Francisella tularensis LVS Induction of Prostaglandin Biosynthesis by Infected Macrophages Requires Specific Host Phospholipases and Lipid Phosphatases

Aaron R. Navratil, Ashley M. Brummett, Joshua D. Bryan, Matthew D. Woolard
Department of Microbiology and Immunology, Louisiana State University Health Sciences Center at Shreveport, Shreveport, Louisiana, USA

Francisella tularensis induces the synthesis of prostaglandin E2 (PGE2) by infected macrophages to alter host immune responses, thus providing a survival advantage to the bacterium. We previously demonstrated that PGE2 synthesis by F. tularensis-infected macrophages requires cytosolic phospholipase A2 (cPLA2), cyclooxygenase 2 (COX-2), and microsomal prostaglandin E synthase 1 (mPGES1). During inducible PGE2 synthesis, cPLA2 hydrolyzes arachidonic acid (AA) from cellular phospholipids to be converted to PGE2. However, in F. tularensis-infected macrophages we observed a temporal disconnect between Ser505-cPLA2 phosphorylation (a marker of activation) and PGE2 synthesis. These results suggested to us that cPLA2 is not responsible for the liberation of AA to be converted into PGE2 by F. tularensis-infected macrophages. Utilizing small-molecule inhibitors, we demonstrated that phospholipase D and diacylglycerol lipase were required for providing AA for PGE2 biosynthesis. cPLA2, on the other hand, was required for macrophage cytokine responses to F. tularensis. We also demonstrated for the first time that lipin-1 and PAP2a contribute to macrophage inflammation in response to F. tularensis. Our results identify both an alternative pathway for inducible PGE2 synthesis and a role for lipid-modifying enzymes in the regulation of macrophage inflammatory function.

Francisella tularensis is a Gram-negative facultative intracellular bacterium and the causative agent of tularemia, a disease capable of causing a high level of mortality in humans. The most severe form of the disease is pneumonic tularemia. Inhalation of as few as 10 organisms can cause disease which has a >30% case fatality rate if untreated (1, 2). The low infectious dose and high morbidity and mortality of F. tularensis infections led to the observation of the organism (3, 4). There is no FDA-approved vaccine available to prevent tularemia. Thus, the CDC has classified F. tularensis as a category A select agent.

F. tularensis invasion and growth within macrophages are critical for disease manifestation (5). The ability of this bacterium to alter host immune responses is also important to bacterial survival in the host and disease pathogenesis. The significance of altering host immune responses is highlighted by the observation that F. tularensis strains with mutations in clpB grow within host macrophages in vitro but fail to alter host immune responses in vivo and are easily eradicated by the host (6). Numerous F. tularensis-mediated immune evasion mechanisms have been identified (reviewed in reference 7). One important mechanism of Francisella (Francisella novicida, F. tularensis live vaccine strain [LVS], and F. tularensis Schu S4) immune evasion that we have identified is the ability of F. tularensis to induce the biosynthesis of prostaglandin E2 (PGE2) by infected macrophages (8, 9). The immunological function of PGE2 is context dependent and can exhibit pro- or anti-inflammatory properties. However, during infectious disease, the activity of PGE2 is mostly anti-inflammatory, whereby it suppresses the production of inflammatory cytokines (10, 11). In vitro, F. tularensis induction of PGE2 synthesis by infected macrophages inhibits T cell proliferation and Tₜ₇₉ phenotypic development, reducing the ‘T cells’ ability to produce proinflammatory cytokines such as gamma interferon (IFN-γ) (12). IFN-γ synthesis by T cells is critical for clearance of F. tularensis from the host (13, 14). PGE2 also reduces macrophage surface expression of major histocompatibility complex (MHC) class II by ubiquitination-mediated degradation (15). Increased levels of PGE2 are detected in the lungs of mice with respiratory tularemia (8). The inhibition of PG synthesis during respiratory tularemia results in a decreased bacterial burden and an increased number of Francisella specific IFN-γ+ T cells (12). Thus, the ability of F. tularensis to induce activation of the PG synthetic pathway modulates host immune responses and provides a survival advantage for the bacteria.

PGE2 is a lipid signaling molecule derived from arachidonic acid (AA). AA is a short-lived metabolite in host cells as it is immediately converted into a bioactive eicosanoid or reincorporated into phospholipids. Activation of the canonical inducible PGE2 synthetic pathway by lipopolysaccharide (LPS) or zymosan typically requires the liberation of AA by group IVA phospholipase A2 (cPLA2α) (16). This liberated AA is oxidized by cyclooxygenase 2 (COX-2) to form PGH2 (17). PGH2 is isomerized to PGE2 by microsomal prostaglandin E synthase (mPGES1). We previously demonstrated that F. tularensis-induced PGE2 synthesis by macrophages requires cPLA2, COX-2, and mPGES1 (8, 18), yet we observed a temporal disconnect between cPLA2 activation and the time at which increases in COX-2 protein and PGE2 synthesis are observed. AA release and utilization by COX-2 are highly coupled events, and this apparent disconnect between cPLA2 activation and the increase in COX-2 protein led us to hypothesize that an alternative pathway utilizing other phospholipases was required for supplying AA to the inducible PGE2 biosynthetic pathway in F. tularensis-infected macrophages.
The canonical PGE\(_2\) biosynthetic pathway involves cPLA\(_2\) liberation of AA directly from phospholipid membranes. However, AA can be obtained via alternative lipid metabolism pathways using diacylglycerol (DAG). Phospholipase D (PLD) and phosphatidate phosphohydrolase (PAP) or phospholipase C (PLC) can liberate DAG from host phospholipid membranes. DAG can then be converted to AA by the diacylglycerol (DAGL) and monoacylglycerol lipase; MAGL, monoacylglycerol lipase; COX-2, cyclooxygenase 2; mPGES1, microsomal prostaglandin E synthase 1.

