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Cutting Edge: Pulmonary *Legionella pneumophila* Is Controlled by Plasmacytoid Dendritic Cells but Not Type I IFN

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Plasmacytoid dendritic cells (pDCs) are well known as the major cell type that secretes type I IFN in response to viral infections. Their role in combating other classes of infectious organisms, including bacteria, and their mechanisms of action are poorly understood. We have found that pDCs play a significant role in the acute response to the intracellular bacterial pathogen *Legionella pneumophila*. pDCs were rapidly recruited to the lungs of *L. pneumophila*-infected mice, and depletion of pDCs resulted in increased bacterial load. The ability of pDCs to combat infection did not require type I IFN. This study points to an unappreciated role for pDCs in combating bacterial infections and indicates a novel mechanism of action for this cell type. *The Journal of Immunology, 2010, 184: 5429–5433.*

L *egionella pneumophila* is an opportunistic intracellular pathogen in humans and the major cause of Legionnaires’ disease. A combination of innate and adaptive immune responses are critical in controlling *L. pneumophila* infection in mice, with IFN-γ (1), MyD88 (2, 3), and T cells playing major roles (4). Several in vitro studies have also investigated the role of type I IFN (IFN-I) in restricting *L. pneumophila* colonization of cells. For example, mouse bone marrow-derived macrophages deficient in the IFN-IR (IFNAR) are more permissive for *L. pneumophila* replication, and the pretreatment of permissive macrophages with IFN-I inhibits growth of the bacteria (5–7). Also, IFN-β expression is induced upon *L. pneumophila* infection of mouse macrophages and in a human alveolar basal epithelial cell line (7–10). One aim of this work was to investigate the role of IFN-I in limiting *L. pneumophila* infection in vivo, as this had not been thoroughly investigated.

A major cell type that produces IFN-I is the plasmacytoid dendritic cell (pDC), a cell type with distinct functional characteristics compared with conventional Ag-presenting DCs (11–13). pDCs may traffic through some tissues in the steady state, but they are rapidly recruited by infection and inflammatory mediators (13). The role of pDCs in different types of infections is still unclear. pDCs produce large quantities of IFN-I in response to viral infections (14, 15); however, some studies have also begun to explore the contribution of pDCs to nonviral immune responses. For instance, pDCs are able to recognize bacterial RNA, resulting in the production of IFN-I (16), pDCs also produce IFN-I when exposed to *Borrelia burgdorferi* in vitro (17). Finally, pDCs can activate CD4 T cells via Ag presentation and secretion of IL-12 in response to *Toxoplasma gondii* infection (18). As IFN-I may also be an effector molecule used in the clearance of intracellular bacterial infections, such as *L. pneumophila*, we studied the role of pDCs in a mouse model of *L. pneumophila* infection. We demonstrated that pDCs do indeed play a role in combating *L. pneumophila*, although IFN-I was not required for this effect.

**Materials and Methods**

**Bacteria strains and culture conditions**

*L. pneumophila* strains 130b (ATCC BAA-74) and JR32 and the flagellin mutant Δ*flaA* JR32 (19) were used for mouse infection studies. GFP-expressing L. pneumophila strain 130b was used for all in vitro infection studies. *gfp* was expressed from the plasmid pMip, a derivative of the cloning vector pMAM207, which was introduced into *L. pneumophila* 130b by electroporation as described previously (20). All bacterial strains were cultured in ACES-buffered yeast extract broth or on buffered charcoal yeast extract agar.

**Mice**

A strain mice, C57BL/6 and IFNAR2−/− (21) mice, were obtained from the Department of Microbiology and Immunology or the Bio21 Molecular Science and Biotechnology Institute (University of Melbourne, Parkville, Victoria, Australia) or the Animal Resource Centre (Canning Vale, Western Australia, Australia). All mice were housed under specific pathogen-free conditions, and experiments were conducted with approval from The University of Melbourne Animal Ethics Committee.

