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Immunological and Genetic Evidence for a Crucial Role of IL-10 in Cutaneous Lesions in Humans Infected with *Leishmania braziliensis*[1]

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In populations exposed to *Leishmania braziliensis*, certain subjects develop skin ulcers, whereas others are naturally protected against cutaneous leishmaniasis. We have evaluated which cytokines are most crucial in the development of skin lesions. We found that active lesions occur in subjects with polarized Th2 or mixed Th1/Th2 responses, both associated with elevated IL-10 production. IL-10 was strongly associated ($p = 0.004$, odd ratio (OR) = 6.8, confidence interval = 1.9–25) with lesions, excluding IFN-γ, IL-12, TNF, IL-13, and IL-4 from the regression model. IL-10 was produced by blood monocytes and $CD4^+CD25^+$ T lymphocytes (mostly $Foxp3^+$). However, we did not observe any difference between the number of these cells present in the blood of subjects with active lesions and those present in resistant subjects. Genetic analysis of the *IL10*−819C/T polymorphism, located in the *IL10* promoter, showed that the C allele increased the risk of lesions (OR = 2.5 (1.12–5.7), $p = 0.003$). Functional analysis of these variants showed allele-specific binding of nuclear factors. The *IL10*-819C/C genotype was associated with higher levels of IL-10 than C/T and T/T genotypes. These observations demonstrate an important role for IL-10 in skin lesions in humans infected with *L. braziliensis,* and identify circulating monocytes and Tregs as principal sources of IL-10 in these patients. *The Journal of Immunology,* 2008, 180: 6139–6148.

Leishmaniasis is a parasitic disease caused by protozoan flagellates that belong to the *leishmania* genus. Diseases range from mild skin ulcers (localized cutaneous leishmaniasis (CL)[4]; LCL) to severe mucocutaneous destruction of the face (mucocutaneous leishmaniasis; MCL) or to deadly visceral disease (Kala Azar). The outcome of infection depends mainly on the strain of *Leishmania* and on the host immunological response, which is in part influenced by host genetic background. *Leishmanias* develop inside the macrophage and IFN-γ and Th1 cytokines are essential in protecting against all *Leishmania* infections (1–4). Conversely, the induction of a Th2 cytokine response is associated with susceptibility to *L. major* (5–7). IL-12 has the essential role of redirecting an early IL-4 response toward a strong Th1 response (8, 9). IL-13 has also been involved in the mechanisms of susceptibility. The growth of certain *Leishmania* substrains is better controlled in IL-13Rα-deficient than in IL-4-deficient mice (10) and *IL13* transgene expression suppresses IL-12 and IFN-γ expression, and makes the normally resistant C57BL/6 mouse strain susceptible to *L. major* infection, even in the absence of IL-4 expression (11). Strong expression of IL10 by APCs increases the susceptibility of mice to *L. major* infections (12); furthermore, injecting *IL4* and *IL10* transgenes suppressed IL-12 and the Th1 response and increased lesion development in resistant mice infected with a small number of parasites (13). Moreover, $IL10^{-/-}$ BALB/c mice develop smaller or fewer lesions than their control littermates after being infected with high doses of parasites (14). Similar results were reported with *L. donovani* using $IL10^{-/-}$ mice, mice overexpressing the IL10 transgene or mice with IL-10R blockade (15, 16): resistance was dependent on a greater production of IFN-γ and NO, and treatment with anti-IFN-γ Abs increased the susceptibility of $IL10^{-/-}$ BALB/c mice (15, 16). Studies in $IL\text{-}10^{-/-}$ mice and in mice infected with a virulent form of *L. major* isolated from a patient with nonhealing lesions have also demonstrated that IL-10 produced by $CD4^+$ T cells are essential for the long term persistence of *L. major* at the site of infection after spontaneous healing of dermal lesions in C57BL/6 mice (17).

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[4] Abbreviations used in this paper: CL, cutaneous leishmaniasis; LCL, localized CL; rCL, subjects resistant to CL; Lb, *Leishmania braziliensis*; EMSA, electrophoretic mobility shift assay; MCL, mucocutaneous leishmaniasis; MAF, minor allele frequeny; aCL, subjects with active CL; hCL, subjects with healed CL lesions.

Table I. *Study subjects*

Phenotype	*n*	Gender (M/F)	Age (Years) (SEM)	Years in Endemic Region (SEM)	Criteria of Selection
rCL	22	16/6	45.6 (3.8)	32.4 (3.9)	>20 yrs in endemic area, IFN^+ in Lb-stimulated cultures of PBMC, no lesion
hCL[a]	62	42/20	41.2 (2.3)	29.4 (2)	hCL, >10 Glucantime injections
aCL IFN+	18	13/5	33.3 (3.5)	27.3 (8.6)	aCL, IFN^+, cured with Glucantime
aCL IFN^-	8	4/4	33.1 (7.6)	16 (9)	aCL, IFN^-, cured with Glucantime

[a] Four out if five of the hCL subjects had been tested with *Leishmania* Ag (Montenegro test) at the time of the diagnosis and those tested were DTH^+ at 48 h.

Nonhealing lesions in humans infected with *L. major*, have been associated with low levels of IFN-γ and high levels of IL-4 and, in contrast, healing lesions were associated with elevated levels of IFN-γ and low levels of IL-4 (18) in PBMCs. IFN-γ, IL-1, IL-12p40, TNF-α, TGF-β, and IL-10 mRNA were detected in the lesions of healing subjects, whereas IL-4 mRNA was only found in a few biopsies (19); in these cases, lesion healing was again associated with elevated levels of IFN-γ and low levels of IL-4. Nevertheless, large numbers of parasites were observed in lesions in the presence of IFN-γ transcripts, suggesting that the protective effects of IFN-γ were partly neutralized.

Other *Leishmania* strains that do not grow well in mice cause more severe cutaneous damage than *L. major*. Most lesions caused by Lb infection do not heal without treatment, though patients mount a good DTH response to *Leishmania* Ags. The nonhealer phenotype of LCL patients has been associated with the production of a mixed Th1/Th2 cytokine response that is polarized toward Th1 in mucocutaneous CL (MCL) (20) and toward Th2 in diffuse CL (21, 22). The role of IL-4 in LCL is not clear, as there is no report of a strong polarization toward Th2 in LCL subjects. PBMC from MCL patients produce less IL-10, respond less to IL-10 and TGF-β (20), and show lower IL-10 receptor expression than PBMCs from LCL subjects (23). These findings suggest that MCL might be aggravated by uncontrolled Th1 mediated inflammation due to low IL-10 and TGF-β production. The cytokines, IFN-γ, TNF, IL-1α, IL-10, and TGF-β, were also detected in *L. mexicana* lesions (24) and there was an increase in IL-10 and TGF-β in late lesions (24); thus, the protective effects of IFN-γ may be neutralized by IL-10 and/or TGF-β. Also, observations in *L. amazonensis* lesions support an aggravating role for IL-13 (25).

