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Mouse Macrophages Are Permissive to Motile *Legionella* Species That Fail To Trigger Pyroptosis▽

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***Legionella pneumophila*, a motile opportunistic pathogen of humans, is restricted from replicating in the lungs of C57BL/6 mice. Resistance of mouse macrophages to *L. pneumophila* depends on recognition of cytosolic flagellin. Once detected by the NOD-like receptors Naip5 and Ipaf (Nlrc4), flagellin triggers pyroptosis, a proinflammatory cell death. In contrast, motile strains of *L. parisiensis* and *L. tucsonensis* replicate profusely within C57BL/6 macrophages, similar to flagellin-deficient *L. pneumophila*. To gain insight into how motile species escape innate defense mechanisms of mice, we compared their impacts on macrophages. *L. parisiensis* and *L. tucsonensis* do not induce proinflammatory cell death, as measured by lactate dehydrogenase (LDH) release and interleukin-1β (IL-1β) secretion. However, flagellin isolated from *L. parisiensis* and *L. tucsonensis* triggers cell death and IL-1β secretion when transfected into the cytosol of macrophages. Neither strain displays three characteristics of the canonical *L. pneumophila* Dot/Icm type IV secretion system: sodium sensitivity, LAMP-1 evasion, and pore formation. Therefore, we postulate that when *L. parisiensis* and *L. tucsonensis* invade a mouse macrophage, flagellin is confined to the phagosome, protecting the bacteria from recognition by the cytosolic surveillance system and allowing *Legionella* to replicate. Despite their superior capacity to multiply in mouse macrophages, *L. parisiensis* and *L. tucsonensis* have been associated with only two cases of disease, both in renal transplant patients. These results point to the complexity of disease, a product of the pathogenic potential of the microbe, as defined in the laboratory, and the capacity of the host to mount a measured defense.**

Legionella pneumophila is a Gram-negative bacterium that opportunistically infects alveolar macrophages of the mammalian lung. Although 50 species of *Legionella*, comprising more than 70 serogroups, have been identified, only a subset have been associated with disease (39). The most prevalent cause of disease are serogroup 1 strains of *Legionella pneumophila*, which account for >75% of cases around the world. Non-*L. pneumophila* species contribute 5 to 10% of the disease burden, primarily in immunocompromised hosts. For example, the non-*L. pneumophila* species *L. parisiensis* and *L. tucsonensis* were each isolated from two renal transplant patients receiving immunosuppressive therapy (38, 69). Nevertheless, *L. parisiensis* strains can replicate in the human monocytic U937 cell line, in primary macrophages from C57BL/6 mice or guinea pigs, and in *Acanthamoeba castellanii* (3, 31, 33, 52). Whether these species use similar strategies to those of *L. pneumophila* to establish a replication niche or to evade the innate immune system is not known, since neither *L. parisiensis* nor *L. tucsonensis* has been studied in detail.

Macrophages are key phagocytic defenders of the innate immune system that scout tissues for foreign materials, including pathogens. An important component of their surveillance are pattern recognition receptors that reside on the macrophage surface or within its cytoplasm. The Toll-like receptors (TLRs), present on the surfaces of many cell types, recognize microbe-associated molecular patterns (MAMPs), such as lipopolysaccharide, peptidoglycan, lipoproteins, microbial nucleic acids, and flagellin (2, 36). Much like the TLRs in function, the nucleotide-binding oligomerization domain (NOD-like) receptors (NLRs) monitor the cytoplasm (2, 78). Detection of microbial products by these receptors initiates a signaling cascade that culminates with the secretion of proinflammatory mediators that recruit other lymphocytes to respond to an infection (78).

NLRs participate as components of a protein complex known as the inflammasome. Several different inflammasomes have been characterized based on the MAMPs that initiate their formation and activation. For example, Nalp1, Nalp3, and the more extensively studied NOD proteins each interact with muramyl dipeptides of peptidoglycan (2, 78). *N*LR family *a*poptosis *i*nhibitory *p*rotein 5 (Naip5; also called Birc1e), which restricts replication of *L. pneumophila* in mouse macrophages (21, 23, 80), is composed of three domains: (i) an amino-terminal baculoviral inhibitor of apoptosis repeats, (ii) a central NOD domain, and (iii) carboxy-terminal leucine-rich repeats (LRRs) (32). Studies of other NLR proteins indicate that the LRR region is critical for recognition of microbial products, which then triggers oligomerization of the NLR with other inflammasome components through the NOD domain. Subsequently, activation of downstream signaling is carried out by the amino-terminal domains, such as the caspase recruitment domains (32). Unlike the NODs, the Nalps and Naip5 control posttranslational processing and secretion of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 (43). Once formed, the inflammasome recruits and activates caspase-1, which in turn processes pro-IL-1β and pro-IL-18 into their

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mature forms before their release into the extracellular space. Recruitment and activation of caspase-1 are mediated directly or through adapter proteins, such as ASC and Ipaf (1, 85). This proinflammatory reaction is a key element of pyroptosis, the "fiery" cell death seen in response to infection by the pathogens *Salmonella enterica*, *Shigella flexneri*, and *L. pneumophila* (11).

L. pneumophila serogroup 1 has become a model intracellular organism to study both bacterial replication and host detection of pathogens (64). The pathogen requires a type IV secretion system to establish a replication vacuole in human and mouse macrophages, a key determinant of infection (29, 42, 74). However, mice can restrict *L. pneumophila* infection when the NLR proteins Naip5 and Ipaf detect flagellin (14, 20, 33, 49, 57, 81, 85). While C57BL/6 mice do not support *L. pneumophila* replication beyond the first 24 h (83), this bacterium replicates profusely in macrophages derived from the A/J mouse strain (82). The A/J *Naip5* allele is hypofunctional, based on experiments using transgenic complementation, RNA knockdown, and *Naip5*$^{-/-}$ mutant mice (21, 37, 80). A/J and *Naip5* null mutant mice are defective for inflammasome activation during *L. pneumophila* infection (37, 49, 57). Likewise, *L. pneumophila* mutants that lack either flagellin (*flaA*) or type IV secretion (encoded by multiple *dot*/*icm* genes) do not stimulate pyroptosis. Whether components of the inflammasome directly bind flagellin is not yet known, but *flaA* mutant *L. pneumophila* subverts Naip5-mediated defenses and replicates in C57BL/6 macrophages (49, 57) and human macrophages (73).

