Members of the genus *Leishmania* are protozoan intracellular parasites that cause a large spectrum of diseases in humans, including cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML), visceral leishmaniasis (VL), and mixed forms. The clinical presentation depends on the infecting species and the host’s immune response (23). American CL usually presents with one or more ulcerated skin lesions that may persist for months or years and eventually self-cure, leaving atrophic scars. In CL, treatment is indicated to accelerate the cure rate, reduce the size of scars, prevent relapses, and prevent dissemination to mucosae (42). In contrast, ML is a debilitating disease with a poor response to treatment. In ML, the pretreatment levels of cytokine mRNAs did not discriminate between responders and nonresponders; however, treatment was more often accompanied by a reduction in the levels of T-cell and cytokine mRNAs in responders than in nonresponders. Overall, these findings point to complex relationships among New World *Leishmania* parasites, skin and mucosal immune responses, and treatment outcome. The persistence of high levels of IL-10 in CL is characteristically associated with a poor response to treatment.

The mechanisms linking the immune response to cutaneous and mucosal leishmaniasis (CL and ML, respectively) lesions and the response to treatment are incompletely understood. Our aims were to prospectively assess, by quantitative reverse transcription-PCR, the levels of mRNA for gamma interferon, tumor necrosis factor alpha, interleukin-10 (IL-10), IL-4, and IL-13, as well as the presence of T cells (CD2) and macrophages (CD68), in CL and ML lesions and to follow their changes in response to treatment with pentavalent antimonials. The leishmanin skin test (LST) was performed on all CL and ML patients before treatment. The patient population included individuals living in areas of Peru where the disease is endemic, i.e., 129 with CL and 43 with ML. Compared to CL patients, the LST induration size was larger, the levels of all cytokine mRNAs but IL-10 were higher, T-cell mRNA was similar, and macrophage mRNA was lower in ML patients. The proportion of CL patients with an LST induration size of >8 mm was higher among responders to treatment. In CL, the pretreatment levels of cytokine mRNAs did not discriminate between responders and nonresponders; however, treatment was more often accompanied by a reduction in the levels of T-cell and cytokine mRNAs in responders than in nonresponders. Furthermore, the production of cytokines per T cell and macrophage decreased with treatment but IL-10 production remained high in nonresponders. Overall, these findings point to complex relationships among New World *Leishmania* parasites, skin and mucosal immune responses, and treatment outcome. The persistence of high levels of IL-10 in CL is characteristically associated with a poor response to treatment.
hanced replication in IFN-γ-stimulated murine macrophages (2, 35, 40). Furthermore, *L. braziliensis* causes small, nonulcerative, and self-healing skin swelling in both C57BL/6 and BALB/c mice and results in skin ulcers only in IL-12−/− or STAT4−/− mice (38).

The precise role of Th1 and Th2 polarized responses in the healing and persistence of human leishmaniasis remains unclear (23). When semiquantitative PCR detection methods have been used, type I cytokine and TNF-α mRNAs were detected not only in localized and recent but also in diffuse and chronic CL lesions. In contrast, type 2 cytokine mRNAs were preferentially identified in the latter (9, 20, 24, 34). Further, a correlation was found between high intraleSIONAL levels of IL-10 and a poor response to treatment in *L. guyanensis* infection (7). The mucosal forms are characterized by an exacerbated cell-mediated immune response and high intraleSIONAL levels of TNF-α, IFN-γ, and IL-4, leading to extensive tissue destruction but not to parasite clearance (3, 5, 34, 47). A defect in IL-10 receptor expression was shown to correlate with high inflammatory responses in ML (14). Finally, Treg cells and high IL-10 production potential have been described in the skin of individuals infected with *L. braziliensis* (10).

Within the framework of LeishnaDrug-R, a multicenter study on ShV treatment failure in leishmaniasis, we prospectively searched for immunological determinants of treatment outcome among Peruvian patients. We asked whether the leishmanin skin test (LST) and the pattern of cytokines present in the skin and mucosal lesions could predict treatment outcome. We used real-time quantitative PCR (Q-PCR)-based methods to detect the levels of prototypic type 1 and 2 cytokine mRNAs, as well as of CD2 and CD68 mRNAs as surrogates for the presence of T cells and macrophages, respectively, before and 10 days after treatment initiation.

