induced upregulation of iNOS. Interestingly, the effects of the Th2 cells were much higher than individual Th2 cytokines alone, suggesting a synergistic effect of multiple factors produced by Th2 cells. Furthermore, it was only the hepatic arginase I isoform that was induced by Th2 cells, and not the extrahepatic arginase II. This was consistent with our findings since only arginase I is highly represented in the suppressive macrophage library and appears to be upregulated by parasite implantation.

From the preliminary analysis of the subtractive library, we established that only 10-20% of the clones are genuinely differentially expressed. Although imperfect, this is likely to be a substantial enrichment of differentially expressed genes. In order to conserve sequencing resources, we will screen the recombinant clones for differential expression before sequencing them individually. This is unfortunately a more labour intensive process than simply sequencing all the clones from the subtractive library.

The results from the subtractive library are gratifying because the data obtained so far from the preliminary characterisation of the subtractive library seems to be consistent and complementary with the EST strategy. The genomic information that we have obtained so far is on an extremely small scale and firm conclusions cannot yet be drawn from our dataset. However, the dataset as it is opens the way for a more detailed analysis of IL-4 dependent genes in these suppressive macrophages. Due to the time limitations of this PhD, this represents the final touch to a 3 year period of work. However, it seems more like the beginning, rather than an end of a scientific study. Thus, the future direction of this work will be discussed in greater detail in the discussion

The original aim of this project was the identification of the suppressive molecule(s) that are involved in the process of proliferative suppression using a molecular strategy. The data presented in this chapter as well as the next chapter illustrates that by taking this approach we are beginning to understand a lot more about the biology of our suppressive macrophages than was originally intended. This has offset our original trepidation about whether taking a molecular approach was the most suitable way towards identifying the suppressive mechanism, compared to taking a biochemical approach. There was a substantial likelihood that the suppressive molecule(s) were not expressed on IL-$4^{-/-}$ macrophages as a result of biochemical differences instead of differences in gene transcription. The molecular approach was chosen due to the availability of technical expertise in our surrounding environment. Although we might not ultimately identify the suppressive mechanism using this approach, at least we would have been able to glean some exciting information about alternatively activated macrophages. With the revolution in proteomics coming around the corner, we could be in a position where we could take advantage of this alternative, but equally powerful, biochemical approach towards identifying the suppressive molecule in the near future.

Chapter 8

DOES AAMΦ PLAY A ROLE IN EOSINOPHIL RECRUITMENT?

(The work described in this chapter was the result of a close collaboration between Franco Falcone, Xingxing Zang and myself)

Summary

The recruitment of eosinophils is a common feature of Th2 mediated inflammatory responses against nematode infections as well as allergic responses such as airway inflammatory diseases. By characterising gene expression in AAMΦ recruited by *Brugia malayi* using an expressed sequence tag (EST) strategy, we found a dramatic upregulation of a novel eosinophil chemotactic factor (Ym1/ECF-L), which correlates with the recruitment of eosinophils by the parasite. Recently, it was found that *B. malayi* secretes a homologue (*Bm*-MIF-1) of the human proinflammatory cytokine macrophage migration inhibitory factor (MIF). Administeration of *Bm*-MIF-1 on its own was able to reproduce the effects of live implanted adult parasites, leading both to a marked recruitment of eosinophils and the upregulation of Ym1/ECF-L. When the enzymatic activity of *Bm*-MIF-1 was abolished by mutating the aminoterminal Proline to Glycine (*Bm*-MIF-1G), this abolished the induction of Ym1/ECF-L in macrophages as well as the recruitment of eosionophils. This data suggests that macrophages could mediate the crosstalk between parasites and recruited eosinophils.

Introduction

Infection with nematode parasites is associated with an immune response resulting in elevated IgE, systemic eosinophilia and mast cell proliferation (Maizels et al., 1993). These features reflect a polarized type-2 T-cell response with overproduction of the cytokines IL-4, IL-5 and IL-13, which is reminiscent of Th2 mediated allergic airway inflammation (Wills-Karp, 1999). Another well established feature of the mammalian immune response to nematode infection is the recruitment of eosinophilic granulocytes to host tissues characterised by intense, focussed eosinophilic infiltrates often associated with extensive tissue damage. Despite the importance of this phenomenon, the molecular basis of eosinophil recruitment in helminth infection is still poorly understood (Maizels et al., 1993). It is currently thought that the recruitment of eosinophils to the tissues may be the result of a complex host-parasite interaction in which the parasite induces the production of chemotactic factors (such as chemokines) and cytokines by host cells and/or secretes products that directly target host granulocytes.

After living for 3 weeks in the peritoneal cavity of mice, *Brugia malayi* induces an almost 10 fold increase in total cell numbers ($3.1 \pm 0.8 \times 10^7$) recruited into the peritoneal cavity of implanted mice, in comparison to control mice ($3.8 \pm 1.1 \times 10^6$). The peritoneal exudate cells from implanted mice is composed of a mixed population consisting mainly of macrophages ($74.3 \pm 3.5\%$) and eosinophils ($11.7 \pm 0.5\%$) (MacDonald et al., 1998). There is a 40 fold increase in the total number of eosinophils in implanted mice ($1.7 \pm 0.8 \times 10^6$) in comparison to control mice ($4 \pm 2 \times 10^4$). This situation is reminiscent of a condition caused by this parasite in a number of infected individuals known as Tropical Pulmonary Eosinophilia, which is characterised by a marked infiltration of eosinophils into the lung tissues (Ottesen and Nutman, 1992).

In order to determine the kinetics of cell recruitment into the peritoneal cavity of mice, Andrew MacDonald performed a time course experiment to monitor cell recruitment after exposure to *B. malayi* for different periods of time (Figure 1A & B).

There was a striking neutrophilia observed immediately after implantation, which reduced steadily until neutrophils were no longer detectable 14 days after implantation. In contrast, eosinophil infiltration was observed slightly later, with numbers peaking at 7 days after implantation and remaining stable until the experiment was terminated at 21 days. The number of macrophages increased steadily over time to reach maximum levels after 2 to 3 weeks.

To assess if the recruitment of eosinophils is dependent on the Th2 cytokines IL-4 and IL-5, Andrew MacDonald implanted parasites into mice genetically deficient for IL-4 (Figure 1C) and IL-5 (Figure 1D) for 3 weeks before recovering the PEC. As expected, there was significantly fewer eosinophils recruited into the peritoneal cavity of IL-$4^{-/-}$ mice. The recruitment of some eosinophils probably occurs via compensatory mechanisms (e.g. via IL-13). IL-$5^{-/-}$ mice failed to recruit significant number of eosinophils to the site of parasite implantation. This data shows that the recruitment of eosinophils in this nematode parasite model is partly dependent on IL-4 and completely dependent on the presence of host IL-5.

