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Critical Role for IL-13 in the Development of Allergen-Induced Airway Hyperreactivity¹

David M. Walter,* Jennifer J. McIntire,* Gerald Berry,† Andrew N. J. McKenzie,‡
Debra D. Donaldson,§ Rosemarie H. DeKruyff,* and Dale T. Umetsu^{2*}

Airway hyperresponsiveness to a variety of specific and nonspecific stimuli is a cardinal feature of asthma, which affects nearly 10% of the population in industrialized countries. Eosinophilic pulmonary inflammation, eosinophil-derived products, as well as Th2 cytokines IL-13, IL-4, and IL-5, have been associated with the development of airway hyperreactivity (AHR), but the specific immunological basis underlying the development of AHR remains controversial. Herein we show that mice with targeted deletion of IL-13 failed to develop allergen-induced AHR, despite the presence of vigorous Th2-biased, eosinophilic pulmonary inflammation. However, AHR was restored in IL-13^{-/-} mice by the administration of recombinant IL-13. Moreover, adoptive transfer of OVA-specific Th2 cells generated from TCR-transgenic IL-13^{-/-} mice failed to induce AHR in recipient SCID mice, although such IL-13^{-/-} Th2 cells produced high levels of IL-4 and IL-5 and induced significant airway inflammation. These studies definitively demonstrate that IL-13 is necessary and sufficient for the induction of AHR and that eosinophilic airway inflammation in the absence of IL-13 is inadequate for the induction of AHR. Therefore, treatment of human asthma with antagonists of IL-13 may be very effective. *The Journal of Immunology*, 2001, 167: 4668–4675.

The prevalence of asthma has increased dramatically in industrialized nations and currently affects as many as 10% of individuals, including 15 million Americans (1–3). The pathological features of asthma include chronic airway inflammation, with excess airway mucus production and increased production of chemokines and Th2 cytokines (IL-4, IL-5, IL-9, and IL-13) by T cells, eosinophils, and mast cells. Moreover, asthma is characterized by the presence of airway hyperreactivity (AHR)³ to a variety of specific and nonspecific stimuli. Although many components of airway inflammation, including eosinophil derived factors, Th2 cytokines IL-13, IL-4, and IL-5, as well as elevated serum IgE are associated with the development of AHR, the specific mechanisms by which AHR develops remains controversial. In many studies, particularly those in murine models, the development of AHR was dependent on neither airway eosinophilia nor IgE (4–6). Administration of recombinant IL-13 has been shown to induce the development of AHR (7), but other studies suggest that either IL-13 or IL-4 can induce AHR (8) or that IL-13 and IL-5 together or IL-13 and IL-4 together are required for the induction of AHR (9). To address this issue more definitively, we used several approaches and examined mice with targeted deletion

of either the IL-13 or the IL-4 genes or both the IL-13 and IL-4 genes, to assess the role of IL-13 in the development of allergen-induced AHR and inflammation.

Materials and Methods

Animals

BALB/c-ByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IL-13^{-/-} mice were generated from 129 × C57BL/6 mice, with the disruption in exon 1 (10), and were backcrossed for seven generations onto the BALB/c strain. IL-4^{-/-}/IL-13^{-/-} mice were generated from 129 × C57BL/6 mice (11) that were backcrossed for seven generations onto the BALB/c strain. DO11.10 mice, which are transgenic for TCR recognizing OVA peptide 323–339 and backcrossed to BALB/c were kindly provided by Dr. D. Loh and were bred in our facilities. DO11.10/IL13^{-/-} were generated by crossing IL-13^{-/-} mice with DO11.10 mice and backcrossing the F₁ progeny (IL-13^{+/-}/DO11.10^{+/-}) to IL-13^{-/-} mice. The resulting N2 progeny were screened by PCR to identify IL-13^{-/-} mice. The IL-13^{-/-} mice were then screened by flow cytometry for mice containing CD4⁺, KJ1-26 TCR⁺ cells. These mice were termed DO11.10/IL13^{-/-} mice. Animals were used between 7 and 12 wk of age and were age and sex matched within each experiment. The Stanford University Committee on Animal Welfare approved all animal protocols.

Monoclonal Abs

mAbs were purified from ascites fluid by ammonium sulfate precipitation and ion exchange chromatography. We used the following hybridomas: R46A2 (anti-IFN-γ mAb), obtained from American Type Culture Collection (Manassas, VA); XMG1.2 (anti-IFN-γ mAb), generously provided by Dr. T. Mosmann (University of Rochester, NY); BVD4-1D11 (anti-IL-4), and BVD6-24G2 (anti-IL-4 mAb), obtained from Dr. M. Howard (Corixa, Redwood City, CA); C17.8 (anti-IL-12 mAb), obtained from Dr. G. Trinchieri (Schering-Plough Laboratory of Immunological Research, Dardilly, France); EM95 (rat anti-mouse IgE mAb), kindly provided by Dr. R. Coffman (DNAX Research Institute, Palo Alto, CA). Anti-OVA mAbs were produced as described previously (12). Anti-38C13 idiotype mAb 4G10 (rat IgG2a) (13) was used as control. Hybridoma cells producing the anti-clonotypic Ab KJ1-26 were provided by Dr. P. Marrack (National Jewish Medical Center, Denver, CO).

