The Nod-Like Receptor Family Member Naip5/Birc1e Restricts Legionella pneumophila Growth Independently of Caspase-1 Activation

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Similar to Ipaf and caspase-1, the Nod-like receptor protein Naip5 restricts intracellular proliferation of Legionella pneumophila, the causative agent of a severe form of pneumonia known as Legionnaires’ disease. Thus, Naip5 has been suggested to regulate Legionella replication through the activation of caspase-1. In this study, we show that cytosolic delivery of recombinant flagellin activated caspase-1 in A/J macrophages carrying a mutant Naip5 allele, and in C57BL/6 (B6) macrophages congenic for the mutant Naip5 allele (B6-Naip5A/J), but not in Ipaf−/− cells. In line with these results, A/J and B6-Naip5A/J macrophages induced high levels of caspase-1 activation and IL-1β secretion when infected with Legionella. In addition, transgenic expression of a functional Naip5 allele in A/J macrophages did not alter Legionella-induced caspase-1 activation and IL-1β secretion. Notably, defective Naip5 signaling renders B6-Naip5A/J macrophages permissive for Legionella proliferation despite normal caspase-1 activation. These results indicate that the restriction of intracellular Legionella replication is more complex than previously appreciated and requires both Ipaf-dependent caspase-1 activation as well as functional Naip5 signaling. The Journal of Immunology, 2007, 178: 8022–8027.

Legionella pneumophila (Legionella) is a Gram-negative bacterium that causes Legionnaires’ disease, a human multisystem illness characterized by severe pneumonia (1). Annually, 8,000–18,000 people with legionellosis are hospitalized in the United States and fatality rates vary from 5% to as much as 30% (2). The pathogen invades and replicates within different eukaryotic cells, including mammalian phagocytes (1). Virulent strains of Legionella are able to prevent the normal course of phagocytosis, including the inhibition of acidification of phagosomes, the prevention of phagosome-lysosome fusion, and the suppression of oxidative burst (3–5). These changes allow the creation of a replicative vacuole that is associated with ribosome-studded membranes and supports the multiplication of the bacteria (6, 7). In contrast to human macrophages, macrophages from most inbred mouse strains are restrictive to Legionella replication with the notable exception of A/J mice (8). The mouse strain-specific variation in permissiveness to Legionella replication is controlled by Lgn1, an autosomal recessive locus on chromosome 13 (9–11). Genetic studies in mice identified the Naip5/Birc1e gene as the Legionella susceptibility factor (12–14). There are 14 amino acid substitutions between the C57BL/6 (B6) and A/J Naip5 proteins (15). Naip5 is a member of the Nod-like receptors (NLRs)3 a protein family involved in the cytosolic recognition of pathogen-associated molecular patterns (16, 17). The Naip5-related NLR family member Ipaf is required for caspase-1 activation in response to the cytosolic presence of Legionella flagellin (18). Whereas Ipaf contains an N-terminal caspase recruitment domain, Naip5 harbors three adjacent baculovirus inhibitors of apoptosis repeats (BIRs) at its N terminus, followed by the centrally located NOD and C-terminal leucine rich repeats that characterize NLR proteins (16). At a low multiplicity of infection (MOI), caspase-1- and Ipaf-deficient macrophages show an increased susceptibility to Legionella intracellular replication due to an impaired fusion of Legionella-containing phagosomes (LCPs) with lysosomes (18). Under similar conditions, Naip5 was recently reported to promote the fusion of the LCPs and lysosomes, thus antagonizing the ability of Legionella to remodel its phagosome into specialized replicative vacuoles (19). Based on the findings that A/J, Ipaf−/−, and caspase-1-deficient macrophages allow intracellular replication of Legionella, a model that links Naip5 to the caspase-1 pathway has been proposed (20–22). In this model, infection with Legionella activates Naip5 by delivering flagellin through its type IV secretion system, which then induces Ipaf-mediated caspase-1 activation and cell death to restrict Legionella replication (20–22). However, these studies were not conclusive in that they used a high MOI to assess caspase-1 activation, which is associated with early macrophage cell death (20, 22). Furthermore, caspase-1 activation was measured indirectly using the fluorescent peptide inhibitor FAM-YVAD-fluoromethyl ketone (22), which targets several

3 Abbreviations used in this paper: NLR, Nod-like receptor; BAC, bacterial artificial chromosome; BMDM, bone marrow-derived macrophage; LCP, Legionella-containing phagosome; MOI, multiplicity of infection; SLO, streptolysin O; WT, wild type.