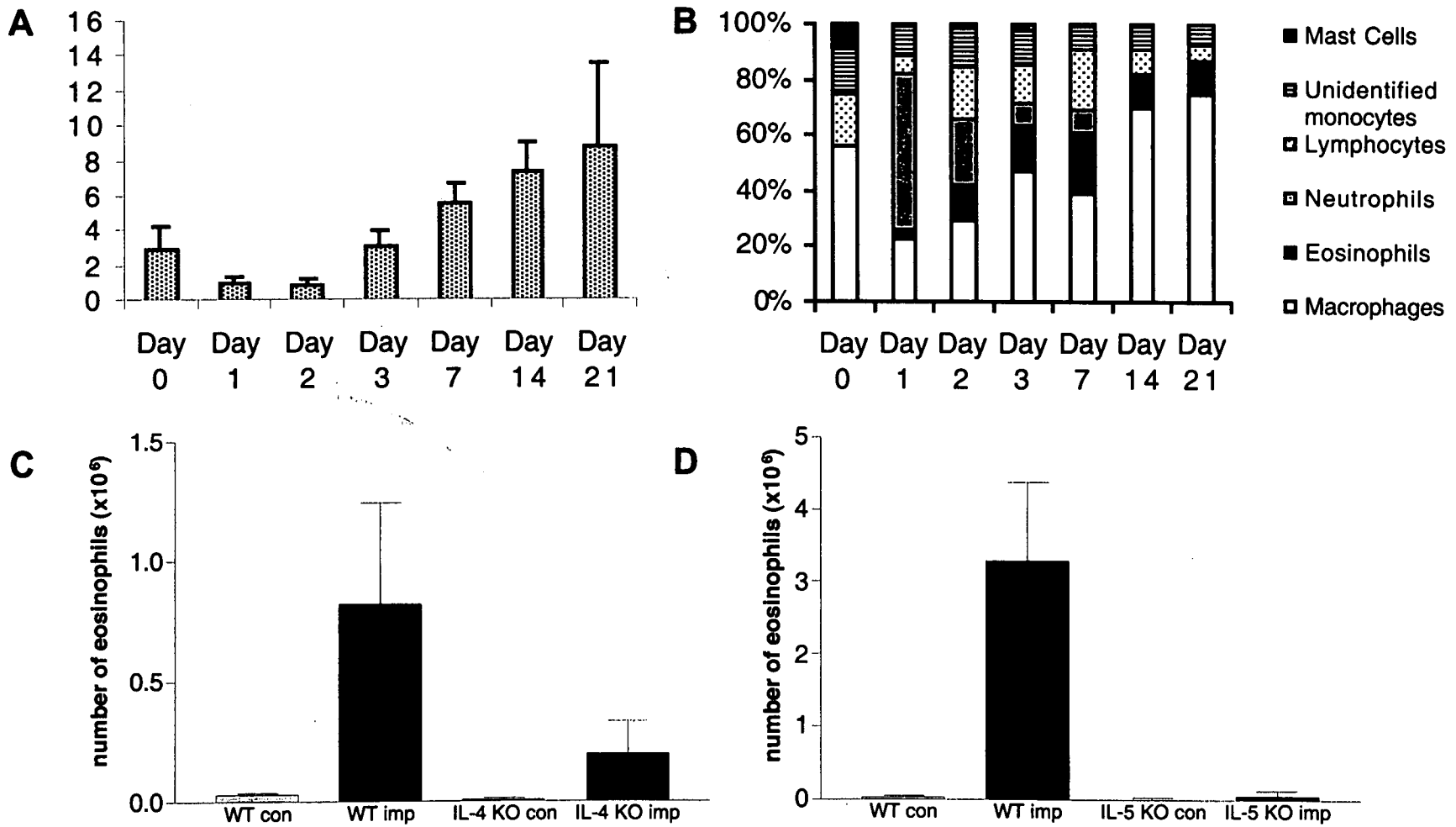


Figure 1: *B. malayi* induces eosinophil recruitment via IL-4 and IL-5 dependent mechanisms. (A) Time course of cell recruitment into the peritoneal cavity after parasite implantation. (B) Cellular composition of PEC populations during a time course of cell recruitment. (C) Eosinophil recruitment into the peritoneal cavity of implanted IL4-/- mice in comparision to WT mice. (D) Eosinophil recruitment into IL-5 -/- mice in comparison to WT mice.

In this chapter we present evidence for a link between the recruitment and activation of AAMΦ by this nematode parasite and the recruitment of eosinophils to the site of inflammation. Furthermore, we show that a cytokine homologue secreted by the nematode parasite (*Bm*-MIF) is directly involved in activating macrophages and recruiting eosinophils.

Macrophage migration inhibitory factor (MIF) was identified as a cytokine with the ability to inhibit monocyte migration *in vitro*. More recently, it has emerged as an important cytokine involved in innate immunity and the control of an inflammatory response. Interestingly, a homologue of this cytokine has recently been identified in *Brugia malayi* (Pastrana et al., 1998) and has been shown to be chemotactic for human monocyte/macrophages (Pastrana et al., 1998; Zang et al.,). An unusual feature of this cytokine is that both the mammalian and nematode forms have enzymatic activity (Zang et al.,). Although the natural substrate is not known, human MIF has been shown to have tautomerase activity on 2 synthetic substrates. The tautomerase activity of the human MIF is crucially dependent on the conserved proline residue at position 2 (Swope et al., 1998). Mutation of proline to glycine at this residue substantially reduces catalytic activity as well as neutrophil priming activity (Swope et al., 1998). Xingxing Zang has shown that mutation of the same proline in Bm-MIF-1 also abolishes exzymatic activity of this molecule (Zang et al.,). In this chapter, we show that the *B. malayi* homologue of this molecule induces eosinophil recruitment *in vivo* when injected into the peritoneal cavity of mice and the mutation of the conserved proline residue to glycine eliminates this activity. We also find that Bm-MIF activate macrophages to produce a novel eosinophil chemotactic factor (ECF-L) that is also known as Ym1 (Jin et al., 1998; Owhashi et al., 2000).

Owhashi et.al. had previously shown that an eosinophil chemotactic factor was produced by $CD8^+$ T lymphocytes in an antigen specific manner after infection with Schistosoma japanicum and Toxocara canis (Owhashi et al., 1998; Owhashi and Nawa, 1987). This factor was biochemically purified and shown to be the same protein as Ym1 (Owhashi et al., 2000). ECF-L/Ym1 was shown to be chemotactic for

eosinophils both *in vitro* as well as *in vivo*. Apart from eosinophils, ECF-L/Ym1 also attracted T cells and bone marrow cells *in vitro* (Owhashi et al., 2000). Interestingly, a separate group has found that eosinophilic crystals found in the lungs of viable motheaten mice (me^v/me^v) were composed of Ym1 protein (Guo et al., 2000). These crystals were found in the cytoplasm of alveolar macrophages and are thought to be similar to the Charcot-Leyden crystals in humans, which are also associated with eosinophil-rich inflammation of the lungs. In this study we show that Th2 driving nematodes induce the dramatic upregulation of Ym1/ECF-L in macrophages and is associated with the recruitment of eosinophils. All these data suggests that Ym1/ECF-L might be an important link between Th2 cytokine responses, macrophage activation and eosinophil chemotaxis.

Results

Ym1/ECF-L is dramatically upregulated in macrophages exposed to *B. malayi*

In the previous chapter, we described the characterisation of gene expression in AAMΦ by an EST strategy. The most striking finding was the extremely high representation of a gene known as Ym1 (Jin et al., 1998) in this library, accounting for 8.5 % of the cDNA clones. This gene has subsequently been identified as a eosinophil chemotactic factor by Owhashi et. al. (Owhashi et al., 2000). The discovery that a novel eosinophil chemotactic factor accounted for a startlingly high proportion of the genes expressed in peritoneal macrophages of parasite-implanted mice was extremely provocative. Thus we decided to verify this finding by RT-PCR analysis of peritoneal cells from mice implanted with *B. malayi* (Figure 2). In resident peritoneal cells from control mice, Ym1 was routinely detected at a low, basal level (lane 6). In comparison, implantation of mice with adult *Brugia malayi* resulted in the dramatic upregulation of Ym1 expression in peritoneal cells (Figure 2A,lane 7). This result was observed in every individual mouse (n=10) that we have analysed by RT-PCR.

Since eosinophil recruitment as well as the immune response to parasitic nematodes is driven by Th2 cytokines, we assessed whether the implantation of parasites into mice deficient for IL-4 or IL-5 would have any effect on Ym1 induction. To ensure that Ym1 was expressed in macrophages we used biotin conjugated α-F4/80 antibodies and streptavidin conjugated microbeads to purify $F4/80^{+}$ macrophages for the RT-PCR analysis. As expected, macrophages from $IL\text{-}5^{-/-}$ mice showed the same induction of Ym1 as wildtype mice, since the initial observation of Ym1 expression was originally made through a cDNA library made from $IL\text{-}5^{-/-}$ macrophages. IL-4 deficient mice also showed the same dramatic upregulation of Ym1 in macrophages.

These data showed that the induction of Ym1 in macrophages is not dependent on the Th2 cytokines IL-4 and IL-5. It also showed that induction of Ym1 alone was not sufficient to recruit eosinophils to the site of inflammation since eosinophil recruitment is significantly reduced in $IL\text{-}4^{-/-}$ mice and completely absent in $IL\text{-}5^{-/-}$ mice.

Bm-MIF-1 induces upregulation of Ym1/ECF-L/ECF-L

Recently, Pastrana et al. found that *Brugia malayi* secretes a homologue of the human cytokine macrophage migration inhibitory factor (MIF) (Pastrana et al., 1998), and these parasitic products had measurable effects on human monocytes and macrophages. We asked whether *Brugia malayi* MIF (*Bm*-MIF-1) could be responsible for the induction of Ym1/ECF-L seen in implanted mice. To mimic the long term effect of parasite secretions, we gave 9 i.p. injections of 1μg recombinant, endotoxin-free *Bm*-MIF-1 over a period of three weeks (Figure 3). As a control for the experimental procedure, we treated the control group with a corresponding volume of sterile PBS. Three days after the last injection, the animals were sacrificed and the cells recruited to or resident in the peritoneal cavity were analysed by RT-PCR.

