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Sustained Activation of Akt and Erk1/2 Is Required for *Coxiella burnetii* Antiapoptotic Activity▽

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Coxiella burnetii **is an obligate intracellular bacterial pathogen that directs biogenesis of a lysosome-like, parasitophorous vacuole in mammalian cells. We recently reported that *C. burnetii* inhibits apoptotic cell death in macrophages, presumably as a mechanism to sustain the host for completion of its lengthy infectious cycle. In the current study, we further investigated *C. burnetii* manipulation of host cell signaling and apoptosis by examining the effect of *C. burnetii* infection on activation of 15 host proteins involved in stress responses, cytokine production, and apoptosis. *C. burnetii* infection of THP-1 human macrophage-like cells caused increased levels of phosphorylated c-Jun, Hsp27, Jun N-terminal protein kinase, and p38 at 2 h postinfection (hpi), and this activation rapidly decreased to near basal levels by 24 hpi. The prosurvival kinases Akt and Erk1/2 (extracellular signal-regulated kinases 1 and 2) were also activated at 2 to 6 hpi; however, the phosphorylation of these proteins increased coincident with *C. burnetii* replication through at least 72 hpi. Sustained phosphorylation of Akt and Erk1/2 was abolished by treatment of infected cells with rifampin, indicating their activation is a *C. burnetii*-directed event requiring pathogen RNA synthesis. Moreover, pharmacological inhibition of Akt or Erk1/2 significantly decreased *C. burnetii* antiapoptotic activity. Collectively, these results indicate the importance of *C. burnetii* modulation of host signaling and demonstrate a critical role for Akt and Erk1/2 in successful intracellular parasitism and maintenance of host cell viability.**

A common property of intracellular bacterial pathogens is their ability to manipulate host cell signaling cascades to promote biogenesis and maintenance of a privileged vacuolar niche for replication. In particular, intracellular pathogens are especially adept at regulating host cell death and cytokine/chemokine production to ensure a growth compartment that is protected from the host innate immune response (5, 12, 42). Elucidation of these host-pathogen interactions has provided insight into novel mechanisms of signaling pathway regulation and enhanced our understanding of the complex interplay between intracellular pathogens and their host (4).

Coxiella burnetii is an obligate intracellular bacterium and the etiological agent of human Q fever. The pathogen is transmitted by aerosols and initially infects alveolar mononuclear phagocytes in vivo. Q fever normally manifests as an acute, debilitating, influenza-like illness, but chronic infection can result in severe endocarditis (27). Environmental stability (15), aerosol transmission (27), and a low infectious dose that approaches one organism (32) have resulted in classification of *C. burnetii* as a Centers for Disease Control and Prevention category B select agent with potential for illegitimate use.

In the host cell, *C. burnetii* directs maturation of a parasitophorous vacuole (PV) that displays lysosomal characteristics, including moderately acidic pH (pH ~ 5), active acid hydrolases, and lysosome-associated membrane proteins (2, 16, 45). The PV membrane is also cholesterol rich and decorates with lipid raft proteins, which are predicted to influence the stability and fusogenicity of the vacuole (17). In the PV, *C. burnetii* converts from an environmentally stable, nonreplicative small-cell variant (SCV) morphological form to a replicatively proficient large-cell variant (LCV) form (7, 15). LCVs replicate to high numbers with a doubling time of 12 h during an exponential growth phase lasting ~4 days (7). At ~1 week postinfection, LCVs begin to transition back to SCVs, presumably as a mechanism to ensure a stable population of infectious organisms following demise of the host cell.

During *C. burnetii*'s lengthy infectious cycle, the organism actively regulates host cell processes to ensure development of a stable niche for growth. *C. burnetii* protein synthesis is needed for early interactions between the PV and autophagosomes, a process that potentially delivers nutrients that may influence transition of dormant SCVs to replicative LCVs (37). *C. burnetii* also directs PV fusion with lysosomes, which provides the moderately acidic conditions required for pathogen metabolism (14). Finally, *C. burnetii* proteins inhibit apoptotic death of epithelial cells and macrophages (24, 46). In macrophages, we recently demonstrated that *C. burnetii* antagonizes cell death triggered by both staurosporine and tumor necrosis factor α, inducers of intrinsic (mitochondrial mediated) and extrinsic (death receptor mediated) apoptosis, respectively (46). *C. burnetii* antiapoptotic activity results in dramatically reduced processing of caspase-3, caspase-9, and poly-ADP(ribose) polymerase (PARP), hallmarks of the apoptotic process (20). Along with reduced activation of the caspase cascade, *C. burnetii*-infected cells also display an antiapoptotic transcriptional program that results in increased production of A1/Bfl-1, an antiapoptotic mitochondrial protein, and c-IAP2, a

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caspase inhibitor. Intracellular pathogens that inhibit cell death typically block several components of the apoptotic pathway (1, 3, 23, 31); however, the critical early step(s) antagonized by *C. burnetii* is currently unknown.

Bacterial pathogens commonly manipulate host prosurvival phosphorylation cascades that inhibit caspase-mediated apoptosis (4). To better understand the regulation of host signaling by *C. burnetii*, we examined the phosphorylation state of 15 mammalian signaling proteins during infection. The prosurvival proteins Akt and Erk1/2 (extracellular signal-regulated kinases 1 and 2) showed sustained activation while c-Jun, Hsp27, Jun N-terminal protein kinase (JNK), and p38 were transiently phosphorylated early during infection. Inhibition of Akt or Erk1/2 antagonized *C. burnetii* antiapoptotic activity. Thus, *C. burnetii* modulates multiple host cell signaling pathways to inhibit apoptosis and establish a protected replication niche.

