



Immune Profile of the Nasal Mucosa in Patients with Cutaneous Leishmaniasis

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ABSTRACT Localized skin lesions are characteristic of cutaneous leishmaniasis (CL); however, *Leishmania (Viannia)* species, which are responsible for most CL cases in the Americas, can spread systemically, sometimes resulting in mucosal disease. Detection of *Leishmania* has been documented in healthy mucosal tissues (conjunctiva, tonsils, and nasal mucosa) and healthy skin of CL patients and in individuals with asymptomatic infection in areas of endemicity of *L. (V.) panamensis* and *L. (V.) braziliensis* transmission. However, the conditions and mechanisms that favor parasite persistence in healthy mucosal tissues are unknown. In this descriptive study, we compared the cell populations of the nasal mucosa (NM) of healthy donors and patients with active CL and explored the immune gene expression signatures related to molecular detection of *Leishmania* in this tissue in the absence of clinical signs or symptoms of mucosal disease. The cellular composition and gene expression profiles of NM samples from active CL patients were similar to those of healthy volunteers, with a predominance of epithelial over immune cells, and within the CD45⁺ cell population, a higher frequency of CD66b⁺ followed by CD14⁺ and CD3⁺ cells. In CL patients with molecular evidence of *Leishmania* persistence in the NM, genes characteristic of an anti-inflammatory and tissue repair responses (*IL4R*, *IL5RA*, *POSTN*, and *SATB1*) were overexpressed relative to NM samples from CL patients in which *Leishmania* was not detected. Here, we report the first immunological description of subclinically infected NM tissues of CL patients and provide evidence of a local anti-inflammatory environment favoring parasite persistence in the NM.

KEYWORDS persistence, Th1/Th2, asymptomatic infection, cutaneous leishmaniasis, nasal mucosa

The subclinical persistence of *Leishmania* after clinical resolution of symptomatic disease and the establishment of naturally occurring asymptomatic infections are characteristic of human leishmaniasis (1–4). Molecular and clinical evidence of parasite persistence has been particularly shown during *Leishmania (Viannia)* species infections. Evidence of this is the detection of parasite kinetoplast DNA (kDNA) and RNA molecules in apparently unaffected tissues, such as oropharyngeal mucosa, healthy skin, and blood of cutaneous leishmaniasis (CL) patients (5–7), as well as the development of mucosal disease or reactivation of cutaneous lesions after several years of primary exposure to infection (8–10).

In areas of *L. (V.) panamensis* endemicity in Colombia, detection of *Leishmania* in healthy tissues of asymptotically infected individuals and of patients with active CL has been estimated to occur in at least 40% of the population, even after treatment and clinical resolution of disease (2, 11). Similarly, in regions of *L. (V.) braziliensis* endemicity in Brazil, molecular detection of *Leishmania* in healthy mucosal tissues was reported in

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7.8% of CL patients and more frequently found in individuals with signs of more severe cutaneous disease (i.e., more or larger skin lesions) (7).

Active mucosal leishmaniasis is characterized by scarcity of parasites in the mucosal lesions and a predominant and exuberant Th1 immune response mediating the pathology (12). The appearance of mucosal lesions typically follows active cutaneous leishmaniasis occurring several years before. This, and the presence of *Leishmania* in healthy mucosal tissues (in the absence of clinical manifestations of disease), indicates that parasite migration from the inoculation site/cutaneous lesion to the nasopharyngeal mucosa does not explain, by itself, the pathogenesis of mucosal leishmaniasis.

Evidence of the participation of host immune responses in the pathogenesis of dermal leishmaniasis includes the development of cutaneous lesions at distal sites after local trauma (13–15) or immunosuppression, involvement of deregulated immune responses in chronic disease (including mucosal leishmaniasis), and more frequent mucosal compromise in immunodeficient individuals (16, 17). For infections caused by some *Leishmania* (*Viannia*) species, it has been suggested that the presence of *Leishmania* RNA virus (LRV) can promote pathology through enhancement of the proinflammatory response (18–20); however, whether LRV contributes to *Leishmania* migration to distal tissues remains unknown. Together, this suggests that perturbation of the immune homeostasis and the systemic spread of *Leishmania* parasites interplay to trigger and develop “metastatic” cutaneous or mucosal lesions.

