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Overlapping and independent contributions of MMP2 and MMP9 to lung allergic inflammatory cell egression through decreased CC chemokines

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Abstract

The mechanisms that initiate allergic lung inflammation are relevant to expression of diseases such as asthma, but the factors underlying resolution of inflammation are equally important. Previously, we demonstrated the importance of matrix metalloproteinase 2 (MMP2) for airway egression of lung eosinophils, a critical anti-inflammatory mechanism without which mice are rendered highly susceptible to lethal asphyxiation. Here we show that leukocyte MMP9 is the dominant airway MMP controlling inflammatory cell egression. The allergic lung phenotype of MMP9^{-/-} mice was similar to WT and was not altered by concomitant deletion of the MMP2 gene (double knockout; dko). However, inflammatory cells accumulated aberrantly in the lungs of allergen-challenged MMP9^{-/-} and dko mice and fewer eosinophils and neutrophils were present in bronchoalveolar lavage. These aberrant cellular trafficking patterns were explained by disruption of transepithelial chemokine gradients, in MMP2^{-/-} mice affecting only eotaxin (CCL11), but in MMP9^{-/-} and dko mice involving eotaxin, MARC (CCL7), and TARC (CCL17). Thus, by establishing multiple transepithelial chemokine gradients, MMP9 is broadly implicated in the resolution of allergic inflammation, an essential protective mechanism that overlaps with a more limited role played by MMP2.

Keywords

chemotaxis; cytokines; gelatinase B; matrix metalloproteinase-9

Allergic inflammatory cells migrate from the bone marrow and blood to the lung in response to T helper cell type 2 (Th2)-mediated immune response. Factors that determine inflammatory cell migration are complex, but critical molecules include cell adhesion molecules, cytokines, chemokines, and their receptors (1–3). Experimental models have shown that induction of allergy causes massive recruitment of lymphocytes, eosinophils, mast cells, or neutrophils to the lung and that this response may represent an aberrant reply to the antigenic stimulation by the host (4–6).

Mice sensitized with allergen, much like patients with an acute episode of asthma, develop an intense inflammatory lung disease that is characterized by lung eosinophilia, increased airway glycoprotein production, and increased responsiveness to acetylcholine bronchoprovocation—termed airway hyperresponsiveness—all of which contribute to airway obstruction and predispose to fatal asphyxiation (7–9). Allergens containing fungal proteases implicated in human airway disease are unusually potent in their ability to elicit allergic airway inflammation in mice and therefore are ideal for use in experimental models of asthma (10). In response to these and other allergens, we and others have shown that Th2 cell-dependent interleukin (IL)-13 and IL-4 orchestrate much of the pathophysiologic sequelae of allergic lung inflammation, especially airway obstruction (9,11).

Endogenous proteases are strongly expressed at sites of inflammation, and matrix metalloproteinases (MMPs), a major family of zinc-dependent enzymes, in particular are secreted by inflammatory and non-inflammatory cells in the lung (12–15). The MMP family members, initially identified as zinc-dependent proteinases, can digest various components of the extracellular matrix. However, several new roles have been described for them, which include cleavage of transmembrane proteins; tissue factors embedded within the matrix, for example, latent growth factors and their binding proteins; chemotactic molecules; and cytokines (16,17). Some MMPs also facilitate inflammatory cell recruitment across the epithelium and endothelial basement membrane (18–20).

MMP2 and MMP9, two gelatinases that are up-regulated in asthma and by the cytokines IL-4 and IL-13, have similar substrate profiles, domain organizations, and structure but differ in their tissue expression (21). MMP2-deficient mice display a defect in clearance of allergic inflammatory cells and are prone to asphyxiation (22). Although recruitment of inflammatory cells was intact in MMP2 deficient mice, an aberrant chemotactic gradient prevented egression of eosinophils into the alveolar lumen. Furthermore, metalloproteinases are necessary for the migration of dendritic cells because emigration of dermal dendritic cells from skin explants of MMP9-deficient mice or in mice treated with anti-MMP2 or -MMP9, are markedly impaired, whereas the generation of contact hypersensitivity responses remained intact (23). Immune cells recruited to sites of allergic inflammation express MMP9, but the physiological role of this enzyme in allergic lung disease remains unclear. We hypothesized that, much like MMP2, the closely related protease MMP9 may play an important role in facilitating the clearance of pre-apoptotic inflammatory cells in the allergic lung. We show here that MMP9 and MMP2 affect clearance of lung inflammatory cells but differ in the profile of chemokine gradients they control. Thus, in addition to the many adhesion molecules and chemokines required, multiple MMPs are essential for coordinating lung cellular trafficking and the resolution of allergic inflammation.

MATERIALS AND METHODS

Mice

MMP9 and MMP2 null mice (24,25) (C57BL/6 background) were bred in the AALAC-accredited transgenic animal facility at Baylor College of Medicine. MMP9/MMP2 double null (dko) mice were generated from F2 and F3 crosses of single null mice. Gelatin gel zymography using 1 μ l of tail blood was used to determine the presence or absence of MMP9 or MMP2 and MMP9 (dko) proteins in experimental mice. Wild-type (WT) C57BL/6 mice were purchased from the Baylor vivarium. All mouse studies were conducted in accordance with institutional animal care and use guidelines.

