Nitric Oxide Limits the Expansion of Antigen-Specific T Cells in Mice Infected with the Microfilariae of *Brugia pahangi*

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Infection of BALB/c mice with the microfilariae (Mf) of the filarial nematode *Brugia pahangi* results in an antigen-specific proliferative defect that is induced by high levels of NO. Using carboxyfluorescein diacetate succinimyl ester and cell surface labeling, it was possible to identify a population of antigen-specific T cells from Mf-infected BALB/c mice that expressed particularly high levels of CD4 (CD4^{hi}). These cells proliferated in culture only when inducible NO synthase was inhibited and accounted for almost all of the antigen-specific proliferative response under those conditions. CD4^{hi} cells also expressed high levels of CD44, consistent with their status as activated T cells. A similar population of CD4^{hi} cells was observed in cultures from Mf-infected gamma interferon receptor knockout (IFN- γ R^{-/-}) mice. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling staining revealed that the CD4⁺ T cells from Mf-infected wild-type mice were preferentially susceptible to apoptosis compared to CD4⁺ T cells from IFN- γ R^{-/-} mice. These studies suggest that the expansion of antigen-specific T cells in Mf-infected mice is limited by NO.

The immune response in human lymphatic filariasis is characterized by the in vitro suppression of antigen-specific proliferation and gamma interferon (IFN- γ) production. These responses are most profoundly affected in individuals with circulating microfilariae (Mf; the first larval stage) and are most difficult to restore within this group (29). Treatment with microfilaricidal drugs such as ivermectin restores responsiveness, suggesting a direct role for Mf in generating suppression (15). Furthermore, the results of a recent study of seasonal transmission demonstrated that in a subset of infected individuals, IFN- γ production correlates inversely with the density of circulating Mf (30).

Infected individuals display a spectrum of pathology, the etiology of which is complex but which is in part related to the worm burden and the type and intensity of the immune response (25). The majority of individuals with circulating Mf display few outward signs of disease, contrasting with individuals who have cleared Mf from the circulation, who more often display the chronic pathology associated with filarial infection. Thus, it has been suggested that the development of more vigorous immune responses and the loss of circulating Mf may predispose to the development of inflammatory pathology (20). In this sense, active downregulation of proinflammatory responses may be a key element in preventing the development of pathology and prolonging the patent phase of infection.

The use of mouse models has allowed more detailed analysis of the responses elicited by distinct life cycle stages of *Brugia* spp. and has revealed a variety of mechanisms by which filarial parasites can modulate host responses (18). Intriguingly, single-stage infection with Mf (whether by the intraperitoneal,

subcutaneous, or intravenous route) uniquely elicits development of a Th1-like IFN- γ -dominated response (16, 17, 24, 26). It has been demonstrated that infection with Mf is associated with an antigen (Ag)-specific proliferative defect in the mouse that is dependent upon the induction of NO by IFN- γ (22). Furthermore, suppression is associated with the NO-mediated apoptosis of CD4⁺ T cells. This contrasts with the situation seen following infection with L3 (the infective third-stage larvae), which elicit a Th2 response and low levels of apoptosis (13). While NO is well recognized as an antimicrobial effector molecule, it may also function in a regulatory capacity during the development and ultimate resolution of an immune response. A growing body of evidence from models of both infectious and autoimmune disease suggests that NO can actively limit the development of potentially pathological inflammatory responses (5, 6, 9, 32). NO can exert negative regulatory effects by several means, either directly via induction of macrophage or T-cell apoptosis (23, 28) or indirectly via inhibition of interleukin-12 (IL-12) production (10). As with many aspects of its biological function, the outcome of exposure to NO in terms of apoptosis is highly context dependent and varies with the concentration and the cell type involved. However high-level NO production is most often associated with proapoptotic effects. While the mechanisms by which NO induces apoptosis remain incompletely defined, potential targets include inhibition of proteasome activity, downregulation of Bcl-2, and modulation of Fas/Fas ligand expression (7, 21, 31).

