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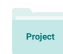

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Immune response to LinB13, a *Lutzomyia intermedia* salivary protein correlates with disease severity in tegumentary leishmaniasis

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Summary

Herein we observed an association between humoral and cellular immune response to the sand fly salivary protein rLinB-13 and disease severity in tegumentary leishmaniasis. This study brings evidence that immunity to rLinB-13 influences disease outcome in *L. braziliensis* infection**.**

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Abstract

Background. We have previously shown that seropositivity to rLinB-13, a salivary protein from *Lutzomyia intermedia*, predicted sand fly exposure and was associated with increased risk of developing cutaneous leishmaniasis (CL). **Methods.** Herein, we investigated the cellular immune response to saliva from *Lu. intermedia*, using rLinB-13 as a surrogate antigen in naturally exposed individuals presenting positive serology to LinB-13. We also investigated the response to rLinB-13 in leishmaniasis patients, displaying active ulcers and positive PCR for *L. braziliensis*. **Results.** Peripheral blood mononuclear cells (PBMCs) stimulated in vitro with rLinB-13 secreted elevated levels of IL-10, IL-4, IL-1β, IL-1α, IL-6 and chemokines (CCL3, CCL4, CCL5 and CXCL5). CL and disseminated leishmaniasis (DL) patients displayed a significantly higher IgG response to rLinB-13, compared to healthy subjects and anti-rLinB-13 IgG was positively correlated with the number of lesions in DL patients. Positive serology to rLinB-13 was also associated with chemotherapy failure. PBMCs from DL patients stimulated with rLINB-13 secreted significantly higher levels IL-10 and IL-1β compared to CL individuals. **Conclusions.** In this study, we observed an association between humoral and cellular immune response to the sand fly salivary protein rLinB-13 and disease severity in tegumentary leishmaniasis. This study brings evidence that immunity to rLinB-13 influences disease outcome in *L. braziliensis* infection and results indicate that positive serology to rLinB-13 IgG can be employed as marker of DL, an emerging and severe form of disease caused by *L. braziliensis*.

Keywords: Leishmania braziliensis, cutaneous leishmaniasis, disseminated leishmaniasis, sand fly saliva, Disease severity

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INTRODUCTION

Tegumentary leishmaniasis (TL) constitutes a group of neglected diseases caused by protozoa of the genus *Leishmania*. *Leishmania* (Viannia) *braziliensis* is the most important species causing TL in Brazil, including three clinical forms: cutaneous leishmaniasis (CL), usually presented by a single ulcer with well-delimited raised borders; mucosal leishmaniasis (ML), characterized by chronic lesion that affects primarily the nasal mucosa; and disseminated leishmaniasis (DL), a severe and emerging TL form characterized by more than 10 and up 1000 cutaneous lesions [1, 2] Moreover, about 20% of individuals living in *L. braziliensis* endemic areas exhibit a positive delayed type hypersensitivity (DTH) response to soluble *Leishmania* antigens (SLA), without any active or prior history of leishmaniasis and are defined as having subclinical (SC) infection [3].

Leishmania transmission occurs during the blood meal of infected sand flies which bite the host and co-inject parasites, salivary proteins and other vector derived factors [4]. Sand fly salivary proteins induce vasodilation, inhibit platelet aggregation and prevent blood clotting, facilitating blood feeding [4]. Salivary proteins are also immunogenic, inducing humoral and cellular response in mammals [5-7]. Exposure to sand fly salivary proteins has been linked to protective and pathologic immune responses, depending on the combination of sand fly and *Leishmania* species. For example, previous exposure to *Phlebotomus papatasi* [8,9] or *Lutzomyia longipalpis* sand flies [10] induces a Th1 response and protects against CL or visceral leishmaniasis (VL), respectively, in murine models. Accordingly, individuals experimentally exposed to *Lu. longipalpis* developed a Th1 immune response to salivary proteins, that was associated with parasite killing [11]. On the contrary, pre-exposure to *Lu. intermedia*, one of the main vectors of *L. braziliensis* in Brazil, induces a mixed immune response and enhances CL in mice [6]. Individuals naturally exposed to *Lu. intermedia* develop an IL-10 dominant immune response to salivary proteins, promoting parasite replication in vitro, and increasing risk of CL development [12].

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Previously, we characterized the salivary proteins repertoire of *Lu. intermedia* and investigated the humoral response to these antigens in naturally exposed individuals. We showed that antibodies to the salivary protein rLinB-13 acts as a marker of exposure to this sand fly and, importantly, that the presence of anti-rLinB-13 IgG increases the risk of developing CL in prospective cohort [13]. Following up on these findings, herein we sought to characterize the immune response to rLinB-13 in naturally exposed individuals and in patients representing the TL clinical spectrum.

MATERIALS AND METHODS

Study design and selection of individuals

This study was conducted in Corte de Pedra, a TL endemic area in the southeastern part of Bahia state, Brazil. Corte de Pedra is surrounded by an Atlantic Forest region, and it is an area of high *L. braziliensis* transmission by *Lu. intermedia*. In the first part of the study participants included residents of Corte de Pedra (n=11), without active TL or previous history of any type of *Leishmania* infection, as described elsewhere [13,14]. Control individuals (n=10) consisted of residents of a non-endemic area (unexposed), where *Lu. intermedia* does not occur. In the second part of the study, sera from TL patients [CL (n= 28), ML (n= 17), DL (n= 46), SC (n= 33)] and control individuals (n=30) from a non-endemic area were evaluated. CL and ML patients had typical leishmaniasis lesions and DL was defined by more than 10 acneiform, papular, and ulcerated lesions affecting at least 2 different regions of the body [2]. The diagnosis was confirmed by documentation of DNA of *L. braziliensis* in biopsied lesions [15]. For investigation of the cellular response to rLinB-13, we recruited CL (n=10) and DL (n=11) patients. Patients were treated with meglumine antimoniate (Sb^v) (Sanofy Aventis) (20 mg/kg/day for 20 days for CL and 30 days for DL). Patients were evaluated every 30 days for response to therapy and the final cure rate was assessed on day 90, after initiation of chemotherapy. Cure was defined as complete re-epithelialization of lesions, without raised borders. This study was approved by the Ethics Committee of the Federal University of Bahia Medical School and informed consent was obtained from all participants.

