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HOST RESPONSE AND INFLAMMATION

Overlapping Roles for Interleukin-36 Cytokines in Protective Host Defense against Murine *Legionella pneumophila* Pneumonia

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ABSTRACT *Legionella pneumophila* causes life-threatening pneumonia culminating in acute lung injury. Innate and adaptive cytokines play an important role in host defense against *L. pneumophila* infection. Interleukin-36 (IL-36) cytokines are recently described members of the larger IL-1 cytokine family known to exert potent inflammatory effects. In this study, we elucidated the role for IL-36 cytokines in experimental pneumonia caused by *L. pneumophila*. Intratracheal (i.t.) administration of *L. pneumophila* induced the upregulation of both IL-36α and IL-36γ mRNA and protein production in the lung. Compared to the findings for *L. pneumophila*-infected wild-type (WT) mice, the i.t. administration of *L. pneumophila* to IL-36 receptor-deficient (IL-36R$^{-/-}$) mice resulted in increased mortality, a delay in lung bacterial clearance, increased *L. pneumophila* dissemination to extrapulmonary organs, and impaired glucose homeostasis. Impaired lung bacterial clearance in IL-36R$^{-/-}$ mice was associated with a significantly reduced accumulation of inflammatory cells and the decreased production of proinflammatory cytokines and chemokines. *Ex vivo*, reduced expression of costimulatory molecules and impaired M1 polarization were observed in alveolar macrophages isolated from infected IL-36R$^{-/-}$ mice compared to macrophages from WT mice. While *L. pneumophila*-induced mortality in IL-36α- or IL-36γ-deficient mice was not different from that in WT animals, antibody-mediated neutralization of IL-36γ in IL-36$\alpha^{-/-}$ mice resulted in mortality similar to that observed in IL-36R$^{-/-}$ mice, indicating redundant and overlapping roles for these cytokines in experimental murine *L. pneumophila* pneumonia.

KEYWORDS IL-36, *Legionella pneumophila*, macrophages, pneumonia

Legionella species are ubiquitous, flagellated Gram-negative facultative intracellular bacilli (1). Typically, humans are infected by inhalation or aspiration of contaminated aerosols from modern technologies and environmental sources, such as water-supply facilities and hot springs (2). Although the genus encompasses more than 50 species and 70 serogroups, *Legionella pneumophila* serotype 1 is the major cause of pneumonia in humans (3). Once it is within the alveolus, *L. pneumophila* is ingested by resident alveolar macrophages and replicates intracellularly (4–6). *L. pneumophila* can cause a broad spectrum of disease, ranging from Pontiac fever to Legionnaires' disease (LD), which is characterized by often severe and life-threatening pneumonia. The rate of mortality from LD remains high, with the rate of mortality from health care-associated disease being nearly 40% to 50% and the rate pf mortality from community-acquired

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disease being approximately 20% (7). Additionally, the incidence of LD in the United States increased by nearly 300% from 2000 to 2014 (8). Host risk susceptibility factors include advanced aged, smoking (9), chronic lung disease, underlying malignancy (1, 10, 11), and treatment with immunosuppressive agents, such as glucocorticoids (12), or anti-tumor necrosis factor alpha (TNF-α) (13). Therefore, cellular immunity and the production of cytokines play important roles in protection against LD. In particular, Th1-type cytokines, such as gamma interferon (IFN-γ), interleukin-12 (IL-12), and IL-17, are important factors in resistance to *L. pneumophila* disease (14–18).

In addition to the aforementioned mediators, the IL-1 family of cytokines is central to protective mucosal immunity in *Legionella* pneumonia. *L. pneumophila* induces caspase-1 activation in macrophages (19, 20). This early event is a key for initiation of the innate immune response of macrophages, as activated caspase-1 accelerates pyroptosis of host cells (21) and promotes the cleavage and secretion of active IL-1β and IL-18 (22). It has previously been shown that the restriction of *L. pneumophila* growth in bone marrow-derived macrophages is dependent on caspase-1 activation (20). IL-1β triggers the production of MyD88-dependent chemokines, such as CXCL1 and CXCL2, by airway epithelial cells, resulting in the rapid and robust recruitment of neutrophils into the lung (23). Moreover, IL-1α is also an initiator of neutrophil recruitment and an initiator of the inflammatory response to *L. pneumophila* in the lung (24, 25).

The IL-1 family of cytokines includes seven agonistic cytokines, three receptor antagonists, and one anti-inflammatory member (26). IL-36 cytokines are members of the larger IL-1 family and include three agonist proteins (IL-36α, IL-36β, and IL-36γ) and the antagonist protein IL-36 receptor antagonist (IL-36Ra) (27). IL-36 agonists bind to the IL-36 receptor (IL-36R; formerly known as IL-1R-related protein 2 [IL-1Rrp2]), and this complex recruits IL-1R3, which was previously known as the IL-1R accessory chain (IL-1RAcP) and which is shared with the IL-1 type I and the IL-33 receptors (27, 28). IL-36R is widely expressed on many different cells, including murine bone marrow-derived dendritic cells (BMDCs), $CD4^+$ cells, mononuclear phagocytes, and various epithelial cells of tissues, such as skin, lung, and digestive tract tissue (29–31). The complex of IL-36 and receptor proteins activates downstream inflammatory signaling pathways, such as nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK), in an MyD88-dependent fashion, like other IL-1 family cytokines (27). IL-36 cytokines are abundantly expressed by a variety of cells, such as activated epithelial cells, macrophages, dendritic cells (DC), and $CD4^+$ T cells (27, 31–35). IL-36 agonist cytokines mediate broad proinflammatory effects, including cytokine production, inflammatory cell recruitment, DC maturation, and T helper 1 and IL-17-producing T cell polarization (27, 35, 36). IL-36 research has mainly focused on skin disorders, especially psoriasis, and the role of IL-36 cytokines in lung biology is not fully understood (37). We have previously shown that IL-36γ assumes a central role in lung innate mucosal immunity against the extracellular bacterial pathogens *Streptococcus pneumoniae* and *Klebsiella pneumoniae*, whereas mice that lack IL-36R are protected against the lung injury and mortality caused by influenza virus (38, 39).

In the current study, we tested the hypothesis that IL-36 receptor agonist cytokines shape host immunity in the lung caused by *L. pneumophila* pneumonia. We found that IL-36 cytokines are expressed in the lung during *L. pneumophila* pneumonia and that these cytokines mediate effective lung bacterial clearance, inflammatory cell influx/activation, and cytokine expression. Notably, IL-36 cytokines are required for the effective M1 polarization of macrophages. Moreover, our data indicate that IL-36α and -γ cytokines can function in an overlapping fashion during *L. pneumophila* pneumonia.

