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Epilysin (MMP-28) Restrains Early Macrophage Recruitment in Pseudomonas aeruginosa Pneumonia

Anne M. Manicone,2*,† Timothy P. Birkland,*,† Michelle Lin,*,† Tomoko Betsuyaku,‡ Nico van Rooijen,§ Jouko Lohi,¶ Jorma Keski-Oja,¶ Ying Wang,*,† Shawn J. Skerrett,† and William C. Parks*†

Several members of the matrix metalloproteinase (MMP) family function in various processes of innate immunity, particularly in controlling leukocyte influx. Epilysin (MMP-28) is expressed in numerous tissues and, in adult mice, it has the highest expression in lung, where it is detected in bronchial epithelial cells (Clara cells). Epilysin is also expressed by bone marrow-derived macrophages, but not by alveolar macrophages, suggesting that its expression by macrophages is dependent on localization and differentiation. To assess the role of this MMP, we generated epilysin-null (Mmp28−/−) mice. Although epilysin is constitutively expressed in normal tissues, Mmp28−/− mice have no overt phenotype. However, using a murine model of Pseudomonas aeruginosa pneumonia, we found that Mmp28−/− mice had an early increase in macrophage recruitment into the lungs, as well as enhanced bacterial clearance and reduced pulmonary neutrophilia, which we predicted were due to accelerated macrophage influx. Macrophage depletion in WT and Mmp28−/− mice confirmed a role for macrophages in clearing P. aeruginosa and regulating neutrophil recruitment. Furthermore, we observed that macrophages derived from Mmp28−/− mice migrated faster than did wild-type cells to bronchoalveolar lavage fluid from P. aeruginosa-treated mice of either genotype. These observations indicate that epilysin functions as an intrinsic negative regulator of macrophage recruitment by retarding the chemotaxis of these cells. The Journal of Immunology, 2009, 182: 3866–3876.

The innate immune system evolved as a rapid response to defend against pathogens and includes ready-to-go bactericidal and proinflammatory processes and the activities of both resident cells and infiltrating leukocytes (1). As the barrier separating the environment from internal tissues, epithelia are key effectors that respond to challenge and control various immune processes (2). For example, in response to infection or injury, epithelial cells produce various proteins and other factors that govern (both promote and restrain) the influx of inflammatory cells, particularly neutrophils and macrophages. Macrophages, which comprise a diverse group of cells, are critical effector cells that bridge innate and adaptive immune responses (3). Both resident macrophages and infiltrated monocytes can directly kill bacteria and, via their ability to release a range of bioactive factors, such as chemokines, TNF-α, and more, shape both the pattern and duration of an acute inflammatory reaction (4). Additionally, as professional APCs, macrophages are essential effectors of lymphocytic responses linking innate and adaptive immunity. Thus, identifying the products and pathways that control macrophage recruitment and activity is central to a clearer understanding of immune functions.

Matrix metalloproteinases (MMPs)3 have emerged as key effector enzymes regulating distinct stages of inflammation, and recent insights from in vitro and mouse models of human disease indicate that MMPs evolved to serve important functions in innate immunity (5, 6). The expression of MMPs in injured or inflamed tissues has led to the general concept that these proteinases mediate proinflammatory functions. Indeed, several MMPs promote leukocyte influx by modulating cytokine or chemokine activity (5–13). For example, work done in our laboratory demonstrated that matrilysin (MMP-7) promotes neutrophil influx across an epithelial barrier via shedding the transmembrane proteoglycan syndecan-1 complexed with KC, a murine CXC chemokine (9). Collagenase-2 (MMP-8) processes the CXC chemokine LIX to a more active form, predicting that it functions to stimulate neutrophil influx (14). Similarly, gelatinases A (MMP-2) and B (MMP-9) regulate the trafficking of specific inflammatory cells from the peribronchovascular bundle into the alveolar space via affecting the formation of CC chemokine gradients (8, 15). Epilysin (MMP-28), the newest and apparently last member of the mammalian MMP family, was cloned in our laboratory from human keratinocyte and testis cDNA libraries (16, 17). Like other MMPs, epilysin contains a prodomain, a zinc-binding catalytic domain, and a hemopexin-like domain. Additionally, epilysin contains a furin activation sequence and, hence, it is activated within the secretion pathway by cleavage of its prodomain (18). In human tissue, epilysin mRNA is expressed at high levels in the testis, lung, heart, GI tract, and in wounded epidermis (18). In mice, epilysin mRNA is produced in placenta, heart, uterus, testis,

3 Abbreviations used in this paper: MMP, matrix metalloproteinase; BALF, bronchoalveolar lavage fluid; BMDM, bone marrow-derived macrophage; CCSP, Clara cell secretory protein; ES, embryonic stem; LCM, laser capture microdissection; qRT-PCR, quantitative RT-PCR; WT, wild type.

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gastrointestinal tract, and lung, which has the highest level of expression. In tissue and cell cultures, epilysin is predominantly expressed by epithelium, including keratinocytes, placental villi, and airway epithelial cells (16, 18, 19). In addition to its predominant expression by epithelium, bone marrow-derived macrophages (BMDM) also express epilysin.