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and the molecular mechanisms that restrict Legionella-mediated caspase-1 activation. Thus, the restriction of intracellular replication inside macrophages even in the presence of flagellin- and Ipaf-dependent caspase-1 activation is independent of Naip5 but requires Ipaf and flagellin. Nevertheless, defective Naip5 signaling results in the proliferation of Legionella at an MOI of 1 for h, extracellular bacteria were washed away, and macrophages were further incubated for 2 h. Lysates were immunoblotted with an anti-caspase-1 Ab. Arrows denote procaspase-1 (p45) and its processed large subunit (p20). Results are representative of three independent experiments. CTRL, Control.

Bacteria and infection
Infections were performed with the thymidine auxotrophic L. pneumophila Philadelphia-1 strain Lp02 or a flaA-deficient mutant of Lp02. Bacteria were grown for 48 h at 35°C in N-(2-acetamido)-2-aminothanesulfonic acid-buffered (Sigma-Aldrich) charcoal yeast extract broth (BCYE) supplemented with 100 mg/ml thymidine and another 24 h in ACES-buffered yeast extract broth (BYE) supplemented with 100 mg/ml thymidine. The bacteria were subsequently used to infect BMDMs at a MOI of 1 (or 0.5 when examining bacterial intracellular multiplication). Following low-speed centrifugation, BMDMs were incubated with the bacterial suspensions for 60 min at 37°C, washed three times to remove nonphagocytosed bacteria, and incubated further in IMDM supplemented with 10% heat-inactivated-FBS and 100 mg/ml thymidine without antibiotics for up to 48 h. Macrophage-associated bacteria were measured as CFUs following the lysis of macrophages with distilled water and the plating of cell lysates onto BCYE agar plates. For cytokine measurements, BMDMs were either left untreated or pretreated with 50 ng/ml Salmonella typhimurium LPS (InvivoGen) for 4 h before infection and infected at a MOI of 1 as described above.

Western blotting
BMDMs were washed in cold PBS and lysed in Nonidet P-40 buffer (10 mM HEPES (pH 7.4), 142.5 mM KCl, 0.2% Nonidet P-40, and 1 mM EGTA supplemented with a protease mixture inhibitor tablet (Roche) and 1 mM DTT. Samples were cleared from membranes by centrifugation at 20,000 × g for 10 min at 4°C. Lysates were separated by electrophoresis on SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane by semidry blotting in a buffer containing 25 mM Tris-HCl (pH 8.0), 190 mM glycine, and 20% methanol. Membranes were incubated with an Ab against caspase-1 (3ZD; provided by the National Cancer Institute, Bethesda, MD), or GAPDH (Millipore), followed by incubation with a HRP-conjugated secondary Ab against rabbit or mouse IgG (Jackson ImmunoResearch Laboratories). Immunoactive proteins were visualized with the ECL method (Pierce).

Measurement of cytokines
Mouse IL-1β and TNF-α was measured in culture supernatants with ELISA kits (R&D Systems).

Materials and Methods
Mice and macrophages
The mouse congenic line BcA75 that expresses the permissive Birc1e/Naip5 allele of A/J origin on the genetically permissive background of B6 mice (14) and Ipaf-deficient (Ipaf<sup>−/−</sup>) mice (18, 24) have been reported. Transgenic A/J mice expressing a restrictive Naip5 allele from B6 mice have been described (12). Bone marrow-derived macrophages (BMDMs) were isolated from femurs of 6- to 12-wk-old mice and were cultured in IMDM containing 10% heat-inactivated FBS, 20% L cell-conditioned medium, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After 5 days of incubation, cells were collected and plated in 6-well plates or in 24-well plates in IMDM containing 10% heat-inactivated FBS and 100 mg/ml thymidine without antibiotics. Macrophages were cultured for an additional 24 h before use. In some experiments, BMDMs were permeabilized with streptolysin O (SLO) (Sigma-Aldrich), as previously described (25), to deliver recombinant flagellin (InvivoGen) into the cytosol. The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals (Ann Arbor, MI).

Measurement of cytokines

**FIGURE 1.** Naip5 is dispensable for caspase-1 activation by cytosolic flagellin. A–E, BMDMs from B6 (A and E), Ipaf<sup>−/−</sup> (B), A/J (C), and B6-Naip5<sup>−/−</sup> (D and E) mice were either left untreated (CTRL, for control) or incubated with SLO (5 μg/ml), purified flagellin (Fla) (2 μg/ml or as indicated), or SLO together with flagellin (SLO+Fla; 2 μg/ml or as indicated) for 10 min. Macrophages were then washed extensively and incubated further for 2 h before the cell extracts were prepared and immunoblotted for caspase-1 activation. Arrows denote procaspase-1 (p45) and its processed large subunit (p20). Results are representative of three independent experiments.

