Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production

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Interleukin (IL)-13 is a pleiotropic cytokine produced in large quantities by activated CD4+ Th2 lymphocytes. To define further its potential *in vivo* effector functions, the Clara cell 10-kDa protein promoter was used to express IL-13 selectively in the lung, and the phenotype of the resulting transgenic mice was characterized. In contrast to transgene-negative littermates, the lungs of transgene-positive mice contained an inflammatory response around small and large airways and in the surrounding parenchyma. It was mononuclear in nature and contained significant numbers of eosinophils and enlarged and occasionally multinucleated macrophages. Airway epithelial cell hypertrophy, mucus cell metaplasia, the hyperproduction of neutral and acidic mucus, the deposition of Charcot-Leyden–like crystals, and subepithelial airway fibrosis were also prominently noted. Eotaxin protein and mRNA were also present in large quantities in the lungs of the transgene-positive, but not the transgene-negative, mice. IL-4, IL-5, granulocyte-macrophage colony-stimulating factor, and monocyte chemoattractant protein-5 were not similarly detected. Physiological evaluations revealed significant increases in baseline airways resistance and airways hyperresponsiveness (AHR) to methacholine in transgene-positive animals. Thus, the targeted pulmonary expression of IL-13 causes a mononuclear and eosinophilic inflammatory response, mucus cell metaplasia, the deposition of Charcot-Leyden–like crystals, airway fibrosis, eotaxin production, airways obstruction, and nonspecific AHR. IL-13 may play an important role in the pathogenesis of similar responses in asthma or other Th2-polarized tissue responses.

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Introduction

The cytokine secretion pattern of mouse CD4+ T cells has been shown to contain two major subsets of helper cells: Th1 cells, which secrete interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-β; and Th2 cells, which secrete IL-4, IL-5, IL-6, IL-10, and IL-13 (1–3). Although not as clearly defined, Th1-like and Th2-like cells and Th1- and Th2-polarized inflammatory responses have also been described in humans (1). The Th1 responses have been implicated in cell-mediated immune responses such as those seen in sarcoidosis and tuberculosis (4). The Th2 responses play a key role in IgE-mediated inflammation and responses characterized by activated mast cells and/or eosinophils, such as asthma, atopy, and immune responses to parasites and some fungi (1, 5–8). Studies of the effector molecules that mediate the tissue effects of Th2-polarized inflammation have focused predominantly on IL-4 and IL-5. This is the result of investigations that demonstrated that these cytokines play a crucial role in the generation of Th2 responses in a variety of animal modeling systems (9–14). Additional support came from studies demonstrating that IL-4 plays a key role in CD4+ T-cell commitment to a Th2 phenotype and the induction of IgE production, whereas IL-5 regulates eosinophil development, activation, and tissue recruitment (1–3, 9, 10, 13, 15, 16). Recently, however, a variety of studies (5, 7, 17–19) have demonstrated that IL-4 and IL-5, alone and in combination, are not able to account totally for the effects of Th2 cells at sites of inflammation. The other mediators that are involved and their *in vivo* effector profiles, however, have not been adequately defined.

The complexity of Th2 inflammation can be appreciated in studies of asthma and other disorders. The asthmatic airway is characterized by chronic inflammation, eosinophil infiltration, and varying degrees of subepithelial fibrosis, mucus hyperproduction, and goblet cell hyperplasia (20–23). Airways hyperresponsiveness (AHR), an exaggerated bronchospastic response to nonspecific agonists such as methacholine, is believed to be caused by these changes and to represent a hallmark physiological abnormality underlying the asthmatic diathesis (24–26). Multiple lines of evidence suggest that allergen-specific CD4+ Th2 cells play an essential role in initiating and generating these abnormalities (8, 17, 27). They have also demonstrated that IL-4 and IL-5 cannot completely

account for these tissue and physiological responses. For example, in experiments using passively transferred *in vitro* polarized and activated Th2 cells, IL-4 plays an important role in T-cell recruitment from the vascular space and the migration of eosinophils from the lung into the airway, but it cannot account for the ability of Th2 cells to stimulate mucus elaboration or induce AHR to methacholine (17, 18). In addition, the AHR seen in antigen-sensitized and -challenged BALB/c mice is mediated by a $CD4^+$ T cell–dependent and IL-4– and IL-5–independent activation pathway (19). A similar inability of IL-4 and IL-5 to account for Th2 immune responses to nematodes in the gastrointestinal (GI) tract has been described (5, 7).

IL-13 is a pleiotropic 12-kDa protein product of a gene on chromosome 5 at q31 that is produced in large quantities by appropriately stimulated $CD4^+$ Th2 cells (28, 29). It has a variety of effects that are relevant to asthma and other Th2-dominated inflammatory disorders, including the ability to induce IgE production (30), CD23 expression (31), and endothelial cell VCAM-1 expression (32), and activate and inhibit the apoptosis of eosinophils (28). IL-13 and IL-4 have overlapping effector profiles (28). This overlap is at least partially due to the shared use of receptor components in the multimeric IL-13 and IL-4 receptor complexes (28, 33). There are, however, a number of important functional differences between IL-4 and IL-13. They include differences in the ability of these cytokines to drive the differentiation of naive cord blood T cells to a Th2 phenotype, support the *in vitro* proliferation of activated human or mouse T cells, regulate prostaglandin biosynthesis, induce IFN-γ production, contribute to nematode-induced tissue inflammation, regulate epithelial electrolyte secretion, prolong eosinophil survival, and stimulate eosinophil chemotaxis (7, 28, 34–38). In addition, IL-13 and IL-4 can be produced by different cells (39) and are differentially regulated by mediators such as IFN-α (40). The exaggerated production of IL-13 is well documented in atopic and nonatopic asthma (41–43), atopic dermatitis (44, 45), allergic rhinitis (46), and chronic sinusitis (47). IL-13 also plays an important role in the pathogenesis of type II granulomatous responses (48). The *in vivo* effector functions of IL-13 in these settings are, however, poorly understood. In addition, the *in vivo* consequences of chronic IL-13 production have not been described.

