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RIPK1 and PGAM5 Control *Leishmania* Replication through Distinct Mechanisms

Nivea Farias Luz,*,† Sakthi Balaji,* Kendi Okuda,‡ Aline Silva Barreto,§ John Bertin,¶ Peter J. Gough,‖ Ricardo Gazzinelli,‡,¶ Roque P. Almeida,§ Marcelo T. Bozza,# Valeria M. Borges,† and Francis Ka-Ming Chan*,**

Leishmaniasis is an important parasitic disease found in the tropics and subtropics. Cutaneous and visceral leishmaniasis affect an estimated 1.5 million people worldwide. Despite its human health relevance, relatively little is known about the cell death pathways that control *Leishmania* replication in the host. Necroptosis is a recently identified form of cell death with potent antiviral effects. Receptor interacting protein kinase 1 (RIPK1) is a critical kinase that mediates necroptosis downstream of death receptors and TLRs. Heme, a product of hemoglobin catabolism during certain intracellular pathogen infections, is also a potent inducer of macrophage necroptosis. We found that human visceral leishmaniasis patients exhibit elevated serum levels of heme. Therefore, we examined the impact of heme and necroptosis on *Leishmania* replication. Indeed, heme potently inhibited *Leishmania* replication in bone marrow–derived macrophages. Moreover, we found that inhibition of RIPK1 kinase activity also enhanced parasite replication in the absence of heme. We further found that the mitochondrial phosphatase phosphoglycerate mutase family member 5 (PGAM5), a putative downstream effector of RIPK1, was also required for inhibition of *Leishmania* replication. In mouse infection, both PGAM5 and RIPK1 kinase activity are required for IL-1β expression in response to *Leishmania*. However, PGAM5, but not RIPK1 kinase activity, was directly responsible for *Leishmania*-induced IL-1β secretion and NO production in bone marrow–derived macrophages. Collectively, these results revealed that RIPK1 and PGAM5 function independently to exert optimal control of *Leishmania* replication in the host. *The Journal of Immunology*, 2016, 196: 000–000.

Leishmaniasis is a vector-borne parasitic disease caused by protozoan of the genus *Leishmania*. *Leishmania* parasites exist as extracellular flagellated promastigotes in sandflies. Upon entry into mammalian hosts, they exist as intracellular amastigotes (1). In humans, disease manifestation varies depending on the *Leishmania* species and can range from asymptomatic infections, self-healing cutaneous lesions, to life-threatening infections involving visceral organs. For instance, *Leishmania amazonensis* and *L. major* are both known to cause cutaneous infections, whereas *L. infantum chagasi* (*L. infantum*) causes visceral leishmaniasis (VL). VL can be extremely debilitating, with significant morbidity and mortality if not promptly treated (2). In addition, disease progression is influenced by host immune response (3). Macrophages are widely believed to play critical roles in the control of *Leishmania* infection (4). Interestingly, they also serve as major reservoirs in which *Leishmania* amastigotes replicate.

The host immune response that controls Leishmaniasis is not fully understood. The inflammatory cytokine IL-1β is critical for innate immune defense against many different pathogens. Production of mature IL-1β in macrophages requires two signals: NF-κB–driven de novo synthesis of pro–IL-1β and inflammasome-mediated cleavage of pro–IL-1β into the mature cytokine. Mice that are deficient in the essential inflammasome sensor NLRP3, the adaptor ASC, or the IL-1β cleavage protease caspase 1 exhibited impaired control of replication of many *Leishmania* species (5). The protective effect of IL-1β is mediated by NO (5), which stimulates macrophage cell death. In contrast with IL-1β, NLRP3 inflammasome-mediated pro–IL-18 processing and secretion exacerbates the disease by skewing the reaction toward a Th2-biased response in susceptible BALB/c mice (6). In addition to IL-1β and IL-18, the stress-induced enzyme heme oxygenase-1 (HO-1) has been shown to facilitate *Leishmania* replication by limiting inflammatory cytokine expression (7). Another key function of HO-1 is to cleave heme, which results in generation of free iron, carbon monoxide, and biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. Heme release is found in intracellular pathogen infections such as *Plasmodium falciparum*

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; GSK, GlaxoSmithKline; HO-1, heme oxygenase-1; MOI, multiplicity of infection; Nec-1, necrostatin-1; NSA, necrosulfonamide; PGAM5, phosphoglycerate mutase family member 5; RIPK, receptor interacting protein kinase; VL, visceral leishmaniasis; WT, wild type.

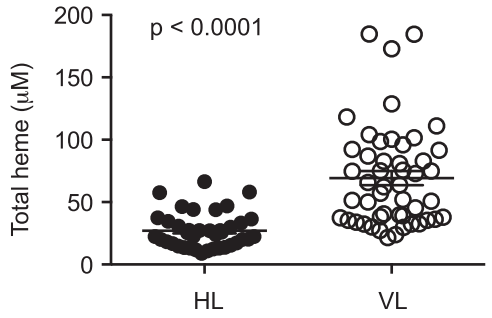

FIGURE 1. Human VL patients exhibit elevated serum concentrations of heme. Serum samples obtained from patients with VL (n = 49) and healthy control subjects (HC; n = 39) from an endemic area in the Northeast of Brazil. Total heme in plasma samples was estimated by a colorimetric determination. Mann–Whitney U test was used to verify statistical difference between HC and VL. Circles represent individual values and black bars represent median values.

and *Plasmodium vivax*, the causative agents of malaria (8, 9). Severe VL is also characterized by hematological alterations and spontaneous bleeding associated with marked inflammatory imbalance (10). These observations suggest that heme may similarly influence the outcome of *Leishmania* infection.

