

RICK/RIP2 Mediates Innate Immune Responses Induced through Nod1 and Nod2 but Not TLRs¹

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RICK is a kinase that has been implicated in Nod1 and Nod2 signaling. In addition, RICK has been proposed to mediate TLR signaling in that its absence confers reduced responses to certain bacterial products such as LPS. We show here that macrophages and mice lacking RICK are defective in their responses to Nod1 and Nod2 agonists but exhibit unimpaired responses to synthetic and highly purified TLR agonists. Furthermore, production of chemokines induced by the bacterial dipeptide γ -D-glutamyl-*meso*-diaminopimelic acid was intact in MyD88 deficient mice but abolished in RICK-null mice. Stimulation of macrophages with muramyl dipeptide, the Nod2 activator, enhanced immune responses induced by LPS, IFN- γ , and heat-killed *Listeria* in wild-type but not in RICK- or Nod2-deficient macrophages. Finally, we show that the absence of RICK or double deficiency of Nod1 and Nod2 was associated with reduced cytokine production in *Listeria*-infected macrophages. These results demonstrate that RICK functions in innate immunity by mediating Nod1 and Nod2 signaling but not TLR-mediated immune responses. *The Journal of Immunology*, 2007, 178: 2380–2386.

Detection of microbes is mediated by the recognition of conserved and unique pathogen structures by specific host pattern-recognition molecules, such as the TLRs and nucleotide-binding oligomerization domain (NOD)³-like receptors (NLRs)³ (1, 2). TLRs mediate bacterial recognition at the cell surface or endosomes and induce host immune responses by inducing the secretion of proinflammatory cytokines and costimulatory surface molecules (1). In contrast, NLRs (also called NODs, NOD-LRR, or Caterpillar) induce innate immune responses through cytosolic recognition of bacterial components (2, 3). Two NLR family members, Nod1 and Nod2, sense bacterial molecules produced during the synthesis and/or degradation of peptidoglycan (PGN) (4–7). Nod1 recognizes PGN-related molecules containing the amino acid *meso*-diaminopimelic acid that are produced by most Gram-negative and specific Gram-positive bacteria (5, 7). In contrast, Nod2 is activated by muramyl dipeptide (MDP), which is a conserved structure in virtually all types of PGN (4, 6). Once acti-

vated, Nod1 and Nod2 induce gene transcription through the NF- κ B transcription factor and the MAPK signaling pathways (4–8).

RICK (also called RIP2 and CARDIAK) is a caspase-recruitment domain (CARD)-containing kinase that has been implicated in Nod1 and Nod2 signaling. Mice deficient in RICK exhibit increased susceptibility to systemic infection with *Listeria monocytogenes* (9, 10), indicating that RICK plays a role in host defense against intracellular bacteria. However, the mechanism by which RICK regulates *Listeria* infection remains poorly understood. Stimulation of Nod1 or Nod2 by their specific bacterial activators causes the recruitment of RICK and the association of RICK with Nod1 or Nod2 via CARD-CARD interactions (11, 12). RICK directly binds to I κ B kinase (IKK) γ and activates the IKK complex by promoting the ubiquitinylation of IKK γ and stimulating the kinase activity of the two other components of the IKK complex, IKK α and IKK β (11, 13). Transfection of plasmids expressing Nod1 or Nod2 do not activate NF- κ B in mouse embryo fibroblasts derived from mice deficient in RICK, suggesting that RICK is critical for signaling via Nod1 and Nod2 (10). However, the role of RICK in mediating Nod1 and Nod2 responses under more physiological conditions such as those induced by microbial stimuli in macrophages or in the animal remains to be investigated. In addition, RICK-deficient macrophages exhibited reduced responses when stimulated with lipoteichoic acid (LTA; TLR2 agonist), LPS (TLR4 agonist), and polyinosinic-poly-citidylic acid (poly(I:C); a TLR3 agonist), but not CpG DNA (TLR9 agonist) (9, 10, 14). These studies suggested that RICK is involved in TLR signaling. However, many preparations of bacterial components contain Nod1- and Nod2-stimulating molecules (4, 5, 7, 15, 16) and the presence of such contaminants could also explain reduced TLR signaling in RICK-deficient macrophages. In the present report, we have studied the role of RICK in TLR signaling in macrophages and in the animal. In addition, we examined the contribution of RICK to immune responses induced by Nod1 and Nod2 activation and *Listeria* infection.

