Repeated Exposure to *Lutzomyia intermedia* Sand Fly Saliva Induces Local Expression of Interferon-Inducible Genes Both at the Site of Injection in Mice and in Human Blood

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**Abstract**

During a blood meal, *Lutzomyia intermedia* sand flies transmit *Leishmania braziliensis*, a parasite causing tegumentary leishmaniasis. In experimental leishmaniasis, pre-exposure to saliva of most blood-feeding sand flies results in parasite establishment in absence of any skin damages in mice challenged with dermotropic *Leishmania* species together with saliva. In contrast, pre-immunization with *Lu. intermedia* salivary gland sonicate (SGS) results in enhanced skin inflammatory exacerbation upon co-inoculation of *Lu. intermedia* SGS and *L. braziliensis*. These data highlight potential unique features of both *L. braziliensis* and *Lu. intermedia*. In this study, we investigated the genes modulated by *Lu. intermedia* SGS immunization to understand their potential impact on the subsequent cutaneous immune response following inoculation of both SGS and *L. braziliensis*. The cellular recruitment and global gene expression profile was analyzed in mice repeatedly inoculated or not with *Lu. intermedia*. Microarray gene analysis revealed the upregulation of a distinct set of IFN-inducible genes, an immune signature not seen to the same extent in control animals. Of note this INF-inducible gene set was not induced in SGS pre-immunized mice subsequently co-inoculated with SGS and *L. braziliensis*. These data suggest the parasite prevented the upregulation of this *Lu. intermedia* saliva-related immune signature. The presence of these IFN-inducible genes was further analyzed in peripheral blood mononuclear cells (PBMCs) sampled from uninfected human individuals living in a *L. braziliensis*-endemic region of Brazil thus regularly exposed to *Lu. intermedia* bites. PBMCs were cultured in presence or absence of *Lu. intermedia* SGS. Using qRT-PCR we established that the IFN-inducible genes induced in the skin of SGS pre-immunized mice, were also upregulated by SGS in PBMCs from human individuals regularly exposed to *Lu. intermedia* bites, but not in PBMCs of control subjects. These data demonstrate that repeated exposure to *Lu. intermedia* SGS induces the expression of potentially host-protective IFN-inducible genes.

**Introduction**

*L. braziliensis* protozoan parasites induce a broad spectrum of disease including cutaneous lesions and visceral leishmaniasis the latter being fatal if not treated. *L. braziliensis* parasites can be transmitted by *Lu. intermedia* sand flies in Central and South America where they are the leading cause of American cutaneous and mucocutaneous leishmaniasis. During a blood meal, the host is exposed to a variety of sand fly factors. Sand fly saliva contains many pharmacological agents aimed at obtaining the optimal amount of blood for nutrition, egg development and survival. In addition, the proteophosphoglycan gel which is synthesized by the parasites inside the fly midgut can exacerbate cutaneous leishmaniasis [1,2]. Individuals living in an endemic region are bitten by both uninfected and infected sand flies, and thus are repeatedly being exposed to sand fly saliva, leading progressively to the induction of an immune response to saliva. In Brazil, *Lu. intermedia* is the predominant sand fly species harboring *L. braziliensis* [3,4] and in Corte de Pedra, Bahia, the endemic area studied in this report, both *Lu. intermedia* and *Lu. whitmani* sand fly species exist sympatrically with fluctuations reported for these populations [5].

The role of sand fly salivary factors is also important in the establishment of infection and thus the outcome of disease. Salivary factors include mediators that circumvent the host’s hemostatic responses by preventing blood clotting, vasoconstriction and platelet aggregation for optimal feeding [6,7]. Sand fly...
Saliva is immunogenic and the immune response to salivary antigens modulates the microenvironment at the site of the bite with an impact on the development of disease. Co-inoculation of antigens modulates the microenvironment at the site of the bite saliva is immunogenic and the immune response to salivary extracts. Among the genes highly induced were the interferon-inducible genes known to contribute to resistance against parasite infections. These genes were also induced in blood cells of human individuals that were naturally pre-exposed to bites of Lutzomyia intermedia sand flies. Interestingly, subsequent infection with Leishmania braziliensis blocked the induction of these genes in mice. These data show that the induction of potentially protective genes by insect saliva can be altered by the infecting parasite. This should be considered when including salivary components in a vaccine.

