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Human Airway Eosinophils Respond to Chemoattractants with Greater Eosinophil-Derived Neurotoxin Release, Adherence to Fibronectin, and Activation of the Ras–ERK Pathway When Compared with Blood Eosinophils

Mary Ellen Bates,* Julie B. Sedgwick, † Yiming Zhu,* Lin Ying Liu, † Rose G. Heuser,* Nizar N. Jarjour, † Hirohito Kita, ‡ and Paul J. Bertics*

Human blood eosinophils exposed ex vivo to hematopoietic cytokines (e.g., IL-5 or GM-CSF) subsequently display enhanced responsiveness to numerous chemoattractants, such as chemokines, platelet-activating factor, or FMLP, through a process known as priming. Airway eosinophils, obtained by bronchoalveolar lavage after segmental Ag challenge, also exhibit enhanced responsiveness to selected chemoattractants, suggesting that they are primed during cell trafficking from the blood to the airway. Earlier work has shown that chemoattractants stimulate greater activation of ERK1 and ERK2 following IL-5 priming in vitro, thus revealing that ERK1/ERK2 activity can be a molecular readout of priming under these circumstances. Because few studies have examined the intracellular mechanisms regulating priming as it relates to human airway eosinophils, we evaluated the responsiveness of blood and airway eosinophils to chemoattractants (FMLP, platelet-activating factor, CCL11, CCL5, CXCL8) with respect to degranulation, adherence to fibronectin, or Ras–ERK signaling cascade activation. When compared with blood eosinophils, airway eosinophils exhibited greater FMLP-stimulated eosinophil-derived neurotoxin release as well as augmented FMLP- and CCL11-stimulated adherence to fibronectin. In airway eosinophils, FMLP, CCL11, and CCL5 stimulated greater activation of Ras or ERK1/ERK2 when compared with baseline. Ras activation by FMLP in blood eosinophils was also enhanced following IL-5 priming. These studies are consistent with a model of in vivo priming of eosinophils by IL-5 or related cytokines following allergen challenge, and further demonstrate the key role of priming in the chemoattractant-stimulated responses of eosinophils. These data also demonstrate the importance of the Ras–ERK signaling pathway in the regulation of eosinophil responses to chemoattractants in the airway. Human airway eosinophils respond to several chemoattractants with increased activation of the Ras–ERK cascade, eosinophil-derived neurotoxin release, and adherence to fibronectin relative to blood eosinophils. The Journal of Immunology, 2010, 184: 000–000.

A llergic asthma is generally described as an inflammatory disease of the airways and is characterized by increased numbers of eosinophils in the affected tissues. Considerable evidence supports the view that eosinophils are key effector cells in asthma (reviewed in Ref. 1). Eosinophils acquired from the airway of human allergic or allergic asthmatic subjects 48 h after segmental bronchoprovocation with Ag (SBP-Ag) display a phenotype that is altered in several respects relative to their counterparts in the peripheral blood. Among the phenotypic changes observed in airway eosinophils are as follows: 1) altered expression of several membrane receptors and cell surface epitopes (2–6); 2) increased capacity to produce superoxide anion and to adhere to endothelial cells when stimulated with the chemoattractant FMLP (2, 7); and 3) the increased expression of genes encoding proteins related to cell survival or responsiveness to the inflammatory microenvironment (4, 6). Although airway eosinophils display an enhanced capacity to respond to several ligands relevant to allergic inflammation, compared with blood eosinophils, the mechanisms regulating this change in phenotype are poorly understood and are one of the aims of the current study.

Human eosinophils may encounter a variety of ligands for chemoattractant receptors in the asthmatic airway, such as chemokines (8), lipid-derived mediators (9, 10), and ligands of formyl peptide receptors (FPRs) (11, 12). As a consequence of in vitro exposure to chemoattractants, eosinophils display altered adherence to matrix proteins and cells (2, 7, 13, 14), undergo directed migration (15, 16), release preformed enzymes and cytotoxic proteins (17, 18), synthesize reactive oxygen species (2, 19), elaborate arachadonic acid metabolites (20, 21), and release cytokines and chemokines (22–24). Therefore, numerous studies have documented that stimulation of eosinophil chemoattractant receptors can have profound effects on the accumulation of eosinophils in the airway and their cytotoxic effector functions in the inflammatory milieu.

In leukocytes and other mammalian cells, a variety of heterotrimeric G protein-coupled receptors mediates responsiveness to chemoattractants. In addition, the responsiveness to chemoattractants can be further modulated by other factors present in the inflammatory microenvironment. In particular, IL-5 and related cytokines...
Eosinophils were purified from heparinized peripheral blood of volunteer subjects supplying blood for eosinophil studies, which ranged in age from 18 to 55 y (St. Louis, MO). Human serum albumin, tissue fibronectin, and CXCL8 from R&D Systems (Minneapolis, MN) and GM-CSF from Peprotech were used. Human FPR2 (a rabbit polyclonal Ab) was a generous gift from Arena and relevant isotype controls (BD Biosciences, San Jose, CA). Anti-mAbs to the human FPR1, ERK2, dually phosphorylated ERK1/ERK2, and control proteins (usually ERK1/ERK2) in the same samples. Images were captured using x-ray film or digital photography, scanned, and quantified using densitometry and NIH Image software. Signal intensities from immunoblotting with anti-phospho-ERK1/2 Abs were normalized to the signal from the corresponding loading control.

