

# Immunologic Markers of Protection in *Leishmania* (*Viannia*) *braziliensis* Infection: A 5-Year Cohort Study

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**Background.** The control of *Leishmania braziliensis* by individuals with subclinical infection (SC) are unknown.

**Methods.** A cohort of 308 household contacts (HCs) of patients with cutaneous leishmaniasis (CL) was established in 2010 in an endemic area and followed up for 5 years. Whole-blood cultures stimulated with soluble *Leishmania* antigen and a *Leishmania* skin test (LST) were performed in years 0, 2, and 4. The identification of the lymphocyte subsets secreting interferon (IFN)  $\gamma$  and the ability of monocytes to control *Leishmania* were determined.

**Results.** During follow-up, 118 subjects (38.3%) had evidence of *L. braziliensis* infection. Of the HCs, CL was documented in 45 (14.6%), 101 (32.8%) had SC infection, and 162 (52.6%) did not have evidence of exposure to *L. braziliensis*. The ratio of infection to disease was 3.2:1. IFN- $\gamma$  production, mainly by natural killer cells, was associated with protection, and a positive LST result did not prevent development of disease. Moreover, monocytes from subjects with SC infection were less permissive to parasite penetration and had a greater ability to control *L. braziliensis* than cells from patients with CL.

**Conclusions.** Protection against CL was associated with IFN- $\gamma$  production, negative LST results, impaired ability of *Leishmania* to penetrate monocytes, and increased ability to control *Leishmania* growth.

**Keywords.** subclinical leishmaniasis; household contacts; *Leishmania braziliensis*; cutaneous leishmaniasis; IFN- $\gamma$ ; *Leishmania* skin test.

Cutaneous leishmaniasis (CL) is the most common clinical presentation of tegumentary leishmaniasis caused by *Leishmania* (*Viannia*) *braziliensis* [1]. The lesions are typically characterized by a single ulcer with raised borders predominantly located in the lower limbs. The delayed-type hypersensitivity test to soluble *Leishmania* antigen (SLA), the *Leishmania* skin test (LST), has a high sensitivity for the diagnosis of CL and is also used to identify individuals exposed to the parasite in the absence of disease [2–4]. Of healthy individuals without a history of CL living in endemic areas, 10%–15% have a positive LST result [3, 5]. More recently, we showed that in vitro interferon (IFN)  $\gamma$  production increases the sensitivity to detect exposure to *L. braziliensis* [6]. Individuals with a positive LST result or evidence of IFN- $\gamma$  production without clinical manifestations of CL are classified as having subclinical (SC) *L. braziliensis* infection [6–8].

The main mechanism of *Leishmania* killing is macrophage activation induced by IFN- $\gamma$  [9–11]. The importance of a T-helper 1 immune response to control parasite growth and dissemination

is well documented in leishmaniasis [12, 13]. However, in *L. braziliensis* infections, the parasite persists, and both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell activation have been linked to disease [14, 15]. There is a direct correlation between the frequency of CD4<sup>+</sup> T cells expressing IFN- $\gamma$  and tumor necrosis factor (TNF) and the size of the ulcer [14]. Moreover, IFN- $\gamma$  and TNF levels are higher in mucosal leishmaniasis than in CL [16]. Finally, there is a direct correlation between the frequency of CD8<sup>+</sup> T cells and CD8<sup>+</sup> T cells expressing granzyme and the intensity of the inflammatory response in CL ulcers [17]. Because individuals with SC infection produce less IFN- $\gamma$ , TNF, and granzyme B than patients with CL [18], one possibility is that control of *L. braziliensis* infection in these subjects is related to the innate immune response.

To better understand the immunologic factors involved in the protection against disease due to *L. braziliensis*, we established a cohort in 2010 of household contacts (HCs) of patients with CL who had no personal history of CL. In the present study, we determined the incidence of CL and identified host factors associated with SC infection or CL in subjects exposed to *L. braziliensis*.

