Interleukin-9 influences chemokine release in airway smooth muscle: role of ERK

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Interleukin-9 influences chemokine release in airway smooth muscle: role of ERK. *Am J Physiol Lung Cell Mol Physiol* 284: L1093–L1102, 2003. First published February 14, 2003; 10.1152/ajplung.00300.2002.—Interleukin (IL)-9 is a pleiotropic cytokine that has been proposed as a candidate gene for asthma. As IL-9 expression is correlated with airway hypersensitivity in animals, we examined the effects of IL-9 on cultured human airway smooth muscle (HASM) cells. IL-9 alone had no effect on IL-8 release, but at concentrations of >30 ng/ml, IL-9 significantly increased IL-8 release induced by TNF-α. IL-9 increased phosphorylation of extracellular signal-regulated protein kinase (ERK, p42 and p44) in a concentration- and time-dependent fashion, and U-0126 (10 μM), which inhibits ERK phosphorylation, abolished the synergism between TNF-α and IL-9 on IL-8 release. IL-9 alone had no effect on eotaxin release into HASM cell supernatants but at concentrations of ≥10 ng/ml caused an ~50% increase in release of eotaxin evoked by IL-13 (10 ng/ml). U-0126 blocked the synergism between IL-9 and IL-13 on eotaxin release. IL-9 had no effect on cyclooxygenase-2 (COX-2) expression or PGE2 release and did not augment the COX-2 expression that was induced by IL-1β. Our results indicate that airway smooth muscle is a target for IL-9 and that IL-9 amplifies the potential for these cells to recruit eosinophils and neutrophils into the airways by a mechanism involving ERK.

Eotaxin; interleukin-8; tumor necrosis factor-α; interleukin-1β; cyclooxygenase-2; interleukin-13; human airway smooth muscle cells; extracellular signal-regulated protein kinase

Asthma is a chronic inflammatory disorder characterized by reversible airway obstruction, bronchial hyporesponsiveness, and infiltration of the airways by activated inflammatory cells, particularly eosinophils, T lymphocytes, and mast cells. There is evidence that helper type 2 (Th2) cells and the cytokines they release play a central role in the pathophysiology of asthma (45). While the Th2 cytokines IL-13, IL-4, and IL-5 have been the focus of much of the research in this area, evidence is now emerging that the Th2 cytokine IL-9 can also play an important role in allergic airway disease. Both the IL-9 and IL-9 receptor α (IL-9Rα) genes are in regions of the genome that have been linked either to asthma or to airway responsiveness (16, 20, 44). In addition, expression of both IL-9 and the IL-9Rα are increased in the airways of asthmatics compared with normal subjects (4, 49). The physiological effects of IL-9 also suggest a role for this cytokine in asthma. IL-9 was initially identified as a growth and differentiation factor for T-helper cells and mast cells (17, 46). IL-9 promotes the differentiation of eosinophils from their progenitors and their survival in vitro and regulates the cell surface expression of IL-5 receptor on human peripheral blood eosinophils (11, 32). In addition, IL-9 also causes mucus hypersecretion by stimulating goblet cell metaplasia and the expression of mucin genes (29, 31).

IL-9 also appears to play a role in regulating airway hyporesponsiveness in animal models. For example, IL-9 expression in the lungs of different strains of mice is highly correlated with the degree of innate airway responsiveness observed in these strains; genetically hyporesponsive mice have no undetectable levels of IL-9 protein, whereas hyperresponsive mice have robust IL-9 protein levels in the lung (27, 40). IL-9 transgenic mice exhibit increased bronchial responsiveness (34, 52), while administration of an IL-9 antibody markedly attenuates bronchial hyperresponsiveness induced by allergen sensitization and challenge in mice (21).

