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Heme Oxygenase-1 Promotes the Persistence of *Leishmania chagasi* Infection

Nívea F. Luz,[*,†] Bruno B. Andrade,[‡] Daniel F. Feijó,[§] Théo Araújo-Santos,[*,†] Graziele Q. Carvalho,[*,†] Daniela Andrade,[*,†] Daniel R. Abánades,[*,†] Enaldo V. Melo,[¶] Angela M. Silva,[¶] Cláudia I. Brodskyn,[*,†,‖] Manoel Barral-Netto,[*,†,‖] Aldina Barral,[*,†,‖] Rodrigo P. Soares,[#] Roque P. Almeida,[¶,‖] Marcelo T. Bozza,[§] and Valéria M. Borges[*,†,‖]

Visceral leishmaniasis (VL) remains a major public health problem worldwide. This disease is highly associated with chronic inflammation and a lack of the cellular immune responses against *Leishmania*. It is important to identify major factors driving the successful establishment of the *Leishmania* infection to develop better tools for the disease control. Heme oxygenase-1 (HO-1) is a key enzyme triggered by cellular stress, and its role in VL has not been investigated. In this study, we evaluated the role of HO-1 in the infection by *Leishmania infantum chagasi*, the causative agent of VL cases in Brazil. We found that *L. chagasi* infection or lipophosphoglycan isolated from promastigotes triggered HO-1 production by murine macrophages. Interestingly, cobalt protoporphyrin IX, an HO-1 inductor, increased the parasite burden in both mouse and human-derived macrophages. Upon *L. chagasi* infection, macrophages from *Hmox1* knockout mice presented significantly lower parasite loads when compared with those from wild-type mice. Furthermore, upregulation of HO-1 by cobalt protoporphyrin IX diminished the production of TNF-α and reactive oxygen species by infected murine macrophages and increased Cu/Zn superoxide dismutase expression in human monocytes. Finally, patients with VL presented higher systemic concentrations of HO-1 than healthy individuals, and this increase of HO-1 was reduced after antileishmanial treatment, suggesting that HO-1 is associated with disease susceptibility. Our data argue that HO-1 has a critical role in the *L. chagasi* infection and is strongly associated with the inflammatory imbalance during VL. Manipulation of HO-1 pathways during VL could serve as an adjunctive therapeutic approach. *The Journal of Immunology*, 2012, 188: 4460–4467.

Visceral leishmaniasis (VL) continues to be a major health threat worldwide and is classified as one of the most neglected diseases by the World Health Organization. VL is a chronic infection clinically characterized by progressive fever, weight loss, splenomegaly, hepatomegaly, anemia, and spontaneous bleeding associated with marked inflammatory imbalance (1). The hallmark of this disease is thought to be a lack of cellular immune responses against the parasite and high systemic levels of IFN-γ and IL-10 (2). The New World *Leishmania infantum chagasi* is the major species implicated in the VL in Brazil. *Leishmania* parasites are obligate intracellular protozoa that replicate preferentially inside macrophages (3). It is well known that *L. chagasi* is able to evade pro-oxidative responses and other macrophage effectors mechanisms (4), possibly hampering the activation of adaptive immune responses against infection (5). During parasite–host interactions, complex signaling pathways are triggered by the recognition of key molecules from parasite (4). In this context, lipophosphoglycan (LPG), a glycoconjugate expressed on the surface of *Leishmania* parasites and TLR2 agonist (6, 7), has been implicated in the modulation of a wide range of innate immune functions. Those may include resistance to complement, attachment and entry into macrophages, protection against proteolytic damage within acidic vacuoles (8), inhibition of phagosomal maturation (9), modulation of NO and IL-12 production (10–13), inhibition of protein kinase C (14), induction of neutrophil extracellular traps (15), and induction of protein kinase R (16). However, specific aspects of how the parasites regulate some protective responses are still unknown. Moreover, it is not fully understood whether LPG from *Leishmania* is the major regulator of the effectors pathways associated with the protective responses against this protozoan.

Excess of heme is very hazardous for the cells, and we have previously shown that heme suppresses some anti-inflammatory mediators in human malaria caused by *Plasmodium vivax* (17). Heme oxygenase-1 (HO-1) is a stress-responsive enzyme that

*Centro de Pesquisas Gonçalo Moniz/Fundação Oswaldo Cruz, Salvador 40295-001, Brazil; †Universidade Federal da Bahia, Salvador 40110-060, Brazil; ‡Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; §Departamento de Imunologia, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-590, Brazil; ¶Department of Medicine, University Hospital, Universidade Federal de Sergipe, Aracaju 49010-390, Brazil; ‖Instituto Nacional de Ciência e Tecnologia de Investigação em Imunologia, Salvador, Bahia 40110-100, Brazil; and #Centro de Pesquisas René Rachou/Fundação Oswaldo Cruz, Belo Horizonte 30190-002, Brazil

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Address correspondence and reprint requests to Dr. Valéria M. Borges, Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, 121, Candeal, Salvador, Bahia 40295-001, Brazil. E-mail address: vborges@bahia.fiocruz.br

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Abbreviations used in this article: BMM, bone marrow-derived macrophage; CoPP, cobalt protoporphyrin IX; DHE, dihydroethidium; HC, healthy control; HO-1, heme oxygenase-1; LPG, lipophosphoglycan; PPARγ, peroxisome proliferator-activated receptor γ; PTX, pentoxifylline; ROC, receiver-operator characteristic; ROS, reactive oxygen species; SOD-1, Cu/Zn superoxide dismutase; VL, visceral leishmaniasis; WT, wild-type.

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metabolizes heme and releases free iron, carbon monoxide, and biliverdin, which rapidly undergoes conversion to bilirubin (18). Recently, the HO-1 isoform encoded by the *Hmox1* gene has emerged as a key regulator of inflammation by its anti-inflammatory, cytoprotective, antiapoptotic, and antiproliferative effects. Interestingly, HO-1 seems also to modulate innate as well as adaptive immunity (19). Studies have emphasized the participation of HO-1 in host-tolerance mechanisms facing infections by means of its heme detoxifying activity (20). Therefore, HO-1 can overcome the pathogenesis of a variety of immune system-mediated inflammatory conditions, such as malaria (20, 21), ischemia/reperfusion injury (22), intrauterine fetal growth restriction (23), sepsis (24, 25), graft rejection (26, 27), and sickle hemoglobin (28). Intriguingly, the immunomodulatory effects of HO-1 can drive both beneficial and detrimental consequences in the host immunity against infections agents (reviewed in Ref. 29). In fact, HO-1 protects *Plasmodium*-infected hepatocytes, thereby promoting the establishment of those parasites (30). In contrast, HO-1 enhances bacterial clearance during polymicrobial sepsis caused by cecal ligation and puncture (24), arguing that this antioxidant enzyme plays an important role in the antimicrobial process without inhibiting the inflammatory response (i.e., resistance to infection).

Despite the recognition of the importance of HO-1 in immunoregulatory mechanisms, the direct role of this enzyme in the host cell–*Leishmania* interplay has not been addressed. Pham and colleagues (31) reported that during infection of macrophages with *L. pifanoi*, HO-1 is involved in the suppression of superoxide production by inducing heme degradation, which hampers the maturation of $gp91^{phox}$, a subunit of NADPH oxidase enzyme complex. However, it was not clear whether this event had any impact in parasite survival or cytokine production.

In the current study, we report that both *L. chagasi* and LPG isolated from promastigotes induce HO-1 expression in murine macrophages. Interestingly, stimulation of macrophages with cobalt protophorphyrin IX (CoPP), a pharmacologic inductor of HO-1, resulted in a significant increase of the parasite burden. Upon *L. chagasi* infection, bone marrow-derived macrophages (BMMs) from $Hmox1^{-/-}$ mice showed lower parasite loads then macrophages from wild-type (WT) mice. Finally, we found that HO-1 is strongly associated with human VL in a cohort of patients from a highly endemic area in Brazil. These results represent the first evidence, to our knowledge, for the importance of HO-1 in regulating host immune responses to *L. chagasi* infection. Our study opens up new perspectives suggesting that HO-1 might be a therapeutic target for human VL.