Recent studies show that free heme induces murine macrophage necroptosis (11), a recently described form of inflammatory cell death that has important protective functions in certain viral infections (12–15). Hence HO-1 may facilitate *Leishmania* replication by limiting heme-induced necroptosis, thereby preserving the parasite replication reservoir. Two serine/threonine kinases, the receptor interacting protein kinase (RIPK) 1 and RIPK3, are critical adaptors for necroptosis (16). Recently, the mitochondrial phosphatase phosphoglycerate mutase family member 5 (PGAM5) was identified as a downstream effector of necroptosis (17). In addition, PGAM5 can also function downstream of RIPK3 to control cytokine expression in NKT cells independent of necroptosis (18). However, the roles of heme, RIPKs, PGAM5, and necroptosis have not been investigated in *Leishmania* infection.

In this study, we report that high levels of heme were detected in the sera of human patients with VL. In response to high doses of heme, human and mouse macrophages underwent RIPK1- and RIPK3-dependent necroptosis. High doses of heme inhibited *L. infantum* replication in macrophages. Although RIPK1 kinase inhibitors restored parasite replication, neither RIPK3 kinase inhibitors nor MLKL inhibitor rescued *L. infantum* replication in heme-treated macrophages. Importantly, RIPK1 kinase activity also inhibited *Leishmania* replication in the absence of heme, because enhanced *Leishmania* replication was detected in macrophages expressing kinase inactive RIPK1 and in wild type (WT) macrophages treated with RIPK1 kinase inhibitors. In addition to RIPK1, the mitochondrial phosphatase PGAM5 was also required for optimal control of *Leishmania* replication in macrophages. Mechanistically, we showed that PGAM5 promotes IL-1β production, which in turn stimulates NO production. By contrast, RIPK1 regulates *Leishmania* replication independent of IL-1β. Collectively, our results identified RIPK1 and PGAM5 as two novel host factors that control *Leishmania* replication through distinct mechanisms.

Materials and Methods

Determination of heme concentration in human VL

Plasma was collected between 2010 and 2012 from symptomatic individuals (n = 49) and sex-matched endemic healthy control subjects (n = 39) in Aracaju, Sergipe, Brazil. The baseline characteristics of the study participants are shown in Luz et al. (7). Total heme in plasma samples was estimated by a colorimetric determination at 400 nm using the QuantiChrom Heme Assay Kit (BioAssay Systems, Hayward, CA), according to the manufacturer's protocol.

Mouse infection

All experimental procedures were approved and conducted according to the Institutional Animal Care and Use Committee of the University of the University of Massachusetts Medical School. *Ripk1*$^{kd/kd}$ (*Ripk1*$^{K45A/K45A}$) have been described before (19). Characterization of the *Pgam5*$^{-/-}$ mice will be published in a different manuscript. For in vivo infection with *Leishmania*, mice were infected with 4×10^6 *L. amazonensis* per footpad in a volume of 20 μl. Swelling of the footpad was measured weekly by digital calipers (Fisher Scientific) and compared with the uninfected footpad. For quantification of *L. amazonensis* parasite load, footpads from infected mice were harvested 10 wk postinfection, homogenized, and serially diluted (1:2) in 96-well plates in complete Schneider media. After 7 d of incubation at 26°C, the number of viable parasites was calculated from the highest dilution at which parasites were observed.

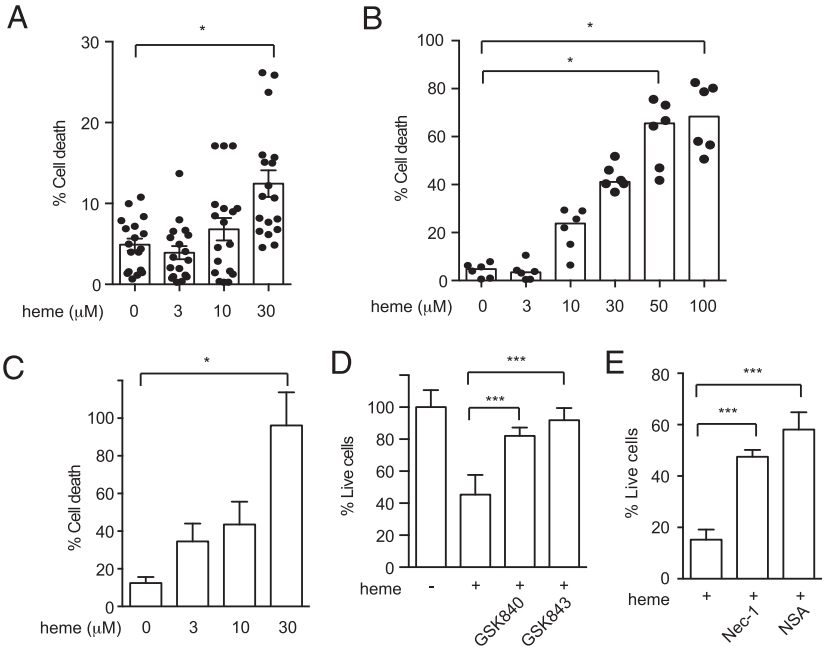

FIGURE 2. Heme induces RIPK1-RIPK3-MLKL–dependent necroptosis in human macrophages. (**A**–**C**) Heme induced dose-dependent cell death in (A) human PBMCs, (B) primary human monocyte-derived macrophages, and (C) PMA-differentiated THP-1 cells. (**D** and **E**) THP-1 cells were pretreated with 10 μM necrostatin-1, 5 μM NSA, or the RIPK3 kinase inhibitors from GlaxoSmithKline (GSK) GSK'840 and GSK'843 (2 μM) for 1 h before treatment with 30 μM heme. Three independent experiments. Results shown are representative of mean of triplicates ± SEM. *$p < 0.001$, ***$p < 0.05$.

For assessment of cytokine production, footpads from mice were homogenized in 1 ml PBS supplemented with protease inhibitors. The footpad extracts were analyzed for TNF and IL-1β by ELISA (BD Biosciences). For ex vivo analysis of IL-1β and TNF production, single-cell suspensions were prepared from the spleen of mice infected after 10 wk. After RBC lysis, 1×10^6 cells resuspended in 1 ml medium were plated in 24-well plates. Cells were stimulated with 50 μg/ml *L. amazonensis* particulate Ag. The supernatants were harvested after 48 h and analyzed by ELISA.