## METHODS

### Area of Study and Establishment of a Cohort

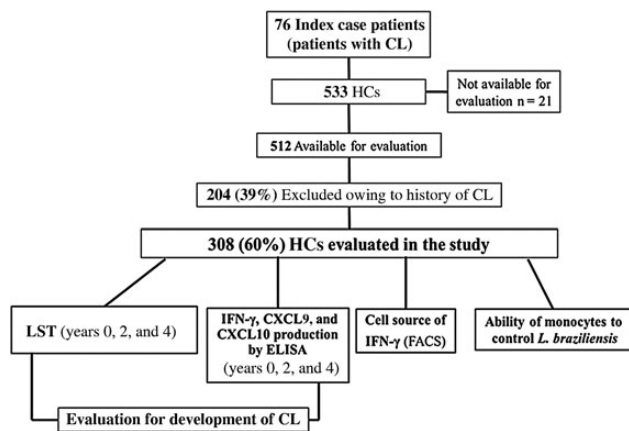
This study was conducted in the village of Corte de Pedra, located in the southeast of Bahia, Brazil. This is a well-known area of *L. braziliensis* transmission that registers the highest incidence

Received 8 March 2016; accepted 5 May 2016; published online 18 May 2016.

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The Journal of Infectious Diseases® 2016;214:570–6

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**Figure 1.** Study design of a cohort of household contacts (HCs) in a *Leishmania braziliensis* endemic area and immunologic studies performed. Immunologic studies were performed in years 0, 2, 4, and development of cutaneous leishmaniasis (CL) was monitored during the whole duration of the cohort study. Abbreviations: ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; IFN, interferon; LST, *Leishmania* skin test.

rates of CL in Brazil. The cohort was established in January 2010, with HCs of 76 index case patients with a diagnosis of CL, within 1 year before the initiation of the study. At that time, 533 HCs were identified. Of those, 225 were excluded from the study, 204 with a history of CL and 21 who could not be located. Therefore, 308 individuals without a history of CL participated in this study (Figure 1).

### Experimental Design

The 308 participants received an identification card, a history of CL was obtained, and a physical examination was performed, looking for cutaneous ulcers and scars indicative of CL. Subjects were instructed to seek medical attention at the Health Center of Corte de Pedra if lymphadenopathy, papular lesions, or cutaneous ulcers developed. In addition, 1 visit was performed annually for active surveillance of CL. Those suspected of having CL had the diagnosis confirmed by parasite isolation or a positive polymerase chain reaction result for *L. braziliensis* [19].

At study entrance, LST and in vitro cytokine production were performed. These immunologic studies were repeated at years 2 and 4. We determined the source of IFN- $\gamma$  in a convenience sample of a limited subset of HCs who produced IFN- $\gamma$  and remained free of disease. In addition, we evaluated the ability of the monocytes to control *L. braziliensis* in a subset of HCs with evidence of immune response that remained free of the disease and in HCs who developed CL. Of the 308 HCs, 19 underwent immunologic studies only in the first 2 evaluations, and 15 had immunologic data only for the first evaluation because of refusal to undergo blood collection ( $n = 21$ ) or relocation to another town ( $n = 13$ ). Patients who relocated were contacted by phone to inquire about the presence of CL lesions. CL developed in none of these 34 subjects during follow-up.

This research was approved by the Ethics Committee of the Federal University of Bahia and the Weill Cornell Medical College Institutional Review Board. Informed consent was obtained from each participant or his or her parents.

### LST and In Vitro Cytokine and Chemokine Production

The LST was conducted using SLA, as described elsewhere [20]. The reaction was considered positive when the diameter was  $\geq 5$  mm. For cytokine production, heparinized peripheral blood (10 mL) was collected immediately before the LST. Aliquots of 1 mL of whole blood were dispensed into a 24-well tissue plate. Cultures were left unstimulated or were stimulated with SLA at 10  $\mu\text{g}/\text{mL}$  and incubated at 37°C at 5% carbon dioxide ( $\text{CO}_2$ ) for 72 hours. Plasma supernatants were collected and the levels of IFN- $\gamma$ , interleukin 10, CXCL9, and CXCL10 were quantified by means of enzyme-linked immunosorbent assay, using commercially available reagents (BD OpTEIA). Results were obtained using the plate reader Emax (Molecular Devices) and expressed in picograms per milliliter.

Subjects were censored at the time of development of CL. If there was evidence of exposure to *L. braziliensis*, the last available chemokine values were analyzed. Similarly, the last available chemokine levels were analyzed for subjects without evidence of exposure to *L. braziliensis*.