TABLE 1. Host signaling proteins in the Bio-Plex assay

Protein	Phosphorylated residue(s)
Akt	S473
c-Abl	Y245
c-Jun	S63
Erk1/2	T202/Y204, T185/Y187
Hsp27	S78
JNK	T183/Y185
MEK1	S217/S221
p38	T180/Y182
p53	S15
p70 S6 kinase	T421/S424
$p90^{RSK}$	T359/S363
PDGF receptor β[a]	Y751
Src	Y416
Stat3	S727
Tyk2	Y1054/Y1055

[a] PDGF, platelet-derived growth factor.

MATERIALS AND METHODS

Mammalian cell culture and *C. burnetii*. Virulent *C. burnetii* Nine Mile phase I (RSA493) and avirulent phase II (clone 4; RSA439) isolates were propagated in African green monkey kidney (Vero) cells (CCL-81; ATCC, Manassas, VA) and purified as previously described (6, 39). Human monocyte-like (THP-1) cells (TIB-202; ATCC) were maintained in RPMI 1640 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (Gibco) at 37°C in 5% CO_2. All cells were used between passages 10 and 20. Prior to infection, THP-1 cells were incubated in the presence of 200 nM phorbol 12-myristate 13-acetate (PMA; EMD Biosciences, San Diego, CA) for 24 h to induce differentiation into an adherent, macrophage-like cell. Before *C. burnetii* infection, PMA-containing medium was removed from cell cultures, and cultures were replenished with fresh medium lacking PMA. Alveolar macrophages from cynomolgus macaques (*Macaca fascicularis*) were isolated by bronchial lavage and cultured in Dulbecco's minimal essential medium as previously described (46). In all experiments, cells were infected with *C. burnetii* phase I or phase II isolates at a multiplicity of infection (MOI) of 250 or 25, respectively, by addition of organisms to cell culture medium. (This time point was considered 0 h postinfection [hpi].) These MOIs result in a high percentage of infected THP-1 cells (9, 10, 46), with the lower MOI of phase II organisms employed because they are roughly 10-fold more infectious for cultured cells than phase I *C. burnetii* cells (45). When needed, rifampin (final concentration, 10 μg/ml) was added to cell cultures along with the *C. burnetii* inoculum.

Bio-Plex assay. *C. burnetii*-infected or uninfected THP-1 cells were lysed by a freeze-thaw cycle in Bio-Plex lysis buffer (Bio-Rad, Hercules, CA). Lysates were incubated overnight with Bio-Plex beads coupled to phospho-specific antibodies. Beads were washed, incubated with biotin-labeled secondary antibodies for 30 min, and then incubated with a streptavidin reporter for 10 min. All steps were performed at room temperature. Internal bead fluorescence, indicative of each distinct signaling protein, and fluorescence intensity were determined using a Bio-Plex Array Reader (Bio-Rad).

Immunoblot analysis. *C. burnetii*-infected cells cultured in six-well plates were directly lysed in buffer containing 50 mM Tris, 5 mM EDTA, 1% sodium dodecyl sulfate, and a phosphatase inhibitor cocktail (Sigma, St. Louis, MO) followed by 10 passages through a 26-gauge syringe needle. The protein concentration of each sample was determined using a detergent-compatible protein assay (Bio-Rad). Total protein (10 μg/lane) was separated by 10% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). After membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS; 150 mM NaCl, 100 mM Tris-HCl, pH 7.6) containing 0.1% Tween 20 and 5% nonfat milk, they were incubated overnight at 4°C in TBS-Tween 20 containing primary antibodies directed against the phosphorylated and nonphosphorylated forms of Akt, Erk1/2, c-Jun, Hsp27, JNK, p38, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Danvers, MA). Membranes were then washed and incubated for 1 h at room temperature in TBS-Tween 20 containing anti-rabbit or anti-mouse immunoglobulin G secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology). Reacting proteins were detected via enhanced chemiluminescence using ECL Pico reagent (Pierce, Rockford, IL).

Signaling pathway inhibition and apoptosis induction. Akt was inhibited by incubating infected cells with the class I phosphatidylinositol-3-kinase (PI3K) antagonists LY294002 (10 μM) or wortmannin (150 nM). Erk1/2 was inhibited by treatment of infected cells with the mitogen-activated protein kinase kinase 1 and 2 (MEK1/2) antagonist U0126 (20 μM). Inhibitors were added along with the *C. burnetii* inoculum and were present throughout the time course of infection. Intrinsic apoptosis was induced by addition of staurosporine (500 nM) to cell cultures at 48 hpi (48). All inhibitors and staurosporine were obtained from EMD Biosciences.

Cell death assay. Cell viability was assessed using a colorimetric Cell Counting Kit-8 (Dojindo Laboratories, Gaithersburg, MD), as previously described (46). Briefly, infected or uninfected THP-1 cells in 96-well plates with or without LY294002 or U0126 treatments were incubated with staurosporine for 4 or 8 h. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] reagent was then added to wells, and incubation continued for an additional 4 h at 37°C. Following measurement of the A_{450} of cell cultures, cell viability was calculated using the following formula: $[(A_{test} - A_{background})/(A_{control} - A_{background})] \times 100$, where A is the absorbance at 450 nm, the background value represents medium alone, and the control value represents untreated cells.

