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# Increases in c-Jun N-Terminal Kinase/Stress-Activated Protein Kinase and p38 Activity in Monocyte-Derived Macrophages following the Uptake of *Legionella pneumophila*

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***Legionella pneumophila*, the causative agent of Legionnaires' disease, infects and replicates within a variety of eukaryotic cells. The purpose of the current study was to examine host cell signaling events immediately following uptake and early in the endocytic process (less than 1 h) following the phagocytosis of *L. pneumophila*. This examination focused on the protein kinase signal pathways to identify any aberrant signal(s) induced by *L. pneumophila* within its host, as a means to alter the normal endocytic pathway. The mitogen-activated protein kinase cascades are of interest due to their involvement in cellular regulation. The experiments were carried out with monocyte-derived macrophages (MDMs). All three mitogen-activated protein kinase cascades were activated when MDMs were inoculated with either *Legionella* strain (wild-type strain AA100 or *dotA* mutant GL10) or an *Escherichia coli* control. Whereas the avirulent treatments, GL10 and *E. coli*, exhibited a leveling off or a return to near basal levels of phosphorylation/activity of c-Jun N-terminal kinase by 60 min, the virulent strain AA100 exhibited a significantly increased level of activity through 60 min that was greater than that seen in GL10 ($P = 0.025$) and *E. coli* ($P = 0.014$). A similar trend was seen with p38 phosphorylation. Phosphorylation of mitogen-activated protein/ERK kinase (MEK) was decreased in strain AA100 compared to *E. coli*. Inhibition of the activity of either the stress-activated protein kinase/c-Jun N-terminal kinase or p38 pathway significantly decreased the ability of legionellae to replicate intracellularly, suggesting the necessity of these two pathways in its intracellular survival and replication.**

*Legionella pneumophila*, the causative agent of Legionnaires' disease, infects and replicates within a variety of eukaryotic cells. During the disease process, the primary host cells are thought to be alveolar macrophages and monocytes (18, 22, 32). In monocytes, phagocytosis of the bacterium is promoted in the presence of opsonizing antibodies and complement (35, 48), although nonopsonic phagocytosis of *L. pneumophila* is also well described (40). In one method of uptake, a "phagocyte pseudopod" coils around the bacterium as it is internalized, initiating an unusual mechanism known as coiling phagocytosis (20). Coiling phagocytosis may allow for the exclusion of MHC class II and I from the site of attachment (7). It has also been shown that legionellae can be taken up by conventional phagocytosis (6).

During normal phagocytosis of bacteria, the phagosome undergoes a complex series of biochemical changes; including acquisition of late endocytic organelle markers such as rab7, LAMP1, and LAMP2 as well as the loss of early endocytic markers, such as rab5 (46). Vacuolar ATPases accumulate in the phagosomal membrane as it matures and these may be involved in acidification of the phagosome (46). Subsequently, phagosomal compartments acquire proteases and antibacterial compounds by fusion with organelles (i.e., lysosomes) in the endocytic pathway (2, 46).

Following the phagocytosis of *L. pneumophila*, mitochondria and rough endoplasmic reticulum surround the phagosome (19). Phagosomes containing *L. pneumophila* do not fuse with the lysosomes and remain nonacidic, of which both factors are important for their intracellular survival (19, 21). Recruitment of rough endoplasmic reticulum around the phagosome has been linked to macrophage-specific infectivity loci (mil) (15). A large bacterial membrane complex that forms a type IV secretion system, coded for by the *dot* (defect in organelle trafficking) and *icm* (intracellular multiplication) genes, is required to alter the endocytic pathway within the macrophage (3, 27). Studies have shown that *L. pneumophila* requires the expression of DotA to establish an intracellular site for replication (25, 41). Bacteria expressing DotA are routed through an alternate endocytic pathway in which phagosomal markers, LAMP1, LAMP2, cathepsin D, and CD3 proteins are excluded from the phagosomal membrane and lysosomal membrane markers, such as Rab7, are also absent. However, Sturgill-Koszycki and Swanson have shown that by 18 h postuptake, endosomes containing *L. pneumophila* exhibit late endosomal markers and characteristics, suggesting that phagosome-lysosome fusion does occur late in the replication cycle (43). This alteration in cellular function by *L. pneumophila* suggests that the intracellular signaling pathways responsible for endosomal maturation may have been altered.

Mitogen-activated protein (MAP) kinase cascades play a key role in the regulation of host cell gene expression as well as cytoplasmic activities (5, 29, 38). Three parallel mitogen-activated protein kinase cascades have been described and include the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/

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SAPK), and the p38 mitogen-activated protein kinases (p38s). The ERKs are typically activated by mitogenic factors, whereas JNK/SAPK and p38 are activated by stress-inducing agents or proinflammatory cytokines (26). Tse et al. (47) and Blumenthal et al. (4) have both shown the requirement of both p38 signaling, through the use of specific kinase inhibitors, and the reduction of ERK activation for the intracellular survival and virulence of *Mycobacterium avium* in human macrophages. A correlation has also been shown between the induction of apoptosis in gastric epithelial cells by *Helicobacter pylori* and an increase in SAPK/JNK activity (13). Interactions between bacteria and host mitogen-activated protein kinase signaling pathways have also been seen in the induction of host cell apoptosis by a variety of bacteria, including *Yersinia enterocolitica* (12) and *Pseudomonas aeruginosa* (23).