Because several MMPs function in immunity, we hypothesized that epilysin may have a regulatory role in the tissue response to environmental challenge. To test this, we generated epilysin-null mice (Mmp28−/−). Similar to many other MMP-null mice, Mmp28−/− mice revealed no overt phenotype. However, as we report herein, when challenged with Pseudomonas aeruginosa, a relevant respiratory pathogen, Mmp28−/− mice had accelerated macrophage recruitment compared with wild-type (WT) mice. Additionally, these knockout mice had enhanced bacterial clearance and reduced pulmonary neutrophilia, which we predict are two potential consequences of enhanced macrophage influx. Macrophage depletion in WT and Mmp28−/− mice confirmed a role for macrophages in clearing P. aeruginosa and regulating neutrophil recruitment. Furthermore, we observed that BMDM, which express epilysin, migrate at a slower rate when compared with BMDM cultured from Mmp28−/− mice. These observations indicate that epilysin functions as an intrinsic negative regulator of macrophage recruitment by retarding the chemotaxis of macrophages.

Materials and Methods

Mice

We cloned the mouse Mmp28 gene from the SV129 genome (gene ID 118453) (20). To construct the homologous extensions of the targeting construct, we isolated a 4.8-kb EcoRI-XhoI fragment containing the 3′ terminus of intron 1, exons 2 and 3, and −4 kb of intron 3 and a 2.1-kb XmaI-HindIII fragment containing the 3′ untranslated end of exon 8 and downstream sequence (Fig. 1A). A pgk-neomycin resistance cassette was inserted between the two arms and a diptheria toxa (dt) cassette was ligated to the 3′ end. The targeting construct was linearized with XhoI (the XhoI site at the 5′ end of the short arm was deleted by ligating to the SalI site in the neomycin cassette).

Transfection of 129X1/SvJ (RV4) embryonic stem (ES) cells was done by lipofection with Mouse Embryonic Stem Cell Core of the Siteman Cancer Center at Washington University (St. Louis, MO). To screen ES cell clones, genomic DNA was isolated and digested with BamHI. Southern blotting of the digests was done using a 694-bp probe to the 3′ terminal end of intron 1, upstream of the targeting construct (Fig. 1A). Selected ES cells were injected into C57BL/6 blastocysts by the Mouse Models Core at Washington University. Genotyping of mouse tail DNA was done by PCR and confirmed by Southern hybridization (Fig. 1B). For PCR, we used two forward primers, one specific to the WT locus (5′GTTGAAAGTGCCCATGCCCAGTG′) and the other to the target construct (5′GAGGAGTTGCTGCATGAGCATTGGTCGGAG3′), and a common reverse primer (5′CACATTCTCCCTGCTGTCATGCTC3′) to generate a 259 nt product from the WT es episine gene and a 324 nt product from the mutated gene (Fig. 1C). Male chimeric mice were bred to female C57BL/6 mice to generate germline heterozygotes, which were then bred to yield homozygous nulls. Mmp28−/− mice were then backedcrossed for 10 generations with C57BL/6 mice from Taconic. Wild-type and Mmp28−/− mice (C57BL/6Nac background) were housed in microisolator cages under specific pathogen-free conditions. Animal experiments were approved by the Institutional Animal Care and Use Committees at Washington University and at the University of Washington (Seattle, WA). Litter-matched mice of both genotypes were used for all studies.

Exposure models

P. aeruginosa strain PAK, a nonmucoid, flagellated strain originally obtained from Dr. Stephen Lory (Harvard University), was grown in Luria-Bertani broth at 37°C, collected, and centrifuged at 1500 × g. The supernatant was stored at −80°C for cytokine levels. The right lung was lavaged for determination of cell counts and differential and total protein and chemokine/cytokine levels. Postlavage, the right lung was inflated to 15 cm H2O with 4% paraformaldehyde and embedded in paraffin. In separate experiments, the left lung was collected and homogenized in TRIzol B (Invitrogen) for RNA extraction per the manufacturer’s protocol. To assess bacteremia, spleens were harvested and homogenized in PBS for quantitative bacterial culture.

In macrophage depletion experiments, P. aeruginosa strain PAK was administered via intranasal inoculation using 1 × 107 organisms/lung. Bacteria were grown overnight in Luria-Bertani broth at 37°C, collected, and quantified during the stationary phase. Bacteria were resuspended in PBS, and 50 μl was administered via intranasal inoculation in mice sedated with 425 mg/kg Avertin (2,2,2-tribromoethanol).

Macrophage depletion

The clodronate-encapsulated liposomes and PBS-encapsulated liposomes were prepared as described (22). Clodronate was a gift of Roche Diagnostics. Phosphatidylcholine was obtained from Lipoid, and cholesterol was purchased from Sigma-Aldrich. Clodronate-encapsulated liposomes were delivered both intranasally (50 μl) and i.p. (200 μl) to deplete alveolar macrophages and circulating monocytes, respectively. PBS-encapsulated liposomes were delivered in a similar fashion as a control. Forty-eight hours after treatment, two to four mice in each group were harvested to determine efficacy of macrophage and monocyte depletion by performing cell counts and differential of bronchoalveolar lavage fluid (BALF) anduffy coats. In a separate set of experiments, mice (macrophage-depleted and undepleted WT and Mmp28−/−, 10 mice/group) received intranasal instillation of 107 P. aeruginosa PAK strain. These mice were then harvested at 4 and 24 h for BALF cell counts, differential, histology, and bacterial counts.

