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Toll-Like Receptor 3 (TLR3) Is Engaged in the Intracellular Survival of the Protozoan Parasite *Leishmania (Leishmania) amazonensis*

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ABSTRACT The protozoan parasite *Leishmania (L.) amazonensis* infects and replicates inside host macrophages due to subversion of the innate host cell response. In the present study, we demonstrate that TLR3 is required for the intracellular growth of *L. (L.) amazonensis*. We observed restricted intracellular infection of TLR3$^{-/-}$ mouse macrophages, reduced levels of IFN1β and IL-10, and increased levels of IL-12 upon *L. (L.) amazonensis* infection, compared with their wild-type counterparts. Accordingly, *in vivo* infection of TLR3$^{-/-}$ mice with *L. (L.) amazonensis* displayed a significant reduction in lesion size. *Leishmania (L.) amazonensis* infection induced TLR3 proteolytic cleavage, which is a process required for TLR3 signaling. The chemical inhibition of TLR3 cleavage or infection by CPB-deficient mutant *L. (L.) mexicana* resulted in reduced parasite load and restricted the expression of IFN1β and IL-10. Furthermore, we show that the dsRNA sensor molecule PKR (dsRNA-activated protein kinase) cooperates with TLR3 signaling to potentiate the expression of IL-10 and IFN1β and parasite survival. Altogether, our results show that TLR3 signaling is engaged during *L. (L.) amazonensis* infection and this component of innate immunity modulates the host cell response.

KEYWORDS TLR3, *Leishmania*, IFN1β, IL-10, PKR, macrophages

*L*eishmania parasites subvert the host cellular immune response by interfering with various signaling pathways, which leads to the repression of macrophage functions (1–3). Several grades of anergic responses have been described in patients with cutaneous leishmaniasis due to *L. (L.) amazonensis* infection (4). Human infections caused by this species exhibit restricted interferon gamma (IFN-γ) production and display interleukin-10 (IL-10), transforming growth factor beta (TGF-β), and type I interferon beta (IFN1β) expression (5, 6). These observations have been corroborated in murine infection studies (7, 8).

We have previously demonstrated that *L. (L.) amazonensis* induces the expression and activation of double-stranded RNA-induced protein kinase (PKR) in infected macrophages. This mechanism plays an important role in mediating the production of IL-10 that favors the parasite intracellular growth (9). Our additional work has demonstrated that the levels of both PKR and IFN1β are increased upon *L. (L.) amazonensis* infection in a TLR2-dependent manner. The axis TLR2/PKR/IFN1β enhances parasite load through superoxide dismutase 1 (SOD1) expression, and associated decrease in reactive oxygen species (ROS) production, due to the enhanced expression of erythroid 2-related factor 2 (Nrf2)-dependent genes. (5, 10).

The intracellular vesicular RNA sensor Toll-like receptor 3 (TLR3) also recognizes dsRNA and restricts several viral infections (11) while also playing a role in other physiological processes, as a "danger" signaling receptor (12). In addition to dsRNA from viruses, cellular

RNAs originated from necrotic cells, probably harboring secondary structures (13), can serve as TLR3 ligands (14). Additionally, TLR3 is expressed in the endoplasmic reticulum in resting cells, endosomes, and other compartments in response to ligand stimulation (15). The dsRNA recognition occurs in late endosomes or acidified endolysosomes, where the inactive TLR3 is cleaved by cathepsins into its active form (16). Endosomal acidification is required not only for cleavage, but also to activate the cleaved fragment of TLR3 (17).

TLR3 signaling requires the recruitment of the adaptor protein TRIF (18, 19), which then leads to the activation of the transcription factors NF-κB (nuclear factor-κB) and IRF (interferon regulatory factor 3) followed by the expression of proinflammatory cytokines, type I IFN, and IFN-responsive genes (20–22). Additionally, it has been demonstrated that the activation of TLR3 in Kupffer cells leads to IL-10 production (23). TLR3 also regulates IL-10 production through the PI3K/AKT signaling pathway in response to *Mycobacterium bovis* Bacillus Calmette-Guérin BCG (24).

Experimental and clinical studies demonstrated that the presence of the endosymbiont *Leishmania* RNA virus (LRV) is associated with the severity of mucocutaneous leishmaniasis due to the infection with parasites belonging to the *Viannia* subgenus, including *Leishmania (V.) guyanensis* (25–27). TLR3 senses dsRNA from LRV in endosomes, triggering cytokine production, including type I IFN, and exacerbating the disease (27, 28). Moreover, TLR3-dependent IFNI production induces autophagy, which results in NLRP3 and ASC degradation, limiting NLRP3 inflammasome activation and its role in restricting *Leishmania* replication (29).

Even though the role of TLR3 in *Leishmania* infection through LRV ́s dsRNA sensing is well known, it is not known if its activation occurs in an LRV-independent way. In the present work, we reveal the engagement of TLR3 signaling and the corresponding expression of IL-10 and IFN1β in *L. (L.) amazonensis*-infected macrophages in an LRV1-independent way. We also investigated the role of a *Leishmania*-encoded proteases in TLR3 processing and the putative cooperation between TLR3 and PKR signaling upon parasite infection. Our data highlight the importance of dsRNA-dependent signaling mediated by vesicular TLR3 and cytoplasmic PKR in the adaptation of parasite intracellular life in macrophages, during evolution.

RESULTS

TLR3 contributes to intracellular *L. (L.) amazonensis* growth and *in vivo* infection. We aimed to investigate the role of TLR3 during *in vitro* macrophage infection and the development of lesions in infected mice. When we analyzed 48 h postinfection, where we can address the balance of replication and death, we observed a significant reduction in both percentage of infected macrophages and number of parasites per cell, which reflected an important reduction in the infection index (Fig. 1A to C). The analyses of TLR3$^{-/-}$ murine macrophages *in vitro* showed that the percentage of infected macrophages (Fig. 1B) and the number of parasites per macrophage (Fig. 1C) are not significantly different between wild-type (WT) and TLR3$^{-/-}$ macrophages during the first 4 h of infection, suggesting that the uptake of parasites is not affected by TLR3 deficiency.

The significance of TLR3 in *L. (L.) amazonensis* infection was also assessed *in vivo*. The development of footpad lesions was reduced in TLR3$^{-/-}$ mice compared to wild-type mice (Fig. 1D). Additionally, we observed a significant reduction in parasite load in the footpad of TLR3$^{-/-}$ animals after 5 weeks of infection (Fig. 1E).

