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A CRUCIAL ROLE OF BASOPHIL mMCP-11 IN ALLERGY

Basophil tryptase mMCP-11 plays a crucial role in IgE-mediated, delayed-onset allergic inflammation in mice

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Key points

- Mice deficient for basophil tryptase mMCP-11 showed ameliorated IgE-mediated allergic inflammation with reduced leukocyte infiltration.
- This is the first demonstration that the basophil-derived protease plays a crucial role in allergic inflammation.

Abstract

Recent studies have identified non-redundant roles for basophils in immune responses including allergy and protective immunity. It is well known that activated basophils release granule contents such as histamine and proteases as do mast cells. However, the functional significance of basophil-derived proteases remains poorly understood in contrast to those released from mast cells. Here we generated mice deficient for mouse mast cell protease 11 (mMCP-11) that is preferentially expressed by basophils rather than mast cells. In spite of normal development of basophils, mMCP-11-deficient mice showed ameliorated IgE-mediated chronic allergic inflammation (IgE-CAI) with reduction of cutaneous swelling, microvascular permeability and leukocyte infiltration in the skin lesion, compared to WT mice. Repeated administration of recombinant mMCP-11 in the skin induced infiltration of leukocytes, including basophils, in a tryptase activity-dependent manner. The transwell migration assay *in vitro* suggested that mMCP-11-mediated proteolytic products of a serum protein(s) promoted migration of basophils, eosinophils and macrophages via a G protein-coupled receptor(s). Thus, basophil tryptase mMCP-11 is a crucial effector molecule for the induction of IgE-CAI-type allergic inflammation. This is the first demonstration that the basophil-derived protease plays a significant role *in vivo*.

Introduction

Basophils are the rarest granulocytes and represent less than 1% of peripheral blood leukocytes. They were often considered erroneously as minor relatives or blood-circulating precursors of tissue-resident mast cells because of some similarity between them.¹⁻³ It is now accepted well that basophils and mast cells are distinct cell lineages.⁴ Recent studies have identified crucial roles for basophils, distinct from those played by mast cells, in immune responses including allergy and protective immunity.⁵⁻⁸ In response to various stimuli, basophils can readily secrete large quantities of cytokine IL-4 that plays versatile roles in immunity through regulating other cells.⁹ In contrast to IL-4, functional roles of other molecules produced by basophils in immune responses remain ill-defined.

Both basophils and mast cells possess basophilic granules stained in violet with basic aniline dyes, as described first by Paul Ehrlich more than 130 years ago. These granules store preformed materials such as histamine and proteases, and release them upon activation.^{1,2} Ten serine proteases have been identified in murine mast cells, including 6 chymases (mouse mast cell protease (mMCP)-1, mMCP-2, mMCP-4, mMCP-5, mMCP-9, mMCP-10) and 4 tryptases (mMCP-6, mMCP-7, mMCP-11, mTMT).¹⁰⁻¹⁴ mMCP-11 is the most recently discovered member of the mouse mast cell tryptase family and the most distantly related to other members in terms of the amino acid sequence.¹⁵ We previously demonstrated that among the tryptase family, mMCP-11 is preferentially expressed by basophils rather than mast cells whereas mMCP-6 and mMCP-7 are expressed by mast cells but not basophils.¹⁶ None of mast cell chymases are expressed by basophils while the expression of granzyme B-like protease mMCP-8 is confined to basophils.^{16,17} Thus, even though basophils and mast cells possess apparently similar-looking basophilic granules, the repertoire of serine proteases stored in granules is distinct between them, suggesting that

mMCP-8 and mMCP-11 might be involved in non-redundant functions of basophils. We previously reported that the subcutaneous injection of recombinant mMCP-11 induces edematous skin swelling with increased microvascular permeability, suggesting that mMCP-11 may contribute to the development of basophil-mediated inflammatory responses.¹⁸

The nonredundant role of basophils *in vivo* has been clearly demonstrated in the IgE-CAI response.¹⁹⁻²¹ A single intradermal administration of allergens in the ear skin of IgE-sensitized mice induces three consecutive waves of ear swelling. The first two waves are mast cell-dependent, immediate-type allergic reactions with early- and late-phase ear swelling. The third wave of ear swelling starts on day 2 with a peak on day 4 postchallenge, accompanied by massive infiltration of eosinophils, neutrophils, monocytes and macrophages in the skin lesion. We designated this delayed-onset response IgE-CAI.¹⁹ Mast cells are dispensable for IgE-CAI while basophil depletion abolishes the development of IgE-CAI even though basophils represent only 1 or 2% of infiltrates in the skin lesion. Thus, basophils but not mast cells play an essential role in IgE-CAI.¹⁹ However, it remains to be determined how basophils elicit allergic inflammation in the IgE-CAI response.

In the present study, we generated mMCP-11-deficient (KO) mice to clarify the functional role of mMCP-11 *in vivo*. KO mice showed ameliorated IgE-CAI response with reduction of swelling, vascular permeability and leukocyte infiltration in the skin lesion even though their basophil development remained unimpaired. *In vitro* experiments suggested that mMCP-11-mediated proteolytic products of a serum protein(s), rather than mMCP-11 itself, trigger migration of leukocytes, including basophils, via a G protein-coupled receptor(s). Thus, the present study has uncovered the crucial role for basophil tryptase mMCP-11 in allergic inflammation.

Materials and Methods

Mice

C57BL/6 mice (7-9 weeks old) were purchased from Japan SLC. To generate mMCP-11-deficient mice, the targeting vector for replacing the exons 1, 2 and 3 of the *Mcpt11* (*Prss34*) gene with the loxP-PGK-Neomycin^R-loxP cassette (Gene Bridges) was constructed by Red/ET recombination method (Gene Bridges), and transfected into C57BL/6 ES cells (UNITECH Co., Ltd.). The resultant knock-out mice were further crossed with CAG-Cre mice (provided by J. Miyazaki, Osaka University)²² to remove floxed Neomycin^R cassette from the *Mcpt11* allele. All animal studies were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Antibodies and recombinant mMCP-11

The following Abs were purchased from BioLegend: biotinylated anti-CD49b; FITC-conjugated anti-CD49b, anti-F4/80; PE-conjugated anti-FcεRIα, anti-CD45 (30-F11); APC-conjugated anti-CD200R3, anti-Ly-6G; Pacific Blue-conjugated anti-CD117, anti-CD11b. PE-conjugated anti-Siglec-F (E50-2440) was from BD Biosciences. Anti-mMCP-11 and anti-mMCP-8 were established as reported previously.¹⁶ IgE (IGELb4, ATCC-Tib141) specific to 2,4,6-trinitrophenol (TNP) and anti-CD16/32 (2.4G2) were prepared in our laboratory. Recombinant mMCP-11 and its protease-dead mutant (Ser²⁰⁰ was substituted with Ala) were prepared as described previously.¹⁸

Generation of bone marrow-derived basophils

Mouse bone marrow-derived basophils (BMBAs) were generated as described previously.¹⁶ Briefly, bone marrow cells were cultured in the presence of 300 pg/ml recombinant mouse IL-3 (BioLegend) for 7 days, followed by purification of CD49b⁺ cells using the IMag system

with biotinylated anti-CD49b (DX5) and streptavidin-conjugated magnetic particles (BD Biosciences). The purity of c-kit⁺CD49b⁺CD200R3⁺ BMBAs was >95% after magnetic purification.

Induction of IgE-CAI and passive cutaneous anaphylaxis (PCA)

IgE-CAI was induced as described previously.¹⁹ In brief, mice were sensitized with intravenous injection of 300 µg anti-TNP IgE, and challenged on the following day with intradermal injection of 10 µg TNP-OVA and control OVA into the right and left ear, respectively. Ear thickness was measured at a 24 h-interval for 7 days (Δ ear thickness; right - left). In some experiments, mice were treated with topical administration of 200 µg SC560, meloxicam, 100 µg celecoxib, or vehicle (ethanol) alone once a day during the development of IgE-CAI. For Evan Blue leakage analysis, mice were intravenously injected with 0.5% Evans blue dye in 100 µl PBS on day 3 of the IgE-CAI response. Two hours after the dye injection, ears were excised and incubated in 0.7 ml formamide at 63°C for overnight to extract the dye leaked into the skin. The amount of the dye in the extracts was determined by a spectrophotometer at 620 nm. PCA was induced as previously described with some modifications.²³ Briefly, mice were locally sensitized with intradermal injection of 100 ng anti-TNP IgE and control PBS into the right and left ear, respectively. On the following day, mice were challenged with intravenous injection of 200 µg TNP-OVA in 200 µl of PBS containing 0.25% Evans blue dye. Thirty minutes later, the treated ears were excised and subjected to measurement of leaked dye.

Flow cytometric, histopathological and Western blot analyses

Single cell suspensions were obtained from ear skins by treatment with 125 U/ml collagenase (Wako) at 37°C for 2 h. After pretreatment with anti-CD16/32 (2.4G2) and normal rat serum

to avoid the non-specific binding of irrelevant Abs, cells were stained with indicated combination of Abs on ice for 30 min, and analyzed by FACSCanto (BD Biosciences). Each cell lineages were defined as follows: basophils (c-kit⁺CD49b⁺CD200R3⁺), eosinophils (Siglec-F⁺SSC^{high}), neutrophils (Ly-6G⁺), monocyte-macrophages (F4/80⁺CD11b⁺ among cells in which both eosinophils and neutrophils were excluded). For histopathological examination, ear specimens were fixed and embedded in paraffin, and sections were stained with hematoxylin and eosin. For Western blot analysis, BMBAs were lysed with RIPA buffer containing protease inhibitor cocktail (Thermo Scientific), and total cell lysates were subjected to SDS-PAGE, followed by immunoblot analysis with indicated antibodies.

Quantitative PCR

Total RNA was extracted from tissues or isolated cells by RNeasy Mini Kit (QIAGEN), followed by cDNA synthesis with reverse transcription using oligo dT and random primers. Quantitative PCR of the cDNA was performed by using following primer sets: 5'-CTGGCTCCTGTTCTCAGTCT-3' and 5'-GCTGTGCTCCATGTCGTAGAG-3' for *Mcpt11* (*Prss34*); 5'-CACTGGTCAATGACATCATGCTCC-3' and 5'-GTCAGACAAAGTGCAATTGGCCAAC-3' for *Mcpt8*; 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-TGTAGACCATGTAGTTGAGGTCA-3' for *Gapdh*. Relative gene expression levels were calculated using standard curves generated by serial dilutions of each cDNA standard and normalized by *Gapdh* expression levels.

In vitro stimulation of basophils

BMBAs were sensitized with 1 µg/ml anti-TNP IgE for overnight, and then stimulated with 300 ng/ml (for degranulation assay) or 400 ng/ml (for measurement of cytokines) of TNP-OVA or control OVA. Degranulation of BMBAs was determined by measurement of

β -hexosaminidase release as previously described.²⁴ For measurement of cytokine production, BMBAs sensitized with IgE were stimulated with antigens for 24 h. The concentration of IL-4 and IL-6 in the culture supernatants was measured by ELISA (BioLegend) and cytometric bead array (BD Biosciences), respectively.

Transwell migration assay

Leukocytes used for this assay were prepared as follows. Neutrophils were prepared from the bone marrow by using 62% Percoll gradient as described previously.²⁵ Eosinophils were isolated from the peritoneum of mice that had been treated for 7 days with daily intraperitoneal administration of IL-5.²⁶ Macrophages were isolated from the peritoneum of mice that had been treated with intraperitoneal administration of 1 ml of 4% thioglycollate broth 3 days before. BMBAs were used as basophils. The transwell apparatus (Kurabo) consisted of the upper and lower chambers separated by a membrane with 3 μ m (for neutrophils) or 5 μ m (for other cell types) pore size. Leukocytes (5×10^5 cells) were placed into the upper chamber while 10 μ g/ml recombinant mMCP-11, its protease-dead mutant, control BSA or chemokines (MIP-2 100 ng/ml, Eotaxin 200 ng/ml, or MCP-1 200 ng/ml) was included in culture medium of the lower chamber. Ninety minutes (for neutrophils) or 2 h (for other cell types) after incubation at 37°C, the number of cells migrating into the lower chamber was counted. In some experiments, cells were pretreated with 200 ng/ml (for BMBAs) or 100 ng/ml (for other cell types) of pertussis toxin (PTX, Sigma-Aldrich) for 1 h before the transwell migration assay. Recombinant tetramerized human β -tryptase was purchased from Promega. Bovine trypsin was from Sigma-Aldrich.

Statistical analysis

Statistical analysis was performed with unpaired Student's t test. A p value <0.05 was

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considered statistically significant.

