The Journal of Immunology

RESEARCH ARTICLE | AUGUST 01 1999

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J Immunol (1999) 163 (3): 1611–1618.
https://doi.org/10.4049/jimmunol.163.3.1611

Activation of Mitogen-Activated Protein Kinase Regulates Eotaxin-Induced Eosinophil Migration[1]

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Eotaxin is a potent eosinophil chemoattractant that plays an important role in regulating eosinophil tissue levels both in healthy individuals and in diseases associated with significant eosinophil infiltrates, such as the allergic inflammation observed in asthma. Here, we demonstrate that treatment of eosinophils with eotaxin induces the phosphorylation of the mitogen-activated protein kinases (MAPKs) p42 and p44, leading to kinase activation. Blockade of MAPK activation by the MAPK kinase inhibitor PD98059 leads to a dramatic decrease in eotaxin-induced eosinophil rolling in vivo and chemotaxis in vitro. This blockade in the leukocyte migration process is consistent with the observed inhibition of actin polymerization and rearrangement within the eosinophil following treatment with MAPK inhibitor. It is suggested, therefore, that the intrinsic mechanism of eotaxin-induced eosinophil rolling and migration involves activation of the p42/p44 MAPK, possibly through regulation of the cytoskeletal rearrangements necessary for chemotaxis. ***The Journal of Immunology,*** **1999, 163: 1611–1618.**

The eosinophil is a proinflammatory granulocyte implicated in the exacerbation of allergic inflammation as well as protection against parasitic infection. Eosinophils constitute 1–3% of peripheral blood leukocytes in healthy individuals, but may accumulate in the circulation or localize in various organs of patients with parasitic infections or allergic diseases (1–4). Eosinophils possess secretory granules that contain multiple cytotoxic proteins, including major basic protein and eosinophil peroxidase. Additionally, eosinophils produce reactive oxygen metabolites, lipid mediators of inflammation and cytokines, and are therefore capable of causing severe host tissue damage upon activation (1–4).

Numerous CC chemokines attract eosinophils, including eotaxin, eotaxin-2, MCP-2,[3] MCP-3, MCP-4, RANTES, and MIP-1α (5). Eotaxin is unique, however, because in vivo protein administration studies have demonstrated that it is chemotactic primarily for eosinophils (6, 7). CCR3 appears to be the primary eotaxin receptor, although eotaxin has recently been shown to bind, but not to activate, CXCR3 (8–12). Both receptors are seven-transmembrane-spanning G protein-coupled receptors (8–12).

Endothelial cell-bound chemokines are thought to provide a gradient to circulating leukocytes, such as eosinophils, that induces a signaling cascade culminating in migration toward the source of the chemoattractant (13, 14). Binding of chemokines, including eotaxin, to their receptors leads to activation of heterotrimeric G proteins that initiate multiple signaling events, including a transient intracellular rise in Ca^{2+}, and tyrosine phosphorylation (6, 10, 11). Furthermore, it has been shown for certain chemokines that receptor binding activates the ERK (extracellular signal-regulated kinase)/MAPK pathway in multiple cell types (15–21). Three families of MAPKs have been identified, the ERKs (which include p44 (ERK1) and p42 (ERK2)), c-Jun amino-terminal kinase, and the p38 subgroup of kinases, and can be distinguished by their tripeptide dual phosphorylation motif required for activation (22). MAPK kinases or ERK kinases (MEKs) are dual specificity kinases that can phosphorylate ERK1 and ERK2 on tyrosine and threonine residues, thereby inducing ERK activity (23, 24). The MEK1 and MEK2 inhibitor PD98059 can specifically block activation of ERK/MAPK (25).

The chemotaxis process in response to chemokine stimulation of leukocytes requires cytoskeletal rearrangement involving both microtubules, which impart directionality and stabilize the cell (26), and the actin-myosin system, which is thought to provide the contractile forces necessary for directional migration (27). Additionally, changes in the actin-myosin complex regulate the avidity and membrane localization of multiple adhesion molecules, including very late Ag-4 (VLA-4) and L-selectin (CD62L) (28–31). Thus, cytoskeletal remodeling has a direct effect on leukocyte rolling, firm adhesion, and transendothelial migration by controlling the location and affinity state of cell surface adhesion molecules for their counter-receptors as well as cell shape.

Because eosinophils play a pivotal role in the pathogenesis of allergic diseases, a better understanding of the signal transduction pathways, which regulate eosinophil chemotaxis and their sequestration to sites of inflammation in the tissue, is of considerable importance. Since a number of chemoattractants can activate MAPK in different cell types, we wanted to explore whether the chemokine eotaxin could activate the p42/p44 MAPK signaling pathway. We demonstrate in this study that eotaxin stimulation of eosinophils leads to p42/p44 MAPK phosphorylation and activation that can be inhibited by the MEK antagonist PD98059. Functionally, eosinophils stimulated with eotaxin undergo MAPK-dependent rolling and chemotaxis, changes in the filamentous actin

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Received for publication March 1, 1999. Accepted for publication May 14, 1999.

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[1] This work was supported in part by National Institutes of Health Grant AI 35796 (to P.S.).

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[3] Abbreviations used in this paper: MCP, monocyte chemoattractant protein; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK ERK kinase; VLA-4, very late Ag-4; MBP, myelin basic protein; RF, rolling fraction; F-actin, filamentous actin; CFDA, 5- and 6-carboxyfluorescein diacetate.

 0022-1767/99/$02.00

content and cellular distribution patterns, and increased CD11b cell surface expression. The significance of these results indicate that a critical step involved in eotaxin-induced eosinophil rolling and migration is the activation of ERK1 and ERK2, which play a key role in regulating the actin cytoskeleton rearrangements required for chemotaxis.

Materials and Methods

Materials

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. The animal care and use committee approved all animal experimentation before implementation.

