# F4/80$^+$ Alternatively Activated Macrophages Control CD4$^+$ T Cell Hyporesponsiveness at Sites Peripheral to Filarial Infection[1]

**Matthew D. Taylor, Anjanette Harris, Meera G. Nair, Rick M. Maizels,[2] and Judith E. Allen[2]**

**Both T cells and APC have been strongly implicated in the immune suppression observed during filarial nematode infections, but their relative roles are poorly understood, particularly in regard to timing and locality of action. Using *Litomosoides sigmodontis* infection of susceptible BALB/c mice, we have studied the progression of filarial immunosuppression leading to patent infection with blood microfilaremia. Patent infection is associated with decreased immune responsiveness in the draining thoracic lymph nodes (tLN) and intrinsically hyporesponsive CD4$^+$ T cells at the infection site. We now show that we are able to separate, both in time and space, different suppressive mechanisms and cell populations that contribute to filarial hyporesponsiveness. *L. sigmodontis* infection recruited a F4/80$^+$ population of alternatively activated macrophages that potently inhibited Ag-specific CD4$^+$ T cell proliferative responses even in the presence of competent naive APC. T cell responsiveness was partially restored by neutralizing TGF-$\beta$, but not by blocking IL-10 or CTLA-4 signaling. During prepatent infection, the macrophage population was restricted to the infection site. However, once infection became patent with systemic release of microfilariae, the suppressive macrophage activity extended peripherally into the tLN. In contrast, the hyporesponsive CD4$^+$ T cell phenotype remained localized at the infection site, and the tLN CD4$^+$ T cell population recovered full Ag responsiveness in the absence of suppressive macrophages. Filarial immunosuppression, therefore, evolves over time at sites increasingly distal to infection, and the mechanisms of filarial down-regulation are dependent on proximity to the infection site.** ***The Journal of Immunology,*** **2006, 176: 6918–6927.**

Chronic human filarial infections are synonymous with down-regulation of the host immune response. This response has led to the hypothesis that the long-lived survival of filarial worms within immunocompetent hosts is due to the parasites' ability to suppress protective immune responses (1–3). In human infection, this effect is evident in a loss of Ag-specific T cell proliferative responses (4, 5) and lowered production of both Th1 (IFN-$\gamma$) and Th2 (IL-5) effector cytokines (6, 7). The isolation of T cells with regulatory characteristics from humans infected with *Onchocerca volvulus*, *Loa loa*, and *Wuchereria bancrofti* indicates that regulatory T cells are involved in filarial suppression (8, 9), and during *Litomosoides sigmodontis* infection of mice, they play an important role in promoting filarial survival (10). Regulatory T cells, however, are not the only cell type or mechanism involved in modulating the response to infection.

There is clear evidence during human infection that APC function is compromised (11, 12), and rodent models of filarial infection have demonstrated a variety of mechanisms by which filarial parasites immunosuppress their host (13–19). Mosquito-borne *Brugia malayi* L3 larvae are able to down-regulate expression of MHC class I and class II on human Langerhans cells and to inhibit their ability to activate T cells in vitro (20), whereas the same parasites, if injected into the murine peritoneal cavity, induce suppressive nematode-elicited macrophages (NeM$\phi$)[3] that are able to block T cell proliferative responses (21). Thus, from the moment the parasite enters mammalian tissue, it is able to inhibit the priming of the immune response by APC, and influence the development of T cell immunity.

Immune suppression may deepen over the course of infection, and adult parasites are themselves strongly immunosuppressive; implantation of a single juvenile adult female of *L. sigmodontis* is sufficient to suppress host immunity and delay clearance of i.v. injected microfilariae (Mf) (22). Similarly, implantation of *B. malayi* adults into the peritoneal cavity recruits NeM$\phi$ that profoundly inhibit the proliferative responses of T cells (23). These macrophages have an alternatively activated phenotype demonstrated by up-regulated expression of arginase, Fizz-1, and Ym1 as well as reduced proinflammatory chemokine expression (24) and are likely to play a strong anti-inflammatory role at the infection site.

In human studies, filarial immunosuppression is most marked in microfilaremic individuals who carry healthy adult worms and circulating Mf (5). The time of onset of microfilaremia is also associated with maximal immune suppression in a range of animal models (17, 25–28). In agreement with these findings, studies in mice using i.v. injection of Mf have shown that they induce apoptosis of T cells in a NO-dependent manner (29–31). Mf Ags have

Institute of Immunology and Infection Research, Ashworth Laboratories, University of Edinburgh, Edinburgh, United Kingdom

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[2] Address correspondence and reprint requests to Dr. Rick M. Maizels, Institute of Immunology and Infection Research, Ashworth Laboratories, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, U.K.; E-mail address: rick.maizels@ed.ac.uk or Dr. Judith E. Allen, Institute of Immunology and Infection Research, Ashworth Laboratories, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, U.K.; E-mail address: j.allen@ed.ac.uk

[3] Abbreviations used in this paper: NeM$\phi$, nematode-elicited macrophage; Mf, microfilariae; DC, dendritic cell; GITR, glucocorticoid-inducible TNFR; PleC, pleural exudate cell; LsAg, *Litomosoides sigmodontis* whole worm Ag; tLN, thoracic lymph node.



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also been shown to inhibit the in vitro maturation and function of human dendritic cells (DC), resulting in impaired T cell priming (32–34). Together, these studies indicate that filarial suppression acts on multiple cell types within the immune response, and that additional mechanisms of suppression may by invoked by each stage of the life cycle. Due to the absence of a permissible filarial parasite of mice, most studies have been limited to individual stages of the life cycle. However, it is important to consider that the immune response against the adult stage is defined by prior immune interactions with the larval stages. Similarly, responsiveness toward the adult stage will strongly influence immunity to the subsequent Mf stage.

The discovery that the filarial nematode *L. sigmodontis* will follow its full natural course of infection within certain mouse strains (35) now provides an immunological model to study these events in the context of full infection. Only with this model can one ask which immunoregulatory events allow the infective L3 stage to migrate from skin entry sites to the pleural cavity, to develop there into mature adult parasites, and to establish a patent infection with Mf circulating within the bloodstream. Using *L. sigmodontis* infection of susceptible BALB/c mice, we have already shown that regulatory T cells increase over the course of infection and are directly responsible for preventing parasite killing (10). Furthermore, once infection achieves patency, $CD4^+$ T cells at the infection site become hyporesponsive to both antigenic and mitogenic stimulation. Distal to the infection site, the immune responses within the draining LN decrease. This suggests that the onset of Mf release is accompanied by a yet more profound degree of immunosuppression.

In this study, we now show that the mechanisms of filarial immunosuppression evolve over time at sites increasingly distal to infection, and that different mechanisms act at different sites. Specifically, we demonstrate that prepatency, *L. sigmodontis* NeM$\phi$ are restricted to the infection site, between the pleural membranes (pleural cavity) within the thoracic cavity, resulting in localized suppressive activity. However, as infection becomes patent and Mf are released systemically, the suppressive activity of NeM$\phi$ extends peripherally into the draining lymph nodes. In contrast, the hyporesponsive phenotype developed at patency by the pleural cavity $CD4^+$ T cell population (10) remained restricted to the site of infection and was not reflected in the draining lymph nodes.

