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Matrix Metalloproteinase 9 Activity Enhances Host Susceptibility to Pulmonary Infection with Type A and B Strains of *Francisella tularensis*[1]

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A striking feature of pulmonary infection with the Gram-negative intracellular bacterium *Francisella tularensis*, a category A biological threat agent, is an intense accumulation of inflammatory cells, particularly neutrophils and macrophages, at sites of bacterial replication. Given the essential role played by host matrix metalloproteinases (MMPs) in modulating leukocyte recruitment and the potentially indiscriminate destructive capacity of these cells, we investigated whether MMP-9, an important member of this protease family released by neutrophils and activated macrophages, plays a role in the pathogenesis of respiratory tularemia. We found that *F. tularensis* induced expression of MMP-9 in FVB/NJ mice and that the action of this protease is associated with higher bacterial burdens in pulmonary and extrapulmonary tissues, development of more extensive histopathology predominated by neutrophils, and increased morbidity and mortality compared with mice lacking MMP-9 (MMP-9$^{-/-}$). Moreover, MMP-9$^{-/-}$ mice were able to resolve infection with either the virulence-attenuated type B (live vaccine strain) or the highly virulent type A (SchuS4) strain of *F. tularensis*. Disease resolution was accompanied by diminished leukocyte recruitment and reductions in both bacterial burden and proinflammatory cytokine production. Notably, neutrophilic infiltrates were significantly reduced in MMP-9$^{-/-}$ mice, owing perhaps to limited release of Pro-Gly-Pro, a potent neutrophil chemotactic tripeptide released from extracellular matrix through the action of MMP-9. Collectively, these results suggest that MMP-9 activity plays a central role in modulating the clinical course and severity of respiratory tularemia and identifies MMPs as novel targets for therapeutic intervention as a means of modulating neutrophil recruitment. *The Journal of Immunology,* **2007, 178: 1013–1020.**

Francisella tularensis exists in two clinically relevant forms, the European biovar B (*holarctica*) that produces acute though mild self-limiting infections and the more virulent U.S. biovar A (*tularensis*) which often is associated with respiratory tularemia and a more severe clinical course (1). The first reference to pleuropulmonary tularemia is a 1924 report by Verbrycke (2), wherein a number of nodules were described in the lungs of a dying patient. Subsequent studies have revealed that cellular infiltrates within these nodules are composed primarily of neutrophils and macrophages with scattered lymphocytes, red cells, desquamated epithelial cells, and plasma cells (3, 4). Aerosol challenge with a virulence-attenuated type B (live vaccine strain (LVS)[3]) and the highly virulent type A (SchuS4) strain of *F. tularensis* elicit a comparable clinical picture in the lungs of infected mice (5–7). Pulmonary infection with either of these strains causes inflammation of peribronchiolar tissues and is characterized by the exudation of dense infiltrates of neutrophils and macrophages into the bronchiolar lumen. Organisms can be found inside macrophages as early as 20 min postinfection (PI) and focal bronchiolitis is apparent by 24 h at which point considerable replication of bacteria has ensued (8, 9). It is likely that the explosive replicative capacity of *F. tularensis* contributes to the greater morbidity and mortality associated with pulmonary infection.

It is generally recognized that pro- and anti-inflammatory cytokines play a critical role in modulating the activation state and effector functions of innate inflammatory cells which migrate into sites of bacterial infection. In addition to cytokines, matrix metalloproteinases (MMPs) and a number of potent chemokines along with complement and extracellular matrix (ECM) components are generated within the inflammatory milieu. It is the coordinate action of these molecules which orchestrate leukocyte extravasation into the inflammatory focus (10–14). MMPs comprise a large family of Zn^{2+}- and Ca^{2+}-dependent endopeptidases whose capacity to degrade or process ECM is associated with tissue remodeling, chronic inflammation, tumor cell metastasis, and more recently, the progression of various infectious diseases (10, 15, 16). MMP-9, an important member of this protease family, is a 92-kDa type B gelatinase that is secreted as an inactive proenzyme by both

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[3] Abbreviations used in this paper: LVS, live vaccine strain; PI, postinfection; MMP, matrix metalloproteinase; ECM, extracellular matrix; PGP, Pro-Gly-Pro; MH, Mueller-Hinton; i.n., intranasal; BAL, bronchoalveolar lavage; BALF, BAL fluid; MTD, median time to death; ESI-LC-MS/MS, electron spray ionization-liquid chromatography-mass spectrometry/mass spectrometry.

neutrophils and macrophages (10, 17). This pro-MMP-9, subsequently activated by proteolytic cleavage, degrades both ECM (type I, IV, V, VII, XI collagens, fibrin, and laminin) and nonmatrix substrates (18, 19). MMP-9 acts to establish a concentration gradient of CXCL8 (formerly human IL-8 or murine KC) which directs the diapedesis of neutrophils across vascular endothelium (10, 13), activates proinflammatory cytokines such as TNF-α and IL-6 (10), and is associated with tissue destruction (16).

Chemokines such as KC and MIP-2 initiate the influx of neutrophils into sites of infection. In turn, these cells release collagenase and MMPs (e.g., MMP-9) to generate chemotactic fragments from ECM proteins that amplify the recruitment of inflammatory cells (10, 17). In 1995, Pfister et al. (20) demonstrated that one such fragment, Pro-Gly-Pro (PGP), is chemotactic for neutrophils and likely results from the hydrolysis of collagen. More recently, it was shown that intratracheal instillation of PGP stimulates neutrophil infiltration into the lungs of C57BL/6 mice (21). Currently, it is thought that upon its generation, PGP contributes to the maintenance and extension of the neutrophil influx during periods of declining chemokine levels (e.g., during late-stage disease).

There is growing appreciation of the role played by MMPs in infectious disease, particularly their importance in orchestrating the recruitment of innate inflammatory cells and regulating their effector functions subsequent to cellular activation (22). Therefore, we evaluated the role of MMP-9 in the pathogenesis of respiratory tularemia relying upon the well-characterized mouse model of infection (6, 23–26). In the present study, we propose that MMP-9-mediated regulation of the matrix environment, which coordinates the influx of neutrophils, plays a more destructive than protective role in host defense against pulmonary infection with *F. tularensis*. This notion is supported by the higher bacterial burden, increased histopathology, and greater susceptibility to infection seen in wild-type mice compared with those deficient for MMP-9.

