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Human anti-saliva immune response following experimental exposure to the visceral leishmaniasis vector, *Lutzomyia longipalpis*

Vera Vinhas[1], Bruno B. Andrade[1], Fábio Paes[1], Andréa Bomura[1], Jorge Clarencio[1], José C. Miranda[1], André Báfica[2], Aldina Barral[1,3] and Manoel Barral-Netto[1,3]

[1] Centro de Pesquisas Gonçalo Moniz (FIOCRUZ) and Faculdade de Medicina da Bahia, Universidade Federal da Bahia, Bahia, Brazil
[2] Immunobiology Section, Laboratory of Parasitic Diseases, NIAID, National Institutes of Health, Bethesda, USA
[3] Instituto de Investigação em Imunologia, Instituto do Milênio, Bahia, Brazil

Experiments in animals verified that phlebotomine saliva enhances *Leishmania* infection, and vaccination with saliva prevents disease. We have shown that individuals from an endemic area of visceral leishmaniasis displayed robust antibody responses to saliva from the vector *Lutzomyia longipalpis*, which correlated with anti-parasite cell-mediated immunity. Here, we explored human anti-saliva responses following exposure to sand flies, using an *in vivo* bite model in which normal volunteers were exposed four times to 30 laboratory-reared *Lu. longipalpis*. Following the third exposure, normal volunteers developed diverse dermatological reactions at the site of insect bite. Serum from normal volunteers displayed high levels of anti-salivary gland sonicate IgG1, IgG4 and IgE as well as several salivary gland proteins. Furthermore, following *in vitro* stimulation with salivary gland sonicate, there was an increased frequency of $CD4^{+}CD25^{+}$ and $CD8^{+}CD25^{+}$ T cells as well as IFN-γ and IL-10 synthesis. Strikingly, 1 year after the first exposure, PBMC from the volunteers displayed recall IFN-γ responses that correlated with a significant reduction in infection rates using a macrophage-lymphocyte autologous culture. Together, these data suggest that human immunization against sand fly saliva is feasible and recall responses are obtained even 1 year after exposure, opening perspectives for vaccination in man.

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Introduction

Leishmaniases are important diseases caused by *Leishmania* parasites which are transmitted by phlebotomine sand fly vectors. Visceral leishmaniasis is a life-threatening condition caused mainly by *Leishmania (L.) chagasi*, a parasite transmitted by the sand fly *Lutzomyia* (*Lu.*) *longipalpis* in the New World. Female sand flies are hematophagous and acquire the parasite while taking a blood meal in a vertebrate host. The parasites develop from amastigotes to extracellular promastigotes in the phlebotomine gut, multiply, and transform to infective metacyclic prosmastigotes which can be transmitted to another vertebrate when the fly attempts to take a blood meal [1–3].

During blood meal probing, sand flies inject saliva into their hosts. Their saliva contains a number of molecules that modulate the host's hemostatic, inflam-

Correspondence: Dr. Manoel Barral-Netto, Centro de Pesquisas Gonçalo Moniz (FIOCRUZ), Rua Waldemar Falcão, 121, Candeal, CEP: 40295-001 Salvador–Bahia, Brazil
Fax: +55-71-3176-2279
e-mail: mbarral@cpqgm.fiocruz.br

Abbreviations: **DTH:** delayed-type hypersensitivity · **IR:** immune response · **SGS:** salivary gland sonicate

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matory, and immune responses (IR), probably representing an evasion mechanism to circumvent the host's homeostatic system [4]. Such effects seem to be important to create a favorable microenvironment on host's skin to establishment of parasites transmitted by the blood-sucking insects [5]. Virtually, all these effects of blood-sucking insect saliva on host physiology are ascribed to a limited number of molecules which may be also immunogenic and elicit host-specific IR [1, 6–10].

A number of reports using animal models have shown that mice pre-exposed to phlebotomine saliva, or some of its components, are protected against *Leishmania* infection *in vivo* [8, 11–15], thus suggesting that salivary proteins could be used as targets for vaccination against leishmaniasis [16, 17]. However, the anti-saliva IR as well as its possible implications on *Leishmania* infection following exposure to sand flies in humans have not been fully characterized.

We have previously shown that serum samples from children living in an area endemic for visceral leishmaniasis displayed IgG Ab against salivary gland sonicate (SGS), a crude saliva extract from *Lu. longipalpis*, a vector of *L. chagasi* [18, 19]. Intriguingly, this serological response correlated with a delayed-type hypersensitivity (DTH) reaction against *L. chagasi* [18, 19]. Furthermore, there was a simultaneous appearance of anti-saliva humoral response and anti-*L. chagasi* cell-mediated immunity [19], favoring the hypothesis that induction of IR against SGS may influence the generation of an IR against leishmaniasis. Nevertheless, *in vitro* experiments demonstrate that SGS from *Lu. longipalpis* [20, 21] or other sand fly species [22] modulates pro-inflammatory cytokine production as well as co-stimulatory molecule expression in human antigen-presenting cells [23], suggesting that SGS can impair host response against *Leishmania*. Although these data suggest that saliva components influence IR *in vitro* as well as *in vivo* in *Leishmania*-infected patients, the response to saliva in uninfected subjects *in vivo* has not been evaluated.

In attempt to characterize immunological patterns following sand fly saliva exposure, we have utilized an *in vivo* bite model in which human subjects were exposed to laboratory-reared *Lu. longipalpis*. Immunological analysis is focused on the host anti-saliva Ab production and cytokine expression as well as its kinetics related to dermatological manifestations. In addition, we identified a number of salivary molecules recognized by sand fly-exposed volunteers, which could be used as a target for future vaccines.

