

Mouse Mast Cell Protease 4 Is the Major Chymase in Murine Airways and Has a Protective Role in Allergic Airway Inflammation¹

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It is widely established that mast cells (MCs) have a harmful role in asthma, for example by secreting various proinflammatory substances stored within their secretory granule. However, in this study, we show that one of the substances stored within MC granule, chymase, in fact has a protective role in allergic airway inflammation, indicating that MCs may possess both harmful and protective activities in connection with this type of disease. Wild-type (WT) mice and mice lacking mouse MC protease 4 (mMCP-4), a chymase that is functionally homologous to human chymase, were sensitized and challenged with OVA, followed by the assessment of airway physiology and inflammatory parameters. Our results show that the airway hyperresponsiveness was significantly higher in mMCP-4^{-/-} as compared with WT mice. Moreover, the degree of lung tissue inflammation was markedly higher in mice lacking mMCP-4 than in WT controls. Histological analysis revealed that OVA sensitization/challenge resulted in a marked increase in the thickness of the smooth muscle cell (SMC) layer and, notably, that the degree of SMC layer thickening was more pronounced in mMCP-4^{-/-} animals than in WT controls, thus indicating that chymase may have an effect on airway SMCs. In support of this, mMCP-4-positive MCs were located in the close vicinity of the SMC layer, mainly in the upper airways, and mMCP-4 was shown to be the major chymase expressed in these MCs. Taken together, our results indicate that chymase present in the upper airways protects against allergic airway responses, possibly by regulating SMCs. *The Journal of Immunology*, 2009, 183: 0000–0000.

Allergic asthma is a complex disease characterized by reversible airflow obstruction, airway hyperresponsiveness (AHR),³ and airway inflammation and remodeling. Mast cells (MCs) are key effector cells in allergic diseases, and they may contribute to asthma pathology in several ways (1). MCs can be activated by different types of stimuli, e.g., by allergen-induced cross-linking of the high-affinity IgE receptor, FcεRI, and this may result in degranulation (2). Upon MC degranulation, a broad range of preformed bioactive substances is released, including histamine, cytokines, proteoglycans, and various proteases,

with the latter group consisting of chymases, tryptases, and carboxypeptidase A (CPA) (3).

A role of MCs in asthma has been suggested for many years, supported by a multitude of findings (1, 4, 5). MC degranulation is often seen in asthmatic lungs, and various MC mediators can be found in bronchoalveolar lavage (BAL) fluid from asthmatic patients. Furthermore, a strong infiltration of MCs into the airway smooth muscle cell (SMC) layer is a prominent feature of asthma, especially in allergic asthma (6), and is associated with AHR (7). Involvement of MCs in allergic asthma is also supported by animal studies. Thus, mice lacking functional MCs are less responsive in models of asthma in which allergic airway inflammation is induced by administration of OVA without external adjuvant (8–11).

Although a role for MCs in allergic asthma is now widely recognized, it is still unclear by which mechanism MCs contribute to airway responses. One plausible scenario would be that the preformed proteases that are released upon MC degranulation contribute by provoking different types of airway responses. In line with this idea, MC tryptase has been shown to induce airway inflammation and AHR, and tryptase inhibitors can reduce these types of responses (12–14). Also, chymase has been implicated in inflammatory responses, as shown by the ability of chymases to induce eosinophil and neutrophil tissue infiltration (15–17). Moreover, a chymase polymorphism has been linked to allergic asthma (18), although it is not clear in which way this polymorphism affects chymase levels and/or activity. Most reports implicate MC proteases as proinflammatory agents, but it cannot be excluded that they, in addition, may have protective functions. In fact, it has been shown that the presence of chymase in small airways correlates with preserved airway function in severe asthmatics, thus suggesting a protective role of chymase (19).

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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; CAE, chloroacetate esterase; C_L, lung compliance; CPA, carboxypeptidase A; FGF, fibroblast growth factor; i.n., intranasal; MC, mast cell; mMCP, mouse mast cell protease; PDGF, platelet-derived growth factor; R_L, lung resistance; SMC, smooth muscle cell; WT, wild type.

In this study, we addressed the role of MC chymase in allergic asthma by comparing the inflammatory responses and airway reactivity in wild-type (WT) mice with those in mice lacking mouse mast cell protease 4 (mMCP-4). Of the several murine MC chymases (mMCP-1, -2, -4, -5, and -9), mMCP-4 has similar tissue distribution, substrate specificity, and heparin-binding properties as human chymase, and is therefore likely to be its functional counterpart (20–22). We show that mice lacking mMCP-4 develop markedly elevated AHR as compared with WT counterparts, indicating that chymase has a protective role in allergic airway responses.

Materials and Methods

Mice and immunization protocol

Mice deficient in mMCP-4 (mMCP-4^{-/-}) (20) were backcrossed for 13 generations to the C57BL/6J genetic background. Experimental groups were age and sex matched, and WT littermates (mMCP-4^{+/+}) were used as controls. Animals were bred and maintained in the animal facility at Uppsala Biomedical Centre. All experiments were approved by the local ethical committee. Eight- to 12-wk-old mice were immunized i.p. with 10 μ g of OVA (Sigma-Aldrich) in 150 μ l of PBS on days 1, 3, 6, 8, 10, 13, and 15. Control mice were nonimmunized. On days 31, 34, and 36, all mice were challenged with 20 μ g of OVA in 20 μ l of PBS intranasally (i.n.). Separate experiments included mice that were immunized i.p. with OVA, but not challenged i.n. with OVA.

Airway reactivity and bronchoalveolar lavage

One or 2 days after the last Ag challenge, mice were anesthetized, tracheostomized, and coupled to a FlexiVent ventilator (23, 24). Airway responsiveness to incremental doses of i.v. methacholine was assessed. Results are presented as lung resistance (R_L) and lung compliance (C_L). Results were pooled from seven separate and matched experiments, and data were obtained from a total of 10 mice per group. BAL fluid was collected by rinsing the lungs twice with 1 ml of HBSS, and the total number of cells was determined. Cytospin slides were prepared and stained with May-Grünwald and Giemsa, followed by differential count of at least 200 cells per slide.

