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Leishmania amazonensis downregulates macrophage iNOS expression via Histone Deacetylase 1 (HDAC1): a novel parasite evasion mechanism

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ABSTRACT

The induced expression of nitric oxide synthase (iNOS) controls the intracellular growth of *Leishmania* in infected macrophages. Histones deacetylases (HDACs) negatively regulate gene expression through the formation of complexes containing transcription factors such as NF-κB p50/50. Herein, we demonstrated the occupancy of p50/p50_HDAC1 to iNOS promoter associated with reduced levels of H3K9Ac. Remarkably, we found increased levels of HDAC1 in *L. amazonensis*-infected macrophages. HDAC1 upregulation was not found in *L. major*-infected macrophages. The parasite intracellular load was reduced in HDAC1 knocked-down macrophages, which presented increased nitric oxide levels. HDAC1 silencing led to the occupancy of CBP/p300 to iNOS promoter and the rise of H3K9Ac modification. Importantly, the immunostaining of skin samples from hiporeactive cutaneous leishmaniasis patients infected with *L. amazonensis,* revealed high levels of HDAC1. In brief, *L. amazonensis* induces HDAC1 in infected macrophages, which contribute to parasite survival and is associated to hiporeactive stage found in *L. amazonensis* infected patients.

INTRODUCTION

Leishmaniasis is an emerging parasitic disease caused by several species of *Leishmania* and affects ~12 million people worldwide with approximately 2 million new infections per year [1]. Depending on the species and the host immune response, diverse clinical manifestations can be observed [2].

Leishmania amazonensis is endemic in Brazil and is the causative agent of local cutaneous leishmaniasis (LCL) and a more severe form, the anergic diffuse cutaneous leishmaniasis (ADCL). The immunological response is associated to a T-cell hyposensitivity pole and patients present a nonreactive profile characterized by negative delayed type hypersensitivity (DTH) reaction to *Leishmania* skin test, as well as a marked Th2-type immune response [3, 4, 5]. *In vitro* infections

corroborate the *in vivo* immune repressive scenario, which is characterized by the suppression of the inherent initial response, noted by the inhibition of pro-inflammatory molecules such as IL12, IFNα, TNFα, IL1α [6, 7].

The production of Nitric Oxid (NO) by iNOS is crucial to control *Leishmania* parasites at all stages of infection [8]. In murine cutaneous leishmaniasis, iNOS was shown to be indispensable for the cell innate response to *L. major* and for the resolution of skin lesions [9]. Patients with visceral leishmaniasis caused by *L. chagasi* also expressed iNOS at higher levels in bone marrow [10]. *L. amazonensis* activates the NF-κB transcriptional repressor homodimer (p50/p50), which binds to iNOS promoter and determines its transcriptional repression. This regulation seems to be species-specific since *L. major* activates a different NF-κB complex, which in fact supports iNOS expression when activated [11].

Covalent post-translational modifications of histones such as acetylation and phoshorylation control gene expression in eukaryotes. The acetylation of histones allows the remodeling of the chromatin in order to expose regulatory DNA sequences to transcriptional regulators. Two classes of enzymes, histone acetyltransferases (HATs) and HDACs are responsible to maintain the acetylation status of histones [12, 13]. Different NF-κB complexes have been demonstrated to recruit and interact with specific members of HDAC family [14, 15]. In particular, p50 NF-κB subunit interacts with HDAC1 protein forming a complex described as repressome and induces the transcriptional downregulation of several NF-κB-dependent genes [16, 17, 18].

Here, we report for the first time that *L. amazonensis* induces the upregulation of HDAC1 expression in infected macrophages both *in vitro* and in human clinical samples. HDAC1 in turns is effectively recruited by p50/p50 NF-κB complex to iNOS promoter leading to the deacetylation of histone H3K9 and the downregulation of iNOS gene and consequent NO levels. Altogether, these

events contribute for parasite survival and progression of the infection and highlight the importance of epigenetic modifications induced by the parasite to effectively manipulate the cell host response.

RESULTS

HDAC1 binds to p50/p50 NF-κB forming a repressor complex in iNOS promoter during *L. amazonensis* infection

L. amazonensis infection leads to NF-κB p50/50 activation throughout PI3K/Akt pathway [19] and this homodimer is responsible for the host iNOS downregulation during the infection. Several reports have demonstrated the role of HDAC1 in the formation of chromatin repressome complexes. Moreover, HDAC1 binds to NF-κB p50/p50 homodimer in several promoters leading to transcriptional repression [16, 17, 18].

To address the hypothesis that HDAC1 was recruited to the iNOS promoter, we have carried out ChIP assays of infected macrophages. Our data show an increased occupancy of HDAC1 to NF-κB binding sites of iNOS promoter (Figure 1A). Importantly, HDAC1 induction seems to be *L. amazonensis* specific, since no changes in HDAC1 levels were observed in *L. major* infection (Figure 1B). Re-ChIP analysis confirmed that HDAC1 and p50/p50 NF-κB integrate the same repressor complex in the iNOS promoter during *L. amazonenis* infection (Figure 1C).

The levels of macrophage infection are depicted in supplementary figure 1 A and B. It has been demonstrated that GP63 from several *Leishmania* species may alter the macrophage nuclear envelope nuclear affecting the translocation of the transcription factors NF-κB and AP-1 [20] (Isnard et al., 2015). To verify the efficiency of extraction and eliminate artefactual CHIP differences, the nuclear levels of H3 were evaluated during the infection. There were no differences in nuclear histone H3 levels in our assays (supplementary figure 1C).

Corroborating the enrichment of HDAC1 at iNOS promoter, we observed decreased levels of acetylated histone 3 (Lys 9), an epigenetic marker associated with transcriptional activation (Figure 1D). Chromatin immunoprecipitation of histone H3 was carried out in the normalization of H3K9Ac (supplementary figure 1D).

***L. amazonenis* induces macrophage HDAC1 expression**

The CHIP-HDAC1 results prompted us to investigate whether *L. amazonensis* would induce the cellular levels of HDAC1 in macrophages. By western blot we observed an increase of both nuclear and total HDAC1 protein levels in early times of infection (Figure 2A and 2B respectively). Thus, the expression of HDAC1 increases during infection and it seems to be *L. amazonensis* specific, since no changes were observed in HDAC1 expression due to *L. major* infection (Figure 2D).

The overall histone deacetylase activity was also measured during macrophage infection (Figure 2C). We can observe a slight, but significative increase of total deacetylase activity in 5 h infection, probably due to the raise of HDAC1 expression. During 18 h of infection, the HDAC activity was not altered corroborating the unaltered HDAC1 nuclear levels verified in 18 h of infection (supplementary figure 2).

***L. amazonensis* infection promotes Sp1 binding to HDAC1 promoter**

We have carried out qPCR assays in infected macrophages to analyze the HDAC1 RNA levels. Figure 3A shows the increase of HDAC1 mRNA levels in 4 hours of infection (Figure 3A). Then, we decided to test the activity of the HDAC1 promoter during the infection. The Luciferase gene reporter assay revealed the upregulation of HDAC1 promoter (Figure 3B), suggesting its induction by transcription factors.

The human HDAC1 promoter displays several putative transcriptional factors binding sites including Sp1, Sp3, AP1, C/EBP and AP2 [21]. Since *L. amazonesis* induces Sp1 activation [22], we addressed the question whether this parasite upregulates HDAC1 promoter via this transcriptional factor. Accordingly, we observed by ChIP assays, that *L. amazonensis* induces Sp1 occupancy in HDAC1 promoter suggesting its participation in the activation of the cognate promoter (Figure 3C). We also investigated the contribution of AP1 on HDAC1 expression during *L. amazonensis* infection. We verified the effect of the specific AP-1 inhibitor, SR 11302, on the HDAC1 expression in our model. Figure 5D and 5E show the inhibition of HDAC1 expression when infected macrophages were pretreated with the AP1 inhibitor. These results strongly suggest that AP1 plays a role in HDAC1 expression.

HDAC1 knocking down correlates with increased NO production and reduced parasite load

Since we have observed increased levels of nuclear HDAC1 and the enrichment of HDAC1 in NF-κB binding sites of iNOS promoter during the infection, we decided to verify the role of this enzyme in iNOS repression. To address this question, we successfully silenced HDAC1 in THP-1 cells throughout shRNA lentivirus transduction using three shHDAC1 different constructions (Figure 4A). The construction shHDAC1 (3) was selected to proceed with the subsequent experiments.