Unlike *L. pneumophila* serogroup 1, many other species of *Legionella* do replicate in C57BL/6 macrophages. These include strains of *L. micdadei*, *L. bozemanii*, *L. dumoffii*, *L. feeleii*, *L. longbeachae*, *L. birminghamensis*, *L. maceachernii*, *L. sainthelensi*, and *L. parisiensis*, as well as three non-serogroup 1 strains of *L. pneumophila* (7, 33, 48). With the exception of *L. longbeachae* (6, 7), the expression of flagellin and type IV secretion by these strains has not been reported. Therefore, we exploited two non-*L. pneumophila* strains to test the model that mouse macrophages rely on cytosolic flagellin to restrict growth of intracellular *Legionella*. We show that two motile strains of *L. parisiensis* and *L. tucsonensis* evade replication restriction by C5BL/6 murine macrophages, despite the host cells' ability to detect their divergent bacterial flagellin when it contaminates the cytosol. Mouse macrophage restriction of replication correlates with three activities of the canonical *L. pneumophila* Dot/Icm type IV secretion system, namely, sodium sensitivity, late endosome and lysosome evasion, and phagosome perforation.

MATERIALS AND METHODS

Bacterial strains and culture. *L. pneumophila* Lp02 (*thyA hsdR rpsL*), derived from the Philadelphia 1 strain, the *dotA* mutant (Lp03), and the *flaA* mutant have been described previously (10, 50). *L. parisiensis* and *L. tucsonensis* were gifts from Cary Engleberg (University of Michigan) that were obtained from the Centers for Disease Control and Prevention (59). Species identity was confirmed by *mip* sequencing (55), using the forward primer Legmip_f [5′-GGG(AG)ATT(ACG)TTTATGAAGATG A(AG)A(CT)TGG-3′], the reverse primer Legmip_r [5′-TC(AG)TT(ATCG)GG(AT G)CC(ATG)AT(ATCG)GG(ATCG)CC(ATG)CC-3′] (where parentheses indicate a mixed-base site), and the *Legionella mip* sequence database (http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/1195733805138). Additional species confirmation was obtained by blue-white fluorescence (72), which was observed when *L. parisiensis* and *L. tucsonensis*, but not *L. pneumophila*, were exposed to a handheld UV light.

Strains maintained at −80°C in glycerol stocks were colony purified onto *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES; Sigma)-buffered charcoal-yeast extract agar (CYE) supplemented with 100 μg/ml of thymidine (CYET). Bacterial strains were cultured in ACES-buffered yeast extract broth (AYE) supplemented with thymidine (100 μg/ml) (AYET) at 37°C with aeration. *Legionella* organisms were subcultured in AYET from an overnight primary culture and grown to the exponential and postexponential phases for experimentation. Exponential-phase cultures (E; replicative) were defined as those having an optical density at 600 nm (OD_{600}) of 0.5 to 2.0, while post-exponential-phase (PE; transmissive) cultures were defined as those having an OD_{600} of 3.0 to 4.0, with high motility. Cultures were defined as motile when >75% of bacteria in a field of ≥100 cells showed rapid, directed movement when wet mount preparations were observed by phase-contrast microscopy.

Macrophage culture. Bone marrow-derived macrophages were isolated from the femurs of female C57BL/6 and A/J mice (Jackson Laboratories) and *Naip*$^{-/-}$ *Ipaf*$^{-/-}$ mutant mice (generous gift of Russell Vance) as previously described (67). Macrophages were maintained in RPMI supplemented with 10% fetal bovine serum (RPMI-FBS; Gibco) and were plated at the density indicated for each assay.

Intracellular bacterial growth. The efficiencies of binding, entering, and surviving within macrophages of *L. pneumophila* were assessed as described previously (13). C57BL/6 macrophages were plated in 24-well tissue culture plates at a density of 2.5×10^5 macrophages per well. Cells were allowed to adhere overnight before incubation with bacteria and were infected at a 1:1 ratio with transmissive bacteria for 2 h at 37°C. Extracellular bacteria were removed by rinsing the macrophage monolayer three times with 0.5 ml of RPMI-FBS, a medium that is not permissive for *Legionella* replication, at 37°C. Intracellular bacteria were quantified by lysing monolayers with 2% saponin (Sigma) in phosphate-buffered saline (PBS) and plating triplicate aliquots on CYET. The CFU added at 0 h was determined by diluting the infection inoculum with PBS and plating cells on CYET. PBS did not affect the viability of the *Legionella* isolates (data not shown). The initiation of infection was calculated from triplicate samples by the following equation: (CFU from lysates at 2 h)/(CFU added at 0 h) × 100.

To quantify replication of bacteria in macrophages at 24-h intervals, cells were infected at a multiplicity of infection (MOI) of 1 as described above. At the indicated times, cells were lysed by treating monolayers with 2% saponin (Sigma) in PBS. Lysates were prepared from triplicate samples and plated on CYET for CFU enumeration.

Cytotoxicity. Contact-dependent cytotoxicity was quantified as the percentage of macrophages killed during a 1-h incubation with *L. pneumophila*. C57BL/6 mouse macrophages were cultured at a density of 8×10^4 per well in 96-well tissue culture plates. Transmissive bacteria suspended in RPMI-FBS at various ratios were coincubated with the macrophages for 1 h at 37°C. After bacteria were washed away, the monolayers were subsequently incubated with 10% (vol/vol) Alamar Blue (Trek Diagnostics) in RPMI-FBS for 4 h to overnight. The redox-specific absorbance resulting from the reduction of Alamar Blue to its reduced form by viable macrophages was measured with a SpectraMax 250 spectrophotometer (Molecular Devices) to determine the OD_{570} and OD_{600}. The percentage of viable macrophages was calculated in triplicate from the standard curve, with the slope of the plot of A_{570}/A_{600} determined for triplicate samples of six known densities of uninfected macrophages in the range of 10^3 to 8×10^4 macrophages per well. The actual MOI was determined by plating duplicate samples of the infection inocula onto CYET.