**Materials and Methods**

**Ethical Statement**

For animal studies, all animal protocols were approved by the Swiss Federal Veterinary Office and experiments were performed adhering to ethical guidelines established by this office. Recommendations in the guidelines for the care and use of laboratory animals were obtained from the Department of Security and Environment of the state of Vaud, Switzerland. The protocol has been approved by the Ethics and Veterinary Office of Regulations of the state of Vaud (SAV), Switzerland under the administrative authorization number 1266-5. For human studies, written informed consent was obtained from all enrolled subjects; all procedures were approved by the Ethical Committee of the Federal University of Bahia.

**Mice**

Female BALB/c mice were purchased from Charles River (Lyon, France), housed under pathogen-free conditions in the BIL Epalinges Center and used for experiments between 6–8 weeks old.

**Sand Flies and SGS Preparation**

Adult Lu. intermedia female sand flies were captured in Corte de Pedra, Bahia, Brazil. Entomological gathering was done on private land with permission from owners for the study to be conducted on their land and within their residences. Lu. intermedia sand flies were morphologically identified according to the identification key proposed by Young and Duncan [14]. Sand fly salivary glands were dissected and stored in groups of 20 pairs in 20 mL NaCl (150 mM), Heps buffer (10 mM; pH 7.4) at ~70°C. Immediately before use, salivary glands were disrupted by ultrasonication in 1.5 mL conical tubes. Tubes were centrifuged at 10,000 x g for 2 min, and the resultant supernatant (SGS) was used for the studies. All SGS batches were below the limit of detection for endotoxin activity (<0.01 EU/µg) using the LAL QCL-1000 assay (Lonza, Portsmouth, NH).

**Parasites and Infections**

L. braziliensis (MHOM/BR/01/BA788 strain) parasite which does not contain the Leishmania RNA virus [15] was used for experiments. The parasites were maintained in vitro in BALB/c mice and grown in vitro in M199 media (GIBCO, Paisley, UK) supplemented with 10% FCS (PA Laboratories, Pasching, Austria), 4% HEPES (Amimed) and 2% antibiotics (penicillin, streptomycin, neomycin) (GIBCO). For infections, 1x106 stationary phase promastigotes with or without SGS (equivalent of 1 pair of Lu. intermedia salivary glands) in 10 µL PBS were injected intradermally into the ear.

**Sand Fly Saliva Immunizations**

Mice were immunized with salivary gland sonicate supernatant (SGS) as previously described [13]. BALB/c mice (at least 3–5 per group) were immunized 3 times with SGS (equivalent to 1 pair of Lu. intermedia salivary glands) or PBS in 10 µL in the right ear at 2-week intervals. After 2 weeks, the opposing left ear was challenged with SGS (equivalent to 1 pair of Lu. intermedia salivary glands) in the presence or absence of 1x106 stationary phase L. braziliensis promastigotes. Ear lesion size was monitored weekly and measured using a caliper. To determine cellular content, ears were digested 2 weeks after challenge in the left ear using 0.2 mg/ml Liberase TL (Roche, Rotkreuz, Switzerland) for 2 h at 37°C followed by FACS analysis [16].

**Flow Cytometry**

For cell surface molecules, mAb 24G2 was used to block FcRs and cells were stained using α-F4/80-biotin, α-Ly6C-FITC, α-Ly6G-APC/Cy7 (clone 1A8), α-MHCII-Alexa Fluor 700 from BioLegend (San Diego, CA) and α-CD11b-eFluor 450, α-CD11c-Anti-CD11c, respectively.
PE/Cy5, α-DEC205-APC, α-pan-NK CD49b-PE (clone DX5) and streptavidin-PE/Cy7 from eBioscience (San Diego, CA). All cell events were acquired on an LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo (Tree Star, Ashland, OR).