Several studies have been performed on cutaneous leishmaniasis patients, but it is still unclear what the crucial mechanisms are that account for the difference of susceptibility between resistant subjects and subjects who develop cutaneous lesions. In this study, we re-evaluated cytokine production in subjects infected with *Leishmania braziliensis* (Lb); in particular, we investigated the cytokines that have been reported to play a critical role in experimental *Leishmania* infections (IFN-γ, TNF, IL-12, IL-4, IL-13, and IL-10). We made two original observations: first, the cytokine response of a third of LCL subjects is strongly polarized toward Th2; second, IL-10 is the cytokine that is the most strongly associated with active lesions, in comparison to cytokines in endemic resistant subjects or healed subjects. Thus, we investigated which cell types produce IL-10 and searched for definitive evidence demonstrating the crucial role of IL-10 in cutaneous lesions. Finally, our genetic analysis clearly demonstrated that IL-10 plays a key-aggravating role.

Materials and Methods

Study subjects

The study was conducted in a population living in two villages and various 'fazendas' (farms) located on the border or in the middle of the Atlantic forest in the state of Bahia. This region is endemic for cutaneous leishmaniasis caused by Lb. CL has been present for >30 years and micro epidemics still occur on a low endemic background in this region. Transmission occurs close to and within the cacao plantations, which are usually grown in the shade of Atlantic forest trees. The conditions under the cacao are ideal for sandfly breeding. The reservoir-hosts of Lb are wild animals from the forest, but domestic animals, such as mules and dogs, have also become reservoirs. We interviewed 3500 subjects to select study subjects and clinically examined all individuals who declared previous leishmaniasis infection.

Subjects included in the immunological study

Mostly farmers or farmers' children — are described in Table I. We did not have ethical authorization to test study subjects for HIV infection. No study subjects were receiving treatment for HIV infection. Patients with active lesions were diagnosed with CL if they had characteristic CL ulcers and responded to Glucantime treatment. Healed scars were considered to be indicative of CL if the patients had been diagnosed with CL at the local health center by physicians and responded to Glucantime treatment. CL study subjects who had been tested for *Leishmania* Ags (Montenegro's skin test), were positive for this test; ~20% CL subjects had not been tested because Ag preparation was not available for diagnosis at time of CL onset. We did not include: 1) subjects with healed scars older than 4 years; 2) subjects who had self-diagnosed CL or had taken traditional medicine, or subjects who healed without treatment. These strict criteria were followed to minimize wrong diagnoses (lesions as a result of other causes) of patients with old lesions. In this study, subjects with no lesions, but who produced IFN-γ in Lb-stimulated PBMC cultures and had lived for $\geq$20 years in the endemic area, were referred to as rCL; subjects with healed lesions were hCL, and those with active lesions (aCL). These study groups are described in Table I; the aCL group was divided into subjects producing or not producing IFN-γ in Lb extract-stimulated PBMC cultures.

Subjects included in the genetic study

The transmission disequilibrium test was performed on 140 trios (one affected case and both parents) from 84 families: 45 families with one case, 25 with 2 cases, 11 with 3 cases and 3 with 4 cases. The study design allowed detection of a disease allele (allele frequency = 0.6; associated risk = 1.35) with powers of 67% (type I error rate α = 0.01) and 51.4% (α = 0.003). Affected cases were subjects with active or healed lesions (recruited using the same criteria as for aCL and hCL subjects, but based on information obtained over the past 15 to 20 years). Confirmed active or past cases were included if they and their parents gave a 5 ml blood sample (<2% refusals). If only one parent was present, the other parent's genotype was inferred from two siblings. Trios (one affected case with both parents) with false parenthood were excluded. This genetic study included all recruited trios with no further exclusion during the study. Resistant cases were not tested. Parent clinical phenotype was not used in the analysis.

Parasite and Ag

Lb was isolated from a dog from the endemic area, which belonged to a family with various active CL cases; it was characterized by isoenzymative analysis. The isolate was grown in Schneider medium supplemented with 2 mM L-glutamine, 40 μg/ml gentamicin and 20% FCS (Invitrogen). *Leishmania* extract was prepared from stationary-phase promastigotes, harvested after the third or fourth passage. Parasites were washed four times in PBS (pH 7.2), and the pellet was resuspended at a concentration of 10^8 promastigotes/ml in sterile water. The suspension was rapidly frozen (−70°C) and thawed (37°C) five times. The Lb was adjusted with 10× PBS and stored at −70°C until use.

Cell cultures

PBMCs were isolated by blood centrifugation on Ficoll-Paque (Amersham Biosciences) (400 × *g*, 20 min at room temperature), were washed three times in RPMI 1640 medium (Invitrogen), and were resuspended

Table II. *Polymorphisms analyzed in this work by restriction enzyme digestion*

Polymorphisms[a]	Primers	Restriction Enzymes	Digestion Fragments (bp)
−3575 T/A	F, 5′-GGTTTTCCTTCATTTGCAGC-3′	Apo I	A: 228
	R, 5′-ACACTGTGAGCTTCTTGAGG-3′		T: 121 + 107
−2763 C/A	F, 5′-GGTCAGGAGATCGAGACCATC-3′	Tsp509 I	C: 106 + 76
	R, 5′-GATCTCAGCTCACTGCAAGC-3′		A: 182
−1352 G/A	F, 5′-CATCTGGGTCCATGGCTACT-3′	Tspr I	A: 296 + 35
	R, 5′-GGCGAGGAGTGTTGCTCTAC-3′		G: 173 + 123 + 35
−1082 A/G	F, 5′-CCAAGACAACACTACTAAGGCTCCTTT-3′	Xag I	A: 280 + 97
	R, 5′-GCTTCTTATATGCTAGTCAGGTA-3′		G: 253 + 97 + 27
−819 C/T	F, 5′-CAACTTCTTCCACCCCATCTTT-3′	Mae III	C: 217 + 175 + 85
	R, 5′-GTGGGCTAAATATCCTCAAAGTT-3′		T: 392 + 85
−592 C/A	F, 5′-CAACTTCTTCCACCCCATCTTT-3′	Rsa I	C: 311 + 116 + 42 + 8
	R, 5′-GTGGGCTAAATATCCTCAAAGTT-3′		A: 240 + 116 + 71 + 42 + 8
+470 T/G	F, 5′-TCCTGCTGGCGCTCTATACT-3′	Ava II	G: 277 + 22
	R, 5′-GCATCTTCACCTCGAATCGG-3′		T: 299
+3917 T/C	F, 5′-ATAAATTAGAGGTCTCCAAAATCG-3′	Nla III	T: 111
	R, 5′-GAGGTATCAGAGGTAATAAATATTCCA-3′		C: 86 + 25

[a] IL10 polymorphisms genotyping: primer sets, restriction enzymes restriction fragment lengths. F, Forward; R, reverse.

in Dulbecco's Eagle Modified medium (Invitrogen), supplemented with 50 μM (2-ME), 2 mM L-glutamine, 40 μg/ml gentamicin and 5% FCS (Invitrogen). We cultured 2×10^6 cells per well per ml in a 24-well microplate in the presence of 5 μg/ml PHA (Sigma-Aldrich) or 5 μg/ml Lb Ags, or in the presence of medium alone. Plates were incubated at 37°C in a 5% CO_2 atmosphere. Supernatants were collected at 24 and 96 h. The supernatants were then centrifuged, and stored at −70°C for analysis of cytokine production. Cytokine levels were measured in supernatants of resting PBMCs, Lb-stimulated-PBMCs, or PHA-stimulated PBMCs from rCL, hCL, and aCL subjects. IL-12p70, TNF, IL-4, and IL-10 levels were measured after 24 h and IFN-γ levels after 96 h of culture. Cytokine levels in PHA-stimulated cultures were evaluated at 72 h. The aCL group was split into aCL IFN$^+$ and aCL IFN- subgroups, as described in the results. The numbers of subjects included in each group for the immunological study were: rCL (20), hCL (22), aCL IFN$^+$ (18), aCL IFN$^-$ (8).

