

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

Requirement for In Vivo Production of IL-4, But Not IL-10, in the Induction of Proliferative Suppression by Filarial Parasites¹

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

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

We investigated the possibility that filariae down-regulate host immune responses by interfering with effective APC function. To delineate the role of the APC, we tested their competence in a defined model Ag system. Mice were implanted in the peritoneal cavity with adult *B. malayi*, and the ability of adherent peritoneal cells to present Ag ex vivo was evaluated (11). We found that when live but not dead parasites were used, the ability of the

D10.G4 Th2 cell clone to proliferate in response to its cognate Ag (conalbumin) was completely blocked. However, levels of Ag-cytokine production were intact, and even elevated, relative to control peritoneal exudate cells (PEC),³ indicating that there was no defect in Ag processing. When low numbers of infection-derived PEC were mixed with an excess of normal irradiated splenocytes, profound proliferative suppression was still observed. This demonstrated that suppression was an active process and not due to an absence of costimulatory molecules or reduced numbers of effective APC. Moreover, while suppression could be transferred with supernatants from cultured PEC, this was not due to soluble factors typically associated with “suppressor macrophages” such as prostaglandins or nitric oxide (11).

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

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

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

Materials and Methods

Parasite material

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

Mouse infection model

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

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

Cultures and cell lines

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

Proliferation and cytokine assays

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

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

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

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

Cell sorting and analysis

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

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

Statistical analysis

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

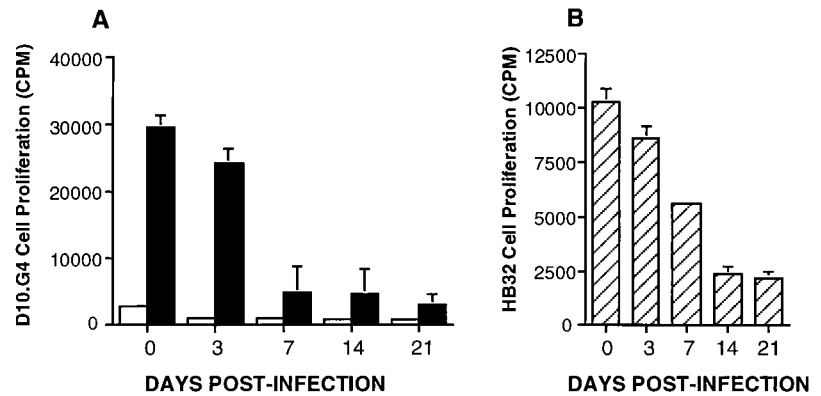
Results

Time course of suppression by adult *B. malayi*

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

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

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



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

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

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

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

Correlation of IL-4 response and suppression

Analysis of Ag-IL-4 production by D10.G4 cells showed that although cellular proliferation was ablated in the presence of both adult and L3 implant-derived PEC, cytokine production was enhanced (Fig. 2B). This argues against the possibility that the re-

duced proliferation of the D10.G4 cells was due to ineffective Ag presentation, reduced numbers of APC, or heightened cell death.

