

Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology

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IL-9 is a pleiotropic cytokine with multiple functions on many cell types involved in the pathology of human asthma. The constitutive overexpression of IL-9 in the lungs of transgenic mice resulted in an asthma-like phenotype. To define the contribution of IL-9 to lung inflammation we generated transgenic mice in which lung-specific expression of the IL-9 transgene is inducible by doxycycline. Transgene induction resulted in lymphocytic and eosinophilic infiltration of the lung, airway epithelial cell hypertrophy with mucus production, and mast cell hyperplasia, similar to that seen in mice that constitutively expressed IL-9 in their lungs. Various cytokines, including IL-4, IL-5, and IL-13, were expressed in the lung in response to IL-9. Blockade of IL-4 or IL-5 following IL-9 induction reduced airway eosinophilia without affecting mucus production. In contrast, neutralization of IL-13 completely abolished both lung inflammation and mucus production. These findings suggest that pathologic changes in the lung require additional signals beyond IL-9, provided by IL-4, IL-5, and IL-13, to develop fully.

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Introduction

Airway inflammation is thought to play a central role in the pathogenesis of allergic asthma (1). However, the precise roles that different inflammatory cells and mediators play in the development of pathologic and physiologic changes seen in the lungs of asthmatics are still not understood. Various inflammatory cell types, including T cells, B cells, eosinophils, macrophages, and mast cells, are involved in the complex immune response to antigen in the airway (2). In particular, activated CD4⁺ T cells of the Th2 subset seem to play a major role in initiation and maintenance of the allergic inflammation (3). Production of cytokines such as IL-4, IL-5, IL-9, IL-10, and IL-13 by Th2 cells is believed to be implicated in the development of asthmatic features, including lung eosinophilia, mucus hypersecretion, mast cell hyperplasia, and bronchial hyperresponsiveness (4).

IL-9, a Th2 cell-derived cytokine (5), was first described in the mouse as a T cell and mast cell growth factor (6, 7). Implication of IL-9 in the pathology of asthma came first from genetic studies in humans that linked asthma, atopy, and bronchial hyperresponsiveness to chromosomal region 5q31-q33, which contains several genes that are implicated in bronchial inflammation associated with asthma, including IL-9 (8, 9). Although the human gene(s) predisposing to asthma have not yet been identified from that chromosomal region, a number of candidate genes have been proposed; among these is IL-9 (8, 10). Support for a role of

IL-9 came from genetic studies in mice that mapped bronchial hyperresponsiveness to a region on chromosome 13 where the IL-9 gene is located in mice (10). These data were further sustained by the finding that bronchial hyperresponsiveness in different inbred strains of mice was correlated with IL-9 mRNA levels in the lung. A potentially important role of IL-9 in the pathogenesis of asthma has been supported recently by multiple findings that have revealed the pleiotropic effects of IL-9 on various cell types associated with allergic asthma, including mast cells, B cells, eosinophils, and airway epithelial cells. Studies in humans and animals have shown the involvement of IL-9 in lung eosinophilia, mucus hypersecretion, Ig production, and pulmonary mastocytosis (11–20). These findings have been strongly supported by our studies with transgenic mice that constitutively express IL-9 selectively within their lungs (21). These mice developed many features that resembled human asthma, including eosinophilic and lymphocytic inflammation of the lung, mucus hypersecretion, subepithelial fibrosis, mast cell hyperplasia, and bronchial hyperresponsiveness.

To study basic mechanisms that are involved in the development of lung inflammation and mucus production in response to IL-9 expression we created doxycycline-inducible, lung-specific IL-9 transgenic mice. We show that, following transgene induction, these mice develop pathologic changes in the lungs similar to those in transgenic mice that constitutively expressed IL-9 in

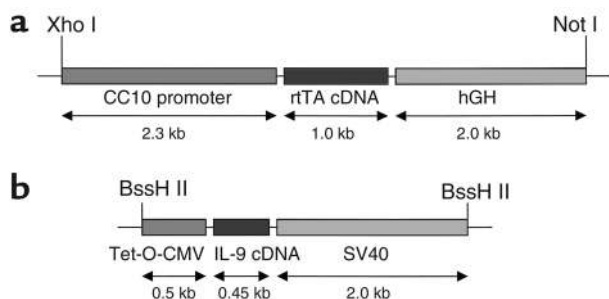


Figure 1
Genetic constructs used for the generation of transgenic mice with regulated overexpression of IL-9 by doxycycline. The inducible, lung-specific expression system consists of two separate constructs. (a) The first construct contains the rtTA cDNA under the control of the lung epithelial cell-specific, rat CC10 promoter and the hGH intronic and polyadenylation sequences. (b) In the second construct the IL-9 cDNA is linked to the tet-O-CMV promoter and the SV 40 small t intron and polyadenylation sequence.

