

Novel role for the double-stranded RNA-activated protein kinase PKR: modulation of macrophage infection by the protozoan parasite *Leishmania*

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ABSTRACT The evolution of *Leishmania* infection depends on the balance between microbicidal and suppressor macrophage functions. Double-stranded RNA (dsRNA)-activated protein kinase R (PKR), a classic antiviral protein, is able to regulate a number of signaling pathways and macrophage functions. We investigated the possible role of PKR in the modulation of *Leishmania* infection. Our data demonstrated that *Leishmania amazonensis* infection led to PKR activation and increased PKR levels. Consistently, in macrophages from PKR knockout 129Sv/Ev mice and RAW-264.7 cells stably expressing a dominant-negative (DN) construct of PKR (DN-PKR), *L. amazonensis* infection was strongly reduced. The treatment of infected macrophages with the synthetic double-stranded RNA poly(I:C), a potent PKR inductor, increased *L. amazonensis* intracellular proliferation. This effect was reversed by 2-aminopurine (2-AP), a pharmacological inhibitor of PKR, as well as by the expression of DN-PKR. NO release induced by dsRNA treatment was inhibited by *L. amazonensis* through NF- κ B modulation. PKR activation induced by dsRNA also resulted in IL-10 production, whose neutralization with specific antibody completely abrogated *L. amazonensis* proliferation. Our data demonstrated a new role of PKR in protozoan parasitic infection through IL-10 modulation.—Pereira, R. M. S., Teixeira, K. L. D., Barreto-de-Souza, V., Calegari-Silva, T. C., De-Melo, D. B., Soares, D. C., Bou-Habib, D. C., Silva, A. M., Saraiva, E. M., Lopes, U. G. Novel role for the double-stranded RNA-activated protein kinase PKR: modulation of macrophage infection by the protozoan parasite *Leishmania*. *FASEB J.* 24, 617–626 (2010). www.fasebj.org

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DOUBLE-STRANDED RNA (dsRNA)-dependent protein kinase R (PKR) has been identified as an anti-

ral protein in pathways regulated by type I interferons (IFNs) (1–3) because of its ability to inhibit protein synthesis through the phosphorylation of the eukaryotic translation initiation factor eIF-2- α , its main downstream substrate (4, 5). PKR can be activated by viral dsRNA intermediates generated during a viral infection as well as by the synthetic polyribonucleotide duplex poly(rI):poly(rC) [poly(I:C)] (2, 6, 7).

In addition to dsRNA, PKR can be activated by a number of other stimuli, including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IFN- γ , and the bacterial product lipopolysaccharide (LPS) (2, 8–10). PKR is also capable of regulating the activation of key transcriptional cellular components, such as nuclear factor (NF)- κ B (11, 12).

PKR plays a pivotal role in the innate signaling pathway inasmuch as it is activated by proinflammatory stimuli and is important in NF- κ B activation. Poly(I:C)-induced macrophage activation is regulated by PKR, which controls inducible nitric oxide synthase (iNOS), IL-1 α and IL-1 β mRNA expression, nitrite formation, and IL-1 release (13). Moreover, the role of PKR in the regulation of the anti-inflammatory cytokine IL-10 in macrophages has recently been reported (14). The infection of primary human blood monocytes by bacillus Calmette-Guérin stimulates a PKR-dependent induction of cytokine expression, including TNF- α , IL-6, and IL-10 (15); this effect highlights the significant role this kinase performs in antibacterial defense. However, the participation of PKR in the control or in the pathogenesis

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of parasitic infections diseases has not yet been addressed.

Leishmaniasis is a well-studied model of protozoan parasitic infection because of its global incidence, affecting roughly 12 million people on all five continents (16). *Leishmania* spp. are transmitted by sandfly vectors in the promastigote form. In vertebrate hosts, leishmaniae, which are mainly found inside macrophages, assume the amastigote form (17). The balance between the microbicidal and suppressor functions exhibited by macrophages in association with parasitic adaptive strategies dictates disease progression.

The production of NO by iNOS (or NOS2) is one of the main effector mechanisms for *Leishmania* elimination by macrophages. This enzyme is induced by IFN- γ and TNF- α , which are cytokines produced by T CD4⁺Th1 cells (18, 19). However, the cytokines associated with Th2 responses, such as IL-4, IL-10, and TGF- β , not only inhibit the IFN- γ -mediated killing of parasites (20, 21), but also directly favor the growth of *Leishmania* inside macrophages through the production of polyamines (22–24).

Leishmaniasis progression in humans and mice has been associated with IL-10 immunomodulatory activity (25, 26). The primary role of IL-10 during *Leishmania* infection may be to limit macrophage activation (20). In *Leishmania amazonensis* infection, IL-10 limits leishmanicidal activity in the host, despite not determining the phenotype of the Th response (27).

In this work, we investigated the role of PKR in the progression of macrophage infection by *L. amazonensis*. Our data demonstrate a novel PKR function, demonstrating its importance in the context of infection by protozoan parasites.

MATERIALS AND METHODS

Reagents and antibodies

Phorbol-12-myristate-13-acetate (PMA), polyinosinic:polycytidylic acid [poly(I:C)], and 2-aminopurine (2-AP) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Anti-NF- κ B, anti-PKR, and anti-CDK2 antibodies and NF- κ B oligonucleotides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-PKR, anti-eIF2 α , and phospho-eIF2 α antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Neutralizing anti-human antibodies, anti-IL-10, and the human isotype control IgG2b were obtained from eBioscience (San Diego, CA, USA).

Cell lines

THP-1 and RAW 264.7 cells were cultured in DMEM with high glucose and L-glutamine (Invitrogen, Carlsbad, CA, USA) and supplemented with 1 mM pyruvate (only in the case of THP-1), 10% heat-inactivated FBS (Cultilab, Campinas, SP, Brazil), 100 U/ml penicillin, and 100 μ g/ml streptomycin in an incubator at 37°C with 5% CO₂. Before infection, THP-1 cells were differentiated to macrophages with 40 ng/ml PMA for 3 d and left to repose for an additional 3 d. RAW 264.7 cells expressing either PKR K296R

(RAW-DN-PKR cells) or an empty vector (RAW-bla cells) were generated as described previously (28).