Immunization protocols

BALB/c (IL-13^{+/+}) or IL-13^{-/-} mice were sensitized to OVA using an established protocol for the induction of AHR in BALB/c mice (14). Briefly, 50 μg OVA adsorbed to 2 mg aluminum potassium sulfate (alum) were administered i.p. to IL-13^{+/+} or IL-13^{-/-} on day zero. On day 14, 50

*Division of Immunology and Allergy, Department of Pediatrics, and †Department of Pathology, Stanford University, Stanford, CA 94305; ‡Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom; and §Immunology Department, Genetics Institute, Cambridge, MA 02140

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² Address correspondence and reprint requests to Dr. Dale T. Umetsu, Department of Pediatrics, Room G309, Stanford University, Stanford, CA 94305-5208. E-mail address: umetsu@stanford.edu

³ Abbreviations used in this paper: AHR, airway hyperreactivity; i.n., intranasal; PAS, periodic acid-Schiff; Penh, enhanced pause.

μg OVA in alum was given i.p., and 50 μg OVA in 50 μl normal saline were administered intranasally (i.n.). On days 25, 26, and 27, the mice were boosted again with OVA i.n. One day after the last intranasal challenge (day 28), AHR was measured in conscious mice after inhalation of increasing concentrations of methacholine (see below). Two days after the last challenge (day 29), mice were bled and then sacrificed, lungs were removed for histology, and bronchial lymph nodes were isolated for *in vitro* culture. For experiments in Figs. 4 and 5, a shorter, but comparable, established immunization protocol was used (15). OVA (50 μg adsorbed to alum) was administered i.p. on day 0. On days 7, 8, and 9, 50 μg OVA in 50 μl normal saline were given i.n. Control mice received i.p. injections of alum alone and OVA i.n. as described above. AHR was measured 1 day later (day 10). On day 11, mice were bled and then sacrificed as described above.

Preparation of OVA-specific Th2 cells

OVA-specific Th2 lines were generated from spleen of DO11.10 (OVA TCR transgenic) IL13^{+/+} mice and DO11.10 IL13^{-/-} mice. T cells were purified from spleen by negative selection on anti-Ig-coated plates, followed by positive selection using MACS CD4 microbeads (Miltenyi Biotec, Auburn, CA). T cells were cultured at 5×10^5 /well with 4×10^6 irradiated spleen cells as APCs and OVA (50 $\mu\text{g}/\text{ml}$; Sigma, St. Louis, MO). To generate Th2 lines, rIL-4 (4 ng/ml) and anti-IL-12 mAb (10 $\mu\text{g}/\text{ml}$) were added to the cultures. After 7 days, cells were washed, counted, and restimulated at 3×10^5 /well with APC and Ag, under the same conditions used for the initial stimulation. Th cell lines were used for transfer into SCID mice 7 days after restimulation. The cytokine profiles of the Th2 lines were confirmed by direct examination of Con A supernatants generated from these lines on the day of transfer, using ELISA.

Transfer of OVA-specific Th2 cells

Th2 cell lines were transferred into histocompatible SCID mice. To facilitate homing of the T cell lines to the lungs, SCID mice were given 50 μg OVA in 50 μl normal saline i.n. 1 day before i.v. transfer of cells. Cultured Th2 cells were harvested and washed with PBS, and 2.5×10^6 cells were adoptively transferred i.v. into the mice. Control mice received only i.n. OVA. One and 2 days after the adoptive transfer of cells, OVA was again administered i.n. AHR was determined 24 h after the last i.n. dose of Ag was administered.

Treatment of mice with anti-cytokine and depletion mAb

Mice undergoing sensitization to OVA were injected i.p. with 1 mg mAb XMG1.2 (for IFN- γ depletion) every 5 days, starting 1 day before administration of OVA i.p. The Abs were present throughout the course of immunization and assessment of AHR.

Treatment of mice with rIL-13

Purified rIL-13 (7) was administered i.n. to IL-13^{-/-} mice to determine whether it would restore the development of AHR. Mice undergoing sensitization to OVA with were given 2 μg rIL-13 i.n. on days 7, 8, and 9 in 50 μl normal saline with or without 50 μg OVA (Fig. 4B). Naive mice were given 2 μg rIL-13 i.n. for 3 consecutive days in 50 μl normal saline before measuring AHR on the 4th day.

Measurement of AHR

AHR was assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole body plethysmograph (model PLY 3211; Buxco Electronics, Troy, NY), as described previously (14).

Determination of lung histology

Animals were sacrificed by CO₂ asphyxiation. The lungs were removed, washed in PBS, inflated, and fixed in 10% neutral buffered formalin, sectioned 5 μm thick, routinely processed, embedded in paraffin wax, and stained with H&E. Intracytoplasmic and luminal mucin was assessed by periodic acid-Schiff (PAS) and digested PAS stains.

Restimulation of lymph node cells *in vitro*

Cells (3.0×10^6 in a 24-well plate, for cytokine ELISAs or 5.0×10^5 in a 96-well plate, for proliferation) isolated from bronchial lymph nodes were restimulated *in vitro* in the presence or absence of 100 $\mu\text{g}/\text{ml}$ OVA.

For ELISA, supernatants were harvested after 4 days for determination of IL-4, IL-5, IL-13, and IFN- γ levels. For proliferation, cultures were incubated for 4 days and pulsed with 1 μCi [³H]thymidine for the last 18 h.

Cytokine ELISAs

ELISAs were performed as previously described (16). The mAb pairs used were as follows, listed by capture/biotinylated detection mAb: IFN- γ , R4-6A2/XMG1.2; IL-4, 11B11/BVD6-24G2; IL-12, C17.8/C15.6; IL-5, TRFK5/TRFK4. Reagents for detection of murine IL-13 were purchased from R&D Systems (Minneapolis, MN).

OVA-specific Igs assay

Mice were bled, and serum levels of OVA-specific IgG1, IgG2a, and IgE Abs were measured using modified OVA-specific ELISAs as previously described (17). Anti-OVA IgG1 and IgG2a mAbs 6C1 and 3A11, respectively, were used as standards for the quantification of each IgG subclass. The standard for the OVA-specific IgE ELISA was sera from mice hyperimmunized with OVA that had been quantitated for IgE.