Mouse and human cell culture

Bone marrow–derived macrophages (BMDMs) were generated from femoral bone marrow cells by culture for 7 d in DMEM supplemented with 20% L929 conditioned medium as described before (20). Human primary monocyte-derived macrophages were generated from PBMCs from healthy volunteers. After Ficoll Paque (GE Healthcare) density centrifugation, PBMCs were plated at 2×10^6 cells/well on 96-well plate. After 1 h, nonadherent cells were removed, and adherent cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine and 10% FBS for 7 d. THP1 cells were stimulated with 200 nM PMA (Sigma-Aldrich) for 3 d, followed by culture in medium without PMA for another 2 d.

NO production and cell death assay

BMDMs (10^5 cells/well), PBMCs (2×10^5 cells/well), monocyte-derived macrophages (2×10^5 cells/well), and promonocytic THP-1 cells (2×10^5 cells/well) were cultured with 3, 10, or 30 μM heme. Twelve hours later, release of lactate dehydrogenase was measured in cell-free culture supernatant by the cytotoxicity detection kit (Roche Applied Science). Where it is indicated, THP-1 cells were pretreated with RIPK1 or RIPK3 inhibitors for 1 h before treatment with heme. BMDM cell viability was assessed by CytoTox96 Non-Radioactive Cytotoxicity Assay. In vitro IL-1β production by BMDMs was measured by ELISA after stimulation with 200 ng/ml ultrapure LPS (Invivogen) for 3 h, followed by 10 μM nigericin for another 3 h. NO production was measured in the supernatants of *L. infantum*–infected BMDMs pretreated with 200 ng/ml LPS, then stimulated with 100 U/ml IFN-γ; in some experiments, 10 ng/ml rIL-1β was added along with IFN-γ treatment. Supernatants were harvested after 48 h and nitrite production was measured using Griess reaction.

Leishmania *infection in BMDMs*

L. amazonensis (strain IFLA/BR/67/PH8), *L. major* (strain MHOM/IL/81/Friedlin), and *L. infantum* (MCAN/BR/89/Ba262) promastigotes were grown in Schneider medium (Sigma-Aldrich) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. Stationary-phase promastigotes were used in all experiments. BMDMs and THP-1 cells were plated on coverslips 1 d before infection. Cells were infected with parasites at early stationary phase (multiplicity of infection [MOI] = 5 for *L. amazonensis* and *L. major*, MOI = 10 for *L. infantum*). After 4 h, cells were washed and further cultured for 72 h. In some experiments, 30 μM heme was added 4 h postinfection. Cells were fixed with methanol and stained with Diff-Quick (Thermo Scientific). The number of amastigotes per 100 macrophages was enumerated by counting on a light microscope. In addition, parasite load was determined by light microscopy and the production of viable promastigotes in Schneider medium. In brief, 72 h postinfection, cell culture medium was replaced by Schneider medium and the plates were then kept at 24°C. Seven days later, proliferating extracellular motile promastigotes were counted in a Neubauer hemocytometer. For THP-1 cells in vitro infection, PMA-differentiated cells were treated for 1 h with GlaxoSmithKline (GSK) RIPK1 and RIPK3 inhibitors before infection with promastigotes.

Statistical analyses

All results shown are mean ± SEM. Statistical significance was determined by unpaired Student *t* test with Welch's correction unless otherwise stated in the figure legends.

Results

Elevated serum heme level is a hallmark of human VL

Heme release is a pathological consequence of hemolytic intracellular pathogen infection such as malaria (21). Heme impairs PGE_2 and TGF-β production by human mononuclear cells via Cu/Zn superoxide dismutase (9). *Leishmania* promastigotes have been shown to degrade hemoglobin, which could lead to release of heme (22). Interestingly, HO-1, a key catabolic enzyme that neutralizes the cytotoxicity of heme (23), has been shown to promote *Leishmania* replication (7). We therefore asked whether *Leishmania* infection would result in heme release. Indeed, we found that the serum of human VL patients had significant increase in heme levels compared with healthy control subjects (Fig. 1). This suggests that heme release is a possible contributing factor to this severe form of leishmaniasis.

Heme induces necroptosis in human macrophages

Leishmania parasites express the heme transporter LHR1 that is essential for virulence in macrophages and mice (24). This suggests that heme promotes *Leishmania* replication. In contrast, we previously showed that heme causes RIPK1- and RIPK3-dependent necroptosis in mouse macrophages (11). To reconcile these two observations and to determine the sensitivity of human cells to heme, we treated human PBMCs from different healthy donors with different doses of heme. We found that human PBMCs were resistant to low doses of heme (3–10 μM); they underwent cell death in response to higher doses of heme (30 μM) (Fig. 2A). By contrast, human monocyte-derived macrophages were more sensitive to