Peritoneal macrophages

Thioglycolate-elicited peritoneal macrophages from wild-type (WT) or PKR-knockout (KO) 129Sv/Ev mice (29) were obtained by injecting 10 ml of PBS into the peritoneal cavity. The cell suspension was washed in PBS one time and then resuspended in serum-free DMEM. Cells were plated on glass coverslips at 2×10^5 /well in 24-well polystyrene plates and incubated for 1 h at 37°C in a 5% CO₂ atmosphere. Nonadherent cells were washed out with PBS, and the adherent cell population was incubated for 1 d in DMEM containing 10% FBS for subsequent *Leishmania* infection assays.

Human monocyte culture

Human monocyte-derived macrophages were obtained from peripheral blood mononuclear cells (PBMCs) isolated by density gradient centrifugation (Histopaque; Sigma-Aldrich) from buffy coat preparations of healthy blood donors as described previously (30). In brief, 1×10^6 PBMCs were plated onto 8-well Permanox chamber slides (Nalge Nunc, Rochester, NY, USA) in DMEM containing 10% human serum (Sigma-Aldrich) and penicillin-streptomycin (Sigma-Aldrich). Cells were maintained at 37°C in 5% CO₂ for 6–7 d for monocytic differentiation into macrophages. Nonadherent cells were washed out with PBS, and the macrophages were maintained for 3 d in DMEM containing 10% human serum before infection studies.

Parasites and infection

L. amazonensis (WHOM/BR/75/Josefa) and *Leishmania major* (LV-39 MHRO/Sv/59/P) were used in this study. Promastigotes were grown at 26°C in Schneider's insect medium (Sigma-Aldrich) supplemented with 10% FBS, 10% human urine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Promastigotes from 4- to 5-d stationary cultures were used for experiments throughout. Cells were infected for 18 h at 37°C with *Leishmania* stationary-phase promastigotes with a parasite:cell ratio of 5:1 to evaluate amastigote proliferation inside macrophages. Noninternalized promastigotes were washed out, fresh medium was added, and cultures were maintained at 37°C in 5% CO₂ for 3 d. Poly(I:C), the dsRNA stimulus, was added to infected macrophage cultures immediately after the removal of noninternalized *Leishmania* parasites. In some experiments, cells were either pretreated with 2-AP before the addition of poly(I:C) or cotreated with blocking anti-mouse antibodies anti-IL-10 or its control isotype plus poly(I:C). Infected macrophages were counted by light microscopy. The infection index was calculated by multiplying the percentage of infected macrophages by the average number of parasites per macrophage in Giemsa-stained slides. Alternatively, promastigote production from infected cells was evaluated as follows: after 3–4 d of infection, nonadherent cells were removed, adherent cells were extensively washed, and 0.5 ml of Schneider's insect medium containing 20% FBS and 10% human urine were added to the wells. After 5–7 d at 26°C, the growing extracellular motile promastigotes derived from infected macrophages were counted.

Immunoblotting

Cells (10^6) were washed twice with ice-cold PBS and then lysed in 60 μ l of lysis buffer (50 mM Tris-HCl, pH 7.5; 5 mM

EDTA; 10 mM EGTA; 50 mM NaF; 20 mM β -glycerophosphate; 250 mM NaCl; 0.1% Triton X-100; and 1 μ g/ml BSA) to which a 1:1000 dilution of protease inhibitor cocktail II (Calbiochem, Gibbstown, NJ, USA) was added. Proteins were subjected to electrophoresis in 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). After blocking with 5% nonfat dry milk in TBS with 0.1% of Tween-20 (TBST), blots were incubated for 1 h with (1:1000 diluted) primary antibodies in blocking solution as mentioned previously, followed by anti-rabbit or anti-mouse horseradish peroxidase-conjugate IgG (1:3000). The membranes were then submitted to 3 washings with TBST, and proteins were detected by the ECL chemiluminescent detection system (Amersham Biosciences).

Semiquantitative RT-PCR

Cells were harvested, and total RNA was obtained with the SV Total RNA Isolation Kit (Promega Corp., Madison, WI, USA). First-strand cDNA synthesis was performed in a reaction containing Superscript II (Invitrogen), a mix of dNTPs, and oligo(dT), as described by the manufacturer. RT-PCR was performed using PKR primers (sense 5'-GACCTTCCTGACATGAAAGAA-3' and antisense 5'-AACTATTTTGTCTGTTCTCAGG-3') or actin primers (sense 5'-GTTGCTATCCAGGCTGTGC-3' and antisense 5'-GCATCCTGTCGGC-AATGC-3'), 2.5 U of GoTaq DNA polymerase (Promega), and 1.5 mM MgCl₂, in an appropriate buffer, at an annealing temperature of 50°C and 25 amplification cycles. PCR products were fractionated onto 1.3% agarose gel, stained with ethidium bromide, and photographed in a UV transilluminator.

Electrophoretic mobility shift assay (EMSA) and supershift for NF- κ B

Nuclear cell extracts were obtained as described elsewhere (31). EMSA was performed by incubating 3 μ g of nuclear protein extract with 40,000 cpm of ³²P end-labeled double-stranded NF- κ B oligonucleotide consensus (Santa Cruz Biotechnology) for 30 min at 25°C. The binding mixture included 1 μ g of poly(dI-dC):poly(dI-dC) in binding buffer (10 mM HEPES, pH 7.9; 4% glycerol; 1 mM DTT; 1 mM EDTA; and 0.1 μ g of BSA). The DNA-protein complex was separated from the free probe in 4% native polyacrylamide gel and then dried and visualized by PhosphorImage analysis (Molecular Dynamics, Sunnyvale, CA, USA). In supershift assays, nuclear cell extracts were incubated with 0.5 μ g of antibodies against the NF- κ B subunits p65 (sc-109), p50 (sc-114), c-Rel (sc-71), RelB (sc-226), and p52 (sc-298) (Santa Cruz Biotechnology) for 1 h on ice before incubation with the probe.

