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The Basophil-specific Protease mMCP-8 Provokes an Inflammatory Response in the Skin with Microvascular Hyperpermeability and Leukocyte Infiltration*[S]

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Hidemitsu Tsutsui, Yoshinori Yamanishi[1], Hiromi Ohtsuka, Shingo Sato, Soichiro Yoshikawa, and Hajime Karasuyama

From the Department of Immune Regulation, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), Tokyo 113-8519, Japan

Edited by Luke O'Neill

Basophils have often been erroneously considered to be minor relatives or blood-circulating precursors of tissue-resident mast cells because of some phenotypic similarity between them, including basophilic secretory granules in the cytoplasm. However, recent studies revealed that the repertoire of serine proteases stored in secretory granules is distinct in them. Particularly, mouse mast cell protease 8 (mMCP-8) is specifically expressed by basophils but not mast cells despite its name. Therefore, mMCP-8 is commonly used as a basophil-specific marker, but its functional property remains uncertain. Here we prepared recombinant mMCP-8 and examined its activity *in vitro* and *in vivo*. Purified recombinant mMCP-8 showed heat-sensitive proteolytic activity when α-tubulin was used as a substrate. One intradermal shot of mMCP-8, not heat-inactivated, induced cutaneous swelling with increased microvascular permeability in a cyclooxygenase-dependent manner. Moreover, repeated intradermal injection of mMCP-8 promoted skin infiltration of leukocytes, predominantly neutrophils and, to a lesser extent, monocytes and eosinophils, in conjunction with up-regulation of chemokine expression in the skin lesion. These results suggest that mMCP-8 is an important effector molecule in basophil-elicited inflammation, providing novel insights into how basophils exert a crucial and non-redundant role, distinct from that played by mast cells, in immune responses.

Basophils are the least common granulocytes, representing only ~0.5% of blood-circulating leukocytes, and therefore have often been neglected in immunological studies (1–3). However, recent studies revealed that basophils play crucial roles, distinct from those by mast cells, in immune responses, including allergic inflammation and protective immunity against parasitic infections (4–7). We previously demonstrated that basophils, but not mast cells, are responsible for the development of IgE-mediated chronic allergic inflammation (IgE-CAI)[2] even though basophils account for only ~2% of cellular infiltrates in the skin lesion, whereas other leukocytes, including eosinophils and neutrophils, are abundant there (8). Basophil depletion before the allergen challenge abolished the development of IgE-CAI, confirming the essential role of basophils (9, 10). Intriguingly, basophil ablation during the progress of IgE-CAI resulted in attenuated skin swelling together with decreased numbers of eosinophils and neutrophils, besides basophils, in the skin lesion, suggesting that basophils may contribute to the recruitment of these proinflammatory cells to the skin lesion (9). Nevertheless, it remains to be determined which molecules derived from basophils are involved in the development of allergic inflammation, including the recruitment of other leukocytes.

Basophils and mast cells are sometimes mixed up, and basophils have been erroneously considered to be minor relatives or blood-circulating precursors of tissue-resident mast cells because of some phenotypic similarity between them, including basophilic secretory granules in their cytoplasm (1–3). Both types of cells store serine proteases in secretory granules and release them in response to various stimuli, such as IgE plus allergens (11–20). Notably, recent studies revealed that the repertoire of serine proteases stored in basophilic granules is distinct in basophils and mast cells. Among the mouse mast cell protease (mMCP) family members, mMCP-8 has been shown to be expressed specifically by basophils but not mast cells, despite its name (14, 20). In contrast, chymases, mMCP-6, and mMCP-7 were expressed only by mast cells but not basophils (20).

mMCP-8 was originally cloned from cDNA of the mouse mastocytoma tumor cell lines and identified as a new subfamily member of murine mast cell serine proteases that does not belong to the authentic chymase and tryptase subfamilies and is rather closely related to cathepsin G and T cell granzymes (13, 21, 22). mMCP-8 showed high sequence similarity with mouse granzyme B in the region critical for substrate specificity, but its physiological substrate(s) is/are still unidentified (13, 23). Because of its unique expression profile confined to basophils, mMCP-8 has been commonly utilized as a specific marker for

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[S] This article contains supplemental Figs. S1–S4.

