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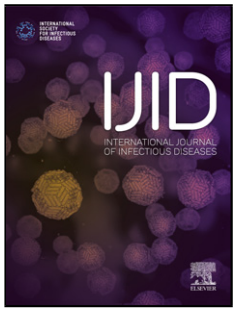

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Anti-Leishmania IgG is a marker of disseminated leishmaniasis caused by *Leishmania braziliensis*

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Running title: Anti-Leishmania antibodies in *L. braziliensis*.

Highlights

- Anti-leishmania IgG is marker of disseminated leishmaniasis
- Anti-leishmania IgG correlates with Th1 immune response in disseminated leishmaniasis
- Anti-leishmania IgG2 correlates with number of lesions in disseminated leishmaniasis

ABSTRACT

Background: In this study we determine the accuracy of anti-*Leishmania* IgG and IgG subclasses to distinguish clinical forms of American tegumentary leishmaniasis (ATL), and the relationship between antibodies levels with cytokine production and severity of ATL.

Methods: Participants were 40 cutaneous leishmaniasis (CL), 20 mucosal leishmaniasis (ML), 20 disseminated leishmaniasis (DL) patients and 20 subjects with subclinical *L. braziliensis* infection (SC). Diagnosis was performed by DNA of *L. braziliensis* or IFN-γ production in SC. IgG and subclasses of IgG to soluble *Leishmania* antigen, as well cytokine levels in supernatants of mononuclear cells were detected by ELISA.

Results: IgG were detected in 95%, 95% and 100% of CL, ML and DL patients, respectively. Higher levels of anti-*Leishmania* IgG and IgG2 were seen in DL compared to CL, ML and SC. ROC analysis confirmed the ability of IgG to distinguish DL from the other clinical forms. A direct correlation was observed between IgG titers and levels of IFN-γ and CXCL10 in CL and DL, and IgG2 antibodies correlated with the number of lesions in DL.

Conclusions: High Anti-*Leishmania* IgG and IgG2 levels are characteristic of DL, and while IgG was correlated with pro-inflammatory cytokines, IgG2 was direct correlated with number of lesions.

Keywords: Cutaneous leishmaniasis; Disseminated leishmaniasis; Leishmania braziliensis; IgG; IgG2; disease severity

Introduction

American Tegumentary leishmaniasis (ATL) are a group of neglected diseases caused by protozoa of *Leishmania* genus and continues to pose a health problem worldwide. In Brazil, ATL is mainly caused by *Leishmania (Viannia) braziliensis* and approximately 30.000 new cases are reported annually. *L. braziliensis* infection results in a spectrum of clinical forms including cutaneous leishmaniasis (CL), typically characterized by a single well-limited ulcer with raised borders (Romero et al., 2001); mucosal leishmaniasis (ML), that predominantly affects nasal mucosa and may damage facial structures causing a disfiguring disease (Lessa et al., 2012); and disseminated leishmaniasis (DL), which is an emerging severe form of ATL characterized by multiple acneiform, papular and ulcerated lesions usually affecting whole body (Turetz et al., 2002). Moreover, in areas where *L. braziliensis* is endemic, around 22% of subjects presents a positive delayed-type hypersensitivity reaction to soluble Leishmania antigen (SLA), usually known as Leishmania skin test (LST) and/or produce IFN-γ when their lymphocytes are stimulated with SLA but do not develop disease. These individuals are classified as having subclinical (SC) *L. braziliensis* infection (Muniz et al., 2016). The diagnosis of ATL is performed by identification of amastigotes or documentation of *L. braziliensis* DNA in tissues biopsied from the lesions (Bittencourt and Barral, 1991; Weirather et al., 2011). Nevertheless, in areas of *L. braziliensis* transmission, due to the high cost of molecular test, low number of parasites in lesions and lack of facilities, the LST is commonly used to identify patients with typical ATL lesions (Carvalho et al., 1985b). However, we recently found that CL patients with typical lesions may present negativity on LST (Carvalho et al., 2020) and the test is also negative in 50% of DL patients (Carvalho et al., 1994; Machado et al., 2015). While Enzyme-Linked

Immunosorbent Assays (ELISA) to SLA are commonly used to diagnosis of visceral leishmaniasis (VL), in ATL it is less used due to the lack specificity as cross-reactivity with other species of the Trypanosomatidae family is high (Roffi et al., 1980; Kalter, 1994; Chiaramonte et al., 1999; Daltro et al., 2019). Therapy for ATL in Latin American is performed mainly with meglumine antimoniate (Sb^v) and depending of the leishmania species and the region were the study is performed failure rate over 40% is observed in CL and ML, and in over 70% of DL patients (Prates et al., 2017; Machado et al., 2007; Machado et al., 2011).

Leishmania control relies on a Th1 immune response as IFN-γ is the main macrophage activating cytokine for leishmania killing. The Th1 response is important for macrophage activation and consequently *Leishmania* killing. However, as in *L. braziliensis* infection parasites may not be eradicated, there is a persistent stimulation of the immune response with overproduction of pro-inflammatory cytokines that cause tissue damage and ulcer development (Melby et al., 1994; Louzir et al., 1998, Bacellar et al., 2002, Carvalho et al., 2013). Actually, histopathologic analysis of the lesions are characterized by an inflammatory infiltrate and paucity of parasites (Saldanha et al., 2012). While the role of cellular response is massively studied, the contribution of humoral immunity in pathology or protection is not clear. In *L. infantum* infection antibody titers have been associated with high parasite burden and severity of *Leishmania* infection (Braz et al., 2002; Teixeira-Neto et al., 2010). In another disease also caused by an intracellular pathogen, the *Mycobacterium leprae*, antibodies levels are associated with lepromatous leprae, the disfiguring and severe form of the disease (Leturiondo et al., 2019). Increase in B cell and immunoglobulin transcripts are also observed in diffuse cutaneous leishmaniasis (DCL) a disease

in the New World associated with *L. amazonensis*, that is characterized by multiple lesions (Christensen et al., 2019).

Here, we evaluated humoral immune response to SLA in CL, ML, DL and SC. Our data indicate that higher levels of IgG and IgG2 antibodies are present in patients with DL, and that anti-*L. braziliensis* IgG2 is directly correlated with the number of lesions in DL patients.

