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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 stasis at a population level.

In vivo in human filarial infections. Moreover, we have established baseline ex vivo frequencies of effector and Tregs at homeostasis. 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: 000–000.

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 skew 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 T 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+CD25+CD127− 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...
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 (Tien-nguebou 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* microfilariaemia of 62% and 63%. At the time of the study, stool examinations for ova and parasites revealed *Hymenolepis nana* in four subjects and *Enteroobius 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 any undetected helminth infections. 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 categorized into helper subsets based on the cytokines they secreted. For instance, Th1 consisted of CD4+IFN-γ+IL-10+TNF-α+IL-17A-IL-5-IL-4-, CD4+IFN-γ-IL-10+TNF-α-IL-17A-IL-5-IL-4-, and CD4+IFN-γ-IL-10+TNF-α-IL-17A-IL-5-IL-4-; Th17 consisted of CD4+IL-17A+TNF-α-IL-10+IFN-γ-IL-5-IL-4-, CD4+IL-17A+IL-10-IFN-γ-IL-5-IL-4-, CD4+IL-17A+IL-10-IFN-γ-IL-17A-IL-5-IL-4-, and CD4+IL-17A+IL-10-IFN-γ-IL-17A-IL-5-IL-4-, Th2 cells consisted of CD4+IL-4-IL-5-IL-10-IFN-γ-IL-17A-, CD4+IL-4-IL-5-IL-10-IFN-γ-IL-17A-, CD4+IL-4-IL-5-IL-10-IFN-γ-IL-17A-, and CD4+IL-4-IL-5-IL-10-IFN-γ-IL-17A-; aTreg/T1 consisted of CD4+IL-10-IL-4-IL-5-IFN-γ-IFN-α-IL-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 (42). The Fil+, nFil−, and Fil+ subjects (Fig. 1). The frequencies...
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 subsets expansion in filarial (blood-borne systemic helminth) infection in its steady state. We found that the frequency of CD4+ cells producing the cytokines listed in 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.

**Frequency of nTregs**

Several studies have reported upregulation of Foxp3 expression during filarial infection (34, 44). Thus, to determine the frequency of nTregs in filiaria-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. 3A). These nTregs were further subtyped based on CTLA-4 expression (CD152) and/or production of IL-10 (Fig. 3B). 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. 3B).

**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 coexpressing any other cytokine assessed) were significantly in 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.
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. 4B), 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. 5C), whereas for Fil+, both CD4+CD25− low and aTreg/Tr1s were significantly correlated with IL-10 production \((r = 0.68, p = 0.002; \text{and } r = 0.87, p < 0.0001, \text{respectively})\) (Fig. 5B, 5D).

### 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. 6A) cells and those CD8+ cells producing IL-10 (Fig. 6B) only were significantly higher in Fil+ compared with Fil− individuals (GMF O: 0.247 versus 0.079; \(p = 0.002\) and \(p < 0.0001, \text{respectively}\)).

### 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 helmint 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...
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-4+IL-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+CD25FoxP3+) cells are the major producer of IL-10 at steady state. Moreover, these IL-10–producing cells were primarily αTreg/Tr1s (CD4+CD25+FoxP3+CD127 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 αTreg/Tr1s cells strongly correlated with IL-10 protein production as measured concurrently.

![FIGURE 3](http://www.jimmunol.org/) 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.

![FIGURE 4](http://www.jimmunol.org/) CD4+CD25−/low cells are the main source of IL-10 in filarial infection (A), and αTreg/Tr1s are the main source of IL-10 (B). A, Frequencies of CD4+CD25−IL-10+ (circles) and CD4+CD25+IL-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 αTreg/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.
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⁺ cells 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...
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
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References