To verify the role of HDAC1 in the course of the infection, HDAC1 knocked-down THP1 cells were infected with *L. amazonensis* for 4 h to evaluate the parasite uptake and for 48 h to analyze the survival of the parasites. It can be observed that there is no alteration in the uptake of the *Leishmania* (figure 4B). However, HDAC1 silencing impaired the parasite load compared to scrambled control (Figure 4C) probably due to the high production of nitric oxide in HDAC1 knocked-down cells (Figure 4D). The supplementary Figure 3 shows no alteration in cell viability during the

infection of shScr and shHDAC1 cells. These experiments corroborate the role of HDAC1 in iNOS donwregulation and its importance in the progression of the infection.

HDAC1 regulates the occupancy of CBP-300 and H3K9Ac levels at iNOS promoter during *L. amazonensis* infection

Further, we aimed to characterize the occupancy by the CBP-300 (a Histone Acetylase Enzyme) suffered by the iNOS promoter and the associated levels of H3K9Ac residues in HDAC1 knocked-down THP1cells. Figure 5A shows the increased occupancy of CBP/p300 to the iNOS promoter during the infection. Accordingly, it was observed that H3K9Ac levels were increased in *L. amazonensis* infected HDAC1 knocked-down macrophages (Figure 4B). Chromatin immunoprecipitation of histone H3 was used in the normalization of H3K9Ac ChIP results (supplementary Figure 4). These data corroborate the notion that HDAC1 plays a pivotal role in iNOS downregulation due to *L. amazonensis* infection by preventing the histone acetyltransferase (CBP/p300) binding to iNOS promoter and further acetylation of H3K9 residue.

Cutaneous lesions of patients infected with *L. (L.) amazonensis* $^{DTH-}$ exhibit high levels of HDAC1 expression

We aimed to compare the HDAC1 expression in two distinct clinical and immunopathological spectrum of cutaneous leishmaniasis caused by *L. (Leishmania) amazonensis* and *L. (Vianna) braziliensis*. *L. amazonensis* infections are characterized by a hyposensitivity polo $^{DTH-}$ and it is implicated with ADCL while *L. braziliensis* infections present a hypersensitivity polo $^{DTH+}$ and may cause mucocutaneous leishmaniasis (MCL). Moreover, both species can also induce an intermediary

form between the central LCL and the two polar MCL and ADCL, the borderline disseminated cutaneous leishmaniasis (BDCL), which is distinguished by a partial inhibition of T-cell response [3, 4].

Histological sections from biopsies obtained from lesions of a total of 30 patients with ADCL, BDCL and LCL caused by *L. (L.) amazonensis* (hyposensitivity polo^{DTH-}) and LCL, LCM by *L. (V.) braziliensis* (hyposensitivity polo^{DTH+}) were submitted to immunohistochemical analysis. The epidemiological information is described in Table 1. By comparing the cell population according to the *Leishmania* species that caused American Cutaneous Leishmaniasis (ACL) (Figure 6), it was verified that there was a higher density of HDAC1-expressing cells ($P<0.0001$) in samples from patients with ACL *L. amazonensis* (hyposensitivity pole/ DTH-) when compared to patients with LCL *L. braziliensis* (hypersensitivity pole/ DTH+). The negative controls are shown in Supplementary Figure 5. These findings corroborate our *in vitro* data and highlight the role of the HDAC1 in human cutaneous infection caused by *L. amazonensis*.

DISCUSSION

While signaling pathways and transcriptional regulators act on a limited subset of genes, epigenetic regulators tend to control gene expression in a global manner, having a concomitant effect on cell functions [23]. Covalent post-translations modifications of histones are one of the mechanisms whereby cells modify the chromatin status and play a major role in eukaryotic gene regulation [24]. Epigenetic changes in the host cell during infection, known as patho-epigenetics, it is an emerging area of research for many aspects of infections disease with particular relevance in the regulation of intracellular infections.

Several intracellular pathogens induce chromatin modifications and promote changes in the gene expression of the host (25). Studies have shown that some virus infections lead to modulation of HDACs, resulting in changes in gene expression profile favoring infection [26, 27, 28]. Other

groups have shown that epigenetic manipulations of the host cell are promoted by intracellular bacteria [29- 33]. There is a growing body of evidence suggesting that protozoan parasites such as *Leishmania*, *Toxoplasma*, and *Theileria* manipulate host cells via epigenetic modification of host gene expression [34].

A balance between the ability of the immune response to activate Leishmania-infected macrophages and the ability of the parasite to resist cytotoxic mechanisms of macrophage activation determines parasite persistence within the macrophages. The parasite may adopt mechanisms to cope with oxidative stress [35, 36]. A hallmark of *L. amazonensis* infection is the downregulation of macrophage activation enabling parasite survival and replication [37- 39].

In the present work, we demonstrated a sophisticated evolutive mechanism involving epigenetic modifications in iNOS promoter during the infection. We have showed that *L. amazonensis* induces the upregulation of HDAC1 and determines the silencing of iNOS promoter through p50-HDAC1 complex with a concomitant reduction in the levels of H3K9Ac. The importance of these events for the course of the infection was verified in HDAC1 knocked-down macrophages where an impaired growth of the intracellular parasites was observed. In an attempt to test HDACs inhibitors, *L. amazonensis* infected macrophages were treated with Trichostatin A or MS-275, but no reproducible results were obtained, probably due to the difficulty to specifically inhibit HDAC1 (data not shown).

High levels of NO, which is involved in the killing of parasite, have been observed in lesions of patients infected with *L. tropica* [40]. However, skin biopsies from patients infected with *L. mexicana* showed that the expression of iNOS was most prominent in lesions with small numbers of parasites whereas lesions with a high parasite burden contained considerably fewer iNOS-positive cells [41]. Corroborating these findings, we verified that NO levels were increased in *L. amazonensis*

HDAC1 knocked-down infected macrophages, which exhibited reduced parasite load compared to infected control cells.

Clinical samples from patients infected with *L. amazonensis* $^{DHT-}$ clearly showed a strong expression of HDAC1 compared with the samples from patients infected with *L. braziliensis*$^{DHT+}$. A hallmark feature of the immunological response in *L. braziliensis* infections is a strong Th1 type immune response to soluble *Leishmania* antigen (SLA), demonstrated by a positive DTH reaction to the *Leishmania* skin test, as well as lymphocyte proliferation and high levels of IFN-γ and TNF-α [42, 43]. On the other side, *L. amazonensis* infections present a suppressive Th1 type immune response, negative DTH reaction and high levels of IL-4 [3]. HDAC1 increased expression in lesions from patients infected with *L. amazonensis* correlates with its immunopathological response, due to negatively regulation of iNOS expression. Moreover, HDAC1 may have a wide role in the "silent" pro-inflammatory scenario during the infection. RNAseq assays are in progress to address this question, using *L. amazonensis*- infected HDAC1 knocked-down macrophages in an attempt to find new HDAC1 targets relevant for the infection.

The enhancement of HDAC1 levels appears to be an ingenious strategy employed by this intracellular parasite to reduce the host defense, by means of decreasing the gene expression of, probably, a repertoire of genes involved in the macrophage response to infections. A similar regulatory mechanism was demonstrated in *Mycobacterium tuberculosis* infection. It was verified the increase of HDAC1 expression with a reduction in the levels of histone H3-acetylation at IL-12B promoter in macrophages containing virulent *M. tuberculosis* [33]. Garcia-Garcia [29] showed that the intracellular bacteria, *Anaplasma phagocytophilum,* led to silencing of host defense gene expression. It was shown that HDAC1 expression, activity and binding to the defense gene promoters significantly increased during infection, which resulted in decreased histone H3 acetylation in infected cells. Another work showed the upregulation of HDAC4 in *L. donovani*

infected macrophages, but the relevance for the gene expression regulation was not evaluated [38]. In *Pseudomonas aeruginosa* infection, the bacteria-induced infection tolerance seems to be regulated *via* HDAC1. Therefore, it was verified a hypo-acetylation of lysine 1 of histone H3 at pro-inflammatory cytokine promoter [44].

The regulation of human HDAC1 promoter has been described in colon cancer cells where it is overexpressed. Two important binding sites for the transcription factor Sp1 at HDAC1 promoter have been reported. The one located -240 region seems to regulate HDAC1 promoter induction whereas the -40 site is related to the basal transcription activity [21]. Based on this, we investigated in our model whether Sp1 regulates HDAC1 promoter. Our data strongly suggests that Sp1 contributes to the upregulation of HDAC1 expression in infected macrophages. This data is in agreement with the reported findings that *L. amazonensis* is able to induce the activation of Sp1. In fact, Yang [22] showed that in *L. amazonensis* induces ERK1/2 activation, which phosphorylates histone H3 at the IL-10 promoter favoring the binding of Sp1 to IL-10 promoter and leading to its activation. We also tested the role of AP1 in the context of HDAC1 induced activation. Our results showed that the inhibition of AP1 reduces the levels of increased HDAC1 levels during the infection. Since there are some discrepancies on the characterization of AP1 putative binding sites on the HDAC1 promoter, other experiments are in progress to address the actual binding to HDAC1 promoter.

