Lipoxin A₄, a 5-lipoxygenase pathway metabolite, modulates immune response during acute respiratory tularemia

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ABSTRACT
Respiratory infection with Francisella tularensis (Ft) is characterized by a muted, acute host response, followed by sepsis-like syndrome that results in death. Infection with Ft establishes a principally anti-inflammatory environment that subverts host-cell death programs to facilitate pathogen replication. Although the role of cytokines has been explored extensively, the role of eicosanoids in tularemia pathogenesis is not fully understood. Given that lipoxin A₄ (LXA₄) has anti-inflammatory properties, we investigated whether this lipid mediator affects host responses manifested early during infection. The addition of exogenous LXA₄ inhibits PGE₂ release by Ft-infected murine monocytes in vitro and diminishes apoptotic cell death. Tularemia pathogenesis was characterized in 5-lipoxygenase-deficient (Alox5⁻/⁻) mice that are incapable of generating LXA₄. Increased release of proinflammatory cytokines and chemokines, as well as increased apoptosis, was observed in Alox5⁻/⁻ mice as compared with their wild-type counterparts. Alox5⁻/⁻ mice also exhibited elevated recruitment of neutrophils during the early phase of infection and increased resistance to lethal challenge. Conversely, administration of exogenous LXA₄ to Alox5⁻/⁻ mice made them more susceptible to infection thus mimicking wild-type animals. Taken together, our results suggest that 5-LO activity is a critical regulator of immunopathology observed during the acute phase of respiratory tularemia, regulating bacterial burden and neutrophil recruitment and production of proinflammatory mediators and increasing morbidity and mortality. These studies identify a detrimental role for the 5-LO-derived lipid mediator LXA₄ in Ft-induced immunopathology. Targeting this pathway may have therapeutic benefit as an adjunct to treatment with antibiotics and conventional antimicrobial peptides, which often have limited efficacy against intracellular bacteria. J. Leukoc. Biol. 101: 000–000; 2017.

Introduction
Tularemia is characterized by the absence of select protective proinflammatory responses, such as production of TNF, IL-1β, and IL-6 during the first 72 h of infection followed by development of a sepsis-like syndrome. This sepsis-like syndrome is characterized by an exaggerated cytokine "storm" or hypercytokinemia and the release of damage-associated molecular patterns, such as S100A9 and HMGB1 [1], which contribute to the extensive tissue pathology associated with tularemia. The mechanisms by which Ft initiates these host responses and, in so doing, establishes and maintains an intracellular niche to promote its own survival is incompletely understood.

We and others [2, 3] have postulated that the ability of Ft to establish a principally anti-inflammatory environment in the lung is a critical element of its host-evasive strategy. In a recent study, Ft was shown to induce the generation of both tDCs and Tregs with concomitant production of anti-inflammatory mediators (e.g., IL-10 and TGF-β) [3]. However, it is still unknown how this anti-inflammatory environment is “cultivated” by Ft during the early phase of the disease, how it affects the ability of bacteria to replicate exponentially, and how it influences the mortality rate of infected individuals. Recently, it was reported that a lethal infection with Ft drives recruitment of large numbers of immature myeloid cells and MDSCs to the lungs. These cells fail to mature and die, leading to subsequent necrotic lung damage, and subsequent host death [4]. However, which factors influence
the development and recruitment of these MDSCs during the early phase of the disease remains undetermined.

There is a growing body of evidence regarding the crucial role of lipid mediators, such as lipoxins and prostanooids in the immune-regulation of inflammation-associated diseases [5]. Lipoxins (e.g., LXA₄) are a class of anti-inflammatory/proresolving lipid mediators derived from lipoxygenase-mediated metabolism of AA [6]. LXA₄ is an endogenous lipid mediator that displays multilevel control of processes relevant in acute inflammation via specific and selective actions. Recently, a growing list of counter-regulatory actions have been attributed to lipoxins, which includes inhibition of proinflammatory cytokine and chemokine production and inhibition of chemotaxis, among others [7]. Pathogens take advantage of this regulatory pathway to establish a less-toxic environment in which to survive and replicate. During infection with Mycobacterium tuberculosis, Alox5⁻/⁻ mice, which are deficient in their capacity to generate LXA₄, exhibited elevated expression of IL-12 and IFN-γ, accompanied by lower bacterial burden in the lungs when compared with wild-type mice [7]. Although lipoxins have been reported to repress proinflammatory responses during the chronic phase of tuberculosis, their role in acute bacterial infections has yet to be defined.

Prostanoids (e.g., PGE₂) are lipid mediators generated from AA by the enzymatic action of cyclooxygenases [5]. During Mtb infection, macrophages infected with an attenuated strain activate PGE₂ production, which prevents necrosis and, instead, leads to apoptosis. In contrast, virulent strains of Mtb inhibit PGE₂ production and, hence, apoptosis. The balance between PGE₂ and LXA₄ determines the cellular fate of the macrophages during this chronic infection [8]. The finding that prostaglandin E synthase-deficient mice are more susceptible to Mtb infection suggests that induction of PGE₂ and the ensuing apoptotic death of macrophages are critical to limiting mortality associated with tuberculosis [9]. With regard to tularemia, PGE₂ released by Ft-infected macrophages blocks T cell proliferation [10] and generation of IFN-γ⁰ T cell targets [11] as well as stimulating IL-10 production by macrophages in vitro [12]; however, its role in regulating host cell-death programs is yet to be revealed.

Herein, we sought to elucidate the role of LXA₄, a metabolite of the Alox5 pathway, in the pathogenesis of respiratory tularemia. In wild-type mice, LXA₄ dampens the release of proinflammatory cytokines and chemokines, impedes recruitment of neutrophils to the site of pulmonary infection, and enhances susceptibility to disease, as evidenced by increased morbidity and mortality after i.n. infection with Ft LVS. In contrast, mice deficient for Alox5, which fail to produce LXA₄ and another 5-LO pathway metabolite, leukotriene B₄ (LTB₄), are more resistant to disease than their wild-type counterparts. However, administration of exogenous LXA₄ alone to Alox5⁻/⁻ mice significantly increased bacterial burden in the lungs and decreased the mean time to death (MTD) and survival of animals challenged with a lethal dose of Ft. Thus, this study reveals a major role for Alox5-dependent LXA₄ in the in vivo immune modulation of Ft infection and suggests this pathway is a potential target for therapeutic intervention in respiratory tularemia.

