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# Comparative Evaluation of Lesion Development, Tissue Damage, and Cytokine Expression in Golden Hamsters (*Mesocricetus auratus*) Infected by Inocula with Different *Leishmania (Viannia) braziliensis* Concentrations

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The golden hamster (*Mesocricetus auratus*) is a susceptible model to *Leishmania (Viannia)* spp.; however, available studies employ different infection protocols, which account for clinical and pathological presentation differences. Herein, *L. (V.) braziliensis* preparations were standardized to contain 10<sup>4</sup>, 10<sup>5</sup>, or 10<sup>6</sup> parasites to determine an optimal inoculum that ensured cutaneous lesions without causing a disseminated infection in hamsters. Lesion development was followed for 105 days by size measurements, and skin, draining lymph node, spleen, and sera were investigated to check parasite load, spleen visceralization, cytokine expression, histopathological changes, and anti-*Leishmania* IgG levels. The lesion emergence time was inversely proportional to the parasite concentration in the inocula. Animals infected by 10<sup>4</sup> parasites presented nodular lesions, while those infected with 10<sup>6</sup> parasites often exhibited ulcerated lesions. The differences in the final lesion sizes were observed between 10<sup>4</sup> and 10<sup>5</sup> inocula or 10<sup>4</sup> and 10<sup>6</sup> inocula. High *IFNG* expression, anti-*Leishmania* IgG levels, and parasite load occurred independently of the inoculum used. A mild inflammatory skin involvement was observed in animals infected with 10<sup>4</sup> parasites, while extensive tissue damage and parasite spleen visceralization occurred with 10<sup>5</sup> and 10<sup>6</sup> parasites. These results indicate that inocula with different concentrations of parasites generate differences in the time of lesion emergence, clinical presentation, and systemic commitment, despite high and similar *IFNG* expression and parasite load. This suggests that a modulation in the immune response to different parasite numbers occurs in an early phase of the infection, which could dictate the establishment and magnitude of the chronic phase of the disease.

Leishmaniasis has several characteristics that are responsible for the different clinical forms observed over the course of an infection in humans. An important factor is the diversity of the species that cause disease, which includes clonal differences within the same species that lead to clinical variants (1–3). Another determinant is the absolute parasite numbers that infect the host, which can influence the infection outcome in combination with the immunological and genetic characteristics of the host (4, 5).

Parasites from the *Viannia* subgenus, *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) guyanensis*, are the most widespread species in the Americas that cause cutaneous leishmaniasis. Most of the knowledge regarding the immunopathogenesis of *L. braziliensis* infection comes from studies performed in human patients and asymptomatic individuals (2, 6, 7). Despite the impact of American tegumentary leishmaniasis (ATL), few experimental studies have been developed for *L. braziliensis* infections (8, 9). This can be attributable mostly to the resistance of common laboratory mice strains to infection by these *Leishmania* species (10, 11). BALB/c mice have been widely used to study Old World cutaneous leishmaniasis, but long-term lesions do not develop when they are infected with *L. braziliensis* (8, 12). The lack of an adequate experimental model to reproduce the human *L. braziliensis* infection is a limiting factor for the development of biological and pharmacological approaches to address ATL.

Golden hamsters have proven to be an excellent model for cutaneous leishmaniasis given their high susceptibility to the *Vi-*

*annia* species and the ability to reproduce many of the clinical and histopathological characteristics of human cutaneous leishmaniasis (13–15). Considering that hamsters present an outbred genetic background, it is expected that individual characteristics have an important role in different clinical outcomes of the disease, in such a way that they may reproduce immune responses observed in the human disease. Despite these advantages, few studies have involved *L. braziliensis* infection in the hamster model, and the protocols vary among them in terms of isolate and inoculum size (13, 16, 17). However, even when an infection is established with the same parasite numbers and *L. braziliensis* strain, the lesion development is variable. Moreover, although high inocula such as 10<sup>6</sup> parasites warrant lesion development, they also lead to visceralization, an occurrence that is not observed

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in human ATL (15). It is known that the biological characteristics of the parasites used in infections, such as the passage number *in vitro*, growth phase, developmental stage, or the metacyclic form index, can impact the clinical course of the experimental infections with *Leishmania* (18, 19). In both mice and hamsters, another factor that influenced lesion onset and size was the absolute parasite numbers in the inoculum (16, 20).

In the present study, we standardized conditions for the generation of inocula with different parasite numbers in order to investigate the parasite concentration that more closely reproduces the cutaneous leishmaniasis observed in human and the immunopathological aspects associated with these infections in the hamster model. We had hypothesized that different parasite numbers in the inoculum would induce different clinical presentations and tissue damage degrees and also lead to spleen visceralization differences. For this study, hamsters were infected with  $10^4$ ,  $10^5$ , and  $10^6$  *L. braziliensis* parasites in a well-defined inoculum condition and were evaluated by clinical and immunopathological alterations.

We showed that in the chronic phase, the animals that were infected with a lower parasite inoculum ( $10^4$ ) developed a disease phenotype that produced smaller lesions and less histopathological damage, although there was no difference in terms of tissue parasite load, IgG levels, or gamma interferon (IFN- $\gamma$ ) and interleukin 10 (IL-10) gene expression in comparison with that in animals infected with the  $10^5$  or  $10^6$  parasite inoculum.

## MATERIALS AND METHODS

**Animals and ethics statements.** Adult female outbred golden hamsters (*Mesocricetus auratus*) (6 to 10 weeks old), weighing 80 to 90 g, obtained from the animal facilities of the Fundação Oswaldo Cruz, were used. Sixty infected animals were separated into three groups in four independent experiments ( $n = 5$  animals per group) according to the inoculum size, and 10 uninfected animals were used as the control. This study was approved by the Ethics Committee on Animal Use (CEUA) of Fundação Oswaldo Cruz—FIOCRUZ, with protocol number LW 11/11.

**Parasites for infection and immunological studies.** *Leishmania* (*Viannia*) *braziliensis* (MCAN/BR/98/R619) was maintained in Schneider's *Drosophila* medium (Sigma Chemical Co., USA) supplemented with 20% fetal bovine serum (Life Technologies, Brazil), L-glutamine (1 mM; Life Technologies, Brazil), and antibiotics (200 U/ml penicillin and 200  $\mu$ g/ml streptomycin; Sigma Chemical Co., USA). Parasites in the stationary growth phase from the third *in vitro* passage were washed in sterile phosphate-buffered saline (PBS) and counted on a hemocytometer. Inocula were prepared in a total volume of 30  $\mu$ l of PBS containing  $1 \times 10^4$ ,  $1 \times 10^5$ , or  $1 \times 10^6$  parasites for intradermal inoculation into the dorsal hind paw of hamsters. Standardization of the inoculum included using the same parasite frozen stock to prepare cultures, culturing for three passages, determination of the percentage of metacyclic forms, determined by the complement lysis test as previous described (19), and measuring the pH of the culture medium by using a pH indicator strip (Merck, Darmstadt, Germany). The percentage of metacyclic forms ranged from 44% to 51.6%, and the pH ranged from 5.5 to 6.5. For immunological studies, *Leishmania* (*Viannia*) *braziliensis* (MHOM/BR/75/2903) parasites from stationary growth phase (Lb-Ag) were prepared as previous described (21) and kept at  $-20^\circ\text{C}$  in a final concentration of 1 mg/ml.

