

Janus Kinase 3 Activity Is Necessary for Phosphorylation of Cytosolic Phospholipase A2 and Prostaglandin E2 Synthesis by Macrophages Infected with *Francisella tularensis* Live Vaccine Strain

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Francisella tularensis, the causative agent of tularemia, modulates the host immune response to gain a survival advantage within the host. One mechanism of immune evasion is the ability of *F. tularensis* to induce the synthesis of the small lipid mediator prostaglandin E2 (PGE₂), which alters the host T cell response making the host more susceptible to *Francisella* growth. PGE₂ is synthesized by a tightly regulated biosynthetic pathway following stimulation. The synthesis of PGE₂ begins with the liberation of arachidonic acid (AA) from membrane phospholipids by cytosolic phospholipase A2 (cPLA₂). AA is subsequently converted to the unstable intermediate PGH₂ by cyclooxygenase-2 (COX-2), and PGH₂ undergoes an isomerization reaction to generate PGE₂. Our objective was to identify *F. tularensis*-activated host signaling pathways that regulate the activity of the enzymes in the PGE₂-biosynthetic pathway. In this study, we show that cPLA₂, p38 mitogen-activated protein kinase (MAPK), and Janus kinase 3 (JAK3) signaling are necessary for *F. tularensis*-induced PGE₂ production. Inhibition of JAK3 activity reduced the phosphorylation of cPLA₂ and COX-2 protein levels. In addition, JAK3 regulates cPLA₂ phosphorylation independent of transcription. Moreover, p38 MAPK activity is required for *F. tularensis*-induced COX-2 protein synthesis, but not for the phosphorylation of cPLA₂. This research highlights a unique signaling axis in which JAK3 and p38 MAPK regulate the activity of multiple enzymes of the PGE₂-biosynthetic pathway in macrophages infected with *F. tularensis*.

Francisella tularensis is a highly virulent Gram-negative facultative intracellular bacterium and the causative agent of tularemia. The inhalation of as few as 10 *F. tularensis* organisms is capable of causing pneumonic tularemia (1). Pneumonic tularemia is the most severe form of tularemia, with a case fatality rate of 30% if untreated (1, 2). *F. tularensis* is classified by the Centers for Disease Control and Prevention as a category A select agent due to its low infectious dose, its ease of dissemination, and the high morbidity and mortality rate associated with the disease. There is currently no FDA-approved vaccine.

F. tularensis invades and replicates within numerous host cell types. The ability of *F. tularensis* to invade and replicate within macrophages is paramount to its survival within the host, as *F. tularensis* mutants that fail to replicate in macrophages are avirulent (3). However, modulation of the host immune response by *F. tularensis* is also important to bacterial survival within the host (4). Numerous mechanisms of *F. tularensis*-mediated immune evasion have been identified (reviewed in reference 5). *F. tularensis* can inactivate complement, modify its lipopolysaccharide (LPS) structure to minimize recognition by TLR4, disrupt NADPH assembly to inhibit a respiratory burst, induce the synthesis of anti-inflammatory mediators by the host, and alter macrophage differentiation. One immune evasion mechanism we identified is the ability of both *F. tularensis* subsp. *tularensis* Schu S4 and *F. tularensis* subspecies *holarctica* LVS (live vaccine strain) to induce the synthesis of prostaglandin E2 (PGE₂) from infected macrophages (6, 7). Prostaglandins are a family of small lipid mediators that have both anti- and proinflammatory functions depending on the cellular context (8, 9). *F. tularensis* LVS-induced PGE₂ downregulates major histocompatibility complex (MHC) class II expression on infected macrophages via a ubiquitin-dependent mechanism (10). *F. tularensis* LVS-induced PGE₂ also blocks T cell proliferation and skews the T cell response away from a Th1- to a

Th2-like response *in vitro* (7). Proinflammatory Th1 cells that secrete gamma interferon (IFN- γ) and/or tumor necrosis factor alpha (TNF- α) are critical for both clearance of *F. tularensis* from the host and the generation of long-term immunity (11). Blocking the synthesis of PGE₂ by administering the cyclooxygenase inhibitor indomethacin increases the number of *F. tularensis*-specific IFN- γ ⁺ T cells and decreases bacterial burden in the lungs of mice infected with *F. tularensis* LVS (12). These results suggest that the ability of *F. tularensis* to stimulate the synthesis of PGE₂ provides the organism with a growth advantage within the host.

PGE₂ is not stored within host cells but rather is synthesized by a cell through a tightly regulated biosynthetic pathway. Induction of the canonical inducible PGE₂-biosynthetic pathway requires the liberation of arachidonic acid (AA) from cell membrane phospholipids by cytosolic phospholipase A2 (cPLA₂) (13, 14). Free AA is then oxidized to PGH₂ by cyclooxygenase-2 (COX-2). PGH₂ is isomerized to PGE₂ by a terminal prostaglandin E synthase. COX-2 is essential for *F. tularensis*-induced PGE₂ synthesis. However, the importance of cPLA₂ in the production of PGE₂ in macrophages infected with *F. tularensis* is unknown. In this study, we determined that cPLA₂ activity is critical for the synthesis of PGE₂ in macrophages infected with *F. tularensis*.

Different regulatory processes control each enzyme of the in-

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ducible PGE₂-biosynthetic pathway. Phosphorylation of cPLA₂ on Ser⁵⁰⁵ by mitogen-activated protein kinases (MAPKs) results in increased enzymatic activity of cPLA₂ (15). COX-2 is regulated at the transcriptional and posttranscriptional levels (16); thus, an increase in COX-2 protein levels typically correlates with an increase in activity. Though the PGE₂-biosynthetic pathway has been extensively studied, the eukaryotic signaling pathways that regulate the activation of cPLA₂ and COX-2 are poorly understood, particularly within the context of bacterial infection. The objective of this study was to identify macrophage signaling pathways that mediate the activity of the key enzymes in the PGE₂-biosynthetic pathway and ultimately the synthesis of PGE₂ in macrophages infected with *F. tularensis*.

In the present study, we demonstrate that increased COX-2 protein levels, cPLA₂ phosphorylation, and PGE₂ synthesis in *F. tularensis* LVS-infected murine macrophages are dependent on Janus kinase 3 (JAK3). Importantly, JAK3 regulates the phosphorylation of cPLA₂ through a transcription-independent mechanism. Additionally, we demonstrate that p38 MAPK activity is necessary for enhanced COX-2 protein levels but not for increased phosphorylation of cPLA₂ in macrophages infected with *F. tularensis*. To our knowledge, we provide the first evidence that JAK3 can positively regulate the PGE₂-biosynthetic pathway.

MATERIALS AND METHODS

Bacteria. *F. tularensis* LVS (ATCC 29684; American Type Culture Collection) was used in this study. Bacteria were grown on brain heart infusion (BHI) agar supplemented with 1% hemoglobin and 1% IsoVitaleX. To prepare bacterial inoculations, bacteria were removed from an overnight lawn grown on BHI agar and resuspended in sterile phosphate-buffered saline (PBS) at an optical density at 600 nm (OD₆₀₀) of 1 (equivalent to 5 × 10⁹ CFU/ml). Appropriate dilutions were made in sterile phosphate-buffered saline (PBS) to obtain the desired bacterial inoculum. To determine if the pharmacological inhibitors used in this study affected *F. tularensis* LVS growth in broth, we performed a growth assay in a 96-well plate in the absence or presence of 50 μM JANEX-1, 15 μM SB203580, or 5 μM pyrrophenone using a Spectramax 190 plate reader. OD₆₀₀ readings were taken every 5 min for 12 h.

Mice. Female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animals used in this study were maintained under specific-pathogen-free conditions in the American Association for Laboratory Animal Care (AALAC)-accredited Louisiana State University Health Sciences Center (LSUHSC) animal medicine facilities. All work was approved by LSUHSC Animal Care and Use Committee (ACUC).

Generation and culture of bone marrow-derived macrophages. Bone marrow cells from female C57BL/6J mouse femurs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 30% L-cell conditioned medium, 20% fetal bovine serum (Atlas Biologicals), 1% L-glutamine (HyClone), 1% penicillin-streptomycin (ATCC), 1% sodium pyruvate (HyClone), and 0.2% sodium bicarbonate for 6 days at 37°C and 5% CO₂. Sixteen hours prior to *F. tularensis* inoculation of bone marrow-derived macrophages (BMDMs), the medium was replaced with antibiotic-free RPMI 1640 medium (HyClone) supplemented with 1% L-glutamine (HyClone), 1% sodium pyruvate (HyClone), 0.1% beta-mercaptoethanol, and 10% fetal bovine serum (Atlas Biologicals). On the day of inoculation, nonadherent cells were removed by a PBS wash, and BMDMs were removed from petri dishes by treatment with 1 mM EDTA in PBS (pH 7.6).

Cell culture. The RAW264.7 murine macrophage-like cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 1% L-glutamine (HyClone), 1% penicillin-streptomycin (ATCC), 1% sodium pyruvate (HyClone), 0.1% beta-mercaptoethanol, and 10% fetal bovine serum (Atlas Biologi-

cal). Sixteen hours prior to *F. tularensis* inoculation, the medium was replaced with antibiotic-free RPMI (HyClone) supplemented with 1% L-glutamine (HyClone), 1% sodium pyruvate (HyClone), 0.1% beta-mercaptoethanol, and 10% fetal bovine serum (Atlas Biologicals).

