Expansion of Foxp3$^+$ Regulatory T Cells in Mice Infected with the Filarial Parasite *Brugia malayi*[1]

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Many helminths, including *Brugia malayi*, are able to establish long-lived infections in immunocompetent hosts. Growing evidence suggests that the immune system's failure to eliminate parasites is at least partially due to the effects of regulatory T cells (Tregs). To test whether parasites may directly stimulate host regulatory activity, we infected mice with two key stages of *B. malayi*. Both mosquito-borne infective larvae and mature adults i.p. introduced were found to preferentially expand the proportion of CD25$^+$Foxp3$^+$ cells within the CD4$^+$ T cell population. The induction of Foxp3 was accompanied by raised CD25, CD103, and CTLA-4 expression, and was shown to be an active process, which accompanied the introduction of live, but not dead parasites. CTLA-4 expression was also markedly higher on Foxp3$^-$ cells, suggesting anergized effector populations. Peritoneal lavage CD4$^+$CD25$^+$ cells from infected mice showed similar suppressive activity in vitro to normal splenic "natural" Tregs. Both *B. malayi* larvae and adults were also able to induce Foxp3 expression in adoptively transferred DO11.10 T cells, demonstrating that filarial infection can influence the development of T cells specific to a third party Ag. In addition, we showed that induction was intact in IL-4R-deficient animals, in the absence of a Th2 or alternatively activated macrophage response. We conclude that filarial infections significantly skew the balance of the host immune system toward Treg expansion and activation, in a manner dependent on live parasites but independent of a concomitant Th2 response. *The Journal of Immunology,* **2008, 181: 6456–6466.**

Human filarial parasites such as *Brugia malayi* establish long-term, stable infections, causing lymphatic filariasis and onchocerciasis in over 150 million people worldwide (1, 2). Lymphatic filariasis covers a spectrum of disease states from asymptomatic carrier (microfilaremic) to chronic lymphatic dysfunction and elephantiasis (3, 4). Notably, the asymptomatic subjects, who carry high levels of circulating transmission-stage microfilariae, display a muted immunological response (5–8), failing to mount parasite-specific T cell proliferative and cytokine responses. The degree of suppression is accentuated with higher parasite loads (9), and can be reversed by anti-filarial chemotherapy (10, 11). Peripheral T cell populations in infected individuals show elevated expression of CTLA-4 and Foxp3, suggesting an increase in effector T cell anergy and regulatory T cell (Treg)[3] activity (12, 13). Taken together, these data indicate an active suppression of Ag-specific responses, dependent directly on the presence of live parasites.

Tregs are a recently recognized subset of CD4$^+$ T cells that actively repress immune effector cells in a range of settings from autoimmunity to infectious diseases (14–16). It is an attractive, but unproven hypothesis that human filarial infections activate the Treg pathway (17–20). Evidence that Tregs may be involved in down-modulating human responses to filarial infection stemmed originally from the prominence of IL-10 in hyporesponsive microfilaraemic carriers (21), and the ability of anti-IL-10 and anti-TGF-β to restore the Ag-specific proliferative response in these individuals (17, 22). More recently, the isolation of Ag-specific Tr1 clones from a microfilaraemic patient (17, 23), and a study demonstrating generalized elevation of Treg-associated gene transcription in filarial patients (13), have lent further support to this interpretation. To date, however, there has been no evidence that human filarial parasites directly induce or activate the regulatory arm of the adaptive immune system.

Mouse models of filarial infection have illustrated many parallels with human infection (24), and work with natural parasites of rodents supports the hypothesis that host immunity is compromised by Treg activity (25–27). In *Litomosoides sigmodontis*–infected mice, Tregs expand in number at the site of infection, whereas the effector population displays a hyporesponsive Th2 phenotype. Hyporesponsiveness can be broken by depletion of the Tregs using anti-CD25 and costimulation of the suppressed Th2 cells using anti-glucocorticoid-induced TNFR or anti-CTLA-4 (25, 26). In *Acanthocheilonema viteae*, another rodent filarial parasite, an excretory/secretory product known as ES-62 has been shown to have down-regulatory effects, including producing a hypoproliferative defect in B and T cells (28, 29). Finally, in a parasite closely related to *B. malayi* (*B. pahangi*), restimulation of splenocytes from infected mice expanded a population of CD25$^+$CTLA-4$^+$ cells, with higher expression of mRNA for the Treg master transcription factor, Foxp3 (30).

Although *B. malayi* cannot complete its life cycle in immunocompetent laboratory mice (31–33), its short-term survival in rodents offers the opportunity to study the initial response to a major human pathogen. In most mouse strains, the mosquito-borne third-stage infective larvae (L3) will survive for 1–3 wk, whereas adult

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[3] Abbreviations used in this paper: BMDC, bone marrow-derived dendritic cell; Treg, regulatory T cell.

