MAJOR ARTICLE

Matrix Metalloproteinase-13 as a Target for Suppressing Corneal Ulceration Caused by *Pseudomonas aeruginosa* Infection

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Purpose. *Pseudomonas aeruginosa* keratitis is characterized by severe corneal ulceration. This study investigated whether matrix metalloproteinase-13 (MMP13) is involved in *P. aeruginosa*–induced corneal ulceration and whether it therefore can be targeted for preventing *P. aeruginosa* keratitis.

Methods. MMP13 expression in *P. aeruginosa*–infected C57BL/6 mouse corneas was assessed by quantitative polymerase chain reaction and immunohistochemistry analyses. An MMP13-inhibitor (MMP13i) was either injected subconjunctivally prior to or coapplied topically with gatifloxacin 16 hours after infection. Disease severity was assessed by corneal imaging, clinical scoring, bacterial burden, neutrophil infiltration, and CXCL2 expression. Corneal damage and infiltration were also determined by immunohistochemistry analysis and whole-mount confocal microscopy.

Results. *P. aeruginosa* infection induced an increased expression of MMP13 in mouse corneas from 6 to 24 hours after infection in a Toll-liked receptor 5–dependent manner. Subconjunctival injection of MMP13i prior to *P. aeruginosa* inoculation significantly decreased keratitis severity, as evidenced by preserved epithelium integrity and intact basement membrane, leading to reduced bacterial dissemination to the stroma. Furthermore, topical coapplication of MMP13i with gatifloxacin greatly improved disease outcomes, including accelerated opacity dissolution; decreased inflammation, cellular infiltration, and collagen disorganization; and basement membrane preservation.

Conclusions. Elevated MMP13 activity may contribute to *P. aeruginosa* keratitis through basement membrane degradation, and its inhibition could potentially be used as an adjunctive therapy to treat microbial keratitis and other mucosal infections.

Keywords. bacterial keratitis; MMP13; basement membrane; Toll-like receptors; corneal ulceration.

Bacterial keratitis is a sight-threatening disease that is most commonly associated with extended contact lens wear [1]. *Pseudomonas aeruginosa* is the common causative agent of keratitis [2]. To cause keratitis, microbes must traverse the epithelial layer and cross the basement membrane to reach the stroma [3, 4]. *Pseudomonas aeruginosa* keratitis usually presents as a rapidly progressing condition, with distinct inflammatory epithelial edema and stromal ulceration, that can lead to significant stromal tissue destruction and loss of vision [5]. These consequences are largely caused by the host's inflammatory responses, although bacterial toxins and exoproducts may contribute to *P. aeruginosa* keratitis [5]. Hence, it is of importance to identify pathogenic factors and to delineate their mechanisms of action, which may lead to the development of new therapeutic strategies for treating bacterial keratitis.

Innate immunity is the first line of defense against a wide range of pathogens at mucosal surfaces. Studies from our laboratory demonstrated that the activation of Toll-like receptor 5 (TLR5) with flagellin prior to pathogen inoculation significantly improves the clinical outcomes of microbial keratitis in C57BL/6 mouse corneas [6, 7]. This protection is related to its ability to enhance innate responses, as manifested by dampened proinflammatory cytokine expression and augmented antimicrobial peptide production in response to infection,

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particularly in corneal epithelial cells [7–9]. To comprehensively identify genes altered by flagellin pretreatments, we recently performed a whole genome complementary DNA microarray and found that matrix metalloproteinase-13 (MMP13) is one of the most profoundly affected genes at 6 hours after infection, and flagellin-pretreatment significantly dampens infection-induced MMP13 expression. Hence, a decrease in MMP13 activity might contribute to flagellin-induced protection against *P. aeruginosa* keratitis [10, 11].

MMP13 belongs to a large family of zinc-dependent neutral endopeptidases that are collectively capable of degrading extracellular matrix. MMP13 is one of the collagenases that degrade native collagen fibrils in vivo and is thought to execute the rate-limiting function in extracellular matrix reorganization, which is essential for morphogenesis and tissue remodeling [12] and corneal wound healing [13]. While all collagenases cleave type I, II, and III collagens, MMP13 (collagenase-3) cleaves collagen type IV, X, and XIV, as well [12, 14, 15]. Because of its exceptionally wide substrate specificity, MMP13 expression is limited to physiological situations in which rapid and effective remodeling of collagenous extracellular matrix is required [16, 17]. In the cornea, MMP13 has been shown to be expressed only at the basal layer of healing corneal epithelium [13, 18], suggesting its potential involvement in remodeling the underlying basement membrane. Moreover, overexpression and/or activation of MMP13 has been linked to the excessive degradation of extracellular matrix in osteoarthritic cartilage, rheumatoid synovium, chronic cutaneous ulcers, intestinal ulcerations, and chronic periodontitis [19–23]. As such, there are many MMP13-specific inhibitors designed to treat osteoarthritis and rheumatoid arthritis without the side effects often associated with many nonselective MMP inhibitors [24, 25]. These inhibitors may also have the potential to be used for ameliorating infection-induced tissue damage and ulceration.

In this study, we investigated MMP13 expression in C57BL/6 mouse corneas in response to *P. aeruginosa* infection and demonstrated that its inhibition decreased the severity of keratitis and significantly reduced the bacterial burden. We also used an MMP13 inhibitor (MMP13i) as an adjunctive therapy to antibiotics used to treat *P. aeruginosa* keratitis. Our data suggest that increased MMP13 activity contributes to basement membrane breaching and *P. aeruginosa* stromal invasion and that MMP13i might be used as an adjuvant therapy to reduce basement membrane damage and the stromal destruction associated with microbial keratitis.

MATERIALS AND METHODS

Animals

Wild-type C57BL6 mice (age, 8 weeks; weight, 20–24 g) were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were treated in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The institutional animal care and use committee of Wayne State University (Detroit, Michigan) approved all animal procedures.

Infection Procedure and MMP13i Treatment

For each experiment, 5 eyes were used for each group, and the experiments were repeated twice. C57BL/6 mouse corneas were subconjunctivally injected with 5 μL of a 1:1000 ratio of phosphate-buffered saline (PBS) to dimethyl sulfoxide or with 10 μg/mL MMP13i ($C_{22}H_{20}F_2N_4O_2$; EMD Millipore) 6 hours prior to *P. aeruginosa* inoculation. This compound inhibits the activity of MMP13 (median inhibitory concentration, 8 nm) but not other MMPs. Bacterial inoculation in epithelial injured corneas was performed as described earlier [26]. To determine whether MMP13i prevents ulceration in infected corneas, gatifloxacin ophthalmic solution was used to dissolve MMP13i (25 μg/mL), and 5 μL was instilled into mouse corneas after *P. aeruginosa* inoculation, starting 16 hours after infection and continuing every 2 hours thereafter during the first and second day of treatment and every 4 hours during the third day of treatment.

Clinical Examination

Clinical examination was performed with corneal photographing and clinical scoring as described previously [26]. Ocular disease was graded using clinical scores (0–12), according to the scoring system we adapted from Wu et al [6, 26, 27].

