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# Alveolar Macrophages and Neutrophils Are the Primary Reservoirs for *Legionella pneumophila* and Mediate Cytosolic Surveillance of Type IV Secretion

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***Legionella pneumophila*, an intracellular pathogen responsible for the severe pneumonia Legionnaires' disease, uses its *dot/icm*-encoded type IV secretion system (T4SS) to translocate effector proteins that promote its survival and replication into the host cell cytosol. However, by introducing bacterial products into the host cytosol, *L. pneumophila* also activates cytosolic immunosurveillance pathways, thereby triggering robust proinflammatory responses that mediate the control of infection. Thus, the pulmonary cell types that *L. pneumophila* infects not only may act as an intracellular niche that facilitates its pathogenesis but also may contribute to the immune response against *L. pneumophila*. The identity of these host cells remains poorly understood. Here, we developed a strain of *L. pneumophila* producing a fusion protein consisting of β-lactamase fused to the T4SS-translocated effector RalF, which allowed us to track cells injected by the T4SS. Our data reveal that alveolar macrophages and neutrophils both are the primary recipients of T4SS-translocated effectors and harbor viable *L. pneumophila* during pulmonary infection of mice. Moreover, both alveolar macrophages and neutrophils from infected mice produced tumor necrosis factor and interleukin-1α in response to T4SS-sufficient, but not T4SS-deficient, *L. pneumophila*. Collectively, our data suggest that alveolar macrophages and neutrophils are both an intracellular reservoir for *L. pneumophila* and a source of proinflammatory cytokines that contribute to the host immune response against *L. pneumophila* during pulmonary infection.**

*Legionella pneumophila* is a Gram-negative bacterium found ubiquitously in freshwater environments, where it is often found in association with its natural host, protozoan amoebae (1). *L. pneumophila* recently has become a human pathogen due to modern technologies, such as cooling towers and air conditioners, which can aerosolize freshwater contaminated with *L. pneumophila* (2–4). Humans then can inhale these contaminated droplets, allowing *L. pneumophila* to gain access to the pulmonary airway. *L. pneumophila* infection can lead to a severe bacterial pneumonia known as Legionnaires' disease (2), with mortality rates approaching 30% (5).

Once in the lung, *L. pneumophila* encounters a specialized subset of pulmonary phagocytes called alveolar macrophages (6). Following phagocytosis, the *Legionella*-containing phagosome avoids endocytic maturation and bacterial degradation and is converted into an endoplasmic reticulum (ER)-derived vacuole that supports bacterial replication (7). To establish infection, *L. pneumophila* utilizes its type IV secretion system (T4SS), encoded by the *dot/icm* genes, to translocate approximately 300 effector proteins into the host cell cytosol (8–15). Many of these effector proteins are thought to be involved in recruiting ER-derived vacuoles to the *Legionella*-containing vacuole or prevent endocytic maturation (15). Other effector proteins modulate host cell processes such as autophagy or host protein synthesis (16–20). These virulence activities ultimately prevent destruction of *L. pneumophila* and allow for its replication within host cells. The T4SS is essential for the ability of *L. pneumophila* to survive and replicate within host cells, as *L. pneumophila* mutants lacking a functional T4SS do not replicate and reside in phagosomes that mature along a canonical endocytic pathway (10, 11).

While the Dot/Icm T4SS is essential for *L. pneumophila* to survive intracellularly and to cause disease, cytosolic immune surveillance systems activate host defense responses to T4SS activity that are critical for the control of *L. pneumophila* infection (21). For example, the NAIP5/NLRC4 inflammasome detects T4SS-dependent delivery of flagellin, leading to the caspase-1-dependent secretion of interleukin-1 (IL-1) family cytokines and pyroptotic cell death (22–24). Cytosolic detection of T4SS activity also is required for the robust secretion of inflammasome-independent cytokines, such as tumor necrosis factor (TNF) (25–27). The IL-1 family cytokines and TNF are critical for host defense against *L. pneumophila* (20, 28–30). Thus, the cells that interact with *L. pneumophila* in the lung and receive T4SS-translocated effectors may have a dual role during *in vivo* infection, in that they can enable intracellular survival of the pathogen and also contribute directly to the immune response by detecting T4SS-translocated products. However, the identities of the pulmonary cell types that interact with *L. pneumophila* and receive T4SS-translocated effectors are poorly understood.

Alveolar macrophages are thought to be the primary cell type infected by *L. pneumophila* and to support bacterial replication *in vivo* (31). However, it is unknown whether other immune phago-

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cytes in the lung, such as neutrophils, inflammatory monocytes, or dendritic cells, also receive T4SS-translocated effectors and contribute to the immune response or support *L. pneumophila* survival. Previous studies have demonstrated that in addition to alveolar macrophages, *L. pneumophila* can be detected in neutrophils during pulmonary infection (30). Neutrophils are thought to be highly bactericidal, and their presence in the lung and airway space during pulmonary *L. pneumophila* infection correlates with lower bacterial burden (20, 28, 32–34). Whether *L. pneumophila* can survive within neutrophils and translocate T4SS effectors into these cells during pulmonary infection is unknown. *L. pneumophila* can be taken up by a wide variety of cell types *in vitro*, such as neutrophils, bone marrow-derived dendritic cells, type I and type II alveolar epithelial cells, endothelial cells, and plasmacytoid dendritic cells (35–39). However, the efficiency of *L. pneumophila* replication within these cell types varies greatly, and whether these cell types are injected by the T4SS or productively infected *in vivo* is unknown. Thus, we decided to investigate which cell types receive T4SS-translocated effectors and, as a result, may support *L. pneumophila* survival and contribute to cytosolic immunosurveillance during pulmonary infection.

Using a fluorescence resonance energy transfer (FRET)-based reporter of T4SS translocation, we were able to detect effector translocation into macrophages, dendritic cells, and airway epithelial cells *in vitro*. We also demonstrate that only T4SS-injected cells contain viable *L. pneumophila*, whereas infected cells that have not received T4SS effectors do not contain viable bacteria. *In vivo*, alveolar macrophages and neutrophils in the airway space and lung tissue were the primary recipients of T4SS-translocated effectors and harbored viable bacteria. Consistent with the critical role of immune sensing of T4SS activity in triggering host cytokine production, alveolar macrophages and neutrophils from mice infected with T4SS-competent *L. pneumophila*, but not T4SS-deficient bacteria, secreted the cytokines TNF and IL-1α, which are known to be important for immune-mediated clearance of infection (28–30). We did not observe T4SS-mediated injection into other lung cell populations, including airway epithelial cells and dendritic cells, suggesting that these cells are not a primary intracellular niche for *L. pneumophila* and do not directly participate in cytosolic immunosurveillance of T4SS activity during lung infection. Collectively, our data indicate that alveolar macrophages and neutrophils play a dual role as both an intracellular niche and immune mediator during pulmonary *L. pneumophila* infection.

## MATERIALS AND METHODS

**Ethics statement.** All experiments performed in this study were done so in accordance with the Animal Welfare Act (AWA) and the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (40). The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all procedures (protocols 803465, 803459, and 804928).