Cytokine titration

Microplates for TNF, IFN-γ, IL-4, IL-10, IL-12 (IL-12p70), and IL-13 titration (Nunc) were sensitized overnight with 100 μl of 2 μg/ml specific capture mAb (Mabtech and BD Pharmingen). Nonspecific binding was prevented by incubating plates with 2% BSA (Sigma-Aldrich) in PBS. Plates were re-incubated overnight with 100 μl of a 1/2 dilution of culture supernatants in 2% BSA-PBS and with recombinant human cytokines for standard curve (Mabtech and BD Pharmingen). Plates were then washed four times with PBS and 0.05% Tween 20 (Sigma-Alrich) and incubated for 2 h at 37°C with 2 μg/ml appropriated biotinylated anti-cytokine detection mAb (Mabtech and BD Pharmingen). Plates were then washed and incubated for 2 h at 37°C with alkaline phosphatase-conjugated streptavidin. Finally, plates were washed four times and enzymatic activity was developed by incubating them with p-nitrophenyl phosphate (Sigma-Aldrich). Absorbance was read at 405 nm in a microplate reader (Bio-Rad Laboratories). Sensitivity was 4 pg/ml (TNF), 2 pg/ml (IFN-γ), 1 pg/ml (IL-4), 10 pg/ml (IL-10), 2 pg/ml (IL-12p70), and 5 pg/ml (IL-13).

Purification of CD4$^+$CD25$^+$ T cell subpopulations

CD4$^+$CD25$^+$T cells isolated from PBMCs were purified using the CD4$^+$CD25$^+$ Regulatory T Cell Isolation kit (Human), according to the protocol provided by the manufacturer (Miltenyi Biotec). In brief, cells were suspended in PBS supplemented with 2 mM EDTA and 0.5% BSA at a density of 10^7 cells in 90 μl of buffer and 10 μl of biotin-Ab mixture. The cells were incubated at 4–8°C for 10 min. Then, 20 μl of anti-biotin microbeads was added and incubated for 15 min at 4°C. CD4$^+$ cells were washed through the column, whereas non-CD4$^+$ cells were retained on the column. The CD4$^+$ cells were resuspended at a density of 10^7 cells in 90 μl of buffer and 10 μl of CD25 microbeads. The cells were incubated with the beads at 4–8°C for 15 min. Unbound cells were washed through the column, whereas the CD4$^+$CD25$^+$ T cell fraction retained on column was eluted by removing the column from the magnetic field and flushing out the cells with 1 ml elution buffer. CD4$^+$CD25$^+$ T cells, after being detached, were washed and used immediately.

Suppression assay for Treg

5×10^4 CD4$^+$CD25$^+$ T cells purified from the blood of study subjects were cultured in 96-well flat-bottom tissue culture plates (Falcon) with 5×10^5 PBMC from two nonrelated donors. Cells were cultured for 5 days at 37°C in a 5% CO2 incubator. [H^3]Thymidine (0.5 μCi) was then added to each well and cells were cultured for an additional 16 h. Cells were harvested and the radioactivity incorporated into the DNA was measured using a beta counter (Beckman Coulter). Data were from triplicate cultures. Percentage of suppression = 100 [(counts in cultures without Treg) − (counts in cultures with Treg)]/counts in cultures without Treg.

Flow cytometry: immunostaining and data acquisition

Ab purchased from BD Pharmingen consisted of FITC-anti-CD4 (IV T114), FITC-anti-CD14, and PE-Cy5-anti-CD25 (A053). PE-anti-IL-10 (JES3–9D7) and PE-anti-Foxp3 (236A/E7) were purchased from eBioscience and appropriated isotypes controls were used. All abs were used according to manufacturer's instructions. PBMCs isolated from the blood of patients were dispensed (5×10^5cells/tubes) into 5 ml polystyrene tubes (Falcon) and washed once with cold buffer (PBS - 5%BSA) by centrifugation at $400 \times g$ for 10 min at 20°C. Cell pellets were resuspended in 100 μl PBS-BSA buffer and were mixed with either FITC anti-CD4 and PE-Cy5 anti-CD25 mAb or anti CD14-FITC for surface labeling. The cells were incubated for 30 min in the dark at 4°C. Samples were then washed three times in buffer by centrifugation at $300 \times g$ for 5 min at 20°C. The cells were then fixed and permeabilized with freshly prepared fixation/permeabilization working solution from the Foxp3 Staining buffer set (eBioscience) for 30 min at 4°C, and washed twice with 2 ml. After the last wash, cell pellets were resuspended in 100 μl 1× permeabilization buffer and mixed with PE-anti-IL-10 or PE-anti-Foxp3 mAb at 4°C in the dark. After 30 min of incubation, the cells were washed twice with 2 ml 1× permeabilization buffer and then resuspended in 1% paraformaldehyde in PBS Dulbeccos (Sigma-Aldrich) for analysis. A total of 20,000 events/tube were acquired using a FACScalibur flow cytometer (BD Biosciences). CellQuest software provided by the manufacturer was used for data acquisition and analysis.

Genotyping IL10 polymorphisms

Genomic DNA was extracted using an Autogen NA2000 (Geneworks) according to manufacturer's procedure. *IL10*-1352 (rs1800893), *IL10*-1082 (rs1800896), *IL10*-819 (rs1800871), and *IL10*-592 (rs1800872) were genotyped using PCR-RFLP assays. PCR primer sequences, restriction enzymes and resulting fragments lengths for each allele are listed (Table II). Genotyping of all other SNPs was assessed using TaqMan probe assays (Applied Biosystems). Primers and probes were provided by Applied Biosystems (sequences remain confidential). Each reaction contained 12.5 ng of genomic DNA, TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of each primer and 200 nM of each fluorescently labeled hybridization probe in a total volume of 5 μl. PCR was conducted in an ABI Prism Sequence Detection System 7900 (Applied Biosystems) using the

Table III. *Cytokines produced by PBMCs of rCL, hCL, or aCL*[a]

