RESEARCH ARTICLE

Receptor interacting protein-2 contributes to host defense against *Anaplasma phagocytophilum* infection

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Received 4 April 2012; revised 18 June 2012; accepted 18 June 2012.
Final version published online 23 July 2012.

DOI: 10.1111/j.1574-695X.2012.01001.x

Editor: Peter Timms

Keywords
Rip2; *Anaplasma phagocytophilum*; NLR; IFNγ.

Abstract

The Gram-negative obligate intracellular bacterium *Anaplasma phagocytophilum* is the causative agent of human granulocytic anaplasmosis (HGA), an emerging tick-borne infectious disease occurring worldwide. HGA is generally self-limiting; however, the underlying mechanisms, particularly the innate immune pathways that mediate the immune clearance of *A. phagocytophilum*, are less understood. We herein report an unexpected role for Receptor interacting protein-2 (Rip2), the adaptor protein for the Nod-like receptors (NLRs), Nod1/Nod2, in the host immune response against *A. phagocytophilum* infection. Although *A. phagocytophilum* genome is reported to lack the genes encoding the known ligands of Nod1 and Nod2, its infection upregulated the transcription of Rip2 in human primary neutrophils. Our results revealed that Rip2-deficient mice had significantly higher bacterial load than wild-type controls throughout the infection period. In addition, the Rip2-deficient mice took strikingly longer duration to clear *A. phagocytophilum* infection. Detailed analysis identified that interferon gamma (IFNγ) and interleukin (IL)-18 but not IL-12, macrophage inflammatory protein-2, and KC response were diminished in *A. phagocytophilum*-challenged Rip2-deficient mice. Together, these results revealed that Rip2 plays important roles in the immune control of *A. phagocytophilum* and may contribute to our understanding of the host response to Rickettsiales.

Introduction

The tick-borne obligate intracellular Gram-negative bacterium *Anaplasma phagocytophilum* primarily colonizes within human neutrophils and causes the illness human granulocytic anaplasmosis (Carlyon & Fikrig, 2003). The infected individuals may develop a range of symptoms from mild to severe and include fever, malaise, headache, septic shock-like syndromes, hemorrhage, coagulopathy, respiratory distress syndrome, acute abdominal syndromes, acute renal failure, heart failure and myocarditis, opportunistic infections, and neuropathies (Bakken & Dumler, 2000). Thrombocytopenia and leucopenia are common in *A. phagocytophilum*-infected individuals. The infection can become fatal in aged- and immune-compromised subjects.

Although *A. phagocytophilum* is among the most prevalent tick-borne pathogens in the USA (von Loewenich

et al., 2004), how this bacterium interacts with mammalian immune system is still unclear. Generally, the *A. phagocytophilum* infection is self-limiting in immunocompetent subjects (Dumler *et al.*, 2005). The laboratory model for *A. phagocytophilum* infection is mouse, which often clears infection within 10–12 days (Sun *et al.*, 1997; Hodzic *et al.*, 1998; Borjesson & Barthold, 2002). Both T and B cells have been shown to play important roles in the control and clearance of *A. phagocytophilum* (Telford *et al.*, 1996; Bunnell *et al.*, 1999; von Loewenich *et al.*, 2004). A key role for $CD4^+$ T cells and T-helper 1 (Th1) response is widely established in the immune response to the infection of *A. phagocytophilum* (Pedra *et al.*, 2007a, b; Birkner *et al.*, 2008). It has been shown that interferon gamma (IFNγ), IL-12, and IL-18 play important roles in the early clearance of *A. phagocytophilum* (Akkoyunlu & Fikrig, 2000; Pedra *et al.*, 2007a, b). Immune effector components such as iNOS and phagocyte NADPH oxidase were dispensable for the *in vivo* clearance of *A. phagocytophilum* infection (Banerjee *et al.*, 2000). Although some of the critical players of adaptive response required for controlling *A. phagocytophilum* are known, our understanding of the innate immune components involved in the recognition and clearance of this bacterium is rather incomplete. Interestingly, prominent anti-bacterial innate immune detection system tool-like receptor (TLR)2, TLR4, and their adaptor MyD88 are shown to play no role in the immune response to *A. phagocytophilum* infection (von Loewenich *et al.*, 2004). A recent study demonstrated that signaling through the Nod-Like Receptor (NLR) family member NLRC4 (also known as IPAF/ICE Protease-Activating Factor), its adaptor ASC, and Caspase-1 is critical for the control of *A. phagocytophilum* infection during the early phase of infection (Pedra *et al.*, 2007a, b). However, mice deficient in NLRC4, ASC, and Caspase-1 were still able to clear *A. phagocytophilum* infection by day 6, indicating there are additional pathogen detection and clearance systems involved in the innate and adaptive immune response to *A. phagocytophilum* infection. Thus, the mechanisms involved in the immune detection and clearance of *A. phagocytophilum* remain unresolved and warrant more studies.

