

Impact of Engagement of FcεRI and CC Chemokine Receptor 1 on Mast Cell Activation and Motility*

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CC chemokines participate in the recruitment and activation of immune cells through CC chemokine receptors (CCRs). Here, we report that cross-talk between CCR1-mediated signaling pathway and FcεRI-mediated signaling pathway affects degranulation positively but affects chemotaxis of mast cells adversely. Costimulation via FcεRI engagement with IgE/antigen and CCR1 engagement with recombinant human CCL3 synergistically enhanced degranulation in rat basophilic leukemia-2H3 cells expressing human CCR1 (RBL-CCR1). Interestingly, FcεRI engagement inhibited CCL3-mediated chemotaxis and membrane ruffling of RBL-CCR1 cells. Small GTP-binding proteins of the Rho family, Rac, Cdc42, and Rho control chemotaxis by mediating the reorganization of the actin cytoskeleton. Both a Rho inhibitor C3 exoenzyme and a Rho kinase (ROCK) inhibitor Y-27632 inhibited chemotaxis of RBL-CCR1 cells toward CCL3, indicating that activation of the Rho/ROCK signaling pathway is required for the CCL3-mediated chemotaxis of the cells. Costimulation with IgE/antigen and CCL3 enhanced Rac and Cdc42 activation but decreased ROCK activation in RBL-CCR1 cells compared with that in the cells stimulated with CCL3 alone. These results suggest that costimulation via FcεRI and CCR1 engagements induced 1) inhibition of membrane ruffling, 2) decreased ROCK activation, and 3) reciprocal imbalance between Small GTP-binding proteins of the Rho family, which result in the inhibition of chemotaxis of RBL-CCR1 cells. The cross-talk between FcεRI-mediated signaling pathway and CCR-mediated signaling pathway would induce optimal activation and arrested chemotaxis of mast cells, thus contributing to allergic inflammation.

Mast cells and basophils play an important role in IgE-associated allergic disorders and immune responses to parasites. FcεRI cross-linking is a key event in the activation of

mast cells and basophils. The FcεRI is composed of three subunits: an α chain, a β chain, and a pair of γ chains, joined by a disulfide bond. The association of allergen with IgE bound to FcεRI induces the transphosphorylation of cytoplasmic domains in the β- and γ-chains called immunoreceptor tyrosine-based activation motifs via Lyn activation, which trigger signaling cascades leading to activation of kinases, phosphatases, or GTPases (1, 2). FcεRI-mediated signaling induces a variety of events such as degranulation, increased gene transcription, and cytoskeleton rearrangement in mast cells and basophils (3, 4).

Apart from the classical FcεRI-mediated mechanism, mast cells and basophils are also activated by other substances such as chemokines and histamine-releasing factors (5, 6). Chemokines are a superfamily of small, structurally related cytokine molecules characterized by their ability to affect trafficking of immune cells. Some chemokines, such as CCL3 (macrophage inflammatory protein-1α), CCL5 (RANTES), or CCL11 (eotaxin-1) have been reported to activate mouse, rat, or human mast cells and basophils *in vitro* (7–9) and *in vivo* (10, 11). In allergic inflammatory tissues, abundant expression of chemokines such as CCL3, CCL5, and CCL11 is observed both in the acute and late phase reactions (12, 13). It is therefore likely that FcεRI and chemokine receptor engagement either occurs simultaneously or in relatively rapid succession in mast cells and basophils *in vivo*.

Recently, Laffargue *et al.* (14) have shown that CCL3 or CCL5 significantly enhanced FcεRI-mediated degranulation via phosphatidylinositol 3-kinase γ-mediated signaling pathway in bone marrow-derived mast cells. The simultaneous engagement of FcεRI and CC chemokine receptor (CCR)¹ appears to be important for optimal degranulation of mast cells *in vitro* and physiologically relevant levels of mast cell activation *in vivo*. However, it has not yet been established whether and how the simultaneous engagement of FcεRI and CCR affects events other than degranulation of mast cells.

In this paper, we show that costimulation via FcεRI and CCR1 engagement synergistically enhances degranulation but inhibits CCL3-mediated chemotaxis using rat basophilic leukemia (RBL)-2H3 cells. Family members of the Rho-like GTPases, Rac, Cdc42, and Rho control cell motility by mediating the reorganization of the actin cytoskeleton (15–17). The costimulation via FcεRI and CCR1 resulted in the imbalance of

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¹ The abbreviations used are: CCR, CC chemokine receptor; DNP, dinitrophenyl; HSA, human serum albumin; MBS, myosin-binding subunit of myosin phosphatase; RBL, rat basophilic leukemia; ROCK, Rho kinase; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; Ag, antigen (DNP-HSA).