Materials and Methods

Reagents

CoPP (Frontier Scientific, Logan, UT) was dissolved in 0.1 N NaOH and RPMI 1640 medium (Invitrogen, Carslbad, CA) and adjusted to concentrations of 50 μM for in vitro assays. Rosiglitazone and GW9662 were obtained from Cayman Chemical (St. Louis, MO) and dissolved in DMSO from ACROS Organics (New Jersey, NJ). RPMI 1640 medium, L-glutamine, penicillin streptomycin, and dihydroethidium (DHE) were from Invitrogen. The following primary Abs were used: anti-mouse HO-2 from R&D Systems (Minneapolis, MN) and anti-mouse β-actin from Cell Signaling Technology (Ann Arbor, MI). Schneider's insect medium, *Escherichia coli* LPS (serotype 0127:b8), IFN-γ, and pentoxifylline (PTX) were purchased from Sigma-Aldrich (St. Louis, MO). Human HO-1 ELISA kit was from Assay Designs (Ann Arbor, MI), and mouse HO-1 ELISA kit was from Takara Bio (Madison, WI). The Cu/Zn superoxide dismutase (SOD-1) Protein ELISA kit was purchased from Calbiochem (San Diego, CA). Cell Proliferation Kit II XTT was from Roche Applied Science (Indianapolis, IN), and Cytometric Bead Array mouse inflammation kit was from BD Biosciences (San Jose, CA).

Parasites and LPG

L. infantum chagasi (MCAN/BR/89/Ba262) (referred throughout the text as *L. chagasi*) promastigotes at stationary phase were cultured at 24°C in Schneider's insect medium supplemented with 20% inactive FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. LPG was isolated and purified from Ba262 *L. chagasi* strain following previously described protocol (13).

Mice

Inbred male or female C57BL/6 mice aged 8–10 wk were obtained from the animal facility of the Centro de Pesquisas Gonçalo Moniz/Fundação Oswaldo Cruz, Salvador, Brazil. All experimental procedures were approved and conducted according to the Animal Care Committee of the Centro de Pesquisas Gonçalo Moniz (L-IGM-024/2009). $Hmox1^{-/-}$ mice bone marrow, a generous gift from Dr. Miguel Soares (Instituto Gulbenkian de Ciência, Oeiras, Portugal), was isolated by crushing femur bones from 10–15-wk-old WT or $Hmox1^{-/-}$ from SCID or BALB/c mice.

Mouse and human macrophages

C57BL/6 mice were injected i.p. with 3% thioglycolate solution. Four days after injection, peritoneal lavage was performed using 8 ml RPMI 1640 medium supplemented with 1% Nutridoma-SP, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. BMMs from $Hmox1^{-/-}$ and $Hmox1^{+/+}$ mice from both BALB/c and SCID strains were cultured in RPMI 1640 medium containing 20% FBS, gentamicin, HEPES, and 20% L929-conditioned medium for 7 d. Human monocytes were isolated from peripheral blood of healthy donors through Ficoll gradient centrifugation and plastic adherence; cells were cultivated in RPMI 1640 medium supplemented with 10% FBS for 7 d to obtain differentiated macrophages in vitro. In some experiments, total PBMCs from normal blood donors were infected with *L. chagasi* in the presence of 1 mM PTX, an inhibitor of TNF-α production, for 12 h.

Infection assays

Macrophages (3×10^5) were seeded onto glass coverslips in 24-well plates. Cells were allowed to adhere for 2 h at 37°C and 5% CO_2; nonadherent cells were removed by washing each well with sterile saline. *L. chagasi* promastigotes in early stationary phase were added to macrophage cultures at a macrophage/parasite ratio of 1:10 in RPMI 1640 medium supplemented with 10% inactive FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Macrophages (1×10^6) were seeded on 48-well plates in the experiments in which the cell lysate or supernatants were used for HO-1 or cytokine measurements. The pharmacological modulation of HO-1 during the *L. chagasi* infection was performed in presence of CoPP, an inductor of HO-1 production (32). After 4 h, infected cells were washed to remove extracellular parasites, fresh medium was replaced with the same stimuli, and plates were returned to the incubator until the desired time. After 4, 24, 48, and 72 h, supernatants were harvested and cleared by centrifugation and stored at −20°C. Cells on glass coverslips were fixed with methanol and stained by Diff-Quick (American Scientific Products, McGraw Park, IL). Intracellular amastigotes were counted under light microscopy in 200 macrophages per slide in a blind fashion manner. Results are shown as amastigote number per 100 macrophages and percentage of infected macrophages in relation to control (group infected only with *L. chagasi*). Alternatively, intracellular load of *L. chagasi* was estimated by production of viable promastigotes in Schneider medium as described previously (33). Briefly, after 72 h of infection, RPMI 1640 medium was replaced by Schneider medium supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and the plate was then kept at 24°C. Intracellular survival of *L. chagasi* was quantified by counting proliferating extracellular motile promastigotes in a Neubauer hemocytometer until the seventh day after the medium replacement. To rule out a possible toxicity induced in cells or parasites treated with porphyrins, we performed a cell viability assay using XTT (Roche Applied Science). In some experiments, macrophages were incubated with LPS (100 ng/ml) or pretreated overnight with an antagonist (5 μM GW9662) or an agonist (10 μM rosiglitazone) of the peroxisome proliferator-activated receptor γ (PPARγ).

HO-1 measurement by ELISA

Murine and human macrophages were infected with *L. chagasi* or treated with LPG and HO-1 was measured in supernatants or cell lysates obtained through the use of lysis buffer available in murine and human HO-1 ELISA kit, following the manufacturer's instructions.

Measurements of the inflammatory mediators

TNF-α, IL-6, MCP-1, and IL-10 were measured in cell supernatants using a cytometric bead array mouse inflammation kit. NO production was measured in the supernatants of *L. chagasi*-infected macrophages pretreated with 100U/ml IFN-γ by the Griess method, as described elsewhere (34, 35). Intracellular reactive oxygen species (ROS) levels were measured by staining with the oxidative fluorescent dye probe DHE 5 μM (Invitrogen/Molecular Probes, Grand Island-NY) for 30 min at 37°C and then analyzed using flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA) using the FL2 emission filter. Data were displayed as histograms, and the geometric median fluorescence intensity was evaluated using FlowJo software (Tree Star, Ashland, OR).

HO-1 measurements in VL serum samples

Serum samples were obtained from patients with VL (n = 52) and sex-matched endemic healthy controls (HC) (n = 42) from an endemic area in the Northeast of Brazil. The baseline characteristics of the study participants are shown in the Supplemental Table I. The patients with VL were followed up and HO-1 was measured before the antileishmanial therapy and after the treatment was finished due to clinical cure. HO-1 was measured using a human ELISA kit following the manufacturer's instructions. This study was approved by Institutional Review Board of Federal University of Sergipe, Brazil, where the field study was performed. All clinical investigations were conduced according to the Declaration of Helsinki. Written informed consent was obtained from all participants or legal guardians.

Statistical analysis

Each experiment was performed at least three times, and at least five mice were used in each experimental group. Data are reported as mean ± SD of representative experiments and were analyzed using GraphPad Prism Software 5.0 (GraphPad, San Diego, CA). After performing a normality test, Kruskal–Wallis nonparametric test followed by Dunn's posttest or linear trend analysis were used to evaluate statistical significance among the groups. In some assays, comparisons between two groups were explored using the Mann–Whitney U test. Correlations among HO-1 and IL-10, TNF-α, and IL-6 were performed using the Spearman test. The Wilcoxon matched pairs test was performed to estimate statistical significance before and after the antileishmanial treatment. Receiver-operator characteristic (ROC) curves with C-statistics were used to establish the threshold value of HO-1 able to discriminate between VL and HC. A logistic regression model adjusted for age was also applied to check the strength of the association between HO-1 systemic concentrations and the occurrence of VL. A p value <0.05 was considered statistically significant.

