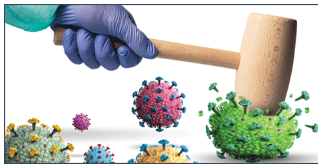

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Regulatory Networks Induced by Live Parasites Impair Both Th1 and Th2 Pathways in Patent Lymphatic Filariasis: Implications for Parasite Persistence[1]

Subash Babu,[2]* Carla P. Blauvelt,* V. Kumaraswami,† and Thomas B. Nutman*

Patent lymphatic filariasis is characterized by a profound down-regulation of immune responses with both parasite Ag-specific tolerance and bystander suppression. Although this down-regulation is confined to the Th1 arm of the immune system in response to parasite Ag, we hypothesized a more generalized suppression in response to live parasites. Indeed, when we examined the cytokine profile of a cohort of filaria-infected ($n = 10$) and uninfected ($n = 10$) individuals in response to live infective-stage larvae or microfilariae of *Brugia malayi*, we found significant impairment of both Th1 and Th2 cytokines characterized by diminished production of IFN-γ, TNF-α, IL-4, IL-5, and IL-10 in infected patients. The molecular basis of this impaired Th1/Th2 response was examined, and we identified three major networks of immunoregulation and tolerance. First, impaired induction of T-bet and *GATA-3* mRNA underlies the Th1/Th2 deficiency in infected individuals. Second, regulatory networks, as evidenced by significantly increased expression of *Foxp3* (natural regulatory T cell marker) and regulatory effectors such as TGF-β, CTLA-4, PD-1, ICOS, and indoleamine 2,3-dioxygenase play an important role in immunosuppression. Third, the compromise of effector T cell function is mediated by the enhanced induction of anergy-inducing factors *cbl-b*, *c-cbl* (*cbl* is abbreviation for Casitas B lymphoma), *Itch*, and *Nedd4*. Indeed, blocking CTLA-4 or neutralizing TGF-β restored the ability to mount Th1/Th2 responses to live parasites and reversed the induction of anergy-inducing factors. Hence, we conclude that a profound impairment of live parasite-specific Th1 and Th2 immune responses occurs in lymphatic filariasis that is governed at the transcriptional level by a complex interplay of inhibitory mediators. *The Journal of Immunology,* 2006, 176: 3248–3256.

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From an immunologic perspective, the most intriguing and most common clinical manifestation of lymphatic filariasis is the asymptomatic (or subclinical) condition associated with the presence of circulating microfilariae (Mf)[3] and/or parasite Ag. In contrast, a large number of individuals remain free of infection in many filaria-endemic regions despite longstanding (or lifelong) exposure to the infective stage of the parasites (1). Based on studies using crude, somatic parasite extracts, patent filarial infection has been associated with impaired parasite-specific proliferative and Th1 responses (IL-2 and IFN-γ) with relatively normal (or enhanced) parasite Ag-specific Th2 cytokine (IL-4, IL-5, and IL-10) production and frequencies of Th2 cells ($CD4^+$ T cells expressing IL-4 or IL-5) (2).

Although most of the immunologic studies have focused on the immune response to parasite Ag, little is known about the immune response induced by live parasites. The type of immune response engendered by live parasites (in contrast to Ag) is more likely to play a role in determining individual susceptibility to infection. In addition, characterization of the in vitro immune responses to live filarial parasites would provide a better understanding of the physiologic nature of the host-parasite interaction that occurs in vivo. Indeed, we have determined previously that T cells from parasite-naive individuals mount a significant proinflammatory response to live infective-stage larvae (L3), but not to L3 Ag (3). Moreover, given the complexity of the life cycle of the filarial parasites, it becomes important to study the immune response not only to live parasites, but also to their different stages. Examination of stage-specific induction of cytokines in human lymphatic filariasis revealed that Ag derived from Mf-containing Ag (Mf and adult females) induced cytokine responses that differed from those of Ag derived from stages that did not contain Mf (4), suggesting an important role for Mf in establishing or maintaining chronic infection. In a nonpermissive mouse model of Brugian filariasis, adult worms and Mf stages have been shown to stimulate contrasting cytokine responses (5) as well.

Therefore, we characterized the immune response of a group of patently infected (INF; $n = 10$) and uninfected (UN; $n = 10$) individuals to the two important life cycle stages of the filarial parasite: live L3 and live Mf, as well as *Brugia malayi* adult worm Ag (BmA). Using ELISA and intracellular flow cytometry, we compared the cytokine response of INF to UN induced by live parasites and parasite Ag. Moreover, having hypothesized the involvement of 1) Th1 and Th2 regulators, *T-bet* (6), and *GATA-3* (7); 2) $Foxp3^+$, natural regulatory T cells (T_{reg}) (8); 3) regulatory networks of tolerance TGF-β (9), CTLA-4 (10, 11), PD-1 (10, 12), ICOS (10, 11), indoleamine 2,3-dioxygenase (IDO) (13), and suppressors of cytokine signaling (*SOCS*) genes (14); and 4) adaptive tolerance/anergy networks-E3 ubiquitin ligase family, Casitas B

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[3] Abbreviations used in this paper: Mf, microfilaria; BmA, *Brugia malayi* adult worm Ag; cbl, Casitas B lymphoma; GM, geometric mean; *GRAIL*, gene related to anergy in lymphocytes; IDO, indoleamine 2,3-dioxygenase; INF, infected; L3, live infective-stage larvae; SOCS, suppressor of cytokine signaling; T_{reg}, regulatory T cell; UN, uninfected.

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lymphoma (cbl)-b, *c-cbl Itch*, gene related to anergy in lymphocytes (*GRAIL*), and *Nedd4* (15, 16) as underlying mechanisms of cytokine modulation, we were able to identify those networks playing a significant and overlapping role in modulating the immune response in patent lymphatic filariasis. In addition, the induction of regulatory pathways is directly linked to cytokine impairment and tolerance networks because blocking CTLA-4 and neutralizing TGF-β resulted in restoration of cytokine responses and failure of tolerance pathway induction.

Materials and Methods

Study population

We studied a group of 10 INF and 10 UN in an area endemic for lymphatic filariasis in Tamil Nadu, South India (Table I). The INF were diagnosed as circulating filarial Ag positive by both the immunochromatographic test filarial Ag test (Binax) and the Trop Bio Og4C3 ELISA (Trop Bio), and they were Mf positive by night blood examination. Each of the UN was filarial Ag negative and had no history or signs/symptoms of filarial infection. BmA-specific IgG4 levels in INF ranged from 1389 to 7830 with a geometric mean (GM) of 4176.7 pg/ml, while no BmA-specific IgG4 was detected in UN. BmA-specific total IgG in INF ranged from 97.4 to 643.8 (GM = 280.2) ng/ml and from 4.3 to 350.5 (GM = 40.1) ng/ml in UN. BmA-specific IgG4 and IgG ELISA were performed exactly as described previously (17). For costimulation blocking and cytokine neutralization studies, we used cells from filarial INF patients studied at the National Institutes of Health. All individuals were examined as part of a clinical protocol approved by both Institutional Review Boards of the National Institutes of Allergy and Infectious Diseases and the Tuberculosis Research Center, and informed consent was obtained from all participants.