Rac, Cdc42, and Rho, which affected reorganization of the actin cytoskeleton that is normally required for optimal chemotaxis. The implications of these data on the biology of mast cell- or basophil-driven inflammatory responses and anti-inflammatory drug design are also discussed.

EXPERIMENTAL PROCEDURES

Cell Culture—RBL-2H3 cells expressing human CCR1 (RBL-CCR1) (18) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 1 mg/ml G418. Tissue culture media and cell culture supplies were from Invitrogen.

Degranulation Assay—Cells (3×10^5 cells/ml) were cultured with or without 25 ng/ml anti-dinitrophenyl (DNP) IgE mAb (SPE7; Sigma) in 24-well plates overnight. After washing, the cells were stimulated with 10.0 ng/ml dinitrophenyl human serum albumin (DNP-HSA; Sigma) and/or various concentrations of recombinant human CCL3 (R&D Systems Europe, Ltd., Abingdon, UK) in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin for 20 min. After the stimulation, β -hexaminidase activity in the supernatants was measured with *p*-nitrophenyl *N*-acetyl- β -D-glucosamide (Sigma) in 0.1 M sodium citrate buffer (pH 4.5) for 60 min at 37 °C. The reaction was stopped by the addition of 0.2 M glycine buffer (pH 10.5). The release of 4-*p*-nitrophenol was detected by absorbance at 405 nm. Total β -hexaminidase activity was determined by lysing the cells in Dulbecco's modified Eagle's medium containing 0.1% Triton.

Chemotaxis Assay—The assay was performed using 24-well Transwell™ cell culture chambers with 8.0- μ m pore size (Corning Glass). After collecting with a cell scraper, cells (1×10^6 cells/ml) were sensitized with anti-DNP-IgE in a 15-ml tube for 6 h. In some experiments, the cells were also incubated with C3 exoenzyme (Sigma) for 2 h or Y-27632 (Sigma) for 30 min. After washing, the sensitized cells (2×10^4 cells) in 100 μ l of Dulbecco's modified Eagle's medium containing 0.5% bovine serum albumin were seeded in the upper compartment, and the lower compartment was filled with 600 μ l of the same medium containing recombinant human CCL3 and/or DNP-HSA. The chambers were incubated for 100 min at 37 °C in 5% CO₂. The reaction was stopped with cold PBS containing 2% paraformaldehyde. The number of cells that passed through the membrane was quantified by staining with Giemsa stain solution (BDH Laboratory Supplies, Dorset, UK) and counting the number of cells present in at least eight microscope fields ($\times 40$ objective).

Scanning Electron Microscopy Analyses—RBL-CCR1 cells were allowed to adhere to glass coverslips and sensitized with anti-DNP-IgE overnight. After stimulation with DNP-HSA and/or recombinant human CCL3, the cells were fixed with 0.07 M sodium cacodylate-HCl buffer (pH 7.4) containing 3% glutaraldehyde and 1% paraformaldehyde. The cells were then washed with cacodylate buffer (pH 7.4), osmicated with a 1% aqueous solution of osmium tetroxide, rinsed in deionized water, dehydrated through ascending alcohols (1 $\times$ 10 min at 50–90% and 3 $\times$ 10 min at 100%), critical point-dried, and sputter-coated with gold prior to observation in a JEOL Model 6100 Scanning Electron Microscope operating at 15 kV.

Immunostaining—After sensitization and stimulation as described above, the cells were fixed with 4% paraformaldehyde for 20 min. The cells were then washed with PBS and permeabilized with PBS containing 0.2% Triton X-100. After blocking with PBS containing 0.5% bovine serum albumin, the cells were stained with 0.1 μ g/ml TRITC-conjugated phalloidin (Sigma) for 30 min.