**Bacterial strains and plasmids.** All experiments used *Legionella pneumophila* serogroup 1 strains. For *in vitro* studies, macrophages, dendritic cells, and epithelial cells were infected with Lp02 (*rpsL*, *hsdR*, *thyA*), a thymidine auxotroph derived from strain Lp01, or a Δ*dotA* or Δ*flaA* isogenic mutant strain (10). For *in vivo* studies, mice were infected with the JR32-derived (*rpsL*, *hsdR*) Δ*dotA* or Δ*flaA* isogenic mutant strain (22, 41). For *in vivo* experiments requiring cell sorting, the aforementioned Lp02 strains were used. For *in vitro* and *in vivo* studies, *L. pneumophila* was cultured on charcoal yeast extract agar containing 6.25 μg/ml chloramphenicol for 48 h at 37°C prior to infection (42, 43). For studies requiring motile *L. pneumophila*, 48-h cultures grown on CYE agar were grown overnight in AYE broth containing chloramphenicol with shaking at 37°C until >50% of the bacteria were observed to be motile by light microscopy. Plasmids encoding M45-tagged β-lactamase-RalF fusion protein or M45-tagged β-lactamase were generated as follows. Briefly, the pJB1806 plasmid (RSF1010 ori, *td*Δ*I*, Amp$^r$, Cm$^r$) first was modified by cloning the *icmR* promoter and M45 epitope tag into the EcoRI and BamHI sites (44). The mature TEM-1 β-lactamase gene (BlaM) then was amplified from a *Yersinia pseudotuberculosis* YopE-BlaM-encoding plasmid using primers that introduced a 5′ BglII site (5′-AATAAGATCTTGCACCCAGAAAC GCTGGTG-3′) and 3′ BamHI site (5′-GCCTCACTGATTAAGCATTGG GGGATCCAATA-3′) (45). The resulting PCR product was digested with BglII and BamHI and cloned into the BamHI site of the pJB1806 P*icmR*:M45 plasmid to create a plasmid encoding M45-tagged β-lactamase. To generate the plasmid encoding a translational fusion of M45-β-lactamase-RalF, RalF was amplified from Lp01 genomic DNA using primers that introduced BamHI sites at the 5′ and 3′ ends (5′-AATAGGATCCG GCATCCAGAAATTGAAAAAGCCC-3′) and (5′-GAAAAAGGTAGAC AATTAAAATTTTAAGGATCCAATA-3′). The resulting PCR product was digested with BamHI and cloned into the BamHI site downstream of the gene encoding M45-BlaM. The resulting plasmids then were electroporated into *L. pneumophila*, and transformed colonies were selected for with chloramphenicol (8).

**Mice.** C57BL/6 mice were purchased from Jackson Laboratories. Mice were maintained in accordance with the guidelines of the University of Pennsylvania Institutional Animal Use and Care Committee. For infections, 8- to 12-week-old female mice were anesthetized by intraperitoneal injection of a ketamine-xylazine-phosphate-buffered saline (PBS) solution at a dose of 100 mg ketamine/kg of body weight and 10 mg/kg xylazine. Mice then were infected intranasally with 40 μl of a bacterial suspension containing $5 \times 10^6$ CFU *L. pneumophila* or PBS vehicle control. At the indicated time points after infection, mice were sacrificed. To isolate lung airway cells, bronchoalveolar lavage was performed 3 to 5 times with 1 ml of cold PBS each time. Lungs then were excised and digested for 30 min at 37°C with occasional shaking in 5 ml of PBS containing 5% fetal bovine serum (FBS), 250 U/ml of collagenase IV (Worthington Biochem), and 20 U/ml DNase I (Roche). Lungs then were mechanically homogenized, and a single-cell suspension was obtained. To determine bacterial burden, lungs were mechanically homogenized in sterile, distilled $H_2O$, and a portion of the lysate was spread onto CYE plates containing either chloramphenicol or streptomycin.

**Cell culture.** For macrophages, C57BL/6 mouse bone marrow cells were differentiated in RPMI containing 30% L929 cell supernatant and 20% FBS at 37°C, 5% $CO_2$ in a humidified incubator. The macrophages were replated in RPMI containing 15% L929 cell supernatant and 10% FBS (28). For dendritic cells, bone marrow cells were differentiated in RPMI containing 10% FBS, 50 μM β-mercaptoethanol, 2 mM L-glutamine, and 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech) (46). Semiadherent dendritic cells then were isolated and replated in medium lacking GM-CSF. A549 cells (ATCC) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS (47). For infections, cells were treated with 10 ng/ml LPS from *E. coli* strain 055:B5 (Sigma), 10 μl of bacterial suspension, or 10 μl of PBS vehicle control.

**Flow cytometry, fluorescence-based imaging flow cytometry, and cell sorting.** For *in vitro* experiments, infected cells were lifted and loaded with CCF4-AM (Invitrogen) per the manufacturer's instructions. Cells then were washed and treated with Live/Dead fixable dead cell stain (Invitrogen). Bone marrow-derived dendritic cells were stained with antibodies specific for CD11c and major histocompatibility complex class II (MHC-II; eBioscience). To stain for intracellular *L. pneumophila*, cells were fixed with BD Cytofix, permeabilized with BD Phosflow perm buffer III (BD Biosciences), and then stained with a rabbit polyclonal antibody against *L. pneumophila* followed by a rabbit-specific secondary antibody tagged to a fluorophore (Invitrogen). For *in vivo* studies, lung and airway

cells were loaded with CCF4-AM and treated with the Live/Dead stain. Cells then were stained with antibodies specific for the cell surface antigens CD45, CD11c, Ly6G, Ly6C, NK1.1 (BioLegend), MHCII, CD19, CD3ε, CD31, CD326 (eBioscience), Siglec F, CD11b, and Ter119 (BD Biosciences). Data were collected on an LSR II flow cytometer (BD Biosciences), and postcollection data were analyzed using FlowJo (TreeStar). For fluorescent imaging experiments, data and images were collected on an Amnis ImageStream$^X$ Mark II, and data were analyzed using IDEAS software (EMD Millipore). Cells were gated on live singlets that had retained the CCF4-AM dye. Cell sorting experiments were performed on a FACSAria II flow cytometer (BD Biosciences).

**Enzyme-linked immunosorbent assay (ELISA).** Harvested supernatants from cultured cells or bronchoalveolar lavage specimens were assayed using capture and detection antibodies specific for IL-1α and TNF (BioLegend).

**Immunoblot analysis.** *Legionella pneumophila* cells expressing the appropriate reporter plasmids were harvested from a 2-day heavy patch and lysed. Lysates then were subjected to SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and probed with an anti-M45 epitope monoclonal antibody (48).

**Statistical analysis.** The plotting of data and statistical analysis were performed using GraphPad Prism software. Statistical significance was determined using the unpaired, two-tailed Student's *t* test or one-way analysis of variance (ANOVA) with Tukey's posttest. Differences were considered significant if the *P* value was $<0.05$.

