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Matrilysin Shedding of Syndecan-1 Regulates Chemokine Mobilization and Transepithelial Efflux of Neutrophils in Acute Lung Injury

Qinglang Li,[1] Pyong Woo Park,[2] Carole L. Wilson,[1] and William C. Parks[1,3]

[1]Departments of Pediatrics, Medicine, and Cell Biology and Physiology
Washington University School of Medicine
Saint Louis, Missouri 63110

[2]Section of Infectious Diseases
Department of Medicine
Baylor College of Medicine
Houston, Texas 77030

Summary

The influx of inflammatory cells to sites of injury is largely directed by signals from the epithelium, but how these cells form chemotactic gradients is not known. In matrilysin null mice, neutrophils remained confined in the interstitium of injured lungs and did not advance into the alveolar space. Impaired transepithelial migration was accompanied by a lack of both shed syndecan-1, a heparan sulfate proteoglycan, and KC, a CXC chemokine, in the alveolar fluid. KC was bound to shed syndecan-1, and it was not detected in the lavage of syndecan-1 null mice. In vitro, matrilysin cleaved syndecan-1 from the surface of cells. Thus, matrilysin-mediated shedding of syndecan-1/KC complexes from the mucosal surface directs and confines neutrophil influx to sites of injury.

Introduction

Despite their specialization to serve distinct functions, the epithelia of different tissues respond similarly to injury and infection and regulate inflammation by equivalent mechanisms. By forming a barrier and releasing antimicrobial products, epithelia provide the first line of defense against invading pathogens. Following injury, epithelial cells initiate a programmed series of coordinated responses, such as proliferation, migration, and differentiation, to restore tissue integrity. By their production of chemoattractants, adhesion molecules, and other proteins, epithelial cells recruit and confine inflammatory cells to sites of injury. Though seemingly divergent processes, the epithelial programs regulating repair, defense, and inflammation may have coevolved, particularly with respect to the genes selectively induced. After all, injury provides an opportunity for infection, and infection can lead to injury, and both events are proinflammatory. Hence, many of the epithelial products associated with any one of these events are likely common to all. Matrilysin (MMP-7), a matrix metalloproteinase (MMP), is an example of a protein that functions in defense, repair, and as shown here, inflammation.

Matrilysin is produced by the epithelium of several non-injured, non-inflamed tissues, such as lung, liver, and breast (Wilson and Matrisian, 1998). The expression of matrilysin in healthy epithelium suggests that it functions in a common homeostatic process among epithelia, which seems to be defense against microorganisms. In mice, matrilysin activates intestinal pro-α-defensins, a family of antimicrobial peptides, and due to the lack of mature α-defensins, matrilysin null mice have an impaired ability to battle enteric pathogens (Wilson et al., 1999). Another function of epithelial-derived matrilysin is generating soluble Fas ligand (Mitsiades et al., 2001; Powell et al., 1999), a processing event that has been linked to tumor progression (Crawford et al., 2002; Fingleton et al., 2001). In addition to being produced in intact tissues, matrilysin is expressed in migrating epithelium in injured airway (Dunsmore et al., 1998) and intestine (Saarialho-Kere et al., 1996), suggesting that this MMP also functions in epithelial repair. Indeed, in wild-type tissue, trachea wounds repair rapidly, whereas wounds in matrilysin null tissue show no evidence of epithelial migration, even several days post injury (Dunsmore et al., 1998). In fact, matrilysin-deficient mice have the most severe wound repair defect among the MMP knockout mice generated to date (Parks, 1999; Parks and Shapiro, 2001).

Because matrilysin is needed for the closure of mucosal wounds, we reasoned that matrilysin null mice would be more susceptible to severe tissue injury. After all, if re-epithelialization is impaired, then excessive fibrosis, due to continued exudation of interstitial fluids, granulation tissue formation, and fibroblast migration, would ensue. We tested this idea using the bleomycin-induced model of lung fibrosis. Bleomycin, instilled intratracheally, causes rapid and extensive alveolar epithelia damage, followed by severe, persistent inflammation and fibrosis. Unexpectedly, matrilysin null mice were markedly protected against the lethal and, to a lesser extent, fibrotic effects of bleomycin-induced injury, and this protection was associated with a significant reduction in the number of neutrophils that moved into the alveolar space. We demonstrate that matrilysin generates a transepithelial gradient of KC, a CXC chemokine, by ectodomain shedding of syndecan-1, the predominant heparan sulfate proteoglycan on epithelia. Our findings identify in vivo a specific sheddase for a syndecan, a substrate for matrilysin, and syndecan-1 as a binding partner for KC. They also reveal an in vivo mechanism of how the epithelium controls and confines inflammation to sites of injury through the activity of three components: matrilysin, syndecan-1, and KC.

Results

Matrilysin Deficiency Confers Protection against Severe Lung Injury

Matrilysin mRNA and protein were barely detectable in control mouse lungs (Figures 1A and 1E). Following instillation with 0.04 U bleomycin, matrilysin mRNA was induced and increased over the next 15 days (Figure 1A), and prominent staining for the protein was seen on the surface of alveolar epithelial cells, particularly those

