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NOD1 and NOD2 regulation of pulmonary innate immunity to *Legionella pneumophila*

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Abstract

The role of nucleotide-binding oligomerization domain-1 (NOD1) and nucleotide-binding oligomerization domain-2 (NOD2), cytoplasmic receptors which detect bacterial cell wall molecules, in pulmonary innate immune responses is poorly understood. We determined that both NOD1 and NOD2 detect heat-killed *Legionella* and stimulate NF- κ B and IFN- β promoter activity using an *in vitro* luciferase reporter system. We next infected NOD1- and NOD2-deficient animals with aerosolized *Legionella pneumophila*. At 3 days post infection, *Nod1*^{-/-} mice had impaired bacterial clearance compared to WT controls. In addition, at 4 h and 24 h, *Nod1*^{-/-} mice had impaired neutrophil recruitment to the alveolar space. In contrast, increased lung neutrophils were seen in the *Nod2*^{-/-} animals at 24 h. Analysis of cytokine production at 4 h post infection revealed a significant decrease in proinflammatory cytokines in the *Nod1*^{-/-} animals when compared to WT animals. In contrast, increased 4-h proinflammatory cytokines were seen in the *Nod2*^{-/-} animals. Furthermore, the lungs of both *Nod1*^{-/-} and *Nod2*^{-/-} mice had significantly increased pro-inflammatory cytokine levels at 24 h, suggesting possible suppressive roles for later stages of infection. Together, our data suggest that although both NOD1 and NOD2 can detect *Legionella*, these receptors modulate the *in vivo* pulmonary immune response differently.

Keywords

Legionella pneumophila; NOD1; NOD2; Pneumonia

Introduction

The immune response to intracellular pathogens in the lung initially involves detection of the organisms through a set of receptors located on the cell surface or endosomal compartment (Toll-like receptors (TLR)) or in the cytoplasm (Nod-like receptors (NLR)) and retinoic acid inducible gene I-like receptors (RLR). Based on the type of foreign material (dsRNA, peptidoglycan, lipopolysaccharide) and location (extracellular, endosomal, cytoplasm), pathogens stimulate distinct sets of receptors to activate the immune response. *Legionella pneumophila* (Lp), an organism known to persist within water-borne amoeba, usually infects humans as a terminal host after exposure to contaminated water systems [1]. Lp replicates within the phagolysosome of the macrophage and secretes bacterial products into the cytosol of the cell through a type IV secretion system (T4SS)

which is known to translocate both DNA and proteins that impair the destruction of the organism [2].

Previous work has identified several innate immune receptors that are responsible for the detection of Lp in the murine model of infection. NAIP5 (Baculoviral inhibitor of apoptosis repeat-containing 1e protein (Birc1e)), NLR4 (IL-1b converting enzyme-protease activating factor (Ipa)), and caspase-1 have been shown to be important in restriction of Lp replication both *in vivo* and *in vitro* [3–6]. TLR5, TLR2, and TLR9 detect Lp and regulate the *in vivo* immune response to Lp [7–10]. Mice deficient in myeloid differentiation factor 88 (Myd88), an adaptor protein for many TLR, are highly susceptible to *in vivo* Lp infection with lack of an early immune response, inability to control bacterial replication, and enhanced mortality [8,10]. More recently, receptor-interacting serine-threonine kinase (RIP2), an adaptor for nucleotide-binding oligomerization domain-1 (NOD1) and nucleotide-binding oligomerization domain-2 (NOD2), was found to regulate Lp replication in the lung, but only on a *Myd88*^{-/-} genetic background [11]. Since Lp is known to replicate intracellularly and can translocate substances to the cytosol via its type IV secretion system, we hypothesized that the cytosolic NLR may be important in control of the innate immune response to Lp.

NOD1 and NOD2 are cytosolic receptors that detect the microbial cell wall byproducts diaminopimelate (DAP) and muramyl dipeptide (MDP), respectively [12,13]. Both NOD1 and NOD2, which contain caspase-1 recruitment domains, activate NF- κ B signaling through RIP2 kinase [14]. NOD2 is expressed mainly in myeloid cells and is important in the immune response to pathogenic organisms including *Mycobacterium tuberculosis* and *Toxoplasma gondii* [15,16]. NOD1 is expressed in both epithelial and myeloid derived cells, and contributes to recognition of a variety of pathogens [17–20]. However, little is known of the *in vivo* role of NOD1 and NOD2 in host defense.