**MATERIALS AND METHODS**

**Inhibitors.** The inhibitors used in these studies were pyrrophenone (for cPLA\(_2\)), CAY10590 (for sPLA\(_2\)), Edelfosine (for phosphatidylinositol [PI]-PLC), IZL184 (for MAGL), and indomethacin (for COX-2) (all purchased from Cayman Chemical); propranolol (for PAP), RH80267 (for DAGL), MJ33 (for iPLA\(_2\)), U73122 (for PLC), and cytochalasin D (for actin polymerization) (all purchased from Sigma-Aldrich); and VU0155056 (for PLD1/2) (purchased from Avanti Polar Lipids, Inc.) and indomethacin (for COX-2) (all purchased from Sigma-Aldrich); and indomethacin (for COX-2) (all purchased from Sigma-Aldrich).

**Cell lines.** The murine macrophage cell line RAW 264.7 (ATCC TIB-71) was cultured at 37°C, 5% CO\(_2\), in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Atlas), 2 mM L-glutamine, 1 mM sodium pyruvate (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin (ATCC), and 50 μM β-mercaptoethanol (Fisher Scientific). RAW 264.7 cells were placed in supplemented antibiotic-free DMEM 24 hours prior to inoculation.

**Bacteria.** The Francisella tularensis live vaccine strain (LVS) (29684; American Type Culture Collection) was used in these studies. Bacteria were grown on chocolate agar at 37°C. Bacteria from lawn growth were isolated with a cotton swab and diluted into phosphate-buffered saline (PBS) to a concentration of 5 × 10\(^6\) CFU per ml. Bacteria were then diluted into PBS and used for inoculation of macrophages.

For experiments that tested the effect of inhibitors on bacterial growth in broth, F. tularensis LVS from chocolate agar was inoculated into brain heart infusion (BHI)-LB agar supplemented with IsoVitalex and grown overnight at 37°C with shaking at 200 rpm. One hundred sixty microliters of broth and 40 μl of overnight LVS culture were added to each well. Wells were then left untreated or treated with vehicle or inhibitor. The absorbance of each well was measured at 600 nm every 5 min over the course of 12 h at 37°C with 120 s of plate shaking before each measurement.

**Mice.** Six-week-old, 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 AALAC Louisiana State University Health Science Center (LSUHSC) animal medicine facilities. All work was approved by the LSUHSC Animal Care and Use Committee (ACUC).

**BMDM generation.** Murine bone marrow-derived macrophages (BMDMs) were generated by flushing the bone marrow from the femurs of female 6- to 10-week-old C57BL/6 mice and incubating these cells for 7
days in complete DMEM with 1929 fibroblast conditioned medium at 37°C and 5% CO₂. Twenty-four hours prior to inoculation, plates containing BMDMs were switched to antibiotic-free RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. On the day of inoculation, medium was removed and cells were washed twice with sterile phosphate-buffered saline (PBS; HyClone) to remove nonadherent cells. Cells were removed from the plate by incubation with 10 mM EDTA, pH 7.6, in PBS (8).

**Infection of cells and treatment with inhibitors.** RAW 264.7 cells or BMDMs were plated in a 96-well plate at a concentration of 1.5 × 10⁵/well and allowed to adhere for 2 h at 37°C. Cells were inoculated at a multiplicity of infection (MOI) of 200:1. Two hours postinoculation, extracellular bacteria were removed and killed by the addition of fresh medium containing 50 μM gentamicin for 1 h. Cells were then washed twice with fresh medium, and fresh, antibiotic-free complete medium was added. Plates were then incubated at 37°C for a further 20 h. Cells were pretreated 1 h prior to infection with inhibitor or vehicle (DMSO or H₂O) where indicated. Inhibitors were readded after each wash. Supernatants were collected 24 h postinfection and exposed to UV radiation for 10 min to inactivate any viable bacteria.

**Enzyme immunoassay.** PGE₂ was measured using a commercially available PGE₂ immunoassay kit (Assay Designs) according to the manufacturer’s instructions. Cytokine concentrations were determined using a Milliplex cytokine panel (Millipore) according to the manufacturer’s instructions.

**Arachidonic acid release assay.** The arachidonic acid release assay was performed as published with modification (21, 22). Briefly, 5.0 × 10⁵ RAW 264.7 cells or BMDMs were plated into a 24-well plate and incubated for 20 h in antibiotic-free, complete DMEM containing 1% FBS and 0.25 μCi/ml tritiated arachidonic acid ([³H]AA; Perkin-Elmer). Cells were then washed twice with serum-free, antibiotic-free complete RPMI 1640 containing 0.5 mg/ml bovine serum albumin (BSA) (Fisher Scientific). Five hundred microliters of antibiotic-free complete RPMI 1640 was added for the assay. Macrophages were pretreated with inhibitors for 1 h prior to inoculation with *F. tularensis* LVS or stimulation with LPS. Macrophages were then mock treated or inoculated with *F. tularensis* LVS. Two hours following treatment, culture medium was replaced with complete RPMI 1640 containing 50 μg/ml gentamicin. Supernatants and cell lysates were collected at indicated time points postinoculation, and AA release was detected by liquid scintillation (ScintiVerse BD cocktail; Fisher Scientific) and expressed as a ratio of radioactivity (counts per minute) in cell lysates versus total radioactivity present in well (cell lysates plus supernatant).

**Intracellular growth assay.** RAW 264.7 cells or BMDMs were plated and incubated as described above. Macrophages were gently lysed in 100 μl of 0.05% SDS in PBS for 5 min to release intracellular bacteria. Bacteria were enumerated by 10-fold serial dilutions in PBS and plating on chocolate agar plates. Plates were incubated at 37°C for 2 to 3 days until single colonies appeared.