### Cell Culture of Peripheral Blood Mononuclear Cells and Flow Cytometry Analysis (Fluorescence-Activated Cell Sorting)

Peripheral blood mononuclear cells from 5 individuals with SC infection and 10 patients with CL were obtained by density gradient centrifugation using heparinized whole blood over Ficoll-Paque Plus medium (GE Healthcare). After washing, cells were resuspended in Roswell Park Memorial Institute 1640 medium (Gibco BRL) supplemented with 10% human AB Rh<sup>+</sup> serum (Sigma Chemical Co) and antibiotics. Cells ( $5 \times 10^5/\text{mL}$ ) were stimulated with SLA (5  $\mu\text{g}/\text{mL}$ ) and cultured at 37°C and 5%  $\text{CO}_2$  for 6 hours. Monensin (Sigma) was added at 25  $\mu\text{mol}/\text{mL}$  during the last 4–5 hours of culture to inhibit protein transportation. Cells were then harvested, stained with fluorochrome-conjugated anti-human CD56, CD8, and CD4 antibodies (BD Biosciences) for 20 minutes, washed, fixed, and permeabilized, as described elsewhere [14]. Cells were stained for IFN- $\gamma$  for 30 minutes, washed with Perm/Wash (BD Biosciences) buffer and resuspended in phosphate-buffered saline (pH 7.2) plus 1% bovine albumin. A minimum of 500 000 events were evaluated for each sample. A FACS Canto II flow cytometer (BD Pharmingen) was used, and analysis was performed using FlowJo software version X (Tree Star).

### Evaluation of *L. braziliensis* Control by Monocytes

An isolate of *L. braziliensis* was cultured in Schneider medium supplemented with fetal bovine serum (FBS). Peripheral blood mononuclear cells ( $1 \times 10^6$  cells per tube) from individuals with SC infection and patients with CL were infected with *L. braziliensis* promastigotes in the stationary phase at a ratio of 5:1 cells and incubated at 37°C in 5%  $\text{CO}_2$ . After 2 hours, extracellular

parasites were removed after centrifugation. The cells were placed in Roswell Park Memorial Institute 1640 medium supplemented with FBS and incubated for another 24, 48, and 72 hours. After these periods of time, the percentage of infected cells and the number of intracellular parasites were determined by microscopic evaluation of 100 monocytes, after May-Grunwald-Giemsa staining from cytocentrifuge preparations. To quantify variable parasites in *L. braziliensis*-infected monocytes after 72 hours, the culture medium was replaced by 0.5 mL of Schneider medium (Sigma-Aldrich) supplemented with 10% FBS and cultured for 5 additional days.

### Statistical Analysis

Data are expressed as mean (standard deviation [SD]) or median (interquartile range). The Mann-Whitney test was used to compare continuous variables between 2 groups, and the Student *t* test and Kruskal-Wallis test to compare them among 3 groups. The Fisher exact test was used to compare proportions. Analyses were conducted using Prism 4 software (GraphPad) and IBM SPSS Statistics 20 software. Differences were considered statistically significant at  $P < .05$ . To determine the incidence of CL, we used person-year analysis, taking into account the number of persons observed as well as the duration of the observation period. We also calculated the relative risk (MedCalc software; version 15.8).

## RESULTS

### Demographic, Epidemiologic and Immunologic Features of HCs of CL Patients According to Disease Outcome

Of the 308 HCs, 45 developed CL, 101 had SC *L. braziliensis* infection, and 162 had no evidence of exposure to *L. braziliensis*.

Table 1 summarizes the demographic, epidemiologic, and immunologic features of the 3 groups of subjects. Subjects who developed CL were younger than the others groups and were predominantly students. Production of CXCL9 and CXCL10 in cultures stimulated with *L. braziliensis* was higher ( $P < .005$ ) in those who had evidence of exposure to *L. braziliensis*, independent of the outcome of the infection.

### Frequency of HCs Who Remained Free of Disease or Developed CL According to Positivity of the LST or IFN- $\gamma$ Production

Figure 2 details the number of HCs with evidence of immune response based on a positive LST result or IFN- $\gamma$  production, as well as the number who developed CL or remained free of disease. Of the 308 HCs followed up for 5 years, 118 (25.5%) had evidence of immune response, including 53 (17.2%) with a positive LST result, of whom 30 (56.6%) produced IFN- $\gamma$ . We also found that 65 (25.5%) of the 308 HCs had evidence of IFN- $\gamma$  production despite a negative LST result. Using both tests as evidence of *L. braziliensis* infection increased the likelihood of detecting exposure to *L. braziliensis* by 2.2-fold compared with using LST alone. Moreover, there was only a fair concordance between the 2 tests (kappa, 0.24; 95% confidence interval, .12-.35).

