Myeloid Differentiation Primary Response Gene (88)– and Toll-Like Receptor 2–Deficient Mice Are Susceptible to Infection with Aerosolized *Legionella pneumophila*

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Background. Toll-like receptors (TLRs) are a family of proteins that orchestrate innate immune responses to microbes. Although pathogens are typically recognized by multiple TLRs, the specific roles of individual TLRs in mediating host protection during in vivo infection are not well understood.

Methods. We compared the roles of myeloid differentiation primary response gene (88) (MyD88), which regulates signaling through multiple TLRs, and TLR2 in mediating resistance to aerosolized *Legionella pneumophila* infection in vivo.

Results. In comparison with wild-type mice, MyD88-deficient ($MyD88^{-/-}$) mice had dramatically higher bacterial counts in the lungs, with decreased neutrophil counts in the bronchoalveolar lavage fluid as well as absent cytokine and chemokine production at early time points. By day 6 of infection, the $MyD88^{-/-}$ mice developed organizing pneumonia with dissemination of *L. pneumophila* to the lymph nodes and spleen. $TLR2^{-/-}$ mice were also more susceptible to *L. pneumophila,* with higher bacterial counts in the lung. However, $TLR2^{-/-}$ mice produced proinflammatory cytokines, recruited neutrophils to the lung alveoli, and cleared the infection without progression to organizing pneumonia and disseminated disease.

Conclusions. These results suggest that a MyD88-dependent pathway is required for eradication of *L. pneumophila* and prevention of organizing pneumonia. TLR2 mediates partial resistance to *L. pneumophila,* which indicates that additional MyD88-dependent, TLR2-independent pathways are essential for full protection.

Although lower respiratory tract infections cause significant morbidity and mortality in the United States, the immunologic factors that mediate host susceptibility to pneumonia are poorly understood [1]. Toll-like receptors (TLRs) constitute a family of transmembrane proteins that differentially recognize pathogen-associated molecular patterns and initiate inflammatory signaling pathways [2–5]. Several recent in vivo pneumonia studies have demonstrated that TLRs are critical regulators of the pulmonary immune response [6–9]. We have found that myeloid differentiation primary response gene (88) (MyD88), an adaptor protein in the TLR pathway, is essential for pulmonary host defense against *Pseudomonas aeuruginosa* but not *Staphylococcus aureus* [10]. The results of our study suggested that the consequences of MyD88 deficiency are pathogen and tissue specific. Together, these initial studies indicate a critical role for TLRs in pulmonary innate immunity; yet, a number of important questions remain unanswered. First, what are the relative roles of individual TLRs in mediating lung immune responses? Second, which TLR-dependent effector molecules and cellular mechanisms are the critical mediators of immunity? Finally, what are the roles of MyD88-dependent and -independent pathways in regulating the in vivo immune response?

Legionella pneumophila is a flagellated gram-negative bacterium that causes community-acquired pneumonia [11, 12]. *L. pneumophila* has several features that distinguish it from other pulmonary pathogens, including intracellular replication in macrophages, a flagellum,

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an unusual lipopolysaccharide (LPS) structure, and fastidious growth requirements [11, 13]. These features suggest that the immune response to *L. pneumophila* will differ in comparison with those to other lung pathogens. Previous data have indicated a role for interferon (IFN)–γ, tumor necrosis factor (TNF)–α, interleukin (IL)–12, and nitric oxide in host defense against *L. pneumophila* during in vivo infection [14–19]. Murine macrophages exhibit levels of in vitro resistance to *Legionella* replication that are dependent on the strain of mouse, ranging from the highly susceptible A/J strain to the resistant C57Bl/6 strain. We and others have used in vitro studies to demonstrate that *L. pneumophila* is recognized by several TLRs, including TLR2, TLR5, and probably also TLR4 [13, 20–23]. By means of human genetic studies, we recently found that polymorphisms in TLR4 and TLR5 are associated with altered susceptibility to legionnaires disease [20, 24]. Although murine in vitro studies suggest that TLR4 mediates recognition of heat-killed *L. pneumophila* in dendritic cells, in vivo studies have not indicated a prominent role for TLR4 in controlling bacterial clearance or inflammation [21, 25]. The role of TLR2 in mediating protection against in vivo *L. pneumophila* infection has not, to our knowledge, been examined. In the present study, we examined the roles of MyD88 and TLR2 in host protection, on a nonpermissive C57Bl/6 background, against murine in vivo *L. pneumophila* infection and hypothesized that these proteins regulate distinct aspects of the immune response.

