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Sharon A. McClellan
Wayne State University

Xi Huang
Wayne State University

Ronald P. Barrett
Wayne State University

Shahrzad Lighvani
Wayne State University

Yunfan Zhang
Wayne State University

See next page for additional authors

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Purpose. The purpose of this study was to determine the role of matrix metalloproteinases (MMP) in *Pseudomonas aeruginosa* keratitis.

Methods. Gene array and selective real-time PCR examined MMP expression in the cornea of susceptible (C57BL/6, B6) versus resistant (BALB/c) mice before and after infection; zymography tested enzyme activity for MMP-2 and -9. Clinical score, Langerhans cell (LC), and Neutrophil (PMN) quantitation were done in recombinant (r) MMP-9, antibody neutralized, and MMP-9$^{-/-}$ mice. The chemotactic potential of MMP-9 was tested in a Boyden chamber assay; light and transmission microscopy and immunostaining for collagen IV and MMP-9 were used to examine the effects and the source of MMP-9 after infection. ELISA was used to assess IL-1β and MIP-2 levels.

Results. Gene array (confirmed by PCR) revealed sixfold more MMP-9, and zymography showed greater enzyme activity in the infected cornea of B6 over BALB/c mice. rMMP-9 injection of BALB/c mice enhanced, whereas MMP-9 antibody neutralization in B6 mice and its absence in MMP-9$^{-/-}$ mice decreased corneal disease. MMP-9$^{-/-}$ and antibody neutralized mice had fewer LCs in cornea; rMMP-9-treated mice had more. A myeloperoxidase (MPO) assay showed a similar pattern for PMN. MMP-9 was not chemotactic for LC or PMN. The basement membrane was more intact in MMP-9$^{-/-}$ over wild-type infected mice and correlated with staining for collagen IV; PMN was a source of MMP-9. IL-1β and MIP-2 were increased in rMMP-9 but decreased in MMP-9 antibody neutralized and MMP-9$^{-/-}$ over control groups.

Conclusions. MMP-9 regulates immune function in cornea by proteolysis, potentiating *P. aeruginosa* keratitis by degrading collagen IV and upregulating chemotactic cytokines/chemokines IL-1β and MIP-2. (*Invest Ophthalmol Vis Sci.* 2006;47:256–264) DOI:10.1167/iovs.05-1050

Pseudomonas aeruginosa is a gram-negative organism associated worldwide with bacterial keratitis, particularly in extended-wear contact lens users.[1] Most complications of bacterial keratitis are structural alterations of the cornea, but secondary glaucoma and cataract also may occur. These responses are largely caused by the host's inflammatory response, but the influence of bacterial toxins, toxicity from treatment, and increased antibiotic resistance[2] remain of importance. In the United States, microbial keratitis is most frequently associated with complications from contact-lens wear. With an incidence of 25,000 to 30,0000 cases annually, and the cost of treatment estimated at between $15 and $30 million, the disease poses a considerable medical and economic impact.[3]

Animal models of disease, produced by topical application of bacteria after wounding the corneal epithelium, by intrastromal inoculation, or by placement of a contaminated contact lens or suture on the cornea[4] have contributed to our understanding of disease pathogenesis. In mice, corneal infection induced experimentally with this pathogen causes corneal perforation in B6 (susceptible) Th1 responder mice,[5] whereas BALB/c (resistant), a Th2 dominant strain,[5] resolves the infection.[6] Extensive studies using these models have provided information regarding the role of inflammatory cells (e.g., Neutrophils [PMN], T and natural killer cells, macrophages, and Langerhans cells [LCs]), as well as cytokines and chemokines in modulating inflammation, innate immunity, and Th1 versus Th2 responses[7–11] to *P. aeruginosa* in the eye. However, the potential role of matrix metalloproteinases (MMP) in immunomodulation of corneal infection and inflammation remains little explored.[12–15]

MMPs are a family of protein-cleaving enzymes that degrade extracellular matrix and basement membrane components.[16] The most widely studied members of the MMP family in eye tissues are the gelatinases MMP-2 and MMP-9, because they preferentially degrade basement-membrane components such as type IV collagen.[17] Recently, MMP-9 also has been found to process cytokines and chemokines, and thus is a prototypic example of the regulation of immune functions through proteolysis.

In the present study, we investigated the expression pattern of several MMPs in the cornea of susceptible B6 versus resistant BALB/c mice. Our data suggest that MMP-9 has an important role in corneal pathogenesis, that it is required for the transmigration of PMN and LC into the infected cornea, that it does not provide a direct chemotactic signal, that its effect is indirect through regulation of cytokines IL-1β and MIP-2, and that PMNs are a major source of the enzyme.

Materials and Methods

Infection of Mice

Eight-week old female C57BL/6 (B6), BALB/c, MMP-9 knockout (–/–) and FVB wild-type (WT) mice (Jackson Laboratory, Bar Harbor, ME) were used. For infection, the cornea was scarified as described before[6] and a 5 μL bacterial suspension containing 1.0×10^6 colony forming units of *P. aeruginosa* strain 19,660 (American Type Culture Collection [ATCC]) was applied. Disease was monitored from 1 to 5 days postinfection (p.i.)[7] and mice were killed at 1, 5, or 6 days p.i. Animals were treated in compliance with the Association for Research in Vision

From the Department of Anatomy/Cell Biology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, Michigan.

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Corresponding author: Linda D. Hazlett, Anatomy/Cell Biology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, MI 48201; lhazlett@med.wayne.edu.

and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Ocular Response to Infection

Disease was graded as before,[18] from 0 to 4, with 0 being clear or slight opacity partially covering the pupil and +4, corneal perforation. A clinical score was recorded for each mouse for statistical comparison (n = 10/group/treatment).

Gene Array and Real-Time PCR

Data for the gene array analysis of MMPs was from a previous study and was analyzed as before.[9] For real-time PCR, total RNA was isolated from individual uninfected corneas at 1 and 5 days p.i. (n = 5/time/group) by using total RNA isolation reagent (RNA STAT-60; TEL-TEST Inc., Friendswood, TX). Reverse transcription was done by using 0.5 μg of oligo(dT)$_{12-18}$ primer with 1 μg of total RNA and heating to 65°C for 5 minutes, then immediately chilling on ice. After addition of a 200-U/μL aliquot of M-MLV reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA), the mixture was heated to 37°C for 1 hour. PCR was done using 1 μL of cDNA in 25 μL PCR reaction by using primers for β-actin: 5′-GATTACTGCTCTGGCTCCTAGC-3′ (sense) and 5′-GACTCATCGTACTCCTGCTTGC-3′ (antisense), MMP-9: 5′-CTCTACAGAGTCTTTGAGTCCGGCAG-3′ (sense) and 5′-TAC GGAACTTCCAGTACCAACCGTC-3′ (antisense), and MMP-2: 5′-ACACTGGGACCTGTCACTCC-3′ (sense) and 5′-CCAAATAAACCGGTCCTTGA-3′ (antisense). Samples were run twice using a cycler system (Cepheid Smart Cycler System; Cepheid Inc., Sunnyvale, CA). A standard curve of fivefold serial dilutions of cDNA was used with a correlation coefficient ($R^2 > 0.99$). The fold difference in expression of MMP-2 and -9 in 1 day and 5 days p.i. versus normal cornea was calculated after each C_T was normalized to β-actin.