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Preparation of Lu. intermedia Salivary Gland Sonicate (SGS) and Soluble Leishmania Antigen (SLA)

Adult *Lu. intermedia* sand flies were captured in Corte de Pedra, Brazil. Sand fly identification and Salivary Gland Sonicate (SGS) preparation were performed as described elsewhere [16]. SLA was produced with an isolate of *L. braziliensis* from a CL patient as previously described [1].

Expression and purification of Lu. intermedia rLinB-13

rLinB-13 was produced by transfection of 293-F cells (Invitrogen) with VR2010-TOPO plasmid coding for rLinB-13 salivary protein as previously described [13].

Analysis of anti–rLinB-13 antibodies

IgG response to *Lu. intermedia* SGS and to rLinB-13 was determined by Enzyme-Linked Immunosorbent Assay (ELISA) as described [13]. rLinB-13 was used at 1 µg/ml. The cut-off value was established employing sera from HS (n = 30) from a non-endemic area and was determined as the mean optical density (OD) value plus 2.5 standard deviations.

Cell Culture and cellular immune response evaluation

Peripheral Blood Mononuclear Cells (PBMC) were obtained from heparinized venous blood layered over a Ficoll Hypaque gradient (GE Healthcare). Cells were washed and resuspended in RPMI 1640 medium (GIBCO) supplemented with 10% human AB serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin (all Invitrogen). Cells (3×10^6/mL) were plated in 24-well plates and stimulated with rLinB-13 (10 µg/mL), or rLinB-13 plus SLA (5 µg/mL), for 72 hours at 37°C and 5% CO_2. Cellular immune response was evaluated by MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel (Merck), R&D MMP and TIMP Luminex kits (R&D Systems, Minneapolis, MN) and sandwich ELISA (R&D Systems), according to manufacturers´ instructions and results were expressed as pg/mL.

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Statistical Analysis

Comparisons between 2 groups were performed by the Mann-Whitney *U* test and comparisons among 3 or more groups by the Kruskal-Wallis test followed by Dunn's multiple comparison ad hoc tests. The Wilcoxon paired test was used to assess differences between variables in the same subjects. A heatmap using log10-transformed and z-score normalized values of parameters measured in PBMC supernatants was built and an unsupervised two-way hierarchical cluster analysis (Ward's method) was used to test whether cellular responses from rLinB-13-exposed and unexposed individuals could be clustered separately. In this analysis, Dendrograms represent Euclidean distance. The Spearman correlation test was used for analysis between anti-rLinB-13 IgG with clinical parameters, and between IgG levels and cellular response. Receiver operator characteristics (ROC) curve analysis was used to evaluate the ability of anti-rLinB-13 IgG levels to distinguish *L. braziliensis* clinical spectrum. Analyses were conducted using GraphPad Prism version 8.0 for Windows, and differences were considered significant at $P < .05$.

RESULTS

IgG responses to Lu. intermedia salivary gland sonicate and rLinB-13 in naturally exposed individuals exposed

Initially, we selected a group of Corte de Pedra residents to confirm exposure to *Lu. intermedia* bites by measuring IgG to SGS and to rLinB-13. All subjects from TL-endemic area displayed positive serology to *Lu. intermedia* SGS and to rLinB-13 whereas unexposed control subjects from a non-endemic area did not (**Figure 1A and B**). We also observed that the IgG response to rLinB-13 is stable as reactivity was similar over time in endemic area residents (**Figure 1C**). These results confirm that residents of a *L. braziliensis* transmission area, are naturally and constantly exposed to *Lu. intermedia* sand fly bites.

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Recall response to rLinB-13 in cells from individuals naturally exposed to Lu. intermedia

Next, we evaluated the cellular immune response to rLinB-13. PBMCs from exposed and unexposed individuals were stimulated in vitro with rLinB-13 and culture supernatants were assayed by Luminex. Stimulation with rLinB-13 induced a mixed response as indicated by the presence of regulatory (IL-10 and IL-4) and inflammatory cytokines (IL-1β, IL-1α and IL-6), chemokines (CCL3, CCL4, CCL5 and CXCL5) as well as matrix metalloproteinases. Hierarchical clustering showed that the immune response profile of exposed individuals is distinct from non-exposed controls (**Figure 2A**). Moreover, higher levels of regulatory IL-10 and Th2-related IL-4 were observed in naturally exposed individuals compared to unexposed controls (**Figure 2B**). Of note, IL-1α, IL-1β, and IL-6 levels were also increased in individuals seropositive to rLinB-13 (**Figure 2B**). Lastly, exposed individuals displayed higher CCL3, CCL4, CCL5, and CXCL5 levels compared to unexposed controls (**Figure 2C).** We observed that levels of matrix metalloproteinase 1 (MMP-1) and 3 (MMP-3) were higher in *Lu. intermedia* exposed individuals compared to unexposed controls. On the other hand, tissue inhibitor of metalloproteinases 3 (TIMP3) was lower in exposed individuals compared to controls (**Supplementary Figure 1**). Collectively, we found a strong positive correlation between presence of IgG to rLinB-13 and production of immune mediators (**Supplementary Figure 2**), indicating that exposure to *Lu. intermedia* salivary antigens also modulates the cellular response.