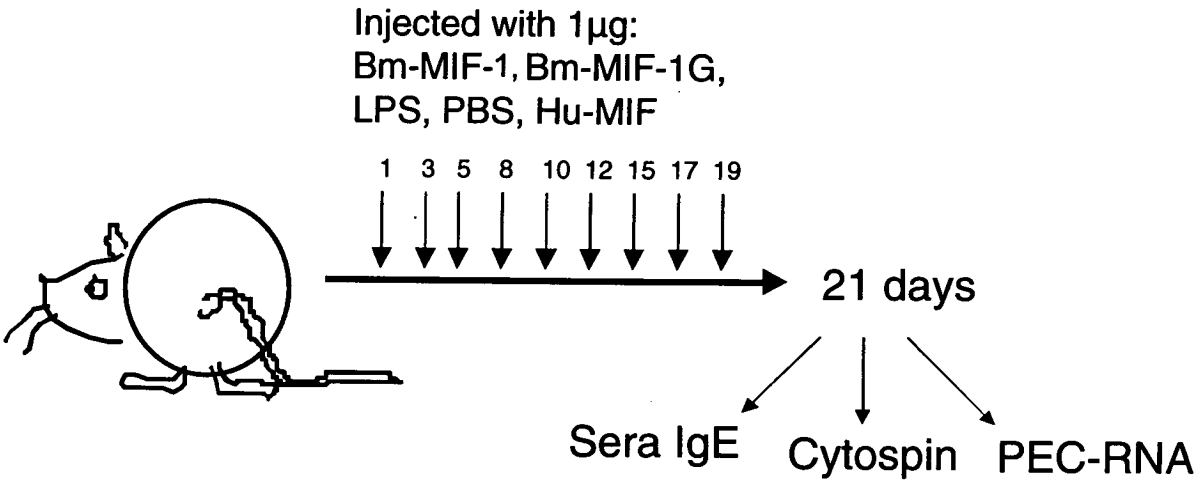


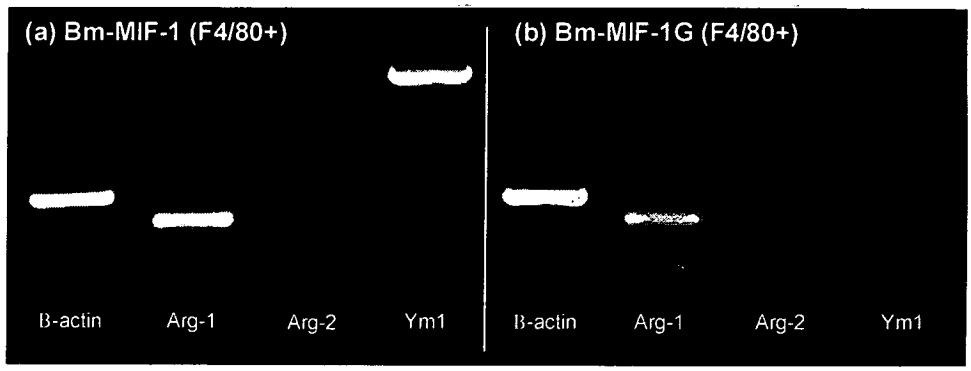
Figure 3: Experimental procedure to assess the *in vivo* effects of Bm-MIF-1. 1µg of BmMIF-1, BmMIF-1G and human MIF were injected i.p. into BALB/C mice 3 times a week for 3 weeks (9 injections), after which the mice were killed by cardiac puncture and PEC were harvested for Cytospin and RT-PCR analysis. Sera were collected from treated mice for analysis of antibodies.

Figure 4 shows the RT-PCR analysis performed with RNA isolated from F4/80+ macrophages purified from the peritoneal cells of mice after treatment. *Bm*-MIF-1 effectively induced Ym1/ECF-L, whereas Ym1/ECF-L was only detected at a low basal level in the PBS-treated mice. Since mammalian MIF share an enzymatic activity with the parasitic MIF (Pastrana et al., 1998; Zang et al.,), which can be effectively destroyed by mutating the N-terminal Pro to Gly, we treated mice with the recombinant mutant *Bm*-MIF-1G with an identical protocol. Interestingly, *Bm*-MIF-1G did not induce YM1/ECF-L, indicating that enzymatic activity, (or alternatively, the presence of an intact N-terminal Pro) is a prerequisite for effective induction of Ym1/ECF-L. We do not think that the dramatic difference between the two recombinant forms could be due to an artefact such as a different folding pattern of the mutated recombinant protein, but truly reflects the effects of the enzymatically active cytokine homologue which is secreted by live *Brugia malayi* parasites.

Before treatment of the animals, the native and the mutated recombinant forms of Bm-MIF were removed of endotoxin contaminants (below the detection limit of the Limulus assay). However, in order to rule out an effect of LPS contamination in inducing Ym1, we also treated a group of mice with LPS. As shown in Figure 4B, LPS was not effective at inducing Ym1 in the treated mice.

We next examined whether the three-week treatment course with rBm-MIF-1 is necessary for effective induction of YM1/ECF-L/ECF-L and/or eosinophilia (Figure 5). Therefore, we treated mice with one single dose (10 μg) or three doses of 1 μg each spread over one week. The single injection of a high dose of rBm-MIF-1 had no effect (data not shown), whereas the three consecutive injections effectively induced Ym1 (Figure 5). Again, neither PBS nor Bm-MIF-1G induced Ym1/ECF-L (Figure 5). A high dose of LPS (10μg) also did not induce Ym1 (data not shown).

A



B

	Bm-MIF-1	*Bm*-MIF-1G	PBS	LPS
no. Ym1+ /total no.	14/19	2/11	2/9	1/5

Figure 4: RT-PCR analysis of Ym1 expression from i.p. injected mice. After 35 rounds of amplification the PCR reaction was run on a 1% agarose gel for visualisation.
(A) F4/80^{+} macrophages were purified from the PEC of mice that have been injected with *Bm*-MIF-1(a) or *Bm*-MIF-1G(b), and gene expression was analysed by RT-PCR. Macrophages from *Bm*-MIF-1 injected mice show a significant upregulation of Ym1 as well as arginase I (but not arginase II), in comparison to *Bm*-MIF-1G injected mice.
(B) PEC from i.p. injected mice were analysed individually by RT-PCR. Ym1+ mice are defined as having Ym1 PCR products that were greater than 50% the intensity of actin PCR products by densitometry.