Isolation of PBMC

Heparinized blood was collected, and PBMC was isolated by Ficoll diatrizoate gradient centrifugation (lymphocyte separation medium; ICN Biomedicals). Erythrocytes were lysed using ACK lysis buffer (BioSource International). Cells were then washed and cryopreserved in a medium containing RPMI 1640 (BioWhittaker), 10% heat-inactivated FCS (Harlan Bioproducts for Science), and 20% DMSO (Fisher Scientific). Serum was collected and stored for further analysis.

Live parasites and parasite Ag

L3 and Mf were obtained from J. McCall (University of Georgia, Athens, GA). The L3 and Mf were washed repeatedly in RPMI 1640 with antibiotics and cultured at 37°C in 5% CO_2. Soluble BmA was made from *B. malayi* adult worms (provided by J. McCall), as described previously (18).

Reagents for flow cytometry

Ab used for analysis were purchased from BD Biosciences. The Ab used in the study were as follows: FITC-labeled anti-human CD14 and CD4; PE-labeled anti-human IFN-γ, TNF-α, IL-4, IL-5, IL-10, and IL-13; PerCP-labeled anti-human CD19 and CD8; and allophycocyanin-labeled anti-human CD3.

In vitro culture

PBMC were cultured with live L3 (5/well) or live Mf (50,000/well) or BmA (5 μg/ml) in 24-well tissue culture plates (Corning Glass) at concentrations of 5×10^6/well. After 24 h, culture supernatants were collected and analyzed for cytokines. For costimulation blocking experiments, we cultured cells with live L3 and CTLA-4 Ig (10 μg/ml), ICOS Ig (10 μg/ml), or control Ig (10 μg/ml) (Ancell). For cytokine neutralization, we cultured cells with live L3 and anti-TGF-β (5 μg/ml), anti-IL-10 (5 μg/ml), or isotype control mouse IgG2b (5 μg/ml) (R&D Systems).

Table I. *Characteristics of the study population*

	INF (n = 10)	UN (n = 10)
Median age (range)	28 (20–50)	28.5 (28–45)
Gender: male/female	7/3	8/2
Pathology/Mf status	None/+	None/−
Wuchereria bancrofti circulating Ag levels U/ml[a] (median)	254–49,069 (6,428.7)	< 32 (< 32)
BmA-specific IgG (μg/ml) (GM)	97.4–643.8 (280.2)	4.3–350.5 (39.4)
BmA-specific IgG4 (ng/ml) (GM)	1,389–7,830 (4,176.7)	0 (0)

[a] The lower limit of the assay detection was 32 U/ml.

Flow cytometry

After 24 h, cells were fixed in 4% paraformaldehyde and permeabilized in PBS/0.1% BSA/0.1% saponin for intracellular detection of cytokines. Staining of surface markers was done concurrently. Fluorescence was measured on a FACSCalibur (BD Biosciences) using 50,000 gated lymphocytes.

ELISA

The levels of cytokines (n = 10 in each group) in the culture supernatants were measured using Searchlight Multiplex ELISA technology (Pierce). The cytokines analyzed were IFN-γ, TNF-α, IL-4, IL-5, IL-10, and IL-13. Net cytokine production was calculated by subtracting unstimulated cytokine levels from stimulus (L3 or Mf)-induced cytokine levels.

RNA preparation

PBMC were lysed using the reagents of a commercial kit (QIAshredder; Qiagen). Total RNA was extracted according to the manufacturer's protocol (RNeasy Mini kit; Qiagen), and RNA was dissolved in 50 μl of RNase-free water.

cDNA synthesis

RNA (1 μg) was used to generate cDNA using TaqMan reverse transcription reagents, according to the manufacturer's protocol (Applied Biosystems). Briefly, random hexamers were used to prime RNA samples for reverse transcription using MultiScribe reverse transcriptase.

Real-time RT-PCR

Real-time quantitative RT-PCR was performed in an ABI 7700 sequence detection system (Applied Biosystems) using TaqMan Assays on Demand reagents for TGF-β, IDO, CTLA-4, PD-1, ICOS, *T-bet*, *GATA-3*, *Foxp3*, *cbl-b*, *c-cbl*, *Itch*, *Nedd4*, *CIS*, *SOCS-1–5*, *SOCS-7*, IFN-γ, TNF-α, IL-4, IL-5, and an endogenous 18S ribosomal RNA control. Relative transcripts were determined by the formula: $1/2^{(\mathrm{CT\ target}\ -\ \mathrm{CT\ control})}$, in which CT is the threshold cycle during the exponential phase of amplification.

Statistical analysis

Comparisons were made using the nonparametric Mann-Whitney U test, and p values were determined using the Holm correction method for multiple comparisons. All statistics were performed with StatView 5 software (SAS Institute).

Results

Live parasite induced diminished production of both Th1 and Th2 cytokines in filaria INF individuals

To determine the cytokine profile induced by live parasites compared with that induced by parasite Ag, we cultured PBMC from INF (n = 10) and UN (n = 10) with live parasites (L3 or Mf) or parasite Ag (BmA) and measured the levels of selected cytokines in the culture supernatants 24 h later. The baseline levels of IFN-γ, TNF-α, IL-4, IL-5, IL-13, and IL-10 were not significantly different between the two groups (data not shown); however, the net cytokine production of IFN-γ (GM 2.95 vs 76.46 pg/ml; p = 0.034), TNF-α (GM 34.56 vs 446.56 pg/ml; p = 0.036), IL-4 (GM 3.53 vs 15.02 pg/ml; p = 0.004), IL-5 (GM 0.33 vs 3.93 pg/ml; p = 0.004), and IL-10 (GM 1.58 vs 29.09 pg/ml; p = 0.009), but not IL-13 (GM 17.15 vs 67.67 pg/ml), was significantly lower in response to live L3 in INF compared with UN (Fig. 1*A*). Similarly, the net cytokine production of IFN-γ (GM 2.53 vs 17.39 pg/ml; p = 0.045), TNF-α (GM 23.26 vs 283.75 pg/ml; p = 0.001), IL-4 (GM 1.37 vs 7.16 pg/ml; p = 0.024), IL-5 (GM 0.56 vs 2.38 pg/ml; p = 0.012), and IL-10 (GM 2.49 vs 13.4 pg/ml; p = 0.002), but not IL-13 (GM 12.32 vs 65.21 pg/ml), was significantly lower in response to live Mf in INF (Fig. 1*B*). In contrast, the net cytokine production of IFN-γ (GM 2.30 vs 3.15 pg/ml), TNF-α (GM