In brief, we are proposing a model in which we demonstrate unique aspects of epigenetic changes in the host cell during *L. amazonensis* infection due to HDAC1 increased expression. This study can provide clues to understand the diversity of clinical manifestations in cutaneous leishmaniasis and may pave the way for novel therapeutic interventions.

MATERIALS AND METHODS

Cell culture

The human monocytic leukemia cell line THP-1 (ATCC: TIB202TM) was cultured in RPMI (Gibco) medium supplemented with 10% fetal bovine serum (Sigma),. These cells were differentiated to macrophages with 40ng/mL PMA (Sigma) for 72 h. Mouse macrophage leukemia cell line RAW 264.7 (ATCC: TIB-71) and Human embryonic kidney cell line HEK-293FT (Life Technologies) were cultured in DMEM (Gibco) medium supplemented with 10% fetal bovine serum. HEK-293FT cells were maintained in medium containing 500μg/mL geneticin.

Parasites, culture conditions and infection

L. (L) amazonensis (WHOM/R/75/Josefa) and *L. (L) major* strain LV39 (MRHO/ Sv/ 59/ P) were maintained *in vitro* in Schneider Insect Medium (Sigma) supplemented with 10% of fetal bovine serum. The infection was carried out with stationary phase promastigotes at a parasite-to-cell ratio of 5:1 in triplicate. Differentiated THP1 or RAW cell lines were plated on glass coverslips and cultured overnight at 37°C. The cultures were washed and infected for 4 h at 37°C, followed by washing, fixation with methanol, and Giemsa staining. The number of intracellular parasites was determined by counting ≥100 cells/replicate under a light microscope. For the survival assays, the cells were infected for 4 h, washed, and further cultured for 48 h. The AP1 inhibitor, SR 11302, (iAP1) (CAS 160162-42-5, Santa Cruz Biotechnology) was used as a pre-treatment (1.5μM) for 30min .

Quantitative Real Time PCR

Total RNA of THP-1 differentiated cells was extracted with RNeasy® Plus (Quiagen) and 1μg total RNA was reverse-transcribed into the first-strand cDNA with ImProm (Promega) and oligo(dT) 12–18 primer, according to the manufacturer's instructions. The following pairs of primers were used to determinate human HDAC1 mRNA levels: 5' CAAGTACCACAGCGATGACTACATTAA-3' and

Reverse: 5'-GCTTGCTGTACTCCGACATGTT-3' GAPDH Forward 5'- TGCACCACCACCTGCTTAGC- 3´ and GAPDH Reverse 5' GGCATGGACTGTGGTCATGAG- 3' were used for normalization. Real time quantitative PCR (qPCR) was carried out via the Applied Biosystems 7500 detection system using Power SYBR Green PCR Master Mix (Applied Biosystems). All qPCR experiments were performed at least 3 times. All expression ratios were computed via the ΔΔCt method.

Immunoblotting

Differentiated THP-1 or RAW 264.7 cells were infected with *L. amazonensis* and total and nuclear protein extract were obtained. For total extract the cell pellet was ressuspended in lysis buffer (Tris-HCL pH 7,4 50 mM; EDTA 0.1mM; NaF 50mM; β- glycerophosphate 10mM; NaCl 150mM; Triton X-100 1%; glycerol 10%; sodium orthovanadate 1mM; protease inhibitor) and incubated during 10min on ice. For the nuclear protein extraction, the cell pellet was ressupended in A buffer (HEPES pH 7.9 10mM; KCl 10mM; EDTA 0.1mM; EGTA 0.1mM; protease inhibitor) and incubated during 15min on ice. The samples were submitted to centrifugation (1810g) during 5min at 4ºC. The nuclear pellet was washed with A buffer, ressuspended in C buffer (HEPES pH 7.9 20mM; NaCl 0.4M; EDTA 1mM; EGTA 1mM; glycerol 20%; protease inhibitor) and incubated on ice during 30min and centrifuged (12000g) for 10min. The nuclear proteins were collected in the supernatant. Both nuclear and total proteins were subjected to electrophoresis in 10% SDS-polyacrylamide gels. Blots were separately incubated with primary antibody against lamin A/C (Cell Signaling 2032), HDAC1 (Santa Cruz Biotechnology, sc-6298), β-actin (Sigma- Aldrich A2228), histone H3 (#9715, cell signaling) and αtubulin (Santa Cruz Biotechnology, sc-12462).

Chromatin immunoprecipitation assay - ChIP

ChIP analysis was carried out according to the Simple ChIP Enzimatic Chromatin IP Kit protocol (Cell Signaling). RAW 264.7 cells or differentiated THP-1 cells were submitted to ChIP assay as described [19]. The chromatin was immunoprecipitated with of anti-p50 (Millipore – 06-886) anti-

HDAC1 (sc-6298), anti-H3Ac (#9671S, cell signaling), anti-CBP/p300 (sc-369X), anti- histone H3 (#9715, cell signaling) antibodies at 4°C under rotation for 16 h. The DNA isolated from immunoprecipitated material was amplified by real time PCR using SyberGreen for murine iNOS promoter: Foward 5`-ACACAGACTAGGAGTGTCCATCATGA-3` and Reverse: 5`-ACAAGACCCAAGCGTGAGAGGCCTCA- 3` and human iNOS promoter Forward: 5'-GAGGGCTTTCCCAGAACCAAG-3' and Reverse: 5'-GGCTACTGACCCAGCAGTTC-3'. For human Sp1 promoter (-240 site) the following primers were used: Forward: 5'-TCAGAAAGTTGGCCTCCGATC-3'and Reverse: 5'-CTGGGCCTGTACCAAGGTCTG-3'. As a control, 1/50 of digested input chromatin was similarly processed and analyzed in the absence of immunoprecipitation. To calculate the input percentage of the samples, input was adjusted to 100% ($IP_{adj} = C_{t\ avr}(IP) - \log_2 50$), followed by application of the formula $100 \times 2^{[IPadj - Ct\ avr(IP)]}$, where IP_{adj} is adjusted input, and $C_{t\ avr}(IP)$ is average C_t of input.

Lentivirus transduction

THP-1 cells knock-down for HDAC1 expression was obtained by lentivirus transduction described previously [45]. The human shRNA HDAC1 constructions (shRNA 1 - TRCN0000004816; shRNA 2 - TRCN0000195672; shRNA 3 - TRCN0000197176 and the non-target shRNA (SHC002) (Scrambled) from Broad Institute, USA were purchased from Sigma-Aldrich. After 6 days of transduction, THP-1 cells were selected with 1μg/mL of puromycin for 12 days.

Determination of nitrite

Griess reaction was employed to analyze the nitrite (NO_2^-) content as an indicator of NO production in supernatant cultures of THP1 cells. As a standard, the absorbance at 540nm was then measured following the incubation of supernatants the solution containing N-[naphthyl] ethylenediamine dihydrochloride (Need), sulfanilamide and phosphoric acid.

Luciferase reporter assay

THP-1 cells were transfected with 0.5 μg of luciferase reporter plasmid HDAC1-luc (S718920, Switch Gear) using Nucleofector™ Technology (Lonza, Basel, Switzerland). For normalization of the luciferase readout, the plasmid pRL-CMV (Promega) was used. The cells were lysed according to the Dual Luciferase System protocol (Promega), and analyzed using the GloMax®-Multi detection system (Promega Corp., Madison, WI, USA).

HDAC activity

Differentiated THP-1 cells were infected with *L. amazonensis* and nuclear extract proteins were obtained as described. The extract was submitted to HDAC activity colorometric assay kit from BioVision (#K331).

