T-bet, GATA-3, and Foxp3 Expression and Th1/Th2 Cytokine Production in the Clinical Outcome of Human Infection with *Leishmania* (Viannia) Species

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**Background.** T cell differentiation determines susceptibility and resistance to experimental cutaneous leishmaniasis, yet mixed Th1/Th2 responses characterize the clinical spectrum of human infection with *Leishmania* (Viannia) species.

**Materials and Methods.** To discern the interrelationship of T cell differentiation and outcome of human infection, we examined factors that regulate T cell differentiation and Th1/Th2 cytokine responses in asymptomatic infection, active and historical chronic and recurrent cutaneous leishmaniasis. T-bet, GATA-3, Foxp3, and cytokine gene expression were quantified by real-time polymerase chain reaction and correlated with interleukin 2, interferon γ, tumor necrosis factor α, interleukin 4, interleukin 13, and interleukin 10 secretion during in vitro response to live *Leishmania panamensis*.

**Results.** Higher GATA-3 expression than T-bet expression occurred throughout the 15 days of coculture with promastigotes; however, neither transcription nor secretion of interleukin 4 was detected. A sustained inverse correlation between GATA-3 expression and secretion of proinflammatory cytokines interferon γ and tumor necrosis factor α was observed in asymptomatic infection. In contrast, higher T-bet expression and a higher ratio of T-bet to GATA-3 characterized active recurrent disease. Down-regulation of T-bet and GATA-3 expression and increased interleukin 2 secretion, compared with control subjects, was directly correlated with Foxp3 expression and interleukin 13 secretion in chronic disease.

**Conclusions.** Regulation of the inflammatory response rather than biased Th1/Th2 response distinguished asymptomatic and recalcitrant outcomes of infection with *Leishmania viannia* species.
Figure 1. Schematic summary of the research strategy to evaluate transcription factor expression and cytokine response profile in relation with outcome of infection with *Leishmania* (*Viannia*) species. ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-γ; IL, interleukin; PBMC, peripheral blood mononuclear cell; qPCR, quantitative polymerase chain reaction; TNF-α, tumor necrosis factor α.

MATERIALS AND METHODS

Study description. T-bet, GATA-3, and Foxp3 expression were examined in recurrent and chronic cutaneous leishmaniasis representing “nonhealing” clinical outcomes and asymptomatic infection as “clinical resistance” (Figure 1). Transcription and secretion of Th1-associated and Th2-associated cytokines were concomitantly evaluated. DTH reactions elicited by intradermal inoculation or in vitro lymphocyte proliferation in response to *Leishmania* antigens are indicative of infection. Specific DTH response is detected in a proportion of endemically exposed individuals in the absence of clinical history or physical evidence of either healed or active leishmaniasis, providing a marker of asymptomatic infection. Historic “nonhealing” disease, based on documented prior parasitologically confirmed chronic or recurrent lesions, was included in the study to determine whether the pattern of cytokines and regulatory mechanisms operating during active disease would be reelicited after resolution of disease.

The rationale of our approach was that an integrated analysis of transcription factors regulating T cell differentiation at the level of gene expression and Th1/Th2 cytokine production in “nonhealing” and “resistant” clinical phenotypes could elucidate the immunological mechanisms that drive the pathogenesis of recalcitrant disease or lead to clinical resistance.

Study population. Thirty-nine individuals—22 men and 17 women—from 18 to 67 years of age participated in the study. Six study groups, each containing 6–7 participants of either sex, were defined as follows: (1) The nonimmune control group were individuals without a history...
of exposure to transmission and a negative leishman skin test or in vitro proliferative response to Leishmania antigen, (2) the asymptomatic infection group were residents of areas where transmission of cutaneous leishmaniasis is endemic who did not have active or scarred lesions attributable to leishmaniasis and had a positive Montenegro skin test result or in vitro proliferative response, (3) the active chronic disease group were patients with active parasitologically confirmed lesions of >3 months duration who had not initiated treatment, (4) the active recurrent disease group were patients with active lesions who had documented prior parasitologically proven lesions that had healed and who had not initiated treatment for their current active lesions, (5) the historical chronic disease group were individuals currently without active lesions who had a documented clinical history of parasitologically confirmed lesions of >3 months duration, and (6) the historical recurrent disease group were individuals currently without active lesions who had a documented clinical history of parasitologically confirmed recurrent disease.

