Sensitized Mast Cells Migrate Toward the Agen: A Response Regulated by p38 Mitogen-Activated Protein Kinase and Rho-Associated Coiled-Coil-Forming Protein Kinase

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Sensitized Mast Cells Migrate Toward the Agen: A Response Regulated by p38 Mitogen-Activated Protein Kinase and Rho-Associated Coiled-Coil-Forming Protein Kinase

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Although mast cells accumulate within the mucosal epithelial layer of patients with allergic rhinitis and bronchial asthma, the responsible chemotactic factors are undefined. We investigated whether mast cells sensitized with Ag-specific IgE migrate toward the Ag. MC/9 mast cells sensitized with anti-DNP IgE migrated toward DNP-conjugated human serum albumin. This migration was directional, and the degree was stronger than that induced by stem cell factor. IL-3 and stem cell factor-dependent cultured mast cells derived from mouse bone marrow also migrated toward the Ag. Subsequent migration mediated by the FceRI was significantly inhibited by incubating the cells with Y-27632, a Rho-associated coiled-coil-forming protein kinase inhibitor, or with SB203580, a p38 mitogen-activated protein kinase (MAPK) inhibitor. Both p38 MAPK and MAPK-activated protein kinase (MAPKAPK)2 were activated following FceRI aggregation, and activation of MAPKAPK2 was almost completely inhibited by 10 μM SB203580. Wortmannin or a low concentration of SB203580 partially inhibited MAPKAPK2, but did not block mast cell migration. In contrast, Y-27632 did not affect the activation of MAPKAPK2. These results indicate that Ag works not only as a stimulant for allergic mediators from IgE-sensitized mast cells, but also as a chemotactic factor for mast cells. Both p38 MAPK activation and Rho-dependent activation of Rho-associated coiled-coil-forming protein kinase may be required for FceRI-mediated cell migration. The Journal of Immunology, 2001, 167: 2298–2304.

Materials and Methods

Cells and reagents

The MC/9 mouse mast cell clone, obtained from the American Type Culture Collection (Manassas, VA), was maintained by passage in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Life Technologies), 50 μM 2-ME (Life Technologies), and 5 ng/ml mouse rIL-3 (provided by KIRIN Brewery, Yokohama, Japan). Mouse bone marrow obtained from the femurs of female BALB/c mice was cultured in

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DMEM supplemented with 10% FBS, 50 μM 2-ME, 100 μg/ml streptomycin, 100 U/ml penicillin, 0.5 μg/ml amphotericin B, 5 mg/ml IL-3, and 10 ng/ml recombinant mouse SCF (PeproTech, London, U.K.). After 4 wk of culture, >95% of nonadherent cells contained granules that stained positively with toluidine blue. These cells are referred to as IL-3- and SCF-dependent mouse bone marrow–derived cultured mast cells (IL-3/SCF-BMMC). Bovine myelin basic protein, rabbit anti-MAPKAPK2 polyclonal Ab, and MAPKAPK2 substrate peptide (TTYADFIASGRTGR) were obtained from Upstate Biotechnology (Lake Placid, NY). POLYclonal anti-extracellular signal-regulated kinase (ERK)2/C-14 agarose conjugate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-p38 MAPK polyclonal Ab was purchased from Cell Signaling Technology (Beverly, MA). Recombinant protein G agarose and protein A agarose were purchased from Zymed Laboratories (San Francisco, CA). DNP-conjugated human serum albumin (DNP-HSA), DNP-conjugated lysine (DNP-lysine), and actinomycin D were obtained from Seikagaku Kogyo (Tokyo, Japan). DNP-conjugated human serum albumin (DNP-HSA), Mouse monoclonal anti-DNP IgE was obtained from Seikagaku Kogyo (Tokyo, Japan). The chambers were incubated for 1 h at 37°C in a moist 5% CO2 atmosphere. Mast cell movement was quantified by counting the number of mast cells in the bottom well using a Fuchus Rosenthal calculator (Erma, Tokyo, Japan). IL-3/SCF-BMMC were cultured with 500 ng/ml anti-DNP IgE in complete culture medium for 18 h. The cell viability and characteristics of IL-3/SCF-BMMC were maintained by complete culture medium. Thereafter, cells were washed three times with 0.1% BSA-DMEM. IL-3/SCF-BMMC suspended at 1 × 10^6 cells/200 μl in 0.1% BSA-DMEM were placed in the top wells, and DNP-HSA (10 ng/ml) was applied to the bottom wells. Cells that had migrated were counted after 6 h.