Immunohistochemistry

Thirty patients were diagnosed with ACL in the ambulatory section of the Prof. Dr. Ralph Lainson Leishmaniasis Laboratory of the Evandro Chagas Institute, Para State, Brazil based on clinical, epidemiological, parasitological and immunological features. Biopsies from patients were deparaffinized in xylene for 10 minutes and hydrated in descending series of alcohol. The epitope retrieval was performed in hot citrate bath (10 mm/pH 6.0) for 40 minutes. Then, endogenous peroxidase was blocked using a solution of hydrogen peroxide 3% and the slides were incubated in a humid chamber, overnight at 4°C, with goat polyclonal antibody raised against a peptide mapping at the C-terminus of HDAC1 human (1:50, C-19; SC 6298, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in PBS 1% BSA. For development of the reaction, the LSAB kit (Dako, Carpinteria, CA, USA) and diaminobenzidine (Sigma, St. Louis, MO, USA) in PBS containing 3% hydrogen peroxide were used. All reactions were performed with positive and negative controls. Negative controls were constituted by the use of normal goat serum (1;25,000, X0907, Agilent/Dako,Glostrup, Denmark) at the same protein concentration as the primary antibody and omission of the primary antibody. At least ten sequential images of each histological section were acquired using a system composed of microscope AxioShop 2 Plus, color video camera connected to computer with AxionVision 4.2

software (Carl Zeiss, San Diego, CA, USA). Immunolabeled cells were quantified by counting in the software and cell densities (cells/mm2) were calculated.

Cell Viability Assay

The MTT reagent 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (M2128) (Sigma) was used according [46].

Statistical analyses

Statistical analyses were performed using the Prism program (GraphPad, San Diego, CA, USA) and Student's *t* test for independent samples or One-way ANOVA, with the Bonferroni *post hoc* test.

Ethics Statement

The methods carried out in this work are in accordance with the guidelines approved by the University of São Paulo Research Ethical Committee – Faculty of Medicine (São Paulo, SP, Brazil), under de number n° 003/14.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article

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FIGURE LEGENDS

FIGURE 1. HDAC1-p50 complex downregulates iNOS promoter during *L. amazonensis* infection. RAW 264.7 cells were infected for 5 h with *L. amazonensis* or *L. major* as indicated; and the chromatin immunoprecipitation assay was performed with the antibodies: anti-HDAC1 (A and B) and anti-H3K9Ac (D). In Re-ChIP assay (C) the anti-HDAC1 antibody in the first reaction and the collected complex was precipitated with anti-p50 for the second immunoprecipitation. The immunoprecipitated chromatin was amplified by real time PCR using specific primers to κB-binding sites in the iNOS gene promoter. The H3K9Ac results were normalized with H3 immunoprecipitation. Experiments were performed 3 independent times in triplicates. Graphs are representative of 1 experiment and indicate means ± SD of experiments performed in triplicate. Statistical analyses were performed using Unpaired t- test. *** p =0.0003; ** p =0.0079; * p = 0.0265.

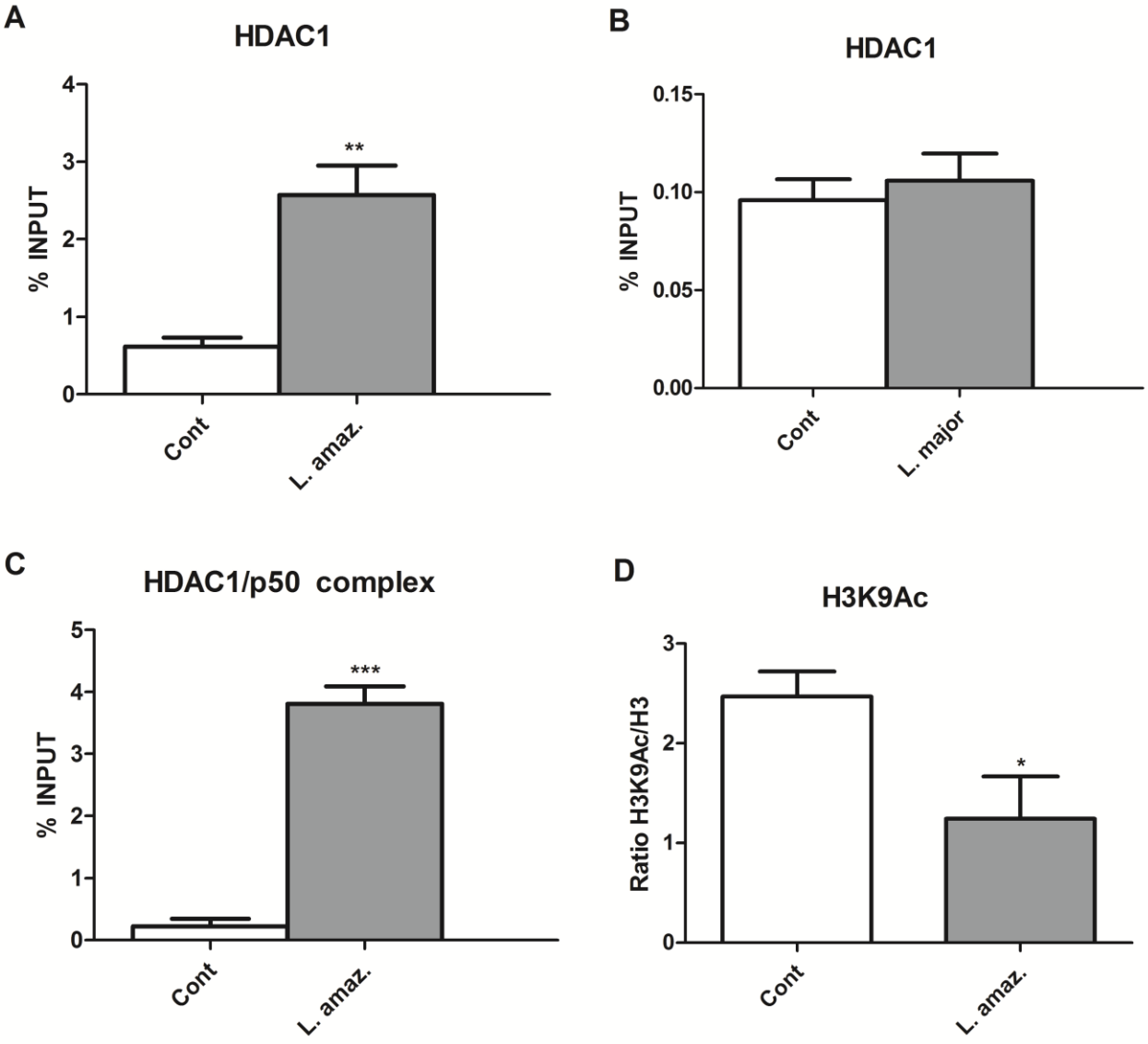


FIGURE 2. HDAC1 expression is increased in *L. amazonensis*-infected macrophages. THP-1 cells were differentiated and infected with promastigotes of *L. amazonensis* or *L. major* as indicated. The nuclear (A) and total (B and D) protein extracts were obtained and subjected to Western blot using the anti-HDAC1. Anti- lamin A/C and anti-tubulin were used as endogenous control. C) Histone deacetylase activity was carried out with nuclear extracts from differentiated THP-1 cells not infected (control) or infected with *L. amazonensis* as indicated. Relative levels of HDAC1, were calculated by densitometry and are indicated at bottom. Experiments were performed 3 independent times in triplicates. Graphs are representative of 1 experiment and indicate means ± SD of experiments performed in triplicate. Statistical analyses were performed using One-Way ANOVA. ** $p < 0.0001$.

