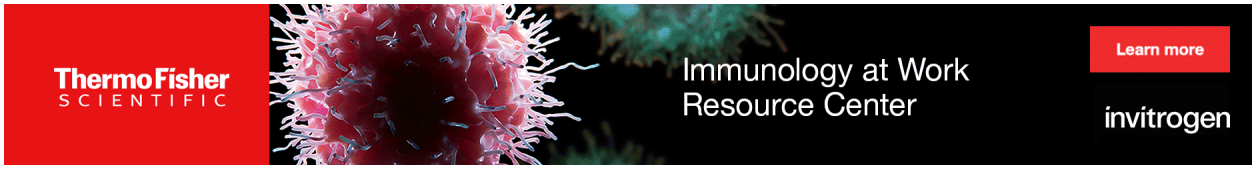

The Journal of Immunology

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Altered Inflammatory Responses in TLR5-Deficient Mice Infected with *Legionella pneumophila*[1]

Thomas R. Hawn,[2]* William R. Berrington,* Ian A. Smith,* Satoshi Uematsu,† Shizuo Akira,† Alan Aderem,‡ Kelly D. Smith,* and Shawn J. Skerrett*

Legionella pneumophila **(Lp), an important cause of morbidity and mortality from pneumonia, infects alveolar macrophages (AMs) and is recognized by several TLRs as well as Birc1e (NAIP5) and IL-1 converting enzyme-protease activating factor. We examined the role of TLR5 during the murine response to aerosolized Lp infection. At 4 h after infection, *Tlr5*$^{-/-}$ mice had lower numbers of polymorphonuclear neutrophils (PMNs) in their broncho-alveolar lavage fluid in comparison to wild-type (WT) mice. At 24 and 72 h, the PMN recruitment was similar. WT mice infected with a flagellin-deficient strain (LpFlaA−) also showed an impaired early PMN response at 4 h compared with those infected with the WT strain. There was no consistent difference in bacterial counts at any of the time points when comparing the *Tlr5*$^{-/-}$ and WT mice. However, at 6 days after infection, the *Tlr5*$^{-/-}$ mice had increased leukocytic infiltrates in the alveolar and peribronchial interstitial spaces that were consistent with organizing pneumonia. We also examined the role of TLR5 during macrophage infection. In contrast to bone marrow-derived macrophages, AMs secreted TNF-α after stimulation with purified flagellin. In addition, WT, but not *Tlr5*$^{-/-}$, AMs produced TNF-α after stimulation with Lp. Live LpFlaA− did not induce TNF-α secretion in AM. These results suggested that AMs recognize Lp flagellin and that a majority of the Lp-induced TNF-α response is TLR5-mediated. Thus, TLR5 mediates recognition of Lp in AMs and performs a distinct role during the in vivo pulmonary immune response through regulation of early PMN recruitment and subsequent later development of pneumonia.** ***The Journal of Immunology,*** **2007, 179: 6981–6987.**

Although pneumonia causes significant morbidity and mortality worldwide, the immunologic factors that mediate host susceptibility to lower respiratory tract infections are poorly understood (1). *Legionella pneumophila*, an important cause of community-acquired pneumonia, has several features that distinguish it from other pulmonary pathogens including intracellular replication in macrophages, a flagellum, an unusual LPS structure, and fastidious growth requirements (2, 3). *L. pneumophila* is detected in macrophages by two innate immune receptor families: the Nod-like receptors (NLRs)[3] and TLRs. Murine macrophages exhibit strain-dependent levels of in vitro resistance to *Legionella* replication, ranging from the highly susceptible A/J strain to the resistant C57BL/6 strain, and are regulated by the *LgnI* locus (4–6). Studies of this locus led to the identification of an NLR protein called NAIP5 (NLR family, apoptosis inhibitory protein 5; also called Birc1e or Baculoviral inhibitor of apoptosis repeat-containing protein 1e), a cytoplasmic protein that mediates recognition of *Legionella*. Recent studies suggest that NAIP5 restricts growth of *Legionella* through recognition of flagellin (7–9). IL-1 converting enzyme-protease activating factor (IPAF), also called Nlrc4 (NLR family, CARD domain containing 4), CARD12 (caspase recruitment domain protein 12), and CLAN (CARD LRR and NACHT-containing protein) is another NLR that has recently been found to regulate *Legionella* replication in macrophages (9, 10). Similar to NAIP5, IPAF mediates intracellular sensing of flagellin and activation of caspase-1 (10–12).

TLRs constitute a family of transmembrane proteins that recognize microbes and initiate inflammatory signaling pathways (13–16). TLRs recognize microbes at the plasma membrane or within organelles, such as phagosomes and endosomes, while NLRs mediate pathogen recognition in the cytosol. We, and others, have used in vitro and in vivo studies to demonstrate that *L. pneumophila* is recognized by several TLRs including TLR2, TLR4, TLR5, and TLR9 (17–24). Using human genetic studies, we previously found that a common stop codon polymorphism in TLR5, a receptor for bacterial flagellin, abrogates signaling and is associated with altered susceptibility to Legionnaires' Disease (20). The immunologic mechanisms underlying this increased susceptibility are currently unknown. In addition, we recently demonstrated that *Myd88*$^{-/-}$ mice, which have impaired signaling through all TLRs, except TLR3, are highly susceptible to in vivo infection with *L. pneumophila* (21). In contrast, *Tlr2*$^{-/-}$ mice are only partially susceptible in comparison to wild type (WT) animals. It is currently unknown which additional TLRs mediate host resistance to *L. pneumophila* in vivo.