Zymography

Gelatin zymography was performed as described before[12] on individual normal corneas (n = 5/time/group) at 1 and 6 days p.i. from B6 and BALB/c mice. Briefly, corneal samples were homogenized in 250 μL lysis buffer (25 mM Tris-HCl, pH 7.5; 100 mM NaCl; and 1% v/v Nonidet P-40) containing 10 μg/mL aprotinin, 2 μg/mL leupeptin, and 4 mM benzamidine. Samples were frozen at −80°C and when used were diluted in ×4 sample buffer to appropriate concentration (standards at 0.5 ng/lane and corneal samples at 100 μg/lane) and were loaded onto 10% polyacrylamide gels containing 0.1% gelatin. Gels were run for 90 minutes at 100 v, then the separated enzymes were renatured by incubating the gels in 2.5% Triton X-100 for 30 minutes at room temperature to remove SDS, then overnight (16 to 20 hours) in developing buffer with calcium for gelatin digestion. To visualize the activated MMPs, gels were stained in 0.5% stain (Coomassie brilliant blue; Sigma, St. Louis, MO) for 1 hour, then destained in 10% methanol and 5% acetic acid until clear bands showing enzyme activity were visible. MMP-2 and -9 standards (R&D Systems, Minneapolis, MN) were run in parallel to compare digested bands. To confirm that the bands were metalloproteinases, separate gels were incubated overnight in buffer lacking calcium and containing 20 mM EDTA. Gels were incubated overnight at 37°C and, after the incubation period, were stained and destained as described above.

Antibody and Recombinant Protein Treatment

To neutralize MMP-9 in susceptible B6 mice, 10 μg of mouse anti-human MMP-9 antibody (Ab) (Calbiochem, San Diego, CA) was injected subconjunctivally 1 day before infection. An additional 150 μg was injected intraperitoneally (i.p.) on the day of infection (day 0), and on days 2 and 4 p.i. Control mice were injected with mouse IgG. Resistant BALB/c mice were given 1 μg of rMMP-9 protein (R&D Systems) subconjunctivally 1 day before infection and an additional 1 μg i.p. on both 1 and 3 days p.i. Control BALB/c mice received similar PBS injections.

Myeloperoxidase Assay

An myeloperoxidase (MPO) assay quantitated PMN in the cornea of Ab- versus control-treated B6, recombinant- versus PBS-treated BALB/c, and FVB (WT) versus MMP-9$^{-/-}$ mice (n = 5/group) at 5 days p.i. as described.[11] One unit of MPO activity is equivalent to ~2 × 10^5 PMN/mL.[19]

LC Staining

Epithelial sheets were harvested at 6 days p.i. from IgG versus Ab neutralized B6, PBS versus rMMP-9-treated BALB/c, and FVB versus MMP-9$^{-/-}$ mice (n = 3/group) and stained with adenosine diphosphate (ADP) as described.[20,21] Representative areas were photographed at ×25 on a microscope (Axiophot; Carl Zeiss, Morgan Instruments, Cincinnati, OH). LCs were counted on prints of the photographed fields and numbers are represented as number of LC per field ± SEM.

Cell Culture and Chemotaxis

LC Culture. LCs (XS52 cell line) were cultured (all reagents from Gibco, Carlsbad, CA) in complete RPMI (with 10% fetal calf serum [FCS]) supplemented with 0.5 ng/mL murine recombinant granulocyte macrophage–colony-stimulating factor and 5% fibroblast (NS line, cultured in complete Roswell Park Memorial Institute medium 1640 [RPMI] with 10% FCS) supernatant. Cells that lifted from confluent cultures were collected and resuspended in migration medium (RPMI 1640 with 0.5% BSA) to a concentration of 1 × 10^6 cells/mL. These cells expressed DEC-205 and did not stain with antibodies against B7 to 1 or B7 to 2[10] costimulatory molecules, indicating that they are immature (data not shown).

Chemotaxis Assay. A 48-well microchemotaxis chamber (Neuroprobe, Bethesda, MD) with an 8-μm-pore-size filter (coated [0.5 mg/mL] with collagen IV) separating the upper and lower chambers was used to test the chemotactic effect of MMP-9. RPMI 1640/0.5% BSA served as the migration medium and also provided a negative control. A total of 30 μL each of RANTES (positive control, 10^{-6} M; Sigma), RPMI 1640/0.5% BSA, (negative control), or MMP-9 (0.1 and 0.01 μg) were put in the lower wells, and 50 μL of an LC cell suspension (50,000 cells/well) was placed in the chamber upper wells and incubated at 37°C with 5% CO_2 for 30 to 45 minutes. Nonmigrated cells were wiped off the back side of the filter after staining (Diff-Quick; Fisher Diagnostics, Middletown, VA). The stained filter was placed on a microscope slide and allowed to dry. Migrated cells were counted for each well, and data were expressed as the mean number of migrating cells ± SEM.

PMNs were induced into the peritoneal cavity of B6 and BALB/c mice, harvested as described,[22] diluted to 10,000 to 20,000 cells/well (purity >90%) and similarly tested in chemotaxis assays by using uncoated polycarbonate filters with a 3.0-μm pore size. The chemoattractants were similar to above, with the exception that MIP-2 (0.001 μg; R&D Systems) was used as the positive control. Migrated cells were counted as above.