Laser capture microdissection (LCM)

At 24 or 72 h after LPS instillation, mice were sacrificed and lungs were inflated with diluted Tissue-Tek OCT (Sakura Finetek), 50% (v/v) in RNase-free PBS containing 10% sucrose, and immediately frozen on dry ice. Frozen sections (7 μm) were processed and stained as described in detail (23). LCM was done to retrieve cells within 100 μm of the bronchoalveolar junction using the PixCell II system (Arcturus Engineering) with the following parameters: laser diameter, 30 μm; pulse duration, 5 ms; and amplitude, 50 mW, as described previously (23, 24).

Approximately 10,000 laser bursts were used to collect cells from each mouse. After the samples were captured on transfer films (CapSure HistoLaser, LCM caps, LCM2011; Arcturus Engineering), nonspecific attached components were removed using adhesive tape (CapSure cleanup pad, LCM2026; Arcturus Engineering).

Quantitative RT-PCR (qRT-PCR)

Total RNA from lung was isolated with TRIzol (Invitrogen) and from LCM-retrieved bronchial epithelial cells using an RNAsesy Mini kit (Qiagen). The quantity and quality of RNA were determined using an RNA LabChip kit (Agilent Technologies) or a NanoDrop spectrophotometer. Primers and TaqMan probes (FAM dye-labeled) for MMP-7, -10, -12, -14, and -28, Clara cell secretory protein (CCSP), β2-microglobulin, hypoxanthine phosphoribosyltransferase, or GAPDH were added to cDNA synthesized from 5 μg of total RNA with a High-Capacity cDNA Archive Kit (Applied Biosystems), and product amplification was measured with an ABI HT7900 Fast real-time PCR system. The threshold cycle (Ct) was obtained from duplicate samples and averaged. The ∆Ct was the difference between the average Ct for the specific cDNAs. The ∆∆Ct was the average ∆Ct at a given time point minus the average ∆Ct of day 0 (uninfected) samples. The data are expressed as relative quantification, which is the fold change and is calculated as 2−ΔΔCt.

Cytokine and chemokine analysis

We used the Luminox platform and a multiplex fluorescent bead array system (Bio-Rad) to quantify levels of nine factors (IL-1β, TNF-α, K, MCP-1, IL-10, IL-6, IL-4, IFN-γ, and TNF-α) in BALF and whole-lung homogenates, using reagents purchased from R&D Systems. Commercially available ELISAs for MIP-1α and MIP-3α were purchased from R&D Systems and performed in a 96-well format per the manufacturer’s
Macrophages cultures

BMDM were derived from both WT and Mmp28−/− mice. Bone marrow from the femur and tibia was harvested under sterile technique. The marrow was recovered from the femur and tibia by brief centrifugation, and the pellet was resuspended in 500 μl of HBSS. Lysis of RBCs was performed using commercially available lysis buffer (eBioscience) per the manufacturer’s protocol. After lysis, the cells were washed in PBS, resuspended in RPMI 1640, and counted with a Beckman Coulter flow cytometer and CXP software.

Leukocyte count and differential

For assessment of monocyte depletion 48 h after clodronate treatment, whole blood (0.7 ml/mouse) was obtained by cardiac puncture using a 25-gauge needle. The blood was diluted in an equal volume of PBS, layered on top of Histopaque 1077, and centrifuged per the manufacturer’s protocol (Sigma-Aldrich). The monolayer of cells at the interface was removed, washed in PBS, and counted with a hemocytometer. Differentials were done on 30,000 cells cytospun onto slides and stained with LeukoStat (Fisher Scientific) (300 cells counted in random fields). Other cellular aliquots were incubated with anti-mouse CD16/CD32 mAb (BD Biosciences) at a concentration of 1 μg/10^6 cells for 10 min, and then anti-mouse CD11b-FITC mAb (BD Biosciences) at a concentration of 1 μg/10^6 cells for 60 min. After washing the cells in PBS plus 0.5% BSA, they were analyzed using a Beckman Coulter flow cytometer and CXP software.

Chemotaxis studies

Macrophage migration toward BALF was assessed with a fluorescence-based assay using 96-well chemotaxis chambers containing polycarbonate filters with 5-μm pores (ChemoTx; Neuro Probe). BMDM were incubated in RPMI 1640 containing 5 μg/ml calcein AM (Molecular Probes) and then washed and suspended at 4 × 10^5 cells/ml in phenol red-free RPMI 1640 (Sigma-Aldrich). Duplicate chamber wells were filled with BALF obtained from 8–10 WT or 8–10 Mmp28−/− mice exposed to aerosolized P. aeruginosa. Wells containing PBS were used to determine chemokinesis. The
chemotaxis membrane was applied and labeled macrophages (1 × 10^5 cells/25 μl) were placed directly onto the membrane. The chambers were incubated for 50 min at 37°C, nonmigrating cells on the top side of the membrane were removed, and fluorescence was determined in a fluorescence microtiter plate reader (CytoFluor II; PerSeptive Biosystems) in the bottom-read position. A standard curve from serial dilutions of macrophages was generated to determine the number of cells migrating to the bottom well. Chemokinesis, or random migration, with PBS in the bottom well was calculated and subtracted from all values when calculating percentage migration. In a separate experiment, WT and Mmp28^-/- BMDM were stimulated with 100 ng of Escherichia coli LPS strain O111:B4 (Sigma-Aldrich) or medium alone 24 h before harvest. Triplicate chamber wells were filled with BALF obtained from a WT mouse instilled with P. aeruginosa 24 h before harvest. LPS-stimulated and medium-alone WT and Mmp28^-/- BMDM were placed on top of the membrane as described above and assessed for chemotaxis after 50 min of incubation at 37°C.