RESULTS

IL-36 cytokines are induced in the lungs after i.t. *L. pneumophila* administration. Initial experiments were performed to assess the *in vivo* expression of IL-36 cytokines in the lungs of wild-type (WT) mice infected with *L. pneumophila* (Suzuki strain). After intratracheal (i.t.) administration of *L. pneumophila* (2.2×10^7 CFU), pul-

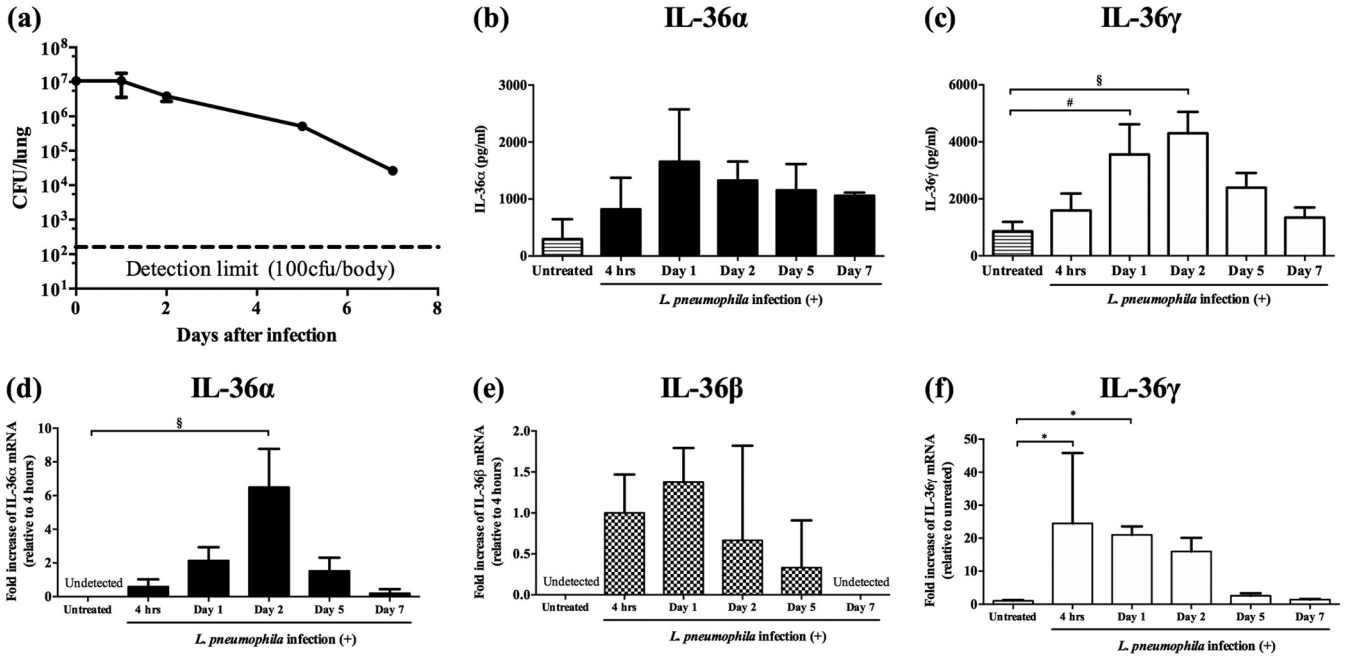

FIG 1 IL-36 cytokine induction in lung after i.t. *L. pneumophila* administration. (a) Numbers of *L. pneumophila* CFU in lung. WT mice were administered *L. pneumophila* (2.2×10^7 CFU) i.t., the lungs were harvested, and the number of CFU was determined at 4 h and 1, 2, 5, and 7 days after infection. (b, c) The levels of IL-36α (b) and IL-36γ (c) cytokines in whole-lung homogenates determined by ELISA. (d to f) mRNA expression of IL-36α (d), IL-36β (e), and IL-36γ (f) at 4 h and 1, 2, 5, and 7 days after *L. pneumophila* administration. Data are expressed as the fold increase compared with the levels in the lungs of uninfected mice or at 4 h after infection if gene expression in uninfected mice was not detected. Data are shown as the means ± SD for three or four mice per group. Results are representative of those from two independent experiments. Significant differences for the indicated comparisons were determined by use of a *post hoc* Bonferroni correction for multiple comparisons. *, $P < 0.05$; #, $P < 0.01$; §, $P < 0.001$.

monary bacterial burdens in WT mice peaked at day 1 of infection and decreased with time (Fig. 1a). All IL-36 cytokine (IL-36α, -β, and -γ) mRNA levels in the lung were upregulated, reaching peak levels within the first 48 h and then declining thereafter (Fig. 1d to f). IL-36α and -γ protein levels were also elevated, peaking at day 2 or before (Fig. 1b and c). We were unable to measure IL-36β protein levels, as no commercially available or home-grown enzyme-linked immunosorbent assay (ELISA) currently exists. Collectively, these data indicate that IL-36 cytokines are induced in the lungs of animals during experimental *L. pneumophila* pulmonary infection.

Decreased survival in IL-36R$^{-/-}$ mice but not IL-36$\alpha^{-/-}$ and -$\gamma^{-/-}$ mice with *L. pneumophila* infection. To determine if IL-36 cytokines are required for protection against *Legionella* infection, WT, IL-36α-deficient (IL-36$\alpha^{-/-}$), IL-36$\gamma^{-/-}$, and IL-36R$^{-/-}$ mice were inoculated i.t. with *L. pneumophila* (8.0×10^6 to 2.2×10^7 CFU) and assessed for survival to day 21. The survival rates of IL-36$\alpha^{-/-}$ and -$\gamma^{-/-}$ mice were not significantly different from those of WT mice (Fig. 2a and b). Conversely, IL-36R$^{-/-}$ mice had significantly reduced survival compared to similarly treated WT mice (Fig. 2c; 67% versus 22% at day 21; $P < 0.05$). To determine if IL-36α and -γ cytokines might play overlapping roles during *Legionella* infection, WT and IL-36$\alpha^{-/-}$ mice were treated with polyclonal anti-IL-36γ-specific antibody or vehicle (normal saline) 2 h prior to i.t. administration of *L. pneumophila* (7.3×10^7 CFU). Additional controls included IL-36$\gamma^{-/-}$ and IL-36R$^{-/-}$ mice. As observed earlier, the rate of survival of IL-36$\alpha^{-/-}$ and IL-36$\gamma^{-/-}$ mice did not differ from that of *L. pneumophila*-infected WT mice. Similarly, treatment of WT mice with polyclonal IL-36γ-specific antibody did not alter survival compared to that for the control animals. However, the survival rate of IL-36$\alpha^{-/-}$ mice administered anti-IL-36γ antibody tended to be reduced compared to that of WT mice receiving normal saline and was indistinguishable from that observed in infected IL-36R-deficient mice (Fig. 2d). These findings indicate a redundant protective role for IL-36α and IL-36γ in *L. pneumophila* pulmonary infection.