Results

IL-13^{-/-} mice do not develop AHR

To examine the role of IL-13 in the development of allergen-induced AHR, we sensitized and challenged IL-13^{-/-}, IL-4^{-/-}/IL-13^{-/-}, and IL-4^{-/-} mice with OVA using a standard protocol. Fig. 1A demonstrates that control wild-type IL-13^{+/+} mice sensitized and challenged i.n. with OVA developed significant AHR, elicited with increasing concentration of methacholine. However, IL-13^{-/-} mice sensitized and challenged in the same manner with OVA had almost normal airway responsiveness. In contrast, sensitization and challenge of IL-4^{-/-} mice resulted in significant

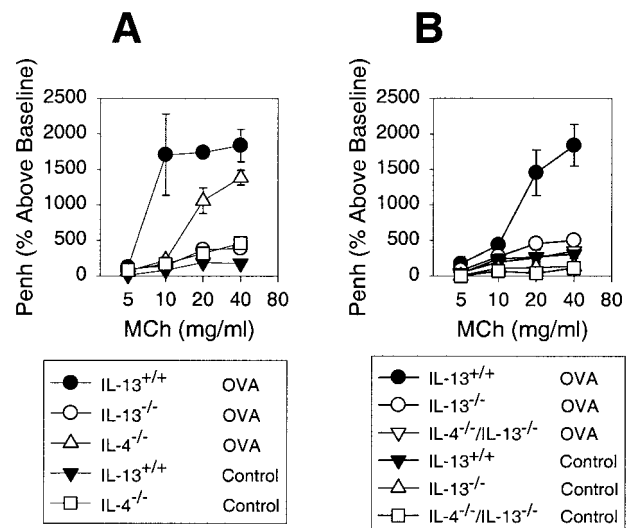


FIGURE 1. OVA-sensitized and -challenged IL-13^{-/-} and IL-4^{-/-}/IL-13^{-/-} mice fail to develop AHR. *A* and *B*, Mice were sensitized and challenged with OVA using the 28-day protocol. One day after the last OVA challenge, AHR was measured in response to increasing concentrations of methacholine (MCh) in conscious mice placed in a whole body plethysmograph (see *Materials and Methods*). Peak enhanced pause (Penh) values were calculated, and data were expressed as mean percent above baseline (saline-induced AHR) \pm SEM. Baseline Penh values are as follows: *A*, IL-13^{+/+}/OVA, 0.49 ± 0.03 ; IL-13^{-/-}/OVA, 0.61 ± 0.03 ; IL-4^{-/-}/OVA, 0.55 ± 0.02 ; IL-13^{+/+}/control, 0.63 ± 0.01 ; and IL-4^{-/-}/control, 0.48 ± 0.01 . *B*, IL-13^{+/+}/OVA, 0.50 ± 0.03 ; IL-13^{-/-}/OVA, 0.51 ± 0.03 ; IL-4^{-/-}/IL-13^{-/-}/OVA, 0.57 ± 0.04 ; IL-13^{+/+}/control, 0.49 ± 0.02 ; IL-13^{-/-}/control, 0.52 ± 0.05 ; and IL-4^{-/-}/IL-13^{-/-}/control, 0.51 ± 0.01 . Only IL-13^{+/+} and IL-4^{-/-} mice developed AHR in response to OVA sensitization and challenge. These two experiments are representative of five independent experiments.

AHR, as we have previously reported (18). In some experiments, as shown in Fig. 1, the degree of AHR that developed in the IL-4^{-/-} mice was slightly less than that observed in wild-type mice, suggesting that IL-4 might play a minor role in the development of AHR. In contrast, mice that lacked both IL-4 and IL-13 (IL-4^{-/-}/IL-13^{-/-}) when sensitized and challenged with OVA had normal airway reactivity (Fig. 1B). Control wild-type IL-13^{+/+} mice that received OVA i.n. without prior sensitization with OVA in alum i.p., as expected, did not develop AHR (Fig. 1), nor did mice that received i.n. saline develop AHR (data not shown). These results indicated that the presence of IL-13, but not IL-4, was critical for the development of AHR.

IL-13^{-/-} mice develop airway inflammation, without mucus production

The failure of IL-13^{-/-} mice to develop AHR was not due to the inability these mice to develop airway inflammation. Examination of tissues taken from the lungs of OVA-sensitized and -challenged IL-13^{-/-} mice had intense airway inflammation, with significant eosinophilia (Fig. 2B). Mucus production, however, was greatly reduced in the IL-13^{-/-} mice, as reflected by a great reduction in the epithelial cell cytoplasmic content, and reduced PAS (magenta) staining for mucus (Fig. 2B, insets). In contrast, airway inflammation in wild-type IL-13^{+/+} mice sensitized and challenged with

