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Multiple MyD88-dependent responses contribute to pulmonary clearance of *Legionella pneumophila*

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Abstract

MyD88-dependent signaling is important for secretion of early inflammatory cytokines and host protection in response to *Legionella pneumophila* infection. Although TLR2 contributes to MyD88-dependent clearance of *L. pneumophila*, TLR-independent functions of MyD88 could also be important. To determine why MyD88 is critical for host protection to *L. pneumophila* the contribution of multiple TLRs and IL-18 receptor (IL-18R)-dependent IFN-γ production in a mouse. Mice deficient for TLR5 or TLR9, or deficient for TLR2 along with either TLR5 or TLR9, were competent for controlling bacterial replication and had no apparent defects in cytokine production compared to control mice. MyD88-dependent production of IFN-γ in the lung was mediated primarily by NK cells and required IL-18R signaling. Reducing IFN-γ levels did not greatly affect the kinetics of *L. pneumophila* replication or clearance in infected mice. Additionally, IFN-γ-deficient mice did not have a susceptibility phenotype as severe as the MyD88-deficient mice and were able to control a pulmonary infection by *L. pneumophila*. Thus, MyD88-dependent innate immune responses induced by *L. pneumophila* involve both TLR-dependent responses and IL-18R-dependent production of IFN-γ by NK cells, and these MyD88-dependent pathways can function independently to provide host protection against an intracellular pathogen.

Introduction

The mammalian innate immune system has the capacity to rapidly sense and respond to invading microorganisms and represents a first line of defense that provides protection from potentially lethal infections (Janeway and Medzhitov, 2002). Host pattern recognition receptors, such as Toll-like receptors (TLRs), initiate innate immune responses by detecting conserved molecular patterns on microbes (Janeway and Medzhitov, 2002). TLRs propagate signaling cascades that control transcriptional activation of genes encoding early inflammatory cytokines, maturation of dendritic cells, and the initiation of the adaptive immune response (Medzhitov *et al.*, 1997). The intricate details on how most pathogens are recognized by the TLR system and the downstream effector responses that ultimately lead to pathogen clearance remain poorly understood.

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The Gram-negative bacterium *Legionella pneumophila* is a model organism used to investigate the initiation of innate immunity to intracellular bacterial pathogens. *L. pneumophila* is ubiquitous in the environment and lives as a parasite of freshwater protozoa (Fields, 1996). When aerosols containing *L. pneumophila* are inhaled by humans, bacteria that enter the lung can infect and replicate within alveolar macrophages (Horwitz and Silverstein, 1980; Fraser *et al.*, 1977; McDade *et al.*, 1977). *L. pneumophila* infections can result in a severe pneumonia known as Legionnaires' disease (Fraser *et al.*, 1977).

L. pneumophila survival inside macrophages requires the Dot/Icm secretion apparatus, a type IV secretion system that is essential for intracellular replication and pathogenesis (Roy *et al.*, 1998; Segal *et al.*, 1998; Vogel *et al.*, 1998; Marra *et al.*, 1992). The Dot/Icm secretion apparatus is used to deliver bacterial proteins into the cytosol of the host cell (Luo and Isberg, 2004; Nagai *et al.*, 2002) that modulate transport of the *L. pneumophila*-containing vacuole by preventing the rapid fusion of this compartment with host endosomes and promoting vacuole fusion with vesicles of the host secretory pathway (Kagan and Roy, 2002). *L. pneumophila* replicate within an endoplasmic reticulum-derived vacuole, are released after consuming the host cell, and this cycle is repeated following bacterial uptake by neighboring macrophages (Derre and Isberg, 2004; Kagan *et al.*, 2004; Kagan and Roy, 2002; Swanson and Isberg, 1995; Horwitz, 1983).

L. pneumophila have the capacity to replicate to high numbers inside macrophages, however, most immune-competent hosts efficiently eliminate *L. pneumophila* from the lung, which is why most infections are asymptomatic. In mice, replication of *L. pneumophila* in the lung is usually suppressed after two days of infection (Archer and Roy, 2006; Brieland *et al.*, 1994), suggesting that the innate immune response is highly efficient at controlling the infection. Macrophages that encounter *L. pneumophila* produce IL-12 and IL-18, cytokines that play a role in activating natural killer (NK) and NK T cells to produce interferon-gamma (IFN-γ) (Nagarajan and Kronenberg, 2007; Nakanishi *et al.*, 2001; Lauwerys *et al.*, 2000; Zhang *et al.*, 1997). Host resistance to *L. pneumophila* infection involves the production of IFN-γ (Archer and Roy, 2006; Sporri *et al.*, 2006; Brieland *et al.*, 2000), which activates numerous antimicrobial pathways in macrophages that are effective at controlling *L. pneumophila* replication and killing intracellular bacteria (Neild *et al.*, 2005; Nash *et al.*, 1988; Bhardwaj *et al.*, 1986). IFN-γ-deficient and IFN-II-receptor-deficient mice have defects in controlling *L. pneumophila* replication in the lung (Shinozawa *et al.*, 2002), further suggesting that activated macrophages are involved in host protection.

Initiation of the innate immune response to *L. pneumophila* is predicted to involve TLRs that respond to microbial products. Multiple TLR proteins have been implicated in the innate immune response to *L. pneumophila* (Newton *et al.*, 2007; Archer and Roy, 2006; Hawn *et al.*, 2006; Akamine *et al.*, 2005; Hawn *et al.*, 2003; Girard *et al.*, 2002). TLR2-dependent recognition of bacterial peptidoglycan and lipopeptides has been shown to play a significant role in stimulating a protective response that limits *L. pneumophila* replication in mice (Archer and Roy, 2006; Hawn *et al.*, 2006). Infected TLR2-deficient mice were shown to have significantly higher numbers of *L. pneumophila* in the lung compared to TLR2-sufficient mice (Archer and Roy, 2006; Hawn *et al.*, 2006). Higher bacterial loads corresponded with reduced cytokine levels in bronchoalveolar lavage fluid (BALF) taken from the lung of TLR2-deficient mice and reduced cytokine production by TLR2-deficient macrophages infected *ex vivo* (Archer and Roy, 2006; Hawn *et al.*, 2006). In contrast, TLR4-mediated responses do not appear to be important for host detection of *L. pneumophila* (Archer and Roy, 2006; Akamine *et al.*, 2005; Lettinga *et al.*, 2002). Previous studies have shown that the lipid A moiety of *L. pneumophila* lipopolysaccharide (LPS) is a weak TLR4 agonist (Girard *et al.*, 2002). Additionally, macrophages from TLR4-deficient mice show no defect in cytokine secretion when infected by *L. pneumophila* and the rate of

L. pneumophila clearance from the lung of TLR4-deficient mice is not significantly different from the rate determined for TLR4-sufficient mice (Archer and Roy, 2006; Lettinga *et al.*, 2002).

It is predicted that other TLR proteins are involved in the initiation of the innate immune response directed against *L. pneumophila*. It has been shown that TLR5 responds to *L. pneumophila* flagellin and that TLR5 contributes to an early stage of neutrophil recruitment to the lung in response to infection (Hawn *et al.*, 2007). TLR9 detection of released bacterial CpG DNA has been suggested to play a role in IL-12 p40 production by dendritic cells infected with *L. pneumophila* (Newton *et al.*, 2007). Thus, it is likely that *in vivo* regulation of the innate immune response to *L. pneumophila* involves the contribution of multiple TLR proteins as well as pathogen recognition receptors belonging to other protein families.