### Mouse Ear Pinna Processing for mRNA Isolation and Microarray Analysis

Ears were harvested 2 weeks after challenge, homogenized using a tissue lyser (Qiagen, Hilden, Germany) and mRNA was extracted by the RNeasy Plus Mini kit (Qiagen). For microarray analysis, RNA was harvested from ears 2 weeks post inoculation and for each sample condition, three independent sets of 200 ng of total RNA were isolated and used as a template for probe generation using an Ambion WT expression kit (Applied Biosystems, Foster City, CA) and the cDNA was fragmented and labeled with WT DNA terminal labeling kit (Affymetrix, Santa Clara, CA). Biotinylated sense strand fragments were hybridized to Affymetrix Mouse Gene 1.0 ST GeneChips using the Hybridization Control and Hybridization Wash and Stain kits at 45°C for 18 h. The stained array was scanned using an Affymetrix GeneChip Scanner 3000 7G to generate the CEL files. The chip data were imported with Partek Genomics Suite 6.5 (Partek, Inc., St. Louis, MO), normalized and summarized using the RMA (Robust Multiarray Average) algorithm. The relative log expression was examined to ensure that the data were properly corrected by normalization and that there were no outliers. Scatter plots were generated using Matlab 2012a (MathWorks, Natick, MA) and DataGraph 3.0 (Visual Data Tools Inc., Chapel Hill, NC). To identify expression changes between genotypes, a one-way ANOVA with contrast was performed by using the methods-of-moments.

### Processing of Human Blood Samples for Quantitative Real-Time PCR

Quantitative real-time PCR was carried out using random 9-mers, M-MLV reverse transcriptase RNase H− (Promega, Madison, WI) and SYBR green on a LightCycler 480 system (Roche). The primer sequences are listed in Table S1. Thermal cycle conditions consisted of a two-min initial incubation at 95°C and 45 cycles at 95°C for 10 sec, 60°C for 10 sec and 72°C for 10 sec. The results were normalized to the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) using the comparative method was used where gene expression cycle threshold (Ct) values were normalized to HPRT expression as determined by the equation ΔCt = Ct (target gene) − Ct (HPRT). Fold change was determined by 2−ΔΔCt, where ΔΔCt = ΔCt (SGS)−ΔCt (medium) [18].

### Results

Repeated pre-exposure of BALB/c mice to *Lu. intermedia* SGS enhances susceptibility to *L. braziliensis* infection with a lesion beginning at 3 weeks post-infection (Fig. 1A and 1B) in line with previously published results [12]. Thus, we wanted to determine if differences in cellular recruitment due to pre-immunization with *Lu. intermedia* SGS prior to infection could explain the differences in disease status. Therefore, mice were repeatedly pre-immunized with SGS or inoculated with PBS and both groups were challenged with SGS in the contralateral ear. We examined the cellular infiltrate of the ear two weeks after SGS challenge, when the adaptive immune response is ongoing and the parasite has typically already established infection, despite a lack of detectable differences in lesion size. At this point, no significant differences were observed in the total number of cells, or the numbers of neutrophils, macrophages or DCs in the ears of mice pre-immunized with SGS compared to those inoculated with PBS (Fig. S1).

As a result, we hypothesized that alterations in gene expression in response to repeated exposures to *Lu. intermedia* SGS may be modulating the local skin microenvironment, impacting the innate and adaptive immune responses and thus the outcome of disease. To examine the effect of SGS pre-immunization at the inoculation site, we carried out a microarray analysis in mice that were pre-immunized with SGS or inoculated with PBS and later challenged with SGS in the opposing ear dermis. The ear pinna was processed and analyzed two weeks after the last SGS challenge (Fig. 1C).