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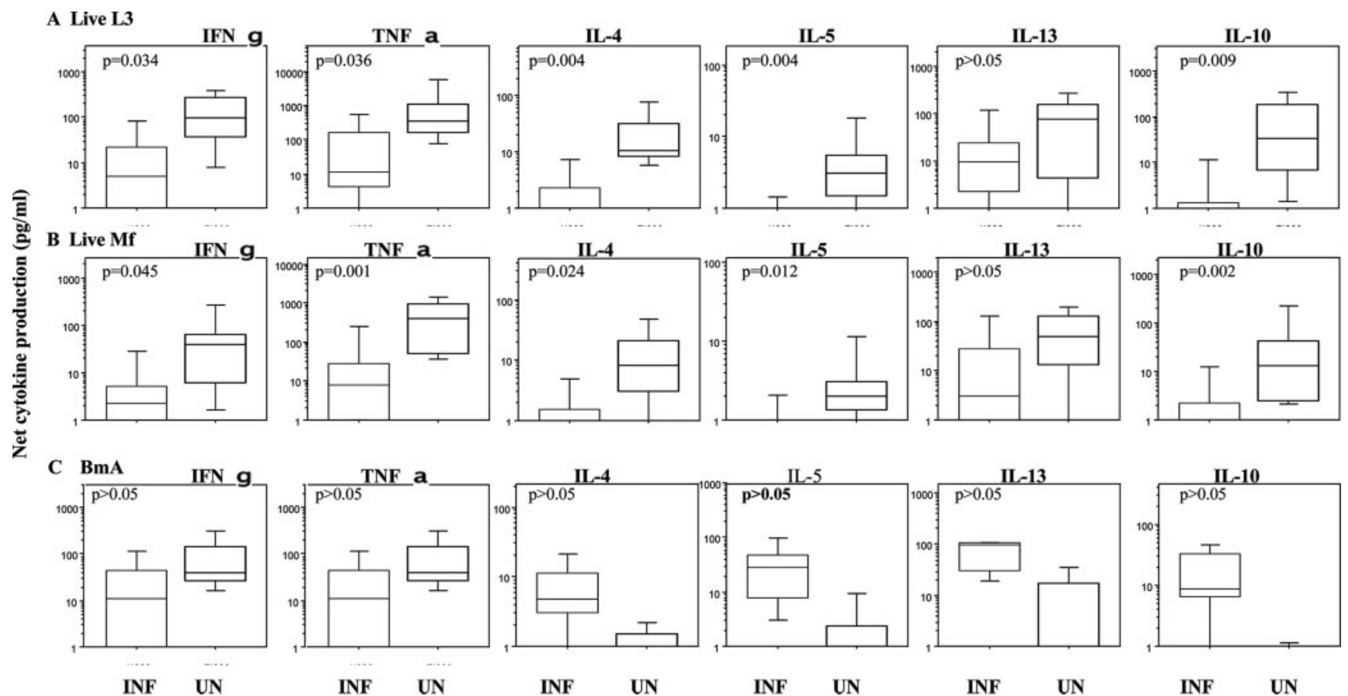

FIGURE 1. Th1 and Th2 cytokine production in INF and UN individuals. PBMC from INF ($n = 10$) and UN ($n = 10$) stimulated with *A*, live L3 (5/ml); *B*, live Mf (50,000/ml); or *C*, BmA (5 μg/ml). Net production of cytokines (in pg/ml) calculated by subtracting baseline level from level following parasite stimulation. Results expressed as box plots; horizontal lines represent the 25th, 50th, and 75th percentiles; vertical lines represent the 10th and 90th percentiles of data. Values of p were calculated using the Mann-Whitney U test and Holm correction for multiple comparisons.

29.0 vs 54.94 pg/ml), IL-4 (GM 7.58 vs 1.69 pg/ml), IL-5 (GM 18.63 vs 9.46 pg/ml), IL-13 (GM 58.36 vs 19.95 pg/ml), and IL-10 (GM 10.04 vs 0.56 pg/ml) was not significantly different in the two groups in response to BmA (Fig. 1*C*), although the trend for each was similar to that described previously (19).

Frequency of Th1 and Th2 cells is lower in filaria INF individuals

To assess the contribution of $CD4^+$ and $CD8^+$ T cells as well as monocytes and B cells to impaired cytokine production, we measured the frequency of $CD4^+$, $CD8^+$, $CD14^+$, and $CD19^+$ cells expressing the respective cytokines. $CD8^+$ T cells expressed IFN-γ, IL-4, and IL-5 in response to live L3 and Mf, but the frequencies were not significantly different between the two groups (data not shown). $CD14^+$ monocytes expressed TNF-α and IL-10, but again the frequencies were not significantly different between the two groups (data not shown). The cell type primarily responsible for differences in cytokine expression were the $CD4^+$ T cells, and the frequency of $CD4^+$ T cells expressing IFN-γ (GM 0.84 vs 1.99%; $p = 0.009$), TNF-α (GM 0.30 vs 1.42%; $p = 0.028$), IL-4 (GM 0.73 vs 1.75%; $p = 0.009$), IL-5 (GM 0.18 vs 1.60%; $p = 0.009$), and IL-10 (GM 0.68 vs 1.48%; $p = 0.028$), but not IL-13 (GM 1.12 vs 0.89%), was significantly lower in response to live L3 in INF compared with UN (Fig. 2*A*). Similarly, in response to live Mf, the frequency of $CD4^+$ T cells expressing IFN-γ (GM 0.89 vs 2.6%; $p = 0.034$), TNF-α (GM 1.49 vs 3.98%; $p = 0.036$), IL-4 (GM 0.60 vs 1.52%; $p = 0.004$), IL-5 (GM 0.36 vs 1.2%; $p = 0.004$), and IL-10 (GM 0.73 vs 1.45%; $p = 0.009$), but not IL-13 (GM 1.32 vs 0.4%), was significantly diminished in INF (Fig. 2*B*).

Impaired expression of T-bet *and* GATA-3 *and augmented expression of* Foxp3 *in INF*

To identify the potential molecular basis of the impaired Th1 and Th2 response in INF, we examined the expression levels of *T-bet*, *GATA-3*, and *Foxp3* at baseline and following live L3 and live Mf stimulation. Baseline expression levels of *T-bet* and *GATA-3* were not significantly different between INF and UN (data not shown); however, following stimulation with live L3, a significant impairment in the induction of *T-bet* ($p = 0.0163$; GM fold change over control of 0.8 in INF vs 3.22 in UN) and *GATA-3* ($p = 0.009$; 0.41 vs 1.99) was associated with patent infection (Fig. 3*A*). Similarly, in response to live Mf, a significant impairment in the induction of T-bet ($p = 0.009$; 0.55 vs 1.3) and *GATA-3* ($p = 0.0163$; 0.85 vs 3.18) was observed (Fig. 3*B*). In contrast, both baseline (data not shown) and live L3 ($p = 0.0163$; 3.48 vs 0.88)- but not live Mf ($p = 0.1172$; 2.1 vs 0.55)-stimulated levels of *Foxp3* were significantly higher in INF compared with UN (Fig. 3).

