Reactive Oxygen Species Regulate Caspase-11 Expression and Activation of the Non-canonical NLRP3 Inflammasome during Enteric Pathogen Infection

Christopher R. Lupfer1*, Paras K. Anand1, Zhiping Liu1, Kate L. Stokes1, Peter Vogel2, Mohamed Lamkanfi3,4, Thirumala-Devı Kanneganti1*

1 Department of Immunology, St. Jude Children’s Research Hospital, Memphis, Tennessee, United States of America, 2 Veterinary Pathology Department, St. Jude Children’s Research Hospital, Memphis, Tennessee, United States of America, 3 Department of Medical Protein Research, Vlaams Instituut voor Biotechnologie, Ghent, Belgium, 4 Department of Biochemistry, Ghent University, Ghent, Belgium

Abstract

Enteropathogenic and enterohemorrhagic bacterial infections in humans are a severe cause of morbidity and mortality. Although NOD-like receptors (NLRs) NOD2 and NLRP3 have important roles in the generation of protective immune responses to enteric pathogens, whether there is crosstalk among NLRs to regulate immune signaling is not known. Here, we show that mice and macrophages deficient in NOD2, or the downstream adaptor RIP2, have enhanced NLRP3- and caspases-11-dependent non-canonical inflammasome activation in a mouse model of enteropathogenic Citrobacter rodentium infection. Mechanistically, NOD2 and RIP2 regulate reactive oxygen species (ROS) production. Increased ROS in Rip2-deficient macrophages subsequently enhances c-Jun N-terminal kinase (JNK) signaling resulting in increased caspase-11 expression and activation, and more non-canonical NLRP3-dependent inflammasome activation. Intriguingly, this leads to protection of the colon epithelium for up to 10 days in Rip2-deficient mice infected with C. rodentium. Our findings designate NOD2 and RIP2 as key regulators of cellular ROS homeostasis and demonstrate for the first time that ROS regulates caspase-11 expression and non-canonical NLRP3 inflammasome activation through the JNK pathway.

Introduction

Enteropathogenic and enterohemorrhagic Escherichia coli (EPEC and EHEC) infections in humans are a severe cause of morbidity and mortality, especially in developing countries [1]. To study these infections, Citrobacter rodentium is used as an enteric bacterial pathogen of mice that triggers similar inflammatory responses to those observed in humans infected with EPEC and EHEC [2]. As with EPEC and EHEC in humans, C. rodentium infection in the mouse results in bacterial attachment and effacing lesion formation in the lumen of the colon [3]. Clearance of C. rodentium from the host tissues requires contributions from humoral and Th1 immune responses [4–6]. However, innate immunity is also important for early bacterial control [7,8] and for the initiation of adaptive immunity [9,10].

Innate immunity to pathogens depends on a limited set of germ-line encoded pattern recognition receptors (PRRs) that sense conserved pathogen motifs. Innate receptors initiate inflammation through activation of pro-inflammatory transcription factors such as NF-κB, by promoting activation of proinflammatory caspases in inflammasomes, and by initiating programmed cell death of infected cells. The intracellular NOD-like receptors (NLRs) NOD1 and NOD2 recognize peptidoglycan fragments from bacterial cell walls in the cytosol, which results in pro-inflammatory NF-κB and MAP-kinase pathway activation via the adaptor RIP2 [11–13]. However, pathogen infection is frequently accompanied by ion fluxes and vacuolar membrane damage elicited by the action of microbial toxins and effectors of specialized bacterial secretion systems [14–17]. Thus, while conserved bacterial peptidoglycan fragments are initially recognized by receptors NOD1 and NOD2, the perturbations inflicted over the course of infection can activate NLRP3.

NLRP3 is the most studied inflammasome-associated NLR. Signaling events initiated by Toll-like receptors (TLRs) contribute to NF-κB mediated up-regulation of NLRP3 and pro-IL-1β for robust activation [18]. No specific pathogen-derived ligand is known to bind and activate NLRP3. Instead, NLRP3 is activated in response to diverse stimuli including microbial, environmental and metabolic...
Author Summary

Caspase-11 is required for NLRP3 inflammasome activation and cell death in response to certain gram-negative bacterial infections like Citrobacter rodentium. However, how C. rodentium drives caspase-11 expression and activation is not well understood. Here, we demonstrate that the NOD2-RIP2 pathway regulates reactive oxygen species production and c-Jun N-terminal kinase signaling to control caspase-11 expression and subsequent activation of caspase-11 and the NLRP3 inflammasome during C. rodentium infection. In the absence of NOD2-RIP2 signaling, increased inflammasome activation results in lower bacteria numbers in the colon and less tissue damage during the early stages of infection.

