Legionella pneumophila Type II Secretion Dampens the Cytokine Response of Infected Macrophages and Epithelia^{∇†}

Kessler McCoy-Simandle,¹ Catherine R. Stewart,¹ Jenny Dao,¹ Sruti DebRoy,¹ Ombeline Rossier,¹ Paul J. Bryce,² and Nicholas P. Cianciotto^{1*}

Department of Microbiology and Immunology¹ and Division of Allergy and Immunology,²
Northwestern University Medical School, Chicago, Illinois 60611

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The type II secretion (T2S) system of *Legionella pneumophila* is required for the ability of the bacterium to grow within the lungs of A/J mice. By utilizing mutants lacking T2S (*lsp*), we now document that T2S promotes the intracellular infection of both multiple types of macrophages and lung epithelia. Following infection of macrophages, *lsp* mutants (but not a complemented mutant) elicited significantly higher levels of interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- α), IL-10, IL-8, IL-1 β , and MCP-1 within tissue culture supernatants. A similar result was obtained with infected lung epithelial cell lines and the lungs of infected A/J mice. Infection with a mutant specifically lacking the T2S-dependent ProA protease (but not a complemented *proA* mutant) resulted in partial elevation of cytokine levels. These data demonstrate that the T2S system of *L. pneumophila* dampens the cytokine/chemokine output of infected host cells. Upon quantitative reverse transcription (RT)-PCR analysis of infected host cells, an *lspF* mutant, but not the *proA* mutant, produced significantly higher levels of cytokine transcripts, implying that some T2S-dependent effectors dampen signal transduction and transcription but that others, such as ProA, act at a posttranscriptional step in cytokine expression. In summary, the impact of T2S on lung infection is a combination of at least three factors: the promotion of growth in macrophages, the facilitation of growth in epithelia, and the dampening of the chemokine and cytokine output from infected host cells. To our knowledge, these data are the first to identify a link between a T2S system and the modulation of immune factors following intracellular infection.

The aquatic bacterium *Legionella pneumophila* is the primary agent of Legionnaires' disease, a potentially fatal form of pneumonia (38). *L. pneumophila* is especially pathogenic for the immunocompromised, the elderly, and smokers. Recent studies highlight the significance of travel-associated Legionnaires' disease and an increasing incidence of legionellosis (79). In natural and man-made waters, Gram-negative *L. pneumophila* survives planktonically, in biofilms, and as an intracellular parasite of protozoa (115). Infection occurs after the inhalation of contaminated droplets that originate from a variety of aerosol-generating devices as well as potable waters (33, 86). In the lung, *L. pneumophila* multiplies in resident alveolar macrophages, although persistence might also involve growth in alveolar epithelia and extracellular survival (82). Much of the pathogenesis and ecology of *L. pneumophila* is mediated by secreted factors, including protein and nonprotein molecules (2, 25, 27, 44, 57). For secreting proteins into the extracellular milieu and/or target host cells, *L. pneumophila* uses both the type II secretion (T2S) and the type IV secretion (T4S) system, large membrane-spanning apparatuses that are each composed of at least 10 component proteins (27, 82).

T2S promotes the physiology and ecology of many bacteria as well as the virulence of various pathogens (28). T2S substrates first translocate across the inner membrane and into the periplasm by the Sec or Tat pathway, and then a pseudopilus acts to push the proteins through a dedicated outer membrane pore (27, 58). For environmental *L. pneumophila*, an intact T2S system (known as Lsp) is required for both intracellular growth in amoebal hosts and extracellular survival in low-temperature water samples (27, 52, 61, 106). Following their intratracheal inoculation into A/J mice, *lsp* mutants do not increase in numbers in the lung, indicating that Lsp is also critical for *L. pneumophila* infection of and pathogenesis in mammalian hosts (27, 34, 96). The fact that these mutants, but not a complemented derivative, are partly impaired in growth within U937 cells, a human macrophage-like cell line, indicates that the *in vivo* importance of T2S is partly due to its role in the intracellular infection of alveolar macrophages (96). Lsp promotes the export of at least 25 proteins, 17 enzymatic activities, and a surfactant that facilitates surface translocation (27, 88, 111). Although the T2S substrate(s) that is required for macrophage infection has not been identified, a metalloprotease and RNase have been linked to infection of amoebae and a chitinase has been linked to optimal lung infection (34, 94, 95). Adding significantly to our appreciation of the medical significance of T2S, we now document that *L. pneumophila* Lsp (i) promotes the intracellular infection of lung epithelial cells, (ii) dampens the cytokine output from infected macrophages and epithelia, and (iii) limits the levels of cytokine transcripts in infected macrophages.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Northwestern University Medical School, 320 East Superior St., Chicago, IL 60611. Phone: (312) 503-0385. Fax: (312) 503-1339. E-mail: n-cianciotto@northwestern.edu.

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TABLE 1. *L. pneumophila* strains used in this study

Strain(s)	Description	Reference(s) or source
130b	Clinical isolate, serogroup 1	96, 111
NU258	<i>lspDE</i> mutant of 130b	96
NU275	<i>lspF</i> mutant of 130b	96
NU275(pMF)	<i>lspF</i> -complemented NU275	96
GG105	<i>dotA</i> mutant of 130b	111
AA200	<i>proA</i> (<i>mshA</i>) mutant of 130b	73, 95
AA200(p <i>SproA</i>)	<i>proA</i> -complemented AA200	This study
NU254	<i>map</i> mutant of 130b	5
NU262, NU263	<i>lipA</i> mutants of 130b	6
NU265, NU266	<i>lipB</i> mutants of 130b	6
NU267	<i>lipA lipB</i> mutant of 130b	6
NU268	<i>plcA</i> mutant of 130b	6
NU270	<i>plaA</i> mutant of 130b	43
NU318	<i>chiA</i> mutant of 130b	34
NU324	<i>lapA lapB</i> mutant of 130b	95
NU328	<i>sma</i> mutant of 130b	94
NU353	<i>celA</i> mutant of 130b	88
NU367, NU368	<i>plcA</i> mutants of 130b	This study
NU369, NU370	<i>plcB</i> mutants of 130b	This study
NU371, NU372	<i>plcA plcB</i> mutants of 130b	This study
NU373, NU374	<i>lipA lipB</i> mutants of 130b	This study