Although there is evidence that NOD1 and NOD2 detect Lp [21], the functional consequences remain poorly understood. Lp-induced cytokine production in NOD1 and/or NOD2-deficient macrophages is selectively impaired [22], yet NOD1 deficiency does not allow for permissive replication in murine macrophages [23]. Although these results suggest that NOD1 and NOD2 detect Lp microbial components, further studies are needed to determine the contribution of NOD1 and NOD2 to the host response *in vivo*. *Nod2*^{-/-} mice are more susceptible to both *Listeria monocytogenes* and *Yersinia pseudotuberculosis* with *in vivo* models of GI infection [24,25]. NOD2 is also important for survival and IFN- γ production in a murine model of *T. gondii* [16]. NOD1 is also required for IFN- γ -mediated elimination of *Trypanosoma cruzi* [26]. Both NOD1 and NOD2 promote clearance of the intracellular pathogen *Chlamydia pneumoniae* from the lung [27] and NOD2 mediates survival and adaptive immunity to *M. tuberculosis* [28,29]. The role of NOD1 and NOD2 activation during *in vivo* Lp infection has not been examined.

Here, we show that Lp induces pro-inflammatory cytokines in a NOD1- and NOD2-dependent manner. In addition, we demonstrate that NOD1 regulates the pulmonary cytokine response and phagocytic recruitment during Lp infection. Furthermore, at 3 and 10 days, delayed clearance of Lp is seen in mice lacking NOD1 receptor. These data suggest that detection of Lp by NOD1 receptor is important in the host response to this intracellular pathogen and delayed clearance at 10 days may suggest NOD1 alters the adaptive immune response.

Results

To determine whether Lp signals through NOD1 and NOD2, we co-transfected human embryonic kidney (HEK) cells with either human NOD1 or NOD2 expression vectors simultaneously with either endothelial-leukocyte adhesion molecule (ELAM) or an IFN- β promoter firefly luciferase reporter construct (Fig. 1A–D). Transfected cells were stimulated with heat killed Lp FlaA (deficient in flagellin protein), to avoid stimulation through endogenously expressed TLR5 (the receptor for bacterial flagellin) in the presence of transfection reagent to optimize cytoplasmic delivery [30]. We found that overexpression of human NOD1 and NOD2 stimulated Lp dependant ELAM promoter activity after 24 h when compared to empty vector control (Fig. 1A and B). Only NOD1 was able to stimulate promoter activity at the highest MOI. In addition overexpression of both NOD1 and NOD2 stimulated Lp dependant IFN- β promoter activity, as compared to empty vector control. As controls, MDP and L-alanyl- γ -D-glutamyl-meso-DAP (Tri-DAP) activated ELAM and IFN- β promoter activity through NOD2 and NOD1, respectively. Relative levels of induction using NOD1 purified stimuli Tri-DAP were considerably less due to higher baseline stimulation in both empty vector controls and untreated cells due to known expression of NOD1 in HEK cells. These data suggest that both human NOD1 and NOD2 proteins can detect *Legionella in vitro*.

To examine the *in vivo* role of NOD1 and NOD2 in pulmonary host defense to intracellular pathogens, we used a murine model of airborne infection with Lp. At 4 and 24 h after infection, no significant differences in Lp CFU were seen between WT and *Nod1*^{-/-} and *Nod2*^{-/-} animals (Fig. 2A and B). At 72 h, however, a significant increase in Lp CFU was seen in *Nod1*^{-/-} animals (mean \pm SEM: 8.9×10^4 CFU/lung $\pm 2.6 \times 10^4$) compared to WT animals (1.7×10^4 CFU/lung $\pm 3.9 \times 10^3$) (Fig. 2C). There were no significant CFU differences observed in *Nod2*^{-/-} animals compared to WT. Lastly, at 10 days, there was late defect in clearance in the *Nod1*^{-/-} mice that trended toward significant ($p = 0.054$) (Fig. 2D). To determine whether the CFU difference was due to differences in apoptotic cell death, we examined lungs at 4 and 24 h for terminal deoxynucleotidyl transferase dUTP nick end labeling and saw no difference between *Nod1*^{-/-} animals and WT controls (our unpublished observations). These results suggest that NOD1 regulates clearance of Lp from the lung after aerosolized exposure.