## RESULTS

**A reporter system tracks translocation of type IV secretion system effectors by *Legionella pneumophila* into mammalian cells.** *L. pneumophila* uses its T4SS to translocate effector proteins into the cytosol of host cells. To track this translocation, we constructed a plasmid in which the well-characterized *L. pneumophila icmR* promoter drives transcription of a gene encoding a translational fusion of the mature TEM-1 BlaM and the well-characterized T4SS effector protein RalF and introduced this plasmid into *L. pneumophila* (see Fig. S1A and B in the supplemental material) (49–51). We chose RalF because it is translocated into the cytosol of infected cells immediately following the intimate interaction of *L. pneumophila* with host cells (52, 53). Following infection of host cells by bacterial strains expressing the BlaM-RalF fusion protein, the enzymatic activity of translocated BlaM-RalF was detected in host cells by means of the membrane-permeable BlaM substrate CCF4-AM (51). CCF4-AM consists of coumarin joined to fluorescein by a β-lactam ring. When excited at 409 nm, fluorescence resonance energy transfer (FRET) between coumarin and fluorescein results in green fluorescence emission at 518 nm. T4SS-injected BlaM will cleave the CCF4-AM substrate in the host cytosol and eliminate FRET, resulting in blue fluorescence emission at 447 nm.

We generated *L. pneumophila* strains expressing either BlaM or BlaM-RalF and infected C57BL/6 bone marrow-derived macrophages (BMDM) with these strains for 8 h. Following infection, the cells were loaded with CCF4-AM and analyzed by flow cytometry to determine whether blue fluorescence emitted by cleaved CCF4-AM was detected (see Fig. S1C in the supplemental material). Approximately 20 to 25% of macrophages infected with BlaM-RalF-expressing wild-type (WT) *L. pneumophila* and *L. pneumophila* lacking flagellin (Δ*flaA* mutant), which evade NAIP5 inflammasome responses, were positive for blue fluorescence resulting from cleaved CCF4-AM, but this was not the case following infection with strains lacking a functional T4SS (Δ*dotA* mutants). This indicates that CCF4-AM is efficiently cleaved only by BlaM-RalF translocated by T4SS-sufficient bacteria and that BlaM-RalF remaining within bacteria does not generate a detectable signal in this assay. The frequencies of injected cells in WT and Δ*flaA* mutant *L. pneumophila* infections were comparable, although the frequency of injection was consistently lower in WT infections (Fig. 1A and 2A; also see Fig. S1C). The robust detection of injection by WT *L. pneumophila* is surprising considering that a higher percentage of vacuoles containing WT *L. pneumophila* fail to avoid rapid endocytic maturation and that flagellin induces NAIP5-dependent cell death in C57BL/6 macrophages (22, 23, 54–56). Following infection with *L. pneumophila* strains expressing BlaM alone, we found that a much lower percentage of macrophages became positive for blue CCF4-AM fluorescence compared to macrophages infected with *L. pneumophila* expressing BlaM-RalF (see Fig. S1C). Importantly, this small percentage of CCF4-AM positive cells still was dependent on infection with T4SS-sufficient bacteria, suggesting that BlaM lacking a canonical T4SS signal sequence is inefficiently delivered into the host cytosol by the T4SS.

**T4SS-injected host cells contain viable *Legionella pneumophila*.** The T4SS is essential for the survival of *L. pneumophila* within host cells. To determine whether cells injected with BlaM-RalF contain *L. pneumophila*, we infected BMDMs with these reporter strains and loaded the cells with CCF4-AM. After loading, the macrophages were fixed, permeabilized, and stained with an antibody specific for *L. pneumophila* (Fig. 1A). Infection with all three strains (the WT and Δ*dotA* and Δ*flaA* mutants) of *L. pneumophila* resulted in macrophages staining positive for the presence of bacteria. Ninety to 100% of cells that were positive for BlaM-RalF injection also were positive for *L. pneumophila* staining in both the WT and Δ*flaA* strains. With both strains, we detected a subset of cells that was positive for *L. pneumophila*, but translocation of BlaM-RalF was not within a detectible range, revealing heterogeneity in BlaM-RalF translocation at the single-cell level. The percentage of cells positive for *L. pneumophila* but negative for BlaM-RalF translocation could result from bacteria that failed to successfully translocate T4SS effectors into the host cell, either because they were nonviable, were not in the transmissive phase, or failed to efficiently evade rapid endocytic maturation.

To determine whether the *L. pneumophila* organisms associated with injected or uninjected macrophages were intact or degraded, we analyzed these macrophages with fluorescence-based imaging flow cytometry (Fig. 1B; also see Fig. S2A in the supplemental material). The majority of macrophages infected with the Δ*dotA* strain showed dim *L. pneumophila* staining, with multiple small puncta present per cell (Fig. 1C). Because the Δ*dotA* mutants are unable to evade endocytic maturation due to their lack of a functional T4SS, punctate staining could result from bacteria that were degraded. Alternatively, punctate staining could represent uninfected cells that had phagocytosed bacterial debris. When we infected macrophages with the *L. pneumophila* Δ*flaA* mutant encoding a functional T4SS, again we could identify T4SS-injected and uninjected cells. Many of the uninjected cells stained positive for intracellular *L. pneumophila* (see Fig. S2B), but the majority of these cells exhibited dim, punctate staining similar to the staining seen for Δ*dotA* mutant-infected macrophages (Fig. 1B and C). This may represent cells containing bacteria that had not successfully evaded endocytic maturation or uninfected cells that had phagocytosed bacterial debris. In contrast, the majority of injected cells showed a single bright punctum of *L. pneumophila*