Fluorescence microscopy. THP-1 cells on 12-mm glass coverslips were infected with *C. burnetii* in the presence or absence of LY294002 or U0126 as described above. Following a 2-h treatment with staurosporine, cells were fixed and permeabilized with 100% ice-cold methanol for 3 min and then blocked for 1 h in phosphate-buffered saline (PBS; 1 mM KH_2PO_4, 155 mM NaCl, 3 mM Na_2HPO_4, pH 7.4) containing 5% bovine serum albumin. Cells were then incubated with rabbit anti-cleaved PARP (Cell Signaling Technology) and guinea pig anti-*C. burnetii* antibodies. Following a 1-h incubation, cells were washed three times in PBS and then incubated in PBS containing AlexaFluor-488 anti-rabbit and AlexaFluor-594 anti-guinea pig immunoglobulin G antibodies (Molecular Probes, Carlsbad, CA) for 1 h. Host and bacterial DNA were stained with DRAQ5 (Alexa Corporation, Lausen, Switzerland). Confocal fluorescence microscopy was conducted with a modified Perkin-Elmer UltraView spinning disk confocal system connected to a Nikon Eclipse TE2000-S microscope. Images were acquired with a 60× (1.4 numerical aperture) oil immersion objective (Nikon, Melville, NY) and a Photometrics Cascade II:512 digital camera (Princeton Instruments, Trenton, NJ) using Metamorph software (Molecular Devices, Downingtown, PA). A total of 100 cells were examined to determine the percentage of cleaved PARP-positive (apoptotic) cells in each cell culture.

RESULTS

***C. burnetii* infection causes transient phosphorylation of c-Jun, Hsp27, JNK, and p38 and sustained activation of Akt and Erk1/2.** To analyze the effect of *C. burnetii* infection on host cell signaling, a Bio-Plex assay was performed. This multiplex system can determine the phosphorylation state of multiple proteins in a single well of a 96-well plate. The 15 proteins analyzed in this study and their respective phosphorylated

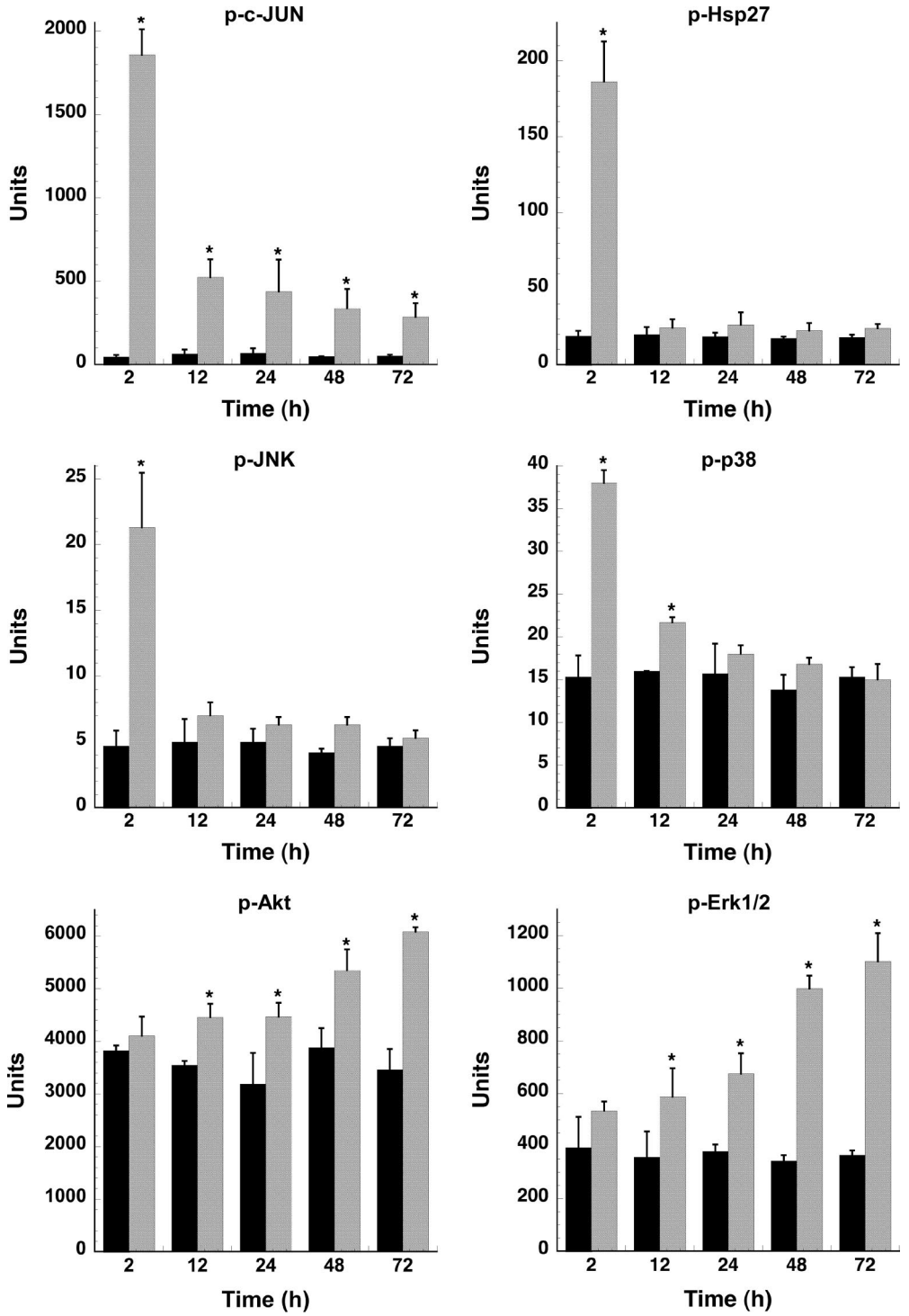

FIG. 1. *C. burnetii* infection induces phosphorylation of host proteins involved in cell survival. Lysates of mock- or *C. burnetii* phase II-infected THP-1 cells were harvested at the indicated times and processed for the Bio-Plex assay as described in Materials and Methods. Protein phosphorylation levels of infected cell lysates (gray bars) were compared to those of uninfected cell lysates (black bars). Phosphorylation of c-Jun, Hsp27, JNK, and p38 was transient, while phosphorylation of Akt and Erk1/2 increased through 72 hpi. Samples were analyzed in triplicate, and results are representative of those found in two independent experiments. Error bars represent the standard deviation from the mean. An asterisk indicates a P value of <0.05 in comparison to uninfected control cells as determined by a Student's t test. p, phosphorylated.