To verify whether the mechanism of resistance to infection in TLR3$^{-/-}$ macrophages by *L. (L.) amazonensis* could be reversed by the addition of type I IFN, peritoneal macrophages from WT or TLR3$^{-/-}$ mice were treated or not with 1,000U/mL recombinant IFN-α after 18 h postinfection and the infection index was determined after 48 h of treatment (Fig. 1F). As expected, the treatment favored the infection of *L. (L.) amazonensis* in WT macrophages, confirming a regulatory role for type I IFNs in this infection model. The fact that the treatment of TLR3$^{-/-}$ macrophages with recombinant IFN-α partially favored the infection indicates that other inflammatory mediators induced by TLR3 activation are also needed to restore the infection index seen in IFN-α -treated WT macrophages.

These results demonstrated the impact that TLR3 signaling exerts on the infection both *in vitro* and *in vivo*.
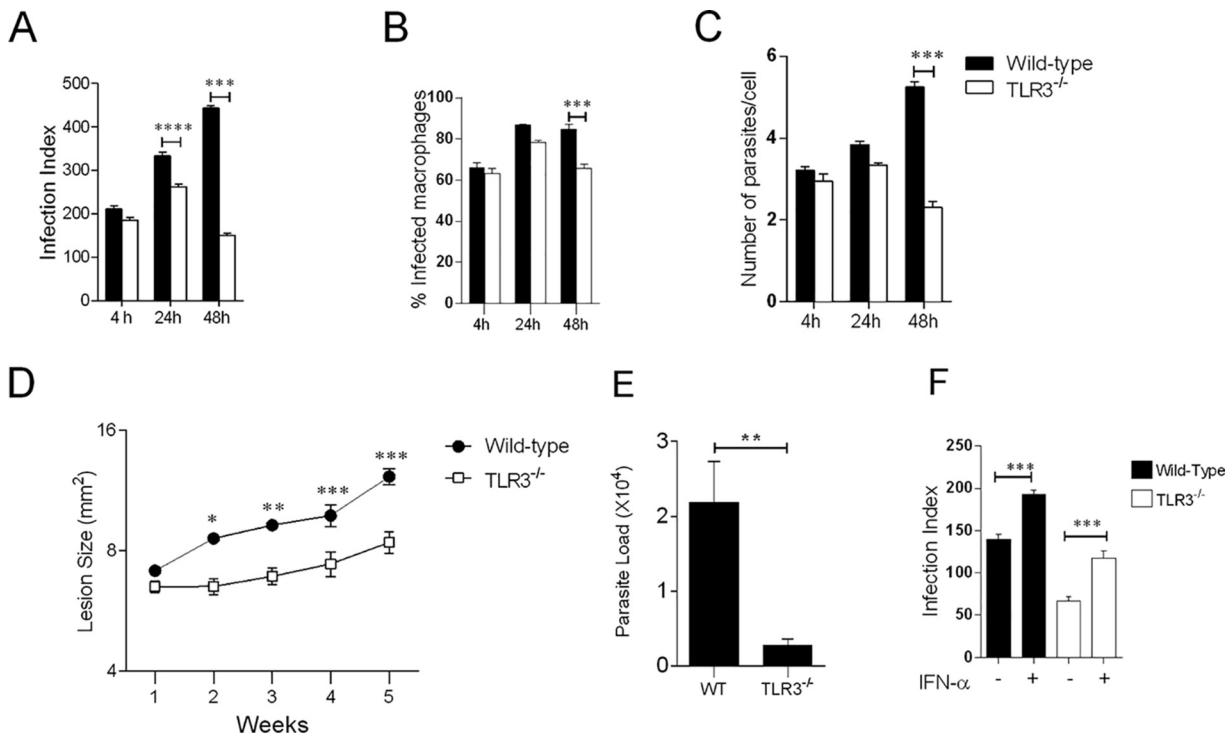
FIG 1 TLR3 affects the intracellular survival of *L. (L.) amazonensis* and is required for parasite burden. Peritoneal macrophages from wild-type and TLR3$^{-/-}$ C57BL/6 mice were infected with stationary-phase promastigotes of *L. (L.) amazonensis* at a ratio of 5 parasites/cell. At 4 and 48 h p.i., one hundred Giemsa-stained cells were inspected, and the infection index was calculated (percentage of infected macrophages multiplied by the average number of amastigotes per macrophage). (A) The percentages of infected macrophages (B) and the number of parasites/cells (C) were evaluated. Four mice per group and values are representative of results of three separate experiments. The lesion size (D) and the titer of parasites (E) of the footpad was determined 5 weeks p.i. with *L. (L.) amazonensis* (5×10^5 promastigotes) in TLR3$^{-/-}$ or WT C57BL/6 mice. (F) IFN1α was added to the cultures 18 h postinfection, and the infection index was determined at 48 h p.i. Asterisks indicate significant differences between groups by ANOVA, with **, $P < 0.0017$ or ***, $P < 0.0001$ ($n = 3$).

TLR3 is required for the expression of cytokines involved in the control of *L. (L.) amazonensis* infection. Since the production of IL-10 and IFN1β has been associated with the intracellular proliferation of *L. (L.) amazonensis* amastigotes (5, 9), we investigated the TLR3-dependence for these cytokines' expression in *L. (L.) amazonensis*-infected macrophages. As previously described, *L. (L.) amazonensis* induced the expression of IFN1β in WT macrophages. However, a drastic reduction in IFN1β was observed in infected TLR3$^{-/-}$ cells (Fig. 2A).

Since TLR3 signaling leads to IRF3 activation, we investigated the levels of phosphorylated IRF3 (pIRF3) (Ser 396) in infected macrophages. As the expression of IFN1β occurred 4 h postinfection (p.i.), analyses of pIRF3 levels were performed at 2 h and 24h post infection. We observed an increase in the accumulation of pIRF3 in WT macrophages infected by *L. (L.) amazonensis* at both time points. In TLR3$^{-/-}$ macrophages, pIRF3 was still observed upon infection, but at significantly lower levels compared to that observed in WT macrophages (Fig. 2B).