Consistent with the hypothesis that multiple TLR proteins are involved in the host response to *L. pneumophila* infection, it has been shown that mice lacking the adapter protein MyD88 are highly susceptible to *L. pneumophila* pulmonary infection (Archer and Roy, 2006; Hawn *et al.*, 2006), which is in contrast to TLR2-deficient animals that exhibit only a minor defect in bacterial clearance (Archer and Roy, 2006; Hawn *et al.*, 2006). MyD88-deficient mice have a defect in controlling *L. pneumophila* replication in the lung, which often leads to bacterial dissemination and death (Archer and Roy, 2006; Hawn *et al.*, 2006). MyD88 plays a critical role in the inflammatory cytokine response directed against *L. pneumophila*. Both macrophages and mice lacking MyD88 fail to produce IL-12 and IFN-γ in response to infection (Archer and Roy, 2006; Sporri *et al.*, 2006). Given the large difference between MyD88-deficient mice and TLR2-deficient mice in their ability to control *L. pneumophila* replication in the lung, here we set out to identify additional receptors that contribute to the MyD88-dependent responses important for pulmonary clearance of *L. pneumophila*

Results

L. pneumophila infection of TLR9-deficient mice

MyD88-deficient mice are more susceptible to pulmonary infection by *L. pneumophila* than TLR2-deficient mice (Archer and Roy, 2006; Hawn *et al.*, 2006), suggesting an important role for other TLRs in providing protection against *L. pneumophila* infection. TLR9-deficient mice and heterozygous littermates were infected intranasally with a clinical isolate of *L. pneumophila* to determine whether a difference requiring TLR9 in the MyD88-dependent innate immune response in the lung could be detected. Measurements of *L. pneumophila* colony forming units (CFUs) in the lung did not reveal any significant difference in the rate of bacterial replication or clearance when TLR9-deficient mice were compared with heterozygous TLR9-sufficient littermate control mice (Fig. 1A). Bacterial growth and clearance in both groups of mice were similar to that from A/J mice (data not shown). Inflammatory cytokine levels were measured in the BALF from infected mice at day 2 and day 3 post infection. No significant difference in IL-12 p40, IL-6, or IFN-γ was detected in the lung of *L. pneumophila*-infected TLR9-deficient mice compared to TLR9-sufficient controls (Fig. 1B). These results indicate that a TLR9-deficiency does not impact the overall magnitude of the MyD88-dependent inflammatory response stimulated during *L. pneumophila* infection.

Considering that TLR2 has been shown to have a measurable role in stimulating the innate immune response to *L. pneumophila*, the ability of TLR9-deficient mice to induce a response to *L. pneumophila* could result primarily from TLR2-mediated signaling. To elucidate whether TLR9 has an important *in vivo* role in stimulating innate immune responses to *L. pneumophila*, the gene deletions were combined to generate a mouse that was deficient in both TLR2 and TLR9. Thus, TLR9-dependent responses could be

uncoupled from TLR2-dependent responses. TLR2/9-deficient mice and TLR2-deficient littermate controls were infected intranasally with *L. pneumophila* and bacterial CFUs were measured over a 7-day period. TLR2/9-deficient mice exhibited no significant difference in bacterial loads in the lung compared to TLR2-deficient littermate control mice over the course of infection (Fig. 1C). As observed with the TLR9-deficient mice, levels of IL-12 p40, IL-6, and IFN-γ in BALF from TLR2/9-deficient mice at day 2 and day 3 post infection were similar to levels measured in TLR2-deficient littermates (Fig. 1D), suggesting that important TLR9-dependent cytokine responses are not being masked by stimulation of TLR2.

In a *Mycobacterium tuberculosis* mouse model, Bafica *et al*. found that TLR9-deficient mice exhibited a severe decrease in survival only when animals were challenged with high bacterial loads (Bafica *et al.*, 2005). Thus, we investigated whether TLR9 plays a role in host survival during high bacterial exposure. TLR2/9-deficient mice and TLR2-deficient littermates were infected with 10^7 CFU of *L. pneumophila* and mice were observed over a 10-day period (Fig. 1E). Mortality was low in both groups of mice and no increase in mortality was observed for mice deficient in both TLR2 and TLR9 compared to TLR2-deficient control mice (Fig. 1E). Surviving mice were sacrificed at day 10 post infection and bacterial CFUs were measured in lung lysates. No significant difference was observed between the two groups of mice (data not shown). These data suggest that either TLR9 signaling does not contribute greatly to the MyD88-dependent recognition and restriction of *L. pneumophila* growth in the lung of infected mice or that there are TLR proteins in addition to TLR2 that effectively mask the contribution of TLR9 to the innate immune response.

L. pneumophila infection of TLR5-deficient mice

To examine whether TLR5 contributes significantly to the innate immune response that limits *L. pneumophila* replication in the lung, TLR5-deficient mice and heterozygous littermates were infected intranasally with *L. pneumophila* and bacterial CFUs were measured over a 7-day period (Fig. 2A). No significant difference was detected in the number of *L. pneumophila* in the lung of TLR5-deficient mice compared to heterozygous littermates (Fig. 2A). The levels of IL-12 p40, IL-6, and IFN-γ measured in BALF at day 2 and day 3 post infection were also similar between the two groups of mice (Fig. 2B) and were similar to results from A/J mice (data not shown). These data indicate that TLR5 alone does not contribute substantially to a protective innate immune response against *L. pneumophila* infection.

Mice deficient in both TLR2 and TLR5 were generated to determine whether TLR2 was masking a protective TLR5-dependent response in the lung. Upon intranasal inoculation with *L. pneumophila*, we found that the kinetics of bacterial replication and clearance in the TLR2/5-deficient mice were similar to that of the control TLR2-deficient mice (Fig. 2C). In addition, no statistically significant differences were observed in the levels of IL-12, IL-6, or IFN-γ in the BALF isolated from the two groups of mice (Fig. 2D). Peritoneal macrophages deficient for TLR2 produced less TNF-α than TLR2-sufficient control cells, however, no difference was observed for macrophages deficient in TLR2 compared to macrophages deficient in both TLR2 and TLR5 (Fig. 2E). Infecting macrophages with a *L. pneumophila* strain lacking flagellin (Δ*flaA*) also did not affect cytokine production (Fig. 2E). These data indicate that either in the presence or absence of TLR2 signaling, *L. pneumophila* stimulation of TLR5 does not greatly impact the magnitude or profile of the innate immune response in the lung and that TLR5-deficient mice have no major defect in *L. pneumophila* resistance.

L. pneumophila infection in mice deficient in TLR2, TLR5, and TLR9 signaling

One possible explanation for the ability of TLR2/9 or TLR2/5 double knockout mice to control *L. pneumophila* replication in the lung is that TLR9 and TLR5 may play redundant roles in activating innate immune responses. Since the absence of flagellin will prevent TLR5 activation following *L. pneumophila* infection, we decided to test whether TLR2-deficient mice are more susceptible to *L. pneumophila* infection if signaling is eliminated from both TLR9 and TLR5 by infecting TLR2/9-deficient mice with a *L. pneumophila* Δ*flaA* mutant that does not produce flagellin. These data show that the TLR2/9-deficient mice and TLR2-deficient mice had equivalent numbers of the *L. pneumophila* Δ*flaA* mutant in the lung at all time points examined (Fig. 3A). Bacterial CFUs and IL-12 p40 levels were significantly higher in the lung of TLR2/9-deficient mice infected with the Δ*flaA* mutant of *L. pneumophila* compared to mice infected with the flagellin-producing strain at day 2 and day 3 post infection (Fig. 3B and 3C). It is unlikely that these differences result from a defect in TLR5 signaling because no dissimilarities were observed when bacterial clearance and cytokine production were analyzed in TLR2/5-deficient mice infected with flagellin-producing *L. pneumophila* and compared to TLR2-deficient control mice (Fig 2C and 2D). It has been previously reported that *L. pneumophila* flagellin is required for stimulation of an Ipaf-dependent pathway of caspase-1 activation (Amer *et al.*, 2006;Molofsky *et al.*, 2006;Ren *et al.*, 2006). Thus, the enhanced replication of the flagellin-deficient *L. pneumophila* and corresponding higher cytokine levels most likely results from the absence of caspase-1-mediated responses by infected macrophages in the lung. Cytokine production was also examined *ex vivo* using bone marrow-derived macrophages (BMMs) from TLR2/9-deficient mice and TLR2-deficient littermates infected with either *L. pneumophila* WT or the isogenic Δ*flaA* strain (Fig. 3D). In agreement with the *in vivo* data, there was no reduction in IL-12 p40 production by BMMs infected with the *L. pneumophila* Δ*flaA* mutant, and in fact higher cytokine levels were detected in the supernatants from macrophages infected with flagellin-deficient *L. pneumophila* (Fig. 3D). In addition, TNF-α production was similar for alveolar macrophages isolated from the lungs of mice deficient in both TLR2 and TLR9 following infection with either WT or Δ*flaA L. pneumophila* (Fig. 3E). Considering that MyD88-deficient macrophages do not produce detectable levels of IL-12 or TNF-α upon *L. pneumophila* infection (Archer and Roy, 2006;Hawn *et al.*, 2006), these data indicate that when signaling from the three principal TLRs predicted to respond to *L. pneumophila* is abrogated, MyD88-dependent production of several key inflammatory cytokines is still observed, which could in part explain why TLR2, TLR5, and TLR9 signaling is not essential for the MyD88-dependent pulmonary clearance of *L. pneumophila* and indicate that other TLR proteins or MyD88-dependent pathogen sensors are responding to *L. pneumophila* when TLR2, TLR5, and TLR9 signaling is eliminated.