**BMDM activation**

BMDM were isolated and cultured as described above. Macrophages were transferred to 12-well plates at a density of 1 × 10^5 cells/well. The cells were stimulated with 100 ng of E. coli LPS strain O111:B4 or infected with 5 × 10^6 CFU of P. aeruginosa PAK strain in DMEM culture medium for 2 h at 37°C, 5% CO2. After 2 h, cells were washed twice with PBS containing 0.1% gentamicin (Invitrogen) and incubated with DMEM, 0.1% gentamicin. Cells were collected at 24, 48, or 72 h for qRT-PCR. Unstimulated macrophages served as a control.

**Data analysis**

Results are expressed as means ± SEM. Statistical significance was determined using Student’s t test. Differences were considered significant if the p value was <0.05.

**Results**

**Epilysin-null mice**

The targeting construct was designed to delete exons 4–8, which include the catalytic domain (Fig. 1A). The construct contained expression cassettes for neomycin resistance (positive selection) and diphtheria toxin sensitivity (negative selection). After homologous recombination, exons 4–7 and the full coding portion of the exon 8 were excised, and any transcript produced would contain only exons 1–3 (coding for the proregion only). We screened 144 neomycin-resistant ES cell clones, and of these, 4 indicated homologous recombination (Fig. 1B). Chimeric mice were bred to C57BL/6 mice to generate germline heterozygotes. These mice were then backcrossed to yield homozygous null (Mmp28^-/-) mice (Fig. 1C). These mice have been backcrossed to C57BL/6N/Tac mice for 10 generations. Homozygous Mmp28^-/- mice are healthy with no defects in fertility, litter size, weight gain (at least up to 6 mo), behavior, or tissue morphology. Additionally, their airways appear normal with the expected proportion of ciliated and Clara cells. As verified by RT-PCR, no epilysin mRNA is detected in these mice (data not shown).

**Epilysin is expressed by Clara cells and macrophages**

In mice, epilysin is expressed in a number of adult tissues, with the highest levels seen in lung (18). By immunofluorescence, epilysin localized to the airway epithelium (Fig. 2A) and colocalized with CCSP (Fig. 2B), indicating that this MMP is produced by Clara cells. To confirm these findings, lung tissue from Mmp28^-/- mice did not demonstrate staining in the airway epithelia (Fig. 2A). Furthermore, we collected an enriched population of Clara cells using LCM of cells at the bronchoalveolar junction (which is comprised of ~90% Clara cells). qRT-PCR for CCSP verified an enrichment of Clara cells (data not shown), and qRT-PCR for Mmp28 confirmed that Clara cells express epilysin mRNA (Fig. 2C). In contrast, matrilysin (MMP-7) is seen only in ciliated and basal cells and not in Clara cells (25–27), indicating that these two epithelial MMPs serve nonoverlapping functions in lung biology.

Analysis of mRNA by qRT-PCR collected from freshly isolated alveolar macrophages revealed no expression of epilysin (Ct value of 35.1 ± 0.23). Additionally, no immunofluorescence signal was seen in alveolar macrophages stained with an epilysin Ab (data not shown). However, resting BMDM did express epilysin, albeit at relatively low levels (Ct value of 31.59 ± 1.18). These findings suggest that epilysin is expressed by a subset of macrophages, and that localization or differentiation within the tissue may change epilysin expression patterns.

**Disparate control of epilysin expression by epithelial cells and macrophages in response to infection**

We examined the temporal expression of epilysin and other MMPs in response to P. aeruginosa infection by qRT-PCR. Lungs from four to five mice per genotype were collected and processed for RNA at 0, 4, 24, 48, and 96 h after pulmonary infection with
P. aeruginosa. Unlike matrilysin (MMP-7), stromelysin-2 (MMP-10), macrophage metalloelastase (MMP-12), and membrane type-1 MMP (MMP-14), which are all up-regulated or induced in response to airway infection (27, 28), epilysin was rapidly down-regulated >4-fold within 4 h postinfection (Fig. 3). Although its mRNA levels dropped markedly, the Ct values at 24 h (25.69 ± 0.48) indicated that expression of epilysin was still maintained at relatively high levels. By 96 h postinfection, by which time the bacteria were cleared (see Fig. 7) and the neutrophilic inflammation had resolved (data not shown), the expression of epilysin rebounded, approaching preinfection levels (Fig. 3A). Similarly, in response to P. aeruginosa, we saw reduced signal for epilysin protein by immunofluorescence. Both the number of positive airway cells and intensity of staining decreased during infection (Fig. 2D). We did not observe differences in mRNA expression of MMP-7, -10, or -14 mRNAs in either naive or infected lungs of Mmp28−/− and WT mice (data not shown).

