

Evidence That Formation of Vimentin·Mitogen-activated Protein Kinase (MAPK) Complex Mediates Mast Cell Activation following FcεRI/CC Chemokine Receptor 1 Cross-talk*[§]

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Masako Toda^{†1,2}, Chuan-Hui Kuo^{†1}, Satty K. Borman[§], Ricardo Micheler Richardson[¶], Akihito Inoko^{||}, Masaki Inagaki^{||}, Andrea Collins[‡], Klaus Schneider^{§3}, and Santa Jeremy Ono^{†**4}

From the [†]Division of Allergy and Immunology, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, the ^{**}University of Cincinnati College of Medicine, Cincinnati, Ohio 45221-0097, the [§]Department of Computational and Structural Chemistry, GlaxoSmithKline Research and Development, Gunnels Wood Road, Stevenage SG1 2NY, United Kingdom, the [¶]Julius L. Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham, North Carolina 27707, and the ^{||}Division of Biochemistry, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan

Background: CC chemokine ligand 2 (CCL2) recruits leukocytes in inflammatory tissues.

Results: Vimentin, a cytoskeletal protein, interacted with phosphorylated MAPKs, was critical for CCL2 production in mast cells activated via FcεRI and a CC chemokine receptor.

Conclusion: Vimentin was involved in optimal CCL2 production in mast cells.

Significance: This work contributes to understanding of mechanisms for chemokine production in mast cells, which are therapeutic targets for allergic inflammation.

Accumulating evidence points to cross-talk between FcεRI and CC chemokine receptor (CCR)-mediated signaling pathways in mast cells. Here, we propose that vimentin, a protein comprising type III intermediate filament, participates in such cross-talk for CCL2/monocyte chemoattractant protein 1 (MCP-1) production in mast cells, which is a mechanism for allergic inflammation. Co-stimulation via FcεRI, using IgE/antigen, and CCR1, using recombinant CCL3/macrophage inflammatory protein-1α (MIP-1α), increased expression of phosphorylated, disassembled, and soluble vimentin in rat basophilic leukemia (RBL)-2H3 cells expressing human CCR1 (RBL-CCR1 cells) and bone marrow-derived murine mast cells, both models of mucosal type mast cells. Furthermore, co-stimulation enhanced production of CCL2 as well as phosphorylation of MAPK. Treating the cells with p38 MAPK inhibitor SB203580, but not with MEK inhibitor PD98058, reduced CCL2 production, suggesting that p38 MAPK, but not ERK1/2, plays a critical role in the chemokine production. Immunoprecipitation analysis showed that vimentin interacts with phosphorylated ERK1/2 and p38 MAPKs in the co-simulated cells. Preventing disassembly of the vimentin by aggregating vimentin filaments using β,β'-imino-

dipropionitrile reduced the interaction of vimentin with phosphorylated MAPKs as well as CCL2 production in the cells. Taken together, disassembled vimentin interacting with phosphorylated p38 MAPK could mediate CCL2 production in mast cells upon FcεRI and CCR1 activation.

Mast cells play an important role in IgE-associated allergic disorders and immune responses to parasites, and FcεRI cross-linking is a key event in activating mast cells. The association of allergen with IgE bound to FcεRI trigger signaling cascades leading to activation of kinases, phosphatases, and GTPases, which subsequently induce a variety of events, such as degranulation, cytoskeleton rearrangement, increased gene transcription, and cytokine/chemokine production, in the activated mast cells (1, 2).

Besides the classical FcεRI-mediated mechanism, mast cells are also activated by chemokines (3, 4). A superfamily of small, structurally related cytokine molecules, chemokines are characterized by their ability to affect trafficking of leukocytes. Some chemokines, such as CCL2/MCP-1 (monocyte chemoattractant protein 1), CCL3/MIP-1α (macrophage inflammatory protein-1α), CCL5/RANTES (regulated upon activation, normal T-cell expressed and secreted), and CCL11/eotaxin-1, have been reported to activate mouse, rat, or human mast cells (3–6). Abundant expression of these CC chemokines and accumulation of leukocytes has also been observed in allergic inflammatory tissues (7–9). It is very likely that FcεRI and CCR⁵ engagements occur either simultaneously or in relatively rapid succession in mast cells *in vivo*.

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[†] Both authors contributed equally to this work.

² To whom correspondence may be addressed: Junior Research Group 1 "Experimental Allergology," Paul-Ehrlich-Institut, Paul-Ehrlich-Str. 59, 63225 Langen, Germany. Tel.: 49-6103-77-5407; Fax: 49-6103-77-1258; E-mail: Masako.Toda@pei.de.

³ Present address: Central Analytical Dept., Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany.

⁴ To whom correspondence may be addressed: Division of Allergy and Immunology, Dept. of Pediatrics, Cincinnati Children's Hospital Medical Center; University of Cincinnati College of Medicine, 2614 McMicken Circle, 210 Van Worner Hall, P.O. Box 210097, Cincinnati, OH 45221-0097. Tel.: 513-556-2588; Fax: 513-556-7861; E-mail: onosa@ucmail.uc.edu.

⁵ The abbreviations used are: CCR, CC chemokine receptor; Ag, antigen; BMMC, bone marrow-derived murine mast cell; DNP, dinitrophenyl; DNP-HSA, DNP-conjugated human serum albumin; IDPN, β,β'-iminodipropionitrile; RBL, rat basophilic leukemia; IPG, immobilized pH gradient; rCCL3, recombinant CCL3.

We previously found that CCL3 acts as a co-stimulator for FcεRI-mediated degranulation in conjunctival mast cells using CCL3-deficient mice (10). Moreover, CCL3 synergistically enhanced FcεRI-mediated degranulation and gene expression of cytokines and chemokines (e.g. *IL-6* and *CCL7/MCP-3*) in a rat basophilic leukemia 2H3 cell line (RBL-2H3) expressing CC chemokine receptor 1 (CCR1), a receptor for CCL3, and bone marrow-derived murine mast cells (11–14). These observations indicate that (i) the simultaneous engagement of FcεRI and CCR1 is important for optimal activation of mast cells *in vitro* and physiologically relevant levels of mast cell activation *in vivo*, and (ii) there is a cross-talk between the FcεRI-mediated and CCR1-mediated signaling cascades.

In this paper, we also found that CCL3 synergistically enhanced FcεRI-mediated CCL2 production in RBL-CCR1 cells and bone marrow-derived murine mast cells (BMMCs). CCL2 is a chemoattractant to induce migration of monocytes, T cells, and eosinophils (3, 9). Increased expression of CCL2 protein in inflammatory tissues of allergic patients has been observed (3, 7–9, 15). Targeting chemokine(s) is a strategy to establish new anti-inflammatory drugs for treatment of allergic diseases. To better understand the molecular mechanisms for chemokine production involved in the complex response of mast cell activation, we investigated the proteins involved in the cross-talk between FcεRI-mediated and CCR1-mediated signaling pathways.