Previous work in this laboratory by Coxon et al. (10) examined the signals involved in phagocytosis of *L. pneumophila* by human monocytes and showed that tyrosine kinase and protein kinase C activities are necessary at the site of bacterial uptake. This study concluded that the tyrosine phosphorylation uptake signals and-actin polymerization during phagocytosis of opsonized legionellae are normal and are generally that which would be seen during uptake of most opsonized bacteria. The purpose of the current study was to examine in more detail host cell signaling events immediately following uptake and early in the endocytic process (less than 1 h) following the phagocytosis of nonopsonized *L. pneumophila*. This examination focused on the protein kinase signal pathways to identify any aberrant signal(s) induced by *L. pneumophila* within its host, as a means to alter the normal endocytic pathway. The mitogen-activated protein kinase cascades are of interest due to their involvement in cellular regulation. We sought to examine whether *L. pneumophila* alters its endosomal environment by altering host cell signal pathways early within the infection, specifically those of the mitogen-activated protein kinase cascades, to delay the maturation of the phagosome.

## MATERIALS AND METHODS

**Bacterial strains.** *L. pneumophila* strains AA100 and GL10 were from the laboratory of Yousef Abu Kwaik (University of Kentucky Chandler Medical Center). The virulent *Legionella pneumophila* strain AA100 is a clinical isolate characterized previously (15). The avirulent strain GL10, a *dotA* mutant defective in intracellular replication, has also been previously described (15). The *Escherichia coli* strain used, K-12, was a common laboratory strain grown on Trypticase soy agar (Becton-Dickinson, Cockeysville, Md.) plates. Frozen aliquots of both *Legionella pneumophila* strains were washed in distilled $H_2O$ to remove freezing medium and plated on standard buffered charcoal yeast extract (BCYE) agar plates (Becton-Dickinson, Cockeysville, Md.) for 3 days before use in any experiments.

**Macrophage cell culture.** Monocyte-derived macrophages (MDMs) were isolated and cultured based upon previously published methods (34). Briefly, blood was collected from normal, healthy human volunteers and peripheral blood mononuclear cells were isolated over a Ficoll density gradient (Fico-Lite-LymphoH, Atlanta Biologicals, Norcross, Ga.). Peripheral blood mononuclear cells were plated at a density of $5 \times 10^6$ cells/ml in Teflon six-well plate inserts in RPMI (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% [vol/vol] nonessential amino acids, 10 mM HEPES) and incubated at 37°C for 3 days. Cell monolayers were then scraped and washed in cold Hanks' balanced salt solution before plating in a 24-well plate at a density of $5 \times 10^6$ cells/well. Monolayers were then incubated for 2 days in RPMI with 10% fetal bovine serum before nonadherent cells were removed by washing with cold Hanks' balanced salt solution. The resulting MDMs were allowed to rest by incubating an additional 1 to 2 days in RPMI lacking serum before use. Viability was assessed by trypan blue dye exclusion and monocyte enrichment was established with the alpha-naphthyl acetate (nonspecific esterase) (Sigma USA) following the manufacturer's inserts. Criteria for further cell usage were set at >98% viability, >95% purity, and a resulting cell density of $\approx 5 \times 10^5$ cells per well.

**Intracellular replication protocol.** To study the intracellular survival and multiplication of *L. pneumophila* strains AA100 and GL10 in MDMs, bacteria were taken from BCYE agar plates, washed, resuspended in RPMI at $10^8$ bacteria/ml and added to MDM monolayers at a multiplicity of infection (MOI) of 1:1 or 30:1 (bacteria/cell). Plates were centrifuged at 1,500 rpm (Sorval RT 6000D) for 20 min at 20°C to aid in bacterial adhesion to the monolayer. After centrifugation, plates were incubated at 37°C for 2 h to allow bacterial uptake, followed by the addition of gentamicin at a concentration of 25 μg/ml for 30 min. Monolayers were then washed and the zero time wells were lysed with sterile distilled $H_2O$, while all remaining wells were overlaid with RPMI and incubated at 37°C until the appropriate time for lysing (24 and 48 h). Cell lysates were plated on BCYE agar for 3 days and resulting *Legionella* colonies were counted.