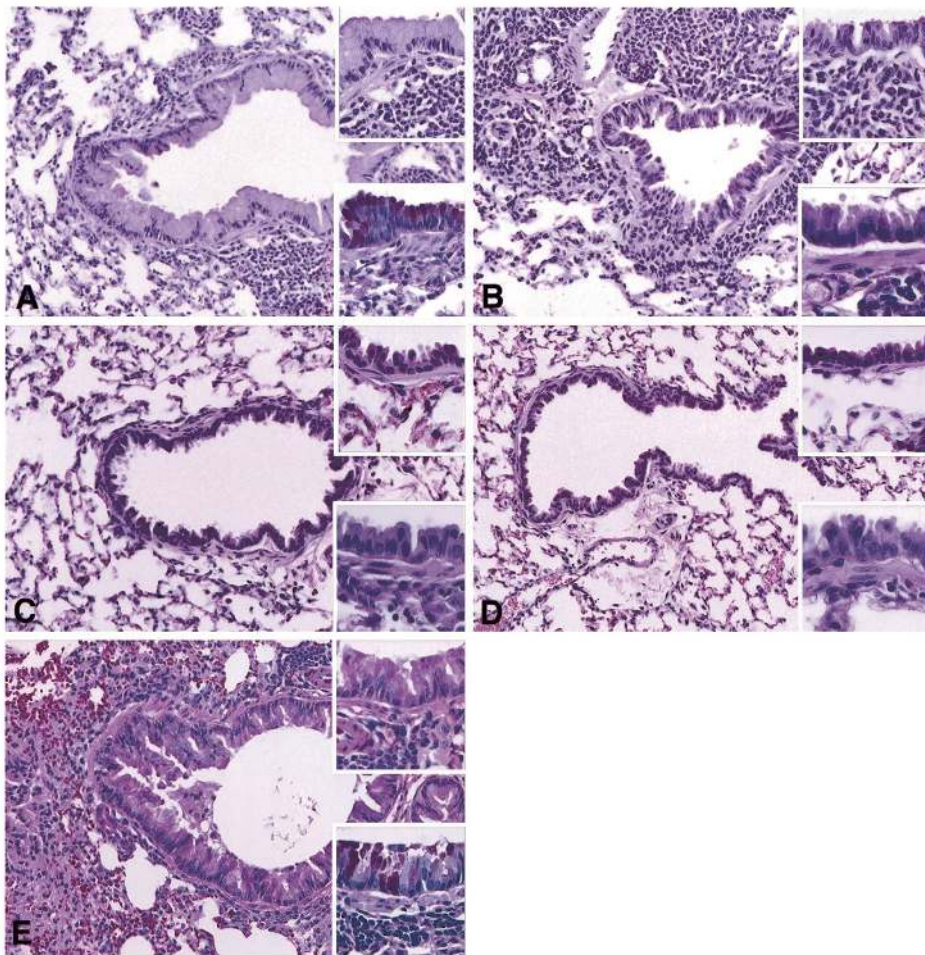


FIGURE 2. Lung histology of IL-13^{+/+}, IL-13^{-/-}, and IL-4^{-/-}/IL-13^{-/-} mice treated as indicated below. Mice were sensitized and challenged with OVA using the 28-day protocol. Two days after the last OVA challenge, the lungs were removed and fixed in formalin. The lungs were then embedded in paraffin wax, cut 5 μ m thick, and stained with H&E and PAS. **A**, IL-13^{+/+} mice treated with OVA i.p. and OVA i.n. show airway inflammation with cellular infiltrates in the peribronchiolar and perivascular tissue spaces. The inflammatory cells are predominantly eosinophils (H&E, $\times 200$). *Upper inset*, High power magnification showing mucosal cells of the bronchioles with abundant intracytoplasmic mucin and numerous eosinophils around the airways (H&E, $\times 400$). *Lower inset*, High power magnification showing abundant mucin within epithelial cells staining bright magenta (PAS, $\times 400$). **B**, IL-13^{-/-} mice treated with OVA i.p. and OVA i.n. developed dense perivascular and peribronchiolar infiltrates composed of a mixture of eosinophils and lymphocytes (H&E, $\times 200$). *Upper inset*, The epithelial cells lining the airways display scant intracytoplasmic mucin, and the inflammation is of mixed cell types (H&E, $\times 400$). *Lower inset*, High power magnification showing that mucin is absent in epithelial cells specifically stained for mucin (PAS, $\times 400$). **C**, IL-4^{-/-}/IL-13^{-/-} mice treated with OVA i.p. and OVA i.n. show normal lung histology (H&E, $\times 200$). *Upper inset*, Bronchiolar lining cells without intracytoplasmic mucin or inflammation is present (H&E, $\times 400$). *Lower inset*, High power magnification showing that mucin is absent in epithelial cells specifically stained for mucin (PAS, $\times 400$). **D**, IL-13^{-/-} mice treated with OVA i.n. also show normal lung histology (H&E, $\times 200$). *Upper inset*, The absence of airway inflammation and changes in the epithelium is confirmed at high power magnification (H&E, $\times 400$). *Lower inset*, High power magnification showing that mucin is absent in epithelial cells specifically stained for mucin (PAS, $\times 400$). **E**, IL-4^{-/-} mice sensitized and challenged with OVA show intense airway inflammation, consisting primarily of eosinophils and mononuclear cells (H&E, $\times 200$). *Upper inset*, High power magnification showing mucosal cells of the bronchioles with abundant intracytoplasmic mucin and numerous eosinophils around the airways (H&E, $\times 400$). *Lower inset*, High power magnification showing abundant mucin within epithelial cells staining bright magenta (PAS, $\times 400$).

