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The Type II Secretion System of *Legionella pneumophila* Dampens the MyD88 and Toll-Like Receptor 2 Signaling Pathway in Infected Human Macrophages

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ABSTRACT Previously, we reported that mutants of *Legionella pneumophila* lacking a type II secretion (T2S) system elicit higher levels of cytokines (e.g., interleukin-6 [IL-6]) following infection of U937 cells, a human macrophage-like cell line. We now show that this effect of T2S is also manifest upon infection of human THP-1 macrophages and peripheral blood monocytes but does not occur during infection of murine macrophages. Supporting the hypothesis that T2S acts to dampen the triggering of an innate immune response, we observed that the mitogen-activated protein kinase (MAPK) and nuclear transcription factor kappa B (NF-κB) pathways are more highly stimulated upon infection with the T2S mutant than upon infection with the wild type. By using short hairpin RNA to deplete proteins involved in specific pathogen-associated molecular pattern (PAMP) recognition pathways, we determined that the dampening effect of the T2S system was not dependent on nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible protein I (RIG-I)-like receptors (RLRs), double-stranded RNA (dsRNA)-dependent protein kinase receptor (PKR), or TIR domain-containing adaptor inducing interferon beta (TRIF) signaling or an apoptosis-associated speck-like protein containing a CARD (ASC)- or caspase-4-dependent inflammasome. However, the dampening effect of T2S on IL-6 production was significantly reduced upon gene knockdown of myeloid differentiation primary response 88 (MyD88), TANK binding kinase 1 (TBK1), or Toll-like receptor 2 (TLR2). These data indicate that the *L. pneumophila* T2S system dampens the signaling of the TLR2 pathway in infected human macrophages. We also document the importance of PKR, TRIF, and TBK1 in cytokine secretion during *L. pneumophila* infection of macrophages.

KEYWORDS *Legionella pneumophila*, TLR2, cytokine, innate immunity, macrophage, type II secretion

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Legionella pneumophila, a Gram-negative bacterium that is widespread in aquatic habitats, is the principal agent of Legionnaires' disease pneumonia (1–6). In the lungs, *Legionella* bacteria invade and grow in resident macrophages and then trigger severe inflammation (2). In macrophages, *L. pneumophila* evades the degradative lysosomal pathway and replicates to large numbers within a membrane-bound vacuole, the *Legionella*-containing vacuole (LCV) (7, 8). Two protein secretion systems, Lsp type II secretion (T2S) and Dot/Icm type IV secretion (T4S), play major roles in the pathogenesis of *L. pneumophila* (9, 10). In T2S, protein substrates are first translocated across the inner membrane, and upon the action of the T2S pilus-like apparatus, they then exit the bacterial cell through a specific outer membrane pore (11). Using proteomics and enzymatic assays, we have shown that the T2S system of *L. pneumo-*

phila secretes >25 proteins, including 18 confirmed enzymes and novel proteins, which, in some instances, appear to be unique to *Legionella* (12, 13). In the aquatic environment, T2S promotes *L. pneumophila* survival at low temperatures and is critical for infection of at least four genera of amoebae (13–15). In mammalian hosts, T2S contributes to both intracellular infection of macrophages and the destruction of lung tissue. *L. pneumophila lsp* mutants that lack T2S have an ~10-fold reduction in intracellular growth in both U937 cells, a human macrophage-like cell line, and murine macrophages obtained from A/J mice (16–18). Data from our laboratory have also shown that this reduction in CFU is not due to an entry defect or increased degradation through the phagosome-lysosome pathway but is instead due to a replication defect in LCVs at 4 to 12 h postentry (19).

During intracellular infection of macrophages, *L. pneumophila* triggers the production of cytokines (20). As is often the case with bacterial infections, this process is initiated in part by the recognition of pathogen-associated molecular patterns (PAMPs) by host surface or endosomal Toll-like receptors (TLRs), cytosolic nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible protein I (RIG-I)-like receptors (RLRs), and inflammasomes (21–23). Upon PAMP recognition, signal transduction events activate the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, causing the transcriptional activators NF-κB and activator protein 1 (AP-1) to induce cytokine gene transcription (23). For the most part, the pathways with which *L. pneumophila* interacts upon infection were discovered through studies in mice, using either infection of mice lacking the relevant innate immune pathway or *in vitro* infections of bone marrow-derived (BMD) macrophages obtained from these knockout mice (22, 24). However, a difference in the interactions of *L. pneumophila* with the innate immune system in human cells versus murine cells, i.e., the role of NAIP5 in restricting *L. pneumophila* growth in murine cells but not in human cells, has been reported (25, 26).

Interestingly, human U937 macrophages infected with *L. pneumophila lsp* mutants that lack T2S (but not a complemented *lsp* mutant) produce higher levels of cytokines than do U937 cells infected with the wild-type (WT) strain (16). We also observed that *lsp* mutant-infected macrophages contain elevated levels of cytokine (e.g., interleukin-6 [IL-6]) mRNAs (16). The increase in the levels of cytokines seen with the T2S mutant was not a result of there being modestly fewer CFU in the mutant monolayers, since fewer CFU, whether of the WT or the *lsp* mutant, result in lower, not higher, cytokine levels. Also, when a nonreplicating *dotA* T4S mutant, which is delivered to the degradative lysosomal pathway, was examined, lower, rather than higher, levels of cytokines were seen (16). Finally, as noted above, the *lspF* mutant is not trafficked to the phagolysosome in either human or murine macrophages (19). Based on these data, we posited that *L. pneumophila* T2S dampens host signal transduction and cytokine gene transcription. Here, we show, among other things, that T2S limits signaling through the MyD88-TLR2 pathway, affecting both NF-κB and MAPK activation. Interestingly, the dampening effect of T2S was not observed in murine macrophages, indicating that PAMP recognition is more distinct between human and mice than previously reported.

RESULTS

T2S dampens the cytokine response of multiple types of human macrophages but not murine macrophages. We previously demonstrated that *L. pneumophila* T2S mutants triggered more cytokine production following infection of U937 cells than did the parental WT strain 130b (16). To validate these results, we assessed the effect of T2S on IL-6 production by the human macrophage-like cell line THP-1 as well as peripheral blood mononuclear cells (PBMCs) obtained from human volunteers. As is the case for U937 cells, THP-1 cells and PBMCs are widely utilized for studying the interaction between *L. pneumophila* and macrophages (27–31). For both of these host cells, infection with an *lspF* mutant that lacks T2S resulted in levels of IL-6 that were significantly higher than those obtained following infection with WT strain 130b (Fig. 1A and B). Furthermore, the magnitudes of the effect of T2S were comparable

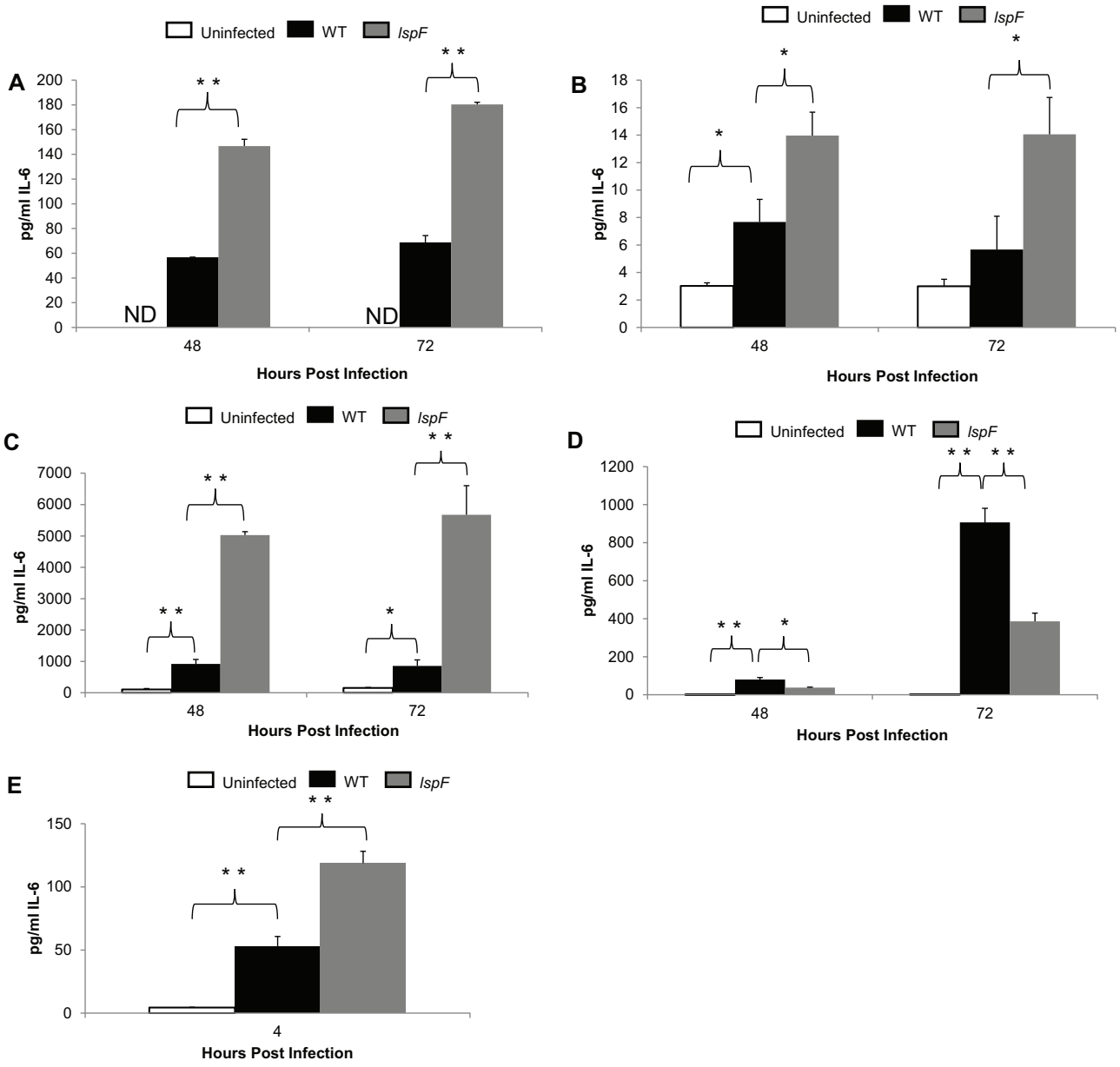

FIG 1 IL-6 output from various types of macrophages infected with *L. pneumophila* WT and T2S mutant strains. (A to D) THP-1 cells (A), PBMCs (B), U937 cells (C), and A/J BMD macrophages (D) were either not infected or infected with WT strain 130b or *lspF* mutant strain NU275 at an MOI of 0.5 (A to C) or 1.0 (D), and at 48 and 72 h postinoculation, the levels of IL-6 in the culture supernatants of the monolayers were determined by an ELISA. (E) U937 cells were either not infected or infected with WT strain 130b or *lspF* mutant strain NU275 at an MOI of 10, and at 4 h postinoculation, the levels of secreted IL-6 were determined. For panels A, C, D, and E, data are the means and standard deviations of results from triplicate wells and are representative of results from at least three independent experiments. The PBMC experiments were done twice with at least duplicate wells. Asterisks indicate those points at which the IL-6 levels elicited were significantly different between two samples (*, $P < 0.05$; **, $P < 0.01$ [by Student's *t* test]). ND, not detected.