Materials and Methods

Mice

Wild-type and congenic MMP-9$^{-/-}$ FVB/NJ mice (The Jackson Laboratory) were housed in the Animal Resources Facility (Albany Medical College, Albany, NY). Food and water were provided ad libitum. All animal procedures conformed to the Institutional Animal Care and Use Committee guidelines. All experiments were conducted using equal numbers of male and female mice of 4–8 wk of age per group.

Bacteria

F. tularensis LVS (ATCC 29684; American Type Culture Collection) was provided by Dr. K. Elkins (U.S. Food and Drug Administration, Bethesda, MD). *F. tularensis* SchuS4, originally isolated from a human case of tularemia (27), was obtained from the U.S. Army Medical Research Institute for Infectious Diseases (Frederick, MD). All experiments using SchuS4 were conducted within a Centers for Disease Control-certified ABSL-3/BSL-3 facility at Albany Medical College. The bacteria were cultured on modified Mueller-Hinton (MH) agar plates or in modified MH broth (Difco Laboratories) supplemented with ferric pyrophosphate and IsoVitalex (BD Biosciences) and maintained as previously described (28, 29).

Infection

All infection experiments used groups of 6–10 mice that were monitored for survival or euthanized at designated time points PI. An aliquot of *F. tularensis* was thawed, diluted in sterile PBS and mice were inoculated intranasally (i.n.) with 10^1 and 10^2 CFU of *F. tularensis* SchuS4 or 10^3 and 10^4 CFU of *F. tularensis* LVS in a volume of 20 μl of PBS (10 μl/nare); actual dosages were confirmed by plating. Before i.n. inoculation, animals were deeply anesthetized via i.p. injection of a mixture of ketamine (20 mg/ml) and xylazine (1 mg/ml). Sham-inoculated controls received an equal volume of uninoculated MH broth diluted in PBS. For survival experiments, mice were examined twice daily for morbidity and mortality for a period of 14 days and the median survival was calculated for each group. Euthanized mice were necropsied at various times PI and lung, liver, and spleen were excised aseptically.

Collection of bronchoalveolar lavage (BAL) fluid (BALF)

Following infection as described above, BAL was performed on euthanized mice using a 1-cc syringe fitted with a flexible capillary tube attached to a 23 G needle. The capillary tubing was inserted into the anterior portion of the trachea of the mouse. The lungs were slowly perfused with 1 ml of PBS (37°C) taking care to avoid hyperinflation of the lungs. Once instilled, the volume of PBS is recovered using the same syringe and the process repeated once with a new syringe and an additional 1 ml volume of PBS. Aliquots of BALF recovered from individual animals were pooled and stored short-term (<24 h) at 4°C or long-term (>24 h) at −80°C. The volume of lavage fluid recovered is recorded for each mouse and the number of cells contained in the BALF, as enumerated by hemacytometry, was normalized on the basis of the volume recovered. Total protein concentrations in BALF samples were measured using a commercially available assay (Bio-Rad).

Electron spray ionization-liquid chromatography-mass spectrometry/mass spectrometry (ESI-LC-MS/MS)

BALF samples were run on an MDS Sciex API-4000 Q-Trap (Applied Biosystems) equipped with a Shimadzu HPLC (Shimadzu). This instrumentation has a limit of detection in the range of 10–20 pg/ml. The HPLC is performed using a Develosil RP-Aqueous C30 (2.0 × 150 mm, 5 μm) column (Phenomenex) with a 20–100% gradient of water containing 0.1% formic acid:acetonitrile. Gradients are run at 0.2 ml/min for 6 min followed by a 4-min equilibration. Mass transitions for acetylated PGP were observed at 312-112 and 312-140. Mass transitions for nonacetylated PGP were observed at 270-70 and 270-116. Peak areas were integrated and quantified based upon standard curves determined using known amounts of both forms of PGP under these conditions.

MMP-9 zymography

Gelatinase activity was assayed by the method of Hibbs et al. (30). Briefly, 50 μg of total protein from lung homogenates was incubated with 1 ml of sterile H_2O and 100 μl of gelatin-agarose beads (Sigma-Aldrich) overnight at 4°C to enrich for MMPs. Beads then were centrifuged at 10,000 × *g* for 1 min, resuspended in nonreducing Laemmli sample buffer (lacking 2-ME) and incubated for 30 min at room temperature (samples are not boiled before loading). After centrifugation at 14,000 × *g* for 2 min, equal volumes of the eluate were resolved on a 7.5% nonreducing SDS-PAGE containing 4 mg/ml gelatin (Sigma-Aldrich). Following electrophoresis, gels were washed three times with 50 mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$ and subsequently incubated for 24 h at 37°C in the same buffer containing 1% Triton X-100. This denaturation/renaturation step promotes MMP activity without proteolytic cleavage of pro-MMP-9. Gelatin activity was visualized by staining gels with 0.5% Coomassie blue and destaining with methanol/acetic acid; band intensities were quantified using a Fluorochem 8000 Imaging system (Alpha Innotech). Zymography results were confirmed using a mouse pro-MMP-9 ELISA (R&D Systems).

Bacterial burden

Portions of lung (20 mg), liver (30 mg), and spleen (5 mg) were homogenized after the addition of 0.5 ml of PBS containing protease inhibitor mixture (Roche Diagnostics) using a mechanical homogenizer (Mini Bead Beater; Biospec Products) and sterile inert Zirconia beads. Homogenates were spun for 10 s in a microcentrifuge at 1000 rpm and 10-fold serial dilutions of clarified supernatant were made in sterile PBS. Ten 1-μl aliquots of each dilution were spotted onto duplicate MH chocolate agar plates as described elsewhere (28, 29). Quantification was done by counting the colonies on the plates after 48–72 h incubation at 37°C and results were expressed as $\log_{10}$ CFU/ml. The remaining lung homogenate was spun in a microcentrifuge at 14,000 rpm for 20 min and the clarified supernatant was used immediately to assay for the presence of cytokines and to measure MMP-9 activity.