Together, these studies indicate a critical role for TLRs and NLRs in the immune response to *Legionella*; yet, a number of important questions remain unanswered. First, does TLR5 mediate a distinct in vivo immune response despite the presence of several

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[3] Abbreviations used in this paper: NLR, Nod-like receptors; IPAF, IL-1 converting enzyme-protease activating factor; FliC, Flagellin C from *S. typhimukium*; WT, wild type; BCYE, buffered charcoal yeast extract; Lp, *Legionella pneumophila*; BAL, bronchoalveolar lavage; BMDM, bone marrow-derived macrophage; AM, alveolar macrophage; FlaA, Flagellin A from *L. pneumophila*.

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additional TLRs which mediate *L. pneumophila* recognition? Second, does TLR5 recognition of flagellin mediate a distinct host response in the presence of NAIP5 and IPAF? To answer these questions, we examined the role of flagellin and TLR5 in the murine host response to in vivo infection with aerosolized *L. pneumophila*.

Materials and Methods

Reagents, bacteria, cells, and mice

RPMI Medium 1640, L-glutamine, and penicillin-streptomycin were from Invitrogen and DMEM was from JRH Biosciences. Ultrapure LPS was from *Salmonella minnesota* R595 (List Biological Laboratories). Flagellin C (FliC) was purified from *Salmonella typhimurium* as previously described from strain TH4778, which is fljB-/fliC+ (25, 26). Flagellin A (FlaA) was purified from *L. pneumophila* Corby strain by the same method with bacteria grown as described below on buffered charcoal yeast extract (BCYE) agar plates. Bone marrow was harvested from mice and grown in DMEM supplemented with 10% heat-inactivated FCS (HyClone), 20% L-cell conditioned medium, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Bone marrow-derived macrophages (BMDMs) were used after 4–10 days of culture. To obtain alveolar macrophages (AMs), the trachea was cannulated and lavaged four times with 800 μl of 0.85% saline/0.6 mM EDTA as previously described (27). Cells were then plated in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. After a 4-h adherence step, the cells were washed six times with PBS before stimulation immediately or the next day.

Model of pneumonic legionellosis

The University of Washington and Institute for Systems Biology's Institutional Animal Care and Use Committees approved all animal protocols. *Tlr5*$^{-/-}$ mice (strain designation B6.129P2-*Tlr5*^{tm1Aki}) were derived and backcrossed to a C57BL/6 background for eight generations as previously described (28). WT control mice were from a C57BL/6 background (The Jackson Laboratory). *L. pneumophila*, Philadelphia 1 (ATCC 33152) was stored as previously described (29, 30). *L. pneumophila* Corby (serogroup 1) and *L. pneumophila* Corby FlaA− were gifts from K. Heuner (31). BCYE medium was prepared with α-ketoglutarate as previously described (32). Bacteria were inoculated from a frozen stock onto BCYE agar for 4 days at 35°C, harvested by rinsing plates with PBS, pelleted by centrifugation, and resuspended in PBS to a concentration of 1×10^{10} CFU/ml (estimated by OD at 540 nm and confirmed by quantitative culture) (20). The mice were exposed to bacterial aerosols generated by twin jet nebulizers (Salter Laboratories) for 30 min in a whole animal exposure chamber, as described (27, 33). Immediately after infection (to determine bacterial deposition) and at subsequent time points, mice were euthanized and exsanguinated. The trachea was cannulated and the right lung was lavaged with 0.85% saline/0.6 mM EDTA to determine cell counts and differentials, as described (9). The left lung was homogenized in PBS and the spleen was homogenized in Mueller-Hinton broth and then each tissue was serially diluted in Mueller Hinton broth for quantitative culture on BCYE agar. The remaining lung homogenate was mixed 1:1 with lysis buffer containing 2× protease inhibitor mixture (Roche Diagnostics), incubated for 30 min on ice, clarified by centrifugation at 2500 rpm, and then the supernatant was saved at −80°C.

Histology

To prepare organs for histology, the lung was inflated to 15 cm pressure with 4% paraformaldehyde, fixed in the same solution, embedded in paraffin, and then 4 μm sections were generated. Sections stained with hematoxylin and eosin were examined by a pathologist blinded to mouse genotype. The quantification of the percentage of inflamed lung was derived from the examination of 10 high-power fields.

Protein analysis

Cytokine levels in lung homogenates were determined in a multiplex fluorescent bead array system. Analytes were captured by Ab-coated, fluorochrome-embedded microspheres and detected by biotin-streptavidin-PE using reagents purchased from R&D Systems or a flow-based sorting and detection platform (Luminex 100). For single cytokine analysis, levels were determined with a sandwich ELISA technique (Duoset, R&D Systems).

Statistical analysis

For in vivo CFU count analysis, comparisons between groups were made with a Mann-Whitney *U* test because small sample sizes precluded an assumption of normal distribution. For cytokine measurements and cell counts, Student's *t* test was used. A value of $p \leq .05$ was considered significant. Statistics were calculated with Prism version 4.03 (GraphPad).

Results

Aerosolized lung infection of mice with FlaA− L. pneumophila

To understand the role of the pulmonary innate immune response to flagellin during an in vivo infection, we infected A/J mice with *L. pneumophila* WT (LpWT) and LpFlaA−, a strain with a deletion of the gene encoding flagellin. After aerosolized infection, we determined lung CFUs at 4, 24, 72, and 144 h after infection. There was a modest increase in CFUs in the mice infected with the FlaA− mutant in comparison to WT at 72 and 144 h after infection (median CFU (interquartile range) of 72 h: LpWT 6×10^3 ($4.2 \times 10^3 - 1.1 \times 10^4$) vs LpFlaA− 2.7×10^4 ($1.8 \times 10^4 - 6.8 \times 10^4$), $p = 0.007$); 144 h: LpWT 7.0×10^1 ($2.1 \times 10^1 - 2.7 \times 10^3$) vs LpFlaA− 5.5×10^3 ($4.1 \times 10^3 - 7.7 \times 10^3$), $p = 0.001$) (Fig. 1*A*). There were no statistically significant differences in CFUs at the earlier time points. We then examined the recruitment of neutrophils and mononuclear cells to the lungs at various time points after infection. In bronchoalveolar lavage (BAL) fluid from mice infected with LpWT, there was vigorous recruitment of polymorphonuclear neutrophils (PMNs) to the lungs 4 h after infection (mean ± SEM $1.9 \times 10^3 \pm 4.9 \times 10^2$ cells/lung) (Fig. 1*B*). In contrast, mice infected with LpFlaA− had a severely blunted PMN response with nearly 10-fold lower PMNs ($2.1 \times 10^2 \pm 1.4 \times 10^2$ cells/lung, $p = 0.0002$). There was no difference in the number of PMNs in BAL fluid at 24 and 72 h after infection (Fig. 1*C*). There was also no difference in the number of mononuclear cells recruited to the lungs at any time point (Fig. 1*B* and data not shown). Together these results suggested that host recognition of flagellin is critical for early neutrophil recruitment to the lungs, and the subsequent control of bacterial replication between 72 and 144 h postinfection.