across the macrophage types, ranging from ~3-fold for THP1 cells and PBMCs to ~5-fold for U937 cells (Fig. 1C). As we previously observed when infecting U937 cells (16, 18), the T2S mutant displayed modestly impaired growth in THP-1 cells and PBMCs (see Fig. S1 in the supplemental material). However, the increases in cytokine levels observed after infection with the *lspF* mutant were unlikely to have been a manifestation of there being fewer CFU in the monolayer, since fewer legionellae typically result in lower, not higher, cytokine levels (16). Taken together, these data suggested that the T2S system of *L. pneumophila* is able to dampen the cytokine output of multiple types of human macrophage-like cells.

To determine if the influence of T2S on cytokine production extends beyond human cell types, we performed infection assays using BMD macrophages obtained from A/J

mice. Interestingly, infection with the mutant lacking T2S resulted in less, not more, IL-6 in murine macrophages (Fig. 1D). Thus, the dampening effect of *L. pneumophila* T2S on cytokine expression is not manifest in all species' macrophages and may be specific to human host cells.

Since all of our assessments of secreted cytokines had been done at 24, 48, or 72 h postinoculation (Fig. 1A to C) (16), we tested whether the dampening effect of T2S can be detected early in the intracellular infection process. To this end, we amplified the number of infected macrophages by increasing the dose of inoculating bacteria such that the ratio of legionellae to host cells was 10 rather than 0.5. Since U937 cells gave the most robust IL-6 response overall (compare Fig. 1C to A and B), we did this experiment, as well as subsequent ones, utilizing this macrophage type. When the supernatants of infected U937 cells were then sampled at 4 h postinoculation, we again observed significantly more IL-6 expression following infection with the *lspF* mutant than following infection with WT strain 130b (Fig. 1E). These data suggested that the dampening effect of T2S on the cytokine response begins during the initial rounds of infection within the macrophage monolayer.

The differences in cytokine levels that we observed could be explained by either a natural dampening of the macrophage's innate immune response by the T2S system of WT bacteria or an artificial hyperactivation of innate immunity by the *lspF* mutant as a result of possible increased lysis. The finding that the higher cytokine levels associated with mutant strains are not evident in all species of macrophages suggested that they are not an unspecific by-product of the *lspF* mutation. The finding that the *lspF* mutant does not traffic more readily to the phagolysosome than the WT further suggests that it is not subject to greater degradation (19). Nonetheless, to determine if the *lspF* mutant undergoes increased lysis upon infection of human macrophages, we infected U937 cells with WT and mutant bacteria expressing cell-associated green fluorescent protein (GFP) and then assayed levels of GFP released from bacteria. We observed similar levels of GFP when we compared the lysates obtained from WT-infected cells to those obtained from mutant-infected cells (see Fig. S2 in the supplemental material), indicating that the T2S mutant does not undergo more lysis. Additional support for this conclusion comes from the fact that some but not all innate immune pathways are affected by the loss of T2S (see below). Thus, we concluded that the T2S system actively diminishes the expression of IL-6 by human macrophages infected with *L. pneumophila*.

T2S decreases stimulation of the MAPK and NF-κB pathways. Given that NF-κB and AP-1 are transcription factors that regulate the activation and secretion of proinflammatory cytokines, we hypothesized that T2S inhibits signaling through one or both of these innate immunity pathways. Thus, U937 cells were infected with WT strain 130b or the *lspF* mutant and examined for levels of phosphorylated p38, stress-activated protein kinase (SAPK)/Jun amino-terminal kinase (JNK), and extracellular signal-regulated kinase 1/2 (ERK1/2), three adaptors within the MAPK pathway that are activated during *L. pneumophila* infection of murine and human macrophages (32–34). In all three cases, WT infection resulted in increased levels of phosphorylated proteins relative to those of the uninfected control (see Fig. S3A to S3C in the supplemental material). Compatible with our hypothesis, levels of phosphorylated p38 and SAPK/JNK were significantly higher during infection with mutant bacteria than during infection with the WT (Fig. 2A and B). In contrast, the levels of phosphorylated ERK1/2 were higher in WT-infected cells (Fig. 2C). This net effect suggested that signaling directed at the AP-1 transcription factor is negatively affected by the T2S system. Hence, components of the AP-1 transcription factor, i.e., c-Jun and activating transcription factor 2 (ATF2), were also examined for their phosphorylation status. As expected, WT infection resulted in increased levels of the activated AP-1 components relative to those of the uninfected control (Fig. S3D and S3E). Moreover, both c-Jun and ATF2 had decreased levels of phosphorylation during strain 130b infection versus *lspF* mutant infection (Fig. 2D and E), implying decreased AP-1 activation during WT infection. Together, these

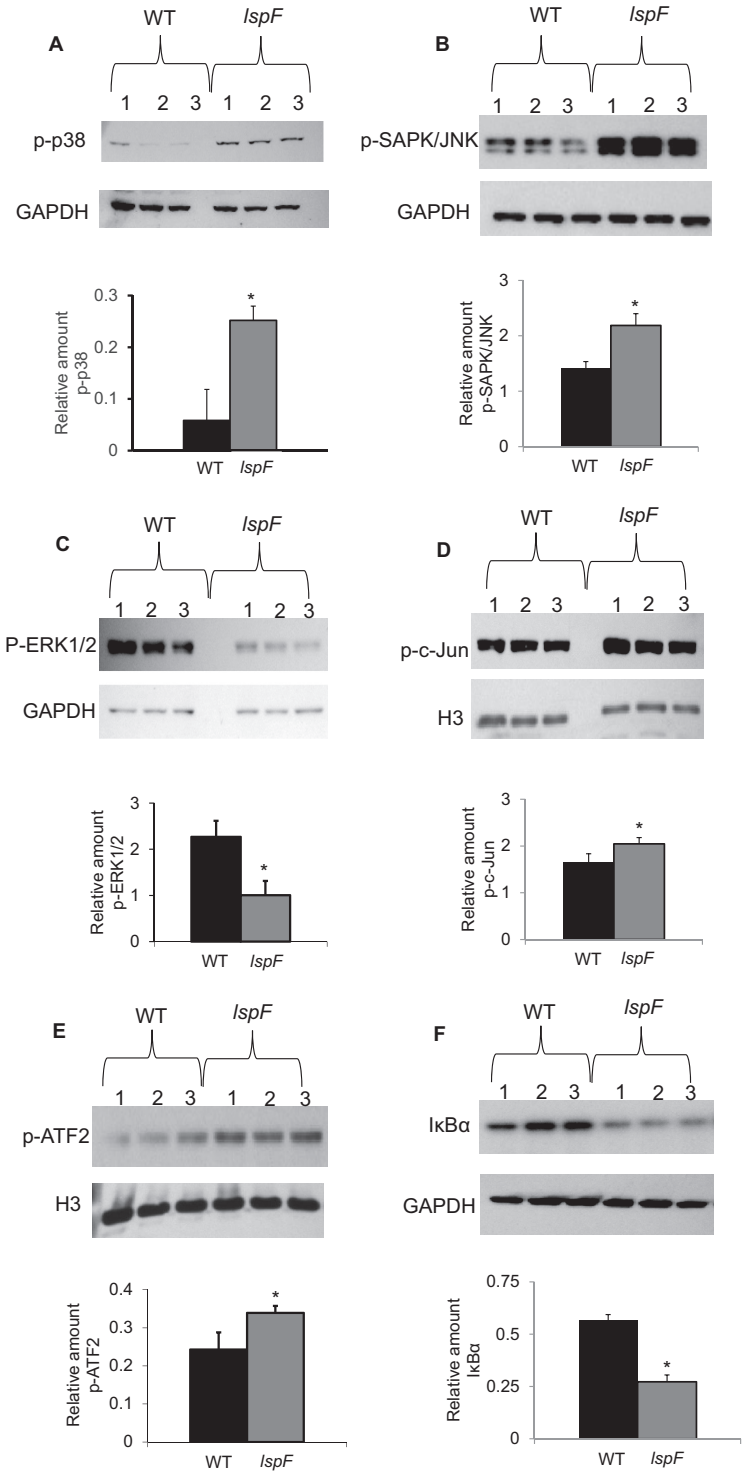

FIG 2 Activation levels of proteins in the MAPK and NF-κB pathways induced by infection of U937 cells with *L. pneumophila* WT and T2S mutant strains. U937 cells were infected with either WT strain 130b or *lspF* mutant strain NU275, and at 4 h postinoculation, infected cell lysates ($n = 3$) were examined by immunoblotting for the presence of p-p38 (A), p-SAPK/JNK (B), p-ERK1/2 (C), p-c-Jun (D), p-ATF2 (E), and IκBα (F). The graph that appears below each of the immunoblot images depicts the relative levels of the proteins, as normalized against GAPDH or H3 controls, in WT-infected cells (black bars) versus mutant-infected cells (gray bars). Data are the means and standard deviations of results from triplicate wells and are representative of results from three independent experiments. Asterisks indicate points at which values for the samples from WT-infected cells were significantly different from those for samples from mutant-infected cells (*, $P < 0.05$ by Student's *t* test).

data indicated that signaling through the MAPK pathway is dampened by the *L. pneumophila* T2S system.