**Clinical evaluation of *Leishmania* (*Viannia*) *braziliensis* infection.** The clinical evolution of lesions was monitored weekly from day 10 up to 105 days postinfection, by measuring the paw dorsal-ventral thickness with a digital thickness gauge (Mitutoyo America Corporation, São Paulo, Brazil) and expressing the data in millimeters. The lesion size was determined by the difference between the thickness of the infected and the noninfected paws of the same animal. Discrepancies in lesion sizes

were determined by the variance coefficient ( $VC = [\text{standard deviation}/\text{mean}] \times 100$ ). Clinical aspects of the lesions were qualified as nodular lesions, nodular and ulcerated lesions, or ulcerated ones. The macroscopic aspect of paws was also registered by digital photographs.

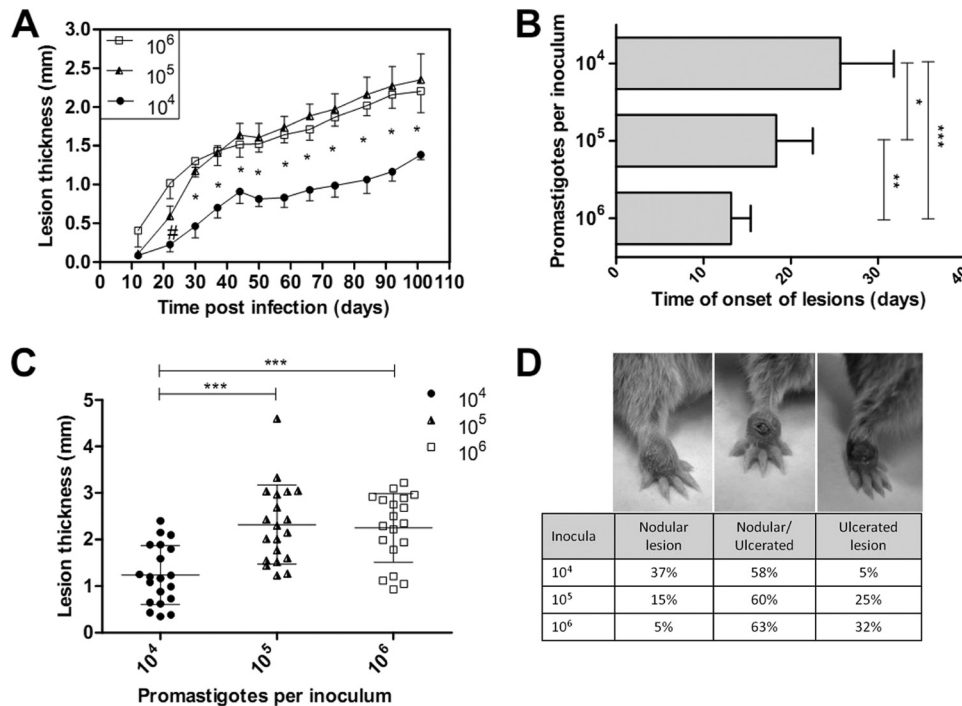
**Quantification of parasite load from infected skin and popliteal lymph node.** Parasite loads from fragments of infected skin and adjacent lymph nodes were determined by a limiting dilution assay (LDA) as previously described (22). Briefly, fragments were placed onto a stainless steel screen containing 1 ml of Schneider's medium (Sigma, USA). The tissue was macerated with a pistol. Twenty microliters of the cell suspension was diluted into 180  $\mu$ l of supplemented Schneider's medium in a Nunclon Delta 96-well microwell plate (Thermo Scientific, Denmark) in quadruplicate. From the first well, serial dilutions were made (dilution factor of 1:10). The plates were then incubated at  $26^\circ\text{C}$  and evaluated weekly for the presence of parasites over a period of 30 days. Results were obtained from the average from the last four wells where viable parasites were observed divided by the weight of fragments and then expressed as the number of parasites per gram of tissue.

**Quantification of anti-*Leishmania* antibodies.** The levels of anti-*Leishmania* IgG were determined in plasma samples through an enzyme-linked immunosorbent assay (ELISA) as previously described (21) with some adaptations. Briefly, *L. braziliensis* parasite (MHOM/BR/1975/M2903) soluble antigen (40  $\mu$ g/ml of PBS) was added (50  $\mu$ l/well) to a polystyrene flat-bottom microtiter plate (Nunc-Immuno plate; Roskilde, Denmark) and incubated overnight in a humidified chamber. After plates were washed, 50  $\mu$ l of plasma samples (diluted 1:5,000) from infected and uninfected (negative controls,  $n = 5$ ) animals was added in duplicate, and the plate was incubated at room temperature. Horseradish peroxidase-labeled goat anti-hamster IgG (1:5,000) was used as a detector system (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The results were expressed as ELISA index (EI), obtained by the mean of sample absorbance divided by the mean of negative-control absorbance. The cutoff was determined by the receiver operator characteristic curve (ROC) method, based on the best relationship between sensibility and specificity.

**Macroscopic analysis of spleens.** In order to evaluate the enlargement of spleens as a result of the presence of nodules in the parenchyma, which represents visceralization of *Leishmania*, spleens were submitted to a macroscopic inspection and weighed on a precision balance.

**Skin and spleen histopathological analysis.** Fragments from the skin of the infected paws and spleens were fixed in 10% buffered formalin and processed for paraffin embedding. Sections of 4- $\mu$ m thickness were stained with hematoxylin and eosin and then observed by light microscopy (Nikon Eclipse E600 microscope; Tokyo, Japan). Images were captured by a CoolSNAP-ProcCF camera and displayed by ImagePro Plus 4.5.1.29 (Media Cybernetics, Maryland, USA). Results from the skin histopathological analysis were expressed by score criteria as previously described (23), based on a semiquantitative analysis that evaluated the intensity of each histopathological feature: granuloma extension, presence of vacuolated macrophages, *Leishmania* amastigotes, Schaumann bodies, and necrosis. The scoring ranged from no observation (score = 0) to slight (score = 1), moderate (score = 2), or intense (score = 3) observation. Final results were given as means from the four independent experiments per inoculum, each one representing the sum of individual animals' scores. Dissemination of parasites from the inoculation site to spleens was confirmed by visualization of *Leishmania* or histopathological alterations (presence of granuloma) through histopathological analysis of spleens.