## Materials and Methods

### *Mice, parasites, and immunization*

Female BALB/c and DO11.10 mice were bred in-house or purchased from Harlan Sprague-Dawley and maintained in individually ventilated cages. Mice were used at age 6–8 wk, and all animal work was conducted in accordance with the Animals (Scientific Procedures) Act 1986. The *L. sigmodontis* life cycle was maintained in gerbils using the mite vector *Ornithonyssus bacoti* (36). Mice were infected with a dose of 25 larvae (L3) s.c. Parasites were recovered from the pleural cavity by lavage of the thoracic cavity with 10 ml of cold AIM V medium (Invitrogen Life Technologies). *L. sigmodontis* whole worm Ag (LsAg) was prepared as the PBS-soluble fraction of homogenized adult male and female worms. For immunization, mice received 10 mg of LsAg emulsified in CFA s.c. in the footpad. For in vivo Ab treatments, mice were injected i.p. 28 days postinfection with either 2 mg of rat IgG (Sigma-Aldrich) or 1 mg of anti-CD25 (PC61) in combination with 1 mg of anti-glucocorticoid-inducible TNFR (anti-GITR, DTA-1).

### *Cell purifications*

The parathymic, posterior mediastinal, and paravertebral lymph nodes (tLN) draining the thoracic cavity were dissociated, washed in AIM V medium, and resuspended in RPMI 1640 (Invitrogen Life Technologies) with 0.5% mouse serum (Caltag-MedSystems), 100 U/ml penicillin, 100 $\mu$g/ml streptomycin (Invitrogen Life Technologies), and 2 mM L-glutamine (Invitrogen Life Technologies). Popliteal lymph nodes were taken from LsAg-immunized mice, and spleen cells from naive DO11.10 mice. Pleural exudate cells (PleC) were isolated from the thoracic lavage fluid. $CD4^+$ T cells and CD4-depleted APC were isolated by CD4 MicroBead magnetic cell sorting (Miltenyi Biotec) as per manufacturer's instructions, except that HBSS-0.25% mouse serum was used as the separation medium, and 15 $\mu$g of rat IgG per $10^7$ cells was used as a block. Thoracic lymph node $CD4^+$ T cell purities were >95%. $Gr1^+$ and $Gr1^-$ cells were purified using PE-conjugated anti-Gr1 (RB6-8C5; BD Pharmingen) in conjunction with anti-PE microbeads (Miltenyi Biotec). $F4/80^+$ and $F4/80^-$ cells were purified using biotinylated anti-F4/80 (A3-1; Caltag-MedSystems) in conjunction with streptavidin microbeads (Miltenyi Biotec). DC were purified from $F4/80^-$ cell fractions using anti-CD11c microbeads (Miltenyi Biotec). B cells were purified positively with anti-CD19 microbeads, or negatively using anti-CD43 microbeads (Miltenyi Biotec). Macrophage purities are shown in *Results*. B cells were >90% pure, $CD11c^+$ cells were 47–52% pure.

### *In vitro cultures*

Whole tLN cells, whole DO11.10 spleen cells, and irradiated CD4-depleted tLN cells were used at $5 \times 10^5$ cells/well. For irradiation, cells received 30 Gy. Irradiated spleen cells from naive mice were used as an APC source at $5 \times 10^5$ cells/well when compared with CD4-depleted tLN cells, and $1 \times 10^6$ cells/well when used in combination with purified APC. $CD4^+$ T cells and irradiated B cells were used at $1 \times 10^5$ cells/well. Irradiated DC and $F4/80^+$ cells were used at $5 \times 10^4$ cells/well. For in vitro restimulations, cells were cultured with medium alone or 10 $\mu$g/ml LsAg for 72 h followed by addition of 1 mCi/well [*methyl*-$^3$H]thymidine per well for 16 h to measure proliferation. DO11.10 cells were restimulated with 0.5 $\mu$g/ml OVA peptide (ISQAVHAAHAEINEAGR) from Advanced Biotechnology Centre (Imperial School of Medicine, London, U.K.). In some experiments anti-IL-10R (1B1.3), whole anti-CTLA-4 (UC10-4F10-11), or anti-CTLA-4 $F(ab')_2$ were added to cultures at the concentrations indicated in the text. Anti-CTLA-4 $F(ab')_2$ were produced from whole Ab using the ImmunoPure Fab kit (Pierce). Both whole anti-CTLA-4 and CTLA-4 $F(ab')_2$ were shown to competitively block the binding of PE-conjugated anti-CTLA-4 (BD Pharmingen) to splenic CD4 T cells. The neutralizing anti-TGF-$\beta$ Ab (1D11.16) was titrated from 0.01 to 100 $\mu$g/ml. Results shown are for a concentration of 50 $\mu$g/ml at which maximum reversal of suppression occurred. For suppression assays using the EL-4 thymoma cell line, $1 \times 10^5$ PleC or $1 \times 10^6$ tLN cells were adhered to 96-well flat-bottom tissue culture plates (Nunc) for 2 h at 37°C and separated into nonadherent and adherent fractions. EL-4 cells were added at $1 \times 10^4$ cells/well and after 48 h cultures were pulsed with thymidine as described. The adherent tLN cells were negligible in number and so results are only shown for the nonadherent population.

### *Flow cytometry, ELISA, and cytospins*

For flow cytometry, nonspecific binding was blocked with 4 $\mu$g of rat IgG/$1 \times 10^6$ cells, and the following Abs applied: purified rat anti-Gr1 (RB6-8C5) in combination with FITC-conjugated goat $F(ab')_2$ anti-rat IgG (Caltag-MedSystems), allophycocyanin-conjugated rat anti-CD4 (RM4-5), FITC-conjugated hamster anti-CD11c (HL3), PE-conjugated rat anti-B220 (RA3-6B2), FITC-conjugated rat anti-CD19 (1D3), TriColor-conjugated rat anti-F4/80 (A3-1, Caltag-MedSystems). All staining was compared against the relevant isotype controls to verify specificity. Flow cytometric acquisition and analysis was performed using a FACSCalibur running CellQuest Pro software. Standard IL-4 ELISA was performed using 11B11 and BVD6-24G2 anti-IL-4 Ab pairs. ELISA detection was performed using ExtrAvidin-alkaline phosphatase conjugate (Sigma-Aldrich) in conjunction with Sigma Fast *p*-nitrophenyl phosphate tablet substrate (Sigma-Aldrich). Cytospins were prepared using $2 \times 10^5$ cells/slide and were stained using the Diff-Quik staining kit (Dade Behring) as per the manufacturer's instructions. Abs were purchased from BD Pharmingen unless otherwise stated.

### *Real-time PCR*

Cell populations were resuspended in TRIzol (Invitrogen Life Technologies) and RNA extraction was performed as per the manufacturer's instructions. After treatment with DNase1 (Ambion), cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Stratagene). Quantification of arginase-1 mRNA was performed by real-time PCR using the LightCycler (Roche Diagnostics). The arginase-1 forward and reverse primer sequences were CAGAAGAATGGAAGAGTCAG (exon 3) and CAGATATGCAGGGAGTCACC (exon 5), respectively. PCR amplifications for arginase 1 were performed in 10 $\mu$l containing 1 $\mu$l of cDNA, 0.3 $\mu$M primers, 4 $\mu$M $MgCl_2$, and the LightCycler DNA SYBR Green I mix under the following conditions: 30 s denaturation at 95°C, 5 s annealing of

primers at 55°C and 12 s elongation at 72°C, for 50 cycles. The fluorescent DNA binding dye was monitored after each cycle at 86°C. Expression of β-actin was used as a control, and PCR conditions are as described. β-Actin forward and reverse primer sequences were TGGAATCCTGTG GCATCCATGAA and TAAAACGCAGCTCAGTAACAGTC, respectively. Five serial dilutions of cDNA pooled from each sample were used to create standard curves for β-actin and arginase 1 (in arbitrary units) against which expression of β-actin and arginase 1 mRNA in the samples was quantified. Arginase 1 mRNA levels were then expressed as a ratio to β-actin mRNA expression.

