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At Homeostasis Filarial Infections Have Expanded Adaptive T Regulatory but Not Classical Th2 Cells

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Despite the well-documented immune suppression associated with human helminth infections, studies characterizing the immune response at the single-cell level are scanty. We used multiparameter flow cytometry to characterize the type of effector (Th1, Th2, and Th17) and regulatory (natural T regulatory cells [nTregs] and adaptive Treg cells [aTreg/type 1 regulatory cells (Tr1s)]) $CD4^+$ and $CD8^+$ T cells in filaria-infected (Fil^+) and -uninfected (Fil^-) individuals at homeostasis (in the absence of stimulation). Frequencies of $CD4^+$ lymphocytes spontaneously producing IL-4, IL-10, and IL-17A were significantly higher in Fil^+, as were those of $IL\text{-}10^+/IL\text{-}4^+$ double-producing $CD4^+$ cells. Interestingly, frequencies of Th17 and aTreg/Tr1s but not classical Th1 or Th2 cells were significantly increased in Fil^+ compared to Fil^- individuals. Although the frequency of nTreg was increased in Fil^+, IL-10 was overwhelmingly produced by $CD4^+CD25^-$ cells. Moreover, the concentration of IL-10 produced spontaneously in vitro strongly correlated with the integrated geometric mean fluorescence intensity of IL-10–producing aTreg/Tr1s in Fil^+. Together, these data show that at steady state, IL-10–producing aTreg/Tr1 as well as nTreg and effector Th17 $CD4^+$ cells are expanded in vivo in human filarial infections. Moreover, we have established baseline ex vivo frequencies of effector and Tregs at homeostasis at a population level. ***The Journal of Immunology*****, 2010, 184: 5375–5382.**

Among the major neglected tropical diseases, parasitic helminth infections affect more than one third of the world's population (1) and are a major source of morbidity and disability (2–4). Murine studies have clearly shown that proinflammatory innate responses, as well as mixed type 1/type 2 adaptive responses, predominate early in infection with tissue-invasive helminth parasites, such as schistosomes or filariae. Typically, however, at the time of patency (when egg laying occurs or microfilariae appear), type 1 responses are markedly downregulated, presumably allowing for expanded (or upregulated) type 2 responses (5–8). These data have found parallels in cross-sectional T cell analyses of patients with filarial or schistosome infection from acutely infected expatriates (9–12) as well as from chronically infected endemic populations (13–15). Techniques such as limiting dilution analysis (16) or ELISPOTS (17, 18) have demonstrated that there were lower frequencies of parasite-specific T cells capable of proliferating and producing IL-2 and IFN-γ in patients with patent lymphatic filariasis. In the overwhelming majority of the above-mentioned studies (and given the limitations of detection up to this point), it was difficult to detect measurable frequencies of cytokine-producing T cells in the absence of Ag or mitogen stimulation.

The IL-10–dominated regulatory environment induced in chronic helminth (and particularly in filarial) infections (19, 20) modulates the entire repertoire (Th1/Th2/Th17) of $CD4^+$ effector cell responses indiscriminately and skews the Ab responses away from production of IgE and toward production of IgG4 (21–23). Not only does this regulated response have major consequences for the quality and quantity of Th cell subsets, but it also modulates quite dramatically the response to both bystander Ags and allergens (24–26). Although termed a modified Th2 response by some (25, 27–30), in large part because of the lack of Th2-associated pathology, this moniker is most appropriate for tissue responses in easily polarized inbred animal model systems rather than for systemic responses to helminth infection in humans (18, 26, 31–33) in which mixed and/or regulatory cytokine responses predominate. Although the focus of this immunomodulation during helminth infections has been on IL-10, there also appear to be significant contributions from TGF-β, CTLA-4, the PD1/PDL axis, and natural T regulatory cells (nTregs) (34–38).

The overwhelming majority of studies characterizing the immune response during tissue-invasive helminth infection in humans has measured cytokines in culture supernatants or in serum/plasma of infected individuals or assessed mRNA expression (39). More than a decade ago, using three-color intracellular cytokine staining, it was demonstrated that the frequency of $CD4^+CD27^-$ cells producing either IL-4 or IL-5 alone was higher in filaria-infected individuals than in normal controls following stimulation with PMA and ionomycin (40). With the accumulating data suggesting that IL-10 and nTregs play important roles in the immune modulation seen in chronic helminthiasis (35, 37, 40), Mitre and colleagues (41) investigated the source of IL-10 in expatriate patients infected with

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Abbreviations used in this paper: aTreg, adaptive T regulatory cells; CI, confidence interval; Fil^+, filaria-infected; Fil^-, filaria-uninfected; GMFo, geometric mean frequency; GM, geometric mean; Hb, hemoglobin; iGMFI, integrated geometric mean fluorescence intensity; Mp, *Mansonella perstans*; NS, not significant; nTreg, natural T regulatory cell; Tr1, type 1 regulatory cell; Treg, regulatory T cell; Wb, *W. bancrofti*.

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the filarial parasites *Loa loa, Onchocerca volvulus*, or *Wuchereria bancrofti* and showed that the IL-10 was produced primarily by $CD4^+CD25^-$ T cells and not by $CD4^+CD25^+$ Tregs.