Results

Non-canonical NLRP3 inflammasome activation is restricted by the NOD2-RIP2 pathway. To determine the extent of NOD2-RIP2 modulation, we examined whether this pathway also modulates the NLRC4 inflammasome. To answer this question, we infected WT, Nod2- and Rip2-deficient cells with Salmonella typhimurium to activate the NLRC4-inflammasome. However, comparable caspase-1 activation was observed in S. typhimurium infected WT, Nod2-deficient and Rip2-deficient cells (Figure 2A-B). In agreement, IL-18 levels were similar (Figure 2C) suggesting that the NOD2-RIP2 pathway specifically restricts the NLRP3 inflammasome. The NLRC4 inflammasome is activated upon recognition of S. typhimurium flagellin [34,35]. However, flagellin-deficient S. typhimurium Afdj/fljfc activates the NLRP3 inflammasome [29]. Consequently, Nod2- or Rip2-deficient BMDM infected with the S. typhimurium Afdj/fljfc strain displayed enhanced caspase-1 activation and IL-18 production over wildtype BMDM (Figure 2D-F). These results suggest that NOD2 and RIP2 specifically modulated the NLRP3 inflammasome in this model while the NLRC4 inflammasome was not affected by the absence of the NOD2-RIP2 signaling axis.

Glyburide, a type 2 diabetes drug, has been shown to specifically inhibit the NLRP3 inflammasome in response to microbial and crystalline stimuli [36]. To further verify that the enhanced caspase-1 activation observed in Nod2- and Rip2-deficient BMDM is the result of elevated NLRP3 inflammasome activation, we exposed Rip2-deficient BMDM to glyburide following C. rodentium infection. Consistent with the requirement for NLRP3, we observed increased caspase-1 activation in Rip2-deficient cells and this was markedly abrogated in the presence of glyburide (Figure 2G,H). In agreement, enhanced IL-18 levels in Rip2-deficient BMDM were significantly decreased upon treatment with glyburide (Figure 2I). In contrast, glyburide treatment did not affect caspase-1 activation in Rip2-deficient cells infected with S. typhimurium (Figure 2J,K). Consequently, IL-18 levels were also unaffected (Figure 2L). These data indicate that NOD2-RIP2 signaling specifically modulates NLRP3 activation and does not regulate all inflammasomes.

Rip2-deficient cells show increased ROS production

To determine the cause of increased NLRP3 inflammasome activation during C. rodentium infection, we examined the possibility that there is a global dysregulation of cytokines and inflammation in Rip2-deficient BMDM. However, there was no difference in IL-6 production and TNF-α levels were lower in Rip2-deficient cells (Figure 3A) suggesting that a global increase in inflammation is not responsible for increased NLRP3 inflammasome activation in this model.

Elevated production of reactive oxygen species (ROS) has previously been associated with increased NLRP3 inflammasome activation [37]. In particular, mitochondrial-derived ROS production was suggested to provoke NLRP3 activation [38], and we previously demonstrated that Rip2-deficient cells have defects in autophagy that lead to the accumulation of damaged mitochondria [39]. LC3 is an autophagy-associated protein that is localized in the cytosol under steady state conditions, but relocates to autophagosomes when autophagy occurs [40]. During C. rodentium infection of WT or Rip2-/- BMDM, the number of autophagosomes was examined using confocal microscopy by counting GFP-LC3+ puncta per cell (Figure 3B-C). Alternatively, we used flow cytometry to measure the fluorescence intensity of GFP-LC3 after permeabilizing cells so that only autophagosome membrane bound GFP-LC3 remained (Figure 3D). In both cases, we found that autophagy was impaired in Rip2-/- BMDM compared to WT cells after C. rodentium infection. Furthermore, Rip2-deficient BMDM exhibited elevated levels of mitochondria-
derived superoxide when infected with *C. rodentium* and stained with the mitochondrial specific ROS sensor MitoSOX (Figure 3E,F). Finally, Rip2²⁻²⁻/²⁻ BMDM treated with the ROS scavenger N-acetyl-L-cysteine (NAC) had reduced caspase-1 activation and IL-18 production compared to WT cells (Figure 3G–I). Thus, the presence of dysfunctional mitochondria in