## Results

### L. sigmodontis *infection recruits a suppressive cell population to the pleural cavity, which expands into the draining lymph nodes following the onset of patency*

Implantation of adult *B. malayi* worms into the peritoneal cavity of mice has been shown to recruit an adherent population of NeMϕ, which are capable of suppressing the Ag-specific proliferation of primary T cells, as well as the Ag-independent proliferation of EL-4 thymoma T cells (21, 37). Similarly, a population of peritoneal exudate cells capable of inhibiting the proliferative responses of spleen cells to mitogen is recruited following implantation of adult *L. sigmondontis* worms (22). To determine whether a similarly suppressive cell population is recruited to the pleural cavity during a natural *L. sigmodontis* infection, we isolated the adherent PleC from BALB/c mice 40 days (prepatency) and 60 days (postpatency) postinfection. The adherent cells taken both pre- and postpatency were able to inhibit the proliferation of the T cell thymoma, EL-4 (Fig. 1, *left*), indicating the recruitment of a NeMϕ population.

The onset of patency in *L. sigmodontis* infection in BALB/c mice heralds a profound inhibition of both Ag-specific and mitogen-driven T cell proliferative responses (day 60), compared with those seen prepatency (day 40), within tLN draining the pleural cavity (10). To determine whether a suppressive cell population was also acting in the tLN, we cocultured tLN cells from infected and naive mice with the EL-4 thymoma cells, taking the nonadherent fraction, as the tLN from both naive and infected mice contained negligible numbers of adherent cells. tLN cells isolated from infected mice with patent microfilaremia (at day 61) were able to inhibit the proliferation of EL-4 cells,. However, prepatent (day 40) tLN cells failed to show a suppressive phenotype (Fig. 1, *right*). Interestingly, the one mouse with tLN cells that did not show suppressive ability at day 61 was infected with only one adult male worm and had no detectable Mf, and so did not have a patent infection. Thus, although prepatent *L. sigmodontis* infection recruits a suppressive cell population to the pleural cavity, this population is only detectable in the tLN postpatency.

### *Depressed immune responsiveness of lymph node T cells is due to a suppressive non-CD4$^+$ APC population*

The presence of an Ag nonspecific suppressive cell population in the draining lymph nodes indicated that the decreased proliferation seen in unfractionated tLN cells at patency (10) is due to the action of a non-CD4$^+$ T cell population. To directly demonstrate the role of APC in the proliferative suppression seen in the lymph node we compared the ability of APC from naive with APC from infected BALB/c mice to restimulate CD4$^+$ T cells. CD4$^+$ T cells were purified from pooled tLN 60 days postinfection, and from the popliteal lymph nodes of mice immunized with LsAg as a source of T cells from a noninfected environment. The CD4$^+$ T cells were restimulated in vitro with LsAg using either irradiated CD4-depleted spleen cells from naive mice, or irradiated CD4-depleted tLN cells from 60-day infected mice. When CD4$^+$ T cells from infected mice were cultured with APC from the tLN of infected mice they showed low levels of Ag-specific proliferation, consistent with findings for unfractionated tLN cells. However, their ability to proliferate in response to Ag was restored when they were cultured with splenic APC from naive mice (Fig. 2*A*). Similarly, culturing CD4$^+$ T cells purified from immunized mice with APC from infected mice reduced their ability to proliferate in response to Ag. This effect was not due to differences in Ag presentation capabilities between spleen and tLN cells because identical results were seen when tLN cells from naive mice were used as an APC source (Fig. 2*B*). Thus, T cell unresponsiveness in the tLN at patency is mediated through an APC population.

To test whether the APC population actively inhibited Ag-specific T cell proliferation, we restimulated CD4$^+$ T cells purified from infected mice with LsAg in the presence of APC combined from both naive and infected animals. APC from infected animals were able to inhibit the Ag-specific proliferation of CD4$^+$ T cells despite the presence of functional APC from naive animals, indicating that the infected APC population has a dominant suppressive capacity (Fig. 2*C*). Similar to findings with the *B. malayi* implant model (18), Ag-specific production of IL-4 (Fig. 2*D*) and IL-5 (data not shown) was not inhibited. Thus, the suppressive phenotype was limited to inhibition of proliferation and the ability of the suppressive tLN APC to process and present Ag was apparently intact.

Unfractionated tLN cells isolated prepatency (day 40) showed strong levels of Ag-specific proliferation (10), consistent with the failure of D40 tLN cells to block EL-4 proliferation (Fig. 1, *right*). Indeed, although CD4$^+$ T cells from infected mice restimulated with postpatent APC showed significantly reduced proliferative responses, restimulation with prepatent (day 40) APC yielded

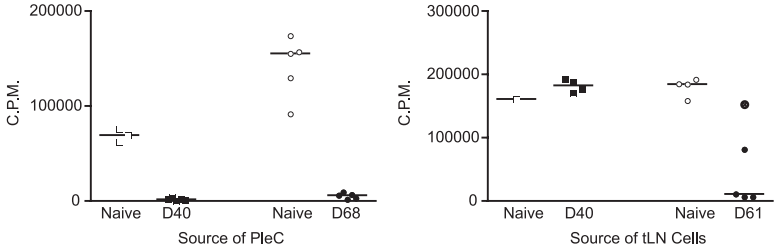


**FIGURE 1.** *L. sigmodontis* infection recruits a suppressive cell population to both the pleural cavity and tLN. *Left*, Proliferative responses of EL-4 cells incubated with adherent cells isolated at day 40 (squares) and day 68 (circles) postinfection from the pleural cavity of naive (open symbols) and infected mice (closed symbols). Symbols represent individual mice and lines represent median values. Results are representative of three independent experiments. *Right*, Proliferative responses of EL-4 cells incubated with nonadherent tLN cells isolated at day 40 (squares) and day 61 (circles) from naive (open symbols) and infected (closed symbols) mice. Data are shown for the nonadherent cell fraction as negligible cell numbers were retained in the adherent tLN fraction, and consistent with the lack of adherent cells no suppressive activity was seen. One infected mouse at day 61 did not have a patent infection (large circle). Cells from the naive tLN at day 40 were pooled and symbols represent pooled value. Symbols for all other groups represent individual mice. Line represents median values. Results are representative of three independent experiments.