Stimulation of macrophages with *F. tularensis* LVS, interleukin 4 (IL-4), or lipopolysaccharide (LPS). Stimulation was carried out in either a 96-well plate (2 × 10⁵ cells/well), a 24-well plate (1.5 × 10⁶ cells/well), or a 12-well plate (10⁶ cells/well). Macrophages were allowed to adhere for 2 h prior to their stimulation with 100 ng/ml LPS, 30 ng/ml IL-4 or *F. tularensis* LVS at a multiplicity of infection (MOI) of 200:1 (bacteria to macrophages). One hour prior to stimulation, macrophages were treated with specific inhibitor compounds. After treatment of macrophages with the particular stimuli, the plate was spun for 5 min at 300 × g to allow closer contact and more efficient infection by *F. tularensis* LVS. The plate was maintained in an incubator at 37°C and 5% CO₂ throughout the experiment. For experiments in which spent medium was collected at 24 h postinoculation to determine the concentration of PGE₂, *F. tularensis* LVS was cocultured with macrophages for 2 h and then macrophages were treated with 50 μg/ml gentamicin for 1 h to kill extracellular bacteria. The macrophages were then washed twice with fresh antibiotic-free RPMI 1640 medium. Antibiotic-free RPMI 1640 medium was added to the macrophages and collected at 24 h postinoculation. For experiments in which cellular lysates were collected beyond 2 h postinoculation, macrophages were treated with penicillin-streptomycin to kill intracellular *F. tularensis* at 2 h postinoculation.

Pharmacological inhibitors. The inhibitors and the final concentration of each inhibitor used in this study were 5 μM pyrrophenone (Cayman Chemical), 15 μM SB203580 (Calbiochem), 50 μM JANEX-1 (Cayman Chemical), 10 μM ruxolitinib (Cayman Chemical), 50 μM AG490 (Calbiochem), and 2 μg/ml actinomycin D (Sigma). All inhibitors were dissolved in DMSO. Working concentrations of 100× of all inhibitors were made in complete medium, and 1/100 of the 100× inhibitor solution was added to the appropriate wells to get the inhibitor to the final concentration used throughout this study. Inhibitors were added 1 h prior to inoculation and were maintained throughout the experiment.

Live/dead fixable dead-cell stain. A total of 10⁶ macrophages were put into a 15-ml conical tube. The cells were treated 1 h after being put into the conical tube with 5 μM pyrrophenone, 15 μM SB203580, or 50 μM JANEX-1. One hour after inhibitor treatment, the cells were either uninfected or inoculated with *F. tularensis* LVS at an MOI of 200:1. At 2 h postinoculation, cells were treated with 50 μg/ml gentamicin for 1 h and then washed once with fresh antibiotic-free RPMI 1640 medium. At 20 h postinoculation, the cells were washed once with PBS and resuspended in PBS, and 1 μl of live/dead yellow fluorescent reactive dye (Life Technologies) was added to each conical tube for 30 min at room temperature protected from light. Cells were washed twice with PBS and resuspended in 500 μl fluorescence-activated cell sorting (FACS) fixative. The percentage of live and dead cells was determined by flow cytometry.

Lentiviral delivery of short-hairpin RNA (shRNA). Short-hairpin RNAs (shRNAs) directed toward JAK3 were delivered into RAW264.7 macrophages using Mission lentiviral transduction particles (Sigma-Aldrich). Lentiviral particles were used to deliver JAK3 shRNA into RAW264.7 macrophages. Twenty-four hours after addition of the lentivirus, macrophages were put under puromycin selection (5 μg/ml) for 48 h to select for cells expressing the JAK3 shRNA. To confirm that JAK3 gene expression was reduced in these cells, we examined the expression levels of JAK3 mRNA by RT-PCR. Briefly, RNA was extracted from cells following the RNA STAT-60 protocol for RNA extraction. cDNA was synthesized from DNA-free RNA using a cDNA synthesis kit (Bio-Rad). Samples of cDNA were subjected to reverse transcription-PCR (RT-PCR) using specific primers for JAK3 with hypoxanthine phosphoribosyltransferase (HPRT) as an internal control. The primer sequences used for amplifying JAK3 were 5'-GGCGTGGCGGTTAGTAAAGAA-3' (forward) and 5'-CCCCCTATCTAGTCTCACCT-3' (reverse), and those for

HPRT were 5'-CGTCTTGCTCGAGATGTGATG-3' (forward) and 5'-TTTATAGCCCCCTTGAGCAC-3' (reverse).

Bacterial uptake and intracellular replication of *F. tularensis* LVS in macrophages. A total of 10^5 RAW264.7 macrophages were plated in each well of a 96-well plate. We followed the protocol described above for stimulation of macrophages with *F. tularensis* LVS in the presence of 15 μ M SB203580, 50 μ M JANEX-1, and 5 μ M pyrrophenone. To determine bacterial uptake and replication, infected macrophages were lysed at 6 h postinoculation (uptake) and 24 h postinoculation (replication) with PBS containing 0.5% SDS. Ten-fold serial dilutions were performed in PBS, and dilutions were plated on chocolate agar plates. The plates were incubated for 2 days at 37°C in 5% CO₂, and colonies were counted and CFU/ml were calculated.

Western blot analyses. At the appropriate time points, host cells were lysed based on the number of live cells. A ratio of 10 μ l of lysis buffer to 2×10^5 live host cells was used throughout this study. The denaturing lysis buffer used in this study was 1 \times NuPage LDS sample buffer containing 100 mM dithiothreitol (DTT) (Life Technologies), 1 \times protease inhibitor cocktail (Thermo Scientific), 1 \times phosphatase inhibitor cocktail 1 (Sigma-Aldrich), and 1 \times phosphatase inhibitor cocktail 3 (Sigma-Aldrich). To minimize the potential dilution effect bacterial protein could have on the measurement of host protein levels, we loaded equivalent numbers of lysed host cells per sample rather than equivalent amounts of protein. Samples were sonicated and boiled prior to gel loading. A total of 10 μ l of whole-cell lysate was loaded per well of the gel. Proteins in cell lysates were separated by 4 to 12% SDS-PAGE (Life Technologies) utilizing a Life Technologies XCell SureLock electrophoresis chamber system at constant amperage (50 mA). Proteins were transferred onto an Immobilon-FL transfer membrane (Millipore) using a semidry blotter run at a constant 20 V for 1 h at room temperature. Membranes were blocked in LiCor blocking buffer (LiCor Biosciences) and then incubated with primary antibodies against alpha/beta tubulin (1:2,000), p38 MAPK (1:1,000), phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) (1:1,000), phospho-STAT6 (Tyr⁶⁴¹) (1:1,000) (all from Cell Signaling Technology), COX-2 (1:500) (Cayman Chemical Company), cPLA₂ (1:1,000), or phospho-cPLA₂ (Ser⁵⁰⁵) (1:500) (both from Santa Cruz Biotechnology). Membranes were washed in PBS containing 0.1% Tween-20 and then incubated in anti-rabbit IgG (IRDye 680 LT) (1:20,000) secondary antibody (LiCor Biosciences). Bands were detected using the LiCor Odyssey infrared imaging system. Band densities were determined using the LiCor Odyssey infrared imaging system software. For each sample, we normalized the band intensity of the protein of interest for that sample to the tubulin loading control for that same sample by using the following equation: band intensity of protein of interest/band intensity of tubulin. Once we had normalized the band intensity for the protein of interest in each sample, we then used these normalized values to make comparisons between samples to determine changes in protein levels of our proteins of interest.

PGE₂ ELISA. PGE₂ concentration in supernatants was determined using a commercially available PGE₂ enzyme-linked immunosorbent assay (ELISA) kit (ENZO Life Sciences, Farmingdale, NY) according to the manufacturer's protocol.

Statistical analyses. Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test. Western blot protein intensities were first normalized to those of infected untreated samples and then analyzed to remove technical variability associated with Western blot analysis. The GraphPad Prism 5.0 software program was used for analysis. Statistical significance was determined by a *P* value of <0.05.

RESULTS

Cytosolic phospholipase A2 is required for *F. tularensis* LVS-induced PGE₂ synthesis by macrophages. We previously demonstrated that *F. tularensis* induces the synthesis of PGE₂ by infected macrophages (6, 7). It is unknown if cPLA₂ is required for the synthesis of PGE₂ by macrophages infected with *F. tularensis*. Therefore, we wanted to examine if cPLA₂ activity is necessary for

the production of PGE₂ by macrophages infected with *F. tularensis*. Bone marrow-derived macrophages (BMDMs) were treated with the well-characterized cPLA₂ inhibitor pyrrophenone 1 h prior to being inoculated with *F. tularensis* LVS at a multiplicity of infection (MOI) of 200:1 (bacteria to macrophages). At 24 h postinoculation, supernatants were collected, and the concentration of PGE₂ was determined by ELISA. Treatment with pyrrophenone significantly reduced *F. tularensis* LVS-induced PGE₂ synthesis (Fig. 1A), suggesting that cPLA₂ activity is required for increased PGE₂ synthesis by macrophages infected with *F. tularensis*. To ensure that the decrease in PGE₂ production observed with pyrrophenone treatment was not due to an adverse effect of the inhibitor on the bacteria or the macrophages, we examined *F. tularensis* LVS growth in broth, macrophage uptake of LVS, intramacrophage growth of LVS, and macrophage viability with pyrrophenone treatment. Treatment with pyrrophenone did not prevent LVS growth in broth culture (Fig. 1B), macrophage uptake of LVS, or intramacrophage growth of LVS in RAW264.7 macrophages (Fig. 1C). Lastly, we did not observe decreased cell viability in *F. tularensis*-infected RAW264.7 macrophages or BMDMs treated with pyrrophenone (Fig. 1D). These data strongly imply that cPLA₂ is necessary for the synthesis of PGE₂ by macrophages infected with *F. tularensis*.

