Transcriptional Control of Impaired Th1 Responses in Patent Lymphatic Filariasis by T-Box Expressed in T Cells and Suppressor of Cytokine Signaling Genes

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T-bet (T-box expressed in T cells) and GATA-3 are transcription factors that play a critical role in the development of Th1 and Th2 cells, as do genes of the SOCS (suppressor of cytokine signaling) family, albeit indirectly. Another transcription factor, Foxp3, is a master regulator of natural regulatory T cells (Tregs). To identify the role of these factors in impaired Th1 responses of patent filarial infection, analysis of cytokine, SOCS, and transcription factor mRNA expression was performed on purified T cells of filaria-infected individuals ($n = 6$) and uninfected controls ($n = 6$). As expected (and in contrast to cells of uninfected individuals), there was a significant depression of gamma interferon (IFN-γ) and a concomitant increase in interleukin-4 (IL-4), IL-5, and IL-10 mRNA expression following stimulation with parasite antigen (BmA) but not with a polyclonal T-cell (anti-CD3) stimulus. T-bet (but not GATA-3) was expressed at significantly lower levels in cells of filaria-infected individuals in response to BmA compared with those from the uninfected group, accounting, at least partially, for the diminished IFN-γ expression. Second, we found no significant differences in expression of Foxp3 between the two groups, although induction of Foxp3 expression correlated with induced expression levels of IL-10, implicating Tregs in the IL-10 expression seen. Finally, parasite-specific T-cell expression of SOCS-1, SOCS-5, and SOCS-7 was significantly diminished among infected patients; in contrast, expression of SOCS-3 increased. Our data therefore indicate that the impaired Th1 responses observed in patent lymphatic filariasis are associated with decreased expression of T-bet, SOCS-1, SOCS-5, and SOCS-7 and increased expression of SOCS-3 in T cells.

Lymphatic filariasis, a global disease affecting over 129 million people worldwide, is caused by the nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. Among the various clinical manifestations associated with infection, the most intriguing is the asymptomatic (or subclinical) form, characterized by either microfilaremia and/or circulating parasite antigen. The antigen-specific immunologic T-cell profile of these individuals suggests normal or enhanced Th2-like CD4$^+$ responses (interleukin-4 [IL-4], IL-5, IL-10) but impaired IL-2 and gamma interferon (IFN-γ) (Th1-like) responses, an impairment that does not extend to responses to most nonparasite antigens or to mitogens (15). In contrast to these infected individuals (INF), uninfected but parasite-exposed individuals (UN) are characterized by the ability to mount robust Th1 responses to filarial antigens (15).

The molecular basis of Th cell differentiation is the differential expression of transcription factors and the epigenetic modifications of cytokine genes and gene loci. T-bet (T-box expressed in T cells; for Th1 cells expressing IFN-γ) and GATA-3 (for Th2 cells expressing IL-4) are the key transcription factors for T-cell subset differentiation and memory (20). T-bet is expressed in Th1 cells, and its absence leads to elimination of IFN-γ production (26). GATA-3, in contrast, is critical for development of Th2 responses and can negatively regulate induction of Th1 responses (17, 28). Thus, a balance between T-bet and GATA-3 is important for the control of Th1/Th2 polarization.

Natural regulatory T cells (CD25$^+$) have been postulated to play an important role in establishment of chronic filarial infection (13), and Foxp3 has been shown to be a critical regulator of the development and function of regulatory T cells (6). Suppressor of cytokine signaling (SOCS) proteins are a family of molecules that also act as negative regulators of CD4$^+$ T-cell differentiation and maintenance by inhibiting components of the cytokine signaling cascade through direct binding to the signaling complex or by preventing access to the signaling complex (1).

In this study, we have examined the role of T-bet, GATA-3, Foxp3, and the SOCS genes in the impairment of Th1 and establishment of Th2 responses in lymphatic filarial infection. We demonstrate, based on mRNA expression, that INF (compared with UN) exhibit an impaired Th1 response (decreased IFN-γ expression) and an elevated Th2 response (increased IL-4, IL-5, and IL-10 expression) associated with a significant decrease in T-bet, SOCS-1, SOCS-5, and SOCS-7 expression and a significant increase in SOCS-3 expression in response to parasite antigen. Although no differences were observed in the baseline or antigen-induced levels of Foxp3 between INF and

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TABLE 1. Characteristics of the study population

Parameter	INF ($n = 6$)	UN ($n = 6$)
Median age (range), yr	35 (25–50)	24 (22–50)
Gender (male/female)	4/2	4/2
Pathology	None	None
W. bancrofti circulating antigen levels (median), U/ml[a]	1,506–32,000 (15,297)	<32 (<32)
BmA-specific IgG concn (geometric mean), µg/ml	36.3–461.0 (131.3)	12.6–296.0 (25.6)
BmA-specific IgG4 concn (geometric mean), ng/ml	377–5,630 (1,514.1)	0 (0)

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

UN, the levels of Foxp3 induction significantly correlated with the levels of IL-10 induction. This study suggests an important role for transcription factors and SOCS genes in regulating the Th1/Th2 response in a human parasitic infection.