**Immunoblot analysis.** BMDMs or RAW 264.7 cells were lysed in NuPAGE LDS sample buffer (Invitrogen). Protein concentration was determined by RC/DC protein assay (Bio-Rad). Equivalent amounts of protein were separated at 200 V for 45 min in MES buffer or [0.1% SDS, 1 mM EDTA, pH 7.7] or MES [50 mM 2-(4-morpholinio)-ethanesulfonic acid, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.3] running buffer was used to run the gel (Invitrogen). Proteins were separated at 200 V for 45 min in MES buffer or 55 min in MOPS buffer. Semidry transfer was performed on the gel for 45 min at 20 V onto a polyvinylidene difluoride (Immobilon-FL) membrane (EMD Millipore). The membranes were blocked for 1 h at room temperature with Li-Cor blocking buffer (Li-Cor Biosciences). Primary antibodies were diluted in 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with the blots overnight at 4°C with rocking. Secondary antibodies were diluted in 5% milk in TBST plus 0.01% SDS. The membranes were washed three times, 15 min each, with TBST while rocking after incubation with each antibody. The blot images were acquired with an Odyssey Western blot imaging system according to the manufacturer’s instructions. The fluorescent secondary antibody for the Odyssey imaging system is light sensitive; therefore, all manipulations with this antibody were protected from light. Densitometry was performed using Li-Cor Odyssey analysis software (Odyssey). Bands of interest were normalized to β-actin for statistical analysis. Antibodies were as follows: COX-2 and mPGES1 (both were purchased from Cayman Chemical); cPLA₂; Ser505 p-cPLA₂, DAGL, and PLD (purchased from Santa Cruz Biotechnology); lipin-1 and PAP2a (purchased from Novus Biologicals); β-actin (purchased from Sigma); and IRDye 680LT donkey (polyclonal anti-rabbit IgG secondary and blocking buffer (purchased from Li-Cor Biosciences). All antibodies were diluted 1:500 with the exception of β-actin and IRDye secondary antibody (1:20,000).

**shRNA knockdown.** Lentiviral short hairpin RNA (shRNA) delivery particles (Sigma-Aldrich) for *ppap2a* and *lipin1* were used to generate stable knockdown in the RAW 264.7 macrophage cell line. RAW 264.7 cells were grown to ~30% confluence, and 5 × 10⁵ cells were plated in a 6-well plate. Protamine sulfate (8 μg/ml) was added to increase virus binding. Ten microliters of virus was added to each well, and plates were incubated for 24 h at 37°C. Medium was then switched to fresh DMEM and incubated at 37°C for 24 h. Cells were placed under selection for 48 h by adding 5 μg/ml puromycin. Fresh selection medium was applied every 24 h until all cells in the virus control well had died. Cells were transferred to T25 flasks under selection.

**Cell death.** Macrophage cell death was determined by flow cytometry using the LIVE/DEAD Fixable Yellow dead cell stain kit (Molecular Probes) according to the manufacturer’s instructions. Mean fluorescence intensities (MFIs) of each group were compared.

**Reverse transcription-PCR (RT-PCR).** RNA was extracted with RNA Stat60 reagent (Tel-Test). RNA extract was treated with 1 U/μl RQ1 DNase (Promega), followed by cDNA synthesis using the Superscript cDNA synthesis kit. cDNA was amplified by PCR with primers specific to each gene. Primer sequences were obtained from the Harvard Primer Bank (Lpin1 and Hprt) or designed with the NCBI primer design tool (Ppp2a). Primer sequences were as follows: murine Lpin1, 5′-CATGCTTCCGAA AGTCCCTCA-3′ and 5′-GGTTATCTTTGCGTGCAACCT-3′; murine Ppp2a, 5′-TACAAGGCATACCCCTTCCTCA-3′ and 5′-ACCTCGAGAAGGCCCCACAT-3′; murine Hprt, 5′-GCTGACCTGCTGGTACCTTA A-3′ and 5′-TGTACATTACAGTAGCTTCTCAGTCTGA-3′.

**Statistical analysis.** GraphPad Prism 5.0 was used for analysis. Statistical significance was determined using a one-way analysis of variance (ANOVA) with a Dunnett posttest, P ≤ 0.05. Bar graphs display means of pooled experiments ± standard errors of the means (SEMs). All experiments were performed a minimum of three times, and the data presented are pooled from all experiments.

**RESULTS**

*Francisella tularensis* LVS induces dephosphorylation of cPLA₂.

We previously demonstrated that Ser505 cPLA₂ phosphorylation peaks and returns to baseline (2 hours after inoculation) before either an increase in COX-2 protein (~4 h after inoculation) or detectable biosynthesis of PGE₂ (~10 to 12 h after inoculation) in *F. tularensis* LVS-infected macrophages (18). Since cPLA₂ phosphatase was not measured more than 2 h after inoculation in our previous study, the possibility for a biphasic pattern of cPLA₂ phosphorylation that correlates with PGE₂ biosynthesis still remains. Therefore, cPLA₂ phosphatase in murine bone marrow-derived macrophages (BMDMs) was quantified 1, 4, 10, and 20 h after inoculation. In agreement with our previous report, cPLA₂ phosphatase peaked 1 h after inoculation with *F. tularensis* LVS. Furthermore, no increase in phosphorylation was detected later than 2 h after inoculation (Fig. 2a). LPS stimulation
cPLA₂, in contrast, resulted in prolonged elevation of cPLA₂ phosphorylation, up to 10 h posttreatment (Fig. 2a). Similar results were observed in the F. tularensis LVS-infected RAW 264.7 macrophage cell line (Fig. 2b).

It is possible for cPLA₂ to be active independently of Ser505 phosphorylation and liberate AA from the phospholipid membrane (23). To determine if cPLA₂ was still liberating AA that could be converted into PGE₂ by F. tularensis LVS-infected macrophages, we decided to monitor liberation of AA using a standard arachidonic acid release assay. In this assay, macrophages are incubated with radiolabeled arachidonic acid that becomes incorporated into host lipid membranes. After stimulation, radioactivity in the supernatant and cell fraction was measured at 1, 4, 10, and 20 hours postinoculation by liquid scintillation and expressed as percent release (n = 3). A significant increase from untreated group; *, significant reduction from F. tularensis LVS-infected group (P < 0.05). For clarity, the data from some experiments were presented in multiple figures. However, these experiments were run simultaneously and as such utilize the same controls (uninfected, infected, and COX-2 inhibitor treated). This includes panels c and d and Fig. 5a and b. Graphs are means of pooled experiments ± SEMs.
Inhibitors used in this study