Results

Sand fly bites induced diverse skin reactions patterns

Following the first exposure to uninfected sand fly bites, normal volunteers developed two main different patterns of skin reaction. Two hours after exposure, three volunteers (1–3) developed lesions represented by an erythematous irregularly shaped area with small hemorrhagic points (immediate response). Twenty-four hours later, these individuals exhibited small papules with a gradual decline of the reaction (Fig. 1, volunteers 1 and 3). Similar aspects were presented after the second (Table 1) or third exposure (Fig. 1) although the presence of papules was much less intense or absent. The three other volunteers displayed a mild reaction immediately after the first exposure and 24 h later developed indurated small nodules (delayed response), with similar aspects presented after the second (Table 1) or third exposure (Fig. 1, volunteers 5 and 6).

Lu. longipalpis-exposed individuals produce IgG and IgE anti-sand fly saliva

A single exposure to 30 sand flies was sufficient to induce an anti-SGS IgG response in two out of the six normal volunteers (Fig. 2A). After two exposures, a clear anti-SGS IgG response was apparent in four out of the six exposed individuals. Further exposures led to further

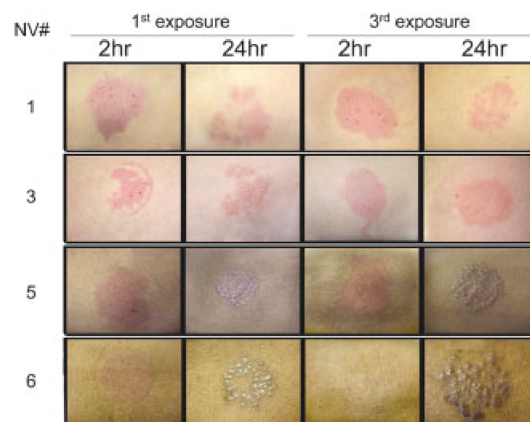

Figure 1. Morphological aspects of skin reactions in volunteers exposed to uninfected sand fly bites. Normal volunteers from non-endemic areas for leishmaniasis were experimentally exposed to uninfected sand fly bites. Two and 24 h after the first and third exposures, pictures were taken to compare skin lesions. Pictures only from volunteers 1, 3, 5, and 6 are presented for comparison. Volunteer 2 presented similar aspects of skin lesions to volunteers 1 and 3; the same was observed for volunteer 4 skin lesions with regard to volunteers 5 and 6.

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Table 1. Semi-quantitative analysis of skin reactions of normal volunteers to experimental *Lu. longipalpis* exposure

Normal volunteer	Exposure	Reaction diameter 24 h[a)] (mm)	Erythema intensity[b)] 24 h[a)]	Micropapule intensity[b)] 24 h[a)]	Nodule intensity[b)] 24 h[b)]
1	1st	12.7	+++	+	0
	2nd	11.7	++	+	0
	3rd	20.4	+	+	0
2	1st	9.8	++	++	0
	2nd	10.1	++	++	0
	3rd	12.5	++	+	0
3	1st	10.3	++	++	0
	2nd	10.7	+	++	0
	3rd	11.9	+++	+	0
4	1st	11.4	+	+	+
	2nd	10.0	+	+	++
	3rd	12.4	++	++	++
5	1st	12.5	+	+	++
	2nd	13.1	+	+	+++
	3rd	12.4	+	+	+++
6	1st	12.3	+	+	++
	2nd	12.2	+	++	+++
	3rd	22.5	+	+	+++

a) Data were collected 24 h after each experimental sand fly exposure.
b) 0 to +++: semi-quantitative intensity degree of skin reactions; 0 = reaction not detected by dermatological examination; + = mild; ++ = moderate; +++ = intense.

elevations of the median OD anti-SGS IgG Ab levels, but one volunteer remained unresponsive throughout the whole period of observation (Fig. 2A). IgG1 seems to be the principal IgG isotype related with the humoral response observed (Fig. 2B), as normal volunteer IgG4 (Fig. 2C) and IgG2 (not shown) levels were very low at all points analyzed.

A similar trend, albeit delayed, was observed with anti-SGS IgE Ab (Fig. 2D). A single exposure did not induce anti-SGS IgE Ab in any volunteer and only two of them produced these Ab after two exposures. Despite a constant elevation of median OD values of anti-SGS IgE Ab, two out of six volunteers remained with pre-exposure values even after four exposures to 30 sand flies. Moreover, volunteers who developed immediate skin reactions presented higher IgE levels and lower IgG levels than those with delayed skin responses, as demonstrated by the IgG/IgE ratio in Fig. 2F.

Salivary sand flies proteins recognized by *Lu. longipalpis*-exposed individuals

To assess whether normal volunteers exposed to sand flies were able to recognize salivary proteins, we performed Western blot analysis using serum from normal volunteers obtained at different time points following the first exposure (Fig. 3). All except one volunteer (normal volunteer 2) recognized SGS bands at day 60. Among them, a band of 45 kDa was more intensely recognized, followed by bands of 44, 43, and 35 kDa as well as a 17-kDa band. In all five positive cases, responses were found to be less intense at day 180 (Fig. 3).

