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## Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2-deficiency

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### Abstract

Clearance of recruited immune cells is necessary to resolve inflammatory reactions. We show here that matrix metalloproteinase 2 (MMP2), as part of an interleukin 13 (IL-13)-dependent regulatory loop, dampens inflammation by promoting the egress of inflammatory cells into the airway lumen. MMP2<sup>-/-</sup> mice showed a robust asthma phenotype and increased susceptibility to asphyxiation induced by allergens. However, whereas the lack of MMP2 reduced the influx of cells into bronchoalveolar lavage (BAL), numerous inflammatory cells accumulated in the lung parenchyma. BAL of MMP2<sup>-/-</sup> mice lacked normal chemotactic activity, whereas lung inflammatory cells from the same mice showed appropriate chemotactic responses. Thus, MMP2 establishes the chemotactic gradient required for egression of lung inflammatory cells and prevention of lethal asphyxiation.

The allergic lung is characterized by airway obstruction, including airway hyperresponsiveness (AHR), which is defined as enhanced smooth muscle constrictive responses to provocative challenge. The principal anatomical features of these physiological changes include the accumulation of inflammatory cells, especially eosinophils, within the lung and goblet cell metaplasia of the airway epithelium, which induces a mucus-secreting phenotype<sup>1</sup>. Although a minor cellular constituent, T helper type 2 (T<sub>H</sub>2) cells are the immunologically dominant cell type that underlie allergic lung disease<sup>2-7</sup>.

T<sub>H</sub>2 cells expand and are recruited to the lung in response to inhaled allergens. All T<sub>H</sub>2 cytokines contribute to experimental allergic lung disease, however, interleukin 4 (IL-4) is required for T<sub>H</sub>2 development, immunoglobulin E (IgE) synthesis and atopic reactions based on type 1 hypersensitivity mechanisms<sup>8-10</sup>. In contrast, IL-13—which is closely related to IL-4, and whose receptor includes the  $\alpha$  chain of the IL-4 receptor (IL-4R $\alpha$ )<sup>11-13</sup>—induces many of the usual features associated with asthma in mice<sup>14</sup>. The mechanism(s) by which IL-13 induces the asthma phenotype are unclear, but IL-13 likely represents a bridge that links immune cells with several non-hematopoietic lung tissues<sup>15</sup>. This suggests that IL-13, and to a lesser extent IL-4, may directly elicit allergic airway disease by stimulating airway epithelial and smooth muscle cells<sup>16</sup>. Additional communication between immune and parenchymal cells, perhaps *via* cytokines, blunt harmful immune responses and initiate repair mechanisms. However, the mechanisms that limit allergic inflammatory responses are poorly understood.

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Matrix metalloproteinases (MMPs) are up-regulated during allergic inflammation<sup>17</sup> and may participate in the pathogenesis of several lung diseases<sup>17–21</sup>. MMPs also facilitate inflammatory cell recruitment across the endothelial basement membrane<sup>22,23</sup>. We examined here the immune-mesenchymal cross-talk that occurs during allergic inflammation as well as the anti-inflammatory role of MMP2, which represents an essential link in an IL-13–dependent regulatory loop that dampens allergic inflammation.

## Results

### MMP2 activity in allergic lung inflammation

We induced stereotypical asthma in BALB/c mice with ovalbumin (OVA)<sup>3</sup>. BALB/c mice that were immunized and intranasally challenged with OVA to induce the allergic lung phenotype showed exaggerated airway closure, or hyperresponsiveness (AHR), in response to acetylcholine provocation as well as pronounced airway eosinophilia, increased titers of serum antigen-specific IgE and up-regulation of T<sub>H</sub>2 cytokines in bronchoalveolar lavage (BAL) (Fig. 1). These features are characteristic of human allergic asthma, which is induced by a wide variety of allergens<sup>24</sup>. Examination of BAL from saline-challenged BALB/c control mice showed that MMP2 was constitutively expressed in the airways of these mice. However, mice with the asthma phenotype showed a fivefold increase in both active and inactive (pro-) MMP2 (Fig. 1b and data not shown). These observations suggested that enhanced expression of MMP2 is a feature of the experimental asthma phenotype.

### Effect of IL-13 on MMP2 and asthma development

We next determined the mechanisms that regulate MMP2 expression during acquisition of the asthma phenotype in mice that were sensitized to OVA or had received intranasal (i.n.) recombinant IL-13 (rIL-13). We found that both OVA-sensitized mice and mice challenged with rIL-13 showed enhanced expression of MMP2 protein in the BAL (Figs. 1b and 2a) and mRNA in lung (Fig. 2b); this was not observed in control tissue. This finding was specific to the lung because northern blot analysis of liver from these mice showed no MMP2 mRNA expression (data not shown). Using *in situ* hybridization, we found that MMP2 mRNA was primarily expressed in cells of mesenchymal origin (Fig. 2c–f). These observations showed that MMP2 is up-regulated and activated during asthma induction and that IL-13 alone can induce this.