amino acid residues are listed in Table 1. PMA-differentiated THP-1 cells, which biologically mimic monocyte-derived macrophages (22), were used in these experiments. This cell line has been used to model the interaction between macrophages and intracellular pathogens, including *Mycobacterium tuberculosis* and *C. burnetii* (22, 46). As shown in Fig. 1, lysates of THP-1 cells infected with *C. burnetii* for 2 h contained phosphorylated c-Jun, Hsp27, JNK, and p38. This activation was

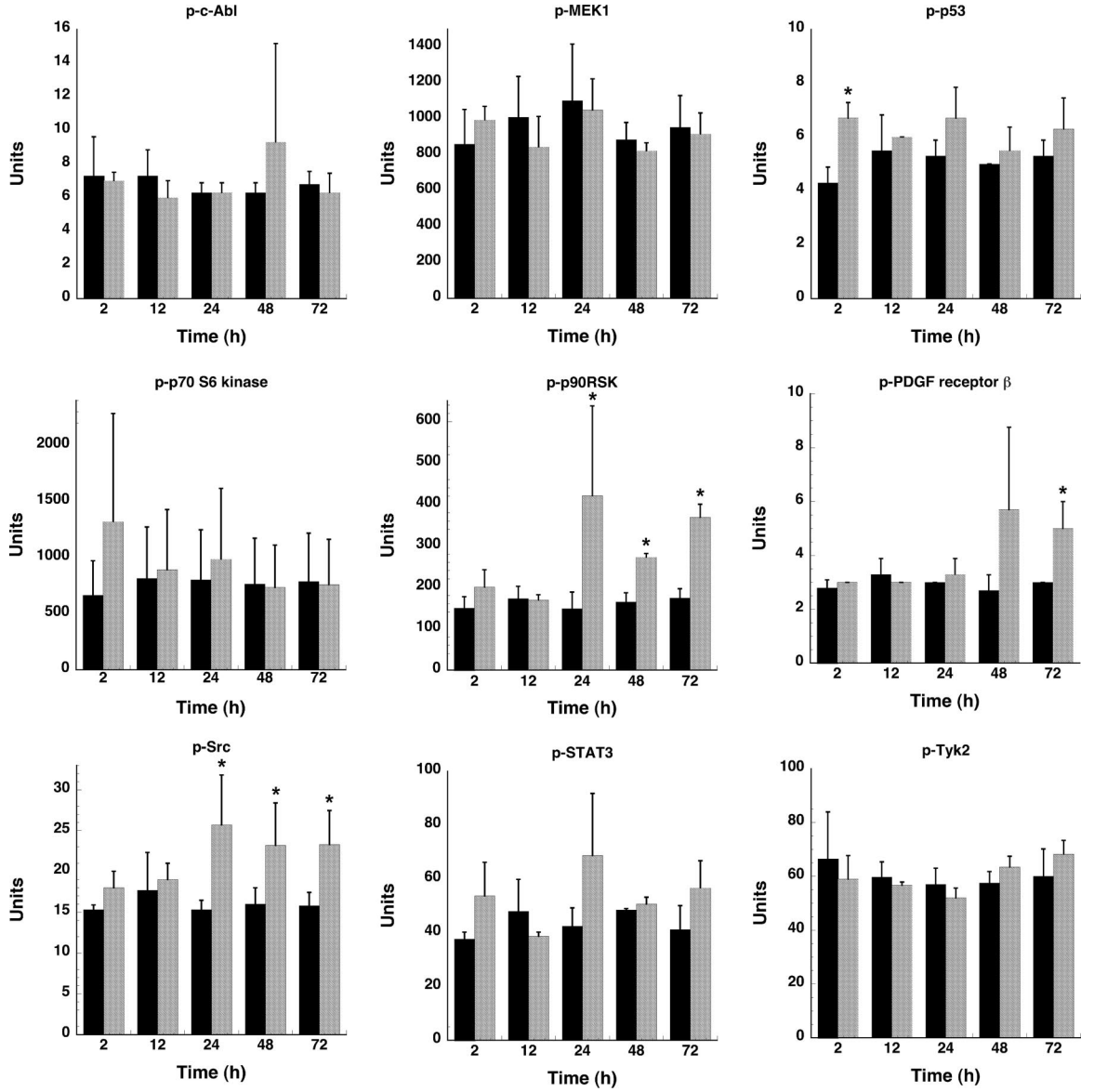

FIG. 2. Phosphorylation levels of seven proteins involved in cell survival responses are relatively unchanged during *C. burnetii* infection. Lysates of mock- and *C. burnetii* phase II-infected THP-1 cells were harvested at the indicated times postinfection and processed for the Bio-Plex assay as described in Materials and Methods. Protein phosphorylation levels of infected cell lysates (gray bars) were compared to those of uninfected cell lysates (black bars). Only $p90^{RSK}$ and Src showed slightly elevated phosphorylation at 24 to 72 hpi. p, phosphorylated. Samples were analyzed in triplicate, and results are representative of those found in two independent experiments. Error bars represent the standard deviation from the mean. An asterisk indicates a P value of <0.05 in comparison to uninfected control cells as determined by a Student's t test.

transient as phosphorylation in most cases returned to basal levels by 24 hpi. c-Jun was an exception: although levels of phosphorylated protein decreased dramatically by 12 hpi, they remained higher than levels in uninfected cells through 72 hpi. These short-lived responses are likely associated with the initial phagocytic uptake of *C. burnetii*. *C. burnetii* infection also induced Akt and Erk1/2 activation at 12 hpi (Fig. 1); however, these proteins remained activated through 72 hpi, with phosphorylation levels increasing coincident with *C. burnetii* replication. Sustained activation suggests that downstream signaling by these proteins occurs throughout the *C. burnetii* infectious process. The phosphorylation status of c-Abl, MEK1, p53, p70 S6 kinase, platelet-derived growth factor receptor β, STAT3, and Tyk2 remained unchanged throughout the 72-h course of infection, while $p90^{RSK}$ and Src showed slightly elevated phosphorylation levels at 24 to 72 hpi (Fig. 2).