ELISA

Analysis of OVA-specific IgE in sera was performed using Mouse IgE ELISA Quantification Kit (Bethyl Laboratories). Serum samples were diluted 1/3, and anti-OVA IgE Abs were quantified with biotinylated OVA (2 μ g/ml), prepared as described (25), followed by incubation with 0.05 U of streptavidin-peroxidase conjugate (Roche Diagnostics). For OVA-specific IgG ELISA, serum samples diluted 1/1000 were added to OVA-coated microtiter plates, and bound IgG was detected with horseradish-conjugated anti-mouse IgG (dilution 1/1000; GE Healthcare). For enzyme detection, 3,3',5'-tetramethylbenzidine liquid substrate (Sigma-Aldrich) was used.

Histology

The left lung lobes were fixed in 4% paraformaldehyde, paraffin embedded, and cut into 6- μ m sections. Samples were deparaffinized and stained with H&E, toluidine blue, or chloroacetate esterase, as described earlier (26). Trachea was embedded in OCT and kept in -70°C before cutting 6- μ m sections. Lung tissue inflammation was evaluated by blinded analyses of numbers of infiltrating cells per defined field of H&E-stained lung sections from five to seven animals per group. Cells were counted at $\times 400$ magnification, using an eyepiece fitted with a square graticule that covered a field of view measuring 0.06 mm². Tissue inflammation in larger vs smaller airways was evaluated by separately counting the cells in regions around primary and secondary airways, or the cells around terminal bronchioles, respiratory bronchioles, and alveoli, respectively. The numbers of cells in at least three randomly selected regions for each individual were determined, and mean values were used for statistical calculations. The thickness of the smooth muscle layer was evaluated by blinded scoring from 0 to 5 of regions around the upper bronchus, resulting in an average score for each section. Number of MCs was evaluated by counting toluidine blue-stained cells in the connective tissue and smooth muscle layer underneath the epithelia of the primary bronchi. Chymotryptic activity was detected by blinded scoring from 0 to 4 of the chloroacetate esterase-staining intensity of MCs around the primary bronchi.

Western blot

Tissue extracts from the upper (one-fourth) part of the left lung lobe were homogenized in PBS/1 mM EDTA/1% Triton X-100 and centrifuged at 13,400 rpm at 4°C for 20 min, and supernatants were collected for analyses of fibronectin, smooth muscle α -actin, and β -actin. The resulting pellets were extracted with PBS/1 mM EDTA/1% Triton X-100 containing 2.0 M NaCl, and supernatants were used for analyses of mMCP-1, mMCP-2, mMCP-4, mMCP-5, mMCP-6, CPA, and β -actin. Samples were separated under reducing conditions on 12% SDS-PAGE gels (or 8% SDS-PAGE gels for fibronectin). Western blots were performed using Abs to smooth muscle α -actin (Sigma-Aldrich; 1/750 dilution), β -actin (Santa Cruz Biotechnology), antisera to fibronectin (a gift of S. Johansson, Uppsala University, Uppsala, Sweden; 1/250 dilution), or antisera to mMCP-1, mMCP-2, mMCP-4, mMCP-5, mMCP-6, and CPA (26, 27) (1/500 dilution). Purified mMCP-4 (28), mucosal-type bone marrow-derived MCs (26), or peritoneal cell-derived MCs (29) were used as positive controls for mMCP-4, mMCP-2, and mMCP-5, respectively. Western blot signals for smooth muscle α -actin were visualized and quantified using the Image Reader V 1.7E software. Data were measured as pixels/mm², and results were obtained by dividing the values for smooth muscle α -actin samples by the values of corresponding β -actin control.

In vitro cleavage

Cleavage of platelet-derived growth factor (PDGF)-BB (provided by C.-H. Heldin, Uppsala University, Uppsala, Sweden) and fibroblast growth factor (FGF)-2 (PeproTech) was performed using 10 μ g of substrate and 0.5 μ g of purified mMCP-4 (28) in PBS/0.05% Triton X-100. Reactions (37°C) were interrupted at 20 min, 2 h, or 6 h by addition of SDS-PAGE sample buffer, followed by boiling for 5 min. Samples were run under reducing conditions on a 12% SDS-PAGE gel. Results were obtained by AgNO₃ staining.

Statistical analysis

Statistical differences were determined with Student's *t* test for differential count and ELISA, with Mann-Whitney *U* test for histology and densitometry, and with two-way ANOVA for airway reactivity measurements, using GraphPad Prism 4.0 (GraphPad). All values are displayed as means $\pm$ SEM.

Results

Enhanced allergic airway inflammation in mMCP-4^{-/-} mice

To investigate the role of mMCP-4 in allergic airway inflammation, we compared the OVA-induced accumulation of cells in BAL and lung tissue of WT and mMCP-4^{-/-} mice. Sensitization and challenge with OVA resulted in a prominent increase of total BAL cells in both mMCP-4^{-/-} and WT mice, mainly due to accumulation of eosinophils (Fig. 1A). However, the numbers of OVA-induced BAL eosinophils, neutrophils, macrophages, and lymphocytes did not differ significantly between mMCP-4^{-/-} and WT mice (Fig. 1A). Lung histology demonstrated a perivascular and peribroncheal infiltration of leukocytes, mainly eosinophils, in animals that had been both sensitized and challenged with OVA, whereas control animals (challenged only) showed no substantial signs of inflammation (Fig. 1B). The tissue around larger airways (primary and secondary bronchi) displayed a higher density of inflammatory cells compared with that around smaller airways (Fig. 1, C and D). Although OVA-induced tissue inflammation was evident in both WT and mMCP-4^{-/-} mice, a significant increase in the number of infiltrating cells around both larger and smaller airways was seen in the absence of mMCP-4 (Fig. 1, B–D), thus suggesting a role for chymase as a negative regulator of allergen-induced airway inflammation.