MATERIALS AND METHODS

Bacterial strains. *L. pneumophila* strain 130b (ATCC strain BAA-74, also known as AA100 or Wadsworth) is a clinical isolate that served as our wild-type strain (96). Previously described mutants of strain 130b that are defective in protein secretion are listed in Table 1. NU367 and NU368 are two new mutants of strain 130b that specifically lack the T2S-dependent PlcA protein (10) (Table 1). To obtain these mutants, the *plcA* gene plus flanking regions was amplified from strain 130b genomic DNA using primers CS36 (5'-AGCGCGAATCAGA ACGCAGGTAAT) and CS37 (5'-ACCCTCTATATCCAATCGGCGCA). The amplified 2.2-kb fragment was then cloned into pGEM-T Easy (Promega, Madison, WI). The resultant plasmid, pGplcA, was digested with ClaI, which cuts twice within *plcA*, resulting in a 600-bp deletion, and then was ligated to a kanamycin resistance gene isolated from pMB2190 after HincII digestion, producing pGplcCK2. Mutated *plcA* was introduced into strain 130b by transformation as previously described (111). Verification of the mutant genotype was carried out by PCR with primers CS35 and CS36. NU369 and NU370 are additional new mutants of strain 130b that are inactivated in a gene (designated lpg1455 in the *L. pneumophila* genome database) that encodes a secreted protein which is homologous to the T2S-dependent PlcA (6, 34, 49) (Table 1). Finally, NU371 and NU372 are double mutants in which both *plcA* and *plcB* are inactivated, and NU373 and NU374 are new double mutants lacking both *lipA* and *lipB* (Table 1). All of the new 130b mutants were constructed by standard allelic exchange, as was described above for the *plcA* mutants and as we have done in the past for many other mutants (88). To construct p*SproA* for complementation of the *proA* mutant AA200 (Table 1), a plasmid containing *proA* cloned into pGEM-T Easy (95) was digested with ApaI and SacI, and then the DNA fragment containing only *proA* and its promoter was cloned into the low-copy-number vector pSBK29 (89). Chloramphenicol-resistant p*SproA* was introduced into *L. pneumophila* by electroporation (95). Past studies have shown how low-copy-number vectors related to pSBK29 can be used for complementation in *L. pneumophila* (39, 93).

Legionellae were cultured at 37°C in buffered yeast extract (BYE) broth or on buffered charcoal yeast extract (BCYE) agar (96). *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA), a host for recombinant plasmids, was grown on LB agar (96). The following antibiotics were added to the media at the indicated concentrations (μg per ml): ampicillin, 100 for *E. coli*; chloramphenicol, 6 for *L. pneumophila* and 30 for *E. coli*; gentamicin, 2.5 for *L. pneumophila* and 5 for *E. coli*; and kanamycin, 25 for *L. pneumophila* and 50 for *E. coli*. Chemicals were from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

Infection assays. To assess *L. pneumophila* growth in macrophages, intracellular infection assays were performed as previously described using the human U937 cell line (ATCC CRL-1593.2) and the murine MH-S alveolar macrophage cell line (ATCC CRL-2019), as well as bone marrow-derived (BMD) macrophages and explanted alveolar macrophages from 6- to 8-week-old A/J mice that

had been obtained from the Jackson Laboratory, Bar Harbor, ME (2). Monolayers containing 2.5×10^5 to 10×10^5 macrophages were infected with bacteria at a multiplicity of infection (MOI) equal to 0.5 (for U937 cells), 1 (for MH-S cells and the BMD macrophages), or 5 (for the alveolar cells), incubated for 2 h to allow bacterial entry, and then washed three times with medium to remove unincorporated bacteria. At 0, 24, 48, or 72 h postinoculation, the infected monolayers were lysed and serial dilutions were plated on BCYE agar in order to determine the numbers of legionellae (CFU). In some of the experiments using BMD macrophages, the host cells were pretreated for 15 h with differing amounts of recombinant mouse gamma interferon (IFN-γ) (R & D Systems, Minneapolis, MN). In other experiments aimed at assessing the impact of cytokine levels on bacterial intracellular growth, U937 cells were treated with antibodies (eBioscience, San Diego, CA) directed against the receptor for either tumor necrosis factor alpha (TNF-α) (i.e., antibody BMS106) or interleukin 6 (IL-6) (BMS135). The blocking antibodies were added at a concentration of 10 μg/ml and maintained throughout infection with *L. pneumophila*. To examine *L. pneumophila* growth in lung epithelial cells, the human A549 cell line (ATCC CCL-185), the human WI-26 VA4 cell line (ATCC CCL-95.1), and the murine TC-1 cell line (ATCC CRL-2785) were infected as previously described (2, 29). Monolayers containing 2.5×10^5 to 5×10^5 epithelial cells were inoculated with bacteria at an MOI of 10, and then the course of the infection was analyzed as noted above. *L. pneumophila* infection of HeLa cells (kindly provided by Paul Hoffman, University of Virginia) was performed in a similar fashion. *L. pneumophila* did not grow when inoculated into tissue culture media alone (data not shown), indicating that changes in numbers of bacterial CFU in the various macrophage or epithelial monolayers were a consequence of intracellular growth. Finally, *L. pneumophila* growth in lungs was determined after intratracheal inoculation of legionellae into A/J mice (Jackson Laboratory, Bar Harbor, ME), as described before (2, 34, 96). Animal experiments were approved by the Animal Care and Use Committee of Northwestern University.

ELISAs for cytokines and chemokines. U937 and A549 cell monolayers were infected with bacteria as noted above. After 0, 24, 48, or 72 h of incubation, supernatants were collected from the infected wells and centrifuged at $1,000 \times g$ for 10 min. The amounts of cytokines and chemokines in the tissue culture supernatants were measured by an enzyme-linked immunosorbent assay (ELISA), as has been done before by others (24). ELISA kits (Ready-Set-Go) for the detection of human TNF-α, IL-1β, IL-6, IL-8, IL-10, and MCP-1 were obtained from eBioscience, and assays were done according to the manufacturer's instructions. To monitor cytokine levels in infected lungs, A/J mice were infected as noted above, and then at 24 h postinoculation, lung homogenates (cleared by centrifugation at $1,000 \times g$ for 10 min to remove cellular debris) were examined for IL-6 and TNF-α using murine ELISA kits (eBioscience).

