Chapter

Biofilm-Derived *Legionella pneumophila* Evades Activation of the NLRC4 Inflammasome

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Abstract

Legionella pneumophila, a causative agent of Legionnaire's disease, replicates in human alveolar macrophages and establishes infection in humans. There is no human-to-human transmission and the main source of infection is L. pneumophila biofilms established in air conditioners, water fountains and hospital equipment. The biofilm structure provides protection for the organism from disinfectants and antibacterial agents. *L. pneumophila* infection in humans is characterized by a subtle initial immune response, giving time for the organism to establish infection before the patient succumbs to pneumonia. Planktonic *L. pneumophila* elicits a strong immune response in murine but not in human macrophages which typically control infection. Interactions between planktonic *L. pneumophila* and murine or hu-

man macrophages have been studied for years, yet the interface between biofilm-derived *L. pneumophila* and macrophages has not been explored. Here, we demonstrate that biofilm-derived *L. pneumophila* replicates significantly more in murine macrophages than planktonic bacteria. In contrast to planktonic L. pneumophila, biofilm-derived *L. pneumophila* downregulate flagellin expression, do not activate caspase-1 or 7 and trigger less pyroptosis. In addition, while planktonic *L. pneumophila* is promptly delivered to lysosomes for degradation in murine macrophages, biofilm-derived bacteria were enclosed in a vacuole that did not fuse with lysosomes. This study advances our understanding to the innate immune response to biofilm-derived *L. pneumophila* and closely reproduces the natural mode of infection in human.

Keywords

Bone Marrow Derived Macrophages; Human Monocyte Derived Macrophages; Lysosomes; Lactate Dehydrogenase; Legionella Pneumophila

Introduction

Legionella pneumophila (L. pneumophila) is a Gram negative aerobic bacterium with fastidious growth requirements. It is found as bacterial communities encased in extracellular polymeric matrices called biofilms [1]. Biofilms have been recognized as one of the most important key factors of survival and proliferation of *L. pneumophila* in warm, humid environments like showers, streams, whirlpools, air conditioners, cooling towers, fountains and spa baths [2-7]. These biofilms have been identified as a source of infection in susceptible hosts who inhale aerosols of contaminated water containing L. pneumophila. In the lung environment within alveolar macrophages, *L. pneumophila* replicate within phagosomes, and ultimately lyse the host macrophages and invade other macrophages causing a type of walking pneumonia called Legionnaire's disease or Legionellosis [8,9]. More than 20,000 cases of Legionellosis are reported yearly in the U.S with no person-to-person transmission [10].

The murine innate immune response to planktonic *L. pneumophila* has been studied extensively. In this model, the NOD like receptors (NLRs) are mediators in sensing the presence of microbes or their factors in the host cytosol which, in turn, initiates signaling cascades that mediate the production of inflammatory cytokines, recruitment of phagocytic cells and direction of the innate and acquired immune responses. One of the major NLRs contributing to the restriction of *L. pneumophila* infection in murine macrophages is NLRC4 [11]. NLRC4 detects flagella monomers in a process that is dependent upon bacterial type IV secretion system. Then, activation of caspase-7 downstream of caspase-1 results in fusion of *L. pneumophila* containing vacuoles with lysosomes and bacterial restriction [12-14]. Conversely, human monocytes do not activate this response upon *L. pneumophila* infection, and phagosomes containing *L. pneumophila* evade fusion with lysosomes resulting in replication of this bacterium within ER-like phagosomes [15,16]. Collectively, these observations lead us to hypothesize that perhaps the murine macrophage response to biofilm-derived *L. pneumophila* might more faithfully reproduce the response of human macrophages to this pathogen than do planktonically derived bacteria.

In this study we demonstrate that biofilm-derived *L. pneumophila* replicates significantly more than planktonic bacteria in murine macrophages due to lack of flagellin expression. Biofilm-derived *L. pneumophila* did not cleave caspase-1 or caspase-7, evaded fusion with lysosomes and induced less cell death. To this end, our study is the first to characterize the innate immune response to biofilm-derived L. pneumophila. Thus, using biofilm-derived *L. pneumophila* to study the innate immune response to infection recapitulates natural infection in human.