### Detection of active, GTP-bound Ras

Active Ras was affinity precipitated from eosinophil cell lysates, as previously described (33). Briefly, following stimulation with FMLP, cells were lysed, vortexed, and clarified by centrifugation to remove insoluble cellular debris. The clarified lysates were incubated with glutathione-conjugated agarose beads coupled to a GST-fusion protein carrying the Ras binding domain of Raf-1. The beads were washed five times with lysis buffer, and the captured (active) Ras was visualized by SDS-PAGE and immunoblotting with anti-Ras mAb. Images were captured by x-ray film or digital photography, scanned, and quantified using densitometry (NIH Image software).

### Adherence to fibronectin

Purified blood or airway eosinophils were incubated in fibronectin-coated tissue culture plates for 30 min following the addition of various chemoattractants. After vigorous washing to remove nonadherent cells, eosinophil adhesion was measured as residual eosinophil peroxidase activity of the adherent cells (34).

### Degranulation

Purified blood and airway eosinophils were cultured in HBSS plus 0.03% gelatin with various chemoattractants in 24-well tissue culture plates for 4 h at 37°C in 5% CO2 (3). The cell-free culture supernates were frozen at −80°C until assayed for EDN by RIA (3). Total cellular EDN was measured for each cell type, and data were expressed as percentage of total EDN.

### Statistical analysis

Data are presented in toto, or summarized as medians (25 and 75 percentiles) for data that are not normally distributed, or as mean (SEM) for normally distributed data, as indicated in the figure legends. A paired Student’s t test or Wilcoxon signed rank test was used to compare results between chemoattractant-stimulated and control-stimulated samples, or between blood and BAL samples acquired from the same subject at the same time. In some cases, the data were log transformed prior to statistical analysis. A two-tailed p value of <0.05 was considered significant.

### Results

#### Effect of chemoattractants on EDN release from blood and airway eosinophils

Previous studies have demonstrated that chemoattractants stimulate the release of the granule protein EDN from eosinophils (17), and elevated levels of EDN are present in BAL fluid after allergen challenge (35, 36). However, EDN release by airway eosinophils

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exposed to chemoattractants has not been examined. To determine whether chemoattractants have a similar effect on degranulation of airway eosinophils as blood eosinophils, EDN release was measured from eosinophils purified from blood and BAL fluid 48 h after SBP-Ag. The eosinophils from both compartments were purified and then stimulated with FMLP, PAF, CCL5, or CCL11, and the percentage of total EDN release was evaluated in the supernates (3). As can be seen in Fig. 1, FMLP and PAF stimulated release of EDN from both blood and airway eosinophils, which was significantly higher than the release stimulated by control buffer. However, CCL5 and CCL11 did not promote significant EDN release above levels stimulated by control buffer alone. Interestingly, EDN release was significantly greater from airway eosinophils than from blood eosinophils following stimulation with control buffer and with FMLP, CCL11, and CCL5, but not PAF (Fig. 1). This increase did not appear to be the result of a greater mass of EDN protein in BAL cells because the two cell populations contained comparable cellular amounts of EDN. Total cellular EDN (mean [SEM]) of blood and airway eosinophils was 2540 (330) ng/10⁶ cells and 2280 (271) ng/10⁶ cells, respectively, and the difference between the samples was not statistically significant. These data demonstrate that both basal and FMLP-stimulated EDN release is greater in airway eosinophils than in blood eosinophils, suggesting that airway eosinophils have an increased inflammatory capacity when stimulated with some, but not all, chemoattractants.

**Eosinophil adherence to fibronectin**

Fibronectin, a component of the extracellular matrix, is increased in BAL fluid 48 h following SBP-Ag (37). The adherence of blood eosinophils to fibronectin-coated wells is enhanced by stimulation with chemoattractants in IL-5– or GM-CSF–primed blood eosinophils and greater in eosinophils from atopic individuals (data not shown) (38, 39). Furthermore, adherence to fibronectin, which is mediated by integrins, enhances eosinophil viability, increases degranulation, and modulates the activity of several intracellular signaling molecules (40–44). Following in vitro stimulation with FMLP or CCL11, unprimed blood eosinophils showed no significant increase in adhesion to fibronectin (Fig. 2A). In contrast, airway eosinophils were significantly more adherent, both at baseline and following stimulation with chemoattractants, than matched blood eosinophils isolated 48 h post–SBP-Ag (Fig. 2A; p < 0.05, n = 5).

