

# Analysis of Signal Transduction Pathways Regulating Cytokine-Mediated Fc Receptor Activation on Human Eosinophils<sup>1</sup>

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IgG can be potent stimulants of eosinophil activation since interaction with IgA or IgG-coated particles can lead to eosinophil degranulation. We have investigated the comparative roles of mitogen-activated protein (MAP) kinases (MAPKs; ERK1/2 and p38) and phosphatidylinositol-3 kinase (PI3K) in the priming and regulation of Fc receptor functioning on human eosinophils utilizing a MAPK kinase (MEK) inhibitor (PD98059), a p38 inhibitor SB203580, and the widely used PI3K inhibitors wortmannin and LY294002. We demonstrate that priming of human eosinophils with Th2-derived cytokines, IL-4 and IL-5, differentially activate phosphotyrosine-associated PI3K and ERK and p38 MAP kinases. This activation can be inhibited by pre-incubation with wortmannin or LY294002, PD98059, and SB203580, respectively. Analysis of the effects of the inhibitors on rosette formation between human eosinophils and IgA- or IgG-coated beads revealed that activation of MEK was not required for IgA binding after priming with IL-4 or IL-5. However, inhibition of MEK did inhibit IL-5-primed binding of IgG-beads. The rosette formation of primed eosinophils with IgA-beads could be completely inhibited by wortmannin and LY294002 treatment, demonstrating a critical role for PI3K. Interestingly, inhibition of the p38 pathway also resulted in a complete blockade of IgA rosette formation. This work demonstrates regulatory control by inside-out signaling of Fc receptors by various cytokines on human eosinophils. Thus in vivo the local production of Th2-derived cytokines will regulate the effector functions of Fc receptors. *The Journal of Immunology*, 1998, 161: 6768–6774.

Eosinophilic granulocytes play an important role in host defense against parasites, and the regulation of their activation through the interaction with cytokines and chemoattractants is critical in regulating their functioning. Eosinophils are also implicated in the pathogenesis of a variety of inflammatory diseases which are associated with tissue damage, for example allergic asthma (1, 2). Although the exact role of eosinophils in allergic inflammation remains to be elucidated, increased numbers are regularly observed in peripheral blood of patients with allergic asthma. Furthermore, in these individuals, eosinophils exhibit a preactivated or "primed" state, indicated by an increased responsiveness toward distinct stimuli (3, 4). In addition, eosinophil-derived products are found in lung tissue, such as active eosinophil cationic protein and major basic protein. The release of such toxic mediators results in destruction and damage of the respiratory epithelial tissue and can lead to airway hyperresponsiveness (5, 6). Also, production of toxic oxygen metabolites during the so-called respiratory burst and synthesis and secretion of bioactive lipid mediators contribute to airway hyperreactivity (7–9).

Despite much knowledge concerning the effector functions of eosinophils in vivo, relatively little is known about the processes that lead to activation of eosinophils in the bronchial compartment. Several mechanisms are involved in these processes, including ac-

tivation via receptors for adhesion molecules, complement factors, and Igs (10). These receptors are potent signaling molecules in vitro, albeit optimal only after priming with cytokines or chemotaxins (11, 12).

Binding of cytokines to specific transmembrane receptors results in intracellular increases in tyrosine phosphorylation and activation of distinct signal transduction pathways (13). In vitro, priming of eosinophils with IL-5 potentiates effector functions such as respiratory burst activation, migration, and platelet-activating factor release (14–17). Although compared with IL-5 little is known about effects of IL-4 priming on eosinophil function, it can also serve as a priming agent for eosinophil chemotaxis (18). Furthermore, functioning of IgA Ig receptors on eosinophils is strongly modulated by both IL-5 and IL-4 (19), albeit with different activation kinetics.

The receptor for Ig A is a possible candidate for final eosinophil activation at allergic inflammatory sites, since IgA is abundantly present on mucosal surfaces and IgA-coated particles potentially induce eosinophil degranulation (20–22). Receptors for IgA are described on many cell types, including monocytes, neutrophils, and eosinophils (23–25). At present not much is known about the activation and functioning of Fc $\alpha$ Rs on eosinophils in contrast with the better defined Fc $\gamma$ RII (26). Binding of IgG-coated magnetic beads or IgG-coated erythrocytes to eosinophils is sensitive for priming by IL-5 and granulocyte-macrophage CSF, but not influenced by priming with IL-4 (19, 27).

To investigate which signal transduction pathways are involved in the regulation of Fc receptors on human eosinophils, we have studied the activation of phosphatidylinositol 3-OH kinase (PI3K),<sup>3</sup> and mitogen-activated protein kinases (MAPK); i.e., extracellular regulated

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<sup>3</sup> Abbreviations used in this paper: PI3K, phosphatidylinositol 3-OH kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular regulated kinase; HSA, human serum albumin; MBP, myelin basic protein; MEK, MAPK kinase.

kinase (ERK)-1, ERK2, and p38 kinase, in freshly isolated eosinophils from normal donors. Specific inhibitors of those kinases were used to study the involvement of particular signaling pathways in the activation of Fc $\alpha$ R and Fc $\gamma$ RII on eosinophils.