[3]Correspondence: parks_w@kids.wustl.edu

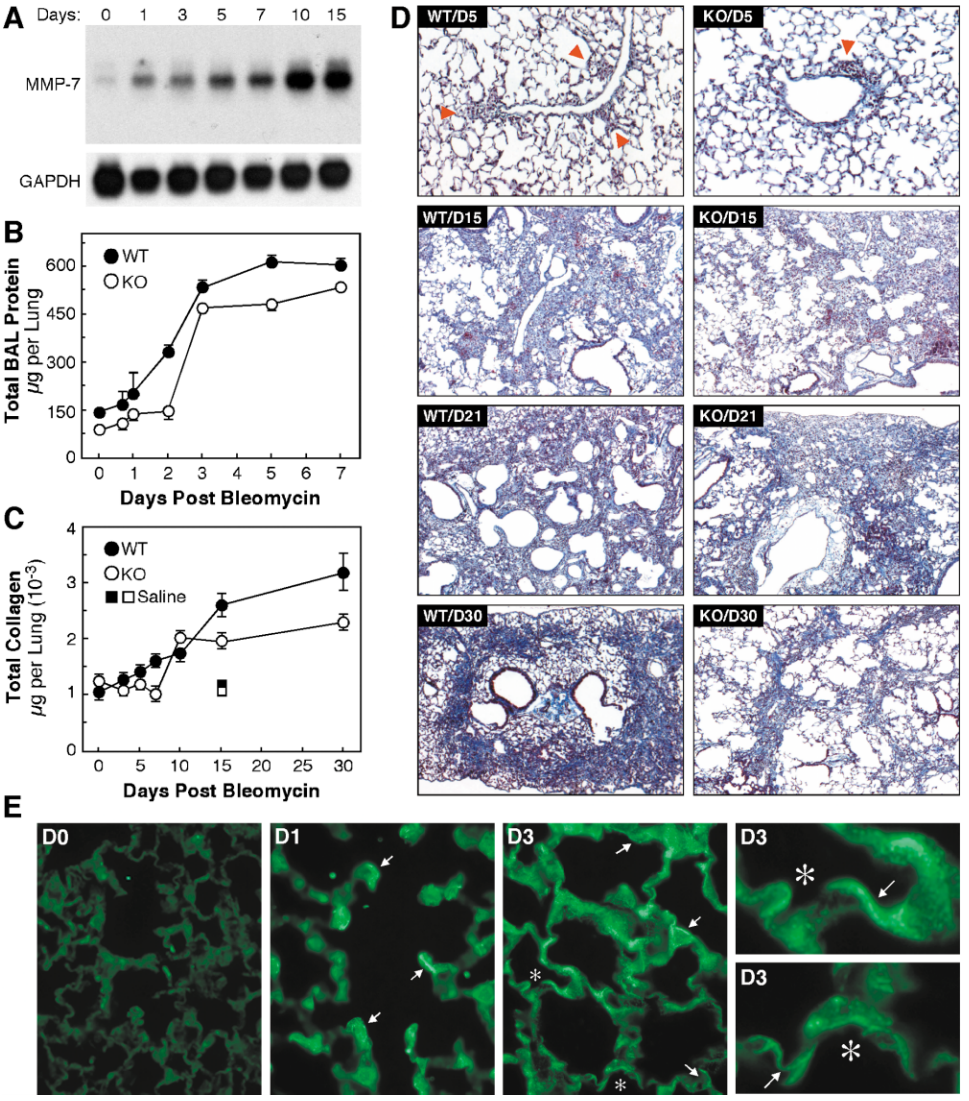

Figure 1. Response to Instilled Bleomycin

WT and MAT−/− mice were instilled intratracheally with 0.04 U bleomycin or saline.

(A) Matrilysin (MMP-7) and GAPDH mRNAs were detected by RT-PCR/Southern hybridization of RNA from control (day 0) and bleomycin-instilled WT lungs. Only baseline signal was detected in saline-instilled mice. No product was seen in control reactions lacking reverse transcriptase (data not shown).

(B) BAL was collected from WT and MAT−/− (KO) lungs, and total protein was quantified. Data are the mean ± SD ($n = 6$). Other than at 16 hr and 1 day, the amount of protein in MAT−/− BAL was significantly less than that in WT BAL at all points ($p < 0.02$).

(C) Hydroxyproline content was assayed in the insoluble matrix. Data were converted to total collagen and are the mean ± SD ($n = 3$–8 mice per point).

(D) Lung sections were stained with Masson's trichrome. At 5 days post-bleomycin, perivascular infiltrates (arrowheads) were seen in both WT and MAT−/− (KO) lungs. In WT mice, infiltrates had progressed from the perivascular space into surrounding alveoli, but in MAT−/− lungs, the infiltrating cells accumulated within an expanded perivascular compartment (arrowhead).

(E) Sections of control and bleomycin-instilled WT lungs were stained with a rat monoclonal against matrilysin and a FITC secondary antibody. Only background fluorescence was seen in control sections (D0). Signal for matrilysin protein was seen in alveolar epithelial cells (arrows) at 1 (D1) and 3 (D3) days post-bleomycin. Fluorescence for matrilysin was strongest along the cell perimeter and on cell extensions spreading over denuded tissue, such as marked by asterisks in the D3 image. These areas are shown under higher magnification at the far right.

bordering denuded regions (Figure 1E). To assess the role of matrilysin in mucosal injury, we compared the effects of bleomycin-instillation between wild-type (WT) and matrilysin null (MAT−/−) mice. MAT−/− mice are healthy, with normal growth, reproduction (Wilson et al., 1997, 1999), and lung morphology. Due to mucosal injury, protein in broncho-alveolar lavage (BAL) increased over the first week post-bleomycin, but BAL protein levels were lower in MAT−/− mice compared to WT animals (Figure 1B).

Bleomycin mediates focal injuries, and discreet sites of inflammation and nascent fibrosis were seen in both WT and MAT−/− mice at 5-days postinstillation (Figure 1D). Total insoluble collagen, a marker of fibrosis, increased progressively following bleomycin but was not markedly different between MAT−/− and WT mice over

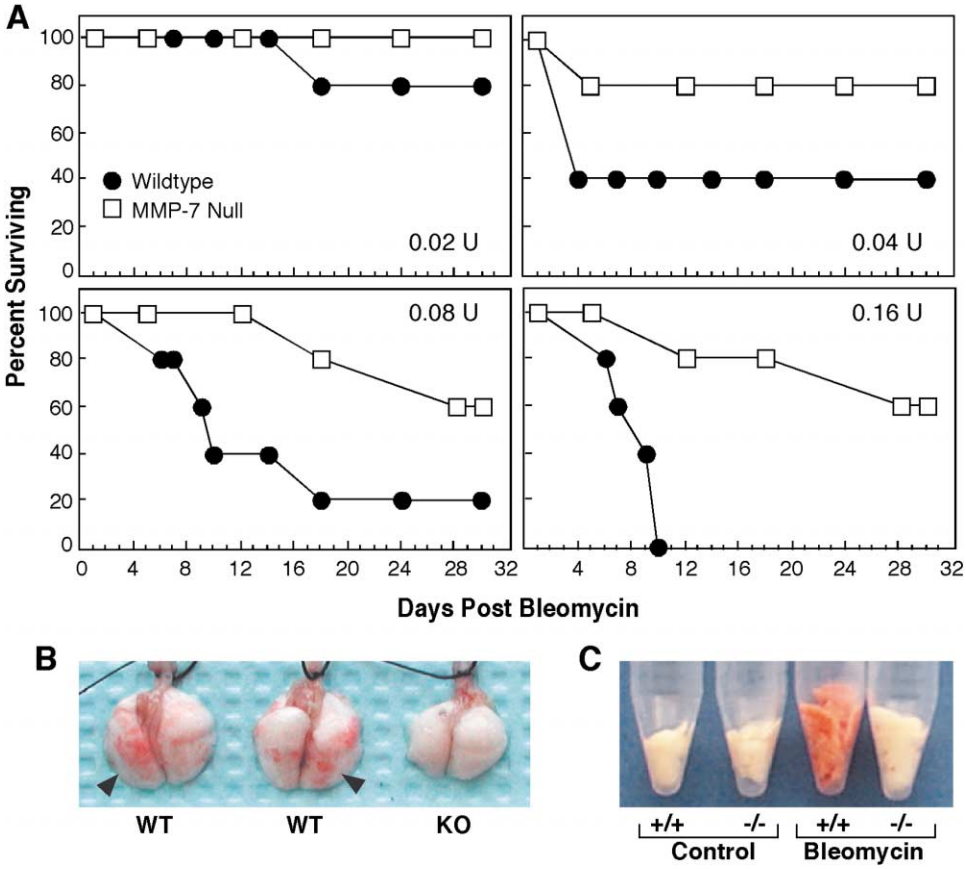

Figure 2. Reduced Mortality in MAT−/− Mice

(A) WT and MAT−/− mice (n = 10) were instilled with the indicated concentrations of bleomycin, and mortality was followed over the next 30 days.