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

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

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

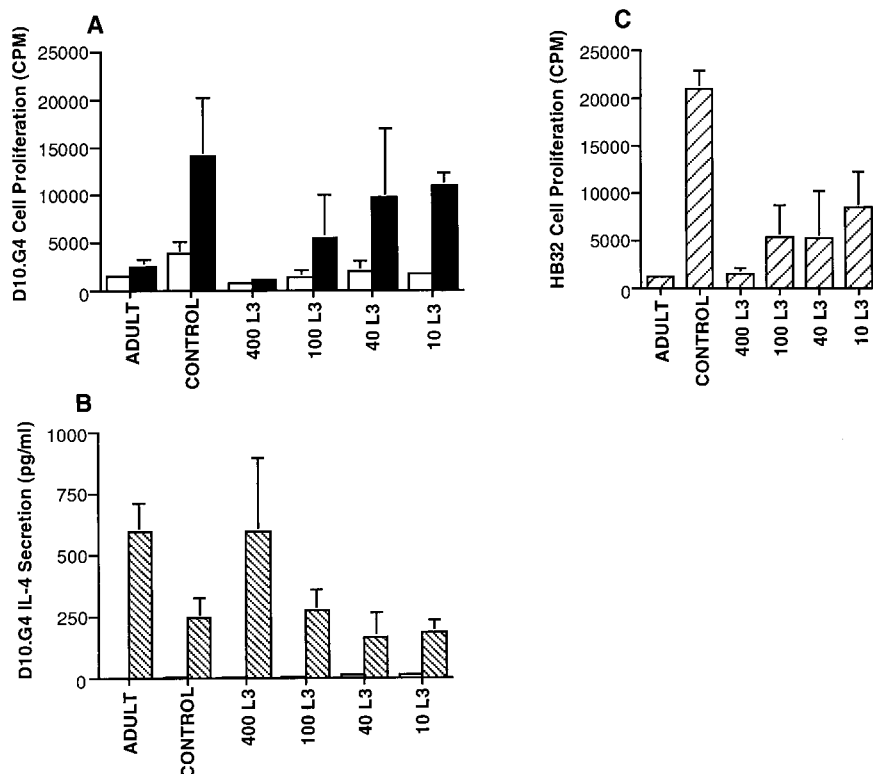
Because of the correlation between splenic IL-4 production and the generation of PEC that block proliferation, we chose to investigate the hypothesis that IL-4 plays a role in the development of the suppressive PEC, by the administration of neutralizing Ab to IL-4 (11B11) at the time of parasite implant.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

Table I. L3 induction of proliferative suppression^a

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

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

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



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

To more firmly establish the role of IL-4 in the induction of PEC suppression, mice genetically deficient for the production of IL-4 (IL-4^{-/-}) were implanted with adult *B. malayi* parasites. The ability to stimulate Ag proliferation was compared in PEC taken from knockout and wild-type C57BL/6 animals implanted with *B. malayi*. Cellular proliferation as measured by [³H]TdR incorporation showed that in contrast to wild-type mice, IL-4^{-/-} mice implanted i.p. with adult *B. malayi* parasites failed to generate adherent PEC that blocked the proliferation of either OVA-specific T cells (Fig. 5A) or the B cell hybridoma, HB32 (Fig. 5B). Assessment of splenocyte cytokine responses showed that adult parasite-implanted IL-4^{+/+} mice produced elevated levels of parasite-specific

IL-4 relative to uninfected controls. As expected, no IL-4 was produced by IL-4^{-/-} mice (data not shown).

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

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

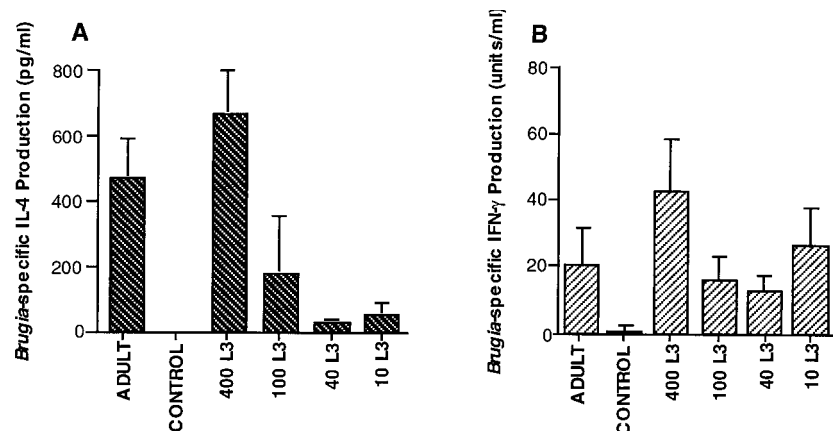


FIGURE 3. L3-induced splenocyte cytokine production. IL-4 production (A) or IFN-γ production (B) by splenocytes from control or L3 implanted CBA/Ca mice stimulated with BmA as measured by NK bioassay and ELISA, respectively. Background levels of cytokine production without the addition of BmA were in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