Eosinophil isolation

Eosinophils were purified from the peripheral blood of allergic donors (32). Anticoagulated blood was diluted 1/1 in PBS (Mediatech, Tustin, CA) and 0.1 mM EDTA and layered onto Percoll (density, 1.087 g/ml; pH 7.4; Pharmacia Biotech, Uppsala, Sweden). Following centrifugation, mononuclear cells were aspirated and RBC were lysed using 150 mM NH_4Cl. The cells were centrifuged again, and the resulting cell pellet was incubated with anti-CD16 microbeads (Miltenyi Biotech, Burlingame, CA) to deplete neutrophils. Following Ab incubation, the cells were magnetically sorted using the MACs system (Miltenyi Biotech) to retain the positively stained neutrophils. The eluate was then washed, and viability was assessed by staining cells with trypan blue. Cytospin slides were stained with Diff-Quik (Baxter Healthcare, Miami, FL) to determine eosinophil purity, which was routinely >95%. Further RBC lysis was performed if necessary using 0.2% NaCl. For the in vivo experiments, eosinophils were fluorescently labeled with carboxyfluorescein diacetate (Molecular Probes, Eugene, OR) as previously described (33). Eosinophils were resuspended in PBS containing 0.01% glucose at a concentration of 1×10^7 cells/ml and kept at room temperature in the dark until use.

Western blot analysis

Eosinophils were resuspended at 1×10^7 cells/ml in PBS/0.1% BSA (fraction V, low endotoxin). Stimulation with eotaxin (R&D Systems, Minneapolis, MN) was conducted at 37°C and was halted by addition of cold PBS. The cells were then centrifuged at 4°C and resuspended in lysis solution consisting of 1% Nonidet P-40 (Calbiochem, La Jolla, CA), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM sodium orthovanadate. The cells were vortex mixed briefly, incubated on ice for 15 min, and subsequently centrifuged to pellet insoluble material. The supernatants were isolated, and boiled for 5 min in 2× Laemmli sample buffer, then loaded onto SDS/Tris-glycine polyacrylamide gels (Novex, San Diego, CA). Protein was transferred to a nitrocellulose membrane using the XCELL II system (Novex). Following transfer, the nitrocellulose membrane was washed briefly in water, and nonspecific protein binding sites were blocked by incubating the membrane in 2% BSA, 0.5% OVA, 2.5% nonfat dry milk (Bio-Rad, Hercules, CA), 10 mM Tris (pH 8), 150 mM NaCl, and 0.2% thimerserol for 1 h at 25°C. The membrane was incubated at 4°C overnight with p42/p44 anti-phospho-MAPK Ab (New England Biolabs, Beverly, MA) diluted 1/1000. The immunoblots were subsequently washed in TTBS (20 mM Tris-HCl (pH 7.6), 137.5 mM NaCl, and 0.1% Tween-20), three times for 5 min each time, and incubated with peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry, Gaithersburg, MD) diluted 1/5000 for 1 h at 25°C. The filter was washed again three times for 5 min each time in TTBS, three times for 5 min each time in TBS (20 mM Tris-HCl (pH 7.6) and 137.5 mM NaCl), and once for 5 min in H_20. Phospho-MAPK was visualized using the chemiluminescent peroxidase substrate Super Signal Ultra (Pierce, Rockford, IL). For subsequent blotting the membrane was stripped using 7 M guanidine, 50 mM glycine, 50 μM EDTA, 100 mM KCl, and 20 mM 2-ME, pH 10.8, for 10 min at 25°C. The anti-MAPK blot was conducted in the same manner, except the primary Ab was a rabbit IgG anti-human ERK-1 (p44) at 1 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA), which cross-reacts with ERK-2 (p42).

In vitro kinase assay

Eosinophils were stimulated as described, and the cell pellets were resuspended in 20 μl of lysing solution (1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.25% deoxycholate, 5 mM EDTA, 1 mM PMSF, 10 μg/ml each of leupeptin and aprotinin, 1 mM sodium orthovanadate, 1 mM EGTA, 100 μM β-glycerophosphate, 10 mM sodium fluoride, and 1 mM tetrasodium phosphate) and incubated on ice for 20 min. The lysates were centrifuged to remove cell debris, 4 μg of agarose-conjugated ERK1 and ERK2 polyclonal Abs (Santa Cruz Biotechnology) was added, and the cell lysate was incubated rotating for 1 h at 4°C. Immunoprecipitated ERK1 and ERK2 were washed once with lysing solution, once in lysing solution containing 0.5 M NaCl, and once in MAPK washing buffer (25 mM Tris-HCl (pH 7.4), 40 mM $MgCl_2$, 137 mM NaCl, and 10% glycerol). ERK enzyme activity was assessed in an in vitro kinase assay using 20 μl of buffer (42.5 mM HEPES, 42.5 mM $MgCl_2$, 0.21 mM ATP, 50 mM myelin basic protein (MBP; Upstate Biotechnology, Lake Placid, NY) and 50 μCi of [γ-^{32}P]ATP (>3000 Ci/mmol sp. act.; Amersham, Arlington Heights, IL)) for 30 min at 30°C. Following incubation, an equal volume of 2× Laemmli sample buffer (Novex) was added, the samples were boiled for 5 min, cooled on ice, and loaded on 16% Tris-glycine gels (Novex). Proteins were then transferred to Immobilon P membranes (Millipore), and phosphorylation of the MBP substrate was visualized by autoradiography. Equal loading of the immunoprecipitates was subsequently assayed by Western analysis of the membrane using ERK1 and ERK2 polyclonal Abs (Santa Cruz Biotechnology).