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target</th>
<th>Conc (µM)</th>
<th>LVS growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJ33</td>
<td>iPLA₂</td>
<td>10</td>
<td>↔</td>
</tr>
<tr>
<td>Pyrophophene</td>
<td>cPLA₂</td>
<td>5</td>
<td>↔</td>
</tr>
<tr>
<td>CAT0590</td>
<td>sPLA₂</td>
<td>20</td>
<td>↔</td>
</tr>
<tr>
<td>D609</td>
<td>PC-PLC</td>
<td>30</td>
<td>↓</td>
</tr>
<tr>
<td>Edelfosine</td>
<td>PI-PLC</td>
<td>30</td>
<td>↔</td>
</tr>
<tr>
<td>Propranolol</td>
<td>PAP</td>
<td>100</td>
<td>↑</td>
</tr>
<tr>
<td>RHC80267</td>
<td>DAGL</td>
<td>25</td>
<td>↔</td>
</tr>
<tr>
<td>JZL184</td>
<td>MAGL</td>
<td>0.3</td>
<td>↔</td>
</tr>
<tr>
<td>VU0155056</td>
<td>PLD1/2</td>
<td>10</td>
<td>↓</td>
</tr>
<tr>
<td>Bromoerololac</td>
<td>iPLA₂</td>
<td>1</td>
<td>↓</td>
</tr>
<tr>
<td>U73122</td>
<td>PLC</td>
<td>5</td>
<td>↔</td>
</tr>
</tbody>
</table>

*The inhibitors used in the study are listed with their target, concentration, and effect on F. tularensis LVS growth in broth. Inhibitors that decreased LVS growth in broth were removed from the study.

Symbols: ↔, no change in bacterial growth; ↓, decreased bacterial growth; ↑, increased bacterial growth.

There was a significant release of AA by infected macrophages at 10 and 20 h after inoculation that was dependent upon cPLA₂ activity (Fig. 2c). However, the inhibition of AA release by cPLA₂ observed at these time points was only partial, suggesting that other enzymes may be contributing to AA-containing lipids secreted by F. tularensis LVS-infected macrophages. The results obtained with the RAW 264.7 macrophage cell line differed in that in addition to the later release of AA (10 and 20 h after inoculations), we also observed a significant increase in AA release at 1 h after inoculation (Fig. 2c). This difference between BMDMs and RAW 264.7 macrophages could be a result of higher baseline levels of c-PLA₂ in RAW 264.7 cells (Fig. 2b). Taken together, these data suggest to us that production of AA-derived lipid metabolites is partially dependent upon cPLA₂ activity. To more precisely address the contribution of cPLA₂-dependent liberation of AA that is used specifically for PGE₂ biosynthesis, we decided to use an “AA-addback” approach. We hypothesized that the addition of AA to culture supernatants following cPLA₂ inhibition would restore PGE₂ biosynthesis by F. tularensis-infected macrophages. BMDMs or RAW 264.7 macrophages were pretreated with inhibitors and then inoculated with F. tularensis LVS. Three hours after inoculation, 5 µM AA was added to the culture medium of macrophages. The anti-PGE₂ antibody used in the PGE₂ ELISA can cross-react with AA (0.1% as reported by Enzo product literature). Thus, to ensure that we were not just detecting AA in our AA-addback groups, we included an indomethacin-treated control. Indomethacin inhibits COX-1/2, blocking PGE₂ production. Exogenous AA restored LPS-induced PGE₂ synthesis when cPLA₂ was inhibited (data not shown) but was unable to restore PGE₂ biosynthesis when cPLA₂, or COX-1/2 was inhibited (Fig. 2d). Though these results do not definitively suggest that cPLA₂ does not supply AA to be converted into PGE₂, they do support our view that at the very least enzymes in addition to cPLA₂ are likely required for providing AA that is converted into PGE₂ in F. tularensis LVS-infected macrophages.

**Multiple lipases are required for PGE₂ biosynthesis in response to F. tularensis LVS.** To determine if other lipid-modifying enzymes could be contributing to F. tularensis-induced PGE₂ biosynthesis, we utilized a variety of small-molecule inhibitors to specifically inhibit the activities of the enzymes listed in Table 1. We have previously observed that some small-molecule inhibitors designed to inhibit eukaryotic enzymes can interfere with growth of F. tularensis LVS in broth. To determine if the inhibitors used in this study affected bacterial viability, we performed a 12-hour broth growth assay in the presence of selected inhibitors. We quantified bacterial growth as a function of broth turbidity by monitoring absorbance at 600 nm (Fig. 3a). The calcium-independent phospholipase A2 (bromoenol lactone) and phospholipase C (D609) inhibitors inhibited bacterial growth in broth and were eliminated from the study. To determine if the listed enzymes were involved in F. tularensis LVS-induced PGE₂ biosynthesis, we treated BMDMs or RAW 264.7 macrophages 1 h prior to inoculation. Twenty-four hours after inoculation, the concentration of PGE₂ in supernatants was determined. This preliminary
screen identified the requirement of PLD, DAGL, and PAP activity for *F. tularensis* LVS-induced PGE$_2$ synthesis by infected macrophages (data not shown). After the initial screen, we identified inhibitor concentrations for the PLD1/2 inhibitor VU0155056 (10 μM) and the DAGL/β inhibitor RHC80267 (25 μM) that retained specificity for the intended target (no reported off-target effects) and significantly reduced PGE$_2$ biosynthesis by infected macrophages (21, 24, 25). There are no specific inhibitors for the different phosphatidic acid phosphatase (PAP) enzymes found in macrophages; as such, we used the pan-PAP inhibitor propranolol (22, 26). Due to the nonspecific nature of propranolol, we used shRNA to confirm the results obtained with this inhibitor. The PAP shRNA results are presented below. The inhibition of cPLA$_2$, PAP, DAGL, or PLD activity 1 h prior to inoculation of BMDMs or RAW 264.7 macrophages with *F. tularensis* LVS resulted in significantly less detectable PGE$_2$ in the supernatants (Fig. 3b). Vehicle alone did not induce the synthesis of PGE$_2$ from macrophages (data not shown). To ensure that decreased PGE$_2$ synthesis was not due to increased macrophage cell death induced by the inhibitor, we determined the percentage of dead cells when inhibitors were added to uninfected or infected cells. Inhibitor treatment alone did not induce cell death of either BMDMs or RAW 264.7 macrophages (Fig. 3c). Furthermore, infection of inhibitor-treated BMDMs or RAW 264.7 macrophages did not result in increased cell death compared to untreated, infected macrophages. Thus, it seems unlikely that the changes in PGE$_2$ biosynthesis observed with inhibitor-treated macrophages were due to inhibitor-induced cell death of macrophages. In fact, propranolol treatment resulted in decreased BMDM cell death compared to untreated infected BMDMs and decreased PGE$_2$ synthesis. Finally, we do not see increased cell death in infected or infected and treated macrophages until 12 h after inoculation, which is after we begin to see the increase in COX-2 and PGE$_2$ levels (data not shown). These data suggest that the enzymes PAP, DAGL, and PLD contribute to PGE$_2$ biosynthesis by *F. tularensis* LVS-infected macrophages.

