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Divergent roles for macrophages in lymphatic filariasis

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SUMMARY

Macrophages have long been recognized as important cells associated with filarial infection but their function as effectors and/or suppressors has not been elucidated. Recent advances in our understanding of the role that macrophages may play in lymphatic filariasis have come from in vitro *studies and mouse models of filarial infection. Based on these new findings, we hypothesize 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 induce type 2 cytokines and the differentiation of 'alternatively' activated macrophages that downregulate 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.*

Keywords Brugia*, alternatively activated macrophage, nematode*

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INTRODUCTION

Macrophages are the key effector cell 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 interferon (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 (1). 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 being important cells in the killing of parasites, and in the pathology associated with dying worms and, quite possibly, the immune hyporesponsiveness associated with infection. In this review, we briefly discuss the evidence to date that macrophages have important functions in filarial infection and subsequently describe in detail the work that has led us to believe that macrophages may be critical mediators of immune regulation in lymphatic filariasis.

MACROPHAGES AS EFFECTOR CELLS IN FILARIAL INFECTION

Macrophage activation/parasite killing

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 *Acanthocheilonema viteae* microfilaria (mf) in the presence of immune sera (2). The same result is seen with *Brugia pahangi* in jirds and mice where adherent macrophages are able to kill mf in the presence of antimicrofilarial sera (3,4). 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 *Brugia*

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malayi mf can be killed *in vitro* by IFN-γ activated macrophages and this appears to be mediated at least in part by nitric oxide (NO) (5,6). In contrast, H_2O_2 appears to have little effect on *Brugia* although it does affect *Onchocerca* mf (5,7). Despite the ability of activated macrophages to kill these parasites *in vitro*, filarial nematodes are remarkably resistant to a lethal hit and killing requires sustained exposure to NO (6). Adult parasites appear to be even more resistant to macrophage mediated damage than the larval stages (6), perhaps due to the production of antioxidant enzymes (7). 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 (8).

In vivo, *B. pahangi* L3 induce activation of macrophages in the peritoneal cavity of jirds. These macrophages have significantly increased phagocytic and microbicidal activity (9). Furthermore, in both the lymphatics and the peritoneal cavity of jirds, macrophages are the dominant cell type associated with the granulomatous lesion that forms around the adult parasites and microfilaria, and are observed adherent to the parasite surface (10). 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 in susceptibility in mice are abolished when macrophage activity is blocked by carbon particle injection (11). There is considerable cumulative evidence that macrophages together with granulocytes are important in parasite destruction. However, there is little information *in vivo* about the factors 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.

Macrophages in filarial pathology

The studies described above 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 (12). The pathological granulomatous lesions that develop around dying parasites are predominantly comprised of eosinophils and macrophages (13,14) suggesting that macrophages activated by parasite infection may damage host tissue.

More recently, Taylor *et al*. (15) showed that intracellular bacteria (*Wolbachia*) present in *B. malayi* could play a role in inflammatory pathology. They found that soluble worm debris from *B. malayi* could stimulate macrophages to produce pro-inflammatory cytokines such as tumour necrosis factor (TNF), interleukin (IL)-1 and NO in an *in vitro* assay. Using a macrophage cell line that was defective in binding lipopolysaccharides (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 demonstrated that the proinflammatory stimulus in their system was due to the presence of LPS. Taylor *et al*. (15) hypothesize that dying (but not live worms) release LPS. Thus, the endosymbiotic bacteria of filarial parasites may have a role in the pathology that accompanies chemotherapy, as well as the fever-like symptoms associated with acute filarial disease. However, there is still little evidence *in vivo* that endosymbiotic bacteria can provide a pro-inflammatory stimulus.