Chemotaxis assay

All chemotaxis assays were performed in a 48-well modified Boyden chamber (Neuroprobe, Cabin John, MD) (34). Eosinophils were resuspended in DMEM (Mediatech) with 10% FBS (HyClone, Logan, UT) at 2×10^6 cells/ml. Twenty-five microliters of medium or medium containing eotaxin (R & D Systems) at varying concentrations was added to the bottom of the chamber. A 5-μm pore size polyvinylene difluoride polycarbonate filter (Osmotics, Livermore, CA) was overlayed, and 1×10^5 eosinophils were added to the upper chamber. Where indicated eosinophils were preincubated with PD98059 (Calbiochem) at 10 μM for 15 min at 25°C. Cells were allowed to migrate for 1 h at 37°C, after which time the filter was washed twice in PBS (Mediatech), fixed in methanol for 5 min, and stained in Diff-Quik (Baxter). Migrating cells were quantified by microscopy, manually counting four fields under high power (×400).

Animal preparation and superfusion of the mesenteric vascular bed with eotaxin

The circulation of 5- and 6-carboxyfluorescein diacetate (*CFDA*)-labeled human eosinophils in postcapillary venules of IL-1β-stimulated mesenteric blood vessels of New Zealand White rabbits was examined by intravital microscopy as described previously (33, 35). The ability of eotaxin to alter the flux of rolling eosinophils in inflamed blood vessels was determined as previously described (36). Between 6 and 10 h after IL-1β stimulation (i.p.), portions of the exposed mesentery were superfused with 50 nM eotaxin or vehicle alone using a constant flow infusion syringe pump (Harvard Apparatus, South Natick, MA). The flow of syringe pump was adjusted to match the flow of superfusion buffer. CFDA-labeled eosinophils ($0.2–0.5 \times 10^7$ cells/ml) were administered into the mesenteric circulation 5–10 min after the superfusion had ensued. These cells were injected successively through the side branch of the terminal superior mesenteric artery cannulated with a PE10 polyethylene catheter, and their ability to roll in the mesenteric circulation was determined.

The ability of PD98059 to alter the flux of eotaxin-induced eosinophil rolling in IL-1β-stimulated mesenteric venules was determined. Eosinophils were incubated ex vivo with increasing concentrations of PD98059 (0–37 μM) for 15 min at room temperature before injecting them into the rabbit mesentery, which was continuously superfused with eotaxin (50 nM). As a control, eosinophils were treated with vehicle alone.

Intravital microscopy and image analysis

The passage of CFDA-labeled eosinophils in the inflamed venules was made visible by stroboscopic epi-illumination as previously described, and the images were recorded using an *SVHS* video recorder (33). The video images were analyzed off-line by manually counting the total number of CFDA-labeled eosinophils passing through a reference point in a venular segment (total flux). The tapes were rewound, and only those cells found to be visibly rolling along the venular wall were counted (rolling flux). The rolling fraction (RF) was calculated as the percentage of rolling cells in the total flux of eosinophils passing through a venular segment during a given injection. The interaction between eosinophils and venular endothelium in vivo before and after treatment with the MAPK inhibitor PD98059 was analyzed by Student's *t* test using a statistical software package (SigmaStat, Jandel Scientific). For all tests, p values less than 5% were considered significant. Data are presented as the mean ± SD.

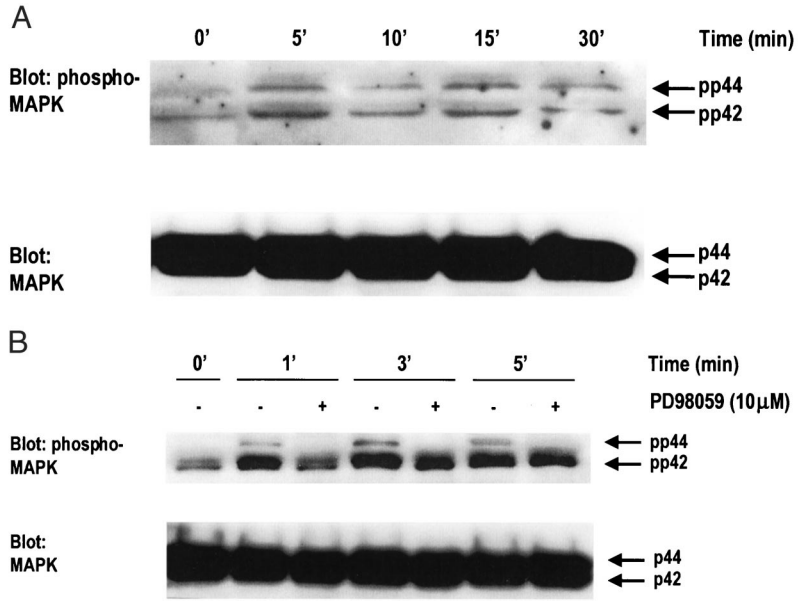

FIGURE 1. Western blot analysis of phosphorylated p42 and p44 MAPK in eotaxin-stimulated eosinophils. Eosinophils were isolated and stimulated with 100 nM eotaxin for the indicated times at 37°C. *A*, The eosinophil lysates were resolved on 12% SDS-PAGE gels and were hybridized with Abs specific for the phosphorylated forms of p42 and p44 MAPK. The immunoblot was reprobed with Abs specific for ERK1 and ERK2 to show equal loading of cell lysate in each lane. *B*, Eosinophils were isolated from a different donor and were either untreated (−) or pretreated (+) with 10 μM of the MEK inhibitor PD98059 for 15 min before being stimulated with 100 nM eotaxin for the indicated time course. The lysates were electrophoresed through a 10% SDS-PAGE gel, and the immunoblot was hybridized with specific phospho-MAPK Abs indicative of active MAPK. To control for equal loading in each lane, the immunoblot was reprobed with ERK1- and ERK2-specific Abs. Experiment 1A was repeated twice, and experiment 1B was repeated three times, and representative Western blots are shown.