Of the 45 HCs (14.6%) who developed CL, 19 had CL in year 1, ten in year 2, nine in year 3, four in year 4, and three in year 5. Among those who developed CL, 8 (17.8%) had a positive LST result before developing signs of the disease. In contrast, only 2 HCs (3.0%) who produced IFN- $\gamma$  despite a negative LST result went on to develop CL. The remaining 35 (18.4%) who developed CL had a negative LST result and did not produce IFN- $\gamma$ .

**Table 1. Demographic, Epidemiologic, and Immunologic Findings in Household Contacts (n = 308) of Patients With CL, According to the Development of CL or SC *Leishmania braziliensis* Infection<sup>a</sup>**

Variable	CL (n = 45)	SC Infection (n = 101)	Controls (n = 162)	<i>t</i>
Age, mean (SD), y	19 (9)	25 (15) <sup>b,c</sup>	20 (12)	0.017
Male sex, No. (%)	25 (56)	52 (52)	75 (47)	0.505
Occupation, %				
Agriculture	13	24	21	0.039
Domestic	16	32	22	
Students and others	71	45	58	
Time in the endemic area, mean (SD), y	20 (9)	25 (14) <sup>d</sup>	20 (11)	0.018
Chemokine data, median (IQR)				
CXCL9 (medium)	4976 (3000-14 941)*	5307 (2413-10 019)*	4228 (1864-7068)*	0.036
CXCL9 (SLA)	8912 (3252-26 158)*	11320 (5116-40 258)*	5257 (2772-8216)* <sup>e,f</sup>	<0.001
CXCL10 (medium)	407 (0-1764)*	591 (334-1067)*	634 (374-1279)*	0.285
CXCL10 (SLA)	713 (260-28 434)*	1203 (574-6189)*	784 (441-1622)* <sup>g,h</sup>	0.014

Abbreviations: CL, cutaneous leishmaniasis; IQR, interquartile range; SC, subclinical leishmaniasis; SD, standard deviation; SLA, soluble *Leishmania* antigen.

<sup>a</sup> The Student *t* test was used to compare continuous variables, the Fisher exact test to compare proportions, and the Kruskal-Wallis test to compare chemokine values.

<sup>b</sup>  $P = .04$  for CL vs SC infection.

<sup>c</sup>  $P = .004$  for SC infection vs control.

<sup>d</sup>  $P = .005$  for SC infection vs control.

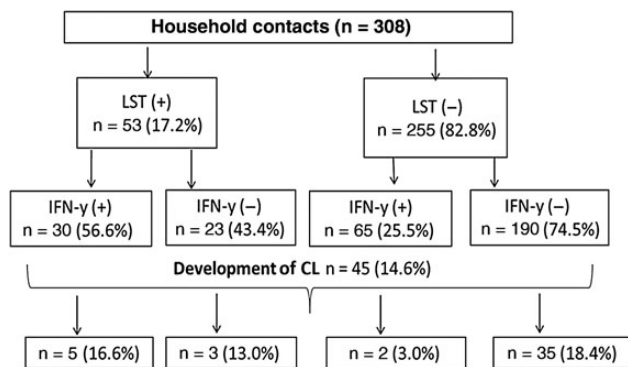
<sup>e</sup>  $P < .001$  for SC infection vs control.

<sup>f</sup>  $P = .03$  for CL vs control.

<sup>g</sup>  $P = .002$  for SC infection vs control.

<sup>h</sup>  $P = .004$  for CL vs control.

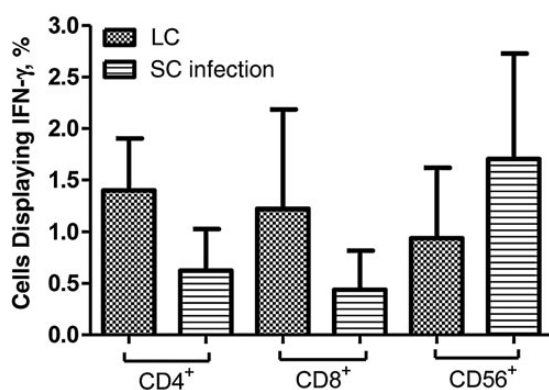
\* The chemokine's values are expressed by pg/mL.