In situ hybridization (ISH)

The labeling of the MMP9 antisense RNA probe for in situ hybridization on prepared paraffin sections of lung tissue was done as described previously (15). Briefly, the plasmid (pSP65) was linearized, and [³⁵S]UTP (1000 Ci/nmol, Amersham)-labeled probes were transcribed from the SP6 promoter using a transcription kit (Promega, Madison, WI). The probes were fractionated with Sephadex G-50 (Pharmacia, Uppsala, Sweden), precipitated with ethanol, mixed with hybridization mixture, and placed on the pretreated sections. The sections were incubated overnight at 55°C, washed in high stringency conditions, and dipped in autographic emulsion (Kodak NTB2). After exposure for 4–8 days, the emulsion was developed and the sections were counterstained with hematoxylin and mounted.

Allergen and rIL-13 challenge

Aspergillus fumigatus allergen was prepared from a clinical isolate of *A. fumigatus* and mixed with 25 µg of chicken egg ovalbumin (OVA; Sigma, St. Louis, MO) in phosphate buffered saline (PBS) as described and will be referred to as complete *Aspergillus* allergen (CAA; 22). A total of 5 CAA challenges were administered to MMP9^{-/-}, dko, or WT C57BL/6 mice every 4 days as described (26). Because data collected from saline-challenged MMP9^{-/-}, dko, heterozygote (MMP9^{+/-}/MMP2^{+/-}), and wild-type mice were identical (data not shown), we show for the sake of clarity only one saline control group per experiment. Further, allergen-challenged MMP9^{+/-} and WT C57BL/6 mice showed identical responses (data not shown), thus data from WT is compared with the MMP9^{-/-} and dko mice. WT C57BL/6 mice were administered 3.5 µg of rIL-13 or saline intranasally twice daily for 3 consecutive days.

Analysis of the asthma phenotype

All data were collected 24 h following the final allergen challenge. Airway hyperresponsiveness (AHR) is a measure of the exaggerated tendency of airways to constrict in response to nonspecific stimuli as seen in asthma. AHR is assessed as a significant decrease in the concentration of acetylcholine chloride required to induce a 200% increase in respiratory system resistance (PC₂₀₀) and was estimated based on provocative concentration of acetylcholine causing increase in lung resistance (PC₂₀₀) (26). Briefly, mice were anesthetized and paralyzed with etomidate and pancuronium bromide, respectively. Intubated mice were maintained inside a whole body plethysmograph connected to a rodent ventilator and provided with 100% oxygen. After establishing a stable baseline for respiratory system resistance (R_{rs}), as determined by continuously quantitating DPt/Vt (where DPt = change in tracheal pressure and Vt = gas flow) at 70% tidal volume, we administered increasing doses of acetylcholine chloride intravenously over 1 s in escalating doses. The provocative concentration of acetylcholine in milligrams per gram that caused a 200% increase in R_{rs}, designated PC₂₀₀, was calculated by linear interpolation of appropriate dose-response curves.

Bronchoalveolar lavage cytology, total IgE, lung histopathology, and BAL mucoglycoprotein and chemokines profiles by ELISA were determined as described with slight modifications (10,22). Bronchoalveolar lavage (BAL) cells were collected by serially instilling and withdrawing 1 ml aliquots of PBS from the tracheal cannula. Cells were washed and enumerated, and aliquots of 10⁵ cells were centrifuged onto glass slides, stained by using modified Giemsa, and were used to determine the absolute numbers of BAL cells.

Serum was prepared from whole blood collected at the time of death for determination of total IgE levels by using a monoclonal antibody-based ELISA. Wells of microtiter plates (Dynex, Inc., Chantilly, VA) were precoated with anti-IgE and blocked with 5% bovine serum albumin in PBS with 0.05% Tween 20 (Bio Rad Laboratories, Hercules, CA). Serum diluted 1:5 and 1:50 was then added and incubated for 1 h at 37°C. Plates were washed, and biotinylated IgE-specific antibody was added for an additional hour of incubation. Plates were washed again,

and streptavidin-conjugated alkaline phosphatase was added for 30 min at room temperature. After a final wash, plates were developed with 0.5M nitrophenyl phosphate in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma). Data are expressed as optical density readings at 405 nM.

For histologic analysis, whole lungs were infused via the trachea with 4% formalin until distended and fixed for 24 h. The tissues were embedded in paraffin, and 4–5 mm sections were cut and stained with hematoxylin and eosin or periodic acid-Schiff for viewing by light microscopy.

Peribronchovascular cell infiltration assay

Total inflammatory cells within a $200 \times 200 \mu\text{m}^2$ area of bronchovascular bundles (a standard anatomic landmark of the lung) were counted by light microscopy of hematoxylin and eosin (H&E)-stained sections. To ensure comparable analyses between different groups, we analyzed randomly selected medium-sized airways (≈ 100 micrometer airway diameter). A total of at least 10 different lung fields from three mice in each experimental group were visualized under high power.

