ESMO IMMUNO-ONCOLOGY
Annual Congress
GENEVA SWITZERLAND
7-9 DECEMBER 2022

The Journal of Immunology

The Differential Role of Extracellular Signal-Regulated Kinases and p38 Mitogen-Activated Protein Kinase in Eosinophil Functions

This information is current as of August 5, 2022.

Tetsuya Adachi, Barun K. Choudhury, Susan Stafford, Sanjiv Sur and Rafeul Alam

J Immunol 2000; 165:2198-2204; ;
doi: 10.4049/jimmunol.165.4.2198
http://www.jimmunol.org/content/165/4/2198

References This article **cites 57 articles**, 38 of which you can access for free at:
http://www.jimmunol.org/content/165/4/2198.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2000 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on August 5, 2022

The Differential Role of Extracellular Signal-Regulated Kinases and p38 Mitogen-Activated Protein Kinase in Eosinophil Functions[1]

Tetsuya Adachi, Barun K. Choudhury, Susan Stafford, Sanjiv Sur, and Rafeul Alam[2]

The activation of eosinophils by cytokines is a major event in the pathogenesis of allergic diseases. We have investigated the activation of mitogen-activated protein (MAP) kinases and their functional relevance in eosinophil differentiation, survival, degranulation, and cytokine production. IL-5 induced phosphorylation and activation of extracellular signal-regulated kinases (ERK) and p38 MAP kinases in eosinophils. PD98059, a MAP/ERK kinase inhibitor, blocked phosphorylation of ERK1/2 in a dose-dependent manner. SB202190, a p38 inhibitor, blocked p38-dependent phosphorylation of activating transcription factor-2. To study the importance of the MAP kinases on eosinophil differentiation, we cultured mouse bone marrow cells with IL-3 and IL-5 in the presence of the inhibitors. SB202190 dramatically inhibited eosinophil differentiation by 71%. PD98059 was less potent and reduced eosinophil differentiation by 28%. Both inhibitors marginally inhibited eosinophil survival only at the highest doses. Prolonged incubation of eosinophils with IL-5 induced significant eosinophil-derived neurotoxin release. Both PD98059 and SB202190 nearly completely inhibited (87% and 100% inhibition, respectively) IL-5-stimulated eosinophil-derived neurotoxin release in a dose-dependent manner. Next, we examined the effect of the MAP kinase inhibitors on eosinophil production of the cytokine macrophage-inflammatory protein (MIP)-1α. PD98059 blocked C5a- but not ionomycin-induced MIP-1α production (59% inhibition at 50 μM concentration). In contrast, SB202190 nearly completely inhibited (99%) C5a-induced MIP-1α production. Further, it blocked ionomycin-stimulated production by 66%. Our results suggest that both p38 and ERK1/2 MAP kinases play an important role in eosinophil differentiation, cytokine production, and degranulation. The p38 MAP kinase plays a greater role than ERK1/2 in eosinophil differentiation and cytokine production. *The Journal of Immunology,* **2000, 165: 2198–2204.**

The mechanism of asthma is chronic inflammation of airways caused by immune cells such as T cells and eosinophils (1). The activation of eosinophils by cytokines and chemoattractants is a critical event in the allergic inflammatory response of the tissue. IL-5 is the principal regulatory cytokine that modulates eosinophil functions. During an allergic response, it stimulates differentiation of eosinophils from bone marrow cells resulting in blood eosinophilia (2). Blood eosinophils are recruited into tissue by chemoattractants such as C5a, platelet-activating factor, and CC chemokines (3). Once in the tissue, IL-5 prolongs survival of eosinophils by delaying their apoptosis (4). At the site of inflammation, eosinophils release cytotoxic products, including granular proteins and oxygen radicals, leading to epithelial damage (5). We have elucidated major signaling pathways of IL-5 in eosinophils. We have demonstrated the activation of Lyn and Janus kinase 2 (Jak2)[3] tyrosine kinases and the transduction of signals via the mitogen-activated protein (MAP) kinase and STAT pathways (6, 7). For better understanding of the mechanism of eosinophil activation, it is of paramount interest to investigate functional relevance of signaling molecules. Previously, we and others have shown that Lyn and Jak2 are important for the maintenance of eosinophil survival (8–11), whereas Raf-1 is crucial for degranulation and up-regulation of adhesion molecules as well as survival (8).

The MAP kinase cascade is one of the most frequently studied signal transduction systems and is known to participate in multiple cellular functions, such as proliferation, differentiation, survival, and locomotion (12). Five distinct MAP kinase cascades have been described in mammalian cells, including the extracellular-regulated kinase 1 and 2 (ERK1/2), the c-Jun N-terminal kinase (JNK), the p38 MAP kinase, ERK3, and ERK5. The MAP kinases are dual phosphorylated on threonine-tyrosine residues by distinct MAP kinase kinases. ERK1/2 are activated by a variety of growth factors and play a critical role in mitogenesis (13). JNK and p38 are typically activated by cellular stress or proinflammatory cytokines that are known to induce cell death (14–19). Recent studies have demonstrated the activation of JNK and p38 in response to hemopoietic growth factors as well (20–23), suggesting that these molecules are involved in both pro- and anti-apoptotic signals, depending on the stimuli. A few studies have investigated the functional role of MAP kinases in eosinophils using a MAP/ERK kinase (MEK) inhibitor (PD98059) and a p38 inhibitor (SB203580). PD98059 did

Department of Internal Medicine, Division of Allergy and Immunology, University of Texas Medical Branch, Galveston, TX 77555

Received for publication November 18, 1999. Accepted for publication May 30, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the National Institutes of Health (AI35713) and the John Sealy Memorial Foundation of the University of Texas Medical Branch. T.A. is the recipient of the Texas Allergy and Immunology Society Memorial Foundation Research Award and the University of Texas Medical Branch Technology Committee Grant.

[2] Address correspondence and reprint requests to Dr. Rafeul Alam, University of Texas Medical Branch, Department of Internal Medicine, Division of Allergy and Immunology, MRB 8.104, Galveston, TX 77555-1083. E-mail address: ralam@utmb.edu

[3] Abbreviations used in this paper: Jak2, Janus kinase 2; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; EDN, eosinophil-derived neurotoxin; ATF, activating transcription factor; MBP, major basic protein; MIP, macrophage inflammatory protein; MEK, MAP/ERK kinase.

Copyright © 2000 by The American Association of Immunologists 0022-1767/00/$02.00

Downloaded from http://www.jimmunol.org/ by guest on August 5, 2022

not block eosinophil survival induced by GM-CSF, indicating that ERK1/2 are not critical for anti-apoptotic signal (11). Bracke et al. (24) studied rosette formation between IL-5-stimulated eosinophils and IgA- or IgG-coated beads and found that the Ras-ERK pathway regulates FcγRII activation, whereas FcαR is activated by the phosphatidylinositol-3 kinase-p38 pathway. However, the role of MAP kinases in other important functions of eosinophils, such as differentiation or degranulation, remains unknown.