Light Microscopy and Transmission Electron Microscopy

Eyes were enucleated from FVB and MMP-9$^{-/-}$ mice at 5 days p.i. (n = 2/group), rinsed in PBS, and fixed in 1% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M Sorensen phosphate buffer (pH 7.4; 1:1:1) at 4°C for 3 hours. Specimens were dehydrated in graded ethanols and embedded in plastic, sectioned, and photographed as described.[23]

Immunostaining

Eyes were enucleated at 5 days p.i. from FVB and MMP-9$^{-/-}$ mice (n = 3/group), along with the uninfected contralateral eyes, embedded in optimum cutting temperature compound, and frozen in liquid nitrogen. Sections (10 μm) were collected onto poly-lysine coated slides and were placed at 37°C for 2 to 4 hours. Slides were fixed in cold acetone for 2 minutes and air-dried. Nonspecific reactivity was blocked with 0.01M PBS containing 1% BSA, 0.05% Tween 20, and 2% normal

TABLE 1. Expression Pattern of Genes in Cornea

Gene Class		Average Fold Differences	
Access No.	Name	BALB/c: 1 day p.i. vs. *n*	B6: 1 day p.i. vs. *n*
NM_008610	MMP-2	1.5	1.2
NM_010809	MMP-3	3.4	10.8
NM_008611	MMP-8	3.4	4.6
NM_013599	MMP-9	5.5	32.8
NM_019471	MMP-10	9	10.2
NM_008606	MMP-11	2.2	1.3

Affymetrix microarray x3 separate experiments. p.i., postinfection.

goat serum for 30 minutes at room temperature (RT). Slides were incubated overnight at 4°C with rabbit anti-type IV collagen Ab (Research Diagnostics, Flanders, NJ) diluted 1:50 with blocking buffer. After a 30-minute incubation with 0.3% hydrogen peroxide, biotin conjugated goat anti-rabbit IgG (H+L) secondary Ab (1:100; Jackson ImmunoResearch, West Grove, PA) with 0.1 M Tris-HCl was applied, and the slides were incubated for 1 hour at RT. Sections were washed and incubated with a 1:100 dilution of peroxidase conjugated modified egg white avidin (ExtrAvidin; Sigma) for 30 minutes, then positive staining was visualized with metal-enhanced diaminobenzidine (Pierce, Rockford, IL).

Immunostaining also identified the cell source of MMP-9. These eyes from B6 ($n = 3$ eyes) mice were enucleated at 3 days p.i., fixed in 0.01 M sodium periodate, 0.05 M lysine, and 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) overnight at 4°C and processed for paraffin embedding as described before.[24] Slides were processed, and nonspecific binding was blocked with 1.5% normal rabbit serum and 2% BSA in PBS-saponin, drained dry, and incubated with a goat anti-human MMP-9 Ab (1:200) for 2 hours, and then with a biotinylated secondary rabbit anti-goat Ab (1:100) for 1 hour. Slides were rinsed and incubated with modified egg white avidin (1:100; ExtrAvidin; Sigma) for 30 minutes in a moist chamber, rinsed with Tris-HCL buffer and drained dry. Control sections were incubated similarly but with the omission of the primary Ab. For routine morphology, slides were deparaffinized and rehydrated as for immunostaining, allowed to dry, stained with 1% methyl green for 2 minutes at RT, rinsed, and mounted as above.

ELISA Analysis of Cytokines

Cytokine protein levels were determined by using ELISA kits (R&D Systems). Corneas were harvested at 5 days p.i. ($n = 5$/group/time), individually homogenized in 1.0 mL PBS with 0.5% hexadecyltrimethylammonium bromide (HTAB) with a glass micro tissue grinder (Fisher Scientific, Hanover Park, IL) and centrifuged at 13,000*g* for 10 minutes. Sample supernatants were diluted (1:10 for IL-1β; 1:50 for MIP-2), and a 50-μL aliquot was assayed. The reported sensitivity of these assays is <1.5 pg/mL for MIP-2, and <3.0 pg/mL for IL-1β.

Statistical Analysis

The difference in clinical score at each experimental time point between two groups was tested by the Mann–Whitney *U* test. An unpaired, two-tailed Student's *t*-test was used to determine the significance for mRNA, MPO, LC number, and for chemotaxis data. Differences were considered significant at $P \leq 0.05$. Each experiment was repeated at least once, and data from a typical experiment are shown.

RESULTS

Gene Expression

Analysis of gene array data for MMPs in cornea at 1 day p.i. (Table 1), showed that fold increases in gene expression were greatest between the two mouse strains for MMP-9 (sixfold more in B6 over BALB/c mouse cornea), with little difference between the others (MMP-2, -3, -4, -10. or -11).

RT-PCR

RT-PCR (Figs. 1A, 1B) confirmed the gene array data for MMP-9 (Fig. 1B) at 1 day p.i. ($P = 0.004$), which was elevated when compared with levels in the normal cornea and also showed that, at 5 days p.i., there was no difference in mRNA levels between the two groups ($P = 0.31$). RT-PCR did not confirm the pattern of MMP-2 (similar fold increases over normal in both groups of mice) seen in gene array analysis, however. MMP-2 was decreased in both groups when compared with normal levels ($P = 0.01$). At 5 days p.i., BALB/c over B6 mice had significantly ($P = 0.0004$) increased levels of mRNA for MMP-2.

Zymography

Zymography (Fig. 2) showed that the normal cornea of B6 and BALB/c mice did not exhibit detectable latent or active enzyme levels of MMP-9 or MMP-2 (lanes 3 and 4) at the protein concentration tested. At 1 day p.i., both groups of mice showed both latent and active MMP-9 (lanes 5 and 6) but not MMP-2. By 6 days p.i., (lanes 7 and 8) BALB/c mice showed similar levels of MMP-9 in the cornea when compared with 1 day p.i., while activity appeared increased in the B6 cornea. The digested bands were confirmed as metalloproteinases (including degraded MMP-9 in the B6 cornea), because they were not detectable (lane 9, from a separately run gel) when calcium was removed by using EDTA.

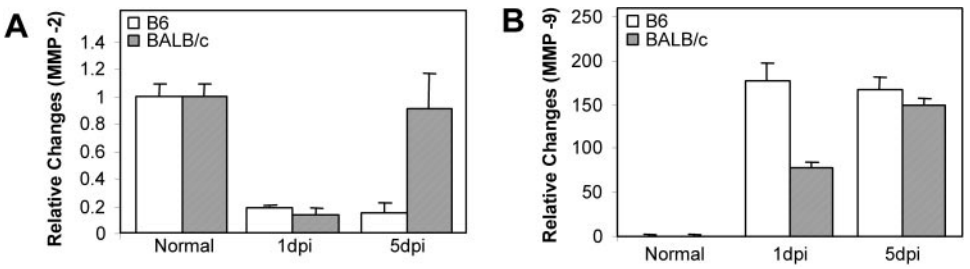

FIGURE 1. Real-time RT-PCR for MMP-2 (**A**) and MMP-9 (**B**) mRNA levels in B6 vs. BALB/c normal cornea and at 1 and 5 days p.i. The MMP-9 data confirms the pattern of gene expression shown in Table 1 at 1 day p.i. and were significant ($P = 0.004$); differences were not significant at 5 days p.i. ($P = 0.31$). The MMP-2 data does not confirm the pattern shown in Table 1 at 1 day p.i.; the data were significant at 1 day ($P = 0.01$) and 5 days ($P = 0.004$) p.i.