Results

Generation of mMCP-11-deficient mice

To explore the role of mMCP-11 *in vivo*, we have established mMCP-11-deficient (KO) mice by means of targeting the exons 1, 2 and 3 of the mMCP-11-coding gene *Prss34* (Figure 1A). c-kit⁺CD49b⁺CD200R3⁺FcεRIα⁺ basophils were detected in the bone marrow, spleen, and peripheral blood of KO mice as many as in wild-type (WT) mice (Figure 1B-C). The number of other hematopoietic cells including mast cells was also comparable between WT and KO mice (Supplemental Figure 1). When cultured with IL-3, bone marrow cells isolated from WT and KO mice generated comparable numbers of c-kit⁺CD49b⁺CD200R3⁺ basophils (bone marrow-derived basophils, BMBAs) (Supplemental Figure 2), suggesting unimpaired development of basophils in KO mice. The lack of mMCP-11 expression in KO basophils was confirmed at both mRNA and protein levels (Figure 1D-E) while the expression of basophil-specific mMCP-8 remained intact in KO basophils (Figure 1D-E). When stimulated with IgE plus antigens, KO BMBAs degranulated (Figure 1F) and produced cytokines IL-4 and IL-6 (Figure 1G) as much as did WT BMBAs. Moreover, WT and KO BMBAs could migrate toward chemokine MIP-2 to a comparable extent (Figure 1H). Thus, the deletion of the *Prss34* gene did not seem to give any adverse effect on the homeostasis and FcεRI- or chemokine-mediated activation of basophils.

mMCP-11 KO mice show ameliorated IgE-CAI response with the reduction in swelling and microvascular permeability of the skin

We previously reported that basophils crucially contribute to the development of IgE-CAI in the ear skin,¹⁹ and that basophil depletion abolishes the IgE-CAI response but not the mast cell-mediated immediate-type reaction.²⁰ However, it remained to be determined what

molecules derived from basophils are responsible for the development of IgE-CAI. To examine the role of mMCP-11 in IgE-CAI, WT and KO mice were passively sensitized with hapten TNP-specific IgE and challenged with intradermal injection of the corresponding allergen TNP-OVA or control OVA. In both mouse strains, the TNP-OVA administration induced ear swelling with a peak on day 4 postchallenge while the extent of ear swelling in KO mice was approximately half of that in WT mice (Figure 2A). Moreover, the increase of microvascular permeability in the skin lesion was significantly lower in KO mice (Figure 2B). The increase of permeability in WT mice was suppressed by the treatment with meloxicam (COX-2 inhibitor) but not SC560 (COX-1 inhibitor) whereas neither of them significantly affected the permeability in KO mice (Figure 2C), suggesting that mMCP-11 increased permeability in the skin lesion through COX-2. In accordance with this, IgE-CAI ear swelling in WT but not KO mice was significantly inhibited by the treatment with COX-2 inhibitor, either meloxicam or celecoxib (Figure 2D and Supplemental Figure 3). The tryptase activity of mMCP-11 remained unaffected by these inhibitors (data not shown). Thus, basophil-derived mMCP-11 appeared to contribute to ear swelling in the IgE-CAI response in part by means of COX-2-mediated vascular hyperpermeability.

Cellular infiltration in the IgE-CAI skin lesion is decreased in KO mice

Histopathological examination of ear skin specimens indicated the reduction of cellular infiltration in the IgE-CAI skin lesion of KO mice compared to WT mice (Figure 3A). In accordance with this, flow cytometric analysis revealed that the number of total cells isolated from the skin lesion, mainly CD45⁺ hematopoietic cells, was reduced in the KO skin lesion to less than half of that in the WT skin lesion (Figure 3B-C). Even though KO basophils by themselves showed unimpaired migration toward MIP-2 *in vitro* (Figure 1H), the number of basophils infiltrating the IgE-CAI skin lesion was significantly reduced in KO mice (Figure

3C). This was also the case for the number of eosinophils, neutrophils and monocytes/macrophages in the skin lesion of KO mice (Figure 3C). Thus, mMCP-11 appeared to be involved in the infiltration and accumulation of these leukocytes in the IgE-CAI skin lesion.

We previously reported that one single cutaneous injection of 10 µg recombinant mMCP-11 elicited transient ear swelling with increased microvascular permeability whereas no significant infiltration of leukocytes was detected in the injection site.¹⁸ We assumed that this difference between the IgE-CAI skin lesion and the mMCP-11-injected skin could be attributed to different duration of mMCP-11 action. In contrast to the single injection, mMCP-11 likely accumulates in the IgE-CAI skin lesion because basophils are continuously recruited there and activated by allergens to release mMCP-11. To mimic this situation *in vivo*, we treated WT mice with repeated intradermal injection of recombinant mMCP-11 in the ear skin. Three times injection of mMCP-11 at a 24 h-interval indeed elicited the infiltration and accumulation of neutrophils, eosinophils, basophils and monocytes/macrophages (Figure 4) along with transient ear swelling induced by each single injection (Supplemental Figure 4A). The extent of mMCP-11-induced ear swelling was not affected by depletion of basophils, demonstrating that exogenous mMCP-11 can induce this reaction in a basophil-independent manner (Supplemental Figure 4A-B). Importantly, mMCP-11 carrying a mutation in the active site of tryptase did not show such a leukocyte recruiting ability (Figure 4), indicating that the protease activity of mMCP-11 is essential for this function.

Proteolytic products generated by mMCP-11 elicit leukocyte migration via a G protein-coupled receptor *in vitro*

To explore mechanisms underlying the mMCP-11-mediated leukocyte recruitment, we first examined the possibility that mMCP-11 can directly stimulate leukocytes to induce their

migration, by using the transwell migration assay. Basophils, eosinophils, neutrophils, macrophages were individually placed in the upper chamber while mMCP-11 was included in the lower chamber. Enzymatically active but not inactive mMCP-11 elicited transwell migration of eosinophils, basophils, and macrophages, but not neutrophils (Figure 5A), demonstrating that the protease activity of mMCP-11 is essential for triggering their migration. In contrast, no significant migration of T or B cells was detected under these conditions (Supplemental Figure 5). Moreover, the addition of meloxicam to the transwell culture showed no significant effect on the mMCP-11-induced basophil migration (data not shown), suggesting that mMCP-11 elicited leukocyte migration in a COX-2-independent manner. Of note, the pretreatment of eosinophils, basophils and macrophages with pertussis toxin almost completely abolished the mMCP-11-induced migration (Figure 5B). These results suggested that mMCP-11 functioned as a protease and triggered leukocyte migration through a G protein-coupled receptor(s).

To clarify how the protease activity of mMCP-11 contributes to the leukocyte migration, we added a protease inhibitor, nafamostat, to the mMCP-11-containing culture medium at the different timing in the transwell migration assay (Figure 6A). As expected, the migration of basophils, eosinophils and macrophages was strongly reduced to a level as low as 10-15% of control, when nafamostat was added simultaneously with mMCP-11 to the lower chamber (compare Experiments 1 and 2 in Figure 6A). In contrast, when nafamostat was added to the lower chamber to inactivate mMCP-11 after the culture medium had been incubated with mMCP-11 at 37°C for 2 h prior to the transwell migration assay, leukocyte migration was elicited to an extent as much as 60-75% of control (Experiment 3 in Figure 6A). In addition, serum depletion from culture medium completely abolished the basophil migration induced by mMCP-11 but not by MIP-2 (Figure 6B). These results strongly suggested that mMCP-11 acted on a serum protein(s) present in culture medium, and its

proteolytic product rather than mMCP-11 itself promoted leukocyte migration. The less extent of migration in Experiment 3 compared to Experiment 1 could be attributed to the degradation of proteolytic products and no supply of new products during the transwell migration assay due to the nafamostat-mediated inactivation of mMCP-11 in Experiment 3. Importantly, the leukocyte migration in Experiment 3 was abolished by the pretreatment of leukocytes with pertussis toxin (Figure 6C), implying that proteolytic products generated by mMCP-11 induced leukocyte migration via a G protein-coupled receptor(s).

Discussion

Previous study illustrated that basophils and mast cells display distinct repertoire of serine proteases stored in secretory granules.¹⁶ Among the tryptase members of the mMCP family, mMCP-11 is preferentially expressed by basophils rather than mast cells whereas mMCP-6 and mMCP-7 are expressed only by mast cells in mice.¹⁶ Extensive investigation on mMCP-6 and mMCP-7 demonstrated that they play important roles in protective immunity against bacterial and parasitic infections^{27,28} and also in the development of inflammatory diseases such as autoimmune arthritis^{29,30} and colitis.³¹ In contrast, the functional significance of basophil tryptase mMCP-11 remained enigmatic. We showed in the present study that mMCP-11 is a crucial effector molecule in the development of the IgE-CAI allergic inflammation. This is the first demonstration that the basophil-derived protease plays a significant role *in vivo*.

mMCP-11 KO mice showed much less severe IgE-CAI response, compared to WT mice, characterized by milder ear swelling with reduced microvascular hyperpermeability and leukocyte infiltration. We previously reported that basophil ablation during the progress of IgE-CAI resulted in drastic reduction of eosinophils and neutrophils infiltrating the skin lesion, concomitant with basophil elimination from there, suggesting the possible contribution of basophils to the eosinophil and neutrophil infiltration.^{20,21} The present study clearly demonstrated that basophil-derived mMCP-11 induces the infiltration of leukocytes, including basophils, eosinophils, neutrophils and macrophages. Basophil infiltration in the IgE-CAI skin lesion was still observed even in KO mice albeit to a lesser extent than in WT mice. Therefore, mMCP-11 is not essential for basophil infiltration in IgE-CAI. Our findings rather suggest the following scenario: when basophils migrate into the skin lesion and get activated by allergens, they release mMCP-11 which in turn recruits more and more basophils

together with other leukocytes. If this is the case, the ablation of mMCP-11 in basophils should reduce the infiltration of leukocytes, including basophils, just as observed in KO mice.

The detailed molecular mechanism by which mMCP-11 triggers the leukocyte migration remains to be determined in future studies, as for the case of mast cell tryptase and chymase-induced chemotaxis of leukocytes.³²⁻³⁵ Nevertheless, our findings in the present study illustrated that the protease activity of mMCP-11 is essential for it, and a G protein-coupled receptor(s) expressed on leukocytes is involved in the migration. mMCP-11 does not seem to directly act on leukocytes to induce their migration. Most likely, mMCP-11 cleaves a serum protein(s), and its proteolytic product attracts basophils, eosinophils, and macrophages via a G protein-coupled receptor(s). In contrast, no significant migration of neutrophils was induced by mMCP-11 in the transwell migration assay while intradermal administration of mMCP-11 elicited neutrophil infiltration in the skin. In the latter case, quantitative PCR analysis showed slightly upregulated expression of neutrophil-attracting chemokines such as CXCL1 and CXCL2 among others but without statistical significance (data not shown). The possible contribution of mMCP-11-elicited production of chemokines in the skin lesion to the migration of neutrophils and other leukocytes needs to be further explored.

mMCP-11 is highly expressed by all basophils, and also by a small subset of mast cells albeit to a much lesser extent.¹⁶ As for the case of basophils, the number of mast cells in the skin and peritoneum of KO mice was comparable to that of WT mice (Supplemental Figure 1), suggesting the unimpaired development of mast cells in the absence of mMCP-11. Moreover, the extent of microvascular leakage in the IgE-mediated passive cutaneous anaphylaxis response was comparable between KO and WT mice (Supplemental Figure 6), indicating intact degranulation of KO mast cells. In accordance with this, the mast cell-dependent, immediate-type (early- and late-phase) ear swelling ahead of the IgE-CAI

response was normally elicited in KO mice (data not shown) in contrast to the amelioration of IgE-CAI. Thus, mMCP-11 did not seem to contribute to the mast cell-mediated immediate-type reactions, as far as we examined, while the functional significance of mMCP-11 in mast cell-mediated, chronic inflammation, if any, remains to be investigated.

The hSPL-4 gene on human chromosome 16p13.3 is assigned to be the human ortholog of mMCP-11 gene located on mouse chromosome 17A3.3.¹⁵ However, the hSPL-4 gene possesses a premature translational-termination codon, and do not encode enzymatically active enzyme, unlike the mMCP-11 gene. Of note, human basophils express α - and β -tryptases, albeit to a lesser extent than do mast cells,^{36,37} suggesting that α - and β -tryptases might be functional ortholog of mMCP-11 in human basophils. Indeed, repeated intradermal injection of human β -tryptase elicited leukocyte infiltration into the skin of mice (Supplemental Figure 7). β -tryptase also induced transwell migration of basophils *in vitro* via a G protein-coupled receptor(s) in a manner dependent on its protease activity and the presence of serum (Supplemental Figures 8-9) as did mMCP-11. Importantly, trypsin showed little or no such ability of basophil migration (Supplemental Figure 8A) in spite of higher tryptase activity than β -tryptase and mMCP-11 as assessed by using a standard tryptase substrate (data not shown), suggesting that not all typtases might share this property of mMCP-11.