Because previous studies demonstrated that integrin-mediated eosinophil adherence stimulated by chemoattractants was sensitive to inhibition of the MEK/ERK signaling cascade (40, 45), we assessed the effect of the MEK1 and MEK2 antagonist, UO126 (10 μM), on FMLP- and CCL11-stimulated adherence of airway eosinophils to fibronectin. This concentration of UO126 effectively inhibits FMLP-stimulated ERK1/ERK2 activation in blood and airway eosinophils (21) (data not shown). As can be seen in Fig 2B, UO126 significantly inhibited the stimulus-induced adherence of airway eosinophils to fibronectin-coated wells to levels comparable to unstimulated airway eosinophils. These data suggest that the MEK/ERK signaling cascade is an intracellular regulator of chemoattractant-stimulated adherence to fibronectin in airway eosinophils.

**Expression of receptors for chemoattractant ligands on blood and airway eosinophils**

One mechanism that may contribute to the enhanced responsiveness of airway eosinophils to chemoattractants is the modulation of cell surface receptor expression. Therefore, we evaluated whether airway eosinophils exhibit greater expression of cell surface receptors for ligands, such as CCL11 and FMLP, which have been shown in previous studies (7, 35) or in this work (Figs. 1, 2A) to stimulate the differential responses of eosinophils obtained from the peripheral and airway compartments. Given that CCL11 binds predominantly to CCR3 in eosinophils (46), it is noteworthy that the cell surface expression of CCR3 and numerous other chemokine receptors on human blood and airway eosinophils has been found to be significantly lower on airway eosinophils relative to blood eosinophils (47, 48). However, the expression of receptors binding FMLP on airway eosinophils has not been previously reported. In leukocytes, FMLP is believed to act primarily through two receptors, FPR1 and FPR2.

![Figure 1: EDN release by purified blood and airway eosinophils.](image)

**Figure 1.** EDN release by purified blood and airway eosinophils. Release of EDN was measured 4 h after in vitro exposure of blood (●) and airway (○) eosinophils from the same subjects to buffer alone, 100 nM FMLP, 100 ng/ml CCL11, 100 ng/ml CCL5, or 100 nM PAF. Boxes represent medians with 25 and 75% quartiles; whiskers are 10 and 90 percentiles. n = 15–21. Total cellular EDN (mean [SEM]) of blood and airway eosinophils was 2543 (330) ng/10⁶ cells and 2283 (271) ng/10⁶ cells, respectively, and was not significantly different between the two populations. *p < 0.05 compared with spontaneous (buffer-stimulated) release; #p < 0.05 relative to blood cells.

![Figure 2: Adherence of purified blood or airway eosinophils to fibronectin.](image)

**Figure 2.** Adherence of purified blood or airway eosinophils to fibronectin. Blood or airway eosinophils from the same subjects were incubated for 30 min in tissue culture wells precoated with 10 μg/ml tissue fibronectin in the presence of control buffer, 100 nM FMLP, or 100 ng/ml CCL11. Data are summarized as the mean (SEM). A, Relative adherence of blood eosinophils (●) or airway eosinophils (○); n = 5. *p < 0.005 versus blood cells. B, Purified airway eosinophils were preincubated with 0 μM (●) or 10 μM (○) of the MEK antagonist UO126 for 30 min and subsequently assessed for adherence to fibronectin, as described above. *p < 0.02, 0 μM UO126 versus 0.10 μM UO126, n = 5.
Our previous work has revealed that, in blood eosinophils, the mRNA for these two receptors is expressed at very low levels (see online supplement in Ref. 4). We examined the expression of FPR1 protein on blood and airway eosinophils of three patients by flow cytometry and observed very low cell surface expression relative to blood neutrophils from the same subjects. In addition, FPR1 expression was not significantly different between blood and airway eosinophils (Fig. 3A; \( p = \text{NS}, n = 3 \)). A polyclonal antiserum to FPR2, which immunodetects a protein of expected size in blood and airway eosinophils and THP-1 monocytes by immunoblot (Fig. 3B), was used to evaluate cell surface FPR2 protein by flow cytometry. FPR2 was abundantly detected on the surface of THP-1 monocytes (data not shown), but was expressed at low levels on blood eosinophils, and the surface expression was slightly, but significantly greater on airway eosinophils than in the blood eosinophils from the same patients (Fig. 3C; \( p < 0.02, n = 5 \)).

Taken together, these data suggest that the enhanced responsiveness of airway eosinophils to FMLP may result, at least in part, from marginally greater cell surface expression of FPR2.