Methods

Area of study and Case definition

This study was performed in Corte de Pedra, an endemic area of ATL with high *L. braziliensis* transmission, located in southeastern of Bahia state, Brazil. Participants were 40 patients with CL, 20 with ML, 20 with DL and 20 individuals with SC *L. braziliensis* infection, all of them living in the endemic area and 20 healthy subjects (HS) from a non-endemic region. This is a cross sectional study comparing total anti-*L. braziliensis* IgG and IgG subclasses in different clinical forms of *L. braziliensis* infection. CL patients had 1 to 3 typical cutaneous ulcers. ML have a previous story of CL and presence of ulcerated lesions in the mucosa and DL had more than 10 acneiforms, papular and ulcerated lesions present in at least to distinct parts of the body. The diagnosis confirmation was performed by detection of DNA for *L. braziliensis* in biopsied tissues obtained from the skin or mucosal lesions or by identification of amastigotes in the biopsied tissues. Subjects with SC *L. braziliensis* infection were household contacts of CL patients who deny previous history of CL but have a positive LST or production of IFN-γ in supernatants of whole blood cells stimulated with SLA (Muniz et al., 2016). ATL patients were treated with Sb^{v} 20 mg/kg/day used for 20 days for CL and ML, and

for 30 days for DL group. The study was approved by the ethics committee of the Federal University of Bahia Medical School and all patients signed an informed consent (2.114.874, /06/12/2017).

Antigen and Leishmania skin test

Soluble *Leishmania* antigen (SLA) was prepared as previously described (Reed et al., 1986). For the LST 25 µg in 0.1 ml of SLA was inoculated in the forearm and induration was determined 48 hours post inoculation. A positive LST was considered when the induration was equal or greater than 5 mm.

ELISA for antibody detection:

Anti-*Leishmania* serology was performed by ELISA as previously described by (Badaró et al., 1986) with some adaptations. For this, 96-well plates (NUNC Maxisorp) were coated with SLA (10 ug/mL) in carbonate buffer (0.45M $NaHCO_3$, 0.02M Na_2CO_3, pH 9.6) for 18 h at 4C. After four washes with PBS-0.5% Tween, the plates were blocked for 1 hour at 37C with PBS Tween 0.5% plus 10% Fetal bovine serum (FBS) (GIBCO). Sera were diluted 1:50 in PBS-Tween 0.5% plus 10% FBS and incubated for 1 hour at room temperature. After a round of washing, the wells were incubated for 1 hour at room temperature with anti- human IgG, IgG1, IgG2, IgG3, IgG4 conjugated to peroxidase (Sigma, Louis, MO) at a 1:10000, 1:4000, 1:15000, 1:4000, 1:10000 dilutions, respectively. Again, the plates were washed and incubated for 30 minutes with 100 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma). 50 µl of 4N H_2SO_4 were added to each well to stop the reaction. Plates were read at 450 nm. The ELISA cutoff value was established as the mean optical density (OD) value plus 2.5 standard deviations, using serum samples from healthy volunteers (n=20) from a

nonendemic area. The serological experiments were repeated twice yielding similar results.

Determination of Cytokines and Chemokines:

Peripheral blood mononuclear cells (PBMC) were isolated from heparin-treated venous blood by ficoll-hypaque gradient centrifugation and stimulated with SLA as previously described (Bacellar et al., 2002). Briefly, after washing three times in 0.9% NaCl, cells were re-suspended in RPMI 1640 culture medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 IU/ml of penicillin and 100 µg/ml of streptomycin. Cells were adjusted to $3x10^6$ cells/ml, put in 24-well plates, and stimulated with SLA (5 µg/ml). After incubation for 72 hours at 37°C and 5% CO^2, supernatants were collected and stored at –20º C. The levels of IFN-γ, CXCL-9 and CXCL-10 were measured by ELISA (BD Bioscience) sandwich method and the results were expressed as pg/ml.

Statistical analysis.

Receiver Operator Characteristics (ROC) curve analysis was used to evaluate the ability of IgG levels to distinguish ATL clinical forms. Comparisons between 2 groups were performed by Mann–Whitney test and those among 3 or more groups by Kruskal–Wallis test followed by Dunn multiple comparison tests. For correlation analysis between immunoglobulins with clinical data and cytokines and chemokines, the Spearman correlation test was used. Analyses were conducted using GraphPad Prism version 5.0 for Windows (GraphPad Software), and differences were considered significant at $P < .05$.

Results

Demographic and clinical features in TL patients and SC individuals

The demographic and clinical features of the different groups of subjects participating of the study are shown in Table 1. Patients with DL and ML were older than cases of CL and of subjects with SC *L. braziliensis* infection (P<.01). Overall, there was a predominance of male individuals in all groups (P>.05). The median number of lesions was higher in DL than CL (P<.001). The positivity in LST was lower in DL group than CL and ML patients (P<0.01). Figure 1 illustrates clinical presentations typical of ATL caused by *L. braziliensis*.

Humoral response in ATL patients and SC individuals

L. braziliensis infection is linked to several clinical forms of disease ranging from classical CL to DL, an emerging severe form. Overall, sera from DL patients presented a higher IgG reactivity to SLA. Positive serology was observed in 95%, 95%, and 100% of CL, ML and DL, respectively (Figure 2A). ROC analysis confirmed that the IgG levels distinguished DL patients from others clinical forms with high accuracy (Figure 2B-C). All ATL patients showed a higher anti-leishmania IgG levels than SC individuals or healthy controls (Figure 2A).

Next, we evaluated IgG subclasses in the same serum panel. With regards to IgG subclasses, IgG1 and IgG4 were the most predominant is subclass detected in ATL patients (Figure 3A-D). However, IgG2 was the better tool to discriminate DL patients from others clinical forms. Indeed, DL patients presented a significantly high IgG2 levels than CL individual (P<0.001). While only 10% and 30% of CL and ML patients respectively displayed positive IgG2 serology, 85% of DL patients showed IgG2 to SLA (Figure 3B). Moreover, only serum from DL and ML patients presented higher IgG2 levels than SC individuals (Figure 3B). ROC analysis showed that IgG2 can distinguishing DL from CL (AUC = 0.90, $p < 0.0001$) and DL from ML (AUC = 0.83, $p = 0.0004$) (Figure 4).