**MATERIALS AND METHODS**

**Patients.** Patients were recruited at the Institute of Tropical Medicine Alexander von Humboldt (ITMAvH) in Lima, Peru. The diagnosis of CL or ML was confirmed by direct microscopic observation of parasites on stained smears, by positive culture, or by positive PCR-based detection. The LeishnaDrug-R study protocol was approved by the ethical committees of ITMAvH and the Institute of Tropical Medicine, Antwerp, Belgium. An addendum to the LeishnaDrug-R protocol for immunological investigations was approved by the ITMAvH ethical committee. Written informed consent was obtained from all individuals. The evolution of lesions was clinically assessed at the end of treatment and during subsequent follow-up visits for 12 months as described previously (4). Only skin or mucosal biopsy samples from patients with a clear clinical outcome upon the completion of a course of ShV were eligible for quantitative reverse transcription-PCR analysis. First-line treatment was based on 20 mg/kg/day of ShV (sodium stibogluconate or meglumine antimoniate) given intravenously for 20 days in CL and 30 days in ML. Skin biopsy samples were also taken from 10 healthy individuals (healthy control group) attending a plastic surgery clinic in Geneva, Switzerland.

**LST.** Leishmanin antigens were prepared at ITMAvH as described previously (26). Forty-eight hours after intradermal injection of the antigen (0.1 ml), induction size was measured. The test was considered positive when the diameter of the induration was ≥5 mm.

**RNA preparation from skin and mucosal biopsy samples.** Biopsies were performed by punch (4 mm) at the margins of skin or mucosal lesions at day 0 (before treatment) and at day 10 of treatment. The samples were stabilized in 1 ml of RNAlater reagent (Qiagen) and stored at −80°C until processing. Total RNA was isolated with TRIzol reagent (Life Technologies) according to the manufacturer’s protocol and suspended in 30 μl RNase-free water. All samples were DNase treated with the DNA-free kit (Ambion) to remove possible contaminating genomic DNA. The quality of the resulting RNA was determined with the RNA 6000 Nano Labchip kit on a Bioanalyzer 2100 (version A02.12 SI 292; Agilent Technologies).

**Real-time Q-PCR.** One microgram of total RNA was reverse transcribed with SuperScript II (Invitrogen) and random primers. The cDNA obtained was diluted 1:24 and subsequently amplified with TaqMan assay reagents (Universal PCR Master Mix Buffer) in a final volume of 10 μl with primer sets for the human IFN-γ, IL-4, IL-10, IL-13, TNF-α, CD2, CD68, glyceraldehyde-3-phosphate dehydrogenase, and 18S rRNA from Applied Biosystems (Foster City, CA) and human TATA binding protein (TBP), glucuronidase B (GusB), and 5-aminolevulinic acid synthase 1 (ALAS-1) from the Genomic Platform (NCCR Frontiers in Genetics, University of Geneva). Reactions were run on the ABI PRISM 7900HT sequence detection system (Applied Biosystems) with the following thermal profile: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Negative controls for cDNA synthesis (i.e., without reverse transcriptase) were included for each gene. O-PCR efficiencies for all genes were higher than 90%, as assessed by standard curves based on cDNA serial dilutions. All reactions were done in triplicate, and the arithmetic average cycle threshold (CT) was used for data analysis.

**Data analysis and statistics.** The interassay variability of the internal standards was 0.7% for IFN-γ, 0.8% for IL-4, 0.9% for IL-10, 0.5% for IL-13, 1.1% for TNF-α, 1.4% for CD2, 2.0% for CD68, 1.7% for TBP, 0.9% for GusB, and 0.5% for ALAS-1. The (nonnormalized) relative quantities were calculated with the formula E−ΔCT, with E equaling the O-PCR efficiencies determined and ΔCT corresponding to the Ct of the standard sample minus the Ct of the test sample. The standard sample was prepared by mixing equal amounts of cDNA originating from 15 different skin biopsy samples. These raw (nonnormalized) relative quantities for all genes were processed with the geNorm VBA add-in for MS Excel to determine the three most stably expressed genes (those for TBP, GusB, and ALAS-1), which were subsequently used to determine the normalization factor for each sample as described by Vandesompele et al. (46).