Determinations of Bacteria Load, CXCL2 Expression, and Polymorphonuclear Leukocyte (PMN) Infiltration

As previously described [6], the corneas were excised from the enucleated eyes, minced, and homogenized in 100 μL of PBS with a TissueLyser (Retch). The homogenates were divided into 2 parts. The first part was subjected to the counting of bacterial colonies. Aliquots (100 μL) of serial dilutions were plated onto *Pseudomonas* isolation agar plates in triplicate. The plates were incubated overnight at 37°C, and bacteria colonies were counted. The second part was used to measure CXCL2 expression, using an enzyme-linked immunosorbent assay (ELISA; R&D Systems), and myeloperoxidase (MPO) activity [26].

RNA Extraction and Real-Time Polymerase Chain Reaction (PCR)

For RNA isolation, corneal epithelial cells were scraped off of the cornea by use of a dulled blade and were frozen in liquid nitrogen immediately. RNA was extracted from the collected corneal epithelial cells, using the RNeasy Mini Kit (Qiagen). Complementary DNA was generated with oligo(dT) primer, followed by analysis using real-time PCR with Power SYBR Green PCR Master Mix (AB Applied Biosystems), based on β-actin levels. Primer sequences were as follows: for MMP3, GCTGCCATTTCTAATAAAGA and GCACTTCCTTTCACAAA; for MMP10, CATTGGAATCCCGAGCCTGAA and GGGTAGCCTGCTTGGACYTCAT; for MMP-12, ATCAGAAAGTGGGTTGTAGC and

GAAGGCAGACCAGGACAC; and for MMP13, TGATGAAA CCTGGACAAGC and CTGGACCATAAAGAAACTGAA.

Immunohistochemistry (IHC) Analysis

Mouse eyes were enucleated, embedded in Tissue-Tek OCT compound, and frozen in liquid nitrogen. Six-micrometer-thick sections were cut and mounted to polylysine-coated glass slides. After fixation for 10 minutes in 4% paraformaldehyde, slides were blocked with PBS containing 2% bovine serum albumin (BSA) for 1 hour at room temperature. Sections were then incubated with collagen III (Abcam; 1:200), collagen IV (EMD Millipore; 1:200), α-smooth muscle actin (α-SMA; Sigma; 1:500), NK1.1 (BD Pharmingen; 1:100), and NIMP-R14 (Hycult Biotech; 1:100), followed by a secondary antibody, FITC-conjugated goat anti-rabbit immunoglobulin G (IgG; Jackson Immunoresearch; 1:100). FITC-conjugated rabbit anti–*P. aeruginosa* (ThermoFisher) was diluted 1:50 and used for *P. aeruginosa* detection in corneal sections. Slides were mounted with DAPI-mounting medium (Vector Labs). Controls were similarly treated with rat IgG. All IHC images from an experiment were taken with identical exposures without autocorrection.

Whole-Mount Confocal Microscopy

Excised corneas were fixed in 4% paraformaldehyde and stored at 4°C until further processing. Before staining, radial incisions were made to produce 6 pie-shaped wedges. Corneas were washed in PBS, incubated in 20 mM prewarmed ethylenediaminetetraacetic acid for 30 minutes at 37°C, followed by blocking with PBS containing 0.2% Triton X-100 and 1% BSA for 1 hour at room temperature. After blocking, the corneas were incubated overnight at 4°C with 100 μL of rabbit anti-mouse collagen IV (EMD Millipore) and mouse anti–α-SMA antibodies (Sigma) diluted in PBS with 1% BSA. After washing, the corneas were incubated with 100 μL of Cy3-conjugated anti-rabbit and FITC-conjugated anti-mouse antibodies diluted in PBS with 1% BSA for 1 hour, followed by 5 washes in PBS. Stained whole mounts were examined under a confocal microscope (TCS-SP8; Leica, Germany). Optical sections of confocal epifluorescence images were acquired with a 20× objective lens, using image-acquisition software (LAS AF Lite). All acquired images, each taken at approximately 2-μm intervals, were merged and viewed en face. LAS AF Lite was used both to convert images to tiff files and to 3-dimensional images.

Statistical Analysis

Data were presented as mean values ± SD. For between-group comparisons, an unpaired, 2-tailed Student *t* test was used to determine statistical significance of differences in fungal counts, cytokine ELISA findings, and the MPO assay. A nonparametric Mann–Whitney *U* test was performed to determine the statistical significance of differences in clinical scores. Experiments were repeated at least twice to ensure reproducibility, and differences were considered statistically significant at *P* values of $< .05$. The results from one experiment were not compared with those of a repeated experiment because the severity of keratitis may differ slightly among experiments.

RESULTS

MMP13 Expression in C57BL/6 Mouse Corneas in Response to *P. aeruginosa* Infection

Our genome-wide complementary DNA array study [28] revealed that, among all MMPs, MMP13 expression was most highly induced in response to PA01 challenge and that this upregulation was dampened by flagellin pretreatment, which protects the cornea from microbial keratitis. The expression pattern suggests a detrimental role of this enzyme in bacterial keratitis. To assess the expression of MMP13 during the course of early infection in the mouse cornea, we performed IHC analysis at different times after bacterial inoculation. The IHC analysis revealed that, while there was little staining in the corneas of uninfected controls and at 3 hours after infection, MMP13 was detected at the entire epithelial layer of the *P. aeruginosa*–infected corneas 6 hours after infection (Figure 1). At 12 hours after infection, strong MMP13 staining was detected in the edematous corneal epithelium, with many MMP13-positive infiltrated cells in the stroma. Twenty-four hours after infection, the stroma became edematous with deteriorated epithelium; MMP13 staining was defused in epithelium-stroma junctions.

MMP13 Expression Is Related to TLR5 Levels in the Corneal Epithelia

Our previous study revealed that, in the cornea, flagellin-induced protection against *Candida albicans* infection was TLR5 dependent. To determine whether infection-induced MMP13 expression is TLR5 dependent, we subconjuctivally injected TLR5 small interfering RNA (siRNA) or nonspecific siRNA control and confirmed that TLR5 levels in TLR5 siRNA–treated corneal epithelial cells were downregulated (Figure 2*A*). MMP13 expression was markedly induced, and flagellin-pretreatment suppressed the elevated expression in corneal epithelial cells of *P. aeruginosa*–infected corneas treated with control siRNA at 6 hours after infection (Figure 2*A*). In TLR5 siRNA–treated corneas, infection-induced MMP13 expression was dampened, while the inhibitory effects of flagellin were diminished in corneal epithelial cells. TLR5 downregulation also resulted in increased keratitis severity (Figure 2*B*), bacterial load (Figure 2*C*), and PMN infiltration (Figure 2*D*), with or without flagellin pretreatment.