L. pneumophila-induced IFN-γ production by NK cells in the lung requires MyD88

A second explanation for why mice lacking multiple MyD88-dependent TLRs predicted to be involved in *L. pneumophila* recognition remained resistant to infection and did not have a defect in clearance similar to mice deficient in MyD88 is that the severe defect observed in MyD88-deficient mice could be primarily due to the requirement for MyD88 in innate immune signaling pathways that do not require TLRs. The receptors for both IL-1 and IL-18 require MyD88 for signaling (Adachi *et al.*, 1998), and MyD88-dependent signaling through the IL-18 receptor (IL-18R) has been shown to be important for the production of IFN-γ by NK cells (Adachi *et al.*, 1998). Thus, MyD88-deficient mice might be highly sensitive to infection by *L. pneumophila* primarily due to a pronounced defect in IFN-γ production in the lung following infection. For this reason, the importance of MyD88-dependent IFN-γ production by NK cells in the lung was examined to determine if this is an event essential for pulmonary clearance of *L. pneumophila*.

Lymphocytes were examined by flow cytometry to determine whether IFN-γ production by NK cells in the lung during *L. pneumophila* infection required MyD88 signaling (Fig. 4A). IFN-γ-producing NK cells were abundant in the lung of control MyD88-sufficient mice but not in the lung of MyD88-deficient mice at day 2 post infection, indicating that MyD88 is important for *L. pneumophila* stimulation of IFN-γ production by NK cells in the lung (Fig. 4A). IL-18 was detected in the BALF of MyD88-deficient mice (Fig. 4B), indicating that the production and secretion of IL-18 in response to *L. pneumophila* infection occurs independently of MyD88. Higher IL-18 levels were likely a result of increased bacterial numbers in MyD88-deficient mice. Thus, the defect in IFN-γ production by NK cells in the lung of MyD88-deficient mice infected with *L. pneumophila* is not due to a defect in IL-18 production.

To test whether IL-18R signaling was required for IFN-γ production during *L. pneumophila* infection, IL-18R1-deficient and MyD88-deficient mice of a C57BL/6 background as well as control C57BL/6 mice were infected intranasally with the *L. pneumophila* Δ*flaA* mutant. The flagellin-deficient *L. pneumophila* strain was used to evade a cell death pathway in C57BL/6 mice triggered by the host proteins Naip5 and Ipaf. This cell death pathway was not operational in the MyD88- and TLR-deficient mice used in previous experiments because these mice were homozygous for the nonfunctional *Naip5* gene from A/J mice (Molofsky *et al.*, 2006; Ren *et al.*, 2006). These data show that IFN-γ levels are abrogated in the BALF of IL-18R1-deficient mice infected with *L. pneumophila* compared to the control mice (Fig. 5A, left panel), whereas, IL-12 p40 levels were not reduced (Fig. 5A, middle panel). Despite the large defect in IFN-γ production observed for the IL-18R-deficient mice, there was no significant increase in the number of *L. pneumophila* in the lung of these mice compared to control mice (Fig. 5A, right panel). Rather, a significant increase in IL-12 levels and a decrease in CFUs occurred in the IL-18R-deficient mice. In addition, the kinetics of L. pneumophila clearance from the lungs were similar in IL-18R1-deficient mice compared to WT mice over a 7-day period (Fig. 5B).

Analysis of cells in the BALF by flow cytometry confirmed that IFN-γ production by NK cells from infected IL-18R1-deficient mice was impaired (Fig. 5C). The defect in IFN-γ production by the IL-18R1-deficient NK cells, however, appeared to be less severe than the defect observed for MyD88-deficient NK cells, suggesting IL-18R1 signaling is important but not essential for *in vivo* production of IFN-γ in response to *L. pneumophila* infection (Fig. 5C). These data clearly indicate that while NK cells are major producers of IFN-γ in the lung during *L. pneumophila* infection and IL-18 signaling is required for optimal IFN-γ production by NK cells, the defect in IFN-γ production resulting from elimination of the IL-18 response does not have a significant impact on *L. pneumophila* clearance from the lung.

NK cell-independent clearance of *L. pneumophila* from the lung

There could be low levels of IFN-γ production by NK cells in the lung of IL-18R1-deficient mice that would be sufficient to activate alveolar macrophages, which might explain why the IL-18R1-deficient mice exhibited no difference in susceptibility to *L. pneumophila* infection compared to control mice. To further elucidate the role of NK cells in clearance of *L. pneumophila* from the lung, α-asialo GM1 was used to deplete NK cells prior to infection with *L. pneumophila*. Analysis of cells in the lung by flow cytometry confirmed that NK cells were efficiently depleted in mice treated with α-asialo GM1 (data not shown). The depletion of NK cells resulted in a significant reduction in the amount of IFN-γ in the lung of mice infected with *L. pneumophila* compared to non-depleted mice, providing independent evidence that NK cells produce the majority of IFN-γ during infection (Fig. 6A). Despite the reduction in IFN-γ, the numbers of *L. pneumophila* recovered from the lung of NK-depleted mice were similar to the numbers recovered from the non-depleted mice

(Fig. 6B). These data indicate that NK cells are not essential for clearance of *L. pneumophila* from the lung and suggest the existence of MyD88-dependent mechanisms of clearance that are independent of IFN-γ.

MyD88-dependent responses in the lung can protect against *L. pneumophila* infection independent of IFN-γ

To determine whether the MyD88-dependent mechanisms of *L. pneumophila* clearance from the lung require IFN-γ, we compared the replication of *L. pneumophila* in the lung of MyD88-deficeint mice and IFN-γ-deficient mice. Bacterial numbers were measured in the lung of C57BL/6-derived mice infected with the *L. pneumophila* Δ*flaA* strain (Fig. 7). Similar to what has been shown previously, there was robust replication of *L. pneumophila* in the lung of MyD88-deficient mice over the first several days and then bacterial numbers remained high over a 10-day period of infection (Fig. 7A). In control mice, *L. pneumophila* replication was less robust after infection and efficient bacterial clearance was observed. At most time points examined, the numbers of *L. pneumophila* recovered from the IFN-γ-deficient mice were significantly higher compared to the control WT mice and significantly lower compared to MyD88-deficient mice (Fig. 7A). *L. pneumophila* numbers in the spleens (Fig. 7B, left panel) and livers (Fig. 7B, right panel) of IFN-γ-deficient mice were significantly lower at day 3 and day 5 post infection compared to MyD88-deficient mice (Fig. 7B). At day 10 post infection *L. pneumophila* was not detected in the spleens and livers of IFN-γ-deficient mice, whereas, MyD88-deficient mice still showed signs of systemic bacterial infection. Additionally, most of the MyD88-deficient mice died between day 8 and day 10 post infection, whereas, mortality was low for the IFN-γ-deficient mice (Fig. 7C). These experiments were repeated using a clinical strain of *L. pneumophila*, and both WT mice and IFN-γ-deficient mice cleared the infection from the lung over a 10-day period, whereas, high levels of bacteria were maintained in the lung of MyD88-deficient mice (data not shown). These data demonstrate that defects in IFN-γ production were not solely responsible for the enhanced susceptibility of MyD88-deficient mice to pulmonary infection by *L. pneumophila*.

Discussion

The facultative intracellular pathogen *L. pneumophila* initiates an inflammatory response upon encountering alveolar macrophages in the lung (Hawn *et al.*, 2007; Archer and Roy, 2006; Hawn *et al.*, 2006; Brieland *et al.*, 1998; Brieland *et al.*, 1995). Multiple studies demonstrate that the production of inflammatory cytokines and subsequent clearance of *L. pneumophila* from the lung require signaling pathways controlled by MyD88 (Archer and Roy, 2006; Hawn *et al.*, 2006). In this study, we have investigated the role of MyD88 in the response to *L. pneumophila* in the lung and demonstrate that several signaling pathways utilize MyD88 to activate antimicrobial effector mechanisms that protect against infection with *L. pneumophila*.

We examined the contribution of TLR9 in the host response to *L. pneumophila* infection. TLR9 recognizes unmethylated CpG motifs from bacteria and viruses inside endocytic vesicles (Hemmi *et al.*, 2000), suggesting that TLR9 could play a significant role in the innate immune response by detecting DNA released from *L. pneumophila* that were unsuccessful in avoiding fusion with lysosomes. When comparing TLR9-deficient mice infected with *L. pneumophila* to littermate control mice, we were unable to detect a difference in the levels of cytokines in BALF or a defect in bacterial clearance. This was unexpected given previous results showing that bone marrow-derived dendritic cells and macrophages were unable to produce IL-12 p40 in response to *L. pneumophila* when the acidification of endosomes was inhibited using chloroquine (Newton *et al.*, 2007). Because treating cells with chloroquine can block TLR9-dependent recognition of CpG DNA

(Newton *et al.*, 2007), the suggestion from these inhibitor studies was that TLR9 could play an important role in *L. pneumophila* detection. Similar to previous results (Newton *et al.*, 2007), we have observed a defect in IL-12 p40 production by TLR2-deficient BMMs treated with chloroquine after infection with *L. pneumophila* (data not shown). Although IL-12 p40 production is decreased when TLR2-deficient BMMs are treated with chloroquine, here we show that IL-12 p40 production after *L. pneumophila* infection of BMMs deficient in both TLR2 and TLR9 was not significantly different compared to control TLR2-deficient BMMs, indicating that treatment of cells with chloroquine interferes with the detection of *L. pneumophila* products by pattern-recognition receptors other than TLR9.