Next, we examined recruitment of inflammatory cells to the pulmonary airspaces by performing bronchoalveolar lavage on WT and *Nod1*^{-/-}, and *Nod2*^{-/-} animals. At 4 h, we saw significantly impaired recruitment of PMN in the *Nod1*^{-/-} (Mean \pm SEM: 2.6×10^5 PMN/lung $\pm 6.3 \times 10^4$) animals compared to WT (5.5×10^5 PMN/lung $\pm 1.1 \times 10^5$) (Fig. 3D). At 24 h, these differences persisted, although the magnitude was smaller (Fig. 3E) (*Nod1*^{-/-}, $2.0 \times 10^6 \pm 1.4 \times 10^5$; WT, $2.5 \times 10^6 \pm 1.4 \times 10^5$). Interestingly, at the same 72-h time point where increased CFU of Lp was present, *Nod1*^{-/-} animals showed a borderline increased level of PMN recruited to the alveolar space compared to WT controls (Fig. 3F, $p = 0.07$). For *Nod2*^{-/-} animals, increased PMN were recruited to the bronchoalveolar space at 24 h compared to WT animals. In addition, no significant differences were seen in total monocytic recruitment to the lung following infection at 4, 24, and 72 h in the NOD1- or NOD2-deficient animals (Fig. 3A–C).

We examined histologic lung sections from 24- and 72-h time points to determine if visual differences were seen in lung samples. Six lungs from 24 h (Fig. 4A) and 72 h (Fig. 4B) were scored in ten separate high-powered fields for percentage of airspace involved. Significant decreases in inflammation were seen in NOD1-deficient animals at 24 h ($p = 0.01$, $n = 6$) compared to WT controls (Table 1). Only a trend toward a decrease in cellular infiltration was seen at 72 h ($p = 0.11$) in the NOD1-deficient animals when compared to

WT controls. These data suggest that NOD1 deficiency impairs recruitment of inflammatory cells to the lung during Lp infection.

We next measured levels of cytokines and chemokines to examine the mechanism of NOD1-mediated protection. Cytokine levels from lung homogenates from WT, *Nod1*^{-/-}, and *Nod2*^{-/-} animals were measured for TNF α , IL-1 β , IL-6, KC, IL-18, and MCP-1 to determine if there were significant differences in WT compared to *Nod1*^{-/-} and *Nod2*^{-/-} animals (Fig. 5). At 4 h, there was significantly decreased production of IL-1 β (WT 1.00 ± 0.06 versus NOD1 0.68 ± 0.06 (mean \pm SEM)), KC (WT 1.00 ± 0.12 versus NOD1 0.72 ± 0.05), and trend toward decreased TNF α (WT 1.00 ± 0.07 versus NOD1 0.78 ± 0.09 , $p = 0.06$) in the *Nod1*^{-/-} animals, when compared to WT controls (Fig. 5A, C, and G). In contrast, at 4 h, there was no change in IL-6, IL-18, or MCP-1 levels in the *Nod1*^{-/-} animals (Fig. 5B, H, and I). At 24 h, *Nod1*^{-/-} animals exhibited significantly increased levels of IL-6 production (WT 1.00 ± 0.06 versus NOD1 1.35 ± 0.13) compared to WT controls and a trend toward increased TNF α production (WT 1.00 ± 0.08 versus NOD1 1.36 ± 0.19 , $p = 0.06$). The only significant change seen in the *Nod2*^{-/-} animals compared to WT controls at 4 h was a significantly increased production of IL-6 (WT 1.00 ± 0.36 versus NOD2 1.49 ± 0.66) and MCP-1 (WT 1.00 ± 0.12 versus NOD2 2.04 ± 0.49). In addition, significant increases were seen in *Nod2*^{-/-} animals compared to WT in IL-1 β (WT 1.00 ± 0.19 versus NOD2 1.49 ± 0.43), IL-6 production (WT 1.00 ± 0.11 versus NOD2 1.49 ± 0.20), and MCP-1 production (WT 1.00 ± 0.10 versus NOD2 1.55 ± 0.21) at 24 h (Fig. 5E, F, and L). In addition, IFN- γ was analyzed at the 24-h, 72-h and 10-day time points and only minimal production was seen in lung homogenates (our unpublished observations). The levels of IFN- γ were not different when comparing WT, *Nod1*^{-/-}, and *Nod2*^{-/-} mice. These data demonstrate an early impaired production of proinflammatory cytokines KC and IL-1 β seen in the absence of NOD1 protein and a later increase in proinflammatory markers (IL-1 β , IL-6, and MCP-1) in *Nod2*^{-/-} and (IL-6) *Nod1*^{-/-} animals.