---

**Figure 1. Enhanced inflammasome activation in *Nod2*²⁻²⁻ and *Rip2*²⁻²⁻ BMDMs.** BMDMs were generated from WT, *Nod2*²⁻²⁻ and *Rip2*²⁻²⁻ mice and infected with 20MOI of *Citrobacter rodentium* for 18 h. (A–F) Combined supernatant and lysates were examined by Western blot for caspase-1 cleavage (casp-1p20) visually (A,D) and by densitometry (B,E), or (C,F) supernatants were examined for IL-18 secretion by ELISA. (G) BMDM were infected with *C. rodentium* and assayed for intracellular growth at the indicated times post-infection. (A–F) Data are representative of five independent experiments with n = 2–3 wells per experiment. (B,C,E,F,G) Data are shown as the mean ± SEM. (*, p<0.05; **, p<0.01; ***, p<0.001). doi:10.1371/journal.ppat.1004410.g001
infected with 1MOI Salmonella typhimurium for 4 h and combined supernatant and lysates were examined by Western blot for caspase-1 cleavage (asp-1p20) visualy (A) and by densitometry (B). (C) Supernatants were examined 4 h after S. typhimurium infection for IL-18 secretion. (D–F) WT, Nod2−/− and Rip2−/− BMDM were infected with 10MOI S. typhimurium Δfljb/flc mutant for 18 h. Caspase-1 activation and IL-18 were examined as in panels A–C. (G–I) WT and Rip2−/− BMDM were infected with 20MOI of C. rodentium for 2 h and Rip2−/− cells were subsequently treated with the NLRP3 specific inhibitor glyburide or mock treated as a control. Caspase-1 activation and IL-18 were examined as in panels A–C. (J–L) WT and Rip2−/− BMDM were infected with 1MOI of S. typhimurium and Rip2−/− cells were simultaneously treated with the NLRP3 specific inhibitor glyburide or mock treated as a control. Caspase-1 activation and IL-18 were examined as in panels A–C. (A–L) Data are representative of three independent experiments with n = 2–3 wells per experiment and displayed as the mean ± SEM. (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

doi:10.1371/journal.ppat.1004410.g002

Rip2−/− cells leads to enhanced ROS production and thereby increased NLRP3 inflammasome activation.

Caspase-11 activation is upregulated in Rip2-deficient cells

Enteric pathogens such as C. rodentium are known to activate the NLRP3 inflammasome by a non-canonical pathway, which additionally requires caspase-11 for its activation [26]. We therefore postulated that during C. rodentium infection increased ROS production might act upstream of caspase-11 as well as caspase-1. Therefore, we sought to examine the role of caspase-11 in the present study. First, we determined that activation of caspase-11 is enhanced in Rip2−/− BMDM [Fig. 4A,B]. Next, we treated Rip2−/− BMDM with the ROS scavenger NAC following C. rodentium infection and observed that caspase-11 activation was significantly diminished compared to mock-treated Rip2−/− BMDM controls [Fig. 4C,D]. We also found that caspase-11 activation (p30 band) correlated with the expression of p43/p38 (pro-caspase-11). Indeed, densitometric analysis showed that C. rodentium infected Rip2−/− BMDM had higher expression of pro-caspase-11 and that treatment with NAC inhibited pro-caspase-11 expression [Fig. 4E]. Mechanistically, we examined whether the increased caspase-11 seen in Rip2−/− BMDM was the result of defects in protein turnover; however, treatment of WT and Rip2−/− BMDM 4 h after infection with the proteasome inhibitor MG-132 resulted in accumulation of caspase-11 in both WT and Rip2−/− BMDM [Fig. 4F–G]. Instead, Casp11 mRNA was higher in Rip2−/− BMDM compared to WT BMDM [Fig. 4H]. These results indicate that RIP2 regulates caspase-11 at the level of mRNA expression, possibly through enhanced ROS production.

Exogenous ROS enhances caspase-11 expression and activation in WT cells

To further confirm the role of enhanced ROS production leading to increased inflammasome activation in Rip2−/− BMDM, we examined the effects of treating WT BMDM with exogenous ROS. WT BMDM were infected with C. rodentium and treated with H2O2 as a ROS source 6 h after infection. Although H2O2 treatment had no effect on IL18 mRNA levels following C. rodentium infection, we detected significantly more IL-18 expression in the culture medium [Fig. 5A,D]. In agreement, caspase-1 activation was also increased following H2O2 treatment [Fig. 5C]. Importantly, H2O2 treatment of WT cells resulted in increased mRNA and protein expression of caspase-11 by qRT-PCR and Western blot respectively [Fig. 5D–F]. Our results indicate that ROS produced during C. rodentium infection can enhance caspase-11 expression and subsequently potentiate activation of the non-canonical inflammasome.