Understanding the conditions that favor parasite persistence in healthy mucosal tissues is of major clinical and epidemiological relevance due to the risk of developing clinical signs and symptoms of mucosal disease and the challenges associated with it. These include the clinical complications of mucosal leishmaniasis (21, 22), challenging diagnosis (23), the inherent difficulties of treatment in terms of access, toxicity, and costs (24), and finally, the unknown participation of subclinically infected individuals in transmission and endemicity of CL.

In this study, we explored the immunological components that could favor the asymptomatic presence of *Leishmania* (*Viannia*) spp. in human nasal mucosal tissues. Our results provide the first immune profile (in terms of cellular composition and gene expression signatures) of clinically healthy nasal mucosa of CL patients and reveal insights into the relationships between the local immune environment and the asymptomatic presence of *Leishmania* in this tissue.

RESULTS

Effect of time after sampling on cell frequency and viability. Downstream analysis of samples obtained from the nasal mucosa is challenging due to the high frequency of dead cells captured in the mucosal lining, as well as the diverse local microbiota, both of which can confound molecular and cytometry-based assays. Therefore, we sought to characterize the inter-individual variability in the recovery of immune cell populations and cellular frequencies in our study population (Table 1) and the impact of time-to-sample processing, in order to evaluate the feasibility of collecting samples at different clinical sites. Using nasal mucosa curette microsampling, the absolute number of cells recovered was on average 1.38×10^5 (range 2.5×10^4 to 4.6×10^5 cells/ml; $n = 23$) with a frequency of cell death of $42\% \pm 21\%$, evidencing between-donor variability.

To explore the impact of time before sample processing, duplicate nasal curette samples were taken from four healthy donors; one of the samples was processed immediately and the other was stored at 4°C and processed after 8 h of storage. Absolute cell counts decreased >60% in samples processed 8 h after collection; however, similar proportions of viable and nonviable cells were found in both groups (Fig. 1A). Notably, the relative frequencies of the immune cell populations analyzed (CD45⁺) remained similar in both sample types, while the frequency of epithelial cells decreased after 8 h of storage (Fig. 1B). These results favor the characterization of nasal mucosal cell populations immediately after sampling.

TABLE 1 Demographic characteristics of study participants^a

Variable	Value for:		
	Healthy donors	CL patients	Total
Subjects, no. (%)	19 (45.2)	23 (54.8)	42
Sex, no. (%)			
Female	4 (21.1)	3 (13.0)	7 (16.7)
Male	15 (78.9)	20 (87.0)	35 (83.3)
Median age, yrs (range)	28 (19–59)	30 (20–53)	28 (19–59)
Ethnic group, no. (%)			
Afro-Colombian	3 (15.8)	8 (34.8)	11 (26.2)
Mestizo	15 (78.9)	12 (52.2)	27 (64.3)
Other	1 (5.3)	3 (13)	4 (9.6)
Avg time of evolution of the cutaneous lesion in months (SD)	NA	1.91 (1.01)	
<i>Leishmania</i> species, no. (%)			
<i>L. (V.) panamensis</i>	NA	14 (61)	
<i>L. (V.) braziliensis</i>	NA	1 (4.3)	
<i>L. (V.) guyanensis</i>	NA	1 (4.3)	
Not isolated ^b	NA	7 (30.4)	
LRV status, no. (%)			
Positive	NA	0 (0)	
Negative	NA	16 (100)	

^aCL, cutaneous leishmaniasis; NA, not applicable.

^bStrains were not isolated either because the culture was contaminated or because the patient did not consent for parasite isolation.

The cellular composition and immune gene expression profiles of nasal mucosa from active CL patients are similar to those of healthy donors. Characterization of cell populations by flow cytometry showed a higher proportion of EPCAM⁺ epithelial cells (51% ± 22%) recovered from healthy donors ($n = 9$) than CD45⁺ leukocytes (26% ± 7%) (Fig. 2A). Within the CD45⁺ population, the most abundant immune cells were CD66b⁺ granulocytes, accounting for 77% ± 21%, followed by CD14⁺ monocytes (14% ± 14%) and CD3⁺ T cells (7% ± 5%) (Fig. 2B). The cellular composition of nasal mucosa of patients with active CL ($n = 5$) was similar to that of healthy volunteers in terms of epithelial and CD45⁺ cell frequencies (Fig. 2A), as well as specific immune cell types within the CD45⁺ population (Fig. 2B).