Humoral response correlates with pro-inflammatory cytokine production

PBMCs from ATL patients caused by *L. braziliensis* secrete high levels of pro-inflammatory cytokines, but this strong Th1 response is not sufficient to eliminate Leishmania infection. Indeed, strong inflammatory response is linked to immunopathology in ATL. Here IFN-γ, CXCL-9 and CXCL-10 levels were measured in supernatants of PBMC of 19 CL patients and of 14 DL patients. The median and interquartile of IFN-γ in CL was 2109pg/ml (402-2569) and in DL 374pg/ml (226-1006), P<.005. The CXCL9 was 25979pg/ml (6870-82040) and 22750pg/ml (988-63110), P>.05 and CXCL10 11170pg/ml (30-15710) and 11090pg/ml (1418-20673), P>.05 for CL and DL patients, respectively. We next evaluated whether humoral response correlates with Th1 cytokines, and chemokines (CXCL-9 and CXCL-10) in peripheral cells stimulated with SLA. Overall, we found a strong positive correlation between IgG levels and IFN-γ ($r = 0.75$, $p = 0.003$) in DL patients but not in CL group (R=0.21, P=0.3) (Figure 5A and C). There was also a direct correlation of IgG levels and CXCL-10 production in both CL (R=0.5, P=0.02) and DL (R=0.61, P=0.002) (Figure 5B and D). No correlation was observed among humoral response and TNF, IL-10 and CXCL-9 levels (data not shown). In addition, we did observe any correlations between IgG2 titers and levels of IFN-γ or CXCL-10 among CL and DL patients.

Correlation of humoral immune response with number of lesions in DL

The number of lesions in DL patients may range from 10 to up than 1000. It is known that the number of lesions in CL is associated with severe disease and failure to Sb^{v} therapy (Llanos-Cuentas et al., 2008). As the majority of reports of DL are series of cases, correlation between number of lesions and clinical outcome or immune response have not been established. Here we asked

whether humoral response correlates with clinical features in DL patients. We found a positive correlation between IgG2 levels and number of lesions in DL patients, but no correlation was observed among total IgG and number of lesions (Figure 6A and B).

Discussion

The diagnosis of ATL is based on the presence of typical lesions associated with identification of amastigotes or parasite DNA in biopsied skin or mucosal tissue. Anti-leishmania antibodies are produced in high levels and the sensitivity is high for diagnosis of ATL, but serology against SLA or recombinant proteins is not useful to differentiate clinical forms of ATL. While the pathogenesis of ATL have been strongly associated with an exaggerated Th1 immune response, there is no evidence that antibodies may participate in the immunopathology or control of leishmania infection. Here, we showed that serology to SLA for detection of anti-leishmania IgG as well IgG isotypes are associated with different clinical forms of ATL and may be a marker of severity of *L. braziliensis* infection as it was associated with ML and DL and with high number of lesions in DL patients. While in VL high production of antibodies is associated with a poor Th1 immune response, here we found that IgG levels were correlated CXCL10 levels in CL and DL and with IFN-γ in DL patients.

In the present study we compare anti-leishmania IgG and IgG isotypes in patients with different clinical forms of *L. braziliensis* infection. One important finding was the observation that IgG antibodies were higher in DL than in all others clinical forms of the disease and the ROC curve showed that anti-leishmania IgG is able to distinguish DL from ML and CL patients. High anti-

Leishmania IgG antibody titers were detected in CL, ML and DL patients but only 1 (5%) of the subjects with SC *L. braziliensis* infection had IgG anti-SLA detected. In *L. infantum* infection antibodies are used to identify both subjects with subclinical *L. infantum* infection and patients with VL (Badaró et al., 1986). In such cases antibody production is higher in VL than in SC *L. infantum* infected subjects and in canine VL there is a direct correlation between anti-leishmania antibodies and parasite load (Braz et al., 2002; Teixeira-Neto et al., 2010). In CL the parasite burden is low, and it is likely that in SC *L. braziliensis* infected subjects the low antibody production is due to low parasite burden. We have previously shown that subjects with SC *L. braziliensis* infection displayed a low Th1 immune response (Follador et al., 2002) and here we documented that anti-*Leishmania* antibodies are also not produced or produced in low levels in SC subjects.

It has been shown that IgG3 levels may be a predictor of cure or failure of therapy for CL (Fagundes-Silva et al., 2012). Here we showed that anti-leishmania IgG2 antibodies are more elevated in DL than in others clinical forms of *L. braziliensis* infection. While IgG1 and IgG3 antibodies followed the similar pattern of total IgG and were elevated in CL, ML, DL but not in SC infected subjects, anti-leishmania IgG4 antibodies, a subclass associated with a Th2 immune response was observed in high titers in all clinical forms and was also detected in subjects with SC infection. IgG4 is induced by IL-4 and IL-13 and both IgE and IgG4 associated with a Th2 immune response. We have previously shown that anti-leishmania IgE antibody is observed in CL due to *L. braziliensis*, a disease characterized by a strong Th1 immune response (Sousa-Atta et al., 2002). Our documentation that anti-leishmania IgG4 was detected in all clinical

forms of ATL indicates that both Th1 and Th2 immune response are present in *L. braziliensis* infection.

The role of anti-Leishmania antibodies in host parasite interaction and consequently in the pathogenesis of ATL has not been well studied. In VL, high production of antibodies is associated with progression of *L. infantum* infection to active VL and while high levels of antibody are produced in VL, PBMC of these patients failed to produce IFN-γ upon stimulation with SLA (Carvalho et al., 1985a). Furthermore, high anti-*Leishmania* IgG in human VL correlate with peak parasitemia, and with negative LST, and successful treatment resulted in decreased antibody titers and a restoration of LST (Miles et al., 2005). In such case, the poor Th1 response in vivo was associated to IgG ability to induce IL-10 production from macrophages (Miles et al., 2005). In addition to VL there are other intracellular infections as those caused *M. leprae* or *Paracoccidiodes braziliensis*, that severe disease is associated with antibody production and poor Th1 immune response (Leturiondo et al., 2019; Singer-Vermes et a., 1993) One of the first observation regarding the relationship between antibodies and impaired T cell response was the documentation that appearance of antibodies coincided with suppression of cell mediated immunity in animals immunized via the anterior champs of the eye (Kaplan and Streilein, 1977). Patients with diffuse cutaneous leishmaniasis (DCL), a disease characterized by multiple nodules lesions with macrophages full of amastigotes, have a weak anti-*Leishmania* Th1 immune response (Barral et al.,1995; Oskam et al., 1999). The human host transcription analysis of the cutaneous lesions in DCL showed up regulation of transcripts encoded B cell or immunoglobulin genes (Christensen et al., 2019) and there was a positive correlation between *Leishmania* transcripts in lesions

