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## IL-13 may mediate allergen-induced hyperresponsiveness independently of IL-5 or eotaxin by effects on airway smooth muscle

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**Eum, Seok-Yong, Karim Maghni, Barbara Tolloczko, David H. Eidelman, and James G. Martin.** IL-13 may mediate allergen-induced hyperresponsiveness independently of IL-5 or eotaxin by effects on airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 288: L576–L584, 2005. First published November 24, 2004; doi: 10.1152/ajplung.00380.2003.—IL-13 is a mediator of allergen-induced airway hyperresponsiveness (AHR). The aim of this study was to evaluate whether eotaxin and IL-5 were implicated in the effects of IL-13 on allergen-induced AHR or whether IL-13 may exert its effects through direct actions on airway smooth muscle (ASM). To study this question airway inflammation and AHR were induced in mice by sensitization and subsequent challenge on three successive days with ovalbumin. A monoclonal anti-IL-13 antibody administered before each challenge significantly reduced AHR without affecting airway eosinophilia. No changes of mRNA in BAL and lung tissues or protein levels in BAL of IL-5 or eotaxin were found following anti-IL-13 treatment. Combined injection of monoclonal anti-IL-5 and antieotaxin antibodies before each antigen challenge blocked airway eosinophilia but failed to reduce AHR. IL-13 induced calcium transients in cultured murine ASM cells and augmented the calcium and contractile responses of these cells to leukotriene D<sub>4</sub>. These results suggest that IL-13 plays an important role in allergen-induced AHR and is important in the early phases of the inflammatory process. Its effects on AHR are mediated independently of IL-5 and eotaxin and may involve a direct effect on ASM to augment its responsiveness.

asthma; interleukin-13; airway eosinophilia; calcium signals; mice

T CELLS OF THE TH<sub>2</sub> PHENOTYPE are closely associated with eosinophilic airway inflammation and airway hyperresponsiveness (AHR), hallmarks of allergic asthma. Th<sub>2</sub> cells secrete a characteristic profile of cytokines, including IL-4, IL-5, IL-6, IL-10, and IL-13. IL-13 is a pleiotropic cytokine that is produced by activated CD4<sup>+</sup> T lymphocytes (2), and there is growing evidence of its importance in causing several of the key features of asthma such as goblet cell hyperplasia, mucous hypersecretion, eosinophilia, airway fibrosis, and AHR (33). IL-13 has been linked to AHR by several lines of evidence. Overexpression of IL-13 in the airway epithelial cells of the mouse is associated with AHR (33). Exogenous IL-13 evokes AHR, whereas the systemic administration of a soluble IL-13 alpha2-IgG-Fc fusion protein inhibits allergen-induced AHR in the mouse (31). Finally, IL-13 gene-deleted mice fail to develop allergen-induced AHR, but AHR is restored by the administration of recombinant IL-13 (30).

The mechanisms of action of IL-13 that could account for its role in allergen-induced AHR are not clear. The induction of AHR by IL-13 seems to be dependent on STAT6 expression by

airway epithelium (15), but the links to airway smooth muscle (ASM), the effector of airway narrowing, have not been made. IL-13 could potentially mediate its effects indirectly through proinflammatory actions. Pulmonary overexpression of IL-13 causes an increase in airway eosinophilia and a marked increase in eotaxin synthesis (33). Eotaxin, unlike all other eosinophil chemoattractants, is eosinophil specific (14, 22) and also has a potent and selective effect in mobilizing bone marrow eosinophils into the blood (21). The effects of IL-13 on eotaxin production are somewhat selective in that other eosinophilia-promoting cytokines such as IL-5 and granulocyte-macrophage colony-stimulating factor are not similarly affected (33). When IL-13 is administered to IL-5- and eotaxin-deficient naïve mice, the AHR induced is attenuated compared with wild-type animals, suggesting a partial dependence of IL-13's actions on these cytokines (32). It is unclear to what extent IL-13 interacts with eotaxin and IL-5 in the generation of AHR following sensitization and challenge with allergen. We hypothesized that the mediation of allergen induced AHR by IL-13 does not necessarily involve eosinophilic airway inflammation but may result from direct effects on ASM. IL-13 has been described to affect the responses of ASM cells and intestinal smooth muscle segments to contractile agonists (1, 9, 26). If IL-13 were able to enhance ASM responses to cysteinyl-leukotrienes (cys-LTs), this could have an important bearing on the mechanisms of allergen-induced AHR, given the evidence that cys-LTs are of key importance in AHR in murine models of allergic asthma (5). To address these issues, we used a murine model of allergic asthma to explore some of the potential pathways through which IL-13 causes AHR. We used antibodies against IL-5 and eotaxin to block the participation of these cytokines in AHR, and we tested the effects of IL-13 on the contractile responses of murine ASM cells in culture to leukotriene D<sub>4</sub> (LTD<sub>4</sub>), by examining intracellular calcium responses and cell contractions.

### MATERIALS AND METHODS

**Immunization and antigen challenge of mice.** Male BALB/c mice of 8–10 wk (24–25 g) were purchased from Harlan Sprague-Dawley, (Indianapolis, IN) and immunized twice at 7-day intervals with 100 µg of ovalbumin (OVA) sc in 0.4 ml of a 4-mg/ml suspension of Al(OH)<sub>3</sub>. One week after the second injection, mice were challenged once or three times daily with 10 µg of OVA in 50 µl of sterile saline intranasally under light anesthesia. All procedures were approved by an Institutional Animal Care Committee.

**Evaluation of airway responsiveness.** Airway responsiveness was measured 48 h after the final OVA challenge. Mice were anesthetized

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(8 mg/kg xylazine and 70 mg/kg pentobarbital ip), tracheostomized, paralyzed (0.5 mg/kg doxacurium ip), and placed on a small animal ventilator (Flexivent; SCIREQ, Montreal, Qc, Canada). Animals were ventilated at a respiratory frequency of 150 breaths/min, tidal volume of 6 ml/kg, and a positive end-expiratory pressure of 1.5 hPa. After a standard volume history, small amplitude volume oscillations at frequencies of 0.9, 4.8, and 10.4 Hz were applied at constant lung volume to the tracheal opening for 16 s and respiratory system resistance ( $R_{rs}$ ) was measured (7). Preliminary experiments indicated that responses at 0.9 Hz exceed those at other frequencies so that only results for this frequency of oscillation are reported. Methacholine was injected through the jugular vein every 5 min at doses of 10, 33, 100, and 330  $\mu$ g/kg. After each injection the changes in  $R_{rs}$  were computed over a 16-s interval from flow, volume, and tracheal pressure signals, and the peak response was identified and retained for analysis.

**Procedures for bronchoalveolar lavage.** After the measurement of airway responses, bronchoalveolar lavage (BAL) was performed using phosphate-buffered saline (PBS). The first 0.5 ml of BAL was kept for ELISA, and the subsequent eight volumes of 0.5 ml were used for other measurements. Total cell numbers were counted with a standard hemacytometer. The cytosin slides of BAL cells were prepared using a Cytospin model II cytocentrifuge (Shandon, Pittsburgh, PA) and stained with May-Grunwald-Giemsa stain. Differential cell counts were determined by light microscopy from a count of at least 200 cells. Absolute cell numbers were also calculated.

**Treatments with anti-IL-13 or combined antibodies against anti-IL-5 and antieotaxin.** Monoclonal mouse anti-IL-13 antibody (R&D Systems, Minneapolis, MN) was injected (0.1 mg/kg iv) into immunized mice 30 min before antigen challenge. To see the effect of the timing of administration of a single injection of anti-IL-13, we injected the same dose of antibody once intravenously 30 min before the first or before the last of three challenges. This concentration of anti-IL-13 has been shown to effectively block OVA-induced AHR (6). Monoclonal mouse anti-IL-5 and antieotaxin antibodies (R&D Systems) were injected simultaneously into immunized mice at 0.1 and 0.8 mg/kg iv, respectively, 30 min before each antigen challenge. These antibody concentrations were shown to be effective in preventing allergen-induced airway eosinophilia (6). The evaluation of BAL and AHR was performed 48 h after the last challenge.