Macrophages in natural resistance

Further evidence for macrophages as important players in the control of filarial infection come from studies with *xid* mice (CBA/N) that have a defect in the Bruton's tyrosine kinase (*Btk*) gene. These studies have shown that CBA/N mice have a reduced ability to clear mf and are more susceptible to infection with vector-derived larvae compared to wild-type CBA/J mice (16–19). 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 antigen presenting cells (APCs) of myeloid origin (20). Mukhopadhyay *et al*. (21) 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 downregulatory effects of NO on IL-12 induction. (21). Furthermore, T cell responses to mf antigen in CBA/N mice are skewed toward a more type 1 immune response and Mukhopadhyay *et al*. (17) have shown, using adoptive transfer experiments, that these alterations in cytokine responses are due to macrophages rather than B cells. This data contrasts with the recent proposal that B-1 cells are responsible for resistance to filarial infection in mice (18). Thus, the long held view that the inability of *xid* mice to rapidly clear their microfilaria is due to defective B cell function may need to be reconsidered and the contribution of macrophages reevaluated.

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MACROPHAGES AS SUPPRESSOR CELLS IN FILARIAL INFECTION

One of the most consistent findings in both human (22–24) and animal (12,25–28) studies is that individuals infected with filarial parasite exhibit profound defects in lymphocyte proliferation. In humans, this is primarily an antigen-specific defect. However, in animal studies, both antigen-specific and nonspecific components have been identified in the proliferative suppression observed following infection. Several studies in the experimental jird model (26,28) demonstrated that the 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 (29). Although not directly demonstrated to be macrophages, the cells described in these studies are likely to be macrophages or monocytes and strongly implicate a role for macrophages in the immune suppression observed over the course of filarial infection. Using an intraperitoneal (i.p.) infection model in jirds, Nasarre *et al*. (30) found that chronic infection with *B. pahangi* led to the deactivation of macrophages, as measured by their ability to kill *Toxoplasma* and produce TNF. The emergence of deactivated macrophages correlated with a reduction in the systemic granulomatous inflammatory response that they observed in the jird. These data suggest that the recruitment of anti-inflammatory macrophages can down modulate immunopathology.

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 mononuclear cells has been implicated in the downregulation of T cell responses observed during human filarial infection (31) and the spontaneous release of high levels of IL-10 by adherent mononuclear cells has been associated with the hyporesponsive state (32). Osborne and Devaney (33) 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.

IL-4 dependent antiproliferative cells in the mouse

We previously set out to determine whether exposure to filarial parasites caused an alteration in APC function that could lead to the defect in antigen-specific T cell proliferation associated with infection (34). We were unable to observe a direct effect of parasites on APC *in vitro*. However, 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. Furthermore, using a range of lymphocyte cell lines, we found that the antiproliferative effect was remarkably nonspecific and that cell division was blocked in a broad range of target cells whether stimulated with antigen or mitogen. The suppressive APC were even able to block the proliferation of transformed cell lines (34).

Notably, exposure to mf alone did not generate the antiproliferative cell. Because mf initially induce IFN-γ while L3 and adult stages induce high levels of early IL-4 (35,36), we hypothesized that induction of suppressor cells may require IL-4. Using neutralizing antibodies to IL-4 and mice genetically deficient in IL-4, it was possible to demonstrate that recruitment and/or development of these antiproliferative cells required host IL-4 (37). Interestingly, IL-10 had no effect in these studies since full suppression was observed in IL-10-deficient mice. Furthermore, 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 (34). Although IL-10 is not responsible for the antiproliferative effect of these cells, there is good evidence from other studies to suggest that it is an important component of the downregulatory effects in lymphatic filariasis (33,38).

More recently, we were able to demonstrate that the cell type responsible for these suppressive effects is indeed a macrophage (39). Purification of cells using antibodies to the macrophage marker F4/80 replicates the results with whole peritoneal cell populations. Although IL-4 deficient mice recruit fewer macrophages than wild-type mice (40), this does not account for the failure of IL-4–/– peritoneal exudate cells to block proliferation because purified macrophages from IL-4 deficient mice are still nonsuppressive (unpublished data). The identification of a macrophage phenotype entirely dependent on IL-4 has led us to speculate that these cells represent an *in vivo* manifestation of the *in vitro* derived 'alternatively activated macrophage' which is discussed in detail below.