Increased expression of TGF-β and IDO in INF

To identify potential extrinsic factors regulating the impaired Th1 and Th2 response in INF, we examined the expression levels of TGF-β and IDO at baseline and following live L3 and live Mf stimulation. Baseline expression levels of TGF-β and IDO were not significantly different between INF and UN (data not shown). Expression of TGF-β was significantly increased in INF upon stimulation with live L3 ($p = 0.0163$; GM fold change over control of 2.56 in INF vs 0.56 in UN), but not following live Mf ($p = 0.0758$; 1.35 vs 0.69) (Fig. 4). Similarly, as shown in Fig. 4, expression of IDO was significantly increased in INF in response to both live L3 ($p = 0.009$; 5.08 vs 0.59) and live Mf ($p = 0.009$; 2.78 vs 0.43).

Increased expression of negative costimulatory molecules in INF

Because CTLA-4 and PD-1 are known to inhibit T cell function and ICOS is a costimulatory molecule essential for certain Th2 responses (10, 11), we examined the expression levels of these molecules at baseline and following live L3 and live Mf stimulation (Fig. 5). Baseline expression levels of CTLA-4, PD-1, and

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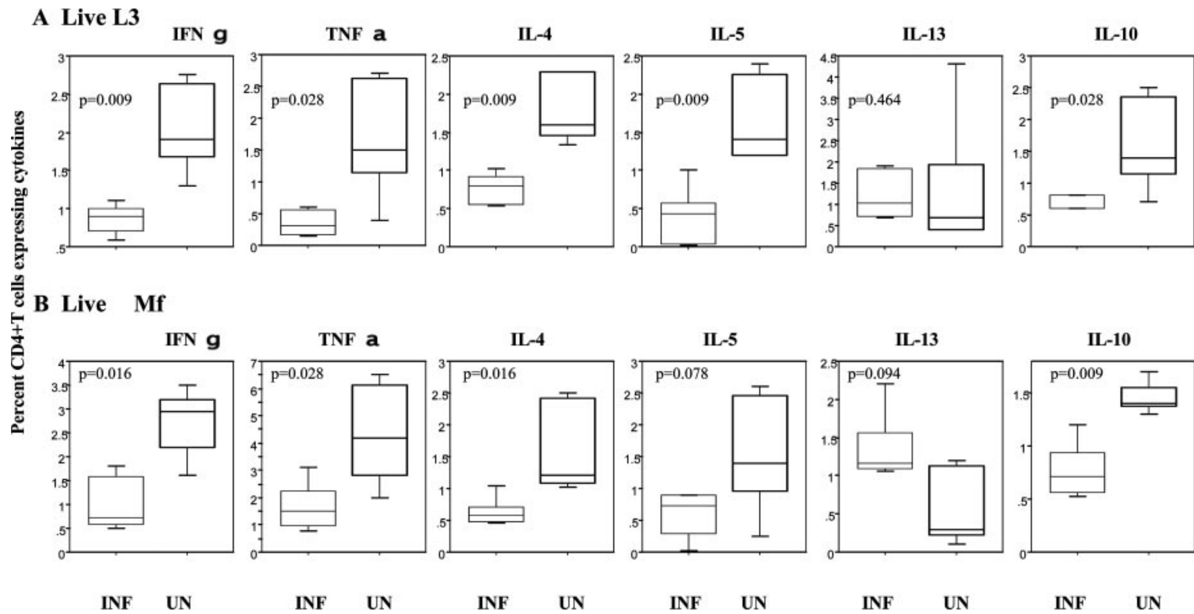

FIGURE 2. Frequency of $CD4^+$ T cells expressing Th1 and Th2 cytokines in INF and UN individuals. PBMC from INF ($n = 5$) and UN ($n = 5$) stimulated with *A*, live L3 (5/ml), or *B*, live Mf (50,000/ml) examined by intracellular flow cytometry. The frequency of cells expressing cytokine expressed as value following subtraction of baseline frequency. Results expressed as box plots; horizontal lines represent the 25th, 50th, and 75th percentiles; vertical lines represent the 10th and 90th percentiles of data. Values of *p* were calculated using the Mann-Whitney *U* test and Holm correction for multiple comparisons.

ICOS were not significantly different between INF and UN (data not shown). The expression of CTLA-4 was significantly increased in INF upon stimulation with live L3 ($p = 0.0163$; GM fold change over control of 8.24 in INF vs 1.27 in UN) and live Mf ($p = 0.0472$; 2.02 vs 0.55). Similarly, the expression of PD-1 was significantly increased in INF in response to live L3 ($p = 0.0472$; 6.42 vs 1.37), but not live Mf ($p = 0.6015$; 1.91 vs 1.76). Notably, expression of ICOS was also significantly increased in INF upon stimulation with live L3 ($p = 0.0283$; 5.09 vs 1.19) and live Mf ($p = 0.0472$; 1.5 vs 0.35).

Expression of SOCS *genes is not different between INF and UN*

To study the role of *SOCS* gene family members, known negative regulators of Th1 and Th2 differentiation (14), we examined the expression of *CIS*, *SOCS-1*, *SOCS-2*, *SOCS-3*, *SOCS-4*, *SOCS-5*, and *SOCS-7* in PBMC of INF and UN. Neither baseline nor live parasite-stimulated *SOCS* expression differed between the groups studied: *CIS* (GM fold change of 1.82 in INF vs 2.56 in UN for L3 and 0.66 in INF vs 1.01 in UN for Mf); *SOCS-1* (1.31 vs 1.88 and 0.75 vs 2.95); *SOCS-2* (1.59 vs 1.47 and 2.13 vs 1.83); *SOCS-3* (1.18 vs 2.99 and 0.56 vs 0.73); *SOCS-4* (1.18 vs 1.88 and 0.49 vs 0.35); *SOCS-5* (1.33 vs 1.9 and 0.48 vs 0.63); and *SOCS-7* (0.61 vs 0.81 and 0.24 vs 0.32) (data not shown).

