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Cooperation between Multiple Microbial Pattern Recognition Systems Is Important for Host Protection against the Intracellular Pathogen *Legionella pneumophila*▽†

Kristina A. Archer,[1]‡ Florence Ader,[1,2] Koichi S. Kobayashi,[3] Richard A. Flavell,[4] and Craig R. Roy[1]*

Section of Microbial Pathogenesis, Yale University School of Medicine, Boyer Center for Molecular Medicine, 295 Congress Avenue, New Haven, Connecticut 06536[1]; Lyon 1 University, Inserm U851, Finovi-Bacterial Pathogenesis and Innate Immunity, National Reference Center for Legionella, Lyon, F-69008, France[2]; Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts[3]; and Department of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06536[4]

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Multiple pattern recognition systems have been shown to initiate innate immune responses to microbial pathogens. The degree to which these detection systems cooperate with each other to provide host protection is unknown. Here, we investigated the importance of several immune surveillance pathways in protecting mice against lethal infection by the intracellular pathogen *Legionella pneumophila*, the causative agent of a severe pneumonia called Legionnaires' disease. Rip2 and Naip5/NLRC4 signaling was found to contribute to the innate immune response generated against *L. pneumophila* in the lung. Elimination of Rip2 or Naip5/NLRC4 signaling in MyD88-deficient mice resulted in increased replication and dissemination of *L. pneumophila* and higher rates of mortality. Irradiated wild-type mice receiving bone marrow cells from pattern recognition receptor-deficient mice displayed *L. pneumophila* infection phenotypes similar to those of donor mice. Rip2 and Naip5/NLRC4 signaling provided additive effects in protecting MyD88-deficient mice from lethal infection by *L. pneumophila*, with the contribution of Naip5/NLRC4 being slightly greater than that of Rip2. Thus, activation of the Rip2, MyD88, and Naip5/NLRC4 signaling pathways triggers a coordinated and synergistic response that protects the host against lethal infection by *L. pneumophila*. These data provide new insight into how different pattern recognition systems interact functionally to generate innate immune responses that protect the host from lethal infection by activating cellular pathways that restrict intracellular replication of *L. pneumophila* and by recruiting to the site of infection additional phagocytes that eliminate extracellular bacteria.

To respond to diverse populations of microbes, the mammalian innate immune system utilizes germ line-encoded pattern recognition receptors (PRRs) that detect conserved molecular patterns associated with pathogens (38). The ectodomains of transmembrane Toll-like receptor (TLR) are involved in detecting microbes outside cells and within vacuoles, and the adapter protein MyD88 is used by many TLRs to transduce extracellular signals into functional responses (38). In contrast, the nucleotide-binding domain, leucine-rich repeat (NLR) proteins constitute a surveillance mechanism capable of responding to microbial products delivered into the host cytosol (27). The Nod1 and Nod2 proteins are PRRs that detect microbial products present in the cytosol and in response activate NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways through an adapter serine-threonine kinase called Rip2 (11, 18, 25, 26, 28, 29, 33, 44, 46, 50).

The Gram-negative bacterium *Legionella pneumophila* is a useful model for investigating the initiation of the innate immune response. *L. pneumophila* persists in the environment as a parasite of freshwater protozoans (15); however, upon gaining access to the mammalian respiratory system through contaminated aerosols, the bacteria can infect and replicate within alveolar macrophages (17, 24, 37). Failure to treat infected individuals, especially those who are immunocompromised, with antibiotics can lead to the development of a severe pneumonia known as Legionnaires' disease (17, 37). Following phagocytosis by a macrophage, *L. pneumophila* generates a unique vacuole that evades fusion with lysosomes and accumulates endoplasmic reticulum (ER) protein markers, features that allow the compartment to support intracellular replication (12, 22, 23, 30, 56). *L. pneumophila* is able to perform this task by utilizing a type IV secretion system encoded by the *dot* and *icm* genes (36, 48, 57). The Dot/Icm secretion apparatus delivers bacterial proteins into the host cell cytosol that modulate normal endosomal trafficking and prevent lysosome-mediated killing of the bacteria (31, 41).

The proteins TLR2, TLR5, and TLR9 have been shown to recognize *L. pneumophila* during engulfment at the cell surface or in an early endosomal compartment (2, 6, 7, 19–21, 43). Mice deficient in TLR2 have a subtle defect in clearance of *L. pneumophila* from the lung after infection (6, 20). Surprisingly, defects in TLR5 and TLR9 signaling do not exacerbate this TLR2 defect significantly (5), suggesting that TLR signaling

* Corresponding author. Mailing address: Section of Microbial Pathogenesis, Yale University School of Medicine, Boyer Center for Molecular Medicine, 295 Congress Avenue, New Haven, CT 06536. Phone: (203) 737-2408. Fax: (203) 737-2630. E-mail: craig.roy@yale.edu.

‡ Present address: Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA.

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alone is not essential for host protection against *L. pneumophila* infection. Mice deficient for MyD88 have a profound defect in interleukin-12 (IL-12) and gamma interferon (IFN-γ) production (5, 6, 20, 54) and display high numbers of *L. pneumophila* CFU in the lungs compared to control mice (6, 20). MyD88 is required for signaling pathways stimulated by TLRs and for pathways activated by the IL-1 family of receptors (1), which is the likely reason why a deficiency in MyD88 results in a more severe *L. pneumophila* susceptibility phenotype than a deficiency in the three primary TLRs stimulated by *L. pneumophila*. Macrophages and NK cells have been implicated as cell types that utilize MyD88 for an *in vivo* response to *L. pneumophila* (5, 6, 20, 54); however, it remains to be determined which cell types play a protective role in the MyD88-dependent response.

In addition to activating MyD88-dependent pathways, virulent *L. pneumophila* activates cytosolic pattern recognition systems. The flagellin protein produced by *L. pneumophila* signals through the NLR proteins Naip5 and NLRC4 (also known as IPAF and CARD12), resulting in the activation of caspase-1 and other pathways that restrict intracellular replication of *L. pneumophila* in mouse macrophages (4, 34, 40, 45, 58). Increased replication of *L. pneumophila* in the lungs is observed after infection of mice deficient in Naip5 or NLRC4 signaling (4, 10, 34, 58); however, these mice are still able to clear the infection over a period of several days. The finding that *L. pneumophila* activates a Rip2-dependent signaling pathway in macrophages that mediates IκB degradation and NF-κB nuclear translocation suggests that the NLR proteins Nod1 and Nod2 are also involved in detection (35, 52). Whether Rip2 signaling is important for host protection against *L. pneumophila*, however, has not been addressed.

The ability of multiple pathogen recognition systems to respond to *L. pneumophila* makes this an attractive model to investigate whether these different signaling pathways play functionally independent or synergistic roles in stimulating the host defense to this intracellular pathogen. In this study, we used a mouse model of Legionnaires' disease to investigate the role of multiple microbial recognition systems in providing host protection against this intracellular pathogen.

MATERIALS AND METHODS

Bacterial strains. *L. pneumophila* serogroup 1 strain JR32 (47), an *flaA* mutant (JR32 Δ*flaA*) (45), and serogroup 1 clinical isolate F2111 (13) were used in this study. *L. pneumophila* strains were cultured on charcoal-yeast extract (CYE) agar (14) for 2 days and then cultured overnight in *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract (AYE) broth (10 g/liter yeast extract, 10 g/liter ACES, 0.4 g/liter L-cysteine HCl-H_2O, 0.135 g/liter ferric nitrate) prior to use in experiments. For enzyme-linked immunosorbent assay (ELISA) studies and *in vivo* growth assays, bacteria were grown to an optical density at 600 nm of 1 in AYE broth. For *ex vivo* growth assays, bacteria were grown to an optical density of 3.4 in AYE broth.