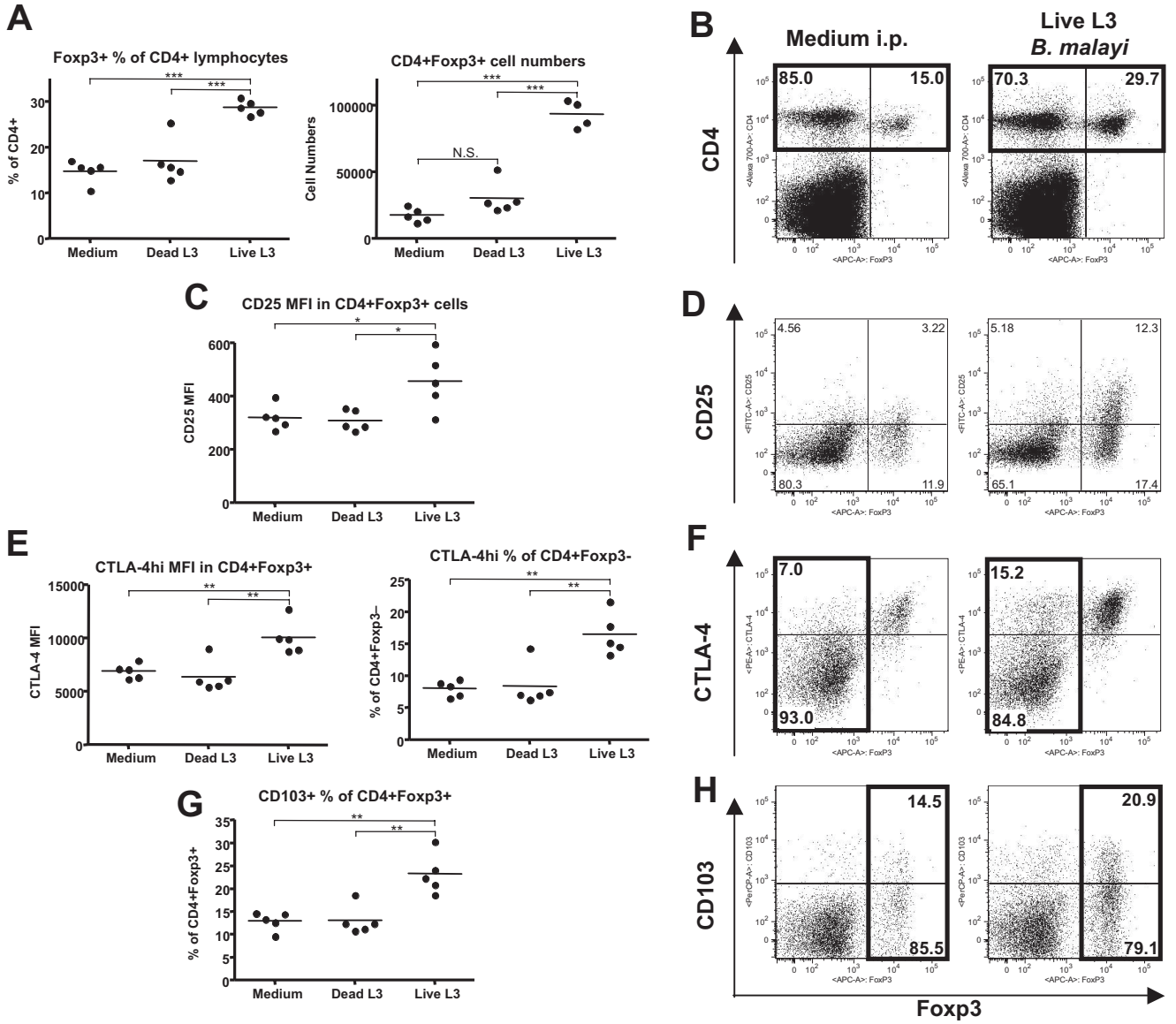

FIGURE 1. Expression of Foxp3, CD25, CTLA-4, and CD103 following infection with *B. malayi* L3. A total of 50 *B. malayi* L3 larvae, or controls of heat-killed L3 *B. malayi*, or medium alone, were injected i.p. into BALB/c male mice, and peritoneal lavage cells taken 7 days later. Flow cytometric staining for CD4, CD25, CD103, Foxp3, and intracellular CTLA-4 was then conducted. Data shown are representative of five repeat experiments. *A*, Percentage of $CD4^+$ peritoneal lavage (PL) cells that express Foxp3 after live *B. malayi* L3 infection (*left*) and total $CD4^+Foxp3^+$ peritoneal lavage cell numbers in the same mice (*right*), compared with cells from mice receiving control injections. Note that baseline levels of Foxp3 expression among peritoneal lavage $CD4^+$ populations are around 15%, compared with ~10% in splenic $CD4^+$ T cells stained under the same conditions. *B*, Representative plots of CD4 and Foxp3 levels on peritoneal lavage cells, gated on lymphocytes. $Foxp3^+$ percentages of $CD4^+$ lymphocytes shown in *A* (*left*) are calculated from proportion of $CD4^+Foxp3^+$ cells (top right quadrant) within the total $CD4^+$ population (both top quadrants, heavy border). *C*, Mean fluorescence intensity (MFI) of CD25 staining on $CD4^+Foxp3^+$ cells, following *B. malayi* L3 infection. *D*, Representative plots of CD25 and Foxp3 levels on peritoneal lavage $CD4^+$ cell populations. *E*, Mean fluorescence intensity (MFI) of CTLA-4 on $CD4^+Foxp3^+$ cells (*left*) and $CD4^+Foxp3^-$ cells that express CTLA-4 (*right*), following *B. malayi* L3 infection. *F*, Representative plots of CTLA-4 and Foxp3 levels on peritoneal lavage $CD4^+$ cell populations. CTLA-4^{high} percentages of $CD4^+Foxp3^-$ lymphocytes shown in *E* (*right*) are calculated from proportion of $CD4^+$CTLA-$4^{high}Foxp3^-$ cells (top left quadrant) within the total $Foxp3^-$ population (both left quadrants, heavy border). *G*, Percentage of $CD4^+Foxp3^+$ cells that express CD103 following *B. malayi* L3 infection. *H*, Representative plots of CD103 and Foxp3 levels on peritoneal lavage $CD4^+$ cell populations. $CD103^+$ percentages of $CD4^+Foxp3^+$ lymphocytes shown in *G* are calculated from proportion of $CD4^+CD103^+Foxp3^+$ cells (top right quadrant) within the total $CD4^+Foxp3^+$ population (both right quadrants, heavy border). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

worms transplanted into the peritoneal cavity live for 2–3 mo (34), in both cases inducing a highly polarized Th2 response (30, 32, 35). Moreover, infection of either stage into the peritoneal cavity results in a recruitment of well-characterized leukocyte populations (33), including suppressive alternatively activated macrophages (36). Within this timeframe, therefore, infection of mice with the human parasite sheds invaluable light on how the mammalian immune response reacts to, and may be manipulated by, filarial infections.

The results presented in this study show an expansion of $CD4^+Foxp3^+$ Tregs, with a concurrent up-regulation of CD103, CTLA-4, and CD25 in mice infected with either stage of *B. malayi*. Selective Treg outgrowth was dependent on live parasites, as no increase in Treg proportions was seen in the presence of killed