Measurement of Rac and Cdc42 Activation—Rac1 and Cdc42 activation were assessed by pull-down assay (19). RBL-CCR1 cells (5×10^6 cells for Rac measurement or 1.0×10^7 for Cdc42 measurement) were cultured in complete medium overnight and were sensitized with anti-DNP-IgE mAb in Dulbecco's modified Eagle's medium containing 1.0% fetal bovine serum for 6 h. The cells were then stimulated with CCL3 and/or DNP-HSA. After washing with cold PBS, the cells were lysed with lysis buffer (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 1 mM dithiothreitol, and 5% glycerol), and an equal amount of lysate was incubated with glutathione *S*-transferase-p21-activated kinase binding domain-coupled glutathione-Swell Gel (Pierce) for 60 min at 4 °C. Proteins complexed to the gels or beads were recovered, resuspended in sample buffer, and heated at 95 °C for 5 min, after which they were resolved by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed with mouse anti-RAC1 mAb (Pierce) or mouse anti-Cdc42 mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and detected using horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce) and

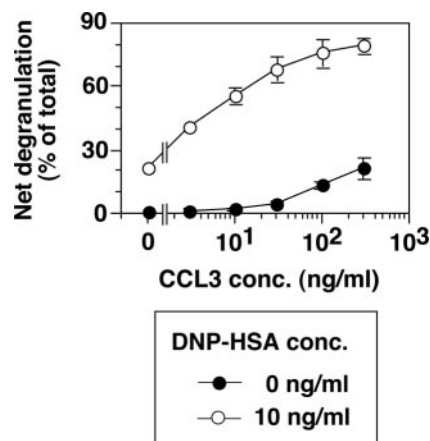


FIG. 1. Costimulation via FcεRI and CCR1 enhanced degranulation of RBL-CCR1 cells. After sensitization with anti-DNP-IgE, RBL-CCR1 cells were stimulated with 10.0 ng/ml DNP-HSA and various concentrations of CCL3 or with CCL3 alone for the degranulation assay. The data are representative of two separate experiments.

enhanced chemiluminescent kit (Pierce).

Measurement of Rho-associated Coiled-coil-forming Protein Kinase (ROCK) Activation—ROCK activation was assessed by CycLex Rho-kinase assay kit (Cyclex Co., Ltd.). After the sensitization with anti-DNP IgE and stimulation with CCL3 and/or DNP-HSA, 1.0×10^7 of RBL-CCR1 cells was lysed with lysis buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin, 1 mM NaF, 1 mM Na₃VO₄, 1% β -mercaptoethanol). The lysates were incubated with reaction buffer (50 mM Tris/HCl, pH 7.5, 100 mM EGTA, 10 mM magnesium acetate, 1% β -mercaptoethanol, 0.1 mM ATP) in wells coated with myosin-binding subunit of myosin phosphatase (MBS) for 60 min at 30 °C. After washing, horseradish peroxidase-conjugated anti-phospho-MBS threonine-696 specific antibody was applied into wells and incubated for 1 h at room temperature. Tetramethylbenzidine was used as a substrate, and the absorbance was read at 450 nm after the enzyme reaction was stopped with 0.5 N H₂SO₄.

To examine total ROCK in lysates, the lysates were resolved by 8.0% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed with goat anti-ROCKII mAb (Santa Cruz Biotechnology) and detected using horseradish peroxidase-conjugated rabbit anti-goat antibody (Santa Cruz Biotechnology) and enhanced chemiluminescent kit.

Statistical Analyses—Statistical significance was determined using analysis of variance.

RESULTS

Costimulation via FcεRI and CCR1 Engagement Enhances Degranulation but Inhibits Chemotaxis of RBL-CCR1 Cells—Because the expression level of CCR1, a receptor of CCL3, on wild type RBL-2H3 cells was low, we used RBL-2H3 cells stably expressing human CCR1 (RBL-CCR1) to examine the effect of costimulation via FcεRI and CCR1 engagement on mast cell events. As shown in Fig. 1, the costimulation via FcεRI and CCR1 synergistically enhanced degranulation in RBL-CCR1 cells. FcεRI-mediated stimulation using the anti-DNP IgE/DNP-HSA (Ag) system under optimal conditions induced $21.4 \pm 0.8\%$ of total degranulation in RBL-CCR1. Simultaneous stimulation with IgE/Ag and 300 ng/ml CCL3 induced $80.1 \pm 2.1\%$ of total degranulation in RBL-CCR1, whereas stimulation with 300 ng/ml CCL3 alone induced $22.1 \pm 5.0\%$ of degranulation (Fig. 1). The results indicate that there is cross-talk between the CCR1-mediated signaling pathway and FcεRI-mediated signaling pathway in RBL-CCR1 cells. Therefore, we used the RBL-CCR1 cells as a model to examine the effect of simultaneous engagement of FcεRI and CCR on events other than degranulation of mast cells.