[1] To whom correspondence should be addressed: Dept. of Immune Regulation, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Tel.: 81-3-5803-5162; Fax: 81-3-3814-7172; E-mail: yamanishi.mbch@tmd.ac.jp.

[2] The abbreviations used are: IgE-CAI, IgE-mediated chronic allergic inflammation; mMCP, mouse mast cell protease; COX, cyclooxygenase; Ab, antibody; Q-PCR, quantitative PCR; HI, heat-inactivated.

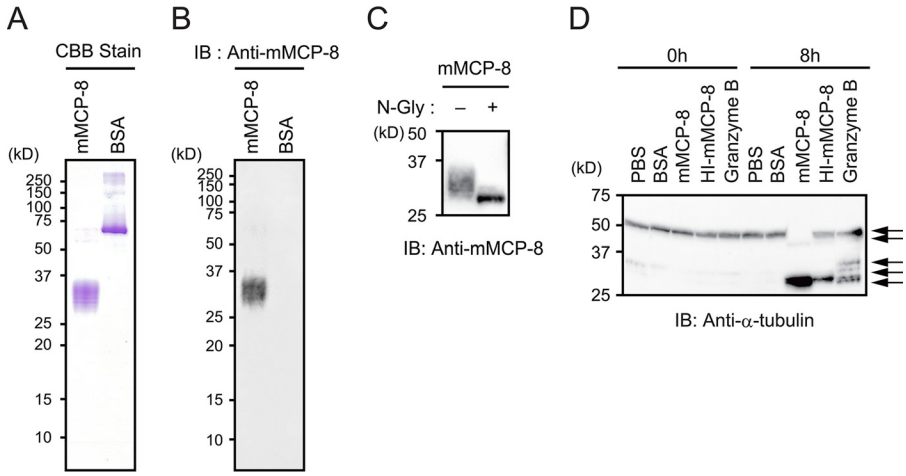

FIGURE 1. **Preparation and characterization of recombinant mMCP-8 protein.** *A* and *B*, recombinant mMCP-8 protein purified from culture supernatant of mMCP-8-transduced Sf9 cells was resolved by SDS-PAGE, followed by detection with Coomassie Brilliant Blue (*CBB*) staining (*A*) or immunoblotting (*IB*) using an mMCP-8-specific mAb (*anti-mMCP-8*, *B*). BSA was used for control experiments. *C*, purified recombinant mMCP-8 was treated with 100 units/ml *N*-glycosidase (*N-Gly*) at room temperature overnight or left untreated, followed by immunoblot analysis using anti-mMCP8 mAb. *D*, total cell lysates of NIH3T3 cells were incubated at room temperature for 8 h in the presence or absence of the indicated proteins at a concentration of 10 μg/ml. After incubation, the cell lysates were subjected to immunoblot analysis with anti-α-tubulin polyclonal Ab. The bands corresponding to the full-length α-tubulin protein and its degradation products are indicated by *arrows*. The same set of cell lysates without incubation is shown as *0 h*. Data shown are representative of at least three independent experiments.

murine basophils both *in vitro* and *in vivo*. For instance, a monoclonal antibody specific for mMCP-8 (TUG8) is a useful tool to detect tissue-infiltrating basophils in tissue sections (20, 24). Gene targeting of the *Mcpt8* locus encoding mMCP-8 protein has been used to generate basophil-deficient or basophil reporter mice (24–26). Nevertheless, the functional property of mMCP-8 remains to be clarified, in contrast to extensive studies of the mast cell-specific proteases mMCP-1, mMCP-6, and mMCP-7 (27–33).

In this study, we prepared recombinant mMCP-8 and characterized its activity *in vitro* and *in vivo*. Our results illustrated that mMCP-8 is an important effector molecule to induce an inflammatory response with microvascular hyperpermeability and leukocyte infiltration. As far as we are aware, this is the first study to demonstrate the function of the basophil-specific protease mMCP-8 *in vivo*.