## Materials and Methods

### Reagents

Percoll was obtained from Pharmacia (Uppsala, Sweden). FMLP and OVA were purchased from Sigma (St. Louis, MO). Human serum albumin (HSA) and purified human serum IgG (without traces of other Igs) were from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Purified human serum IgA (>20 mg/ml) was obtained from Cappel (Malvern, PA). It contained no detectable trace of IgG, IgM, or non-Ig serum proteins. Human rIL-5 was a gift from Dr D. Fattah (Glaxo/Wellcome, Stevenage, U.K.). IL-4 was a gift from Dr F. Kalthoff (Sandoz Forschungsinstitut, Vienna, Austria). Rabbit polyclonal ERK1 (C-16), ERK2 (C-14), and p38 MAPK (C-20) antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antiphosphotyrosine polyclonal MAPK and p38 MAPK Abs were obtained from New England Biolabs (Beverly, MA). Pharmacological inhibitors wortmannin, LY294002, PD98059, and SB203580 were purchased from BioMol (Plymouth Meeting, PA).

### Isolation of eosinophils

Blood was obtained from healthy volunteers from the Red Cross Blood Bank (Utrecht, The Netherlands). Mixed granulocytes were isolated from the buffy-coat of 500 ml blood anticoagulated with 0.4% (w/v) trisodium citrate (pH 7.4) as previously described (28). Mononuclear cells were removed by centrifugation over isotonic Percoll (1.078 g/ml). After lysis of the erythrocytes with an ice-cold NH<sub>4</sub>Cl solution, the granulocytes were washed and resuspended in RPMI 1640 (Life Technologies, Paisley, U.K.) with 0.5% (w/v) HSA.

Granulocytes were incubated for 30 min at 37°C to restore the initial density of the cells. Thereafter, the cells were washed and resuspended in PBS supplemented with 0.5% HSA and 13 mM trisodium citrate, and incubated with FMLP (10 nM) for 10 min at 37°C to decrease the specific gravity of the neutrophils, but not that of the eosinophils. Subsequently, eosinophils were obtained by centrifugation (20 min at 1000  $\times$  g) over isotonic Percoll (density 1.084 g/ml, layered on percoll with a density of 1.1 g/ml), washed, and resuspended in incubation buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, supplemented with 5 mM glucose, 1.0 mM CaCl<sub>2</sub>, and 0.5% (w/v) HSA). Purity of eosinophils was 97% ( $\pm$ 0.5 SEM), and recovery was usually 80–90%. This procedure leads to the isolation of relatively unprimed eosinophils compared with conventionally used isolation procedures with immunomagnetic beads (29).

### MAPK activity

Eosinophils were isolated as described above and incubated at 37°C for 30 min. After pretreatment with or without PD98059, SB203580, LY294002, or wortmannin and stimulation with IL-4 or IL-5, cells ( $2 \times 10^6$  cells) were washed twice in ice-cold PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM  $\beta$ -glycerophosphate, 1% Triton X-100) supplemented with Na<sub>3</sub>VO<sub>4</sub> (1 mM), aprotinin (10  $\mu$ g/ml), leupeptin (10  $\mu$ g/ml), and 1 mM PMSF. Lysates were precleared for 30 min at 4°C with protein A-Sepharose beads and subsequently, MAPK was immunoprecipitated with 1  $\mu$ g of ERK1, ERK2, or p38 polyclonal antisera for 1 h at 4°C on a rotating wheel. Protein-A Sepharose was then added for a further 1 h at 4°C. After washing twice with lysis buffer with phosphatase inhibitors, samples were washed twice with kinase buffer (30 mM Tris-HCl, pH 8.0, 20 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 10  $\mu$ M rATP, 10  $\mu$ M MBP) without ATP. Precipitates were then incubated in 25  $\mu$ l kinase buffer with 0.3  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP for 20 min at 30°C. Reaction was stopped by the addition of 5 $\times$  Laemmli sample buffer. Samples were separated by electrophoresis on 15% SDS-polyacrylamide gels. MBP phosphorylation was detected by autoradiography.

### p38 MAPK phosphorylation

Eosinophils were isolated as described above and incubated at 37°C for 30 min in incubation buffer. For detection of phosphorylation of p38 MAPK, eosinophils ( $0.5 \times 10^6$  per condition) were washed twice in ice-cold PBS after stimulation and lysed in lysis buffer (1% Triton-X100, 50 mM Tris-Cl, pH 8.0, 100 mM NaCl) with phosphatase inhibitors and subsequently boiled for 5 min after addition of 5 $\times$  sample buffer. Total cell lysates were analyzed on 15% SDS-polyacrylamide gels. Proteins were transferred to

Immobilon-P and incubated with blocking buffer (Tris buffered saline/Tween 20 (TBST) supplemented with 1 mM EDTA and 0.6% BSA) with polyclonal phospho-p38 MAPK antisera. Detection was with ECL (Amersham, Rainham, U.K.). Blots were reprobated with p38 MAPK Ab.