Quantitative reverse transcription (RT)-PCR determination of cytokine gene expression. At 4 and 24 h postinoculation, RNA was purified from the infected monolayers using the RNeasy minikit (Qiagen, Hilden, Germany). cDNA was then synthesized using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The cDNA was amplified by PCR using primers specific for IL-6, IL-8, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Applied Bioscience, Carlsbad, CA), an ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA), and specific TaqMan probes (Applied Biosystems) (50). GAPDH was used as a housekeeping gene for analysis of changes in cycle threshold values; the fold induction above the induction of the uninfected control was determined based on changes in the cycle threshold values (50).

RESULTS

T2S promotes *L. pneumophila* infection of multiple types of macrophages. We previously determined that *L. pneumophila* *lspDE*, *lspF*, and *lspG* mutants lacking an intact T2S system are defective by ca. 10-fold in their infection of U937 cells (96). To validate the results obtained from using U937 cells, we assessed the intracellular growth capacity of an *lspF* mutant in three more types of macrophages that are also known to support *L. pneumophila* intracellular growth; i.e., the MH-S mouse alveolar macrophage cell line (78, 118), A/J mouse BMD macrophages (75), and explanted A/J mouse alveolar macrophages (36, 46). In all cases, the T2S mutant had a reduced infectivity that was comparable to what has been observed with U937 cells; e.g., there was a 14-fold-reduced recovery of the mutant

FIG. 1. Intracellular infection of MH-S cell macrophages by wild-type and T2S mutant *L. pneumophila*. MH-S cells were inoculated with wild-type strain 130b (black diamonds), *lspF* mutant NU275 (white squares), or complemented mutant NU275 (pMF) (black squares), and then the number of CFU in each well was determined at various times postinfection. Results are the means and standard deviations of results from triplicate wells and are representative of three independent experiments. The differences in CFU recovery between the mutant and the wild type or the complemented mutant were significant at 20 and 40 h postinfection (Student's *t* test, $P < 0.05$).

from MH-S cells and BMD macrophages (Fig. 1 and 2 and data not shown). As exemplified by the results of MH-S cell infection shown in Fig. 1, the reintroduction of *lspF* into the mutant restored its capacity to fully infect. The complemented *lspF* mutant also behaves as the wild type does upon infection of U937 cells (96). A number of studies have noted the importance of IFN- γ -activated macrophages during *L. pneumophila* infection, including the pneumonia that follows intratracheal inoculation of A/J mice (19, 36). Therefore, we next compared the *lspF* mutant and its wild-type parent for their ability to survive within BMD A/J macrophages that had been treated with IFN- γ . Both strains were inhibited by the IFN treatment and in similar dose-dependent manners (Fig. 2). Thus, the defect of the *lspF* mutant relative to the wild type was still evident. This defect did not vary significantly with IFN- γ levels, although in monolayers that had been treated with 100 ng of IFN- γ , there was a trend toward a larger defect at 72 h postinoculation than in untreated monolayers. Together, these data indicate that the T2S system of *L. pneumophila* is required for optimal intracellular infection of multiple types of macrophages, including explanted murine phagocytes. By performing additional *in vivo* experiments, we confirmed, however, that the *lspF* mutant's defect in murine macrophages is still modest compared to its inability to increase in numbers within infected murine lungs (see Fig. S1 in the supplemental material). These data suggest that *L. pneumophila* T2S is also involved in events that go beyond replication in the alveolar macrophage niche.

T2S promotes *L. pneumophila* growth within alveolar epithelial cells. Various studies have identified alveolar epithelial cells as a potential niche for *L. pneumophila* growth during lung infection (29, 40, 74, 83, 84, 85, 101). Therefore, we examined the ability of T2S mutants to infect and grow within alveolar epithelial cell lines, namely, the human A549 type II epithelial line, the human WI-26 VA4 type I epithelial line,

FIG. 2. Intracellular infection of BMD macrophages by wild-type and T2S mutant *L. pneumophila*. A/J mouse BMD macrophages that had been treated or not treated with the indicated concentrations of IFN- γ were inoculated with wild-type strain 130b (black bars) or *lspF* mutant NU275 (white bars), and then the number of CFU in each well was determined at various times postinfection. Bacterial growth is graphed as the increase in CFU number (in log units) from the time of inoculation to 48 h (top graph) or 72 h (bottom graph) postinoculation. Data are the means and standard deviations of results from triplicate wells and are representative of at least three independent experiments. The fold differences in CFU recovery between 130b- and NU275-infected monolayers, as indicated by the numbers over the brackets, were significant at both 48 and 72 h postinfection (Student's *t* test, $P < 0.05$). However, the magnitude of the differences between the strains did not change significantly as a result of the IFN- γ treatments ($P > 0.05$).

and the murine TC-1 line. Over the 3-day course of the assays, wild-type strain 130b increased 100-fold to 1,000-fold within the A549 and WI-26 monolayers, a net growth comparable to that observed in previous studies (Fig. 3A and B). The strain grew equally well in TC-1 cells (Fig. 3C); this experiment represented the first documentation of *L. pneumophila* growth in TC-1 cells. Both the *lspF* mutant and an *lspDE* mutant displayed an impaired ability to grow in alveolar epithelial monolayers, as evidenced by their reduced recoveries (5- to 10-fold) at 24, 48, and 72 h postinoculation in all three cell lines (Fig. 3). The fact that a defect was not evident at the first time point ($t = 0$), even after the infected monolayers were treated with gentamicin in order to kill any residual extracellular bacteria (data not shown), indicated that the mutants are not impaired for entry into these epithelial cells. The defects at 24,

FIG. 3. Intracellular infection of lung epithelial cells by wild-type and T2S mutant *L. pneumophila*. The A549 human alveolar type II epithelial cell line (A), WI-26 VA4 human alveolar type I epithelial cell line (B), and TC-1 murine alveolar epithelial cell line (C) were inoculated with wild-type strain 130b (black diamonds), *lspF* mutant NU275 (white squares), *lspDE* mutant NU258 (white triangles), or complemented mutant NU275 (pMF) (black squares), and then the number of CFU in each well was determined at various times postinfection. Results are the means and standard deviations of results from quadruplicate wells and are representative of three independent experiments. The differences in CFU recovery between the mutant and wild type or the complemented mutant were significant at 24, 48, and 72 h postinfection (Student's *t* test, $P < 0.01$).

