NO Contributes to Proliferative Suppression in a Murine Model of Filariasis

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Infection of BALB/c mice with microfilariae (mf) of Brugia pahangi leads to the suppression of antigen (Ag)-specific proliferative responses in the spleen. The proliferative defect is dependent on inducible nitric oxide synthase (iNOS) activity, since inhibition of iNOS with either l-N-nitroarginine (l-NNA) or aminoguanidine reversed defective proliferation. Splenocytes from mf-infected animals produce high levels of gamma interferon (IFN-γ) upon in vitro restimulation with Ag, and experiments in IFN-γ receptor-deficient (IFN-γR−/−) mice demonstrated that signaling via the IFN-γR is essential in the induction of NO production and subsequent proliferative suppression. Restimulation of splenocytes from mf-infected animals with an extract of Acanthocheilonema viteae, a related filarial worm which lacks endosymbiotic bacteria, also resulted in NO production and proliferative suppression, demonstrating that lipopolysaccharide of bacterial origin is not essential to the induction of iNOS activity. These results extend previous observations that infection with different life cycle stages of Brugia leads to the development of differentially polarized immune responses and demonstrate one method by which these differences may exert their effects on the proliferative potential of cells from infected animals.

Lymphatic filariasis is a major tropical disease, caused by nematode worms of the genera Wuchereria and Brugia and affecting an estimated 128 million individuals worldwide; around one-fifth of the world’s population lives in areas where the infection is endemic (28). The adult worms inhabit the lymphatics of the definitive host, where they can survive for extended periods. Adult females produce millions of microfilariae (mf), or first-stage larvae, which circulate in the peripheral blood. If ingested by a susceptible mosquito, mf develop to the third larval stage (L3), which reinitiates the cycle of infection when the infected mosquito next takes a blood meal.

Human filarial infection is characterized by a dominant Th2 response and a defective antigen (Ag)-specific T-cell proliferative response (34, 35, 39, 47). Although the proliferative defect was first described only in microfilaraemic individuals (34), the defect is now known to extend to other clinical groups (47). However, proliferative unresponsiveness is most pronounced in microfilaraemic individuals and is most difficult to restore in these patients following chemotherapy (41). In contrast, T cells from patients with chronic pathology, who are generally amicrofilaraemic, have relatively strong parasite-specific proliferative responses (23). Attempts to reverse the proliferative defect of T cells from Brugia malayi-infected individuals by using a variety of immunomodulators or neutralizing antibodies were largely unsuccessful, although some effect was noted with recombinant interleukin-2 (rIL-2) (40). In Wuchereria bancrofti infection, peripheral blood mononuclear cells from microfilaraemic individuals produce large amounts of spontaneous and Ag-specific IL-10 in vitro (22). Several studies have shown that neutralization of IL-10 (14, 22) or transforming growth factor β (14) enhanced Ag-specific proliferative responses, suggesting that regulatory cytokines may contribute to impaired T-cell responses. More recent studies using peripheral blood mononuclear cells from W. bancrofti-infected individuals demonstrated that the source of parasite Ag used for in vitro restimulation was an important determinant of proliferative unresponsiveness: culture with Ag from mixed-sex adult worms or mf down regulated proliferative responses while culture with adult male antigen had no such effect (21). These studies imply a role for the mf in the proliferative suppression. Furthermore, in the jird model of infection, loss of proliferative responsiveness accompanies the onset of mf production (16). Although a number of mechanisms have been proposed which could account for this hyporesponsiveness (24), the exact nature of the proliferative suppression is still not fully understood.

Down regulation of proliferative immune responses is a hallmark of several different parasitic infections (4, 7, 42) and presumably reflects a mechanism by which parasite survival is promoted. The mechanisms underlying this defect vary from organism to organism, but various mediators such as inducible nitric oxide synthase (7, 20), pro- and anti-inflammatory cytokines (26, 33, 46), and T-cell apoptosis (17, 18) have been implicated in mediating suppression. In this study, we have used the BALB/c mouse infected with the mf of Brugia pahangi to further investigate the nature of the Ag-specific proliferative suppression associated with infection.

MATERIALS AND METHODS

Mice and infection protocols. Six-week-old male BALB/c mice (purchased from Harlan Olac, Oxford, United Kingdom) were used in most experiments. IFN-γR−/− mice on the 129Sv background were provided by Allan Mowat, University of Glasgow, while the wild-type controls were purchased from Harlan. All mice were maintained in filter-top cages. The mice were injected intravenously (i.v.) via the tail vein with 105 mf or 50 L3 of Brugia pahangi in Hanks balanced salt solution (HBSS; Gibco-BRL) or with an equal volume of HBSS alone. B. pahangi mf were obtained by HBSS peritoneal lavage of jirds infected for >3 months. mf were separated from host cells by centrifugation over Histopaque 1077 (Sigma), washed twice in HBSS, and counted. L3 were harvested from Aedes aegypti (refm) at day 9 postinfection (p.i.) as previously described (9).