PBMC from *Lu. longipalpis*-exposed individuals produce cytokines following SGS stimulation

To investigate whether sand fly saliva modulates cytokine production as well as activation markers by PBMC, cells from *Lu. longipalpis*-exposed volunteers (2 months after first exposure) were stimulated with the salivary extract *in vitro*. PBMC from unexposed volunteers were used as control. As shown in Fig. 4, SGS-stimulated PBMC display increased frequency of either $CD4^+$ (Fig. 4A, B) or $CD8^+$ T cells (Fig. 4C, D) expressing CD25. Increased percentages of $CD25^+$ T cells were observed in all volunteers, albeit at different levels and modest in one of them. As shown in Fig. 5,

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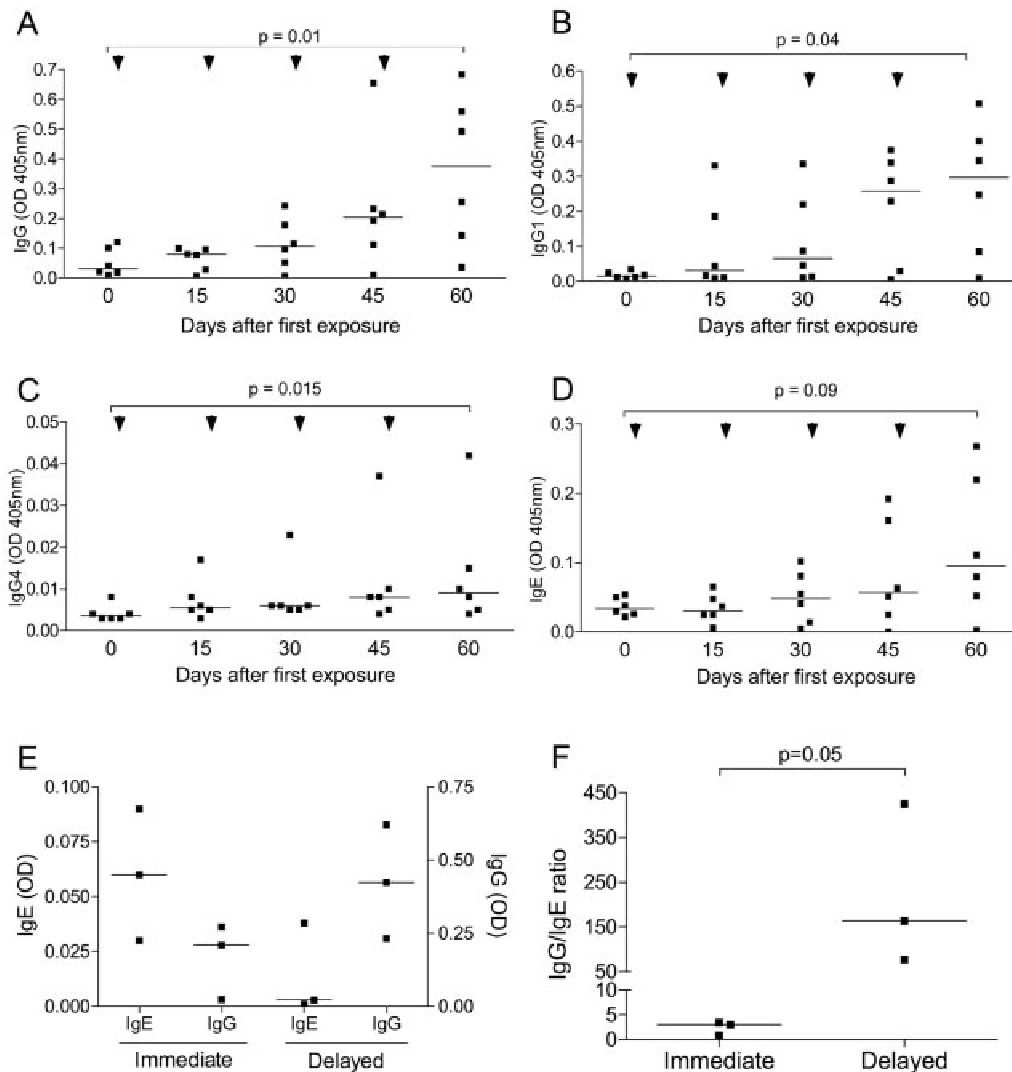

Figure 2. Anti-saliva humoral response. (A–D) Kinetics of anti-sand fly saliva Ab serological levels of normal volunteers after experimental exposures to uninfected sand fly bites. Arrows indicate exposure to 30 sand flies. Mann–Whitney test was used to compare Ab levels at 0 (pre-exposure) and 60 days after first sand fly exposure; p values are expressed in each panel. (E) IgG and IgE levels comparison in volunteers who developed immediate or delayed cutaneous skin reactions after sand fly bites exposures. (F) IgG/IgE levels ratio in immediate and delayed group. Mann–Whitney test was used to compare Ig ratios between immediate or delayed group ($p<0.05$). Experiments were performed at least three times. Each point represents a volunteer and bars represent medians. Note different scales between graphics.

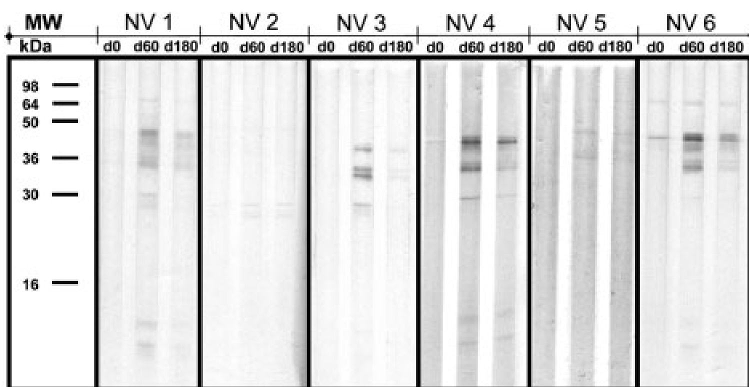

Figure 3. *Lu. longipalpis* salivary proteins recognized by human serum samples from normal volunteers experimentally exposed to sand fly bites. Western blot of *Lu. longipalpis* salivary proteins reacted to volunteer's serum samples before first exposure (0), 60 days, and 180 days after the first exposure. These data are representative of two independent experiments.