(B and C) Lungs of WT and MAT−/− (KO) mice were harvested 16 or 24 hr after instillation with 0.08 U (B) or 0.16 U (C) bleomycin and were perfused with saline under airway pressure. Arrowheads mark a few areas of hemorrhage seen on the surface of the WT lungs.

the first 10 days postinstillation (Figure 1C). At later times, collagen accumulated at a slightly reduced rate in MAT−/− animals. Regardless, extensive fibrosis was seen at 15–30 days postinstillation in all mice (Figure 1D). Consistent with the collagen levels, alveolar fibrosis was typically less extensive in MAT−/− mice compared to WT animals, particularly at later times.

The BAL protein and collagen data indicated that bleomycin caused less injury in MAT−/− mice compared to WT mice. To explore further this unexpected response, we instilled mice with various concentrations of bleomycin and tracked their survival over the next 30 days. At all doses, MAT−/− mice were protected against bleomycin-induced lethality. At the higher doses (0.08 and 0.16 U), most WT mice were dead by 10 days after instillation, while most MAT−/− mice were still alive (Figure 2A). Furthermore, within 24 hr of instillation with 0.08 or 0.16 U bleomycin, hemorrhage (Figures 2B and 2C), and cell debris (Figures 3A and 3B) were evident in WT lungs and BAL, respectively, but not in samples from MAT−/− mice.

Impaired Neutrophil Influx

We did not think that the slight reduction in fibrosis in the later phases led to protection against bleomycin-induced lethality. Rather, we reasoned that matrilysin regulates a potentially lethal process during the acute phase of injury. TNFα, a key factor mediating bleomycin-induced fibrosis (Ortiz et al., 1998; Piguet et al., 1989), is activated by matrilysin in cultured macrophages (Haro et al., 2000). However, we found no difference in the levels of total or active TNFα in BAL, lung extract, or blood between WT and MAT−/− mice at any time after bleomycin (data not shown). Matrilysin can release Fas ligand (Powell et al., 1999); however, a role for Fas ligand-mediated apoptosis in bleomycin-induced fibrosis is controversial (Aoshiba et al., 2000; Kuwano et al., 1999). Regardless, we detected no difference in the levels of soluble Fas ligand or the number of apoptotic cells between bleomycin-instilled WT and MAT−/− mice (data not shown). The prominent phenotype in MAT−/− mice was impaired transepithelial influx of neutrophils.

In bleomycin-treated WT lungs, inflammatory cells infiltrated from the perivascular space into the lumen of nearby alveoli (Figure 1D). In contrast, inflammatory cells in MAT−/− lungs were huddled within the perivascular space and, for the most part, had not moved into surrounding alveoli (Figure 1D). We then examined earlier times using a higher dose of bleomycin (0.16 U) to maximize differences between mouse strains. In BAL from WT mice 16 hr post-bleomycin, we saw several neutro-

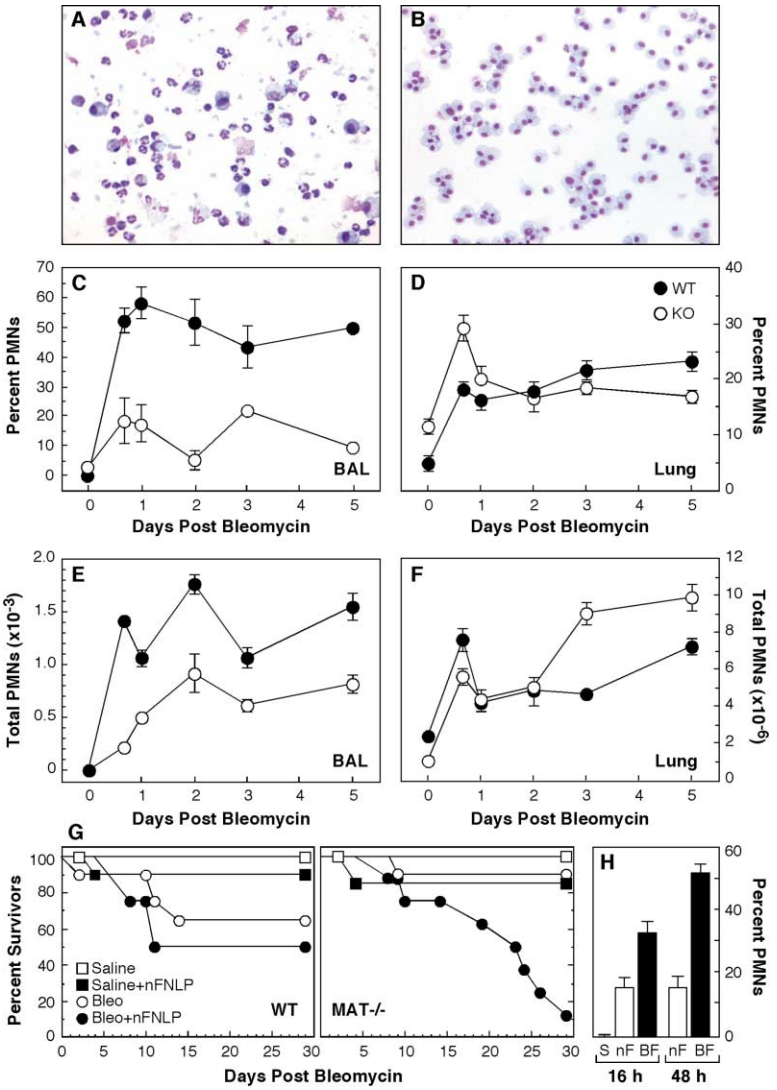

Figure 3. Reduced Neutrophil Influx into the Alveolar Space of MAT−/− Mice

Lungs and BAL from WT and MAT−/− (KO) mice were harvested from control animals (0 days) and at various times (16 hr to 5 days) postinstillation with 0.16 U bleomycin.

(A) At 16 hr post-bleomycin, BAL from WT mice contained numerous neutrophils, as well as a few macrophage and cell debris, (B) whereas BAL from MAT−/− mice contained an abundance of macrophages, few neutrophils, and essentially no debris.

(C–F) Leukocytes were isolated from BAL and lung extracts and counted. Neutrophils (PMNs) are expressed as a percent of total leukocytes (C and D) or as total cells per lung (E and F). Data are the mean ± SD (n = 6).

(G) WT and MAT−/− mice (n = 10) were instilled with saline or 0.04 U bleomycin with or without nFNLP, and mortality was assessed over the next 29 days.

(H) Leukocytes were isolated at 16 and 48 hr from the BAL of MAT−/− mice instilled with saline (S), nFNLP (nF), or bleomycin and nFNLP (BF) and counted.

phils, some macrophages, and cell debris (Figure 3A). However, in the 16 hr BAL of MAT−/− mice, we saw few neutrophils, an abundance of macrophages, and essentially no debris (Figure 3B). From 1–5 days post-bleomycin, the number of neutrophils recovered in BAL—expressed as either percent of total leukocytes or as total neutrophils—was consistently less in MAT−/− mice than in WT mice (Figures 3C and 3E).

