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Matrix Metalloproteinase-9 Deficiency Results in Enhanced Allergen-Induced Airway Inflammation[1]

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Matrix metalloproteinases (MMPs) are a large family of endopeptidases that proteolytically degrade extracellular matrix. Many different cells produce MMP-9, and levels have been shown to be up-regulated in patients with allergic asthma. The aim of this study was to investigate the in vivo role of MMP-9 during allergen-induced airway inflammation. Acute allergic pulmonary eosinophilia was established in MMP-9 knockout (KO) and wild-type (WT) control mice by sensitization and challenge with OVA. Cell recruitment was significantly increased in both bronchoalveolar lavage (BAL) and lung tissue compartments in MMP-9 KO mice compared with WT mice. This heightened cell recruitment was primarily due to increased eosinophils and Th2 cells in the BAL and lung tissue of MMP-9 KO mice in comparison with WT controls. Moreover, levels of the Th2 cytokines, IL-4 and IL-13, and the chemokines eotaxin/CCL11 and macrophage-derived chemokine/CCL22 were substantially increased in MMP-9 KO mice compared with WT after OVA challenge. Resolution of eosinophilia was similar between MMP-9 KO and WT mice, but Th2 cells persisted in BAL and lungs of MMP-9 KO mice for longer than in WT mice. Our results indicate that MMP-9 is critically involved in the recruitment of eosinophils and Th2 cells to the lung following allergen challenge, and suggest that MMP-9 plays a role in the development of Th2 responses to allergen. ***The Journal of Immunology*****, 2004, 172: 2586–2594.**

One of the characteristic features of the asthmatic reaction is the accumulation of leukocytes within the lung (1). These leukocyte infiltrates are composed of a variety of different cells of the immune system, most commonly eosinophils and lymphocytes (2). Their accumulation in the lung necessitates their travel from the peripheral circulation, through the vascular endothelium, through the lung parenchymal areas, and ultimately, to the bronchial and bronchiolar spaces. This process involves the complex interplay of a series of molecules including adhesion molecules and chemokines (reviewed in Ref. 3).

Matrix metalloproteinases (MMPs)[3] are a large family of proteinases with functional and structural similarities. Their function is thought to be critical for multiple fundamental biological processes (4). MMPs are central to the regulation of extracellular matrix, and can cleave the majority of extracellular matrix proteins, including collagen and elastin. They can also cleave several circulating, cell surface, and pericellular proteins, enabling them to regulate cell behavior in multiple ways (4). These mechanisms include the alteration of cell-matrix and cell-cell interactions; release, activation, or inactivation of autocrine or paracrine signaling molecules; and the potential activation or inactivation of cell surface receptors (5). The ultimate effects of these mechanisms include digestion of structural proteins, as well as modification of cellular functions, including cell migration.

To minimize excessive tissue damage, the production and activation of MMPs is a tightly controlled and regulated process. Normal tissues do not store MMPs, and their constitutive expression is minimal (6). MMPs are transcriptionally regulated by multiple mediators, including growth factors, cytokines, and extracellular matrix components (5, 7). The MMPs are secreted as inactive proenzymes, and their proteolytic activity is regulated within tissues by zymogen activation and inhibition by enzymes such as the tissue inhibitors of metalloproteinases or by binding to plasma proteins such as α_2-macroglobulin (8, 9).

MMPs and their inhibitors are expressed during acute and chronic asthma, and are thought to contribute to the pathogenesis via their influence on the function and migration of inflammatory cells as well as matrix deposition and degradation. In particular, MMP-9 (gelatinase B) is a 92-kDa (103-kDa in mice) type IV collagenase that is present in low amounts in normal lung, but is up-regulated in a variety of inflammatory lung diseases, including asthma (10). Raised levels of MMP-9 have been shown in bronchoalveolar lavage (BAL) fluid, blood, and sputum from individuals with allergic asthma (11–13). In addition, expression of MMP-9 is enhanced when patients have spontaneous exacerbations or in response to local instillation of allergen in the airway (14). As acute inflammation resolves, MMP-9 levels return toward baseline. Corticosteroids have been shown to down-regulate MMP expression and to enhance the levels of tissue inhibitors of metalloproteinases (15). MMP-9 is expressed by many inflammatory leukocytes, as well as multiple lung resident cells, such as bronchial epithelial cells, endothelial cells, smooth muscle cells, and fibroblasts after stimulation (reviewed in Ref. 10). MMP-9 could

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[3] Abbreviations used in this paper: MMP, matrix metalloproteinase; AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; KO, knockout; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIP, macrophage-inflammatory protein; WT, wild type.

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potentially promote the movement of cells across basement membranes due to its ability to cleave collagen IV (16, 17). Even though it is clear that enhanced airway inflammation in asthma is associated with increased expression of MMPs, whether specific inhibitors of MMP function could reduce airway injury and facilitate orderly healing in asthma is still unknown.

Although the expression of MMP-9 in airway inflammation has been documented, its role in the immune process is less clear. Inhibition of MMPs in a mouse model of allergic inflammation reduced inflammation; however, the study used a broad spectrum inhibitor of MMP function, so the specific contribution of MMP-9 could not be assessed (18). In contrast, we have determined the contribution of MMP-9 to the development of pulmonary inflammation and airway hyperreactivity by using MMP-9-deficient mice and a well-characterized mouse model of allergic inflammation encompassing the salient features of the human disease. Allergen challenge to the airways of sensitized MMP-9-deficient mice resulted in heightened airway inflammation in comparison with wild-type (WT) controls. These data suggest that MMP-9 plays an important role in cell migration through the lung after allergen challenge.

Materials and Methods

Mice

Mice deficient in MMP-9 and WT controls were generated, as described previously (19). MMP-9 knockout (KO) mice on a 129SvEvC57B6 background were bred in Imperial College (London, U.K.) animal facility and used at 6–8 wk of age (25–30 g).

Induction of allergic airway disease

Groups of MMP-9-deficient mice and WT control mice were sensitized using OVA (Sigma-Aldrich, Poole, U.K.) at a concentration of 0.01 mg/mouse in 0.2 ml of alum (Au-Gel-S; Serva Electrophoresis, Heidelberg, Germany) i.p. on days 0 and 12. Control mice received the same volume of PBS in alum. All groups of mice were challenged daily with 5% OVA (aerosolized for 20 min) via the airways between days 18 and 23. Mice were sacrificed by exsanguination under terminal anesthesia at 24 h after the final OVA challenge, and the following parameters were analyzed: airway hyperreactivity, pulmonary inflammation, IgE production, Th2 cytokine and chemokine production, and lymph node proliferation. To examine the kinetics of resolution of inflammation, another group of mice was sensitized and challenged in the same way, but were sacrificed 6 days after the final OVA challenge.