MMP13 Inhibition Reduces the Severity of *P. aeruginosa* Keratitis

To assess the potential involvement of MMP13 in *P. aeruginosa* keratitis, we used a MMP13-specific inhibitor (MMP13i) and monitored the progression of *P. aeruginosa* keratitis at the early stages of infection. In vitro, the inhibitor exhibited no bactericidal

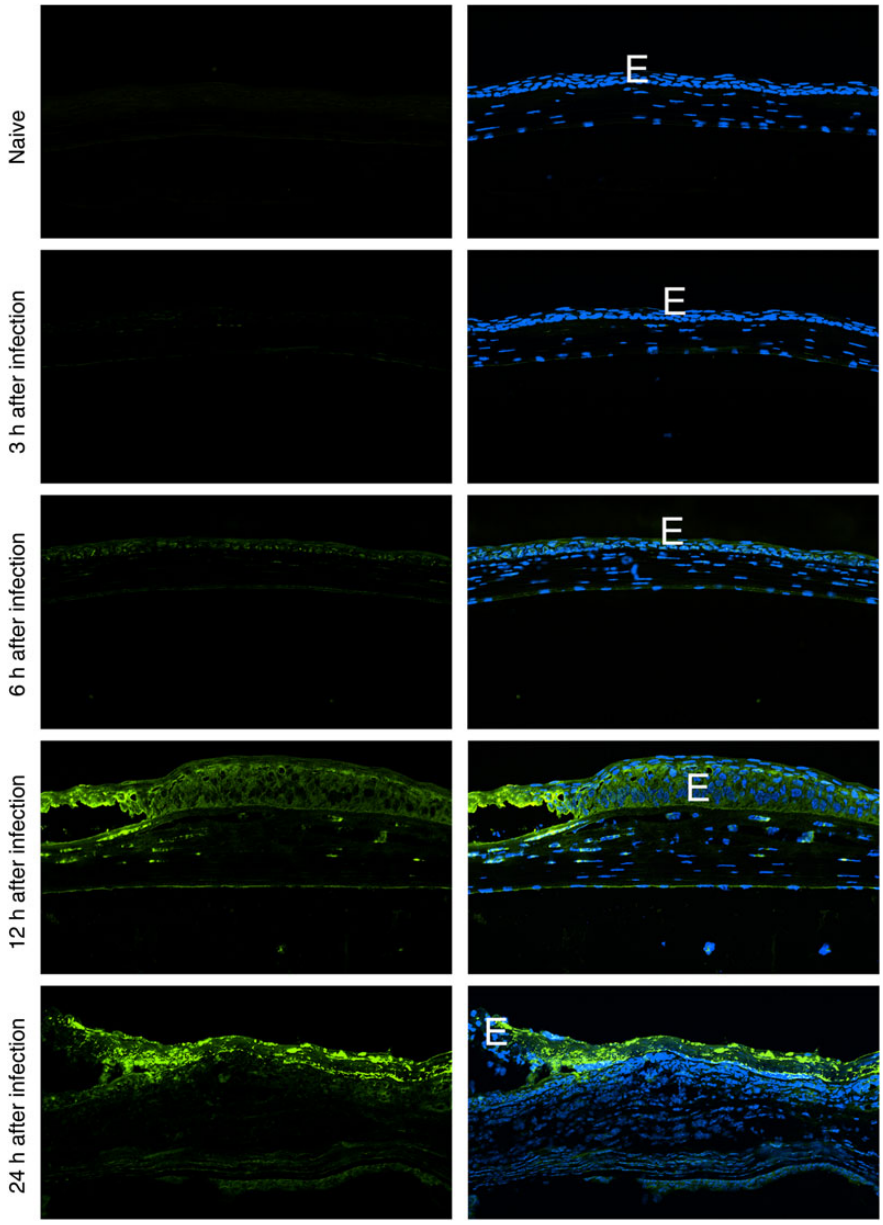

Figure 1. Matrix metalloprotein-13 (MMP13) expression in C57BL/6 mouse cornea during *Pseudomonas aeruginosa* infection. Three 1-mm incisions in C57BL/6 mouse corneas were made with a needle, and corneas were inoculated at the injured site with a 5-μL suspension containing 1×10^4 colony-forming units of ATCC19660. At the indicated time after infection, the corneas were excised and subjected to immunohistochemistry analysis, using anti-MMP13 antibody and DAPI for nuclear staining. The results are representative of 2 independent experiments. Five corneas were used per experiment, and at least 3 were sectioned, stained, and examined. Abbreviation: E, epithelium.

activity (data not shown). At 1 day after infection, there were significant increases in the severity of keratitis in the control eyes (clinical score, 4), compared with MMP13i-treated corneas, which showed slight signs of inflammation/infection (clinical score, 1; Figure 3*A*). However, no significant difference in bacterial burden was observed in the control versus MMP13i-treated corneas 1 day after infection (Figure 3*B*). Consistent with suppressed keratitis, MMP13i treatment also decreased PMN infiltration (Figure 3*C*) and CXCL2 expression (Figure 3*D*) at this time point. Three days after infection, the entire cornea of the control mice had thick opacification and neovascularization (clinical score 10), whereas MMP13i-treated corneas had significantly less opacification but apparent surface irregularity (clinical score 5; Figure 3*A*). While the bacterial burden in the control corneas continued to increase from 1 to 3 days after infection, it remained largely unchanged in MMP13i-treated corneas (Figure 3*B*). Interestingly, while the levels of CXCL2 elevated moderately, PMN infiltration was increased in MMP13i-treated