### Preparation of Ig-coated magnetic dynabeads

Serum IgG and serum IgA were coated to uncoated magnetic dynabeads (M-450, Dynal, Norway). Beads were washed twice with PBS (pH 8.5) and brought to a concentration of 45 mg/ml. Proteins were added at a final concentration of 1 mg/ml to the beads and mixed overnight at 4°C. The next day the beads were washed with borate buffer (0.5 M NaCl, 0.2 M H<sub>3</sub>BO<sub>3</sub>, and 0.02 M NaOH, pH 8.6) and blocked with 0.1 M lysine monohydrochloride (pH 8.6) in borate buffer for 2 h at room temperature. After two washes with 0.1 M acetate buffer (pH 4), beads were washed once with PBS with 1% (w/v) BSA. Until use, the beads were stored at 4°C in PBS/BSA at a concentration of 30 mg/ml ( $4 \times 10^8$  beads/ml). Before the rosette assay, beads were resuspended in 20% (w/v) HSA and left for 20 min at room temperature.

### Rosette assay

Purified eosinophils were washed with Ca<sup>2+</sup>-free incubation buffer containing 0.5 mM EGTA and brought to a concentration of  $8 \times 10^6$  cells/ml. A 50  $\mu$ l cell suspension ( $0.4 \times 10^6$  cells) was incubated at 37°C. For priming, IL-5 and IL-4 were added 1:10, with final concentrations of  $10^{-9}$  M. Cells were incubated with IL-4 for 5 min and with IL-5 for 15 min at 37°C. After priming, the beads were added in a ratio of 3.5 beads/cell. Cells and beads were mixed briefly and pelleted for 15 s at 100 rpm. Normally, eosinophils were incubated with beads during 30 min at 37°C. In the case of IL-4 primed eosinophils, beads and cells were incubated together for only 20 min. Longer incubation resulted in lower levels of binding, due to the transient effect of IL-4 priming, as described previously (19).

After incubation cells were resuspended vigorously and rosettes were evaluated under a microscope. All cells that had bound two beads or more were defined as rosettes. One hundred cells were scored, and the number of beads that were bound to the cells was counted. The amount of beads bound to a total of 100 cells (bound and unbound to beads) was designated as the rosette index. Previously we have demonstrated that the rosetting method with magnetic beads is very specific since 1) there is no appreciable background binding of eosinophils to control beads coated with OVA and 2) relevant blocking mAbs against Fc receptors inhibit the response (19).

### Inhibition of rosette assays with specific PI3K, MAPK kinase (MEK), and p38 inhibitors

For priming-inhibition studies, cells were pre-incubated with specific inhibitors before priming with cytokines. Cells were incubated with PI3K inhibitors wortmannin and LY294002 for 15 min at final concentrations of 20 nM and 1  $\mu$ M, respectively. The p38 inhibitor SB203580 was incubated for 15 min at a concentration of 1  $\mu$ M, while incubation with the MEK inhibitor PD98059 was for 30 min at a concentration of 10 or 50  $\mu$ M.

### Statistical analysis

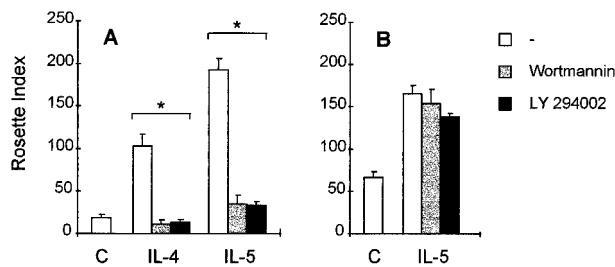
Results were expressed as means  $\pm$  SE. Statistical analysis was performed by using paired and unpaired Student's *t* tests. *p* values < 0.05 were considered as statistically significant.

## Results

### PI3K inhibitors, wortmannin and LY294002, inhibit Ig binding to Fc $\alpha$ R but not to Fc $\gamma$ RII

IgA and IgG receptors on human eosinophils have been shown to be modulated differentially by priming with Th2-derived cytokines IL-4 and IL-5 (19). Stimulation of human eosinophils with optimal concentrations of both IL-5 ( $10^{-9}$ ) and IL-4 ( $10^{-8}$ ) leads to a time-dependent, transient activation of the lipid kinase PI3K, as measured in antiphosphotyrosine immunoprecipitates (30). Preincubation with 30 nM wortmannin, a relatively specific PI3K inhibitor, resulted in a complete inhibition of PI3K activation in eosinophils stimulated with IL-5.