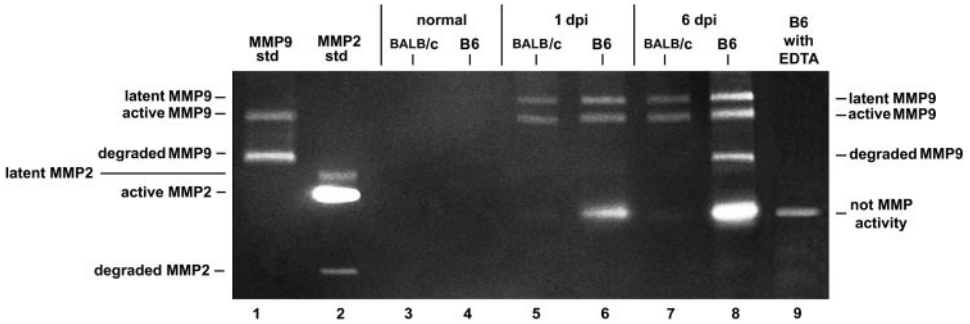

FIGURE 2. Zymography of MMP-2 and MMP-9 activity in normal cornea and at 1 and 6 days p.i. No enzyme activity was detected in the normal cornea of either mouse strain. At 1 day p.i., MMP-9 activity was detected in both groups of mice (appeared slightly increased in B6 over BALB/c). By 6 days p.i., activity was unchanged in BALB/c cornea but enhanced in the cornea of B6 mice. No MMP-2 (latent or active) was detected in either group of mice. *Lane 9* (from a separate gel) shows that the detected latent and active enzyme activity for MMP-9 are Ca+ dependent (confirming that they are metalloproteinases) and that no activity is detected when Ca+ is chelated with EDTA. MMP standards in *lanes 1 and 2* (0.5 ng/lane) and corneal samples in *lanes 3 to 9* (100 μg/lane).

Clinical Score

Based on these data, suggesting that MMP-9 gene and protein levels were greater in the cornea of susceptible versus resistant mice, BALB/c mice were injected with rMMP-9, whereas B6 mice were injected with an anti-MMP-9 Ab; MMP-9 deficient mice also were tested and compared with WT controls. Clinical scores (Figs. 3A, 3B, 3C) showed that BALB/c mice injected with rMMP-9 versus PBS had significantly increased disease at 1, 3, and 5 days p.i., $P = 0.01$, 0.004, and <0.0001, respectively (Fig. 3A). In B6 mice injected with Ab against MMP-9, the reverse was apparent. Less disease was seen in these mice compared with IgG-injected control animals at 1, 3, and 5 days p.i., $P = 0.02$, 0.05, and 0.004, respectively (Fig. 3B). Infection of MMP-$9^{-/-}$ versus WT FVB mice confirmed the Ab neutralization data and showed that MMP-$9^{-/-}$ mice showed a decreased level of disease; none of the infected corneas of knockout mice had perforated at 5 days p.i., while a significant number of WT corneas had ($P = 0.009$, 0.0008, and 0.0004, for 1, 3, and 5 days p.i., respectively; Fig. 3C).

LC

Because LCs are one of the most efficient types of migrating antigen presenting cells, we tested whether injecting rMMP-9 into BALB/c, Ab neutralizing MMP-9 in B6 and MMP-9 deficiency in the knockout mice changed the number of LCs in the cornea at 6 days p.i. (Figs. 4A, 4B, 4C). Injection of rMMP-9 versus PBS significantly increased the number of LC in the infected BALB/c cornea (Fig. 4A, $P < 0.0001$). In MMP-9 Ab neutralized and knockout animals, LC were reduced significantly compared with their appropriate controls (Fig. 4B, $P < 0.0001$ and Fig. 4C, $P = 0.0003$).

PMN

Because PMNs are the most numerous cells responding to *P. aeruginosa* corneal infection, they were quantitated by using an MPO assay at 5 days p.i. in the cornea of the three groups of mice (rMMP-9, Ab-neutralized, and knockout mice) (Figs. 5A, 5B, 5C). PMNs were increased in the BALB/c cornea (Fig. 5A, $P < 0.0001$) after rMMP-9 treatment. In contrast, in Ab neutralized B6 mice (Fig. 5B) and in knockout (Fig. 5C) over their respective controls, MPO activity was decreased significantly (Fig. 5B, $P = 0.01$; Fig. 5C, $P = 0.03$).

Chemotaxis

We tested whether MMP-9 induced chemotaxis in the two cell populations (Figs. 6A, 6B, 6C). When compared with the neg-

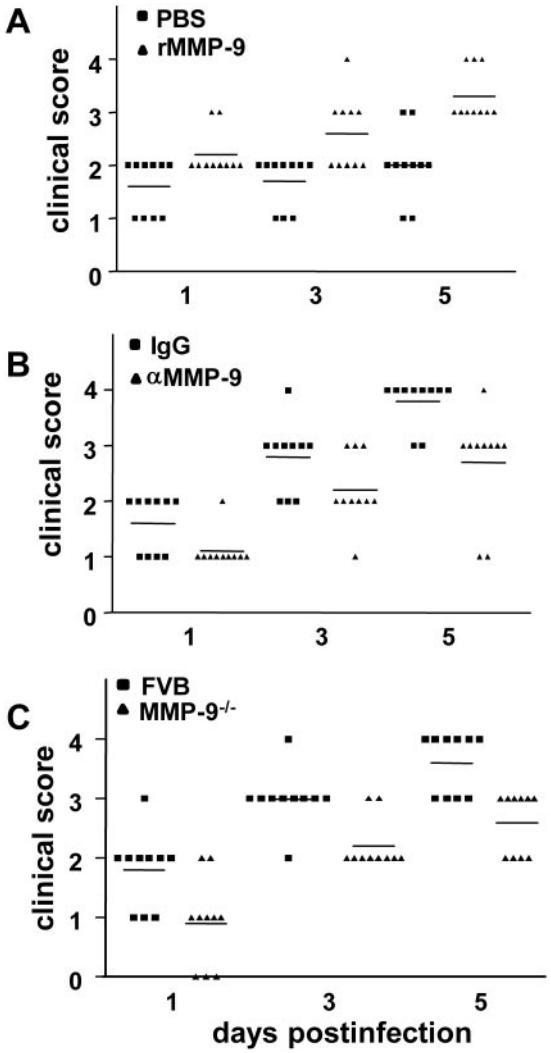

FIGURE 3. Clinical scores are shown for rMMP-9 treated BALB/c mice (**A**), for anti-MMP-9 Ab-treated B6 mice (**B**), and for MMP-$9^{-/-}$ and FVB WT controls (**C**). Significant differences were detected at 1, 3, and 5 days p.i. (**A**) $P = 0.01$, 0.004, <0.0001; (**B**) $P = 0.02$, 0.05, 0.004; (**C**) $P = 0.009$, 0.0008, 0.0004.