Exclusion criteria for all groups were as follows: age below 18 years, presence of immunosuppressive disease, history of immunosuppressive pharmacotherapy, pregnancy, unwillingness to participate, or unwillingness to have human immunodeficiency virus testing. Eighteen Leishmania isolates recovered from active and historical patients at the time of diagnosis were identified as L. (V.) panamensis.

This study was approved and monitored by CIDEIM and Yale University institutional review boards for the ethical conduct of research involving human subjects in accordance with national and international guidelines. Blood samples of up to 40 mL were obtained by venipuncture. All patients with active lesions were referred for treatment following guidelines of the Colombian Ministry of Social Protection.

Parasites. L. (V.) panamensis strain MHOM/COL/85/1166 was harvested during stationary phase on day 6 of culture for in vitro evaluation of the response of peripheral blood mononuclear cells (PBMCs).

Isolation and culture of mononuclear cells. PBMCs were isolated by centrifugation over Ficoll-hypaque 1.077 (Sigma-Aldrich), and resuspended at 2 × 10^6 cells/mL in Roswell Park Memorial Institute 1640 medium (RPMI 1640) (Sigma-Aldrich) containing 10% fetal calf serum (GIBCO). Live promastigotes were used to generate a Th2-biased control response. Up-regulation of T-bet and GATA-3 expression was confirmed by quantitative real-time polymerase chain reaction (PCR).

RNA extraction and complementary DNA synthesis. RNA was extracted from PBMCs using RNeasy Micro kit (Qiagen), resuspended in 18 µL RNase free water, and stored at −80°C. RNA quality was verified by agarose gel electrophoresis of each sample before preparing complementary DNA, which was generated within 24 h of RNA extraction using random hexamers and M-MLV RT (SuperScript III First-Strand Synthesis System for reverse-transcription PCR, Invitrogen).

Transcription factor and Th1/Th2 cytokine complementary DNA cloning. Complementary DNA encoding human T-bet, GATA-3, Foxp3, β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA, IFN-γ, tumor necrosis factor α (TNF-α), interleukin 10 (IL-10), and interleukin 13 (IL-13) were cloned into Escherichia coli to generate standard curves for quantitative real-time PCR. Total messenger RNA from Th1- and Th2-polarized PBMCs was used to generate complementary DNA by amplification with Taqman primer/probe gene expression assays (Applied Biosystems). Purified PCR products were cloned into PCR 2.1-TOPO vector (Invitrogen) and transformed into competent E. coli DH5-α (Invitrogen). Target DNA–positive colonies were selected by colony PCR using T7/M13 primers. Plasmids were purified using the Wizard Plus Minipreps DNA Purification System (Promega) and concentrations estimated by absorbance at 260 nm. The miniprep product was serially diluted from 10^2 to 10^8 copies and amplified by quantitative real-time PCR.
Validation and selection of housekeeping gene. Three housekeeping genes, 18S ribosomal RNA, GAPDH, and β-actin, were evaluated for normalization of transcription factor and cytokine gene expression. The efficiency of amplification of 18S ribosomal RNA, GAPDH, and β-actin ranged between 1.89 and 1.95. β-Actin was selected for normalization on the basis of the level of expression relative to the genes of interest and low variability under the study conditions (mean cycle threshold ± SE, 24.6 ± 0.8).