Materials and Methods

Mice

RICK knockout (KO), Nod1 KO, and Nod2 KO have been described (5, 8, 10). RICK KO, Nod1 KO, and Nod2 KO mice were in a C57BL/6 background. MyD88 KO mice in C57BL/6 background were a gift from S.

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³ Abbreviations used in this paper: NOD, nucleotide-binding oligomerization domain; NLR, nucleotide-binding oligomerization domain-like receptor; BMDM, bone marrow derived macrophage; CARD, caspase-recruitment domain; HKLM, heat-killed *Listeria monocytogenes*; iE-DAP, γ -D-glutamyl-*meso*-diaminopimelic acid; IKK, I κ B kinase; KC, keratinocyte-derived chemokine; KO, knockout; LTA, lipoteichoic acid; MDP, muramyl dipeptide; PGN, peptidoglycan; poly(I:C), polyinosinic-polycitidylic acid; WT, wild type.

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Akira (Osaka University, Osaka, Japan). Mice deficient in Nod1 and Nod2 were generated by crossing Nod1 KO and Nod2 KO mice and intercrossing the F₁ generation. C57BL/6J mice were purchased from The Jackson Laboratory. Mice were housed in specific pathogen-free facility. Animal studies were approved by the University of Michigan Committee on Use and Care of Animals (Ann Arbor, Michigan).

Reagents and bacterial culture

Ultrapure LPS from *Escherichia coli* 0111:B4 and *S. minnesota*, monophosphoryl lipid A, LTA, poly I:C, and imiquimod were purchased from Invivogen. LPS preparations denoted as A (from *E. coli* 055:B5), B (from *Salmonella typhimurium*), and C (from *Klebsiella pneumoniae*) were purified by TCA extraction and purchased from Sigma-Aldrich. Lipid A extracted from *S. minnesota* and *E. coli* were purchased from Sigma-Aldrich. Synthetic lipid A was a gift from Dr. K. Fukase (Osaka University, Osaka, Japan). MDP (Ac-(6-*O*-stearoyl)-muramyl-Ala-D-Glu-NH₂) was purchased from Bachem. *N*-myristoyl (C₁₄) γ -D-glutamyl-*meso*-diaminopimelic acid (iE-DAP; in a modified form denoted KF1B), a Nod1 activator, has been described (17). The amount of Nod1 and Nod2 stimulatory activity in LPS preparations was determined as described (15, 18) and given as units per microgram of weight. One unit of the Nod1 and Nod2 stimulatory activity is equivalent to that of 1 ng of synthetic iE-DAP and MDP, respectively. The *L. monocytogenes* strain 10403S was a gift from Dr. M. O'Riordan (University of Michigan, Ann Arbor, MI). Single colonies were inoculated into 5 ml of brain-heart infusion medium and grown overnight at 37°C with shaking. A 1/10 dilution of the overnight culture was prepared and allowed to grow at 37°C with shaking to A₆₀₀ = 0.5, which corresponds to ~10⁹ CFU/ml. Bacteria were diluted to the desired concentration and used in subsequent experiments.

Stimulation of BMDM with ligands and bacteria

Bone marrow derived macrophages (BMDMs) were prepared as previously described (19). Briefly, bone marrow cells were cultured for 5 days with IMDM supplemented with 30% L929 supernatant containing macrophage-stimulating factor, glutamine, sodium pyruvate, 10% heat-inactivated FBS (Invitrogen Life Technologies), and antibiotics. The cells were stimulated with various ligands singly or in different combinations at the concentrations described in the figures for 24 h. In addition, the cells were treated with heat-killed *L. monocytogenes* (HKLM) or live bacteria. Culture supernatants were collected and assayed for cytokine production.

Measurement of NO and cytokines

Nitrite and mouse cytokines were measured in culture supernatants using Griess reagent (20) and ELISA kits from R&D Systems, respectively.