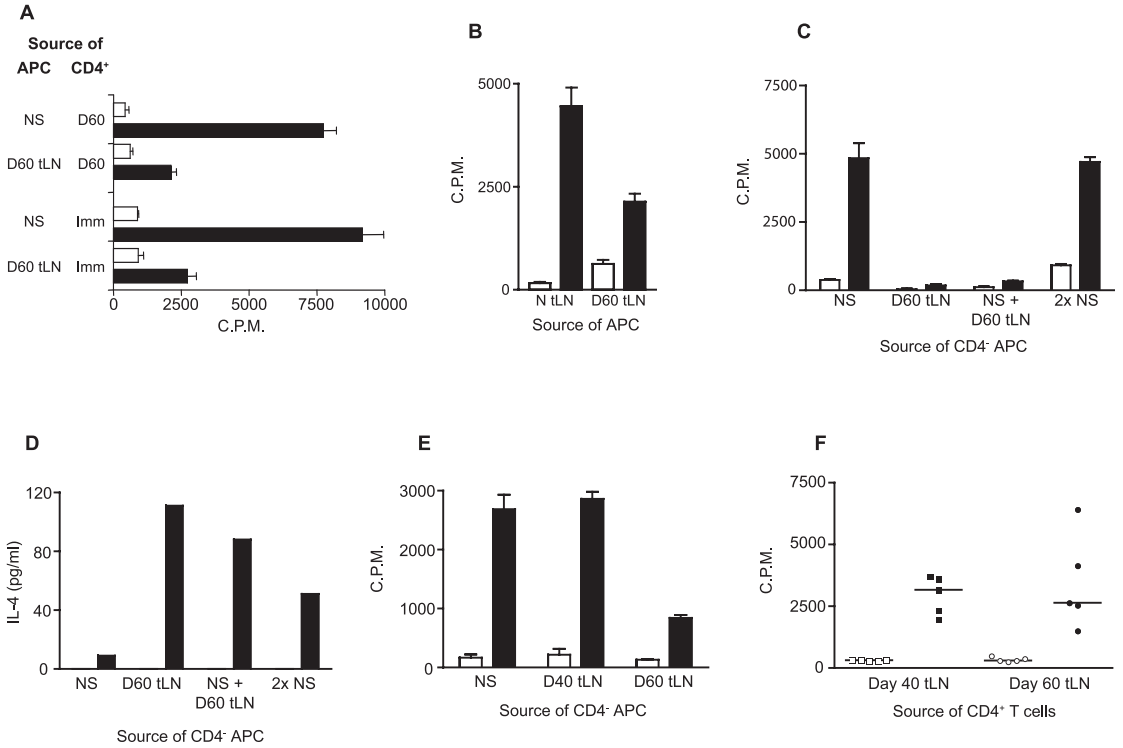


**FIGURE 2.** Ag unresponsiveness within the tLN is due to active suppression by a non-CD4$^+$ APC population. *A*, Proliferative responses of pooled CD4$^+$ T cells purified from the tLN of *L. sigmodontis*-infected mice 60 days postinfection (D60) or from the popliteal lymph node of mice immunized with LsAg in CFA (Imm) and restimulated with medium alone (□) or LsAg (■). CD4-depleted spleen cells from naive mice (NS) or from the tLN of 60 day infected (D60 tLN) were used as APC. Results are representative of three independent experiments. Error bars represent SD of triplicate cultures. *B*, Proliferative responses of CD4$^+$ T cells purified from the tLN of *L. sigmodontis*-infected mice 60 days postinfection and restimulated with medium alone (□) or LsAg (■) using tLN cells from naive or 60 day infected mice as APC. Error bars represent SD of triplicate cultures. *C*, Proliferative responses of CD4$^+$ T cells purified from the tLN of *L. sigmodontis*-infected mice 60 days postinfection and restimulated with medium alone (□) or LsAg (■). CD4-depleted spleen cells from naive mice (NS) and from the tLN of 60 day infected mice (D60 tLN) were used as APC either individually or in combination. As a control for APC number, double the number of naive spleen cells was used (2× NS). Results are representative of two independent experiments. Error bars represent SD of triplicate cultures. *D*, IL-4 production by CD4$^+$ T cells purified from the tLN of *L. sigmodontis*-infected mice 60 days postinfection and restimulated with medium alone (□) or LsAg (■). CD4-depleted spleen cells from naive mice (NS) and from the tLN of 60-day infected mice (D60 tLN) were used as APC either individually or in combination. As a control for APC number, double the number of naive spleen cells was used (2× NS). Results are representative of two independent experiments. *E*, Proliferative responses of CD4$^+$ T cells purified from the tLN of *L. sigmodontis*-infected mice 60 days postinfection and restimulated with medium alone (□) or LsAg (■). CD4-depleted spleen cells from naive mice (NS), or from the tLN of infected mice taken 40 days (D40 tLN) or 60 days (D60 tLN) postinfection were used as APC. Error bars represent SD of triplicate cultures. *F*, Proliferative responses of CD4$^+$ T cells purified 40 (squares) or 60 (circles) days postinfection from the tLN of infected mice and restimulated with medium alone (open symbols) or LsAg (closed symbols) using irradiated spleen cells from naive mice as APC. Symbols represent individual mice and a line represents the median values.

equivalent levels of Ag-specific proliferation to those induced by APC from naive mice (Fig. 2*E*). This further demonstrates that before patency, down-regulatory APC are detectable only at the site of infection, whereas after the onset of patency the inhibitory APC phenotype is present in the draining lymph nodes.

At the infection site, in concert with the suppressive adherent cell population, an intrinsic defect in Ag responsiveness within the pleural cavity CD4$^+$ T cell compartment develops between pre- and postpatency (10). To test the possibility that two levels of suppression are also acting within the tLN at patency, purified tLN CD4$^+$ T cells from day 40 and day 60 infected mice were challenged with LsAg presented by naive, rather than infected, APC (Fig. 2*F*). CD4$^+$ T cells purified postpatency did not show a loss of Ag responsiveness compared with prepatent CD4$^+$ T cells and thus, in contrast to the CD4$^+$ T cells at the infection site, tLN CD4$^+$ T cells do not become intrinsically hyporesponsive as infection becomes patent. The immune suppression seen within the peripheral tLN is therefore mediated entirely through non-CD4$^+$ APC.

### *The suppressive APC are F4/80$^+$ NeMϕ with an alternatively activated phenotype*

An important feature of *L. sigmodontis* infection is a significant expansion in F4/80$^+$ macrophages at patency, both at the site of infection (naive, 2.3–7.8 × 10$^5$; day 60, 1.0–8.9 × 10$^6$; $p < 0.01$ Mann-Whitney *U* test) and in the tLN (naive, 0.2–5.9 × 10$^4$; day 61, 4.3–11.3 × 10$^6$; $p < 0.01$ Mann-Whitney *U* test). To determine whether macrophages were responsible for the observed suppression, F4/80$^+$ cells were enriched from the pleural cavity and tLN of infected mice, and from the spleens of naive mice. CD4$^+$ T cells purified from the tLN of infected mice were then challenged with LsAg in the presence or absence of different F4/80$^+$ populations, together with irradiated naive splenic APC. F4/80$^+$ cells enriched from both tLN and pleural cavity 60 days postinfection were able to inhibit CD4$^+$ T cell Ag-specific proliferation (Fig. 3*A*). The pleural cavity F4/80$^+$ cell fraction was more suppressive than F4/80$^+$ cells from the tLN, perhaps reflecting the higher proportion of F4/80$^{high}$ macrophages within the pleural cavity fraction (39%) compared with the