Results

L. chagasi *infection enhances HO-1 expression by mouse macrophages*

Crescent concentrations of HO-1 protein were detected in mouse peritoneal macrophages infected with *L. chagasi* compared with uninfected cells (Fig. 1A, 1B), with significant trend to increase over time postinfection (linear trend $p < 0.0001$) in either cell-culture supernatants (Fig. 1A) or cell lysates (Fig. 1B). In addition, infected macrophages treated with 50 μM CoPP, an inductor of HO-1 (32), amplified the production of HO-1 (Fig. 1C, 1D), when compared with *L. chagasi*-infected macrophage alone. Because HO-1 is a microsomal enzyme, and we were able to detect HO-1 protein in the culture supernatants, cell death could be occurring during in vitro infection. To rule out an important toxic effect of CoPP in cell culture, we tested whether treatment with this porphyrin could affect cell viability. The stimulus was not significantly toxic for either infected macrophages (Supplemental Fig. 1A) or *Leishmania* parasites (Supplemental Fig. 1B). As expected, HO-2, the constitutive form of HO, remained unchanged in both infected macrophages or with CoPP treatment (data not shown). Therefore, we conclude that mouse macrophages display consistently high amounts of HO-1 upon infection with *L. chagasi*.

HO-1 promotes L. chagasi *infection in macrophages*

Once inside macrophages, *Leishmania* parasites may circumvent various host defense mechanisms to survive. Because HO-1 induction is associated with the persistence of *Plasmodium* in the liver (30) and also favors *Mycobacterium tuberculosis* inside macrophages during a latent infection via induction of the dormancy-associated genes (36), we hypothesized that the elevated concentrations of HO-1 detected in infected macrophages treated with CoPP could bring key benefits for the *Leishmania*. To address this question, we infected murine macrophages in vitro in presence of CoPP and evaluated the parasite load. Our experiments revealed that induction of HO-1 led to an increased in the percentage of infected macrophages (Fig. 2A), number of intracellular amastigotes (Fig. 2B), and viability of *L. chagasi* inside macrophages (Fig. 2C), whereas inhibition of HO-1 activity by Tin protoporphyrin had no impact on the parasite burden (data not shown). Interestingly, BMMs from *Hmox1*$^{-/-}$ mice from either BALB/c (Fig. 2D) or SCID (Fig. 2E) mouse strains presented significantly reduced parasite burden compared with the strain-matched WT. Infected BMMs from *Hmox1*$^{-/-}$ mice from both genetic backgrounds incubated or not with CoPP displayed reduced *Leishmania* viability at different time points postinfection, confirming that the primary effect of CoPP on parasite survival within macrophages was due to the induction of HO-1 (Fig. 2D, 2E). These results are consistent with the idea that induction of HO-1 by infected macrophages is a key event promoting *Leishmania* survival.

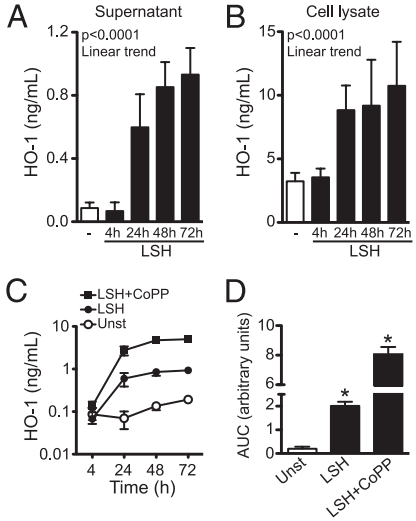

FIGURE 1. *Leishmania* infection induces HO-1 by macrophages. C57BL/6 peritoneal macrophages were infected with *L. chagasi* (LSH) at a multiplicity of infection of 10. HO-1 production was analyzed in cell-culture supernatant (**A**) and cell lysate (**B**) at 4, 24, 48, and 72 h postinfection. Data were compared using one-way ANOVA with linear trend posttest. Macrophages infected with *L. chagasi* promastigotes in the presence of 50 μM CoPP were tested for HO-1 release in cell-culture supernatants at different time points poststimulation (**C**). HO-1 release was induced by CoPP in response to *L. chagasi* infection (**D**), as showed by the area under the curve (AUC); Mann–Whitney U test was used for the pairwise comparisons. Data are from one representative experiment out of three experiments performed with at least five mice per experimental group. Bars represent mean ± SD. *$p < 0.05$.

HO-1 regulates the production of proinflammatory mediators by L. chagasi*-infected macrophages*

It is well established that the essential mechanisms of protection against *Leishmania* involves activation of macrophages and production of proinflammatory cytokines, ROS (37, 38), and NO (39). In this study, we addressed whether the role of HO-1 in promoting *Leishmania* persistence within macrophages involves regulation of proinflammatory cytokines and/or oxidative stress. Induction of HO-1 by CoPP decreased production of TNF-α (Fig. 3A), but did

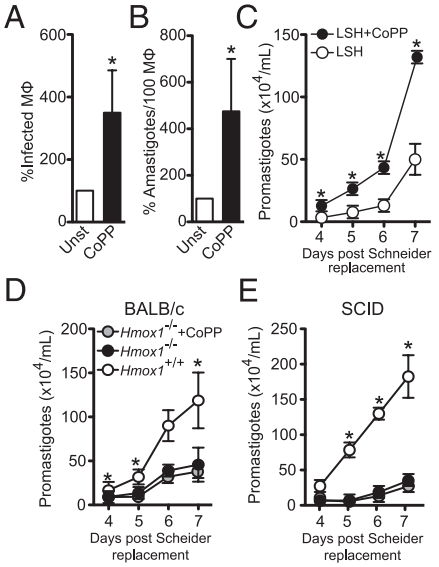

FIGURE 2. HO-1 promotes *Leishmania* persistence in infected macrophages. Macrophages were infected in vitro with *L. chagasi* (multiplicity of infection 10) in the absence or presence of 50 μM of CoPP. The parasite load was measured by optical microscopy at 72 h postinfection as described in *Materials and Methods*. The percentage of *L. chagasi* (LSH)-infected macrophages (MΦ) (**A**) and the number of amastigotes per 100 macrophages (**B**) are displayed as percentage of control (group infected only with *L. chagasi*). (**C**) After 72 h of infection, RPMI 1640 medium was replaced by Schneider (*Leishmania* medium), and extracellular *L. chagasi* promastigotes were counted following 4, 5, 6, and 7 d. Data were analyzed using the Kruskal–Wallis test. Bars represent mean ± SD. BMMs from *Hmox1*$^{-/-}$ mice from both BALB/c (**D**) and SCID (**E**) genetic backgrounds or from strain-matched WT mice were infected with *L. chagasi* and CoPP. Parasite load was measured by the Schneider method; after 72 h of infection, the RPMI 1640 medium was replaced by Schneider medium, and extracellular *L. chagasi* promastigote number was measured 4, 5, 6, and 7 d postreplacement. Data were evaluated using Kruskal–Wallis test. Points and error lines represent mean ± SD. Data are from one representative experiment out of three experiments performed with at least five mice per experimental group. $*p < 0.05$.