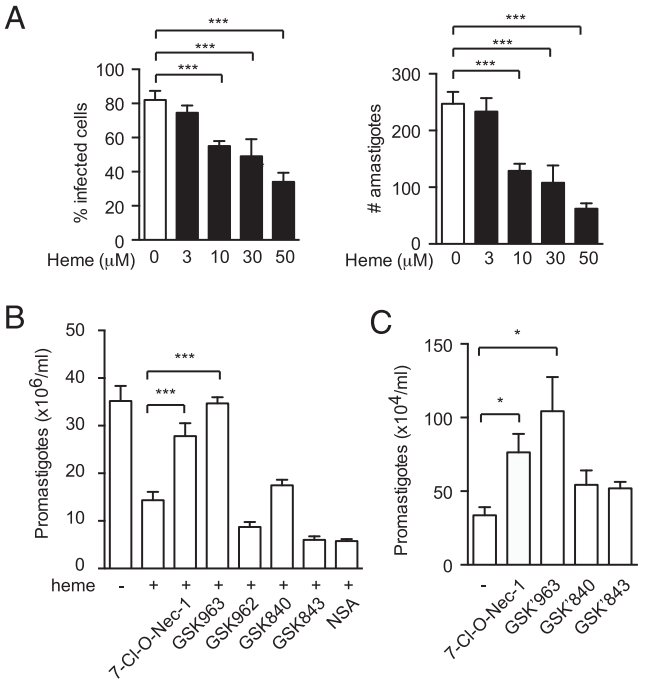

FIGURE 3. RIPK1 kinase activity regulates *Leishmania* replication-independent heme-induced necroptosis. (**A**) High doses of heme inhibit *Leishmania infantum* replication. BMDMs were infected in vitro with *L. infantum* (MOI 10) and treated with different doses of free heme (3, 10, 30, and 50 μM). Parasite load was counted by optical microscopy at 72 h postinfection as described in *Materials and Methods*. The percentage of *L. infantum*–infected macrophages (*left*) and the number of amastigotes per 100 macrophages (*right*) are shown. Results shown are mean of triplicates ± SEM. ANOVA with Bonferroni's posttest was used to evaluate statistical significance. ***$p < 0.05$. (**B**) RIPK1 kinase inhibitors, but not RIPK3 or MLKL inhibitors, reversed heme-induced inhibition of *Leishmania infantum* replication in THP-1 cells. PMA-differentiated THP-1 cells were pretreated with the indicated inhibitors for 1 h, followed by infection with *Leishmania infantum* for 4 h before stimulation with 30 μM heme. Parasite load was determined 24 h postinfection. (**C**) RIPK1 kinase activity regulates *Leishmania infantum* replication in THP-1 cells independent of heme-induced necroptosis. PMA-differentiated THP-1 cells were pretreated with the indicated inhibitors for 1 h before infection with *Leishmania infantum.* Parasite load was determined 24 h postinfection. Three independent experiments were performed. Results shown are mean of triplicates ± SEM. *$p < 0.001$, ***$p < 0.05$.

heme, with detectable cell death starting at 10 μM heme (Fig. 2B). The human monocytic cell line THP-1 differentiates into macrophages when stimulated with the phorbol ester PMA. Similar to primary PBMCs and macrophages, PMA-treated THP-1 cells also underwent high-dose (30 μM) heme-induced cell death in a dose-dependent manner (Fig. 2C). Heme-induced cell death of THP-1 cells was inhibited by the RIPK1 kinase inhibitor necrostatin-1 (Nec-1), the RIPK3 kinase inhibitors GSK'840 and GSK'843 (25), and the MLKL inhibitor necrosulfonamide (NSA) (Fig. 2D, 2E). Because RIPK1, RIPK3, and MLKL are key adaptors for necroptosis (15), we conclude that high doses of heme triggered classical necroptosis in human macrophages.

Leishmania *replication is inhibited by heme and RIPK1 kinase activity*

We next asked whether high-dose heme-induced necroptosis might inhibit *Leishmania* replication. Indeed, we found that heme blunted parasite replication in a dose-dependent manner in BMDMs (Fig. 3A). Consistent with the requirement for heme for replication, low-dose (3 μM) heme did not impair *L. infantum* replication (Fig. 3A). To determine whether the inhibition of parasite replication was due to necroptosis, we treated infected THP-1 cells with different necroptosis inhibitors. The serine/threonine kinases RIPK1 and RIPK3 are crucial for heme-induced necroptosis (11). As expected, the RIPK1 kinase inhibitors 7-Cl-*O*-Nec-1 (26) and GSK'963, but not the inactive enantiomer GSK'962, reversed high-dose heme-induced inhibition of *Leishmania* replication (Fig. 3B). Surprisingly, the RIPK3 inhibitors GSK'840 and GSK'843 (25), and the MLKL inhibitor NSA (27), had no effects on heme-induced inhibition of *Leishmania* replication (Fig. 3B). These results suggest that RIPK1 kinase activity might regulate *Leishmania* replication independent of necroptosis. Indeed, the RIPK1 inhibitors 7-Cl-*O*-Nec-1 and GSK'963 strongly enhanced *L. infantum* replication in THP-1 cells even in the absence of heme (Fig. 3C). By contrast, the RIPK3