48, and 72 h were evident not only when the entire contents of the infected wells were sampled, as shown in Fig. 3, but also when the tissue culture supernatants were removed prior to our plating of the lysates of infected epithelia. Therefore, the reduced numbers of CFU that were obtained for the *lsp* mutants were a manifestation of impaired intracellular growth. To confirm that the reduced infectivity exhibited by the *lsp* mutants was due to the loss of Lsp rather than a second-site mutation, we tested a complemented *lspF* mutant and found that it infected the lung epithelial lines analogously to the wild type (Fig. 3). The *lspF* mutant was also impaired in its infection of HeLa cells (data not shown), a non-lung epithelial line that also supports *L. pneumophila* growth (51). In sum, these data demonstrate that an intact T2S system is required for optimal infection of epithelial cells by *L. pneumophila*. Thus, *Legionella* T2S likely promotes pulmonary infection by facilitating intracellular growth in both alveolar macrophages and lung epithelia. To our knowledge, this is the first demonstration of a T2S system promoting bacterial growth in epithelial cells.

We previously determined that the T2S system of *L. pneumophila* strain 130b mediates the secretion of at least 25 proteins, many of which exhibit enzymatic activities (27, 88). In an attempt to identify a secreted factor that is responsible for the importance of T2S in infection of epithelia, mutants of strain 130b that are specifically defective for each of the currently known T2S-dependent exoenzymes (Table 1) were tested for their infectivity of A549 cells. Mutants AA200, NU254, NU270, NU367, NU268, NU267, NU328, NU353, NU318, and NU324, which are defective for *proA*, *map*, *plaA*, *plaC*, *plcA*, *lipA* and *lipB*, *srnA*, *celA*, *chiA*, and *lapA* and *lapB*, respectively, all grew as well as the wild type did (data not shown). These data indicate that the ProA metalloprotease, Map acid phosphatase, PlaA lysophospholipase A, Plac glycerophospholipid:cholesterol acyltransferase, PlcA phospholipase C, LipA and LipB lipases, SrnA RNase, CelA endoglucanase, ChiA chitinase, and LapA and LapB aminopeptidases are not required for infection of lung epithelia, just as past studies (27, 88) had found them not to be required for infection of macrophages. We also tested the *plcB* mutant NU369, which lacked a secreted protein that is highly similar to PlcA and had not been included in previous infection studies. However, this mutant also grew normally within A549 cells as well as within U937 cells (data not shown). Thus, it appears that yet-to-be-defined T2S substrates are required for optimal intracellular infection of lung epithelia. Alternately, it is also possible that there exists functional redundancy among the known T2S-dependent substrates.

T2S dampens the cytokine and chemokine output of *L. pneumophila*-infected macrophages and epithelia. The Lsp mutant's defects in macrophages and epithelia, even when considered in combination, appeared insufficient to fully explain the strain's inability to increase its numbers in the lung. Thus, we hypothesized that *L. pneumophila* T2S influences processes in addition to growth in permissive host cells. Coincident with *L. pneumophila* intracellular growth are the production and secretion of cytokines and chemokines by infected host cells (82). Since these molecules play critical roles in establishing the immune response necessary for controlling lung infection, we next compared the wild type and the T2S mutants for their ability to elicit cytokines/chemokines from infected U937 cells.

FIG. 4. Cytokine/chemokine output from macrophages infected with wild-type, T4S mutant, and T2S mutant *L. pneumophila*. U937 cells were either not infected (dotted bars) or infected (MOI = 0.5) with wild-type strain 130b (black bars), *dotA* mutant GG105 (diagonally striped bars), or *lspF* mutant NU275 (white bars), and then at the indicated times, the levels of IL-6 (A), TNF- α (B), IL-10 (C), IL-8 (D), IL-1 β (E), and MCP-1 (F) in culture supernatants were determined by ELISA. Data are the means and standard deviations of results from triplicate wells and are representative of at least three independent experiments. The absence of some data bars in panel B is simply a reflection of the exceedingly low levels of TNF- α that were detected in some of the monolayers. Asterisks indicate those time points when the cytokine/chemokine levels elicited by the *lspF* mutant were significantly (Student's *t* test, $P < 0.05$) above those produced by wild-type infection.

Infection of macrophages with wild-type 130b led to a release of IL-6, IL-8, TNF- α , IL-10, and IL-1 β at levels that were significantly above those of the uninfected controls (Fig. 4A to E). These data were compatible with the results of previous studies that had used other types of macrophages and other wild-type strains (1, 9, 15, 23, 30, 66, 67, 69, 75, 80, 87, 103, 120). In the case of IL-6, IL-8, and IL-10, expression levels above those of uninfected controls were evident at 24 h, whereas for TNF- α and IL-1 β , the effect of *L. pneumophila* was more easily seen at 48 h. Except with IL-10, elevated levels of expression were maintained until the end of the experiment at

72 h. Among the different cytokines, IL-8 showed the highest levels in the infected culture supernatants. TNF- α and IL-1 β exhibited intermediate levels, and IL-6 and IL-10 were induced to the lowest levels. When experiments were done using the wild type inoculated at different MOIs, the amount of IL-6 elicited correlated with the size of the bacterial load in the infected monolayer, as might be expected (Fig. 5). As an additional confirmation of the validity of our assays, a nonreplicating Dot/Icm mutant of strain 130b that lacks T4S failed to trigger sustained production of IL-6, TNF- α , IL-10, and IL-1 β (Fig. 4), as had been observed in the past (23, 103, 120). The

FIG. 5. Cytokine output from U937 cells infected with differing doses of wild-type *L. pneumophila*. U937 cells were infected with wild-type strain 130b at an MOI equal to 0.05 (gray triangles or bar), 0.5 (black squares or bar), or 5.0 (white diamonds or bar), and then at 24 and 48 h postinoculation, the numbers of CFU (inset) and amounts of secreted IL-6 within the infected wells were determined. Data are the means and standard deviations of results from triplicate wells and are representative of at least two independent experiments. At 24 and 48 h, IL-6 levels triggered by infections begun with an MOI of 5.0 were significantly greater than those produced by infections with the two lower doses of bacteria (Student's *t* test, $P < 0.05$). An MOI of 0.5 in turn elicited higher levels of the cytokine at 48 h than did an MOI of 0.05 (Student's *t* test, $P < 0.05$).

dot mutation did not greatly affect the elicitation of IL-8 (Fig. 4D). Previous studies using macrophages had not assayed the effect of Dot/Icm on IL-8 production, but a result akin to ours was obtained upon *L. pneumophila* infection of A549 cells (101). Wild-type infection of the U937 cells also led to a reduction in the level of the chemokine MCP-1, relative to that in the uninfected control (Fig. 4F). This effect, which was not previously addressed in the literature, was *dot* dependent (Fig. 4F). These data suggest that *L. pneumophila* has various effects on the cytokine/chemokine output of infected macrophages.