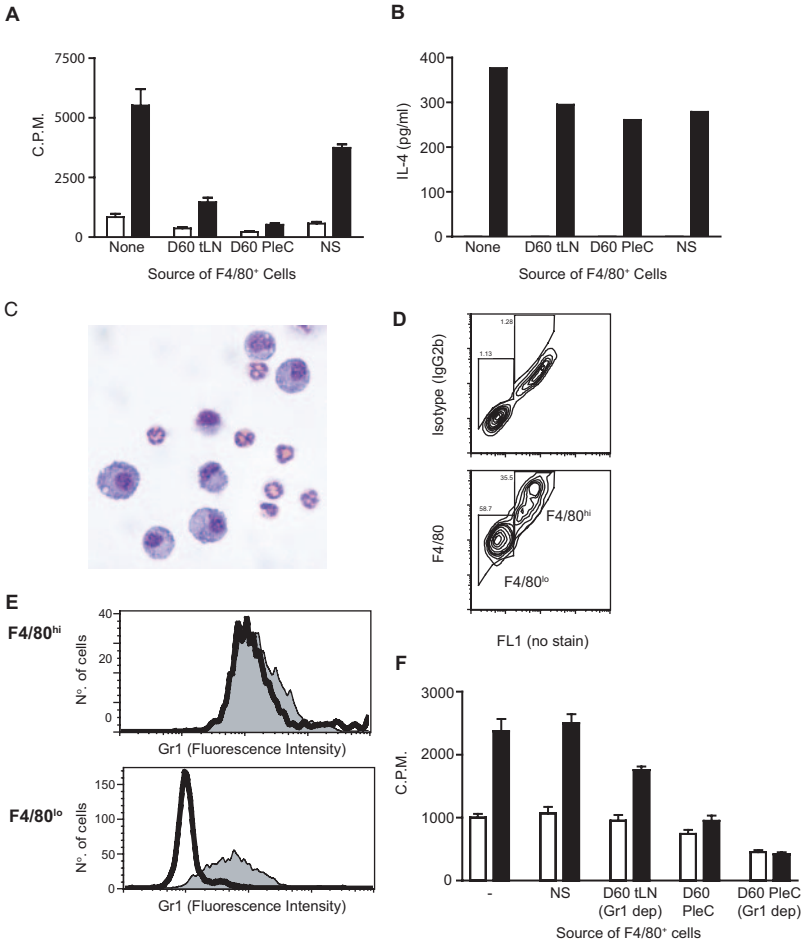


**FIGURE 3.** The inhibition of $CD4^+$ T cell responses by tLN cells and PleC are mediated by $F4/80^+$ macrophages. *A*, $CD4^+$ T cells were isolated from the tLN of 60-day infected mice and restimulated in vitro with medium alone (□) or LsAg (■) using naive spleen APC in the presence or absence of $F4/80^+$ cells purified from the spleens of naive mice, or from the tLN or pleural cavity of 60-day infected mice. Proliferation was measured via thymidine incorporation. Error bars represent SD of cultures in triplicate and results are representative of three independent experiments. *B*, $CD4^+$ T cells were isolated from the tLN of 60-day infected mice and their production of IL-4 measured in response to restimulation with medium alone (□) or LsAg (■) using naive spleen APC in the presence or absence of $F4/80^+$ cells purified from the spleens of naive mice, or from the tLN or pleural cavity of 60-day infected mice. Results are representative of three independent experiments. *C*, Cytospin of the $F4/80^+$ cell fraction purified from the pleural cavity of 60-day infected mice by F4/80 magnetic separation. Magnification at ×400. *D*, Expression of F4/80 by cells purified from the pleural cavity of day 60 infected mice by F4/80 magnetic separation. Contour plots show staining for F4/80 or its isotype control. To resolve the $F4/80^{high}$ and $F4/80^{low}$ populations, the staining for F4/80 and its isotype is plotted against background autofluorescence (no stain). *E*, Expression of Gr1 by $F4/80^{high}$ and $F4/80^{low}$ populations. F4/80 purified cells from the pleural cavity of day 60 infected mice were costained for F4/80 verses Gr1 or its isotype control. Histograms are gated on the $F4/80^{high}$ and $F4/80^{low}$ cell populations. Gray shaded histogram represents Gr1 staining and open black line histogram represents isotype control. *F*, Proliferative responses of $CD4^+$ T cells isolated from the tLN of 60-day infected mice and restimulated in vitro with medium alone (□) or LsAg (■) using naive spleen APC in the presence or absence of Gr1-depleted $F4/80^+$ cells from naive spleen, Gr1-depleted $F4/80^+$ cells from day 60 tLN, $F4/80^+$ cells from whole day 60 PleC, or Gr1 depleted $F4/80^+$ cells from day 60 PleC. Error bars represent SD of cultures in triplicate.

tLN fraction (26%). Despite the strong suppression of Ag-specific proliferative responses by both $F4/80^+$ populations, Ag-specific production of cytokines was unaffected, in terms of IL-4 (Fig. 3*B*) as well as IL-5, IL-10, and IFN-γ (data not shown).

The low percentage of macrophages within the F4/80-purified fractions was due to expression of F4/80 by eosinophils, as the majority of copurifying nonmacrophage cells from the pleural cavity (57%) and tLN (47%) were $F4/80^{low}Gr1^+$ eosinophils as defined by cytospins and flow cytometry. Fig. 3*C* demonstrates that the two cell populations in the pleural cavity $F4/80^+$ fraction were eosinophils and macrophages, which by flow cytometry matched the percentages of $F4/80^{low}$ and $F4/80^{high}$ cells, respectively (Fig. 3*D*). When the $F4/80^{high}$ and $F4/80^{low}$ populations were costained for expression of Gr1, they were found to be $Gr1^-$ and $Gr1^+$, respectively (Fig. 3*E*). To confirm that the $F4/80^+$ macrophages were mediating the suppressive effect, a Gr1 depletion was performed before the F4/80 purification. Prior depletion of $Gr1^+$ cells increased the purity of macrophages in the pleural cavity $F4/80^+$ fraction from 49.0 to 78.5% and decreased the eosinophil component from 45.7 to 20.2% as defined by cytospins and F4/80 staining. The increased percentage of $F4/80^+$ macrophages was accompanied by enhanced suppressive activity, arguing that the $F4/80^+$ macrophage population is the suppressive cell population (Fig. 3*F*). Similarly, $F4/80^+$ cells from infected tLN were still suppressive following depletion of $Gr1^+$ cells (Fig. 3*F*).

Alternatively activated macrophages can be characterized by their expression of arginase 1, Ym1, and Fizz1 (24, 38). We have previously demonstrated that unfractionated PleC from *L. sigmodontis*-infected mice express Ym1 and Fizz1 (39). In this study, we extend those results to show that the expression of arginase 1 mRNA is up-regulated in both unfractionated and adherent PleC populations, indicating the presence of NeMϕ bearing an alternatively

activated phenotype (Fig. 4, *A* and *B*). F4/80$^+$ PleC from infected mice include both F4/80$^{high}$ Gr1$^-$ macrophages and F4/80$^{low}$Gr1$^+$ eosinophils (Fig. 3, *C* and *D*). Arginase 1 expression by the pleural cavity NeM$\phi$ population was assessed by real-time PCR with mRNA from F4/80$^+$ cells (47% F4/80$^{high}$ with 48% F4/80$^{low}$) and Gr1-purified cells (88% Gr1$^+$). The F4/80$^+$ fraction showed 9-fold higher levels of arginase 1 mRNA than the Gr1$^+$ cells (Fig. 4*C*), demonstrating that the F4/80$^+$ PleC express arginase 1 and that expression is primarily by macrophages rather than eosinophils. tLN cells isolated from mice 60 days postinfection also showed increased arginase 1 expression (Fig. 4*D*) consistent with increased Ym1 and Fizz1 expression by these cells (39); however, it was not possible to measure expression in the F4/80$^+$ fraction due to insufficient cell recoveries.