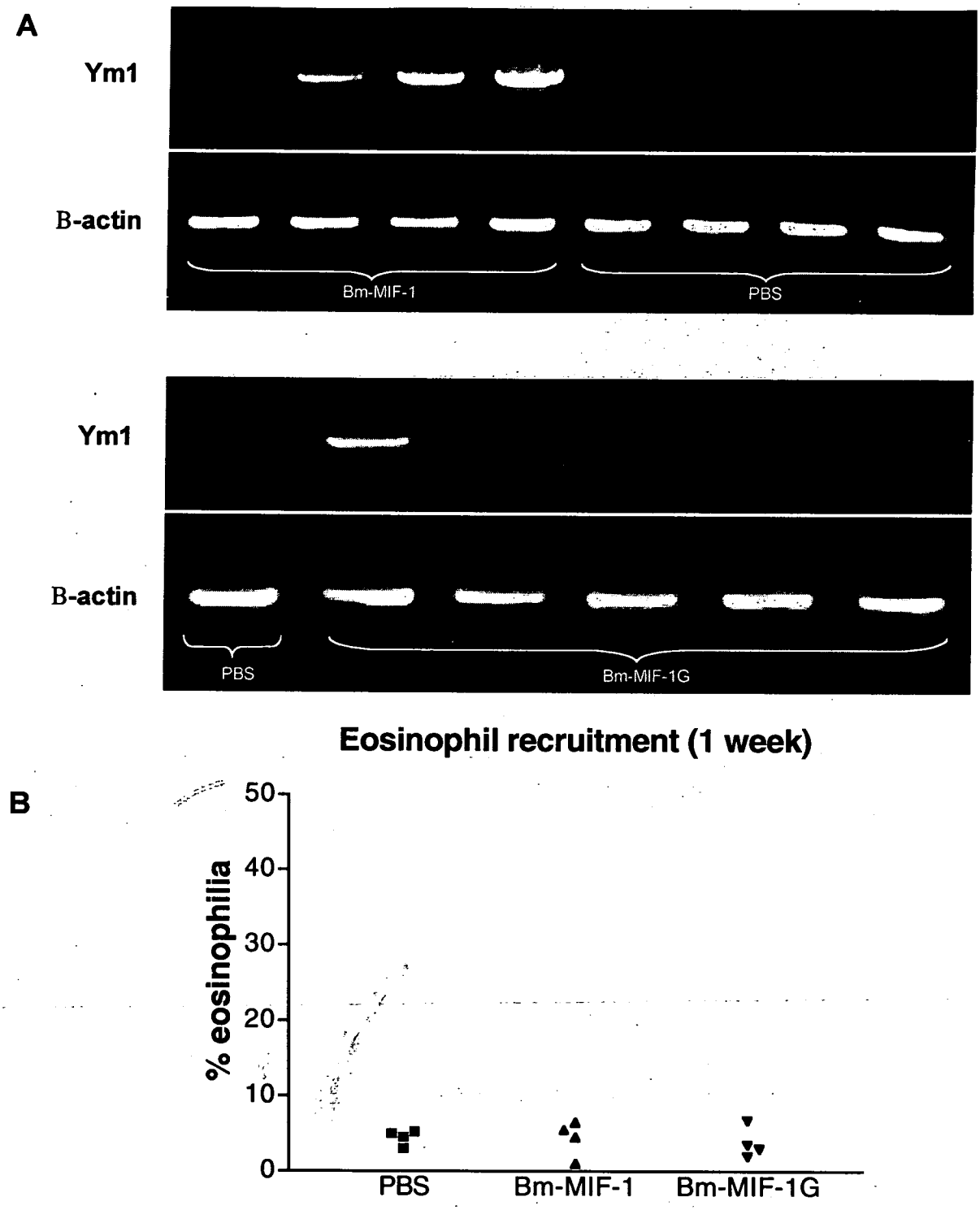


Figure 5: 1 week treatment of Bm-MIF-1 induced Ym1 expression but not eosinophil recruitment. (A) PEC from mice injected i.p. with 3 doses of Bm-MIF-1, Bm-MIF-1G or PBS over a 1 week period was analysed by RT-PCR for Ym1 expression and (B) by cytospin analysis for eosinophil recruitment. Each lane represents an individual experimental animal.

Bm-MIF-1 induces recruitment of eosinophils

The dramatic increase in Ym1 transcription should correspond to a significant rise in the production of Ym1/ECF-L protein. Since Ym1/ECF-L recruits eosinophils *in vitro* as well as *in vivo* (Owhashi et al., 2000), we asked if there was any relationship between recruitment of eosinophils to the peritoneal cavity and the induction of Ym1 gene expression. If there was an increase in eosinophil recruitment, we wanted to know if this was dependent on the enzymatic activity of *Bm*-MIF. Hence, we examined stained cytospins of cells derived from the peritoneal cavity of mice treated with either PBS, *Bm*-MIF-1 or *Bm*-MIF-1G and assessed the percentage of eosinophil granulocytes. Figure 6 shows that the 3-wk treatment of mice with r*Bm*-MIF-1 leads to an average of about 3 fold increase of peritoneal eosinophils compared with PBS or r*Bm*-MIF-1G treated mice ($p=0.0001$). Interestingly, although the 1-wk treatment with r*Bm*-MIF-1 effectively induced YM1/ECF-L, these mice did not display any increased eosinophil recruitment (Figure 5). This result points to a more complex relationship between *in vivo* induction of Ym1 and the recruitment of eosinophils to the tissues.

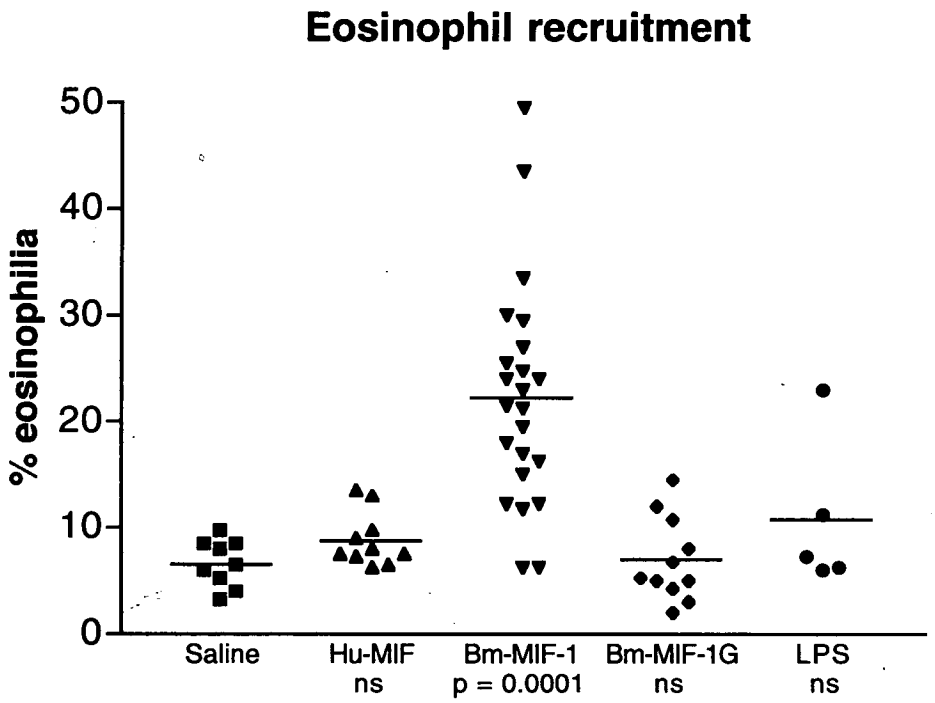


Figure 6: Bm-MIF-1 (but not Bm-MIF-1G) induces eosinophil recruitment into the peritoneal cavity. The cell composition of PEC (from mice injected i.p. over a 3 week period) was determined by microscopy of cyto-centrifuge preparations stained with Diff Quik (A). about 500 cells were counted from randomly selected fields per cyto-centrifuge preparation. Data presented is pooled from several independent experiments. Each data point represents an individual animal, with the horizontal bar representing the mean of the group. .

Discussion

Our results suggest that a parasite homologue of MIF could activate macrophages to recruit eosinophils at the local site of inflammation. Mammalian MIF has not been reported to have eosinophil recruitment activity (whether directly or indirectly), although it is produced by human eosinophils (Rossi et al., 1998). We are currently investigating whether this is a yet undiscovered function for MIF, which we have identified by studying the parasite homologue. Our preliminary studies suggests that human MIF does not induce eosinophil recruitment or induction of ECF-L/Ym-1 expression (Figure 6). However, we are not confident of a positive biological effect of our purchased batch of human MIF. Nonetheles, these data suggests that macrophages could provide a crucial link between parasitic infections and eosinophil chemotaxis. Since the production of MIF-like enzymatic activity has been demonstrated in a variety of parasites (Pennock et al., 1998), ECF-L/Ym-1 induction by parasites could be a wide spread mechanism that results in tissue eosinophilia.

It is interesting to note the homology of ECF-L/Ym1/ECF-L with bacterial, plant and mammalian chitniases (Owhashi et al., 2000). Guo et al. found a 3- to 5-fold increase in chitinase activity in the broncho-alveolar lavage fluids from me^{v}/me^{v} mice, which correlated with the Ym1/ECF-L protein band in native electrophoresis gels (Guo et al., 2000). On the other hand, the sequence of ECF-L/Ym1/ECF-L suggests that it may not possess chitinase activity due to the replacement of two residues (Glu and Asp by Gln and Asn), which are perfectly conserved in active chitinases and are considered essential for chitinase (Owhashi et al., 2000). The biological relevance of this chitinase homology and/or activity remains unclear.