Here, we identified phosphorylated vimentin, a cytoskeletal protein, as the major protein species up-regulated in the co-stimulated mast cells with IgE/Ag and CCL3. Interestingly, vimentin interacted with mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 MAPK. Furthermore, our findings suggest that vimentin is a component for optimal production of CCL2 in the FcεRI- and CCR1-engaged mast cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Monolayer cultures of RBL-CCR1 cells (rat basophilic leukemia RBL-2H3 cells expressing human CCR1) (16) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 12% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mg/ml geneticin.

To generate primary BMMCs, low density mononuclear cells (LDMNCs) were isolated from BALB/c mice (Jackson Laboratories) and cultured in RPMI1640 containing 10% FBS, 4 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids, and 50 μM 2-mercaptoethanol in the presence of 5 ng/ml of recombinant murine IL-3 (Pepro-Tech) for 4–6 weeks (12, 17). The purity of BMMCs exceeded 80%, which was determined by flow cytometric analysis after staining c-Kit and FcεRI on the cell surface. Tissue culture media and cell culture supplements were from Invitrogen.

Chemokine Production Assay—RBL-CCR1 cells (3.0×10^6 cells/ml) and BMMCs (1.0×10^6 cells/ml) were sensitized with 10 and 100 ng/ml anti-DNP IgE monoclonal antibody (SPE7, Sigma-Aldrich) overnight, respectively. These cells were then treated with or without the p38 MAPK inhibitor SB203580 (Calbiochem) or MEK inhibitor PD98058 (Calbiochem) for 1 h

or β,β'-iminodipropionitrile (Sigma-Aldrich), inhibitor of soluble vimentin formation, for 1 h and subsequently stimulated with DNP-conjugated human serum albumin (DNP-HSA from Sigma-Aldrich) and/or human rCCL3 (R&D Systems) in DMEM containing 2% FCS for 2–6 h. The concentrations of rat and murine CCL2 in the culture supernatant were measured by ELISA (Peprotech and eBioscience, respectively).

Measurement of Phosphorylated ERK1/2 and p38 MAPK by ELISA—After sensitization with anti-DNP-IgE mAb, cells were stimulated with rCCL3 and/or DNP-HSA for 5 min and lysed in 10 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate. The levels of total and phosphorylated ERK and p38 kinase in cell lysates were measured by ELISA (Invitrogen) following the manufacturer's instructions.

Two-dimensional Gel Electrophoresis—RBL-CCR1 cells (1.5×10^6 cells/ml) were sensitized with anti-DNP IgE mAb overnight and stimulated with 10 ng/ml DNP-HSA and 100 ng/ml rCCL3 for 5 min. The cells were washed with cold PBS and lysed in a buffer containing urea (8 M), CHAPS (4%, w/v), DTE (65 mM), resolytes 3.5–10 (2%, v/v), 2.5 μg/ml DNase I, 2.5 μg/ml RNase, 50 mM NaF, 1 mM Na₃VO₄, protease inhibitors (Complete, Roche Applied Science), and a trace of bromophenol blue. The total lysates were loaded on the first dimensional separation with a sigmoidal immobilized pH gradient (IPG) from pH 4.0 to 7.0. After equilibration, the IPG gel strips were transferred onto the second dimension vertical gradient slab gels and run with the Laemmli SDS-discontinuous system. Proteins were detected using Coomassie staining. Two-dimensional gel electrophoresis and gel staining were performed by the Proteomics Core Facility at the University of Geneva.

Protein Identification by MALDI-TOF MS—Protein spots were excised from the polyacrylamide gel, reduced with dithiothreitol, treated with iodoacetamide (Sigma-Aldrich) for carboxyamidation of the cysteine residues, and digested *in situ* with trypsin (sequencing grade; Roche Applied Science), according to the method of Shevchenko *et al.* (18, 19). An aliquot of the liquid surrounding the gel pieces was mixed with an equal volume of matrix solution (60% (v/v) acetonitrile, 0.5% (v/v) trifluoroacetic acid (Applied Biosystems), 6 mg/ml α-cyano-4-hydroxycinnamic acid (Bruker Daltonics GmbH, Bremen, Germany)). One microliter of the mixture was spotted immediately onto a 384-well stainless steel MALDI-TOF target and allowed to dry. Peptide calibration standard solution (Bruker Daltonics) was spotted in a similar manner adjacent to the samples.

The mass spectra were recorded on an Ultraflex I (Bruker Daltonics) MALDI mass spectrometer. Peptides were selected for fragmentation analysis by LIFT-MS/MS sequencing (20). The spectra were interpreted using FlexAnalysis and Biotoools software (Bruker Daltonics), and the data were searched against non-redundant protein sequence databases using the program MASCOT (21). Proteins were identified with 0.2-Da accuracy and a minimum of four matching peptides.

Extraction of Soluble Vimentin—The method of Valgeirsdottir *et al.* (22) for extracting soluble vimentin was slightly modified. Briefly, after washing with ice-cold PBS, cells were lysed in

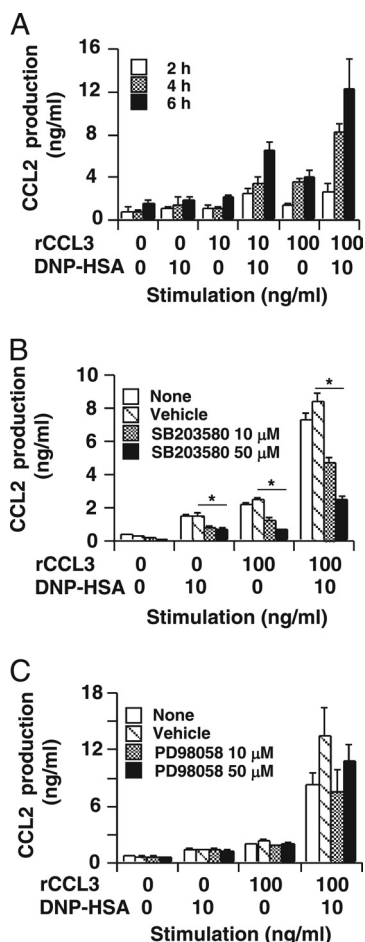


FIGURE 1. Synergistic CCL2 production in RBL-CCR1 cells co-stimulated via FcεRI and CCR1. A, after sensitization with anti-DNP-IgE mAb, RBL-CCR1 cells were stimulated with 10 ng/ml DNP-HSA and/or various concentrations of rCCL3 for 2, 4, or 6 h before determining CCL2 production by ELISA. B, to inhibit p38 MAPK activation, the cells were incubated with 10 or 50 μM SB203580 for the last 1 h of sensitization. C, to inhibit ERK1/2 activation, the cells were incubated with 10 or 50 μM PD98058 for the last 1 h of sensitization. The cells were then stimulated with 10 ng/ml DNP-HSA and/or 100 ng/ml rCCL3 for 6 h. The plotted data of mean CCL2 concentrations in culture supernatants measured by ELISA are representative of three independent experiments. Error bars, S.D. *, $p < 0.01$.

PBS (pH 7.4) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM NaF, 1 mM Na₃VO₄, and protease inhibitors.