**Tissue cytokine mRNA expression by real-time RT-PCR.** The samples (skin of paws and popliteal lymph nodes) were collected in RNAlater (Ambion, Life Technologies, Carlsbad, CA, USA) and frozen at  $-20^\circ\text{C}$  until use. Total RNA was extracted from 20 to 30 mg of tissue using the RNeasy kit (Qiagen, Austin, TX, USA). All RNA samples were treated with RQ1 RNase-free DNase (Promega Corporation, Madison, WI, USA), quantified using the Pico 200 microliter spectrophotometer (Picodrop Ltd., Saffron Walden, United Kingdom), and kept at  $-80^\circ\text{C}$  until they were ready to be used. RNA (0.8  $\mu$ g) was reverse transcribed using the



**FIG 1** Evaluation of the golden hamster skin lesions that were infected with *Leishmania (Viannia) braziliensis* at three different inoculum concentrations ( $10^4$ ,  $10^5$ , and  $10^6$  parasites). The lesion sizes were measured by evaluating the dorsal-ventral thickness differences between the infected and uninfected paws. These data are expressed in millimeters. (A) Skin lesion development kinetics through the 105 days following the infections. The graph represents four independent experiments (median and standard error;  $n = 59$  animals). (B) The onset of clinical skin lesions for each inoculum concentration, given in days postinfection (mean  $\pm$  SD); (C) final skin lesion measurements 105 days after infection (mean  $\pm$  SD;  $n = 59$  animals;  $P < 0.0001$ ); (D) final lesion clinical aspect images 105 days after infection. Left, a nodular lesion; middle, a nodular and an ulcerated lesion; right, an ulcerated lesion. #, a significant difference was observed only between the  $10^4$  and  $10^6$  parasite-infected groups ( $P < 0.05$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA, USA). For real-time PCR assays of each target gene, 2  $\mu$ l cDNA was used to a final reaction volume of 15  $\mu$ l, in duplicate, using 7.5  $\mu$ l of TaqMan universal PCR mastermix (Applied Biosystems, USA) in ABI Prism 7500 Fast real-time PCR equipment (Applied Biosystems, USA). The sequences and concentrations of primers and probes for the hamster target genes as well as reverse transcription-quantitative PCR (RT-qPCR) cycling conditions used in this study were previously described (24). Results were calculated by relative quantification using the comparative threshold cycle method ( $\Delta\Delta C_T$ ), as previously described (25), having as the endogenous control the expression of the GAPDH golden hamster constitutive gene (5' to 3', forward, GTGGAGCCAAGAGGGTCATC; reverse, GGTTACACCCATCACAACAT; probe, 5'-FAM-TCTCCGCACCTTCTGCTGATGCC-3'-TAMRA (GenBank accession no. DQ403055.1), where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine. Subsequently, we calculated the  $\Delta\Delta C_T$ -based on the calibrator, represented by uninfected animals. Final results were expressed by  $2^{-\Delta\Delta C_T}$ , representing the number of times that there was a change in cytokine gene expression in relation to the calibrator (fold change).

**Statistical analysis.** The results were expressed as the means  $\pm$  standard deviations and medians with interquartile ranges. Statistical tests were performed with GraphPad Prism software version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Comparison between experimental groups was performed using one-way analysis of variance (ANOVA). A parametric or nonparametric test was selected according to the distribution of the raw data, followed by a posttest analysis for multiple groups as appropriate. Correlation analysis was performed based on Spearman's or Pearson's rank correlation test, according to data distribution. Significant differences were considered when  $P$  values were  $< 0.05$ .

**RESULTS**

**The *Leishmania (Viannia) braziliensis* parasite concentration in the inoculum influenced the skin lesion clinical course.** The lesion development kinetics over 105 days revealed a chronic and progressive course of evolution. None of the infected animals evolved with a spontaneous resolution of their lesions. The lesion sizes in the animals infected with  $10^4$  parasites were significantly smaller than those in animals inoculated with  $10^5$  and  $10^6$  parasites in all measurements that were conducted over the entire clinical observation period ( $P < 0.05$ ). These two last groups showed similar lesion sizes at most points of the kinetic curve (Fig. 1A). The parasite inoculum concentration directly influenced the lesion time onset with an inverse relationship (Fig. 1B). Clinical detection of lesions was observed later in the animals infected with  $10^4$  parasites (mean of  $25.7 \pm 6.1$  days postinfection) than in the animals infected with  $10^5$  (mean =  $18.3 \pm 4.2$  days postinfection) or  $10^6$  (mean =  $13.2 \pm 2.2$  days postinfection) parasites. Significant differences were observed between the  $10^4$  and  $10^5$  groups ( $P < 0.05$ ), between the  $10^5$  and  $10^6$  groups ( $P < 0.01$ ), and between the  $10^4$  and  $10^6$  groups ( $P < 0.0001$ ). Additionally, the mean lesion size at the endpoint was significantly lower in the animals infected with  $10^4$  parasites ( $1.24 \pm 0.63$  mm; median = 1.18 mm;  $n = 20$  animals;  $P < 0.0001$ ) than in those infected with  $10^5$  parasites ( $2.32 \pm 0.85$  mm; median = 2.22 mm;  $n = 20$  animals) or  $10^6$  parasites ( $2.25 \pm 0.74$  mm; median = 2.35 mm;  $n = 19$  animals) (Fig. 1C).



The clinical aspects of the lesions from the animals infected with  $10^4$  parasites differed from those in animals infected with  $10^5$  and  $10^6$  parasites. The animals infected with  $10^4$  parasites exhibited a higher percentage of nodular lesions than the latter groups, whereas  $10^5$  and  $10^6$  parasite-infected animals exhibited more nodular and ulcerated lesions, which was considered a more destructive outcome (Fig. 1D). Notably, all the animals inoculated with either  $10^5$  or  $10^6$  parasites presented clinical lesions, and only one animal that was infected with  $10^4$  parasites (5%) did not present any visible signs of a skin lesion.

The variability pattern for the lesion sizes was determined by a variance coefficient that was calculated between each experiment for a given group. From the four experiments in which the animals were infected with  $10^4$  parasites, the lesion sizes presented a heterogeneous pattern (VC > 30%). This pattern was observed in two experiments that used  $10^5$  and  $10^6$  parasite inocula, while the other two experiments in each group showed an intermediary pattern (VC between 15% and 30%). None of the experimental comparisons displayed a homogenous pattern (VC < 15%) for either of the inoculum groups.