TLR9-deficient mice in a BALB/c background were recently shown to be more susceptible to infection with *L. pneumophila* (Bhan *et al.*, 2008). The inability to detect a role for TLR9 in this study is likely due to the genetic background of the mice. BALB/c mice express an *lgn1* allele that restricts *L. pneumophila* replication in macrophages, which could impact the magnitude and course of infection and permit a role for TLR9 to be uncovered. The other important difference is that BALB/c mice mount a Th2-biased effector response that is less favorable for clearing most intracellular bacterial infections (Reiner and Locksley, 1995). Thus, it is possible that during *L. pneumophila* infection of BALB/c mice the contribution of a single TLR can be more readily detected in a virulence assay. Overall, these data indicate that TLR9 is stimulated during *L. pneumophila* infection, but the importance of TLR9 in providing host protection is likely to be determined by other genetic factors.

Our data examining TLR5-deficient mice homozygous for the permissive A/J *lgn1* allele showed that cytokine levels and replication of *L. pneumophila* in the lung was not affected in the absence of TLR5. These data are similar to findings from a recent study that showed no major differences in protection or in cytokine levels in the lung after *L. pneumophila* infection of non-permissive C57BL/6 mice deficient in TLR5 (Hawn *et al.*, 2007). Several studies have demonstrated that TLR5 can detect the *L. pneumophila* flagellin protein (Hawn *et al.*, 2007; Hawn *et al.*, 2003), indicating that TLR5 should be involved in the innate immune response to *L. pneumophila*. Although no obvious defect in cytokine levels have been observed in TLR5-deficient mice after *L. pneumophila* infection, previous data have indicated that neutrophil migration into the lung is delayed slightly in TLR5-deficient mice (Hawn *et al.*, 2007). The innate immune defect in TLR5-deficient mice, however, is compensated for by other MyD88-dependent responses, resulting in a similar overall response that controls *L. pneumophila* infection in the lung.

Since previous data suggested that multiple TLRs are capable of detecting *L. pneumophila*, we examined infection of the lung by *L. pneumophila* under conditions in which three MyD88-dependent TLRs capable of responding to *L. pneumophila* were rendered inoperable. A protective innate immune response was generated in the lung of TLR2/9 double knockout mice infected with flagellin-deficient *L. pneumophila*. The cytokine response and susceptibility of the TLR2/9-deficient mice infected with a flagellin-deficient strain of *L. pneumophila* were similar to the response in WT animals and any differences we were able to detect in multiple TLR-deficient mice were quite modest compared to the severe defects in the innate immune response demonstrated by the MyD88-deficient mice.

The inability to phenocopy MyD88-deficient mice by eliminating multiple TLRs indicate that *L. pneumophila* is likely recognized by additional TLRs and/or possibly other MyD88-dependent pathogen sensors. MyD88 is involved in the signaling of IL-1R and IL-18R (Adachi *et al.*, 1998), however, there was no appreciable difference in the kinetics of bacterial clearance by WT and IL-18R1-deficient mice. In addition, caspase-1-deficient mice, which are deficient in production of IL-1β and IL-18, do not exhibit enhanced bacterial growth in macrophages (Zamboni *et al.*, 2006) or in the lung (data not shown)

when homozygous for the permissive *lgn1* allele. It is possible that the protective response to *L. pneumophila* is mediated by a MyD88-dependent receptor that has yet to be tested. There remain several candidate TLRs to investigate. Although LPS from *L. pneumophila* has been shown to be a weak agonist of TLR4 (Girard *et al.*, 2002), it remains possible that low levels of TLR4 signaling contributes to the innate immune response and is sufficient to control infection in the absence of the several other TLRs. It is feasible that there are additional TLRs that might play a role in responding to intracellular bacterial pathogens such as *L. pneumophila*. These would include TLR7 and TLR8, which are thought to respond primarily to viral pathogens (Heil *et al.*, 2004; Kawai *et al.*, 2004), and TLR11 shown to recognize uropathogenic bacteria (Zhang *et al.*, 2004) and a profillin-like molecule from *Toxoplasma gondii* (Yarovinsky *et al.*, 2005).

Considering that NK cells require MyD88 to respond to cytokines and produce IFN-γ production *ex vivo* (Adachi *et al.*, 1998), it was possible that the susceptibility of MyD88-deficient mice to *L. pneumophila* infection was related primarily to a defect in macrophage activation resulting from a lack of IFN-γ production by NK cells. We addressed whether MyD88 is required to stimulate IFN-γ production and if MyD88-mediated responses could be protective in the absence of IFN-γ-mediated activation of macrophages. Lymphocytes in the lung were found to produce IFN-γ following *L. pneumophila* infection and the majority of cells producing IFN-γ were determined to be NK cells. IFN-γ secretion by these NK cells in the lung of infected mice required MyD88, which is consistent with previous observations indicating that NK cells require MyD88-dependent signaling through the IL-18 receptor (Nakanishi *et al.*, 2001; Lauwerys *et al.*, 2000). Our findings also support a study showing that IFN-γ production by splenic NK cells in response to intravenous infection with *L. pneumophila* requires MyD88 (Sporri *et al.*, 2006).

It was found that depleting NK cells prior to infection with *L. pneumophila* greatly reduced IFN-γ levels in the BALF, further implicating NK cells as being the principle producers of IFN-γ in the lung. NK cell-depleted mice did not have significantly higher numbers of *L. pneumophila* in the lung and were able to effectively restrict bacterial replication in the lung. Similar results were obtained using IL-18R-deficient mice, where NK cells were found to be defective in the production of IFN-γ. We conclude from these data that a severe defect in NK cell production of IFN-γ in the lung is not the primary reason that MyD88-deficient mice are extremely sensitive to pulmonary infection by *L. pneumophila*. Importantly, these data are in contrast to what was observed using an intravenous model of infection (Sporri *et al.*, 2006), where NK cell depletion prior to *L. pneumophila* infection resulted in a significant increase in bacterial numbers in the spleen compared to control mice, suggesting there are differences in the immune mechanisms controlling *L. pneumophila* in the lung compared to a systemic model of infection. These data indicate that in addition to regulating IFN-γ production by NK cells, there are other MyD88-dependent mechanisms responsible for controlling infection by *L. pneumophila* in the lung.

The observation that *L. pneumophila* replication was controlled in the lung of mice after eliminating IFN-γ production by NK cells prompted us to reexamine the importance of IFN-γ in the protection to *L. pneumophila*. Bacterial replication was more robust in the IFN-γ-deficient mice than what was observed for NK cell-depleted or IL-18R-deficient mice. These data indicate that in addition to NK cells, there are other cell populations that can respond to *L. pneumophila* infection and produce sufficient amounts of IFN-γ to control infection in the lung. Dendritic cells, γδ T cells and NK T cells are all likely candidates for producing low levels of IFN-γ in the lung following *L. pneumophila* infection (Nagarajan and Kronenberg, 2007; Carding and Egan, 2002; Stober *et al.*, 2001).

We found that MyD88-deficient mice were more susceptible to *L. pneumophila* infection than mice deficient in IFN-γ. Overall, these data indicate the existence of MyD88-dependent pathways that function independently of IFN-γ that are sufficient to activate innate immune responses to protect mice from pulmonary infection by *L. pneumophila*. One MyD88-dependent response that is likely to be important in the IFN-γ-independent innate immune defense is TNF-α production since mice depleted of TNF-α have impaired clearance of *L. pneumophila* from the lungs (Skerrett *et al.*, 1997). (Janeway and Medzhitov, 2002) Cell autonomous responses impaired in the MyD88-deficient hosts are also likely to enhance susceptibility to infection. For example, there are differences in the kinetics of phagosome maturation and in *L. pneumophila*-induced apoptosis that are observed for macrophages deficient in MyD88 (Losick and Isberg, 2006; Blander and Medzhitov, 2004), which could interfere with events that are important for bacterial clearance. The use of IFN-γ-deficient mice will ultimately provide a useful system to begin investigating the importance of additional MyD88-dependent processes in host protection to intracellular pathogens.