In the present study, we investigated the participation of ERK1/2 and p38 MAP kinase in eosinophil differentiation, survival, degranulation, and cytokine production. The results reveal that they have distinct roles in eosinophil functions. Most strikingly, p38 MAP kinase, but not ERK1/2, is essential for eosinophil differentiation from bone marrow stem cells.

Materials and Methods

Reagents

RPMI 1640, IMDM, and antibiotic-antimycotic were obtained from Life Technologies (Grand Island, NY), and FCS was obtained from Atlanta Biologicals (Norcross, GA). Percoll and RIA kit for eosinophil-derived neurotoxin (EDN) were purchased from Pharmacia (Piscataway, NJ). C5a, cytochalasin B, ionomycin, propidium iodide, Chromotrope 2R, and peroxidase-conjugated anti-mouse IgG Ab were obtained from Sigma (St. Louis, MO). The mouse mAb against phospho-ERK, rabbit polyclonal anti-ERK2, anti-p38 Abs, HRP-conjugated goat anti-rabbit Ab, protein A/G Plus agarose, and activating transcription factor-2 (ATF-2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-phospho-p38 Ab was obtained from New England Biolabs (Beverly, MA). The polyclonal rabbit anti-mouse major basic protein (MBP) Ab was a kind gift from Dr. Gerald J. Gleich (Mayo Clinic, Rochester, MN). The Alexa 488-conjugated goat anti-rabbit IgG Ab was purchased from Molecular Probes (Eugene, OR). The source of SB202190 and PD98059 was Calbiochem (La Jolla, CA). Anti-CD16 immunomagnetic beads were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Human IL-5 and murine IL-3 were purchased from PeproTech (Rocky Hill, NJ). Murine IL-5 and an ELISA kit for macrophage inflammatory protein-1α (MIP-1α) were obtained from R&D Systems (Minneapolis, MN). Enhanced chemiluminescence detection system, Hybond ECL nitrocellulose membrane, and [γ-^{32}P]ATP were obtained from Amersham (Arlington Heights, IL). The source of cold ATP was Pharmacia & Upjohn (Uppsala, Sweden). Gel/Mount was purchased from Biomedia (Foster City, CA).

Eosinophil purification

Peripheral blood was obtained from subjects with mild to moderate eosinophilia (6–12%). Some of the blood donors have allergic rhinitis, whereas others are healthy subjects. Subjects with allergic rhinitis were off all medications 24 h before blood donation. Eosinophils were isolated by sedimentation with 6% hydroxyethyl starch followed by centrifugation on a 1.088 Percoll density gradient according to the method of Hansel et al. (25) The cells were further purified by negative selection using anti-CD16 immunomagnetic beads and the magnetic cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). Eosinophils ($>$98% purity and $>$95% viability) were then suspended in RPMI 1640 in tubes coated with 3% HSA. Eosinophils from individual donors were used separately for various experiments. In experiments using PD98059 or SB202190, eosinophils were treated with the inhibitor dissolved in DMSO. The highest final concentration of DMSO was 0.1%. A short-term incubation (30 min) of eosinophils with this concentration of DMSO and the inhibitors did not affect eosinophil survival.

Preparation of cytosolic extracts and immunoprecipitation

Purified eosinophils (1–2 $\times$ 10^6 cells) were incubated with and without either PD98059 or SB202190 for 30 min at 37°C followed by stimulation with 10 ng/ml of human IL-5 for 5 min. The reaction was terminated by addition of 9 volumes of ice-cold HBSS containing 1 mM Na_3VO_4. The cells were pelleted by centrifugation and lysed in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% Triton X-100, 10% glycerol, 1 μg/ml of aprotinin, leupeptin, and pepstatin). After 20 min on ice, detergent-insoluble materials were removed by centrifugation at 4°C at 12,000 $\times$ *g*. The protein concentration was determined using bicinchoninic acid assay (Pierce Chemical, Rockford, IL).

For immunoprecipitation, the cell lysates were prepared using the above-described lysis buffer without glycerol. After preclearing with 20 μl of the protein A/G Plus agarose for 30 min, the lysates were incubated with the appropriate Ab (1–2 μg for each sample) for 1 h followed by the incubation with 20 μl of protein A/G Plus agarose for 2 h at 4°C. The beads were washed three times with the cold lysis buffer. The whole cell lysates or the immunoprecipitates were boiled in 2$\times$ Laemmli reducing buffer for 4 min.

Immunocomplex kinase assay

The immunoprecipitates were assayed for p38 kinase by measuring the phosphotransferase activity for ATF-2. The kinase reaction was performed by incubating the immunoprecipitates in 40 μl of kinase buffer (10 mM HEPES, 50 mM NaCl, 10 mM $MgCl_2$, 100 μM Na_3VO_4, 500 μM dithiothreitol, 25 mM β-glycerophosphate) containing 2.5 μM ATP, 10 μCi [γ-^{32}P]ATP, and 12.5 μg/ml ATF-2 for 30 min at 30°C. After centrifugation, the reaction was stopped by boiling the supernatant with equal amount of 2$\times$ Laemmli buffer. The kinase reaction products were then applied to SDS-PAGE and autoradiography.

Gel electrophoresis and Western blotting

SDS-polyacrylamide gels were prepared according to the Laemmli protocol and used for Western blotting. The concentration of polyacrylamide was 8–10% depending on the m.w. of the protein of interest. Gels were blotted onto Hybond membranes for Western blotting using the enhanced chemiluminescence system. Blots were incubated in a blocking buffer containing 10% BSA in TBST buffer (20 mM Tris-base, 137 mM NaCl, pH 7.6, 0.05% Tween 20) for 1 h followed by incubation in the primary Ab (0.1 μg/ml) for 1–2 h. After washing three times in TBST buffer, blots were incubated for 30 min with a HRP-conjugated secondary Ab (0.04 μg/ml) directed against the primary Ab. The blots were developed with the enhanced chemiluminescence substrate according to the manufacturer's instruction. In some experiments, blots were reprobed with another Ab after stripping in a buffer of 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-ME, and 2% SDS at 50°C for 30 min.

Murine bone marrow cell culture

In vitro liquid culture was performed as described elsewhere with modifications (26). OVA-sensitized BALB/c mice were sacrificed, and the femurs were removed. The bone marrow cavity was flushed with saline to obtain cells. The bone marrow cells (5 $\times$ 10^5 cells/ml) were suspended in IMDM. These cells were incubated with and without the inhibitors for 30 min at 37°C followed by further culture in the presence of 1 ng/ml of murine IL-3 and 6 ng/ml of murine IL-5 plus 10% FCS for 1 wk. After harvesting, the total cell count was obtained, and the remaining cells were used for cytospin preparations. These preparations were stained with Wright–Giemsa stain for counting the number of eosinophils.