**IL-13, IL-5, and eotaxin levels in BAL fluids.** The levels of IL-13, IL-5 and eotaxin in BAL fluids (BALF) were determined by ELISA. Briefly, 96-well ELISA plates were coated with 50  $\mu$ l of an anti-mouse IL-13 monoclonal antibody (R&D Systems) at 4  $\mu$ g/ml, IL-5 monoclonal antibody (TRFK-5, R&D Systems) at 2  $\mu$ g/ml, or with 100  $\mu$ l of an anti-mouse eotaxin polyclonal antibody (R&D Systems) at 0.2  $\mu$ g/ml in 0.1 M  $\text{Na}_2\text{HPO}_4$  (pH 9.0) at 4°C overnight. The plates were blocked with PBS containing 10% fetal bovine serum (200  $\mu$ l/well) for 1 h at room temperature and then were washed with PBS containing 0.05% Tween 20 before incubation with the BAL supernatants at 100  $\mu$ l/well for 2 h at room temperature. The plates were then washed with PBS/Tween 20 and were incubated with biotinylated anti-mouse IL-13 antibody (R&D Systems) at 0.2  $\mu$ g/ml, IL-5 monoclonal antibody (TRFK-4, Pharmingen) at 1  $\mu$ g/ml, or anti-mouse eotaxin polyclonal antibody (R&D Systems) at 25 ng/ml for 1 h at room temperature. For detection, the plates were incubated with 100  $\mu$ l of streptavidin-horseradish peroxidase (Pharmingen) for 30 min and washed again with PBS/Tween 20. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) substrate solution containing  $\text{H}_2\text{O}_2$  was added to the wells, and the plates were read at optical density of 405 nm. Under these conditions, these assays were sensitive to <10 pg/ml of murine IL-5, eotaxin, and IL-13.

**RT-PCR analysis.** Total RNA was extracted from BAL cells and lung tissues with TRIzol (Invitrogen, Mississauga, ON, Canada) as previously described (17). RNA pellets were dissolved in RNase- and DNase-free-tested water (Ambion, Austin, TX). Strand cDNA was made in a 20- $\mu$ l reaction, by use of 2  $\mu$ g of total RNA as template,

oligo(dT)<sub>12-18</sub> primer, and Superscript II enzyme in the presence of BSA acetylated (Invitrogen) and RNAGuard ribonuclease (Pharmacia Biotech, Quebec, Canada) as enzyme inhibitors. The PCR mixture (final concentration) consisted of 1.5 mM  $\text{MgCl}_2$ , 1 $\times$  PCR buffer, 0.2 mM dNTP mixture, 2.5 units Platinum Taq polymerase (Invitrogen), 20 pmol of the upstream and downstream primers, as well as the synthesized cDNA strand. The primers used were 5'-ACTCT-CAGCTGTGTCTGGG-3' (sense) and 5'-GCCCACTCTGTACT-CATCAC-3' (antisense) for mouse IL-5, 5'-TTCTATTCCTGCT-GCTCACG-3' (sense) and 5'-TTATGGTTTGGAGTTTGGAG-3' (antisense) for mouse eotaxin and 5'-GGTCAACCCACCAGTGT-TCTTCG-3' (sense) and 5'-GTGCTCTCCTGAGCTACAGAAGG-3' (antisense) for cyclophilin. The samples were amplified in a Programmable Thermal Controller (PTC-100; MJ Research, Watertown, MA) with 1 min of denaturation at 92°C, 2 min of annealing at 56°C, and 3 min of extension at 72°C for IL-5 and eotaxin, and 1 min of denaturation at 92°C, 2 min of annealing at 60°C, and 3 min of extension at 72°C for the housekeeping gene cyclophilin. The linear range of PCR cycles for the amplification of IL-5 and eotaxin was over 30, 32, 35, and 38 PCR cycles; beginning of saturation at 40 cycles. For cyclophilin, the linear range of PCR cycles was over 26, 28, and 30 PCR cycles; beginning of saturation at 32 cycles. Therefore, mRNAs for IL-5 and eotaxin were amplified at 35 cycles and cyclophilin at 28 cycles. The PCR products were visualized by ethidium bromide staining after gel-agarose (2%) electrophoresis, and the correct size of the bands was determined by comparison with DNA molecular weight markers (Roche Molecular Biochemicals, Montreal, Qc, Canada). The efficiency of the PCR primers designed for IL-5 and eotaxin was initially tested in PCR assays using RNA extracted from mouse lung tissues. A negative PCR control was included for the RT reaction (no RNA). PCR primers were synthesized and purified by fast protein liquid chromatography at the Sheldon Biotechnology Centre (Montreal, Qc, Canada).

**Murine airway smooth cell cultures and calcium responses to IL-13 and LTD<sub>4</sub>.** Tracheal smooth muscle cells were isolated from the tracheae of two mice and cultured following previously described methods for the rat (27). Briefly, the cells were enzymatically dissociated with 0.05% elastase type IV and 0.2% collagenase type IV and cultured in 1:1 Dulbecco's modified Eagle's medium-Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS), 0.2%  $\text{NaHCO}_3$ , and 1% penicillin-streptomycin in the presence of 5%  $\text{CO}_2$ . Cell culture reagents were purchased from Invitrogen. First- or second-passage cells were rendered quiescent by incubation in medium containing 0.5% FBS for 4 days before experiments. Confirmation of a smooth muscle phenotype was based on typical morphology, positive smooth muscle-specific  $\alpha$ -actin staining, negative keratin staining, and contractile responses to agonists. For the measurement of calcium cells were loaded with the  $\text{Ca}^{2+}$ -sensitive dye, fura-2 AM (Molecular Probes, Eugene, OR) according to the previously described methods (27) and imaged using an intensified charge-coupled device camera (IC200) and PTI software at a single emission wavelength (510 nm) with a double excitatory wavelength (340 and 380 nm). Fluorescence ratio (340/380) was measured in cells stimulated with IL-13 (0.5, 5, and 50 ng/ml) and LTD<sub>4</sub> (1  $\mu$ M) or appropriate vehicle. Intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ) was calculated according to the formula of Grynkiewicz et al. (10). Each experimental group consisted of 102–115 cells.

**Murine ASM contractile responses to IL-13 and LTD<sub>4</sub>.** Changes in cell surface area in cells stimulated by IL-13 and LTD<sub>4</sub> were measured, and the decrease in cell area was used to estimate cell contraction. Cells were plated on homologous cell substrate, as previously described (24) and rendered quiescent by serum deprivation for 48 h. Images of cells stimulated with IL-13 (50 ng/ml) or vehicle were acquired every 2 s for 5 min and every min for additional 5 min using a video camera (Hamamatsu Photonics, Hamamatsu City, Japan) mounted on a microscope equipped with Nomarski optics (Nikon



Diaphot, Nikon, Tokyo, Japan) and PTI software. The surface area of the cells was measured before and 10 min after stimulation.

**Statistical analysis.** The data are presented as cumulative frequency curves for contraction experiment and as means  $\pm$  SE for the remaining experiments. Statistical comparisons for calcium experiments were performed using Dunnett and Student's *t*-tests, for contraction experiments using Kolmogoroff-Smirnoff test, and for remaining experiments one-way analysis of variance followed by Student's *t*-test.