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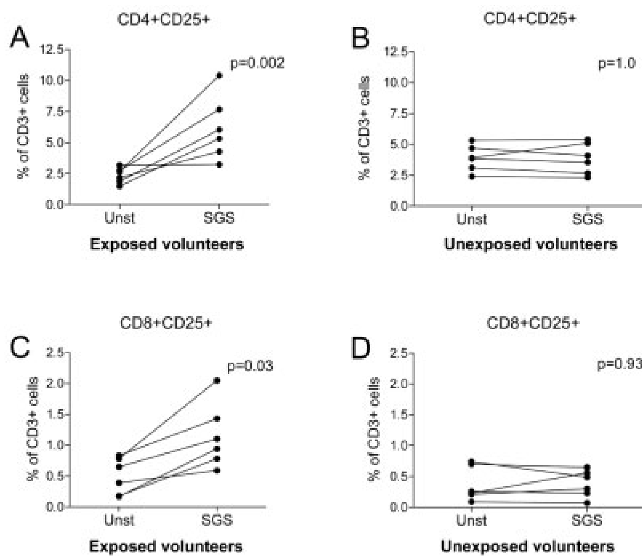

Figure 4. Volunteers' T cell frequencies 2 months after first exposure to sand fly bites. Volunteer's PBMC were stimulated *in vitro* with SGS for 48 h 2 months after the first exposure to sand fly bites. Frequency of $CD4^+$ (A, B) or $CD8^+$ (C, D) T cells expressing CD25 from PBMC was assessed by flow cytometry. Non-parametric Mann–Whitney test was used to check statistically significant differences; *p* values are expressed in each panel. Experiments were performed at least three times.

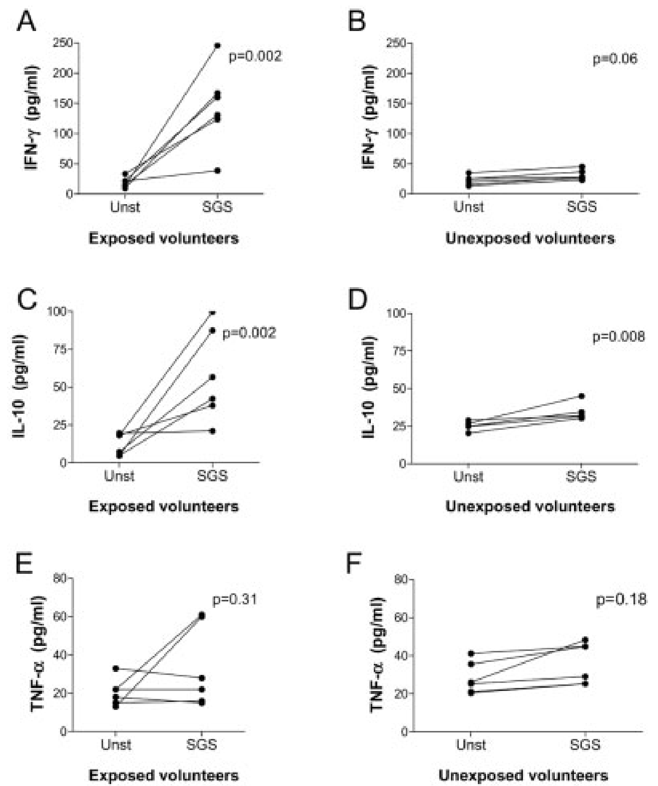

Figure 5. Volunteers' cytokine production 2 months after first exposure to sand fly bites. Volunteer's PBMC were stimulated *in vitro* with SGS for 48–72 h, 2 months after the first exposure to sand fly bites. (A–F) Supernatant levels of IFN-γ (72 h after stimulation), IL-10 (48 h after stimulation), and TNF-α (48 h after stimulation) were determined by ELISA. Non-parametric Mann–Whitney test was used to check statistically significant differences; *p* values are expressed in each panel. Experiments were performed at least three times. Note different scales between graphics.

SGS-stimulated PBMC from exposed volunteers were able to produce IFN-γ and IL-10. The observed augmentation in cytokine synthesis upon treatment with SGS was consistently seen in all volunteers, although one subject exhibited modestly increased levels of the analyzed cytokines. Alteration of TNF-α levels were not consistent and did not reach statistical significance (Fig. 5E–F).

Recall immune response elicited by *Lu. longipalpis* bites

One year after their first exposure to sand fly bites, volunteers were boosted by a further session of *Lu. longipalpis* bites. Seven days thereafter, PBMC were obtained and stimulated *in vitro* with SGS for 96 h. A post-boost recall IR was attained even 1 year after the first exposure to sand fly bites as cells from all volunteers produced IFN-γ when stimulated with SGS (Fig. 6A). A recall humoral response was also achieved. IgG1 and IgG4 isotypes levels were significantly higher than before the new exposure (Fig. 6B).

Lymphocytes from *Lu. longipalpis*-exposed individuals limit parasite burden in macrophages

Since PBMC from sand fly-exposed individuals presented an increased synthesis of IFN-γ, a major cytokine involved in the control of leishmaniasis [24], we further accessed whether lymphocytes from these individuals would be able to influence *L. chagasi in vitro* infection in macrophages by an autologous cell culture system. Macrophages from one unexposed volunteer and two normal volunteers collected 1 year after first exposure to sand fly bites were infected *in vitro* with *L. chagasi* and co-cultured for 92 h in the presence or absence of autologous lymphocytes and/or SGS. Since normal volunteers 3 and 6 were the highest IFN-γ producers following exposure to SGS, we decided to use their cells in this experiment.