### Lack of MMP exacerbates lung inflammatory cells

To understand the *in vivo* role MMP2 plays in the asthma model, we first administered a synthetic inhibitor of MMPs, GM6001, to OVA-sensitized BALB/c mice at the time of i.n. OVA challenge. Although GM6001 treatment did not alter the asthma phenotype (Fig. 3a and data not shown), we observed >60% dose-dependent inhibition of inflammatory cell egression with concomitant accumulation of inflammatory cells in the lung parenchyma (Fig. 3b). Because GM6001 inhibits a number of MMPs<sup>25</sup> we next examined the lung phenotype of MMP2-deficient mice<sup>26</sup>. Because MMP2<sup>-/-</sup> mice were crossed onto the C57BL/6 (B6) background, which is resistant to the OVA-induced asthma phenotype<sup>3</sup>, we did these experiments using complete aspergillus antigen (CAA)—which is derived from the pathogenic fungus *Aspergillus fumigatus*—as an allergen. CAA challenge in B6 mice induces all the key features of asthma that are observed in OVA-challenged BALB/c mice<sup>5,27</sup>: marked increases in AHR; serum total IgE and BAL mucin content (Fig. 4a–e); peribronchovascular inflammation and goblet cell metaplasia (Fig. 4f–i); and MMP2 protein and mRNA expression (data not shown). In addition, compared to saline-challenged controls, CAA-challenged MMP2<sup>-/-</sup> or GM6001-treated B6 mice showed ~50% fewer BAL cells, with eosinophils mainly being affected (Fig. 4e). These data suggest that MMP2 accounts for much of the GM6001-induced decrease in BAL inflammatory cells.

### MMP2 is required for inflammatory cell egression

There were no marked differences in total blood leukocyte counts during CAA challenge of wild-type and MMP2<sup>-/-</sup> mice ( $6.7 \times 10^3 \pm 0.4 \times 10^3$  versus  $5.3 \times 10^3 \pm 0.1 \times 10^3$ , respectively;  $n=4$ ). However, MMP2 did play a key role in the regulation of lung inflammatory cell trafficking. If influx of inflammatory cells did not increase, then efflux should decrease. Thus, we counted the total number of inflammatory cells that were present in 40,000  $\mu\text{m}^2$  areas centered on medium-sized bronchovascular bundles (airway diameters  $\sim 100 \mu\text{m}$ ; see Fig. 4h for scale). Compared to wild-type mice, we consistently found a three- to fivefold increase in inflammatory cells present in the parenchyma of CAA-challenged MMP2<sup>-/-</sup> and GM6001-treated mice (Fig. 4j). Thus, the absence of active MMP2 resulted in excess accumulation of inflammatory cells in the lung.

This increased inflammation in the lung parenchyma was accompanied by excessive amounts of T<sub>H</sub>2 cytokines. Using RNase protection assay (RPA), we found that the expression of IL-4, IL-5, IL-6, IL-9 and IL-13 mRNA was markedly up-regulated in wild-type mice that were challenged with antigen. In the absence of MMP2, expression of IL-4 and IL-13 mRNA was 2.7- and 4.2-fold higher, respectively, than in MMP2-sufficient mice (Fig. 5a and data not shown). Similarly, using quantitative real-time polymerase chain reaction (PCR), we found that expression of IL-4 and IL-13 mRNA was highly up-regulated in the lungs of MMP2<sup>-/-</sup> mice that had been challenged with antigen (Fig. 5b). Together, these data indicate that a principal function of MMP2 during allergic lung inflammation is to facilitate the egression of allergic inflammatory cells into the airway lumen and, in its absence, these cells accumulate massively in the lung parenchyma.

### Lethal susceptibility to allergic lung inflammation

We next examined the physiological relevance of our findings by monitoring the survival of MMP2<sup>-/-</sup> and wild-type mice after i.n. CAA challenge. We observed a sharp rise in the mortality of MMP2<sup>-/-</sup> mice after the second challenge (Fig. 5c). Mortality rates continued to increase in MMP2<sup>-/-</sup> mice after the third i.n. administration of CAA and resulted in 100% mortality by the fourth challenge (Fig. 5c). The susceptibility of MMP2<sup>-/-</sup> mice to death after acute allergen challenge was markedly different from that of wild-type mice ( $P < 0.008$ ). Asphyxiation was the cause of death, as treatment with oxygen for 10 min before and after challenge reduced mortality to 20% and 0% in MMP2<sup>-/-</sup> and wild-type mice, respectively (Fig. 5d).

### Chemotactic activity in CAA-challenged MMP2<sup>-/-</sup> mice

We reasoned that reduced inflammatory cell egression in MMP2<sup>-/-</sup> mice was caused either by defective chemotaxis of the recruited lung inflammatory cells or a lack of chemotactic activity in the MMP2<sup>-/-</sup> BAL. We found the latter to be the case because inflammatory cells extracted from whole-lung homogenates from MMP2<sup>-/-</sup> or wild-type mice showed similar chemotaxis in response to wild-type BAL (Fig. 6a). However, a marked reduction in chemotaxis was observed when the same cell populations were tested with BAL from MMP2<sup>-/-</sup> mice, which indicated that chemotactic activity was deficient in MMP2<sup>-/-</sup> BAL (Fig. 6a). In addition, the recombinant mouse chemokines CCL7 (also known as MCP-3, 100 ng/ml) and CCL11 (also known as eotaxin, 10–100 ng/ml) induced similar chemotactic responses from MMP2<sup>-/-</sup> or wild-type lung inflammatory cells (Fig. 6a and data not shown). Finally, we determined the concentration in BAL of three chemokines—CCL7, CCL11 and CCL17 (also known as TARC)—relevant to allergic lung disease<sup>28,29</sup>. We found comparable concentrations of CCL7 and CCL17 in the BAL of MMP2<sup>-/-</sup> mice and wild-type controls. However, the amount of CCL11 detected in the BAL of MMP2<sup>-/-</sup> was one-third that of wild-type mice (Fig. 6b).