Overall, there were few differences in the global gene expression patterns between mice that were repeatedly pre-exposed to SGS and challenged with SGS compared to those inoculated with PBS and challenged with SGS. However, hierarchical clustering analysis revealed that 95 genes were increased and 60 genes were decreased in response to SGS pre-immunization compared to control mice (Fig. 1D and 1E). Of the 155 transcripts modulated...
Figure 1. Gene categories modulated by SGS pre-immunization. BALB/c mice were inoculated 3 times in the right ear pinna every 2 wks with SGS from 1 pair of *Lu. intermedia* salivary glands and then challenged 2 wks later with *Lu. intermedia* SGS plus $1 \times 10^6$ *L. braziliensis* parasites. (A) Lesion development was monitored weekly. Each point is the mean ± SEM of 5 animals per group. (B) Lesion images of ear pinna at 8 wks p.i. and these data are representative of two independent experiments. (C) BALB/c mice were pre-immunized (Imm) 3 times in the right ear every 2 wks with *Lu. intermedia* SGS or PBS and then challenged (Chl) in the left ear 2 wks later with *Lu. intermedia* SGS. The challenged left ears were collected after 2 wks.
by SGS pre-immunization, only 49 transcripts have been annotated, or ascribed to a specific gene, and the rest are classified as hypothetical or unknown. Despite the majority of these genes being classified as hypothetical or unknown, many of the transcripts that were differentially expressed in response to SGS pre-sensitization are known to play a role in immune processes like antigen presentation and signaling as well as transcripts encoding for cytokines, chemokines and their receptors (Fig. 1F).

Of the 49 annotated genes differentially regulated with SGS pre-immunization, the microarray analysis revealed all but one of these annotated genes was increased upon SGS challenge in mice pre-immunized with SGS compared to those inoculated with PBS (Table 1). Of the 49 annotated genes, 4 transcripts had greater than 2-fold expression in SGS pre-exposed mice compared to controls; the gene most highly expressed in SGS pre-immunized mice compared to PBS-inoculated animals was CXCL9. Mpeg1, a transcript indicative of macrophage presence, as well as IL-1r1 and TLR13 which are members of the toll-like superfamily of receptors, were also significantly elevated in response to SGS challenge in SGS pre-exposed mice compared to controls. The microarray results revealed an especially high frequency (14.3% of the annotated genes) of genes induced in response to SGS pre-immunization to be IFN-inducible genes including immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs) [19–25].

Given the surprisingly large proportion of the modulation of IFN-inducible genes in mice pre-immunized with SGS compared to control mice, we carried out real-time qPCR for IFN-inducible genes as well as genes associated with IFN-induced responses on a biological replicate experiment to confirm the findings of the microarray analysis. Cells from mice pre-sensitized with SGS and challenged with SGS had a higher expression of Ifit1, Irgm1 and Irgm2 compared to mice inoculated with PBS and challenged with SGS. Of note, despite these differences, challenge with SGS in mice pre-immunized or not with SGS had a higher expression of these genes compared to naïve mice, suggesting SGS inoculation of SGS pre-exposed mice is a protective response as Irgm1 expression of IFN-inducible genes, which are typically associated with a protective response as Irgm1−/− animals are highly susceptible to Leishmania infection (mentioned as data not shown in [21]). However, pre-immunization with Lu. intermedia SGS has been reported to enhance L. braziliensis infection [12]. To evaluate the effect of the parasite on the local immune response induced by pre-immunization with SGS, mice were repeatedly pre-exposed to SGS or PBS and challenged with SGS in the presence or absence of L. braziliensis parasites. Gene expression profiling studies were carried out 2 weeks later. Mice that were pre-sensitized with SGS and challenged with SGS alone significantly upregulated the expression of Ifit1, Irgm1, Irgm2, Stat1 and CXCL9 compared to PBS pre-inoculated mice in line with our microarray data (Fig. 4).

However, the mice that were pre-exposed to SGS and challenged with SGS and L. braziliensis did not significantly upregulate the expression of these genes compared to controls (Fig. 4 and Table S2). Taken together, these data suggest that L. braziliensis parasites modulate host gene expression at the site of infection creating a more hospitable environment for parasite establishment which is associated with increased lesion development.