Measurement of cytoplasmic free Ca2+ ([Ca2+]i)

MC9 cells were incubated for 20 min with 1 μM fura 2 acetoxymethyl ester in 0.1% BSA-DMEM. [Ca2+]i was estimated as a change in the fluorescence of the fura 2-loaded cells, as previously described (12).

Kinase assay of MAPKAPK2

MC9 cells (3 × 10^6) were lysed in buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM Na2VO4, 0.1% (v/v) 2-ME, 1% Triton X-100, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 50 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, and 5 μg/ml leupeptin). The lysates were incubated with 5 μg/ml rabbit anti-MAPKAPK2 Ab for 2 h at 4°C. Recombinant protein G agarose was added to the lysates and incubated for 1 h at 4°C. The immunoprecipitates were washed twice with lysis buffer and once with kinase buffer (20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na2VO4, and 1 mM DTT). After the final wash, 30 μl kinase assay buffer containing 10 μCi [γ-32P]ATP, 150 μM cold ATP, 23 mM MgCl2, and 10 μg substrate peptide (KKNRNTLSVA) was added per sample. The samples were incubated for 30 min at 30°C, and then the reaction was stopped by adding 30 μl of excess phosphoric acid. Samples (25 μl) were loaded onto phosphocellulose paper (Whatman, Maidstone, U.K.), and phosphorylation of the substrate peptide was determined by liquid scintillation counting.

Kinase assay of c-Jun amino-terminal kinase (JNK)

GST-c-Jun fusion protein was prepared, and kinase activity was measured as previously described (13-16).

FIGURE 1. Mast cells migrate toward Ag and SCF. A, MC9 cells sensitized with anti-DNP IgE (1 × 10^6 cells) were placed in the upper wells and separated from the lower wells by polycarbonate filters. Ag (0–100 ng/ml DNP-HSA) was added to the lower wells. Unsensitized MC9 cells were also applied to the upper wells, and 10 ng/ml DNP-HSA was added to the lower wells. Chambers were incubated for 4 h at 37°C under moist 5% CO2. Mast cell movement was quantified by counting numbers of cells that migrated to the lower wells. B, Unsensitized MC9 cells (1 × 10^6 cells) were placed in the top wells, and SCF (0–100 ng/ml) was applied to the bottom wells. After a 4-h incubation, migrated cells were counted. C, Sensitized or unsensitized MC9 cells (1 × 10^6 cells) were placed in the upper wells. Ag (10 ng/ml DNP-HSA) or SCF (10 ng/ml) was added to the lower wells. Chambers were incubated for 1–6 h, and migrated cells were counted. D, IL-3/SCF-BMMC sensitized with anti-DNP IgE (1 × 10^6 cells) were applied to the top wells, and Ag (0–100 ng/ml DNP-HSA) was added to the lower wells. Migrated cells were counted after a 6-h incubation.
Kinase assay of p38 MAPK

The activity of p38 kinase was assayed as previously described using activating transcription factor-2 as the substrate (14–16), and anti-phospho-p38 MAPK (1/100 dilution) was used for immunoprecipitation.

Kinase assay of ERK2

The kinase activity of ERK2 was assayed in vitro as previously described (13–16) using myelin basic protein as the substrate, and anti-ERK2 agarose conjugate was used for immunoprecipitation.

Results

Sensitized mast cells migrate toward the Ag

Sensitized MC/9 cells migrated toward the bottom wells in a dose-dependent manner after 4 h. Cell migration reached maximal levels at 10 ng/ml DNP-HSA. In contrast, unsensitized MC/9 cells did not migrate toward the bottom wells containing 10 ng/ml DNP-HSA (<200/well) (Fig. 1A). The degree of cell migration decreased with excess Ag. Unlike when the bottom wells contained Ag, few sensitized MC/9 cells migrated toward the bottom wells when DNP-HSA was added to the top wells (1,000 vs 81,200 migrated cells/well). These results suggest that the migration was directional. Ag-induced cell migration was significantly increased after 4 h with a 1- to 2-h lag (Fig. 1C).