Tlr5$^{-/-}$ *mice fail to recruit neutrophils to the lungs early after infection*

We next sought to determine whether the neutrophil recruitment defect was attributable to TLR5 or another host recognition pathway. To assess whether NAIP5 was involved in early neutrophil recruitment, we infected C57BL/6 mice, which have an intact NAIP5 locus in comparison to the A/J strain. Similar to the A/J strain, C57BL/6 mice infected with LpWT had a vigorous recruitment of PMNs to the lungs 4 h after infection (mean ± SEM: $1.1 \times 10^3 \pm 2.9 \times 10^2$ cells/lung) (Fig. 1*D*). In contrast, LpFlaA− infected mice had a blunted PMN response (mean ± SEM: $2.8 \times 10^1 \pm 1.0 \times 10^1$ cells/lung, $p = 0.0003$) (Fig. 1*D*). There was no difference in recruitment of PMNs at 24 and 72 h after infection or of monocytes at any time points (Fig. 1*D* and data not shown). Because there was a similar PMN recruitment defect in A/J and C57BL/6 mice, we speculated that the NAIP5 locus was not regulating this response.

To determine whether TLR5 regulated PMN recruitment, we infected *Tlr5*$^{-/-}$ mice on a C57BL/6 background with LpWT. There was a modest increase in CFUs in the *Tlr5*$^{-/-}$ mice infected with the LpWT mutant in comparison to WT mice at 4 h after infection (median CFU (interquartile range) at 4 h: WT mice 3.0×10^5 ($2.4 \times 10^5 - 4.1 \times 10^5$) vs *Tlr5*$^{-/-}$ 5.9×10^5 ($4.9 \times 10^5 - 8.2 \times 10^5$), $p = 0.008$) (Fig. 1*E*). There were no statistically significant differences in CFUs at any later time points. We then examined the recruitment of neutrophils and mononuclear cells to the

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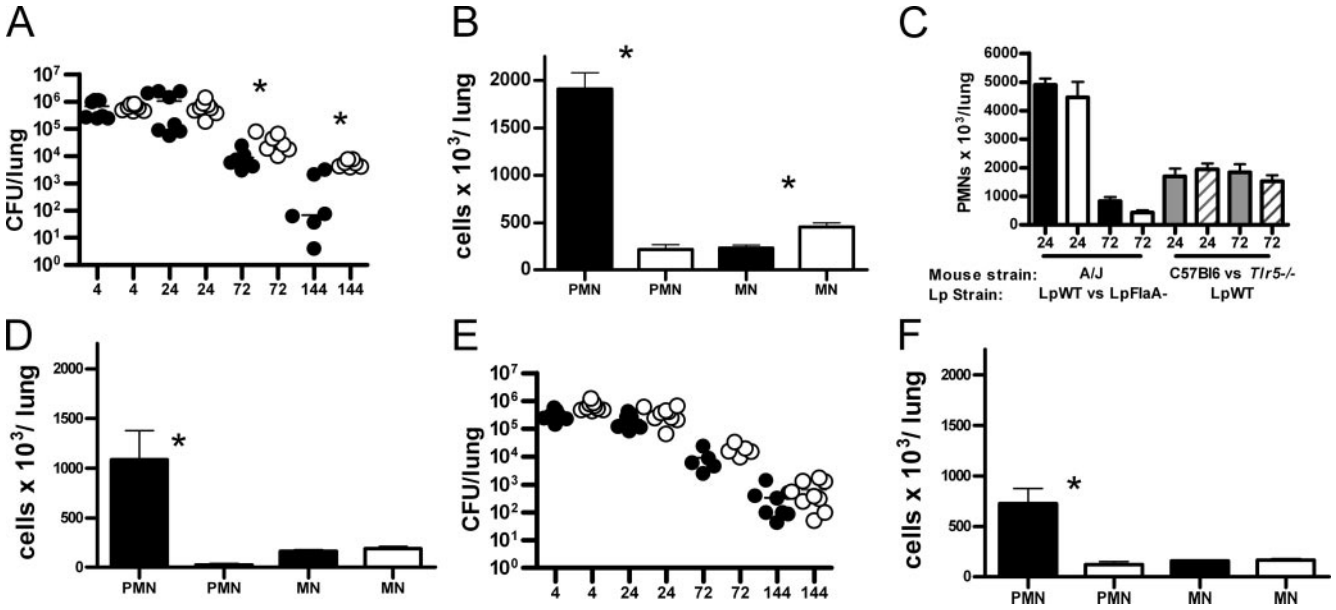