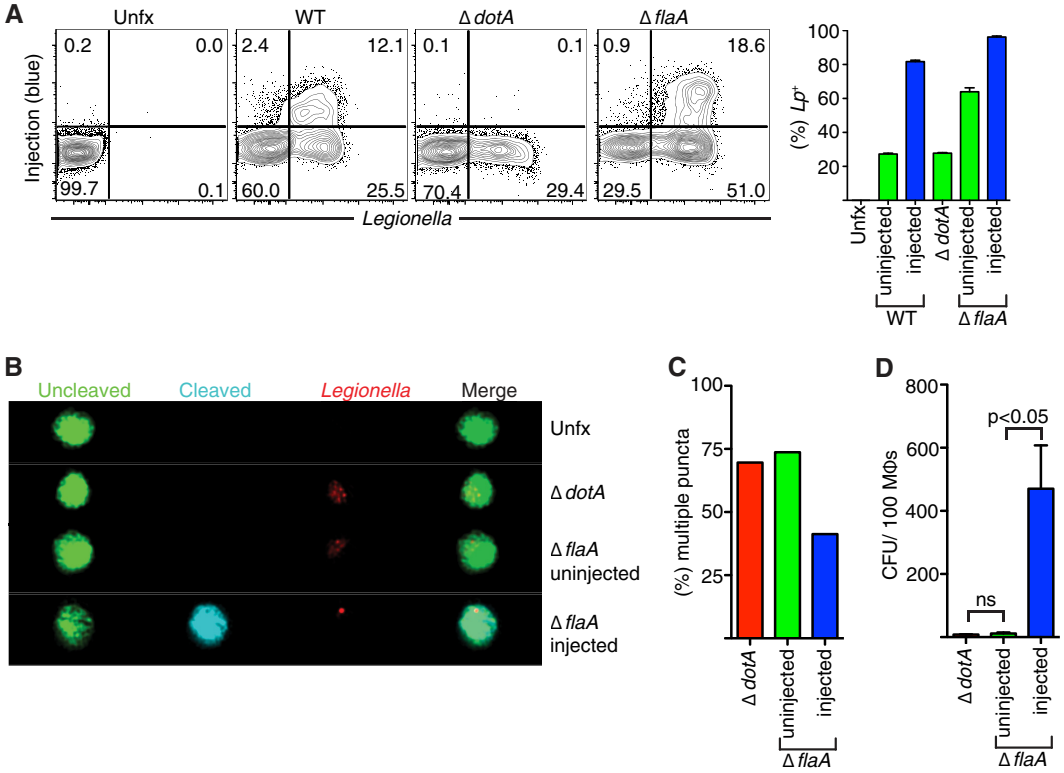


**FIG 1** Viable *Legionella pneumophila* organisms are associated predominantly with macrophages positive for T4SS-dependent translocation. (A) BMDMs were left untreated (Unfx) or were infected with the WT, Δ*dotA*, or Δ*flaA* strain for 8 h at an MOI of 5. Cells then were loaded with CCF4-AM, fixed, permeabilized, and stained with a polyclonal antibody against *L. pneumophila*. Cells were analyzed by flow cytometry. The percentages of cells positive for *L. pneumophila* as determined by staining and cells positive for T4SS injection are denoted within the gates and quantified in the bar graph shown. (B and C) Cells were treated as described for panel A but were infected at an MOI of 10 for 4 h and analyzed using an Amnis ImageStream imaging flow cytometer. (D) BMDMs were left untreated or infected with the Δ*dotA* or Δ*flaA* mutant strain for 8 h at an MOI of 5 in the presence of exogenous thymidine (100 μg/ml) and loaded with CCF4-AM. Samples infected with the Δ*dotA* mutant were sorted in bulk for loaded cells. Cells infected with the Δ*flaA* mutant then were sorted based on uncleaved or cleaved CCF4-AM signal, lysed, and plated on CYE plates. CFU then were enumerated. Bar graphs show means only or means ± standard errors of the means (SEM) from triplicate samples. Results are representative of 2 independent experiments. ns, not significant.

staining, indicating the presence of an intact bacterium that had not been transported to a hydrolytic compartment.

To test whether injected macrophages contain viable *L. pneumophila*, we sorted infected macrophages that were either positive or negative for the cleaved CCF4-AM signal, lysed the macrophages, and enumerated bacterial CFU in these distinct cell populations (Fig. 1D). T4SS-injected cells recovered from an *L. pneumophila* Δ*flaA* mutant infection contained the vast majority (nearly 6 bacteria for every injected BMDM) of viable *L. pneumophila* as determined by CFU count. Uninjected cells from the same infection contained a minimal number of viable *L. pneumophila* (less than 1 bacterium for each uninjected BMDM), comparable to the number of viable bacteria recovered from a Δ*dotA* mutant infection. Similar results were obtained with the JR32 strains of *L. pneumophila* (see Fig. S1D and E in the supplemental material). To exclude the possibility that the uninjected cells contained viable *L. pneumophila* that lost the BlaM-RalF reporter plasmid encoding chloramphenicol resistance, we also plated cell lysates in the presence or absence of chloramphenicol (see Fig. S3A). The CFU obtained on plates with and without chloramphenicol were indistinguishable, suggesting that the plasmid is stably maintained during *in vitro* infection in the absence of antibiotics. Collectively, our data indicate that viable bacteria are associated primarily with cells that have received translocated BlaM-Ralf, whereas uninjected cells either are not infected or contain nonviable bacteria.

**Translocation by the type IV secretion system can be detected in dendritic cells and alveolar epithelial cells *in vitro*.** *L. pneumophila* can infect a variety of cell types *in vitro*, including dendritic cells and airway epithelial cells (35–37). Thus, we examined whether T4SS-mediated translocation into these cell types could be detected using the β-lactamase reporter system. At a given MOI, compared to infected BMDMs, we detected a much lower frequency of T4SS-mediated injection into bone marrow-derived dendritic cells (BMDCs) infected with WT or Δ*flaA* mutant *L. pneumophila* (Fig. 2A and B). We also infected A549 cells, an alveolar epithelial cell line, and detected a low frequency of injection into these cells (Fig. 2C).

Other researchers have noted an increase in bacterial uptake by host cells when *L. pneumophila* is grown under conditions that promote bacterial motility (22). Indeed, infection of macrophages with motile *L. pneumophila* resulted in a large increase in the frequency of injected macrophages, as the percentage of injected cells increased from 10% to more than 80% (Fig. S4B). In contrast, in A549 cells, we did not observe an increase in injection regardless of bacterial motility, in that 1.3% of cells infected with nonmotile or motile *L. pneumophila* were injected (see Fig. S4A). For all cell

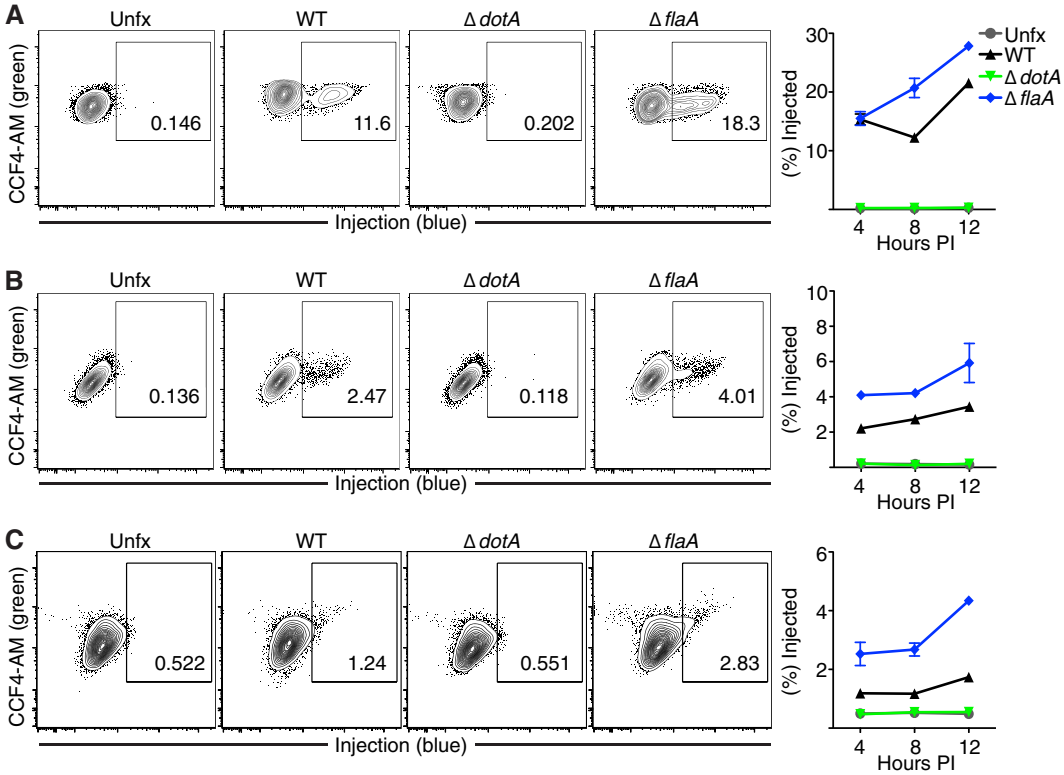


**FIG 2** *Legionella pneumophila* T4SS-dependent translocation is detected in dendritic cells and alveolar epithelial cells during in vitro infection. BMDMs (A), bone marrow-derived dendritic cells (BMDCs) (B), and A549 cells (C) were left untreated or were infected with the WT, Δ*dotA*, or Δ*flaA* strain for 4, 8, or 12 h at an MOI of 5. Cells then were loaded with CCF4-AM and analyzed for injection by flow cytometry. Representative plots show injection 8 h postinfection (PI). Graphs show means ± SEM from triplicate wells. Results are representative of 2 independent experiments ($n = 3$).

types, the percentage of cells injected by the T4SS of *L. pneumophila* increased over time (Fig. 2). In all instances, cleaved CCF4-AM signal required the expression of a functional T4SS, suggesting that the β-lactamase reporter operates in a T4SS-dependent manner in a variety of cell types. As we observed more robust injection into macrophages and dendritic cells than into nonphagocytic alveolar epithelial cells, these data suggest that both increased cell contact and efficient uptake by professional phagocytes contribute to the ability of *L. pneumophila* to efficiently translocate effector proteins.