with the levels of host B cell transcripts encoding IgG (Gonçalves et al., 2020). However, in the present study we did not find that antibody titers were associated with a poor inflammatory response measured by detection for the pro-inflammatory cytokines IFN-γ, CXCL9 and CXCL10 in CL and DL patients. Actually, there was a strong positive correlation between IgG levels and IFN-γ in DL and also a direct correlation between IgG and CXCL10 in CL and DL.

There was no previous evidence that antibody titers in ATL are associated with severity of disease. However, it is known that the number of B cells in CL lesion enhances from the early phase of the disease when a papular lesion is present, to the chronic phase characterized by the appearance of the classical ulcers (Saldanha et al., 2017). Moreover, in skin lesions from DL patients the number of B cells is higher than $CD4^+$ T cell (Mendes et al., 2013). It is known that the number of parasites in DL ulcers is similar to the one observed in CL ulcers (Mendes et al., 2013). However, as DL patients may present 100 up to more than 1000 lesions, the parasite burden is higher in DL than in CL. Here we showed a direct correlation between IgG2 antibodies and number of lesions in DL patients. As there was no association between IgG2 antibody titers and illness duration, this suggests that IgG2 antibodies may be related to parasite burden and severity of the DL. A positive correlation between parasite load and anti-Leishmania IgG2 isotypes was documented in dogs with active VL caused by *L. infantum* (Teixeira-Neto et al., 2010). As the number of lesions in CL is associated with failure to therapy (Llanos-Cuentas et al., 2008; Suprien et al., 2020) and anti-leishmania IgG2 was associated with number of lesions, the high antibody titers in DL may be a marker of failure to therapy. Nevertheless, studies with large number of patients should be performed to better determine this association.

In the present study we showed that anti-Leishmania IgG and IgG2 antibodies are markers of DL, a disease characterized by multiple cutaneous lesions and high parasite burden. However, antibodies levels were not associated with a poor Th1 immune response as anti-leishmania IgG antibodies were direct correlated with IFN-γ and CXCL10 production. DL caused by *L. braziliensis* is an emerging ATL clinical form usually confounded with other diseases and linked to poor response to therapy. Thus, we encourage the use of IgG anti-*Leishmania* as differential diagnosis of DL, especially in endemic areas where access to molecular tests is scarce. Furthermore, early diagnosis of DL may be important for therapeutic management.

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Potential conflicts of interest.

The authors declare no competing financial interests. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

References

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, https://doi.org/10.1128/IAI.70.12.6734-6740.2002.

Badaró R, Reed SG, Barral A, Orge G, Jones TC. Evaluation of the micro enzyme-linked immunosorbent assay (ELISA) for antibodies in American visceral leishmaniasis: antigen selection for detection of infection-specific responses, https://doi.org/10.4269/ajtmh.1986.35.72.

Barral A, Costa JM, Bittencourt AL, Barral-Netto M, Carvalho EM. Polar and subpolar diffuse cutaneous leishmaniasis in Brazil: clinical and immunopathologic aspects. Int J Dermatol 1995;34(7):474-9, https://doi.org/10.1111/j.1365-4362.1995.tb00613.x.

Bittencourt AL, Barral A. Evaluation of the histopathological classifications of American cutaneous and mucocutaneous leishmaniasis. Mem Inst Oswaldo Cruz 1991;86(1):51-6, https://doi.org/10.1590/S0074-02761991000100009.

Braz RF, Nascimento ET, Martins DR, Wilson ME, Pearson RD, Reed SG, Jeronimo SM. The sensitivity and specificity of Leishmania chagasi recombinant K39 antigen in the diagnosis of American visceral leishmaniasis and in differentiating active from subclinical infection. Am J Trop Med Hyg 2002;67(4):344-8, https://doi.org/10.4269/ajtmh.2002.67.344.

Carvalho AM, Guimarães LH, Costa R, et al. Impaired Th1 response is associated therapeutic failure in patients with cutaneous leishmaniasis caused by Leishmania braziliensis. J Infect Dis 2020 ;jiaa374, https://doi.org/10.1093/infdis/jiaa374.

Carvalho AM, Magalhães A, Carvalho LP, Bacellar O, Scott P, Carvalho EM. Immunologic response and memory T cells in subjects cured of tegumentary leishmaniasis. BMC Infect Dis 2013;13:529, https://doi.org/10.1186/1471-2334-13-529.

[a]Carvalho EM, Badaró R, Reed SG, Jones TC, Johnson WD Jr. Absence of gamma interferon and interleukin 2 production during active visceral

leishmaniasis. J Clin Invest 1985;76(6):2066-9, https://doi.org/10.1172/JCI112209.

Carvalho EM, Barral A, Costa JM, Bittencourt A, Marsden P. Clinical and immunopathological aspects of disseminated cutaneous leishmaniasis. Acta Trop 1994;56(4):315-25, https://doi.org/10.1016/0001-706X(94)90103-1.

[b]Carvalho EM, Johnson WD, Barreto E, Marsden PD, Costa JL, Reed S, Rocha H. Cell mediated immunity in American cutaneous and mucosal leishmaniasis. J Immunol 1985;135(6):4144-8.

Chiaramonte MG, Frank FM, Furer GM, Taranto NJ, Margni RA, Malchiodi EL. Polymerase chain reaction reveals Trypanosoma cruzi infection suspected by serology in cutaneous and mucocutaneous leishmaniasis patients. Acta Trop 1999;72(3):295-308, https://doi.org/10.1016/S0001-706X(99)00005-4.