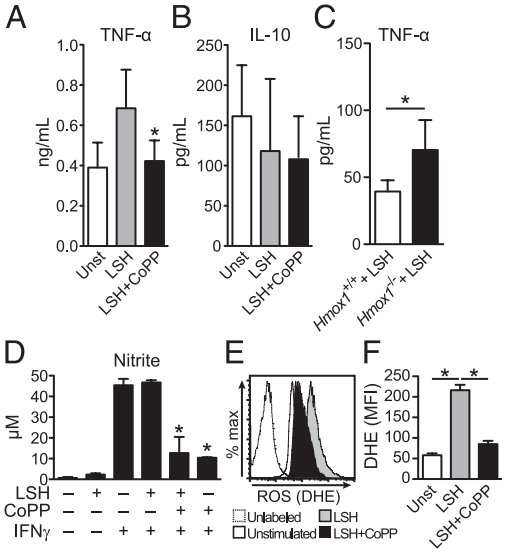

FIGURE 3. HO-1 regulates production of proinflammatory mediators by macrophages infected with *Leishmania*. Quantification of TNF-α (**A**) and IL-10 (**B**) in the supernatants from macrophages infected in vitro with *L. chagasi* in the presence or absence of 50 μM CoPP. (**C**) TNF-α production by BMMs from WT or *Hmox1*$^{-/-}$ mice infected in vitro with *L. chagasi*. (**D**) Murine peritoneal macrophages were stimulated with IFN-γ (100 U/ml) during in vitro infection in the presence or absence of 50 μM CoPP, and supernatants were harvested after 48 h; nitrite was measured using Griess reaction. Representative histograms (**E**) and median fluorescence intensity (MFI) (**F**) for ROS in macrophages infected with *L. chagasi* in the presence or absence of CoPP evaluated by flow cytometry using the probe DHE. (F) MFI of DHE-stained macrophages. Data are from one representative experiment out of three experiments performed with at least five mice per experimental group. Bars represent mean ± SD. Kruskal–Wallis test was used to compare experimental groups. $*p < 0.05$.

not change the amounts of IL-10 (Fig. 3B) at 24 h post-*L. chagasi* infection. BMMs from *Hmox1*$^{-/-}$ (SCID genetic background) produced higher amounts of TNF-α than WT cells upon infection (Fig. 3C), reinforcing the participation of HO-1 in the modulation of TNF-α production. Moreover, macrophages primed with IFN-γ presented significantly diminished NO production when treated with CoPP (Fig. 3D). Additionally, we tested if the modulation of the proinflammatory mediators induced by HO-1 could be robust enough to revert the inflammatory profile of infected macrophages primed with LPS. Indeed, induction of HO-1 by CoPP consistently increased parasite burden (Supplemental Fig. 2A) and reduced production of TNF-α (Supplemental Fig. 2B), NO (Supplemental Fig. 2E), IL-6 (Supplemental Fig. 2F), and MCP-1 (Supplemental Fig. 2G), but not IL-10 (Supplemental Fig. 2C) by these cells. Thus, a higher IL-10/TNF-α ratio was found in infected macrophages primed with LPS upon induction of HO-1 (Supplemental Fig. 2D). In addition, CoPP treatment reduced *L. chagasi*-induced ROS production by macrophages (Fig. 3E, 3F). These data suggest that HO-1 promotes *Leishmania* survival within macrophages by precluding inflammation and oxidative stress.

L. chagasi *promastigotes and LPG induce HO-1 independently of PPARγ activation*

LPG from the cell surface of *Leishmania* promastigotes has been described as a major virulence factor (40), involved in the ability to induce lesions in mice (41) and activation of immune cells (15). To test if this virulence factor from the parasite surface could be able to induce HO-1, we incubated macrophages with LPG isolated from the *L. chagasi* strain Ba262. Indeed, LPG was capable of inducing HO-1 production by mouse macrophages (Fig. 4B). HO-1 expression is transcriptionally regulated by PPARγ (42). Because PPARγ is induced by *L. donovani* and could exacerbate the disease in a chronic experimental model of VL (43), we determined whether PPARγ was involved in the induction of HO-1 by *L. chagasi* and its LPG. Interestingly, induction of HO-1 by macrophages stimulated with *L. chagasi* (Fig. 4A) or LPG (Fig. 4B) persisted in the presence of a PPARγ antagonist (GW 9662) or an agonist (rosiglitazone). Thus, the induction of HO-1 does not seem to be regulated by PPARγ in the context of *L. chagasi* infection.

HO-1 promotes Leishmania *persistence in human macrophages*

To verify if our results were reproducible in human macrophages, we performed in vitro infections with *L. chagasi* in the presence of CoPP. Induction of HO-1 favored increased percentage of infected macrophages (Fig. 5A), number of intracellular amastigotes (Fig. 5B), and the viability of *L. chagasi* inside macrophages (Fig. 5C). With an attempt to verify a potential mechanism by which the induction of HO-1 favors *L. chagasi* persistence inside the human macrophages, we tested surrogates of the oxidative stress. Treatment of the cells with CoPP led to reduction in the production of superoxide radicals triggered by *L. chagasi* infection (Fig. 5D). Interestingly, some antioxidant mechanisms of HO-1 require interplay with another class of antioxidant enzyme, the superoxide

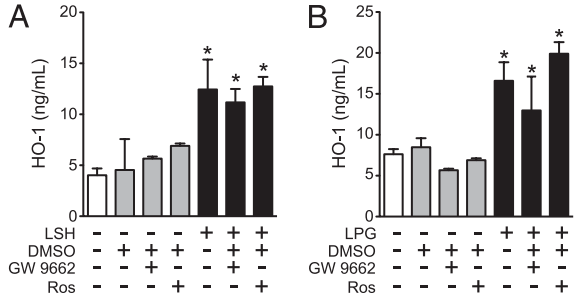

FIGURE 4. LPG from *Leishmania* induces HO-1 in macrophages. Murine peritoneal macrophages were pretreated with PPARγ agonist (5 μM rosiglitazone) or antagonist (10 μM GW9662) following interaction with *L. chagasi* promastigotes (LSH) (**A**) or LPG (**B**). HO-1 was measured in cell lysates at 48 h poststimulation. Data are from one representative experiment out of three experiments performed with at least five mice per experimental group. Bars represent mean ± SD. Kruskal–Wallis test was used to compare the experimental groups. *$p < 0.05$ when compared with untreated macrophages.

dismutase (44, 45). Therefore, we investigated the association between the induction of HO-1 and SOD-1. Notably, SOD-1 has been shown to favor *Leishmania* growth in human macrophages (35). We found hereby that the induction of HO-1 by CoPP upon *L. chagasi* infection is also associated with induction of SOD-1 protein (Fig. 5E) in human macrophages. These findings argue that induction of HO-1 favors *Leishmania* survival by its antioxidant activities per se and also by the induction of superoxide dismutases, which are potent scavengers of superoxide anions generated during infection.

HO-1 is strongly associated with human visceral leishmaniasis

The ultimate goal of our current investigation was to investigate whether HO-1 was associated with the human disease caused by *L. chagasi* infection. We then evaluated serum samples obtained from patients with VL and sex-matched endemic controls from a highly endemic area in the northeast of Brazil. The baseline characteristics of the study participants are shown in Supplemental Table I. Patients with VL presented higher serum concentrations of HO-1 compared with healthy individuals ($p < 0.0001$; Fig. 6A). A logistic regression adjusted for age revealed that HO-1 is indeed strongly associated with VL (odds ratio: 30.28; 95% confidence interval: 12.72–45.01; $p < 0.0001$). The systemic concentrations of HO-1 were significantly reduced 15–30 d after the antileishmanial treatment ($p < 0.01$; Fig. 6B). An analysis using ROC curves revealed that HO-1 could be used to discriminate patients with VL from those uninfected in the cohort studied (Fig. 6C). In individuals with VL, HO-1 presented positive correlation with IL-10 ($r = 0.56$; $p < 0.0001$; Fig. 6F), a major factor involved in the clinical severity of VL (46, 47). HO-1 was also correlated with diverse markers of systemic inflammation, such as TNF-α ($r = 0.23$; $p = 0.0117$; Fig. 6D), IL-6 ($r = 0.58$; $p < 0.0001$; Fig. 6G), and IL-8 ($r = 0.44$; $p < 0.0001$; Fig. 6H). These results reinforce the notion that HO-1 is associated with *L. chagasi* in humans and that HO-1 may be linked to susceptibility to human VL. Given that HO-1 is a stress-responsive gene, this finding is consistent with the notion that the disease is associated with increased oxidative stress, thereby explaining the increased level of expression of this enzyme. As expected, after the antileishmanial treatment, systemic levels of TNF-α were reduced ($p < 0.001$; Fig. 6E) and no longer correlated with HO-1 ($r = 0.4920$; $p = 0.0625$). The next step was to test whether it is possible to interfere with HO-1 expression by inhibiting proinflammatory cytokines in human cells. We found that treatment of human PBMC with PTX, a pharmacological TNF-α inhibitor with wide clinical use, resulted in reduction of HO-1 production upon infection with *L. chagasi* (Supplemental Fig. 3A, 3B).