***Legionella pneumophila* translocates bacterial effectors into alveolar macrophages and neutrophils during pulmonary infection.** Our data suggest that the *L. pneumophila* T4SS can translocate effectors into alveolar epithelial cells, dendritic cells, and macrophages during *in vitro* infection. During pulmonary infection, replicating *L. pneumophila* can be detected in alveolar macrophages (31), indicating that alveolar macrophages receive T4SS-translocated effectors. However, whether alveolar epithelial cells, dendritic cells, and other cell types receive T4SS-translocated effectors *in vivo* has not been investigated. To identify the cells that receive translocated effectors during a permissive model of *in vivo* infection, we intranasally infected C57BL/6 mice with the *L. pneumophila* Δ*flaA* mutant expressing BlaM-RalF, as WT *L. pneumophila* does not establish a productive infection in mice that encode a functional NAIP5 allele (see Fig. S3D in the supplemental material) (22, 23). In this model, similar to WT *L. pneumophila* infection of A/J mice expressing a hypomorphic NAIP5 allele, the lungs of C57BL/6 mice exhibit an approximately 1-log increase in Δ*flaA* mutant CFU by 24 to 48 h postinfection (hpi) (see Fig. S3C and D) (57). The mice subsequently are able to control infection, with minimal bacterial CFU detected in the lungs by 5 days postinfection (see Fig. S3C and D) (23). Expression of the plasmid containing BlaM-RalF did not affect the replication of the Δ*flaA* mutant *in vivo* (see Fig. S3C). After intranasal inoculation with *L. pneumophila*, we performed bronchoalveolar lavage to isolate cells from the airway space at various time points and loaded them with CCF4-AM to detect T4SS-mediated injection of BlaM-RalF. At 4 h postinoculation, we detected T4SS-mediated translocation of β-lactamase activity in nearly 50% of cells recovered from the airway of mice infected with the Δ*flaA* mutant (Fig. 3A). Greater than 95% of the T4SS-injected cells were alveolar macrophages, as indicated by their expression of CD11c and Siglec F (58, 59). Similar results were obtained with WT *L. pneumophila* at this time point (data not shown). Consistent with our *in vitro* data, we did not observe injection of BlaM-RalF in mice infected with the Δ*dotA* strain, which is unable to translocate effectors into host cells and cannot establish a productive infection *in vivo*.

At later times postinfection, we detected recruitment of a large population of neutrophils to the airway space of Δ*flaA* mutant-infected mice that did not occur in mice infected with the Δ*dotA* mutant (see Fig. S5A and B in the supplemental material), consistent with previous studies indicating that neutrophil recruitment is T4SS dependent (20, 28, 30, 60). When we identified cells injected by *L. pneumophila* in the airway space at 24 hpi, we again

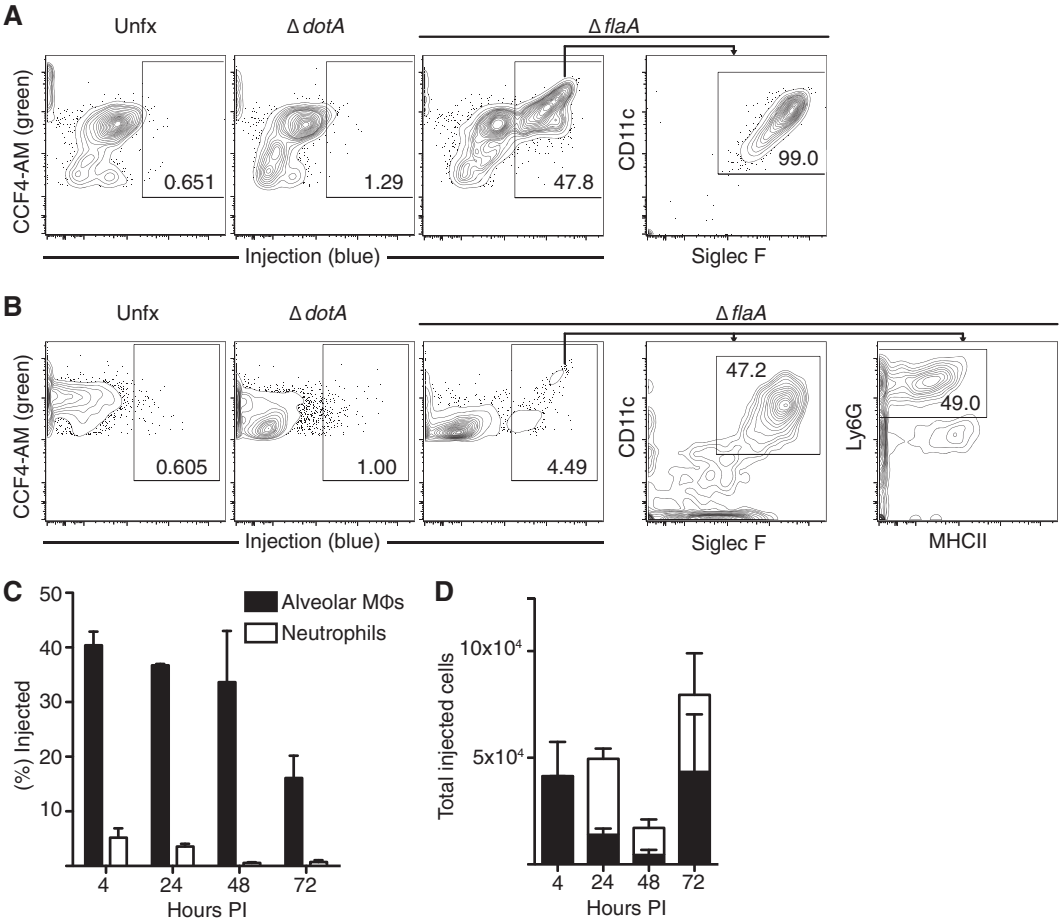


**FIG 3** Alveolar macrophages and neutrophils in the airway space are injected by the *Legionella pneumophila* T4SS. C57BL/6J mice were infected intranasally with PBS vehicle control or the Δ*dotA* or Δ*flaA* mutant. At 4 (A) or 24 (B) h postinfection, cells in the airway space were isolated, enumerated, loaded with CCF4-AM, and stained for cell surface markers. Cells then were analyzed for injection by flow cytometry and cell surface marker expression. Results are representative of 2 independent experiments, with $n = 4$ mice per group. (C and D) Alveolar macrophages (MΦ) and neutrophils from the airway space of mice infected with the Δ*flaA* strain were isolated at 4, 24, 48, or 72 h postinfection and loaded with CCF4-AM. The percentage (C) and total number (D) of cells injected by *L. pneumophila* in each population were quantified. Graphs show means ± SEM ($n = 3$ to 4 mice per group).