In conclusion, we generated mMCP-11-deficient mice in the present study and demonstrated that basophil tryptase mMCP-11 plays a crucial role in the development of the IgE-CAI allergic response through the promotion of microvascular leakage and leukocyte infiltration in a tryptase activity-dependent manner. This study has cast new light on the functional significance of proteases produced and released by basophils.

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Authorship

Contribution: M.I. and K.T. performed experiments; H.D. contributed to the initial study; M.F. and S.Y. generated mMCP-11-deficient mice; S.S. generated rmMCP-11; S.Y. provided helpful suggestions; M.I., Y.Y. and H.K. wrote the manuscript; Y.Y. and H.K. designed and supervised the study.

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References

1. Galli SJ. Mast cells and basophils. *Curr Opin Hematol*. 2000;7(1):32-39.
2. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol*. 2010;125(2 Suppl 2):S73-80.
3. Falcone FH, Haas H, Gibbs BF. The human basophil: a new appreciation of its role in immune responses. *Blood*. 2000;96(13):4028-4038.
4. Huang H, Li Y, Liu B. Transcriptional regulation of mast cell and basophil lineage commitment. *Semin Immunopathol*. 2016;38(5):539-548.
5. Sokol CL, Medzhitov R. Emerging functions of basophils in protective and allergic immune responses. *Mucosal Immunol*. 2010;3(2):129-137.
6. Siracusa MC, Comeau MR, Artis D. New insights into basophil biology: initiators, regulators, and effectors of type 2 inflammation. *Ann N Y Acad Sci*. 2011;1217:166-177.
7. Karasuyama H, Mukai K, Obata K, Tsujimura Y, Wada T. Nonredundant roles of basophils in immunity. *Annu Rev Immunol*. 2011;29:45-69.
8. Voehringer D. Protective and pathological roles of mast cells and basophils. *Nat Rev Immunol*. 2013;13(5):362-375.
9. Yamanishi Y, Karasuyama H. Basophil-derived IL-4 plays versatile roles in immunity. *Semin Immunopathol*. 2016;38(5):615-622.
10. Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. *Immunol Rev*. 2007;217:141-154.
11. Miller HR, Pemberton AD. Tissue-specific expression of mast cell granule serine proteinases and their role in inflammation in the lung and gut. *Immunology*. 2002;105(4):375-390.

12. Hunt JE, Friend DS, Gurish MF, et al. Mouse mast cell protease 9, a novel member of the chromosome 14 family of serine proteases that is selectively expressed in uterine mast cells. *J Biol Chem*. 1997;272(46):29158-29166.
13. Hallgren J, Pejler G. Biology of mast cell tryptase. An inflammatory mediator. *FEBS J*. 2006;273(9):1871-1895.
14. McNeil HP, Adachi R, Stevens RL. Mast cell-restricted tryptases: structure and function in inflammation and pathogen defense. *J Biol Chem*. 2007;282(29):20785-20789.
15. Wong GW, Yasuda S, Morokawa N, Li L, Stevens RL. Mouse chromosome 17A3.3 contains 13 genes that encode functional tryptic-like serine proteases with distinct tissue and cell expression patterns. *J Biol Chem*. 2004;279(4):2438-2452.
16. Ugajin T, Kojima T, Mukai K, et al. Basophils preferentially express mouse Mast Cell Protease 11 among the mast cell tryptase family in contrast to mast cells. *J Leukoc Biol*. 2009;86(6):1417-1425.
17. Poorafshar M, Helmby H, Troye-Blomberg M, Hellman L. MMCP-8, the first lineage-specific differentiation marker for mouse basophils. Elevated numbers of potent IL-4-producing and MMCP-8-positive cells in spleens of malaria-infected mice. *Eur J Immunol*. 2000;30(9):2660-2668.
18. Yamagishi H, Mochizuki Y, Hamakubo T, et al. Basophil-derived mouse mast cell protease 11 induces microvascular leakage and tissue edema in a mast cell-independent manner. *Biochem Biophys Res Commun*. 2011;415(4):709-713.
19. Mukai K, Matsuoka K, Taya C, et al. Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity*. 2005;23(2):191-202.
20. Obata K, Mukai K, Tsujimura Y, et al. Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood*. 2007;110(3):913-920.

21. Egawa M, Mukai K, Yoshikawa S, et al. Inflammatory monocytes recruited to allergic skin acquire an anti-inflammatory M2 phenotype via basophil-derived interleukin-4. *Immunity*. 2013;38(3):570-580.
22. Sakai K, Miyazaki J. A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission. *Biochem Biophys Res Commun*. 1997;237(2):318-324.
23. LiHua L, Yoshikawa S, Ohta T, et al. Large particulate allergens can elicit mast cell-mediated anaphylaxis without exit from blood vessels as efficiently as do small soluble allergens. *Biochem Biophys Res Commun*. 2015;467(1):70-75.
24. Nei Y, Obata-Ninomiya K, Tsutsui H, et al. GATA-1 regulates the generation and function of basophils. *Proc Natl Acad Sci U S A*. 2013;110(46):18620-18625.
25. Izawa K, Kitaura J, Yamanishi Y, et al. Functional analysis of activating receptor LMIR4 as a counterpart of inhibitory receptor LMIR3. *J Biol Chem*. 2007;282(25):17997-18008.
26. Karasuyama H, Melchers F. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. *Eur J Immunol*. 1988;18(1):97-104.
27. Thakurdas SM, Melicoff E, Sansores-Garcia L, et al. The mast cell-restricted tryptase mMCP-6 has a critical immunoprotective role in bacterial infections. *J Biol Chem*. 2007;282(29):20809-20815.
28. Shin K, Watts GF, Oettgen HC, et al. Mouse mast cell tryptase mMCP-6 is a critical link between adaptive and innate immunity in the chronic phase of *Trichinella spiralis* infection. *J Immunol*. 2008;180(7):4885-4891.
29. McNeil HP, Shin K, Campbell IK, et al. The mouse mast cell-restricted tetramer-forming tryptases mouse mast cell protease 6 and mouse mast cell protease 7 are

critical mediators in inflammatory arthritis. *Arthritis Rheum*. 2008;58(8):2338-2346.

30. Shin K, Nigrovic PA, Crish J, et al. Mast cells contribute to autoimmune inflammatory arthritis via their tryptase/heparin complexes. *J Immunol*. 2009;182(1):647-656.

31. Hamilton MJ, Sinnamon MJ, Lyng GD, et al. Essential role for mast cell tryptase in acute experimental colitis. *Proc Natl Acad Sci U S A*. 2011;108(1):290-295.

32. Huang C, Friend DS, Qiu WT, et al. Induction of a selective and persistent extravasation of neutrophils into the peritoneal cavity by tryptase mouse mast cell protease 6. *J Immunol*. 1998;160(4):1910-1919.

33. Tani K, Ogushi F, Kido H, et al. Chymase is a potent chemoattractant for human monocytes and neutrophils. *J Leukoc Biol*. 2000;67(4):585-589.

34. Huang C, De Sanctis GT, O'Brien PJ, et al. Evaluation of the substrate specificity of human mast cell tryptase beta I and demonstration of its importance in bacterial infections of the lung. *J Biol Chem*. 2001;276(28):26276-26284.

35. Terakawa M, Tomimori Y, Goto M, Hayashi Y, Oikawa S, Fukuda Y. Eosinophil migration induced by mast cell chymase is mediated by extracellular signal-regulated kinase pathway. *Biochem Biophys Res Commun*. 2005;332(4):969-975.

36. Foster B, Schwartz LB, Devouassoux G, Metcalfe DD, Prussin C. Characterization of mast-cell tryptase-expressing peripheral blood cells as basophils. *J Allergy Clin Immunol*. 2002;109(2):287-293.

37. Jogie-Brahim S, Min HK, Fukuoka Y, Xia HZ, Schwartz LB. Expression of alpha-tryptase and beta-tryptase by human basophils. *J Allergy Clin Immunol*. 2004;113(6):1086-1092.

Figure legends

Figure 1. Generation of mMCP-11-deficient mice

(A) A strategy of gene targeting for the generation of mMCP-11-deficient mice. (B) Flow cytometric analysis of indicated surface markers on bone marrow cells isolated from wild-type (WT) and mMCP-11-deficient (KO) littermate mice. c-kit-negative cells were gated and displayed. (C) The number of basophils in the bone marrow, spleen, and peripheral blood of WT and KO mice (mean $\pm$ SD, n=3 each). (D) Quantitative PCR analysis for the expression of *Mcpt11* and *Mcpt8* mRNAs in c-kit⁻CD49b⁺CD200R3⁺ basophils isolated from the bone marrow of WT and KO mice. (E) Western blot analysis for the expression of indicated proteins in bone marrow-derived basophils (BMBAs) generated from WT and KO mice. (F-G) BMBAs generated from WT (open bars) and KO (filled bars) mice were sensitized with anti-TNP IgE and then stimulated with TNP-OVA or control OVA. In F, the extent of degranulation was examined by using β -hexosaminidase release assay (mean $\pm$ SD, n=3). In G, the concentration of cytokines IL-4 and IL-6 in culture supernatants was measured by using ELISA and cytometric bead array, respectively (mean $\pm$ SD, n=3). (H) Migration ability of WT (open bars) and KO (filled bars) BMBAs in response to chemokine MIP-2 (100 ng/ml) or control PBS was examined by using transwell migration assay. The number of cells migrated from the upper to lower chamber during 2 h-culture was counted (mean $\pm$ SD, n=3 each). Data shown in B-H are representative of at least 3 independent experiments. n.s., not significant.

Figure 2. mMCP-11-deficient mice show ameliorated IgE-CAI response in the skin

WT and KO mice were sensitized with anti-TNP IgE and challenged with intradermal administration of TNP-OVA or control OVA in their ears to induce IgE-CAI. (A) Time course of ear swelling (Δ ear thickness: TNP-OVA - OVA) is shown (mean $\pm$ SD, n=4 each).

(B) Three days after the allergen challenge, mice were treated with intravenous injection of Evans blue, and 2 h later their ears were subjected to photography followed by the measurement of leaked dye (mean $\pm$ SD, n=3-4 each). (C-D) WT and KO mice were treated, once a day during IgE-CAI, with topical administration of SC560, meloxicam or vehicle (ethanol) alone. In C, mice were subjected to the Evans blue leakage analysis as in B. Δ Evans blue leakage (TNP-OVA – OVA) is shown (mean $\pm$ SD, n=3-4 each). In D, time course of ear swelling (Δ ear thickness: TNP-OVA – OVA) is shown (mean $\pm$ SD, n=6 each). Data shown in A-D are representative of at least 3 independent experiments. *p<0.05; **p<0.01; ***p<0.001; n.s., not significant.

Figure 3. Cellular infiltration in the IgE-CAI skin lesion is decreased in mMCP-11-deficient mice

WT and KO mice were treated as in Fig. 2 to induce IgE-CAI. (A) Hematoxylin and eosin-stained specimens of IgE-CAI skin lesions on day 3 postchallenge. Scale bars, 200 μ m. (B-C) The number of total, CD45⁺ cells, and indicated cell types isolated from the TNP-OVA- (filled bars) or control OVA-administered (open bars) ear skin on day 3 postchallenge is shown (mean $\pm$ SD, n=5-6 each). Data shown are representative of three independent experiments. *p<0.05, **p<0.01.

Figure 4. Repeated intradermal administration of recombinant mMCP-11 in the ear skin induces leukocyte infiltration in a protease activity-dependent manner

WT mice were treated three times at a 24 h-interval with intradermal administration of 10 μ g wild-type (WT, black bars), inactive mutant (MT, gray bars) mMCP-11 or control BSA (white bars) each time. The number of total, CD45⁺ cells, and indicated cell types isolated from the ear skin at 4 h postchallenge is shown (mean $\pm$ SD, n=3 each). Data shown are

representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$.

Figure 5. mMCP-11 induces leukocyte migration *in vitro*

The ability of mMCP-11 to induce leukocyte migration was examined by using the transwell system. Indicated types of cells (5×10^5 cells) were placed in the upper chamber while WT (black bars) or MT (gray bars) mMCP-11, control BSA (white bars) or indicated chemokines (hatched bars) was included in culture medium of the lower chamber. (A) The number of cells recovered from the lower chamber after 1.5 h (for neutrophils)- or 2 h (for other cell types)-incubation at 37°C is shown (mean $\pm$ SD, $n=3$ each). (B) Indicated types of cells were pretreated with pertussis toxin (PTX, vertical striped bars) or control PBS (black bars) for 1 h prior to the transwell migration assay, and analyzed as in A (mean $\pm$ SD, $n=3$ each). Data shown in A-B are representative of at least three independent experiments. ** $p < 0.01$; *** $p < 0.001$; n.s., not significant.