The observations that IL-9 can increase bronchial responsiveness and that the IL-9 receptor is expressed on human bronchial smooth muscle cells (12) suggest that IL-9 may have direct effects on airway smooth muscle. IL-9 has been shown to influence eotaxin release in lung epithelial cells (6), IL-8 release in neutrophils (1), and cyclooxygenase-2 (COX-2) expression in mouse bone marrow-derived mast cells (38). Consequently, we examined the effects of IL-9 on eotaxin and IL-8 release into human airway smooth muscle (HASM) cell supernatants, as well as the effects of IL-9 on COX-2 expression and PGE2 release.
In hematopoietic cells, binding of IL-9 to the IL-9Rα results in dimerization of the receptor with the IL-2R common γ chain (γc), resulting in activation of janus kinases (JAKs) that are constitutively associated with the receptor and consequent JAK phosphorylation of both receptor components (3, 5, 57). Phosphorylation of the receptors creates docking sites for a variety of signaling molecules, including insulin receptor substrate 1 and 2 (IRS-1/2), as well as signal transducer and activator of transcription (STAT) 1, 3, and 5. Interaction of these moieties with the receptor leads to their phosphorylation and activation, resulting in STAT-dependent gene transcription, as well as activation of ERK (30). Because ERK is involved in the induction of IL-8, eotaxin, and COX-2 by other cytokines in HASM cells (13–15, 23, 37), we examined the hypothesis that IL-9 causes ERK phosphorylation in cultured HASM cells that contributes to its effects on cytokine release.

METHODS

Cell culture. Human tracheas were obtained from lung transplant donors, in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. A segment of trachea just proximal to the carina was dissected under sterile conditions, and smooth muscle cells from the trachealis were isolated and placed in culture as previously described (42). Cells were plated in culture flasks at 10⁴ cells/cm² in Ham’s F-12 media supplemented with 10% FBS, penicillin (10⁵ U/ml), streptomycin (1 mg/ml), amphotericin B (2 mg/ml), NaOH (12 mM), CaCl₂ (1.7 μM), l-glutamine (2 mM), and HEPES (25 mM). Media were replaced every 3–4 days. Cells were passaged with 0.25% trypsin and 1 mM EDTA every 10–15 days. Media were replaced every 3 days. Cells were then serum deprived and supplemented with 0.25% trypsin and 1 mM EDTA every 10–14 days. Confluent cells were serum deprived and supplemented with 5.7 μg/ml insulin and 5 μg/ml EDTA every 10–14 days. Cells from 20 different donors studied in passages 4–7 were used in the studies described below.

Western blotting. Confluent HASM cells plated in six-well plates (10⁵ cells/well) were serum deprived and treated with cytokines. Medium was removed, and cells were washed with PBS and then lysed in 200 μl of extraction buffer (10 mM Tris-HCl buffer with 50 mM NaCl, 50 mM NaF, 10 mM β-serine, 1 μM EDTA, 1 μM EGTA, 1% SDS, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 10⁻² U/ml aprotinin). Cells were scraped off and passed through a 25% gauge needle. Cell lysates were mixed with an appropriate volume of loading buffer [0.062 M Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.01% bromphenol blue] and then boiled for 5 min. Solubilized proteins (10–30 μg/lane) were separated by SDS-PAGE on 12% Tris-glycine gel (Invitrogen, Carlsbad, CA) under nonreducing conditions and transferred electrophoretically to a nitrocellulose membrane in transfer buffer (Pierce, Rockford, IL). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 3 h at room temperature. The blots were probed with the appropriate primary antibodies, at the dilutions suggested by the manufacturer. Blots were washed and incubated in TBS-0.1% Tween 20–5% nonfat dry milk containing horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h. The proteins were subsequently visualized by light emission on film with enhanced chemiluminescent substrate (Pierce, Rockford, IL). The protein bands were quantified using a laser densitometer, and band density values were expressed in arbitrary optical density (OD) units. Anti-ERK and anti-phospho-ERK (p42 and p44) antibodies were purchased from New England Biolabs (Beverly, MA). Phosphorylated ERK standards were also obtained from New England Biolabs. Anti-COX-2 and COX-2 standards were purchased from Oxford Biomedical Research (Oxford, MI).

Eotaxin and IL-8 release. HASM cells were plated in 24-well plates. After reaching confluence, cells were serum deprived for 48 h and then treated with cytokines for 24 h. The supernatant was removed and stored at −20°C until subsequent assay. Eotaxin and IL-8 release were analyzed by ELISA (R&D Systems, Minneapolis, MN), following the instructions of the manufacturer. In experiments involving inhibition of ERK, cells were treated with U-0126 (10 μM) or vehicle (0.01% DMSO) 2 h before addition of cytokines. Note that U-0126 virtually abolished ERK phosphorylation under all conditions (control and cytokine treated) examined.