**Phospholipase activity is not required for bacterial entry.** Phospholipases can be involved in membrane rearrangement and phagocytosis (27, 28). Thus, the reduction in detectable PGE$_2$ by inhibitor-treated infected macrophages could be due to inhibition of bacterial uptake. We first wanted to determine if bacterial uptake was required for *F. tularensis* LVS-induced PGE$_2$ synthesis. The actin polymerization inhibitor cytochalasin D (CD) has been extensively used to examine F. tularensis entry into host macrophages as it inhibits *F. tularensis* LVS uptake into macrophages (29). Thus, to determine if macrophage uptake of *F. tularensis* is required for the induction of PGE$_2$ biosynthesis, we treated BMDMs with CD (10 μM) 1 h prior to inoculation. This concentration of CD did not affect macrophage cell death (data not shown). Inhibition of actin rearrangement blocked F. tularensis-induced PGE$_2$ biosynthesis by infected macrophages (Fig. 4a). Actin rearrangement is not required for normal inducible PGE$_2$ biosynthesis as LPS-induced PGE$_2$ biosynthesis is unaffected by CD treatment (30). These data suggest that macrophage uptake of *F. tularensis* LVS is required for induction of PGE$_2$ biosynthesis. Although growth within the macrophage is not required for the induction of PGE$_2$ biosynthesis (9), it is a key component of *F. tularensis* pathogenesis. Therefore, we wanted to determine the effects of phospholipase inhibition on *F. tularensis* uptake and intramacrophage growth. To determine if cPLA$_2$, PLD, DAGL, or PAP activity was required for BMDM or RAW 264.7 uptake of *F. tularensis* LVS or intramacrophage growth, macrophages were treated with inhibitors 1 h prior to inoculation. Cells were lysed 6 h (to determine uptake) or 24 h (to determine intramacrophage growth) after inoculation, and a CFU assay was performed. The inhibition of cPLA$_2$, PAP, DAGL, or PLD activity did not affect either bacterial entry or intramacrophage growth (Fig. 4b and c). These results suggest that *F. tularensis* entry into host macrophages is required for the induction of PGE$_2$ biosynthesis and that the activities of cPLA$_2$, PAP, DAGL, or PLD are dispensable for both bacterial uptake and intramacrophage growth.

**Exogenous arachidonic acid restores PGE$_2$ biosynthesis.** PLD binds phospholipid membranes to release phosphatidic acid. Thus, to determine if PLD was playing a role in the liberation of AA that could potentially contribute to PGE$_2$ biosynthesis, BMDMs were inoculated with *F. tularensis* LVS in the presence or absence of the PLD inhibitor. Twenty hours after inoculation, radioactivity in the supernatant and cell fraction was measured. We chose 20 h after inoculation as this time corresponds with PGE$_2$ synthesis in *F. tularensis*-infected macrophages. Inhibition of PLD results in reduced AA release compared to untreated infected macrophages (Fig. 5a). These data suggest that PLD is contributing to the pool of AA-derived lipids that are secreted by macrophages following *F. tularensis* LVS infection.

The precursor lipids that PAPs and DAGL modify can be dis-
Culture medium. PGE2 in the supernatant was quantified by enzyme-linked immunoassay (ELISA).

Three hours after inoculation, AA uptake and incorporation into phospholipid (PLD) or DAGL activity, not cPLA2, contributes AA that is converted into PGE2 during F. tularensis infection of macrophages.

**PAP inhibition decreases macrophage COX-2 protein levels during infection.** The regulation of COX-2 activity is at the transcriptional and posttranscriptional level with increases or decreases in cellular COX-2 protein levels correlating with overall cellular COX-2 activity (31, 32). Treatments that alter COX-2 protein levels are likely to influence signal transduction events that regulate either the expression of pgs2 (COX-2) or degradation of the transcript/protein. Inhibiting PAP reduces LPS-induced increases in COX-2 protein levels as well as PGE2 synthesis by macrophages through a reduction in cellular levels of DAG, a critical secondary messenger (33). Thus, we hypothesized that inhibiting PAP activity would reduce F. tularensis-induced increases in COX-2 protein levels. We also wanted to determine if cPLA2, PLD, or DAGL activity influenced COX-2 protein levels in F. tularensis-infected macrophages. To determine this, we pretreated RAW 264.7 macrophages with inhibitors followed by inoculation with F. tularensis LVS. We then assessed COX-2 protein levels in total cell extracts 20 h after inoculation by Western blotting analysis. The RAW 264.7 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. We demonstrated that inhibiting macrophage PAP activity led to decreased COX-2 protein levels while inhibition of cPLA2 activity led to increased COX-2 protein levels (Fig. 6). Because COX-2 controls the rate-limiting step in the biosynthesis of PGE2, a 50% reduction in COX-2 protein levels will impact the amount of PGE2 that can be synthesized by a cell. However, the reduction in COX-2 protein is unlikely to be sufficient to result in complete loss of PGE2 synthesis observed in propranolol-treated F. tularensis LVS-infected macrophages. Moreover, the observation that the inhibition of PAPs or cPLA2 results in altered COX-2 protein levels suggests that these enzymes may be involved in signaling pathways that contribute to macrophage function during F. tularensis infection.