Immunoprecipitation—For immunoprecipitation to detect phosphorylation of tyrosine, serine, and threonine residues in vimentin, the cells were lysed in PBS containing 5 mM EDTA, 2% SDS, 10% glycerol, 2.5 μg/ml DNase I, 2.5 μg/ml RNase, 50 mM NaF, 1 mM Na₃VO₄, and protease inhibitors. The lysates were diluted 20-fold with PBS containing 1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, and protease inhibitors. The lysates were incubated with protein A/G Plus-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit anti-vimentin mAb (clone H54; Santa Cruz Biotechnology). Proteins complexed to the agarose gels were recovered, resuspended in sample buffer, and analyzed by immunoblotting. For immunoprecipitation to detect proteins associated with vimentin, the cells were lysed in a buffer used for extracting soluble vimentin. The lysates were incubated with protein G-agarose (Santa Cruz Biotechnology, Inc.) and monoclonal mouse anti-vimentin mAb V9 (Sigma-Aldrich).

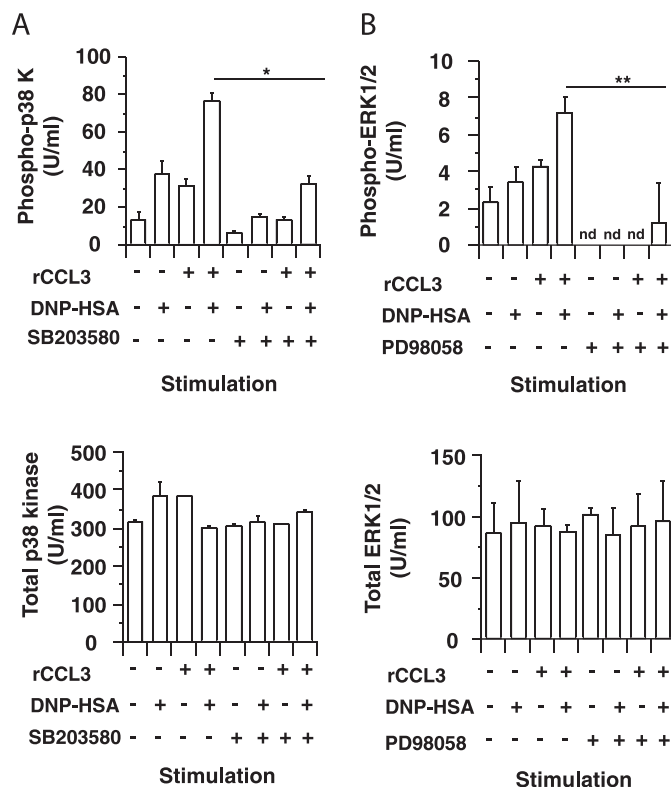


FIGURE 2. Increased phosphorylation of p38 MAPK and ERK1/2 in RBL-CCR1 cells stimulated via FcεRI and CCR1. After sensitization with anti-DNP-IgE mAb, RBL-CCR1 cells were stimulated with 10 ng/ml DNP-HSA and/or 100 ng/ml rCCL3 for 5 min. To inhibit p38 kinase and ERK1/2 activation, the cells were incubated with 50 μM SB203580 or 50 μM PD98058 for the last 1 h of sensitization. Levels of phosphorylated and total p38 kinase (A) and ERK1/2 (B) protein in total cell lysates were measured by ELISA. The data are representative for three independent experiments. nd, not detectable (<1.0 unit (U)/ml). Error bars, S.D. *, $p < 0.01$; **, $p < 0.05$.

Immunoblotting—The lysates or the proteins immunoprecipitated with anti-vimentin antibodies were suspended in sample buffer, loaded onto 12% polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. The membranes were probed with primary antibodies, detected using appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc.), and enhanced with a chemiluminescent kit (Pierce). Mouse anti-vimentin phospho-Ser-55 mAb (4A4) (23), rabbit anti-vimentin phospho-Ser-71 mAb (TM71) (24), mouse anti-vimentin phospho-Ser-6 (MO6) or vimentin phospho-Ser-82 mAb (MO82) (25), rabbit anti-vimentin mAb (H54), rabbit anti-phospho-ERK1/2 mAb (Cell Signaling Technology), and rabbit anti-phospho-p38 MAPK mAb (Cell Signaling Technology) were used as the primary antibodies.

RESULTS

CCL2 Production and MAPK Activation Are Enhanced in FcεRI- and CCR1-activated RBL-CCR1 Cells—CCL2 plays a critical role in activation and accumulation of leukocytes in allergic inflammatory tissues (3, 7–9, 15). To examine whether cross-talk between FcεRI- and CCR-mediated signaling pathways in mast cells is involved in expression of this chemokine, we used RBL-2H3 cells expressing human CCR1, a model cell line of mucosal type mast cells.

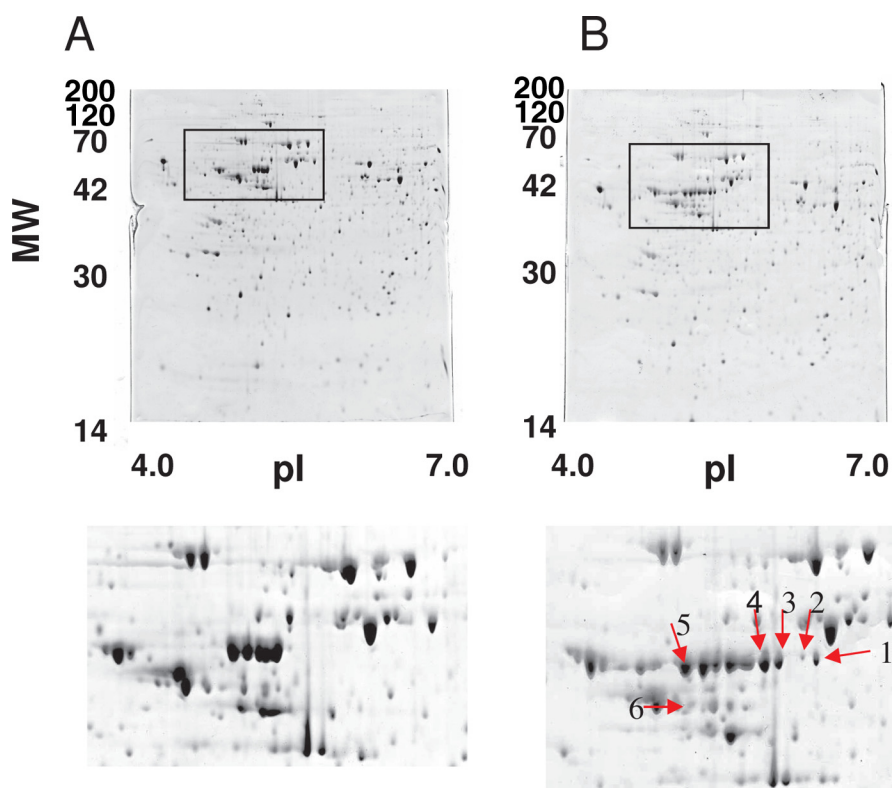


FIGURE 3. **Differential protein expression analysis in RBL-CCR1 cells stimulated via FcεRI and CCR1.** After sensitization with anti-DNP-IgE, RBL-CCR1 cells were stimulated with or without 10 ng/ml DNP-HSA and 100 ng/ml rCCL3 for 5 min. Total cell lysates of the unstimulated cells (A) or total cell lysates of the stimulated cells (B) were subjected to two-dimensional PAGE and stained with Coomassie Brilliant Blue. Protein spots up-regulated in a gel are shown with the red arrows. MW, molecular weight.