The normalized relative quantity (RQ) or expression level was assessed for each cytokine, CD2, and CD68 in biopsy samples taken at days 0 and 10 of treatment. For each skin sample, the ratios of the RQs of the various cytokines and the RQ of CD2 or CD68 were computed to estimate the production of cytokine per T cell or macrophage, respectively.

Statistical analysis was performed with the SPSS software, version 11.0, and GraphPad Prism, version 4. Categorical variables were compared by using cross-tabulations and chi-square tests, whereas numerical variables (means) were compared by using analysis of variance or the Student t test at a critical α level of 0.05. All P values were two sided.

**RESULTS**

**Clinical outcome and patient group classification.** Two hundred twenty-one patients with CL (155 males and 66 females, aged 3 months to 85 years with ± standard deviation, 29 ± 19 years), were included in the LeishnaDrug-R study between April 2003 and December 2004. Of the 221 enrolled patients with CL, 129 (58%) had a biopsy sample of a skin lesion taken before treatment. No skin biopsy was performed on 92 (42%) patients for various reasons (e.g., patient’s refusal, lesion on the face). Exclusion criteria for performing mRNA studies on skin biopsy samples were incomplete treatment or treatment with drugs other than ShV (n = 9), unclear clinical outcome (n = 34), and nonavailability of biopsy material (n = 22; 7 before treatment, 15 at day 10 of treatment). CL patients were subdivided into three groups. Group A included patients definitely cured (n = 62), group B included patients with treatment failure (n = 11), and group C included patients with previous failure to respond to antimonial therapy (n = 8). In group A, 37 random samples were tested at day 0, of which 21 were tested at day 10 of treatment. In group B, all 11 samples were tested at day 0 and 7 were tested at day 10 (no biopsy material was available at day 10 for 4 of them).

Forty-three patients with ML, 40 males and 3 females aged 9 to 72 (mean ± standard deviation, 39 ± 15) years, were
enrolled in the study. Twenty-two (51%) had a biopsy of a mucosal lesion done at baseline. After the exclusion of 11 patients with incomplete or non-SbV-based treatment (n\textsuperscript{-11005}/110) or with an unclear clinical outcome (n\textsuperscript{-11005}/4), patients subsequently cured formed group D (n\textsuperscript{-11005}/8) and patients with subsequent treatment failure formed group E (n\textsuperscript{-11005}/3). All but one had a previous history of untreated CL. We tested biopsy samples taken at day 10 of treatment from 10 patients, 7 in group D and 3 in group E. Skin biopsy samples from healthy individuals undergoing abdominoplastic surgery for esthetic reasons and living in Geneva (Switzerland) were included as normal controls (group H, n\textsuperscript{-11005}/10).

**LST.** The LST was performed before treatment on 188 patients with CL (85%). After the exclusion of patients with a prior history of leishmaniasis, with treatment differing from SbV, or with an unclear clinical outcome, 163 patients were included in the analysis. The proportions of positive LST results were 89.5 and 96.7% (P\textsuperscript{-11005}/0.31) of 133 patients definitely cured and 30 patients with treatment failure, respectively. The proportion of patients with an LST induration size of ≥8 mm was significantly higher (P\textsuperscript{-11005}/0.008) in patients definitely cured (66.2%) than in patients with treatment failure (40%). The LST was performed on 36 patients with ML (84%). A positive reaction was found in 34 (94.4%) of the 36 patients. In patients with a positive LST result, the induration size was significantly larger in ML patients (median, 13 mm; range, 5 to 35 mm) than in CL patients (median, 9 mm; range, 5 to 30 mm; P\textsuperscript{-11005}/0.001). Thus, in CL patients, LST positivity does not predict the response to treatment but induration size may do so.