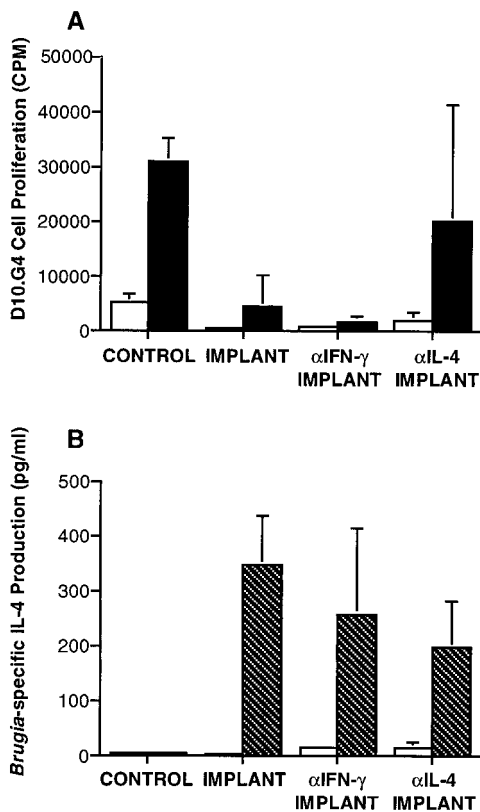


FIGURE 4. Treatment of mice with neutralizing Ab to IL-4. *A*, Proliferation of D10.G4 cells stimulated in the presence of PEC from control or 2-wk parasite-implanted CBA/Ca mice that had been treated at the time of implant with 2 mg/mouse anti-IL-4 or anti-IFN- γ Ab or with no Ab. Cultures were incubated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

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

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

Having established that *in vivo* production of IL-4 is necessary for the induction of competent suppressor cells by filarial implant, and given the importance of IL-10 as a down-regulatory cytokine influenced by IL-4, we decided to investigate the role of IL-10 *in vivo* in the induction phase of suppression. C57BL/6 mice defi-

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

Recruitment of cells into the peritoneal cavity

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

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

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

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

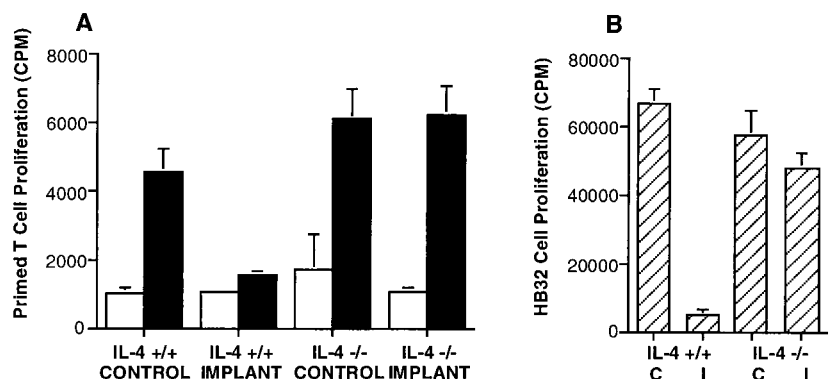


Table II. Neutralization of IL-4 and IL-10 in vitro^a

Source of PEC	A. HB32 Proliferation (cpm)			
	Medium	Conalbumin	Medium + anti-IL-4	Conalbumin + anti-IL-4
Control	13,459 ± 5,244			
Adult implant	2,636 ± 1,214			
	B. D10.G4 Proliferation (cpm)			
	Medium	Conalbumin	Medium + anti-IL-4	Conalbumin + anti-IL-4
Control	9,583 ± 490	45,685 ± 2,398	6,129 ± 258	10,255 ± 216
Adult implant	1,106 ± 484	13,917 ± 5,721	731 ± 106	1,496 ± 399
	C. D10.G4 Proliferation (cpm)			
	Medium	Conalbumin	Medium + anti-IL-10	Conalbumin + anti-IL-10
Control	2,692 ± 554	29,598 ± 1,750	4,434 ± 449	52,286 ± 4,829
Adult implant	697 ± 57	2,885 ± 1,579	781 ± 223	2,028 ± 984

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

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

Discussion

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

IL-4 production is likely to be one of the first events to occur following exposure to helminth parasites (24). Production of IL-4 is greatly expanded in *Brugia* infected mice (9, 10, 12), and in this study we see a direct correlation between parasite-specific splenocyte IL-4 production and PEC-mediated suppression of lymphocyte proliferation at each dose of L3. IL-4^{-/-} mice were unable to generate a suppressive PEC population, formally demonstrating that IL-4 is required. We cannot yet determine whether IL-4 alone is sufficient for suppressive PEC development or whether a mature

