Leishmania infantum-chagasi activates SHP-1 and reduces NFAT5/TonEBP activity in the mouse kidney inner medulla

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Zhou X, Wang H, Koles NL, Zhang A, Aronson NE. Leishmania infantum-chagasi activates SHP-1 and reduces NFAT5/TonEBP activity in the mouse kidney inner medulla. Am J Physiol Renal Physiol 307: F516–F524, 2014. First published July 2, 2014; doi:10.1152/ajprenal.00006.2014.—Visceral leishmaniasis patients have been reported to have a urine concentration defect. Concentration of urine by the renal inner medulla is essentially dependent on a transcription factor, NFAT5/TonEBP, because it activates expression of osmoprotective genes betaine/glycine transporter 1 (BGT1) and sodium/myo-inositol transporter (SMIT), and water channel aquaporin-2, all of which are imperative for concentrating urine. Leishmania parasites evade macrophage immune defenses by activating protein tyrosine phosphatases, among which SHP-1 is critical. We previously demonstrated that SHP-1 inhibits toxicity-dependent activation of NFAT5/TonEBP in HEK293 cells through screening a genome-wide small interfering (si) RNA library against phosphatases (Zhou X, Gallazzini M, Burg MB, Ferraris JD. Proc Natl Acad Sci USA 107: 7072–7077, 2010). We sought to examine whether Leishmania can activate SHP-1 and inhibit NFAT5/TonEBP activity in the renal inner medulla in a murine model of visceral leishmaniasis by injection of female BALB/c mice with a single intravenous dose of 5 × 10^7 L. chagasi metacyclic promastigotes. We found that SHP-1 is expressed in the kidney inner medulla. L. chagasi activates SHP-1 with an increase in stimulatory phosphorylation of SHP-1-Y536 in the region. L. chagasi reduces expression of NFAT5/TonEBP mRNA and protein as well as expression of its targeted genes: BGT1, SMIT, and aquaporin-2. The culture supernatant from L. chagasi metacyclic promastigotes increases SHP-1 protein abundance and potently inhibits NFAT5 transcriptional activity in mIMCD3 cells. However, L. chagasi in our animal model has no significant effect on urinary concentration. We conclude that L. chagasi, most likely through its secreted virulence factors, activates SHP-1 and reduces NFAT5/TonEBP gene expression, which leads to reduced NFAT5/TonEBP transcriptional activity in the kidney inner medulla.

ERK1/2; urinary concentration; aquaporin-2; virulence factors; leishmaniasis

Visceral Leishmaniasis (VL) is a disseminated infection transmitted by the bite of infected sand flies and caused by parasite species Leishmania infantum-chagasi (L. chagasi) and Leishmania donovani. It is estimated that one to two million people worldwide are VL infected, with ~500,000 new cases and 50,000 deaths each year, marking this as a parasitic disease with a high mortality rate second only to malaria (2). Macrophages are a first line of immune defense against Leishmania infection and paradoxically also one of the primary reservoirs of Leishmania. To survive and propagate inside the phagocytes, Leishmania subvert the signaling activation of macrophages by activating protein tyrosine phosphatases PTP1B, TC-PTP, and SHP-1, and other inhibitory pathways, among which SHP-1 is critical (28, 50). Activation of SHP-1 inhibits MAP kinase, JAK-STAT, TLR, and IFN-γ pathways, which leads to reduction of the generation of reactive oxygen species and nitric oxide, thus inhibiting innate immune responses (28, 50).

Renal involvement during VL infection is described in both humans (13, 14, 31, 42, 43) and dogs (3, 12). A majority of VL patients have a defect in concentrating urine (13, 32). A urine concentration defect is also described in Brazilian cutaneous leishmaniasis patients (42, 43). The kidney inner medulla plays an essential role in concentrating urine. Nuclear factor of activation of T cells 5 (NFAT5), also named tonicity-responsive enhancer binding protein (TonEBP), is the newest member of the Rel family of transcription factors. It is expressed in a variety of tissues and regulates an assortment of functions, including immune responses (6, 7, 20), cardiac development (37), atherosclerosis (23), and ischemia-induced brain injury (36). In the kidney inner medulla, NFAT5 is known for its activation of expression of osmoprotective genes such as betaine/glycine transporter 1 (BGT1) and sodium/myo-inositol transporter (SMIT) (9, 58), and genes necessary for water traffic like aquaporin-2 (AQP2) (27, 30), aquaporin-1 (29), and urea transporters (27, 39). These genes are essential for the renal inner medulla to concentrate urine, a primary mechanism to maintain body fluid balance. Through screening a genome-wide phosphatase small interfering (si) RNA library, we previously demonstrated that SHP-1 inhibits hyperosmoticity-induced activation of NFAT5 in HEK293 cells (63). These data raise questions concerning whether SHP-1 is expressed in the kidney inner medulla and Leishmania activate SHP-1 and inhibit NFAT5 in that region, leading to the clinically observed urine concentration defect. The present study addresses these questions in a murine model of L. chagasi VL.