**Detection and quantification of intralesional cytokine mRNA before treatment.** To identify immunological parameters capable of predicting response to treatment, we prospectively compared the relative mRNA levels of inflammatory cytokines in the skin and mucosal lesions across different groups of leishmaniasis patients before treatment and in healthy controls. In CL patients at baseline, the skin lesion levels of IFN-γ, TNF-α, IL-4, IL-13, and IL-10 mRNAs were similar in groups A and B. However, TNF-α (difference of normalized RQ = 0.301; 95% confidence interval [CI], 0.069 to 0.533 [P\textsuperscript{-11005}/0.02]) and IL-13 (difference of normalized RQ = 1.7; 95% CI, 0.856 to 2.544 [P\textsuperscript{-11005}/0.001]) mRNA levels were significantly higher in patients with previous treatment failure (group C) than in group A (Fig. 1). All CL lesional tissues were positive for these cytokines. In contrast, cytokines were inconsistently detected in healthy skin, of which 40% of the samples were positive for IFN-γ, 10% were positive for IL-4, 80% were
positive for IL-10, 80% were positive for IL-13, and 90% were positive for TNF-α. When detected, IFN-γ, IL-4, IL-10, and TNF-α mRNA levels were significantly lower in healthy skin than in CL lesions (IFN-γ, IL-4, and IL-10, \( P < 0.001; \) TNF-α, \( P = 0.001 \)) (Fig. 1).

Interestingly, pretreatment levels of IFN-γ, IL-4, IL-13, and TNF-α mRNAs were higher in ML than in CL lesions (\( P \leq 0.001 \)), whereas IL-10 levels were lower (\( P = 0.01 \)). Among ML patients, those who would fail treatment (group E) had significantly higher levels of TNF-α (difference of normalized RQ = 0.57; 95% CI, 0.05 to 1.09 \( [P = 0.03] \)) than did those subsequently cured (group D) (Fig. 1).

Overall, these data indicate that there is a large degree of heterogeneity in the levels of cytokines detected in mucocutaneous lesions of leishmaniasis patients which reflect the tissue site of the lesion and, at least in part, previous and future responses to treatment.

Detection and quantification of intralesional cytokine mRNA at day 10 of treatment. We then assessed the impact of 10 days of treatment with SbV on cytokine mRNA levels in skin and mucosal lesions. As expected, the mRNA levels of all cytokines decreased with treatment; however, interesting differences were observed between CL individuals subsequently cured (group A) and those with subsequent treatment failure (group B) since all cytokine levels decreased more in group A than in group B, a difference that was statistically significant for IFN-γ \( (P = 0.021) \) and IL-13 \( (P = 0.032) \) (Fig. 2). As observed in patients with CL, also in ML patients all cytokine mRNA levels decreased with treatment, but within the limited number of individuals tested, no differences between responders and nonresponders were found (not shown). The sharper decline in the IL-13 mRNA levels of CL individuals subsequently cured, together with higher baseline levels of IL-13 mRNA in CL individuals with previous treatment failures, indicates a possible role for IL-13 in lesion persistence.

T cells and macrophages in CL and ML patients and healthy controls. To assess the presence and amounts of T cells and macrophages in the skin, we quantified CD2 and CD68 gene expression levels as surrogate markers for T cells and macrophages, respectively. Relative expression levels of CD2 at day 0 of treatment were similar across all of the groups of leishmaniasis patients, while in healthy skin they were much lower (\( P < 0.001 \)). Of interest, the relative expression levels of CD2 decreased significantly (\( P = 0.025 \)) after 10 days of treatment only in group A and not in the others (Fig. 3A). In contrast to CD2, the relative expression levels of CD68 at day 0 were lower in ML than in CL patients (Fig. 3B); however, the CD68 levels did not significantly change with treatment, whatever the outcome was (Fig. 3C).

Relationship between T-cell and macrophage numbers and cytokines in lesional tissues. We then addressed the question of whether cytokine levels in the skin samples were dependent on T-cell and/or macrophage numbers, making the simplified assumption that only CD2- and/or CD68-positive cells contribute to the production of the cytokines we studied. This was the case for IFN-γ \( (r^2 = 0.617) \) TNF-α \( (r^2 = 0.793) \), and IL-10 \( (r^2 = 0.677) \), which correlated significantly with relative CD2 quantities, and for TNF-α \( (r^2 = 0.650) \) and IL-10 \( (r^2 = 0.678) \), which correlated with relative CD68 quantities. The correlation was poor, however, for IL-4 and IL-13. More interestingly, by assessing the amount of cytokines produced per cell, we observed that the production of IL-10 per T cell decreased significantly in CL responders but not in CL nonresponders (Fig. 4). This was in sharp contrast to IFN-γ and TNF-α, which decreased or tended to decrease in both groups of CL patients (Fig. 4). Similarly, the production of IL-10 per macrophage decreased significantly with treatment only in CL responders, while it did not in CL nonresponders (Fig. 5). Overall, these data indicate that in CL, the failure to respond to treatment is preceded by a failure to reduce the number of T cells, which furthermore continue to produce high levels of IL-10.