AHR in the absence of mMCP-4

To analyze whether the increase in tissue inflammation in the absence of mMCP-4 was accompanied by an increased AHR, invasive lung mechanics measurements were performed 1 or 2 days after the last OVA challenge. OVA-sensitized and OVA-challenged mMCP-4^{-/-} mice exhibited significantly enhanced R_L as compared with OVA-sensitized/challenged mMCP-4^{+/+} mice

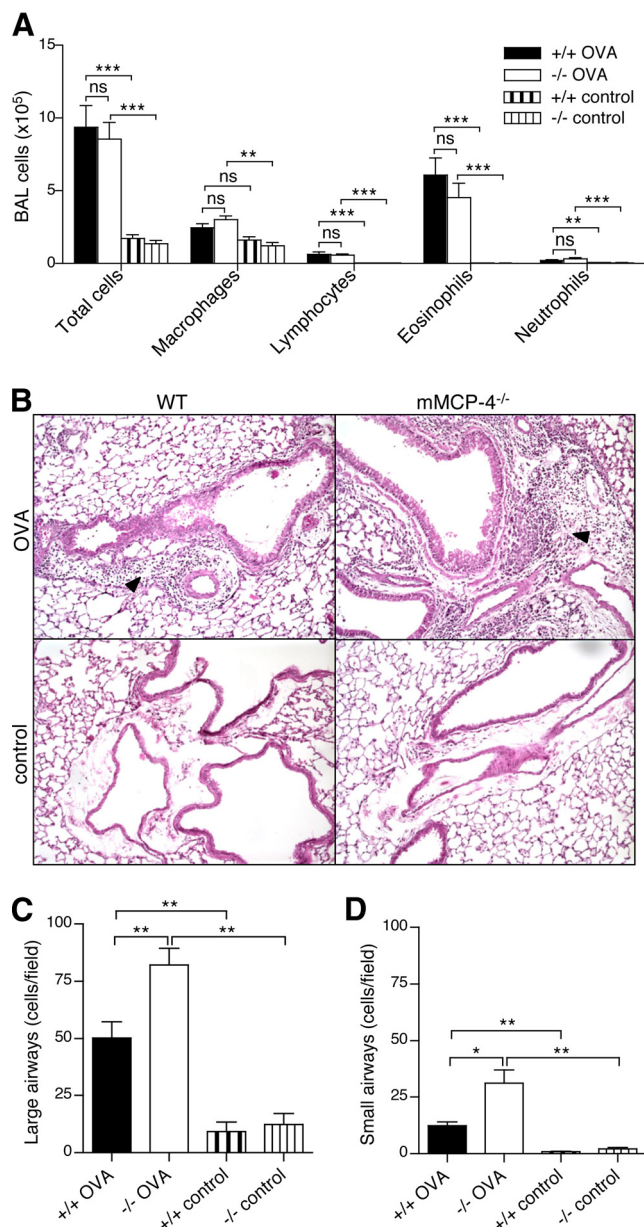


FIGURE 1. Allergic airway inflammation in BAL and lung tissue of mMCP-4^{-/-} and WT mice. Mice were sensitized with OVA or were non-sensitized (controls). All mice received three i.n. instillations with 20 μ g of OVA. **A**, Differential counts of leukocytes in BAL fluid ($n = 17$ – 23 , data pooled from 8 independent experiments). Data are presented as mean number of cells + SEM. Differences between groups were analyzed by Student's t test, as follows: *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$. **B**, Lung histology of representative sections from OVA-immunized mice (upper panels) and controls (lower panels). Arrows indicate leukocyte infiltrates. Original magnifications were $\times 100$. Lung tissue inflammation around larger (**C**) or smaller (**D**) airways was evaluated by blinded counting of number of infiltrating cells per field, as described in *Materials and Methods* ($n = 5$ – 7 , data pooled from 3 independent experiments). Data are presented as mean number of infiltrating cells/field + SEM. Data were analyzed by Mann-Whitney U test, and significant differences are indicated, as follows: *, $p < 0.05$ or **, $p < 0.01$.

($p < 0.01$), as well as compared with nonsensitized controls ($p < 0.001$; Fig. 2A). Thus, induction of AHR was clearly seen in OVA-sensitized/challenged mMCP-4^{-/-} mice, but was undetectable in the corresponding WT mice. These results strongly suggest that the presence of mMCP-4 in WT mice protects from AHR in this

model of allergic airway inflammation. In line with the increased R_L , OVA-sensitized/challenged mMCP-4^{-/-} mice displayed a significant decrease in C_L as compared with nonimmunized mMCP-4^{-/-} controls at the dose of 1 mg/kg methacholine ($p < 0.05$; Fig. 2B).

Normal allergic sensitization in mMCP-4^{-/-} mice

The increased airway inflammation and AHR seen in mMCP-4^{-/-} mice could potentially be explained by a deficient control of the humoral immune response during OVA sensitization. To study the effects of mMCP-4 on Ab production, we measured the systemic OVA-specific IgE and IgG levels in all groups of mice. Mice presensitized with OVA had significantly increased serum levels of OVA-specific IgE (Fig. 3A) and IgG (Fig. 3B) compared with nonsensitized controls. However, no significant differences in the levels of OVA-specific IgE or IgG were observed when comparing WT and mMCP-4^{-/-} animals (Fig. 3). This finding does not support a major role of mMCP-4 in the sensitization process.

Localization of MCs in upper airways

We evaluated the presence of MCs in the airways by toluidine blue staining of lung tissue sections. In agreement with previous observations (30), MCs were only rarely seen in the tissue around smaller airways, either in control or sensitized/challenged animals (data not shown). In contrast, MCs were more abundant in the tissue around the larger upper airways, in particular in regions close to the primary bronchus (Fig. 4A) and trachea (supplemental data).⁴ MC numbers and distribution were similar in WT and mMCP-4^{-/-} animals, and there was no obvious difference in the degree of MC degranulation when comparing OVA-sensitized/challenged mice and control mice (data not shown). Furthermore, no difference in numbers of MCs around the primary bronchus or trachea was observed between sensitized/challenged animals vs controls (Fig. 4C, and data not shown). Notably, MCs were particularly abundant in the tissue below the epithelium and the smooth muscle layer of the primary bronchus, and MCs in this region were frequently seen in close vicinity to the SMC layer (Fig. 4, A and B).

mMCP-4 is the major source of chymotryptic activity in lung MCs

To detect chymotrypsin-like activity in the upper airways, we used the chloroacetate esterase assay, resulting in red stain where active chymase is present. As shown in Fig. 4, B and D, chymase activity was readily detected in WT MCs, whereas mMCP-4^{-/-} MCs exhibited a severely reduced chymase activity, as demonstrated by the faint staining. This indicates that mMCP-4 accounts for a major part of the chymase activity stored within lung MCs, i.e., other chymases such as mMCP-1, mMCP-2, mMCP-5, or mMCP-9 contribute to a lesser extent to the total chymotrypsin-like activity in MCs of the upper airways. Moreover, OVA sensitization/challenge did not significantly affect the chymase activity of WT or mMCP-4^{-/-} lung MCs, thus arguing against the possibility of a compensatory up-regulation of other chymases during allergic airway inflammation in mMCP-4^{-/-} mice (Fig. 4D).