Mice. C57BL/6 (stock number 000664) mice were purchased from Jackson Laboratories. MyD88$^{-/-}$ (1) and Rip2$^{-/-}$ (33) mice in a C57BL/6 background have been described previously. MyD88$^{-/-}$ mice were provided by R. Medzhitov, and Rip2$^{-/-}$ mice were provided by R. Flavell. For experiments using mice with the nonfunctional *Naip5* gene, MyD88$^{-/-}$ and Rip2$^{-/-}$ mice in a mixed 129/SvJ × C57BL/6 background were mated with A/J mice to generate mice that were homozygous for the A/J *Naip5* allele.

Bone marrow-derived macrophages (BMMs). Bone marrow was collected from the femurs and tibiae of mice. Cells were plated on petri dishes and incubated at 37°C in RPMI 1640 medium containing 20% fetal bovine serum (FBS), 30% macrophage colony-stimulating factor (M-CSF)-conditioned medium, and 1% penicillin-streptomycin. At day 6, cells were harvested and resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 5% M-CSF. Cells were then plated in 24-well tissue culture-treated plates and incubated at 37°C. M-CSF was obtained from an L-929 fibroblast cell line (ATCC).

***Ex vivo* macrophage infections and growth assays.** BMMs were added to 24-well plates at a concentration of 2×10^5 cells/well. The cells were infected with either the *L. pneumophila* JR32 wild-type (WT) or JR32 Δ*fla* strain at a multiplicity of infection (MOI) of 5 and incubated at 37°C. BMMs were washed at 1 h postinfection with warm Dulbecco's phosphate-buffered saline (PBS) to remove extracellular bacteria. Then either the BMMs were lysed immediately with sterile H_2O (day 0) or fresh medium was added until the cells were harvested at 24, 48, and 72 h postinfection. Cell lysates were plated on CYE agar to determine the number of bacterial CFU. Each data point is the value for one mouse for which the average bacterial content of three independent wells was determined. The fold differences were determined by dividing the values obtained at 24, 48, and 72 h by the values obtained on day 0. *Ex vivo* growth assays were repeated at least once, and the results obtained were similar.

***In vivo* mouse infections.** Mice were anesthetized by intraperitoneal injection of a ketamine (100 mg/kg)-xylazine (10 mg/kg)-PBS solution and infected intranasally with wild-type *L. pneumophila* strain JR32, the isogenic Δ*flaA* derivative of JR32, or the clinical isolate F2111 in 40 μl of PBS. For *in vivo* bacterial growth assays, mice were euthanized by CO_2 either at 4 h postinfection (day 0) or at the times indicated in the figures. Lungs, spleens, or livers were harvested and placed in 10 ml of sterile double-distilled H_2O and homogenized using a PowerGen 125 handheld homogenizer (Fisher) for 30 s. Lysates were plated on CYE agar to determine the numbers of bacterial CFU. Each data point in the figures is the CFU count for a single mouse. All experiments were repeated at least one time independently, and similar results were obtained. The lower limit of detection in the assay was 100 CFU of *L. pneumophila*.

To determine cytokine levels in bronchoalveolar lavage fluid (BALF), mice were intranasally infected with either 1×10^6 or 4×10^4 CFU of *L. pneumophila* Δ*fla*. Mice were euthanized by intraperitoneal injection of a ketamine (250 mg/kg)-xylazine (25 mg/kg)-PBS solution. The mouse lungs were lavaged once with 500 μl of PBS, and the BALF was stored at −80°C. IL-12 p40, IL-6, and IFN-γ levels in the BALF were determined by ELISA using BD Pharmingen IL-12 (p40/p70), IL-6, and IFN-γ reagents, respectively. Monocyte chemoattractant protein-1 ([MCP-1] BD OptEIA kit; BD Pharmingen) and keratinocyte-derived chemokine [KC] DuoSet Mouse KC; R&D Systems) levels were also determined by ELISA.

Lymphocyte isolation and intracellular cytokine staining. Lymphocytes were isolated from lungs by digesting minced lungs for 1 h in a 37°C shaking incubator in collagenase buffer (RPMI 1640 medium, 100 U/ml collagenase I [Gibco], 5% fetal bovine serum, 0.1% $CaCl_2$, 0.1% $MgCl_2$). Lysates were put through a 70-μm-pore-size nylon cell strainer (BD Falcon), and cells were isolated using a Percoll (Sigma) gradient. Red blood cells were lysed with red blood cell lysing buffer (Sigma). Isolated lymphocytes were resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS, 1% FBS, 0.025% sodium azide) and blocked with purified rat anti-mouse CD16/CD32 (BD Pharmingen). Cells were stained with rat anti-mouse CD11b (Caltag Laboratories), rat anti-mouse Ly-6C/G (Gr-1) (Caltag Laboratories), and rat anti-mouse F4/80 (Caltag Laboratories). Neutrophils were gated as CD11b$^+$, Gr-1$^+$, and F4/80$^-$. Inflammatory monocytes were gated as CD11b$^+$, Gr-1$^+$, and F4/80$^+$. Data were collected using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Generation of bone marrow-chimeric mice. Recipient C57BL/6 (expressing the Ly5.1 antigen) mice were lethally irradiated with two doses of 500 rads over a 5-h period using a ^{137}Cs γ-source and reconstituted intravenously with 1×10^7 bone marrow cells from donor C57BL/6 (expressing the Ly5.2 gene) wild-type, MyD88$^{-/-}$, or Rip2$^{-/-}$ MyD88$^{-/-}$ mice. Mice were placed on Sulfatrim (40 mg/ml sulfamethoxazole–8 mg/ml trimethoprim) for 8 weeks following transplantation of bone marrow cells, and chimerism was confirmed by flow cytometry after differential staining of blood lymphocytes for Ly5.1 and Ly5.2. Mice were infected as described above.

Statistical analysis. A two-tailed, Mann-Whitney *U* test was used to analyze the significance of differences in means between groups. Survival curves were generated using the Kaplan-Meier method, and the significance of differences was calculated by a log rank test. Differences were considered statistically significant if the *P* value was <0.05.