Histopathology

Lungs, liver, and spleen from *F. tularensis* LVS-infected and sham-inoculated mice sacrificed at day 9 were excised and fixed in 10% neutral-buffered formalin. Lungs were inflated by instillation of PBS into the trachea before fixation. Tissues were processed using standard histological methods to obtain 5-μm-thick paraffin sections and were stained with H&E. For some experiments, the extent of collagen deposition and structural

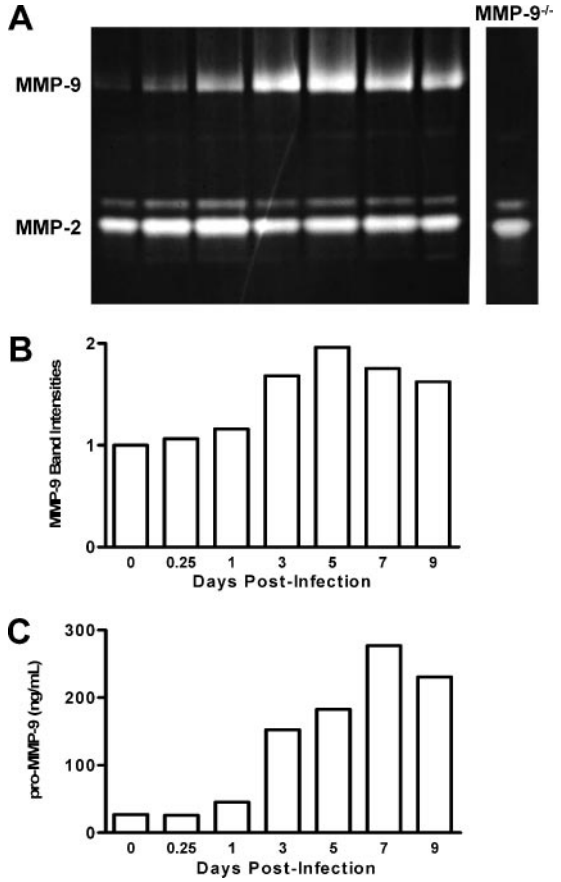

FIGURE 1. *F. tularensis* induces in vivo production of MMP-9. FVB/NJ mice were infected with 10^3 CFU of *F. tularensis* LVS, sacrificed at various time points and lung homogenates were assayed by gelatin zymography for the presence of MMP-9 and -2 (*A*). MMP-$9^{-/-}$ mice served as a negative control. MMP-9 band intensities were normalized to MMP-2 levels and shown as a fold increase above baseline levels (*B*). Pro-MMP-9 levels were measured by commercial ELISA (*C*). Results are derived from lung homogenates pooled from four to six individual animals and are representative of three independent experiments (12–18 mice total).

integrity of fibrillar collagen was assessed in lung sections following Masson's Trichrome staining using the Accustain Trichrome stain kit (Sigma-Aldrich). Lungs also were assessed microscopically using a histopathologic scoring system and a numerical score for each animal was assigned by adding the subscores from nine parameters as described elsewhere (29). Each of these parameters was graded from 0 to 3 and then combined for a maximum cumulative score of 27. Lung scores were based on the extent and degree of peribronchiolar/bronchiolar inflammation, bronchial lumen exudation, extent of perivascular infiltration, the frequency and extent of necrotizing/organizing bronchopneumonic patches, the degree of alveolar inflammation, and the type and numbers of inflammatory cells (i.e., neutrophils and/or macrophages) involved in peribronchovascular areas and in the lung parenchyma. Liver sections were evaluated with respect to the quantity and quality (discrete/nondiscrete) of granuloma formation, their distribution and cellular composition. Spleen sections were similarly evaluated for granulomatous lesions within the red and white pulp.

Differential cell counting

The percentage of neutrophils infiltrating the lungs of infected mice was determined by modified Wright's staining of leukocytes isolated from enzymatically digested tissue. Briefly, lungs were perfused with sterile PBS, collected aseptically, finely cut with a scalpel, and incubated for 60 min at 37°C with digestion buffer (PBS containing 0.1% BSA, 0.01 M $MgCl_2$, and 7 mM NaN_3) to which 250 μg/ml DNase I and 3.3 mg/ml collagenase A was added. Cell suspensions were passed through a 70-μm nylon cell strainer (BD Biosciences) and cytospun onto polylysine-coated slides. Slides were prepared using a standard, modified Wright's staining protocol and leukocytes were enumerated by light microscopy.

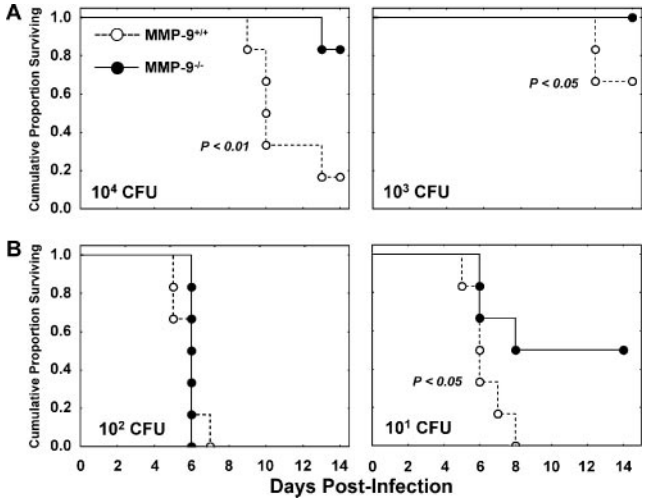

FIGURE 2. MMP-9 deficiency enhances host resistance to *F. tularensis* infection. MMP-$9^{+/+}$ and MMP-$9^{-/-}$ mice were inoculated i.n. with 10^3 and 10^4 CFU of *F. tularensis* LVS (*A*) or 10^1 and 10^2 CFU of *F. tularensis* SchuS4 (*B*) and monitored for morbidity and mortality. Results are expressed as Kaplan-Meier curves and *p* values determined using log-rank test. The results shown are representative of three independent experiments ($n = 6$ mice per group or 18 mice total).