Immunocytochemical staining

The immunocytochemical staining for intracellular MBP was performed according to previously described methods with modifications (27). Cultured murine bone marrow cells were dropped on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) and dried at room temperature for 2 h. The slides were fixed in ice-cold acetone for 15 min at 4°C and washed three times in PBS. Normal goat serum (10%) was applied to the slides for 30 min to block nonspecific binding. Polyclonal rabbit anti-mouse MBP Ab diluted in PBS (1:125) was added to the slides and incubated in the Shandon Coverplate System (Shandon, Pittsburgh, PA) for 45 min at room temperature. Then, the slides were washed three times in PBS. To reduce background caused by the secondary Ab, the slides were soaked in 1% Chromotrope 2R for 30 min at room temperature followed by washing with PBS three times. Alexa 488-conjugated goat anti-rabbit IgG Ab (20 μg/ml) was added to the slides and incubated for 1 h at 37°C in a dark chamber. Following three washes with PBS, the slides were coverslipped with Gel/Mount to prevent the fluorescence from fading. Slides were photographed with a fluorescence microscope (Nikon, Tokyo, Japan).

Eosinophil survival assay

Purified eosinophils (5 $\times$ 10^5 cells/ml) were suspended in RPMI 1640 with 10% FCS and treated with or without the inhibitors for 30 min at 37°C. After the incubation, the cells were cultured with 1 ng/ml human IL-5 for 3 days. The viability of the cultured eosinophils was assessed by counting propidium iodide-stained dead cells.

EDN release

A 96-well plate was coated with 3% HSA in HBSS for 2 h at 37°C and washed three times with HBSS before use. Purified eosinophils (5 $\times$ 10^5

Downloaded from http://www.jimmunol.org/ by guest on August 5, 2022

cells/ml) were suspended in RPMI 1640 with 0.1% HSA. The cells were preincubated with or without the inhibitors for 30 min at 37°C followed by the stimulation with 100 ng/ml of human IL-5 for 4 h. The supernatants were separated by centrifugation, and the concentration of EDN was measured by RIA.

MIP-1α production

Purified eosinophils (10^6 cells/ml) were suspended in RPMI 1640 with 10% FCS. After the treatment with or without the inhibitors for 30 min at 37°C, eosinophils were stimulated with either 100 nM C5a with 5 μg/ml cytochalasin B or 1 μM ionomycin for 24 h. The supernatants were separated by centrifugation, and MIP-1α concentration was measured by ELISA.

Statistical analyses

Results were expressed as mean ± SD. Data were analyzed for statistical significance using ANOVA and Student's *t* test.

Results

Effect of PD98059 on the activation of ERK1/2 by IL-5

IL-5 has been shown to stimulate ERK1/2 in eosinophils (6). PD98059 is a selective inhibitor of MEK1 (IC_{50} = 2–7 μM) and MEK2 (IC_{50} = 50 μM), which blocks phosphorylation and activation of ERK1/2 (28, 29). To test its effect on ERK1/2 phosphorylation, eosinophils were preincubated with or without various concentrations of PD98059 followed by stimulation with IL-5. The cytosolic extracts were subjected to Western blotting with anti-phospho-ERK Ab. As shown in Fig. 1, IL-5 increased the phosphorylation of both ERK1 (*upper band*) and ERK2 (*lower band*). Particularly, the band of phosphorylated ERK2 showed motility shift. PD98059 inhibited ERK1/2 phosphorylation in a dose-dependent manner. Reprobing the membrane with the anti-ERK2 Ab revealed that same amount of protein was loaded on the gel.

Effect of SB202190 on the activation of p38 by IL-5

We also examined the phosphorylation of p38 MAP kinase in IL-5-stimulated eosinophils. Western blotting with the anti-phospho-p38 Ab showed p38 phosphorylation by IL-5 as reported elsewhere (Fig. 2*A*) (24). SB202190, an analogous compound of SB203580, is a specific p38 inhibitor that blocks the kinase activity of p38 (IC_{50} = 0.28 μM) (30, 31). For this reason, we tested the effect of SB202190 on p38 activity using in vitro kinase assay. The eosinophil lysates were immunoprecipitated with an anti-p38 Ab followed by the immunocomplex kinase assay using ATF-2 as the substrate. As shown in Fig. 2*B*, IL-5 stimulated p38 kinase activity, which was blocked by the pretreatment of eosinophils with SB202190. The Western blotting of the same immunoprecipitates with the anti-p38 Ab showed equal amounts of the protein.

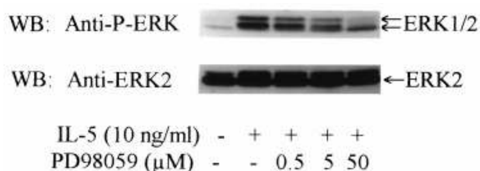


FIGURE 1. The effect of PD98059 on IL-5-induced threonine/tyrosine phosphorylation of ERK1/2. Eosinophils were incubated in the presence or absence of PD98059 for 30 min and stimulated with or without IL-5 for 5 min. The lysates of the cells were subjected to electrophoresis and Western blotting with anti-phospho-ERK1/2 Ab. ERK1/2 activation after IL-5 stimulation was significantly inhibited by PD98059. Reprobing the membrane with the anti-ERK2 Ab revealed that same amount of protein was loaded on the gel.

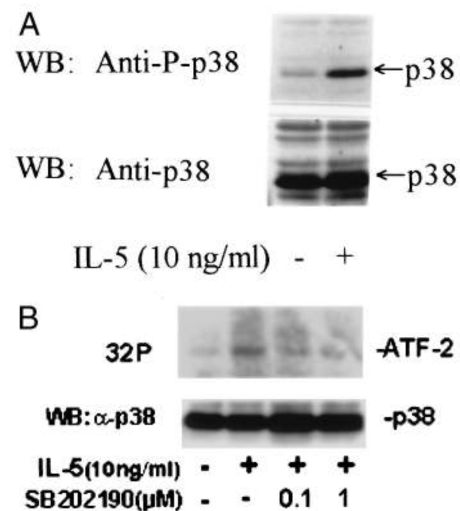


FIGURE 2. IL-5 stimulated p38 activation in eosinophils. *A*, Threonine/tyrosine phosphorylation of p38 MAP kinase in eosinophils stimulated with IL-5. Eosinophils were lysed followed by electrophoresis and Western blotting with anti-phospho-p38 Ab. IL-5 stimulated p38 activation in eosinophils. Reprobing the membrane with the anti-p38 Ab revealed that same amount of protein was loaded on the gel. *B*, The effect of SB202190 on IL-5-induced kinase activity of p38 MAP kinase. Eosinophils were incubated in the presence or absence of SB202190 for 30 min and stimulated with or without IL-5 for 5 min. The lysates of the cells were immunoprecipitated with anti-p38 Ab. The immunocomplex was subjected to in vitro kinase assay using ATF-2 as the substrate followed by electrophoresis and autoradiography. The p38 activity after IL-5 stimulation was significantly inhibited by SB202190. Western blotting with anti-p38 Ab using the same immunoprecipitates revealed that each sample had same the amount of protein.