their lungs. We successfully used this inducible system to further define the role of IL-9 and other Th2 type cytokines in airway eosinophilia and mucus production. The blockade of selected cytokines during IL-9 transgene expression revealed that the development of lung pathology, in particular eosinophilia and mucus production induced by IL-9, was dependent on the expression of several Th2 cytokines, including IL-4, IL-5, and IL-13.

Methods

Generation of transgenic mice. Transgenic mice were generated in which lung-specific expression of the IL-9 transgene is externally inducible by doxycycline, a tetracycline derivative. The production of these mice required two separate constructs. The first construct contained the cDNA for the reverse tetracycline transactivator (rtTA) under the control of the rat CC10-promoter and the human growth hormone (hGH) intronic and polyadenylation sequences (Figure 1a). The CC10-rtTA-hGH construct was obtained by XhoI and NotI digestion from a vector described previously (22). The second construct contained the IL-9 cDNA, kindly provided by the Genetics Institute (Andover, Massachusetts, USA), under the control of the CMV promoter linked to the tet-O sequences and the SV 40 small t intron and polyadenylation sequence (Figure 1b). To generate this construct the 443-bp IL-9 cDNA was isolated by EcoRI digestion of plasmid pCC10-IL9-SV40 (21) and cloned into the EcoRI site of plasmid pBluescript II KS (Stratagene, La Jolla, California, USA). The IL-9 cDNA was released from this vector by SpeI and HindIII digestion and cloned into the SpeI and HindIII sites of plasmid ptet-splice (23) to form plasmid ptet-O-IL9. The tet-O-CMV-IL-9-SV40 construct was released from this plasmid by BssHIII digestion. For microinjection, both constructs were separated by electrophoresis through a 1% SeaKem GTG agarose gel (FMC Corp., Rockland, Maine, USA), isolated by electroelution, and further purified as

described previously (24). Both constructs were simultaneously injected into (C3H × C57BL/6) F₂ eggs as described previously (25). Positive founder animals for both constructs were identified by PCR analysis of tail DNA. PCR was performed for the IL-9 transgene and the rtTA transgene as described previously (21, 22). Animals positive for both transgenes were backcrossed onto strain B10.D2. Expression of the IL-9 transgene was confirmed by measuring IL-9 levels in lung lavage fluid from transgene positive animals, which were kept for 14 days on doxycycline-supplemented food (2.3 g doxycycline/kg dry food).

Lung lavage, tissue fixation, and staining. Mice were anesthetized by methoxyflurane inhalation and then sacrificed by carbon dioxide inhalation. Lung lavage and cell enumeration was performed as described previously (21). The lungs were excised completely from the chest, inflated with 1 ml of 10% formalin, and immersed in 10% formalin. Tissue processing and various histological stainings were performed by the Yale Medical School Research Histology, Department of Pathology.

Cytokine assay. Quantitation of IL-9 levels in lung lavage fluid was performed by ELISA as described previously (21). The detection limit for IL-9 in this ELISA was 0.4 ng/ml.

RNase protection assay. For RNA isolation, lung tissue was removed from the animal, immediately quick-frozen in liquid N₂, and stored at -70°C until use. Frozen lung tissue was then homogenized in TRIzol Reagent (Invitrogen, Carlsbad, California, USA) and total RNA was isolated following the manufacturer's instructions. RNase protection assay was performed using the RiboQuant Multi-Probe RNase Protection Assay System according to the manufacturer's instructions (BD Bioscience, San Diego, California, USA). Ten micrograms of total lung RNA of each sample was used for hybridization with the [α -³²P] UTP-labeled mouse cytokine multiprobe template set mCK-1b (templates for IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-3, IFN- γ , L32, and GAPDH). Protected fragments were separated using the QuickPoint Gel System according to the manufacturer's instructions (Novex, San Diego, California, USA). Gels were dried for 30 minutes at 80°C and exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, New York, USA).