A significant decrease in IL-10 expression was also observed in infected TLR3$^{-/-}$ cells (Fig. 2C). As predicted, infected TLR3$^{-/-}$ macrophages expressed high levels of IL-12 (Fig. 2D), which is associated with the activation of macrophages and the killing of amastigotes (30). Thus, TLR3 signaling seems to be crucial for both parasite proliferation in macrophages and IFN1β and IL-10 production.

To determine whether the LRV was present in the *L. (L.) amazonensis* used in our study, we extracted total RNA from the promastigotes of *L. (L.) amazonensis* and two strains of *L. (V.) guyanensis* LRV (*L.g*.LRV^{high} and *L.g*.LRV^{low}). A fraction of the RNA from both *Leishmania* species was treated with S1 nuclease, an endonuclease that specifically degrades single-stranded nucleic acids, and thereafter, we analyzed the RNA (treated and untreated with S1) by agarose gel electrophoresis (26). We observed a fragment of 5.3 kb corresponding to the genome size of the viral dsRNA detected in *L. (V.) guyanensis*. However, we did not detect dsRNA in the *L. (L.) amazonensis* strain, in which only rRNA was detected. The fragment
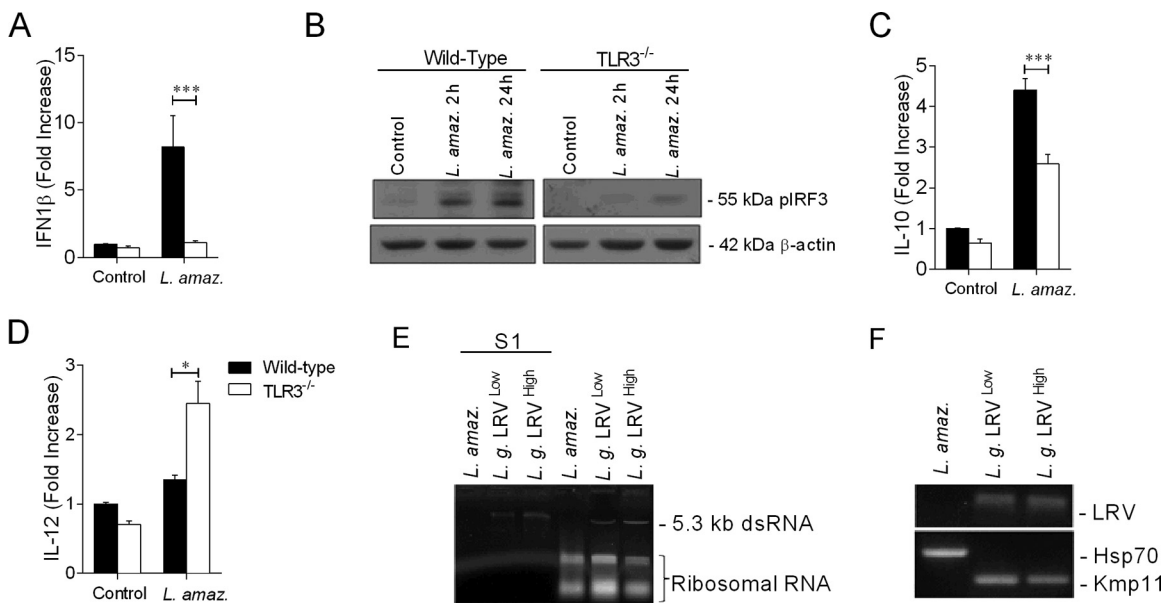
FIG 2 Cytokine expression is dependent on the TLR3 signaling pathway. Peritoneal macrophages from wild-type and TLR3$^{-/-}$ C57BL/6 mice were kept uninfected (control) or infected with stationary-phase promastigotes of *L. (L.) amazonensis* at a ratio of 5 parasites/cell. At 4 h p.i., total RNA was extracted and analyzed by quantitative real-time PCR for IFN1β, (A), IL-10 (C), and IL-12 (D). Protein extracts from macrophages infected by *L. (L.) amazonensis* or not infected (B) were submitted to Western blot analysis using anti-phospho-IRF3 (Ser 396) or anti-β-actin (endogenous control) antibodies, as depicted in the figure. The total stationary-phase promastigote RNA from cultures of *L. (L.) amazonensis* and *L. (V.) guyanensis* LRV high and LRV low was extracted, and a portion of this total RNA was treated with S1 nuclease. Then, 4 mg of RNA (treated and untreated) was separated on a 1.2% agarose gel (E). LRV transcripts were also detected by semiquantitative PCR. As endogenous controls, we used hsp70 and kmp11 for *L. (L.) amazonensis* and *L. (V.) guyanensis*, respectively (F). Asterisks indicate significant differences between groups by ANOVA, with *, $P < 0.05$; **, $P < 0.0017$; or ***, $P < 0.0001$ ($n = 3$).

corresponding to the viral genome could also be visualized after treatment with the S1 nuclease (Fig. 2E). LRV1 RNA was also detected by RT-PCR in the strains of *L. (V.) guyanensis*, but not in *L. (L.) amazonensis* (Fig. 2F), confirming the absence of viral dsRNA in the *L. (L.) amazonensis* strain used. These results suggest that TLR3 activation in our system is mediated by other sources of RNA, such as dead macrophages, or parasites (14), which needs to be further investigated.

TLR3 processing is induced due to *L. amazonensis* infection, and the expression of IFN1β and the cleavage of TLR3 are reduced in macrophages infected by cysteine peptidase B (CPB)-deficient (Dcpb) mutant *L. (L.) mexicana*. We questioned the mechanisms through which TLR3 favors infection in murine macrophages. The proteolytic processing of TLR3 by cysteine cathepsins is required for TLR3 activation and downstream signaling (16). Therefore, we addressed whether *L. (L.) amazonensis* infection would lead to TLR3 processing by Western blotting. We detected a fragment of approximately 70 kDa in infected macrophages at 4 and 24 h, indicating TLR3 processing upon *L. (L.) amazonensis* infection (Fig. 3A). The inhibition of TLR3 processing by leupeptin, a serine/cysteine peptidase inhibitor, abrogated the cognate signaling cascade by interfering with the activation of transcription factors and cytokine expression in other models (16). When we pretreated macrophages with leupeptin, TLR3 processing induced by infection was partially inhibited (Fig. 3B), as shown by densitometry (Fig. 3C).