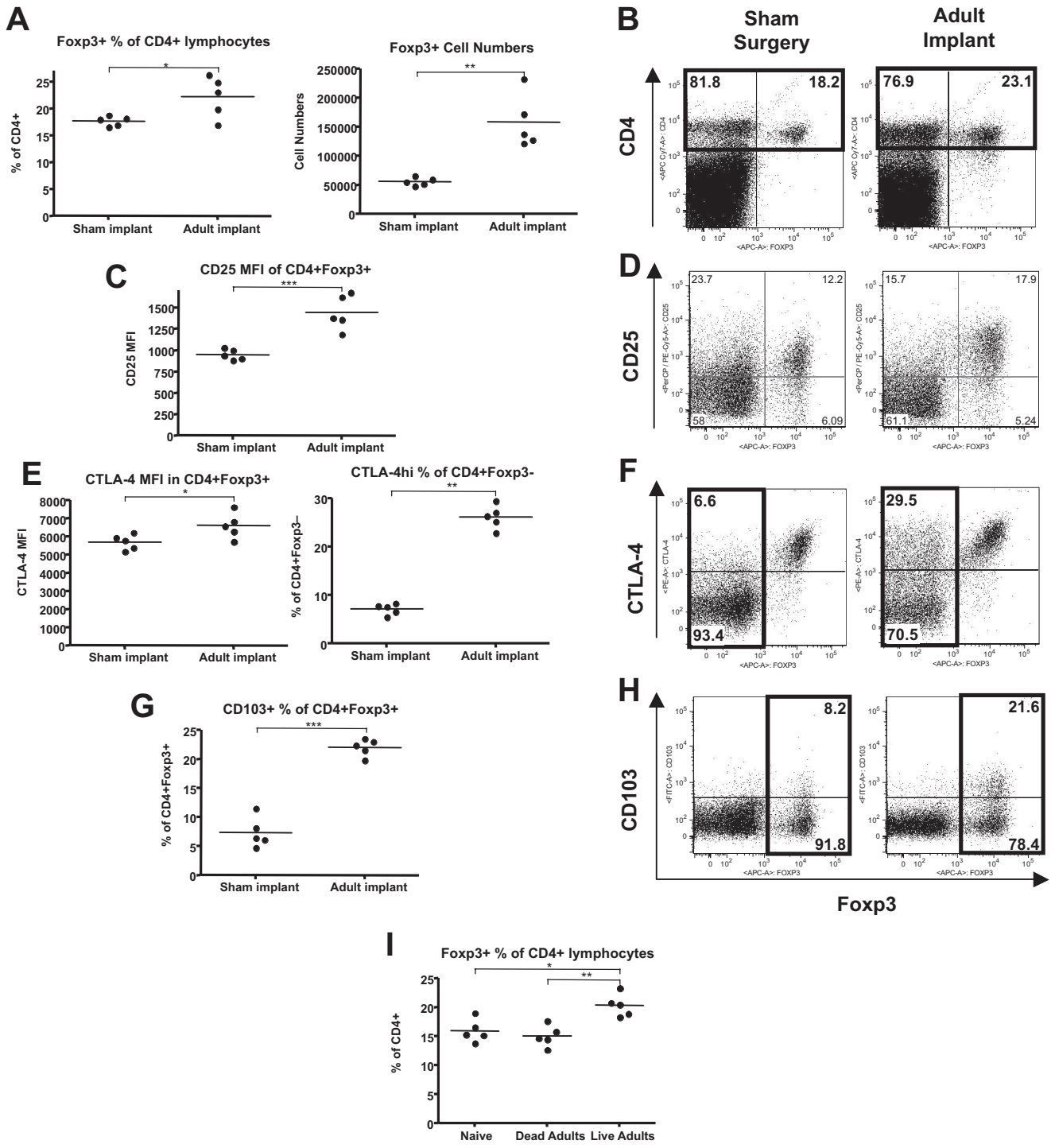

FIGURE 2. Expression of Foxp3, CD25, CTLA-4, and CD103 following infection with *B. malayi* adult parasites. Groups of 4 female and 1 male adult *B. malayi* were surgically implanted into the peritoneal cavity of BALB/c male mice, and peritoneal lavages taken 7 days later from infected mice and from sham surgery controls. Flow cytometric staining for CD4, CD25, CD103, Foxp3, and intracellular CTLA-4 was then conducted. Data shown are representative of three repeat experiments. *A*, Percentage of $CD4^+$ peritoneal lavage cells that express Foxp3 after sham implant or adult *B. malayi* implantation (*left*) and total $CD4^+Foxp3^+$ peritoneal lavage cell numbers in the same mice (*right*). *B*, Representative plots of CD4 and Foxp3 levels on peritoneal lavage cells, gated on lymphocytes. $Foxp3^+$ percentages of $CD4^+$ lymphocytes shown in *A* (*left*) are calculated from proportion of $CD4^+Foxp3^+$ cells (top right quadrant) within the total $CD4^+$ population (both top quadrants, heavy border). *C*, CD25 mean fluorescence intensity (MFI) after sham implant or adult *B. malayi* implantation. *D*, Representative plots of CD25 and Foxp3 levels on peritoneal lavage $CD4^+$ cell populations. The number in each quadrant indicates the percentage within the $CD4^+$ population. *E*. Mean fluorescence intensity of CTLA-4 on $CD4^+Foxp3^+$ cells (*left*) and $CD4^+Foxp3^-$ cells that express CTLA-4 (*right*), after sham implant or adult *B. malayi* implantation. *F*, Representative plots of CTLA-4 and Foxp3 levels on peritoneal lavage $CD4^+$ cell populations. CTLA-4^{high} percentages of $CD4^+Foxp3^-$ lymphocytes shown in *E* (*right*) are calculated from proportion of $CD4^+CTLA\text{-}4^{high}Foxp3^-$ cells (top left quadrant) within the total $Foxp3^-$ population (both left quadrants, heavy border). *G*, Percentage of $CD4^+Foxp3^+$ cells that express CD103 after sham implant or adult *B. malayi* implantation. *H*, Representative plots of CD103 and Foxp3 levels on peritoneal lavage $CD4^+$ cell populations. $CD103^+$ percentages of $CD4^+Foxp3^+$ lymphocytes shown in *G* are calculated from proportion of $CD4^+CD103^+Foxp3^+$ cells (top right quadrant) within the total $CD4^+Foxp3^+$ population (both right quadrants, heavy border). *I*, Foxp3 induction in peritoneal lavage cells from mice implanted with dead or live adult *B. malayi*, compared with naive animals. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

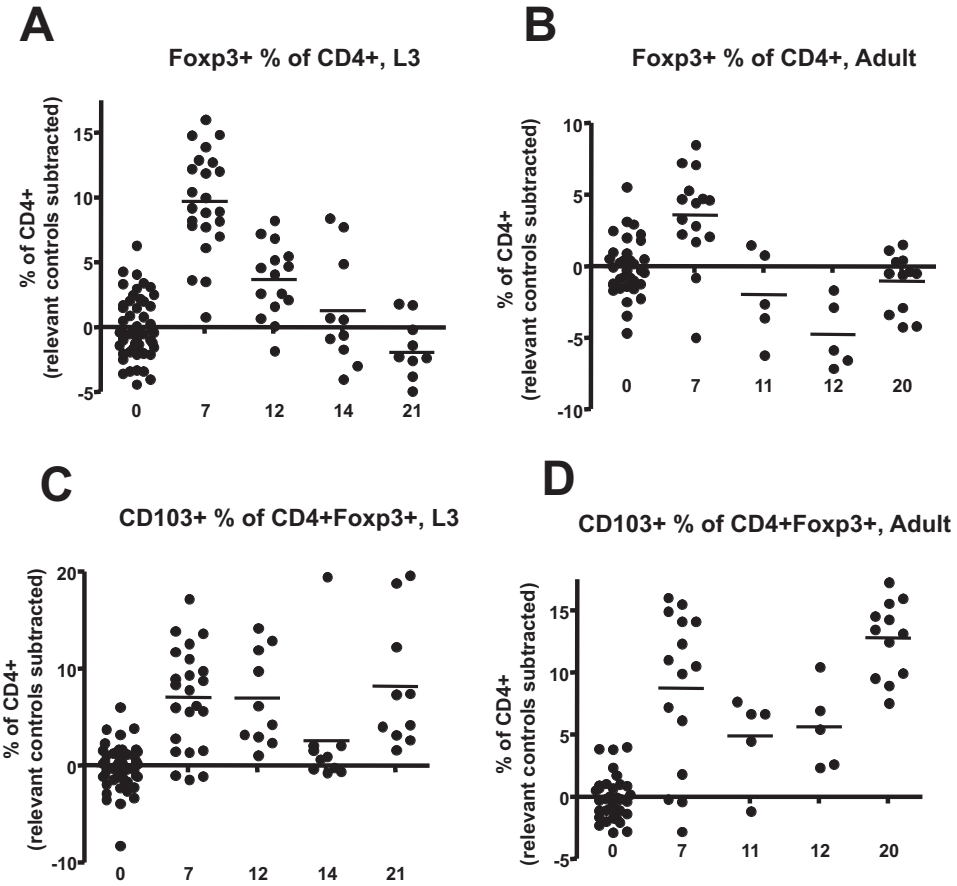

FIGURE 3. Kinetics of expression of Treg markers following infection with *B. malayi*. Infections of BALB/c males with *B. malayi* L3 larvae or adults were conducted with peritoneal lavage cells harvested at days 7, 12, 14, and 21 or days 7, 11, 12, and 20 postinfection, respectively. Peritoneal lavage populations were analyzed by flow cytometric analysis for CD4, Foxp3, and CD103. Experiments from each time point were analyzed separately by Student's *t* test, with *p* values determined for each. Horizontal bar represents mean cell levels. *A*, Percentage of $CD4^+$ cells that express Foxp3 after L3 *B. malayi* infection (*p* values for each experiment: day 7, 0.0097, 0.0011, 0.0018, and 0.0000061; day 12, 0.0051, 0.042, and 0.29; day 14, 0.32 and 0.96; day 21, 0.98 and 0.022). *B*, Percentage of $CD4^+$ cells that express Foxp3 after adult *B. malayi* implant (*p* values for each experiment: day 7, 0.032 and 0.0076; day 11, 0.25; day 12, 0.059; and day 20, 0.15 and 0.71). *C*, Percentage of $CD4^+Foxp3^+$ cells that express CD103 after L3 *B. malayi* infection (*p* values for each experiment: day 7, 0.0058, 0.00029, 0.000027, 0.0014, and 0.11; day 12, 0065, 0.048, and 0.29; day 14, 0.17 and 0.45; and day 21, 0.0085 and 0.0036). *D*, Percentage of $CD4^+Foxp3^+$ cells that express CD103 after adult *B. malayi* implant (*p* values for each experiment: day 7, 0.0000041 and 0.60; day 11, 0.038; day 12, 0.0086; and day 20, 0.00012 and <0.0001). The percentage of $Foxp3^+$ cells within uninfected control $CD4^+$ populations varied between 10.5 and 25.6%, with a mean value of 17.7%. For each time point shown, relevant mean control levels were subtracted from all replicates. Data presented includes experiments conducted in other areas of this study.

