Legionella pneumophila translocated translation inhibitors are required for bacterial-induced host cell cycle arrest

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The cell cycle machinery controls diverse cellular pathways and is tightly regulated. Disruption of cell division plays a central role in the pathogenesis of many disease processes. Various microbial pathogens interfere with the cell cycle machinery to promote host cell colonization. Although cell cycle modulation is a common theme among pathogens, the role this interference plays in promoting diseases is unclear. Previously, we demonstrated that the G1 and G2/M phases of the host cell cycle are permissive for Legionella pneumophila replication, whereas S phase provides a toxic environment for bacterial replication. In this study, we show that L. pneumophila avoids host S phase by blocking host DNA synthesis and preventing cell cycle progression into S phase. Cell cycle arrest upon Legionella contact is dependent on the Icm/Dot secretion system. In particular, we found that cell cycle arrest is dependent on the intact enzymatic activity of translocated substrates that inhibit host translation. Moreover, we show that, early in infection, the presence of these translation inhibitors is crucial to induce the degradation of the master regulator cyclin D1. Our results demonstrate that the bacterial effectors that inhibit translation are associated with preventing entry of host cells into a phase associated with restriction of L. pneumophila. Furthermore, control of cyclin D1 may be a common strategy used by intracellular pathogens to manipulate the host cell cycle and promote bacterial replication.

Legionella pneumophila | cell cycle | translation | intracellular growth | innate immunity

The bacterium Legionella pneumophila is the causative agent of Legionnaires’ disease (1, 2). The natural hosts of L. pneumophila are amoebae, with human disease resulting from pathogen replication within alveolar macrophages (1). To sustain intracellular replication, L. pneumophila uses the Icm/Dot type IV secretion system (3, 4), which introduces more than 300 Icm/Dot-translocated substrate (IDTS) proteins into the host cell cytosol (5). These IDTSs manipulate key host pathways to allow biogenesis of the Legionella-containing vacuole (LCV) (6–8). Establishment of this replication niche and maintenance of vacuole integrity are two critical steps in supporting intracellular growth and avoiding pathogen recognition by cytosolic innate immune sensing (9). The inability to form the LCV or maintain its integrity results in severe bacterial growth defects and rapid pathogen clearance, respectively (10). Our knowledge of host factor restriction of L. pneumophila intracellular growth has been greatly enhanced by studies of the targets of the bacterial translocated substrates. For instance, studies on L. pneumophila mutants defective for maintaining LCV integrity have allowed significant breakthroughs in identifying the key players in caspase 11-dependent pyroptosis (11).

The eukaryotic cell cycle can be divided into four distinct phases: G1, S, G2, and M (12). Cells in G1 phase commit to proliferation, and DNA replication occurs in S phase. Following DNA replication, cells cycle into the G2 phase. Transition from G2 to M results in new daughter cells. Control of the cell cycle is critical for regulating a number of central processes such as cell differentiation and death, and is tightly controlled by cyclin-dependent Ser/Thr kinases and their cyclin partners (13). Failure to regulate these proteins in any step of the cell cycle process can lead to catastrophic effects, including uncontrolled cellular growth, such as in cancer (14).

Microbial pathogens can exert cell cycle control on host targets. Notably, a class of proteins called cyclomodulins has been identified that are targeted into the host cell cytosol and interfere with progression through the cell cycle (15, 16). There is also evidence supporting a role for pathogens in modulating tumor progression (17), although the role of such control in supporting disease is still unknown. Recently, studies performed in our laboratory determined that host cell cycle regulatory proteins control L. pneumophila growth (18). We demonstrated that the G1 and G2/M phases of the host cell cycle are permissive for bacterial replication, whereas S phase provides a toxic environment for bacterial replication. L. pneumophila that attempts to initiate replication in S phase shows poor viability as a result of a failure to control vacuole integrity that leads to cytosolic exposure of the bacterium and bacterial cell lysis resulting from cytoplasmic innate immune surveillance (11, 18).

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Significance

The host cell cycle regulatory proteins control Legionella pneumophila growth. In particular, bacterial replication is depressed in S phase. This indicates that bacterial control of the host cell cycle can limit exposure of the pathogen to antimicrobial events that are cycle-specific. Here we uncovered bacterial factors that induce host cell cycle arrest by inhibiting host protein synthesis and preventing S-phase transition. These data are consistent with S-phase toxicity serving as an important antimicrobial response that limits growth of some intracellular pathogens. Moreover, identification of microbial factors that block cell cycle progression and uncovering host cell cycle partners are candidates for future drug development. Our data point to a unifying role of the cell cycle in multiple disease processes.