Figure 6. Proteolytic products generated by mMCP-11 induce leukocyte migration via a G protein-coupled receptor(s)

(A) The effect of nafamostat on mMCP-11-elicited leukocyte migration was examined in the transwell migration assay. mMCP-11 was added to the lower chamber together with nafamostat (Experiment 2) or control vehicle DMSO alone (Experiment 1), and then the upper chamber containing leukocytes was placed onto the lower chamber to start the migration assay. In Experiment 3, nafamostat was added to the lower chamber after the culture medium in the lower chamber had been incubated with mMCP-11 at 37°C for 2 h prior to the transwell migration assay. The number of cells recovered from the lower chamber at the end of the migration assay is shown as in Figure 5 (mean $\pm$ SD, $n=3$ each). (B) The transwell migration assay of BMBAs was performed as in Figure 5 by using culture medium

sufficient (including 10% FCS) or deficient (including 0.1% BSA) for serum in both upper and lower chambers. The number of cells migrating into the lower chamber after 2 h-incubation at 37°C is shown (mean $\pm$ SD, n=3 each). (C) As in Experiment 3 shown in A, nafamostat was added to the lower chamber after the culture medium had been incubated with mMCP-11 for 2 h prior to the transwell migration assay while indicated types of cells were pretreated with PTX or control PBS for 1 h and transferred to the upper chamber. The number of cells recovered from the lower chamber at the end of the migration assay is shown (mean $\pm$ SD, n=3 each). Data shown in A-C are representative of at least three independent experiments. **p<0.01; ***p<0.001; n.s., not significant.

Figure 1

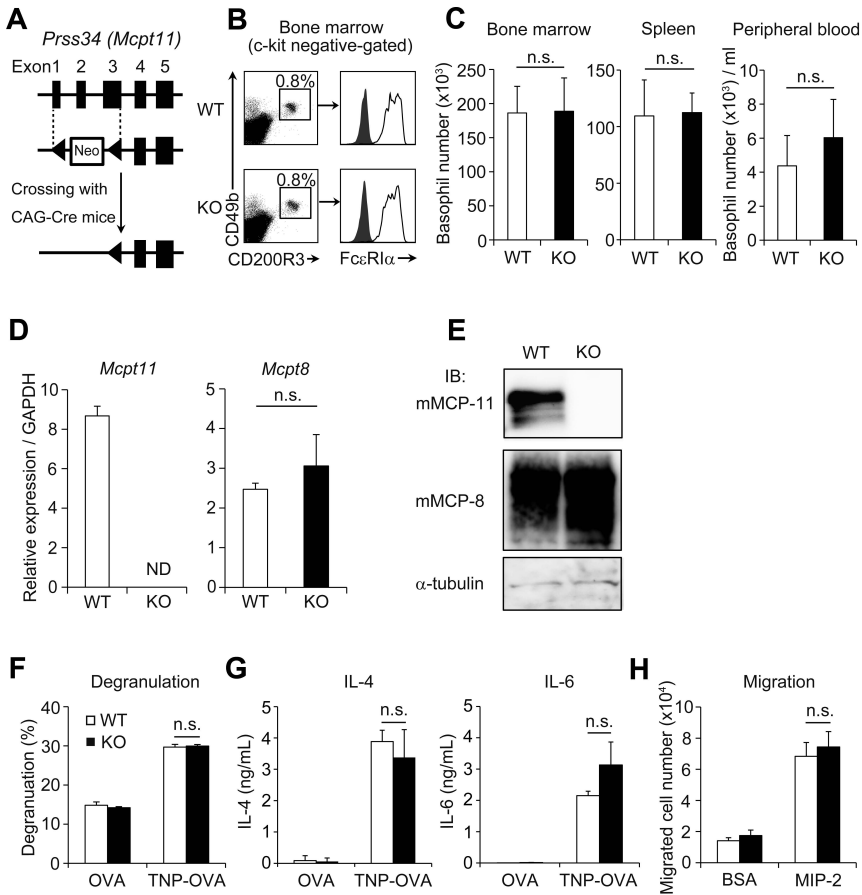


Figure 2

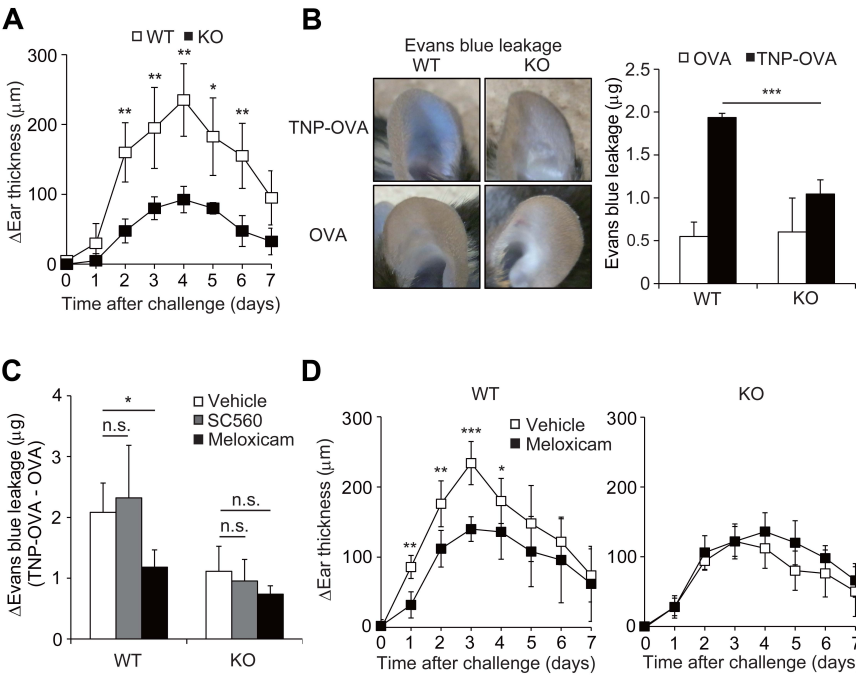


Figure 3

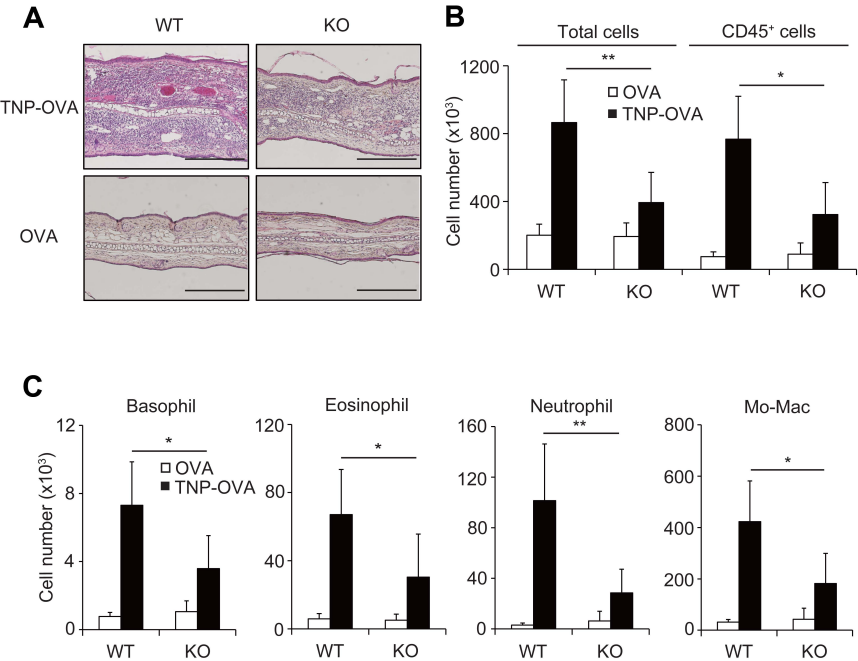


Figure 4

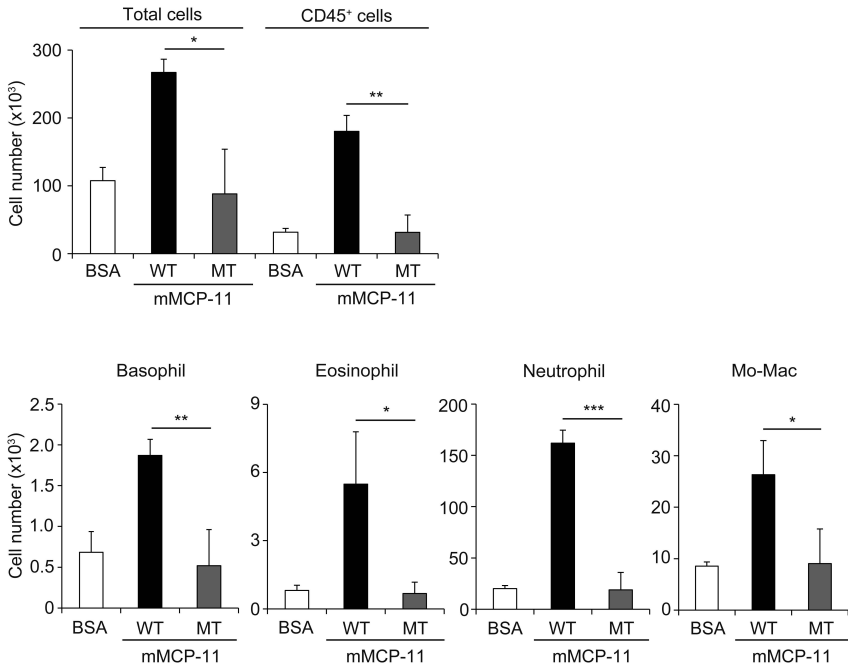
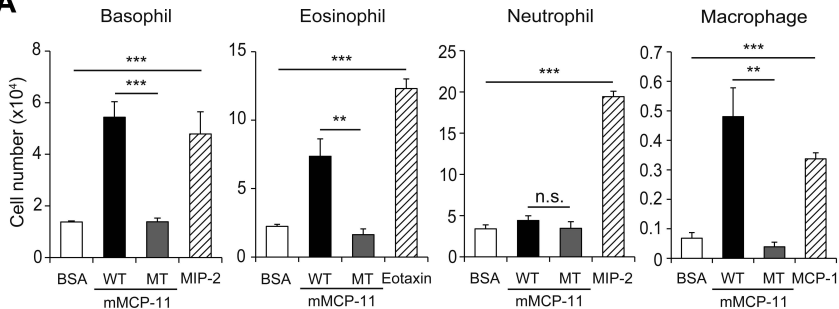


Figure 5

A



B

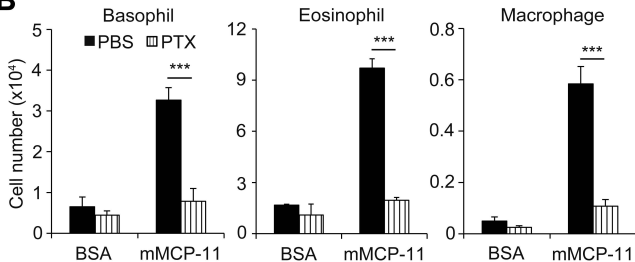
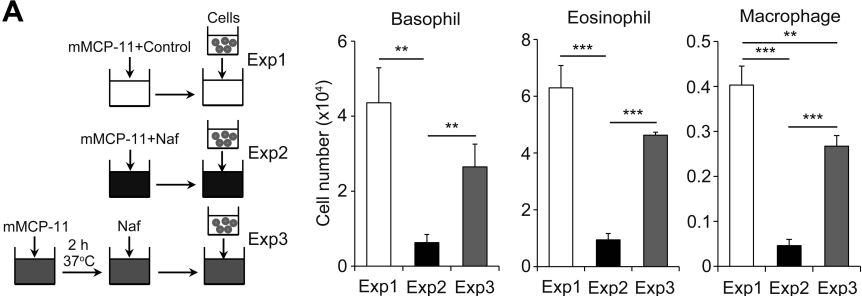
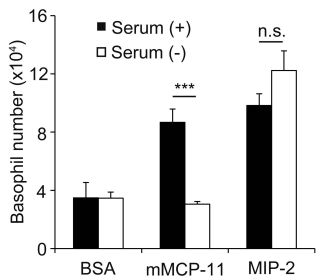


Figure 6

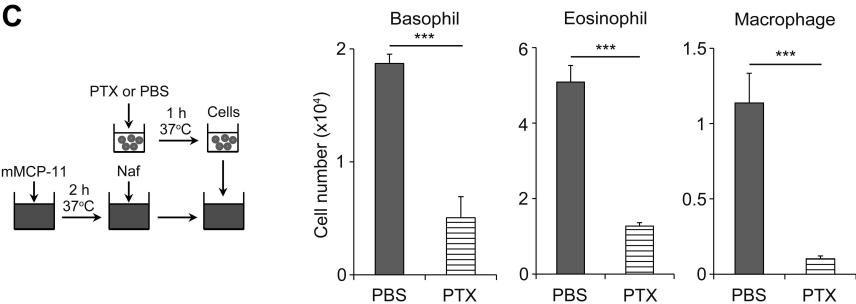
A



B



C



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A CRUCIAL ROLE OF BASOPHIL mMCP-11 IN ALLERGY

Basophil tryptase mMCP-11 plays a crucial role in IgE-mediated, delayed-onset allergic inflammation in mice

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Key points

- Mice deficient for basophil tryptase mMCP-11 showed ameliorated IgE-mediated allergic inflammation with reduced leukocyte infiltration.
- This is the first demonstration that the basophil-derived protease plays a crucial role in allergic inflammation.