**Skin lesions and lymph nodes presented high parasite loads that were independent of the inoculum concentration of *Leishmania (Viannia) braziliensis*.** Although there was an observed trend for increased skin and popliteal lymph node parasite loads according to inocula, it was not statistically significant. The animals infected with  $10^4$  parasites presented with skin lesions that had a median of  $1 \times 10^5$  parasites/g, whereas the animals infected with  $10^5$  parasites had  $3.6 \times 10^5$  parasites/g, and in those infected with  $10^6$  parasites, the observed median was  $11.0 \times 10^5$  parasites/g (Table 1, Fig. 2A). The adjacent lymph nodes also did not present a significant difference between the median values of the three groups:  $10^4 = 7.4 \times 10^5$  parasites/g,  $10^5 = 2.9 \times 10^5$  parasites/g, and  $10^6 = 2.6 \times 10^5$  parasites/g (Table 1, Fig. 2B). The skin parasite load, however, was positively correlated with the lesion size ( $r = 0.57, P < 0.0001$ ). Of note in Fig. 2C, a region is shown that is delineated by dotted lines in which 58.7% of the animals were infected with either of the three inocula presented and had lesions with a measured size between 1.2 and 2.6 mm (interquartile range, 25% to 75%), which was independent of the inoculum and final parasite load.

**Different *Leishmania (Viannia) braziliensis* numbers in inoculum produced similar anti-*Leishmania* species IgG levels.** All of the animal groups presented high anti-*Leishmania* IgG levels that were independent of different parasite inoculum concentrations, with no significant statistical difference between them. The animals that developed small lesions or did not develop lesions had low IgG levels or produced no IgG, respectively. A weak but positive correlation was observed between lesion sizes and anti-*Leishmania* IgG levels ( $r = 0.31, P = 0.02$ ; data not shown). The average antibody levels (ELISA index) for the  $10^4, 10^5$ , and  $10^6$  parasite-infected groups were  $20.1 \pm 10.8$  (median = 19.4),  $25.2 \pm 12.1$  (median = 22.7), and  $26.6 \pm 11.2$  (median = 27.6), respectively (Fig. 2D).

**The spleen weights varied with the highest parasite inoculum concentration and were correlated with lesion severity.** As spleen enlargement is a common finding observed in *L. braziliensis*-infected hamsters (15), which show the presence of nodules in the parenchyma of the organ, we evaluated spleen weights at the endpoint of infection. The animals infected with  $10^4$  parasites did

TABLE 1 Summary of the clinical, parasitological, and immunological feature numerical data of the golden hamsters at 105 days postinfection with different *Leishmania (Viannia) braziliensis* parasite numbers<sup>a</sup>

Control or parasite no.	Fold change									
	Skin lesion parasite load (no. of parasites/g of tissue)	Lymph node parasite load (no. of parasites/g of tissue)	Spleen wt (g)	Skin IFN- $\gamma$ expression	Lymph node IFN- $\gamma$ expression	Skin IL-10 expression	Lymph node IL-10 expression	% spleen visceralization of <i>Leishmania</i>		
Control			0.240 (0.204–0.265)	1.0 (0.1–3.1)	1.5 (0.9–1.6)	1.0 (0.2–2.7)	0.9 (0.7–2.3)			
$10^4$	$10^5$ (0.08 $\times 10^5$ – $7.7 \times 10^5$ )	$7.4 \times 10^5$ (0.003 $\times 10^5$ – $161 \times 10^5$ )	0.289 (0.257–0.361)	229 (137.8–1,158)	39.3 (28.6–55.9)	7.3 (1.9–14)	1.2 (0.7–2.5)	16 (4/25)		
$10^5$	$3.6 \times 10^5$ (1.8 $\times 10^5$ – $10^6$ )	$2.9 \times 10^5$ (0.028 $\times 10^5$ – $19 \times 10^5$ )	0.326 (0.273–0.697)	723.6 (357.4–1,502)	30.2 (20.6–45)	3.7 (2.4–8.8)	2.6 (0.57–5.5)	23.3 (7/30)		
$10^6$	$11.0 \times 10^5$ (0.2 $\times 10^5$ – $30 \times 10^5$ )	$2.6 \times 10^5$ (0.27 $\times 10^5$ – $24 \times 10^5$ )	0.380 (0.270–0.547)	454.8 (247–975)	41.1 (24.1–46.9)	5.3 (1.6–12.8)	3.2 (2.0–8.2)	69.2 (18/26)		

<sup>a</sup> Data are median (interquartile ranges) and percentage (no. of affected animals/no. of total animals analyzed) values.

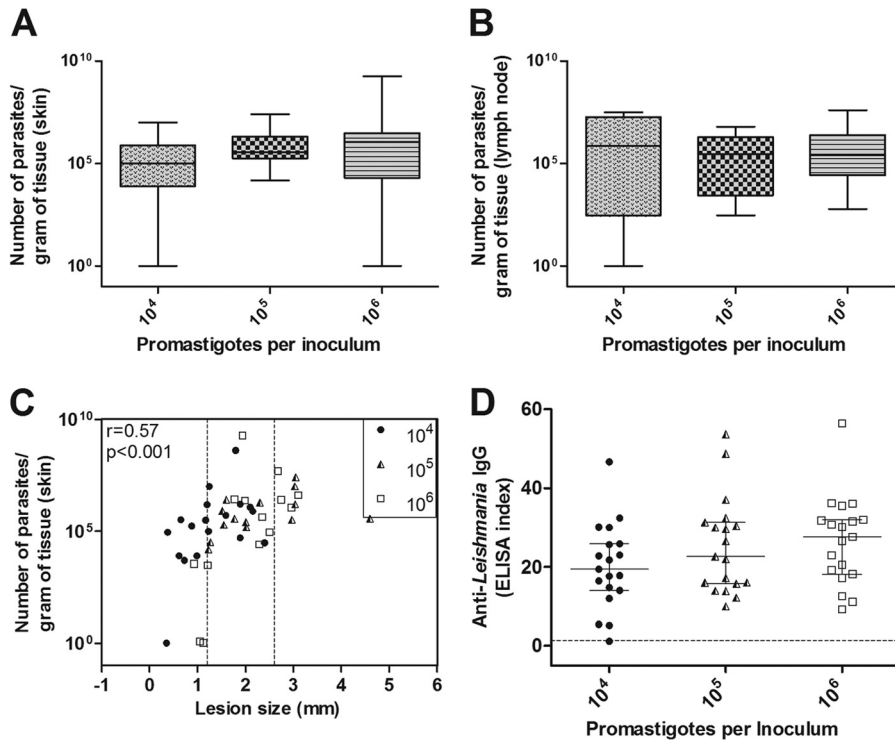


FIG 2 The parasite load and anti-*Leishmania* immunoglobulin levels in the golden hamsters that were infected with different *Leishmania* (*Viannia*) *braziliensis* concentrations ( $10^4$ ,  $10^5$ , and  $10^6$  parasites) after 105 days of infection. The skin parasite (A) and lymph node parasite (B) loads are shown. (C) The correlation between skin parasite loads and lesion sizes (the medians and interquartile ranges are shown). The dotted lines represent intersection regions in which the lesions that were generated by the three different inocula measured between 1.2 and 2.6 mm (58.7% of the total animals). (D) Anti-*Leishmania* IgG serum levels. The dotted line represents the cutoff value. Each point represents one animal, and the error bars represent the medians and interquartile ranges ( $r$  = correlation coefficient;  $p$  = significance level).