Experimental procedures

Bacterial strains

The *L. pneumophila* serogroup 1 clinical isolate F2111 (Edelstein and Edelstein, 1989), the serogroup 1 strain (JR32) (Sadosky *et al.*, 1993), and the *flaA* mutant (JR32 ΔFlaA) (Ren *et al.*, 2006) were cultured on charcoal-yeast extract (CYE) agar (Feeley *et al.*, 1979) for 2 days and then cultured overnight in ACES-buffered yeast extract (AYE) broth (Feeley *et al.*, 1979) 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.

Mice

MyD88$^{-/-}$ (Adachi *et al.*, 1998) and TLR2$^{-/-}$ (Takeuchi *et al.*, 1999) mice homozygous for the *lgn1* allele were described previously (Archer and Roy, 2006). TLR9$^{-/-}$ (Hemmi *et al.*, 2000) mice in a 129/Ola X C57BL/6 background and TLR5$^{-/-}$ (Feuillet *et al.*, 2006) mice in a 129/SvJ X C57BL/6 background were mated with A/J mice to generate mice that were homozygous for the *lgn1* allele as described previously (Archer and Roy, 2006). MyD88$^{-/-}$ mice in a C57BL/6 background were obtained from Ruslan Medzhitov. C57BL/6, IL-18R1$^{-/-}$ (# 004131) and IFN-γ$^{-/-}$ (# 002287) mice were purchased from Jackson Laboratories.

Ex vivo cytokine production assays

To isolate bone marrow-derived macrophages (BMMs), bone marrow was collected from the femurs and tibiae of mice. Cells were plated and harvested as described previously (Archer and Roy, 2006). BMMs were added to 24-well tissue culture-treated plates at a concentration of 2.5×10^5 cells/well. To isolate alveolar macrophages, mice were lavaged four times with 800 μl of PBS. Cells were resuspended in RPMI 1640 with 10% fetal bovine serum and 1% penicillin-streptomycin and added to 96-well tissue culture-treated plates at a concentration of 5×10^4 cells/well. After 4 h at 37°C, adherent cells were washed with warm PBS and RPMI 1640 with 10% fetal bovine serum was added. To isolate peritoneal macrophages, the peritoneal cavity of mice was lavaged three times with 10 ml of PBS. Cells were resuspended in RPMI 1640 with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated for three days at 37°C. Adherent cells were plated in 96-well plates at a concentration of 1.5×10^5 cells/well. Cells were infected with *L. pneumophila* strain JR32 or JR32 Δ*flaA* at a multiplicity of infection of 5 and incubated at 37°C. Supernatants were collected 24 hours post infection. For BMMs, IL-12 p40 was measured by ELISA using BD Pharmingen IL-12 (p40/p70). Each data point represents the value for

cells from one mouse from which the average cytokine content of three independent wells was determined. For alveolar and peritoneal macrophages, TNF-α was measured by ELISA using reagents from R & D systems. Each data point represents the cytokine content of one well from cells pooled from six mice.

In vivo mouse infections

Mice were anesthetized by subcutaneous injection of a ketamine (100 mg/kg)-xylazine (10 mg/kg) PBS solution and infected intranasally with 10^6 *L. pneumophila* strain F2111, JR32 or JR32 Δ*flaA* in 40 μl of PBS. For *in vivo* bacterial growth assays, mice were euthanized by CO_2 either four hours post infection (day 0) or at the indicated time points and lungs, spleens or livers were harvested as previously described (Archer and Roy, 2006). In each *in vivo* experiment, three mice were used per group unless otherwise stated. Each data point in the figures below represents the CFU count for a single mouse. The lower limit of detection in this assay was 100 CFU of *L. pneumophila*. For bronchoalveolar lavage experiments, mice were euthanized with a ketamine (250 mg/kg)-xylazine (25 mg/kg) PBS solution and 500 μl of PBS was used to lavage lungs. IL-12 p40, IL-6, and IFN-γ production was measured by ELISA using BD Pharmingen IL-12 (p40/p70), IL-6, and IFN-γ reagents, respectively.

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, 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 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 FACS buffer (PBS, 1% fetal bovine serum, 0.025 % sodium azide) and blocked with purified rat anti-mouse CD16/CD32 (BD Pharmingen) and stained on the surface with R-Phycoerythrin-conjugated rat anti-mouse CD49B (DX5) (BD Pharmingen) and biotin-conjugated rat anti-mouse NKG2A/C/E (BD Pharmingen). For intracellular staining of IFN-γ, cells were fixed and permeabilized for 20 min on ice using 50 μl of BD Cytofix/Cytoperm solution (BD). Cells were washed in 1X Perm/Wash buffer and then stained for 1 h on ice with FITC-conjugated-IFN-γ (Caltag Laboratories). Data was collected using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Natural killer cell depletion assays

$MyD88^{-/-}$ or $MyD88^{+/-}$ mice were injected intraperitoneally with 580 μg of anti-asialo GM1 (Wako Pure Chemical Industries, Ltd.) in 300 μl of PBS (total volume) or 300 μl PBS one day prior to intranasal infection with 10^6 *L. pneumophila* strain F2111. Mice were administered second and third doses of anti-asialo GM1 at days one and three post infection. Lymphocytes were isolated from lungs as described above. No significant numbers of NK cells were detected in depleted mice.

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 the log-rank test. Differences were considered statistically significant at $P < 0.05$.

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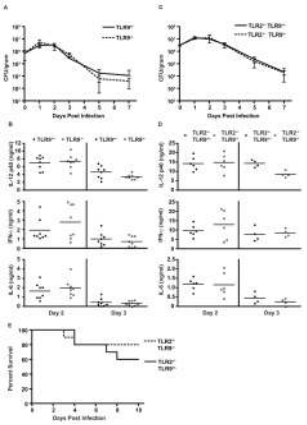

Fig. 1.

L. pneumophila infection of TLR9-deficient mice. A. TLR9-deficient mice ($TLR9^{-/-}$) and heterozygous littermates ($TLR9^{+/-}$) were infected with 10^6 *L. pneumophila* intranasally. Three mice were sacrificed at the indicated days post infection and average number of CFU ± S.D. detected in TLR9-deficient (dotted line) and TLR9-sufficient mice (solid line) are indicated. B. BALF from infected mice was assayed for the indicated cytokines at day 2 and day 3 post infection by ELISA. Each point represents data from a single TLR9-deficient (open circles) or a TLR9-sufficient mouse (solid circles). The lines indicate the means calculated from the data for the two groups of mice. There was no statistical significance between the two groups of mice ($P > 0.05$). C. TLR2-deficient mice that were either deficient in TLR9 ($TLR2^{-/-}$ $TLR9^{-/-}$) or sufficient for TLR9 ($TLR2^{-/-}$ $TLR9^{+/-}$) were infected with *L. pneumophila* intranasally. Three mice were sacrificed at the indicated days post infection and average number of CFU ± S.D. detected in TLR9-deficient (dotted line) and TLR9-sufficient mice (solid line) are indicated. D. BALF from infected mice was assayed for the indicated cytokines at day 2 and day 3 post infection by ELISA. Each point represents data from a single $TLR2^{-/-}$ $TLR9^{-/-}$ (open circles) or a $TLR2^{-/-}$ $TLR9^{+/-}$ mouse (solid circles). The lines indicate the means calculated from the data for the two groups of mice. There was no statistical significance between the two groups of mice ($P > 0.05$). E. $TLR2^{-/-}$ $TLR9^{+/-}$(solid line) and $TLR2^{-/-}$ $TLR9^{-/-}$ mice (dotted line) were infected intranasally with 10^7 *L. pneumophila* and survival was monitored (n = 10 animals/group). The graph represents the percentage of mice surviving at the time points indicated. There was no statistical difference between the two groups of mice ($P > 0.05$).