In this study, we set out to determine whether Ag-specific T cells in cultures from Mf-infected mice were preferentially susceptible to NO, and if so, to investigate the possible mechanisms by which NO targets a specific cellular population. Using a combination of CFSE and cell surface labeling, it was possible to identify a population of Ag-specific T cells from Mf-infected mice that expressed particularly high levels of CD4 (CD4^{hi}). In cultures from these mice, the CD4^{hi} population accounted for almost all of the Ag-specific proliferative response when inducible NO synthase (iNOS) activity was in-

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hibited. Infection of IFN- γ receptor knockout (KO) (IFN- γ R^{-/-}) mice (unable to respond with high-level NO production) also facilitated the expansion of Ag-specific T cells in vitro. The increased expansion of CD4 cells from IFN- γ R^{-/-} mice was accompanied by a decrease in the levels of T-cell apoptosis in these cultures. These findings provide a further example of how NO may limit the expansion of proinflammatory responses in a mouse model of infectious disease.

MATERIALS AND METHODS

Mice and infection protocols. BALB/c and 129Sv mice (6 to 8 weeks old) were obtained from Harlan UK (Bicester, United Kingdom), and IFN- γ R^{-/-} mice were bred at the University of Glasgow. All mice were maintained in filter-top cages. The mice were infected intravenously in the tail vein with either 10⁵ Mf or 50 L3 of *Brugia pahangi* or received Hanks Balanced Salt Solution only. The numbers of parasites used for infection were based on previous studies (13, 22). Mf were harvested from the peritoneums of infected gerbils (*Meriones unguiculatus*), while L3 were isolated from infected *Aedes aegypti* mosquitoes exactly as described previously (13). In all experiments, five animals per group were used.

Spleen cell preparation and culture. The mice were killed on day 12 postinfection (p.i.) by CO₂ inhalation, and the 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 μ g of streptomycin per ml; all from Gibco/BRL) by passage of the spleens through Nytex mesh (Cadisch and Sons, London, United Kingdom). The 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. The cells were incubated in RPMI plus 10% heat-inactivated fetal calf serum (FCS; Gibco/BRL) at a concentration of 10⁷ per well in 24-well plates in the presence of 10 μ g of adult *B. pahangi* Ag per ml (cross-reactive with both Mf and L3). In some experiments, cells were cultured with Ag and the iNOS inhibitor aminoguanidine (AMG; Calbiochem, Nottingham, United Kingdom) at 500 mM and then processed for fluorescence-activated cell sorter (FACS) analysis. At various times, cells were removed and surface stained with antibodies to CD4 (clone L3-T4; allophycocyanin [APC] labeled), CD44 (phycoerythrin labeled), Fas (fluorescein isothiocyanate labeled), or appropriate isotype-matched controls (all from Pharmingen) prior to FACS analysis. The cells were stained with 2 μ g of fluorochrome-conjugated monoclonal antibody per sample in a final volume of 100 μ l.

CFSE labeling. Splenocytes from five mice per group were labeled with CFSE *ex vivo*. In brief, 5 $\times$ 10⁷ cells from each mouse were washed twice in 5 ml of sterile phosphate-buffered saline (PBS) and then incubated in 5 mM CFSE (Molecular Probes) at 5 $\times$ 10⁷ per ml for 8 min at room temperature. The reaction was stopped by the addition of 5 ml of RPMI containing 20% FCS, and then all samples were washed twice in RPMI containing 10% FCS. The stained cells were plated out at a concentration of 10⁷ per ml as described above in medium only or with Ag at 10 μ g per ml in the presence or absence of 500 mM AMG. After 96 h of incubation, the cells were stained with either an anti-mouse CD4 APC (L3-T4) conjugate or an isotype-matched control antibody (R35-95) (both from Pharmingen).

Flow cytometry analysis using TUNEL. Splenocytes from control uninfected, Mf-infected, and L3-infected mice were cultured with Ag (10 μ g per ml) for 48 h. The cells were washed in PBS, fixed in 1% paraformaldehyde at room temperature for 15 min, washed, resuspended in 75% ice-cold ethanol, and held at -20°C for at least 18 h. Terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) was performed using an APO-DIRECT kit (Pharmingen). Briefly, fixed cells were labeled with fluorescein isothiocyanate-dUTP in the presence of TdT enzyme at 37°C for 2 h. The cells were washed twice and analyzed by flow cytometry using a Becton Dickinson FACS-can. The controls for TUNEL included cells labeled in the absence of TdT enzyme. Cell surface labeling was carried out following TUNEL with an APC-conjugated anti-CD4 antibody (Pharmingen), the cells were washed twice in PBS, and then a total of 10,000 to 20,000 events of interest were analyzed. Lymphocytes were gated by physical parameters (size and granularity), and the TUNEL staining profile of CD4-positive cells was analyzed using CellQuest Software. The experiments were performed on cells from individual animals (five per group) and were reproducible (repeated at least three times).