To exclude the possibility that an unknown factor generated from MC/9 cells following FcεRI aggregation acts as a chemotactic factor for mast cells, the supernatant from Ag-stimulated MC/9 cells was applied to the bottom wells. MC/9 cells sensitized with anti-DNP IgE were stimulated by DNP-HSA (10 ng/ml) for 1, 2, or 4 h, and the supernatant was obtained by centrifugation and filtration. Unsensitized MC/9 cells did not migrate at all (<200/well) to the bottom wells containing supernatants from cultures stimulated for 1, 2, and 4 h with DNP-HSA (10 ng/ml). Incubating sensitized MC/9 cells for 15 min with a large excess of monovalent hapten (DNP-lysine) disrupted multivalent binding and inhibited subsequent cell migration induced by multivalent Ag (DNP-HSA) in a dose-dependent manner. Cell migration was completely blocked by 1 mM DNP-lysine (<200/well) (Fig. 2A). Supernatants derived from Ag-stimulated culture incubated for 1, 2, and 4 h with DNP-HSA (10 ng/ml) were placed in the bottom wells. The top wells contained sensitized MC/9 cells in the presence of 1 mM DNP-lysine. Sensitized MC/9 cells hardly migrated (<200/well) to the bottom wells under these conditions. To further confirm that Ags can cause migration in the absence of chemotactic factors in the medium, we examined the chemotactic ability of sensitized MC/9 cells to Ag in the presence of a high dose of actinomycin D (5 μg/ml). Sensitized MC/9 cells were incubated with actinomycin D for 30 min and placed in the top wells. Actinomycin D (5 μg/ml) and a control vehicle (0.1% ethanol) were added to both wells, and DNP-HSA was added to the bottom wells. Ag-induced cell migration was not inhibited by actinomycin D after incubation for 1, 2, and 4 h (Fig. 2B).

Another unique receptor on mast cells is c-Kit, a transmembrane tyrosine kinase receptor (17, 18). The cognate ligand for c-Kit, SCF, promotes the growth, differentiation, and activation of mast cells (19, 20). SCF has chemotactic activity for mast cells (4, 5) and, when applied to the bottom wells, also induced cell migration. Migrated cells reached a maximal level at 10 ng/ml after 4 h (Fig. 1B). However, the degree of cell migration was weaker than that induced by the Ag (Fig. 1C).

The MC/9 mast cell line has been used as a model of mast cells to investigate intracellular signaling and function (13–15, 21, 22). To examine the generality of this phenomenon, we investigated whether IL-3/SCF-BMMC migrate toward Ag as well as MC/9 cells. Sensitized IL-3/SCF-BMMC migrated toward the bottom wells in a dose-dependent manner after a 6-h incubation (Fig. 1D).

Few sensitized IL-3/SCF-BMMC stimulated by DNP-HSA in the top wells migrated toward the bottom wells (640 vs 11,200 migrated cells/well).

Effects of wortmannin, a MAPK/ERK kinase (MEK)1 inhibitor, PD98059, and the p38 MAPK inhibitor, SB203580, on FcεRI- or c-Kit-mediated cell migration

We showed that three members of the MAPK family, JNK, p38 MAPK, and ERK, are activated by FcεRI or c-Kit-mediated cell migration.

To exclude the possibility that an unknown factor generated from MC/9 cells following FcεRI aggregation acts as a chemotactic factor for mast cells, the supernatant from Ag-stimulated MC/9 cells was applied to the bottom wells. MC/9 cells sensitized with anti-DNP IgE (5 × 10^6 cells/ml) were incubated in the presence or absence of DNP-lysine (10–1,000 μM) for 15 min. Cells (1 × 10^6) were then placed in the upper wells, and Ag (10 ng/ml DNP-HSA) was applied to the bottom wells. Migrated cells were counted after 4 h. The number of migrated cells was standardized by comparison with the number of migrated cells in the absence of DNP-lysine. Data are shown as means ± SD (n = 4).