Since the role of eosinophils in the host response against filarial parasites is unclear, it is not known whether this effect represents an immune evasion strategy of the parasite, or is a byproduct of an alternative effect. We have also not established whether there is a direct or indirect relationship between the action of *Bm*MIF-1, the activation of macrophages to produce ECF-L/Ym-1 and the recruitment of eosinophils. Our results suggest more complex relationship between ECF-L/Ym-1

induction and eosinophil recruitment since the induction of ECF-L/Ym-1 alone is not sufficient to recruit eosinophils. Eosinophil recruitment only occurs after a long term treatment with *Bm*-MIF-1 and does not occur in IL-$5^{-/-}$ mice, whereas ECF-L/Ym-1 can be induced after 1 week and is also induced in IL-$5^{-/-}$ mice. Further, we also found induction of ECF-L/Ym-1 in thioglycolate-injected animals (data not shown), which do not recruit eosinophils.

In this chapter, we have shown how the characterisation of gene expression in macrophages recruited by *B. malayi* using a genomic strategy via an expressed sequence tag (EST) analysis has serendipitously led to the identification of a novel relationship between macrophage activation by MIF, and the recruitment of eosinophils by macrophage derived Ym1/ECF-L. This illustrates the advantages of working in a functional post genomic era of biology. EST analysis was introduced for the purpose of gene discovery on a large scale. However, by doing a small EST analysis of a relatively uncharacterised cell type, we have identified an interesting relationship between a parasite gene product (also identified as part of the much larger filarial genome EST project), host derived macrophages and eosinophils recruitment.

Chapter 9

DISCUSSION

Macrophages are key effector cells in many bacterial, protozoan and viral infections. Indeed one of the most critical functions of a type 1 immune response is the activation of macrophages by IFN-γ. This activation is required for disease resolution following infection with a large number of intracellular pathogens, most clearly illustrated in murine models of leishmaniasis (Reiner and Locksley, 1995). The functional importance of macrophages in helminth infection in general and lymphatic filariasis in particular is far less apparent. However, the presence of large numbers of macrophages at the site of infection in experimental models of lymphatic filariasis has long implicated macrophages as important cells in the killing of parasites, the pathology associated with dying worms and quite possibly the immune hypo-responsiveness associated with infection. The work described in this thesis, as well as previous work, has led us to believe that macrophages may be critical mediators of immune regulation in lymphatic filariasis.

Using a mouse model, we have established that the filarial parasite *Brugia malayi* can recruit suppressive macrophages that are dependent on IL-4 *in vivo*. This has strongly suggested that they represent an *in vivo* manifestation of the recently defined category of alternatively activated macrophages (AAMΦ). Molecular characterisation of genes expressed in parasite induced macrophages supports this classification since many genes known to be upregulated by IL-4 were expressed in these macrophages. Furthermore, we have shown for the first time that these AAMΦ can drive Th2 cell differentiation, consistent with suggestions that they play an important role in the regulation of the immune response. In this discussion, we would like to put our findings into the general context regarding what is known about macrophages in filarial infection. We would like to hypothesise that while dead or dying worms induce the "classical" activation of macrophages and a subsequent pro-inflammatory response, live and healthy worms secrete products that induces type 2 cytokines and induces the differentiation of "alternatively" activated macrophages that

down-regulate an inflammatory response. Thus, the balance between the 'classical' and 'alternative' activation pathways of macrophages could be an important factor in inflammatory pathology associated with filariasis. In this discussion, I will review all the work we have done and also the filarial literature that has led to this hypothesis. I will also present a strategy for the future in terms of characterising the AAMΦ that we have found.

Macrophages as effector cells in filarial infection

Considerable *in vitro* evidence exists to suggest that macrophages are effective at killing the larval stages of filarial parasites. For example, resident macrophages from the peritoneal cavity of rats have been shown to kill *A. viteae* microfilaria in the presence of immune sera (Haque et al., 1980). The same result is seen with *Brugia pahangi* in jirds and mice where adherent macrophages are able to kill MΦ in the presence of anti-microfilarial sera (Karavodin and Ash, 1982; Oxenham et al., 1984). One mechanism by which macrophages are able to damage an organism that they cannot engulf appears to be through the release of reactive oxygen species and nitric oxide derivatives. Two separate groups have shown that *B. malayi* MΦ can be killed *in vitro* by IFN-γ activated macrophages and this appears to be mediated least in part by nitric oxide (NO) (Taylor et al., 1996b; Thomas et al., 1997). In contrast, H_2O_2 appears to have little effect on *Brugia* although it does affect *Onchocerca* MΦ (Ou et al., 1995; Taylor et al., 1996a). Despite the ability of activated macrophages to kill these parasites *in vitro*, filarial nematodes are remarkably resistant to a lethal hit and killing required sustained exposure to NO (Thomas et al., 1997). Adult parasites appear to be even more resistant to macrophage mediated damage than the larval stages (Thomas et al., 1997) perhaps due to the production of antioxidant enzymes (Ou et al., 1995). The evidence that filarial parasites have evolved numerous strategies to counter oxidative attack highlights the importance of reactive oxygen species as a means of immune attack (Selkirk et al., 1998).

In vivo, *B. pahangi* L3 induce activation of macrophages in the peritoneal cavity of jirds. These macrophages have significant increases in phagocytic and

microbicidal activity (Jeffers et al., 1984). Further, in both the lymphatics and the peritoneal cavity of jirds macrophages are the dominant cell type associated with granulomatous lesion that forms around the adult parasites and microfilaria. The macrophages are observed to be adherent to the parasite surface (Jeffers et al., 1984). More direct evidence *in vivo* for a role of macrophages in resistance to infection is found with studies that demonstrate that age and sex-related differences mice susceptibility are abolished when macrophage activity is blocked by carbon particle injection. (Nakanishi et al., 1989). There is considerable cumulative evidence that macrophages together with granulocytes are important in parasite destruction. However, there is little information about the factors *in vivo* that activate macrophages to kill these large extracellular pathogens and despite the evidence that NO producing IFN-γ activated-macrophages can kill parasites *in vitro*, their importance *in vivo* remains unresolved.

These studies suggest that macrophages can be activated to kill filarial parasites but as is so often the case, the line between protection and pathology is barely distinguishable. The same evidence that suggests macrophages can be activated to kill parasites suggests that these cells may be responsible for some of the pathology associated with filariasis. Inflammatory lesions in the lymphatic vessels of individuals infected with filarial parasites are commonly observed in both human and animal studies (Rao et al., 1996). The pathological granulomatous lesions that develop around dying parasites are predominantly comprised of eosinophils and macrophages (Vickery et al., 1991) suggesting that macrophages activated by parasite infection may damage host tissue.

More recently, Taylor et.al. produced an important study to show that the intracellular bacteria (Wolbachia) present in *Brugia* could play an important role in inflammatory pathology (Taylor et al., 2000). They found that soluble worm debris from *Brugia malayi* could stimulate macrophages to produce pro-inflammatory cytokines such as TNF, IL-1 and NO in an *in vitro* assay. Using a macrophage cell line that was defective in binding LPS due to the lack of surface CD14, and also a mouse strain that had a mutation in the Toll-like receptor 4 (another LPS receptor), they

elegantly demonstrated that the proinflammatory stimulus was due to the presence of LPS. Taylor et al. hypothesize that dying (but not live worms) release LPS. This could account for pathology that accompanies chemotherapy as well as the fever-like symptoms associated with acute filarial disease (Taylor et al., 2000).

Further evidence for macrophages as important players in the control of filarial infection come from studies using xid mice. CBA/N mice have a defect in the Bruton's tyrosine kinase (Btk) gene and a number of studies have shown that they have a reduced ability to clear Mf as compared to wild-type CBA/J (Al-Qaoud et al., 1998; Mukhopadhyay et al., 1999). The assumption has generally been that the phenotype observed is due to defects in B cell signalling and development. However, Btk is expressed in both B cells and APCs of myeloid origin. Mukhopadhyay et al. (Mukhopadhyay et al., 1999) have demonstrated that CBA/N mice have significant alterations in macrophage function that could play an important role in filarial infection. Macrophages from CBA/N mice have a reduced capacity to produce nitric oxide but an enhancement of IL-12 production perhaps due to the down regulatory effects of NO on IL-12 induction. (Mukhopadhyay et al., 1999). Further, T cell responses to Mf antigen in CBA/N mice are skewed toward a more type 1 immune response and Mukhopadhyay et al. have shown by adoptive transfer experiments that these alterations in cytokine responses are due to macrophages rather than B cells. So the long held view that the inability of xid mice to rapidly clear their microfilaria is due to defective antibody production may need to be reconsidered and the contribution of macrophages reevaluated.