Lung inflammatory cell extraction and chemotaxis assay

Inflammatory cells were extracted from lungs of sensitized WT mice or mice deficient in MMP9 and were used in chemotaxis assays as we have described previously (22). Briefly, BAL inflammatory cells were discarded, the lung was then minced and pressed through a fine nylon mesh, and the crude cell suspension was purified by lysing red blood cells followed by selection of live inflammatory cells by differential centrifugation through 1-Step lymph-prep media (Accurate Chemical and Scientific Corporation; Westbury, New York). Viable inflammatory cells (macrophages, eosinophils, lymphocytes, and neutrophils) were resuspended to a final working concentration of 0.5×10^6 cell/ml in RPMI for use in the chemotaxis assays. Inflammatory cell chemotaxis was measured by transfilter assays in 48-well chemotaxis chambers (Neuro Probe, Inc. Gaithersburg, MD) as previously described (22). The chemotactic activity of the BAL fluid from WT or MMP9^{-/-} ($n=5$) mice was assessed against each of the lung inflammatory cell populations in duplicate. Serum-free media, BAL from WT and MMP9^{-/-}, were placed in the lower wells of the chemotaxis chambers and separated from cell suspension in the upper wells. The total number of cells migrating into the chamber filters was enumerated by microscopy, and data were expressed as the total cells/filter.

ELISA

BAL chemokines and glycoproteins were quantified from BAL fluid stored at -80°C following removal of cells. BAL CCL11, CCL7, CCL17, CXCL3, and CXCL10 were quantified by using standard chemokine sandwich ELISAs based on commercially obtained antibodies and protocols (BD PharMingen, San Diego, CA). For OVA, specific antibody isotypes, biotinylated anti-mouse IgE, IgG1, and IgG2a antibodies were purchased from Caltag Laboratories (Burlingame, CA). For detection of BAL glycoproteins, including mucins, biotinylated jacalin lectin was purchased from Vector Laboratories (Burlingame, CA) and used as described previously (10).

Gelatin gel zymography and MMP9 activation

Bronchoalveolar lavage fluid was collected as above. Samples (5 μl) of BAL were added to nondenaturing loading buffer and separated in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels containing 0.02% gelatin (15). SDS was removed by three 20-min washes with 2.5% Triton X-100 before incubation for 24 h at 37°C in developing buffer (50 mM TrisHCl pH 8; 5 mM CaCl₂; 0.02% NaN₃). Gelatin gels were then fixed and stained with 50% methanol and 10% acetic acid containing 0.3% w/v Coomassie Blue. MMP9 (gelatinase B)

activity appears as two clear bands at 98 kDa (pro-gelatinase B) and 92 kDa (active gelatinase B). Purified murine MMP9 (15ng; Chemicon, Temecula, CA) was activated by incubation at 37°C for 2 h with the organomercurial compound APMA at 1 mM, and the active and zymogen forms of this enzyme were loaded into the gelatin gels for size comparison.

RNA isolation and real-time PCR

Total cellular RNA was extracted from lung tissue by using RNeasy Lysis Buffer (Qiagen, Crawfordsville, IN) according to the manufacturer's protocol. A two-step real-time reverse transcription (RT) polymerase chain reaction (PCR) was used to determine the relative expression of mRNA using the ABI Perkin Elmer Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). cDNA was generated from the 1 µg of RNA by using multiscribe reverse transcriptase, random hexamers, magnesium chloride, dNTP's, reaction buffer and RNase inhibitor (all from Applied Biosystems, PE) in a final volume of 100 µL. cDNA (10 ng) per sample were analyzed for expression of IL-4, IL-13, and 18S in a 50 µL reaction mixture by using PDARS (PreDeveloped Assay Reagents, Applied Biosystems, Foster City, CA) comprising 20× concentrated solutions of primers and 6FAM-labeled probe used at a concentration of 1× in the final reaction mixture with TaqMan Universal Master Mix. 18S was detected by using 20× target mix (primers/probe [FAM]). Relative quantifications of 18S rRNA, IL-4, IL-13, and mRNA were calculated by using the comparative threshold cycle number for each sample fitted to a five-point standard curve (ABI Prism 5700 User Bulletin #2, PE Applied Biosystems). Expression levels for the mRNAs in each sample were normalized to 18S rRNA. The samples were assayed three times for the target genes, and the average of the readings was used to calculate the relative level of IL-4, IL-13, and mRNA in the tissue. The means and standard errors were calculated for each group.