To address more comprehensively and under more physiologic conditions the effector and regulatory environment associated with filarial infections, in which peripheral cells are constantly interacting with blood-borne microfilariae and parasite Ags of all stages, we used multiparameter flow cytometry (surface and intracellular staining) to demonstrate that filarial infections are associated (at homeostasis) with in vivo expansion of Th17, adaptive T regulatory cells (aTreg)/Tr1s, and nTregs, but not Th1 or classical Th2 cells. We have, in addition, demonstrated that the major T cell source of IL-10 in vivo is a subset of $CD4^+$ cells ($CD4^+CD25^-FoxP3^-$) that, in the absence of stimulation, does not also express IL-4, IL-5, IL-17A, TNF-α, or IFN-γ. Coupling measurements of integrated geometric mean fluorescence intensity (iGMFI) with IL-10 protein expression ex vivo suggests that both increased frequencies and per-cell expression of IL-10 by aTreg/type 1 regulatory cells (Tr1s) is the hallmark of patent filarial infection.

Materials and Methods

Study population

The study was carried out in two Malian villages (Tienéguébougou and Bougoudiana) endemic for *W. bancrofti* and *Mansonella perstans* with prevalences of circulating bancroftian filarial antigenemia (TropBio, Queensland, Australia) of 56% and 36%, respectively (42), and prevalences of *M. perstans* microfilaremia of 62% and 63%. At the time of the study, stool examinations for ova and parasites revealed *Hymenolepsis nana* in four subjects and *Enterobius vermicularis* in one subject. No other helminth eggs or larvae were detected. Six weeks prior to the immunological assessments, all individuals received mebendazole and praziquantel to treat potential occult helminth infection. The study was approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board and the Ethical Committee of the University of Mali and is part of an ongoing larger clinical trial (NCT00471666). Informed consent was obtained from all participants. All study participants were treated with albendazole and ivermectin at the end of the study as part of the Malian National Program for the Elimination of Lymphatic Filariasis.

Thirty-five volunteers were enrolled in the study; 18 individuals were positive for circulating filarial Ag (filaria-infected individuals [Fil^+]) (TropBio ELISA) and/or detectable *W. bancrofti* or *M. perstans* microfilariae as detected by calibrated thick smear (total volume 60 μl) of peripheral blood samples drawn between 10 PM and 2 AM. The other 17 individuals had a negative test for circulating filarial Ag (filaria-uninfected [Fil^-]), and no microfilariae were seen. All subjects were from the same two villages with no differences in socioeconomic status, educational level, or environmental exposure between the two groups apart from their filarial infection status.

Whole blood culture

Heparinized blood samples collected from study subjects in the village at night were transported at ambient temperature to the laboratory in Bamako for processing. Whole blood (1 ml) was used for leukocyte counts and differentials using an automated cell counter (Beckman Coulter, Fullerton, CA), and another 1 ml was diluted with an equal volume of RPMI 1640 supplemented with penicillin/streptomycin (100 U/100 mg/ml), L-glutamine (2 mM), and HEPES (10 mM) (all from Invitrogen, Carlsbad, CA) and incubated at 37°C for 24 h in a CO_2 incubator with 5% CO_2. Brefeldin A (Sigma-Aldrich, St. Louis, MO) (20 μg/ml final concentration) was added to the samples after 12 h of incubation. Culture supernatants were collected and stored at −70°C until assayed for cytokines by Luminex (Austin, TX).

Cell preparation for flow cytometry

Cells were prepared for flow cytometry as described by Mitre et al. (41). Briefly, the pellet was washed once in cold PBS and lysed with 1× BD lysis buffer (BD Pharmingen, San Diego, CA), then washed again with cold PBS. The cells were then fixed with 4% paraformaldehyde (Fluka, Sigma-Aldrich) in PBS for 10 min, washed in PBS containing 0.1% BSA (Sigma-Aldrich), resuspended in PBS/10% DMSO (Thermo Fisher Scientific, Waltham, MA), and cryopreserved at −70°C until used for flow cytometry.

Flow cytometry

Cryopreserved fixed cells were thawed, washed twice with PBS containing 1% BSA, permeabilized for 15 min, and washed twice more in permeabilization buffer (eBioscience, San Diego, CA). Permeabilized cells were blocked for 15 min with PBS containing 1% BSA, 10% (v/v) FcR blocking reagent (Miltenyi Biotec, Auburn, CA), and 10 μg/ml human IgG (Sigma-Aldrich). For the effector panel, cells were stained with mouse anti-human CD3-allophycocyanin-Cy5.5, CD4-allophycocyanin-Cy7, IL-17A–PerCP-Cy5.5, TNF-α–Pacific blue, IFN-γ–PE-Cy7 (eBioscience), rat anti-human IL-4–FITC, rat anti-human IL-10–allophycocyanin (eBioscience), mouse anti-human CD8-Qdot605 (Invitrogen), and rat anti-human IL-5–PE (BD Pharmingen). For the regulatory panel, cells were stained with mouse anti-human CD3-allophycocyanin-Cy5.5, CD4-PE-Cy5, rat anti-human IL-10–Pacific blue (eBioscience), mouse anti-human CD25-allophycocyanin-Cy7, CD127-PE, CD152 (CTLA-4)-allophycocyanin, and Foxp3-Alexa Fluor 488 (BD Pharmingen). Samples were acquired on a BD LSRII (BD Pharmingen) and analyzed using FlowJo (Tree Star, Ashland, OR).