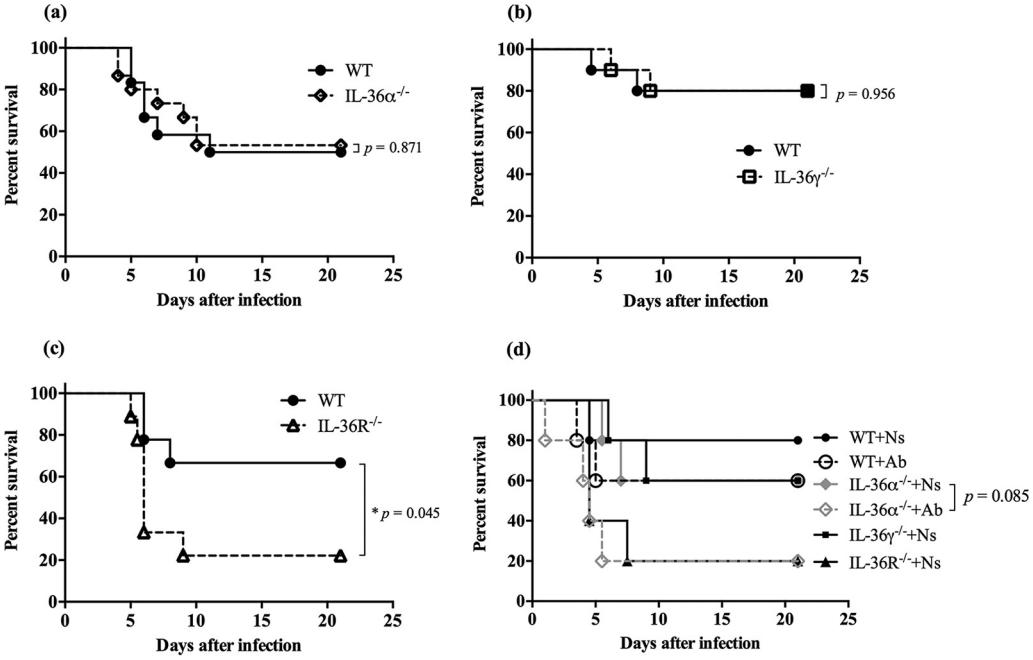

FIG 2 IL-36 cytokines impact survival in *Legionella* pneumonia. (a) Survival curves for WT ($n = 12$) and IL-36$\alpha^{-/-}$ ($n = 15$) mice infected with *L. pneumophila*. Mice were infected i.t. with 1.5×10^7 CFU to 2.2×10^7 CFU of *L. pneumophila*. Results from three independent experiments were combined. (b) Survival curves for WT ($n = 10$) and IL-36$\gamma^{-/-}$ ($n = 10$) mice infected with *L. pneumophila*. Mice were infected intratracheally with 8.0×10^6 CFU to 7.3×10^7 CFU of *L. pneumophila*. Results from two independent experiments were combined. (c) Survival curves for WT ($n = 9$) and IL-36R$^{-/-}$ ($n = 9$) mice infected with L. pneumophila. Mice were infected intratracheally with 8.0×10^6 CFU to 2.2×10^7 CFU of *L. pneumophila*. Results from two independent experiments were combined. (d) Survival curves for *L. pneumophila* (7.3×10^7 CFU)-infected WT mice treated with normal saline (Ns), WT mice treated with antibody (Ab), IL-36$\alpha^{-/-}$ mice treated with normal saline, IL-36$\alpha^{-/-}$ mice treated with antibody, IL-36$\gamma^{-/-}$ mice treated with normal saline, and IL-36R$^{-/-}$ mice treated with normal saline ($n = 5$ to 10). Animals were passively immunized with either normal saline or antibody and intratracheally infected with *L. pneumophila* at 2 h after immunization. Results are representative of those from two independent experiments. *, $P < 0.05$ compared with WT mice, as determined by the Mantel-Cox test.

Deficiency of IL-36R receptor signaling results in impaired lung bacterial clearance and increased systemic dissemination. Reduced survival was observed in IL-36R$^{-/-}$ mice but not in IL-36$\alpha^{-/-}$ or IL-36$\gamma^{-/-}$ mice, so for all subsequent experiments, responses were assessed in IL-36R mutant mice and compared to those in WT animals. To determine the mechanism(s) responsible for increased lethality, WT and IL-36R$^{-/-}$ mice were administered *L. pneumophila* (2.4×10^6 to 3.9×10^7 CFU), lungs were harvested at days 2, 4, and 6 after infectious challenge, and the bacterial CFU was quantitated. IL-36R$^{-/-}$ mice had significantly higher numbers of bacteria in the lung at days 2, 4, and 6 than the WT controls (Fig. 3a to c). Furthermore, the difference in lung bacterial burdens widened with time and was approximately 3.4-fold higher in IL-36R$^{-/-}$ mice than in WT mice at day 6 (Fig. 3c; $P < 0.01$). Moreover, the number of bacterial CFU in spleen was significantly higher at day 2 (Fig. 3d; 90.0 ± 28.06 CFU versus 415.0 ± 117.2 CFU; $P < 0.05$), and the presence of *L. pneumophila* in the spleens of IL-36R$^{-/-}$ mice persisted to days 4 and 6 (Fig. 3e and f).

To determine if differences in lung injury could account for the increased lethality and bacterial dissemination in IL-36R-deficient mice, we measured the albumin concentration in bronchoalveolar lavage (BAL) fluid as a measure of alveolar-capillary membrane integrity and compared it with that in WT mice at days 2 and 4 (Fig. 3g). Interestingly, BAL fluid albumin concentrations were lower in IL-36R$^{-/-}$ mice than in WT mice at day 2, whereas no differences in albumin levels were noted at day 4 (day 2, 0.42 ± 0.10 mg/ml versus 0.18 ± 0.03 mg/ml [$P < 0.05$]; day 4, 0.47 ± 0.09 mg/ml versus 0.40 ± 0.11 mg/ml [$P = 0.63$]). We also assessed the systemic response to infection by measuring hepatic and kidney injury and glucose homeostasis. The levels of alanine aminotransferase (ALT) in IL-36 R$^{-/-}$ mice tended to be higher than that in WT