Statistical analysis. The unpaired Student's *t* test was used to determine the statistical significance of differences between groups. A *P* value of <0.05 was considered to be a significant difference.

RESULTS

Inhibition of NO allows the expansion of a population of CD4^{hi} T cells in vitro. CD4 T cells from Mf-infected BALB/c mice fail to proliferate in response to restimulation with parasite Ag unless NO production in these cultures is inhibited (22). Intriguingly, while restoring the proliferative capacity of these cells, iNOS inhibition also caused a distinct alteration in the CD4 staining profile of cells from Mf-infected animals. Cells from infected and uninfected animals were removed from Ag-stimulated culture after 96 h and surface stained prior to FACS analysis. In the presence of AMG, a discrete tertiary peak of brightly staining CD4^{hi} cells could be clearly identified, as well as CD4⁻ and CD4⁺ lymphocyte populations (Fig. 1A, top panel). In contrast, in the absence of AMG, only low levels of CD4^{hi} cells were observed (Fig. 1A, top panel). In cultures of cells from L3-infected animals (Fig. 1A, middle panel), the expansion of the CD4^{hi} subpopulation was not influenced by the addition of AMG, reflecting both the proliferative capacity and the relative lack of NO production in these cultures (note the convergence of dashed and solid lines). Such distinct CD4^{hi} populations were not observed among cells from uninfected control animals (Fig. 1A, bottom panel), in which they accounted for <2% of total lymphocytes (Fig. 1B). Indeed, only among cells from Mf-infected animals did iNOS inhibition consistently and significantly increase the percentage of CD4^{hi} cells (Fig. 1B). In the experiment shown, the mean number of CD4^{hi} cells as a percentage of total lymphocytes rose from 1.31 $\pm$ 0.96 to 7.06 $\pm$ 1.5 upon iNOS inhibition among cells from Mf-infected animals (*P* = 0.012), while it was not significantly altered in other groups.

It was interesting that the CD4^{hi} population appeared most distinctly among cells from Mf-infected animals (Fig. 1), while in cultures from L3-infected mice, the CD4^{hi} population appeared as a "shoulder" on the CD4⁺ population. The discrete nature of the CD4^{hi} cells from Mf-infected mice reflected the significantly higher mean fluorescence intensity (MFI) of these cells compared to those derived from L3-infected animals (*P* < 0.05 for MFI of Mf versus L3 or control; data not shown). CD4^{hi} cells were not observed in the splenocyte population from any experimental group immediately *ex vivo*.

CD4^{hi} population contains Ag-reactive cells. CD4^{hi} cells displayed increased forward and side scatter characteristic of activated lymphocytes, and it has recently been reported that T cells upregulate surface expression of CD4 following encounters with their specific Ag (27). In order to determine whether the cells that proliferate in response to Ag were CD4^{hi}, splenocytes from Mf-infected, L3-infected, and uninfected control animals were labeled with CFSE and cultured in the presence or absence of AMG for 96 h prior to surface staining and FACS analysis. In AMG-supplemented cultures from Mf-infected mice, the majority of CD4^{hi} cells had divided (Fig. 2A), and their division accounted almost entirely for proliferation within the whole CD4 population. In contrast there was little evidence of proliferation in the CD4-normal population (Fig. 2A).

In the presence of AMG, the percentages of CD4^{hi} cells that had divided were remarkably similar in cultures from Mf-infected (63.9% $\pm$ 3.4%) and L3-infected (67.5% $\pm$ 2.9%) animals and were significantly higher than that of control animals

(13.3% $\pm$ 2.5%; $P \leq 0.001$ in both cases) (Fig. 2B). Cells taken from medium-only cultures were routinely analyzed, and under no circumstance tested (with or without AMG) did they display significant evidence of proliferation or expansion of a CD4^{hi} population.