Western blot

For analysis of phosphorylation of $\text{I}\kappa\text{-B}\alpha$, p38, ERK, and JNK, cells were stimulated with LPS or MDP, harvested, and lysed in buffer containing 1% Nonidet P-40 supplemented with complete protease inhibitor mixture (Roche) and 2 mM DTT. Lysates were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with primary Abs. Abs against mouse $\text{I}\kappa\text{-B}\alpha$, p38, ERK1/2, and JNK (phosphorylated and unphosphorylated forms) were purchased from Cell Signaling. Proteins were detected by ECL.

In vivo induction of cytokines

Mice were injected i. p. with 20 μg of LPS or 50 μg of KF1B and blood samples were collected at 3, 6, and 24 h after injection. Sera were isolated by centrifugation at 3,000 rpm for 10 min and submitted to ELISA for analysis of keratinocyte-derived chemokine (KC) and IL-6.

Results

Cytokine responses to TLR agonists are unimpaired in RICK-deficient macrophages

To assess the contribution of RICK to TLR-mediated immune responses, macrophages from wild-type (WT) and RICK-deficient mice were stimulated with several preparations of LPS. We found the production of IL-6 was reduced in RICK-deficient macrophages in response to LPS containing relatively high amounts of Nod1 ligand, but not in response to LPS preparations with low levels of Nod1 and Nod2-stimulating activity (Fig. 1A). Highly purified LPS from *E. coli* and *S. minnesota* induced comparable levels of IL-6 and TNF- α in WT and RICK-deficient macrophages

(Fig. 1B). In contrast, the response elicited by LPS was abrogated in macrophages lacking TLR4 (Fig. 1C), indicating that such LPS preparations mediate cytokine responses through TLR4. To verify these results, we stimulated macrophages with monophosphoryl lipid A and synthetic lipid A, the essential component of LPS required for TLR4 signaling (1). In addition, we tested lipid A preparations from *E. coli* and *S. minnesota*. As we observed with LPS preparations, IL-6 production induced by natural and synthetic lipid A was unimpaired in RICK-deficient macrophages (Fig. 1, D and E). Moreover, secretion of IL-6 induced by LTA (TLR2 agonist), poly(I:C) (TLR3 agonist), and imiquimod (TLR7 agonist) was comparable in WT and RICK-deficient macrophages (Fig. 1F). These results indicate that RICK does not contribute to IL-6 and TNF- α production by macrophages in response to TLR agonists.

RICK is required for NF- κ B and MAPK activation in response to MDP but not LPS

LPS and MDP induce the secretion of proinflammatory cytokines through transcriptional gene responses via NF- κ B and MAPK activation (1, 4, 6). To determine the role of RICK in NF- κ B and MAPK activation, macrophage extracts were prepared at different times after LPS or MDP stimulation and immunoblotted with Abs that recognize activated forms of NF- κ B, ERK, JNK, and p38. By 15 min of stimulation, LPS induced phosphorylation and degradation of $\text{I}\kappa\text{-B}\alpha$ as well as phosphorylation of p38, ERK, and JNK, which was unaffected in RICK-deficient macrophages (Fig. 2A). Such activation of NF- κ B and MAPKs in response to LPS was greatly inhibited or abolished in TLR4-null macrophages (Fig. 2A). In contrast, phosphorylation of $\text{I}\kappa\text{-B}\alpha$, p38, ERK, and JNK induced by MDP was abrogated in RICK-deficient macrophages (Fig. 2B). These results indicate that RICK is required for signaling induced through MDP, the Nod2 ligand, but not LPS.