Eosinophil differentiation from bone marrow cells

The differentiation of eosinophils from stem cells occurs stepwise. Lineage-committed stem cells initially require IL-3 for their proliferation. Subsequent stimulation with IL-5 leads to the differentiation of eosinophils (2). Previous studies have shown that a combination of IL-3 and IL-5 stimulates eosinopoiesis in vitro (26). We have used an in vitro liquid culture system using bone marrow cells from mouse according to the protocol described previously (32). In this protocol, mice receive two i.p. injections of OVA in alum 2 wk apart. One week later, mice are exposed to OVA aerosol 1 h daily for 5 days a week for two consecutive weeks. The mice are sacrificed 1–2 wk later. This protocol of allergic sensitization of mice significantly increases the sensitivity of bone marrow stem cells to IL-5. The percent of in vitro-differentiated eosinophils increases from about 10% in nonsensitized mice to 30% in sensitized mice. We studied the functional role of MAP kinases in eosinophil differentiation using bone marrow cells from sensitized mice. Murine bone marrow cells were incubated with IL-3 and IL-5 for 1 wk followed by cytospin preparations for Wright–Giemsa staining. Murine eosinophils were recognized by the typical circular nucleus and the presence of eosinophilic granules. The total cell and eosinophil counts after 1 wk were 46 ± 6 and 14 ± 2 × 10^4 cells, respectively (n = 3). Approximately 32% of the total cells were of eosinophilic lineage (Fig. 3*A*). The differentiation of eosinophils was confirmed using immunocytochemistry with an anti-MBP Ab (Fig. 3*B*). Next, we studied the effect of PD98059 and SB202190 on eosinophil differentiation. The number of eosinophils as well as total cells was partially reduced by the highest concentration (50 μM) of PD98059 (Fig. 4*A*). In contrast, SB202190 potently inhibited eosinophil differentiation (Fig. 4*B*), which was reduced by 71%. Interestingly, the total cell count was

Downloaded from http://www.jimmunol.org/ by guest on August 5, 2022

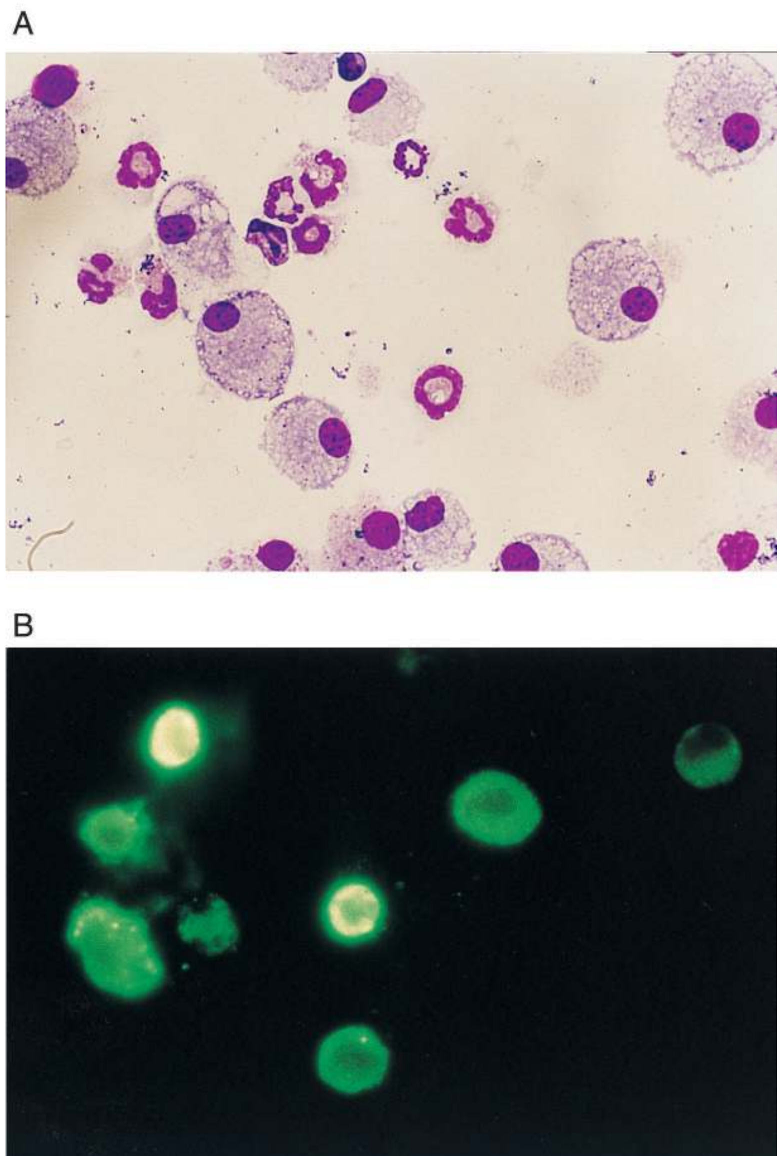


FIGURE 3. Differentiation of eosinophils from bone marrow stem cells. Mouse bone marrow cells from immunized animals were cultured in the presence of IL-3 and IL-5 for 7 days. Cytocentrifuge preparations of the cells were then processed with Wright–Giemsa stain (*A*) or immunocytochemically with an anti-MBP Ab (*B*). *A* shows multiple cells with circular nucleus and pink granules located both inside and outside the circle. There are a few cells of monocytic and neutrophilic lineage. *B* shows two brightly fluorescent MBP-containing eosinophils. A few faintly stained other cells are also present.

Downloaded from http://www.jimmunol.org/ by guest on August 5, 2022

actually increased, due to the proliferation of monocytes (data not shown). Similar results were obtained with another inhibitor of p38, SB203580. Eosinophil differentiation was 23 ± 1.5% in control cultures. This differentiation was reduced to 16 ± 3.3%, 11.6 ± 2.1%, and 11.3 ± 1.8% in the presence of 1, 5, and 10 μM concentration of SB203580. The difference at 5 and 10 μM concentrations of the inhibitor was statistically significant ($p < 0.006$, Student's *t* test). These results suggest that p38 has a predominant role in eosinophil differentiation from stem cells.

Eosinophil survival and degranulation

Next, we investigated the role of MAP kinases in the maintenance of eosinophil survival. As shown in Fig. 5, most of the eosinophils underwent apoptosis after 3 days without IL-5 (9% viable cells). In contrast, the eosinophil viability was prolonged to 89 ± 2% after stimulation with IL-5 ($n = 3$). Both PD98059 (50 μM) and SB202190 (10 μM) have only marginal effects on IL-5-stimulated eosinophil survival at the highest concentrations (Fig. 5, *A* and *B*), indicating that MAP kinases do not play an important role in eosinophil survival. We also studied the effect of the inhibitors on eosinophil degranulation. IL-5 itself induces degranulation at high concentrations, whereas it causes priming of cells at low concentrations (5, 33). The EDN release without and with IL-5 was 214 ± 65 and 454 ± 80 ng/10^6 cells, respectively ($n = 3$). Both PD98059 and SB202190 significantly inhibited IL-5-stimulated EDN release from eosinophils (Fig. 6, *A* and *B*). Taken together, both ERK1/2 and p38 MAP kinase are important for eosinophil degranulation but not for survival.