In contrast, the expression pattern of epilysin by BMDM differed from that of epithelial cells. Both bacteria (P. aeruginosa) or LPS (from E. coli) up-regulated epilysin expression by BMDM (Fig. 4A). We did not detect epilysin expression in resident macrophages in the lung by immunohistochemistry (data not shown), but by 48 h after infection with P. aeruginosa, 33.3 ± 5.2% of pulmonary macrophage express epilysin based on coimmunofluorescence with MAC-2 and epilysin Abs (Fig. 4B). Collectively, these data indicate disparate regulation of epilysin by epithelium and leukocytes and suggest different roles of epilysin in epithelial and leukocyte biology. Furthermore, the decrease in epilysin mRNA expression in the injured lung likely reflects the contribution of epithelial-derived epilysin. Although epilysin is expressed in cultured BMDM, the fact that it is not detected in resident macrophages in naive lung suggests that multiple factors may interact to regulate its expression, and bacterial stimulation is one factor.

**Altered macrophage recruitment in Mmp28−/− mice**

Because many MMPs function in immunity (6), we assessed whether epilysin has immunoregulatory functions. To test this idea, we compared the inflammatory response between WT and Mmp28−/− mice challenged with P. aeruginosa. Mice were exposed to aerosolized P. aeruginosa to achieve a deposition of ~5 × 105 bacteria/lung. The lungs were harvested at 4 and 24 h postinfection for BALF cell counts, differential, histology, bacterial counts, and cytokine analysis. At 4 h postinfection, we detected no significant difference in the total number of cells in BALF from Mmp28−/− and WT mice (Fig. 5A). While 79–94% of the total cells where neutrophils at this time, their numbers did not differ significantly between genotypes (Fig. 5B). However, at 4 h postinfection, Mmp28−/− mice had a markedly enhanced (6-fold) increase in BALF macrophages compared with infected WT mice (Fig. 5, C and D). Importantly, uninfected WT and Mmp28−/− mice did not differ in their macrophage numbers in BALF or whole-lung extracts, and they also had similar numbers of circulating monocytes (data not shown). These data indicate that the increased number of macrophages recovered from the lungs of infected Mmp28−/− mice reflected an accelerated influx and not an increased population of resident macrophages.

By 24 h postaerosolization, the increase in total BALF cells observed in WT mice was significantly blunted in Mmp28−/− mice (7.14 ± 0.82 × 106 vs 4.97 ± 0.54 × 106 cells in WT vs Mmp28−/− mice, respectively), which was due to a lesser increase in neutrophils (Fig. 5, A and B). At this time, we no longer saw a difference in the recovery of BALF macrophage between genotypes.

Evaluation of the lung histology by immunohistochemistry for a macrophage-specific marker, MAC-2 (29), demonstrated increased influx of macrophages in Mmp28−/− compared with WT mice (16.4 ± 4.2 vs 7.2 ± 2.4 cells/160 mm², p < 0.05) (Fig. 6). In our evaluation of lung sections, labeled with a neutrophil-specific marker, we saw no evidence that neutrophils in Mmp28−/− mice...
were labeled with the Vectastain ABC kit, and colorimetric detection was
secondary Ab was HRP-conjugated donkey anti-rat Ab. Secondary Abs
stained with anti-mouse MAC-2 Ab (left) or anti-neutrophil Ab (right).

Lung histology demonstrated no evidence of lung injury (data not shown). Immunostaining with MAC-2 Ab confirmed a reduction of pulmonary macrophages (Fig. 8A).

Enhanced P. aeruginosa clearance in Mmp28−/− mice

Infected Mmp28−/− mice had enhanced P. aeruginosa clearance from their lungs at 4 h (Fig. 7A). However, by 24 h postinfection, both WT and Mmp28−/− mice had efficiently cleared the bacteria. There was no significant bacteremia between genotypes as measured by the recovery of live bacteria from homogenates of spleen (Fig. 7B).

Macrophages are required for P. aeruginosa clearance

Our data suggest that macrophages contribute to clearance of P. aeruginosa from the lung and, possibly as a result of this, neutrophil recruitment. To assess if accelerated macrophage recruitment in Mmp28−/− mice led to faster bacterial clearance and reduced neutrophil influx to the lungs of infected Mmp28−/− mice, we depleted alveolar and circulating monocytes from WT and Mmp28−/− mice by intranasal and i.p. administration of clodronate coupled with liposomes. Control mice received the liposome carrier by both routes. Clodronate is a common agent used to deplete selected populations of macrophages and monocytes in vivo and, when coupled with liposomes, it allows for the intracellular introduction and accumulation of clodronate in macrophages, resulting in apoptosis (30–32).

Forty-eight hours after clodronate treatment, mice were harvested to determine the efficacy of macrophage depletion in both the lung and circulation. We observed a 10-fold reduction in alveolar macrophage numbers (Fig. 8A) and a partial reduction in circulating monocytes, particularly a reduction in CD11bhigh monocytes (Fig. 8B and C). Neutrophil influx into the lungs did not differ significantly between both clodronate and liposome-only groups (Fig. 8A), and the lung histology demonstrated no evidence of lung injury (data not shown). Immunostaining with MAC-2 Ab confirmed a reduction of pulmonary macrophages (Fig. 8A).
We instilled *P. aeruginosa* into the lungs of macrophage-depleted (clodronate) WT and *Mmp28*−/− mice and liposome-only (control) WT and *Mmp28*−/− mice. Compared with control WT mice, macrophage-depleted WT mice had increased neutrophil influx into BALF at 4 and 24 h (Fig. 9A), yet impaired bacterial clearance at 4 h (Fig. 9C). Increased neutrophils and impaired bacterial clearance were also observed in *Mmp28*−/− mice treated with clodronate (Fig. 9, A and C). Furthermore, bacterial clearance at 4 h and neutrophil recruitment at 24 h did not differ between WT and *Mmp28*−/− macrophage-depleted mice (Fig. 9, A and C). These results indicate that macrophages aid in the clearance of *P. aeruginosa* from the lung and modify the inflammatory response to these bacteria, and they provide further evidence that accelerated macrophage recruitment in *Mmp28*−/− mice contributes to faster bacterial clearance and reduced neutrophil recruitment.