B, MC/9 cells sensitized with anti-DNP IgE (5 × 10^6 cells/ml) were incubated with 5 μg/ml actinomycin D or control vehicle (0.1% ethanol) for 30 min. Thereafter, cells (1 × 10^6) were placed in the upper wells, and Ag (10 ng/ml DNP-HSA) was applied to the bottom wells. Actinomycin D (5 μg/ml) or control vehicle was added to both top and bottom wells. Migrated cells were counted after 1, 2, and 4 h. The number of migrated cells was standardized by comparison with the number of migrated cells in control vehicle after 4 h. Data are shown as means ± SD (n = 4).

FIGURE 2. Effect of excess monovalent hapten and high dose of actinomycin D on Ag-induced mast cell migration. A, MC/9 cells sensitized with anti-DNP IgE (5 × 10^6 cells/ml) were incubated in the presence or absence of DNP-lysine (10–1,000 μM) for 15 min. Cells (1 × 10^6) were then placed in the upper wells, and Ag (10 ng/ml DNP-HSA) was applied to the bottom wells. Migrated cells were counted after 4 h. The number of migrated cells was standardized by comparison with the number of migrated cells in the absence of DNP-lysine. Data are shown as means ± SD (n = 4).
activation (Fig. 4, A). MAPK inhibitor, SB203580, inhibited FcεRI activation (data not shown), in agreement with our previous findings (15). These results suggested that ERK activation is not required for mast cell migration. In contrast to SB203580, wortmannin failed to alter the ERK activation (Fig. 4, A and B). This suggests that FcεRI-mediated JNK activation is not required for cell migration (Fig. 3A). In contrast to signaling through FcεRI, wortmannin failed to alter the c-Kit-mediated activation of JNK and p38 MAPK (Fig. 4, C and D). The MEK1 inhibitor, PD98059, did not affect FcεRI- or c-Kit-mediated migration of MC/9 cells (Fig. 3B), but significantly inhibited ERK activation (data not shown), in agreement with our previous findings (15). These results suggested that ERK activation is not required for mast cell migration. In contrast, the p38 MAPK inhibitor, SB203580, inhibited FcεRI- and c-Kit-mediated migration in a dose-dependent manner. SB203580 (10 μM) significantly inhibited FcεRI- and c-Kit-mediated migration (81 and 76%, respectively). The inhibitory effect of SB203580 on cell migration was more significant at 10 μM than at 1 μM (Fig. 3C).

FIGURE 3. Effects of wortmannin, a MEK1 inhibitor, PD98059, and a p38 MAPK inhibitor, SB203580 on mast cell migration. MC/9 cells sensitized with anti-DNP IgE or unsensitized MC/9 cells (5 × 10^6 cells/ml) were incubated with 100 nM wortmannin (15 min) (A), 30 μM PD98059 for 30 min (B), 0.1–10 μM SB203580 (30 min) (C), or control vehicle (0.01–0.1% DMSO). Thereafter, cells (1 × 10^6 cells) were placed in the top wells, and Ag (10 ng/ml DNP-HSA) or SCF (10 ng/ml) was added to the bottom wells. SB203580, PD98059, wortmannin, or control vehicle was added to both top and bottom wells. Migrated cells were counted after 4 h. The number of migrated cells in the presence of SB203580, PD98059, and wortmannin was standardized by that in control vehicle. Data are shown as means ± SD (n = 6).

Effects of the p38 MAPK inhibitor, SB203580, on MAPKAPK2 activation

In contrast to SB203580, wortmannin slightly blocked mast cell migration and partially inhibited the FcεRI-mediated activation of p38 MAPK. Therefore, we further examined how p38 MAPK regulates mast cell migration. Activation of p38 MAPK results in the phosphorylation of heat shock protein (HSP)25/27, which may modulate F-actin polymerization in several types of cells (23–25). Because SB203580 is a reversible inhibitor of p38 MAPK but not of the phosphorylation of p38 MAPK regulated by MAPK kinase (MKK)3 or MKK6, it is difficult to evaluate the degree of inhibitory effect of SB203580 on p38 MAPK activity in intact cells using lysates (26). Therefore, we assessed the activation of MAPKAPK2 because this enzyme is phosphorylated and activated by p38 MAPK (8–10). MC/9 cells sensitized with anti-DNP IgE were challenged by DNP-HSA or SCF. MAPKAPK2 activity reached maximum levels at 5 min. MAPKAPK2 activation was mediated more by FcεRI than by c-Kit (Fig. 5A). Fig. 5B shows that MAPKAPK2 activation through either FcεRI or c-Kit was inhibited by SB203580 in a dose-dependent manner, and that susceptibility through either receptor did not differ. SB203580 inhibited MAPKAPK2 activation in MC/9 cells almost completely at 10 μM and considerably (60–70%) at 1 μM.