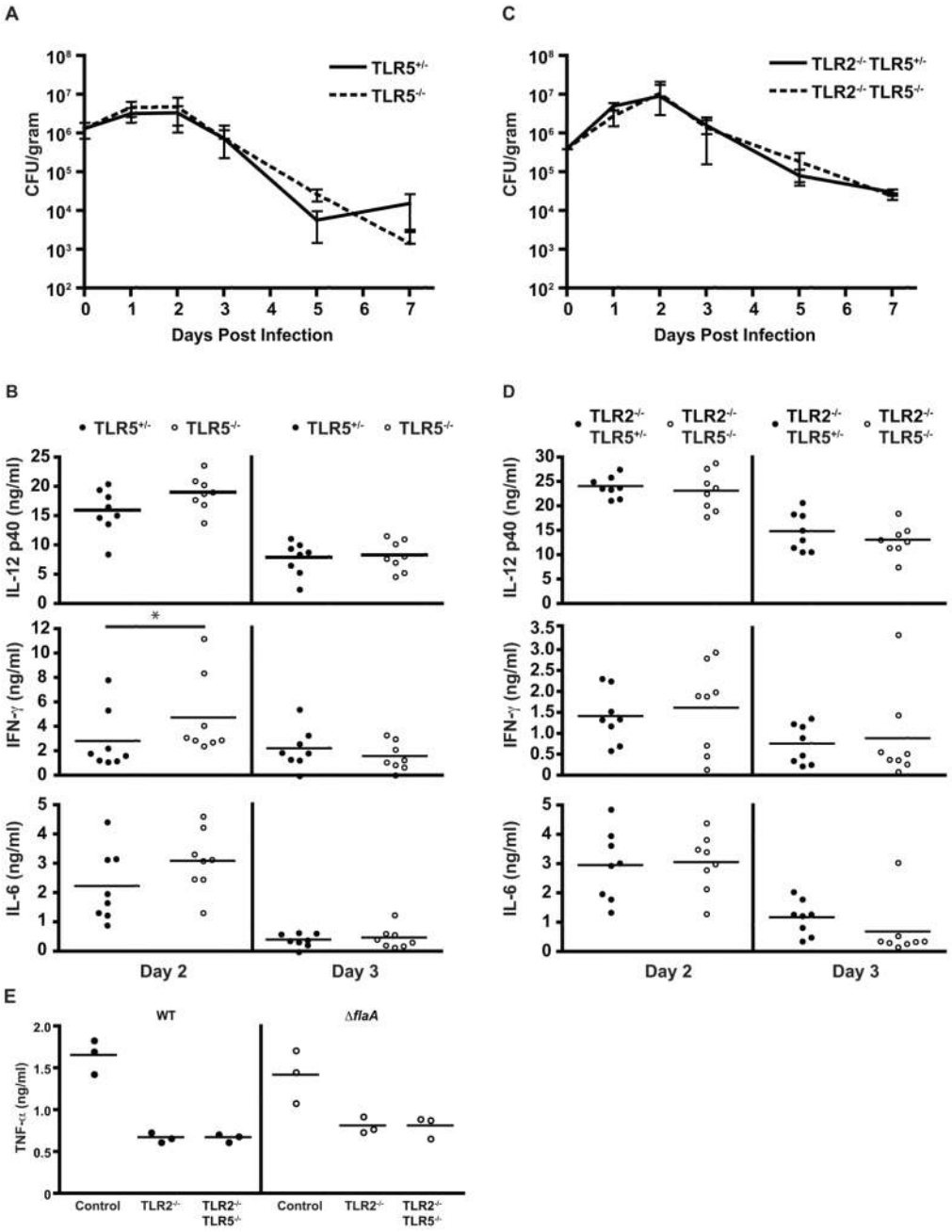

Fig. 2.

L. pneumophila infection of TLR5-deficient mice. A. TLR5-deficient mice ($TLR5^{-/-}$) and heterozygous littermates ($TLR5^{+/-}$) were infected with 10^6 *L. pneumophila* intranasally. Three mice were sacrificed at the indicated days post infection and average number of CFU ± S.D. detected in TLR5-deficient (dotted line) and TLR5-sufficient mice (solid line) are indicated. B. BALF from infected mice was assayed for the indicated cytokines at day 2 and day 3 post infection by ELISA. Each point represents data from a single TLR5-deficient (open circles) or a TLR5-sufficient mouse (solid circles). The lines indicate the means calculated from the data for the two groups of mice. There was no statistical significance between the two groups of mice ($P > 0.05$) except for IFN-γ at day 2 post infection (*$P <$

0.05). C. TLR2-deficient mice that were either deficient in TLR5 ($TLR2^{-/-}$ $TLR5^{-/-}$) or sufficient for TLR5 ($TLR2^{-/-}$ $TLR5^{+/-}$) were infected with *L. pneumophila* intranasally. Three mice were sacrificed at the indicated days post infection and average number of CFU ± S.D. detected in TLR5-deficient (dotted line) and TLR5-sufficient mice (solid line) are indicated. D. BALF from infected mice was assayed for the indicated cytokines at day 2 and day 3 post infection by ELISA. Each point represents data from a single $TLR2^{-/-}$ $TLR5^{-/-}$ (open circles) or a $TLR2^{-/-}$ $TLR5^{+/-}$ mouse (solid circles). The lines indicate the means calculated from the data for the two groups of mice. There was no statistical significance between the two groups of mice ($P > 0.05$). E. Peritoneal macrophages from $TLR5^{+/-}$ (Control), $TLR2^{-/-}$, and $TLR2^{-/-}TLR5^{-/-}$ mice were incubated with either WT (solid circles) or Δ*flaA* (open circles) *L. pneumophila*. Supernatants were collected 24 h post infection, and TNF-α levels were measured by ELISA. Each data point represents the cytokine content of one well from cells pooled from six mice.

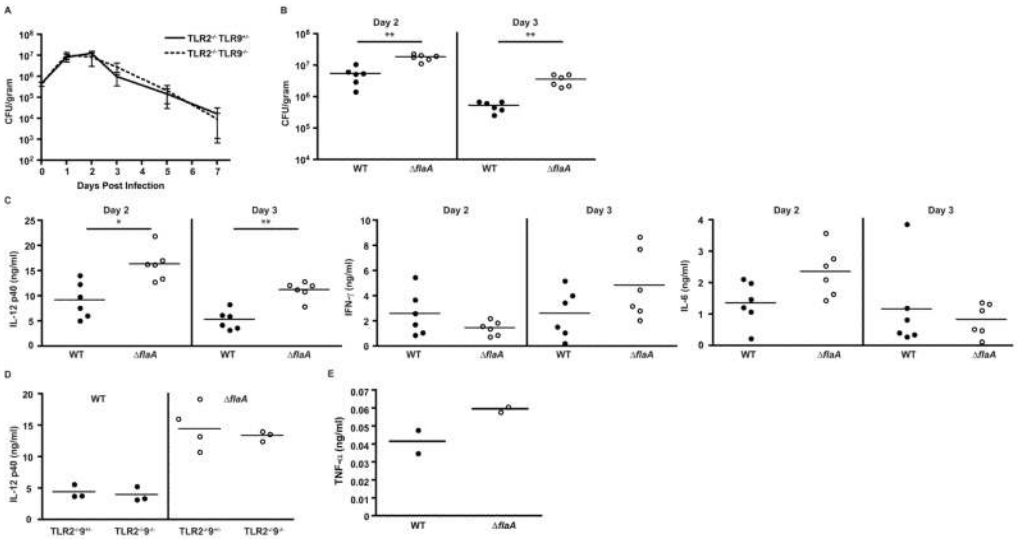

Fig. 3.

A protective innate immune response to *L. pneumophila* is induced in mice deficient in TLR2, TLR5 and TLR9 signaling. A. TLR2-deficient mice that were either TLR9-deficient ($TLR2^{-/-}TLR9^{-/-}$) or TLR9-sufficient ($TLR2^{-/-}$ $TLR9^{+/-}$) were infected with 10^6 CFU of the *L. pneumophila* Δ*flaA* strain intranasally. Three mice were sacrificed at the indicated days post infection and average number of CFU ± S.D. detected in TLR9-deficient (dotted line) and TLR9-sufficient mice (solid line) are indicated. B. $TLR2^{-/-}$ $TLR9^{-/-}$ mice were infected with either WT or Δ*flaA L. pneumophila.* Mice were sacrificed at day 2 and day 3 post infection and bacteria were measured in the lung. The number of WT (closed circles) and Δ*flaA* (open circles) *L. pneumophila* detected in the lung are plotted for individual mice sacrificed at the times indicated. The lines indicate the average number of CFUs for each strain at each time point (** $P < 0.005$). C. BALF from infected $TLR2^{-/-}TLR9^{-/-}$ mice was assayed for the indicated cytokines at day 2 and day 3 post infection by ELISA. Each point represents data from a single mouse infected with either WT (solid circles) or Δ*flaA L. pneumophila* (open circles) (*$P < 0.01$; **$P < 0.005$). D and E. BMMs from $TLR2^{-/-}TLR9^{-/-}$ and $TLR2^{-/-}TLR9^{+/-}$ mice (D) or alveolar macrophages from $TLR2^{-/-}TLR9^{-/-}$ mice (E) were incubated with either WT (solid circles) or Δ*flaA* (open circles) *L. pneumophila*. Supernatants were collected 24 h post infection, and IL-12 p40 (D) or TNF-α(E) levels were determined by ELISA. For BMMs, all data points represent the average cytokine concentration determined from three wells infected independently.