Results

Preparation and Characterization of Recombinant mMCP-8—To explore the biological functions of mMCP-8, we first prepared recombinant mMCP-8 proteins by using a baculovirus-mediated expression system. SDS-PAGE analysis of mMCP-8 proteins purified from culture supernatants of mMCP-8-transduced Sf9 cells demonstrated that purified proteins had an apparent molecular mass of 29–36 kDa (Fig. 1*A*). Purified proteins, but not control BSA, were reacted with the mMCP-8-specific mAb TUG8 in an immunoblot assay (Fig. 1*B*), indicating that they were indeed mMCP-8. Treatment of purified proteins with *N*-glycosidase F reduced their apparent molecular mass to 27 kDa (Fig. 1*C*), in accordance with previous reports that mMCP-8 is an *N*-glycoprotein (13, 23).

We next sought to check the protease activity of recombinant mMCP-8 proteins. Although mMCP-8 substrates remain unknown, mMCP-8 shows sequence similarity with mouse granzyme B in the region critical for substrate specificity (13). A previous report that granzyme B could cleave α-tubulin (34) prompted us to assess the protease activity of mMCP-8 by using α-tubulin as a tentative substrate. In accordance with the previous study (34), incubation of NIH3T3 cell lysates with granzyme B, but not control BSA, resulted in the appearance of proteolytic fragments of α-tubulin, as detected by immunoblotting with an α-tubulin-specific antibody (Fig. 1*D*). Incubation with recombinant mMCP-8 proteins reduced the apparent molecular mass of α-tubulin from 52 to 28 kDa (Fig. 1*D*). Of note, this activity of mMCP-8 was attenuated by heat treatment of mMCP-8 proteins (Fig. 1*D*), suggesting that recombinant mMCP-8 proteins had heat-sensitive protease activity.

Intradermal Administration of Recombinant mMCP-8 Induces Cutaneous Swelling with Increased Microvascular Permeability—One intradermal shot of 10 μg of recombinant mMCP-8, but not control BSA, into the ear skin of mice induced skin swelling with a peak at 4 h post-injection, followed by gradual attenuation until 24 h post-injection (Fig. 2*A*). This ear swelling-inducing activity of mMCP-8 was dose-dependent up to 10 μg (Fig. 2*B*), but no further increase of ear swelling was observed when 20 μg or more mMCP-8 was injected (data not shown). Accordingly, we used 10 μg of recombinant mMCP-8 to induce ear swelling in the following experiments. Heat treatment of recombinant mMCP-8 attenuated the ear swelling-inducing activity of mMCP-8 (Fig. 2*C*), suggesting that the protease activity of mMCP-8 played an important role in the induction of ear swelling.

Evans blue dye leakage analysis revealed that intradermal administration of recombinant mMCP-8, but not control BSA, induced an increase in microvascular permeability in the skin lesion (Fig. 3*A*). This hyperpermeability was abolished when the ear skin was pretreated with indomethacin (Fig. 3*A*), suggesting that mMCP-8 increased microvascular permeability via COX activation. Indomethacin treatment also abolished the mMCP-8-induced ear swelling (Fig. 3*B*). Meloxicam, a COX-2 inhibitor, showed a similar inhibitory effect (supplemental Fig. S1).