We hypothesized that IL-13 plays an important role in the pathogenesis of the inflammation and remodeling in the asthmatic airway and/or other sites of Th2 cell polarization. To address this hypothesis, studies were undertaken to characterize the potential *in vivo* effector functions of this important cytokine. This was accomplished by targeting IL-13 to the murine lung and characterizing the phenotype of the resulting transgenic offspring. These studies demonstrate that IL-13 induces tissue inflammation, mucus hyperproduction, goblet cell hyperplasia, subepithelial airway fibrosis, Charcot-Leyden–like crystal deposition, airways obstruction, and AHR on methacholine challenge. They also demonstrate that these effects may be mediated, at least in part, by the ability of IL-13 to selectively induce the respiratory production of eotaxin.

Methods

Isolation of murine IL-13 cDNA. Total cellular RNA from mouse Th2 D10 cells was a gift from A. Ray (Yale University). These cells were cultured and stimulated with PMA (25 ng/ml) and dibutyryl cAMP (1 mM), and total cellular RNA was isolated using Trizol Reagent (Life Technologies Inc., Gaithersburg, Maryland, USA) as described previously (49). Reverse transcription (RT)-PCR was performed with the RT-PCR kit purchased from Promega Inc. (Madison, Wisconsin, USA) using the following primers: 5′-ATT AAG CTT CTA CAG CTC CCT GGT TCT CTC-3′ and 5′-ATT GGA TCC TCA TTA GAA GGG GCC GTG-3′. These primers were designed to incorporate *Hin*dIII and *Bam*HI restriction enzyme sites. The RT-PCR conditions were as follows: for RT incubation at 48°C for 45 min; for PCR 95°C for 8 min; for cycling 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min for 35 cycles, and a final extension at 72°C for 10 min.

Production and identification of transgenic mice. To study the respiratory effector functions of IL-13, we took advantage of the high density of Clara cells in the murine respiratory epithelium (50, 51) and used the Clara cell 10-kDa protein (CC10) promoter to target the expression of IL-13 to the airway. The approach that was used has been described previously by our laboratory (50, 52). The 2.3-kb rat CC10 promoter was a gift of B. Stripp and J. Whitsett (University of Cincinnati, Cincinnati, Ohio, USA) (53). Construct (pKS-CC10-rtTA-hGH), which contains the CC10 promoter, reverse tetracycline transactivator (rtTA), and human growth hormone (hGH) intronic and polyadenylation sequences, was prepared as described previously (52). PCR cloning was used to synthesize and amplify the cDNA-encoding murine IL-13 from total RNA extracted from mouse Th2 D10 lymphocytes. This PCR amplification product was then subcloned into pKS-CC10-rtTA-hGH, replacing rtTA with IL-13, yielding the construct pKS–CC10–IL-13–hGH. After the fidelity of the junction areas and the IL-13 cDNA were confirmed by sequencing, the *Xho*I–*Not*I fragment containing CC10 promoter, mouse IL-13 cDNA, and hGH 3′ untranslated region (Fig. 1) was digested and isolated from the vector by electrophoresis. The DNA was purified through an Elutip-D column following the manufacturer's instructions (Schleicher and Schuell Inc., Keene, New Hampshire, USA) and dialyzed against microinjection buffer (0.5 mM Tris-HCl, 25 mM EDTA; pH 7.5). Transgenic mice were generated in (CBA x C57BL/6) F2 eggs using standard pronuclear injection as described previously (52, 54).

The presence or absence of the transgene in the resulting animals and their progeny was determined using tail DNA and Southern blot analysis with ^{32}P-labeled murine IL-13 cDNA as a probe or PCR. When PCR was used, primers were used that spanned the IL-13–hGH junction that was unique to our transgene. The primers were: 5′-CAA AAC TGC TCA GCT ACA CAA AG-3′ and 5′-GAG CTG TTT GTT TTT CTC TCT CC-3′. The following PCR protocol was used: 95°C for 8 min; 35 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min.

Bronchoalveolar lavage and quantification of IL-13 levels. Mice were euthanized; the trachea was isolated by blunt dissection; and small caliber tubing was inserted and secured in the airway. Three successive volumes of 0.75 ml of PBS with 0.1% BSA were then instilled and gently aspirated and pooled. Each bronchoalveolar lavage (BAL) fluid sample was centrifuged, and the supernatants were stored at –70°C until use. The levels of IL-13 were determined immunologically using a commercial ELISA per the manufacturer's instructions (R&D Systems Inc., Minneapolis, Minnesota, USA).

Northern analysis. Total cellular RNA from lungs or a variety of other mouse tissues were obtained using Trizol Reagent (GIBCO BRL, Grand Island, New York) per the manufacturer's

instructions. The RNA was then fractionated by formaldehyde-agarose gel electrophoresis as described previously (55). IL-13 gene expression was quantitated by probing with ^{32}P-labeled murine IL-13 cDNA. Eotaxin and monocyte chemoattractant protein-5 (MCP-5) mRNA were assessed with similarly labeled murine cDNA encoding these moieties. Equality of sample loading and transfer was assessed by stripping and reprobing the membrane with a cDNA encoding β-actin or glyceraldehyde phosphate dehydrogenase (GAPDH).

Ribonuclease protection assay. Ribonuclease protection assays were performed using the RiboQuant kit purchased from PharMingen (San Diego, California, USA). The mCK-1 template kit was used. Assays were performed according to the instructions provided by the manufacturer.

Histological evaluation. Animals were sacrificed via cervical dislocation; median sternotomy was performed; and right heart perfusion was accomplished with calcium- and magnesium-free PBS to clear the pulmonary intravascular space. The heart and lungs were then removed *en bloc,* inflated with 1 cc neutral buffered 10% formalin, fixed overnight in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained. When extrathoracic organs (skin, small intestine, uterus, liver, spleen, and muscle) were being evaluated, tissues were obtained, fixed, and sectioned in a similar fashion. Hematoxylin and eosin (H&E), Mallory's trichrome, periodic acid-Schiff with diastase (PAS), alcian blue at pH 2.5, PAS/alcian blue, modified Congo red, and Papanicolaou stains were performed in the Research Pathology Laboratory at Yale University.