When the U937 cells were infected with the *lspF* mutant, we observed levels of IL-6, TNF- α , IL-10, IL-8, IL-1 β , and MCP-1 that were significantly greater than those obtained after infection with the wild type (Fig. 4). The difference between 130b and its *lspF* mutant was generally evident after 48 h of incubation and uniformly present by 72 h. The mutant's altered phenotype was most easily observed when IL-6 was assayed, with the difference between the wild type and mutant being ca. 5-fold at 48 and 72 h (Fig. 4A). The mutant phenotype was evident when the bacterial inocula were prepared from cultures grown on standard BCYE agar (as for Fig. 4 and 5) or grown in BYE broth (data not shown). We do not believe that this effect was simply a manifestation of there being modestly fewer bacteria in those wells infected with the mutant than in those inoculated with the wild type, since, as noted above, fewer CFU generally correlate with lower, not higher, levels of cytokines. Also, when additional experiments were performed using a 10-fold-higher MOI for the *lspF* mutant, we still observed an elevation in cytokine levels. A complemented *lspF*

mutant elicited levels of IL-6, TNF- α , IL-10, IL-8, IL-1 β , and MCP-1 that were equivalent to those produced by the wild type (Fig. 6), confirming the finding that the elevation in cytokines/chemokines exhibited by the *lspF* mutant was due to the loss of Lsp rather than a second-site mutation. Supporting this conclusion, an *lspDE* mutant behaved similarly to the *lspF* mutant (Fig. 6).

Since previous studies have shown that *L. pneumophila* infection of lung epithelial lines also triggers the secretion of chemokines/cytokines (24, 63, 101, 116), we investigated the connection between T2S and the elicitation of IL-6, IL-8, and MCP-1 from A549 cells. Infection with the *lspF* mutant once again resulted in levels of chemokines/cytokines that were elevated compared to those produced after infection with the wild type, although the effect seen here was less dramatic than what had been observed using macrophages (Fig. 7). When the lungs of infected A/J mice were examined for levels of IL-6 and TNF- α , infection with the *lspF* mutant once again resulted in significantly higher levels of cytokines than were produced after wild-type infection (Fig. 8). Taken together, these results, coupled with our earlier data, provide an explanation for the inability of the Lsp mutants to infect the lung; i.e., the impact of T2S on lung infection is a combination of at least three factors: the promotion of growth in macrophages, the facilitation of growth in epithelia, and the dampening of the chemokine and cytokine output from infected host cells. To our knowledge, these data are the first to identify a link between T2S and the modulation of immune factors following intracellular infection.

TNF- α can preferentially limit the intracellular growth of a T2S mutant. Previous studies determined that TNF- α can act, in autocrine fashion, to render macrophages less permissive for bacterial growth (17, 68, 105). Thus, we hypothesized that the reduced intracellular growth of the T2S mutant within macrophages might be due, at least in part, to the high levels of TNF- α that are made when this mutant infects. To address this hypothesis, we examined whether the addition of antibodies directed against the TNF- α receptor could reverse to some degree the growth defect of the *lspF* mutant in U937 cells. Although this antibody treatment did not affect the recovery of wild-type bacteria, it did lead to significantly increased outgrowth of the T2S mutant (Fig. 9A). In 5/5 trials, the blockade of the TNF- α receptor produced a significant increase in the levels of mutant CFU compared to levels in the control lacking antibody, with the average increase being 1.5-fold. In contrast to these results, the addition of antibodies against the IL-6 receptor did not have any effect on the recovery of the mutant or wild type (Fig. 9B). These data indicate that, at least in the case of TNF- α , modulation of the cytokine response by T2S can directly impact the ability of *L. pneumophila* to grow within host cells.

Proteins secreted via T2S influence cytokine output from infected macrophages. To begin to identify secreted factors that are required for the dampening effect of T2S on cytokine output, U937 cells were infected with mutants that are specifically defective for the individual T2S-dependent effectors (Table 1), and then the levels of IL-6 present in culture supernatants were determined. Mutants of strain 130b that are defective for either *map*, *plaA*, *celA*, *srnA*, *lapA*, *lapB*, *plcA*, or *plcB* elicited IL-6 to the same level that the wild type did

FIG. 6. Cytokine/chemokine output from macrophages infected with T2S mutant and complemented T2S mutant *L. pneumophila*. U937 cells were either not infected (dotted bars) or infected (MOI = 0.5) with wild-type strain 130b (black bars), the *lspF* mutant NU275 (white bars), the complemented *lspF* mutant NU275(pMF) (gray bars), or the *lspDE* mutant NU258 (striped bars), and then at 48 and 72 h postinoculation the levels of IL-6 (A), TNF- α (B), IL-10 (C), IL-8 (D), IL-1 β (E), and MCP-1 (F) in culture supernatants were determined by ELISA. Data are the means and standard deviations of results from triplicate wells and are representative of at least three independent experiments. Asterisks indicate those time points when the cytokine/chemokine levels elicited by the *lspF* and *lspDE* mutants were significantly (Student's *t* test, $P < 0.05$) above those produced by wild-type infection.