Cytokine ELISA systems

The concentration of IL-10 in the culture supernatant was measured *via* ELISA systems purchased from BD Biosciences (San Jose, CA, USA).

Determination of nitrite concentration

Nitrite, the end product of NO metabolism, was measured from 50 μ l of cell culture supernatants by using Griess reagent, as described elsewhere (32).

Luciferase reporter assay

For measurement of NF- κ B transcriptional activity, RAW 264.7 (2×10^5 cells/well) cells were seeded onto 24-well plates and

transfected using Lipofectamine 2000 reagent (Invitrogen). Plasmids used in the assays were pTK-3XNS and pTK-3XS (2 μ g) (kindly provided by Dr. David Geller, University of Pittsburgh, Pittsburgh, PA, USA). The plasmid pRL-CMV (Promega) was used for luciferase activity normalization. After infection and treatment, the cells were then washed with PBS, lysed according to the dual luciferase system protocol (Promega), and analyzed in the TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA).

Statistical analyses

Data were analyzed by Student's *t* test for independent samples using Prism 5 software (GraphPad, San Diego, CA, USA). Data are expressed as the average of 3 determinations, and significant differences were indicated for *P* < 0.05.

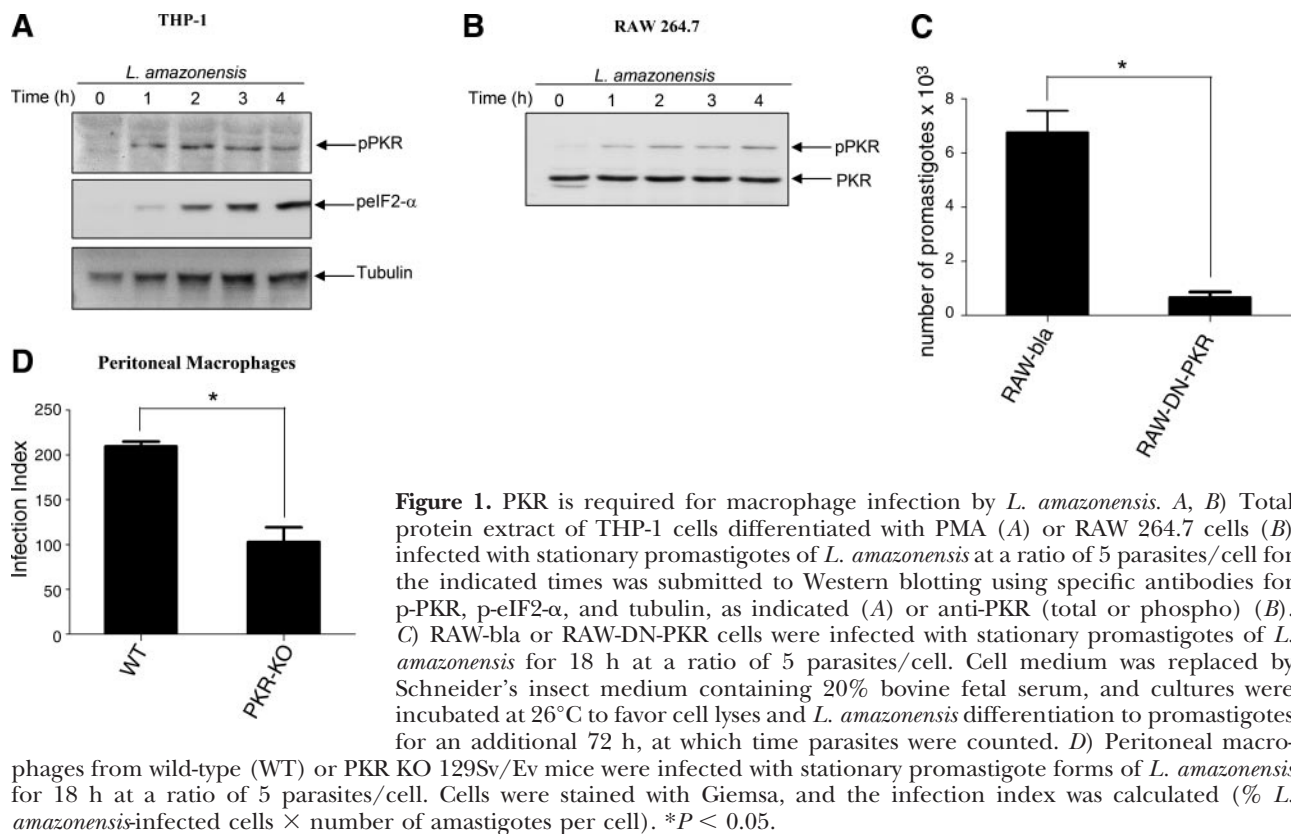
RESULTS

L. amazonensis-activated PKR exhibits a prominent role in macrophage infection

As a way to determine the possible role of PKR in *Leishmania* infection, the ability of *L. amazonensis* to induce the activation of this kinase was investigated. Our results demonstrated that, at the first hours of infection, stationary-phase *L. amazonensis* promastigotes were able to induce PKR phosphorylation in human (differentiated THP-1) and murine macrophages (RAW 264.7) (Fig. 1A, B). Accordingly, the PKR substrate eIF2- α was also phosphorylated (Fig. 1A). These results suggest that *L. amazonensis* infection leads to PKR activation. In view of the finding that *L. amazonensis* itself was capable of inducing PKR phosphorylation, it was decided to address the role of this kinase as a regulator of *Leishmania* infection. For this purpose, we used RAW 264.7 cells expressing the dominant-negative form of PKR (RAW-DN-PKR) or blasticidin-resistant RAW cells transfected with an empty vector (RAW-bla) as control. As can be seen in Fig. 1C, *L. amazonensis* infection was markedly reduced in RAW-DN-PKR cells. The infection was also reduced in peritoneal macrophages derived from PKR-KO mice (Fig. 1D). Taken together, these results demonstrate that PKR signaling is activated by *L. amazonensis* and that it is required to increase parasitic growth in macrophages.