**cPLA2 activity contributes to macrophage cytokine/chemokine production in response to F. tularensis LVS.** The observation that cPLA2 inhibition altered COX-2 protein levels in F. tularensis-infected macrophages suggested to us that cPLA2 could be contributing to other aspects of macrophage function in addition to COX-2 synthesis. To investigate this possibility, we decided to examine the contribution of phospholipase activity to macrophage cytokine/chemokine production in response to F. tularensis LVS. We performed a cytokine bead array to measure the concentration of cytokines, chemokines, and growth factors secreted by infected macrophages when the activities of cPLA2, PLD, or DAGL were inhibited. As a control for prostaglandin-dependent changes in F. tularensis-induced cytokine production, we inhibited COX-1/2 activity as well. BMDMs were left untreated or pretreated with inhibitors for 1 h prior to mock inoculation or inoculation with F. tularensis LVS at an MOI of 200:1. Twenty hours after inoculation, supernatants were analyzed for cytokines, chemokines, and growth factors. As has been previously reported (34), F. tularensis LVS infections of BMDMs resulted in increased granulocyte colony-stimulating factor (G-CSF), interleukin-10 (IL-10), IL-1β,

---

**FIG 5** Phospholipase D (PLD) and diacylglycerol lipase contribute arachidonic acid (AA) for PGE2 biosynthesis. (a) RAW 264.7 macrophages labeled with [3H]AA. Inhibiting cPLA2 or PAP did not affect uptake of [3H]AA (data not shown). Taken together, these data support our AA release results and suggest that macrophage PLD and DAGL activity, not cPLA2, contributes AA that is converted into PGE2 during F. tularensis infection of macrophages.
IL-6, IL-10, IL-12p70, IP-10, monocyte chemoattractant protein 1 (MCP-1), MIP-1α, MIP-1β, MIP-2, and RANTES (data not shown). The inhibition of cPLA2 activity significantly reduced the concentrations of the cytokines IL-6 and IL-1β and the chemokines IP-10, MCP-1, MIP-1α, and MIP-1β (Fig. 7). The inhibition of DAGL, PLD, or COX-1/2 did not significantly alter macrophage cytokine/chemokine secretion. We also analyzed cytokine/chemokine secretion from infected propranolol-treated BMDMs. Supernatants from these cells had elevated concentrations of numerous cytokines and chemokines. However, as demonstrated above, the treatment of infected macrophages with propranolol increases cell survival, making it likely that the observed increase in cytokine secretion was due to a greater number of live macrophages (data not shown). These data suggest that cPLA2 activity contributes to macrophage function independently of PGE2 synthesis, though the mechanism by which cPLA2 affects macrophage function during *F. tularensis* LVS infection is still under investigation.

**Lipin-1 and PAP2a are required for increases in COX-2 protein and PGE2 biosynthesis.** There are two distinct classes of PAP enzymes that possess phosphatidate phosphohydrolase activity. PAP1 enzymes are magnesium-dependent phosphohydrolases, while PAP2 enzymes are magnesium independent (35). Due to the nonspecific nature of propranolol (it inhibits both type 1 and type 2 PAPs [22]) and its targeting of β-adrenergic receptors (36), we decided to use shRNA to confirm the contribution of PAP enzymes to *F. tularensis* LVS-induced PGE2 synthesis. The best-characterized PAP enzymes are lipin-1 and PAP2a (LPP1). PAP2a is a 31-kDa, transmembrane, broad-specificity phosphohydrolase involved in lipid signaling (37). Lipin-1 is a 97-kDa protein involved in de novo lipid synthesis, lipid signaling, and adipocyte development and has a more specific substrate preference for phospha-
Host Lipases Alter Francisella-Infected Macrophages

August 2014 Volume 82 Number 8 iai.asm.org

3307

FIG 8 shRNA knockdown of macrophage PAP2a or lipin-1 reduced PGE₂ biosynthesis and COX-2 by Francisella tularensis-infected macrophages. (a) Steady-state mRNA was collected from wild-type (WT) RAW 264.7, knockdown, or nontarget shRNA cell lines, and knockdown was confirmed by RT-PCR. (b) RAW 264.7 knockdown or nontarget cell lines were inoculated with Francisella tularensis LVS at an MOI of 200:1. PGE₂ in the supernatant was quantified by enzyme-linked immunosorbent assay 24 hours after inoculation (n = 3). (c) Knockdown or nontarget cell lines were inoculated with Francisella tularensis LVS at an MOI of 200:1. Whole-cell lysates were collected 20 hours after inoculation, and COX-2 protein was quantified by Western blotting. Data are expressed as a fold change in COX-2 band intensity compared to its respective uninfected control (n = 4). (d) RAW 264.7 knockdown or nontarget cell lines were inoculated with Francisella tularensis LVS at an MOI of 200:1. TNF-α in the supernatant was quantified by enzyme-linked immunosorbent assay at 24 hours postinoculation (n = 3). (e) Knockdown or nontarget cell lines were inoculated with Francisella tularensis LVS at an MOI of 200:1. At 6 and 24 hours postinoculation, macrophages were lysed and intramacrophage bacteria were quantified (n = 3). An asterisk denotes a statistical difference compared to the nontarget (NT) shRNA-infected group (P ≤ 0.05). Graphs are means of pooled experiments ± SEMs.

tidic acid (PA) than does PAP2a (38). Using shRNA, we generated RAW 264.7 macrophage cell lines that were depleted of lipin-1 or PAP2a. We had difficulty quantifying PAP2a or lipin-1 protein levels in whole-cell extracts by Western blotting due to poor antibody specificity. Therefore, we, like others (39, 40), used mRNA transcript levels of lipin-1 and PAP2a to confirm knockdown of intended targets (Fig. 8a).