Physiological evaluation of mice. Age- and gender-matched littermate mice were evaluated physiologically using invasive and noninvasive assessment techniques.

Noninvasive physiological assessment. The baseline resistance and AHR in unrestrained, conscious animals was assessed by barometric plethysmography using whole-body plethysmography (Buxco Electronics Inc., Troy, New York, USA). The techniques that were used were those described by Hamelmann *et al.* (56) and Kline *et al.* (57). In brief, mice were placed into whole-body plethysmographs and interfaced with computers using differential pressure transducers. Measurements were made of respiratory rate, tidal volume, and enhanced pause (P_{enh}). Airways resistance is expressed as $P_{enh} = [(T_e/0.3\ T_r) - 1] \times [2\ P_{ef}/3\ P_{if}]$, where P_{enh}= enhanced pause, T_e = expiratory time (seconds), T_r = relaxation time (seconds), P_{ef} = peak expiratory flow (ml), and P_{if} = peak inspiratory flow (ml/s). Increasing doses of methacholine were administered by nebulization for 120 s, and P_{enh} was calculated over the subsequent 5 min.

Invasive physiological assessment. Physiological assessments were also performed in anesthetized and tracheostomized mice using techniques described previously by our laboratory (50, 58). With these techniques, the changes in the lung volumes of anesthetized and tracheostomized mice were measured plethysmographically by determining the pressure in a plexiglass chamber using an inline microswitch pressure transducer. Flow was measured by differentiation of the volume signal, and transpulmonary pressure was determined via a second microswitch pressure transducer placed in line with the plethysmograph and animal ventilator. Resistance was then calculated. The resistance of the tracheotomy catheter was routinely eliminated. Baseline measurements of pulmonary resistance were obtained by ventilating the mouse in the plethysmograph at volumes of 0.4 ml at a rate of 150 breaths per minute (settings shown previously to produce normal arterial blood gases in the species). Bronchial reactivity was assessed using noncumulative methacholine challenge procedures as described previously by our laboratory (50). In this procedure, increasing concentrations of methacholine in PBS were administered by nebulization (20 one-ml breaths) using a Devilbiss Aerosonic nebulizer (model 5000; Somerset, Pennsylvania, USA) that produces particles 1–3 μm in diameter. Pulmonary resistance was calculated precisely 1 min later. Stepwise increases in methacholine dose were then given until the pulmonary resistance, in comparison to the baseline level, had at least doubled. All animals received serial threefold increases in methacholine from 1 mg/ml to 100 mg/ml. The data are expressed as the PC_{100} (provocative challenge 100), the dose at which pulmonary resistance was 100% above the baseline level as calculated by linear regression analysis.

Statistical analysis. Values are expressed as means ± SEM. Unless otherwise noted, group means are compared with the Student's two-tailed unpaired *t* test using the StatView software for the Macintosh (Abacus Concepts Inc., Berkeley, California, USA).

Results

Generation of transgenic mice. To generate a number of independent lines of transgenic mice in which IL-13 is selectively expressed in the lung, constructs containing the CC10 promoter and murine IL-13 were prepared. The CC10 promoter was used because previous studies from our laboratory (50, 52) and others (59) have demonstrated its ability to target genes to pulmonary tissues. Standard microinjection was used, tail DNA was isolated, and the presence or absence of IL-13 transgenic sequences was determined via Southern blot analysis and PCR. Three animals contained the appropriate transgenic construct. They were subsequently back-crossed with C57BL/6 mice, yielding transgene-negative and -positive progeny. All three lines expressed IL-13 and had qualitatively similar phenotypes. Based on the levels of IL-13 expression, a line that produced modest quantities of IL-13 (line 1) and a line that produced larger quantities of IL-13 (line 2) were chosen for further analysis.

Assessment of transgene expression. Studies were undertaken to determine whether IL-13 was appropriately expressed in pulmonary tissues. This was done by quantitating the levels of IL-13 protein in the BAL fluid of transgene (+) and (–) animals, comparing the levels of IL-13 mRNA in the lungs and extrapulmonary organs of transgene (+) and (–) animals, and examining the light microscopic appearance of extrapulmonary organs in transgene (+) mice. The BAL fluid from transgene (+) line 1 animals contained modest amounts of IL-13 (20–50 pg/ml). Higher levels of IL-13 were noted in the BAL fluid from transgene (+) line 2 animals (1.9–2.1 ng/ml). IL-13 was not detected in the BAL fluid from transgene (–) littermate control animals (data not shown). In accordance with these findings, IL-13 mRNA was readily detected in the total RNA from the lungs from line 2 mice, detected at a lower level in the RNA from lungs from line 1 mice, and unable to be detected in pulmonary tissues from transgene (–) animals (Fig. 2, and data not shown). In addition, transgene-induced IL-13 mRNA was not noted, and histological abnormalities were not appreciated on H&E analysis of a variety of visceral tissues from transgene (+) animals (data not shown). This demonstrates that the CC10 promoter selectively targeted IL-13 to the lungs of these mice.

Histologic analysis. In contrast to transgene (–) littermate animals, the lungs from the transgene (+) mice consistently manifest an inflammatory response that was diffusely noted around small and large airways and in nearby parenchyma (Fig. 3). This inflammatory response was more pronounced in the high-expressing line 2 compared

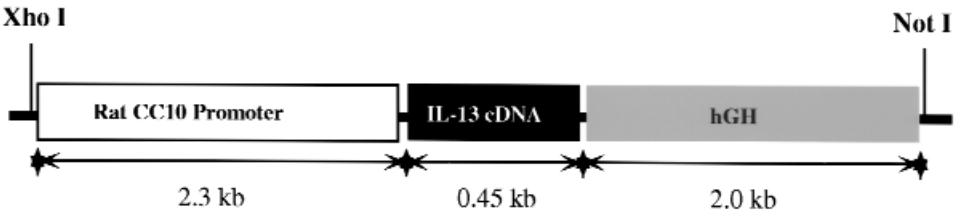

Figure 1
Schematic illustration of the construct in the IL-13 transgenic mice. *hGH*, human growth hormone; *IL*, interleukin.

with the low-expressing line 1 animals. In addition, it was milder in the 1- to 2-month-old animals and more prominent in older (2–4 months old) transgene (+) mice. In all cases, it was predominantly mononuclear in nature and contained moderate numbers of enlarged, occasionally multinucleated, macrophages that contained granular and crystalline intracellular material(s) (Fig. 3). Significant numbers and clusters of eosinophils could also be appreciated on the H&E and Congo red stains (Fig. 3). Pockets of degenerating inflammatory cells could also be appreciated, many of which were eosinophils.