MATERIALS AND METHODS

Parasites and Parasite Supernatant

L. chagasi (parasite strain generously provided by Dr. Mary E. Wilson, University of Iowa) was grown at 26°C in DMEM, supplemented with 20% heat-inactivated FBS serum (GIBCO), 25 mM HEPES, 50 μM 2-mercaptoethanol, and 20 μg/ml of gentamicin (60) or HOMEM (5). The parasite supernatant was obtained by centrifuging cultured metacyclic promastigotes (~1.2 × 10^7/ml parasites between passage 4 and passage 19) at 3,500 rpm at room temperature for 10 min twice. The supernatant can be stored at 4°C for at least 1 wk without losing the inhibitory effect on NFAT5 transcriptional activity.

Animals and Animal Infection

BALB/c mice (female, 5 wk old), purchased from Jackson Laboratory, were handled according to the procedures approved by the

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Uniformed Services University Institutional Animal Care and Use Committee. The mice were treated with a single tail vein injection of PBS (control) or 5 × 10⁵ metacyclic promastigotes of L. chagasi for 4 wk before they were euthanized. Both kidney inner medullas including the papillae of each mouse were quickly dissected on ice. In some cases, the liver and kidney cortex were also used. Analyses of protein phosphorylation and abundance, mRNA abundance, and SHP-1 activity were performed under blinded conditions.

**Limiting Dilution Assay**

Limiting dilution culture was performed as described previously (8, 54). Briefly, liver, spleen, and kidney inner medulla tissue was harvested aseptically and homogenized in HO-MEM containing 10% FCS (5). Five-fold serial dilutions were incubated at 26°C. After culturing for 17–19 days, the highest dilution yielding growth of viable L. chagasi promastigotes was determined using a phase-contrast microscope.

**Immunohistochemistry**

The whole kidney was fixed with 10% formalin in neutral buffer (Thermo Scientific) overnight and then paraffin embedded and sliced in the university histology laboratory. Immunohistochemistry was performed according to the procedures described in the Cell Signaling Technology website (http://www.cellsignal.com/common/content/content.jsp?d=ihc-paraffin-signalstain). Briefly, tissues were deparaffinized and rehydrated with xylene, ethanol, and water sequentially. Antigen was unmasked by boiling slides in sodium citrate buffer, EDTA for 1 h. Slides were then incubated with a rabbit anti-AQP-2 antibody at a 1:50 dilution (SC-287, Santa Cruz Biotechnology) or with additional secondary anti-rabbit antibody and imaged with a confocal microscope (LSM710). The images from the inner medulla were captured with a 63 lens. The inner medulla were captured with a ×63 lens, whereas the images from the cortex were taken with a ×40 lens, because they were too weak to be captured with the ×63 lens.

**SHP-1 Activity Assay**

The inner medullas were homogenized in 10 mM triethanolamine, pH 7.4, and 250 mM sucrose plus a protease inhibitor tablet (Roche), 2.0 µM NaF, and 2.0 µM NaVO₃. The protein concentrations in homogenates were measured with a BCA assay (Pierce). Protein samples (18 µg/lane in most cases) were fractionated in Bolt 4–12% Bis-Tris Plus gel and then transferred to nitrocellulose membranes (Invitrogen) according to the manufacturer’s protocol. The membranes were first blocked with Odyssey Blocking Buffer for 60 min at room temperature (Li-Cor) and then incubated with primary antibodies overnight at 4°C. The antibodies against ERK1/2-P, ERK1/2, STAT3-Y705-P, STAT3, AKT-S473-P, AKT1, GSK-3β-P, GAPDH were purchased from Cell Signaling Technology (catalog nos. 9101, 9102, 9145, 4904, 4060, 2920, 9336, 9315, and 2118, respectively). The anti-SHP-1 and actin antibodies were purchased from Santa Cruz Biotechnology (catalog nos. SC-287 and SC-1615, respectively). The antibodies against SHP-1-Y536-P and SHP-1-S591-P were purchased from ECM Biosciences (catalog nos. SP-1571 and SP-1531, respectively). The anti-AQP2 antibody is a generous gift of Dr. Mark A. Knepper (National Heart, Lung, and Blood Institute). After incubation with primary antibodies and a brief wash with PBS plus 0.1% Tween 20, the membranes were incubated with Alexa Fluor-conjugated secondary antibodies at room temperature for 60 min. Protein expression and phosphorylation were analyzed by an Odyssey Infrared Imager (Li-Cor).