The total neutrophils isolated from lung tissue of bleomycin-instilled mice was about 3–4 logs greater than the number found in BAL (Figures 3E and 3F) and was not markedly different between WT and MAT−/− mice (Figures 3D and 3F). However, the percent of neutrophils in the lung at 16 hr and 1 day post-bleomycin was slightly greater in MAT−/− mice than in WT animals (Figure 3D), consistent with more macrophages moving into the alveolar space. Furthermore, about 20%–40% more neutrophils were detected in MAT−/− lungs at 3–5 days postinstillation with 0.16 U bleomycin (Figure 3F). These data were confirmed by measuring myeloperoxidase activity in lung extracts (data not shown). The number of circulating neutrophils, monocytes, and other leukocytes did not differ between WT and MAT−/− mice (data not shown). These data indicate that although roughly the same number of neutrophils emigrated into MAT−/− and WT lungs soon after injury, these cells had an impaired ability to advance from the interstitium into the lumenal compartment in the absence of matrilysin. Because matrilysin is not expressed by murine neutrophils, macrophages, or interstitial cells, the defect in neutrophil migration must be due to an epithelial-derived process.

Forced Neutrophil Influx Restores Lethality

To assess if the protective response seen in MAT−/− mice was due to impaired transepithelial influx of neutrophils, we instilled n-formyl-nle-leu-phe (nFNLP), a potent neutrophil chemotactic peptide, with 0.04 U bleomycin. At this dose, about 30%–50% of WT and 10%–20% of knockout mice died within 15 days (Figures 2A and 3G). When coinstilled with nFNLP, bleomycin led to the death of essentially all MAT−/− mice, but this combination only modestly increased the lethality of WT animals (Figure 3G). In bleomycin-treated MAT−/− mice, nFNLP

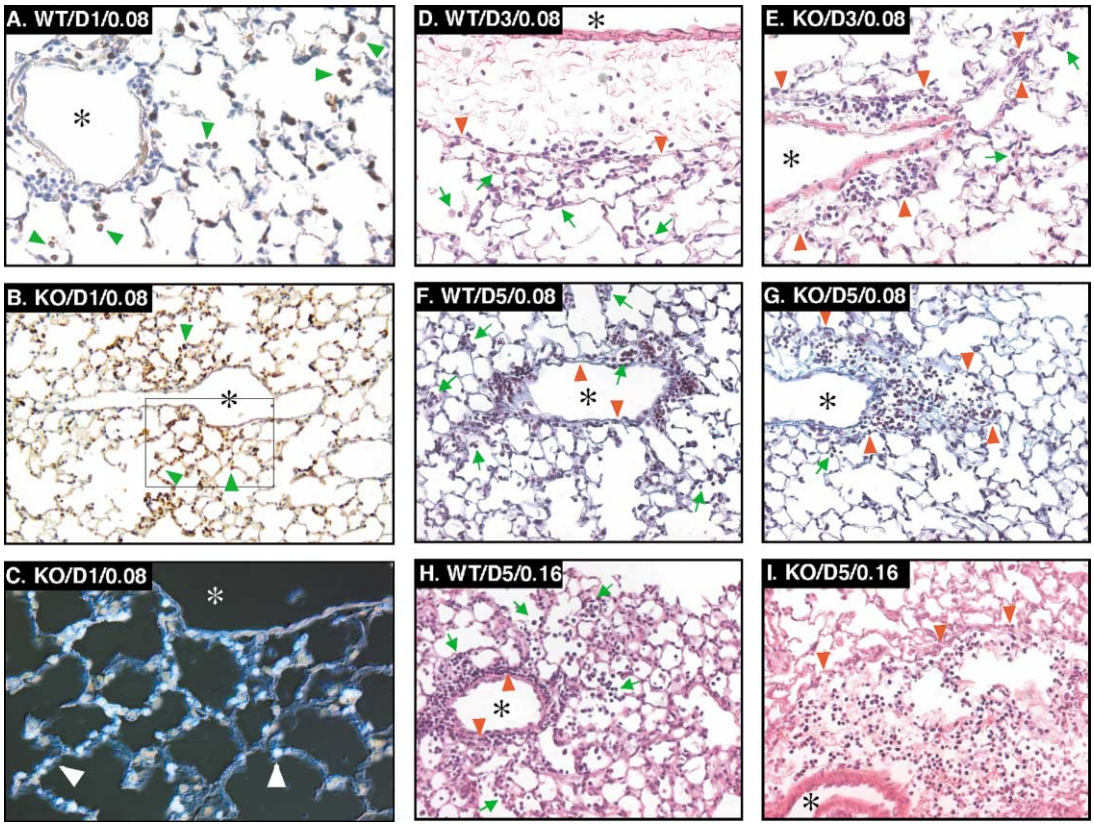

Figure 4. Limited Neutrophil Migration in MAT−/− Mice

WT and MAT−/− mice were instilled 0.08 or 0.16 U bleomycin, and lungs were harvested 1–5 days later, fixed, and embedded. In each image, the lumen of a nearby venule is marked (*).

(A–C) Sections of day 1 lungs were incubated with GR-1, an antibody specific for murine neutrophils. In WT lungs, many neutrophils (brown stain; green arrowheads) were seen unattached within the alveolar space. In MAT−/− lungs, several neutrophils were seen but only in close apposition to the alveolar walls and not free within the lumen. Under higher magnification (C), stained neutrophils (cyan in this reverse image of the boxed area in B) were seen within the alveolar wall.

(D–I) Sections of day 3 and 5 lungs were stained with hematoxylin/eosin or Masson's trichrome (F and G). In all images, the border between the perivascular and alveolar compartments is marked by red arrowheads; neutrophils that have migrated into the alveoli are indicated by green arrows. In WT lungs, some neutrophils were seen within the perivascular space, but most had moved well into alveolar area. In MAT−/− mice, neutrophils were confined to the perivascular space.

mediated an influx of neutrophils comparable to that seen in bleomycin-treated WT mice (Figure 3H). These findings demonstrate that with an appropriate signal neutrophils from MAT−/− mice can move through tissue as effectively as do WT cells. In addition, once neutrophils were recruited into the alveolar space, MAT−/− mice were more susceptible to injury, as we originally predicted.

Confined Influx of Neutrophils into MAT−/− Lungs

To localize neutrophils, we stained lung sections with GR-1, an antibody specific for murine neutrophils. In WT lungs, neutrophils were mostly seen within the lumen of alveoli (Figure 4A). Because lungs were lavaged before fixation, the number of neutrophils seen in the alveolar space under represents the total amount present. In MAT−/− lungs, many more neutrophils were detected, but few of these were seen free within the alveolar lumen (Figure 4B). Instead, they were tightly apposed to the alveolar walls and under high magnification (Figure 4C) were seen confined within the alveolar walls and not on the lumenal side of the mucosal surface.

At 3 and 5 days post-bleomycin, a similar yet morphologically distinct phenotype was seen. In WT mice, neutrophils (and lymphocytes) had migrated from the perivascular space into the parenchymal compartment and were seen loose within the alveolar lumen (Figures 4D and 4F, green arrows). By 5 days postinstillation, more neutrophils had moved into lung tissue and had infiltrated deeper into the alveolar compartment, and an even more profound inflammatory response was seen in WT lungs instilled with 0.16 U bleomycin (Figure 4H). In contrast, infiltrating neutrophils were confined to an expanded perivascular space in 3- and 5-day MAT−/− lungs, and only occasional cells were seen within the alveolar areas (Figures 4E, 4G, and 4I).