Cytokines	Culture	rCL	hCL	aCL INF$^+$	aCL IFN$^-$
IFN-γ	–	16 (5)	13 (7)	<5	<5
IFN-γ	Lb	834 (218)	652 (170)	578 (114)[a]	<5[a,b]
IFN-γ	PHA	2072 (145)	2164 (161)	1314 (196)	1202 (395)
IL-12	–	<5	4.9 (3.5)	76.4 (27.5)	10.5 (6.9)
IL-12	Lb	13.9 (7.8)	14.2 (7.2)	129 (46.8)[a,b]	30.1 (20.1)
IL-12	PHA	26.1 (9.9)	43.0 (17.3)	95.4 (38.6)	95.4 (40.7)
TNF	–	9 (8)	104 (58)	311 (97)[a,b]	333 (95)[a,b]
TNF	Lb	41 (26)	127 (70)	459 (170)[a,b]	364 (97)[a,b]
TNF	PHA	380 (63)	855 (282)	1498 (486)	1736 (616)
IL-4	–	<5	1.4 (1.2)	18.4 (4.4)[a,b]	32.3 (3.1)[a,b]
IL-4	Lb	2.9 (1.4)	5.0 (1.9)	22.4 (5.1)[a,c]	38.0 (4.2)[a,c]
IL-4	PHA	83.8 (13.1)	63.3 (10.7)	98.8 (11.2)	55.5 (11.4)
IL-13	–	4 (2)	54 (46)	14 (8)	15 (11)
IL-13	Lb	101 (48)	63 (16)	204 (124)	17 (9)
IL-13	PHA	1189 (124)	1068 (94)	1959 (296)[d]	1358 (371)
IL-10	–	89 (24)	124 (42)	700 (160)[a,b]	588 (193)
IL-10	Lb	85 (23)	152 (41)	467 (76)[a,b]	518 (125)[c,d]
IL-10	PHA	3491 (526)	2763 (511)	4303 (520)	2923 (587)[b]

[a] Cytokine levels were measured in supernatants of resting PBMCs (-), *Leishmania* extract (Lb) stimulated-PBMCs, or PHA-stimulated PBMCs from rCL, hCL, and aCL subjects. IL-12, TNF, IL-4, IL-10 were evaluated after 24 h and IFN-γ after 96 h of culture. PHA-stimulated cultures were evaluated at 72 h. Data are presented as the arithmetic mean of two determinations (SEM). aCL group was split into aCL IFN$^+$ and aCL IFN$^-$ subgroups, as described in *Results*. Statistical analysis was carried out using non-parametric ranking tests. Cytokine levels in rCL and hCL groups were not statistically different ($p > 2.10–3$). Number of subjects in each group: rCL (20), hCL (22), aCL IFN$^+$ (18), aCL IFN$^-$ (8).

$^{(a,b)}$ vs $^{(c,d)}$, $p < 10^{-4}$ ($<10^{-3}$) for comparison between aCL groups and rCL and hCL, respectively. There were no statistically significant differences between rCL and hCL groups.

following conditions: 50°C for 2 min, 95°C for 10 min and 40 cycles of amplification (95°C denaturation for 15 s, 60°C annealing/extension for 1 min).

Genotyping was performed without knowing the subject clinical status to ensure quality control. *IL10*-1082 and *IL10*-592 were also genotyped by TaqMan assays using ABI Prism 7900 Sequence Detection System (Applied Biosystem) with recommended protocols; similar results were found by both methods. *IL10* gene sequencing was performed on PCR-amplified genomic DNA from 11 individuals (7 hCL and 4 rCL). Both strands were sequenced using an ABI prism Big Dye Terminator cycle sequencing system with an ABI prism 310 automatic sequencer (Applied Biosystem). Sequences were analyzed with Chromas software v 2.01 (Technelysium). We defined correlation groups within the *IL-10* gene: eight polymorphisms with minor allele frequency (>0.2) covering the sequenced region were genotyped in a sample of 80 unrelated adults from the study population. Genotyping was conducted using PCR-RFLP.

EMSA

Nuclear extract preparation

Nuclear extracts were prepared with the NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Pierce).

EMSA

Complementary single-stranded oligonucleotides (5′-biotinylated or -unbiotinylated) were commercially synthesized to span 10 bp on either side of the variant nucleotide, as follows: *IL10*-819T forward, 5′- AGGTGAT GTAA*T*ATCTCTGTGCCTCAG −3′; *IL10*-819T reverse, 5′-CTGAG GCACAGAGAT*T*TACATCACCT-3′; *IL10*-819C forward, 5′-AGGT GATGTAA*C*ATCTCTGTGCCTCAG-3′; and *IL10*-819C reverse, 5′-CTGAGGCACAGAGAT*G*TTACATCACCT-3′.

Complementary strands were annealed by combining each oligonucleotide, by placing in a boiling water bath for 5 min, and by allowing the reaction to cool to room temperature. DNA-protein binding reactions were conducted in a 20 μl reaction mixture consisting of 10 μg nuclear protein extract, 10 mM Tris, 50 mM KCl, 1 mM DTT (pH 7.5), 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/μl poly(dI:dC), 0.05% Nonidet P-40, 20 fmol 5′biotin end-labeled probe, and 2–10 pmol cold probe. The DNA-protein binding reactions were incubated at room temperature for 20 min. The reactions were loaded onto an 8% nondenaturing polyacrylamide gel, and run for 4 h at 140 V. Finally, the DNA was transferred onto nylon N$^+$ membranes (Amersham Biosciences). The membranes were baked at 80°C for 20 min. The blot was visualized with the Light Shift Chemiluminescence EMSA kit (Pierce) according to the manufacturer's instruction.

Statistical analysis

Linkage disequilibrium patterns and deviation from Hardy-Weinberg equilibrium were analyzed for unaffected parents using algorithms implemented in the software HAPLOVIEW (26).

Family based tests of association between *IL10* polymorphisms and CL were performed using the software FBAT, version 1.7.2 (27). Additionally, for alleles demonstrating potential association with CL, odds ratio were estimated using conditional logistic regression as described in (28). Conditional logistic analysis was performed using the PHREG procedure implemented in the SAS software v9.2 (SAS Institute, Cary, NC). Finally, to account for both the small sample size and the multiplex nature of our families (two issues that may inflate type I error when using asymptotic distribution of the FBAT test statistic), empirical *p* values were generated by means of 1,000,000 Monte Carlo permutations for each polymorphism found to have a significant effect.

Correlation groups ($r^2 = 0.8$) were evaluated from our genotyping data using Haploview.

Univariate comparison of cytokine levels was conducted using nonparametric ranking tests. Multivariate regression analysis of the cytokine data was performed as described in (29) to assess their weight in the risk of active lesions. Forward conditional analysis was used and cytokine classes were defined by the median; in cases of the median being below detection levels, cytokine classes were defined by the threshold of detection for the cytokine (IL-4, 5 pg/ml; IL-12, 5pg/ml).