Christensen SM, Belew AT, El-Sayed NM, Tafuri WL, Silveira FT, Mosser DM. Host and parasite responses in human diffuse cutaneous leishmaniasis caused by L. amazonensis. PLoS Negl Trop Dis 2019;13(3):e0007152, https://doi.org/10.1371/journal.pntd.0007152.

Daltro RT, Leony LM, Freitas NEM, et al. Cross-Reactivity Using Chimeric Trypanosoma cruzi Antigens: Diagnostic Performance in Settings Where Chagas Disease and American Cutaneous or Visceral Leishmaniasis Are Coendemic. J Clin Microbiol 2019;57(8):e00762-19, https://doi.org/10.1128/JCM.00762-19.

Fagundes-Silva GA, Vieira-Goncalves R, Nepomuceno MP, et al. Decrease in anti-Leishmania IgG3 and IgG1 after cutaneous leishmaniasis lesion healing is correlated with the time of clinical cure. Parasite Immunol 2012;34(10):486-91, https://doi.org/10.1111/j.1365-3024.2012.01379.x.

Follador I, Araújo C, Bacellar O, Araújo CB, Carvalho LP, Almeida RP, Carvalho EM. Epidemiologic and immunologic findings for the subclinical form of Leishmania braziliensis infection. Clin Infect Dis 2002;34(11):E54-8, https://doi.org/10.1086/340261.

Goncalves R, Christensen SM, Mosser DM. Humoral immunity in leishmaniasis – Prevention or promotion of parasite growth? Cytokine 2020; 2(4):100046, https://doi.org/10.1016/j.cytox.2020.100046.

Kalter DC. Laboratory tests for the diagnosis and evaluation of leishmaniasis. Dermatol Clin 1994;12(1):37-50, https://doi.org/10.1016/S0733-8635(18)30200-6.

Kaplan HJ, Streilein JW. Immune response to immunization via the anterior chamber of the eye. I. F. lymphocyte-induced immune deviation. J Immunol 1977;118(3):809-14.

Lessa HA, Lessa MM, Guimarães LH, et al. A proposed new clinical staging system for patients with mucosal leishmaniasis. Trans R Soc Trop Med Hyg;106(6):376-81, https://doi.org/10.1016/j.trstmh.2012.03.007.

Leturiondo AL, Noronha AB, do Nascimento MOO, Ferreira CO, Rodrigues FDC, Moraes MO, Talhari C. Performance of serological tests PGL1 and NDO-LID in the diagnosis of leprosy in a reference Center in Brazil. BMC Infect Dis 2019;19(1):22, https://doi.org/10.1186/s12879-018-3653-0.

Llanos-Cuentas A, Tulliano G, Araujo-Castillo R, et al. Clinical and parasite species risk factors for pentavalent antimonial treatment failure in cutaneous leishmaniasis in Peru. Clin Infect Dis 2008;46(2):223-31, https://doi.org/10.1086/524042.

Louzir H, Melby PC, Ben Salah A, Marrakchi H, Aoun K, Ben Ismail R, Dellagi K. Immunologic determinants of disease evolution in localized cutaneous leishmaniasis due to Leishmania major. J Infect Dis 1998;177(6):1687-95, https://doi.org/10.1086/515297.

Machado PR, Ampuero J, Guimarães LH, et al. Reappraisal of the immunopathogenesis of disseminated leishmaniasis: in situ and systemic immune response. Trans R Soc Trop Med Hyg 2011;105(8):438-44, https://doi.org/10.1016/j.trstmh.2011.05.002.

Machado PR, Lessa H, Lessa M, Guimarães LH, Bang H, Ho JL, Carvalho EM. Oral pentoxifylline combined with pentavalent antimony: a randomized trial for

mucosal leishmaniasis. Clin Infect Dis 2007;44(6):788-93, https://doi.org/10.1086/511643.

Machado PR, Rosa ME, Guimarães LH, Prates FV, Queiroz A, Schriefer A, Carvalho EM. Treatment of Disseminated Leishmaniasis With Liposomal Amphotericin B, Clin Infect Dis 2015;61(6):945-9, https://doi.org/10.1093/cid/civ416.

Melby PC, Andrade-Narvaez FJ, Darnell BJ, Valencia-Pacheco G, Tryon VV, Palomo-Cetina A. Increased expression of proinflammatory cytokines in chronic lesions of human cutaneous leishmaniasis. Infect Immun 1994;62(3):837-42, doi: 10.1128/IAI.62.3.837-842.1994.

Mendes DS, Dantas ML, Gomes JM, et al. Inflammation in disseminated lesions: an analysis of CD4+, CD20+, CD68+, CD31+ and vW+ cells in non-ulcerated lesions of disseminated leishmaniasis. Mem Inst Oswaldo Cruz 2013;108(1):18-22, https://doi.org/10.1590/S0074-02762013000100003.

Miles SA, Conrad SM, Alves RG, Jeronimo SM, Mosser DM. A role for IgG immune complexes during infection with the intracellular pathogen Leishmania. J Exp Med 2005;201(5):747-54, https://doi.org/10.1084/jem.20041470.

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, https://doi.org/10.1093/infdis/jiw196.

Oskam L, Nieuwenhuijs JL, Hailu A. Evaluation of the direct agglutination test (DAT) using freeze-dried antigen for the detection of anti-Leishmania antibodies in stored sera from various patient groups in Ethiopia. Trans R Soc Trop Med Hyg 1999;93(3):275-7, https://doi.org/10.1016/S0035-9203(99)90021-4.

Prates FV, Dourado ME, Silva SC, et al. Fluconazole in the Treatment of Cutaneous Leishmaniasis Caused by Leishmania braziliensis: A Randomized Controlled Trial. Clin Infect Dis 2017;64(1):67-71, https://doi.org/10.1093/cid/ciw662.

Reed SG, Badaró R, Masur H, et al. Selection of a skin test antigen for American visceral leishmaniasis. Am J Trop Med Hyg 1986;35(1):79-85, https://doi.org/10.4269/ajtmh.1986.35.79.

Roffi J, Dedet JP, Desjeux P, Garré MT. Detection of circulating antibodies in cutaneous leishmaniasis by enzyme-linked immunosorbent assay (ELISA). Am J Trop Med Hyg 1980;29(2):183-9, https://doi.org/10.4269/ajtmh.1980.29.183.