We previously reported that *A. phagocytophilum* infection upregulates Rip2 (Sukumaran *et al.*, 2005), the adaptor molecule of the cytoplasmic pattern recognition receptor (PRR) Nod1 and 2, (Magalhaes *et al.*, 2011) in immune cells. Nod1 and 2 belong to NLR family of proteins and are known to detect bacterial membrane-derived peptidoglycan components iE-DAP and muramyl dipeptide (MDP), respectively (Inohara & Nunez, 2003; Franchi *et al.*, 2009). Following peptidoglycan detection, Nod1/Nod2 recruit and associate with the adaptor protein Rip2, triggering proinflammatory signaling pathways via NF-κB and the mitogen-activated protein kinases p38, JNK, and ERK (Kobayashi *et al.*, 2002). Activation of Nod1 and Nod2 also triggers proinflammatory responses, leading to the induction of cytokine and chemokines. Interestingly *A. phagocytophilum* genome does not encode genes for the synthesis of iE-DAP and MDP (Dunning Hotopp *et al.*, 2006). Therefore, it is intriguing that Rip2 is activated during *A. phagocytophilum* infection. We hypothesized that Rip2 may play a role in the immune response to *A. phagocytophilum*, through a potentially unknown mechanism. In this study, we investigated whether Rip2 plays any role in *A. phagocytophilum* infection clearance and demonstrate that Rip2 plays an unexpected role in controlling the *A. phagocytophilum* infection.

Materials and methods

Anaplasma phagocytophilum propagation

The *A. phagocytophilum* HZ strain (Prof. Yasuko Rikihisa, Ohio State University) was used in this study (Sukumaran *et al.*, 2005). The *A. phagocytophilum* was propagated in the promyelocytic cell line (HL-60; ATCC, 240 CCL; Sukumaran *et al.*, 2005). A previously reported method was used to propagate and purify *A. phagocytophilum* (Carlyon *et al.*, 2004). Briefly, equal volume of *A. phagocytophilum*-infected and *phagocytophilum*-uninfected HL-60 cells were mixed and diluted 1/5 with RPMI 1640 medium with 10% fetal bovine serum. For the purification of cell-free *A. phagocytophilum,* maximally infected cells were pelleted by centrifugation for 10 min at 400 ***g***, lysed first by six passage through a 25-gauge needle, followed by another six passages through a 27-gauge needle. After lysis, the cell debris was removed by centrifugation at 300 ***g*** for 5 min, and the supernatant containing host cell free bacterium was used for the *in vitro* (human neutrophils) and *ex vivo* (mouse splenocytes) infection studies.

Infection of neutrophils with *A. phagocytophilum in vitro*

The infection of neutrophils was made as reported earlier (Sukumaran *et al.*, 2005). Briefly, suspensions of freshly prepared *A. phagocytophilum* (isolated from 5×10^6 heavily infected HL-60 cells) were added to neutrophils (10^6 neutrophils mL^{-1}) maintained in the ultra-low attachment six-well plates (Corning Inc., NY) and incubated at 37 °C in 5% CO_2 for 4 h. The percentage of infected neutrophils was confirmed by immunofluorescence microscopy, and the number of *A. phagocytophilum* isolates per neutrophil was calculated as previously described (Carlyon *et al.*, 2004). The ratio of bacteria to neutrophils was ~5 : 1. At various time points post-infection, the cells

were recovered by centrifugation at 210 ***g***, RNA was isolated using RNAeasy kit (Qiagen) and cDNA was made using iSCRIPT kit (Biorad). The Rip2 levels were determined by SYBR Green dye-based quantitative real-time PCR (qRT-PCR), with primers detecting Rip2 (GenBank accession AF064824), and normalized with human β actin gene. Rip2 was amplified using the following primers: Forward: 5′-CCATCCCGTACCACAAGCTC-3′ and Reverse: 5′-GCAGGATGCGGAATCTCAAT-3′. The primers used for amplifying human β actin gene were as follows: Forward: 5′-TGATATCGCCGCGCTCGTCGTC-3′ and Reverse: 5′-GCCGATCCACACGGAGTACT-3′.