MATERIALS AND METHODS

Study population. We studied a cohort of 12 individuals in an area endemic for *W. bancrofti* infection in Tamil Nadu, South India (Table 1). This included six asymptomatic INF and six UN. INF were positive by both the ICT filarial antigen test (Binax, Portland, ME) and the Bio Og4C3 enzyme-linked immunosorbent assay (Trop Bio Pty. Ltd., Queensland, Australia) for circulating filarial antigen. Each of the UN was filarial antigen negative and had no history or signs/symptoms of filarial infection. *B. malayi* antigen (BmA)-specific immunoglobulin G4 (IgG4) levels in INF ranged from 377 to 5,630 pg/ml, with a geometric mean (GM) of 1,514.1 pg/ml, while no BmA-specific IgG4 was detected in the UN. BmA-specific total IgG in INF ranged from 36.3 to 461 (GM = 131.3) ng/ml and from 12.6 to 196 (GM = 25.6) ng/ml in UN, likely indicating exposure. The BmA-specific IgG4 and IgG enzyme-linked immunosorbent assays were performed exactly as described previously (9). All individuals were examined as part of a clinical protocol approved by the Institutional Review Boards of both the National Institute of Allergy and Infectious Diseases and the Tuberculosis Research Center, and informed consent was obtained from all participants.

Isolation of PBL. Heparinized blood was collected, and peripheral blood lymphocytes (PBL) were isolated by Ficoll diatrizoate gradient centrifugation (LSM; ICN Biomedicals, Aurora, OH). Erythrocytes were lysed using ACK lysis buffer (Biosource, Camarillo, CA).

Parasite preparation. Soluble BmA was made from *B. malayi* adult worms (provided by J. McCall, University of Georgia, Athens) as described previously (14). The endotoxin level of the final soluble BmA was <0.1 EU/ml using the QCL-1000 chromogenic LAL test kit (BioWhittaker, Walkersville, MD).

In vitro culture and T-cell purification. PBL were cultured with BmA (5 µg/ml) in 24-well tissue culture plates (Corning Inc., Corning, NY) at a concentration of 5×10^6/well. After 24 h of culture, $CD3^+$ T cells were negatively selected by column purification using the T-cell negative selection kit (Miltenyi-Biotec, Auburn, CA). T cells were >98% pure in all our experiments as estimated by flow cytometry.

RNA preparation. T cells were lysed using the reagents of a commercial kit (QIAshredder; QIAGEN, Valencia, CA). 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, Foster City, CA). Briefly, random hexamers were used to prime RNA samples for reverse transcription using MultiScribe reverse transcriptase.

Real-time reverse transcription-PCR. Real-time quantitative reverse transcription-PCR was performed in an ABI 7700 sequence detection system (Applied Biosystems) using TaqMan assays-on-demand reagents (Applied Biosystems) for IFN-γ, tumor necrosis factor alpha (TNF-α), IL-8, IL-4, IL-5, IL-10, T-bet, GATA-3, Foxp3, cytokine-inducible SH2 domain-containing protein (CIS), SOCS-1, SOCS-2, SOCS-3, SOCS-4, SOCS-5, SOCS-7, and an endoge-

nous 18S rRNA control. Relative transcripts were determined by the formula $1/2^{(CT_{target} - CT_{control})}$, where CT is the threshold cycle during the exponential phase of amplification.

Statistical analysis. Comparisons were done using the nonparametric Mann-Whitney test. All statistics were performed with StatView 5 (SAS Institute, Cary, NC).