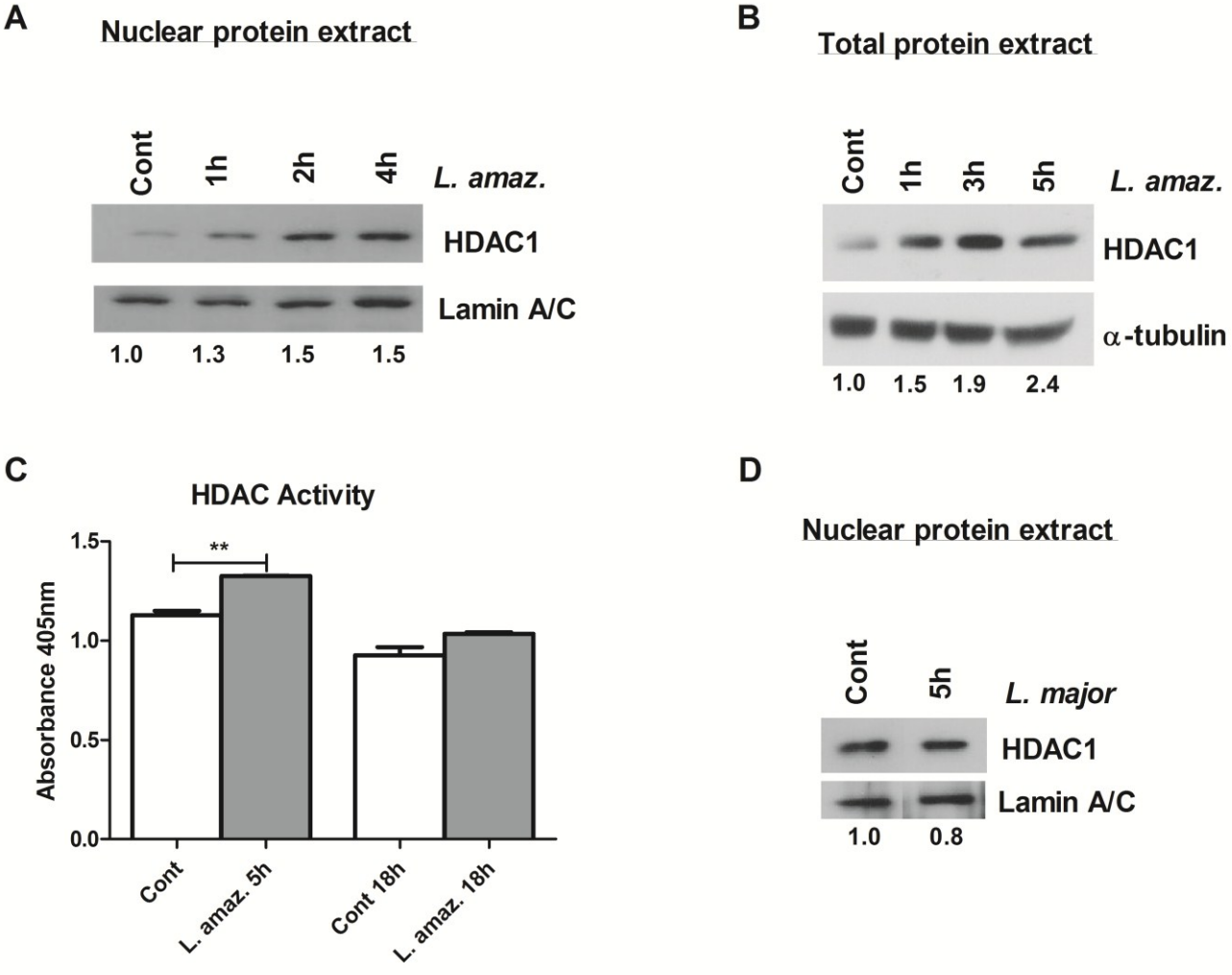


FIGURE 3. *L. amazonensis* increases HDAC1 promoter activity. THP-1 cells were differentiated and infected with promastigotes. A) The total RNA was extracted at 4h of infection and cDNA synthesis were performed. The samples were subjected to qPCR as previously described. (B) Differentiated THP1 cells were transiently transfected with the reporter plasmid pHDAC1-LUC and were infected for 24 h. Non-infected cells were used as negative controls for basal luciferase activity. (C) Differentiated THP1 cells were infected for 5 h with *L. amazonensis*; and the chromatin immunoprecipitation assay was performed with the antibodies anti-Sp1. The immunoprecipitated chromatin was amplified by real time PCR using specific primers to Sp1-binding sites located at the HDAC1 gene promoter. Differentiated THP1 cells were infected with *L. amazonensis* for 4 h or pre-treated with 1.5μM of AP1 inhibitor (iAP1) during 30min or DMSO. The total RNA (D) was extracted and cDNA synthesis were performed. The samples were subjected to qPCR to verify HDAC1 transcript levels and β-actin was used as an endogenous control. The total protein extracts (E) were obtained and subjected to Western blot using the anti-HDAC1 and anti- β-actin as endogenous control. Relative levels of HDAC1, calculated by densitometry, are indicated at bottom. Experiments were performed 3 independent times in triplicates. Experiments were performed 3 independent times in triplicates. Graphs are representative of 1 experiment and indicate means ± SD of experiments performed in triplicate. Statistical analyses were performed using Unpaired t- test. * p = 0.0039; ** p = 0.0019; *** p = 0.0008. Graphs are representative of 1 experiment and indicate means ± SD of experiments performed in triplicate. Statistical analyses were performed using Unpaired t- test. * p = 0.0039; ** p = 0.0019; *** p = 0.0008. The One-Way ANOVA analyses was used in D; *** p =0.0004

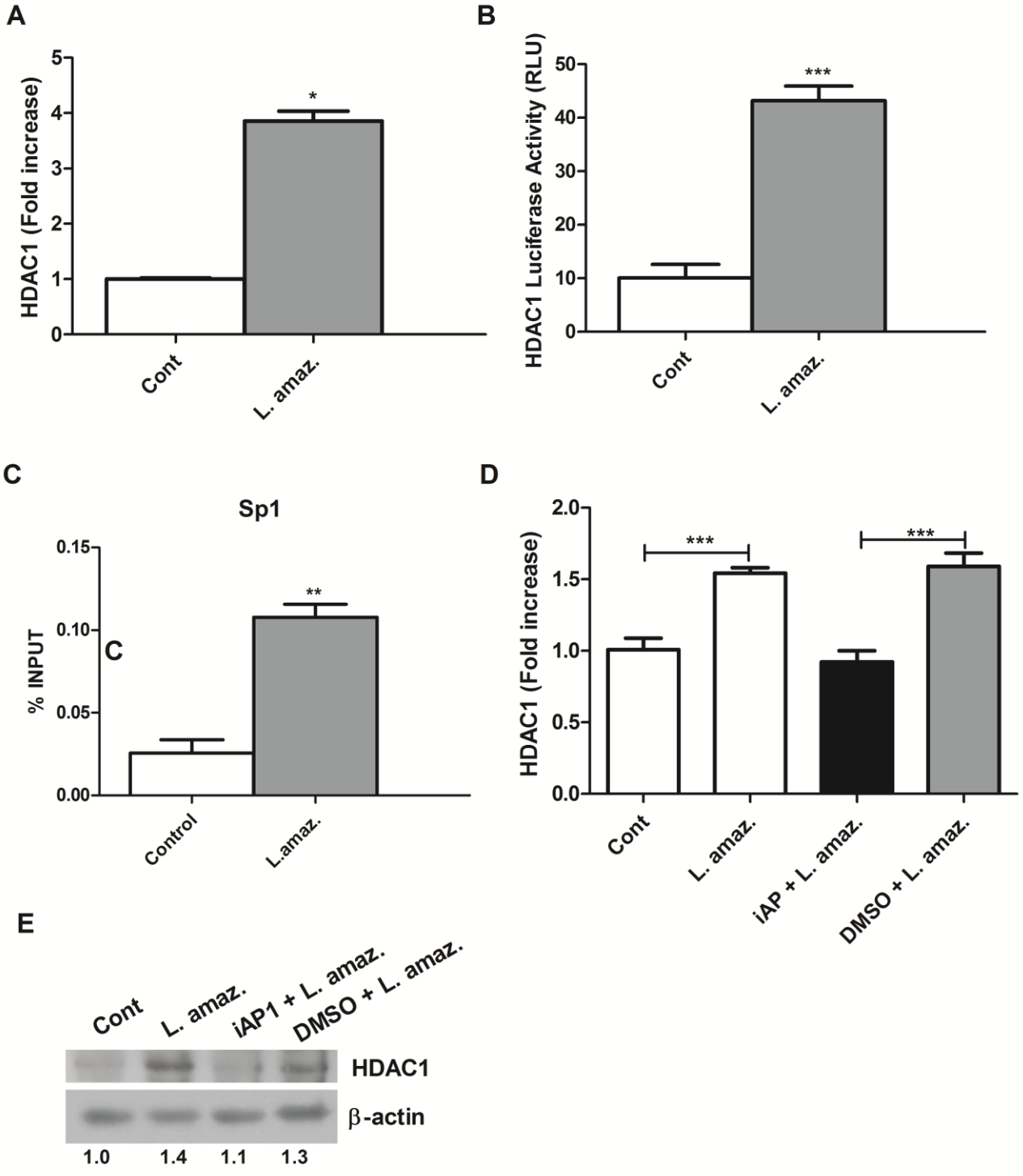
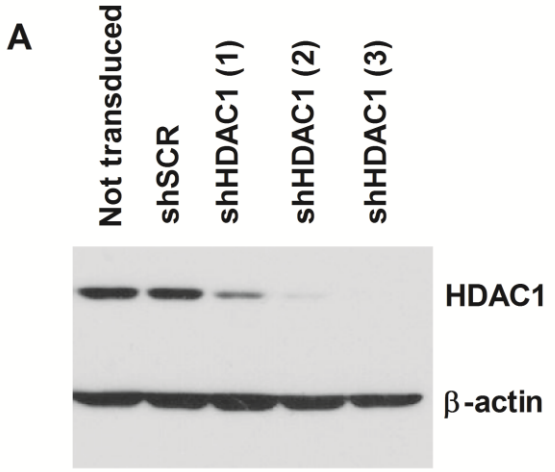
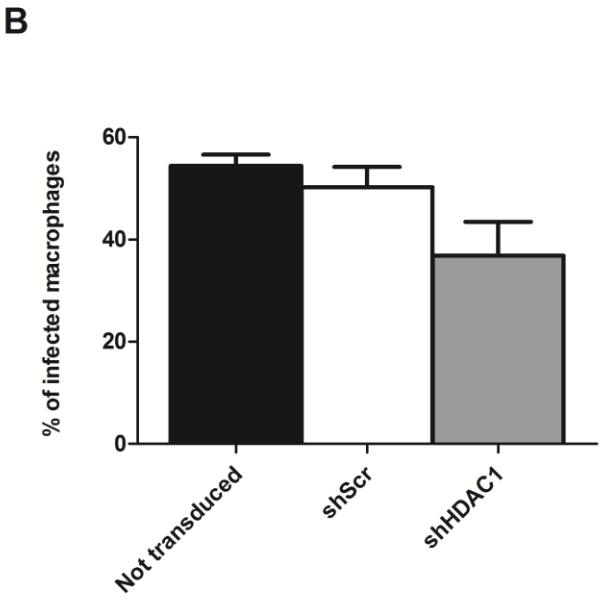
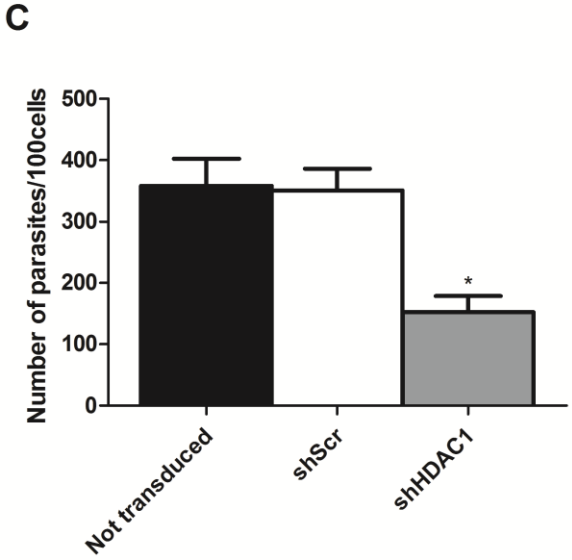