Abstract

Recent studies have identified non-redundant roles for basophils in immune responses including allergy and protective immunity. It is well known that activated basophils release granule contents such as histamine and proteases as do mast cells. However, the functional significance of basophil-derived proteases remains poorly understood in contrast to those released from mast cells. Here we generated mice deficient for mouse mast cell protease 11 (mMCP-11) that is preferentially expressed by basophils rather than mast cells. In spite of normal development of basophils, mMCP-11-deficient mice showed ameliorated IgE-mediated chronic allergic inflammation (IgE-CAI) with reduction of cutaneous swelling, microvascular permeability and leukocyte infiltration in the skin lesion, compared to WT mice. Repeated administration of recombinant mMCP-11 in the skin induced infiltration of leukocytes, including basophils, in a tryptase activity-dependent manner. The transwell migration assay *in vitro* suggested that mMCP-11-mediated proteolytic products of a serum protein(s) promoted migration of basophils, eosinophils and macrophages via a G protein-coupled receptor(s). Thus, basophil tryptase mMCP-11 is a crucial effector molecule for the induction of IgE-CAI-type allergic inflammation. This is the first demonstration that the basophil-derived protease plays a significant role *in vivo*.

Introduction

Basophils are the rarest granulocytes and represent less than 1% of peripheral blood leukocytes. They were often considered erroneously as minor relatives or blood-circulating precursors of tissue-resident mast cells because of some similarity between them.¹⁻³ It is now accepted well that basophils and mast cells are distinct cell lineages.⁴ Recent studies have identified crucial roles for basophils, distinct from those played by mast cells, in immune responses including allergy and protective immunity.⁵⁻⁸ In response to various stimuli, basophils can readily secrete large quantities of cytokine IL-4 that plays versatile roles in immunity through regulating other cells.⁹ In contrast to IL-4, functional roles of other molecules produced by basophils in immune responses remain ill-defined.

Both basophils and mast cells possess basophilic granules stained in violet with basic aniline dyes, as described first by Paul Ehrlich more than 130 years ago. These granules store preformed materials such as histamine and proteases, and release them upon activation.^{1,2} Ten serine proteases have been identified in murine mast cells, including 6 chymases (mouse mast cell protease (mMCP)-1, mMCP-2, mMCP-4, mMCP-5, mMCP-9, mMCP-10) and 4 tryptases (mMCP-6, mMCP-7, mMCP-11, mTMT).¹⁰⁻¹⁴ mMCP-11 is the most recently discovered member of the mouse mast cell tryptase family and the most distantly related to other members in terms of the amino acid sequence.¹⁵ We previously demonstrated that among the tryptase family, mMCP-11 is preferentially expressed by basophils rather than mast cells whereas mMCP-6 and mMCP-7 are expressed by mast cells but not basophils.¹⁶ None of mast cell chymases are expressed by basophils while the expression of granzyme B-like protease mMCP-8 is confined to basophils.^{16,17} Thus, even though basophils and mast cells possess apparently similar-looking basophilic granules, the repertoire of serine proteases stored in granules is distinct between them, suggesting that

mMCP-8 and mMCP-11 might be involved in non-redundant functions of basophils. We previously reported that the subcutaneous injection of recombinant mMCP-11 induces edematous skin swelling with increased microvascular permeability, suggesting that mMCP-11 may contribute to the development of basophil-mediated inflammatory responses.¹⁸

The nonredundant role of basophils *in vivo* has been clearly demonstrated in the IgE-CAI response.¹⁹⁻²¹ A single intradermal administration of allergens in the ear skin of IgE-sensitized mice induces three consecutive waves of ear swelling. The first two waves are mast cell-dependent, immediate-type allergic reactions with early- and late-phase ear swelling. The third wave of ear swelling starts on day 2 with a peak on day 4 postchallenge, accompanied by massive infiltration of eosinophils, neutrophils, monocytes and macrophages in the skin lesion. We designated this delayed-onset response IgE-CAI.¹⁹ Mast cells are dispensable for IgE-CAI while basophil depletion abolishes the development of IgE-CAI even though basophils represent only 1 or 2% of infiltrates in the skin lesion. Thus, basophils but not mast cells play an essential role in IgE-CAI.¹⁹ However, it remains to be determined how basophils elicit allergic inflammation in the IgE-CAI response.

In the present study, we generated mMCP-11-deficient (KO) mice to clarify the functional role of mMCP-11 *in vivo*. KO mice showed ameliorated IgE-CAI response with reduction of swelling, vascular permeability and leukocyte infiltration in the skin lesion even though their basophil development remained unimpaired. *In vitro* experiments suggested that mMCP-11-mediated proteolytic products of a serum protein(s), rather than mMCP-11 itself, trigger migration of leukocytes, including basophils, via a G protein-coupled receptor(s). Thus, the present study has uncovered the crucial role for basophil tryptase mMCP-11 in allergic inflammation.

Materials and Methods

Mice

C57BL/6 mice (7-9 weeks old) were purchased from Japan SLC. To generate mMCP-11-deficient mice, the targeting vector for replacing the exons 1, 2 and 3 of the *Mcpt11* (*Prss34*) gene with the loxP-PGK-Neomycin^R-loxP cassette (Gene Bridges) was constructed by Red/ET recombination method (Gene Bridges), and transfected into C57BL/6 ES cells (UNITECH Co., Ltd.). The resultant knock-out mice were further crossed with CAG-Cre mice (provided by J. Miyazaki, Osaka University)²² to remove floxed Neomycin^R cassette from the *Mcpt11* allele. All animal studies were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Antibodies and recombinant mMCP-11

The following Abs were purchased from BioLegend: biotinylated anti-CD49b; FITC-conjugated anti-CD49b, anti-F4/80; PE-conjugated anti-FcεRIα, anti-CD45 (30-F11); APC-conjugated anti-CD200R3, anti-Ly-6G; Pacific Blue-conjugated anti-CD117, anti-CD11b. PE-conjugated anti-Siglec-F (E50-2440) was from BD Biosciences. Anti-mMCP-11 and anti-mMCP-8 were established as reported previously.¹⁶ IgE (IGELb4, ATCC-Tib141) specific to 2,4,6-trinitrophenol (TNP) and anti-CD16/32 (2.4G2) were prepared in our laboratory. Recombinant mMCP-11 and its protease-dead mutant (Ser²⁰⁰ was substituted with Ala) were prepared as described previously.¹⁸

Generation of bone marrow-derived basophils

Mouse bone marrow-derived basophils (BMBAs) were generated as described previously.¹⁶ Briefly, bone marrow cells were cultured in the presence of 300 pg/ml recombinant mouse IL-3 (BioLegend) for 7 days, followed by purification of CD49b⁺ cells using the IMag system

with biotinylated anti-CD49b (DX5) and streptavidin-conjugated magnetic particles (BD Biosciences). The purity of c-kit⁺CD49b⁺CD200R3⁺ BMBAs was >95% after magnetic purification.

Induction of IgE-CAI and passive cutaneous anaphylaxis (PCA)

IgE-CAI was induced as described previously.¹⁹ In brief, mice were sensitized with intravenous injection of 300 µg anti-TNP IgE, and challenged on the following day with intradermal injection of 10 µg TNP-OVA and control OVA into the right and left ear, respectively. Ear thickness was measured at a 24 h-interval for 7 days (Δ ear thickness; right - left). In some experiments, mice were treated with topical administration of 200 µg SC560, meloxicam, 100 µg celecoxib, or vehicle (ethanol) alone once a day during the development of IgE-CAI. For Evan Blue leakage analysis, mice were intravenously injected with 0.5% Evans blue dye in 100 µl PBS on day 3 of the IgE-CAI response. Two hours after the dye injection, ears were excised and incubated in 0.7 ml formamide at 63°C for overnight to extract the dye leaked into the skin. The amount of the dye in the extracts was determined by a spectrophotometer at 620 nm. PCA was induced as previously described with some modifications.²³ Briefly, mice were locally sensitized with intradermal injection of 100 ng anti-TNP IgE and control PBS into the right and left ear, respectively. On the following day, mice were challenged with intravenous injection of 200 µg TNP-OVA in 200 µl of PBS containing 0.25% Evans blue dye. Thirty minutes later, the treated ears were excised and subjected to measurement of leaked dye.

Flow cytometric, histopathological and Western blot analyses

Single cell suspensions were obtained from ear skins by treatment with 125 U/ml collagenase (Wako) at 37°C for 2 h. After pretreatment with anti-CD16/32 (2.4G2) and normal rat serum

to avoid the non-specific binding of irrelevant Abs, cells were stained with indicated combination of Abs on ice for 30 min, and analyzed by FACSCanto (BD Biosciences). Each cell lineages were defined as follows: basophils (c-kit⁺CD49b⁺CD200R3⁺), eosinophils (Siglec-F⁺SSC^{high}), neutrophils (Ly-6G⁺), monocyte-macrophages (F4/80⁺CD11b⁺ among cells in which both eosinophils and neutrophils were excluded). For histopathological examination, ear specimens were fixed and embedded in paraffin, and sections were stained with hematoxylin and eosin. For Western blot analysis, BMBAs were lysed with RIPA buffer containing protease inhibitor cocktail (Thermo Scientific), and total cell lysates were subjected to SDS-PAGE, followed by immunoblot analysis with indicated antibodies.

Quantitative PCR

Total RNA was extracted from tissues or isolated cells by RNeasy Mini Kit (QIAGEN), followed by cDNA synthesis with reverse transcription using oligo dT and random primers. Quantitative PCR of the cDNA was performed by using following primer sets: 5'-CTGGCTCCTGTTCTCAGTCT-3' and 5'-GCTGTGCTCCATGTCGTAGAG-3' for *Mcpt11* (*Prss34*); 5'-CACTGGTCAATGACATCATGCTCC-3' and 5'-GTCAGACAAAGTGCAATTGGCCAAC-3' for *Mcpt8*; 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-TGTAGACCATGTAGTTGAGGTCA-3' for *Gapdh*. Relative gene expression levels were calculated using standard curves generated by serial dilutions of each cDNA standard and normalized by *Gapdh* expression levels.

In vitro stimulation of basophils

BMBAs were sensitized with 1 µg/ml anti-TNP IgE for overnight, and then stimulated with 300 ng/ml (for degranulation assay) or 400 ng/ml (for measurement of cytokines) of TNP-OVA or control OVA. Degranulation of BMBAs was determined by measurement of

β -hexosaminidase release as previously described.²⁴ For measurement of cytokine production, BMBAs sensitized with IgE were stimulated with antigens for 24 h. The concentration of IL-4 and IL-6 in the culture supernatants was measured by ELISA (BioLegend) and cytometric bead array (BD Biosciences), respectively.

Transwell migration assay

Leukocytes used for this assay were prepared as follows. Neutrophils were prepared from the bone marrow by using 62% Percoll gradient as described previously.²⁵ Eosinophils were isolated from the peritoneum of mice that had been treated for 7 days with daily intraperitoneal administration of IL-5.²⁶ Macrophages were isolated from the peritoneum of mice that had been treated with intraperitoneal administration of 1 ml of 4% thioglycollate broth 3 days before. BMBAs were used as basophils. The transwell apparatus (Kurabo) consisted of the upper and lower chambers separated by a membrane with 3 μ m (for neutrophils) or 5 μ m (for other cell types) pore size. Leukocytes (5×10^5 cells) were placed into the upper chamber while 10 μ g/ml recombinant mMCP-11, its protease-dead mutant, control BSA or chemokines (MIP-2 100 ng/ml, Eotaxin 200 ng/ml, or MCP-1 200 ng/ml) was included in culture medium of the lower chamber. Ninety minutes (for neutrophils) or 2 h (for other cell types) after incubation at 37°C, the number of cells migrating into the lower chamber was counted. In some experiments, cells were pretreated with 200 ng/ml (for BMBAs) or 100 ng/ml (for other cell types) of pertussis toxin (PTX, Sigma-Aldrich) for 1 h before the transwell migration assay. Recombinant tetramerized human β -tryptase was purchased from Promega. Bovine trypsin was from Sigma-Aldrich.

Statistical analysis

Statistical analysis was performed with unpaired Student's t test. A p value <0.05 was

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considered statistically significant.