## Discussion

Our data highlight the importance of luminal clearance of inflammatory cells in preventing lethal asphyxiation and the role that MMP2 plays in this process. Both fibroblasts and smooth muscle cells—which are lung cells of mesenchymal origin—participate in the asthmatic phenotype, which is regulated by the signaling cascade initiated by T<sub>H</sub>2 cytokines, in particular IL-13. Lack of MMP2 during allergen challenge resulted in increased expression of several T<sub>H</sub>2 cytokines, exacerbated inflammatory cell infiltration of the lung and a decrease in the chemoattractant activity of these inflammatory cells in the BAL. Paradoxically, these findings were accompanied by decreased recruitment of the same inflammatory cells into the BAL, with eosinophils representing the inflammatory cell that was most affected by this aberrant cellular trafficking pattern. However, consistent with these findings, we discovered a selectively decreased concentration of CCL11—a potent chemoattractant for eosinophils<sup>30,31</sup>—in the BAL of MMP2<sup>-/-</sup> mice. Thus, IL-13 initiates secondary gene-activation programs in the mesenchyme, which are essential for the clearance of eosinophils, by providing the necessary chemotactic gradient required to prevent the harmful accumulation of these cells.

The term airway remodeling is often applied to describe the range of anatomical changes observed in the lungs during acute and chronic allergic inflammation<sup>1,32</sup>. Although variously defined, airway remodeling includes changes such as goblet cell metaplasia and mucus gland hyperplasia, subepithelial collagen deposition, smooth muscle hyperplasia and/or hypertrophy, airway mucus plugging, peribronchovascular accumulation of inflammatory cells and other changes that are believed to contribute to the airway obstruction of asthma. Our results establish a key contribution made by the lung parenchyma to the lung-remodeling paradigm. We found that the clearance of allergic inflammatory cells from the lung parenchyma was critically influenced by parenchymally derived MMP2. Our findings were confirmed in two different mouse strains, using different allergens and methods for inactivating MMP2. Our data define a unique mechanism by which parenchymal inflammatory cells egress into the airway lumen that requires generation of an MMP2-dependent trans-epithelial chemotactic gradient. Because the phenotype of MMP2<sup>-/-</sup> mice largely reproduced that of GM6001-treated mice, MMP2 is likely the most important MMP regulating parenchymal inflammatory cell flux *via* this mechanism. However we cannot exclude contributions made by MMP9 (also known as gelatinase B) and MMP14 (also known as MT1-MMP), which are also expressed in the lung. Lung neutrophil emigration and egression induced by lipopolysaccharide remain intact in the absence of MMP9<sup>33</sup>. However this finding does not exclude a potential role played by MMP9 in the allergic setting. Indeed, whereas CCL11 is particularly affected by a lack of MMP2, deficiency in other MMPs (for example MMP9) reduces formation of the airway trans-epithelial gradient of distinct chemokines (F. K and K. R., unpublished data). This work provides the impetus to evaluate the role of other MMPs in allergic and other lung inflammatory settings.

Like most other features of experimental asthma, MMP2 expression is controlled through IL-13. Lung fibroblasts, smooth muscle and other parenchymal cells contribute significantly to the total production of lung MMP2. Importantly, rIL-13 directly stimulated MMP2 mRNA and protein, which provides an essential link between the pulmonary mesenchyme and cytokine-producing T<sub>H</sub>2 cells that underlie allergic lung disease. Such a connection further establishes IL-13 as a general mediator of T<sub>H</sub>2 -dependent physiological effects<sup>14,15,18</sup>.

Our findings suggest that both eosinophils and cells that produce T<sub>H</sub>2 cytokine accumulate in the lungs in the absence of MMP2. Although excess eosinophils could account for the aberrant cytokine expression patterns observed, lymphocytes are the major producers of T<sub>H</sub>2 cytokine mRNA in murine lungs after antigen challenge (D. C., unpublished data) and are, therefore, a more likely source. T<sub>H</sub>2 cells and eosinophils are strongly implicated in the pathogenesis of

allergic diseases such as asthma, and their accumulation—together with their excess cytokine products—would be expected to correlate with more severe disease.

The mechanisms that regulate clearance of recruited leukocytes from the lung are not fully understood, but may include several routes, including apoptosis and phagocytosis, lymphatic recirculation and cell egression into the airway lumen<sup>34,35</sup>. *In vitro*, clearance of apoptotic neutrophils is mediated through phagocytosis by macrophages<sup>36</sup>. Both lung and gut are frequently involved with allergic inflammation and, not surprisingly, their lumens are sites of inflammatory cell clearance<sup>37,38</sup>. In acute allergic airway inflammation, eosinophils and macrophages constitute >80% of total recovered leukocytes<sup>3,5,14</sup>. Although allergic inflammation may be harmful to any organ, the host is particularly susceptible to lung involvement due to the potentially lethal effects of the inflammatory cells on gas exchange<sup>39</sup>. We showed that 100% mortality occurred in MMP2<sup>-/-</sup> mice compared to 20% in wild-type mice ( $P<0.008$ ). The vastly improved survival chances that administration of oxygen conferred showed that death resulted from asphyxiation, which was consistent with the aberrant accumulation of lung parenchymal inflammatory cells observed in MMP2<sup>-/-</sup> mice. Asphyxiation is the major cause of mortality in human asthma<sup>40,41</sup>, which highlights the relevance of this asthma model and the critical role played by MMPs in clearing inflammatory cells and preventing death. Because we measured similar AHR in allergen-challenged MMP2<sup>-/-</sup> and wild-type mice, differences in smooth muscle or other contractile cell function cannot explain the increased mortality observed.

Inflammatory cell concentration in BAL diminished in MMP2<sup>-/-</sup> mice despite peribronchovascular cell accumulation, thus we conclude that MMP2 is essential for the egression of recruited inflammatory cells into the airway lumen. This impairment was due to the failure to establish an appropriate chemokine gradient that extended into the BAL of MMP2<sup>-/-</sup> mice. The MMP2<sup>-/-</sup> inflammatory cells responded normally to the BAL from wild-type mice, which showed that the appropriate chemokines were lacking. MMP2 may also indirectly contribute to altered cell recruitment. Several chemokines, including CCL7, are physiological substrates for MMP2<sup>42</sup> and the cleavage products of CCL7 behave as receptor antagonists for the chemokine receptors CCR1, CCR2 and CCR3<sup>42</sup>. Although we did not find marked differences in CCL7 concentrations between the BAL of wild-type and MMP2<sup>-/-</sup> mice, this suggests that other MMPs may contribute to cleavage of CCL7.

