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Nonhematopoietic Cells Are Key Players in Innate Control of Bacterial Airway Infection

Salomé LeibundGut-Landmann,* Kerstin Weidner,* Hubert Hilbi,† and Annette Oxenius*

Airborne pathogens encounter several hurdles during host invasion, including alveolar macrophages (AMs) and airway epithelial cells (AECs) and their products. Although growing evidence indicates pathogen-sensing capacities of epithelial cells, the relative contribution of hematopoietic versus nonhematopoietic cells in the induction of an inflammatory response and their possible interplay is still poorly defined in vivo in the context of infections with pathogenic microorganisms. In this study, we show that nonhematopoietic cells, including AECs, are critical players in the inflammatory process induced upon airway infection with *Legionella pneumophila*, and that they are essential for control of bacterial infections. Lung parenchymal cells, including AMs, are not infected themselves by *L. pneumophila* in vivo but rather act as sensors and amplifiers of inflammatory cues delivered by *L. pneumophila*-infected AM. We identified AM-derived IL-1β as the critical mediator to induce chemokine production in nonhematopoietic cells in the lung, resulting in swift and robust recruitment of infection-controlling neutrophils into the airways. These data add a new level of complexity to the coordination of the innate immune response to *L. pneumophila* and illustrate how the cross talk between leukocytes and nonhematopoietic cells contributes to efficient host protection. The Journal of Immunology, 2011, 186: 3130–3137.

Mucosal surfaces are the entry sites of airborne and foodborne pathogens, which overall constitute a major continuous microbial challenge for the host. Mucosal tissues have adapted to confront this assault by various means: during steady-state conditions (i.e., in absence of pathogenic microbes), epithelial cells, together with their mucous lining and the constitutive expression of antimicrobial substances, represent a physical and biological barrier for commensal microorganisms. The innate defense system in the lung consists of: 1) airway epithelial cells (AECs) and their products (the airway surface liquid, antimicrobial substances, cytokines, and chemokines); 2) alveolar macrophages (AMs), which are strategically positioned at the luminal surface of the epithelial lining being responsible for efficient phagocytic uptake and clearance of microbes; and 3) dendritic cells (DCs), which were shown to capture particulate Ags through transepithelial luminal dendrites (1–3).

Infections with pathogens in the lung lead to the induction of a local inflammatory response. The fact that AECs outnumber AMs by far renders their involvement in initiating or amplifying the innate immune response very likely. Indeed, a number of studies have shown that AECs can express a variety of membrane-bound or cytosolic pattern recognition receptors (PRRs) and are directly responsive to pathogen-associated molecular patterns (PAMPs) (1, 3, 4). However, most studies have analyzed either isolated primary AECs or lung epithelial cell lines in in vitro experiments, and it remains unclear whether AECs respond comparably in the intact lung tissue in presence of airway surface liquid and AMs, which are more broadly equipped with PRRs. Moreover, it is conceivable that there is significant cross talk between AECs and AMs during an inflammatory response after airway infection with pathogenic bacteria.

We have used an airway infection model with the Gram-negative bacterium *Legionella pneumophila*, the causative agent of Legionnaire’s disease, to assess in vivo the importance of nonhematopoietic cells in the lung and their communication with AMs to initiate an inflammatory response, which, in turn, is critical for rapid control of *L. pneumophila* infection (5–9). On airway infection, *L. pneumophila* infect AMs, and in contrast to extracellular bacteria, *L. pneumophila* resist intracellular lyosomal degradation by means of their icm/dot type IV secretion system (T4SS) (10). Within their host cells, *L. pneumophila* establish and reside within a “Legionella-containing vacuole” that is permissive for intracellular multiplication (11). Hence the induction of an inflammatory response that recruits other immune cells to the site of infection is necessary to eventually control this pulmonary bacterial infection.

