Matrix Metalloproteinase-8 Facilitates Neutrophil Migration through the Corneal Stromal Matrix by Collagen Degradation and Production of the Chemotactic Peptide Pro-Gly-Pro

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Matrix metalloproteinase (MMP)-8 and MMP-9 play several roles in inflammation, including degradation of extracellular matrix (ECM) components and regulation of cytokine activity. To determine the roles of MMP-8 and MMP-9 in a neutrophil-dependent inflammatory response, we used a murine model of corneal inflammation in which LPS is injected into the corneal stroma. In contrast to wild-type mice, we found that i) lipopolysaccharide (LPS)-injected CXCR2^{-/-} corneas had impaired neutrophil infiltration and did not express either MMP-8 or MMP-9; ii) neutrophil migration through the central cornea was impaired in *Mmp8*^{-/-}, but not *Mmp9*^{-/-}, mice; iii) neutrophil migration was inhibited in collagenase-resistant mice; iv) the chemotactic Pro-Gly-Pro (PGP) tripeptide that binds CXCR2 was decreased in CXCR2^{-/-} mice; v) PGP production was impaired in *Mmp8*^{-/-} corneas; and vi) neutralizing anti-PGP antibody did not inhibit neutrophil infiltration in *Mmp8*^{-/-} mice. We found no effects of MMP-8 on LPS-induced CXC chemokine (LIX, or CXCL5)-induced neutrophil recruitment or on LPS-induced CXC chemokine production. Together, these studies indicate that neutrophils contribute to the production of both MMP-8 and MMP-9 in LPS-injected corneas and that MMP-8 regulates neutrophil migration through the dense collagenous ECM of the corneal stroma by generating chemotactic PGP dur-

ing inflammation. (Am J Pathol 2008, 173:144–153; DOI: 10.2353/ajpath.2008.080081)

Neutrophils play an essential role in the clearance of microbial pathogens; however, neutrophil degranulation and release of proteases and other cytotoxic mediators can also cause tissue damage. In the cornea, neutrophil degranulation leads to destruction of stromal matrix integrity, neovascularization, and loss of corneal clarity and visual acuity, and, in severe cases, can result in blindness.^{1–6} Furthermore, clinical disease is decreased or absent in mice in which neutrophil infiltration to the cornea is impaired by systemic depletion or in the absence of adhesion molecules or chemokine receptors, which inhibit neutrophil recruitment to the cornea.^{7–9}

Matrix metalloproteinases (MMPs) are a family of Zn²⁺-dependent endopeptidases that regulate cell-cell and cell-matrix interactions by several means, including modulating cell behavior through the activation, inactivation, or release of adhesion molecules, growth factors and receptors, cytokines, and extracellular matrix proteolysis.¹⁰ Thus, MMP activity is associated with many physiological and pathological processes, including inflamma-

This work was supported by the National Institutes of Health (grants EY10320 and EY11373 to E.P., and grants HL077783 and HL090999 to J.E.B.), by the Research to Prevent Blindness (RPB) Foundation and the Ohio Lions Eye Research Foundation, by Prevent Blindness Ohio Foundation (M.L.), and by Cystic Fibrosis Foundation grant R464-CR02 (to J.E.B.). E.P. is also a recipient of an RPB Senior Investigator Award. C.M.O. is supported by a Canada Research Chair in Metalloproteinase Proteomics and Systems Biology, with research grants from the Canadian Institutes of Health Research as well as with a Centre Grant from the Michael Smith Research Foundation. Funds for the purchase of mass spectrometers and the operation of the UAB Mass Spectrometry Shared Facility came from the following NIH grants to U.A.B.: S10 RR19231, P30 CA13148, P50 AT00477, U54 CA100949, P30 AR050948, and P30 DK74038.

Accepted for publication April 15, 2008.

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tion. In corneal inflammation, human case studies and animal models of keratitis have reported an increase in MMP-1, -2, -8, -9, and -13 expression and activity that correlates with disease progression. Furthermore, MMP-8 and MMP-9 are associated with neutrophil infiltration of the corneal stroma.^{5,6,11–15}

Of the 23 human and 24 murine MMPs currently known, neutrophils contain MMP-8, MMP-9, and MMP-25; where MMP-8 is preformed in specific granules, MMP-9 is in tertiary granules, and MMP-25 is in specific, tertiary granules, secretory vesicles, and on the plasma membrane. When neutrophils are activated, MMPs are released from their granules to the extracellular space and localized to the membrane surface where they are resistant to tissue inhibitors of MMPs.^{16–20} Although these MMPs have *in vitro* activity for collagens, basement membrane components, cytokines, and chemokines, the role of MMP-8 and MMP-9 in neutrophil migration and function *in vivo* has yet to be fully elucidated.^{21,22}

In the present study, we examined the role of MMP-8 and MMP-9 on neutrophil migration using an lipopolysaccharide (LPS)-induced model of corneal inflammation. We used mice deficient in either MMP-8 or MMP-9 to determine their function on neutrophil infiltration. As MMP-8 cleaves pro-forms of lipopolysaccharide-induced CXC chemokine (LIX, or CXCL5), synthetic analogs of full-length and truncated LIX were also used to examine whether MMP-8 promoted neutrophil migration through chemokine processing. In addition, we used collagenase-resistant Col1a1^{tm1Jae} mice, in which the main site of collagen type I cleavage is mutated to establish whether neutrophil migration involves collagen processing. Finally, we examined the role of the chemotactic Pro-Gly-Pro (PGP) tripeptide on neutrophil migration through the corneal stroma and the involvement of MMP-8 in generation of this PGP fragment.

Materials and Methods

Animals and Reagents

Mice deficient in *Mmp8* on a C57BL/6J × 129 S background were generated as described.²³ Dr. Robert Fairchild (Cleveland Clinic Foundation, Cleveland, OH) provided mice deficient in *Mmp9* on a C57BL/6 background and heterozygous BALB/c-Cmkar2^{tm1Mwm} (CXCR2^{+/-}) that were bred to obtain homozygous (CXCR2^{-/-}) and wild-type (CXCR2^{+/+}) littermates. Wild-type C57BL/6J × 129S and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Heterozygous Col1a1^{tm1Jae} (Col1a1^{+/+}) mice were provided by Dr. Robert Weinreb (University of California-San Diego, San Diego, CA) and bred to establish homozygous resistant (Col1a1^{tr}) and wild-type (Col1a1^{+/+}) mice. Highly purified LPS (TLR4-specific) from *Escherichia coli* K12 was purchased from InVivoGen (San Diego, CA). Rat anti-mouse NIMP-R14 antibody was produced and purified as described.²⁴ Rat anti-mouse MMP-9 antibody, mouse KC, MIP-2, and LIX enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D Systems (Minneapolis, MN). Rabbit

anti-human MMP-8 antibody was obtained from BioMol International, LP (Plymouth Meeting, PA). Goat anti-actin antibody, donkey anti-goat IgG-HRP, goat anti-rabbit IgG-HRP, and chicken anti-rat IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Synthetic LIX (1–92), LIX(5–92), and LIX(5–79) were generated as described.²³