In addition to NeM$\phi$, both B cells and DC from the lymph nodes of *B. malayi*-implanted mice have been shown to express Ym1 and Fizz1, indicating some functional cross-over (39). To determine whether these cell types also had a role in suppressing proliferation, CD11c$^+$ DC and CD19$^+$ B cells were purified from the tLN of *L. sigmodontis*-infected mice. Neither DC nor B cells from *L. sigmodontis*-infected mice were able to inhibit Ag-specific proliferation (Fig. 5) or production of IL-4, IL-5, IL-10, or IFN-$\gamma$ (data not shown) of CD4$^+$ T cells isolated from *L. sigmodontis*-infected mice.

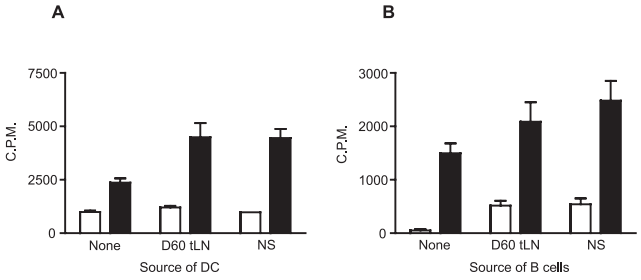


**FIGURE 5.** Neither DC nor B cells from infected mice suppress T cell responses. *A*, Proliferative responses of CD4$^+$ T cells isolated from the tLN of 60-day infected mice and restimulated in vitro with medium alone (□) or LsAg (■) using spleen cells from naive mice as APC in the presence or absence of CD11c$^+$ DC purified from the tLN of 60-day infected mice, or from the spleens of naive mice. Error bars represent SD of cultures in triplicate. Results are representative of three independent experiments. *B*, Proliferative responses of CD4$^+$ T cells isolated from the tLN of 60-day infected mice and restimulated in vitro with medium alone (□) or LsAg (■) using spleen cells from naive mice as APC in the presence or absence of CD43$^-$ B cells purified from the tLN of 60-day infected mice, or from the spleens of naive mice. Error bars represent SD of cultures in triplicate. Results are representative of three independent experiments.

### *Suppression is IL-10 and CTLA-4-independent, but is partially dependent on TGF-$\beta$*

CTLA-4 is a known inhibitor of T cell activation and proliferation (40), and during *L. sigmodontis* infection its intracellular expression is up-regulated on CD4$^+$ T cells within the pleural cavity and tLN (10). To determine whether F4/80$^+$ macrophage suppression of T cell proliferation involves CTLA-4, tLN cells were isolated from infected mice 60 days postinfection and restimulated in vitro with LsAg in the presence of varying concentrations of anti-CTLA-4 whole IgG or anti-CTLA-4 F(ab′)$_2$. Neither anti-CTLA-4 whole IgG (Fig. 6*A*), nor anti-CTLA-4 F(ab′)$_2$ (Fig. 6*B*) were able to restore Ag-specific proliferative responses, indicating that the suppressive effect is not mediated through CTLA-4.

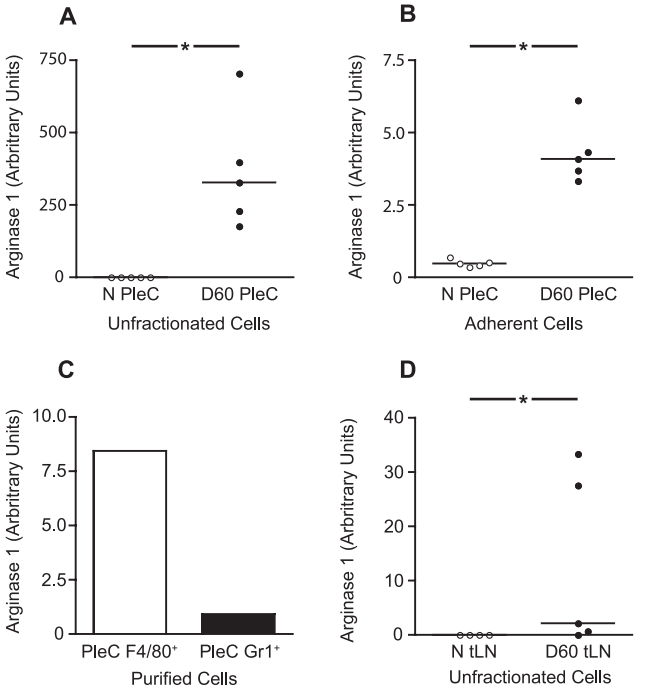


**FIGURE 4.** NeM$\phi$ express the alternatively activated macrophage marker arginase 1. Expression of arginase 1 by unfractionated PleC (*A*) and adherent PleC (*B*) isolated from naive and infected mice 60 days postinfection. *C*, Expression of arginase 1 by F4/80$^+$ and Gr1$^+$ cells purified from pooled PleC isolated from 60-day infected mice. *D*, Expression of arginase 1 by unfractionated tLN cells isolated from naive and infected mice 60 days postinfection. Individual naive mice (open symbols) and infected mice (closed symbols) are shown. *, $p < 0.01$ by Mann-Whitney *U* test.

IL-10 and TGF-$\beta$ have been implicated as a mediators of filarial suppression in several studies (41–43), however, the proliferative block exerted by *B. malayi* recruited NeM$\phi$ has been shown to be IL-10- and TGF-$\beta$-independent (21, 44). To confirm whether IL-10 plays a role in the proliferative suppression seen in the tLN of *L. sigmodontis*-infected mice, whole tLN cells were isolated 60 days postinfection and restimulated in vitro with LsAg in the presence of varying concentrations of a neutralizing Ab against the IL-10R. Neutralizing the IL-10R did not restore the Ag-specific proliferative responses toward LsAg (Fig. 6*C*), although it did increase IFN-$\gamma$ production (Fig. 6*D*), indicating that the proliferative suppression is IL-10-independent. To investigate whether *L. sigmodontis*-recruited NeM$\phi$ inhibit T cell proliferation through TGF-$\beta$, a blocking anti-TGF-$\beta$ Ab was used to neutralize TGF-$\beta$ in DO11.10 spleen cell cultures restimulated in vitro with OVA peptide in the presence or absence of F4/80$^+$ cells purified from the pleural cavity of *L. sigmodontis*-infected mice 60 days postinfection. Adding D60 F4/80$^+$ PleC to the DO11.10 spleen cell cultures suppressed Ag-specific proliferation by 98.6%, and this suppression was reduced to 85% when TGF-$\beta$ was neutralized (Fig. 6*E*). Thus, suppression by *L. sigmodontis*-recruited NeM$\phi$ is partially dependent on TGF-$\beta$, however, TGF-$\beta$ is not a major suppressive mechanism as TGF-$\beta$ neutralization only restored proliferation to a maximum of 14% of the control level.