Altered chemokines and cytokines in Mmp28−/− mice

Given the above differences in macrophage and neutrophil recruitment, we assessed if levels of neutrophil and macrophage chemokines and other cytokines in BALF and whole-lung homogenates differed between *P. aeruginosa*-infected WT and *Mmp28*−/− mice. For this, we used a multiplex cytokine/chemokine assay on a Luminex platform. Nine cytokines (IL-1β, TNF-α, KC, MIP-2, MCP-2, IL-10, IL-12, GM-CSF, and IFN-γ) were assessed from BALF and lung homogenates from WT and *Mmp28*−/− mice at 4 and 24 h postinfection. The levels of two additional macrophage chemokines, MIP-1α and MIP-3α, in BALF and lung homogenates were assessed by ELISA.

Consistent with reduced neutrophilia in *Mmp28*−/− mice (Fig. 5B), we found slightly reduced levels of the CXC chemokines KC and MIP-2 in both BALF and lung homogenates from *Mmp28*−/− mice at 4 h postinfection (Fig. 10). Because KC and MIP-2 levels...
were reduced in both the alveolar (BALF) and tissue compartments, it is likely that the lack of epilysin affected chemokine expression and not the formation of chemokine gradients. Importantly, we found that the levels of MCP-1, a potent macrophage chemokine, in the BALF or tissue homogenates did not differ between infected WT and Mmp28<sup>−/−</sup> mice. There was no difference in MIP-1α levels, and there was a slight decrease in MIP-3α at 4 h in the Mmp28<sup>−/−</sup> BALF compared with WT. Thus, the rapid and early influx of macrophages seen in the Mmp28<sup>−/−</sup> mice was apparently not mediated by an increase in chemokine expression or altered chemokine gradient.

We also found that TNF-α, IL-6, and GM-CSF were increased in Mmp28<sup>−/−</sup> BALF at 4 h, but not 24 h. Because these cytokines are produced by macrophages, the increased levels at 4 h may reflect the increased number of pulmonary macrophages in the Mmp28<sup>−/−</sup> mice. We did not detect any significant differences in IL-1β, IL-10, IL-12, or IFN-γ (not shown).

**Enhanced chemotaxis of Mmp28<sup>−/−</sup> BMDM**

The chemokine data suggested that the more rapid influx of macrophages into Mmp28<sup>−/−</sup> was not due to altered levels of chemotactic activity. We then assessed if the accelerated macrophage recruitment seen in vivo was due to enhanced migratory ability of Mmp28<sup>−/−</sup> macrophage. For this, we compared the chemotaxis of BMDM from WT and Mmp28<sup>−/−</sup> mice to move toward unconcentrated, cell-free BALF obtained from 8–10 P. aeruginosa-infected WT or 8–10 Mmp28<sup>−/−</sup> mice (PA BALF).

Although the chemotactractive activity in PA BALF was modest, we observed a small decrease in the chemotaxic activity in PA BALF from Mmp28<sup>−/−</sup> compared with WT mice (Fig. 11A). This diminished activity in the Mmp28<sup>−/−</sup> PA BALF may be due to a greater clearance of chemokines and other bacterial factors by the augmented macrophage numbers. In agreement with the chemokine data, these findings indicate that epilysin does not restrain macrophage influx by affecting the activity of chemotactic factors.

Interestingly, we observed an enhanced ability of the Mmp28<sup>−/−</sup> BMDM to migrate to PA BALF of either genotype (Fig. 11), and no difference in chemokinosis or random migration to PBS (not shown). Although P. aeruginosa or E. coli LPS stimulated epilysin expression by BMDM (Fig. 5), up-regulation of this proteinase did not further affect BMDM migration (Fig. 11B). These data suggest that the basal level of epilysin expression is sufficient to govern the migratory ability of BMDM. Furthermore, because BMDM express epilysin and because they would not be exposed to epithelial-derived epilysin in this culture model, these data also indicate that epilysin retards macrophage influx by a cell-autonomous mechanism.

**Discussion**

The broad expression of epilysin in naive tissue and the rapid down-regulation of total levels in response to infection, unlike that of many other MMPs, suggest that this proteinase serves a unique role among its family members. Although the constitutive expression of epilysin in many tissues suggests that this proteinase functions in homeostasis, Mmp28<sup>−/−</sup> mice, similar to other MMP-deficient mice, have no overt phenotype in the unchallenged state. We have found, however, increased deposition of elastin in the lungs of Mmp28<sup>−/−</sup> mice compared with WT animals (our unpublished observations), suggesting that the basal expression of epilysin may function in the epithelial control of interstitial matrix production. Regardless of this phenotype, the null mice are overtly normal.