PGF₂α release. Cells were plated in 24-well plates, grown to confluence, and serum deprived for 24 h. Cells were then either left untreated or were treated with IL-9 (30 ng/ml) in the presence or absence of IL-1β (2 ng/ml). After approximately 22 h later, cell medium was removed, cells were washed with PBS, and 0.5 ml of fresh serum-free, hormone-supplemented media was added, at which point the cells were either left untreated or were treated with arachidonic acid (AA; 10⁻⁵ M). After a 15-min incubation at 37°C, the supernatant was harvested and stored at −20°C until assayed with a PGF₂α enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Transfection of HASM cells. Following passage, HASM cells were grown in complete medium for 72 h (60–80% confluence) in six-well plates. Before transfection, the medium was changed to serum-free, hormone-supplemented media. HASM cells were transfected with 0.5 μg of an IL-8 promoter-firefly luciferase reporter construct and with 0.5 μg of a β-galactosidase control vector or with 0.5 μg of an eotaxin promoter-firefly luciferase reporter construct and 0.5 μg of a Renilla luciferase control vector. β-Gal and Renilla luciferase were used to normalize for differences in transfection efficiency from well to well. The constructs were transfected with Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer’s protocol. For the measurement of IL-8 promoter activity, 24 h after transfection, the cells were either left untreated or were treated with TNF-α (0.5 or 1.0 ng/ml) in the presence or absence of IL-9 (50 ng/ml). For the measurement of eotaxin promoter activity, 24 h after transfection, the cells were either left untreated or were treated with IL-13 (10 ng/ml) in the presence or absence of IL-9 (30 ng/ml). Twenty-four hours later, the cells were lysed with reporter lysis buffer (Promega, Madison, WI) and harvested. For IL-8 promoter experiments, the samples were assayed for luciferase activity by scintillation counting and for β-galactosidase activity by spectrophotometry with the β-galactosidase enzyme assay system (Promega). For eotaxin promoter experiments, firefly and Renilla luciferase activity were sequentially measured in a luminometer using the Dual-Luciferase Reporter Assay System (Promega).

The IL-8 promoter-reporter construct was made by amplifying a 1,421-bp fragment of human genomic DNA by PCR with the primers 5'-CACAGTGTGGGCAAATTCAC and 5'-GTTCTTCCGTCGTTTTT. The PCR product was purified by agarose gel electrophoresis and Qiaex II gel extraction (Qiagen, Valencia, CA) and cloned using the pCR 2.1 vector (Invitrogen) in accordance with the manufacturer’s instructions. Smal and BglII restriction sites were added to the cloned promoter fragment by PCR with the primers 5'-
IL-9 has been shown to be capable of inducing IL-8 release from neutrophils (1). To determine whether IL-9 might have similar effects in airway smooth muscle, we examined the effects of IL-9 on the concentration of IL-8 measured in HASM cell supernatants. IL-9, even at concentrations as high as 50 ng/ml, had no effect on IL-8 release. IL-8 release averaged 0.39 ± 0.11 ng/ml (n = 15) in untreated cells and 0.44 ± 0.09 ng/ml (n = 9) in cells treated with IL-9 (50 ng/ml for 24 h). In contrast, TNF-α (0.3 ng/ml for 24 h) increased IL-8 to 5.4 ± 0.37 ng/ml (n = 6) (P < 0.05). However, IL-9 did cause a concentration-related increase in the release of IL-8 that was induced by TNF-α (0.3 ng/ml) (Fig. 1). Effects were significant at IL-9 concentrations of ≥30 ng/ml. IL-9 (50 ng/ml for 24 h) also enhanced the release of IL-8 that was induced by higher concentrations of TNF-α (10 ng/ml for 24 h): IL-8 averaged 12.0 ± 1.3 ng/ml in supernatants of cells treated with TNF-α alone and 19.6 ± 3.6 ng/ml in supernatants of cells treated with TNF-α + IL-9 (P < 0.05).