We and others have previously shown that in vivo *L. pneumophila*-induced inflammation critically depends on both MyD88 signaling on the host side and on a functional T4SS on the bacterial side (6–9, 12). This is in contrast with in vitro experiments with isolated cells where the *L. pneumophila* T4SS plays only a minor role for the production of various proinflammatory cytokines (13). This apparent discrepancy between in vitro and in vivo experiments may be caused by the involvement of various cell subsets of hematopoietic and nonhematopoietic origin par-
participating in a communication network in vivo, with subset-specific signal requirements. Furthermore, the dual dependence on the *L. pneumophila* T4SS and host MyD88 signaling implies that a combination of cytoplasmic recognition events of T4SS-secreted PAMPs and host cell-receptor signaling events via MyD88 is required to initiate an inflammatory response in vivo.

TLR2, TLR5, and TLR9 have all been shown to recognize *L. pneumophila*. However, the strong dependence of the induction of inflammation on the adaptor protein MyD88 cannot be explained solely by the recognition of *L. pneumophila* through these receptors. Mice deficient in these receptors or mice infected with a TLR5 ligand-deficient *L. pneumophila* strain show only subtle or no defects in pathogen control (6–9), whereas MyD88-deficient mice display high bacterial counts in the lung compared with control mice (6–9, 14). In addition to being required for signaling pathways stimulated by TLRs, MyD88 is essential for signaling events activated by the IL-1 family of receptors (15). The different MyD88-dependent signaling pathways that may be activated concomitantly during *L. pneumophila* infection may, however, function in different cell types. It has been shown recently that MyD88 is required in cells of the hematopoietic compartment for efficient control of airway *L. pneumophila* infection (14). However, the question whether MyD88 may also play a role in non-hematopoietic cells has not been addressed so far.

In this article, we show proof for this hypothesis and unravel an intricate cross talk between lung-resident nonhematopoietic cells and AMs: the initial recognition event of *L. pneumophila* occurs in AMs that phagocytose *L. pneumophila* and in response secrete IL-1β via T4SS-dependent activation of caspase-1. IL-1β is then sensed by nonhematopoietic cells, including AECs (which by themselves are not infected in vivo), and triggers MyD88-dependent chemokine production leading to the rapid recruitment of neutrophils, monocytes, and DCs. Thus, nonhematopoietic cells were identified as essential amplifiers of lung inflammation, which actively promote the recruitment of migratory innate immune cells, rendering them critical contributors to host protection from pathogenic airway infections.

**Materials and Methods**

*Mice, bacteria, and infections*

C57BL/6, myd88<sup>−/−</sup> (15), il-1<sup>−/−</sup> (16), caspase-1<sup>−/−</sup> (17), H-2K<sup>b</sup>-tsA58 (18), and H-2K<sup>b</sup>-tsA58 × myd88<sup>−/−</sup> mice were bred at the ETH Zürich or purchased from Janvier Elevage and used at 7–16 wk of age (age-matched within experiments). All mice were backcrossed on C57BL/6 except the H-2K<sup>b</sup>-tsA58 transgenic mice, which were on a mixed Sv129 × C57BL/6 background. All animal experiments were performed in accordance with institutional policies and have been reviewed by the cantonal veterinary office. For generation of chimeric mice, C57BL/6, myd88<sup>−/−</sup>, il-1<sup>−/−</sup>, and caspase-1<sup>−/−</sup> mice were gamma-irradiated (950 Rad) and reconstituted with 1 × 10<sup>8</sup> bone marrow cells derived from C57BL/6 or myd88<sup>−/−</sup> mice. Experiments were performed after at least 8 wk of reconstitution. The chimera was checked in some control animals, and it was verified, in particular, that the hematopoietic compartment was of donor origin (95% reconstitution in the hematopoietic compartment and >85% in AMs; data not shown). Normal cytocrine production (IL-12p40, Supplemental Fig. 1B, 1B, Fig. 5C) in B6 > Myd88<sup>−/−</sup> chimeras further proved, on a functional level, that engraftment had taken place.