Fluorescence minus one controls were used to draw the gates for positive events. The frequency of multifunctional cells was determined using Boolean gating, and only categories with at least 10 events were considered positive. In addition to the frequency of cytokine-producing cells, the GMFI was determined to assess the quality of cytokine produced. The iGMFI was used to determine the amount of cytokine produced and was calculated by multiplying the frequency of cytokine-producing cells by the GMFI (43). CD4 cells were categorized into helper subsets based on the cytokines they produce. For instance, Th1 consisted of $CD4^+IFN\text{-}\gamma^+IL\text{-}10^+TNF\text{-}\alpha^+IL\text{-}17A^-IL\text{-}5^-IL\text{-}4^-$, $CD4^+IFN\text{-}\gamma^+IL\text{-}10^+TNF\text{-}\alpha^-IL\text{-}17A^-IL\text{-}5^-IL\text{-}4^-$, and $CD4^+IFN\text{-}\gamma^+IL\text{-}10^-TNF\text{-}\alpha^-IL\text{-}17A^-IL\text{-}5^-IL\text{-}4^-$; Th17 consisted of $CD4^+IL\text{-}17A^+TNF\text{-}\alpha^+IL\text{-}10^+IFN\text{-}\gamma^-IL\text{-}5^-IL\text{-}4^-$, $CD4^+IL\text{-}17A^+TNF\text{-}\alpha^+IL\text{-}10^-IFN\text{-}\gamma^-IL\text{-}5^-IL\text{-}4^-$, $CD4^+IL\text{-}17A^+IL\text{-}10^+TNF\text{-}\alpha^-IFN\text{-}\gamma^-IL\text{-}5^-IL\text{-}4^-$, and $CD4^+IL\text{-}17A^+TNF\text{-}\alpha^-IL\text{-}10^-IFN\text{-}\gamma^-IL\text{-}5^-IL\text{-}4^-$; TNF-α consisted of $CD4^+TNF\text{-}\alpha^+IL\text{-}10^+IFN\text{-}\gamma^-IL\text{-}17A^-IL\text{-}5^-IL\text{-}4^-$, and $CD4^+TNF\text{-}\alpha^+IL\text{-}10^-IFN\text{-}\gamma^-IL\text{-}17A^-IL\text{-}5^-IL\text{-}4^-$; Th2 consisted of $CD4^+IL\text{-}4^+IL\text{-}5^+IL\text{-}10^+IFN\text{-}\gamma^-TNF\text{-}\alpha^-IL\text{-}17A^-$, $CD4^+IL\text{-}4^+IL\text{-}5^+IL\text{-}10^-IFN\text{-}\gamma^-TNF\text{-}\alpha^-IL\text{-}17A^-$, $CD4^+IL\text{-}4^+IL\text{-}5^-IL\text{-}10^-IFN\text{-}\gamma^-TNF\text{-}\alpha^-IL\text{-}17A^-$, $CD4^+IL\text{-}5^+IL\text{-}4^-IL\text{-}10^-IFN\text{-}\gamma^-TNF\text{-}\alpha^-IL\text{-}17A^-$; aTreg/T1 consisted of $CD4^+IL\text{-}10^+IL\text{-}4^-IL\text{-}5^-IFN\text{-}\gamma^-TNF\text{-}\alpha^-IL\text{-}17A^-Foxp3^-$ and nTregs were $CD4^+CD25^+Foxp3^+CD127^-$.

Absolute numbers of cells in various subpopulations were calculated based on the frequency of the various T cell populations obtained from flow cytometry and the total lymphocyte count obtained from the complete blood count and differential.

Statistical analyses

The Mann-Whitney and Wilcoxon signed-rank tests were used for paired and unpaired analyses, respectively; Spearman test was used for correlations, and p values were corrected for multiple comparisons using the Holm's correction. All analyses were performed using Prism version 5.0 (GraphPad, San Diego, CA).

Results

Study population

The study was carried out in two Malian villages where *W. bancrofti* and *M. perstans* are coendemic. Thirty-five individuals (17 Fil^- and 18 Fil^+) were enrolled in the study. Apart from the differences in their filarial status, there were no differences between the two groups in terms of demographics or measured hematologic parameters (Table I). All Fil^+ subjects had detectable microfilariae (*M. perstans*, $n = 12$, and/or *W. bancrofti*, $n = 9$); all those with *W. bancrofti* microfilariae were also positive for circulating *W. bancrofti* filarial Ag (42). The Fil^- subjects were repeatedly negative for microfilariae and circulating filarial Ag.

Frequency of $CD4^+$ cytokine-producing cells

The frequency of $CD4^+$ cytokine-producing cells in the absence of stimulation was determined by whole blood flow cytometry and compared between Fil^- and Fil^+ subjects (Fig. 1). The frequencies of $CD4^+$ cells producing IL-4, IL-10, and IL-17A were significantly higher in Fil^+ compared with Fil^- subjects ($p = 0.02$, $p < 0.0001$, and $p = 0.02$, respectively). Not only were the frequencies of cells higher in Fil^+ than in Fil^- subjects, but the absolute

Table I. *Study population*

	Filarial Status		
	Negative (n = 17)	Positive (n = 18)	p Value
Gender (male/female)	14/3	14/4	NS
Age in yr (GM range)	14.4 (11–17)	14.1 (11–18)	NS
No. positive for Wb circulating Ag	0	9	N/A
GM Ag level ub U/ml (95% CI)	0	320.4 (56.7–1810)	
No. positive for Mp microfilaremia	0	12	N/A
GM mf/ml (95% CI)	0	33 (18–60)	
GM WBC at 103/μl (range)	6.8 (3.5–10.5)	7.5 (4.9–11.8)	NS
GM Hb as g/dl (range)	11.7 (9.4–13.5)	12.18 (9.9–15.5)	NS

Hb, hemoglobin; Mp, *Mansonella perstans*; NS, not significant; Wb, *W. bancrofti*.

numbers of IL-4–producing $CD4^+$ cells [geometric mean (GM) (range), 126 (0–1113) versus 82 (6–536); p = 0.02), IL-10–producing CD4 cells [1514 (353–6818) versus 342 (24–1200); p = 0.0006], and IL-17A–producing CD4 cells [875 (174–2945) versus 371 (10–2797); p = 0.04] were significantly higher in Fil^+ compared with Fil^- subjects (Table II). Of interest, there were no differences in either frequencies or absolute numbers of $CD4^+$ cells producing IFN-γ, TNF-α, or IL-5 between the two groups.