A second facet of innate immunity that controls cytokine transcription and is triggered by *L. pneumophila* infection in murine and human cells is signaling through NF-κB (32, 33, 35–39). Thus, we assessed levels of IκBα, the inhibitor of NF-κB that keeps the transcription factor sequestered in the cytoplasm of the cell. WT-infected cells displayed decreased IκBα levels relative to those of the uninfected control (see Fig. S3F in the supplemental material). Increased levels of IκBα were detected in U937 cells infected with WT strain 130b compared to cells infected with the *lspF* mutant (Fig. 2F), indicating that T2S limits the levels of NF-κB in the nuclei of *L. pneumophila*-infected macrophages. In sum, the dampening of both the MAPK and NF-κB pathways would explain, at least in part, why cytokine levels are lower in WT-infected cells than in T2S mutant-infected cells.

NLR and RLR signaling is necessary for optimal IL-6 secretion during *L. pneumophila* infection of human macrophages but is not required for the dampening effect of T2S on cytokine production. The noninflammasome NLRs NOD1 and NOD2 are stimulated during *L. pneumophila* infection. This has been shown in the context of lung infection of knockout mice and infected BMD macrophages obtained from knockout mice as well as a human epithelial cell line (33, 40–43). The activation of the MAPK and NF-κB pathways is in part dependent upon NOD signaling, which is contingent upon RIP2 (44, 45). Thus, in order to assess the impact of NOD signaling on *L. pneumophila* infection of human macrophages as well as T2S-mediated dampening, we made a stable RIP2 knockdown U937 cell line with a 72% decrease in the protein level, as shown by immunoblotting (Fig. 3A). We infected these cells, along with control cells expressing a nontargeting short hairpin RNA (shRNA) ("scramble") plasmid, with WT strain 130b as well as the T2S *lspF* mutant and then checked for both bacterial growth and IL-6 secretion. While a previous study reported an increase in WT *L. pneumophila* growth in the lungs of NLR-deficient mice (41), we did not find any impact of the NLR pathway on the intracellular CFU of either WT or T2S mutant bacteria (see Fig. S4A in the supplemental material). Knockdown of RIP2, however, resulted in a decrease in IL-6 production following 24 h of WT infection (Fig. 3B, top), showing, for the first time, that RIP2 is necessary for optimal cytokine stimulation during *L. pneumophila* infection of human macrophages. However, the ratio of cytokine output of WT-infected cells to that of T2S-mutant infected cells did not change as a result of the knockdown (Fig. 3B, bottom, for values normalized to the scramble controls; see also Fig. S5A for absolute values), indicating that the NLR pathway is not required for the T2S-dependent dampening of the cytokine response.

In addition to triggering NLRs, *L. pneumophila* stimulates the RLR pathway, which is also linked to the type I interferon (IFN) response. This is based on studies using BMD macrophages from knockout mice and knockdown of RLR pathway adaptors in human epithelial cells (46–52). In order to judge the influence of RLR signaling on *L. pneumophila* infection of human macrophages as well as T2S-mediated dampening of innate immunity, we examined infected U937 cells that were knocked down for receptors and adaptors in the RLR pathway. Three stable knockdown cell lines were created with a 91% decrease for RIG-I, a 98% decrease for MDA-5, or a 52% decrease for MAVS (Fig. 4A). These knockdown cells were normal for WT and T2S mutant growth (see Fig. S4B to S4D in the supplemental material). Since *L. pneumophila*-infected U937 cells did not exhibit an induction of IFN-β transcripts or the secretion of detectable levels of IFN-β (data not shown), we surmised that the type I interferon response is not germane to the effect that we are studying, and therefore, we continued to use IL-6 as our marker for cytokine output. While the knockdown of the receptors RIG-I and MDA-5 did not alter cytokine signaling following WT infection (Fig. 4B and C, top), the knockdown of the downstream adaptor MAVS decreased the IL-6 output from WT-infected cells (Fig. 4D, top), demonstrating, for the first time, the importance of MAVS in cytokine signaling by *L. pneumophila*-infected human macrophages. However, the knockdown of RIG-I and MDA5 and their adaptor MAVS did not decrease the ratio of the IL-6 output from

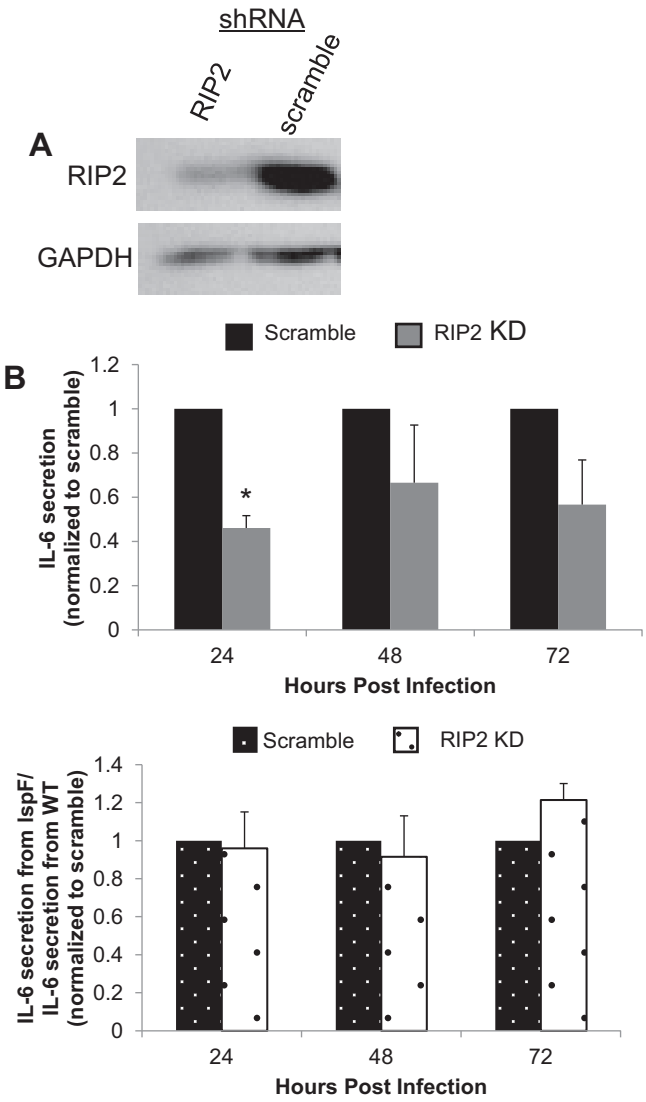

FIG 3 Effect of RIP2 depletion on cytokine production triggered by *L. pneumophila* WT and *lspF* mutant strains in macrophages. (A) Lysates obtained from U937 cells containing either an shRNA targeting RIP2 or a nontargeting scramble shRNA were subjected to immunoblot analysis using an anti-RIP2 antibody (top) or an anti-GAPDH antibody (bottom) as a loading control. (B, top) Scramble U937 cells or RIP2 knockdown (KD) U937 cells were infected with WT strain 130b, and the levels of IL-6 in culture supernatants at 24, 48, and 72 h postinoculation were then determined by an ELISA. IL-6 levels elicited upon infection of scramble cells were set to a value of 1, and levels in infected knockdown cells were normalized to the value for scramble cells. (Bottom) Scramble U937 cells or RIP2 knockdown U937 cells were infected with WT strain 130b or *lspF* mutant strain NU275, and IL-6 levels in culture supernatants at 24, 48, and 72 h were then determined by an ELISA. The graph shows the ratio of IL-6 levels from mutant-infected cells to IL-6 levels from WT-infected cells. The IL-6 ratio obtained from infection of scramble cells was set to a value of 1, and the ratio from infection of RIP2 knockdown cells was normalized to the value for scramble cells. Graphs show the average IL-6 levels ($n = 3$) pooled from three independent experiments, with standard errors. Asterisks indicate points at which the values for samples from knockdown cells were significantly different from those for samples from scramble cells (*, $P < 0.05$ by Student's *t* test).

mutant-infected cells to that from WT-infected cells (Fig. 4B to D, bottom, for values normalized to the scramble control; see also Fig. S5B to S5D for absolute values). The ratio for MAVS knockdown macrophages actually increased, perhaps because the T2S system secretes a PAMP or secretes an enzyme that generates a PAMP that stimulates this pathway. Overall, this data set indicates that the RLR pathway is not required for the T2S-dependent dampening of IL-6.