(Fig. 10A), indicating that the Map phosphatase, PlaA lyso-phospholipase A, CeiA endoglucanase, SraA RNase, LapA and LapB aminopeptidases, and PlcA phospholipase C and its paralog PlcB are not required for limiting cytokine expression. In 5/7 trials, infection with the *chiA* mutant NU318 or the *plaC* mutant NU367 resulted in levels of IL-6 that were slightly below, rather than above, that of the wild type (Fig. 10B). These data suggest that the chitinase and acyltransferase might play minor roles in the elicitation of the cytokine response. In

7/7 experiments, the *lipA lipB* double mutant NU267 gave significantly greater levels of IL-6 than the wild type (Fig. 10C), and similar results were obtained when the newly made *lipA lipB* double mutants NU373 and NU374 were tested (data not shown). However, when mutants lacking either LipA or LipB were tested, none gave IL-6 levels that were statistically greater than that elicited by the wild type (Fig. 10C and data not shown). Thus, neither LipA nor LipB alone is required for the effect of T2S on cytokine output, but the two lipases might

FIG. 7. Cytokine/chemokine output from lung epithelial cells infected with wild-type and T2S mutant *L. pneumophila*. A549 cells were either not infected (dotted bars) or infected (MOI = 10) with wild-type strain 130b (black bars), *lspF* mutant NU275 (white bars), or *lspDE* mutant NU258 (striped bars [in panel A only]), and then at the indicated times the levels of IL-6 (A), IL-8 (B), and MCP-1 (C) in culture supernatants were determined by ELISA. Data are the means and standard deviations of results from triplicate wells and are representative of at least three independent experiments. Asterisks indicate points when the cytokine and chemokine levels elicited by the *lspF* and *lspDE* mutants were significantly (Student's *t* test, $P < 0.05$) above those produced by wild-type infection.

have overlapping functions which have a modest effect on cytokines that is evident only when the double mutants are examined. The data set that was most compelling was obtained when a mutant lacking the T2S-dependent metalloprotease ProA was examined. In 10/10 trials, infection with the mutant AA200 gave a level of cytokine that was significantly greater

FIG. 8. IL-6 and TNF- α levels in lungs infected with wild-type (WT) and T2S mutant *L. pneumophila*. A/J mice were intratracheally inoculated with equal numbers of either wild-type 130b (black bars) or the *lspF* mutant NU275 (white bars), and then at 24 h postinoculation, the levels of IL-6 and TNF- α per CFU in lung homogenates were determined by ELISA. Data are the means and standard deviations obtained for 9 to 10 infected animals and are representative of at least three independent experiments. The differences in cytokine levels between the wild-type- and mutant-infected lungs were significantly different (Student's *t* test, $P < 0.05$).

than that produced by infection with the wild type (Fig. 10C). A complemented mutant containing *proA* cloned into a low-copy-number vector (as well as the wild type harboring the same *proA* plasmid) did not exhibit this phenotype and produced levels of IL-6 that were comparable to those of the wild type (Fig. 10D). These data indicate that ProA, directly or indirectly, dampens the cytokine output of infected macrophages. They also support our supposition that the elevated chemokine/cytokine levels that follow infection by *Lsp* mutants are not simply an artifact of a potential cell-associated (envelope) change in the mutant. Since the *proA* mutant did not elicit a cytokine output that was as great as that produced by the *lspF* mutant (Fig. 10C and D), we posit that more yet-to-be-defined effectors are involved in limiting the cytokines/chemokines produced by host cells.

T2S limits the levels of cytokine mRNAs. To start to discern the mechanism(s) by which T2S and its effectors influence chemokine and cytokine output, we employed quantitative RT-PCR to monitor the levels of the cytokine transcript within wild-type- versus *lspF* mutant-infected U937 cells. At 24 h postinoculation, the transcript levels of IL-6 were ca. 5-fold greater in the mutant-infected monolayers than in the wild-

FIG. 9. Effect of anticytokine antibodies on *L. pneumophila* growth in macrophages. Strain 130b (WT) and mutant NU275 (*lspF*) were used to infect U937 cells that had been treated in standard fashion (black bars) or exposed to antibodies (white bars) directed against either the TNF- α receptor (α -TNFR) (A) or the IL-6 receptor (B), and then at 0 and 48 h, the numbers of CFU in the monolayers were determined. Bacterial growth is graphed as the increase in numbers of CFU (in log units) from the time of inoculation to 48 h postinoculation. Data are the means and standard deviations of results from triplicate wells and are representative of at least three independent experiments. (A) The fold difference in mutant recovery between the untreated and treated monolayers indicated by the number over the bracket was significant (Student's *t* test, $P < 0.05$).

type-infected monolayers (Fig. 11A). IL-8 mRNA levels were also significantly greater, although the effect was less dramatic (Fig. 11B). These data imply that the increases in cytokines that we had observed for *Lsp* mutant-infected monolayers were due, at least in part, to greater levels of cytokine mRNA. Thus, the *L. pneumophila* T2S system appears to, directly or indirectly, limit the transcription of cytokine genes and/or the stability of cytokine mRNA. Infection with a *proA* mutant did not result in a change in IL-6 transcript levels (Fig. 11B), implying that the effect of *ProA* on cytokine expression is at the posttranscriptional level. Taken together, these data suggest that some T2S-dependent effectors influence transcriptional events but that others, such as *ProA*, act at a subsequent step in cytokine expression.

DISCUSSION

T2S contributes to the natural history and pathogenesis of Legionnaires' disease in multiple ways. First, as previously determined, T2S is critical for *L. pneumophila* growth in water

and in amoebal hosts (52, 61, 95, 96, 107). Second, T2S is required for the ability of *L. pneumophila* to colonize the lung. Third, at least one of the known T2S-dependent substrates (52, 61, 96), *ProA*, mediates lung damage independently of *L. pneumophila* growth (31, 73). Here, we document that T2S promotes infection of the lung by at least two mechanisms. In the first case, *Lsp* facilitates bacterial growth in macrophages and epithelia, the two cell types that are present in the alveoli at the time of infection. Future studies will need to identify which secreted protein(s) is responsible for this effect, in the way that *ProA* and *SrnA* have been linked to *L. pneumophila* growth in amoebae (94, 95). In the second case, T2S dampens the cytokine/chemokine output from infected host cells. This effect is due, at least in part, to T2S-dependent effectors, because cells infected with the *ProA* mutant exhibited an increase in cytokine output relative to that of cells infected with the wild type. Since dampening of the cytokine/chemokine response would have a major impact on innate immune responses, it is now possible to understand why *L. pneumophila* T2S mutants are dramatically impaired *in vivo*, despite being only modestly impaired during *in vitro* intracellular infections. Although the promotion of growth in macrophages and epithelia and the dampening of the cytokine and chemokine response explain much of the importance of T2S for infection of the lung, more processes are likely at work. For example, the *ChiA* chitinase is not required for intracellular growth or the dampening of the cytokine response, but it still promotes *L. pneumophila* survival in the lung (34). Overall, *L. pneumophila* is providing significant new insights into how T2S promotes pathogenesis and host cell interactions. We suspect that many of the observations made here will have implications for other bacterial pathogens that express T2S but have not been investigated to a similar extent (27, 28).