CCL3 induces a chemotaxis response of RBL-CCR1 cells, with the maximum motility observed at a concentration of 1.0

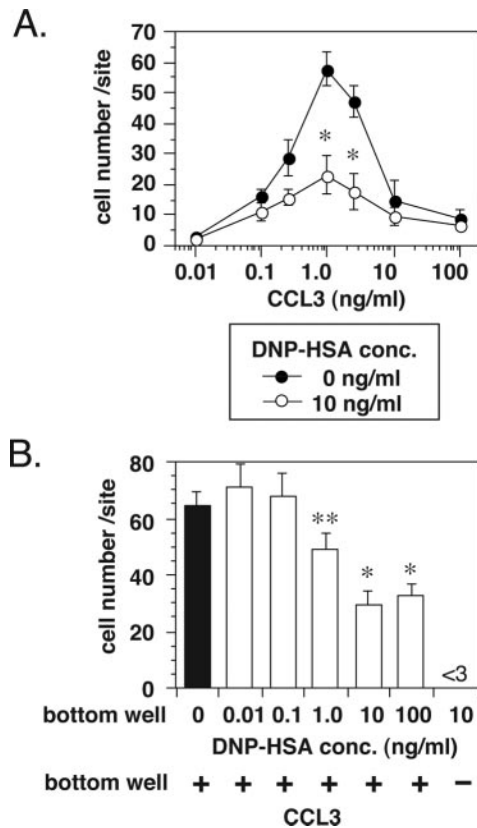


FIG. 2. FcεRI engagement inhibits CCL3-induced chemotaxis of RBL-CCR1 cells. After sensitization with anti-DNP IgE, RBL-CCR1 cells were applied to upper wells, and various concentrations of CCL3 were applied to the bottom wells. To examine the effect of DNP-HSA stimulation on chemotaxis, 10 ng/ml DNP-HSA was applied to both upper wells and bottom wells (A). The sensitized cells were applied in the upper wells, and 1.0 ng/ml CCL3 and various concentrations of DNP-HSA were applied to the bottom wells (B). Unstimulated cells (negative control) did not exhibit marked chemotaxis (3 ± 2 cells/visual site). The results are $\pm$ S.E. from three experiments. *, $p < 0.001$; **, $p < 0.01$.

ng/ml (Fig. 2A). Next, we examined whether costimulation with Ag and CCL3 affects the chemotaxis of RBL-CCR1 cells. Interestingly, the chemotaxis was decreased when RBL-CCR1 cells were sensitized with anti-DNP IgE mAb and costimulated with Ag and CCL3. Because the gradient of allergen proteins would be formed in tissues, especially in surface of tissues, we examined the effect of Ag stimulation with or without gradient on CCL3-mediated chemotaxis of RBL-CCR1 cells by application of Ag to the upper and/or the bottom wells of the chamber for chemotaxis assay. The inhibition was observed when Ag was applied to the upper and/or the bottom wells (Fig. 2, A and B). Comparable chemotaxis toward CCL3 was observed between IgE-sensitized and IgE-nonsensitized RBL-CCR1 cells, indicating that IgE sensitization itself does not affect the CCL3-mediated chemotaxis of RBL-CCR1 cells (data not shown). The chemotaxis of RBL-CCR1 cells toward Ag alone was not observed after incubation for 100 min (Fig. 2B). The results indicate that FcεRI engagement inhibits CCL3-mediated chemotaxis of RBL-CCR1 cells.

FcεRI Engagement Inhibits CCL3-mediated Ruffling of RBL-CCR1 Cells—The rearrangement of the actin cytoskeleton is a crucial component of chemotaxis (15–17). The inhibitory effect of FcεRI engagement on chemotaxis suggests that costimulation with Ag and CCL3 affect reorganization of actin cytoskeleton of RBL-CCR1 cells. We therefore examined the effects of the costimulation on actin reorganization of RBL-CCR1 cells by immunostaining with TRITC-labeled phalloidin. As shown in