Cytokine measurements

Lung homogenates were assayed for the presence of pro- and anti-inflammatory cytokines using Cytometric Bead Array flex sets (BD Pharmingen) which allow for simultaneous measurement of MCP-1, KC, TNF-α, IL-6, IFN-γ, IL12-p70, and IL-10 levels. Flow cytometric analysis was performed using a FACSArray flow cytometer (BD Immunocytometry Systems (BDIS)). Data were acquired and analyzed using BD FACSArray software and FCAP Array software, version 1.0 (BDIS), respectively. The limits of detection for MCP-1, KC, TNF-α, IL-6, IFN-γ, IL-12p70, and IL-10 are 29, 16.2, 17.1, 6.5, 5.2, 9.2, and 16.4 pg/ml, respectively. Murine MIP-2 was measured using a commercially available kit (BioSource International) following the manufacturer's instructions.

Statistical analysis

A log-rank test was used to determine the level of significance for the Kaplan-Meier survival analyses. All other results were expressed as mean ± SEM and comparisons between the groups were made using one-way ANOVA followed by Bonferroni's correction, nonparametric Mann-Whitney *U* test, or Student's *t* test. Differences between control and experimental groups were considered significant at a $p < 0.05$ level.

Results

F. tularensis *LVS induces expression of MMP-9*

To begin evaluating the role of MMP-9 in tularemia pathogenesis we determined whether F. tularensis has the capacity to promote its release during infection. Lungs were excised from mice at various times following i.n. infection and tissue homogenates were assayed by gelatin zymography which specifically reveals MMP-9 and MMP-2 expression. It was observed that MMP-9 levels were substantially elevated above sham-inoculated controls by day 3 and remained so for the duration of the experiment (Fig. 1, *A* and *B*). In contrast, MMP-2 levels were unaltered from baseline. As expected, no MMP-9 expression was associated with lung homogenates recovered from mice with a targeted mutation of the *mmp9* gene (Fig. 1*A*). A more quantitative way of measuring MMP-9 levels than gel zymography is by ELISA, therefore, this latter methodology was used to corroborate the findings presented in Fig. 1, *A* and *B*. As measured by ELISA, the pattern of pro-MMP-9 expression in lung homogenates was similar to that observed in zymograms, beginning at day 3 PI and continuing through day 9 (Fig. 1*C*).

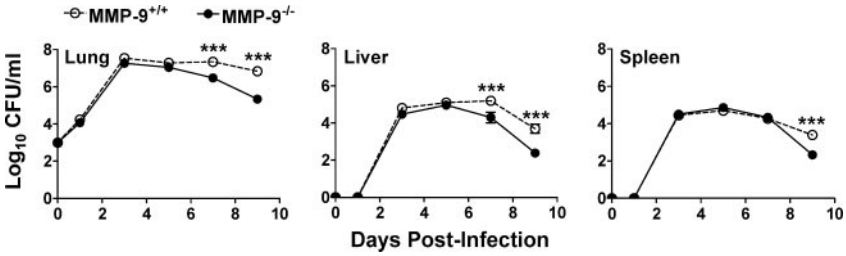

FIGURE 3. MMP-9$^{-/-}$ mice harbor significantly fewer bacteria in affected tissues. MMP-9$^{+/+}$ and MMP-9$^{-/-}$ mice were inoculated i.n. with 10^3 CFU of *F. tularensis* LVS. At the times indicated, mice were sacrificed and homogenates of the lungs, liver, and spleen were plated for determination of bacterial burden. Results shown are the mean ± SEM and are representative of two independent experiments ($n = 6$ mice per time point or 12 mice total). ***, $p < 0.001$ using the one-way ANOVA.

F. tularensis-*induced MMP-9 activity is associated with greater morbidity and mortality*

MMP-9 activity may engender extensive cellular infiltration of the lungs by neutrophils and macrophages, the latter of which supports bacterial replication and thus would increase susceptibility to tularemia. Further, the combination of degradative molecules released by activated neutrophils and MMP-induced tissue destruction contributes to pathophysiological changes in the lung. To test these notions, MMP-9$^{+/+}$ and congenic MMP-9$^{-/-}$ mice were infected i.n. with 10^3 and 10^4 CFU of *F. tularensis* LVS and their relative susceptibility was evaluated on the basis of the cumulative proportion of mice surviving and the median time to death (MTD) of the experimental group. MMP-9$^{+/+}$ mice began to die in response to a 10^4 CFU dose 9 days PI. The MTD for this group was 10 days and 83% of the mice were dead by day 14 (Fig. 2*A*). In contrast, no mortality was recorded in the MMP-9$^{-/-}$ group until day 13 and by day 14 only 17% of the mice had succumbed to infection with a MTD of >14 days, a significant difference compared with the wild-type controls ($p < 0.01$). Following infection with 10^3 CFU of *F. tularensis* LVS, 33% of the MMP-9$^{+/+}$ mice died while all of those deficient for MMP-9 survived ($p < 0.05$) (Fig. 2*A*).

Given the highly virulent nature of type A strains of *F. tularensis* and their potential for use as a biological threat agent, it was of interest to determine whether MMP-9 deficiency could alter the course of disease caused by infection with SchuS4. When mice were inoculated with 10^1 CFU of *F. tularensis* SchuS4, 100% of the MMP-9$^{+/+}$ mice succumbed to infection by the eighth day with a MTD of 6 days. In contrast, only 50% of the MMP-9$^{-/-}$ mice died by day 14 with a MTD of 11 days (Fig. 2*B*). However, no difference was observed between the two groups when challenged with 10^2 CFU suggesting that greater numbers of *F. tularensis* can overcome the resistance phenotype conferred by MMP-9 deficiency. All mice surviving challenge with either the LVS or SchuS4 strain were sacrificed at day 21 and infection was confirmed by ELISA-based serological measurement of anti-*F. tularensis*-specific IgG Ab levels (our unpublished data). These results demonstrate that MMP-9$^{-/-}$ mice exhibit a diminished morbidity and mortality associated with pulmonary infection of *F. tularensis*. Importantly, MMP-9 deficiency does not alter the mRNA levels of other MMPs or tissue inhibitors of metalloproteases (31), nor are compensatory changes in the enzymatic activity of other MMPs observed in MMP-9$^{-/-}$ animals (32). Thus, the more resistant phenotype in this model of *F. tularensis* infection can be attributed to the lack of MMP-9 activity. To our knowledge, this is the first report of a host gene whose absence enhances resistance to challenge with a type A strain of *F. tularensis*.