Our data highlight the importance of luminal clearance of inflammatory cells in preventing lethal asphyxiation and the role that MMP2 plays in this process. In the absence of MMP2, the rapid accumulation of cells in the lung parenchyma—as early as 5 days after i.n. antigen challenge—showed that the luminal route may be the most important route for clearing lung allergic inflammatory cells. MMP inhibitors block transmigration of IL-5-activated eosinophils through an artificial basement membrane<sup>43</sup>. Our data show that extravasation of inflammatory cells across the endothelial basement membrane occurs independently of the MMPs required for luminal clearance. Thus, the processes of extravasation and luminal clearance, although obviously related, rely on distinct mechanisms, with MMP2 playing a key role only in luminal clearance.

Our results are in agreement with a published study of the role played by MMPs in limiting BAL cell recruitment<sup>19</sup>; however, we have extended this observation to the entire lung and demonstrate the accumulation of cells in the parenchyma. Our data show that the principal effect of MMP inhibition is reduced trafficking of parenchymal inflammatory cells into the airway lumen, with little or no effect on inflammatory cell recruitment or production. Thus, inhibition of MMPs does not preclude lung inflammatory cell recruitment, only cell egress. Neither the presence nor absence of MMPs markedly hinders the development of the pathognomonic features of asthma. Because parenchymal inflammatory cells continue to



contribute to tissue damage, allergic features may actually worsen in the absence of MMPs, obviating MMP inhibition as a useful therapy for asthma and other allergic diseases<sup>19</sup>. Indeed, after only 3 weeks of allergic lung inflammation, MMP2<sup>-/-</sup> mice showed greatly increased susceptibility to the asphyxiating potential of acute allergen challenge. Clarification of the role of other MMPs will further elucidate the pathogenesis of allergic inflammation and may provide additional therapeutic insight into diseases such as asthma.

## Methods

### Mice

MMP2<sup>-/-</sup><sup>26</sup> (on a B6 background), BALB/c and B6 mice were bred in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited transgenic animal facility at Baylor College of Medicine.

### Allergens and other reagents

Chicken egg OVA (Sigma, St. Louis, MO) was precipitated in alum (OVA-alum) as described<sup>3</sup>. CAA was prepared from a clinical isolate of *Aspergillus fumigatus* as described<sup>5</sup>. Mice were given 50, 150 or 250 mg/kg of GM6001 (AMS Scientific, Concord, CA)<sup>25</sup> by i.p injection 1 h before i.n. challenge with OVA or CAA. Murine rIL-13 was from Peprotech (Rocky Hill, NJ).

### Allergen and IL-13 challenge

All mice on the BALB/c background were sensitized with 25 µg of OVA-alum, which was administered intradermally three times at 7-day intervals. Starting on day 21, sensitized mice received 25 µg of i.n. OVA daily for 5 days, as described<sup>14</sup>. Because mice on the B6 background are insensitive to OVA<sup>3</sup>, we used CAA instead of OVA to immunize MMP2<sup>-/-</sup>, MMP2<sup>-/+</sup> or wild-type B6 mice every 4 days, as described<sup>5</sup>. Unless otherwise specified, mice were treated in an inhalation chamber with 100% oxygen for 10 min before and after i.n. CAA challenges. Because MMP2<sup>-/+</sup> and wild-type B6 mice showed identical responses (data not shown), data from wild-type mice was compared to that from MMP2<sup>-/-</sup> mice. Additional groups of mice were given 3.5 µg of i.n. rIL-13 twice daily for 3 days<sup>14</sup>.

### Analysis of the asthma phenotype

All data were collected 24 h after the final allergen challenge. AHR was measured based on the provocative concentration of acetylcholine that caused a 200% increase in lung resistance (PC<sub>200</sub>)<sup>3</sup>. Bronchoalveolar lavage cytology, OVA-specific and total IgE, lung histopathology and lung cytokine profiles obtained by ELISA were determined as described<sup>3</sup>. Mucin was quantified using the mucin-binding lectin Jacalin (Calbiochem, La Jolla, CA). Aliquots (40 µl) of BAL diluted 1:1000 and 1:10,000 were added to the individual wells of microtiter ELISA plates and incubated for 2 h at 37 °C. Plates were washed, blocked with 5% bovine serum albumin (BSA) and 0.002% biotinylated Jacalin added. After incubation for 1 h at 37 °C, plates were washed, then developed with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, Westgrove, PA) and nitrophenylphosphate (Sigma) and quantified by comparison with a mucin (Sigma) standard curve.

### Gelatin gel zymography

Zymography was used to detect the presence of MMP2 in BAL with standard protocols<sup>33</sup>. Briefly, 5 µl samples of BAL were added to nondenaturing loading buffer and separated in 10% SDS-polyacrylamide gels that contained 0.02% gelatin. 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 TrisCl at pH 8, 5 mM CaCl<sub>2</sub> and 0.02% NaN<sub>3</sub>) with or without 10 mM of 1'10-

phenanthroline (Sigma). Gelatin gels were then fixed and stained with 50% methanol and 10% acetic acid that contained 0.3% w/v Coomassie Blue. The optical density of clear bands was determined by densitometry with Quantity 1 software (Bio-Rad, Richmond, CA).