To determine the normal bacterial uptake and removal rate of the MDMs, monolayers were inoculated with serum-opsonized *Escherichia coli* at an MOI of 1:1. Serum opsonization of *E. coli* was performed by incubation in RPMI with 30% pooled human sera for 30 min at 37°C, before washing the bacteria, and resuspension in RPMI with 10% fetal bovine serum at the final concentration. All plates were centrifuged at 1,500 rpm for 20 min at 20°C to aid in bacterial adhesion to the monolayers. The monolayers were then gently rinsed with warm RPMI (minus fetal bovine serum) and the time zero wells were lysed with cold sterile distilled $H_2O$. At predetermined time points (15, 30, 45, and 60 min), subsequent wells were lysed and serially plated on TSA plates. After 24 h of incubation the CFU per time point was determined.

**Infection protocol for mitogen-activated protein kinase activity.** MDM monolayers were inoculated with either *L. pneumophila* strain AA100, GL10, or *E. coli* at an MOI of 30:1. This MOI was used in an attempt to maximize the signal induced by *L. pneumophila* infection, while minimizing any cytopathogenic effects due to the presence of the bacteria. All bacteria used in the mitogen-activated protein kinase assays were nonopsonized. At various predetermined time points the monolayers were lysed with 30 μl of a cold immunoprecipitation buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM disodium EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride). The standardization of the protein concentration within the cell lysates was then carried out in triplicate with a BCA assay kit (Promega) following the manufacturer's protocol.

**Western blot.** Cell lysates were then diluted with treatment buffer (125 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 20% glycerol, 1% β-mercaptoethanol, and 0.003% bromophenol blue) containing 200 μM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate to yield 5 μg of protein per 15 μl of sample. Samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% minigels. Proteins were transferred to nitrocellulose membranes (0.45 μ pore size) with a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad).

Western blotting was then performed on each membrane with a primary antibody specific for the phosphorylation-state specific form of the protein of interest followed by a secondary horseradish peroxidase-conjugated antibody. Antibody-bound proteins were detected with an enhanced chemiluminescence ECL Western blotting analysis system (Amersham Corp.) and the membranes were exposed to Kodak X-Omat LS x-ray film. Films were scanned for band density with the Fluor-S MultiImager and accompanying Quantity One software (Bio-Rad, Hercules, Calif.). Loading controls consisted of reprobing of the membranes with non-phosphorylation-specific antibodies or an anti-β-actin antibody. Membranes were stripped by incubating the membrane in a stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tri-HCl, pH 6.7) for 30 min at 50°C. Antibodies used included anti-phospho-/total-MAP/ERK kinase 1/2 (MEK1/2), anti-phospho- and anti-total-ERK1/2, anti-phospho- and anti-total-p38, anti-phospho- and anti-total-SAPK/JNK (Cell Signaling Technology, Beverly, Mass.), and anti-β-actin (murine) for loading control (Sigma).

**Kinase assays.** Kinase activity of p38, ERK, and JNK was assayed with nonradioactive assay kits purchased from Cell Signaling Technologies (Cell Signaling Technology, Beverly, Mass.). Assays were performed according to enclosed manufacturer's procedures. Lysates were taken from $1 \times 10^6$ cells with the following treatments: untreated control inoculated with either *Legionella* strain AA100, GL10, or *E. coli* at an MOI of 30:1. Protein concentrations were normalized as mentioned above. In short, 200 μl of lysates were incubated overnight with the 15 μl of immobilized antibodies to the protein of interest (ERK, p38, or JNK) at 4°C with gentle rocking. Lysates were microcentrifuged (13,000 rpm/16,000 x *g*) to pellet immobilized antibody-protein complexes. The resulting pellets were washed to remove inhibitors and a kinase reaction buffer containing ATP, and

the substrate of interest (recombinant peptide rELK, rATF, or c-Jun; Cell Signaling Technology) was added. The substrates used were recombinant peptides corresponding to the activation domain of the transcription factor proteins. Pellet and kinase buffer were incubated at 30°C for 30 min before the reaction was terminated by the addition of 3x treatment buffer (Cell Signaling Technologies). Kinase reaction results were visualized with Western-blotting techniques mentioned above. Antibodies for the phosphorylated rATF and c-Jun substrates were included with the kits.

**Mitogen-activated protein kinase inhibitor assays.** MDM monolayers were treated with inhibitors of MEK, PD98059 (Sigma) or U0126 (Cell Signaling Technologies); inhibitor of p38, SB203580 (Biomol Research Laboratories, Inc.); inhibitor of SAPK/JNK, SP600125 (Biomol); or inhibitor of phagocytosis, cytochalasin D (Sigma), at previously published concentrations (8, 28). *L. pneumophila* AA100 was then added at an MOI of 1:1 before the plates were centrifuged at 1,500 rpm for 20 min at 10°C. The monolayers were then treated as above with gentamicin and cells lysed at 24 and 48 h. Cell lysates were plated on BCYE agar for 3 days and resulting *Legionella* colonies were counted. No evidence of inhibitor cytotoxicity was evident following addition of inhibitors by trypan blue exclusion test for viability (>95%), by a caspase 3 activity assay (>93%, ENZChek caspase-3 assay kit 2, Molecular Probes, Eugene, Oreg.), and by a mitochondrial membrane potential assay (>98% MitoCapture apoptosis detection kit, Apotech).