Consistent with the Bio-Plex results, immunoblotting demonstrated that c-Jun, Hsp27, JNK, and p38 phosphorylation levels were dramatically increased at 2 hpi, followed by a decline to near basal levels by 24 hpi (Fig. 3). Total levels of c-Jun, Hsp27, JNK, and p38 remained constant during the same time course. In contrast to this transient activation, Akt was phosphorylated at both S473 and T308 at 6 to 12 hpi, and phosphorylation was sustained through 48 hpi (Fig. 4A). Phos-

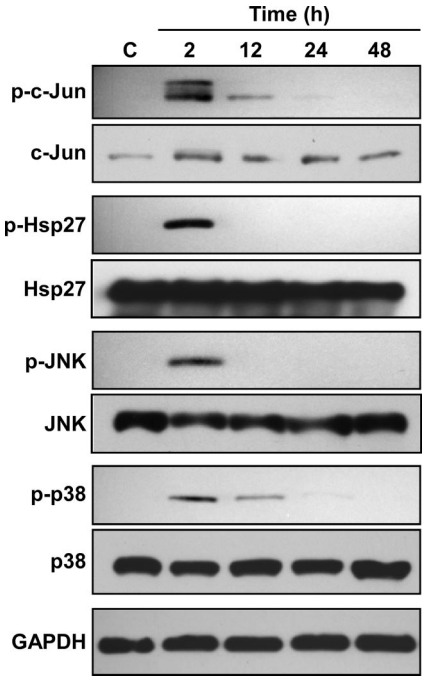

FIG. 3. Immunoblotting confirms transient phosphorylation of c-Jun, Hsp27, JNK, and p38 during *C. burnetii* infection. Lysates were harvested from mock- and *C. burnetii* phase II-infected THP-1 cells at the indicated times postinfection and subjected to immunoblot analysis. A representative immunoblot is shown for each protein. Mock-infected THP-1 cell lysates were used as a negative control (lane C). Phosphorylation levels of c-Jun, Hsp27, JNK, and p38 increased at 2 hpi, followed by a decrease to levels near those of uninfected cells by 12 to 24 hpi. In contrast, total levels of c-Jun, Hsp27, JNK, and p38 remained constant across the same time course. GAPDH levels were probed as a loading control. p, phosphorylated.

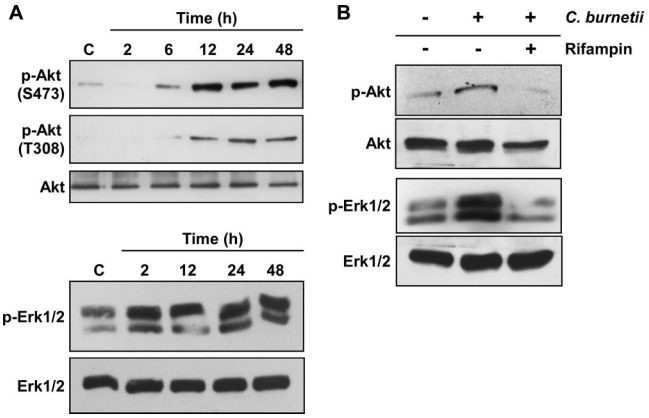

FIG. 4. Sustained *C. burnetii* activation of Akt and Erk1/2 is dependent on bacterial protein synthesis. Lysates were harvested from mock- and *C. burnetii* phase II-infected THP-1 cells at the indicated times postinfection and subjected to immunoblot analysis. In each panel, a representative immunoblot is shown for each protein. Mock-infected THP-1 cell lysates were used as a negative control (lanes C). (A) Akt and Erk1/2 were phosphorylated early after infection and remained activated through 48 hpi. (B) THP-1 cells were infected for 48 h in the presence or absence of rifampin to inhibit *C. burnetii* RNA synthesis. Cell lysates from mock- and *C. burnetii*-infected THP-1 cells were subsequently analyzed for phosphorylated Akt and Erk1/2 by immunoblotting. Phosphorylation of each protein was substantially reduced in rifampin-treated, infected cells. p, phosphorylated.

phorylation of both S473 and T308 is required for full Akt activity (26), indicating complete activation of the kinase. Erk1/2 was also activated early after *C. burnetii* infection (2 hpi), with phosphorylation levels sustained through 48 hpi (Fig. 4A). The total levels of both Akt and Erk1/2 remained constant throughout the 48-h infection. These results are consistent with the Bio-Plex data indicating that multiple host cell signaling pathways are differentially activated during *C. burnetii* infection. To determine whether de novo *C. burnetii* protein synthesis is required for Akt and Erk1/2 activation, infected THP-1 cells were treated with rifampin to inhibit bacterial RNA synthesis. As shown in Fig. 4B, increased levels of phosphorylated Akt and Erk1/2 were not observed at 48 hpi in infected THP-1 cells treated with rifampin. These results indicate that Akt and Erk1/2 activation are *C. burnetii*-driven processes. Akt and Erk1/2 phosphorylation levels were also elevated at 48 hpi in THP-1 cells infected with virulent *C. burnetii* Nine Mile phase I, indicating that activation is not strictly associated with avirulent organisms (Fig. 5A), and during *C. burnetii* Nine Mile phase II infection of monkey primary alveolar macrophages, indicating activation also occurs in primary cells (Fig. 5B).