Apoptosis assay

The Fluorescein-fragEL™ DNA Fragmentation (Oncogene Research Products Cambridge, MA) detection kit was used to label the free 3'-OH groups at the end of DNA fragments in BAL cytopins, as well as paraffin-embedded lung tissue following manufacture recommended protocol. Early apoptotic inflammatory cell characterization was accomplished by two-color flow cytometry by using combinations of the following monoclonal antibodies (mAbs): FITC-conjugated anti-annexin-V (BD PharMingen), and PI (BD PharMingen) intensity on PE channel. Freshly isolated lung inflammatory cells were resuspended to 1×10^7 cells/ml, and 50 µl of cells were incubated with antibodies to annexin-V. Cells were washed and resuspended in 300 µl of RPMI and expression of annexin-V, and PI labeled cells was detected by flow cytometry (Beckman Coulter Epic XL, FL; Fullerton, CA). Data were analyzed by using FlowJo software (Tree Star, Inc., San Carlos, CA) and are presented as the mean $\pm$ SEM of the percentages of annexin-V-positive, and PI-negative cells in each group ($n=5$).

Statistics

All data are representative of at least three independent experiments with four to five mice in each in vivo experiment and are expressed as means $\pm$ SEM. Significant differences ($P \leq 0.05$) are expressed relative to saline-challenged control (*) or CAA-challenged WT mice (**) using a Student's *t*-test (log PC₂₀₀) or a Mann-Whitney U test (all others). Cell migration was expressed as mean $\pm$ SEM of cell numbers per 10 high-power field (400×). Results were assessed for statistical significance by using ANOVA and the Bonferroni multiple-comparison tests. *P* values <0.05 were considered statistically significant.

RESULTS

MMP9 is expressed by recruited leukocytes during allergic lung inflammation

We have previously shown that MMP2 (gelatinase A, 72 kD Type IV collagenase) is constitutively expressed in the airways of naïve and allergen-challenged mice but is also up-regulated with inflammation (22). Many of the characteristic features of asthma seen in allergen-challenged mice, such as goblet cell hyperplasia and AHR, are also seen in mice that receive rIL-13 (9). Thus, to understand the lung expression and distribution of the other major gelatinase, MMP9 (gelatinase B, 92 kD gelatinase) in mice that exhibit the asthma phenotype, we assessed whole lung bronchoalveolar lavage and mRNA in complete *Aspergillus* allergen (CAA)-challenged and mice treated with intranasal rIL-13 for three days. By gelatin zymography, MMP9 was induced in bronchoalveolar lavage fluid of mice that were treated with CAA and mice that were given rIL-13 (Fig. 1*a*). Under the same conditions, quantitative real-time PCR analysis demonstrated marked up-regulation of mRNA, which suggests that regulation of MMP9 was largely transcriptionally controlled (Fig. 1*b*).

Although most studies agree that MMP9 is expressed minimally in alveolar macrophages under normal conditions, there remains much controversy as to the spatio-temporal regulation of lung MMP9 under pathological conditions. For example, under pathological conditions, both the presence and absence of MMP9 in the lung airway epithelium has been reported (27–29). In contrast to MMP2, lungs of naïve mice were devoid in expression of MMP9 mRNA (Fig. 1*c*, top panel). However, following airway immunization with CAA, MMP9 expression was dramatically enhanced in the peribronchovascular regions of the lung where allergic inflammatory cells typically concentrate (Fig. 1*c*, middle panel). Close examination of these low-power images indicated that the constitutive lung tissues, especially the airway epithelium, did not express MMP9. This impression was confirmed on high-power images that clearly revealed intense MMP9 mRNA expression in macrophages and other recruited inflammatory cells, which spared entirely the epithelium and mesenchymal cells, i.e., fibroblasts and endothelium (Fig. 1*c*, bottom panel). Although the epithelium also does not express MMP2, the MMP9 expression pattern is otherwise in striking contrast to MMP2 expression, which, in mice exhibiting the identical asthma phenotype, is strongly expressed in mesenchymal cells (22). Thus, although both MMP2 and MMP9 are up-regulated under similar conditions, different cell types differentially express them under naïve and inflammatory conditions. Mesenchymal lung cells express MMP2, and recruited inflammatory cells express MMP9.

Recruitment of allergic inflammatory cells and AHR are independent of MMP9 and MMP2

Although the many biochemical similarities between MMP2 and MMP9 suggest functional redundancy, the entirely different lung tissue distributions of these enzymes implicate distinct functions in vivo. To understand the role of MMP9 and the potential synergistic effects of MMP9 and MMP2 in allergic lung disease, we assessed the asthma phenotype in WT and MMP2-deficient mice by using CAA (22,26). Similar to MMP2-deficient mice, wild-type (WT), MMP9^{-/-} and MMP9/MMP2 dko mice immunized with CAA showed characteristic allergic and obstructive features, including similar degrees of airway hyperresponsiveness, mucin, and glycoprotein hypersecretion and elevated serum IgE levels (Fig. 2*b–d*). In contrast, total bronchoalveolar lavage (BAL) cells, which were notably increased in allergen-challenged WT mice, were significantly reduced in both MMP9 and dko mice (Fig. 2*e, f*). Differential cell count of the BAL cells revealed significant reduction in total number of eosinophils and neutrophils in the lavage fluid of MMP9^{-/-} and dko mice (Fig. 2*f*). Thus, several features of the asthma phenotype were preserved in the absence of MMP2 and MMP9, but lack of either or both enzymes profoundly disrupted trafficking of inflammatory cells into the airway. Although decrease in BAL inflammatory cells was also observed in MMP2^{-/-} mice, it was entirely accounted for by fewer eosinophils. Our observed decrease in neutrophils and

eosinophils in the BAL of mice deficient in MMP9 reveals similar but non-overlapping specificity of MMP9 in comparison with MMP2.