***F. tularensis* infection of macrophages increases COX-2 protein levels, cPLA₂ phosphorylation, and PGE₂ synthesis.** The kinetics of PGE₂ synthesis following infection of macrophages with *F. tularensis* has not been examined. Determining the kinetics of PGE₂ synthesis will allow us to more easily investigate the eukaryotic signaling pathways responsible for regulating PGE₂ production in *F. tularensis*-infected macrophages. Therefore, we performed a time course experiment to determine when the concentration of PGE₂ begins to increase in *F. tularensis*-infected macrophages. RAW264.7 cells and BMDMs were inoculated with *F. tularensis* LVS at an MOI of 200:1. At 0, 1, 4, 6, 12, 18, and 24 h postinoculation, supernatants were collected, and the concentration of PGE₂ in each sample was determined by ELISA. In RAW264.7 macrophages, we observed a significant increase in PGE₂ synthesis by 6 h postinoculation (Fig. 2A). In contrast, infected BMDMs did not synthesize a significant amount of PGE₂ until 18 h postinoculation (Fig. 2B). One would hypothesize that an increase in the synthesis of PGE₂ would correlate with increased activation of the enzymes in the PGE₂-biosynthetic pathway. To test this hypothesis, we examined the kinetics of COX-2 protein synthesis and cPLA₂ phosphorylation.

COX-2 is tightly regulated at the transcriptional and posttranscriptional levels and there is no evidence of posttranslational modifications regulating COX-2 activity. Therefore, an increase in the level of COX-2 protein within a cell correlates with an increase in activity. To determine COX-2 protein levels in *F. tularensis*-infected macrophages, RAW264.7 macrophages were inoculated with LVS at an MOI of 200:1. The RAW264.7 murine macrophage cell line was used in these experiments because the level of COX-2 protein in BMDMs is too low to be detected by Western blotting. At 0, 1, 4, 6, 12, 18, and 24 h postinoculation, whole-cell lysates were collected and examined for COX-2 protein levels by Western blotting. COX-2 protein levels increased in a time-dependent manner with a significant increase in COX-2 protein levels occurring at 4 h postinoculation and peaking at 12 h postinoculation (Fig. 2C).

cPLA₂ is regulated posttranslationally by phosphorylation on

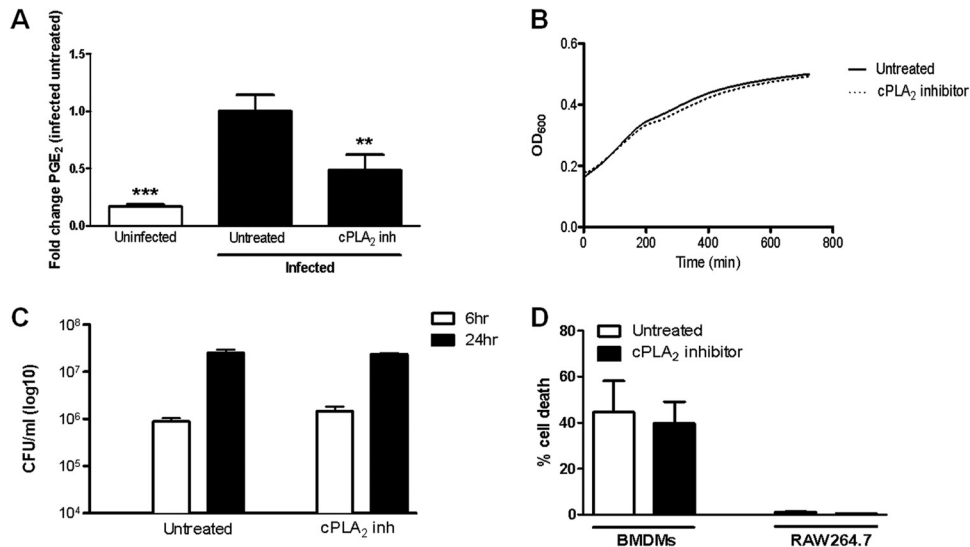


FIG 1 Inhibition of cPLA₂ significantly decreases *F. tularensis* LVS-induced PGE₂ synthesis by macrophages. BMDMs were either untreated or pretreated for 1 h with 5 μM pyrrophenone (cPLA₂ inhibitor) prior to inoculation with *F. tularensis* LVS at an MOI of 200:1. The inhibitor was maintained throughout the experiment. At 24 h postinoculation, supernatants were collected, and PGE₂ concentration was determined by ELISA. The results are the means and standard errors of the means (SEM) from four independent experiments. (B) *F. tularensis* LVS was grown in BHI-IsoVitalX broth in a 96-well plate in the absence or presence of 5 μM pyrrophenone for 12 h, with OD₆₀₀ readings taken every 5 min. Results are representative of three independent experiments. (C) RAW264.7 macrophages were either untreated or pretreated for 1 h with 5 μM pyrrophenone prior to inoculation with *F. tularensis* LVS at an MOI of 200:1. Cells were lysed for 6 h and 24 h postinoculation to determine the number of viable bacteria (CFU). (D) RAW264.7 macrophages and BMDMs were either untreated or pretreated for 1 h with 5 μM pyrrophenone prior to inoculation with *F. tularensis* LVS at an MOI of 200:1. At 20 h postinoculation, a live/dead staining was performed. The results are the percent cell death from three independent experiments. Statistical differences (**, $P \leq 0.01$; ***, $P \leq 0.001$) compared with infected untreated controls were determined by Dunnett's multiple comparison test.

Ser⁵⁰⁵. Therefore, an increase in cPLA₂ phosphorylation on Ser⁵⁰⁵ indicates an increase in cPLA₂ activity (17). We demonstrated that cPLA₂ activity is required for *F. tularensis*-induced PGE₂ synthesis (Fig. 1A). To determine if cPLA₂ is phosphorylated in macrophages infected with *F. tularensis*, BMDMs were inoculated with LVS at an MOI of 200:1 and whole-cell lysates were collected at 0, 15, 30, 60, and 120 min postinoculation. Whole-cell lysates were subjected to Western blot analysis using an antibody that recognizes cPLA₂ phosphorylated on Ser⁵⁰⁵. *F. tularensis* induced a time-dependent increase in cPLA₂ phosphorylation on Ser⁵⁰⁵ with significant phosphorylation of cPLA₂ occurring as early as 30 min postinoculation and peaking at 60 min postinoculation (Fig. 2D). The level of cPLA₂ phosphorylation returns to uninfected levels by 2 h postinoculation. These data show that *F. tularensis* infection of macrophages increases cPLA₂ phosphorylation and COX-2 protein levels which are required for the synthesis of PGE₂.

JAK3 and p38 MAPK activities are required for *F. tularensis*-induced PGE₂ synthesis by primary murine macrophages. p38 MAPK regulates PGE₂ synthesis in nonmacrophage cells, including lung epithelial cells infected with *Haemophilus influenzae* (18). *F. tularensis* stimulates the phosphorylation and activation of p38 MAPK following infection of macrophages (19). This led us to hypothesize that p38 MAPK is involved in regulating PGE₂ synthesis by macrophages infected with *F. tularensis*. To test this hypothesis, we utilized a well-characterized inhibitor of p38 MAPK, SB203580 (20). BMDMs were treated with 15 μM SB203580 prior to inoculation with LVS at an MOI of 200:1. At 24 h postinoculation, cell supernatants were collected, and PGE₂ concentration was determined by ELISA. Treatment with SB203580 significantly decreased *F. tularensis*-induced PGE₂ synthesis compared to un-

infected untreated macrophages (Fig. 3A). Similar results were observed in *F. tularensis*-infected RAW264.7 macrophages (data not shown). These data suggest that p38 MAPK activity is necessary for the synthesis of PGE₂ by macrophages infected with *F. tularensis*.

Janus kinases (JAKs) can regulate the synthesis of PGE₂ in human colonic epithelial cells and human endometrial cancer cells (21, 22). We wanted to determine if JAKs were necessary for *F. tularensis*-induced PGE₂ synthesis in macrophages. In mammals, there are four members of the JAK family of tyrosine kinases: JAK1, JAK2, JAK3, and tyrosine kinase 2 (Tyk2). To identify which if any JAKs are necessary for *F. tularensis*-induced PGE₂ synthesis in macrophages, we utilized pharmacological inhibitors of multiple members of the JAK family of tyrosine kinases. Treatment of BMDMs with AG490 (JAK2/JAK3 inhibitor) significantly reduced the synthesis of PGE₂ by *F. tularensis*-infected macrophages. Conversely, treatment with ruxolitinib (JAK1/JAK2 inhibitor) did not decrease PGE₂ production (Fig. 3B). These data suggested to us that JAK3 is the primary JAK involved in regulating the synthesis of PGE₂ by *F. tularensis*-infected macrophages. To test this idea, we utilized a well-characterized inhibitor of JAK3, JANEX-1 (23). BMDMs were treated with 50 μM JANEX-1 prior to inoculation with LVS at an MOI of 200:1. At 24 h postinoculation, supernatants were collected, and PGE₂ concentration was determined by ELISA. Treatment with JANEX-1 markedly reduced *F. tularensis*-induced PGE₂ synthesis by macrophages (Fig. 3C). These data were recapitulated in the RAW264.7 macrophage cell line (data not shown). These data suggest that JAK3 activity is necessary for the synthesis of PGE₂ by macrophages infected with *F. tularensis*.