In control cultures (*i.e.* an unexposed volunteer), SGS in the presence or absence of lymphocytes did not influence amastigote cell numbers in infected macrophages (Fig. 7). In contrast, in both normal volunteers, co-culture with SGS plus lymphocytes significantly reduced the number of amastigotes in macrophages (parasite counts were reduced by 58.0% in normal volunteer 3 and 67.3% in normal volunteer 6, compared with untreated infected macrophages; $p<0.05$), while

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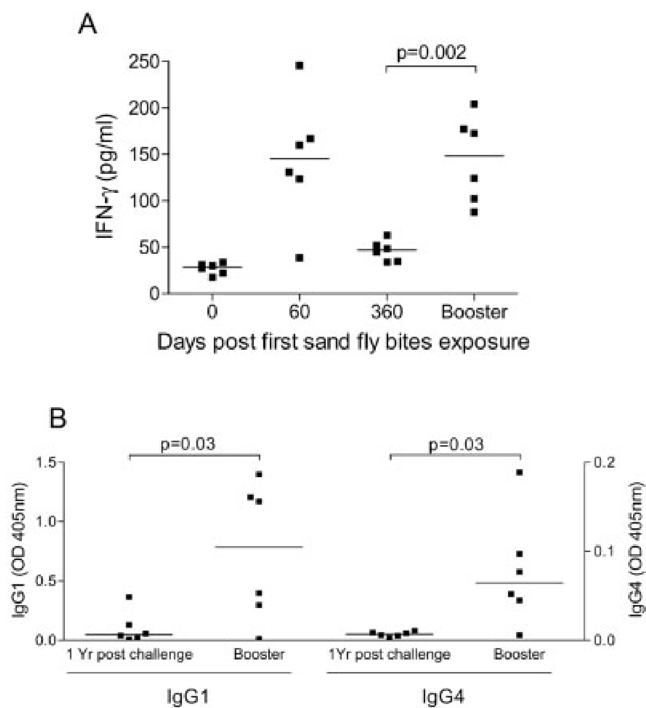

Figure 6. Analysis of normal volunteers' IR to SGS 1 year after the first exposure to sand fly bites. One year after their first exposure to sand fly bites, all volunteers were boosted by a further session of exposure to 20 *Lu. longipalpis*. Seven days after boost, PBMC were obtained and stimulated *in vitro* with SGS; 96-h supernatant IFN-γ levels were determined and were compared to cytokine levels 1 day before boost (A). IgG1 (left axis) and IgG4 (right axis) serological levels 1 year after volunteer's first exposure to sand fly bites and levels 15 days after the boost are compared (B). Each point represents a volunteer and bars represent medians. Non-parametric Mann–Whitney test was used to check statistically significant differences; *p* values are expressed in each panel. Experiments were performed two times. Note different scales between IgG1 OD and IgG4 OD graphics.

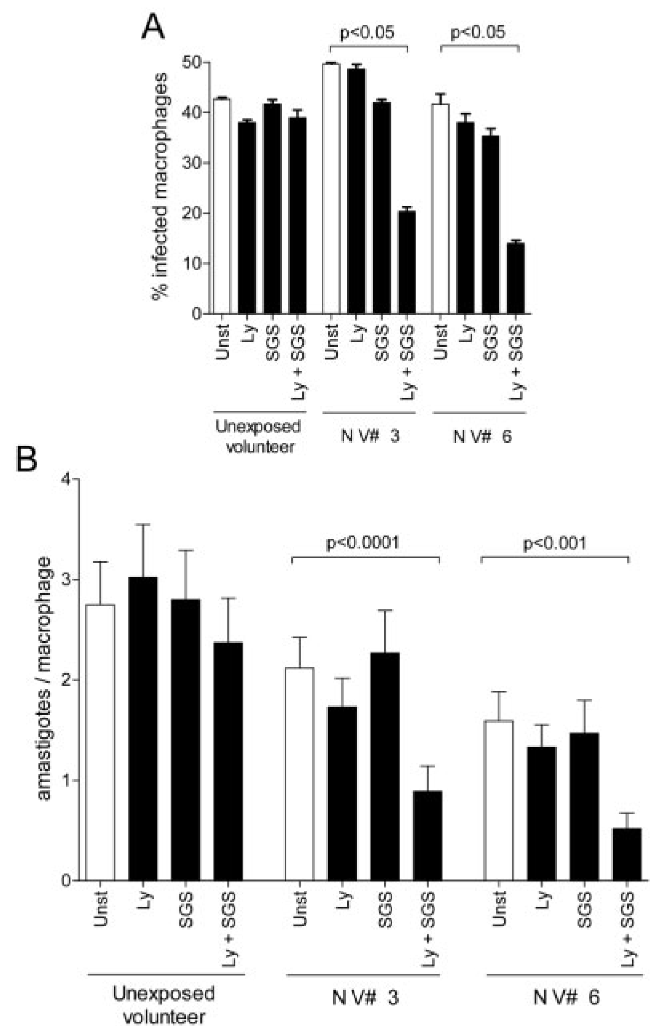

Figure 7. Analysis of macrophage *in vitro* infection with *L. chagasi*. Monocytes were isolated from PBMC of one unexposed volunteer or two normal volunteers 1 year after first exposure to sand fly bites. Macrophages were infected with *L. chagasi* promastigotes (*Leishmania*/macrophage = 5:1) for 4 h and then cultured in the presence or absence of SGS (2 μg/mL), with or without autologous lymphocytes (lymphocyte/macrophage = 2:1). Infection rate was evaluated after 4 days, when cover slips were washed and stained with hematoxilin and eosin, and intracellular amastigotes were counted in 200 macrophages. Results are shown as percent of infected cells (A) and mean of amastigote number per macrophage (B). Bars represent means and SEM. Kruskal–Wallis test was used to compare infection rates in macrophages cultures; *p* values are expressed in each panel; Unst = Unstimulated, Ly = Lymphocyte.

neither SGS nor lymphocytes alone were able to significantly influence infection in the two analyzed normal volunteers (Fig. 7).