MIP-1α production in eosinophils

Eosinophils appear to amplify allergic inflammation in the tissue by producing cytokines. We investigated the role of MAP kinases in eosinophil cytokine production. Our preliminary studies indicated that IL-5 was not a potent stimulant for this purpose. Previously, C5a in combination with cytochalasin B was shown to elicit cytokine release from eosinophils (34). Ionomycin was also employed as a nonphysiologic stimulus because of its potent activity on cytokine production (35, 36). We and others have reported the production of MIP-1α by ionomycin-stimulated eosinophils and basophils (37, 38). For this reason, we examined the role of MAP kinases in eosinophil MIP-1α production. In neutrophils, ERK1/2 and p38 are known to be activated by C5a (39–41) or ionomycin (42). Consistent with those results, 0.1 μM C5a with 5 μg/ml cytochalasin B or 1 μM ionomycin stimulated MIP-1α production from eosinophils (Fig. 7). The amount of MIP-1α without stimuli, with C5a plus cytochalasin B, and with ionomycin

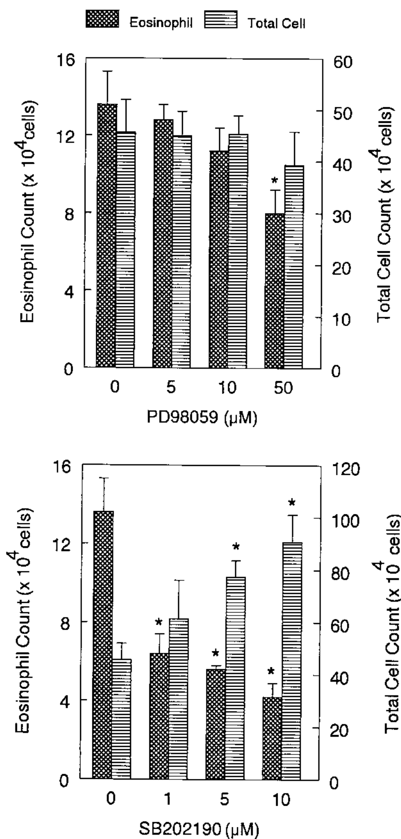


FIGURE 4. The effect of PD98059 and SB202190 on eosinophil differentiation of murine bone marrow cells. The cells were incubated with the inhibitors or medium containing DMSO (vehicle) for 30 min, and then IL-3 and IL-5 were added. The cells were harvested after 1 wk, and the total cell count was obtained. After the cytospin preparation, the number of eosinophils was counted using Wright–Giemsa stain. Data are expressed as means ± SD ($n = 3$). PD98059 partially reduced eosinophil differentiation at the highest concentration. SB202190 dramatically inhibited eosinophil but stimulated monocyte differentiation. *, $p < 0.05$ vs medium (ANOVA).

were 71 ± 70, 998 ± 275, and 4340 ± 1297 pg/10^6 cells, respectively ($n = 3$). We examined the effect of C5a and cytochalasin B on ERK1/2 phosphorylation in eosinophils. C5a induced significant phosphorylation of ERK1/2, whereas cytochalasin B had no effects (Fig. 8). There was a modest increase in phosphorylation when the two agents were combined. PD98059 significantly inhibited MIP-1α production from eosinophils stimulated with C5a/cytochalasin B, but not with ionomycin (Fig. 7*A*). In contrast, both C5a- and ionomycin-induced MIP-1α release were nearly completely blocked by SB202190 (Fig. 7*B*). These results indicate that the utilization of the ERK1/2 or p38 pathway for MIP-1α production depends upon the nature of the stimuli. Further, the p38 MAP kinase plays a greater role in eosinophil MIP-1α production.

Discussion

In this study, we have examined the functional relevance of two species of MAP kinases, i.e., ERK1/2 and p38, in eosinophil signaling. We have demonstrated that ERK1/2 is important for eosinophil degranulation and cytokine production, whereas p38 is critical for eosinophil differentiation, degranulation, and cytokine production. More importantly, the p38 MAP kinase appears to play a greater role in regulating eosinophil function than ERK1/2. Neither of the MAP kinases is necessary for the maintenance of eosinophil survival. This is the first report on the essential role of MAP kinases, especially p38, in eosinophil differentiation.

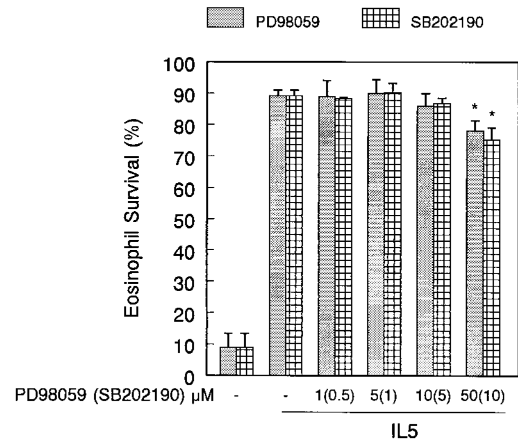
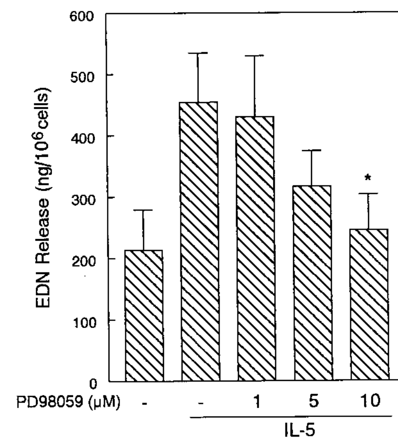


FIGURE 5. The effect of PD98059 and SB202190 on IL-5-stimulated eosinophil survival. Eosinophils were incubated with the inhibitors or medium containing DMSO (vehicle) for 30 min. The cells were further cultured with or without IL-5 for 72 h, and then the viability of eosinophils was assessed by propidium iodide staining. Data are expressed as means ± SD ($n = 3$). Both PD98059 and SB202190 had a small but significant effect at the highest concentration. *, $p < 0.05$ vs medium (ANOVA).



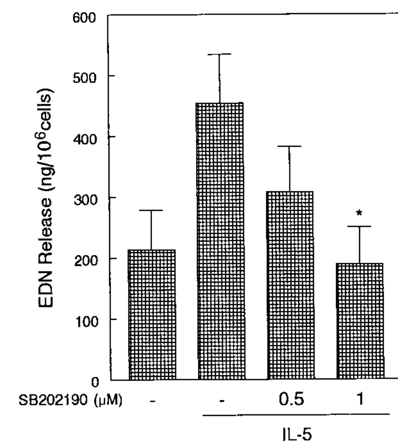


FIGURE 6. The effect of PD98059 and SB202190 on EDN release. Eosinophils were incubated with the inhibitors or medium containing DMSO (vehicle) for 30 min. Then cells were stimulated with or without IL-5 for 4 h, and the supernatants were obtained. EDN concentration in the supernatant was measured by RIA. Data are expressed as means ± SD ($n = 3$). Both PD98059 and SB202190 significantly affected EDN release from eosinophils. *, $p < 0.05$ vs medium (ANOVA).