In other cell types, the ability of TNF-α to induce IL-8 expression occurs in part through NF-κB activation and the subsequent effects of this transcription factor on the IL-8 promoter (28, 54). Moreover, in other cell types IL-9 has been shown to induce binding of nuclear proteins to NF-κB consensus sequences (47). Consequently, we examined the ability of IL-9 (50 ng/ml for 24 h) alone and in combination with TNF-α to induce NF-κB activation by transfecting HASM cells with an NF-κB luciferase reporter construct (Fig. 2). TNF-α caused a concentration-dependent increase in NF-κB activation. IL-9 also caused a small but significant (P < 0.05) increase in NF-κB activity in untreated cells but did not significantly augment the ability of TNF-α to activate NF-κB. We also examined the effect of IL-9 alone and in combination with TNF-α on IL-8 promoter activity (Fig. 3). TNF-α for 24 h caused a concentration-related increase in luciferase activity in HASM cells transfected with an IL-8 promoter luciferase reporter construct. However, there was no effect of IL-9 (50 ng/ml for 24 h) alone on promoter activity, and IL-9 did not augment the IL-8 promoter activity that was induced by TNF-α.

Because ERK is involved in TNF-α-induced release of IL-8 from HASM cells (14), and because IL-9 induces ERK activation in other cell types (30), we examined the hypothesis that ERK plays a role in the synergistic effects of IL-9 and TNF-α on IL-8 release. To do so, we first examined the ability of IL-9 to induce ERK activation. Figure 4 shows the time course of IL-9 (30 ng/ml)-induced ERK phosphorylation in HASM cells. Representative results from a single HASM cell donor are shown in Fig. 4A, and densitometric results from multiple donors are shown in Fig. 4B. IL-9 caused ERK phosphorylation that peaked ~15 min after addition of cytokine with a fourfold increase over baseline and then returned to basal values by 4 h. In contrast, Western blots for nonphosphorylated ERK indicated that there was no effect of IL-9 on the expression of ERK per se (data not shown). ERK phosphorylation occurred in a concentration-dependent manner at IL-9 concentrations of ≥30 ng/ml (Fig. 5). We also examined the effect of U-0126 (10 μM), an inhibitor of the MEK enzyme that phosphorylates ERK (8), on IL-8 release in HASM cells stimulated with TNF-α (0.3 ng/ml for 24 h), alone or in combination with IL-9 (30 ng/ml for 24 h) (Fig. 6). U-0126 had no effect on IL-8 release in unstimulated cells but significantly reduced IL-8 re-
lease in cells treated with TNF-α either alone or in combination with IL-9 (*P < 0.05). In control cells, IL-8 release was greater in cells treated with the combination of TNF-α and IL-9 than in cells treated with TNF-α alone (*P < 0.05 by paired t-test). However, treatment with U-0126 abolished this synergism: in cells treated with U-0126, IL-8 release induced by TNF-α in combination with IL-9 was not different from that induced by TNF-α alone. In contrast to the effects of U-0126 on TNF-α-induced IL-8 release, U-0126 did not affect TNF-α-stimulated IL-8 promoter activity (Fig. 7).

We used Western blotting with phospho-ERK antibodies to determine whether IL-9 and TNF-α synergized at the level of ERK activation (Fig. 8). TNF-α caused a much greater phosphorylation of ERK than...
IL-9. When the two cytokines were administered simultaneously, the amount of ERK phosphorylation was similar to that observed with TNF-α/H9251 alone. Den- sitometric analysis on blots from four cell donors indicated no signifi-
cant difference between TNF-α/H9251-treated and TNF-α/H9251/H11001 IL-9-treated cells (90.8 ± 39.2 vs. 69.3 ± 19.3% of phospho-ERK standard, respectively, for p42, and 32.2 ± 22.7 vs. 22.8 ± 16.8% of phospho-ERK standard, respectively, for p44).

Overexpression of IL-9 in the lungs or exogenous administration of IL-9 in a murine model results in increased recovery of eotaxin into bronchoalveolar lavage (BAL) fluid, as well as increased eotaxin mRNA expression (6). Furthermore, HASM cells have been shown to be an important source of eotaxin in the lung (10). Consequently, we examined the effect of IL-9 on the concentration of eotaxin measured in HASM cell supernatants. IL-9 alone had no effect on eotaxin concentration. Eotaxin concentrations averaged 3.43 ± 0.35 ng/ml (n = 16) in unstimulated cells and 3.48 ± 0.42 ng/ml (n = 14) in supernatant of cells stimulated with IL-9 (50 ng/ml for 24 h). In contrast, IL-13 (10 ng/ml) caused a marked and signifi-
cant increase in eotaxin to 13.3 ± 2.7 ng/ml (n = 20) (P < 0.01). However, when IL-9 was combined with IL-13, IL-9 augmented the ability of IL-13 to increase eotaxin (Fig. 9). The effect was statistically significant at IL-9 concentra-
tions of 10 and 30 ng/ml. IL-9 at any concentration had no signifi-
cant effect on IL-4-induced eotaxin release (data not shown).