Macrophages as suppressor cells in filarial infection

One of the most consistent findings in both human (Maizels and Lawrence, 1991; Ottesen et al., 1977; Piessens et al., 1980) and animal (Baize et al., 1997; Lammie and Katz, 1984; Miller et al., 1991; Prier and Lammie, 1988; Rao et al., 1996) studies is that individuals infected with filarial parasite exhibit profound defects in cellular proliferation. In humans, this is primarily an antigen-specific defect. However, in animal studies both antigen-specific and non-specific components have been

identified in the proliferative suppression observed following infection. Several studies in the experimental jird model (Lammie and Katz, 1984; Prier and Lammie, 1988) demonstrated that removal of plastic-adherent cell populations was able to reverse the proliferative defect. Similar findings have been described for human peripheral blood in which an adherent phagocytic mononuclear cell mediated proliferative suppression (Piessens et al., 1980). Although not directly demonstrated to be macrophages, the cells described in these studies are likely to be either macrophages or monocytes and strongly implicated a role for macrophages in the immune suppression observed over the course of filarial infection. Using an intraperitoneal (i.p.) infection model in jirds, C. Nasarre found that chronic infection with *B. pahangi* led to the deactivation of macrophages, as measured by their ability to kill *Toxoplasma* and produce TNF-α (Nasarre et al., 1998). The emergence of deactivated macrophages correlates with a reduction in the systemic granulomatous inflammatory response that they observe in the jird. This data suggests that the recruitment of anti-inflammatory macrophages could down modulate immuno-pathology.

IL-10 is a downregulatory cytokine produced by macrophages as well as lymphocytes that can directly counter the pro-inflammatory effects of IFN-γ. IL-10 produced by blood monoculear cells has been implicated in the down-regulation of T cell responses observed during human filarial infection (Mahanty and Nutman, 1995) and the spontaneous release of high levels of IL-10 by adherent mononuclear cells has been associated with the hypo-responsive state. Osborne and Devaney (Osborne and Devaney, 1999) have further demonstrated that IL-10 produced by an adherent splenocyte population can suppress T cell proliferative responses in *B. pahangi* infected mice. Taken together, these results suggest that IL-10, potentially produced by monocyte/macrophages, may regulate the immune response during filarial infection.

Several years ago, my supervisor, Dr. Judith Allen set out to determine whether exposure to filarial parasites caused an alteration in antigen presenting cell (APC) function that could lead to the defect in antigen-specific T cell proliferation

associated with infection(Allen et al., 1996). There wasn't a direct effect of parasites on APC *in vitro*. However, she found that when adult or larval stages of the parasite were implanted in the peritoneal cavity of mice, a population of adherent cells were recruited *in vivo* that when used as APC *in vitro* prevented the proliferation of a T cell clone. Interestingly, despite the profound effect on cell division, T cells were able to produce normal or elevated levels of antigen-specific cytokine. Further, using a range of lymphocyte cell lines she found that the anti-proliferative effect was remarkably non-specific and that cell division was blocked in a broad range of target cells whether stimulated with antigen or mitogen (Allen et al., 1996). Anti-proliferative cell could not be generated when animals were exposed to MΦ alone. As Mf induces IFN-γ, while L3 and adult stages induce high levels of early IL-4 (Lawrence et al., 1994; Osborne and Devaney, 1998) the induction of suppressor cells may require IL-4.

Subsequently, a PhD student Andrew S. MacDonald, used neutralizing antibodies to IL-4 and mice genetically deficient in IL-4, to establish that recruitment and/or development of these anti-proliferative cells required host IL-4 (MacDonald et al., 1998). Interestingly, IL-10 had no effect in these studies as full suppression was observed in IL-10 deficient mice. Further, studies with neutralizing antibodies to IL-10 and TGF–β or inhibitors of nitric oxide, H_2O_2 or prostaglandins also were unable to reverse the proliferative defect (Allen et al., 1996).

In this thesis, I have been able to demonstrate that the cell type responsible for these suppressive effects is indeed a macrophage. Purification of cells using antibodies to the macrophage marker F4/80 is able to replicate the results with whole peritoneal cell populations. Although IL-4 deficient mice recruit slightly fewer macrophages than wild type mice, this does not account for the failure of IL-$4^{-/-}$ PEC to block proliferation, since purified macrophages from IL-4 deficient mice are still non-suppressive.

The combination of *in vitro* studies, animal models and field studies strongly suggests that macrophages associated with Th2 cytokines could play a vital role in the proliferative defect that is associated with filarial infection. The recognition that anti-

inflammatory macrophages are often associated with Th2 cytokines is an emerging concept in macrophage biology. The phenotypic differences between such "alternatively activated" macrophages from "classically activated" macrophages could account for the different roles that macrophages seem to play in filarial infection. Thus, the relationship between parasite clearance/coexistence, immune pathology/suppression with the balance of Th1 and Th2 cytokines could reflect the type of macrophages that is induced by infection.

A balance of cytokines and macrophages

Whereas the Th1/Th2 paradigm has been well accepted as playing a central role in our interpretation of immune responses, the concept that these opposing cytokine responses might act on macrophages in different ways has only recently attracted the attention of a wider audience. While macrophages activated by interferon-γ play a critical role in destroying intracellular pathogens (Kaufmann, 1993), macrophages can also be activated by type 2 cytokines (particularly IL-4 and IL-13) to develop a phenotype quite distinct from that of IFN-γ activated macrophages (Doyle et al., 1994; Gordon, 1999; Stein et al., 1992). These cells have been termed "alternatively activated macrophages" (AAMΦ) to distinguish them from classically activated macrophages (CAMΦ) (Stein et al., 1992). This alternative activation pathway is not simply a down-regulation of the IFN- γ mediated effects but a developmental pathway in its own right (Gordon, 1999). Thus, it is apparent that macrophages, like T cells and dendritic cells (Rissoan et al., 1999), represent a heterogeneous population of cells with distinct bioiogical roles. As with other cells of the immune system, this heterogeneity in function and developmental pathway is dependent on the cytokine microenvironment. We believe that this duality (or plurality) of macrophage function also exists within the context of lymphatic filariasis and could play a key role in the balance between parasite clearance and immuno-pathology.

Since the evidence for macrophages as effector cells in filiariasis is correlated with such inflammatory factors as NO, we think that these fall into the category of CAMΦ. The suppressive AAMΦ observed in our mouse model do not produce NO. Consistent with NO being a key effector molecule in macrophage function, probably the clearest distinction between the 2 categories of macrophages is the different metabolic pathways for L-arginine (Modolell et al., 1995). Under type 1 conditions, inflammatory macrophages produce NO as a result of upregulating inducible NO synthase (iNOS), which catalyzes the L-arginine substrate (MacMicking et al., 1997). Under type 2 cytokine conditions, arginase is upregulated instead of iNOS (Munder et al., 1998; Munder et al., 1999). The induction of arginase leads to the catalysis of L-arginine into L-ornithine and urea. The induction of either iNOS or arginase is usually associated with the suppression of the opposing enzyme, indicating a competitive nature in these alternative states of macrophage metabolism.

Recent *in vitro* studies on the alternative arginase pathway showed that Th2 T cell clones were considerably more powerful at inducing arginase activity than the addition of Th2 cytokines alone (Munder et al., 1998). A different group has shown that peritoneal macrophages from strains of mice that were more inclined to mount a Th1 (C57/BL6, B10.D2) response, preferentially produce NO, whereas macrophages from strains that were biased towards Th2 responses appeared to inhibit NO production via TGF-β. Consistent with this arginase/iNOS paradigm, the EST project (chapter 7) showed that the AAMΦ that are recruited by live adult *B.malayi* implantation have highly upregulated expression of hepatic arginase I, but not extrahepatic arginase II. This is was consistent with studies using bone marrow derived macrophages, which respond to Th2 stimuli by specifically inducing arginase I, but not affecting the constitutive expression of arginase II (Munder et al., 1999).

Both CAMΦ and AAMΦ have been shown to suppress proliferation of lymphocytes. It is important to recognise that although NO is a clear indicator of classical activation, it is also involved in the inhibition of T cell proliferation. In the presence of inhibitors of NO synthesis, or in the absence of iNOS, proliferation of T cells and production of inflammatory cytokines can be enhanced significantly

(MacMicking et al., 1997). Infection of mice with the protozoan, *Trypanosoma brucei* also leads to generation of suppressive macrophages that act via NO and prostaglandins (Mabbott et al., 1995; Schleifer and Mansfield, 1993b; Sternberg and Mabbott, 1996a).