**Figure 2.** Development of cutaneous leishmaniasis (CL) or subclinical *Leishmania braziliensis* infection in household contacts of patients with CL. The data indicate the number of individuals who had evidence of immune response based on positivity of the *Leishmania* skin test (LST) and interferon (IFN)  $\gamma$  production during the 5 years of follow-up and the number of household contacts who developed CL. Parenthetical plus and minus signs denote positive and negative findings, respectively.

### Incidence of Cutaneous Leishmaniasis According to the Immunologic Response to Leishmania Antigen

We calculated the incidence of CL according to the immune response to SLA: IFN- $\gamma$  production, positive LST results, and both negative LST and negative IFN- $\gamma$  results (Table 2). This analysis showed that although the risk of developing CL was similar in the LST-positive and LST-negative/IFN- $\gamma$ -negative groups, the risk was reduced by 85% in those who had only IFN- $\gamma$  production when compared with the group without evidence of immune response. When we compared the frequency of CL in HCs who were IFN- $\gamma$  positive with those who were LST positive, HCs who were LST positive had a 6.2-fold greater chance of developing CL than those who had only IFN- $\gamma$  production.



**Figure 3.** Cell source of interferon (IFN)  $\gamma$  production by flow cytometry. The graph shows the total frequency of CD4<sup>+</sup>, CD8<sup>+</sup>, and CD56<sup>+</sup> cells from household contacts (n = 5) and patients with cutaneous leishmaniasis (CL) (n = 10) expressing IFN- $\gamma$  after stimulation with soluble *Leishmania* antigen. Peripheral blood mononuclear cells from household contacts and patients with CL were stimulated with 5  $\mu$ g of soluble *Leishmania* antigen, and the frequency of cells expressing IFN- $\gamma$  was determined by fluorescence-activated cell sorting. Abbreviation: SC, subclinical.

### Analysis of Cell Source of IFN- $\gamma$

Flow cytometry analysis was conducted to determine the source of IFN- $\gamma$  in subjects who developed SC infection and belonged to the IFN- $\gamma$ -positive/LST-negative group and in patients with CL (Figure 3). The main sources of IFN- $\gamma$  in subjects with SC infection were natural killer (NK) cells (1.7%) followed by CD4<sup>+</sup> T (0.62%) cells and CD8<sup>+</sup> T cells (0.43%); in patients with CL, the main sources were CD4<sup>+</sup> T cells (1.4%), followed by CD8<sup>+</sup> T cells (1.2%) and NK cells (0.94%).

### Ability of Monocytes From Individuals With SC Infection to Control *L. braziliensis* Infection

The monocytes from individuals with SC infection and patients with CL were infected with *L. braziliensis*, and the number of infected cells and the parasite load were evaluated using optical microscopy (Figure 4). The mean (SD) percentages of infected cells were similar after 2 hours of infection in both groups (55  $\pm$  15% in SC infection and 47  $\pm$  17% in CL). However, there was a significant decrease in the mean (SD) frequency of infected monocytes from individuals with SC infection (35  $\pm$  8.6% [ $P$  < .05] and 12  $\pm$  4% [ $P$  < .01] after 24 and 72 hours, respectively). In cells from patients with CL there was no difference ( $P$  > .05) in the mean (SD) frequency of infected monocytes at 24 hours (55  $\pm$  22.5) and 48 hours (63  $\pm$  28). At 72 hours there was a decrease ( $P$  < .05) in the frequency of infected cells (29  $\pm$  7) (Figure 4A). To assess whether the decrease in the parasite load was related to *Leishmania* killing, we evaluated the viability of promastigotes in culture of infected monocytes from patients with CL and individuals with SC infection. The mean (SD) number of motile promastigotes in Schneider medium was higher in lysed monocytes from patients with CL (23  $\pm$  6.4) than in those from subjects with SC infection (9.6  $\pm$  16);  $P$  < .01.

Although the percentages of infected cells were similar in the 2 groups after 2 hours of infection, the mean (SD) number of amastigotes per 100 cells at 2 hours in monocytes from individuals with SC infection was lower than that in patients with CL (55  $\pm$  15 vs 175  $\pm$  82, respectively;  $P$  < .05). Moreover, although in monocytes from subject with SC infection the number of amastigotes per 100 cells decreased significantly at 24 and 48 hours, in patients with CL there was a slight increase in the number of parasites in this period of time (Figure 4B).