In other cell types, the ability of IL-13 to induce eotaxin occurs in part through effects on the eotaxin promoter (33). To determine whether the synergism between IL-13 and IL-9 occurred at the level of eotaxin transcription, we examined the effects of these cyto-

![Graph of IL-8 release](image1)

![Graph of IL-8 promoter activity](image2)

![Graph of eotaxin release](image3)
kines alone and in combination on eotaxin promoter activity (Fig. 10). IL-13 (10 ng/ml for 24 h) caused a significant increase in normalized luciferase activity in cells transfected with an eotaxin promoter-firefly luciferase reporter construct, whereas IL-9 (30 ng/ml for 24 h) had no effect on its own and did not augment the ability of IL-13 to increase eotaxin promoter activity.

We and others (15, 37) have previously reported that ERK activation is, in part, required for IL-13-induced release of eotaxin from HASM cells. Because our data (Figs. 4 and 5) indicated that IL-9 caused ERK activation, we examined the effect of U-0126 (10 μM) on eotaxin release in HASM cells stimulated with IL-13 (10 ng/ml for 24 h), alone or in combination with IL-9 (30 ng/ml for 24 h) (Fig. 11). U-0126 had no effect on eotaxin release in unstimulated cells but significantly reduced eotaxin release in cells treated with IL-13 either alone or in combination with IL-9. As reported above, IL-9 significantly increased IL-13-induced eotaxin release in control cells. However, treatment with U-0126 abolished this synergism: in cells treated with U-0126, eotaxin release induced by IL-9 in combination with IL-13 was not different from that induced by IL-13 alone. To determine whether the synergism between IL-13 and IL-9 occurred at the level of ERK activation, we used Western blotting to examine ERK phosphorylation (Fig. 12). As described above, both IL-9 and IL-13 induced ERK phosphorylation. However, when the two cytokines were administered simultaneously, the effect on ERK phosphorylation was additive rather than synergistic. ERK phosphorylation in cells treated with IL-9 and IL-13 was not significantly different from the sum of ERK phosphorylation induced by the two cytokines individually.

IL-9 has also been shown to synergize with other ligands in the induction of COX-2 expression in other cells (38). Because AA metabolites have been shown to modulate IL-8 release by HASM cells (43), because IL-9 caused ERK activation (Figs. 4, 5), and because we have previously reported that COX-2 expression is...
dependent on ERK activation (23), we examined the effects of IL-9 on COX-2 expression in HASM cells from four different donors. IL-9 (50 ng/ml for 24 h) did not induce COX-2 expression, although IL-1β (2 ng/ml for 24 h) had a marked effect. IL-9 also had no effect on the expression of COX-2 that was induced by IL-1β (data not shown). Similarly, IL-9 alone had no effect on the concentration of PGE2 in HASM cell supernatants and did not alter IL-1β-induced PGE2 release either in unstimulated cells (Fig. 13) or in cells treated with AA (data not shown).

**DISCUSSION**

Our results indicate that in HASM cells, IL-9 is capable of activating ERK and amplifying the release of eotaxin and IL-8 caused by other cytokines. Furthermore, the effects of IL-9 appear to be at least in part mediated by ERK. To our knowledge, this is the first report of functional responses to IL-9 in airway smooth muscle cells in any species.