### *Killing of* L. sigmodontis *can occur in the presence of APC suppression*

The potent suppressive ability of the NeM$\phi$ indicated that they may inhibit the host's protective immune responses, favoring parasite survival. To test their relative importance in controlling parasite numbers, we used *L. sigmodontis*-infected BALB/c mice that had been induced to kill their adult parasites through neutralization of CD4$^+$ regulatory T cells by cotreatment with anti-CD25 and

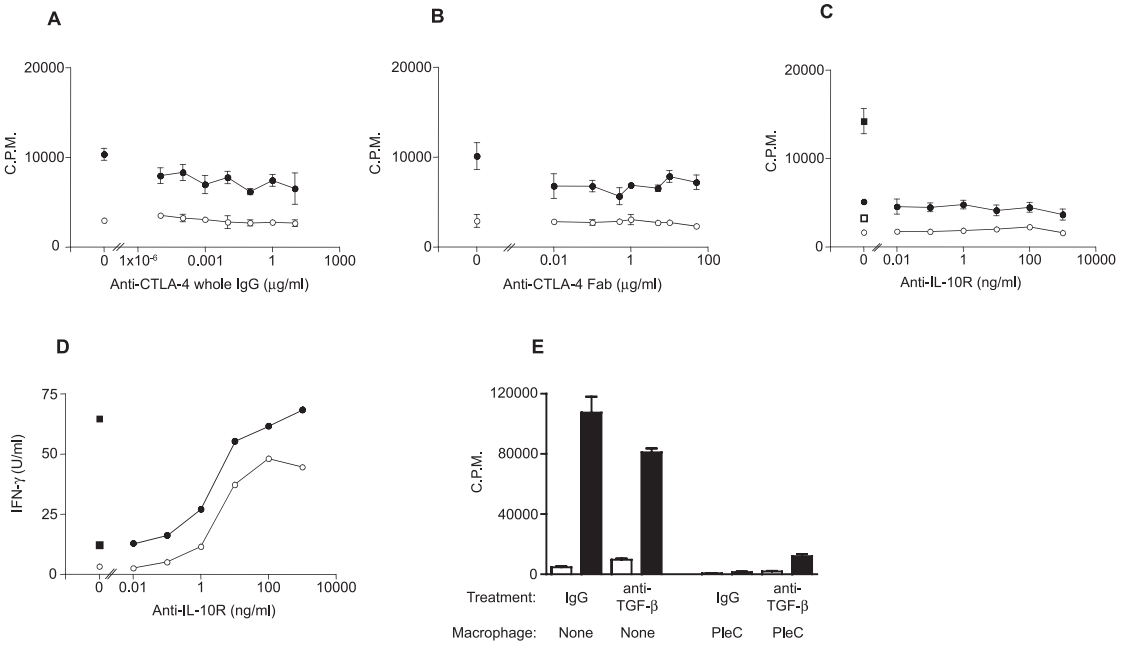


**FIGURE 6.** Suppression of T cell responses is independent of both IL-10 and CTLA-4, but partially dependent on TGF-$\beta$. *A* and *B*, Proliferative responses of tLN cells isolated 60 days postinfection and restimulated in vitro with medium alone (open symbols) or LsAg (closed symbols) in the presence of varying concentrations of whole anti-CTLA-4 Ab (*A*) or anti-CTLA-4 F(ab′)$_2$ (*B*). Error bars represent the SD of cultures in triplicate. Proliferative (*C*) and IFN-$\gamma$ (*D*) responses of pooled tLN cells isolated 60 days postinfection and restimulated in vitro with medium alone (○) and LsAg (●) with varying concentrations of a neutralizing Ab against the IL-10R. As a positive control, pooled tLN cells isolated from 40-day infected mice were restimulated with medium alone (□) and LsAg (■). Cells were pooled from at least five mice. Error bars for proliferation represent the SD of cultures in triplicate. *E*, Proliferative response of DO11.10 spleen cells restimulated in vitro with OVA peptide in the presence or absence of day 60 pleural cavity F4/80$^+$ purified macrophages with 50 $\mu$g/ml neutralizing anti-TGF-$\beta$ Ab or control rat IgG. Error bars represent SD of cultures in triplicate. Results are representative of two experiments.

anti-GITR Abs (10). Adherent PleC isolated from mice cotreated with anti-CD25 and anti-GITR, or control treated with rat IgG, were tested for their ability to inhibit the proliferation of EL-4 cells. Cotreatment of infected mice with anti-CD25 and anti-GITR did not affect the suppressive phenotype of the adherent PleC population, despite a 72% reduction in the numbers of adult parasites (Fig. 7). As Ab cotreatment did not impact on the suppressive macrophage population, these data demonstrate that killing of adult filariae can be induced by intervening against CD4$^+$ T cell-mediated regulation, even in the presence of suppressive macrophages.

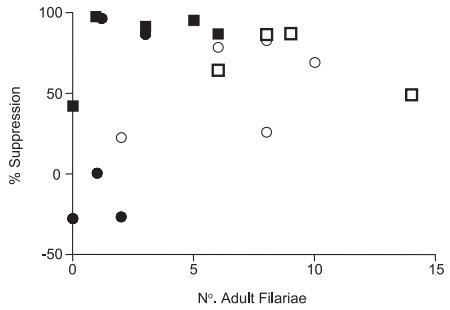


**FIGURE 7.** Killing of adult parasites can occur in the presence of the suppressive macrophage population. Percentage of suppression of EL-4 cell proliferation by adherent PleC isolated from *L. sigmodontis*-infected mice treated with IgG (open symbols) or with combined anti-CD25/anti-GITR (closed symbols) plotted against parasite burden. Cells were isolated 60 days postinfection, and Ab treatments were given on day 28. Percentage suppression was calculated against the average proliferation of EL-4 cells in the presence of adherent PleC from naive mice. Square and circle symbols denote two independent experiments, and each symbol represents individual animals.

## Discussion

Chronic human filarial infections are synonymous with suppression of the host immune response, indicating that the parasites' long-lived nature is due to their ability to turn off host protective immunity (11, 45). Studies in humans and animal models have demonstrated that immune inhibition extends to many arms of the immune response, including DC (33, 46), macrophages/monocytes (12, 47), and T cells (7, 48), and that parasites secrete numerous molecules to aid in this task (45). These and other studies indicate that the mechanisms of filarial suppression differ according to the stage of the life cycle, and that patency is a turning point at which maximal suppression occurs (17, 25–28). Using *L. sigmodontis* infection of susceptible BALB/c mice to follow the development of filarial infection to patency, we have previously demonstrated that immune regulation takes hold as the infection progresses. In particular, there is expansion of regulatory T cell activity following larval entry (10), and as infection becomes patent the CD4$^+$ T cell population in the pleural cavity develops a hyporesponsive phenotype, while Ag-specific recall responses within the draining lymph nodes are reduced.

We now demonstrate that although immune regulation within the CD4$^+$ T cell compartment is a critical determinant of filarial survival (10), the inactivation of CD4$^+$ effector T cells into a hyporesponsive phenotype remains restricted to the infection site, and does not explain immune suppression within the draining lymph nodes. We present evidence that beyond the site of infection, immune regulation is mediated by a suppressive F4/80$^+$ population of NeM$\phi$ acting through a partially TGF-$\beta$-dependent, but

IL-10- and CTLA-4-independent mechanism. The activity of this NeM$\phi$ population evolved over the course of infection, being restricted to the infection site during prepatency, and spreading into the draining lymph nodes as infection became patent. Thus, at patency, two independent mechanisms of suppression operate in the pleural cavity, an intrinsic defect in the Ag responsiveness of the $CD4^+$ T cell population and inhibition of proliferation by NeM$\phi$. In contrast, in the draining lymph nodes, immune suppression is restricted to the MeM$\phi$ population.