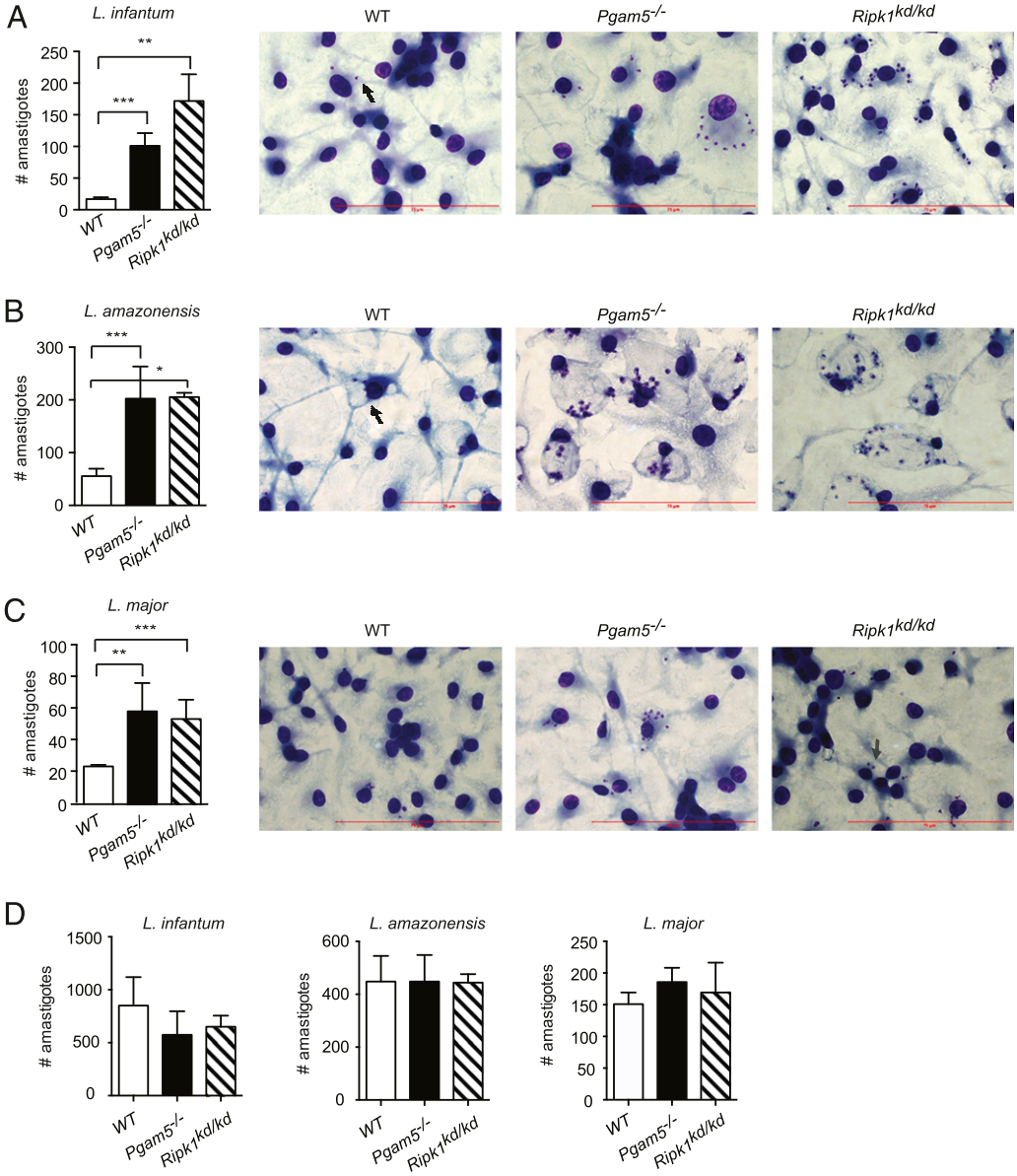

FIGURE 4. RIPK1 and PGAM5 control *Leishmania* replication in mouse BMDMs. (**A**–**C**) WT, *Pgam5*$^{-/-}$, and *Ripk1*$^{kd/kd}$ BMDMs were infected with (A) *Leishmania infantum*, (B) *Leishmania amazonensis*, or (C) *Leishmania major.* Amastigotes were counted 72 h postinfection. Representative images of amastigotes in infected BMDMs using Diff-Quick staining were shown on the right. Red scale bars, 75 μm. (**D**) RIPK1 and PGAM5 do not affect infectivity. Intracellular parasites were counted 4 h postinfection with the indicated parasites. Three independent experiments were performed. Results shown are mean of triplicates ± SEM. *$p < 0.001$, **$p < 0.01$, ***$p < 0.05$.

inhibitor GSK'843 had no effect on *L. infantum* replication (Fig. 3C). These results show that although high doses of heme can inhibit parasite replication through necroptosis, RIPK1 kinase activity also inhibits *L. infantum* replication independent of necroptosis.

The putative RIPK1 downstream effector PGAM5 regulates Leishmania *replication*

The effect of the RIPK1 kinase inhibitors on *Leishmania* replication prompted us to examine the underlying mechanism. Because pharmacological inhibitors can have off-target effects, we first confirmed the role of RIPK1 kinase activity in *Leishmania* replication using BMDMs derived from knock-in mice expressing the kinase inactive RIPK1 mutant RIPK1-K45A (*Ripk1*$^{kd/kd}$) (19). Similar to THP-1 cells treated with RIPK1 inhibitors, the number of *L. infantum* amastigotes in infected *Ripk1*$^{kd/kd}$ BMDMs was far greater than that of WT BMDMs (Fig. 4A). Similar defects were observed in *Ripk1*$^{kd/kd}$ BMDMs infected with two other strains of *Leishmania*, *L. amazonensis* (Fig. 4B) and *L. major* (Fig. 4C). Importantly, no significant differences in intracellular parasite were detected 4 h postinfection (Fig. 4D), indicating that the increase in parasite replication in *Ripk1*$^{kd/kd}$ BMDMs was not due to differences in infection efficiency. Rather, these results indicate that RIPK1 kinase activity restricts *Leishmania* replication after entry into macrophages.

The mitochondrial phosphatase PGAM5 has been reported to act downstream of RIPK1 and RIPK3 in necroptosis (17). Although subsequent studies have challenged the role of PGAM5 in necroptosis (28, 29), PGAM5 has been shown to mediate other RIPK1 functions (30). Indeed, we found that replication of *L. infantum* (Fig. 4A), *L. amazonensis* (Fig. 4B), and *L. major* (Fig. 4C) was also increased in *Pgam5*$^{-/-}$ BMDMs. The increase in parasite replication was not due to changes in infection rate, because a similar number of parasites was found in WT and *Pgam5*$^{-/-}$ BMDMs 4 h postinfection (Fig. 4D).