Romero GA, De Farias Guerra MV, Paes MG, de Oliveira Macêdo V. Comparison of cutaneous leishmaniasis due to Leishmania (Viannia) braziliensis and L. (V.) guyanensis in Brazil: clinical findings and diagnostic approach. Clin Infect Dis 2001;32(9):1304-12, https://doi.org/10.1086/319990.

Saldanha MG, Queiroz A, Machado PRL, de Carvalho LP, Scott P, de Carvalho Filho EM, Arruda S. Characterization of the Histopathologic Features in Patients in the Early and Late Phases of Cutaneous Leishmaniasis. Am J Trop Med Hyg 2017;96(3):645-652, https://doi.org/10.4269/ajtmh.16-0539.

Singer-Vermes LM, Caldeira CB, Burger E, Calich LG. Experimental murine paracoccidioidomycosis: relationship among the dissemination of the infection, humoral and cellular immune responses. Clin Exp Immunol ;94(1):75-9, https://doi.org/10.1111/j.1365-2249.1993.tb05980.x.

Sousa-Atta ML, Salamé GS, D'Oliveira A Jr, Almeida RP, Atta AM, Carvalho EM. Immunoglobulin E antileishmanial antibody response in cutaneous leishmaniasis. Clin Diagn Lab Immunol 2002;9(1):101-4, https://doi.org/10.1128/CDLI.9.1.101-104.2002.

Suprien C, Rocha PN, Teixeira M. Clinical Presentation and Response to Therapy in Children with Cutaneous Leishmaniasis. Am J Trop Med Hyg 2020;102(4):777-781, https://doi.org/10.4269/ajtmh.19-0531.

Teixeira Neto RG, Giunchetti RC, Carneiro CM, et al. Relationship of Leishmania-specific IgG levels and IgG avidity with parasite density and clinical signs in canine leishmaniasis. Vet Parasitol 2010;169(3-4):248-57, https://doi.org/10.1016/j.vetpar.2010.01.023.

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, https://doi.org/10.1086/345772.

Weirather JL, Jeronimo SMB, Gautam S, et al. Serial Quantitative PCR Assay for Detection, Species Discrimination, and Quantification of Leishmania spp. in Human Samples. J Clin Microbiol 2011;49(11): 3892–3904, https://doi.org/10.1128/JCM.r00764-11.

Table 1. Demographic and clinical aspects from patients with American tegumentary leishmaniasis.

	Cutaneous leishmaniasis (n=40)	Mucosal leishmaniasis (n=20)	Disseminated leishmaniasis (n=20)	Subclinical infection (n=20)	P value
Age, y median (range)	25 (15-57)	51 (5-88)	49 (19-65)	24 (9-56)	P<.05[a]
Male gender, No. (%)	29 (75)	13 (65)	16 (80)	11 (55)	P>.05[b]
Illness duration, d median (range)	32 (15-90)	-	37 (15-120)	-	P>.05[c]
Leishmania skin test, No. (%)	40 (100%)	20 (100%)	13 (65%)	20 (100%)	P<.001[b]
Lesion size, mm^2 median (range)	131 (11-1759)	-	188 (7-3140)	-	P>.05[c]
Number of lesions (range)	1 (1-4)	-	53.5 (14-800)	-	P<.001[c]
Cure on day 90, No. (%)	27 (67.5)	11 (55)	10 (50)	-	P>.05[b]

[a]Kruskal Wallis test

[b]Chi-square test

[c]Mann-Whitney test

Figure 1. Clinical presentation of ATL caused by *L. braziliensis*. A, 31-year-old male with CL presenting ulcerated lesion measuring 29X26mm on the right leg at 30 days of illness. B and C, 65-year-old male patient with ML, previous history of cutaneous ulcer on the right leg for 8 months and nasal obstruction and mouth pain for 6 months. Large ulcer on the soft palate (B) and nasal septum perforation (C). D and E, 30-year-old male patient with DL presenting >100 mixed lesions (acneiform, crusted papules, superficial nodules and few ulcerations) on the face, trunk and limbs.

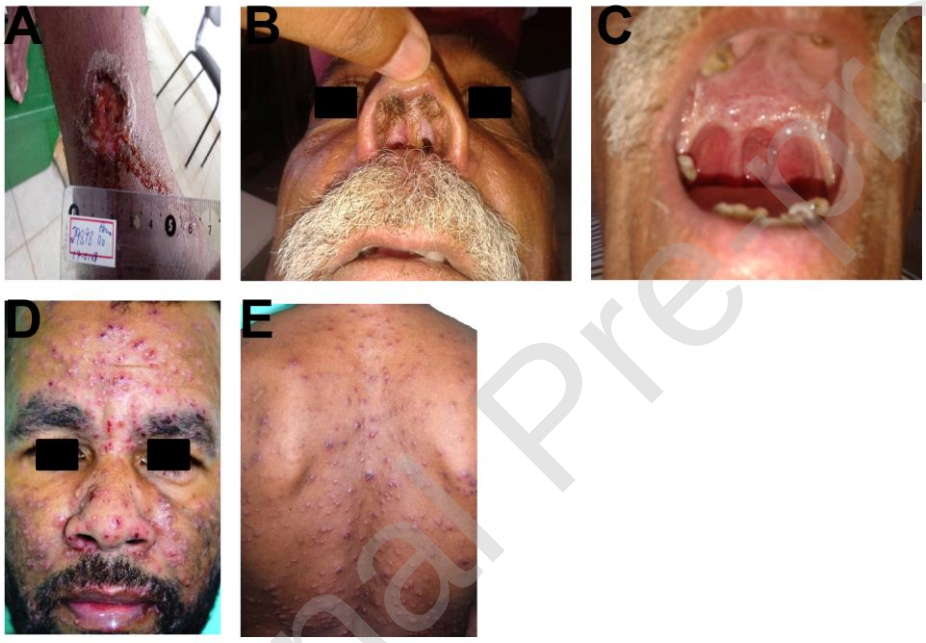
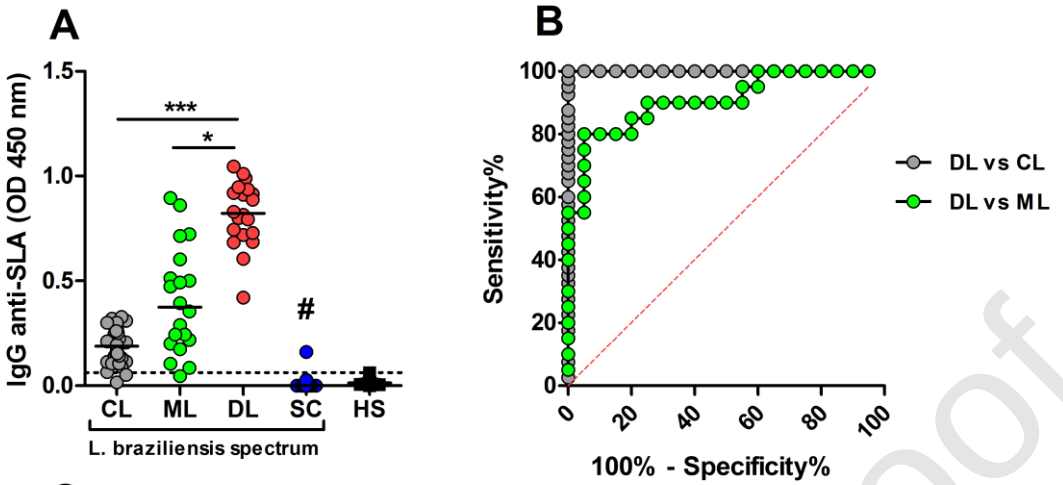