Discussion

Studies have emphasized that the cytoprotective enzyme HO-1 plays a pivotal role in maintaining cellular homeostasis during inflammation (48), and its expression is increased in a variety of pathological conditions (29). The protective actions of HO-1 during infection are usually associated with the reduction in immunopathology caused by the oxidative stress (49, 50). Indeed, a higher expression of HO-1 results in diminished damage of cells and tissues even at the relatively high infection burden, what is called tolerance to infection (20, 51). In this study, we show that HO-1 seems to affect host effector molecules that drive resistance to infection, such as inflammatory cytokines and free radicals, which favors *Leishmania* persistence. Our results also suggest that HO-1 may play an essential dual role during *Leishmania* infection. On one hand, HO-1 can protect host tissues against injury by damping excess of inflammation. On the other hand, induction of HO-1 protects the parasite against the host defense. Similarly, upon *Plasmodium* infection, induction of HO-1 seems to be an

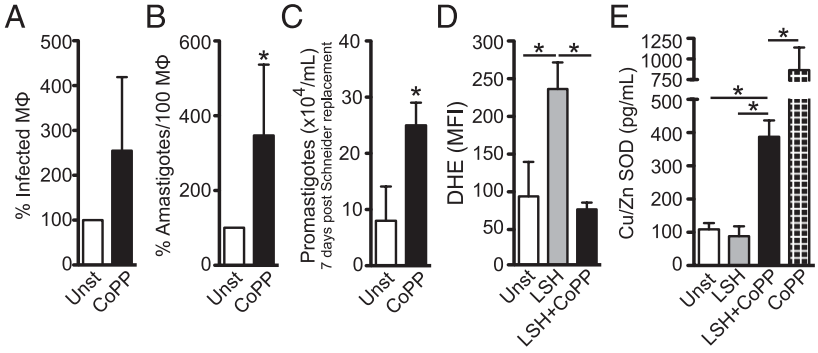

FIGURE 5. HO-1 promotes *Leishmania* persistence in infected human macrophages. Monocyte-derived human macrophages infected with *L. chagasi* were cultured with or without 50 μM CoPP for 72 h, and the percentage of infected macrophages (**A**) and number of intracellular amastigotes (**B**) were quantified. Results shown in (A) and (B) are displayed as percentage of control (group infected only with *L. chagasi*). (**C**) Intracellular survival of *L. chagasi* amastigotes was quantified by transformation of proliferating extracellular motile promastigotes in Schneider's medium. Each bar represents the mean ± SD of six to eight donors. Differences were estimated using Mann–Whitney *U* test. (**D**) Median fluorescence intensity (MFI) for ROS in macrophages infected with *L. chagasi* in the presence or absence of CoPP, evaluated by flow cytometry using the probe DHE. (**E**) Cu/Zn SOD expression of protein in cell-culture supernatant of those cells was quantified by ELISA. Each bar represents the mean ± SD. Kruskal–Wallis nonparametric test followed by Dunn's posttest was used to evaluate statistical significance. *$p < 0.05$.

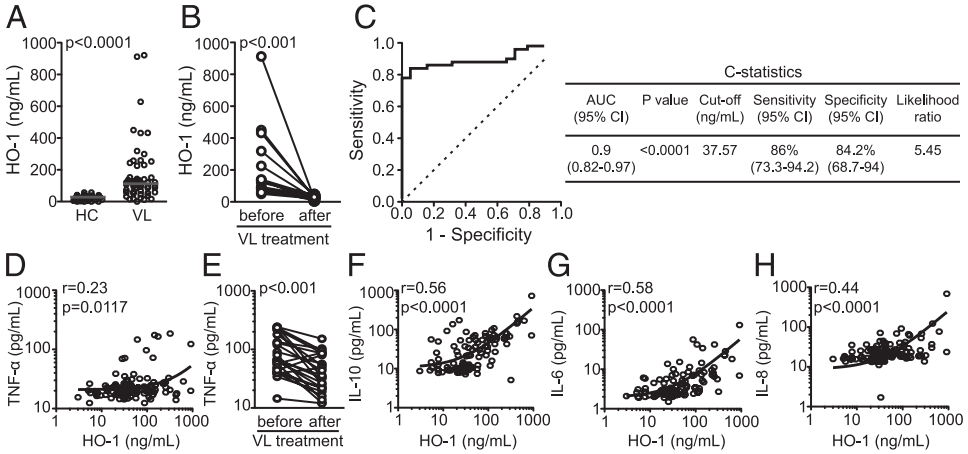

FIGURE 6. HO-1 is strongly associated with human visceral leishmaniasis. Serum samples were obtained from individuals at an endemic area in the Northeast of Brazil. Serum levels of HO-1 were measured in healthy endemic controls (HC; $n = 42$) and in patients with VL ($n = 50$) at admission to a reference hospital (**A**) and after 15 d of the antileishmanial treatment (**B**). (**C**) An ROC curve was used to evaluate the power of serum HO-1 to discriminate VL patients from the healthy endemic controls. Concordance (C)-statistics are illustrated in the table (*right panel*) and were used to verify the validation of the ROC curves and the discrimination power of HO-1. Mann–Whitney *U* test was used to verify differences between HC and VL. Wilcoxon matched-pairs signed rank test was performed to calculate the statistical significance in HO-1 and TNF-α (**E**) serum levels before and after treatment. Correlations between systemic concentrations of HO-1 and TNF-α (**D**), IL-10 (**F**), IL-6 (**G**), and IL-8 (**H**) were calculated using the Spearman test. The values of *p* and *r* are illustrated in each graph. AUC, Area under the curve.

obligatory step in the liver stage by controlling host innate inflammatory responses and protecting infected hepatocytes from cell death (21, 30). To date, little is known about the role of HO-1 in *L. chagasi* pathogen burden, and our data demonstrate that HO-1 enhances *L. chagasi* survival within host macrophages and that HO-1 is a highly associated with human VL. We propose that HO-1 plays two major roles following *L. chagasi* infection: 1) prevents damage to host cells; and 2) decreases the host ability to limit the intracellular growth of the parasite.

Hmox1 gene expression involves multiple pathways, including redox-dependent and -independent signaling molecules and immune mediators (52–54). Most of the studies that tried to address the role of HO-1 in infectious diseases have been focused on the anti-inflammatory effects, especially during malaria infection. Indeed, Pamplona and colleagues (20) have demonstrated that infection of BALB/c mice with *Plasmodium berghei* causes upregulation of HO-1 in the brain. It has been also suggested that *P. berghei* infection upregulates HO-1 in hepatocytes in vivo and peritoneal macrophages in vitro (30). In the current study, we observed that *L. chagasi* infection induced HO-1 in mouse macrophages, whereas concentrations of HO-2, the constitutive HO isoform, remained unchanged upon infection or treatment with CoPP. The obvious further step was to try to identify what molecule from the parasite would be able to trigger HO-1. A natural candidate would be LPG, which is the major *Leishmania* parasite surface molecule and has been implicated in *Leishmania* survival within mammalian macrophages in vitro (41, 55). Interestingly, we found that LPG was able to strongly induce HO-1 in mouse macrophages. Although tempted, we cannot assume that LPG is the most important trigger for HO-1 induction, because we did not rule out other *Leishmania* surface proteins. The absence of available mutants for *L. chagasi* still underlines the need for further experiments. After the identification of a possible trigger, we tested whether PPARγ, a known transcription factor of upstream *Hmox1* gene activation (42), would be involved in *L. chagasi*-induced HO-1 expression. PPARγ has been implicated in alternative activation of macrophages upon infection with *L. major*, which favors parasite survival (56) and interferes with adaptive immunity to exacerbate the pathogenesis of experimental VL (43). Our experiments performed using macrophages cocultured with an agonist or an antagonist of PPARγ showed no alteration in HO-1 induction by either *L. chagasi* or LPG, suggesting that PPARγ does not seem to be the major orchestrator of *Hmox1* induction in this experimental model. Thus, the specific transcription factors upstream of HO-1 that are induced by *Leishmania* or LPG remain to be determined.