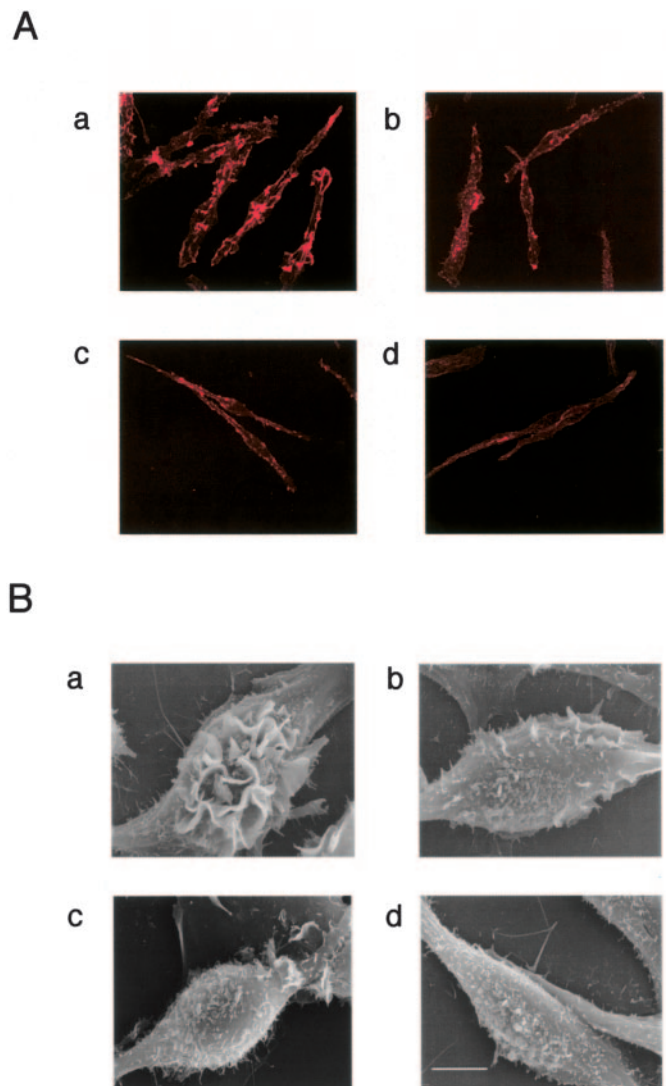


FIG. 3. FcεRI engagement inhibits CCL3-induced membrane ruffling of RBL-CCR1 cells. After sensitization with anti-DNP IgE, RBL-CCR1 cells were stimulated with 1.0 ng/ml CCL3 (a), 1.0 ng/ml CCL3 and 10.0 ng/ml DNP-HSA (b), or 10.0 ng/ml DNP-HSA (c) or were unstimulated (d). The cells were then fixed and processed for polymerized actin staining with TRITC-conjugated phalloidin (A) or for a scanning electron microscope (B). Bar, 5.0 μ m. The data are representative of three separate experiments with more than 40 cells analyzed in each.

Fig. 3A (a), RBL-CCR1 cells stimulated with 1.0 ng/ml CCL3 (optimal concentration for chemotaxis) dramatically showed membrane ruffling at 90 s. In contrast, RBL-CCR1 cells costimulated with 10.0 ng/ml Ag (optimal condition for degranulation) and 1.0 ng/ml CCL3 or stimulated with Ag alone exhibited less membrane ruffling compared with CCL3-stimulated cells (Fig. 3A, b and c).

To examine the morphological changes in stimulated cells in more detail, we observed the cells using scanning electron microscopy. The most extensive membrane ruffling was observed in RBL-CCR1 cells at 90 s after stimulation with 1.0 ng/ml CCL3 (Fig. 3B (a)) or 10.0 ng/ml CCL3 alone (data not shown). When RBL-CCR1 cells were costimulated with 10.0 ng/ml Ag and 1.0 ng/ml CCL3, ruffling response of RBL-CCR1 cells was reduced (Fig. 3B (b)), compared with that of cells stimulated with CCL3 alone (Fig. 3B (a)). Ag-stimulated cells also exhibited less membrane ruffling compared with CCL3-stimulated cells (Fig. 3B (c)). In unstimulated cells, obvious morphological change was not observed at 90 s (Fig. 3B (d)). The results indicate that FcεRI engagement inhibits pro-