FIGURE 4. HDAC 1 knocked-down expression impairs *L. amazonensis* growth in infected macrophage. A) Western blot of total protein extract obtained form differentiated THP-1 stably knocked-down for HDAC 1 expression. Monocytic THP-1 were not transduced or transduced with lentivirus carrying the construction: shScr (Scrambled), shHDAC 1 (1), shHDAC 1(2) and shHDAC 1 (3). Differentiated THP-1 cells knocked-down for HDAC 1 (3) were infected with *L. amazonensis* promastigotes. At 48h post-infection, the percentual of infected macrophage (B) and the number of parasites/ 100 cells (C) were evaluated. D) Differentiated THP-1 cells shScr or shHDAC 1 were infected with *L. amazonensis* and after 48h pos-infection the nitric oxide was measured in the culture cell supernatant as described in the Experimental Procedures. Experiments were performed 3 independent times in triplicates. Graphs are representative of 1 experiment and indicate means ± SD of experiments performed in triplicate. Statistical analyses were performed using One-Way ANOVA. ** $p < 0.0014$; * $p < 0.0046$.







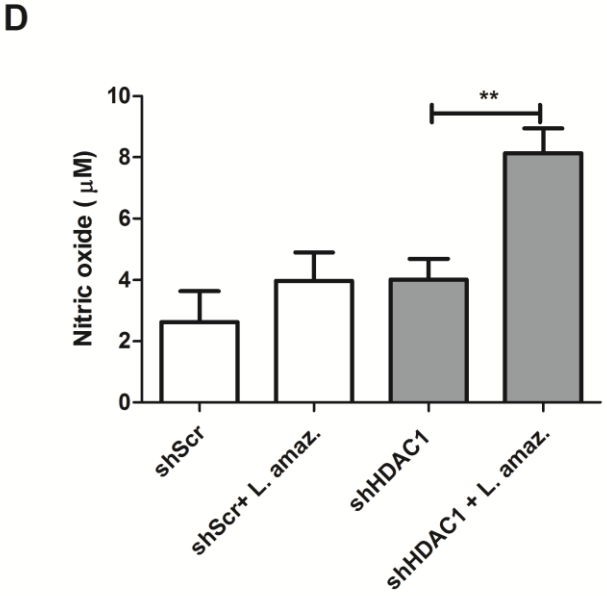


FIGURE 5. HDAC1 expression is important to determine the occupancy of CBP-p300 and the levels of H3K9Ac in iNOS promoter during *L. amazonensis* infection. Differentiated THP-1 cells shScr or shHDAC 1 were infected with *L. amazonensis*. At 5h of infection, the chromatin immunoprecipitation assay was performed with the antibodies: CBP-p300 (A) and anti-H3K9Ac (B). The immunoprecipitated chromatin was amplified by real time PCR using specific primers to κB-binding sites in the iNOS gene promoter. The H3K9Ac results were normalized with H3 immunoprecipitation. Experiments were performed 3 independent times in triplicates. Graphs are representative of 1 experiment and indicate means ± SD of experiments performed in triplicate. Statistical analyses were performed using One-Way ANOVA. *** p <0.0001.

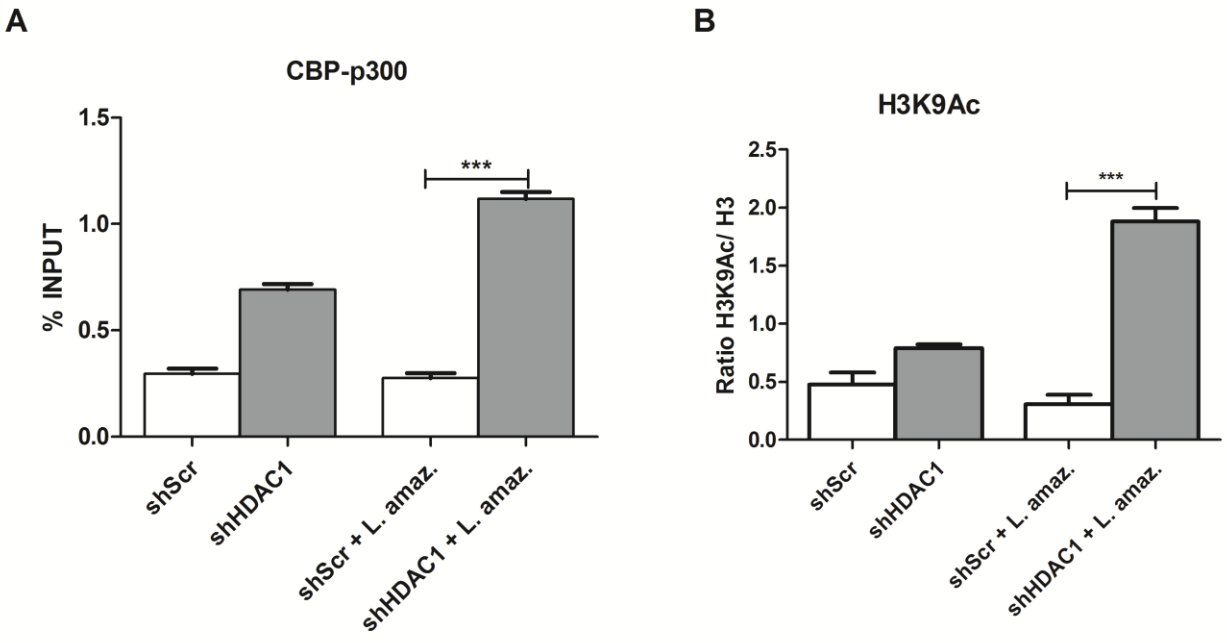
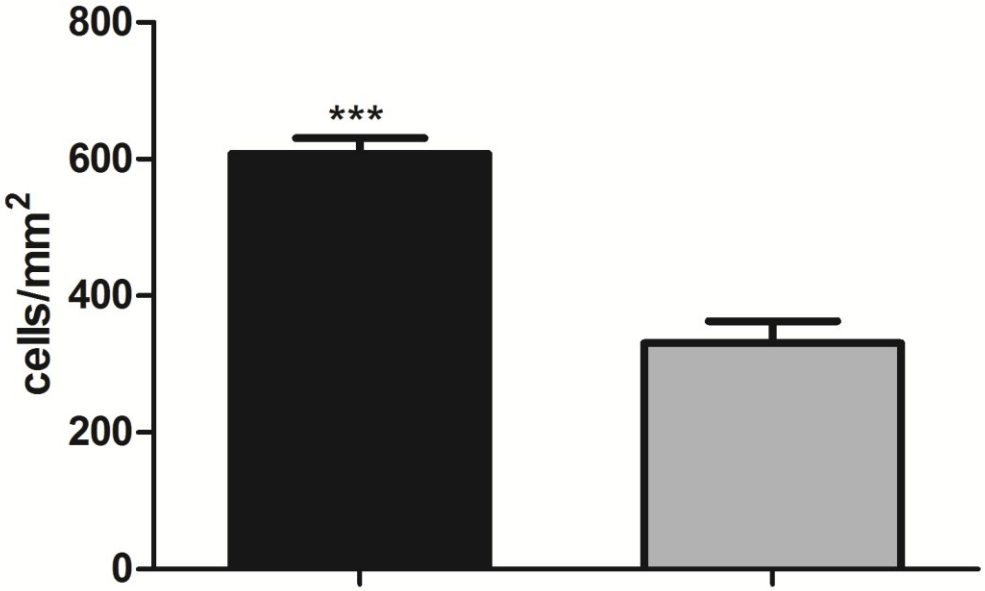