**FIGURE 2.** A mutant Naip5 allele does not affect Legionella-induced caspase-1 activation. BMDMs from B6 (A), Ipaf<sup>−/−</sup> (B), A/J (C), and B6-Naip5<sup>−/−</sup> (D) mice were infected with WT or flagellin-deficient (Fla<sup>A−</sup>) Legionella at an MOI of 1 for h, extracellular bacteria were washed away, and macrophages were further incubated for 2 h. Lysates were immunoblotted with an anti-caspase-1 Ab. Arrows denote procaspase-1 (p45) and its processed large subunit (p20). Results are representative of three independent experiments. CTRL, Control.
Results

Cytosolic delivery of purified flagellin induces caspase-1 activation independently of Naip5

Flagellin and Ipaf are known to be essential for Legionella-induced activation of caspase-1 (18, 20, 21). To assess the requirement of Naip5 for flagellin-induced caspase-1 activation, macrophages were stimulated with purified flagellin in the presence or absence of SLO, a protein that allows the delivery of exogenous molecules into the cytosol of living cells (25). In line with previous reports (18, 24), the flagellin/SLO-induced caspase-1 activation seen in B6 macrophages (Fig. 1A) was abrogated in Ipaf−/− cells (Fig. 1B). The low amount of SLO used to deliver flagellin to the cytosol was necessary but not sufficient to activate caspase-1 (Fig. 1). Unexpectedly, A/J macrophages activated caspase-1 when flagellin was delivered into their cytosol (Fig. 1C). Moreover, caspase-1 activation proceeded normally in B6 macrophages congenic for the mutant Naip5 allele from A/J mice (B6-Naip5A/J) (Fig. 1D), indicating that Naip5 is dispensable for flagellin-induced caspase-1 activation. To rule out the possibility that the flagellin concentration used (2 μg/ml) was saturating, we analyzed the activation of caspase-1 in response to different concentrations of flagellin. The dose-dependent caspase-1 activation levels observed in B6-Naip5A/J macrophages were identical with those in B6 control cells (Fig. 1E). These results show that Naip5 is not required for flagellin-induced activation of caspase-1.

Naip5 does not control Legionella-induced caspase-1 activation

To verify the results obtained with purified flagellin, we infected macrophages with wild-type (WT) or flagellin-deficient (FlaA−) Legionella. As reported (18, 20, 21), WT Legionella activated caspase-1 in B6 macrophages but not in Ipaf-deficient cells (Fig. 2, A and B). Moreover, flagellin was required because Legionella flaA mutants did not induce caspase-1 activation (Fig. 2). To assess the role of Naip5 in Legionella-induced caspase-1 activation, we analyzed B6-Naip5A/J and A/J macrophages infected with WT or flagellin-deficient Legionella. Consistent with the results obtained with purified flagellin, caspase-1 activation proceeded normally in the latter macrophage populations when infected with WT Legionella (Fig. 2, C and D). Moreover, the kinetics of caspase-1 activation was identical in B6, A/J, and B6-Naip5A/J, with activation detectable as early as 1 h postinfection (data not shown). Flagellin-deficient bacteria did not activate caspase-1 in B6-Naip5A/J or A/J macrophages (Fig. 2, C and D), consistent with the observation that flagellin is required for Ipaf-mediated caspase-1 activation.

Functional Naip5 is not essential for IL-1β secretion in response to Legionella infection

The maturation and release of IL-1β is critically dependent on the presence of active caspase-1. Therefore, we next determined the production of mature IL-1β after the infection of macrophages with Legionella.
with WT and mutant Legionella. To facilitate the analysis of mature IL-1β, macrophages were first primed with LPS for 4 h to induce pro-IL-1β production, and the levels of secreted IL-1β were measured at several time points following infection. As expected, the infection of B6 macrophages with WT Legionella led to the production of significant levels of mature IL-1β (Fig. 3A). Similar to uninfected cells, macrophages infected with flagellin-deficient Legionella did not secrete detectable levels of IL-1β in the culture supernatant (Fig. 3). Notably, IL-1β production was severely impaired in Ipaf−/− macrophages whereas significant levels of mature IL-1β were measured in the culture supernatants of B6-Naip5A/J and A/J macrophages (Fig. 3, B–D). Unlike mature IL-1β, TNF-α levels were comparable in the different Legionella-infected macrophage populations (Fig. 3E). Therefore, Naip5 is not essential for Legionella-induced IL-1β production, in contrast to Ipaf. As the levels of IL-1β in the supernatant of B6 macrophages were higher compared with those of B6-Naip5 A/J and A/J macrophages (Fig. 3), we tested whether this could reflect differential pro-IL-1β levels due to interstrain differences in LPS responsiveness rather than caspase-1 activation. Densitometric quantification of the levels of pro-IL-1β relative to GAPDH in LPS-primed macrophages revealed that the induction of pro-IL-1β was ~40–50% lower in Naip5A/J and A/J macrophages than in B6 macrophages (Fig. 3F). Thus, the differences in Legionella-induced IL-1β levels between B6-Naip5A/J or A/J and B6 macrophages appear to be due to differential induction of pro-IL-1β and not to caspase-1 activation. These results are consistent with the previously reported interstrain differences in LPS-induced IL-1β production between B6 and A/J macrophages (27) and genetic studies that mapped this difference to a locus on mouse chromosome 1 (28).