Results

Analysis of IFN-γ production shows heterogeneity among endemic subjects without lesions and among subjects with active lesions

Cytokines produced in PBMC cultures of endemic subjects, without past or present history of CL, were first examined to define a group of endemic resistant subjects. Scars from healed cutaneous lesions due to Lb persist for life; therefore, subjects who have never presented with CL can be easily identified. A third of PBMC cultures of endemic subjects (who have lived ≥20 years in an area of high Lb transmission) without lesions produced IFN-γ, and the proportion of IFN$^+$ cultures increased with longer residency in the endemic area (data not shown). Endemic subjects producing IFN-γ in culture after specific stimulation with Lb extracts are likely to have been exposed to infection but have no detectable lesions; in

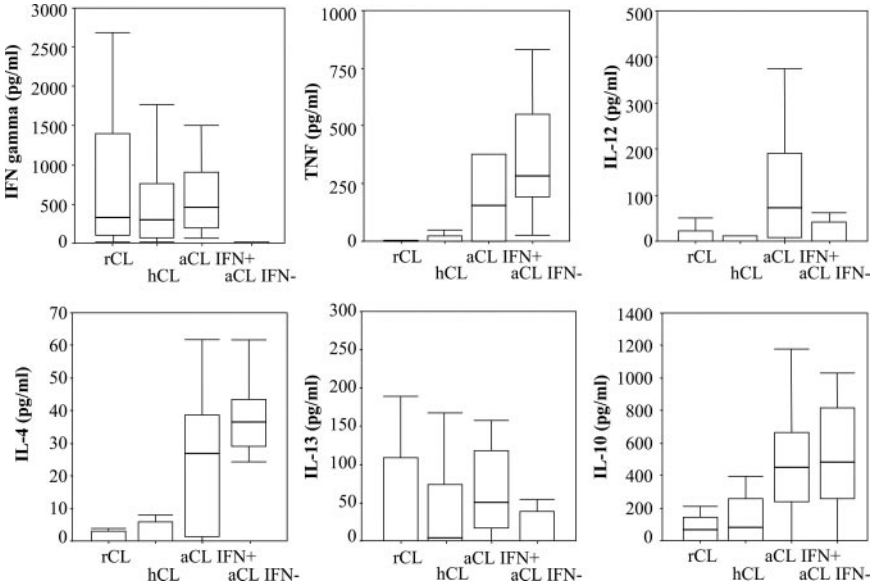

FIGURE 1. Th1 and Th2 cytokine in cultures of PBMCs from rCL, hCL, and aCL subjects. PBMCs were cultured as indicated in the methods and cytokines were measured in supernatants of Lb-stimulated cultures, as indicated in the Table III legend. The figure depicts the titers recorded (in pg/ml) from Lb-stimulated cultures only. Data are given as medians, boxes are 75%, and bars 95%. Comparisons using nonparametric tests indicated $p < 10^{-3}$ for IFN-γ (gp 1 vs gp 3; 1,3; 1,4; 2,4; 3,4); IL-12 (1, 3); TNF (1, 3, 1, 4, 2, 3, 2, 4); IL-4 (1, 3, 1, 4, 2, 3, 2, 4); IL-10 (1, 3, 2, 3, 1, 4, 2, 4), gp1,2,3,4 referred to rCL (1), hCL (2), aCL IFN$^+$ (3) and aCL IFN$^-$ (4).

addition, previous studies in experimental models (see Introduction) have associated IFN-γ with protection against *Leishmania* infection. Thus, in this study, IFN-γ$^+$ endemic subjects with no past or present lesions due to *Leishmania* were considered to be resistant to the development of lesions. It was less clear whether subjects without scars but not producing IFN-γ were resistant and therefore, they were not included in this study.

Similar analysis was performed on cultures of aCL and hCL. PBMCs of all but one subject with a past history of CL produced IFN-γ. By contrast, 8 of 26 aCL subjects did not produce IFN-γ in Lb-stimulated cultures. Thus, aCL subjects were grouped as aCL IFN$^+$ and aCL IFN- subgroups.

Production of IFN-γ, IL-12 (IL-12p70), TNF, IL-4, IL-13, and IL-10 in PBMC cultures of rCL, hCL, aCL IFN$^+$, and aCL IFN$^-$ subjects

The cytokines, IFN-γ, IL-12, TNF, IL-4, IL-13, and IL-10, produced in resting, Lb-stimulated and PHA-stimulated PBMC cultures from the four study groups are shown in Table III and in Fig. 1. No statistically significant differences in cytokine levels were observed between rCL and hCL subjects; this was consistent with endemic IFN-γ$^+$ subjects without lesions being resistant to the development of the lesions. Lb-stimulated cultures from aCL IFN$^+$ subjects produced significantly less IFN-γ and more TNF,

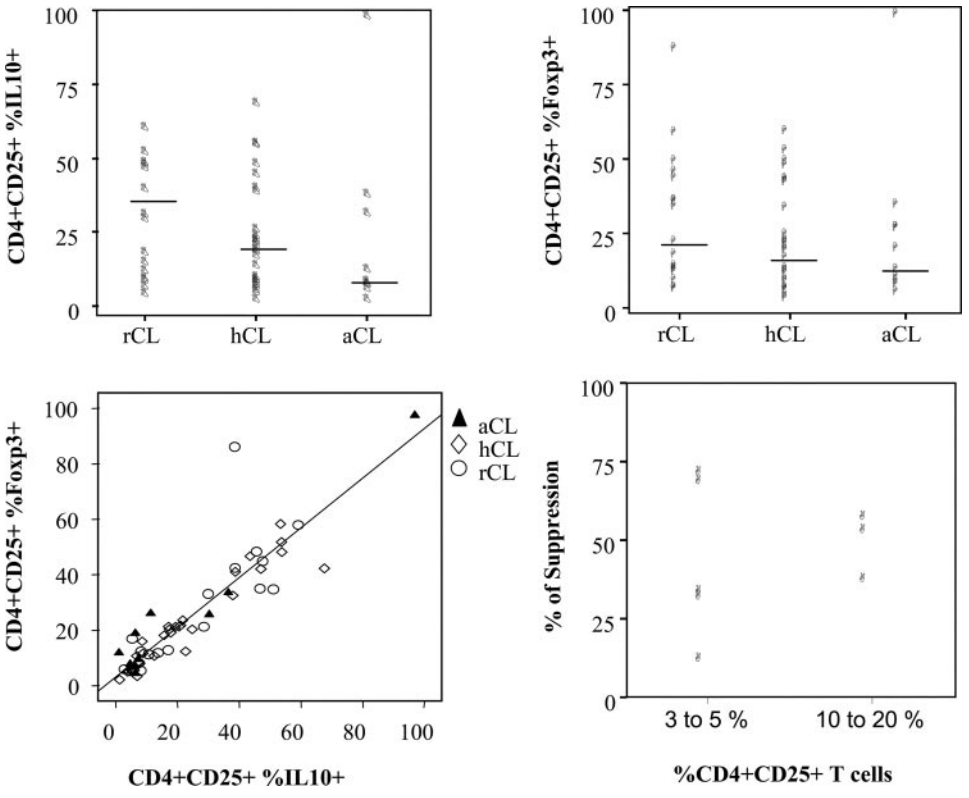

FIGURE 2. Characterization of CD4$^+$CD25$^+$ cells in the blood of study subjects. *Upper figures*, Percentage of IL10$^+$ cells among CD4$^+$CD25$^+$ cells and percentage of Foxp3$^+$ cells among CD4$^+$CD25$^+$ cells. *Lower-left figures*, correlation between IL10$^+$ and Foxp3$^+$ among CD4$^+$CD25$^+$ cells. *Lower-right figure*, percent suppression of MLR by various proportions (3–20%) of CD4$^+$CD25$^+$ cells isolated from study subjects. Staining of PBMCs was conducted as indicated in the methods. Number of study subjects were aCL (10), hCL (34), and rCL (15) subjects. Median CD4$^+$CD25$^+$IL-10$^+$ values were 6.9 (aCL), 16.4 (hCL), and 28.6 (rCL). Median CD4$^+$CD25$^+$Foxp3$^+$ values were 15.4 (aCL), 17 (hCL), and 21.2 (rCL).