Infection of mice and quantification of *A. phagocytophilum* in the peripheral blood

Wild-type C57BL/6, mice were purchased from Jackson Laboratory (Bar Harbor, ME). Rip2 null mice (backcrossed to C57BL/6 for at least 10 generations) have been previously described (Kobayashi *et al.*, 2002, 2005). For the *in vivo* infection, 4–12 weeks age, sex matched, and mice maintained under specific pathogen-free conditions (such as maintenance in barrier-filtered cages, given standard laboratory diet, and sterile water *ad libitum* throughout the study) were used. The experiments were carried out according to the guidelines of the Yale University institutional animal care and research committee.

For the *in vivo* infection studies, 100 μL of blood from *A. phagocytophilum* HZ strain-infected *rag1*-null mice (25% neutrophil infection) was used as the inoculum to infect immunocompetent C57BL/6 or Rip2$^{-/-}$ mice, by intra peritoneal injection. To determine the infection level in *rag1*-null mice, Giemsa staining of the blood cells was performed, followed by quantification of the percentage of granulocytes containing morulae (*A. phagocytophilum* aggregates; Borjesson & Barthold, 2002).

For the quantification of the *A. phagocytophilum* level in the peripheral blood, 100 uL of peripheral blood from mice was incubated twice with 900 μL of erythrocyte lysis buffer (Sigma-Aldrich) at room temperature for 20 min, followed by DNA was extraction using DNeasy Tissue Kit (Qiagen). The bacterial levels were determined by SYBR Green dye-based qRT-PCR, with primers detecting *A. phagocytophilum* 16S rRNA gene (GenBank accession M73224), and normalized with mouse actin gene (GenBank accession X03672) as described before (Pedra *et al.*, 2007a, b). Mouse actin was amplified using the following primers: Forward: 5′-AGAGGGAAATCGTGCGTGAC-3′ and Reverse: 5′-CAATAGTGATGACCTGGCCGT-3′. The primers used to amplify the 16S rRNA gene of *A. phagocytophilum* were as follows: Forward: 5′-CCATTTCTAG TGGCTATCCCATACTAC-3′ and Reverse: 5′-TCGAAC GGATTATTCTTTATAGCTTG-3′.

Measurement of cytokines

The protein standards and antibody pairs for IL-12p40/p70, IL-2, macrophage inflammatory protein-2 (MIP-2), KC, and IFNγ were purchased from BD Pharmingen, and IL-18 from MBL. Retro-orbital bleeding was performed at the desired time points from wild-type and Rip2$^{-/-}$ mice, and the ELISA experiments for cytokine measurement were carried out as described previously (Sukumaran *et al.*, 2005).

Anaplasma phagocytophilum restimulation assays

For the *ex vivo* splenocyte restimulation assays, the spleens were first removed from euthanized mice 6 days post-infection with *A. phagocytophilum*. The splenocytes (10^6 cells) were prepared following RBC lysis and restimulated with live purified *A. phagocytophilum* (10^8 bacteria) *in vitro* for 18 h, and the culture supernatant was used to perform ELISA for cytokine measurements. *Anaplasma phagocytophilum* was propagated and purified as described in the Materials and methods section. Uninfected spleen cells were used as negative controls.

Statistical analysis

We performed statistical analysis using unpaired Student's *t*-test. *Anaplasma phagocytophilum* genome copy numbers present in the peripheral blood of wild-type and gene-deficient mice were compared, and values of $P \leq 0.05$ were considered statistically significant.

Results

Anaplasma phagocytophilum infection upregulates Rip2 expression

In this study, we first investigated the potential induction of Rip2 by *A. phagocytophilum* in human primary neutrophils. For this, we infected human primary neutrophils with *A. phagocytophilum* and quantified the levels of Rip2 using quantitative real-time PCR (qRT-PCR). As shown in Fig. 1, there was a fourfold increase ($P < 0.05$) in Rip2 transcripts at 4-h post-infection with *A. phagocytophilum*. This observation was consistent with our earlier study. We further determined the time course of the transcription of Rip2. It was observed that, as shown in Fig. 1, up-regulation of Rip2 transcription starts as early as 2-h post-infection and continued to increase over a period of 4 h, and reaches a plateau thereafter. In conclusion, *A. phagocytophilum* infection results in the up-regulation of transcription of Rip2.