identified alveolar macrophages as being positive for T4SS-mediated injection, but we also could identify injected cells that expressed high levels of Ly6G and were negative for MHC-II (Fig. 3B). We determined that these injected Ly6G$^+$ cells were neutrophils, as they expressed low levels of Ly6C, a cell surface marker highly expressed on inflammatory monocytes (see Fig. S2C) (61, 62). The frequency of injected neutrophils was much lower than that of injected alveolar macrophages (Fig. 3C). However, due to the large influx of neutrophils, the total number of injected neutrophils was comparable to or greater than the total number of injected alveolar macrophages at 24, 48, and 72 hpi (Fig. 3D).

As we could detect robust T4SS-mediated injection of BlaM-RalF into cells of the airway space, we wanted to determine whether cells within the lung interstitium were injected by *L. pneumophila* as well. Notably, we again observed T4SS-mediated injection into alveolar macrophages and neutrophils within lung homogenates (Fig. 4A). As in the airway space, we detected a large influx of neutrophils into the lung tissue of Δ*flaA* mutant-infected mice but not in mice infected with the Δ*dotA* strain of *L. pneumophila* (see Fig. S5C and D in the supplemental material). Although *in vitro* we observed T4SS-mediated injection into bone marrow-derived dendritic cells as well as A549 alveolar epithelial cells (Fig. 2B and C), we did not detect injection into lung dendritic cells or CD326$^+$ airway epithelial cells, suggesting that *L. pneumophila* does not efficiently infect or translocate effectors into these cell types during a permissive mouse model of infection (Fig. 4A). We also did not observe injection into inflammatory monocytes, plasmacytoid dendritic cells, eosinophils, B cells, T cells, NK cells, or endothelial cells within the lung tissue at any time assayed postinfection (see Fig. S2C and D in the supplemental material). The frequency of T4SS-injected neutrophils in the lung tissue was much lower than that seen in alveolar macrophages, similar to what we observed in the airway space (Fig. 4B). At 4 hpi, the majority of cells receiving T4SS-translocated effectors in the lung tissue were alveolar macrophages, but at later times, many of the T4SS-injected cells were neutrophils (Fig. 4C). Importantly, *L. pneumophila* recovered at 48 and 72 hpi retained the reporter plasmid, indicating that the plasmid is stably maintained during *in vivo* infection even in the absence of antibiotic selection (see Fig. S3B). A previous study examining a nonpermissive model of C57BL/6 mice infected with WT *L. pneumophila* also found that CD45-negative cells or lung epithelial cells did not appear to have

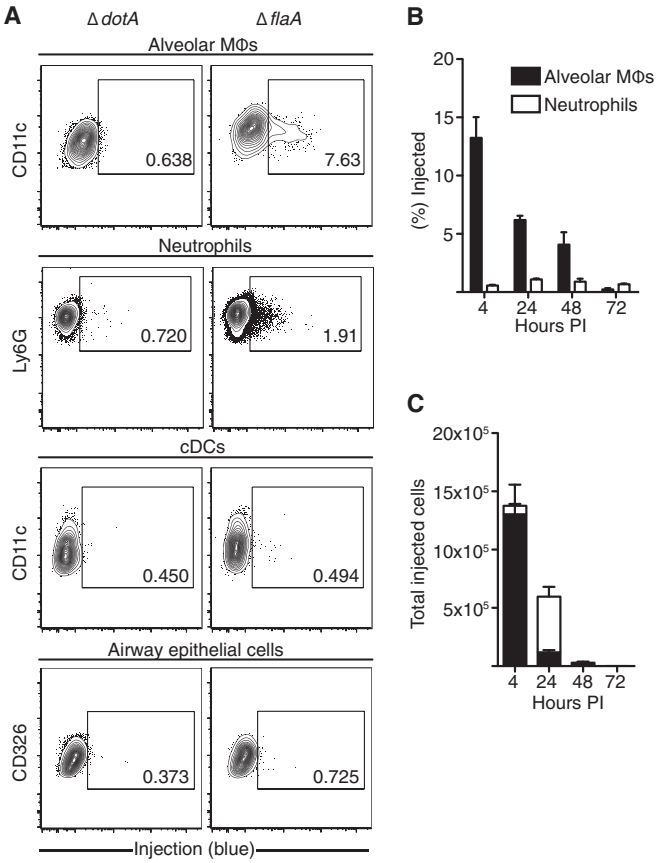


**FIG 4** *Legionella pneumophila* T4SS injects alveolar macrophages and neutrophils in the lung. (A) Mice were infected intranasally with the *L. pneumophila* Δ*dotA* or Δ*flaA* mutant for 24 h. Lung cells then were isolated, loaded with CCF4-AM, and stained. Flow plots are pregated on the denoted cell populations. Results are representative of 3 independent experiments, with $n = 4$ mice per group. (B and C) Mice were infected with the Δ*flaA* mutant, and lung cells were isolated at various times postinfection. The percentage (B) and total number (C) of cells injected by *L. pneumophila* in each population were quantified. Graphs show means ± SEM ($n = 3$ to 4 mice per group). cDC, conventional dendritic cell.