Quantitative PCR. Gene expression was determined by using a LightCycler FastStart DNA MasterPLUS (Roche Diagnostics) and primers and probes of the Taqman Gene Expression Assay described above and Fast Start DNA Master Hybridization Probes (Roche Diagnostics). Messenger RNA copy number was calculated using the LightCycler integrated Quantitative Analysis Program software (version 4.0; Roche Diagnostics). Each experiment was internally controlled using ≥1 standard curve and calibrant of known concentration (10⁴ copies/μL). Results were expressed as normalized values of the ratios of cytokine to β-actin and transcription factor to β-actin complementary DNA. Ratio of T-bet/β-actin to GATA-3/β-actin was also determined as an indicator of Th1/Th2 differentiation [26, 27, 32, 33].

Cytokine detection. IL-4, IL-10, TNF-α, and IFN-γ were quantified in supernatant samples obtained from cultures at 72 h using the BDTM CBA human Th1/Th2 cytokine kit II (BD

Figure 2. T-bet and GATA-3 expression in peripheral blood mononuclear cells (PBMCs) from patients with active or historic cutaneous leishmaniasis, asymptptomatically infected residents of endemic areas, and healthy control subjects cocultured during 8 h, 24 h, and 6 days with live promastigotes of Leishmania (Viannia) panamensis. All data were normalized to β-actin. P values were calculated using a Kruskal-Wallis (K-W) test and Dunn procedure. * P < .05. AC, active chronic; AR, active recurrent; AS, asymptomatic; C, healthy control; HC, historic chronic; HR, historic recurrent.
Figure 3. Interrelation of Foxp3 expression, interleukin 2 (IL-2), and interleukin 13 (IL-13) production by peripheral blood mononuclear cells (PBMCs) in cutaneous leishmaniasis. A, Foxp3 expression at 24 h in PBMCs from patients in the active chronic group (AC), the active recurrent disease group (AR), the asymptptomatically infected individuals (AS), the healthy control group (C), the historic chronic group (HC), and the historic recurrent group (HR), cultured with live promastigotes of *Leishmania (Viannia) panamensis*; Kruskal-Wallis Tests, Dunn *P* < .05. Data were normalized to β-actin. B, Net IL-2 secretion. C, Correlation between IL-2 secretion and Foxp3 expression. D, Correlation between IL-2 at 24 h and IL-13 secretion at 72 h.

**Pharmingen** and BD FACSCalibur cytometer. The sensitivity of the kit was 2.6 pg/mL for IL-4, 2.8 pg/mL for TNF-α, and 7.1 pg/mL for IFN-γ. Because concentrations frequently exceeded the upper limit of the CBA kit, IFN-γ, IL-10, and IL-13 were also measured by enzyme-linked immunosorbent assay (ELISA) [34]. IL-2 levels were determined in culture supernatant obtained at 24 h and 72 h using a human ELISA set (BD Pharmingen).

**Data management and statistical analyses.** Analyses of the expression of T-bet, GATA-3, Foxp3, and cytokine production in the different clinical groups were conducted using a non-parametric Kruskal-Wallis test for independent samples. Multiple comparisons were performed using the Dunn procedure. Correlation analysis between the ratios of T-bet/β-actin to GATA-3/β-actin and Foxp3/β-actin and cytokine production were conducted using the Spearman coefficient. *P* < .05 was considered statistically significant. SPSS software (version 7.0; SPSS) was used for all analyses.

**RESULTS**

**T-bet and Gata-3 Expression**

Differences in expression of T-bet and GATA-3 among the study groups were significant (*P* < .01) by 8 h and sustained at 6 days (Figure 2). Both T-bet and GATA-3 were down-regulated in PBMCs from patients with history of chronic or recurrent leishmaniasis. Differences among study groups were evident in the ratio of expression of T-bet to GATA-3 at 24 h (*P* = .019), day 6 (*P* < .003), and day 15 (*P* < .006) (data not shown).

Overall, GATA-3 expression was higher than T-bet expression and varied significantly among the study groups at 8 h, 24 h, and 72 h and at 6 days (Figure 2). Healthy control donors presented the highest GATA-3 expression over the 15 days of observation following exposure to promastigotes (data not found).