MMP9^{-/-} and MMP2/MMP9 dko mice show increased inflammatory cells in lung parenchyma

Our previous studies with MMP2^{-/-} mice showed that this enzyme is required neither for recruitment to the lung of inflammatory cells nor for their localization to bronchovascular bundles (22). Indeed, preservation of most of the asthma phenotype, especially airway obstruction, which requires efficient Th2 homing to the lung, is itself an indication that MMP2 was not required for Th2 homing. Additional stigmata of the asthma phenotype, including goblet cell metaplasia, were also fully expressed in airways of MMP-deficient animals (data not shown). We therefore reasoned that, because much of the asthma phenotype was preserved in MMP9^{-/-} and dko mice, allergic inflammatory cell recruitment to lung would not be reduced. Pathologic examination of lungs indicated that, relative to saline-challenged mice, lack of MMP9 or MMP9 and MMP2 did not impair lung recruitment of inflammatory cells or their ability to localize to the peribronchovascular space following allergen challenge (Fig. 3a). Rather, we noted larger and more organized aggregates of inflammatory cells clustering tightly around bronchioles and arterioles of MMP-deficient animals, which suggests that either cellular recruitment was increased or egression of cells into the lavage is diminished (arrowheads, Fig. 3a). Interestingly, we noted a second abnormality unique to MMP9^{-/-} and dko mice: Alveolar inflammation, which was present diffusely in WT mice (arrows, Fig. 3a, b), was two- to threefold higher and was more confined to the perivascular bundle in these mice. We sought additional evidence for aberrant intrapulmonary cellular trafficking in mice lacking MMPs. Characteristic biochemical features of allergic inflammatory cells, particularly Th2 cells or eosinophils, include their selective production of the cytokines IL-4 and IL-13. In the absence of MMP9, expression of IL-4 and IL-13 mRNA was two- and threefold higher, respectively, by real-time PCR, relative to WT mice following allergen challenge (Fig. 3c), in keeping with the larger number of inflammatory cells. Together these findings indicate that, in MMP9^{-/-} and dko mice, the excess inflammatory cells that accumulate abnormally around bronchovascular bundles in the lung parenchyma produce IL-4 and IL-13.

Excess lung apoptotic cells in the absence of MMP9 and MMP2

An essential aspect of any inflammatory response is the prompt disposition of effete immune cells. Unnecessary inflammatory cells most likely prolong potentially deleterious immune responses; furthermore, lysed or apoptotic cells can incite additional tissue injury and inflammation. There were virtually no apoptotic cells in the BAL or parenchyma of saline-challenged animals. Compared with WT mice, MMP9-deficient mice challenged with CAA showed a reduction in the absolute number of apoptotic cells in their bronchoalveolar lavage as determined by TUNEL assay (Fig. 4a). In contrast, there was a massive accumulation of apoptotic cells in the lungs of allergen-challenged MMP9^{-/-} mice, compared with lungs of allergen-challenged WT mice (Fig. 4a).

We assessed isolated whole lung cells for the fraction undergoing apoptosis. Flow cytometry was used to identify live cells [propidium iodide (PI) negative] that were in the early phase of apoptosis (preapoptotic cells; annexin-V positive; Fig. 4b). By these criteria, preapoptotic cells were 60–70% more abundant in lungs of MMP9 and dko mice relative to WT (Fig. 4c). Further, the fraction of dead (PI positive) and apoptotic (annexin-V positive) cells in lung parenchyma was approximately doubled in MMP9 and MMP2/MMP9 mice (data not shown). Similar to the effect of MMP2 deletion, these findings demonstrate that, in the absence of MMP9 and MMP9/MMP2, inflammatory cells accumulate excessively in lung parenchyma and, instead of being efficiently cleared through the airway, many undergo apoptosis in situ.

Decreased CCL11, CCL17, and CCL7 concentrations in BAL but not lung tissue of MMP9^{-/-} and dko mice