MATERIALS AND METHODS

Model of pneumonic legionellosis. The University of Washington and the Institute for Systems Biology's Institutional Animal Care and Use Committees approved all animal protocols. MyD88-deficient (MyD88$^{-/-}$) and TLR2$^{-/-}$ mice were derived on a 129SvJ $\times$ C57Bl/6 background and backcrossed for 6–8 generations with C57Bl/6 [26, 27]. *L. pneumophila* strain Philadelphia 1 (ATCC 33152) bacteria were stored as described elsewhere [18, 19]. Buffered charcoal yeast extract (BCYE) medium was prepared with α-ketoglutarate as described elsewhere [28]. Bacteria were inoculated from a frozen stock onto BCYE agar for 4 days at 35°C, harvested by rinsing plates with PBS, pelleted by centrifugation, and resuspended in PBS to a concentration of 1×10^{10} cfu/mL (estimated by optical density at 540 nm and confirmed by quantitative culture) [20]. The mice were exposed to bacterial aerosols generated by twin jet nebulizers (Salter Labs) for 30 min in a whole-animal exposure chamber, as described elsewhere [10, 29]. Immediately after infection (to determine bacterial deposition) and at subsequent time points, mice were killed and exsanguinated. The trachea was cannulated, and the right lung was lavaged with 0.85% saline/0.6 mmol/L EDTA to determine cell counts and differentials, as described elsewhere [9]. The left lung was homogenized in PBS, and the spleen was homogenized in Mueller-Hinton broth; each tissue was then serially diluted in Mueller Hinton broth for quantitative culture on BCYE agar. The remaining lung homogenate was mixed 1:1 with lysis buffer containing 2$\times$ protease inhibitor cocktail (Roche Diagnostics), incubated for 30 min on ice, and clarified by centrifugation at 1000 *g;* the supernatant was then saved at -80°C.

Histologic analysis. To prepare organs for histologic analysis, the lung was inflated to 15 cm of pressure with 4% paraformaldehyde, fixed in the same solution, and embedded in paraffin, and then 4-μm sections were generated. Sections stained with hematoxylin-eosin were examined by a pathologist blinded to mouse genotype. The quantification of the percentage of inflamed lung was derived from the examination 10 high-power fields. For immunohistochemical analysis, an indirect immunoperoxidase technique was used. Deparaffinized sections were sequentially incubated with the primary antibody (rabbit anti-*Legionella* antibody [Biodesign] or control rabbit IgG antibody [Santa Cruz Biotechnology]), the secondary biotinylated goat anti-rabbit antibody (Vector Laboratories), and, finally, with avidin-biotin–horseradish peroxidase (ABC-Elite) complex (Vector). The immunoreaction was visualized by use of 3,3′-diaminobenzidine (Sigma) with nickel chloride enhancement.

Protein analysis. Cytokine levels in lung homogenates were determined in a multiplex fluorescent bead array system. Analytes were captured by antibody-coated, fluorochrome-embedded microspheres and detected by biotin-streptavidin-phycoerythrin, using reagents purchased from R&D Systems and a flow-based sorting and detection platform (Luminex 100).

Statistical analysis. Comparisons between groups were made with a Mann-Whitney *U* test, because small sample sizes precluded an assumption of a normal distribution. $P \leqslant .05$ was considered to be significant. Statistics were calculated with SPSS software (version 11.5.0; SPSS).

RESULTS

Failure to control* L. pneumophila *pneumonia in MyD88$^{-/-}$ mice. To assess the role of the TLR pathway during in vivo infection, we infected wild-type C57Bl/6 mice and MyD88$^{-/-}$ mice with aerosolized *L. pneumophila.* After infection, wild-type mice cleared *L. pneumophila* from the lungs (figure 1*A*). In contrast, not only did MyD88$^{-/-}$ mice fail to clear *L. pneumophila* from the lungs, but they permitted net bacterial replication. By 144 h after infection, the median bacterial counts in the lungs differed by >4 $\log_{10}$ between the 2 groups (wild-type mice, 2.0×10^2 cfu/lung; MyD88$^{-/-}$ mice, 2.9×10^6 cfu/lung; $P = .06$, Mann-Whitney *U* test). Spleen cultures also demonstrated greater systemic dissemination of infection in the MyD88$^{-/-}$ mice than in wild-type mice (144 h after infection: wild-type mice, 0 cfu/spleen; MyD88$^{-/-}$ mice, 3.0×10^4 cfu/spleen; $P = .06$, Mann-Whitney *U* test). In addition, at the 144-h time point, 1 of 3 remaining MyD88$^{-/-}$ mice died, and