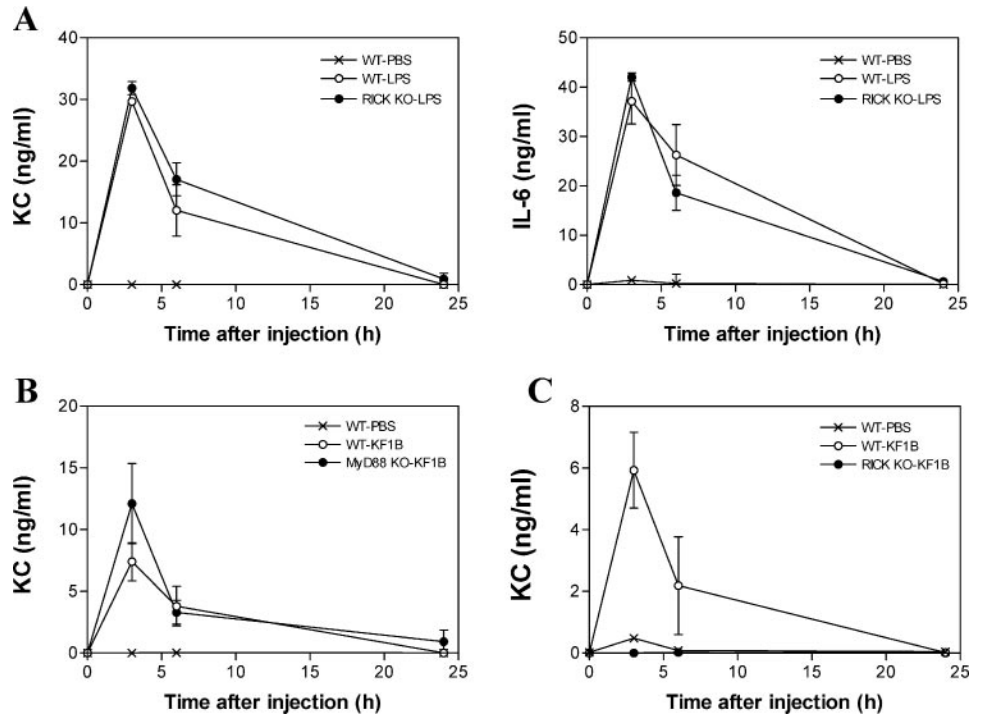
Differential requirement of RICK and MyD88 for chemokine and cytokine production in response to the bacterial dipeptide iE-DAP and LPS in vivo

To assess the requirement for RICK in TLR4 and Nod1 signaling in vivo, WT and RICK-deficient mice were administered LPS or KF1B, a modified iE-DAP dipeptide and the bacterial activator of Nod1 (17). Injection of LPS i. p. induced comparable levels of KC and IL-6 in WT and RICK-deficient mice (Fig. 3A), which is consistent with the results presented in Figs. 1 and 2. To determine the role of MyD88, a critical mediator of TLR activation, in Nod1-induced responses, WT and MyD88-null mice were injected with KF1B i. p. and the levels of KC were determined in serum at different times postinjection. We found no significant differences in the production of KC between WT and MyD88-deficient mice (Fig. 3B). In contrast, the production of KC induced by KF1B was abolished in RICK-deficient mice when compared with WT mice (Fig. 3C). Together, these studies indicate that RICK is required for Nod1-mediated signaling but does not contribute to the production of KC or IL-6 induced by LPS in vivo. Furthermore, MyD88 is dispensable for Nod1-mediated inflammatory responses in mice.

RICK is required for MDP-mediated enhancement of immune responses in macrophages

RICK has been shown to be required for Nod1- and Nod2-induced NF- κ B activation in plasmid DNA transfection experiments using mouse embryonic fibroblasts (10). However the role of RICK under more physiological conditions such as those induced by Nod2 activation in macrophages is unclear. The stimulation of mouse macrophages with MDP alone does not induce the production of

FIGURE 3. Differential requirement of RICK and MyD88 for chemokine and cytokine production in response to the bacterial dipeptide iE-DAP and LPS in vivo. *A–C*, WT ($n = 4$) and RICK-KO mice ($n = 4$) were injected i.p. with 20 μg of purified LPS (*A*) or 50 μg of KF1B, a modified iE-DAP dipeptide (*C*). In addition, WT ($n = 4$) and MyD88-KO mice ($n = 4$) were also injected i.p. with 50 μg of KF1B (*B*). Blood was collected at indicated time points and serum was prepared. Serum levels of KC and IL-6 were measured by ELISA. Results are presented as the mean \pm SEM.



macrophages with low number of HKLM to assess the enhancement of the response by MDP stimulation. We found that stimulation with MDP enhanced the production of IL-6 and TNF- α in response to HKLM (Fig. 5, *B* and *C*). Notably, the enhancement in cytokine production induced by MDP was abolished in RICK-deficient macrophages (Fig. 5, *B* and *C*). To verify that the requirement for RICK was mediated via Nod2, we assessed cytokine production in macrophages deficient in Nod2 in response to HKLM. As it was observed with RICK-deficient macrophages, the enhancement of IL-6 production induced by MDP was abrogated in Nod2-deficient macrophages (Fig. 5*D*). These results indicate that MDP enhances the immune response to HKLM and that this response is mediated by RICK and Nod2.