Frequency of $CD4^+$ cells producing multiple cytokines

The ability to categorize $CD4^+$ cell populations into subsets has provided a paradigm that has implicated helminth infection (and allergens) in Th2 expansion. Thus, we used multiparameter flow cytometry ex vivo in whole blood to examine the nature of T cell subset expansion in filarial (blood-borne systemic helminth) infection in its steady state. We found that the frequency of $CD4^+$ T cells producing only IL-4, IL-10, or IL-17A (single producers) were significantly expanded in Fil^+ compared with Fil^- (p = 0.0025, $p < 0.0001$, and p = 0.0064, respectively). In addition, the frequencies of $CD4^+/IL10^+/IL\text{-}4^+$ cells (so-called double producers [GM frequency (GMFo): 5.78×10^{-4} versus 1.49×10^{-5}; p = 0.006]) were also increased (Fig. 2). There were no differences in the levels of other single or multiple cytokine-producing CD4 cells between the two groups (Fig. 2). As can also be seen in Fig. 2, cytokine-producing $CD4^+$ T cells, when grouped into Th1, Th2, Th17, TNF-α, and aTreg/Tr1s based on patterns of cytokine production, the proportion of $CD4^+$ cells that were Th17 or aTreg/Tr1 $CD4^+$ cells (Fig. 2) was significantly higher in Fil^+ compared with Fil^- subjects (9% versus 12%; p = 0.002 and 8% versus 24%; $p < 0.0001$, respectively). Surprisingly, filarial infection was not associated with an expansion of total Th2 cells, nor did the absence of filarial infection (Fil^-) cause a relative expansion of Th1 cells.

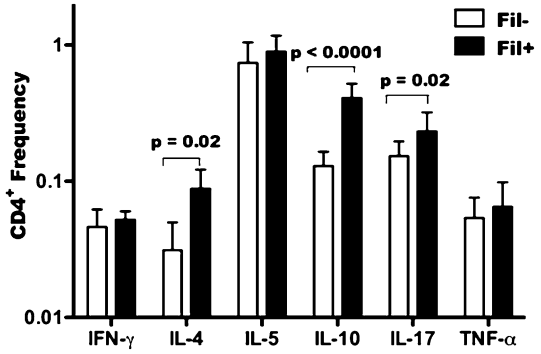

FIGURE 1. Filarial infection is associated with higher frequency of $CD3^+CD4^+$ cells producing IL-4, IL-17A, and IL-10. Frequencies of cytokine-producing $CD3^+CD4^+$ cells are shown in Fil^- (white bars) and Fil^+ (black bars) individuals with bars representing the GM and 95% confidence interval (CI) of the $CD4^+$ cells producing the cytokines listed on the x-axis.

Frequency of nTregs

Several studies have reported upregulation of Foxp3 expression during filarial infection (34, 44). Thus, to determine the frequency of nTregs in filaria-infected individuals, whole blood was stained using a panel of mAbs chosen to identify Treg subsets. When the frequency of nTregs ($CD3^+CD4^+CD25^+Foxp3^+CD127^-$) was compared between the Fil^+ and Fil^- groups (Fig. 3), the frequency of nTreg was significantly higher in Fil^+ compared with Fil^- (GMFo: 0.27 versus 0.036; $p < 0.0001$) (Fig. 3*A*). These nTregs were further subtyped based on CTLA-4 expression (CD152) and/or production of IL-10 (Fig. 3*B*). There were no differences in the frequencies of nTreg coexpressing CTLA-4 and IL-10 between the Fil^+ and Fil^- groups, whereas the frequencies of nTregs producing IL-10 alone (not expressing CTLA-4) were significantly higher in Fil^+ compared with Fil^- (GMFo: 2.2×10^{-2} versus 2.5×10^{-4}; $p < 0.0001$) (Fig. 3*B*).

$CD4^+$ sources of IL-10

The regulatory cytokine IL-10 has been shown to be significantly elevated in filarial infection, and many studies have reported its association with immune hyporesponsiveness during filarial infection (reviewed in Ref. 45). As shown in Figs. 1, 2, and Table II, we found that the frequency of all $CD4^+$ cells producing IL-10 and the frequency of $CD4^+$ cells producing IL-10 only (and not co-expressing any other cytokine assessed) were significantly increased in the Fil^+ compared with the Fil^- group. Therefore, we compared the frequencies of various subpopulations of $CD4^+$ T cells producing IL-10 in Fil^+ and Fil^- groups. Within an individual's cell populations, the frequency of $CD4^+CD25^{-/low}$ cells producing IL-10 was significantly higher than the frequency of $CD4^+CD25^{+/high}$ producing IL-10 (p = 0.0015 for Fil^+ and p = 0.0002 for Fil^- individuals) (Fig. 4*A*). When the proportion of the frequencies of IL-10–producing effector ($CD4^+CD25^{-/low}$) and regulatory ($CD4^+CD25^{+/high}$) cells was compared between the two groups, not surprisingly, Fil^+ patients had greater numbers of both IL-10–producing effector and regulatory cells as compared with Fil^- subjects (GMFo: 4.6×10^{-3} versus 4.3×10^{-4} and 2.1×10^{-2} versus 2.8×10^{-3}; p = 0.0010 and p = 0.0023, respectively) (Fig. 4*A*).