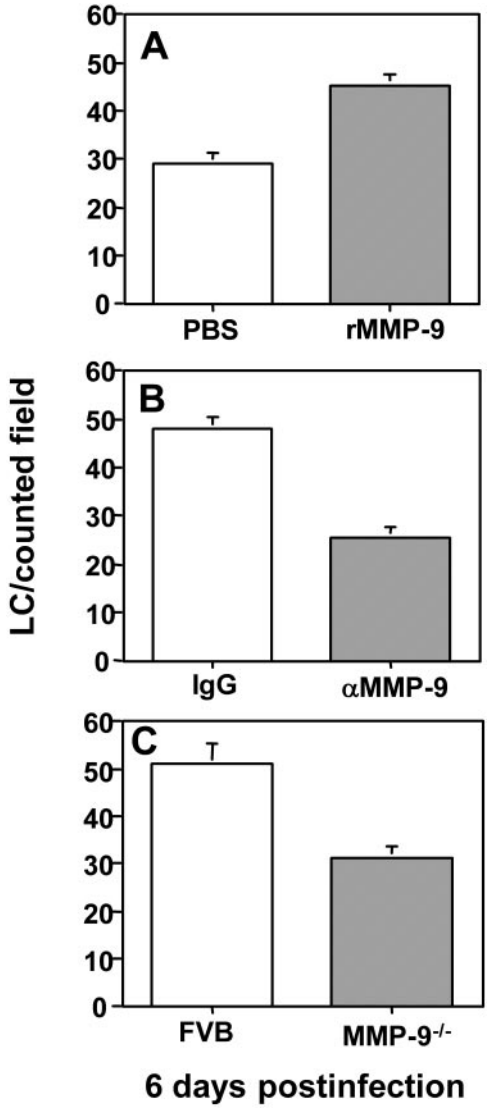

FIGURE 4. Quantitation of LC (stained by ADPase) in BALB/c mice treated with rMMP-9 (**A**), B6 mice treated with anti-MMP-9 Ab (**B**), and MMP-9$^{-/-}$ mice and their WT control (**C**). LC were significantly increased ($P < 0.0001$) in BALB/c cornea after rMMP-9 treatment; B6 mice treated with aMMP-9 Ab showed a decreased ($P < 0.0001$) number of LC over controls. MMP-9$^{-/-}$ mice also showed fewer ($P = 0.0003$) LC than FVB WT mice. Fifteen fields were counted for each group.

ative media control MMP-9 at high (0.1 μg) or low (0.01 μg) concentration had no chemotactic activity for LC (Fig. 6A, $P = 0.64$ and 0.1). RANTES served as the positive control. PMN from B6 and BALB/c mice were tested similarly for chemotaxis to MMP-9 (Fig. 6B upper and lower graphs). When compared with the media control, there was no difference in chemotaxis of PMN at either concentration of MMP-9 (as above) for either B6 ($P = 0.13$ and 0.41) or BALB/c ($P = 0.2$ and 0.3) mice. MIP-2 was the positive control.

Light Microscopy and Transmission Electron Microscopy

The corneas of infected MMP-9$^{-/-}$ and WT (FVB) control mice were examined microscopically at 5 days p.i. (Figs. 7A, 7B, 7C, 7D). MMP-9$^{-/-}$ mice (Fig. 7B) exhibited a more intact basal lamina beneath the peripheral corneal epithelium than WT mice (Fig. 7A). PMNs were numerous, and when examined at the TEM level, cells were observed closely associated with a more intact basal lamina (Fig. 7D) in the knockout mouse cornea and with regions where the basal lamina was interrupted in the FVB (Fig. 7C) control.

Immunostaining

To examine collagen IV integrity, immunostaining was performed; the data are shown in Figures 8A, 8B, 8C, 8D). Staining for collagen IV was more even and uninterrupted in the peripheral cornea in the MMP-9$^{-/-}$ (Fig. 8B) over WT (Fig. 8A) cornea compared favorably with the immunostaining pattern in the normal, uninfected cornea (Fig. 8C). No staining was observed in the absence of the primary antibody (Fig. 8D).

Immunostaining was used to determine the cell source of MMP-9 in B6 cornea at 3 days p.i. (Figs. 9A, 9B, 9C, 9D, 9E, 9F). Staining for MMP-9 was associated with cells marginated within blood vessels in the paracentral cornea (Figs. 9A, 9C) and in the anterior chamber (Fig. 9E). Routine morphology of these two

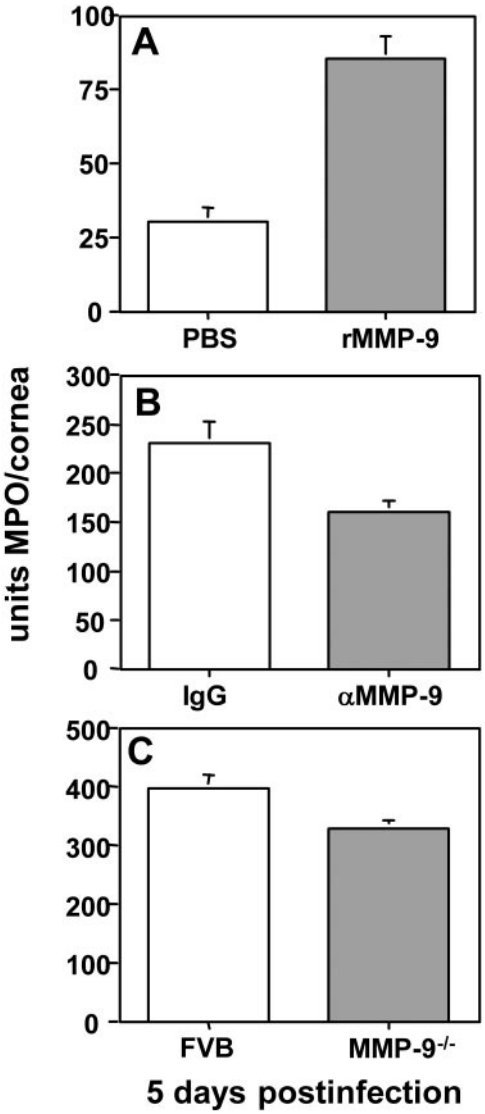

FIGURE 5. MPO (PMN) activity in the cornea 5 days p.i. in BALB/c mice after rMMP-9 treatment (**A**), B6 mice after anti-MMP-9 Ab treatment (**B**), and in MMP-9$^{-/-}$ vs. control mice (**C**). (**A**) PMN were increased ($P < 0.0001$) in BALB/c cornea after rMMP-9 over control treatment; (**B**) PMN were reduced significantly ($P = 0.01$) after anti-MMP-9 Ab treatment of B6 mice and (**C**) in MMP-9$^{-/-}$ over WT controls ($P = 0.03$)