The *L. pneumophila* strains used in this study were JR32 (wild type Philadelphia-1) (19), JR32-GFP (20), GS211 (Δt, icmt<sup>−</sup>) deletion mutant lacking a functional Icm/Dot T4SS (21), and ΔT-GFP (20). *L. pneumophila* was grown for 3 d on charcoal yeast extract agar plates. Liquid cultures were inoculated in ACES yeast extract medium at an OD<sub>600</sub> of 0.1 and grown for 21 h at 37°C. Chloramphenicol (5 μg/ml) was added to maintain plasmids.

Mice were infected intranasally with 5 × 10<sup>6</sup> CFU *L. pneumophila* (grown on plates) in 20 μl prewarmed PBS that were applied directly onto the nostril of anesthetized mice using a Gilson pipette. Bronchoalveolar lavage (BAL) was collected by flushing the airways with 1 ml PBS after sublethal anesthesia of mice with xylazine (0.38 mg), ketamine (1.9 mg), and acepromazine (57 μg), and perfusion of the blood circuit with PBS. Cells were recovered from the BAL by centrifugation, and cell-cleared BAL was stored at −20°C for further analyses. CFU counts were determined by plating dilutions of BAL and homogenized lung tissue onto charcoal yeast extract agar plates.

**Cells**

Bone marrow-derived macrophages (BMDM) were generated by plating bone marrow in complete medium (RPMI 1640, 10% FCS, 2 mM glutamine, nonessential amino acids, 50 μM 2-ME, 1 mM sodium pyruvate, 10 mM HEPES and antibiotics) supplemented with 20% L929-cell conditioned medium. On day 4 of culture, the cells were further supplemented with complete BMDM medium. After a total of 6–7 d of culture, the adherent macrophages were harvested and seeded in 96-well plates at 1 × 10<sup>5</sup> cells/well, and 1 d later, cells were infected with the indicated multiplicity of infection of *L. pneumophila* from liquid cultures. Infection was synchronized by centrifugation, and the infected cells were incubated at 37°C for 6 h (for in vitro cross-talk experiments and protein measurements), or for 3 h in case of mRNA analysis.

Long-term cultures of primary alveolar epithelial cells (imAECs) were isolated from lungs of H-2K<sup>b</sup>-tsA58 and H-2K<sup>b</sup>-tsA58 × myd88<sup>−/−</sup> mice by FACS, based on their CD45<sup>−</sup>CD31<sup>−</sup> phenotype. Long-term cultures of these cells were established by culturing the sorted cells in complete medium supplemented with 100 U/ml IFN-γ at 33°C. IFN-γ was removed and cells were grown at 37°C during 3 d before experiments, and cells were seeded in 96-well plates at 5 × 10<sup>4</sup> cells/well 1 d before stimulation. For in vitro cross-talk experiments, BAL from infected C57BL/6 mice or the supernatant of *L. pneumophila*-infected BMDM was transferred onto AECs for 15 h, and chemokines were analyzed in the supernatant. To prevent direct infection of AECs by cotransferred bacteria, we added 10 μg/ml gentamicin to the transferred BMDM supernatants. IL-1R antagonist (IL-1Ra) was added where indicated.

**Reagents**

All Abs for FACS and FACS analysis were from Biot-Legend or BD Biosciences. The podoplanin-specific Ab (clone 8.1.1) was a kind gift from Prof. Cornelia Halin (ETH, Zurich, Switzerland). The neutrophil-depleting NimpR14 Ab was a kind gift of Dr. Nancy Hogg (Cancer Research UK, London, U.K.). One hundred micrograms of Ab was injected i.p. daily from 1 d before infection to 3 d postinfection. A goat anti-mouse anti–IL-1β Ab from R&D Systems in combination with an HRP-conjugated anti-goat Ab was used for Western blotting according to standard protocols. IL-1Ra (Kinetic/AnaKrina; Amgen Europe B.V.) was used at 1–10 μg/ml rIFN-γ was produced from X63-IFN-γ-transfected cells (a kind gift from Prof. Hans Hengartner, University of Zurich). Gentamicin was from AppliChem.