ROS regulates caspases-11 through a JNK mediated pathway

Our data indicate that ROS is capable of regulating caspase-11 expression. However, it remains unclear what pathways are regulated by ROS that subsequently affect caspase-11 expression. As a TLR4-TRIF-IFN-β pathway has been established for expression of caspase-11 [30,31], we examined the level of type-I interferons, but found they were similar between WT, Rip2−/−, and Nod2−/− BMDM [Fig. 6A]. One pathway that is responsive to ROS is the c-Jun N-terminal kinase [JNK pathway [41]. We next infected WT BMDM with C. rodentium, treated them with H2O2 and examined the effects on JNK1/2 phosphorylation. H2O2 treatment resulted in increased JNK phosphorylation. H2O2 treatment resulted in increased JNK phosphorylation compared to mock treated cells [Fig. 6B]. Similarly, Rip2−/− BMDM infected with C. rodentium alone had substantially more JNK1/2 phosphorylation than WT cells [Fig. 6C]. Finally, we infected WT or Rip2−/− BMDM with C. rodentium and then treated Rip2−/− BMDM with the JNK inhibitor SP600125. We observed that caspase-11 levels were reduced in conjunction with reduced JNK1/2 activation [Fig. 6D]. From these results we propose that in the absence of NOD2-RIP2 signaling, defective autophagy results in the accumulation of ROS and the subsequent enhancement of JNK signaling. This ultimately leads to an increase in caspase-11 expression and non-canonical inflammasome activation [Fig. 6E].

Increased non-canonical inflammasome activation provides protection against C. rodentium induced colitis

To determine the physiological relevance of our proposed pathway, we examined Rip2-deficient mice for IL-18 production and inflammasome activation in the colon during C. rodentium induced colitis. On day 10 post-infection, caspase-11 and caspase-1 activation were much higher in colon lysates of Rip2−/− mice compared to WT controls [Fig. 7A,B]. IL-18 activation was significantly increased by Western blot and IL-18 levels were higher in colon lysates taken from Rip2−/− mice compared to WT controls [Fig. 7C,D]. In contrast, bacterial loads in the colon were reduced in Rip2−/− versus WT mice [Fig. 7E]. In agreement, colon tissue examined on day 10 after infection showed increased numbers of bacteria adherent to the mucosal surface in WT compared to Rip2−/− mice [Fig. 7F]. Intriguingly, colon sections from WT mice also showed more hyperplasia and inflammation and the extent of colon area that was inflamed was more pronounced with WT mice compared to Rip2−/− mice [Fig. 7F,G]. The stool of Rip2−/− mice also had fewer C. rodentium CFU at both day 7 and day 10, highlighting the protective effect of increased inflammasome activation during the innate immune phase of infection [Fig. 7H]. These results demonstrate that the NOD2-RIP2 pathway plays an important role in regulating the non-canonical NLRP3 inflammasome in vivo during C. rodentium-induced colitis. Of note, previous reports have demonstrated an essential role for adaptive immunity in the eventual clearance of C. rodentium [9,42]. In agreement with these studies, on day 14 after infection, mice deficient in RIP2 signaling had a clearance defect in the stool [Fig. 7H]. Finally, to verify that increased inflammasome activation and IL-18 production were responsible for the lower bacterial numbers on day 7 or 10, we infected WT, Rip2−/−, and Rip2−/− ×Il18−/− mice with C.rodentium.
ROS Regulates Casp-11 Pathway

(A) Graph showing IL-6 and TNF-α levels in WT and Rip2−/− mice.

(B) Immunofluorescence images of DAPI and GFP-LC3 in WT and Rip2−/− mice.

(C) Graph showing LC3 puncta per cell in WT and Rip2−/− mice with Citrobacter infection.

(D) Graph showing LC3-GFP levels in WT and Rip2−/− mice with Citrobacter infection over time.

(E) Flow cytometry analysis of MitoSOX in Uninfected and Citrobacter 18h infected WT and Rip2−/− mice.

(F) Graph showing MitoSOX levels in WT and Rip2−/− mice with Citrobacter infection.

(G) Western blot analysis of procasp-1 and casp-1 p20 in WT and Rip2−/− mice with Citrobacter infection and NAC treatment.

(H) Graph showing p20 relative intensity in WT, Rip2−/−, and Rip2−/− + NAC.