By utilizing multiple mutants derived from a virulent clinical isolate of *L. pneumophila*, we have demonstrated that T2S is able to limit (but not abolish) the output of IL-6, TNF- α , IL-1 β , and IL-8 from both infected U937 cell macrophages and A549 epithelial cells. IL-6, TNF- α , IL-1 β , and IL-8 (and its murine equivalent) are major proinflammatory cytokines modulating both local and systemic inflammatory responses, helping to recruit and activate leukocytes and thereby fostering both the further production of cytokines and increased bactericidal activity (71, 104). TNF- α and IL-1 β also activate endothelia to trigger vasodilation and increase the permeability of blood vessels, allowing the import of serum proteins and leukocytes into infected sites (71). IL-6, TNF- α , and IL-1 β also trigger hepatocytes to release acute-phase proteins, which, in turn, activate complement and promote opsonization (71). Thus, the limitation of these four cytokines is predicted to greatly enhance *L. pneumophila* growth *in vivo* as well as the associated pathology. Support for our hypothesis comes from multiple sources. First, IL-6, TNF- α , IL-1 β , and IL-8 are in fact all induced upon lung infection by *L. pneumophila*, based on the examination of patients with Legionnaires' disease and experimentally infected A/J mice (20, 26, 41, 109, 112, 113). *In vivo* induction of IL-6, TNF- α , and IL-1 β was also examined and documented when *L. pneumophila* was used to infect the lungs of C57BL/6, BALB/c, and BDF1 mice (7, 15, 45, 48, 53, 103). Second, when the levels of TNF- α and its receptor are experimentally manipulated, there is a corresponding change

FIG. 10. IL-6 output from macrophages infected with mutants of *L. pneumophila* lacking individual T2S-dependent effectors. (A to D) U937 cells were infected (MOI = 0.5) with wild-type strain 130b, the *lspF* mutant NU275, or mutants specifically lacking the indicated T2S substrate(s), and then at 72 h postinoculation, the levels of IL-6 in culture supernatants were determined by ELISA. Data are the means and standard deviations of results from triplicate wells and are representative of at least three independent experiments. Asterisks indicate those mutants that elicited a cytokine level that was significantly different (Student's *t* test, $P < 0.05$) from that triggered by the wild type. Related to the data in panel A, mutants NU268, NU369, and NU370, lacking just *plcA* or *plcB*, behaved similarly to *plcA plcB* double mutants, including strain NU371, which is shown in the figure, and did not show a phenotype that was distinct from that of the wild type (data not shown).

in *L. pneumophila* growth in the lungs of A/J mice and other animals (16, 20, 47). Third, addition of TNF- α renders macrophages less permissive for *L. pneumophila* and neutrophils more bactericidal (17, 68, 105). Fourth, in humans, anti-TNF agents given to treat inflammatory diseases are a risk factor for Legionnaires' disease (12, 56). Fifth, neutrophils, whose levels in infected tissue are controlled by these cytokines, contribute to the clearance of *L. pneumophila* from the lungs of A/J mice and other infected animals, albeit in a manner that goes beyond direct bactericidal activity (42, 45, 53, 110, 114). Sixth, mice that have limited cytokines because of a mutation that abolishes a Toll-like receptor(s) (TLR) show increased susceptibility to *L. pneumophila* lung infection (8, 9, 48, 54). Finally, an *L. pneumophila pilD* mutant that lacks T2S along with several other factors elicits more exudation after inoculation into the lungs of guinea pigs than wild-type *L. pneumophila* (61, 96). Thus, *L. pneumophila* growth in the lungs is likely potentiated by an ability of T2S to dampen the output of IL-6, TNF- α , IL-1 β , and IL-8 from macrophages and epithelia. The mechanisms for this enhanced growth are predicted to include the maintenance of permissive macrophages (as supported by the enhanced growth of the T2S mutant that we observed when U937 cells were treated with anti-TNF- α receptor), limitations on killing by neutrophils, and subversion of complement, among other possibilities.

We also observed that *L. pneumophila* T2S dampens (but does not abolish) the output of IL-10 and MCP-1 from infected macrophages and epithelia, and previous studies have shown that IL-10 and MCP-1 are induced *in vivo* upon *L. pneumophila* infection (7, 13, 21, 41, 109). However, MCP-1 is known for being a chemoattractant for monocytes and macrophages (37), i.e., potential host cells for *Legionella*, and IL-10 is an anti-inflammatory cytokine, suppressing the expression of the Th1 cytokines (70, 87, 119). Therefore, it seems that by dampening IL-10 and MCP-1, T2S reduces, rather than enhances, *L. pneumophila* growth. But, it is quite likely that the limiting of IL-6, TNF- α , IL-1 β , and IL-8 by T2S outweighs the effects of IL-10 and MCP-1. Thus, the overall impact of T2S is still one of growth promotion in the lung. Although we focused our analysis on IL-6, TNF- α , IL-1 β , IL-8, MCP-1, and IL-10, it is quite possible that T2S influences other cytokines or chemokines that are produced by macrophages or epithelia. Indeed, *L. pneumophila* infection of A/J mouse lungs also results in the production of IL-4, IL-12, IL-18, IFN- γ , keratinocyte chemoattractant (KC), MIP-2, and lipopolysaccharide (LPS)-induced CXC chemokine (LIX) (18, 19, 26, 36, 109, 112, 114), and *in vitro* infections revealed the additional induction of MCP-3, MIP-1 α , Mip-1 β , IL-2, IL-5, IL-7, IL-13, and IL-17 as well as prostaglandins and defensins (78, 84, 98, 101). In addition, *L. pneumophila* infection triggers the secretion of type I IFNs (62,

FIG. 11. Cytokine transcripts from macrophages infected with wild-type and T2S mutant *L. pneumophila*. U937 cells were either not infected or infected with wild-type 130b (black bars in panels A to C), *lspF* mutant NU275 (white bars in panels A to B), or *proA* mutant AA200 (checkered bars in panel C), and then at 4 and 24 h postinoculation, the levels of IL-6 (A and C) and IL-8 (B) transcripts were determined by quantitative RT-PCR. Plotted on the y axis is the fold induction of the cytokine transcript in infected cells over the level of induction in the uninfected control. Data are means and standard deviations of results from triplicate wells and are representative of at least three independent experiments. Asterisks indicate statistically significant differences (Student's *t* test, $P < 0.05$) between the wild type and *lspF* mutant.