**FACS**

Lungs were digested for 30 min at 37°C with Collagenase I and DNAse I, and a single-cell suspension was then prepared by forcing the organ through a 70-μm strainer. Cells from the BAL and lung were stained by standard procedures using fluorescence-labeled Abs. Cell populations were defined as CD45<sup>+</sup> CD11c<sup>−</sup> F4/80<sup>−</sup> Ly6C<sup>−</sup> Ly6G<sup>−</sup> (neutrophils), CD45<sup>−</sup> CD11c<sup>−</sup> F4/80<sup>−</sup> Ly6C<sup>−</sup> Ly6G<sup>−</sup> (inflammatory monocytes), and CD45<sup>−</sup> CD11e<sup>−</sup> F4/80<sup>−</sup> autofluorescence<sup>+</sup> (AMs).

**Cytokine and chemokine measurements**

The concentration of CXCL1, CXCL2, CCL2, CCL3, IL-12p40, and TNF-α in cell culture supernatants and in the BAL was determined by ELISA. Secreted IL-1β levels were quantified by cytometric bead array (BD Biosciences) according to the manufacturer’s instructions. IL-1β mRNA was quantified by real-time PCR using SensiMix Plus SYBR kit (Quanta), a RotorGene 3000 Thermal Cycler (Corbett Research), and the following primers: IL-1β forward, 5′-caaccaacagtagattctcag-3′; IL-1β reverse, 5′-gattccacacactctccagctgca-3′; β-actin forward, 5′-cttcatgctcctggttc-3′; β-actin reverse, 5′-ctttcagctgggtctg-3′. Values were normalized with respect to β-actin and were expressed relative to the levels of uninfected controls.

**Statistical analysis**

The two-tailed unpaired *t* test was applied for statistical analysis: *p* < 0.05; **p** < 0.01; ***p*** < 0.001.
Results

L. pneumophila-induced inflammation in the airways is MyD88 and T4SS dependent

In a previous study, we found that L. pneumophila-induced inflammation upon systemic infection is dependent on both the bacterial T4SS and pathogen recognition via a MyD88-controlled pathway (8). This finding also holds true for L. pneumophila-induced inflammation in the airways of mice infected via the intranasal route (Fig. 1) (5, 9). We show here that the production of proinflammatory cytokines was induced, and that large numbers of leukocytes were recruited into the airways of L. pneumophila-infected mice (Fig. 1A, 1B). Neutrophils could be detected in the BAL as early as 4 h postinfection, and their numbers increased with time, whereas inflammatory monocytes and DCs appeared later (Fig. 1A, 1B, and data not shown). In contrast, AM numbers in the BAL remained unchanged upon infection (Fig. 1A, 1B). The infiltration of neutrophils, inflammatory monocytes, and DCs coincided with the production of chemokines in the BAL (Fig. 1C). Neutrophil-attracting CXCL1 and CXCL2 were detected at the onset of neutrophil infiltration, whereas the monocyte- and DC-attracting chemokines CCL2, CCL3, and CCL20 reached their maximum levels only at later time points (Fig. 1C and data not shown). The production of proinflammatory cytokines and chemokines, and the recruitment of inflammatory leukocytes to the airways in response to infection were dependent on both host MyD88 (Fig. 1A, Supplemental Fig. 2A) (9) and the bacterial T4SS (Fig. 1B, 1C, Supplemental Fig. 2B). Infection with L. pneumophila is controlled rapidly by the host innate immune response (5, 6, 8, 9), and neutrophils may play a critical part. Consistent with this notion, we show that neutrophil-depleted mice (neutrophil depletion efficacy shown in Supplemental Fig. 3) had a strong defect in controlling L. pneumophila in the lung and the BAL (Fig. 1D). The concurrent recognition of L. pneumophila via MyD88-dependent and cytoplasmic mechanisms is thus essential not only for induction of the inflammatory response but also for the reduction of the pathogen load and for host protection.