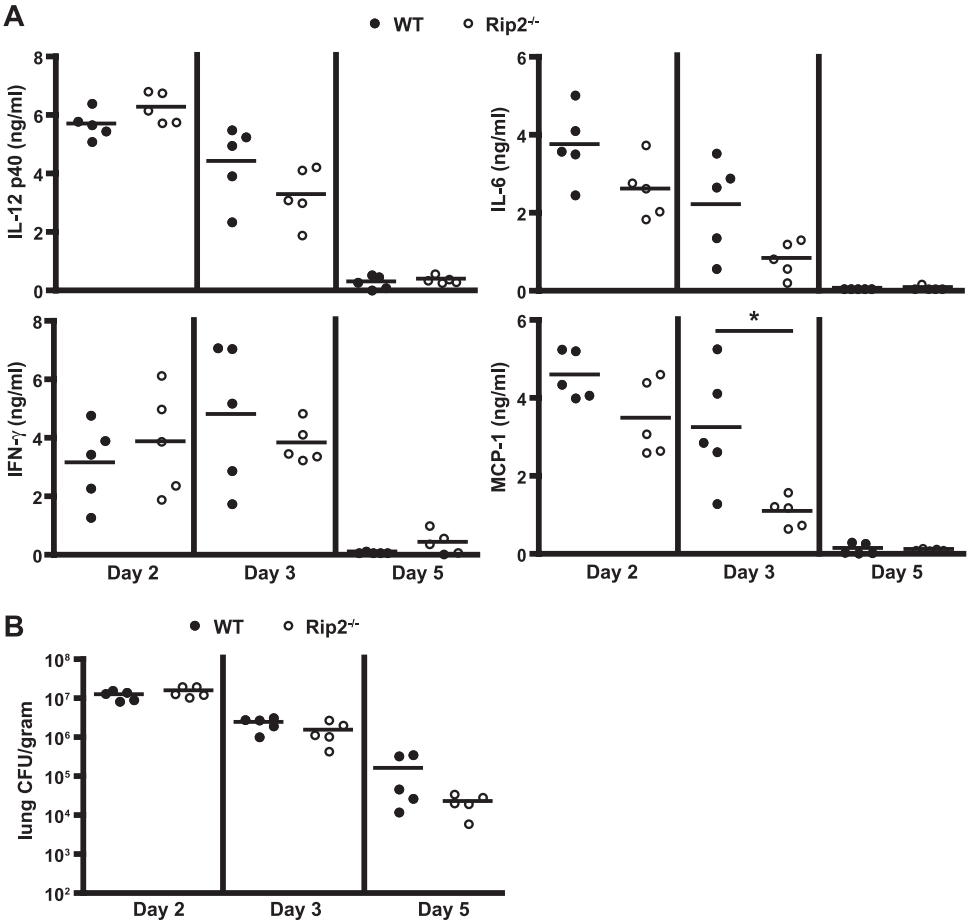

FIG. 1. *L. pneumophila* activates Rip2-dependent pathways *in vivo*. WT and Rip2-deficient ($Rip2^{-/-}$) mice in a C57BL/6 background were given a high intranasal dose (1×10^6 CFU) of *L. pneumophila* Δ*flaA*. For each group, five mice were sacrificed at days 2, 3, and 5 postinfection as indicated below each graph. (A) BALF from infected mice was assayed for the indicated cytokines by ELISA. Each circle represents data obtained from a single mouse. The lines indicate the mean values calculated from the data for the two groups of mice. *, $P < 0.05$. (B) Bacterial numbers were determined from lung lysates at the indicated time points. There was no statistical significance between the two groups of mice ($P > 0.05$).

RESULTS

***L. pneumophila* activates Rip2-dependent pathways *in vivo*.** The activation of NF-κB by a Rip2-dependent pathway was shown following *L. pneumophila* infection of MyD88-deficient macrophages *ex vivo* (35, 52), suggesting that Nod1 and Nod2 are able to detect the presence of *L. pneumophila* products within the host cell cytosol (52). To determine whether Rip2 signaling is important for *in vivo* responses to *L. pneumophila*, wild-type (WT) and Rip2-deficient mice in the C57BL/6 background were infected intranasally with a flagellin-deficient (Δ*flaA*) strain of *L. pneumophila*. The *L. pneumophila* Δ*flaA* strain was used to evade activation of the Naip5 and NLRC4 signaling pathways. Mice were sacrificed at days 2, 3, and 5 postinfection, and inflammatory cytokine levels were measured in the BALF by ELISA. No significant difference was observed in the levels of IL-12 p40, IFN-γ, or IL-6 in the lung of Rip2-deficient mice compared to WT mice (Fig. 1A). A significant decrease was observed consistently in the levels of the chemokine MCP-1 (Fig. 1A), indicating a role for Rip2 in the detection of *L. pneumophila in vivo*. To determine whether the absence of Rip2 results in enhanced *L. pneumophila* replication in the lung, bacterial numbers were measured. No significant difference was detected in the number of *L. pneumophila* cells in the lung of Rip2-deficient mice compared to WT mice, and the rate of clearance from the lungs was also similar (Fig. 1B). To control for the possibility that the nonmotile phenotype exhibited by *L. pneumophila* deficient in flagellin might influence these results, the Rip2 deletion was introduced into mice that were deficient in Naip5 signaling as a result of being homozygous for the hypomorphic *Naip5* allele from the A/J mouse (34). Similar results were obtained using Rip2-deficient mice having a defect in Naip5 signaling following infection with a clinical isolate of *L. pneumophila* that produces flagellin (see Fig. S1 in the supplemental material). Thus, bacterial motility and the production of flagellin did not affect the Rip2-dependent response.

Rip2 is important for suppression of *L. pneumophila* replication in MyD88-deficient mice. Because MyD88-dependent signaling contributes significantly to the innate immune response directed against *L. pneumophila*, host responses mediated by Rip2 may be masked by MyD88-dependent signaling

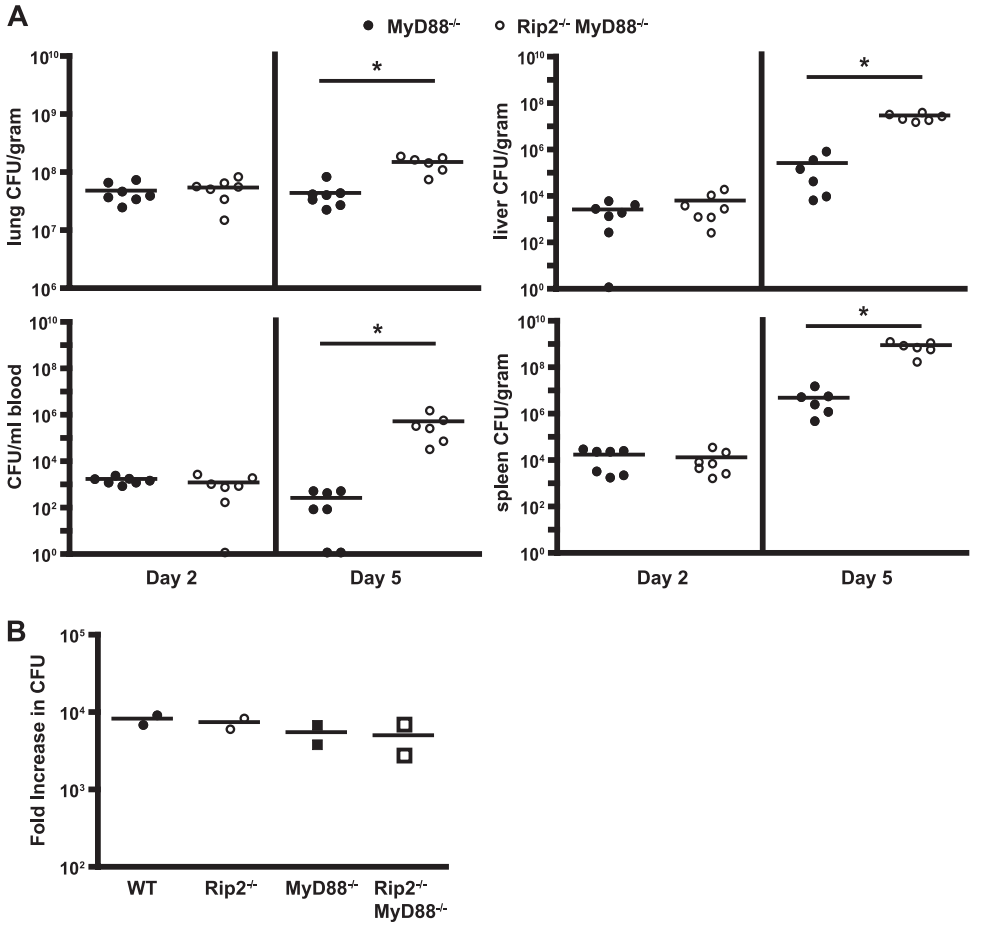

FIG. 2. Rip2 is important for suppression of *L. pneumophila* replication in MyD88-deficient mice. (A) MyD88-deficient ($MyD88^{-/-}$) and Rip2/MyD88-deficient ($Rip2^{-/-}MyD88^{-/-}$) mice in a C57BL/6 background were given a low intranasal dose (4×10^4 CFU) of *L. pneumophila* Δ*flaA*. At day 2 and day 5 postinfection, seven mice from each group were sacrificed, and bacterial numbers were determined from the lung, blood, liver, and spleen. Each point represents data from a single mouse. The lines indicate the mean values calculated from the data for the two groups of mice. The difference between the two groups of mice was statistically significant at day 5. *, $P < 0.005$. (B) BMMs from WT (filled circles), $Rip2^{-/-}$ (open circles), $MyD88^{-/-}$ (filled squares), and $Rip2^{-/-}$ $MyD88^{-/-}$ (open squares) mice were infected with *L. pneumophila* Δ*flaA*. The numbers of bacterial CFU were determined at 1 h and 48 h postinfection. Intracellular growth is expressed as the fold increase in the number of CFU detected over this period. Each point represents data for BMMs derived from a single mouse. All data points represent the average increase in the number of bacterial CFU determined from three wells infected independently. Each line indicates the mean calculated from the data for the two different mice.