Expression of genes known to mediate T cell anergy/tolerance

E3 ubiquitin ligases are a family of molecules, including *cbl-b*, *c-cbl, Itch*, *GRAIL*, and *Nedd4*, that are induced predominantly in anergic T cells (16). Because T and B cell responses in INF resemble a state of anergy/adaptive tolerance (20), we examined the expression pattern of E3 ubiquitin ligases in PBMC of INF and

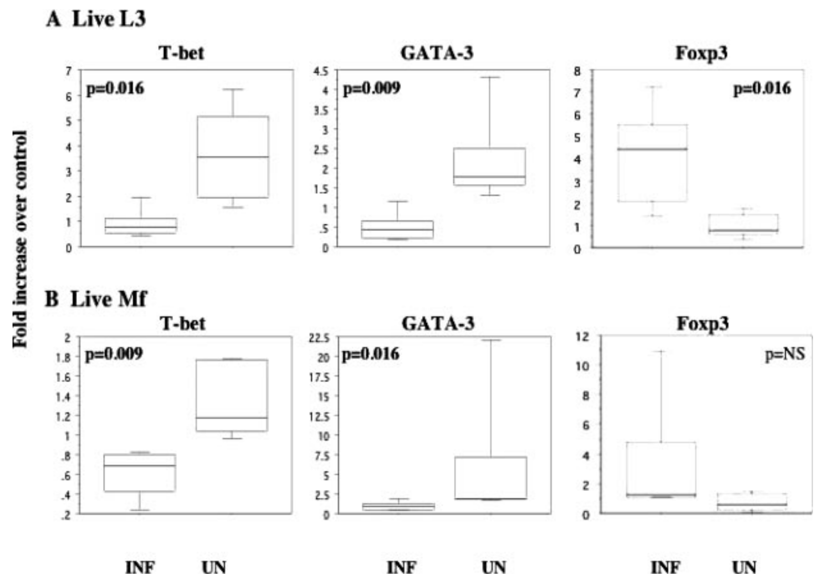

FIGURE 3. Expression of *T-bet*, *GATA-3*, and *Foxp3* in filarial infections. *A*, PBMC expression of *T-bet*, *GATA-3*, and *Foxp3* mRNA following 24-h stimulation with live L3 depicted as fold change over medium control. *B*, PBMC expression of *T-bet*, *GATA-3*, and *Foxp3* mRNA following 24-h stimulation with live Mf depicted as fold change over medium control. Results expressed as box plots; horizontal lines represent the 25th, 50th, and 75th percentiles; vertical lines represent the 10th and 90th percentiles of data. Values of *p* were calculated using the Mann-Whitney *U* test.

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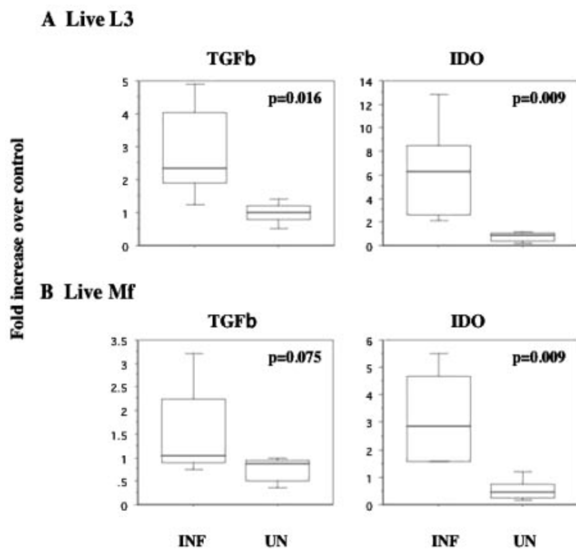

FIGURE 4. Expression of TGF-β and IDO in filarial infections. *A*, PBMC expression of TGF-β and IDO mRNA following 24-h stimulation with live L3 depicted as fold change over medium control. *B*, PBMC expression of TGF-β and IDO mRNA following 24-h stimulation with live Mf depicted as fold change over medium control. Results expressed as box plots; horizontal lines represent the 25th, 50th, and 75th percentiles; vertical lines represent the 10th and 90th percentiles of data. Values of p were calculated using the Mann-Whitney U test.

UN. Baseline expression of all the members with the exception of GRAIL was detected in both groups of individuals and was not significantly different between the two groups under study (data not shown). Live L3 induced a significant up-regulation of *cbl-b* ($p = 0.0472$; GM fold change of 2.21 in INF vs 0.6 in UN); *c-cbl* ($p = 0.0283$; 1.82 vs 1.05); *Itch* ($p = 0.009$; 2.1 vs 0.78); and *Nedd4* ($p = 0.0163$; 3.01 vs 0.82) (Fig. 6*A*). Similarly, live Mf induced a significantly differential expression of *cbl-b* ($p = 0.009$; GM fold change of 0.89 in INF vs 0.35 in UN), *Itch* ($p = 0.0163$; 0.93 vs 0.43), and *Nedd4* ($p = 0.0163$; 1.55 vs 0.46), but not *c-cbl* ($p = 0.0758$; 1.13 vs 0.59) (Fig. 6*B*). Thus, both live L3 and Mf can induce genes associated with T cell anergy/adaptive tolerance in INF.

CTLA-4 blockade reverses cytokine impairment and down-regulates E3 ubiquitin ligases in INF

To determine the importance of costimulatory molecules in the induction of cytokine and Th1/Th2 transcription machinery down-regulation as well as in the induction of E3 ubiquitin ligases, we stimulated PBMC from INF with live L3 in the presence of CTLA-4 Ig or ICOS-Ig or control Ig. In the presence of CTLA-4 blockade, we found a significant difference in the induction of IFN-γ ($p = 0.0039$, GM fold increase of 1.99 vs the baseline of 1), TNF-α ($p = 0.0039$, 2.66), IL-4 ($p = 0.0039$, 3.79), and IL-5 ($p = 0.0039$, 2.16) mRNA by live L3 compared with control (Fig. 7). This difference is manifested at the transcription factor level as both *T-bet* ($p = 0.0039$, 3.27) and GATA-3 ($p = 0.0039$, 2.95) were significantly increased in response to live L3 upon CTLA-4 blockade. No significant difference in the expression of *Foxp3* was noted. In addition, the E3 ubiquitin ligase family of anergic factors that are highly induced in response to live L3 is significantly diminished in the presence of CTLA-4 blockade (Fig. 7). Thus, c-cbl ($p = 0.0039$, GM fold change of 0.49 vs baseline of 1), *cbl-b* ($p = 0.0039$, 0.45), and *Itch* ($p = 0.0039$, 0.65), but not *Nedd4*, were significantly decreased in the presence of CTLA-4 Ig. Interestingly, ICOS Ig did not alter the expression of any of the above factors.