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Cell cycle progression plays an important role in the intracellular growth of *L. pneumophila*, as emphasized by specific S-phase toxicity toward this pathogen (18). Therefore, bacterial control of the host cell cycle can limit exposure of the pathogen to antimicrobial events that are cycle-specific. Indeed, *L. pneumophila* can arrest the host cell cycle, which is an effective strategy to avoid S-phase toxicity (18, 19). The exact mechanism and the bacterial and host factors that contribute to this cell cycle block remain unknown. Here we show that *Legionella* block of cell cycle progression is dependent on bacterial translocated substrates that interfere with host cell translation. These data provide a mechanism for *L. pneumophila* that allows control of the host cell cycle in multiple cell types.

**Results**

**Host Cell Cycle Arrest Is Dependent on *Legionella* Translocated Substrates.** We previously demonstrated that S phase provides a toxic environment for *L. pneumophila* growth and that S phase-infected cells do not progress through the cell cycle after *L. pneumophila* challenge (18). Therefore, avoidance of S phase has the potential to protect this pathogen from antimicrobial events. To determine if *L. pneumophila* has the capacity to arrest the host cell cycle independently of the phase, we used the double-thymidine block method to synchronize HeLa cells and determine if *L. pneumophila* blocks cycle progression in a specific phase. Synchronized populations were released from block at time points corresponding to G1 and G2/M and challenged with WT *L. pneumophila-GFP* or a mutant lacking the Icm/Dot type 4 secretion system (T4SS; *dotA3*). Following bacterial challenge, the DNA content of infected cells was compared with that of uninfected cells [Fig. 1, compare GFP<sup>+</sup> (green lines) vs. GFP<sup>−</sup> (black lines)] by flow cytometry. The uninfected bystander G1- and G2/M-synchronized HeLa cells could progress through the cell cycle as measured by accumulation of DNA content over time (Fig. 1, black lines). For instance, bystander G1-synchronized cells accumulated DNA by 13 h post infection (hpi), and, by 16 hpi, a G2/M population was visible, indicating that these cells progressed through the cell cycle (Fig. 1A, Middle, black lines). The same pattern applied for G2/M-synchronized cells that were bystanders at 16 hpi (Fig. 1B, Middle, black lines). In contrast, cells harboring *L. pneumophila* arrested and did not progress through the cell cycle. This was true for G1- and G2/M-synchronized cells (Fig. 1, Middle, green lines). Interestingly, cells infected with the *dotA3* mutant strain progressed normally through the cell cycle. Comparing uninfected cells vs. those challenged with *dotA3* showed no significant change in DNA content (Fig. 1, Right). Taken together, these data confirm that blocking the host cell cycle is a conserved theme in *L. pneumophila* pathogenesis that relies on the IDTSs and is independent of the cell cycle phase encountered by the pathogen.

**Legionella Translocated Protein Synthesis Inhibitors Are Required for Host Cell Cycle Arrest.** The IDTSs support intracellular growth of *Legionella* in amoebal species and macrophages and are required for virulence (20–22). Previous studies have shown that protein synthesis in G1 phase is a necessary step to progress through the mammalian cell cycle (23), and chemical inhibition of protein synthesis by pharmacological agents such as cycloheximide (CHX) leads to cell cycle arrest (24). *Legionella* has been shown to target host translation elongation through secretion of protein synthesis inhibitors. These inhibitors include the *Legionella* glucosyltransferases Lgt1–3 as well as SidI and SidL. The Lgt1–3 proteins glucosylate host elongation factor eEF1A (25, 26). SidI targets eEF1A and eEF1By by an unknown mechanism (27), and the mechanism of action of SidL is yet to be determined (28). Challenge of host cells with the *L. pneumophila* Δ5 strain, lacking all five of these bacterial protein synthesis inhibitors, results in increased host protein translation compared with cells challenged with the WT strain in the first 4 h of infection (29). To further explore the ability of *Legionella* to block cell cycle progression in a biologically relevant cell type, we determined the proliferation rate of macrophages after *L. pneumophila* challenge by measuring incorporation of the deoxynucleotide analog EdU into newly synthesized DNA. To this end, RAW macrophages were challenged with *L. pneumophila-GFP* strains WT, *dotA3*, or Δ5, and DNA synthesis was measured by using EdU incorporation in the GFP<sup>+</sup> (infected) and GFP<sup>−</sup> (bystander) populations (Fig. 2A and B). Macrophages harboring WT *L. pneumophila* showed a large decrease in DNA synthesis compared with cells harboring the *dotA3* or Δ5 strains. Strikingly, cells harboring the two mutant strains each had a distinct EdU<sup>+</sup> population that overlapped with uninfected cells, indicating a large population of infected cells that show effective cell cycle progression (Fig. 2A and B). Approximately 45% of the uninfected cells showed proliferation based on EdU incorporation, which was similar to what was observed in response to the *dotA3* mutant (Fig. 2B). In contrast, ~5% of the cells harboring the WT strain showed evidence of proliferation, and this was increased ~7x by introducing the Δ5 mutation (Fig. 2B).