(I) Graph showing IL-18 levels in WT, Rip2−/−, and Rip2−/− + NAC.
rodentium and examined bacterial loads on day 7 after infection. As before, Rip2\(^{-/-}\) mice had significantly lower bacterial numbers compared to WT mice. However, the deletion of IL-18 in Rip2\(^{-/-}\) \times IL18\(^{-/-}\) mice resulted in significantly increased bacterial burden compared to the Rip2\(^{-/-}\) mice (Figure 7I).

Discussion

Regulation of the non-canonical NLRP3 inflammasome is still not well understood. It is clear that pathogen entry into the cytosol is required for caspase-11 activation [17,43,44]. In addition, caspase-11 activation requires guanylate binding protein (GBP) for activation in response to cytosolic LPS [45]. However, the exact upstream LPS sensor has yet to be identified [46,47]. Although the exact activation mechanism involved is not clear, our lab and others demonstrated that expression of caspase-11 via a TLR4-TRIF-IFN-\(\beta\)-dependent mechanism is necessary for non-canonical NLRP3 inflammasome activation during C. rodentium infection [30,31]. Our current findings show that ROS produced during C. rodentium infection, or added exogenously to infected cells as \(\text{H}_2\text{O}_2\), is capable of regulating caspase-11 expression. In agreement, treatment with the ROS scavenger NAC dampened caspase-11 expression and activation, and subsequently reduced caspase-1 activation through the non-canonical NLRP3 inflammasome. Mechanically, Rip2\(^{-/-}\) cells display enhanced ROS production following C. rodentium infection, and our data define a pathway where ROS acts through JNK signaling to increase caspase-11 expression and the subsequent activation of the non-canonical NLRP3 inflammasome.

Similar to C. rodentium, we found that infection with flagellin-deficient S. typhimurium \(\Delta\text{fljBfljC}\) resulted in augmented inflammasome activation in Nod2\(^{-/-}\) and Rip2\(^{-/-}\) BMDM. Since activation of the NLRC4 inflammasome was comparable between WT and Rip2-deficient BMDM infected with WT S. typhimurium, these data suggest that NOD2-RIP2 mediated regulation of ROS and JNK activation specifically modulates the non-canonical NLRP3 inflammasome and not NLRC4.

In addition to the mechanistic insight provided by our in vitro studies, caspase-1 activation has never been examined in Rip2\(^{-/-}\) mice in response to C. rodentium. Our findings demonstrate the NOD2-RIP2 pathway helps suppress the non-canonical NLRP3 inflammasome in vivo during C. rodentium induced colitis. In the absence of RIP2, there is increased caspase-11 and caspase-1 activation and higher levels of processed IL-18. These increases are associated with reduced bacterial burden and protection of the colon epithelium during the innate immune phase of infection [47–50]. Our findings are thus in line with our previous publications, which show that NLRP3 inflammasome activation and IL-18 production are important for the control of bacterial burden and disease during C. rodentium infection [48]. The importance of IL-18 in the protection seen in this model was also confirmed through the use of Rip2\(^{-/-}\) \times IL18\(^{-/-}\) mice. Although IL-18 deletion in Rip2\(^{-/-}\) mice did not entirely abolish the protective phenotype, this is likely due to pyroptosis or other functions of caspase-11, such as lysosome-phagosome fusion [49].

Other reports have examined NOD2 signaling in vivo. However, these reports focused on the effects of NOD2-RIP2 signaling on the adaptive immune response. Intriguingly, similar results to ours were published for the ubiquitin ligase pellino3, which is required for NOD2-RIP2 signaling. At early time points, Pel3\(^{-/-}\) mice had reduced bacterial burden similar to our model, but after d14, these mice showed a clearance defect [42]. On the other hand, Nod2\(^{-/-}\) mice displayed reduced production of the chemokine CCL2, which ultimately lead to impaired adaptive immunity and impaired clearance of C. rodentium after d14 of infection [9]. Collectively, these findings suggest that increased NLRP3 inflammasome activation provides protection early during the innate immune phase, but the defects in adaptive immunity that are also present in Nod2\(^{-/-}\), Rip2\(^{-/-}\), or Pel3\(^{-/-}\) mice ultimately lead to an inability of these mice to resolve the infection.