77, 85), and it has been argued that, upon infection of macrophages, retinoid-inducible gene 1 (RIG-I) and MDA5 act as cytosolic sensors of *Legionella* RNA in order to initiate IRF3-dependent induction of IFN- β (76). Thus, investigations into the many other host factors that are induced upon intracellular infection by *L. pneumophila* will provide an even fuller appreciation of the impact of T2S on the innate immune response.

Based upon our qRT-PCR analysis of IL-6 and IL-8 in wild-type- versus *lspF* mutant-infected macrophages, *L. pneumophila* T2S dampens the levels of cytokine mRNA in infected host cells. Because the *proA* mutant did not show altered transcript levels but did exhibit a partially impaired ability to dampen cytokine output, some of the effect of T2S must also occur at a posttranscriptional level. A number of mechanisms may explain the effect of T2S on cytokine mRNA. In the first scenario, T2S, directly or indirectly, reduces the recognition of pathogen-associated molecular patterns (PAMPs) by host surface or endosomal TLRs and/or cytosolic nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and thereby leads to a dampening of signal transduction events that result in optimal transcription of the cytokine genes. In the case of *L. pneumophila*, TLR2 recognizes lipopolysaccharide (LPS) and lipoprotein, TLR5 flagellin, TLR9 CpG sequences in DNA, NOD1 and NOD2 peptidoglycans, and Naip5 and Ipaf flagellin (1, 3, 7, 9, 13, 23, 30, 45, 48, 53, 54, 60, 65, 75, 81, 92, 102, 103, 117, 120). In the second scenario, T2S compromises a step(s) in the signal transduction pathways that result after TLR/NLR ligation, including transcription itself. For *L. pneumophila* infection of macrophages and epithelial cells, signal transduction includes the NF- κ B pathway, multiple mitogen-activated protein kinase (MAPK) pathways, and the transcriptional activators NF- κ B and AP-1 (7, 11, 65, 100, 101, 103, 116). Finally, as a third scenario to explain our RT-PCR data, a T2S activity might decrease the stability of some of the cytokine mRNAs. As possible explanations for the posttranscriptional effect of *Lsp*, a T2S-dependent effector(s) may limit cytokine mRNA translation, reducing the secretion of the cytokine and/or degrading the cytokine after it is secreted. In the case of *L. pneumophila*, NLRs promote the formation of inflammasomes, in which activated caspase-1 cleaves IL-1 β , IL-18, and caspase-7 from their proforms to their mature forms (3, 4, 23, 120). Thus, it is possible that the effect of T2S on IL-1 β also involves reductions in the inflammasome pathway.

In an attempt to explain the posttranscriptional impact of ProA on cytokines, the simplest hypothesis is that the protease directly degrades the immune factors. Indeed, ProA is a metalloprotease with broad substrate specificity, and previous studies have shown that purified ProA can degrade TNF- α as well as other host proteins, including the cytokine IL-2 and the acute-phase protein α -1-antitrypsin (32, 55, 72). How ProA (or any of the other relevant T2S effectors, for that matter) reaches its target is a key question to consider. On the one hand, ProA might be delivered into the tissue culture supernatant upon lysis of the infected host cell and then degrade cytokine that had been previously secreted. As a variation on this theme, ProA might be exported from the infected host cell prior to or in the absence of host cell lysis, akin to the recently described export of *Salmonella enterica* serovar Typhi CdtB from infected epithelia (108). On the other hand, ProA might be secreted by legionellae that (temporarily) reside extracellularly after lysis of the infected host cell. Finally, since ProA is expressed in infected macrophages and can be seen to some extent in the host cytoplasm (91), the effector may degrade the cytokine while both reside in the intracellular space.

Another question is the identities of the secreted effectors that mediate the effect of T2S on cytokine gene transcription and any posttranscriptional effects that are not linked to ProA.

The fact that the T2S output of *L. pneumophila* contains more than 25 proteins, including novel proteins that have no similarity to known proteins (27, 34, 88), identifies many candidates for future investigation. Based upon our current observations with the *lipA lipB* double mutants, some T2S-dependent effectors will likely prove to be functionally redundant, influencing cytokines without being absolutely required. In light of our findings with the *chiA* and *plaC* mutants, other T2S substrates might stimulate, rather than dampen, the cytokine response. As previously published (23, 64, 103, 120) and as confirmed here, the ability of *L. pneumophila* to induce a cytokine response is dependent, to a large extent, on the bacterium's Dot/Icm T4S system. Thus, part of the effect of T2S on cytokine and chemokines could be indirect, involving a dampening of the T4S system. Overall, the effect of T2S on cytokine and chemokine output is a combination of multiple factors that target different aspects of the host cell in different ways.

In recent years, there have been notable examples of other bacteria, including other intracellular parasites, influencing the cytokine/chemokine response (14, 90, 97). *Brucella* species produce TcpB (also known as Btp1), which inhibits TLR2 and TLR4 action by inducing the degradation of the signaling adapter MAL (MyD88 adaptor-like), or TIRAP (TIR domain-containing adaptor protein) (35). In *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*, the type III secretion (T3S) system secretes YopJ/YopP, which inhibits the activation of the NF- κ B transcription factors by acetylating I κ B kinase (IKK), a key protein in cytosolic signal transduction (14, 90, 97). Another *Yersinia* effector, YopK, prevents inflammasome activation in response to the T3S system (22). In the case of *Shigella flexneri*, two T3S effectors, OspB and OspF, conspire to disable signal transduction pathways by, among other things, acting within the nucleus upon chromatin remodeling (14, 97, 121). Finally, for *Salmonella enterica* serovar Typhimurium, the T3S effector SseL is a deubiquitinase that suppresses the ubiquitination of I κ B α , which is necessary for NF- κ B activation (59). The effects of *L. pneumophila* T2S and its substrate ProA on cytokines are comparable in magnitude to those in previous reports; e.g., OspF and YopJ have ca. 2- and 4-fold effects on IL-8 expression, respectively, and SseL has a 2- to 5-fold impact on TNF- α and IL-1 β (59, 99, 121). Thus, *L. pneumophila* and T2S-dependent effectors can now be added to the growing list of bacterial factors that inhibit the proinflammatory, innate immune responses of infected host cells.

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