The failure of *L. pneumophila* Δ5 to block cell cycle progression was further verified in the amoebal species *Dictyostelium discoideum* by prelabeling amoebal cells with eFluor cell proliferation dye and measuring dilution of the label as a consequence of

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**Fig. 1.** *L. pneumophila* Icm/Dot-dependent arrest of the host cell cycle is independent of cell cycle phase. (A and B) HeLa cells were synchronized by the double thymidine block method and challenged with WT or *dotA3 L. pneumophila-GFP*. At various times after uptake, cells were collected and analyzed by flow cytometry to determine cell cycle profile (Materials and Methods): 11.5 h (A) and 6.5 h (B) after release. (Left) Images with black filled distributions display cell cycle profiles at the time of contact with bacteria. “hpi” refers to the total time of contact with bacteria. Uninfected cells are indicated by black lines and infected cells by green lines.
cell division. Amoebal cells harboring *L. pneumophila* were blocked from proliferation, and this block was partially relieved by the Δ5 mutation (Fig. 2C). The ability to block proliferation of RAW cells was dependent on the protein synthesis inhibitors (Fig. 2D). The defect in blocking proliferation of RAW macrophages observed with the Δ5 strain could be reversed by complementation with a plasmid encoding Lgt3 (Fig. 2D). Complementation was not observed in cells challenged with a mutant version of Lgt3 (Lgt3*) that harbored a point mutation in a catalytic residue (Fig. 2D). These data indicate that inhibition of host cell proliferation by *L. pneumophila* requires the presence of translocated protein synthesis inhibitors, and that the catalytic activity of one of these was sufficient to block proliferation.

**Legionella Effectors Trigger the Loss of Cyclin D1.** The commitment to start a new round of DNA replication is tightly regulated (30). Transition from G1 to S phase is maintained mostly by D-type cyclins and cyclin E, which are controlled by the activity of their partner cyclin-dependent kinases (31). These complexes control the activation of a transcriptional network that promotes entry into S phase. D-type cyclins function throughout G1 phase whereas cyclin E shows more activity at the G1/S phase. Based on their central role in cell cycle progression, overexpression of D-type cyclins is often associated with cancer, and inhibition leads to cell cycle arrest (32). For this reason, we analyzed the dynamics of cyclin levels in response to *L. pneumophila* challenge of macrophages. The transcription of cyclin D1 (*Ccnd1*) and cyclin E1 (*Ccne1*) genes did not show significant changes compared with uninfected or dotA3-challenged cells (Fig. 3A). To confirm that there was no control of these cyclins at the transcriptional level, macrophages were challenged with the WT or Δ5 strains, and the response of *Ccnd1* was compared with that of Egr1, a gene known to be transcriptionally activated in response to protein synthesis inhibition by *L. pneumophila* (Fig. 3B) (33). As a positive control, transcription of *Il6* was analyzed, as *Il6* is known to respond to *L. pneumophila* independently of the protein synthesis inhibitors (33). Whereas a response to *L. pneumophila* was observed for *Il6* and a protein synthesis inhibitor-dependent response was observed for Egr1, no significant change was observed in the expression of *Ccnd1* in Δ5 compared with WT infected cells (Fig. 3B). Therefore, transcriptional regulation of G1 cyclins is an unlikely mechanism for *Legionella*-dependent host cell cycle arrest (Fig. 2).