Preparation and culture of spleen cells. At 12 d p.i., the mice were sacrificed by CO2 inhalation and their spleens were removed aseptically. Single-cell suspensions were prepared in RPMI (RPMI 1640 Dutch modification with 5 mM HEPES, 5 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml [all from Gibco-BRL]) by passage of the spleens through Nytex mesh (Cadiach and Sons, London, United Kingdom). Erythrocytes were lysed in 0.83%
NH₄Cl (pH 7.2), the remaining cells were washed twice in RPMI, and the numbers of viable lymphocytes were assessed by trypan blue exclusion. Cells were resuspended to a concentration of 10⁵ per ml in RPMI plus 10% fetal calf serum (Gibco). Splenocytes (5 x 10⁵ per well) were plated out in triplicate wells in 96-well half-area plates (Costar) in the presence or absence of 10 μg of adult Ag per ml (a soluble extract of B. pahangi adult worms prepared by homogenization on ice). The cells were also stimulated with 1 μg of concanavalin A (Sigma) per ml to assess polyclonal responses. The plates were incubated at 37°C under 5% CO₂, and proliferation was assessed by [³H]thymidine incorporation during the last 16 h of culture. For analysis of cytokine and NO₂⁻ production, cells were incubated in 1-ml cultures (10⁷ per ml) under identical conditions and supernatants were harvested at the time points indicated.

**RESULTS**

Ag-specific proliferative responses of splenocytes from mf-infected animals are down regulated in vitro. BALB/c mice were infected i.v. with 10⁵ mf or 50 L3 or were injected with HBSS. At 12 days p.i., spleens were removed and restimulated in vitro with an extract of adult B. pahangi. The results presented in Fig. 1 show the Ag-stimulated proliferative responses of splenocytes from each group of animals, at two time points of in vitro restimulation. At 48 h, splenocytes from both groups of infected mice showed Ag-specific proliferation (Fig. 1A), but by 96 h of culture, Ag-stimulated cells from mf-infected animals were routinely incorporated fewer cpm than did cells cultured in medium only (P = 0.00015). When expressed as stimulation indices, cells from mf-infected animals routinely displayed a stimulation index of <1 after 96 h of Ag-stimulated culture. In contrast, Ag-specific proliferation of cells from L3-infected animals was maintained throughout the period observed (Fig. 1B). Polyclonal responses were not affected in any group of animals as assessed by concanavalin A stimulation. This experiment has been repeated on multiple occasions with equivalent results.

Analysis of cytokine secretion profiles in i.v.-infected mice demonstrated very similar results to those reported previously with mice infected by the subcutaneous route (32) or the intraperitoneal route (15). The only cytokine produced by splenocytes from mf-infected mice at 12 days p.i. was IFN-γ (Fig. 1C), while cells from L3-infected mice produced IL-4, IL-5, and IL-10 but no IFN-γ (data not shown).

rIL-2 fails to restore defective Ag-specific proliferation. The survival of activated T cells in vitro is supported by cytokines such as IL-2 and IL-4, and, while T cells may be exposed to high levels of these growth factors during the initial stages of response to Ag, their concentration decreases as the response progresses. Thus, T cells may die as a result of reduced levels of such growth factors (reviewed in reference 25). Previous studies in the jird-B. pahangi model of infection have shown that splenocytes from microfilaremic jirds were unable to proliferate or produce significant levels of IL-2 in response to in vitro restimulation with parasite Ag (16, 36). Since Ag-stimulated splenocytes from mf-infected BALB/c mice produce only low or undetectable levels of IL-2 in vitro (data not shown), we investigated whether a lack of IL-2 was limiting proliferation in these cultures. Ag-stimulated cells from mf-infected and unin-
animals (increased Ag-specific proliferation of cells from mf-infected L3-infected or uninfected control animals. As shown in Fig. 1C, addition of AMG to Ag-stimulated cultures of cells from mf-infected animals also caused a significant increase in IFN-γ production compared with Ag-only cultures (P = 0.02).