Downloaded from http://www.jimmunol.org/ by guest on August 5, 2022

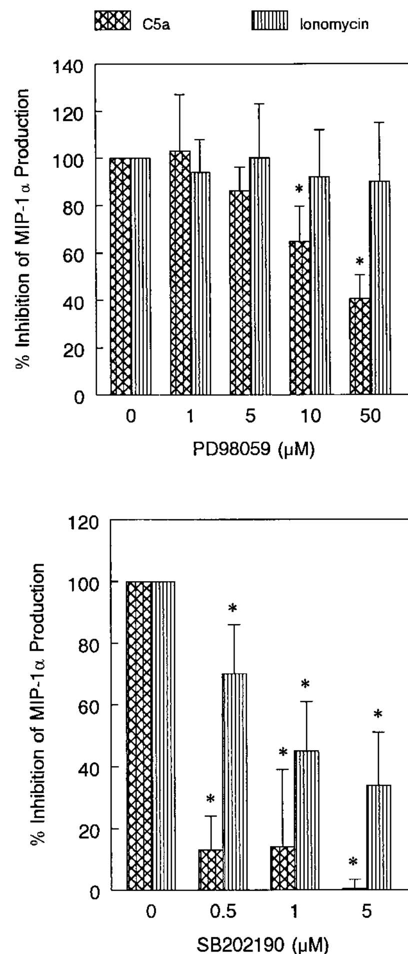


FIGURE 7. The effect of PD98059 and SB202190 on MIP-1α production from eosinophils. Cells were incubated with the inhibitors or medium containing DMSO (vehicle) for 30 min followed by the stimulation either with C5a plus cytochalasin B, or with ionomycin for an additional 24 h. MIP-1α concentration in the cultured supernatant was measured by ELISA. Data are expressed as means $\pm$ SD ($n = 3$). PD98059 significantly inhibited MIP-1α production from eosinophils that were stimulated with C5a, but not with ionomycin. Both C5a- and ionomycin-induced MIP-1α release was significantly abrogated by SB202190. $*$, $p < 0.05$ vs medium (ANOVA).

The involvement of MAP kinase pathway in both cell proliferation and differentiation has initially been shown in nonhemopoietic cells (43). Nerve growth factor treatment of PC12 cells induces sustained ERK activation associated with neurite outgrowth and cessation of cell division, whereas the treatment of PC12 cells with epidermal growth factor induces transient ERK activation and cell proliferation. Several groups have subsequently studied the role of MAP kinase in differentiation of hemopoietic cells. The treatment of K562 cells with phorbol esters or CMK cells with stem cell factor induces differentiation of megakaryocytes (44, 45). In these cells, the importance of MAP kinase for magakaryocytic differentiation has been shown by expression of constitutively active MEK or using PD98059. Recently, Nagata et al. (46) have found that JNK and p38, but not ERK1/2, play a crucial role in erythropoietin-induced erythroid differentiation. These findings are, in part, consistent with our results of IL-5-induced eosinophil differentiation. The receptors for both erythropoietin and IL-5 associate with Jak2 and Lyn (47, 48) and transduce signals through distinct MAP kinase cascades (20–23, 49). p38 is responsible for the phosphorylation and activation of several transcription factors, including ATF-2, Max, and CHOP, which are not substrates for ERK1/2 (50–52). Therefore, these transcription factors may have a critical role in differentiation of eosinophils or erythroid cells, although the exact mechanism remains to be elucidated.

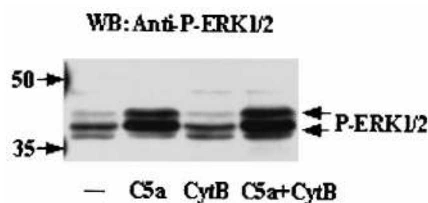


FIGURE 8. The effect of C5a and cytochalasin B on ERK1/2. Purified eosinophils were incubated with buffer (−), 100 nM C5a, 5 μg/ml cytochalasin B (CytB), or the combination of C5a (100 nM) and cytochalasin B (5 μg/ml) for 3 min and then lysed and Western blotted with the anti-phospho-ERK1/2 Ab. The blot shows that C5a but not cytochalasin B induces phosphorylation of ERK1/2 in eosinophils. However, cytochalasin B modestly augments C5a-induced ERK1/2 phosphorylation.

The Ras-ERK pathway is involved in the prevention of cell death in certain factor-dependent cell types. In this study, we show that ERK1/2 is not important for the maintenance of eosinophil survival. This is consistent with other recent publications (11). Interestingly, Raf-1 is critical for preventing eosinophils from apoptosis (8). The mechanism of inhibition of apoptosis can be explained by the divergence of the downstream signaling pathway of Raf-1 from the MAP kinase pathway. Bcl-2, an anti-apoptotic protein, through its ability to associate with Raf-1, translocates this kinase from the cytosol to the mitochondrial membrane (53). Once there, Raf-1 interacts with regulatory proteins of the mitochondrial membrane and contributes to the prevention of apoptosis (54). However, the exact mechanism of action of Raf-1 in this process remains unclear.

C5a and fMLP are potent chemoattractants for granulocytes. A recent study has shown that C5a and fMLP promote eosinophil IL-8 production in the presence of cytochalasin B (34). In agreement with the foregoing, we have shown that C5a stimulates eosinophil production of MIP-1α. Both PD98059 and SB202190 inhibited C5a-induced MIP-1α production. Interestingly, only SB202190 but not PD98059 blocked ionomycin-stimulated MIP-1α production by eosinophils. The results suggest that the ionomycin-stimulated signaling pathway mainly involves the p38 MAP kinase. Previously, MAP kinases have been shown to variably affect the production of cytokines by other cells. While PD98059 did not inhibit IgE-mediated TNF-α production from MC/9 murine mast cells, it did so from the rat basophilic leukemia cell line RBL-2H3 (55, 56). In another study, SB203580 attenuated IL-8 production from TNF-α-stimulated neutrophils (42).

In conclusion, we have defined an essential role of ERK1/2 and p38 MAP kinases in specific functions of eosinophils. It must be emphasized that we have used mostly IL-5 for stimulation of eosinophils. Therefore, our conclusions may not applicable to other activators. Nonetheless, IL-5 is the most important regulator of eosinophilopoiesis and p38 MAP kinase appears to critically regulate this process. Excessive production of eosinophils and their subsequent influx into the airways and other target organs are characteristic features of asthma and allergic diseases. In this regard, we have recently shown that both p38 and ERK1/2 play an important role in eosinophil chemotaxis in response to eotaxin (57). Based upon these findings, we believe that inhibitors of MAP kinases will be useful for treatment of allergic inflammation. Indeed, while this manuscript was under review, it was reported that SB 239063, a new inhibitor of p38 MAP kinase, reduced inflammatory cytokine production and eosinophilic inflammation in the airways in vivo in a mouse model of asthma (58).