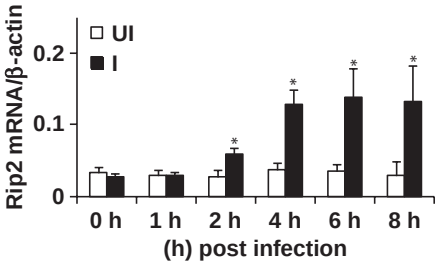

Fig. 1. Rip2 transcription is induced during *Anaplasma phagocytophilum* infection. RNAs from 1, 2, 4, 6, and 8 h infected (I) and uninfected (UI) neutrophils were used to perform qRT-PCR. The results are expressed as number of copies of Rip2 mRNA/human β-actin. The results are expressed as mean ± SD from three independent experiments. The statistical significance was calculated using Student's *t*-test and $*P < 0.05$.

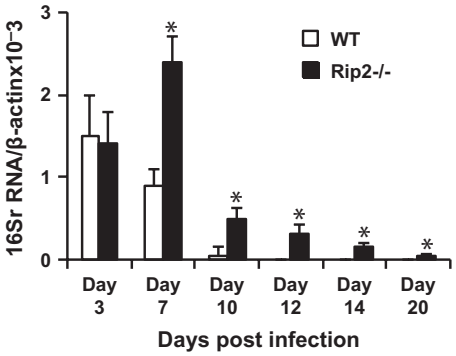

Fig. 2. Rip2 deficiency caused increased load and delayed clearance of *Anaplasma phagocytophilum* in peripheral blood of mice. Rip2$^{-/-}$ and WT ($n = 15$) mice were infected with *A. phagocytophilum*, and the bacterial load was determined in the peripheral blood (by measuring the levels of bacterial 16S rRNA gene normalized to mouse β actin) at the indicated time points using qRT-PCR analysis as detailed in methods section. The experiment was repeated three times, and the result from a representative experiment is shown. The results are expressed as median ± SD. The statistical significance was calculated using Student's *t*-test and $*P < 0.05$.

Rip2 is important for the immune control of *A. phagocytophilum* infection *in vivo*

As we observed an increase in the transcription of Rip2 in *A. phagocytophilum*-infected cells, and Rip2 has a known role in controlling intracellular bacterial infections, we addressed the important question whether Rip2 has any functional role in the immune defense and clearance of *A. phagocytophilum* infection *in vivo*. For this, we infected Rip2$^{-/-}$mice with *A. phagocytophilum* intra-peritoneally, and monitored bacterial load and infection clearance kinetics in the blood using qRT-PCR detecting *A. phagocytophilum* 16S rRNA gene. Previous studies have shown that in the C57BL/6 murine model of *A. phagocytophilum* infection, bacterial load increases from day 2 onwards, reaching peak at 3–4 days, followed by infection decline and clearance by 10–12 days (von Loewenich *et al.*, 2004). Strikingly, genetic deletion of Rip2 noticeably altered *A. phagocytophilum* infection pattern in the mouse model. As shown in Fig. 2, Rip2$^{-/-}$ mice showed an increased *A. phagocytophilum* burden, compared with gene sufficient controls. In addition, there was a prominent delay in the kinetics of clearance of *A. phagocytophilum* in the blood of Rip2$^{-/-}$ animals, compared with wild-type controls (Fig. 2). In wild-type C57BL/6 mice, *A. phagocytophilum* burden peaked at day 3 and then rapidly declined and there was complete clearance by day 12. In contrast, even though there was comparable bacterial load in Rip2$^{-/-}$ and in wild-type mice at day 3, the *A. phagocytophilum* load was consistently higher in Rip2$^{-/-}$ mice than in wild-type controls during the entire course of infection following day 3. Contrast to that in wild type, in Rip2$^{-/-}$ mice, the *A. phagocytophilum* burden progressively increased with a peak bacterial load reaching at day 7 post-infection, with eightfold ($P < 0.05$) more bacterial load than in wild-type mice. While wild-type animals cleared infection completely by 10–12 days, Rip2$^{-/-}$ mice had a very prolonged infection, and the bacteria could be detected even at day 20.