TABLE 1
Identified proteins in RBL-CCR1 cells co-stimulated via FcεRI and CCR1

Spot no.	Protein name	Species	NCBI accession no.	Theoretical pI	Theoretical mass	Experimental pI	Experimental mass	Sequence coverage
					<i>kDa</i>		<i>kDa</i>	%
1	Vimentin	Rat	14389299	5.1	53.77	5.35	51.56	56
2	Vimentin	Rat	14389299	5.1	53.77	5.39	51.25	61
3	Vimentin	Rat	14389299	5.1	53.77	5.26	50.378	73
4	Vimentin	Rat	14389299	5.1	53.77	5.2	50.78	74
5	Vimentin	Rat	14389299	5.1	53.77	4.97	50.78	75
6	Vimentin	Rat	14389299	5.1	53.77	4.98	42.04	56

We found that co-stimulation with IgE/Ag (DNP-HSA) and rCCL3 synergistically enhanced CCL2 production in RBL-CCR1 cells (Fig. 1A). However, this CCL2 production was attenuated by the p38 MAPK inhibitor SB203580 (Fig. 1B) but not by the MEK inhibitor PD98058, which inhibits activation of ERK1/2 (Fig. 1C). These results suggest that p38 MAPK but not ERK1/2 kinase plays a role in FcεRI- and CCR1-mediated CCL2 production in RBL-CCR1 cells. Co-stimulation with IgE/Ag and rCCL3 also enhanced phosphorylation of p38 MAPK and ERK1/2 in the cells, but SB203580 and PD98058 abolished this stimulation, respectively (Fig. 2). The results indicate that cross-talk between FcεRI- and CCR1-mediated signaling pathways synergistically enhances MAPK activation as well as subsequent CCL2 production in RBL-CCR1 cells.

Protein Expression Analysis of FcεRI- and CCR1-activated RBL-CCR1 Cells Revealed Vimentin Proteins—CCL2 is a therapeutic target in allergic diseases (9, 26). To elucidate the molecular mechanisms for CCL2 production in mast cells, we set out to identify proteins involved in the cross-talk between

FcεRI- and CCR1-mediated signaling pathways. Therefore, we analyzed total cell lysates of RBL-CCR1 cells non-stimulated or co-stimulated with IgE/Ag plus rCCL3 by two-dimensional electrophoresis. We observed clear up-regulation of six protein species (Fig. 3) and identified rat vimentin (accession number P31000) as the major component of all of these spots using MALDI-TOF MS (Table 1). Vimentin has multiple phosphorylation sites, which would explain the various isoforms observed migrating in a horizontal line across the gel. It is likely that spot 6, with a lower molecular weight, is a truncated fragment of vimentin.

Vimentin Was Disassembled in FcεRI- and CCR1-activated RBL-CCR1 Cells—Vimentin is a major structural component of intermediate filaments that create cell rigidity and shape (27). Upon phosphorylation, vimentin regulates the disassembly of these intermediate filaments (28–32). Vimentin also organizes signaling process in a phosphorylation-dependent manner (33, 34). To obtain insights into the roles of vimentin in mast cell activation, we examined phosphoryla-

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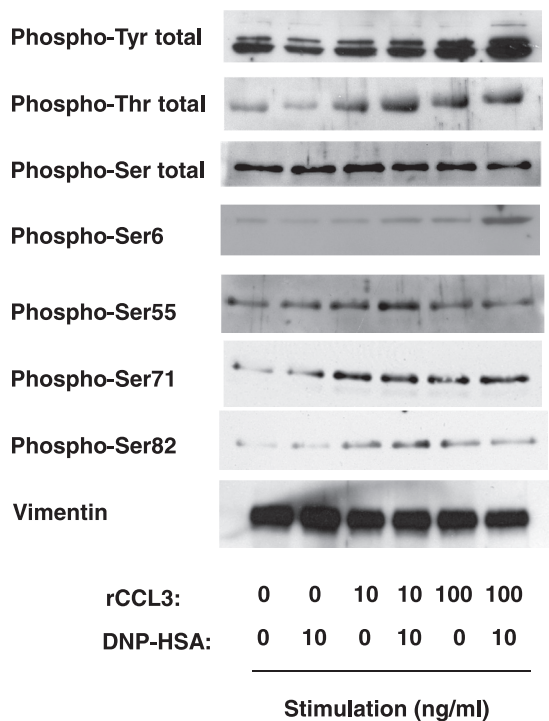


FIGURE 4. Phosphorylation of vimentin in RBL-CCR1 cells stimulated via FcεRI and CCR1. After sensitization with anti-DNP-IgE mAb, RBL-CCR1 cells were stimulated with 10 ng/ml DNP-HSA and/or various concentrations of rCCL3 for 5 min. Total cell lysates of non-stimulated or the stimulated cells were subjected to immunoprecipitation with anti-vimentin mAb. The immunoprecipitated proteins were analyzed by immunoblotting using antibodies against phosphotyrosine; phosphothreonine; phosphoserine; phosphorylated vimentin at Ser-6, -55, -71, or -82; or vimentin. The data are representative of two independent experiments.

tion of vimentin in FcεRI- and/or CCR1-activated RBL-CCR1 cells.

We therefore extracted total proteins of unstimulated and stimulated cells using a lysis buffer containing 2% SDS and 10% glycerol and diluted it using a buffer containing 1% Nonidet P-40 for immunoprecipitation. We then analyzed the phosphorylation status of vimentin by immunoprecipitating the protein and detecting different phosphospecies by immunoblotting with specific anti-phosphotyrosine, phosphothreonine, and phosphoserine antibodies. The total tyrosine phosphorylation of vimentin in RBL-CCR1 cells was enhanced in an rCCL3 concentration-dependent manner, when the cells were co-stimulated with the chemokine and IgE/Ag. In contrast, high levels of serine and threonine phosphorylation were observed in the cells co-stimulated with 10 ng/ml rCCL3 and/or 10 ng/ml IgE/Ag (Fig. 4). The highest levels of phosphorylation at serines 55, 71, and 82 in the N terminus of vimentin were detected when the cells were stimulated with 10 ng/ml rCCL3 and 10 ng/ml IgE/Ag. Interestingly, appreciable levels of phosphorylation at Ser-6 required co-stimulation with 100 ng/ml rCCL3 and 10 ng/ml IgE/Ag (Fig. 4).