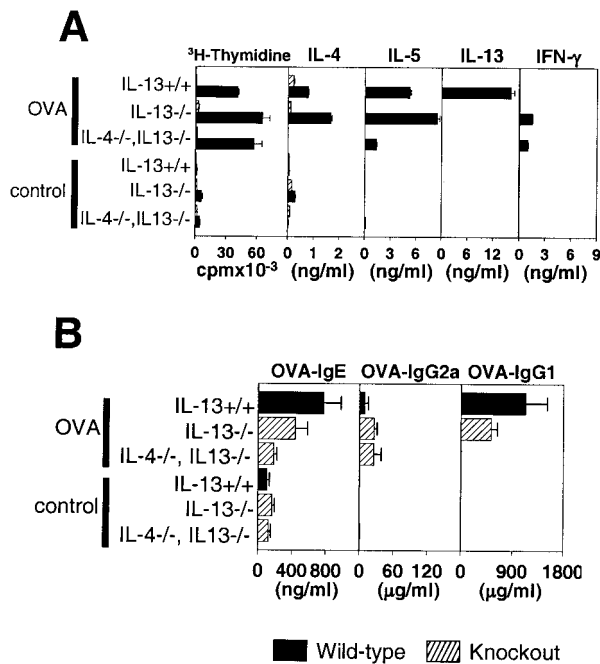


FIGURE 3. A) IL-13^{-/-} mice produce IL-4, IL-5, and IFN-γ but not IL-13. Two days after the last OVA challenge, bronchial lymph node cells were removed and cultured in the presence of 100 μg/ml OVA (■) or medium only (▨). Proliferation was then determined by [³H]thymidine incorporation, and IL-4, IL-5, IL-13, and IFN-γ cytokine levels were measured in the supernatants by ELISA. The IL-13^{-/-} mice produce IL-4, IL-5, and IFN-γ, but not IL-13. B, Serum levels of OVA-specific IgE, IgG2a, and IgG1 Abs were determined by ELISA.

OVA was intense with significant eosinophilia as well as mucus production (Fig. 2A). The IL-4^{-/-}/IL-13^{-/-} mice sensitized and challenged with OVA had essentially normal lung histology (Fig. 2C), as did control IL-13^{-/-} mice that received i.n. OVA without prior sensitization to OVA (Fig. 2D), whereas IL-4^{-/-} mice sensitized and challenged with OVA developed significant airway inflammation and mucus production (Fig. 2E). These results together indicated that the intense airway inflammation with eosinophilia that developed in IL-13^{-/-} mice was not sufficient for the induction of AHR and that IL-13, but not IL-4, was required for the development of AHR.

IL-13^{-/-} mice produce IL-4 and IL-5 but not IL-13

We examined the cytokines produced in bronchial lymph node cells in IL-13^{-/-} mice sensitized and challenged with OVA. Fig. 3A shows that lymph node cells from all mice sensitized and challenged with i.n. OVA proliferated vigorously in response to OVA. The proliferating T cells from wild-type IL-13^{+/+} mice produced substantial quantities of IL-13, as well as large quantities of IL-4 and IL-5, but no detectable IFN-γ. The T cells from OVA sensitized and challenged IL-13^{-/-} mice developed a similar Th2 profile with high levels of IL-4 and IL-5 but, as expected, no IL-13. The Th2 response in these IL-13^{-/-} mice was associated with the production of OVA-specific IgE and IgG1, although these levels were slightly lower than that observed in wild-type IL-13^{+/+} mice (Fig. 3B). In addition, T cells from IL-13^{-/-} mice sensitized and challenged with OVA produced detectable levels of IFN-γ. In contrast, T cells from IL-4^{-/-}/IL-13^{-/-} mice sensitized and challenged with OVA produced low levels of IFN-γ and IL-5 and no IL-4 or IL-13. This was associated with production of background