Although inflammation and cell death are important for bacterial clearance [25], they also increase the likelihood of bacterial dissemination to other tissues and induction of severe inflammatory responses by increased tissue damage and released cellular contents [50–52]. Therefore, the sequential and carefully orchestrated activation of inflammatory pathways is essential for pathogen clearance as well as maintaining homeostasis in the host. Initial recognition of specific peptidoglycan fragments from bacterial cell walls by the NOD2-RIP2 pathway, results in pro-inflammatory NF-\(\kappa\)B and MAP-kinase activation within minutes of infection [53,54]. However, if this initial inflammatory burst is inadequate, then subsequent inflammatory pathways, such as the non-canonical NLRP3 inflammasome, are initiated. One common feature of pathogens that activate the non-canonical NLRP3 inflammasome is activation proceeds with slower kinetics than during canonical activation. In all instances, non-canonical NLRP3 inflammasome activation is observed only after a time-period of 12 to 16 h [26,28,30,31], whereas canonical activation requires less than 1 h [21,22,55]. Based on our results, we propose that NOD2-RIP2 signaling initially suppresses or delays non-canonical NLRP3 inflammasome activation by preventing or removing mitochondrial damage. However, extended assault from C. rodentium eventually overcomes these mechanisms and leads to ROS generation and activation of the non-canonical NLRP3 inflammasome [20,39,56]. In the absence of NOD2 or RIP2, increased mitochondrial damage and ROS production leads to elevated JNK activation, and in turn to augmented non-canonical NLRP3 inflammasome activation. During C. rodentium infection in vivo, this impaired initial bacterial colonization of the colon and provided protection of the epithelium up to days 10 after infection. However, in other colitis models, increased inflammasome activation may result in increased damage to the colon tissue if left unchecked. The NOD2-RIP2 pathway has been associated with Groh’s disease in humans [57] and plays an essential role in several mouse models of colitis [58–62]. As Rip2\(^{-/-}\) deficiency leads to enhanced inflammasome activation, it will be of interest to
examine caspase-1 activation in other models where inflammation is a key factor and where the NOD2-RIP2 axis is involved.

Materials and Methods

Ethics statement

Experiments were conducted under protocols approved by the St. Jude Children’s Research Hospital Committee on Use and Care of Animals (Protocol #482) and were performed in accordance with institutional policies, AAALAC guidelines, the AVMA Guidelines on Euthanasia (CO2 asphyxiation following by cervical dislocation), NIH regulations (Guide for the Care and Use of Laboratory Animals), and the United States Animal Welfare Act (1966).

Mice

All mice were maintained at SJCRH and were backcrossed for at least 10 generations onto the C57BL/6J (B6) background. Nod2+/−, Rip2+/−, and Rip2+/−×Il18−/− mice have been reported previously [39,63,64]. WT-GFP-LC3+ and Rip2+/−×GFP-LC3+ mice were generated by crossing WT or Rip2+/− mice with LysM-Cre+ AlgsflpFlpF+ GFP-LC3+ mice [39] and selecting progeny that only contained the GFP-LC3 transgene but not LysM-Cre or AlgsflpFlpF. All mice were housed in the SJCRH animal resource center, which is a specific pathogen free (SPF) and AAALAC accredited facility.

Bacterial infection, caspase-1 activation and IL-18 production

WT, Nod2−/− and Rip2−/− bone marrow derived macrophages (BMDM) were differentiated in complete IMDM containing 5% CO2 for 5 days. Citrobacter rodentium (ATCC 51459), Salmonella typhimurium (SL1344), Salmonella typhimurium Δfljib/fljib were grown in LB broth overnight with shaking at 37°C. Next day, the bacteria were subcultured to mid-log phase, containing 10% heat-inactivated FBS and supplemented with M-CSF (200 μg/mL, Sigma) or glyburide (200 μM, Sigma) treatment, BMDMs were infected with 20MOI of C. rodentium for 2 h and Rip−/− cells were subsequently treated with NAC or mock treated as a control. Samples were collected at 18 h and caspase-11 activation was examined as in panels A–B. (E) Densitometry was performed on pro-Caspase-11 p43 band from 3 independent experiments. (F–G) WT and Rip2−/− BMDM were infected with 20MOI of C. rodentium for 4 h and cells were subsequently treated with 1 μM MG-132 or mock treated as a control. Samples were collected at the indicated time points and pro-caspase-11 levels examined by Western blot and densitometry. (H) WT and Rip2−/− BMDM were infected with 20MOI of C. rodentium and RNA collected at the indicated time points and analyzed by qRT-PCR for fold induction and normalized to GAPDH. (A-H) Data are representative of three independent experiments with n = 2–3 wells per experiment and displayed as the mean ± SEM. (**, p<0.01; ***, p<0.001).