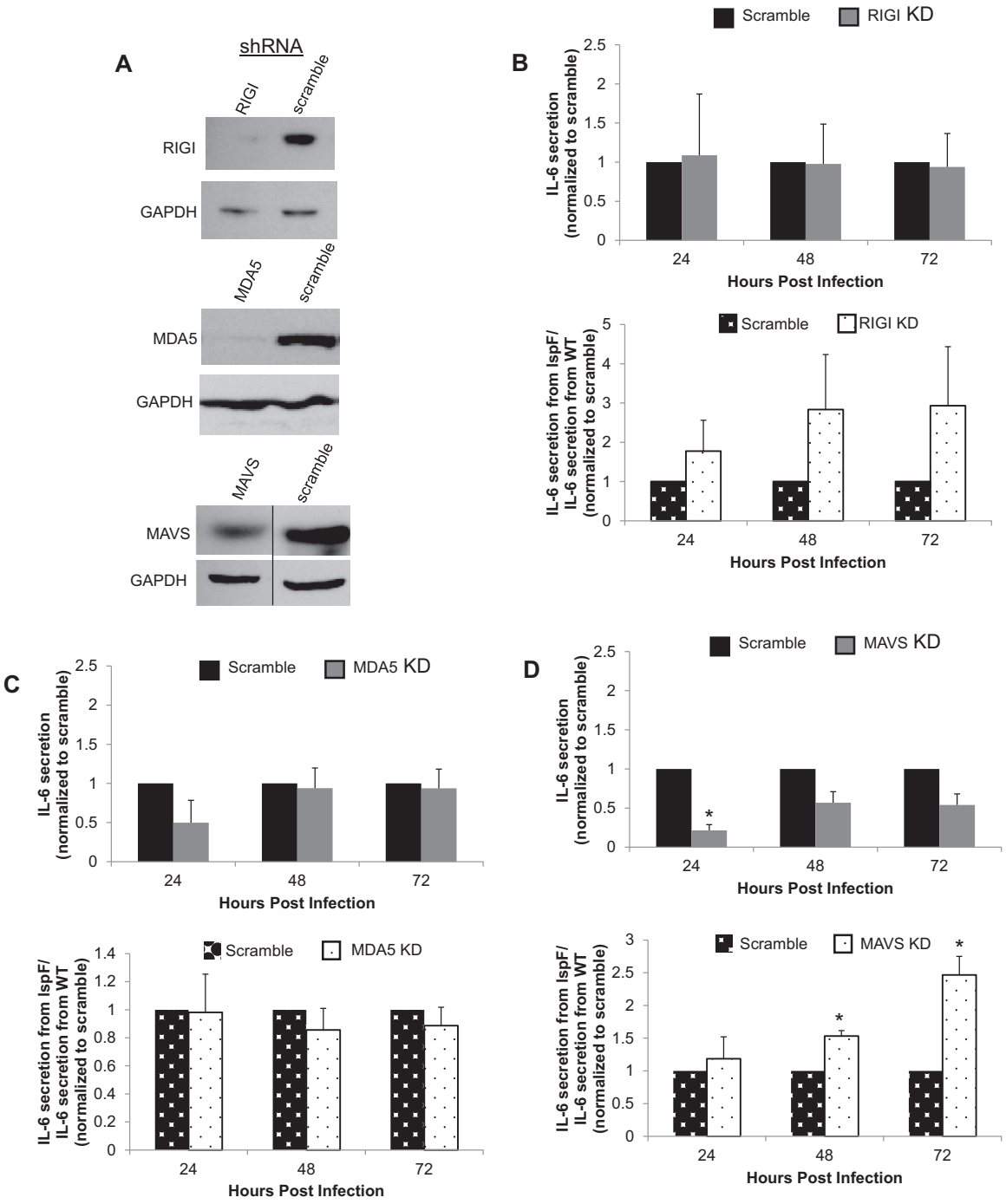

FIG 4 Effect of RIG-I, MDA-5, or MAVS depletion on cytokine production triggered by *L. pneumophila* WT and *lspF* mutant strains in macrophages. (A) Lysates obtained from U937 cells containing either an shRNA targeting RIG-I, MDA5, or MAVS or a nontargeting scramble shRNA were subjected to immunoblot analysis using an anti-RIG-I, anti-MDA5, or anti-MAVS antibody (top) or an anti-GAPDH antibody (bottom) as a loading control. Although the samples used to assay MAVS knockdown were examined on the same immunoblot, they were not in adjacent lanes, and therefore, we cropped out the intervening lane in order to prepare the figure. (B to D, top) Scramble U937 cells or RIG-I (B), MDA-5 (C), or MAVS (D) knockdown cells were infected with WT strain 130b, and the levels of IL-6 in culture supernatants were then determined by an ELISA. IL-6 levels elicited upon infection of scramble cells were set to a value of 1, and levels from infected knockdown cells were normalized to the value for scramble cells. (Bottom) Scramble cells or RIG-I (B), MDA-5 (C), or MAVS (D) knockdown cells were infected with WT strain 130b or *lspF* mutant strain NU275, and IL-6 levels in culture supernatants were then determined. The graph shows the ratio of IL-6 levels from mutant-infected cells to IL-6 levels from WT-infected cells. The IL-6 ratio obtained from infection of scramble cells was set to a value of 1, and the ratio from infection of knockdown cells was normalized to the value for scramble cells. Graphs show the average IL-6 levels ($n = 3$) pooled from three independent experiments, with standard errors. Asterisks indicate points at which values for samples from knockdown cells were significantly different from those for samples from scramble cells (*, $P < 0.05$ by Student's t test).

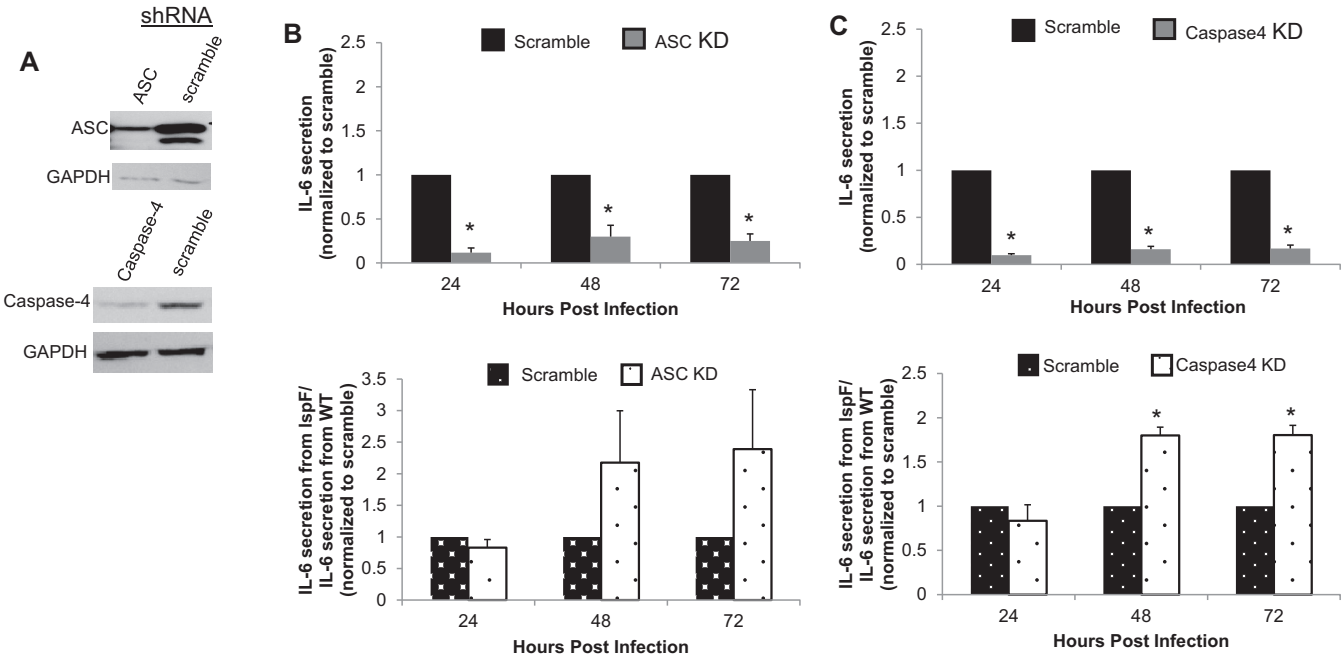

FIG 5 Effect of ASC and caspase-4 depletion on cytokine production triggered by *L. pneumophila* WT and *lspF* mutant strains in macrophages. (A) Lysates obtained from U937 cells containing either an shRNA targeting ASC or caspase-4 or a nontargeting scramble shRNA were subjected to immunoblot analysis using an anti-ASC or anti-caspase-4 antibody (top) or an anti-GAPDH antibody (bottom) as a loading control. (B and C, top) Scramble U937 cells or ASC (B) or caspase-4 (C) knockdown U937 cells were infected with WT strain 130b, and the levels of IL-6 in culture supernatants were then determined. IL-6 levels elicited upon infection of scramble cells were set to a value of 1, and levels from infected knockdown cells were normalized to the value for scramble cells. (Bottom) Scramble U937 cells or ASC (B) or caspase-4 (C) knockdown U937 cells were infected with WT strain 130b or *lspF* mutant strain NU275, and IL-6 levels in culture supernatants were then determined. The graph shows the ratio of IL-6 levels from mutant-infected cells to IL-6 levels from WT-infected cells. The IL-6 ratio obtained from infection of scramble cells was set to a value of 1, and the ratio from infection of knockdown cells was normalized to the value for scramble cells. Graphs show the average IL-6 levels ($n = 3$) pooled from three independent experiments, with standard errors. Asterisks indicate points at which the values for samples from knockdown cells were significantly different from those for samples from scramble cells (*, $P < 0.05$ by Student's *t* test).

The inflammasome in human macrophages is not required for the dampening effect of T2S on cytokine production. During *L. pneumophila* infection of murine macrophages, activation of the inflammasome occurs through NAIP5/NLRC4, triggered by flagellin, as well as through an apoptosis-associated speck-like protein containing a CARD (ASC)-dependent pathway, resulting in the production of cytokines via an IL-1 autocrine loop (22, 23, 53–56). Humans lack the NAIP5 allele present in murine cells (57); however, the canonical ASC-dependent inflammasome is still activated upon *L. pneumophila* infection of human macrophages (58). Furthermore, it has been shown, via gene knockdown in human macrophages, that noncanonical inflammasome activation occurs through caspase-4, which serves as an intracellular lipopolysaccharide (LPS) receptor (59). In order to ascertain the role of the ASC-dependent inflammasome and caspase-4 in the dampening effect of the *L. pneumophila* T2S system, we created two stable U937 knockdown cells lines with a 73% decrease of both ASC and caspase-4 levels (Fig. 5A). The growth of WT and T2S mutant *L. pneumophila* bacteria in U937 cells was unaffected by the gene knockdowns (see Fig. S4E and S4F in the supplemental material). The knockdown of either the ASC or caspase-4 pathway decreased IL-6 production following WT infection (Fig. 5B and C, top). This agrees with data from previous studies that showed that both ASC and caspase-4 are stimulated during *L. pneumophila* infection of human macrophages (59, 60), although our data are the first documentation of the role of ASC and caspase-4 in IL-6 secretion. The knockdown of ASC or caspase-4 did not decrease the ratio of the IL-6 output from mutant-infected cells to that from WT-infected cells (Fig. 5B and C, bottom, and Fig. S5E and S5F). If anything, the ratio between the T2S mutant and the WT increased when either protein was knocked down, suggesting that a PAMP released by or generated as a result of the T2S system stimulates the ASC or caspase-4 pathway. Nonetheless, given that no

decrease in the ratio of IL-6 stimulation from T2S mutant- versus WT-infected cells was seen, we concluded that the dampening effect of the T2S system does not require ASC- or caspase-4-mediated inflammasome responses.

PKR is necessary for optimal IL-6 secretion during *L. pneumophila* infection of human macrophages; however, cytokine dampening by T2S is independent of PKR. Double-stranded RNA (dsRNA)-dependent protein kinase receptor (PKR) is an intracellular RNA receptor that can stimulate proinflammatory cytokines. This protein has been implicated mainly in the host response to viral infection (61); however, it also plays a role in the immune response to bacterial products (62). One study using a PKR inhibitor showed that blocking of the PKR pathway in murine BMD macrophages does not affect the activation of the MAPK pathway during *L. pneumophila* infection (63); however, to our knowledge, PKR has not been studied in relation to *L. pneumophila* infection of human macrophages. In order to test the impact of this understudied pathway in a human model, we created a stable PKR knockdown in the U937 cell line with a 55% decrease in protein expression (Fig. 6A). The loss of PKR decreased the secretion of IL-6 following WT infection (Fig. 6B, top), indicating that PKR is involved in optimal cytokine secretion in response to *L. pneumophila* infection of human macrophages. However, PKR knockdown did not decrease the difference in IL-6 stimulation between WT-infected and T2S mutant-infected macrophages (Fig. 6B, bottom; see also Fig. S5G in the supplemental material). The loss of PKR also did not affect the levels of WT or T2S mutant growth during intracellular infection (Fig. S4G).