IgG response to rLinB-13 correlates with severity of tegumentary leishmaniasis

TL patients displayed higher IgG levels to rLinB-13 compared to unexposed individuals (**Figure 3A**). Importantly, when we stratified TL patients according to the clinical manifestation, we observed that antibodies to rLinB-13 were present in 89% of patients with CL, 100% of ML patients and 100% of DL patients while antibodies to rLinB13 were only present in 21% of SC patients and 3% of control individuals. In terms of the magnitude of antibody response, sera from DL patients presented higher IgG reactivity to rLinB-13 compared to CL patients and SC individuals (**Figure 3B**). Lastly, ROC analysis confirmed that anti-rLinB-13 IgG levels could be used to distinguish DL patients

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from other clinical presentations and discriminated SC subjects with high accuracy (**Figure 3C and 3D**). These data show that a prominent humoral response to rLinB-13 is associated with DL, a severe form of TL in which the rate of failure to SB^v treatment is over 70% [17].

Larger CL ulcers are associated with poor response to SB^v therapy [18]. When we stratified CL patients by median lesion size, patients presenting ulcer size >177 mm^2 had a significantly higher anti-rLinB-13 IgG response compared to patients in which ulcers were < 177 mm^2 (**Figure 4A**). However, we did not find a correlation between lesion size and IgG levels to rLinB-13 in CL patients nor could the presence of IgG to rLinB-13 be associated with treatment failure (**Figure 4B, 4C and 4D**). In DL patients, the number of lesions may range from 10 to more than 1000 and this parameter is also an indicator of disease severity. Herein, DL patients presenting >30 lesions (median number) had a significantly higher antibody response to rLinB-13 compared to patients that had < 30 lesions, and importantly, a strong positive correlation was found between number of lesions and rLinB-13 IgG levels (**Figure 4E and 4F**). Importantly, DL patients who presented failed response to chemotherapy exhibited elevated levels of anti-LinB-13 IgG, and ROC analysis showed that IgG to rLinB-13 predicts treatment outcome with 75% accuracy (**Figure 4G and 4H**). Collectively, these results indicate that seropositivity to rLinB-13 correlates with disease severity in TL and suggest that the humoral response to salivary antigens may play an yet unidentified role in the pathogenesis of TL.

Cellular response to rLinB-13 in cutaneous and disseminated leishmaniasis patients

We further compared the cytokine response to rLinB-13 in exposed individuals (without active TL or history of disease) *versus* CL and DL patients. Again, the IgG response to rLinB-13 in DL patients was higher than the response displayed by CL patients (**Supplementary Figure 3**). Overall, we observed a significantly lower production of IL-1α, IL-1β and IL-6 in both CL and DL patients compared to the exposed individuals (**Figure 5A-C**). Of note, stimulation of PBMCs from TL patients

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with rLinB-13 induced higher IL-1β secretion in comparison to control (medium) cultures (**Figure 5B**). Higher levels of IL-10 were also found in PBMCs from TL patients also stimulated with rLinB-13 compared to control cultures (**Figure 5D**). Moreover, IL-10 production was similar in DL patients and in *Lu. intermedia*-exposed individuals (without active TL or history of disease). Comparing CL and DL, DL patients produced significantly more IL-1β and IL-10 and significantly less IL-6, in response to rLinB-13, compared CL patients (**Figure 5**). We also found a positive correlation between anti-rLinB-13 IgG and IL-10 levels in DL patients (**Supplementary Figure 4**). Lastly, addition of rLinB-13 to SLA-stimulated cell cultures led to increased production of IL-10 in DL patients (**Figure 6B**) but not in CL patients (**Figure 6A**). On the contrary, TNF, IFN-γ, and IL-1β levels remained unaltered.

DISCUSSION

Immune response to sand fly salivary components has been associated with protection or susceptibility to *Leishmania* infection, depending on the sand fly vector involved [6,10-12,19,20]. Herein, we show that the response to one salivary antigen, *Lu. Intermedia's* rLinB-13, is linked to clinical severity in TL caused by *L. braziliensis*. We show that individuals naturally exposed to *Lu. intermedia* sand flies, without history of TL, as well as those with active clinical disease develop a humoral response and cellular immune response to rLinB-13, the latter characterized by the production of IL-10 and IL-1Β.

LinB-13 is an antigen 5-related salivary protein, abundant and well conserved across sand fly species [13,21]. The presence of antibodies to rLinB-13 identifies individuals naturally exposed to the vectors *Lu. intermedia* (New World) and *Phlebotomus orientalis* (Old World) [15,22]. We have previously shown that IgG to rLinB-13 is a biomarker of CL development in an endemic area of leishmaniasis caused by *L. braziliensis* as individuals seropositive to this protein present a higher risk of developing disease [13]. Here, we expanded these observations to show that higher anti-rLinB-13 IgG titers in peripheral blood are also detected in patients across the TL spectrum compared with

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unexposed, healthy subjects. Similar data has been reported with *Lu. intermedia* SGS, and with salivary antigens from distinct sand flies [6,20,23,24]. Moreover, the presence of antibodies to rLinB-13 correlated with larger CL lesions, similar to a study performed in Saudi Arabia, with individuals presenting anti-*Ph. papatasi* antibodies [24]. Lesion size is a risk factor for Sb^v failure in CL and the presence of larger lesions also correlates with longer healing time in CL [18,25]. However, we did not find any association between anti-rLinB-13 IgG and chemotherapy failure in CL patients.

DL is a severe and emergent clinical form of *L. braziliensis* infection [26]. Since the first description of DL in 1986, the frequency of this clinical form has increased about 20-fold in Corte de Pedra, where this study was conducted [26]. Previously, we showed that anti-SLA IgG levels are higher in DL compared to CL patients [27]. We now demonstrate that IgG levels to rLinB-13, a sand fly salivary protein, are also heightened in DL patients and ROC analysis uncovered that anti-rLinB-13 IgG distinguishes DL patients from those with ML or CL. Additionally, a positive correlation between anti-rLinB-13 IgG and number of lesions was found for DL patients. Cross-reactivity between salivary antigens and *Leishmania* antigens has never been documented, to our knowledge, and all of the well characterized sand fly salivary proteins have no sequence similarity to any Leishmania protein in current databases [28]. Thus, we do not believe that the greater antigen load in DL patients enhances preexisting immunity to rLinB-13 nor that exposure to *Lu. intermedia* saliva drives or induces polyclonal B cell activation. While it is widely acknowledged that polyclonal B cell activation is a hallmark of visceral leishmaniasis [29], it has not been documented in TL caused by *L. braziliensis*, including in patients with disseminated leishmaniasis.