**Effect of chemoattractants on ERK1/ERK2 activation**

Because chemoattractant-stimulated adherence to fibronectin (Fig. 2B) (40, 45) and degranulation (18) can be attenuated by inhibition of MEKs 1 and 2, the upstream activators of ERK1/ERK2, we examined the effect of various chemoattractants on ERK1/ERK2 phosphorylation in blood and airway eosinophils. Previous studies from our laboratory had revealed that priming of blood eosinophils with IL-5 enhanced the capacity of FMLP to stimulate ERK1 and ERK2 activity in blood eosinophils (21). Therefore, we hypothesized that airway eosinophils would respond to FMLP in a similar manner to in vitro primed blood eosinophils and demonstrate increased activity of ERK1/ERK2. Purified blood eosinophils were primed with control buffer or 1 nM IL-5 for 1 h. Subsequently, airway eosinophils as well as unprimed and primed blood eosinophils were stimulated with control buffer or 100 nM FMLP. As can be seen in Fig. 4A, low levels of immunodetectable phospho-ERK1/ERK2 are present in unprimed blood eosinophils stimulated by FMLP (Fig. 4A, lane 2 versus lane 1). IL-5 priming enhances the phosphorylation of ERK1/ERK2 stimulated by FMLP (Fig. 4A, lane 4 versus lane 2), as described previously (21). By comparison, FMLP was a powerful activator of ERK1/ERK2 in airway eosinophils (Fig. 4A, lane 6 versus lane 5) even in the absence of prior in vitro exposure to IL-5. These data demonstrate that, although the three cell populations have similar mass of total ERK1/ERK2 protein (Fig. 4A, lower immunoblot panel), airway eosinophils have a greater capacity to activate ERK1/ERK2 in response to FMLP than either unprimed or IL-5–primed blood eosinophils. The immunoblot density of the images in Fig. 4A was determined, expressed as a ratio of dually phosphorylated ERK1/ERK2 (pERK) density to ERK1/ERK2 density, and plotted in Fig. 4B. The densitometric quantification shows greater pERK1/2 labeling in both the medium-treated and FMLP-treated lysates of airway eosinophils relative to blood eosinophils (Fig. 4B). When the FMLP-stimulated activation of ERK1/ERK2 was examined in blood and airway eosinophils of multiple subjects 48 h following SBP-Ag, and summarized by densitometry in a similar manner as for Fig. 4A and 4B, airway eosinophils showed significantly higher ERK1/ERK2 phosphorylation in response to 100 nM FMLP than their blood-derived counterparts (Fig. 4C; \( n = 7 \)).

Because different methods of cell purification were used to isolate blood and airway eosinophils, we assessed whether the robust responsiveness of airway eosinophils can be attributed to the technique of purification via Percoll density gradient centrifugation. To address this issue, we conducted an analysis of ERK1/ERK2 phosphorylation by intracellular FACS following in vitro stimulation of the cells with FMLP (see Supplemental Information, Supplemental Figs. 1–3). This technique permits the evaluation of eosinophil ERK1/ERK2 phosphorylation in mixed populations of cells by allowing for the identification of eosinophils based on their size, light-scattering properties, and green autofluorescence (Supplemental Fig. 1). We observed that the robust activation of ERK1/ERK2 was seen in airway eosinophils following stimulation with FMLP, even without prior purification of the airway cells by centrifugation through a Percoll bilayer (Supplemental Fig. 2). This robust activation was markedly greater than that seen in blood eosinophils purified by immunomagnetic negative selection, as outlined in Materials and Methods.

**FIGURE 3.** Expression of FPRs on blood and airway eosinophils. A, Expression of cell surface receptors for FPR1 was evaluated by flow cytometry, summarized as the MCF with labeling by isotype control Ab subtracted. Values are plotted for three separate donors and, as an aid to visualization, the blood and airway eosinophil expression levels for each subject are connected with a line. The mean values for each cell type are shown as a large ●. \( p = \text{NS}. \) B, FPR2 was evaluated in lysates of human blood and airway eosinophils for two separate donors, from blood eosinophils from two additional donors, and in THP-1 monocytes as a positive control. Lysate representing equal numbers of each eosinophil preparation was loaded into gel lanes. FPR2 was abundantly detected on the size in blood and airway eosinophils and THP-1 monocytes by flow cytometry. FPR2 was abundantly detected on the surface of THP-1 monocytes (data not shown), but was expressed at low levels on blood eosinophils, and the surface expression was slightly, but significantly greater on airway eosinophils than in the blood eosinophils from the same patients (Fig. 3C; \( p < 0.02, n = 5 \)).

Taken together, these data suggest that the enhanced responsiveness of airway eosinophils to FMLP may result, at least in part, from marginally greater cell surface expression of FPR2.

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Because chemoattractant-stimulated adherence to fibronectin (Fig. 2B) (40, 45) and degranulation (18) can be attenuated by inhibition of MEKs 1 and 2, the upstream activators of ERK1/ERK2, we examined the effect of various chemoattractants on ERK1/ERK2 phosphorylation in blood and airway eosinophils. Previous studies from our laboratory had revealed that priming of blood eosinophils with IL-5 enhanced the capacity of FMLP to stimulate ERK1 and ERK2 activity in blood eosinophils (21). Therefore, we hypothesized that airway eosinophils would respond to FMLP in a similar manner to in vitro primed blood eosinophils and demonstrate increased activity of ERK1/ERK2. Purified blood eosinophils were primed with control buffer or 1 nM IL-5 for 1 h. Subsequently, airway eosinophils as well as unprimed and primed blood eosinophils were stimulated with control buffer or 100 nM FMLP. As can be seen in Fig. 4A, low levels of immunodetectable phospho-ERK1/ERK2 are present in unprimed blood eosinophils stimulated by FMLP (Fig. 4A, lane 2 versus lane 1). IL-5 priming enhances the phosphorylation of ERK1/ERK2 stimulated by FMLP (Fig. 4A, lane 4 versus lane 2), as described previously (21). By comparison, FMLP was a powerful activator of ERK1/ERK2 in airway eosinophils (Fig. 4A, lane 6 versus lane 5) even in the absence of prior in vitro exposure to IL-5. These data demonstrate that, although the three cell populations have similar mass of total ERK1/ERK2 protein (Fig. 4A, lower immunoblot panel), airway eosinophils have a greater capacity to activate ERK1/ERK2 in response to FMLP than either unprimed or IL-5–primed blood eosinophils. The immunoblot density of the images in Fig. 4A was determined, expressed as a ratio of dually phosphorylated ERK1/ERK2 (pERK) density to ERK1/ERK2 density, and plotted in Fig. 4B. The densitometric quantification shows greater pERK1/2 labeling in both the medium-treated and FMLP-treated lysates of airway eosinophils relative to blood eosinophils (Fig. 4B). When the FMLP-stimulated activation of ERK1/ERK2 was examined in blood and airway eosinophils of multiple subjects 48 h following SBP-Ag, and summarized by densitometry in a similar manner as for Fig. 4A and 4B, airway eosinophils showed significantly higher ERK1/ERK2 phosphorylation in response to 100 nM FMLP than their blood-derived counterparts (Fig. 4C; \( n = 7 \)).