We next investigated the role of translational regulation of the G1 cyclins upon *Legionella* infection. Cyclin D1 and cyclin E1 levels were measured in postsorted cells harboring the *L. pneumophila*-GFP strains (Fig. 3C–E, +) and compared with bystander cells without bacteria (Fig. 3C–E, −). Macrophages harboring WT showed loss of cyclin D1 compared with bystander cells (Fig. 3C and D), whereas cells harboring dotA3 or Δ5 showed no appreciable change in cyclin D1 levels (Fig. 3C and D). In contrast, we did not observe significant change in the levels of cyclin E1 protein in cells harboring *Legionella* (Fig. 3E). To further verify that host translation inhibition by *Legionella* is sufficient to induce a proliferation block, we challenged macrophages with Δ5 in the presence of the translation inhibitor CHX (Fig. 3F and G). CHX treatment resulted in a complete proliferation block in macrophages challenged with Δ5 (Fig. 3F) and was sufficient to recapitulate the loss of cyclin D1 observed in WT *Legionella* infection (Fig. 3G). These results support a model whereby cell cycle arrest and destabilization of cyclin D1 by *L. pneumophila* requires the translocation and enzymatic activity of the translation inhibitor effectors.

**Ectopic Expression of a Single *L. pneumophila* Glucosyltransferase Is Sufficient to Block Host Cell Proliferation.** To further study the role of the Lgt proteins in blocking the host cell cycle, we transfected
HEK293 cells with plasmids expressing Lgt3, Lgt1, or catalytically inactive Lgt3 (Lgt3*). To determine effects on translation, the incorporation of the methionine analog azidohomoalanine (AHA) was determined during a 2-h labeling, followed by detection via orthogonal linkage to an alkenyl fluorescent probe (34). Cells transfected with Lgt3 or Lgt1 showed inhibition of protein translation compared with cells transfected with a control vector expressing GFP. In control cells, ∼43.5% of the cells were in the high-translation population compared with 11.1% and 9.59% in the Lgt3- and Lgt1-transfected cells, respectively (Fig. 4 A and B). This inhibition was similar to CHX treatment, which is a known translation inhibitor (35), and in agreement with previous papers that measured translation rates in cells expressing Lgt proteins by using a radiolabeling assay (Fig. 4 A and B) (33, 36). Translation inhibition in transfected cells was dependent on Lgt enzymatic activity, as cells transfected with a catalytically inactive Lgt3 (plgt3*) showed translation rates similar to cells harboring a plasmid encoding GFP (42.75% and 43.5%, respectively; Fig. 4 A and B).

We next tested the link between translation inhibition and cell cycle arrest. To this end, HEK293 was transfected with Lgt1, Lgt3, or the inactive Lgt3* and cell cycle arrest was measured based on EdU incorporation into DNA and steady-state cyclin D1 levels (Fig. 4 C and D). Cells transfected with Lgt1 or Lgt3 showed a clear cell cycle arrest compared with cells expressing a control GFP or the inactive Lgt3 (Fig. 4 C and D). Furthermore, Lgt-transfected cells showed decreased expression of cyclin D1 protein (Fig. 4 D), whereas cotransfection of HA-cyclin D1 with Lgt3 in HEK293 did not rescue loss of cyclin D1 or the arrest of cell proliferation caused by Lgt3 (Fig. 4 E and F). These data demonstrated that Lgt3 alone is sufficient to cause cell cycle arrest.