**Quantitative PCR**

Tissues were sonicated in an ice-cold RNAzol RT kit (Molecular Research Center). Total RNA was measured by NanoDrop 8000 (Thermo Scientific). cDNAs were synthesized with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). mRNAs were quantified with a SYBR Green PCR kit (QuantiFast, Qiagen) in Applied Biosystems 7900HT. The primers for each gene are listed in Table 1. These primers were designed with a GenScrip online program. Two hundred nanograms total inner medulla RNA/reaction was used. 18S rRNA was used to control for the amount of cDNA used in each analysis, but mRNA abundance was not normalized to 18S rRNA. 18S rRNA primers and probes were purchased from Applied Biosystems. Fold-difference in mRNA abundance between conditions was calculated, as described previously (17).

**Cell Culture, Cell Transfection, and Luciferase Activity Assay**

mIMCD3 cells (47) were cultured in DMEM plus 10% FBS at 37°C supplemented with 5% CO₂-95% air and used between passage 14 and 24. The osmotic response element (ORE), which is the NFAT5-targeted DNA sequence luciferase reporter, and the mutated ORE luciferase reporter, in which the ORE was mutated to prevent NFAT5 binding, were described previously (17). The reporter plasmids were transfected into mIMCD3 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol for 48 h. Then, SHP-1 activity is expressed by the readings at A630 normalized to immunoprecipitated SHP-1.

**Western Blot Analysis**

Tissues were homogenized in 10 mM triethanolamine, pH 7.4, and 250 mM sucrose plus a protease inhibitor tablet (Roche), 2.0 µM NaF, and 2.0 µM NaVO₃. The protein concentrations in homogenates were measured with a BCA assay (Pierce). Protein samples (18 µg/lane in most cases) were fractionated in Bolt 4–12% Bis-Tris Plus gel and then transferred to nitrocellulose membranes (Invitrogen) according to the manufacturer’s protocol. The membranes were first blocked with Odyssey Blocking Buffer for 60 min at room temperature (Li-Cor) and then incubated with primary antibodies overnight at 4°C. The antibodies against ERK1/2-P, ERK1/2, STAT3-Y705-P, STAT3, AKT-S473-P, AKT1, GSK-3β-P, GAPDH were purchased from Cell Signaling Technology (catalog nos. 9101, 9102, 9145, 4904, 4060, 2920, 9336, 9315, and 2118, respectively). The anti-SHP-1 and actin antibodies were purchased from Santa Cruz Biotechnology (catalog nos. SC-287 and SC-1615, respectively). The antibodies against SHP-1-Y536-P and SHP-1-S591-P were purchased from ECM Biosciences (catalog nos. SP-1571 and SP-1531, respectively). The anti-AQP2 antibody is a generous gift of Dr. Mark A. Knepper (National Heart, Lung, and Blood Institute). After incubation with primary antibodies and a brief wash with PBS plus 0.1% Tween 20, the membranes were incubated with Alexa Fluor-conjugated secondary antibodies at room temperature for 60 min. Protein expression and phosphorylation were analyzed by an Odyssey Infrared Imager (Li-Cor).