Discussion

In the present work, we have shown that human volunteers developed both humoral and cell-mediated IR against sand fly saliva following exposure to laboratory-reared *Lu. longipalpis* bites. Inflammatory reaction has been observed in mice following *Lu. longipalpis* bites [25]. Interestingly, while three volunteers developed hemorrhagic points followed by small papules, the other three volunteers exhibited very mild early-phase reactions and later developed small nodular lesions. Volunteers who developed papules tended to present a higher serum IgE production, in contrast to those who developed nodules, which produced increased amounts of IgG. Nevertheless, bullous reactions after contacts with *Cimex lectularius* bites have been related to specific IgE Ab [26]. In addition, anti-saliva IgE has been implicated in allergic reactions to *Aedes albopictus* bites [27]. Local phenomena not represented in the peripheral blood may also explain such differences. Following mosquito bites, mast

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cells have been demonstrated to play critical role in inducing T cell and dendritic cell recruitment within draining lymph nodes influencing adaptive IR [28]. Ethical issues precluded the investigation of responses at bite sites.

Despite the absence of histopathological analysis, dermatological evaluation of the skin reactions suggested that the nodules observed at the bite sites in some normal volunteers seem to be reminiscent of an inflammatory cell infiltration, and may be a DTH-like reaction. *Lu. longipalpis* saliva was implicated in increased MCP-1 expression and macrophage influx in a murine air pouch model [29]. In experiments performed in mice, saliva from *Lu. longipalpis* has been shown to induce cell recruitment into the site of injection, differing in mice of diverse genetic background [29]. Induction of a DTH-like reaction by sand fly saliva is thought to be a survival strategy. The DTH-like reaction induced by sand flies seems to facilitate blood feeding at the site of inflammation, which presents a larger blood flow than normal skin [30]. Although some *Lu. longipalpis* salivary components present immunomodulatory and anti-inflammatory properties [7, 21, 31], the development of DTH-like reactions to *Lu. longipalpis* SGS exposure in experimental models [25] and in individuals from endemic areas [19] suggests that these components elicit host-specific IR.

Following experimental exposure to sand fly bites, normal volunteers presented an increase in the frequency of $CD25^+CD4^+$ or $CD25^+CD8^+$ peripheral lymphocytes, suggesting that T cells are becoming activated by sand fly salivary antigens. On the other hand, $CD4^+CD25^+$ regulatory T cells may play important role in leishmaniasis [32] and seem to be stimulated by sand fly saliva [33, 34]. In the present report, the marked production of pro-inflammatory cytokines such as IFN-γ by normal volunteer's PBMC after SGS stimulation *in vitro* suggests that the T cells display an effector phenotype.

Besides lymphocytes, other cells, like macrophages, may contribute to these increased levels of such cytokines. Thus, it is feasible to propose that these activated cells may be migrating to the site of sand fly bites, interacting with local immune cells, and generating DTH response. In agreement with this evidence, an enhanced influx of lymphocytes together with macrophages, neutrophils, basophils, and eosinophils at the site of sand fly bites has been reported in *Lu. longipalpis*-exposed mice *in vivo* [25]. As described previously, a *Lu. longipalpis* salivary component named maxadilan can influence immune cell activation by reducing pro-inflammatory cytokine production and stimulating IL-10 secretion and IR deviation to Th2 pattern [20, 35, 36]. Specific anti-maxadilan IR achieved after several exposures to *Lu. longipalpis* bites may abrogate or reduce its immunomodulatory effects. Altogether, these findings allow us to hypothesize that saliva from *Lu. longipalpis* influences host cell trafficking, cytokine production, as well as Th1-mediated response against *L. chagasi* at the site of infection in humans. But this needs to be directly tested.

Prior studies investigating host humoral responses against ectoparasite saliva has revealed an important role for IgG, with major contribution of isotype IgG1, IgM, and IgE [4, 37–40]. Our group has also investigated humoral response against sand fly saliva in a murine model [25], and in children living on an endemic area of visceral leishmaniasis [18]. In both reports, IgG levels significantly correlated with cellular IR, serving as epidemiological marker of sand fly exposure.

Individuals naturally exposed in an endemic area predominantly recognize a salivary protein of 45 kDa, followed by proteins of 44, 43, 35, 17, and 16 kDa [19]. Such findings agree with the observations of the present study. At 60 days after their first bite exposure, a salivary protein of 45 kDa was more intensely recognized, followed by bands of 44, 43, and 35 kDa and a protein of 17 kDa. Combined, these data indicate the most strongly immunogenic molecules of *Lu. longipalpis* saliva to man. As for their use as vaccine candidate, however, a note of caution is needed. We have no information on the type of response these proteins induce when used as a single product, nor of its capacity to favor a protective anti-*Leishmania* response. Nevertheless, these results suggest that such immunogenic proteins could serve as possible targets in future vaccine designs.