PKR activation increases intracellular growth of *L. amazonensis*

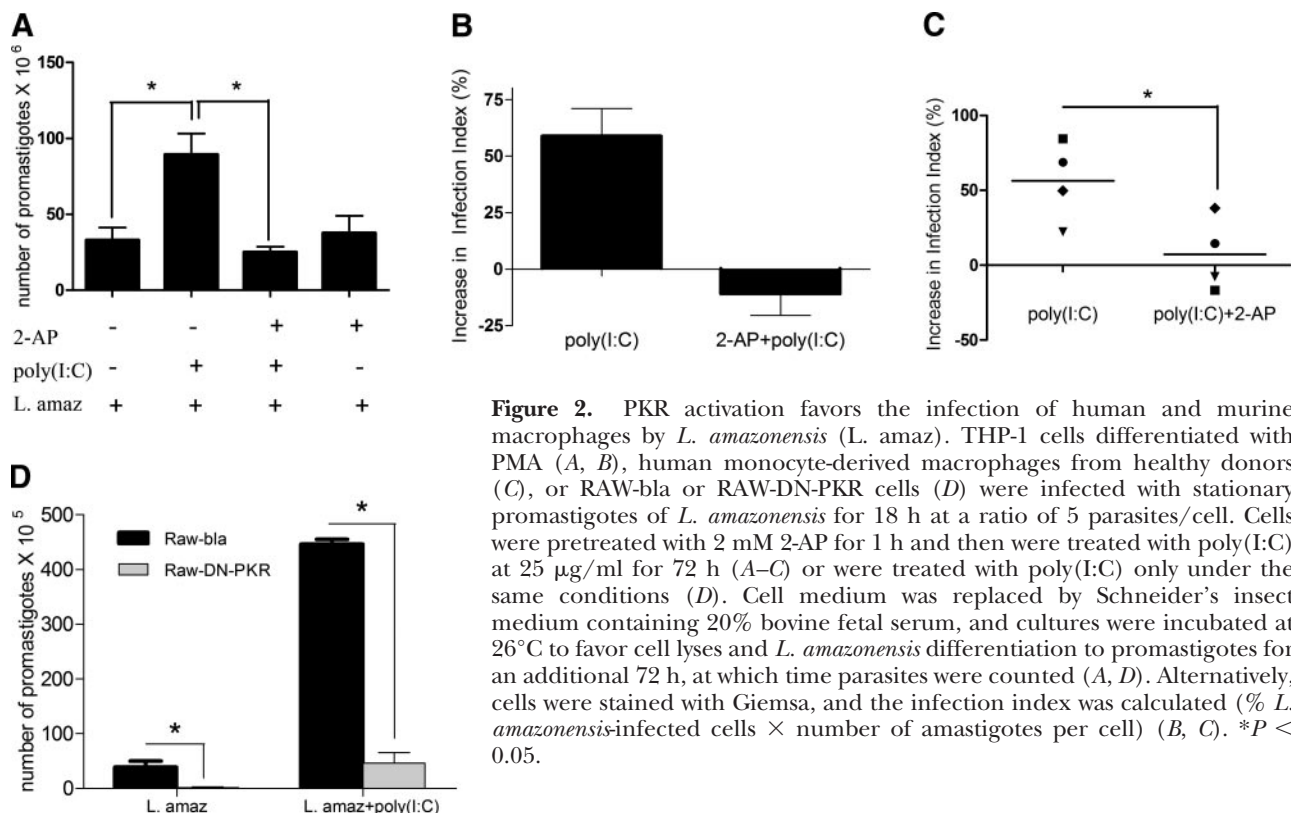
Poly(I:C), a classic PKR inducer, was used to evaluate the effect of PKR activation on the infection of macrophages by *Leishmania*. Human differentiated THP-1 cells were infected with stationary-phase *L. amazonensis* promastigotes for 18 h and then were treated with poly(I:C) for 72 h to induce PKR activation. It was observed that poly(I:C) treatment caused a significant increase in promastigote production, which was prevented by treatment with 2 mM 2-aminopurine (2-AP), a pharmacological inhibitor of PKR (Fig. 2A). A similar result was obtained when the infection index of THP-1 in Giemsa-stained cells was



determined (Fig. 2*B*). These observations were further confirmed in primary cells using human monocyte-derived macrophages (Fig. 2*C*). Additional experiments with macrophages from 3 other donors

were evaluated. Consistently, poly(I:C) favored *L. amazonensis* infection (data not shown).

To confirm the role of PKR in intracellular *Leishmania* growth, we then used RAW-DN-PKR cells. Figure 2*D*



shows that expression of DN-PKR strongly reduces intracellular proliferation of *L. amazonensis* in infected cells treated with poly(I:C), supporting the notion that PKR activation promotes an increase in intracellular proliferation of this parasite in macrophages.

L. amazonensis modulates PKR levels

To confirm PKR activation and its increased levels in our study model, Western blot assays were performed. It was observed that poly(I:C) treatment of differentiated THP-1 cells induced PKR phosphorylation both in infected and uninfected cells (Fig. 3A).