Because different methods of cell purification were used to isolate blood and airway eosinophils, we assessed whether the robust responsiveness of airway eosinophils can be attributed to the technique of purification via Percoll density gradient centrifugation. To address this issue, we conducted an analysis of ERK1/ERK2 phosphorylation by intracellular FACS following in vitro stimulation of the cells with FMLP (see Supplemental Information, Supplemental Figs. 1–3). This technique permits the evaluation of eosinophil ERK1/ERK2 phosphorylation in mixed populations of cells by allowing for the identification of eosinophils based on their size, light-scattering properties, and green autofluorescence (Supplemental Fig. 1). We observed that the robust activation of ERK1/ERK2 was seen in airway eosinophils following stimulation with FMLP, even without prior purification of the airway cells by centrifugation through a Percoll bilayer (Supplemental Fig. 2). This robust activation was markedly greater than that seen in blood eosinophils purified by immunomagnetic negative selection, as outlined in Materials and Methods.
Furthermore, when blood eosinophils were stimulated by FMLP and assessed for ERK1/ERK2 phosphorylation in a mixed population of leukocytes obtained after centrifugation through a Percoll bilayer (the technique routinely used to purify airway eosinophils), their activation was not significantly different from eosinophils isolated from the same blood donor using immunomagnetic negative selection (Supplemental Fig. 3). Taken together, these findings support the idea that the enhanced ERK1/ERK2 activation detected in airway eosinophils following SBP-Ag is an intrinsic property of the cells from the airway compartment and does not appear to be a feature of the different isolation methods used to acquire the purified populations of cells.

We next determined whether this potent activation of ERK1 and ERK2 was a general feature of airway eosinophil responsiveness to chemoattractants, as was demonstrated for blood eosinophils following in vitro priming with IL-5 (21). In that study, IL-5–primed blood eosinophils displayed greater ERK activation when stimulated with FMLP, CCL5, or CXCL8 than did unprimed blood eosinophils. Therefore, blood and airway eosinophils from the same patient 48 h after SBP-Ag were stimulated for 2 min with various agonists of chemoattractant receptors, as well as PMA, a potent stimulus of ERK1/ERK2 phosphorylation in eosinophils (50). PMA stimulated marked and approximately equivalent activation in blood and airway eosinophils (p = NS, n = 12). In this experiment, the chemoattractants FMLP, CCL5, and CXCL8 stimulated greater ERK1/ERK2 activation in airway eosinophils than in blood eosinophils (Fig. 5A, upper immunoblot panel), although the mass of ERK1/ERK2 protein was similar in all samples (Fig. 5A, lower immunoblot panel). Identical experiments using blood and airway eosinophils from multiple subjects stimulated with FMLP, CXCL8, CCL5, or CCL11 revealed that ERK1/ERK2 activation in airway eosinophils was equal to or greater than that in blood eosinophils in the majority of subjects and with most stimuli, although there was some heterogeneity in the responses, both in control-stimulated and chemoattractant-stimulated eosinophils.

**FIGURE 4.** ERK1/ERK2 phosphorylation in blood and airway eosinophils stimulated with FMLP. Purified blood eosinophils were stimulated for 2 min with control buffer or 100 nM FMLP, and cell lysates were prepared. SDS-PAGE gels were loaded with protein equivalent to 10⁶ cells/lane and immunoblotted with antiserum raised against the dually phosphorylated activation motif of ERK1/ERK2 (pERK1/2) as well as total ERK1/ERK2 (ERK1/2). A, Blood eosinophils were primed for 1 h with 0 nM (lanes 1, 2) or 1 nM (lanes 3, 4) IL-5, and then the blood eosinophils and airway eosinophils (lanes 5, 6) were stimulated for 2 min with 0 nM (lanes 1, 3, 5) or 100 nM (lanes 2, 4, 6) FMLP. Lysates were immunoblotted for anti–phospho-ERK1/2 (upper panel) and anti-ERK1/2 (lower panel). B, The phosphorylation of ERK1/ERK2 seen in the representative experiment of A above was quantified by densitometry and the data normalized to the loading control (ERK1/2). Relative densities for blood eosinophils are plotted with ▪, and for airway eosinophils with □. C, ERK1/ERK2 phosphorylation stimulated with FMLP or control buffer was evaluated on both blood and airway eosinophils of seven subjects, quantified as the normalized density, in the same way as Fig. 3B above, and plotted with a different symbol for each subject. As an aid to visualization, the blood and airway eosinophil densitometry values for each subject are connected with a line. The population mean for each cell/treatment group is shown as a larger ●. *p < 0.05 for blood versus airway eosinophils following FMLP stimulation.