We further identified an association between the presence of anti-rLinB-13 IgG and failure to Sb^v therapy, predicting the outcome of treatment. Although Sb^v is highly toxic, it is still the first line of treatment for TL patients in Brazil, and a failure rate >70% has been reported for DL patients [17]. Thus, we propose that the detection of high titers of anti-rLinB-13 IgG antibodies, before the appearance of multiple lesions, could, perhaps, identify patients who will progress to DL. This proposition needs validation in the field, in a prospective manner. Lastly, early identification of DL

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patients who may not respond to Sb^v therapy, by use of serology to rLinB13, could be useful in therapeutic management in endemic areas.

Individuals exposed to *Lu. intermedia* presented increased levels of CCL3, CCL4, CCL5 and CXCL5, which is consistent with previous findings showing that either sand fly SGS or sand fly bites induce potent cellular recruitment [30,31,32]. We also observed an increased production of MMP1 and MMP3 in individuals naturally exposed to *Lu. intermedia* which was paralleled by lower levels of TIMP3. Upregulated expression of MMP1 and MMP3 has been documented in CL lesions [33]. MMPs are enzymes involved in both degradation and synthesis of extracellular matrix proteins and are related to both tissue injury and wound healing [34]. Elevated MMP3 and decreased TIMP3, as seen here, may contribute to tissue damage and disease severity. These data indicate that natural exposure to *Lu. intermedia* sand fly saliva and, consequently to rLinB-13, promotes MMPs and chemokine expression leading to cellular recruitment, which may ultimately contribute to parasite establishment, resulting in lesion development.

Previously, we have documented the development of mixed cellular response to *Lu. intermedia* saliva in naturally exposed individuals, similarly to data reported on other sand fly species [7,11,12,35]. Of note, IL-10 was the dominant cytokine produced in response to both stimulation with *Lu. intermedia* and to *P. papatasi* saliva [12,36]. Here, the recall response to rLinB-13 recapitulated the elevated IL-10 production observed in naturally exposed individuals, without symptoms of disease, but we now extend this finding to TL patients. In fact, IL-10 production was significantly higher in DL compared to CL patients and a positive correlation was found between production of this regulatory cytokine and anti-rLinB-13 IgG. Furthermore, while IL-10 levels, secreted by PBMCs in response to rLinB-13, were lower in CL patients compared to *Lu. intermedia* exposed individuals, no difference was observed in IL-10 production comparing DL patients and exposed individuals, without disease. IL-10 is linked to *Leishmania* proliferation and disease progression in mice and in human disease [37,38]. Indeed, co-culture of lymphocytes stimulated with *Lu. intermedia* saliva plus autologous *L. braziliensis*–infected macrophages significantly

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increased infection rates in vitro, and this effect was reverted by addition of IL-10 neutralizing antibody [12]. We also showed that anti-rLinB-13 IgG was associated with lower frequency of a positive Montenegro skin test in individuals naturally exposed to *Lu. intermedia* [13]. We suggest that IL-10 produced in response to vector saliva and to rLinB-13 favors *L. braziliensis* establishment in TL patients. This effect may also impair the effector response to *Leishmania* antigens, again favoring severe disease development. However, we can't rule out that increased cytokines response in DL patients is secondary to higher Leishmania antigen load possibly observed in this clinical form. Interestingly, rLinB-13 also increased IL-10 production in PBMCs from DL patients stimulated with SLA whereas the production of pro-inflammatory mediators remained unaltered.

IL-1β is a proinflammatory cytokine related to NLRP3 inflammasome activation and linked to immunopathology and disease severity in experimental and human *Leishmania* infection [39,40]. In contrast, NLRP3 inflammasome and IL-1β production is associated with nitric oxide (NO) induction, promoting parasite control in mice infected with *L. amazonensis* [41]. As NO seems to be less important to *L. braziliensis* killing by human cells [42], it is possible, that increased levels of IL-1β induced upon stimulation with rLinB-13 contributes to TL pathology.

Herein, we demonstrated an association between the humoral response to rLinB-13, a sand fly salivary protein, and disease severity in TL caused by *L. braziliensis.* We also show that the cellular response to rLinB-13 is characterized by a mixed cytokine profile with high levels of IL-10 and IL-1β in individuals naturally exposed to the vector and in TL patients. The excessive production of IL-10, which enhances parasite survival, as well as of pro-inflammatory mediators may contribute to the immunopathology of TL. Our results bring an advancement in the field as identification of patients at high risk of failing antimonial therapy, using antibodies to rLinB-13, may guide the choice for alternative therapy regimes, preventing exposure to these toxic compounds.