The presence of mMCP-4 in the larger airways of WT mice was also confirmed by Western blot analyses of the upper (one-fourth) part of the left lung lobe or of the trachea (Fig. 4E and supplemental data). In contrast, mMCP-4 protein was below the level of

⁴ The online version of this article contains supplemental material.

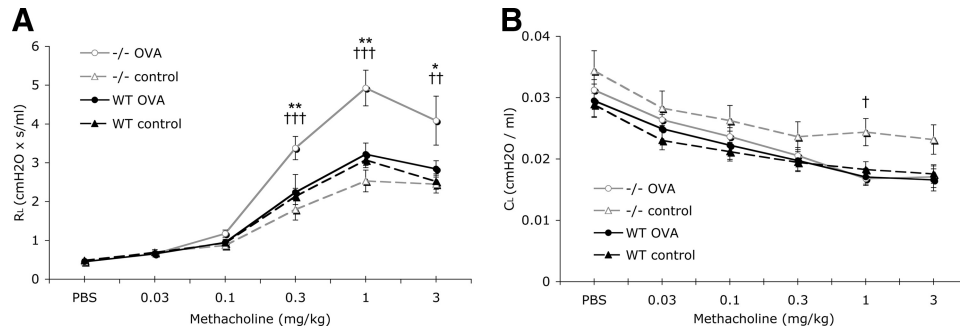


FIGURE 2. AHR to methacholine in WT and mMCP-4^{-/-} mice. Lung mechanics measurements were performed 24 or 48 h after last i.n. challenge of OVA-sensitized (OVA) or nonsensitized (control) mice. **A**, R_L was significantly enhanced in OVA-immunized mMCP-4^{-/-} mice both vs controls ($p < 0.001$) and vs OVA-immunized WT mice ($p < 0.01$), as determined by two-way ANOVA. **B**, C_L . Statistical significances for individual doses are shown, as follows: *, $p < 0.05$ or **, $p < 0.01$ of OVA-immunized mMCP-4^{-/-} mice vs OVA-immunized WT mice; †, $p < 0.05$; ††, $p < 0.01$; or †††, $p < 0.001$ of OVA-immunized groups vs controls; not significant, $p > 0.05$ (data not shown). Data were obtained from 7 independent experiments with a total of 10 mice per group.

detection in lower parts of the lungs (data not shown). As expected, mMCP-4 protein was not detected in tissue from mMCP-4^{-/-} animals (Fig. 4E and supplemental data). In a previous study, it was demonstrated that peritoneal MCs from mMCP-4^{-/-} mice display normal morphology, normal IgE-induced MC degranulation, and normal levels of other granule-stored proteases, i.e., mMCP-5, mMCP-6, and CPA (20). To confirm that mMCP-4^{-/-} airway MCs exhibit an unaltered phenotype, we in this study analyzed for the presence of mMCP-1, mMCP-2, mMCP-5, mMCP-6, and CPA in upper airways. No substantial difference between mMCP-4^{-/-} mice and WT controls could be detected, although a minor tendency of higher levels of mMCP-1 and mMCP-6, and lower levels of mMCP-2, was seen in the absence of mMCP-4 (Fig. 4E). These findings demonstrate that there are no substantial compensatory alterations of the major MC proteases in the absence of mMCP-4.

Increased OVA-induced smooth muscle thickening in primary bronchi of mMCP-4^{-/-} mice

The localization of chymase-containing MCs mainly around the upper airways prompted us to further analyze the local tissue response at this site. Considering the profound effect of mMCP-4 deficiency on AHR (Fig. 2), it was of particular interest to evaluate OVA-induced alterations in the SMC layer. Histological analyses revealed a substantial OVA-induced thickening of the SMC layer

of the primary bronchi of mMCP-4^{-/-} mice, whereas only a modest, nonsignificant thickening of the SMC layer was seen in the WT mice (Fig. 5). The increased smooth muscle thickness in mMCP-4^{-/-} mice occurs only in response to the complete asthma protocol (OVA sensitization and challenge), because neither sensitization alone nor i.n. challenge alone had any effect on the smooth muscle thickness of the primary bronchi (Fig. 5, and supplemental data). This is in line with a role for mMCP-4 only during local allergic airway inflammation, i.e., when mMCP-4 is released upon MC degranulation during the effector phase of the disease. Moreover, the SMC layer thickness of OVA-sensitized/challenged mMCP-4^{-/-} mice was significantly increased as compared with that in OVA-sensitized/challenged WT mice (Fig. 5B). To further address the effect of mMCP-4 in tissue remodeling, we analyzed total levels of SMC α -actin in the upper (one-fourth) part of the lungs by Western blot analysis. As shown in Fig. 6, A and B, there was a significantly lower level of total SMC α -actin in the (one-fourth) upper part of lung tissue from mMCP-4^{-/-} control mice compared with that in WT controls, despite no obvious histological differences in the airway smooth muscle compartment (Fig. 5, and data not shown). We cannot exclude that there is an altered lung tissue homeostasis and/or a generally reduced SMC α -actin expression in the absence of mMCP-4 that is not dependent on allergic inflammation. Notably, there was a tendency of an allergen-induced increase in levels of total SMC α -actin in mMCP-4^{-/-} mice, whereas the opposite tendency was seen for WT mice (Fig. 6B). Although these tendencies are compatible with an increased SMC response to allergen sensitization/challenge in the absence of mMCP-4, the differences between groups did not reach statistical significance. Thus, despite a clear effect of mMCP-4 on the smooth muscle thickening of the primary bronchus (Fig. 5A), there was no substantial effect of mMCP-4 on the OVA-induced increase of total SMC α -actin levels of the upper (one-fourth) part of the lungs (including SMC around both small and large airways and blood vessels). Taken together, these observations suggest a role for mMCP-4 in the local control of allergen-induced tissue remodeling, i.e., in the control of SMC hyperplasia and/or hypertrophy of primary airways.