In conclusion, Rip2$^{-/-}$ mice showed a significantly higher bacterial load at all time points following day 3 and a prominent delay of infection clearance extending up to day 20. These data identify an unexpected but important role for Rip2 in the immune clearance of *A. phagocytophilum* infection.

Rip2 is dispensable for the MIP-2 and KC production in response to *A. phagocytophilum* infection

We next sought to find out the impaired immune and inflammatory mechanisms that resulted in the increased susceptibility of Rip2$^{-/-}$ mice to *A. phagocytophilum* infection. For this, we first investigated whether Rip2 plays any role in the signature inflammatory host response typically manifested during *A. phagocytophilum* infection, by analyzing the levels of major chemokines and cytokines associated with *A. phagocytophilum* infection (Akkoyunlu & Fikrig, 2000; Akkoyunlu *et al.*, 2001; Pedra *et al.*, 2007a, b) in the knock-out mouse model. One of the key products of human and mouse neutrophils are chemokines. Therefore, we first determined whether Rip2 contributes to the IL-8 chemokine response during *A. phagocytophilum* infection by measuring the levels of murine IL-8 homologues MIP-2 and KC (Scorpio *et al.*, 2004). We infected Rip2$^{-/-}$ mice with *A. phagocytophilum* intra-peritoneally and measured the levels of MIP-2 and

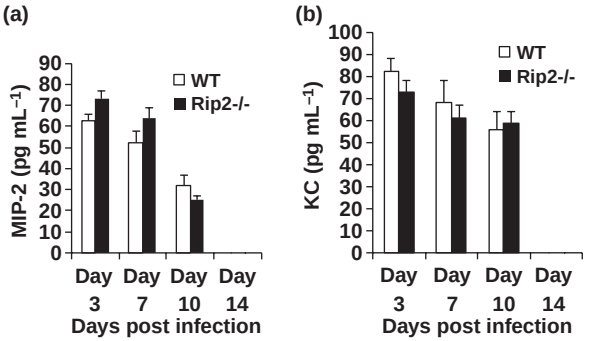

Fig. 3. Rip2 is not involved in the MIP-2 and KC response to *Anaplasma phagocytophilum* infection. Sera from wild-type ($n = 5$) or Rip2$^{-/-}$ ($n = 5$) mice were harvested at 3, 7, 10, and 14 days post-infection, pooled, and the levels of MIP-2 (a), and KC (b) were measured using ELISA. The results represent mean ± SD of samples from three independent experiments. The statistical significance was calculated using Student's *t*-test.

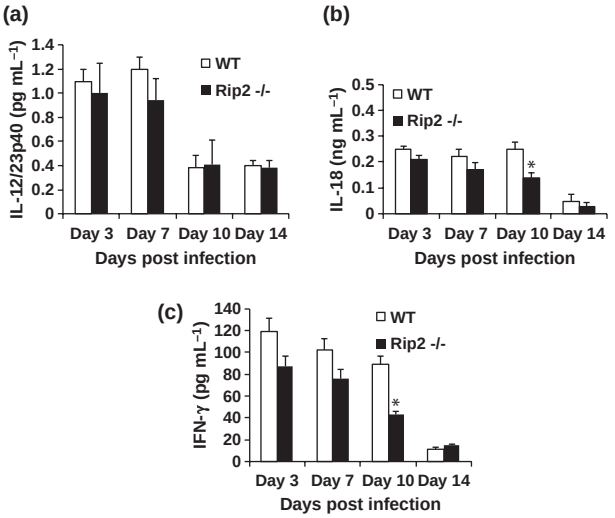

Fig. 4. Rip2 deficiency resulted in altered immune response to *Anaplasma phagocytophilum* infection. Sera from wild-type ($n = 6$) or Rip2$^{-/-}$ ($n = 6$) mice were harvested at 3, 7,10, and 14 days post-infection, pooled, and the levels of IL-12 (a), IL-18 (b), and IFNγ (c) were measured using ELISA. The results are expressed as mean ± SD of samples from three independent experiments. The statistical significance was calculated using Student's *t*-test and $*P < 0.05$.

KC, in the mice sera. The results showed that both MIP-2 and KC levels were unaltered in *A. phagocytophilum*-infected Rip2$^{-/-}$ mice (Fig. 3a and b) compared with wild-type controls at all time points tested. These results indicate that Rip2 is not involved in the chemokine response during *A. phagocytophilum* challenge.