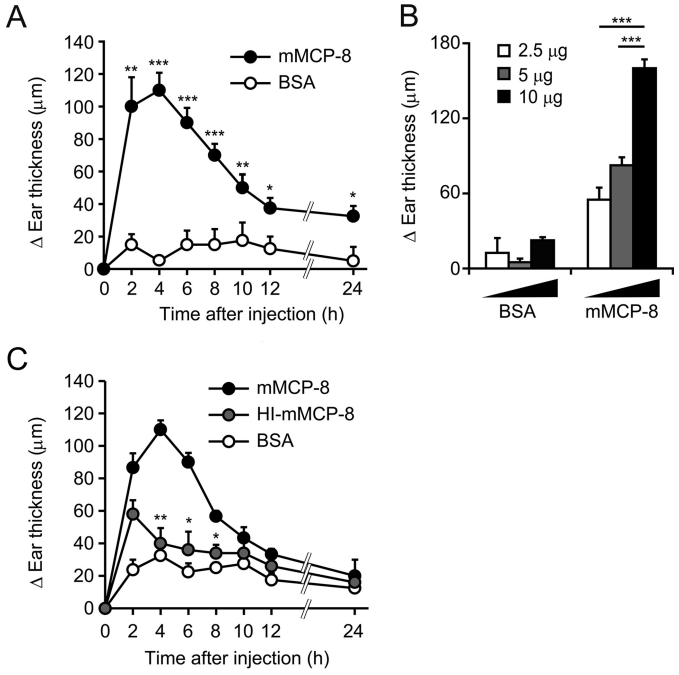

FIGURE 2. **Intradermal administration of mMCP-8 provokes a transient cutaneous swelling.** C57BL/6 mice were challenged with intradermal administration of mMCP-8, HI-mMCP-8, or control BSA into their ears (right, mMCP-8; left, BSA). *A*, time course of ear swelling (ΔEar thickness, each time points – 0-h point) after challenge with 10 μg of the indicated proteins (mean ± S.E., $n = 4$ each). *B*, ear swelling was measured 4 h after challenge with the indicated doses of proteins (mean ± S.E., $n = 4–8$ each). *C*, time course of ear swelling after challenge with 10 μg of the indicated proteins (mean ± S.E., $n = 3–8$ each). Data shown are representative of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

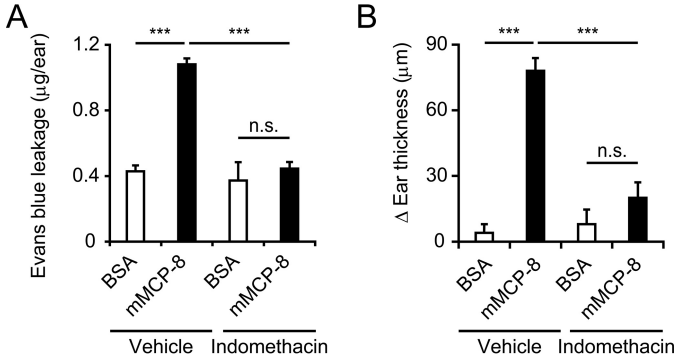

FIGURE 3. **Intradermal administration of mMCP-8 induces vascular hyperpermeability in the skin via COX activation.** *A* and *B*, C57BL/6 mice were pretreated with topical application of 200 μg of indomethacin or vehicle (ethanol) alone and then challenged with intradermal administration of mMCP-8 or control BSA into their ears. Three hours post-challenge, mice were treated with intravenous injection of Evans blue, and, 2 h later, their ears were subjected to measurement of leaked dye into the skin (mean ± S.E., $n = 5$ each) (*A*). Four hours post-challenge, ear swelling (ΔEar thickness, each time point – 0-h point) was measured (mean ± S.E., $n = 5$ each) (*B*). Data shown are representative of three independent experiments. ***, $p < 0.001$; *n.s.*, not significant.

Thus, mMCP-8 appeared to induce cutaneous swelling through COX-mediated microvascular hyperpermeability.

Repeated Intradermal Administration of mMCP-8 Induces Leukocyte Infiltration in the Skin—We then examined whether mMCP-8 triggers inflammation with leukocyte infiltration and accumulation. One intradermal shot of mMCP-8 resulted in no detectable accumulation of $CD45^+$ hematopoietic cells in the