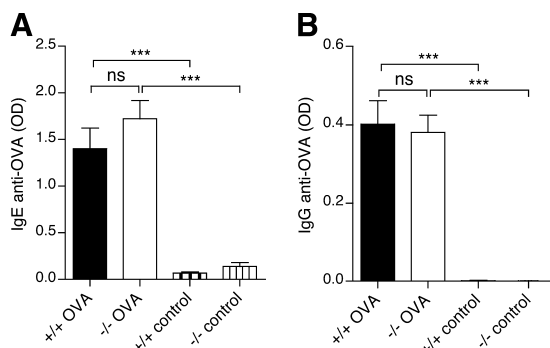


FIGURE 3. Normal OVA-specific Ab production in mMCP-4^{-/-} mice. Sera from OVA-sensitized/challenged (OVA) or nonsensitized (control) WT or mMCP-4^{-/-} mice were collected after allergen challenge. Samples were analyzed by ELISA for OVA-specific IgE (A) and OVA-specific IgG (B). Results are displayed as OD values + SEM. ***, $p < 0.001$ of OVA-immunized groups vs controls. Data were pooled from eight independent experiments ($n = 17$ –23).

Degradation of SMC mitogens and fibronectin by mMCP-4

To investigate possible mechanisms by which mMCP-4 could regulate SMCs, we examined whether mMCP-4 can cleave purified SMC mitogens. Indeed, purified mMCP-4 cleaved both PDGF-BB and FGF-2 (Fig. 6, C and D). Another potential substrate for mMCP-4 could be fibronectin. Fibronectin has previously been

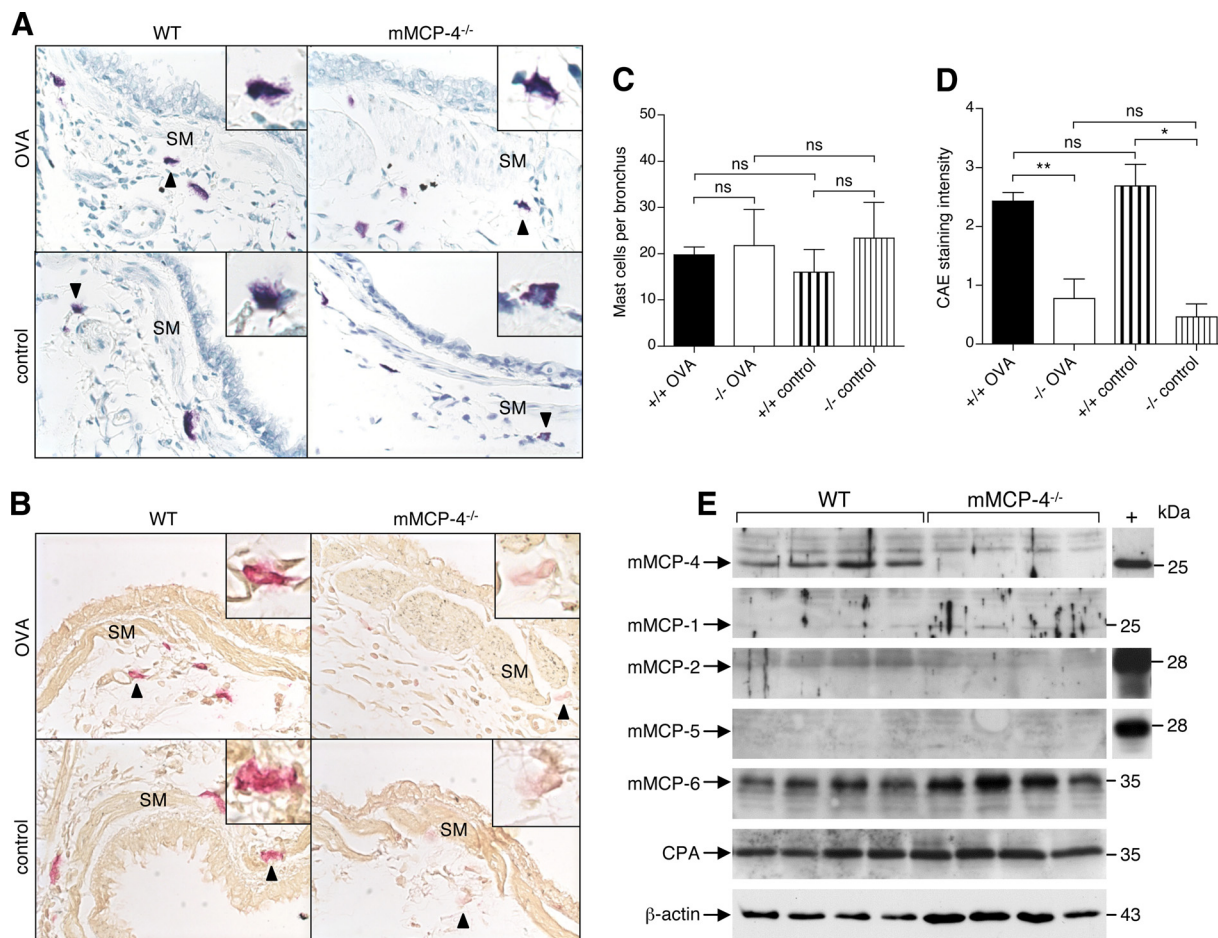


FIGURE 4. Chymase-containing MCs around primary bronchi. Lung tissue sections were collected from OVA-sensitized/challenged (OVA; $n = 5-7$) or nonsensitized (control; $n = 3-4$) WT and mMCP-4^{-/-} mice. **A**, Sections of primary bronchi stained with toluidine blue. **B**, Sections of primary bronchi stained with chloroacetate esterase. Arrows indicate MCs shown in higher magnification in inserts (original magnification of $\times 400$). The smooth muscle layer is indicated (SM). **C**, Total number of MCs around the primary bronchus of each section, shown as mean $\pm$ SEM. **D**, Chloroacetate esterase staining intensity, as determined by blinded scoring of all visible MCs/section (a mean number of 20 MCs for WT mice and 7.7 MCs for mMCP-4^{-/-} mice was scored). Results are shown as mean $\pm$ SEM. Data were analyzed by Mann-Whitney U test, as follows: *, $p < 0.05$; **, $p < 0.01$. **E**, Western blot analyses of mMCP-1, mMCP-2, mMCP-4, mMCP-5, mMCP-6, and CPA in upper lung tissue of naive WT and mMCP-4^{-/-} mice. Levels of β -actin are shown as a loading control. The following positive controls (+) were used: purified peritoneal mMCP-4 (28), mucosal-type bone marrow-derived MCs (26) for mMCP-2, or peritoneal cell-derived MCs (29) for mMCP-5.