RICK contributes to cytokine responses induced by live Listeria via Nod1 and Nod2

The early recognition of *Listeria* involves activation of TLRs at the plasma membrane and endosomes as well as in the cytosol via NLRs and receptors involved in nucleic acid recognition (22–24). To assess the contribution of RICK in the cytokine response induced by live *Listeria*, WT and RICK-deficient macrophages were infected with the bacterium and the production of cytokines was determined 12 and 24 h postinfection. We found reduced levels of IL-6 and TNF- α in RICK-deficient macrophages after *Listeria* infection when compared with WT macrophages (Fig. 6, *A* and *B*). *Listeria* produces PGN molecules containing both iE-DAP and

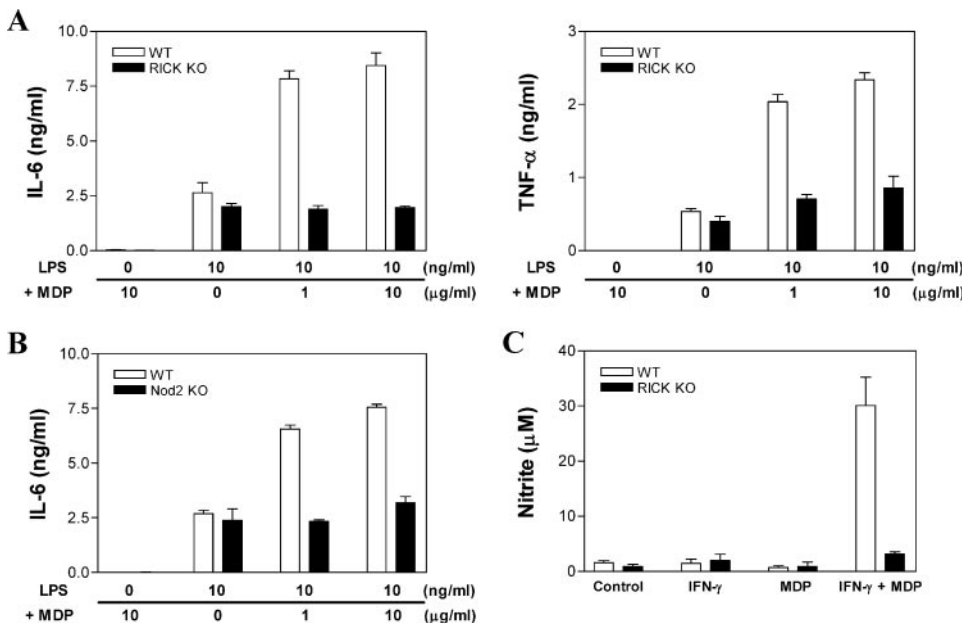
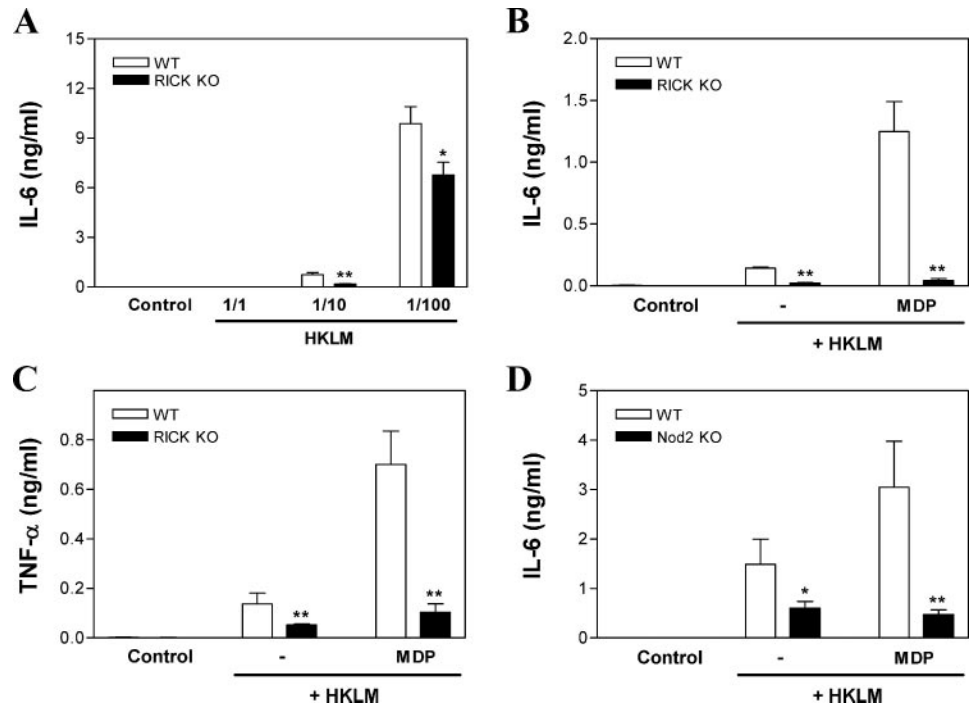