Airway hyperreactivity (AHR)

Airway responsiveness was measured in mice 24 h after the final OVA challenge by recording respiratory pressure curves by whole body plethysmography (Buxco Technologies, Troy, NY) in response to inhaled methacholine (Sigma-Aldrich) at concentrations of 3–100 mg/ml for 1 min, as described previously (20). Results are shown for Penh after allergen challenge in WT mice and MMP-9 KO mice sensitized to PBS/alum and OVA/alum.

Cell recovery

Airway lumen. BAL was performed, as described (21). Briefly, the airways of the mice were lavaged three times with 0.4 ml of PBS via a tracheal cannula. BAL fluid was centrifuged (700 × *g*, 5 min, 4°C); cells were counted, then pelleted onto glass slides by cytocentrifugation (5×10^4 cells/slide). Differential cell counts were performed on Giemsa (Shandon, Runcorn, U.K.)-stained cytospins. Percentages of eosinophils, lymphocytes/monocytes, neutrophils, and macrophages were determined by counting their number in eight high power fields (×40 magnification; total area 0.5 mm^2 per area) randomly selected, and then dividing this number by the total number of cells per high power field. To obtain absolute numbers of each leukocyte subtype, these percentages were multiplied by the total number of cells obtained in the lavage fluid. All differential counts were performed blind and in a randomized order at the end of the study by the same observer.

Lung parenchyma. To disaggregate the cells from the lung tissue, one lobe (100 mg) of lung was incubated (37°C) for 1 h in digest reagent: 30 mg/ml collagenase (type D; Boehringer Mannheim, Lewes, U.K.), 5 mg/ml DNase (type 1; Boehringer Mannheim, Lewes, U.K.), 100 U/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, Paisley, U.K.) in RPMI 1640/10% FCS. The recovered cells were filtered through a 70-μm nylon sieve (Falcon; Marathon Lab Supplies, London, U.K.), washed twice, resuspended in RPMI 1640/10% FCS, and counted in a hemocytometer. Cytocentrifuge preparations were prepared and Giemsa stained, and differential counts were performed as for BAL.

Staining of BAL and lung cells for flow cytometric analysis

Suspensions of BAL and lung tissue cells were stained in PBS containing 1% FCS and 0.01% sodium azide. To reduce nonspecific binding, cells were incubated with rabbit serum (Sigma-Aldrich) for 15 min before staining. The following Abs were used: anti-mouse CD3e (PerCP conjugated), anti-mouse CD4 (allophycocyanin conjugated), anti-mouse CD8 (FITC conjugated), anti-mouse T1/ST2 (FITC conjugated), and anti-TIM-3. All Abs were purchased from BD PharMingen (Oxford, U.K.) apart from anti-T1/ST2, which was purchased from Morwell Diagnostics (Zurich, Switzerland). Flow cytometric analysis was performed using a FACSCalibur (BD PharMingen, Oxford, U.K.).

Histology

Lung sections from the different experimental groups of mice were prepared and analyzed, as described (22). Briefly, lungs were fixed in 10% neutral buffered Formalin and paraffin embedded, and sections (4 μm) were stained with H&E, according to standard protocols.

Ex vivo culture of peribronchial lymph node cells

Peribronchial lymph nodes were recovered from mice, and single cell suspensions were prepared. Lymph node cells were cultured in triplicate at 5×10^5/well in flat-bottom 96-well plates in the presence of medium alone (RPMI 1640/10% FCS), 50 μg/ml OVA, or 5 μg/ml Con A for 72 h. Supernatants were then harvested for cytokine analysis, and the cells were incubated for an additional 18 h in the presence of 1 μCi/well [^{3}H]thymidine (ICN Pharmaceuticals, Costa Mesa, CA) to quantify cell proliferation. [^{3}H]Thymidine incorporation data in cpm were converted to stimulation index (fold increase = cpm with OVA/cpm of medium-only condition).

Cytokine analysis

Cytokines were analyzed in BAL samples and lung tissue homogenate supernatants and lung-draining lymph node cell culture supernatants. Lung tissue (100 mg) was homogenized in 2 ml of HBSS and centrifuged (800 × *g*, 10 min), and the supernatant was collected. Paired Abs for murine IFN-γ and IL-4 (BD PharMingen), IL-5 (Endogen, Buckingham, U.K.), and eotaxin/CCL11 (R&D Systems, Abingdon, U.K.) were used in standardized sandwich ELISAs, according to the manufacturer's protocol. Macrophage-derived chemokine (MDC) levels were measured using a sandwich ELISA generated by coating ELISA plates with anti-mouse MDC (a gift from ICOS, Bothell, WA) and detecting bound Ab with biotinylated anti-mouse MDC (R&D Systems) against a standard curve generated using rMDC (R&D Systems). ELISA kits to measure IL-13, monocyte chemoattractant protein-1 (MCP-1)/CCL2, macrophage-inflammatory protein-1α (MIP-1α)/CCL3, and RANTES/CCL5 were purchased from R&D Systems.

IgE

Levels of total IgE were measured in serum by ELISA using paired Abs, according to the manufacturer's instructions (BD PharMingen). Levels of anti-OVA IgE were measured in serum by ELISA, as described previously (22).

Data analysis

Data are expressed as mean ± SEM. Statistical significance was tested using a Kruskal Wallis test for differences between groups and a Mann-Whitney *U* test to determine significance between WT and KO groups after OVA challenge. Significance was accepted when $p < 0.05$.

Results

Airway hyperreactivity after OVA challenge in MMP-9-deficient mice

Airway hyperreactivity is a characteristic feature of the pulmonary allergic response, and has been associated with recruitment of inflammatory cells to the lung. To determine whether deficiency in MMP-9 has a direct effect on the development of airway dysfunction, AHR was measured by whole body plethysmography 24 h

following the final serial OVA challenge in MMP-9 KO and WT mice. Fig. 1 shows that there was no difference in the response to methacholine in unsensitized (alum/PBS) WT mice or MMP-9 KO mice. Both WT and MMP-9 KO mice showed increased Penh following allergen challenge. A significant increase in Penh was observed in sensitized and challenged MMP-9 KO mice compared with WT controls, at a dose of 30 mg/ml methacholine.