RT-PCR analysis. Total lung RNA for RT-PCR was isolated as described above. Reverse transcription was performed using 5 μ g of total RNA in a first-strand cDNA synthesis reaction with SuperScript II RNaseH Reverse Transcriptase as recommended by the manufacturer (Invitrogen). One microliter of the resulting RT product was used for PCR amplification. Primers used for PCR of IL-4 cDNA were as follows: 5'-CCC CAG CTA GTT GTC ATC C-3', 5'-TGA TGC TCT TTA GGC TTT CC-3' (26). The primer sequences are located within the first and fourth exon of the murine IL-4 gene and do not amplify genomic DNA (data not shown). Primers used for HPRT cDNA were: 5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3', 5'-TCG GAT ATC CGG TCG GAT GGG AG-

3' (27). PCR conditions were one cycle of 95°C for 3 minutes, 35 cycles of 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. Amplification of the appropriate cDNA results in a 390-bp fragment for IL-4 and a 300-bp fragment for HPRT. PCR products were separated in 1% agarose gels.

In vivo blockade of selected cytokines during transgene expression. Four to five inducible IL-9 transgene-positive mice per group, 6 weeks of age, were housed with or without doxycycline-supplemented food over a period of 14 days. During the time of transgene expression, mice in different groups received either no Ab's, isotype control Ab's (GL113, rat IgG1, see ref. 28; human IgG, see ref. 29), anti-IL-4 Ab's (11B11, rat IgG1; ref. 30), sIL-13R α 2-IgG-Fc fusion protein (29) (kindly provided by D. Donaldson, Genetics Institute), anti-IL-5 Ab's (TRFK-5, rat IgG1; ref. 31), or a combination thereof. Ab's were injected intraperitoneally at day 1 and day 7 (1 mg in 0.1 ml PBS for 11B11, TRFK-5, and GL113) or every other day (0.2 mg in 0.1 ml PBS for sIL-13R α 2-IgG-Fc and human IgG). At day 14 mice were sacrificed and the lungs lavaged and removed for histology.

Statistical analysis. Values are expressed as means plus or minus SD. The data were normally distributed, and group means were compared with the Student two-tailed, unpaired *t* test using Excel 5 for Apple Macintosh (Microsoft Corporation, Redmond, Washington, USA).

Results

Generation of transgenic mice. We generated several independent lines of transgenic mice in which lung-specific expression of the IL-9 transgene is inducible by dietary doxycycline. The two separate constructs necessary for microinjection to produce these mice are shown in Figure 1. This inducible transgenic system had been described previously for IL-11 transgenic mice (22) and is therefore not explained here in detail. Briefly, in the presence of doxycycline rtTA, which is under the control of the lung epithelial cell-specific CC10 promoter, is able to bind in trans to the tet-O and mediate transactivation of the IL-9 transgene from the CMV promoter. In the absence of doxycycline, binding of the rtTA does not occur and transcription of the IL-9 transgene is not activated. We obtained 23 founder animals that carried both transgene constructs, and 12 of them were backcrossed onto strain B10.D2 for analysis of transgene expression. Progeny received from ten of these founder animals expressed detectable levels of IL-9 in lung lavage fluid after transgene induction by doxycycline. One of these lines was chosen for the following experiments and backcrossed at least five times onto strain B10.D2.

Induction of IL-9 transgene expression. To study if an inducible IL-9 transgene expression system would result in the same histological changes in the lung as seen in transgenic mice that constitutively expressed IL-9 in the lung (21), IL-9 transgene-positive and transgene-negative mice were housed with or without doxycycline-supplemented food over a period of 14 days. At three different time points animals were sacrificed and

the lungs lavaged and removed for histology. At day 1, 7, and 14, without induction of the IL-9 transgene, there was no IL-9 detectable by ELISA in lung lavage fluid from either IL-9 transgene-negative or transgene-positive animals. IL-9 was detectable only in lung lavage fluid from transgene-positive animals at day 7 (0.556 ± 0.333 ng/ml) and at day 14 (0.913 ± 0.301 ng/ml) in the presence of doxycycline.