### RPA and quantitative PCR

Cytokine mRNA was measured using total RNA (10 µg) by RNase protection with a commercial kit (PharMingen, San Diego, CA). Briefly, mouse cytokine plasmid-1 (mCK-1, PharMingen) was used as a template to synthesize the antisense RNA probe and was labeled with [ $\alpha$ -<sup>32</sup>P]-UTP. A total of  $1 \times 10^6$  cpm of radiolabeled probe was used to hybridize to 15 µg of total RNA extracted from lungs. A two-step reverse-transcription PCR was used to determine the relative expression of mRNA with the Perkin Elmer Prism 5700 Sequence Detection System (Applied Biosystems, Norwalk, CT). Ten nanograms of cDNA per sample were generated from 1 µg of RNA; this was analyzed for the expression of IL-4, IL-13 and 18S rRNA with Taqman predeveloped assay reagents (Applied Biosystems). Relative quantities of 18S rRNA and IL-4 and IL-13 mRNA were calculated using the comparative threshold cycle number for each sample fitted to a five-point standard curve. The expression of mRNA for each sample was normalized to 18S rRNA. The samples were analyzed three times for the target genes, and the average of the readings was used to calculate the relative abundance of IL-4 and IL-13 mRNA in the tissue.

### Northern blot analysis and *in situ* hybridization

Northern blot analysis of total RNA isolated from lungs of mice after i.n. immunization with allergens, rIL-13 (Peprotech) or saline was done as described<sup>44</sup>. We used RNA probes prepared from the plasmid vector pSP65<sup>45</sup> for MMP2. The labeling of the probes and *in situ* hybridization on prepared paraffin sections of lung tissue were done as described<sup>46</sup>.

### Inflammatory cell egression

BAL samples were collected and cell counts done as described above. Immunized BALB/c mice ( $n=5$ ) received either PBS (vehicle) or 50, 150 or 250 mg/kg of the MMP inhibitor GM6001 intraperitoneally 1 h before i.n. OVA challenge. For each dose of GM6001, the BAL cell count was expressed relative to that of the vehicle-treated group according to the following formula: (total number of BAL inflammatory cells from GM6001- and OVA-challenged mice/total number of BAL inflammatory cells from PBS- and OVA-challenged mice) $\times 100$ .

### Peribronchovascular cell infiltration assay

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

### Survival experiment

CAA was given to MMP2<sup>-/-</sup> and wild-type mice every 4 days as described<sup>5</sup> without ( $n=10$ ) or with ( $n=20$ ) 100% oxygen, which was administered in an inhalation chamber for 10 min both before and after treatment. Mortality within 30 min of antigen challenge was recorded for each group and the percentage survival recorded with Kaplan-Meier coordinates. No additional mortality beyond the 30-min period was observed.

## Lung inflammatory cell extraction and chemotaxis assay

Inflammatory cells were extracted from lungs of sensitized wild-type or MMP2-deficient mice and used in chemotaxis assays. Briefly, BAL inflammatory cells were discarded by injecting into the lung and withdrawing, *via* the tracheal cannula, 1 cc aliquots of PBS for a total of 3 cc of BAL. The lung was then minced and pressed through a fine nylon mesh. The crude cell suspension was purified by lysing the red blood cells, which was followed by the selection of live inflammatory cells by differential centrifugation through 1-Step lymph-prep media (Accurate Chemical & Scientific Corporation, Westbury, NY). Viable inflammatory cells (macrophages, eosinophils, lymphocytes and neutrophils) were resuspended to a final working concentration of  $1 \times 10^6$  cell/ml in RPMI and were used in the chemotaxis assays. Inflammatory cell chemotaxis was measured by transfilter assays in 48-well chemotaxis chambers (Neuro Probe, Gaithersburg, MD) as described<sup>47,48</sup>. The chemotactic activity of the BAL fluid from wild-type or MMP2<sup>-/-</sup> mice ( $n=5$ ) was assessed against each of the lung inflammatory cell populations in duplicate. Serum-free media, BAL from wild-type and MMP2<sup>-/-</sup> mice, CCL7 (100 ng/ $\mu$ l) and CCL11 (100 ng/ $\mu$ l, both from R&D Systems, Minneapolis, MN) were placed in the lower wells of the chemotaxis chambers and separated from cell suspension in the upper wells. The total number of cells that migrated into the chamber filters was counted by microscopy and data were expressed as the total cells/filter.

## Chemokine concentration assay

Standard antibody-based ELISAs were used to measure concentrations of CCL7 (polyclonal mouse anti-CCL7), CCL11 (polyclonal mouse anti-CCL11) and CCL17 (monoclonal mouse anti-CCL17) in the BAL fluid of wild-type or MMP2<sup>-/-</sup> ( $n=5$ ) mice. All capture antibodies, their corresponding biotin-conjugated detection antibodies and recombinant proteins were from R&D Systems.

## Statistics

Data are mean $\pm$ s.e.m and are representative of at least three *in vivo* independent experiments that used four or five mice in each. Significant differences are expressed relative to saline-challenged control ( $*P \leq 0.05$ ) or CAA-challenged wild-type ( $**P \leq 0.05$ ) mice using Student's *t*-tests for the PC<sub>200</sub> values or Mann-Whitney U-tests for all other data. Cell migration was expressed as the mean $\pm$ s.e.m. of the numbers cell observed per ten high-power fields ( $\times 400$ ). Results were assessed for statistical significance with ANOVA and the Bonferroni multiple comparison tests.