IL-1β secretion. C57BL/6 macrophages were seeded in 24-well plates at a density of 1×10^6 and were either left untreated or pretreated overnight with 50 ng/ml of lipopolysaccharide (LPS) (Invivogen). Prior to transfection, cells were washed with serum-free medium and then infected with bacteria at an MOI of 5 or transfected with Profect P1 and crude flagellin preparation (CFP) complexes. Contact of the bacteria was promoted by centrifuging the plates at 250 × *g* for 5 min. After a 2-h incubation, the concentration of IL-1β in the supernatants was determined by enzyme-linked immunosorbent assay (ELISA) (eBioscience).

CFPs. CFPs were obtained from wild-type (WT) and *flaA* mutant *L. pneumophila*, *L. parisiensis*, and *L. tucsonensis* essentially as described previously (50). Broth cultures were centrifuged at 8,000 × *g* for 20 min at 4°C to collect the bacteria. Supernatants were discarded, and the bacterial pellets were resuspended in 50 ml of sterile PBS. To shear flagella from the bacteria, suspensions were vortexed at high speed for 5 to 10 min and then centrifuged as before to remove bacteria from the suspension. To remove any remaining bacteria, supernatants were collected and filtered through a 0.45-μm filter. Filtered supernatants, containing flagellin, were ultracentrifuged at 100,000 × *g* for 3 h at 4°C,

and the resulting supernatants were discarded. The pellet was resuspended in 1 ml sterile PBS and analyzed by SDS-PAGE and Coomassie blue staining. Protein concentration was determined by the Bradford assay (Pierce). To disassemble flagella and denature the flagellin protein, the CFPs were incubated at 70°C for 15 min. To degrade the flagellin, CFPs were incubated with 1 μg/ml proteinase K (Sigma) for 1 h at 37°C. Proteinase K was then inactivated by incubation at 75°C for 15 min.

Immunoblotting. To evaluate flagellin production, CFPs were boiled for 5 min in Laemmli buffer and resolved by SDS-PAGE, and then the samples were either stained with Coomassie blue or transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in BLOTTO (Tris-buffered saline with 0.05% Tween 20 [TBST] containing 5% nonfat milk) and incubated at 4°C overnight with monoclonal antibody 2A5, specific to *L. pneumophila* flagellin (50), diluted 1:100 in BLOTTO. Membranes were then washed 5 times in TBST and incubated with secondary goat anti-mouse conjugated to horseradish peroxidase (Pierce), diluted 1:10,000 in BLOTTO, for 1 h at room temperature (RT) with shaking. Membranes were washed as before and developed using the West Pico enhanced chemiluminescence (ECL) system (Pierce).

DNA hybridization. Genomic DNA from each *Legionella* strain was transferred to a positively charged nylon membrane by use of a Bio-Dot SF (Bio-Rad) apparatus. Flagellin-specific probes were labeled with digoxigenin-dUTP (DIG-dUTP) (Roche) by PCR amplification and detected by nonradioactive CSPD [disodium 3-(4-methoxyspiro-{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1.]decan}-4-yl) phenylphosphate; Roche] chemiluminescence detection. Hybridization was performed under low-stringency conditions at 42°C. Films were analyzed using Image J software (NIH).

Toxicity of cytosolic flagellin. C57BL/6 macrophages were plated in 96-well plates at densities of 5×10^4 and 8×10^4. The toxicity of cytosolic flagellin was analyzed by incubating the protein transfection reagent Profect P1 (Targeting Systems) with either native, heat-treated, or proteinase K-treated CFP at the concentration indicated. Suspensions were centrifuged onto macrophages and incubated at 37°C for 2 h. After incubation, the supernatants were assayed for the cytosolic enzyme lactate dehydrogenase (LDH), using the CytoTox96 nonradioactive cytotoxicity assay (35). Profect P1 without flagellin preparations or with proteinase K served as a background control.

Sodium sensitivity. Bacterial cultures were grown to the phase indicated and plated in serial dilutions on CYET alone or CYET containing 100 mM NaCl. After incubation at 37°C for 4 days, CFU were counted and sodium sensitivity expressed as follows: [(CFU in CYET+NaCl)/(CFU in CYET)] × 100.

Immunofluorescence microscopy. The interaction between *L. pneumophila* and late endosomes and lysosomes was assessed by studying colocalization of the *L. pneumophila* phagosome with LAMP-1 (17). A total of 2.0×10^5 macrophages per 12-mm glass coverslip were exposed to PE-phase *Legionella* at an MOI of 0.5 in RPMI-FBS, centrifuged at 200 × *g* for 5 min at RT, and then incubated at 37°C for 1.5 h in RPMI-FBS containing 100 μg/ml thymidine. After two rinses with 37°C RPMI-FBS, cultures were fixed with 37°C periodate-lysine-paraformaldehyde (44) containing 4.5% sucrose for 30 min at RT. LAMP-1 was stained by rat monoclonal antibody 1D4B at a dilution of 1:500 followed by Oregon Green-conjugated anti-rat serum (Molecular Probes) diluted 1:2,000, bacteria were labeled using an *L. pneumophila*-specific antibody (a kind gift of Ralph Isberg [10]) diluted 1:2,000 followed by Texas Red-conjugated anti-rabbit serum (Molecular Probes) diluted 1:2,000, and DNA was labeled with 0.1 μM 4′,6-diamidino-2-phenylindole (DAPI) in PBS. Samples were analyzed with a Zeiss Axioplan 2 epifluorescence microscope equipped with a 100× Plan-Neofluor objective with a numerical aperture of 1.3. At least 100 vacuoles containing *L. pneumophila* were scored in each of three experiments.

EtdBr permeability. To minimize the potential contribution of caspase-1-dependent pore formation, the coculture period was limited to 1 h and *Naip5* mutant macrophages were obtained from A/J mice (22, 49). Bacteria were cultured to the PE phase until they were highly motile. Macrophages plated at 2.5×10^5 per glass coverslip were infected at an MOI of 50 for 1 h. Coverslips were inverted onto a 5-μl drop of PBS containing 25 mg/ml ethidium bromide (EtdBr) and 5 mg/ml acridine orange placed on the surface of a glass slide. Samples were scored immediately by fluorescence microscopy as described previously (35).

Statistics. The significance of quantitative differences was determined using one-way analysis of variance (ANOVA).