Table IV. *Genetic associations between IL10 alleles and CL*[a]

SNP	*rs*		Frequency	Informative Families	S	E	*p*	PMC[b]
Alleles associated with CL								
−3575 T/A	1800890	T	0.770	33	32	32.8	0.8	
−2763 C/A	6693899	C	0.711	27	19	20.7	0.6	
−1352 G/A	1800893	G	0.672	31	21	25.2	0.3	
−1082 A/G	1800896	A	0.693	32	24	28.7	0.3	
−819 C/T	1800871	C	0.640	30	31	20.8	0.003	0.004
−592 C/A	1800872	C	0.649	30	30	20.8	0.007	0.011
+470 G/T	3024490	G	0.652	29	31	20.3	0.004	0.002
+735 G/T	3024491	G	0.720	30	29	28.6	0.9	
+1136 G/A	1518111	G	0.633	31	27	20.3	0.045	0.035
+1548 C/T	1554286	C	0.660	26	24	15.7	0.011	0.007
+2068 C/G	1878672	C	0.726	31	26	28.5	0.5	
+3917 T/C	3024496	T	0.638	32	20	22.9	0.4	

[a] SNPs were identified by sequencing IL10 in subjects from aCl and rCl groups; correlation groups ($r^2 > 0.8$) are depicted in Fig. 3. Each SNP was genotyped in the whole cohort of 140 trios. Data were analyzed using FBAT under a dominant model. The number of transmission under the hypothesis of no association ("E") and the number of transmission that has been observed ("S") are shown and indicates whether a given allele is associated with protection (observed < expected) or aggravation (observed > expected).

[b] Empirical *p* values (PMC) were generated by means of 1,000,000 Monte Carlo permutations for each of the significant polymorphism.

IL-4, and IL-10 than those from rCL subjects. Lb-stimulated cultures from aCL IFN$^-$ subjects (IFN nonproducers) produces more TNF, IL-4, and IL-10 than Lb-stimulated rCL cultures. Similar results were also recorded in resting cultures: TNF, IL-4, and IL-10 levels were higher in resting cultures from aCL IFN$^+$ subjects than in rCL resting cultures; and TNF and IL-4 levels were higher in resting cultures from aCL IFN$^-$ subjects than in resting rCL cultures. This indicates that the in vitro production of these cytokines partly resulted from stimuli received in vivo.

Similar observations were made for the comparison between aCL and hCL cultures to those between aCL and rCL cultures: aCL IFN$^+$ cultures stimulated with Lb produced more TNF, IL-4, and IL-10 than hCL cultures; aCL IFN$^-$ cultures also produced more TNF, IL-4, and IL-10 than hCL cultures. The comparison of cytokine levels in resting cultures of hCL with those in resting cultures of aCL IFN$^+$ or aCL IFN$^-$ also gave significant differences for TNF, IL-4, and IL-10 levels.

We then asked which cytokines were more closely associated with active lesions. Thus, we evaluated the risk of subjects developing a lesion (in comparison with resistant subjects) as a function of age, gender, IFN-γ, IL-12p70, IL-4, IL-10, and TNF by logistic regression analysis. Cytokine levels were treated as qualitative variables. rCL and hCL subjects were considered as a single resistant group, as they did not differ significantly for any of the cytokines. If only aCL IFN$^+$ subjects were considered, IL-10 provided the best association with active lesions ($p = 0.004$, OR = 9.3, CI = 2–43) and no other cytokine could be entered into the model in the presence of IL-10. The best regression model included both IL-10 ($p = 0.004$, OR = 6.8, 1.9–25) and IL-4 ($p = 0.009$, OR = 4.4, 1.4–13.5) when all aCL subjects (aCL IFN$^+$ and aCL IFN$^-$) were grouped.

IL-10 is produced by monocytes and CD4$^+$CD25$^+$ T lymphocytes, including CD4$^+$CD25$^+$FoxP3$^+$ T lymphocytes

We analyzed which cells produce IL-10 in the PBMC cultures. We found that both monocytes (CD14$^+$) and CD4$^+$ lymphocytes were IL-10$^+$. Two to five percent of blood monocytes were IL10$^+$ and this proportion did not vary between study groups. Among CD4$^+$ T lymphocytes, only CD25$^+$ lymphocytes stained IL-10$^+$ indicating that IL-10 producing cells were either Tregs or activated T cells. We could not, for technical reasons, stain simultaneously for all four markers, CD4, CD25, IL-10, and Foxp3. The percentage of IL-10$^+$ and Foxp3$^+$ cells in CD4$^+$CD25$^+$ T lymphocytes from aCL, rCL, and hCL are shown on Fig. 2. No statistically significant differences in IL-10$^+$ or in Foxp3$^+$CD4$^+$CD25$^+$ cells were observed between the three groups though there was a trend for smaller proportion of IL-10$^+$ or Foxp3$^+$ cells in CD25$^+$CD4$^+$ T lymphocytes in the blood of aCL subjects; the percentage of IL-10$^+$ and Foxp3$^+$ cells among CD25$^+$ cells were well correlated (r2 = 0.82, $p = 10-6$, Fig. 2, *bottom left*), indicating that most IL-10$^+$CD4$^+$CD25$^+$ cells were Foxp3$^+$. In eight experiments, CD4$^+$CD25$^+$ T cells purified from the blood of study subjects suppressed an MLR reaction in vitro from 11 to 70% (median = 40%) (Fig. 2, *bottom left*).

The risk of skin ulcer is increased in subjects with polymorphisms in the IL-10 promoter

We sought additional evidence for the role of IL-10 in CL by evaluating whether polymorphisms in IL-10 could modulate the risk of disease. We analyzed 140 CL Brazilian subjects with active or healed lesions and their parents. The FBAT method tests

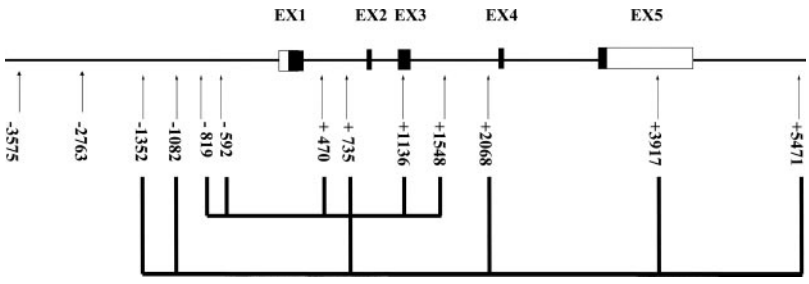

FIGURE 3. Most polymorphisms in *IL10* are distributed in two correlation groups. Only polymorphisms with MAF > 0.2 were analyzed and are shown. Polymorphisms belonging to the same correlation group ($r^2 > 0.8$) are linked by a line.