**MATERIALS AND METHODS**

**Bacteria**

Ft LVS (ATCC 29684; ATCC, Manassas, VA, USA) was kindly provided by Dr. Karen Elkins (U.S. FDA, Bethesda, MD, USA). Bacteria were cultured in BHIB, as described previously [3, 13]. In brief, a single colony picked from a MH broth-agar plate was used to initiate a 5-ml BHIB culture that was maintained for 12 h at 37°C while shaking at 220 rpm. These “starter” cultures were then used to inoculate (1:200) a 100-ml BHIB culture that was maintained for 12-16 h under the same culture conditions. Bacteria were harvested when cultures achieved an early log-phase A600nm OD of 0.2; at which point, CFUs per milliliter were determined by serial dilution and colony plating. Both fresh, bacterial cultures and frozen aliquots stored in liquid nitrogen elicited identical responses when used in the in vitro and in vivo studies.

**Mice**

Wild-type and Alox5⁻/⁻ mice both on a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in the specific pathogen-free Animal Resources Facility of Albany Medical College (Albany, NY, USA) or in the Trudeau Institute Animal Breeding Facility (Saranac Lake, NY, USA). All mice used in these experiments were between 6- and 8-weeks of age and were matched for age and sex within each individual experiment. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). The protocols were approved by the Institutional Animal Care and Use Committee of Albany Medical Center and the Trudeau Institute.

**Infection of mice**

All infection experiments used groups of 6–10 mice that were monitored for survival or were euthanized at designated time points postinfection. Before i.n. inoculation, animals were deeply anesthetized via intraperitoneal injection of a cocktail of ketamine (20 mg/ml) and xylazine (1 mg/ml) or through metered inhalation of 2.5% isoflurane. After dilation in sterile BHIB, either 250 CFU or 1 × 10⁷ CFU of Ft LVS in a volume of 20 μl was instilled dropwise (10 μl per nare). Actual dosages received by the mice were confirmed by colony plating. Sham-inoculated controls received an equal volume of sterile BHIB. Euthanized mice were necropsied at various times postinfection, and lungs were perfused with PBS and excised aseptically. The smaller lobe of the lung was used for preparation of lung homogenate for cytokine analysis and determination of bacterial burden, as described previously [3], and the remainder was used for isolation of single-cell suspensions for flow cytometry analysis. For survival experiments, mice were examined twice daily for morbidity and mortality for a period of 21 d, and the MTD and median survival was calculated for each group. MK-886 (5 mg/kg) and LXA₄ (2.5 μg/kg) were administered i.n. at various time points during infection.

**Immunohistochemistry**

Lung samples were processed as previously described [3]. In brief, lungs from sham-inoculated and Ft-infected mice sacrificed at different times postinfection were inflated by tracheal instillation of PBS before fixation in 10% neutral-buffered formalin. Tissues were processed using standard histologic methods to obtain 5-μm-thick paraffin sections that were stained following a standard H&E procedure.

**Isolation and differentiation of BMDMs**

Bone marrow progenitor cells were isolated from femurs to enrich for BMDMs, as previously described [3, 13]. Suspension cells (1 × 10⁶) were

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maintained in 10-cm² bacteriologic Petri dishes (Falcon; BD Biosciences, Franklin Lakes, NJ, USA) in BDM medium for 3–4 d. Cell monolayers were recovered using ice-cold PBS and scraping. Single-cell suspensions were used immediately or frozen in liquid nitrogen with 90% FBS and 10% DMSO for use in future experiments.

**Lipid body staining**

Cells were recovered from the BALF of Fl LVS-infected and sham-inoculated mice at 2 d postinfection. Cells (5 × 10⁵) were incubated for 1 h at 37°C with Nile Red (Sigma-Aldrich, St. Louis, MO, USA), in 1/10 000 PBS + 25 FBS from a stock solution of 0.1 mg/ml in acetone [14]. After incubation, cells were washed twice in Ca²⁺/Mg²⁺-free PBS, cytosplon onto slides, and fixed in 3.7% formaldehyde at room temperature for 10 min. Cells were visualized using a Leica TCS SP5 Inverted Laser Scanning Confocal Microscope and Leica Applications Suite Advanced Fluorescence Software version 2.6.0.7266.

**Ultraperformance LC-MS/MS for lipid mediator analysis**

Sample analysis was performed using an ACQUITY system (Waters, Milford, MA, USA) equipped with a tandem quadrupole mass spectrometer with an electrospray ionization source. Separation was performed on a C18-column (2.1 x 50 mm) packed with 1.7-µm particles (BEH C18; Waters). The mobile phases consisted of 1) water containing 0.1% (v/v) formic acid and 5.0% acetonitrile, and 2) acetonitrile containing 0.1% (v/v) formic acid and 5.0% water. The mobile phase flow rate was 0.1 ml/min. The gradient program started from 35% of phase 2, which was maintained for 2.2 min, was increased to 100% of phase 2 for 7.5 min, and was reversed to the initial composition of 35% phase 2 from 7.5 min to 9.0 min. Full loop injection was used to introduce 10 µl of sample into the system. All aspects of system operation and data acquisition were controlled using Empower v2 software (Waters) with automated data processing. PGE₂ EIA kit-monoclonal operation and data acquisition were controlled using Empower v2 software (Waters) with automated data processing. PGE₂ EIA kit-monoclonal (Cayman Chemical Company, Ann Arbor, MI, USA) was used to quantify the levels of PGE₂ and LXA₄ ELISA kit (MyBioSource, Inc., San Diego, CA, USA) was used to quantify the levels of LXA₄ in the in vitro cell culture supernatants or the tissue homogenates according to the manufacturer’s instructions wherever implied.

An LC-MS/MS method operating in MRM mode was developed that allows for the characterization and simultaneous quantification of LXA₄, LTB₄, PGF₂α, and 8-iso-PGF₂α-d₄ (internal standard). In vivo and in vitro media samples were spiked with 8-iso-PGF₂α-d₄ for the evaluation of the analytical procedure. LC-MS/MS analyses of media samples were performed using a system consisting of a Prominence HPLC with a refrigerated auto sampler and a SCIEX API 4000 (Shimadzu, Gaerloch, ON, Canada) triple-quadrupole mass spectrometer. The separation was carried out on a Synergi Hydro-RP column (250 x 2.0 mm inside diameter; Phenomenex, Torrance, CA, USA), which was eluted with a linear gradient of acetonitrile (solvent phase 2) (containing 0.1% formic acid) in aqueous 0.1% formic acid (solvent phase 1) with a flow rate of 0.2 ml/min. The gradient started initially at 10% phase 2, followed by a rise to 80% phase 2 at 11 min, to 100% phase 2 at 14 min, and was then re-equilibrated to a 10% phase 2 solvent for the next sample. LC-ESI/MS/MS was operated in MRM mode, with the precursor ion to product ion transitions from m/z 351/115 and 351/217 (lipoxin A₄), m/z 335/195 and 335/129 (leukotriene B₄), m/z 351/271 and 351/189 (PGF₂α), and 357/157 and 357/313 (8-iso-PGF₂α-d₄). The column effluent was introduced into the mass spectrometer using ESI in the negative-ion mode. The lower limit of detection for the standards LXA₄, LTB₄, and PGF₂α was estimated to be 0.1, 0.1, and 0.5 ng/ml, respectively.