Lipin-1- and PAP2a-depleted cell lines were used to investigate the contribution of these enzymes to Francisella tularensis LVS-induced COX-2 protein levels and PGE₂ synthesis. We infected the RAW 264.7 shRNA knockdown cells with Francisella tularensis at an MOI of 200:1 and then quantified PGE₂ in supernatants and COX-2 protein levels 20 h after inoculation. Depletion of either lipin-1 or PAP2a resulted in a significant decrease in both COX-2 protein and detectable PGE₂ compared to control RAW 264.7 macrophages (nontarget shRNA) (Fig. 8b and c). These data confirm results that we obtained with propranolol and suggest that both lipin-1 and PAP2a enzymes contribute to Francisella tularensis-induced PGE₂ biosynthesis by infected macrophages. We decided to also examine the contribution of lipin-1 and PAP2a to the macrophage inflammatory response toward Francisella tularensis LVS by quantifying tumor necrosis factor alpha (TNF-α) concentration in supernatants from infected macrophages. The loss of lipin-1 or PAP2a resulted in decreased TNF-α secretion (Fig. 8d). The loss of PGE₂, COX-2, and TNF-α is unlikely to be due to either an alteration of bacterial uptake by macrophages or a lack of intramacrophage growth as there was no difference between control macrophages and depleted macrophages in these aspects (Fig. 8e). These results suggest that PAP2a and lipin-1 contribute to signal transduction events that regulate aspects of macrophage function during infection (Fig. 8e).

DISCUSSION

As seen with other intracellular bacterial pathogens, Francisella tularensis infection of macrophages leads to increased biosynthesis of PGE₂ by these host cells, and the synthesis of prostanoids is beneficial to the bacterium and detrimental to the host (12, 41–43). We also established that the activities of the major enzymes in...
the inducible PG biosynthetic pathway (cPLA2, COX-2, and mPGES1) are indispensable for *F. tularensis* LVS-induced PGE2 biosynthesis (8, 18). However, there is a temporal disconnect between the activation of cPLA2 (via phosphorylation) and COX-2 protein increases during *F. tularensis* LVS infection. This suggested to us that other enzymes besides cPLA2 might also contribute to the liberation of AA that is converted into PGE2, by *F. tularensis* LVS-infected macrophages. In this study, we demonstrate that the phospholipase PLD and the lipase DAGL likely supply AA to be converted into PGE2, by *F. tularensis* LVS-infected macrophages. Furthermore, we provide evidence that cPLA2, and the PAPs lipin-1 and PAP2a regulate macrophase-mediated inflammation during *F. tularensis* LVS infection. *F. tularensis* LVS AcpA has been reported to have phospholipase C activity (44). However, we have already demonstrated that this enzyme is not required for *Francisella*-induced PGE2 synthesis (9), and from the fact that none of the inhibitors used in this work inhibited either broth growth or intramacrophage growth, it seems unlikely that the results observed are due to inhibition of bacterial phospholipase activity. As such, this study provides evidence that modulation of macrophage lipid metabolism by *F. tularensis* LVS contributes not only to eicosanoid production but also to macrophage-mediated inflammation.

cPLA2 is considered the primary enzyme responsible for the liberation of arachidonic acid that contributes to eicosanoid production during inflammation; this includes the production of prostaglandins (45). We have previously demonstrated the requirement of cPLA2 for *F. tularensis* LVS-induced PGE2 synthesis; however, the observed temporal disconnect between cPLA2 activation and detectable PGE2 synthesis suggested that cPLA2 was not supplying AA that was being converted into PGE2 in these infected macrophages (18). Our results demonstrating that cPLA2 inhibition only partially blocks late AA release or that the addition of exogenous AA does not restore PGE2 biosynthesis by cPLA2-inhibited infected macrophages would support this model. There is evidence that arachidionate distribution within macrophages can influence the generation of eicosanoids, including PGE2 (46). During the Lands cycle, arachidonate is liberated by cPLA2 and reincorporated into phospholipids by coenzyme A (CoA)-dependent acyltransferases (47, 48). However, the addition of exogenous AA should overcome even this step and restore PGE2 biosynthesis, and this was not observed in cPLA2-inhibited macrophages. Further studies examining the cellular localization of cPLA2 during *F. tularensis* infection are required to better understand the spatial-temporal regulation of this enzyme and its contribution to *F. tularensis*-induced PGE2 biosynthesis. While exogenous AA did not restore PGE2 biosynthesis in *F. tularensis* LVS-infected macrophages when cPLA2 activity was inhibited, it did restore PGE2 biosynthesis when the activity of PLD or DAGL was inhibited. These results point to an alternative pathway of AA liberation involving PLD and DAGL in *F. tularensis* LVS-infected macrophages. Alternative enzymatic pathways that liberate AA for conversion into prostanooids have been noted in neurons (20) but, as far as we know, not in macrophages. The immediate lipid product generated by PLD or DAGL activity is not AA. However, 2-acylglycerol can be generated from the PLD-generated metabolite PA by the sequential activities of PAP and DAGL. 2-Arachidonoyl glycerol can also serve as a substrate for COX-2 (49) and be converted into PGE2-glycerol (PGE2-G). Therefore, it is possible that some of the PGE2 that we detect from *F. tularensis*-infected macrophages may actually be PGE2-G derived from 2-arachidonoyl glycerol. PGE2-G may have an enhanced anti-inflammatory capacity compared to that of PGE2, as it better inhibits LPS-induced NF-kB activity, and this may suggest why the induction of the inducible PGE2 synthesis pathway in macrophages by *F. tularensis* LVS so effectively inhibits T cell responses (50). Detailed mass spectrometric analysis of the lipid species present within macrophages during *F. tularensis* infection will be required to determine if PGE2-G is indeed present. Regardless, our data suggest that cPLA2 is likely not providing precursor metabolites to be converted into PGE2 in *F. tularensis* LVS-infected macrophages, while PLD and DAGL activities are.

Although our data suggest that cPLA2 is likely not contributing AA to be converted into PGE2 in *F. tularensis* LVS-infected macrophages, they do demonstrate that *F. tularensis* LVS induction of cPLA2 activity contributes to the inflammatory state of these infected macrophages. The inhibition of cPLA2 reduces *F. tularensis* LVS-induced cytokine responses while enhancing *F. tularensis* LVS-induced COX-2 protein levels. Interestingly, these responses are not recapitulated by inhibition of COX-1/2 activity, suggesting that these effects are mediated by eicosanoids not of the prostaglandin family. cPLA2-derived AA can contribute to the production of leukotrienes, which have immunomodulatory activity in macrophages (51, 52). If cPLA2 does contribute to macrophage proinflammatory responses, then the dephosphorylation of cPLA2 that we observed would likely abrogate these proinflammatory responses. At some time later, the activation of an alternative pathway for liberation of AA, specifically PLD and DAGL, would allow for the production of anti-inflammatory prostaglandins without the requirement for cPLA2 activity. This modulation of cPLA2, PLD, and DAGL activity by *F. tularensis* LVS could result in the inhibition of detrimental proinflammatory eicosanoids while still allowing for the production of anti-inflammatory eicosanoids in vivo, thus providing a survival advantage. The inhibition of cPLA2, PLD, and DAGL does not affect *F. tularensis* LVS intramacrophage growth, suggesting that the responses mediated by these enzymes are more important for altering the in vivo milieu to create a favorable environment for bacterial survival. Future experiments will be required to better dissect how different eicosanoids influence *F. tularensis* LVS infections in vivo and the possible roles for cPLA2, PLD, and DAGL in those responses. Regardless, our data suggest that alteration of cPLA2, PLD, and DAGL activity contributes to *F. tularensis* LVS alteration of macrophage function.