## RESULTS

**Characterization of the MDM model for the intracellular growth of *L. pneumophila*.** To determine the permissibility of MDMs for intracellular replication, monolayers were inoculated with *L. pneumophila* wild-type strain AA100, *dotA* mutant strain GL10, or *E. coli* at an MOI of 1:1 (bacteria:cell). This minimal MOI was used in order to exhibit the greatest increase in bacterial numbers with the lowest cytopathogenicity to the cell monolayer. A representative growth curve is seen in Fig. 1A where AA100 exhibited a 1.7 ± 0.2 log increase in bacterial numbers by 24 h and a 2.3 ± 0.3 log increase by 48 h. The mutant strain GL10 exhibited intracellular survival, but no increase in bacterial numbers at either 24 or 48 h time points. When macrophages were inoculated with *E. coli* (Fig. 1B), a reduction of bacterial numbers of approximately 40% of original adherent bacterial numbers (time zero minutes) was seen by 30 min and a reduction of 70 ± 0.5% was observed at 60 min. Similar results were exhibited when the MOI used was 30:1, with a slightly greater cytopathogenic effect of AA100 on the MDMs at 48 h (data not shown).

**Western blot and kinase assay analysis of individual mitogen-activated protein kinase cascades following bacterial infection and uptake. (i) SAPK/JNK pathway.** Western blot analysis for JNK phosphorylation (Fig. 2A) detected two isoforms (JNK1 and JNK2) which followed a general pattern of basal phosphorylation at time zero, increasing through 5, 10, and 15 min, with maximal phosphorylation by 30 min. When comparing treatments, no differences in JNK phosphorylation were evident up to 30 min. At 60 min, a decrease in phosphorylation is seen in both *L. pneumophila* strains, with an even greater decrease in the phosphorylation level in the *E. coli* treatment (returning to near basal levels). To confirm normalization of protein levels, membranes were reprobed for β-actin and showed similar amounts in all treatments. The kinase activity of JNK (Fig. 2B and 2C) was measured by the increase in phosphorylation of a recombinant protein substrate, c-Jun. Activity exhibited for JNK in treated MDMs was evident between 10 to 15 min in all three treatments. Both GL10 and *E. coli* exhibited an increase in activity through 30 min with a plateau of activity that remained at 60 min. However, the activity of the AA100 treatment showed an increasing level of activity through 60 min that was significantly greater than that seen in GL10 ($P = 0.025$) and *E. coli* ($P = 0.14$) when the results of four experiments were averaged (Fig. 2C).

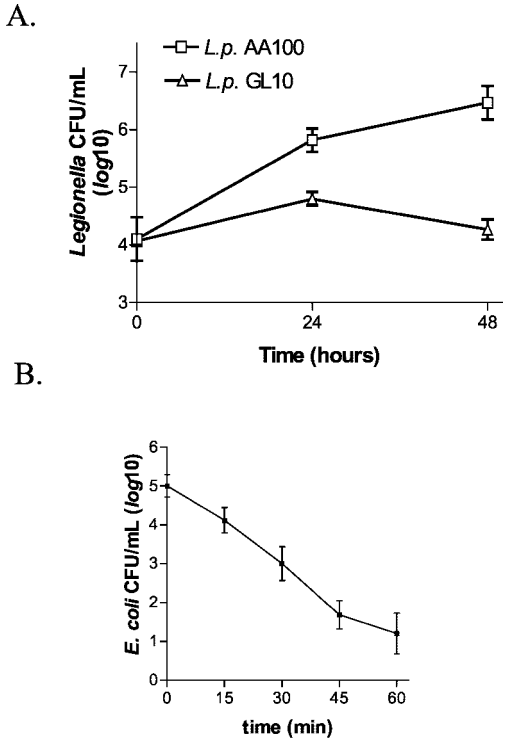


FIG. 1. Intracellular growth curves of *Legionella pneumophila* virulent strain AA100 and avirulent mutant GL10 in MDMs. (A) A 48-h time course exhibiting an approximate 2.5 log increase in AA100 (□) CFU compared to the no growth exhibited by GL10 (△) in MDMs. (B) Kill curve of *E. coli* in MDMs, approximate 70% reduction in bacterial CFU by 60 min.

**(ii) MEK/ERK pathway.** The phosphorylation pattern of MEK for both *Legionella* strains exhibited an increase above basal by 15 min that carries through 30 min with a return to near basal level by 60 min (Fig. 3A). In comparison, the *E. coli* treated cells exhibited a pattern of MEK phosphorylation that was sustained through 60 min. A significant difference ($P = 0.005$) is seen between *Legionella pneumophila* AA100 and *E. coli* at 60 min when averaged over five experiments (Fig. 3A and 3C). ERK phosphorylation across all three treatments was similar, with phosphorylation increasing through 30 min and decreased at 60 min (Fig. 3B). Due to problems with stripping of the membranes, a β-actin loading control was used for both MEK and ERK phosphorylation Western blots. ERK activity was measured with a recombinant ELK substrate and showed results similar to the ERK Western blot (data not shown). When multiple experiments were analyzed, no significant difference was found between the treatments in both ERK phosphorylation and ERK activity. As seen in Fig. 3A and 3B, multiple isoforms for both MEK (MEK1 and MEK2) and ERK (p42/p44) were detected on the Western blots.