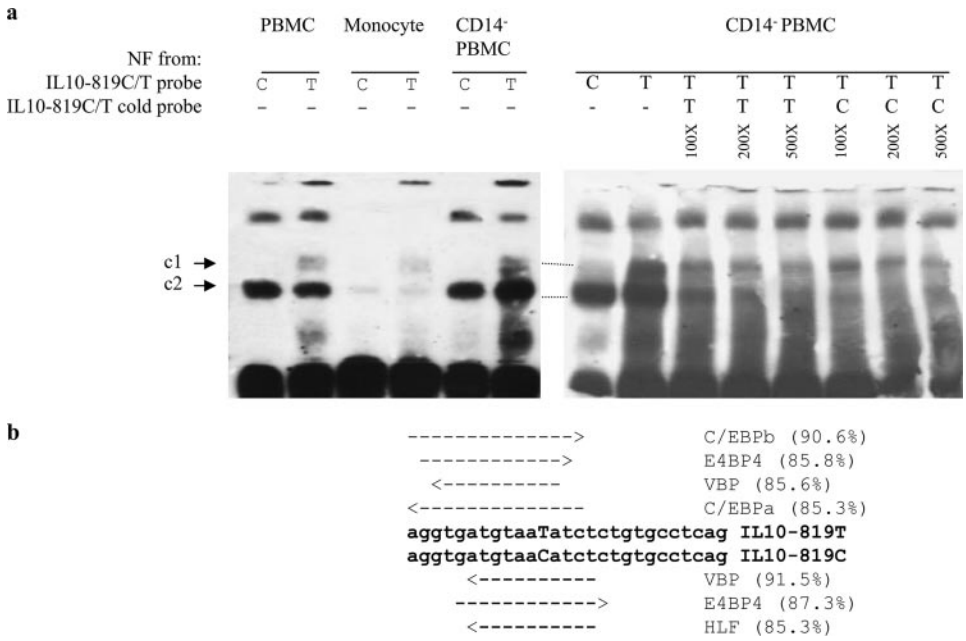

FIGURE 4. IL10−819C/T creates new binding sites for nuclear factors from PBMC, monocytes and CD14- mononuclear cells. *a*, EMSA were performed in vitro according to the protocol described in the methods section. The experiments were performed with nuclear extracts from PBMCs, monocytes, and CD14$^-$ mononuclear cells. Competitive reactions were done with 2–10 pmol of cold probe. *b*, In silico analysis evaluating whether this polymorphism could have created or altered some DNA-protein interactions. This analysis was performed on the website (www.cbrc.jp/research/db/TFSEARCH.html). An 85% threshold score (matrix similitude) was used in this analysis. The matrix similitude scores are indicated in brackets.

whether one allele is more frequently transmitted from a heterozygous parent to an affected child than expected under the hypothesis of independence between the locus and the disease. We first analyzed three polymorphisms in the *IL10* promoter that had been associated with inflammatory diseases: *IL10*-592A/C, *IL10*-819C/T, and *IL10*-1082A/G (Table IV). These polymorphisms were in Hardy Weinberg equilibrium in the study population. The *IL10*-819C allele was transmitted ($p = 0.003$) more frequently to subjects with CL than was expected under the hypothesis of no association. *IL10*-1082A was less frequent in affected children, but this was not statistically significant ($p = 0.3$).

HapMap predicted that −819 T/C and −1082 A/G were strongly correlated with several other SNPs ($r^2 > 0.8$); therefore, we sequenced IL10 in seven aCL and four resistant subjects and determined which polymorphisms were strongly correlated ($r^2 > 0.8$). Twenty-five polymorphisms were detected, of which two had not been previously reported (data not shown). Only common polymorphisms with a minor allele frequency (MAF) > 0.2 were considered in the analysis. Ten polymorphisms had a MAF > 0.2 in the sequenced sample. Five polymorphisms with a MAF > 0.2 were located in the promoter region. We recorded two SNP correlation groups (Fig. 3) in our Brazilian cohort: group 1 was comprised of −819 C/T, −592 C/A, +470 G/T, +1136 G/A + 1548 C/T; and group 2 was comprised of −1352 G/A, −1082 A/G, +735 G/T, +2068 C/G, and +3917 C/T (Fig. 3). All polymorphisms in group 1 were associated with CL, as expected from their high correlation. By contrast, no polymorphisms in group 2 showed association with CL (Table IV).

Monte Carlo simulations were performed to further test the associations; the results confirm the associations (last column of Table IV). Odds ratios were determined using reconstructed case controls: OR (95% CI) for the polymorphism 819 was CC+CT vs TT: 2.53 (1.12–5.69).

Functional analysis of −819 C/T and −592 A/C IL10 promoter polymorphisms

We used EMSA to analyze the *IL10*-819C/T and *IL10*-592A/C polymorphisms, located in the promoter region. This analysis showed allele-specific NF binding for −819 C/T (Fig. 4*a*). The T allele bound nuclear factors (complex c1) that were not bound by the C allele. A second complex (complex c2), which was not allele-specific, was also detected. Complex c1 was displaced by the homologous T allele probe and, to some extent, by the C probe. NFs in complex c1 were produced by PBMCs, monocytes and CD14$^-$ PBMC. These findings were consistent with in silico analysis of these SNPs, which predicted allele-specific NF binding for −819 C/T (Fig. 4*b*). No allele-specific NF binding was detected using EMSA for *IL10*-592 A/C (data not shown). These observations suggest that −819 C/T is a functional polymorphism. We also analyzed the effects of this polymorphism on IL-10 production in LPS- and hyaluronic acid-stimulated cultures of PBMCs from healthy subjects from the Marseille blood bank. We found that −819 C/C was associated with higher levels of IL-10 production than the −819 C/T and T/T genotypes (Table V).

Table V. *IL10−819CC is associated with increased production of IL-10 by PBMC from healthy blood donors*[a]

	IL10-819 CC ($n = 11$)			*IL10*-819 CT and TT ($n = 17$)			
	Arithm mean	S.E.M	Median	Arithm mean	S.E.M	Median	p Value[b]
LPS (3 μg/ml)	187.0	87.6	63	29.4	11.7	7	0.03
HA (1 μg/ml)	157.8	76.3	29	34.8	13.5	14	0.05
none	44.3	25	33	13.9	4.3	5	0.1

[a] PBMC were stimulated with 3 μg/ml LPS or 1 μg/ml Hyaluronic acid. IL-10 was measured in 48 h supernatants.
[b] Student T test.

Discussion

There is a considerable amount of data in experimental models on the immunology of *Leishmania* infections. This is partly due to the availability of genetically modified mice, allowing a fine genetic dissection of the immune response. Human leishmaniasis studies are less advanced and there are still key unresolved questions relating to disease mechanisms in most severe *Leishmania* infections. In this study, we have combined immunological and genetic approaches to evaluate which components of the cytokine response contribute most to the development and persistence of lesions caused by Lb.