Actin polymerization assay

Eosinophils were resuspended in DMEM (Mediatech) and 10% FBS (HyClone) at a concentration of 5×10^5 cells/sample (37). A portion of the eosinophils was treated with PD98059 (Calbiochem) at 10 μM for 30 min at 25°C. The cells were then stimulated with 100 nM eotaxin (R & D Systems) for the indicated times at 37°C. Cells were fixed with 4% paraformaldehyde for 10 min. Cells were washed twice in PBS (Mediatech) and subsequently permeabilized with 0.1% Triton X-100 (Calbiochem) for 5 min. Cells were washed again twice in PBS. Incubating the cells in PBS/1% BSA for 10 min blocked nonspecific binding sites. Oregon Green phalloidin (325 nM; Molecular Probes) was added to samples and incubated for 20 min. Cells were washed twice in PBS and resuspended in PBS/1% BSA. Samples were analyzed by flow cytometry using a FACScan II analyzer (Becton Dickinson, Mountain View, CA).

Actin staining of eosinophils

Freshly isolated eosinophils were incubated in the presence or the absence of eotaxin and/or the MEK inhibitor PD98059 for various time points. Following stimulation at 37°C, the cells were fixed in 3.7% formaldehyde for 10 min and permeabilized with 0.1% Triton X-100 (Calbiochem). The eosinophils were subsequently stained with rhodamine-labeled phalloidin according to the manufacturer's protocol (Molecular Probes).

Flow cytometric analysis

Isolated eosinophils were prepared and stained for flow cytometry as previously described (38). Briefly, the cells were incubated on ice for 30 min with unconjugated isotype control Abs to block nonspecific binding sites (PharMingen, San Diego, CA). The cells were then washed in 2 ml of FACS buffer (PBS containing 1% BSA and 0.1% sodium azide). The cells were subsequently incubated on ice and stained with 10 μl of the appropriate Ab, which were obtained from PharMingen. The Abs used for this study include CD18 (clone 6.7), CD62L (clone Dreg 56), CD11a (clone G43-25B), CD11b (clone ICRF44), CD11c (clone B-ly6), CD4 (clone RPA-T4), CD49d (clone 9F10), CD49e (clone VC5), CD49f (clone GoH3), HLA-DR, DP, and DQ (clone TU39). The cells were washed again in cold FACS buffer and analyzed using a FACScan II analyzer (Becton Dickinson) and CellQuest software (Becton Dickinson).

Results

MAPKs ERK1 and ERK2 are phosphorylated in response to eotaxin stimulation of human eosinophils

It has been previously shown that stimulation of a number of different hemopoietic cell types with chemoattractants leads to the activation of p42/p44 MAPKs (15–21). Therefore, we investigated whether eotaxin induced MAPK activation in eosinophils. Phosphorylation of ERKs is essential for inducing kinase activity. Accordingly, eosinophils were incubated with 100 nM eotaxin for varying lengths of time, and cell lysates were subjected to immunoblotting using Abs specific for the phosphorylated forms of ERK1 (p44) and ERK2 (p42) (Fig. 1, *A* and *B*). A low basal level of ERK phosphorylation was detectable in unstimulated eosinophils, particularly in p42 MAPK. Following eotaxin treatment, ERK phosphorylation increased dramatically, with peak phosphorylation observed between 5 and 15 min (Fig. 1*A*). The ERKs returned to nearly basal levels of phosphorylation by 30 min (Fig. 1*A*). Because p42 and p44 are rapidly phosphorylated, we wanted to further examine earlier time points. Eosinophils from separate donors were isolated, and a fraction was pretreated with 10 μM of the MEK inhibitor PD98059 for 15 min. The eosinophils were then stimulated with 100 nM eotaxin for 1, 3, or 5 min (Fig. 1*B*). Eotaxin induced the phosphorylation of p42 and p44 MAPK as early as 1 min poststimulation, and this was blocked by PD98059. We again noticed baseline phosphorylation of p42 MAPK, and this intrinsic level of activation was not down-regulated by the PD98059 pretreatment of eotaxin-treated cells (Fig. 1*B*). These data suggest that eotaxin rapidly induces the phosphorylation of the MAPKs p42 and p44 in human eosinophils.

Eotaxin-induced activation of ERK1 and ERK2 in eosinophils is both dose and time dependent

As an independent measure of MAPK activation, we directly monitored MAPK activity in eosinophils. Eosinophils were treated with eotaxin, ERK1 and ERK2 were then immunoprecipitated, and their kinase activity was assessed in vitro. A slight baseline kinase activity was observed; however, eotaxin greatly increased MAPK activity at all concentrations tested over a 15-min stimulation period, particularly at the 10- and 100-nM concentrations (Fig. 2, *left panels*). This correlated with an increase in the amount of phospho-MAPK detected by immunoblotting. Consistent with the phosphorylation results shown in Fig. 1*B*; a dose of 10 nM eotaxin maximally induced MAPK activity as early as 1 min (Fig. 2, *right panels*). Since phosphorylation and activation of ERKs are mediated by MEK, the MEK-specific inhibitor, PD98059 (25), should inhibit eotaxin-induced MAPK activation. As shown in Fig. 2, PD98059 blocked kinase activity following stimulation with 10 nM eotaxin at 15 min, demonstrating that p42/p44 MAPK is phosphorylated and activated via a MEK-dependent signaling pathway.