parasites. Treg induction was also seen within a bystander Ag-specific T cell population using an adoptive transfer model, indicating the induction is not restricted to parasite-specific cells. We also show that Treg expansion is independent of alternatively activated macrophage induction, as it is retained in IL-$4R^{-/-}$ mice. Thus, we show that the human filarial parasite has the inherent ability to stimulate Treg activity, providing further support for the hypothesis that these cells play an important role in the human infection process.

Materials and Methods

Animals

Male BALB/c, DO11.10, and IL-$4R^{-/-}$ (on a BALB/c background) mice, 6- to 10-wk-old, were bred and housed according to Home Office guidelines. Male adult jirds (*Meriones unguiculatus*) carrying a patent *B. malayi* infection were used as a source of adult worms and microfilariae.

Parasites

B. malayi L3 were obtained from *Aedes aegypti* mosquitoes that had been fed on human blood containing microfilariae 12–14 days previously, and 50 L3/mouse were i.p. injected in RPMI 1640 (Invitrogen). L3 were killed as controls by heating to 65°C for 10 min. Adult *B. malayi* parasites were retrieved from the peritoneal cavity of patently infected jirds and separated into groups of 4 females with 1 male for implantation. Where dead adults were required, parasites were killed by freeze-thawing. Adult parasites were then surgically implanted into the peritoneal cavity.

Peritoneal lavage

At indicated time points, mice were sacrificed using terminal anesthesia and bled out. Ice-cold RPMI 1640 with 10% FCS (Invitrogen) was then used to wash out peritoneal lavage cells. The cells were then adhered to plates for 1–3 h at 37°C (to remove adherent macrophages), and nonadherent cells stained for flow cytometry of T cell markers. For characterization of macrophage and eosinophil populations, samples of unseparated cells were taken for staining, and the remainder adhered to plates before staining for T cell markers. L3 recovered from the peritoneum were also counted at this point. Spleens were also taken, homogenized through nylon filters, RBC lysed (using RBC lysis buffer; Sigma-Aldrich) and restimulated with *B. malayi* adult homogenate for 72 h, 37°C. Supernatants were then taken and used in cytokine ELISA.

Flow cytometry

One million peritoneal lavage cells were stained with Alexa Fluor 700 (BioLegend) or allophycocyanin-Cy7-conjugated (eBioscience) anti-CD4,

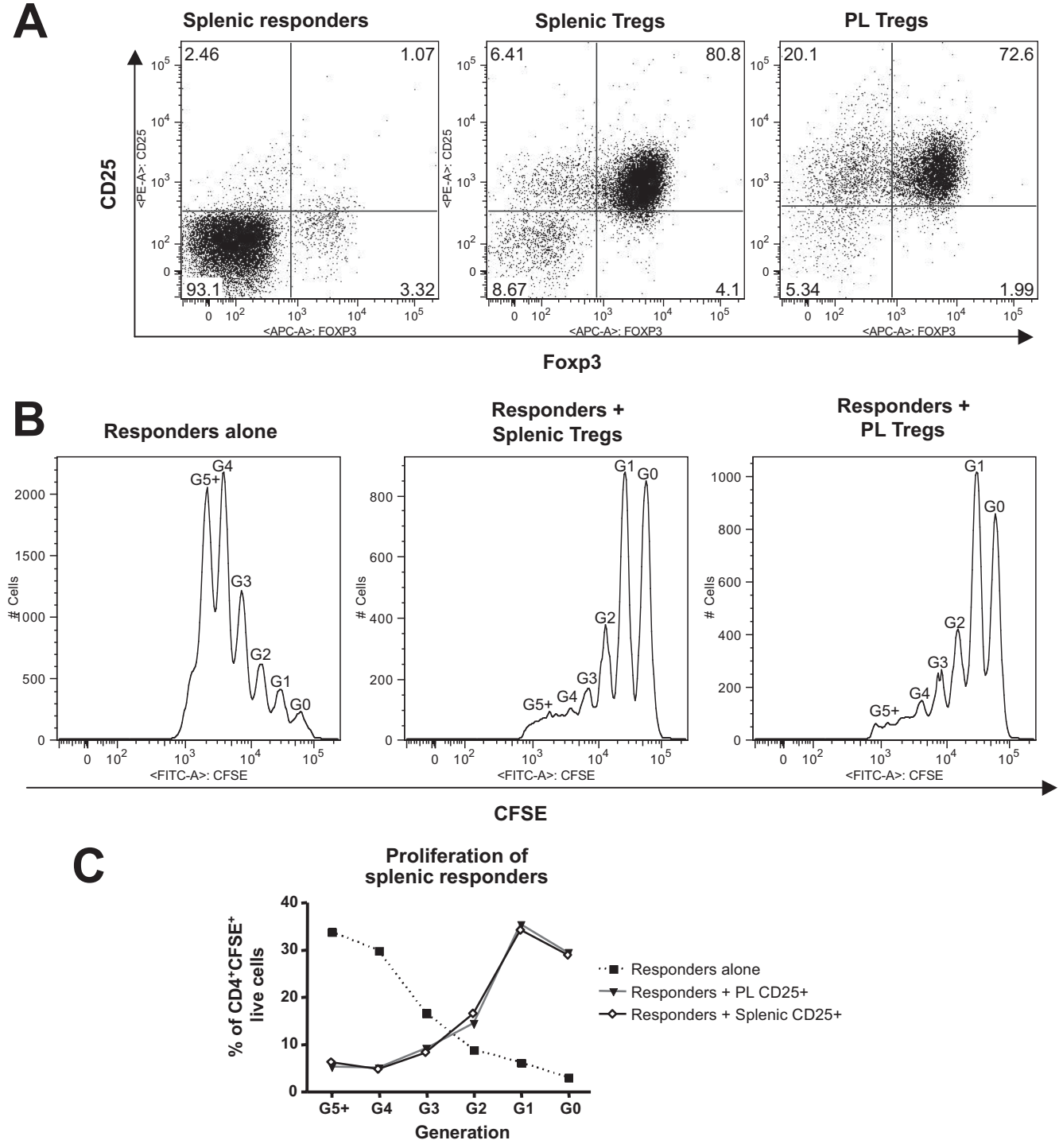

FIGURE 4. In vitro suppression by $CD4^+CD25^+$ peritoneal T cells from infected mice. Peritoneal lavage (PL) cells from mice infected for 7 days with 50 *B. malayi* L3 larvae, and naive splenocytes from control mice, were prepared by positive MACS selection for CD4, then FACS-sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations. Splenic naive $CD4^+CD25^-$ cells (responders) were then polyclonally stimulated in the presence or absence of $CD4^+CD25^+$ cells from either naive or *B. malayi*-infected mice. *A*, Foxp3 and CD25 expression in the FACS-purified populations. *B*, Representative plots of CFSE staining on the CFSE-stained $CD4^+CD25^-Foxp3^-$ responder population. *C*, Proportions of labeled live $CD4^+$ cells in each generation, as gated by CFSE dilution.

biotinylated or FITC-conjugated anti-CD103 (BD Pharmingen), biotinylated, FITC-, or PE-conjugated anti-CD25 (BD Pharmingen), biotinylated KJ1-26 (DO11.10 clonotypic mAb), PerCP- or allophycocyanin-conjugated streptavidin (BD Pharmingen), PE-conjugated anti-Siglec F (BD Pharmingen), or biotinylated anti-F4/80 (BioLegend). Where required, cells were then permeabilized using the eBioscience Foxp3 staining kit, and stained with allophycocyanin or PE-conjugated Foxp3 (eBioscience) and PE-conjugated CTLA-4 (BD Pharmingen). Relevant isotype controls were used on all samples. Samples were acquired on a BD Biosciences LSR II flow cytometer and analyzed using FlowJo software (Tree Star).