Figure 2. IgG anti-SLA in *L. braziliensis* clinical spectrum. A, Total immunoglobulin (Ig) G response in *L. braziliensis* clinical spectrum (CL (n=40); ML (n=20); DL (n=20); and SC (n=20), 20 HS were used as controls. B, ROC curve analysis of IgG levels to distinguish DL patients from CL and ML subjects. C, 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 interval (CI). Circles represent

individual values; horizontal lines, median optical density (OD) values; dotted line in A, cutoff level. *P < .05, ***P < .0001.



C

ROC curve	AUC	Cut-off	Sens (95% CI)	Spec (95% CI)	P value
DL vs CL	1	0.37	100 (91.1-100)	100 (83.1-100)	<0.0001
DL vs ML	.90	0.60	80 (56.3-94.2)	95 (75.1-99.8)	<0.0001

Figure 3. IgG subclasses anti-SLA in *L. braziliensis* clinical spectrum. IgG subclasses response in *L. braziliensis* clinical spectrum (CL (n=40); ML (n=20); DL (n=20); and SC (n=20), 20 HS were used as controls. A, IgG1. B, IgG2. C, IgG3. D, IgG4. Circles represent individual values; horizontal lines, median optical density (OD) values; dotted line, cutoff level. *P < .05; **P < .01, ***P < .0001.

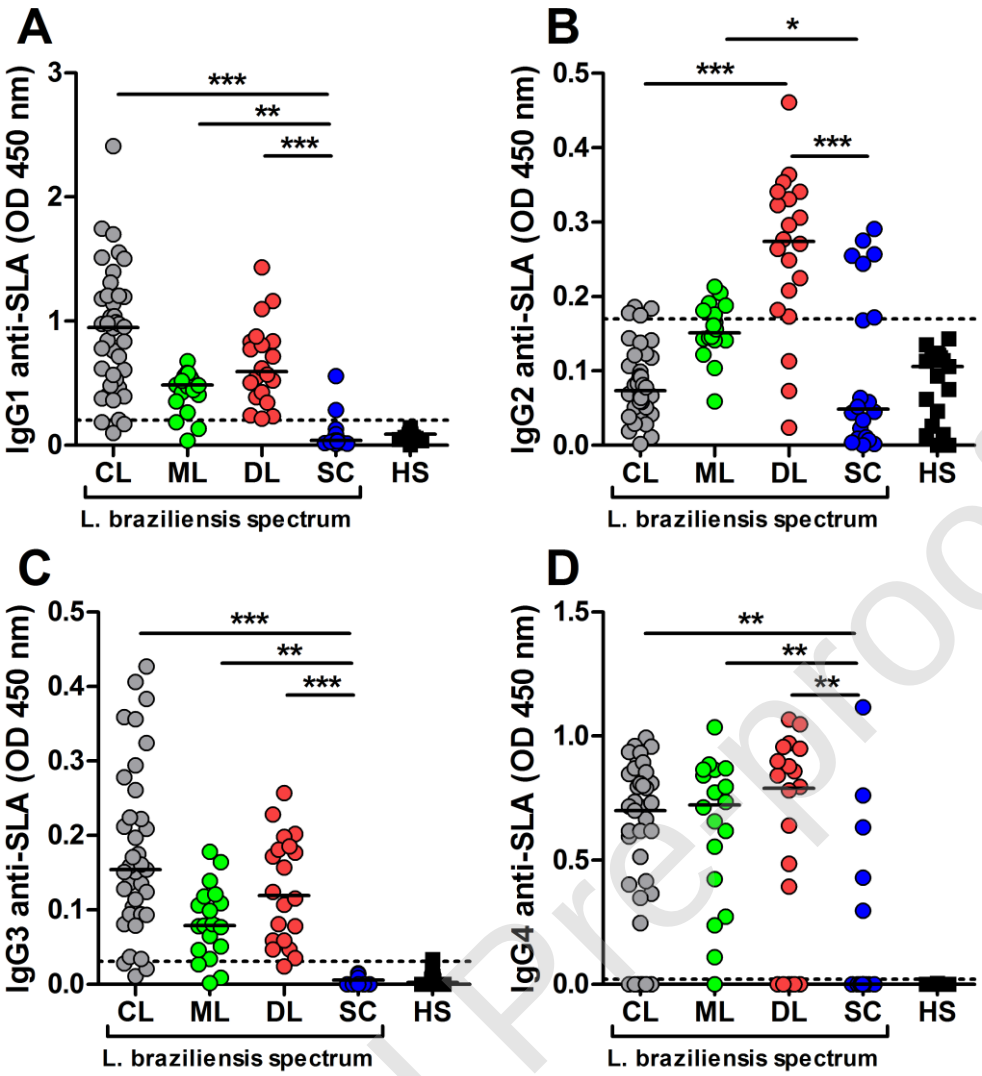
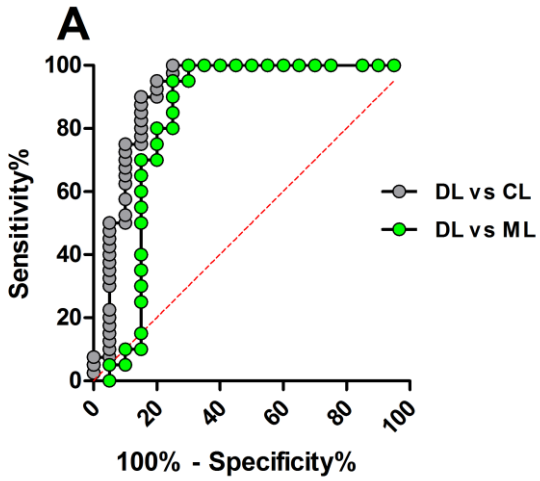