Fig. 3. Legionella translocated protein synthesis inhibitors induce the rapid degradation of cyclin D1 in infected macrophages. (A) Lack of transcriptional effect on cyclin expression. Quantitative PCR analysis of Ccd1 and Cose1 transcripts in RAW 264.7 macrophages following L. pneumophila challenge for 2 h. Results are normalized based on Gapdh expression and presented as relative to uninfected cells. (B) RAW 264.7 macrophages were challenged with L. pneumophila WT, dotA3, or Δ5 for 2 h, and transcript levels of Ccd1, Ile6, and Egr1 were measured by quantitative PCR. Results are presented as relative to uninfected population. (C–E) Reduced steady-state levels of cyclin D1 in response to L. pneumophila requires protein synthesis inhibition. RAW 264.7 macrophages were infected with WT, dotA3, or Δ5 L. pneumophila expressing GFP for 2 h and sorted based on fluorescence by flow cytometry. Following infection, the levels of cyclin D1 (C and D) and cyclin E1 (E) in infected (GFP*) and bystander (GFP*) populations were measured by immunoblot. Graph in D indicates fold change of cyclin D1 in GFP* compared with GFP* cells. (F) RAW 264.7 macrophages were challenged with L. pneumophila WT or Δ5 in the absence or presence of 50 μg/mL CHX for 1 h and then incubated with 20 μM EdU for an additional 2 h. At 3 hpi, cells were harvested and analyzed for DNA synthesis by flow cytometry. (G) RAW 264.7 macrophages were infected with L. pneumophila WT or Δ5 as in F, and levels of cyclin D1 in GFP* population were measured by immunoblot. Statistical analyses were performed by unpaired t test (∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.001).
FZR1 Silencing Partially Restores DNA Synthesis in Infected Cells. Based on our results showing that overexpression of cyclin D1 did not affect cell cycle arrest in cells expressing *Legionella* translation inhibitors (Fig. 4), we further explored the mechanism of cyclin D1 degradation in cells harboring *L. pneumophila*. To determine the rate of cyclin D1 turnover, lysates from infected cells treated with CHX were taken at different time points after infection, and cyclin D1 levels were determined by immunoblotting (Fig. 5A). Cyclin D1 degradation was accelerated in infected cells and resulted in more than 60% reduction in protein levels after 1 h compared with 20% in uninfected cells (Fig. 5A). Lowered steady-state levels were caused by proteasomal degradation, based on incubation in the presence of the inhibitor MG132. Treatment with MG132 before infection was sufficient to stabilize cyclin D1 in cells harboring WT *L. pneumophila*, as there was no detectable change in cyclin D1 levels in infected cells compared with bystander cells following treatment (Fig. 4B). These results are consistent with the documented role of ubiquitination in regulating cyclin D1 levels (37–39) and in agreement with a previous study demonstrating that MG132 treatment stabilizes cyclin D1 in cells treated with CHX (40).

Levels of cell cycle proteins are maintained through the action of two ubiquitin ligases, the SCF complex (SKP1–CUL1–F-box protein) and the APC/C (anaphase-promoting complex/cyclosome) complexes, with both targeting specific substrates to the 26S proteasome (41, 42). The APC/C activity is dependent on two coactivators, CDC20 and CDC20-related protein FZR1 (also known as CDH1; Fig. 5C). Although APC\(^{\text{Cdc20}}\) is important to complete mitosis, association of the APC\(^{\text{FZR1}}\) complex is important throughout the G1/S phase checkpoint (43), with FZR1 knock-downs resulting in early S-phase entry and truncated G1 phase (44). For this reason, FZR1 was knocked down in macrophages to determine if depletion reduced the cell cycle arrest phenotype after challenge with *L. pneumophila* (Fig. 5D). In agreement with

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**Fig. 4.** Ectopic expression of the LGTs is sufficient to block host translation and proliferation. (A and B) Transfected Lgts block translation. HEK 293T cells were transfected with GFP, Lgt1, Lgt3, or Lgt3* (catalytically dead) for 24 h. At 3 h before harvest, cells were methionine-starved for 1 h and incubated with 50 μg/mL CHX as appropriate. Cells were then incubated with AHA for 2 h, and de novo protein synthesis was measured by orthogonal chemistry and flow cytometry. (C) Lgts are sufficient to block proliferation. HEK 293T cells were transfected as in A. At 2 h before harvest, cells were incubated with 20 μM EdU, and de novo DNA synthesis was measured as in B. (D) Lowered steady-state levels of cyclin D1 in the presence of Lgts. HEK 293T cells were transfected as in A, and cyclin D1 levels were measured by immunoblot. (E and F) Overproduced cyclin D1 does not overcome Lgt inhibition. Cotransfection of Lgt3 or Lgt3* inactive enzyme with pcDNA3 harboring HA-cyclin D1 protein. At 24 h posttransfection, the levels of remaining cyclin D1 were measured by immunoblot (E). (F) The percentages of proliferating cells were measured by EdU incorporation as in C. Statistical analyses were performed by unpaired t test (*P < 0.05, **P < 0.01, and ***P < 0.001).