Generation of PGP Antibody

Rabbit immunoglobulin (Ig) against PGP was produced and purified by EZ Biolab (Westfield, IN). Briefly, rabbits were immunized with *N*-acetyl (α)-PGP (N- α -PGP) coupled to keyhole limpet hemocyanin via the peptide's carboxyl terminus and Ig was purified by ammonium sulfate precipitation. Anti-PGP Ig at 25 μ g/ml neutralized 100% and 75%, respectively, of N- α -PGP chemotactic activity and 100% and 80% of PGP chemotactic activity, whereas anti-PGP Ig at 25 μ g/ml had no inhibitory effect on IL-8 chemotactic activity.

Murine Model of LPS Keratitis

Four- to 8-week-old female mice were anesthetized by intraperitoneal injection with 0.4 ml of a 1.2% solution of 2,2,2-tribromoethanol containing 2.5% 2-methyl-2-butanol (tertiary amyl alcohol; Sigma-Aldrich, St. Louis, MO) dissolved in distilled water. After creating a tunnel into the corneal stroma using a 33-gauge needle, 200 ng of LPS in 2 μ l of Hanks' buffered salt solution (HBSS; Invitrogen, Carlsbad, CA) was injected into the central corneal stroma via a separate 33-gauge needle attached to a 5- μ l glass syringe (Hamilton Co., Reno, NV) as described previously.^{25,26} Control corneas were injected with 2 μ l of HBSS. For neutralization of PGP, a 2.5- μ l mixture containing 50 μ g of rabbit anti-PGP or control IgG and 200 ng of LPS in HBSS was injected into the central cornea. A dose response of anti-PGP was performed in wild-type corneas to determine the effective inhibitory concentration of antibody, and whether the antibody has any intrinsic proinflammatory activity. All mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Histology and Immunohistochemistry

Eyes were immersed in OCT (Tissue-Tek, Sakura, Torrance, CA) and snap-frozen in liquid nitrogen. Cryosections (5 μ m) taken from the center of the eye were stained as previously described for neutrophils using the rat anti-mouse mAb NIMP-R14, which is specific for neutrophils.²⁷ Briefly, sections were fixed in 4% formaldehyde for 30 minutes, rinsed in 0.05 M Tris buffer solution, pH 7.6. Sections were then incubated with the 8 μ g/ml NIMP/R14 for 2 hours. After washing, sections were incubated for 45 minutes with 5 μ g/ml FITC-conjugated anti-rat IgG (H+L; Vector Laboratories, Burlingame, CA). Sections were mounted in VectaShield Mounting Medium containing DAPI (Vector Laboratories). Neutrophils per 5- μ m section were counted (from limbus to limbus at ×400) by

fluorescence microscopy (Olympus Optical Co. Ltd., Tokyo, Japan). As the mouse cornea is approximately 3 mm in diameter, the peripheral regions of the corneas were defined as 0.5 mm from the limbus, and the central region was defined as the central 2 mm of the cornea.

SDS-PAGE, Western and Gelatin Zymography

Corneas were excised after injection of LPS or HBSS at indicated times and mechanically disrupted in 1× cell lysis buffer (Cell Signaling Technologies, Danvers, MA) containing 1 mmol/L PMSF using a TissueLyzer (QIAGEN, Valencia, CA). After disruption, protein concentration was determined using the BCA method (Pierce, Rockford, IL), and 10 µg was loaded onto a 10% polyacrylamide gel with or without 0.1% gelatin for zymography or Western blot analysis, respectively.

For Western blot analysis, the gel was transferred to nitrocellulose membrane for 2 hours at 90 V. Membranes were blocked in 5% milk (Bio-Rad, Hercules, CA) and incubated with primary antibodies, rabbit anti-human MMP-8 (1:1000), rat anti-mouse MMP-9 (1:500), or actin (1:2000) diluted in 1% milk/PBS-Tween overnight at 4°C. We used the corresponding secondary antibodies, goat anti-rabbit IgG-HRP, donkey anti-rat IgG-HRP, and chicken anti-IgG-HRP, and reactivity was detected using an ECL kit (GE Healthcare Biosciences Corporation, Piscataway NJ) according to manufacturer's directions.

For gel zymography, the gel was incubated in 2.5% Triton X-100, pH 8.0 for 30 minutes at room temperature. The gel was then equilibrated for 30 minutes at room temperature in 1× Zymogram Developing buffer (Bio-Rad) and incubated in fresh Zymogram Developing buffer overnight at 37°C. To visualize the gelatinolytic activity, the gel was placed in 0.5% Coomassie Blue R-250 (Bio-Rad) for 30 minutes at room temperature and destained in a solution of 50% methanol, 10% acetic acid, and 40% water.

Quantification of PGP

PGP was measured by liquid chromatography followed by electrospray ionization mass spectrometry with multi-

ple reaction monitoring (ESI-LC/MS/MS) using an MDS Sciex (Applied Biosystems, Foster City, CA) API-4000 spectrometer equipped with a Shimadzu HPLC apparatus. HPLC was done using a 2.1 × 150 mm Develosil C30 column (A, 0.1% formic acid; B, acetonitrile with 0.1% formic acid). The gradient was 0 minutes to 0.6 minutes at 80% A/20% B and 0.6 to 5 minutes up to 100% B. Background was removed by flushing with 100% isopropanol with 0.1% formic acid. Positive electrospray mass transitions are monitored at 270–70 and 270–116 for PGP.

Statistical Analysis

Statistical analysis was performed with an unpaired *t*-test (Prism; GraphPad Software, San Diego, CA). *P* < 0.05 was considered significant.