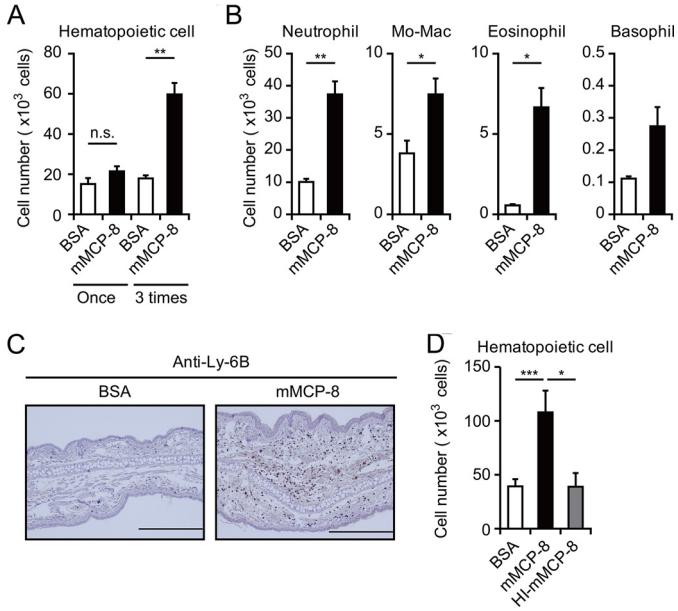

FIGURE 4. **Repeated intradermal administration of mMCP-8 induces leukocyte accumulation in the ear skin.** C57BL/6 mice were challenged once or three times at 24 h-intervals with intradermal administration of 10 μg of mMCP-8, HI-mMCP-8, or control BSA in the ear skin. The ears of treated mice were excised 6 h after the last challenge. (*A*, *B*, and *D*) The number of $CD45^+$ hematopoietic cells (*A* and *D*) and the indicated cell types (*B*) isolated from the ear skin are shown (mean ± S.E., $n = 3–6$ each). *C*, immunohistochemical analysis using anti-Ly-6B to detect neutrophils and a certain subset of monocytes/macrophages was performed. *Scale bars* = 200 μm. Data shown are representative of three independent experiments. *Mo-Mac*, monocytes/macrophages. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *n.s.*, not significant.

swollen skin lesion (Fig. 4*A*). We assumed that, in basophil-mediated inflammation such as IgE-CAI (8, 9), basophils continuously infiltrate the skin lesion and release mMCP-8 one after another. To mimic this situation in the inflammation site, we repeatedly treated mice with intradermal mMCP-8 at 24-h intervals. After three administrations of mMCP-8, we definitely detected an accumulation of $CD45^+$ hematopoietic cells in the skin lesion by using flow cytometry, predominantly neutrophils and, to a lesser extent, monocytes/macrophages and eosinophils compared with administration of control BSA (Fig. 4, *A* and *B*). In accordance with this, immunohistochemical analysis of the skin section revealed an accumulation of $Ly\text{-}6B^+$ cells in skin treated with mMCP-8 but not control BSA (Fig. 4*C*). The number of leukocytes accumulating in the skin lesion increased in a manner dependent on the dose of mMCP-8 injected (supplemental Fig. S2). Importantly, heat-inactivated mMCP-8 did not display such a leukocyte-recruiting ability (Fig. 4*D*), indicating that the protease activity of mMCP-8 is essential for leukocyte recruitment by mMCP-8.

mMCP-8 Up-regulates Chemokine Expression in the Skin—To understand the mechanism underlying the mMCP-8-elicited leukocyte infiltration, we first examined the possibility that mMCP-8 acts directly on leukocytes to promote their migration. To this end, we set up a transwell migration assay in which leukocytes were placed in the upper chamber, whereas mMCP-8, control BSA, or a relevant chemokine was included in the lower chamber. No significant migration of neutrophils, macrophages, eosinophils, or basophils into the mMCP-8-containing chamber was detected, whereas relevant chemokines