Absence of MMP-9 results in increased leukocyte recruitment to the airways after allergen challenge

Recruitment of inflammatory leukocytes to the lung occurs as a consequence of airway challenge with allergen in sensitized mice. We analyzed cell recruitment to the airway lumen by performing BAL and in the lung tissue by recovering leukocytes from lung tissues by collagenase digestion. The number and phenotype of infiltrating leukocytes were then determined. There were significantly more cells in both the airway lumen (Fig. 2*A*) and the lung tissue (Fig. 2*B*) of MMP-9 KO mice compared with WT mice. Differential cell counts revealed that BAL and lung tissue eosinophil numbers were significantly higher in OVA-treated MMP-9 KO mice in comparison with WT mice. Similarly, numbers of lymphocytes, monocytes, and macrophages were also increased in the BAL and lungs of MMP-9 KO mice after challenge. There was no difference in neutrophil recruitment to either compartment.

Pulmonary inflammation is enhanced in the absence of MMP-9

Pulmonary inflammation after allergen challenge is characterized by peribronchiolar and perivascular inflammatory cell infiltrates. To investigate the extent and anatomical localization of pulmonary inflammation, we examined sections of lung isolated from WT and MMP-9 KO mice 24 h after the final serial allergen challenge. Challenge with OVA induced characteristic widespread peribronchiolar and perivascular inflammation in comparison with alum-treated mice, which was primarily eosinophilic in nature (Fig. 3). However, MMP-9 KO mice had significantly enhanced inflammation when compared with WT mice, reflecting the increased cell counts documented in Fig. 2. No differences in lung histology were observed between alum-treated WT and MMP-9 KO mice.

Pulmonary Th2 cytokine secretion is increased in the absence of MMP-9

Production of cytokines such as IL-4, IL-5, and IL-13 is a feature of a robust Th2 response and is characteristic of allergic pulmonary reactions. Levels of Th2-type cytokines were measured in lung homogenates and BAL fluid in WT and MMP-9 KO mice after allergen challenge. Levels of IL-4 and IL-13 were significantly increased in the MMP-9 KO mice compared with the WT

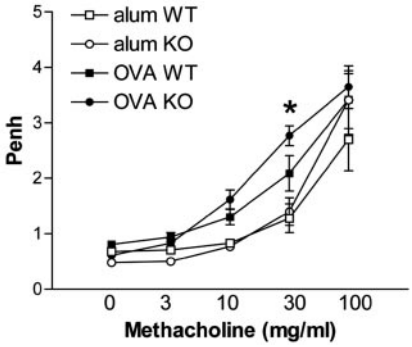

FIGURE 1. Effect of MMP-9 deficiency on airway hyperreactivity. AHR was measured 24 h after the final OVA challenge using a Buxco system in which mice were exposed to increasing concentrations of methacholine (3–100 mg/ml). Values are expressed as mean ± SEM, $n = 8$–20/group in three separate experiments, and ∗, $p = 0.05$ in comparison with WT OVA-treated mice.

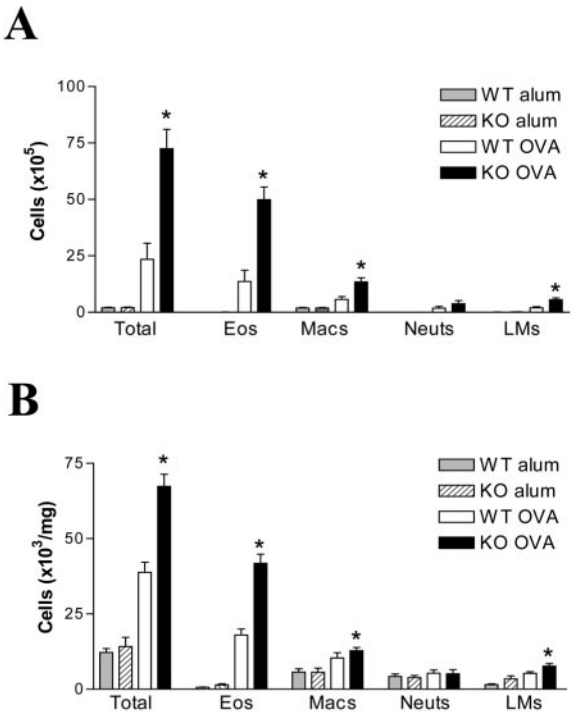

FIGURE 2. Effect of MMP-9 deficiency on airway inflammation. Differential cell counts in BAL (*A*) and lung tissue digest (*B*) from WT alum control mice and OVA-sensitized mice and MMP-9 KO alum-treated mice and OVA-sensitized MMP-9 KO mice. Mice were sacrificed 24 h after the final OVA challenge, and BAL and lung tissue digest cells were isolated, as described in *Materials and Methods*. Values are expressed as mean ± SEM, $n = 8$–18/group from three separate experiments, and ∗, $p < 0.05$ in comparison with WT OVA-treated mice.

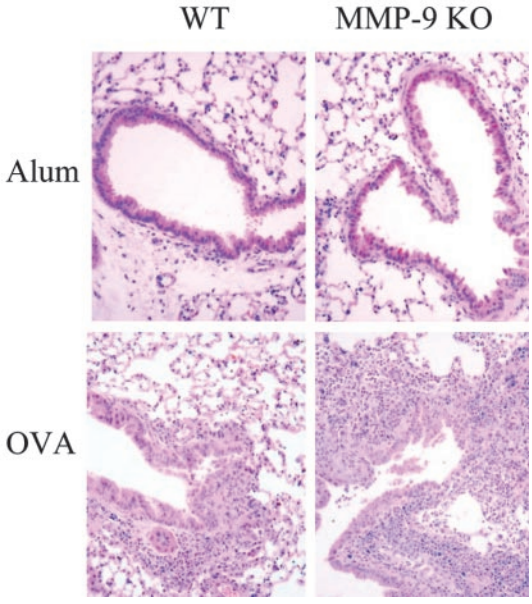

FIGURE 3. OVA-induced lung inflammation in MMP-9 KO and WT mice. Representative photomicrographs of H&E-stained lung sections from WT alum control mice and OVA-sensitized mice and MMP-9 KO alum-treated mice and OVA-sensitized MMP-9 KO mice are shown (original magnification ×10).

mice, in both BAL and lung tissue compartments (Fig. 4, *A* and *C*). MMP-9 KO mice also showed increased IL-5 levels in the BAL after challenge. Interestingly, the MMP-9 KO mice had increased lung IL-5 levels even before challenge with OVA (Fig. 4*B*), although after OVA challenge KO mice had significantly more IL-5 in their lungs than WT mice. In contrast, there was reduced secretion of the Th1-type cytokine IFN-γ in the BAL of MMP-9 KO mice. There was no difference between IFN-γ levels in lung homogenates of any of the groups, before or after OVA challenge.