As an alternative marker of activation, CD44 expression on CD4⁺ cells from Mf-infected, L3-infected, and uninfected control animals was assessed following in vitro restimulation in the presence or absence of AMG. In cultures from Mf-infected mice, CD4^{hi} cells were expanded in the presence of AMG, and this population consistently expressed higher levels of CD44 than normal CD4⁺ cells (Fig. 3, compare the plots with and without AMG), further confirming their status as activated T cells. There was no significant difference between the levels of CD44 expression by CD4^{hi} cells from Mf-infected and L3-infected animals (data not shown). Similar results were obtained in replicate experiments.

CD4^{hi} cells are expanded in Mf-infected IFN- γ R KO mice. As the expansion of CD4^{hi} cells in cultures from Mf-infected BALB/c mice was dependent upon the inhibition of iNOS, we determined whether a similar population of CD4^{hi} cells would be expanded in the absence of IFN- γ -induced NO production. For this purpose, IFN- γ R^{-/-} mice were infected with Mf, and splenocytes were analyzed 12 days p.i. By 96 h of culture, CD4 cells made up a greater percentage of total lymphocytes in cultures from Mf-infected IFN- γ R^{-/-} mice than in cultures of their equivalent wild-type counterparts (Fig. 4) ($P < 0.05$), in keeping with the superior proliferative responses of total splenocytes from IFN- γ R^{-/-} mice observed previously (22). When the CD4^{hi} population was analyzed, there was a significantly greater expansion of these cells in Mf-infected IFN- γ R^{-/-} mice than in Mf-infected wild-type mice (Fig. 5). In fact, the numbers of CD4^{hi} cells in cultures from Mf-infected IFN- γ R^{-/-} mice were very similar to those in cultures from BALB/c mice in the presence of AMG. In cultures from Mf-infected IFN- γ R^{-/-} mice, the numbers of CD4^{hi} cells correlated well with the overall increase in CD4⁺ cells ($r = 0.95$). In this background strain of mouse (129Sv), the CD4^{hi} cells appeared as a shoulder on the CD4-normal population rather than as a distinct peak, as observed in Mf-infected BALB/c mice.

Levels of apoptosis are reduced in cultures from Mf-infected IFN- γ R^{-/-} mice. Previous results have shown that proliferative suppression is associated with high levels of NO-mediated apoptosis of CD4 T cells in cultures from Mf-infected BALB/c

FIG. 1. iNOS inhibition allows the Ag-driven expansion of CD4^{hi} T cells in vitro. (A) The histograms show the CD4 staining profile of splenocytes from Mf-infected (top), L3-infected (middle), and uninfected control (bottom) BALB/c mice after 96 h of Ag-stimulated culture in the presence (dashed line) or absence (solid line) of 500 mM AMG. Note the tertiary peak of CD4^{hi} staining in cells from Mf-infected mice. The graphs show individual animals from each group, but the results were consistent within the groups (Fig. 1B) and in replicate experiments. (B) iNOS inhibition significantly increased the percentage of total lymphocytes expressing high levels of CD4 only in Ag-stimulated splenocytes from Mf-infected animals. The results show the mean plus standard deviation of five mice per group. Open bars, cells cultured in the presence of 10 μ g of *B. pahangi* Ag per ml; solid bars, cells cultured in Ag plus 500 mM AMG; *, significantly different from unsupplemented cultures; HBSS, Hanks balanced salt solution.

FIG. 2. The CD4^{hi} population contains Ag-reactive cells. (A) CFSE-labeled splenocytes from infected and uninfected BALB/c mice were restimulated in vitro with Ag in the presence of 500 mM AMG. After 96 h of culture, dividing cells (CFSE low) were clearly visible among the total CD4⁺ population (solid line). However, the majority of divisions had occurred within the CD4^{hi} population (boldface line) and not the CD4-normal population (dashed line). Note the overlay between CD4^{hi} cells (boldface line) and the total CD4⁺ population (solid line). The FACS plot shows cells from an individual animal, but the results were consistent within groups (Fig. 2B) and between experiments. (B) Percentages of CD4^{hi} T cells that have divided in response to Ag in cultures from Mf- or L3-infected mice or control mice supplemented with 500 mM AMG. All data represent the mean plus standard deviation of five mice per group. *, significantly different from uninfected control mice; HBSS, Hanks balanced salt solution.