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NOTES

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REFERENCES

1. Bacellar O, Lessa H, Schriefer A, et al. Up-regulation of Th1-type responses in mucosal leishmaniasis patients. Infect Immun **2002**; 70(12):6734-40.
2. Turetz ML, Machado PR, Ko AI, et al. Disseminated leishmaniasis: a new and emerging form of leishmaniasis observed in northeastern Brazil. J Infect Dis **2002**; 186(12):1829-34.
3. Follador I, Araújo C, Bacellar O, et al. Epidemiologic and immunologic findings for the subclinical form of Leishmania braziliensis infection. Clin Infect Dis **2002**; 34(11):E54-8.
4. Oliveira F, de Carvalho AM, de Oliveira CI. Sand-fly saliva-leishmania-man: the trigger trio. Front Immunol **2013**; 4:375.
5. Barral A, Honda E, Caldas A, et al. Human immune response to sand fly salivary gland antigens: a useful epidemiological marker?Am J Trop Med Hyg **2000**; 62(6):740-5.
6. de Moura TR, Oliveira F, Novais FO, et al. Enhanced Leishmania braziliensis infection following pre-exposure to sandfly saliva. PLoS Negl Trop Dis **2007**; 1(2):e84.
7. Oliveira F, Traoré B, Gomes R, et al. Delayed-type hypersensitivity to sand fly saliva in humans from a leishmaniasis-endemic area of Mali is Th1-mediated and persists to midlife. J Invest Dermatol **2013**; 133(2):452-9.
8. Belkaid Y, Kamhawi S, Modi G, et al. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva

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preexposure on the long-term outcome of Leishmania major infection in the mouse ear dermis. J Exp Med **1998**; 188(10):1941-53.

9. Kamhawi S, Belkaid Y, Modi G, Rowton E, Sacks D. Protection against cutaneous leishmaniasis resulting from bites of uninfected sand flies. Science **2000**; 290(5495):1351-4.
10. Gomes R, Teixeira C, Teixeira MJ, et al. Immunity to a salivary protein of a sand fly vector protects against the fatal outcome of visceral leishmaniasis in a hamster model. Proc Natl Acad Sci U S A **2008**; 105(22):7845-50.
11. Vinhas V, Andrade BB, Paes F, et al. Human anti-saliva immune response following experimental exposure to the visceral leishmaniasis vector, Lutzomyia longipalpis. Eur J Immunol **2007**; 37(11):3111-21.
12. Carvalho AM, Cristal JR, Muniz AC, et al. Interleukin 10-Dominant Immune Response and Increased Risk of Cutaneous Leishmaniasis After Natural Exposure to Lutzomyia intermedia Sand Flies. J Infect Dis **2015**; 212(1):157-65.
13. Carvalho AM, Fukutani KF, Sharma R, et al. Seroconversion to Lutzomyia intermedia LinB-13 as a biomarker for developing cutaneous leishmaniasis. Sci Rep **2017**; 7(1):3149.
14. Muniz AC, Bacellar O, Lago EL, et al. Immunologic Markers of Protection in Leishmania (Viannia) braziliensis Infection: A 5-Year Cohort Study. J Infect Dis **2016**; 214(4):570-6.
15. Weirather JL, Jeronimo SMB, Gautam S, et al. Serial quantitative PCR assay for detection, species discrimination, and quantification of

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Leishmania spp. in human samples. J Clin Microbiol **2011**; 49(11):3892-904.

16. de Moura TR, Oliveira F, Rodrigues GC, et al. Immunity to Lutzomyia intermedia saliva modulates the inflammatory environment induced by Leishmania braziliensis. PLoS Negl Trop Dis **2010**; 4(6):e712.
17. Machado PRL, Rosa MEA, Luís H Guimarães LH, et al. Treatment of Disseminated Leishmaniasis With Liposomal Amphotericin B. Clin Infect Dis **2015**; 61(6):945-9.
18. Suprien C, Rocha PN, Teixeira M, et al. Clinical Presentation and Response to Therapy in Children with Cutaneous Leishmaniasis. Am J Trop Med Hyg **2020**; 102(4):777-781.
19. Oliveira F, Rowton E, Aslan H, et al. A sand fly salivary protein vaccine shows efficacy against vector-transmitted cutaneous leishmaniasis in nonhuman primates. Sci Transl Med **2015**; 7(290):290ra90.
20. Marzouki S, Ahmed MB, Boussoffara T, et al. Characterization of the antibody response to the saliva of Phlebotomus papatasi in people living in endemic areas of cutaneous leishmaniasis. Am J Trop Med Hyg **2011**; 84(5):653-61.
21. de Moura TR, Oliveira F, Carneiro MW, et al. Functional transcriptomics of wild-caught Lutzomyia intermedia salivary glands: identification of a protective salivary protein against Leishmania braziliensis infection. PLoS Negl Trop Dis **2013**; 7(5):e2242.
22. Sumova P, Sima M, Spitzova T, et al. Human antibody reaction against recombinant salivary proteins of Phlebotomus orientalis in Eastern Africa. PLoS Negl Trop Dis **2018**; 12(12):e0006981.

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23. Rohousova I, Ozensoy S, Ozbel Y, Volf P. Detection of species-specific antibody response of humans and mice bitten by sand flies. Parasitology **2005**; 130(Pt 5):493-9.

24. Mondragon-Shem K, Al-Salem WS, Kelly-Hope L, et al. Severity of old world cutaneous leishmaniasis is influenced by previous exposure to sandfly bites in Saudi Arabia. PLoS Negl Trop Dis **2015**; 9(2):e0003449.

25. Oliveira F, Bafica A, Rosato AB, et al. Lesion size correlates with Leishmania antigen-stimulated TNF-levels in human cutaneous leishmaniasis. Am J Trop Med Hyg **2011**; 85(1):70-3.

26. Jirmanus L, Glesby MJ, Guimarães LH, et al. Epidemiological and clinical changes in American tegumentary leishmaniasis in an area of Leishmania (Viannia) braziliensis transmission over a 20-year period. Am J Trop Med Hyg **2012**;86(3):426-33.

27. Magalhães A, Carvalho LP, Costa R, et al. Anti-Leishmania IgG is a marker of disseminated leishmaniasis caused by Leishmania braziliensis. Int J Infect Dis **2021**; 106:83-90.

28. Oliveira F, Jochim RC, Valenzuela JG, et al. Sand flies, Leishmania, and transcriptome-borne solutions. Parasitol Int **2009**; 58(1):1-5.