**(iii) p38 pathway.** The pattern evident in p38 signaling (Fig. 4A and 4B) demonstrated phosphorylation by 5 min that increased through 30 min but was reduced at 60 min. A signifi-

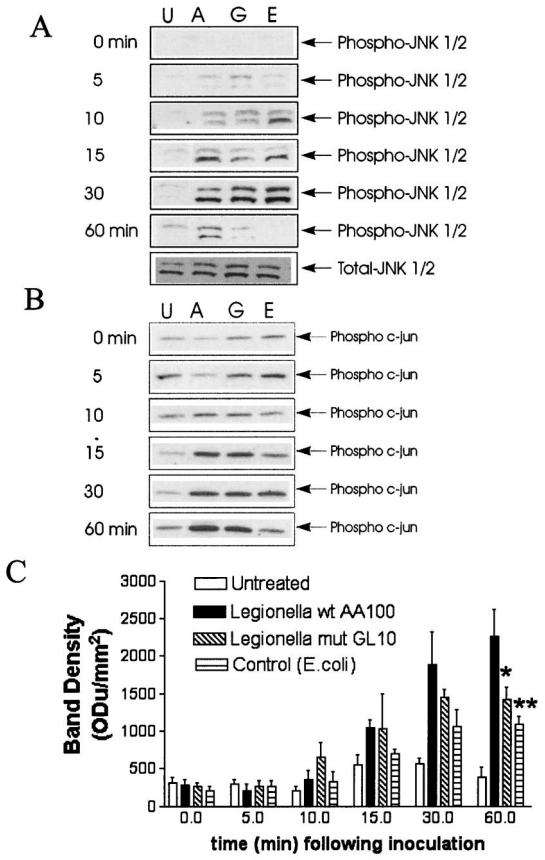


FIG. 2. SAPK/JNK phosphorylation and kinase activity is significantly increased following uptake of *L. pneumophila* AA100. (A) Representative Western blot of JNK phosphorylation over a 60-min time course. Infected MDMs (U, untreated; A, AA100; G, GL10; and E, *E. coli*) were analyzed for SAPK/JNK phosphorylation and kinase activity. (B) JNK kinase activity measured by determining increase in phosphorylation of recombinant protein substrate (c-Jun) through Western blot analysis; representative Western blot of kinase assay showing increased activity in AA100 at 60 min. (C) Graphic representation of data averaged from multiple experiments showed significant increase in JNK activity in AA100 over GL10 ($P = 0.025$) (*) and over *E. coli* ($P = 0.014$) (**) ($N = 4$).

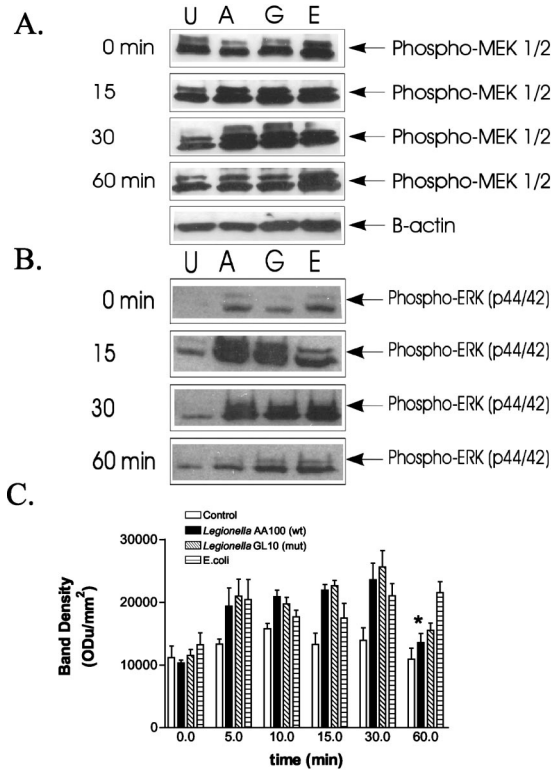


FIG. 3. MEK phosphorylation is significantly decreased in *L. pneumophila* treated MDMs. (A) Representative Western blot of MEK phosphorylation over a 60-min time course, with only pertinent time points shown. Treated MDMs (U, untreated; A, AA100; G, GL10; and E, *E. coli*) were analyzed for MEK phosphorylation. (B) Representative Western blot of ERK phosphorylation as a measure of MEK activity; only pertinent time points (0, 15, 30, and 60 min) are represented. (C) Graphic representation of MEK phosphorylation data averaged from multiple experiments illustrates significant decrease in MEK phosphorylation ($P = 0.004$) with AA100 treatment (*) ($N = 5$).