### Table 1. List of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>F4/80</td>
<td>5′-CCAGGGAGCTGAGGCAGGAGACCTGGGAAA-3′</td>
<td>5′-TGAGACTCATCTCCCTTTGACAC-3′</td>
</tr>
<tr>
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<td>5′-CCCGGAGCCACACATACCGAGACCTGGGAAA-3′</td>
<td>5′-CCCGGAGCCACACATACCGAGACCTGGGAAA-3′</td>
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<tr>
<td>NFAT5</td>
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<td>5′-GAGCGAACGACGAGATCGCGAGAATGG-3′</td>
</tr>
<tr>
<td>AQP2</td>
<td>5′-GATCGCGAGACGAGATCGCGAGAATGG-3′</td>
<td>5′-GATCGCGAGACGAGATCGCGAGAATGG-3′</td>
</tr>
<tr>
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<td>5′-GATCGCGAGACGAGATCGCGAGAATGG-3′</td>
</tr>
<tr>
<td>SMIT</td>
<td>5′-GATCGCGAGACGAGATCGCGAGAATGG-3′</td>
<td>5′-GATCGCGAGACGAGATCGCGAGAATGG-3′</td>
</tr>
</tbody>
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AQP2, aquaporin-2.
**Inner medulla**

![Image](image1.png)

**Cortex**

![Image](image2.png)

*Fig. 1. SHP-1 is expressed in the female BALB/c mouse kidney inner medulla and cortex. The SHP-1 antibody from rabbits was used at 1:50 dilution. The SHP-1 antibody competition binding assay was performed in the presence of 50× SHP-1 peptide. The rabbit anti-aquaporin-2 (AQP2) antibody was used at 1:500 dilution.*

After mice were infected with *L. chagasi* or injected with PBS (control) for 4 wk, they were placed in metabolic cages (Hatteras Instruments) and acclimated to a water-replete gelled food diet containing 4 g powdered food (OpenSource Diet), 0.09 g agar, and 5 ml water-20 g body wt \(^{-1}\)·24 h\(^{-1}\) (61) for 48 h. Subsequently, the 24-h urine samples were collected with the same diet and same ration. Then, mice were fed with the water-restricted food diet of 4 g powdered food, 0.05 g agar, and 1 ml water-20 g body wt \(^{-1}\)·24 h\(^{-1}\) for 24 h (61). Urine samples were collected under mineral oil. Mice were weighed before and after water restriction.

**Statistical Analysis**

Results are expressed as means ± SE. The reading from the first mouse in the control group was set to one, and all other results were normalized to that value. Statistical analyses were performed by a nonpaired *t*-test (animal studies) or paired *t*-test (cell culture studies). *P* ≤ 0.05 is considered significant.

**RESULTS**

**L. chagasi Is Expressed in the Renal Inner Medulla**

As the first step in studying the possible effect of SHP-1 on NFAT5 in the kidney inner medulla, we demonstrate that SHP-1 is expressed in the inner medulla, with high expression in the collecting duct epithelial cells (Fig. 1A), as supported by immunohistochemical staining of AQP2, a specific marker of the collecting duct (Fig. 1D). SHP-1 is also expressed in the renal cortex (Fig. 1E). In contrast to the concentrated expression in the basolateral peritubular plasma area of the inner medulla, expression of SHP-1 in the cortex is diffusive.

**L. chagasi Activates SHP-1 in the Kidney Inner Medulla**

We found that *L. chagasi* increases SHP-1 activity in the inner medulla (Fig. 2A). As an additional test, we examined the phosphorylation of ERK1/2, which has been shown to be a regulatory target of SHP-1 in experimental visceral leishmaniasis (19) as well as in nonleishmaniasis models (55), and phosphorylation of STAT3-Y705, which is dephosphorylated by SHP-1 in other models (38, 57), but has not been demonstrated for *L. chagasi* by SHP-1 in other models (38, 57), but has not been demonstrated...
strated in leishmaniasis. We found that *L. chagasi* significantly reduces ERK1/2-P (Fig. 2B) but has no significant effect on macrophage infiltration (D) in the kidney inner medulla. The female BALB/c mice (4 wk old) were injected with a single dose of 5 × 10⁵ parasites/mouse through tail veins for 4 wk before they were euthanized for analyses. A: SHP-1 was immunoprecipitated with agarose beads-conjugated rabbit anti-SHP-1 (SC-287, Santa Cruz Biotechnology) and then incubated with 100 μM phosphor-tyrosine peptide substrate (12–217, Millipore). The released inorganic phosphate was measured at A630 (44). SHP-1 activity is expressed by normalization of spectrophotometric reading at A630 to the amount of immunoprecipitated SHP-1. B–D: evaluation by Western blotting. *P < 0.05 vs. group injected with PBS (control); n = 4 for A and n = 5 for B–D.