Chemokine Compartmentalization

Based on these findings, we reasoned that matrilysin regulates the activity of an epithelial-derived neutrophil

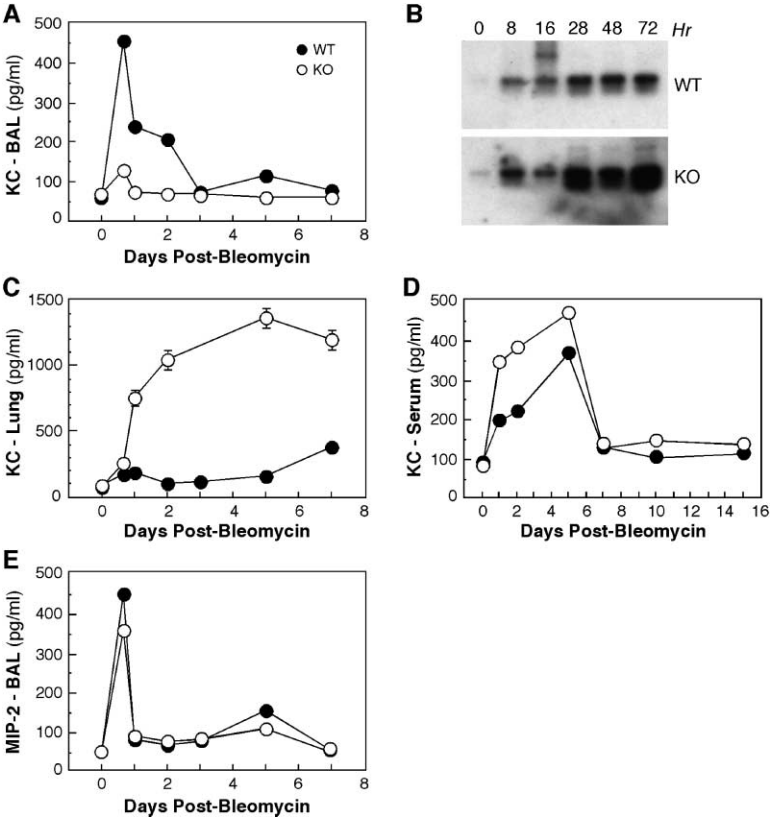

Figure 5. Impaired KC Compartmentalization in MAT−/− Lungs

WT and MAT−/− mice ($n = 6$) were instilled with 0.16 U bleomycin.

(A and E) BAL, (C) lung homogenates, and (D) sera were harvested from control animals and at 16 hr to 15 days postinstillation. KC and MIP-2 protein levels were determined by ELISA, shown as the mean ± SE.

(B) Total RNA was isolated at 0 to 72 hr postinstillation, and KC mRNA was assessed by RT-PCR/Southern hybridization.

chemotactic factor. We assessed the level of KC, a murine functional homolog of IL-8 and a structural homolog of human GROα. KC, an 8 kDa member of the CXC chemokine family, is a potent and essential neutrophil chemoattractant (Wiekowski et al., 2001) and is expressed by epithelial, endothelial, and interstitial cells in a variety of inflammatory conditions, including models of lung injury and infection (Heeckeren et al., 1997; Mehrad et al., 1999). In response to injury, KC is rapidly expressed and appears before many other chemokines (Endlich et al., 2002; Rovai et al., 1998).

KC levels in BAL from WT lungs were markedly elevated within 12 hr after bleomycin instillation and returned to controls levels by 3 days (Figure 5A), likely due to clearance. In contrast, KC protein remained at or near to baseline levels in BAL from bleomycin-treated MAT−/− mice (Figure 5A). Bleomycin-induced changes in other chemokines, namely MIP-2 (Figure 5E), eotaxin, and MIP-1α, did not differ between MAT−/− and WT mice (data not shown). Based on mRNA levels, reduced KC protein in the alveolar space of MAT−/− mice was not due to decreased gene expression (Figure 5B), and elevated levels in the serum indicated that endothelial-derived KC was essentially equal in both mouse strains. Of significance, KC protein levels remained fairly constant in lung extracts from WT mice but accumulated in lungs of MAT−/− mice (Figure 5C).

Lack of Syndecan-1 Shedding in MAT−/− Mice

These data indicate that matrilysin acts on a protein that facilitates the distribution of KC from the interstitium to the lumen. This protein is not KC; KC is not a substrate for matrilysin (data not shown). To identify a candidate substrate, we relied on four key bits of published information. (1) IL-8, the human homolog of KC, must be associated with heparan sulfate to have neutrophil chemotactic activity (Webb et al., 1993) and to bind its receptor, CXCR2, with high affinity (Hoogewerf et al., 1997). (2) Heparan sulfate is required for the IL-8-mediated efflux of neutrophils through the endothelium of venules (Middleton et al., 1997). (3) Matrilysin docks to heparan sulfate proteoglycans, and this association enhances enzyme activity (Yu and Woessner, 2000). (4) Syndecan proteoglycans, the principal source of cell-surface heparan sulfate, are shed in response to injury and inflammation by a yet to be described metalloproteinase (Bernfield et al., 1999; Fitzgerald et al., 2000). Syndecans, of which there are four gene products in mammals, are type I transmembrane proteoglycans found on essentially all adherent cells and have been shown in vitro to interact with a variety of proteins, including chemokines (Park et al., 2000b). Of these, syndecan-1 is abundantly expressed on the basal surface of lung epithelial cells. Thus, we explored if syndecan-1 was the matrilysin substrate involved in KC compartmentalization.

In untreated WT and MAT−/− mice, equivalent signal for syndecan-1 was seen in the columnar epithelium of conducting airways and the cuboidal type II cells of the alveoli (Figure 6A, D0), and similar levels of the proteoglycan were detected in detergent extracts of whole lung (Figure 6B, Lung). At 1 and 2 days post-bleomycin, total syndecan-1 levels remained relatively unchanged in both WT and MAT−/− lungs (Figure 6B, Lung). Be-