**FIGURE 5.** ERK1/ERK2 phosphorylation in blood and airway eosinophils stimulated by additional chemoattractants. A, Purified blood eosinophils (lanes 1–5) and airway eosinophils (lanes 6–9) were stimulated for 2 min with buffer control (lanes 1, 6), 100 nM FMLP (lanes 2, 7), 10 nM CCL5 (lanes 3, 8), 10 ng/ml CXCL8 (lanes 4, 9), or 50 nM PMA (lane 5). The cell lysates were immunoblotted for pERK1/ERK2 (pERK1/2, upper panel) and ERK1/ERK2 (ERK1/2, lower panel) to validate the presence of equal mass of ERK1/ERK2 protein in the various cell preparations. B, ERK1/ERK2 phosphorylation was evaluated on both blood and airway eosinophils from five subjects following stimulation for 2 min with control buffer, 10 nM CCL5, or 10 nM CCL11. ERK1/ERK2 phosphorylation was quantified by densitometry and normalized by the densitometry of the corresponding ERK1/2 immunoblot. Each subject’s data are plotted with a different symbol, and the values for the blood and airway eosinophils are connected by a line as an aid to visualization. The population mean for each cell/treatment category is shown as a ●. Data were log transformed prior to statistical analysis to compensate for lack of normalcy in the distribution. *p < 0.05.
It is noteworthy that ligands that bind CCR3, namely CCL11 and CCL5, stimulate significantly greater ERK1/ERK2 phosphorylation in airway cells than in blood eosinophils (Fig. 5B; p < 0.05, n = 5). An identical conclusion was reached based on four additional experiments in which, due to the limited availability of post-SBP-Ag blood cells, blood eosinophils from an unrelated donor, who did not undergo SBP-Ag, were compared with BAL eosinophils from the Ag-challenged subjects (data not shown).

**Ras activation in blood and airway eosinophils following FMLP stimulation**

To evaluate a potential mechanism of the priming described above, we examined the ability of IL-5 priming to affect the activation of the small m.w. G protein Ras, given that Ras is an upstream activator of the ERK kinase cascade in many cellular contexts, including FMLP- (45) and IL-5–stimulated blood eosinophils (33, 45, 50). Blood eosinophils were primed for 1 h with 0 or 100 pM IL-5 and subsequently stimulated with FMLP (100 nM for 2 or 5 min). Active, GTP-bound Ras was affinity precipitated from the cell lysates by incubation with the Ras binding domain of Raf-1 coupled to agarose, as previously described (33). Although both primed and unprimed blood eosinophils contained a small amount of GTP-Ras in the absence of FMLP stimulation, the mass of active Ras was increased by FMLP stimulation (p < 0.05) and enhanced by IL-5 priming, as pictured in Fig. 6A and quantified in Fig. 6B (p < 0.004, n = 5; Fig. 6B). Furthermore, airway eosinophils stimulated with FMLP showed greater Ras activation than did blood eosinophils acquired at the same time in all of the five subjects tested (Fig. 7; n = 5). These data demonstrate that the increased activation of ERK1 and ERK2 seen in airway eosinophils and in blood eosinophils following IL-5 priming is accompanied by increased accumulation of GTP-bound Ras. These findings, therefore, support a model of airway eosinophil function that encompasses in vivo priming of eosinophil responses to chemoattractants, possibly because of exposure to IL-5 or related cytokines during migration to, or residence in, the airway.

**Discussion**

Previous studies have shown that airway eosinophils respond to chemoattractants with greater adherence, migration, and superoxide anion generation than do blood eosinophils (2, 7, 51). This process is recapitulated in many respects by treating blood eosinophils with IL-5 or related cytokines prior to exposure to chemoattractants, a process known as priming (7). Given the prominent occurrence of chemoattractants in the airway lumen following Ag challenge, the current study was initiated to further examine important biological responses of airway and blood eosinophils to chemoattractants, including degranulation and adherence to fibronectin (Figs. 1, 2). In addition, because of our previous work indicating that eosinophil priming with IL-5 and related cytokines is associated with enhanced activity of the Ras–ERK signaling cascade (21) (data not shown), we tested the hypothesis that the enhanced responsiveness to chemoattractants seen in airway eosinophils following SBP-Ag is associated with increased activity of the ERK1/ERK2 signal transduction cascade following chemoattractant exposure. We report in this study that multiple chemoattractants activate ERK1 and ERK2 in airway eosinophils to a greater extent than blood eosinophils and in a manner similar to that seen in IL-5–primed blood eosinophils (Figs. 4, 5). Furthermore, the enhanced activation of the ERK1/ERK2 signaling cascade observed in airway eosinophils was associated with a parallel increase in Ras activation (Fig. 7), also a feature of IL-5–primed blood eosinophils (Fig. 6). This report therefore suggests that airway eosinophils are primed in vivo and that this process is an important mechanism regulating eosinophil responsiveness to chemoattractants in the airway. Furthermore, this study extends our previous observations (21) suggesting that, as with in vitro-primed eosinophils, the greater activation of the Ras–ERK signaling cascade may be an intracellular marker of priming.