RESULTS

Filarial antigen but not anti-CD3 induces impaired IFN-γ responses in INF. To assess the expression pattern of Th1 and Th2 cytokines at the mRNA level, we examined the T-cell expression of IFN-γ, TNF-α, IL-8, IL-4, IL-5, and IL-10 following 24-h stimulation of peripheral blood mononuclear cells with BmA. Although baseline T-cell expression of the various cytokines assessed was not significantly different between the two groups (data not shown), we found a significant impairment of IFN-γ induction in INF ($P = 0.016$), with a GM change over the control of 1.05-fold in INF compared with 8.25-fold in UN. We also observed a significant increase in INF T-cell expression of IL-4 ($P = 0.0009$; 2.5 versus 0.25), IL-5 ($P = 0.0009$; 32.2 versus 0.67), and IL-10 ($P = 0.047$; 16.3 versus 1.2) (Fig. 1A) compared with the expression in UN. No significant differences in the expression levels of TNF-α (0.5 versus 2.1) and IL-8 (0.4 versus 0.8) were observed between the two groups (Fig. 1A). In contrast to BmA stimulation, anti-CD3 stimulation resulted in no significant differences in the induction levels of IFN-γ (36 versus 163), TNF-α (2.6 versus 12), IL-8 (2.6 versus 4), IL-4 (0.9 versus 1.4), IL-5 (5.3 versus 2.6), or IL-10 (13 versus 44) between the two groups studied (Fig. 1B).

Filarial antigen induces impaired expression of T-bet in INF. To identify the potential molecular basis of the impaired Th1 response in INF, we examined the T-cell expression levels of T-bet, GATA-3, and Foxp3 following BmA or anti-CD3 stimulation (Fig. 2). Baseline expression levels of T-bet, GATA-3, or Foxp3 were not significantly different between INF and UN (data not shown); however, following stimulation with BmA, a significant impairment in the induction of T-bet ($P = 0.039$; GM change over the control of 0.8-fold in INF versus 1.8-fold in UN) but not GATA-3 ($P = 0.336$; 0.7 versus 0.9) or Foxp3 ($P = 0.336$; 1.2 versus 1.5) was observed in INF (Fig. 2A). Upon anti-CD3 stimulation, T-bet (2.0 versus 3.7), GATA-3 (0.8 versus 0.9), and Foxp3 (1.8 versus 1.9) levels were not significantly different between the two groups studied (Fig. 2B).