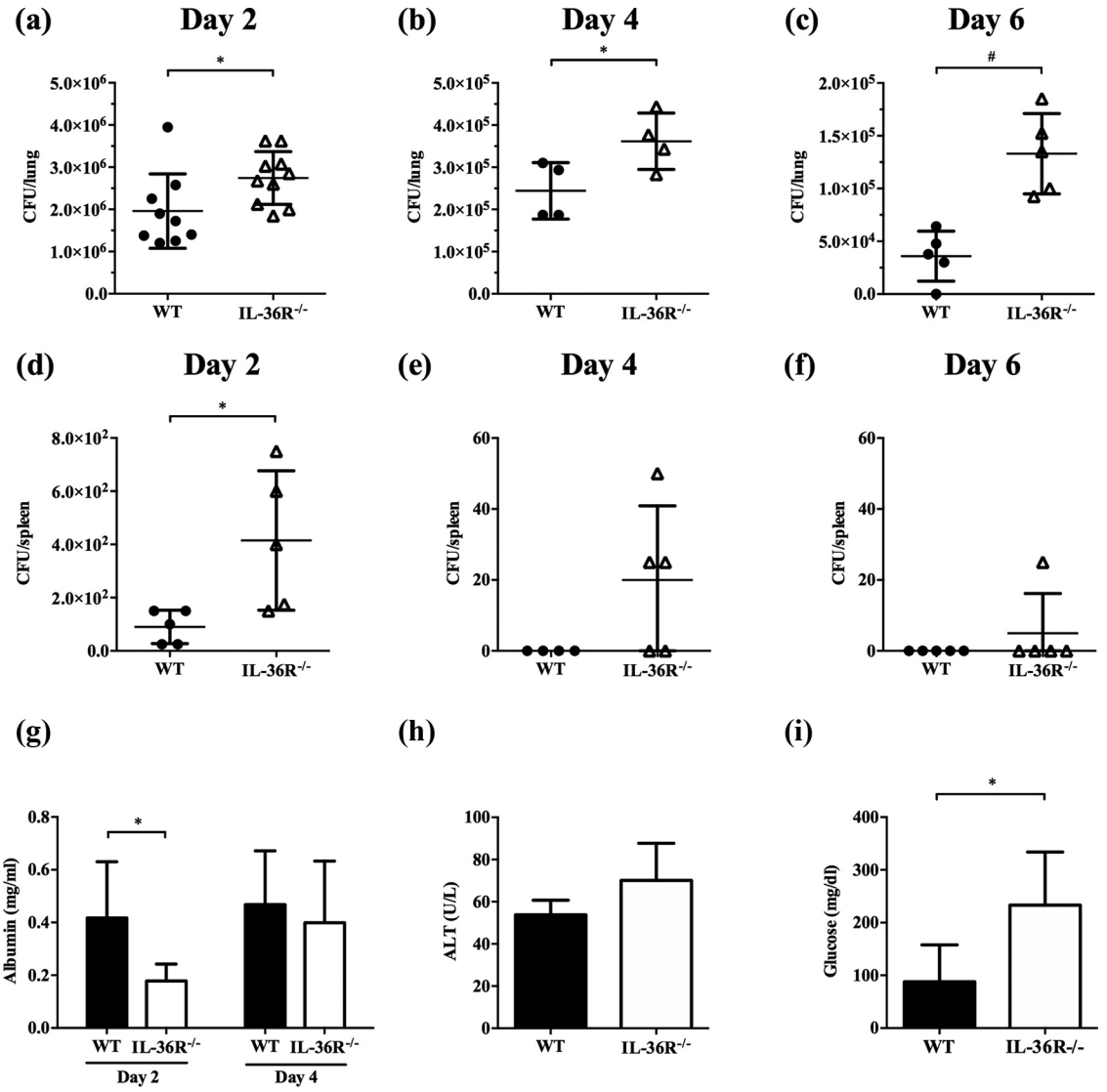

FIG 3 IL-36R gene deletion alters bacterial clearance and dissemination in *L. pneumophila* pneumonia. WT and IL-36R$^{-/-}$ mice were i.t. infected with 2.4×10^6 to 3.9×10^7 CFU of *L. pneumophila*. (a to c) The numbers of CFU in the lungs were assessed by serial dilution at 2 (a), 4 (b), and 6 (c) days after infection ($n = 4$ to 10 mice per group). (d to f) The numbers of CFU in the spleen were determined at 2 (d), 4 (e), and 6 (f) days after infection ($n = 4$ or 5 mice per group). (g) Lung permeability was quantified by determination of the albumin concentration in BAL fluid collected at 2 and 4 days postinfection ($n = 5$ per each group). (h, i) Blood samples were collected from the right ventricle at 4 days after infection, and the levels of ALT (h) and glucose (i) were determined ($n = 4$ to 7). Data are shown as the means ± SD. Results are representative of those from two or three independent experiments. Significant differences from WT mice for the indicated comparisons were determined by two-tailed Student's *t* test. *, $P < 0.05$; #, $P < 0.01$.

mice, but the difference was not significant (Fig. 3h; ALT concentrations, 70.14 U/liter versus 53.75 U/liter; $P = 0.11$). However, the glucose levels in IL-36 R$^{-/-}$ mice were significantly higher than those in WT mice (Fig. 3i; 233.3 mg/dl versus 87.8 mg/dl; $P > 0.05$). These results suggest that IL-36 R$^{-/-}$ mice experienced a more severe systemic inflammatory response, as indicated by impaired glucose homeostasis, during *L. pneumophila* pneumonia.

Reduced lung leukocyte accumulation in IL-36R$^{-/-}$ mice with *L. pneumophila* pneumonia. We next quantified the differences in alveolar leukocyte accumulation in WT and IL-36R$^{-/-}$ mice with *L. pneumophila* pneumonia at days 2 and 4 after bacterial administration. No differences in the total numbers of BAL fluid cells, polymorphonuclear leukocytes (PMN), and monocytes/macrophages were noted between uninfected WT and IL-36R$^{-/-}$ mice at the baseline. In WT mice, a substantial increase in BAL fluid

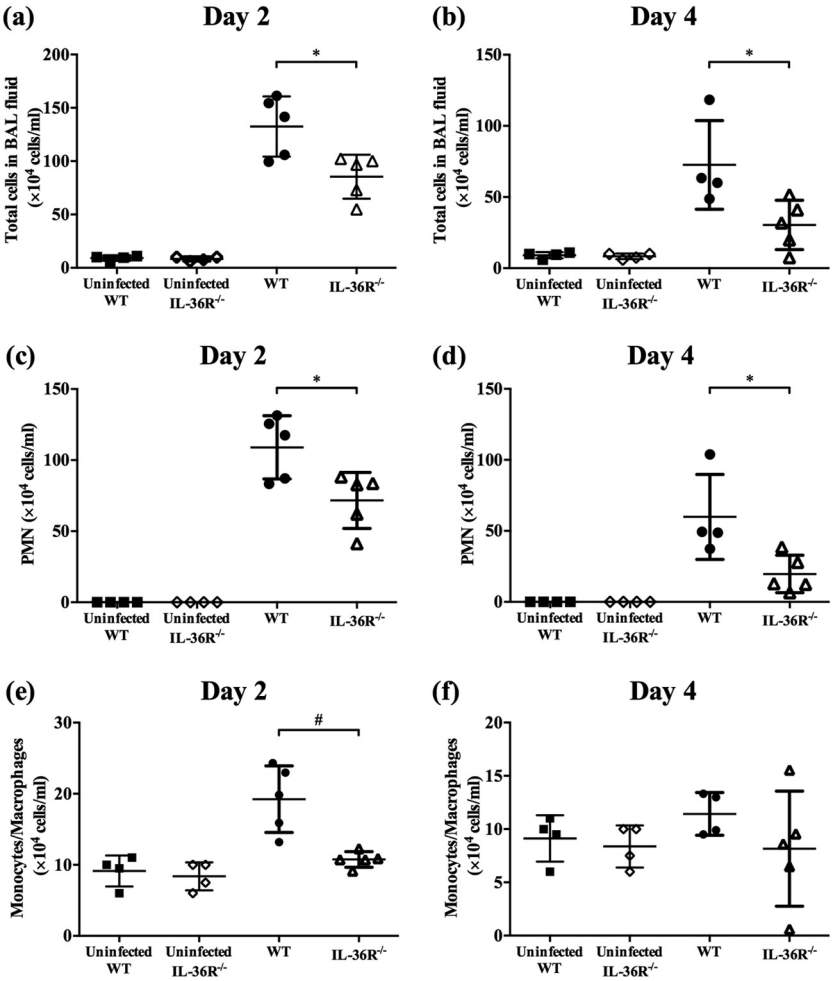

FIG 4 IL-36R gene deletion alters lung leukocyte accumulation during *L. pneumophila* pneumonia. (a to f) BAL fluid samples were collected at 2 (a, c, e) and 4 (b, d, f) days after infection. (a and b) The numbers of total cells in BAL fluid were determined using a hemocytometer. (c to f) After these were counted, samples were stained with modified Wright stain and the numbers of neutrophils (c and d) and monocytes/macrophages (e and f) were counted. Each group consisted of five mice. Data (means ± SD) are representative of those from two independent experiments. * $P < 0.05$ compared with WT mice; #, $P < 0.01$ compared with WT mice.

total cells, PMN, and monocytes/macrophages was noted after i.t. *L. pneumophila* administration. The total number of lung leukocytes in the BAL fluid during infection was significantly lower in IL-36R$^{-/-}$ mice than in WT mice at both days 2 and 4 (Fig. 4a and b; day 2, 132.55 ± 12.62 × 10^4 cells/ml versus 85.47 ± 9.26 × 10^4 cells/ml [$P < 0.05$]; day 4, 133.50 ± 17.70 × 10^4 cells/ml versus 82.98 ± 10.58 × 10^4 cells/ml [$P < 0.05$]). Moreover, the number of neutrophils and monocytes/macrophages in BAL fluid was significantly reduced in IL-36R$^{-/-}$ mice compared with WT mice at day 2 (Fig. 4c and e; neutrophils, 109.0 ± 9.96 × 10^4 cells/ml versus 71.6 ± 8.82 × 10^4 cells/ml [$P < 0.05$]; monocytes/macrophages, 19.24 ± 2.10 × 10^4 cells/ml versus 10.75 ± 0.50 × 10^4 cells/ml [$P < 0.01$]). These results indicate an important role for IL-36 receptor signaling in the accumulation of inflammatory cells at the site of infection.

Reduced *ex vivo* alveolar macrophage activation but not neutrophil activation in IL-36R$^{-/-}$ mice with *L. pneumophila* pneumonia. We next compared the cell surface expression of CD86 and CD62L to assess the activational state of monocytes/macrophages and PMN, respectively, in infected WT and IL-36R$^{-/-}$ mice by flow cytometry. Macrophage populations were defined by CD11b$^+$ and F4/80^{high} cells, and neutrophils were defined by CD11b$^+$ and Ly6G^{high} cells. At days 2 and 4, the mean

expression of the costimulatory molecule CD86 was significantly reduced in cells recovered from IL-36R$^{-/-}$ mice compared to cells recovered from WT animals (Fig. 5a), whereas there were no significant differences in the mean expression of CD62L by neutrophils recovered from infected WT and IL-36R$^{-/-}$ mice (Fig. 5b).