Nonhematopoietic cells contribute critically to L. pneumophila-induced inflammation and bacterial control

Epithelial cells are greatly abundant among the stromal cells in the lung and are strategically positioned at the interface between the luminal airspace and the lung tissue. Given these features of AECs and their previously described capacity to respond directly to various microbial stimuli (1, 3, 4) and specifically to L. pneumo-
in L. pneumophila infection in vitro (22), we next evaluated the role of nonhematopoietic cells, including AECs, in host protection from L. pneumophila infection. To this end, we generated chimeric mice, wherein all nonhematopoietic cells including epithelial cells were deficient of MyD88, whereas all hematopoietic cells were of wild-type origin. These chimeric mice showed a strong defect in bacterial control upon L. pneumophila infection (Fig. 2A), indicating that nonhematopoietic cells, including most likely AECs, indeed are critically involved in controlling L. pneumophila airway infection in an MyD88-dependent manner. In fact, nonhematopoietic cells were essential for the early production of chemokines and the swift attraction of neutrophils to the airspace of the lung (Fig. 2B, 2C). Importantly, these results do not exclude a comparably critical role of MyD88 signaling in hematopoietic cells for control of airway L. pneumophila infection, as has been previously shown in chimeric mice lacking MyD88 selectively within hematopoietic cells (14) (data not shown).

Nonhematopoietic cells including AECs could produce chemokines either in response to direct recognition of L. pneumophila or in response to inflammatory mediators released by other cells such as L. pneumophila-infected AMs. In support of the first hypothesis, lung epithelial cell lines and imAECs could be infected by L. pneumophila and produced chemokines in response to L. pneumophila infection in vitro (unpublished observations). However, in vivo, we did not find any evidence for direct infection of CD45– nonhematopoietic cells or specifically of lung epithelial cells (Fig. 2D; N. Joller and A. Oxenius, unpublished observations). Rather, GFP+ cells in the lung of mice infected with a rGFP-expressing L. pneumophila strain were exclusively CD45+ F4/80+ CD11c+ AMs at early time points postinfection (Fig. 2D). One day postinfection, infiltrating neutrophils and monocytes also turned GFP+, but we consistently failed to detect L. pneumophila in nonhematopoietic CD45– cells in the lung (Fig. 2D and data not shown). The discrepancy between our in vitro and in vivo findings highlights the complexity of the intact lung and emphasizes the relevance to study host–pathogen interactions in vivo where the integrity of the host tissue is retained.

AM-derived IL-1β induces chemokine production in epithelial cells

Our findings that nonhematopoietic cells are not directly infected in vivo by L. pneumophila is consistent with a model in which they respond to inflammatory mediators rather than sensing L. pneumophila directly. Given the MyD88 dependence of the response of the nonhematopoietic cells, we hypothesized that IL-1R– or IL-18R–dependent events rather than TLR-mediated signaling may be involved. To assess the IL-1β and IL-18 responsiveness of nonhematopoietic cells in the lung, we focused on alveolar type I cells, which constitute ∼95% of the internal lung surface and are therefore prominent representatives of nonhematopoietic cells in the lung. Indeed, CXCL1 production could be induced in vitro in imAECs by IL-1β (Fig. 3A), but not by IL-18 (data not shown). Consistent with this finding, IL-18R–deficient mice do not exhibit defects in control of airway L. pneumophila infection (data not shown) (7). Furthermore, imAECs did also produce chemokines in response to stimulation with supernatants from L. pneumophila-infected BMDM (Fig. 3B, 3C) or with BAL of L. pneumophila-infected mice (Fig. 3D). The dependence on IL-1β of this response was demonstrated by the lack of chemokine production in MyD88-deficient imAECs and by the efficient blocking of the response in wild type imAECs by the IL-1Ra (Fig. 3C, 3D). Of note, in vitro L. pneumophila infection of BMDM also resulted in production of low levels of CXCL1, consistent with previously published data of L. pneumophila-infected BMDM (23) or BMDM stimulation with specific TLR ligands (24, 25). However, the level of CXCL1 production in BMDM was at least one order of magnitude lower than the amount produced by imAECs stimulated with supernatants from L. pneumophila-infected BMDM (Fig. 3C and data not shown). The superior in vivo relevance of chemokine production by nonhematopoietic cells was further demonstrated by the finding that CXCL1 and CXCL2 contents in the BAL of L. pneumophila-infected chimeric mice harboring wild type hematopoietic cells (including AMs) in hosts with selective MyD88 or IL-1R deficiency in nonhematopoietic cells were severely reduced (Figs. 2B, 4A).