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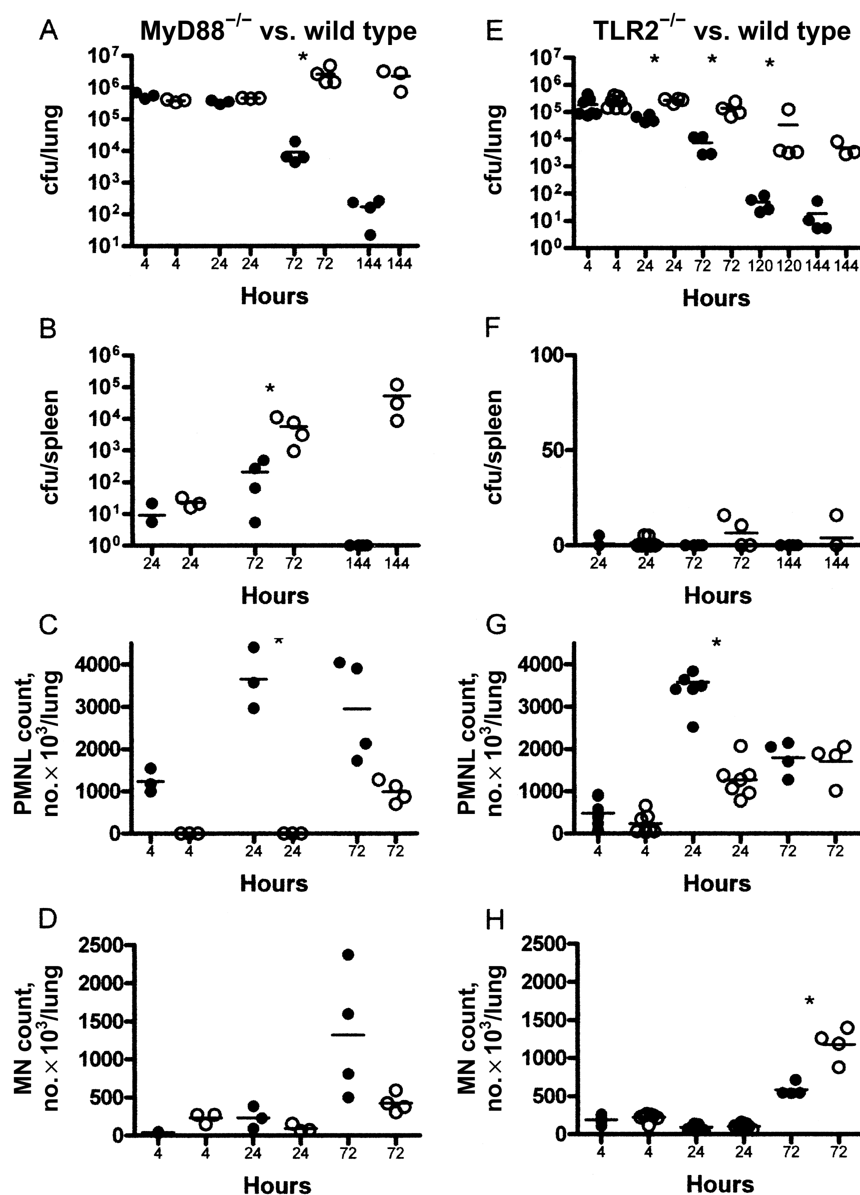

Figure 1. Greater susceptibility of myeloid differentiation primary response gene (88)–deficient (MyD88$^{-/-}$) and Toll-like receptor 2–deficient (TLR2$^{-/-}$) mice to in vivo infection with *Legionella pneumophila.* Mice were infected with aerosolized *L. pneumophila* and killed at the indicated time points, to determine the no. of bacterial colony forming units in lung and spleen. Bronchoalveolar lavage (BAL) specimens were obtained for cell counts. *A, B, E,* and *F,* Bacterial colony-forming units in lung (*A* and *E*) and spleen (*B* and *F*). *C, D, G,* and *H,* Cell counts in BAL, indicating neutrophils (*C* and *G*) and mononuclear cells (*D* and *H*). Data for each mouse are plotted with the median, represented by a horizontal bar. Results represent 1 experiment with MyD88$^{-/-}$ mice and the combined results of 2 experiments with TLR2$^{-/-}$ mice. In all panels, C57Bl/6 wild-type mice are denoted by black circles; white circles denote MyD88$^{-/-}$ mice in panels A–D and denote TLR2$^{-/-}$ mice in panels E–H. MNs, mononuclear cells; PMNLs, polymorphonuclear leukocytes. *$P \leq .05$, wild-type vs. knockout strains (Mann-Whitney *U* test).

the other 2 would have required euthanasia for inactivity and respiratory distress. Thus, the infection was ultimately lethal for MyD88$^{-/-}$ mice. In contrast, none of the wild-type mice died, and all appeared to be healthy at the end of the experiment. Two additional experiments demonstrated that MyD88$^{-/-}$ mice also failed to clear lower inocula of *L. pneumophila* (data not shown). These results indicate that MyD88 is required for the containment and elimination of *L. pneumophila* from the lungs.

Impaired pulmonary leukocyte recruitment and cytokine responses to* L. pneumophila *in MyD88$^{-/-}$ mice. We next obtained bronchoalveolar lavage (BAL) fluid from infected mice and determined its cellular composition. Early neutrophil recruitment was evident in BAL samples harvested from wild-type

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mice (figure 1*C*). In contrast, BAL samples from MyD88$^{-/-}$ mice contained very few neutrophils 4 or 24 h after infection (median polymorphonuclear leukocyte [PMNL] count at 24 h: wild-type, 3.6×10^6 cells/lung; MyD88$^{-/-}$, 1.25×10^3 cells/lung; $P \leqslant .05$, Mann-Whitney *U* test). Similarly, the accumulation of bronchoalveolar mononuclear cells 72 h after infection in wild-type mice was blunted or absent in MyD88$^{-/-}$ mice (figure 1*D*). Thus, MyD88 is required for early neutrophil recruitment and later mononuclear cell expansion after inhalation of *L. pneumophila.*

We next examined whether the altered recruitment of leukocytes to the lungs was associated with differences in intrapulmonary levels of cytokines and chemokines (figure 2). We examined a range of cytokines and chemokines that have previously been shown to be critical for the in vivo immune response to *L. pneumophila* or are known to be mediated by the TLR pathway. These included proinflammatory cytokines (TNF-α and IL-6); CXC chemokines that recruit neutrophils to the lungs (macrophage inflammatory protein [MIP]–2 and keratinocyte-derived cytokine [KC]); and IFN-γ, an NK cell– and T cell–derived cytokine that activates macrophages to eliminate intracellular pathogens. Although wild-type mice had detectable levels of TNF-α, IL-6, KC, and MIP-2 at 4 and 24 h after infection, the MyD88$^{-/-}$ mice did not produce any of these cytokines at levels significantly above the background levels found in uninfected mice. Because of small sample sizes, the differences in cytokine and chemokine levels did not reach statistical significance when the MyD88$^{-/-}$ and wild-type mice were compared. IFN-γ levels in MyD88$^{-/-}$ mice were not different from those in wild-type mice at any of the time points (figure 2*E*).