Effect of MMP-9 deficiency on the development of humoral immune responses

Elevated levels of IgE have been reported to be important in the development of an allergic pulmonary response. We measured total IgE and OVA-specific IgE in sera from MMP-9 KO and WT mice following allergen challenge. Both groups of mice showed enhanced production of total and Ag-specific IgE following allergen challenge (Fig. 5). MMP-9 KO mice had raised levels of both total and OVA-specific IgE, although only levels of OVA-specific IgE were significantly elevated compared with WT mice.

Resolution of inflammation in the absence of MMP-9

To investigate the potential role of MMP-9 in the resolution of OVA-induced airway inflammation, we examined mice on day 30, 6 days after the final allergen challenge. By day 30, levels of Penh in OVA-challenged mice from both MMP-9 KO and WT groups had declined such that there was no longer a significant difference between OVA-challenged and control mice (data not shown). Similarly, levels of eosinophil numbers had decreased in both MMP-9 KO and WT mice by day 30.

To investigate the mechanism for increased cell recruitment to the lung in the absence of MMP-9, levels of chemokines thought to be involved in the pulmonary allergic response, eotaxin/CCL11, RANTES/CCL5, MCP-1/CCL2, and MIP-1α/CCL2, were measured in lung homogenate supernatants (23). MMP-9 KO mice produced significantly more eotaxin/CCL11 in both the lavage and lung tissue in comparison with WT mice following allergen challenge (Fig. 6, *C* and *D*). In contrast, there was no significant difference in levels of MCP-1/CCL2, MIP-1α/CCL3, and RANTES/CCL5 between MMP-9 KO and WT mice following OVA

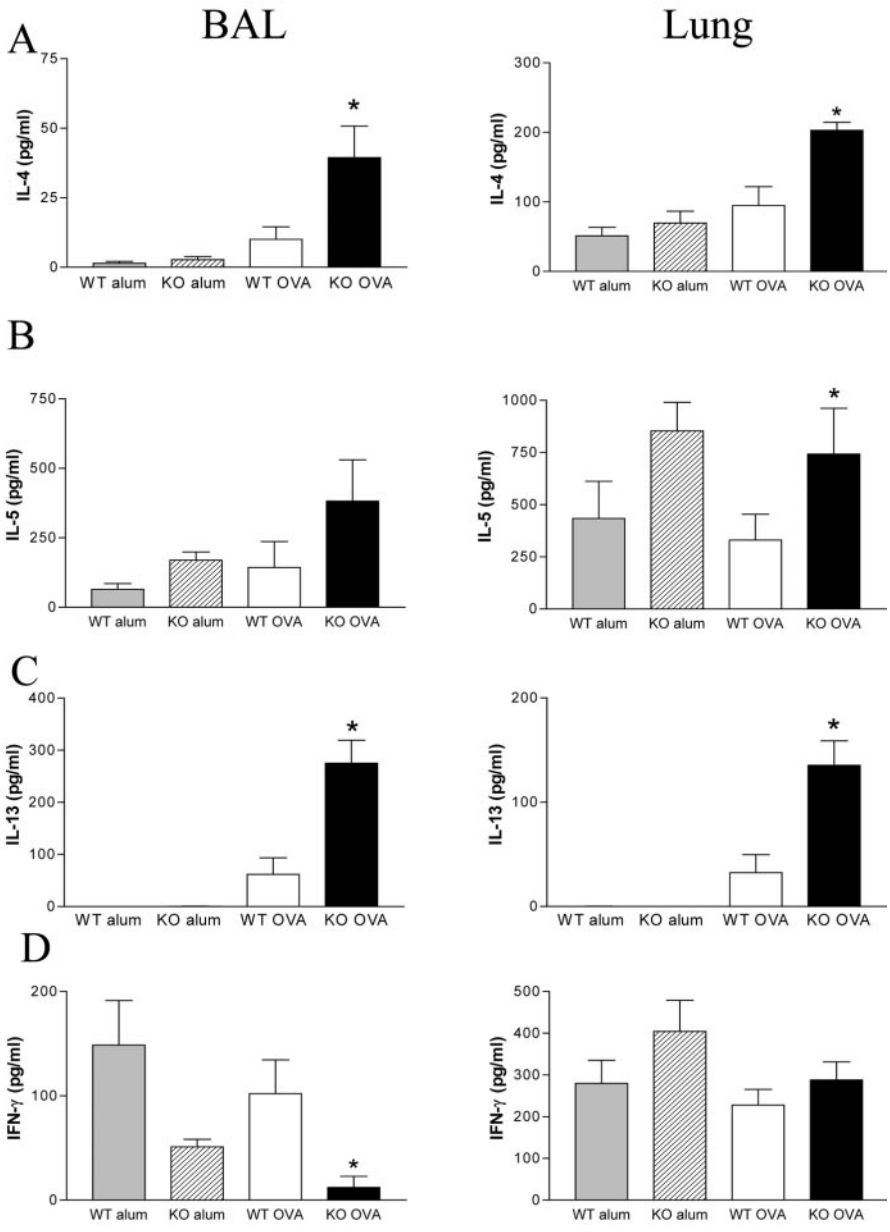

FIGURE 4. Effect of MMP-9 deficiency on cytokine production. IL-4 (*A*), IL-5 (*B*), IL-13 (*C*), and IFN-γ (*D*) levels were measured in BAL fluid (*left panel*) and lung homogenate supernatant (*right panel*) by ELISA in samples from WT alum mice (stippled bars), MMP-9 KO alum mice (▨), OVA-treated WT mice (□), and OVA-treated MMP-9 KO mice (■) sacrificed 24 h after the final OVA challenge. Data are expressed as mean ± SEM, $n = 4$–12/group from three separate experiments, and $*$, $p < 0.05$ in comparison with WT OVA-treated mice.