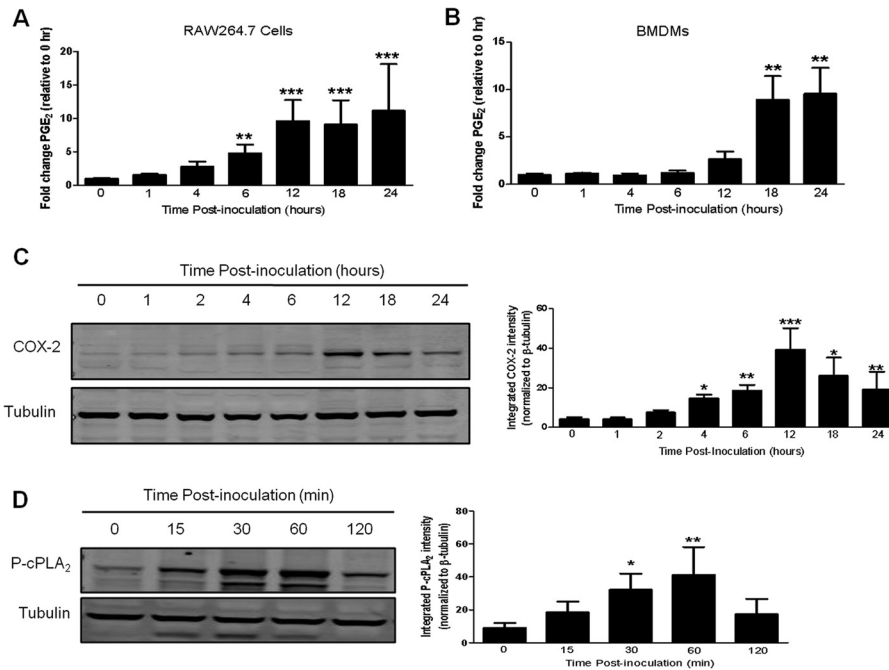


FIG 2 *F. tularensis* infection of macrophages increases PGE₂ synthesis, COX-2 protein levels, and cPLA₂ phosphorylation. (A and B) RAW264.7 macrophages (A) or BMDMs (B) were inoculated with LVS at an MOI of 200:1. At 0, 1, 4, 6, 12, 18, and 24 h postinoculation, supernatants were collected, and PGE₂ concentrations were determined. The data are the means and SEM from three independent experiments. (C) RAW264.7 macrophages were inoculated with LVS at an MOI of 200:1. At 0, 1, 2, 4, 6, 12, 18, and 24 h postinoculation, whole-cell lysates were collected and analyzed for COX-2 protein levels by Western blotting (left). The band intensities (right) were measured in each experiment using LiCor Odyssey infrared imaging system software and were normalized to the loading control (tubulin). The graph shows the band intensities from three independent experiments. (D) BMDMs were inoculated with LVS at an MOI of 200:1. At 0, 15, 30, 60, and 120 min postinoculation whole-cell lysates were collected and analyzed for cPLA₂ phosphorylated on Ser⁵⁰⁵ by Western blotting (left). The band intensities (right) were measured in each experiment using LiCor Odyssey infrared imaging system software and were normalized to the loading control (tubulin). The graph shows the band intensities from three independent experiments. Statistical differences (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) compared to the 0-h sample were determined by Dunnett's multiple comparison test.

We determined that treatment with JANEX-1 or SB203580 did not significantly affect LVS growth in broth (Fig. 3D), macrophage uptake of LVS, or intramacrophage growth of LVS in RAW264.7 cells (Fig. 3E). Treatment of RAW264.7 macrophages with JANEX-1 did result in a slight increase in cell death (up from ~5% to ~12%) (Fig. 3F). We do not believe that this slight increase in cell death in JANEX-1-treated RAW264.7 cells can account for the significant decrease in PGE₂ production in infected macrophages. Alternatively, treatment of *F. tularensis*-infected BMDMs with JANEX-1 or SB203580 actually enhanced cell survival of BMDMs (Fig. 3F). These data confirm our view that macrophage cell death is not the cause of decreased PGE₂.

Due to the potential for off-target effects of JANEX-1, we wanted to confirm that JAK3 was required for *F. tularensis*-induced PGE₂ synthesis via a genetic approach. We utilized lentivirus-delivered vectors to express JAK3 short-hairpin RNA (shRNA) to generate RAW264.7 macrophage cell lines with reduced JAK3 gene expression. As we had difficulty in detecting JAK3 protein levels, we decided to demonstrate JAK3 depletion by examining JAK3 gene expression. To verify that JAK3 gene expression was in fact reduced in our cells expressing JAK3 shRNA, we performed reverse transcription-PCR (RT-PCR) using primers specific to the JAK3 gene. We observed a decrease in JAK3 transcript levels in our JAK3 shRNA-expressing cells (Fig. 3G). Cells expressing JAK3 shRNA were inoculated with *F. tularensis* LVS at an MOI of 200:1. At 24 h postinoculation, supernatants were col-

lected, and a PGE₂ ELISA was performed to determine the concentration of PGE₂. In cells expressing JAK3 shRNA, we observed a significant decrease in PGE₂ production compared to wild-type RAW264.7 macrophages (Fig. 3H). A nontarget (NT) shRNA vector control plasmid was expressed as a negative control.

Unfortunately, we observed that the JAK3-depleted cells quickly recovered their ability to synthesize PGE₂ in response to *F. tularensis* after cell culture passage due to some unknown compensatory mechanism. Because of this compensation, it was not possible to generate enough JAK3-depleted macrophages to recapitulate the data from all of the experiments performed throughout this study in the JAK3-depleted macrophages. However, since we performed six independent lentiviral transfections to generate JAK3-depleted macrophages that all resulted in the same phenotype upon initial infection, decreased PGE₂ synthesis, this strongly suggests to us that JAK3 is involved in *F. tularensis*-induced PGE₂ production. Taken together, these data demonstrate that JAK3 activity is critical for the synthesis of PGE₂ by *F. tularensis*-infected macrophages.

p38 MAPK activity is required for increased COX-2 protein levels, but not for enhanced phosphorylation of cPLA₂ in macrophages infected with *F. tularensis*. p38 MAPK has been reported to regulate both the phosphorylation of cPLA₂ and COX-2 protein levels in macrophages (24). We first investigated if p38 MAPK was required for the phosphorylation of cPLA₂ in macrophages infected with *F. tularensis*. BMDMs were treated with 15