Based on the similarities between cell frequencies in the NM of CL patients and healthy donors, we explored whether differences could be evidenced at the activation/functional level. PCR arrays were performed on a total of 12 samples: 4 mucosal samples from healthy donors and 8 from CL patients without any clinical sign or symptom of mucosal leishmaniasis. Of the 8 patients with active CL, *Leishmania* was detected (by amplification of the *Leishmania* 18S rDNA gene product) in the mucosal tissue of 4 patients (referred to as CLⁿ⁺) and was undetected in the other 4 (CLⁿ⁻). Good-quality NM RNA was evidenced by well-defined and abundant 18S and 28S bands in the bio analyzer profile (Fig. S2 in the supplemental material). Lower molecular weight bands (not degradation products) were also detected, likely corresponding to RNA of the commensal microbial flora (Fig. S2). Based on a cutoff value of a 1.5-fold change in gene expression, 5 of the 84 immune genes evaluated (Table S2) were differentially expressed (but not statistically significant) between samples from healthy donors ($n = 4$) and CL patients ($n = 8$). Expression of *IL-33* decreased while expression of *ADRB2*, *FCεR1A*, *IL5RA*, and *SATB1* increased in CL patients compared to samples from healthy donors (Table S3).

Gene expression profiles of NMs of CL patients with molecular evidence of *Leishmania* persistence differ from that of patients without detectable parasites. The gene expression profile of the nasal mucosa of CLⁿ⁺ patients (those with active CL

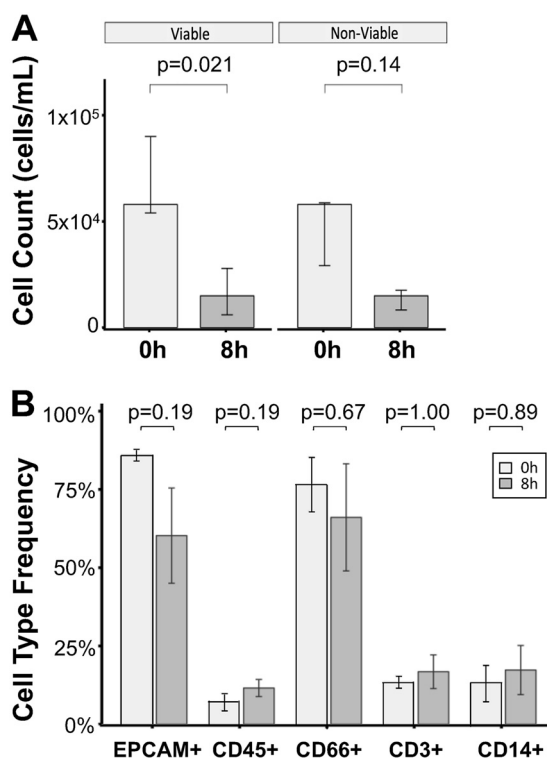


FIG 1 Time after collection of NM samples affects cell recovery. Effect of sample processing time on (A) cell count and viability and (B) cell type frequency in NM samples ($n = 4$) processed immediately after sampling, at 0 h (light gray), and 8 h (gray) after sampling and storage at 4°C (B). Frequencies reported for CD66b⁺, CD14⁺ and CD3⁺ populations were calculated based on the total number of CD45⁺ cells in each sample. The Wilcoxon test was used to estimate statistical significance and P values.

and molecular evidence of *Leishmania* parasites in the NM, but without any clinical signs or symptoms of mucosal disease) was contrasted to that of CLⁿ⁻ patients (those with active CL and no detection of *Leishmania* in the NM) and summarized in Table 2. Higher levels of expression of 14 genes (*ADRB2*, *ALOX5*, *BCL6*, *FCεR1A*, *IFNγR2*, *IL12A*, *IL4R*, *IL5RA*, *KIT*, *LTB4R*, *POSTN*, *SATB1*, *STAT6*, and *TGFβ1*) were found in samples from CLⁿ⁺ patients, with differences in expression between 1.5-fold and 7.1-fold compared against samples from CLⁿ⁻ patients (Table 2). Of these, expression of *IL4R*, *IL5RA*, *POSTN*, and *SATB1* was significantly increased in NM samples from CLⁿ⁺ patients, while *STAT6* and *BCL6* were at the limit of significance ($P = 0.057$) (Table 2).