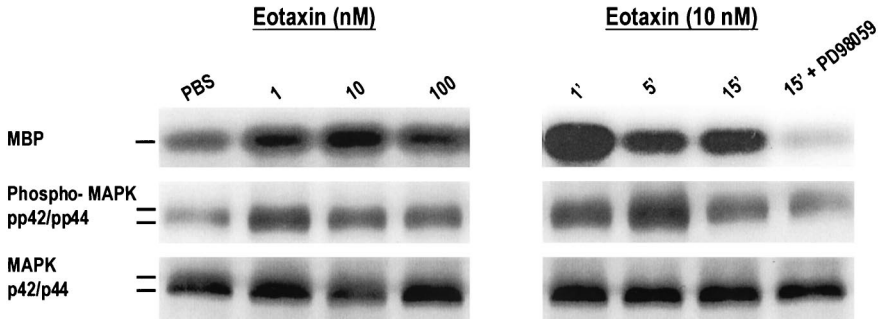

FIGURE 2. In vitro analysis of p42/p44 MAPK activity in eotaxin-stimulated eosinophils. Eosinophils were stimulated with increasing amounts of eotaxin for 15 min (*left panels*) or 10 nM eotaxin for the indicated times with or without 10 μM PD98059 (*right panels*). ERKs were immunoprecipitated from fresh eosinophil lysates, and kinase activity was measured in an in vitro kinase assay using MBP as a substrate. Following the in vitro kinase assay, lysates were resolved on 16% SDS-PAGE gels and transferred to membranes. The *top panel* is an autoradiograph of the ERK in vitro kinase assay substrate myelin basic protein, and the *middle* and *bottom panels* are Western blots. The immunoblot was probed with specific pp42/pp44 phospho-MAPK Abs (*middle panel*). The blot was then rehybridized with anti-p42/p44 MAPK Abs to control for equal loading (*bottom panels*). The data shown are representative of four independent experiments.

Collectively, these data demonstrate that p42/p44 MAPK activation in eosinophils is induced by eotaxin in a time- and dose-dependent manner.

Blockade of MAPK p42 and p44 activity inhibits chemotaxis

Since eotaxin induces MAPK activation and triggers chemotaxis (6, 10, 11), blockade of MAPK activation may have functional consequences for eosinophils. The effect of MAPK inhibition on eotaxin-mediated chemotaxis of eosinophils was examined by incubating the cells in a modified Boyden chamber with various concentrations of eotaxin with or without 10 μM of the MEK inhibitor PD98059. Eotaxin induced eosinophil migration in a dose-dependent manner, which was maximal at 10 nM eotaxin (Fig. 3, *A* and *B*). Strikingly, addition of PD98059 (Fig. 3, *A* and *B*) almost completely abrogated eotaxin-induced eosinophil chemotactic migration, suggesting a role for the ERK pathway in regulating eosinophil chemotaxis in response to eotaxin stimulation. Treatment of eosinophils with 100 ng/ml pertussis toxin before eotaxin stimulation blocked all chemotaxis (Fig. 3*B*), confirming earlier observations that CCR3 is linked to the $G_i\alpha$ family of G proteins (11).

The MEK inhibitor PD98059 inhibits in vivo eosinophil rolling in response to eotaxin stimulation

Given that in vitro eosinophil chemotaxis in response to eotaxin was strongly inhibited by the blockade of MAPK activation, we wanted to investigate whether earlier steps in the leukocyte migration pathway were similarly affected. This was addressed by examining the behavior of fluorescently labeled human eosinophils to roll in inflamed blood vessels of the rabbit mesentery by intravital microscopy. CFDA-labeled human eosinophils were observed to efficiently roll along the walls of IL-1β-stimulated postcapillary venules (RF, 16.4 $\pm$ 6.7%). However, superfusion of the mesentery with 50 nM eotaxin resulted in a 3-fold increase in the flux of rolling eosinophils (RF, 48.3 $\pm$ 7.2; Fig. 4*A*). Pretreatment of eosinophils with the MEK inhibitor PD98059 for 15 min resulted in a dose-dependent decrease in the flux of rolling eosinophils (Fig. 4*B*). Maximum inhibition of rolling was observed when eosinophils were treated with PD98059 at a concentration of 37 μM ($\sim$60% inhibition; $p < 0.01$ vs control). The rolling of vehicle-treated eosinophils was not effected. Together with the in vitro data, this suggests that the p42/p44 MAPK pathway regulates multiple steps (rolling and chemotaxis) in the migration process of eotaxin-stimulated eosinophils.

The MEK inhibitor PD98059 inhibits actin polymerization in eosinophils upon eotaxin stimulation

Actin polymerization controls a number of processes regulating eosinophil migration. First, the polymerization of actin provides the contractile forces necessary for chemotaxis of the cell (27). Second, the actin cytoskeleton regulates the affinity state of multiple adhesion molecules toward their counter-receptors and their positioning on the eosinophil microvilli (28–31). Therefore, we examined the effect of eotaxin-induced MAPK activation on actin

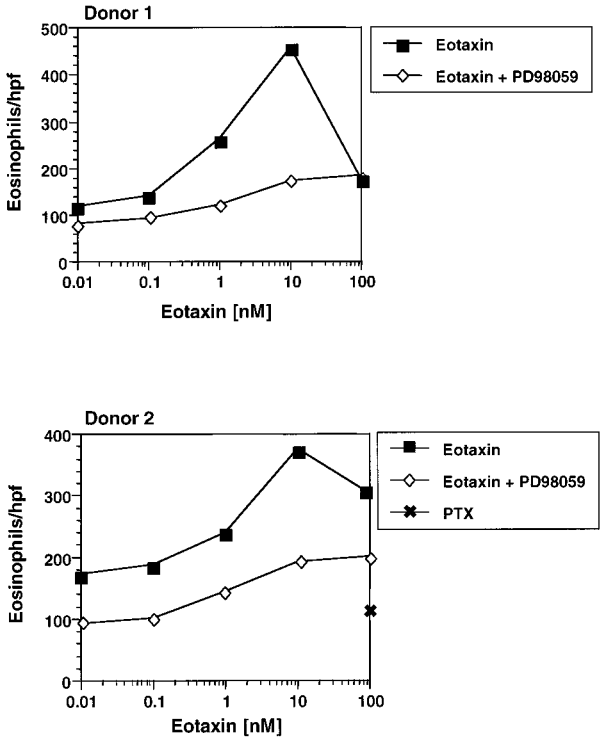

FIGURE 3. Migration of eosinophils in response to eotaxin. Each treatment was performed in duplicate, four high power fields were counted per treatment (eight high power fields per treatment), and the average number of migrated cells per high power field is shown. The experiment was repeated six independent times with different donors, and two representative chemotaxis assays are presented. As a control, eosinophils were preincubated with 100 ng/ml pertussis toxin for 1 h before stimulation with 100 nM eotaxin (*B*).