## RESULTS

**Effect of anti-IL-13 antibody on airway inflammation and BALF IL-5 and eotaxin levels after a single antigen challenge in immunized mice.** First we wished to examine the role of IL-13 in the early events associated with the induction of airway inflammation in response to a single allergen exposure. For this purpose we administered anti-IL-13 antibody before a single OVA challenge, and BAL was evaluated 24 and 48 h after challenge. Eosinophil and neutrophil numbers in BAL were increased after OVA challenge at 24 h ( $5.4 \pm 1.3$  and  $5.4 \pm 0.7 \times 10^4$  cells/ml, respectively) and at 48 h ( $9.3 \pm 1.5$  and  $1.2 \pm 0.3 \times 10^4$  cells/ml, respectively) compared with saline-challenged control mice ( $0.0 \pm 0.0$  and  $0.3 \pm 0.1 \times 10^4$  cells/ml, respectively,  $P < 0.001$ ; Fig. 1). Macrophages were also significantly increased ( $14.4 \pm 2.2$  and  $9.7 \pm 1.3 \times 10^4$  cells/ml at 24 and 48 h, respectively;  $P < 0.005$ ) compared with saline-challenged control mice ( $5.6 \pm 0.6 \times 10^4$  cells/ml, Fig. 1). Lymphocytes did not change significantly at either time point. Administration of anti-IL-13 reduced eosinophil and neutrophil numbers at 24 h ( $2.1 \pm 0.6$  and  $2.3 \pm 0.4 \times 10^4$  cells/ml, respectively,  $P < 0.05$ ), but there were no changes at 48 h (Fig. 1). No significant reduction of macrophages was observed.

IL-5 levels were increased in the BALF of OVA-challenged mice at 24 and 48 h compared with sham-challenged controls. IL-5 was higher at 24 h than at 48 h. The administration of anti-IL-13 before challenge did not affect these values (Fig. 2A). Eotaxin was also higher at 24 h after challenge than at 48 h, but there was no OVA-specific increase in eotaxin, which was as high in sham-challenged animals as in OVA-challenged animals. There was no effect of anti-IL-13 treatment on eotaxin levels (Fig. 2B).

**Effect of anti-IL-13 antibody on airway inflammation and AHR after multiple antigen challenges in immunized mice.** A single antigen challenge produced no significant alteration of the responsiveness to methacholine measured 48 h after the exposure so that the effects of neutralizing IL-13 on AHR could not be tested for single allergen exposure (data not shown). In contrast to the effects of single challenge, mice developed AHR following three consecutive OVA challenges so that the effects of anti-IL-13 administered before each of three antigen challenges on BAL and measurements of AHR were evaluated at 48 h after the last challenge.

The intensity of airway inflammation following three OVA challenges was much greater than after a single challenge. Total cell and eosinophil numbers in BALF were increased in OVA-challenged mice ( $62.5 \pm 9.8$  and  $35.8 \pm 8.1 \times 10^4$  cells/ml, respectively) compared with saline-challenged animals ( $12.4 \pm 2.9$  and  $0.3 \pm 0.1 \times 10^4$  cells/ml,  $P < 0.001$ ; Fig. 3A). The BAL total cell and eosinophil numbers after treatment with anti-IL-13 ( $47.1 \pm 7.3$  and  $24.4 \pm 4.4 \times 10^4$  cells/ml,

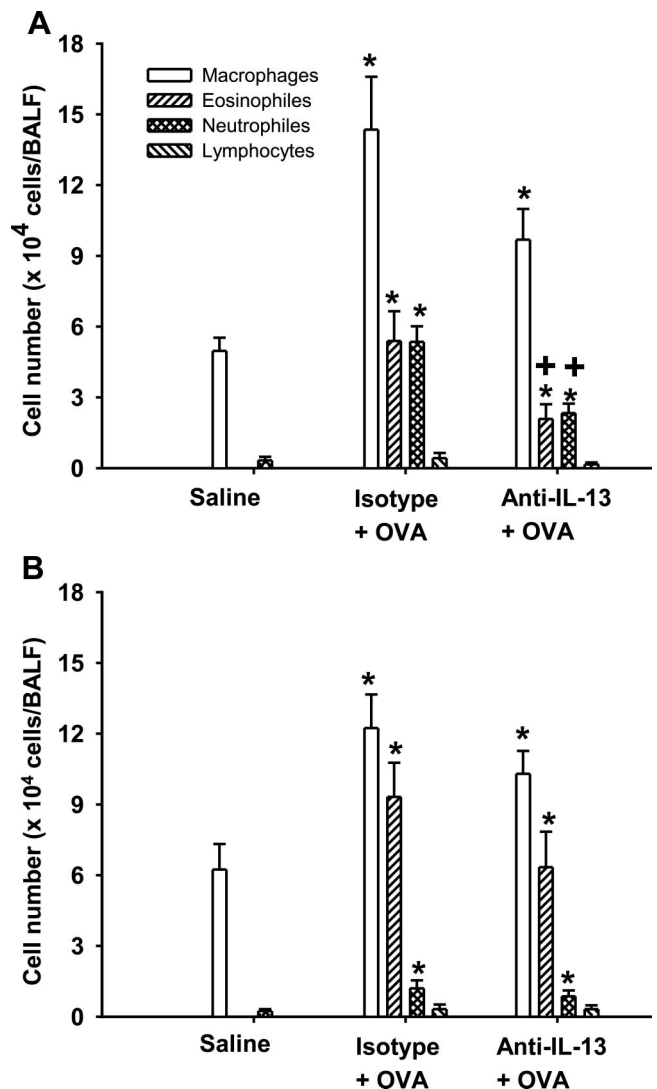


Fig. 1. Effect of a single antigen challenge on the bronchoalveolar lavage (BAL) fluid (BALF) leukocytes determined at 24 h (A) and at 48 h (B) in immunized BALB/c mice. Saline animals were ovalbumin (OVA) sensitized and saline challenged. Isotype+OVA animals were OVA sensitized and OVA challenged but received IgG isotype irrelevant antibody before challenge, whereas anti-IL-13+OVA animals were OVA sensitized and OVA challenged but received anti-IL-13 before challenge. Each bar represents the mean ( $\pm$ SE) of 5–6 mice. \*Significant differences between OVA-challenged group and saline control; +significant differences between IL-13-treated group and isotype controls.

respectively) were not significantly reduced compared with isotype-treated and OVA-challenged mice (Fig. 3A). Lymphocytes were significantly increased after OVA challenge and also were not affected significantly by anti-IL-13 administration (data not shown).

AHR was significantly reduced in mice treated with anti-IL-13 before each OVA challenge compared with animals treated with isotype antibody (Fig. 3B). When anti-IL-13 antibody was given before the first of the three challenges it failed to prevent the development of AHR. However, when antibody was injected before the last of the three OVA challenges, AHR was significantly reduced (data not shown). The injection of anti-IL-5 and antieotaxin antibodies did not significantly alter OVA-induced AHR (Fig. 4).