Our data herein suggest that both NOD1 and NOD2 can detect Lp, but only NOD1 regulates *in vivo* bacterial clearance at 72 h. In addition, NOD1-deficient animals display early decreases in PMN recruitment to the alveolar space of the lung at 4 and 24 h and NOD2-deficient animals display a significant increase in PMN recruitment at 24 h. NOD1- and NOD2-deficient mice also show altered pulmonary inflammatory cell infiltration and cytokine responses to *Legionella*.

Discussion

In our aerosolized animal model, we identified higher Lp CFU in *Nod1*^{-/-} mice compared to WT controls. Delayed bacterial clearance of Lp has been a characteristic of other knockout systems. *Myd88*^{-/-} and to a lesser extent *Tlr2*^{-/-} animals have impaired bacterial clearance that is associated with delayed inflammatory cytokine production and decreased early neutrophil recruitment to the lungs [7,10]. *Tlr9*^{-/-} and *Tlr5*^{-/-} deficient animals, however, show little difference in Lp clearance compared to WT controls (unpublished observations) [7,9]. In addition, NAIP5 and NLRC4 limit growth of *Legionella* both *in vitro* and *in vivo* through the detection of intracellular flagellin [3,31]. The mechanism of delayed *Legionella* clearance in *Nod1*^{-/-} infected lung may be due to multiple factors. One possible explanation could be that *Nod1*^{-/-} animals have impaired early recruitment of PMN to the alveolar space leading to later impaired Lp phagocytic clearance. Alternatively, NOD1 may either directly or indirectly regulate replication of Lp in macrophages. Studies in bone marrow derived macrophages suggest, however, that NOD1 does not regulate Lp replication through direct detection [23]. Interestingly RIP2-deficient animals show little difference in organism clearance, suggesting the mechanism of increased CFU seen in *Nod1*^{-/-} animals may be due to a RIP2-independent mechanism [11]. Whether the mechanism of Lp clearance by NOD1

is due to increased phagocytic killing *versus* impaired replication in cells containing NOD1 is currently unknown.

Recruitment of neutrophils to the lung may be important in clearance of *Legionella* and help to develop a protective Th1 response to the pathogen [32]. In addition, inhibition of chemotactic receptors important for neutrophil recruitment has been associated with enhanced mortality of mice infected with Lp [33]. Impaired early neutrophil recruitment was previously observed in the lungs of *Myd88*^{-/-}, and to a lesser extent in *Tlr2*^{-/-} and *Tlr5*^{-/-} deficient animals [9,10]. In our model, we demonstrated that decreased PMN recruitment and impaired Lp clearance in the *Nod1*^{-/-} animals was associated with decreased early IL-1 β , and KC levels in the lungs of *Nod1*^{-/-} mice as compared to WT controls. Impaired production of KC (CXCL1) may account for the impaired PMN recruitment seen in *Nod1*^{-/-} mice [34]. Also, NOD may be important in regulation of IL-1 β not only by inducing pro-IL-1 β transcription but also by activating caspase-1 directly to cleave pro-IL-1 β to the active form [35,36]. At 24 h, we also observed increased IL-6 levels and a trend toward increased TNF α in the *Nod1*^{-/-} lung in comparison to WT mice. These data suggest that NOD1 regulates suppression of later proinflammatory cytokine signaling. Together, our data suggest that NOD1 detection of Lp contributes to early cytokine and chemokine responses, early recruitment of PMN, and effective clearance of Lp from the lungs.