### DISCUSSION

Understanding how the infections behave in naturally exposed individuals who do not develop disease is crucial to determine the mechanisms behind the control of the infection and may contribute to vaccine development. Herein, we showed that evidence of both innate and adaptive immune response are markers of exposure to *L. braziliensis*, but control of the infection was due to the innate immune response. We extend our previous observation that IFN- $\gamma$  production supplements the LST to detect exposure to infection, showing that the addition of measuring IFN- $\gamma$  production to the LST increased the detection of

**Table 2. Incidence Rates and Relative Risk of CL According to the Host's Immune Response in a Cohort of Household Contacts of Patients With CL**

Group	Incidence Cases of CL	Person-Years of Observation	Incidence Rate per 100 Person-Years (95% CI)	Relative Risk (95% CI) <sup>a</sup>
LST positive	8	138	5.79 (2.50–11.42)	0.96 (.38–2.10)
LST negative and IFN- $\gamma$ positive	2	214	0.93 (.11–3.37)	0.15 (.02–.58)
LST and IFN- $\gamma$ negative	35	578	6.05 (4.21–8.42)	...

Abbreviations: CI, confidence interval; CL, cutaneous leishmaniasis; IFN, interferon; LST, *Leishmania* skin test.

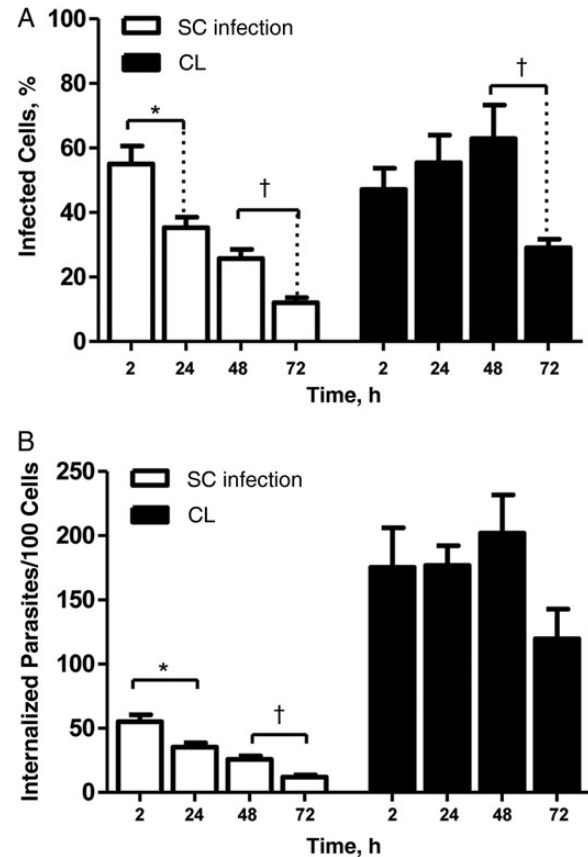
<sup>a</sup> Relative risks were calculated using the group with both negative LST and negative IFN- $\gamma$  findings as the reference group (denominator).

*L. braziliensis* infection by 2.2-fold. The ratio of infection to disease was 3.2:1. Development of CL was more frequent among younger subjects, who were predominantly students. Furthermore, protection against the development of CL was associated with IFN- $\gamma$  production rather than with a positive LST result. We also found that monocytes play a key role in the control of *L. braziliensis* infection. NK cells were the major source of IFN- $\gamma$  in individuals with SC infection, and monocytes from these subjects were less permissive to the infection and control *Leishmania* growth more effectively than cells from those who developed CL.

In an area of *Leishmania major* transmission, it has been shown that a positive LST result was associated with a decrease in the incidence of CL [21]. In the current study, we showed that the incidence rate of CL in individuals who had a positive LST result is 6.2 times higher than the rate in those who had only IFN- $\gamma$  production, indicating that evidence of IFN- $\gamma$  production reduced the risk of developing CL by 85%. So, IFN- $\gamma$  production early in the infection may participate in the control of parasite growth and thereby prevent the development of CL.