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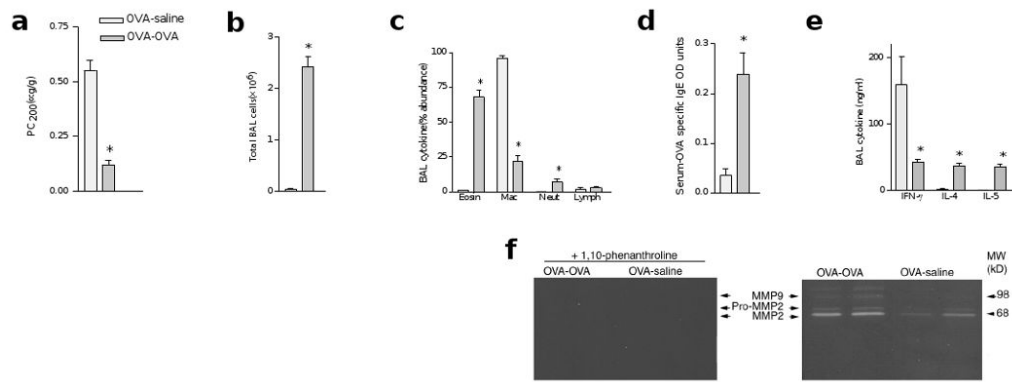
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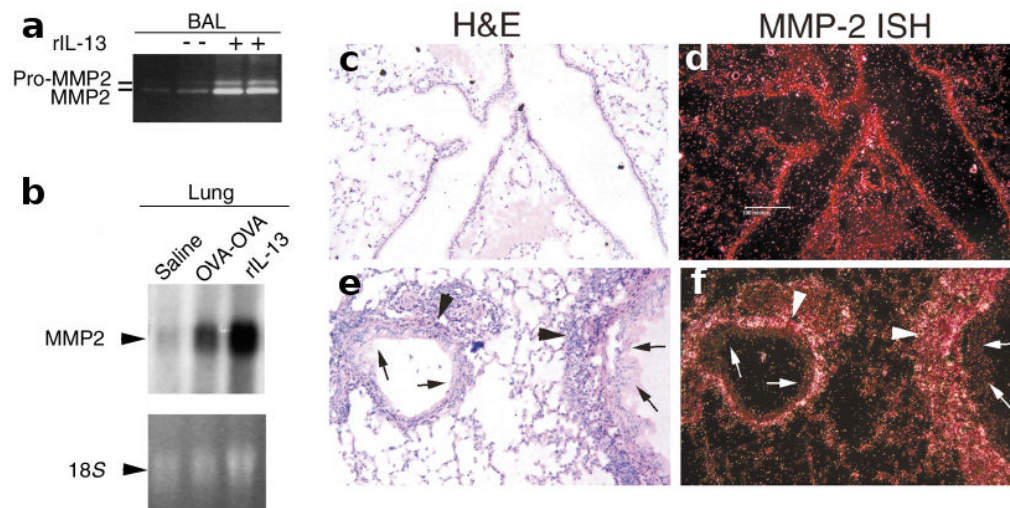
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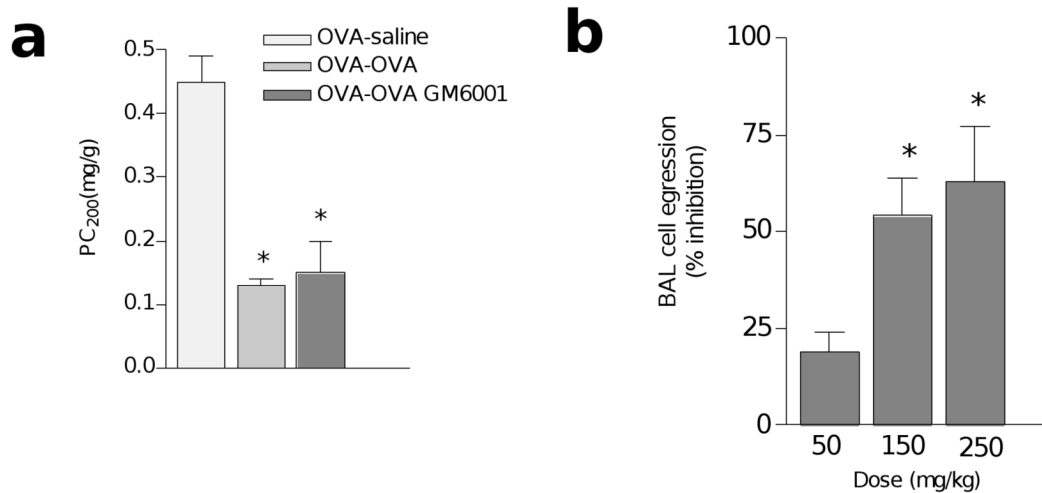
**Figure 1. OVA challenge of BALB/c mice induces a robust asthma phenotype and increased MMP2 activity in BAL**

Mice were immunized intraperitoneally with OVA-alum and subsequently challenged intranasally with either saline (OVA-saline) or OVA (OVA-OVA). **(a)** AHR, assessed as PC<sub>200</sub>. **(b)** Total number of cells recovered from BAL. **(c)** The abundance of eosinophils (Eosin), macrophages (Mac), neutrophils (Neut) and lymphocytes (Lymph) in BAL, as assessed by modified Giemsa staining. **(d)** Serum OVA-specific IgE, as determined by ELISA. **(e)** IFN- $\gamma$ , IL-4 and IL-5 concentrations in BAL fluid, as determined by ELISA. **(f)** Detection of BAL MMP activity by zymography. (Right) More active MMP2 (68 kD) and MMP9 (98 kD) were expressed in OVA-OVA-treated mice ( $n = 2$  mice for each condition). The higher molecular weight bands indicated the presence of relatively inactive (pro-MMP) zymogens (left). Protease activity was neutralized by the addition of 1,10-phenanthroline, a zinc chelator, which confirmed the identity of the MMPs.