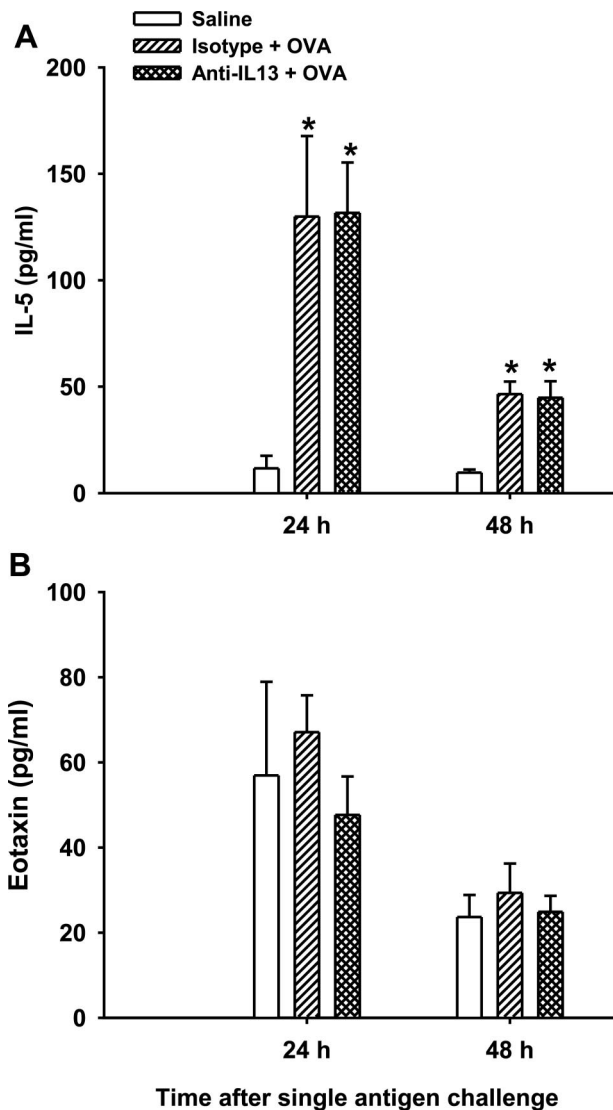


Fig. 2. IL-5 (A) and eotaxin (B) were measured by specific ELISA after a single OVA or saline challenge. The groups correspond to those described in the legend of Fig. 1. Each bar represents the mean ( $\pm$ SE) of 5–10 mice. \*Significant differences compared with saline control ( $P < 0.005$ ).

*Effects of anti-IL-13 on the mRNA levels of IL-5 and eotaxin detected by RT-PCR in mice after multiple antigen challenges.* The levels of IL-5 and eotaxin mRNA were evaluated using RT-PCR to amplify transcripts in BAL cells and lung tissues. BAL cells and lung tissues were harvested 48 h after the last of three challenges. In BAL cells of saline-challenged mice, the IL-5 mRNA levels were almost undetectable, but in lung tissues of some animals, detectable IL-5 mRNA levels were observed. Significantly increased levels of IL-5 mRNA were detected in both BAL cells and lung tissues after antigen challenges compared with saline-challenged mice. Anti-IL-13-treated mice showed similar levels of IL-5 mRNA to OVA-challenged mice (Fig. 5).

Eotaxin mRNA was almost undetectable in BAL cells whether harvested from OVA- or saline-challenged mice (results not shown). In contrast, eotaxin mRNA was readily detectable in lung tissues, but there were no differences be-

tween expression in lung tissues of saline-challenged and OVA-challenged mice. Furthermore, there was no effect of anti-IL-13 treatment observed on eotaxin expression (Fig. 6).

*Effect of anti-IL-13 antibody on IL-5, eotaxin, and IL-13 levels in BALF after three antigen challenges in immunized mice.* IL-5, eotaxin, and IL-13 levels were also measured 48 h after the last of three antigen challenges, at which time AHR was demonstrable. The IL-5 levels in BALF harvested from antigen-challenged mice were significantly higher ( $107.5 \pm 14.1$  pg/ml,  $P < 0.05$ ) than in sham-challenged mice ( $55.2 \pm 7.4$ ). The levels of eotaxin were also increased but to a similar degree in both sham-challenged ( $95.8 \pm 25.9$ ) and OVA-challenged mice ( $91.2 \pm 15.2$ ). Both IL-5 and eotaxin levels were much higher than at the 48-h time point after a single OVA exposure. Neither IL-5 nor eotaxin was affected by anti-IL-13 treatment ( $106.8 \pm 6.9$  and  $101.6 \pm 20.0$ , respectively). IL-13 levels in the BALF were comparable in the

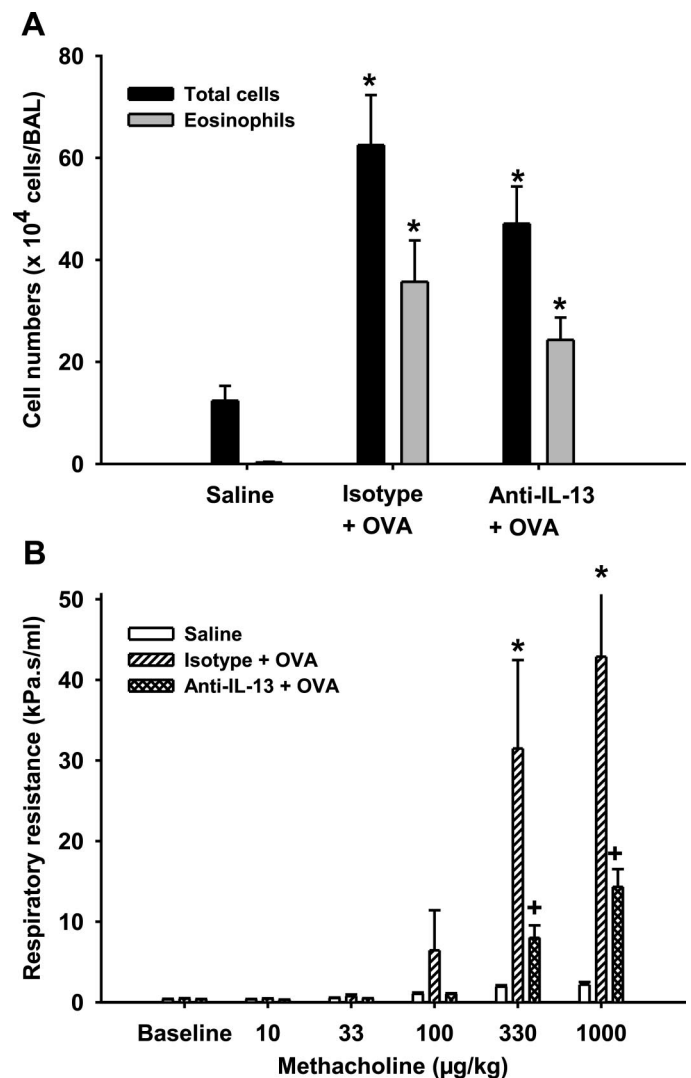


Fig. 3. Total cell and eosinophil counts following 3 saline or OVA challenges (A). The groups correspond to those described in the legend of Fig. 1. \*Significant difference from saline-challenged group. Respiratory system resistance following intravenous injections of methacholine is shown (B). \*Significant differences compared with saline control; + differences compared with isotype control. Each value represents mean  $\pm$  SE of 6–7 mice.

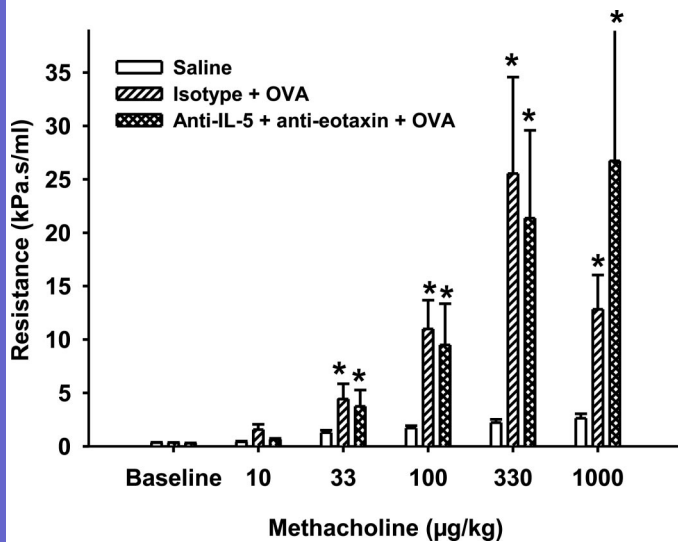


Fig. 4. Respiratory system resistance following intravenous injections of methacholine is shown. Means  $\pm$  SE are shown. \*Significant differences compared with saline. Each group comprised 6–7 mice.