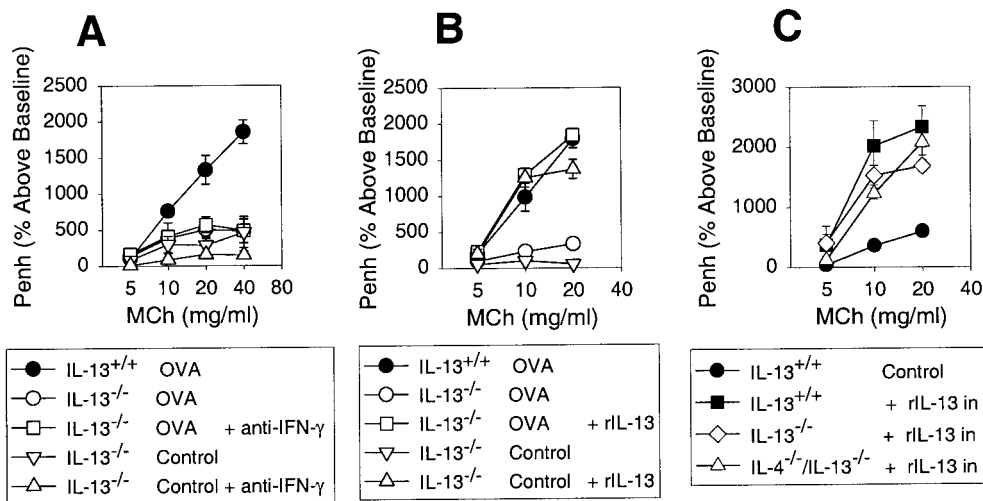


FIGURE 4. A) Blocking IFN-γ production in IL-13^{-/-} mice fails to restore their ability to develop AHR. Throughout the 9-day protocol, IL-13^{-/-} mice were given 1 mg anti-IFN-γ i.p. to block production of IFN-γ. One day after the last OVA challenge, AHR was measured. Peak Penh values were calculated, and data were expressed as mean percent above baseline (saline-induced AHR) ± SEM. Baseline Penh values are as follows: IL-13^{+/+}/OVA, 0.47 ± 0.03; IL-13^{-/-}/OVA, 0.49 ± 0.02; IL-13^{-/-}/OVA + anti-IFN-γ, 0.45 ± 0.02; IL-13^{-/-}/control, 0.52 ± 0.07; and IL-13^{-/-}/control + anti-IFN-γ, 0.64 ± 0.08. Only IL-13^{+/+} mice developed AHR in response to OVA sensitization and challenge. This experiment is representative of two independent experiments. Therefore, the production of IFN-γ in IL-13^{-/-} mice is not the cause for their inability to develop AHR. B, rIL-13 is sufficient to induce AHR in unsensitized mice. OVA-sensitized and -challenged mice, as well as control mice that received only i.n. OVA, were given 2 μg rIL-13 i.n. daily for the last 3 days of the immunization protocol. On day 10, AHR was measured. Baseline Penh values are as follows: IL-13^{+/+}/OVA, 0.47 ± 0.01; IL-13^{-/-}/OVA, 0.47 ± 0.01; IL-13^{-/-}/OVA + rIL-13, 0.52 ± 0.02; IL-13^{-/-}/control, 0.57 ± 0.03; and IL-13^{-/-}/control + rIL-13, 0.52 ± 0.02. Mice that received rIL-13 developed AHR, regardless of sensitization. C, Naive IL-13^{+/+}, IL-13^{-/-}, and IL-4^{-/-}/IL-13^{-/-} were given rIL-13 in normal saline i.n. for 3 consecutive days. On the 4th day, AHR was measured. Baseline Penh values are as follows: IL-13^{+/+}/control, 0.50 ± 0.03; IL-13^{+/+}/rIL-13, 0.46 ± 0.03; IL-13^{-/-}/rIL-13, 0.49 ± 0.01; and IL-4^{-/-}/IL-13^{-/-}/rIL-13, 0.50 ± 0.02. The experiments in B and C are representative of two independent experiments. These results indicate that IL-13 is sufficient to induce AHR, without sensitization and/or challenge with an Ag.

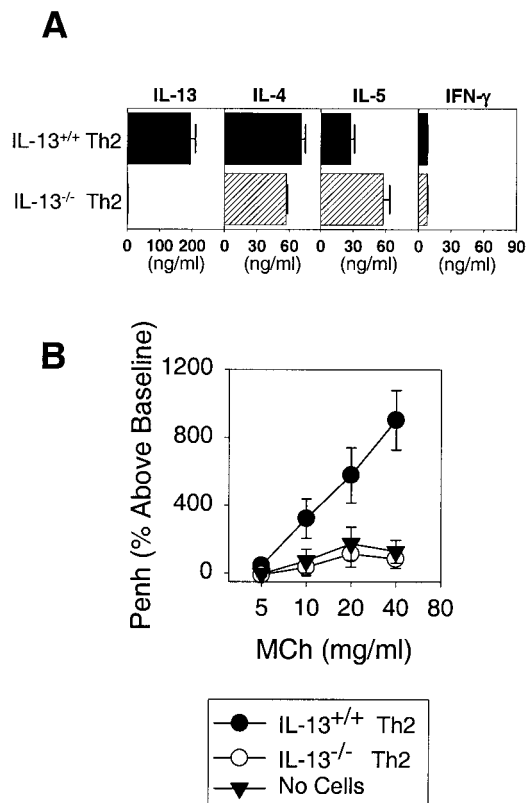


FIGURE 5. OVA-specific Th2 cell lines generated from IL-13^{-/-} mice do not induce AHR. *A*, Con A supernatants generated from the cell lines on the day of transfer were examined using ELISA to determine the cytokine profile. *B*, One day after the last OVA challenge, AHR was measured. Peak Penh values were calculated, and data were expressed as mean percent above baseline (saline-induced AHR) ± SEM. Baseline Penh values are as follows: DO11.10/IL-13^{+/+} Th2, 0.49 ± 0.03; DO11.10/IL-13^{-/-} Th2, 0.47 ± 0.02; and control, 0.53 ± 0.01. This experiment is representative of two independent experiments. Only SCID mice that received DO11.10/IL-13^{+/+} Th2 cells developed AHR in response to i.n. OVA.

levels of OVA-specific IgE but increased amounts of OVA-specific IgG2a. Together these results indicated that the OVA-sensitized and -challenged IL-13^{-/-} mice failed to develop AHR despite the generation of a polarized OVA-specific Th2 response, associated with increased production of OVA-specific IgE, and significant airway inflammation.

IL-13^{-/-} mice treated with anti-IFN-γ do not develop AHR

Because OVA-sensitized and -challenged IL-13^{-/-} mice produced more IFN-γ than did sensitized and challenged wild-type IL-13^{-/-} mice, we asked whether IL-13^{-/-} mice could develop AHR when

the IFN-γ was neutralized. Therefore, we administered a neutralizing anti-IFN-γ mAb during the immunization protocol. As expected, OVA-sensitized and -challenged IL-13^{+/+} mice developed significant AHR, whereas IL-13^{-/-} mice, similarly sensitized and challenged, had almost normal airway reactivity (Fig. 4A). Treatment with the anti-IFN-γ mAb failed to reverse the inability of IL-13^{-/-} mice to develop AHR. Treatment of OVA sensitized and challenged IL-13^{+/+} mice with anti-IFN-γ mAb had no significant effect on AHR (data not shown). When bronchial lymph node cells from these mice were cultured in the presence of OVA, cells from the anti-IFN-γ-treated group showed greatly reduced levels of IFN-γ production as compared with cells from the untreated IL-13^{-/-} mice. Levels of IFN-γ were similar to those in the wild-type BALB/c control, indicating that the anti-IFN-γ treatment inhibited the development of IFN-γ production in the IL-13^{-/-} mice (data not shown). Production of IL-4, IL-5, IL-12, and IL-13 was not affected by the anti-IFN-γ treatment (data not shown). Thus, the development of AHR in IL-13^{-/-} mice was not inhibited by increased IFN-γ production.