Downloaded from http://www.jimmunol.org/ by guest on August 5, 2022

References

1. Gleich, G. J. 1990. The eosinophil and bronchial asthma: current understanding. *J. Allergy Clin. Immunol. 85:422.*
2. Yamaguchi, Y., T. Suda, J. Suda, M. Eguchi, Y. Miura, N. Harada, A. Tominaga, and K. Takatsu. 1988. Purified interleukin-5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J. Exp. Med. 167:43.*
3. Kita, H., C. R. Adolphson, and G. J. Gleich. 1998. Biology of eosinophils. In *Allergy, Principles & Practice*, 5th Ed. E. Middleton, C. E. Reed, E. F. Ellis, N. F. Adkinson, J. W. Yunginger, and W. W. Busse, eds. Mosby, St. Louis, p. 242.
4. Yamaguchi, Y., T. Sudo, S. Ohta, K. Tominaga, Y. Miura, and T. Kasahara. 1991. Analysis of the survival of mature eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils. *Blood 78:2542.*
5. Kita, H., D. A. Weiler, R. I. Abu-Ghazaleh, C. J. Sanderson, and G. J. Gleich. 1992. Release of granule proteins from eosinophils cultured with IL-5. *J. Immunol. 149:629.*
6. Pazdrak, K., D. Schreiber, P. Forsythe, L. Justement, and R. Alam. 1995. The signal transduction mechanism of IL-5 in eosinophils: the involvement of lyn tyrosine kinase and the ras-raf 1-MEK-MAP kinase pathway. *J. Exp. Med. 181:1827.*
7. Pazdrak, K., S. Stafford, and R. Alam. 1995. The activation of the Jak-STAT1 signaling pathway by IL-5 in eosinophils. *J. Immunol. 155:397.*
8. Pazdrak, K., B. Olszewska-Pazdrak, S. Stafford, R. Garofalo, and R. Alam. 1998. Lyn, Jak2, and Raf-1 kinases are critical for the anti-apoptotic effect of interleukin 5, whereas only Raf-1 kinase is essential for eosinophil activation and degranulation. *J. Exp. Med. 188:421.*
9. Yousefi, S., D. C. Hoessli, K. Blaser, G. B. Mills, and H. U. Simon. 1996. Requirement of Lyn and Syk tyrosine kinases for the prevention of apoptosis by cytokine in human eosinophils. *J. Exp. Med. 183:1407.*
10. Simon, H. U., S. Yousefi, B. Dibbert, F. Levi-Schaffer, and K. Blaser. 1997. Anti-apoptotic signals of granulocyte-macrophage colony-stimulating factor are transduced via Jak2 tyrosine kinase in eosinophils. *Eur. J. Immunol. 27:3536.*
11. Miike, S., A. Nakao, M. Hiraguri, K. Kurasawa, Y. Saito, and I. Iwamoto. 1999. Involvement of JAK2, but not PI 3-kinase/Akt and MAP kinase pathways, in anti-apoptotic signals of GM-CSF in human eosinophils. *J. Leukocyte Biol. 65:700.*
12. Lewis, T. S., P. S. Shapiro, and N. G. Ahn. 1998. Signal transduction through MAP kinase cascades. *Adv. Cancer Res. 74:49.*
13. Robinson, M. J., and M. H. Cobb. 1997. Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol. 9:180.*
14. Derijard, B., M. Hibi, I. H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R. J. Davis. 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell 76:1025.*
15. Kyriakis, J. M., P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett. 1994. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature 369:156.*
16. Slutt, H. K., T. Barrett, B. Derijard, and R. J. Davis. 1994. Signal transduction by tumor necrosis factor mediated by JNK protein kinases. *Mol. Cell. Biol. 14:8376.*
17. Han, J., J. D. Lee, L. Bibbs, and R. J. Ulevitch. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science 265:808.*
18. Rouse, J., P. Cohen, S. Trigon, M. Morange, A. Alanso-Llamazares, D. Zamanillo, T. Hunt, and A. R. Nebreda. 1994. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell 78:1027.*
19. Raingeaud, J., S. Gupta, J. S. Rogers, M. Dickens, J. Han, R. J. Ulevitch, and R. J. Davis. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem. 270:7420.*
20. Nagata, Y., E. Nishida, and K. Todokoro. 1997. Activation of JNK signaling pathway by erythropoietin, thrombopoietin, and interleukin-3. *Blood 89:2664.*
21. Foltz, I. N., and J. W. Schrader. 1997. Activation of the stress-activated protein kinases by multiple hematopoietic growth factors with the exception of interleukin-4. *Blood 89:3092.*
22. Nagata, Y., T. Moriguchi, E. Nishida, and K. Todokoro. 1997. Activation of p38 MAP kinase pathway by erythropoietin and interleukin-3. *Blood 90:929.*
23. Foltz, I. N., J. C. Lee, P. R. Young, and J. W. Schrader. 1997. Hematopoietic growth factors with the exception of interleukin-4 activate the p38 mitogen-activated protein kinase pathway. *J. Biol. Chem. 272:3296.*
24. Bracke, M., P. J. Coffer, J. W. Lammer, and L. Koenderman. 1998. Analysis of signal transduction pathways regulating cytokine-mediated Fc receptor activation on human eosinophils. *J. Immunol. 161:6768.*
25. Hansel, T. T., I. J. M. de Vries, T. Iff, S. Rihs, M. Wandzilak, S. Betz, K. Blaser, and C. Walker. 1991. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J. Immunol. Methods 145:105.*
26. Takamoto, M., and K. Sugane. 1995. Synergism of IL-3, IL-5, and GM-CSF on eosinophil differentiation and its application for an assay of murine IL-5 as an eosinophil differentiation factor. *Immunol. Lett. 45:43.*
27. Sur, S., D. G. Glitz, H. Kita, S. M. Kujawa, E. A. Peterson, D. A. Weiler, G. M. Kephart, J. M. Wagner, T. J. George, G. J. Gleich, and K. M. Leiferman. 1998. Localization of eosinophil-derived neurotoxin and eosinophil cationic protein in neutrophilic leukocytes. *J. Leukocyte Biol. 63:715.*
28. Dudley, D. T., L. Pang, S. J. Decker, A. J. Bridges, and A. R. Saltiel. 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA 92:7686.*
29. Alessi, D. R., A. Cuenda, P. Cohen, D. T. Dudley, and A. R. Saltiel. 1995. PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J. Biol. Chem. 270:27489.*
30. Lee, J.C., J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, D. Green, D. McNulty, M. J. Blumenthal, J. R. Heys, S. W. Landvatter, et al. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature 372: 739.*