To further assess differences in macrophage activation during infection, alveolar macrophages were isolated *ex vivo* from BAL fluid collected from WT or IL-36R$^{-/-}$ mice at 48 h after *L. pneumophila* administration. We compared the constitutive mRNA expression levels of cytokines in association with M1 and M2 activation. The expression levels of inducible nitric oxide synthase (iNOS), TNF-α, and IFN-γ mRNA in macrophages from IL-36R$^{-/-}$ mice were significantly lower than those in macrophages from WT mice, and IL-1β mRNA expression levels tended to be lower in macrophages from IL-36R$^{-/-}$ mice (Fig. 5c and e to g; $P = 0.05$ for IL-1β). Conversely, arginase-1 (Arg-1) mRNA expression was greater in macrophages from IL-36R$^{-/-}$ mice than in macrophages from WT mice (Fig. 5d). These results implicate IL-36 receptor signaling in lung macrophage polarization (40).

Delay and/or decrease in expression of protective cytokines and chemokines in IL-36R$^{-/-}$ mice during *Legionella* pneumonia. Finally, we examined whether IL-36 receptor signaling regulated the expression of important cytokines and chemokines involved in protective innate and acquired pulmonary immune responses. As shown in Fig. 6 and Table 1, a reduction in whole-lung mRNA expression and the protein levels of relevant cytokines and chemokines, respectively, was observed in infected IL-36R$^{-/-}$ mice compared to their WT counterparts. Specifically, decreased expression of IL-1β, IL-6, and IL-17 mRNA was found at day 2 postinfection, reductions in IL-12p40 mRNA expression were found at day 4, and decreased CXCL2/macrophage inflammatory protein 2 (MIP-2) mRNA levels were noted at both days 2 and 4 after *L. pneumophila* administration in IL-36R$^{-/-}$ mice compared to infected WT mice. Interestingly, we also observed abrogated mRNA expression of IL-36α, -β, or -γ at both 2 and 4 days after bacterial challenge, suggesting an autocrine loop of induction *in vivo*. Significant reductions in cytokine proteins mirrored the changes in mRNA expression for all proteins except the IL-36α protein, the levels of which were not reduced in IL-36R$^{-/-}$ mice. As mentioned earlier, we were unable to quantitate IL-36β levels due to the lack of an ELISA.

DISCUSSION

The current study demonstrates that IL-36 receptor signaling is required for protection against *L. pneumophila* pneumonia. Specifically, i.t. *L. pneumophila* challenge resulted in the early and sustained expression of multiple IL-36 agonist cytokines in lung. We observed an increase in mortality in IL-36R$^{-/-}$ mice with *L. pneumophila* pneumonia. Lethality in these animals was associated with a higher lung bacterial burden and increased bacterial dissemination to extrapulmonary organs, although less early lung injury was found in IL-36R$^{-/-}$ mice. Moreover, no increased lethality was noted in mice deficient in either IL-36α or IL-36γ, as IL-36R agonist cytokines appeared to be able to compensate for the absence of one another.

Several recent studies have shown the importance of IL-36 cytokines in immune responses against infection. However, we still know very little regarding the biology of these cytokines, especially in the lung. Previous studies have indicated that the transcription products of IL-36γ are upregulated in bronchial epithelial cells by stimulation with proinflammatory cytokines or bacterium- or virus-associated molecular patterns (37, 41). Moreover, we have shown that IL-36γ contributes to lung innate mucosal immunity in bacterial pneumonia due to the Gram-positive pathogen *S. pneumoniae* and the Gram-negative pathogen *K. pneumoniae* (38). Our data indicated that either IL-36α or IL-36γ, but not both, is required for host defense against *L. pneumophila* infection, a finding which differs somewhat from observations made in experimental pneumonia due to *S. pneumoniae* or *K. pneumoniae*. A possible explanation for this discrepancy is that *S. pneumoniae* and *K. pneumoniae* have capsular polysaccharides on their surface and are extracellular bacteria, whereas *L. pneumophila*