Epithelial alterations were also noted in the conducting and smaller airways of the transgene (+), but not the transgene (–), animals. These changes included nuclear enlargement and airway epithelial cell hypertrophy (Fig. 3). The cellular enlargement was associated with and possibly due to the intracellular accumulation of refractile materials in the epithelial cell cytoplasm (Fig. 3). Papillary hyperplasia was also noted in older animals (data not shown).

An additional interesting finding was the presence of long, thin, rectangular, and needlelike crystals in the alveoli and occasionally the airways of the transgene (+) animals. These crystals stained with eosin but not with PAS or Congo red (Fig. 3, and data not shown). In accordance with reports on human Charcot-Leyden crystal protein (60), they also stained positively on the Papanicolaou evaluations (data not shown). The crystals were seen in small numbers in young animals (4–6 weeks old) and accumulated in impressive quantities in older line 2 animals. In these older animals, they were noted to be associated with a pericrystaline refractile amorphous material, and, on occasion, to fill local alveoli totally (data not shown). Alveolar wall rupture, air space enlargement, and the focal organization of crystalline material into fibrotic foci reminiscent of Masson bodies could also be appreciated (data not shown).

Effect on airway mucus. Studies were undertaken to understand further the relationship between the airway epithelial cell hypertrophy that was noted and airway mucus production. This was done by comparing the PAS, alcian blue, and alcian blue/PAS staining in transgene (–) and transgene (+) mice. At all time points, PAS and/or alcian blue staining cells could not be appreciated, or were extremely rare, in the airways of the transgene (–) animals. In contrast, PAS, alcian blue, and alcian blue/PAS staining cells were prominent in the airways of the transgene (+) mice (Fig. 4, and data not shown). These studies clearly demonstrate that IL-13 stimulates the production of neutral and acidic mucus and induces goblet cell hyperplasia in these animals.

Effect on airway fibrosis. Masson's trichrome stains were used to evaluate the presence and distribution of collagen in the lungs from transgene (–) and transgene (+) animals. As can be seen in Fig. 5, a small amount of wispy blue staining collagen could be appreciated in and near the airway wall, and loosely packed collagen could be appreciated in the bronchovascular bundles of transgene (–) mice. In contrast, enhanced collagen deposition was readily appreciated in the subepithelial regions and, to a lesser extent, the adventitia of small and large airways of the transgenic animals (Fig. 5). This accumulation was most prominent in the airways from the older (3–4 months old) compared with the younger (1–1.5 months old) transgenic animals.

IL-13 induction of pulmonary cytokines. To gain additional insight into the mechanism(s) by which IL-13 generated the phenotypic features that were noted, enzyme-linked immunosorbent assay (ELISA), Northern blot, and ribonuclease protection assays were used to determine whether IL-13 stimulated the production of a number of cytokines that are believed to be important in the pathogenesis of asthma. These studies demonstrated that significant levels of eotaxin protein and mRNA could be appreciated in the BAL fluid and lungs of the transgene (+), but not the transgene (–), animals (Fig. 6). Significant levels of IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) protein and IL-4, IL-5, and MCP-5 mRNA were not able to be detected in lungs from transgene (+) mice using similar approaches (Fig. 6, and data not shown).

Physiological evaluation. Studies were also undertaken to characterize the effects of IL-13 on airway physiology. These studies demonstrated that the baseline airways resistance of 1-month-old transgene (–) and line 1 transgene (+) animals were similar. The baseline resistance of 1-month-old line 2 animals was greater than the controls. This difference approached but did not achieve statistical significance (data not shown). In contrast, evaluations of older (2–3 months old) animals demonstrated a clear increase in baseline airway resistance (Table 1). In addition, the transgene (+) mice manifest exaggerated bronchospastic responses to methacholine. These alterations were easily detected with the invasive (Table 1) and noninvasive (Fig. 7) assessment methodologies. In accordance

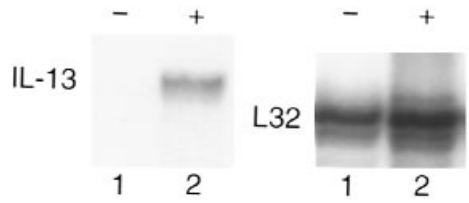

Figure 2
Ribonuclease protection analysis of IL-13 gene expression in transgene (+) mice. Total RNA was isolated from the lungs from transgene (+) (lane *2*) and (–) mice, and the levels of IL-13 and L32 mRNA were quantitated by ribonuclease protection as described in Methods.