**Cell death analysis by TUNEL and PI-Annexin V staining**

Lungs were perfused with sterile PBS and isolated from sham and Fl-infected mice. Single-cell suspensions were prepared as follows. Dissected lung tissue was incubated in 0.7 mg/ml collagenase IX (Sigma-Aldrich) and 10 U rDNase I (Roche Diagnostics, Mannheim, Germany). Following digestion for 30 min. at 37°C, the lung cells were passed through a cell strainer, collected by centrifugation (250 g. for 10 min.), and resuspended in assay buffer. These cells then were stained using the DeadEnd Fluorometric TUNEL System (Promega Corporation, Madison, WI, USA) according to the manufacturer’s instructions and analyzed by flow cytometry for evidence of apoptosis. PI-Annexin V staining was performed using a kit (BD Pharmingen; BD Biosciences) according to the manufacturer’s protocol. Flow cytometric analysis was performed on a BD FACSCan™ II cytometer (BD Biosciences), and data were analyzed using FlowJo software version 7.6.1 (Tree Star, Ashland, OR, USA).

**Genotyping**

The genotyping of the Alox5-/- mice was performed as described by the Jackson Laboratories (http://jaxmice.jax.org/strain/002778.html).

**Cytokine measurements**

Cytokine levels were measured in recovered culture supernatant using MILLIPLEX MAP Mouse Cytokine/Chemokine bead assay (EMD Millipore, St. Charles, MO, USA). Data were acquired and analyzed using the Magpix-Luminex instrument (Luminex, Austin, TX, USA) with the software xPONENT 4.1 (Luminex) and Masterplex 2010 (Hitachi Solutions America, San Bruno, CA, USA), respectively.

**Flow cytometry**

Single-cell suspensions were prepared from the lungs of sham and Fl-infected mice, as described above, and were surface stained with the Abs indicated below. To identify Treg, lung cells were first incubated with Abs directed against CD4 and CD25, followed by intracellular staining with anti-FoxP3 or an isotype control Ab. Multiparameter FACS analysis was performed on a BD FACSCan™ II cytometer (BD Biosciences), and data were analyzed using FlowJo software version 7.6.1 (Tree Star). The cells were gated based on forward and side scatter characteristics and with respect to specific surface markers. Specific cell populations are graphed as a percentage of the total cells recovered from uninfected or infected lungs at various times. In addition to presenting the percentage of specific cell types observed, the total number of cells was calculated. Mouse APC-anti-CD11c (clone N418), APC/Cy7-anti-CD11b (clone M1/70), PE-F4/80 (clone BM8), Pacific blue-anti-Gr-1 (clone RB6-8C5), FITC-anti-CD4 (clone GK1.5), APC-anti-CD25 (clone PC61), and PE-anti-FoxP3 (clone E13D3) Abs were purchased from BioLegend (San Diego, CA, USA).

**Statistical analysis**

Where applicable, all results are expressed as means ± sem from 2 or more independent experiments. Depending upon the distribution of the data set, comparisons between groups were made using a parametric ANOVA test with Bonferroni’s posttest or a nonparametric Kruskal-Wallis test with Dunn’s posttest. Differences between control and experimental groups were considered significant at α = 0.05 level.

**RESULTS**

**Fl elicits PGE₂ and LXA₄ during chronic and acute infection, respectively**

Woolard et al. [11] reported that, after initiation of respiratory tularemia, PGE₂ was induced above baseline at d 7 and beyond. Release of PGE₂ led to a blockade of T cell proliferation and promotion of a Th₂-like response, resulting in modulation of adaptive immunity during Fl infection [10] However, PGE₂ and LXA₄ also are known to modulate infection-induced inflammation. Thus, we wanted to evaluate whether these lipid mediators had a role during the early or acute phases (≤72 h) of tularemia with the potential to modulate innate immunity. Because lipid mediators are generated within lipid bodies [14], we tested whether such structures formed within the BALF cells isolated from...
from mice on d 2 postinfection. **Figure 1A** shows that cells from *Ft*-infected mice treated with the intracellular lipid staining dye Nile Red contained markedly increased numbers of lipid bodies when compared with those from sham-inoculated mice. In addition, BALF was isolated from mice at different time points after infection and was analyzed for the presence of PGE2 and LXA4. PGE2 did not rise above basal levels until d 5 postinfection (Fig. 1B). However, levels of LXA4 rose significantly soon after initiation of infection, peaking at d 2 and returning to baseline by d 5 (Fig. 1C). We also characterized the in vitro capacity of initiation of infection, peaking at d 2 and returning to baseline (Supplemental Fig. 1, LXA4. PGE2 did not rise above basal levels until d 5 postinfection and LXA4 (C) during the course of homogenates by mass spectrometry. Data are presented as the means of these lipid mediators were comparable. Given that LXA4 is associated with an anti-inflammatory environment and modulation of cell-death pathways [15], we explored whether LXA4 might reciprocally regulate PGE2 production during the course of *Ft* infection.

**Exogenous LXA4 down-regulates the release of PGE2 and inhibits cell death in *Ft*-infected BMDMs**

Because we found dissimilar kinetics suggesting an inverse relationship between the production of PGE2 and LXA4, we next tested whether LXA4 could directly regulate the production of PGE2 from BMDMs in response to *Ft* infection. As shown in **Fig. 2A**, addition of a physiologically relevant dose of exogenous LXA4 down-regulated the release of PGE2 by *Ft*-infected BMDMs. Whether LXA4 was provided 16 h before (data not shown) or concomitant with *Ft* infection, release of PGE2 by the BMDMs was diminished.

Because *Ft* infection can delay apoptosis to provide a more conducive environment for bacterial replication [15, 16], we investigated whether LXA4 inhibited apoptosis of *Ft*-infected BMDMs. As shown in **Fig. 2B**, the addition of exogenous LXA4 significantly inhibited cell death as assessed by TUNEL assay. Such inhibition of cell death might contribute to preserving the replicative niche of the bacterium during early phase tularemia.