Although CAMΦ have some down-regulatory capacity, their prime function is microbial destruction. In contrast, the primary function of AAMΦ may be the dampening down or regulation of immune responses. Goerdt and Orfanos suggest that the production of anti-inflammatory cytokines is a key feature of APC activated under type 2 conditions (Goerdt and Orfanos, 1999). Immuno-suppressive macrophages induced under Th2 conditions have been described both *in vitro* as well as *in vivo*. Human monocyte/macrophages co-cultured in the presence of IL-4 and glucocorticoids *in vitro*, suppress proliferation of T cells (Schebesch et al., 1997). Alveolar and placental macrophages which have known suppressive effects exist under type 2 conditions and are likely to represent *in vivo* examples of AAMΦ (Goerdt and Orfanos, 1999). In the context of helminth infection, macrophages which actively down-regulate granulomatous lesions surrounding schistosome eggs have been very well characterised (Stadecker, 1999). The formation of granulomas around schistosome eggs is dependent on $CD4^+$ T cells. The initial response to the egg is a type 1 pro-inflammatory response with the induction a large poorly circumscribed granuloma. Macrophages with an alternatively-activated phenotype appear to then rapidly down-regulate the inflammatory response and promote a switch to a type 2 response. This is mediated primarily by IL-10. The subsequent granulomotous lesion is smaller in size and more compact, causing reduced damage to host tissue (Stadecker, 1999).

Although IL-10 and TGF–β are supposed to be involved in AAMΦ mediated suppression (Goerdt and Orfanos, 1999), there is clearly an alternative mechanism by which AAMΦ are able to suppress cellular proliferation that is not understood. Studies of human AAMΦ induced *in vitro* in the presence of IL-4 and glucocorticoids demonstrated the suppression was independent of IL-10, NO or prostaglandins

(Schebesch et al., 1997). This was highly consistent with our previous work on suppressive macrophages induced by filarial infection that had also ruled out these potential mediators (Allen et al., 1996). Andrew MacDonald had also found that suppression required cell-to-cell contact and does not occur across a membrane. He also showed that AAMΦ are capable of blocking cellular proliferation when fixed with paraformaldehye demonstrating that a soluble mediator is not responsible. Furthermore, I described in chapter 5 how we had found that the macrophages induced by filarial infection not only suppressed lymphocyte proliferation but suppressed the proliferation of a large number of human tumour cell lines.

Our findings bear a remarkable similarity to the macrophages described by Lee et al. using an aerosol challenge transgenic mice system (Lee et al., 1999). Lee et.al. showed that lung parenchymal T cells failed to proliferate ex vivo, but retained their ability to produce Th1/Th2 cytokines. Interstitial $F4/80^+$ macrophages were responsible for this proliferative suppression since the depletion of $F4/80^+$ macrophages restored the proliferation of T cells ex vivo. Further, this suppression could not occur across a transwell membrane suggesting that these macrophages also act in a contact dependent manner. As with our peritoneal cells these macrophages are associated with eosinophilia suggesting a type 2 cytokine environment. These macrophages also seemed to augment IL-4 and IL-5 production of Th2 cells, while suppressing the proliferation of both Th1 and Th2 cells.

The combination of evidences suggests that Th2 responses can drive the activation/differentiation of a macrophage population that inhibits proliferation of cells in the local vicinity via a novel receptor mediated mechanism. The ability of these macrophages to block cellular proliferation over a broad range of cell types and strongly suggests the engagement of a highly conserved receptor. A key direction of future studies on AAMΦ will be the identification of this anti-proliferative mechanism.

Apart from a role in immune suppression, Geordt et. al. also proposed that AAMΦ, like DC2, could induce naïve T cells to differentiate in Th2 cells. CAMΦ that are typically associated with cellular immunity have been shown to promote Th1 cell

differention (Desmedt et al., 1998). However, the production of TGF–β and IL-10 by AAMΦ would suggest that these cells in contrast might help to promote Th2 responses, either directly or by the inhibition of type 1 responses. In chapter 6, I described how we have found that the AAMΦ that are induced by live adult *Brugia malayi* can indeed drive Th2 differentiation of naïve T cells from pigeon cytochrome c (PCC) specific TCR transgenic (PCC-tg) mice. This observation showed that macrophages can be both the targets of T cell cytokines as well as regulators of T cell differentiation. While there have been reports describing dendritic cell subsets that differ in their capacity to stimulate the differentiation of naïve T cells (Iwasaki and Kelsall, 1999; Rissoan et al., 1999; Stumbles et al., 1998), this was one of the first studies to show that macrophages can also stimulate Th2 differentiation. Although it is still unclear how filarial parasites initiate a Th2 response, it is unlikely that AAMΦ play an early role in this process. Since AAMΦ are only induced under existing Th2 response, it probably plays a more downstream role of suppressing Th1 cells and thus stabilising the Th2 response.

Future work

Generation of a AAMΦ gene expression database

The definition of AAMΦ has been based on the expression of a number of molecules that appear to be upregulated in the presence of IL-4 and/or IL-13 (Doyle et al., 1994; Goerdt and Orfanos, 1999; Munder et al., 1998; Munder et al., 1999; Stein et al., 1992). These include the macrophage mannose receptor, arginase and the C10 chemokine. Since AAMΦ are considered to be important in down-modulating certain pathogenic immune responses, further clarification of their cellular and molecular repertoires may help in developing novel immuno-therapeutic strategies. The ability to generate large numbers of these cells with *B. malayi in vivo* provides us with sufficient material to enable a detailed characterisation of the genes that are expressed by these cells. One of the main aims of the future is to identify the molecules that are involved in suppressing proliferation of other cells, as well as driving Th2 differentiation of naïve T cells.

I would like to take advantage of the recent explosion in genomic technology and use several complementary approaches to advance our understanding of AAMΦ. First, we could survey genes from a recently constructed subtractive cDNA library to identify genes in AAMΦ regulated by IL-4. Secondly, we could use DNA microarray technology to analyse expression of genes in AAMΦ, complementing the identification of potentially novel genes in the subtractive library. Thirdly, we would like to expand expressed sequence tag (EST) projects from IL-$4^{+/+}$ and IL-$4^{-/-}$ AAMΦ that would provide meaningful comparisons with other EST datasets, including the EST data from the subtractive library. The data generated from this type of study will define for the first time, *in vivo* IL-4 dependent genes that are uniquely expressed or highly up-regulated by parasite induced macrophages. It will also provide a thorough survey of gene expression of AAMΦ generated *in vivo* and thus greatly enhance our knowledge of macrophage biology. Finally, it may reveal the receptor that is involved in macrophage mediated proliferative inhibition.

In chapter 7 we have described the construction of a subtractive library by using 'driver' RNA from non-suppressive IL-4 deficient PEC to subtract away 'tester' RNA from suppressive AAMΦ. This yielded a population of 10-20,000 recombinants enriched in putative suppression generating transcripts. A preliminary assay of 160 randomly selected clones from this library has revealed that between 10-20% of the recombinants are differentially expressed between the 2 macrophage populations. I would like to identify and sequence more clones (around 1000) from this library in order to generate a detailed profile of IL-4 dependent genes that are expressed in AAMΦ that are induced by *B. malayi in vivo*.

With modern high speed robotics, we could plate out the subtractive library and pick the colonies into 384 well plates with a robotic picker. These clones could then be gridded onto nylon membranes, which now represent a clone macroarray. This can be probed with radioactively labelled cDNA made from either suppressive AAMΦ RNA or non-suppressive IL-4 deficient AAMΦ in order to identify the genuinely differentially expressed clones. These can then be sequenced rapidly from the PCR product. Alternatively, we are exploring the possibility of constructing a DNA microarray from the subtractive library. This would save considerable amounts of time

for the identification of differentially expressed clones. Since a DNA microarray maker has just become available in Edinburgh, we could be able to exploit this facility.