Several studies have identified cysteine proteinases (CPs) as virulence factors prevalent in species of the *L. (L.) mexicana* complex, especially in the murine infection model used for most of the studies (31–34). Among the CPs studied in *Leishmania*, CPB is the major virulence factor for species of the *L. (L.) mexicana* complex, due to the reduced ability of CPB-deficient parasites to infect and induce lesions (35). We decided to utilize a genetic model to analyze the role of the parasite CBP on TLR3 cleavage. To confirm the deletion of CPB in our *L. (L.) mexicana* strain, we performed an enzymatic inhibition assay with lysates of stationary-phase promastigotes (Fig. 3D). Analysis of CP activity with the Z-Phe-Arg-MCA substrate showed that lysates of Δcpb parasites were less efficient in hydrolyzing the substrate compared to lysates of wild-type parasites. The treatment of *L. (L.) mexicana* with E-64 reduced
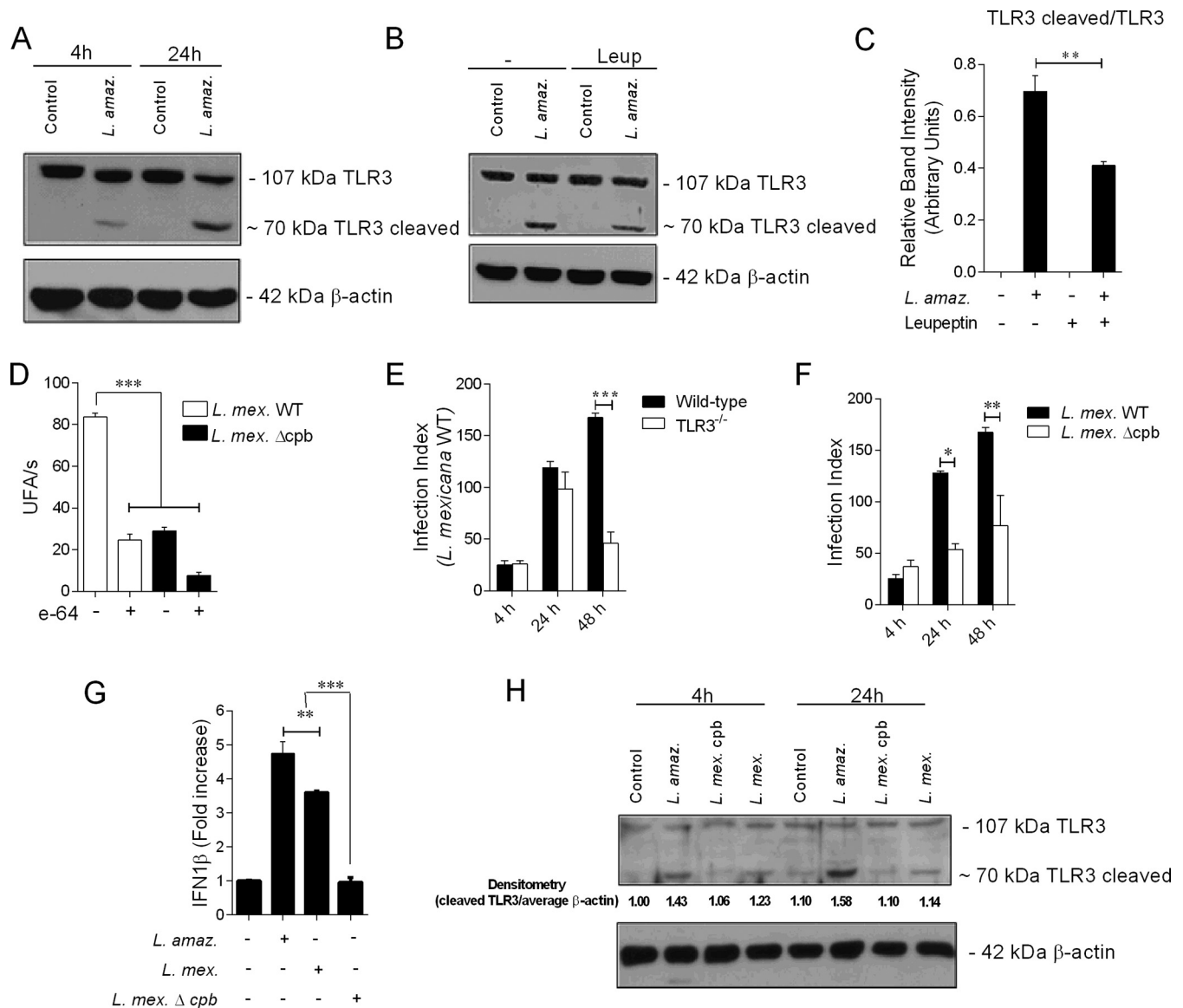
FIG 3 The activity of cysteine protease B is partially required for the cleavage of TLR3 and expression of IFN1β in infected macrophages. Peritoneal macrophages from wild-type or TLR3$^{-/-}$ C57BL/6 mice pretreated with 1 mM leupeptin or not were infected with stationary-phase promastigotes of *L. (L.) amazonensis* at a ratio of 5 parasites/cell. Protein extracts obtained 4 h and 24 h p.i. were submitted to Western blot analysis using anti-TLR3 or anti-β-actin (endogenous control) antibodies, as depicted in the figure (A and B). Densitometric analysis of the blots for TLR3 (C). Lysates of stationary-phase promastigotes of *L. (L.) mexicana* and *L. (L.) mexicana* Δcpb were tested for their ability to hydrolyze Z-Phe-Arg-7-starch-4-methylcoumarin (ZFR-AMC) in assay buffer with or without cysteine protease inhibitor E-64. The reaction rate was measured in a spectrofluorometer. Measurements were plotted as arbitrary fluorescence units (UFA) (D). Peritoneal macrophages from wild-type and TLR3$^{-/-}$ C57BL/6 mice were infected with stationary-phase promastigotes of *L. (L.) mexicana* (E), or *L. (L.) mexicana* WT or Δcbp at a ratio of 5 parasites/cell. At 4, 24, or 48 h p.i., one hundred Giemsa-stained cells were inspected, and the infection indexes were evaluated. Peritoneal macrophages from wild-type and TLR3$^{-/-}$ C57BL/6 mice were infected with stationary-phase promastigotes of *L. (L.) amazonensis*, *L. (L.) mexicana*, or *L. (L.) mexicana* Δcpb at a ratio of 5 parasites/cell. At 4 h p.i., total RNA was extracted and analyzed by quantitative real-time PCR for IFN1β (G), and protein extracts of 4 and 24 h p.i. were submitted to Western blot analysis using anti-TLR3 and anti-β-actin (endogenous control) antibodies, as depicted in the figure together with the densitometry (H). Asterisks indicate significant differences between groups by ANOVA, with *, $P < 0.05$; **, $P < 0.0017$; or ***, $P < 0.0001$ ($n = 3$).

the substrate's hydrolysis, similar to the CPB-null mutant parasites. As the deletion is done only for a CP, the residual activity may result from others CPs present in the parasite.