To investigate the involvement of PI3K in the cytokine-induced Fc receptor activation on eosinophils, we performed rosette assays in the presence of wortmannin or LY294002, which both inhibit



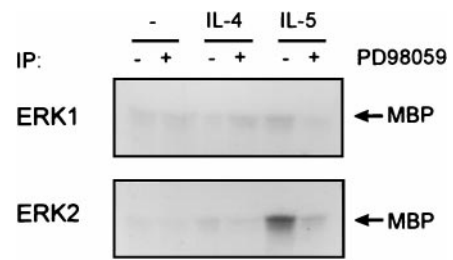
**FIGURE 1.** Effect of PI3K inhibitors, wortmannin and LY294002, on the rosette formation of cytokine primed eosinophils with Ig-coated beads. Freshly isolated eosinophils from normal donors were pretreated for 15 min at 37°C with buffer (open bars), 20 nM wortmannin (gray bars), or 1  $\mu$ M LY294002 (filled bars) and subsequently stimulated at 37°C with IL-4 ( $10^{-9}$  M) or IL-5 ( $10^{-9}$  M) for 2 min or 15 min, respectively. After stimulation, IL-4- and IL-5-primed cells were incubated for 20 and 30 min, respectively, with magnetic dynabeads coated with human serum IgA (A) or incubated with IgG beads (B) for IL-5-primed cells. Binding of Ig beads to unprimed cells is presented by the control bars. Binding of beads is expressed as rosette index (number of beads/100 cells). Mean values are presented  $\pm$  SE ( $n = 4$ ). A paired Student's *t* test was used to perform a statistical analysis of each condition and the control. Statistical analysis of each priming condition and the effect of inhibitors on this priming revealed that values indicated with \* differed significantly ( $p < 0.05$ ).

PI3K activity through distinct mechanisms. Freshly isolated eosinophils from normal donors only bind very weakly human serum IgA-coated beads. Only 8% of the cells are able to form rosettes (19). However, as shown previously, priming of eosinophils with IL-4 or IL-5 dramatically changes the ability of these cells (50–60%) to bind IgA-coated beads (Fig. 1A, open bars) (19). The binding of IgG-coated beads to unprimed cells (25%) is also increased by pre-incubation with IL-5, but IL-4 does not significantly change the capacity of eosinophils to bind IgG-beads (19). As shown in Fig. 1A, blocking of PI3K activation with wortmannin (gray bars) or LY294002 (filled bars) completely inhibited the rosette formation of IgA-coated beads with IL-4 or IL-5 primed eosinophils. We have previously demonstrated that the concentrations of wortmannin and LY294002 used here inhibit PI3K in human eosinophils (30). This suggests a role for PI3K in the cytokine-induced priming of IgA receptor functioning. In contrast, the ability of IL-5 primed eosinophils to bind IgG beads is not influenced by incubation with wortmannin or LY294002. (Fig. 1B). These results show that activation of the PI3K pathway is involved in the specific priming of IgA-binding, but not crucial for IL-5 induced priming of IgG-binding.

#### Phosphorylation and activation of MAPKs, ERK1 and ERK2, in eosinophils after stimulation with Th2-derived cytokines IL-4 and IL-5

We have also studied the activation of ERK MAPKs to determine their role in Fc receptor stimulation. First, we analyzed the ability of IL-4 and IL-5 to induce activation of MAPKs ERK1 and ERK2, which are both downstream targets of p21<sup>ras</sup> signal transduction.

To measure the kinase activity of both ERK1 and ERK2 after stimulation, we specifically immunoprecipitated ERK1 or ERK2 and performed a kinase assay as described in *Materials and Methods*. As shown in Fig. 2, ERK1 kinase activity was minimally affected by stimulation with IL-4 or IL-5, which is in agreement with the unaltered levels of phosphorylation of ERK1 in eosinophils (30). Kinase activity of ERK2 was clearly induced by IL-5 ( $10^{-9}$  M) stimulation but remained unaffected by IL-4 ( $5 \times 10^{-9}$  M) (Fig. 2). We have utilized a pharmacological inhibitor of the p21<sup>ras</sup>-ERK pathway, PD98059, which has been described as a



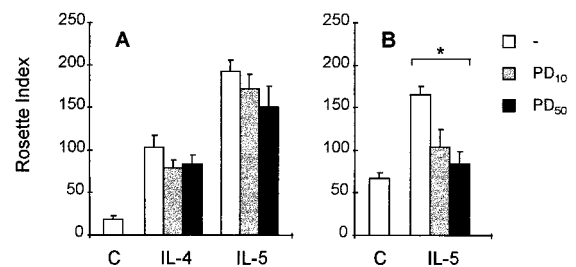
**FIGURE 2.** Effect of cytokines on the activation of MAPKs, ERK1 and ERK2, in eosinophils and the effect of MEK inhibitor PD98059. Eosinophils were incubated for 30 min at 37°C in the absence or presence of 50  $\mu$ M PD98059 and subsequently stimulated with IL-4 ( $5 \times 10^{-9}$  M) or IL-5 ( $10^{-9}$  M) for 5 or 15 min, respectively. After stimulation the cells were lysed and the MAPKs were specifically immunoprecipitated with either an ERK1 (A) or ERK2 (B) Ab. Kinase activity was analyzed by the phosphorylation of the substrate MBP, which was detected by autoradiography. The experiment shown is representative of at least three experiments.

specific inhibitor of MEK, the upstream kinase mediating activation of ERK1 and ERK2 by p21<sup>ras</sup> (31, 32). Pretreatment of eosinophils with PD98059 (50  $\mu$ M) resulted in an inhibition of ERK2 activation after IL-5 stimulation as measured by MBP phosphorylation (Fig. 2, lower panel, lane 6).