DISCUSSION

The objective of the present work was to assess whether the pattern of cytokines detected in the skin and mucosal lesions and the LST could predict clinical responses to antimonial treatment among Peruvian patients with cutaneous leishmaniasis. Altogether, we assessed whether CL and ML are characterized by different patterns of cytokine expression. While no single assessed parameter could definitively predict the response to treatment, we found that the proportion of CL...
individuals with an LST induration size of >8 mm was higher among patients subsequently cured by treatment. Furthermore, the levels of the cytokines studied decreased or tended to decrease more in CL patients who would respond to treatment than in those who would not. In this respect, the persistence in the lesions of T cells producing high levels of IL-10 was distinctively characteristic of CL patients nonresponsive to treatment. Additional findings included higher levels of TNF-α and IL-13 mRNAs in CL patients with previous treatment failure and higher levels of IFN-γ, TNF-α, IL-13, and IL-4 mRNAs and lower levels of macrophages (CD68) and IL-10 mRNA in ML patients than in CL patients.

We found a high rate of LST positivity in both responder and nonresponder patients, and in contrast to a previous study conducted in Brazil (32), a negative LST was not associated with an increased risk of treatment failure. Thus, despite the significantly higher proportion of strong reactions (induration diameter of >8 mm) found in CL patients subsequently cured, the LST is not sufficiently discriminative to reliably predict treatment outcome.

To the best of our knowledge, this is the first study in which Q-PCR was used to prospectively assess the levels of inflammatory cytokines and T-cell and macrophage numbers in New World Leishmania infection in relation to the response to treatment. The assumption we made that CD2 and CD68 mRNA levels directly reflect the number of T cells and macrophages may be criticized insofar as in vivo modulation of these markers has not yet been investigated. However, in an in vitro assessment, we observed an excellent correlation between CD2 and CD68 mRNA levels and the numbers of purified T cells and macrophages, respectively (not shown). Previous work in French Guyana, taking advantage of semiquantitative cytokine determination, pointed to high intralesional IL-10 mRNA expression in CL patients as a predictor of unrespon-
siveness to treatment (7). Indeed, IL-10 has been associated with *Leishmania* susceptibility for its capacity to downregulate Th1 responses (7, 28, 29, 31). While we did not find higher levels of IL-10 in lesions of CL patients with previous treatment failure (group C) than in those who would respond to treatment (group A), the persistence of IL-10 production by T cells and macrophages was characteristic of nonresponders among CL patients. In addition, IL-10 levels in diseased skin were found to be much higher than in healthy skin. IL-10 can be secreted by T lymphocytes, macrophages, and stromal cells.

FIG. 4. Changes in cytokine production per T cell during treatment from day 0 to day 10. Cytokine production per T cell was assed as the ratio of the relative gene expression of a cytokine versus the relative gene expression of CD2, a marker for T cells, only for the cytokines IL-10, IFN-γ, and TNF-α, which show a correlation with CD2 expression of >0.5. Group A, CL patients definitively cured by treatment; group B, CL patients with treatment failure.