RESULTS

Flagellated strains of *L. parisiensis* and *L. tucsonensis* replicate in C57BL/6 macrophages. The innate immune system of C57BL/6 mice restricts *L. pneumophila* by detecting cytosolic flagellin (37, 49, 57). Like *L. pneumophila*, when cultured to the stationary phase, *L. parisiensis* and *L. tucsonensis* displayed rapid, directed movement that was apparent by light microscopy. To investigate whether other flagellated species of *Legionella* are restricted by mouse macrophages, we compared the intracellular growth of *L. parisiensis* and *L. tucsonensis* to that of wild-type and *flaA* mutant *L. pneumophila*. Macrophages were infected with stationary-phase bacteria, and then replication was assessed at 24-h intervals by quantifying CFU. As expected, the number of *L. pneumophila* organisms increased 10-fold over the first 24 h, but subsequently, the infection was suppressed (Fig. 1). In contrast, the yield of *L. parisiensis* increased 100-fold over a 2-day period in primary C57BL/6 macrophages, a pattern similar to that of nonflagellated *L. pneumophila flaA* mutants and consistent with their capacity to replicate in a variety of professional phagocytes (3, 31, 33, 52). Likewise, motile *L. tucsonensis* effectively infected C57BL/6 macrophages, replicating to CFU yields similar to those of *L. parisiensis*. Therefore, evasion of the innate defenses of C57BL/6 macrophages is not unique to nonflagellated bacteria, since two motile strains of non-*L. pneumophila Legionella* species established robust infections.

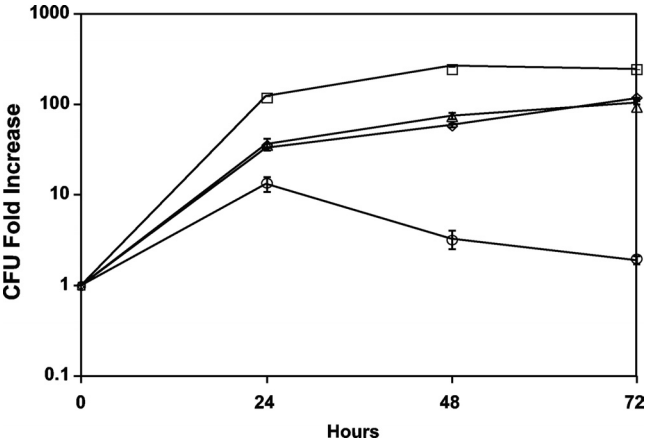


FIG. 1. Flagellated strains of *L. parisiensis* and *L. tucsonensis* evade C57BL/6 macrophage restriction of replication. The intracellular growth in C57BL/6 macrophages of *L. pneumophila* (○), *flaA* mutant *L. pneumophila* (□), *L. parisiensis* (◇), and *L. tucsonensis* (△) was determined by quantifying CFU at the times shown and dividing by the CFU associated with cells at 2 h. The values shown are the means ± standard deviations (SD) calculated for triplicate samples in one experiment that is representative of three or more independent trials.

***L. parisiensis* and *L. tucsonensis* do not trigger pyroptosis.** *L. pneumophila* elicits pyroptosis by a mechanism that requires flagellin, since *flaA* mutants are not cytotoxic and do not induce IL-1β secretion from macrophages (49, 57). To assess whether flagellated *L. parisiensis* and *L. tucsonensis* fail to trigger an innate immune response, we analyzed macrophage cytotoxicity and secretion of the proinflammatory cytokine IL-1β. When infected for 1 h at a high MOI, PE-phase *L. pneumophila* was cytotoxic; fewer than 35% of macrophages were viable (Fig. 2A). In contrast, nearly 100% of mouse macrophages cultured with *L. parisiensis* and *L. tucsonensis* were viable, a pattern similar to that for macrophages incubated with flagellin-deficient *flaA* mutants of *L. pneumophila*. Fur-

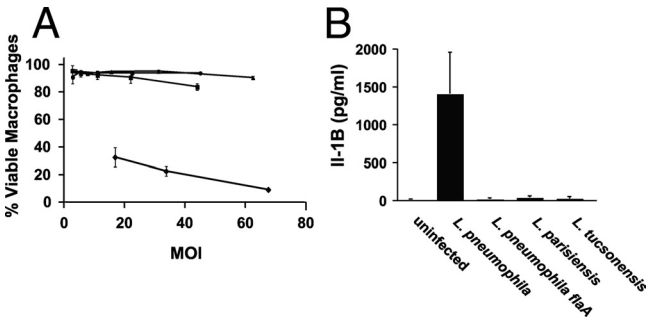
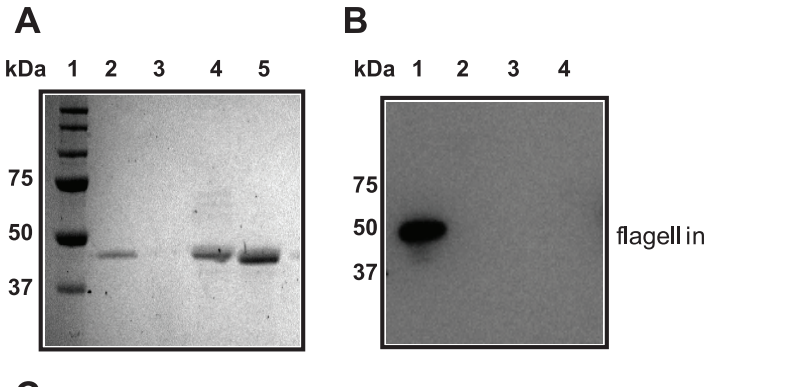


FIG. 2. *L. parisiensis* and *L. tucsonensis* strains do not trigger cell death or IL-1B secretion. (A) C57BL/6 macrophages were incubated for 1 h with twofold dilutions of *L. pneumophila* (◆), *flaA* mutant *L. pneumophila* (■), *L. parisiensis* (▲), and *L. tucsonensis* (●), and viability was quantified by Alamar Blue reduction. Results from one experiment, representative of three, are shown. (B) Macrophages pretreated with LPS were infected with the strain indicated for 2 h, and secreted IL-1β was quantified. Results shown are means ± standard errors (SE) for duplicate wells in three experiments.

thermore, after a 2-h incubation, *L. parisiensis* and *L. tucsonensis* triggered secretion of only negligible amounts of IL-1β from macrophages, whereas *L. pneumophila* induced the release of 1,500 pg/ml (Fig. 2B). Increasing the incubation period or the MOI had no effect on the amount of IL-1β secreted (data not shown). Therefore, *L. parisiensis* and *L. tucsonensis* fail to induce pyroptosis in mouse macrophages, despite their motility.