Rip2 is required for IL-18 but not IL-12 production during *A. phagocytophilum* challenge

Earlier studies have shown that the pro-inflammatory cytokines such as IL-12 and IL-18 were important components in the immunity against *A. phagocytophilum* infection by inducing optimal production of IFN-γ and Th1 immune responses (Pedra *et al.*, 2007a, b). To determine whether Rip2 plays any role in the cytokine response, we studied the serum levels of major NF-κB-dependent cytokine IL-12, and inflammasome-dependent cytokine IL-18, in Rip2-deficient mice following *A. phagocytophilum* challenge. As shown in Fig. 4a, there was no significant difference in the levels of IL-12 in Rip2$^{-/-}$ animals. However, *A. phagocytophilum*-infected Rip2$^{-/-}$ mice showed a modest but significant reduction (1.78-fold, $P < 0.05$) in IL-18 levels at day 10 (Fig. 4b). This evidence indicates that Rip2 plays a differential role in the cytokine response to *A. phagocytophilum* challenge.

Rip2 deficiency impairs IFNγ production during *A. phagocytophilum* infection

Earlier studies have shown that mice deficient in either the production or signaling of IFNγ had a prominent delay in the immune clearance of *A. phagocytophilum* (Akkoyunlu & Fikrig, 2000; Dumler *et al.*, 2000; Lepidi *et al.*, 2000; Martin *et al.*, 2000; Wang *et al.*, 2004; Pedra *et al.*, 2007a, b, 2008; Birkner *et al.*, 2008). Moreover, the level of IL-18 (also known as interferon-gamma inducing factor) was reduced in the serum of Rip2-deficient mice during *A. phagocytophilum* infection. Therefore, we next analyzed whether Rip2 impacts on the IFNγ production during *A. phagocytophilum* infection. For this, Rip2$^{-/-}$ animals were infected with *A. phagocytophilum*, and the IFNγ response was measured. As shown in Fig. 4c, Rip2$^{-/-}$ mice displayed significantly reduced IFNγ levels in the sera at day 10 post-infection. The IFNγ levels in Rip2$^{-/-}$ animals were 2.1-fold ($P < 0.05$) less than that in the infected wild-type mice, indicating a potential role for Rip2 in the IFNγ response to *A. phagocytophilum*.

To further confirm the role of Rip2$^{-/-}$ in the cytokine response to *A. phagocytophilum* infection, we performed *ex vivo* experiments. As Th1-type immune response is important in the immune response to *A. phagocytophilum* infection, we isolated murine splenocytes from infected Rip2-deficient mice, re-stimulated with *A. phagocytophilum ex vivo* and measured the levels of IFNγ and also IL-2. The results of these experiments identified a reduction in the production of both IFNγ (Fig. 5a) and IL-2 (Fig. 5b) by the splenocytes of Rip2$^{-/-}$ mice in response to *A. phagocytophilum* infection. When splenocytes from *A. phagocytophilum*-infected wild-type and Rip2$^{-/-}$ mice were restimulated, the IFNγ response in Rip2$^{-/-}$ splenocytes was

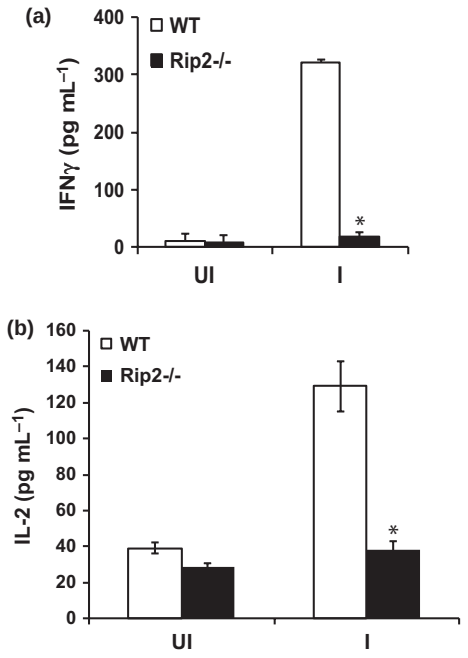

Fig. 5. Splenocytes from *Anaplasma phagocytophilum*-infected Rip2$^{-/-}$ mice have reduced IFNγ and IL-2 secretion during restimulation with *A. phagocytophilum*. Splenocytes collected from wild-type ($n = 5$) or Rip2$^{-/-}$ ($n = 5$) mice were stimulated with live *A. phagocytophilum* for 18 h, and IFNγ (a) and IL-2 (b) levels were measured using ELISA. The results are expressed as mean ± SD of five mice from each group. The experiment was repeated twice with similar results. The statistical significance was calculated using Student's *t*-test and $*P < 0.05$. UI, uninfected samples and I, *A. phagocytophilum*-infected samples.