Although the lack of phenotype in unchallenged Mmp28<sup>−/−</sup> mice might imply compensation by another MMP, we have found neither altered nor aberrant expression of other epithelial MMPs. An alternative possibility is that epilysin functions in innate immunity and, hence, a phenotype would not be revealed until this system is challenged. Indeed, when we infected Mmp28<sup>−/−</sup> mice with P. aeruginosa, we observed accelerated macrophage recruitment into their lungs by 4 h, compared with the infiltration seen in...
that the endogenous expression of epilysin by macrophages is suf-
icient to retard their early recruitment into the lung. Indeed, in
both in vivo and cell-based models, epilysin-dependent effects on
macrophage influx and chemotaxis were evident at early times: 4 h
in vivo and <1 h in isolated cells.

Of the many MMPs that function in immunity, most do so by
cleaving specific proteins, often resulting in a gain-of-function ac-
tivity for the target substrate (5). Authentic and potential MMP
substrates that regulate inflammatory responses include antimicro-
bial peptides, cytokines, chemokines, chemokine receptors, and
accessory proteins that bind, retain, or concentrate chemokines (6).
For example, IL-1β can be activated by several MMPs (MMP-2,
-3, and -9) (33) and MMP-7 and MMP-12 have been reported to
activate latent TNF-α in isolated macrophages (12, 34, 35). In
addition to cytokines, several MMPs process chemokines making
them more or less potent (14, 36–38) or by generating chemoa-
tractive peptides from precursor proteins (39). For example, the
N-terminal domain of CXCL8 (IL-8) and LIX (the mouse equivalent
of CXCL5 and CXCL6) are processed by MMP-9 and MMP-8, re-
spectively, resulting in products that have more potent chemoat-
tractant activities than do the full-length molecules (14, 36). MMPs
can also affect chemokine gradients by shedding accessory proteins
that bind or restrain chemokines. Work in our laboratory demonstrated
that matripsin promotes neutrophil influx into the alveolar space via
shedding of transmembrane proteoglycan syndecan-1 complexed
with KC, a murine CXC chemokine (9).

MCP-1 is the predominant macrophage chemokine regulating
monocyte recruitment to the lung via its interaction with its recep-
tor, CCR2 (40). Based on our observations of accelerated macro-
phage recruitment in infected Mmp28−/− mice, we examined the
lung and bronchoalveolar compartments for altered MCP-1 levels
or gradients. At both 4 and 24 h, we found similar levels of MCP-1

WT mice. At 24 h postinfection, we saw no difference in macro-
phage recruitment between genotypes, likely reflecting redundant
mechanisms involved in their later recruitment. Furthermore, bac-
terial clearance was accelerated and neutrophil recruitment was
blunted in Mmp28−/− mice, which we predict are consequences of
accelerated macrophage recruitment. Supporting this idea, we
found that macrophage depletion in WT and Mmp28−/− mice re-
sulted in impaired 4 h clearance of P. aeruginosa with increased 4
and 24 h neutrophil recruitment to the lung. Overall, our results
suggest that epilysin serves as a negative regulator of macrophage
recruitment by inhibiting the chemotaxis of macrophages.

In naïve lung, epilysin is expressed by airway Clara cells and is
not expressed by resident pulmonary macrophages. Epilysin is also
constitutively expressed by BMDM. In response to infection, ex-
pression of epilysin by epithelial cells is rapidly down-regulated,
but it is up-regulated in pulmonary macrophages and BMDM.
These findings indicate disparate regulation of epilysin by epithe-
lium and leukocytes and suggest different roles of epilysin in ep-
ithelial and leukocyte biology. Furthermore, our findings indicate
that the endogenous expression of epilysin by macrophages is suf-
ficient to retard their early recruitment into the lung. Indeed, in
both in vivo and cell-based models, epilysin-dependent effects on
macrophage influx and chemotaxis were evident at early times: 4 h
in vivo and <1 h in isolated cells.

Of the many MMPs that function in immunity, most do so by
cleaving specific proteins, often resulting in a gain-of-function ac-
tivity for the target substrate (5). Authentic and potential MMP
substrates that regulate inflammatory responses include antimicro-
bial peptides, cytokines, chemokines, chemokine receptors, and
accessory proteins that bind, retain, or concentrate chemokines (6).
For example, IL-1β can be activated by several MMPs (MMP-2,
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or gradients. At both 4 and 24 h, we found similar levels of MCP-1
in the BALF and whole-lung homogenate between the Mmp28−/− and WT mice. A similar finding was observed for other macrophage chemokines, MIP1-α and MIP3-α. Thus, the rapid and early influx of macrophages seen in the Mmp28−/− mice was apparently not mediated by chemokine production or gradient formation.