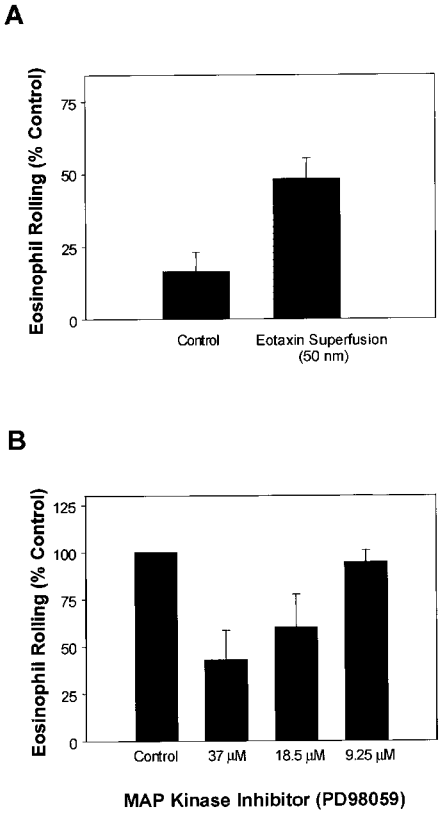

FIGURE 4. In vivo analysis of eotaxin-stimulated eosinophil rolling in IL-1β-stimulated postcapillary venules using intravital microscopy. Human eosinophils were labeled with CFDA and administered to the rabbit mesenteric circulation through a cannulated side branch of the terminal mesenteric artery. *A*, The percentage of rolling eosinophils in inflamed postcapillary venules before (control) and during continuous superfusion of the rabbit mesentery with 50 nM eotaxin. *B*, The effect of MEK inhibitor PD98059 treatment on the rolling flux of eosinophils during continuous superfusion of the rabbit mesentery with 50 nM eotaxin. Human eosinophils were pretreated with different concentrations of PD98059 for 15 min before administration to the IL-1β-treated mesenteric circulation. Greater amounts of PD98059 were used for the in vivo experiments due to the dilution factor once injected into the blood circulation. Eosinophils were isolated from multiple donors (five donors and five rabbits; three to five venules per rabbit were examined), and the bars represent the mean ± SD.

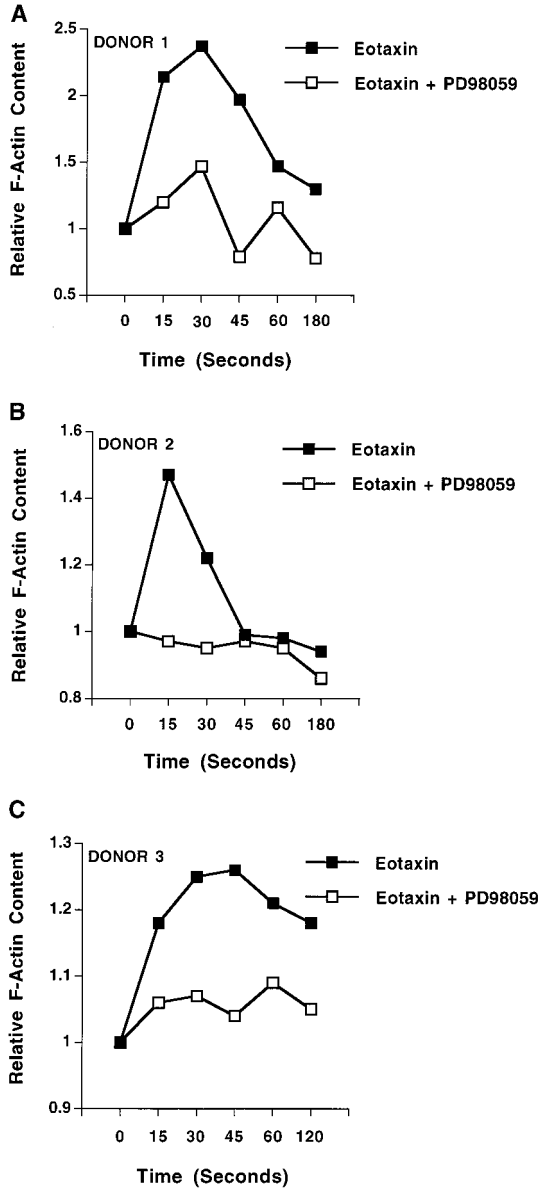

FIGURE 5. Eotaxin-induced actin polymerization is inhibited by the MEK-specific inhibitor PD98059 in eosinophils. The filamentous actin content was measured in eosinophils stimulated with 100 nM eotaxin with or without 10 μM PD98059 for the indicated times. Oregon Green phalloidin was used to stain the cells, and the mean F-actin content was then determined by flow cytometry. Data are expressed as the ratio of the mean channel fluorescence between stimulated and unstimulated cells. The data shown are from eosinophils isolated from three different donors (*A*, *B*, and *C*) and are representative of five independent experiments. The average inhibition of F-actin polymerization induced by 10 μM PD98059 treatment at the peak time point was 59.1% in five experiments.

polymerization by using the MEK inhibitor PD98059. Eotaxin-induced actin polymerization was strongly inhibited, as there was a 59.1% decrease in the F-actin content of the PD98059 and eotaxin-stimulated eosinophils compared with that of eotaxin alone-treated eosinophils (Fig. 5).

We further investigated the effect on actin polymerization and distribution in eosinophils following eotaxin treatment by microscopic examination of actin-stained cells. The MEK inhibitor PD98059 appeared to have little or no effect on intracellular actin localization in untreated eosinophils (Fig. 6, *upper right panel*). In contrast, the addition of 10 nM eotaxin dramatically changed both the actin distribution and polymerization into bundles after just 45 s (Fig. 6, *lower left panel*). This effect on intracellular actin rearrangement was blocked by PD98059 (Fig. 6, *lower right panel*). Thus, actin polymerization and redistribution in eosinophils appear to be MAPK-regulated processes. This suggests that MAPK activation could regulate eosinophil migration by controlling both the location on the cell membrane and avidity of adhesion molecules as well as the changes in cell structure necessary for diapedesis to occur.