Then, we performed *in vitro* infection assays with the *L. mexicana* CBP null mutants. Our results showed that, as with *L. (L.) amazonensis*, TLR3 also influences the intracellular survival and proliferation of *L. (L.) mexicana*. The percentage of infected WT-derived macrophages was higher than TLR3$^{-/-}$-derived macrophages after 24 and 48 h p.i. (Fig. 3E). Therefore, we used *L. (L.) mexicana* Δcpb previously generated and characterized (36, 37) as a tool to study the role of parasite CPB proteins in TLR3 signaling.

We also tested the infection ability of *L. (L.) mexicana* Δ*cpb* in murine macrophages and observed no significant differences in the entry between wild-type *L. (L.) mexicana* or *L. (L.) mexicana* Δ*cpb* (see time point of 4 h p.i., Fig. 3F). However, survival (24 h) and proliferation (48 h) were compromised by the absence of CPB.

We also observed a reduction in the expression of IFN1β transcripts in macrophages infected with *L. (L.) mexicana* Δ*cpb* compared to wild-type parasites (Fig. 3G). To analyze the role of CPB on TLR3 processing and consequently on its activation, extracts from macrophages infected with *L. (L.) amazonensis*, *L. (L.) mexicana*, or *L. (L.) mexicana* Δ*cpb* were submitted to Western blot assays. Although the cleavage of TLR3 in response to *L. (L.) mexicana* infection was moderate compared to *L. (L.) amazonensis*, the 70 kDa cleavage product could be readily detected. In macrophages infected with *L. (L.) mexicana* Δ*cpb*, there was a reduction of the TLR3 cleavage product (Fig. 3H), associating the parasite cysteine peptidase B to TLR3 activation. The inhibition of macrophages' serine/cysteine peptidase by leupeptin led to a similar profile in reducing the infection index by *L. amazonensis*, the decrease of IFN1β and IL-10 expression, and the increase of IL-12 (Fig. S1A to D, respectively).

TLR3 and PKR signaling cooperate to parasite growth and IFN1β and IL-10 expression. A common feature of PKR and TLR3 activation is the induction of IFN1 and IL-10 expression. As previously reported (5, 9), *L. (L.) amazonensis* induces PKR signaling, culminating in the production of IFN1β and IL-10, leading to intracellular parasite proliferation. We asked whether TLR3 and PKR perform redundant functions during the infection or play a cooperative role. To address this question, we inhibited PKR with 300 nM of the PKR inhibitor (iPKR) and measured the expression of IFN1β and IL-10 along with the infection index in TLR3$^{-/-}$ macrophages. The deletion of only one dsRNA sensor, TLR3, was sufficient to reduce the infection index by approximately half. Even though the inhibition of PKR in infected TLR3$^{-/-}$ macrophages resulted in a statistically significant difference, the same reduction was observed for DMSO (dimethyl sulfoxide) treatment (Fig. 4A).

The expression of IFN1β induced by *L. (L.) amazonensis* was reduced equally in TLR3$^{-/-}$ and WT macrophages treated with iPKR (Fig. 4B). Similar results were obtained with regard to IL-10 expression (Fig. 4C). These results suggest that PKR and TLR3 are on the same signaling pathway to induce expression of IFN1β and IL-10, there being no further decrease after the inhibition of both pathways. The expression of IL-12 was increased both in the absence of TLR3 and after treatment of cells with iPKR (Fig. 4D). However, the inhibition of PKR signaling in infected TLR3$^{-/-}$ macrophages showed a further increase in IL-12 expression (Fig. 4D).

DISCUSSION

TLR3 binds to dsRNA, which is a common viral replication intermediate (38, 39). Endocytosed viral dsRNA particles are recognized by TLR3, while free dsRNA is detected by cytoplasmic RNA sensors, such as PKR. The common downstream event of both TLR3 and PKR sensors involves the activation of the transcription factors NF-κB and IRF3 (40, 41), as well as the production of type I IFN, which is a pivotal component of the antiviral cell response (42, 43). The involvement of TLR3 signaling in controlling inflammation and autoimmunity has been described (44, 45), unveiling additional physiological roles for RNA sensors than the antiviral response.