*L. chagasi* Increases Phosphorylation of SHP-1-Y536 in the Kidney Inner Medulla and Cortex

The activity of SHP-1 is regulated by phosphorylation of its C terminus. Phosphorylation of the tyrosines, such as Y536, usually activates the phosphatase, whereas phosphorylation of the S591 often does the opposite (46). We observed that *L. chagasi* significantly increases phosphorylation of SHP-1-Y536 (SHP-1-Y536-P) in the renal inner medulla (Fig. 3A), whereas it has no significant effect on SHP-1-S591-P (Fig. 3B).

To determine whether the effect of *L. chagasi* on SHP-1-Y536-P is unique to the inner medulla or generalized, we examined the effect of the parasite on SHP-1-Y536-P in the renal cortex and found a similar effect (Fig. 3C). We also found that expression of SHP-1 in the cortex is much more variable than that in the inner medulla, in addition to its diffusive pattern (Fig. 1E). We conclude that *L. chagasi* increases SHP-1-Y536-P in the renal inner medulla and cortex, suggest-

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Fig. 2. *Leishmania chagasi* increases SHP-1 activity (A), reduces phosphorylation and protein abundance of ERK1/2 (B) and protein abundance of STAT3 (C), and has no significant effect on macrophage infiltration (D) in the kidney inner medulla. The female BALB/c mice (4 wk old) were injected with a single dose of 5 × 10⁵ parasites/mouse through tail veins for 4 wk before they were euthanized for analyses. A: SHP-1 was immunoprecipitated with agarose beads-conjugated rabbit anti-SHP-1 (SC-287, Santa Cruz Biotechnology) and then incubated with 100 μM phosphor-tyrosine peptide substrate (12–217, Millipore). The released inorganic phosphate was measured at A630 (44). SHP-1 activity is expressed by normalization of spectrophotometric reading at A630 to the amount of immunoprecipitated SHP-1. B–D: evaluation by Western blotting. *P < 0.05 vs. group injected with PBS (control); n = 4 for A and n = 5 for B–D.

Fig. 3. *L. chagasi* increases phosphorylation of SHP-1-Y536 in the kidney inner medulla (A) and cortex (C) and has no significant effect on phosphorylation of SHP-1-S591 in the inner medulla (B). *L. chagasi* does not significantly affect SHP-1 protein abundance in either of these regions. *P < 0.05, **P < 0.005 vs. group injected with PBS (control); n = 5.
ing that the parasite activates SHP-1 through increasing SHP-1-Y536-P.

**L. chagasi Reduces Expression of NFAT5 Gene and NFAT5-Targeted Genes in the Kidney Inner Medulla**

*L. chagasi* significantly reduces mRNA and protein abundance of NFAT5 in the inner medulla (Fig. 4A). *L. chagasi* also significantly reduces mRNA abundance of NFAT5-targeted genes BGT1, SMIT, and AQP2 and protein abundance of AQP2 (Fig. 4, B and C). We could not examine the effect of the parasite on protein abundance of BGT1 or SMIT, because we did not find a satisfactory antibody against either of them. We conclude that *L. chagasi* inhibits NFAT5 transcriptional activity in the inner medulla.

**The L. chagasi Supernatant Increases SHP-1 Protein Abundance and Inhibits NFAT5 Transcriptional Activity in mIMCD3 Cells**

The failure to detect parasites in the kidney led us to examine whether *L. chagasi* activates SHP-1 and inhibits NFAT5 activity through its secreted virulence factors. We found that the supernatant from culturing the parasite significantly increases SHP-1 protein abundance by 48% and has no significant effect on SHP-1-S591-P (Fig. 5, A and B). We could not detect SHP-1-Y536-P with the antibody either from ECM Bioscience or from Abcam (data not shown). The supernatant reduces the ORE luciferase reporter activity by 75%. In contrast, the supernatant only reduces the mutated ORE reporter activity by 22%, and the effect does not reach statistical significance (Fig. 5C), indicating that the supernatant inhibits NFAT5 transcriptional activity through a specific mechanism.

The supernatant has no significant effect on NFAT5 protein abundance (Fig. 5, A and C). In contrast to the effect of *L. chagasi* in the kidney, the supernatant increases ERK1/2-P. However, the result cannot be semiquantitated, because the ERK1/2-P signal in some cells treated with the DMEM culture medium is too weak to be read reliably (Fig. 5A). We conclude that the *L. chagasi* culture supernatant inhibits NFAT5 transcriptional activity associated with an increase in the SHP-1 protein level in mIMCD3 cells.