One important point demonstrated here, not previously shown in man, is the recall IR observed 1 year after first experimental exposure to sand fly bites. The significant IFN-γ secretion and the Ab production (IgG1 and IgG4) after a further boost with *Lu. longipalpis* bites suggest that an efficient IR against sand fly saliva could be achieved through a feasible, albeit not yet solidified, vaccine. Consistent with these results, the sand fly saliva exposure seems to favor *Leishmania* killing *in vitro*, as SGS reduced parasite load in human macrophages. These findings could open new prophylactic perspectives in leishmaniasis.

Materials and methods

Volunteers

Six volunteers (four males), with ages ranging from 20 to 28 years (Table 2), were used in the study. The volunteers were negative for both *Lu. longipalpis* saliva and *Leishmania* exposure as measured by an absence of Ab against SGS and a DTH response to the parasite. The study was approved by the Research Ethics Committee of FIOCRUZ-Bahia, and all

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Table 2. Volunteer characteristics and sand fly bites estimation

Normal volunteer	Sex	Age (years)	Range of sand flies fed (mean)[a)]
1	Male	20	03–29 (19.5)
2	Male	28	22–30 (26.5)
3	Male	27	04–26 (14)
4	Female	25	14–24 (17.5)
5	Female	24	11–23 (18.75)
6	Male	27	10–23 (17.75)

a) Normal volunteers were experimentally exposed four times to 30 laboratory-reared sand flies. Numbers represent the range and the mean numbers of sand flies fed considering all exposures of each volunteer.

individuals included in this report signed an informed consent form before enrollment.

Sand flies

Sand flies (colony obtained from Cavunge, Bahia, Brazil, 12° 15′ 0S; 39° 19′ 60W), were reared at FIOCRUZ-Bahia on a fermented mixture of rabbit chow, rabbit feces, and fish food as described previously [41]. Salivary glands from 3- to 7-day-old adult female flies were dissected and transferred to 10 or 20 μL of 10 mM HEPES pH 7.0, 0.15 mM NaCl in 1.5-mL polypropylene vials, usually in groups of 20 gland pairs in 20 μL of HEPES saline, or individually in 10 μL of HEPES saline. Salivary glands were kept at –70°C until needed, when they were disrupted by sonication using a Branson Sonifier 450 homogenizer (Branson, Danbury, CT). SGS were centrifuged at 10 000 × *g* for 4 min; the supernatants were used for experiments. SGS preparation was shown to be free of LPS contamination as there was no difference in TNF-α production by PBMC from normal volunteers stimulated with SGS with or without addition of polymyxin (data not shown).

Exposure to *Lu. longipalpis* bites

Volunteers were experimentally exposed four times, with 15-day intervals, to 30 laboratory-reared uninfected sand flies for 30 min each time. At each exposure, captured sand flies were cautiously transferred to a small home-made cylindrical plastic recipient which has an open orifice. This device was attached to the volunteers' arm by a black adhesive strip (because of sand fly feeding behavior). One year after their first exposure to sand fly bites, all normal volunteers were boosted by a further session of exposure to 20 *Lu. longipalpis*. The number of sand flies fed in each exposure varied from three to 30 (Table 2). Before each exposure (days 0, 15, 30, and 45) and at 60, 180, and 360 days after the first exposure, 20 mL of peripheral blood was obtained in order to assess anti-SGS IR.

Semi quantitative analyses of skin reactions

Two and 24 h after experimental exposures to sand flies, normal volunteers' skin reactions were dermatologically examined by a dermatologist. Digital pictures of skin reactions with magnifying lens were taken and a semi-quantitative analysis was performed regarding reaction diameter, erythema, micropapules, and nodules intensity.

Anti-*Lu. longipalpis* saliva serology

Normal volunteer sera were collected at different times after the first sand fly exposure and conserved in –70°C. Serological tests of all samples were performed in a single experiment. ELISA was performed as described elsewhere [18]. Briefly, plates were coated with SGS equivalent to five pairs of salivary glands/mL in carbonate buffer overnight at 4°C, then washed with PBS/0.05% Tween and blocked with PBS/0.1% Tween plus 0.05% BSA. Sera were diluted 1:100 with PBS/0.05% Tween and incubated overnight at 4°C. After further washings, the wells were incubated with alkaline phosphatase-conjugated anti-human IgG (Sigma-Aldrich, St. Louis, MO) at a 1:1000 dilution. Following another washing cycle, the color was developed with p-nitrophenylphosphate. The reactions were blocked with NaOH and read at 405 nm using Soft Max-Pro Software v5 (Molecular Devices Corporation, Sunnyvale, CA) ELISA reader.

Quantification of anti-*Lu. longipalpis* saliva IgG subtypes

Quantification of anti-*Lu. longipalpis* saliva IgG subtypes was performed on Maxisorb plates (Nunc, Roskilde, Denmark) as previously described [19]. In brief, *Lu. longipalpis* SGS (5 or 15 μg/mL) was bound to plates, and free sites blocked as above. Sera were tested at 1:100 for IgG1 and 1:50 for IgG4. Anti-IgG1 (HP6069) was used at 1:1000, and anti-IgG4 (HP6023) at 1:500. Alkaline phosphatase-labeled goat anti-mouse IgG was used, developed, and read as above.