**TABLE 1.** MyD88 expression in nonhematopoietic cells is essential for efficient control of L. pneumophila infection. A. Irradiation chimeras were generated by reconstituting C57BL/6 or myd88+/− mice with C57BL/6 bone marrow. Bacterial counts were determined in the lung and BAL 5 d postinfection with L. pneumophila strain JR32. Each symbol depicts one mouse, and the experiment is representative of two independent repeats. B and C. Chemokine levels (B) and neutrophil counts (C) were determined in the BAL of infected chimeras 4 h postinfection with L. pneumophila JR32. Data are mean and SD of three mice per group (one uninfected mouse) and are representative of three independent experiments. D. C57BL/6 mice were infected with GFP-expressing L. pneumophila strain JR32 or AT, and the cells that have taken up bacteria in the lung were identified 4 h postinfection by FACS. GFP+ cells within the scatter-gated population (left column), CD45 expression on GFP+ cells (middle column), and F4/80+ CD11c+ expression on the CD45+ population (right column). *p < 0.05, **p < 0.01, ***p < 0.001 (two-tailed unpaired t test).
The relevance of IL-1R signaling for chemokine production by nonhematopoietic cells was further underlined by the finding that chimeric mice in which nonhematopoietic cells selectively lacked the IL-1R showed a strong defect in chemokine production at early time points after L. pneumophila infection (Fig. 4A). This condition resulted in a strong decrease in neutrophil recruitment (Fig. 4B) and impaired control of L. pneumophila (Fig. 4C). Furthermore, comparable impairment of bacterial control was observed in L. pneumophila-infected IL-1R−/− mice (Fig. 4D).

We finally assessed which cells produced the IL-1β that acted on nonhematopoietic cells in the airways of L. pneumophila-infected mice. Whereas caspase-1 (which is involved in IL-1β processing) was necessary for L. pneumophila-induced inflammation (Fig. 5A, 5B), it was not required in nonhematopoietic cells because chimeric mice lacking caspase-1 selectively in nonhematopoietic cells showed no defect in chemokine production and leukocyte infiltration into the airways (Fig. 5C, 5D). Furthermore, chimeric mice with MyD88 deficiency in nonhematopoietic cells produced normal levels of IL-1β in response to L. pneumophila infection (Fig. 5E). These findings suggested that the hematopoietic compartment was the source of IL-1β. Indeed, we detected IL-1β in AMs from L. pneumophila-infected mice (Fig. 5F). In addition, IL-1β production was dependent on the expression of MyD88 in hematopoietic cells (Fig. 5F). This was further corroborated by the fact that MyD88-deficient macrophages were unable to express IL-1β protein, as shown previously (12), and mRNA in response to L. pneumophila infection in vitro (Fig. 5G, 5H). This