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**Fig. 5.** FZR1 silencing partially restores entry into S phase in response to *L. pneumophila*. (A) Challenge with *L. pneumophila* destabilizes cyclin D1. Densitometry analysis of cyclin D1 in uninfected RAW 264.7 macrophages and cells challenged with *L. pneumophila* in the presence of 10 μg/mL CHX. (B) Proteasome inhibition stabilizes cyclin D1. Immunoblot analysis of cyclin D1 in RAW 264.7 macrophages pretreated for 60 min with 10 μM of the proteasome inhibitor MG132, challenged with WT *L. pneumophila* for 2 h, and sorted by flow cytometry. (C) Schematic representation of the APC/C association with its coactivators in different phases of the cell cycle (Left). (Right) Efficiency of targeting Fzr1 with siRNA based on quantitative RT-PCR. (D) Depletion of FZR1 partially rescues proliferation of *L. pneumophila*-targeted cells.RAW 264.7 macrophages treated with siRNA against Fzr1 or nontargeting siRNA (NT) were challenged with *L. pneumophila*-GFP for 1 h, incubated with 20 μM EdU for an additional 2 h, and analyzed for de novo DNA synthesis by flow cytometry. (Top) Macrophages in absence of bacteria. Horizontal lines are used to represent gates that separate bystander from infected cells (based on lack of GFP fluorescence), and vertical lines are used to show percentage of S-phase cells based on the scans. (Bottom) Macrophages after 3 h total of infection based on gates determined above. Infected cells are those found in the GFP*+* gate. Sol et al.
previous studies, uninfected cells with FZR1 knockdown resulted in an increase in S-phase cells in the uninfected population (19.5–33.72%; Fig. 5D). Interestingly, we observed a slight increase in S-phase population in infected cells treated with siRNA against FZR1 compared with nontargeting control, consistent with a role for FZR1 in mediating cell cycle arrest resulting from translation inhibition by *L. pneumophila* (Fig. 5D, Bottom).

**Discussion**

Manipulation of the host cell cycle is a common strategy used by bacteria and viruses to support intracellular growth (45, 46). Previous work showed that the intracellular pathogen *L. pneumophila* modulates the host cell cycle in a T4SS-dependent manner (18, 19). In particular, we previously found that *L. pneumophila* growth was enhanced in G1 and G2 phases, whereas S phase was found to be linked to defective pathogen growth and loss of LCV membrane integrity (18). These results indicate that bacterial control of the host cell cycle can limit exposure of the pathogen to antimicrobial events that are cycle-specific, such as avoiding S phase that is highly restrictive for *L. pneumophila* intracellular growth. It should be noted that the natural hosts of *Legionella*, amoebae, are presumably growth-arrested at the time of bacterial contact as a result of nutrient limitation in their common reservoirs, such as cooling towers. This may provide an opportunity for *L. pneumophila* to lock the host in a state that can promote bacterial growth even when the amoebae respond to nutrient acquisition, such as when they graze on microbial prey. In mammalian hosts, growth arrest is in dynamic conflict with the ability to control intracellular replication, as entry into S phase can be considered an antimicrobial strategy to block intracellular replication. For example, a myeloid precursor responding to growth factor stimulation provides a hostile environment for *L. pneumophila*, as contact with the bacterium during S phase will restrict replication and result in degradation of the LCV with ensuing bacteriolysis.

In this study, we have shown that host cell cycle arrest by *Legionella* is dependent on the Icm/Dot T4SS and is independent of the cell cycle phase at the time of bacterial contact (Fig. 1). In particular, the host proliferation block was found to rely on the activity of five translocated translation inhibitors, with the function of one of these sufficient to cause cell cycle arrest. It should be noted that the Lgt arrest protein synthesis at the elongation step by glucosylation of serine 53 in eEF1A. We found that, early in infection, these translocated substrates are critical for blocks in proliferation and host DNA synthesis (Figs. 2–4). Furthermore, their presence and enzymatic activity induced the rapid degradation of the G1/S master regulator, cyclin D1, which prevented cells from entering S phase (Figs. 3 and 4). Chemical inhibition of protein synthesis by CHX was sufficient to cause cell cycle arrest in the presence of the ΔS strain lacking the five bacterial protein synthesis inhibitors, indicating that the host translation machinery is likely the *L. pneumophila* effector target that causes arrest (Fig. 3). In addition, ectopic expression of the LGTs was sufficient to block DNA synthesis and led to cell cycle arrest in transfected cells (Fig. 4). These data support a model in which the translation block by *Legionella* is the primary cause of host cell cycle arrest and provides an important selective pressure for retention of this activity. It should be pointed out from previous work that the cell cycle blockade caused by RNAi depletion of elongation factors confers no cell cycle phase specificity, with the distribution of DNA content in depleted cells appearing similar to that in proliferating cells (18, 47). This is identical to the result observed in this work. Elongation inhibition clearly provides a strategy to block entry into S phase as well as prevent mitosis (Fig. 1), which may be similarly restrictive of intracellular growth. As we previously noted, although it seems counterintuitive to encode a protein that allows a block in S phase, in amoeba, this phase is extremely short and difficult to identify experimentally (18). The limited opportunities for interaction with the natural host during S phase may also limit the opportunity for negative selection.