Methods

Bacterial Strains

L. pneumophila strain JR32 a wild-type strain; the *dotA* mutant, a JR32-derived strain defective in the Dot/Icm Type IV secretion sys-

tem; *flaA* mutant is deficient in flagellin, were kindly provided by Dr. Howard Shuman, University of Chicago. *L. pneumophila* expressing Green Fluorescent Protein (GFP) was used for microscopy. *L. pneumophila* strains were grown as previously described.

L. pneumophila Growth

L. pneumophila strains were grown on buffered charcoal yeast extract (BCYE) plates at 37°C. Three days later, the bacteria were resuspended into 5 ml of *L. pneumophila* broth with additives (ferric nitrate, L-cysteine, thymidine) and vortexed 100X at high speed. For biofilm formation, a bacterial suspension with an optical density (OD) at 600nm of 3.5 was diluted to 1:2500 in supplemented broth and 200 µl of this suspension was inoculated into each well of an 8-well chamber slide (Thermo Scientific Lab Tek chambered coverglass with cover #155411 and/or #177402). Slides were incubated at 37°C, 5% CO_2 incubator with humidified atmosphere, static. Biofilms were fed by delivery of fresh medium to one side of the chamber slide well, very slowly to prevent disruption of the biofilm, every 24 hours for 6 days using 100 µl of *L. pneumophila* medium. *L. pneumophila* was grown for planktonic culture as previously described [11].

Murine Macrophage Infection

WT mice were purchased from Jackson laboratory. C57BL/6 BMDMs were prepared from the femurs of five to eight-week-old mice as previously described [13,17]. Isolation and preparation of the human monocyte-derived macrophages (hMDMs) from peripheral blood was carried out as previously described [18,19]. Planktonic infections were from the post-exponential culture as previously described [11,13]. Infection from biofilm derived bacteria was carried as below. Briefly, on day 7 the medium was aspirated and transferred to a 50 mL tube and biofilms were scraped from the chamber slide wells. Chambers were washed 2X with 200 µl of fresh LP medium-containing L-cystiene, ferric nitrate and thymidine and drained into the 50 ml tube. Collected biofilms were vortexed 100x then the OD at 600nm of the collected suspension was used to calculate the desired

multiplicity of infection. Equivalent inocula to planktonic bacteria were used for infection.

Confocal Laser Scanning Microscope Visualization

On day seven, biofilms were washed gently with 200µl of sterile saline (0.9% sodium chloride) (Hospira 0409-4888-10), then stained with Live/Dead BacLight Bacterial Viability Kit (Invitrogen #7007) for 15 min at room temperature protected from the light. Wells were washed 2x with sterile saline then 200µl of 10% formalin was added for 24 hrs to fix the biofilm and kept at room temperature protected from the light with before it was visualized using inverted confocal Zeiss LSM 510 META microscope with a 63X water objective. Z-stacks were captured every 1 µm.

Enzyme-Linked Immunosorbent Assay (ELISA)

L. pneumophila JR32 and *dotA* strains from exponential and biofilm cultures were used to infect murine macrophages for 24 hours. Supernatants were collected and centrifuged at 1200 RPM for 10 min and kept at -80°C. Ninety six well plates were coated with the capture antibody overnight. The next day, plates were washed with ELISA wash buffer, blocked with bovine serum albumin 1% (BSA) then incubated with the standards and samples for 2 hrs. Plates were washed 3x, and incubated with the secondary antibody for 1hr. Subsequently HRP was supplied to each well, stopped with 1N HCl. The absorbance was measured at 450nm.

Western Blot

Macrophage extracts were prepared following infection with either planktonic or biofilm JR32 or *dotA* mutant and immunoblotted with caspase-1, caspase-7, or β-actin antibodies (caspase-1 1:3000, caspase-7 1:300). Blots were washed and the corresponding secondary antibody was added. For flagellin Western blot, one OD were pelleted and resuspended in SDS-containing sample buffer from planktonic or biofilm grown bacteria. Eighteen µl were loaded on 12% SDS-PAGE

gel. Blot was probed with α-*flaA* (1:100) followed by the secondary antibody, donkey anti-rabbit (1:5000). Films were washed 10X and developed after adding the ECL Western Blotting Detection Reagent (GE Healthcare Amersham).