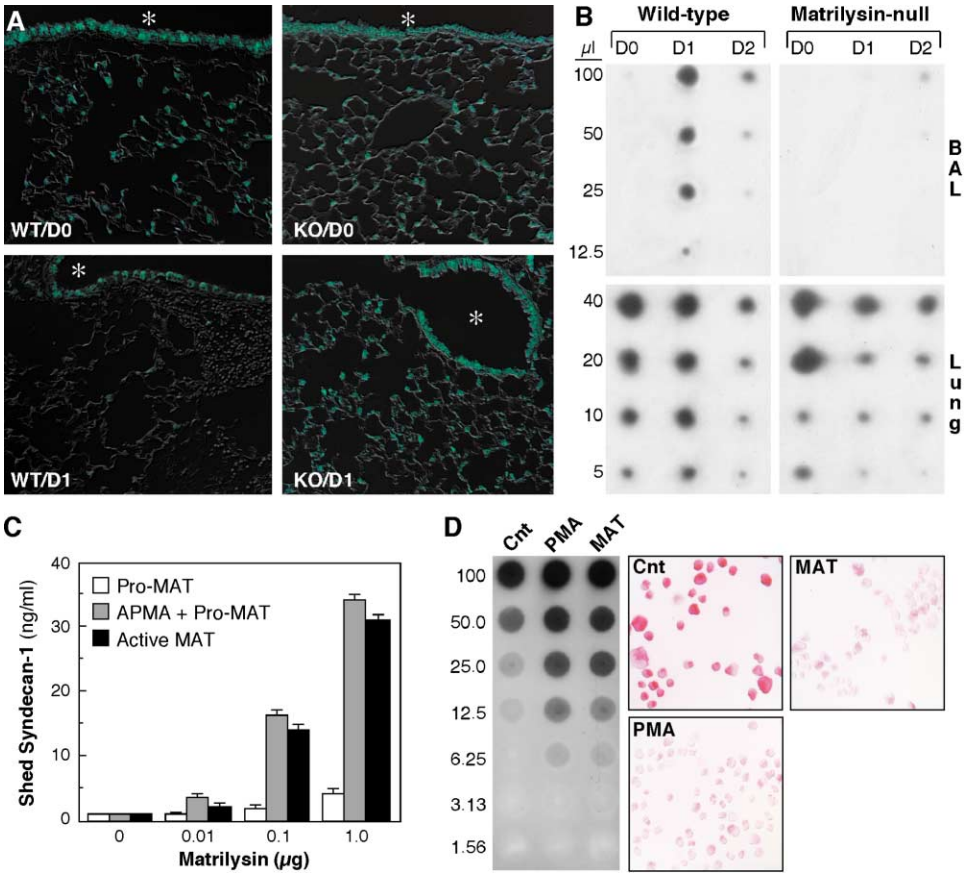

Figure 6. Lack of Syndecan-1 Shedding in MAT−/− Mice

(A) Lungs were harvested from WT and MAT−/− (KO) mice before (D0) and 1 day (D1) postinstillation with 0.16 U bleomycin. Sections were stained with antibody 281-2 against mouse syndecan-1 ectodomain. Images were viewed using DIC optics and inverted. In each image, the lumen of a conducting airway is marked (*).

(B) Serial dilutions (μl) of BAL and lung homogenates from WT and MAT−/− controls (D0) and from mice at 1 and 2 days post-bleomycin were immunoblotted with antibody 281-2 for shed syndecan-1. Similar results were obtained using either cationic or nitrocellulose membranes, suggesting that shed syndecan-1 in WT BAL is bound to a protein.

(C) Attached NMuMG cells were incubated with the indicated amounts of pro-matrilysin, APMA-activated pro-matrilysin, or active matrilysin. Shed syndecan-1 was quantified by dot immunoblot assay using pure mouse syndecan-1 as a standard. Data are mean ± SE ($n = 3$).

(D) HB-215 cells were incubated with 1 μg/ml active matrilysin or 1 μM phorbol ester (PMA). Shed syndecan-1 was assayed in serial dilutions of 100 μl medium by dot immunoblot. The cells were spun onto slides and stained without permeabilization for syndecan-1 ectodomain. Increased recovery of shed syndecan-1 ectodomains in the medium was accompanied by a loss of the proteoglycan on the cell surface.

cause bleomycin causes focal injuries mostly in the alveolar compartment, we did not expect to see a noticeable change in total lung syndecan-1. However, in bleomycin-treated WT lungs, we found numerous and extensive areas of alveoli lacking staining for syndecan-1 and slightly reduced signal in conducting airways (Figure 6A, WT/D1). Complementary to these observations, we detected a marked increase in soluble syndecan-1 in BAL at 1 day post-bleomycin, and these levels were reduced at day 2 (Figure 6B), likely indicating clearance by the mucociliary apparatus or by endocytosis.

In bleomycin-treated MAT−/− mice, however, both the pattern and intensity of syndecan-1 staining did not change compared to that seen in day 0 control lungs (Figure 6A) and soluble syndecan-1 was not detected in BAL samples (Figure 6B). Syndecan-1 in WT BAL was not detected with an antibody to the cytoplasmic domain (results not shown), verifying that the ectodomain was shed and that the proteoglycan was not released intact from damaged cells. We did not detect any difference in the levels of shed syndecan-4 (data not shown), another syndecan expressed by lung epithelia (Park et al., 2001). To assess if matrilysin directly sheds syndecan-1, we added the enzyme to cultures of syndecan-1-expressing cells. APMA-activated pro-matrilysin and active matrilysin, but not pro-matrilysin, cleaved syndecan-1 from normal murine mammary gland (NMuMG) cells (Figure 6C). Active matrilysin also shed syndecan-1 from the surface of HB-215 mouse myeloma cells similar to the release mediated by phorbol ester (Figure 6D), a syndecan shedding agonist (Fitzgerald et al., 2000).

KC Associates with Shed Syndecan-1

We used two immunoprecipitation approaches to assess if KC interacts with shed syndecan-1. In BAL samples from WT mice digested with heparinases and chondroitinase ABC and blotted with a monoclonal antibody

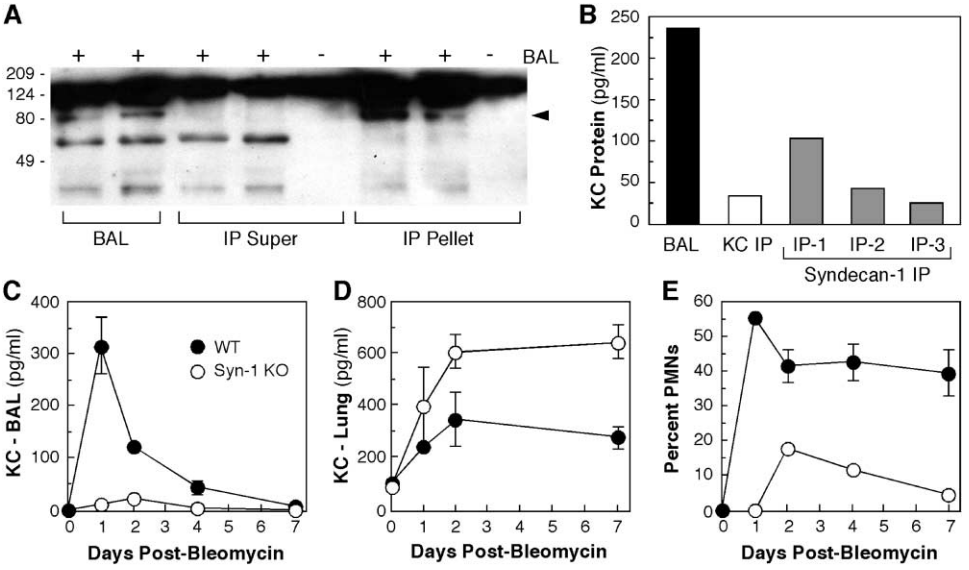

Figure 7. Syndecan-1/KC Interaction

(A) KC was immunoprecipitated (IP) from BAL of WT mice 1 day post-bleomycin. BAL and post-IP supernatants and pellets were electrophoresed and blotted for syndecan-1 ectodomain. The arrowhead indicates the band specifically coprecipitated with KC.

(B) KC and syndecan-1 were immunoprecipitated from BAL of WT mice 1 day post-bleomycin. Two additional syndecan-1 IPs were done on the post-IP supernatant. The level of KC protein in the BAL and in the post-IP supernatants was quantified by ELISA.

(C–E) WT and SYN1−/− mice ($n = 3$) were instilled with 0.15 U bleomycin. KC levels in BAL (C) and lung homogenates (D) were determined by ELISA.