Flagellins from *L. parisiensis* and *L. tucsonensis* are divergent. To verify that *L. parisiensis* and *L. tucsonensis* encode flagellin, we used conventional methods to isolate flagellin from *L. pneumophila* and then analyzed the crude preparations (CFPs) by SDS-PAGE and Western blot analysis. *L. parisiensis* and *L. tucsonensis* produce proteins consistent with the molecular size of *L. pneumophila* flagellin (Fig. 3A). However, CFPs from *L. parisiensis* and *L. tucsonensis* did not react with anti-*L. pneumophila* flagellin antibody (Fig. 3B), indicating divergence at the protein level. A recent DNA array study of several *Legionella* species also reported divergence of *flaA* in *L. parisiensis* and *L. tucsonensis*, since probes representing the *flaA* locus of three *L. pneumophila* strains did not hybridize to *L. parisiensis* or *L. tucsonensis* genomic DNA (15). Indeed, several attempts using conventional and degenerate PCR to amplify the complete flagellin gene from these species were unsuccessful. To estimate the extent of this divergence, we generated a labeled probe for each of three regions of *L. pneumophila flaA* and analyzed the probe homology to genomic DNA by dot blot hybridization. *L. parisiensis* DNA hybridized weakly at the N terminus and the core region, whereas *L. tucsonensis* was most divergent in the core region (Fig. 3C). Significant homology was observed for *L. parisiensis* and *L. tucsonensis* in the C-terminal region, as >90% hybridization was evident in the region that also harbors the *L. pneumophila* flagellin carboxy-terminal "death" domain (37). However, a smaller probe designed to encode the final 51 amino acids of *L. pneumophila* flagellin (bp 1275 to 1428) consistently showed



Relative Hybridization

L. pneumophila	100%	100%	100%
L. parisiensis	67%	58%	90%
L. tucsonensis	95%	59%	95%

FIG. 3. Characterization of crude flagellin preparations. Flagellin samples prepared in parallel from broth cultures were separated by SDS-PAGE and analyzed by Coomassie blue staining (A) of *L. pneumophila* (lane 2), *flaA* mutant *L. pneumophila* (lane 3), *L. parisiensis* (lane 4), and *L. tucsonensis* (lane 5) or Western analysis, using anti-*L. pneumophila* flagellin antibody (B), of *L. pneumophila* (lane 1), *flaA* mutant *L. pneumophila* (lane 2), *L. parisiensis* (lane 3), and *L. tucsonensis* (lane 4). Positions of molecular mass standards are shown (lane 1 in panel A). (C) Relative genomic hybridization under low-stringency conditions with DNA probes for the indicated regions of *L. pneumophila flaA*.

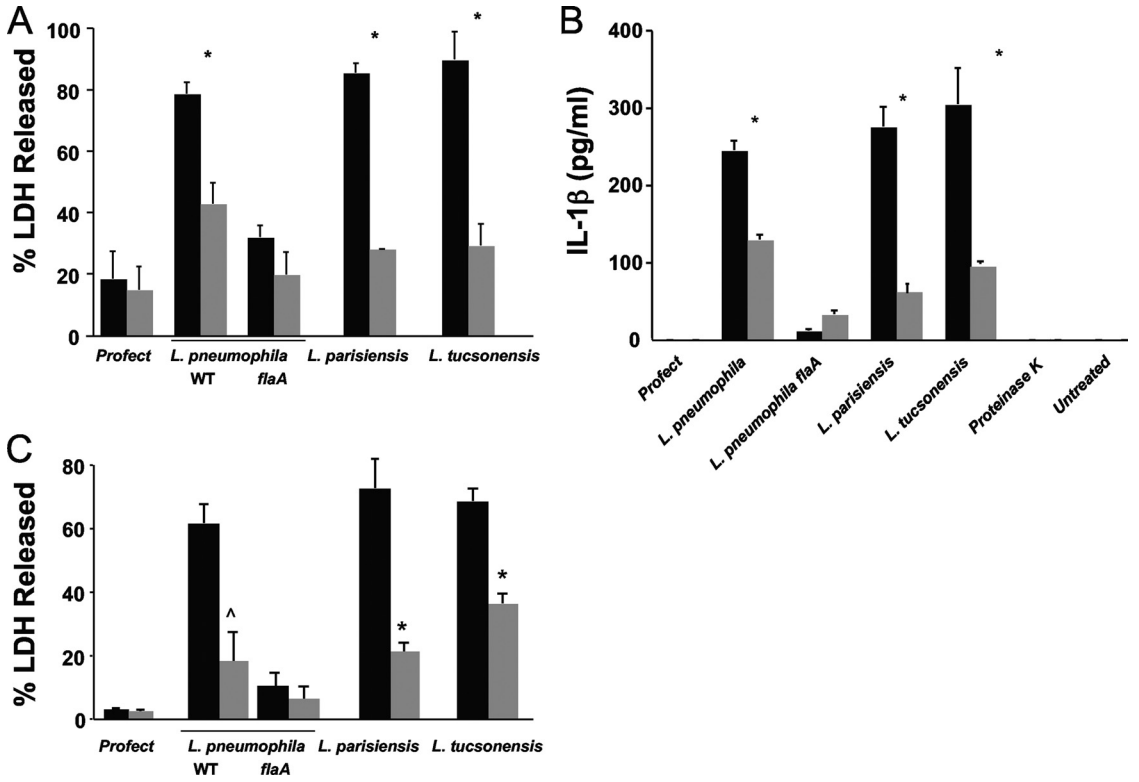


FIG. 4. Flagellin preparations from *L. parisiensis* and *L. tucsonensis* trigger macrophage cell death. To determine whether flagellins from *L. parisiensis* and *L. tucsonensis* trigger pyroptosis, the amounts of LDH (A) and IL-1β (B) released from C57BL/6 macrophages transfected with heat-treated crude flagellin (1.25 μg; black bars) or treated with proteinase K (gray bars) and incubated for 2 h were quantified. The data shown are means ± SE for three experiments, where asterisks indicate statistical differences from *flaA* mutant *L. pneumophila* ($P < 0.05$ using one-way ANOVA). (C) To determine whether the pyroptosis response to cytosolic flagellin required the NLR proteins Naip5 and Ipaf, LDH released from macrophages derived from C57BL/6 or *Naip5*$^{-/-}$ *Ipaf*$^{-/-}$ double mutant mice was quantified as in panel A. Asterisks indicate statistical differences ($P < 0.01$) compared to C57BL/6 macrophages exposed to WT *L. pneumophila*, calculated using one-way ANOVA; the carat indicates that $P = 0.016$.

less hybridization for *L. parisiensis* and *L. tucsonensis* genomic DNA than for *L. pneumophila* DNA (data not shown). Therefore, by analogy to escape from TLR5 recognition by several species of motile bacteria (4), it remained formally possible that the divergence observed for the flagellin genes and proteins of *L. parisiensis* and *L. tucsonensis* was sufficient to circumvent detection by the cytosolic surveillance machinery.