Table I. *Histological scores reflecting the degree of* F. tularensis-*induced lung pathology*

Days PI	MMP-9$^{+/+}$	MMP-9$^{-/-}$
1	5.2 ± 1.8	4.1 ± 1.1
3	16.1 ± 0.6***	7.0 ± 0.5
5	17.0 ± 0.4***	9.1 ± 0.6
7	18.8 ± 0.8***	10.8 ± 0.9
9	23.6 ± 0.2***	9.6 ± 1.3

***, $p < 0.001$.

F. tularensis *LVS-infected MMP-9$^{-/-}$ mice have a significantly lower bacterial burden in pulmonary and extrapulmonary tissues*

Based upon the greater resistance observed in MMP-9$^{-/-}$ animals, we postulated this disease phenotype might be associated with a lower bacterial burden in infected tissues. In fact, the number of bacteria found in tissues from MMP-9$^{-/-}$ mice were 1–1.5 $\log_{10}$ CFU lower than that found in MMP-9$^{+/+}$ mice (Fig. 3). In lung

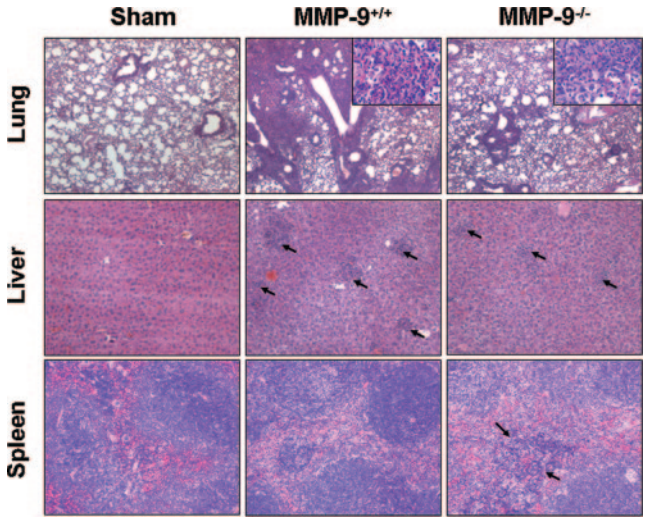

FIGURE 4. MMP-9$^{-/-}$ mice exhibit less severe histopathological changes than their wild-type counterparts. Lungs, liver, and spleen of MMP-9$^{+/+}$ and MMP-9$^{-/-}$ mice were evaluated at day 9 post-i.n. inoculation with 10^3 CFU of *F. tularensis* LVS. Sham-inoculated FVB/NJ mice served as a control. *Inset* in MMP-9$^{+/+}$ lung panel shows a necrotic lesion containing neutrophilic debris. *Inset* in MMP-9$^{-/-}$ lung panel has an accumulation of macrophages with evidence of only minimal neutrophilic infiltration. Arrows in liver panels indicate nascent granulomas and those in the spleen indicate an area of intense lymphoproliferation that is absent in the spleens of infected MMP-9$^{+/+}$ mice. Magnification is as follows: lung (×40), liver and spleen (×100), and *insets* (×200).

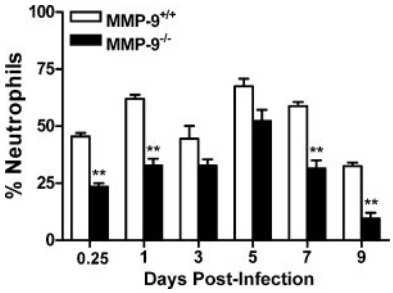

FIGURE 5. MMP-9 deficiency impairs neutrophil recruitment into the lungs of mice infected with *F. tularensis.* Differential cell counting was performed on leukocytes liberated from the enzymatically digested lungs of MMP-$9^{+/+}$ and MMP-$9^{-/-}$ mice inoculated i.n. with 10^3 CFU of *F. tularensis* LVS. Results represent the mean percentage ± SEM of neutrophils determined for each group and are cumulative results of two independent experiments ($n = 3$ mice per group or 6 mice total). **, $p < 0.01$ using the one-way ANOVA.

and liver, a significant difference in bacterial burden was observed between groups as early as 7 days PI, while splenic burdens did not differ significantly until the ninth day. In other experiments, it was determined that, unlike in MMP-$9^{+/+}$ mice, *F. tularensis* LVS and SchuS4 were cleared entirely from the tissues of surviving MMP-$9^{-/-}$ animals by day 21 PI (our unpublished data).

MMP-$9^{-/-}$ mice develop less severe pathology in lung, liver, and spleen in response to F. tularensis *LVS*

MMP-$9^{-/-}$ mice infected i.n. with 10^3 CFU of *F. tularensis* LVS underwent histological evaluation to determine whether increased survival and a lower bacterial burden was associated with diminished tissue pathology (Table I and Fig. 4). Beginning at day 3 and continuing throughout the course of infection, MMP-$9^{-/-}$ mice exhibited less severe histopathological changes in the lungs compared with their MMP-$9^{+/+}$ counterparts ($p < 0.001$) (Table I). The difference in mean histopathological scores was greatest at day 9 PI (MMP-$9^{+/+}$ mice, 23.6 ± 0.2 vs MMP-$9^{-/-}$, 9.6 ± 1.3) as corroborated by the extent of inflammation in the lungs of infected wild-type vs MMP-$9^{-/-}$ mice (Fig. 4). Wild-type lungs showed severe pathology characterized by infiltration of macrophages and lymphocytes around the majority of bronchi/bronchioles and accompanying blood vessels. Hypertrophy of bronchiolar walls, thickening of the alveolar septa and exudation of macrophages and neutrophils within the alveolar lumen were a consistent finding. Additionally, many discrete to diffuse disseminated necrotic pneumonic patches predominated by neutrophils (see *inset*) were seen within the lung parenchyma. The alveoli around such necrotic patches contained fibrinous exudate admixed with neutrophilic debris and macrophages. In marked contrast, the lungs of MMP-$9^{-/-}$ mice showed a lesser degree of pathological changes in the airways and parenchyma (Fig. 4). The inflammatory reaction showed mild to moderate interrupted cuffs of mononuclear cells (lymphocytes, macrophages, and fibroblasts) and only slight thickening of alveolar walls. Few chronic pneumonic patches comprised almost exclusively of macrophages and fibroblasts were seen mostly adjacent to inflamed airways and blood vessels. One of the more intriguing histopathological features that distinguished the MMP-$9^{-/-}$ mouse from its wild-type counterpart is the decreased number of neutrophils in the lungs of the former (see *inset*).