16.8-fold less than ($P < 0.05$) that in wild-type animals. We also observed a 3.4-fold reduction ($P < 0.05$) in the IL-2 response in Rip2$^{-/-}$ splenocytes when restimulated with *A. phagocytophilum*. These results collectively identify the notable defect in Th1-like response, in Rip2-deficient mice in response to *A. phagocytophilum* infection, indicating that Rip2 likely acts as a contributor to the immune response against this bacterium.

Discussion

Although mammals often successfully clear the infection by *A. phagocytophilum*, the underlying innate immune mechanisms, particularly the PRRs and innate immune pathways that initiate its clearance, are still a mystery. A previously published gene microarray-based study from our group analyzing transcriptional response of human neutrophils to *A. phagocytophilum* indicated that the transcription of Rip2 is upregulated during infection (Sukumaran *et al.*, 2005). Following up on this earlier observation, in this study, we identified that Rip2, the key adaptor molecule mediating signaling from the cytoplasmic PRRs Nod1/2, plays an important role in the control of *A. phagocytophilum* infection.

Rip2 has been previously shown to play an essential role in the immunity against various intracellular pathogens including *Listeria monocytogenes* (Chin *et al.*, 2002), *Legionella pneumophila* (Archer *et al.*, 2010), *Chlamydophila pneumonia* (Shimada *et al.*, 2009), *Mycobacteirum tuberculosis* (Shimada *et al.*, 2009), and extracellular pathogens such as *Escherichia coli* (Balamayooran *et al.*, 2011).

Compared with wild-type mice, Rip2$^{-/-}$ mice were significantly more susceptible to *A. phagocytophilum* infection and had a markedly higher bacterial burden at all time points following day 3 post-infection. The deficiency of Rip2 also resulted in delayed clearance of *A. phagocytophilum* from the blood in the mouse model. While wild-type mice took 10–12 days to clear *A. phagocytophilum* infection, Rip2$^{-/-}$ mice took ~20 days to clear infection. Over all the role of Rip2 became prominent during the early mid-phase of the immune response to *A. phagocytophilum* infection. The fact that the Rip2 knockout mice were still able to clear the infection suggests that there may be other un-identified pathways that may be important in the clearance of *A. phagocytophilum*.

A major inflammatory chemokine that is heavily induced during *A. phagocytophilum* infection is IL-8 in humans (Akkoyunlu *et al.*, 2001) and murine IL-8 homologues (MIP-2 and KC) in mice (Scorpio *et al.*, 2004). These chemokines are suggested to be important in *A. phagocytophilum* pathogenesis by inducing trafficking of neutrophils to the sites of infection via binding to receptors in neutrophils. Signaling through Rip2 can activate NF-κB target genes such as IL-8 (Buchholz & Stephens, 2006, 2008). Furthermore, Rip2 has been previously shown to play a crucial role in neutrophil recruitment *in vivo* (Magalhaes *et al.*, 2011). But interestingly, our study found that the levels of the chemokines (MIP-2 and KC) were un-affected in *A. phagocytophilum*-infected Rip2$^{-/-}$ mice hinting the possibility that these chemokines may not play any role in the immune defect manifested by Rip2$^{-/-}$ mice.

IFNγ, another inflammatory cytokine, has been shown to play a very critical role in early host defense to *A. phagocytophilum* infection (Akkoyunlu & Fikrig, 2000; Dumler *et al.*, 2000; Lepidi *et al.*, 2000; Martin *et al.*, 2000; Wang *et al.*, 2004; Pedra *et al.*, 2007a, b, 2008; Birkner *et al.*, 2008). Our study observed a defect in the IFN-γ and IL-18 levels in Rip2-deficient mice during *A. phagocytophilum* infection. Both *in vivo* and *ex vivo* data showed that the levels of IFNγ, a major inflammatory cytokine, were significantly lower in Rip2$^{-/-}$ mice. These results are consistent with the previous reports that IFN-γ plays a major role in the immune pathology and