Macrophage Cytotoxicity Assay

Percentage of macrophage necrosis was determined by measuring the release of host cell cytoplasmic lactate dehydrogenase (LDH) using the cytotoxicity detection kit (Roche Applied Science) to the specification of the manufacturer. BMDMs were infected with JR32 or the *dotA* mutant from either planktonic or biofilm culture for 4 or 24 hours at an MOI of 0.5. Supernatants were collected, LDH reagent was added, the plate was incubated and the reaction was stopped. The OD was read at 490 and 630nm and the cytotoxicity percentage was calculated according to this equation: % cytotoxicity = [exp value-low control/high control-low control] x 100.

L. pneumophila Colocalization with Lysotracker

L. pneumophila JR32 from exponential or biofilm culture were used to infect macrophages plated in 24 well plate containing sterilized coverslips. Lysotracker red (1:500) was added to each well, incubated and fixed. 4', 6-diamidino-2-phenylindole (DAPI) was added diluted at 1:5000. Coverslips were mounted on slides and viewed with Olympus Flow View FV10i CLSM. Three hundred bacteria were counted from 2 coverslips.

AContact-Dependent Hemolysis

Sheep RBCs (sRBCs) were diluted in RPMI, and washed three times by centrifugation for 10 min at 2000 x g until the supernatant did not show any signs of hemolysis; the cells were counted on hemocytometer chamber. Reactions were set up in a final volume of 1 ml with final concentrations of 1x 10^7 sRBCs/ 1ml. The sRBCs were incubated with the planktonic or biofilm bacteria at an MOI of 20 and incubated for 3hrs at 37°C as previously described [20, 21].

Statistical Analysis

All experiments were performed at least 3 independent times and yielded similar results. In some instances, experiments were done one time in triplicates or quadruplicates and yielded similar results. Comparisons of groups for statistical difference were done using Student's two tailed t-test. P value ≤ 0.05 was considered significant.

Results

Dot/Icm type IV Secretion System was required for *L. pneumophila* Biofilm Formation

To reproduce biofilm formation *in vitro*, wild-type *L. pneumophila* (JR32) and *dotA* mutant were grown for 7 days at 37°C in 8 well chambered coverslips and fed with *L. pneumophila* media every 24 h. On the seventh day, biofilms were stained with Live/Dead stain then observed by confocal microscopy. *L. pneumophila* JR32 produced a thick biofilm with a maximum height of ~120 μm and exhibited filamentous structures while the *dotA* mutant which lacks a functional type IV secretion system produced a thinner and less filamentous biofilm (Figure 1A). The depths of these biofilms were 120 μm and 60 μm respectively.

The pore forming activity of *L. pneumophila* has been shown to contribute to cytotoxicity [21] and the ability of the organism to egress from the host cell after cessation of intracellular replication. To examine if biofilm derived *L. pneumophila* exhibited a functional type IV secretion system, contact-dependent hemolysis of RBCs was performed to examine pore-forming activity as previously described [21]. Triton-x100, heat killed bacteria were used as positive and negative control respectively. The *flaA* mutant lacks the flagellin but exhibits functional type IV secretion system. Our data demonstrated that both JR32 planktonic and biofilm-derived bacteria were proficient in lysing the RBCs, suggesting that biofilm-derived *L. pneumophila* exhibit a functional type IV secretion system (Figure 1B).

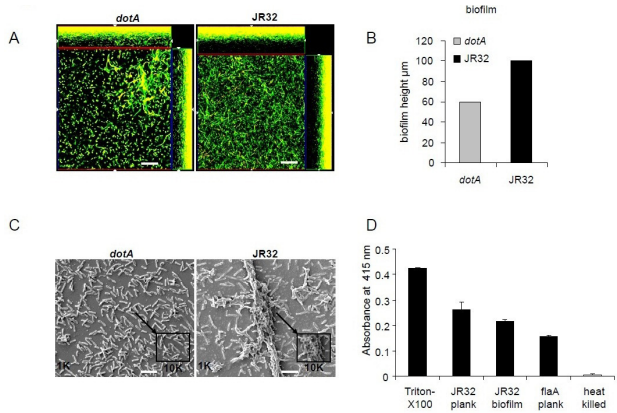

Figure 1: Dot/Icm type IV secretion system is required for L. pneumophila biofilm formation.
(A) Representative images showing the Live/Dead staining of WT L. pneumophila biofilm (top) or the type IV secretion mutant (*dotA*). Images were captured using inverted confocal Zeiss LSM 510 META microscope with a 63X water objective. Z-stacks were captured every 1 µm. Red color indicates dead bacteria while green indicates live ones. (B) Pore-forming activity of L. pneumophila as determined by contact-dependent hemolysis of sRBC and measured at A415. Y axis denotes the absorbance of hemoglobin at 415 nm. Data are presented as means ± S.D of two independent experiments. No significant difference in sRBCs lysis is observed between planktonic and biofilm derived L. pneumophila.