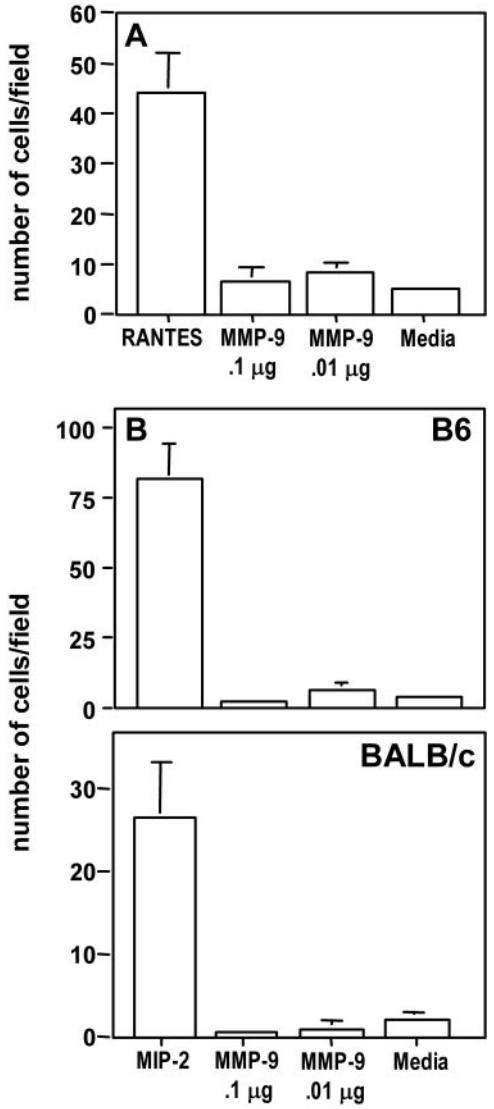

FIGURE 6. (**A**) Chemotaxis assay for cell migration of LC (cell line XS52). RANTES was the positive and media were the negative control. MMP-9 had no significant chemotactic activity for LC ($P = 0.64$ and 0.1 compared with media control at 0.1 and 0.01 mg, respectively). (**B**) Chemotaxis assay for cell migration of PMN isolated from B6 vs. BALB/c mice. MIP-2 was the positive and media were the negative control. MMP-9 at 0.1 and 0.01 mg/mL showed no significant PMN chemotactic activity in B6 (**B**, *upper panel*; $P = 0.13$ and 0.41) or BALB/c (**B**, *lower panel*; $P = 0.2$ and 0.3) mice over media control.

areas (Figs. 9C, 9E) showed the distinct multi-lobulated pattern characteristic of PMN nuclei, confirming their identity. Control sections in which the primary Ab was omitted from the reaction were negative for staining (Figs. 9B, 9D, 9F).

ELISA

To test MMP-9 interactions with cytokines and chemokines in modulating corneal disease, protein analysis of IL-1β and MIP-2 was done 5 days p.i. after rMMP-9 treatment of BALB/c (Figs. 10A, 10D), Ab neutralization of B6 (Figs. 10B, 10E) and in MMP-9$^{-/-}$ (Figs. 10C, 10F) and appropriate control groups of mice. Injection of rMMP-9 in BALB/c mice significantly enhanced both IL-1β (Fig. 10A) and MIP-2 (Fig. 10D) levels in the infected cornea ($P < 0.0001$ for both). Ab neutralization of MMP-9 in B6 mice significantly decreased levels of both IL-1β (Fig. 10B, $P = 0.008$) and MIP-2 (Fig. 10E, $P = 0.02$). Endogenous absence of MMP-9 also resulted in reduced IL-1β (Fig. 10 C, $P < 0.0001$) and MIP-2 (Fig. 10F, $P = 0.03$) levels in knockout compared with WT mice.

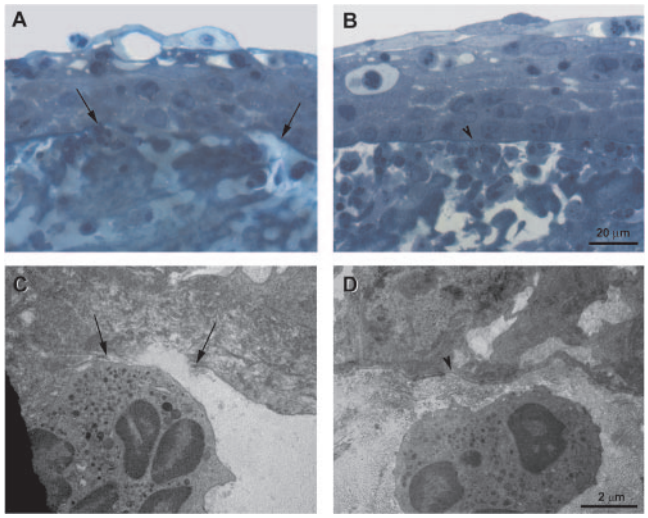

FIGURE 7. Light (**A**, **B**) and transmission EM (**C**, **D**) at 5 days p.i. The peripheral cornea of a FVB WT mouse (**A**, **C**) shows disrupted basement membrane (*arrows*) associated with an inflammatory cell infiltrate. The MMP-9$^{-/-}$ mouse cornea (**B**, **D**) exhibits an intact basement membrane (*arrowhead*), despite the heavy cellular (mostly PMN) infiltrate. Magnification: (**A**, **B**) ×550; (**C**, **D**) ×6000

DISCUSSION

MMPs are secreted enzymes with major functions in the degradation and remodeling of extracellular matrix[25] and in the pathology of inflammatory diseases of the connective tissues.[26] In the eye, corneal degradation and ulceration is often observed after exposure to infectious agents such as *P. aeruginosa* or herpes simplex virus, after chemical or thermal injury to the cornea, or in association with diseases such as rheumatoid arthritis or vitamin A deficiency.[27–32] In *P. aeruginosa* corneal infection, Kernacki et al.[12] showed that when compared with susceptible (cornea perforates) nonimmunized mice, resistant immunized animals displayed lower levels of

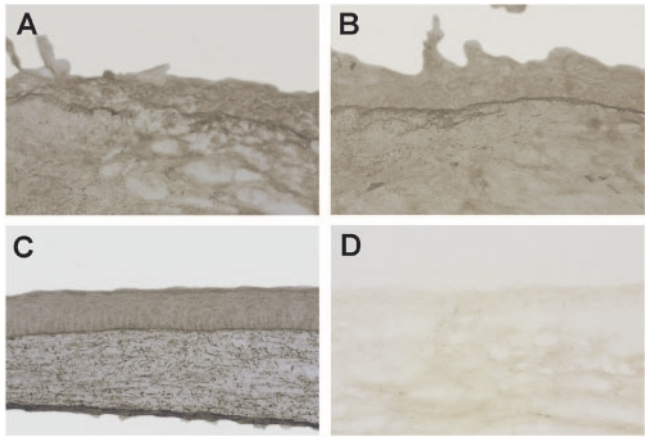

FIGURE 8. Staining for collagen type IV in FVB WT (**A**) vs. MMP-9$^{-/-}$ mouse (**B**) cornea at 5 days p.i. Type IV collagen is disrupted in the basement membrane of the WT (**A**) vs. the knockout mouse (**B**) cornea. Type IV collagen also shown in the cornea of an uninfected, control FVB mouse (**C**). An antibody control (normal serum) for immunostaining (**D**) is shown using 5 day p.i. cornea. No staining was detected. Magnification, ×75.