29. Kumar R, Nylén S. Immunobiology of visceral leishmaniasis. Front Immunol **2012**; 3:251.

30. Anjili CO, Mbati PA, Mwangi RW, et al. The chemotactic effect of Phlebotomus duboscqi (Diptera: Psychodidae) salivary gland lysates to murine monocytes. Acta Trop **1995**; 60(2):97-100.

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31. Peters NC, Egen JG, Secundino N, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science **2008**; 321(5891):970-4.

32. Guimaraes-Costa AB, Shannon JP, Waclawiak I, et al. A sand fly salivary protein acts as a neutrophil chemoattractant. Nat Commun **2021**; 12(1):3213.

33. Campos TM, Passos ST, Novais FO, et al. Matrix metalloproteinase 9 production by monocytes is enhanced by TNF and participates in the pathology of human cutaneous Leishmaniasis. PLoS Negl Trop Dis **2014**; 8(11):e3282.

34. Elkington PTG, O'Kane CM, Friedland JS. The paradox of matrix metalloproteinases in infectious disease. Clin Exp Immunol **2005**; 142(1):12-20.

35. Lakhal-Naouar I, Mukbel R, DeFraites RF, et al. The human immune response to saliva of Phlebotomus alexandri, the vector of visceral leishmaniasis in Iraq, and its relationship to sand fly exposure and infection. PLoS Negl Trop Dis **2021**; 15(6):e0009378.

36. Abdeladhim M, Ahmed MB, Marzouki S, et al. Human cellular immune response to the saliva of Phlebotomus papatasi is mediated by IL-10-producing CD8+ T cells and Th1-polarized CD4+ lymphocytes. PLoS Negl Trop Dis **2011**; 5(10):e1345.

37. Kane MM,Mosser DM. The role of IL-10 in promoting disease progression in leishmaniasis. J Immunol **2001**; 166(2):1141-7. doi: 10.4049/jimmunol.166.2.1141.

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38. Vouldoukis I, Bécherel PA, Riveros-Moreno V, et al. Interleukin-10 and interleukin-4 inhibit intracellular killing of Leishmania infantum and Leishmania major by human macrophages by decreasing nitric oxide generation. Eur J Immunol **1997**; 27(4):860-5.
39. Novais FO, Carvalho AM, Clark ML, et al. CD8+ T cell cytotoxicity mediates pathology in the skin by inflammasome activation and IL-1β production. PLoS Pathog **2017**; 13(2):e1006196.
40. Carvalho AM, Novais FO, Paixão CS, et al. Glyburide, a NLRP3 Inhibitor, Decreases Inflammatory Response and Is a Candidate to Reduce Pathology in Leishmania braziliensis Infection. J Invest Dermatol **2020**; 140(1):246-249.e2.
41. Lima-Junior DJ, Costa DL, Carregaro V, et al. Inflammasome-derived IL-1β production induces nitric oxide-mediated resistance to Leishmania. Nat Med **2013**; 19(7):909-15.
42. Carneiro PP, Conceição J, Macedo M, Magalhães V, Carvalho EM, Bacellar O. The Role of Nitric Oxide and Reactive Oxygen Species in the Killing of Leishmania braziliensis by Monocytes from Patients with Cutaneous Leishmaniasis. PLoS One **2016**; 11(2):e0148084.

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FIGURES LEGENDS

Figure 1. IgG response to *Lu. intermedia* saliva and rLinB-13 in naturally exposed individuals. (A) Anti-SGS IgG response in residents of a non-endemic area (n = 10) (purple circles) and a TL-endemic area (n = 11) (yellow circles). (B) anti-rLinB-13 IgG in the same individuals from A. (C) Kinetics of anti-rLinB-13 IgG in the same population from a TL-endemic area. Circles represent individual values; horizontal lines, median optical density (OD) values; dotted line, cutoff level. ***P < .0001.

Figure 2. Screening of cellular immune response to rLinB-13 in naturally exposed individuals. PBMC from residents of a non-endemic area (n = 10) (purple) and a TL-endemic area (n = 11) (yellow) were stimulated with rLinB-13 for 72 hours, and immune molecules were determined by multiplex Luminex assay. (A) Heat map based on immune molecules levels. Levels of cytokines (B) and chemokines (C) in exposed and unexposed subjects. An usupervised two-way hierarchical cluster analysis (Ward's method) using dendrograms as Euclidean distance was employed to investigate whether the overall expression profile of log10-transformed z-score normalized values of the indicated parameters could be used to distinguish responses from rLinB-13-exposed vs. that from unexposed individuals. Data were represented as mean and standard deviation (C and D). *P < .05, ***P < .0001.

Figure 3. IgG response to rLinB-13 in *L. braziliensis* clinical spectrum. (A) Anti-rLinB-13 IgG response in TL patients (n = 91) and HS from non-endemic area (n = 30). (B) anti-rLinB-13 IgG in *L. braziliensis* clinical spectrum, [CL (n = 28), ML (n = 17), DL (n = 46), SC (n = 33)] and HS (n = 30) as controls. (C) ROC curve analysis of IgG levels to distinguish patients with DL from subjects with CL, ML and SC. (D) Detailed information obtained from each ROC curve is shown: Area Under Curve (AUC), P values of the ROC curves, the cut-off values chosen, and sensitivity and specificity with the 95% confidence

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interval (CI). Circles represent individual values; horizontal lines, median optical density (OD) values; dotted line in A and B, cutoff level. **P < 0.001, ***P < 0.0001.

Figure 4. Anti-rLinB-13 IgG is associated with disease severity in TL. (A) Anti-rLinB-13 IgG in CL patients with lesions greater or smaller than 117 mm^2. (B) Correlation between IgG to rLinB-13 and lesion size in CL patients. (C) Anti-rLinB-13 IgG between CL patients who did not respond to treatment versus cure patients (D) ROC curve analysis of IgG levels distinguishes CL patients who did not respond to treatment from cured patients (E) Anti-rLinB-13 IgG in DL patients with more than 30 lesions and less than 30 lesions. (F) correlation between IgG and number of lesions. (G) Anti-rLinB-13 IgG between DL patients who did not respond to treatment versus cured patients. (H) ROC curve analysis of IgG levels distinguishes DL patients who did not respond to treatment from cured patients. Circles represent individual values; horizontal lines, median optical density (OD) values. *P<.05, ***P<.0001.