Results

LPS Induces Expression of MMP-8 and MMP-9 in the Cornea

Our previous studies using this model of LPS-induced corneal inflammation showed that neutrophil recruitment to the corneal stroma was first detected at 6 hours, peaked at 18 to 24 hours, and rapidly declined thereafter.²⁵ As we are interested in regulation of neutrophil recruitment and migration through the corneal stroma, we examined MMP expression at 4 hours and 18 hours. Corneas of C57BL/6 mice injected with either saline or LPS were excised and were either processed for MMP-8 and MMP-9 expression by Western blot, or assayed for gelatinase activity by gel zymography. Figure 1A shows that MMP-8 and MMP-9 were not detected at 4 hours, but were elevated at 18 hours in LPS-injected corneas in comparison to saline controls. Gelatinolytic activity is shown in Figure 1B. The pro- and active forms of MMP-2 gelatinolytic activity (72 and 62 kDa, respectively), were observed in all samples at both time points, indicating constitutive expression. MMP-9 is released from neutrophils in three different forms: 92-kDa monomers, 200-kDa homodimers, and 120-kDa

Figure 1. Expression of MMP-8, MMP-9, and gelatinase activity during corneal inflammation. Corneas were injected intrastromally with 0.2 µg LPS or saline (HBSS). After 4 hours or 18 hours, corneas were dissected and sonicated. **A:** MMP-8 and MMP-9 protein levels were examined by SDS-PAGE and Western blot, using β-actin as loading controls. **B:** Cornea lysates from C57BL/6 mice were also analyzed by gelatin zymography for gelatinase activity. **C:** Gelatinase activity in wild-type and MMP9^{-/-} mice 18 hours after LPS injection. Results are derived from two pooled corneas, and are representative of one of three experiments. Naive: normal mouse corneas. Monomeric MMP-9 is 92 kDa (arrowhead); MMP-2, expressed constitutively, is 65 kDa.

complexes of MMP-9 covalently bound to neutrophil gelatinase-B-associated lipocalin (NGAL), a 25-kDa member of the lipocalin family of transport molecules.²² Monomeric MMP-9 (92 kDa) gelatinolytic activity was confirmed using cornea samples from LPS-injected wild-type and *Mmp9*^{-/-} mice (Figure 1C). These results indicate that intrastromal injection of LPS induces MMP-8 and MMP-9 expression in the cornea, and that MMP-9 is enzymatically active.

MMP-8 and MMP-9 Activity Is Absent in Corneas from LPS-Injected CXCR2^{-/-} Mice

Although resident cells in the cornea can be induced to express MMPs,^{28–30} neutrophils contain pre-formed MMP-8 and MMP-9 in secondary and tertiary granules, respectively.²⁰ To determine the role of neutrophils in MMP expression, LPS or saline was injected into the corneas of CXCR2^{-/-} mice, which have impaired neutrophil recruitment to the cornea in other murine models of keratitis.^{7,9} After 18 hours, either the eyes were processed for immunohistochemistry to quantify neutrophils in the cornea, or corneas were dissected and disrupted to examine the expression of MMP-8 and MMP-9 by Western blot or assayed by gelatin zymography.

We found that whereas corneas of wild-type mice had a pronounced neutrophil infiltrate after LPS injection, neutrophil migration to corneas of CXCR2^{-/-} mice was completely ablated (Figure 2A). In addition, corneas of CXCR2^{-/-} mice did not express MMP-8 or MMP-9 (Figure 2B), or display gelatinase activity in contrast to wild-type corneas (Figure 2C). Taken together, these data indicate that CXCR2 and neutrophils are essential for expression of MMP-8 and MMP-9 and for gelatinolytic activity in LPS-treated corneas.

Figure 2. Neutrophil migration and expression of MMP-8, MMP-9, and gelatinase activity in CXCR2^{-/-} mice during LPS-induced corneal inflammation. Corneas of wild-type and CXCR2^{-/-} mice were injected intrastromally with 0.2 μ g of LPS or HBSS. **A:** At 18 hours, eyes were enucleated, sectioned, and immunostained with NIMP/R14. The number of neutrophils was determined by direct counting of 5- μ m sections. Results are mean $\pm$ SEM of five individual corneas. **B:** LPS-injected corneas were sonicated, and MMP-8 and MMP-9 protein expression was analyzed by Western blot. **C:** Gelatinase activity by gel zymography. Note expression of MMP-8 and monomeric MMP-9 (92 kDa) and gelatinase activity in wild-type, not CXCR2^{-/-} mice, whereas 65-kDa MMP-2 is expressed in all mice. Samples are from two pooled corneas, and results are representative of two repeat experiments with similar results.

Neutrophil Migration Is Impaired in *Mmp8*^{-/-} but Not *Mmp9*^{-/-} Mice

To evaluate whether MMP-8 and MMP-9 contribute to neutrophil migration, corneas of wild-type, *Mmp8*^{-/-}, and *Mmp9*^{-/-} mice were injected with either HBSS or LPS. After 8 hours or 18 hours, eyes were processed for immunohistochemistry, and neutrophil infiltration to the peripheral and central cornea was determined, which represent neutrophils transmigrating from limbal vessels to the corneal stroma (periphery) and migration through the cornea to the site of injury (central), respectively.

The effect of MMP-9 deficiency is shown in Figure 3A. We found no difference in peripheral, central, or total neutrophil accumulation between *Mmp9*^{-/-} corneas and wild-type corneas either at 8 hours or 18 hours after

Figure 3. Neutrophil migration through the corneal stroma of *Mmp8*^{-/-} and *Mmp9*^{-/-} mice. Corneas of wild-type (black bars), *Mmp8*^{-/-} or *Mmp9*^{-/-} (gray bars) mice were injected intrastromally with 0.2 μ g of LPS. At 8 hours and 18 hours, eyes were enucleated, sectioned and immunostained for neutrophils using NIMP/R14. The number of neutrophils was determined in peripheral and central regions of the cornea by direct counting of 5 μ m sections. **A:** *Mmp8*^{-/-} mice. Data are representative of one of two experiments with five corneas per group (mean $\pm$ SEM). **B:** *Mmp9*^{-/-} mice. Data are representative of three repeat experiments with five corneas per group (mean $\pm$ SEM). **C:** Representative 5- μ m sections of the corneal stroma show FITC neutrophils (green) in the central region of wild-type mice and *Mmp8*^{-/-} corneas (blue = DAPI).

intrastromal injection. In marked contrast, *Mmp8*^{-/-} corneas showed a significant reduction in total and central neutrophil numbers compared with wild-type controls after 8 hours, and a significant reduction in central neutrophils after 18 hours (Figure 3B), indicating that MMP8 has an essential role in neutrophil migration through the corneal stroma. Figure 3B shows decreased neutrophils in the central corneal stroma of *Mmp8*^{-/-} after 18 hours in representative corneal sections. There was no difference in neutrophil numbers in the perivascular regions of peripheral, limbal vessels.

Taken together, these results indicate that MMP-8, but not MMP-9, regulates neutrophil migration into the cornea.