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 observation that cytokines have opposing effects on macrophages has only recently attracted the attention of a wider audience. Macrophages activated by

IFN-γ play a critical role in destroying intracellular pathogens through the production of pro-inflammatory mediators such as NO (41,42). However, macrophages can also be activated by type 2 cytokines (particularly IL-4 and IL-13) to develop a distinct phenotype (43–45). These cells have been termed 'alternatively activated macrophages' (AAMΦ) to distinguish them from macrophages 'classically activated' (CAMΦ) by IFN-γ or LPS (45). The alternative activation pathway is not simply a down-regulation of IFN-γ-mediated effects but a developmental pathway in its own right (44). Thus, it is apparent that macrophages, similar to T cells and dendritic cells (46), represent a heterogeneous population of cells with distinct biological 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 immunopathology.

NO and arginase

The evidence that macrophages can function as effector cells against filarial parasites is correlated with inflammatory mediators such as NO and, thus, they fall into the category of CAMΦ. In contrast, the suppressive AAMΦ we observe in the *B. malayi* mouse model do not produce NO. Consistent with NO as a key effector molecule in macrophage function, the clearest distinction between the two categories of macrophages is the different metabolic pathways for L-arginine (47). Under type 1 conditions, inflammatory macrophages produce NO as a result of up-regulating inducible NO synthase (iNOS), which catalyses the L-arginine substrate (48). Under type 2 cytokine conditions, arginase is up-regulated instead of iNOS. 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 (49). Although there was a synergistic effect of adding combinations of Th2 cytokines (e.g. IL-4 with IL-10), this did not account for the level of arginase induction by Th2 cell supernatants, suggesting that additional molecules are involved. A different study has shown that peritoneal macrophages from strains of mice that were more inclined to mount a Th1 response (C57/BL6, B10.D2) preferentially produce NO, whereas macrophages from strains that were biased towards Th2 responses appeared to inhibit NO production via TGF-β (50).

Consistent with this arginase/iNOS paradigm, we have recently found that the AAMΦ that are recruited by live adult *B.malayi* implantation have highly upregulated expression of arginase I, but not arginase II (unpublished observations). This is 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 (51). In addition, we have observed upregulated expression of other markers previously associated with AAMΦ, such as the murine CC chemokine, C10 (52), strengthening the classification of these filarial-activated macrophages as AAMΦ.

Mechanism of proliferative suppression

Both CAMΦ and AAMΦ have been shown to suppress the proliferation of lymphocytes. Although NO produced by CAMΦ has significant cytotoxic activity, it can also inhibit cellular proliferation in a nontoxic manner. 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 (48). Classically activated 'suppressor' macrophages have been demonstrated in mouse models of African trypanosomiasis where infection with *Trypanosoma brucei* leads to the generation of macrophages that inhibit T cell proliferation via NO and prostaglandins (53–55).

Although CAMΦ have some downregulatory 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 (56) suggest that the production of anti-inflammatory cytokines is a key feature of APC activated under type 2 conditions. Although IL-10 and TGF-β are involved in AAMΦ mediated suppression (56) there are additional mechanisms by which AAMΦ are able to suppress cellular proliferation that are not well understood. Human monocyte/macrophages cocultured in the presence of IL-4 and glucocorticoids *in vitro* suppress proliferation of T cells through a mechanism that is independent of IL-10, NO or prostaglandins (57).

This was highly consistent with our work on suppressive macrophages induced by filarial infection that had also ruled out these potential mediators (34). We have recently found that suppression requires cell-to-cell contact and does not occur across a membrane. Even more striking, AAMΦ are capable of blocking cellular proliferation when fixed with paraformaldehye (39) demonstrating that a soluble mediator is not responsible. Furthermore, we found that the

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macrophages induced by filarial infection acted not only on murine lymphocytes, but also suppressed the proliferation of a large number of human tumour cell lines (39).

These findings bear a remarkable similarity to the macrophages described by Lee *et al*. (58) using an aerosol challenge transgenic mice system. 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*. Furthermore, this suppression could not occur across a transwell membrane suggesting that these lung macrophages also act in a contact dependent manner. As with our peritoneal cells, this lung model is associated with eosinophilia, suggesting a type-2 cytokine environment. These interstitial macrophages also seemed to augment IL-4 and IL-5 production by Th2 cells, while suppressing the proliferation of both Th1 and Th2 cells.

The evidence from two disparate systems 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 the *B. malayi*-induced macrophages to block cellular proliferation over a broad range of cell types strongly suggests the engagement of a highly conserved receptor. A key direction of future studies on AAMΦ will be the identification of this antiproliferative mechanism.