#### Inhibition of MEK by PD98059 prevents binding of IgG but not IgA to primed eosinophils

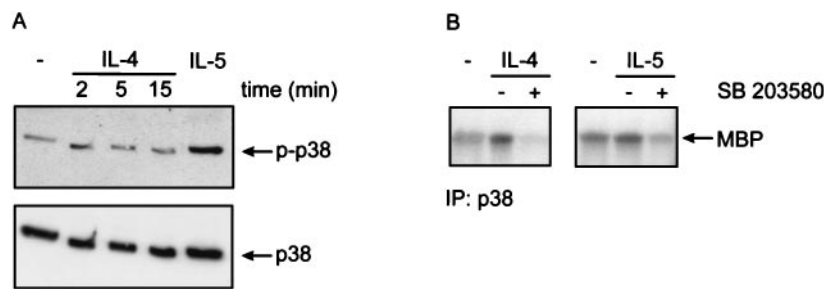
It is clear that ERK2 is activated in eosinophils after stimulation with IL-5 and this activation can be inhibited by PD98059. We thus studied the effect of the MEK inhibitor on Ig-rosette formation with eosinophils to investigate whether the activation of ERKs is involved in the modulation of Ig receptors.

Interestingly, whereas PI3K appeared to be critical for binding of IgA beads (Fig. 1A), activation of ERKs is not required for Fc $\alpha$ R activation. Binding of IgA-coated beads to primed eosinophils was not inhibited by preincubation with 10  $\mu$ M of the MEK inhibitor, PD98059 (Fig. 3A, gray bars). Even a higher concentration, 50  $\mu$ M, which completely inhibits ERK activation (Fig. 2) does not significantly effect rosette formation with IgA beads (Fig. 3A, filled bars). However, 30-min incubation with PD98059 before



**FIGURE 3.** Effect of MEK inhibitor PD98059 on the rosette formation of cytokine primed eosinophils with Ig-coated beads. Eosinophils were pretreated for 30 min at 37°C in the absence (open bars) or presence of 10  $\mu$ M (gray bars) or 50  $\mu$ M (filled bars) PD98059 and subsequently stimulated at 37°C with IL-4 ( $10^{-9}$  M) or IL-5 ( $10^{-9}$  M) for 2 min or 15 min, respectively. After stimulation primed cells were incubated with magnetic dynabeads coated with human serum IgA (A) or incubated with IgG beads (B). The open bar at left represents binding of Ig beads to unprimed cells as a control. Binding of beads is expressed as rosette index (number of beads/100 cells). Mean values are presented  $\pm$  SE ( $n = 4$ ). A paired Student's *t* test was used to perform a statistical analysis of each condition and the control. Values indicated with \* differed significantly ( $p < 0.05$ ) from values obtained with cells that were not pretreated with inhibitors (open bars).





**FIGURE 4.** Phosphorylation and activation of p38 MAPK in eosinophils after stimulation with Th2-derived cytokines IL-4 and IL-5. *A*, After stimulation of the cells with either IL-4 or IL-5 ( $2 \times 10^6$  per condition) for indicated time, eosinophils were washed with ice-cold PBS, lysed in lysis buffer, and heated for 5 min after addition of  $5\times$  sample buffer. Phosphorylation of p38 was detected using a polyclonal antiphospho-p38 antiserum for Western blot analysis (*upper panel*) and the blot was reprobed with a polyclonal anti-p38 Ab (*lower panel*). The experiment shown is representative of at least three experiments. *B*, Kinase assays were performed with specifically immunoprecipitated p38 MAPK of IL-4 ( $5 \times 10^{-9}$  M; 5 min) or IL-5 ( $10^{-9}$  M; 15 min) stimulated cells in the presence or absence of  $1 \mu\text{M}$  of SB203580. Kinase activity was analyzed by the phosphorylation of the substrate MBP, which was detected by autoradiography. The experiment shown is representative of three experiments.

IL-5 stimulation resulted in a 60% decrease of IgG binding. Levels of IgG binding to PD98059 treated and IL-5-stimulated cells were reduced to the levels of IgG binding to unprimed eosinophils (Fig. 3*B*).