The suppressive NeM$\phi$ recruited by *L. sigmodontis* infection had similar characteristics to the "alternatively activated" peritoneal cavity NeM$\phi$ in mice implanted with adult *B. malayi* parasites (18, 21, 23, 24, 37), and their alternatively activated status was confirmed by their expression of arginase 1. Their lack of expression of Gr1 suggests they are distinct from alternatively activated $Gr1^+$ myeloid suppressor cells described in other systems (49–51). The alternatively activated phenotype is driven by the Th2 responses of the infected host, and NeM$\phi$ are recruited by a range of different helminth infections (18, 21, 23, 24, 37). Filarial infection induces a Th2 response from first contact with the infective L3 stage (52), and *B. malayi* L3 are able to recruit NeM$\phi$ to the peritoneal cavity within 7 days (21). Thus, although the most profound suppression is associated with patency, it is not surprising that NeM$\phi$ are recruited locally to the site of *L. sigmodontis* infection from an earlier point. Interestingly, although Th2 responses are seen in the draining lymph nodes from day 12 of *L. sigmodontis* infection and are equally strong at days 40 and 60 (10), the presence of a Th2 cytokine response was not sufficient in itself to induce the suppressive NeM$\phi$ response in the tLN. The dissemination of suppressive activity to the lymph nodes only occurred when Mf, released from gravid adult females, exit the pleural cavity and circulate through the blood and draining lymph nodes. Hence, the presence of NeM$\phi$ peripheral to the infection site may be triggered by the newly systemic nature of infection.

The functional role of NeM$\phi$ within parasitic infections is still not fully clear. Their ability to suppress T cell responses suggests a role in down-regulating inflammatory responses and limiting pathology during chronic infections, whereas their specialized profile of gene expression (24) indicates further undiscovered functions. Given their potent suppressive capability, a major question is whether NeM$\phi$ have an influential role in restraining host immunity to parasitism, either through direct inhibition of T cell proliferation, or by driving the T cell toward a regulatory or unresponsive phenotype. Although T cells have been shown to recover full functionality once NeM$\phi$ have been removed from culture and thus the inhibitory effect of the NeM$\phi$ is transient (44), the effect of repeated stimulation by inhibitory NeM$\phi$ has not been investigated, and $F4/80^+$ macrophages have recently been shown to induce $CD8^+$ regulatory T cell activity (53). Significantly, although NeM$\phi$ inhibited the proliferative responses of T cells, IL-4 and IL-5 cytokine responses remained relatively unaffected. These cytokines are known to be important for protective immunity toward both adult parasites and Mf (54, 55), suggesting that the generation of NeM$\phi$ in vivo may not have a direct impact on parasite survival. Interestingly, it was found that killing of adult *L. sigmodontis* parasites induced through the neutralization of regulatory T cell activity, occurred despite the presence of the suppressive NeM$\phi$. This indicates that, although multiple levels of suppression do exist, specifically targeting T cell-mediated regulation is sufficient to restore protective immunity. Further in vivo studies, however, will be required to ascertain whether NeM$\phi$ contribute to the generation of regulatory T cells or T cell hyporesponsiveness during infection.

The marked correlation between the location of functional NeM$\phi$ and that of the parasite during the different stages of infection indicates a close interaction. Alternatively activated macrophages play an important role in controlling pathology during infection with *Schistosoma mansoni* (56), and are associated with tissue repair indicating a role in wound healing (39, 57). If the NeM$\phi$ do not promote *L. sigmodontis* survival, their role in vivo maybe to limit and repair potentially damaging inflammatory responses. While the adult parasites reside solely within the pleural cavity and the inflammatory response and damage is localized, the NeM$\phi$ would only be required at the infection site. As Mf begin to migrate through the lymph nodes and bloodstream, potentially inducing more widespread inflammatory responses, then NeM$\phi$ activity would need to expand to follow the parasites' migration. Alternatively, NeM$\phi$ may play an effector role, either directly through granuloma formation or indirectly through the recruitment of eosinophils by secretion of Ym1 (58) again requiring them to closely follow the migration of the Mf.

The intrinsic responsiveness of the $CD4^+$ T cell populations was determined by their proximity to the infection site, as pleural cavity $CD4^+$ T cells become hyporesponsive (10), whereas those in the tLN retained their ability to respond to Ag. Interestingly, the hyporesponsive phenotype of the pleural cavity $CD4^+$ T cells is associated with greatly increased expression of CTLA-4 and GITR, whereas the tLN $CD4^+$ T cells only show slight increases in CTLA-4 and GITR expression (10). Hyporesponsiveness is therefore associated with a highly activated phenotype, and greatly increased expression of the inhibitory receptor CTLA-4, indicating a potential role for CTLA-4 in the development of filarial-induced $CD4^+$ T cell hyporesponsiveness. It is likely, therefore, that the $CD4^+$ T cells at the infection site represent a resident population of effector T cells that have been chronically exposed to both antigenic challenge and the down-regulatory environment induced by infection resulting in their hyporesponsiveness. In contrast, the lymph nodes are provided with a continual stream of newly activated effector cells from the naive T cell pool, potentially maintaining the Ag responsiveness of the tLN T cell population. In the draining lymph nodes, the presence of NeM$\phi$ could either benefit the parasite by inhibiting the expansion of these "fresh" effector cells, or benefit the host by polarizing them toward a protective Th2 phenotype (44).

Although the suppressive effects of the NeM$\phi$ are highly potent in dissociated cell cultures, their function in vivo will be dependent on their location within the lymph node microenvironment, particularly as their main suppressive mechanism appears to be contact-dependent (18, 37). Recently, alternatively activated macrophages recruited by infection with *Taenia crassiceps* or *S. mansoni* have been shown to up-regulate expression of PD-L1 and PD-L2, and mediate their suppressive effect through interactions of these ligands with PD-1 expressed on T cells (59, 60). Thus, suppression in vivo by NeM$\phi$ will likely require direct interactions with their target cell. The ability of T cells to respond to *L. sigmodontis* in vivo will therefore be defined by the APC type they encounter within the lymph nodes, of which the suppressive NeM$\phi$ only form a minority. To have an impact on the generation or maintenance of T cell responses within the tLN would require the NeM$\phi$ to preferentially migrate into the T cell areas and out-compete other APC for T cell interactions. Alternatively, if NeM$\phi$ are more involved in controlling pathology in situ or as effector cells, then they would be expected to accumulate around the sites of migrating Mf, and may have little influence in the T cell areas. To fully understand the function of NeM$\phi$ it will therefore be important in the future to follow their movements and interactions with other cell types in vivo.

Immunosuppression during *L. sigmodontis* infection therefore consists of several independent and overlapping players, $CD4^+$ T cells (including both regulatory and effector cells) and $F4/80^+$ NeM$\phi$. These different mechanisms of suppression vary both temporally and spatially with a large part of their progression linked to the onset of patency. The expanded profile of peripheral immune suppression at patency may mark the transition from a localized to a systemic infection, rather than an abrupt induction of new suppressive mechanisms by the parasite. Understanding how filarial suppression develops according to locality to the infection site is of particular importance for human studies where sampling is often restricted to peripheral blood populations. Immune suppression during human infection may, therefore, only be detectable once infection has become systemic, and may not be representative of the regulatory pathways at the actual infection site. The findings described in this study, along with continuing study of the *L. sigmodontis* model, will provide a framework for understanding and integrating the data on filarial-induced suppression seen in T cells and APC of humans and animals.

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## Disclosures

The authors have no financial conflict of interest.

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