The excess accumulation of lung parenchymal inflammatory cells combined with diminished cellularity of the bronchoalveolar lavage seen in MMP9^{-/-} and dko mice suggested that the normal trafficking of recruited lung cells into the airway lumen had been disrupted, favoring their accumulation in parenchyma. We showed previously (22) that a similar abnormality seen in MMP2^{-/-} mice was related to attenuation of the normal trans-epithelial chemokine gradient that determines egression of inflammatory cells. Interestingly, of several chemokines relevant to allergic lung inflammation, only CCL11 (eotaxin) was affected by lack of MMP2, which suggests an important but limited contribution of this metalloproteinase to cellular egression. To understand the contribution of MMP9 and MMP9/MMP2 to transepithelial chemokine gradient formation, we quantified CCL11, CCL7 (MCP-3/MARC), CCL17 (TARC), CXCL10 (IP-10), and CXCL3 (KC) in BAL of WT, MMP9^{-/-}, and dko mice challenged with allergen. In contrast to MMP2^{-/-} mice, concentrations of all three CC chemokines were 30–70% less than those of the corresponding chemokines of WT mice (Fig. 5a–c). Furthermore, these alterations in CC chemokine concentration were confined to the airway because total lung protein levels of these chemokines did not differ significantly between WT and MMP9 allergen-instilled animals (data not shown). Because we noted a decrease in transepithelial migration of neutrophils in MMP9 and dko mice, we also measured KC (CXCL3) and IP-10 (CXCL10). We did not detect KC or IP-10 proteins in the BAL of the naïve or allergen challenged WT, MMP9, or dko mice (data not shown).

Normal chemotaxis of MMP9-deficient inflammatory cells

Migration and chemotaxis of neutrophils and eosinophils in MMP9-deficient mice may be affected by the loss of this enzyme. Therefore, we assessed the chemotactic ability of inflammatory cells obtained from lungs of allergen-challenged WT and MMP9-deficient mice in response to BAL from the same mice. We found that lung inflammatory cells showed similar chemotaxis in response to WT BAL regardless of their genotype (Fig. 5d). However, a significant reduction in chemotaxis was observed when the same cells were tested against the BAL of MMP9^{-/-} mice, indicating that chemotactic activity is deficient in MMP9^{-/-} BAL (Fig. 5d). These findings are consistent with the hypothesis that, MMP9—like MMP2—controls the formation of transepithelial CC chemokine gradients that control the egression (chemotaxis) of inflammatory cells.

DISCUSSION

This study provides significant new insights into the biology of metalloproteinases and their roles in regulating allergic inflammation. We have shown that MMP9 is up-regulated in the lung during allergic inflammation but that its expression is confined primarily to leukocytes. In the absence of MMP9, most features of the asthma phenotype were preserved, including airway hyperresponsiveness, serum IgE responses, and airway mucin and glycoprotein secretion, confirming that neither MMP9 nor MMP2 (22) is required for initiating allergic inflammation and its attendant disease. In contrast, allergen-challenged MMP9^{-/-} mice showed decreased numbers of eosinophils and neutrophils in bronchoalveolar lavage and excess dead and apoptotic inflammatory cells in the lung parenchyma. Although MMP2 is produced by pulmonary fibroblasts and smooth muscle cells, whereas MMP9 is produced by the responding inflammatory cells, these features are all shared by MMP9^{-/-} and MMP2^{-/-} mice, confirming that the major function of lung gelatinases during allergic inflammation is facilitating egression through the airway of recruited inflammatory cells (22).

These new studies extend our prior findings with MMP2^{-/-} mice by showing that MMP9—not MMP2—is the dominant airway MMP controlling cellular trafficking. Whereas only

eosinophils were affected by lack of MMP2, eosinophils and neutrophils were reduced in bronchoalveolar lavage of MMP9^{-/-} mice. Further, MMP9 null inflammatory cells were capable of normal chemotactic responses; thus the loss of MMP9 did not affect cell migration. However, instead of a single Th2 chemokine gradient (CCL11/eotaxin) being affected as in MMP2^{-/-} mice, multiple transepithelial Th2 CC chemokine gradients (CCL11, CCL7, and CCL17) were disrupted by lack of MMP9. Combined deletion of MMP2 and MMP9 (dko mice) had no additive effect on the MMP9^{-/-} phenotype alone, confirming the overriding importance of MMP9 in this model. We found that peripheral blood leukocyte counts were unaffected by MMP deficiency, but BAL cell numbers were lower. These findings suggest that cells simply accumulate aberrantly in the parenchyma because they are hindered from their egression into the BAL. We believe that the decrease in cellular migration into the BAL fluid reflects abnormal processing of the chemokines, precluding their secretion into the BAL, as justified by our finding of decreased eotaxin (MMP2^{-/-}) and TARC (MMP9^{-/-}) in BAL, but normal levels of these chemokines in lung parenchyma. The increased inflammatory cell death seen in the lung of MMP9 and dko mice, the presumed result of effete cells lingering in situ longer than normal, further supports the existence of a mechanism essential for cell egression, which is controlled through MMPs. We conclude, therefore, that egression of inflammatory cells into the airway, a function essential to maintaining gas exchange and preventing fatal asphyxiation, is controlled primarily by recruited cells themselves through MMP9.