FIGURE 4. RICK is required for the cooperation of Nod2 with LPS and NO production by MDP. *A–C*, BMDMs from WT, RICK-KO (*A*), and Nod2-KO mice (*B*) were stimulated with LPS or MDP alone and with their combination. Supernatant was collected at 24 h after stimulation and IL-6 or TNF- α levels were measured. In addition, BMDMs from WT and RICK-KO mice were treated with MDP in the presence or absence of IFN- γ (1 $\mu\text{g/ml}$; Sigma-Aldrich) and NO production was determined in the supernatant at 48 h after stimulation (*C*). Results are from one representative experiment of three independent experiments and presented as the mean \pm SD.

FIGURE 5. MDP enhances cytokine responses induced by heat-killed *Listeria* in a RICK- and Nod2-dependent manner. *A*, BMDMs from WT and RICK KO mice were stimulated with HKLM at the indicated macrophage:bacterium ratio. *B–D*, BMDMs from WT, RICK-KO (*B* and *C*), and Nod2-KO mice (*D*) were stimulated with HKLM at a macrophage:bacterium ratio of 1:10 in the presence and absence of MDP. Supernatant was collected at 24 h after stimulation and IL-6 (*B*) or TNF- α levels (*C*) were measured by ELISA. Results are from one representative experiment of three independent experiments and presented as the mean \pm SD. Control indicates uninfected. *, $p < 0.05$; **, $p < 0.01$; statistically significant differences between WT and mutant macrophages.



MDP motifs, the activators of Nod1 and Nod2, respectively. We first tested the role of Nod1 in the response to *Listeria* by an analysis of Nod1-deficient macrophages. There was no significant contribution of Nod1 in the IL-6 response to *Listeria* infection (Fig. 6C). We next tested the production of cytokines in WT and mutant mice deficient in Nod2 or both Nod1 and Nod2. The results re-

vealed that Nod1 and Nod2 contribute in a redundant manner to the immune response to *Listeria* in that the deficiency of both proteins, but not Nod1 or Nod2 alone, resulted in a significant reduction in IL-6 production when compared with WT macrophages (Fig. 6, *B* and *C*). To compare the contribution of RICK and double Nod1/Nod2 deficiency, we infected macrophages lacking RICK,