Filarial antigen induces significantly impaired expression of SOCS-1, SOCS-5, and SOCS-7 and enhanced expression of SOCS-3. To assess the role of SOCS genes in regulating differential cytokine responses, we measured the expression levels of CIS, SOCS-1, SOCS-2, SOCS-3, SOCS-4, SOCS-5, and SOCS-7 in T cells of INF and UN before or after stimulation with BmA or anti-CD3. We observed that CIS, SOCS-1 to SOCS-5, and SOCS-7 were expressed in T cells and that the baseline level of expression was not significantly different between the two groups (data not shown). Upon stimulation with BmA, INF exhibited a significant increase in expression of SOCS-3 ($P = 0.0163$), with an average GM increase over the control of 1.9-fold compared with 1.1-fold in UN. In contrast, the induced expression of SOCS-1 ($P = 0.0104$; 0.9 versus 1.9), SOCS-5 ($P = 0.0104$; 0.6 versus 1.6), and SOCS-7 ($P = 0.0374$;
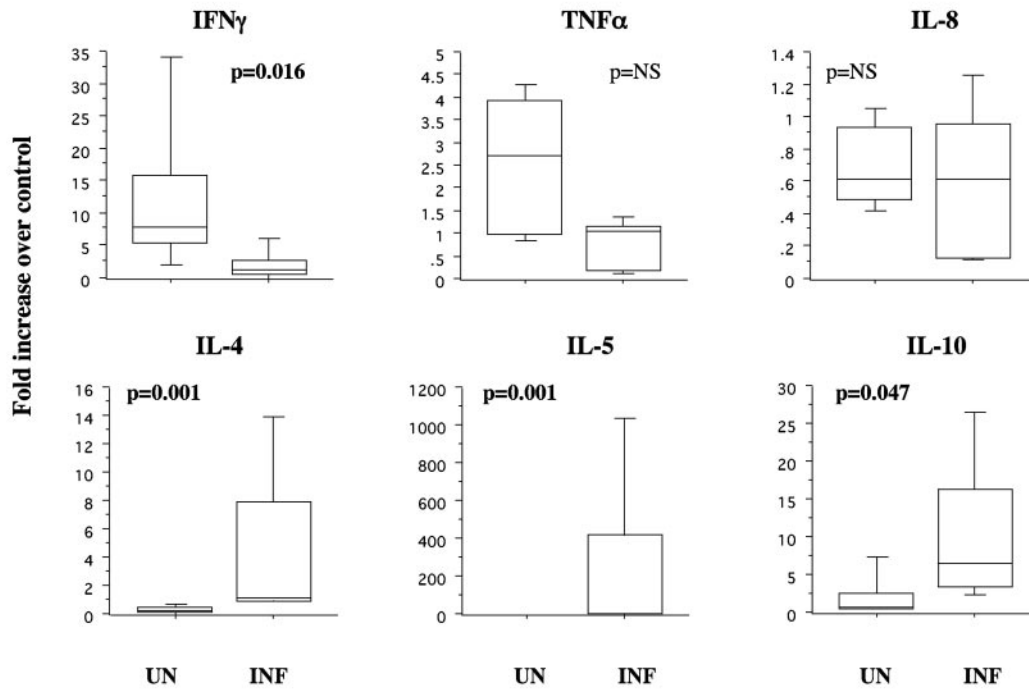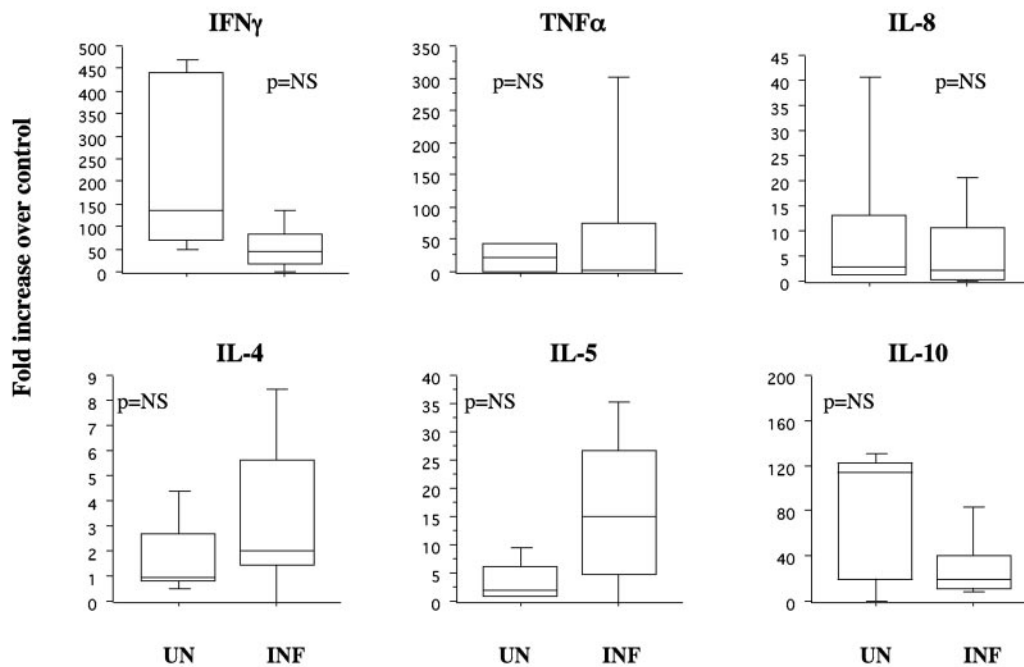
FIG. 1. Th1 and Th2 cytokine mRNA expression in INF and UN. (A) T-cell expression of IFN-γ, TNF-α, IL-8, IL-4, IL-5, and IL-10 levels after 24 h of stimulation with BmA is depicted as the n-fold change over the medium control in UN (n = 6) and INF (n = 6). (B) T-cell expression of IFN-γ. TNF-α, IL-8, IL-4, IL-5, and IL-10 levels after 24 h of stimulation with anti-CD3 is depicted as the n-fold change over the medium control. The results are expressed as box plots with the horizontal lines representing the 25th, 50th, and 75th percentiles and the vertical lines representing the 10th and 90th percentiles of the data. P values were calculated using the Mann-Whitney test. NS, not significant.
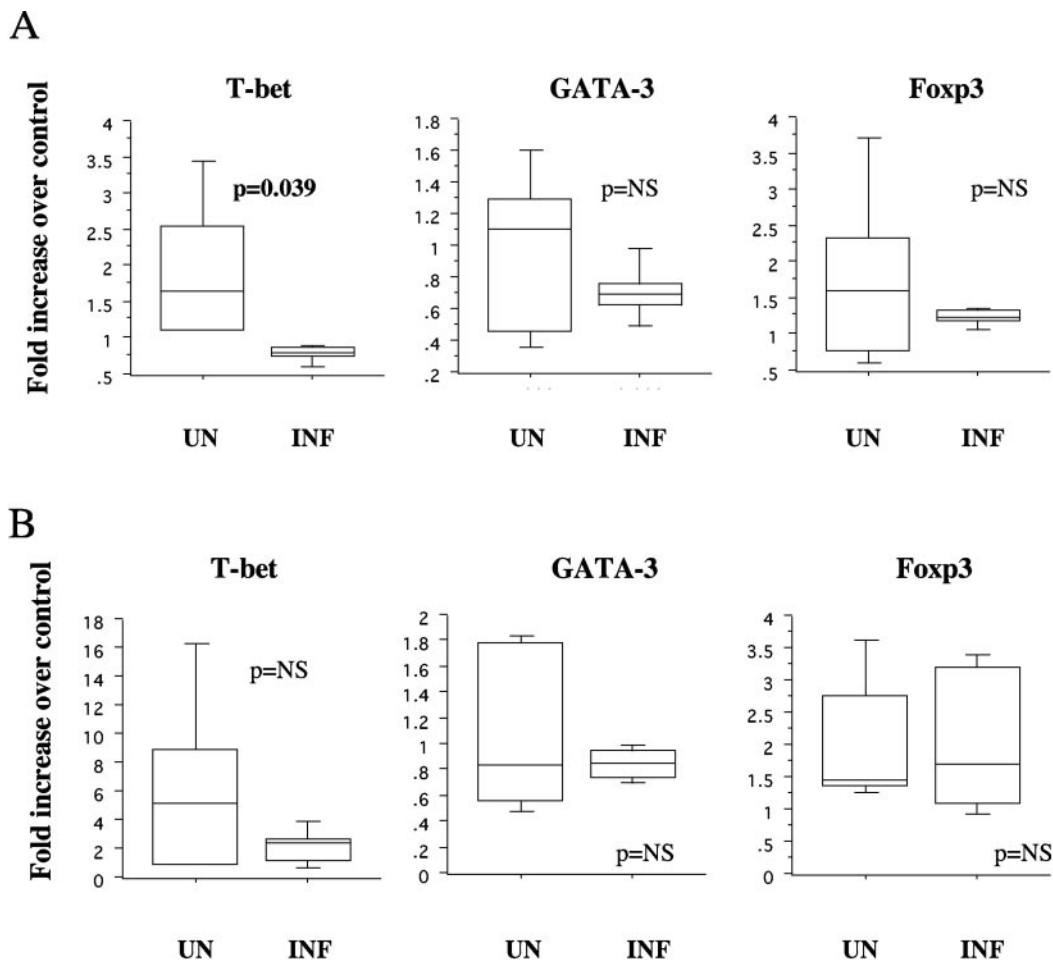
A