Development of organizing pneumonia after failure to contain* L. pneumophila *in the intra-alveolar space in MyD88$^{-/-}$ mice. We performed a histologic analysis of the lungs, to further understand the mechanisms of increased susceptibility in MyD88$^{-/-}$ mice (figure 3). At day 3, wild-type mice had leukocytic infiltrates in the bronchiolar lumina, interstitial peribronchial spaces, and alveolar spaces (figure 3*A*–3*C*). Most of the leukocytes were neutrophils, with smaller numbers of alveolar macrophages. The bronchiolar epithelial cells demonstrated an activated appearance, characterized by enlarged reactive nuclei and increased cytoplasm (figure 3*B*). At 6 days after infection, inflammation was less evident and had essentially resolved in the wild-type mice, in which there was only focal and minimal peribronchial inflammation; the bronchial epithelium had also returned to a less activated state, with smaller nuclei and less prominent cytoplasm (figure 3*J*–3*L*). At 3 days after infection, the MyD88$^{-/-}$ mice also had leukocytes in the interstitial peribronchial space (figure 3*D* and 3*E*). However, few inflammatory cells were in the alveolar spaces, consistent with the cell counts in BAL fluid (figure 3*F*). We quantified the percentage of inflamed lung at 6 days after infection and found that 3.3% of lungs from wild-type mice and 53% of lungs from MyD88$^{-/-}$ mice showed evidence of pneumonia. By 6 days after infection, the lungs of MyD88$^{-/-}$ mice demonstrated organizing pneumonia with dense leukocyte infiltrates in the peribronchial interstitial space, as well as in the bronchial lumina and alveolar spaces (figure 3*M*–3*O*). In addition, several air spaces were filled with fibrous and myxoid matrix material in association with numerous macrophages and fibroblasts—features that are characteristic of the organizing phase of pneumonia. Similarly, reactive changes persisted in the bronchial epithelium

We used immunohistochemical analysis with an anti–*L. pneumophila* antibody to localize the bacteria in the infected mice. In wild-type mice at 3 days after infection, *L. pneumophila* was found almost exclusively in alveolar macrophages within the alveoli, with minimal staining in the lymph nodes (figure 4*A*, 4*G*, and 4*M*). At 6 days after infection, few alveolar macrophages stained positive for *L. pneumophila,* which is consistent with the bacterial counts and clearing of the infection (figure 4*B*). In contrast to wild-type mice, MyD88$^{-/-}$ mice had widespread staining in the alveoli, bronchial lumen, interstitial peribronchial spaces, and lymph nodes at 3 days after infection (figure 4*D*, 4*J*, 4*N*, and 4*O*). The bacteria had disseminated beyond the alveoli into the lung interstitium and the mediastinal lymph node. At 6 days after infection, there was diffuse staining of the entire lung, as well as persistence of *L. pneumophila* in the lymph node (figure 4*E* and 4*K*). As a negative control, lung and lymph node sections stained with normal rabbit serum did not have any positive-staining cells (figure 4*C*, 4*F*, 4*I*, and 4*L*).

Overall, these results suggest that MyD88$^{-/-}$ mice have an early defect in neutrophil recruitment to the alveolar space and an inability to clear and contain the infection. *L. pneumophila* disseminates beyond the lung, causing persistent inflammation that culminates in the development of organized pneumonia at 6 days after infection. In contrast, wild-type mice have a robust initial response to infection with neutrophilic alveolar pneumonitis and are able to contain the infection within the alveolar space.

Higher susceptibility to pneumonic legionellosis—but no development of organizing pneumonia—in TLR2$^{-/-}$ mice. Since TLR2$^{-/-}$ macrophages have blunted cytokine responses in response to *L. pneumophila* stimulation, we hypothesized that TLR2$^{-/-}$ mice would manifest impaired resistance to *L. pneumophila* in vivo [22]. We found that elimination of *L. pneumophila* from the lungs was significantly delayed in TLR2$^{-/-}$ mice, in comparison with wild-type control mice (median bacterial count at 72 h after infection: wild-type, 7.4×10^3 cfu/lung; TLR2$^{-/-}$ 1.2×10^5 cfu/lung; $P \leqslant .05$, Mann-Whitney *U* test) (figure 1*E*). However, in contrast to MyD88$^{-/-}$ mice, TLR2$^{-/-}$ mice were able to partially clear bacteria from the lung and had only transient dissemination of bacteria to the spleen (fig-