(E) Neutrophils (PMNs) in BAL were counted and expressed as a percent of total leukocytes. Data are the mean ± SE.

against syndecan-1 ectodomains, we detected a band of about 80 kDa, the size of the syndecan-1 ectodomain core protein and non-specific bands at about 60 and 40 kDa (Figure 7A, BAL). BAL samples were then immunoprecipitated with an anti-KC antibody, and the pellets and supernatants were treated with heparinases and chondroitinase ABC and blotted for syndecan-1. In these samples, the 80 kDa syndecan-1 core protein was selectively coimmunoprecipitated with KC (Figure 7A, IP Pellet) but was absent in the non-precipitated supernatant (Figure 7A, IP Super). In addition, KC was effectively cleared from WT BAL with anti-KC antibodies and by immunoprecipitating consecutively with anti-syndecan-1 antibodies (Figure 7B). Together, these results demonstrate that KC is bound to syndecan-1 ectodomains in BAL of bleomycin-instilled WT mice.

To determine if syndecan-1 is required for KC mobilization, we examined syndecan-1 null (SYN1−/−) mice. Similar to MAT−/− mice, SYN1−/− are normal with no overt phenotype in unchallenged animals (Alexander et al., 2000; Park et al., 2001). As we saw in MAT−/− mice (Figure 5A), KC levels in BAL of bleomycin-instilled SYN1−/− mice remained at or near baseline levels (Figure 7C), and the levels in lung tissue were elevated compared to WT (Figure 7D). In addition and again similar to MAT−/− mice (Figure 3C), many less neutrophils were recovered from the BAL of SYN1−/− mice than of WT mice (Figure 7E). Elevated myeloperoxidase levels in lung homogenates did not differ between SYN1−/− and WT bleomycin-instilled mice were similar (data not shown) indicating that neutrophil movement into the lung was not impaired. Thus, lung alveolar inflammation is attenuated in the absence of syndecan-1 or matrilysin. These findings provide further evidence that matrilysin, syndecan-1, and KC coordinate and confine inflammation to sites of injury.

Discussion

Our study describes a mechanism in which three epithelial components—a secreted proteinase (matrilysin), a cell-bound proteoglycan (syndecan-1), and a chemokine (KC)—act coordinately to direct inflammatory cells to injury sites. We identified syndecan-1, the predominant heparan sulfate proteoglycan on epithelia, as a substrate for matrilysin in vivo and in vitro and demonstrate that shed syndecan-1 binds to and regulates the activity of the chemokine KC in injured mouse lung. We propose that in response to injury, epithelial cells synthesize, secrete, and deposit KC (and possibly other chemokines) onto the preexisting syndecan-1 molecules. Matrilysin is also induced by injury and secreted by wound-edge epithelia (Figure 1E) and, as suggested by the findings of others, may anchor to the heparan sulfate chains of cell surface proteoglycans (Yu and Woessner, 2000; Yu et al., 2002). Our data indicate that matrilysin cleaves the syndecan-1 core protein to release the ectodomain-KC complex. The shed complex is then transported, either actively or passively, to the apical surface creating a chemotactic gradient guiding neutrophils into the alveolar space.

Recently, two groups reported that matrilysin is prominently expressed by the epithelium in human pulmonary fibrosis (Cosgrove et al., 2002; Zuo et al., 2002), confirming our earlier findings (Dunsmore et al., 1998). Zuo et al. (2002) also showed a slight reduction in fibrosis in

MAT−/− mice at 14 days post-bleomycin, and we obtained essentially the same data at 15 days (Figure 1C). Although they concluded that matrilysin null mice are protected against bleomycin-induced fibrosis, our results indicate that the prominent and relevant phenotype in MAT−/− mice is an impaired efflux of neutrophils into the alveolar space. We conclude that the slight reduction in collagen deposition is a minor phenotype, likely a downstream consequence of less acute alveolar injury.

MAT−/− mice were protected against bleomycin-mediated lethality, particularly before significant fibrosis was detected (i.e., before day 15). Based on the number of cells recovered from tissue and on histologic analyses of lungs, neutrophils, as well as other inflammatory cells, in MAT−/− mice had no problem effluxing from the vasculature into the tissue and migrating through the interstitial space to the basal epithelial border. Because matrilysin is expressed by injured mucosa but not by neutrophils or interstitial cells, we expected a phenotype related to epithelial function. Our findings showed that neutrophils in MAT−/− lungs were stuck at the epithelial-matrix interface at 1 day post-bleomycin and accumulated in perivascular spaces over the next few days. Forcing neutrophils into the alveolar space by coinstillation of a bacterial chemotactic peptide, nFNLP, led to complete mortality of bleomycin-treated MAT−/− mice (Figure 3G), indicating that the protection seen in the knockout mice was due to confinement of neutrophils to the subepithelial compartment. Thus, epithelial-derived matrilysin regulates the transepithelial advancement of neutrophils during the initial stages of injury, but to understand this mechanism, we had to identify the enzyme's substrate.

The reduced levels of KC in BAL concomitant with an accumulation of the chemokine in the lungs of bleomycin-treated MAT−/− mice provided an important clue to uncovering the mechanism by which matrilysin regulates neutrophil movement. KC is a potent neutrophil chemoattractant, and like matrilysin, it is rapidly induced in epithelial cells in response to injury and infection. Complementary to our findings, immunoablation of KC in lung injury models significantly reduces tissue damage, presumably due to reduced neutrophil influx (Piccolo et al., 1999; Yoshidome et al., 1999). Furthermore, in various models of injury, infection, and inflammation, the influx and transepithelial migration of neutrophils are significantly impaired in mice deficient for the KC receptor CXCR2 or in mice treated with anti-CXCR2 antibodies (Del Rio et al., 2001; Godaly et al., 2000; Hall et al., 2001; Kielian et al., 2001; Olszyna et al., 2001). Together with our data, these findings indicate that KC is critical for directing the movement of neutrophils in tissues. Compartmentalization of other chemokines, such as MIP-2, was similar between WT and MAT−/− mice. Interestingly, neutralization of MIP-2 attenuates bleomycin-induced pulmonary fibrosis independently of neutrophil infiltration (Keane et al., 1999), which may explain why MAT−/− mice still develop significant fibrosis.

Modulation of chemokine activity and compartmentalization by a metalloproteinase is not unprecedented. Various MMPs cleave monocyte chemoattractant proteins resulting in either inactive or antagonistic chemokines (McQuibban et al., 2000, 2002). Gelatinase-A (MMP-2) facilitates the formation of a transepithelial gradient of interleukin-13 in allergen-challenged lung (Corry et al., 2002). The substrate MMP-2 cleaves to create this gradient is not known. Because KC was not a substrate for matrilysin, we hypothesized that this MMP acts on a molecule that shuttles the chemokine from the basal surface of the epithelium into the alveolar space. Our results indicate that syndecan-1 is the substrate responsible for establishing a transepithelial chemotactic gradient of KC within the injured lung. Syndecan-1 is shed by metalloproteinase cleavage of the ectodomain at a site near the transmembrane domain (Fitzgerald et al., 2000) and occurs in cells and tissues in response to injury, various inflammatory mediators, and virulence factors (Bernfield et al., 1999; Park et al., 2000b). Like the expression of matrilysin, shedding of syndecan-1 is a common response of challenged epithelia. The lack of syndecan-1 shedding in MAT−/− mice following bleomycin injury, along with our in vitro studies showing that matrilysin sheds syndecan-1 ectodomains from cultured epithelial cells, indicates that this MMP is a physiologic sheddase of this transmembrane proteoglycan.