B

FIG. 2. Expression of T-bet, GATA-3, and Foxp3 in filarial infections. (A) T-cell expression of T-bet, GATA-3, and Foxp3 following 24 h of stimulation with BmA is depicted as the *n*-fold change over the medium control. (B) T-cell expression of T-bet, GATA-3, and Foxp3 following 24 h of stimulation with anti-CD3 is depicted as the *n*-fold change over the medium control. The results are expressed as box plots with the horizontal lines representing the 25th, 50th, and 75th percentiles and the vertical lines representing the 10th and 90th percentiles of the data. *P* values were calculated using the Mann-Whitney test. NS, not significant.

1.1 versus 2.7) was significantly lower in INF (Fig. 3A). The expression levels of SOCS-2 (1.7 versus 1.5), SOCS-4 (1.6 versus 1.3), and CIS (1.9 versus 2.1) were not significantly different between the two groups. Moreover, upon anti-CD3 stimulation, the expression levels of CIS (1.9 versus 2.9), SOCS-1 (2.0 versus 2.8), SOCS-2 (4.3 versus 3.7), SOCS-3 (0.9 versus 1.5), SOCS-4 (1.1 versus 1.7), SOCS-5 (0.6 versus 0.4), and SOCS-7 (0.7 versus 0.5) were not significantly different between the two groups (Fig. 3B).

Foxp3 induction in T cells correlates with IL-10 expression. Because Foxp3 is known to be exclusively expressed in natural regulatory T cells (Tregs) (6) and because Treg production of IL-10 plays an important role in T-cell downmodulation in filariasis (21), we examined the correlation between the increase in Foxp3 and the increase in IL-10 in both INF and UN (*n* = 12). As shown in Fig. 4, we observed a significant correlation between the antigen-induced levels of Foxp3 and IL-10 in this cohort of individuals (*P* = 0.0012; *r* = 0.979), indicating that the presence of antigen-reactive regulatory T cells can be directly correlated to the production of IL-10.