The livers of MMP-$9^{+/+}$ mice showed rarefied hepatocytes around the central veins, prominent Kupffer cells and stray lymphocytes in the sinusoids (Fig. 4). Many discrete microgranulomas (more than five per field) were found disseminated in different hepatic lobules adjacent to the vessels in the portal areas and a few within the lobules. These granulomas consisted of macrophages (of mostly >50 cells), neutrophils, few lymphocytes, red cells, and occasional apoptotic hepatocytes. Hepatocytes surrounding the granulomas were swollen and had eosinophilic cytoplasm. In contrast, liver changes in MMP-$9^{-/-}$ mice were considerably less severe as evidenced by the presence of minute and fewer numbers of microgranulomas (two to three per field) having loosely arranged macrophages (mostly 10–15 cells) and lymphocytes.

The spleen of MMP-$9^{+/+}$ mice revealed hypertrophy of the majority of the white pulp with pale areas and an increased number of marginal zone cells (Fig. 4). The red pulp was moderately engorged and had mild to moderate neutrophil, macrophage, and lymphocytic infiltration. MMP-$9^{-/-}$ mice showed considerably fewer hypertrophied splenic follicles with pale areas, while the red pulp contained reduced numbers of macrophages and no infiltrating neutrophils.

The lungs of F. tularensis*-infected MMP-$9^{-/-}$ mice harbor quantitatively fewer neutrophils than are found in MMP-$9^{+/+}$ mice*

In addition to differences in the overall extent of cellular infiltration, histological evaluation suggested that MMP-9 deficiency results in neutrophils representing a lower percentage of the total leukocyte population in the lungs. To quantify the percentage of different inflammatory cell types in the lung, we determined cell numbers after modified Wright's staining of leukocytes recovered from enzymatically digested tissues. As seen in Fig. 5, as early as 6 h PI neutrophils represented a significantly lower percentage of

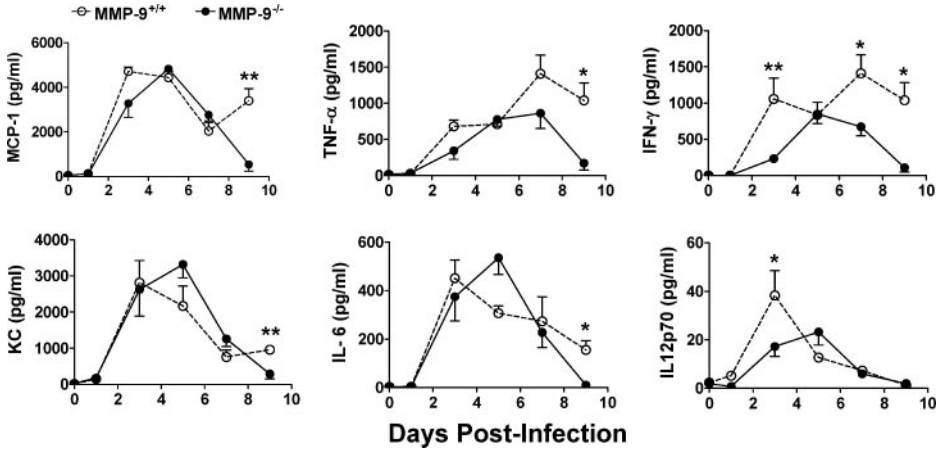

FIGURE 6. MMP-$9^{+/+}$ mice produce higher levels of proinflammatory cytokines than those deficient for MMP-9. MMP-$9^{+/+}$ and MMP-$9^{-/-}$ mice were inoculated i.n. with 10^3 CFU of *F. tularensis* LVS. At the times indicated, mice were sacrificed and homogenates of the lungs were used to measure cytokine levels with sham inoculated mice serving as controls. Results shown are the mean ± SEM and are representative of two independent experiments ($n = 6$ mice per time point or 12 mice total). *, $p < 0.05$, **, $p < 0.01$ using the one-way ANOVA.

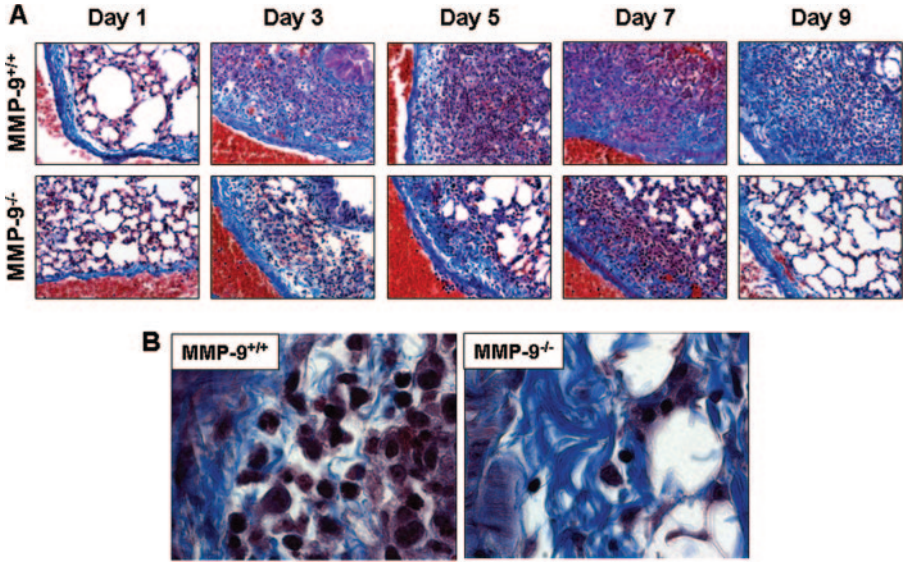

FIGURE 7. MMP-$9^{-/-}$ mice exhibit less intense and self-limiting inflammatory cell recruitment and minimal collagen degradation in response to *F. tularensis* infection. Lung sections of MMP-$9^{+/+}$ and MMP-$9^{-/-}$ mice inoculated i.n. with 10^3 CFU of *F. tularensis* LVS were stained with Masson's Trichrome. Sections were evaluated on the basis of leukocyte recruitment and collagen deposition at various times PI (*A*, magnification, ×200), and the integrity of collagen fibrils on the fifth day (*B*, magnification ×1000, oil immersion).