Serine phosphorylation at the N terminus appears to regulate the disassembly of vimentin filaments (28–32). It has also been shown that disassembly of vimentin filaments convert the protein into a soluble form (29–32). Vimentin reportedly interacts with phosphorylated MAPK in neurons

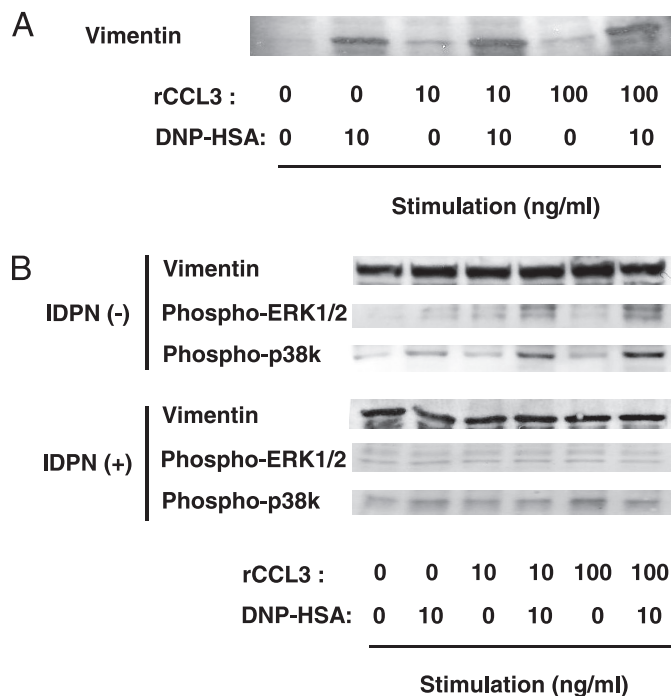


FIGURE 5. Interaction of vimentin and phosphorylated MAPK in RBL-CCR1 cells stimulated via FcεRI and CCR1. After sensitization with anti-DNP-IgE, RBL-CCR1 cells were stimulated with 10 ng/ml DNP-HSA and/or various concentrations of rCCL3 for 5 min. *A*, after the stimulation, soluble forms of vimentin were extracted and analyzed by immunoblotting using anti-vimentin Abs. *B*, the cells were incubated with β,β' -iminodipropionitrile (IDPN (+)) to promote aggregation of vimentin intermediate filaments for the last 1 h of sensitization. Soluble forms of vimentin in the cells were extracted and subjected to immunoprecipitation using anti-vimentin mAb and analyzed by immunoblotting using anti-phosphorylated ERK1/2 mAb, anti-phosphorylated p38 kinase mAb, or anti-vimentin Abs. The data are representative of three independent experiments.

and adipocytes (35–37). Hence, we hypothesized that (i) FcεRI and CCR1 activation induces vimentin disassembly and that (ii) the disassembled and soluble vimentin interacts with activated MAPKs and is thus involved in CCL2 production in mast cells. To test these hypotheses, we examined levels of vimentin filament disassembly by detecting amounts of soluble vimentin in activated RBL-CCR1 cells. Cell lysates were prepared with lysis buffer containing 1% Nonidet P-40, which extracts soluble forms of vimentin (22). Indeed, co-stimulation with rCCL3 and IgE/Ag enhanced formation of soluble vimentin in RBL-CCR1 cells (Fig. 5A). Taken together, FcεRI and CCR1 engagement induces vimentin phosphorylation, which in turn increases soluble vimentin levels in these activated RBL-CCR1 cells.

Vimentin Interacted with Phosphorylated MAPKs in FcεRI- and CCR1-activated RBL-CCR1 Cells—Next, we examined whether vimentin interacts with MAPKs and is involved in CCL2 production in RBL-CCR1 cells upon FcεRI and CCR1 stimulation. Immunoprecipitation with anti-vimentin antibody was performed using extracts of the cells stimulated with IgE/Ag and/or rCCL3, which contained soluble vimentin. The immunoprecipitated samples were separated on one-dimensional SDS-PAGE followed by Western blotting with anti-phospho-p38 MAPK or anti-phospho-ERK1/2 mAb. We detected association of vimentin with both phosphorylated p38 kinase and ERK1/2 in the extract from the stimulated cells (Fig.

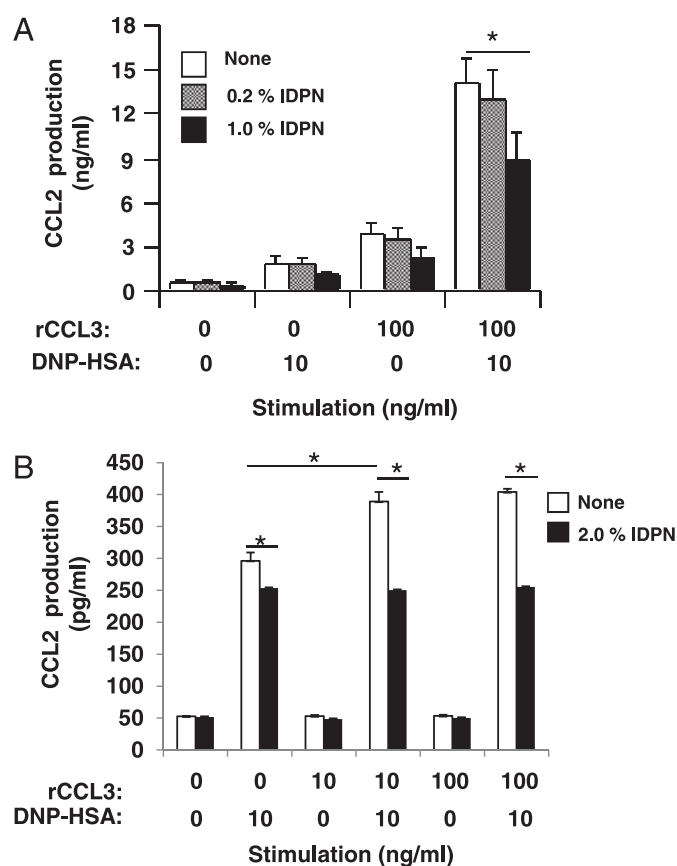


FIGURE 6. Reduced CCL2 production by β,β' -iminodipropionitrile treatment in RBL-CCR1 cells and BMMCs stimulated via Fc ϵ RI and CCR1. RBL-CCR1 cells (A) and BMMCs (B) were sensitized with anti-DNP-IgE and were incubated with IDPN for the last 1 h of sensitization. The cells were then stimulated with 10 ng/ml DNP-HSA and/or 10 or 100 ng/ml rCCL3. Culture supernatants were harvested after stimulation. Concentrations of CCL2 in the supernatant were measured by ELISA. The plotted CCL2 concentration data are representative of three independent experiments. Error bars, S.D. *, $p < 0.01$.

5B). These results suggest that vimentin may interact with phosphorylated p38 MAPK and ERK1/2 in activated RBL-CCR1 cells.