31. Jiang, Y., C. Chen, Z. Li, W. Guo, J. A. Gegner, S. Lin, and J. Han. 1996. Characterization of the structure and function of a new mitogen-activated protein kinase (p38β). *J. Biol. Chem. 271:17920.*
32. Adachi, T., S. Stafford, S. Sur, and R. Alam. 1999. A novel Lyn-binding peptide inhibitor blocks eosinophil differentiation, survival, and airway eosinophilic inflammation. *J. Immunol. 163:939.*
33. Horie, S., G. J. Gleich, and H. Kita. 1996. Cytokines directly induce degranulation and superoxide production from human eosinophils. *J. Allergy Clin. Immunol. 98:371.*
34. Miyamasu, M., K. Hirai, Y. Takahashi, M. Iida, M. Yamaguchi, T. Koshino, T. Takaishi, Y. Morita, K. Ohta, T. Kasahara, and K. Ito. 1995 Chemotactic agonists induce cytokine generation in eosinophils. *J. Immunol. 154:1339.*
35. Kita, H., T. Ohnishi, Y. Okubo, D. Weiler, J. S. Abrams, and G. J. Gleich. 1991. Granulocyte/macrophage colony-stimulating factor and interleukin 3 release from human peripheral blood eosinophils and neutrophils. *J. Exp. Med. 174:745.*
36. Moqbel, R., Q. Hamid, S. Ying, J. Barkans, A. Hartnell, A. Tsicopoulos, A. J. Wardlaw, and A. B. Kay. 1991. Expression of mRNA and immunoreactivity for the granulocyte/macrophage colony-stimulating factor in activated human eosinophils. *J. Exp. Med. 174:749.*
37. Li, H., T. Sim, J. A. Grant, and R. Alam. 1996. The production of MIP-1α by human basophils. *J. Immunol. 157:1207.*
38. Costa, J. J., K. Matossian, M. B. Resnick, W. J. Beil, D. T. W. Wong, J. R. Gordon, A. M. Dvorak, P. F. Weller, and S. J. Galli. 1993. Human eosinophils can express the cytokines tumor necrosis factor α and macrophage inflammatory protein-1α. *J. Clin. Invest. 91:2673.*
39. Thompson, H. L., C. J. Marshall, and J. Saklatvala. 1994. Characterization of two different forms of mitogen-activated protein kinase kinase induced in polymorphonuclear leukocytes following stimulation by *N*-formylmethionyl-leucyl-phenylalanine or granulocyte-macrophage colony-stimulating factor. *J. Biol. Chem. 269:9486.*
40. Knall, C., S. Young, J. A. Nick, A. M. Buhl, G. S. Worthen, and G. L. Johnson. 1996. Interleukin-8 regulation of the Ras/Raf/mitogen-activated protein kinase pathway in human neutrophils. *J. Biol. Chem. 271:2832.*
41. Huang, R., J. P. Lian, D. Robinson, and J. A. Badwey. 1998. Neutrophils stimulated with a variety of chemoattractants exhibit rapid activation of p21-activated kinases (Paks): separate signals are required for activation and inactivation of Paks. *Mol. Cell. Biol. 18:7130.*
42. Zu, Y. L., J. Qi, A. Gilchrist, G. A. Fernandez, D. Vazquez-Abad, D. L. Kreutzer, C. K. Huang, and R. I. Sha'afi. 1998. p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-α or FMLP stimulation. *J. Immunol. 160:1982.*
43. Marshall, C. J. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell 80:179.*
44. Whalen, A. M., S. C. Galasinski, P. S. Shapiro, T. S. Nahreini, and N. G. Ahn. 1997. Megakaryocytic differentiation induced by constitutive activation of mitogen-activated protein kinase kinase. *Mol. Cell. Biol. 17:1947.*
45. Melemed, A. S., J. W. Ryder, and T. A. Vik. 1997. Activation of the mitogen-activated protein kinase pathway is involved in and sufficient for megakaryocytic differentiation of CMK cells. *Blood 90:3462.*
46. Nagata, Y., N. Takahashi, R. J. Davis, and K. Todokoro. 1998. Activation of p38 MAP kinase and JNK but not ERK is required for erythropoietin-induced erythroid differentiation. *Blood 92:1859.*
47. Witthuhn, B. A., F. W. Quelle, O. Silvennoinen, T. Yi, B. Tang, O. Miura, and J. N. Ihle. 1993. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell 74:227.*
48. Chin, H., A. Arai, H. Wakao, R. Kamiyama, N. Miyasaka, and O. Miura. 1998. Lyn physically associates with the erythropoietin receptor and may play a role in activation of the Stat5 pathway. *Blood 91:3734.*
49. Todokoro, K., M. Sugiyama, E. Nishida, and K. Nakaya. 1994. Activation of mitogen-activated protein kinase cascade through erythropoietin receptor. *Biochem. Biophys. Res. Commun. 203:1912.*
50. Derijard, B., J. Raingeaud, T. Barrett, I. H. Wu, J. Han, R. J. Ulevitch, and R. J. Davis. 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science 267:682.*
51. Zervos, A. S., L. Faccio, J. P. Gatto, J. M. Kyriakis, and R. Brent. 1995. Mxi2, a mitogen-activated protein kinase that recognizes and phosphorylates Max protein. *Proc. Natl. Acad. Sci. USA 92:10531.*
52. Wang, X. Z., and D. Ron. 1996. Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP kinase. *Science 272:1347.*
53. Wang, H. G., U. R. Rapp, and J. C. Reed. 1996. Bcl-2 targets the protein kinase Raf-1 to mitochondria. *Cell 87:629.*
54. Gajewski, T. F., and C. B. Thompson. 1996. Apoptosis meets signal transduction: elimination of a BAD influence. *Cell 87:589.*
55. Ishizuka, T., N. Terada, P. Gerwins, E. Hamelmann, A. Oshiba, G. R. Fanger, G. L. Johnson, and E. R. Gelfand. 1997. Mast cell tumor necrosis factor α production is regulated by MEK kinases. *Proc. Natl. Acad. Sci. USA 94:6358.*
56. Zhang, C., R. A. Baumgartner, K. Yamada, and M. A. Beaven. 1997. Mitogen-activated protein (MAP) kinase regulates production of tumor necrosis factor-α and release of arachidonic acid in mast cells. *J. Biol. Chem. 272:13397.*
57. Kampen, G., S. Stafford, T. Adachi, T. Jinquan, S. Quan, J. A. Grant, P. Skov, L. Poulsen, and R. Alam. 2000. Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. *Blood 95:1911.*
58. Underwood, D. C., R. R. Osborn, C. J. Kotzer, J. L. Adams, J. C. Lee, E. F. Webb, D. C. Carpenter, S. Bochnowicz, H. C. Thomas, D. W. P. Hay, and D. E. Griswold. 2000. SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. *J. Pharmacol. Exp. Ther. 293:281.*

Downloaded from http://www.jimmunol.org/ by guest on August 5, 2022