#### Th2-derived cytokines IL-4 and IL-5 stimulate phosphorylation of p38 MAPK in eosinophils

Although p38 MAPK was initially described as being activated by a variety of cellular stresses, it is now also known to be activated in response to various inflammatory cytokines (33–35). We thus studied the stimulation of p38 MAPK phosphorylation in human eosinophils. Phosphorylation of p38 after stimulation with IL-4 and IL-5 was detected by Western blot analysis with an activation-specific Ab that only interacts with Tyr<sup>182</sup>-phosphorylated p38. As shown in Fig. 4*A*, *upper panel*, a phosphorylated form of p38 is clearly detected in eosinophils that are stimulated for 15 min with IL-5. However, stimulation with IL-4 induced a rapid but very weak, transient phosphorylation of p38 MAPK. In all donors tested, the phosphorylation of p38 had already diminished after 5-min stimulation with IL-4. This in contrast with p38 phosphorylation by IL-5 that remained for at least 25 min (data not shown). Reprobing of the blot with p38 antiserum (Fig. 4*A*, *lower panel*) shows an equal amount of p38 protein in all lanes. Activation of p38 MAPK is also shown in Fig. 4*B*. Although the level of p38 MAPK activity in unprimed cells was variable (compare first lanes of both panels in Fig. 4*B* and first lane in Fig. 6), an increase of p38 MAPK activity was detected both in IL-4- and IL-5-stimulated cells. Cytokine induced p38 MAPK activation could be blocked completely by  $1 \mu\text{M}$  of SB203580 (Fig. 4*B*, *right lane* of both panels). This inhibitor, a pyridinyl imidazole compound, is a highly selective inhibitor of p38 activity and has been used extensively as a tool to evaluate p38 MAPK-dependent events in vivo (36).

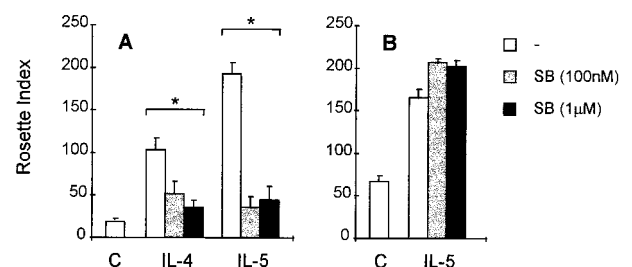
#### Inhibition of p38 by SB203580 prevents binding of IgA but not IgG to primed eosinophils

To study a potential role for the activation of p38 kinase in activation of Fc receptors by Th2-derived cytokines, we studied the binding capacity of primed eosinophils after addition of SB203580. As shown in Fig. 5, inhibition of p38 in eosinophils results in a striking difference in binding capacities of the cells to either IgA or IgG beads. The effect of priming with both IL-4 and IL-5 on IgA binding appears to be abolished by incubation with SB203580. The effect of IL-4 priming on IgA rosette formation (Fig. 5*A*) is dramatically decreased by pre-incubation with a 100

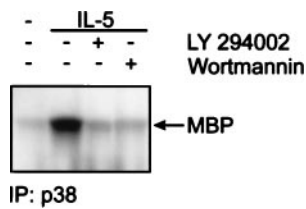
nM concentration of SB203580 (Fig. 5*A*, *middle panel*, gray bar), and a higher concentration ( $1 \mu\text{M}$ ) of the inhibitor totally blocks the effect of IL-4 on rosette formation (Fig. 5*A*, *middle panel*, filled bar). IL-5-primed binding of IgA beads is already completely inhibited by the lower concentration (100 nM) of SB203580 (Fig. 5*A*, *right panel*, gray bar). In contrast, IL-5-induced modulation of Fc $\gamma$ R2 on eosinophils is not affected by pre-treatment with SB203580 (Fig. 5*B*).

#### Inhibition of p38 by LY294002 and wortmannin

As suggested by Figs. 1 and 5, both activation of PI3K and p38 MAPK are involved in the modulation of Fc $\alpha$ R on human eosinophils. Since inhibition of either PI3K or p38 MAPK activity resulted in a complete loss of IgA rosette formation, we questioned whether PI3K and p38 MAPK may be components of a single signal transduction pathway activated upon Th2-cytokine stimulation. We investigated this possibility by performing p38 MAPK assays with IL-5-primed eosinophils that were pretreated with the PI3K inhibitors LY294002 and wortmannin. As shown in Fig. 6, activation of p38 MAPK by IL-5 is blocked by inhibiting PI3K activation by both LY294002 ( $1 \mu\text{M}$ ) and wortmannin (30 nM),



**FIGURE 5.** Effect of the p38 MAPK inhibitor SB203580 on the rosette formation of cytokine primed eosinophils with Ig-coated beads. Cells were pretreated for 15 min at  $37^\circ\text{C}$  in the absence (open bars) or presence of 100 nM (gray bars) or  $1 \mu\text{M}$  (filled bars) SB203580 and subsequently stimulated at  $37^\circ\text{C}$  with IL-4 ( $10^{-9}$  M) or IL-5 ( $10^{-9}$  M), for 2 min or 15 min, respectively. Both IL-4- and IL-5-primed eosinophils were incubated with IgA-coated beads for 20 and 30 min, respectively (*A*). For rosette assay with IgG-coated beads only IL-5-stimulated cells were used and compared with binding of Ig beads to unprimed eosinophils. Rosette index is the measure of binding of the beads (number of beads/100 cells). Mean values are presented  $\pm$  SE ( $n = 3$ ). Statistical analysis of each priming condition and the effect of inhibitors on this priming revealed that values indicated with \* differed significantly ( $p < 0.05$ ).