pathways. To investigate this possibility, mice deficient in both Rip2 and MyD88 were generated in the C57BL/6 background and infected with *L. pneumophila* Δ*flaA*. At day 2 postinfection, there was no significant difference between the number of *L. pneumophila* Δ*flaA* CFU in the Rip2- and MyD88-deficient (Rip2/MyD88-deficient) mice and in the MyD88-deficient mice (Fig. 2A). At day 5, however, the Rip2/MyD88-deficient mice had slightly higher CFU counts in the lungs, liver, spleen, and blood than mice deficient in only MyD88 (Fig. 2A). This difference was statistically significant. Rip2/MyD88-deficient mice infected with motile wild-type *L. pneumophila* also had higher CFU counts than MyD88-deficient mice 5 days after infection, indicating that the contribution of Rip2 was independent of flagellin sensing by the Naip5/NLRC4 pathway (see Fig. S2 in the supplemental material). These data suggest that Rip2 signaling contributes to restricting *L. pneumophila* replication *in vivo* when MyD88-dependent responses are absent.

To determine whether higher levels of replication in Rip2/MyD88-deficient mice result from an intrinsic defect in the ability of macrophages to restrict *L. pneumophila* multiplication, bone marrow-derived macrophages (BMMs) from WT, Rip2-deficient, MyD88-deficient, and Rip2/MyD88-deficient mice were infected with *L. pneumophila* Δ*flaA*, and intracellular growth was monitored for 3 days. *L. pneumophila* replication in macrophages lacking Rip2 was comparable to that in MyD88-deficient and WT cells (Fig. 2B). Similar results were observed using macrophages deficient in Naip5 signaling and infected with motile *L. pneumophila* producing flagellin (see Fig. S3 in the supplemental material). Thus, the enhanced replication of *L. pneumophila* observed in the Rip2/MyD88-deficient mice was not due to intrinsic differences in the ability of naive macrophages derived from these mice to support intracellular replication.

Cellular recruitment defects in Rip2/MyD88-deficient mice after *L. pneumophila* infection. The cytokine IL-6 and chemo-

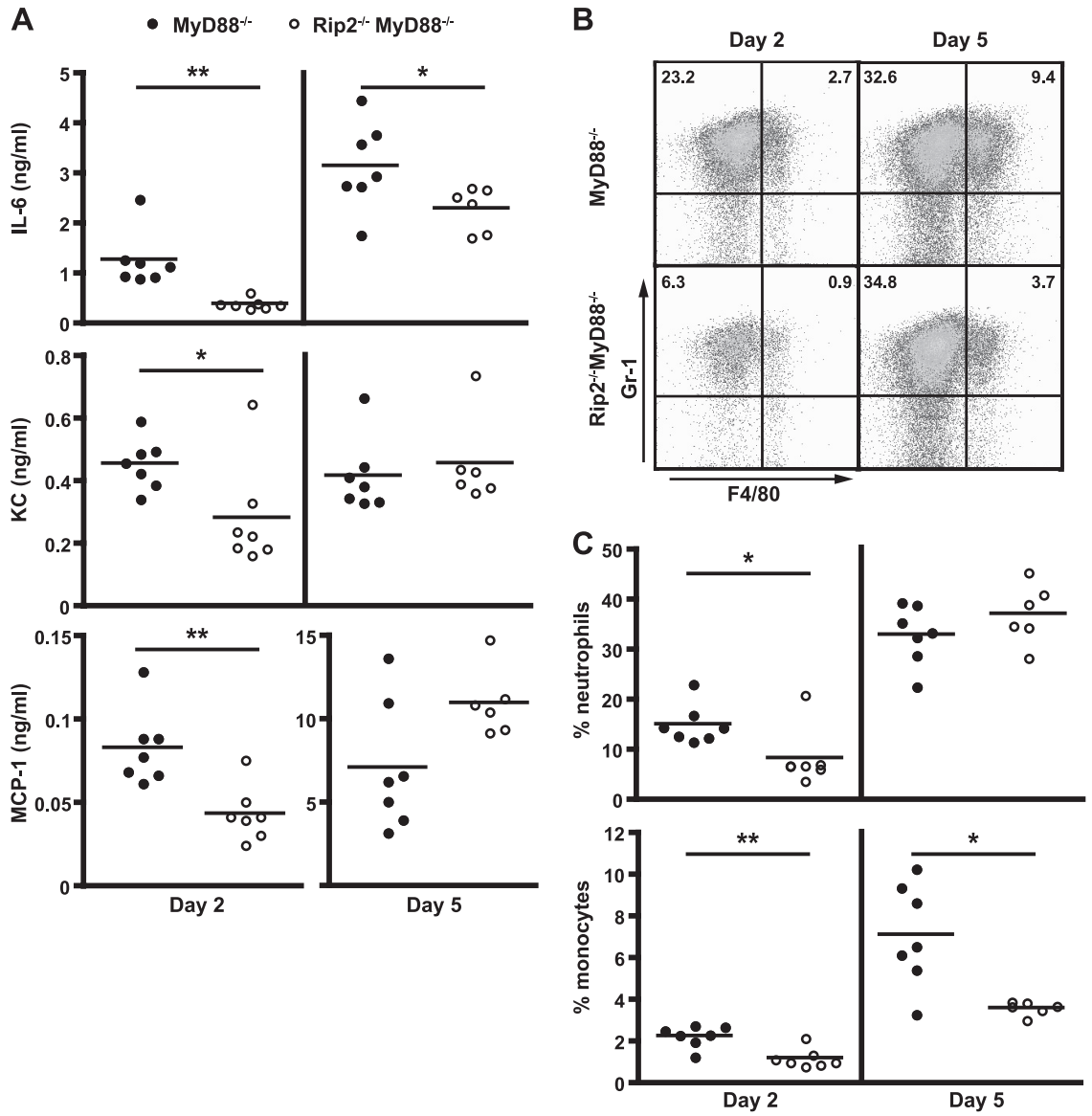

FIG. 3. Cellular recruitment defects in Rip2/MyD88-deficient mice after *L. pneumophila* infection. MyD88$^{-/-}$ and Rip2$^{-/-}$ MyD88$^{-/-}$ mice in a C57BL/6 background were given a low intranasal dose (4×10^4 CFU) of *L. pneumophila* Δ*flaA*. Seven mice from each group were sacrificed at days 2 and 5 postinfection. (A) BALF from infected mice was assayed for the indicated cytokines by ELISA. Each point represents data from a single mouse. The horizontal lines indicate the mean values calculated from the data for the two groups of mice. (B and C) Cell suspensions from isolated lungs were stained for CD11b, Gr-1, and F4/80 and examined by flow cytometry. (B) Representative plots indicating the frequency of neutrophils (CD11b$^+$, Gr-1$^+$, and F4/80$^-$; upper-left quadrant) and inflammatory monocytes (CD11b$^+$, Gr-1$^+$, and F4/80$^+$; upper-right quadrant) from an individual MyD88$^{-/-}$ or Rip2$^{-/-}$ MyD88$^{-/-}$ mouse sacrificed at day 2 or day 5. (C) Average percentage of neutrophils or inflammatory monocytes from individual MyD88$^{-/-}$ or Rip2$^{-/-}$ MyD88$^{-/-}$ mice sacrificed at day 2 or day 5. Plots for mouse groups are arranged as in panel A. *, $P < 0.05$; **, $P < 0.005$.