To assess the per-cell production of IL-10 in hopes of identifying the major contributing sources to the IL-10 pool, we calculated the iGMFI, a parameter that encompasses the number (frequency of cytokine-producing cells) and the quantity of the cytokine

Table II. *Absolute numbers of lymphocytes, CD4$^+$ lymphocytes, and CD4$^+$ lymphocytes producing IFN-γ, IL-4, IL-5, IL-10, IL-17A, and TNF-α*

	Filaria$^-$ [GM (Range)]	Filaria$^+$[GM (Range)]	*p* Value[a]
Lymphocytes	1.9×10^6(1.29×10^6–2.6×10^6)	1.79×10^6(8.59×10^5–3.89×10^6)	>0.05
CD4$^+$ cells	264,700 (9,280–1.29×10^6)	444,535 (151,200–915,200)	>0.05
CD4$^+$IFN-γ$^+$	45 (0–588)	31 (0–556)	>0.05
CD4$^+$IL-4$^+$	82 (6–536)	126 (0–1113)	**0.02**
CD4$^+$IL-5$^+$	2,556 (59–78,412)	3,970 (847–14,460)	>0.05
CD4$^+$IL-10$^+$	342 (24–1200)	1514 (353–6818)	**0.0006**
CD4$^+$IL-17A$^+$	371 (10–2797)	875 (174–2945)	**0.04**
CD4$^+$TNF-α$^+$	226 (9–1397)	443 (70–6955)	>0.05

[a]Numbers in boldface indicate significant *p* values.

produced (GMFI) (43). In Fil$^+$ subjects aTreg/Tr1 and Th2 cells produced most of the IL-10, whereas in Fil$^-$ individuals, IL-10 was produced almost exclusively by aTreg/Tr1s (Fig. 4*B*), albeit at a relatively low frequency.

The relationship between per-cell production (based on iGMFI) of IL-10 and the IL-10 protein actually produced spontaneously was next examined (Fig. 5) in each individual for each of the relevant CD4$^+$ cell subsets. As can be seen, for Fil$^-$ subjects only, the iGMFI of nTregs was significantly correlated with spontaneous ex vivo IL-10 production ($r = 0.82$; $p = 0.006$) (Fig. 5*C*), whereas for Fil$^+$, both CD4$^+$CD25$^{-/low}$ and aTreg/Tr1s were significantly correlated with IL-10 production ($r = 0.68$, $p = 0.002$; and $r = 0.87$, $p < 0.0001$, respectively) (Fig. 5*B*, 5*D*).

Frequency of CD8-producing cytokines

There were no differences in frequencies of cytokine-producing CD8 cells (either singly or in combination) between Fil$^+$ and Fil$^-$ subjects, with the exception of IL-10 (Fig. 6). Indeed, both the total number of CD8$^+$IL-10$^+$ (Fig. 6*A*) cells and those CD8$^+$ cells producing IL-10 (Fig. 6*B*) only were significantly higher in Fil$^+$ compared with Fil$^-$ individuals (GMFo: 0.247 versus 0.079; $p = 0.002$ and $p < 0.0001$, respectively).

Discussion

Based largely on data from murine studies, parasitic helminths are generally felt to induce type 2 (Th2) CD4$^+$ responses in their host (46). Unlike some geohelminths, tissue-invasive helminths (such as filarial parasites) are long lived, produce subclinical infection in most patients, and induce a state of parasite-specific T cell hyporesponsiveness or anergy (47). Although immune responses to lymphatic filarial parasites may be biased toward Th2 (30) as assessed in vitro in response to stimulation, several studies have reported no difference in the Th2 responses between Fil$^-$ and Fil$^+$ subjects living in endemic areas (10, 11, 47). In fact, immune responses induced by lymphatic filariasis in humans are characterized by IL-10 and other regulatory processes that downregulate immune responses not only to filarial Ags but to other Ags as well (17, 26, 32). In addition, these regulatory molecules modulate both Th1 and Th2 responses (34).

The majority of studies investigating the immune response against helminth infections have used immunoassays to assess cytokine production in culture supernatants, and fewer studies have investigated the immune responses induced by helminth parasites at a single-cell level. Using a combination of only a few Abs, Elson et al. (40) found that CD4$^+$ cells from helminth-infected individuals produced measurable IL-4 and IL-5 either alone or in combination in response to PMA/ionomycin, and they also found that CD4$^+$ production of IL-4 or IL-5 and IFN-γ was mutually exclusive. Using similar technology, de Boer et al. (48) found that in response to stimulation with *Brugia malayi* adult worm Ag, the majority of T cells from four individuals from a filaria-endemic region of the world (and presumed filarial exposure) produced IL-4 and IL-13 and that coexpression of these Th2 cytokines with IFN-γ was rare. Thus, a comprehensive analysis of the immune response induced by helminth infection at a single-cell level has not been done.