leukocytes in MMP-$9^{-/-}$ mice than in their wild-type counterparts. This difference also was observed at 1, 7, and 9 days PI and just missed statistical significance at days 3 and 5. In contrast, the percentage of macrophages comprising the inflammatory infiltrate was unaltered by MMP-9 deficiency and lymphocytes represented a larger percentage of the total cell population compared with MMP-$9^{+/+}$ mice (our unpublished data).

MMP-$9^{-/-}$ mice exhibit an altered profile of cytokine production following i.n. challenge with F. tularensis *LVS*

The elaboration of pro- and anti-inflammatory mediators is a reflection of the host immune response to pathogens. To explore whether the production of immunomodulators is altered by MMP-9 deficiency, we determined the levels of MCP-1, KC, TNF-α, IL-6, IFN-γ, IL-12p70, and IL-10 at various time points post-i.n. inoculation with 10^3 CFU of *F. tularensis* LVS, a dosage found to be nonlethal during the first week of infection. Given the initial "centralized" nature of the immune response to pulmonary infection, we chose to measure tissue-resident levels of these cytokines/chemokines. As seen in Fig. 6, MCP-1, KC, TNF-α, and IL-6 were similarly elevated in both MMP-$9^{+/+}$ and MMP-$9^{-/-}$ mice until day 9 at which point their levels returned to baseline in MMP-$9^{-/-}$ mice, but remained elevated in their wild-type counterparts. Production of another potent neutrophil chemoattractant, MIP-2, mirrored that of KC insofar as no difference was observed between mice until day 9 PI at which point levels of this chemokine were higher in wild-type animals (our unpublished data). Also in MMP-$9^{+/+}$ mice, IFN-γ levels rose faster, achieved higher levels and remained elevated as compared with that found in MMP-$9^{-/-}$ mice where IFN-γ release returned to preinfection levels by day nine PI (Fig. 5). *F. tularensis* LVS stimulated IL-12p70 release in both groups of mice, however, only at day 3 were the levels higher in the MMP-$9^{+/+}$ than in MMP-$9^{-/-}$ mice. It is worth noting that the level of IL-10 in both mouse genotypes remained below detectable limits under these experimental conditions.

MMP-9 deficiency impairs degradation of collagen whose deposition is increased during F. tularensis *infection*

Respiratory infection of mice by *F. tularensis* LVS results in considerable deposition of collagen beneath the basement membrane and throughout the extracellular space surrounding the bronchioles and venules of the lung (Fig. 7*A*). By day 9 PI, both deposited collagen and dense cellular infiltrates remain prominent features of inflammation in the lungs of wild-type mice. In contrast, a lesser degree of collagen deposition and many fewer inflammatory cells are observed in MMP-$9^{-/-}$ mice and this inflammatory response

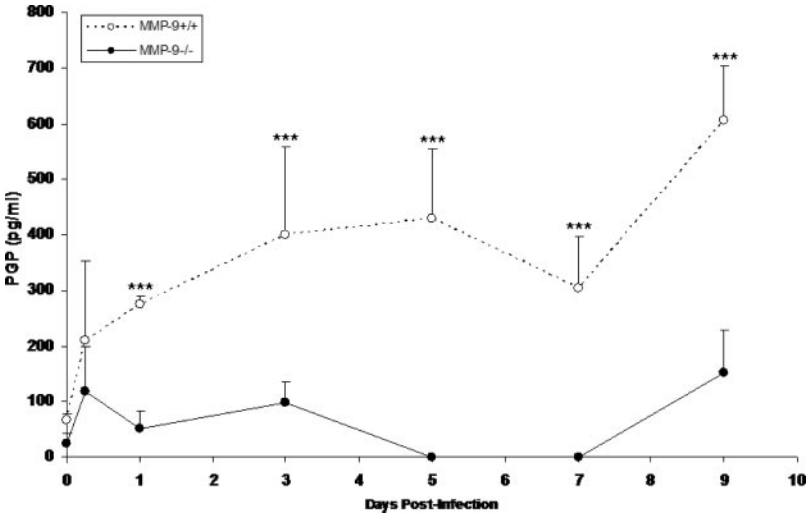

FIGURE 8. Significantly less PGP is generated in the *F. tularensis*-infected lungs of MMP-$9^{-/-}$ mice. BALF was recovered from mice infected i.n. with 10^3 CFU of the LVS at different times PI and was assayed by ESI-LC-MS/MS for the presence of PGP. Results shown are the mean ± SEM (n = 6 mice per time point). ***, $p < 0.001$ using the one-way ANOVA.

to *F. tularensis* appears self-limiting as it is almost entirely resolved by the ninth day of infection. Another striking and consistent difference is that the structural integrity of collagen fibrils in MMP-9$^{+/+}$ mice indicates extensive proteolytic cleavage into ECM fragments while dense collagen bundles are essentially intact within the lungs of mice lacking MMP-9 (Fig. 7*B*).

Infection of mice with F. tularensis *LVS induces the MMP-9-dependent generation of PGP*

Based upon the findings presented in Fig. 7*B*, one would predict that PGP levels in the lungs of wild-type mice would be higher than in their MMP-9$^{-/-}$ counterparts. To determine whether this is the case, BALF was recovered from mice infected i.n. with 10^3 CFU of the LVS at different times PI and were assayed by ESI-LC-MS/MS for the presence of PGP. Consistent with the lack of MMP-9 activity and the intact state of collagen fibrils, there was significantly less PGP in the lungs of MMP-9$^{-/-}$ mice at days 1, 3, 5, 7, and 9 PI compared with their wild-type counterparts ($p <$ 0.0001, 0.0236, 0.0037, 0.0083, and 0.0034, respectively) (Fig. 8).