In Contrast to Human Macrophages, Biofilm-Derived *L. pneumophila* Replicated significantly more and Induced Less Murine Macrophages than the Planktonic Bacteria

Murine macrophages are restrictive to planktonic *L. pneumophila* replication. However, the murine macrophages response to biofilm derived *L. pneumophila* is not known. We therefore examined the intracellular replication of biofilm derived L. pneumophila. In contrast to the restriction of planktonic bacteria in WT macrophages, the biofilm derived bacteria replicated significantly more as indicated by the colony forming units (CFUs) over time (48-96 hr) (Figure 2A).

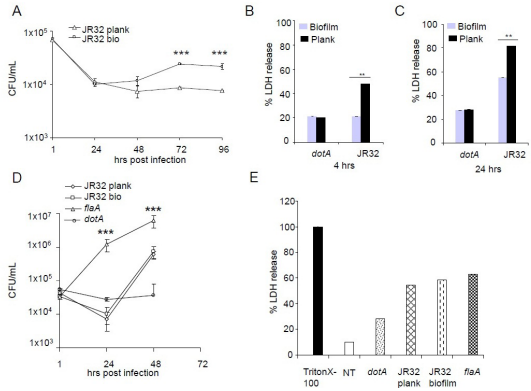

Figure 2: In contrast to human macrophages, biofilm-derived L. pneumophila replicated significantly more and induced less murine macrophages than the planktonic bacteria.
(A) BMDMs were infected with planktonic or biofilm-derived L. pneumophila at an MOI of 0.5. CFUs were scored at 1, 24, 48, 72 and 96 hr. Data are presented as mean ± S.D of two independent experiments. Asterisks indicate significant differences (***P<0.001). BMDMs were not infected (NT) or infected with *L. pneumophila* JR32 (planktonic or biofilm) or the *dotA* mutant at an MOI of 0.5 for (B) 4 or (C) 24 hr. The fold change in LDH release was measured from the overall population of macrophages. Data are presented as means ± S.D of two independent experiments. Asterisks indicate significant differences (**P<0.01).
(D) The hMDMs were infected with *L. pneumophila* strain JR32 (planktonic or biofilm). CFUs were quantified at 1, 24 and 48 hr post infection. Data are representative of one experiment and presented as means ± S.D of quintuplicate samples. No significant difference was detected. (E) The hMDMs were not infected (NT) or infected with *L. pneumophila* JR32 (planktonic or biofilm), *dotA* or *flaA* mutant at an MOI of 0.5 for 4 or 24 hr. The fold change in LDH release was measured from the overall population of macrophages. Data are representative of one experiment and presented as mean ± S.D of triplicate samples. No significant change was observed between the JR32 or legA9 mutant.

Caspase-1 mediates pyroptotic cell death thereby restricting *L. pneumophila* replication inside murine macrophages [13,17]. Macrophage death can be detected by measuring the cytoplasmic enzyme activity released by dead cells using LDH cytotoxicity assay. Our data demonstrated a significant decrease in LDH release at 4 and 24 hrs

post infection of biofilm-derived L. pneumophila, respectively, when compared to planktonic bacteria (Figure 2B & C). These data suggested that biofilm-derived *L. pneumophila* induced less cell death in murine macrophages than did planktonic-derived L. pneumophila.

Human are permissive to *L. pneumophila* replication as a result of lack of caspase-1 and 7 activation and downregulation of the NLRC4 inflammasome members [22, 23]. We evaluated the intracellular growth of planktonic and biofilm derived *L. pneumophila* in hMDMs. The biofilm derived *L. pneumophila* did not show significant differences in replication within hMDMs when compared to the planktonic one (Figure 2D). Furthermore, we tested macrophage death by measuring % LDH released after 24 hr of infection. Our data did not demonstrate a significant difference in LDH release at 24 hrs post infection of biofilm-derived *L. pneumophila* respectively (Figure 2E). These data suggest that biofilm-derived *L. pneumophila* behave very similar to planktonic *L. pneumophila* in hMDMs. Tritonx-100 treatments induced maximum LDH release, while un-treated (NT) were used as a negative control. The *dotA* mutant lacks the type IV secretion system and induced less LDH release. The *flaA* mutant lacks flagellin and release LDH.