Neutralization of TGF-β restores cytokine responses in INF

To determine the role of TGF-β in the induction of T cell hyporesponsiveness, we stimulated PBMC from INF ($n = 6$) with live L3 in the presence of anti-TGF-β or anti-IL-10 or control Ab. We found that TGF-β neutralization resulted in a significant increase in the induction of IFN-γ ($p = -0.0039$, GM increase of 3.87 vs baseline of 1), TNF-α ($p = 0.0039$, 2.52), IL-4 ($p = 0.0039$, 2.16), and IL-5 ($p = 0.0039$, 4.11) mRNA (Fig. 8). This is mirrored by a significant increase in *T-bet* ($p = 0.0039$, 4.61) and *GATA-3* ($p = 0.0039$, 6.41), but not *Foxp3*. Unlike CTLA-4 blockade, blocking TGF-β had no effect on *c-cbl*, *cbl-b*, *Itch*, or *Nedd4* mRNA. Moreover, in this system, IL-10 had played little (if any) role in the live parasite-induced hyporesponsiveness, as IL-10 neutralization was unable to reverse the responses studied in INF (Fig. 8).

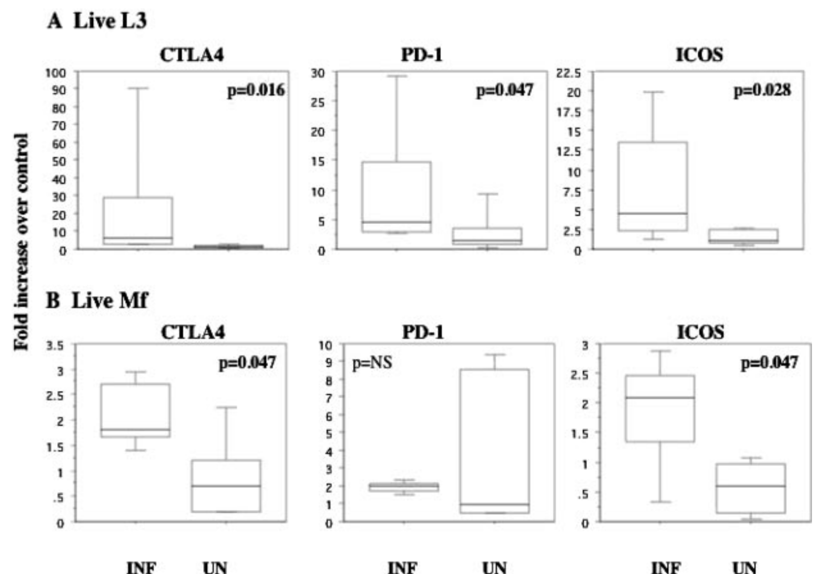

FIGURE 5. Expression of CTLA4, PD-1, and ICOS in filarial infections. *A*, PBMC expression of CTLA-4, PD-1, and ICOS mRNA following 24-h stimulation with live L3 depicted as fold change over medium control. *B*, PBMC expression of CTLA-4, PD-1, and ICOS mRNA following 24-h stimulation with live Mf depicted as fold change over medium control. Results expressed as box plots; horizontal lines represent the 25th, 50th, and 75th percentiles; vertical lines represent the 10th and 90th percentiles of data. Values of p were calculated using the Mann-Whitney U test.

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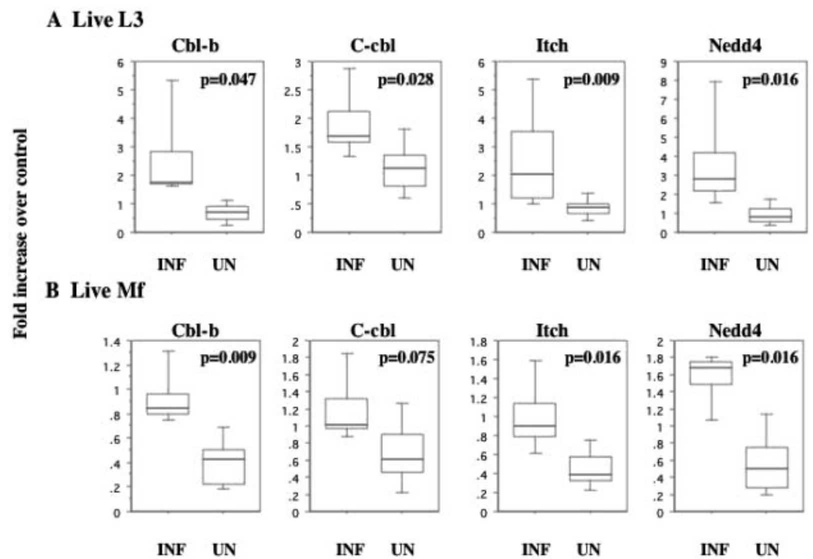

FIGURE 6. Expression of *cbl-b*, *c-cbl*, *Itch*, and *Nedd4* in filarial infections. *A*, PBMC expression of *cbl-b*, *c-cbl*, *Itch*, and *Nedd4* mRNA following 24-h stimulation with live L3 depicted as fold change over medium control. *B*, PBMC expression of *cbl-b*, *c-cbl*, *Itch*, and *Nedd4* mRNA following 24-h stimulation with live Mf depicted as fold change over medium control. Results expressed as box plots; horizontal lines represent the 25th, 50th, and 75th percentiles; vertical lines represent the 10th and 90th percentiles of data. Values of *p* were calculated using the Mann-Whitney *U* test.

Discussion

Lymphatic filariasis is a longstanding, chronic infection with complex life cycle stages and host-parasite interactions. High parasite loads are accompanied by a state of immunologic tolerance to parasite Ag, at least in the Th1 arm of adaptive immunity (2). Although Th2 responses are thought to be a characteristic feature of filarial infections, not all studies reveal a clear Th1/Th2 dichotomy in filarial infections (21, 22). Another feature in the studies examining the immune responses in filarial infections is the exclusive use of parasite Ag as the stimulating agent in vitro. The use of crude, soluble Ag provides vital information toward understanding the pathogenesis of infection and host-parasite interactions, but more information can be gleaned by studying in vitro immune responses to live parasites. Indeed, live parasites and parasite Ag elicit qualitatively different responses in T cells of naive individuals (3). Moreover, different life cycle stages of the parasite have been shown to elicit different immune responses (5), indicating the need to elucidate immune responses to both the L3 stage, which is crucial in initiating infection, and the Mf stage, which is crucial in maintaining chronic infection.