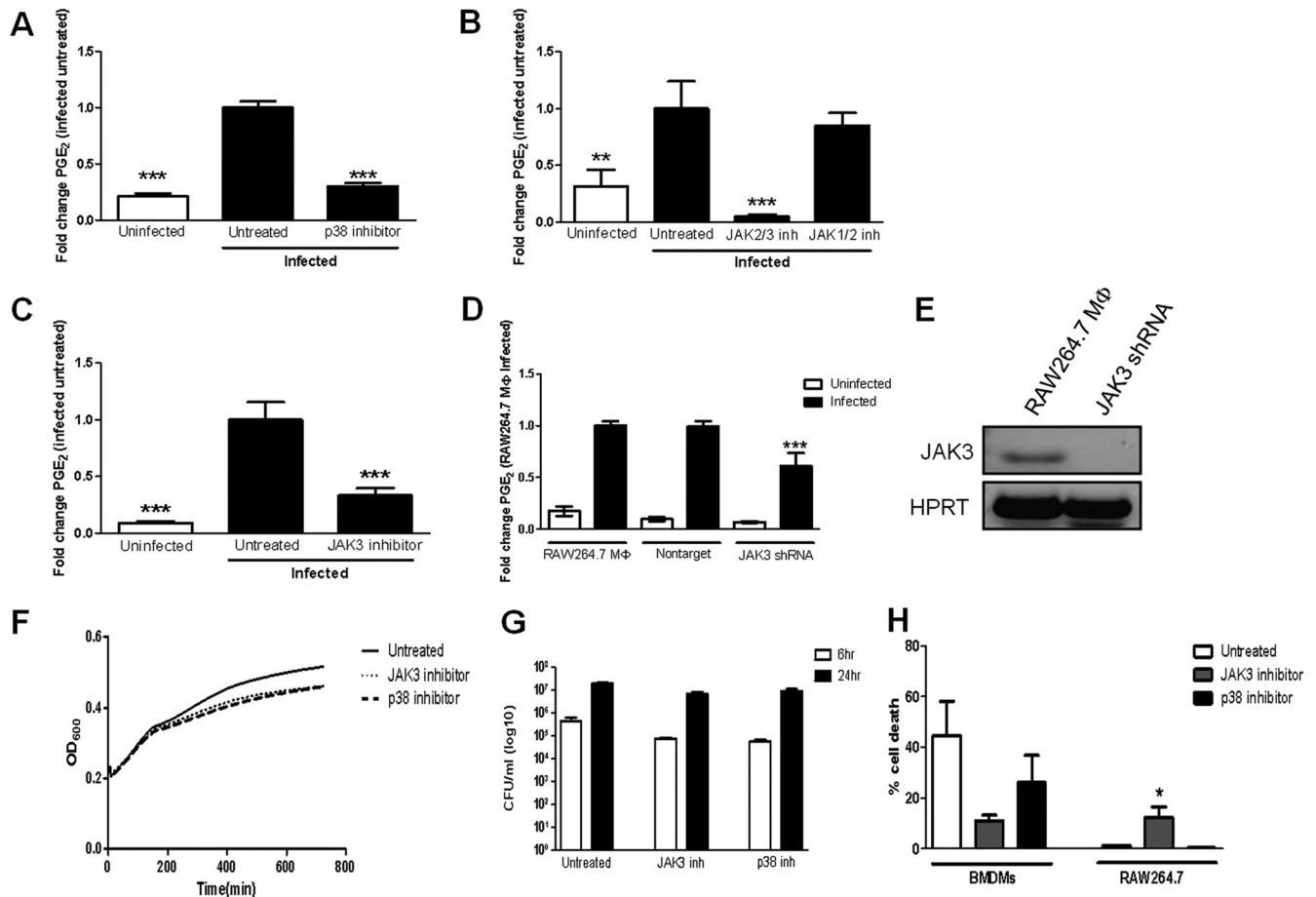


FIG 3 Inhibition of JAK3 and p38 MAPK decreases *F. tularensis* LVS-induced PGE₂ synthesis by macrophages. BMDMs were either untreated or pretreated for 1 h with 15 μ M SB203580 (p38 MAPK inhibitor) (A), 50 μ M AG490 (JAK2/3 inhibitor) (B), 400 nM ruxolitinib (JAK1/2 inhibitor) (B), or 50 μ M JANEX-1 (JAK3 inhibitor) (C) prior to inoculation with *F. tularensis* LVS at an MOI of 200:1. At 24 h postinoculation, supernatants were collected, and PGE₂ concentration was determined by ELISA. Results are the means and SEM from nine (A), three (B), five (C), and five (D) independent experiments. (E) RT-PCR was performed using cDNA synthesized from RNA extracted from wild-type RAW264.7 macrophages and RAW264.7 macrophages expressing JAK3 shRNA. (F) *F. tularensis* LVS was grown in BHI-IsoVitaleX broth in a 96-well plate in the absence or presence of 15 μ M SB203580 or 50 μ M JANEX-1 for 12 h, with OD₆₀₀ readings taken every 5 min. Results are representative of three independent experiments. (G) RAW264.7 macrophages were either untreated or pretreated for 1 h with 15 μ M SB203580 or 50 μ M JANEX-1 prior to inoculation with *F. tularensis* LVS at an MOI of 200:1. Cells were lysed at 6 h and 24 h postinoculation to determine the number of viable bacteria (CFU) on chocolate agar medium. (H) RAW264.7 macrophages and BMDMs were either untreated or pretreated for 1 h with 15 μ M SB203580 or 50 μ M JANEX-1 prior to inoculation with *F. tularensis* LVS at an MOI of 200:1. At 20 h postinoculation, a live/dead staining was performed. The results are the means from three independent experiments. Statistical differences (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) compared with infected untreated or wild-type macrophages (D) were determined by Dunnett's multiple comparison test.

μ M SB203580 prior to inoculation with LVS at an MOI of 200:1. Whole-cell lysates were collected at 1 h postinoculation, and a Western blot analysis was performed to determine the level of cPLA₂ phosphorylated on Ser⁵⁰⁵. Inhibition of p38 MAPK did not alter the phosphorylation state of cPLA₂ (Fig. 4A), suggesting that p38 MAPK activity is not required for the phosphorylation of cPLA₂ in *F. tularensis*-infected macrophages. We next examined if p38 MAPK controlled COX-2 protein levels in macrophages infected with *F. tularensis*. RAW264.7 macrophages were untreated or treated with the p38 MAPK inhibitor SB203580 prior to inoculation with LVS at an MOI of 200:1. Whole-cell lysates were collected at 4 h and 18 h postinoculation to allow us to examine COX-2 protein levels at an early time point as well as a late time point. We found that inhibition of p38 MAPK resulted in a signif-

icant decrease in COX-2 protein levels at both 4 h and 18 h postinoculation (Fig. 4B and C). These data demonstrate that p38 MAPK activity is necessary for *F. tularensis*-induced COX-2 protein synthesis in macrophages. Overall, these data suggest that p38 MAPK activity contributes to the synthesis of PGE₂ in *F. tularensis*-infected macrophages by increasing COX-2 protein levels but not by increasing the phosphorylation of cPLA₂.

JAK3 activity is necessary for both increased COX-2 protein levels and cPLA₂ phosphorylation in macrophages infected with *F. tularensis*. JAKs are capable of mediating cPLA₂ expression and activation in response to growth factors and interferon in vascular smooth muscle cells and HeLa cells (25, 26). Therefore, we first wanted to determine if JAK3 activity is necessary for the phosphorylation of cPLA₂ in macrophages infected with *F. tularensis*.

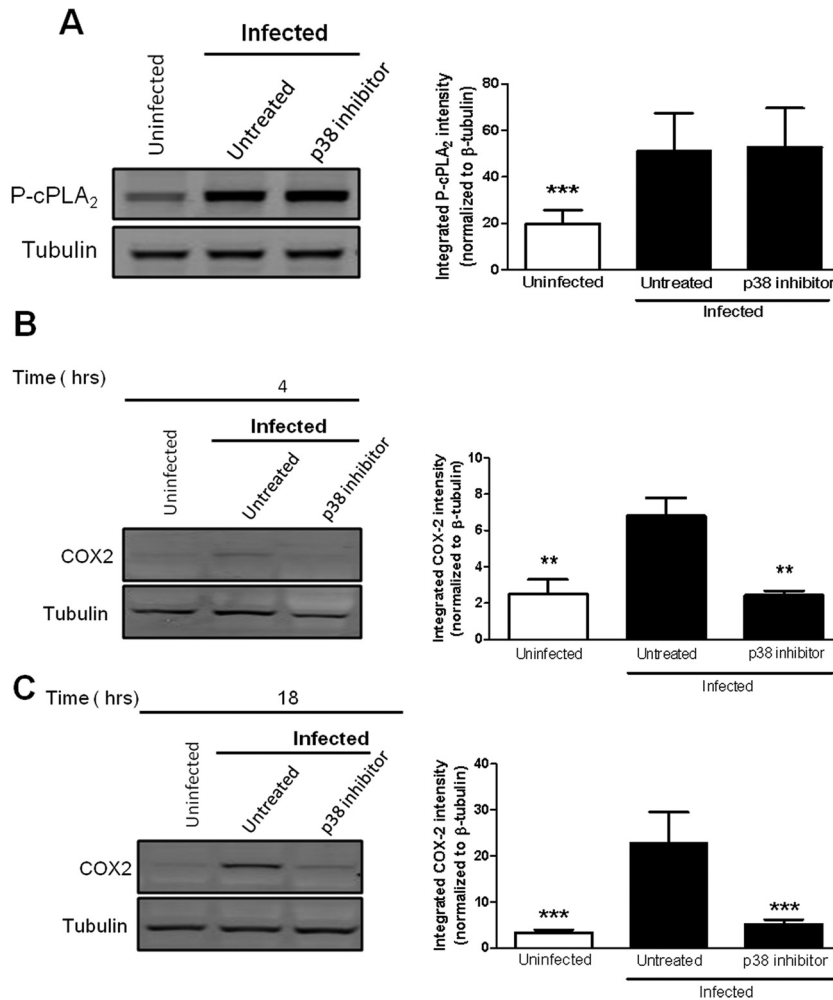


FIG 4 Inhibition of p38 MAPK decreases COX-2 protein levels but not cPLA₂ phosphorylation. (A) BMDMs were either untreated or pretreated with 15 μM SB203580 (p38 MAPK inhibitor) for 1 h prior to inoculation with LVS at an MOI of 200:1. At 1 h postinoculation, whole-cell lysates were collected and analyzed for cPLA₂ phosphorylated on Ser⁵⁰⁵ by Western blotting (left). The band intensities (right) were measured in each experiment using LiCor Odyssey infrared imaging system software and were normalized to the loading control (tubulin). The graph presents data from three independent experiments. (B and C) RAW264.7 cells were either untreated or pretreated with 15 μM SB203580 for 1 h prior to inoculation with LVS at an MOI of 200:1. At 2 h postinoculation, the cells were incubated in RPMI containing penicillin-streptomycin. At 4 h (B) and 18 h (C) postinoculation, whole-cell lysates were collected and analyzed for COX-2 protein levels by Western blotting (left). The band intensities (right) were measured in each experiment and were normalized to the loading control tubulin. The graphs present data from three independent experiments. Statistical differences (**, $P \leq 0.01$; ***, $P \leq 0.001$) compared to infected untreated controls were determined by Dunnett’s multiple comparison test.

BMDMs were untreated or treated with 50 μM JANEX-1 prior to inoculation with LVS at an MOI of 200:1. Whole-cell lysates were collected at 1 h postinoculation and examined for cPLA₂ phosphorylation on Ser⁵⁰⁵ by Western blotting. Treatment with JANEX-1 significantly decreased cPLA₂ phosphorylation on Ser⁵⁰⁵ (Fig. 5A), suggesting that JAK3 signaling is important for *F. tularensis*-induced cPLA₂ phosphorylation.