DISCUSSION

Patent lymphatic filariasis is characterized by diminished antigen-specific proliferative, IFN-γ, and IL-2 responses (8) with generally intact Th2-like responses. Thus, stimulation of peripheral blood mononuclear cells from INF by BmA resulted in the production of IL-4, IL-5, and IL-10 but not of IFN-γ or IL-2 and in decreased T-cell proliferation (7, 16). This phenomenon is highly antigen specific, because stimulation of lymphocytes of these same INF with polyclonal stimuli (e.g., mitogens [16] or anti-CD3) or nonfilarial antigens (16) demonstrates normal production of both Th1- and Th2-like cytokine production. In contrast, UN in endemic areas usually exhibit robust Th1 responses and normal T-cell proliferation (2). Although the phenomenon of antigen-specific Th1 suppression in lymphatic filariasis has been well described for more than a decade, the molecular machinery underlying this regulation is still not clear. Filarial parasites have profound regulatory effects on antigen-presenting cells with downregulation of both macrophage and dendritic cell functions, which
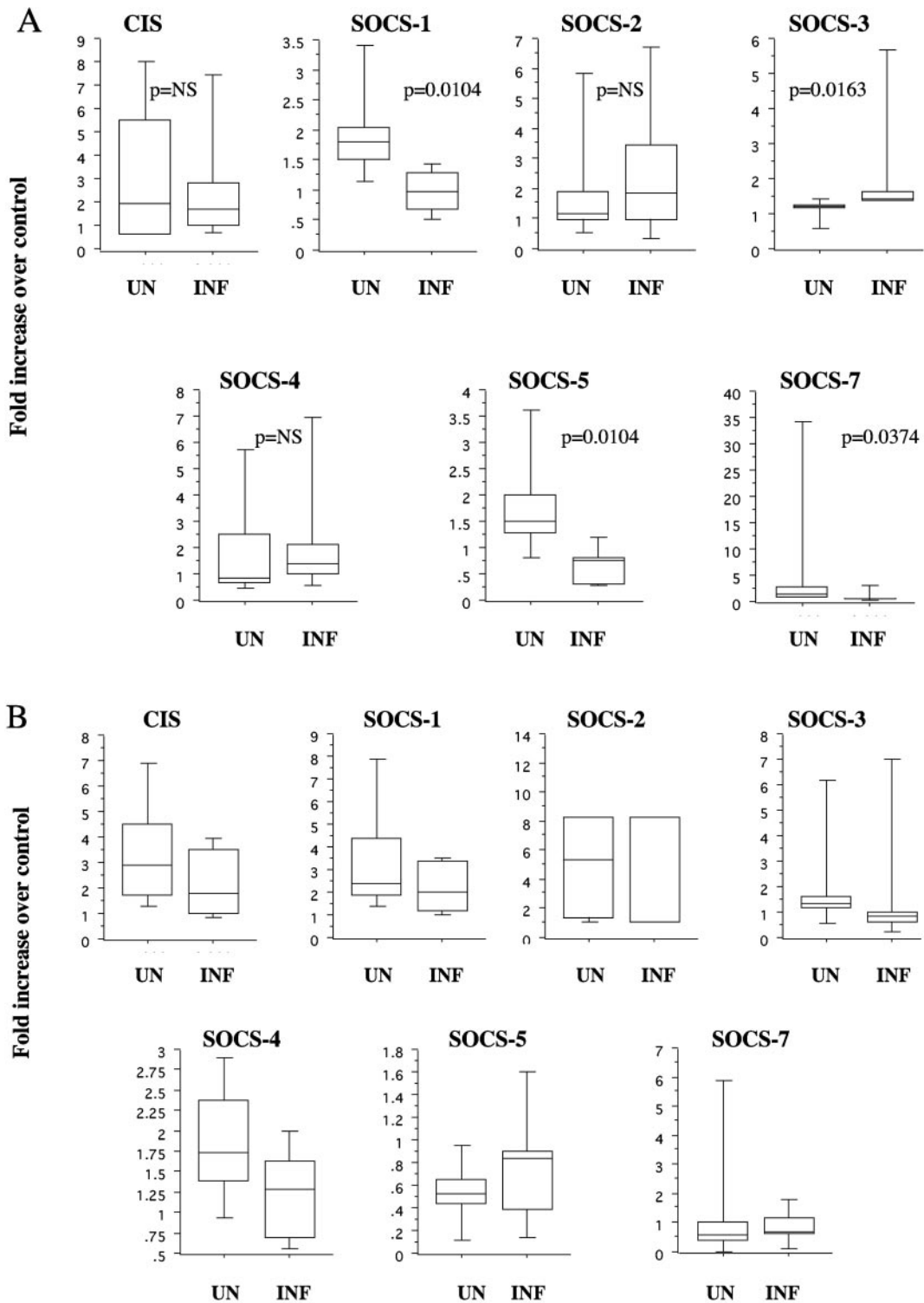
FIG. 3. Expression of CIS, SOCS-1, SOCS-2, SOCS-3, SOCS-4, SOCS-5, and SOCS-7 in filarial infections. (A) T-cell expression of CIS, SOCS-1 to SOCS-5, and SOCS-7 following 24 h of stimulation with BmA is depicted as the n-fold change over the medium control. (B) T-cell expression of CIS, SOCS-1 to SOCS-5, and SOCS-7 following 24 of h stimulation with anti-CD3 is depicted as the n-fold change over the medium control. The results are expressed as box plots with the horizontal lines representing the 25th, 50th, and 75th percentiles and the vertical lines representing the 10th and 90th percentiles of the data. P values were calculated using the Mann-Whitney test. NS, not significant.
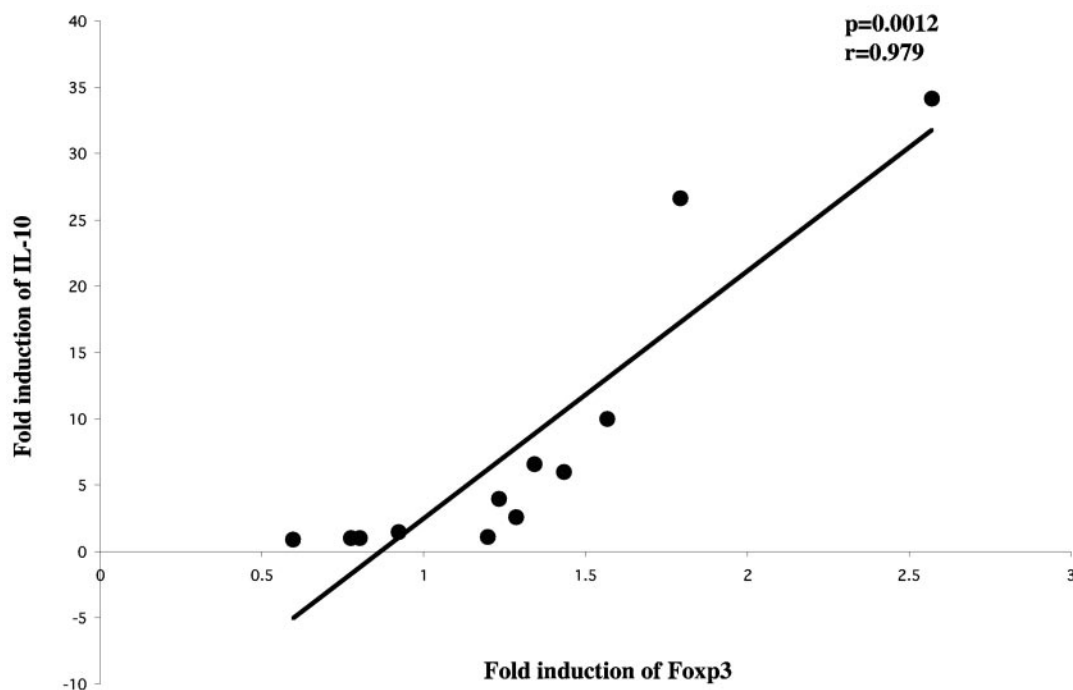
FIG. 4. Correlation between Foxp3 induction and IL-10 in endemic individuals. The *n*-fold induction of Foxp3 by BmA in the cohort of INF and UN (*n* = 12) was measured and plotted against the *n*-fold induction of IL-10 mRNA expression by BmA. Spearman rank correlation was used to measure the correlation between the variables.