**Foxp3 Gene Expression**

On the basis of the kinetics of Foxp3 expression under the experimental conditions, transcription was evaluated at 24 h. Foxp3 gene expression was low (an order of magnitude lower than GATA3 expression) yet varied significantly among the clinical and control groups after exposure to *L. panamensis* (*P* = .006), being highest in the healthy control group (Figure 3A). Foxp3 was down-regulated in historic chronic disease and the historic recurrent disease groups, compared with other study groups.

**Cytokine Secretion**

Exposure of PBMCs to promastigotes from patients with active or historical disease and asymptomatic infection induced the secretion of both Th1 and Th2 cytokines (Figure 4). However, no secreted IL-4 was detected. When cytokine production was analyzed without distinction of clinical outcome, IL-2, TNF-
Figure 4. Net cytokine secretion by peripheral blood mononuclear cells (PBMCs) from patients with active and historic cutaneous leishmaniasis, asymptomatically infected residents of areas where the disease is endemic, and healthy control subjects cultured for 72 h with live promastigotes of *Leishmania (Viannia) panamensis*. *P* values were calculated using a Kruskal-Wallis test and Dunn procedure. *P* < .05. Net production was calculated by subtraction of background (PBMC plus medium) from cytokine production in the presence of live promastigotes. AC, active chronic disease group; AR, active recurrent group; AS, asymptomatic individuals group; C, healthy control group; HC, historic chronic disease group; HR, historic recurrent disease group; IFN-γ, interferon γ; IL, interleukin; TNF-α, tumor necrosis factor α.

α, IFN-γ, and IL-13 levels were all positively correlated (r = 0.527–0.885; *P* = .001), whereas IL-10 secretion correlated only with TNF-α (r = 0.543; *P* = .001) and IFN-γ levels (r = 0.620; *P* = .001).

Cytokine Gene Expression

Transcription of IL-4 was 3–4 orders of magnitude lower than IFN-γ, TNF-α, IL-13, and IL-10. When all participants were included in the analysis, the transcription and secretion of IFN-γ (r = 0.572; *P* = .001), TNF-α (r = 0.328; *P* = .048), and IL-13 (r = 0.370; *P* = .022) were positively correlated. In contrast, transcription and production of IL-10 were inversely correlated (r = −0.350; *P* = .031), indicating distinct regulatory control of this cytokine.

Relationship of Transcription Factor Expression and Th1/Th2 Cytokine Transcription and Secretion in Infection and Disease

Asymptomatic infection. A consistently high and significant negative correlation was observed between GATA-3 expression and proinflammatory cytokines TNF-α and IFN-γ during recall response of PBMCs from asymptomatically infected individuals. This relationship prevailed over the entire period of observation from 8 h to 15 days after exposure to *L. panamensis* (Figure 5A, 5B). Conversely, the ratio of T-bet/β-actin to GATA-3/β-actin was positively correlated with TNF-α production at all time points (r = 0.886; *P* = .019 at 8 h and 24 h; r = 0.829, *P* = .042 at 6 days; r = 0.943, *P* = .005 at 15 days; data not shown) and with IFN-γ at 6 and 15 days (r = 0.812; *P* = .050). Secretion of these proinflammatory cytokines was also positively correlated during asymptomatic infection (r = 0.899, *P* = .015). The coordinate production of these 2 cytokines and correlation with
Figure 5. Inverse correlation between the ratio of GATA-3 to β-actin expression and the level of secretion of tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) in patients with asymptomatic infection with Leishmania (Viannia) species. A, Regression analysis of TNF-α secretion (pg/mL), vs GATA-3 expression at 8 h, 24 h, 6 days, and 15 days of culture of peripheral blood mononuclear cells (PBMCs) with live promastigotes of L. (V) panamensis. B, Regression analysis of IFN-γ secretion at 8 h, 24 h, 6 days, and 15 days of culture of PBMCs with live promastigotes of L. (V) panamensis.

dest the ratio of T-bet/β-actin to GATA-3/β-actin is consistent with a regulated proinflammatory response.