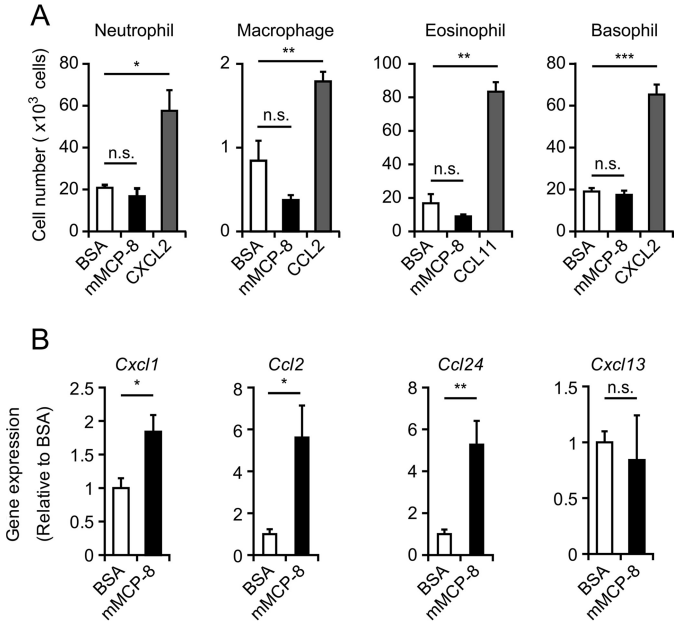

FIGURE 5. **mMCP-8 fails to induce leukocyte migration *in vitro*, whereas it enhances gene expression of chemokines in the ear skin *in vivo*.** *A*, the ability of mMCP-8 to induce leukocyte migration was examined by using the transwell system *in vitro*. The indicated types of cells (5×10^5 cells) were placed in the upper chamber, whereas mMCP-8 (*black columns*), control BSA (*white columns*), or the indicated chemokines (*gray columns*) were included in the culture medium of the lower chamber. The number of cells recovered from the lower chamber after 1.5-h incubation (for neutrophils and macrophages) or 2 h incubation (for eosinophils and basophils) at 37 °C are shown (mean ± S.E., $n = 4$ each). *B*, C57BL/6 mice were challenged three times at 24 h-intervals with intradermal administration of 10 μg of mMCP-8 or control BSA in the ear skin. The ears of treated mice were excised 6 h after the last challenge and subjected to Q-PCR analysis to access the gene expression of the indicated chemokines (mean ± S.E., $n = 9$ each). Data shown are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *n.s.*, not significant.

induced their migration (Fig. 5*A*). This observation prompted us to explore another possibility: that mMCP-8 acts on skin-resident cells to induce their production of chemokines, which, in turn, attract leukocytes. Indeed, three administrations of mMCP-8 in the skin up-regulated expression of mRNAs encoding the chemokines *Cxcl1*, *Ccl2*, and *Ccl24* (Fig. 5*B*), which are known to induce chemotaxis of neutrophils, monocytes/macrophages, and eosinophils, respectively (35), whereas no significant up-regulation of the B cell chemoattractant *Cxcl13* was detected. Up-regulated expression of CCL2 in the mMCP-8 injection site was detected at the protein level (supplemental Fig. S3*A*). Moreover, CCR2 (receptor of CCL2)-deficient mice showed reduced accumulation of monocytes/macrophages in the skin lesion compared with wild-type mice (supplemental Fig. S3*B*), suggesting a contribution of the CCL2-CCR2 axis to the migration of monocytes/macrophages to the mMCP-8 injection site. Of note, pretreatment of the ear skin with meloxicam prior to each mMCP-8 injection showed no apparent impact on leukocyte accumulation or chemokine expression (supplemental Fig. S4), in contrast to the COX-dependent edematous response (supplemental Fig. S1).

Discussion

Mcpt8 is the only known gene that is selectively expressed by mouse basophils (14, 20). Therefore, this gene and its product mMCP-8 are commonly used as basophil-specific markers to identify basophils and generate engineered mice with basophil-specific modification (14, 20, 24–26) even though the biological function of mMCP-8 remains unknown. In this study, we demonstrated that mMCP-8 can provoke an inflammatory response in the skin with increased microvascular permeability and leukocyte infiltration in a protease activity-dependent manner. Considering that basophils play a crucial role in the development of inflammation, including IgE-CAI (8, 9), the basophil-specific protease mMCP-8 could be an important effector molecule involved in the induction of such inflammation.

mMCP-8-elicited cutaneous swelling with microvascular hyperpermeability was almost completely inhibited by indomethacin treatment, indicating that COX-mediated production of prostaglandins likely contributes to the formation of edematous swelling. Although the exact mechanism underlying the mMCP-8-mediated COX activation remains to be determined, the inability of heat-inactivated mMCP-8 in this function suggested that proteolytic cleavage of a protein(s) on target cells may trigger the induction or activation of COX. One intradermal shot of mMCP-8 induced edematous swelling in the skin with no apparent infiltration of leukocytes, whereas three consecutive injections resulted in accumulation of leukocytes. Therefore, mMCP-8-elicited microvascular hyperpermeability in the skin did not seem to directly contribute to leukocyte extravasation and accumulation in the skin. Because the expression of leukocyte-attracting chemokines was up-regulated in the skin lesion after three injections of mMCP-8, it is likely that mMCP-8 at certain amounts persisting for a while activated skin-resident cells to produce chemokines in a protease activity-dependent manner. mMCP-4 and human chymase reportedly show chemotactic activity that directly attracts leukocytes *in vitro* (36, 37). As far as we examined in the transwell migration assay, mMCP-8 showed no such activity.