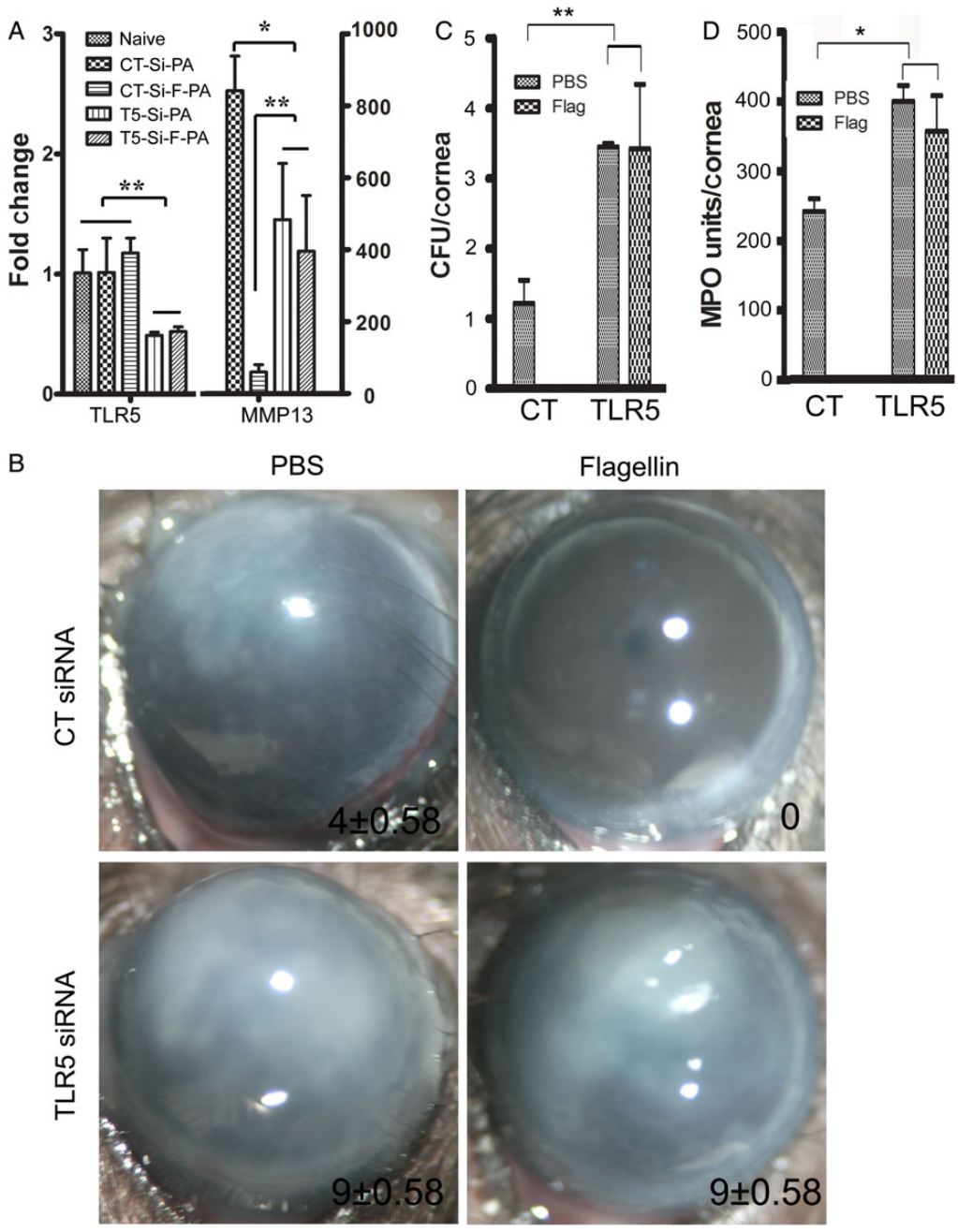

Figure 2. *Pseudomonas aeruginosa* infection–induced expression of matrix metalloprotein-13 (MMP13) is dependent on Toll-like receptor 5 (TLR5). C57BL/6 mouse corneas were pretreated with flagellin or phosphate-buffered saline (PBS) 24 hours before infection. Six hours before infection, small interfering RNAs (siRNAs; 5 μL; 10 μM) were injected into the subconjunctival space, followed by PA inoculation at 0 hours. Six hours after infection, 5 mice were euthanized, and their corneas were excised and subjected to real-time polymerase chain reaction analysis for TLR5 and MMP13 (*A*). Eyes were photographed and scored (using a 12-point system) 2 days after infection (*B*). The number in each panel is the mean clinical score (± standard deviation [SD]) for the presented eye. Mice were then euthanized, and their corneas were excised and subjected to fungal plate counting, with the results presented as colony-forming units (CFU; *C*) and myeloperoxidase (MPO) activity (*D*). Results in panels *B–D* are mean values (+ SD) and are representative of 2 independent experiments (5 mice each). $^{*}P < .05$ and $^{**}P < .01$, by 1-way analysis of variance. Abbreviation: CT, control.

corneas, with even higher levels in the control corneas 3 days after infection (Figure 3*C* and 3*D*). All parameters for keratitis assessed at 3 days after infection were decreased in MMP13i-treated corneas, compared with the controls.

MMP13 Inhibition Preserves the Integrity of the Corneal Epithelium

As the epithelial basement membrane has been proposed to be a biological barrier that bacteria must penetrate [3, 29, 30], we

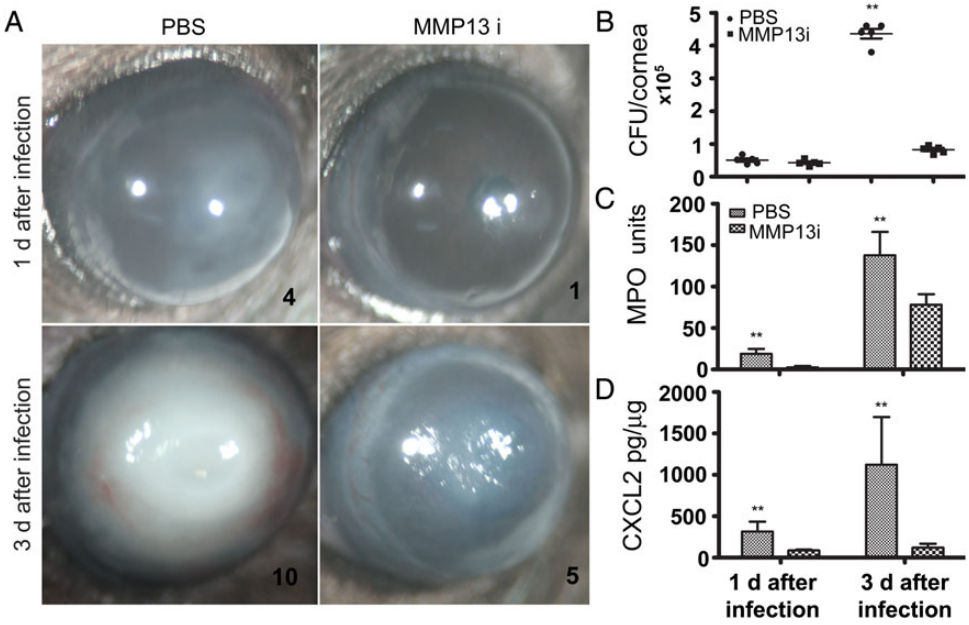

Figure 3. Pretreatment with matrix metalloprotein-13 inhibitor (MMP13i) is effective in the protection against *Pseudomonas aeruginosa* keratitis. C57BL/6 mouse corneas were injected subconjunctivally with 5 μL of phosphate-buffered saline (PBS) or MMP13 inhibitor (10 μg/mL) 6 h prior to bacterium inoculation with topical application of 1.0×10^4 colony-forming units (CFU) of *P. aeruginosa* strain ATCC19660 in 5 μL of PBS. The infected corneas were photographed and scored 1 and 3 days after infection (*A*); the number in each panel is the clinical score for the presented eye. Bacterial load assays (*B*), myeloperoxidase (MPO) activity assays (*C*), and CXCL2 enzyme-linked immunosorbent assays (*D*) were performed 1 and 3 days after infection. An unpaired, 2-tailed Student *t* test was performed to compare bacterial load, MPO activity, and CXCL2 levels between MMP13-pretreated mice to the PBS control groups (n = 5 mice/group). Results are representative of 3 independent experiments. $^{**}P < .01$.