The importance of TLR3 in *L. (V.) guyanensis* infection has previously been described (27, 28). According to these reports, during *L. (V.) guyanensis* infection, the TLR3 pathway is activated, leading to increased tissue damage due to the upregulation of proinflammatory cytokines (27). These observations were related to a high *Leishmania* RNA viral (LRV1) load, which may occur in isolates of the subgenus *Viannia* (46). This response occurs due to recognition of viral dsRNA by TLR3, which after recruitment of TRIF increases the secretion of inflammatory cytokines and chemokines such as IL-6 (27), polarizing naive T cells into Th17 cells, promoting parasite dissemination and metastatic lesions (27). The parasite survival and disease severity promoted by *Leishmania* harboring LRV1 in mice is also due to the caspase-11 block. This process is dependent on TLR3 and expression of ATG5, leading to inhibition of NLPR3 activation that can lead to subversion of innate immunity (29). While TLR3 favors infections caused by some species of *Leishmania*, the opposite was observed for *Neospora*
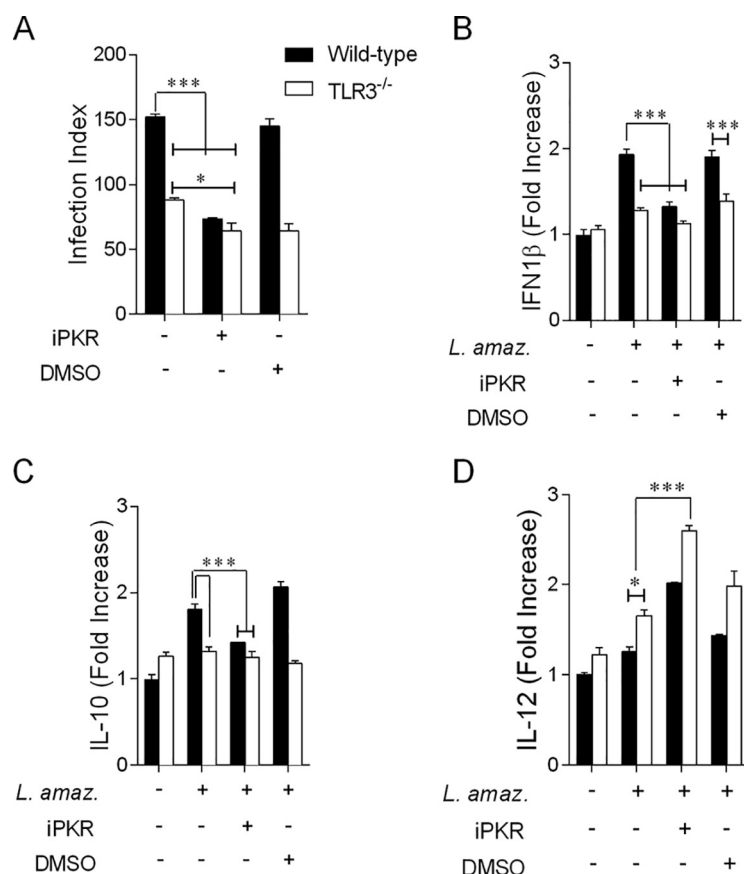
FIG 4 TLR3 and PKR signaling act cooperatively in the expression of IFN1β and IL-10 and in the parasite burden of infected macrophages. Peritoneal macrophages from wild-type or TLR3$^{-/-}$ C57BL/6 mice pretreated with 300 mM iPKR or not were infected with stationary-phase promastigotes of *L. (L.) amazonensis* at a ratio of 5 parasites/cell. At 48 h p.i., one hundred Giemsa-stained cells were inspected, and the infection index was calculated (percentage of infected macrophages multiplied by the average number of amastigotes per macrophage) (A). At 4 h postinfection, total RNA was extracted and analyzed by quantitative real-time PCR for IFN1b (B), IL-10 (C), and IL-12 (D). Asterisks indicate significant differences between groups by ANOVA, with *, $P < 0.05$; **, $P < 0.0017$; or ***, $P < 0.0001$ ($n = 3$).

caninum. In a recent publication, it was shown that the TLR3-TRIF signaling pathway enhances the Th1 immune response, increasing resistance against infection by this protozoan (47). These findings were corroborated by a recent paper that demonstrated that TLR3 absence improved macrophage activity in pneumonia induced by *Klebisella pneumonia* (48).

We have previously demonstrated the role of PKR, a nonendosomal dsRNA sensor, in *L. (L.) amazonensis* infections (5, 9). *Leishmania (L.) amazonensis* activates PKR-dependent signaling, thus favoring infection due to the production of IFN1β (5) and IL-10 (9). Additionally, we observed enhanced *L. (L.) amazonensis* infection by the Phlebovirus-dependent activation of PKR/IFN1β/IL-10 axis (49). The role of PKR in *Leishmania* infection appears to be species specific. For instance, *L. (L.) major* infection is restricted upon PKR activation (50). These results clearly demonstrated the engagement of RNA sensor molecules in the progression of the infection by obligatory intracellular protozoan parasites, which are corroborated by the recent observations that PKR controls the infection of the unrelated species *Toxoplasma gondii* (51). These findings prompted us to address the role of the endosomal dsRNA sensor TLR3 in *L. (L.) amazonensis* infection.

In the present work, we demonstrate that *L. (L.) amazonensis* activates the TLR3 pathway and by so doing further increases the infection. A reduced infection index in TLR3$^{-/-}$ macrophages and impaired *in vivo* infection in TLR3$^{-/-}$ mice were observed. Consistently, we observed the reduction in IL-10 and the increase in IL-12 expression in infected TLR3$^{-/-}$ macrophages. Similar results have been reported in *Mycobacterium tuberculosis* infection,

in which IL-10 production was also impaired in infected TLR3$^{-/-}$ mice and increased levels of IL-12 were observed (25). A recent report also demonstrates the role of TLR3 as a negative regulator of the proinflammatory response during the infection by the fungus *Paracoccidioides brasiliensis* (52). IFN1β expression is induced in *L. (L) amazonensis* infection or LPG treatment in a TLR2- and PKR-dependent way, which favors the parasite growth by modulating oxidative stress (5, 10). This finding is consistent with the observation that lesions from patients with anergic cutaneous leishmaniasis exhibit high PKR and IFN1 expression (5). Our results showed a reduction in IFN1β expression in infected TLR3$^{-/-}$ macrophages, which may partially explain the parasite load reduction.

TLR3 activation by *L. (L) amazonensis* in our model happens in the absence of detectable viral particles since we confirmed the absence of dsRNA in our *L. (L.) amazonensis* strain. The activation of TLR3 could be originated from RNA from diverse origins, such as dead parasites or exosomes containing RNA. We cannot rule out that RNAs originated from dead macrophages could serve as ligands for TLR3, as it has been previously demonstrated that RNA released from or associated with necrotic cells can stimulate this innate receptor and induce immune activation (14). This phenomenon is highly relevant during cutaneous leishmaniasis, as tissue destruction caused by direct macrophage damage by the parasite (53) or by the action of cytokines and cytotoxic cells takes place *in vivo* (54, 55).

We further investigated the mechanisms of TLR3 activation by *L. (L) amazonensis*. It is critical to consider that the proteolytic cleavage of this receptor is required for NF-κB and IRF3 activation, cytokine production, dimerization, and endosomal localization (16, 52), and that macrophages infected with *L. (L.) amazonensis* harbor some enzymes within the parasitophorous vacuole, such as acid phosphatase, arylsulfatases, and cathepsins (B, D, H and L). Our results showed that TLR3 generates cleavage products with a size compatible with its activation in *L. (L.) amazonensis*-infected macrophages. Macrophages pretreated with leupeptin presented partial reduction of TLR3 cleavage after infection. The effect of leupeptin in parasite intracellular growth, inhibition of IFN1β and IL-10 expression, and promotion of IL-12 expression was very evident. However, it seems that the two phenomena are not fully associated. The effect of leupeptin might be also mediated via inhibition of additional proteolytic events in the macrophage that are unrelated to TLR3 activation but required for parasite growth (which in turn is likely fully associated with the production of IFN1β and IL-10).