mice (13). Interestingly, when the levels of apoptosis in cultures from Mf-infected IFN- γ R^{-/-} mice and wild-type controls were compared, CD4 T cells from the KO animals showed significantly lower levels of apoptosis than those from the equivalent wild-type mice (Fig. 6) ($P < 0.05$), consistent with the absence of NO in these cultures and the greater proliferative capacity of CD4 cells.

To assess whether the expansion of activated CD4^{hi} CD44^{hi} T cells was limited by IFN- γ -induced NO production in vivo, cells from Mf-infected IFN- γ R^{-/-} mice and the equivalent wild-type counterparts were analyzed directly ex vivo. No differences were observed in the levels of CD4 or CD44 expression on CD4⁺ T cells taken ex vivo 12 days p.i. (data not shown).

CD4^{hi} cells display upregulation of Fas expression. In an attempt to determine whether differential levels of Fas expression accounted for the elevated levels of apoptosis seen among cells from Mf-infected animals, cells from infected and uninfected BALB/c mice were removed from Ag-stimulated culture (with and without AMG) and dual stained for CD4 and Fas expression. Consistently low levels of Fas staining were seen among the CD4⁺ population as a whole regardless of culture conditions or infection status. However when normal CD4⁺ cells and CD4^{hi} cells were gated separately, it was apparent that CD4^{hi} cells expressed comparatively high levels of Fas (Fig. 7). While this difference was most marked among cells

from Mf-infected animals, there was no consistent or significant difference between the levels of Fas expression in cells from Mf-infected and L3-infected animals, suggesting that the high levels of apoptosis in cultures from Mf-infected mice are unlikely to be the result of increased Fas expression.

DISCUSSION

The results of this study show that NO plays a key role in regulating CD4 T-cell expansion in splenocyte cultures from Mf-infected mice and add to a growing number of studies that have implicated NO-mediated apoptosis in limiting proinflammatory responses in various mouse models of disease (5, 6, 9, 32, 33). Inhibition of NO production either by AMG or by infection of IFN- γ R^{-/-} mice allows expansion of a population of CD4^{hi} T cells. By CFSE labeling, it was possible to show that the CD4^{hi} cells are Ag specific, as almost all the CD4 T cells which proliferated in response to Ag were contained within this population. Only very low levels of CD4^{hi} cells (~1%) were observed in Mf-infected wild-type mice in the absence of AMG. Ag-specific CD4^{hi} cells have previously been described following immunization of mice with sperm whale myoglobin (27), and the increased expression of CD4 has been used to enrich for Ag-specific T cells in nonobese diabetic mice (19). While the implication of upregulated CD4 expression is not fully established, it is likely to have functional significance. It

FIG. 3. CD4^{hi} T cells upregulate expression of CD44. Ag-stimulated splenocytes from Mf-infected BALB/c mice were cultured in the presence (AG+AMG) or absence (AG) of 500 mM AMG for 96 h prior to being stained with CD4 and CD44. The FACS plots shown were gated on CD4⁺ lymphocytes, and the quadrants were set to show CD4^{hi} CD44^{hi} cells (upper right quadrant). The data represent cells from individual animals, but the results were consistent within groups and in replicate experiments.

has been proposed that CD4 itself may act as a coreceptor through association with the T-cell receptor, leading to enhanced signaling (12), or that it may act to increase the avidity of T-cell-major histocompatibility complex interactions (14). Either mechanism could amplify responsiveness following an encounter with a specific Ag, favoring the rapid expansion of responder cells. Notably, in no experimental group were CD4^{hi} T cells observed ex vivo. While autoreactive T cells were not visible as a discrete population, Lejon and Fathman (19) were able to isolate them from NOD mice by selecting the brightest 1% of CD4⁺ cells. This suggests that upregulation of CD4 expression is not merely an in vitro artifact, although the degree of enhancement may depend upon the strength of the antigenic stimulus. Alternatively, the apparent absence of CD4^{hi} cells in the spleen ex vivo may reflect changes in their migratory potential as a function of their highly activated effector status.