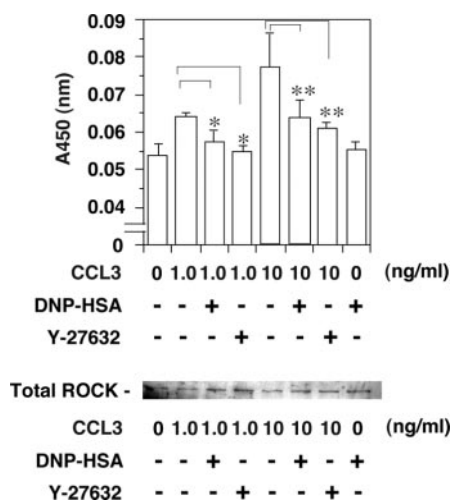


FIG. 4. Costimulation via FcεRI and CCR1 inhibits ROCK activation in RBL-CCR1 cells. After sensitization with anti-DNP IgE for 6 h, RBL-CCR1 cells were stimulated with various concentration of CCL3 and/or 10.0 ng/ml of DNP-HSA for 1 min. Activated ROCK in lysate from the stimulated cells was examined by measuring the degree of phosphorylation of MBS using an enzyme-linked immunosorbent assay system. To examine the effect of ROCK inhibitor on the ROCK activation, the cells were treated with Y-27632 for the final 30 min of the IgE sensitization. To examine the total volume of ROCK, the lysates were subjected to electrophoresis and Western blotting with anti-ROCKII mAb. The data are representative of three separate experiments. *, $p < 0.05$; **, $p < 0.01$.

nounced CCL3-induced ruffling response of RBL-CCR1 cells.

Costimulation via FcεRI and CCR1 Decreases ROCK Activation but Enhances Rac and Cdc42 Activation in RBL-CCR1 Cells—Small GTP-binding proteins of the Rho family, Rho, Rac, and Cdc42 control chemotaxis by mediating the reorganization of the actin cytoskeleton (15–17). It has been reported that Rho-dependent activation of ROCK was required for chemotaxis of mice mast cells toward Ag (20). Hence, we examined activation of ROCK, Rac1, and Cdc42 in RBL-CCR1 cells after stimulation with CCL3 and/or Ag.

ROCK activation was examined by measuring the kinase activity to phosphorylate MBS. The kinase activity was detected in the lysate derived from CCL3-stimulated RBL-CCR1 cells at 1 min after the stimulation. As shown in Fig. 4, treatment of RBL-CCR1 cells with Y27632, a ROCK inhibitor, decreased the kinase activity, confirming that ROCK activation was involved in the phosphorylation of MBS. When RBL-CCR1 cells were costimulated with Ag and 1.0 or 10 ng/ml of CCL3, the kinase activity was reduced, compared with that in cells stimulated with CCL3 alone.

Rac1 and Cdc42 activation in RBL-CCR1 cells was detected by a pull-down assay (19). The maximum Rac1 and Cdc42 activation was detected at 1 min after stimulation of RBL-CCR1 cells with CCL3. Consistent with the chemotaxis assay, the optimal concentration of CCL3 for Rac1 and Cdc42 activation was 1.0 ng/ml (Fig. 5A). Interestingly, the Rac1 and Cdc42 activation in RBL-CCR1 cells was rather enhanced by the costimulation with 1.0 ng/ml CCL3 and Ag. These results indicate that costimulation with Ag and CCL3 inhibits ROCK activation but enhances Rac and Cdc42 activation in RBL-CCR1 cells.

It has been reported that ROCK-related signaling antagonizes Rac activity in fibroblasts (21). Therefore, we examined whether inhibition of ROCK affects Rac activation in RBL-CCR1 cells using Y27632. As shown in Fig. 5B, Rac activation in CCL3-stimulated RBL-CCR1 cells was enhanced by treatment with Y27632. The result suggests that the ROCK-related signaling pathway antagonizes Rac activity in RBL-CCR1 cells.

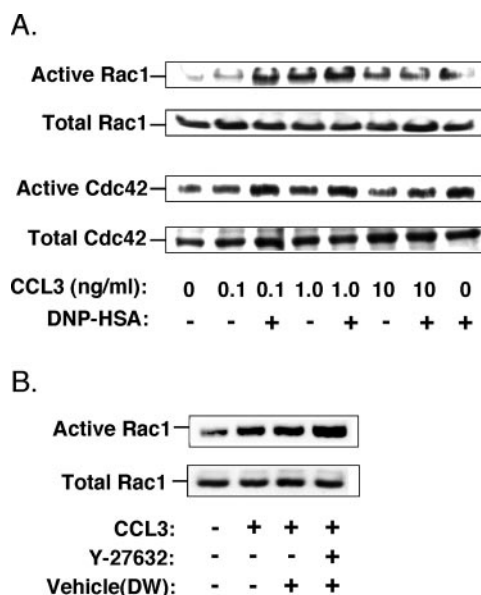


FIG. 5. Costimulation via FcεRI and CCR1 enhances Rac and Cdc42 activation in RBL-CCR1 cells. After sensitization with anti-DNP IgE for 6 h, RBL-CCR1 cells were stimulated with various concentrations of CCL3 and/or 10.0 ng/ml of Ag for 1 min (A). To examine the effect of ROCK inhibitor on Rac activation, the cells were treated with Y-27632 (10 μ M) for the final 30 min of the IgE sensitization. The cells were then stimulated with 1.0 ng/ml CCL3 (B). Activated Rac or Cdc42 was measured by pull-down assay. The data are representative of three separate experiments.