BALF of sham-challenged animals ( $111.3 \pm 22.3$ ) and in mice after OVA challenge ( $94.0 \pm 10.9$ ). The levels were unaffected by anti-IL-13 treatment ( $94.1 \pm 16.7$ ).

**Effect of IL-13 on calcium transients in cultured murine ASM cells exposed to LTD<sub>4</sub>.** To evaluate the possibility that IL-13 may have direct effects on ASM that could account for its role in OVA-induced AHR, we placed ASM cells in culture and measured changes in  $[Ca^{2+}]_i$  evoked by IL-13 alone and in conjunction with LTD<sub>4</sub>. Figure 7A shows an example of a single cell response to IL-13 or the vehicle followed by stimulation with LTD<sub>4</sub>. Both IL-13 and LTD<sub>4</sub> caused an increase in  $[Ca^{2+}]_i$ . Prolonged recording of IL-13-stimulated cells revealed that there was no secondary peak at the time corresponding to LTD<sub>4</sub> application (Fig. 7A). IL-13 (5 and 50 ng/ml) evoked rapid, significant  $Ca^{2+}$  mobilization (from baseline level of  $104 \pm 6$  and  $104 \pm 6$  nM to  $134 \pm 11$  and  $214 \pm 12$  nM, respectively; Fig. 7B). Application of the vehicle had no effect. Stimulation of control (treated with vehicle for IL-13) ASM cells with 1  $\mu$ M LTD<sub>4</sub> resulted in a very modest  $[Ca^{2+}]_i$  increase (from  $95 \pm 4$  to  $120 \pm 11$  nM). However, addition of IL-13 (5 or 50 ng/ml) 2 min before LTD<sub>4</sub> stimulation caused augmentation of the response (from  $105 \pm 7$  to  $143 \pm 14$  nM and from  $147 \pm 8$  to  $214 \pm 12$  nM, respectively; Fig. 7B).

**Effect of IL-13 on cultured ASM cell contraction.** To investigate whether IL-13-induced  $Ca^{2+}$  mobilization and enhancement of  $Ca^{2+}$  responses to LTD<sub>4</sub> affected ASM contraction and/or cytoskeletal reorganization, changes in cell shape were monitored after addition of 50 ng/ml IL-13 or an appropriate vehicle followed by application of LTD<sub>4</sub> or its vehicle. Although IL-13 caused a decrease in cell area that was greater than the vehicle and in some cells very substantial, the effect was somewhat inconsistent so that the difference did not reach statistical significance. However, the decrease in cell area in cells stimulated with both IL-13 and LTD<sub>4</sub> was significantly different from all control groups (Fig. 8).

## DISCUSSION

In this study we have demonstrated that IL-13 has an important role in the induction of AHR in the BALB/c mouse sensitized and exposed to allergen on three consecutive days. Anti-IL-13 antibody abrogated the allergen-induced AHR but had only modest effects on the inflammatory response to allergen challenge. Inhibition of eosinophilia and neutrophilia by anti-IL-13 was observed but only at 24 h after challenge, whereas by 48 h the effect was no longer present. There was an allergen-specific increase in IL-5 expression that was unaffected by anti-IL-13. Eotaxin expression increased in BALF after airway challenges but did so in a nonspecific manner, showing a comparable increase after saline and after OVA, and, like IL-5, eotaxin expression was unaffected by anti-IL-13 antibody. However, IL-13 had direct effects on murine ASM in culture, causing  $Ca^{2+}$  transients and enhancing  $Ca^{2+}$  responses to LTD<sub>4</sub>. These findings indicate an alternative pathway by which IL-13 may contribute to the development of AHR after allergen challenge.

Experiments published to date strongly support the central role of IL-13 in the development of AHR in allergic asthma. IL-13 gene-deleted mice fail to develop allergen-induced AHR (30), and the inhibition of IL-13 through the administration of

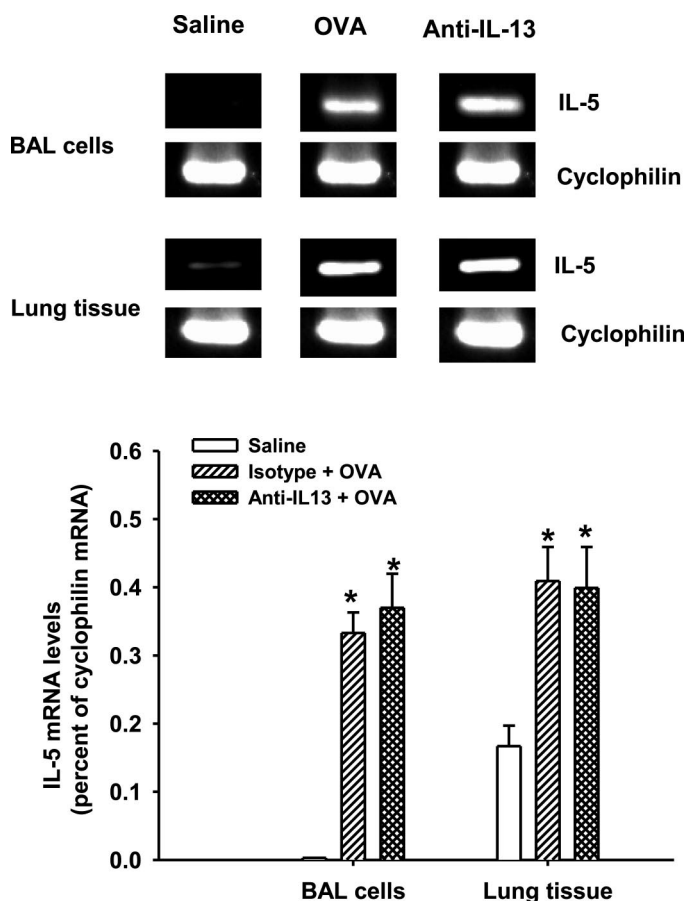


Fig. 5. RT-PCR analysis of IL-5 mRNA expression in BAL cells and lung tissue. The BAL cells and tissue were harvested 48 h after saline or antigen challenge. Four animals were used at each time point. Cyclophilin was used as a housekeeping gene. Top: agarose gel; bottom: results of densitometry for IL-5 mRNA compared with the density of cyclophilin mRNA. \* $P < 0.05$  vs. saline control.

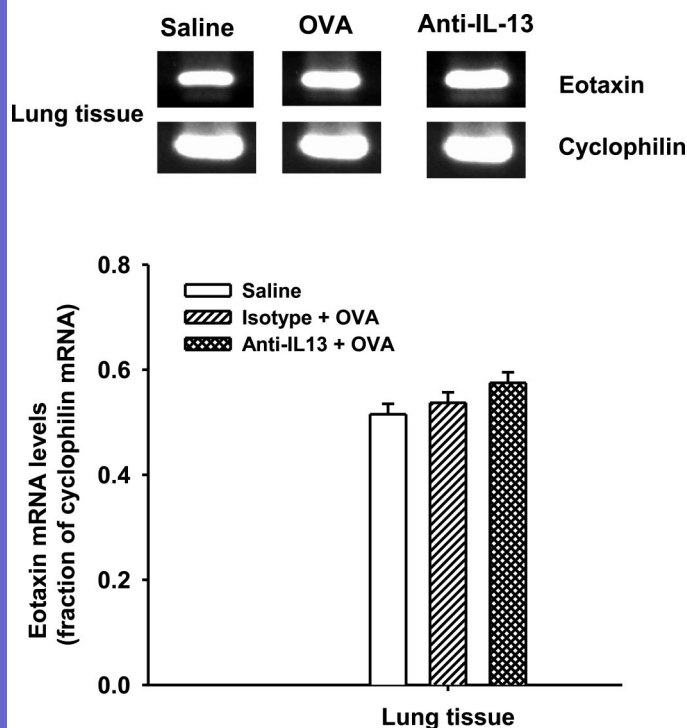


Fig. 6. RT-PCR analysis of eotaxin mRNA expression in lung tissues after saline or OVA challenge. Four animals were used at each time point. *Top*: agarose gel; *bottom*: results of densitometry for eotaxin mRNA compared with the density of the mRNA for the housekeeping gene, cyclophilin.

a soluble form of the IL-13 receptor (IL-13R $\alpha_2$ ) reverses OVA-induced AHR in mice (31) and guinea pigs (20). In the current study, a neutralizing antibody directed against IL-13 also inhibited the development of AHR after OVA challenge. As a cytokine produced by Th2 cells, IL-13 has actions that promote allergic inflammation. Repeated administration of IL-13 to naïve mice causes eosinophilic inflammation (31). Overexpression of IL-13 in the airway epithelium also causes eosinophilic inflammation (33). What is not clear is which, if any, proinflammatory effects of endogenously produced IL-13 are important for the development of AHR.