**Alox5*/−*/ mice do not produce significant levels of LXA4 and LTB4 in response to *Ft* infection**

Because *Ft* modulates production of LXA4 during the course of infection, we further analyzed the effect of the absence of 5-LO in the host. Mice deficient in 5-LO were incapable of synthesizing LXA4 [17]. Before being used in the study, disruption of the *alox5* gene was confirmed by genotyping (Supplemental Fig. 2). To explore the effect of the absence of the 5-LO pathway on the induction of various lipid mediators in response to *Ft* infection, clarified lung homogenates collected from *Ft*-infected mice at various time points were analyzed for the presence of PGE2, LXA4, and LTB4 (Supplemental Fig. 3). The levels of PGE2 were not found to be significantly different in Alox5*/−*/ and Alox5*/+/+ mice at various time points before (time zero) or during the course of infection, although a trend toward increased production was observed by d 7 in the Alox5*/−*/ mice. Compared with wild-type mice, Alox5*/−*/ animals failed to produce significant amounts of LXA4 and LTB4 above baseline (Supplemental Fig. 3). In contrast, infection of wild-type mice elicited LXA4 during the acute phase of the disease, whereas LTB4 levels rose, with a kinetic similar to that observed for PGE2, later in the course of infection (d 5 and beyond).

**BMDMs from Alox5*/−*/ mice exhibit significantly elevated levels of PGE2 and increased apoptosis**

To evaluate the effect of the deficiency of the Alox5*/−*/ on the production of PGE2 by BMDMs, which have been reported to be a source of PGE2 [10] in response to *Ft* infection, we infected BMDMs isolated from wild-type and Alox5*/−*/ mice with *Ft*. The collected supernatants were examined for the presence of PGE2 by ELISA. As shown in **Fig. 3A**, significantly more PGE2 was produced in the absence of 5-LO than in its presence. Furthermore, 5-LO deficiency was associated with increased...
Absence of the 5-LO pathway increases production of proinflammatory cytokines and chemokines during *Ft* infection

Having established that Alox5 deficiency caused elevated levels of PGE2 and increased cell death in BMDMs, we next wanted to explore its effect on the levels of proinflammatory cytokines and chemokines elicited during the course of tularemia. Although they did not reach statistical significance, both TNF (Fig. 4A) and IL-6 (Fig. 4C) levels showed a trend toward increase in Alox5+/− BMDMs. However, a significant increase in IL-1β was seen in Alox5+/− BMDMs as compared with their wild-type counterparts (Fig. 4B). As BMDMs are not a significant source of IL-10, *Ft* infection failed to elicit this anti-inflammatory cytokine from cells of either genotype (Fig. 4D). Finally, significantly more KC and MCP-1 were produced at 24 h, but not at 6 h, by Alox5+/− BMDMs as compared with wild-type cells (Fig. 4E and F).

**Figure 2. Addition of exogenous LXA₄ ablates PGE₂ secretion by *Ft*-infected BMDMs.** (A) Wild-type C57BL/6 BMDMs (2.5 × 10⁵ cells/well) were seeded into 24-well plates and infected with *Ft* LVS at an MOI of 100. Exogenous LXA₄ (1 μM) was added at the time of infection to the cells. Supernatants were collected after incubation for 24 h at 37°C and were analyzed for the presence of PGE₂. ***P < 0.01, **P < 0.001. (B) Wild-type C57BL/6 BMDMs (1 × 10⁶ cells/well) were infected with *Ft* LVS at an MOI of 100. Exogenous LXA₄ (1 μM) was added to cells at the time of infection, and after incubation for 24 h at 37°C, the cells were stained with TUNEL to quantify the percentage of cells undergoing apoptosis. ***P < 0.001. Data are presented as the means ± SEM from 3 independent experiments. All results shown were subjected to 1-way ANOVA with Bonferroni’s posttest.

*Ft*-induced cell death (Fig. 3B), as evaluated by TUNEL staining. Next, we evaluated whether intracellular replication of LVS in the BMDMs was altered by Alox5 deficiency. As shown in the Supplemental Fig. 4, there were no significant differences in bacterial numbers between the wild-type and the Alox5−/− BMDMs at 6, 24, and 48 h. Regarding the relationship between LVS replication and the effect of LXA₄ on this process, we treated Alox5−/− BMDMs with exogenous LXA₄ and observed a significant reduction in bacterial burden at these same time points. Thus, as shown in Fig. 2A, the decreased production of PGE₂ by *Ft*-infected wild-type BMDMs exposed to exogenous LXA₄ may reflect decreased intracellular LVS replication. In addition to TUNEL staining, *Ft*-induced cell death in wild-type and the Alox5−/− BMDMs was quantified by PI-Annexin V staining because this method is a more specific means of characterizing apoptosis (Supplemental Fig. 5). At 24 h postexposure to *Ft*, a greater percentage of Alox5−/− BMDMs underwent apoptosis than their wild-type counterparts (45 vs. 20%, respectively; a 1.25-fold change). At 48 h, the difference was 39 vs. 14%, a 1.79-fold change. However, by 72 h of in vitro culture, no significant difference was observed between the 2 genotypes, but cell death, even in uninfected controls, rose to 40% by that point.

**Figure 3. Alox5−/− BMDMs secrete significantly more PGE₂ in response to *Ft* infection.** (A) Wild-type (Alox5+/+) and Alox5−/− C57BL/6 BMDMs (2.5 × 10⁵ cells/well) were seeded into 24-well plates and infected with *Ft* LVS at an MOI of 100. Cells were incubated at 37°C, and supernatants were collected at various times and analyzed for the presence of PGE₂. ***P < 0.001. (B) Wild-type (Alox5+/+) and Alox5−/− C57BL/6 BMDMs (1 × 10⁶ cells/well) were seeded into 6-well plates and infected with *Ft* LVS at an MOI of 100 and were incubated for 24 h at 37°C. The cells were stained with TUNEL to quantify the percentage of undergoing apoptosis. ***P < 0.001. Data are presented as the means ± SEM from 3 independent experiments. All results shown were subjected to One-way ANOVA with Bonferroni’s Posttest. Asterisks immediately above columns indicate a significant difference compared with their corresponding control while asterisks above a bracket indicate a significant difference between 2 experimental groups.
Increased PGE₂ production and cell death is observed in Ft-infected Alox5⁻/⁻ mice

After defining a crucial role for the 5-LO pathway in regulating PGE₂ production by, and cell death of, BMDMs, we extended our findings to the murine model of respiratory tularemia. As shown in Fig. 5A, Ft-infected Alox5⁻/⁻ mice produced significantly greater amounts of PGE₂ by d 6 postinfection as compared with their wild-type counterparts. Cell death was quantified by the TUNEL assay and was found to be significantly greater in the Ft-infected Alox5⁻/⁻ mice by d 6 as compared with the wild-type mice (Fig. 5B). Although there was a trend toward greater TUNEL positivity in the lungs of Alox5⁻/⁻ mice on d 2, the difference compared with wild-type animals did not reach statistical significance. Importantly, the observations made in vivo in Ft-infected mice were consistent with the in vitro findings.