Elevated Chemokine Production in *Mmp8*^{-/-} Mice

We previously showed that CXC chemokines CXCL1/KC and CXCL5/LIX mediate neutrophil recruitment to the cornea stroma in LPS keratitis.²⁵ As a first approach to understanding the underlying mechanism of MMP-8 dependent neutrophil infiltration, we examined the role of MMP-8 in LPS-induced ELR+ CXC chemokine expression. LPS-injected corneas were evaluated for chemokine production at 4 and 24 hours. Although we anticipated no effect or reduced CXC production in the absence of MMP-8, we found that KC and MIP-2 were significantly elevated in MMP-8-deficient corneas compared with wild-type corneas at 4 hours, which occurs before neutrophil infiltration, but there was no difference in chemokine production at 24 hours, which is the peak of neutrophil infiltration in the cornea (Figure 4). The production of LIX was not significantly altered in expression at 4 or 24 hours. Although elevated chemokine production is often associated with an increase in neutrophil infiltration, these findings occur together with impaired neutrophil infiltration in corneas of *Mmp8*^{-/-} mice, and indicate that impaired neutrophil migration in *Mmp8*^{-/-} mice is not due to increased CXC chemokine production.

Impaired Neutrophil Infiltration in MMP-8-Deficient Mice Is Independent of LIX Cleavage

As we showed above that MMP-8 does not impair total chemokine production, we next examined whether MMP-8 affects chemokine activity. MMP-8 can cleave various substrates, including pro-inflammatory and chemotactic cytokines, where the site of proteolysis determines their activation state. Overall and colleagues^{23,31} demonstrated that MMP-8 processes CXCL5/LIX at both the N terminus and C terminus, but only the N-terminal cleavage enhanced its chemotactic activity, and that MMP-8 proteolysis of LIX is a critical mechanism for neutrophil chemotaxis in an air pouch model of inflammation. Because we previously showed that LIX is important in neutrophil recruitment to the corneal stroma,²⁵ and as a second approach to understanding the role of MMP-8 in LPS keratitis, we determined whether the impaired neutrophil migration in the corneas of *Mmp8*^{-/-} mice is due to compromised LIX activity.

Synthetic analogs of the full-length and cleaved forms of LIX were injected into the corneal stroma of wild-type and *Mmp8*^{-/-} mice and, after 18 hours, eyes were processed for immunohistochemistry, and neutrophils in the corneal stroma were enumerated as previously stated. We found that neutrophil infiltration to the corneal stroma was induced by full-length LIX(1-92); however, in MMP-8-deficient mice, the response was significantly impaired compared to wild-type controls (Figure 5), consistent with a role for MMP-8 in LIX processing. Unexpectedly, neutrophil infiltration was also significantly impaired in response to the truncated forms of LIX [LIX(5-92) and LIX(5-79)] in *Mmp8*^{-/-} corneas compared to wild-type controls (Figure 5). These data indicate that as with LPS-induced neutrophil infiltration to the corneal stroma, MMP-8 also has a role in LIX-mediated neutrophil infiltration; however, unlike the air pouch model, the role of MMP-8 in the cornea is due to functions other than, or in addition to, LIX processing.

Figure 4. CXC chemokine production in LPS-injected *Mmp8*^{-/-} and wild-type corneas. Corneas of wild-type and *Mmp8*^{-/-} mice were injected intrastromally with 0.2 μ g of LPS. After 4 hours and 24 hours, corneas were disrupted by sonication, and KC, LIX, and MIP-2 were measured by ELISA. The limit of detection for each cytokine was 15 pg/ml. Results are the mean $\pm$ SEM of five individual corneas per time point, and are representative of three repeat experiments with similar results.

Figure 5. Neutrophil infiltration in response to synthetic LIX peptides in *Mmp8*^{-/-} and wild-type corneas. Corneas of *Mmp8*^{-/-} and wild-type mice were injected intrastromally with 100 ng of synthetic full-length LIX peptides (1–92), and truncated peptides LIX(5–92) and LIX (5–79). After 18 hours, eyes were enucleated, sectioned, and immunostained with NIMP/R14. The total number of neutrophils was determined in the cornea by direct counting of 5- μ m sections. Results are representative of two repeat experiments with four individual corneas per group (mean + SEM).

Neutrophil Infiltration Through the Corneal Stroma Is Associated with Collagen Degradation

As a third approach to examining the mechanism of MMP-8-dependent neutrophil migration through the corneal stroma, we examined the role of collagen type I, which is a primary substrate of MMP-8. Degradation of collagen I is initiated between the conserved site of Gly775 and Ile776 of the α 1(I) chain and Gly775 and Leu776 of the α 2(I) chain.^{32,33} The extracellular matrix of the corneal stroma is arranged to allow maximal light penetration, and comprises a highly organized array of primarily type 1 collagen fibrils separated by keratan sulfate proteoglycans.³⁴ To determine whether neutrophil migration through the corneal stroma involves collagen degradation, LPS was injected into the corneas of collagenase-resistant mice (*Col1a1*^{tm1Jae}), which have a targeted mutation encoding amino acid substitutions Gln774Pro, Ala777Pro, and Ile776Met in the helical domain of the collagen type I (I) chain, thereby preventing collagen cleavage.³⁵

As shown in Figure 6, there was no difference in the number of neutrophils in the peripheral region of the corneas of *Col1a1*^{+/+} mice compared with littermate controls. In contrast, neutrophil migration to the central corneal stroma was significantly impaired in *Col1a1*^{+/+} mice compared with littermate controls, and the total number of neutrophils was significantly lower. These findings indicate that collagen degradation has a demonstrable role in neutrophil migration through the corneal ECM but apparently not in extravasation.

LPS Induces Neutrophil- and MMP-8-Dependent Generation of the Chemotactic Peptide PGP

In addition to dense collagen matrices serving as a physical barrier requiring proteolysis for an accessible path of

Figure 6. Neutrophil infiltration to the corneas of collagenase-resistant mice. Corneas of *Col1a1*^{+/+} or *Col1a1*^{+/+} mice were injected intrastromally with 0.2 μ g of LPS. After 18 hours, eyes were enucleated, and 5- μ m sections were immunostained with NIMP/R14. The number of neutrophils was assessed in peripheral and central regions of the cornea by direct counting. Results are representative of one of three experiments with four corneas per group (mean + SEM).

migration, several collagen-derived peptides also exhibit pronounced neutrophil chemotactic activity.³⁶ One such peptide, PGP, originally described in rabbit corneas subjected to alkali injury and hypothesized to derive from collagen, has been shown to activate neutrophils through the CXCR2 receptor.^{37,38} To determine whether PGP levels are increased during LPS-induced corneal inflammation, cornea tissue lysates and anterior chamber fluid were collected after intrastromal injection of saline or LPS, and were analyzed for the presence of PGP by ESI-LC/MS/MS. As shown in Figure 7A, naïve and HBSS-injected corneas had high levels of PGP, and there was no significant increase in LPS-injected corneas.