The results of the current study clearly demonstrate that IL-13 may influence the degree of eosinophilia and neutrophilia following allergen challenge. However, the effects appear to be modest at most. The inhibitory effects of an anti-IL-13 antibody on inflammation were evident only at 24 h after a single OVA challenge, and by 48 h the effects were no longer significant. When three challenges were performed, the administration of anti-IL-13 antibody before each of three OVA challenges was unable to significantly attenuate the airway inflammatory response. Consistent with our findings is the report that a single administration of IL-13 to mice causes a transient eosinophilia, whereas the inhibition of eosinophilia by IL-13R $\alpha_2$  is overcome by repeated allergen exposures (31). These findings raise the question as to the extent to which IL-13 affects AHR by modulating inflammation.

The possibility that IL-5 and eotaxin might have a role in allergen-induced AHR downstream of IL-13 has received some attention (32). IL-13 has been shown to stimulate the production of eotaxin from airway epithelial cells (33) and ASM cells (12, 19) and to induce upregulation of mRNA expression and

release of IL-5 protein from cultured human ASM cells (9). Eotaxin has been previously implicated in allergen-induced AHR (8) and IL-5 linked to increased responsiveness of IgE-sensitized ASM (9). Our study suggests that IL-13's role in AHR is independent of IL-5 and eotaxin for two reasons. First, the neutralization of IL-5 and eotaxin did not alter OVA-induced AHR, and, second, the levels of these cytokines were not affected by anti-IL-13 antibody. These results are consistent with the findings of Yang and colleagues (32), who have shown that exogenous IL-13 can induce AHR in mice that are IL-5 and eotaxin deficient. OVA-specific Th2 cells derived from IL-13-deficient mice fail to transfer AHR to OVA-challenged mice despite producing IL-4 and IL-5 (30), also suggesting that the dependence of AHR on IL-13 is not mediated by other Th2 cytokines.

There is evidence that IL-13 synthesis is, in part, dependent on IL-5 and eotaxin expression. Mattes and colleagues (18) have found that mice that are gene deleted for both IL-5 and eotaxin do not develop AHR and have reduced IL-13 production by CD4<sup>+</sup> T cells. The transfer of IL-13-producing anti-

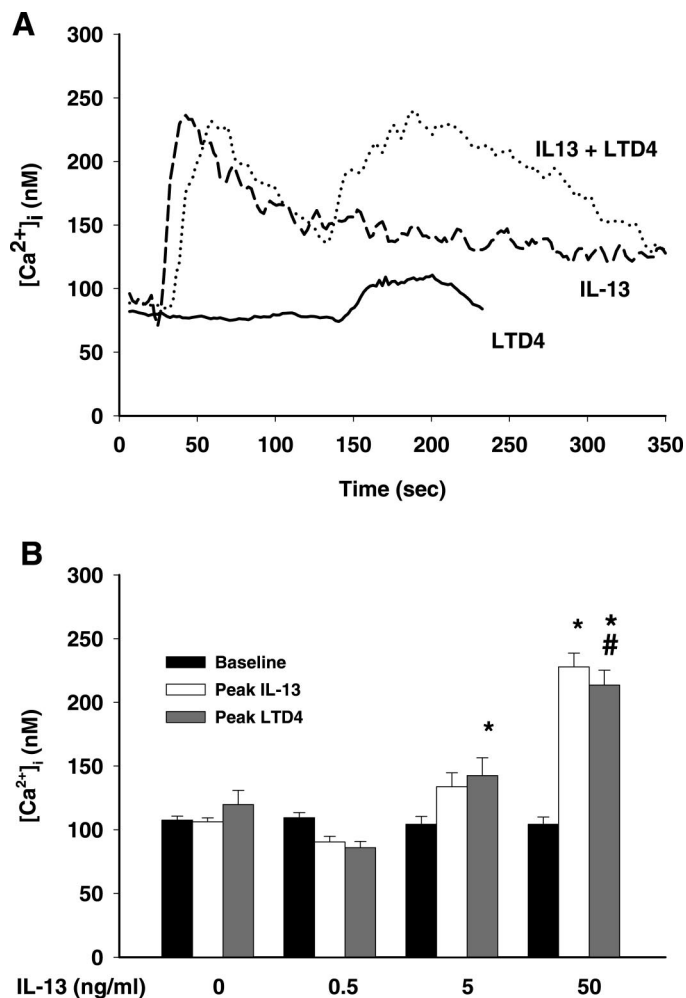
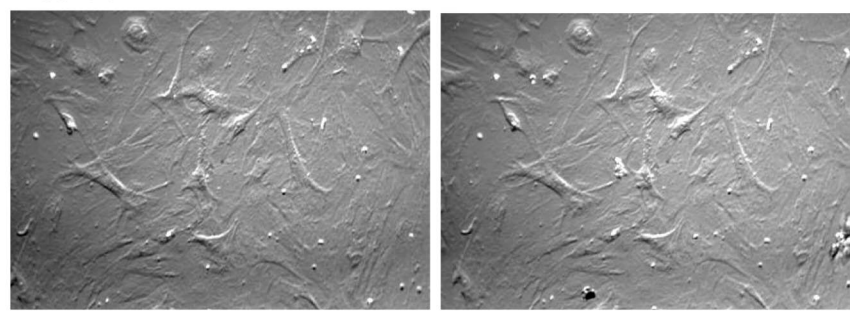


Fig. 7. Calcium responses of cultured airway smooth muscle (ASM) cells to IL-13 and IL-13 or appropriate vehicle followed by 1  $\mu$ M leukotriene D<sub>4</sub> (LTD<sub>4</sub>). *A*: representative example. Each curve represents a single cell. *B*: mean results from 3 independent experiments. \**P* < 0.01 vs. baseline, #*P* < 0.01 vs. LTD<sub>4</sub> response following application of the vehicle. Each value represents the mean  $\pm$  SE of 102–115 cells.