CCL2 Production in CCR1- and Fc ϵ RI- activated RBL-CCR1 Cells Was Decreased by Preventing Formation of Soluble Vimentin—To further examine whether vimentin filaments are required for the phosphorylation of MAPKs and production of CCL2, RBL-CCR1 cells were treated with β,β' -iminodipropionitrile (IDPN), which induces aggregation of vimentin intermediate filaments and prevents formation of soluble vimentin (37, 38). The IDPN-treated cells were then stimulated with rCCL3 and/or IgE/Ag. As expected, soluble vimentin amounts decreased in the stimulated RBL-CCR1 cells after treatment with IDPN (Fig. 5B). Notably, Fc ϵ RI- and CCR1-stimulated phosphorylation of p38 MAPK and ERK1/2 was not reduced in the IDPN-treated RBL-CCR1 cells (supplemental Fig. S1). Due to the low amount of soluble vimentin, the levels of associated MAPKs with the vimentin were lower in the IDPN-treated cells (Fig. 5B). Furthermore, IDPN treatment reduced CCL2 production in Fc ϵ RI- and CCR1-activated RBL-CCR1 cells (Fig. 6A), suggesting involvement of soluble vimentin in the chemokine production.

Finally, we verified the involvement of vimentin in Fc ϵ RI/CCR1-stimulated CCL2 production in BMMCs, a model of primary mast cells. Stimulating BMMCs with IgE/Ag plus rCCL3 enhanced formation of soluble vimentin, phosphorylation of MAPKs, and CCL2 production (Fig. 6B and supplemental Fig. S2). Furthermore, pretreatment of BMMCs with IDPN reduced CCL2 production upon IgE/Ag and rCCL3 stimulation (Fig. 6B).

DISCUSSION

Here, we identified vimentin as a component involved in the cross-talk between Fc ϵ RI- and CCR1-mediated signaling pathways in mast cells. Fc ϵ RI and/or CCR1 activation of RBL-CCR1 cells induced phosphorylation and subsequent disassembly of vimentin. Importantly, the disassembled and soluble vimentin interacted with phosphorylated ERK1/2 and p38 MAPKs in the stimulated RBL-CCR1 cells. p38 MAPK plays a role in Fc ϵ RI- and CCR1-mediated CCL2 production in RBL-CCR1 cells. Conversely, inducing aggregation of vimentin filaments by IDPN reduced the levels of disassembled vimentin and hence the interactions with phosphorylated MAPKs and production of CCL2 in mast cells. These results suggest that vimentin could play a role in optimal CCL2 production in mast cells.

Vimentin has been shown to interact with phosphorylated ERK1/2 in several cells, such as neurons and adipocytes (35, 37). Perlson *et al.* (36) observed that vimentin binds directly to phosphorylated ERK1/2 but not to phosphorylated p38 MAPK and non-phosphorylated forms of these MAPKs. We observed that soluble vimentin interacted not only with phosphorylated ERK1/2 but also with phosphorylated p38 MAPK in Fc ϵ RI- and CCR1-activated RBL-CCR1 cells. Because vimentin is capable of interacting with a variety of proteins involved in cell signaling cascades (31, 39), phosphorylated p38 MAPK might indirectly interact with vimentin by binding to other vimentin-binding proteins. In neurons, it has been shown that the complex of vimentin and phosphorylated ERK1/2 moves from the cytoplasm to the nucleus (35). Translocation of MAPKs into the nucleus is a mechanism for the gene expression of cytokines and chemokines. Disassembled vimentin, which is generated by Fc ϵ RI and/or CCR1 stimulation, may act as a shuttle protein for the MAPKs to enter into the nucleus in mast cells, as observed in neurons (see the hypothetical model in Fig. 7).

p38 MAPK has been shown to play a critical role in calcium ionophore-, cytokine-, or Fc ϵ RI-mediated CCL2 production in cultured mast cells, such as BMMC and a human leukemic mast cell line, HMC-1 (40–42). Consistent with these previous studies, p38 MAPK is apparently involved in Fc ϵ RI- and CCR1-mediated CCL2 production in RBL-CCR1 cells because inhibiting p38 MAPK reduced CCL2 production. Co-stimulation with rCCL3 and IgE/Ag enhanced phosphorylation of ERK1/2 and p38 MAPK in RBL-CCR1 cells and BMMCs. Such enhanced phosphorylation of p38 kinase and the association of the activated kinase with the disassembled vimentin could lead to its translocation into the nucleus and the enhanced CCL2 production we observed. Although vimentin interacts with phosphorylated ERK1/2, only p38 MAPK is required for CCL2 production in RBL-CCR1 cells. This may be due to

Role of Vimentin in CCL2 Production in Mast Cells

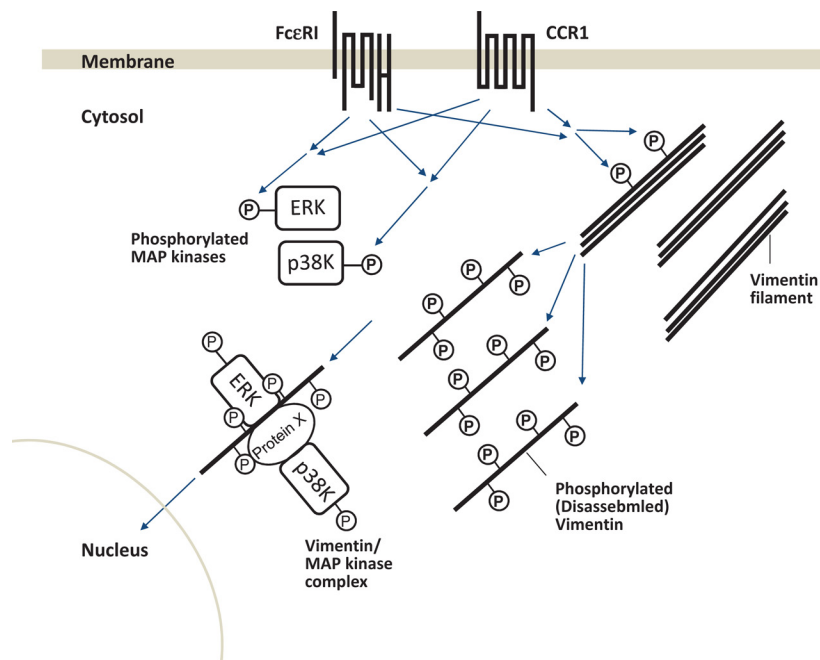


FIGURE 7. **Hypothetical model for FcεRI and CCR1 mediated signaling pathways in mast cells.** FcεRI and CCR1 activation synergistically induces phosphorylation of ERK1/2, p38 MAPK and vimentin in mast cells. Phosphorylation of vimentin induces disassembly of the filament binding protein. The disassembled vimentin interacts with phosphorylated ERK1/2 directly or with phosphorylated p38 MAPK indirectly via other vimentin-binding protein(s) (protein X). Vimentin may then act as a shuttle protein for the activated MAPKs to translocate them into the nucleus.

enhancer and promoter regions in the CCL2 gene that only p38 MAPK may regulate. It has been shown that ERK1/2 and p38 MAPK activate a different set of substrates and transcription factors (43). Future studies on the enhancer/promoter region in the CCL2 gene would elucidate a detailed molecular mechanism for p38 MAPK-mediated chemokine production in mast cells.