The CD4^{hi} population also expressed high levels of the activation marker CD44. While the role of CD44 as an adhesion molecule via interactions with its principal ligand, hyaluronan, is well established, CD44 also plays a role in costimulation and T-cell proliferation (8, 11) and promotes IFN- γ production in *Toxoplasma gondii* infection (3). A recent study identified an additional role for CD44 in the regulation of T-cell apoptosis in a mouse model of hepatitis (4). In that study, T cells from CD44^{-/-} mice were shown to be more resistant to activation-induced cell death than T cells from wild-type mice. Transfer of these cells from KO to wild-type mice resulted in more severe hepatic disease and continued cytokine production, related to the enhanced survival of these cells. It was speculated that CD44 expression might target T cells for apoptosis. In the present study, it was demonstrated that the CD4^{hi} population

is consistently CD44^{hi}, but further studies will be required to determine if this population is preferentially susceptible to apoptosis.

We show that while the CD4^{hi} population accounts for most of the proliferative response in cultures from Mf-infected

FIG. 4. IFN- γ R-mediated signaling is necessary to suppress T-cell expansion. Splenocytes from Mf-infected (Mf) and uninfected (hbss [Hanks balanced salt solution]) wild-type (WT; 129Sv) and IFN- γ R^{-/-} (KO) mice were restimulated with Ag in vitro for 96 h prior to being stained for CD4 expression and FACS analysis. The CD4⁺ population was significantly expanded in cultures derived from Mf-infected IFN- γ R^{-/-} compared to Mf-infected wild-type mice. All data represent the mean plus standard deviation of five mice per group. *, significantly different from equivalent wild-type counterparts.

FIG. 5. Increased expansion of the CD4^{hi} population among cells from Mf-infected IFN- γ R^{-/-} mice. Splenocytes from Mf-infected wild-type mice (top) and IFN- γ R^{-/-} mice (bottom) were cultured for 96 h in the presence of Ag and then stained for CD4 expression. The gates were set to show the percentage of splenocytes that were CD4⁺ and the percentage of cells that were CD4^{hi} and reveal the greater expansion of both CD4⁺ and CD4^{hi} T cells in the absence of IFN- γ -mediated signaling. The plots show cells from individual animals, but the results were consistent within groups and between experiments.

BALB/c mice in the absence of NO, it was similarly expanded in mice deficient in IFN- γ signaling. In these animals, the levels of CD4 T-cell apoptosis were also significantly lower than in Mf-infected wild-type mice, presumably reflecting the absence of NO in these cultures and the enhanced proliferative capacity of the Ag-specific CD4 T-cell population. These data suggest that Ag-specific T cells are the targets of NO-mediated apoptosis upon in vitro restimulation with Ag. Given these results, it was of interest to investigate the mechanism by which a specific cellular population is targeted for apoptosis. To this end, we examined the expression of one of the best-characterized death receptors, Fas. Elevated levels of Fas expression were seen only within the CD4^{hi} population, but there were no significant differences in Fas expression between CD4^{hi} T cells from L3- and Mf-infected mice, despite the markedly increased levels of apoptosis in cultures from Mf-infected mice

FIG. 6. Expansion of CD4 T cells is associated with lower levels of T-cell apoptosis. Cells from Mf-infected (Mf) and uninfected (HBSS [Hanks balanced salt solution]) IFN- γ R^{-/-} mice were restimulated with Ag in vitro for 48 h prior to TUNEL staining and FACS analysis. The levels of apoptosis in the CD4⁺ population from Mf-infected wild-type and IFN- γ R^{-/-} mice or from uninfected control mice were measured by TUNEL staining. CD4⁺ T cells from Mf-infected IFN- γ R^{-/-} mice displayed significantly lower levels of apoptosis than those from their wild-type counterparts ($P < 0.05$). All data represent the mean plus standard deviation of five animals per group. Solid bars, wild-type mice; open bars, IFN- γ R^{-/-} mice. *, significant difference in levels of apoptosis between cells from Mf-infected IFN- γ R^{-/-} mice and wildtype mice.