Leishmania virus was not detected in *Leishmania* strains isolated from patients with or without molecular evidence of parasite presence in the NM. To explore putative relationships between *Leishmania* RNA virus (LRV) and the presence of *Leishmania* in healthy mucosal tissues of CL patients, or the differential inflammatory gene expression profiles found between patient groups, we evaluated the presence of LRV in isolates from study participants. Among the 23 CL patients (Table 1), *Leishmania* strains were isolated from 16 patients [14 were *L. (V.) panamensis*; 1 was *L. (V.) guyanensis*; and 1 was *L. (V.) braziliensis* (Table 1)]. Despite the good quality of extracted RNA, evidenced by the amplification of the β-tubulin gene product (Fig. S3A), none of the isolates was positive for LRV, even among strains isolated from CL patients with molecular evidence of *Leishmania* in the NM or *L. (V.) braziliensis* or *L. (V.) guyanensis* strains (Fig. S3B).

DISCUSSION

Localized skin pathologies (ulcers, nodules, papules, etc.) are characteristic of CL. However, *Leishmania* (*Viannia*) species [*L. (V.) braziliensis*, *L. (V.) panamensis*, and *L. (V.) guyanensis*] can spread systemically and cause disease, evidenced by mucosal and

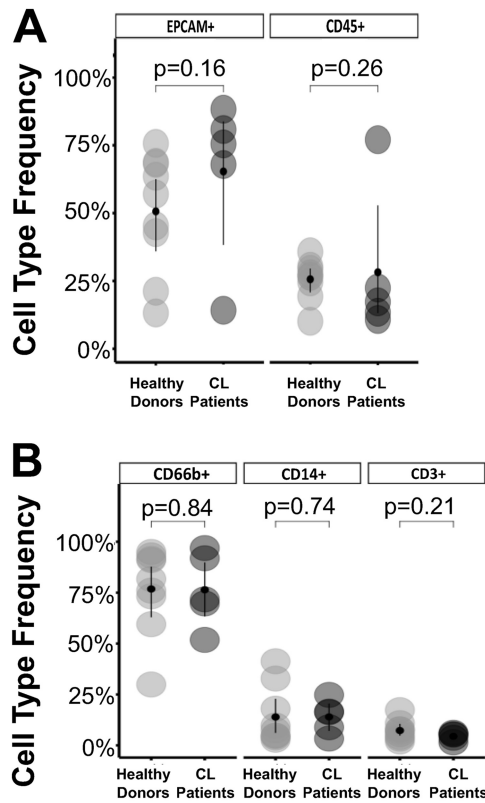


FIG 2 Cellular composition of the nasal mucosa of healthy volunteers and CL patients. Frequency of (A) EPCAM⁺ and CD45⁺ cells and (B) CD45⁺/CD3⁺, CD45⁺/CD14⁺, and CD45⁺/CD66b⁺ populations in NM curette samples from healthy volunteers (n = 9) and CL patients (n = 5). Data are shown as mean ± SD. The Kruskal-Wallis test was used to estimate statistical significance and P values.

satellite lesions, adenopathy, and even reactivation of disease after clinical cure of a prior symptomatic infection (25, 26). Despite efforts to isolate and characterize parasites that migrate (or “metastasize”) to tissues distant from the primary cutaneous lesion, it remains unclear whether specific parasite populations are more avid to migrate to alternative tissues or, rather, whether “metastatic lesions” are an infrequent clinical manifestation of readily occurring systemic parasite spread and persistent infection. In