We chose to study the in vitro immune responses to two important life cycle stages of the parasites: live L3 and live Mf, both of which have critical roles in the human host-mosquito vector interface. We identified a cohort of 20 individuals in South India, each of whom was classified into one of two groups: INF, based on 1) the presence of the diagnostic *W. bancrofti* circulating Ag (23), 2) positive BmA-specific IgG4 (17), and 3) nocturnal Mf; and UN, based on 1) the absence of signs/symptoms of infection (1), 2) the absence of circulating filarial Ag (23), and 3) undetectable BmA-specific IgG4 (17). Of interest, all UN displayed the presence of BmA-specific total IgG, presumably indicating exposure to infection. We first examined production of the Th1 and Th2 cytokines from PBMC of INF and UN in response to live L3 or Mf. Although baseline production did not differ between the two groups, the production of both Th1 (IFN-γ and TNF-α) and Th2 (IL-4, IL-5, IL-10, but not IL-13) cytokines was significantly lower in INF. In contrast, parasite Ag stimulation did not induce a significantly different cytokine response, although heightened Th2 responses were observed in agreement with previous studies in this area (19). This is similar to the cytokine response observed in naive, unexposed

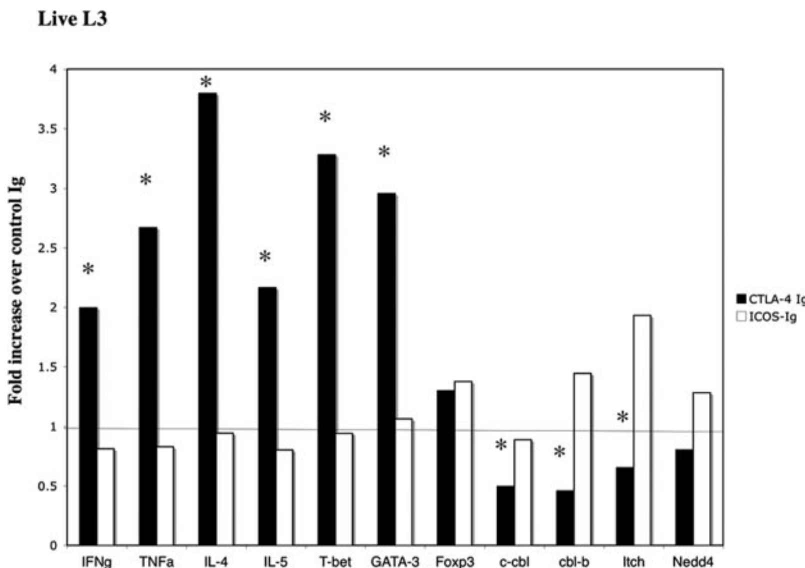

FIGURE 7. Blocking CTLA-4 and ICOS in filarial immune responses. PBMC expression of IFN-γ, TNF-α, IL-4, IL-5, *T-bet*, *GATA-3*, *Foxp3*, *c-cbl*, *cbl-b*, *Itch*, and *Nedd4* mRNA following 24-h stimulation of live L3 in the presence of CTLA-4 Ig (■) and ICOS-Ig (□) is depicted as fold change over L3 stimulation in the presence of control Ig. *, Denotes significant differences, and *p* values were calculated using the Mann-Whitney *U* test.

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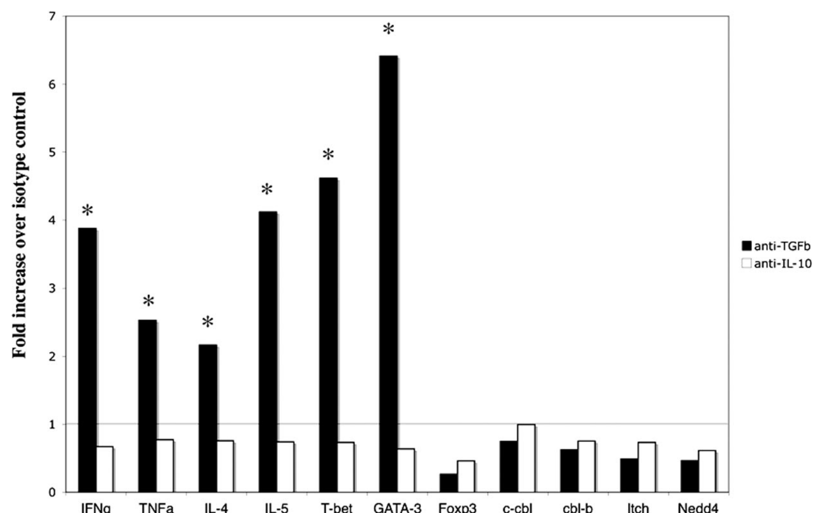

FIGURE 8. Neutralizing TGF-β and IL-10 in filarial immune responses. PBMC expression of IFN-γ, TNF-α, IL-4, IL-5, *T-bet*, *GATA-3*, *Foxp3*, *c-cbl*, *cbl-b*, *Itch*, and *Nedd4* mRNA following 24-h stimulation of live L3 in the presence of anti-TGF-β (■) and anti-IL-10 (□) is depicted as fold change over L3 stimulation in the presence of isotype control Ab. *, Denotes significant differences, and *p* values were calculated using the Mann-Whitney *U* test.

individuals to live parasites, which requires the presence of accessory cells in culture as well as direct contact with live parasites. Interestingly, parasite Ag had no effect in the study, indicating that live parasites and parasite Ags engender qualitatively different responses (3). Thus, the early acute immune response of INF is significantly diminished in terms of both Th1 and Th2 cytokine production in response to live filarial parasites. This state of parasite-induced Th1/Th2 immunosuppression could play a pivotal role in the continued maintenance of high-density infection. It could also partially explain the absence of exuberant immune responses in endemic individuals that are characteristic of infection in expatriates (24). In addition, cytokine impairment appears to be specific to filarial parasites, as we have shown previously that nonparasite (e.g., purified protein derivative) responses in the same group of individuals were unimpaired (25).

To identify the source of the Th1/Th2 cytokines in INF and UN, cell separations and flow cytometry suggested quite clearly that $CD4^+$ T cells expressed each cytokine examined in response to live parasites. In addition, the frequency of $CD4^+$ T cells expressing IFN-γ, TNF-α, IL-4, IL-5, and IL-10 was significantly lower in INF. These data indicate that the profound impairment of Th1/Th2 responses that occurs in INF is predominantly at the $CD4^+$ T cell level and implies that effector T cell function compromise underlies this filaria-induced immunosuppression. Notably, by both ELISA and flow cytometry, we could not detect any significant differences in production of IL-13, a prominent Th2 cytokine. Although basophils have been shown to be major producers of IL-4 in filarial infections (26), we were unable to examine their role, as we were limited to cryopreserved PBMC; basophils unfortunately do not survive cryopreservation.

Multiple mechanisms have been postulated to play a role in pathogen persistence in chronic infections (27, 28). We examined the involvement of various regulatory and adaptive tolerance pathways leading to T cell hyporesponsiveness. The main characteristic of natural T_{reg} is the high expression of Foxp3 transcription factor, which appears to have a key function in programming these cells (8). *Foxp3* expressing natural T_{reg} have been postulated to play an important role in the establishment of chronic filarial infection and Th2 responses (25, 29, 30). Although previously we had found no significant differences in *Foxp3* expression using parasite Ag stimulation (25), live parasite stimulation significantly up-regulates expression of *Foxp3* in INF, indicating heightened T_{reg} activity. Interestingly, *Foxp3* expression is also higher at baseline in INF, indicating the presence of chronic parasite-stimulated regulatory networks occurring in vivo.