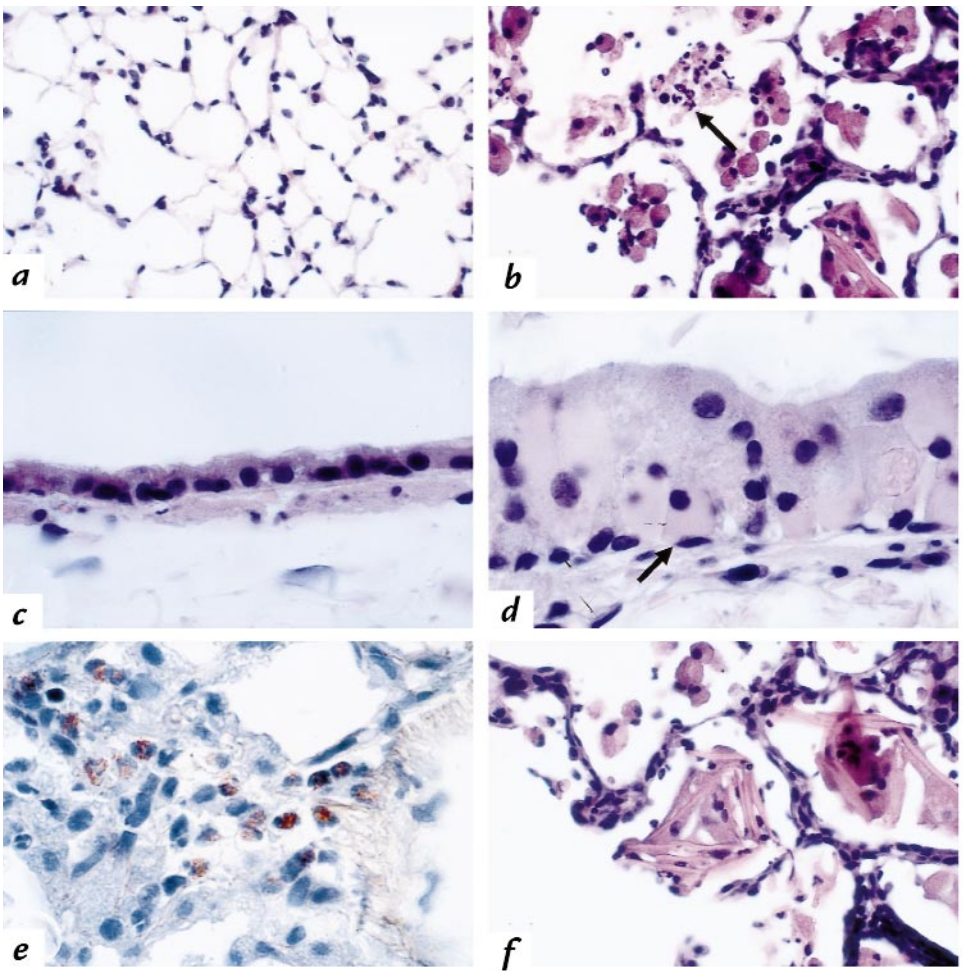

Figure 3
Comparison of the histological features of transgene (+) and (–) mice. Lungs from littermate transgene (–) and (+) mice were obtained, fixed, sectioned, and stained. (***a***) Transgene (–) lung parenchyma. H&E, ×100 (all photographs are original magnification). (***b***) Transgene (+) lung parenchyma. H&E, ×100. (***c***) Transgene (–) airway wall and epithelium. H&E, ×250. (***d***) Transgene (+) airway wall and epithelium. H&E, ×250. (***e***) Transgene (+) lung parenchyma. Congo red, ×250. (***f***) Transgene (+) lung parenchyma. H&E, ×100. A comparison of *a* and *b* illustrates the inflammatory response, macrophage enlargement, and collections of eosinophils (*b, arrow*) in the transgene (+) animals. A comparison of *c* and *d* illustrates the epithelial cell hypertrophy and intracellular refractile material (*d, arrow*) in the transgene (+) animals. *e* illustrates the tissue eosinophils (*small red cells with bilobed nuclei*). *f* illustrates the alveolar crystals in the transgene (+) animals. Eosinophils and crystals were not seen in the lungs from the transgene (–) animals. *H&E*, hematoxylin and eosin.

with the different amounts of IL-13 that were produced by line 1 and line 2 animals, the magnitude of the airways obstruction and AHR in line 2 animals was significantly greater than that in line 1 mice (data not shown).

Discussion

The type of immune response that develops in response to a foreign antigen can be attributed, at least partially, to the heterogeneity of responding $CD4^+$ T-cell populations (61). Th1 and Th2-polarized inflammation represent two contrasting and mutually antagonistic responses of an organism to an immunogenic insult. The specialized effector properties of the Th1 and Th2 cells that mediate these responses are major determinants of the outcomes of a myriad of immune responses including autoimmune, allergic, and infectious diseases (62). To provide additional information about the effector mechanisms in Th2 immunity, we characterized the *in vivo* effector functions of IL-13. To our knowledge, these studies are the first to demonstrate that the chronic overexpression of IL-13 in the lung generates a complex phenotype that recapitulates many of the features of the Th2 response in human asthma. This includes mononuclear and eosinophilic tissue inflammation, epithelial hypertrophy, mucus hypersecretion, goblet cell hyperplasia, subepithelial airway fibrosis, Charcot-Leyden–like crystal deposition, airways obstruction, and AHR to methacholine.

IL-13 and IL-4 are closely related cytokines. The genes encoding IL-13 and IL-4 are located in the same 3,000-kb gene cluster on chromosome 5 and are believed to be the result of a duplication event during evolution (reviewed in ref. 28). IL-4 and IL-13 also have overlapping biologic profiles and share the IL-4 Rα subunit and IL-13 receptor components in their respective multimeric receptor complexes (28, 33). Recent studies, however, have provided mounting evidence of important differences between these molecules. In particular, IL-4 and IL-13 differ in their ability to regulate T-cell proliferation, Th2 cell development, prostaglandin biosynthesis, IFN-γ production, eosinophil survival, eosinophil chemotaxis, and epithelial electrolyte secretion (7, 28, 34–38). IL-4 and IL-13 can also be produced by different cell populations and are differentially regulated by a number

Table 1
Physiologic profile of CC10–IL-13 mice

	Baseline Rrs	(Log) PC_{100}
Transgene (–)	231 ± 10	> 2.0
Transgene (+)	451 ± 60[A]	–0.23 ± 0.25[B]

The baseline respiratory resistance (*Rrs*) and methacholine sensitivity of 2- to 3-month-old transgene (+) line 2 mice and transgene (–) littermate controls were assessed using the invasive assessment methodology as described in Methods. Rrs is expressed as cm H_20/l/s. Methacholine sensitivity is expressed as the log of the PC_{100} (provocative challenge 100). Each value represents the mean ± SEM of a minimum of three animals. [A]$P < 0.022$ vs. transgene (–) control animals. [B]$P < 0.004$ vs. transgene (–) control animals.