**L. chagasi Has No Significant Effect on AKT1-S471-P or GSK-3β-S9-P in the Kidney Inner Medulla**

The effects of *L. chagasi* on SHP-1 and NFAT5 prompted us to examine whether the parasite also affected other signaling pathways that have been demonstrated to regulate NFAT5 under hypotonic conditions. We chose AKT1 and GSK-3β, because they are reported to have a role in leishmaniasis (40, 41) in addition to regulation of tonicity-dependent activation of NFAT5, and AKT1 phosphorylates GSK-3β-S9 in the hypertonic context and in *Leishmania* infection (40, 65). Phosphorylation of AKT1-S471 activates AKT1, whereas phosphorylation of GSK-3β serine 9 inhibits GSK-3β. We found that *L. chagasi* has no significant effect on AKT1-S471-P or GSK-3β-S9-P in the renal inner medulla (Fig. 6).

**Intravenous Injection of 5 × 10⁵ Metacyclic Parasites/Mouse Does Not Induce a Urinary Concentration Defect**

We found that infection of mice with 5 × 10⁵ parasites/mouse does not significantly reduce urinary osmolality when mice were fed with a water-replete diet (control) or water-restricted diet for 24 h (Fig. 7). Water restriction does not significantly reduce body weight (control: from 19.3 ± 0.4 to 19.8 ± 0.4 g; infected: from 19.8 ± 0.4 to 19.2 ± 0.5 g). We conclude that *L. chagasi* at the dose we used has no significant effect on urinary concentration.

**DISCUSSION**

**L. chagasi Activates SHP-1 in the Kidney Inner Medulla**

Pathogens often exploit host signaling pathways to propagate their infection, because the signaling pathways regulate many host cellular defense processes (1). *Leishmania* activate SHP-1 in host macrophages, which in turn inhibit activation of the macrophage immune defense response. The parasites achieve this effect through their secreted virulence factors such as EF-1α, fructose-1,6-bisphosphate aldolase, and GP63 (28, 50). Activation of an enzyme may be accompanied by an increase in its abundance or by a structural change either at the primary, secondary, or tertiary level or at all of these three levels. However, the result cannot be semiquantitated, because the ERK1/2-P signal in some cells treated with the DMEM culture medium is too weak to be read reliably (Fig. 5A).

The result cannot be semiquantitated, because the ERK1/2-P signal in some cells treated with the DMEM culture medium is too weak to be read reliably (Fig. 5A).

![Fig. 4. *L. chagasi* reduces mRNA abundance of BGT1, SMIT, AQP2 (A), and NFAT5 (C) and protein abundance of AQP2 (B) and NFAT5 (C) in the renal inner medulla. *P < 0.05, **P = 0.05 vs. group injected with PBS (control); n = 5.](Image)
SHP-1 comes from macrophage cell line models. 


data not shown). The supernatant has no significant effect on inhibitory SHP-1-Y536-P (46) (Fig. 3 A, B and C). However, our in vivo model is more representative of leishmaniasis in patients than in vitro infection of cell lines. From this model, we demonstrate for the first time that in addition to macrophages, L. chagasi also activates the enzyme SHP-1 in nonphagocytes as well (Fig. 2A). Although SHP-1 is expressed abundantly in immune cells and inhibits immune responses in general (11), it is also expressed in other cells and tissues and regulates various functions like cell growth, cell injury, and glucose homeostasis (15, 22, 34). Therefore, besides subverting macrophage innate immune defense, SHP-1 could also be involved in Leishmania-induced pathological effects in nonphagocytic cells and tissues.

**L. chagasi Inhibits NFAT5 in the Kidney Inner Medulla**

A majority of NFAT5 homozygous knockouts are embryonically lethal (33). Knockout of NFAT5 through multiple breeding procedures was associated with 20-fold increases in L. major parasite burden and splenic dissemination in a mouse species where L. major usually causes localized infection (10). These data suggest that NFAT5 is protective against Leishmania infections and that Leishmania inhibit NFAT5 to propagate in the host. We speculate that NFAT5 could show protection against other parasitic or intracellular infections. Our present studies show that L. chagasi decreases NFAT5 mRNA and protein abundance in the kidney inner medulla (Fig. 4 A). This effect leads to a decrease in expression of its targeted genes, BGT1, SMIT, and AQP2, (Fig. 4, B and C) and is associated with activation of SHP-1 (Fig. 2A). Furthermore, the L. chagasi

levels. However, the intramolecular mechanisms by which Leishmania activate SHP-1 remains unknown. We have shown that L. chagasi significantly increases SHP-1 activity (Fig. 2A), which is associated with an increase in activational SHP-1-Y536-P (46) (Fig. 3A) and has no significant effect on inhibitory SHP-1-S591-P (46) (Fig. 3B). The latter effect is different from the effect of hypertonicity, which inhibits SHP-1 function by increasing SHP-1-S591-P (64). Phosphorylation of Y536 activates SHP-1 possibly by releasing its autoinhibition (46) and increasing the affinity to its substrates (25).