Several studies have shown that IFN- $\gamma$  participates in the activation of phagocytic cells, inducing parasite killing [22, 23], but there are several cell sources of IFN- $\gamma$  [18, 24]. We found that all 3 T-cell subsets, CD56<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup>, produced this cytokine but CD3<sup>-</sup>CD56<sup>+</sup> (NK) cells were the main source of IFN- $\gamma$  in subjects exposed to *L. braziliensis* who did not develop CL. Although in patients with CL the majority of cells expressing IFN- $\gamma$  were CD4<sup>+</sup> and CD8<sup>+</sup> T cells, the CD3<sup>-</sup>CD56<sup>+</sup> (NK cells) were the main source of IFN- $\gamma$  in subjects exposed to *L. braziliensis* who did not develop CL. IFN- $\gamma$  production may have a dual role in *L. braziliensis* infection. In patients with CL, there is a correlation between the frequency of CD4<sup>+</sup> cells producing IFN- $\gamma$  and lesion size [14]. Moreover IFN- $\gamma$  levels are higher in CL than in SC infection [3, 25]. Consequently it is likely that early in the infection IFN- $\gamma$  production is related to protection, whereas in the late phase it is associated with disease. In CL due to *L. braziliensis*, virtually all patients have positive LST results and produce IFN- $\gamma$  in vitro. The documentation that NK cells are the major source of IFN- $\gamma$  in SC infection may also explain the only fair concordance between LST results and IFN- $\gamma$  production in these subjects. In this setting the LST response may be related to T cells, whereas IFN- $\gamma$  is produced by NK cells.

The killing of *Leishmania* is mediated by IFN- $\gamma$  activated macrophages or cytotoxicity induced by CD8<sup>+</sup> T cells or NK cells [26–29]. However, in CL, although CD8<sup>+</sup> T cells induce apoptosis of infected monocytes, *Leishmania* killing is impaired [18, 30]. NK cells may be inflammatory, regulatory, and cytotoxic. Therefore, studies should be designed to evaluate whether, in addition to being an important source of IFN- $\gamma$  in SC, NK cells from these subjects have the ability to kill *L. braziliensis*.



**Figure 4.** Phagocytosis and the ability to control *Leishmania braziliensis* by monocytes from individuals with subclinical (SC) infection. Monocytes from 7 individuals with SC infection and 7 patients with cutaneous leishmaniasis (CL) were infected with *L. braziliensis* at a 5:1 ratio at different times. The number of infected cells (A) and the number of intracellular parasites (B) were determined by microscopic evaluation after May-Grunwald-Giemsa staining from cytocentrifuge preparations. Data are summarized as means and standard deviations. \*  $P < .05$ ; †  $P < .01$  (Mann-Whitney test).

*Leishmania* induces a potent inflammatory response in macrophages. In a previous study, cytokine production and macrophage killing of *L. braziliensis* were compared between HCs with SC infection and patients with CL. Macrophages from subjects with SC infection were less inflammatory and have more ability to kill *Leishmania* than cells from patients with CL [31]. Moreover macrophages from individuals with SC infection are less permissive to *Leishmania* penetration than cells from patients with recurrent CL [32], and in the current study we show that, although the frequencies of monocytes infected by *L. braziliensis* were similar in SC infection and in CL, there was a significant decrease in the percentage of infected cells at all time points. The low number of amastigotes 2 hours after infection indicates a decreased ability of *L. braziliensis* to penetrate in monocytes from individuals with SC infection. After 72 hours of infection there was a decrease in the frequency of infected cells in patients with CL, but this was probably due to the death of the cells and release of viable parasites.

This study has several limitations. For simplicity, we analyzed only 1 chemokine value per subject rather than using survival analyses methods to incorporate the longitudinal data. Because immunologic evaluations were performed every 2 years, the exact time of exposure could not be determined. Indeed, because the knowledge of the role of immune response in the pathogenesis of CL has grown rapidly, some mediators that may play important roles in the development of the disease, such as metalloproteinases, type 1 IFN- $\gamma$ , and interleukin 1 $\beta$ , were not evaluated. Nonetheless, we have made highly relevant observations. Specifically, we have demonstrated that IFN- $\gamma$  production by NK cells and the ability of monocytes to control *Leishmania* were the defense mechanisms preventing subjects exposed to *L. braziliensis* from developing CL.

## Notes

**Acknowledgments.** We thank all household contacts who participated in this study, the undergraduate students for the help with the enzyme-linked immunosorbent assays, and the secretaries of the Serviço de Imunologia, Complexo Hospitalar Universitário Professor Edgard Santos for their assistance in preparing this manuscript.

**Financial support.** This work was supported by National Institutes of Health (grant AI030639).

**Potential conflicts of interest.** All authors: No potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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