Defective proliferation is restored in IFN-γR−/− mice but not by neutralization of IFN-γ activity in vitro. IFN-γ is known to be a potent inducer of NO production (10) and is produced at high levels by cells from mf-infected animals in Ag-stimulated culture (see Fig. 1C). To assess the role of IFN-γ in NO induction in this model, proliferation of splenocytes from mf-infected and uninfected control IFN-γR−/− mice was measured over a time course of in vitro restimulation with Ag and the responses were compared with those of their wild-type (129 Sv) counterparts. As shown in Fig. 4A, after 96 h of culture, Ag-specific proliferative responses of splenocytes from mf-infected KO mice were significantly greater than those from the equivalent wild-type animals (P = 0.02). While high levels of nitrite were found in culture supernatants of cells from mf-infected wild-type mice, background levels of NO2− were detected in cultures of cells from mf-infected KO mice upon in vitro restimulation with Ag (Fig. 4B). No differences were observed in Ag-stimulated cytokine production from the IFN-γR−/− KO mice and their wild-type counterparts, except for the presence of Ag-specific IL-5 in the KO mice (data not shown). Measurement of the IFN-γ levels in these cultures demonstrated that IFN-γ was effectively neutralized at the concentration of MAb used.

LPS is not involved in the induction of Ag-stimulated NO production. The presence of gram-negative microorganisms in filarial worms was first reported by McLaren et al. in 1975 (27), and there is currently a resurgence of interest in these intracellular symbionts. The potential of Wolbachia endosymbionts as targets for chemotherapy, as mediators of pathology, and as modulators of the immune response was recently reviewed by Taylor and Houveraf (44). IFN-γ and lipopolysaccharide (LPS) display synergism as potent stimulators of NO production (10), and it has been demonstrated that sequential exposure to IFN-γ followed by LPS is efficient at stimulating NO production by murine macrophages (19). To investigate the role of bacterial LPS in driving Ag-stimulated NO production in vitro, cells from infected or control mice were restimulated in vitro with an extract of Acanthocheilonema viteae, a related filarial parasite which lacks endosymbionts (44). Elevated levels of NO were produced in response to A. viteae Ag (Fig. 5A), and the proliferative defect was still apparent in cells from mf-infected animals (Fig. 5B). Furthermore, when polymyxin B (2.5 μg/ml) was used as an inhibitor of LPS activity, no effect on the Ag-stimulated production of NO2− by cells from mf-infected animals or on Ag-specific proliferative responses was observed (data not shown). These results suggest that LPS is not involved in generating NO production and the subsequent in vitro proliferative suppression in this model.

DISCUSSION

In this study we have shown that the Ag-specific proliferative defect observed with splenocytes from mf-infected mice is mediated via the IFN-γ dependent induction of NO. In contrast, splenocytes from L3-infected mice produced insignificant lev-
els of NO and gave sustained levels of Ag-specific proliferation. These results build on observations made previously that infection with different life cycle stages of *Brugia* leads to differentially polarized cytokine profiles (15) and demonstrate that these differences can exert their effects on the proliferative potential of cells from infected animals.

Splenocytes from mf-infected animals produced high levels of Ag-specific IFN-γ in vitro restimulation, and experiments with IFN-γR−/− mice demonstrated that signaling via the IFN-γR was essential to the induction of high-level NO production and subsequent proliferative suppression. The Th2 pattern of cytokine secretion by splenocytes from L3-infected animals may act to prevent NO production in these cultures. Both IL-4 and IL-10 down-regulate iNOS activity (6, 12) and have recently been shown to promote an alternative pathway of L-arginine metabolism via arginase rather than iNOS in murine macrophages and dendritic cells (29). Moreover, previous studies using peritoneal exudate cells from mice infected with *B. malayi* mf by the intraperitoneal route demonstrated a role for NO in down regulating the proliferation of a conalbumin-specific T-cell clone (2). This effect was also specific to infection with mf, since although peritoneal implantation of adult worms or L3 elicited a profoundly suppressive Ag-presenting cell population, the suppression was not NO dependent. These results highlight the fact that different life cycle stages of filarial worms have the potential to down regulate proliferative responses by a variety of mechanisms.

In contrast to the results with the IFN-γR−/− mice, neutralization of IFN-γ in vitro had no effect on NO production or proliferation of cells from mf-infected mice. One possible explanation for these apparently contrasting results is that prior activation in vivo by IFN-γ is sufficient to prime for NO production in culture, with other proinflammatory cytokines compensating for a lack of IFN-γ. It is also possible that residual IFN-γ activity was sufficient to drive NO production.