The PAP inhibitor utilized in many studies (33) indiscriminately inhibits the activity of all identified PAPs (22). Thus, we decided to determine which PAPs were involved in *F. tularensis*-induced PGE2 synthesis. Using shRNA to knock down two prominent PAP enzymes, PAP2a and lipin-1, we show that both of these PAPs regulate COX-2 protein levels and PGE2 biosynthesis in response to *F. tularensis*. Lipin-1 can function as a transcriptional coactivator of peroxisome proliferator-activated receptor alpha/gamma (PPARα/γ) as well as NFAT (53, 54) in addition to its PAP activity. Through its nuclear localization and transcriptional coactivation, lipin-1 has also been shown to be essential for adipocyte development, with mutations in the lipin-1 gene producing lipodystrophy and fatty liver disease (55). Given that members of the PPAR family of transcriptional coactivators have been linked to macrophage polarization, we are interested in determining if lipin-1 transcriptional coactivation activity is contributing
to the macrophage phenotypes seen in our study. Lipin-1 has also been demonstrated to play a role in the generation of lipid droplets (LDs) within human macrophages, where knockdown of lipin-1 reduced the size and number of LDs (56). The PAP activity of lipin-1 produces DAG, which can be used to generate triacylglycerols, which are central components of LD structure. However, DAG is a potent inducer of proinflammatory signaling in macrophages (57). Lipin-1 has been suggested to induce COX-2 protein levels via DAG formation; however, these studies used nonspecific inhibitors (33). Our results using lipin-1-depleted macrophages confirm the requirement of lipin-1 for increases in COX-2 protein levels. Future work will determine which activities, either cotransactivator or enzymatic, of lipin-1 contribute to F. tularensis-induced macrophage activation.

PAP2s are membrane-bound, lipid phosphohydrolases that have ectoactivity, i.e., their catalytic active site is present on the outside of cells. PAP2 ectoactivity regulates cell signaling by modifying the concentrations of lipid phosphates versus their dephosphorylated products; this includes the conversion of sphingosine-1 phosphate (SIP) to sphingosine and of lysophosphatidic acid (LPA) to monoacylglycerols. We demonstrate here that PAP2a contributes to F. tularensis-induced macrophage activation. Specifically, the loss of PAP2a reduces F. tularensis-induced COX-2, PGE₂, and TNF-α levels. The loss of PAP2a should increase the extracellular concentration of SIP and LPA. SIP and LPA are potent signaling lipids that can alter macrophage function (58). However, there is not a consensus on whether these lipid mediators have inflammatory or anti-inflammatory control over macrophage responses. SIP enhances macrophage Fcr receptor expression, phagocytosis, and LPS-induced cytokine responses (59–61). LPA stimulation results in calcium mobilization in human monocytes and inhibition of TNF-α production in LPS-treated mice (62, 63). In contrast, SIP can induce an anti-inflammatory macrophage phenotype, while SIP receptor 3 is required for recruitment of anti-inflammatory monocytes to atherosclerotic plaques (64, 65). The contrasting results suggest that the effects of SIP and LPA are context dependent. How PAP2a contributes to SIP and LPA function on macrophages is entirely unknown, though our data suggest that this enzyme does contribute to macrophage function. We are currently investigating levels of LPA and SIP in F. tularensis-infected macrophages and whether these levels are altered in our PAP2a-depleted macrophages. More work is clearly required to begin to decipher the complex relationship between lipid mediators, the enzymes that regulate their concentrations, and the effect that they have on immunologic responses both during normal function and during infections such as tularemia.

We believe that our results suggest an alternative pathway for supplying AA into the PGE₂ biosynthetic pathway during F. tularensis infection of macrophages. We demonstrate that liberation of AA to be converted into PGE₂ in F. tularensis-infected macrophages is likely not due to cPLA₂ activity but occurs via PLD and DAGL activity. cPLA₂ can also contribute to the generation of proinflammatory eicosanoids in addition to anti-inflammatory eicosanoids and may be contributing to the generation of proinflammatory cytokine responses during F. tularensis LVS macrophage infection. In addition, our data also identified the unexpected contribution of PAP2a and lipin-1 to F. tularensis-mediated macrophage activation. Thus, by targeting host lipid signaling pathways, F. tularensis is able to mute proinflammatory responses while maintaining anti-inflammatory eicosanoid production to promote its own survival. Future work will define the molecular mechanisms by which cPLA₂ and the PAPs lipin-1 and PAP2a regulate macrophage function. Understanding the contribution of lipid metabolic pathways toward inflammatory responses could potentially aid in the development of immunomodulatory therapies to treat F. tularensis infections and/or enhance vaccine development.

ACKNOWLEDGMENTS
We thank David McGee, Robert Chervenak, and Kenneth Peterson for helpful conversations and critical comments on the manuscript. This work was supported by the NIH/NIAID grant K22AI83373-2 and a Louisiana State University Health Sciences Center-Shreveport grant-in-aid.

Author contributions were as follows: A.R.N. and M.D.W. designed experiments; A.R.N., J.D.B., and A.M.B. performed experiments; A.R.N. and M.D.W. analyzed data and wrote the paper. There is no conflicting financial interest among the authors.

REFERENCES
Minimal requirements for murine resistance to infection with Francisella tularensis LVS. Infect. Immun. 64:3288–3293.