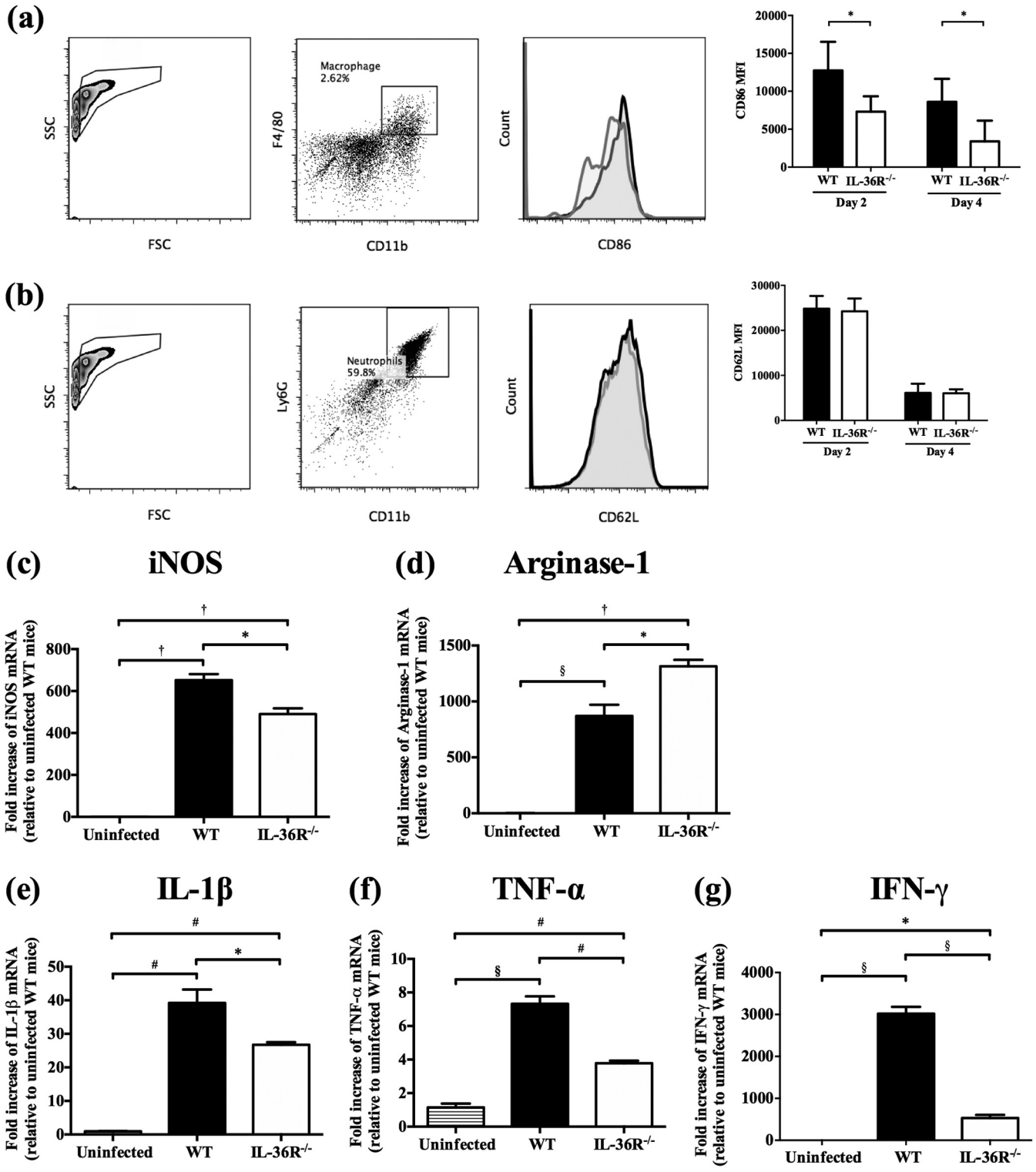

FIG 5 IL-36R gene deletion alters markers of M1 macrophage polarization and cell surface expression of CD86 but not CD62L on neutrophils during *L. pneumophila* pneumonia. (a) BAL fluid samples were collected at 2 and 4 days after infection. Cells in BAL fluid were stained for CD11b, F4/80, and CD86 and evaluated by flow cytometry. Plots were gated on $CD11b^{+}$ and $F4/80^{high}$ cells. A representative histogram and the mean fluorescence intensity are shown for CD86. Black lines, results for a WT mouse; gray lines, results for an IL-36R gene deletion mouse. SSC, side scatter; FSC, forward scatter. (b) Cells in BAL fluid were stained for CD11b, Ly6G, and CD62L and evaluated by flow cytometry. Plots were gated on $CD11b^{+}$ and $Ly6G^{high}$ cells. A representative histogram and the mean fluorescence intensity are shown for CD62L. Each group consisted of five mice. Data (means ± SD) are representative of those from two independent experiments. Black lines, results for a WT mouse; gray lines, results for an IL-36R gene deletion mouse. (c to g) mRNA expression levels of M1 and M2 activation markers in alveolar macrophages isolated at 2 days after *L. pneumophila* administration. (c) iNOS; (d) arginase-1; (e) IL-1β; (f) TNF-α; (g) IFN-γ. Each group consisted of macrophages from four separate mice. Data are shown as the means ± SD. Significant differences for the indicated comparisons were determined by the two-tailed Student's *t* test or a *post hoc* Bonferroni correction for multiple comparisons. *, $P < 0.05$; #, $P < 0.01$; §, $P < 0.001$; †, $P < 0.0001$.

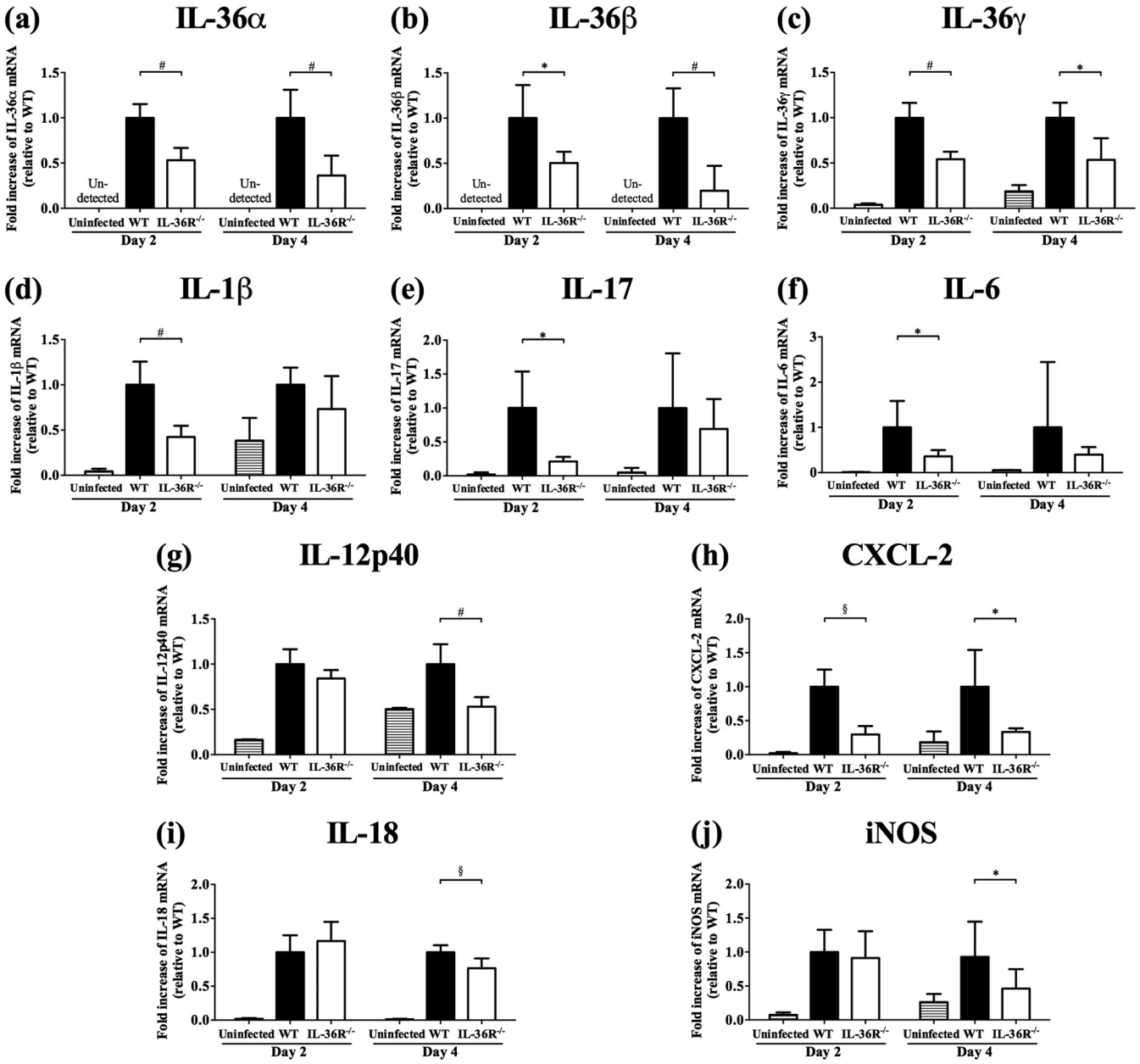

FIG 6 IL-36R gene deletion reduces the production of proinflammatory cytokines and chemokines in mice with *L. pneumophila* pneumonia. Mice were infected i.t. with 2.4×10^6 to 3.9×10^7 CFU of *L. pneumophila*. Lung samples were collected at 2 and 4 days after i.t. *L. pneumophila* administration. (a to j) mRNA expression levels of inflammatory markers. (a) IL-36α; (b) IL-36β; (c) IL-36γ; (d) IL-1β; (e) IL-17; (f) IL-6; (g) IL-12p40; (h) CXCL2; (i) IL-18; (j) iNOS. Each group consisted of 4 to 5 mice. (a to h) Data (means ± SD) are representative of those from two independent experiments. (i and j) Data from two independent experiments were combined. *P* values are for comparison with the results for WT mice and were determined using the two-tailed Student's *t* test. *, $P < 0.05$; #, $P < 0.01$; §, $P < 0.001$.

replicates intracellularly and does not have capsular polysaccharides on its cell surface. We speculate that these bacteria may elicit different cytokine responses depending upon the pathogen-associated molecular patterns (PAMPs) expressed or whether the PAMP exposure is intracellular versus extracellular. To that end, we have previously observed that exposure to *S. pneumoniae* or *K. pneumoniae in vitro* or *in vivo* preferentially induces IL-36γ rather than IL-36α, whereas we found a more balanced expression of both cytokines in response to *L. pneumophila in vivo* or *in vivo*. In contrast to their protective roles in invasive bacterial infections, IL-36 agonist cytokines can mediate deleterious host responses in viral or cytotoxic bacterial pneumonias, as IL-36

TABLE 1 Levels of immunoreactive cytokines and chemokines in whole lung[d]