cant difference was found at 60 min between AA100 and *E. coli* ($P = 0.04$) with a higher level of phosphorylation of p38 with the AA100 (Fig. 4A and 4C). A similar trend was observed between AA100 and Gl10, but the differences did not achieve statistical significance. p38 kinase activity was measured by determining the increase in phosphorylation of a recombinant ATF protein substrate (Fig. 4B and 4D). A similar pattern was observed as with p38 phosphorylation, although the differences between AA100, GL10, and *E. coli* did not achieve statistical significance.

**Mitogen-activated protein kinase inhibitor studies.** MDM monolayers were pretreated with inhibitors of the three mitogen-activated protein kinase cascades or phagocytosis for 30 min prior to inoculation with *Legionella* AA100 with the protocol mentioned above for generating the intracellular growth curves. The Time zero lysates were serially plated and used to determine the affect of the inhibitors on phagocytosis. The inhibitor of microfilament formation, cytochalasin D (20 μM) significantly reduced the uptake of AA100 by approximately 80%. No affects on phagocytic uptake were seen in any of the other inhibitors. At 48 h the remaining monolayers were harvested as before and the bacterial load was determined. The intracellular growth for each treatment was determined by subtraction of the CFU attime zero from the CFU at 48 h. The result indicates the increase in intracellular bacteria above what was measured at time zero.

Inhibition of MEK activity by PD98059 (20 μM) and U0126 (10 μM) exhibited no effect on intracellular replication of legionellae (Fig. 5B). However, a significant reduction in intracellular replication was evident when JNK or p38 was inhibited. Inhibition of the SAPK/JNK pathway with SP600125 (5 μM) resulted in a significant decrease of 30 ± 0.12% or 0.8 log ($P = 0.01$) of intracellular growth and inhibition of p38 activity (SB203580, 10 μM) decreased intracellular growth by 0.7 log or 28 ± 0.08% ($P = 0.006$). A confirmatory kinase assay (Fig. 5C) showed inhibition of MEK activity, measured by ERK phosphorylation, with PD98059 (90%) and U0126 (91%) with a decrease in SAPK/JNK activity, i.e., c-Jun phosphorylation, at 65%; and decreased p38 activity, i.e., ATF phosphorylation, at 75%.