Cytokine dampening by T2S is mediated through the immune adaptor MyD88. Previous studies used knockout mice and BMD macrophages from these knockout mice to demonstrate that during intracellular infection, *L. pneumophila* stimulates TLRs, including both cell surface TLR2 (via the recognition of lipoprotein and LPS) and TLR5 (flagellin) and intracellular TLR9 (CpG oligodeoxynucleotide) (64–69). In order to test, for the first time, the importance of these pathways in human macrophages, we started out by generating a stable knockdown of MyD88 with a 76% decrease in protein expression (Fig. 7A). MyD88 is the adaptor for all the TLRs known to be important for *L. pneumophila* infection (22). While the loss of MyD88 did not appear to impact IL-6 secretion during WT infection (Fig. 7B, top), it resulted in a decrease in the ratios of IL-6 levels stimulated in T2S mutant infection to those in WT infection by 59% and 49% at the 48-h and 72-h time points, respectively (Fig. 7B, bottom; see also Fig. S5H in the supplemental material). In order to confirm that this decrease in the IL-6 secretion ratio was a result of the decrease in the level of MyD88 and not an unintended off-target effect of the V2LHS_152058 shRNA plasmid (Table 1), we used shRNA plasmid V2LHS_152059 to generate a second independent MyD88 knockdown cell line. This cell line displayed a 54% decrease in protein levels (Fig. S6A). Importantly, infection of this cell line also showed a decrease in the difference in the IL-6 output from mutant- versus WT-infected cells (Fig. S6B), validating that MyD88-mediated signaling is dampened by T2S and suggesting that there is an effect on an upstream TLR(s).

In order to obtain further support for the role of MyD88 in dampening, we knocked down TANK binding kinase 1 (TBK1), a second, downstream adaptor in the TLR signaling pathway, among other immune-signaling pathways (70–72). When the protein level of TBK1 was decreased by 87% in a stable knockdown in U937 cells (Fig. 7A), there was a substantial drop in IL-6 levels upon WT infection (Fig. 7C, top), formally documenting the role of TBK1 in human macrophage infection. The finding that the knockdown of TBK1 had a greater effect than did the knockdown of MyD88 (compare Fig. 7B and C) is likely due to the fact that TBK1 is also an adaptor for the RLR pathway as well as many intracellular RNA/DNA receptors (70). Importantly, similarly to our observation with the knockdown of MyD88, the knockdown of TBK1 led to a decrease in the difference between the IL-6 outputs of T2S mutant- and WT-infected macrophages, specifically showing a 44% decrease at 48 h postinfection (Fig. 7C, bottom; see also Fig. S5I in the supplemental material). The loss of MyD88 and TBK1 did not influence the growth of the WT or the T2S mutant in U937 monolayers (Fig. S4H and S4I). Overall, these data indicate that T2S-mediated dampening occurs through both

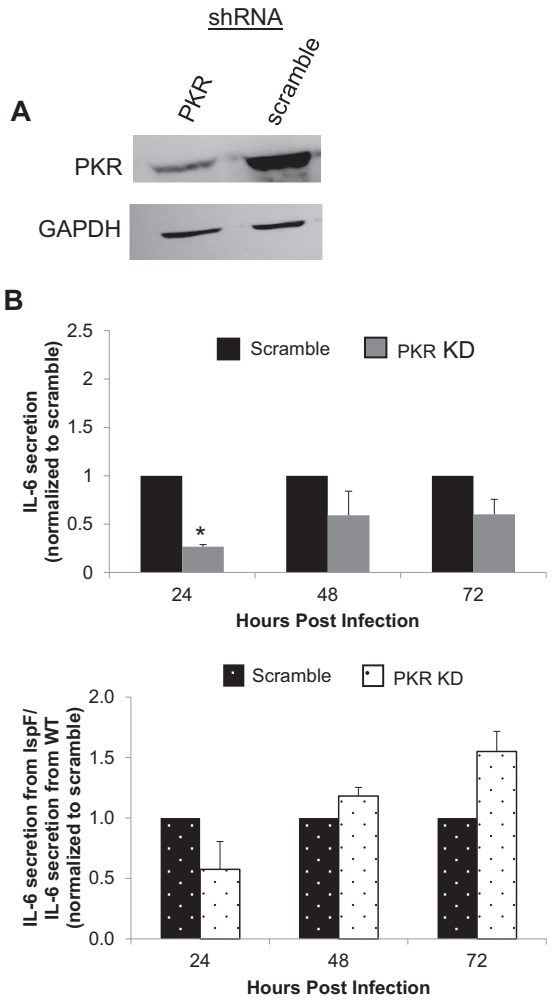

FIG 6 Effect of PKR depletion on cytokine production triggered by *L. pneumophila* WT and *lspF* mutant strains in macrophages. (A) Lysates obtained from U937 cells containing either an shRNA targeting PKR or a nontargeting scramble shRNA were subjected to immunoblot analysis using an anti-PKR antibody (top) or an anti-GAPDH antibody (bottom) as a loading control. (B, top) Scramble U937 cells or PKR knockdown U937 cells were infected with WT strain 130b, and the levels of IL-6 in culture supernatants were then determined. IL-6 levels elicited upon infection of scramble cells were set to a value of 1, and levels from infected knockdown cells were normalized to the value for scramble cells. (Bottom) Scramble U937 cells or PKR knockdown U937 cells were infected with WT strain 130b or *lspF* mutant strain NU275, and IL-6 levels in culture supernatants were then determined. The graph shows the ratio of IL-6 levels from mutant-infected cells to IL-6 levels from WT-infected cells. The IL-6 ratio obtained from infection of scramble cells was set to a value of 1, and the ratio from infection of PKR knockdown cells was normalized to the value for scramble cells. Graphs show the average IL-6 levels ($n = 3$) pooled from three independent experiments, with standard errors. Asterisks indicate points at which the values for samples from knockdown cells were significantly different from those for samples from scramble cells (*, $P < 0.05$ by Student's *t* test).

MyD88 and TBK1, further suggesting an effect on the upstream TLRs known to be involved in *L. pneumophila* infection (i.e., TLR2, TLR5, and/or TLR9).

T2S-mediated dampening of the cytokine response is facilitated through TLR2. In order to begin to determine which TLR(s) is involved in T2S-mediated dampening, we generated a knockdown of TLR2 reducing protein expression by 86% (Fig. 8A). As expected, the gene knockdown reduced the responsiveness of macrophages to purified palmitoyl-3-Cys-Ser-(Lys)4 (Pam_3CSK_4), a known agonist for TLR2 (see Fig. S7 in the supplemental material). This TLR2 knockdown did not lead to a decrease in the IL-6 output during WT infection of U937 cells and in fact led to an increase in the early cytokine output (Fig. 8B, top). However, more significantly, it led to a decrease in the difference in IL-6 outputs from T2S mutant-infected compared to WT-infected macro-

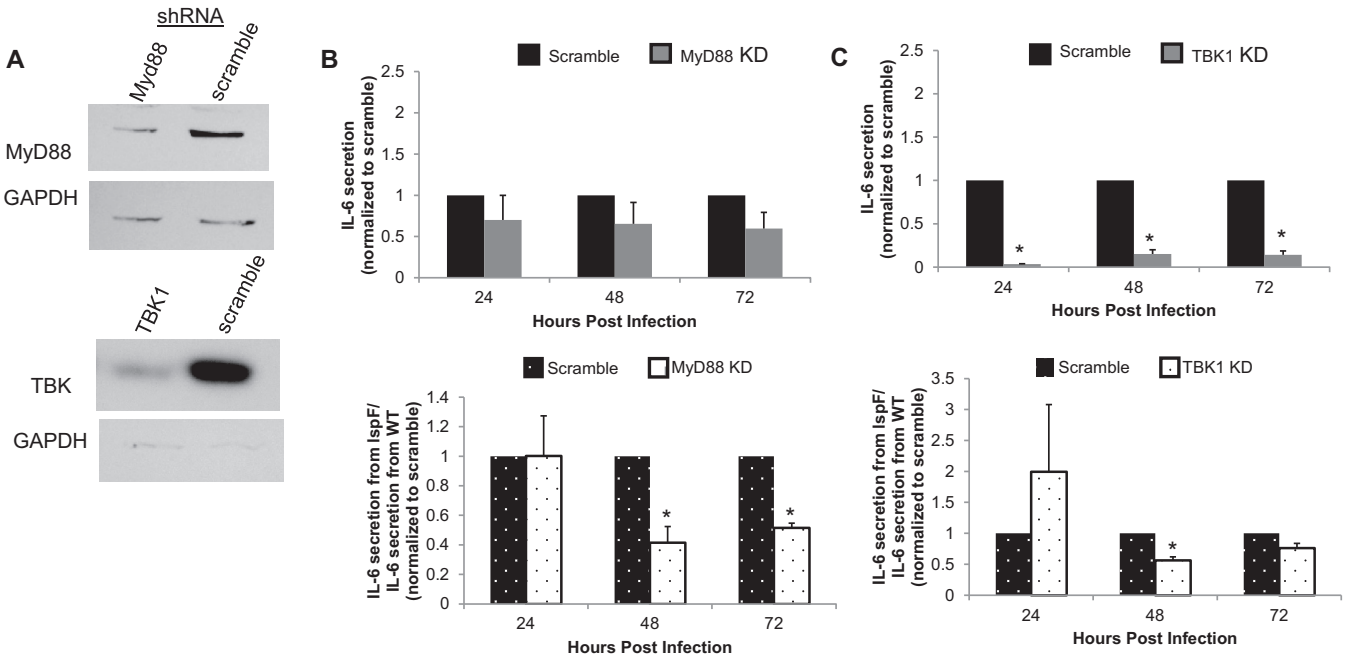

FIG 7 Effect of MyD88 or TBK1 depletion on cytokine production triggered by *L. pneumophila* WT and *lspF* mutant strains in macrophages. (A) Lysates obtained from U937 cells containing either an shRNA targeting MyD88 or TBK1 or a nontargeting scramble shRNA were subjected to immunoblot analysis using an anti-MyD88 or anti-TBK1 antibody (top) or an anti-GAPDH antibody (bottom) as a loading control. (B and C, top) Scramble U937 cells or MyD88 (B) or TBK1 (C) knockdown U937 cells were infected with WT strain 130b, and the levels of IL-6 in culture supernatants at 24, 48, and 72 h postinoculation were then determined by an ELISA. IL-6 levels elicited upon infection of scramble cells were set to a value of 1, and levels from infected knockdown cells were normalized to the value for scramble cells. (Bottom) Scramble U937 cells or MyD88 (B) or TBK1 (C) knockdown U937 cells were infected with the WT or *lspF* mutant strain NU275, and IL-6 levels in culture supernatants at 24, 48, and 72 h from infections were then determined. The graph shows the ratio of IL-6 levels from mutant-infected cells to IL-6 levels from WT-infected cells. The IL-6 ratio obtained from infection of scramble cells was set to a value of 1, and the ratio from infection of knockdown cells was normalized to the value for scramble cells. Graphs show the average IL-6 levels ($n = 3$) pooled from three independent experiments, with standard errors. Asterisks indicate points at which the values for samples from knockdown cells were significantly different from those for samples from scramble cells (*, $P < 0.05$ by Student's *t* test).