We also analyzed the levels of proinflammatory cytokines and chemokines in the Ft-infected, wild-type and Alox5⁻/⁻ mice. As shown in Fig. 6A–C, we observed significantly greater levels of proinflammatory mediators, such as TNF, IL-1β, and IL-6, in the Alox5⁻/⁻ mice on d 6 postinfection as compared with their wild-type counterparts. The levels of IL-10 were found not to be significantly greater between the 2 groups, suggesting that cytokines modulated by the 5-LO pathway and IL-10 are independently regulated (Fig. 6D). Consistent with the observations made in vitro, we found significantly elevated levels of KC and MCP-1 in the Alox5⁻/⁻ mice at d 6 postinfection compared with their wild-type counterparts (Fig. 6E–F).

Figure 4. Increased proinflammatory cytokine and chemokine production is observed by Ft-infected Alox5⁻/⁻ BMDMs. (A-F) Wild-type (Alox5⁺⁺) and Alox5⁻/⁻ C57BL/6 BMDMs (2.5 × 10⁵ cells/well) were infected with Ft LVS at an MOI of 100. Supernatants were collected 24 h postinfection and were assayed for the presence of various cytokines and chemokines with the Luminex assay. *P < 0.05, **P < 0.01, ***P < 0.001. Data are presented as the means ± SEM from 3 independent experiments. All results shown were subjected to 1-way ANOVA with Bonferroni’s posttest. Asterisks immediately above columns indicate a significant difference compared with its corresponding control, whereas asterisks above a bracket indicate a significant difference between 2 experimental groups.

Alox5⁻/⁻ mice possess lower bacterial burdens in various organs

We hypothesized that induction of LXA₄ by Ft creates an anti-inflammatory environment that supports exponential bacterial replication during the early phase of tularemia. Therefore, we next explored whether the bacterial burden in various organs of Ft-infected Alox5⁻/⁻ mice were significantly different from their wild-type counterparts. Both wild-type and Alox5⁻/⁻ mice were infected with Ft, and at various time points, the lungs, liver, and spleen were examined for the presence of bacteria (Fig. 7). Reduced bacterial burdens were observed in the lungs of Alox5⁻/⁻ mice at d 5 and 7 postinfection (Fig. 7A). However, we did not observe significant differences between the groups on d 1 and 3 (Fig. 7A). These results are perhaps not surprising because the wild-type and Alox5⁻/⁻ mice do not distinguish themselves with regard to the kinetics of apoptosis during the early (≤72 h) phase of disease. Thus, the size of the replicative niche in both groups is similar, and differences in bacterial burden would not be anticipated. Significantly reduced bacterial numbers were also observed in the liver of Alox5⁻/⁻ mice on d 5 and 7 as compared with their wild-type counterparts (Fig. 7B). However, in the spleen, although a trend toward decreased bacterial burden was observed on d 5, significantly reduced numbers of bacteria were only seen in the Alox5⁻/⁻ mice on d 7 postinfection.

Because the activation and migration of a variety of leukocytes into the lungs profoundly influences the overall outcome of respiratory tularemia [18], we evaluated the role of 5-LO activity...
MK-886, an inhibitor of the 5-LO pathway, lowers bacterial burden

MK-886 is a potent and selective inhibitor of the 5-LO pathway that has been used in various infectious-disease models to investigate the role of LXA₄ in pathogenesis. We treated *Ft*-infected mice with MK-886 at the time of infection and at 24-h intervals postinfection. To confirm the in vivo effect of MK-886, lung homogenates processed from these infected mice were tested for the presence of LXA₄. In the mice treated with MK-886, levels of LXA₄ were found to be significantly reduced compared with *Ft*-induced peak levels at 2 and 3 d postinfection (Fig. 8A). Next, we analyzed the lungs of these mice for bacterial burden at different time points postinfection. Bacterial burden in the lungs of MK-886–treated mice showed a marked decrease in the numbers of bacteria by d 5 postinfection (Fig. 8B). These observations are consistent with the lower bacterial burdens observed in the Alox5⁻/⁻ mice that are deficient in their capacity to produce LXA₄.

We also analyzed the lung homogenates from both untreated and MK-886–treated mice for the presence of various proinflammatory cytokines and chemokines. MK-886–treated mice showed a trend toward increased production of TNF, IL-1β, and IL-6 on d 5 postinfection (Supplemental Fig. 7A–C). No difference was observed in the levels of IL-10 between the 2 groups (Supplemental Fig. 7D). However, as was found in the lungs of *Ft*-infected Alox5⁻/⁻ mice, the levels of KC and MCP-1 were found to be significantly higher in the MK-886–treated mice than in wild-type mice (Supplemental Fig. 7E–F).

5-LO activity is associated with greater morbidity and mortality during *Ft* infection