**FIGURE 6.** Effect of PI3K inhibitors LY294002 and wortmannin on p38 MAPK activation. After preincubation at 37°C with either buffer, LY294002 (1  $\mu$ M; 15 min) or wortmannin (30 nM; 5 min), eosinophils were subsequently stimulated with IL-5 ( $10^{-9}$  M) for 15 min. A kinase assay was performed with specifically immunoprecipitated p38 MAPK. Detection of the phosphorylation of the substrate MBP by autoradiography was a measurement for kinase activity. The experiment shown is representative of three experiments.

suggesting that indeed PI3K and p38 MAPK are activated in the same signal transduction pathway and that p38 MAPK is activated downstream of PI3K.

## Discussion

The phenotype of activated eosinophils *in vivo* is well described and much is known concerning the importance of the eosinophil in inflammatory diseases such as allergic asthma. It is also clear that cytokines and chemoattractants are important modulators of eosinophil functions *in vivo*, and several signal transduction pathways are activated in eosinophils after stimulation with these agonists (30, 37). However, little is known about the molecular mechanisms that finally lead to activation of eosinophils at specific sites of inflammation. The hypothesis that there might be spatial and temporal regulation of eosinophil priming by different cytokines is an interesting concept to understand eosinophil activation in patients with allergic diseases. As shown previously, receptors for IgA and IgG on human eosinophils are differentially modulated by Th2-derived cytokines IL-4 and IL-5 (19). In this study we show that IL-4 and IL-5 activate Ig receptors by means of different signal transduction pathways.

Binding of IL-5 to its receptor on eosinophils directly results in association and activation of members of the JAK/STAT pathway and phosphorylation and activation of several kinases (37, 38), including the tyrosine kinase Lyn (37) as well as PI3K (30). The IL-4R signals through a complex network of signaling molecules (reviewed in Refs. 39 and 40) including members of JAK/STAT pathway (41); PI3K (42); specific cellular substrates such as insulin receptor substrate-2 (43, 44) and Cbl (45); and protein kinases including Fes (46). In contrast with IL-5, IL-4 is unable to activate the p21<sup>ras</sup> pathway, and ERK1 and ERK2 are not activated by IL-4 stimulation (47). Although tyrosine phosphorylation of multiple proteins and PI3K activation by IL-4 stimulation are described in several cells and cell lines, very little is known about IL-4 signaling in human eosinophils.

As previously described IL-4 and IL-5 are potent activators of PI3K (IL-4 and IL-5) and ERK2 (IL-5) (30), but this is the first report demonstrating p38 MAPK activation in eosinophils. p38 MAPK (also known as CSBP, RK, and HOG1) is a recently identified protein kinase that shares sequence similarity with other MAPKs. p38 MAPK is activated by the dual specific kinase MKK3 following exposure to products of microbial pathogens, environmental stress and pro-inflammatory cytokines (34, 48, 49). It has previously been shown to be activated by cytokines such as TNF- $\alpha$ , IL-1, and granulocyte-macrophage CSF in human neutrophils (36, 50–52).

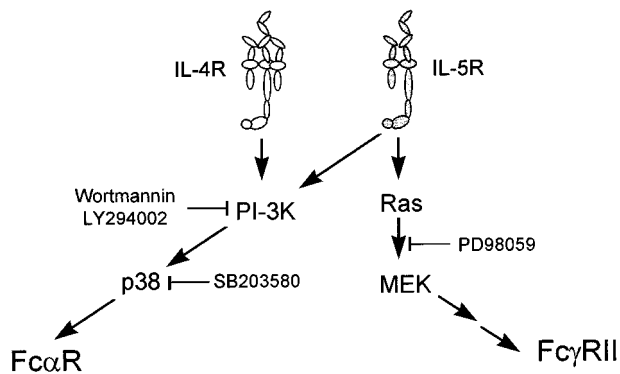
This is the first report to show that IL-4 and IL-5 are able to activate p38 MAPK. Although p38 phosphorylation by IL-5 priming seems to be much stronger, both IL-4 and IL-5 are able to induce p38 kinase activity, as shown in Fig. 4. This demonstrates that downstream of the IL-4 and IL-5 receptors some signals converge at the level of p38 MAPK. The specific nature of these signals remains to be elucidated. To examine if there was a role of p38 in IL-4- and IL-5-induced activation of Fc receptors, we utilized the potent and highly selective inhibitor of p38 kinase, SB203580 (36). This inhibitor has been useful in dissecting signaling pathways involved in inflammatory responses. Our data suggest that p38 activation also plays a role in the activation of Ig receptors on human eosinophils. Cytokine-induced binding of IgA-coated beads to human eosinophils was blocked by inhibition of p38 activation (Fig. 5), since incubation of the cells with SB203580 before IL-4 or IL-5 stimulation leads to a complete inhibition in IgA binding. The kinetics of IL-4 priming on rosette formation with IgA beads correlate with the fast induction of p38 MAPK phosphorylation by IL-4. This is different for IL-5-induced p38 and ERK phosphorylation and IgA-binding, which is much slower and remains activated for a longer duration.