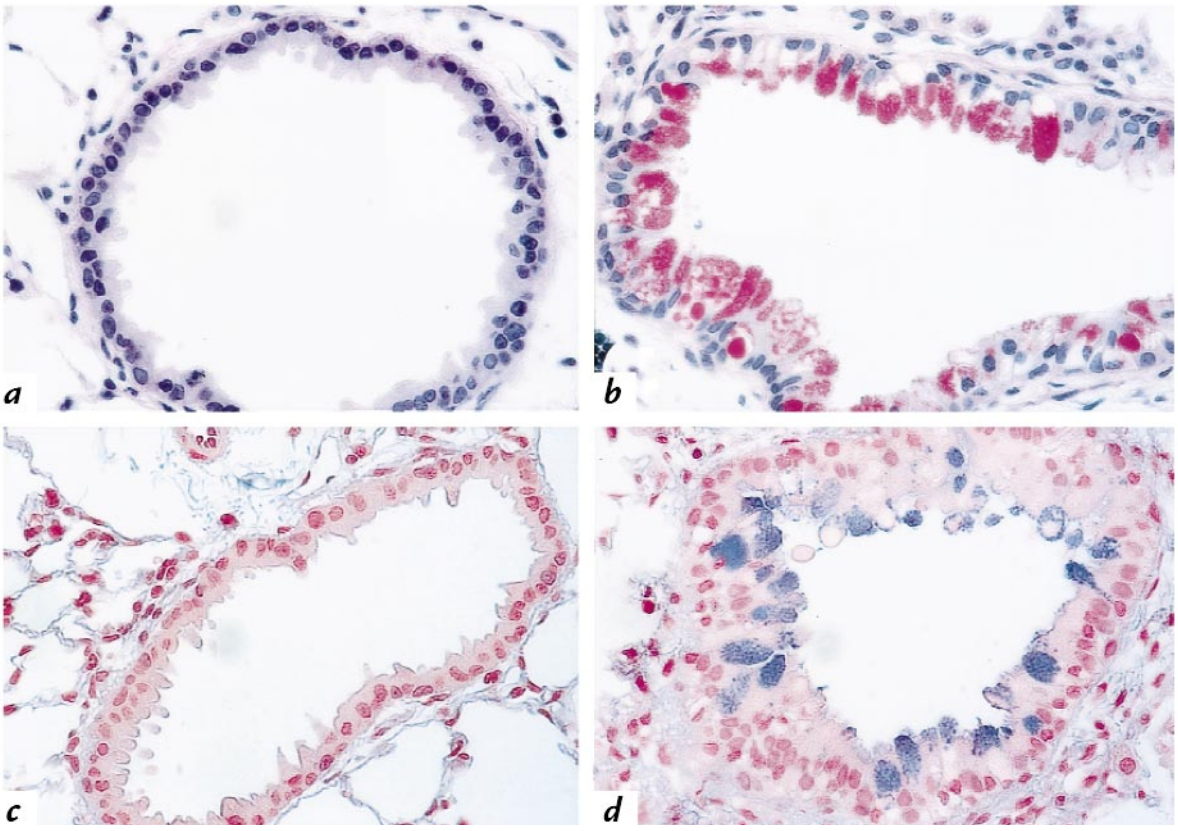

Figure 4
Comparison of mucus production by airway epithelium in transgene (–) and (+) animals. Lungs from littermate transgene (–) (***a*** and ***c***) and (+) (***b*** and ***d***) animals were obtained, fixed, and subjected to PAS (*a* and *b*) and alcian blue (*c* and *d*) staining. ×100. PAS and alcian blue staining cells have purple and blue cytoplasmic inclusions, respectively. *PAS*, periodic acid-Schiff.

of important biologic agents, including IFN-α (40). The distinct roles of IL-4 and IL-13 *in vivo* can also be appreciated in studies that demonstrated that mice that cannot make IL-4 have a markedly different capacity to mount an effective response against nematodes than do mice with a targeted disruption of the IL-4 R gene (which inactivates both the IL-4 and IL-13 receptors) (5). Thus, a complete appreciation of the phenotype of the present IL-13 transgenic animals mandates an understanding of the ways these animals are similar to and differ from mice in which IL-4 is expressed in a similar fashion (59, 63). As expected, comparisons of the IL-13 and the IL-4 transgenic mice reveal a number of similarities. The transgenes in both mice induce an inflammatory response characterized by mononuclear cells, significant numbers of eosinophils, and enlarged and occasionally multinucleated alveolar macrophages. These findings are compatible with the known ability of IL-13 and IL-4 to induce tissue inflammation and stimulate mononuclear phagocytes to form multinucleated structures (64) and the ability of IL-13 to regulate the function of monocytic lineage cells (65). They are also relevant to the pathogenesis of Th2-dominated granulomas such as those seen in schistosomiasis (48). Airway epithelial hypertrophy, mucus hypersecretion, and goblet cell hyperplasia were also noted in both the IL-4 and IL-13 mice (59, 63). This provides a plausible mechanism for the airways obstruction, mucus hypersecretion, and goblet cell hyperplasia that are commonly seen in Th2-polarized inflammatory responses in the airway and GI tracts (7, 21, 63). Prominent differences between the IL-13– and IL-4–expressing mice were also appreciated. In contrast to the reports on the IL-4 mice that did not mention or demonstrate very small degrees of airway fibrosis (63, 66), the IL-13 transgenic mice manifest significant degrees of subepithelial and moderate amounts of adventitial fibrosis. Importantly, the IL-4 mice did not manifest AHR on methacholine challenge (63), whereas the expression of IL-13 induced AHR on methacholine challenge. These findings have impressive implications with respect to the pathogenesis of human asthma.