Akt and Erk1/2 activation are required for *C. burnetii* inhibition of cell death. Akt directs eukaryotic cell survival responses via kinase activity that regulates at least seven different apoptosis-related components (28). Erk1/2 is commonly involved in antiapoptotic events, including phosphorylation and inactivation of proapoptotic Bad (29). To test whether either pathway is involved in *C. burnetii*'s ability to inhibit cell death, THP-1 cells were infected for 48 h in the presence of LY294002 or U0126 and then treated with staurosporine to induce intrinsic apoptosis. Uninfected cells treated with staurosporine for 4 and 8 h displayed 40% and 74% death, respectively. In contrast, infected cells treated with staurosporine for

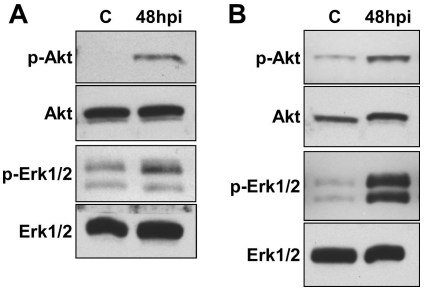

FIG. 5. Akt and Erk1/2 activation occurs irrespective of *C. burnetii* isolate virulence and is observed during infection of monkey primary alveolar macrophages. (A) THP-1 cells were infected with virulent phase I *C. burnetii* for 48 h, and then lysates were probed for phosphorylated Akt and Erk1/2 by immunoblotting. Similar to cells infected with avirulent phase II *C. burnetii*, cells infected with virulent phase I organisms also showed increased phosphorylation of both kinases at 48 hpi compared to uninfected cells. (B) Monkey primary alveolar macrophages isolated by bronchial lavage were infected for 48 h with phase II *C. burnetii*. Lysates were collected and probed by immunoblotting for the phosphorylated forms of Akt and Erk1/2. Similar to experiments using THP-1 cells, primary alveolar macrophages showed increased phosphorylation of Akt and Erk1/2 at 48 hpi. Lanes C, mock-infected control cells; p, phosphorylated.

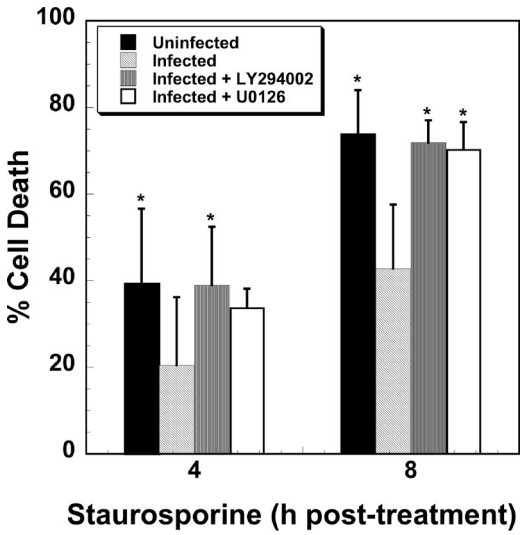

FIG. 6. *C. burnetii* requires Akt and Erk1/2 activation to protect mammalian cells from staurosporine-induced death. THP-1 cells were infected with phase II *C. burnetii* for 48 h in the presence or absence of LY294002 (class I PI3K inhibitor) or U0126 (MEK1/2 inhibitor) and then treated with staurosporine for 4 or 8 h. Cell death was assessed by WST-8 staining. In the presence of either inhibitor, *C. burnetii* was unable to prevent cell death induced by staurosporine. Experiments were performed in triplicate, and error bars represent the standard deviation from the mean. An asterisk indicates a P value of <0.05 in comparison to infected, staurosporine-treated cells as determined by a Student's t test.

4 and 8 h showed 20% and 42% death, respectively. Infected cells incubated in the presence of LY294002, which inhibits PI3K and consequently Akt (43), displayed 39% and 72% death when treated with staurosporine for 4 and 8 h, respectively (Fig. 6). A similar effect was observed when infected cells were incubated with the PI3K antagonist wortmannin (data not shown). Infected cells incubated in the presence of U0126, which inhibits MEK1/2 and consequently Erk1/2 (43), showed 34% and 70% death when treated with staurosporine for 4 and 8 h, respectively. Thus, all inhibitor treatments resulted in levels of death that were similar to those of uninfected cells treated with staurosporine (Fig. 6). Akt and Erk1/2 inhibitor treatments did not demonstrably affect *C. burnetii* entry or growth as PVs containing replicating organisms were indistinguishable between treated and untreated THP-1 cells (Fig. 7).

To confirm that Akt and Erk1/2 activation by *C. burnetii* protects host cells from apoptotic death, we assessed levels of cleaved PARP, an indicator of apoptosis, in THP-1 cells infected for 48 h in the presence of LY294002 or U0126 and then treated with staurosporine for 2 h. Approximately 4% of infected cells were positive for cleaved PARP in the absence of inhibitors. Conversely, ~10% of cells treated with LY294002 or U0126 were positive for cleaved PARP, roughly half the percentage (~20%) observed with uninfected cells (Fig. 7 and data not shown). Collectively, these results indicate that both Akt and Erk1/2 contribute to *C. burnetii*'s antiapoptotic activity.