$CD4^+CD25^+$ suppression assay

$CD4^+$ cells were isolated from a spleen from a naive mouse, and peritoneal lavage cells from L3 *B. malayi*–infected mice (15 mice pooled), using magnetic bead isolation on MACS columns (Miltenyi Biotec), as per the

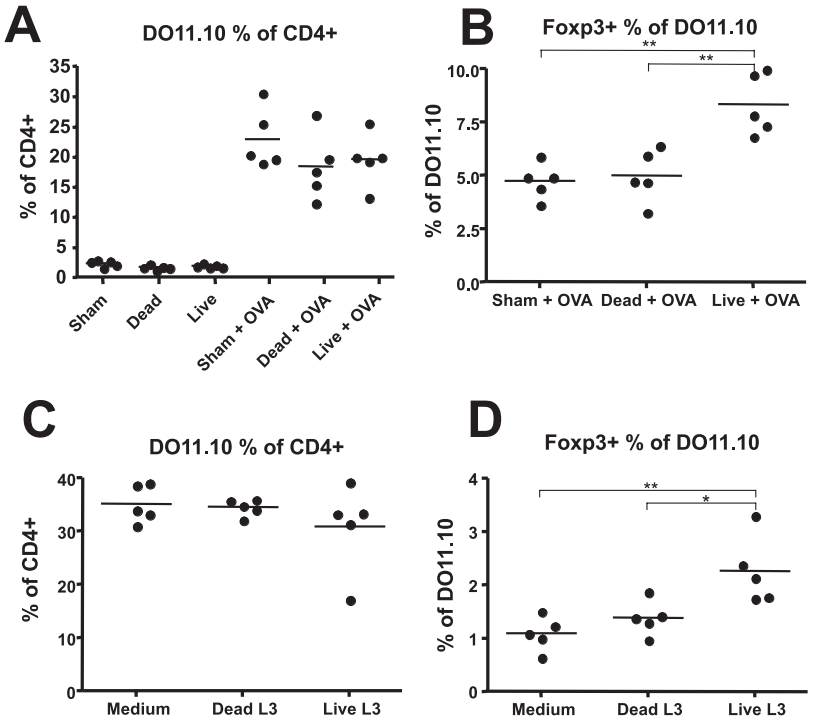

FIGURE 5. Expansion of OVA-specific Treg population during infection. DO11.10 splenocytes were adoptively transferred into BALB/c mice, which 1 day later were infected with *B. malayi* adults (*A* and *B*) or L3 (*C* and *D*). A further day later LPS-matured peptide OVA-loaded BMDCs were injected i.p. At day 7 postinfection, peritoneal lavage (PL) cells were taken and analyzed by flow cytometry for expression of CD4, Foxp3, and KJ1-26 (DO11.10 clonotypic Ab). The adult implant experiment was conducted once, whereas L3 experiment shown is representative of two repeats. Horizontal bar represents mean cell levels. *A*, DO11.10 (KJ1-26$^+$) percentage of the CD4$^+$ population after adult implant. *B*, Foxp3$^+$ percentage of the DO11.10 population after adult implant. *C*, DO11.10 (KJ1-26$^+$) percentage of the CD4$^+$ population after L3 infection. *D*, Foxp3$^+$ percentage of the DO11.10 population after L3 infection. *, $p < 0.05$; **, $p < 0.01$.

manufacturer's instructions. Irradiated CD4$^-$ splenocytes were used as APCs in the suppression assay. CD4$^+$ MACS purified splenocytes and peritoneal lavage cells were stained with Alexa Fluor 700-conjugated anti-CD4 and PE-conjugated anti-CD25. They were then separated on CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells using a BD Biosciences FACSAria. CD4$^+$CD25$^-$ naive splenocytes were then stained with 5 μM CFSE for 5 min and washed into medium. A total of 5×10^4 CD4$^+$CD25$^-$ splenocytes (responders) were then cultured in 96-well round-bottom plates with 5×10^4 CD4$^+$CD25$^+$ cells and 2×10^5 APCs for 4 days, in the presence of 0.1 μg/ml anti-CD3.

Adoptive transfer

Splenocytes were prepared from DO11.10 mice and 5×10^6 cells injected i.p. into groups of BALB/c male mice. The following day mice were infected as before with either L3 or adult *B. malayi*, and a further day later 5×10^5 mature peptide OVA-loaded bone marrow-derived dendritic cells (BMDCs) were injected i.p.; mice were sacrificed 6 days later and peritoneal lavage taken.

BMDC culture

Bone marrow cells were obtained from femurs and tibias of BALB/c mice by flushing the bones with RPMI 1640 containing 10% FCS/1% penicillin-streptomycin/1% L-glutamine. Cells were centrifuged and plated out in nontissue culture petri dishes at a density of 5×10^5/ml in complete RPMI 1640, supplemented with 20 ng/ml GM-CSF. Fresh medium added 3, 6, and 8 days after the start of culture. After 10 days at 37°C, the cells were washed off into complete medium containing 5 ng/ml GM-CSF (PeproTech), 100 ng/ml LPS (Sigma-Aldrich), and 100 μg/ml peptide $OVA_{323-339}$ and incubated overnight. Cells were then washed into PBS before injection.

Macrophage suppression assay

A total of 1×10^5 adherent cells from peritoneal lavage were cultured with 1×10^4 EL-4 cells for 48 h, then pulsed overnight with 1 μCi/well [*methyl*-^{3}H]thymidine to measure proliferation.

Cytokine ELISA

Ab pairs used for cytokine ELISAs were: IL-4 (11B11/BVD6-24G2), IL-5 (TRFK5/TRFK4), IL-10 (JES5-2A5/SXC-1), IFN-γ (R4-6A2/XMG1.2), and IL-13 (38213/polyclonal rabbit anti-IL-13 (PeproTech)). Recombinant murine IL-4, IFN-γ, IL-10, IL-13, and IL-5 (Sigma-Aldrich) were used as cytokine standards. Biotin detection Abs were used with ExtrAvidin/alkaline phosphatase conjugate (Sigma-Aldrich) and Sigma-Aldrich Fast *p*-nitrophenyl phosphate substrate.

Statistical analyses

Statistical analyses were conducted using Prism 4.0b (Graphpad Software). Student's *t* test was used to compare two groups, and one-way ANOVA with a Bonferroni's multiple comparison test was used where groups of three or more were analyzed. When variances were significantly different between groups, data was log-transformed to normalize before analysis.