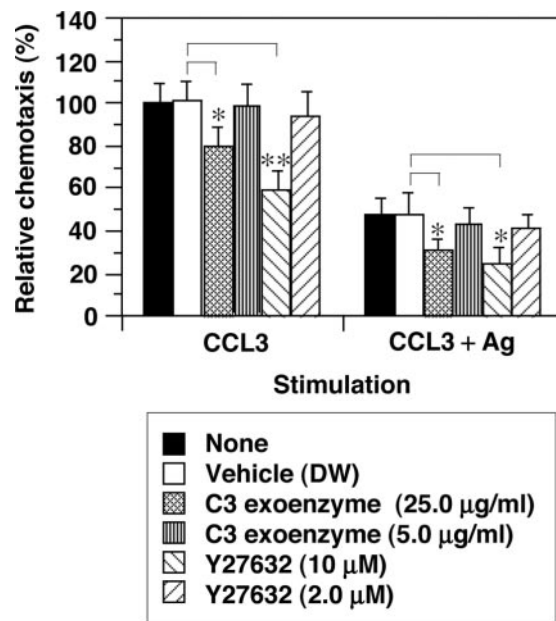


FIG. 6. Rho inhibitor and ROCK inhibitor inhibits chemotaxis of RBL-CCR1 cells toward CCL3 and/or Ag. RBL-CCR1 cells were sensitized with anti-DNP IgE for 6 h. The cells were treated with C3 exoenzyme for the final 4 h or with Y-27632 for the final 30 min of the IgE sensitization. The cells were then applied to upper wells, and 1.0 ng/ml CCL3 and/or 10.0 ng/ml DNP-HSA were applied to the bottom wells. The results are $\pm$ S.E. from three experiments. *, $p < 0.01$; **, $p < 0.001$.

Rho/ROCK Activation Pathway Is Required for CCL3-mediated Chemotaxis of RBL-CCR1 Cells—Next, we examined whether a Rho/ROCK activation pathway was related to the chemotaxis ability of RBL-CCR1 cells in response to CCL3 and/or Ag using C3 exoenzyme, a Rho inhibitor, and Y27632. As shown in Fig. 6, both C3 exoenzyme and Y-27632 inhibited CCL3-induced chemotaxis of RBL-CCR1 cells. The inhibitory

effect of C3 exoenzyme and Y-27632 was also observed in decreased chemotaxis of RBL-CCR1 cells by costimulation with CCL3 and Ag. The inhibition ratio of both inhibitors on chemotaxis upon costimulation with Ag and CCL3 was comparable with that on chemotaxis upon stimulation with CCL3 alone. These results suggest that activation of Rho/ROCK pathway is required for CCL3-induced chemotaxis of RBL-CCR1 cells.

DISCUSSION

In this study, we demonstrate that FcεRI engagement inhibits CCL3-mediated chemotaxis of RBL-2H3 cells expressing CCR1 (RBL-CCR1) (Fig. 2). The movement of cells toward chemoattractants requires actin cytoskeleton rearrangement and polarization such as formation of leading edge and membrane ruffles. Costimulation with Ag and CCL3 decreases ruffle formation of RBL-CCR1 cells (Fig. 3), suggesting that the decreased ruffling response involves the inhibition of chemotaxis by the costimulation. At signal transduction level, CCL3-mediated chemotaxis of RBL-CCR1 cells requires activation of the Rho/ROCK pathway (Fig. 6). We found that costimulation with Ag and CCL3 enhanced Rac and Cdc42 activation but decreased ROCK activation in RBL-CCR1 cells (Figs. 4 and 5). Decreased ROCK activation would be a mechanism by which FcεRI engagement inhibits CCL3-mediated chemotaxis of RBL-CCR1 cells.