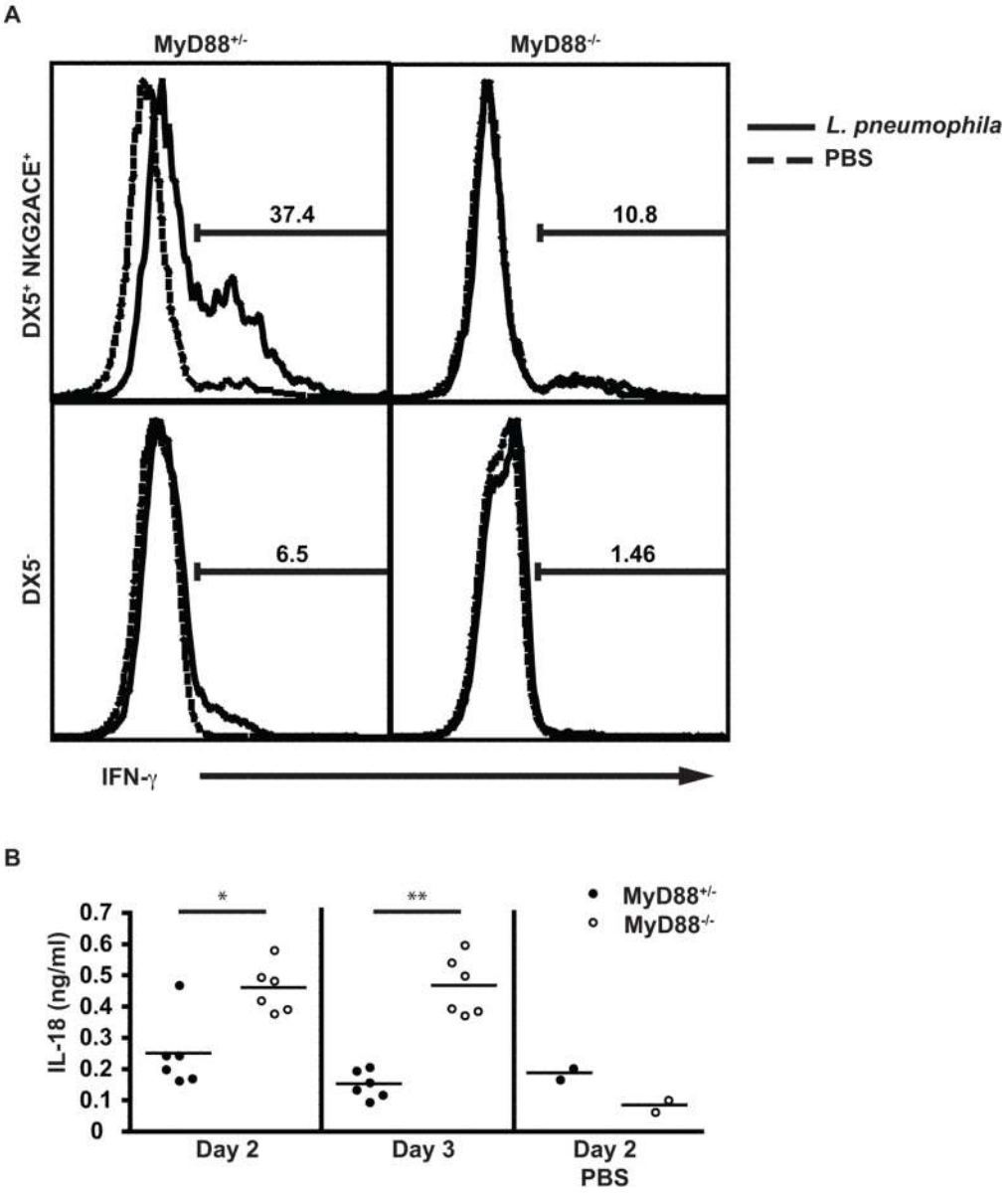

Fig. 4.

IL-18R signaling is important of NK cell production of IFN-γ. A. MyD88$^{+/-}$ (left panels) and MyD88$^{-/-}$ mice (right panels) were either infected intranasally with 10^6 *L. pneumophila* (solid line) or administered PBS intranasally (dotted line). At day 2 post infection, cell suspensions from isolated lungs were stained for DX5, NKG2ACE, and intracellular IFN-γ and examined by flow cytometry. Top panels are DX5$^+$ NKG2ACE$^+$ lymphocytes. Bottom panels are DX5$^-$ lymphocytes. Results are representative of independent data from three mice. B. MyD88$^{+/-}$ and MyD88$^{-/-}$ mice were either infected intranasally with 10^6 *L. pneumophila* or administered PBS intranasally. IL-18 levels in BALF were measured at day 2 and day 3 post infection by ELISA. Each point represents data from a single MyD88$^{-/-}$ (open circles) or a MyD88$^{+/-}$ mouse (solid circles) and the mean value for each group is indicated by the line (*$P < 0.05$, **$P < 0.005$).

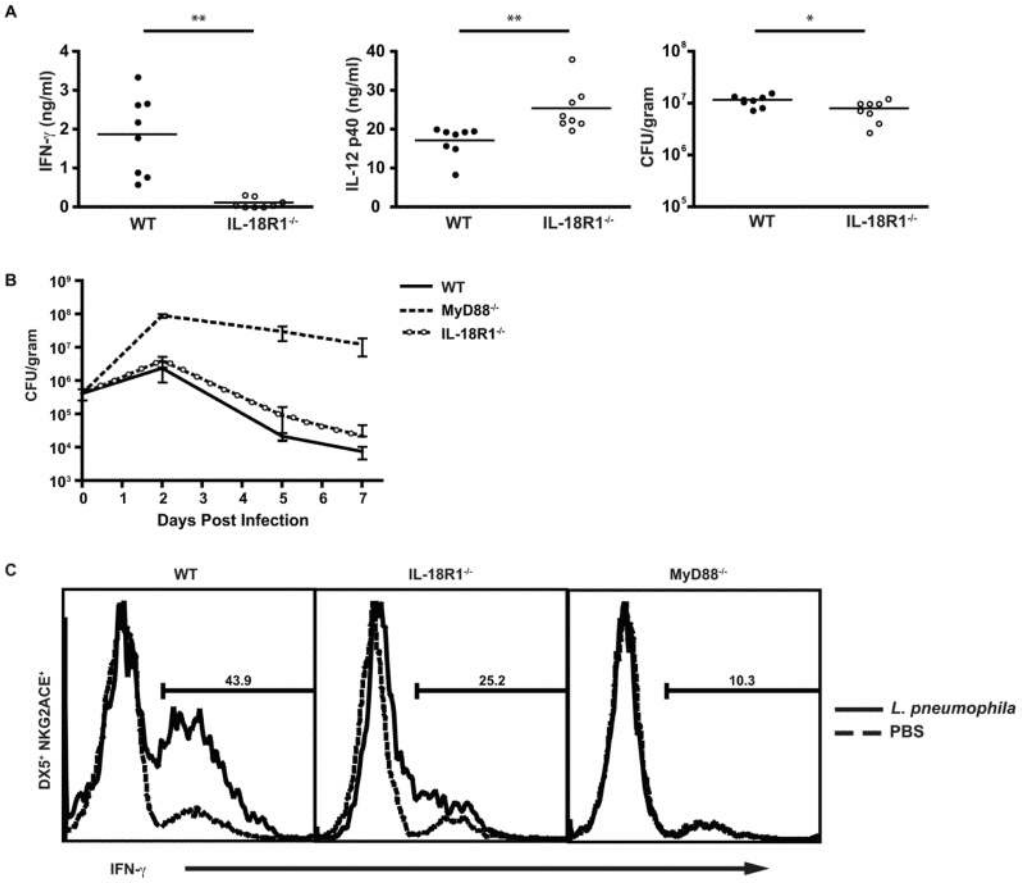

Fig. 5.