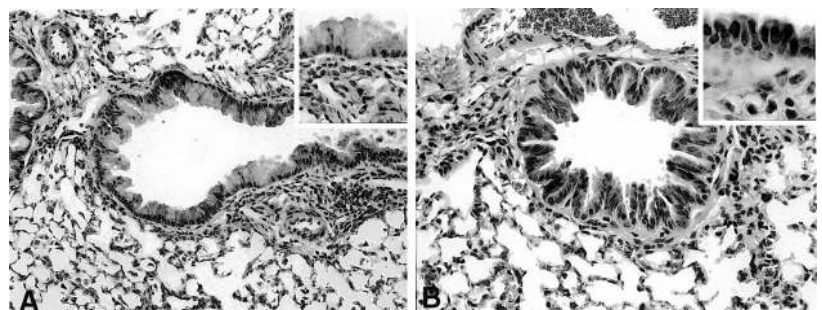
IL-13^{-/-} mice treated with IL-13 develop AHR

To demonstrate that IL-13^{-/-} mice could develop AHR if exogenous IL-13 was provided, we administered rIL-13 during the immunization protocol. Fig. 4B shows that i.n. administration of rIL-13 to IL-13^{-/-} mice induced the development of AHR, whether or not the mice were sensitized with i.p. OVA (Fig. 4B). IL-13^{-/-} mice that received rIL-13 had approximately the same responsiveness to methacholine as did IL-13^{+/+} mice that were sensitized and challenged with OVA, whereas IL-13^{-/-} mice that did not receive exogenous IL-13 failed to develop AHR. Similarly, naive IL-13^{+/+}, IL-13^{-/-}, and IL-4^{-/-}/IL-13^{-/-} treated with rIL-13 i.n. developed severe AHR (Fig. 4C). Although administration of rIL-13 to naive mice caused the development of AHR, examination of their lung histology was remarkable for almost total absence of cellular infiltration and mucus production (data not shown). These data suggest that the administration of exogenous IL-13 can directly activate the pathway that leads to the development of AHR and that this occurs independently of eosinophils, mucus production, and IL-4 expression.

OVA-specific Th2 cell lines generated from IL-13^{-/-} mice do not induce AHR

Because OVA-specific Th2 responses may not develop optimally in vivo in IL-13^{-/-} mice, we examined the capacity of Th2 lines generated ex vivo from IL-13^{-/-} mice to induce AHR when adoptively transferred into SCID mice. The OVA-specific Th2 lines were generated from IL-13^{-/-} mice backcrossed to OVA TCR-transgenic (DO11.10) mice. Fig. 5A shows that the cytokine profiles of these IL-13^{-/-} Th2 cell lines included high levels of IL-4 and IL-5, negligible levels of IFN-γ, and no IL-13. This profile

FIGURE 6. OVA-specific Th2 cell lines generated from IL-13^{-/-} mice induce significant airway inflammation. *A*, DO11.10/IL-13^{+/+} Th2 cells induce inflammatory infiltrates in the peribronchiolar and perivascular spaces and abundant intracytoplasmic mucin (H&E, ×200). *Inset*, High power magnification showing epithelial cells containing pale mucin (H&E, ×400). *B*, DO11.10/IL-13^{-/-} Th2 cells induce inflammatory infiltrates in the peribronchiolar and perivascular spaces but negligible intracytoplasmic mucin production (H&E ×200). *Inset*, Bronchial epithelial cells showing normal appearance (H&E, ×400).



was similar to the profile of a control Th2 cell line generated from IL-13^{+/+} DO11.10 mice, except for IL-13, which was produced in large quantities by the wild-type Th2 line. Thus, except for the lack of IL-13 production in the DO11.10/IL13^{-/-} cell line, both cell lines had similar Th2-like cytokine profiles. Fig. 5B shows that adoptive transfer of the wild-type OVA-specific Th2 cell line resulted in the development of severe AHR in recipient mice. In contrast, mice that received the DO11.10 IL-13^{-/-} Th2 cell line failed to develop AHR. The inability of the DO11.10 IL-13^{-/-} Th2 cell line to induce AHR was not due to a complete lack of functional activity, because the IL-13^{-/-} Th2 cell line induced airway inflammation, consisting of eosinophils and lymphocytes (Fig. 6). However, mucus production induced by the IL-13^{-/-} Th2 cell line was greatly limited compared with that induced by the wild-type Th2 line. In contrast, the degree of mononuclear infiltration appeared to be equivalent between the two cell lines, suggesting that the DO11.10 IL-13^{-/-} Th2 cells migrated to the lungs as well as did the wild-type DO11.10 Th2 cells. These results using Th2 lines differing only in their production of IL-13 confirm our earlier observations that OVA-sensitized and challenged IL-13^{-/-} mice do not develop AHR. These results thus demonstrated that the AHR cannot develop in the absence of IL-13, even in the presence of large quantities of other Th2 cytokines, including IL-4 and IL-5, or in the presence of significant airway eosinophilia.

Discussion

Our results demonstrate that IL-13 is essential for the induction of AHR in a mouse model of asthma, and that airway inflammation alone, as defined by the presence of large quantities of peribronchiolar eosinophils and lymphocytes, in the absence of IL-13, is not sufficient for the development of significant AHR. Thus, IL-13^{-/-} mice sensitized and challenged with allergen failed to develop AHR, although they displayed severe airway inflammation. Moreover, OVA-specific Th2 lines generated from IL-13^{-/-} mice, which produced high levels of IL-4 and IL-5 but not IL-13, failed to induce AHR, demonstrating the essential role of IL-13 in the development of AHR.