FIG. 5. Changes in cytokine production per macrophage from day 0 to day 10 of treatment. Cytokine production per macrophage was assessed as the ratio of the relative gene expression of a cytokine versus the relative gene expression of CD68, a marker for macrophages, only for the cytokines IL-10 and TNF-α, which show a correlation with CD68 expression of >0.5. Group A, CL patients definitively cured by treatment; group B, CL patients with treatment failure.
(14), and our findings suggest that, in vivo, IL-10 may indeed play a major role in CL pathology. Our data are in agreement with high levels of IL-10 produced by peripheral blood mononuclear cells in L. braziliensis-infected individuals with active skin lesions (41). Furthermore, it has been reported that splenic T cells from individuals with Indian visceral leishmaniasis have a high potential for IL-10 production and appear to be responsible for the high levels of IL-10 detected in the serum of infected individuals. Of interest, phenotypic characterization indicated that IL-10-producing T cells belong to a subset distinct from that of Treg cells (30). In contrast, Treg cells (CD4+ CD25+ FoxP3+) with high IL-10 production have been identified in the skin of individuals infected with L. amazonensis (10). Our experimental approach was not meant to differentiate among T-cell subsets in CL patients. Considering the similarity between our findings and those of others, it is, however, tempting to speculate that distinct Old and New World Leishmania species may all specifically induce T cells with high IL-10 production, which would reduce the effectiveness of the immune response, thus favoring parasite survival (33, 45). Accordingly, in experimental VL, a complete cure with amphotericin B in liposomes involves downregulation of IL-10 (6).

Our data show that IL-13 mRNA levels were higher in individuals with previous treatment failure (group C) and decreased significantly in responders (group A) but not in nonresponders (group B). Others have suggested that IL-13 may participate in inducing IL-12 unresponsiveness in T cells and inhibiting the production of proinflammatory cytokines by macrophages, which would subsequently result in increased susceptibility to Leishmania parasites (1, 8, 22). To somehow attenuate the unique role of IL-13 in resistance to treatment, we found also that IFN-γ mRNA levels decreased significantly in treatment responders but not in nonresponders. Thus, based on our data, it is difficult to point at a preferential Th2 response as a unique element causing resistance to treatment in New World CL. Rather, it would appear that both Th1 and Th2 cytokines contribute to pathology. In this respect, it is important to stress that IFN-γ may favor L. amazonensis amastigote replication in macrophages (35). Thus, subtle immunological characteristics may be operationally responsible for treatment outcome. It should, however, be stressed that the observed number of nonresponders to treatment was lower than expected, thus leading to a lack of power of our study for this group of individuals. Within the LeishNaDrug-R study, in the same cohort of Peruvian patients, other parasite and host determinants were found to be of importance for treatment outcome. These included the parasite species but not the in vitro sensitivity of parasites to SbV (4, 19, 39, 48). Furthermore, a short stay (<72 months) in the area of disease transmission was a risk factor for treatment failure, indicating that protective immunity increases with the time of residence in areas where the disease is endemic (19). Similarly, treatment failure was strongly associated with a short (<5 weeks) disease duration, as previously reported (21).

In our study, the mRNA levels of IFN-γ, TNF-α, IL-4, and IL-13, but not those of IL-10, were much higher in ML than in CL. Of interest, macrophages (CD68) were less abundant in ML than in CL, pointing to a higher T-cell-to-macrophase ratio in ML. In keeping with these findings, the LST induration size was significantly larger in ML than in CL. These data are consistent with the hypothesis that in ML a strong uncontrolled inflammatory response eventually leads to intense destruction of host tissues (5, 14). Indeed, mucosal disease has been associated with T-cell hyperresponsiveness (12) and high intraleisonal levels of TNF-α and IFN-γ have been reported (3, 11, 14). To extend these findings, we found that TNF-α mRNA levels were particularly high in ML lesions that would not respond to treatment. The other observation we made of lower levels of IL-10 mRNA in mucosal compared to cutaneous lesions complements the finding by others of decreased IL-10 receptor expression (14). In this respect, it is possible to hypothesize that ML may differ from CL as far as the immune response is concerned because both production of and responsiveness to IL-10 are partially defective in ML, thus unleashing the synthesis of proinflammatory mediators. Why such intense production of TNF-α and IFN-γ results not in parasite control but rather in tissue destruction remains to be ascertained.

In conclusion, our work highlights the complex links between New World Leishmania infection and immune responses in the skin and mucosae. This is made evident by LST responses that showed bigger induration sizes in ML than in CL, consistent with higher levels of inflammatory cytokine mRNAs found in ML. However, CL responders to treatment segregated in a subgroup with a stronger LST response. Furthermore, the data indicate that IL-10 may be particularly abundant in CL lesions and that resistance to treatment may correlate with failure to control IL-10 production.

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