FIGURE 1. Lung neutrophil recruitment is regulated by flagellin recognition during in vivo infection with *L. pneumophila*. Mice were infected with aerosolized *L. pneumophila* and euthanized at the indicated time points to determine bacterial CFU in lung and spleen. BAL specimens were obtained for cell counts. *A*, Bacterial CFU in lung in C57BL/6 mice infected with LpWT (●) or LpFlaA− (○). *B* and *D*, Four-hour BAL cell counts indicating PMNs and mononuclear cells in A/J mice (*B*) or C57BL/6 mice (*D*) infected with LpWT (■) or LpFlaA− (□). *C*, Twenty four- and 72-h PMN BAL counts in A/J mice infected with LpWT (■) or LpFlaA− (□) or in C57BL/6 mice (▨) or *Tlr5*$^{-/-}$ mice (□ with hatch) infected with LpWT. *E*, Bacterial CFU in lung in C57BL/6 mice (●) or *Tlr5*$^{-/-}$ mice (○) infected with LpWT. *F*, Four-hour BAL counts from C57BL/6 mice (■) or *Tlr5*$^{-/-}$ mice (□) infected with LpWT. CFU data for each mouse are plotted with the median represented by a horizontal bar. Results represent combined data of two experiments. *, $p \leq 0.05$ by Mann-Whitney *U* test when comparing WT and knockout strains.

lungs. In BAL fluid from infected WT mice, there was vigorous recruitment of PMNs to the lungs (mean ± SEM: $7.3 \times 10^2 \pm 1.5 \times 10^2$ cells/lung) (Fig. 1*F*). In contrast, *Tlr5*$^{-/-}$ mice had a severely blunted PMN response ($1.6 \times 10^2 \pm 1.1 \times 10^1$ cells/lung, $p < 0.0001$). There was no difference in recruitment of PMNs at 24 and 72 h after infection or of monocytes at any time points (Fig. 1, *C* and *F* and data not shown). Together, these results demonstrate that TLR5 regulates early PMN recruitment in mice infected with *L. pneumophila*.

TLR5, flagellin, and cytokine/chemokine production

We next measured several cytokines and chemokines to determine whether production was regulated by TLR5 or stimulated by flagellin during in vivo infection. We measured several proinflammatory cytokines (TNF-α, IL-6, and IL-1β), chemokines involved in PMN recruitment (KC and MIP2), and cytokines involved in regulating Type 1 T cell mediated immunity (IL-12p40 and IFN-γ). We first compared A/J mice infected with LpWT or LpFlaA− strains and found that TNF-α levels at 4 h after infection were higher in mice infected with the WT strain (mean ± SEM LpWT 569.3 ± 37.1 pg/ml vs LpFlaA− 316.0 ± 32.0 pg/ml, $p = 0.001$, Fig. 2*A*). IL-6 levels were also higher in the WT animals with differences that reached borderline statistical significance (mean ± SEM LpWT 1259.0 ± 191.5 pg/ml vs LpFlaA− 671.8 ± 203.8 pg/ml, $p = 0.05$, Fig. 2*B*). In contrast, there was no difference in

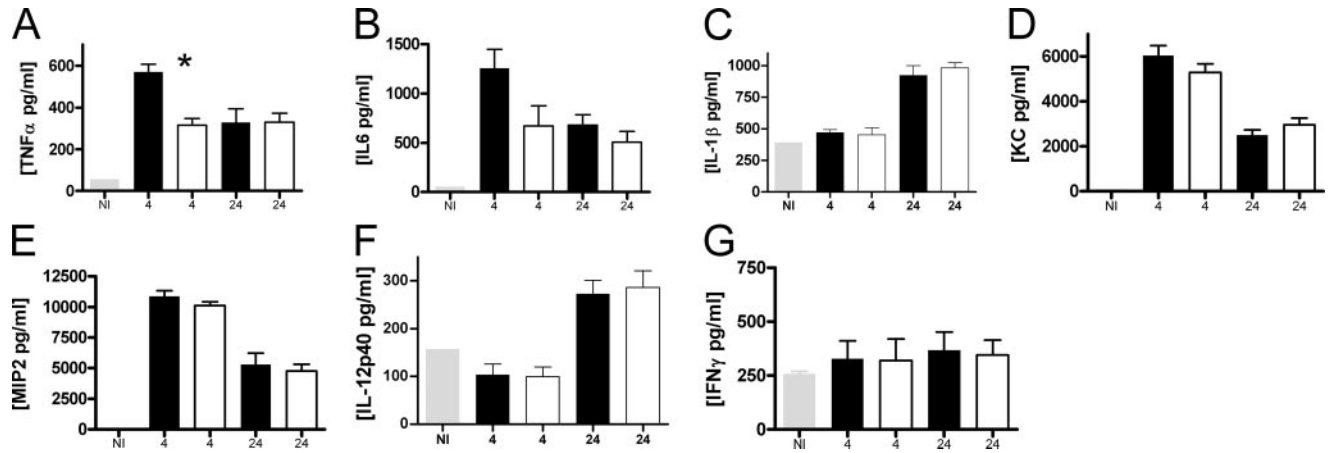

FIGURE 2. Cytokine and chemokine responses in mice after inhalation of WT and FlaA− mutant *L. pneumophila*. Lung homogenates were harvested after infection and assayed for cytokines and chemokines by multiplex microbead array: Data ($n = 4$–9 infected mice or $n = 2$ uninfected controls) represent means plus SEM *, $p \leq 0.05$ by unpaired Student's *t* test. Legend: *A–G*, A/J mice infected with LpWT (■) or LpFlaA− (□). 4 and 24, Time points in hours. NI, Not infected, in gray bars.