postulated that the elevated MMP13 expression may contribute to the basement membrane breakdown during *P. aeruginosa* keratitis. To test this hypothesis, we used IHC analysis to illustrate changes in the basement membrane in *P. aeruginosa*–infected corneas (Figure 4*A*). Three hours after infection, no major differences were observed between MMP13i-treated corneas and control corneas. Six hours after infection, there were more infiltrated cells and less collagen IV staining in the control corneas, compared with MMP13i-treated corneas. Twelve hours after infection, the MMP13i-treated corneas were edematous, although to a much lesser extent than control corneas. Strikingly, while the epithelial sheet was partially detached from the stroma with the disappearance of collagen IV staining in the control cornea, the epithelium remained attached with an intact basement membrane in the MMP13i-treated corneas. Twenty-four hours after infection, the loss of anterior cornea was evident in the control cornea (ulceration), while the MMP13i-treated corneas remained intact with a loose but still attached epithelium (epithelial edema). To determine whether *P. aeruginosa* invaded the stroma, antibody recognizing whole *P. aeruginosa* was used to identify bacteria in the corneas (Figure 4*B*). Twelve hours after infection, bacteria were found both in the epithelia and within the stroma in the control corneas. In MMP13i-treated corneas, where the basement membrane was intact, bacteria were mostly seen within the epithelial layer and in the subbasal epithelial space but not in the underlying stroma. Twenty-four hours after infection, the ulcerated cornea contained abundant *P. aeruginosa*, whereas in MMP13i-treated corneas most bacteria were found along the basement membrane, indicating their limited ability to cross the basement membrane.

The structural changes of the basement membrane in infected and infected/MMP13i-treated corneas were also assessed, using whole-mount confocal microscopy and double staining of collagen IV and α-SMA for myofibroblasts (Figure 5). The naive cornea had an intact basement membrane with no myofibroblasts. *Pseudomonas aeruginosa*–infected corneas investigated 1 day after infection had an area where there was no basement membrane and contained numerous myofibroblasts, which were more concentrated in the areas with residual basement membrane. In MMP13i-treated corneas, the basement membrane was intact, with only a few myofibroblasts observed. Three dimensional reconstructions revealed a healthy basement membrane in the naive cornea without myofibroblasts, patchy basement membranes with numerous myofibroblasts lying underneath in *P. aeruginosa*–infected corneas, and an intact but thin basement membrane with a few myofibroblasts nearby in *P. aeruginosa*–infected and MMP13i-treated corneas.

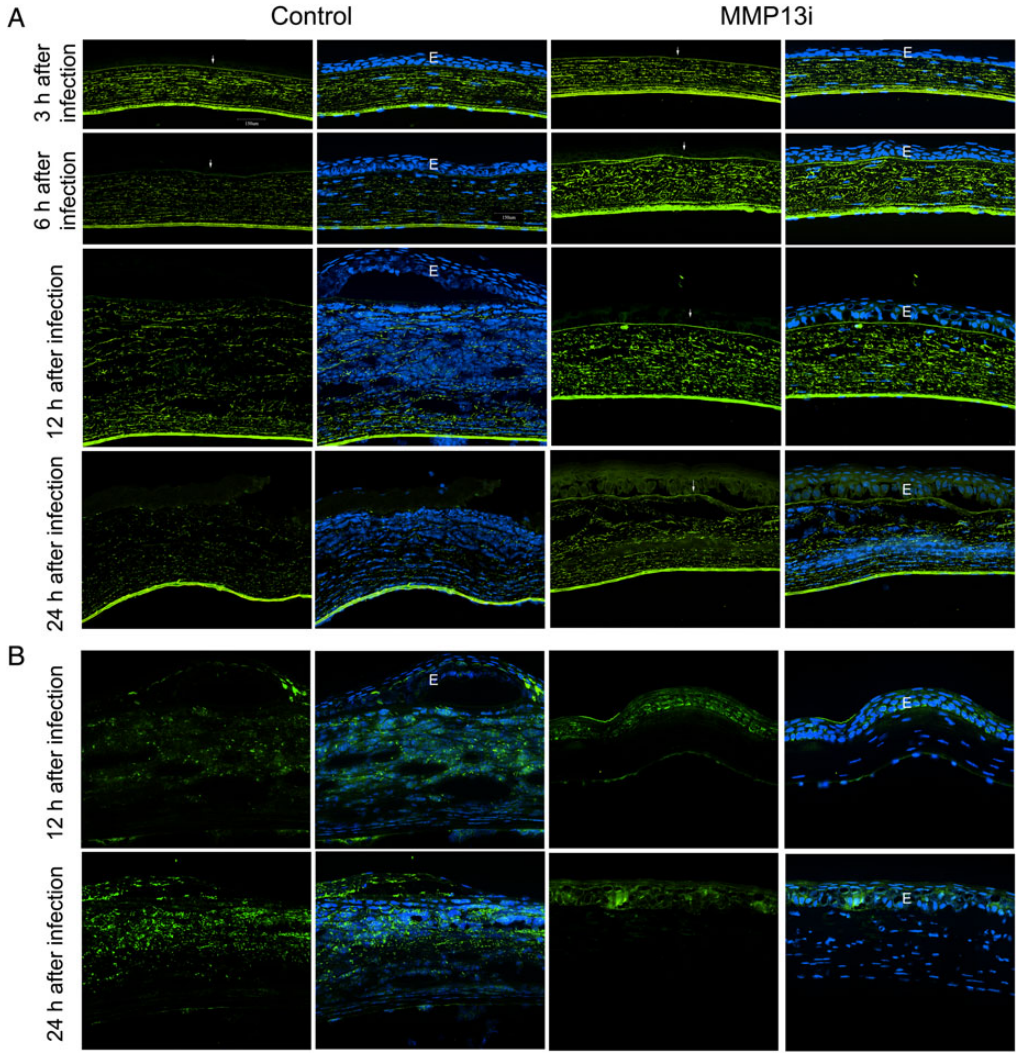

Figure 4. Matrix metalloprotein-13 (MMP13) inhibition preserves the integrity of corneal epithelium (E). C57BL/6 mouse corneas were pretreated and infected with *Pseudomonas aeruginosa* as described in Figure 2. The infected corneas were subjected to immunohistochemistry analysis using anti–collagen IV to illuminate the basement membrane (arrowheads) 3, 6, 12, and 24 hours after infection (*A*). The images were taken with identical exposures without autocorrection and are representative of 3 eyes/group from 2 experiments. The corneal sections analyzed 12 and 24 hours after infection were also stained with *P. aeruginosa* antibody (*B*).