doi:10.1371/journal.ppat.1004410.g004

Figure 4. RIP2 regulates the caspase-11 non-canonical inflammasome. (A–B) WT and Rip2−/− BMDM were infected with 20MOI of C. rodentium for 18 h and combined supernatant and lysates were examined by Western blot for caspase-11 cleavage (casp-11p30) visually (A) and by densitometry (B). (C–D) WT and Rip2−/− BMDM were infected with 20MOI of C. rodentium for 2 h and Rip−/− cells were subsequently treated with NAC or mock treated as a control. Samples were collected at 18 h and caspase-11 activation was examined as in panels A–B. (E) Densitometry was performed on pro-Caspase-11 p43 band from 3 independent experiments. (F–G) WT and Rip2−/− BMDM were infected with 20MOI of C. rodentium for 4 h and cells were subsequently treated with 1 μM MG-132 or mock treated as a control. Samples were collected at the indicated time points and pro-caspase-11 levels examined by Western blot and densitometry. (H) WT and Rip2−/− BMDM were infected with 20MOI of C. rodentium and RNA collected at the indicated time points and analyzed by qRT-PCR for fold induction and normalized to GAPDH. (A–H) Data are representative of three independent experiments with n = 2–3 wells per experiment and displayed as the mean ± SEM. (**, p<0.01; ***, p<0.001).
Figure 5. ROS enhances caspase-11 expression. WT BMDM were infected with 20MOI of C. rodentium for 6 h and mock treated or treated with 50 μM of H₂O₂ as a ROS source. (A) BMDM were collected at 8 h or 18 h after initial infection and examined for Il18 mRNA expression by qRT-PCR. (B) Supernatants were collected 18 h post-infection and IL-18 levels determined by ELISA. (C) WT BMDMs were examined by Western blot for caspase-1 activation. (D–F) Caspase-11 expression was examined at the (D) mRNA level by qRT-PCR or the (E–F) protein level by Western blot and densitometric analysis. (A–F) Data are representative of three independent experiments with n = 2–4 wells per experiment and displayed as the mean ± SD. (*, p<0.05; ***, p<0.001).

doi:10.1371/journal.ppat.1004410.g005
Figure 6. RIP2 regulates caspases-11 expression through a ROS-JNK pathway. (A) WT and Rip2−/− BMDM were infected with 20MOI of C. rodentium for 18 h and supernatants collected and examined for type-I interferon using a reporter cell line (U = Units). (B–C) WT or Rip2−/− BMDM were infected with 20MOI of C. rodentium and mock treated or treated with H2O2. Samples were collected at the indicated times and examined for total JNK or phosphorylated JNK by Western blot. (D) WT or Rip2−/− BMDM were infected with 20MOI of C. rodentium and mock treated or treated with the JNK inhibitor SP600125 (JNKi). Samples were collected at the indicated times and examined for total JNK, phosphorylated JNK and caspase-11 by western blot. (E) Proposed signaling pathway that regulates inflammasome activation. (A) Data are combined from 9 independent experiments and displayed as the mean ± SEM. (B–D) Data are representative of 3 independent experiments. (n.s. = not significant).

doi:10.1371/journal.ppat.1004410.g006
Cells were then examined by fluorescence confocal microscopy for the number of GFP-LC3\(^+\) puncta after staining with Alexafluor 647 phalloidin and mounting with ProLong Gold+DAPI (Life Technologies). Alternatively, cells were scraped from wells and resuspended in FACS buffer and examined by flow cytometry for GFP intensity as a readout of autophagy.

**Citrobacter rodentium** induced colitis

Age and sex matched 6–10 week old mice were infected with 10\(^{10}\) CFU of *C. rodentium* by oral gavage. Stool was collected from mice for CFU at the indicated time points. Mice were euthanized on D10 and colons collected for CFU determination by homogenizing in PBS or for Western blot by homogenizing in RIPA buffer containing protease inhibitors and phosphatase inhibitors (Calbiochem). Protein concentration was normalized by homogenizing in PBS or for Western blot by homogenizing in RIPA buffer containing protease inhibitors and phosphatase inhibitors (Calbiochem). Protein concentration was normalized and samples analyzed by western blot; anti-IL-18 (MBL, 39-3F) anti-caspase-1 p10 (Santa Cruz Biotechnology, A20) and anti-caspase-11 antibodies (Enzo, 4E11) were used. Lysates were additionally examined by ELISA for IL-18 (Ebiosciences) and normalized to total protein. D10 colon samples were also collected, formalin fixed and processed for routine histopathological examination by hematoxylin and eosin staining. Sections were examined by a board certified veterinary pathologist (Peter Vogel) for inflammation, bacterial invasion of epithelial surface, and distribution of inflammation.