Our results indicate that IL-9 causes a concentration- and time-dependent activation of ERK in HASM cells (Figs. 4 and 5). IL-9 has also been shown to induce Ras activation in murine T-helper clones TS 2 and TS 3, which would lead to ERK activation (30). In contrast, IL-9 does not appear to cause ERK activation in human M07e cells (36) and murine HT-2 cells (3), suggesting that this effect can be cell type specific. In hematopoietic cells, binding of IL-9 to the IL-9Rα results in JAK phosphorylation of the IL-9Rα (3, 5, 57), leading to its interaction with IRS-1/2, which then also becomes phosphorylated (57). Phosphorylated IRS-1/2 is capable of interacting with SH-2 domains of the adaptor protein Grb2, which is constitutively complexed to Sos; Sos can in turn activate Ras, leading to Raf activation and subsequent activation of the ERK MAP kinase pathway (39). The adaptor protein 14-3-3ζ also interacts with IL-9Rα and is capable of Raf activation, which could lead to ERK activation (50).

IL-8 is a CXC chemokine that causes neutrophil migration, degranulation, and respiratory burst activity (2). IL-8 was originally identified in supernatants of monocytes, but it has also been shown to be released from HASM cells in response to IL-1β or TNF-α (14, 15, 18, 55). Because IL-9 has been shown to induce release of IL-8 from human polymorphonuclear leukocytes (1), we measured the release of IL-8 into HASM cell supernatants in response to IL-9 in the presence and absence of TNF-α. We found that IL-9 alone had no effect on IL-8 release (Fig. 1). However, IL-9 significantly increased the release of IL-8 induced by TNF-α. In a preliminary report, Gounni et al. (12) also noted effects of IL-9 on IL-8 release from human bronchial smooth muscle cells but observed effects of IL-9 even in the absence of stimulation with other cytokines.

Our results (Fig. 6) confirm previous observations (14) indicating that TNF-α-induced IL-8 release from HASM cells is partly dependent on ERK activation. Our results also indicate that the ability of IL-9 and TNF-α to synergize in the induction of IL-8 by HASM requires ERK activation. We do not know the precise role of ERK in these events. However, previous results from our lab indicate that inhibition of ERK activation causes a small but significant attenuation in IL-1β-induced NF-κB activation in HASM cells (24), and NF-κB activation has been shown to be important for induction of IL-8 in a variety of cell types (28, 41, 54). Furthermore, IL-9 alone induced a small but significant activation of NF-κB in HASM cells (Fig. 2) consistent with its effects in other cell types (47). However, IL-9 did not synergize with TNF-α in the induction of NF-κB (Fig. 2). Furthermore, transient transfection analysis with an IL-8 promoter reporter construct did not provide evidence to support a transcriptional mechanism for the effect of IL-9 (Fig. 3). It is possible that effects of IL-9 on promoter activity may require response elements upstream of the 1,634-bp promoter fragment examined. In addition, an important regulatory role has been ascribed to intronic regions in other genes (25), and such regions were not represented in our promoter fragment. However, we believe it is more likely that IL-9 is acting downstream of transcription, either at the level of mRNA stability or protein translation. The role of ERK in the synergism between TNF-α and IL-9 is also likely to be mediated downstream of transcription, because U-0126 did not affect TNF-α-induced increases in IL-8 promoter activity (Fig. 7) even though it had a very striking effect on TNF-α-induced IL-8 release (Fig. 6). A role for ERK in mRNA stability has been demonstrated for other genes (7, 56), and the IL-8 gene contains AU-rich regions in its 3′-untranslated region that may be important for mRNA stability.

Eotaxin is a chemokine belonging to the CC family and was initially isolated from lung lavage fluid of a guinea pig model of allergic disease (19). It has been shown to be a potent chemoattractant for eosinophils...
both in vitro and in vivo (9, 48). Structural cells in the airways have been identified as important sources of eotaxin. In particular, airway smooth muscle cells constitutively express eotaxin mRNA and protein (10, 37) and recently the Th2 cytokines IL-4 and IL-13 have been identified as important stimuli for the release of eotaxin from these cells (15, 37). The data presented here indicate that another Th2 cytokine, IL-9, synergizes with IL-13 to enhance release of eotaxin, even though IL-9 by itself does not cause release of the chemokine (Fig. 9).