Vimentin seems to be involved in mast cell degranulation (39). It was hypothesized that phosphorylation of vimentin induces disassembly of the intermediate filaments, subsequently increasing the mobility of granules (39, 44, 45). Supporting this hypothesis, degranulation of BMDCs was enhanced by vimentin deficiency (39). In this study, we found that the levels of vimentin disassembly (*i.e.* levels of soluble vimentin) were not associated with degranulation levels in FcεRI and/or CCR1-activated RBL-CCR1 cells (not shown). Degranulation of RBL-CCR1 cells was previously shown to be synergistically enhanced by rCCL3 and IgE/Ag stimulation in a CC chemokine dose-dependent manner; around 55 or 80% degranulation was induced by 10 or 100 ng/ml rCCL3, respectively, together with IgE/Ag (11). However, the level of soluble vimentin in the stimulated cells was almost the same (Fig. 5A). These results suggest that the disassembly of vimentin filaments does not determine the level of degranulation in mast cells.

Vimentin has many phosphorylation sites (27, 28, 31). FcεRI and CCR1 co-stimulation in RBL-CCR1 cells enhanced phosphorylation of tyrosine, serine, and threonine residues of vimentin. It has been shown that phosphorylations at serines 55, 71, 72, and 82 of vimentin are coordinated by several kinases (*i.e.* Rho kinase and Aurora B) and induce disassembly of the protein filaments in mitotic cells (9, 23–25, 33). Here, co-stimulation of the cells with 10 ng/ml rCCL3 plus

10 ng/ml IgE/Ag induced the highest levels of phosphorylation at serines 55, 71, and 82. Interestingly, the levels of soluble vimentin in the cells stimulated with 10 or 100 ng/ml rCCL3 plus 10 ng/ml IgE/antigen were comparable. These results suggest that not only serines 55, 71, 82 but also other phosphorylation sites could regulate the disassembly of vimentin in activated RBL-CCR1 cells. Further study will be necessary to elucidate the mechanisms and roles of vimentin phosphorylation in the signaling cascades and function of mast cells and basophils.

In summary, our results suggest that vimentin could be a component inducing optimal CCL2 production in mast cells. Because increased expression of CCL2 has been observed in tissues of allergic patients, our findings could provide clues to unraveling detailed molecular mechanisms underlying allergic inflammation.

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REFERENCES

1. Abramson, J., and Pecht, I. (2007) Regulation of the mast cell response to the type 1 Fcε receptor. *Immunol. Rev.* **217**, 231–254
2. Gilfillan, A. M., and Tkaczyk, C. (2006) Integrated signaling pathways for mast cell activation. *Nat. Rev. Immunol.* **6**, 218–230
3. Ono, S. J., Nakamura, T., Miyazaki, D., Ohbayashi, M., Dawson, M., and Toda, M. (2003) Chemokines. Roles in leukocyte development, trafficking, and effector function. *J. Allergy Clin. Immunol.* **111**, 1185–1199; quiz 1200
4. Beer, F., Kuo, C. H., Morohoshi, K., Goodliffe, J., Munro, P., Aye, C. C., Dawson, M., Richardson, R. M., Jones, L. H., Ikeda, Y., Nakamura, T., Toda, M., Flynn, T., Ohbayashi, M., Miyazaki, D., and Ono, S. J. (2007) Role of β-chemokines in mast cell activation and type I hypersensitivity reactions in the conjunctiva. *In vivo and in vitro* studies. *Immunol. Rev.* **217**, 96–104