Discussion

Many studies have suggested the anti-saliva response against sand fly species such as P. papatasi or L. longipalpis is detrimental for the establishment of Leishmania infection. In contrast, the Lu. intermedia anti-saliva response does not prevent the development of disease, but rather may modulate the outcome of infection. Studies in a mouse experimental model have demonstrated that L. braziliensis infection alone induces a strong Th1 cell immune response with high levels of IFNγ and elevated numbers of IFNγ-producing CD4+ and CD8+ T cells in the dLN [32–34]. The strong protective immune response characterized by the presence of IFNγ was thought to correlate with the strong resistance to L. braziliensis infection [34–39]. Here, we show that repeated pre-immunizations with Lu. intermedia SGS alters the skin microenvironment and induces the expression of a variety of genes involved in the immune response, especially from the family of IFN-inducible genes. Genomic analysis of the skin of mice pre-immunized with SGS reveals an inflammatory setting with an increase in genes involved in immune responses including antigen presentation and cell signaling. Genes associated with Th1 cell immune responses such as CXCL9, a chemokine linked with the recruitment of Th1 cells, exhibited the greatest fold induction at >5 times over control mice. The IL-7R, also known to influence the Th1 cell immune response, was also elevated following SGS pre-exposure (this study and [40–42]). Of note, cytokines typically associated with a Th2 cell immune response such as Chi31 (Ym1), or regulatory cytokines such as IL-10R2 were also detected at higher levels in SGS pre-immunized mice.

In the periphery IFNγ binds to its receptor and initiates the JAK/STAT signaling pathway leading to the phosphorylation and translocation of STAT1 to the nucleus which induces the transcription of more than 2000 genes including effector molecules.
Table 1. Genes differentially expressed in response to SGS pre-immunization.

| Gene Symbol | Description | Fold Change | p-value |
|-------------|-------------|-------------|---------|
| **Antigen Presentation** | | | |
| CD74 | CD74 antigen (invariant chain) | 1.65 | 0.018 |
| H2-gs10 | MHCI like protein | 1.72 | 0.003 |
| H2Q6 | Histocompatibility 2 | 1.61 | 0.009 |
| Tap1 | Transporter 1 | 1.51 | 0.0006 |
| **Chemokine, cytokines and their receptors** | | | |
| CXCL9 (MIG) | Chemokine ligand 9 | 5.72 | 0.037 |
| IL-1r11 (ST2) | IL-1 receptor-like 1 | 2.58 | <0.05 |
| IL-7r (CD127) | IL-7 receptor | 1.53 | 0.017 |
| IL-10r | IL-10 receptor α | 1.69 | 0.016 |
| **Immune response signaling** | | | |
| CD180 | CD180 antigen | 1.99 | 0.034 |
| Sfpi1 (PU.1) | SFFV proviral integration 1 | 1.54 | 0.038 |
| Sla | Src-like adaptor | 1.55 | 0.027 |
| Stat1 | Signal transducer and activator of transcription 1 | 1.51 | 0.017 |
| TLR13 | Toll-like receptor 13 | 2.48 | 0.049 |
| **IFN-inducible genes** | | | |
| Gbp6 | Guanylate-binding protein 6 | 1.74 | 0.031 |
| Gpb8 | Guanylate-binding protein 8 | 1.86 | 0.037 |
| Ifit1 | IFN-induced protein | 1.62 | 0.007 |
| Iggp1 | IFN-inducible GTPase | 3.38 | 0.049 |
| Irgb1 | Immunity-related GTPase | 1.65 | 0.033 |
| Irgb2 | Immunity-related GTPase | 2.16 | 0.027 |
| **Other immune response genes** | | | |
| Aif1 | Allograft inflammatory factor 1 | 1.84 | 0.012 |
| Chi3l1 (Ym1) | Chitinase 3-like 1 | 1.77 | 0.005 |
| Jgsf6 | Immunoglobulin superfamily member | 1.98 | 0.044 |
| Kird1 | Killer cell lectin-like receptor | 1.66 | 0.030 |
| Mpeg1 | Macrophage expressed gene | 3.23 | 0.044 |
| Nkg7 | Natural killer cell group 7 | 1.67 | 0.040 |
| Pdcd11g | Programmed cell death 1 ligand 2 | 1.96 | 0.035 |
| **Other genes** | | | |
| Apobec1 | Apolipoprotein B | 1.65 | 0.021 |
| Atp8b4 | ATPase 8B | 1.70 | 0.004 |
| Dpep2 | Dipeptidase 2 | 1.52 | 0.040 |
| F10 | Coagulation factor X | 1.88 | 0.038 |
| Fyb | FYN binding protein | 1.94 | 0.010 |
| Havcr2 | Hepatitis A virus cellular receptor | 1.51 | 0.038 |
| Lgals3bp | Lectin, galactoside-binding | 1.50 | 0.042 |
| Mir203 | microRNA 203 | −1.53 | 0.022 |
| Mrgpra9 | MAS-related GPR | 1.54 | 0.031 |
| Msx4a4b | Membrane-spanning 4 domains | 1.99 | 0.020 |
| Myo1f | Myosin 1F | 1.65 | 0.023 |
| Naaa | N-acylethanolamine acid amidase | 1.52 | 0.033 |
| Ptprc | Protein tyrosine phosphatase receptor 1 | 1.91 | 0.046 |
| Samhd1 | SAM domain and HD domain | 1.51 | 0.048 |
| Slfn1 | Schlafen 1 | 1.76 | 0.030 |
| Sp110 | Nuclear body protein | 1.57 | <0.05 |