Figure 5. Cytokine production in response to rLinB-13 in TL endemic area. PBMCs from CL patients (n = 10) (purple), DL patients (n = 11) (salmon) or *Lu. Intermedia* exposed individuals, without TL or history of disease (n= 11) (orange), were cultured in the presence or absence of rLinB-13 for 72 hours and cytokines were determined in culture supernatants by ELISA. Levels of IL-1α (A), IL-1β (B), IL-6 (C), and IL-10 (D). Circles represent individual values; horizontal lines, median values. *P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.0001.

Figure 6. rLinB-13 increases SLA-induced IL-10 levels in DL patients. PBMC from CL (n = 10) and DL (n = 11) patients were stimulated with SLA in the presence or absence of rLinB-13 for 72 hours, and cytokines were determined by ELISA. Levels of IL-10, TNF, IFN-γ, and IL-1β in CL (A) and DL patients (B). Circles represent individual values. **P < 0.001.

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Figure 1

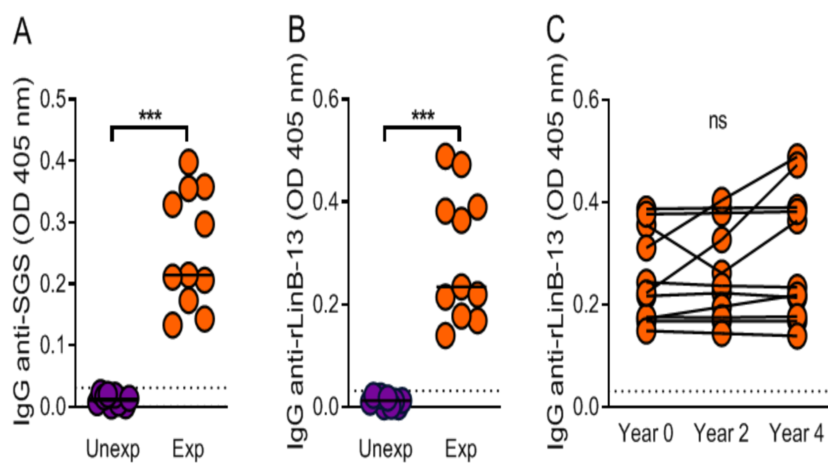

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Figure 2

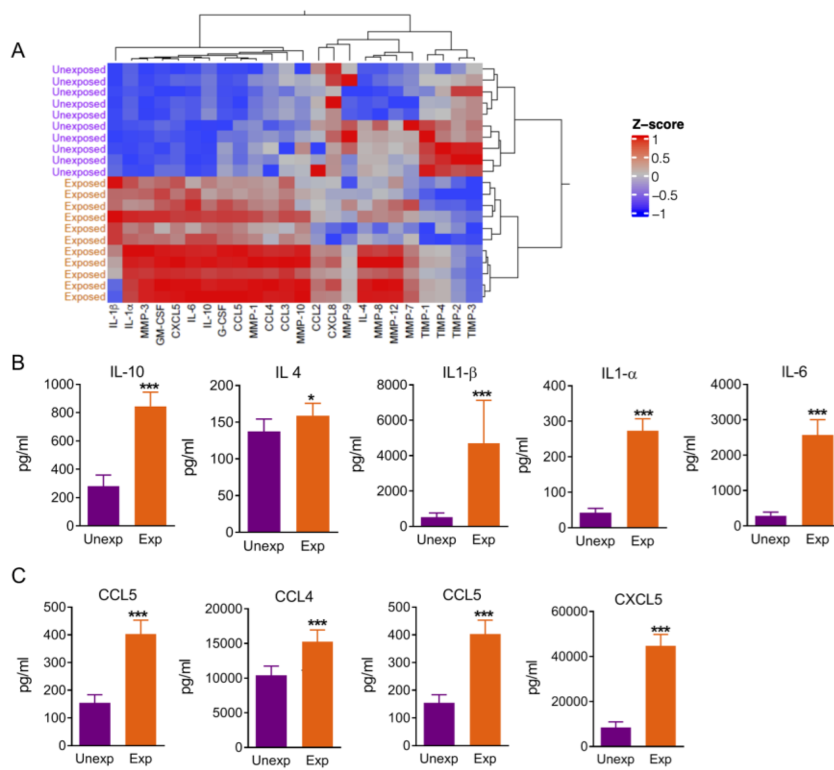

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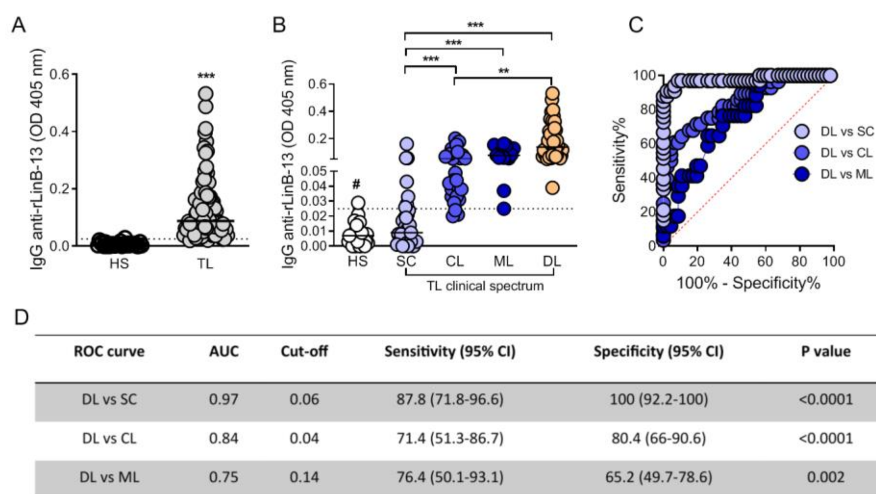
Figure 3