L. pneumophila clearance from the lung can occur independent of IL-18 signaling. A. IL-18R1-deficient (IL-18R1$^{-/-}$) and C57BL/6 wild-type (WT) mice were infected intranasally with 10^6 CFU of the *L. pneumophila* Δ*flaA* strain and mice were sacrificed at day 2 post infection. IFN-γ and IL-12 p40 in BALF was measured by ELISA (left and middle panel, respectively) and bacterial CFU in lung lysates were determined (right panel). Each point represents data from a single IL-18R1$^{-/-}$ (open circles) or WT (solid circles) mouse. The lines indicate the means calculated from the data for the two groups of mice (*$P < 0.05$; **$P < 0.0005$). B. IL-18R1$^{-/-}$, MyD88$^{-/-}$, and WT mice on the C57BL/6 background were infected intranasally with 1×10^6 CFU of the *L. pneumophila* Δ*flaA* strain. Mice were sacrificed in groups of 3 and bacterial numbers were determined from lung lysates at the indicated time points. The average number of CFUs detected for the IL-18R1$^{-/-}$ (line with open circles), MyD88$^{-/-}$ (dotted line), and WT (solid line) mice are indicated. C. WT (left panel), IL-18R1$^{-/-}$ (middle panel), and MyD88$^{-/-}$ (right panel) mice in a C57BL/6 background were infected intranasally with 10^6 *L. pneumophila*Δ*flaA*. At day 2 post infection, cell suspensions from isolated lungs were stained for DX5, NKG2ACE, and intracellular IFN-γ and examined by flow cytometry. Results are representative of independent data from three mice.

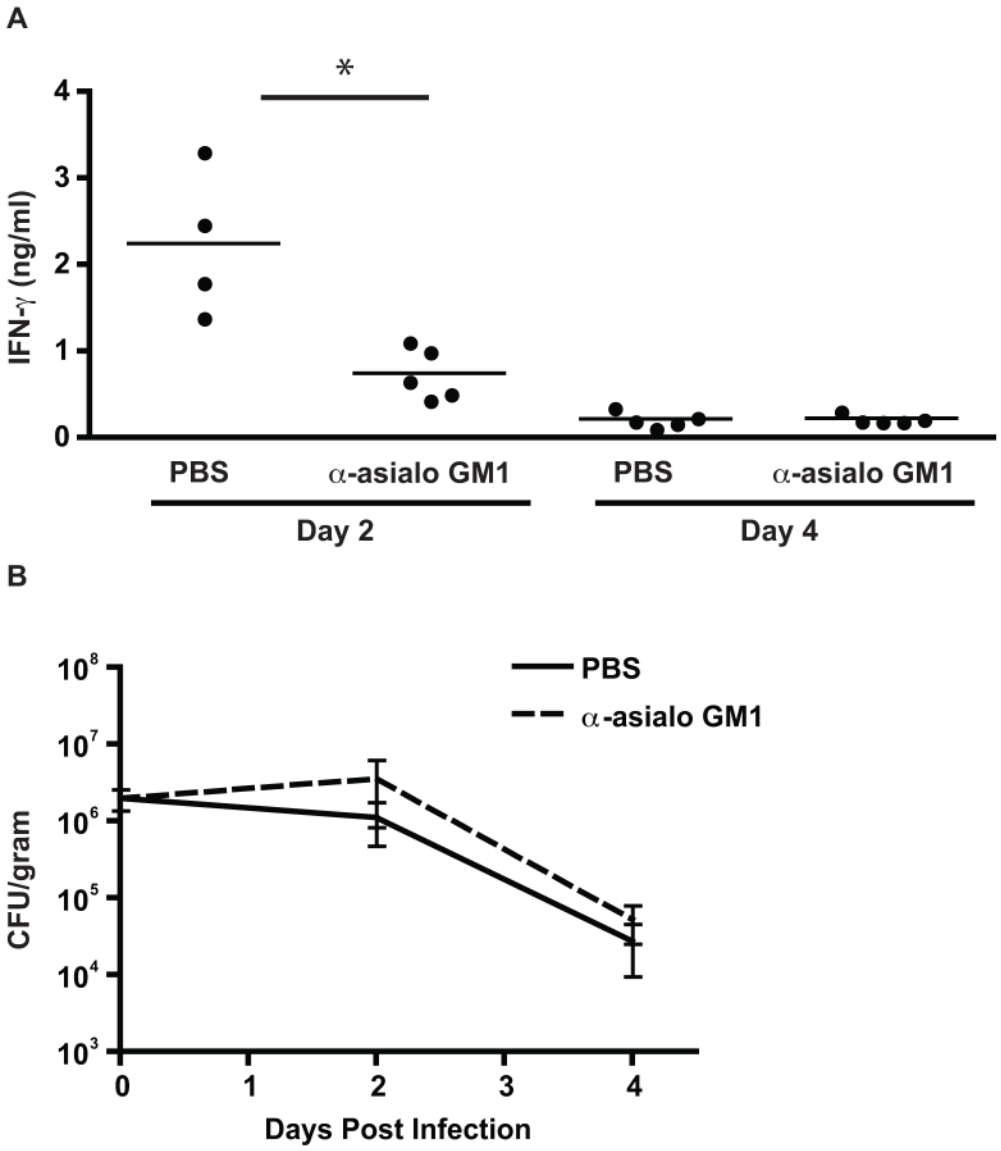

Fig. 6.

L. pneumophila clearance from the lung can occur independent of NK cells. A. MyD88$^{+/-}$ mice were injected with α-asialo GM-1 or PBS one day prior to intranasal infection with 10^6 *L. pneumophila*. IFN-γ in BALF was measured at day 2 post infection by ELISA. The reduction in IFN-γ levels in NK cell-depleted mice compared to non NK-cell depleted mice is significant ($P < 0.05$). B. MyD88$^{+/-}$ mice were injected with α-asialo GM-1 (dashed line) or PBS (solid line) one day prior to intranasal infection with 10^6 *L. pneumophila*. CFUs in lung lysates were determined for at indicated time points. There was no statistical significance between the two groups of mice at day 2 and day 4 post infection ($P > 0.05$).

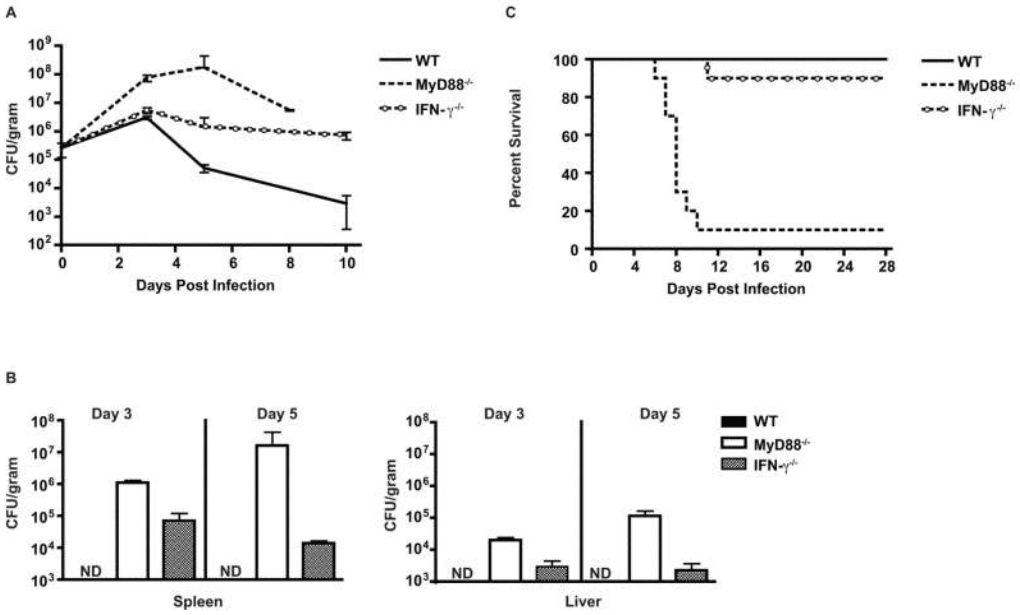

Fig. 7.

IFN-$\gamma^{-/-}$ mice are more resistant to *L. pneumophila* infection than MyD88$^{-/-}$ mice. A. IFN-$\gamma^{-/-}$, MyD88$^{-/-}$, and WT mice on the C57BL/6 background were infected intranasally with 10^6 CFU of the *L. pneumophila*Δ*flaA* strain. Mice were sacrificed in groups of 3 and bacterial numbers were determined from lung lysates at the indicated time points. The average number of CFUs detected for the IFN-$\gamma^{-/-}$ (line with open circles), MyD88$^{-/-}$ (dotted line), and WT (solid line) mice are indicated. B. CFUs were measured from the spleens (left panel) and livers (right panel) of infected mice. The average number of *L. pneumophila* detected for IFN-$\gamma^{-/-}$ (shaded bars), MyD88$^{-/-}$ (open bars), and WT (solid bars) are indicated (ND = not detectable). C. Survival was monitored in infected mice (n = 10 animals/group). The graph represents the percent of surviving mice. The difference between the WT and IFN-$\gamma^{-/-}$ curves versus the MyD88$^{-/-}$ curve was significant ($P < 0.0001$).