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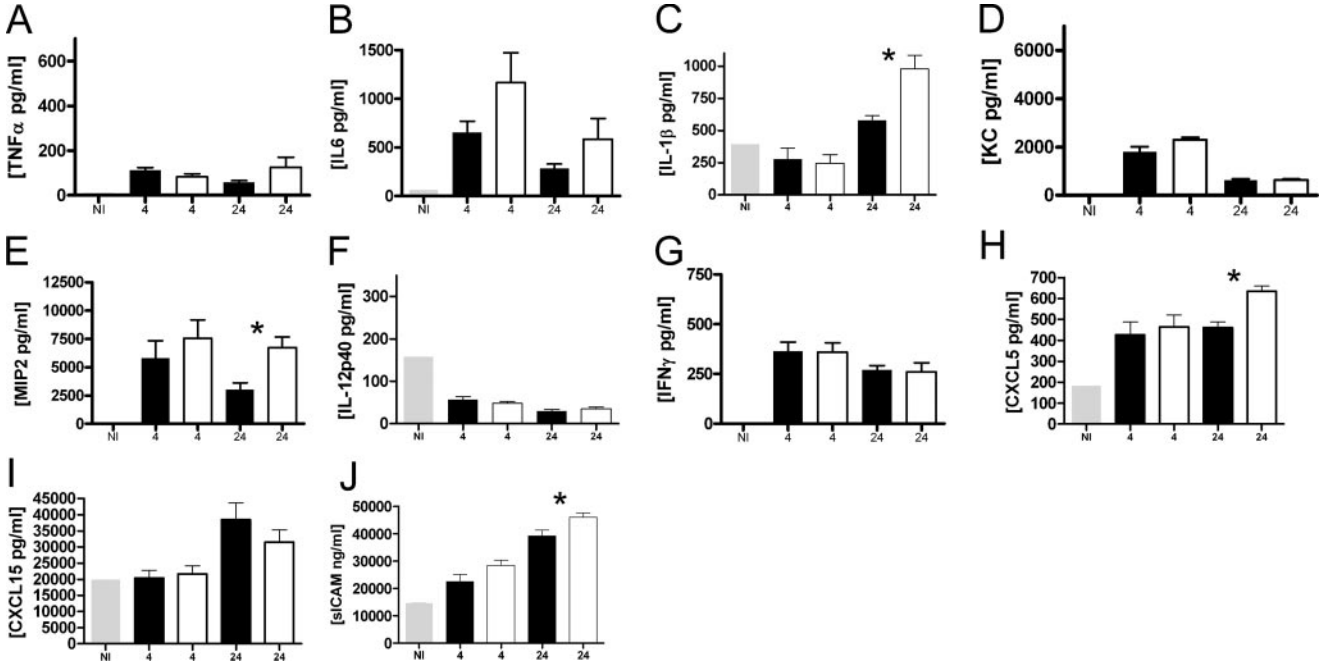

FIGURE 3. Cytokine and chemokine responses in WT and *Tlr5*$^{-/-}$ mice after inhalation of *L. pneumophila*. Lung homogenates were harvested after infection and assayed for cytokines and chemokines by multiplex microbead array: Data (n = 4–9 infected mice or n = 2 uninfected controls) represent means plus SEM ∗, $p \leq 0.05$ by unpaired Student's t test. Legend: *A–J*, LpWT infection of C57BL/6 (■) or *Tlr5*$^{-/-}$ mice (□). 4 and 24, Time points in hours. NI, Not infected, in gray bars.

any of the other cytokines or chemokines tested at 4 or 24 h after infection.

We next compared levels in WT and *Tlr5*$^{-/-}$ mice and found no significant differences 4 h after infection with LpWT. At 24 h after infection, there were higher levels of IL-1β and MIP-2 in the *Tlr5*$^{-/-}$ mice, but not in any other inflammatory molecules (IL-1β mean ± SEM C57BL/6 578.4 ± 37.8 pg/ml vs *Tlr5*$^{-/-}$ 976.5 ± 108.1 pg/ml, p = 0.003, Fig. 3*C*; MIP-2 mean ± SEM C57BL/6 3023.0 ± 583.1 pg/ml vs *Tlr5*$^{-/-}$ 6744.0 ± 932.8 pg/ml, p = 0.004, Fig. 3*E*). Although KC and MIP-2 are two important chemokines that mediate PMN recruitment, there was no difference in their levels at 4 h that could account for the difference in lung PMN levels. We next examined additional chemokines and molecules that regulate PMN recruitment including CXCL5 (LIX, LPS-Induced CXC chemokine), CXCL15 (lungkine), and soluble ICAM. There was no difference in the levels of any of these molecules at 4 h after infection (Fig. 3, *H–J*). At 24 h after infection, there were slight increases in CXCL5 and sICAM levels in the *Tlr5*$^{-/-}$ mice comparison to Wt (CXCL5: mean ± SEM C57BL/6 461.8 ± 25.2 pg/ml vs *Tlr5*$^{-/-}$ 634.1 ± 24.4 pg/ml, p <0.001, Fig. 3*H*; sICAM: mean ± SEM C57BL/6 39,170 ± 2185 pg/ml vs *Tlr5*$^{-/-}$ 45,950 ± 1590 pg/ml, p = 0.025, Fig. 3*J*). Together, these results suggest modest overall alterations in cytokine and chemokine profiles that are regulated by TLR5 and flagellin during in vivo infection.

Tlr5$^{-/-}$ *mice have increased pulmonary inflammation at 6 days after infection*

We next examined whether there were any differences in lung histology in WT and *Tlr5*$^{-/-}$ mice. In particular, we were interested in whether the early alterations in PMN recruitment were associated with pathologic consequences at later time points. We examined lung histology with H&E stains at 24, 72, and 144 h after infection. There were no differences at the earlier time points of 24 and 72 h when comparing WT and *Tlr5*$^{-/-}$ lungs (data not shown). However, at 6 days after infection, the *Tlr5*$^{-/-}$ mice had increased leukocytic infiltrates in the alveolar and peribronchial interstitial spaces (Fig. 4, *C* and *D*). The infiltrates had features that