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that suppress the growth and survival of intracellular pathogens (Phox, iNOS, IDO, NRAMP1, GTPases, Ifits and chemokines) [21]. Remarkably, several IFN-inducible genes as well as the IFN signaling molecule, STAT1, were upregulated at the site Lu. intermedia challenge in mice that were pre-exposed through immunization. For example, p47 GTPases such as Irgm1 (formerly Lrg47) and Irgm2 (Gtpi) and p65 GTPases such as GBP6 and GBP8 were expressed at high levels in pre-immunized mice. Interestingly, IFN-inducible genes were similarly induced in SGS-stimulated PBMCs isolated from humans living in an area endemic for L. braziliensis with active Lu. intermedia sand fly transmission. It was not possible to perform skin biopsy in the human population studied due to ethical considerations; however, the expression of IFN-inducible genes in SGS-stimulated blood cells of individuals naturally exposed to sand fly bites was similar to that observed at the site of SGS challenge in mice. Collectively, these data demonstrate that the induction of IFN-inducible genes by SGS is also occurring in humans.

To our knowledge this is the first report demonstrating an induction in the expression of IFN-inducible GTPases in response to vector saliva. These products have been well characterized for their role in host defense against viruses but they also contribute to resistance against protozoans. Mice deficient for either Irgm1 or many of the other GTPases are highly susceptible to infection with Toxoplasma gondii, Trypanosoma cruzi and Leishmania major, and many mimic the dramatic susceptibility phenotypes seen in IFNγR-deficient mice [21]. It should be noted that IFN-inducible genes are turned on in response to IFNγ but type I IFNs may also contribute, although to a lesser degree [21]. In our analysis neither IFNγ or type I IFNs were elevated in cells from mice pre-sensitized with SGS but this may be a reflection of the time point analyzed (14 days post inoculation).

In this study mice were immunized with SGS to mimic one of the features of natural transmission of Leishmania where individuals are pre-exposed to several sand fly bites prior to deposition of parasites by the sand fly. A high dose of L. braziliensis promastigotes was co-inoculated in mice with SGS in an attempt to reproduce the cell recruitment rapidly observed at the site of infection after a sand fly bite. However, it is important to note that upon a blood meal, the sand fly is inoculating fewer parasites and also regurgitating many other factors including metacyclic promastigotes embedded in a proteophosphoglycan gel in a blood pool [2]. These factors are not all present during needle inoculation of the parasites and SGS. It is clear that further studies using natural sand fly infection will be required for a better understanding of the transmission dynamics during Leishmania infection.

Repeated exposures to Lu. intermedia SGS followed by challenge with L. braziliensis parasites in the presence of SGS leads to an enhanced disease compared to control mice (Fig. 1A and [12]). Interestingly, in this prior study the SGS pre-immunized mice challenged with L. braziliensis plus SGS, and analyzed two weeks later had a lower parasite load compared to mice not immunized.
Thus, in that study, the highest levels of IFN-γ were associated with decreased parasite numbers in vivo suggesting IFN-γ is not the major factor contributing to parasite killing by macrophages at this time point. In our study, increased expression of IFN-inducible genes and of IFN-γ were also detected in the microarray performed in SGS pre-immunized mice and subsequently infected with L. braziliensis and SGS controls challenged with L. braziliensis plus SGS. In this and the previous study, the levels of IFN-γ upon challenge with L. braziliensis and SGS were not elevated following SGS pre-immunization. Thus, other factors may be involved in the transient control of parasite load observed by Moura and colleagues [12]. Furthermore, higher concentrations of IFN-γ were required for optimal parasite killing of L. braziliensis compared to L. major suggesting differences in susceptibilities to IFN-γ-mediated killing between different parasite strains [15]. Nevertheless, following L. braziliensis and SGS co-inoculation, both studies showed increased inflammatory lesions in the group pre-immunized with SGS compared to that injected with PBS.