Additional studies support the proposed link between MMPs and regulation of chemokines as proposed here (16,30). In vitro studies have shown that MMP1, MMP2, MMP-3, and MMP14 (MT1-MMP) cleave CCL7/MCP-3 at an identical site and that these MMPs generally show the same N terminus cleavage site for several members of the MCP chemokine family, stromal cell-derived factor-1 α (SDF-1 α) and SDF-1 β (31,32). Interestingly, truncation of these chemokines results in formation of natural CC chemokine receptor antagonists. Thus, these in vitro studies suggest an important role for MMPs in disrupting chemokine gradients. However, it is important to note that little is known of the molecular interactions between MMPs and chemokines in vivo. Our studies demonstrate that, distinct from the inactivating effects of MMPs on chemokines, MMPs critically regulate formation of active chemokine gradients that nonetheless have anti-inflammatory functions in vivo. In support of our findings, lack of MMP7 impaired transepithelial migration of neutrophils by disrupting formation of a CXC (KC) chemokine gradient in mice instilled with bleomycin (19). How MMPs regulate chemokine gradients in vivo is not understood, but a plausible mechanism may include MMP-mediated proteolysis of the extracellular matrix-associated proteoglycans that are known to bind and regulate chemokines (33). We are currently investigating the role of MMP-mediated syndecan-1 shedding in the formation of chemokine gradient in the BAL of mice that exhibit the asthma phenotype.

Recent data indicate that normal scavenging mechanisms that clear apoptotic cells can inhibit acute inflammatory responses (34–36). Macrophages ingesting apoptotic cells release IL-10, a regulatory cytokine that suppresses Th2 inflammation and inhibits tumor necrosis factor α production (37–39). Conversely, inappropriate accumulation of apoptotic cells results in increased concentrations of proinflammatory chemoattractants, such as IL-8 and IL-1 β (40, 41). We have shown that MMP2 and MMP9 are required for the transepithelial egression of neutrophils and eosinophils, and without these enzymes, cells undergoing apoptosis accumulate massively in the peribronchovascular space, presumably overwhelming the normal capacity of professional phagocytic cells responsible for their clearance. Although phagocytic clearance of potentially deleterious apoptotic cells may suffice during quiescent periods, our data indicate that transepithelial egression is a necessary second means for removal of excess cells during inflammation.

We can only speculate as to why the mesenchyme evolved a separate, but overlapping mechanism, through MMP2, for controlling cellular egression during allergic inflammation that is somewhat specific for eosinophils. At most stages of experimental allergic lung disease, the eosinophil is the predominant infiltrating cell type (42–44). During severe bouts of allergic inflammation, induced by proteolytic allergens as here, or also by migrating parasitic larvae (45,46), astonishing numbers of lung eosinophils may be seen, sufficient in many instances to physically impair gas exchange and substantially increase the risk of asphyxiation (22). Eosinophils also secrete many potentially toxic products that may further impair lung function (47–49). Interestingly, although our data indicate that Th2 cells accumulated abnormally in MMP9^{-/-} mice, this did not lead to a worse asthma phenotype. Therefore, overwhelming eosinophilia generated during allergic lung inflammation, combined with the intrinsic toxicity of these cells, may have provided sufficient evolutionary pressure to ensure development of redundant mechanisms for their disposal.

The experimental asthma model is a technically difficult one, and the importance of carefully controlling critical disease-related variables cannot be overemphasized. Wherever possible, we have assessed relevant endpoints, such as airway hyperresponsiveness, in a manner as consistent as possible with analogous human protocols. Further, to induce the asthma phenotype, we have primarily relied on an allergen derived from a fungal pathogen relevant to human asthma. Nonetheless, our protocols remain flexible, and we have approached our questions from several different perspectives, using multiple strains of mice, allergens and protocols for their administration, and methods to inactivate MMP2 and MMP9. Across these divergent approaches, a fundamental underlying principal of lung MMP biology has emerged, that MMP2 and MMP9 are not required for cellular recruitment and thus induction of disease, only cellular egression and thus disease resolution. We cannot exclude the possibility that, using alternative experimental conditions, lack of MMPs might appear to impair inflammatory cell recruitment to lung as suggested by several studies (50,51). However, because these studies all relied on much shorter airway antigen challenge protocols, we conclude that such effects are transient and that the importance of MMPs to chronic diseases, such as asthma, is more likely defined by their importance in clearance of effete inflammatory cells. Perhaps most important, our findings do not support the use of MMP inhibitors for the treatment of asthma.

Previous studies have indicated that several members of the MMP family may be involved in human chronic obstructive pulmonary disease and experimental models of allergic and non-allergic lung disease (14,52–55). Additional studies of LPS-challenged MMP9^{-/-} mice showed that neutrophil recruitment to the lung was not altered (56); however, in the absence of MMP9, IL-13-induced acute leukocyte egression into the airway was impaired (57). Although further studies are warranted, these findings combined with ours indicate that MMPs may be required during diverse lung disease processes to promote transepithelial egression of inflammatory cells.

Collectively, we have shown that, concomitant with recruitment to the lung of Th2 cells and release of the cytokines IL-4 and IL-13, MMP2 and MMP9 are both up-regulated. Functionally, neither enzyme alone nor in combination is required for initiation of lung inflammation, but both operate cooperatively to facilitate egression through the airway of pre-apoptotic inflammatory cells. Despite their ability to inactivate chemokines in vitro, these enzymes in vivo function to establish the necessary chemokine gradients required for transepithelial egression of effete inflammatory cells. Factors that disrupt the function of MMP2 and MMP9 are likely to exacerbate diseases such as asthma and likely other inflammatory syndromes and are therefore suitable topics for future investigation.