We evaluated the production of cytokines reported to play a key role in experimental leishmaniasis. We firstly recruited a group of subjects without CL lesions or scars who have been living for >20 years in an area associated with high Lb transmission and whose PBMC produced IFN-γ when cultured with *Leishmania* extracts. Positive IFN-γ responses confirmed exposure to infection. Thus, these patients are likely to have controlled a previous infection with no detectable lesion (or very small lesions that have escaped detection and healed spontaneously); these subjects were considered to be resistant to disease. This is further supported by our findings that the PBMCs of these individuals mounted a cytokine response comparable to that of hCL subjects. Previous studies have shown that most patients with healed lesions have developed immunity allowing them to control further infections with Lb. Secondly, we assessed a previously reported observation that lesions fail to heal in most subjects, despite a strong IFN-γ response. However, the response in a third of the subjects with active lesions was strongly Th2 polarized with undetectable IFN-γ and low IL-12 production. Thus, the low production of IFN-γ in these subjects likely contributes to the development and persistence of the lesions, as has been observed in BALB/C mice infected with *L. major*. A critical factor in these patients could be their weak IL-12 response, as IL-12 in mice is essential for redirecting an early IL-4 response toward a Th1 response (8, 9). IL-12 was lower in IFN- aCL cultures than IFN^+ aCL cultures, but this was not significant after correction for multiple comparisons. PBMCs of aCL IFN^- patients and those of aCL patients producing IFN-γ exhibited a strong IL-10 response, which may also interfere with Th1 development. The strong IL-10 response likely reflects in vivo stimulation, as high IL-10 levels were also present in resting cultures. Multivariate analysis allowed us to weight all tested cytokines in subjects with active lesions with respect to resistant subjects. IL-10 was strongly associated with active lesions in PBMC cultures from IFN^- and IFN^+ patients. Indeed, the association was so strong that none of the other cytokines that are thought to play a key role in immunity to *Leishmania* were accepted in the regression models that tested the cytokines' association with lesions. This finding is consistent with results reported in experimental models: these models demonstrate greater protection owing to the removal of either *IL10* or *IL10RA* (14–17, 30) or greater susceptibility caused by the over expression of IL10 (12, 13) in infections by *L. major* or *L. donovani*. IL-10 has not been investigated in human leishmaniasis as extensively as in animal models. This is partly due to the lack of genetic and immunological studies on naturally resistant subjects. Studies on nonhealing lesions as a result of *L. mexicana* infection have shown an association with increased IL-10 and TGF-β production (24) in the presence of IFN-γ; this suggests that IFN-γ is neutralized by IL-10 and/or TGF-β. Severe and rare MCL disease was associated with low IL-10 and TGF-β production (20) and lower IL-10 receptor expression than that observed in LCL (23). Thus, the role of IL-10 in human CL, as a result of Lb infection, was less clear. It was suggested that the absence rather than the high production of IL-10 was the cause of disease due to exaggerated inflammation caused by Th1 cytokines. Acute KA is caused by significant suppression of IFN-γ, and of IL-12 responses associated with production of IL-10 and IL-4 (31, 32). High levels of *IL10* and *IFNG* transcripts have been reported in bone marrow aspirates (33). In addition, adding anti-IL-10 Abs to PBMC cultures from KA patients have been shown to enhance IFN-γ production (32, 34).

This study also showed that *IL10*-819C increases the risk of skin lesion. To establish the causal relationship between *IL10*-819C and CL, we firstly analyzed all polymorphisms present in *IL10* in these patients. We then determined which SNPs were strongly correlated ($r^2 = 0.8$, correlation groups) and tested the association of the SNPs from these groups with CL. We found that the *IL10*-819 correlation group was strongly associated with CL. We performed this analysis using family based association studies, not affected by selection bias. The observed association does not necessarily demonstrate a causative role for the SNPs in the −819 correlation group in disease. We thus performed functional studies to investigate potential functional effects of the promoter polymorphisms, −819 C/T and −592 A/C. The −819 mutation, but not −592 A/C, modified NF binding. The *IL10*-819C/C genotype was associated with increased IL-10 production in LPS- or HA-stimulated. Thus, −819C/C in the *IL10* promoter region is associated with 1) up-regulation of IL-10, 2) modification of NF binding, and 3) an increased risk of lesions. Our genetic studies, together with findings of a strong association with increased IL-10 production and active lesions, establish that this IL-10 variant increases the risk of lesion caused by Lb.

IL-10 may suppress immunity in several ways: IL-10 inhibits the production of reactive nitrogen intermediates by IFN-γ-activated macrophages (35, 36), reduces the production of IL-12 and TNF (37, 38) by activated macrophages, and deviates the Th response toward a Th2 response by acting on costimulatory molecules of APCs (8); and IL-10 also synergizes with TGF-β to inhibit macrophage microbicidal activity (36).

IL-10-producing mouse $CD4^+CD25^+Foxp3^+$ T cells are recruited during experimental infection with *L. major;* these cells down-regulate immunity allowing the persistence of low-level infections and disease reactivation (17, 39). $CD4^+CD25^+$ Tregs are present in *L.braziliensis* lesions (40) and IL-10-producing $CD4^+CD25^-$ Tregs isolated from the spleen of *L. donovani* infected patients enhance parasite growth (41). Thus, we investigated the source of IL-10 in our study subjects and characterized the Tregs. We showed that IL-10 produced in PBMC cultures from all clinical groups was released mostly by monocytes and $CD4^+CD25^+$ T lymphocytes. We cannot exclude that $CD4^+CD25^-$ T lymphocytes could also contribute to IL-10 production, but to a lesser extent than $CD4^+CD25^+$ T lymphocytes. A substantial proportion of the IL-10-producing $CD25^+$ T lymphocytes were $Foxp3^+$. These $CD4^+CD25^+Foxp3^+$ T lymphocytes exerted nonspecific suppression in vitro and were therefore Tregs. Our preliminary data (not shown) suggested that inhibiting IL-10 reduces this Treg-mediated suppression. We tested whether patients with active lesions had higher levels of $CD4^+CD25^+IL\text{-}10^+$ and $CD4^+CD25^+Foxp3^+$ Tregs than resistant subjects or subjects with healed lesions, but found no convincing evidence for this. These findings may be interpreted in several ways: first, the composition of the $IL\text{-}10^+$ T cell population in the blood may not reflect that of the T cells infiltrating the lesions; second, other T cells, including $IL\text{-}10^+$ IFN^+ Th1 cells in mice infected with *Leishmania* and *Toxoplasma* (42, 43), produce IL-10. Furthermore, more recent studies (44–47), published since submission for

publication of this paper, indicate that IL-27 induces mouse Th1 and Th2 $CD25^+$ lymphocytes to produce IL-10 and that Th17 cells produce IL-10. IL-27 does not induce IL-10 in $CD25^+Foxp3^+$ T cells. IL-27, however, together with TGF-β, induces Tr-1 ($CD25^-Foxp3^-IL\text{-}10^+$ T cells). It remains to be determined whether these cell populations are present in subjects infected with Lb, and whether they regulate the infection.

This study bridges an important gap between existing experimental models and immunological human studies, demonstrating that IL-10 contributes significantly to the development of lesions in humans infected with Lb. Our observations relating to $IL\text{-}10^+CD4^+CD25^+$ cells in patient PBMCs also suggest that these cells are important regulators of immunity against *Leishmania* in humans, as observed in mice. This study further illustrates the impact of genetic polymorphisms on human susceptibility to infection. In this regard, polymorphisms in other genes coding for components of the IL-10 pathway must also be evaluated.

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Disclosures

The authors have no financial conflict of interest.

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