phages, with decreases of 49% at 48 h and 60% at 72 h (Fig. 8B, bottom, and Fig. S5J). Indeed, the effect of the TLR2 knockdown was equivalent to that seen with the MyD88 knockdown. The TLR2 knockdown, along with the MyD88 knockdown, also led to a decrease in the difference in IL-8 outputs from T2S mutant-infected compared to WT-infected macrophages at 48 h or 72 h (Fig. S8). Together, these data indicate that the TLR2 pathway is dampened by the T2S system of *L. pneumophila*.

To assess if any of the other TLRs linked to *L. pneumophila* infection were involved in T2S-mediated dampening, we next knocked down TLR9, leading to a 63% decrease in protein expression (Fig. 8A). This knockdown did not lead to a decrease in IL-6 stimulation during WT infection (Fig. 8C, top), nor did it lead to a decrease in the difference in IL-6 outputs from T2S mutant- compared to WT-infected macrophages (Fig. 8C, bottom; see also Fig. S5K in the supplemental material), indicating that TLR9

TABLE 1 Plasmids used for gene silencing in U937 cells

Gene target (alternate name)	Plasmid(s)	Source
RIP2	V2LHS_17021	GE Healthcare
MyD88	V2LHS_152058, V2LHS_152059	GE Healthcare
ASC (Pycard)	V2LHS_190981	GE Healthcare
Caspase-4	V2LHS_112712	GE Healthcare
MDA-5 (IFIH1)	V2LHS_202869	GE Healthcare
MAVS	V2LHS_277487	GE Healthcare
PKR (EIF2AK2)	V2LHS_170553	GE Healthcare
TBK	V2LHS_71334	GE Healthcare
TRIF (TICAM1)	V2LHS_217477	GE Healthcare
TLR2	V2LHS_171342	GE Healthcare
TLR9	TRCN0000056890	Sigma-Aldrich

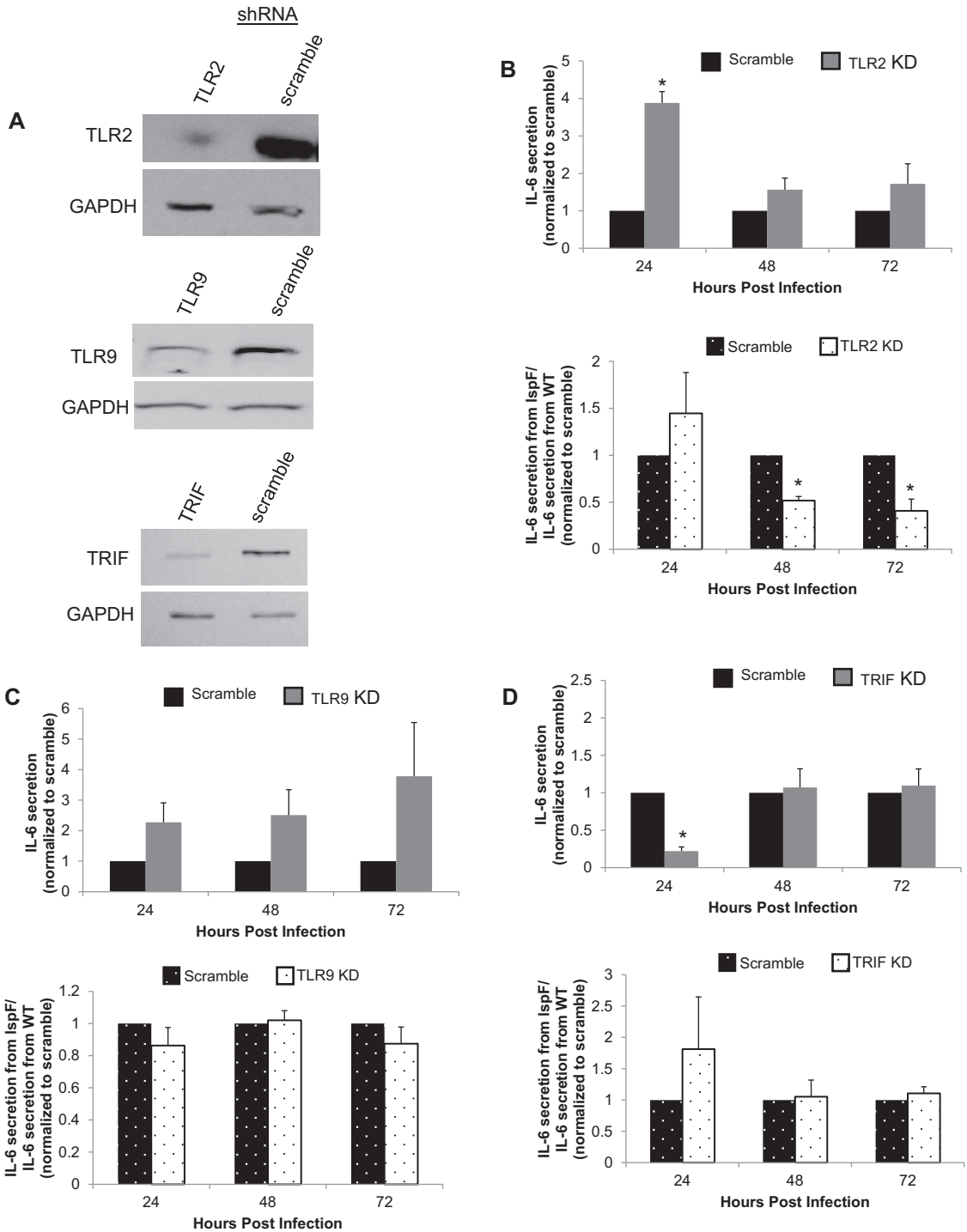

FIG 8 Effect of TLR2, TLR9, or TRIF depletion on cytokine production triggered by infection of macrophages with *L. pneumophila* WT and *lspF* mutant strains. (A) Lysates obtained from U937 cells containing either an shRNA targeting TLR2, TLR9, or TRIF or a nontargeting scramble shRNA were subjected to immunoblot analysis using an anti-TLR2, anti-TLR9, or anti-TRIF antibody (top) or an anti-GAPDH antibody (bottom) as a loading control. (B to D, top) Scramble U937 cells or TLR2 (B), TLR9 (C), or TRIF (D) knockdown U937 cells were infected with WT strain 130b, and the levels of IL-6 in culture supernatants at 24, 48, and 72 h postinoculation were then determined by an ELISA. IL-6 levels elicited upon infection of scramble cells were set to a value of 1, and levels from infected knockdown cells were normalized to the value for scramble cells. (Bottom) Scramble U937 cells or TLR2 (B), TLR9 (C), or TRIF (D) knockdown U937 cells were infected with WT strain 130b or *lspF* mutant strain NU275, and IL-6 levels were then determined by an ELISA. The graph shows the ratio of IL-6 levels from mutant-infected cells to IL-6 levels from WT-infected cells. The IL-6 ratio obtained from infection of scramble cells was set to a value of 1, and the ratio from infection of knockdown cells was normalized to the value for scramble cells. Graphs show the average IL-6 levels ($n = 3$) pooled from three independent experiments, with standard errors (*, $P < 0.05$ by Student's *t* test).

signaling is not dampened by T2S. We next turned our attention to TLR5 but were unable to knock down this TLR in U937 cells. However, since TLR5 is the receptor for the *L. pneumophila* PAMP flagellin (68), we generated an *L. pneumophila flaA lspF* mutant that lacks the T2S system as well as flagellin and then tested its ability to trigger cytokine production. The *flaA lspF* double mutant elicited IL-6 secretion at the same level as that of the *lspF* mutant (Fig. S9), indicating that flagellin is not required for the observed effect of T2S, and therefore, TLR5 is unlikely to be involved in cytokine dampening.

As an additional step toward investigating the possible involvement of other TLRs in cytokine dampening, we knocked down TIR domain-containing adaptor inducing interferon beta (TRIF). The TRIF protein is a downstream adaptor for TLR3 and TLR4. Although TLR3 and TLR4 have not been implicated in signaling during *L. pneumophila* infection of murine cells (66, 73), we considered this a first approach to look at the involvement of these two TLRs in *L. pneumophila* infection of human macrophages. The knockdown of TRIF led to an 81% decrease in the protein level (Fig. 8A) and resulted in a decrease in IL-6 stimulation at the 24-h time point following WT infection (Fig. 8D, top), indicating, for the first time, that TRIF is necessary for optimal IL-6 secretion in human macrophage infection. However, the ratio of IL-6 secreted during T2S mutant infection to that secreted during WT infection remained the same between scramble and knockdown cells (Fig. 8D, bottom; see also Fig. S5L in the supplemental material), indicating that TRIF is not required for T2S-dependent dampening during *L. pneumophila* infection of human macrophages. TRIF was also unimportant for the intracellular growth of the WT and the T2S mutant (Fig. S4J). Overall, our data set indicates that *L. pneumophila* dampens the MyD88- and TBK1-dependent TLR2 pathway in a T2S-dependent manner, leading to a reduction in cytokine levels produced by infected human macrophages.