ROC curve	AUC	Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	P value
DL vs SC	0.97	0.06	87.8 (71.8-96.6)	100 (92.2-100)	<0.0001
DL vs CL	0.84	0.04	71.4 (51.3-86.7)	80.4 (66-90.6)	<0.0001
DL vs ML	0.75	0.14	76.4 (50.1-93.1)	65.2 (49.7-78.6)	0.002

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Figure 4

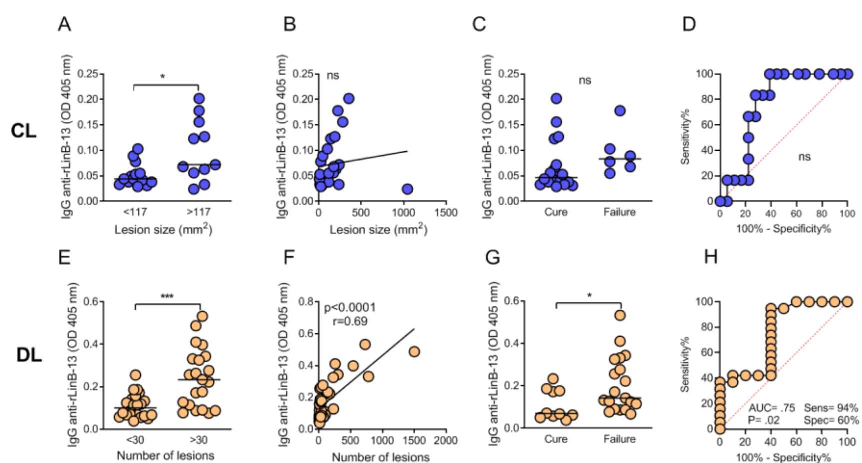

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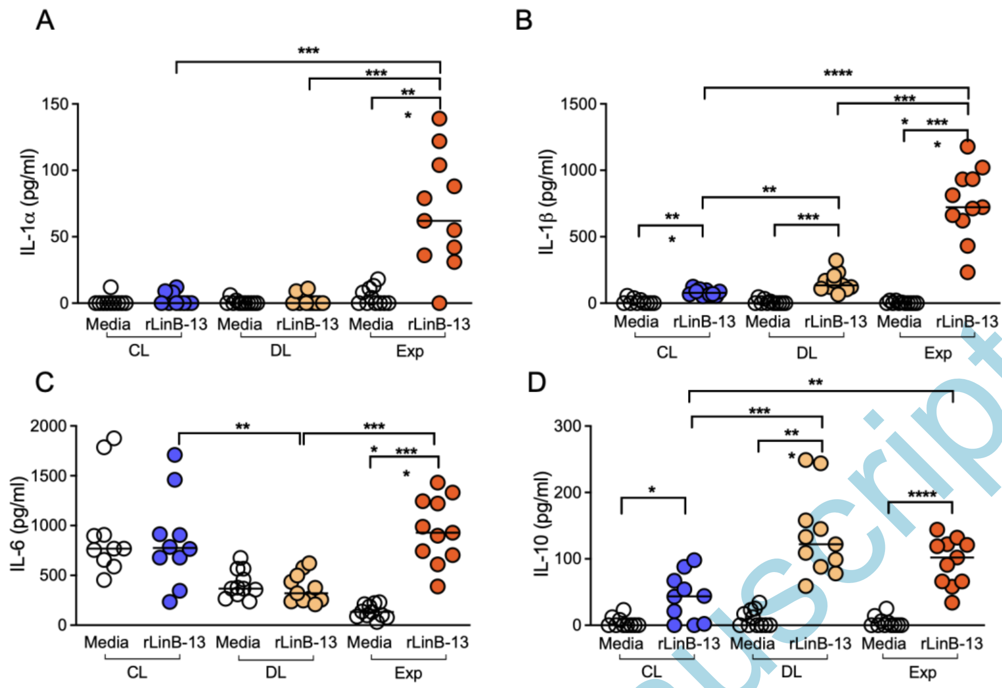
Figure 5



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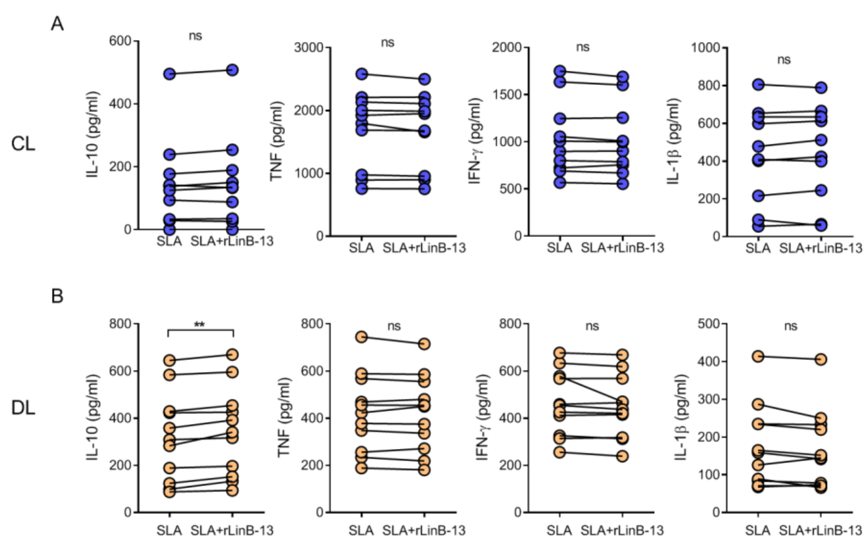

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