Cytokine or chemokine	Concn (pg/ml)			
	Day 2		Day 4	
	WT mice	IL-36R$^{-/-}$ mice	WT mice	IL-36R$^{-/-}$ mice
IL-36α	3,969 ± 525.1	3,341 ± 169.7	6,458 ± 367.2	6,608 ± 432.3
IL-36γ	5,931 ± 1,023	5,522 ± 378.3	5,952 ± 388.3	3,434 ± 466.9[b]
IL-1β	2,555 ± 162.7	1,625 ± 164.7[b]	697.5 ± 144.2	362.0 ± 109.5
IL-17	526.5 ± 63.08	464.7 ± 49.72	713.1 ± 38.32	492.2 ± 50.56[a]
IL-6	1,085 ± 119.3	508.2 ± 60.65[b]	508.0 ± 62.13	306.5 ± 64.54
IL-12	1,316 ± 157.7	1,024 ± 121.5	1,728 ± 85.34	1,260 ± 148.2[a]
CXCL2	3,027 ± 179.5	1,374 ± 119.2[c]	832.6 ± 121.9	242.9 ± 27.59[b]

[a]$P < 0.05$ compared with WT mice using the two-tailed Student's *t* test.
[b]$P < 0.01$ compared with WT mice using the two-tailed Student's *t* test.
[c]$P < 0.001$ compared with WT mice using the two-tailed Student's *t* test.
[d]Each group consisted of four or five mice. Data are the means ± SEM and are representative of those from two independent experiments.

receptor deletion decreases mortality and lung injury in mice with influenza and cytotoxic *Pseudomonas aeruginosa* pneumonia (39, 42). The effects in influenza virus and *P. aeruginosa* infections are contrary to those observed in *L. pneumophila* pneumonia. This disparity can be partially reconciled by the observation that certain proinflammatory cytokines, such as IL-1β and IL-18 in *Pseudomonas* pneumonia and IL-17 in influenza virus pneumonia, mediate detrimental effects, whereas these cytokines contribute to protective immunity against intracellular pathogens, including *L. pneumophila* (43–46).

Lung resident and recruited macrophages are required for the ingestion and eventual eradication of *L. pneumophila* from the lung. Recruited neutrophils also contribute to innate immunity against *L. pneumophila*, in part by elaborating cytokines that drive protective type 1 cytokine responses (14). In this study, we found reduced numbers of leukocytes in the lung airspace of *L. pneumophila*-infected IL-36R-deficient mice compared to the WT controls. This defect in accumulation was most profound for PMN influx, but decreases in monocytes/macrophages were also observed. In addition to reduced numbers of monocytes/macrophages, a reduction in expression of macrophage activation markers was noted in IL-36R$^{-/-}$ mice. Specifically, the costimulatory molecule CD86 is upregulated in DC and macrophages in response to *L. pneumophila* (47, 48), and cell surface expression of macrophage CD86 was reduced in IL-36R$^{-/-}$ mice during *L. pneumophila* infection. Moreover, we observed reduced classical or M1 macrophage activation in mice lacking IL-36 receptor signaling. M1 macrophages are referred to as killer macrophages and produce proinflammatory cytokines, such as TNF-α, IL-1, and IFN-γ, and reactive oxygen intermediates and upregulate iNOS and costimulatory molecules, such as CD86 (40, 49). In contrast, M2 macrophages are characterized by the release of anti-inflammatory and reparative cytokines, upregulate Arg-1, and downregulate iNOS (40, 50). We observed reduced amounts of M1 markers (iNOS, IL-1β, TNF-α, and IFN-γ) and increased amounts of Arg-1 in alveolar macrophages isolated from IL-36R$^{-/-}$ mice *ex vivo* (Fig. 5c to g). This finding suggests that IL-36 cytokines are required for M1 macrophage skewing. Whether this is a direct or an indirect effect of the IL-36 cytokine is not known. In contrast, the genetic deletion of IL-36R did not alter cell surface CD62L expression by recruited PMN, although we cannot exclude the possibility of other functional neutrophil effects, such as oxidant production or protease release. Taken together, it is highly likely that these defects in PMN and monocyte/macrophage influx and impaired M1 macrophage polarization contributed to both defective lung bacterial clearance responses and reduced lung injury (as reflected by lower BAL fluid albumin levels) in mice deficient in IL-36R.

Our findings indicate for the first time that IL-36α and -γ cytokines have overlapping roles in *Legionella* infection *in vivo*. This notion is supported by the finding that antibody-mediated neutralization of IL-36γ in IL-36$\alpha^{-/-}$ mice resulted in a markedly increased mortality compared to that of infected WT mice (Fig. 2d), whereas no

increased mortality was observed in mice with a deletion of the single gene for either IL-36α or IL-36γ alone (Fig. 2a and b). These observations suggest that IL-36α or IL-36γ, but not both, is necessary for effective host defense against *L. pneumophila* pneumonia. There remains a possibility that IL-36β also contributes to anti-*Legionella* host immunity, as this cytokine is upregulated at the mRNA level in *L. pneumophila*-infected mice. Unfortunately, we have not yet produced a neutralizing antibody directed against IL-36β and no commercial source for such an antibody exists, nor do mice with genetic deletions in IL-36β exist. However, the fact that the rate of mortality observed in mice in which both IL-36α and IL-36γ have been inhibited/genetically deleted is similar to that observed in IL-36R$^{-/-}$ mice suggests that at least one of these two agonist cytokines is indispensable.

A reduction of important proximal cytokines (IL-1β, IL-6), neutrophil-active chemokines (CXCL2), type 1 cytokines (IL-12), and IL-17 cytokines was observed in IL-36R-deficient mice during the early and/or later response to *L. pneumophila* administration (Fig. 6; Table 1). This finding is in line with observations in other infection models (38, 39). IL-36 receptor signaling requires MyD88 and activates MyD88-dependent downstream signal transduction cascades, including the NF-κB and MAPK pathways (51). It is possible that the decrease in cytokine expression *in vivo* is in part a consequence of reductions in PMN and monocyte/macrophage influx in lung. However, we and others have shown that IL-36 cytokines can directly induce inflammatory cytokines from macrophages and epithelial cells *in vitro* (39, 52). We also observed attenuated mRNA expression of IL-36 cytokines in *L. pneumophila*-infected IL-36R$^{-/-}$ mice, suggesting an autocrine amplification loop for induction of IL-36 cytokines. While this is the first time, to our knowledge, that this has been reported, *in vivo* observations are consistent with this premise. However, we have a couple of limitations in the present study. First, we did not assess in detail cytokine production *in vitro*, including that by macrophage, type II alveolar epithelial cells, and DC. This should be assessed in future studies. Second, while alterations in cytokine mRNA levels generally tracked the changes in protein levels, this was not the case for IL-36α mRNA and protein expression. The likely explanation for this disparity is the lower sensitivity of the IL-36α ELISA than other cytokine ELISAs and the use of whole-lung homogenates, which can reduce the sensitivity of antigen detection due to the interference of capture antibody binding by a multitude of lung proteins.

The precise cause for the increased mortality in infected IL-36R$^{-/-}$ mice has not been completely defined. We observed a transient systemic dissemination of *L. pneumophila* to the spleen in WT mice by day 2 (Fig. 3d and e), with elimination by day 6, in line with the findings of previous studies (53, 54). By comparison, the splenic bacterial burden was considerably higher in IL-36R$^{-/-}$ mice than in WT mice and persisted even out to 6 days after *L. pneumophila* challenge. Interestingly, the delayed systemic clearance is analogous to that observed in MyD88$^{-/-}$ mice during *L. pneumophila* infection (53), and we have shown the IL-36R signaling requires MyD88 (38). IL-36R-deficient mice also displayed an altered septic response to infection, manifest by altered glucose homeostasis, which occurs as a result of insulin resistance (55), and a trend toward enhanced hepatic injury. We did not observe significant differences in renal function (data not shown). Also, the enhanced mortality in the IL-36R$^{-/-}$ mice was not due to lung injury, as BAL fluid albumin levels were decreased in these animals at 2 days but not at 4 days compared to those in *L. pneumophila*-infected WT mice (Fig. 3f). Thus, we speculate that the higher mortality of IL-36R$^{-/-}$ mice is caused, in part, by bacterial dissemination and the corresponding deleterious inflammatory response.