Based upon the collective findings detailed above one would predict that LXA₄ activity might alter the susceptibility of mice to lethal challenge with *Ft*. To test this notion, wild-type and Alox5⁻/⁻ mice were infected i.n. with 250 or 1 × 10³ CFU of *Ft* and their relative susceptibility was evaluated on the basis of MTD and the cumulative proportion of mice surviving in the different experimental groups. When both groups of mice were infected with 250 CFU of *Ft*, a significant difference was observed in the susceptibility of wild-type and Alox5⁻/⁻ mice (Fig. 9A). Unlike wild-type mice, in which only 37.5% survived to lethal challenge with *Ft*, 87.5% of the Alox5⁻/⁻ mice survived. The MTD was found to be d 12 for the wild-type group, whereas it was ≥21 d (at which point the experiment was terminated) for the Alox5⁻/⁻ group (P < 0.05) (Fig. 9A). Weight loss for the 2 groups of mice was calculated for the course of infection, and no significant difference was observed (Fig. 9B). When both groups of mice were infected with 1 × 10³ CFU of *Ft*, a significant difference was observed in the susceptibility of wild-type and Alox5⁻/⁻ mice (Fig. 9B). Unlike wild-type mice, in which only 37.5% survived the *Ft* challenge, 87.5% of the Alox5⁻/⁻ mice survived. The MTD was found to be d 12 for the wild-type group, whereas it was ≥21 d (at which point the experiment was terminated) for the Alox5⁻/⁻ group (P < 0.05) (Fig. 9A). Weight loss for the 2 groups of mice was calculated for the course of infection, and no significant difference was observed (Fig. 9B). When both groups of mice were infected with 1 × 10³ CFU of *Ft*, wild-type mice started dying at 8 d postinfection; all were dead by d 14, and the MTD was 9.5 d. The Alox5⁻/⁻ mice started dying on d 9; all were dead by day 17, and the MTD was d 11 (data not shown). Thus, it is likely the differences in susceptibility between the 2 groups were masked by the high virulence of the pathogen at a 1 × 10³ CFU challenge dose.

Next, we evaluated whether the increased survival and lower bacterial burden observed in the Alox5⁻/⁻ mice was associated

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**Figure 5.** Alox5⁻/⁻ mice secrete significantly higher levels of PGE₂ in response to *Ft* infection. (A) Wild-type (Alox5⁺/⁺) and Alox5⁻/⁻ C57BL/6 mice were infected with 1000 CFU of *Ft* LVS, and total lung cells were isolated from individual mice at different times. The lung homogenates were analyzed for the presence of PGE₂ by ELISA. *P < 0.05, *P < 0.01 (Student’s t test) (B) Single-cell lung suspensions isolated from wild-type and Alox5⁻/⁻ C57BL/6 mice were infected with 1000 CFU of *Ft* LVS and were stained with TUNEL to quantify the percentage of cells undergoing apoptosis. **P < 0.01, ***P < 0.001, ****P < 0.0001 (Student’s t test). Data are presented as the means ± SEM from 3 independent experiments. All results shown were subjected to 1-way ANOVA with Bonferroni posttest. Asterisks immediately above a bracket indicate a significant difference between 2 experimental groups.

in cellular recruitment. Flow cytometric analysis of cells recovered from the lungs of wild-type and Alox5⁻/⁻ mice showed differences in the frequency and the total numbers of CD11b⁺Gr-1⁺ cells on d 3 postinfection (Supplemental Fig. 6). Alox5⁻/⁻ mice showed (means ± SEM) of 33.04 ± 4.53% CD11b⁺Gr-1⁺ cells as compared with 19.8 ± 2.2% observed in the wild-type mice (P < 0.05) (Supplemental Fig. 6A). The total number of CD11b⁺Gr-1⁺ cells recruited into the *Ft*-infected lungs was also calculated, and the same trend was observed: greater recruitment in the absence of 5-LO by d 3 postinfection (Supplemental Fig. 6B). Another cell-type reported to increase in numbers in the lungs of *Ft*-infected mice are Tregs [3]. To investigate whether the absence of the 5-LO pathway had any effect on Treg lung cells from *Ft*-infected wild-type and Alox5⁻/⁻ mice were analyzed for the presence of Tregs and no differences in the frequency or total number of Tregs were observed by flow cytometry or average numbers of Treg/field, as determined by immunohistological staining of lung tissue sections with fluorescently labeled anti-FoxP3 Ab (data not shown).
with less-severe pathology. To characterize the histopathology in vivo, both groups of mice were either sham-inoculated or infected with *F. tularensis* strain Schu S4, and at various times postinfection PBS-perfused lungs were harvested and H&E-stained sections were evaluated for histopathologic changes. As shown in the Supplemental Fig. 8, we observed comparable evidence of general inflammation in the lungs of wild-type and Alox5−/− mice beginning on d 2, but by day 4, and especially by d 6, the Alox5−/− mice exhibited significantly less tissue pathology. Notably, this difference in the severity of the histopathologic changes is consistent with the greater survival of *Ft*-infected Alox5−/− mice as compared with their wild-type counterparts. Collectively, these findings point to a robust phenotypic difference in the course of *Ft* infection in Alox5+/+ and Alox5−/− animals.

Administration of exogenous LXA4 to Alox5−/− mice renders them more susceptible to infection
To evaluate mechanistically whether the enhanced resistance of the Alox5−/− mice to infection with *Ft* was attributed to the absence of LXA4, we complemented this defect in *Ft*-infected Alox5−/− mice by providing exogenous LXA4 at the time of infection and at 24-h intervals postinfection. As observed in Fig. 10A, the Alox5−/− mice receiving exogenous doses of LXA4 mimicked the wild-type phenotype and exhibited enhanced mortality (50% survival) as compared with the untreated Alox5−/− mice (88% survival). In contrast, only 38% of the infected wild-type mice survived. MTD was calculated to be d 11 for the wild-type group, d 15 for the Alox5−/− + LXA4 group, and was undefined for the untreated Alox5−/− group because not all the animals from this latter group died (Fig. 10A). We also evaluated the bacterial burden in the lungs of all 3 experimental groups at different points postinfection (Fig. 10B). The bacterial burden in the lungs of Alox5−/− mice receiving exogenous LXA4 was comparable to the wild-type mice and significantly greater than that found in the untreated Alox5−/− animals.

DISCUSSION
It has been suggested that rapid production of proinflammatory mediators is critical for the initial control of *Ft* and a variety of other bacteria. Lung inflammation in response to infection with *Ft* LVS and strain Schu S4 is observed as early as 24–48 h postinfection and consists of both cellular (e.g., PMNs and macrophages) and soluble (e.g., IL-10, IL-17, MCP-1, KC, and TGF-β) components. However, proinflammatory mediators, such as TNF, IL-1β, IL-6, IL-12p70, and IFN-γ, are not detected at significant levels until ≥72 h postinfection [3, 13]. The absence of these crucial “protective” cytokines during early infection allows unrestricted bacterial growth, leading to high pathogen burden in sensitive pulmonary and extrapulmonary organs. Later, up-regulation of protective immune defenses is “too little, too late” because the tissue damage that drives development of sepsis during late-phase tularemia has already occurred. Our group has previously reported the presence of tDCs and Treg during the early phase of tularemia, which sustains an anti-inflammatory milieu via mediators such as IL-10 and TGF-β [3]. Herein, we report yet another defense mechanism in the...
host evasive “armamentarium” of Ft: the rapid induction of LXA4 within 24 h of infection. Induction of LXA4 during early infection, which has anti-inflammatory effects, contributes to a local environment that facilitates Ft evasion of killing by innate immune cells and their exponential replication within such cells. Alveolar macrophages and neutrophils are a major source of LXA4 during bacterial infections [19]. During the first 72 h of tularemia, most cells (almost 70%) infected with Ft LVS and Schu S4 are the alveolar macrophages [20], and after d 3, almost 50% of the infected infiltrating cells are neutrophils [20]. Hence, it is conceivable that Ft-induced LXA4 is derived from these infected alveolar macrophages and neutrophils. However, during Ft infection, the absence of 5-LO did not significantly affect the levels of IL-10, suggesting the anti-inflammatory effects of LXA4 are independent of IL-10. To our knowledge, this is the first report of the regulation of immune responses via the 5-LO pathway and LXA4 in the context of an acute bacterial infection.