**Discussion**

Airway infections with pathogenic bacteria need to be rapidly detected and controlled to secure a high degree of organ function, which is essential for sufficient gas exchange. In this article, we show that the swift induction of an inflammatory response including the fast recruitment of infection-controlling neutrophils upon airway infection with the Gram-negative bacterium L. pneumophila critically depends on both hematopoietic and nonhematopoietic cells in the lung. Most importantly, the nonhematopoietic cells in the lung are not merely a physical barrier for the lung tissue, but take an active role in the buildup of the L. pneumophila-induced inflammatory response. We demonstrate that this cross talk between lung AMs and nonhematopoietic cells is chiefly regulated via IL-1β. Infection of alveolar macrophages by L. pneumophila leads to the secretion of IL-1β into the BAL, which is dependent on MyD88 on the host side and on a functional T4SS on the pathogen side. IL-1β then engages IL-1Rs on nonhematopoietic cells, including AECs, which leads to the rapid induction of chemokine synthesis, with the neutrophil recruiting chemokines CXCL1 and CXCL2 being secreted as early as 4 h postinfection. Thus, the dependence of the L. pneumophila-induced inflammatory response on MyD88 is 2-fold: first, MyD88 is essential for IL-1β induction in hematopoietic cells; and second, nonhematopoietic cells depend on MyD88 for IL-1R signaling. This cross talk between hematopoietic and non-

**FIGURE 3.** IL-1R signaling induces chemokine production in nonhematopoietic cells in vitro. A, Wild type imAECs were stimulated with the indicated doses of IL-1β, and chemokine induction was measured by ELISA in the supernatant after 6 h. Data shown are mean and SD of triplicate wells and representative of three independent experiments. B, Schematic overview of the setup of in vitro cross-talk experiments with BMDM and imAECs. C, Supernatant from L. pneumophila JR32-infected BMDM was used to stimulate wild type and myd88−/− imAECs in presence or absence of IL-1Ra, as indicated, and chemokine induction in the imAEC supernatant was measured by ELISA. Data shown are mean and SD of triplicate wells and representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (two-tailed unpaired t test).

**FIGURE 4.** IL-1R signaling induces chemokine production in nonhematopoietic cells in vivo. A–C, Irradiation chimeras were generated by reconstituting C57BL/6 or il-1r−/− mice with C57BL/6 bone marrow. Chemokine levels (A) and neutrophil counts (B) were determined in the BAL of infected chimeras 4 h postinfection with L. pneumophila JR32. C, Bacterial counts in the BAL were determined at 4 and 24 h after L. pneumophila infection in B6 > B6 and B6 > il-1r−/− chimeric mice (left panel), or in nonchimeric il-1r−/− or B6 mice (right panel). Data are mean and SD of four mice per group (one uninfected mouse in A and B) and are representative of two independent experiments each. *p < 0.05, **p < 0.01, ***p < 0.001 (two-tailed unpaired t test).
hematopoietic cells expedites the early recruitment of neutrophils, which, in turn, are crucial effectors in controlling airway *L. pneumophila* infection.

In this study, we have focused on analyzing the very early stages of airway *L. pneumophila* infection where we have dissected an important role of nonhematopoietic cells in orchestrating rapid recruitment of PMNs. However, it is conceivable that other mechanisms may compensate for an involvement of nonhematopoietic cells in PMN recruitment at later stages of infection. For instance, bacterial replication, and hence increased PAMP levels, in turn, are crucial effectors in controlling airway macrophages, and DCs), NK cells, and others (38–40).