shown to be a substrate both for human chymase and for mMCP-4 (31, 32) and, considering that proteolytic fibronectin fragments can be proapoptotic for SMCs (33), it was relevant to examine whether fibronectin is differentially processed in lungs from WT and mMCP-4^{-/-} animals. As shown in Fig. 6A, WT lung tissue contained both intact fibronectin as well as a number of fibronectin degradation prod-

ucts of different sizes. In contrast, mMCP-4^{-/-} lung tissue predominantly contained the intact form of fibronectin. Thus, mMCP-4 appears to regulate the processing of fibronectin in lung tissue. Similar amounts of fibronectin were seen in control and sensitized/challenged animals, and the degree of fibronectin processing was not affected by the allergen sensitization/challenge procedure (Fig. 6A).

FIGURE 5. Increased OVA-induced SMC layer thickening in mMCP-4^{-/-} mice. Lung tissue sections were collected from OVA-sensitized/challenged (OVA; $n = 7$) or nonsensitized (control; $n = 5$) WT and mMCP-4^{-/-} mice and stained with H&E. **A**, Representative sections of the upper primary bronchi, demonstrating the increased thickness of the SMC layer in OVA-sensitized/challenged mMCP-4^{-/-} mice. Arrows indicate smooth muscle layer. Original magnification of $\times 200$. **B**, Smooth muscle layer thickness of the primary bronchi, as determined by blinded scoring. Results are presented as mean $\pm$ SEM. Data were analyzed by Mann-Whitney U test, as follows: *, $p < 0.05$.

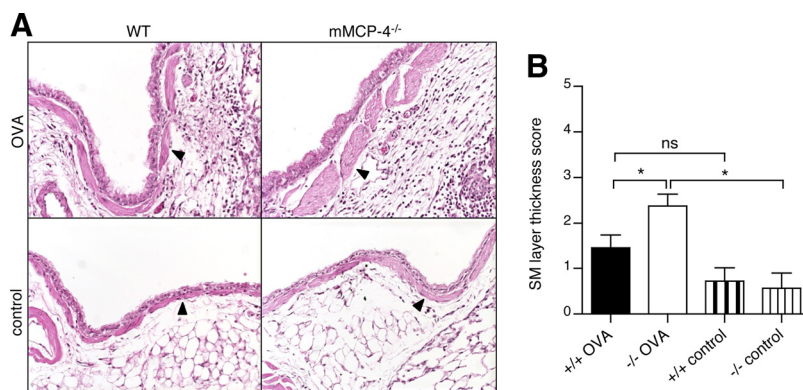
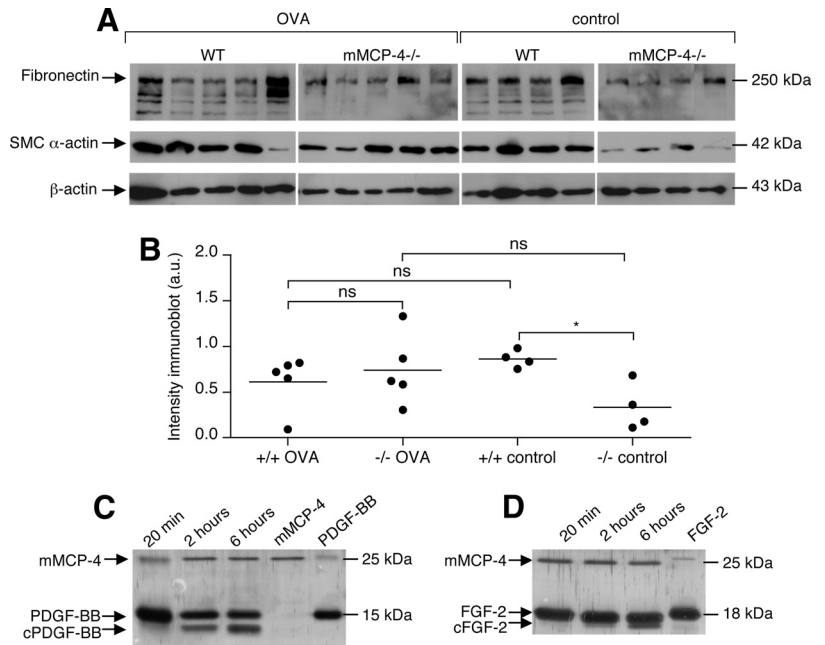


FIGURE 6. Altered tissue remodeling and cleavage of PDGF-BB and FGF-2 by mMCP-4. **A**, Lung homogenates from OVA-sensitized/challenged (OVA) or non-sensitized (control) WT or mMCP-4^{-/-} mice were subjected to Western blot analysis for fibronectin and SMC α -actin. β -actin was used as loading control. **B**, Densitometry of SMC α -actin vs β -actin immunoblot. Results are presented as arbitrary units (a.u.), in which intensity for β -actin was set to 1. Data were analyzed by Mann-Whitney *U* test, as follows: *, *p* < 0.05. **C**, Cleavage of PDGF-BB by purified mMCP-4. **D**, Cleavage of FGF-2 by purified mMCP-4. Cleavage was allowed for 20 min, 2 h, and 6 h, followed by SDS-PAGE of products under reducing conditions.



Discussion

This is the first study in which the role of MC chymase in allergic airway responses is addressed by using a chymase-deficient mouse strain. We show that in response to OVA immunization and challenge, mMCP-4^{-/-} mice develop a more pronounced tissue inflammation, AHR, and SMC remodeling than WT mice, indicating that chymase is a negative regulator of these features. Consequently, chymase does not seem to contribute to the overall detrimental effects of MCs in allergic airway inflammation (11). Instead, chymase could have a crucial role in counteracting the harmful reactions induced by other bioactive substances released by MCs or other inflammatory cells.