Results

B. malayi *i.p. infection induces Treg recruitment to the site of infection*

B. malayi L3 larvae, harvested from infected mosquitoes, were directly tested for their ability to induce a regulatory phenotype in BALB/c CD4$^+$ T cells in vivo. Seven days following i.p. injection of L3, peritoneal lavage cells were analyzed for expression of Treg markers by flow cytometry. Infected mice substantially increased the proportion (Fig. 1, *A*, *left*, and *B*) and number (Fig. 1*A*, *right*) of Foxp3$^+$ CD4$^+$ T cells, compared with controls receiving sham injections or heat-killed parasites. Sharp increases were seen in expression levels of CD25 (Fig. 1, *C* and *D*) and CTLA-4 (Fig. 1*E*, *left*, and *F*) within the CD4$^+$Foxp3$^+$ population. *B. malayi* infection also induced higher levels of CD103 (Fig. 1, *G* and *H*), a marker associated with an activated subset of Tregs, which is important for Treg retention at the suppressive site (37). Within the CD4$^+$Foxp3$^-$ (effector) population, there was a significant increase in CTLA-4 expression (Fig. 1*E*, *right*, and *F*), indicating cells of an anergic phenotype (27).

Adult parasites also induce Foxp3 expression

Adult worms represent the long-lived stage of the parasite in humans, and we were interested to assess their ability to influence T cell phenotypes in vivo. Because L3 parasites do not mature into adult stages in mice, adults harvested from jirds were surgically transplanted into the peritoneal cavity of BALB/c mice (38). Peritoneal T cell populations were analyzed 7 days following

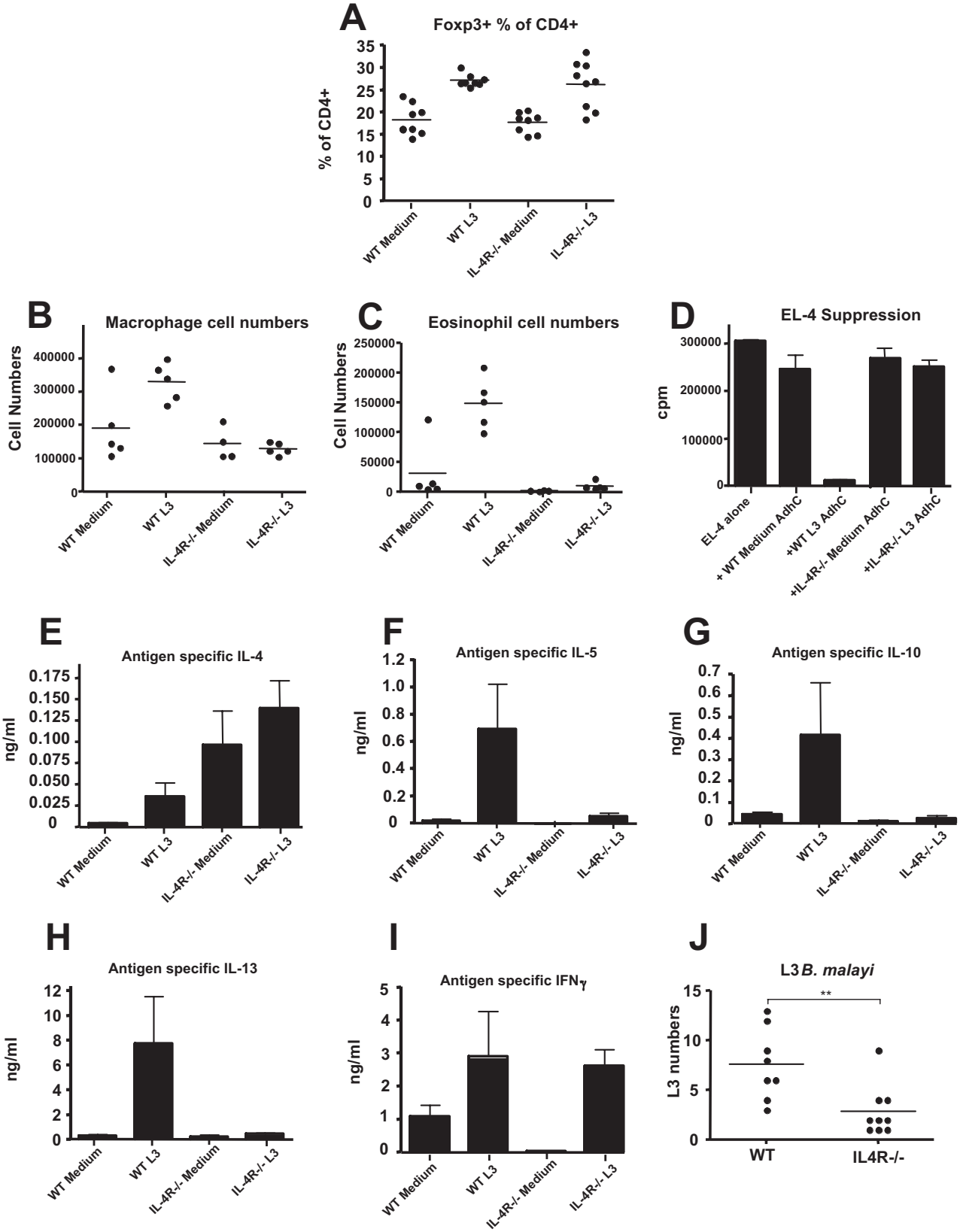

FIGURE 6. Expansion of $CD4^+Foxp3^+$ population is independent of IL-4R-mediated signaling. Wild-type BALB/c or IL-$4R^{-/-}$ mice were injected i.p. with either medium or 50 *B. malayi* L3. Seven days later peritoneal lavage (PL) and spleen cells were taken for analysis. *A*, Peritoneal lavage cells analyzed by flow cytometry for expression of Foxp3, expressed as a percentage of total $CD4^+$ lymphocytes. *B*, Macrophage cell numbers in peritoneal lavage from BALB/c or IL-$4R^{-/-}$ mice given medium or L3. *C*, Eosinophil numbers in peritoneal lavage from BALB/c or IL-$4R^{-/-}$ mice given medium or L3. Horizontal bar represents mean cell levels. *D*, Peritoneal lavage adherent cells (AdhC) from mice given medium or L3, cocultured with EL-4 cells, and assayed for proliferation by tritiated thymidine uptake. *E*, Splenocyte IL-4 recall responses restimulated with *B. malayi* adult homogenate. IL-5 (*F*), IL-10 (*G*), IL-13 (*H*), IFN-γ (*I*) in peritoneal lavage from BALB/c or IL-$4R^{-/-}$ mice given medium or L3. *J*, Recoveries of live L3 larvae from the peritoneal cavity. Data in *A* and *J* pooled from two repeat experiments, whereas other data shown are from a single representative experiment. **, $p < 0.01$.

implantation, in comparison to sham surgery controls. In close similarity to L3 larvae, adult parasites recruited Treg phenotype cells, which were $CD4^+Foxp3^+$ (Fig. 2, *A* and *B*), with up-regulated CD25 (Fig. 2, *C* and *D*) and CTLA-4 (Fig. 2*E*, *left*, and *F*), a subset of which was $CD103^+$ (Fig. 2, *G* and *H*). As with L3-infected mice, the $CD4^+Foxp3^-$ population showed a very marked increase in CTLA-4 expression (Fig. 2*E*, *right*, and *F*). Importantly, increased Foxp3 expression required the presence of live parasites (Fig. 2*I*), as was the case with L3 infections.