To exclude the possibility of SB203580 cytotoxicity, we compared the [Ca^{2+}], increase in sensitized MC/9 cells stimulated by DNP-HSA after a 4.5-h incubation with 10 μM SB203580 to that with a control vehicle. The difference in the [Ca^{2+}], increase between the two conditions was not significant (control vs SB203580, 1055 ± 233 nM vs 1005 ± 242 nM; mean ± SD; n = 6). These results indicate that almost complete inhibition of p38 MAPK and MAPKAPK2 activation is required to block mast cell...
migration, and that partial activation of these kinases is sufficient for MC/9 cells to migrate. Wortmannin, as well as 1 μM SB203580, weakly inhibited mast cell migration as described above, although wortmannin partially inhibited the FceRI-mediated activation of p38 MAPK and MAPKAPK2 (Figs. 4B and 5C). These findings support the notion that the partial inhibition of p38 MAPK and MAPKAPK2 is not sufficient to block mast cell migration.

Effects of Y-27632 on FceRI- or c-Kit-mediated migration

The ROCK inhibitor, Y-27632, significantly inhibited both the FceRI- and c-Kit-mediated migration of MC/9 cells at 10 μM (Fig. 6). Y-27632 inhibits FceRI- or c-Kit-mediated migration but not MAPKAPK2 activation in MC/9 cells. A, MC/9 cells sensitized with anti-DNP IgE or not (5 × 10⁶ cells/ml each) were incubated with 10 μM Y-27632 or control vehicle (distilled water) for 15 min. Cells (1 × 10⁶) were then placed in the upper wells, and Ag (10 ng/ml DNP-HSA) or SCF (10 ng/ml) was applied to the bottom wells. Y-27632 or control vehicle was added to both top and bottom wells. Migrated cells were counted after 4 h. The number of migrated cells in the presence of Y-27632 was standardized by comparison with that in the presence of control vehicle. B, MC/9 cells sensitized or not with anti-DNP IgE (3 × 10⁶ cells each) were incubated for 15 min with Y-27632 or control vehicle and were then stimulated for 5 min by 10 ng/ml DNP-HSA or 10 ng/ml SCF. MAPKAPK2 activities in the cells were measured as described in Materials and Methods and standardized to that in the presence of control vehicle.

FIGURE 7. Putative intracellular mechanism of FceRI-mediated mast cell migration. Aggregation of FceRI in mast cells activated p38 MAPK and MAPKAPK2/3 followed by phosphorylation of HSP25 (HSP27). Such aggregation also activated Rho/ROCK followed by phosphorylation of myosin light chain (MLC) independently of p38 MAPK activation. Activation of both p38 MAPK and ROCK seems to be required for mast cell migration. MP, Myosin phosphatase.
6A). To investigate the mechanism of this action, we measured MAPKAPK2 activation following ligation of FcεRI or c-Kit in the presence or absence of Y-27632. Subsequent FcεRI- or c-Kit-mediated MAPKAPK2 activation was not affected by Y-27632 (Fig. 6B). Y-27632 should inhibit cell migration by inhibiting Rho-dependent ROCK activation. Although the activation of Rho/ROCK is required for mast cell migration, the Rho-dependent intracellular signaling pathway seems to be independent of the p38 MAPK-dependent pathway (Fig. 7).