At first glance, the dampening effect of endogenous mMCP-4 on the OVA-induced tissue inflammation could appear to be in discrepancy with the reported proinflammatory properties of chymases (15–17). However, studies demonstrating proinflammatory effects were frequently conducted by administration of purified chymases. Obviously, the context of an administered chymase, e.g., dose, timing, and localization, is radically different from that of the endogenous counterpart. Furthermore, the physiological form of both human chymase and mMCP-4 is in complex with serglycin proteoglycans, and this association affects chymase function (3). In other approaches, chymase functions have been inferred based on studies using various chymase inhibitors. However, most chymase inhibitors have been developed to act against human chymase, with the activity and selectivity toward the relevant animal chymase being less characterized. Therefore, the use of genetically targeted mice selectively lacking functional chymase may be a more relevant approach for identifying physiological effects of endogenous chymase in different inflammatory settings. The mechanism by which mMCP-4 dampens OVA-induced lung tissue inflammation is not known, but could involve degradation of proinflammatory cytokines and chemokines (34). In addition, because mMCP-4 is needed for proper processing of connective tissue components, as shown previously (20) and in Fig. 6, this may facilitate the transfer of leukocytes from the tissue into BAL. In support of the latter notion, the numbers of OVA-induced BAL eosinophils were similar in WT and mMCP-4^{-/-} mice, despite a significantly lower degree of tissue inflammation in the WT

mice. Although it needs to be firmly established, a possible explanation for the lower ratio of BAL vs tissue inflammation in mMCP-4^{-/-} mice could be that the presence of unprocessed connective tissue components in the absence of mMCP-4 hinders the migration of cells over the lung epithelium. Alternatively, it is possible that impairment of cytokine degradation could lead to an increased cell activation in the mMCP-4^{-/-} mice that renders the cells more adhesive to the tissue.

A major finding in this study was the development of AHR, only in mMCP-4^{-/-} mice, indicating that the presence of mMCP-4 in WT mice protects against AHR in this model. Notably, AHR was totally absent in OVA-sensitized and -challenged WT C57BL/6 mice. Although C57BL/6 mice are known to be low responders in MC-independent protocols involving alum (35, 36), they have been reported to develop a significant AHR in MC-dependent protocols in which external adjuvant is omitted during OVA sensitization (37, 38). We used a protocol that is almost identical with a MC-dependent protocol described by Williams and Galli (9). The absence of AHR in WT C57BL/6 mice in the present study might be explained by the relatively low dose of OVA during i.n. challenge (20 μ g of OVA as in Ref. 9, instead of 200 μ g of OVA as in Refs. 37 and 38). Differences in the assessments of lung mechanics (e.g., dissimilar ventilators and routes of methacholine delivery) might also explain the discrepancies in the levels of AHR between this and other studies.

Strong OVA-specific IgE and IgG responses were seen in both WT and mMCP-4^{-/-} mice, and there were no significant differences in the levels of produced Abs between the two genotypes. This indicates that the OVA sensitization was efficient, and suggests that mMCP-4 does not have a major impact on the generation of adaptive immune responses, when using this immunization protocol. Thus, the increased airway responsiveness of mMCP-4^{-/-} mice most likely depends on mMCP-4-regulated events during the acute phase of this model, rather than during the sensitization phase.

An obvious and important question concerns the mechanism by which mMCP-4 affects AHR. Considering the location of mMCP-4-containing MCs in close association of the SMC layer of the upper bronchi, a direct effect of chymase on SMC remodeling is an

attractive hypothesis. In support of this, we found a higher SMC responsiveness in the absence of mMCP-4, as demonstrated by the OVA-induced thickening of the SMC layer and increase of SMC α -actin only in the lungs of mMCP-4^{-/-} mice. Because the SMC layer most likely has a major impact on airway responses, i.e., bronchoconstriction, an effect of mMCP-4 on SMC responsiveness is well in line with the observed effects on the AHR. It is also interesting to note that infiltration of the SMC layer with MCs appears to be a hallmark of allergic asthma, and that most of the MCs infiltrating the SMC layer are chymase positive (6, 7). Hence, chymase is abundant in the SMC layer of allergic asthmatics and may thus have the potential to influence SMC function in a similar fashion, as was seen in this study.

The exact mechanism by which chymase could affect SMCs is intriguing. One possibility would be that mMCP-4 degrades SMC growth factors, thus counteracting the SMC hyperplasia typically seen in allergic airway inflammation. In line with such a scenario, we demonstrate that two major SMC growth factors, PDGF-BB and FGF-2, indeed are substrates for mMCP-4. Furthermore, Lazaar et al. (39) previously showed that chymase reduces epidermal growth factor-induced proliferation of SMCs. An alternative explanation for how mMCP-4 may control SMC responses would be that the fibronectin fragments generated in the presence of mMCP-4 could be proapoptotic for SMCs (33), thus possibly preventing an exaggerated SMC expansion in response to OVA treatment. However, numerous other potential targets for chymase have been described, many of which could directly or indirectly affect SMCs and AHR, e.g., substance P, vasoactive intestinal peptide, endothelin-1, angiotensin I, IL-13, and C3a (3).

Notably, several data presented in this study demonstrate an effect of mMCP-4 in control mice that had not been sensitized with OVA. For instance, we found a defect in fibronectin degradation in mMCP-4^{-/-} nonsensitized mice as compared with WT counterparts, indicating a role for mMCP-4 in regulating normal lung homeostasis. Moreover, a minor difference in C_L was observed between nonsensitized mMCP-4^{-/-} and WT mice, possibly reflecting an altered lung tissue composition in mMCP-4^{-/-} mice.

We identify in this study mMCP-4 as the major source of chymotryptic activity in the lungs of mice, as demonstrated by the severely reduced chloroacetate esterase (CAE)-staining intensity of mMCP-4^{-/-} lung MCs. The absolute fraction of remaining chymotryptic activity in the absence of mMCP-4 is difficult to evaluate, and is most likely overestimated in Fig. 4D, due to the risk of unintentionally neglecting mMCP-4^{-/-} MCs that are totally CAE negative. Nonetheless, a faint, but evident CAE staining was seen in several mMCP-4^{-/-} MCs. This is in contrast to the total absence of CAE staining observed in mice lacking all chymase activity due to the deletion of the enzyme dipeptidyl peptidase I that activates mMCP-4 as well as other chymases (40). Therefore, the remaining CAE activity in mMCP-4^{-/-} MCs strongly suggests the presence of low amounts of one or several other dipeptidyl peptidase I-dependent chymases in airway MCs. Indeed, we found detectable, but low amounts of mMCP-1, mMCP-2, and mMCP-5 in the lungs that could contribute to CAE staining in the absence of mMCP-4 (Fig. 4E). Moreover, we cannot exclude that airway MCs also express low amounts of cathepsin G, a chymotryptic neutrophil protease that has been reported to be expressed also by MCs (41).