Since some MMPs can cleave chemokines, making them more or less potent, we also examined the chemotactic activity of BALF recovered from P. aeruginosa-treated WT and Mmp28−/− mice (6). Using macrophages cultured from bone marrow from WT and Mmp28−/− mice in a chemotaxis assay, we did not observe an increase in the chemotactic activity from BALF recovered from the Mmp28−/− mice. In fact, we observed a slight decrease in the chemotactic activity in the BALF from Mmp28−/− compared with WT mice. The diminished activity in the Mmp28−/− BALF may be due to greater clearance of chemokines by the augmented macrophage numbers. Based on these findings, it is unlikely that epilysin affected macrophage recruitment via enhanced chemokine gradients. Using this same chemotaxis assay, we observed an enhanced ability of the Mmp28−/− macrophages to migrate to BALF of either genotype. These data indicate that epilysin expression by macrophages retards the ability of macrophages to migrate to chemotactants. Interestingly, we found that P. aeruginosa or E. coli LPS stimulated epilysin expression by BMDM. However, this up-regulation did not further affect BMDM migration, suggesting that the basal levels of this MMP are sufficient to restrain BMDM response to chemotactants. Thus, epilysin may have additional unidentified roles in macrophage biology. Epilysin alters early migration, but later recruitment is likely mediated by redundant or more potent mechanisms.

The chemokine receptor for MCP-1, CCR2, is a critical determinant of monocyte recruitment, and studies of CCR2-deficient mice demonstrate significantly reduced monocyte/macrophage influx in models of peritonitis or lung injury (40, 41). Although we found no evidence of an altered MCP-1 gradient, proteolysis of CCR2 on infiltrating macrophages could slow their advancement at the early stages of inflammation. We examined the expression of CCR2 on macrophages isolated from the peritoneum of naive WT and Mmp28−/− mice and observed no difference in baseline CCR2 levels (not shown). Additionally, we evaluated the expression of CCR2 on our unstimulated BMDM and observed all of our macrophages express this chemokine receptor with no difference in expression between our WT and Mmp28−/− mice (not shown). These data suggest that epilysin does not alter CCR2 expression or CCR2 shedding. Macrophages can express additional chemokine receptors, such as CCR1, CCR5, CCR6, and CXCR3; however, these chemokine receptors appear less important in chemotaxis, as only a small subset of macrophages expresses these receptors (42, 43). Careful analysis of macrophage chemotaxis to various recombinant chemokines may prove useful in further identifying a chemokine receptor that may be an MPP-28 substrate.

Alternatively, epilysin may regulate macrophage influx by shedding an adhesion protein. To migrate across vascular endothelium in vitro, human monocytes utilize sequential interactions of members of the selectin family (L-selectin), α1 integrins (VLA-4 and VLA-5), α2 integrins (CD11c/CD18), and PECAM-1 with endothelial selectins ICAM-1, VCAM-1, and PECAM-1 (44–47). Adhesive interactions on the epithelial side include VLA-4, VLA-5, VLA-6, CD11a, CD11b, CD11c/CD1.8, and CD47 on human monocytes (44). In mice, however, monocyte recruitment into lung is dependent on monocyte expression of CD11a, CD11b, ICAM-1, and VLA-4 (48). Recently, the neural cell adhesion molecule NCAM has been shown to be an intravascular substrate for Xenopus MPP-28 (49), and other Ig domain cell adhesion proteins may also be potential substrates. Other mechanisms, such as delayed apoptosis, could also contribute to increased macrophage numbers in the Mmp28−/− mice; however, they are not involved given these early time points.

In addition to our findings related to epilysin-mediated macrophage recruitment, our study demonstrates a key protective role for macrophages in P. aeruginosa pneumonia. To date, only two studies have examined the role of the alveolar macrophage in P. aeruginosa pneumonia, each with opposing findings. Cheung et al. found no difference in P. aeruginosa clearance from the lung in macrophage-depleted vs control mice (50). However, Kooguchi et al., who achieved greater macrophage depletion than Cheung et al., found that macrophage-depleted mice had delayed clearance of P. aeruginosa, delayed but sustained neutrophil recruitment to the alveolar spaces, and increased lung injury and mortality compared with control mice (30). Our findings are consistent with those of Kooguchi et al. and indicate that macrophages contribute modestly to P. aeruginosa clearance, and reduction of the macrophage population leads to significantly more pulmonary neutrophilia. Although the change in neutrophil influx may be due to other effects mediated by macrophages, delayed bacterial clearance is a likely contributor. In a different model of P. aeruginosa infection in the cornea of macrophage-depleted mice, a similar finding of impaired P. aeruginosa clearance and increased neutrophil recruitment was observed (51). This relationship of the macrophage to neutrophil recruitment is also consistent with our findings of reduced 24 h neutrophil recruitment to the lung in the Mmp28−/− mice. Since loss of neutrophil recruitment in Mmp28−/− mice may be due to altered chemokine gradients, we examined the levels of KC and MIP-2 chemokines in both whole lung and BALF. These neutrophil chemokines were reduced in both compartments (whole lung and BALF), suggesting that epilysin affected KC and MIP-2 expression but not gradient formation. We also examined the lungs of Mmp28−/− mice using immunostaining for neutrophils, and there was no evidence that neutrophils were sequestered elsewhere in the lung. Thus, the reduced neutrophil numbers seen in the BALF of Mmp28−/− mice at 24 h postinfection cannot be attributed to impaired migration through lung tissue. Our findings indicate that the lower BALF neutrophil levels in Mmp28−/− mice are due to blunted chemokine signals, likely a consequence of reduced bacterial replication in Mmp28−/− mice.

Overall, our results indicate that epilysin serves as a negative regulator of early macrophage recruitment, and this novel function may have evolved as a mechanism to restrain unnecessary and untimely inflammation. Future studies will be aimed at substrate identification to determine how epilysin restrains macrophage influx.

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**Disclosures**

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