Our results suggest that the HO-1 expression in macrophages is an important subversion mechanism by which *Leishmania* parasites can escape from the oxidative burst. Similarly, Pham and colleagues (31) have shown that *L. pifanoi* avoids elicitation of superoxide production during their internalization, and this phenomenon is dependent on HO-1 production. However, the authors did not address whether this escape via HO-1 has any impact on infection burden and *Leishmania* survival. Our study expands the current knowledge, as we demonstrate that induction by HO-1 by CoPP markedly increases parasite burden within infected macrophages. We then tested whether the genetic deficiency of HO-1 expression would be associated with modulation of parasite burden. Indeed, we found that lack of HO-1 expression leads to a significant reduction in parasite load and that this effect was correlated with a higher TNF-α production in response to infection, indicating that expression of *Hmox1* gene promotes *Leishmania* survival. In fact, infection burdens in BMMs from *Hmox1*$^{-/-}$ mice treated with CoPP were unaffected, indicating that CoPP acts through HO-1 to favor parasite persistence. We propose that in *L. chagasi* infection, HO-1 plays a similar role as in malaria, in which overexpression of HO-1 increases *P. berghei* liver infection, and that HO-1 is required to protection of infected hepatocytes by controlling the inflammatory responses (30). TNF-α is a central inflammatory cytokine in the induction of macrophage antimicrobial activities (57, 58) and has been associated with disease severity in patients with VL (59, 60). In fact, we found that induction of HO-1 reduced production of TNF-α and ROS upon *L. chagasi* infection of macrophages, consistent with mechanisms of cell protection by HO-1 (48). Furthermore, induction of HO-1 caused reduction of proinflammatory mediators such as NO, MCP-1, and IL-6 by macrophages primed with IFN-γ or LPS. A recent study suggested that LPS might contribute to the cytokine

storm and cellular activation in patients with VL (61). We propose that HO-1 is probably increasing the tolerance to *L. chagasi* infection by reducing the inflammatory status of activated macrophages and that this is probably critical in human VL, which is frequently associated with bacterial coinfection.

Finally, our findings on human macrophages confirm that HO-1 induction increases parasite load and indicate that this mechanism may be important for the pathogenesis of human disease. Patients with VL had higher serum levels of HO-1 than those not infected, and the systemic concentrations of HO-1 were significantly reduced at 15 d post-antileishmanial treatment. HO-1 is primarily thought to be an intracellular enzyme (62), and the increased serum levels of HO-1 suggest that some degree of cell death is occurring during VL. Indeed, knowing the source of extracellular HO-1 is still needed. Our data show that high concentrations of serum HO-1 are associated with a higher chance to have VL in our cohort of patients. Consistent with our results, increased concentrations of HO-1 have already been associated with other diseases such as vasculitis in Henoch-Schonlein purpura (63), hemophagocytic syndromes from hematological conditions (64, 65), type 2 diabetes (66), and prostate cancer (67). Thus, although showing strong associations between HO-1 and VL, our study expands the list of the diseases in which HO-1 potentially plays a fundamental role. In the current study, we also show that systemic concentrations of HO-1 are positively correlated to diverse cytokines, such as TNF-α, IL-6, IL-8, and IL-10. In this context, HO-1 could play a role similar to the one previously described for IL-10, which is anti-inflammatory but strongly associated with inflammatory conditions and also in human VL (2, 46). We found positive correlation between serum HO-1 and TNF-α. Of note, there is increased production of several cytokines and chemokines in VL patients, and much of the response appears to be proinflammatory, as indicated by the elevated plasma protein levels of IL-1, IL-6, IL-8, and TNF-α (reviewed in Ref. 58). Indeed, elevated serum levels of TNF-α have been associated with VL (60). We speculate that the systemic amounts of the proinflammatory cytokines could be even higher in the absence of HO-1. The high levels of TNF-α and cytokine storm, features of VL, could be inducing higher levels of HO-1 as a counterregulatory response. Similar to our results, patients with hemophagocytic syndrome also present a positive correlation between HO-1 expression and serum TNF-α (65), suggesting that this pattern may be common in inflammatory conditions in vivo. Our results argue that it is possible that the susceptibility to infection would be worse in case of the lack or absence of HO-1 in human VL. Whether the systemic concentrations of the cytokines and HO-1 evaluated in this study represent their respective amounts in the tissues in which the infected cells are localized deserves further investigation.

In summary, the current study shows that HO-1 drives *L. chagasi* infection within macrophages by means of its anti-inflammatory properties, reinforcing that *Hmox1* gene is required for *Leishmania* survival and persistence. Moreover, we show that HO-1 is strongly associated with human VL. Therefore, HO-1 may represent an important escape mechanism required for the control of *Leishmania* replication by immune cells and could be used as a therapeutic target to reduce VL severity. Protection against excessive inflammatory response may preclude deleterious effects of VL.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Saha, S., S. Mondal, A. Banerjee, J. Ghose, S. Bhowmick, and N. Ali. 2006. Immune responses in kala-azar. *Indian J. Med. Res.* 123: 245–266.
2. Caldas, A., C. Favali, D. Aquino, V. Vinhas, J. van Weyenbergh, C. Brodskyn, J. Costa, M. Barral-Netto, and A. Barral. 2005. Balance of IL-10 and interferon-gamma plasma levels in human visceral leishmaniasis: implications in the pathogenesis. *BMC Infect. Dis.* 5: 113.
3. Chappuis, F., S. Sundar, A. Hailu, H. Ghalib, S. Rijal, R. W. Peeling, J. Alvar, and M. Boelaert. 2007. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat. Rev. Microbiol.* 5: 873–882.
4. Olivier, M., D. J. Gregory, and G. Forget. 2005. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clin. Microbiol. Rev.* 18: 293–305.
5. Gantt, K. R., T. L. Goldman, M. L. McCormick, M. A. Miller, S. M. Jeronimo, E. T. Nascimento, B. E. Britigan, and M. E. Wilson. 2001. Oxidative responses of human and murine macrophages during phagocytosis of *Leishmania chagasi*. *J. Immunol.* 167: 893–901.
6. Becker, I., N. Salaiza, M. Aguirre, J. Delgado, N. Carrillo-Carrasco, L. G. Kobeh, A. Ruiz, R. Cervantes, A. P. Torres, N. Cabrera, et al. 2003. *Leishmania* lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. *Mol. Biochem. Parasitol.* 130: 65–74.
7. de Veer, M. J., J. M. Curtis, T. M. Baldwin, J. A. DiDonato, A. Sexton, M. J. McConville, E. Handman, and L. Schofield. 2003. MyD88 is essential for clearance of *Leishmania major*: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. *Eur. J. Immunol.* 33: 2822–2831.
8. Bogdan, C., and M. Rollinghoff. 1999. How do protozoan parasites survive inside macrophages? *Parasitol. Today* 15: 22–28.
9. Dermine, J. F., S. Scianimanico, C. Prive, A. Descoteaux, and M. Desjardins. 2000. *Leishmania* promastigotes require lipophosphoglycan to actively modulate the fusion properties of phagosomes at an early step of phagocytosis. *Cell. Microbiol.* 2: 115–126.
10. Brittingham, A., and D. M. Mosser. 1996. Exploitation of the complement system by Leishmania promastigotes. *Parasitol. Today* 12: 444–447.
11. Proudfoot, L., A. V. Nikolaev, G. J. Feng, W. Q. Wei, M. A. Ferguson, J. S. Brimacombe, and F. Y. Liew. 1996. Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of *Leishmania* lipophosphoglycan in murine macrophages. *Proc. Natl. Acad. Sci. USA* 93: 10984–10989.
12. Piedrafita, D., L. Proudfoot, A. V. Nikolaev, D. Xu, W. Sands, G. J. Feng, E. Thomas, J. Brewer, M. A. Ferguson, J. Alexander, and F. Y. Liew. 1999. Regulation of macrophage IL-12 synthesis by *Leishmania* phosphoglycans. *Eur. J. Immunol.* 29: 235–244.
13. Coelho-Finamore, J. M., V. C. Freitas, R. R. Assis, M. N. Melo, N. Novozhilova, N. F. Secundino, P. F. Pimenta, S. J. Turco, and R. P. Soares. 2011. *Leishmania infantum*: Lipophosphoglycan intraspecific variation and interaction with vertebrate and invertebrate hosts. *Int. J. Parasitol.* 41: 333–342.
14. Giorgione, J. R., S. J. Turco, and R. M. Epand. 1996. Transbilayer inhibition of protein kinase C by the lipophosphoglycan from *Leishmania donovani*. *Proc. Natl. Acad. Sci. USA* 93: 11634–11639.
15. Guimaraes-Costa, A. B., M. T. Nascimento, G. S. Froment, R. P. Soares, F. N. Morgado, F. Conceicao-Silva, and E. M. Saraiva. 2009. *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. *Proc. Natl. Acad. Sci. USA* 106: 6748–6753.
16. Vivarini Ade, C., M. Pereira Rde, K. L. Teixeira, T. C. Calegari-Silva, M. Bellio, M. D. Laurenti, C. E. Corbett, C. M. Gomes, R. P. Soares, A. M. Silva, et al. 2011. Human cutaneous leishmaniasis: interferon-dependent expression of double-stranded RNA-dependent protein kinase (PKR) via TLR2. *FASEB J.* 25: 4162–4173.
17. Andrade, B. B., T. Araujo-Santos, N. F. Luz, R. Khouri, M. T. Bozza, L. M. Camargo, A. Barral, V. M. Borges, and M. Barral-Netto. 2010. Heme impairs prostaglandin E2 and TGF-beta production by human mononuclear cells via Cu/Zn superoxide dismutase: insight into the pathogenesis of severe malaria. *J. Immunol.* 185: 1196–1204.
18. Tenhunen, R., H. S. Marver, and R. Schmid. 1968. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc. Natl. Acad. Sci. USA* 61: 748–755.
19. Soares, M. P., I. Marguti, A. Cunha, and R. Larsen. 2009. Immunoregulatory effects of HO-1: how does it work? *Curr. Opin. Pharmacol.* 9: 482–489.
20. Pamplona, A., A. Ferreira, J. Balla, V. Jeney, G. Balla, S. Epiphanio, A. Chora, C. D. Rodrigues, I. P. Gregoire, M. Cunha-Rodrigues, et al. 2007. Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nat. Med.* 13: 703–710.
21. Seixas, E., R. Gozzelino, A. Chora, A. Ferreira, G. Silva, R. Larsen, S. Rebelo, C. Penido, N. R. Smith, A. Coutinho, and M. P. Soares. 2009. Heme oxygenase-1 affords protection against noncerebral forms of severe malaria. *Proc. Natl. Acad. Sci. USA* 106: 15837–15842.