In addition to the proteases expressed by the host cell in response to infection, the genus *Leishmania* also expresses proteases implicated in differentiation, nutrition, host cell infection, and immune response evasion and may be implicated in TLR3 processing. The deletion of the CPB protease, the main virulence factor of the *L. (L.) mexicana* complex (34), partially reduced TLR3 cleavage, as well as the expression of IFN1β in infected macrophages, thus indicating the role of CBP in TLR3 cleavage and signaling. The participation of CPB in the progression of infection may vary according to the genetic background of the animal host (56).

As mentioned, dsRNA can also be recognized by PKR, which, besides inducing type I IFN, acts on the inhibition of protein translation, preventing viral replication (57). PKR signaling is induced during *L. (L.) amazonensis* infection via TLR2 (5). Our current model suggests that endosomal TLR3 is also involved in *L. (L.) amazonensis* infection. We observed that inhibition of TLR3 and PKR signaling can restrain the expression of IFN1β and IL-10 in the same manner, indicating that they may share the same pathway. In the absence of both dsRNA sensors there was a further increase in IL-12 expression, which could impact the fate of the adaptive immune response and generate an inflammatory-prone environment. Thus, in the context of *L. (L.) amazonensis* infection, TLR3 and PKR have a dual role, not only to induce IFN1β and IL-10 required for parasite growth and counteract inflammation, but also to prevent the induction of additional proinflammatory cytokines such as IL-12. Recent work showed similar cooperative roles of PKR and TLR3 toward the expression of IFN1β in hepatocytes (44).

However, an apparent paradox has come to light. Studies have shown that type I IFN induced by *L. (V.) guyanensis* LRV1$^+$ isolates are involved in severe inflammatory reactions leading to mucocutaneous lesions (27, 28, 58). Therefore, how can the opposite pathogenic profile observed in *L. (L.) amazonensis* infections be explained? One possible explanation

might be attributed to the increased IL-10 production mediated by PKR and TLR3 in *L. (L.) amazonensis* infection, which would attenuate the inflammatory response. It has been demonstrated that TLR3 activation is capable of mediating the production of anti-inflammatory cytokines, including IL-10, and thus controlling the inflammatory response in astrocytes (59). Moreover, the production of proinflammatory cytokines requires the activation of the canonical NF-κB pathway. We have shown that *L. (L.) amazonensis* activates the NF-κB homodimer p50/50, which is a transcriptional repressor and thus mitigates the production of proinflammatory mediators (60, 61).

In summary, we have described a novel adaptive mechanism exhibited by *L. (L.) amazonensis* that is devoid of LRV1 but involves TLR3 activation and cooperation with another RNA sensor, PKR. Our findings contribute to the notion that the *Leishmania* species present distinct and sometimes specific programs for interacting with host cells, leading to a variety of parasite proliferation and pathogenesis outcomes. In addition, our results corroborate the idea that TLR3 may be engaged in nonviral infections, which could serve as a basis for further studies.

MATERIALS AND METHODS

Reagents and antibodies. The protease and phosphatase inhibitor cocktail Leupeptin and E-64 were obtained from Sigma-Aldrich (St. Louis, MO). PKR inhibitor (iPKR; CAS-608512-97-6) was obtained from Calbiochem (Millipore Sigma, Billerica, MA). The anti-TLR3 (ab62566) was obtained from Abcam (Cambridge, United Kingdom); anti-pIRF3 (Ser396-4D4G) was obtained from Cell Signaling Technology (Beverly, MA); anti-β-actin (A00702-SZ) was obtained from GenScript Biotech Corp. (Piscataway, NJ); and goat anti-rabbit horseradish peroxidase-conjugated IgG (sc-2030) was obtained from Santa Cruz Biotechnology (Dallas, TX).

Peritoneal macrophages. Thioglycolate-elicited peritoneal macrophages from 6- to 8-week-old wild-type (WT) or TLR3-knockout (TLR3$^{-/-}$) C57BL/6 mice were obtained by injecting 8 mL of PBS into the peritoneal cavity (pool of 4–5 mice per group with male and females numbers matched between WT and TLR3$^{-/-}$). The cell suspension was washed once with PBS and then resuspended in serum-free Dulbecco modified Eagle medium (DMEM). Cells were plated on glass coverslips at 2×10^5/well in 24-well polystyrene plates or at 4×10^6/well in 6-well polystyrene plates and incubated for 1 h at 37°C in a 5% CO$_2$ atmosphere. Nonadherent cells were removed with PBS, and the adherent cell population was incubated for 1 day in high-glucose DMEM and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 g/mL streptomycin for subsequent *Leishmania* infection assays.

Cell treatment. To assess TLR3 receptor processing in *L. (L.) amazonensis* infection, peritoneal macrophages were pretreated for 1 h with 1 mM Leupeptin (16), an inhibitor of serine and cysteine proteases.

To investigate the role of PKR during coinfection, PKR activity was inhibited by pretreatment of the macrophages for 1 h with 300 nM of the PKR inhibitor CAS-608512-97-6 (Millipore) as described (49).

Parasites, culture conditions, and infection. *Leishmania (L.) amazonensis* (WHOM/BR/75/Josefa), *Leishmania (V.) guyanensis*, *Leishmania (L.) mexicana* (MNYC/BZ/62/M379) or *L. (L.) mexicana* Δ*cbp* promastigotes were grown at 26°C in Schneider's insect (Sigma-Aldrich) or HOMEM medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 g/mL streptomycin. Promastigotes from 4- to 5-day stationary cultures were used for all experiments. Cells were infected for 1 h at 35°C with *Leishmania* stationary-phase promastigotes with a parasite:cell ratio of 5:1. Noninternalized promastigotes were washed away, fresh medium was added, and cultures were maintained at 35°C in a 5% CO$_2$ atmosphere for various periods of time. In some experiments, cells were pretreated with 300 nM of the PKR inhibitor or 1 mM leupeptin. Infected macrophages were counted under light microscopy to assess the infection index as follows: 100 Giemsa-stained cells were inspected, and the percentage of infected macrophages was multiplied by the average number of amastigotes per macrophage.