TABLE 2 Differentially expressed genes in NM swab samples from CL patients^a

Gene	CL ⁿ⁻ patients ^b (n = 4)		CL ⁿ⁺ patients ^b (n = 4)		Fold difference (2 ^{-ΔΔCt} CL ⁿ⁻ vs CL ⁿ⁺)	P value ^c
	Avg 2 ^{-ΔCt}	SD	Avg 2 ^{-ΔCt}	SD		
ADRB2	0.007	0.002	0.025	0.030	1.95	0.200
ALOX5	0.036	0.061	0.027	0.024	1.52	0.485
BCL6	0.013	0.001	0.022	0.011	1.61	0.057
FCER1A	0.008	0.006	0.027	0.025	3.03	0.342
IFNGR2	0.015	0.007	0.031	0.024	1.78	0.342
IL12A	0.003	0.001	0.012	0.016	2.29	0.342
IL4R	0.008	0.003	0.018	0.005	2.15	0.028
IL5RA	0.004	0.002	0.012	0.006	2.31	0.028
KIT	0.003	0.002	0.011	0.012	2.21	0.200
LTB4R	0.004	0.004	0.019	0.015	4.72	0.114
POSTN	0.001	0.0006	0.022	0.023	7.09	0.028
SATB1	0.005	0.001	0.047	0.069	3.75	0.028
STAT6	0.009	0.004	0.042	0.038	3.37	0.057
TGFB1	0.001	0.002	0.018	0.029	5.79	0.114

^aCL, cutaneous leishmaniasis; NM, nasal mucosa; SD, standard deviation; CLⁿ⁺, patients with *Leishmania*-positive NM samples; CLⁿ⁻, patients with *Leishmania*-negative NM samples. Boldface indicates significantly different gene expression.

^bGene expression values (2^{-ΔCt}) of differentially modulated genes in NM swab samples.

^cP values were derived from the Mann-Whitney test.

this study, we explored the potential contribution of tissue-specific inflammatory responses in the subclinical presence of *Leishmania* (*Viannia*) in the healthy nasal mucosa of patients with active CL.

Studies from our group and others have demonstrated that *Leishmania* can be detected in healthy tissues such as skin, conjunctiva, tonsils, and nasal mucosa of patients with localized CL (5, 7, 11), as well as in individuals with immunological evidence of asymptomatic infection (Montenegro skin test positivity) (2) in areas of endemicity of *L. (V.) panamensis*, *L. (V.) braziliensis*, and *L. (V.) guyanensis* transmission. Recent findings indicate that in *L. (V.) guyanensis* and potentially *L. (V.) braziliensis* infections, the presence of *Leishmania* RNA virus (LRV) promotes pathogenesis and increased inflammation (18–20). However, whether LRV contributes to *Leishmania* migration from the inoculation site to distant tissues remains unknown, and its role in mucosal pathology remains controversial. Although *L. (V.) panamensis* can also cause muco-cutaneous disease, we did not find any evidence of the presence of LRV in any of the strains isolated from our study participants, indicating that other factors beyond LRV contribute to parasite migration and subclinical persistence in the nasal mucosal tissue.

The frequency of immune cells, as well as activation markers and gene expression signatures, provides information about tissue-specific immune environments. Concurring with previous studies, the cell composition of the nasal mucosa in healthy volunteers was dominated by epithelial cells and, among CD45⁺ cells, higher frequency of granulocytes (CD66b⁺), followed by monocytes (CD14⁺) and CD3⁺ T cells (27–29). Variation in immune cell frequencies in the NM can occur during allergic pathologies or during infection of the nasopharyngeal tract (30–33). For example, seasonal increase of mast cells and eosinophils has been reported in patients with allergic rhinitis who experience obstruction, rhinorrhea, sneezing, itching, and/or postnasal drip due to IgE-mediated inflammatory disease (30, 34). Also, asthma patients present significant increases in lymphocyte, eosinophil, and basophil recovery following antigen challenge (35). Due to the low frequency of CL patients with *Leishmania*-positive mucosal samples (2, 7, 11), we could not assess the relationship between cellular frequencies and parasite persistence in the nasal mucosa. However, similar cell type proportions and immune gene profiles found in NM samples from healthy volunteers and CL patients concur with the absence of clinical signs or symptoms of nasal inflammation or mucosal disease in our patient cohort.

Among CL patients, significantly higher expression of *IL4R*, *SATB1*, *POSTN*, and *IL5RA* was observed, while *STAT6* and *BCL6* approached significance (potentially due to sample size limitations). This six-gene set is suggestive of an anti-inflammatory environment (36–40) involving Th2 cell differentiation (*IL4R*), eosinophil activation (*IL5RA* and *IL4R*—via IgE production), and tissue remodeling (*POSTN*), globally regulated at the transcriptional level via expression of the chromatin regulator *SATB1* and the *STAT6* transcriptional driver of Th2 responses (38–41). Interestingly, higher expression of *BCL6* suggests the presence of T follicular helper (T_{fh}) cells (42) in the NM of CLⁿ⁺ patients, previously shown to participate in IgE production in the context of eosinophilic nasal polyps (43).