**Figure 2. Lung MMP2 is expressed in the presence of allergic inflammation and IL-13**

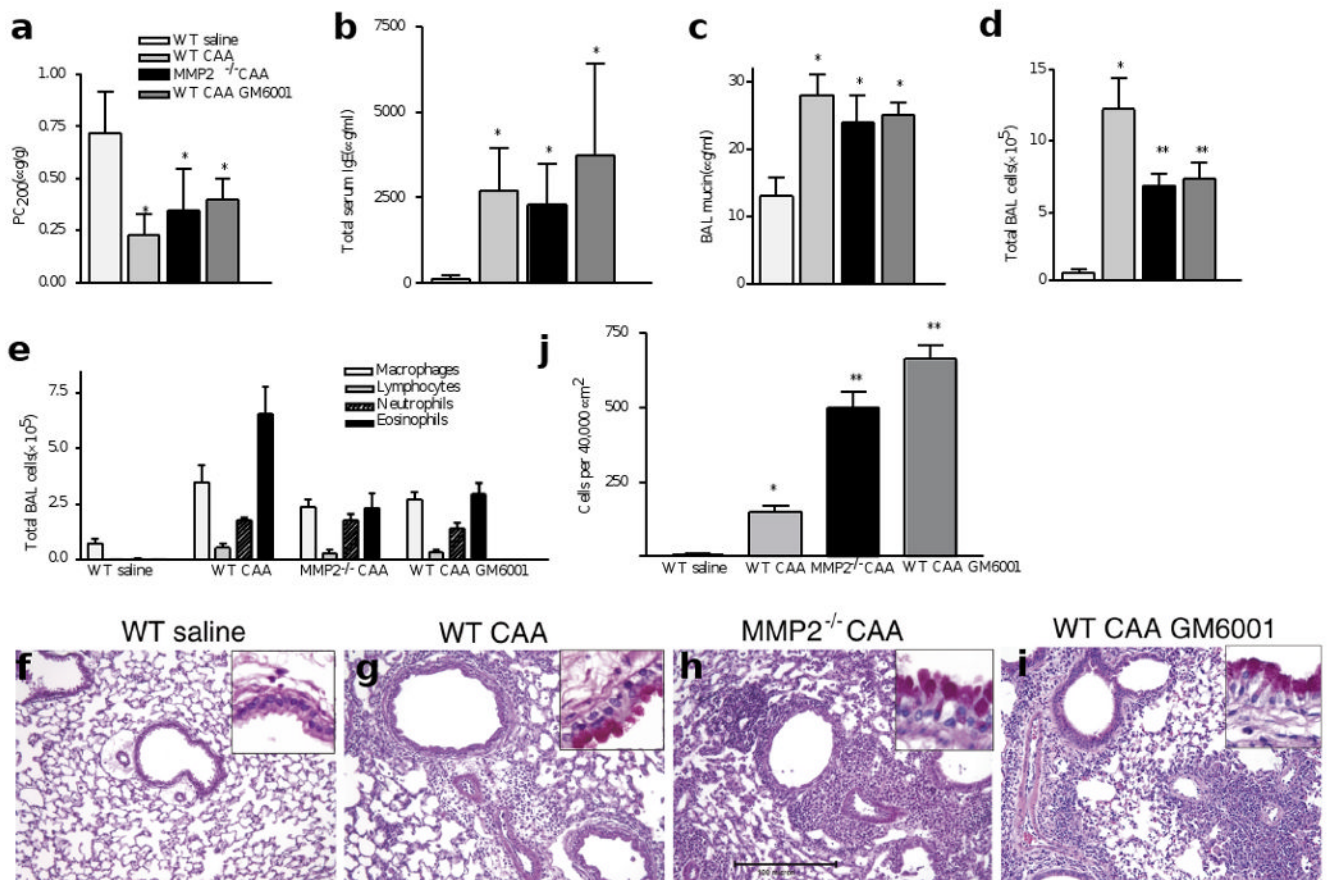
(a) Zymogram showing expression of active MMP2 and pro-MMP2 in BAL in response to i.n. challenge of mice with either saline (–) or rIL-13 (+). (b) Northern blot analysis of MMP2 expression. Total RNA from the lungs of BALB/c mice that were challenged with saline or were immunized and then challenged with either OVA (OVA-OVA) or rIL-13 was probed for MMP2 expression. The MMP2 band (3 kb) and relative loading of total RNA (as indicated by the 18S ribosomal RNA subunit band) are shown. (c–f) Lung MMP2 *in situ* hybridization (ISH). Airway tissue from (c,d) OVA-saline– or (e,f) OVA-OVA–challenged mice were fixed with formalin and stained with H&E; representative samples ( $n=3$ ) are shown. MMP2 mRNA was detected in contiguous sections with a radiolabeled antisense MMP2-specific probe. Arrowheads indicate bright ISH signals, which appear as dark grains in H&E-stained sections (c,e) or white grains in the darkfield views (d,f). Significant amounts of MMP2 mRNA were detected only in lung mesenchyme (arrowheads) that showed active allergic inflammation (e,f); it was largely absent from airway epithelium (arrows). Bar, 100  $\mu$ m.



**Figure 3. Effect of MMP inhibition on AHR and BAL cell egression**

BALB/c mice were immunized with OVA-alum and intranasally challenged with saline or OVA as in Fig. 1. A third immunized group was given the MMP inhibitor GM6001 (150 mg/kg) 1 h before i.n. challenge with OVA (OVA-OVA GM6001). **(a)** AHR, assessed as PC<sub>200</sub>. **(b)** Inhibition of cell egression into BAL relative to OVA-OVA-challenged mice (see Methods). At doses of 250 mg/kg, GM6001 inhibited BAL inflammatory cell recruitment by 65%. \* $P \leq 0.05$  relative to **(a)** saline or **(b)** 50 mg/kg of GM6001.