Dynamics of Treg recruitment in vivo

Similar experiments were conducted between 11 and 21 days postinfection, to appraise dynamic changes in the Treg population. In all cases, the total numbers of $Foxp3^+$ Tregs remained well in excess of control numbers, but as time progressed the $Foxp3^-$ subset expanded, reducing the overall frequency of Tregs within the $CD4^+$ population. Thus, the percentage of $CD4^+$ cells expressing Foxp3 returned to control levels 11–14 days postinfection with *B. malayi* L3 larvae (Fig. 3*A*) or adult worms (Fig. 3*B*). At all time points studied, live L3 larvae or adults and microfilariae could be recovered from the peritoneal cavity of infected mice.

A more enduring change was observed with respect to CD103 expression within the $CD4^+Foxp3^+$ population, which remained elevated at days 11–12 and days 20–21 (Fig. 3, *C* and *D*). These results indicate that following *B. malayi* infection, although in numbers alone the Treg population appears to subside with time, these cells retain higher CD103 expression for the duration of the experiment. This observation may reflect either stable CD103 expression on Tregs activated early in infection, or the dynamic recruitment of further cohorts of $CD103^+Foxp3^+$ Tregs by surviving parasites in vivo. In functional terms, heightened CD103 expression may favor retention of Tregs in the infected tissue, possibly aiding survival of parasites in the peritoneal cavity.

Tregs from peritoneal lavage are functionally suppressive

To ascertain whether peritoneal $CD4^+CD25^+$ T cells are functional Tregs in terms of suppressive capacity, cells were purified from the peritoneal lavage of L3 *B. malayi*-infected animals, and from naive spleens. These were then used in a suppression assay, with naive splenic $CD4^+CD25^-$ cells that had been CFSE-labeled as responder cells, and were stimulated with APCs and anti-CD3. As can be seen in Fig. 4, infected peritoneal lavage Tregs are equivalently suppressive as naive splenic Tregs in this assay, with similar proportions of labeled cells in each division in the presence of $CD4^+CD25^+$ cells from either source (represented graphically in Fig. 4*C*).

B. malayi *can induce Tregs in a bystander response*

Treg expansion following infection could be comprised of either or both "natural" (recognizing self) or "adaptive" populations, and the latter subset could be parasite-specific or reactive to third-party "bystander" epitopes. To test whether *B. malayi* infection was able to stimulate or convert peripheral T cells with nonparasite specificities, we analyzed an OVA-specific response during infection, by phenotyping DO11.10 OVA-specific TCR-transgenic cells in a transfer model. DO11.10 splenocytes were first administered to mice, which were then infected as in previous experiments, and immunized with peptide OVA-loaded mature BMDC by the i.p. route. The use of dendritic cell immunization avoided administration of adjuvants into the same compartment occupied by live parasites.

As can be seen in Fig. 5*A*, transferred DO11.10 cells did not proliferate to infection alone, demonstrating that there was no cross-reaction between OVA and *B. malayi*. With mature peptide OVA-loaded BMDC transfer, however, a robust expansion of DO11.10 T cells was seen within the peritoneal cavity (Fig. 5, *A* and *C*). When levels of Foxp3 within the transferred population of T cells were analyzed, it was seen that live L3 larvae or live adult *B. malayi* infection caused an increase in Foxp3 over levels seen in dead parasite immunization or medium controls (Fig. 5, *B* and *D*). Surprisingly, DO11.10 cell numbers were similar in animals with expanded Foxp3 expression, indicating that in vivo an antiproliferative effect may not be apparent. Because the DO11.10 TCR transgene was not on a RAG-deficient background, this experiment does not formally distinguish between de novo induction from naive peripheral cells, or expansion of the small population of $Foxp3^+$ natural Tregs derived from clones expressing endogenous TCR α-chain products. However, we can conclude that *B. malayi* infection results in accumulation of Treg phenotype cells within populations of T cells specific for third-party Ags at the infection site.

Ablation of IL-4 signaling does not prevent Treg induction

Previous studies have shown that the characteristic suppressive alternatively activated macrophage population that develops in *B. malayi* infection is dependent on IL-4 signaling (36). These suppressive macrophages have been shown to produce the Treg-inducing factor TGF-β (39). IL-4 has also been proposed to promote Foxp3 induction in naive T cells (40), and *B. malayi* larvae and adults elicit powerful IL-4 responses (35, 38). Therefore it could be hypothesized that in IL-4R-deficient ($IL\text{-}4R^{-/-}$) mice, *B. malayi* would be unable to induce expansion in Treg numbers. As can be seen in Fig. 6 *A*, however, Foxp3 induction in L3-infected $IL\text{-}4R^{-/-}$ mice was at least as strong as that observed in wild-type animals despite the absence of macrophage recruitment (Fig. 6*B*) and greatly reduced eosinophil numbers (Fig. 6*C*). Unlike wild-type controls, macrophages from infected $IL\text{-}4R^{-/-}$ mice were unable to suppress proliferation of a standard EL-4 cell line (Fig. 6*D*).

Splenocytes from the $IL\text{-}4R^{-/-}$ mice and controls were also restimulated with *B. malayi* adult Ag and supernatants taken for ELISA. As can be seen in Fig. 6, *E–I*, the Th2 cytokines IL-5 and IL-13 were lacking in the $IL\text{-}4R^{-/-}$ mice; however, higher levels of IL-4 were measured, presumably because no IL-4R-mediated uptake could occur. IL-10 was also absent from the response in the $IL\text{-}4R^{-/-}$ mice, indicating that the early IL-10 response is dependent on IL-4R-mediated signaling, as previously reported (41). Conversely, increased IFN-γ was observed after antigenic stimulation only in $IL\text{-}4R^{-/-}$ mice. Interestingly, a significant decrease in L3 larvae recovered from the peritoneal cavity was seen in mice of the $IL\text{-}4R^{-/-}$ genotype (Fig. 6*J*).

Discussion

The data presented in this study support the hypothesis that helminth parasites amplify the host Treg network, postulated to be a key factor in the ability of parasites to evade host immunity (18–20). In particular, we have shown that both the initial invasive stage (L3 larvae), and the long-lived adult parasite elicit a significant expansion in Treg numbers, and stimulate up-regulation of the activation-associated markers CD25, CTLA-4, and CD103. Enhancement of the $CD4^+$ regulatory T cell population has also been observed in several other helminth infections, including the rodent filaria *L. sigmodontis* (25), the gastrointestinal nematode *Heligmosomoides polygyrus* (42–44), the gut/muscle-dwelling nematode *Trichinella spiralis* (45), and the trematode parasite of man, *Schistosoma mansoni* (46, 47). It is likely that an immunological challenge of any nature will induce some element of Treg

activity, as a homeostatic guard against inappropriate responsiveness; however, in these infections we document a preferential expansion of the regulatory phenotype. Interestingly, the relative proportions of regulatory and effector cells may change over the course of infection (25, 43, 47), perhaps allowing the responder population in due course to overcome regulation and eliminate the parasites.