Because of the high PGP level in naïve corneas, which has been reported previously,³⁸ and given that unbound PGP tripeptide is likely to diffuse across the corneal endothelium into the anterior chamber, we also examined PGP levels at this site. In contrast to whole corneas, PGP levels in anterior chambers of naïve and HBSS-treated mice were low (Figure 7B). Furthermore, intrastromal injection of LPS resulted in significantly elevated PGP levels in the anterior chamber fluid.

To determine whether neutrophils are essential for PGP production in the cornea, we examined PGP levels in the anterior chamber of LPS-injected CXCR2^{-/-} mice, which have impaired neutrophil migration as shown in Figure 2. We found that PGP levels were significantly lower in CXCR2^{-/-} mice compared to wild-type controls (Figure 7C), indicating a significant role for neutrophils in generating PGP.

Finally, to determine whether MMP-8-dependent neutrophil migration involves release of PGP peptides, corneas of MMP-8-deficient and wild-type mice were injected with LPS as before, and anterior chamber fluid was collected after 24 hours. We found that PGP levels were significantly decreased at 24 hours post-stimulation in *Mmp8*^{-/-} mice compared to wild-type controls (Figure

Figure 7. Generation of chemotactic peptides PGP in corneas and anterior chambers of CXCR2^{-/-} and *Mmp8*^{-/-} mice during LPS-induced keratitis. Corneas of wild-type, CXCR2^{-/-}, and *Mmp8*^{-/-} mice were injected intrastromally with 0.2 μ g of LPS or HBSS. Corneas and anterior chamber fluid were collected, and samples were assayed for the presence of PGP by ESI-LC/MS/MS. **A:** PGP in total corneas from naive corneas, or 24 hours after intrastromal injection of HBSS or LPS ($n = 3$). **B:** PGP in pooled anterior chamber fluid from naive mice, or 24 hours after mice were injected intrastromally with HBSS or LPS ($n = 3$). **C:** Anterior chamber fluid from wild-type and CXCR2^{-/-} mice 24 hours after intrastromal injection of LPS ($n = 3$). **D:** Anterior chamber fluid from wild-type and *Mmp8*^{-/-} mice 24 hours after intrastromal injection of LPS ($n = 3$). Results for **A** and **B** are representative of three repeat experiments, and anterior chamber fluid results are representative of two experiments with two pooled corneas per group (**C** and **D**) (mean $\pm$ SEM).

7D), indicating that neutrophils and MMP-8 contribute to the production of PGP.

PGP Generation by MMP-8 Mediates Neutrophil Infiltration to the Corneal Stroma

Although MMP-8 is important in generating PGP, we have yet to determine whether PGP has a role in neutrophil infiltration. To determine the role of PGP in control and *Mmp8*^{-/-} mice, we injected rabbit anti-PGP antibody simultaneously with LPS into the corneal stroma of wild-type and *Mmp8*^{-/-} mice. After 8 hours, mice were sacrificed and the number of neutrophils in the peripheral and central cornea was examined by immunohistochemistry. Figure 8 shows that there is no effect of anti-PGP on the number of neutrophils in the peripheral cornea. In contrast, neutrophil migration to the central stroma was significantly inhibited (40%) in anti-PGP-treated wild-type corneas compared with those injected with control rabbit

IgG, indicating that PGP is important in neutrophil migration through the corneal stroma.

Since MMP-8 contributes to PGP generation in the cornea, we predicted that anti-PGP would have no effect on neutrophil infiltration to the corneas of *Mmp8*^{-/-} mice. As shown in Figure 8, and in contrast to wild-type mice, neutrophil numbers in the central corneal region of anti-PGP-treated *Mmp8*^{-/-} mice were lower than those in IgG-treated controls, but this difference was not statistically significant. Total neutrophil numbers were also lower in anti-PGP-treated wild-type corneas (35% compared with IgG control) but not in *Mmp8*^{-/-} mice (21% compared with IgG controls).

Together, these results imply that i) PGP mediates neutrophil infiltration through the corneal stroma, and ii) PGP has no role in *Mmp8*^{-/-} mice. The latter finding indicates that the underlying mechanism of MMP-8-dependent neutrophil migration through the corneal stroma is by ECM degradation and generation of chemotactic PGP.

Figure 8. Effect of antibody neutralization of PGP on neutrophil infiltration to the corneal stroma of wild-type and *Mmp8*^{-/-} mice. Corneas of wild-type and *Mmp8*^{-/-} mice were injected with 0.2 μ g of LPS together with 50 μ g of anti-PGP antibody. Mice were sacrificed after 8 hours, and corneal sections were immunostained with NIMP/R-14 antibody. Neutrophils in peripheral and central regions of the cornea were quantified by direct counting. Results are representative of two repeat experiments with five corneas per group (mean $\pm$ SEM).

Discussion

Neutrophil migration to the sites of inflammation relies on the coordinated action of chemoattractants and adhesion molecules to extravasate from capillaries and then migrate through the extracellular matrix to the site of the inflammatory stimulus. These mediators include selectins and integrins for endothelial cell adhesion, and ELR+ CXC chemokines for neutrophil recruitment and migration.³⁹ Several of these mediators interact with or are substrates for MMPs. For example, MMP-9 binds to CD18 on neutrophils and appears regulate neutrophil migration *in vitro* and *in vivo*.⁴⁰ In addition, MMP-9 can cleave big endothelin 1, ET-1[1-38], to form the active vasoconstrictor peptide, ET-1[1-32], which up-regulates CD18 and E-selectin, and can enhance neutrophil adhesion to activated endothelial cells.⁴¹ The activity of cytokines such

as IL-1 β and TNF- α , and the chemotactic potential of chemokines IL-8, Gro- α , LIX, and ENA-78 can also be modulated following MMP processing to either potentiate or inhibit inflammation.^{23,42–44}

In the present study, we show that MMP-8 and MMP-9 expression is up-regulated in LPS-treated corneas after 18 hours, which is the peak time of neutrophil infiltration.²⁵ Furthermore, we found that MMP-8 and MMP-9 were not expressed in corneas of CXCR2^{-/-} mice, in which neutrophil recruitment to the corneal stroma was ablated. Taken together with other reports that MMP-8 and MMP-9 are present in neutrophil granules, it is highly likely that neutrophils are the major source of these MMPs. An indirect role for neutrophils may be in activating corneal fibroblasts, as neutrophils or supernatants from neutrophil cultures can induce collagen degradation by cornea fibroblasts embedded in a collagen matrix.⁴⁵

Despite the increased expression of MMP-8 and MMP-9 in association with neutrophil infiltration to the cornea, we found that MMP-8 but not MMP-9 has a significant role in neutrophil migration through the corneal matrix. In the absence of MMP-8, there were significantly fewer neutrophils in the central corneal stroma, but not in the peripheral stroma, indicating that MMP-8 is important in neutrophil migration through the ECM from the peripheral to the central corneal stroma, but appears less likely to mediate extravasation from limbal vessels to the peripheral cornea.