Most of our knowledge of the effect of Leishmania on SHP-1 comes from macrophage cell line models. L. major and L. mexicana activate SHP-1 by cleaving it in a murine macrophage cell line B10R cells (21, 24). L. donovani increases SHP-1 protein abundance in another murine macrophage cell line, RAW264.7 (4). In the present study, we show that the L. chagasi supernatant also increases SHP-1 protein abundance in mIMCD3 cells (Fig. 5, A and B), a mouse inner medullary collecting duct cell line (47). However, we did not observe a significant effect of L. chagasi on SHP-1 protein abundance in the inner medulla or in the cortex (Fig. 3). Besides the differences in Leishmania species and cell types, the duration and route of infection may also play an important role in how SHP-1 is activated. We intravenously infected mice with a single parasite dose and evaluated after 4 wk of infection, whereas in previous reports Leishmania were incubated in vitro with macrophage cell lines for hours and the cells were analyzed within days (4, 21, 24). In our cell culture studies, we also treated mIMCD3 cells with the supernatant only for 22 h. The difference between treatment of cells in vitro and infection of mice in vivo may also explain the different effects on NFAT5 protein abundance and ERK1/2-P observed in mIMCD3 cells and the kidney inner medulla (Figs. 2B, 4A, and 5, A and C). However, our in vivo model is more representative of leishmaniasis in patients than in vitro infection of cell lines. From this model, we demonstrate for the first time that in addition to macrophages, L. chagasi also activates the enzyme SHP-1 in nonphagocytes as well (Fig. 2A). Although SHP-1 is expressed abundantly in immune cells and inhibits immune responses in general (11), it is also expressed in other cells and tissues and regulates various functions like cell growth, cell injury, and glucose homeostasis (15, 22, 34). Therefore, besides subverting macrophage innate immune defense, SHP-1 could also be involved in Leishmania-induced pathological effects in nonphagocytic cells and tissues.
culture supernatant inhibits NFAT5 transcriptional activity accompanied by an increase in SHP-1 protein level in mIMCD3 cells (Fig. 5). SHP-1 is a signaling molecule, not a “housekeeping” gene. The presence of SHP-1 in the inner medulla raises a question as to what function this phosphatase might have. SHP-1 inhibits toxicity-dependent activation of NFAT5 in cell culture (63). Our observations suggest that SHP-1 may inhibit NFAT5 in the inner medulla. Since the optimal function of NFAT5 is essential for the inner medulla to concentrate urine effectively, L. chagasi-induced inhibition of NFAT5 (potentially through the activation of SHP-1) may explain the urine concentration defect observed in >60% of patients with visceral leishmaniasis (13, 32).

Effects of L. chagasi on Other Kinases and STAT3

Hypertonicity activates NFAT5 through multiple pathways (64, 65). This prompted us to examine whether L. chagasi also affects other kinases such as ERK1/2, AKT1, and GSK-3β, which have been demonstrated to regulate toxicity-dependent activation of NFAT5 (48, 56, 65) in the kidney inner medulla. Both increases and decreases in stimulatory ERK1/2-P have been observed in experimental visceral leishmaniasis, depending on the cell type and other experimental conditions (19, 41). We found that L. chagasi reduces stimulatory ERK1/2-P in the kidney inner medulla (Fig. 2B), but the L. chagasi supernatant increases ERK1/2-P despite its inhibition of NFAT5 transcriptional activity in mIMCD3 cells. L. chagasi has a tendency to reduce stimulatory AKT1-S473-P and inhibitory GSK-3β-S9-P (Fig. 5) in the inner medulla, but the effects did not reach statistical significance, because of variations among mice (Fig. 5). L. chagasi significantly reduces protein abundance of ERK1/2 (Fig. 2A), GSK-3β (Fig. 5B), and STAT3 (Fig. 2C). A related species, L. donovani, induces proteasome-mediated degradation of STAT1α in the B10R macrophage cell line, and this effect is independent of SHP-1, but involves PKCα (18). It should be noted that the parasite has no significant effect on STAT3 protein stability in the same study (18). Whether L. chagasi-induced decreases in protein abundance of ERK1/2, GSK-3β, and STAT3 are due to enhanced proteasome degradation remains to be studied. Nevertheless, it remains possible that L. chagasi inhibits NFAT5 through additional signaling pathways.