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The online version of this article contains supplemental material. Abbreviations used in this paper: DC, dendritic cell; IFN-I, type I IFN; IFNAR, type I IFNR; pDC, plasmacytoid dendritic cell; PDCA, plasmacytoid dendritic cell Ag.

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Infection and quantification of L. pneumophila in mice

Mice were anesthetized with ketamine and xylazine and restrained on their backs with the larynx illuminated. A 20-gauge catheter was used to intubate the trachea, and 2.5 × 10^8 CFU L. pneumophila in 25 μl PBS was slowly injected into the lungs followed by an equal volume of air using a blunt Hamilton syringe. For analysis, lungs and spleens were harvested aseptically and homogenized followed by lysis of cells with 0.05% saponin in PBS. Intracellular L. pneumophila were enumerated by serial dilution and plating onto buffered charcoal yeast extract agar.

In vivo depletion of pDCs

Mice were depleted of pDC using the mAb 120G8 (22). Mice were injected i.p. with 1 mg/mouse 120G8 mAb or rat IgG (Sigma-Aldrich, St. Louis, MO) the day before infection and were given additional doses of Ab each day after inoculation until mice were killed (Supplemental Fig. 1). Depletion of pDCs in the lung and spleen of 120G8-treated mice was ∼95% at 72 h post-infection.

Flow cytometry

Single-cell populations from the spleen or lung were generated by enzymatic digestion and labeled with combinations of Abs. Enumeration of cells was calculated based on a known quantity of allophycocyanin-conjugated beads added to each sample. Beads and beads were purchased from BD Pharmingen (San Diego, CA) or eBioscience (San Diego, CA). Cells were analyzed on a CyAN ADP Analyzer (Beckman Coulter, Fullerton, CA) using Summit software.

Enrichment of DCs and macrophages for in vitro infection

DCs were enriched by Nycodenz medium (Nycomed, Oslo, Norway) centrifugation from collagenase-DNase digests of spleen as previously described (23). Macrophages were harvested from mouse peritoneal washes. Cells were incubated with the GFP-expressing L. pneumophila at a multiplicity of infection of 50 at 37°C for 2 h using RPMI 1640 media supplemented with 10% FCS and glutamine in microcentrifuge tubes. DCs were seeded into 96-well tissue culture plates at a density of 1–1.5 × 10^5 cells/well. Macrophages were isolated by adherence purification on coverslips before confocal microscopy. Cells were cultured in RPMI 1640 media for 24 h at 37°C and 5% CO₂ before analysis.

Confocal microscopy

L. pneumophila-infected DCs were labeled with anti-pDC Ag (PDCA)-1 and allowed to adhere onto coverslips coated with anti-MHC class II Ab (eBioscience). L. pneumophila-infected macrophages were labeled with anti-CD45. Cells were fixed in 4% paraformaldehyde, and images were acquired using a Leica LCS SP2 confocal laser-scanning microscope (Leica Microsystems, Deerfield, IL).

Statistical analysis

Two-tailed Mann-Whitney U tests were performed using Prism software (GraphPad, San Diego, CA). Comparisons giving p values ≤0.05 were considered significant.

Results

pDCs are recruited rapidly into the lungs of L. pneumophila-infected mice

To determine if pDCs were recruited during L. pneumophila infection, lung infiltrates were analyzed at 24 h and 72 h after infection. Significant increases in the number of CD45⁺CD11c⁺MHCIIintPDCA-1⁺CD11b⁻F4/80⁻ pDCs were observed at 24 and 72 h after infection compared with mock-infected mice (representative flow cytometric plots are shown in Supplemental Fig. 2; enumeration is shown in Fig. 1). The mean number of pDCs increased by ∼4-fold in the lungs of infected mice at 72 h compared with 24 h, suggesting an active recruitment of pDCs during the course of infection. Recruitment of pDCs into the lung during influenza virus and respiratory syncytial virus infection has been reported (24–26), but this is the first report of pDCs infiltrating the lung during a bacterial infection.