The activation of PKR was confirmed by the detection of phosphorylated eIF2- α , a well-characterized PKR substrate (Fig. 3A). Strikingly, total PKR levels increased in *L. amazonensis*-infected cells. To better evaluate the *Leishmania*-induced increase in PKR levels

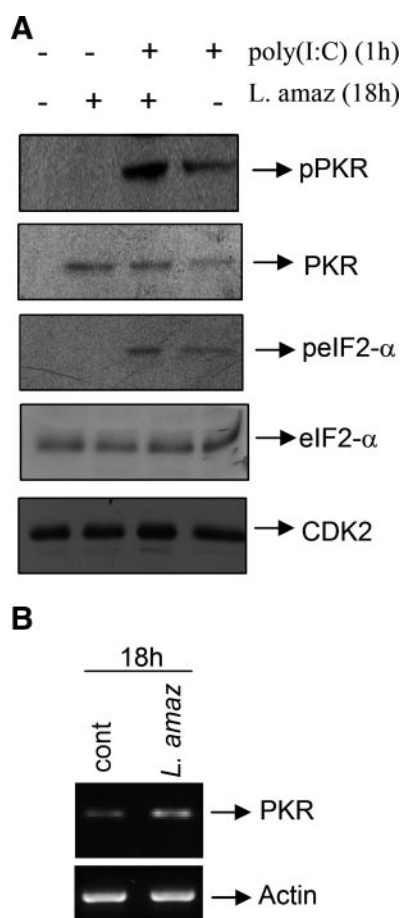


Figure 3. PKR levels are increased after *L. amazonensis* (*L. amaz*) infection. **A**) Total protein extract of THP-1 cells differentiated with PMA, infected with stationary promastigotes of *L. amazonensis* for 18 h at a ratio of 5 parasites/cell, and treated with poly(I:C) at 25 μ g/ml for 1 h was submitted to Western blot using specific antibodies for p-PKR (Thr-446), PKR, p-eIF2- α , eIF2- α , and CDK2. **B**) THP-1 cells differentiated with PMA were infected with stationary promastigotes of *L. amazonensis* for 18 h at a ratio of 5 parasites/cell. Cells were harvested, and total RNA was extracted. RT-PCR was performed using PKR or actin primers.

in infected macrophages, PCR assays were performed. Figure 3B reveals that after 18 h of infection with *L. amazonensis*, there was a notable increase in PKR mRNA levels.

L. amazonensis infection modifies the NF- κ B dimer activated by poly(I:C)

NF- κ B activation plays an important role in cellular responses regulated by PKR. Through EMSA, we confirmed that NF- κ B activation induced by poly(I:C) in THP-1-differentiated cells is dependent on PKR-activation, because it can be clearly demonstrated by the inhibition with 2-AP treatment (Fig. 4A).

Remarkably, when the cells were infected with *L. amazonensis* and treated with poly(I:C), there was a pronounced reduction in the bandshift corresponding to poly(I:C)-induced NF- κ B activation (p65/p50 complex) and an increase in the complex that comigrates with the p50/p50 homodimer activated by *L. amazonensis* (Fig. 4B). This modification in the NF- κ B migration pattern appears to be specific to *L. amazonensis* infection because *L. major* was not able to induce the same pattern (Fig. 4B). A more detailed analysis by supershift revealed that, besides induction of the p50/p50 homodimer, *L. amazonensis* infection also changed the p65/p50 complex activated by poly(I:C) into a higher mobility complex (Fig. 4C), suggesting that the infection with this parasite modifies the NF- κ B proteins. In fact, *L. amazonensis* infection leads to p65 cleavage, as demonstrated by Western blot analysis (Fig. 4D). Conversely, *L. major* infection preserved p65/p50 activation by poly(I:C) (Fig. 4C).

PKR-dependent NO production is inhibited by *L. amazonensis* through NF- κ B subversion

Because PKR also modulates iNOS levels, we evaluated whether poly(I:C)-induced PKR activation promotes the production of NO. As expected, poly(I:C) treatment led to significantly higher NO production in RAW-bla cells. However, *L. amazonensis* was able to inhibit the increase in these levels (Fig. 5A). This finding is congruent with the ability of this parasite to reduce iNOS expression in macrophages (33).

Next, we performed luciferase reporter assays in an attempt to correlate the inhibition of PKR-dependent NO production by *L. amazonensis* with the ability of this parasite to subvert NF- κ B activation and inhibit iNOS expression. RAW 264.7 cells were transiently transfected with a NF- κ B/signal transducer and activator of transcription (Stat1) luciferase reporter construction (pTK-3XNS) containing three copies of the NF- κ B/Stat1 motifs present at -5.8 kb of the iNOS promoter. The day after transfection, cells were infected with *L. amazonensis* and treated with poly(I:C) to induce promoter activity through PKR induction. After 24 h, luciferase activity was assayed. As shown in Fig. 5B, *L.*