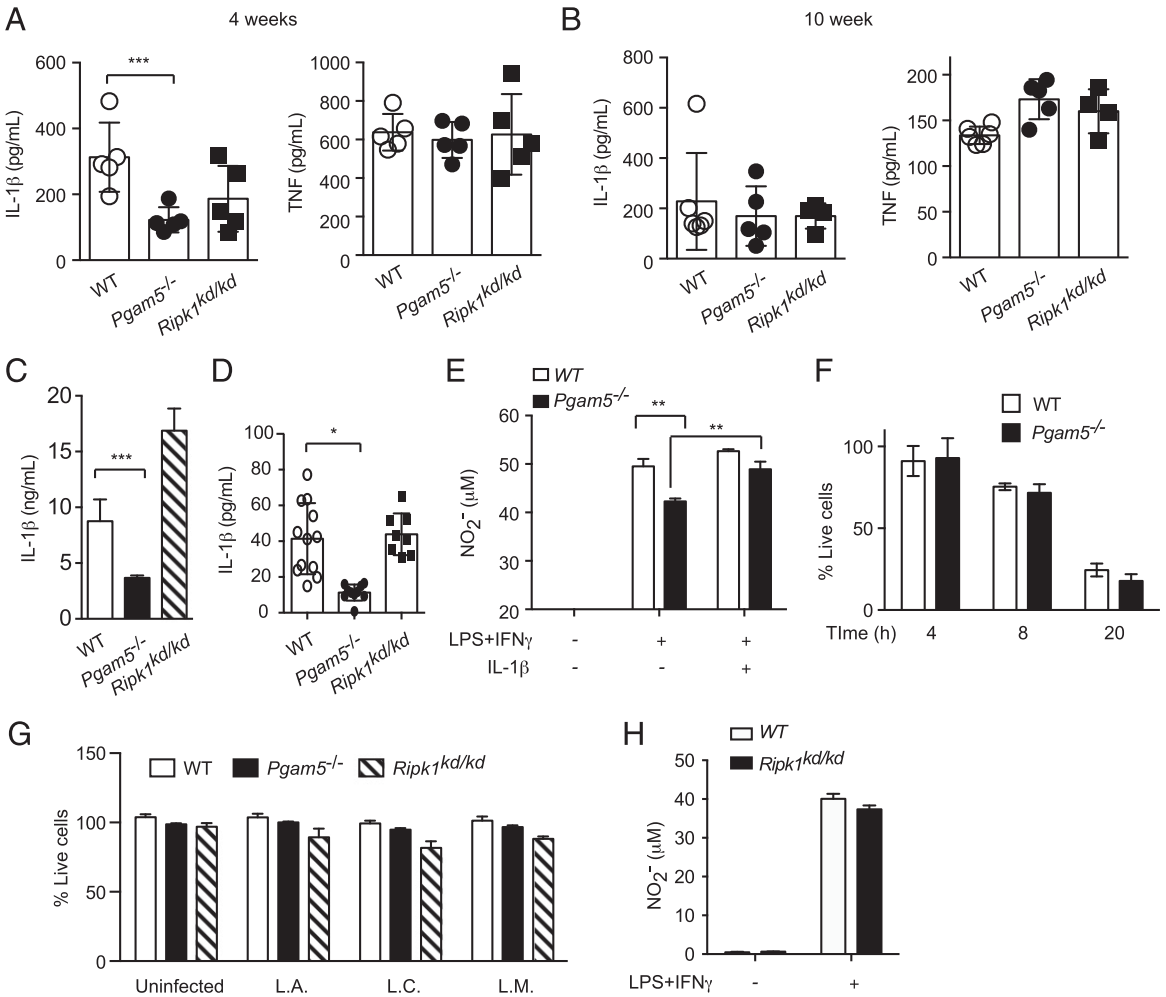

FIGURE 5. RIPK1 and PGAM5 control *Leishmania* through distinct mechanisms. (**A** and **B**) Production of IL-1β, but not TNF, was reduced in *Leishmania*-infected *Pgam5*$^{-/-}$, but not *Ripk1*$^{kd/kd}$, mice. (A) Footpad tissue extracts from mice infected with *Leishmania amazonensis* for 4 wk were stimulated with *Leishmania* particular Ag for 48 h. IL-1β and TNF secretion were determined by ELISA. (B) Footpad tissue extracts from infected mice at 10 wk were examined for IL-1β and TNF expression by ELISA. (**C**) *Pgam5*$^{-/-}$, but not *Ripk1*$^{kd/kd}$, BMDMs are defective for IL-1β secretion. BMDMs were primed with LPS for 3 h followed by 3 h of stimulation with nigericin. IL-1β secretion was determined by ELISA. (**D**) Splenocytes from mice infected with *Leishmania amazonensis* were stimulated with *Leishmania* particular Ag. IL-1β secretion was measured 48 h after stimulation. (**E**) WT and *Pgam5*$^{-/-}$ BMDMs were infected with *L. infantum* for 4 h. Where indicated, BMDMs were also treated with LPS and the indicated cytokines. After incubation for 48 h incubation, NO production was determined as described in *Materials and Methods*. (**F**) PGAM5 deficiency does not affect heme-induced necroptosis. BMDMs were stimulated with 30 μM heme and cell death was determined as described in *Materials and Methods*. (**G**) RIPK1 and PGAM5 do not regulate BMDM survival during *Leishmania* infection. BMDMs were infected with the indicated *Leishmania* strains. Cell death was determined 24 h postinfection. (**H**) WT and *Ripk1*$^{kd/kd}$ BMDMs were infected with *L. infantum* for 4 h. NO production was determined 48 h postinfection. Two independent experiments were performed. Results shown are mean of triplicates ± SEM. *$p < 0.001$, **$p < 0.01$, ***$p < 0.05$.

RIPK1 and PGAM5 controls Leishmania *replication through different mechanisms*

The results in BMDMs suggest that RIPK1 and PGAM5 may act along the same pathway to control *Leishmania* replication. To interrogate this possibility, we examined cytokine response in *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ mice in response to *Leishmania* infection. Because *L. infantum* does not cause productive infection in mice, we used *L. amazonensis* for the in vivo infections. A recent study demonstrated that NLRP3 inflammasome-mediated IL-1β secretion stimulates NO production and killing of *Leishmania* parasites (5). Because both RIPK1 and PGAM5 have been implicated to regulate cell death and pro–IL-1β cleavage (29, 30), we examined IL-1β production in mice infected with the parasites. At 4 wk postinfection, IL-1β expression was reduced in the footpad of infected *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ mice (Fig. 5A). By contrast, TNF expression was normal in *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ mice (Fig. 5A). However, by 10 wk postinfection, the difference in IL-1β was no longer detected in *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ mice (Fig. 5B). These results indicate that RIPK1 and PGAM5 regulate early IL-1β release during *Leishmania* infection.