While NOD2 deficiency was not associated with impaired bacterial clearance in our study, alterations in inflammatory cell recruitment and cytokine responses were seen in *Nod2*^{-/-} compared to WT. At 4 h after infection, increases in MCP-1 and IL-6 were observed in *Nod2*^{-/-} animals compared to WT and at 24 h there was an increase in PMN recruitment and an increase in IL-1 β , IL-6 and MCP-1 levels that was observed in the *Nod2*^{-/-} mice. These data suggest that NOD2 may counteract the proinflammatory response to Lp by decreasing cytokine production at 4 and 24 h and PMN recruitment at 24 h. The mechanism of this early decrease in proinflammatory response by NOD2 to Lp is unknown. One possibility is that through heterotypic association of the caspase-1 recruitment domains, NOD2 protein may inhibit other inflammasomes, such as those containing NAIP5/NLRC4 [37]. Alternatively, NOD2 may modulate IL-1 β production through interaction with RIP2 or TLR-pathway intermediates.

Our study is unique in that it is one of the few to do a side-by-side comparison of both NOD1 and NOD2 in a murine infection model. In comparison, other studies demonstrated unilaterally that NOD1 is important in the gastrointestinal and intravenous immune response to organisms such as *L. monocytogenes* and Salmonella Pathogenicity Island 1-deficient *Salmonella enterica* serovar Typhimurium [38,39]. Furthermore, in isolated lung epithelium, *Moraxella catarrhalis*-induced IL-8 production is in part due to detection by NOD1 [40]. Although replication of *C. pneumoniae* *in vitro* and clearance of organisms has been shown to be dependent upon NOD1 and NOD2, increased *in vivo* mortality was only demonstrated in RIP2 kinase-deficient animals [27]. Lastly, although no significant phenotype was seen in Lp infection with the NOD2-deficient mouse, its importance has been demonstrated in other mouse pulmonary models of intracellular infections such as *M. tuberculosis* [28,29]. Together, these studies suggest that NOD1 and NOD2 regulate distinct aspects of the *in vivo* immune response to pathogens.

Materials and methods

Materials

DMEM media, L-glutamine, penicillin, and streptomycin were obtained from Invitrogen. FCS was from Hyclone Thermo-Fisher (Waltham, MA, USA). FUGENE HD was from Roche Applied Bioscience (Mannheim, Germany). Buffered charcoal yeast extract

components were from Sigma (St. Louis, MO USA) and Difco/BD Biosciences (San Jose, CA, USA).

Bacteria

Lp Corby (serogroup 1) and Lp Corby Flagellin deficient were gifts from K. Heuner [41]. Lp, Philadelphia 1 (American Type Culture Collection – ATCC 33152) was stored as previously described [42,43]. NOD1 and NOD2 expression plasmids were a kind gift from Gabriel Nuñez. *Legionella* were grown in buffered charcoal yeast extract plates and harvested by scraping into PBS. Bacterial concentrations were estimated by correlating OD600 values with CFU counts on agar plates.

Model of murine legionellosis

All experiments were approved by Institutional Animal Care and Use Committee at the University of Washington. *Nod2*^{-/-} mice (strain designation B6.129S1-*Nod2*^{tm1Flv/J}) were derived and backcrossed for eight generations as previously described [44]. *Nod1*^{-/-} mice were derived and backcrossed against eight generations of mice as previously described [12]. WT control mice were from a C57BL/6 background (Jackson Laboratory, Bar Harbor, ME, USA). Lp (Philadelphia strain) used for mouse aerosol infections was grown and prepared as described [9]. The mice were exposed to bacterial aerosols generated by twin jet nebulizers (Salter Laboratories, Arvin, CA, USA) for 30 min in a whole animal exposure chamber as described [45]. At each time point, mice were euthanized with intraperitoneal pentobarbital and exsanguinated by cardiac puncture. The left lung was homogenized for quantitative culture and measurement of cytokines as described [9]. The right lung was lavaged for cell counts [9]. Cytospins were performed on cells from bronchoalveolar lavages and cell types were determined following a modified Wright-Giemsa stain (Diff-Quick, Dade Behring, Dudingon, Switzerland). For histologic preparation, the lung was inflated to 15 cm pressure with 4% paraformaldehyde, fixed in the same solution, embedded in paraffin, and 4-mm sections were generated. Sections stained with hematoxylin and eosin were examined by a pathologist blinded to mouse genotype and time following infection. Inflammation was scored as a percentage of the airspaces involved derived from the examination of ten high-power fields.