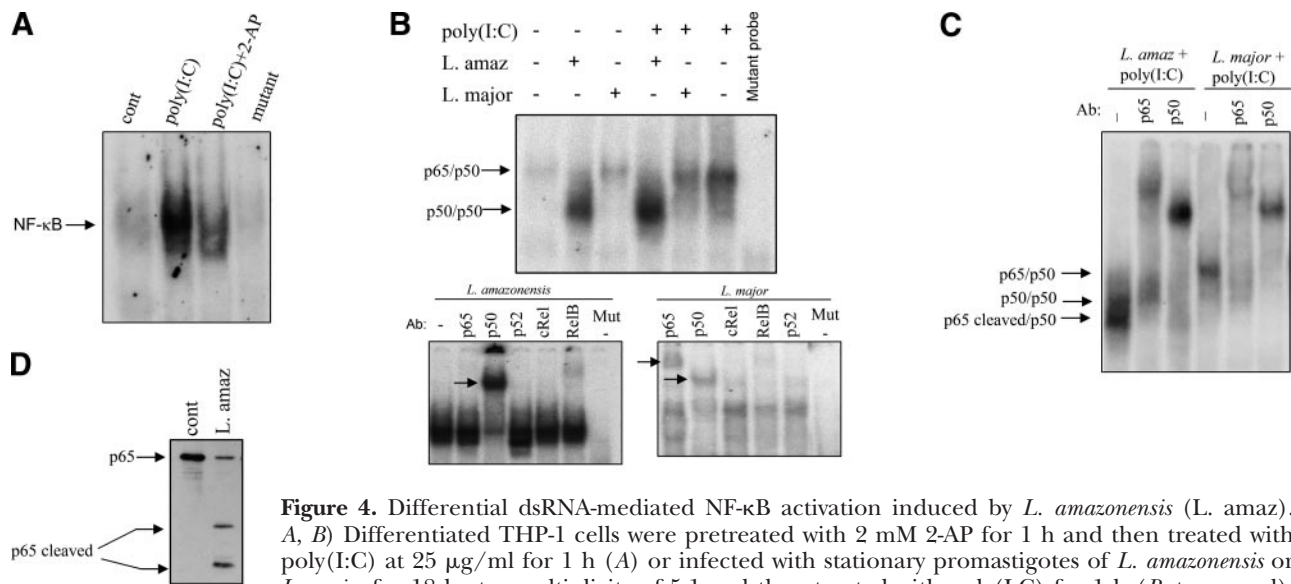


Figure 4. Differential dsRNA-mediated NF-κB activation induced by *L. amazonensis* (*L. amaz*). **A, B**) Differentiated THP-1 cells were pretreated with 2 mM 2-AP for 1 h and then treated with poly(I:C) at 25 μg/ml for 1 h (**A**) or infected with stationary promastigotes of *L. amazonensis* or *L. major* for 18 h at a multiplicity of 5:1 and then treated with poly(I:C) for 1 h (**B**, top panel). Nuclear protein extracts were obtained and submitted to EMSA with oligonucleotide containing the consensus sequence for NF-κB binding. Mut, oligonucleotide mutant for NF-κB. **C**) Extracts were supershifted with specific antibodies for NF-κB subunits. **D**) Total extracts of RAW 264.7 cells infected with stationary promastigotes of *L. amazonensis* for 18 h at a multiplicity of 5:1 were submitted to Western blot with specific antibodies for the p65 subunit of NF-κB.

amazonensis reduced the NF-κB/Stat1-dependent transcription activity induced by poly(I:C). Interestingly, the transient transfection of RAW 264.7 cells with a Stat1 luciferase reporter construction, corresponding to the motifs mapped at 5.2 kb of the iNOS promoter (pTK-3XS), treated as described above (Fig. 5C), revealed that *L. amazonensis* infection did not reduce the Stat1 transcriptional activity induced by this PKR induc-

In human macrophages, poly(I:C) treatment has also induced reactive oxygen species production, but, similarly to the results observed for NO, these levels were

inhibited by *L. amazonensis* infection (Supplemental Fig. 1).

PKR-dependent production of IL-10 favors *L. amazonensis* intracellular replication in macrophages

It was our aim to pursue the mechanisms involved in the *L. amazonensis* intracellular replication favored by PKR. **Figure 6A** shows that *L. amazonensis* infection and/or poly(I:C) treatment induces the secretion of

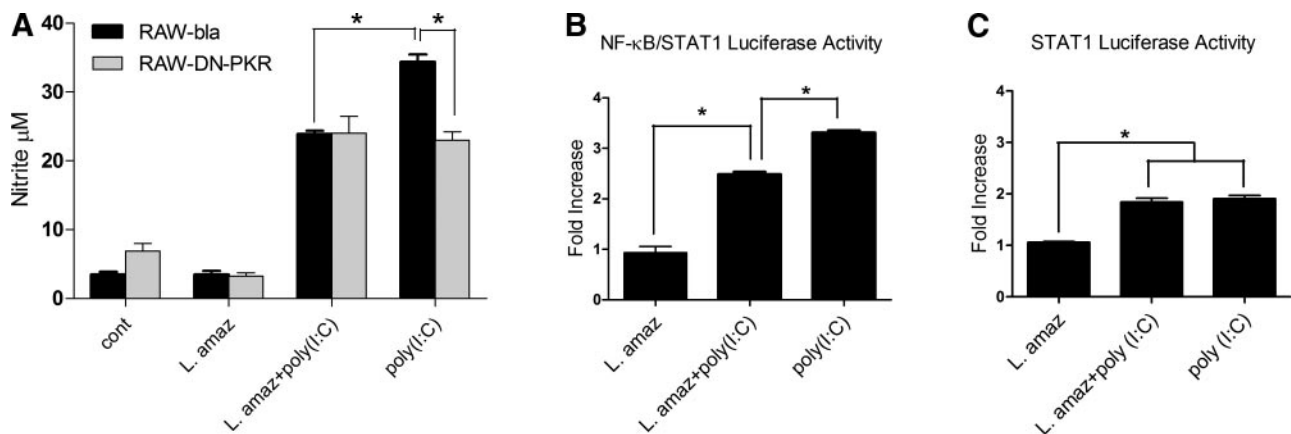


Figure 5. dsRNA-mediated production of NO requires PKR and involves NF-κB activation, but it is inhibited by *L. amazonensis* (*L. amaz*) infection. **A**) RAW-bla or RAW-DN-PKR cells were infected with stationary promastigote forms of *L. amazonensis* for 18 h at a multiplicity of 5:1 and then treated with poly(I:C) at 25 μg/ml for 24 h. Supernatants were collected, and the nitrite concentrations were evaluated by Griess reaction. **B, C**) RAW cells were transiently transfected using a reporter plasmid, pTK-3XNS, containing 3 NF-κB/Stat1 consensus-binding sites upstream of the luciferase reporter gene (**B**) or transfected using a reporter plasmid pTK-3XS containing 3 Stat1 consensus-binding sites upstream of the luciferase reporter gene (**C**). Twenty-four hours after transfection, cells were infected with stationary promastigote forms of *L. amazonensis* for 18 h at a multiplicity of 5:1 and then treated with poly(I:C) at 25 μg/ml. After 24 h, whole-cell lysates were analyzed for luciferase activity. **P* < 0.05.