**Statistics**

All statistical analyses were performed using GraphPad Prism 6.0. Students \(t\)-Test was used for single comparisons and One-Way ANOVA with Dunnett’s or Sidák’s post-hoc test for multiple comparisons. Two-way ANOVA was used to analyze *C. rodentium* growth between groups over time.

**Acknowledgments**

We thank Prajwal Gurung, John Lukens, and Si Ming Man for helpful review of the manuscript. We also thank Richard Flavell (Yale University) for the generous supply of mutant mice.

**Author Contributions**

Conceived and designed the experiments: CRL PKA ZL TDK. Performed the experiments: CRL PKA ZL KLS PV. Analyzed the data: CRL PKA ZL. PV ML TDK. Contributed reagents/materials/analysis tools: TDK. Contributed to the writing of the manuscript: CRL PKA ML TDK.

**References**

1. Kaper JB, Nataro JP, Mobley HL (2004) Pathogenic Escherichia coli. Nat Rev Microbiol 2: 123–130.
2. Borenshtein D, McBee ME, Schauer DB (2008) Utility of the Citrobacter rodentium infection model in laboratory mice. Curr Opin Gastroenterol 24: 32–37.
3. Mundy R, MacDonald TT, Dougan G, Frankel G, Wiles S (2005) Citrobacter rodentium of mice and man. Cell Microbiol 7: 1697–1706.
4. Kamada N, Kim YG, Shaan HP, Vallenance BA, Furuta JL, et al. (2012) Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. Science 336: 1325–1329.
5. Simmons CP, Clare S, Ghaem-Maghami M, Uren TK, Rankin J, et al. (2003) Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri. EMBO Rep 2: 736–742.
6. Pay SE, Tournebize R, Mavris M, Page AL, Li X, et al. (2001) CARD4/CED-4/Apaf-1 cell death family member that activates NF-kappaB. J Biol Chem 276: 12955–12958.
7. Pay SE, Tournebize R, Mavris M, Page AL, Li X, et al. (2001) CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. J Biol Chem 276: 12955–12958.
Pilla DM, Hagar JA, Haldar AK, Mason AK, Degrandi D, et al. (2014) Caspase-11 protects against bacteria that escape the vacuole. Science 339: 975–978.

55. Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, et al. (2005) Development of a peptidoglycan-polysaccharide murine model of Crohn’s disease: effect of genetic background. Inflamm Bowel Dis 11: 1238–1244.

56. Koide M, Tomiyama E, Muraki K, Kobayashi S, Tomita Y, et al. (2010) Deficiency of caspase-11, but not caspase-1 or caspase-12, is protective against experimental sepsis. J Immunol 184: 3428–3434.

57. Kowalczyk J, Day R, Goldwasser J, Hagan JA, Hagar JA, et al. (2009) Caspase-11 agonists identified using an in vitro high-throughput screening assay. J Immunol 182: 5728–5738.

58. Kowalczyk J, Day R, Goldwasser J, Hagan JA, Hagar JA, et al. (2009) Caspase-11 agonists identified using an in vitro high-throughput screening assay. J Immunol 182: 5728–5738.

59. Kowalczyk J, Day R, Goldwasser J, Hagan JA, Hagar JA, et al. (2009) Caspase-11 agonists identified using an in vitro high-throughput screening assay. J Immunol 182: 5728–5738.

60. Kowalczyk J, Day R, Goldwasser J, Hagan JA, Hagar JA, et al. (2009) Caspase-11 agonists identified using an in vitro high-throughput screening assay. J Immunol 182: 5728–5738.

61. Kowalczyk J, Day R, Goldwasser J, Hagan JA, Hagar JA, et al. (2009) Caspase-11 agonists identified using an in vitro high-throughput screening assay. J Immunol 182: 5728–5738.

62. Kowalczyk J, Day R, Goldwasser J, Hagan JA, Hagar JA, et al. (2009) Caspase-11 agonists identified using an in vitro high-throughput screening assay. J Immunol 182: 5728–5738.

63. Kowalczyk J, Day R, Goldwasser J, Hagan JA, Hagar JA, et al. (2009) Caspase-11 agonists identified using an in vitro high-throughput screening assay. J Immunol 182: 5728–5738.

64. Kowalczyk J, Day R, Goldwasser J, Hagan JA, Hagar JA, et al. (2009) Caspase-11 agonists identified using an in vitro high-throughput screening assay. J Immunol 182: 5728–5738.

ROS Regulates Casp-11 Pathway