A previous study using chromogenic substrates and a phage-displayed random nonapeptide library failed to identify the candidates of mMCP-8 substrates (23), and no further study to identify them has been reported, to our knowledge. In this study, we could show the heat-sensitive protease activity of mMCP-8, as assessed by the proteolysis of α-tubulin. Although α-tubulin may not be a physiological substrate of mMCP-8, further analysis of the amino acid sequence of its proteolytic fragments may give a clue to identify real substrates.

In conclusion, we demonstrated in this study that the basophil protease mMCP-8 can elicit an inflammatory response in the skin with microvascular hyperpermeability and leukocyte infiltration. Considering the basophil-restricted expression and pro-inflammatory activity of mMCP-8 shown here, mMCP-8 may contribute to the non-redundant role of basophils, distinct from that played by mast cells, in immune responses, including allergic inflammation and protective immunity against parasitic infections.

Experimental Procedures

Mice—C57BL/6 and BALB/c mice (7–9 weeks old) were purchased from Japan SLC. *Ccr2*$^{-/-}$ BALB/c mice were as described previously (38). All animal studies were approved by

the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Antibodies—The following Abs were purchased from BioLegend: biotinylated anti-CD49b (DX5), FITC-conjugated anti-CD49b (HMα2), anti-Ly-6G (1A8), allophycocyanin-conjugated anti-CD200R3 (Ba13), anti-F4/80 (BM8), phycoerythrin-Cy7-conjugated anti-CD45 (30-F11), allophycocyanin-Cy7-conjugated anti-Gr-1 (RB6–8C5), Pacific Blue-conjugated anti-CD11b (M1/70), and anti-c-Kit (2B8). Anti-Ly-6B.2 (7/4) and phycoerythrin-conjugated anti-Siglec-F (E50–2440) were from AbD Serotec and BD Biosciences, respectively. Rabbit anti-α-tubulin polyclonal Ab (2144S) was from Cell Signaling Technology. Anti-mMCP-8 (TUG8) was established as reported previously (20). Anti-CD16/32 (2.4G2) was prepared in our laboratory.

Preparation of Recombinant mMCP-8—Recombinant mMCP-8 was prepared by using a baculovirus expression system. In brief, the cDNA fragment encoding mMCP-8 tagged with a FLAG peptide between the natural activation peptide (13), and the first residue of the catalytic domain was inserted into the pFastBac1 baculoviral vector (Invitrogen). The resultant vector was transfected into Sf9 cells to produce a recombinant protein according to the protocol of the manufacturer of the BAC-to-BAC expression system (Invitrogen). The recombinant protein was purified by anti-DYKDDDDK (FLAG) tag antibody beads (Wako) from culture supernatants of transfected Sf9 cells, followed by treatment with enterokinase (Novagen) to cleave off their N-terminal sequences containing the natural activation peptide and FLAG peptide. After removal of enterokinase by EKapture agarose (Novagen) and dialysis with PBS, the purity and identity of the recombinant protein were confirmed by SDS-PAGE, followed by detection with Coomassie Brilliant Blue staining (Nacalai Tesque) and immunoblotting with anti-mMCP-8 mAb, respectively. Before use, both recombinant mMCP-8 and control BSA were treated with polymyxin B using Proteus NoEndo Micro Spin Column Kits (Protein Ark) to remove potential contamination of the endotoxin. In some experiments, a heat-inactivated form of mMCP-8 (HI-mMCP8) was prepared by incubating recombinant mMCP-8 at 95 °C for 1 h.

α-Tubulin Cleavage Assay—The α-tubulin cleavage assay was performed as described previously with some modifications (34). Briefly, 4×10^7 NIH3T3 cells were lysed with 100 μl of PBS containing 0.5% Triton X-100. After removal of insoluble components, the lysates were incubated at room temperature for 8 h in the presence or absence of 10 μg/ml mMCP-8, HI-mMCP-8, granzyme B, or control BSA. The resultant lysates were subjected to SDS-PAGE, followed by immunoblotting with anti-α-tubulin polyclonal Ab. The same set of lysates without incubation were utilized for the 0-h experiment as input.

mMCP-8-induced Cutaneous Inflammation—The indicated amounts of mMCP-8 or control BSA in 10 μl of PBS were intradermally administered, once or three times at 24 h-intervals, into the ear skin of C57BL/6 mice (right, mMCP-8; left, BSA). The ear thickness was measured by a dial thickness gauge (Peacock) at the indicated time points, and the degree of ear swelling was determined by ΔEar thickness (each time point − 0-h point). To evaluate vascular permeability, mice were intravenously injected with 0.5% Evans blue dye in 100 μl of PBS at 3 h after the intradermal administration of mMCP-8 or BSA. Two hours after the dye injection, the ears were excised and incubated in 0.7 ml of formamide at 63 °C 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. For flow cytometric or immunohistochemical analysis to assess cell infiltration or gene expression analysis for chemokines, ears were excised 6 h after the last administration of the reagents. In some experiments, HI-mMCP-8 was used.