22. Shen, X. D., B. Ke, Y. Zhai, F. Gao, R. W. Busuttil, G. Cheng, and J. W. Kupiec-Weglinski. 2005. Toll-like receptor and heme oxygenase-1 signaling in hepatic ischemia/reperfusion injury. *Am. J. Transplant.* 5: 1793–1800.
23. Zenclussen, M. L., P. A. Casalis, T. El-Mousleh, S. Rebelo, S. Langwisch, N. Linzke, H. D. Volk, S. Fest, M. P. Soares, and A. C. Zenclussen. 2011. Haem oxygenase-1 dictates intrauterine fetal survival in mice via carbon monoxide. *J. Pathol.* 225: 293–304.
24. Chung, S. W., X. Liu, A. A. Macias, R. M. Baron, and M. A. Perrella. 2008. Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. *J. Clin. Invest.* 118: 239–247.
25. Larsen, R., R. Gozzelino, V. Jeney, L. Tokaji, F. A. Bozza, A. M. Japiassu, D. Bonaparte, M. M. Cavalcante, A. Chora, A. Ferreira, et al. 2010. A central role for free heme in the pathogenesis of severe sepsis. *Science Transl. Med.* 2: 51ra71.
26. Ke, B., R. Buelow, X. D. Shen, J. Melinek, F. Amersi, F. Gao, T. Ritter, H. D. Volk, R. W. Busuttil, and J. W. Kupiec-Weglinski. 2002. Heme oxygenase 1 gene transfer prevents CD95/Fas ligand-mediated apoptosis and improves liver allograft survival via carbon monoxide signaling pathway. *Hum. Gene Ther.* 13: 1189–1199.
27. Camara, N. O., and M. P. Soares. 2005. Heme oxygenase-1 (HO-1), a protective gene that prevents chronic graft dysfunction. *Free Radic. Biol. Med.* 38: 426–435.
28. Nath, K. A., J. P. Grande, J. J. Haggard, A. J. Croatt, Z. S. Katusic, A. Solovey, and R. P. Hebbel. 2001. Oxidative stress and induction of heme oxygenase-1 in the kidney in sickle cell disease. *Am. J. Pathol.* 158: 893–903.
29. Chung, S. W., S. R. Hall, and M. A. Perrella. 2009. Role of haem oxygenase-1 in microbial host defence. *Cell. Microbiol.* 11: 199–207.
30. Epiphanio, S., S. A. Mikolajczak, L. A. Goncalves, A. Pamplona, S. Portugal, S. Albuquerque, M. Goldberg, S. Rebelo, D. G. Anderson, A. Akinc, et al. 2008. Heme oxygenase-1 is an anti-inflammatory host factor that promotes murine plasmodium liver infection. *Cell Host Microbe* 3: 331–338.
31. Pham, N. K., J. Mouriz, and P. E. Kima. 2005. *Leishmania* pifanoi amastigotes avoid macrophage production of superoxide by inducing heme degradation. *Infect. Immun.* 73: 8322–8333.
32. Maines, M. D., and A. Kappas. 1975. Cobalt stimulation of heme degradation in the liver. Dissociation of microsomal oxidation of heme from cytochrome P-450. *J. Biol. Chem.* 250: 4171–4177.
33. Gomes, N. A., C. R. Gattass, V. Barreto-De-Souza, M. E. Wilson, and G. A. DosReis. 2000. TGF-beta mediates CTLA-4 suppression of cellular immunity in murine kalaazar. *J. Immunol.* 164: 2001–2008.
34. Saltzman, B. E. 1954. Colorimetric microdetermination of nitrogen dioxide in the atmosphere. *Anal. Chem.* 26: 1949–1955.
35. Khouri, R., A. Bafica, P. Silva Mda, A. Noronha, J. P. Kolb, J. Wietzerbin, A. Barral, M. Barral-Netto, and J. Van Weyenbergh. 2009. IFN-beta impairs superoxide-dependent parasite killing in human macrophages: evidence for a deleterious role of SOD1 in cutaneous leishmaniasis. *J. Immunol.* 182: 2525–2531.
36. Shiloh, M. U., P. Manzanillo, and J. S. Cox. 2008. *Mycobacterium tuberculosis* senses host-derived carbon monoxide during macrophage infection. *Cell Host Microbe* 3: 323–330.
37. Murray, H. W. 1982. Cell-mediated immune response in experimental visceral leishmaniasis. II. Oxygen-dependent killing of intracellular *Leishmania donovani* amastigotes. *J. Immunol.* 129: 351–357.
38. Novais, F. O., R. C. Santiago, A. Bafica, R. Khouri, L. Afonso, V. M. Borges, C. Brodskyn, M. Barral-Netto, A. Barral, and C. I. de Oliveira. 2009. Neutrophils and macrophages cooperate in host resistance against *Leishmania braziliensis* infection. *J. Immunol.* 183: 8088–8098.
39. Liew, F. Y., S. Millott, C. Parkinson, R. M. Palmer, and S. Moncada. 1990. Macrophage killing of *Leishmania* parasite in vivo is mediated by nitric oxide from L-arginine. *J. Immunol.* 144: 4794–4797.
40. Turco, S. J., G. F. Spath, and S. M. Beverley. 2001. Is lipophosphoglycan a virulence factor? A surprising diversity between *Leishmania* species. *Trends Parasitol.* 17: 223–226.
41. Spath, G. F., L. Epstein, B. Leader, S. M. Singer, H. A. Avila, S. J. Turco, and S. M. Beverley. 2000. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major. Proc. Natl. Acad. Sci. USA* 97: 9258–9263.
42. Kronke, G., A. Kadl, E. Ikonomu, S. Bluml, A. Furnkranz, I. J. Sarembock, V. N. Bochkov, M. Exner, B. R. Binder, and N. Leitinger. 2007. Expression of heme oxygenase-1 in human vascular cells is regulated by peroxisome proliferator-activated receptors. *Arterioscler. Thromb. Vasc. Biol.* 27: 1276–1282.
43. Adapala, N., and M. M. Chan. 2008. Long-term use of an antiinflammatory, curcumin, suppressed type 1 immunity and exacerbated visceral leishmaniasis in a chronic experimental model. *Lab. Invest.* 88: 1329–1339.
44. Turkseven, S., A. Kruger, C. J. Mingone, P. Kaminski, M. Inaba, L. F. Rodella, S. Ikehara, M. S. Wolin, and N. G. Abraham. 2005. Antioxidant mechanism of heme oxygenase-1 involves an increase in superoxide dismutase and catalase in experimental diabetes. *Am. J. Physiol. Heart Circ. Physiol.* 289: H701–H707.