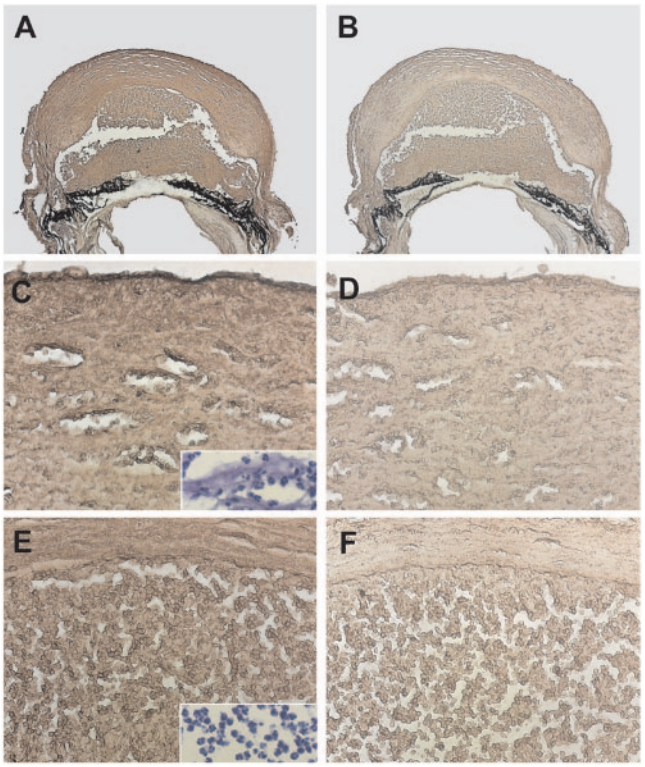

FIGURE 9. Staining for MMP-9 in infected cornea (**A**–**F**). Staining in the B6 mouse infected eye at 3 days p.i. (**A**, **C**, **E**) vs. control sections (**B**, **D**, **F**) shows staining for MMP-9 is the stroma and associated with cells marginated within vessels in the peripheral cornea and in the anterior chamber. Stromal vessels are shown in (**C**) and (**E**), the anterior chamber is shown. *Insets* (**C**, **E**) show that the cells in the stroma and anterior chamber are PMNs. Control sections in which the primary antibody was omitted show no specific staining. Magnification: (**A**, **B**) ×15; (**C**, **D**, **E**, **F**) ×100.

both the active and latent forms of MMP-9. In the present study, gene expression data were analyzed to provide a clue as to which of the MMPs may be of importance in the genetically susceptible B6 versus resistant BALB/c mouse models of *P. aeruginosa* infection and to focus on the immune-associated functions of the MMPs.

The greatest fold increases in MMP-9 were detected by gene array in the infected cornea of B6 (susceptible) over BALB/c (resistant) mice, and the data were confirmed by RT-PCR. Furthermore, latent and active forms of MMP-9 only were detected in the cornea by using zymography. This showed that in the infected cornea, B6 mice appeared to have increased levels of both forms of the enzyme when compared with BALB/c mice. MMP-2 was not detected and, although unlikely, might reflect that the amount of protein loaded was insufficient. Nonetheless, based on these data, we used MMP-9 Ab neutralization and injection of rMMP-9 protein, as well as MMP-9$^{-/-}$ mice to explore further the contribution of MMP-9 to corneal immunopathology. By using a clinical scoring system, rMMP-9–injected resistant BALB/c mice exhibited worsened disease, while Ab-neutralized B6 mice and MMP-9$^{-/-}$ mice had less disease than control groups. These in vivo data are consistent with an in vitro rabbit corneal fibroblast model in which cells treated with pseudomonal exoproteases or lipopolysaccharide (LPS) expressed secreted MMP-9.[33] Various other studies have indicated that MMP-9 (or gelatinase B) is a key player in the mechanisms underlying immunopathology. Active forms of gelatinases 2 and 9 were detected in endogenous corneal ulcer cases in tear fluid, and it was concluded that the active form of gelatinase expression may be related to the severity of ulceration.[34] In other infectious disease models using *Candida albicans*, in vitro infection of human oral mucosal cells induced MMP-2 and MMP-9 gene activation, but importantly as in this study, only the levels of active MMP-9 rose.[35] In marked contrast to these studies, Lee et al.[36] showed that MMP-9, among several other MMPs, is needed for resistance to *P. aeruginosa* corneal and lung infection by using TIMP-1 mutant mice. The conclusion that MMP agonists may be of therapeutic benefit to augment resistance to the bacterium are not consistent with this report nor with extensive past studies with murine and other corneal experimental bacterial infection models.[4]

Whether MMP-9 was crucial for LC migration also was tested. LCs are dendritic cells that capture foreign antigens and migrate with them to regional lymph nodes where they are presented to naïve T cells. These cells have been shown to be critical in the innate immune response to *P. aeruginosa,*[8,10] and increased numbers of cells in the cornea have been associated with corneal perforation. When using the same paradigm of testing as described above, MMP-9 Ab neutralized and knockout mice had fewer LCs in the infected cornea and BALB/c mice injected with rMMP-9 had more, suggesting that MMP-9 may be involved in LC transmigration into the cornea. When tested, however, soluble MMP-9 did not have a chemotactic effect. In this regard, others have reported that membrane-associated MMP-2 and -9 rather than soluble MMPs appear involved in cell migration.[37] Membrane-type MMPs (MT1 to 6) have been detected in the cornea of mice infected with *P. aeruginosa*, and peak expression of all three showed a good correlation with inflammation and stromal destruction,[14,15] but their role in LC chemotaxis remains untested in the cornea. On the other hand, LC (epidermal), as well as other epidermal cells have been shown to be capable of producing MMP-9 and may themselves contribute to proteolysis associated with transmigration of LC in the induction phase of contact dermatitis.[38] Also, it has been shown that MMP-9 may be involved in LC morphologic and phenotypic maturation.[39]

In inflammation, leukocytosis and blood-cell homeostasis are crucial in host defense. Gelatinase B assists in the peripherilization of leukocytes in response to chemokines into sites of inflammation.[40] Use of MMP-9 neutralization and MMP-9$^{-/-}$ animals showed that PMNs are decreased in these two versus control mouse groups. Consistent with these data, PMNs were increased in the cornea of rMMP-9 injected mice, suggesting that MMP-9 contributes to the regulation of PMN transmigration into the cornea. The heavy inflammatory infiltrate, consisting mainly of PMN, also was confirmed by LM and TEM in the cornea of knockout versus control mice and also provided evidence that a more intact basal lamina was present in the cornea of MMP-9$^{-/-}$ versus WT animals. Immunostaining for collagen IV confirmed an interrupted basal lamina in the WT versus MMP-9$^{-/-}$ mouse cornea, possibly contributing to PMN transmigration into the cornea.