JAKs are capable of regulating COX-2 protein levels by regulating COX-2 gene expression (27, 28). Therefore, we next wanted to determine if JAK3 activity was necessary for the increased COX-2 protein levels in macrophages infected with *F. tularensis*. RAW264.7 macrophages were untreated or treated with 50 μM JANEX-1 prior to inoculation with LVS at an MOI of 200:1. Whole-cell lysates were collected at 4 h and 18 h postinoculation and COX-2 protein levels were determined by Western blotting. Treatment with JANEX-1 significantly decreased COX-2 protein

levels at 4 h and 18 h postinoculation (Fig. 5B and C), suggesting that JAK3 activity is important for COX-2 protein synthesis in *F. tularensis*-infected macrophages. Treatment of macrophages with JANEX-1 decreased COX-2 protein levels to a greater extent at 4 h postinoculation than 18 h postinoculation, suggesting that other signaling pathways that are less reliant on JAK3 could be regulating COX-2 protein synthesis at later time points postinfection. Future work will be necessary to understand the mechanism behind this differential regulation of COX-2 protein synthesis. Together, these data demonstrate that JAK3 is important for regulating multiple steps of the PGE₂-biosynthetic pathway, including the phosphorylation of cPLA₂ and COX-2 protein synthesis in *F. tularensis*-infected macrophages.

JAK3 regulation of cPLA₂ phosphorylation is not universal for agonist-induced PGE₂ synthesis in macrophages. We were intrigued by the data suggesting that JAK3 stimulates the phos-

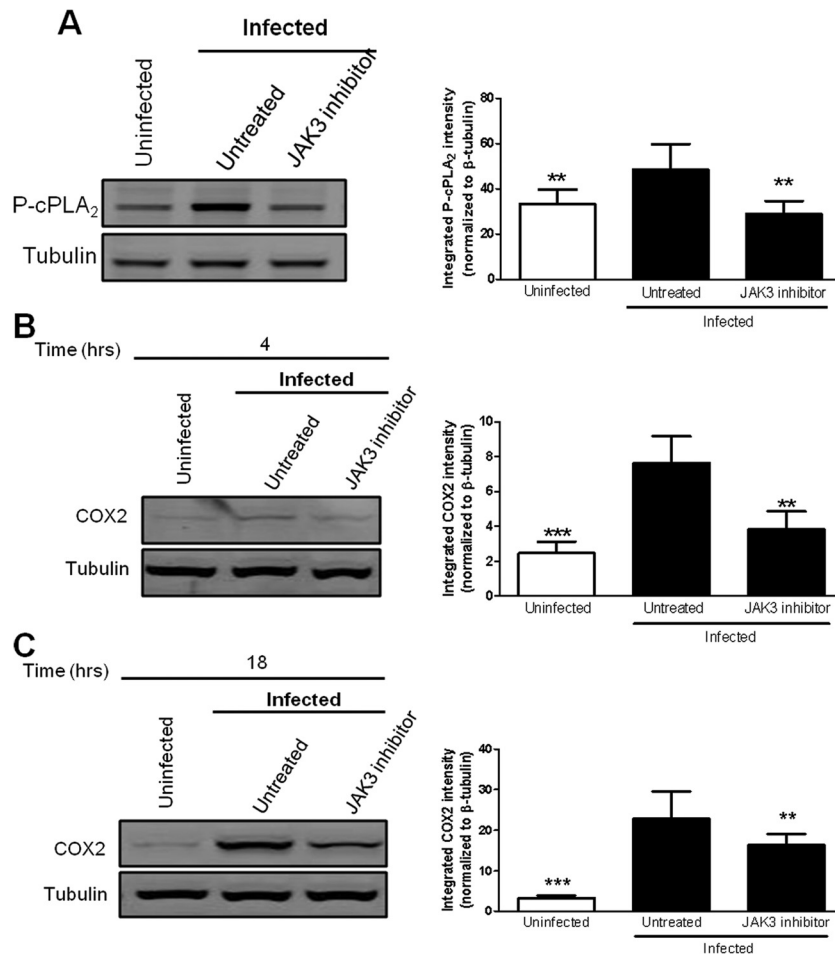


FIG 5 Inhibition of JAK3 decreases COX-2 protein levels and phosphorylation of cPLA₂ in macrophages infected with *F. tularensis*. (A) BMDMs were either untreated or pretreated with 50 μM JANEX-1 (JAK3 inhibitor) for 1 h prior to inoculation with LVS at an MOI of 200:1. At 1 h postinoculation, whole-cell lysates were collected and analyzed for cPLA₂ phosphorylated on Ser⁵⁰⁵ by Western blotting (left). The band intensities (right) were measured in each experiment using LiCor Odyssey infrared imaging system software and were normalized to the loading control (tubulin). The graph presents data from eight independent experiments. (B and C) RAW264.7 macrophages were either untreated or pretreated with 50 μM JANEX-1 (JAK3 inhibitor) for 1 h prior to inoculation with LVS at an MOI of 200:1. After 2 h, the cells were incubated in RPMI containing penicillin-streptomycin. At 4 h (B) and 18 h (C) postinoculation, whole-cell lysates were collected and analyzed for COX-2 protein levels by Western blotting (left). The band intensities (right) were measured in each experiment and were normalized to the loading control (tubulin). The graphs present data from seven (B) and six (C) independent experiments. Statistical differences (**, $P \leq 0.01$; ***, $P \leq 0.001$) compared to infected untreated controls were determined by Dunnett's multiple comparison test.

phorylation of cPLA₂ in *F. tularensis*-infected macrophages. To the best of our knowledge, JAK3 has not been documented to induce the phosphorylation of cPLA₂ in any cell type. JAKs are best characterized for their ability to regulate gene transcription through the phosphorylation and activation of members of the signal transducer and activator of transcription (STAT) family of transcription factors (29). However, the fact that JAK3 activity is necessary for the phosphorylation of cPLA₂ at 1 h postinoculation suggests that JAK3 stimulates cPLA₂ phosphorylation independently of transcription. To test this hypothesis, BMDMs were untreated or treated with the transcription inhibitor actinomycin D prior to inoculation with LVS at an MOI of 200:1. Whole-cell lysates were collected at 1 h postinoculation, and a Western blot analysis was performed to examine cPLA₂ phosphorylation on Ser⁵⁰⁵. Treatment with actinomycin D did not decrease *F. tularensis*-induced phosphorylation of cPLA₂ (Fig. 6A). To confirm that actinomycin D was functional, we examined *F. tularensis*-

induced PGE₂ production by macrophages treated with actinomycin D. The synthesis of PGE₂ requires the generation of new proteins (e.g., COX-2) and is therefore dependent on transcription. As expected, treatment with actinomycin D significantly decreased PGE₂ synthesis compared to infected untreated samples (Fig. 6B). These data indicate that transcription is essential for *F. tularensis*-induced PGE₂ synthesis and that the phosphorylation of cPLA₂ in macrophages infected with *F. tularensis* occurs via a transcription-independent mechanism. Together, these results suggest that JAK3 regulates the phosphorylation of cPLA₂ by a mechanism that is independent of STAT proteins. Furthermore, it suggests that JAK3 activity is critical for the phosphorylation of cPLA₂ either directly or indirectly via an intermediate serine kinase, since JAK3 is a tyrosine kinase. Future work will focus on defining the mechanism of JAK3 phosphorylation of cPLA₂ in macrophages infected with *F. tularensis*.