NOD1 and NOD2 transfection experiments

Human NOD1 and NOD2 constructs (gift from Gabriel Nuñez) were subcloned into the pEF6 expression vector (Promega, Madison, WI, USA). The region of the murine IFN- β promoter (-43 bp to -218 bp upstream the transcription site) containing interferon response factor-1 and NF- κ B binding sites was placed upstream a luciferase reporter construct (pGL2-IFN β) (gift from Pierre-Yves Bochud) (Invitrogen, Carlsbad, CA, USA). pGL2-ELAM was used as previously described [46], and pRL-TK was purchased from Promega. HEK293 cells were transfected with FUGENE HD (Roche Diagnostics, Basel, Switzerland) using the manufacturer recommended protocol. ELAM promoter-firefly luciferase and IFN- β promoter-firefly luciferase reporter constructs were co-transfected with plasmid expression constructs containing human NOD1 and NOD2 along with Renilla luciferase expression constructs driven by the HSV thymidine kinase promoter to control for transfection efficiency. Cells were simultaneously exposed to heat-killed FlaA and WT (Corby strain) Lp and incubated overnight at 37°C in order to potentiate cytoplasmic delivery of the pathogen by the transfection reagent. Firefly luciferase activity was measured after lysis of cells using Dual Luciferase Reporter Assay System as per the manufacturer's recommended instructions (Promega). Total luminescence over one second was measured using luciferin (to measure firefly luciferase) followed by a second reading with coelenterazine (to measure Renilla luciferase activity) with simultaneous administration of an inhibitor to firefly luciferase.

Transfection efficiency of the reporter promoter was adjusted for each well by dividing the relative light units of firefly luciferase by the relative light units of Renilla luciferase.

Protein analysis

For cytokine analysis, *in vivo* lung homogenates were used and levels of specific cytokines were measured by sandwich ELISA (Duoset) (R&D systems, Minneapolis, MN, USA) according to the manufacturer's recommendations.

Statistical analysis

Student's *t*-test was used to assess statistical significance. A value of $p < 0.05$ was considered significant. Statistics were calculated with Prism version 5.0c (GraphPad).

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Abbreviations

DAP	diaminopimelate
ELAM	endothelial-leukocyte adhesion molecule
FlaA	flagellin deficient
HEK293	human embryonic kidney (cell line)-293
Lp	<i>Legionella pneumophila</i>
MDP	muramyl dipeptide
Myd88	myeloid differentiation factor 88
NAIP5	neuronal apoptosis inhibitory protein 5
NOD1	nucleotide-binding oligomerization domain-1
NOD2	nucleotide-binding oligomerization domain-2
NLR	NOD-like receptors
NLRC4	NLR family, CARD domain containing 4
RIP2	receptor -interacting serinethreonine kinase g 4
Tri-DAP	L-alanyl-γ-D-glutamyl-meso-DAP

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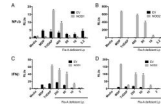


Figure 1.

Heat-killed *Legionella* is detected by NOD1 and NOD2. HEK293 cells were transfected with expression vectors containing human NOD1 (A, C) and NOD2 (B, D) (grey) or empty vector (EV) controls (black). Firefly luciferase reporter constructs for the NF- κ B (ELAM-pGL2) (A, B) and IFN- β promoters (IFN β -pGL2) (C, D) were simultaneously transfected. Cells were then exposed to NOD1 ligand (Tri-DAP, 1 μ g/mL), NOD2 ligand (MDP, 1 μ g/mL), or heat-killed flagellin-deficient Lp (Lp FlaA, Corby strain) at decreasing heat-killed MOI as determined by OD 600 nm. HEK cells were incubated overnight (16 h) and then lysed. Transfection efficiency was compared between wells by co-transfecting the expression vector for *Renilla* luciferase. Data are mean \pm SD, $n = 3$, representative of three experiments. Lp: *Legionella pneumophila*, RLU: relative light units.