Release of cytotoxic granule proteins is an important mechanism through which eosinophils contribute to innate immunological...
defense and may contribute to the pathobiology of airway diseases. Previous studies have shown that the release of EDN by blood eosinophils is stimulated by IL-5 and related cytokines, but that chemokines, such as CCL5 and CXCL8, did not cause the elaboration of significant amounts of EDN or superoxide anion (52). However, some studies suggest that priming with IL-5 at very low concentrations promoted significant release of EDN following stimulation with CCL11, but only after prior exposure of the cells to cytchalasin B (17). To our knowledge, no previous reports have examined granule protein release in airway eosinophils stimulated by chemotact- actants. The finding, that airway eosinophils release greater amounts of the granule protein EDN constitutively and following stimulation with FMLP (Fig. 1), is consistent with the model of in vivo priming of FMLP-stimulated responses and our previous studies showing that airway eosinophils produce markedly greater amounts of superoxide anion than do blood eosinophils following exposure to FMLP (2, 7). As seen in Fig. 1, PAF also stimulated EDN release from both blood and airway eosinophils, but in approxi- mately equal amounts, and in this respect EDN release was also reflective of the behavior of airway eosinophils with respect to super- oxide generation. PAF did not stimulate greater superoxide re- lease from airway eosinophils than from blood eosinophils, al- though there was some heterogeneity in the responses (2). It is notable, therefore, that airway eosinophils are not more responsive to all stimuli because another potent activator of blood eosinophil EDN release, namely IL-5, was less robust in promoting EDN re- lease from airway eosinophils than from blood eosinophils (3). With respect to the chemokines CCL5 and CCL11, however, we did not observe EDN release above that stimulated by buffer alone from either blood or airway eosinophils, demonstrating that following eosinophil transit into the airway, CCR3 ligands are not potent acti- vators of EDN release as are PAF and FMLP.

In current paradigms of allergic airway inflammation, the re- cruitment and retention of eosinophils in the airway is an adherence- dependent process. This model predicts that with the coordinated action of chemoattractants, integrins, and adherence molecules, such as ICAM1 and VCAM, eosinophils migrate to inflammatory foci where adherence to physiological substrates, such as fibronectin, can prolong eosinophil viability and enhance superoxide produc- tion (4, 13, 16, 32, 34, 37, 44, 51, 53–55). The current finding that airway eosinophils show greater adherence to fibronectin than do blood eosinophils following stimulation with FMLP or CCL11 (Fig. 2A) is consistent with studies showing that in vitro priming of eosinophils with IL-5 family cytokines enhances adherence to fibronectin stimulated by CCL11 (data not shown) (38), and that chemoattractant-stimulated adherence is a process dependent on the Ras–MEK–ERK signaling network (Fig. 2).

Earlier investigations have revealed that, even in the absence of in vitro stimulation, airway eosinophils specifically adhere to a wide variety of physiologically relevant ligands, including VCAM, ICAM, albumin, vitronectin, fibrinogen, and laminin. Blood eosin-ophils express at least seven integrin heterodimers, namely α4β1, α6β1, α4β2, α5β2, αβ6, αβ2, and αβ7 (56, 57). Of these receptors, adherence to tissue fibronectin appears to be primarily mediated by αβ1 (58, 59). When the cell surface expression of integrin epitopes was examined on blood and airway eosinophils following SBP-Ag, the α4 subunit was not significantly different in the two eosinophil populations. However, a subpopulation of asth- matic patients did show discernibly increased expression of the β1 subunit in airway eosinophils as well as enhanced labeling by an Ab specific for an activation-sensitive epitope of β1. This subpopulation of asthmatics consisted of those individuals who were characterized by a late-phase reaction to inhaled Ag challenge (60). Therefore, these data suggest that, with respect to adherence to fibronectin, the enhanced cell surface expression of β1 integrin and in vivo cellular priming (which promotes the appearance of an activation epitope for β1 integrins) can cooperate to increase the ability of airway eosinophils to bind fibronectin. This process could then contribute to the enhanced adherence-dependent physiological responses ob- served when the cells are exposed to chemotactactants.