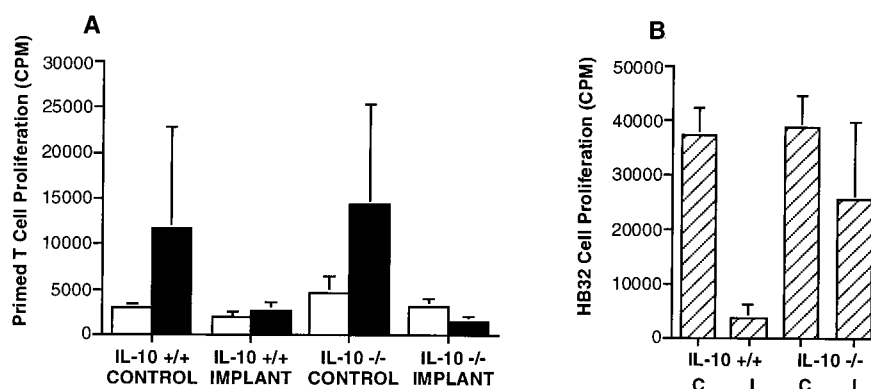
Th2 response must first emerge. However, mice given anti-IL-4 at the time of implantation still develop “Th2” responses but are unable to generate full suppression. This suggests that it is early IL-4 production, and not necessarily Th2 establishment, that may be the essential factor in the development of a proliferative block.

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

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

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

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



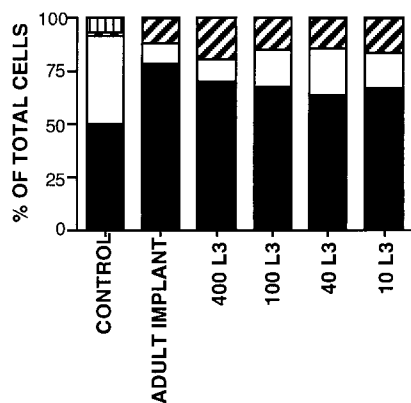


FIGURE 7. Peritoneal cell populations in control and parasite-implemented mice. The cell composition of PEC from control and implanted mice was determined by microscopy. Data shown are mean percentage of mast cells (▩), eosinophils (▨), lymphocytes (□), and macrophages (■). Data shown are mean of three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

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

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

Alternatively, the suppression we describe here may be important only at the site of infection, where potentially damaging host inflammatory responses in the local environment are down-regulated. Adult worms live within the lymphatic vessels for many years, apparently impervious to damage by the host immune system (39). We have found that adult excretory-secretory (ES) ma-

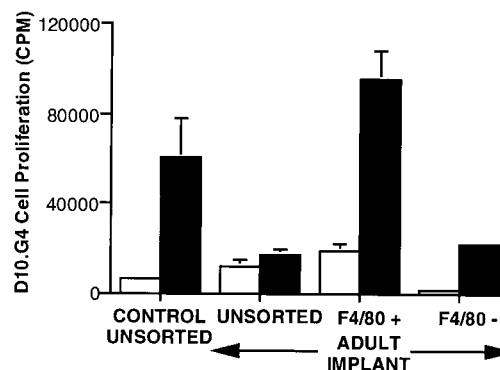


FIGURE 8. FACS sorting of macrophages. Proliferation of D10.G4 cells with media (□) or 50 μ g/ml conalbumin (■) in the presence of unsorted PEC from control or adult parasite-implemented mice or macrophage-enriched (F4/80⁺) or depleted (F4/80⁻) PEC from adult parasite-implemented mice (97% and 98% purity,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

terial injected daily into mice can induce the PEC-mediated proliferative block (unpublished data). Adult parasites are ideally placed within the afferent lymphatics to “bathe” the cells within the lymph nodes with down-regulatory ES. Generation of profound localized proliferative suppression by this mechanism could explain the absence of local inflammation at the site of infection in human filariasis (40–42) and in animal models (43, 44). The profundity of the suppressive mechanism(s) at play in this system is demonstrated by the fact that thus far we have been able to block proliferation of fully differentiated T cell clones, hybridomas, and primary cell lines.

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

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

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

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

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

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

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