**FIGURE 4.** Functional Naip5 does not alter caspase-1 activation and IL-1β production in A/J macrophages. A and B, BMDMs expressing a restrictive Naip5 allele (A/J-Naip5BAC−/−) (B) and control A/J littermates (A) were infected with WT or flagellin-deficient (FlaA−) Legionella at an MOI of 1 for 1 h. Extracellular bacteria were washed away and macrophages were further incubated for 2 h. Lysates were immunoblotted with an anti-caspase-1 Ab. Arrows denote procaspase-1 (p45) and its processed large subunit (p20). Results are representative of three independent experiments. CTRL, control. C, BMDMs were pretreated for 4 h with LPS (50 ng/ml) to induce pro-IL-1β production and then either left uninfected or infected with WT or flagellin-deficient (FlaA−) Legionella at an MOI of 1 for 1 h. Release of mature IL-1β was determined in culture supernatants at the designated time points after infection. Results of triplicate samples are expressed as the mean ± SD. Data are representative of three independent experiments.

**FIGURE 5.** Defective Naip5 signaling renders B6 macrophages permissive for Legionella proliferation. BMDMs from B6 (A), Ipaf−/− (B), A/J (C), and B6-Naip5A/J (D) mice were infected with WT or flagellin-deficient (FlaA−) Legionella at an MOI of 0.5 for 1 h, lysed at the designated time points, and plated for CFU scoring. Values represent the mean CFU of at least three independent experiments ± SD. KO, Knockout.
Expression of a functional Naip5 allele in A/J macrophages does not affect caspase-1 activation or IL-1β secretion induced by Legionella infection

To further assess the role of Naip5 in Legionella infection, we analyzed the effect of the introduction of a functional Naip5 allele in A/J macrophages. Our results showed that the transgenic expression of a functional Naip5 allele from a bacterial artificial chromosome (BAC) did not alter caspase-1 activation (Fig. 4A and B) or the production of mature IL-1β (Fig. 4C) in A/J macrophages infected with WT Legionella as compared with BAC-negative littermates. As expected, flagellin-deficient bacteria were unable to induce caspase-1 activation or IL-1β secretion in both BAC+ and control macrophages. Altogether, our results indicate that Naip5 is dispensable for caspase-1 activation and the release of mature IL-1β from Legionella-infected macrophages, whereas flagellin and Ipaf are required.

Defective Naip5 signaling allows Legionella growth in the presence of normal caspase-1 activation

Our results showed that Naip5 is dispensable for Legionella-induced activation of caspase-1 (Figs. 2–4). Naip5 has been suggested to function upstream of Ipaf and caspase-1 to restrict Legionella replication inside macrophages (20, 22). To test this model directly, we infected macrophages with WT or flaA−mutant Legionella and assessed bacterial growth over time. As expected, B6 macrophages, but not Ipaf−/−cells, restricted the proliferation of WT bacteria (Fig. 5, A and B). Flagellin-deficient Legionella replicated freely in these macrophages. Therefore, the deletion of Ipaf or Legionella flaA is sufficient to evade growth restriction inside macrophages that carry a restrictive Naip5 allele. We next determined bacterial growth in B6-Naip5−/−and A/J macrophages carrying a mutant Naip5 allele. Interestingly, WT Legionella grows inside both macrophage populations (Fig. 5, C and D), although caspase-1 is activated (Fig. 2, C and D). Therefore, mutations in Naip5 allow Legionella growth inside macrophages, although they do not affect caspase-1 activation (Table I). Finally, the proliferation of Legionella flaA−mutants, which evade Ipaf-mediated caspase-1 activation, in macrophages carrying a mutant Naip5 allele is comparable to that of WT bacteria (Fig. 5, C and D), suggesting that the combined loss of caspase-1 activation and Naip5 signaling does not result in higher bacterial loads compared with defective Naip5 signaling alone.