Lipoxins (i.e., LXA4) and prostanoids (i.e., PGE2) are eicosanoids, a family of molecules derived from the catabolism of AA [17, 21, 22]. The role of PGE2 during Ft infection has been reported previously [10–12]. Woolard et al. [11] showed that, depending on the route of infection, there are significant differences in the production of PGE2 in Ft-infected mice, as measured in the BALF between d 7 and 14. During i.n. infection,

Figure 7. Alox5 deficiency is associated with reduced bacterial burdens in the various organs during the course of Ft infection. Wild-type and Alox5−/− C57BL/6 mice were inoculated i.n. with 1000 CFU of Ft LVS. At the times indicated, mice were sacrificed and homogenates of the lungs, liver, and spleen were plated for determination of bacterial burden. *P < 0.05, **P < 0.01. Results shown are the means ± sem and are representative of 3 independent experiments (n = 4 mice/time point; 12 mice total). Asterisks immediately above columns indicate a significant difference compared with its corresponding control, whereas asterisks above a bracket indicate a significant difference between 2 experimental groups.

Figure 8. The inhibitor of the 5-LO pathway, MK-886, results in lower bacterial burden. (A) Wild-type C57BL/6 mice were infected i.n. with Ft LVS alone or with doses of MK-886 (5 mg/kg) at the time of infection and, thereafter, every 24 h. The infected mice were sacrificed at different times as shown and were analyzed for the presence of LXA4 by ELISA. (B) The lung homogenates were prepared and plated for determination of bacterial burden. *P < 0.05, **P < 0.01. Results shown are the means ± sem and are representative of 2 independent experiments (n = 6 mice/time point; 12 mice total). Asterisks immediately above columns indicate a significant difference compared with its corresponding control, whereas asterisks above a bracket indicate a significant difference between 2 experimental groups.

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significant increases in PGE2 correlated with lower numbers of IFN-γ+ T cells in the lung and spleen but increased amounts of Th17 cells. In addition to blocking T cell proliferation [10] and generating IFN-γ+ T cells [11], PGE2 may stimulate Ft-infected macrophages in an autocrine/paracrine fashion to drive the production of a yet unknown, small soluble factor that facilitates IL-10 production by macrophages in vitro [12]. Curiously, the in vitro temporal relationship of Ft-induced PGE2 preceding IL-10 release was the reverse of what was observed during natural infection reported herein and elsewhere [3].

In contrast to PGE2, the role of 5-LO deficiency, and more specifically LXA4, has been largely unexplored in tularemia pathogenesis. LXA4 binds receptors on PMNs, inhibiting their chemotaxis and degranulation and inhibiting PGE2, a proapoptotic agonist, in the context of wound healing and a variety of infectious diseases [5, 9, 23]. For example, induction of LXA4 and the simultaneous inhibition of PGE2 contributes to the virulence of Mtb and the bacterium’s ability to evade innate host defenses [5]. For its part, LXA4, a potent antipapoptotic and pronecrotic agonist, causes irreversible mitochondrial damage, thus resulting in necrosis, which releases Mtb into the surrounding tissue, facilitating dissemination of the pathogen. In addition, it has been reported that impaired LXA4 biosynthesis is associated with exaggerated PMN recruitment [24]. As has been reported by our group and several others [4, 18, 20], excessive neutrophil recruitment and their subsequent death via necrosis contributes to severe tissue damage and death. On d 3, the absence of a functional 5-LO pathway resulted in a statistically significant increase in both the percentage and total number of neutrophils in the lungs of Ft-infected mice. Therefore, the decrease in LXA4 observed in wild-type mice starting on d 3 is consistent with the increased recruitment of neutrophils observed at this same point during active infection.

Godson et al. [25] suggested that LXA4 also promoted phagocytosis of apoptotic neutrophils by macrophages, thereby enhancing disease resolution. In contrast, despite the

Figure 9. 5-LO deficiency enhances host resistance to Ft infection.
Wild-type (Alox5+/+) and Alox5−/− mice were inoculated i.n. with 250 CFU of Ft LVS (A and B, respectively) and were monitored for morbidity and mortality, and weight loss (A and B, respectively). Results are expressed as Kaplan-Meier curves, and P values were determined using the log-rank test. The results shown are representative of 2 independent experiments (n = 8 mice/group; 16 mice total).

Figure 10. Exogenous LXA4 renders the Alox5−/− mice more susceptible to Ft infection. (A) Wild-type (Alox5+/+) and Alox5−/− mice were infected i.n. with 250 CFU of Ft LVS. Alox5−/− mice were either untreated or received doses of LXA4 (2.5 μg/kg) at the time of infection and, thereafter, every 24 h for 3 d. Following initiation of infection, all mice were monitored for morbidity and mortality over 21 d. (B) Lung homogenates were prepared and plated for determination of bacterial burden. *P < 0.05, **P < 0.01. Results are expressed as Kaplan-Meier curves, and P values were determined using the log-rank test. The results shown are representative of 2 independent experiments (n = 8 mice/group; 16 mice total). Asterisks immediately above columns indicate a significant difference compared with its corresponding control, whereas asterisks above a bracket indicate a significant difference between 2 experimental groups.
ability of \( F.t \) to induce rapid production of LXA\(_4\), it does not engender disease resolution. Instead, we postulate that, during acute phase tularemia (\(<72\) h), LXA\(_4\), with known antiapoptotic properties because of its ability to inhibit PGE\(_2\), delays apoptosis. However, cells engorged with \( F.t \) do eventually undergo programmed cell death and, because of defective efferocytosis, as elegantly demonstrated by Mares et al. [26], those cells subsequently disintegrate through a process of secondary necrosis that ultimately drives hypercytokinemia and tissue damage. Regarding the kinetics of LXA\(_4\) and PGE\(_2\) production, our finding that \( F.t \)-infected PGE\(_2\) levels only increase above baseline after LXA\(_4\) levels return to baseline implicates this “crossover” point as the transition between delayed apoptosis and secondary necrosis, which occurs around 72 to 96 h postinfection. In the absence of LXA\(_4\) and in the face of extensive secondary necrosis during late-phase tularemia, it is understandable that disease resolution fails to occur—by this point in the disease process, the normally protective host responses are too little, too late. Importantly, Schwartz et al. [16] demonstrated that both \( F.t \) LVS and Schu S4 can delay apoptosis and prolong the life span of human PMNs. Our findings clearly demonstrate a role for LXA\(_4\) in delaying the death of \( F.t \)-infected, murine macrophages in vitro and during murine pulmonary infection in vivo. It will be interesting to determine whether the action of LXA\(_4\) underlies delayed apoptosis in \( F.t \)-infected human PMNs. Yet to be discovered is what bacterial factors are responsible for the induction of LXA\(_4\) in the context of \( F.t \) infection.