Results

Generation of mMCP-11-deficient mice

To explore the role of mMCP-11 *in vivo*, we have established mMCP-11-deficient (KO) mice by means of targeting the exons 1, 2 and 3 of the mMCP-11-coding gene *Prss34* (Figure 1A). c-kit⁺CD49b⁺CD200R3⁺FcεRIα⁺ basophils were detected in the bone marrow, spleen, and peripheral blood of KO mice as many as in wild-type (WT) mice (Figure 1B-C). The number of other hematopoietic cells including mast cells was also comparable between WT and KO mice (Supplemental Figure 1). When cultured with IL-3, bone marrow cells isolated from WT and KO mice generated comparable numbers of c-kit⁺CD49b⁺CD200R3⁺ basophils (bone marrow-derived basophils, BMBAs) (Supplemental Figure 2), suggesting unimpaired development of basophils in KO mice. The lack of mMCP-11 expression in KO basophils was confirmed at both mRNA and protein levels (Figure 1D-E) while the expression of basophil-specific mMCP-8 remained intact in KO basophils (Figure 1D-E). When stimulated with IgE plus antigens, KO BMBAs degranulated (Figure 1F) and produced cytokines IL-4 and IL-6 (Figure 1G) as much as did WT BMBAs. Moreover, WT and KO BMBAs could migrate toward chemokine MIP-2 to a comparable extent (Figure 1H). Thus, the deletion of the *Prss34* gene did not seem to give any adverse effect on the homeostasis and FcεRI- or chemokine-mediated activation of basophils.

mMCP-11 KO mice show ameliorated IgE-CAI response with the reduction in swelling and microvascular permeability of the skin

We previously reported that basophils crucially contribute to the development of IgE-CAI in the ear skin,¹⁹ and that basophil depletion abolishes the IgE-CAI response but not the mast cell-mediated immediate-type reaction.²⁰ However, it remained to be determined what

molecules derived from basophils are responsible for the development of IgE-CAI. To examine the role of mMCP-11 in IgE-CAI, WT and KO mice were passively sensitized with hapten TNP-specific IgE and challenged with intradermal injection of the corresponding allergen TNP-OVA or control OVA. In both mouse strains, the TNP-OVA administration induced ear swelling with a peak on day 4 postchallenge while the extent of ear swelling in KO mice was approximately half of that in WT mice (Figure 2A). Moreover, the increase of microvascular permeability in the skin lesion was significantly lower in KO mice (Figure 2B). The increase of permeability in WT mice was suppressed by the treatment with meloxicam (COX-2 inhibitor) but not SC560 (COX-1 inhibitor) whereas neither of them significantly affected the permeability in KO mice (Figure 2C), suggesting that mMCP-11 increased permeability in the skin lesion through COX-2. In accordance with this, IgE-CAI ear swelling in WT but not KO mice was significantly inhibited by the treatment with COX-2 inhibitor, either meloxicam or celecoxib (Figure 2D and Supplemental Figure 3). The tryptase activity of mMCP-11 remained unaffected by these inhibitors (data not shown). Thus, basophil-derived mMCP-11 appeared to contribute to ear swelling in the IgE-CAI response in part by means of COX-2-mediated vascular hyperpermeability.

Cellular infiltration in the IgE-CAI skin lesion is decreased in KO mice

Histopathological examination of ear skin specimens indicated the reduction of cellular infiltration in the IgE-CAI skin lesion of KO mice compared to WT mice (Figure 3A). In accordance with this, flow cytometric analysis revealed that the number of total cells isolated from the skin lesion, mainly CD45⁺ hematopoietic cells, was reduced in the KO skin lesion to less than half of that in the WT skin lesion (Figure 3B-C). Even though KO basophils by themselves showed unimpaired migration toward MIP-2 *in vitro* (Figure 1H), the number of basophils infiltrating the IgE-CAI skin lesion was significantly reduced in KO mice (Figure

3C). This was also the case for the number of eosinophils, neutrophils and monocytes/macrophages in the skin lesion of KO mice (Figure 3C). Thus, mMCP-11 appeared to be involved in the infiltration and accumulation of these leukocytes in the IgE-CAI skin lesion.

We previously reported that one single cutaneous injection of 10 µg recombinant mMCP-11 elicited transient ear swelling with increased microvascular permeability whereas no significant infiltration of leukocytes was detected in the injection site.¹⁸ We assumed that this difference between the IgE-CAI skin lesion and the mMCP-11-injected skin could be attributed to different duration of mMCP-11 action. In contrast to the single injection, mMCP-11 likely accumulates in the IgE-CAI skin lesion because basophils are continuously recruited there and activated by allergens to release mMCP-11. To mimic this situation *in vivo*, we treated WT mice with repeated intradermal injection of recombinant mMCP-11 in the ear skin. Three times injection of mMCP-11 at a 24 h-interval indeed elicited the infiltration and accumulation of neutrophils, eosinophils, basophils and monocytes/macrophages (Figure 4) along with transient ear swelling induced by each single injection (Supplemental Figure 4A). The extent of mMCP-11-induced ear swelling was not affected by depletion of basophils, demonstrating that exogenous mMCP-11 can induce this reaction in a basophil-independent manner (Supplemental Figure 4A-B). Importantly, mMCP-11 carrying a mutation in the active site of tryptase did not show such a leukocyte recruiting ability (Figure 4), indicating that the protease activity of mMCP-11 is essential for this function.

Proteolytic products generated by mMCP-11 elicit leukocyte migration via a G protein-coupled receptor *in vitro*

To explore mechanisms underlying the mMCP-11-mediated leukocyte recruitment, we first examined the possibility that mMCP-11 can directly stimulate leukocytes to induce their

migration, by using the transwell migration assay. Basophils, eosinophils, neutrophils, macrophages were individually placed in the upper chamber while mMCP-11 was included in the lower chamber. Enzymatically active but not inactive mMCP-11 elicited transwell migration of eosinophils, basophils, and macrophages, but not neutrophils (Figure 5A), demonstrating that the protease activity of mMCP-11 is essential for triggering their migration. In contrast, no significant migration of T or B cells was detected under these conditions (Supplemental Figure 5). Moreover, the addition of meloxicam to the transwell culture showed no significant effect on the mMCP-11-induced basophil migration (data not shown), suggesting that mMCP-11 elicited leukocyte migration in a COX-2-independent manner. Of note, the pretreatment of eosinophils, basophils and macrophages with pertussis toxin almost completely abolished the mMCP-11-induced migration (Figure 5B). These results suggested that mMCP-11 functioned as a protease and triggered leukocyte migration through a G protein-coupled receptor(s).

To clarify how the protease activity of mMCP-11 contributes to the leukocyte migration, we added a protease inhibitor, nafamostat, to the mMCP-11-containing culture medium at the different timing in the transwell migration assay (Figure 6A). As expected, the migration of basophils, eosinophils and macrophages was strongly reduced to a level as low as 10-15% of control, when nafamostat was added simultaneously with mMCP-11 to the lower chamber (compare Experiments 1 and 2 in Figure 6A). In contrast, when nafamostat was added to the lower chamber to inactivate mMCP-11 after the culture medium had been incubated with mMCP-11 at 37°C for 2 h prior to the transwell migration assay, leukocyte migration was elicited to an extent as much as 60-75% of control (Experiment 3 in Figure 6A). In addition, serum depletion from culture medium completely abolished the basophil migration induced by mMCP-11 but not by MIP-2 (Figure 6B). These results strongly suggested that mMCP-11 acted on a serum protein(s) present in culture medium, and its

proteolytic product rather than mMCP-11 itself promoted leukocyte migration. The less extent of migration in Experiment 3 compared to Experiment 1 could be attributed to the degradation of proteolytic products and no supply of new products during the transwell migration assay due to the nafamostat-mediated inactivation of mMCP-11 in Experiment 3. Importantly, the leukocyte migration in Experiment 3 was abolished by the pretreatment of leukocytes with pertussis toxin (Figure 6C), implying that proteolytic products generated by mMCP-11 induced leukocyte migration via a G protein-coupled receptor(s).

Discussion

Previous study illustrated that basophils and mast cells display distinct repertoire of serine proteases stored in secretory granules.¹⁶ Among the tryptase members of the mMCP family, mMCP-11 is preferentially expressed by basophils rather than mast cells whereas mMCP-6 and mMCP-7 are expressed only by mast cells in mice.¹⁶ Extensive investigation on mMCP-6 and mMCP-7 demonstrated that they play important roles in protective immunity against bacterial and parasitic infections^{27,28} and also in the development of inflammatory diseases such as autoimmune arthritis^{29,30} and colitis.³¹ In contrast, the functional significance of basophil tryptase mMCP-11 remained enigmatic. We showed in the present study that mMCP-11 is a crucial effector molecule in the development of the IgE-CAI allergic inflammation. This is the first demonstration that the basophil-derived protease plays a significant role *in vivo*.

mMCP-11 KO mice showed much less severe IgE-CAI response, compared to WT mice, characterized by milder ear swelling with reduced microvascular hyperpermeability and leukocyte infiltration. We previously reported that basophil ablation during the progress of IgE-CAI resulted in drastic reduction of eosinophils and neutrophils infiltrating the skin lesion, concomitant with basophil elimination from there, suggesting the possible contribution of basophils to the eosinophil and neutrophil infiltration.^{20,21} The present study clearly demonstrated that basophil-derived mMCP-11 induces the infiltration of leukocytes, including basophils, eosinophils, neutrophils and macrophages. Basophil infiltration in the IgE-CAI skin lesion was still observed even in KO mice albeit to a lesser extent than in WT mice. Therefore, mMCP-11 is not essential for basophil infiltration in IgE-CAI. Our findings rather suggest the following scenario: when basophils migrate into the skin lesion and get activated by allergens, they release mMCP-11 which in turn recruits more and more basophils

together with other leukocytes. If this is the case, the ablation of mMCP-11 in basophils should reduce the infiltration of leukocytes, including basophils, just as observed in KO mice.

The detailed molecular mechanism by which mMCP-11 triggers the leukocyte migration remains to be determined in future studies, as for the case of mast cell tryptase and chymase-induced chemotaxis of leukocytes.³²⁻³⁵ Nevertheless, our findings in the present study illustrated that the protease activity of mMCP-11 is essential for it, and a G protein-coupled receptor(s) expressed on leukocytes is involved in the migration. mMCP-11 does not seem to directly act on leukocytes to induce their migration. Most likely, mMCP-11 cleaves a serum protein(s), and its proteolytic product attracts basophils, eosinophils, and macrophages via a G protein-coupled receptor(s). In contrast, no significant migration of neutrophils was induced by mMCP-11 in the transwell migration assay while intradermal administration of mMCP-11 elicited neutrophil infiltration in the skin. In the latter case, quantitative PCR analysis showed slightly upregulated expression of neutrophil-attracting chemokines such as CXCL1 and CXCL2 among others but without statistical significance (data not shown). The possible contribution of mMCP-11-elicited production of chemokines in the skin lesion to the migration of neutrophils and other leukocytes needs to be further explored.

mMCP-11 is highly expressed by all basophils, and also by a small subset of mast cells albeit to a much lesser extent.¹⁶ As for the case of basophils, the number of mast cells in the skin and peritoneum of KO mice was comparable to that of WT mice (Supplemental Figure 1), suggesting the unimpaired development of mast cells in the absence of mMCP-11. Moreover, the extent of microvascular leakage in the IgE-mediated passive cutaneous anaphylaxis response was comparable between KO and WT mice (Supplemental Figure 6), indicating intact degranulation of KO mast cells. In accordance with this, the mast cell-dependent, immediate-type (early- and late-phase) ear swelling ahead of the IgE-CAI

response was normally elicited in KO mice (data not shown) in contrast to the amelioration of IgE-CAI. Thus, mMCP-11 did not seem to contribute to the mast cell-mediated immediate-type reactions, as far as we examined, while the functional significance of mMCP-11 in mast cell-mediated, chronic inflammation, if any, remains to be investigated.

The hSPL-4 gene on human chromosome 16p13.3 is assigned to be the human ortholog of mMCP-11 gene located on mouse chromosome 17A3.3.¹⁵ However, the hSPL-4 gene possesses a premature translational-termination codon, and do not encode enzymatically active enzyme, unlike the mMCP-11 gene. Of note, human basophils express α - and β -tryptases, albeit to a lesser extent than do mast cells,^{36,37} suggesting that α - and β -tryptases might be functional ortholog of mMCP-11 in human basophils. Indeed, repeated intradermal injection of human β -tryptase elicited leukocyte infiltration into the skin of mice (Supplemental Figure 7). β -tryptase also induced transwell migration of basophils *in vitro* via a G protein-coupled receptor(s) in a manner dependent on its protease activity and the presence of serum (Supplemental Figures 8-9) as did mMCP-11. Importantly, trypsin showed little or no such ability of basophil migration (Supplemental Figure 8A) in spite of higher tryptase activity than β -tryptase and mMCP-11 as assessed by using a standard tryptase substrate (data not shown), suggesting that not all typtases might share this property of mMCP-11.

In conclusion, we generated mMCP-11-deficient mice in the present study and demonstrated that basophil tryptase mMCP-11 plays a crucial role in the development of the IgE-CAI allergic response through the promotion of microvascular leakage and leukocyte infiltration in a tryptase activity-dependent manner. This study has cast new light on the functional significance of proteases produced and released by basophils.