In the current study, using measurement of six cytokines simultaneously in unstimulated cells directly from Fil$^+$ or Fil$^-$ subjects, we found that the frequency of CD4$^+$ cells producing either IL-4 alone or both IL-4 and IL-10 was significantly increased in Fil$^+$ patients; however, there were no differences in the frequency (or absolute numbers) of classical Th2 and Th1 cells between

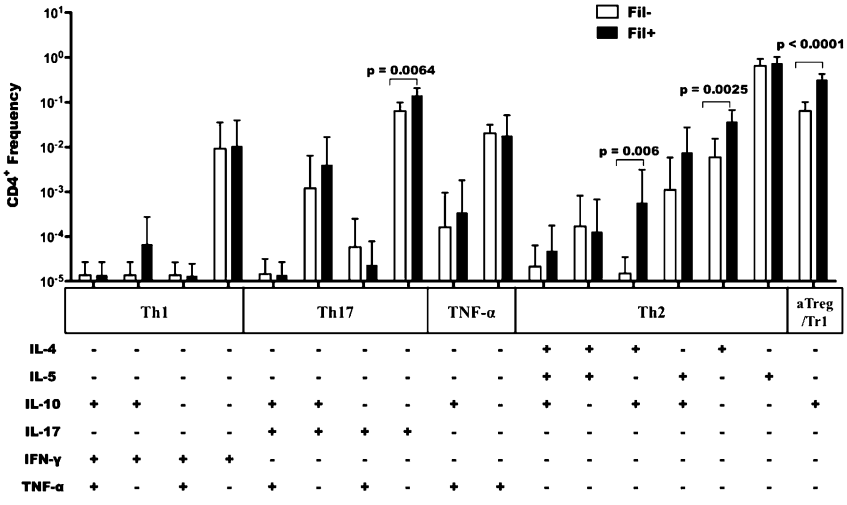

FIGURE 2. Filarial infection is associated with expansion of CD4$^+$ cell populations producing IL-10 or IL-17A alone and CD4$^+$ cells producing both IL-10 and IL-4. The frequency of multiple cytokine-producing CD4 cells was determined using Boolean gating. Bars represent the GM and 95% CI of CD4$^+$ cells producing the combination of cytokines listed below each set of bars in Fil$^-$ (white) and Fil$^+$ (black) populations.

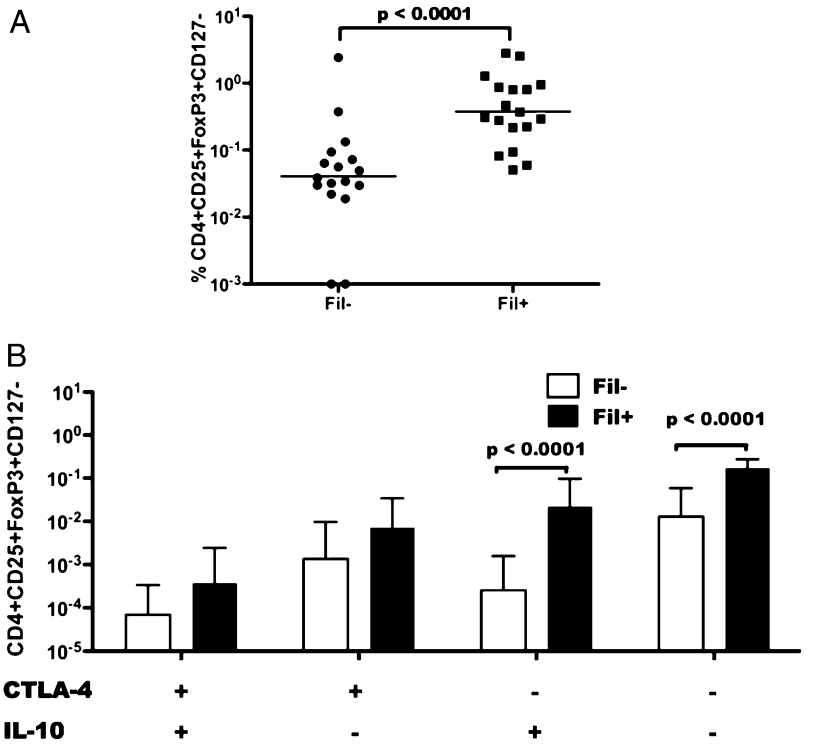

FIGURE 3. Filarial infection is associated with expansion of nTregs (*A*) and those expressing CTLA-4 or IL-10 (*B*). Each dot in *A* represents an individual's frequency of $CD3^+CD4^+CD25^+Foxp3^+CD127^-$ cells; the bar represents the GM for each group. In *B*, the bars represent the GM with the 95% CI of the frequencies of specific cells based on expression of markers listed below each bar.

filarial-infected and -uninfected subjects. The finding of higher frequencies of IL-4–producing $CD4^+$ cells corroborates data from a previous study in which the frequency of $CD4^+IL\text{-}4^+IL\text{-}13^+$ cells producing IL-4 and IL-13 was higher in Fil^+ subjects in Indonesia (48). Despite the higher frequency of IL-4–producing $CD4^+$ cells in Fil^+, there was no difference in the aggregate frequency of all potential Th2 cells between the two groups in the current study. Nevertheless, given the relatively large numbers of IL-5–producing cells in both groups, our results fail to support (in the steady state and in the absence of T cell stimulation) the narrowly defined modified Th2, one that is characterized by both IL-4 and IL-10 cytokine responses but low to absent expression of IL-5 (33).