Quantification of anti-*Lu longipalpis* saliva IgE

Sera were pre-incubated with purified sheep IgG anti-human IgG (RF absorbent; Behring Diagnostics, Marburg, Germany) to eliminate IgG Ab competition. Sera (60 μL) prediluted 1:2 in PBS pH 7.4 were mixed with 60 μL of RF absorbent for 15 min at room temperature and then centrifuged (13 000 rpm, 2 min, 4°C) to remove immune complexes. Supernatants (100 μL) were added to polystyrene microtiter plates (Linbro/Titertek, Aurora, OH) previously coated with 15 μg/mL of SGS (100 μL per well) in carbonate bicarbonate

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buffer overnight at 4°C, washed in PBS/0.05% Tween, and blocked with PBS/0.1% Tween plus 0.05% BSA (2 h, 37°C). After incubation (8 h, 4°C), the wells were washed six times with PBS and incubated with 100 μL of goat anti-human IgE-alkaline phosphatase conjugate (2 h, 37°C). After washing, the reactions were developed (30 min, room temperature), stopped, and read as above.

Western blots

Western blots were performed as described elsewhere [19]. In a large single well of Tris-glycine gels, running with sodium dodecyl sulfate (Novex, San Diego, CA), SGS equivalent of 80 pairs were added (approximately 80 μg of protein) treated with 8% SDS and 4% 2-mercaptoethanol in 0.5 M Tris-HCl buffer pH 6.8, 10% glycerol, and 1% bromophenol blue dye, and heated (5 min, 100°C). The gel was developed with Tris-glycine buffer and transferred to nitrocellulose using a Blot-Module for the XCell II Mini-Cell (Novex). Free sites were blocked with PBS/0.05% Tween plus 0.5% non-fat dried milk (2 h, 37°C). The strips were washed with PBS/0.05% Tween and incubated with sera (diluted 1:100 in PBS/0.05% Tween plus 0.5% non-fat dried milk) overnight at 4°C. Following further washings, strips were incubated with rabbit alkaline phosphatase-conjugated anti-human IgG (1 h, 37°C). Blots were then washed and developed with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Flow cytometry

PBMC were separated from heparinized blood by centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NY) gradients and washed twice with PBS and once with complete medium, RPMI 1640 (GIBCO BRL, Grand Island, NY) supplemented with 2 mmol/L of L-glutamine, 5×10^{-5} mM 2-mercaptoethanol, 100 U/mL of streptomycin (Sigma-Aldrich), and 10% AB human serum (Sigma-Aldrich). Cells were cultured in 5% CO_2 at 37°C. On the second day non-adherent cells (lymphocytes) were collected and resuspended in PBS, 1% BSA, 0.1% sodium azide and blocked with 20% FBS for 30 min on ice. The cells were then double-stained with monoclonal Ab from Becton Dickinson (PE- or FITC-labeled anti-human CD25, clone FTR-D4; PE-labeled anti-human CD8, clone RPA-T8; and FITC labeled anti-human CD4, clone RPA-T4) or respective controls for 1 h and then collected and analyzed using Cell Quest software and a FACSort flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Quantification of cytokine production by volunteers' PBMC

Two months after normal volunteers' first exposure to sand fly bites, blood samples were collected. PBMC were washed as above. Then, one aliquot of PBMC was frozen in liquid nitrogen and another fresh aliquot of 5×10^6 PBMC/mL was co-cultured with SGS (0.5 μg/mL) in 24-well plates. Levels of IL-10 (48 h after stimulation), TNF-α (48 h after stimulation), and IFN-γ (72 or 96 h after stimulation) were determined by ELISA (Pharmingen, San Diego, CA) as previously described [42]. PBMC supernatants were collected at different times after SGS stimulation and conserved in –70°C. Tests of all samples were performed in a single experiment. One year after their first exposure to sand fly bites, volunteers were boosted by a further session of *Lu. longipalpis* bites. Seven days thereafter, PBMC were obtained and stimulated *in vitro* with SGS for 96 h to evaluate IFN-γ production. PBMC from the time point 0 were thawed and stimulated *in vitro* with SGS for 96 h to evaluate IFN-γ production.

Macrophage cultures and *in vitro L. chagasi* infection

One year after exposure, normal volunteers were boosted with sand fly bites and PBMC were collected as described above. Monocytes were isolated by adhesion of 5×10^6 PBMC/mL in 24-well plates for 40 min at 37°C and 5% CO_2. Plates were washed and cells were resuspended in RPMI 1640 (GIBCO BRL) supplemented with 2 mmol/L of L-glutamine, 5×10^{-5} mM 2-mercaptoethanol, 100 U/mL of streptomycin (Sigma-Aldrich), and 10% FBS (GIBCO BRL) for 7 days for macrophage differentiation. *Leishmania chagasi* (MHOM/BR00/MER/STRAIN2) promastigotes were cultured in Schneider's medium supplemented with 20% inactive FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μL/mL streptomycin.

Macrophages were infected with *L. chagasi* (*Leishmania*/macrophage = 5:1) for 4 h, washed twice with PBS, and then cultured in the presence or absence of SGS (2 μg/mL), with or without autologous lymphocytes (lymphocyte/macrophage = 2:1). Infection rate was evaluated after 4 days, when cover slips were washed and stained with hematoxilin and eosin, and intracellular amastigotes were counted in 200 macrophages. Results are shown as percent of infected cells and amastigote number per macrophage (mean and SEM).

Statistical analysis

Increase in IgG or IgE levels at days 0 and 60 and IgG/IgE ratio at day 60 were compared by nonparametric Mann–Whitney test (Fig. 2A–F). The same test was used to compare pre *versus* post levels of Ab, cytokines, and frequencies of $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells (Fig. 6). Kruskal-Wallis statistic with Dunn's multiple comparison tests were used to compare infection rates in macrophage cultures (Fig. 7). $p<0.05$ was established as the minimum significance level.

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