Phenotypic analysis of eotaxin-stimulated eosinophils

To further investigate the role of ERK activation in eotaxin-induced eosinophil migration, we examined the expression of cell surface molecules. Eotaxin treatment of eosinophils increased CD11b cell surface expression; however, PD98059 had a minimal effect on this up-regulation, indicating that the p42/p44 MAPK pathway is probably not involved (Fig. 7). Induction of CD11b expression appeared to be unique in that expression of LFA-1, CD18, CD11c, CD62L, VLA-4, VLA-5, VLA-6, CD4, or HLA class II was not modulated by eotaxin treatment (data not shown).

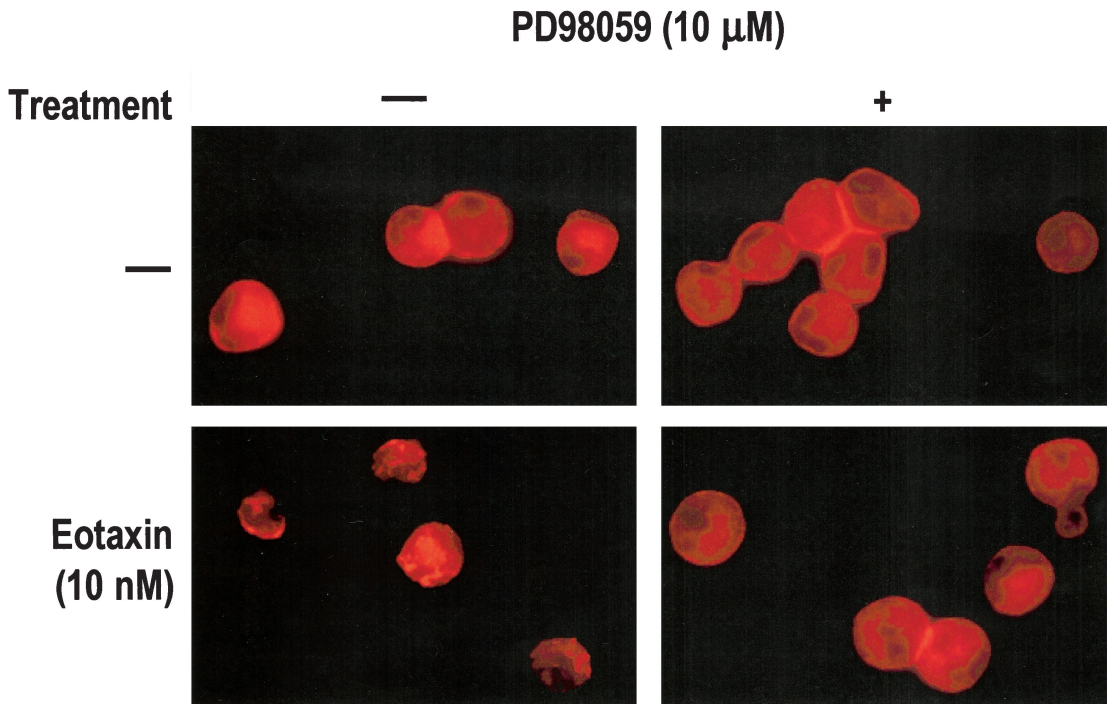

FIGURE 6. Photomicrographs of actin-stained eosinophils. Eosinophils were isolated and were untreated (*upper left*), pretreated with 10 μM PD98059 for 15 min (*upper right*), treated with 10 nM eotaxin for 45 s (*lower left*), or pretreated with 10 μM PD98059 for 15 min and stimulated with 10 nM eotaxin for 45 s (*lower left*). Cells were then fixed, stained with rhodamine-coupled phalloidin, and photographed using a Zeiss Axioskop microscope at ×400. This experiment was repeated three times with similar results, and representative photomicrographs are shown.

As a control, eosinophils treated with 10 ng/ml IL-5 for 45 min shed L-selectin (CD62L; data not shown) (39). CD11b has previously been shown not to play a significant role in eosinophil tethering and rolling in response to chemoattractants (33). Consistent with these previous results, CD11b did not appear to play a major part in in vivo rolling in this study, as eotaxin induced its expression, which was not affected by PD98059 treatment, yet in vivo rolling was substantially decreased by MEK inhibitor treatment (Fig. 4).

Discussion

Eotaxin is a powerful eosinophil chemoattractant, with important roles in immune homeostasis, where it recruits eosinophils to the intestine and thymus (40, 41), and in inflammatory diseases such as asthma, where it attracts eosinophils to the lung (1–4). We show here that eotaxin-mediated chemotaxis of eosinophils in vitro is regulated in large part by its ability to activate the p42/p44 MAPKs. However, eotaxin and other chemokines may also activate other intracellular signaling pathways, leading to functional chemotaxis, as a blockade of MAPK activation strongly reduced, but did not ablate, chemotaxis (16, 19). In contrast to in vitro migration assays, in vivo progression of leukocyte migration requires a sequential series of steps, including rolling along the endothelial wall, firm adhesion to the endothelium, and finally diapedesis (42). Leukocyte rolling and tethering involve the interaction of cell surface receptors expressed by eosinophils with counter-receptors on the endothelial cell surface. The role of p42/p44 MAPK in eosinophil rolling was directly examined within IL-1β-stimulated mesenteric venules under conditions of blood flow by intravital microscopy. Previous results have shown that VLA-4 acts in concert with L-selectin in promoting eosinophil rolling, leading to firm adhesion in vivo (33).