kines KC and MCP-1 are detected at low levels after *L. pneumophila* infection of MyD88-deficient mice (Fig. 3A). To determine whether the higher bacterial numbers observed in the Rip2/MyD88-deficient mice correspond with a defect in production of proinflammatory chemokines or cytokines, MyD88-deficient and Rip2/MyD88-deficient mice were infected intranasally with *L. pneumophila* Δ*flaA*. Mice were sacrificed at days 2 and 5 postinfection, and ELISA measurements of BALF were used to assess the host response to infection. The levels of IL-6 at days 2 and 5 postinfection and of KC and MCP-1 at day 2 postinfection were reduced significantly in Rip2/MyD88-deficient mice compared to MyD88-deficient mice (Fig. 3A). Because the KC and MCP-1 attract neutrophils and inflammatory monocytes to infected tissues, respectively (9, 49), we examined whether the reduction of these chemoattractants in Rip2/MyD88-deficient mice early upon infection correlate with differences in cellular recruitment to the lung of infected mice. Rip2/MyD88-deficient mice had a significant reduction in the proportion of neutrophils (CD11b$^+$, Gr-1$^+$, and F4/80$^-$) in the lung at day 2 postinfection and fewer inflammatory monocytes (CD11b$^+$, Gr-1$^+$, and F4/80$^+$) at days 2 and 5 postinfection than MyD88-deficient mice (Fig. 3B and C). Thus, Rip2 sig-

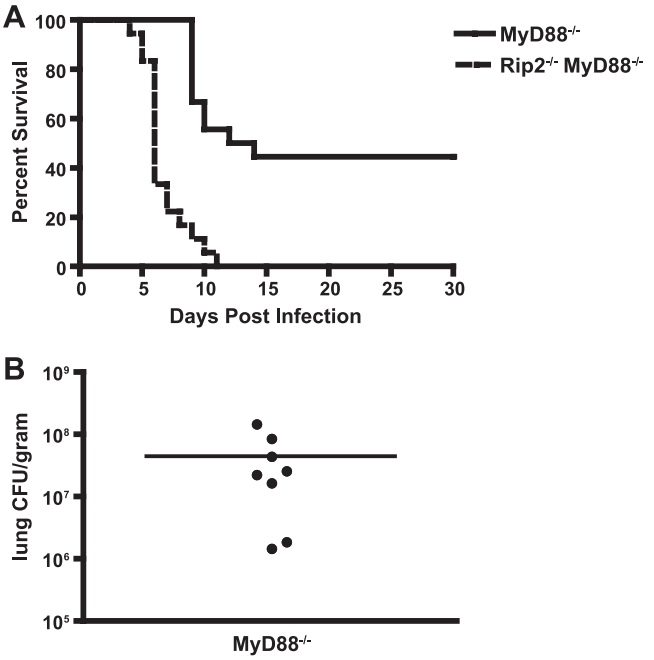

FIG. 4. Mice deficient for both Rip2 and MyD88 are highly susceptible to lethal infection by *L. pneumophila*. MyD88$^{-/-}$ and Rip2$^{-/-}$ MyD88$^{-/-}$ mice in a C57BL/6 background were given a low intranasal dose (4×10^4 CFU) of *L. pneumophila* Δ*flaA*. (A) The graph represents the percentage of MyD88$^{-/-}$ ($n = 19$) and Rip2$^{-/-}$ MyD88$^{-/-}$ ($n = 18$) mice surviving at the time points indicated. The difference between the two groups of mice was significant ($P < 0.0001$). (B) Surviving MyD88$^{-/-}$ mice were sacrificed at day 30 postinfection, and the average number of CFU in the lung was measured. There were no detectible bacteria in either the liver or the spleen of these mice, and there were no surviving Rip2/MyD88-deficient mice to analyze. These data are in contrast to immune-sufficient mice, which did not have detectible bacteria in the lung at day 30 postinfection. Each point represents data from a single MyD88$^{-/-}$ mouse, and the line represents the average from all mice.

naling contributes to cytokine production and cellular recruitment to the lung of *L. pneumophila*-infected mice.

Mice deficient for both Rip2 and MyD88 are highly susceptible to lethal infection by *L. pneumophila*. To investigate whether Rip2 signaling contributes to host protection upon infection by *L. pneumophila*, MyD88-deficient and Rip2/MyD88-deficient mice were infected with *L. pneumophila* Δ*flaA* and monitored for survival. Most of the infected Rip2/MyD88-deficient mice died within the first 7 days, and all of the mice died by day 11 (Fig. 4A). In contrast, all of the infected MyD88-deficient mice were alive at day 9, and 50% of the MyD88-deficient mice survived infection and appeared healthy at day 30. The surviving MyD88-deficient mice were sacrificed, and the numbers of bacteria in the lungs, liver, spleen, and blood were determined. The surviving MyD88-deficient mice had high levels of *L. pneumophila* in the lung, but bacteria were not detected in the liver, spleen, or blood (Fig. 4B). Similar findings were observed for Rip2/MyD88-deficient mice having a defect in Naip5 signaling and infected with motile *L. pneumophila* producing flagellin (see Fig. S4 in the supplemental material). These data indicate that Rip2 signaling in response to *L. pneumophila* activates pathways that provide host protection against infection and that these pathways play a critical role in host survival when MyD88 signaling is disrupted.

Host protection to *L. pneumophila* infection requires activation of innate signaling pathways in bone marrow-derived cells. To elucidate cell types in the lung that require MyD88 and Rip2 signaling to direct a protective host response during infection by *L. pneumophila*, chimeric mice were generated by irradiating wild-type C57BL/6 mice and repopulating these mice with bone marrow cells obtained from either wild-type mice, MyD88-deficient mice, or Rip2/MyD88-deficient mice. Data examining *L. pneumophila* numbers at day 5 postinfection revealed that the chimeric mice had similar phenotypes as their respective bone marrow donor mice (Fig. 5A). Bacterial loads in the lung and spleen were significantly higher in the chimeric mice receiving MyD88-deficient or Rip2/MyD88-deficient bone marrow cells than in control mice receiving wild-type bone marrow cells. Importantly, bacterial colonization in the chimeric mice was not significantly different from that of their respective bone marrow-donor mice. High IL-6 levels were present in BALF from chimeric mice receiving MyD88-deficient or Rip2/MyD88-deficient bone marrow cells but not in BALF from respective donor MyD88-deficient or Rip2/MyD88-deficient mice (Fig. 5B), which indicates that a significant amount of Rip2/MyD88-dependent production of IL-6 is mediated by a radiation-resistant population of cells in the chimeric mice. Thus, even though radiation-resistant cells are contributing to the immune response, innate immune signaling pathways in the bone marrow-derived cells are critical for host protection against *L. pneumophila*.