pDCs are infected by L. pneumophila

L. pneumophila can infect bone marrow-derived DCs, a heterogeneous population that may include pDCs (19, 27–29). To discover if L. pneumophila can infect pDCs, splenocytes from a strain mice were enriched for DCs by density gradient centrifugation and cultured with GFP-expressing L. pneumophila strain 130b. The DC-enriched splenocytes were then labeled with anti–PDCA-1 and anti-CD11c mAb for flow cytometric analysis. The proportion of L. pneumophila-infected cells was determined by analyzing GFP fluorescence in gated populations of pDCs, conventional DCs, and non-DCs (Supplemental Fig. 3). The proportion of L. pneumophila-infected cells was 43.03 ± 2.41% and 36.02 ± 4.55% for pDCs and conventional DCs, respectively, demonstrating that ex vivo-harvested DCs can become host cells for L. pneumophila infection (Fig. 2A). A significantly smaller proportion of non-DCs (10.60 ± 1.43%) was infected by L. pneumophila when compared with pDCs or conventional DCs, suggesting that under these culture conditions, L. pneumophila has a tropism for DCs (Fig. 2A). To determine if L. pneumophila could replicate in pDCs, the number of bacteria in each pDC was counted using confocal microscopy and compared with the number in infected peritoneal macrophages (Fig. 2B). All of these pDCs contained intracellular bacteria rather than bacteria adhering on the outside of the plasma membrane, demonstrating that pDCs can internalize L. pneumophila (Fig. 2C). The mean number of intracellular bacteria in pDCs was significantly lower than in macrophages, a cell type in which L. pneumophila replicate efficiently. Only 13% of pDCs contained >2 bacteria compared with 57% of macrophages, suggesting an impaired ability of L. pneumophila to replicate in pDCs (Fig. 2B).

pDCs restrict L. pneumophila infection in vivo

pDCs are important agents in combating viral infections via the production of IFN-I (14, 15). To determine if pDCs play a role in the immune response against L. pneumophila, A strain mice were depleted of pDCs with mAb 120G8 before infection with L. pneumophila. The details of Ab treatments used for
FIGURE 2. pDCs are infected by L. pneumophila. Splenocytes from A strain mice were enriched for DCs by density gradient centrifugation and cultured with GFP-expressing L. pneumophila 130b for 2 h. Cells were cultured for 24 h before analysis by flow cytometry or confocal microscopy. A, Proportion of L. pneumophila-infected cells. B, DCs and peritoneal macrophages were cultured with GFP-expressing L. pneumophila and the number of intracellular bacteria per cell determined by confocal microscopy. C, Representative images of L. pneumophila-infected pDCs and macrophages. Red, PDCA-1 for pDCs and CD45 for macrophages; green, GFP-expressing L. pneumophila. Scale bar (for both panels), 5 μm. Data are pooled from two to three independent experiments. Mean ± SEM is shown. ***p = 0.0002.

FIGURE 3. pDCs restrict L. pneumophila infection in vivo. A strain mice were injected i.p. with 1 mg/mouse pDC-depleting mAb 120G8 24 h before infection with 2.5 × 10⁶ CFU L. pneumophila 130b. Control mice received rat IgG. Treatment regimes are depicted in Supplemental Fig. 1. Total number of L. pneumophila CFU detected in the lungs of pDC-depleted and control mice are shown. Each circle represents a single mouse. Open circles, mice administered with 120G8 mAb; gray circles, mice administered with rat IgG. Mean ± SEM is shown. Data are pooled from five independent experiments. *p < 0.04; **p < 0.006.

FIGURE 4. IFN-I-mediated responses do not influence colonization of L. pneumophila in infected mice. Mice were infected with 2.5 × 10⁶ CFU Δ flaA L. pneumophila strain. At 48 h after infection, lungs were harvested and the bacteria load determined. Each circle represents a single mouse. A, Total number of L. pneumophila CFU detected in the lungs of infected IFNAR2−/− and C57BL/6 mice are shown. Open circles, IFNAR2−/− mice; gray circles, C57BL/6 mice. Data are pooled from three independent experiments. B, Total number of L. pneumophila CFU detected in the lungs of pDC-depleted and control IFNAR−/− mice. Mice were administered Ab according to Supplemental Fig. 1 ii. Open circles, mice treated with 120G8 mAb; gray circles, mice treated with rat IgG. Data are pooled from two independent experiments. All error bars are mean ± SEM. *p < 0.05.