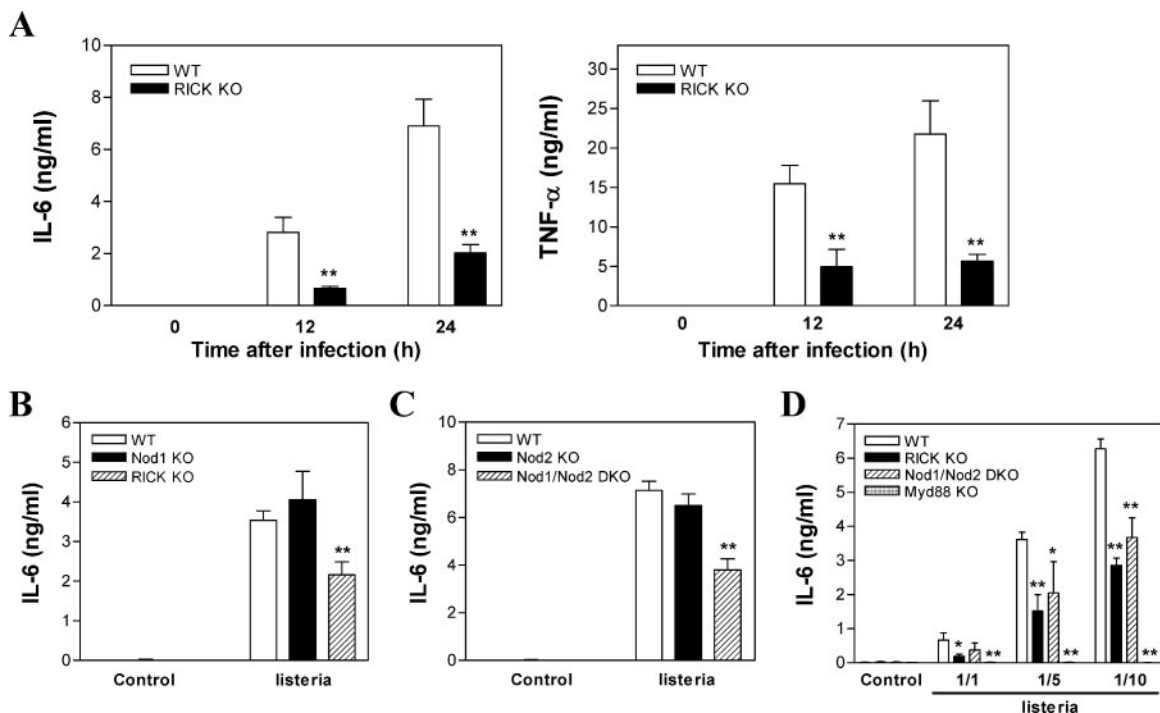


FIGURE 6. RICK contributes to cytokine responses induced by live *Listeria* via Nod1 and Nod2. *A–D*, BMDMs from WT and mutant mice were stimulated with live *L. monocytogenes* at a multiplicity of infection of 10:1 for 12 and 24 h (*A*). BMDMs from WT and Nod-1 (*B*), Nod2-KO, and double Nod1/Nod2-KO (*C*), and WT, RICK-KO, double Nod1/Nod2-KO, and MyD88-KO (*D*) mice were also stimulated with live listeria at a multiplicity of infection of 10:1 for 24 h. Supernatant were collected and IL-6 or TNF- α levels were measured. Results are from one representative experiment of three independent experiments and presented as the mean \pm SD. Control indicates uninfected. *, $p < 0.05$; **, $p < 0.01$; statistically significant differences between WT and mutant macrophages.

both Nod1 and Nod2, or MyD88 in the same experiment and assessed the IL-6 response. Consistent with the results shown in Fig. 6, *B* and *C*, there was a comparable reduction in IL-6 production in macrophages deficient in RICK and Nod1/Nod2 (Fig. 6*D*). The IL-6 response induced by *Listeria* was abolished in macrophages deficient in MyD88 (Fig. 6*D*). These results indicate that RICK pathways contribute to the production of IL-6 in response to *Listeria*, whereas TLR pathways are essential for their response.

Discussion

Initial studies using RICK-deficient mice suggested that this kinase was involved in TLR signaling and that its absence conferred reduced cytokine responses after the stimulation of macrophages with preparations of LPS (9, 10, 14). In the current studies we have studied in detail the role of RICK in TLR signaling. We conclude that RICK is critical for the activation of Nod1 and Nod2 signaling pathways, but we find no direct role for this kinase in TLR signaling. Because PGN molecules capable of stimulating Nod1 and Nod2 are commonly present in LPS preparations (4–6, 15, 16), we tested LPS preparations that are devoid of significant amounts of Nod1 and Nod2 activators and verified these results with natural and synthetic lipid A molecules. In contrast to previous results (10), we also found no significant reduction of IL-6 responses to the synthetic TLR3 agonist poly (I:C). Although the reason for these differences in results is unclear, this could be explained, at least in part, by the fact that our mice were in a pure C57BL/6 background, whereas the previous work was performed in macrophages from mice with a mixed 129/C57BL/6 background. Together, these studies, including macrophage experiments *ex vivo* as well as *in vivo* experiments, support the conclusion that the production of cytokines induced by LPS and other TLR ligands is unimpaired in RICK-deficient mice. Conversely, the production of chemokines induced by the administration of the Nod1 activator *in vivo* was abolished in mutant mice lacking RICK but unimpaired in MyD88-deficient mice *in vivo*. We conclude from these studies that RICK functions in innate immunity as a mediator of Nod1 and Nod2 signaling but independently of TLRs. Yet, RICK was found to be essential for the cooperation between Nod2 and TLR signaling in the production of cytokines. Thus, RICK and TLR signaling pathways, although independent, cooperate for optimal immune responses when the amounts of their specific bacterial activators are limiting. Consistent with this functional cooperation between TLR and RICK signaling is our finding that RICK was more critical in responses to low number of HKLM when the amounts of microbial ligands were limiting and the response suboptimal. The molecular basis for the cooperative effect between RICK and TLRs remains unclear, but it may be caused by cross-induction of critical signaling molecules such as RICK by LPS and MyD88 by Nod1 or Nod2 stimulation (25, 26).