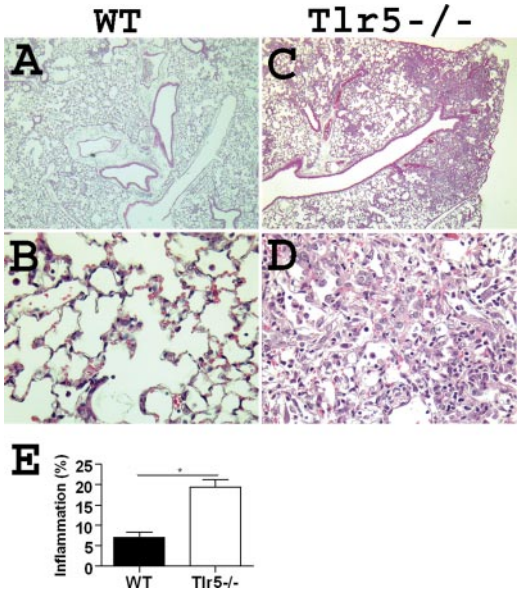

FIGURE 4. Lung histology of mice infected with *L. pneumophila*. Lung sections were stained with hematoxylin and eosin as described in *Materials and Methods*. *A–D*, Lung sections obtained from mice after 6 days of infection. *A* and *C*, ×40 original magnification; *B* and *D*, ×400 original magnification. Photographs are representative of histological sections reviewed from at least two mice at each time point from at least two independent experiments. *E*, Histologic score. The percentage of airway inflammation was estimated from examining 10 high power fields per mouse in four WT and four *Tlr5*$^{-/-}$ mice. ∗, p = 0.002 by Student's t test.

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were consistent with organizing pneumonia with fibrous and myxoid matrix material that was associated with macrophages and fibroblasts. In contrast, the WT mice had significantly less inflammation (Fig. 4, *A* and *B*). We quantified the percentage of the airspace with inflammation and found that the *Tlr5*$^{-/-}$ mice had a 278% increase in comparison to WT mice (C57BL/6 7.1% vs *Tlr5*$^{-/-}$ 19.4%, $p = 0.002$ by Student's *t* test, Fig. 4*E*). Together these results suggest that TLR5 mediates regulatory pathways that promote earlier resolution of pulmonary inflammation.

TLR5 mediates inflammatory response in AMs

To better understand the cellular mechanism of TLR5's regulation of the pulmonary innate immune response, we isolated AMs from BAL fluid for further examination. Previous studies demonstrated that murine BMDMs and peritoneal macrophages do not express TLR5, whereas CD11c^{+} lamina propria cells do express it and are flagellin responsive (28, 34). We first stimulated C57BL/6 murine AMs with purified *S. typhimurium* FliC and found that the cells produced TNF-α in doses that ranged from 11–300 ng/ml (Fig. 5*A*). AMs were also responsive to purified *Legionella* FlaA. In addition, we extracted RNA from AMs and detected TLR5 using real-time PCR (data not shown). As controls, we stimulated BMDMs and RAW 264.7 cells with the same preparation of FliC and found no detectable TNF-α in comparison to stimulation with medium alone (Fig. 5*B*). As an additional control, both AMs and BMDMs were responsive to LPS (Fig. 5*B*). We also stimulated *Tlr5*$^{-/-}$ AMs with FliC and were unable to detect TNF-α (Fig. 5*C*). We next infected AMs with live LpWT and also detected TNF-α (Fig. 5*C*). In contrast, infection with LpFlaA− was not associated with TNF-α production, suggesting that the majority of TNF-α production during infection of AMs is attributable to activation by flagellin. In addition, TNF-α production in LpWT-stimulated AMs was abolished in *Tlr5*$^{-/-}$ cells, suggesting that TLR5, rather than other flagellin sensors, is the major mediator of TNF-α production in AMs stimulated by Lp. We also stimulated cells with heat-killed extracts of Lp and found that LpWT stimulated higher levels of TNF-α in comparison to LpFlaA− in AMs from both C57BL/6 and A/J mice (Fig. 5, *A* and *D*). We next examined whether an additional proinflammatory cytokine (IL-6) or chemokines involved in PMN recruitment (KC and MIP-2) were produced in response to flagellin stimulation. IL-6, MIP-2, and KC were all produced by AMs in response to FliC stimulation (Fig. 5, *E* and *F*). In addition, each of these inflammatory mediators was secreted at higher levels in cells stimulated with LpWT in comparison to LpFlaA−. Together, these results suggest that murine AMs are flagellin responsive in a TLR5-dependent manner. Surprisingly, these data also demonstrate that flagellin is the major stimulus of cytokine and chemokine production in these cells when stimulated by *Legionella*.

Discussion

We report in this manuscript a distinct role for TLR5 during the in vivo immune response to *L. pneumophila*. We found that TLR5 recognition of flagellin regulates early neutrophil recruitment to the lung and influences persistence of airspace inflammation up to 6 days after infection. We further found that recognition of *L. pneumophila* by AMs predominantly occurs through flagellin in a TLR5-dependent fashion. These data address an important question in microbial recognition regarding redundancy among TLRs. *L. pneumophila* is recognized by several TLRs including TLR2, TLR4, TLR5, and TLR9 (17–20, 22–24). Interestingly, *Legionella*