**Active chronic disease.** Although T-bet expression was low, the ratio of T-bet to β-actin was highly and significantly correlated with IL-2 secretion from 24 h to 15 days in active chronic disease (Figure 6). IL-2 secretion was further distinguished in this patient group by being significantly higher than the control group at 24 h (Figure 3B) and positively correlated with Foxp3 expression (Figure 3C) and IL-13 secretion (Figure 3D), which are associated with the development of regulatory T cells.

**Active recurrent disease.** This patient group presented the highest T-bet expression throughout the period of observation and the highest ratio of T-bet/β-actin to GATA-3/β-actin, which was significantly higher than that of healthy control subjects at 24 h, 6 days, and 15 days (data not shown) of culture with L. panamensis (P < .05) (Figure 2). Prominent T-bet transcription was not accompanied by a Th1-biased cytokine response, rather a mixed Th1/Th2 response including correlation of TNF-α and IL-13 transcription (r = 0.829, P = .042). Transcription factor expression was not correlated with cytokine secretion in this group of patients, suggesting dysregulation of the cytokine response.

**Historic recurrent and chronic disease.** Transcription factor expression was remarkably down-regulated in these patient groups. PBMCs from patients who had long recovered from chronic or recurrent disease presented low transcription factor expression. Both GATA-3 (Figure 2) and Foxp3 expression (Fig-
ure 3A) were significantly lower in historic disease than the healthy control group. Transcription of the housekeeping gene β-actin confirmed that gene expression was not impaired. Cytokine gene expression was also low, except IFN-γ, which was significantly higher (P < .05) for historic recurrent disease than healthy controls at 24 h of coculture (data not shown). Despite the low level of gene transcription, historic “nonhealing” disease groups presented the highest levels of secretion of Th1 and Th2 cytokines (Figure 4).

**DISCUSSION**

This study revealed distinctive interrelationships between transcription factors that regulate T cell differentiation, and cytokine expression and secretion during asymptomatic infection, active chronic and recurrent disease, and after clinical resolution of the latter disease presentations. Differences in T cell differentiation in relation with clinical outcomes were not discernible by cytokine gene transcription or secretion alone. The results also established that production of IL-4 was not induced during in vitro recall in human infection with *L. panamensis*; rather, production of IL-13 was induced.

Asymptomatic infection was distinguished by a controlled proinflammatory response that was inversely correlated with GATA-3 transcription, and positively correlated with the ratio of T-bet/β-actin to GATA-3/β-actin. This finding concurs with the report of lower proinflammatory cytokines in asymptomatic infection with *L. braziliensis* [18]. Validation of the inverse relationship between GATA-3 expression and secretion of TNF-α and IFN-γ as a marker of clinical resistance could provide a correlate of protective response.

Although both active chronic and recurrent disease presented mixed Th1/Th2 cytokine responses, concomitant analysis of transcription factor expression yielded evidence of ongoing negative regulation of the proinflammatory response in chronic disease, and positive regulation in recurrent disease. High IL-2 secretion was elicited in PBMCs during active chronic disease, and IL-2 correlated with Foxp3 expression. In contrast, T-bet transcription and the T-bet/β-actin to GATA-3/β-actin ratio were highest in active recurrent disease. These findings indicate involvement of distinct immunoregulatory mechanisms in these 2 “nonhealing” manifestations of human cutaneous leishmaniasis caused by *Leishmania (Viannia)* species. Mucosal leishmaniasis, which is also a nonhealing manifestation if untreated, has been associated with defective regulation of a Th1 type CD4+ mediated response [23].

IL-2 has recently been shown to participate in the induction of the negative transcriptional regulator Foxp3, Treg cell development, expansion, and maintenance [35–37]. The positive correlation between IL-2 and Foxp3 in active chronic cutaneous leishmaniasis that we found in this study is consistent with this finding and encourages the investigation of the participation of Treg cells during chronic cutaneous leishmaniasis. In experimental infection of BALB/c mice with *Leishmania major*, IFN-γ producing CD4+CD25− effector lymphocytes and Treg cells (Foxp3+, CD4+CD25+) producing IL-10 accumulated in the proinflammatory site of infection [38]. IL-10–producing CD4+CD25− Foxp3+ TTh1 cells are also an important source of antiinflammatory IL-10 and contribute to the persistence of *Leishmania* [39]. Although Treg cells have been detected in acute cutaneous leishmaniasis lesions [40], their role in the resolution or perpetuation of human leishmaniasis is unknown.