DISCUSSION

Building upon our previous work (16), we have now demonstrated that *L. pneumophila* T2S dampens the cytokine response of three types of human macrophage-like cells, including cells obtained from human volunteers. Therefore, we strongly suspect that this effect of T2S has implications for human disease caused by *L. pneumophila*. Our new immunoblot data indicate that the previously observed effect of T2S on cytokine gene transcription (16) is due, at least partly, to a dampening of the macrophage's MAPK and NF-κB pathways. Although it is possible that proteins secreted via T2S act directly on components of the MAPK and NF-κB pathways, as has been documented following exposure to various pathogens (21, 74–76), we focused this study on discerning whether T2S diminishes the upstream event of PAMP recognition. By generating a set of human knockdown cell lines, we determined that the NLR, RLR, TRIF, and PKR pathways as well as ASC- and caspase-4-dependent inflammasomes are not required for the T2S dampening effect. However, utilizing a similar experimental approach, we gained evidence for T2S acting on the MyD88-dependent TLR2 pathway. Based on the current literature, this pathway is involved in recognizing *L. pneumophila* LPS (66, 67) as well as a peptidoglycan-associated lipoprotein (77); however, there is still the possibility that there is an as-yet-unidentified PAMP recognized by TLR2. Further support for the *L. pneumophila* T2S system acting upon the TLR2 pathway is the fact that the dampening effect of T2S, although clearly evident in human macrophages, was not manifest for infected murine macrophages. Indeed, the TLR2 pathway in human cells can be different from that in murine cells. Although human TLR2 and murine TLR2 share 84% amino acid similarity in their intracellular domains, they are only 65% similar in their extracellular domains, and thus, the two TLRs differ in their abilities to recognize ligands. For example, while human TLR2 discriminates between the tripalmitoylated peptide P_3CSK_4 and the trilauroylated lipopeptide Lau_3CSK_4 (both of which are synthetic analogs of microbial lipopeptides), responding only to the former, murine TLR2 responds to both of these lipopeptide ligands (78). To our knowledge, the connection

between *L. pneumophila* T2S and the manipulation of innate immunity that we have documented is a novel illustration of the functional importance of a T2S system.

The dampening effect of T2S on the TLR2 pathway in human macrophages could be occurring at a number of levels. In the simplest scenario, a T2S-dependent effector(s) could limit the host recognition of the TLR2 agonists (e.g., LPS and/or lipopeptides) that are present on or released by the bacterium. As a first possible explanation, T2S might be secreting an effector(s) that limits the levels of LPS and lipopeptides or alters their conformation in some way such that they are no longer recognized by human TLR2. It has been theorized that *L. pneumophila* LPS is recognized by TLR2 because it contains long fatty acid chains in its lipid A and because it has a ketone group on the penultimate carbon of one of these fatty acid chains (67). Thus, a secreted effector that either cleaves or otherwise changes the fatty acids in the LPS could be one explanation for how T2S decreases signaling through TLR2. Based on our *in silico* analysis of the *L. pneumophila* genome, there are multiple putative lipases predicted to be secreted by T2S that could be involved in processing and deacylating LPS (12), analogous to lipases from other bacteria (79–83). As a second means by which T2S could diminish PAMP recognition by TLR2, it is possible that a protein effector secreted into the LCV impedes the translocation of LPS and/or lipopeptides out of the LCV and into the macrophage cytoplasm and perhaps ultimately into the extracellular milieu. Although historically, TLR2 has been named a surface TLR, recent findings have challenged this idea and demonstrated intracellular TLR2 colocalizing with its ligand (84, 85). As a final way in which T2S might impede the TLR2 pathway, it is conceivable that a secreted bacterial protein traffics out of the LCV and obstructs recognition by blocking, cleaving, or otherwise altering the host receptor or a downstream adaptor such as MyD88. Although TLR5 and TLR9 also signal through MyD88, the fact that we see a dampening of only the TLR2 pathway could be because there is more stimulation of TLR2 than of TLR5 and TLR9 during *L. pneumophila* infection. Compatible with the possibility of an effector directly targeting the host receptor or adaptor, there is already an example of a T2S-dependent substrate occurring in the cytosol of infected macrophages (86), although this particular effector, the ProA metalloprotease, is not required for the dampening of cytokine gene transcription (16). Thus, we posit the existence of a novel T2S-dependent effector that impedes the functioning of the TLR2 pathway.

Although the dampening effect of T2S on the cytokine output is evident 24, 48, and 72 h after infection of U937 cells (16), the impact of the knockdown of MyD88, TBK1, or TLR2 on the ratio of the amount of IL-6 from mutant infections relative to that from WT infections, although evident at 48 and/or 72 h, was not seen at 24 h (Fig. 7 and 8). This suggests that the dampening effect of T2S is not due solely to an effect on the MyD88-TLR2 signaling pathway. Even at 48 and 72 h, the contribution of MyD88-TLR2 was not the full explanation, as knockdowns resulted in 50 to 60% reductions of T2S-mediated dampening (Fig. 7B and 8B). Taken together, the T2S-dampening effect at earlier time points (e.g., 4 to 24 h) might mainly involve a pathway(s) that is yet to be defined, but at later times (e.g., 48 and 72 h), it is primarily (but not entirely) due to the action on MyD88-TLR2. It is also formally possible that the T2S-dampening effect, particularly at the earlier time points, involves one of the known signaling pathways that we have examined here (i.e., the NLR, RLR, TRIF, and PKR pathways and ASC- and caspase-4-dependent inflammasomes) but that the level of inhibition of any one pathway is low, such that the single-gene knockdowns that we performed did not show an effect. Thus, the identification of the additional target(s) of T2S might require the simultaneous knockdown of multiple signaling pathways.

The dampening effect of T2S on TBK1 also suggests that *L. pneumophila* T2S could limit another PAMP recognition pathway(s), in addition to TLR signaling. In recent years, a number of TBK1-utilizing pathways have been discovered and shown to be involved mostly in the recognition of viral or bacterial nucleic acid that is present in the cytoplasm of infected cells. These pathways include the cGAS (cyclic GMP-AMP synthase) pathway, the MRE11 (meiotic recombination 11 homolog A) pathway, and the DAI (DNA-dependent activator of IFN regulatory factors) pathway (87, 88). Whereas DAI

is not required for the expression of IFN-β triggered by *L. pneumophila* infection of a human lung epithelial cell line (47), cGAS is active during *L. pneumophila* infection of BMD murine macrophages (89). Thus, it is possible that one or more of these TBK1-dependent pathways are also inhibited by T2S during *L. pneumophila* infection of human macrophages.

The decrease of the cytokine response by the T2S system has implications for understanding *Legionella* pathogenesis. In addition to IL-6 and IL-8, which served as the readouts in this study, we previously observed that the T2S system is able to decrease the levels of human tumor necrosis factor alpha (TNF-α) and IL-1β in supernatants of infected macrophages (16). Since these various proinflammatory cytokines fulfill a number of important roles in host defense, including increasing vasodilation, recruiting and activating leukocytes, triggering the release of acute-phase proteins, and activating complement and opsonization (16), we hypothesize that the dampening effect of T2S on TLR2 signaling contributes to the ability of *L. pneumophila* to grow in the human lung and cause pneumonia. Previously, we also determined that the T2S-dependent CelA endoglucanase, ChiA chitinase, LapA and LapB aminopeptidases, LipA and LipB lipases, Map phosphatase, PlaA lysophospholipase A, PlaC glycerophospholipid:cholesterol acyltransferase, PlcA and PlcB phospholipases C, and SrnA RNase are not required for the dampening of the cytokine output, and the ProA metalloprotease, although able to directly degrade cytokines, does not affect the levels of cytokine gene transcripts (16). Our proteomic and *in silico* analyses indicate that the *L. pneumophila* T2S system exports between 25 and 60 proteins, including several putative lipolytic enzymes and a number of novel proteins (12, 13, 15). Thus, future studies will be directed toward, among other things, identifying which T2S-dependent effector(s) is responsible for impeding immune signaling and then determining its molecular mode of action. We suspect that the observations made here by studying *L. pneumophila* T2S will have implications for other pathogens that grow in or otherwise target macrophages.

While the initial impetus for this study was the investigation of the dampening effect of T2S on cytokine production by human macrophages, the generation of cell lines deficient in individual immune pathways led to substantial new information regarding innate immune pathways that operate during (WT) *L. pneumophila* infection. Our data set documented, for the first time, a variety of pathways as being important for optimal cytokine stimulation during *L. pneumophila* infection of macrophages, including the intracellular RNA receptor PKR, the TLR adaptor TRIF, and the RNA/DNA sensor adaptor TBK1. We also demonstrated that many of the signaling pathways known to be involved in *L. pneumophila* infection of murine cells are also operative during infection of human cells, including the noninflammasome NLR pathway through the adaptor RIP2, the RLR pathway through the adaptor MAVS, and the inflammasome through ASC and caspase-4. Finally, the dampening effect of T2S was not seen in murine macrophages, demonstrating that PAMP recognition is more distinct between human and mice than previously reported.

MATERIALS AND METHODS

Bacterial strains, bacteriological media, and chemicals. *L. pneumophila* strain 130b (ATCC BAA-74, also known as AA100) is a clinical isolate that served as our WT and parental strain for mutants (90). Both mutant strain NU275, which lacks a functional *lspF* gene that encodes an inner membrane component of the T2S apparatus, and mutant strain NU347, which lacks a functional *flaA* gene encoding flagellin, were described previously (18, 91). A mutant lacking both *flaA* and *lspF* (NU430) was obtained by introducing pGlspF::Km (18) into NU347 by transformation (91) and then selecting for the acquisition of kanamycin resistance. In order to help monitor the behavior of *L. pneumophila* in macrophages, we utilized previously reported *L. pneumophila* WT and T2S mutant strains with a GFP-expressing plasmid introduced (19). Legionellae were routinely cultured at 37°C in buffered yeast extract (BYE) broth or on buffered charcoal yeast extract (BCYE) agar, which, when appropriate, contained gentamicin or kanamycin (92). Unless noted otherwise, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Catalog numbers are indicated when there is more than one version of a reagent available from the vendor.