Although we did not observe a role for MMP-9 in LPS keratitis, MMP-9 is implicated in neutrophil migration in *Pseudomonas aeruginosa* keratitis and neovascularization in HSV-1 keratitis.^{11,46} Although the differences in these findings have yet to be determined, conflicting reports on the role of MMP-9 also occur in murine models of lung inflammation, with MMP-9 important in respiratory tularemia, but having no role in LPS-induced lung inflammation.^{47–49} Thus, the tissue environment as well as the nature of the initiating stimulus may determine the conditions in which MMP-9 influences neutrophil migration and function.

Our current findings show an important role for MMP-8 in corneal inflammation, although different roles for MMP-8 in models of neutrophil chemotaxis, wound healing, TNF- α -induced acute hepatitis, LPS-mediated acute lung injury, and OVA-induced asthma have been reported.^{16,23,31,50–52} For example, during wound healing, an initial delay of neutrophil infiltration in *Mmp8*^{-/-} mice and a persistent inflammation at later time points was observed.⁵¹ In contrast, neutrophil infiltration is increased in lung inflammation as a result of either LPS or OVA instillation in *Mmp8*^{-/-} mice.^{16,50} One mechanism by which MMP-8 mediates neutrophil chemotaxis is through CXCL5/LIX activation. In a TNF- α -induced model of hepatitis and in an air-pouch model of inflammation, MMP-8 induced LIX chemotactic activity by mobilization of LIX or proteolytic cleavage of precursor LIX, respectively.^{23,52} Although we did not demonstrate LIX proteolysis in the cornea, the possibility that MMP-8-dependent cleavage of LIX acts as a positive feedback mechanism for neutrophil chemotaxis was not evident in our model of corneal inflammation as decreased neutrophil infiltration was

unaffected in *Mmp8*^{-/-} mice injected with precursor or truncated LIX peptides. This could be due to the dense collagenous stromal matrix in which degradation of the collagen facilitates migration to the central stroma and therefore masks any observable MMP-8-dependent LIX chemotactic properties, which is observed in an air pouch where the blood vessels are close to the air space and not separated by significant amounts of collagenous matrix.²³ As we also found that KC and MIP-2 levels were either unaffected or elevated rather than reduced in LPS-injected *Mmp8*^{-/-} corneas, we conclude that these chemokines are unable to rescue LIX chemotactic activities in the *Mmp8*^{-/-} deficient mice.

In contrast to the reported role of MMP-8 on LIX processing,²³ our findings indicate that an important mechanism of action of MMP-8 in the corneal stroma is the generation of the proteolytic cleavage fragment of the extracellular collagen matrix, PGP in response to LPS, as PGP in the anterior chamber was diminished in the absence of MMP-8 during LPS-induced inflammation. The role of collagen degradation in this process is also supported by our observations that collagenase-resistant Col1a1^{tmJae} mice have impaired neutrophil migration to the central cornea.

The presence of PGP in naïve corneas was not associated with neutrophils or other markers of inflammation, most likely because additional inflammatory mediators are required to induce neutrophil migration. Although we have yet to determine the basis for this observation, we postulate that PGP production is due to normal turnover of the very dense collagen matrix in the corneal stroma. Elevated background PGP levels in naïve lung homogenates compared with bronchoalveolar lavage fluid have also been observed (J.E. Blalock, unpublished observations). Furthermore, although neutrophils were occasionally detected in the anterior chamber of LPS-injected corneas, we did not observe a consistent presence at this site, and in the current study, we used anterior chamber fluid as a reflection of PGP levels in the corneal stroma.

Based on results from the current study, and on our previous observations on the role of CXC chemokines,^{7,9} we propose a sequence of events leading to neutrophil infiltration and inflammation in the cornea. Resident stromal fibroblasts and macrophages produce ELR+ CXC chemokines in response to LPS, which recruit neutrophils from peripheral vessels into the corneal stroma.^{7,9} Neutrophils in the ECM release MMP-8, which cleaves collagen and generates the chemotactic PGP peptide. PGP, which also binds to CXCR2, then mediates infiltration of neutrophils through the corneal stroma. In support of a role for PGP, we found that neutralizing anti-PGP antibody significantly inhibited neutrophil infiltration to the LPS treated cornea. Moreover, our data show that anti-PGP had no effect in *Mmp8*^{-/-} mice, which is consistent with the requirement for MMP-8 in proteolytic generation of PGP. A recent study by two of the authors (P.L.J., J.E.B.) showed that PGP generation is a multistep process in which MMP-8 and MMP-9 initially cleave collagen to 30 to 100 amino acid fragments, which are subsequently cleaved by prolyl endopeptidase⁵³. This study

also demonstrated that PGP is elevated in cystic fibrosis patients.⁵³

Although we focused on the role of MMP-8 in generating PGP in the cornea, it is possible that additional MMPs contribute to PGP processing, including MMP-25 produced by neutrophils, and MMP-13 and MMP-14, which can be expressed in the cornea.^{12,54} However, the observations that antibody to PGP inhibits neutrophil infiltration in wild-type mice, but has no effect on neutrophil infiltration to *Mmp8*^{-/-} corneas, indicates that MMP-8 has a predominant role in generating PGP in the cornea. In addition, matrilysin (MMP-7) facilitates chemokine activity in the lungs by cleaving the KC-syndecan complex from the matrix.⁵⁵ Similarly, MMP-8 regulates neutrophil migration and function by mobilizing LIX from the portal veins into the sinusoids of the liver,⁵² and may have a similar role in the cornea that could also explain the increase of chemokines found in the corneas of *Mmp8*^{-/-} mice. An additional role for MMP-8 may be regulation of serine proteinase activity, which has also been implicated in ECM degradation, modulation of the cytokine/chemokine milieu, and bacterial clearance by neutrophils.⁵⁶ Furthermore, MMP-8 can inactivate the serpin, α 1-proteinase inhibitor, which leads to increased serine proteinase activity.¹⁶

In summary, the present study using a model of corneal inflammation indicates that MMP-8 contributes the generation of the proteolytic fragment of the extracellular matrix, PGP, which mediates neutrophil migration through the corneal stroma and subsequent development of corneal inflammation. Furthermore, our observations indicate that there may be different roles for MMP-8 in different connective tissue matrices, as MMP-8-dependent LIX processing predominates in a loose ECM such as the air-pouch model,²³ whereas in a dense ECM such as the corneal stroma, MMP-8 mediates neutrophil migration by collagen degradation and PGP production, which may supercede LIX chemotaxis. By identifying a role for MMP-8 and the underlying mechanisms in this model of corneal inflammation, results of the current study thereby add to our general understanding of the function of MMP-8 in neutrophil migration and inflammation.