As well as ROCK activation, Rac activation is essential for CC chemokine-mediated chemotaxis of RBL-2H3 cells, as shown by inhibition of CCL11-induced chemotaxis via CCR3 with dominant negative Rac (22). Cdc42 also appears to be required for directional motility during chemotaxis of immune cells (23, 24). Interestingly, costimulation with CCL3 and Ag enhanced Rac and Cdc42 activation, although chemotaxis of RBL-CCR1 cells was inhibited by the costimulation. The results indicate that reciprocal balance between Small GTP-binding proteins of the Rho family is important to induce optimal chemotaxis of mast cells and basophils. It has been reported that the Rac- and Cdc42-related signaling pathway antagonizes Rho activity, whereas the Rho-ROCK signaling pathway antagonizes Rac activity in fibroblast (21, 25, 26). In our study, treatment with ROCK inhibitor (Y-27632) enhanced Rac activation in RBL-CCR1 cells. The results could reflect antagonism between Rac/Cdc42-related and Rho/ROCK-related signaling pathways. It is likely that costimulation with CCL3 and Ag enhances Rac and Cdc42 activation, which results in reduced ROCK activation, and *vice versa*.

FcεRI engagement inhibited not only CCL3-mediated chemotaxis but also CCL3-induced membrane ruffling of RBL-CCR1 cells (Figs. 2 and 3). With the Rho GTPase family, Rac is the main regulator of membrane ruffling in RBL-2H3 cells, as shown by a significant decrease of membrane ruffling in the cells expressing dominant negative Rac (27). Considering the role of Rac in membrane ruffling formation, costimulation with CCL3 and Ag was expected to reduce Rac activation in RBL-CCR1 cells. However, Rac activation in the cells was enhanced by the costimulation. Recently, Doughman *et al.* (28) have showed that not only Rac signaling but also phosphatidylinositol 4,5-bisphosphate synthesis by phosphatidylinositol 4-phosphate 5-kinase Iα was required for membrane ruffling. Rac stimulates Arp2/3 complex-induced actin polymerization through WAVE/SCAR proteins, leading to formation of branching actin filament networks found in membrane ruffling (29, 30). Phosphatidylinositol 4,5-bisphosphate produced by phosphatidylinositol 4-phosphate 5-kinase Iα is suggested to be essential for the formation of the actin filament polymerization through the Arp2/3 complex (28). Chatah and Abrams (31) have clearly shown that Rho but not Rac directly induces activation and membrane translocation of the phosphatidylinositol

4-phosphate 5-kinase Iα. Phosphatidylinositol 4-phosphate 5-kinase Iα may not be activated enough for formation of membrane ruffling due to reduced ROCK activation by costimulation with CCL3 and Ag. Further study is required to elucidate the detailed mechanisms involved in the inhibition of membrane ruffling by FcεRI engagement.

The costimulatory effect of CCL3 and Ag upon mast cell degranulation has been observed in bone marrow-derived mast cells (14). In this paper, we confirm and extend the observations by showing that CCR1 can mediate the synergistic effect of CCL3 on degranulation of RBL-CCR1 (Fig. 1). In contrast, the costimulation with CCL3 and IgE/Ag decreased chemotaxis of RBL-CCR1 cells toward CCL3 (Fig. 2). The chemotaxis of mast cells to a site of inflammation and the subsequent release of chemical mediators such as histamine and proteases upon degranulation are crucial to eliciting allergic inflammation. Considering the role of mast cells and basophils in allergic inflammation, these results are consistent with the biology of this response. Mast cells and basophils would migrate toward the chemokine gradient at a site of inflammation but stop and accumulate at the site where allergen concentration is high. Meanwhile, the degranulation of mast cells and basophils is enhanced by the costimulation with CCL3 and allergen at the site where the cells accumulate, thereby focusing the inflammatory response. Our results suggest that costimulation by CCL3 and Ag play important roles in the orchestration and focusing of the allergic response.

In conclusion, we have demonstrated that the cross-talk between the CCR1-mediated signaling pathway and FcεRI-mediated signaling pathway affects not only degranulation but also actin reorganization and chemotaxis of RBL-CCR1 cells. The observed impact of costimulation of mast cells via CCR1 and FcεRI is consistent with a model of chemokine-mediated recruitment followed by antigen "focusing." The molecular basis of the antigen focusing would appear to involve a putative "stop signal" illustrated by impaired polarization and chemotaxis. Once recruited to a site of parasitic infection or allergen exposure, the accumulated mast cells and basophils are optimally activated by antigen and chemokine. A corollary to this work is that CCRs and signal transduction pathways downstream of CCRs may represent a new class of mast cell stability compounds for immunotherapy.

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