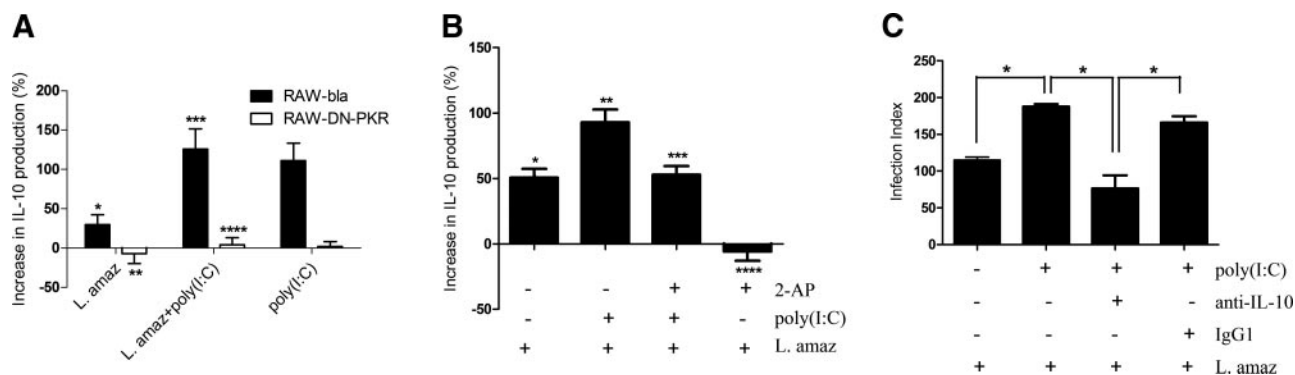


Figure 6. IL-10 production induced by dsRNA and/or *Leishmania* infection has a determinant role in the proliferation of *L. amazonensis* (*L. amaz*). A, B) RAW 264.7 cells transfected with RAW-bla or with RAW-DN-PKR (A) or THP-1 cells differentiated with PMA (B) were infected with stationary promastigotes of *L. amazonensis* for 18 h at a multiplicity of 5:1. Cells were pretreated with 2 mM 2-AP for 1 h and then treated with poly(I:C) at 25 μ g/ml for 48 h (B), or cells, infected or not, were only treated with poly(I:C) under the same conditions (A). Production of IL-10 in supernatants was analyzed by ELISA. C) THP-1 cells differentiated with PMA were infected with stationary promastigotes of *L. amazonensis* for 18 h at a ratio of 5 parasites/cell and treated with poly(I:C) at 25 μ g/ml and anti-IL-10 blocking antibody or IgG1 isotype control for 72 h. Cells were stained with Giemsa, and the infection index was calculated (% *L. amazonensis*-infected cells $\times$ number of amastigotes per cell). Asterisks indicate a significant difference ($P < 0.05$) between groups: A) *untreated $\times$ *L. amaz*, ***L. amaz*-RAW-bla $\times$ *L. amaz*-RAW-DN-PKR, ****L. amaz* $\times$ *L. amaz* + poly(I:C), *****L. amaz* + poly(I:C)-RAW-bla $\times$ *L. amaz* + poly(I:C)-RAW-DN-PKR; B) *untreated $\times$ *L. amaz*, ***L. amaz* $\times$ *L. amaz* + poly(I:C), ****L. amaz* + poly(I:C) $\times$ *L. amaz* + poly(I:C) + 2-AP, *****L. amaz* $\times$ *L. amaz* + 2-AP; C) *groups are indicated.

the suppressor cytokine IL-10 by RAW-bla cells. However, this induction was not observed in RAW-DN-PKR cells. Accordingly, IL-10 production by poly(I:C) or *L. amazonensis* in THP-1 cells was also prevented by 2-AP treatment (Fig. 6B). Treatment with neutralizing anti-IL-10 antibody, but not by its control isotype, was able to impair the increase in *Leishmania* infection promoted by poly(I:C) (Fig. 6C). This result strongly supports the observation that IL-10 is a key secreted factor regulated by PKR that is important to facilitate *Leishmania* replication in macrophages.

DISCUSSION

PKR is a key component of the cell antiviral response that acts by modulating the activity of a number of cellular proteins. In this work, we investigated the role of PKR during protozoan parasite *Leishmania* infection. We observed that *L. amazonensis* is able to induce PKR phosphorylation in human and mouse macrophages (Fig. 1A, B) in short-term infections. This activation favors the intracellular replication of the parasite because in DN-expressing and PKR-deficient macrophages, *L. amazonensis* infection was seen to clearly diminish (Fig. 1C, D). Preliminary *in vivo* assays in our laboratory demonstrated the reduction of *L. amazonensis* infection in 129Sv/Ev PKR KO mice in comparison with the wild type (data not shown), strengthening the relevance of our *in vitro* data in murine *Leishmania* infection.

Using the pharmacological inhibitor 2-AP and by expressing a DN-PKR construct in cells, we observed that PKR activation in response to synthetic dsRNA poly(I:C) treatment promotes an increase in *L. amazonensis* intracel-

lular proliferation in human and murine macrophages (Fig. 2). The effect of PKR appears to be dependent and specific on *Leishmania* species because similar results have been obtained for *Leishmania chagasi* but not for *L. major* infection (data not shown).

To our knowledge, this is the first description of the role played by PKR in parasitic infections. Cheung *et al.* (15) have demonstrated the critical role of PKR in the regulation of cytokine expression on mycobacterial infection, corroborating the fact that PKR is more than an antiviral protein when it is acting in innate immunity. The demonstration that mice expressing the viral protein E3L, which is capable of inhibiting PKR, are more susceptible to *L. major* infection (34) reinforces the putative role of this kinase in modulating *Leishmania* infection and supports our macrophage data with respect to *L. major* (data not shown).

Besides PKR phosphorylation, we also verified increased levels of this kinase induced by *L. amazonensis* infection (Fig. 3B). Considering the positive role of PKR induction in *L. amazonensis* intracellular proliferation, it is expected that the observed increase in PKR levels may provide additional molecules that are able to be phosphorylated in response to stimuli present at the site of infection as a parasitic adaptive mechanism to modulate the host-cell response. Because of the role of type I IFN in inducing PKR expression (4) through the induction of the heterodimeric factor interferon-stimulated gene factor 3 (STAT1, STAT2, and interferon regulatory factor 9) (35–37), it can be hypothesized that the increased PKR levels provoked by *Leishmania* may be due to a possible induction of these cytokines by the parasite.

Susceptibility to *Leishmania* infection may be associated with IL-10 production in humans (26) and murine

models of cutaneous (20, 38) and visceral leishmaniasis (39). In *L. amazonensis* infection, IL-10 is also important in limiting the Th1 response during the acute phase of C57BL/6 mice infection even though IL-10 does not play the same role in the chronic phase of the disease (27). Moreover, IL-10 is related to the reduction of IFN- γ , NO, and IL-12 production in BALB/c mice infected by *L. amazonensis* (40).