not exhibit significant spleen enlargement (median of 0.289 g) in comparison with that of uninfected animals (median of 0.240 g). The spleen weights from both  $10^5$  (median of 0.326 g) and  $10^6$  (median of 0.380 g) parasite-infected animals were higher than those of the controls animals ( $P < 0.05$ ). Notably, seven out of 19 animals in both groups presented spleen weights equal to or larger than 0.500 g. A positive correlation between spleen weight and lesion size was observed ( $r = 0.57$ ,  $P < 0.0001$ ) (Table 1, Fig. 3A and B).

**Infections with different *L. (V.) braziliensis* inoculum concentrations were characterized by high IFN- $\gamma$  expression in skin and lymph node.** High IFN- $\gamma$  expression was observed in the skin of the *L. braziliensis*-infected animals compared with that of the control group ( $P < 0.0001$ ), independent of the inoculum concentrations, and no significant difference was observed between the three infected groups (Table 1, Fig. 4A). The adjacent lymph nodes also presented high IFN- $\gamma$  expression levels compared with those of the uninfected control group ( $P < 0.01$  with the  $10^5$  group

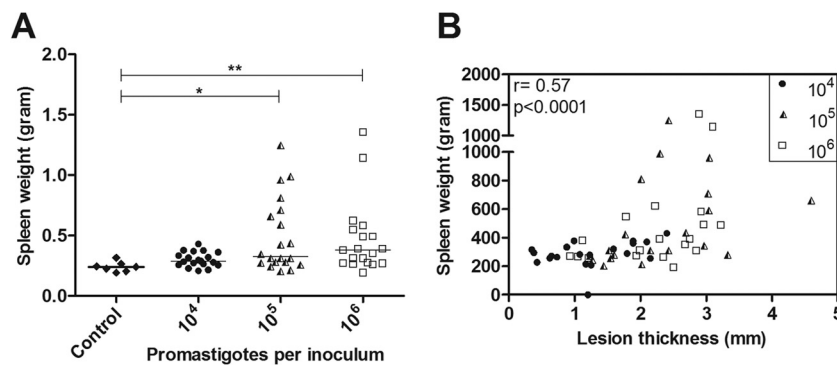
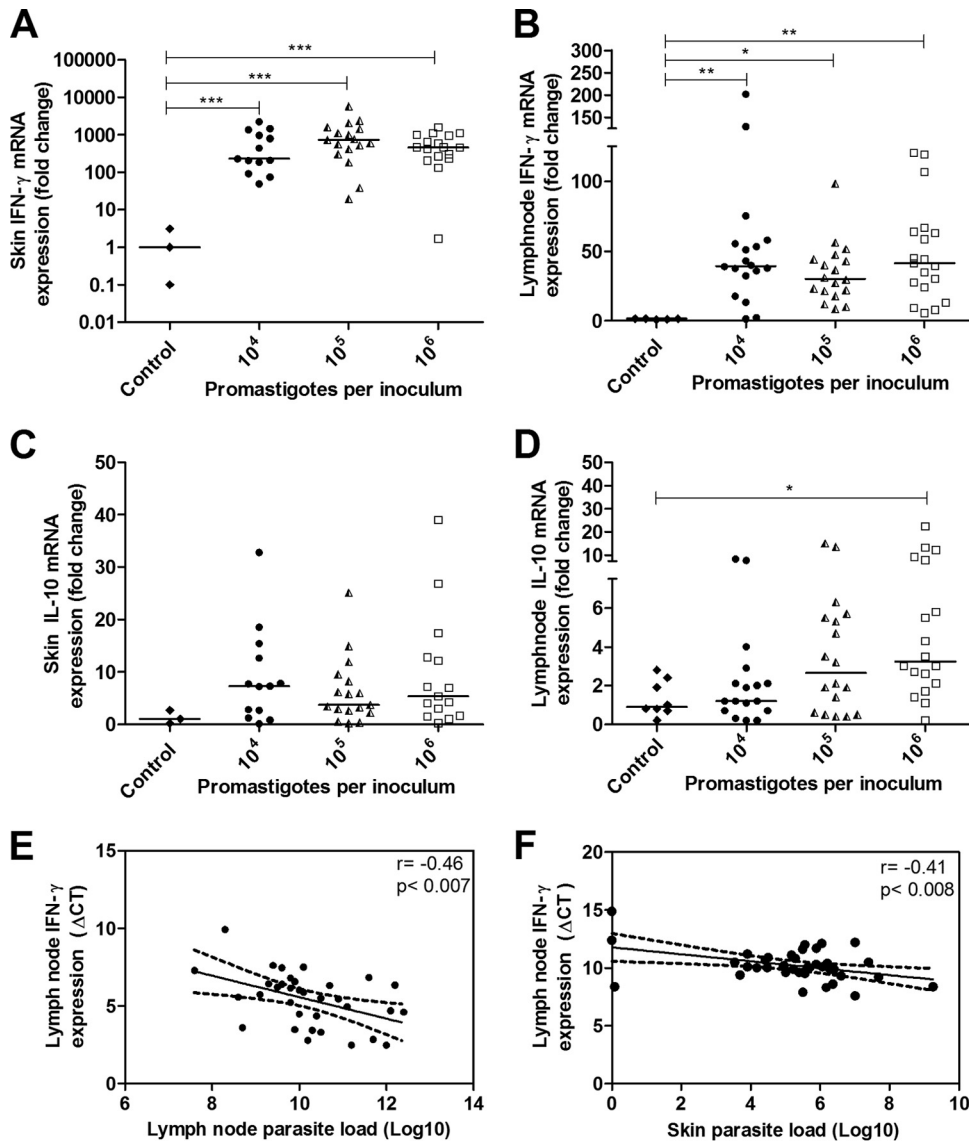


FIG 3 The spleen weights in grams from the golden hamsters that were infected with *Leishmania* (*Viannia*) *braziliensis* at three different inoculum concentrations ( $10^4$ ,  $10^5$ , and  $10^6$  parasites) after 105 days of infection. (A) The spleen weights in the control and in the three inoculum groups. The horizontal bars represent the medians. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . (B) Skin lesion sizes positively correlated with spleen weight (Spearman correlation). Each point represents one animal ( $r$  = correlation coefficient;  $p$  = significance level; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).