5. Tominaga, T., Miyazaki, D., Sasaki, S., Mihara, S., Komatsu, N., Yakura, K., and Inoue, Y. (2009) Blocking mast cell-mediated type I hypersensitivity in experimental allergic conjunctivitis by monocyte chemoattractant protein-1/CCR2. *Invest. Ophthalmol. Vis. Sci.* **50**, 5181–5188
6. Campbell, E. M., Charo, I. F., Kunkel, S. L., Strieter, R. M., Boring, L., Gosling, J., and Lukacs, N. W. (1999) Monocyte chemoattractant protein-1 mediates cockroach allergen-induced bronchial hyperreactivity in normal but not CCR2^{-/-} mice. The role of mast cells. *J. Immunol.* **163**, 2160–2167
7. Sousa, A. R., Lane, S. J., Nakhosteen, J. A., Yoshimura, T., Lee, T. H., and Poston, R. N. (1994) Increased expression of the monocyte chemoattractant protein-1 in bronchial tissue from asthmatic subjects. *Am. J. Respir. Cell Mol. Biol.* **10**, 142–147
8. Holgate, S. T., Bodey, K. S., Janezic, A., Frew, A. J., Kaplan, A. P., and Teran, L. M. (1997) Release of RANTES, MIP-1 α , and MCP-1 into asthmatic airways following endobronchial allergen challenge. *Am. J. Respir. Crit. Care Med.* **156**, 1377–1383
9. Toda, M., Nakamura, T., Ohbayashi, M., Ikeda, Y., Dawson, M., Aye, C. C., Miyazaki, D., and Ono, S. J. (2007) Mechanisms of leukocyte trafficking in allergic diseases. Insights into new therapies targeting chemokines and chemokine receptors. *Expert Rev. Clin. Immunol.* **3**, 351–364
10. Miyazaki, D., Nakamura, T., Toda, M., Cheung-Chau, K. W., Richardson, R. M., and Ono, S. J. (2005) Macrophage inflammatory protein-1 α as a costimulatory signal for mast cell-mediated immediate hypersensitivity reactions. *J. Clin. Invest.* **115**, 434–442
11. Toda, M., Dawson, M., Nakamura, T., Munro, P. M., Richardson, R. M., Bailly, M., and Ono, S. J. (2004) Impact of engagement of Fc ϵ RI and CC chemokine receptor 1 on mast cell activation and motility. *J. Biol. Chem.* **279**, 48443–48448
12. Fifadara, N. H., Aye, C. C., Raghuvanshi, S. K., Richardson, R. M., and Ono, S. J. (2009) CCR1 expression and signal transduction by murine BMDC results in secretion of TNF- α , TGF β -1, and IL-6. *Int. Immunol.* **21**, 991–1001
13. Laffargue, M., Calvez, R., Finan, P., Trifilieff, A., Barbier, M., Altruda, F., Hirsch, E., and Wymann, M. P. (2002) Phosphoinositide 3-kinase γ is an essential amplifier of mast cell function. *Immunity* **16**, 441–451
14. Aye, C. C., Toda, M., Morohoshi, K., and Ono, S. J. (2012) Identification of genes and proteins specifically regulated by costimulation of mast cell Fc ϵ receptor I and chemokine receptor 1. *Exp. Mol. Pathol.* **92**, 267–274
15. Gaga, M., Ong, Y. E., Benyahia, F., Aizen, M., Barkans, J., and Kay, A. B. (2008) Skin reactivity and local cell recruitment in human atopic and nonatopic subjects by CCL2/MCP-1 and CCL3/MIP-1 α . *Allergy* **63**, 703–711
16. Richardson, R. M., Pridgen, B. C., Haribabu, B., and Snyderman, R. (2000) Regulation of the human chemokine receptor CCR1. Cross-regulation by CXCR1 and CXCR2. *J. Biol. Chem.* **275**, 9201–9208
17. Groschwitz, K. R., Ahrens, R., Osterfeld, H., Gurish, M. F., Han, X., Abrink, M., Finkelman, F. D., Pejler, G., and Hogan, S. P. (2009) Mast cells regulate homeostatic intestinal epithelial migration and barrier function by a chymase/Mcpt4-dependent mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 22381–22386
18. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850–858
19. Wilm, M., Shevchenko, A., Houthaave, T., Breit, S., Schweigerer, L., Fotsis, T., and Mann, M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**, 466–469
20. Suckau, D., Resemann, A., Schuereberg, M., Hufnagel, P., Franzen, J., and Holle, A. (2003) A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. *Anal. Bioanal. Chem.* **376**, 952–965
21. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**, 3551–3567
22. Valgeirsdottir, S., Claesson-Welsh, L., Bongcam-Rudloff, E., Hellman, U., Westermark, B., and Heldin, C. H. (1998) *J. Cell Sci.* **111**, 1973–1980
23. Tsujimura, K., Ogawara, M., Takeuchi, Y., Imajoh-Ohmi, S., Ha, M. H., and Inagaki, M. (1994) Visualization and function of vimentin phosphorylation by cdc2 kinase during mitosis. *J. Biol. Chem.* **269**, 31097–31106
24. Yasui, Y., Goto, H., Matsui, S., Manser, E., Lim, L., Nagata, K., and Inagaki, M. (2001) Protein kinases required for segregation of vimentin filaments in mitotic process. *Oncogene* **20**, 2868–2876
25. Ogawara, M., Inagaki, N., Tsujimura, K., Takai, Y., Sekimata, M., Ha, M. H., Imajoh-Ohmi, S., Hirai, S., Ohno, S., and Sugiura, H. (1995) Differential targeting of protein kinase C and CaM kinase II signalings to vimentin. *J. Cell Biol.* **131**, 1055–1066
26. McDonald, J. R., Finck, B. K., McIntosh, L. M., and Wilson, S. E. (2011) Anti-inflammatory approaches that target the chemokine network. *Recent Pat. Inflamm. Allergy Drug Discov.* **5**, 1–16
27. Chang, L., and Goldman, R. D. (2004) Intermediate filaments mediate cytoskeletal cross-talk. *Nat. Rev. Mol. Cell Biol.* **5**, 601–613
28. Inagaki, M., Nishi, Y., Nishizawa, K., Matsuyama, M., and Sato, C. (1987) Site-specific phosphorylation induces disassembly of vimentin filaments *in vitro*. *Nature* **328**, 649–652
29. Eriksson, J. E., He, T., Trejo-Skalli, A. V., Härmälä-Braskén, A. S., Hellman, J., Chou, Y. H., and Goldman, R. D. (2004) Specific *in vivo* phosphorylation sites determine the assembly dynamics of vimentin intermediate filaments. *J. Cell Sci.* **117**, 919–932
30. Li, Q. F., Spinelli, A. M., Wang, R., Anfinogenova, Y., Singer, H. A., and Tang, D. D. (2006) Critical role of vimentin phosphorylation at Ser-56 by p21-activated kinase in vimentin cytoskeleton signaling. *J. Biol. Chem.* **281**, 34716–34724
31. Sihag, R. K., Inagaki, M., Yamaguchi, T., Shea, T. B., and Pant, H. C. (2007) Role of phosphorylation in the structural dynamics and function of types III and IV intermediate filaments. *Exp. Cell Res.* **313**, 2098–2109
32. Chou, Y. H., Opal, P., Quinlan, R. A., and Goldman, R. D. (1996) The relative roles of specific N- and C-terminal phosphorylation sites in the disassembly of intermediate filament in mitotic BHK-21 cells. *J. Cell Sci.* **109**, 817–826
33. Ivaska, J., Pallari, H. M., Nevo, J., and Eriksson, J. E. (2007) Novel functions of vimentin in cell adhesion, migration, and signaling. *Exp. Cell Res.* **313**, 2050–2062
34. Helfand, B. T., Chou, Y. H., Shumaker, D. K., and Goldman, R. D. (2005) Intermediate filament proteins participate in signal transduction. *Trends Cell Biol.* **15**, 568–570
35. Perlson, E., Michaelievski, I., Kowalsman, N., Ben-Yaakov, K., Shaked, M., Seger, R., Eisenstein, M., and Fainzilber, M. (2006) Vimentin binding to phosphorylated Erk sterically hinders enzymatic dephosphorylation of the kinase. *J. Mol. Biol.* **364**, 938–944
36. Perlson, E., Hanz, S., Ben-Yaakov, K., Segal-Ruder, Y., Seger, R., and Fainzilber, M. (2005) Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve. *Neuron* **45**, 715–726
37. Kumar, N., Robidoux, J., Daniel, K. W., Guzman, G., Floering, L. M., and Collins, S. (2007) Requirement of vimentin filament assembly for β 3-adrenergic receptor activation of ERK MAP kinase and lipolysis. *J. Biol. Chem.* **282**, 9244–9250
38. Galigniana, M. D., Scruggs, J. L., Herrington, J., Welsh, M. J., Carter-Su, C., Housley, P. R., and Pratt, W. B. (1998) Heat shock protein 90-dependent (geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton. *Mol. Endocrinol.* **12**, 1903–1913
39. Nahm, D. H., Tkaczyk, C., Fukuishi, N., Colucci-Guyon, E., Gilfillan, A. M., and Metcalfe, D. D. (2003) Identification of Fyn-binding proteins in MC/9 mast cells using mass spectrometry. *Biochem. Biophys. Res. Commun.* **310**, 202–208
40. Teshima, R., Onose, J., Okunuki, H., and Sawada, J. (2000) Effect of Ca²⁺ ATPase inhibitors on MCP-1 release from bone marrow-derived mast cells and the involvement of p38 MAP kinase activation. *Int. Arch. Allergy Immunol.* **121**, 34–43
41. Wong, C. K., Tsang, C. M., Ip, W. K., and Lam, C. W. (2006) Molecular mechanisms for the release of chemokines from human leukemic mast cell line (HMC)-1 cells activated by SCF and TNF- α . Roles of ERK, p38 MAPK, and NF- κ B. *Allergy* **61**, 289–297
42. Gonzalez-Espinosa, C., Odom, S., Olivera, A., Hobson, J. P., Martinez, M. E., Oliveira-Dos-Santos, A., Barra, L., Spiegel, S., Penninger, J. M., and

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- Rivera, J. (2003) Preferential signaling and induction of allergy-promoting lymphokines upon weak stimulation of the high affinity IgE receptor on mast cells. *J. Exp. Med.* **197**, 1453–1465
43. Cargnello, M., and Roux, P. P. (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol. Mol. Biol. Rev.* **75**, 50–83
44. Sahara, N., Siraganian, R. P., and Oliver, C. (1990) Morphological changes induced by the calcium ionophore A23187 in rat basophilic leukemia (2H3) cells. *J. Histochem. Cytochem.* **38**, 975–983
45. Izushi, K., Fujiwara, Y., and Tasaka, K. (1992) Identification of vimentin in rat peritoneal mast cells and its phosphorylation in association with histamine release. *Immunopharmacology* **23**, 153–161