Previous studies revealed that mice deficient in RICK are more susceptible to *i.p.* and *i.v.* infection with *Listeria* than control mice (9). Impaired bacterial clearance was observed by day 3 postinfection, suggesting a role for RICK in innate immunity against *Listeria* (9). However, the mechanism by which RICK contributes to the early response to the bacterium remains poorly understood. We found that RICK-deficient macrophages exhibit reduced production of cytokines in response to *Listeria* infection. These results suggest that RICK promotes host defense, at least in part, through the induction of proinflammatory cytokines during *Listeria* infection. Single deficiency Nod1 or Nod2 did not result in a significant alteration in the cytokine response to *Listeria* infection. However, macrophages lacking both Nod1 and Nod2 exhibited reduced production of cytokines in response to the live bacterium. These results indicate a redundant role for Nod1 and Nod2 in the response

to *Listeria* and suggest that the impairment of the cytokine response observed in RICK-deficient macrophages is mediated by the loss of both Nod1 and Nod2 signaling. We also found that stimulation with MDP, the activator of Nod2, increases the production of cytokines induced by heat-killed *Listeria*, an activity that required both Nod2 and RICK. This finding is consistent with the observation that Nod2 and RICK cooperate with TLR signaling for optimal responses to TLR ligands. The innate immune response to *Listeria*, including cytokine production, also involves TLR signaling particularly through TLR2 (22, 24). Consistent with these findings, we observed that in the absence of RICK there was a considerable residual cytokine response induced by *Listeria* in macrophages. We found that the cytokine response induced by *Listeria* was abolished in MyD88-deficient macrophages, which is consistent with previous studies (22, 27). The latter results appear at odds with a model in which RICK and MyD88 function in parallel signaling pathways. However, the interpretation of the latter results is difficult given that MyD88-null macrophages express lower levels of many TLR-regulated genes, including NF- κ B subunits in the absence of stimulation (28). Although the molecular basis for the reduction of TLR-regulated genes in MyD88-null macrophages remains unclear, it has been suggested that the presence of trace amounts of endotoxin and/or other microbial products in the culture medium may explain these observations (29). Thus, the greatly diminished response of MyD88 KO macrophages to *Listeria* that appears to involve both TLR-MyD88 and RICK signaling pathways could be explained, at least in part, by a generalized low response of MyD88-deficient macrophages to microbial ligands, including to Nod1 and Nod2 ligands *in vitro*. Consistent with this thesis, we found no impairment of the chemokine responses induced by the Nod1 ligand in MyD88 KO mice *in vivo* (Fig. 3). Furthermore, it is well documented that the response of MyD88 KO macrophages is abolished or greatly diminished to *Listeria* *in vitro* but that MyD88 KO mice secrete abundant amounts of cytokines when challenged with *Listeria* *in vivo* (27). Although our *in vivo* results with the Nod1 ligand suggest that MyD88 is not involved in Nod1 signaling, we cannot formally rule out that MyD88 may contribute directly or indirectly to Nod1 and Nod2 signaling. Together, our results and those from several laboratories indicate that both TLR and RICK signaling pathways contribute to the production of proinflammatory cytokines in response to *Listeria*.

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Disclosures

The authors have no financial conflict of interest.

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