The intracellular mechanisms regulating the primed phenotype are most likely multifaceted. Previous work has shown that priming of eosinophils with IL-5 or related cytokines can increase the ability of these cells to undergo degranulation (61), directed migration (30), superoxide anion generation (7), and leukotriene C4 (21) produc- tion in response to selected chemotactactants. The enhancement of these effector functions was found to be accompanied by an in- crease in ERK1/ERK2 activation and an increase in the association of the adapter protein GRB2 with tyrosine-phosphorylated proteins (21). This priming process could be initiated by IL-5 and GM-CSF, but not by other eosinophil-active cytokines, such as IFN-γ, stem cell factor, TNF-α, or IL-4. The current study identifies enhanced Ras activation as an additional potential intracellular marker of priming in vitro and in vivo (Figs. 6, 7).

The effector functions stimulated in eosinophils by a given chemotactactant in the airway are governed by several processes, including the concentration of the ligand in the airway and ex- pression of the ligand’s receptor(s) on the eosinophil cell surface. Previous flow cytometry studies have reported equivalent and very low, or nondetectable, levels of receptors for several of the chemotactactants used in this study (CXCR1, CXCR2, CCR1, and CCR5) on blood and airway eosinophils. Furthermore, markedly decreased expression of CCR3 on human airway eosinophils rel- ative to blood cells was seen (47, 48). Therefore, the enhanced signaling and adherence responses of airway eosinophils to CCL5 and CCL11 are not likely the result of increased cell surface expression of CCR3, suggesting that additional mechanisms, such as in vivo priming, are regulating the behavior of airway eosin- phils. With respect to eosinophil responsiveness to FMLP, two receptors, namely FPR1 and FPR2, have been shown to be expressed and to transduce signals in blood eosinophils (4, 62). Flow cytometric analysis revealed that expression of FPR1 on eosinophils was low relative to expression in neutrophils and ap- proximately equivalently expressed on blood and airway eosino- phils (Fig. 3A). FPR2 was also expressed at low levels on the eosinophil cell surface, relative to its surface expression on THP-1 monocytic cells, and was slightly, but significantly, more abundant on airway eosinophils than blood eosinophils. Therefore, the mechanisms by which priming of FMLP-stimulated responses is achieved most likely encompass multiple intracellular events besides the expression of cognate receptors on the eosinophil cell surface.

It should be noted that priming also contributes to the enhanced responsiveness of additional receptor classes, notably the FcRs for Igs. In this regard, IL-5 and GM-CSF can increase the expression of CD32 mRNA and protein on blood eosinophils (4, 63). IgG is a potent stimulus for eosinophil degranulation, and the ability of blood eosinophils to bind IgG-coated beads is enhanced by in vitro priming with cytokines, a process that also appears to be dependent on the MEK–ERK signaling module (64). Indeed, we have reported that a priming-associated motif of FcyRII (CD32) can be detected with the phage mAb A17 on airway eosinophils (5). Eosinophils in the blood have greater expression of this motif following allergen challenge, and the magnitude of the increase in expression in air- way eosinophils correlates with a measure of the patients’ bronchial hyperresponsiveness (5). Taken together, these previous reports and the current study suggest a model of airway eosinophil activation by chemotactactants and IgG-coated targets that encompasses several
levels of regulation that may include the following: 1) exposure to one or more cytokines; 2) expression of cognate receptors on the eosinophil cell surface; 3) inside-out signaling that modulates receptor affinity for its ligand(s); and 4) postreceptor signaling pathway alteration. Although IgG has not been used as a stimulus in the current study, the model described above predicts that airway eosinophils would demonstrate a greater capacity to bind Ig-coated targets and release cytotoxic granule proteins with subsequent ramifications for tissue homeostasis.

The present work, together with previous studies from our laboratories and others, reveals that agonists of diverse chemotaxant receptors, namely FMLP, CCL11, and CCL5, stimulate greater adherence to several substrates, such as collagen, endothelial cells, and fibronectin (Fig. 2) (2, 7), and that FMLP promotes enhanced superoxide anion release (2, 7) and degranulation (Fig. 1) in airway eosinophils relative to blood-derived eosinophils. Intracellular markers of this enhanced responsiveness to FMLP include the activation of ERK1/ERK2 and the low m.w. G protein Ras, as well as prolonged elevations in intracellular-free calcium concentrations (2). The increased responsiveness to CCL11 and CCL5, seen with respect to activation of ERK1/2 in airway eosinophils, was modest in comparison with that stimulated by FMLP (Fig. 5A), and variable among the subjects examined. It is likely, however, that any increase in ERK1/ERK2 activation promoted in airway eosinophils by CCR3 ligands, resulting from in vivo exposure to priming agents, such as IL-5 and related cytokines (GM-CSF, IL-3), may be partially offset by the concurrent reduction in the cell surface expression of CCR3 (47, 48) detected in airway eosinophils. In contrast, FMLP, a synthetic ligand of the FPRs, is a potent activator of airway eosinophils. Enhanced responsiveness to FMLP and CCR3 ligands by airway eosinophils may be mediated in part by mechanisms impinging on intracellular signaling through the Ras–ERK cascade. Altogether, these studies support a model of in vivo priming of eosinophils by IL-5 or related cytokines following allergen challenge, and reveal an important role of priming and the Ras–ERK network in the differences in chemoattractant-stimulated responses of blood and airway eosinophils.

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