Analysis of the cellular content of lung lavage fluid revealed that cell numbers increased significantly with time in IL-9 transgene-positive mice after transgene induction at day 7 and day 14, compared with transgene-negative and -positive animals that did not receive doxycycline (Figure 2). Differential cell counts on lung lavage cells revealed that the induction of the IL-9 transgene resulted in significantly elevated numbers of macrophages, lymphocytes, and especially eosinophils in the airways, which increased with time of induction (Figure 3). At day 14 after transgene induction about 50% of all cells retrieved by lung lavage from transgene-positive animals were eosinophils. In contrast, mostly macrophages were retrieved from transgene-negative animals (Figure 3). Not unexpectedly, there was also an increase in cell numbers in lung lavage fluid from transgene-positive animals that had been kept without doxycycline-supplemented food (Figure 2). These cell numbers did not, however, significantly increase with time and were about the same at day 7 and day 14 (Figure 2). Differential cell counts demonstrated the presence of increased numbers of macrophages, lymphocytes, and eosinophils (Figure 3). This mild pathology in the airways from transgene-positive animals in the absence of doxycycline reveals a small leakage in the rtTA system that results in low-level expression of the IL-9 transgene, below the detection limit of the ELISA.

Histological assessment of lung tissue stained with hematoxylin and eosin (H&E) demonstrated that the

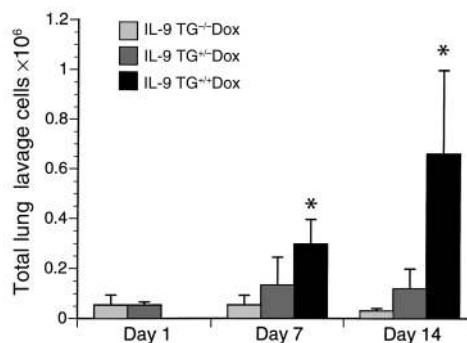


Figure 2

Total cell counts from lung lavage fluid. IL-9 transgene-negative (TG⁻) and -positive (TG⁺) mice, 6 weeks of age, were housed with or without doxycycline-supplemented food over a period of 14 days. At three different time points mice were sacrificed, subjected to lung lavage, and total cell numbers assessed. Data are expressed as mean \pm SD of counts from four to five animals per group. Significant differences in cell numbers compared with that of transgene-negative and -positive mice that did not receive doxycycline are indicated by an asterisk.

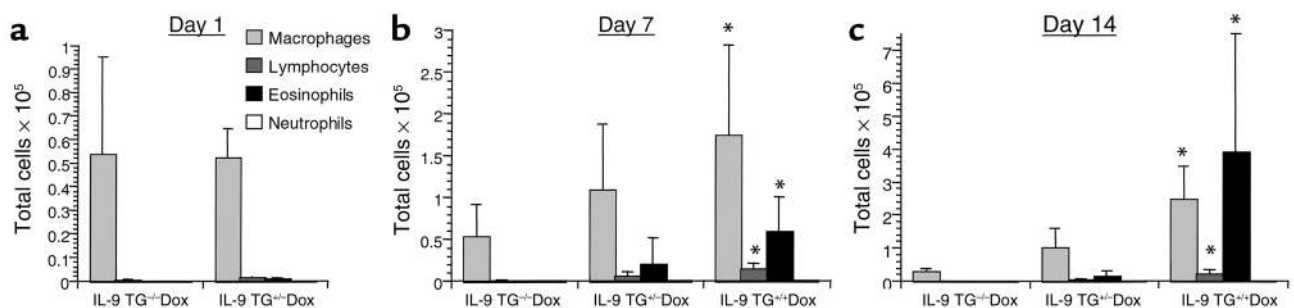


Figure 3

Characterization of inflammatory cells in lung lavage fluid. Differential cell counts were performed on lung lavage cells retrieved from transgene-negative (TG⁻) and -positive (TG⁺) mice that had been housed with or without doxycycline-supplemented food over a period of 14 days. At day 1, 7, and 14, mice were sacrificed and lungs lavaged. Differential cell counts were derived from at least 200 cell counts and are expressed as total cell numbers. Data represent the mean \pm SD of counts from four to five animals per group. Significant differences in cell numbers compared with that of transgene-negative or -positive mice at day 1 that did not receive doxycycline are indicated with an asterisk. Notice the differences in the scale of the y axis between the graphs.