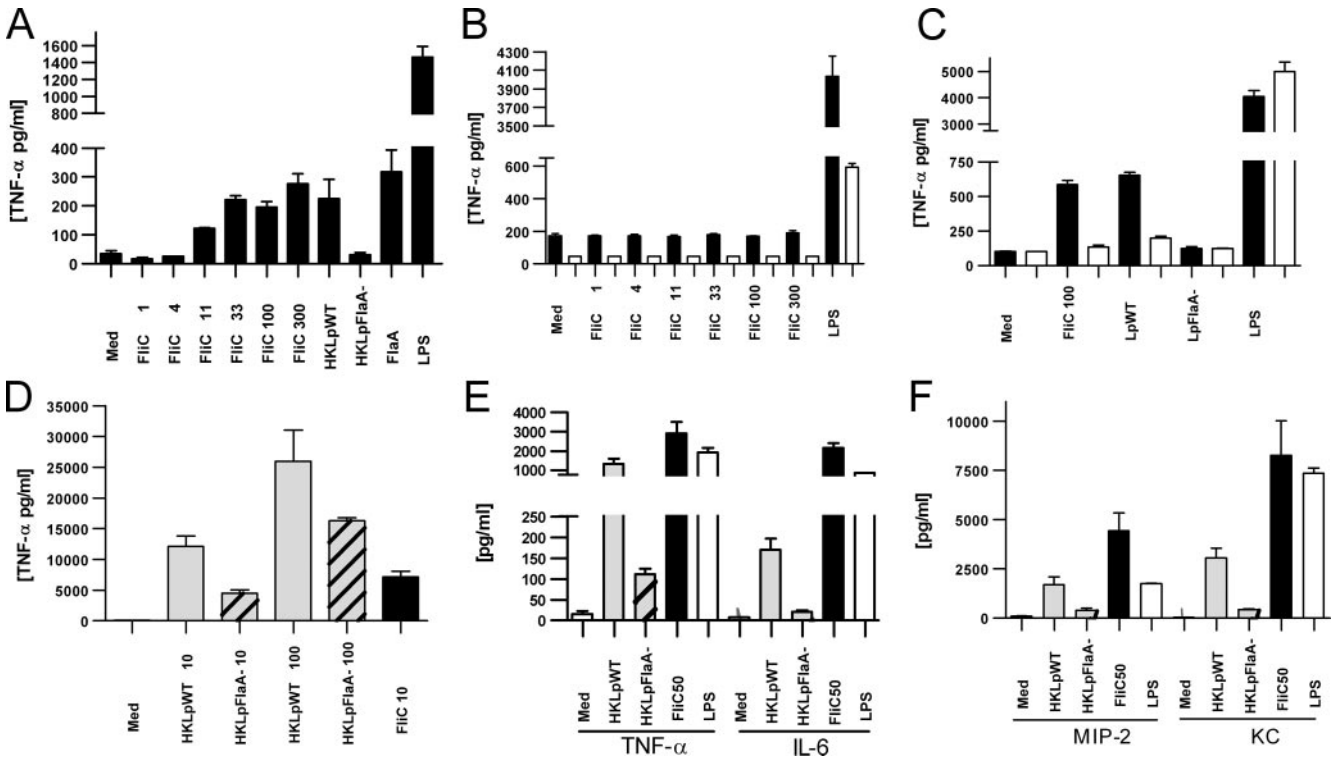

FIGURE 5. Macrophage response to flagellin and *L. pneumophila* stimulation. Macrophages were harvested from mice and stimulated with *S. typhimurium* FliC (in ng/ml), *L. pneumophila* FlaA (at 500 ng/ml), LPS (at 10 ng/ml), live LpWT, or flagellin-deficient *L. pneumophila* (LpFlaA−) (*C*), or heat-killed versions of either (HKLpWT or HKLpFlaA−) (*A* and *D–F*). *A*, C57BL/6 AMs. *B*, RAW 264.7 macrophages (■) or C57BL/6 BMDMs (□). *C*, AMs from C57BL/6 (■) or *Tlr5*$^{-/-}$ mice (□). *D*, A/J AMs, for *Legionella* stimulation, multiplicities of infection of 10 or 100 as indicated. *E* and *F*, C57BL/6 AMs. *D–F*, HKLpWT in gray bars, HKLpFlaA− in gray bars with diagonal marks, FliC in black bars, and LPS in open bars. Mean ± SD is depicted.

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LPS signals predominantly through TLR2, rather than TLR4, suggesting an important role for TLR2 in *Legionella* recognition (18). Consistent with this in vitro data, others have found that TLR4 does not control resistance to in vivo *Legionella* infection (35). In addition, we previously found that *Myd88*$^{-/-}$ and *Tlr2*$^{-/-}$ mice are highly susceptible to in vivo infection with Lp (21). However, we found that the *Tlr2*$^{-/-}$ mice had a partial phenotype in comparison to *Myd88*$^{-/-}$ with a smaller lung CFU difference, no splenic dissemination, and less blunting of lung neutrophil recruitment in comparison to WT animals. We speculated that multiple TLRs were mediating the effects seen in the *Myd88*$^{-/-}$ mice, which have altered signaling for all TLRs, except for TLR3. This speculation was borne out in the present study with evidence of an in vivo phenotype regulated by TLR5 that is distinct from TLR2. Although both strains of mice had impaired neutrophil recruitment, the *Tlr5*$^{-/-}$ exhibited an earlier defect at 4 h after infection while the *Tlr2*$^{-/-}$ mice had a defect at 24 h. The TLR2-dependent recruitment defect was accompanied by decreased levels of KC and MIP-2, which are chemokines that regulate neutrophil recruitment. Interestingly, the *Tlr5*$^{-/-}$ mice had no difference in levels of these cytokines at the 4 h time point, suggesting that TLR5 regulates PMN recruitment through KC and MIP-2-independent pathways. In addition to KC and MIP-2, several other molecules have been implicated in murine neutrophil recruitment, including CXCL5 (or LIX, LPS-Induced CXC chemokine), CXCL15 (lungkine), and ICAM-1 (36–39). We also did not detect any differences in lung concentrations of these molecules in *Tlr5*$^{-/-}$ mice when compared with controls at the early 4 h time point. Together, these results suggest that TLR5 regulates early neutrophil recruitment in response to Lp infection by a mechanism that does not involve chemokines that are currently recognized as the principal regulators of this process. Further study will be required to identify the mechanism underlying this recruitment defect.