**A Control**



**IL-13+LTD<sub>4</sub>**

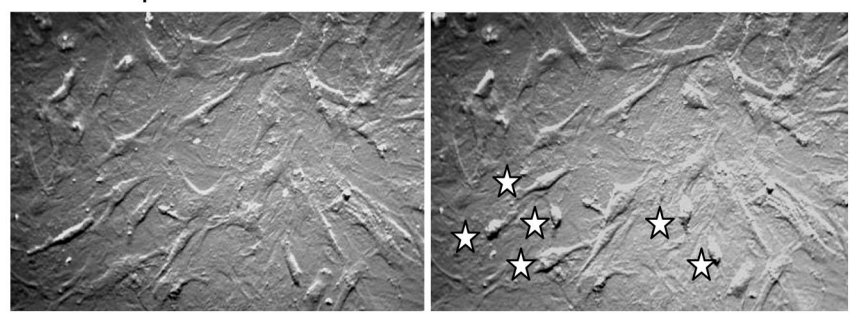
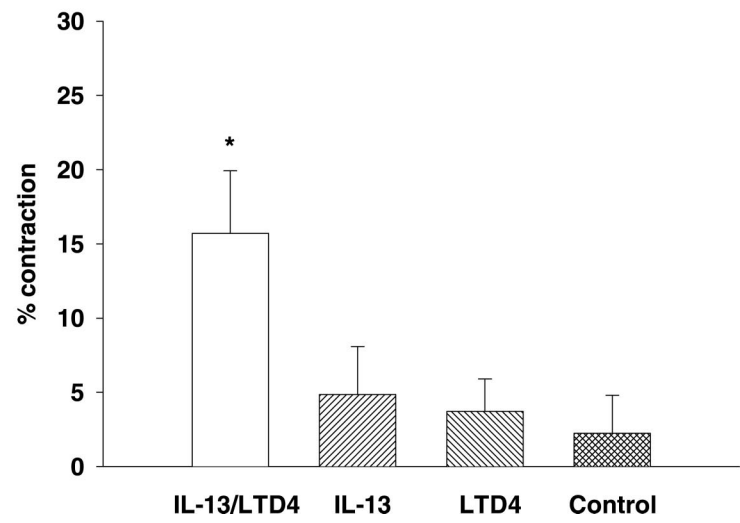


Fig. 8. Contractile responses of cultured ASM cells to IL-13 measured as a change in cell surface area. *A*: representative images of cells before and 10 min after application of 50 ng/ml IL-13. ☆, Contracted cells. *B*: mean responses of 30–40 cells stimulated with vehicle (control) LTD<sub>4</sub>, IL-13, or LTD<sub>4</sub> following IL-13.

**B**



gen-specific CD4<sup>+</sup> T cells to naïve mice restored the IL-13 levels in BALF to normal as well as the ability of the mice to develop AHR after allergen challenge. This effect was mediated through the eosinophil because the adoptive transfer of eosinophils restored the synthesis of IL-13 to normal levels. If one assumes a similar dependence of IL-13 synthesis in the current experiments, it is perhaps surprising that anti-IL-5 and anti-eotaxin antibodies did not reduce AHR. It has been argued that discrepancies between antibody neutralization experiments and the knockout animals lie in the possibility that antibodies against IL-5 and eotaxin may leave a significant residual eosinophilia in the tissues despite low numbers of

eosinophils in the BALF (18, 32). We have previously demonstrated that anti-IL-5 and anti-eotaxin, in the concentrations used in this study, cause both tissue and BAL depletion of eosinophils in the A/J mouse (6). However, in addition to these considerations, there were significant differences in the methods used to evaluate AHR between our study and that of Foster et al. (18, 32).

There is growing evidence that IL-13 may cause AHR independently of eosinophilic airway inflammation. AHR is rapidly induced by the administration of IL-13 to experimental animals (29). Exogenous IL-13 administered intratracheally has been shown to cause AHR as early as 6 h after the



exposure, and this early phase of AHR is independent of inflammation (32). Eosinophilic inflammation develops in allergen-challenged animals despite IL-13 gene deletion and clearly is not sufficient for AHR (30). Several reports indicate that IL-13-dependent AHR may result from a direct effect of IL-13 on ASM. Immunohistochemistry has revealed that both subunits of IL-13 receptor are prominently expressed on bronchial epithelium and ASM of asthmatic subjects (11). Shore and colleagues (16, 19) have confirmed that the IL-4R $\alpha$  chain as well as the IL-13R $\alpha_1$  and  $\alpha_2$  chains are expressed by cultured human ASM cells. These receptors are functional and mediate IL-13-dependent augmentation of contractility to ACh and impaired relaxation to isoproterenol in rabbit tracheal smooth muscle (9) as well as isoproterenol-induced decrease in cell stiffness in cultured human ASM cells (16, 19). A 24-h incubation of murine tracheal segments with IL-13 has also been shown to enhance responses to carbachol and potassium chloride (26).

On the basis of the observation that IL-13 stimulates secretion of eotaxin (12) and IL-5 by ASM cells (9), it has been postulated that the effect of IL-13 on ASM contractility may be mediated by autocrine signaling. However, our data do not support a role for either of these molecules in the mediation of AHR by IL-13 in the mouse. Recently it has been shown that IL-13-mediated augmentation of contractile responses in human ASM is at least partially dependent on increased Ca<sup>2+</sup> mobilization (26), which is in turn related to the increased expression of CD38 (3). In this study of murine cultured ASM cells, IL-13 triggers immediate Ca<sup>2+</sup> mobilization, indicating that there may be significant differences between murine and human ASM where direct IL-13-induced Ca<sup>2+</sup> transients were not observed. Of importance also is the ability of IL-13 to acutely prime ASM for responses to other agonists such as cys-LTs, which are involved in allergen-induced AHR in the mouse (5). Upregulation of the cys-LT1 receptor has been demonstrated on monocytes and macrophages (25) and ASM (4). However, the time course of the effect of IL-13 on responses to LTD<sub>4</sub> is short in the current study, and even if IL-13 can upregulate the Cys-LT1 receptor on ASM it could not account for our observations. The modification of ASM Ca<sup>2+</sup> signaling by IL-13 is an effect that may therefore cause or more likely contribute to bronchospasm in vivo through priming effects on ASM. Although the Ca<sup>2+</sup> transients that we observed were rapid in onset and had the typical appearance associated with phospholipase C/inositol trisphosphate-mediated signaling, further studies are required to determine which messengers are involved. Inositol trisphosphate-mediated signaling is not usually associated with IL-13 stimulation, and this signaling pathway has thus far been described only in monocytes (23). The signaling pathways implicated to date in ASM include a variety of kinases including JAK/STAT, p42/44 ERK, p38, and SAPK/JNK (12, 16, 19). These pathways are not known to participate in Ca<sup>2+</sup> mobilization but perhaps could be responsible for other changes in the contractile signaling in ASM found in other allergic models of AHR. Prominent among these changes is the induction of myosin light chain kinase (13). Whether IL-13 induces such changes in smooth muscle phenotype has not been explored. Our observation that IL-13 on its own does not cause a significant cell contraction despite inducing Ca<sup>2+</sup> transients but augments LTD<sub>4</sub>-induced contraction seems to indicate that IL-13 may

affect Ca<sup>2+</sup> without affecting the other signaling molecules that are activated by contractile agonists such as LTD<sub>4</sub>. Alternatively, contraction mediated by Ca<sup>2+</sup>-independent mechanisms that are a part of the LTD<sub>4</sub> signaling cascade together with increased Ca<sup>2+</sup> mobilization by IL-13 may be sufficient to cause contraction, whereas the increased Ca<sup>2+</sup> signal alone in response to acute IL-13 stimulation alone is not.

In conclusion, IL-13 causes AHR after allergen challenge by mechanisms that appear to be independent of the eosinophil, IL-5, and eotaxin. The apparent lack of involvement of IL-5 in mediating the effects of IL-13 (9) may be related to species differences. Direct effects on ASM are an alternative explanation for its actions, through a direct bronchoconstrictor effect or through an enhancement of the effects of cys-LT. A recent demonstration that exogenous IL-13 causes synthesis of cys-LTs in the murine lung (28) provides further support for the IL-13-cys-LT-ASM axis in the pathogenesis of allergen-induced AHR.