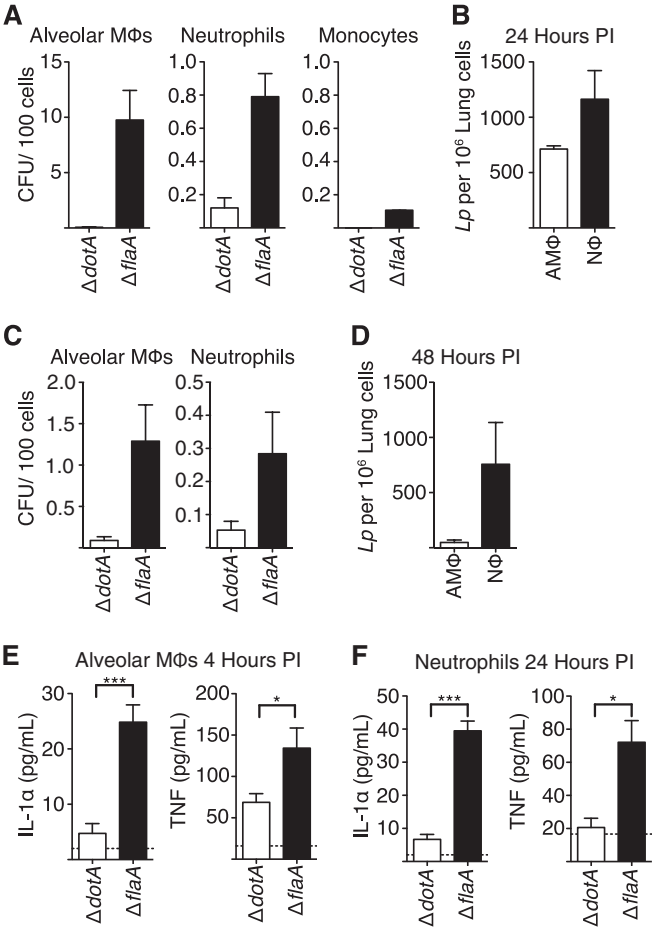


**FIG 5** Alveolar macrophages and neutrophils from infected mice contain viable *Legionella pneumophila* and secrete cytokines. (A and C) Mice were intranasally infected with the Δ*dotA* or Δ*flaA* mutant. At 24 (A) or 48 (C) hours postinfection, the lungs and airway space were homogenized, and then single-cell suspensions were stained for cell surface markers to identify alveolar macrophages, neutrophils, and inflammatory monocytes. These cell populations were sorted using a flow cytometer, lysed, and plated on CYE agar to enumerate *L. pneumophila* CFU. (B) The frequency of viable bacteria per cell type shown in panel A then was multiplied by the frequency of the appropriate cell type found in a total of $10^6$ lung cells. (D) Similarly, the frequency of viable bacteria per cell type shown in panel C was multiplied by the frequency of the appropriate cell type found in a total of $10^6$ lung cells. (E) Alveolar macrophages were isolated by bronchoalveolar lavage 4 h after intranasal infection with the Δ*dotA* or Δ*flaA* mutant and cultured overnight. Supernatants were collected, and TNF and IL-1α concentrations were determined by ELISA. (F) Neutrophils were isolated and sorted at 24 hpi from mice treated as described for panel A and then cultured overnight. Graphs show means ± SEM. *, $P < 0.05$; ***, $P < 0.0005$.

taken up *L. pneumophila*, with alveolar macrophages appearing to be the primary cells infected at early time points postinfection, followed by infection of recruited neutrophils at 1 day postinfection (30), suggesting that in both permissive and nonpermissive mouse models, similar lung cell types are infected.

**Neutrophils and alveolar macrophages in the lungs of infected mice harbor viable *Legionella pneumophila* bacteria and produce cytokines.** Alveolar macrophages are thought to be the primary cell type that is infected by *L. pneumophila* and supports bacterial replication (31). A previous study using a nonpermissive model of C57BL/6 mice infected with WT *L. pneumophila* also found that recruited neutrophils take up *L. pneumophila* in the lung, but whether *L. pneumophila* could translocate effectors into neutrophils or survive within these cells was not examined (30). As we observed that both alveolar macrophages and neutrophils in *L. pneumophila*-infected lungs were injected by the T4SS, we sought to determine whether in addition to alveolar macrophages, neutrophils also contained viable bacteria. Therefore, we sorted total alveolar macrophages and neutrophils from the lungs of mice infected intranasally with *L. pneumophila* and enumerated bacteria from lysed cells. As a comparison, we also sorted total inflammatory monocytes, a population of cells negative for T4SS injection, from these infected mice as well. As expected, alveolar macrophages isolated 24 h after infection contained viable bacteria (Fig. 5A). In contrast, inflammatory monocytes contained very few viable *L. pneumophila* organisms, consistent with the lack of observed T4SS-dependent translocation into these cells (Fig. 5A). Interestingly, neutrophils from Δ*flaA* mutant-infected mice contained viable bacteria at a frequency consistent with the extent of injection, suggesting that injected neutrophils harbor viable bacteria in the airway and lung tissue. Although the absolute fre-

quency of viable *L. pneumophila* in alveolar macrophages was greater than that within neutrophils (nearly 10 bacteria per 100 alveolar macrophages versus 1 bacterium per 100 neutrophils), the higher absolute numbers of neutrophils present during infection results in the unexpected finding that neutrophils actually contain nearly twice as many viable bacteria as alveolar macrophages (Fig. 5B). To examine whether this also was the case at the peak of pulmonary bacterial load, we sorted alveolar macrophages and neutrophils from mice 48 hpi (Fig. 5C and D). As with samples from 24 hpi, although a higher frequency of alveolar macrophages contained viable *L. pneumophila*, in total there were more *L. pneumophila* organisms in neutrophils than alveolar macrophages.

The presence of neutrophils in the airway space during infection correlates with lower bacterial burden thought to be due in part to their potent bactericidal activity (20, 28, 30). However, as our data suggest that *L. pneumophila* cells inject and survive within neutrophils, potentially activating cytosolic immunosurveillance pathways within these cells, we next examined whether or not infected neutrophils also contribute to the T4SS-dependent production of proinflammatory cytokines important for bacterial clearance. To test this, following intranasal infection with either the *L. pneumophila* Δ*dotA* or Δ*flaA* mutant, we measured cytokines secreted by alveolar macrophages isolated at 4 h postinfection or neutrophils isolated at 24 h postinfection. Alveolar macrophages from Δ*flaA* mutant-infected mice secreted TNF and IL-1α, whereas macrophages from Δ*dotA* mutant-infected mice did not. Interestingly, neutrophils from mice infected with the Δ*flaA* mutant also secreted substantial amounts of TNF and IL-1α, whereas neutrophils from mice infected with the Δ*dotA* mutant did not secrete detectable levels of IL-1α and secreted significantly less TNF, which correlates with the lack of detectable cytokine production observed during pulmonary infection with the Δ*dotA* mutant (Fig. 5E and F). Intriguingly, these data demonstrate that in addition to alveolar macrophages, neutrophils also produce proinflammatory cytokines in the context of T4SS-competent *L. pneumophila* infection. This indicates that in addition to their potent bactericidal activity, neutrophils contribute to the control of infection by other immune effector mechanisms, such as cytokine production.

## DISCUSSION

*Legionella pneumophila* uses its T4SS to inject a large number of effector proteins into the cytosol of host phagocytes (63). The T4SS is necessary for intracellular replication and pathogenesis, as *L. pneumophila* mutants lacking a functional T4SS fail to establish a replicative niche and do not cause pathology in mice (8, 11, 20, 30). In addition to being required for *L. pneumophila* pathogenesis, T4SS activity potently activates multiple cytosolic immunosurveillance pathways (25, 64–66). Thus, cells that interact with *L. pneumophila* and receive T4SS-translocated effectors serve as a potential replicative niche but also may contribute to the immune response against *L. pneumophila*. However, the precise identity of such cells is unknown. Therefore, we set out to identify host cells that receive T4SS-translocated effectors during infection with *L. pneumophila*. BlaM reporter systems have been used during *in vivo* infection with *Yersinia pseudotuberculosis* (67, 68), *Yersinia pestis* (69, 70), *Yersinia enterocolitica* (71), *Salmonella enterica* serovar Typhimurium (72, 73), and *Pseudomonas aeruginosa* (74, 75) to detect the translocation of effectors into host cells by the type III and type IV secretion systems. We demonstrate in this study that by using β-lactamase (BlaM) translationally fused to the T4SS-translocated effector protein RalF, we can successfully track injection by the T4SS into host cells during both *in vitro* and *in vivo* infection, and we describe the first use of this BlaM reporter during *in vivo* pulmonary infection with *L. pneumophila*.