Flow Cytometric and Immunohistochemical Analyses—Single-cell suspensions were obtained from ear skins by treatment with 125 units/ml collagenase (Wako) at 37 °C for 2 h. After pretreatment with anti-CD16/32 mAb (2.4G2) and normal rat serum to avoid the nonspecific binding of irrelevant Abs, cells were stained with the indicated combination of Abs on ice for 30 min and analyzed by FACSCanto (BD Biosciences). Each cell lineage was defined as follows: neutrophils (Ly-6G$^+$), eosinophils (Siglec-F$^+$SSC^{high}), monocyte-macrophages (F4/80$^+$CD11b$^+$ among cells in which both eosinophils and neutrophils were excluded), and basophils (c-kit$^-$CD49b$^+$CD200R3$^+$). For immunohistochemical analysis, ear specimens were fixed and embedded in paraffin, and sections were stained with anti-Ly-6B.2 mAb (7/4) in combination with the appropriate secondary Ab and 3,3′-diaminobenzidine, followed by hematoxylin counterstaining (39, 40).

Quantitative PCR (Q-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. Q-PCR of the cDNA was performed by using the following primer sets: 5′-ACTGCACCCAAACCGAAGTC-3′ and 5′-TGGGGACACCTTTTAGCATCTT-3′ for *Cxcl1*, 5′-TTAAAAACCTGGATCGGAACCAA-3′ and 5′-GCATTAGCTTCAGATTTACGGGT-3′ for *Ccl2*, 5′-ATTCTGTGACCATCCCCTCAT-3′ and 5′-TGTATGTGCCTCTGAACCCAC-3′ for *Ccl24*, 5′-CATAGATCGGATTCAAGTTACGCC-3′ and 5′-TCTTGGTCCAGATCACAACTTCA-3′ for *Cxcl13*, and 5′-GGCCCTCGACTCTCGCTTTC-3′ and 5′-TGCCAGGACGCGCTTGT-3′ for *36B4*. Relative gene expression levels were calculated using standard curves generated by serial dilutions of each cDNA standard and normalized by *36B4* expression levels.

Transwell Migration Assay—Neutrophils were prepared from the bone marrow by using a 62% Percoll gradient as described previously (41). Eosinophils were isolated from the peritoneum of mice that had been treated for 7 days with daily intraperitoneal administration of IL-5 (42). 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. Mouse bone marrow-derived basophils were generated as described previously (20). In brief, total bone marrow cells were cultured in the presence of 300 pg/ml recombinant IL-3 (BioLegend) for 7 days, followed by purification with biotinylated anti-CD49b (DX5) in combination with the IMag cell separation system (BD Biosciences). The transwell apparatus (Kurabo) consisted of the upper and lower chambers separated by a membrane with 3-μm (for neutrophils) or 5-μm (for other cell types) pore size. Leukocytes (5×10^5 cells) were

placed into the upper chamber, whereas 10 μg/ml mMCP-8, chemokines (CXCL2 100 ng/ml, CCL11 200 ng/ml, or CCL2 200 ng/ml), or control BSA was included in the culture medium of the lower chamber. Ninety minutes (for neutrophils and macrophages) or 2 h (for eosinophils and basophils) after incubation at 37 °C, the cells migrating into the lower chamber were counted.

Statistical Analysis—Statistical analysis was performed with unpaired Student's *t* test. $p < 0.05$ was considered statistically significant.

Author Contributions—H. T. performed the experiments. H. O. performed the immunohistochemical staining. S. S. generated recombinant mMCP-8. S. Y. provided helpful suggestions. H. T., Y. Y., and H. K. wrote the manuscript. Y. Y. and H. K. designed and supervised the study.

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