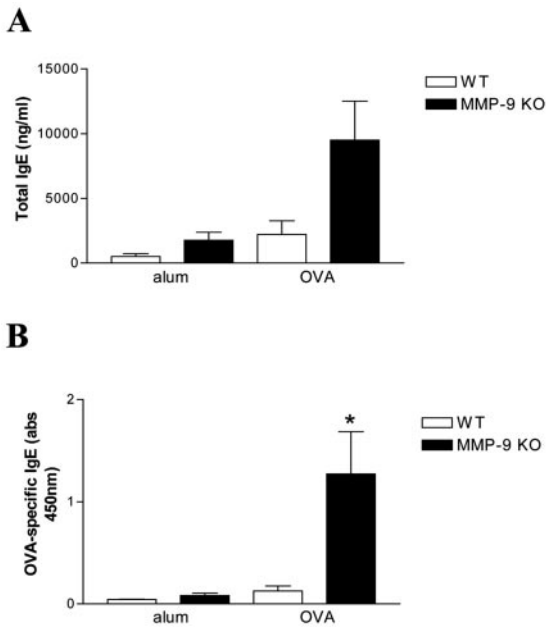

FIGURE 5. Effect of MMP-9 deficiency on serum IgE levels. Mice were sacrificed 24 h after the final OVA challenge, bled via cardiac puncture, serum collected, and analyzed by ELISA for total IgE (*A*) and OVA-specific IgE (*B*). Data are expressed as mean ± SEM, $n = 4–6$/group from two separate experiments, and ∗, $p < 0.05$ in comparison with WT OVA-treated mice.

challenge (data not shown). We examined levels of eotaxin in MMP-9 KO and WT mice on day 30 and found that eotaxin levels remained significantly increased in both the BAL and lung homogenate from MMP-9 KO mice, but had declined to baseline values in WT mice (Fig. 6, *C* and *D*).

Numbers of Th2 cells were also determined in lungs from MMP-9 KO and WT mice, by staining for the Th2 surface marker T1/ST2. Flow cytometric analysis of leukocytes isolated from airways of allergen-stimulated mice showed that on day 24 numbers of $CD4^+T1/ST2^+$ cells were significantly increased in BAL from MMP-9 KO mice compared with WT mice, whereas numbers were comparable between WT and MMP-9 KO mice in the lung tissue digest (Fig. 7, *A* and *B*). By day 30, Th2 cell numbers had decreased in BAL and lungs of WT mice, but numbers remained significantly raised in both BAL and lung tissue compartments in MMP-9 KO mice (Fig. 7, *A* and *B*). We also determined levels of the Th2 chemokine MDC/CCL22 in BAL and lung at both time points from each group of mice. BAL levels of MDC/CCL22 were significantly enhanced in OVA-treated MMP-9 KO mice in comparison with WT mice on day 24, but levels were comparable between OVA-treated MMP-9 KO and WT mice in the lung (Fig. 7, *C* and *D*). On day 30, MDC/CCL22 levels remained increased in MMP-9 KO mice in both BAL and lung tissue digest, although they had declined to baseline levels in WT mice.

Role of MMP-9 in allergen-specific Th2 responses

To compare allergen-specific T cell responses between MMP-9 KO and WT mice, lymphocytes were isolated from the draining lymph nodes of the lung, and proliferative responses to allergen, as well as allergen-stimulated cytokine production were measured. Proliferation to OVA was significantly enhanced in MMP-9 KO mice compared with WT mice on both days 24 and 30, although levels only reached significance on day 24 (Fig. 8*A*). In contrast, responses to Con A were not significantly different between any group at any time point (data not shown). Similarly, OVA-induced production of IL-4 was enhanced in MMP-9 KO mice compared with WT mice on days 24 and 30, although levels only reached significance on day 24 (Fig. 8*B*). On day 24, levels of IFN-γ were significantly increased in MMP-9 KO mice treated with alum in