We observed robust T4SS-mediated injection into alveolar macrophages at 4 h postinfection, consistent with previous observations that these cells are the primary cell type infected by *L. pneumophila* during pulmonary infection in human patients (31). At later time points postinfection, we find that in addition to alveolar macrophages, a large number of the cells injected by *L. pneumophila in vivo* are neutrophils. Most likely this is due to the large influx of neutrophils into the lungs and airway space during infection (60, 76–80). Other researchers have shown that neutrophils contain intracellular *L. pneumophila* in a nonpermissive mouse model of pulmonary infection, but they did not examine whether *L. pneumophila* could survive within neutrophils or whether neutrophils are capable of receiving T4SS-translocated effectors (30). *In vitro* studies have suggested that *Legionella* species are resistant to the highly bactericidal activity of neutrophils but cannot replicate within these cells (39, 81). Thus, we initially presumed that although neutrophils might be injected during *in vivo* infection, the majority of bacteria eventually would be cleared due to a failure to replicate in these cells, and we would not be able to detect large numbers of viable bacteria within these cells. To our surprise, we obtained viable *L. pneumophila* cells in numbers that roughly corresponded to the frequency of injection seen with our reporter system, suggesting that *L. pneumophila* can survive within neutrophils during *in vivo* infection. Unexpectedly, given the large number of neutrophils that enter the lung, the total number of *L. pneumophila* CFU harbored by neutrophils is greater than the total number of *L. pneumophila* CFU found within the alveolar macrophage population 24 and 48 hpi. Given the large numbers of infected neutrophils that we observed, it would be of interest to determine whether *L. pneumophila* could establish an ER-derived vacuole and successfully replicate within neutrophils, as this could represent another intracellular niche for *L. pneumophila*. Most bacteria are thought not to survive or replicate within neutrophils, but there are a few exceptions, including *Neisseria gonorrhoeae* (82), *Anaplasma phagocytophilum* (83, 84), and pathogenic *Escherichia coli* (85).

As we were able to detect robust T4SS-dependent injection only into alveolar macrophages and neutrophils, we conclude that phagocytic cells in the airway space are the primary recipients of T4SS-translocated effectors during pulmonary *L. pneumophila* infection. Whether or not T4SS-injected cells survive infection and traffic to other organs, including lymph nodes, also is unknown. However, previous studies have reported that alveolar macrophages do traffic to lymph nodes when given allergic stimuli (86). Thus, it would be of interest to investigate whether T4SS-injected alveolar macrophages either induce adaptive immunity or participate in the dissemination of infection to other organs (86). Surprisingly, we were unable to detect T4SS-injected conventional dendritic cells during *in vivo* infection. Dendritic cells undergo rapid apoptosis in response to *L. pneumophila* T4SS activity (87), which could account for why we do not detect injection in dendritic cells that is as robust as that of macrophages *in vitro* and *in vivo*. It would be interesting to determine whether more robust injection could be detected in DCs lacking apoptotic regulators,

such as BAX and BAK, that are resistant to *L. pneumophila*-induced apoptosis. However, C57BL/6-derived macrophages undergo rapid pyroptosis in response to WT *L. pneumophila* infection, yet we still detect robust levels of injection in this cell type, suggesting that *L. pneumophila*-induced cell death is an insufficient explanation to account for the lack of detectable injection in DCs (57). Given that phagocytosis is required for T4SS-mediated translocation, another possibility is that dendritic cells in the lung do not efficiently phagocytose *L. pneumophila* (88). Alternatively, pulmonary dendritic cells and *L. pneumophila* may be spatially separated during *in vivo* infection.

Using the A549 alveolar epithelial cell line during *in vitro* infection, we detected a low percentage of T4SS-injected cells under conditions using both nonmotile and motile bacteria. Many researchers utilize A549 cells as a model for *L. pneumophila* infection and can detect productive bacterial replication within these cells (37). However, these studies either use higher MOIs than those used in this study or opsonize the bacteria prior to infection. These discrepancies in technique may explain why we are unable to detect higher percentages of injected alveolar epithelial cells during *in vitro* infection. We also were unable to detect robust T4SS-dependent translocation into airway epithelial cells or other nonphagocytic cells during *in vivo* infection. Utilizing a nonpermissive model of C57BL/6 mice infected with WT *L. pneumophila*, other researchers also found that lung epithelial cells did not appear to contain *L. pneumophila* (30). Our data argue against a direct role for airway epithelial cells in the cytosolic sensing of *L. pneumophila* T4SS activity during pulmonary infection. Airway epithelial cells have been shown to indirectly respond to *L. pneumophila* infection by producing the chemokine CXCL1 in response to IL-1 produced by macrophages (30).

We found that alveolar macrophages secreted TNF and IL-1α 4 h postinfection *in vivo*. Both TNF and IL-1α are important for controlling *L. pneumophila* infection. The inflammasome-regulated cytokines IL-1α and IL-1β are critical for neutrophil recruitment to the lung airway during *L. pneumophila* infection through a mechanism involving the IL-1R-dependent induction of CXCL1 from alveolar epithelial cells (20, 28, 30). It is unclear whether other cells in the lung also produce cytokines so early during infection. However, as IL-1α production *in vivo* is T4SS dependent and we could detect T4SS injection only into alveolar macrophages at 4 h postinfection, our data suggest that during the first few hours of infection, alveolar macrophages are the primary source of IL-1α, consistent with another study indicating that hematopoietic cells are an early source of IL-1α (20).

At 24 h postinfection, we found that neutrophils recruited to the lungs of mice infected with the Δ*flaA* mutant also secrete the proinflammatory cytokines TNF and IL-1α, but not mice infected with the Δ*dotA* mutant. Therefore, these data indicate that, like alveolar macrophages, neutrophils also secrete cytokines in response to cytosolic sensing of T4SS-translocated bacterial products. It has been reported previously that neutrophils can secrete cytokines, but the signaling pathways that control cytokine production and secretion in neutrophils are poorly understood. Neutrophils are known to release TNF-containing granules in response to a variety of stimuli, including various bacterial infections (89, 90). Previous research has demonstrated that neutrophils can release IL-1α in a model of sterile inflammation or IL-1β independently of caspase-1 and caspase-11 in a mouse model of arthritis and during bacterial infection (91–93). In an intravenous infection model of *L. pneumophila* infection, splenic neutrophils were shown to produce IL-18, an IL-1 family cytokine, which induces IFN-γ production from NK cells (94). Previous studies demonstrated that IL-1α secretion is regulated by both inflammasome-dependent and -independent pathways during *in vivo* WT *L. pneumophila* infection (20, 28), but it is unknown which of these pathways are used by macrophages and neutrophils to secrete IL-1α *in vivo*. It would be of interest to determine the host and bacterial components required for release of IL-1 and other cytokines from macrophages and neutrophils in response to *in vivo* infection with *L. pneumophila*.

Overall, our study is the first to define the cell types that receive T4SS-translocated effectors during pulmonary *L. pneumophila* infection. We reveal that both alveolar macrophages and neutrophils receive translocated effector proteins, harbor viable bacteria, and respond to infection by producing inflammatory cytokines. Collectively, our data indicate that alveolar macrophages and neutrophils provide not only an intracellular reservoir for *L. pneumophila* but also an important source of proinflammatory cytokines that contribute to a successful host immune response during pulmonary *L. pneumophila* infection.

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