We next asked whether PGAM5 and RIPK1 kinase activity directly regulate IL-1β secretion. We first examined IL-1β secretion by LPS-primed macrophages stimulated with the NLRP3 inflammasome agonist nigericin. Strikingly, reduced IL-1β secretion was only observed in *Pgam5*$^{-/-}$, but not *Ripk1*$^{kd/kd}$, BMDMs (Fig. 5C). In fact, IL-1β secretion was slightly increased in *Ripk1*$^{kd/kd}$ BMDMs compared with WT BMDMs. To further confirm these results, we isolated splenocytes from mice infected with *L. amazonensis* and stimulated them with *Leishmania* particular Ag ex vivo. Similar to LPS-primed macrophages, IL-1β secretion was reduced in *Pgam5*$^{-/-}$, but not *Ripk1*$^{kd/kd}$, splenocytes (Fig. 5D). A key function of IL-1β during *Leishmania* infection is to induce production of NO, which in turn mediates killing of infected cells (5). Indeed, LPS and IFN-γ–induced NO production was significantly reduced in *Leishmania*-infected *Pgam5*$^{-/-}$ BMDMs (Fig. 5E). Moreover, rIL-1β restored NO production by *Pgam5*$^{-/-}$ BMDMs to WT level (Fig. 5E). The defect in IL-1β secretion by *Pgam5*$^{-/-}$ BMDMs was not due to increased cell death, because *Pgam5*$^{-/-}$ BMDMs were similarly sensitive to heme-induced cell death (Fig. 5F). Moreover, *Leishmania*-infected WT, *Ripk1*$^{-/-}$, and *Pgam5*$^{-/-}$ BMDMs had similar viability (Fig. 5G). Furthermore, *Pgam5*$^{-/-}$ BMDMs were similarly sensitive to heme-induced cell death (Fig. 5F). This indicates that PGAM5 facilitates control of *Leishmania* replication through an IL-1β/NO axis. In contrast, NO production was normal in infected *Ripk1*$^{kd/kd}$ BMDMs (Fig. 5H). Hence RIPK1 and PGAM5 inhibit *Leishmania* replication through distinct mechanisms.

RIPK1 and PGAM5 are required for in vivo control of Leishmania *infection*

We next asked how the early defect in IL-1β production by *Pgam5*$^{-/-}$ and *Ripk1*$^{kd/kd}$ mice might affect inflammation and *Leishmania* replication in vivo. Histological sections of the footpad revealed similar extent of neutrophil infiltration in the footpad

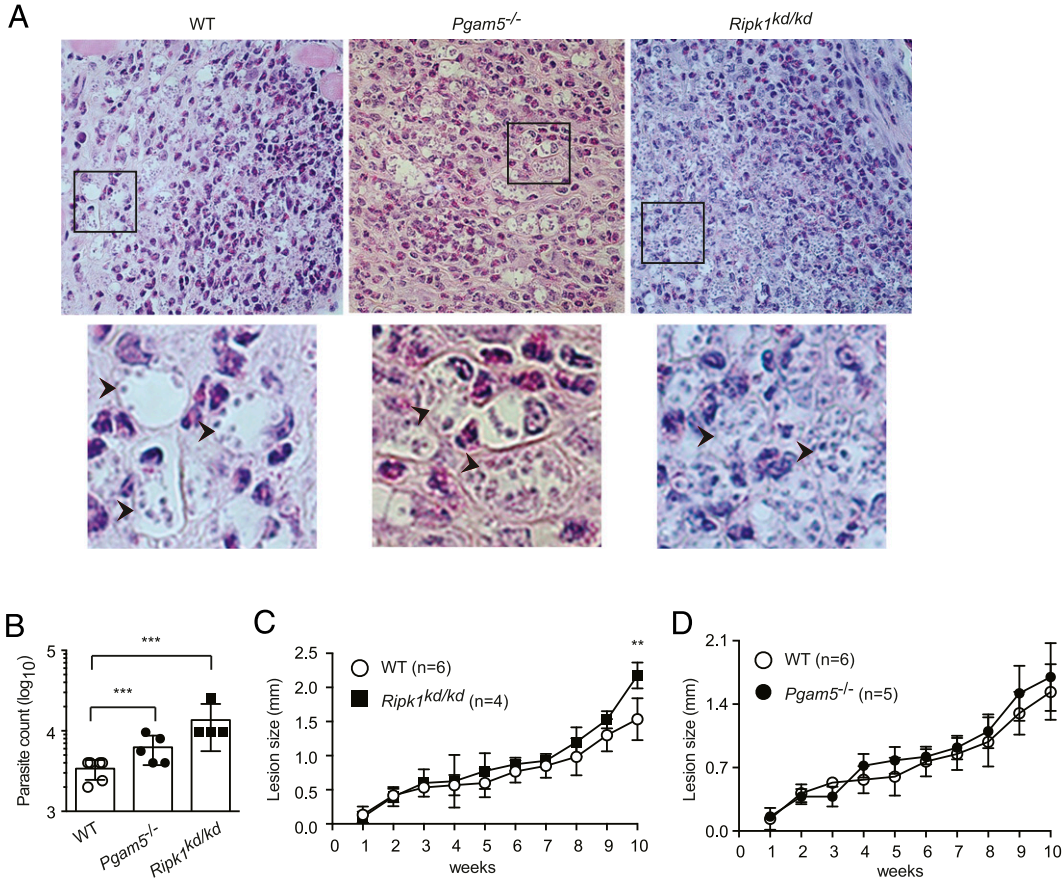

FIGURE 6. RIPK1 kinase activity and PGAM5 are required for in vivo control of *Leishmania* replication. WT, *Ripk1*$^{kd/kd}$, or *Pgam5*$^{-/-}$ mice were infected with *Leishmania amazonensis* in the footpad. (**A**) Histology sections of H&E-stained infected footpad at 5 wk postinfection were examined. Representative images were shown and revealed similar extent of inflammation marked by neutrophilic infiltration in WT, *Ripk1*$^{kd/kd}$, and *Pgam5*$^{-/-}$ mice. Boxed areas were magnified to show intracellular parasites in the vacuoles. (**B**) Parasite load in the footpad was determined 10 wk postinfection. (**C** and **D**) Lesion size on the footpad was monitored weekly. Two independent experiments were performed. $^{**}p < 0.01$, $^{***}p < 0.05$.

of WT, *Ripk1*$^{kd/kd}$, and *Pgam5*$^{-/-}$ mice at 4 wk postinfection (Fig. 6A). Importantly, an increase in the number of amastigotes in intracellular vacuoles was apparent in *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ mice. Indeed, when the numbers of parasites in the infected footpad were counted, a significantly greater number of parasites was found in both *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ mice (Fig. 6B). Moreover, the lesion size in the infected footpad was significantly increased in *Ripk1*$^{kd/kd}$ mice at 10 wk postinfection (Fig. 6C). Surprisingly, lesion size was not significantly increased in *Pgam5*$^{-/-}$ mice (Fig. 6D). These results indicate that although RIPK1 does not directly regulate pro–IL-1β processing and secretion, it has a more prominent role than PGAM5 in the control of *Leishmania* replication in vivo.