Consistent with the observation that IL-10, a cytokine with suppressor activities on cells, can be modulated by PKR in macrophages (14), we have also observed an increase in IL-10 production in *L. amazonensis*-infected RAW 264.7 and THP-1 cells treated with poly(I:C), which was reversed by DN-PKR expression or 2-AP treatment, respectively (Fig. 6A, B). Notably, IL-10 production induced during *L. amazonensis* infection also depends on PKR. The role of IL-10 in PKR-mediated favoring of *L. amazonensis* intracellular proliferation was confirmed in our model by immunoneutralization assays (Fig. 4C). The increased IL-10-dependent susceptibility could be due to the switch from NO to polyamine production in arginine metabolism *via* induction of arginase I activity (23, 41).

Despite regulating the production of mediators that have positive effects on *Leishmania* intracellular proliferation, PKR can also induce the expression of iNOS and consequent production of NO in a poly(I:C)-dependent fashion (13). The control of *Leishmania* infection by NO production *in vivo* and *in vitro* is well documented (19, 42, 43). In our model, it was found that part of NO induction by poly(I:C) is dependent on PKR because NO production is inhibited by DN-PKR expression. Moreover, secreted, PKR-dependent NO levels were inhibited by *L. amazonensis* infection (Fig. 5A), which is consistent with previous results of our group demonstrating the inhibition of iNOS expression and NO production by *L. amazonensis* in a NF- κ B modulation-dependent mechanism (33). In the present work, it was shown that reduction by *L. amazonensis* of poly(I:C)-induced NO production also correlated with the inhibition of NF- κ B-dependent regulation of the iNOS promoter (Fig. 5B, C). Although NO was still produced, it did not appear to impair *L. amazonensis* survival. In fact, it has been observed that, in activated macrophages, *L. amazonensis* is more resistant to killing than *L. major* (44).

EMSA assays made it possible to better understand the mechanisms by which *L. amazonensis* inhibits NF- κ B-dependent iNOS expression induced by poly(I:C). We have confirmed PKR-dependent activation of this transcriptional factor in differentiated THP-1 cells (Fig. 4A). However, *L. amazonensis* subverts this activation through the cleavage of the NF- κ B subunit p65 induced by poly(I:C) (Fig. 4C, D) and induction of the p50/p50 homodimer (Fig. 4B, C), a classic repressor of NF- κ B-dependent transcriptional activity in view of the fact that the p50 subunit does not possess a transactivation domain (45) yet can still associate with histone deacetylase 1 (46). We had already perceived the same effect of *L. amazonensis* infection on LPS-treated macrophages (33). The cleavage of NF- κ B subunits by New Word

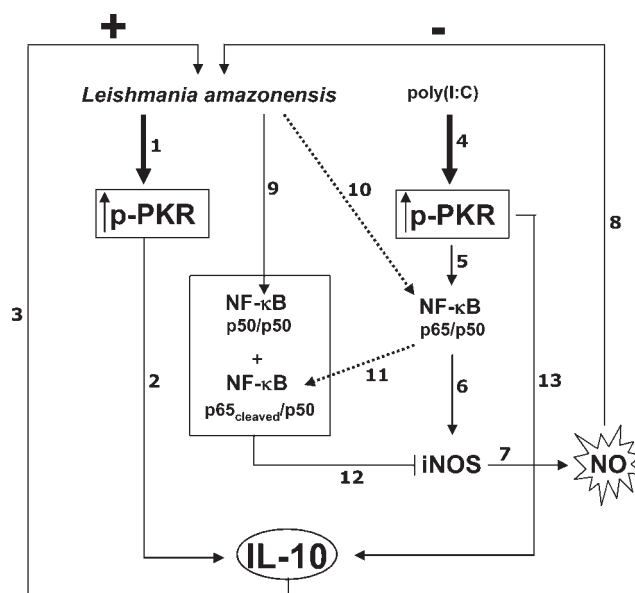


Figure 7. Proposed model of the role of PKR in the *L. amazonensis* infection of macrophages. *L. amazonensis* infection induces PKR phosphorylation and levels increase (1). Activated PKR induces production of IL-10 (2), a classic suppressor cytokine that can favor *Leishmania* intracellular proliferation (3) (+). Poly(I:C) treatment also determines PKR phosphorylation and the increase in PKR levels (4). PKR activation can induce the transactivator dimer of NF- κ B p65/p50 (5), leading to iNOS expression (6) and the consequent production of NO (7), which, in turn, has negative effect on intracellular *Leishmania* proliferation (8) (–). However, *L. amazonensis* is able to induce the NF- κ B p50/p50 repressor homodimer (9) and the cleavage of the p65 NF- κ B transactivator subunit (10, 11), determining the suppression of iNOS production (12) and the consequent reduction of NO intracellular levels (not shown). Activation of PKR by poly(I:C) also induces IL-10 production (13) and has a positive effect on intracellular *Leishmania* proliferation (3).

Leishmania species has also been studied by other groups (47, 48).

Several reports have demonstrated the activation of PKR by other proinflammatory stimuli besides dsRNA, such as LPS, IFN- γ , IL-1 β , and TNF- α (2, 12). Based on these reports, it is possible to visualize the role of other stimuli, perhaps IL-1, TNF- α , or *Leishmania* itself, activating PKR in the context of infection.

To our knowledge, our current findings have demonstrated for the first time that the classic antiviral PKR-dependent response also modulates the protozoan parasitic *L. amazonensis* infection. This effect depends on PKR-induced production of the suppressor cytokine IL-10. Likewise, *L. amazonensis* may negatively modulate NF- κ B activation and the subsequent NO production induced by this kinase (see the proposed model in Fig. 7).

In brief, our results provide new data regarding *Leishmania*-macrophage interaction and suggest PKR as a new target for the pharmacological control of *Leishmaniasis*. FJ

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