DISCUSSION

C. burnetii actively modulates Akt and Erk1/2 prosurvival host signaling pathways during intracellular growth in THP-1 cells and primary alveolar macrophages. Sustained activation of each kinase is observed for at least 3 days following infection, with levels of phosphorylated protein increasing concomitantly with *C. burnetii* replication. Each of these signaling proteins is a well-established regulator of mammalian cell survival responses (28, 29). We previously showed that *C. burnetii* infection potently inhibits apoptosis associated with caspase inhibition and a prosurvival transcriptional program (46). Here, we demonstrate that Akt and Erk1/2 cascades are integral components of *C. burnetii*-directed antiapoptotic signaling.

Sustained prosurvival signaling by Akt and Erk1/2 contributes to decreased host cell apoptosis during *C. burnetii* infection as specific inhibition of either of these kinases antagonizes the ability of *C. burnetii* to protect macrophages from staurosporine-induced apoptotic cell death. Akt is a prosurvival kinase that directly or indirectly inhibits activation of proapoptotic mitochondrial proteins (e.g., Bad) and transcription factors (e.g., p53). Additionally, Akt regulates activation of antiapoptotic transcription factors (e.g., FOXO) and caspase inhibitors (e.g., X-linked inhibitor of apoptosis protein), leading to a prosurvival cellular response (28). Erk1/2 regulates numerous transcription factors, such as Elk-1 and CREB, that enhance expression of antiapoptotic components (29). In addition to *C. burnetii*, Akt- and Erk1/2-mediated cell survival is observed during infection by other intracellular pathogenic bacteria. Activation of Akt by the *Salmonella enterica* type III effector SopB leads to host cell protection from caspase-mediated apoptosis during early stages of intracellular growth in epithelial cells (21). *Chlamydia trachomatis*-directed Akt phosphorylation leads to Bad inactivation and subsequent inhibition of mitochondrial-mediated apoptosis (44). Activation of Erk1/2 by *Helicobacter pylori* and *Neisseria gonorrhoea* results in increased production of antiapoptotic Mcl-1 (30) and degradation of the proapoptotic mitochondrial proteins Bad and Bim (18), respectively. The downstream effects of Akt and Erk1/2 activation in *C. burnetii*-infected cells are unknown. However, slightly elevated levels of $p90^{RSK}$, an Erk1/2 effector that mediates numerous transcriptional events including NF-κB activation (13), are observed at 24 to 72 hpi.

The sustained activation of Akt and Erk1/2 during *C. burnetii* infection is unusual as the pathways regulating these proteins are tightly controlled by the host. Indeed, the kinases are generally rapidly dephosphorylated within minutes of performing their inherent functions (28). For example, *S. enterica* activates Akt only upon entry into nonphagocytic cells (21). Similarly, activation of Akt by *M. tuberculosis* surface-associated lipoarabinomannan is strictly associated with uptake of the pathogen by macrophages (25). Only *C. trachomatis* is reported to sustain phosphorylation of Akt during infection, with an elevated level at 24 hpi that decreases to basal level by 36 hpi (44). With respect to Erk1/2, the kinase is activated by *Legionella pneumophila*, *Mycobacterium avium*, and *S. enterica* at 0.5 to 1 hpi; however, phosphorylation returns to basal levels within 5 hpi, suggesting that Erk1/2 activity is only critical for early infection events (33, 36, 47). In contrast, *Mycobacterium leprae* and *C. trachomatis* induce prolonged Erk1/2 phosphorylation for 30 and 1 day(s) postinfection (dpi), respectively (5, 41), indicating that this pathway is utilized by a subset of intracellular pathogens for postentry infection processes.