FIGURE 6. Skin lesions from patients with ACL caused by *L. (L.) amazonensis*$^{DTH-}$ exhibit higher expression of HDAC1 than *L.(V.) braziliensis*$^{DTH+}$ in immunohistochemistry analysis. Graphic analysis of immunoreactive cells density(mean ± SEM) for HDAC1. (*A and B*) Histological sections from biopsies obtained from lesions of patients with ADCL, BDCL and LCL caused by *L. (L.) amazonensis* (hyposensitivity polo^{DTH-}) and LCL, LCM by *L.(V.)braziliensis* (hyposensitivitypolo^{DTH+}) were submitted to immunohistochemical reaction with antibodies against HDAC1, as described previously. Immunoperoxidase staining of skin lesion with nuclear localization (400x) and positive cell density were obtained. Images are representative of sections from 16 patients

with ACL *L.(L.) amazonensis* (DTH^{-}) and 14 with ACL *L.(V.) braziliensis* (DTH^{+}). Statistically significant differences were determined using a two-tail parametric t- test. *n* = 30. **P*< 0.0001.DTH= delayed-type hypersensitivity.



L.(L)a $^{DTH-}$ (hyposensitivity polo). *L.(V)b* $^{DTH+}$ (hypersensitivity polo).

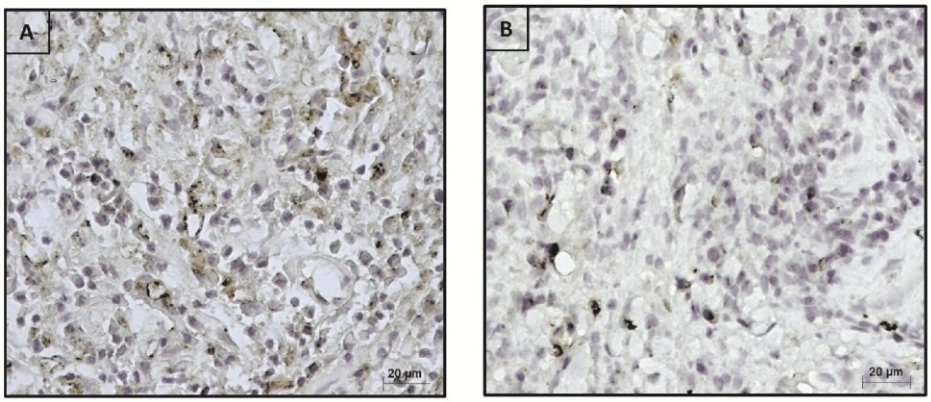
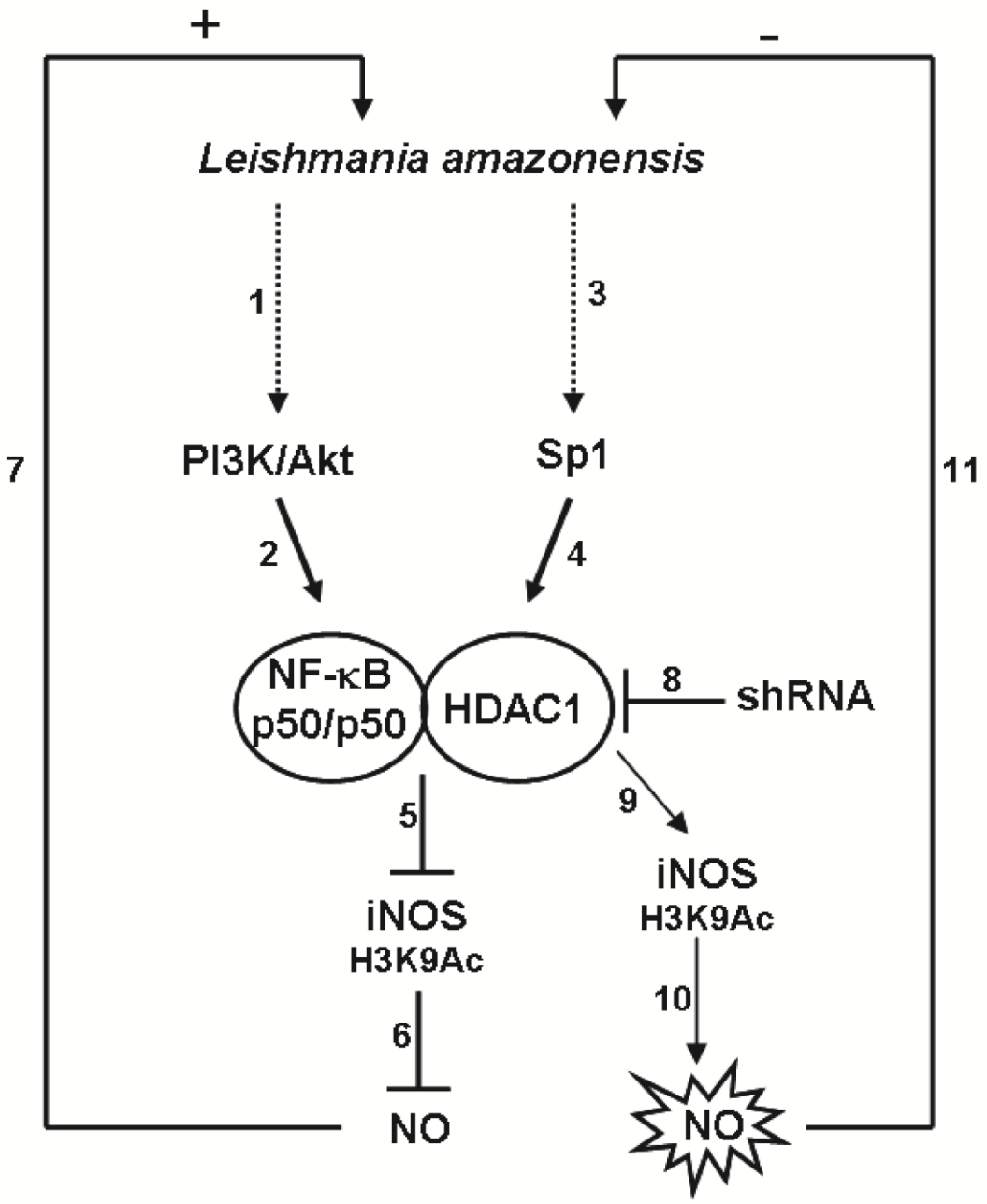


FIGURE 7. Proposed model for HDAC1 in *L. amazonensis* infection. As previously described, *L. amazonensis* infection induces PI3K/Akt pathway (1) to promote the formation of p50/ p50 NF-κB homodimers (2). In this work, we demonstrated that *L. amazonensis* through Sp1 induction (3) increases HDAC1 expression (4). We also verified that the infection induces the formation of HDAC1-p50/p50 complex, which determines iNOS promoter repression associated with the low levels o H3K9Ac (5) and the reduction of NO production (6) favoring the replication the parasites (7). On the other hand, the reduction of HDAC1 expression using shRNA (8), revert the iNOS inhibition and increase the H3K9Ac levels at this promoter (9) leading to NO production (10) and culminates in a negative effect on intracellular *Leishmania* proliferation (11).