Topical MMP13 Inhibitor Prevented Ulceration of *P. aeruginosa*–Infected, Antibiotic-Treated Corneas

The aforementioned results suggest that if MMP13i is applied prior to infection, it is effective in protecting the cornea from *P. aeruginosa* keratitis. This approach, however, is not clinically feasible. We next investigated whether topically applied MMP13i can suppress infection-induced tissue damage while the pathogens are eliminated by antibiotics. Our preliminary data showed that topical gatifloxacin applied 12–24 hours after infection was able to eradicate invading *P. aeruginosa*, but inflammation lingered for several days, hence providing an interval for assessing the effects of MMP13 inhibition on corneal inflammation and its resolution. We chose 16 hours after infection as a starting point to treat *P. aeruginosa* keratitis with topical gatifloxacin in the presence or absence of MMP13i (25 μg/mL). Figure 6 showed the representative micrographs of infected corneas 1 and 3 days after treatment initiation. While gatifloxacin-treated eyes exhibited some dense opacification and subepithelial haze, the eyes treated with MMP13i as adjunctive therapy were mostly clear with some areas of slight opacity (Figure 6*A*). A significant difference was observed 2 and 3 days after treatment initiation between the clinical scores of corneas co-treated with MMP13i and gatifloxacin and those of corneas treated with gatifloxacin (Figure 6*B*). Consistent with reduced corneal opacification, topical MMP13i treatment significantly reduced PMN infiltration, compared with gatifloxacin alone (Figure 6*C*).

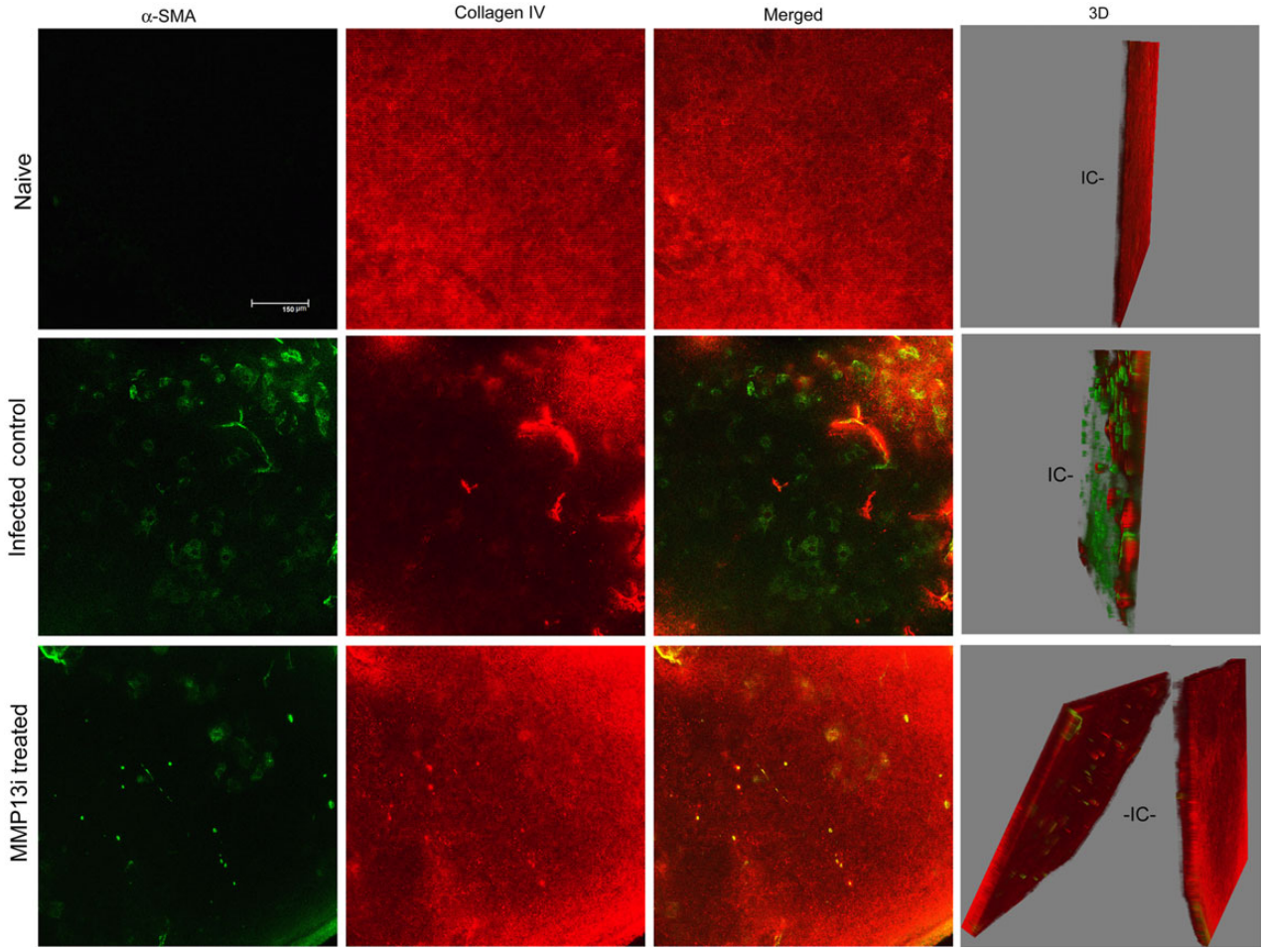

Figure 5. Matrix metalloprotein-13 (MMP13) inhibition preserves the integrity of corneal epithelium, as revealed by corneal whole-mount confocal microscopy. C57BL/6 mouse corneas were infected with 10^4 colony-forming units of ATCC19660, with or without (infected controls) pretreatment with MMP13 inhibitor (MMP13i); naive corneas were used an uninfected control (naive). One day after infection, the corneal whole-mount slide was stained with antibodies to collagen IV (red) and α-smooth muscle actin (α-SMA; green) antibodies. A series of image stacks was collected through the *z*-plane of the tissue. Merged and overlaid images are shown. Optical sections from 2-channel confocal imaging were digitally rendered in 3-dimensions (3D). For each condition, 3 sample corneas were used, and 2 independent experiments were performed. Abbreviation: IC, intracellular face.

MMP13 Inhibitor Preserved the Structural Integrity of Gatifloxacin-Treated Corneas

The corneas obtained 3 days after treatment initiation were subjected to hematoxylin–eosin staining and IHC analysis, with naive and infected, untreated corneas as controls. Hematoxylin–eosin staining (Figure 7*A*) revealed the destruction of untreated, infected corneas, whereas corneas treated with gatifloxacin were edematous with structural alterations at the anterior side of the stroma. MMP13i/gatifloxacin-cotreated corneas were similar to naive corneas, with minimal structural changes and few infiltrated cells in the stroma. IHC analysis revealed that, while the infected, untreated corneas had no definable epithelial layer, with the residual basement membrane arranged in opposite orientation, the basement membrane in gatifloxacin-treated corneas was rough and discontinuous with edematous epithelium, and the cotreated corneas had an intact basement membrane and epithelium, appearing normal (Figure 7*B*). There was no α-SMA staining in the naive corneas; the heavily stained cluster of α-SMA in infected corneas corresponded to residual basement membrane. Corneal epithelial cells and some stromal cells were also α-SMA positive in gatifloxacin-treated corneas but not in cotreated corneas (Figure 7*C*). Collagen III staining was seen in infected corneas as disorganized and spotty (Figure 7*D*). Strong collagen III staining was observed in subepithelial stromal areas, corresponding to loss and light hematoxylin–eosin staining in gatifloxacin-treated corneas, while a trace amount of staining could be detected in the cotreated mouse corneas. In infected control corneas, NIMP-R14, an antibody that recognizes mouse neutrophils, stained strongly in the area with strong collagen III staining (Figure 7*E*), while NK1.1, an antibody that recognizes natural killer cells and natural killer T cells, appeared to be nonspecific (Figure 7*F*). In the edematous gatifloxacin-treated cornea,