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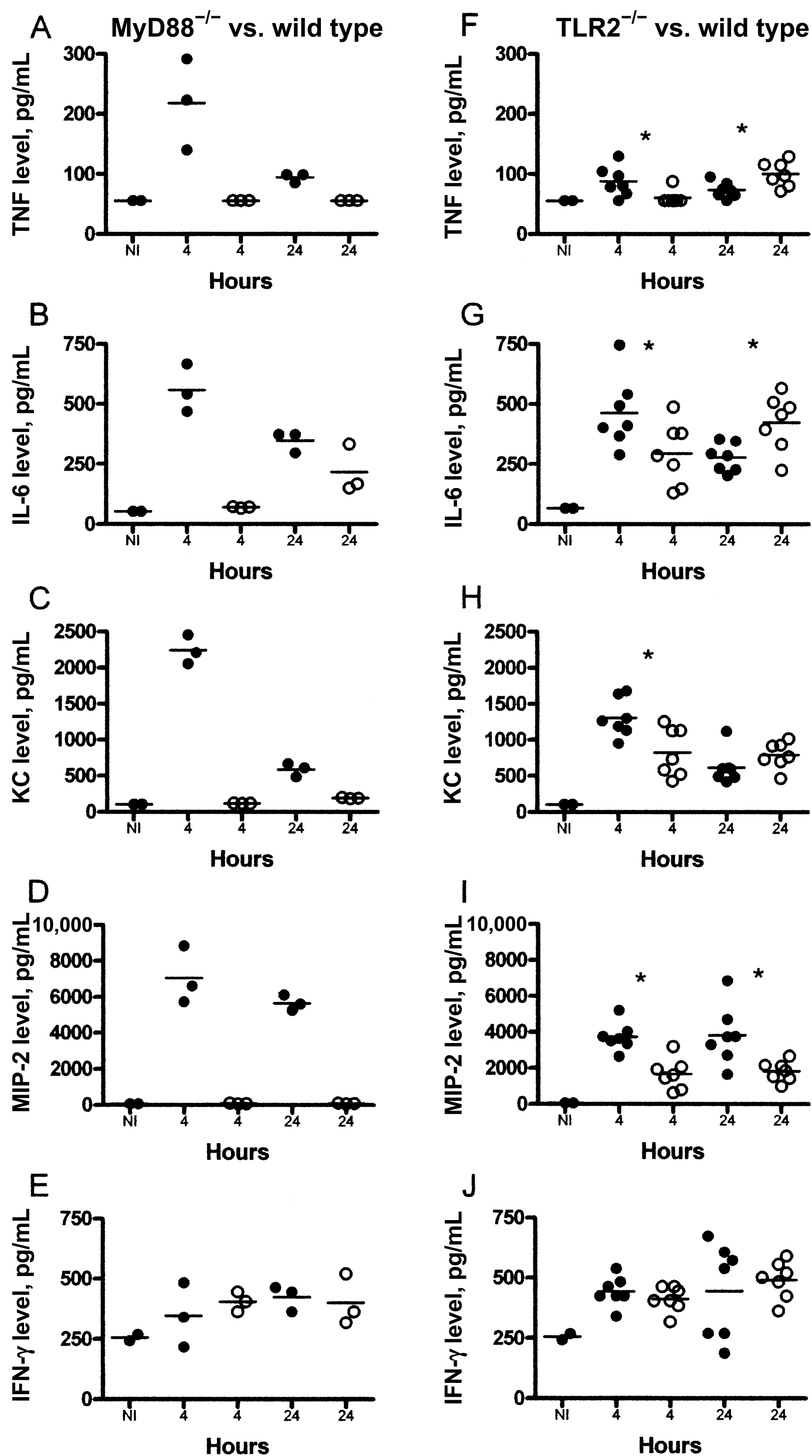

Figure 2. Cytokine and chemokine responses in myeloid differentiation primary response gene (88)–deficient (MyD88$^{-/-}$) and Toll-like receptor 2–deficient (TLR2$^{-/-}$) mice after inhalation of *Legionella pneumophila.* Lung homogenates were harvested after infection and assayed by multiplex microbead array for the following cytokines and chemokines: tumor necrosis factor (TNF)–α (*A* and *F*), interleukin (IL)–6 (*B* and *G*), keratinocyte-derived cytokine (KC) (*C* and *H*), macrophage inflammatory protein (MIP)–2 (*D* and *I*), and interferon (IFN)–γ (*E* and *J*). Data for each mouse (infected mice, $n = 3$–8; uninfected control mice [NI], $n = 2$) are plotted with the median represented by a horizontal bar. In all panels, C57Bl/6 wild-type mice are denoted by black circles; white circles denote MyD88$^{-/-}$ mice in panels A–E and denote TLR2$^{-/-}$ mice in panels F–J. $^*P \leqslant .05$, wild-type vs. knockout strains (Mann-Whitney *U* test).