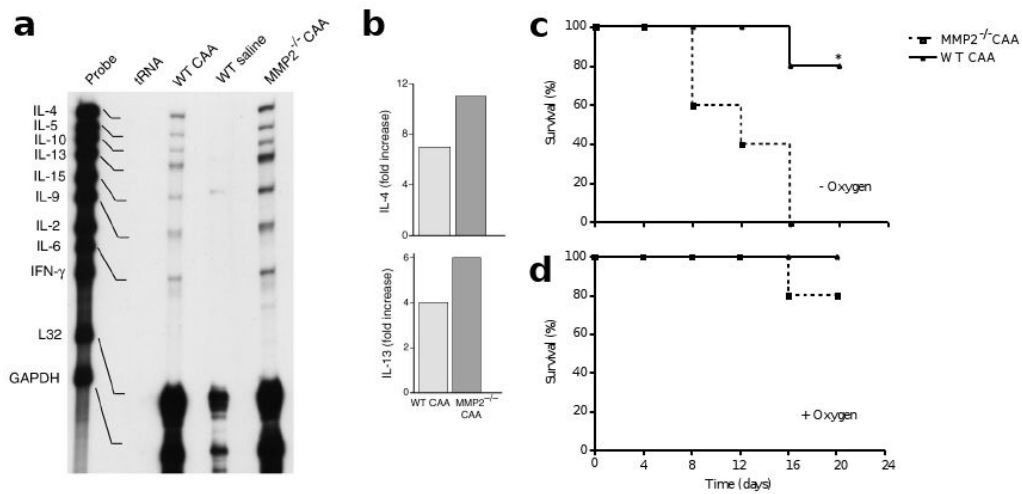




#### Figure 4. Comparison of GM6001 treatment to MMP2 deficiency

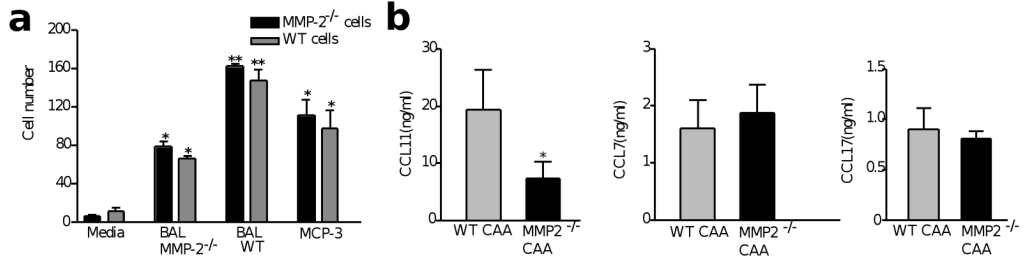
Wild-type and syngeneic MMP2<sup>-/-</sup> mice were challenged intranasally with saline or CAA and responses were compared to those of allergen-challenged WT mice treated with GM6001.

(a) AHR, assessed as PC<sub>200</sub>. (b) Total serum IgE, measured by ELISA. (c) Total BAL mucin. (d) Total BAL cells. (e) BAL cell differential, which indicates the total numbers of individual inflammatory cells. (f–i) Photomicrographs of representative lung bronchovascular bundles stained with H&E. The insets at the upper right depict higher magnification images of the airway epithelium stained with periodic acid-Schiff. (g–i) Abundant mucus-producing goblet cells (pink cytoplasm) are seen lining the airway epithelium of all mice receiving the CAA allergen. (h,i) Relative to the mice challenged with saline, peri-bronchovascular inflammatory cells were visible in the lungs of allergen-challenged mice but were more prominent in MMP2<sup>-/-</sup> and GM6001-treated animals. (j) This was confirmed by counting the cells within 40,000 μm<sup>2</sup> areas. \*P≤0.05 compared to WT saline. \*\*P≤0.05 compared to WT CAA. Bar, 100μm.



**Figure 5. MMP2<sup>-/-</sup> mice aberrantly accumulate TH<sub>2</sub> cytokine mRNA in the lung and show increased susceptibility to lethal asphyxiation**

(a) Total RNA was obtained from lungs of mice treated as in Fig. 4. Equal quantities of RNA (10 ng) were then analyzed for the indicated TH<sub>2</sub> cytokines and housekeeping genes (L32 and GAPDH) by RPA. (b) Specific mRNA species are indicated by dark bands that are shifted slightly downward in the gels relative to the gene probes. Relative to wild-type, all mRNA species were over-represented in CAA-challenged MMP2<sup>-/-</sup> mice, but IL-4 and IL-13 were disproportionately affected. This was confirmed by quantitative real-time PCR which indicates an increase in expression of IL-4 and IL-13 in the absence of MMP2. Mice were given i.n. CAA every 4 days in the (c) absence ( $n=10$ ) or (d) presence ( $n=20$ ) of 100% supplemental oxygen (given for 10 min before and after CAA challenge). Survival was assessed within 30 min of each antigen challenge. tRNA, transfer RNA (used as negative control). \* $P \leq 0.05$  compared to MMP2<sup>-/-</sup>.



**Figure 6. Aberrant chemotaxis of lung inflammatory cells in response to MMP2<sup>-/-</sup> BAL**  
**(a)** The migratory responses of lung inflammatory cells from CAA-challenged wild-type and MMP2<sup>-/-</sup> mice in response to RPMI (media), BAL from CAA-challenged MMP2<sup>-/-</sup> (BAL MMP2<sup>-/-</sup>) or wild-type (BAL WT) mice and recombinant MCP-3 were determined by transfilter assays. The total number of cells that migrated into the chamber filters are shown. Note the normal chemotactic response of MMP2<sup>-/-</sup> cells in response to wild-type BAL. \*P≤0.05 compared to the media control. \*\*P≤0.05 compared to MMP2<sup>-/-</sup> BAL. **(b)** Concentrations of CCL11, CCL7 and CCL17 in BAL from wild-type and MMP2<sup>-/-</sup> mice challenged with CAA, as measured by ELISA. \*P≤0.05 compared to wild-type mice.