IFN-I is not essential for immunity against L. pneumophila

To determine if IFN-I plays a role in the immune response against L. pneumophila, we used mice deficient for the IFN-IR (IFNAR2−/−) on the C57BL/6 background. To obtain robust infection of C57BL/6 mice, we used a flagellin-deficient (Δ flaA) mutant of L. pneumophila. C57BL/6 mice have a more active flagella-sensing Naip5/Birc1e compared with A strain mice and thus are able to clear wild-type bacteria more effectively (30, 31). The Δ flaA mutant was derived from the L. pneumophila JR32 strain, so we verified that infection by L. pneumophila JR32 is restricted by pDCs in a similar manner to the 130b strain in A mice (Supplemental Fig. 4). Subsequently, IFNAR2−/− mice were infected with Δ flaA L. pneumophila. No significant differences in the number of L. pneumophila CFU were detected in the lungs of IFNAR2−/− mice compared with control C57BL/6 mice at 48 h after infection (Fig. 4A).

To prove that the control of L. pneumophila infection by pDCs did not require IFN-I, IFNAR2−/− mice were depleted of pDCs using the 120G8 mAb treatment (Supplemental Fig. 1 ii) before infection with Δ flaA L. pneumophila. Similar to A strain mice at 48 h after infection, the lungs of pDC-depleted IFNAR2−/− mice contained significantly higher numbers of L. pneumophila compared with nondepleted controls (Fig. 4B).

Discussion

Recently, several reports have indicated that L. pneumophila induces IFN-I in infected cells and that IFN-I may help control infection. In this study, we have shown that in a mouse model,
IFN-I does not appear to be required to limit \textit{L. pneumophila} replication during the acute phase of infection (Fig. 4). This work is consistent with a recent report showing that mice deficient in molecules essential to IFN-I production and signaling were colonized to levels similar to that of control mice by \textit{L. pneumophila} (32). Sporri et al. (33) also found IFNAR deficiency did not influence \textit{L. pneumophila} colonization of the spleen in the less physiological i.v. infection model. It seems likely that in normal mice, other cytokines, such as TNF-\alpha and IFN-\gamma, are sufficient to mediate clearance (1). Whether this is true in humans or immunocompromised individuals remains to be determined.

We established that pDCs were recruited to the lung early in infection by \textit{L. pneumophila} (Fig. 1) and that pDCs played a significant role in controlling \textit{L. pneumophila} replication and persistence in mice (Figs. 3, 4). The main reported modes of action of pDCs in combating infection are the secretion of IFN-I and/or the presentation of peptides derived from pathogens to T cells. In this study, we ruled out a requirement for the former mechanism, as IFNAR2\textsuperscript{−/−}, mice depleted of pDCs had increased bacterial load relative to nondepleted IFNAR2\textsuperscript{+/−} mice (Fig. 4B). A T cell-dependent mechanism also seems unlikely given the short time frame of our experiments. The effects of pDCs were apparent within 48 h of infection, which is too early for an adaptive T cell-mediated response. One alternative mechanism is that pDCs produce cytokines other than IFN-I that can be used to combat bacteria. Little attention has been paid to pDC cytokine production other than IFN-I, but pDCs do produce IL-12 (18, 34), and pDCs deficient in IL-12 production are unable to determine if pDCs are involved in the immune response to infection.

Another possible mode of action relates to our discovery that pDCs play a role in combating \textit{L. pneumophila} infection (33, 36). Villadangos et al. (32) have shown that mice deficient in molecules essential to IFN-I production and signaling confers distinct antigen-presenting properties on conventional dendritic cells. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. "Rapid pathogen-induced apoptosis: a mechanism used by dendritic cells to limit intracellular replication of Legionella pneumophila." PLoS Pathog. 4: e1000220.

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