***In vivo* infection.** The cushion footpads of WT or TLR3$^{-/-}$ male C57BL/6 mice were subcutaneously injected with 5×10^6 *L. (L.) amazonensis* stationary-phase promastigotes in PBS. The progression of the lesions was measured weekly with a digital caliper and expressed as the difference between the thickness of the infected and noninfected footpads. At the indicated time points, the mice were euthanized and parasite load on the lesion (paw) was evaluated after 5 weeks p.i. The material obtained from the dissections was macerated in PBS, and the cell suspension was plated at serial dilutions in Schneider's medium (Sigma) supplemented with 10% FBS, 100 U/mL penicillin, and 100 g/mL streptomycin. The parasite titer was evaluated after approximately 7 days of culture (according to the promastigote production time) at 26°C.

All animals were kept in the mouse facility of the Departmento de Imunologia (UFRJ) according to the rules established by CONCEA (Conselho Nacional de Experimentação Animal). The animal experimentation protocols that were used in this research were approved by the UFRJ CEUA (Protocol 046/20).

Protease activity assays. *Leishmania (L.) mexicana* and *L. (L.) mexicana* Δ*cpb* stationary-phase promastigotes were lysed with PBS 1× containing 1% of NP-40. Lysates were tested for their ability to hydrolyze the Z-Phe-Arg-7-amido-4-metilcumarina (Z-FR-AMC) substrate at 30°C in 0.1M sodium acetate, pH 5.2, with 2 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT). The samples were preincubated for 10 min at 30°C in assay buffer only or in the presence of cysteine protease inhibitor E-64 (10 μM). The reaction rate was measured on a spectrofluorometer, with 380 nm as the excitation wavelength and 440 nm as the emission wavelength.

Immunoblotting. Peritoneal macrophages (4×10^6) infected or not by *L. (L.) amazonensis* were washed twice with ice-cold PBS and then lysed in 80 μL of lysis buffer (50 mM Tris-HCl, pH 7.5; 5 mM EDTA; 10 mM EGTA; 50 mM NaF; 20 mM glycerophosphate; 250 mM NaCl; 0.1% Triton X-100; and 1 g/mL BSA) to

which a 1:100 dilution of protease inhibitor cocktail and a 1:50 dilution of phosphatase inhibitor cocktail were added. Proteins were subjected to electrophoresis in 10% SDS-polyacrylamide gels and transferred to a PVDF membrane (Bio-Rad). The following primary antibodies were used in this study: TLR3(Abacm), pIRF3 (Ser396-4D4G, Cell Signaling), β-actin (#4967, Cell Signaling), followed by goat anti-rabbit horseradish peroxidase-conjugated IgG (sc-2030, Santa Cruz Biotech). The membranes were then submitted to 3 washes with Tris-buffered saline with Tween 20 (TBST), and proteins were detected by the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). The bands were quantified by densitometry using Adobe Photoshop CS6.

S1 nuclease assay. Total RNA from stationary-phase *Leishmania* promastigotes (*L. (L.) amazonensis* and *L. (V.) guyanensis*—1×10^7) was extracted via Direct-zol RNA MiniPrep Plus (Zymo). A fraction of the RNA from both *Leishmania* species was treated with S1 nuclease (Promega), and the S1 treated RNA was subjected to agarose electrophoresis according to (26).

Semiquantitative RT-PCR for LRV1. Peritoneal macrophages were harvested, and total RNA was obtained with the Direct-zol RNA MiniPrep Plus Kit (Zymo). First-strand cDNA synthesis was performed in a reaction mixture containing Improm-II Reverse Transcriptase (Promega), a mix of dNTPs, and random primers (Promega), as described by the manufacturer. RT-PCR was performed using LRV1 primers (sense 5'-CTGACTGGACGGGGGGTAAT-3' and antisense 5'-GCGTAAGGGAGTGTTTTG-3'), kmp11 primers (sense 5'- GCC TGG ATG AGG AGT TCA ACA -3' and antisense 5'- GTG CTCCTT CAT CTC GGG -3'), HSP70 primers (sense 5'- CGC GTA CTC GAT GAA GAA CA -3' and antisense 5'- ATC GGG TTG CAT ACG TTC TC -3'), 2.5 U of GoTaq DNA polymerase (Promega), and 1.5 mM MgCl$_2$ in an appropriate buffer at an annealing temperature of 52°C and 40 amplification cycles. PCR products were separated on a 1.2% agarose gel, stained with ethidium bromide, and photographed in a UV transilluminator. The semiquantitative PCR data were normalized to kmp11 and HSP70 amplification.

Real-time quantitative RT-PCR (qRT-PCR). Total RNA from peritoneal macrophages (4×10^6) was extracted via the Direct-zol RNA MiniPrep Plus kit (Zymo). RNA (1 μg) was reverse transcribed to first-strand cDNA with ImProm-II (Promega, Madison, WI, USA) and oligo (dT) 12–18 primers, according to the manufacturer's instructions. The nucleotide sequences of the GAPDH primers are as follows: (sense 5' TTG ACC AAC TGC TTA GC 3' and antisense 5' GGC ATG GAC TGT GGT CAT GAG 3'), IFN1β (sense 5' TCC AAG AAA GGA CGA ACA TTC G 3' and antisense 5' TGA GGA CAT CTC CCA CGT CAA 3'), IL-10 (sense 5' CCC AGA AAT CAA GGA GCA TT 3' and antisense 5' TCA CTC TTC ACC TGC TCC AC 3'), and IL-12 p40 (sense 5' TGT CGC TAA CTC CCT GCA TC 3' and antisense 5' CTG AGG ACA CAT CCC ACT CC 3'). Real-time qRT-PCR was carried out via the Applied Biosystems StepOne detection system (Applied Biosystems) using SYBR green PCR Master Mix (Promega). All qRT-PCR experiments were carried out in 3 independent experiments with 3 internal technical replicates. The qRT-PCR experimental data were normalized to GAPDH amplification as an endogenous control. All expression ratios were determined by the relative gene expression $\Delta\Delta C_t$ method via StepOne 2.0 software 2.0 (Applied Biosystems).

Statistical analysis. Data were analyzed by two-way ANOVA for independent samples followed by a *post hoc* Dunnett's test (comparing to a single control group) or a Tukey's test (with no designated control group), using GraphPad Prism 5 software (San Diego, CA, USA). Data were presented as the mean values $\pm$ standard error of the mean (SEM) of three independent experiments. Comparisons between means were considered to be statistically significant when $P < 0.05$.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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