The protective role of chymase in AHR suggested by this study is well in line with two reports demonstrating positive correlations between lung function and the presence of chymase-containing MCs, either in the small airways of severe asthmatics (19) or in the upper airways of mild to moderate asthmatics (42). However, a striking difference between humans and mice is that MCs are

widely distributed within both the upper and lower airways in humans, whereas MCs are mainly present in the upper airways of mice (30, 43). It could therefore be speculated that the impact of chymase in human airway responses could be even more pronounced than in mouse. Furthermore, it is important to stress that whereas only one chymase is expressed by human MCs, mMCP-4 is only one of several chymase genes that are expressed by murine MCs. Thus, it is possible that other murine chymases could have functions that overlap with those of mMCP-4. In contrast, the severely reduced chymotryptic activity detected in the lung MCs of mMCP-4^{-/-} mice, even during allergic inflammation, argues against a major role of other murine chymases in airway responses. Moreover, there is strong evidence suggesting that mMCP-4 constitutes the relevant functional counterpart of human MC chymase (see above). It is therefore likely that effects of mMCP-4 mimic those of human chymase.

The results presented in this study, combined with previous findings demonstrating a harmful role of MCs in similar models of allergic asthma (8–10), indicate that MCs can possess both protective and deleterious activities during a given disorder. Possibly, harmful and protective effects of MCs could occur during different time frames with, for example, initial activities that promote the airway response being followed by the mounting of protective activities that down-regulate the initial proinflammatory activities. It should also be emphasized that individual MC mediators may have separate roles in different types of inflammation. For example, it was recently reported that mMCP-4 has a harmful role in collagen-induced arthritis, by contributing both to the Ab production and inflammation (44). Apparently, the precise role of MC chymase in an inflammatory setting may thus vary extensively, most likely depending on the repertoire and availability of suitable chymase substrates.

Taken together, the results presented in this study suggest that chymase has a role in counteracting the harmful effects of MCs during allergic airway responses, possibly via limiting tissue inflammation and SMC remodeling.

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Disclosures

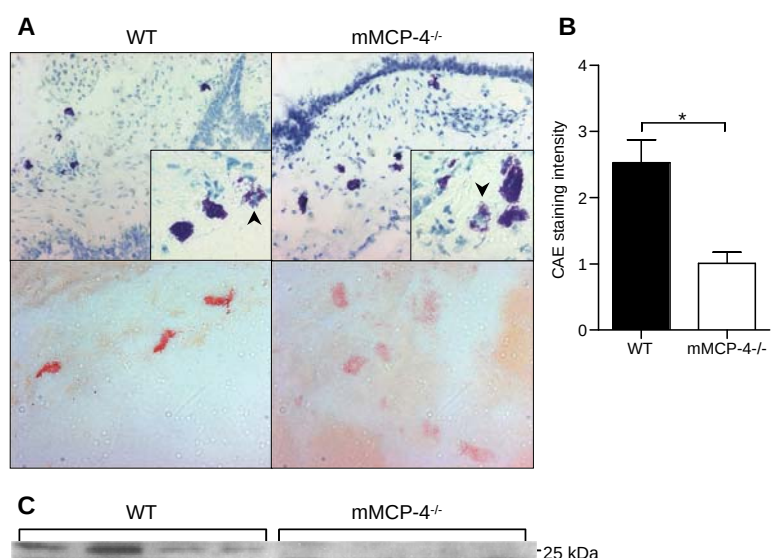
The authors have no financial conflict of interest.

References

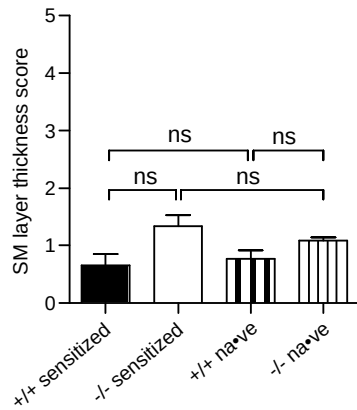
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Supplemental Data (Waern et al.)



Suppl Fig. 1. Chymase-containing MCs in trachea. **A**, Sections of trachea from OVA-sensitized/challenged WT and mMCP-4^{-/-} mice were stained with toluidine blue (upper panels, original magnification of 200x), or chloroacetate esterase (lower panels, original magnification of 400x). Larger magnification (insets in upper panel) showing the presence of degranulated MCs (arrows). **B**, Chloroacetate esterase (CAE) staining intensity in sections of trachea from WT and mMCP-4^{-/-} mice (n= 5). CAE staining intensity was determined by blinded scoring of 10-40 mast cells/section and data is presented as mean intensity/mast cell +SEM. Data were analyzed by Mann Whitney test: **P*<0.05. **C**, Western blot analysis of mMCP-4 in trachea from untreated mice, demonstrating the presence of mMCP-4 in WT trachea.



Suppl Fig. 2. Unaltered thickness of bronchial smooth muscle layer in response to sensitization alone. Lung tissue sections were collected from i.p. OVA-sensitized (sensitized; n=4) or non-sensitized (naïve; n=3) WT (+/+) and mMCP-4^{-/-} (-/-) mice, and stained with hematoxylin and eosin. None of the mice were given intranasal instillations with OVA. Smooth muscle layer thickness of the primary bronchi was determined by blinded scoring. Results are presented as mean score +SEM. Data were analyzed by Mann Whitney test: ns $P>0.05$. Notably, there was a statistically significant difference between “-/- sensitized” mice shown in this figure and “-/- OVA” mice shown in Fig. 5B ($P<0.05$), whereas the difference between “+/+ sensitized” mice and “+/+ OVA” mice (Fig. 5B) was not statistically significant.