Discussion

Loss-of-function mutations in the Naip5 gene reportedly underlie the susceptibility of A/J macrophages to Legionella proliferation (10, 15). In line with this observation, Naip5 was recently reported to be required for the fusion of the LCP with lysosomes, thus antagonizing the ability of Legionella to remodel its phagosome into specialized replicative vacuoles in restrictive macrophages (19). Similarly, caspase-1- and Ipaf-deficient macrophages show an increased susceptibility to Legionella intracellular replication due to an impaired fusion of LCP with lysosomes (18). Mechanistic models in which Naip5 controls the Ipaf-mediated activation of caspase-1 to restrict Legionella growth in infected macrophages have recently been proposed (20, 22). We reasoned that if caspase-1 activation underlies the marked Naip5-dependent susceptibility of A/J mice to Legionella replication, activation of this protease should be abolished or at least strongly impaired in A/J macrophages and B6 macrophages congenic for the mutant Naip5 allele from A/J mice. Our results showed that caspase-1 activation proceeds normally in these macrophages, comparable to macrophages carrying a restrictive Naip5 allele. Moreover, transgenic expression of a restrictive Naip5 allele renders A/J macrophages restrictive for Legionella growth (19) without altering caspase-1 activation or IL-1β secretion. Therefore, the Naip5 mutations that underlie Legionella growth in A/J macrophages must affect another host defense signaling pathway, thus allowing the replication of Legionella in these macrophages even in the presence of caspase-1 activation (summarized in Table I). As reported (18, 20–22), impaired caspase-1 activation (in Ipaf−/−macrophages infected with WT bacteria or in WT macrophages infected with flagellin-deficient Legionella mutants) supports bacterial growth in macrophages with a restrictive Naip5 allele.

The conclusions from our studies are in marked contrast with those derived form studies by Roy and collaborators (22) that concluded that infection with Legionella activates Naip5 by delivering flagellin through its type IV secretion system, which then induces Ipaf-mediated caspase-1 activation and cell death to restrict Legionella replication. There are several differences in the experimental approach used by Roy and colleagues (22) and this report that may explain the discrepancy in the conclusions drawn from the two studies. Whereas we assessed caspase-1 activation at early time points using a low MOI, Roy and colleagues used a high MOI in their studies (22). Notably, we also found similar levels of caspase-1 activation at 1 and 2 h postinfection in B6 and A/J macrophages infected with a high MOI of 10 (data not shown), indicating that infection at high MOI alone does not induce a differential activation of caspase-1 at early time points. However, Roy and colleagues measured caspase-1 activation at rather late time points (8 h), which are associated with macrophage cell death under conditions of high MOI (22). Finally, caspase-1 activation was assessed using an indirect assay based on the fluorescent peptide inhibitor FAM-YVAD-fluoromethyl ketone (22), which is known to target several caspases as well as cathepsins (23). Both caspases and cathepsins are activated and have central roles in the initiation and execution of programmed cell death (29, 30). The low background staining of the fluorescent peptide inhibitor in caspase-1-deficient macrophages may therefore reflect an impaired activation of apoptotic caspases and cathepsins in these cells due to an abolished cell death response rather than the absence of caspase-1 activation per se. Indeed, Legionella-induced cell death is reportedly dependent on the presence and activation of caspase-1 (20, 22). We thus favor the concept that Ipaf-induced caspase-1 activation and Naip5 signaling represent distinct host defense pathways that are essential for the restriction of Legionella proliferation (Fig. 6),

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<th>Mouse Strain</th>
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<tr>
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*Naip55R, Restrictive Naip5 allele; Naip55S, susceptible Naip5 allele; FlaA−, flagellin-deficient Legionella.

Table I. Caspase-1 activation and Legionella growth in macrophages

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although we cannot formally rule out the possibility that Naip5 functions downstream of Ipaf and caspase-1. In this model, Ipaf-mediated caspase-1 activation and signaling through Naip5 are both required to restore fusion of the LCPs with lysosomes, thus preventing the formation of a replicative vacuole. Loss of either signaling pathway results in the inability to fuse LCPs with lysosomes and allows the bacteria to multiply. Although flagellin clearly is responsible for Ipaf-dependent caspase-1 activation, its remains to be determined whether this or another bacterial factor is sensed by Naip5. Answering this question probably will require the identification of a downstream molecular target of Naip5. Altogether, our results show that the restriction of intracellular Legionella replication is more complex than previously appreciated and requires both Ipaf-dependent caspase-1 activation and functional Naip5 signaling.

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Disclosures

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References