**FIG 4** Cytokine mRNA expression as determined by RT-qPCR in the organs of golden hamsters infected with different *Leishmania (Viannia) braziliensis* parasite concentrations ( $10^4$ ,  $10^5$ ,  $10^6$  parasites) at 105 days postinfection. The control group represents the uninfected animals. The results are expressed as relative fold changes between the experimental samples and the skin or lymph nodes of a control animal to which the value 1 was arbitrarily assigned. IFN- $\gamma$  mRNA expression in skin lesions (A) and lymph nodes (B) and IL-10 mRNA expression in skin lesions (C) and lymph nodes (D) are shown. Pearson's correlations between the parasite load and IFN- $\gamma$  expression in the lymph nodes (E) and the parasite load in skin and the IFN- $\gamma$  expression in the lymph node (F) are also displayed. Each point represents one animal, and the error bars represent the medians and interquartile ranges. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

and  $P < 0.0001$  with the  $10^4$  and  $10^6$  groups) (Table 1, Fig. 4B); however, there were no significant differences between the groups. IFN- $\gamma$  expression in the lymph nodes was at least 10 times lower than the IFN- $\gamma$  expression in the skin. The expression of IL-10 in the skin of animals infected with  $10^4$ ,  $10^5$ , and  $10^6$  parasites was similar to that in the uninfected control group (Table 1, Fig. 4C). The lymph nodes from the  $10^6$  parasite-infected group had a 3.5-fold increase in IL-10 levels compared with those of the control group ( $P < 0.05$ ) (Table 1, Fig. 4D). A negative correlation was observed between the lymph node IFN- $\gamma$   $\Delta C_T$  and the lymph node parasite load ( $r = -0.46$ ,  $P < 0.007$ ) (Fig. 4E) and skin parasite load ( $r = -0.41$ ,  $P < 0.008$ ) (Fig. 4F), which indicated that as

parasite loads in the lymph nodes and skin increased, IFN- $\gamma$  expression in lymph nodes also increased.

**A high parasite inoculum concentration contributed to a high degree of tissue damage and was associated with spleen visceralization of dermatotropic *Leishmania*.** The pathological parameters of the *Leishmania* infections and tissue damage were semiquantitatively evaluated and ranked with scores as shown in Fig. 5A. Significant differences were observed between the groups infected with  $10^4$  and  $10^5$  parasites ( $P < 0.05$ ) and between the groups infected with  $10^4$  and  $10^6$  parasites ( $P < 0.05$ ) (Fig. 5A). A positive correlation was observed between lesion sizes and histopathological scores, in which a higher degree of

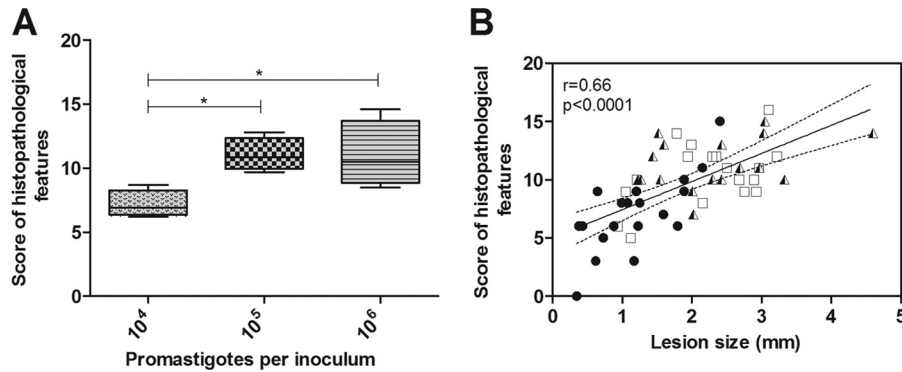


FIG 5 Skin lesion histopathological analyses of golden hamsters infected with different *Leishmania (Viannia) braziliensis* parasite concentrations ( $10^4$ ,  $10^5$ ,  $10^6$  parasites) at 105 days postinfection. (A) Histopathological features measured by scores in the animal groups infected with  $10^4$ ,  $10^5$ , and  $10^6$  parasites (medians and interquartile ranges) are shown. (B) A correlation between the skin histopathological feature scores and the lesion sizes of the animals infected with  $10^4$ ,  $10^5$ , and  $10^6$  parasites. Each point represents one animal. The correlation graph shows a fit line with a confidence curve.  $r$  = correlation coefficient;  $p$  = significance level; \*,  $P < 0.05$ .

tissue damage was associated with a greater lesion size (Fig. 5B). Additionally, an association between the skin parasite load and histopathological features was also observed ( $r = 0.47$ ,  $P < 0.008$ ) (data not shown).

All infected animals displayed a similar histopathological pattern, which was represented by a granulomatous reaction that was surrounded and/or interspaced by neutrophils, eosinophils, lymphocytes, plasma cells, and the occasional presence of small foci of fibrinoid necroses. The *Leishmania* intensity was scarce in the majority of the animals. The main difference, at the histopathological level, between the three different inocula was the degree of tissue damage, which was represented by an extension of the granulomatous inflammatory infiltrates and necroses. This was usually proportionally more important in the higher inocula. Parameters such as the granuloma extension (Fig. 6A and B), vacuolated macrophage intensity (Fig. 6C), presence of amastigotes (Fig. 6C), and the presence of Schaumann bodies (Fig. 6D) were considered pathological alterations due to the *Leishmania* infections.

The histopathological analysis of the spleens of the highest inoculum-infected animals showed, compared with the lower inoculum (see Fig. S1 in the supplemental material), enlarged organs with nodules in the parenchyma (inset in Fig. 6E) and extensive areas with granulomas (Fig. 6E) that sometimes had vacuolated macrophages with intracytoplasmic *Leishmania* (Fig. 6F arrow). *Leishmania* visceralization was observed in 16% of the  $10^4$  (4/25), in 23.3% of the  $10^5$  (7/30), and in 69.2% of the  $10^6$  (18/26) parasite-infected animals (Table 1).