The pathology of active mucosal leishmaniasis is typically associated with a strong proinflammatory T cell immune response and scarcity of parasites at the lesion site (44). Interestingly, a Th2 nasal mucosa microenvironment in CLⁿ⁺ patients may allow permissiveness for asymptomatic parasite persistence in this tissue. This suggests that perturbation of this immunologically permissive environment toward activation of a Th1 response may constitute the triggering event for development of clinically active mucosal disease. Although subpopulations of T cells, B cells, and granulocytes were not phenotypically characterized in our study, these data provide the basis for selection of specific immune cells and their subpopulations to be assessed in the context of *Leishmania* presence in mucosal tissues and evaluated for their contribution to parasite persistence and/or disease.

The relationship between mucosal and systemic immune responses has been

substantially investigated and is the basis of mucosal vaccines and mucosal-targeted immunotherapeutics for autoimmune diseases (45). Primary and repeated exposure of mucosal surfaces to microbial or experimental antigens results in systemically induced tolerance in murine models, primarily through diminished T cell responses and induction of regulatory T cell populations (46). Although the development of systemic tolerance in humans remains controversial, experimental exposure to keyhole limpet hemocyanin (KLH) antigen and food antigens (47) results in diminished systemic T cell immunity and concomitant priming of B cell responses. Interestingly, evidence suggests that prior systemic exposure to the antigen and subsequent mucosal exposure do not result in tolerance (45). Whether nasal exposure to *Leishmania* antigen modifies the systemic response is unknown.

Our results provide evidence of differential local immune environments in CLⁿ⁺ and CLⁿ⁻ patients. Persistent exposure to *Leishmania* antigens in the nasal mucosa of CLⁿ⁺ patients, together with a prior elicited systemic immune response, could favor activation of a local anti-inflammatory environment and the establishment and persistence of the parasite in nasopharyngeal mucosal tissues. The asymptomatic presence of *Leishmania* (*Viannia*) in these tissues could promote selection of drug-resistant parasite populations after treatment of active CL (6) and could represent a risk of reactivation of the cutaneous disease or appearance of mucosal lesions (8). Our study sets the groundwork for exploration of the immune response of apparently unaffected mucosal tissues of CL patients and, potentially, of other infections traditionally considered "localized."

MATERIALS AND METHODS

Ethics statement. This study was reviewed and approved by the Institutional Review Board for Ethical Conduct of Research Involving Human Subjects of the Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM) with approval code CIEIH-1270, in accordance with national and international guidelines. All individuals voluntarily participated in the study and written informed consent was obtained from each participant.

Participants and samples. This study included samples and data from 19 healthy adult volunteers from Cali (an area of nonendemicity for CL) without any history of leishmaniasis and 23 patients with active CL and without any clinical manifestation of mucosal disease (4 retrospectively and 19 prospectively recruited) (Table 1). The distribution and use of samples for each experimental procedure are summarized in Table S1.

Patients were enrolled based on parasitological diagnosis of active CL, having not received prior antileishmanial or immunosuppressive treatment, and having no history of immunological disorders, active inflammatory conditions, or mucosal leishmaniasis. The CL study group included adults between 18 and 60 years of age with time of lesion evolution of ≤ 4 months. Nasal mucosa samples were collected for RNA and DNA extraction by gently rubbing sterile swabs (Catch-All, Epicenter Biotechnologies) on the mucosal surface of all study participants. Swab samples were stored in TRIzol (Invitrogen Corp., Carlsbad, CA) within the first 2 h after sample collection and stored at -80°C until extraction. Nasal curettes (ASL Rhino-Pro, Arlington Scientific) were used to obtain mucosal cell samples for cytometry analyses from healthy donors and CL patients (Table S1). Briefly, the inferior turbinate of each nasal fossa was gently scraped (27, 48–50) and the curettes were stored in 15 ml falcon tubes containing 1 ml of phosphate-buffered saline (PBS), 0.5% fetal bovine serum (FBS), and 2.5 mM ethylenediaminetetraacetic acid (EDTA) (27) until processing, which was performed within 1 h after obtaining the sample. Cells were detached from the curettes by repetitive gentle pipetting. Cells were stained with trypan blue for counting and viability evaluation. Nasal swabs, instead of nasal curettes, were used to collect samples for RNA and DNA extraction to favor greater recovery of nucleic acid material.