Beyond numbers alone, however, both regulatory and nonregulatory populations show important qualitative differences in phenotype. We report that Tregs following *B. malayi* infection show increased levels of CD103, a marker associated with stronger suppressive activity (48), and retention of Treg at the site of infection (37). Our data also indicate significant up-regulation of CD25 and CTLA-4 on the Foxp3$^+$ population, and as these are variously described as either transient activation markers or Treg markers, this response also indicates these Tregs may be activated. In addition, the nonregulatory (Foxp3$^-$) population also show higher CTLA-4 expression, as indeed observed in both *H. polygyrus* (43) and *L. sigmodontis* (26) infections. In the latter case anti-CTLA-4 treatment, in conjunction with anti-CD25 Ab, induces protective immunity (27). It is notable that a very similar profile has been observed in human filarial infections, in which CTLA-4 expression is elevated on the peripheral T cell population, and anti-CTLA-4 Ab treatment uplifts cytokine responsiveness of T cells from filarial patients (13, 49). CTLA-4 expression on CD4$^+$Foxp3$^-$ T cells has also been shown to be necessary for Treg induction through the TGF-β pathway (50)), and so a further possibility is that CD4$^+$CTLA-4$^+$Foxp3$^-$ T cells may be primed for Foxp3 induction later in the course of infection.

In high-intensity human filarial infections, there is strong evidence that immune down-regulation extends beyond parasite specificities, spreading out to affect responses to vaccine Ags in vivo (51–53) and T cell stimulation in vitro (54). A more subtle consequence of such regulatory spreading may be the dampening of allergies in helminth-infected individuals (42, 55, 56). In this context, it is important to know whether *B. malayi* can induce Treg activity beyond the parasite Ag-specific T cell population. Using an adoptive transfer model, we show that DO11.10 T cells specific for a bystander, non-cross-reactive Ag, OVA, show similar expansion of Foxp3 as seen with the endogenous polyclonal T cell population, even though the DO11.10 cells are strongly stimulated with mature BMDCs loaded with cognate peptide. The DO11.10 cells remained strongly Th1 polarized (intracellular cytokine staining showed means of 37–39% of DO11.10 cells expressed IFN-γ, with no significant differences between groups (data not shown)), indicating that the Foxp3 induction could take place even in the presence of a strong Th1 response. Our data do not, at this stage, distinguish whether the parasite is stimulating Foxp3 expression within newly activated naive cells, or simply recruiting a larger number of pre-existing natural Tregs. An important caveat of these results is the wild-type background of the DO11.10 cells used, where a small population of pre-existing Foxp3$^+$ T cells exist, due to low levels of endogenous TCR expression (57). Thus the induction of Foxp3 in the DO11.10 cells seen from this study may either be from expansion of a pre-existing Foxp3$^+$ subset or induction of Foxp3 in naive cells.

A well-documented result of *B. malayi* adult implantation is the recruitment of alternatively activated macrophages to the peritoneal cavity (36, 58). These have been shown to suppress proliferation of Ag-specific T cells through a contact-dependent mechanism (58). However, alternatively activated macrophages do not develop in filarial-infected IL-4R$^{-/-}$ mice (J. E. Allen, unpublished observation), or in STAT6-deficient animals infected with other helminths (59, 60). By showing that *B. malayi* induces Foxp3 within the T cell population even in the absence of IL-4R signaling, we have established that this process is independent of alternatively activated macrophages. In long-term *L. sigmodontis* infections, alternatively activated macrophages are found together with both Foxp3$^+$ Tregs and CTLA-4$^+$ anergic effector T cells (39), although the interdependency of these populations has not been established. In our short-term experimental system, CTLA-4 remained high on Foxp3$^-$ effector cells even in the IL-4R$^{-/-}$ setting, whereas CD4$^+$FoxP3$^+$ cell expression of CD25, CD103, and CTLA-4, were indistinguishable from wild-type (data not shown). Thus, we hypothesize that induction of Foxp3 may be a primary event in the infection process, leading subsequently to effector cell anergy and the generation of alternatively activated macrophages. The possibility that Tregs at the site of infection drive alternatively activated macrophage differentiation is supported by a recent study showing that in humans, alternatively activated macrophages are induced after culture in the presence of activated Tregs (61).

B. malayi L3 larvae, unlike those of *L. sigmodontis* (62), do not survive long enough in immunocompetent mice to develop into adult worms. As it is known that male mice harbor infections for longer than females (63), we compared Treg induction in mice of each gender. Interestingly, the more resistant female mice showed weaker Treg induction, although the difference did not reach statistical significance. Strain differences have also been observed in mouse tolerance of L3 *B. malayi* infection, with BALB/c mice being more susceptible than C57BL/6 (64). Further studies are therefore under way to compare Foxp3 induction in different mouse strains. In this respect, it is interesting to note the lower susceptibility of IL-4R$^{-/-}$ to L3 infection, with a significantly lower number of larvae recovered from the peritoneal cavity. This observation is at odds with a previous study (65), which showed significantly higher worm recovery from L3 *B. malayi*-infected IL-4R$^{-/-}$ mice. This discrepancy could be accounted for by differences in timing, as the previous study did not report a significant difference until 7 wk postinfection. Therefore the possibility remains of increased resistance in IL-4R$^{-/-}$ mice at early time points after infection, followed by a less productive response at later time points. The curtailed IL-10 in the IL-4R$^{-/-}$ genotype draws a parallel with the doubly deficient IL-4$^{-/-}$IL-10$^{-/-}$ construct that was found (unlike IL-4$^{-/-}$ single deficiency) to be resistant to *L. sigmodontis* (66). This outcome could reflect a more potent immune response, in the absence for example of suppressive macrophages, although the lack of an increased neutrophil recruitment or increased Th1 cytokines argues against this possibility. Alternatively, as filarial worms show accelerated growth in an IL-5-over-expressing host (67, 68), the reduction of IL-5 levels in the IL-4R$^{-/-}$ mouse may impact on parasite survival.

The fact that only live parasites can induce Foxp3 within the CD4$^+$ population is consistent with both field and laboratory data linking active infection to immune regulation. For example, in human studies, T cell responsiveness is regained after drug-induced parasite killing (11). Moreover, live L3 larvae have been shown to directly inhibit in vitro responses of human Langerhans cells (69) and peripheral blood lymphocytes (13). These results imply that immunomodulatory products of viable organisms are released within the infected host.

Future work will aim at defining the molecular pathways by which filarial parasites may target the Treg network. Some candidate molecules can already be identified (70). For example, *B. malayi* secretes a homolog of mammalian TGF-β, *Bm*-TGH-2, which has been demonstrated to signal through the mammalian TGF-β receptor (71), a proven pathway for Foxp3 induction (72). Currently, it is not possible to construct genetically deficient parasites to experimentally test the role of candidates such as TGH-2,

but by raising neutralizing Abs, the role of each molecule can be investigated in vivo.

A number of other possible candidates have emerged from recent definition of the *B. malayi* genome (73) and secretome (74), including retinoic-acid binding proteins and enzymes involved in cleaving arachidonic acid to PGE_2. As retinoic acid has been shown to synergize with TGF-β to induce Foxp3 while downregulating TH17 (75), and PGE_2 has been shown to induce Foxp3 in a manner similar to TGF-β (76), these candidates may be worthy of further investigation.

In conclusion, the results presented in this study indicate that, in the mouse at least, filarial parasites expand the frequency and activity of Foxp3-expressing T cells at the site of infection, and that these cells have functional in vitro suppressive capacity indicative of Tregs. As the mouse is not fully susceptible to the parasite, this result suggests that an initial immune subversion mechanism is accompanied by an effector cell response that can eventually outpace regulation and kill the filariae. If a similar dynamic relationship exists in human infections, with regulatory and effector mechanisms in contention, this may help explain the spectrum of immunological and pathological outcomes that ensue in filariasis. Furthermore, by understanding the factors that promote or impede regulatory mechanisms in helminth infection, intervention can aim to reverse this activity and generate an immunologic cure for disease.

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Disclosures

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