## DISCUSSION

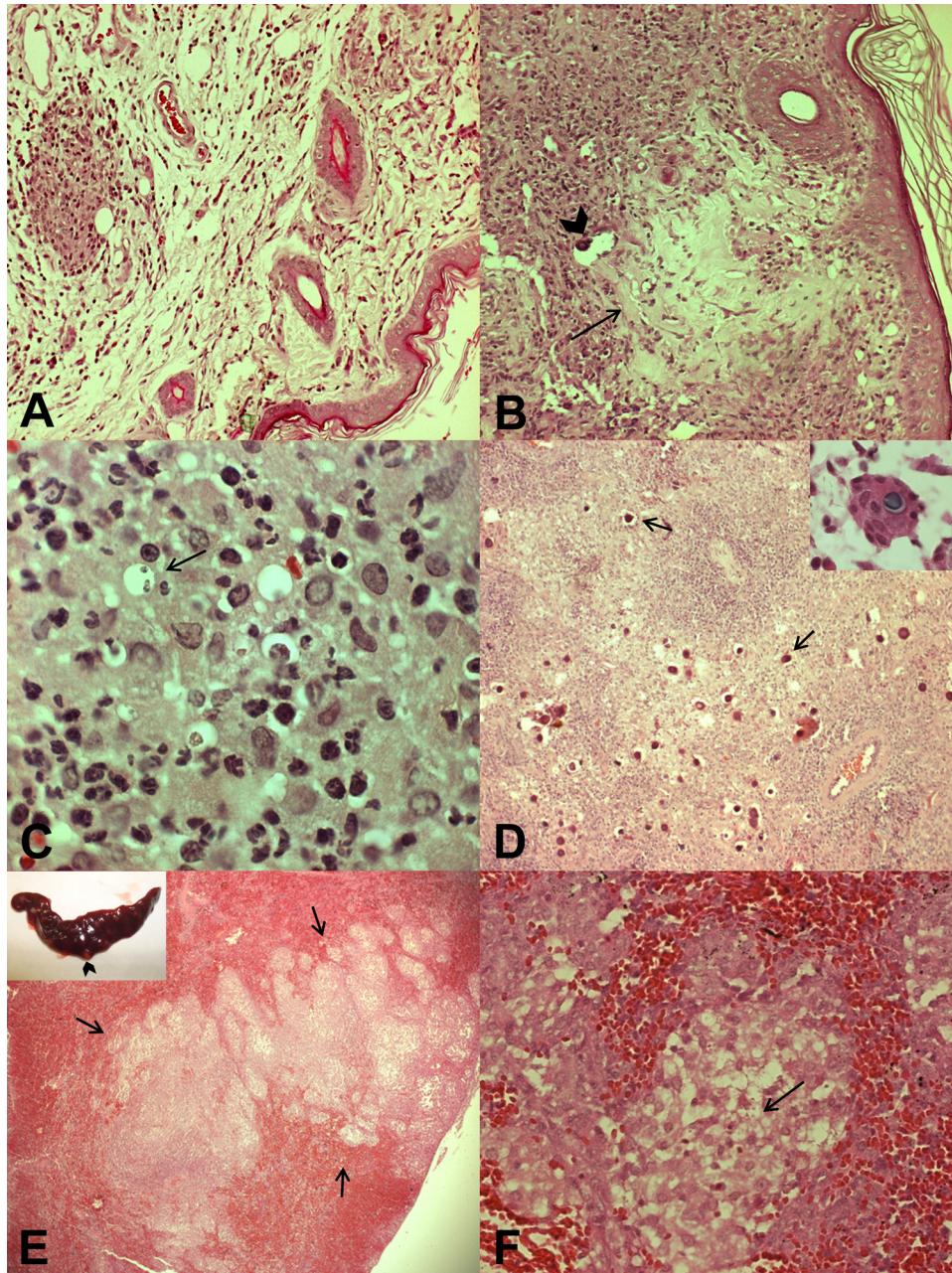
It is known that the parasite number in the infection dose is essential to disease outcomes in both murine (26–28) and hamster (16) models of ATL. The variability of the final lesion sizes and spleen visceralization observed in hamsters infected with  $10^6$  *L. braziliensis* parasites (15) prompted us to ask if such a high inoculum could interfere with the generation of an effective model system that tests the protective effects of vaccine candidates and the effectiveness of new drugs under development. In the present study, we used three different *L. braziliensis* inocula in a standardized protocol to reduce the variability that is inherent to the hamster outbred genetic background and to test the inoculum that ensures infection but does not lead to an exacerbated disease.

The lesion time onset in the *L. braziliensis*-infected hamsters that were studied was inversely proportional to the parasite inoculum concentration ( $10^4$ ,  $10^5$ , and  $10^6$ ), as was observed in previous studies (13, 16). However, at the infection endpoint, after 105 days, we observed that  $10^5$  and  $10^6$  parasite inocula caused the same lesion sizes and that these were significantly larger than lesions in the hamsters infected with  $10^4$  parasites. These results were also reported in an *L. major* infection mouse model (20). Nevertheless, the consequences of each inoculum in the clinical and immunopathologic evolution of lesions produced by *L. braziliensis* in a hamster model of infection are not yet known.

The initial immune response elicited by an infection can be dependent upon the parasite inoculum concentration, which can influence the establishment of the chronic phase. It has been hypothesized that a threshold limit of dermally infected macrophages or of parasites released has not been reached in the early infection phase (known as the silent phase) in animals infected with lower inocula. It may render a quiescent development of parasites that are restricted to macrophages at the inoculation site without stimulation of IL-12 and IFN- $\gamma$  production by lymph node T cells, which leads to lesion development (29). This phenomenon could explain the longer onset of lesions in the  $10^4$  parasite-infected animals observed in the present work. From this same point of view, the  $10^5$  and  $10^6$  parasite-infected animals could have reached this threshold limit earlier, which would elicit cytokine production and lead to earlier lesion development. The lesion size differences observed in the present work with the variance coefficients were also observed in inbred mouse models (20, 30), suggesting that the outbred genetic background of the hamster model is not the main factor that contributes to this variability.

Although the lesion onset was more precocious in the animals that were infected with higher parasite concentration inocula, no differences in the final parasite load (i.e., the chronic phase of infection) were observed between the three groups. The same behavior was observed in the *L. major* mouse model, in which infections with low and high *Leishmania* doses led to an equivalent parasite load during the chronic infection phase (28). It is possible that an excess of parasites could imply an increased *Leishmania*





**FIG 6** Photomicrography of organs from golden hamsters infected with different *Leishmania (Viannia) braziliensis* concentrations ( $10^4$ ,  $10^5$ , and  $10^6$  parasites) at 105 days postinfection. The following are displayed: a localized dermal granuloma that is representative of a  $10^4$  parasite-infected animal (hematoxylin and eosin;  $\times 10$  magnification) (A), a dermal spread granulomatous reaction that is representative of a  $10^6$  parasite-infected animal (arrow, necrosis; arrowhead, Schaumann bodies) (hematoxylin and eosin;  $\times 20$  magnification) (B), macrophage vacuoles with amastigotes (arrow) inside a dermal granuloma of a  $10^6$  parasite-infected animal (hematoxylin and eosin;  $\times 100$  magnification) (C), Schaumann bodies inside a dermal granuloma (arrows) of a  $10^6$  parasite-infected animal (hematoxylin and eosin;  $\times 10$  magnification) and Schaumann bodies inside a dermal multinucleated giant cell of a  $10^6$  parasite-infected animal (inset,  $\times 40$  magnification) (D), granulomas in the spleen parenchyma (arrows) (hematoxylin and eosin;  $\times 10$  magnification) that are representative of macroscopic splenic nodules of a  $10^6$  parasite-infected animal (inset, arrowhead) (E), and splenic vacuolated macrophages that contain *Leishmania*, which are representative of a  $10^6$  parasite-infected animal (arrow) (hematoxylin and eosin;  $\times 100$  magnification) (F).

extracellular death, because the threshold of macrophage infection was achieved.