Discussion

Leishmania parasite causes tegumentary (cutaneous, mucocutaneous, disseminated, and diffuse clinical forms) and visceral pathologies in humans and other animals. Leishmaniasis is endemic in the tropics and subtropics, and thus is a major public health concern in the affected regions (1). Despite its importance, the relationship between host cell death pathways and immune defense against *Leishmania* infection is poorly understood. Certain intracellular pathogens such as *Plasmodium falciparum* and *Plasmodium vivax* elicit the release of heme (9), which can cause oxidative damage, inflammation, and necroptosis (11, 21).

We recently found that heme induces RIPK1- and RIPK3-dependent necroptosis in murine macrophages (11). Inflammatory cell death of macrophages could provide a key signal that stimulates innate immune response during *Leishmania* infection. Damage-associated molecular patterns released from necrotic cells could further stimulate expression of cytokines such as IL-1β to exacerbate tissue inflammation. Given their reported roles in inflammation and necroptosis, we speculated that RIPK1 and PGAM5 might contribute to these disease pathologies in human leishmaniasis. Indeed, we found that human patients with VL exhibited elevated serum levels of heme. High doses of heme could induce necroptosis and inhibit *Leishmania* replication, as we found to be the case in human macrophages. Heme-induced cytotoxicity has also been observed in *Mycobacterium tuberculosis* infection (31). However, low doses of heme did not induce necroptosis or inhibit *Leishmania* replication. This is consistent with the fact that a low dose of heme is required for optimal *Leishmania* growth (24). Free heme is neutralized by HO-1, an enzyme that promotes *Leishmania* replication (7). Thus, at high doses of heme, such as that found in VL, heme-induced necroptosis may prevent excessive parasite replication by overwhelming the neutralizing effect of HO-1. Unfortunately, the lack of mouse model that mimics human visceral leishmaniasis prevented us from directly testing this hypothesis.

RIPK1 and PGAM5 also participate in the control of parasite replication in cutaneous leishmaniasis, a more mild form of the disease. In contrast with their reported roles in apoptosis and necroptosis (17, 32), RIPK1 and PGAM5 did not regulate *Leishmania* replication through cell death. Rather, we found that PGAM5 facilitates optimal IL-1β and NO production in response to *Leishmania* infection. IL-1β has been shown to cooperate with other inflammatory cytokines such as TNF and IFN-γ to enhance NO production by macrophages (5, 33). The IL-1β–NO axis causes macrophage cell death and creates an antimicrobial milieu at the tissue level, both of which are key mechanisms that limit parasite replication (34, 35). Consistent with their reported roles in regulating NLRP3 inflammasome activation (29, 30, 36), IL-1β secretion was reduced in *Leishmania*-infected *Ripk1*$^{kd/kd}$ and *Pgam5*$^{-/-}$ mice during the early phase of the infection. However, we found that *Pgam5*$^{-/-}$, but not *Ripk1*$^{kd/kd}$, splenocytes from infected mice exhibited defective IL-1β secretion in response to *Leishmania* particular Ag. The distinct effects of RIPK1 and PGAM5 in pro–IL-1β processing are also observed in BMDMs (29). The critical role for PGAM5 in IL-1β secretion is consistent with its reported function in inflammasome polymerization (29). However, despite reduced IL-1β secretion and increased parasite load, footpad swelling was not significantly increased in *Leishmania*-infected *Pgam5*$^{-/-}$ mice. This is in contrast with *Ripk1*$^{kd/kd}$ mice, which showed significant increase in footpad swelling compared with WT mice. The relatively mild phenotype of *Pgam5*$^{-/-}$ mice compared with mice lacking core inflammasome components is consistent with the fact that PGAM5 deficiency reduced, but did not abolish, IL-β secretion.

Although IL-1β secretion in the footpad was reduced, IL-1β secretion by splenocytes from *Leishmania*-infected *Ripk1*$^{kd/kd}$ mice was normal. The mechanism by which RIPK1 kinase activity promotes IL-1β expression in vivo is unknown at present. Although the kinase activity of RIPK1 is known to promote cell death by apoptosis and necroptosis (12, 37), infected *Ripk1*$^{kd/kd}$ BMDMs did not exhibit significant difference in cell death when compared with WT BMDMs. Besides promoting cell death, RIPK1 is a known activator of NF-κB and MAPK (38–42). In this regard, it is noteworthy that *L. amazonensis* infection in macrophages activates the p50/p50 homodimer, a NF-κB transcriptional repressor. Inhibition of NF-κB–dependent gene transcription by p50/p50 homodimer has been attributed to cause downregulation of inducible NO synthase expression in *L. amazonensis* infection (43, 44). It is therefore tempting to speculate that RIPK1 may promote canonical NF-κB activation to counteract this inhibitory signal from p50/p50.

The discovery that RIPK1 and PGAM5 control *Leishmania* replication suggests that they may have wider roles in host defense and resistance against other intracellular pathogens. In this light, Roca and Ramakrishnan (45) have recently shown that macrophages inhibit *M. tuberculosis* growth, and that this phenomenon is dependent on RIPK1- and RIPK3-mediated reactive oxygen species production. Thus, RIPK1 and PGAM5 may represent preserved innate immune mechanisms against intracellular pathogens. Identifying the pathogen recognition receptors that stimulate these pathways could lead to better therapeutics against these diseases.

Disclosures

J.B. and P.J.G. are employees of GlaxoSmithKline. The other authors have no financial conflicts of interest.

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