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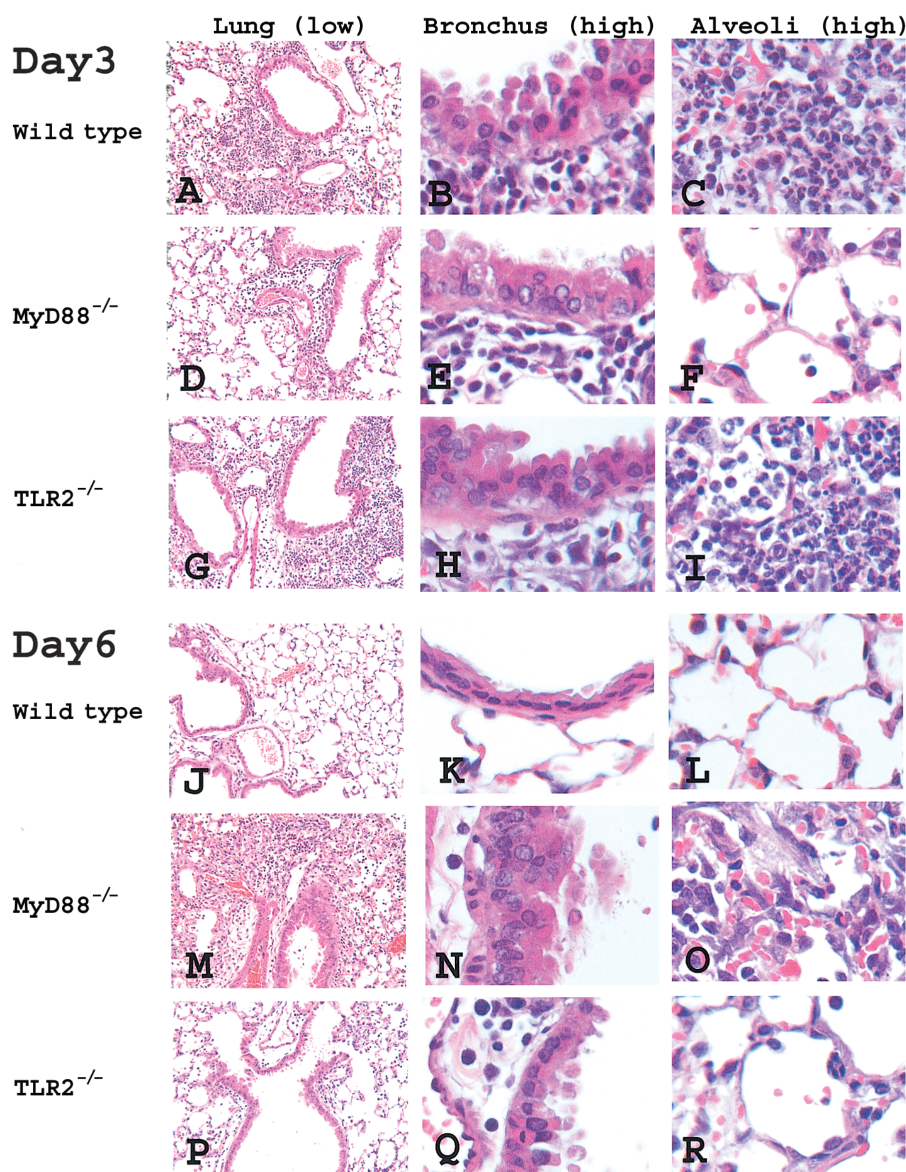

Figure 3. Histologic analysis of lungs from mice infected with *Legionella pneumophila.* Lung sections were stained with hematoxylin-eosin, as described in Materials and Methods. *A–I,* Lung sections obtained from mice after 3 days of infection. *J–R,* Lung sections obtained from mice after 6 days of infection. The mouse strain, relative magnification, and tissue type are labeled. Pictures are representative of histologic sections reviewed from at least 2 mice at each time point from at least 2 independent experiments. The low- and high-magnification designations refer to images taken with 20× and 40× objectives, respectively. The high-magnification images are printed at an image size that optimizes visualization of individual cells. $MyD88^{-/-}$, myeloid differentiation primary response gene (88) deficient; $TLR2^{-/-}$, Toll-like receptor 2 deficient.

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ure 1*F*). Neutrophil recruitment, as measured by BAL, was significantly impaired in $TLR2^{-/-}$ mice at 24 h after infection but not ablated as in $MyD88^{-/-}$ mice (figure 1*G*). Furthermore, expansion of the mononuclear cell population 72 h after infection was greater in $TLR2^{-/-}$ mice than in wild-type control mice (figure 1*H*).

Intrapulmonary cytokine responses to *L. pneumophila* infection were blunted in $TLR2^{-/-}$ mice. Lung homogenate TNF-α, IL-6, MIP-2, and KC levels were reduced at 4 h after infection in the $TLR2^{-/-}$ mice, compared with those in wild-type mice (figure 2*F*–2*J*). At 24 h after infection, IL-6 and MIP-2 responses were blunted in $TLR2^{-/-}$ mice, but TNF-α levels were significantly higher in $TLR2^{-/-}$ mice than in wild-type mice. Overall, there was a partial defect in cytokine production in $TLR2^{-/-}$ mice.

To further understand the mechanism of susceptibility in $TLR2^{-/-}$ mice, we performed a histologic analysis of the lungs. In contrast to $MyD88^{-/-}$ mice, $TLR2^{-/-}$ mice did not have a significant neutrophil recruitment defect in the intra-alveolar space at 3 days after infection. Similar to wild-type mice,