AHR to methacholine is a cardinal feature of asthma (24–26), and methacholine challenge studies are frequently used to establish the diagnosis of asthma in cases in which clinical uncertainty exists. The importance of this physiological abnormality is reflected in the correlation between AHR and the symptoms and treatment requirements, variations in peak expiratory flow, nocturnal awakenings, and risk of sudden death in asthma (25). It is now generally accepted that AHR is caused by the inflammation in the asthmatic airway (24). The mechanism(s) by which asthmatic inflammation leads to AHR is, however, poorly understood. Cohn *et al.* (18) demonstrated that *in vitro* polarized and passively transferred Th2 cells induce AHR in otherwise naive animals via an IL-4–independent pathway. Hogan

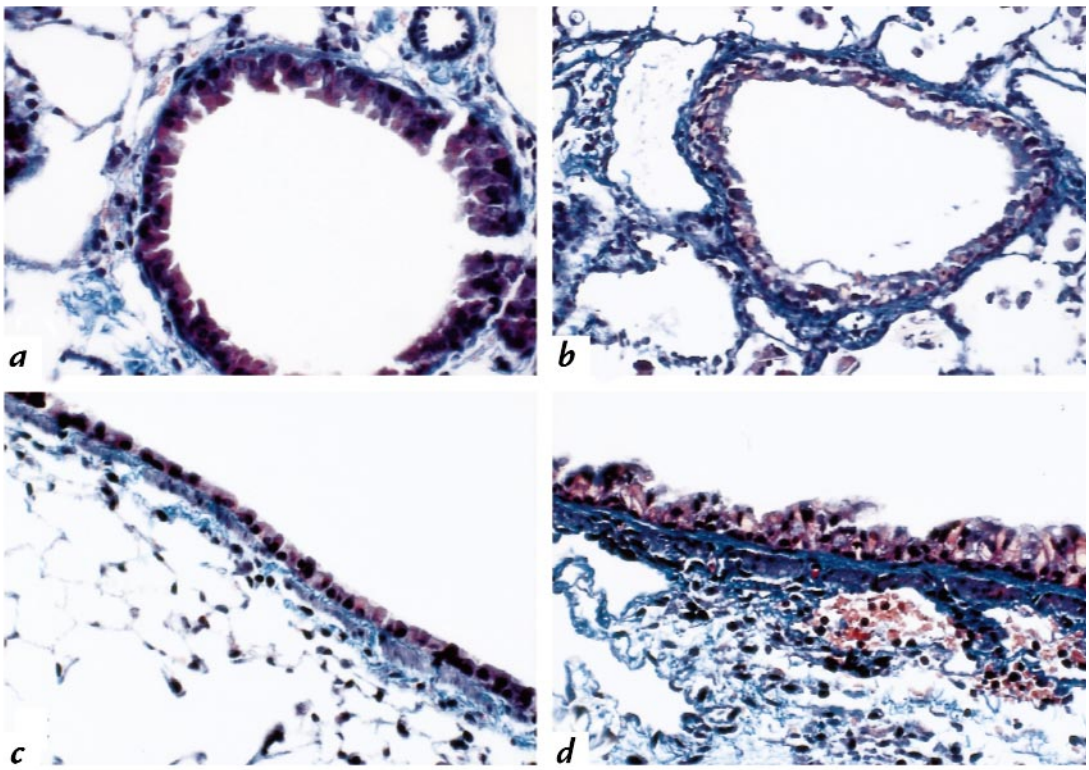

Figure 5
Comparison of the airway collagen in transgene (–) and (+) animals. Lungs from transgene (–) (***a*** and ***c***) and (+) (***b*** and ***d***) animals were obtained, fixed, sectioned, and stained (Masson's trichrome). The blue staining collagen in the small airways (*a* and *b*; ×100) and large airways (*c* and *d*; ×250) are compared.

et al. (19) also demonstrated that the AHR seen in antigen-sensitized and -challenged BALB/c mice is mediated via an IL-4– and IL-5–independent activation pathway. In accordance with these observations, our studies demonstrate that IL-13 is a potent inducer of AHR. This permits the speculation that IL-13 plays a role in the generation of the AHR noted by these investigators. When combined with studies documenting the dysregulation of IL-13 in atopic and nonatopic asthma (41–43), these studies suggest that IL-13 is also an important mediator of AHR in human asthma.

Airway wall remodeling is a prominent feature of the asthmatic airway. It was initially described as "basement membrane thickening" in early studies of asthma fatalities. More recent studies (22, 23) have demonstrated that this thickening is the result of a subepithelial fibrotic response that contains types I, III, and V collagen. Although this response is likely to have important physiological consequences, its pathogenesis has not been adequately defined. These studies are the first to demonstrate that IL-13 is a profibrotic molecule and that the chronic expression of IL-13 leads to the enhanced accumulation of collagenous material in the subepithelial and adventitial regions of the airway. These studies suggest that IL-13 may be an important mediator of the fibrotic remodeling response in the asthmatic airway. They also have implications for a number of other fibrotic pulmonary disorders, as IL-13 is produced in an exaggerated fashion by alveolar macrophages from patients with interstitial fibrosis (65) and is overexpressed in patients with systemic sclerosis (67).

To gain additional insight into the mechanism(s) by which IL-13 might mediate its effects in the lung, studies were undertaken to determine whether IL-13 induced the production of a variety of cytokines that have been implicated in the generation of airway eosinophilia and AHR (68). To our knowledge, these studies are the first to demonstrate that IL-13 is a potent inducer of eotaxin production. They also demonstrate that this induction is at least partially specific for eotaxin, as IL-4, IL-5, GM-CSF, and MCP-5 were not similarly upregulated. Eotaxin was originally described as a chemotactic agent for eosinophils (69). It regulates the levels of basal tissue eosinophilia (70) and interacts as part of a complex organized cascade to produce the impressive levels of eosinophilia noted in murine airways after antigen sensitization and challenge (68). In accordance with the effector functions of eotaxin, we noted collections of eosinophils in the lungs of IL-13 transgenic animals. Eosinophils were not, however, the predominant inflammatory cells in these animals. This may reflect the need for eotaxin to interact with other cytokines, such as IL-5 (12, 71), in generating maximal tissue eosinophil infiltration. The demonstration that IL-13 induces eotaxin provides an important link between Th2 inflammation and this important cytokine. It also raises the possibility that IL-13, via the local induction of eotaxin, primes tissues to respond in an exaggerated eosinophilic fashion to any stimulus that induces the elaboration of IL-5 or other eosinophil active moieties.