A functional hierarchy of pattern recognition receptors generates a protective immune response against *L. pneumophila*. Rip2, MyD88, and Naip5/NLRC4 are activated during infection of macrophages by *L. pneumophila*, and each signaling pathway contributes to host protection. Although MyD88-dependent responses provide protection against flagellin-deficient *L. pneumophila* that fails to activate Naip5/NLRC4, flagellin-deficient *L. pneumophila* replicates to higher numbers and persists longer in the lungs of wild-type mice than isogenic *L. pneumophila* that produces flagellin (4, 5, 10, 34, 40), indicating that the contribution of Naip5/NLRC4 to bacterial clearance is not masked by a strong MyD88-dependent response. This is in contrast to results shown here for Rip2, where no significant difference was observed in *L. pneumophila* numbers when infected Rip2-deficient mice were compared to control wild-type mice. To determine whether the Naip5/NLRC4 pathway and the Rip2 pathway have functionally redundant or independent roles in providing host protection, immune-deficient mice were infected with a high dose of wild-type *L. pneumophila* or an isogenic Δ*flaA* strain, and bacterial colonization and host survival were compared.

Similar to what has been observed in wild-type mice, there were significantly more *L. pneumophila* Δ*flaA* bacteria in the lung of Rip2-deficient mice at day 5 postinfection than in wild-type *L. pneumophila*-infected mice (Fig. 6A). Rip2-deficient mice were able to control infection by either strain, as indicated by a decrease in the number of wild-type *L. pneumophila* and Δ*flaA* bacteria in the lung at day 5 compared to the count at 4 h postinfection (Fig. 6A). Additionally, all of the Rip2-deficient animals survived a high-dose infection by either wild-type *L. pneumophila* or the Δ*flaA* strain (Fig. 6B and C).

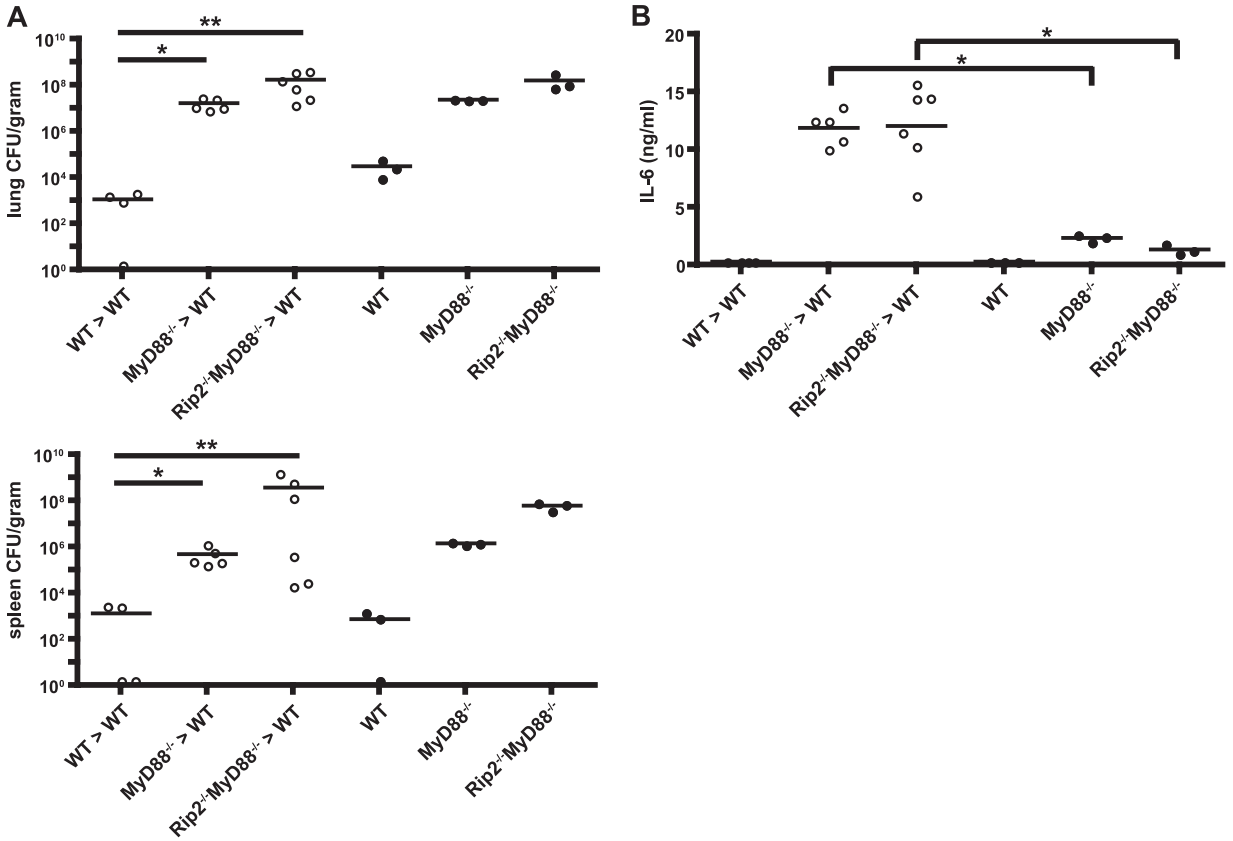

FIG. 5. Host protection from *L. pneumophila* infection requires activation of innate signaling pathways in bone marrow-derived cells. Bone marrow-chimeric mice were generated by irradiating wild-type (WT) mice and reconstituting them with donor bone marrow from either WT (WT>WT), MyD88-deficient ($MyD88^{-/-}$>WT), or Rip2/MyD88-deficient ($Rip2^{-/-}$ $MyD88^{-/-}$>WT) mice. Mice were given a low intranasal dose (4×10^4 CFU) of *L. pneumophila* Δ*flaA*. (A) Bacterial CFU in the lung and spleen was measured at 5 days postinfection for irradiated mice (open circles) receiving bone marrow from wild-type, $MyD88^{-/-}$, or $Rip2^{-/-}$ $MyD88^{-/-}$ donor mice and for mice with the same genetic background as the donors (filled circles). (B) BALF from infected mice was assayed for IL-6. Each point represents data from a single mouse. The lines indicate the means calculated from data for each group of mice. *, $P < 0.05$; **, $P < 0.01$.

In both MyD88-deficient and Rip2/MyD88-deficient mice, bacterial numbers were higher at day 5 than at 4 h postinfection, and the difference between the numbers of wild-type and Δ*flaA* bacteria was not as great as that which was observed in Rip2-deficient mice (Fig. 6A), indicating that MyD88-deficient mice are unable to suppress replication of these strains *in vivo*. Importantly, the majority of MyD88-deficient mice (9/10) survived a high-dose infection by wild-type *L. pneumophila*, whereas most Rip2/MyD88-deficient mice (8/10) died within the first 10 days of infection by this strain (Fig. 6B). Measurements of the number of wild-type *L. pneumophila* bacteria in the lungs of the surviving mice sacrificed 21 days after infection revealed that the Rip2-deficient mice were able to clear bacteria from the lungs, whereas persistent colonization of the lung by wild-type *L. pneumophila* was observed in the MyD88-deficient and Rip2/MyD88-deficient survivors (Fig. 6B). All of the MyD88-deficient and Rip2/MyD88-deficient mice receiving a high dose of the *L. pneumophila* Δ*flaA* strain died (Fig. 6C) although, consistent with what was observed for mice that succumbed following a low-dose infection, the Rip2/MyD88-deficient mice had a shorter mean time to death than the MyD88-deficient mice. Thus, Rip2 and Naip5/NLRC4 have additive and nonoverlapping roles in contributing to host protection following *L. pneumophila* infection.

DISCUSSION

Innate immune responses controlled by MyD88, Naip5/NLRC4, and Rip2 are activated by PRRs that detect bacterially derived molecules. How these systems interact to generate host immunity to bacterial pathogens has been an important question that has not been investigated systematically. Here, we addressed the importance of these pathways in providing protection against an intracellular pathogen using a mouse model of Legionnaires' disease. Our data suggest that these systems are responding independently to different microbial stimuli and that these individual responses are important elements of a complex functional hierarchy that regulates a multifaceted innate immune response, providing host protection against lethal infection by the intracellular pathogen *L. pneumophila*.