Our data also demonstrate that KC is bound to shed syndecan-1 ectodomains and that these complexes are translocated into the alveolar space in a matrilysin-dependent manner. Indeed, KC was detected in the alveolar space of WT but not of MAT−/− or SYN1−/− mice. More important, KC was found in the BAL of WT mice only when syndecan-1 ectodomains were also detected in this compartment—that is, the first 2 days post bleomycin—despite the fact that KC expression continued beyond this time. Association with heparan sulfate enhances both the neutrophil chemotactic activity of IL-8 (Webb et al., 1993), the human functional homolog of KC, and the catalytic activity of matrilysin (Yu and Woessner, 2000). Thus, the heparan sulfate chains of syndecan-1 seem to be the central components of the epithelial machine that directs neutrophils to sites of injury. Importantly, the parallel reduction of KC levels and neutrophils in BAL in MAT−/− and SYN1−/− mice underscore the cooperative function that matrilysin and syndecan-1 serve in regulating chemokine compartmentalization and inflammation to sites of injury.

The observation that MAT−/− mice are protected against the acute injury and subsequent lethal effects of bleomycin does not mean that this MMP is a detrimental proteinase for the host. Indeed, matrilysin functions in mucosal immunity, epithelial migration, and as shown here, controlling neutrophil influx. Neutrophils comprise a major cellular component of host defense; for example, patients with neutropenia and leukocyte adhesion deficiency are susceptible to infection by opportunistic microbial pathogens. However, with extensive injury, such as that produced with high doses of bleomycin, a massive neutrophil influx would cause indiscriminate, severe, and potentially mortal tissue damage. We have described what we believe to be a normal and required repair process, which, if unregulated and exuberant, can be harmful. A lack of neutrophils and, in turn, the oxidative damage they can cause, would be consistent with the protective effect we observed in the MAT−/− mice.

Our data also suggest that the activation state of neu-

trophils is controlled by their location as they move through tissue compartments. Although neutrophils were plentiful in the MAT−/− lungs following bleomycin injury, the increased survival of these mice correlated with the inability of the granulocytes to cross the epithelial barrier. If the neutrophils were induced to advance into the lumenal space, as we did with nFNLP (Figure 3G), then MAT−/− mice died. We interpret these data to mean that once the neutrophils have completed their transepithelial trek into the lumenal space, they received the appropriate signal, possibly from the epithelium, to be activated and initiate an oxidative burst and degranulation. It is reasonable that neutrophil activity would be mostly restrained until the cells reach the lumen, where they would encounter microorganisms. We are now exploring how matrilysin-mediated processes control neutrophil activation.

Experimental Procedures

Lung Injury Model

At 8–10 weeks of age, MAT−/−, SYN1−/−, and WT mice (all C57BL/6 background) mice were instilled intratracheally with 0.02 to 0.16 U bleomycin (MeadJohnson, Princeton, NJ) in 50 μl sterile saline. Control animals were instilled with an equal volume of saline. In some experiments, 1 μM nFNLP (Sigma, St. Louis, MO) was coinstilled with saline or bleomycin.

Sample Collection and Analyses

Lungs were perfused via the right ventricle with 10 ml saline at 30 cm H_20, and BAL was collected by flushing the lung via the trachea 3 times with a 1 ml bolus of saline. The recovered lavage (typically 0.8 ml) was centrifuged at 2000 rpm, 4°C for 10 min. Total protein in BAL was quantified by the Bradford assay. Pelleted cells were suspended at 10^6 cells/ml in DMEM, 0.1% albumin, and a 100 μl aliquot was cytospun onto slides and differentially stained with LeukoStat (Fisher Scientific, Pittsburgh, PA). Leukocytes in lung tissue were isolated by Percoll centrifugation and quantified by LeukoStat staining (Moore et al., 2000). At least 500 cells in at least 5 fields were counted per sample.

RNA was isolated from flushed lungs with RNA-Bee (Tel-Test, Friendswood, TX). Matrilysin and GAPDH mRNAs were detected by RT-PCR/Southern hybridization (Wilson et al., 1995). Hydroxyproline levels in acid hydrolysates of whole lung were quantified by a colorimetric assay (Keane et al., 1999) and converted to total collagen using a conversion factor of 7.14. Myeloperoxidase activity was measured by a colorimetric assay using *o*-dianisidine-HCl. Chemokine and cytokine levels were quantified by ELISA (R&D Systems, Minneapolis, MN). Fas L in BAL was assayed by Western blotting. All assays were done in triplicate.

Histology and Immunostaining

Lungs were perfused-fixed with PBS-buffered formalin through the trachea under 20 cm pressure for 15 min, incubated in fixative at room temperature for 48 hr, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin/eosin or Masson's trichrome or were processed for alkaline phosphatase immunohistochemistry (Vector Laboratories, Burlingame, CA) or immunofluorescence. Rat anti-mouse syndecan-1 ectodomain monoclonal antibody (281-2) (Park et al., 2001) was diluted 1:1,000 and rat anti-mouse Ly6G (GR-1) monoclonal antibody (BD PharMingen, San Diego, CA) was used at 3 μg/ml. Anti-human matrilysin rat monoclonal antibody was used as described (Crawford et al., 2002). Controls sections were processed with preimmune serum.

Syndecan Assays

Syndecans in BAL, tissue extracts, and culture media were blotted onto nitrocellulose or were acidified and blotted onto cationic nylon membranes (Immobilon Ny+, Millipore) and detected by dot immunoblot assay using antibody 281-2, rabbit polyclonal antibody against mouse syndecan-1 cytoplasmic domain, or rat anti-mouse syndecan-4 monoclonal antibody (Ky8.2) as described (Park et al., 2001; Subramanian et al., 1997). Confluent cultures of NMuMG cells in 96-well microtiter plates were incubated for 4 hr in 100 μl medium without or with recombinant pro-matrilysin (Chemicon, Temecula, CA), pro-matrilysin activated with 1 mM APMA, or active matrilysin (Wilson et al., 1999). HB-215 cells (5×10^7 cells/ml; ATCC, Manassas, VA) were incubated in suspension with 1 μg/ml active matrilysin or 1 μM PMA at 37°C for 30 min serum-free medium.

Immunoprecipitation

Lavage (300 μl) was precleared with protein G Sepharose 4B (PGS) and immunoprecipitated with 10 μg rat anti-mouse KC antibody (R&D Systems) or 20 μg 281-2 antibody as described (Dumin et al., 2001). KC remaining in the immunoprecipitation supernatant was quantified by ELISA. In some experiments, syndecan-1 was reimmunoprecipitated twice from the post-wash supernatant. For Western blots of syndecan-1, glycosaminoglycans were removed before electrophesis by treating BAL samples and immunoprecipitates with heparinases and chondroitinase ABC as described (Park et al., 2000a).

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