early clearance of *A. phagocytophilum* infection (Akkoyunlu & Fikrig, 2000), even though it is dispensable for the complete elimination of the pathogen (Birkner *et al.*, 2008). It is also known that an adaptive CD4+ T-cell-mediated response is critical for the complete clearance of *A. phagocytophilum* infection (Birkner *et al.*, 2008). Whether Rip2 plays any role in the CD4+ T-cell response against *A. phagocytophilum* infection needs to be addressed in the future studies. Also, the identity of the specific cell type in which the IFNγ production is impaired during *A. phagocytophilum* infection of the Rip2$^{-/-}$ mouse remains to be determined. As previous reports have shown the importance of natural killer (NK) cells, NKT cells (Choi *et al.*, 2007) and CD4+T cells (Birkner *et al.*, 2008) in the IFNγ production and host defense to *A. phagocytophilum* infection, we speculate that Rip2 pathway could contribute to the IFNγ production by these cell types. Although IL-12 secretion was previously shown to be dependent on Rip2 (Kobayashi *et al.*, 2002), interestingly, there was no difference in IL-12 in Rip2$^{-/-}$ mouse when challenged with *A. phagocytophilum*. But the levels of the inflammasome-dependent cytokine, IL-18, was less in infected Rip2$^{-/-}$ animals. It is also intriguing that even though the bacterial load in Rip2$^{-/-}$ mice became significantly different from controls on day 7 onwards, the difference in the levels of IFNγ and IL-18 in Rip2$^{-/-}$ mice became more prominent only at day 10 post-infection. This hints to the possibility that Rip2 may also have a role independent of IFNγ and IL-18. Future studies are required to delineate the mechanism.

As Rip2 is not a PRR but mediates the innate immune response by acting as an adaptor for the innate immune receptors Nod1 and Nod2, it will be important to identify the receptor that detects *A. phagocytophilum* and signals through Rip2. The role for Rip2 in the immune response to *A. phagocytophilum* is rather unexpected, given the fact that the genome of *A. phagocytophilum* is reported to lack the genes encoding peptidoglycan, the known ligand for Nod1/2. So it is unlikely that the role of Rip2 in the immune response to *A. phagocytophilum* infection is linked to mechanisms involving peptidoglycan sensing. This leaves us with a major question as to which mechanism *A. phagocytophilum* uses to activate Rip2-mediated immune response. Our speculation would be that (1) a non-peptidoglycan microbial component (e.g. virulence factor) or (2) endogenous danger-associated molecular patterns (DAMPs, e.g. High-mobility group box proteins, heparin sulfate, ATP, DNA etc.; Chen & Nunez, 2010) that are released by damaged host cells during infection could potentially play roles in activating Rip2 during *A. phagocytophilum* invasion. As a precedence for the first hypothesis, a *Salmonella* type III secretion system effector was recently identified to activate Nod/Rip2 signaling pathway (Keestra *et al.*, 2011). *Anaplasma phagocytophilum* is also known to possess a functional type IV secretion system (Rikihisa *et al.*, 2010). Previous studies also reported that DAMPs can initiate signaling through innate immune receptors such as TLRs (Yanai *et al.*, 2009) and NLRs (Petrilli *et al.*, 2007; Benko *et al.*, 2008). It is also possible that Rip2 may interact or act synergistically with other molecules that have been previously associated with *A. phagocytophilum* clearance such as the inflammasome NLR proteins NLRC4, Asc, or Caspase 1, thereby leading to the delayed immune clearance. Supporting this possibility are previous reports showing the interaction of Rip2 pathway with Asc and caspase1 pathway (Sarkar *et al.*, 2006; Pan *et al.*, 2007). In addition, the observation that the production of inflammasome-dependent cytokine IL-18 was less in *A. phagocytophilum*-infected Rip2 knockout mice again hints toward a possibility of cross-talk between Rip2 and inflammasome pathways during *A. phagocytophilum* infection. Further studies are required to delineate the underlying mechanisms.

In conclusion, our studies revealed a previously unanticipated role for Rip2$^{-/-}$ in the immune clearance of *A. phagocytophilum*. This study thus provides novel insights into the mechanisms underlying immune response to the infection by *A. phagocytophilum*.

Acknowledgements

The authors would like to thank Debbie Beck for assistance with mice studies. We also thank Prof. Yasuko Rikihisa for providing *A. phagocytophilum* strain HZ for the studies. This work was supported by grants from the NIH. The authors have no conflict of interest to declare.

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