induction of the IL-9 transgene over a period of 14 days resulted in similar pathologic changes in the lungs as seen in mice that constitutively expressed IL-9 (21). After 7 days in the presence of doxycycline, there was infiltration of inflammatory cells visible around airways and blood vessels in lung sections from transgene-positive mice (Figure 4c). This infiltration was even more impressive after 14 days of transgene induction (Figure 4d). Airway epithelial cells on most conducting airways were hypertrophic and stained positive for mucin at day 7 and day 14 after transgene induction (Figure 5, c and d) in lung sections stained with Alcian blue/periodic acid-Schiff (AB/PAS). However, mucus accumulation seemed to be mostly intracellular since mucin levels in lung lavage fluid from IL-9 transgene-positive mice maintained on doxycycline for 14 days were not increased compared with that from transgene-negative mice (data not shown). Intraepithelial mast

cells that were typically found in the airways of transgene-positive mice that constitutively expressed IL-9 in the lungs were only occasionally seen in lung sections stained with toluidine blue from inducible IL-9 transgenic mice at day 7 of transgene expression, but increased in number in lung sections from day 14 (Figure 5f). Inflammatory cell infiltration, airway epithelial cell hypertrophy with mucus accumulation, and intraepithelial mast cells were not found in lung sections from transgene-negative mice at any time point (Figure 4a, Figure 5a). Transgene-positive mice, which were kept without doxycycline-supplemented food, also showed lung pathology. While there was only minor infiltration of inflammatory cells in lung sections visible at day 1, 7, or 14, epithelial cell hypertrophy with mucus accumulation was already present in larger airways and occasionally in smaller airways at day 1 (Figure 5b). Lung sections from day 7 and day 14

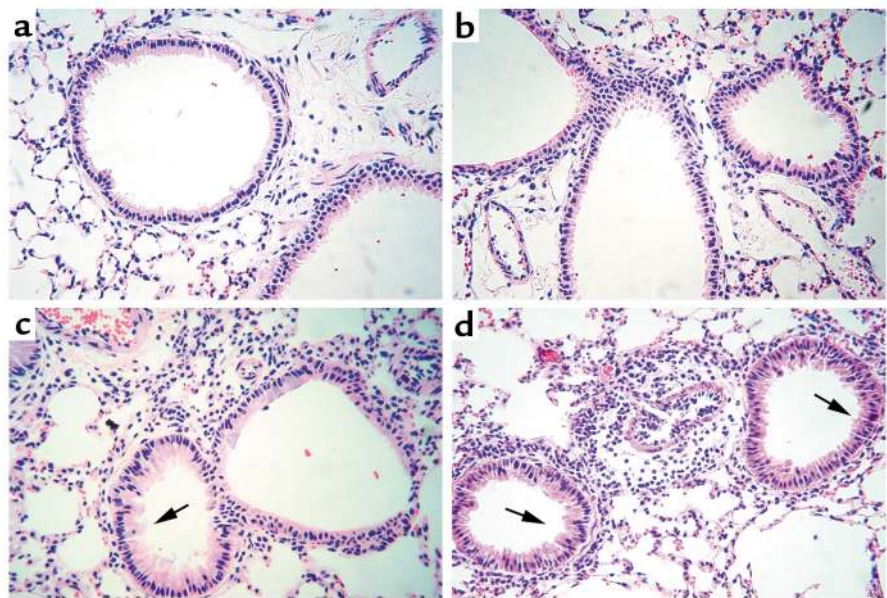


Figure 4

Lung histology of conducting airways and parenchyma. Lung sections from IL-9 transgene-negative (a) and -positive (b-d) mice were stained with H&E for examination using light microscopy. Induction of the IL-9 transgene by doxycycline over a period of 7 days (c) or 14 days (d) resulted in infiltration of lung tissue with inflammatory cells. Furthermore, airway epithelial cells were hypertrophic (arrows). Inflammatory cells were not observed in lung sections from six-week-old transgene-negative (a) or -positive (b) mice without doxycycline treatment. Original magnification $\times 300$.