Although we did not find any difference in the levels of effector Th2 cells between Fil^+ and Fil^- subjects, the frequency of IL-17A–producing cells was significantly increased in Fil^+. These cells have been felt to play an important effector role against extracellular bacteria and in the induction of autoimmunity (49). Although the involvement of Th17 cells in parasitic infections is still largely unstudied, they have been implicated in mediating some of the pathologic consequences of both schistosomiasis (50, 51) and lymphatic filariasis (52).

Filarial infection has long been associated with production of IL-10 and, to a lesser extent, TGF-β (34, 36). The major regulatory cytokine IL-10 can be expressed by many cell types, but in a study in filaria-infected expatriates/travelers, IL-10 was shown to be produced primarily by $CD4^+$ T cells that were also negative for CD25 (41). In the current study, we have more extensively characterized the source of IL-10 and confirmed that $CD4^+CD25^-$ (and not $CD4^+CD25^+FoxP3^+$) cells are the major producer of IL-10 at steady state. Moreover, these IL-10–producing cells were primarily aTreg/Tr1s ($CD4^+CD25^{-/low}$ that do not coexpress IL-4, IL-5, IL-17A, TNF-α, or IFN-γ). In addition, we found that the amount of IL-10 produced by these aTreg/Tr1s cells strongly correlated with IL-10 protein production as measured concurrently

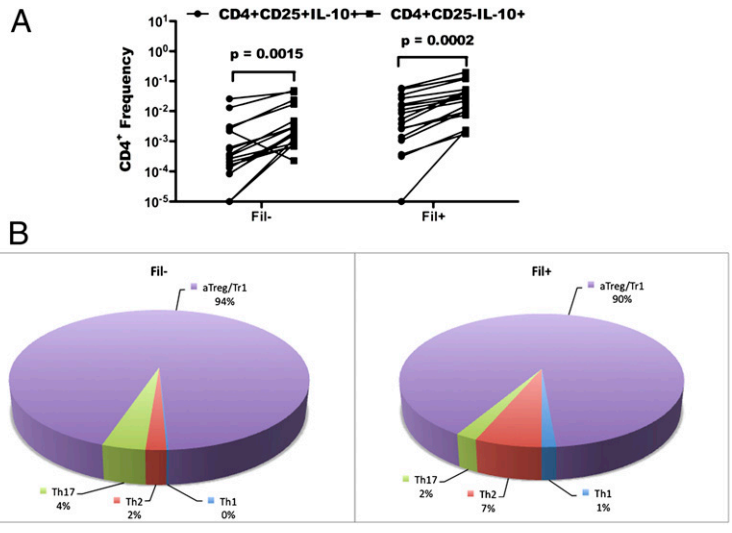

FIGURE 4. $CD4^+CD25^{-/low}$ cells are the main source of IL-10 in filarial infection (*A*), and aTreg/Tr1s are the main source of IL-10 (*B*). *A*, Frequencies of $CD4^+CD25^-IL\text{-}10^+$ (circles) and $CD4^+CD25^+IL\text{-}10^+$ (squares) cells were calculated for each individual and plotted as individual lines for both Fil^+ and Fil^- subjects. *B*, The iGMFI of IL-10–producing Th1, Th2, Th17, and aTreg/Tr1 cells was calculated, and the GM of each cell population was used to plot the pie chart. Each portion of the pie represents the relative contribution of each cell population to the total IL-10 pool in each group.

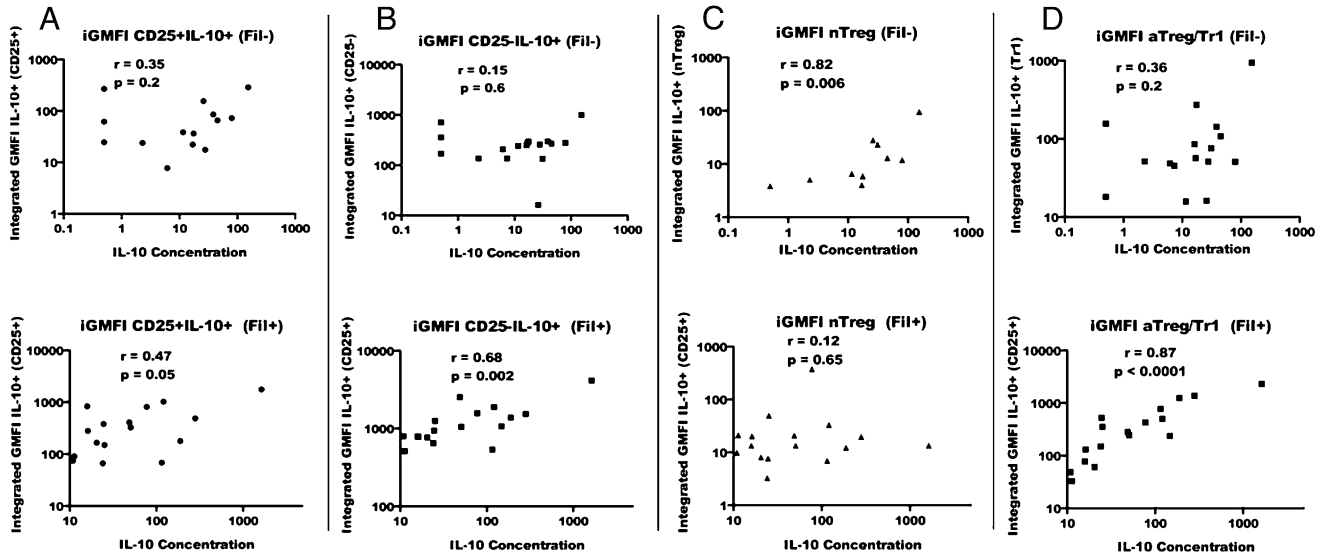

FIGURE 5. In patent filarial infection, the levels of spontaneously produced IL-10 correlate with the iGMFI of IL-10–producing $CD4^+CD25^-$ and aTreg/Tr1s. The iGMFI for IL-10–producing nTreg (*A*), single IL-10–producing $CD4^+$ (aTreg/Tr1) (*B*), $CD4^+CD25^-IL\text{-}10^+$ (*C*), and $CD4^+CD25^+IL\text{-}10^+$ cells (*D*) for Fil^- (*top row*) and Fil^+ (*bottom row*) individuals as a function of IL-10 produced spontaneously. Values of *p* and *r* derive from Spearman rank correlation analyses.