Despite the similarity in the parasite load endpoint between the three inocula, the clinical aspects of lesions and the histopathological features varied in animals infected with the lower inoculum compared to those in animals infected with the highest inoculum.

The animals infected with  $10^4$  parasites presented a higher percentage of nodular lesions than the  $10^5$  and  $10^6$  parasite-infected groups, which presented a higher percentage of nodular and ulcerated lesions. Accordingly, a reduced skin inflammatory infiltrate was observed in the  $10^4$  parasite-infected animal group in comparison with that of the  $10^5$  or  $10^6$  group. In agreement, mice

infected with low and high *L. major* parasite numbers resulted in minor and severe skin pathological damage, respectively (28). These results suggest that parasite replication during the silent infection phase could also dictate the magnitude of the inflammatory reaction in the infection site. Afterward, the parasite-specific effector response, which was triggered by the highest inoculum ( $10^5$  or  $10^6$ ), should sustain an unregulated inflammatory response until the chronic phase occurs. Conversely, it is possible that the longer clinical lesion onset and the less severe tissue damage observed in animals infected with  $10^4$  parasites can be explained by a delay in the capacity for the parasites to reach the replication threshold and, consequently, recruit fewer inflammatory cells to the inoculation site.

Another possible explanation for the differences in tissue damage and clinical presentation by the different parasite inoculum concentrations could be the cytokine profile. Previous reports regarding human *L. braziliensis* infections demonstrated a positive correlation between large and ulcerated lesions with IFN- $\gamma$  and tumor necrosis factor (TNF) levels, which suggests that these cytokines contribute to tissue injury (31). Recent results also have shown that tissue damage in cutaneous lesions can be attributed to CD8<sup>+</sup> granzyme B<sup>+</sup> T cells, while CD4<sup>+</sup> T cells that produced IFN- $\gamma$  did not correlate with lesion size but did correlate with parasite killing (32). In our hamster model, however, high IFN- $\gamma$  levels were expressed during the infection endpoint, independent of the inoculum concentration used, lesion size, or clinical presentation. Notably, no detectable IFN- $\gamma$  expression was observed in a unique animal that was infected with  $10^4$  parasites that did not present a lesion, which reinforces a possible role for IFN- $\gamma$  in lesion development.

The higher IFN- $\gamma$  expression along with the presence of parasites gave rise to the hypothesis that IFN- $\gamma$  was not sufficient to control parasite replication. In hamster visceral leishmaniasis, a diminished capacity for IFN- $\gamma$  to activate macrophages was attributed to limited binding of IFN- $\gamma$  to the IFN- $\gamma$  receptor, which was compensated by increased IFN- $\gamma$  expression (33, 34). Later studies have shown that this impairment in parasite killing was not due to IFN- $\gamma$  inactivity but to a diminished NO production due to low inducible nitric oxide synthase (iNOS) expression and activity, despite a strong observed Th1 response (35, 36). A nonpolarized mixed type 1 and type 2 cytokine pattern, which was conferred by high IFN- $\gamma$ , IL-12p40, IL-4, IL-10, IL-13, and IL-21 levels, was detected in the skin of animals infected with *L. panamensis* during the early infection phase (24). The coexpression of type 1 and type 2 cytokines was also observed in chronic lesions of hamsters infected with *L. panamensis* (14). This inflammatory response can contribute to skin tissue damage; however, the role of other cytokines has to be taken into account. Another explanation for the extensive tissue damage can be low expression or no modulation of IL-10 mRNA, which has been observed in the majority of tissues of all animals and leads to a decreased ability to downregulate IFN- $\gamma$  mRNA. In human leishmaniasis patients, a high IFN- $\gamma$ /IL-10 ratio is associated with more severe clinical forms of the disease, such as mucosal leishmaniasis (37, 38).

Visceralization of dermatropic *Leishmania* spp. distant from the inoculation site is considered a common finding in hamsters and mice that are experimentally infected with inocula that contain a high parasite concentration (13, 15, 17, 39, 40). This also correlated with lesion size and consequently with infection severity (15). In the animals that were inoculated with  $10^4$  parasites, the

involvement of the spleen was observed only in 16% of animals, compared to 70% for animals infected with  $10^6$  parasites.

In the present work, well-organized granulomas that contained amastigotes were easily detectable in the spleens of the *Leishmania*-infected hamsters. We hypothesized that these granulomas have a well-organized formation but are not functional (41), at least in this infection phase, because the amastigotes were not completely cleared. This finding reinforces the presence of the Th1 response that was generated by the hamster, because granuloma formation requires IL-12, IFN- $\gamma$ , and TNF (42). Granuloma formation is also possibly associated with deficient NO generation, which is required to kill parasites within granulomas. Additionally, this is known to occur in hamster models (36).

A positive correlation between anti-*Leishmania* IgG and lesion size is a common finding in dermatropic *Leishmania* species infections in hamster models (14, 15) and in humans (43) and could be considered a biomarker of the active cutaneous disease. Herein, we also observed that the disease was accompanied by high anti-*Leishmania* IgG levels, although there were no differences between the inoculum groups. Because the measurements of antibody levels were made at the infection endpoint, the antibody production and seroconversion kinetics were unknown. Notably, hamsters infected with *L. infantum* presented increased anti-*Leishmania* IgG levels that were associated with time following infections (44). Thus, further studies may help to explain whether the onset of lesions is associated with B lymphocyte activation and antibody production.

In summary, different parasite inoculum concentrations influenced disease onset, clinical presentation, final lesion size, and the degree of tissue damage but did not lead to differences in the final parasite load, cytokine expression, or anti-*Leishmania* IgG levels between the three inocula. These observations indicate that in the early infection phase, during the innate immune response, the differences observed between the different parasite inoculum concentrations dictate the establishment and magnitude of the adaptive immune response, which thus determines the chronic disease phase. The inoculum with  $10^4$  parasites generated more benign lesions and a less systemic commitment than the higher-parasite-number inocula, which was more consistent with ATL presentation in humans.

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