There is evidence for a host-induced translation initiation block in response to bacterial infection that is regulated by the mTOR pathway (29, 33, 36, 48). In addition, there may be uncharacterized translocated substrates that interfere with translation initiation. Ribosome profiling analysis of the WT *L. pneumophila* strain and a strain lacking all known translocated protein synthesis inhibitors provides solid evidence for other proteins that could interfere with translation initiation (49). Even so, it is clear that the translation blocks provided by these other mechanisms of translation inhibition are not sufficient to interfere with cell cycle progression based on our analysis of the ΔS strain (Fig. 2). There is no clear explanation for why an initiation factor block is not sufficient to cause cell cycle arrest. It is known that depletion of translation initiation factors by RNase treatment causes a dramatic arrest in G phase, blocking entry of cells harboring *L. pneumophila* into S phase and stimulating intracellular replication (18, 47). Presumably during *L. pneumophila* growth, the factors that promote translation inhibition allow sufficient breakthrough translation to allow the cell cycle to proceed in their presence.

Control of cyclin D1 function is associated with control of bacterial and viral growth within hosts, but cyclin activity has different consequences depending on the pathogen (45, 50). A clear example is a recent study that indicates that induction of cyclin D1 expression is required for *Salmonella* replication (51), and our results show that *Legionella* accelerates cyclin D1 turnover to drive microbial replication (Fig. 5). Cyclin D1 degradation was directly connected to the enzymatic activity of the Lgt proteins (Fig. 3). As a result of the tight link between cyclin D1 regulation and the cell cycle state, induction of cyclin D1 degradation by *Legionella* could be the key event that blocks entry into S phase in response to the microorganism. It was recently shown that depletion of FZR1 could lead to prolonged S phase (44), consistent with the results presented here (Fig. 5). Regulation of cyclin D1 is a key feature in cellular proliferation, and, as a consequence, cyclin D1 misregulation has been found to be involved in several types of cancer (32). Furthermore, manipulation of the APC/C complex is a common strategy used by viruses (52). Regulation of FZR1 activity could provide a rapid strategy to induce S-phase entry or cause cell cycle arrest upon pathogen contact, which is a common theme in many pathogenic events.

In summary, our results demonstrate that specific Legionella translocated substrates interfere with the host cell cycle, causing cells to show growth arrest at the stage in the cell cycle that encounters the microorganism. These data provide evidence for the key role for cyclin D1 turnover in infected cells, consistent with a role for the APC/C complex in promoting bacterial replication by preventing G1 cells from entering S phase, which restricts *L. pneumophila* growth. Although *Legionella* infects terminally differentiated host macrophages, recent studies show that tissue-resident macrophages can proliferate in situ in response to different triggers, providing potentially antimicrobial reservoirs (53, 54). These data point to a model in which S-phase reentry by macrophages harboring *Legionella* could serve as a poorly appreciated strategy to promote restriction of selected pathogens.

**Materials and Methods**

**Bacteria and Culturing Media.** *L. pneumophila* Philadelphia 1 strains used in this study are described in Table 1. *L. pneumophila* was grown in N-(2-acetamido)-2-aminoethanesulfonic acid-buffered yeast extract (AYE) broth medium supplemented with 100 μg/mL thymidine and maintained on solid medium by using buffered charcoal yeast extract agar (BCYE). Strains carrying pGFP Cm plasmids were maintained on BCYE agar containing 100 μg/mL thymidine and 5 μg/mL chloramphenicol. When appropriate, IPTG was added to a final concentration of 1 mM. Strains carrying pJ8908 plasmid were grown in AYE without thymidine supplementation.

**Mammalian Cell Culture.** RAW 264.7 cells (American Type Culture Collection) were grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% heat-inactivated FBS and 1 mM L-glutamine. HeLa and HEK293T cells were cultivated in DMEM (Gibco) supplemented with 10%
heat-inactivated FBS (Gibco). All cell lines were cultured at 37 °C with 5% CO2.