in parallel culture supernatants (Fig. 5). Although we did not assess the expression of CTLA-4 on the $CD4^+CD25^{-/low}$ cells, in a mouse model of *Schistosoma mansoni,* it has been shown that CTLA-4 expressed by $CD4^+CD25^-$ regulates the effector Th2 response rather than contact- mediated suppression by $CD4^+$ $CD25^+$ cells (53).

We also found that filarial infection was associated with expansion of nTregs. Although several studies had previously reported an increased expression of Foxp3 mRNA in filaria-infected patients (34), this lends support to the notion that nTreg numbers are expanded in filarial infections. These data find parallels in murine studies with *Litomosoides sigmodontis* in which

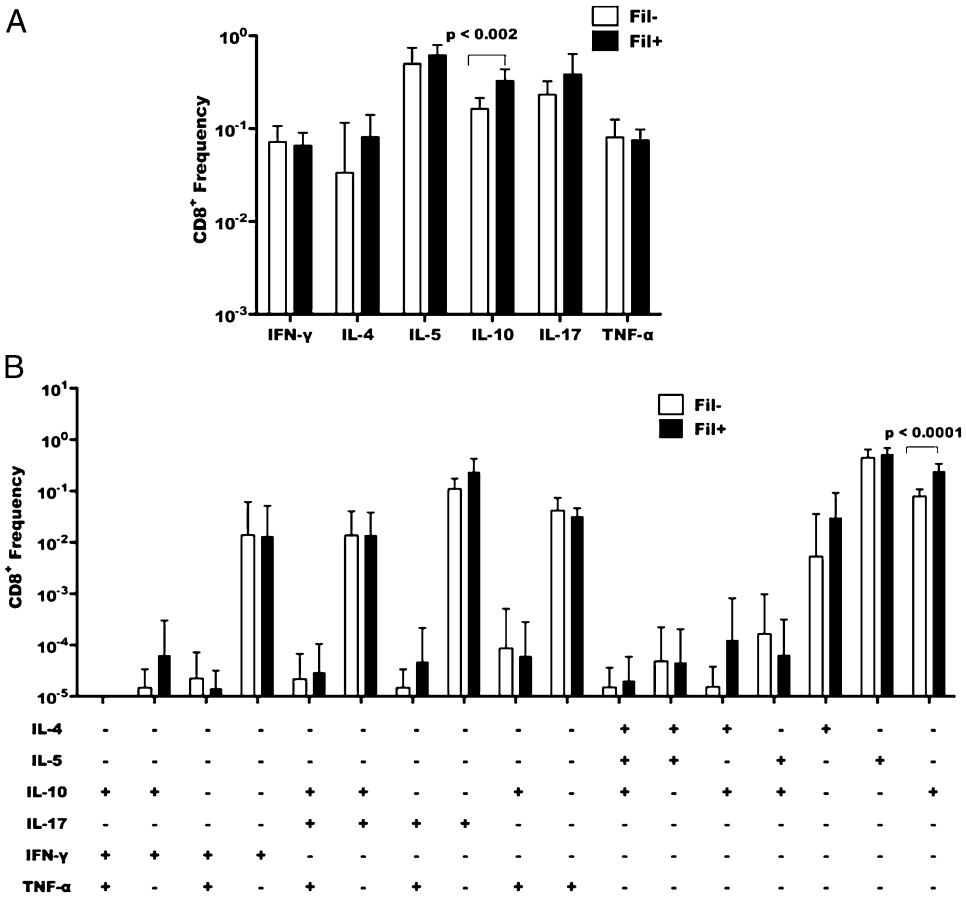

FIGURE 6. CD3/$CD8^+$ cells also contribute to IL-10–producing populations in filaria-infected patients as total $CD8^+$ T cells (*A*) or $CD8^+$ T cells producing only IL-10 (*B*). The frequency of single cytokine-producing $CD8^+$ cells is shown in *A* with the bar representing the GM plus 95% CI for both Fil^- (white) and Fil^+ (black) individuals for each cytokine listed below the bar. *B* shows the frequency of multiple cytokine-producing cells as determined by Boolean gating.

infections are associated with increased frequency of nTregs that regulate effector responses against the parasite (38).

Our results suggest that in humans, filarial parasites induce a regulatory environment with multiple components that likely contribute to downregulation of immune responses both to specific filarial Ags and to bystander Ags. This IL-10–dominated regulatory environment seen ex vivo suggests that chronicity (or long-standing exposure to filarial Ags) is the driving force that these long-lived tissue-invasive and blood-borne parasites use to inhibit (or modulate) effector responses to themselves, other nonfilarial pathogens, and bystander Ags.

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Disclosures

The authors have no financial conflicts of interest.

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