in turn profoundly influence the function of cognate T cells (24). In addition, the regulatory cytokines IL-10 and transforming growth factor beta have been shown to mediate, at least in part, the diminished proliferation and IFN-γ production by T cells in patent filarial infection (7).

To understand the molecular basis of the downregulation of Th1-like responses in filarial infection, we studied the cytokine responses of a cohort of INF and UN to BmA and anti-CD3. Our study relied on semiquantitative measurements of mRNA to infer regulatory mechanisms in filariasis. In confirmation of previous studies (7, 11, 12, 19), BmA stimulation was found to induce a profound diminution at the mRNA level in IFN-γ expression but a significant increase in IL-4, IL-5, and IL-10 expression in INF. We then examined the molecular pathways presumably involved in the differential cytokine responses. T-bet and GATA-3 are important transcription factors that govern the development of Th1 and Th2 responses, respectively (20). T-bet is expressed exclusively in Th1 cells, and its expression is mediated primarily by a STAT-4-dependent pathway (5, 26). Moreover, T-bet-deficient mice have a profound defect in IFN-γ production (27); retroviral introduction of T-bet in classic Th2 cells results in production of IFN-γ and suppression of IL-4 and IL-5 (26). GATA-3, in contrast to T-bet, is expressed during Th2 differentiation in a STAT-6-dependent pathway (5, 28); GATA-3 deficiency eliminates the development and maintenance of Th2 cells (18). GATA-3 has also been shown to downregulate IFN-γ production, suppress Th1 development, and induce IL-4 and IL-5 production (4, 17). Thus, T-bet and GATA-3 not only control the development of Th1 and Th2 populations, respectively, but also antagonize the development of the alternative arm of Th cell development.