Th2 cell induction by AAMΦ

Apart from a role in immune suppression, Geordt & Orfanos (56) also proposed that AAMΦ, similar to type 2 dendritic cells (DC2), could induce naïve T cells to differentiate to Th2 cells. Macrophages are typically associated with the induction of Th1 type responses and have been shown to promote Th1 cell differentiation (59). 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. We have found that the AAMΦ that are induced by live adult *B. malayi* can indeed drive Th2 differentiation of naïve T cells from pigeon cytochrome c (PCC) specific TCR transgenic (PCC-tg) mice (60). We also found that Th2 differentiation was associated with the inhibition of (or failure to stimulate) IFN-γ producing T cells, rather than the induction of IL-4 producing cells during primary stimulation. TGF-β was involved in this naïve T cell/APC interaction because the Th2 driving effect could be reversed with blocking antibodies against TGF-β (but not IL-10).

Our observations show that macrophages can be both the targets of T cell cytokines as well as regulators of T cell differentiation. While there have been reports of dendritic cell subsets that differ in their capacity to stimulate the differentiation of naïve T cells (46), 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 type 2 response, it is unlikely that AAMΦ are a key player in the early induction of Th2 cells since AAMΦ are only induced under existing type 2 conditions. AAMΦ may be more important in downstream events that suppress Th1 cells and thus stabilize the Th2 response.

Divergent roles for macrophages in filarial infection

It is apparent that macrophages exhibit a complexity of functional roles that parallels the Th1/Th2 paradigm. However, as the relative importance of Th1 versus Th2 cells in protection against filarial infection still remains broadly unresolved, it is unlikely that we will immediately discern the roles of CAMΦ versus AAMΦ in parasite destruction. Nonetheless, the evidence that we have discussed suggests a scenario in which, once infection is established, a balance between these two macrophage populations may determine the severity of disease associated with infection.

We have found that AAMΦ can be generated by the daily injections of excretory/secretory material (ES) products from the adult but cannot be generated by the implantation of dead parasites (61). In contrast, Taylor *et al*. (15) have shown that dead/dying worms can cause the release of pro-inflammatory mediators by macrophages. This leads us to suggest the following scenario: Live adult parasites live in the lymphatic system, where they secrete ES products into the afferent lymphatics. Among the ES products are immunomodulatory molecules that induce Th2 responses (60,62) and recruitment of AAMΦ (61). On the death of parasites, LPS from the intracellular bacteria is released, leading to the activation of CAMΦ. The production of cytokines by CAMΦ would drive Th1 cell development as well as inducing inflammatory-mediated damage to the surrounding tissue. However, type 2 responses induced and maintained by living parasites would allow the recruitment of AAMΦ that can control this inflammation while continually promoting a type 2 response. As more parasites are killed, however, the balance could eventually shift towards a strong Th1 cytokine response and the subsequent inflammatory effects could lead to the more severe pathology associated with chronic disease. Most inflammatory responses induced by bacterial LPS are cleared relatively quickly while, in contrast, filarial disease is

characterized by chronic inflammation. This may be due to the slow and continual release of bacterial products by worms dying in individuals with insufficient recruitment of AAMΦ.

The prediction from this model would be that asymptomatic mf$^+$ individuals have more AAMΦ, reflecting more live healthy adult parasites. Elephantiasis patients have too many CAMΦ leading to pathology and potentially more parasite clearance. Endemic normals have a successful balance that allows AAMΦ to control the inflammatory effects of CAMΦ such that parasites can be killed without pathology.

The scenario described here may have considerable similarity to the pattern of responsiveness induced during schistosome egg deposition. 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 of a large poorly circumscribed granuloma. Macrophages with an alternatively activated phenotype appear to then rapidly downregulate the inflammatory response and promote a switch to a type 2 response. This is mediated primarily by IL-10. The subsequent granulomatous lesion is smaller in size and more compact, causing reduced damage to host tissue (63). We would propose that a balance between cytokine responses leads to a balance between the two macrophage populations and this balance is required to avoid the chronic pathology associated with many helminth infections.

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