Cytosolic *L. parisiensis* or *L. tucsonensis* flagellin induces proinflammatory cell death. Pyroptosis in murine bone marrow-derived macrophages is induced when flagellin that presumably has leaked to the cytosol is detected by Naip5 and Ipaf (37, 49, 57). To determine if the divergent *L. parisiensis* and *L. tucsonensis* flagellins elude detection, we introduced crude flagellins from broth-grown cultures into the cytosol and then tested if either could trigger pyroptosis. C57BL/6 mouse macrophages were incubated for 2 h with the protein transfection reagent Profect P1 complexed with CFP obtained from *L. pneumophila*, *L. pneumophila flaA*, *L. parisiensis*, or *L. tucsonensis*, and then the LDH released by intoxicated cells was quantified. Mouse macrophages whose cytosol was transfected with CFPs from *L. pneumophila*, *L. parisiensis*, and *L. tucsonensis* each released >80% of their LDH, significantly more than the quantities measured for transfection reagent alone and the *flaA* mock prep ($P < 0.0001$) (Fig. 4A). The toxicity was due to protein and not some other bacterial product in the CFP, since treatment of the CFP with proteinase K prior to transfection reduced the toxicity (Fig. 4A, gray bars).

A hallmark of pyroptosis is secretion of IL-1β, a result of caspase-1 activation. Not only did the cytosolic CFPs trigger cell death, but each also induced secretion of IL-1β from macrophages. When macrophages were transfected with CFP, ~70% more IL-1β was secreted from those macrophages than from those transfected with CFP pretreated with proteinase K (Fig. 4B, gray bars), and >95% more was secreted than that from macrophages that were treated with *L. pneumophila flaA* mock CFP or that from the negative control samples (Fig. 4B).

As another approach to ascertain whether pyroptosis was primarily a response to the flagellin protein in the CFP or instead a response to some other microbial contaminant, we analyzed macrophages that lacked both Naip5 and Ipaf, the NLRs that collaborate to detect cytosolic flagellin (37, 49, 57). As observed previously for *L. pneumophila* (49), in response to cytosolic CFPs prepared from *L. parisiensis* or *L. tucsonensis*, C57BL/6 macrophages secreted >60% of their LDH (Fig. 4C, black bars), whereas Naip5- and Ipaf-deficient macrophages released significantly less ($P < 0.01$) (Fig. 4C, gray bars). The *L. tucsonensis* CFP elicited more LDH release from the Naip5- and Ipaf-deficient macrophages than did either of the negative