Discussion
Allergic reaction is initiated by FcεRI aggregation in mast cells following the binding of a multivalent Ag to IgE. It is believed that such Ags bind to IgE on mast cells by chance. Mast cells accumulate at sites of inflammation and wound healing (2, 3) and in the epithelial layer of the mucosa of patients with allergic rhinitis and bronchial asthma after natural exposure to pollen or a provocation test (27–32). This area of the mucosa normally has very few, if any, mast cells. Such epithelial mast cells bear surface IgE (33). The chemotactic factors responsible for mast cells in an allergic reaction are undefined, although many chemokines and cytokines, including SCF, RANTES, eotaxin, monocyte chemotactic protein-1, and TGF-β, can elicit mast cell migration (4–7, 34, 35). In the present study, we found that the Ag itself has chemotactic activity for IgE-sensitized mast cells, which suggests that mast cells sensitized with Ag-specific IgE migrate to sites of optimal Ag concentration. This phenomenon is reasonable if mast cells primarily work as host defense cells against parasites and other organisms through FcεRI, although it also may result in the development of allergic states.

The mammalian MAPK family consists of ERK, JNK, and p38 MAPK. We recently showed that FcεRI aggregation or c-Kit ligation activates all three (13–16). These kinases should regulate gene expression by phosphorylating transcription factors. We also showed that phosphatidylinositol 3-kinase and MEK kinases, especially MEK kinase 2, play an important role in cytokine production by Ag-stimulated mast cells, mainly through JNK activation (36). The role of p38 MAPK in mast cells is obscure, although FcεRI-mediated activation of p38 MAPK as well as JNK is regulated by phosphatidylinositol 3-kinase and calcineurin (14–16).

MAPKAPK2, which was originally designated HSP25/27 kinase, is phosphorylated and activated by p38 MAPK (8–10). HSP25/27 phosphorylation by MAPKAPK2/3 induces actin fiber polymerization and is required for the directional migration of many kinds of cells, including human bronchial smooth muscle and endothelial cells (24, 25, 37–41). The p38 MAPK inhibitor, SB203580, inhibits cell migration in response to platelet-derived growth factor, sphingosine 1-phosphate, IL-1β, and TGF-β (42, 43). The activated mutant of MKK6, which activates p38 MAPK, increases cell migration, and either the dominant negative p38 MAPK or an HSP25 phosphorylation mutant blocks cell migration in human bronchial smooth muscle cells (37). The present study shows that MAPKAPK2 was significantly activated following FcεRI or c-Kit ligation, and its activation was abolished by 10 μM SB203580. Although FcεRI-mediated activation of p38 MAPK and MAPKAPK2 was significantly inhibited by wortmannin, it was not sufficient to block mast cell migration. The inhibitory effect of SB203580 on mast cell migration was weaker at 1 μM than at 10 μM. These results suggested that MAPKAPK2 must be completely inhibited to block mast cell migration.

The small GTPase, Rho, controls cell adhesion and motility through reorganization of the actin cytoskeleton (44). Rho in mast cells promotes cortical F-actin disassembly in addition to controlling secretion and actin polymerization, and all of these effects are blocked by the C3 exoenzyme in permeabilized mast cells (45). Several proteins have been identified as Rho effectors, including ROCK (11), which plays a key role in focal adhesion and stress fiber formation and in the Ca2+ sensitization of smooth muscle (46). Y-27632 was originally identified as an inhibitor of two ROCK isoforms and its antihypertensive effect is achieved by preventing ROCK from inhibiting smooth muscle protein phosphatase 1M, the major myosin phosphatase of this tissue. This decreases the phosphorylation of myosin, increases arterial smooth muscle relaxation, and hence increases the dilation of blood vessels (47). Y-27632 also inhibits Rho-mediated cell transformation, tumor cell invasion, and chemotaxis in many types of cells (48–52). Both FcεRI- and c-Kit-mediated migration of mast cells were inhibited by Y-27632 in the present investigation. A Rho/ROCK activation pathway seems to be independent of the p38 MAPK/MAPKAPK2 activation pathway because Y-27632 did not affect MAPKAPK2 activation.

In summary, we demonstrated that the Ag itself induces directional mast cell migration, the process of which is regulated by both p38 MAPK/MAPKAPK2 and Rho/ROCK activation pathways. In allergic conditions such as bronchial asthma and allergic rhinitis, mast cell accumulation in a lesion is important in the development of disease. Our findings provide novel evidence of Ag-induced mast cell migration and should support the development of a therapeutic approach to this phenomenon in allergic states.

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References