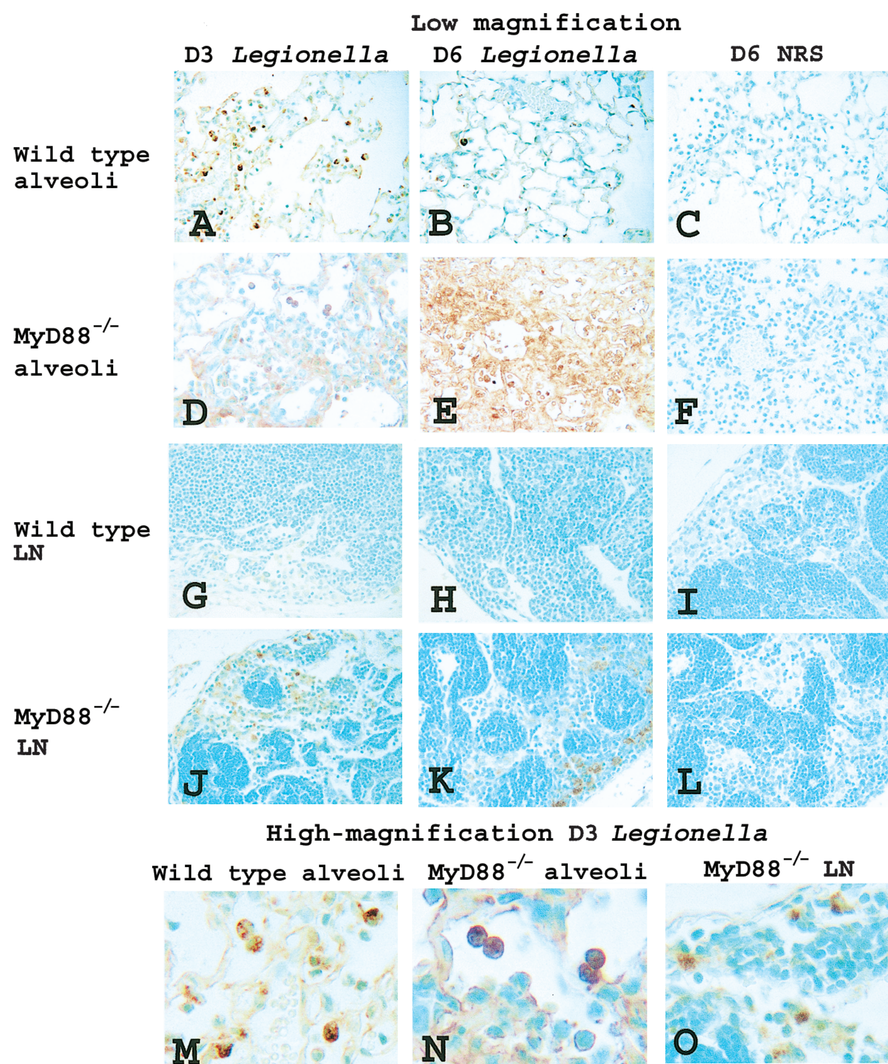

Figure 4. Immunohistochemical analysis of lungs and lymph nodes (LNs) of mice infected with *Legionella pneumophila.* Lung and LN sections from infected mice were stained with anti–*L. pneumophila* antibody (*A, B, D, E, G, H, J, K, M, N,* and *O*) or normal control rabbit serum (*C, F, I,* and *L*). Positive-staining cells are brown. The high-magnification images in panels M, N, and O are contained within the images from panels A, D, and J, respectively. The mouse strain, relative magnification, and tissue type are labeled. Pictures are representative of histologic sections reviewed from at least 2 mice at each time point from at least 2 independent experiments. *L. pneumophila* immunoreaction is indicated by a brown-colored product, and the cells are counterstained with methyl green. MyD88$^{-/-}$, myeloid differentiation primary response gene (88) deficient; NRS, normal rabbit serum; TLR2$^{-/-}$, Toll-like receptor 2 deficient.

TLR2$^{-/-}$ mice had leukocytic infiltrates in the bronchiolar lumina, peribronchial interstitial space, and alveoli (figure 3*G*–3*I*). By 6 days after infection, there were some areas of persistent inflammation in TLR2$^{-/-}$ mice but no evidence of organizing pneumonia. We quantified the percentage of inflamed lung at 6 days after infection and found that 3.6% of wild-type lung tissue and 9.9% of TLR2$^{-/-}$ lung tissue showed parenchymal involvement. Together, these results suggest that TLR2$^{-/-}$ mice are more susceptible than wild-type mice to airborne infection with *L. pneumophila*. However, the phenotype of TLR2$^{-/-}$ mice differs from that of MyD88$^{-/-}$ mice, since recruitment of neutrophils and mononuclear cells to the alveoli is still intact in TLR2$^{-/-}$ mice. In addition, TLR2$^{-/-}$ mice are able to clear the infection and do not develop organizing pneumonia.

DISCUSSION

Although the innate immune system is essential for defending the host against microbes, the degree of redundancy and specificity manifested in vivo among different TLR family members is only partially understood. In this study, we have demonstrated that both MyD88 and TLR2 mediate in vivo host resistance to *L. pneumophila* infection. However, the phenotypes of these 2 mouse strains were different. The immune system

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of MyD88$^{-/-}$ mice failed to recognize *L. pneumophila* in the lungs, and the mice ultimately developed organizing pneumonia by day 6 of infection. In contrast, the TLR2$^{-/-}$ mice had less severely blunted inflammatory responses and were able to contain the infection, albeit with delayed bacterial clearance. Interestingly, the MyD88$^{-/-}$ mice had both a quantitative decrease in PMNL recruitment and a localization defect. The neutrophils in the MyD88$^{-/-}$ mice were predominantly found in the interstitial and peribronchial spaces but not in the alveoli. The absence of intra-alveolar neutrophils was associated with low intrapulmonary levels of the CXC chemokines MIP-2 and KC. In contrast, the TLR2$^{-/-}$ mice had only partially reduced numbers of neutrophils in BAL fluid, no defect of PMNL migration to the alveoli, and no defect in accumulation of mononuclear cells. Together, these results suggest that both TLR2 and MyD88 are important mediators of host resistance to *L. pneumophila* infection. However, the different phenotypes in these mice also indicate that TLR2 is not solely responsible for MyD88-dependent resistance to *L. pneumophila* infection. These data imply that MyD88-dependent, TLR2-independent pathways contribute to cytokine responses, lung inflammation, and bacterial clearance of *L. pneumophila.*