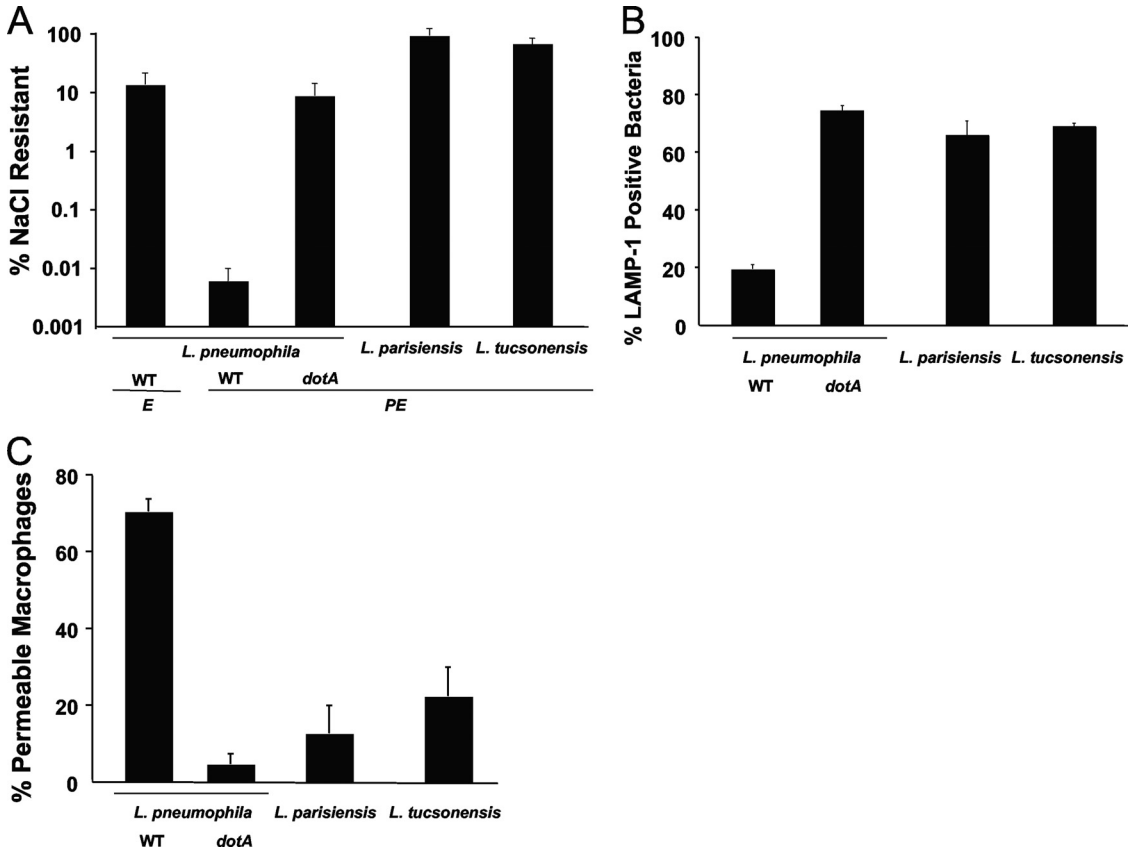


FIG. 5. *L. parisiensis* and *L. tucsonensis* do not display features of the canonical Dot/Icm type IV secretion system. (A) Sodium sensitivity was quantified by plating E- or PE-phase broth cultures on medium with or without 100 mM NaCl and using the formula [(CFU on CYET+NaCl)/(CFU on CYET)] × 100. (B) Colocalization with the late endosomal and lysosomal protein LAMP-1 was quantified by immunofluorescence microscopy after incubating C57BL/6 macrophages for 1.5 h with PE-phase WT *L. pneumophila*, *dotA* mutant *L. pneumophila*, *L. parisiensis*, or *L. tucsonensis*. Results shown are means ± SE for three experiments. (C) Permeability of A/J macrophages after incubation for 1 h with PE-phase *Legionella* was determined by uptake of ethidium bromide. The data shown are means ± SE calculated from three experiments.

controls (Profect P1 and *L. pneumophila flaA* mock CFP), suggesting that in addition to flagellin, other microbial products contributed to the pyroptosis response. Thus, the capacity of *L. parisiensis* and *L. tucsonensis* to replicate in C57BL/6 macrophages (Fig. 1) could not be accounted for fully by postulating that the cytosolic surveillance pathway is blind to their flagellins.

***L. parisiensis* and *L. tucsonensis* do not exhibit type IV secretion-dependent traits.** To activate the inflammasome complex and subsequently induce secretion of IL-1β, *L. pneumophila* requires not only flagellin but also type IV secretion, which is thought to provide a conduit to the cytoplasm (45, 49, 57). Therefore, we next tested whether the cytotoxicity defect of *L. parisiensis* and *L. tucsonensis* is attributed to their type IV secretion systems.

Although a mechanism has not been established, *L. pneumophila* strains that are defective for type IV secretion are resistant to sodium; indeed, several of the *dot*/*icm* type IV secretion mutants were originally isolated based on their ability to grow on high concentrations of NaCl (75). Salt sensitivity of an *L. parisiensis* strain has also been reported (52). Accordingly, as one probe of their type IV secretion systems, we tested the ability of the *L. parisiensis* and *L. tucsonensis* strains to form colonies on medium containing 100 mM NaCl. *L. pneumophila* is sensitive to NaCl in the PE phase of growth, yet exponential-phase bacteria and PE *dotA* mutants are NaCl resistant (13) (Fig. 5A). In contrast to *L. pneumophila*, PE-phase cultures of the strains of *L. parisiensis* and *L. tucsonensis* were resistant to salt. Compared to the *dotA* mutant salt-resistant control, the non-*L. pneumophila* cultures were 10-fold more tolerant to NaCl (Fig. 5A).

A second hallmark of type IV secretion-competent *L. pneumophila* is the ability to evade the late endosomal compartment, which is rich in LAMP-1 (74) (Fig. 5B). However, like *L. pneumophila dotA* mutants, >60% of PE-phase *L. parisiensis* and *L. tucsonensis* cells colocalized with LAMP-1 within 2 h of internalization by C57BL/6 macrophages (Fig. 5B).

A third characteristic of type IV secretion is the ability to perforate macrophage membranes, an activity evident by staining cells, after a 1-h infection, with the fluorescent dyes EtdBr and acridine orange (35). Cells with intact membranes exclude EtdBr; as such, cells with red nuclei are scored as EtdBr permeable and those with green nuclei are scored as alive or EtdBr impermeable. As shown previously, WT *L. pneumophila* permeabilized 70% of macrophages, whereas *L. pneumophila dotA* mutants did not (<10% of macrophages had red nuclei)

(Fig. 5C). Similar to the case with *dotA* mutants, the majority of nuclei from macrophages infected with the *L. parisiensis* strain remained green (Fig. 5C), consistent with the inability of *L. parisiensis* to lyse sheep red blood cells (3). Likewise, the *L. tucsonensis* strain failed to induce macrophage permeability. Taken together, their phenotypes indicate that *L. parisiensis* and *L. tucsonensis* do not express the canonical Dot/Icm type IV secretion system of *L. pneumophila*, which forms pores in macrophage membranes that are thought to provide a conduit for flagellin to the macrophage cytoplasm.

DISCUSSION

The response to cytosolic flagellin by the innate immune machinery of C57BL/6 macrophages results in resistance to *L. pneumophila* infection. Yet other species of *Legionella* establish a productive replication niche in these macrophages (3, 7, 33, 48). We show here that the flagellated species *L. parisiensis* and *L. tucsonensis* replicate in C57BL/6 macrophages and that they both fail to stimulate proinflammatory cell death. Pyroptosis is likely one rapid response by C57BL/6 macrophages that leads to the elimination of infected cells and the recruitment of leukocytes to the site (11, 20). Since stationary-phase *L. parisiensis* and *L. tucsonensis* fail to perforate macrophage membranes, evade LAMP-1, and do not become sodium sensitive, they lack a type IV secretion system like that of *L. pneumophila*, which likely provides a conduit for toxic flagellin to the macrophage cytosol (45, 49, 57). Likewise, motile *L. longbeachae* organisms replicate in C57BL/6 macrophages but lack pore-forming activity, despite carrying at least some of the *dot*/*icm* loci (7). We postulate that without release of flagellin into the cytoplasm, macrophages remain blind to these intracellular *Legionella* organisms and fail to trigger pyroptosis to combat the infection.

Although we have focused here on the early response of macrophages to *L. pneumophila*, host defense is a culmination of many factors. Indeed, wild-type and *flaA* mutant *L. pneumophila* cells are eventually cleared from the lungs of A/J *Naip5* mutant and C57BL/6 mice, respectively, indicating that other immune response pathways contribute to restricting bacterial replication (49). Many studies of infections in animal and human models have shown an important role not only for macrophages but also for other components of the immune system, most notably neutrophils, cytokines, and chemokines (70). For example, gamma interferon (IFN-γ), IFN-α/β, and tumor necrosis factor alpha (TNF-α) each induce macrophage resistance to *L. pneumophila* (12, 18, 51, 53).