In vivo, the exact contribution of parenchymal versus hematopoietic cells to the initiation and control of the immune response within the lung remains elusive and seems to depend on the nature of the inflammatory stimulus (i.e., type of pathogen, isolated PRR agonist) as reported in various studies using chimeric mouse models. When the PRR agonist flagellin was administered intranasally, nonhematopoietic cells control early chemokine production and PMN infiltration via TLR5 signaling (41, 42). In contrast, inhalation of endotoxin induced a cytokine response that depended on MyD88 signaling on nonhematopoietic cells (43), and in a study using TLR4-deficient chimeric mice, expression of TLR4 on hematopoietic cells and macrophages was crucial to recruit neutrophils to the airspace after LPS treatment (44).
Also in the context of bacterial airway infections, non-hematopoietic cells in the lung were previously attributed a role in innate immune defense: pneumonia induced by infection with Klebsiella pneumoniae is controlled by nonhematopoietic cells by directly ingesting and degrading bacteria, and by facilitating their opsonization (45). Furthermore, nonhematopoietic cells in the lung were shown to contribute to cytokine production upon exposure to Bacillus anthracis spores (46) or Haemophilus influenzae infection (47). Finally, in P. aeruginosa-infected mouse lungs, MyD88 in nonhematopoietic cells is essential for early control of infection, whereas at later phases of the infection, MyD88 expression in both parenchymal and hematopoietic cells contributes to control pathogen replication (48).

In this study, we dissected the cross talk between nonhematopoietic and hematopoietic cells during early airway L. pneumophila infection-induced inflammation. This cellular interplay does not rely on direct sensing of PAMPs by nonhematopoietic cells themselves, but rather positions them as potent amplifiers between L. pneumophila-infected AMs and PMN recruitment, the latter being essential for early reduction in pathogen load.

Of interest, a similar cross talk between nonhematopoietic and hematopoietic cells has been described in a murine cutaneous infection model with Staphylococcus aureus, where MyD88- and IL-1R-deficient mice showed impaired neutrophil recruitment to the site of infection. This process is independent of IL-1R/MyD88 in hematopoietic cells, suggesting that IL-1R/MyD88 signaling in skin-resident nonhematopoietic cells is critical to promote neutrophil recruitment (49).

Taken together, our results contribute to the understanding of the early events that control the initiation of the inflammatory response upon L. pneumophila airway infection. Nonhematopoietic cells, including AECs, which form a tight boundary and line the entire airspace of the lung, thus perform many more functions than just that of a physical barrier preventing pathogens from invading the underlying tissues. Our data add an important example to the recently recognized role of nonhematopoietic cells in the lung (but also in other organs such as the gut and the skin) to secrete specific inflammatory mediators, which can have various functions but often promote key decisions in innate and adaptive immune cells (50).

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Supplementary figure legends

**Suppl. Fig. 1.** C57BL/6 mice and chimeras wherein either all non-hematopoietic cells (B6 > MyD88−/−) or all hematopoietic cells (MyD88−/− > B6) were MyD88-deficient were infected with *Lpn* JR32 and IL-12p40 secretion in the BAL was determined 18 hours post-infection. Data are mean and standard deviation of 2 mice per group.

**Suppl. Fig. 2A.** IL-12p40 and TNF-α levels in the BAL of C57BL/6 and *myd88−/−* mice that were infected i.n. with *Lpn* JR32 18 hours previously. Data show the mean and standard deviation of 2 (C57BL/6) and 3 (*myd88−/−*) mice and are representative of 2 independent experiments.

**Suppl. Fig. 2B.** IL-12p40 and TNF-α levels in the BAL of C57BL/6 mice that were infected i.n. with the *Lpn* strains JR32 or ΔT 28 hours previously. Data show the mean and standard deviation of 3 infected mice each and 1 control and are representative of 3 independent experiments.

**Suppl. Fig. 3A.** The efficacy of neutrophil depletion was quantified by flow cytometry at the end of the experiment shown in Fig. 1D. The FACS plots show representative examples of neutrophil (CD45+ Ly6G+ Ly6C+; top panels) and AM populations (CD45+ CD11c+ F4/80+; bottom panels) in the lung of antibody-treated and control mice.

**Suppl. Fig. 3B.** Neutrophil and AM cell counts in the lung of all mice shown in Fig. 1D.

**Suppl. Fig. 4.** Supernatant from *Lpn* JR32-infected wild type and MyD88-deficient BMDM was used to stimulate imAECs and chemokine induction in the imAEC supernatant was measured by ELISA. Data shown are mean and standard deviation of triplicate wells and representative of 2 independent experiments.