In conclusion, our findings demonstrate the importance of IL-36 cytokines in host innate immunity during experimental *L. pneumophila* pneumonia. The role of these cytokines in humans with *L. pneumophila* pneumonia is unknown, but future studies will be especially important, given the pivotal role that these cytokines play in balancing robust protective innate responses with deleterious inflammation and the resultant collateral damage within the lung.

MATERIALS AND METHODS

Mice. Specific-pathogen-free age- and sex-matched C57BL/6 WT mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-36R$^{-/-}$ mice on the C57BL/6 genetic background were provided by Amgen (Thousand Oaks, CA) (56). IL-36$\alpha^{-/-}$ and IL-36$\gamma^{-/-}$ mice bred on a C57BL/6 background were established at the University of Michigan (52). All mice were housed under specific-pathogen-free conditions in the University of Michigan Animal Care Facility. All experiments were conducted according to the University of Michigan's ethical guidelines for animal experiments and safety guidelines for gene manipulation experiments. Animal studies were reviewed and approved by the Committee on Use and Care of Animals at the University of Michigan.

Generation of rabbit anti-mouse polyclonal IL-36-specific antibody. Anti-IL-36γ and anti-IL-36α antibodies were generated in New Zealand White rabbits by immunization with recombinant mouse IL-36γ or IL-36α as described previously (57). The antibody was purified and the titer was measured by ELISA. Purified IgG from nonimmunized rabbits was used as a control. The rabbit anti-mouse IL-36γ antibody produced was neutralizing, whereas the anti-IL-36α antibody was not. The IL-36γ antibody failed to recognize purified recombinant murine IL-36β or IL-36α by ELISA or inhibit the IL-36α-induced production of chemokines by lung macrophages. Likewise, the IL-36α antibody did not recognize purified recombinant murine IL-36γ or IL-36α by ELISA or inhibit the IL-36γ-induced production of cytokines and chemokines by lung macrophages *in vitro* (data not shown).

***L. pneumophila* administration.** The clinical isolate that we used was the *L. pneumophila* Suzuki serogroup 1 strain, stocked at the University of Michigan for all animal experiments (14). A bacterial suspension was prepared as described previously (14). Briefly, *L. pneumophila* was grown for 3 to 4 days at 37°C on buffered charcoal yeast extract (BCYE) agar (BD, Franklin Lakes, NJ) supplemented with L-cysteine (Sigma, St. Louis, MO), ferric nitrate (Sigma), and thymidine (Sigma). A single colony was transferred to *N*-(2-acetamido)-2-aminoethanesulfonic acid (Sigma) buffered yeast extract broth and incubated for 20 to 24 h. One hundred fifty microliters of this incubated bacterial suspension was transferred to 3 ml of new broth and again incubated for 20 to 24 h. We measured the optical density value of this suspension for estimating bacterial numbers, and then we diluted it to the target numbers. The number of CFU was confirmed by plating and incubating the organisms on BCYE agar for 4 days.

Intratracheal administration. Mice were anesthetized by intraperitoneal injection of ketamine and xylazine, and then the trachea was exposed under sterile conditions and 30 μl of inoculum was administered into the trachea via a 26-gauge needle. The skin incision was closed by the use of surgical staples (58).

Bronchoalveolar lavage fluid sampling. Mice were euthanized by CO_2 asphyxiation for the collection of bronchoalveolar lavage (BAL) fluid samples at 2 and 4 days after infection. The trachea was exposed and intubated using a 21-gauge polyethylene catheter. BAL was performed with a total of 3 ml (three aliquots of 1 ml each) of phosphate-buffered saline (PBS) containing EDTA (final concentration, 0.5 mM) (Fisher Scientific). BAL fluid samples were collected from each animal. Leukocyte numbers were determined using a hemocytometer. After counting of the leukocyte numbers, cytospins were prepared (Cytospin 3 centrifuge; Thermo Shandon Ltd., Cheshire, England) from each sample and stained with modified Wright stain. These remaining BAL fluid samples were stored at −80°C until subsequent use.

Whole-lung, spleen, and blood collection. At designated time points, mice were euthanized by CO_2 asphyxiation. The chests were then opened and whole blood was aspirated from the right ventricle and placed into heparinized syringes. The collected blood samples were centrifuged at 2,000 × *g* for 10 min at 4°C and stored at −80°C until assay. ALT and glucose levels in blood samples were measured by the In-Vivo Animal Core (IVAC) of the Unit for Laboratory Animal Medicine (ULAM) at the University of Michigan. The right lower lobe of the lungs was harvested for RNA extraction, immediately snap-frozen in liquid nitrogen, and stored at −80°C until it was used for evaluation of mRNA expression. The other lobes in the lungs and spleen were collected and homogenized under a vented hood with a homogenizer (Omni, Kennesaw, GA) in 1 ml of sterile PBS. Homogenized samples were inoculated on BCYE agar after serial dilution 1:10 in PBS. The remaining portions were centrifuged at 2,000 × *g* for 10 min, and the supernatants from these centrifugations were collected and stored at −20°C until use.

Murine alveolar macrophage isolation from lung. We isolated alveolar macrophages from BAL fluid using the method described by Deng et al. (59). Briefly, BAL fluid was centrifuged at 500 × *g* for 10 min, the cell pellet was resuspended, and the cells were counted to adjust the cell number (10^6 cells/ml). The cells were incubated for 45 min at 37°C on a 24-well tissue culture plate, the wells were washed for elimination of nonadherent cells, and fresh medium was added. After incubation for 18 h, the cells were lysed for RNA analysis. Greater than 90% macrophage purity was demonstrated by flow cytometry.

Flow cytometry analysis. Cells isolated from BAL fluid were stained with fluorescein isothiocyanate-, phycoerythrin-Cy5-, Violet 421-, V500-, or Qdot 605-labeling antibodies to Ly6G, F4/80, CD86, CD11b, or CD62L (L-selectin), respectively (BioLegend, San Diego, CA, or BD Biosciences, San Jose, CA). Flow cytometry was performed using an Attune acoustic focusing cytometer (Thermo Scientific-Applied Biosystems, Foster City, CA) for analysis. Macrophages were identified as CD11b$^+$ and F4/80^{high} cells, and neutrophils were identified as CD11b$^+$ and Ly6G^{high} cells.

Measurement of cytokines by enzyme-linked immunosorbent assay. Murine IL-36α and IL-36γ cytokines were quantitated using mouse sandwich ELISAs developed in our laboratory at the University of Michigan by a modified double-ligand method (39, 52). Other murine cytokines/chemokines (IL-1β, IL-6, IL-12, IL-17, and MIP-2/CXCL2) and albumin were analyzed using mouse DuoSet ELISA kits (R&D

Systems, Minneapolis, MN) or an albumin quantification kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturers' protocols.

RNA isolation and real-time PCR. RNA was isolated by use of the TRIzol reagent (Thermo Fisher, Carlsbad, CA), and real-time quantitative reverse transcription-PCR was performed by use of an AB Step One Plus real-time PCR system (Thermo Scientific-Applied Biosystems). Predesigned primer and probes for targeted molecules were purchased from Integrated DNA Technologies (Coralville, IA). Relative quantitation of mRNA levels was plotted as the fold change compared with the level for untreated control cells or WT mice.

Statistical analysis. Data were analyzed using Prism (version 5) software (GraphPad Software, La Jolla, CA, USA). All data except survival data are presented as the mean ± standard deviation (SD). Statistical significance was determined using the unpaired, two-tailed *t* test or the *post hoc* Bonferroni correction for multiple comparisons. Survival curves were constructed by the Kaplan-Meier method and were analyzed using log-rank (Mantel-Cox) tests. For all tests, differences were defined to be statistically significant if the *P* value was <0.05.

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