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Authorship

Contribution: M.I. and K.T. performed experiments; H.D. contributed to the initial study; M.F. and S.Y. generated mMCP-11-deficient mice; S.S. generated rmMCP-11; S.Y. provided helpful suggestions; M.I., Y.Y. and H.K. wrote the manuscript; Y.Y. and H.K. designed and supervised the study.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

1. Galli SJ. Mast cells and basophils. *Curr Opin Hematol*. 2000;7(1):32-39.
2. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol*. 2010;125(2 Suppl 2):S73-80.
3. Falcone FH, Haas H, Gibbs BF. The human basophil: a new appreciation of its role in immune responses. *Blood*. 2000;96(13):4028-4038.
4. Huang H, Li Y, Liu B. Transcriptional regulation of mast cell and basophil lineage commitment. *Semin Immunopathol*. 2016;38(5):539-548.
5. Sokol CL, Medzhitov R. Emerging functions of basophils in protective and allergic immune responses. *Mucosal Immunol*. 2010;3(2):129-137.
6. Siracusa MC, Comeau MR, Artis D. New insights into basophil biology: initiators, regulators, and effectors of type 2 inflammation. *Ann N Y Acad Sci*. 2011;1217:166-177.
7. Karasuyama H, Mukai K, Obata K, Tsujimura Y, Wada T. Nonredundant roles of basophils in immunity. *Annu Rev Immunol*. 2011;29:45-69.
8. Voehringer D. Protective and pathological roles of mast cells and basophils. *Nat Rev Immunol*. 2013;13(5):362-375.
9. Yamanishi Y, Karasuyama H. Basophil-derived IL-4 plays versatile roles in immunity. *Semin Immunopathol*. 2016;38(5):615-622.
10. Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. *Immunol Rev*. 2007;217:141-154.
11. Miller HR, Pemberton AD. Tissue-specific expression of mast cell granule serine proteinases and their role in inflammation in the lung and gut. *Immunology*. 2002;105(4):375-390.

12. Hunt JE, Friend DS, Gurish MF, et al. Mouse mast cell protease 9, a novel member of the chromosome 14 family of serine proteases that is selectively expressed in uterine mast cells. *J Biol Chem*. 1997;272(46):29158-29166.
13. Hallgren J, Pejler G. Biology of mast cell tryptase. An inflammatory mediator. *FEBS J*. 2006;273(9):1871-1895.
14. McNeil HP, Adachi R, Stevens RL. Mast cell-restricted tryptases: structure and function in inflammation and pathogen defense. *J Biol Chem*. 2007;282(29):20785-20789.
15. Wong GW, Yasuda S, Morokawa N, Li L, Stevens RL. Mouse chromosome 17A3.3 contains 13 genes that encode functional tryptic-like serine proteases with distinct tissue and cell expression patterns. *J Biol Chem*. 2004;279(4):2438-2452.
16. Ugajin T, Kojima T, Mukai K, et al. Basophils preferentially express mouse Mast Cell Protease 11 among the mast cell tryptase family in contrast to mast cells. *J Leukoc Biol*. 2009;86(6):1417-1425.
17. Poorafshar M, Helmby H, Troye-Blomberg M, Hellman L. MMCP-8, the first lineage-specific differentiation marker for mouse basophils. Elevated numbers of potent IL-4-producing and MMCP-8-positive cells in spleens of malaria-infected mice. *Eur J Immunol*. 2000;30(9):2660-2668.
18. Yamagishi H, Mochizuki Y, Hamakubo T, et al. Basophil-derived mouse mast cell protease 11 induces microvascular leakage and tissue edema in a mast cell-independent manner. *Biochem Biophys Res Commun*. 2011;415(4):709-713.
19. Mukai K, Matsuoka K, Taya C, et al. Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity*. 2005;23(2):191-202.
20. Obata K, Mukai K, Tsujimura Y, et al. Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood*. 2007;110(3):913-920.

21. Egawa M, Mukai K, Yoshikawa S, et al. Inflammatory monocytes recruited to allergic skin acquire an anti-inflammatory M2 phenotype via basophil-derived interleukin-4. *Immunity*. 2013;38(3):570-580.
22. Sakai K, Miyazaki J. A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission. *Biochem Biophys Res Commun*. 1997;237(2):318-324.
23. LiHua L, Yoshikawa S, Ohta T, et al. Large particulate allergens can elicit mast cell-mediated anaphylaxis without exit from blood vessels as efficiently as do small soluble allergens. *Biochem Biophys Res Commun*. 2015;467(1):70-75.
24. Nei Y, Obata-Ninomiya K, Tsutsui H, et al. GATA-1 regulates the generation and function of basophils. *Proc Natl Acad Sci U S A*. 2013;110(46):18620-18625.
25. Izawa K, Kitaura J, Yamanishi Y, et al. Functional analysis of activating receptor LMIR4 as a counterpart of inhibitory receptor LMIR3. *J Biol Chem*. 2007;282(25):17997-18008.
26. Karasuyama H, Melchers F. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. *Eur J Immunol*. 1988;18(1):97-104.
27. Thakurdas SM, Melicoff E, Sansores-Garcia L, et al. The mast cell-restricted tryptase mMCP-6 has a critical immunoprotective role in bacterial infections. *J Biol Chem*. 2007;282(29):20809-20815.
28. Shin K, Watts GF, Oettgen HC, et al. Mouse mast cell tryptase mMCP-6 is a critical link between adaptive and innate immunity in the chronic phase of *Trichinella spiralis* infection. *J Immunol*. 2008;180(7):4885-4891.
29. McNeil HP, Shin K, Campbell IK, et al. The mouse mast cell-restricted tetramer-forming tryptases mouse mast cell protease 6 and mouse mast cell protease 7 are

critical mediators in inflammatory arthritis. *Arthritis Rheum*. 2008;58(8):2338-2346.

30. Shin K, Nigrovic PA, Crish J, et al. Mast cells contribute to autoimmune inflammatory arthritis via their tryptase/heparin complexes. *J Immunol*. 2009;182(1):647-656.

31. Hamilton MJ, Sinnamon MJ, Lyng GD, et al. Essential role for mast cell tryptase in acute experimental colitis. *Proc Natl Acad Sci U S A*. 2011;108(1):290-295.

32. Huang C, Friend DS, Qiu WT, et al. Induction of a selective and persistent extravasation of neutrophils into the peritoneal cavity by tryptase mouse mast cell protease 6. *J Immunol*. 1998;160(4):1910-1919.

33. Tani K, Ogushi F, Kido H, et al. Chymase is a potent chemoattractant for human monocytes and neutrophils. *J Leukoc Biol*. 2000;67(4):585-589.

34. Huang C, De Sanctis GT, O'Brien PJ, et al. Evaluation of the substrate specificity of human mast cell tryptase beta I and demonstration of its importance in bacterial infections of the lung. *J Biol Chem*. 2001;276(28):26276-26284.

35. Terakawa M, Tomimori Y, Goto M, Hayashi Y, Oikawa S, Fukuda Y. Eosinophil migration induced by mast cell chymase is mediated by extracellular signal-regulated kinase pathway. *Biochem Biophys Res Commun*. 2005;332(4):969-975.

36. Foster B, Schwartz LB, Devouassoux G, Metcalfe DD, Prussin C. Characterization of mast-cell tryptase-expressing peripheral blood cells as basophils. *J Allergy Clin Immunol*. 2002;109(2):287-293.

37. Jogie-Brahim S, Min HK, Fukuoka Y, Xia HZ, Schwartz LB. Expression of alpha-tryptase and beta-tryptase by human basophils. *J Allergy Clin Immunol*. 2004;113(6):1086-1092.

Figure legends

Figure 1. Generation of mMCP-11-deficient mice

(A) A strategy of gene targeting for the generation of mMCP-11-deficient mice. (B) Flow cytometric analysis of indicated surface markers on bone marrow cells isolated from wild-type (WT) and mMCP-11-deficient (KO) littermate mice. c-kit-negative cells were gated and displayed. (C) The number of basophils in the bone marrow, spleen, and peripheral blood of WT and KO mice (mean $\pm$ SD, n=3 each). (D) Quantitative PCR analysis for the expression of *Mcpt11* and *Mcpt8* mRNAs in c-kit⁻CD49b⁺CD200R3⁺ basophils isolated from the bone marrow of WT and KO mice. (E) Western blot analysis for the expression of indicated proteins in bone marrow-derived basophils (BMBAs) generated from WT and KO mice. (F-G) BMBAs generated from WT (open bars) and KO (filled bars) mice were sensitized with anti-TNP IgE and then stimulated with TNP-OVA or control OVA. In F, the extent of degranulation was examined by using β -hexosaminidase release assay (mean $\pm$ SD, n=3). In G, the concentration of cytokines IL-4 and IL-6 in culture supernatants was measured by using ELISA and cytometric bead array, respectively (mean $\pm$ SD, n=3). (H) Migration ability of WT (open bars) and KO (filled bars) BMBAs in response to chemokine MIP-2 (100 ng/ml) or control PBS was examined by using transwell migration assay. The number of cells migrated from the upper to lower chamber during 2 h-culture was counted (mean $\pm$ SD, n=3 each). Data shown in B-H are representative of at least 3 independent experiments. n.s., not significant.

Figure 2. mMCP-11-deficient mice show ameliorated IgE-CAI response in the skin

WT and KO mice were sensitized with anti-TNP IgE and challenged with intradermal administration of TNP-OVA or control OVA in their ears to induce IgE-CAI. (A) Time course of ear swelling (Δ ear thickness: TNP-OVA - OVA) is shown (mean $\pm$ SD, n=4 each).

(B) Three days after the allergen challenge, mice were treated with intravenous injection of Evans blue, and 2 h later their ears were subjected to photography followed by the measurement of leaked dye (mean $\pm$ SD, n=3-4 each). (C-D) WT and KO mice were treated, once a day during IgE-CAI, with topical administration of SC560, meloxicam or vehicle (ethanol) alone. In C, mice were subjected to the Evans blue leakage analysis as in B. Δ Evans blue leakage (TNP-OVA – OVA) is shown (mean $\pm$ SD, n=3-4 each). In D, time course of ear swelling (Δ ear thickness: TNP-OVA – OVA) is shown (mean $\pm$ SD, n=6 each). Data shown in A-D are representative of at least 3 independent experiments. *p<0.05; **p<0.01; ***p<0.001; n.s., not significant.

Figure 3. Cellular infiltration in the IgE-CAI skin lesion is decreased in mMCP-11-deficient mice

WT and KO mice were treated as in Fig. 2 to induce IgE-CAI. (A) Hematoxylin and eosin-stained specimens of IgE-CAI skin lesions on day 3 postchallenge. Scale bars, 200 μ m. (B-C) The number of total, CD45⁺ cells, and indicated cell types isolated from the TNP-OVA- (filled bars) or control OVA-administered (open bars) ear skin on day 3 postchallenge is shown (mean $\pm$ SD, n=5-6 each). Data shown are representative of three independent experiments. *p<0.05, **p<0.01.

Figure 4. Repeated intradermal administration of recombinant mMCP-11 in the ear skin induces leukocyte infiltration in a protease activity-dependent manner

WT mice were treated three times at a 24 h-interval with intradermal administration of 10 μ g wild-type (WT, black bars), inactive mutant (MT, gray bars) mMCP-11 or control BSA (white bars) each time. The number of total, CD45⁺ cells, and indicated cell types isolated from the ear skin at 4 h postchallenge is shown (mean $\pm$ SD, n=3 each). Data shown are

representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$.

Figure 5. mMCP-11 induces leukocyte migration *in vitro*

The ability of mMCP-11 to induce leukocyte migration was examined by using the transwell system. Indicated types of cells (5×10^5 cells) were placed in the upper chamber while WT (black bars) or MT (gray bars) mMCP-11, control BSA (white bars) or indicated chemokines (hatched bars) was included in culture medium of the lower chamber. (A) The number of cells recovered from the lower chamber after 1.5 h (for neutrophils)- or 2 h (for other cell types)-incubation at 37°C is shown (mean $\pm$ SD, $n=3$ each). (B) Indicated types of cells were pretreated with pertussis toxin (PTX, vertical striped bars) or control PBS (black bars) for 1 h prior to the transwell migration assay, and analyzed as in A (mean $\pm$ SD, $n=3$ each). Data shown in A-B are representative of at least three independent experiments. ** $p < 0.01$; *** $p < 0.001$; n.s., not significant.