The fact that inhibition of either the p38 MAPK or PI3K pathway results in loss of IgA binding after priming demonstrates that both pathways are involved in modulation of the IgA receptor on eosinophils. It is tempting to speculate that p38 and PI3K might be both components of one signal transduction pathway that is activated by both IL-4 and IL-5 and utilized for the priming of Fc $\alpha$ R activation. Since PI3K is a lipid kinase that is activated by translocation to the membrane, it is not likely that it will act downstream of p38. Activation of p38 by PI3K has previously been suggested by Krump et al. (53), and here we show that p38 MAPK activation can indeed be a downstream target of PI3K in eosinophils. Activation of p38 MAPK is dramatically decreased in eosinophils in which PI3K activity is blocked by either LY294002 or wortmannin (Fig. 6), suggesting that under these circumstances PI3K activation is necessary for proper p38 activation.

We have also shown that in contrast with IgA binding, inhibition of PI3K and p38 MAPK activity do not influence the effect of IL-5 on binding of IgG beads, suggesting that neither one of these pathways plays a major role in Fc $\gamma$ RII modulation by IL-5. Modulation of Fc $\gamma$ RII functioning seem to require other signals including the activation of the p21<sup>ras</sup>-ERK pathway, since inhibition of this pathway with PD98059 results in a complete reduction of IgG rosette formation after stimulation with IL-5. Therefore, it is not surprising that IL-4 is not able to induce IgG binding to eosinophils (19), since IL-4 it cannot activate the p21<sup>ras</sup> pathway (54).

In Fig. 7, a model is presented for the hypothesis that selective production of Th2-derived cytokines can lead to activation of different Ig receptors. This model predicts the distinction between Fc $\alpha$ R and Fc $\gamma$ RII activation and the general signal transduction pathways initiated by IL-5 compared with the more restricted activities of IL-4. The control of receptor activation by priming might be a critical process for the final activation of eosinophils. The regulation of Fc receptor affinity on eosinophils is analogous to the activation of integrins by diverse cellular stimuli. Both receptor types are controlled by inside-out signaling generated from cytokine/chemotaxis receptors. Although inside-out signaling is now well established for integrins (55), this is a novel concept for Fc receptors.

Despite the clear phenotype of cytokine-activated eosinophils in the context of Ig binding, not much is known about the mechanisms responsible. Several possibilities can be suggested: 1) a conformational change of the receptor, leading to an affinity switch to



**FIGURE 7.** Model for cytokine-induced signaling events regulating Fc receptor priming on human eosinophils. The presence of specific Th2-derived cytokines can lead to activation of different Ig receptors. For example, the presence of IL-5 in the eosinophil environment might lead to a broad range of effects, including activation of Fc $\alpha$ R and Fc $\gamma$ RII receptors. In contrast, the effects of IL-4 priming are more restricted, leading via activation of p38 MAPK and PI3K to Fc $\alpha$ R activation.

a higher affinity for Ig (56), and 2) membrane expression of receptors that are stored in secretory granules and released upon triggering of the cells with cytokines. It has been described for neutrophils that priming results in rapid mobilization of subcellular granules to cell surface to increase the number of granule associated receptors (57). 3) During activation of leukocytes, co-capping or clustering of integrin and Fc receptors can lead to an avidity switch (58). 4) Cytokine-induced activation of Ig receptors on eosinophils might be caused by inhibition of an inhibitory signal which keeps the receptor in a nonfunctional state. A suppressive regulation has been suggested to explain the default highly activated state of integrin  $\alpha_v\beta_3$  when it is purified, in contrast with the moderate affinity state of cellular  $\alpha_v\beta_3$  (59). The hypothesis of an "inside-out suppressor" is supported by the finding that adding protein kinase C inhibitors to eosinophils leads to increased responsiveness of opsonized particles (60). Additional experiments will be necessary to determine the precise nature of the signals mediating these effects.

In conclusion, activation of Fc receptors appear to be a tightly regulated process of inside-out signaling from cytokine receptors leading to receptor activation. Production of specific cytokines by Th2 lymphocytes leads to either a generalized priming phenotype (IL-5/PI3K, p38 MAPK, and ERK2) associated with activation of Ig receptors and integrin receptors or a restricted phenotype (IL-4/PI3K and p38 MAPK) leading primarily to enhanced IgA receptor functions. Elucidation of the mechanisms generated by the aforementioned receptor-activated signals will lead to insight into the mechanisms of eosinophil priming occurring in allergic diseases in vivo.

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