Possible Explanations for Lack of Effect of L. chagasi on Urinary Concentration Ability

In contrast to the potent inhibition of NFAT5 transcriptional activity by the L. chagasi supernatant from 1.2 × 10⁷/ml metacyclics in mIMCD3 cells, infection of mice with 5 × 10⁵ promastigotes/mouse only modestly inhibited expression of NFAT5-targeted genes and did not cause a defect in urinary concentration. One of the possible reasons may be due to the fact that the dose of parasites we used was 2–20 times lower than what other investigators have reported (49, 59). Another possible explanation is the genetic differences between humans and mice. Visceral leishmaniasis patients often die if they are left untreated, and the infection is chronic and progressive for months to years (53). In contrast, BALB/c mice, which are more susceptible to Leishmania than C57BL/6 mice, will eventually resolve L. chagasi infection themselves (26).

Perspective

We have demonstrated that L. chagasi activates SHP-1 and inhibits NFAT5 activity in the kidney inner medulla in our murine VL model (Fig. 4). However, we did not find evidence that L. chagasi is present in the inner medulla. This raises a question as to how the parasite activates SHP-1 there. Leishmaniasis secrete a large repertoire of molecules into the medium (24, 51). It has been proposed that Leishmania activate SHP-1 through their secreted virulence factors (28, 50). We speculate that L. chagasi may activate SHP-1 in the kidney inner medulla through the same mechanism. This speculation is supported by the observation showing that the L. chagasi culture supernatant increases SHP-1 protein abundance and reduces NFAT5 transcriptional activity in cultured mIMCD3 cells (Fig. 5). Patients with Brazilian cutaneous Leishmaniasis have a urine concentration defect (43). Injection of desmopressin did not raise urine osmolality, suggesting that urine concentration impairment is nephrogenic (43). Since cutaneous Leishmaniasis is a localized infection, it is intriguing whether the Leishmania strains in these patients impair urinary concentration also through their secreted virulence factors.

SHP-1 is often regulated by membrane receptors for cytokines, growth factors, and antigens (11). It is possible that mammals might have the endogenous membrane receptors for circulating Leishmania virulence factors, and the virulence factors bind these receptors, leading to activation of SHP-1. For example, Toll-like receptor 4 is an endogenous receptor for Gram-negative bacterial lipopolysaccharides (45). Leishmania activates Toll-like receptor 2, 4, and other Toll-like receptors. However, activation of these Toll-like receptors leads to a reduction in parasite burdens (10, 16), making these Toll-like receptors unlikely candidates for the receptors that link to activation of SHP-1, which promotes Leishmania propagation. Therefore, the identity of the endogenous membrane receptors for Leishmania circulating virulence factors remains to be defined.

In summary, SHP-1 is abundantly expressed in hematopoietic cells and has been studied extensively in these cells (35,
52). We demonstrate that SHP-1 is also expressed in the cells of the renal inner medulla (Fig. 1) and that L. chagasi activates SHP-1 possibly through increasing phosphorylation of its Y536 in that region (Figs. 2A and 3A). This effect is not contributed to by macrophages, because L. chagasi does not increase macrophage infiltration to the inner medulla (Fig. 2D).

The L. chagasi culture supernatant increases SHP-1 protein abundance and inhibits NFAT5 transcriptional activity in mIMCD3 cells (Fig. 5). L. chagasi, most likely through its secreted virulence factors, inhibits NFAT5 gene expression, which leads to reduced expression of NFAT5-targeted genes in the kidney inner medulla (Fig. 4).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: X.Z. and N.E.A. provided conception and design of research; X.Z., H.W., N.L.K., A.Z., and N.E.A. performed experiments; X.Z. and N.E.A. analyzed data; X.Z. and N.E.A. interpreted results of experiments; X.Z. prepared figures; X.Z. drafted manuscript; X.Z. and N.E.A. edited and revised manuscript; X.Z. and N.E.A. approved final version of manuscript.

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