MyD88-dependent responses to *L. pneumophila* were analyzed previously both in macrophages cultured *ex vivo* and in mouse infection models (5, 6, 20, 54). These studies showed

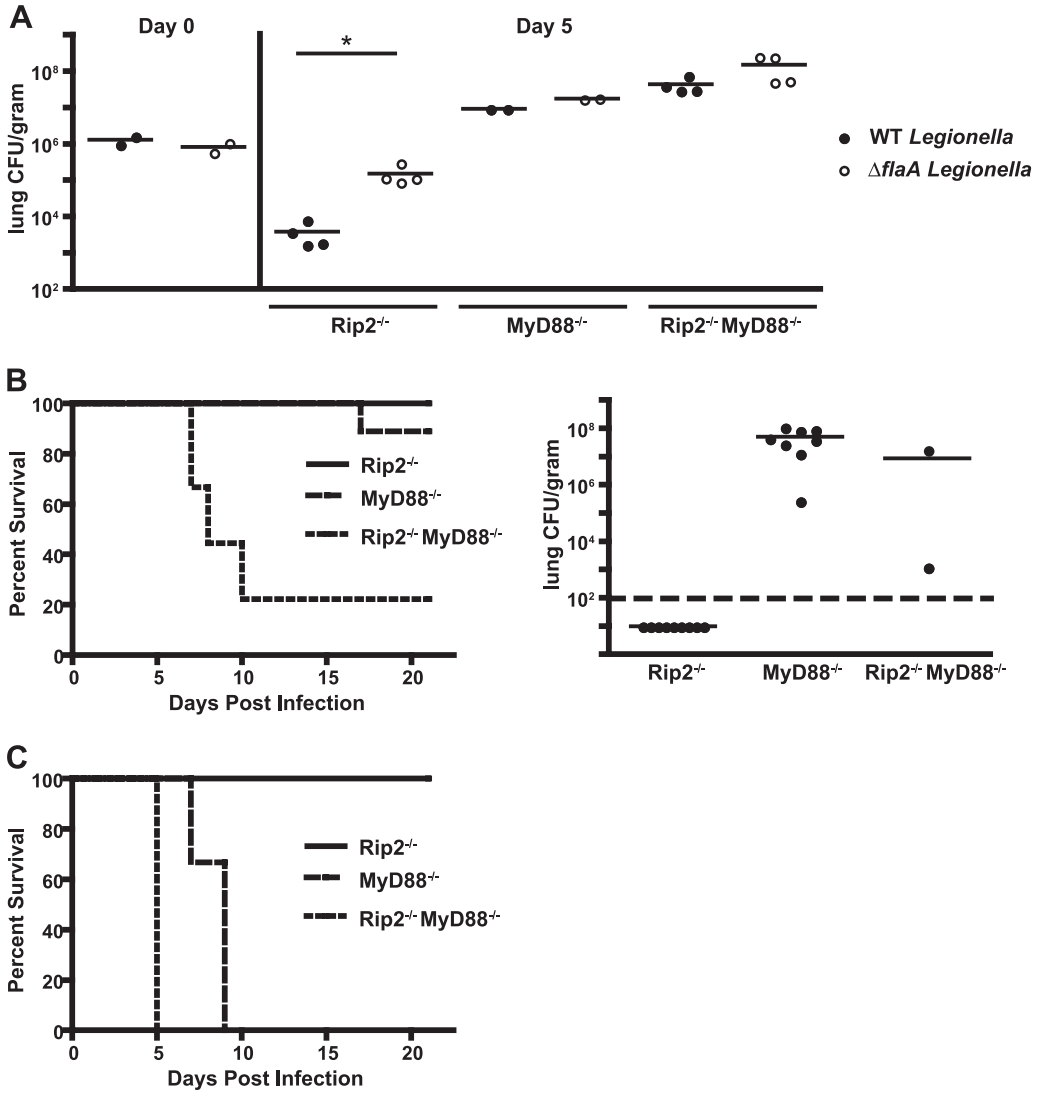

FIG. 6. Cooperative signaling between multiple pattern recognition systems is important for a protective immune response against *L. pneumophila*. Rip2$^{-/-}$, MyD88$^{-/-}$, and Rip2$^{-/-}$ MyD88$^{-/-}$ mice in a C57BL/6 background were given a high intranasal dose (1×10^6 CFU) of either wild-type *L. pneumophila* or *L. pneumophila* Δ*flaA*. (A) At day 5 postinfection bacterial CFU were measured in the lung. Data in the left panel (day 0) represent CFU measured in the lungs of control animals sacrificed 4 h after intranasal infection. Each point represents data from a single mouse. The lines indicate the means calculated from the data for the two groups of mice. The dashed line indicates the lower limit of detection. *, $P < 0.05$ (B) The graph on the left indicates the survival of Rip2$^{-/-}$, MyD88$^{-/-}$, and Rip2$^{-/-}$ MyD88$^{-/-}$ mice after a high intranasal dose (1×10^6 CFU) of wild-type *L. pneumophila* ($n = 9$ mice for each group). The difference between the MyD88$^{-/-}$ and Rip2$^{-/-}$ MyD88$^{-/-}$ mice was significant ($P < 0.01$). The graph on the right shows the number of *L. pneumophila* bacteria in the lungs of mice surviving until day 21. (C) Survival of Rip2$^{-/-}$, MyD88$^{-/-}$, and Rip2$^{-/-}$ MyD88$^{-/-}$ mice after a high intranasal dose (1×10^6 CFU) of *L. pneumophila* Δ*flaA* ($n = 3$ mice for each group). The difference between the MyD88$^{-/-}$ and Rip2$^{-/-}$ MyD88$^{-/-}$ mice was significant ($P < 0.05$).

that MyD88 has a central role in generating a protective innate immune response to *L. pneumophila*. Extracellular *L. pneumophila* and bacteria that reside in endocytic vacuoles initiate MyD88-dependent responses through stimulation of TLR molecules, which mediate macrophage production of several key proinflammatory cytokines and chemokines through the activation of regulatory factors including NF-κB and MAPKs. The cytokines released by macrophages during *L. pneumophila* infection are important for the production of IFN-γ, a process that requires IL-18 activation of an MyD88-dependent pathway for IFN-γ expression by NK and NK T cells. Here, we show that Rip2-deficient mice infected with the *L. pneumophila* Δ*flaA* mutant were able to clear bacteria from the lung, indicating that the MyD88-dependent axis is able to protect the host against *L. pneumophila* infection in the absence of Rip2 and Naip5/NLRC4 stimulation. Thus, the MyD88 signaling module resides near the top of a hierarchical structure of signaling components critical for generating a protective innate immune response against *L. pneumophila*.