Biofilm-derived *L. pneumophila* did not promote caspase-1 or 7 Activation in Murine Macrophages and showed Significantly less IL-1β release as a result of lack of Flagellin Expression

Wild type murine macrophages restrict planktonic *L. pneumophila* replication via caspase-1 activation leading to bacterial clearance [13,17]. Therefore, we tested caspase-1 activation in response to biofilm-derived and planktonic L. pneumophila. The type IV secretion mutant (*dotA*) and *flaA* were used as a negative and positive control respectively. In contrast to planktonic bacteria, biofilm-derived *L. pneumophila* did not promote caspase-1 cleavage (Figure 3A). These data indicates a different murine response to biofilm-derived *L. pneumophila* than the planktonic one.

IL-1β maturation is promoted by caspase-1 in wild type macrophages infected with planktonic L. pneumophila. Therefore, we tested IL-1β release in the supernatants of macrophages infected with either planktonic or biofilm-derived *L. pneumophila* strain JR32 or the *dotA* mutant. Our data showed that murine macrophages infected with biofilm-derived bacteria released 30% less IL-1β than planktonic L. pneumophila-infected macrophages (Figure 3B). This result indicated that the inflammatory response to biofilm-derived *L. pneumophila* in murine macrophages is less than the response elicited by planktonic bacteria.

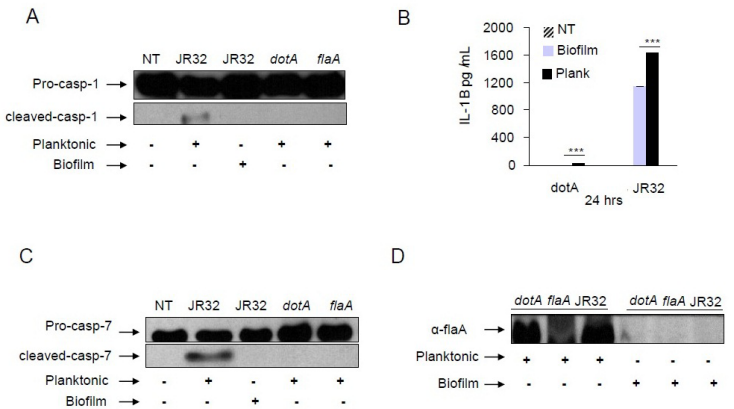

Figure 3: Biofilm-derived *L. pneumophila* did not promote caspase-1 or 7 activation in murine macrophages and showed significantly less IL-1β release as a result of lack of flagellin expression.
WT BMDMs were either not treated (NT) or infected with *L. pneumophila* JR32 (biofilm or planktonic), the *dotA* or the flagellin mutant for 2 hr. (B) level of IL-1β was detected in supernatants of WT infected with JR32 (biofilm or planktonic) or the *dotA* mutant after 24 hr. Data are presented as means ± S.D of one experiment performed in quadruplicate. Asterisks indicate significant differences (***P<0.001). (C) Activation of casp-7 was detected in cell extracts using casp-7 antibody. (D) Western blot with α-flaA antibody, planktonic and biofilm derived *L. pneumophila* were grown as indicated in materials and methods.

Caspase-7 is cleaved by caspase-1 in murine macrophages as a response to planktonic *L. pneumophila* infection and leads to bacterial restriction [13,17]. Therefore, we tested caspase-7 activation of murine macrophages in response to biofilm-derived and planktonic L. pneumophila. In contrast to planktonic bacteria, biofilm-derived *L. pneumophila* did not promote caspase-7 cleavage (Figure 3C). This result indicates that biofilm-derived bacteria do not elicit caspase-7 activation thereby allowing them to evade a restrictive mechanism employed by macrophages. Flagellin mediates restriction of *L. pneumophila* in murine macrophages and *flaA* mutant bacteria replicate significantly more than the parent strain [11]. Since biofilm-derived bacteria replicated significantly in murine macrophages and failed to activate caspase-1 or 7, we reasoned that biofilm derived bacteria might downregulate flagellin expression. Western blot analysis of bacterial lysate showed that biofilm derived bacteria lacks flagellin expression compared to planktonic bacteria (Figure 3D).