\( A.A \) is a common substrate for 5-LO, which yields lipoxins and leukotrienes, and for cyclooxygenases, which yield prostaglandins as part of the COX1/2 pathway. Studies have suggested that the blockade of the COX pathways by anti-inflammatory drugs augments the metabolism of AA through the 5-LO pathway [27]. However, others have reported that, although inhibition of COX-2 shifts AA metabolism toward 5-LO activation, the inverse was not the case, implying that these pathways do not proceed in parallel [28]. In our study, we used the inhibitor MK-886 [29–32] to investigate the effect of blocking the 5-LO pathway during \( F.t \)-infection. Reduced bacterial burden and significantly increased levels of chemokines were observed in the presence of MK-886, which corroborated observations made using mice deficient for 5-LO. It has also been reported that 5-LO activity negatively regulates Th1 responses during \( B.a \) infection in vivo. In the absence of 5-LO pathway, increased expression of the proinflammatory mediators IL-12, IFN-\( \gamma \), and iNOS was observed [33]. In addition, the 5-LO-deficient mice displayed lower bacterial burden in the spleen and liver and demonstrated enhanced resistance to infection with \( F.t \). Similarly, during \( P.b \) infection, 5-LO activity increased the susceptibility of mice to infection and was associated with higher levels of IL-12p70 and IFN-\( \gamma \) [34]. Likewise, \( F.t \)-infected Alox\(_5^\{-\}-\) mice exhibited enhanced levels of proinflammatory mediators, such as TNF, IL-1\( \beta \), IL-6, and the chemokines KC and MCP-1, and greater resistance to death. Interestingly, absence of the 5-LO pathway is also associated with diminished numbers of T\(_{reg} \) during infection with \( P.b \) [34]. T\(_{reg} \) inhibit effector T cell proliferation and cytokine production, and their presence promotes the persistence of \( P.b \) in lesions and pathogen dissemination. Because T\(_{reg} \) increase in number during the course of tularemia [3], we determined whether absence of the 5-LO pathway affected the levels of these important immune suppressor cells. Unlike infection with \( P.b \), no significant differences in T\(_{reg} \) were found between \( F.t \)-infected Alox\(_5^\{-\}-\) mice and their wild-type counterparts. However, we and others [4, 35] recently demonstrated that the cross talk between T\(_{reg} \) and MDSCs is essential to maintaining an immune-suppressive environment. Thus, it will be interesting to explore whether 5-LO deficiency alters the number or repertoire of MDSCs recruited to the lungs during the course of tularemia. Any alteration in the presence or function of these immune suppressor cells could correlate with the LXA\(_4\)-induced changes in cytokine/chemokine production observed in \( F.t \)-infected mice.

Because 5-LO controls the production of other eicosanoids, such as LTB\(_4\), the effects of 5-LO deficiency reported here may not be the direct and/or entire result of a failure to produce LXA\(_4\). However, that argument is challenged by our in vivo results showing that the addition of exogenous LXA\(_4\) was associated with decreased levels of PGE\(_2\) and diminished cell death. These phenomena are recapitulated when comparing and contrasting Alox\(_5^\{-\}-\) mice with wild-type mice with and without administration of the 5-LO pathway inhibitor MK-886. To further confirm the critical role of LXA\(_4\) in regulating the acute response to \( F.t \), we “complemented” 5-LO-deficient mice by providing exogenous LXA\(_4\) i.n. during the course of infection and analyzing the effect of treatment on bacterial burden and the survival of these mice as compared with the wild-type and untreated Alox\(_5^\{-\}-\) mice. The Alox\(_5^\{-\}-\) mice provided exogenous LXA\(_4\) mimicked the wild-type phenotype in terms of increased bacterial burden in the lungs and enhanced morbidity and mortality after infection with \( F.t \). The fact that the addition of LXA\(_4\) alone “rescued” the mice deficient in 5-LO, coupled with the observation that LXA\(_4\) was induced early during the infection (d 1), whereas the levels of LTB\(_4\) were not significantly elevated above background until later time points (d 5 and 7), as was seen with PGE\(_2\), supports our contention that LXA\(_4\), rather than LTB\(_4\), is more likely the primary modulator of acute host response to \( F.t \) infection.

In toto, results presented herein emphasize a previously unappreciated role for LXA\(_4\) in regulating tissue bacterial burden, proinflammatory cytokine/chemokine production, and modulating host cell-death programs by \( F.t \). The initial absence and then presence of “classic” Th1-type proinflammatory cytokines (e.g., TNF, IL-1\( \beta \), IL-6, IL-12p70, and IFN-\( \gamma \)) coincides with both the temporal and reciprocal production of LXA\(_4\), LTB\(_4\), and PGE\(_2\), as the host transitions from the acute to late phase of respiratory tularemia. Given the concomitance of IL-10 and TGF-\( \beta \) production, the presence of T\(_{reg} \), T\(_{reg} \)-induced MDSCs and the induction of LXA\(_4\) during early phase respiratory tularemia, it is intriguing to speculate whether these anti-inflammatory mediators and cells act in either an additive or synergistic fashion to facilitate bacterial survival and replication.
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The authors declare no competing disclosures.

AUTHORSHIP

A.S., T.R., R.B., A.A., J.P., S.B, and F.M.M. performed research; A.S., and T.J.S. designed the research, analyzed the data, and wrote the manuscript.

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DISCLOSURES

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