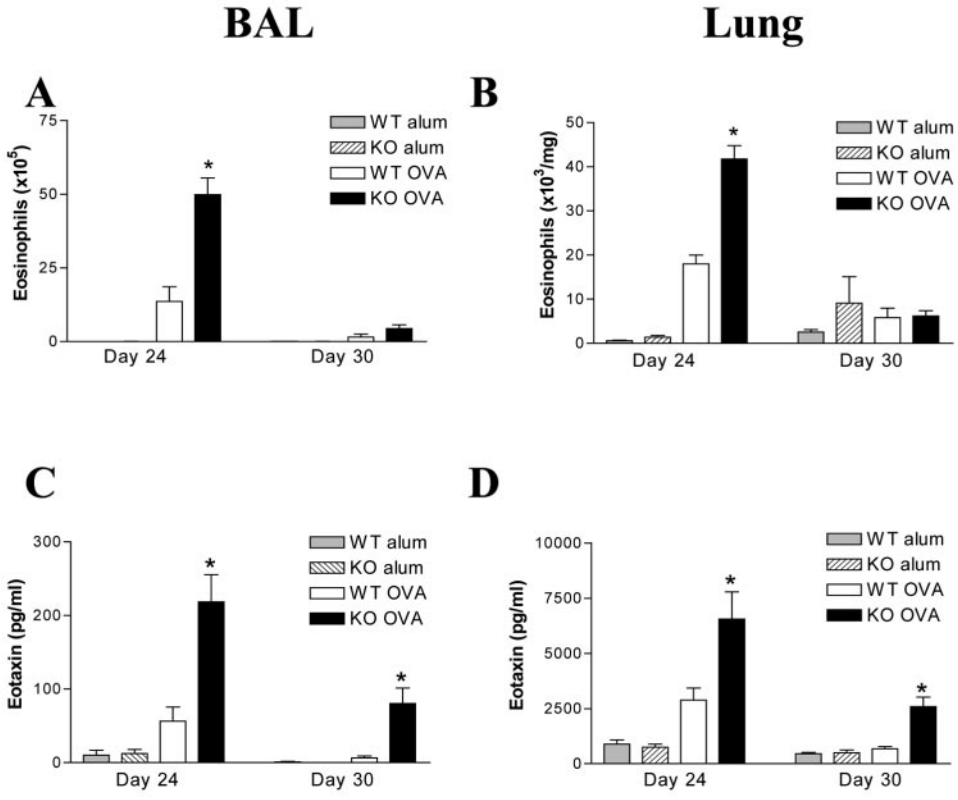

FIGURE 6. Effect of MMP-9 deficiency on resolution of airway eosinophilia and eotaxin/CCL11 production. *A* and *B*, Eosinophil cell counts were performed in BAL (*A*) and lung tissue digest (*B*) from WT alum control mice and OVA-sensitized mice and MMP-9 KO alum-treated mice and OVA-sensitized MMP-9 KO mice. Mice were sacrificed 24 h after the final OVA challenge (day 24) and 6 days later on day 30, and BAL and lung tissue digest cells were isolated, as described in *Materials and Methods*. Values are expressed as mean ± SEM, $n = 8–18$/group from three separate experiments, and ∗, $p < 0.05$ in comparison with WT OVA-treated mice. *C* and *D*, Levels of eotaxin/CCL11 were measured in BAL fluid (*C*) and lung homogenate supernatant (*D*) by ELISA in samples from WT alum control mice and OVA-sensitized mice and MMP-9 KO alum-treated mice and OVA-sensitized MMP-9 KO mice sacrificed 24 h after the final OVA challenge (day 24) and 6 days later on day 30. Data are expressed as mean ± SEM, $n = 7–12$/group, and ∗, $p < 0.05$ in comparison with WT OVA-treated mice.

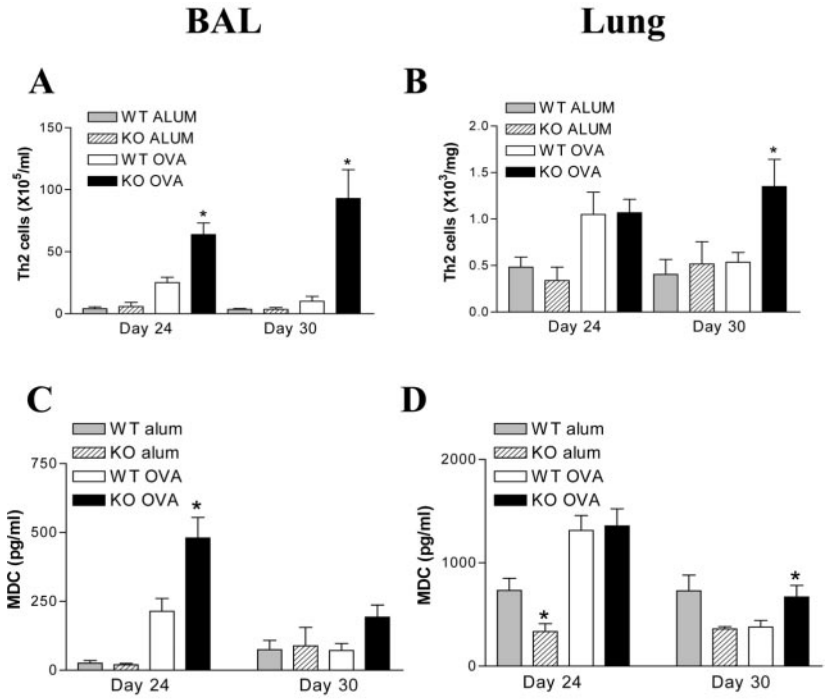

FIGURE 7. Effect of MMP-9 deficiency on Th2 cell recruitment to the airway. *A* and *B*, CD4/T1ST2-positive cells were counted in the BAL (*A*) and lung tissue digest (*B*) by flow cytometric analysis. Mice were sacrificed 24 h after the final OVA challenge (day 24) and 6 days later on day 30, and BAL and lung tissue digest cells were isolated and stained with Abs for flow cytometric analysis, as described in *Materials and Methods*. Values are expressed as mean ± SEM, $n = 4–7$/group, and *, $p < 0.05$ in comparison with WT OVA-treated mice at each time point. *C* and *D*, Levels of MDC/CCL22 were measured in the BAL (*C*) and lung tissue homogenate supernatant (*D*) by ELISA from mice sacrificed 24 h after the final OVA challenge (day 24) and 6 days later on day 30. Data are expressed as mean ± SEM, $n = 4–7$/group, and *, $p < 0.05$ in comparison with WT OVA-treated mice.