While differential host cell interactions have been proposed

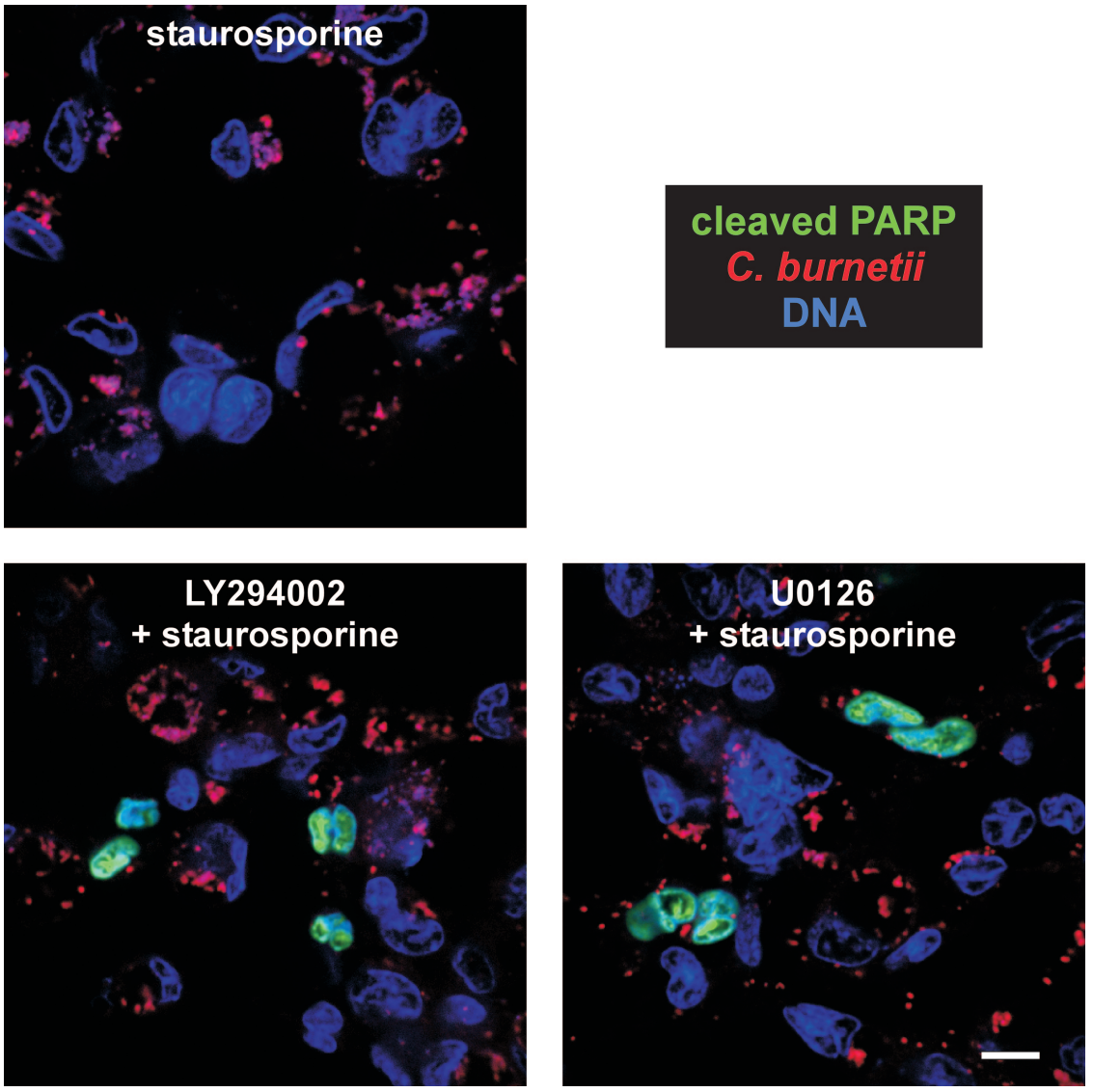

FIG. 7. *C. burnetii* requires Akt and Erk1/2 activation to protect mammalian cells from apoptosis. THP-1 cells were infected with phase II *C. burnetii* in the presence or absence of LY294002 or U0126 for 48 h and then treated with staurosporine for 2 h to induce apoptosis. Cells were fixed and immunostained for confocal fluorescence microscopy using antibodies directed against *C. burnetii* (red) and cleaved PARP (green). THP-1 and bacterial DNA (blue) were stained with DRAQ5. LY294002- or U0126-treated cells showed substantially increased levels of cleaved PARP compared to untreated cells. Bar, 10 μM.

for virulent phase I and avirulent phase II *C. burnetii* organisms (45), we demonstrate that Akt and Erk1/2 activation occurs irrespective of the virulence of *C. burnetii* isolates. This result is consistent with the similar antiapoptotic effects induced by phase variants during infection of THP-1 cells (46). In contrast, virulent and avirulent isolates of some other pathogens, such as *M. tuberculosis*, have been shown to differentially activate Erk1/2 (36).

Shortly following infection (2 hpi), c-Jun, JNK, Hsp27, and p38 are activated in response to *C. burnetii*. However, phosphorylation of these proteins rapidly decreases to nearly basal levels by 24 hpi, suggesting that *C. burnetii* quickly escapes a host response that could potentially alert the immune system to the presence of the pathogen (8, 34, 38, 40). For example, p38 contributes to the normal inflammatory response by regulating interleukin-1 and tumor necrosis factor α production by cells stimulated with endotoxin (8). Transient activation of these proteins is presumably a host cell response associated with phagocytosis of a foreign particle and not specifically directed by *C. burnetii* as significant pathogen metabolism at this time postinfection would not be expected (14). Moreover, similar events are observed early after infection of monocyte-derived macrophages by *L. pneumophila*, indicating that a common set of proteins is activated during initial host cell interactions with intracellular pathogens (47).

Multiple pathways are commonly manipulated by intracellular pathogens to inhibit apoptosis. For example, *L. pneumophila* activates an NF-κB-dependent transcriptional program early during infection (1, 23) and also secretes Dot/Icm type IV secretion system effectors that directly antagonize proapop-

totic BNIP3 and Bcl-rambo (3). *M. tuberculosis* activates Akt, which leads to inactivation of proapoptotic Bad and induction of antiapoptotic NF-κB (11, 25). *Chlamydia* spp. are perhaps the most versatile apoptosis-antagonizing pathogens with NF-κB activation, Akt phosphorylation, and degradation of multiple proapoptotic mitochondrial proteins all occurring during infection of various cell types (31, 44). In addition to sustained phosphorylation of Akt and Erk1/2, *C. burnetii* also activates NF-κB during infection (2 to 24 hpi) (D. E. Voth and R. A. Heinzen, unpublished results). Indeed, NF-κB-dependent transcriptional responses, such as upregulation of the A1/Bfl-1 and c-IAP2 genes, are observed in *C. burnetii*-infected cells that likely potentiate Akt and Erk1/2 control of apoptosis-related genes (46).

Most intracellular pathogens promote a lytic release from host cells within 1 to 3 dpi (19). However, *C. burnetii* has a lengthy infectious cycle, with stationary phase occurring ~6 dpi, after which the pathogen is presumably released to the extracellular milieu following demise of the host cell. Thus, *C. burnetii* has necessarily evolved efficient methods of regulating host survival, including the sustained activation of the Akt and Erk1/2 pathways described here. *C. burnetii* protein synthesis is required for sustained phosphorylation of these proteins; thus, pathogen effectors of activation are likely constitutively produced during infection. Although the *C. burnetii* proteins directing these responses are unknown, they are presumably delivered to the host cytosol via the organism's Dot/Icm type IV secretion system. Indeed, our laboratory along with other investigators has recently identified multiple *C. burnetii* proteins that are translocated into the host cytosol in a Dot/Icm-dependent manner (35; also D. E. Voth et al., unpublished data). Further characterization of *C. burnetii*'s manipulation of host signaling pathways and of the bacterial proteins responsible will provide insight into virulence mechanisms exploited by this obligate intracellular bacterial pathogen.

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