Due to the presence of multiple cells and pathways that are activated in the lung during in vivo infection, it may be difficult to detect alterations in cytokines or chemokines at this early time point that can explain the neutrophil recruitment defect. In light of this possibility, we examined the in vitro role of TLR5 in AMs in more detail. Although previous studies have demonstrated that human leukocytes, including dendritic cells, are responsive to flagellin and express TLR5, murine macrophages and dendritic cells are not uniformly flagellin responsive (34). Bone marrow-derived DCs and macrophages and peritoneal macrophages do not respond to flagellin (28, 34). However, a specialized population of intestinal CD11c^{+} lamina propia cells has been shown to express TLR5 and respond to flagellin and may be involved with detection of pathogenic bacteria in the intestinal lumen (28). In the current study, we found that AMs were highly responsive to low doses of flagellin and expressed TLR5. Surprisingly, we also found that a significant proportion of *Legionella* recognition in these cells is mediated by TLR5 and flagellin, rather than other TLRs. Despite the availability of multiple receptors for Lp detection, AMs appear to rely predominantly on TLR5 for activation of cytokine and chemokine production. Given that *Legionella* is known to activate macrophages through TLR2 (via lipopeptides and its unusual LPS), it is surprising that TLR5-flagellin dominates the in vitro response to Lp in this cell type. Although dominant, TLR5 is not the sole receptor mediating cytokine responses in AMs. For example, heat-killed LpFlaA− extracts were still stimulatory, particularly at higher multiplicities of infection (Fig. 5*D*). Further studies will be needed to fully elucidate the repertoire of TLR-mediated recognition of pathogens in AMs. Together these studies suggest an important role for TLR5 in murine AMs in the recognition of *Legionella* flagellin and a possible explanation for the different phenotypes observed during in vivo infection.

A second distinction between the *Tlr2*$^{-/-}$ and *Tlr5*$^{-/-}$ phenotypes was found in the level of late stage lung inflammation. Both *Tlr2*$^{-/-}$ and *Tlr5*$^{-/-}$ mice had increased inflammation at 6 days after infection. However, the *Tlr2*$^{-/-}$ mice had increased lung CFUs in comparison to WT mice, whereas no difference was detected in the *Tlr5*$^{-/-}$ mice. The increased inflammation in *Tlr2*$^{-/-}$ mice is likely attributable to the increased pathogen load that was observed throughout the infection, including at 6 days after infection. In contrast, the increased inflammation in *Tlr5*$^{-/-}$ mice must arise from separate mechanisms because the CFU loads were nearly identical throughout the time course of the infection. Together, these results suggest that TLR2 and TLR5 regulate overlapping, but distinct aspects of the immune response to *L. pneumophila*.

In addition to redundancy among the TLRs for Lp recognition, there are other innate immune receptors that mediate host immunity. Recent studies indicate that NAIP5 and IPAF mediate recognition of cytosolic Lp flagellin and regulate its growth in macrophages (7–10). Whereas macrophages from A/J mice are permissive for in vitro Lp growth, C57BL/6 macrophages normally restrict Lp replication. This phenotype led to the genetic identification of NAIP5 as being responsible for the differential permissiveness of B6 and A/J macrophages to *Legionella* replication (4–6). Subsequent studies demonstrated that flagellin mutants of Lp were not restricted by B6 macrophages, suggesting that Naip5 mediates recognition of flagellin (7–9). These in vitro phenotypes were found to be Myd88 (and likely also TLR5)-independent. Although Naip5 regulates intracellular multiplication of *Legionella* within macrophages in vitro, its effect on in vivo replication is considerably less dramatic, varying from negligible to ~10-fold differences in lung CFUs (7, 9, 40, 41). In addition to NAIP5, IPAF has also been found to mediate recognition of Lp flagellin with TLR5-independent activation of caspase-1 and restriction of Lp replication (9, 10). IPAF-deficiency resulted in a 10-fold increase in lung CFUs in one study (10). Additional in vivo phenotypes, such as pulmonary neutrophil recruitment and cytokine production, have not been examined in NAIP5 and IPAF-deficient mice to know whether they will differ from those found in *Tlr5*$^{-/-}$ mice. Together, these data suggest that in vitro and in vivo detection of flagellin by NAIP5 and IPAF is distinct from the TLR5/Myd88 pathway and may be associated with different outcomes. However, further studies will be required to clearly compare these phenotypes.

Previous murine in vivo infection models suggest that TLR5 mediates an important role in the immune response to several pathogens. *Tlr5*$^{-/-}$ mice were protected against oral *S. typhimurium* infection in comparison to WT mice, possibly through impaired transport of the pathogen from the intestine to the mesenteric lymph node (28). Interestingly, there was no difference when using an intraperitoneal infection, possibly due to differing roles of peritoneal cells and gastrointestinal mucosal cells in disease pathogenesis. However, when *Tlr4/5*$^{-/-}$ mice were examined, TLR5 mediated a protective role distinct from TLR4 and suggested that TLR redundancy masks some in vivo phenotypes (42). In contrast to *Salmonella* infections, we recently found that *Tlr5*$^{-/-}$ mice were more susceptible to urinary tract infections from transurethral *Escherichia coli* infections (43). The bladder was highly responsive to stimulation with purified flagellin in this study, suggesting that urinary epithelia mediate inflammatory responses via TLR5. An important role for TLR5 in pulmonary epithelia has been observed, including mediation of PMN recruitment to the lungs in mice exposed to intranasal flagellin (42).

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Recruitment of PMNs was observed even in bone marrow chimera studies where WT hemopoietic cells were replaced with *Tlr5*$^{-/-}$ cells, suggesting that the alveolar epithelium mediates TLR5 responses in the lung. Furthermore, *Tlr4/5*$^{-/-}$ mice infected intranasally with *Pseudomonas aeruginosa* were more susceptible in comparison to *Tlr4*$^{-/-}$ and WT mice (42). Together, these studies suggest that epithelial cells mediate TLR5-mediated immunity, including in the lung. We speculate that lung epithelial cells, working together with AMs, are also important mediators of immunity during murine *L. pneumophila* infection.

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Disclosures

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