Graphic Abstract

Leishmania amazonensis infection induces NF-κB p50/p50 and increases the nuclear levels of HDAC1 in macrophages. In the nucleus, p50/50 binds to *nk* promoter sequences and recruits HDAC1, forming a repression complex leading to H3K9 deacetylation and consequent iNOS promoter silencing. The absence of Nitric Oxide production favors the amastigote growth.

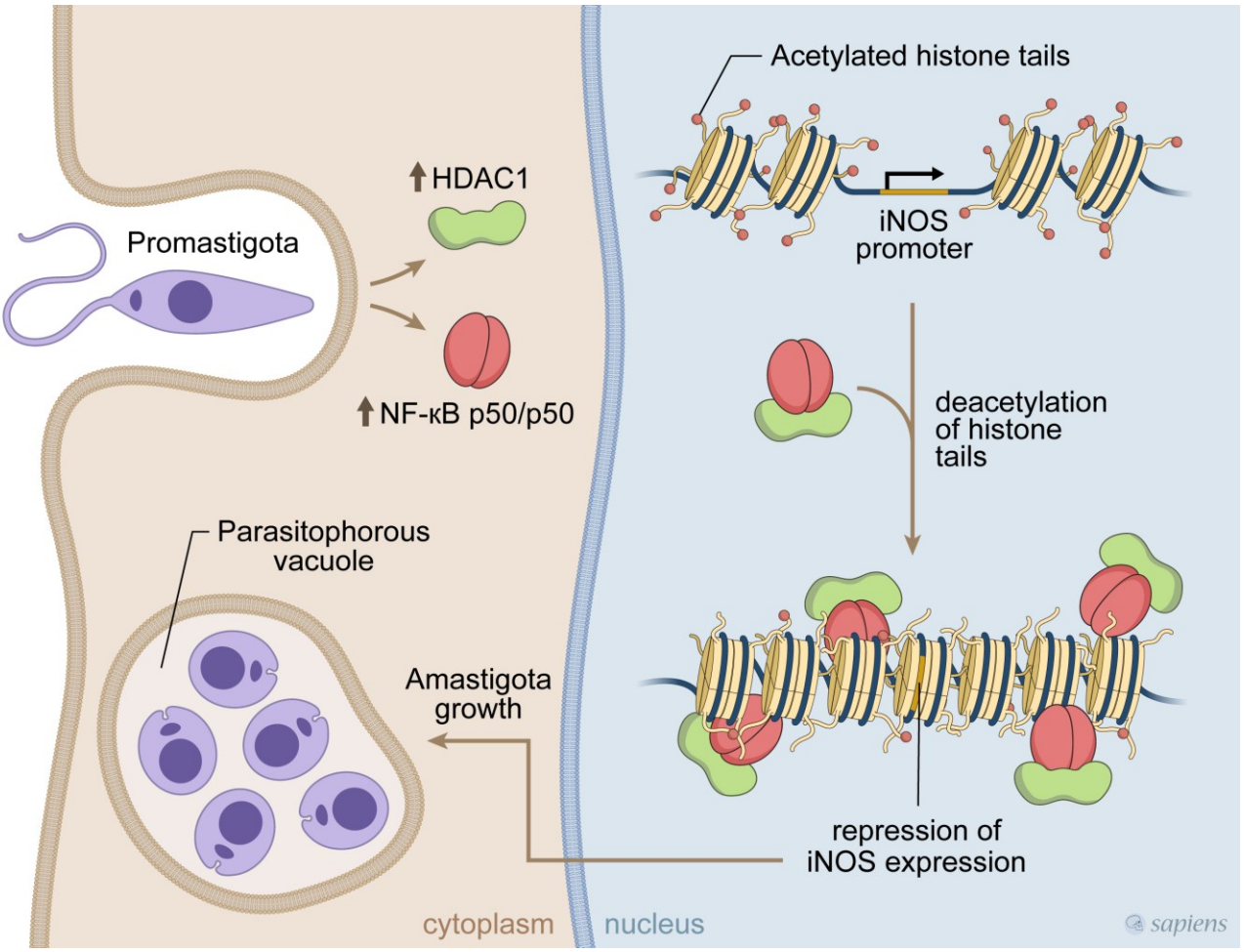


Table 1: Clinical and epidemiological characteristics of patients with American cutaneous leishmaniasis caused by *L. (L.) amazonensis* (hyposensitivity polo DTH-) and *L. (V.) braziliensis* (hypersensitivity polo DTH+)

Hyposensitivity polo	**Patient**	**Clinical form**	**Age (year)**	**Sex**	**State, Country**	**Time of disease (month/ year)**	**Lesion type**	**Number of lesion**	**Parasite demonstration**	**DTH**
La(LCL-)	1	LCL	21	M	Pará, Brazil	6 m	ulcered	1	positive	negative
La(LCL-)	2	LCL	10	M	Pará, Brazil	2 m	ulcered	1	positive	negative
La(LCL-)	3	LCL	41	M	Pará, Brazil	2 m	ulcered	4	positive	negative
La(LCL-)	4	LCL	28	M	Pará, Brazil	9 m	ulcered	1	not performed	positive
La(LCL-)	5	LCL	36	M	Pará, Brazil	6 m	ulcered	1	positive	positive
La(LCL-)	6	LCL	41	M	Pará, Brazil	2 m	ulcered	4	positive	negative
La(LCL-)	7	LCL	47	M	Pará, Brazil	2 m	ulcered	1	positive	negative
La(LCL-)	8	LCL	29	F	Pará, Brazil	5 m	ulcered	1	positive	negative
La(LCL-)	9	LCL	34	M	Pará, Brazil	3 m	ulcered	2	positive	negative
La(BDCL-)	10	BDCL	57	M	Pará, Brazil	1 y	infiltrated and nodular	2	positive	negative
La(BDCL-)	11	BDCL	33	M	Pará, Brazil	2 y	infiltrated and nodular	countless	positive	negative

La (BDCL-)	12	BDCL	49	M	Pará, Brazil	5m	ulcered	3	positive	positive
La (ADCL-)	13	ADCL	42	M	Pará, Brazil	35 y	nodular and vegetative	countless	positive	negative
La (ADCL-)	14	ADCL	31	M	Pará, Brazil	28 y	nodular and vegetative	countless	positive	negative
La (ADCL-)	15	ADCL	59	M	Amapá, Brazil	20 y	Acneform, infiltrated and nodular	countless	positive	negative
La(ADCL-)	16	ADCL	9	M	Pará, Brazil	1 y	vegetative	countless	positive	negative
La(ADCL-)	17	ADCL	45	F	Maranhão, Brazil	20y	nodular and vegetative	countless	positive	negative
La(ADCL-)	18	ADCL	24	M	Maranhão, Brazil	19y	nodular and vegetative	countless	positive	negative
Hypersensitivity polo										
Lb(LCL+)	19	LCL	18	M	Pará, Brazil	3m	ulcered	3	positive	positive
Lb(LCL+)	20	LCL	40	M	Pará, Brazil	17m	ulcered	2	negative	positive
Lb(LCL+)	21	LCL	20	M	Pará, Brazil	2 m	ulcered	1	positive	not performed
Lb(LCL+)	22	LCL	63	F	Pará, Brazil	6 m	ulcered	1	positive	positive

Lb (LCL+)	23	LCL	30	M	Pará, Brazil	3 m	ulcered	1	positive	not performed
Lb (LCL+)	24	LCL	31	M	Pará, Brazil	2 m	ulcered	1	negative	positive
Lb (LCL+)	25	LCL	31	M	Pará, Brazil	5 m	ulcered	2	negative	positive
Lb (LCL+)	26	LCL	46	M	Pará, Brazil	3 m	ulcered	1	positive	positive
Lb (BDCL+)	27	BDCL	48	M	Pará, Brazil	29 m	papulo-ulcerative	6	positive	positive
Lb (BDCL+)	28	BDCL	61	M	Pará, Brazil	3 m	papulo-ulcerative	countless	positive	not performed
Lb (LCM+)	29	MCL	41	M	Pará, Brazil	2 y	infiltrated granulomatous	1	positive	positive
Lb (LCM+)	30	MCL	27	M	Pará, Brazil	1 y	infiltrated granulomatous	2	positive	positive