At baseline, the expression levels of T-bet and GATA-3 did not differ significantly between the two groups under study here. Upon BmA stimulation, however, INF T cells exhibited a profound defect in the ability to upregulate T-bet compared with T cells from UN. This suggests that the impaired IFN-γ production in INF is mediated through the diminished induction of T-bet. Of interest, although INF have higher expression levels of IL-4, IL-5, and IL-10 following stimulation with BmA, GATA-3 expression was not significantly different between the two groups. This suggests that GATA-3 expression might play a less crucial role in the induction of recall Th2 responses to antigen in an established, chronic infection. As expected from the cytokine data, anti-CD3 stimulation abolished the differential expression of T-bet and induced no difference in the GATA-3 expression pattern. Thus, T-cell responses in chronic filarial infections are governed at the transcriptional level by the expression patterns of the transcription factor T-bet.

At the phenotypic level, Tregs have been postulated to play an important role in filarial infections (13). Studies have shown that both Tr1 regulatory cells (21) and CTLA-4 (25) are associated with the impaired Th1 response seen in patent lymphatic filariasis. Because Foxp3 is a specific marker for Tregs (6), we sought to examine the expression patterns of Foxp3 in INF and UN. Our results clearly show no difference in the induction or baseline levels of Foxp3 between the two groups, suggesting that Tregs are not major players of immunoregulation in our system; however, we did observe a significant correlation between IL-10 induction and Foxp3 induction in our studies, indicating that Foxp3-expressing Tregs could mediate the increased IL-10 production in filariasis and hence be indirectly responsible for the regulated Th1 response.

SOCS proteins act as negative feedback factors for cytokine signaling by attenuating the signal transduction from cytokine/cytokine receptor interaction that acts through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways (1). There are eight members of the SOCS protein family: CIS and SOCS-1 through SOCS-7. The SOCS proteins can act to cause negative regulation by at least two different mechanisms: targeting signal transducers for proteosomal degradation and inhibiting the catalytic activity function of enzymes by competing with substrates downstream in the signaling pathway. In addition, SOCS proteins have been implicated in the control of balance between Th1 and Th2 cells (3) through unknown mechanisms. SOCS-3 is predominantly expressed in Th2 cells and inhibits Th1 responses (23); conversely, SOCS-1 (3) and SOCS-5 (22) are expressed predominantly in Th1 cells and inhibit Th2 responses (10, 22). The expression patterns and roles of other SOCS family members in T cells are not well defined. In this study, we examined the expression of CIS, SOCS-1 to SOCS-5, and SOCS-7 and demonstrated that all these SOCS genes are expressed in T cells and that, at baseline, they were not significantly different between the two groups of subjects in the present study. Induction of SOCS-1, SOCS-5, and SOCS-7, however, was significantly impaired in T cells following antigen stimulation in INF but not in UN. This suggests that the Th2 response may be indirectly mediated in INF by downregulation of Th2 inhibitors—SOCS-1, -5, and -7. To our knowledge, the present study provides the first evidence that SOCS-7 may be involved in the Th1/Th2 differentiation process and implies that SOCS-7 might be an inhibitor of Th2 responses. In contrast, SOCS-3 is upregulated significantly in INF, implying a role for SOCS-3 in repression of IFN-γ in filarial infections. Also, SOCS-3 is a known mediator of Th2 responses in allergic asthma and atopic dermatitis (23), and its upregulation in filarial infections indicates that it, too, might play an important role in the Th2 responses of parasitic infections. Interestingly, filarial antigens did not induce an upregulation of cytokine genes, T-bet, GATA-3, Foxp3, or the SOCS genes in a limited number of nonendemic, uninfected controls. This suggests that the Th1 downregulation is the effect of chronic exposure to filarial parasites and not an in vitro effect of the filarial antigens.

In conclusion, the downregulation of IFN-γ in filarial infections appears to be mediated in at least two ways at the molecular level: (i) downregulation of T-bet, which is absolutely essential for Th1 induction, and (ii) upregulation of SOCS-3, which can suppress IFN-γ production. In addition, at the phenotypic level, Foxp3-expressing Treg cells might be playing a role in immunoregulation by providing a source of IL-10, which has been shown to modulate cytokine expression and T-cell proliferation in lymphatic filariasis. In addition, our data show that INF are able to mount Th2 responses not because of increased GATA-3 expression but indirectly through the downregulation of Th1-inducing SOCS-1, SOCS-5, and SOCS-7 genes. Our study thus highlights the complex pattern of molecular transcriptional events that control differential cytokine responses in a chronic, tissue-invasive helminth infection.

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