At the same time, I would like to expand our existing EST datasets (described in chapter 7). Because highly expressed genes appear more frequently in non-normalised cDNA libraries, valuable expression pattern information can be obtained from comparisons of datasets from different libraries. This is the basis of Digital Differential Display (DDD) analysis currently available on NCBI[1]. DDD is a computational method for comparing sequence-based gene representation profiles among individual cDNA libraries or pools of libraries. The accuracy of such comparisons is dependent on the number of available EST from the different libraries.

I would like to sequence at least another 1000 clones from our suppressive AAMΦ cDNA library and another 1000 clones from non-suppressive macrophages. This will allow the identification of important new genes as well as a comparison of gene expression patterns between macrophages activated in the presence or absence of IL-4 *in vivo*. In addition we can compare our dataset to the currently available dataset of 5534 ESTs from the macrophage cell line WEHI-3. Fortunately, we have just received a grant in order to carry out this sequencing project.

I would also like to exploit the recent availability of DNA microarray analysis. Gene microarray analysis is the most time efficient way to characterise expression of known genes or ESTs from our different populations of macrophages. I will compare 1) suppressive parasite induced AAMΦ vs non-suppressive IL-4 deficient AAMΦ in order to identify IL-4 dependent genes that are expressed by AAMΦ and 2) suppressive parasite induced AAMΦ vs resident macrophages in order to identify which genes are upregulated as a result of parasite induction of AAMΦ. Because most DNA arrays contains previously uncharacterised murine ESTs, it may also allow us to identify new genes involved in suppression of cellular proliferation.

In the future, all the sequences produced from the conventional and subtractive cDNA libraries will be submitted to dbest division of GenBank. The AAMΦ EST

[1] (http://www.ncbi.nlm.nih.gov/UniGene/ddd.cgi?ORG=Mm)

sequences will be compared to each other and to other relevant datasets to define 'clusters' of ESTs deriving from single genes. The clustering information, associated expression profiles, and consensus sequences will be collated in a relational database in FileMaker Pro. This will then become available on our website for access to the scientific community.

The data generated from these studies will have an immediate impact on our understanding of macrophage activation in a Th2 environment. It will also provide the foundation for future work in this area and provide an outstanding resource for other investigators working in the field of macrophage biology.

Characterisation of genes expressed by AAMΦ

Apart from molecules that are involved in proliferative suppression as well as Th2 differentiation, we are interested in other IL-4 dependent genes that might be important in the function of AAMΦ. We will begin to characterise these genes using a combination of molecular, cellular and immunological strategies. The nature of future investigations will be subject to the kinds of genes that we identify. Since the cDNA libraries were constructed into a mammalian expression vectors (pCMV-Script & pcDNA3.1), interesting genes can immediately be expressed by transfection into mammalian cells. 2 particularly interesting genes that deserve attention are described below.

PNG1/FIZZ1 is extremely interesting because of its abundant expression as well as its IL-4 dependent induction pattern. Recently, this gene has been described as a major secreted protein found in the lungs of OVA-induced asthmatic mice (Holcomb et al., 2000). We were going to begin by characterising it's expression in different tissue types by probing mouse tissue specific Northern blots, however, the recent publication of this data has changed our analysis strategy. We will use our light cycler quantitative PCR machine (Roche) to verify the differential expression of this gene in different immunological cell types, eg. Th1 vs Th2 cells, B cells, bone marrow cells, thymocytes, splenocytes and etc., because the published study has not characterised it's expression in immune cells. We will then make a Ig-fusion protein, which can be used

for flow cytometry to establish the cell types that have a receptor for this molecule. An Ig-fusion protein would also enable us to immuno-precipitate the receptor for biochemical characterisation as well as for peptide sequencing and cDNA cloning. Blocking studies can also be carried out with Ig-fusion proteins. We have already designed primers to express this small molecule in *E. Coli* using the pET29 vector. Expressed protein can be used to generate polyclonal antibodies that will be useful for western blots, flow cytometry and immunocytochemistry. Future studies, based on the interests of the results we receive from these experiments could include the generation of genetically deficient mice.

We are especially interested in any IL-4 dependent transcription factors (eg. CRIP). The identification of transcription factors that could drive the differentiation of AAMΦ and not the maturation of CAMΦ would be a significant discovery. We can use the strategy of transferring retroviral infected bone marrow cells into lethally irradiated syngeneic mice. This strategy has been used to study the differentiation of stem cells (Chen et al., 1998a; Pui et al., 1999) as well as the transformation activity of certain proto-oncogenes (Pear et al., 1996). We can make retroviral expression constructs expressing candidate transcription factors, which will be transfected into retroviral packaging cell lines and high titre retroviral supernatants can be used to infect bone marrow cells, which will then be transferred into lethally irradiated syngeneic host.

The original purpose of our molecular characterisation strategy for these suppressive macrophages was the identification of the suppressive molecule. Our aims and intentions have changed considerably since the determination of this original objective. The data we obtained with respect to the relationship between Ym1/ECF-L, eosinophils and Bm-MIF-1, fundamentally changed my perspective regarding the most suitable direction to proceed, which broadened from a more focused and direct goal of identifying the suppressive molecule towards a broader aim of understanding the biology of these suppressive macrophages by analysing gene expression.

Using a molecular strategy would not guarantee the identification of the suppressive molecule(s), which could have involved post-translational modifications,

glycosylations and other properties that could not have been identified by differential gene expression. We have not lost sight of this original objective and are considering other non-molecular strategies for identifying the suppressive mechanism. Proteomics for example is in the process of undergoing the same revolution as we have observed in molecular biology over the last few years. Since we already know that the suppressive molecule is likely to be a cell surface molecule, we could biotinylate all the cell surface proteins of suppressive macrophages and non suppressive IL-4$^{-/-}$ macrophages. Biotinylated proteins can then be extracted with streptavidin and run on 2D gels for MALDI analysis. This approach would provide us with information about variations in other cell surface molecules as well as the potential suppressive molecules. Perhaps we might even identify unique cell surface markers that are distinct for suppressive macrophages. This approach would be complementary to our molecular approach, since peptide sequences of unknown proteins could well correspond to novel genes that we would identify through our molecular strategies.

Future goals and opinions

Immunologists today, who are in possession of sophisticated genetic tools to study experimental antigens (e.g. ovalbumin, haptens, KLH, HEL PCC and etc..), often overlook the fact that our immune response evolved primarily for the protection against infectious diseases. From this perspective, my opinions are in agreement with Zinkernagel, in that to understand the process of immunity we must take an evolutionary perspective with emphasis on how protection against infection is achieved (Zinkernagel, 2000). The increasingly sophisticated and complicated transgenic mice, which are mostly specific to experimental antigens instead of infectious organisms, are useful in revealing the extreme limits of the immune system, but might not reflect the general physiology of the immune system. To understand physiological processes *in vivo*, we should perhaps turn to infectious organisms again for guidance. Unfortunately, these experimental models tend to produce messy rather than clean data due to the complex nature of 2 organisms interacting (rather than a one sided interaction with an inanimate antigen), which is probably why they were abandoned by the majority of basic immunologists.

Although Matzinger argues that we must "understand what the immune system does (i.e. transplantation, tumour immunity, autoimmunity), not only what it may have evolved to do (i.e. combat infection)" (Matzinger, 1998), it seems to me that a disproportionate amount of resources is directed towards understanding relatively low level effects, in comparison to understanding what may be more physiologically as well as evolutionarily relevant and affecting a much wider proportion of the worlds population. My research aims in the future will be to work with experimental systems that bear closer resemblance to real life immune responses against infections. Hopefully the immunological tools from basic immunological research will begin to trickle down to fill the voids in infection orientated immunology, especially in the field of parasite immunology.

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APPENDIX: PUBLISHED PAPERS

Loke P, MacDonald AS, Robb AO, Maizels RM, Allen JE *European Journal of Immunology* 2000 Sep;30(9):2669-78 Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell to cell contact.

Loke P, MacDonald AS, Allen JE *European Journal of Immunology* 2000 Apr;30(4):1127-35 Antigen-presenting cells recruited by Brugia malayi induce Th2 differentiation of naive CD4(+) T cells.

Le Goff L, **Loke P**, Ali HF, Taylor DW, Allen JE *Infection & Immunity* 2000 May;68(5):2513-7 Interleukin-5 is essential for vaccine-mediated immunity but not innate resistance to a filarial parasite.

MacDonald AS, **Loke P**, Allen JE *Pathobiology* 1999;67(5-6):265-8 Suppressive antigen-presenting cells in Helminth infection.