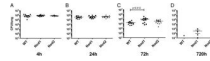


Figure 2.

NOD1-deficient animals show impaired clearance of *Legionella pneumophila* in lungs at 3 days. Mice were infected with aerosolized *L. pneumophila* (Philadelphia strain) and then euthanized at 4 h (A), 24 h (B), 72 h (C), and 240 h (D). Bacterial CFU were determined by serial dilution of tissue homogenates plated on buffered yeast charcoal extract agar. *Nod1*^{-/-} knockout mice (■), WT controls (●), *Nod2*^{-/-} knockout mice (▲). Initial deposition was approximately 1×10^6 Lp CFU *per* lung. A Student's *t*-test was used to determine significance. Bars represent mean \pm SEM range; results combined from three separate experiments with four mice *per* time point ($n = 12$ total) except for the 720-h time point, which was one experiment ($n = 4$).

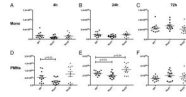


Figure 3.

NOD1 and NOD2 regulate neutrophil recruitment to the lung in Lp-infected mice. Bronchoalveolar lavages were performed on mice infected with *Legionella pneumophila* and euthanized at the indicated time points: 4 h (A, D), 24 h (B, E), and 72 h (C, F). Total neutrophil (PMN) (D, E, F) and monocyte (Mono) (A, B, C) counts were compared in WT (•), *Nod1*^{-/-} (■) and *Nod2*^{-/-} animals (▲). A Student's *t*-test was used to determine significance as represented on the graph. Bars represent mean ± SEM. Combined results from three separate experiments with four mice *per* time point ($n = 12$).

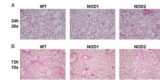


Figure 4. NOD1-deficient mice have impaired inflammatory cell infiltrates at 24 h. Lungs from WT, NOD1- and NOD2-deficient mice were fixed in paraformaldehyde and stained in H and E. Representative slides for 24 h (A) and 72 h (B) are shown. Ten high-power fields for each lung were examined and the percentage of airspace involvement was scored by a pathologist blinded to condition. Student's *t*-test was used to determine significance. Scores are shown in Table 1 (mean \pm SEM, $n = 6$).

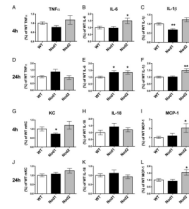


Figure 5.

Cytokine production in lung homogenates of Lp-infected mice. WT (white), *Nod1*^{-/-} (black), and *Nod2*^{-/-} (grey) mice were infected with Lp and at 4 and 24 h after infection lungs were homogenized. Total levels of TNF α (A,D), IL-6 (B,E), IL-1 β (C,F), mKC (G,J), IL-18 (H,K), and MCP-1 (I,L) were measured. To combine experiments, *Nod1*^{-/-} and *Nod2*^{-/-} cytokine levels were normalized to average of WT controls for each cytokine. * $p < 0.05$ compared with WT, ** $p \leq 0.01$ compared with WT. Statistical significance was determined by performing Student's *t*-test. Data combined from three separate experiments with $n = 12$ total for each group except *Nod2*^{-/-} animals, which had $n = 11$. Bars represent mean \pm SEM.

Table 1Decreased inflammation in lungs of *Nod1*^{-/-} mice

Score±SEM	WT	NOD1	NOD2
24h	3.40±0.34	1.88±3.42 ^{a)}	4.44±0.53
72h	13.63±3.75	6.82±3.42 ^{b)}	8.68±0.80

^{a)} $p = 0.01$ by Student's t-test compared to WT controls 9 ($n = 6$).

^{b)} $p = 0.11$ by Student's t-test compared to WT controls ($n = 6$).