Acknowledgments

We thank Denise Hatala and Catherine Doller (Case Western Reserve University) and Jennifer Cox (University of British Columbia) for expert technical assistance.

References

1. Carr DJ, Harle P, Gebhardt BM: The immune response to ocular herpes simplex virus type 1 infection. *Exp Biol Med* (Maywood) 2001, 226:353–366
2. Girgis DO, Sloop GD, Reed JM, O'Callaghan RJ: A new topical model of *Staphylococcus* corneal infection in the mouse. *Invest Ophthalmol Vis Sci* 2003, 44:1591–1597
3. Hazlett LD: Corneal response to *Pseudomonas aeruginosa* infection. *Prog Retin Eye Res* 2004, 23:1–30
4. Hume EB, Dajcs JJ, Moreau JM, Sloop GD, Willcox MD, O'Callaghan

- RJ: *Staphylococcus* corneal virulence in a new topical model of infection. *Invest Ophthalmol Vis Sci* 2001, 42:2904–2908
5. O'Brien TP, Li QJ, Sauerburger F, Reviglio VE, Rana T, Ashraf MF: The role of matrix metalloproteinases in ulcerative keratolysis associated with perioperative diclofenac use. *Ophthalmology* 2001, 108:656–659
6. Rohini G, Murugeswari P, Prajna NV, Lalitha P, Muthukkaruppan V: Matrix metalloproteinases (MMP-8, MMP-9) and the tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2) in patients with fungal keratitis. *Cornea* 2007, 26:207–211
7. Khan S, Cole N, Hume EB, Garthwaite L, Conibear TC, Miles DH, Aliwaga Y, Krockenberger MB, Willcox MD: The role of CXC chemokine receptor 2 in *Pseudomonas aeruginosa* corneal infection. *J Leukoc Biol* 2007, 81:315–318
8. Khatri S, Lass JH, Heinzel FP, Petroll WM, Gomez J, Diaconu E, Kalsow CM, Pearlman E: Regulation of endotoxin-induced keratitis by PECAM-1, MIP-2, and toll-like receptor 4. *Invest Ophthalmol Vis Sci* 2002, 43:2278–2284
9. Hall LR, Diaconu E, Patel R, Pearlman E: CXC chemokine receptor 2 but not C-C chemokine receptor 1 expression is essential for neutrophil recruitment to the cornea in helminth-mediated keratitis (river blindness). *J Immunol* 2001, 166:4035–4041
10. Vu TH, Werb Z: Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev* 2000, 14:2123–2133
11. Lee S, Zheng M, Kim B, Rouse BT: Role of matrix metalloproteinase-9 in angiogenesis caused by ocular infection with herpes simplex virus. *J Clin Invest* 2002, 110:1105–1111
12. Li DQ, Shang TY, Kim HS, Solomon A, Lokeshwar BL, Pflugfelder SC: Regulated expression of collagenases MMP-1, -8, and -13 and stromelysins MMP-3, -10, and -11 by human corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2003, 44:2928–2936
13. Reviglio VE, Hakim MA, Song JK, O'Brien TP: Effect of topical fluoroquinolones on the expression of matrix metalloproteinases in the cornea. *BMC Ophthalmol* 2003, 3:10
14. Xue ML, Wakefield D, Willcox MD, Lloyd AR, Di Girolamo N, Cole N, Thakur A: Regulation of MMPs and TIMPs by IL-1 β during corneal ulceration and infection. *Invest Ophthalmol Vis Sci* 2003, 44:2020–2025
15. Yang YN, Bauer D, Wasmuth S, Steuhl KP, Heiligenhaus A: Matrix metalloproteinases (MMP-2 and 9) and tissue inhibitors of matrix metalloproteinases (TIMP-1 and 2) during the course of experimental necrotizing herpetic keratitis. *Exp Eye Res* 2003, 77:227–237
16. Owen CA, Hu Z, Lopez-Otin C, Shapiro SD: Membrane-bound matrix metalloproteinase-8 on activated polymorphonuclear cells is a potent, tissue inhibitor of metalloproteinase-resistant collagenase and serpinase. *J Immunol* 2004, 172:7791–7803
17. Owen CA, Hu Z, Barrick B, Shapiro SD: Inducible expression of tissue inhibitor of metalloproteinases-resistant matrix metalloproteinase-9 on the cell surface of neutrophils. *Am J Respir Cell Mol Biol* 2003, 29:283–294
18. Pei D: Leukolysin/MMP25/MT6-MMP: a novel matrix metalloproteinase specifically expressed in the leukocyte lineage. *Cell Res* 1999, 9:291–303
19. Faurschou M, Borregaard N: Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 2003, 5:1317–1327
20. Borregaard N, Sorensen OE, Theilgaard-Monch K: Neutrophil granules: a library of innate immunity proteins. *Trends Immunol* 2007, 28:340–345
21. Van Lint P, Libert C: Matrix metalloproteinase-8: cleavage can be decisive. *Cytokine Growth Factor Rev* 2006, 17:217–223
22. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G: Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol* 2002, 37:375–536
23. Tester AM, Cox JH, Connor AR, Starr AE, Dean RA, Puente XS, Lopez-Otin C, Overall CM: LPS responsiveness and neutrophil chemotaxis in vivo require PMN MMP-8 activity. *PLoS ONE* 2007, 2:e312
24. Johnson AC, Heinzel FP, Diaconu E, Sun Y, Hise AG, Golenbock D, Lass JH, Pearlman E: Activation of toll-like receptor (TLR)2, TLR4, and TLR9 in the mammalian cornea induces MyD88-dependent corneal inflammation. *Invest Ophthalmol Vis Sci* 2005, 46:589–595
25. Lin M, Carlson E, Diaconu E, Pearlman E: CXCL1/KC and CXCL5/LIX are selectively produced by corneal fibroblasts and mediate neutrophil infiltration to the corneal stroma in LPS keratitis. *J Leukoc Biol* 2007, 81:786–792
26. Carlson EC, Drazba J, Yang X, Perez VL: Visualization and charac-