NO mediates proliferative suppression in several models of parasitic infection including trypanosomiasis, toxoplasmosis, and cestode infection (4, 20, 42). iNOS is induced in response to proinflammatory cytokines such as IFN-γ, which can also exert cytostatic effects on T cells. In this study, it was possible to distinguish between the suppressive effects of NO and IFN-γ by using inhibitors of iNOS in cultures of cells from BALB/c mice. These experiments demonstrated that proliferative responses could be restored in the presence of very high levels of IFN-γ, as long as iNOS activity was blocked, and confirmed

![Graph A](image1.png)

**FIG. 3.** Proliferative suppression can be reversed by inhibition of iNOS activity. (A and B) Proliferative responses of splenocytes from BALB/c mice given 10⁵ *B. pahangi* mf or HBSS after 96 h of Ag-stimulated culture in the presence or absence of 500 μM AMG (A) or 250 μg of L-NMMA per ml (B). (C) IFN-γ production by splenocytes from mf-infected mice cultured in the presence or absence of AMG at 96 h of culture. All data represent the mean and standard deviation for five mice per group. *, significantly different from unsupplemented cultures *(P < 0.05).*
that the primary role of IFN-γ in mediating proliferative suppression in this model was via iNOS induction.

NO can regulate the development of immune responses either directly by inducing the apoptosis of T cells or Ag-presenting cells (1, 11, 31, 38) or indirectly via the modulation of cytokine secretion, as suggested by recent studies proposing a model in which NO plays both direct and indirect roles in Th1 development (30). Low-level NO has a direct effect on CD4+ T cells, enhancing their capacity for IFN-γ production (13, 30), which activates macrophages to secrete IL-12, a potent promoter of Th1 differentiation (45). Increased production of IFN-γ may then lead to high-level NO production, which in turn can inhibit the production of IL-12 by macrophages, consequently preventing excessive amplification of Th1 cells (30). In the present study, inhibition of iNOS with AMG resulted in significant increases in IFN-γ production in cells from mf-infected mice, suggesting that the negative-feedback mechanism described above may be operative in mf-infected animals. The ability of NO to limit the expansion of the Th1 responses may be particularly important in the context of high levels of circulating Ag, where unchecked proinflammatory responses could lead to pathologic changes.

In other model systems where the effects of NO in mediating proliferative suppression have been investigated, it appears that the direct effects of NO and those mediated via IFN-γ may synergize to promote apoptosis. For example, studies in a murine model of Trypanosoma cruzi demonstrated that both IFN-γ-induced up regulation of Fas expression and NO production contributed to the apoptosis among splenocytes during the acute stage of infection (26). It has also been demonstrated, using human T cells, that NO may induce apoptosis directly and also render cells susceptible to IFN-γ-mediated apoptosis (3). In preliminary experiments we have observed the development of a CD4ψ subpopulation of lymphocytes on inhibition of iNOS activity. By analogy to previous reports, this CD4ψ subset may contain the Ag-reactive T cells (37). Since this population fails to expand in the presence of NO, it is possible that they may be lost via NO-mediated apoptosis. A similar phenomenon has recently been reported for bacille Calmette-Guérin (BCG)-infected mice (8), in which IFN-γ-induced NO production was demonstrated to eliminate responding CD4 T cells.

Although IFN-γ is the only cytokine capable of inducing iNOS activity on its own, it is most potent in this function when acting in concert with LPS (10). In this respect, the presence of LPS in the Ag preparations used for in vitro restimulation may...
assume biological significance. However, we were unable to demonstrate a role for LPS in NO induction in our model. Culture of splenocytes with polymyxin B had no effect on Ag-stimulated proliferative responses or on NO production. Likewise, when mf-primed cells were restimulated in vitro with extracts from the related filarial parasite *A. viteae*, which does not contain endosymbionts, NO production and proliferative suppression were still observed. Recent studies using a macrophage cell line have suggested that inflammatory responses induced by *B. malayi* extract are elicited in response to contamination with LPS of bacterial origin (43). The discrepancy in our results may relate to the readout of the experiments (Ag-specific proliferation versus proinflammatory cytokine production) or the species and/or strain of parasite used (*B. pahangi* versus *B. malayi*), which may vary in their levels of *Wolbachia* contamination.

The results presented in this paper demonstrate that the proliferative defect observed in vitro with cells from mf-infected mice is mediated by NO. Blocking iNOS activity reverses the proliferative defect, demonstrating that cells are not irreversibly committed to undergo activation-induced cell death. Our present studies are aimed at further characterizing the cellular targets of NO-mediated proliferative suppression and determining the effector mechanisms involved.

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