Flagellin is a potent stimulator of innate immune signaling pathways. By analogy to the TLR5 epitope of flagellin, which is conserved across bacterial species, it is likely that the epitope recognized by the NLR cytoplasmic receptors is highly conserved (4, 45, 46, 49, 57, 68, 85). For example, mouse macrophages detect cytosolic flagellins from *Legionella*, *Salmonella*, *Bacillus*, and *Pseudomonas* through the NLRs Ipaf and Naip5 (24, 25, 37, 45, 47, 49, 54, 76). By genomic hybridization, the *flaA* sequences from *L. parisiensis* and *L. tucsonensis* appear to be divergent from the *L. pneumophila flaA* sequence, including in the C-terminal region, which harbors one toxic epitope recognized by Naip5 and required for activation of the inflammasome (37). Nevertheless, *L. parisiensis* and *L. tucsonensis* flagellins can trigger pyroptosis when delivered directly to the cytosol (Fig. 4). Therefore, the divergence of the *L. parisiensis* and *L. tucsonensis* flagellin species that is evident at the DNA and protein levels is not sufficient to account for the lack of cytotoxicity of these bacteria (Fig. 2). Instead, we favor the model that during *L. parisiensis* or *L. tucsonensis* infection, flagellin does not escape from the vacuole to contaminate the cytoplasm.

Bacterial secretion systems are one route to the cytosol for bacterial products (5, 30, 71). For example, *Salmonella* activates the innate immune system not only through TLRs but also through NLRs, since mutants that lack a type III secretion system do not activate NLR signaling (40, 41). Therefore, the cytosolic surveillance system provides macrophages a mechanism to respond specifically to invading pathogens that express virulence factors that breach the phagosome, namely, toxins and specialized secretion systems (11, 19). The cytosolic surveillance system detects *L. pneumophila* bacteria that have a functioning type IV secretion apparatus (49, 57), which the bacteria require to establish replication vacuoles (74). Although translocation of flagellin by *L. pneumophila* through its type IV secretion system has not been demonstrated unequivocally, it is hypothesized that as for *Salmonella*, when virulence effectors are secreted, minute amounts of flagellin are also translocated from the bacterium to the macrophage cytosol (37, 46, 66). Compared to *L. pneumophila*, *L. parisiensis* and *L. tucsonensis* lack phenotypes characteristic of the canonical type IV secretion system.

It remains to be determined whether *L. parisiensis* and *L. tucsonensis* evade detection by the inflammasome due to either incompatibility of their divergent flagellin species with the secretion system, a pore size that is not permissive for flagellin to escape, or a defective secretion system. Genomic DNA hybridization studies of different *Legionella* strains indicate that some, but not all, of the many *dot*/*icm* structural and secreted effector genes are conserved (3, 15; C. Buchrieser, personal communication). In particular, *L. parisiensis* genomic DNA hybridizes to *icmD*, *icmF*, *dotF*, *dotE*, and *lidA*, and *L. tucsonensis* contains sequences similar to *icmD*, *dotL*, *dotN*, *dotD*, *dotC*, and *dotB* (Buchrieser, personal communication). Accordingly, some *L. pneumophila* Dot/Icm components are either highly divergent or absent in *L. parisiensis* and *L. tucsonensis*. In some non-*L. pneumophila Legionella* species, the capacity to replicate intracellularly or to transfer plasmids by conjugation does not correlate with sodium sensitivity (34, 52), a trait conferred by the Dot/Icm type IV secretion system (75). Furthermore, under certain conditions, the canonical Dot/Icm type IV secretion system is dispensable. When amoebae are infected with water- or Ers-treated *dot*/*icm* mutants, the bacteria replicate intracellularly and avoid phagosomal acidification as efficiently as the wild-type parental strain, provided that they encode the homologous Lvh type IV secretion system (8, 9). *L. pneumophila* also utilizes a type II secretion system to replicate proficiently in host cells (60). Thus, legionellae utilize a variety of specialized secretion systems to parasitize macrophages. Some, but not all, of these machineries may inadvertently translocate flagellin to the cytoplasm of host cells.

In humans, *L. pneumophila* infection causes an acute pneumonia and severe inflammation, where lung exudates contain macrophages, polymorphonuclear cells, fibrin, red blood cells,

proteinaceous material, and cellular debris indicative of cell death and cell lysis (28, 79). In mouse macrophages, both flagellin and type IV secretion are key inducers of the inflammatory response to *L. pneumophila*, since the Dot/Icm type IV secretion system can lyse white blood cells, flagellin is proinflammatory, and both factors are required for activation of the inflammasome (35, 49, 50, 58, 61–63, 65, 73). Likewise, studies of both human peripheral blood-derived monocytes and an alveolar epithelial cell line indicate that a Naip- and Ipaf-mediated response to flagellin reduces the yield of intracellular *L. pneumophila* (73). Accordingly, it is plausible that despite their capacity to replicate in a variety of host cells (3, 31, 52), *L. parisiensis* and *L. tucsonensis* are less frequent causes of Legionnaires' disease because their flagellin is not translocated to the macrophage cytoplasm. By this model, the inflammation and cellular damage characteristic of Legionnaires' disease are manifestations of not only bacterial replication but also robust activation of the inflammasome and extensive pyroptosis triggered by flagellin that contaminates the cytosol during type IV secretion. However, it cannot be assumed that mouse and human macrophages utilize similar mechanisms to restrict infection. For example, the human Naip protein has been shown to interact with caspases 3, 7, and 8, whereas mouse Naip5 is predicted to regulate caspase-1 activity (reviewed in reference 70). As another test of this model, it would be important to analyze multiple isolates of each non-*L. pneumophila* species, due to well-documented heterogeneity within species and the genus (3, 6, 7, 15, 16, 26, 27, 33, 34, 48, 56, 77).

Our analysis of non-*L. pneumophila Legionella* species has extended the evidence that pore formation by canonical type IV secretion makes *L. pneumophila* vulnerable to early detection of flagellin by the innate immune system. It is also notable that the potential of legionellae to cause human disease appears to be inversely correlated with the capacity to replicate in macrophages of C57BL/6 mice (3, 7, 33, 39, 48). Accordingly, this study points to the complexity of human disease, a product of both microbial virulence traits, as defined by laboratory assays, and the capacity of the host to mount a measured immune response.

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