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Figure 1. Expression of lung MMP9

a) Mice were challenged with vehicle (saline), CAA, or recombinant IL-13 (rIL-13) as indicated; bronchoalveolar lavage (BAL) and whole lung lysates were assessed for the presence of MMP9 protein by gelatin zymography (top figure) and mRNA by quantitative real-time PCR (bottom graph), respectively. Shown in the zymogram are enhancement of 98 (proenzyme) and 92 (active) kDa MMP9 in CAA or rIL-13 challenge BAL fluid. Purified MMP9 and MMP9 activated with 1 mM of APMA are shown for size and active fragment comparison. *, ** denotes MMP9 dimer and trimer, respectively. Arrowheads point to active (lower kDa) MMP9. **b**) PCR data of the above challenged mice are depicted as fold increase over saline-challenged animals, * $P < 0.05$ relative to saline challenged. **c**) In situ hybridization

(ISH) was performed on lungs of saline-challenged (top panels) and CAA-challenged allergic mice and viewed at low (middle panels) and high (bottom panels), 60 $\times$ magnification. Darkfield images are shown on the left to reveal the punctate ISH signal, and hematoxylin and eosin (H&E) counterstained images of the same sections are shown at the right to reveal tissue morphology. Short arrows point to airway epithelium and endothelial cells that do not express MMP9; long arrows point to inflammatory cells (macrophages and eosinophils) that express MMP9. Relative size is indicated by 200 μ m bar.

Figure 2. Asthma phenotype of MMP^{-/-} mice

a) Wild-type (WT), MMP9^{-/-} and MMP9/MMP2 double knockout (dko) mice were genotyped by gelatin zymography and challenged intranasally with saline or CAA as indicated and assessed for **(b)** airway hyperresponsiveness by PC200, **(c)** mucins and glycoproteins in bronchoalveolar lavage (BAL) by ELISA, and **(d)** total serum IgE by ELISA. **e)** Total and **(f)** differential cell counts from BAL were determined from the same groups of mice. * $P < 0.05$ relative to saline-challenged WT (**e**).

Figure 3. Histopathology and cytokine production in MMP9 and MMP2;MMP9 null lungs
a) Depicted are photomicrographs of representative bronchovascular bundles stained with H&E. Arrows indicate alveolar inflammation that was prominent in WT mice; arrowheads show interstitial, peribronchovascular inflammation that was more prominent in MMP-deficient animals. Relative size is indicated by 200 μm bar. **b)** Relative to WT CAA-challenged mice, three- to fourfold more peribronchovascular inflammatory cells are visible in lungs of allergen-challenged MMP9 and dko mice as confirmed by enumeration of cells within 40,000 μm^2 areas. * $P < 0.05$ relative to CAA challenged WT mice. **c)** Fold difference in IL-4 and IL-13 mRNA production from lungs of the same mice by real time PCR. * $P < 0.05$ relative to saline challenged WT. ** $P < 0.05$ relative to CAA challenged WT.

Figure 4. Accumulation of lung apoptotic cells in MMP9 and MMP2;MMP9 null (dko) mice
a) Fluorescent TUNEL assay was used to identify apoptotic cells in whole lung (Lung) and BAL of wild-type (WT) and MMP9^{-/-} mice. **b)** Two-color flow cytometry was used to identify annexin-V and propidium iodide staining characteristics of unselected whole lung cells from WT, dko, and MMP9^{-/-} mice challenged with allergen. The lower right quadrant defines annexin-V positive, propidium iodide negative (pre-apoptotic) cells. Quadrant numbers represent the percentage of all gated cells within that quadrant. **c)** Average frequency of lung pre-apoptotic cells for allergen-challenged MMP9^{-/-} mice expressed as the percentage increase over wild-type. **P* < 0.05 relative to CAA- challenged wild-type.

Figure 5. Chemokine concentration of and chemotactic response to bronchoalveolar lavage fluid in MMP-deficient mice

Wild-type (WT) mice were challenged with saline or allergen (CAA) and compared with MMP9^{-/-} and dko mice for concentrations of (a) eotaxin, CCL11, (b) MARC, CCL7, and (c) TARC, CCL17 in the bronchoalveolar lavage. * $P < 0.05$ relative to saline-challenged WT.

** $P < 0.05$ relative to CAA-challenged WT. (d) Migratory responses of lung inflammatory cells from CAA-challenged wild-type (WT cells) and MMP9^{-/-} mice (MMP9^{-/-} cells) in response to media (RPMI), and BAL from CAA-challenged MMP9^{-/-} (BAL MMP9^{-/-}) or wild-type (BAL WT) mice were determined in transfilter assays. Data are expressed as the total number of cells migrating into the chamber filters per low power field (10 $\times$). Note the normal chemotactic response of MMP9^{-/-} cells in response to wild-type BAL. *Significantly different compared with MMP9^{-/-} BAL.