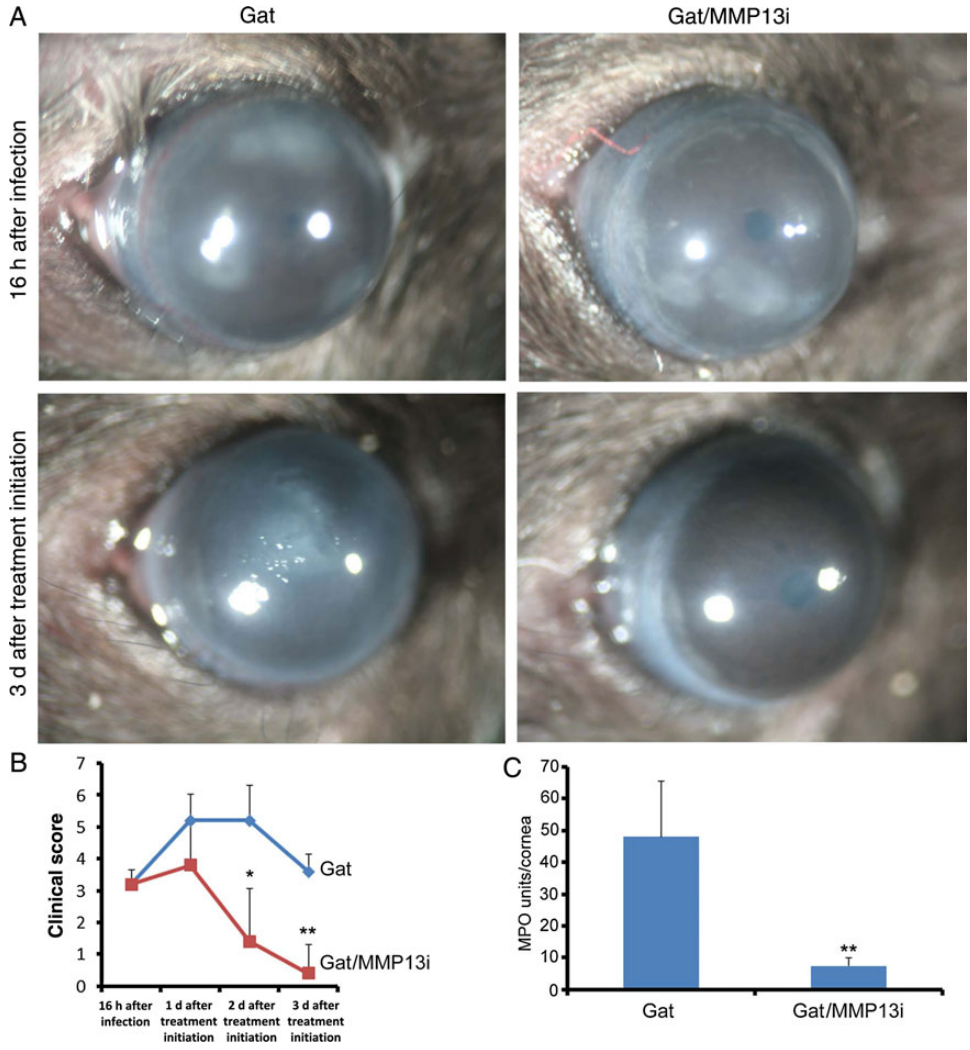

Figure 6. Matrix metalloprotein-13 inhibitor (MMP13i) prevented ulceration in infected corneas. C57BL/6 mouse corneas were inoculated with 1.0×10^4 colony-forming units of *Pseudomonas aeruginosa* ATCC19660. Topical solution containing gatifloxacin (Gat) was used to dissolve MMP13i (25 μg/mL). Topical MMP13i was then applied, starting 16 hours after infection, at 2-hour intervals during the first and second days of treatment and at 4-hour intervals on the third day of treatment. The infected corneas were photographed (*A*) and scored (*B*), and a myeloperoxidase (MPO) activity (*C*) assay was performed at the end of experiment. A nonparametric Mann–Whitney *U* test was performed to compare each retreatment to the phosphate-buffered saline group (n = 5). The results are representative of 3 independent experiments. $^*P < .05$ and $^{**}P < .01$.

PMNs were found just beneath the epithelium, where NK cells were also located. In gatifloxacin/MMP13i-cotreated corneas, there were no NIMP-R14– or NK1.1-positive cells.

DISCUSSION

In this study, we investigated the role of MMP13 in and the effects of its inhibition on the pathogenesis of *P. aeruginosa* keratitis. We demonstrated that MMP13 expression was markedly upregulated in C57BL/6 mouse corneal epithelial cells in response to *P. aeruginosa* infection and that the expression of MMP13 appears to be related to TLR5 levels and the severity of infection. We showed that the inhibition of MMP13 at the early stage of infection greatly reduced the severity of *P. aeruginosa* keratitis, suggesting a pathogenic role of MMP13. We also showed that MMP13 upregulation coincides with basement membrane breakdown. Furthermore, we demonstrated that, while the invading pathogens are being eradicated by antibiotics, MMP13 inhibition decreases corneal opacification, preserves the basement membrane structure and epithelial integrity, and accelerates inflammation resolution. Hence, in addition to treating osteoarthritis [31, 32], MMP13i may also be used as an adjunctive therapy to suppress inflammation-caused tissue damage in infected corneas. Corticosteroids have been used as adjunctive therapy for bacterial corneal ulcers [33]. In light of known side effects of steroids to the eye, our data suggest that MMP13 inhibition and MMP13i have potential as a new strategy and an effective remedy for treating bacterial corneal ulcers.