Discussion

Within the lung, epithelia and alveolar macrophages are resident cellular responders to inhaled bacteria. Activation of these cell types, along with endothelial cells lining the pulmonary vasculature, results in the up-regulation of IL-1β, CXCL8, TNF-α, MCP-1, and members of the selectin, integrin, and Ig superfamily of adhesion molecules. *F. tularensis* LVS potently induces the secretion of CXCL8 by and the expression of adhesion molecules on HUVECs (33). Collectively, these adhesion molecules and chemokines play a critical role in directing the emigration of neutrophils and peripheral blood macrophages into infected tissues. Recruitment of host cells into an inflammatory focus is an important first step in tularemia pathogenesis because *F. tularensis* replicates within macrophages and dendritic cells (34, 35). Leukocyte recruitment is facilitated by the action of a large family of metalloproteinases whose members are responsible for remodeling the ECM, conversion of inactive cytokines/chemokines to their active form, and establishment of chemotactic gradients that direct the movement of leukocytes across epithelial and endothelial barriers (19). One such member of this family is MMP-9, a type B gelatinase released from granular stores within activated neutrophils and also secreted by activated macrophages (17).

Herein, we report that *F. tularensis* LVS triggers an influx of innate inflammatory cells, principally neutrophils and macrophages, in wild-type mice that far exceed the number found in MMP-9$^{-/-}$ mice. The greater density of cells found in the lungs of mice expressing MMP-9 is associated with a higher bacterial burden and prolonged release of a number of proinflammatory cytokines and chemokines, an observation consistent with the fact that activated neutrophils and macrophages are the primary source of these potent immunomodulators. With respect to the production of neutrophil chemoattractants, one of the more unexpected results was the similarity in KC and MIP-2 levels seen in wild-type and MMP-9$^{-/-}$ mice for much of the course of disease. A newly described mechanism for neutrophil recruitment involves the generation of PGP via MMP-9-mediated collagen degradation (21). Sharing sequence homology with CXCL8/KC (21), PGP binds to the same CXCR1/2 receptors on neutrophils as does CXCL8/KC thus directing their movement into inflammatory sites (36, 37). Histological evaluation of collagen integrity in the lungs of *F. tularensis*-infected MMP-9$^{-/-}$ mice suggests limited breakdown of collagen compared with that observed in the lungs of wild-type mice. Entirely consistent with this difference in ECM degradation are the significantly lower levels of PGP found in mice lacking MMP-9 (Fig. 8) and the lower number of neutrophils infiltrating the lung parenchyma of these animals (Fig. 3). The notion that generation of PGP plays an important auxiliary role in neutrophil recruitment during the acute phase of respiratory tularemia is currently under investigation.

In other studies, MMP-9$^{-/-}$ mice exhibit altered patterns of neutrophil migration in a model of allergic lung inflammation (38), less severe Ab-induced arthritis (39), and are protected against lethal endotoxic shock (31). The ability of MMP-9 deficiency to protect mice against challenge with LPS is particularly intriguing and consistent with our findings. Dubois et al. (31) found that LPS stimulates the release of MMP-9 both in vitro and in vivo. Further, the release of preformed stores of MMP-9 by degranulating neutrophils precedes secretion of proinflammatory cytokines such as TNF-α suggesting a direct effect. Although *F. tularensis* possess LPS this molecule is quite impotent as a proinflammatory agonist (40–42), and recently was demonstrated to be incapable of activating neutrophils due to its inability to engage the cell surface (43). The capacity of other *F. tularensis*-associated molecular patterns to trigger the release of granular stores or to stimulate de novo production of MMP-9 is being evaluated. Other bacterial components known to induce MMP-9 activity, which *F. tularensis* also possess (44), include lipoproteins such as those found in *Borrelia burgdorferi*, a Gram-negative bacterium which happens to lack LPS (45).

The release of inflammatory mediators (e.g., cytokines, lytic enzymes, and reactive oxygen and nitrogen species, etc.) from neutrophils and the subsequent degradation of ECM are believed to be key factors contributing to multiple organ failure (46). Elegant studies conducted by Bosio et al. (47), further support the notion that neutrophils play a key pathogenic role in mediating host susceptibility to *F. tularensis* LVS insofar as B cell-deficient mice succumb to infection more readily than their wild-type counterparts. Enhanced susceptibility could not be mitigated by transfer of specific Abs and was not related to differences in macrophage phagocytic/antimicrobial function or cytokine production. Rather, transfer of primed B cells re-established a resistant phenotype and was associated with diminished bacterial burden and decreased neutrophilia, findings consistent with our studies using MMP-9$^{-/-}$ mice. It was postulated that excessive neutrophilia during *F. tularensis* infection of B cell-deficient mice is detrimental and results in marked tissue pathology (47), a notion supported by similar findings in a B cell-deficient murine model of *Leishmania donovani* (48).

Notwithstanding the current study and these other reports, it also is recognized that neutrophils are critical for host defense against lethal challenge with *F. tularensis* (23, 49–51). Considering this dichotomy in the role of neutrophils in tularemia pathogenesis we propose that the host's immune response to *F. tularensis* must strike a fine balance between one which is too muted and thus ineffectual in killing and clearing organisms and one which is overexuberant to the point of causing organ failure and death. This notion of achieving a "just right" response likely holds true for other cellular and soluble mediators of the innate and adaptive immune response to infection as well.

In summary, our study suggests a mechanism whereby MMP-9 production, whose activity may enhance infiltration of *F. tularensis*-infected lungs by neutrophils and macrophages, enhances the morbidity and mortality associated with respiratory tularemia. The former cell type likely contributes to the greater destruction of the lung parenchyma in MMP-9$^{+/+}$ mice while the latter exacerbates this pathology by supporting greater bacterial replication than is found in mice lacking MMP-9. These results argue strongly that MMP-9 plays an important role in modulating the intensity of the inflammatory response to both type A and B strains of *F. tularensis*.

Selective blockade of this ECM-degrading protease through pharmacological or molecular genetic strategies may provide a novel adjunct to antibiotic-based therapeutic intervention in respiratory tularemia.

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Disclosures

The authors have no financial conflict of interest.

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