This increase in disease severity to L. braziliensis infection in SGS pre-immunized mice, corresponds to a silencing of many of the genes turned on by SGS pre-sensitization, including IFN-inducible genes. This suggests that the parasite is actively modulating the host's immune response to the SGS. Interestingly, this observation is consistent with previous findings showing a decreased ratio of IFN-γ/IL-4 production in the dLN of mice pre-exposed to SGS and challenged with parasites [12]. In the same line, the same group further reported that challenge with L. braziliensis plus SGS after SGS pre-immunization also silenced CXCL10, another IFN-inducible gene [13]. Despite differences in the methodology used between these studies (air pouch model in the former studies and needle inoculation in the ear pinna in the current study), the outcomes are going in the same direction. In addition, the impact of SGS on the skin microbiome which was shown to influence skin immunity may also contribute to the phenotype observed [43]. We show here that there is an obvious benefit for the parasite to down-regulate the IRG system expressed in response to SGS pre-exposure to allow for parasite establishment. However, modulation of IFN-inducible genes is most likely not the only mechanism for enhancing disease. It is unclear how the parasite is altering the host's response to the SGS in the present study and this will require further investigation.

In conclusion, we have shown that in both humans and mice, an array of IFN-inducible genes were up-regulated in response to Lu. intermedia SGS pre-exposure. Interestingly, these genes were silenced when the parasite was present during the challenge. Given the marked changes in the skin microenvironment resulting from repeated exposures to Lu. intermedia SGS, and the different outcomes to Leishmania infection, understanding the relationship between pathogens and their homologous vectors is essential. Since SGS proteins from different sand fly species can either exacerbate or protect from disease, subsequent studies will aim to understand how the parasite is modulating SGS impact on the microenvironment [44]. This will help determine risk factors for disease development, markers of exposure and defining potential vaccine candidates.

Supporting Information

Figure S1 SGS pre-immunization does not modify cellular recruitment in response to L. braziliensis inoculation. BALB/c mice were inoculated 3 times in the right ear every 2 wks with 1 pair of Lu. intermedia salivary glands and then challenged 2 wks later in the left ear with L. intermedia SGS. Ears were digested 2 wks post inoculation and cellular content was analyzed by FACS. Cell numbers are shown as the mean +SEM with 5 mice per group. Data are results from one experiment and representative of 2 individual experiments. (TIF)

Table S1 List of the primers used in this study to analyze gene expression by RT-PCR in mouse and human samples. (DOC)

Table S2 List of the genes that were positively (>1.5 fold) or negatively (<1.5 fold) regulated at the site of parasite inoculation in mice immunized with SGS and subsequently infected with L. braziliensis plus SGS, compared to mice pretreated with PBS and infected with L. braziliensis plus SGS. Contralateral ears were isolated for microarray analysis 2 weeks after parasite challenge. The data are presented as the fold change of mice pre-immunized with SGS (3 times every 2 weeks) over mice inoculated with PBS and challenged with L. braziliensis plus SGS. p-values<0.05: statistically significant. (DOC)
significant. The values for IFN-inducible genes (below the IFN-inducible shaded line) are given but they did not vary >1.5 times and were not statistically significant between L. braziliensis samples that were pretreated with SGS or PBS.

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Author Contributions

Conceived and designed the experiments: TW CIdO AB FTC. Performed the experiments: TW AMdC YHLT ACM JCM. Analyzed the data: TW CIdO AMdC FTC. Contributed reagents/materials/analysis tools: ACM JCM AB FTC. Wrote the paper: TW CIdO FTC.

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