Macrophage lines and intracellular infection assays. In order to assess *L. pneumophila* growth within macrophages as well as the effects of bacterial infection on host cell function, we utilized a human U937 cell line (ATCC CRL-1593.2), a human THP-1 cell line (ATCC TIB 202), and BMD macrophages

obtained from 6- to 8-week-old female A/J mice (Jackson Laboratory, Bar Harbor, ME) (16, 27, 93). The preparation of the different macrophages was done as previously described, with the exception of THP-1 cells, which were differentiated by incubation for 24 h in RPMI medium (catalog number 10-040-CV; Corning Mediatech, Inc., Manassas, VA) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 100 ng/ml phorbol myristate acetate (catalog number P8139; Sigma) and at a concentration of 1×10^6 cells/well in a 24-well tissue culture plate (94). For our infection analysis, we also used PBMCs, which had been obtained from healthy human volunteers, as previously described (95). To this end, phosphate-buffered saline (PBS) containing 7.5 U heparin/ml (catalog number H3149; Sigma) was added at three times the volume of the buffy coat (obtained from C. Gunderson and H. Seifert, Northwestern University), and the resulting suspension was centrifuged at $500 \times g$ for 10 min. The cell pellet was resuspended in 0.3 mM EDTA in PBS, and the suspension was then centrifuged at $100 \times g$ for 10 min. After washing of the pellet in this manner two more times, the mononuclear fraction was suspended in RPMI medium containing 15% human serum (catalog number H6914; Sigma), and the cell suspension was then added to tissue culture-treated, 24-well polystyrene plates (Falcon, Corning, NY) and incubated overnight at 37°C. To enrich for the adherent mononuclear cell population, as described previously (28, 96–98), the newly formed monolayers were washed three times to exclude nonadherent cells from the plate, and RPMI medium containing 15% human serum was then added back. The adherent mononuclear cells were then allowed to differentiate in human serum-containing medium for an additional 6 days, as previously described (96, 98).

In order to assess the role of innate immune pathways during infection, we generated a series of stable U937 knockdown cell lines. The generation of these cell lines was done as previously described (19). Briefly, silencing was achieved by transduction with a vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped lentivirus that encodes an shRNA molecule targeting the gene of interest. Table 1 lists plasmids used for gene silencing and their sources. As a control, a nontargeting shRNA plasmid (scramble) was introduced into U937 cells, and the scramble cell line that was obtained was used to compare results from cell lines carrying a target shRNA plasmid. When experiments utilized cell lines carrying shRNA plasmids from GE Healthcare (Table 1), scramble control cells were generated by using plasmid RHS4346 (GE Healthcare, Chalfont, UK). For experiments where cell lines carrying shRNA plasmids from Sigma were used (Table 1), the scramble plasmid SHC016 (Sigma-Aldrich, St. Louis, MO) was utilized. In an attempt to knock down TLR5, we utilized shRNA plasmids V2LHS_171357 and V2LHS_171361 from GE Healthcare and shRNA plasmids TRCN0000430395, TRCN0000431479, TRCN0000056956, TRCN0000056954, and TRCN0000056953 from Sigma-Aldrich, but we were ultimately unable to reduce TLR5 protein levels with these shRNA plasmids.

Monolayers containing 0.25×10^6 to 1×10^6 macrophages were inoculated with bacteria at a multiplicity of infection (MOI) of 0.5 (for WT and knockdown U937 cells, THP-1 cells, and PBMCs) or an MOI of 1 (for murine BMD macrophages), incubated for 2 h to allow bacterial entry, and then washed three times with RPMI medium to remove any remaining extracellular legionellae (16). At 0, 24, 48, and 72 h postinoculation, the infected monolayers were lysed, and dilutions were plated onto BCYE agar in order to determine the number of intracellular CFU (16). In order to examine the effect of *L. pneumophila* on cytokine production, macrophages were infected with WT and mutant bacteria as noted above, and after 24, 48, or 72 h of incubation, the amount of IL-6 or IL-8 in cell-free culture supernatants was measured by using Ready-Set-Go enzyme-linked immunosorbent assay (ELISA) kits (EBioscience, San Diego, CA), as described previously (16). However, to judge IL-6 levels after 4 h of infection, inoculation of U937 cells was done by employing an MOI of 10 in order to increase the number of infected macrophages within the monolayer. To measure IFN-β levels in culture supernatants, we utilized an IFN-β ELISA kit (catalog number 41410-1; R&D Systems, Minneapolis, MN). In order to assess the phenotype of our TLR2 knockdown cells, we stimulated scramble or knockdown cells with 50 ng/ml the purified TLR2 ligand Pam_3CSK_4 (catalog number tlrl-pms; InvivoGen, San Diego, CA) for 24 h and then collected supernatants and assessed IL-6 secretion using the Ready-Set-Go IL-6 ELISA kit.

Immunoblot analysis of macrophage gene knockdown and of the MAPK and NF-κB pathways in infected macrophages. Immunoblot analyses were used both to confirm the knockdown of target proteins in gene knockdown in stable U937 cell lines and to check the activation status of adaptors important in the MAPK and NF-κB pathways. For confirmation of the knockdown, U937 cell monolayers were prepared as usual (16), after which protein levels of shRNA targets were probed. For analysis of the activation of MAPK and NF-κB pathways, U937 cell monolayers were either left uninfected or inoculated with *L. pneumophila* at an MOI of 50, and at 4 h postinoculation, the phosphorylation of proteins involved in immune signaling was determined (99). For both assays, at the indicated time points, the tissue culture medium was removed from the wells containing the monolayers, the wells were washed with PBS, and 100 μl of 4°C radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 2 mM EDTA, 0.2% Triton X-100, 0.3% NP-40 alternative, 25 mM NaF, 0.1 mM Na orthovanadate, 0.25% Na deoxycholate) that contained a protease inhibitor (RIPA-PI) was then added. To prepare a stock solution of RIPA-PI, one tablet of a protease inhibitor (catalog number 04693124001; Roche, Penzberg, Germany) was dissolved in 10 ml of RIPA buffer. U937 cells were resuspended in RIPA-PI by using a cell scraper (catalog number 353085; Fisher Scientific, Pittsburgh, PA), and after the addition of 1 μl of phenylmethylsulfonyl fluoride, the cell suspension was incubated for 10 min on ice. Lysis of the infected cells was completed by passing the sample through a 22-gauge syringe (catalog number 305156; BD Biosciences, San Jose, CA) five times. Next, the lysate was centrifuged at $16,300 \times g$ for 15 min at 4°C in order to remove cellular debris and bacteria. The resulting supernatant containing soluble host proteins was subjected to SDS-polyacrylamide (8 to 12%) gel electrophoresis, and separated proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (catalog number 88518; Thermo Scientific,

Waltham, MA) (99). Next, the blots were incubated for 12 h at 4°C in 5% bovine serum albumin (BSA) (catalog number A4503; Sigma) for the detection of phosphorylated SAPK/JNK (p-SAPK/JNK), p-ERK1/2, and p-ATF2 or in 5% nonfat dry milk (i.e., blotting-grade blocker from Bio-Rad) for probing for p-c-Jun, p-p38, IκBα, TLR2, TLR9, ASC, TBK1, RIP2, MyD88, RIG-I, MAVS, MDA-5, PKR, caspase-4, TRIF, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and H3. For the most part, primary antibodies were added at a concentration of 1:1,000 in Tris-buffered saline containing 5% Tween 20 (catalog number P1379; Sigma) (TBS-T) and 5% BSA and incubated at 4°C for 12 h. However, for the detection of H3 and GAPDH, the primary antibodies were diluted in TBS-T containing 5% nonfat dry milk at concentrations of 1:2,000, and 1:4,000, respectively, and for TLR9, incubation was done for 1 h. All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA), with the exception of anti-GAPDH antiserum, which was obtained from Santa Cruz Biotechnology (Dallas, TX). After a series of washes, the blots were incubated with secondary antibody solutions for 1 h at room temperature. An anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP) conjugate (Cell Signaling) diluted 1:1,000 was used for the detection of all primary antibodies, except for those directed against TLR9, H3, and GAPDH. In the case of H3 and TLR9 detection, the anti-rabbit IgG-HRP conjugate was used at a 1:2,000 dilution, and for the detection of GAPDH, an anti-mouse IgG-HRP conjugate (Cell Signaling) was used at a 1:3,000 dilution. The detection reagents used were ECL (Amersham, Buckinghamshire, UK) for the GAPDH and H3 blots and ECL prime (Amersham) for all other blots, and Amersham Hyperfilm was used to develop the images of the blots (19). Image Lab software (Bio-Rad) was used to quantify the detection signals generated on the immunoblots.

Immunoblot assessment of bacterial lysis within infected macrophages. U937 cell monolayers were inoculated with GFP-expressing *L. pneumophila* strains at an MOI of 50, as noted above, and at 4 h postinoculation, the amount of GFP released into the soluble fraction in infected macrophages was determined by immunoblot analysis. At 4 h, the culture medium was removed from the wells and replaced with 1 ml of fresh medium containing 0.1% saponin in order to lyse the infected U937 cells (but not the intact bacteria contained within them) (100). After dislodging the macrophages from the plate with a cell scraper, the sample was centrifuged in a microcentrifuge tube at 16,300 × *g* for 10 min at 4°C. The pellet containing intact bacteria and large cellular debris was resuspended and lysed in Laemmli buffer (101). Soluble proteins within the supernatant were isolated and concentrated, as previously described (102), and then reconstituted in Laemmli buffer. Cellular and soluble fractions were subjected to SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a PVDF membrane. After incubation of the membranes for 12 h at 4°C in TBS-T containing 5% nonfat dry milk, the membranes were treated with anti-GFP antibody (catalog number 2956s; Cell Signaling) at a dilution of 1:1,000 in TBS-T containing 5% nonfat dry milk for 12 h at 4°C. Following washes, the blots were incubated with an anti-rabbit IgG-horseradish conjugate at a dilution of 1:1,000 in TBS-T containing 5% dry milk for 1 h at room temperature, and they were then developed by using ECL Prime and analyzed as noted above.

Quantitative reverse transcription-PCR. At 24 h postinoculation, RNA was purified from infected U937 cells by using the RNeasy minikit (Qiagen, Hilden, Germany). cDNA was made by using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The cDNA was amplified by PCR using primers specific for IFN-β and GAPDH (Hs02621180_s1 and Hs00266705_g1) (Applied Bioscience, Carlsbad, CA), an ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA), and TaqMan probes (Applied Biosystems). As described previously (16), GAPDH served as a control gene for analysis of changes in cycle threshold values; fold induction above the uninfected control was ascertained based on changes in the cycle threshold values.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00897-16.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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