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#### REFERENCES

1. Akiho H, Blennerhassett P, Deng Y, and Collins SM. Role of IL-4, IL-13, and STAT6 in inflammation-induced hypercontractility of murine smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol* 282: G226–G232, 2002.
2. De Vries JE. The role of IL-13 and its receptor in allergy and inflammatory responses. *J Allergy Clin Immunol* 102: 165–169, 1998.
3. Deepak AD, Dogan S, Walseth TF, Miller SM, Amrani Y, Panettieri RA, and Kannan MS. Modulation of calcium signaling by interleukin-13 in human airway smooth muscle: role of CD38/cyclic adenosine diphosphate ribose pathway. *Am J Respir Cell Mol Biol* 31: 36–42, 2004.
4. Espinosa K, Bosse Y, Stankova J, and Rola-Pleszczynski M. CysLT1 receptor upregulation by TGF- $\beta$  and IL-13 is associated with bronchial smooth muscle cell proliferation in response to LTD<sub>4</sub>. *J Allergy Clin Immunol* 111: 1032–1040, 2003.
5. Eum SY, Maghni K, Hamid Q, Campbell H, Eidelman DH, and Martin JG. Involvement of the cysteinyl-leukotrienes in allergen-induced airway eosinophilia and hyperresponsiveness in the mouse. *Am J Respir Cell Mol Biol* 28: 25–32, 2003.
6. Eum SY, Maghni K, Hamid Q, Eidelman DH, Campbell H, Isogai S, and Martin JG. Inhibition of allergic airways inflammation and airway hyperresponsiveness in mice by dexamethasone: role of eosinophils, IL-5, eotaxin, and IL-13. *J Allergy Clin Immunol* 111: 1049–1061, 2003.
7. Gomes RF, Shen X, Ramchandani R, Tepper RS, and Bates JH. Comparative respiratory system mechanics in rodents. *J Appl Physiol* 89: 908–916, 2001.
8. Gonzalo JA, Lloyd CM, Kremer L, Finger E, Martinez A, Siegelman MH, Cybulsky M, and Gutierrez-Ramos JC. Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J Clin Invest* 98: 2332–2345, 1996.
9. Grunstein MM, Hakonarson H, Leiter J, Chen M, Whelan R, Grunstein JS, and Chuang S. IL-13-dependent autocrine signaling mediates altered responsiveness of IgE-sensitized airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 282: L520–L528, 2002.
10. Grynkiewicz G, Poenie M, and Tsien RY. A new generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440–3450, 1985.
11. Heinzmann A, Mao XQ, Akaiwa M, Kreomer RT, Gao PS, Ohshima K, Umeshita R, Abe Y, Braun S, Yamashita T, Roberts MH, Sugimoto R, Arima K, Arinobu Y, Yu B, Kruse S, Enomoto T, Dake Y, Kawai M, Shimazu S, Sasaki S, Adra CN, Kitaichi M, Inoue H, Yamauchi K,



- Tomichi N, Kurimoto F, Hamasaki N, Hopkin JM, Izuhara K, Shirakawa T, and Deichmann KA. Genetic variants of IL-13 signalling and human asthma and atopy. *Hum Mol Genet* 9: 549–559, 2000.
12. Hirst SJ, Hallsworth MP, Peng Q, and Lee TH. Selective induction of eotaxin release by interleukin-13 or interleukin-4 in human airway smooth muscle cells is synergistic with interleukin-1 $\beta$  and is mediated by the interleukin-4 receptor  $\alpha$ -chain. *Am J Respir Crit Care Med* 165: 1161–1171, 2002.
13. Jiang H, Rao K, Liu X, Liu G, and Stephens NL. Increased Ca<sup>2+</sup> and myosin phosphorylation, but not calmodulin activity in sensitized airway smooth muscles. *Am J Physiol Lung Cell Mol Physiol* 268: L739–L746, 1995.
14. Jose PJ, Griffiths-Johnson DA, Collins PD, Walsh DT, Moqbel R, Totty NF, Truong O, Hsuan JJ, and Williams TJ. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J Exp Med* 179: 881–887, 1994.
15. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, and Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 8: 885–889, 2002.
16. Laporte JC, Moore PE, Baraldo S, Jouvin MH, Church TL, Schwartzman IN, Panettieri RA Jr, Kinet JP, and Shore SA. Direct effects of interleukin-13 on signaling pathways for physiological responses in cultured human airway smooth muscle cells. *Am J Respir Crit Care Med* 164: 141–148, 2001.
17. Maghni K, Taha R, Afif W, Hamid Q, and Martin JG. Dichotomy between neurokinin receptor actions in modulating allergic airway responses in an animal model of helper T cell type 2 cytokine-associated inflammation. *Am J Respir Crit Care Med* 162: 1068–1074, 2000.
18. Mattes J, Yang M, Mahalingam S, Kuehr J, Webb DC, Simson L, Hogan SP, Koskinen A, McKenzie AN, Dent LA, Rothenberg ME, Matthaei KI, Young IG, and Foster PS. Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperreactivity in experimental asthma. *J Exp Med* 195: 1433–1444, 2002.
19. Moore PE, Church TL, Chism DD, Panettieri RA Jr, and Shore SA. IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK. *Am J Physiol Lung Cell Mol Physiol* 282: L847–L853, 2002.
20. Morse B, Sypek JP, Donaldson DD, Haley KJ, and Lilly CM. Effects of IL-13 on airway responses in the guinea pig. *Am J Physiol Lung Cell Mol Physiol* 282: L44–L49, 2002.
21. Palframan RT, Collins PD, Williams TJ, and Rankin SM. Eotaxin induces a rapid release of eosinophils and their progenitors from the bone marrow. *Blood* 91: 2240–2248, 1998.
22. Rothenberg ME, Luster AD, and Leder P. Murine eotaxin: an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. *Proc Natl Acad Sci USA* 92: 8960–8964, 1995.
23. Sozzani P, Cambon C, Vita N, Seguelas MH, Caput D, Ferrara P, and Pipy B. Interleukin-13 inhibits protein kinase C-triggered respiratory burst in human monocytes. Role of calcium and cyclic AMP. *J Biol Chem* 270: 5084–5088, 1995.
24. Tao F, Chaudry S, Tolloczko B, Martin JG, and Kelly SM. Modulation of smooth muscle phenotype in vitro by homologous cell substrate. *Am J Physiol Cell Physiol* 284: C1531–C1541, 2003.
25. Thivierge M, Stankova J, and Rola-Pleszczynski M. IL-13 and IL-4 up-regulate cysteinyl leukotriene 1 receptor expression in human monocytes and macrophages. *J Immunol* 167: 2855–2866, 2001.
26. Tliba O, Deshpande D, Chen H, Van Besien C, Kannan M, Panettieri R, and Amrani Y. IL-13 enhances agonist-evoked calcium signals and contractile responses in airway smooth muscle. *Br J Pharmacol* 140: 1159–1162, 2003.
27. Tolloczko B, Tao FC, Zacour ME, and Martin JG. Tyrosine kinase-dependent calcium signaling in airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 278: L1138–L1145, 2000.
28. Vargaftig BB and Singer M. Leukotrienes mediate murine bronchopulmonary hyperreactivity, inflammation, and part of mucosal metaplasia and tissue injury induced by recombinant murine interleukin-13. *Am J Respir Cell Mol Biol* 28: 410–419, 2003.
29. Venkayya R, Lam M, Willkom M, Grunig G, Corry DB, and Erle DJ. The Th2 lymphocyte products IL-4 and IL-13 rapidly induce airway hyperresponsiveness through direct effects on resident airway cells. *Am J Respir Cell Mol Biol* 26: 202–208, 2002.
30. Walter DM, McIntire JJ, Berry G, McKenzie AN, Donaldson DD, Dekruyff RH, and Umetsu DT. Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. *J Immunol* 167: 4668–4675, 2001.
31. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, and Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258–2261, 1998.
32. Yang M, Hogan SP, Henry PJ, Matthaei KI, McKenzie AN, Young IG, Rothenberg ME, and Foster PS. Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor- $\alpha$  chain and STAT-6 independently of IL-5 and eotaxin. *Am J Respir Cell Mol Biol* 25: 522–530, 2001.
33. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, and Elias JA. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 103: 779–788, 1999.