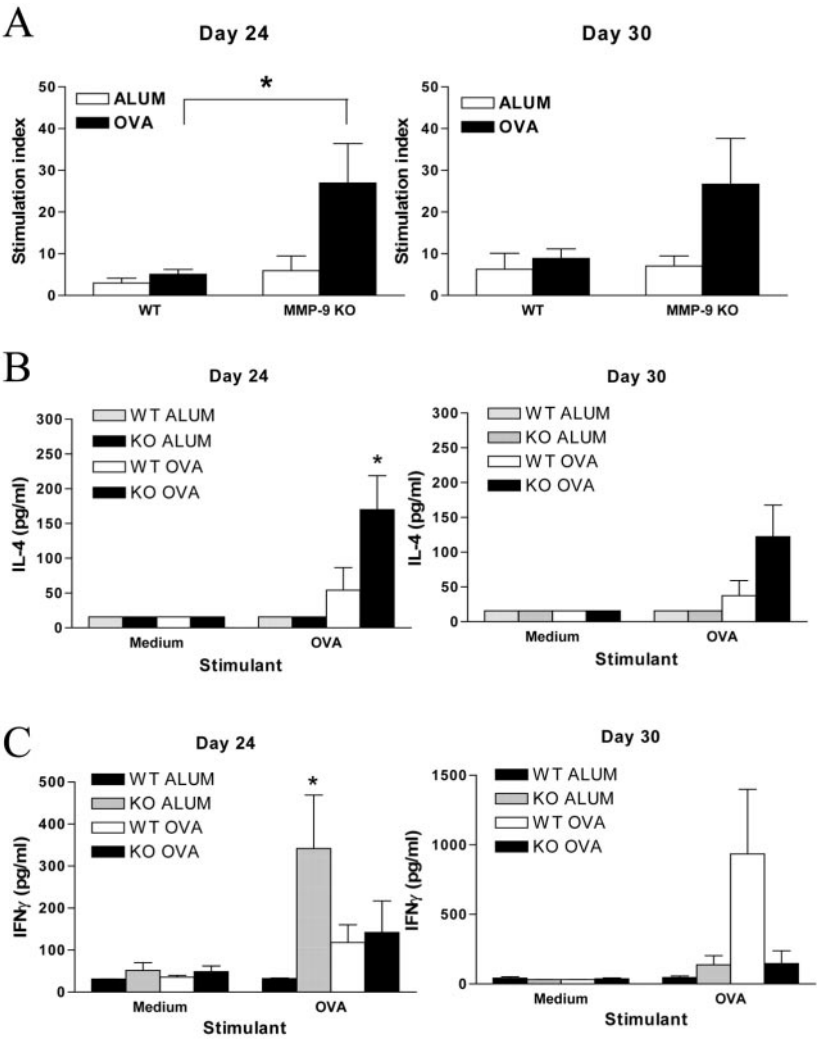

FIGURE 8. Effect of MMP-9 deficiency on allergen-driven T cell responses in the lymph node. Peribronchial lymph nodes were recovered from mice, and single cell suspensions were cultured in triplicate at 5×10^5/well in the presence of medium alone (RPMI 1640/10% FCS) or 50 μg/ml OVA for 72 h from mice sacrificed 24 h after the final OVA challenge (day 24) and 6 days later on day 30. *A*, Cells were incubated for an additional 18 h in the presence of 1 μCi/well [^{3}H]thymidine to quantify cell proliferation. Data are expressed as mean ± SEM, $n = 4–7$ mice/group, and *, $p < 0.05$ in comparison with WT OVA-treated mice. *B* and *C*, Supernatants were harvested for IL-4 (*B*) and IFN-γ (*C*) analysis from mice sacrificed 24 h after the final OVA challenge (day 24) and 6 days later on day 30. Values are expressed as mean ± SEM, $n = 4–7$ mice/group, and *, $p < 0.05$ in comparison with WT OVA-treated mice (IL-4) and WT alum control mice (IFN-γ).

comparison with WT alum mice (Fig. 8*C*). In contrast, no difference was observed in MMP-9 KO and WT mice following OVA challenge. On day 30, ex vivo OVA-induced IFN-γ secretion was only observed in lymphocytes isolated from allergen-challenged MMP-9 WT mice, not MMP-9 KO mice (Fig. 8*C*).

Discussion

MMPs are zinc-dependent endoproteinases with extracellular matrix-degrading activity. Although this function is likely to be important for tissue repair and remodeling, there is a growing body of evidence to suggest that MMPs are important for proteolysis and processing of both cell surface and secreted factors, which alters the biological activity of these substrates (4). One particular function most likely to be affected is the recruitment of cells across endothelial basement membranes. We postulated that MMP-9 might be important for the development of allergic airway inflammation because migration of large numbers of leukocytes is a characteristic pathophysiological feature of this disease. We used KO mice to assess the functional importance of MMP-9 in the development of pulmonary inflammation because it is well documented that levels of MMP-9 are elevated in the blood, BAL fluid, and sputum of allergic asthmatics (11–13). Surprisingly, we found that airway inflammation was significantly heightened in MMP-9 KO mice compared with WT controls. Increased cell accumulation was also associated with enhanced levels of Th2 cytokines, IL-4, IL-5, and IL-13, which are commonly up-regulated following allergen challenge. Moreover, levels of the proeosinophilic chemokine eotaxin/CCL11 and the Th2 chemokine MDC/CCL22 were significantly increased in the MMP-9 KO mice in comparison with WT controls. Allergen-specific Th2 responses were also enhanced in MMP-9 KO mice, with increased BAL Th2 recruitment, lymph node IL-4 production, and T cell proliferation. Moreover, evidence suggested that inflammation persisted longer in the MMP-9 KO mice than in the WT mice after allergen challenge. Our data imply that MMP-9 deficiency influences leukocyte accumulation in the lung either by promoting increased migration of cells, or by reduced resolution of inflammation following allergen challenge.

The MMP family has several points of interaction with the cytokine network. Inflammatory cytokines and growth factors, such as IL-13, can regulate MMP expression (24), but the reverse is also true in that cytokines and their receptors can be substrates for MMP action. In this way, MMPs could influence the progression of inflammation by affecting the function of mediators such as cytokines and chemokines. Indeed, significantly increased production of eotaxin/CCL11 and MDC/CCL22 was observed in MMP-9 KO mice in comparison with WT mice. This effect was specific to eotaxin and MDC, as no increase in levels of other chemokines important during allergen-induced airway inflammation, MCP-1/CCL2, MIP-1α/CCL3, and RANTES/CCL5 was observed (data not shown). It is also possible that MMP-9 has a direct effect on Th2 cytokine generation, and because both IL-4 and IL-13 directly contribute to generation of eotaxin and MDC this would promote recruitment of eosinophils and Th2 cells (25, 26), while increased IL-5 would enhance eosinophil survival (27). Recently, it has been demonstrated that IL-5 can down-modulate its receptor, thereby limiting IL-5-dependent inflammatory conditions. Moreover, this down-modulation could be partially reversed through MMP inhibitors, BB-94 and GM6001, suggesting contribution from an MMP-dependent pathway (28).

MMPs have also been shown to modulate chemokine responses. Thus, MMP-9-induced cleavage of IL-8 enhances the neutrophilic chemotactic activity of IL-8 10-fold (29). In contrast, recent work has suggested that proteolysis of chemokines may provide a mechanism by which chemokine signaling is dampened (30). Several chemokines contain sequences that allow for cleavage by MMPs. For example, MCP-3/CCL7 is a physiological substrate for MMP-2, and studies have shown that cleaved MCP-3/CCL7 binds its cognate receptors, but no longer promotes chemotaxis and instead acts as a general CC chemokine receptor antagonist that dampens inflammation (31). Similarly, other CC chemokines are processed by MMPs to form receptor antagonists with anti-inflammatory properties (30). It is conceivable that in vivo MMP-9 activity affects chemokine function either directly, or by interaction with other MMPs. It has been demonstrated that MMP-7 cleaves syndecan-bound KC, which is required for the entry of neutrophils into the lung during a model of bleomycin-induced lung injury (32). Alternatively, other MMPs may compensate for the absence of MMP-9 and thus may release glycosaminoglycan-bound eotaxin within the lung tissue. Preliminary experiments in our hands have determined that MMP-9 has no direct effect on the ability of eotaxin to recruit eosinophils in vitro (data not shown).