Flow cytometry analysis. Cells were centrifuged (5 min at $440 \times g$), resuspended in fluorescence-activated cell sorting (FACS) buffer and stained with an antibody cocktail containing CD14-FITC (BD, Becton, Dickinson, clone M Φ P9, catalog number 347493), CD3-PerCP (BD, Becton, Dickinson, clone SK7, catalog number 347344), CD45-APC (BD, Becton, Dickinson, clone 2D1, catalog number 340943), CD66b-FITC (eBioscience, Clone G10F5, catalog number 11-0666-42), and EPCAM-PE (BioLegend, clone 9C4, catalog number 324206). After 20 min of incubation at 4°C , the cells were washed with PBS, centrifuged and resuspended in 300 μl of FACS buffer and 50,000 events were acquired on a BD Accuri C6 flow cytometer. The performance of the cytometer was verified using validation beads to ensure a coefficient of variation of $<5\%$ for the top peaks on each fluorescent detector and the forward scatter (FSC). Finally, after acquisition and verification with compensation controls, the settings for the compensation matrix were set the same for each experiment. The data were analyzed using Flowjo V.10 (Treestar) following the gating strategy previously reported for this type of sample, with a minor modification that included the addition of a nonstained control (Fig. S1) (27).

Molecular detection of *Leishmania* spp. in healthy nasal mucosal tissues. DNA was extracted from mucosal swabs and cultured promastigotes (as positive control), using the AllPrep DNA/RNA kit

(Qiagen), and quantified by Nanodrop. *Leishmania* spp. were detected by quantitative PCR (qPCR) amplification of the 18S rDNA fragment from 1.25 μ l of total DNA extracts as previously described (51).

Inflammatory gene expression profile. DNase-treated RNA from nasal swab samples was used for gene expression analysis of 84 inflammatory mediators and receptors (RT² Profiler PCR Array-PAHS-067Z) (Table S2). RNA quality and concentration were evaluated by chip electrophoresis on an Agilent Bioanalyzer 2100 (Agilent Technology, Santa Clara, CA, USA). Expression data were presented as $2^{-\Delta Ct}$ and fold difference calculated by the $\Delta\Delta Ct$ method. Data analysis was performed using the GeneGlobe Data Analysis Center (Qiagen) with a 1.5-fold change cutoff.

Parasite isolation and detection of *Leishmania* RNA virus (LRV). *Leishmania* isolates were obtained by aspiration of the cutaneous lesion border and cultured in semisolid Senekjie's medium. Strains were kept on RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1% glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. *Leishmania* species identification was achieved by indirect immunofluorescence using species-specific monoclonal antibodies (52, 53). Total RNA was extracted from stationary-phase promastigotes (4×10^6 cells) using TRIzol (Invitrogen, USA), resuspended in RNase free water, and quantified in a Nanodrop spectrometer (Thermo Fisher Scientific, USA). cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems). LRV was detected by reverse transcription-quantitative PCR (qRT-PCR) using the primer sets previously described by Zangger et al. (54), as follows: forward 5'-CTG ACT GGA CGG GGG GTA AT-3' and reverse 5'-CAA AAC ACT CCC TTA CGC-3', derived from LRV1-4 genome sequences (GenBank accession number NC_003601). For quality control of *Leishmania* RNA, a 372-bp fragment of the *Leishmania* β -tubulin gene was also amplified. Positive and negative controls for LRV were included in each run: *L. (V.) guyanensis* M5313 (WHI/BR/78/M5313; LRV⁺) and *L. (V.) panamensis* (MHOM/CO/2002/3594 stably transfected with the Luciferase reporter gene, L.p.LUC 001; LRV⁻).

Statistical analyses. Cell counts and viability frequencies were expressed as mean \pm standard deviation (SD) and geometric mean \pm standard error of the mean (SEM), respectively. The distribution of the data was evaluated with the Kolmogorov-Smirnov normality test, and differences in variance analyzed accordingly. Gene expression data were analyzed using a two-tailed Mann-Whitney test. Flow cytometry data were analyzed using the Wilcoxon or Kruskal-Wallis tests as appropriate. All analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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