Elucidating the distinct role of each TLR in the innate immune response is critical for understanding, at an in vivo level, how specific innate immune system defects affect the ability of the host to successfully control infections. The defective recognition and inflammatory responses observed in MyD88$^{-/-}$ mice could potentially be mediated by several TLRs. Previous in vitro studies have indicated that *L. pneumophila* is recognized by TLR2, TLR5, and probably also TLR4 [13, 20–22]. In addition, TLR9 may, potentially, recognize *L. pneumophila* through recognition of bacterial CpG DNA. Published studies have indicated that TLR4$^{-/-}$ mice are not significantly more susceptible to *L. pneumophila* infection and that they efficiently recruit leukocytes to the lungs [25]. This suggests that TLRs other than TLR2 or TLR4—or a combination of several TLRs—are required for neutrophil recruitment. TLR5 is a leading candidate for this role, since we have previously shown that TLR5 recognizes *L. pneumophila* and that human TLR5 deficiency is associated with an increased risk of legionnaires disease [20].

Although *L. pneumophila* is a gram-negative bacterium with LPS, our studies and those of others suggest that TLR2 is more important than TLR4 in mediating innate immune responses to this pathogen in vivo. Previous in vitro studies have indicated that *L. pneumophila* has an atypical LPS structure that may be recognized by TLR2 [13, 20, 22]. It is unusual for a gram-negative bacterium to be predominantly recognized by TLR2 rather than by TLR4, and the in vivo significance of such findings has not been previously described. Our data provide the first in vivo evidence, to our knowledge, that TLR2 mediates essential aspects of the host innate immune response to a gram-negative bacterium. These findings may have implications for other gram-negative bacteria, such as those of the genus *Leptospira,* that have atypical LPS structures that are recognized by TLR2 [30]. Although our murine data and those of others suggest a stronger role for TLR2 than TLR4 in the host response to *L. pneumophila,* we recently demonstrated an association between TLR4 polymorphisms and resistance to legionnaires disease in humans [24]. In addition, Kikuchi et al. [21] found that TLR4 mediates IL-12 production in vitro in murine dendritic cells stimulated with heat-killed *L. pneumophila.* These studies suggest that findings from murine in vivo model systems may not fully recapitulate the role of the same receptors in human innate immunity.

We chose to study the roles of TLR2 and MyD88 in C57Bl/6 mice, which are highly resistant to *L. pneumophila,* to discover genes that can alter this nonpermissive phenotype. Remarkably, the bacterial load of *L. pneumophila* during in vivo infection in MyD88$^{-/-}$ mice was not just increased in comparison with that in wild-type mice but also showed net growth over time (figure 1). Resistance to legionellosis correlates with reduced permissiveness of macrophages for intracellular replication of *L. pneumophila* [31]. In macrophages from C57Bl/6 mice, replication of *L. pneumophila* initiates poorly, proceeds slowly or terminates early, and is associated with premature cell death, in comparison with permissive macrophages from A/J mice [31–36]. Murine macrophage resistance to *L. pneumophila* is controlled by the *Birc1e/Naip5* gene within the Lgn-1 locus on chromosome 13 [33–36]. Although these studies suggest an important role for *Naip5* in the regulation of *L. pneumophila* growth in murine macrophages, there is evidence that additional genes, such as those encoding TLRs, regulate *L. pneumophila* replication independently of Lgn-1. Peritoneal macrophages from C3H/HeJ TLR4 mutant mice were less resistant to *L. pneumophila* than were those from wild-type mice in one study [23]. A separate study that showed that bone marrow–derived macrophages from TLR2$^{-/-}$ mice on a mixed C57BL/6–129 background were permissive for replication of *L. pneumophila,* whereas those from wild-type littermate control mice and TLR4$^{-/-}$ mice were not [22]. The molecular basis for permissive growth of *L. pneumophila* in human macrophages is unknown. Together, these findings suggest that multiple genes regulate the growth of *L. pneumophila* in macrophages in both humans and mice and that studying *L. pneumophila* infections on both permissive and nonpermissive murine backgrounds is essential to the discovery of the full set of genes that regulate a successful immune response. Our data suggest that MyD88 and TLR2 are 2 of these genes in the context of a nonpermissive background.

The molecular and cellular mechanisms underlying the extreme susceptibility of MyD88$^{-/-}$ mice to *L. pneumophila* infection are only partially understood. Previous studies have

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suggested that IFN-γ is critical for an effective immune response to *L. pneumophila* [17, 37–39]. Interestingly, IFN-γ was not decreased in MyD88$^{-/-}$ mice (figure 2), suggesting that the increased susceptibility of MyD88$^{-/-}$ mice may be unrelated to IFN-γ production. The significant difference in the neutrophil recruitment defect observed between MyD88-deficient mice and TLR2-deficient mice and the associated difference in outcome suggests that MyD88-dependent polymorphonuclear leukocyte recruitment is one essential mechanism. These data are consistent with those of previous studies that suggest a critical role for neutrophils during *L. pneumophila* infection [40, 41]. Furthermore, this neutrophil defect is likely to be related to the decreased production of MyD88-dependent chemokines, such as KC. Further experiments will be required to fully delineate the different MyD88-dependent mechanisms that contribute to increased susceptibility to *L. pneumophila* infection.

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