Both natural T_{reg} and inducible T_{reg} (not expressing Foxp3) can act through several mechanisms (31). First, by surface expression of TGF-β or by its secretion, T_{reg} can suppress different stages of the immune response such as T cell expansion, cytokine secretion, and cytolytic activity (9). Indeed, TGF-β is a major immunoregulatory cytokine in filariasis, as demonstrated by the fact that neutralization of TGF-β partially reverses the defective T cell response (32). This study corroborates the role of TGF-β by showing increased induction of TGF-β in response to live parasite in INF; however, a role for IL-10 expression, as has been noted previously (33, 34), was not found in our study. We postulate that this may reflect either no IL-10 involvement in response to live parasites or, more likely, IL-10 induction and involvement that can occur at a later time point.

Second, T_{reg} can act by binding of ligands found on the surface of effector T cells to cell surface molecules such as CTLA-4, PD-1, and ICOS (31). This binding is postulated to result in an outside-in signaling in effector T cells, leading to their suppression (31). In addition, CTLA-4 and PD-1, present on effector T cells, can directly dampen their activity, and ICOS can limit certain Th1 responses (11). A role for CTLA-4 in filarial immunosuppression has already been demonstrated (35), and in this study, we expand these observations and show that the expression of other costimulatory molecules such as PD-1 and ICOS is also significantly increased in INF. Third, T_{reg}, by their expression of CTLA-4, can interact with APC expressing CD80 and CD86, resulting in activation of IDO (31). IDO is responsible for the metabolism of essential amino acid tryptophan, and depletion of tryptophan is associated with decreased activation of T cells (13). We find that expression of IDO is significantly increased in INF PBMC, suggesting the involvement of yet another arm of T_{reg} and APC activity. Because IDO mRNA does not necessarily reflect enzymatic activity, we plan to address the role of IDO activity in our future studies. We therefore conclude that immunoregulation in filariasis occurs in multiple layers with both cytokine/enzyme-mediated and T cell costimulator-mediated pathways operating in response to live parasite stimulation.

The molecular regulation of Th1 and Th2 differentiation is controlled by *T-bet* (6) and *GATA-3* (7), respectively. Because both type 1 and type 2 responses were depressed in INF in response to live parasites, we examined the expression patterns of *T-bet* and

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GATA-3 in INF and UN and found a significant depression of both molecules. Although *GATA-3* is absolutely required for IL-4 induction, its role in IL-13 induction is not clear. Our data suggest that *GATA-3*-independent regulation of IL-13 probably occurs in filarial infections because no impairment of IL-13 was observed in our studies. We have shown previously that parasite Ag-induced Th1 impairment in INF is affected by the *SOCS* gene family members, with *SOCS-1*, *SOCS-5*, and *SOCS-7* being significantly decreased in INF (25). In this study, live parasite stimulation did not induce any significant changes in *SOCS* gene expression. This suggests that *SOCS* molecules play a minimal role in live parasite stimulation, indicating that the live parasite-induced molecular mechanism of inhibition is qualitatively different from parasite Ag-induced pathways.

Finally, we hypothesized that the compromise of effector T cell function could also be mediated by induction of anergy/adaptive tolerance factors in INF. To verify this possibility, we measured the expression of a family of E3 ubiquitin ligases known to be involved in the establishment and maintenance of T cell anergy (15, 16). The family of E3 ubiquitin ligases are negative regulators of immune cell function and facilitate the ubiquitination of activated tyrosine kinases and other signaling proteins and of the signaling chains of the receptors themselves (16). The cbl-b and c-cbl proteins and *GRAIL* belong to the RING family of E3 ubiquitin ligases and are increased as a component of anergic signaling program in T cells (15). The homology to EBAP C terminus family of ligases includes *Itch* and *Nedd4*, which are thought to be important in T cell anergy maintenance (15). We found that expression of *cbl-b*, *c-cbl*, *Itch*, and *Nedd4* was significantly higher in PBMC of INF, indicating that live filarial parasites induce the expression of classical anergy/adaptive tolerance molecules. Notably, both TGF-β (36) and CTLA-4 (37) are known inducers of cbl molecules, implying that the E3 ubiquitin ligases might function as downstream effectors of T_{reg} in filariasis. Unlike HIV infection, in which E3 ubiquitin ligase-mediated T cell hyporesponsiveness is associated with clinical immunodeficiency (38), no clinically apparent immunodeficiency occurs in lymphatic filariasis, suggesting that the live parasite-induced T cell anergy is not associated with global immunodeficiency.

To determine the mechanism by which T cell responses are impaired in response to live parasites, we examined the effect of costimulation blockade of both CTLA-4 and ICOS. Although ICOS blockade had no significant effect on the T cell cytokine and molecular responses, blocking CTLA-4 interaction with its ligands, CD80 and CD86, significantly reversed the suppression of cytokine responses, both Th1 and Th2. The restoration of cytokine responses was associated with an increase in *T-bet* and *GATA-3* transcription factors. Interestingly, CD28 interaction with CD80 and CD86, which is also blocked by CTLA-4 Ig, appears dispensable for recall or memory cytokine responses in filarial infections. In addition, CTLA-4 is also a major player in the induction of live parasite-mediated T cell anergy because blocking CTLA-4 reversed the induction of the E3 ubiquitin ligase family members in INF. The other major players described in T cell hyporesponsiveness in filarial infections are TGF-β and IL-10 (32). Our study shows that TGF-β, but not IL-10, plays a vital role in the impairment of Th1 and Th2 cytokine production. Thus, CTLA-4 and TGF-β, both associated with effector T cell compromise and regulatory T cell activity, play an important role in live parasite-mediated modulation of host immune responses.

In summary, we have demonstrated that there is impairment of both Th1 and Th2 responses in filarial infections following live parasite stimulation. This impairment occurs mainly within the $CD4^+$ T cell population and is regulated by both external and internal factors. Our studies highlight the importance of using live parasite stimulation as means of recapitulating in vivo immune responses in vitro and provide a greater understanding of the complex host-parasite interactions in lymphatic filariasis. Studies in murine models suggest the involvement of both Th1 (39, 40) and Th2 (39) arms of immunity in resistance to filarial parasites. Hence, a compromise in Th1/Th2 effector function could play a pivotal role in establishment and maintenance of chronic, high-density filarial infections without triggering exuberant host immune responses. The identification of the host factors involved in this immunosuppression provides us with novel strategies to combat this dynamic infection, but also highlights the role of negative regulation of immune responses in chronic infections.

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Disclosures

The authors have no financial conflict of interest.

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