We wanted to determine if JAK3-mediated phosphorylation of

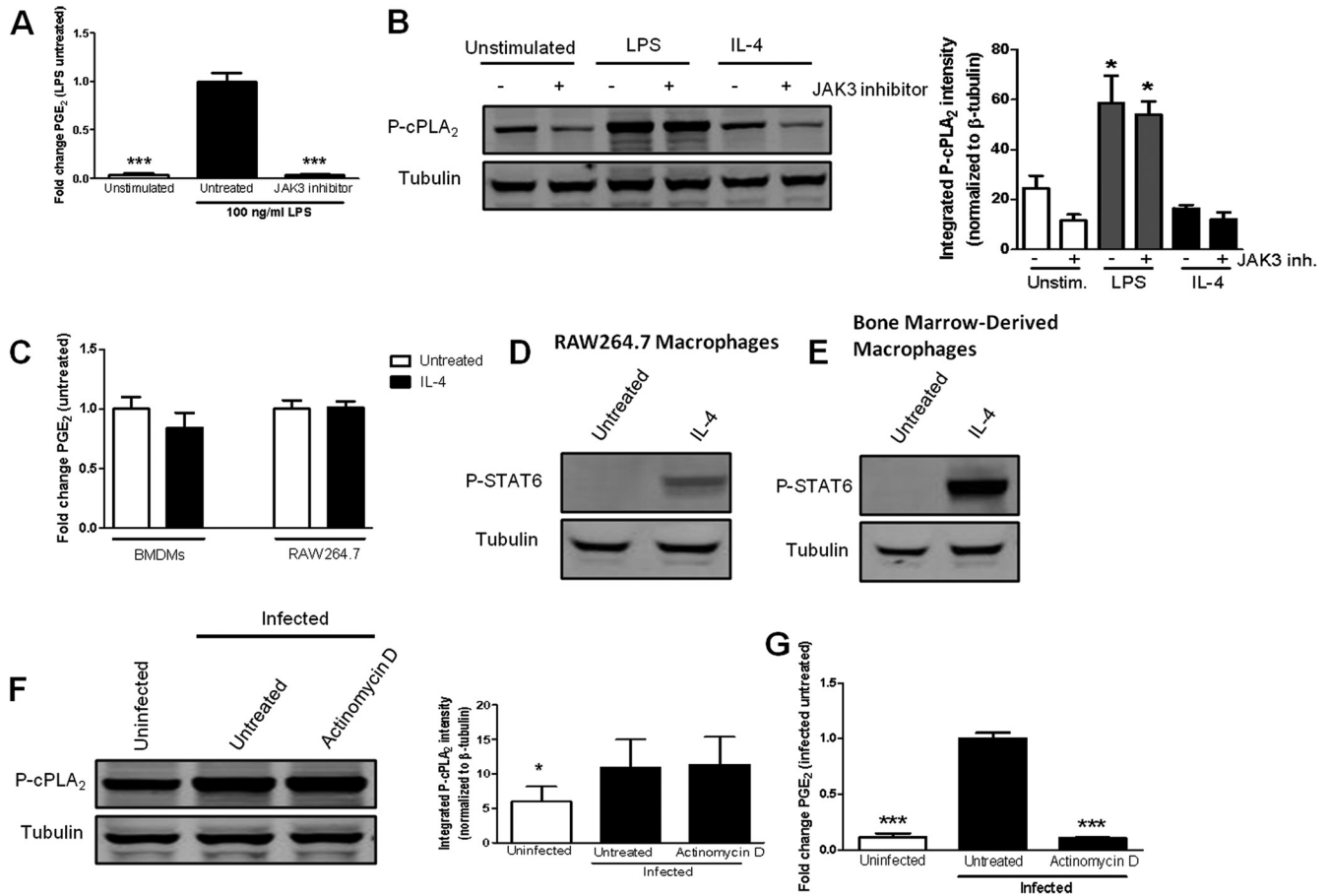


FIG 6 Inhibition of JAK3 does not decrease cPLA₂ phosphorylation in macrophages stimulated with LPS or IL-4. (A) BMDMs were either untreated or pretreated with 50 μM JANEX-1 (JAK3 inhibitor) for 1 h prior to stimulation with 100 ng/ml LPS. JANEX-1 was maintained throughout the experiment. At 24 h postinoculation, supernatants were collected, and PGE₂ concentration was determined by ELISA. The results are the means and SEM from three independent experiments. (B) BMDMs were either untreated or pretreated with 50 μM JANEX-1 (JAK3 inhibitor) for 1 h prior to stimulation with 100 ng/ml LPS or 30 ng/ml IL-4. After 1 h, whole-cell lysates were collected and analyzed for cPLA₂ phosphorylated on Ser⁵⁰⁵ by Western blotting (left). The band intensities (right) were measured in each experiment and were normalized to the loading control (tubulin). The graph presents data from three independent experiments. (C) BMDMs were either untreated or treated with 30 ng/ml IL-4. At 24 h postinoculation, supernatants were collected, and PGE₂ concentration was determined by ELISA. The results are means and SEM from three independent experiments. RAW264.7 macrophages (D) or BMDMs (E) were untreated or treated with 30 ng/ml IL-4. At 1 h postinoculation, whole-cell lysates were collected and analyzed for STAT6 phosphorylated on Tyr⁶⁴¹ by Western blotting. (F) BMDMs were untreated or pretreated with 2 μg/ml actinomycin D for 1 h prior to inoculation with LVS at an MOI of 200:1. At 1 h postinoculation, whole-cell lysates were collected and analyzed for cPLA₂ phosphorylated on Ser⁵⁰⁵ by Western blotting (left). The band intensities (right) were measured in each experiment and were normalized to the loading control (tubulin). The graph presents data from five independent experiments. (G) BMDMs were either untreated or pretreated for 1 h with 2 μg/ml actinomycin D (transcription inhibitor) prior to inoculation with *F. tularensis* LVS at an MOI of 200:1. At 24 h postinoculation, supernatants were collected and PGE₂ concentration was determined by ELISA. Statistical differences (*, $P \leq 0.05$; ***, $P \leq 0.001$) compared to infected untreated (G) or untreated unstimulated (A and B) controls were determined by Dunnett's multiple comparison test.

cPLA₂ is unique to *F. tularensis* or if JAK3 is involved in cPLA₂ phosphorylation in macrophages in response to other stimulants. To address this, BMDMs were untreated or treated with 50 μM JANEX-1 prior to stimulation with 100 ng/ml lipopolysaccharide (LPS) (a known inducer of cPLA₂ phosphorylation) or 30 ng/ml interleukin-4 (IL-4) (a known activator of JAK3) (30, 31). Whole-cell lysates were collected at 1 h poststimulation and examined for cPLA₂ phosphorylation on Ser⁵⁰⁵ by Western blotting. Stimulation of macrophages with LPS resulted in a significant increase in cPLA₂ phosphorylation compared to unstimulated control macrophages. However, treatment with JANEX-1 did not reduce the level of phosphorylated cPLA₂ in macrophages stimulated with LPS (Fig. 6C), which suggests that LPS-induced phosphorylation of cPLA₂ occurs independently of JAK3. LPS is capable of induc-

ing PGE₂ synthesis from BMDMs, and treatment with JANEX-1 decreases PGE₂ synthesis by macrophages stimulated with LPS (Fig. 6D). Based on these data, we believe that *F. tularensis* and LPS utilize distinct signal transduction pathways to induce the synthesis of PGE₂. Conversely, treatment with IL-4 did not increase cPLA₂ phosphorylation (Fig. 6C) or induce the synthesis of PGE₂ from macrophages (Fig. 6E). We wanted to confirm that the IL-4 that we used to stimulate the macrophages activated IL-4 receptor signaling by examining the phosphorylation of STAT6 (a known downstream protein that is phosphorylated following IL-4 receptor engagement) (31). IL-4 stimulation of RAW264.7 macrophages and BMDMs resulted in the phosphorylation of STAT6 (Fig. 6F and G). These data suggest that activation of JAK3 does not always result in cPLA₂ phosphorylation or PGE₂ production.

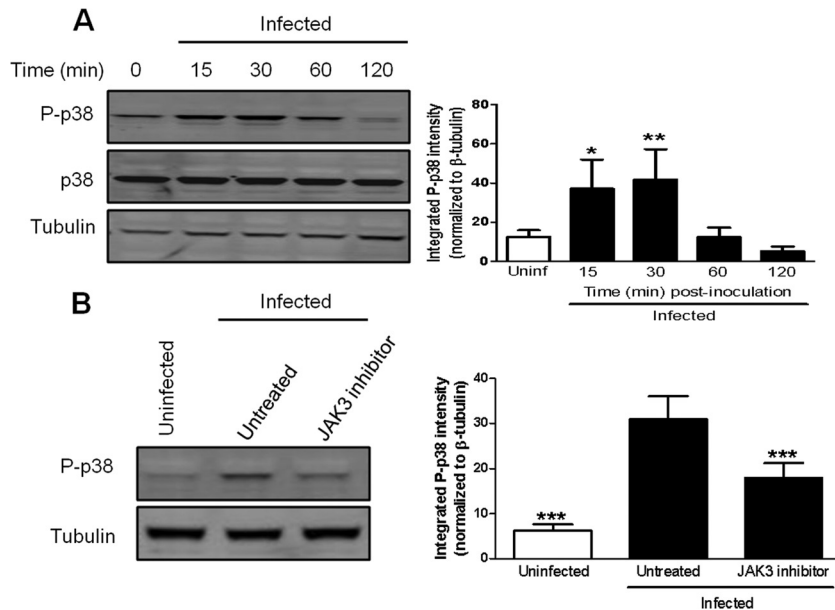


FIG 7 Inhibition of JAK3 decreases p38 MAPK phosphorylation in BMDMs infected with *F. tularensis*. (A) BMDMs were inoculated with LVS at an MOI of 200:1. At 0, 15, 30, 60, and 120 min postinoculation, whole-cell lysates were collected and analyzed for total p38 MAPK or p38 MAPK phosphorylated on Thr¹⁸⁰ and Tyr¹⁸² by Western blotting (left). The band intensities (right) were measured in each experiment and were normalized to the loading control (tubulin). The graph presents data from four independent experiments. (B) BMDMs were either untreated or pretreated with 50 μ M JANEX-1 (JAK3 inhibitor) for 1 h prior to inoculation with LVS at an MOI of 200:1. At 30 min postinoculation, whole-cell lysates were collected and analyzed for p38 MAPK phosphorylated on Thr¹⁸⁰ and Tyr¹⁸² by Western blotting (left). The band intensities (right) were measured in each experiment and were normalized to the loading control (tubulin). The graph presents data from eight independent experiments. Statistical differences (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) compared to infected untreated controls were determined by Dunnett's multiple comparison test.

More importantly, these data support a unique role for JAK3 in regulating the phosphorylation of cPLA₂ and the synthesis of PGE₂ in *F. tularensis*-infected macrophages. Overall, these data suggest that *F. tularensis* activates a signaling pathway to stimulate the synthesis of PGE₂ that is distinct from LPS. In *F. tularensis*-infected macrophages, JAK3 plays a novel role in regulating, likely indirectly, both the phosphorylation of cPLA₂ and COX-2 protein levels, which results in increased PGE₂ production.