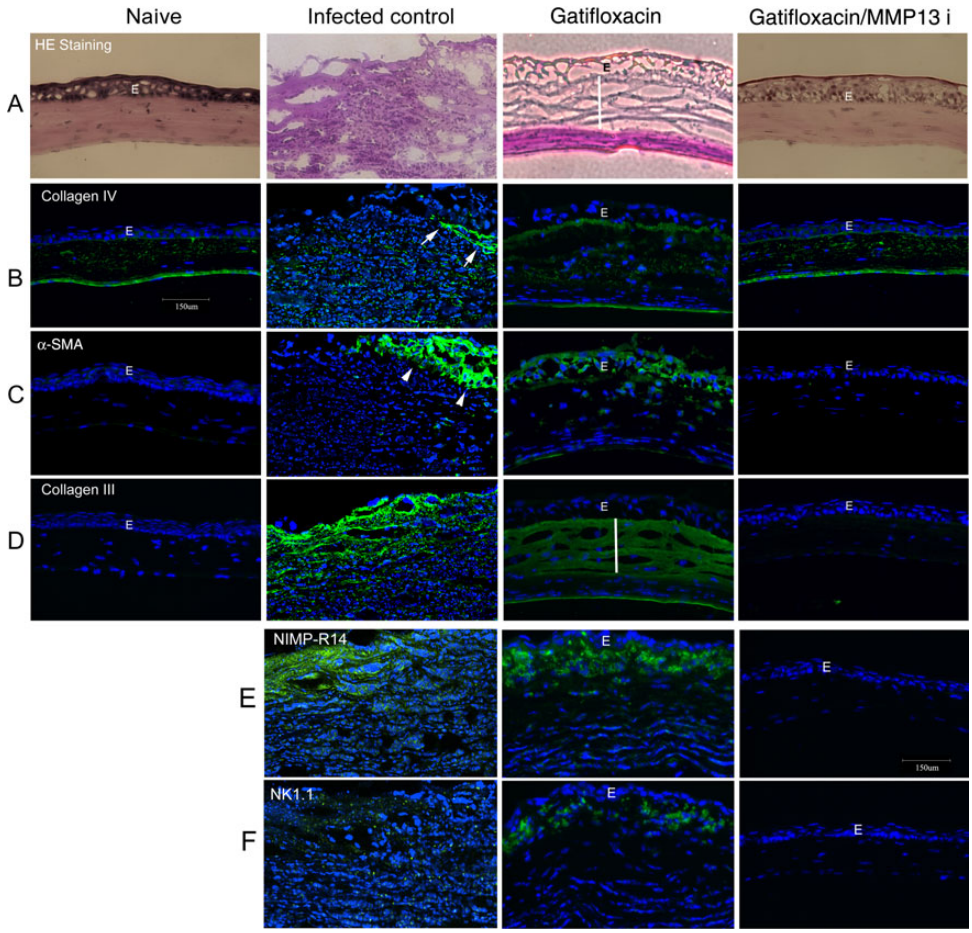

Figure 7. Matrix metalloprotein-13 inhibitor (MMP13i) preserved the structural integrity of gatifloxacin-treated corneas. C57BL/6 mouse corneas were inoculated with 1.0×10^4 colony-forming units of *Pseudomonas aeruginosa* ATCC19660 and treated with gatifloxacin in the presence or absence of MMP13i, as described in Figure 5. Naive corneas and those infected with *P. aeruginosa* but untreated (infected control) were used as controls. The cryosections of the corneas collected 3 days after treatment initiation were subjected to hematoxylin–eosin (HE) staining and immunohistochemistry analysis using collagen IV, collagen III, α-smooth muscle actin (α-SMA), NK1.1, and NIMP-R14 (anti-mouse neutrophil) antibodies with DAPI for nuclear staining. Results are representative of 2 independent experiments; for each condition/time, 5 corneas were used and fixed, and 3 corneas were sectioned, stained, and examined. Arrows, inversed, folded basement membrane; arrowheads, αSMA-positive cells above the basement membrane fragment; E, epithelium; vertical white lines, corresponding sections of loosely stained corneal stroma and collagen III–positive region.

Recent studies after the discovery of TLRs revealed epithelia to be an active and important component of innate immunity at mucosal surfaces [34]. Exposure of epithelial-expressed TLRs resulted in rapid activation of downstream signaling and the production of antimicrobial peptides such as Cramp (LL-37) [35], β-defensins [36], and ELR$^-$-CXC chemokine CXCL10 [26, 37]. These antimicrobial peptides may eradicate invading pathogens [6, 7, 26, 38]. The activated epithelial cells also express another group of injury-responsive genes, the MMPs, for tissue remodeling [18, 39]. In this study, we showed that *Mmp13* was the most highly inducible gene among the MMPs. Other bacterial components, such as the TLR9 ligand CpG-DNA [40, 41], are also known to stimulate MMP13 expression. Hence, the elevated level of expression in infected corneas suggests that this unique collagenase may be involved in the pathogenesis of microbial keratitis.

Using subconjunctival injection of MMP13i, we showed that a preexisting MMP13 inhibitor significantly reduced keratitis severity, including bacterial burden. Since the inhibitor possesses no intrinsic bactericidal activity, it might exert bactericidal activity through its antiinflammatory effects. However, previous animal studies revealed that corticosteroid treatment is associated with increased bacterial growth and the recurrence of *P. aeruginosa* keratitis in the absence of antibiotic treatment [42, 43]. Hence, the observed effects in MMP13i-treated corneas may not directly result from inflammation suppression. Indeed, our study revealed that MMP13i inhibited *P. aeruginosa* proliferation and reduced its pathogenesis in vivo. We postulated that *P. aeruginosa* may use MMP13 to gain access to the stroma by degrading the basement membrane. Our data showed while the control, infected corneas dissolved the basement

membrane, induced bacterial invasion of the stroma and ulceration, MMP13i preserved the basement membrane integrity with few *P. aeruginosa* reached the stroma at 1 day after infection. In an ex vivo model of basement membrane degradation, MMP13, but not MMP1, -2, -3, -7, or -9, was shown to be capable of basement membrane degradation [44]. Thus, we suggest that the elevated level of MMP13 may assist with bacterial spreading into the stroma by degrading collagen IV, creating a path through the otherwise impermissible basement membrane. When bacteria arrive at the stroma, they are likely to escape from the killing network of antimicrobial peptides and the trapping effects of corneal epithelial cells, allowing them to proliferate rapidly. Hence, targeting MMP13 activation may promote bacterial clearance and tissue resistance to microbial infection.

Another important aspect of our study is the use of MMP13i as an adjunctive therapy to treat *P. aeruginosa* keratitis. It is generally believed that although invading *P. aeruginosa* can be readily eliminated by topical antibiotics, persistent inflammation may cause the progression of corneal ulceration. We showed that there was significant basement membrane degradation in infected cornea at 1 day after infection. However, once infection is suppressed and pathogens are eradicated by antibiotics, there would be wound-repair mechanisms for functional recovery, including epithelium reattachment or reepithelialization, both of which might be negatively affected if excessive MMP13 activity remains. Indeed, we observed partial recovery of corneal structure within 3 days of gatifloxacin treatment. This recovery was significantly accelerated in the presence of MMP13i, including preserved epithelial integrity and prevented excessive expression of both collagen III, a marker for fibrosis [45], and α-SMA, a marker for myofibroblasts [46]. As a result, there were minimal signs of keratitis when infected corneas were cotreated with gatifloxacin and MMP13i. Given these beneficial effects and the fact that preclinical trials with broad MMP inhibitors have persistently failed because of toxicity or limited efficacy [47–49], we suggest that MMP13-specific inhibitors may also be used as adjunctive therapy to treat bacterial ulcers in the cornea and other mucosal infections.

Notes

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