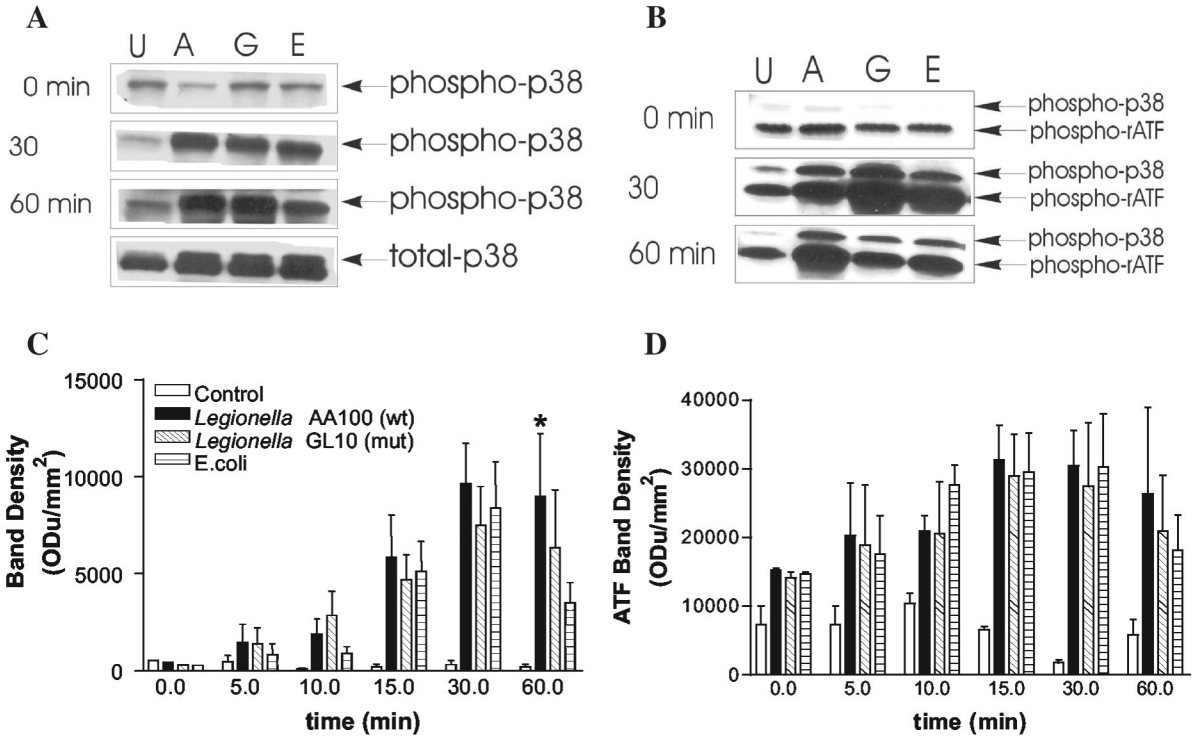


FIG. 4. Increased p38 phosphorylation following the uptake of *L. pneumophila* AA100. (A) Representative Western blot of p38 kinase phosphorylation. U, untreated; A, AA100; G, GL10; and E, *E. coli*. Only pertinent time points (0, 30, and 60 min) are represented. (B) Representative Western blot of p38 kinase assay exhibiting both phosphorylated p38 and the phosphorylated recombinant ATF substrate. (C) Graphic representation of p38 phosphorylation in the AA100 treated MDMs was significantly higher than *E. coli* ($P = 0.04$) at 60 min but no difference was seen between AA100 and GL10 ($N = 4$). (C) Graphic representation of p38 kinase activity averaged from multiple experiments ($N = 4$).

## DISCUSSION

The consensus view of *L. pneumophila* has resulted in a model for bacterial intracellular survival within a monocyte phagosome involving disruption of the normal endocytic pathway, with the resulting recruitment of host cell organelles. Alterations in host cell signaling and cellular functions begin within the first 5 min with the accumulation and attachment of the rough endoplasmic reticulum and mitochondria to the *Legionella*-containing phagosome (45). This organelle recruitment is dependent upon a functioning Dot/Icm transporter (45). Disruption of the Dot/Icm transporter results in the accumulation within the bacteria of DotA protein (31). The purpose of the current study was to examine in more detail host cell signaling events immediately following uptake and early in the endocytic process (less than 1 h) following the phagocytosis of *L. pneumophila*. This examination focused on the protein kinase signal pathways to identify any aberrant signal(s) induced by *L. pneumophila* within the host cell, as a means to alter the normal endocytic pathway.

These experiments were performed with monocyte-derived macrophages (MDMs), a model that has been previously described for *L. pneumophila* (36), and has been used successfully with *M. tuberculosis* (17). The MDM model and protocol described here for *Legionella* growth exhibited typical growth characteristics for *L. pneumophila*, and reduced the variability in culturing of the human primary blood cells and allowed for a reduction in cellular activation preexperimentation.

We have shown that all three mitogen-activated protein kinase cascades were activated when MDMs were inoculated with either *Legionella* strain or the *E. coli* control, but to various degrees. The virulent *Legionella* strain AA100 exhibited a significantly increased level of phosphorylation and activity in SAPK/JNK and p38, with a significant decrease in MEK phosphorylation compared to the avirulent strain or to the *E. coli* control. Chemical inhibitors demonstrated the necessity of both the SAPK/JNK and p38 signals for intracellular growth by the virulent *L. pneumophila*. Work done with *Mycobacterium avium* in murine macrophages and monocyte-derived macrophages has shown that the uptake of virulent *M. avium* will result in the activation of both the extracellular signal-regulated kinase ERK and p38 pathway (4, 47). Peak activation of ERK and p38 was shown to occur at 30 min, with a gradual decrease to basal levels in virulent *Mycobacterium*-treated cells, while less virulent strains exhibited an increased activation for longer periods of time (39).

The specific chemical inhibition of p38 in MDMs did not significantly affect the intracellular replication of *M. avium*, and inhibition of ERK did significantly increase the intracellular growth of some *M. avium* strains (4, 47). In *Mycobacterium avium* infections of murine macrophages the activation of ERK has been shown as necessary for the expression of tumor necrosis factor alpha independently of p38. The reduction in signal in the virulent *Mycobacterium* treatment was thought to correspond to a reduction in host cell expression of immune