The Naip5/NLRC4 axis stimulated by bacterial flagellin was found to provide a level of protection against *L. pneumophila* that was measurable in all of the mouse genetic backgrounds examined in this study. Caspase-1 is activated upon stimulation of the Naip5/NLRC4 signaling pathway, and this response is

important for the secretion of bioactive IL-1β and IL-18 from infected macrophages (4, 10, 34, 40, 45, 53, 58). This connects the Naip5/NLRC4 and MyD88 signaling pathways because increased production of pro-IL-1β is mediated by an MyD88-dependent pathway, and the receptors that respond to IL-1β and IL-18 utilize MyD88 for signaling (1, 39). Naip5/NLRC4 also controls MyD88-independent functions that result in a cell-autonomous response that limits intracellular replication of *L. pneumophila*. Caspase-1, caspase-7, interferon regulatory factor 1 (IRF1), and IRF8 have all been implicated in the cell-autonomous response regulated by Naip5/NLRC4 signaling (3, 4, 10, 16, 34, 40, 45, 53, 58); however, the exact mechanism by which Naip5/NLRC4 restricts replication of *L. pneumophila* is not completely clear. Stimulation of the Naip5/NLRC4 pathway in MyD88-deficient macrophages cultured *ex vivo* is sufficient to restrict replication of *L. pneumophila* (40). In contrast, we observed that in MyD88-deficient mice, wild-type bacteria grew to levels that were similar to those of the *L. pneumophila* Δ*flaA* strain, which indicates that replication of wild-type *L. pneumophila* in the lung was not restricted severely by the Naip5/NLRC4 pathway. Interestingly, the majority of MyD88-deficient mice survived a high-dose challenge by wild-type *L. pneumophila*, whereas most MyD88-deficient mice died after infection with the isogenic Δ*flaA* mutant. From these data we conclude that in the lung the Naip5/NLRC4 axis provides a level of host protection that is independent of MyD88 and that may extend beyond a cell-autonomous response that limits replication in macrophages.

Rip2 was found to be involved in the innate immune response to *L. pneumophila*. Previously, it was shown that Rip2 participates in the activation of NF-κB following infection of macrophages by *L. pneumophila*; however, this activation pathway was masked by the MyD88-dependent pathway of NF-κB activation (35, 52). Consistent with what was observed using mouse macrophages cultured *ex vivo*, Rip2-deficient mice had a very subtle phenotype compared to wild-type mice. IL-6 and MCP-1 levels were slightly diminished in the lungs of Rip2-deficient mice, but no significant differences in bacterial loads or host susceptibility were detected following infection by *L. pneumophila*. Our results using *L. pneumophila* differ slightly from data obtained recently for the intracellular pathogen *Chlamydophila pneumoniae*, where delayed pathogen clearance from the lung and an increased lethality were observed after infection of Rip2-deficient mice (51). This might relate to the fact that *C. pneumoniae* is an obligate intracellular pathogen that continues to evolve in association with mammalian hosts and thereby has acquired mechanisms to interfere with host immune signaling pathways. In contrast, *L. pneumophila* is an organism that normally lives in freshwater and soil and has coevolved with unicellular protozoan hosts, so it would be less likely to interfere with signaling pathways unique to mammalian hosts. Thus, the enhanced importance of Rip2 signaling in the host response to *C. pneumoniae* compared to the response to *L. pneumophila* may reflect differences in the ability of these two organisms to modulate innate immune pathways rather than fundamental differences in the role Rip2 plays in responding to pathogen infection.

Rip2 signaling was found to be very important in host protection in MyD88-deficient mice. MyD88 and Rip2 signaling both converge on the NF-κB activation pathway. Thus, it is not unexpected that Rip2 and MyD88 would have partially overlapping functions. An important difference between these two pathways lies in the mechanism by which they are activated in response to infection by *L. pneumophila*. MyD88-dependent NF-κB activation occurs after TLR engagement by either virulent or avirulent *L. pneumophila*. In contrast, Rip2-dependent NF-κB activation occurs after intracellular infection by *L. pneumophila* encoding a functional Dot/Icm type IV secretion system (52), which presumably translocates into the cytosol bacterial products that activate the PRRs Nod1 and Nod2. Thus, the MyD88 and Rip2 signaling systems cooperate with each other to mediate sustained activation of NF-κB during the course of macrophage infection by virulent *L. pneumophila* and enable the host to distinguish between nonpathogenic microbes and pathogens that have evolved mechanisms to establish direct communication with the host cytosol.

Although a defect in sustained NF-κB activation could account for the difference observed in the bulk levels of IL-6 and MCP-1 in the lungs of infected Rip2-deficient mice, it seems unlikely that the main role for Rip2 would be to increase the cellular output of cytokines. Rather, it seems more likely that Rip2 signaling provides a mechanism that allows infected cells to maintain a cytokine gradient that would enable immune effector cells to migrate efficiently to the location where infected macrophages reside. We hypothesize that for an intracellular pathogen like *L. pneumophila*, which completes a cycle of growth in macrophages rather quickly, the contribution of Rip2 in maintaining a cytokine gradient may not be as important because bacteria will be unable to evade MyD88-dependent detection for an extended period of time. Our hypothesis predicts a more critical role for Rip2 signaling in host protection against pathogens that persist intracellularly for extended periods of time and replicate slowly. This might also contribute to the more pronounced role for Rip2 in mediating protection against *C. pneumoniae.*

Overall, these data suggest that the innate immune response to *L. pneumophila* can be divided into three phases. Initially, macrophages are infected by *L. pneumophila* and initiate the production of cytokines. Previous studies and data using chimeric mice presented here suggest that MyD88, Rip2, and Naip5/NLRC4 all participate in this early process of pathogen recognition by macrophages. Cytokine production by macrophages is important for the recruitment of additional immune effector cells into the lung and for the activation of IFN-γ by NK and NK T cells recruited to the site of infection (5, 54). MyD88 and Rip2 signaling were shown here and in previous studies (5, 6, 20, 52, 54) to mediate responses important for upregulation of cytokine expression, and Naip5/NLRC4 signaling contributes to the processing and secretion of several cytokines. The second phase involves restricting the intracellular replication of *L. pneumophila* in macrophages, which both MyD88 and Naip5/NLRC4 appear to have a direct role in mediating. Activation of macrophages by IFN-γ is clearly one of the most potent and critical effector responses that limit intracellular replication of *L. pneumophila* in macrophages (8, 42), and the most abundant source of IFN-γ produced during infection comes from NK and NK T cells (5, 54). IFN-γ production by these cells involves MyD88-dependent signaling in response to IL-18 (5), which is secreted by macrophages and neutrophils after Naip5/NLRC4-mediated caspase-1 activation

(10, 55). Naip5/NLRC4 also plays a role in activating a cell-autonomous response that limits replication in infected macrophages, but the molecular mechanism by which this occurs remains unknown. The third phase of the innate immune response to *L. pneumophila* is characterized by the recruitment of neutrophils to the site of infection. *L. pneumophila* is unable to replicate within neutrophils, and these highly chemotactic and phagocytic cells play an important role in eliminating extracellular *L. pneumophila* (32). MyD88-dependent production of chemokines contributes significantly to neutrophil recruitment. Rip2-deficient mice infected with *L. pneumophila* were also found to have a defect in chemokine production, and there was a delay in the recruitment of neutrophils to the lung, suggesting that Rip2 and MyD88 signaling plays a cooperative role in directing neutrophils to the site of infection.

In conclusion, these data provide a detailed picture of how different pattern recognition systems function coordinately to generate a robust and efficient innate immune response capable of limiting the replication of an intracellular pathogen. The susceptibility phenotypes observed for immune-deficient mice could be explained by modeling the overall contribution of these pattern recognition receptor systems to the generation of the three phases of a protective immune response. The increase in the total number of *L. pneumophila* bacteria observed in mice deficient in MyD88 or Naip5/NLRC4 signaling would result primarily from an inability of macrophages to restrict bacterial intracellular replication. IFN-γ production in mice deficient in Naip5/NLRC4 signaling eventually compensates for the defect that macrophages from these mice have in restricting intracellular replication of *L. pneumophila*, which explains why bacterial numbers eventually decline in the lung of mice infected with the Δ*flaA* strain when MyD88 signaling remains operational. In the absence of both MyD88 and Naip5/NLRC4 signaling, pattern recognition receptors that signal through Rip2 provide a measure of host protection by assisting in the recruitment of neutrophils. Rapid neutrophil recruitment mediated by Rip2 signaling served to help contain the infection in the lung and prevent progression to a systemic disease that results in host mortality, resulting in mice that showed persistent lung colonization but remained viable 3 weeks after infection.

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