Figure 6. Proteolytic products generated by mMCP-11 induce leukocyte migration via a G protein-coupled receptor(s)

(A) The effect of nafamostat on mMCP-11-elicited leukocyte migration was examined in the transwell migration assay. mMCP-11 was added to the lower chamber together with nafamostat (Experiment 2) or control vehicle DMSO alone (Experiment 1), and then the upper chamber containing leukocytes was placed onto the lower chamber to start the migration assay. In Experiment 3, nafamostat was added to the lower chamber after the culture medium in the lower chamber had been incubated with mMCP-11 at 37°C for 2 h prior to the transwell migration assay. The number of cells recovered from the lower chamber at the end of the migration assay is shown as in Figure 5 (mean $\pm$ SD, $n=3$ each). (B) The transwell migration assay of BMBAs was performed as in Figure 5 by using culture medium

sufficient (including 10% FCS) or deficient (including 0.1% BSA) for serum in both upper and lower chambers. The number of cells migrating into the lower chamber after 2 h-incubation at 37°C is shown (mean $\pm$ SD, n=3 each). (C) As in Experiment 3 shown in A, nafamostat was added to the lower chamber after the culture medium had been incubated with mMCP-11 for 2 h prior to the transwell migration assay while indicated types of cells were pretreated with PTX or control PBS for 1 h and transferred to the upper chamber. The number of cells recovered from the lower chamber at the end of the migration assay is shown (mean $\pm$ SD, n=3 each). Data shown in A-C are representative of at least three independent experiments. **p<0.01; ***p<0.001; n.s., not significant.

Figure 1

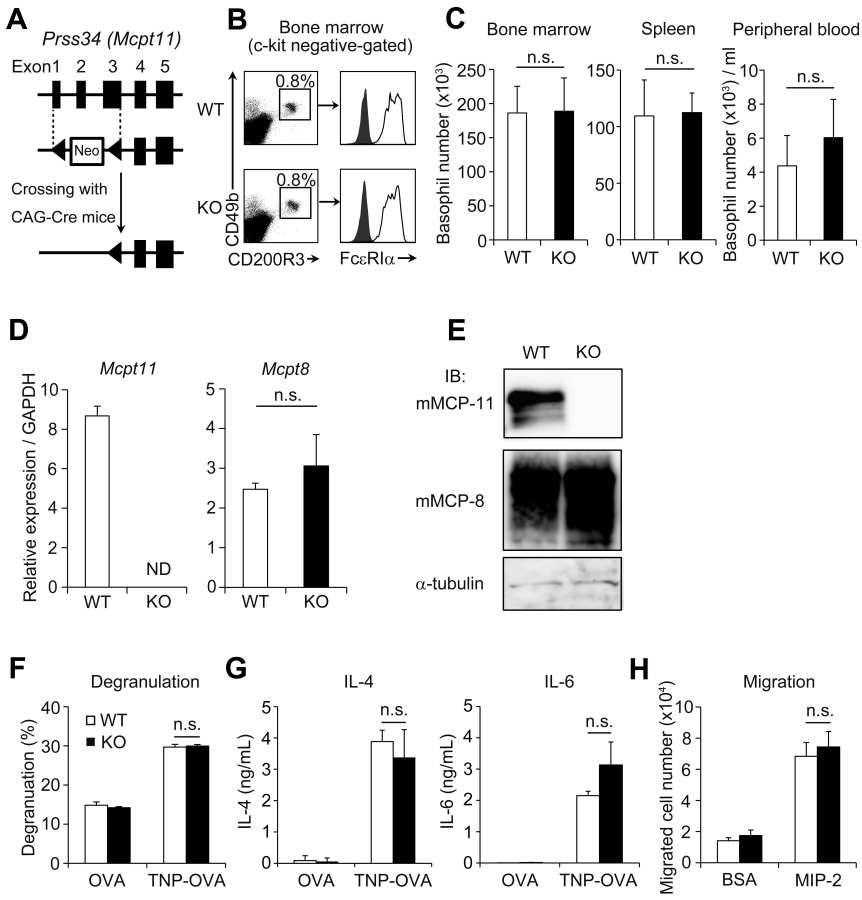


Figure 2

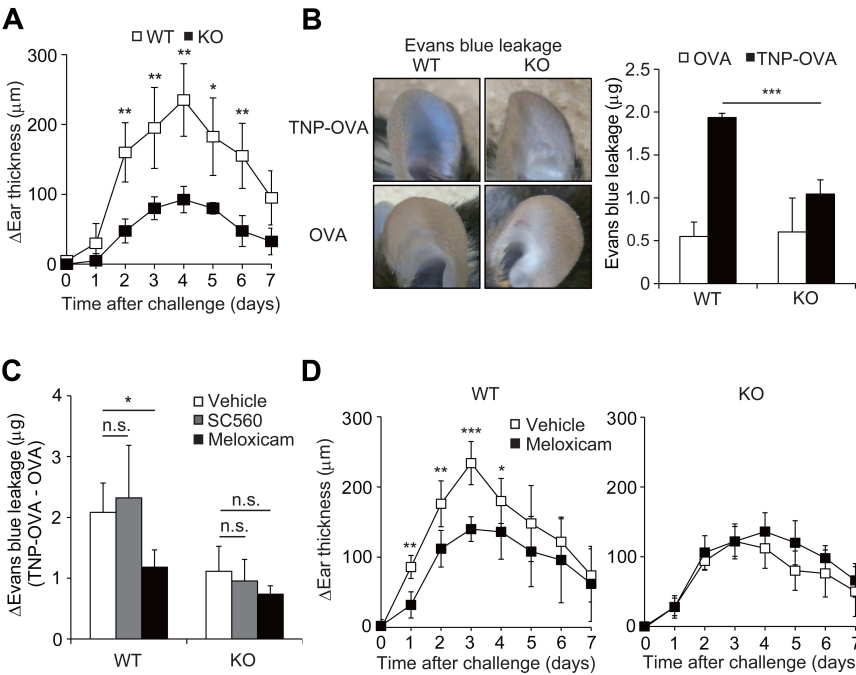


Figure 3

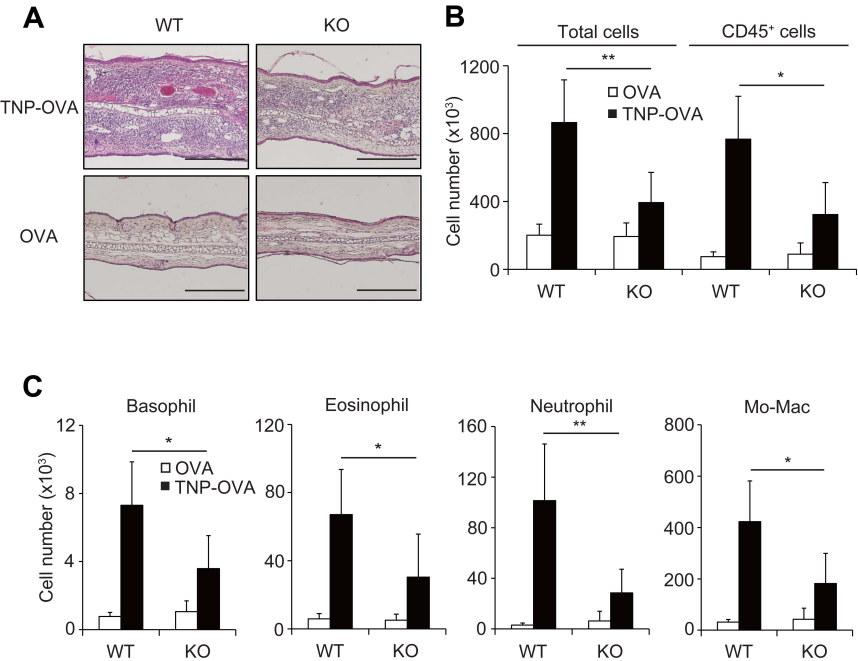


Figure 4

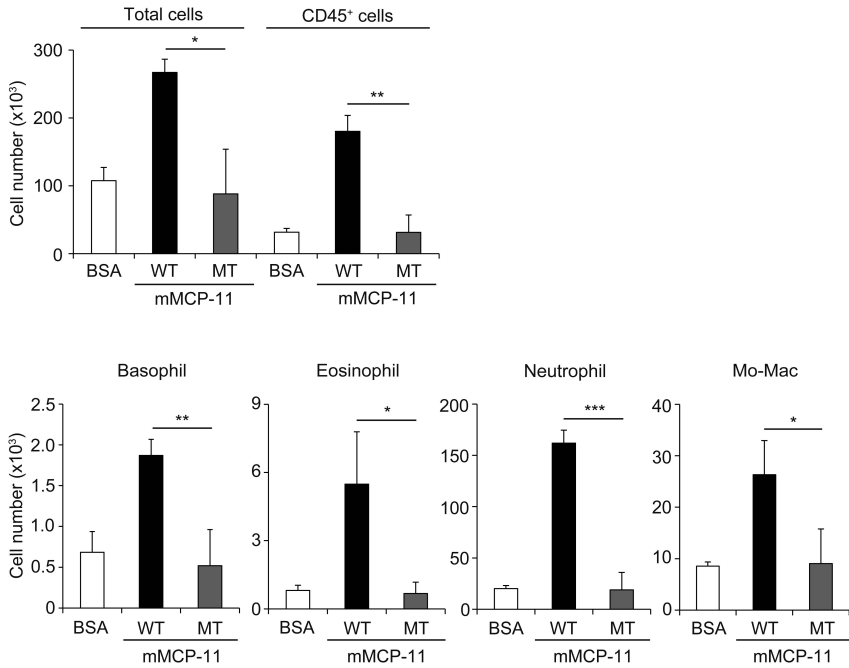
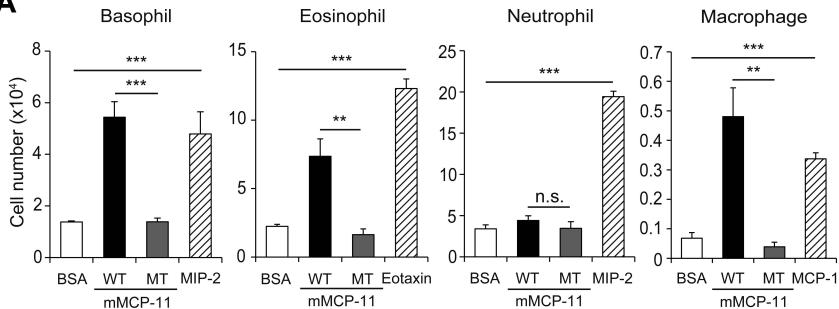


Figure 5

A



B

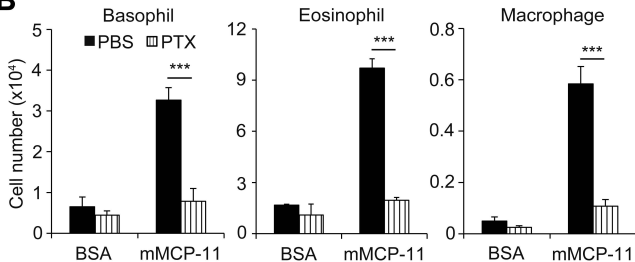
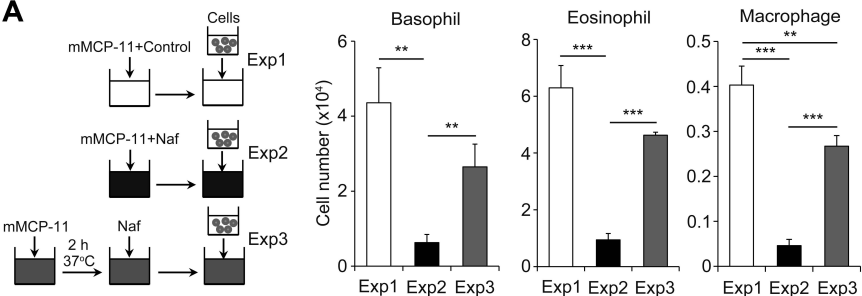
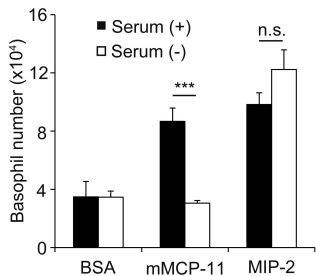


Figure 6

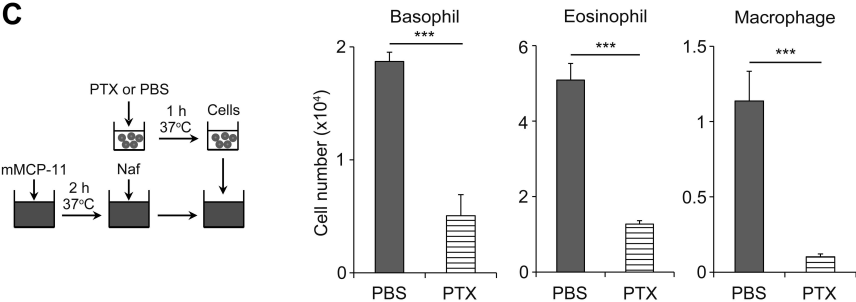
A



B



C





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Basophil tryptase mMCP-11 plays a crucial role in IgE-mediated, delayed-onset allergic inflammation in mice

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