Previous studies using IL-13^{-/-} mice backcrossed only five generations to BALB/c, demonstrated a modulatory and proinflammatory role in airway inflammation for IL-13, the role of which was thought to be redundant with IL-4 (9). In their system with IL-13^{-/-} mice, depletion of IL-5 or IL-4 with neutralizing mAb was required to significantly diminish AHR. Similarly, Grunig et al. (8) demonstrated that administration of either rIL-13 or rIL-4 to rag 1-deficient mice induced AHR, and the effects of these cytokines were mediated through an IL-4 receptor α -chain-dependent pathway. These studies, demonstrating overlapping or complementary roles for IL-4 and IL-13 in the induction of AHR, are consistent with those using STAT6-deficient mice, which lack IL-4 and IL-13 signaling pathways and which do not develop AHR (19, 20).

In our studies, however, using a slightly different protocol to induce AHR and using mice more completely backcrossed to BALB/c (seven generations), we showed that IL-13 alone was both necessary and sufficient for the development of AHR. We demonstrated that IL-4^{-/-} mice have the capacity to develop significant AHR, indicating that although IL-4 contributes to the overall allergic inflammatory response and enhances eosinophil migration into the lungs (21), IL-4 is clearly not essential for the induction of AHR. We confirmed that administration of IL-13 to normal BALB/c mice induced AHR; in addition, we established that administration of IL-13 to IL-4/IL-13 double knockout mice induced AHR. Furthermore, the AHR in naive mice following i.n. admin-

istration of IL-13 developed independently of cellular infiltration and extensive mucus production. Moreover, we demonstrated that OVA-specific IL-13^{-/-}-derived Th2 cells, which produced high levels of IL-4 but not IL-13, were ineffective in inducing AHR. Together, our results using several distinct approaches and several different cytokine-deficient mice indicated that IL-13 in the absence of IL-4 induced AHR and that IL-4 in the absence of IL-13 did not.

IL-13 therefore plays a major role as an effector molecule in asthma. Previous studies in humans demonstrated that IL-13 mRNA and protein levels are elevated in the lungs of atopic and nonatopic asthmatics (22–24), suggesting that overproduction of IL-13 may predispose toward the development of both types of asthma (23). In addition, human asthma has been linked to a region of chromosome 5q and to polymorphisms in the IL-13 gene, which is located at chromosome 5q32 (25–28). Furthermore, mutations in IL-4R α , which is a component of the IL-13 receptor complex, have been linked with asthma (29, 30). Taken together, the mouse and human data suggest that IL-13 is an integral component of the mosaic of factors that lead to the development of asthma. It is clear, however, that there are many cytokines that play a role in the development of asthma, and in contrast to our studies, previous experiments in other laboratories have suggested that IL-5 as well as IL-4 can induce AHR. Possible reasons for these conflicting results include differences in the intensity of immunization and challenge (more intensive regimens may bring out IL-13 independent contributions), genetic modifiers in different murine strains, and different methods used to measure airway responses. Nevertheless, our results using a common mouse strain (BALB/c) and a standard method to induce AHR indicate that IL-13 plays an essential role in the development of AHR and suggest that antagonists of IL-13 may be effective in the treatment of asthma in humans.

The precise mechanism by which IL-13 induces AHR is not clear. IL-13 may increase AHR by directly affecting airway smooth muscle cells. IL-13 induces smooth muscle proliferation *in vitro* (31) and can aid contractions of tracheal smooth muscle cells (32). More recently, airway smooth muscle cells have been shown to express IL-13 receptors, including both components of the IL-13R complex (IL-13R α 1 and IL-4R α) (25). In addition, IL-13 may increase AHR by thickening airway walls because of increased mucus secretion (33, 34). In this regard, administration of IL-13 into the lungs of wild-type or IL-4^{-/-} mice induced mucus production (7, 8); in addition, transgenic mice overexpressing IL-13 had increased mucus production (33–35). Moreover, neutralization of IL-13 using a soluble IL-13 α 2-IgGfC fusion protein reversed allergen-induced increases in mucus production (7), and IL-13^{-/-} mice had a marked reduction in mucus secretion.

IL-13 may have several additional roles in asthma. For example, IL-13 may regulate cytokine synthesis and immune function, as evidenced by an increase in IFN- γ production in IL-13^{-/-} mice, and through the production of IL-13 by NK T cells (36). The increase in IFN- γ production in IL-13^{-/-} mice, however, cannot explain the limited capacity of IL-13^{-/-} mice to generate AHR, because neutralization of IFN- γ did not restore AHR in these mice. Serum IgE levels were slightly decreased in IL-13^{-/-} mice compared with wild-type mice and was enhanced by anti-IFN- γ mAb treatment of these mice, suggesting that IgE production was inhibited by increased IFN- γ synthesis. Alternatively, IL-13 may normally serve to enhance IgE synthesis, particularly because IL-13^{-/-} mice have previously been shown to have reduced serum levels of IgE (10), and overexpression of IL-13 in transgenic mice results in 10–100 fold higher levels of serum IgE levels (34, 35). Finally, IL-13 may have profibrotic activity, acting as a key player

in the development of hepatic fibrosis *in vivo* in normal mice (37), and causing the accumulation of collagen in the subepithelial layers in the lungs of mice overexpressing IL-13 (33, 34).

Our studies also demonstrated that in the absence of IL-13, airway inflammation and eosinophilia was insufficient to cause AHR. The role of eosinophils in causing AHR has been controversial but has been assumed to be a critical determinant of AHR (6, 38–40). However, several studies have shown that AHR can develop independently from the recruitment of eosinophils (4, 41–46). In addition, it has been demonstrated that airway eosinophilia can occur without the development of AHR (7, 47). In contrast, T cells, rather than eosinophils, correlate directly with the development of AHR (7, 8, 45, 48, 49). Our results clearly show that T cells producing IL-13 are critical for the development of AHR, because transfer of IL-13^{+/+} Th2 cells but not IL-13^{-/-} Th2 cells, induced the development of AHR.

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