Gelatinase B was first identified in PMN[41] and later in monocytes and macrophages.[42] In general, these types of cells secrete gelatinase A constitutively, but the expression of gelatinase B requires induction by adequate triggering.[43] IL-1, lectins, double-stranded RNA, and LPS are soluble inducers of MMP-9.[43,44] In the present study, immunostaining showed that PMNs in the cornea (and anterior chamber) were a source of MMP-9 (gelatinase B). PMN gelatinase B has been shown to be released in the extracellular medium as a latent proform and then to be activated mainly by PMN elastase in a model of PMN migration across a Matrigel basement membrane matrix coated onto a filter in a Boyden chamber.[45] However, others have used gelatinase-B-deficient mice and intratracheal LPS challenge and have shown that gelatinase B is not required for PMN emigration in the lungs, the peritoneum, and the skin.[46]

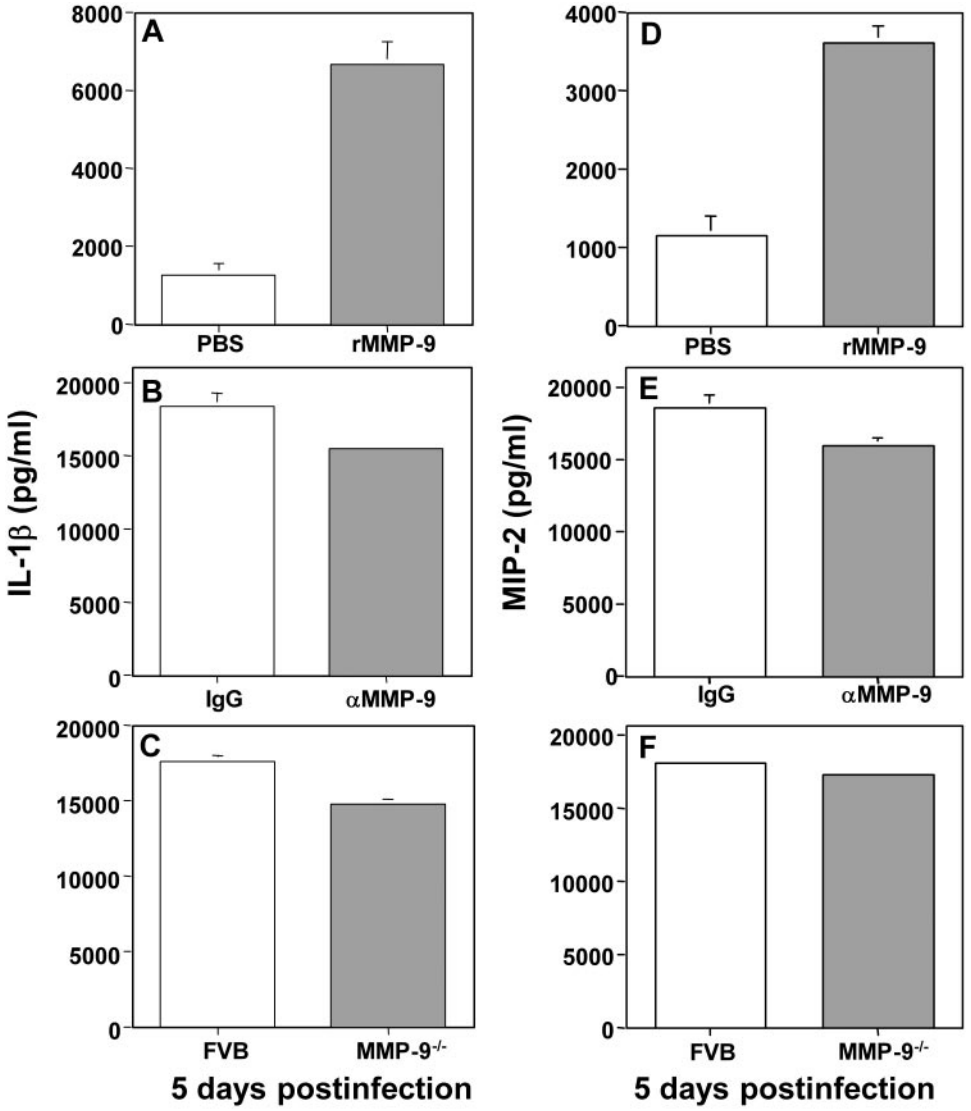

FIGURE 10. ELISA for IL-1β and MIP-2 (**A**–**F**). IL-1β and MIP-2 levels were significantly elevated after rMMP-9 treatment (**A**, **D**; $P < 0.0001$ for both); cytokine levels were reduced significantly after MMP-9 neutralization (**B**, **E**; $P = 0.008$ and $P = 0.02$) and with endogenous absence of the metalloproteinase. $N = 5$ mice/group/time/cytokine. (**C**, **F**; $P < 0.0001$ and $P = 0.03$).

MMP-9 has been shown to function as a regulator and effector in leukocyte biology, because it is able to activate pro-IL-1β into active IL-1β.[47] IL-1β and MIP-2 are important in the susceptibility response of B6 mice[48] and thus were examined in MMP-9–Ab-neutralized and gene-knockout mice. Both groups when compared with control groups had decreased levels for each cytokine that were significant statistically. Nonetheless, the reduced chemokine level in FVB versus MMP-9$^{-/-}$ mice was modest and so the biological relevancy may be suspect. In contrast, rMMP-9-treated mice had enhanced levels for IL-1β and MIP-2, suggesting that MMP-9 proteolysis modulates cytokine expression in the cornea. Because gelatinase B and cytokines both function in the extracellular milieu and might be secreted together at particular sites within tissues, it is not unexpected that they would interact. In fact, others have shown that treatment of mice with an anti-IL-1β antibody before *P. aeruginosa* challenge resulted in a significant reduction of MMP-9. Together with the data from the present study, the evidence suggests that, in the *P. aeruginosa* infected cornea, there is a reciprocal interaction between the two.[49]

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