The prominent expression of T-bet and high ratio of T-bet/β-actin to GATA-3/β-actin elicited during active recurrent cutaneous disease suggests that reactivation of disease may involve a T-bet regulated shift in response from the immunologic homeostasis achieved during asymptomatic infection or following resolution of lesions. Significantly lower cutaneous DTH responses to leishmanin were found in patients with recurrent disease than in patients with chronic disease [3], and immunosuppressive disease can lead to reactivation of cutaneous and mucosal leishmaniasis [41]. However, because inflammatory stimuli also induce activation of leishmaniasis in asymptotically infected humans [12] and experimental models [13, 14], the observed up-regulation of T-bet expression during reactivation could signal an ongoing proinflammatory response.

The profuse secretion of Th1 and Th2 cytokines and low cytokine gene and transcription factor expression elicited by restimulation of PBMCs from donors who had resolved prior chronic or recurrent disease likely corresponds with a long-term memory response. In contrast with primary activation of T helper cells, which requires T cell receptor signaling, costimulation, and differentiation signaling, antigen–experienced cells reexpress cytokines with only T cell receptor stimulation and cytokine memory [42]. Supporting this interpretation, transcription and secretion of cytokines were not correlated in the groups of patients with a prior history of disease.

We did not detect IL-4, the principal cytokine driving the differentiation of specific CD4+ T cells toward Th2 development, at either transcriptional or protein levels in PBMCs from the different clinical groups. In contrast, IL-13 was detected in all clinical groups at both levels of analysis, perhaps due in part to up-regulation of IL-13 production by GATA-3 [43]. IL-4 has been detected with low frequency or been absent in most reports on cytokine response in human cutaneous leishmaniasis [20, 21, 44]. Infecting species of *Leishmania* could conceivably influence the repertoire of cytokines elicited and corresponding regulatory mechanisms; however, this has not been evaluated. Because IL-13 shares many functions with IL-4 [21, 45, 46], it may effectively replace IL-4 in human cutaneous leishmaniasis caused by *L. (Viannia)* species.

IL-4 and IL-13 negatively regulate proinflammatory responses, yet IL-13 together with IL-2 enhances IFN-γ produc-
tion by natural killer cells [47], which produce interleukin 12 and IFN-γ on exposure to live *Leishmania* promastigotes [48]. Concomitantly high levels of IFN-γ, IL-2, and IL-13 observed during the recall response of human PBMCs to live promastigotes might reflect such a synergistic effect. The importance of IL-13 in pathogenesis is substantiated by the conversion of resistant C57BL/6 mice susceptible to *L. major* by overexpression of IL-13 [49]. Evidence that IL-13 contributes to pathogenesis has also been reported for human cutaneous leishmaniasis caused by *Leishmania guyanensis* [21]. In the current study, IL-13 was secreted by PBMCs of participants in all study groups, but correlation of expression and secretion of IL-13 was found only in active chronic and recurrent disease. This finding does not allow conclusions to be drawn regarding the role of IL-13 in these nonhealing presentations. Nevertheless, the relationship of this antiinflammatory cytokine with regulatory T cells merits investigation because IL-13, as well as IL-4, can induce CD25+CD4+ regulatory T cells from CD25−CD4+ precursors in an Ag-dependent manner [48] and regulatory T cells produce IL-13 [50].

This study provides evidence of regulatory differences in the T cell–mediated response in humans during chronic and recurrent disease and asymptomatic infection with *Leishmania Viannia* species. Therapeutic interventions targeting regulatory mechanisms may allow more effective management of clinically susceptible individuals.

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