(13). While these experiments do not implicate Fas expression as a basis for the differential susceptibility to apoptosis of CD4 cells from Mf-infected mice compared to those from L3-infected mice, it remains possible that Fas signaling may be involved via differences in Fas ligand expression on accessory cells.

The development of a strong IFN- γ -dominated response following Mf infection contrasts with the Th2 responses generally associated with helminth infection. In mice implanted with adult *Brugia malayi*, the Th2 bias is associated with expansion of a population of alternatively activated macrophages, which are profoundly suppressive toward many cell types (reviewed in reference 2). However, infection with Mf does not elicit this population whether mice are infected systemically or by the intraperitoneal route (1). These differences may relate to the anatomical compartmentalization of the parasite in its natural host, with the adult worms in the lymphatics and Mf in the peripheral circulation, where the systemic induction of suppression could be disastrous. The molecular mechanisms by which Mf induce IFN- γ are not completely defined, but it is noteworthy that Mf express at least one stage-specific protein, serpin (34), which is known to induce IFN- γ production when injected into mice. It is interesting to speculate whether Mf would also induce IFN- γ and NO in the context of an existing Th2 response, elicited by infection with L3 or adults, as would be the case in a natural infection. In preliminary experiments, we infected BALB/c mice with L3

FIG. 7. CD4^{hi} cells display elevated levels of Fas expression. Cells from Mf-infected (Mf) and L3-infected (L3) animals were restimulated with Ag in the presence of 500 μ M AMG for 96 h prior to surface staining and FACS analysis. CD4-normal and CD4^{hi} cells were gated separately, revealing significantly increased MFI of anti-Fas staining on CD4^{hi} T cells compared to CD4-normal cells in both experimental groups. All data represent the mean plus standard deviation of five animals per group. Open bars, CD4 cells; solid bars, CD4^{hi} cells.

and then 7 days later with Mf and analyzed the responses on day 19 p.i. At this time point, in mice infected with L3 alone, IFN- γ is elicited along with IL-4 and IL-5 (our unpublished data). Intriguingly, rather than enhancing Th1 responsiveness, superinfection with Mf reduced IFN- γ production in Ag-stimulated culture (R. A. O'Connor and E. Devaney, unpublished data). These findings suggest that restimulation *in vivo* consolidates Th2 polarization by downregulating IFN- γ production, but determining whether the *in vivo* mechanism involves NO-mediated apoptosis of Ag-reactive Th1 cells will require additional studies. In a previous report, repeated immunization with Mf extract was shown to result in suppression of Ag-specific IFN- γ responses (26), further suggesting that restimulation *in vivo*, as well as *in vitro*, results in downregulation of Th1 responses. Moreover, additional evidence that a similar scenario may occur *in vivo* comes from TUNEL staining of spleen sections from Mf-infected mice. These spleens contain many apoptotic nuclei compared to those from L3-infected or uninfected control animals (J. Jenson, J. Osborne, R. A. O'Connor, and E. Devaney, unpublished data).

The IFN- γ -induced expression of iNOS and the resulting apoptosis of Ag-reactive T cells is a recurring theme in several mouse models of autoimmune disease (5, 32) and in some models of infectious disease (6), where the uninhibited expansion of proinflammatory cells could lead to pathology. This presumably represents one mechanism by which the immune system maintains homeostasis. Interestingly, Xu et al. (33) recently reported that Ag-specific CD4⁺ T cells are selectively depleted by apoptotic death in a murine model of malaria. In that study, the fate of CFSE-labeled parasite-specific T cells was investigated by adoptive transfer into *Plasmodium*-infected or uninfected mice. CD4 T cells were deleted only in infected animals, while ovalbumin-specific T cells survived. Similarly to our *in vitro* studies, the mechanism of deletion was IFN- γ dependent and did not require Fas expression on the target cells. Most importantly, deletion of these cells was shown to

have a negative impact on immunity to subsequent infection and to favor parasite survival. In human lymphatic filariasis, the microfilariae represent the reservoir of infection in the endemic population, and infected individuals can remain microfilaremic for many years (20). Whether the NO-mediated regulation of T-cell apoptosis described here may be operative in microfilaremic individuals, resulting in the prolonged survival of the parasite and chronic infection, warrants further study.

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