An interesting finding in these studies was the accumulation of crystals in the airways and alveoli of the IL-

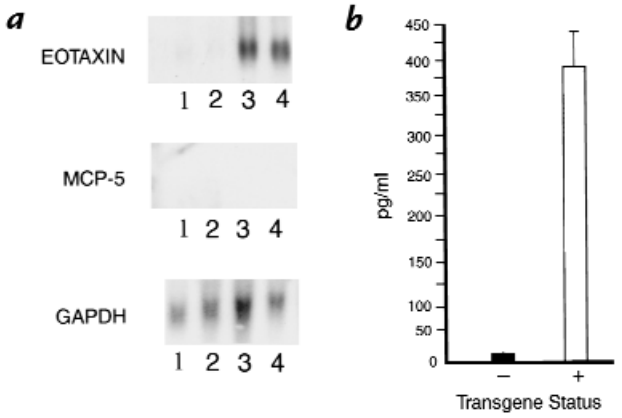

Figure 6
Documentation of eotaxin production in lungs from transgene (+) mice. (***a***) Illustrates the levels of eotaxin, monocyte chemoattractant protein-5 (*MCP-5*), and glyceraldehyde phosphate dehydrogenase (*GAPDH*) mRNA in total lung RNA from transgene (–) (lanes *1* and *2*) and (+) (lanes *3* and *4*) animals (assessed using Northern analysis). Each lane contains RNA from a separate animal. (***b***) Illustrates the levels of eotaxin protein detected by ELISA in BAL fluid from transgene (–) and (+) mice. Each value represents the mean ± SE of assays involving four mice ($P < 0.001$). *BAL*, bronchoalveolar lavage.

13 transgenic animals. These crystals accumulated in a dose- and time-dependent fashion, being rarely seen in line 1 animals, seen in small numbers in young (1 month old) line 2 animals, and seen in massive numbers in older (3–4 months old) line 2 transgenic mice. At present, we cannot state with 100% certainty that these are Charcot-Leyden crystals. We referred to them as Charcot-Leyden–like crystals based on their impressive similarity to the mouse Charcot-Leyden–like crystals described by Huffnagle *et al.* (6), their positive staining with Papanicolaou (60), and their appearance to an expert on Charcot-Leyden crystals (Ackerman, S., personal communication). Interestingly, the magnitude of crystal accumulation exceeded the magnitude of tissue eosinophilia in the lungs of the transgenic mice. This suggests that large numbers of eosinophils are attracted to the lungs of the IL-13 transgenic mice, where they die quickly and deposit granule proteins without being detected by light microscopy. Additional investigation will be required to establish definitely the nature and source of these crystals, their specificity for IL-13– or Th2-induced inflammation, and their contribution to the AHR, subepithelial fibrosis, and other aspects of the phenotype seen in these animals.

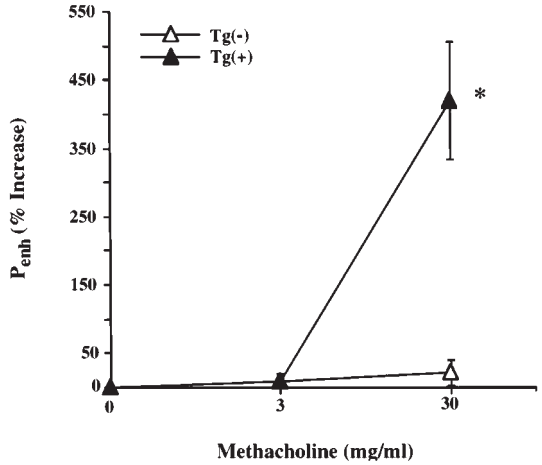

Figure 7
Comparison of the airway physiology of 1-month-old transgene (–) and line 2 transgene (+) mice. The airways resistance of transgene (–) (*open triangles*) and (+) (*filled triangles*) littermate mice was evaluated before and at intervals after methacholine administration using noninvasive methodology as described in the Methods. Enhanced pause (P_{enh}) is expressed as percent increase over baseline values. The noted values represent the mean ± SEM of assays on a minimum of five animals. *$P < 0.01$.

To our surprise, CC10-driven expression of IL-13 caused alveolar as well as airway abnormalities. Although this finding is in accordance with previous studies using the CC10 promoter (50, 52, 58), the mechanism by which CC10-driven transgene expression leads to alveolar abnormalities is poorly understood. It is possible that the CC10 promoter is activated in non-Clara cells. Alternatively, CC10-driven transgenic protein could travel centrifugally and modify the phenotype of alveolar target cells and/or induce paracrine responses that alter alveolar phenotype. Additional investigation will be required to clarify the pathogenesis of this abnormality.

In summary, we used an overexpression transgenic approach to characterize the *in vivo* effector functions of IL-13 in the murine lung. These studies demonstrate that IL-13 causes a modest inflammatory response characterized by mononuclear cell infiltration, significant numbers of eosinophils, and the appearance of enlarged and multinucleated alveolar macrophages. They also demonstrate that IL-13 is a potent inducer of airway epithelial cell hypertrophy, goblet cell hyperplasia, acidic and neutral mucus hyperproduction, subepithelial airway fibrosis, crystal deposition, airways obstruction, and AHR on methacholine challenge. Last, they demonstrate that these effects may be mediated, at least in part, by the ability of IL-13 to induce the selective local production of eotaxin. These studies support the notion that IL-13 plays an important role in the tissue inflammation, mucus hyperproduction, mucus metaplasia, airway fibrosis, Charcot-Leyden crystal deposition, and physiological abnormalities seen in asthma and other Th2-polarized inflammatory responses.

Note added in proof. After the submission of this manuscript, two additional reports were published implicating IL-13 in the generation of tissue eosinophilia, mucus metaplasia, and airways hyperresponsiveness in an acute aeroallergen model of asthma (Grünig, G., *et al.* 1998. *Science*. **282**:2261–2263; Wills-Karp, M., *et al.* 1998. *Science*. **282**:2258–2260). When viewed in combination, these studies support the importance of IL-13 in the pathogenesis of the acute and chronic manifestations of asthma.

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