Phagosomes containing Biofilm-derived *L. pneumophila* were Defective in fusion with Lysosomes

L. pneumophila replication is restricted by caspase-1 and 7 activation that lead to phagosome lysosome fusion which correlates with bacterial degradation [13, 17]. We examined the colocalization of planktonic or biofilm-derived bacteria with lysosomes 1 hr post-infection. Approximately 55% of biofilm-derived *L. pneumophila* resided in lysosomes (Figure 4A & B). Yet, by comparison, 72% of planktonic *L. pneumophila* resided in lysosomes. These results suggested that more biofilm-derived *L. pneumophila* evades lysosomal degradation in macrophages.

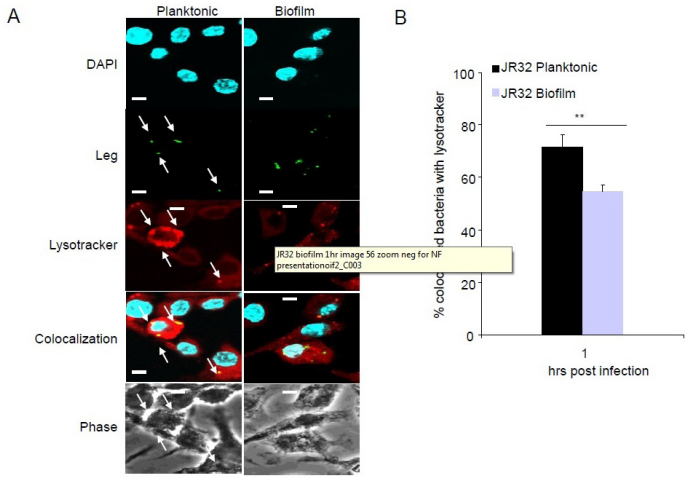

Figure 4: Vacuoles harboring biofilm derived *L. pneumophila* bacteria significantly evade fusion with lysosomes.
(A) Representative images of WT BMDMs infected for 1hr with JR32 planktonic or biofilm bacteria. Nuclei stained blue with DAPI and *L. pneumophila* stained green with L. pneumophila-specific antibody. Lyso-tracker red was used to stain acidified lysosomes. White arrows show *L. pneumophila* colocalization with lysotracker. (B) Percent colocalization of *L. pneumophila* with lysotracker. Images were captured with the 60X objective and magnified 3x, scale bar = 10μm. Data are presented as means ± S.D of three independent experiments. Asterisks indicate significant differences (**P<0.01).

Discussion

Biofilm formation is essential to sustain the survival of *L. pneumophila* in the environment. Eradication of *L. pneumophila* from these settings has been proven difficult as - bacteria within a biofilm exclude most antibacterial agents. Furthermore, most Legionnaire's disease outbreaks have been linked to inhalation of contaminated aerosol from water systems or air conditioners harboring *L. pneumophila* biofilm.

Although innate immune response to *L. pneumophila* from planktonic culture is well studied, the macrophage response to biofilm-derived bacteria is yet to be elucidated. In this study, we examined murine macrophage response to biofilm-derived L. pneumophila. Our data shows that biofilm derived *L. pneumophila* replicates significantly more in murine macrophages than do planktonic bacteria. Unlike planktonic L. pneumophila, caspase-1 and -7 are not activated in response to biofilm-derived L. pneumophila. Caspase-1 and -7 activation modulate the fusion of vacuoles containing *L. pneumophila* with lysosomes. Thus, biofilm derived *L. pneumophila* evades phagosome lysosome fusion by avoiding caspase-1 and 7 activation. Macrophages infected with biofilm-derived bacteria showed significantly less IL-1β release and macrophage death than the ones infected with planktonic bacteria. IL-1β is a pro-inflammatory cytokine activated by caspase-1. Thus, by reducing IL-1β production, biofilm-derived *L. pneumophila* avoid major inflammatory reactions and recruitment of inflammatory cells. We showed that biofilm-derived *L. pneumophila* elicits weaker immune response than planktonic bacteria because it lacks flagellin expression. A better understanding of the innate immune response to biofilm-derived *L. pneumophila* will pave the way for the development of novel diagnosis and treatment strategies.

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