Eotaxin release and pulmonary recruitment of eosinophils caused by allergen sensitization are reduced in IL-9 knockout mice (35) or mice treated with IL-9 antibodies (21). Similarly, transgenic overexpression of IL-9 in the lung or pulmonary administration of exogenous IL-9 results in increased release of eotaxin into BAL fluid, as well as increased eotaxin mRNA expression (6). Pulmonary overexpression of IL-9 also causes an increase in IL-13 expression in the lungs, and IL-13 blocking antibodies completely ablate the pulmonary eosinophilia that is induced by IL-9 expression (53). Our results (Fig. 9) indicate that IL-9 augments the ability of IL-13 to cause eotaxin release from HASM cells. Taken together, the results suggest that smooth muscle cells may be one of the cellular targets for IL-9 that contribute to its ability to promote eotaxin release in the lung and that IL-9 may induce these effects through interactions with IL-13. We and others have reported that ERK is important for the induction of eotaxin by a variety of cytokines in HASM cells (13, 15, 37). Results reported here indicate that the ability of IL-9 to enhance eotaxin release also requires ERK (Fig. 11). We do not yet know the mechanistic basis for the ERK dependence of the effects of IL-9. However, such effects are unlikely to involve activation of transcription factors important for induction of eotaxin expression because synergism between IL-9 and IL-13 was observed at the level of protein expression (Fig. 9), but not at the promoter level (Fig. 10).

It is curious that IL-9 enhanced the ability of IL-13, but not IL-4, to induce eotaxin, because in HASM cells, both IL-13 and IL-4 act at the type II IL-4 receptor, a dimer composed of the IL-4Ra1 and the IL-13Ra1. It is theoretically possible that IL-9 exerts its effects on IL-13 responsiveness by inhibiting IL-13RaII expression. The latter is thought to be a decoy receptor for IL-13, and reductions in its expression would be expected to enhance responses to IL-13 without affecting response to IL-4. However, if IL-9 acted by changing IL-13RaII expression, we would have expected to see an increased effect of IL-13 at every step in the signal transduction pathway. Instead, IL-9 augmented IL-13-induced eotaxin release without inducing any effect on its own, a synergistic effect (Fig. 9), whereas it resulted in only an additive effect on ERK activation when given in conjunction with IL-13 (Fig. 12). We have also reported an effect of IL-13 but not IL-4 on β-adrenergic responses in HASM cells (22), while others have reported differences in the pattern of gene expression induced by these two cytokines (26). We do not know the mechanistic basis for these differences, but it is possible that when IL-13 is bound, the conformation of the receptor is not the same as when IL-4 is bound, leading to differences in the signal transduction pathways activated by the two cytokines.

PGE2 has been reported to increase IL-8 release in HASM cells (43). Because IL-9 promotes the induction of COX-2 in other cell types (38), we considered the hypothesis that IL-9 might be acting to enhance IL-8 release by increasing PGE2 release in HASM cells, either by inducing COX-2 expression or by activating phospholipase A2 (PLA2) and thus increasing synthesis of PGE2 from endogenously expressed COX-1. However, IL-9 had no effect on PGE2 release in HASM cells either alone or in combination with IL-1β (Fig. 13) or when PLA2 was bypassed by addition of AA to the medium. IL-9 also had no effect on COX-2 either alone or in the presence of IL-1β.

In hematopoietic cells, IL-9 signals through a dimer composed of the IL-9Rα and γc (5). The IL-9Rα is expressed in HASM cells (12), but in unstimulated HASM cells, γc cannot be detected either by RT-PCR or by flow cytometry (15, 22). Nevertheless, IL-9 is capable of activating the cells and of inducing physiological responses if coupled with other cytokines. It is possible that γc is expressed in HASM cells but that its expression is below the limit of detection of the assays used. It is also possible that TNF-α induces expression of γc in HASM cells, as has been proposed by others (51), and that this accounts for the synergism between TNF-α and IL-9. Alternatively, it is possible that in nonhematopoietic cells, the IL-9Rα forms dimers with other, as yet unrecognized, receptors.

In summary, we found that in HASM cells, IL-9 enhanced the release of IL-8 and eotaxin induced by other cytokines, and these effects were dependent on ERK activation. Because IL-9 is expressed in the asthmatic airway (49), these results suggest that IL-9 can influence the regulation of chemokine release from HASM cells, thus contributing to the establishment of the inflammatory milieu observed in asthmatic airways.

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REFERENCES
3. Bauer JH, Liu KD, You Y, Lai SY, and Goldsmith MA. Heteromerization of the γc chain with the interleukin-9 receptor