We observed an increase in Th2 cytokine generation in lungs of MMP-9 KO mice compared with WT mice. In addition, there were significantly more Th2 cells in the BAL, but not in lung tissue digest on day 24 in comparison with WT mice. By day 30, 6 days after the final allergen challenge, Th2 cell numbers were higher in BAL and lung of MMP-9 KO mice compared with WT mice. This might be due to increased recruitment of Th2 cells in the MMP-9 KO mice, and certainly this pattern was reflected in the levels of the Th2 chemokine MDC in both BAL and lung. Alternatively, it is possible that Th2 responses to allergen mice might be altered in the MMP-9 KO compared with WT mice. In support of this, we found that lymphocytes from draining lymph nodes of MMP-9 KO showed enhanced allergen-specific proliferation in ex vivo experiments. Moreover, significantly more IL-4, but not IFN-γ, was produced in response to ex vivo allergen stimulation in MMP-9 KO mice compared with WT mice. It is possible that MMP-9 might affect T cell function, and therefore polarization during the sensitization and challenge process, resulting in altered pathology. Indeed, previous studies have shown that MMP-9 mediates cleavage of IL-2Rα, and thus down-regulates the proliferative capacity of T cells (33). MMP-9 might therefore be important in controlling T cell responses to allergen.

The data presented in this work contrast to those from a previous study whereby MMP function was manipulated by the use of a chemical inhibitor (18). Mice were treated with the MMP inhibitor at six hourly intervals during the 24-h period following a single allergen challenge in sensitized mice. This treatment was found to reduce airway inflammation as well as airway hyperreactivity. However, it is difficult to compare our two studies directly because the inhibitor used by Kumagai et al. (18) was broad spectrum in action, having reported effects on MMP-9, MMP-2, MMP-3, MMP-7, and MMP-13 (34). Because MMPs operate in a tightly regulated network with the action of one member being able to influence the action of another, the use of such an inhibitor is relatively uninformative as to the action of individual MMPs.

The most striking finding in our study was the increased cell recruitment to the lung and airways seen in MMP-9 KO mice compared with WT mice following allergen challenge. A recent study showed a similar heightened lung inflammation in the absence of MMP-2 (35). Mice that lacked MMP-2 failed to clear leukocytes from their lung tissue and had an increase in death due to asphyxiation. Several different methods of blocking MMP-2 function were used, including mice genetically deficient in MMP-2 as well as a broad-spectrum synthetic inhibitor of MMP function, GM6001. All methods gave similar results, namely an increase in cells within the lung parenchyma, but decreased cells in the airway lumen, implying that MMP-2 has a major role in clearance or

egression of cells from the lung to the airways. As in our study, this sustained inflammation of the parenchyma was associated with increased Th2 cytokine production. We found that levels of the IL-4, IL-5, and IL-13 protein were enhanced in both BAL and lung. In our model, MMP-9 deficiency was also associated with moderately increased airway hyperreactivity, presumably because of the increased levels of these cytokines, in particular IL-13, which is thought to directly contribute to airway function (36–38). Corry et al. (35) concluded that although MMP-2 was likely to be the most important MMP regulating parenchymal inflammatory cell influx, the potential role of other MMPs, including MMP-9, could not be excluded. Indeed, they stated that deficiency in MMP-9 could affect the formation of transepithelial chemokine gradients. Taken together, our data imply that although MMP-9 and MMP-2 are both important in the recruitment of leukocytes to the lung, it is MMP-2 that is critical for their transepithelial migration, and that maybe the role of MMP-9 is more directed toward resolution of leukocyte recruitment during allergen-induced airway inflammation.

To delineate a possible role for MMP-9 in resolution of inflammation, we looked at recruitment of eosinophils and Th2 cells in mice 6 days after the last allergen challenge (day 30). Although eosinophil numbers had decreased by day 30, Th2 cells were still present in the lungs of OVA-treated MMP-9 KO, but not WT mice. There were still significant amounts of eotaxin and MDC in lung and BAL fluid 6 days after OVA challenge in the MMP-9 KO mice, whereas levels in WT mice had decreased back to baseline. These data imply that resolution of inflammation may be slower in the absence of MMP-9. In support of this idea, a model of contact dermatitis was found to be nonresolving in MMP-9 KO mice (39). In this study, the authors concluded that the failure of MMP-9 KO mice to resolve their injury might be due in part to a failure to generate the regulatory cytokine IL-10. However, we could not detect a difference in IL-10 levels between WT and MMP-9 KO mice in either the lung or the lavage (data not shown).

Another study has looked directly at the contribution of MMP-9 to development of allergic airway disease also by using MMP-9-deficient mice. Surprisingly, the results of this study are in direct contrast to those presented in this work. Cataldo et al. (40) demonstrated that allergen-induced monocytic cell recruitment to the airway was decreased in MMP-9 KO mice when compared with WT mice. However, eosinophil recruitment was unaffected in the absence of MMP-9, with comparable eosinophilia in MMP-9 KO and WT mice. The inflammation induced by the protocol in the study by Cataldo et al. was relatively mild with even the WT mice showing minimal peribronchial or perivascular infiltrates and few eosinophils in the lavage (6.4×10^4/ml). In contrast, our protocol induces a florid eosinophilic inflammation with large numbers of eosinophils in the tissue and lavage (60×10^5/ml). Further supporting our data and providing more evidence that MMP-9 is not absolutely essential for leukocyte migration, it has been shown that neutrophils migrate normally into the lung, skin, and peritoneal cavity of MMP-9 KO mice (41). Moreover, in the study by Cataldo et al. (40), AHR was significantly decreased in MMP-9 KO mice in comparison with WT controls after allergen challenge. However, the AHR was significantly higher in MMP-9 KO mice in the absence of allergen challenge, making the results difficult to interpret. In our study, AHR was only moderately increased in MMP-9 KO mice on day 24, even in the presence of increased eosinophil recruitment. However, pulmonary inflammation, Th2 cytokine production, and eotaxin/MDC generation were all markedly increased in the absence of MMP-9. The differences between our two studies are likely to be due to subtle differences in the induction protocol as well as perhaps the genetic backgrounds of the mice used.

In summary, we have shown that a deficiency in MMP-9 is associated with enhanced cell recruitment to the lung following allergen challenge. These data indicate that MMP-9 is involved in the recruitment of leukocytes to an inflammatory site, by affecting development of allergen-specific T cell responses or perhaps by influencing expression of inflammatory mediators such as cytokines and chemokines. Furthermore, we provide evidence that MMP-9 might be important in the resolution of lung injury following allergen challenge.

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