45. Ahmad, M., X. Zhao, M. R. Kelly, S. Kandhi, O. Perez, N. G. Abraham, and M. S. Wolin. 2009. Heme oxygenase-1 induction modulates hypoxic pulmonary vasoconstriction through upregulation of ecSOD. *Am. J. Physiol. Heart Circ. Physiol.* 297: H1453–H1461.
46. Nylén, S., and D. Sacks. 2007. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. *Trends Immunol.* 28: 378–384.
47. Ansari, N. A., R. Kumar, S. Gautam, S. Nylen, O. P. Singh, S. Sundar, and D. Sacks. 2011. IL-27 and IL-21 are associated with T cell IL-10 responses in human visceral leishmaniasis. *J. Immunol.* 186: 3977–3985.
48. Gozzelino, R., V. Jeney, and M. P. Soares. 2010. Mechanisms of cell protection by heme oxygenase-1. *Annu. Rev. Pharmacol. Toxicol.* 50: 323–354.
49. Ryter, S. W., and R. M. Tyrrell. 2000. The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free Radic. Biol. Med.* 28: 289–309.
50. Pae, H. O., and H. T. Chung. 2009. Heme oxygenase-1: its therapeutic roles in inflammatory diseases. *Immune Network* 9: 12–19.
51. Schneider, D. S., and J. S. Ayres. 2008. Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. *Nat. Rev. Immunol.* 8: 889–895.
52. Ryter, S. W., L. E. Otterbein, D. Morse, and A. M. Choi. 2002. Heme oxygenase/carbon monoxide signaling pathways: regulation and functional significance. *Mol. Cell. Biochem.* 234-235: 249–263.
53. Seldon, M. P., G. Silva, N. Pejanovic, R. Larsen, I. P. Gregoire, J. Filipe, J. Anrather, and M. P. Soares. 2007. Heme oxygenase-1 inhibits the expression of adhesion molecules associated with endothelial cell activation via inhibition of NF-kappaB RelA phosphorylation at serine 276. *J. Immunol.* 179: 7840–7851.
54. Wagener, F. A., H. D. Volk, D. Willis, N. G. Abraham, M. P. Soares, G. J. Adema, and C. G. Figdor. 2003. Different faces of the heme-heme oxygenase system in inflammation. *Pharmacol. Rev.* 55: 551–571.
55. Lodge, R., T. O. Diallo, and A. Descoteaux. 2006. *Leishmania donovani* lipophosphoglycan blocks NADPH oxidase assembly at the phagosome membrane. *Cell. Microbiol.* 8: 1922–1931.
56. Odegaard, J. I., R. R. Ricardo-Gonzalez, M. H. Goforth, C. R. Morel, V. Subramanian, L. Mukundan, A. Red Eagle, D. Vats, F. Brombacher, A. W. Ferrante, and A. Chawla. 2007. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* 447: 1116–1120.
57. Green, S. J., R. M. Crawford, J. T. Hockmeyer, M. S. Meltzer, and C. A. Nacy. 1990. *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha. *J. Immunol.* 145: 4290–4297.
58. Engwerda, C. R., M. Ato, S. Stager, C. E. Alexander, A. C. Stanley, and P. M. Kaye. 2004. Distinct roles for lymphotoxin-alpha and tumor necrosis factor in the control of *Leishmania donovani* infection. *Am. J. Pathol.* 165: 2123–2133.
59. Barral-Netto, M., R. Badaro, A. Barral, R. P. Almeida, S. B. Santos, F. Badaro, D. Pedral-Sampaio, E. M. Carvalho, E. Falcoff, and R. Falcoff. 1991. Tumor necrosis factor (cachectin) in human visceral leishmaniasis. *J. Infect. Dis.* 163: 853–857.
60. Peruhype-Magalhaes, V., O. A. Martins-Filho, A. Prata, A. Silva Lde, A. Rabello, A. Teixeira-Carvalho, R. M. Figueiredo, S. F. Guimaraes-Carvalho, T. C. Ferrari, J. Van Weyenbergh, and R. Correa-Oliveira. 2006. Mixed inflammatory/regulatory cytokine profile marked by simultaneous raise of interferon-gamma and interleukin-10 and low frequency of tumour necrosis factor-alpha(+) monocytes are hallmarks of active human visceral Leishmaniasis due to *Leishmania chagasi* infection. *Clin. Exp. Immunol.* 146: 124–132.
61. Santos-Oliveira, J. R., E. G. Regis, C. R. B. Leal, R. V. Cunha, P. T. Bozza, and A. M. Da-Cruz. 2011. Evidence That Lipopolisaccharide May Contribute to the Cytokine Storm and Cellular Activation in Patients with Visceral Leishmaniasis. *PLoS Negl. Trop. Dis.* 5: e1198.
62. Yoshinaga, T., S. Sassa, and A. Kappas. 1982. The oxidative degradation of heme c by the microsomal heme oxygenase system. *J. Biol. Chem.* 257: 7803–7807.
63. Chen, T., Z. P. Guo, Y. H. Zhang, Y. Gao, H. J. Liu, and J. Y. Li. 2009. Elevated serum heme oxygenase-1 and insulin-like growth factor-1 levels in patients with Henoch-Schonlein purpura. *Rheumatol. Int.* 31: 321–326.
64. Kirino, Y., M. Takeno, M. Iwasaki, A. Ueda, S. Ohno, A. Shirai, H. Kanamori, K. Tanaka, and Y. Ishigatsubo. 2005. Increased serum HO-1 in hemophagocytic syndrome and adult-onset Still's disease: use in the differential diagnosis of hyperferritinemia. *Arthritis Res. Ther.* 7: R616–R624.
65. Miyazaki, T., Y. Kirino, M. Takeno, M. Hama, A. Ushihama, R. Watanabe, K. Takase, T. Tachibana, K. Matsumoto, M. Tanaka, et al. 2010. Serum HO-1 is useful to make differential diagnosis of secondary hemophagocytic syndrome from other similar hematological conditions. *Int. J. Hematol.* 91: 229–237.
66. Bao, W., F. Song, X. Li, S. Rong, W. Yang, M. Zhang, P. Yao, L. Hao, N. Yang, F. B. Hu, and L. Liu. 2010. Plasma Heme Oxygenase-1 Concentration Is Elevated in Individuals with Type 2 Diabetes Mellitus. *PLoS ONE* 5: e12371.
67. Blann, A. D., B. Balakrishnan, P. Ryan, and G. Y. Lip. 2011. Increased levels of plasma haemoxygenase-1 in prostate cancer. *Prostate Cancer Prostatic Dis.* 14: 114–117.