- terization of inflammatory cell recruitment and migration through the corneal stroma in endotoxin-induced keratitis. *Invest Ophthalmol Vis Sci* 2006, 47:241–248
27. Lopez AF, Strath M, Sanderson CJ: Differentiation antigens on mouse eosinophils and neutrophils identified by monoclonal antibodies. *Br J Haematol* 1984, 57:489–494
28. Tao Y, Bazan HE, Bazan NG: Platelet-activating factor induces the expression of metalloproteinases-1 and -9, but not -2 or -3, in the corneal epithelium. *Invest Ophthalmol Vis Sci* 1995, 36:345–354
29. Fini ME, Girard MT, Matsubara M: Collagenolytic/gelatinolytic enzymes in corneal wound healing. *Acta Ophthalmol Suppl* 1992, 26–33
30. Sivak JM, Fini ME: MMPs in the eye: emerging roles for matrix metalloproteinases in ocular physiology. *Prog Retin Eye Res* 2002, 21:1–14
31. Balbin M, Fueyo A, Tester AM, Pendas AM, Pitiot AS, Astudillo A, Overall CM, Shapiro SD, Lopez-Otin C: Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat Genet* 2003, 35:252–257
32. Gioia M, Fasciglione GF, Marini S, D'Alessio S, De Sanctis G, Diekmann O, Pieper M, Politi V, Tschesche H, Coletta M: Modulation of the catalytic activity of neutrophil collagenase MMP-8 on bovine collagen I. Role of the activation cleavage and of the hemopexin-like domain. *J Biol Chem* 2002, 277:23123–23130
33. Pelman GR, Morrison CJ, Overall CM: Pivotal molecular determinants of peptidic and collagen triple helicase activities reside in the S3' subsite of matrix metalloproteinase 8 (MMP-8): the role of hydrogen bonding potential of ASN188 and TYR189 and the connecting cis bond. *J Biol Chem* 2005, 280:2370–2377
34. Michelacci YM: Collagens and proteoglycans of the corneal extracellular matrix. *Braz J Med Biol Res* 2003, 36:1037–1046
35. Liu X, Wu H, Byrne M, Jeffrey J, Krane S, Jaenisch R: A targeted mutation at the known collagenase cleavage site in mouse type I collagen impairs tissue remodeling. *J Cell Biol* 1995, 130:227–237
36. Chang C, Houck JC: Demonstration of the chemotactic properties of collagen. *Proc Soc Exp Biol Med* 1970, 134:22–26
37. Weatherington NM, van Houwelingen AH, Noerager BD, Jackson PL, Kraneveld AD, Galin FS, Folkerts G, Nijkamp FP, Blalock JE: A novel peptide CXCR ligand derived from extracellular matrix degradation during airway inflammation. *Nat Med* 2006, 12:317–323
38. Pfister RR, Haddox JL, Sommers CI, Lam KW: Identification and synthesis of chemotactic tripeptides from alkali-degraded whole cornea. A study of N-acetyl-proline-glycine-proline and N-methyl-proline-glycine-proline. *Invest Ophthalmol Vis Sci* 1995, 36:1306–1316
39. Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994, 76:301–314
40. Stefanidakis M, Ruottula T, Borregaard N, Gahmberg CG, Koivunen E: Intracellular and cell surface localization of a complex between alphaMbeta2 integrin and promatrix metalloproteinase-9 progelatinase in neutrophils. *J Immunol* 2004, 172:7060–7068
41. Fernandez-Patron C, Zouki C, Whittall R, Chan JS, Davidge ST, Filep JG: Matrix metalloproteinases regulate neutrophil-endothelial cell adhesion through generation of endothelin-1[1–32]. *FASEB J* 2001, 15:2230–2240
42. Van den Steen PE, Proost P, Wuyts A, Van Damme J, Opdenakker G: Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood* 2000, 96:2673–2681
43. Schonbeck U, Mach F, Libby P: Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. *J Immunol* 1998, 161:3340–3346
44. Van den Steen PE, Husson SJ, Proost P, Van Damme J, Opdenakker G: Carboxyterminal cleavage of the chemokines MIG and IP-10 by gelatinase B and neutrophil collagenase. *Biochem Biophys Res Commun* 2003, 310:889–896
45. Li Q, Fukuda K, Lu Y, Nakamura Y, Chikama T, Kumagai N, Nishida T: Enhancement by neutrophils of collagen degradation by corneal fibroblasts. *J Leukoc Biol* 2003, 74:412–419
46. McClellan SA, Huang X, Barrett RP, Lighvani S, Zhang Y, Richiart D, Hazlett LD: Matrix metalloproteinase-9 amplifies the immune response to *Pseudomonas aeruginosa* corneal infection. *Invest Ophthalmol Vis Sci* 2006, 47:256–264
47. Betsuyaku T, Shipley JM, Liu Z, Senior RM: Gelatinase B deficiency does not protect against lipopolysaccharide-induced acute lung injury. *Chest* 1999, 116:17S–18S
48. Betsuyaku T, Shipley JM, Liu Z, Senior RM: Neutrophil emigration in the lungs, peritoneum, and skin does not require gelatinase B. *Am J Respir Cell Mol Biol* 1999, 20:1303–1309
49. Malik M, Bakshi CS, McCabe K, Catlett SV, Shah A, Singh R, Jackson PL, Gaggar A, Metzger DW, Melendez JA, Blalock JE, Sellati TJ: Matrix metalloproteinase 9 activity enhances host susceptibility to pulmonary infection with type A and B strains of *Francisella tularensis*. *J Immunol* 2007, 178:1013–1020
50. Gueders MM, Balbin M, Rocks N, Foidart JM, Gosset P, Louis R, Shapiro S, Lopez-Otin C, Noel A, Cataldo DD: Matrix metalloproteinase-8 deficiency promotes granulocytic allergen-induced airway inflammation. *J Immunol* 2005, 175:2589–2597
51. Gutierrez-Fernandez A, Inada M, Balbin M, Fueyo A, Pitiot AS, Astudillo A, Hirose K, Hirata M, Shapiro SD, Noel A, Werb Z, Krane SM, Lopez-Otin C, Puente XS: Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8). *FASEB J* 2007, 21:2580–2591
52. Van Lint P, Wielockx B, Puimege L, Noel A, Lopez-Otin C, Libert C: Resistance of collagenase-2 (matrix metalloproteinase-8)-deficient mice to TNF-induced lethal hepatitis. *J Immunol* 2005, 175:7642–7649
53. Gaggar A, Jackson PL, Noerager BD, O'Reilly PJ, McQuaid DB, Rowe SM, Clancy JP, Blalock JE: A novel proteolytic cascade generates an extracellular matrix derived chemoattractant in chronic neutrophil inflammation. *J Immunol*, 2008, 180:5662–5669
54. Dong Z, Ghabrial M, Katar M, Fridman R, Berk RS: Membrane-type matrix metalloproteinases in mice intracorneally infected with *Pseudomonas aeruginosa*. *Invest Ophthalmol Vis Sci* 2000, 41:4189–4194
55. Li Q, Park PW, Wilson CL, Parks WC: Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell* 2002, 111:635–646
56. Pham CT: Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol* 2006, 6:541–550