**Infection with L. pneumophila.** L. pneumophila strains were grown on plates on charcoal yeast extract agar plates for 36–48 h before infection. Patchers were then resuspended in AYE media with the appropriate supplements and grown overnight. Challenge of host cells was carried out at a multiplicity of infection (MOI) of 50 to allow for pool adhesion by the ΔSflA strains. Following challenge, plates were subjected to centrifugation for 10 min at 400 × g to synchronize the infection. One to 2 h after uptake, cells were washed twice to remove extracellular bacteria and resuspended in fresh medium.

**HeLa Cell Cycle Synchronization.** HeLa cells were synchronized by double thymidine block (55). Briefly, 1 × 106 HeLa cells were incubated with an excess of 2 mM thymidine for 18 h. Cells were washed twice with 1× PBS solution and released by incubation for 8 h in DMEM-FBS without thymidine. Following release, cells were treated with a second dose of 2 mM thymidine. After 14–16 h, cells were collected and replated at 2.5 × 105 cells per well in six-well plates for cytometry or challenge with L. pneumophila at the indicated times after release.

**EdU Incorporation and de Novo DNA Synthesis Detection.** RAW 264.7 cells were plated at 1 × 106 cells per well in six-well plates and then challenged with L. pneumophila at an MOI of 50. At 1 h, cells were washed with 1× PBS solution and incubated with medium containing 20 μM of the deoxynucleotide analog EdU for an additional 2 h. At 3 hpi, cells were collected, and de novo DNA synthesis was measured by orthogonal Click-IT chemistry with a Click-IT EdU Alexa Fluor 647 Assay Kit (Invitrogen) and analyzed by flow cytometry with a BD LSRII analyzer.

**Proliferation Assay in D. discoideum.** To measure cell proliferation in amoebae at 23 °C, stationary-phase D. discoideum AX4 cells were collected and seeded at 2 × 106 cells per well on six-well plates in PBS solution. Cells were incubated in the dark with 10 μg Glucosyltransferase.

**Immunoblotting.** RAW 264.7 cells were plated at a density of 1 × 105 cells per well in 10-cm dishes and then challenged with L. pneumophila-GFP strains at an MOI of 50. When appropriate, cells were incubated for 1 h before Legionella challenge with 50 μg/mL CHX or 10 μM MG132. At 2 hpi, cells were washed with 1× PBS solution and harvested. Cells were fluorescently sorted based on GFP signal by using a BD INFLUX sorter. Postsorted infected cells (GFP+) and bystander cells (GFP-) were subjected to centrifugation and resuspended in RLT lysis buffer (RNeasy kit, QIAAGEN) according to the manufacturer's instructions. RNA was isolated by using the RNeasy kit, and cDNA synthesis was performed by using SuperScript IV VILO Master Mix (Invitrogen). Transcripts were measured by using SYBR green (Applied Biosystems) from cDNA templates using the following primer pairs: Cond1(5'-TGCGGAAAGTTGTGCATCTA and 3'-ACCTTGGAGAAGGACCTGTG), Cond2(5'-CTGGGAAGAAGCCGAGATGAC and 3'-AAGGATGGGTTGGGAGTGT), Inv1(5'-CCTGGATGAACTGCTAGAAGAAA and 3'-AAGAGAGACGGAAAGCTCAA), Inv2(5'-CAACACCCATGAGGACCTGAC and 3'-CCTCTGCTACAATGGGCTCG), and Gapdh(5'-CAAGGTATCCTAGGAGCTGAA and 3'-ACACAGGAGCAGACTAAC). Gene expression levels were quantified by using the 2ΔΔCt method with Gapdh as an endogenous control. All reactions were carried out by using the StepOnePlus real-time PCR system (Applied Biosystems).

**Gene Silencing by siRNA.** All siRNA transfection experiments were performed by using Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's instructions for 72 h. siRNA oligonucleotides were purchased from Dharmacon and were specifically designed to deplete mouse FZR1. Nontargeting siRNA was used as a control.

**Transfection of Legionella Glucosyltransferase.** HEK 293T cells were seeded at 2 × 106 cells per well in a six-well tissue culture plate. Cells were transfected by using Lipofectamine 2000 or 3000 (Invitrogen) according to the manufacturer’s protocol. At 24 hpi, cells were lysed in Laemmli sample buffer.
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