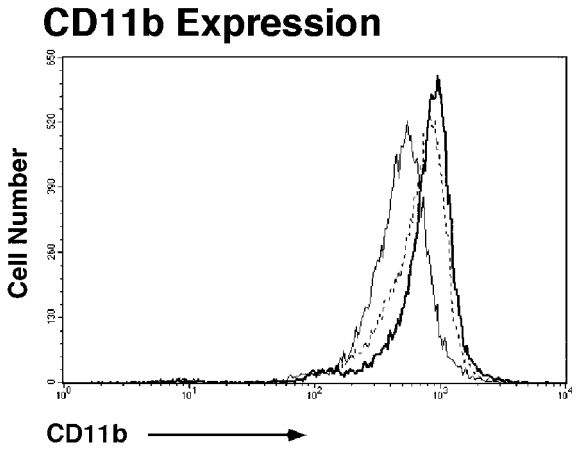

FIGURE 7. Flow cytometric analysis of CD11b expression on untreated (light line) 10 nM eotaxin-treated (dark line) or 10 μM P098059 and 10 nM eotaxin-treated (dashed line) eosinophils for 45 min. The experiment was repeated four times with multiple eosinophil donors, and a representative histogram is shown.

We demonstrate that blockade of p42/p44 MAPK activation strongly inhibits eosinophil rolling in vivo. Once the cell is stably arrested to the endothelium, the leukocyte can diapedesis through the vessel wall and migrate toward the source of the chemoattractant, a process involving cell motility. Our results examining the polymerization and redistribution of the eosinophil actin cytoskeleton in response to eotaxin stimulation point to multiple mechanisms in which MAPK activation can regulate chemotaxis. First, eotaxin-induced MAPK activation induces an immediate rise in the cellular F-actin content of the eosinophil, which may allow for proper adhesion molecule positioning within the microvilli (30). Second, intracellular actin distribution and orientation control the avidity of VLA-4 and L-selectin (CD62L) toward their counter-receptors expressed on the luminal surface of the vessel (28–31). Third, actin rearrangement may govern the physical process of

chemotaxis by directly regulating changes in cell shape and motility (27). The finding that the MEK-specific inhibitor PD98059 constrains rolling and chemotaxis, presumably by impeding ERK activation and ultimately actin reorganization, is consistent with studies showing that cytochalasin B also inhibits cell migration (28). Thus, a critical event controlling eosinophil trafficking in vivo is p42/p44 MAPK-regulated intracellular actin reorganization of the cytoskeleton.

The eosinophils from many of the allergic donors in this study varied in their basal activation state of both ERK1 and ERK2 (data not shown). Because activation of p42/p44 MAPK appears to regulate chemotaxis, this illustrates the difficulty in controlling eosinophil tissue accumulation in allergic diseases. We consistently observed in our studies with eosinophils a baseline phosphorylation of ERK2, previously noted by others (17). Treatment of eosinophils with 10 μM PD98059 did not reduce the basal level of p42 MAPK phosphorylation; however, this concentration of PD98059 maintained specificity for the MEK/ERK pathway. The lack of reduction in the basal level of p42 MAPK phosphorylation may be explained by the fact that PD98059 inhibits MEK2 activity with an IC_{50} of ~50 μM, which is ~20-fold less potent than its effect on MEK1 (IC_{50}, ~2.4 μM) (25). This suggests that the baseline level of phosphorylation of p42 MAPK after pretreatment with PD98059 may be specifically due to the kinase activity of MEK2. This raises the possibility that MEK1 activity is critical for the functional changes observed, and furthermore, that MEK1 or MEK2 may preferentially phosphorylate either ERK1 or ERK2. It has already been shown that the p42 and p44 MAPK isoforms differentially regulate eosinophil function (43). Specifically, activation of p44 MAPK results in the activation of the Elk-1 transcription factor, whereas ERK2 activation stimulates c-Myc (43).

In the broader context of chemokine-regulated inflammation, this study along with others (15–21) raise the question of whether chemokines binding to their receptors in multiple cell types initiates a common signaling pathway leading to ERK1 and ERK2 activation and culminating functionally in chemotaxis. Both CC and CXC chemokines have been shown to activate the p42/p44 MAPKs in eosinophils, T lymphocytes, thymocytes, monocytes, and neutrophils. In addition, the chemokines RANTES, MCP-3, and eotaxin all increase F-actin levels in eosinophils following stimulation (44). Signaling leading to MAPK activation has been best studied in neutrophils stimulated with IL-8 (15). These studies demonstrated that ERK activation followed Ras GTP loading and activation of Raf-1 and B-Raf (15). A role for phosphatidylinositol 3-kinase in regulating p42/p44 MAPK activation was also implicated. Similar observations were made with eosinophils stimulated with MIP-3β (unpublished observations), IL-5, or platelet-activating factor (17), and monocytes treated with MCP-1 (16). Our results with PD98059 demonstrate that MEK-1 and -2 act upstream in the ERK activation cascade, while actin polymerization occurs downstream of p42/p44 MAPK activation. The chemokine pathway leading to ERK activation may be different from those signal transduction pathways triggered by nonchemokine chemoattractants such as C5a and FMLP (45, 46). Specifically, in neutrophils FMLP-induced F-actin polymerization and subsequent chemotaxis are not blocked by the MEK inhibitor PD98059 (47). This again suggests that there multiple signaling pathways leading to leukocyte chemotaxis, a finding suggested by our results using eosinophils.

In summary, understanding the intracellular signaling pathways triggered by chemoattractants that govern eosinophil trafficking to sites of inflammation may offer additional molecular targets for antagonism of eosinophil-mediated allergic diseases. This study demonstrates that eotaxin-stimulated eosinophils activate p42/p44 MAPK, which leads to changes in the intracellular actin organization and polymerization that may directly affect eosinophil chemotaxis.

Acknowledgments

We thank Dr. David Alleva for critical reading of this manuscript, Drs. Dominique Maciejewski-Lenoir and Greg Naeve for technical help, and Lee Ellingson and Joelle Eggold for expert graphics assistance.

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