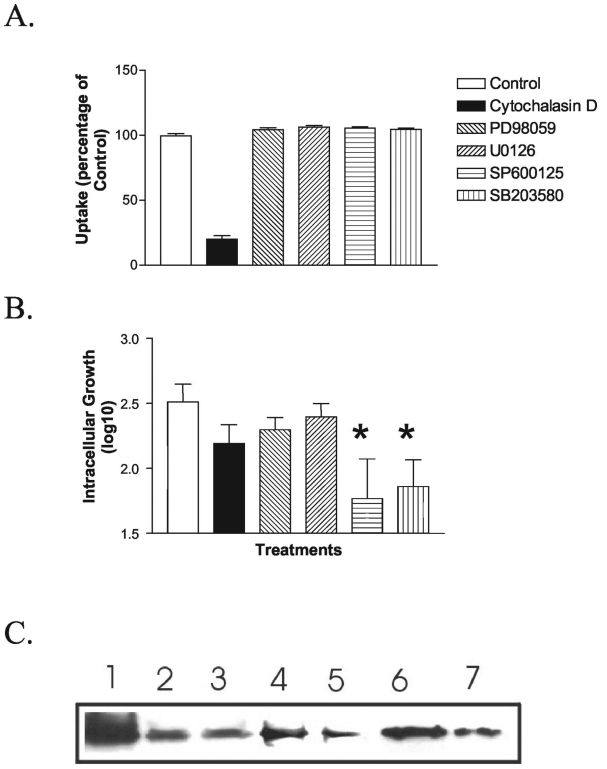


FIG. 5. Inhibition of SAPK/JNK and p38 significantly reduced the intracellular replication of *L. pneumophila* AA100. (A) Mitogen-activated protein kinase inhibitors PD98059 (MEK inhibitor), U0126 (MEK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) were compared to cytochalasin D to determine the effect of inhibitors on phagocytosis. (B) Intracellular growth of AA100 was significantly reduced in SAPK/JNK inhibited (SP600125) ($P = 0.011$) and p38 inhibited (SB203580) ($P = 0.006$) MDMs. (C) Western blot analysis, composite figure of three individual Western blots, of inhibitor activity in MDMs treated for 30 min and activated by incubation with AA100 for 30 min. Lanes: 1, phospho-ERK; 2, PD98059-treated phospho-ERK; 3, U0126-treated phospho-ERK; 4, phospho-c-Jun; 5, SP600125-treated phospho-c-Jun; 6, phospho-ATF; 7, SB203580-treated phospho-ATF.

factors (e.g., tumor necrosis factor alpha, interleukin-1-B, etc.) (39). Conversely, p38 activation is necessary for the expression of interleukin-10 independently of ERK activation (37). To date, there have been no published reports that have examined the activation of the mitogen-activated protein kinase cascades following uptake of virulent *L. pneumophila*, although it has been reported that the expression of tumor necrosis factor alpha, interleukin-1α, interleukin-1β, and interleukin-6 is increased in murine macrophages (30).

The influence of the mitogen-activated protein kinase cascades on the intracellular environment (e.g., organelle recruitment mentioned previously) within the time frame set by these experiments has not been addressed. The current results did not show virulence-associated differences in mitogen-activated protein kinase until 30 to 60 min after infection. While this pathway may represent a possible site for interdiction by legionellae, the level of sensitivity in the procedures utilized in the current study may not have been high enough to determine conclusively if the mitogen-activated protein kinase cascades are involved in the early intracellular environmental changes discussed by Tilney et al. (45). The mechanism by which legionellae is able to modify the intracellular environment has not been identified as of yet, but has been attributed to the *dot/icm* locus. Bacteria with mutations in their Dot/Icm expression have been shown to be nonpathogenic and incapable of intracellular replication, and this has been attributed to defects in their type IV secretion system (9, 42, 49). In the present study, the avirulent mutant was defective in DotA, suggesting that an unidentified product of the *Legionella* type IV secretion system may be injected into the host cells, interacting with the signaling pathways upstream of the mitogen-activated protein kinases.

Previous reports have studied the ability of mutants to remain viable intracellularly, and measured replication as a means of determining the effects of the mutation on pathogenicity. The findings of the current study allow a more discrete correlation between a bacterial expressed protein and a cellular response to be determined. For instance, the hypothesis that a mutation in a particular gene will affect the increased phosphorylation and activity seen in JNK may have more relevance in determining the specific pathogenic mechanisms of legionellae, rather than measuring an end-point such as intracellular replication. The intracellular outcome of an *L. pneumophila* mutant can be varied depending upon the bacterial growth conditions or the host cell model chosen (24, 44). This illustrates the need for a more direct measure of a causal link between the affects of a particular bacterial gene and an aberrant cellular response.

A recent report by Neumeister et al. supports previous work (1, 14, 50) in showing that legionellae has the ability to induce apoptosis early in the infection, followed by necrosis. Neumeister et al. has further shown that the induction of apoptosis is centered on the mitochondrial apoptotic cascade (33). The activation of both the p38 and SAPK/JNK pathways, as seen in the current study, is generally considered pro-apoptotic with ERK activity being anti-apoptotic (11). Thus, it is tempting to speculate that the early activation of SAPK/JNK and p38 pathways by virulent *L. pneumophila* pushes the host cell in the direction of apoptosis. This response would be clearly different from the inhibition of apoptosis observed with other intracellular pathogens such as *Mycobacterium*, *Brucella*, *Chlamydia*, *Rickettsia*, and *Coxiella* species (reviewed in reference 16).

Future studies will examine the effects of the increased phosphorylation and activity seen in the mitogen-activated protein kinase cascades to determine if these signal pathways alter the cellular trafficking machinery and to determine if this increased activity is causal for the induction of apoptosis in the host cell. Investigations into the role of apoptosis in *L. pneumophila* pathogenesis will also examine the protein kinase C pathways, the mitochondrial death pathways, and their effects on caspase-3 activation.

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