Figure 4. ROC curve analysis of IgG2 levels to distinguish DL patients from CL and ML subjects. A, ROC curves were built using IgG2 levels from *L. braziliensis* clinical spectrum (CL (n=40); ML (n=20); and DL (n=20). B, Detailed information obtained from each ROC curve is shown: Area Under Curve (AUC), P values of the ROC curves, the cut-off values chosen, and sensitivity and specificity with the 95% confidence interval (CI).



B

ROC curve	AUC	Cut-off	Sens (95% CI)	Spec (95% CI)	P value
DL vs CL	0.90	0.15	90 (76.3-97.2)	85 (62.1-96.7)	<0.0001
DL vs ML	0.83	0.20	95 (75.1-99.8)	75 (50.9-91.3)	0.0003

Figure 5. Correlation between humoral response and IFN-γ and CXCL-10 production in patients with cutaneous leishmaniasis and disseminated leishmaniasis. A, Correlation between total immunoglobulin (Ig) G response and IFN-γ in CL patients (n = 24). B, correlation between IgG response and CXCL-10 IFN-γ in CL patients (n = 21). C, Correlation between anti-leishmania IgG antibodies and IFN-γ levels in DL (n = 14). D, Correlation between leishmania IgG antibodies and CXCL-10 levels in DL (n = 14). Spearman correlation was used in the analysis.

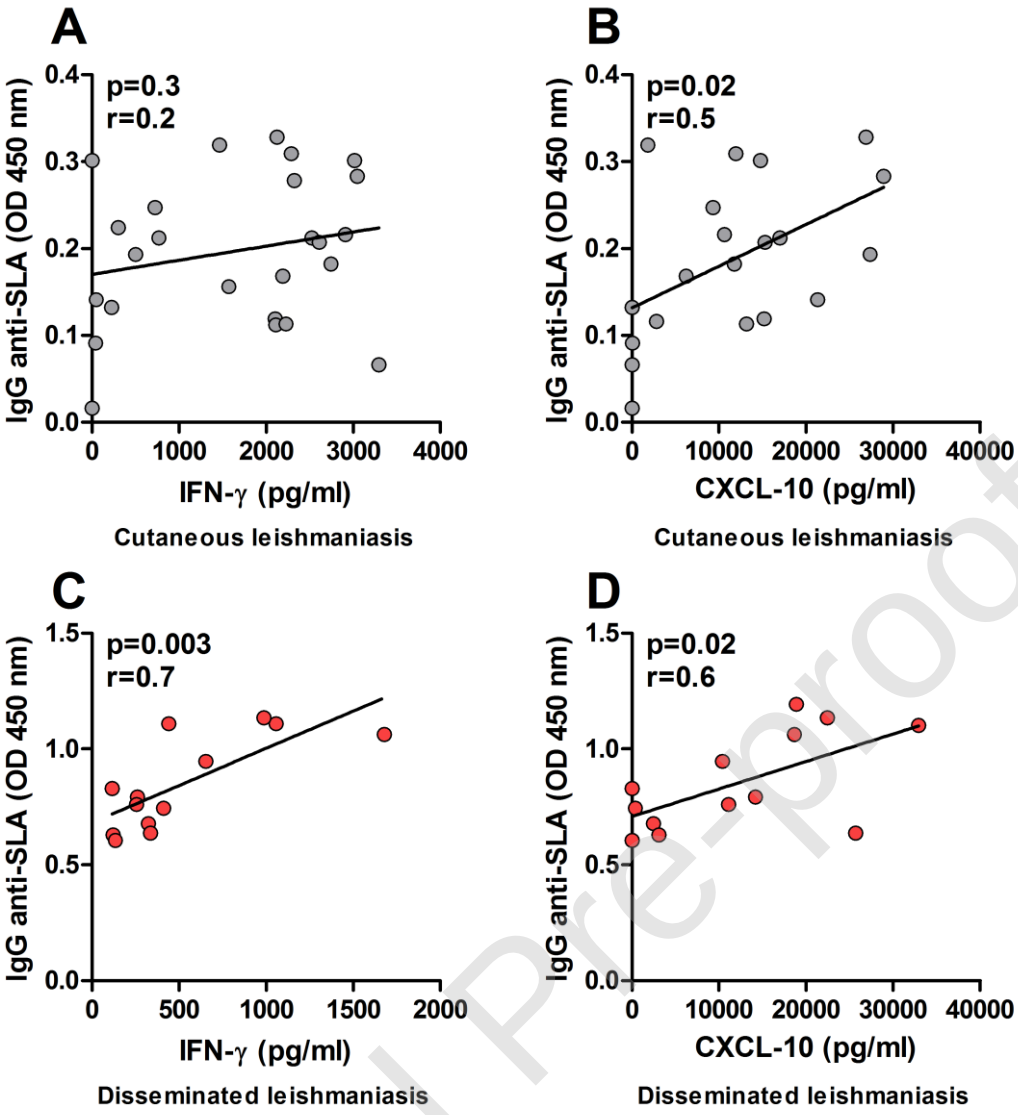


Figure 6. Correlation between humoral response and number of lesions in DL patients. A, Correlation between total immunoglobulin (Ig) G response to SLA and number of lesions in DL patients. B, correlation between IgG2 response and number of lesions in DL patients. Spearman correlation was used in the analysis.