JAK3 activity is required for the phosphorylation of p38 MAPK in murine macrophages infected with *F. tularensis*. Activated JAKs are capable of initiating a multitude of signaling cascades, including major signaling pathways such as JAK/STAT, PI3K/Akt, and p38 MAPK pathways (32, 33). Like activation of other MAPKs, p38 MAPK activation is regulated posttranslationally by phosphorylation. This led us to hypothesize that JAK3 acts upstream of p38 MAPK in a linear pathway and JAK3 activity is required for the phosphorylation of p38 MAPK in *F. tularensis*-infected macrophages. Prior to examining if JAK3 activity is necessary for the phosphorylation of p38 MAPK, we wanted to confirm the previously reported kinetics of p38 MAPK phosphorylation in macrophages infected with *F. tularensis* (19). BMDMs were inoculated with LVS at an MOI of 200:1, and whole-cell lysates were collected at 0, 15, 30, 60, and 120 min postinoculation. Cell lysates were examined for p38 MAPK phosphorylation on Thr¹⁸⁰ and Tyr¹⁸² by Western blotting. There was a significant increase in p38 MAPK phosphorylation as early as 15 min postinoculation, and peak phosphorylation of p38 MAPK occurred by 30 min postinoculation (Fig. 7A). To determine if JAK3 activity was necessary for the phosphorylation of p38 MAPK in *F. tularensis*-infected macrophages, BMDMs were treated with 50 μ M

JANEX-1 prior to inoculation with LVS at an MOI of 200:1. Whole-cell lysates were collected at 30 min postinoculation and subjected to Western blot analysis to determine the levels of phosphorylated p38 MAPK. Treatment with JANEX-1 significantly decreased the phosphorylation of p38 MAPK, as shown by Western blotting (Fig. 7B). These data suggest that JAK3 is activated early by *F. tularensis* and acts upstream of p38 MAPK in a signaling pathway that induces the synthesis of PGE₂ in macrophages.

DISCUSSION

The ability of *F. tularensis* to modulate and subvert the host immune response is critical to its survival within the host. *F. tularensis*-induced synthesis of PGE₂ from infected macrophages is one of several identified mechanisms of *F. tularensis* immune evasion (6, 7). The signaling pathways responsible for regulating the activity of the enzymes in the PGE₂-biosynthetic pathway in *F. tularensis*-infected macrophages are not well defined at this time. In the present study, we demonstrated that JAK3, cPLA₂, and p38 MAPK signaling are required for *F. tularensis* LVS-induced PGE₂ synthesis by infected murine macrophages. p38 MAPK activity was necessary for increased COX-2 protein levels, while JAK3 activity was necessary for the phosphorylation of cPLA₂, the phosphorylation of p38 MAPK, and increased COX-2 protein levels. To the best of our knowledge, this is the first evidence of JAK3 regulating enzymes in the inducible PGE₂-biosynthetic pathway in any eukaryotic cell.

The canonical inducible PGE₂-biosynthetic pathway typically requires cPLA₂ to liberate AA from phospholipids. Liberated AA is then oxidized and isomerized by COX-2 and terminal prostaglandin synthases to produce PGE₂ (34). As seen with other pathogen-

induced PGE₂ synthesis, *F. tularensis* required cPLA₂ for the induction of PGE₂ synthesis by infected macrophages. *F. tularensis* infection resulted in a significant increase in COX-2 protein levels by 4 h postinoculation and a significant increase in detectable PGE₂ by 6 h postinoculation in RAW264.7 macrophages and an increase in PGE₂ production between 12 h and 18 h postinoculation in BMDMs. The phosphorylation of cPLA₂ on Ser⁵⁰⁵ is functionally important for cPLA₂-mediated release of AA (35). We observed peak phosphorylation of cPLA₂ at 1 h postinoculation and a return to baseline phosphorylation by 2 h postinoculation. We did not observe phosphorylation of cPLA₂ after 1 h postinoculation (data not shown). We also did not observe an overlap between cPLA₂ phosphorylation and PGE₂ synthesis. This is surprising, since these are typically closely coupled events. We take these data to suggest that cPLA₂ is not the phospholipase responsible for supplying the AA that is used by the cell to synthesize PGE₂ in *F. tularensis*-infected macrophages. It is not unreasonable to hypothesize that cPLA₂ is not supplying AA, considering that phospholipase D- and phospholipase C-mediated pathways have been implicated in liberating AA (36–38). In fact, we have preliminary data that suggest that phospholipase D is responsible for liberating the AA for PGE₂ synthesis (data not shown). We are currently trying to elucidate an alternative mechanism by which cPLA₂ regulates PGE₂ synthesis independently of liberating AA that is converted into PGE₂. Overall, from these studies we were able to establish the kinetics of cPLA₂ phosphorylation, COX-2 protein synthesis, and PGE₂ synthesis in *F. tularensis*-infected macrophages. By knowing the timing of these events, we can identify the host signal transduction pathways that regulate *F. tularensis*-induced PGE₂ synthesis in macrophages.

The p38 MAPK signaling cascade is one of the primary intracellular pathways activated in response to a variety of extracellular stimuli. Previous studies have shown that p38 MAPK can regulate agonist-induced transcription/translation of COX-2, phosphorylation of cPLA₂, and PGE₂ production (39). p38 MAPK is rapidly phosphorylated following infection of both murine and human macrophages with *F. tularensis*, and the phosphorylation of p38 MAPK begins to decrease by 2 h postinoculation (19), which we also observed. We demonstrated that p38 MAPK activity is needed for the production of PGE₂ by macrophages infected with *F. tularensis*. This is likely due to the fact that p38 MAPK activity is necessary for COX-2 protein synthesis in *F. tularensis*-infected macrophages. We hypothesize that p38 MAPK either regulates the transcription of the COX-2 gene or regulates the stability of COX-2 mRNA. However, at this time, the mechanism behind p38 MAPK regulation of COX-2 gene expression in *F. tularensis*-infected macrophages is unknown.

Our studies uncovered an unexpected signaling pathway involving JAK3 that may be unique to *F. tularensis* LVS-induced PGE₂ synthesis. Specifically, JAK3 activity is necessary for *F. tularensis*-induced PGE₂ synthesis. We hypothesize that JAK3 is involved in two distinct signaling pathways that are activated by *F. tularensis* in murine macrophages. This hypothesis is based on the following observations: (i) JAK3 is involved in the phosphorylation of p38 MAPK and cPLA₂ and increased COX-2 protein levels and (ii) p38 MAPK is involved only in COX-2 protein synthesis, not cPLA₂ phosphorylation. In the first pathway, JAK3 mediates the phosphorylation of cPLA₂ on Ser⁵⁰⁵. However, JAK3 is a tyrosine kinase, suggesting that an intermediate serine kinase is phosphorylated and activated directly by JAK3 and this serine

kinase subsequently phosphorylates cPLA₂. We are currently trying to identify the intermediate serine kinase(s) responsible for phosphorylating cPLA₂ in *F. tularensis*-infected macrophages. The second pathway involves JAK3 phosphorylating p38 MAPK, which results in increased COX-2 protein levels. Regardless of whether there are two distinct pathways, we know that phosphorylation of cPLA₂ and increased COX-2 protein levels are necessary for *F. tularensis*-induced PGE₂ production.

JAK3 typically associates with the common γ chain (γ_c) subunit of cytokine receptors and becomes activated only following cytokine binding to the receptor (40–42). We showed that JAK3 is involved in the phosphorylation of p38 MAPK at 30 min postinoculation and cPLA₂ phosphorylation at 1 h postinoculation. It seems unlikely that a cytokine could be synthesized, be secreted, bind to its receptor on the cell surface, and activate JAK3 in less than 30 min following *F. tularensis* infection. Based on this, we believe that JAK3 is not phosphorylated and activated by cytokine-mediated receptor engagement at this early time postinoculation. We propose that either *F. tularensis* binds a common γ chain-containing cytokine receptor or JAK3 associates with a surface receptor that functions as a pathogen pattern recognition receptor. To our knowledge, there are no studies that have shown *F. tularensis* binding to a cytokine receptor or JAK3 associating with a receptor that does not contain a common γ_c subunit. We are excited about exploring these two possibilities, and clearly, more work is required to define the mechanism of JAK3 activation in macrophages infected with *F. tularensis*.

Our results demonstrate for the first time a novel role for JAK3 in regulating multiple enzymes in the prostaglandin-biosynthetic pathway. We observed in *F. tularensis* LVS-infected murine macrophages that JAK3 is necessary for cPLA₂ phosphorylation, p38 MAPK phosphorylation, increased COX-2 protein levels, and ultimately the synthesis of PGE₂. We propose that *F. tularensis* interacts with a macrophage surface receptor that results in the activation of JAK3 and initiates an intracellular signaling cascade that increases the production of PGE₂. This represents an unexpected bacterial-eukaryotic receptor interaction or the involvement of JAK3 with a host pattern recognition receptor that was previously unknown.

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A.M.B. and M.D.W. designed experiments; A.M.B., A.R.N., and J.D.B. performed experiments; A.M.B. and M.D.W. analyzed data and wrote the paper.

There are no conflicting financial interests among the authors.

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