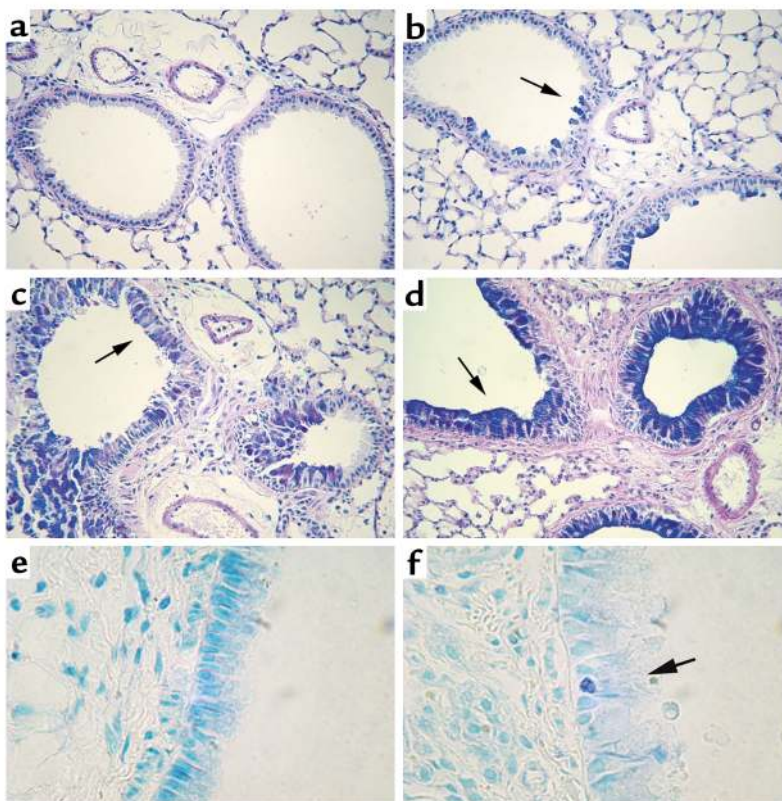


Figure 5

Histologic staining for mucin and mast cells. Lung sections from IL-9 transgene-negative (a) or -positive (b-d) mice were stained with AB/PAS for light microscopic identification of mucin-containing cells. Intense, positive (purple) staining for mucin was observed in hypertrophied airway epithelial cells from IL-9 transgene-positive mice after 7 days (c) (arrow) and 14 days (d) (arrow) of transgene induction by doxycycline. In lung sections from transgene-positive mice that did not receive doxycycline some mucin-positive epithelial cells were also detected (b) (arrow). Airway epithelium from transgene-negative mice did not stain for mucin (a). For the identification of mast cells, lung sections from transgene-negative (e) or -positive (f) mice were stained with toluidine blue. Dark blue-stained mast cell granules (arrow) were present in the airway epithelium from transgene-positive mice that were housed with doxycycline-supplemented food over a period of 14 days (f), but not in transgene-negative mice (e). Original magnification: a-d, $\times 300$; e-f, $\times 600$.

showed epithelial cell hypertrophy in conducting airways of all different sizes with strong positive staining for mucin (data not shown). Intraepithelial mast cells were not observed in any lung sections from transgene-positive mice at any time point without doxycycline treatment (data not shown). However, at any time point the inflammatory response was clearly stronger in the lungs of transgene-positive mice that received doxycycline compared with mice that did not.

Analysis of Th2 cytokine expression in the lungs of inducible IL-9 transgenic mice. Induction of the IL-9 transgene over a period of 14 days resulted in an impressive inflammatory response in the lungs. To study which Th2 cytokine genes in addition to the IL-9 transgene were expressed, RNase protection assay was performed using total lung RNA isolated from transgene-negative and transgene-positive mice that had been kept on doxycycline-supplemented food for 14 days. Using a multitemplate probe for the RNase protection assay, transcripts for the Th2 cytokines IL-5, IL-13, and IL-9 could be detected in lung tissue from transgene-positive mice after transgene induction but not from transgene-negative mice (Figure 6a). There were also transcripts detectable for IFN- γ , a Th1 cytokine, in lung tissue from transgene-positive mice and mRNA for IL-15 in both lung tissue from transgene-positive and -negative mice (Figure 6a). Surprisingly, transcripts for IL-4 could not be detected by the RNase protection assay; RT-PCR was therefore performed. As shown in Figure 6b, specific bands from amplified IL-4 cDNA could be clearly detected using RT-PCR on total lung RNA isolated from transgene-

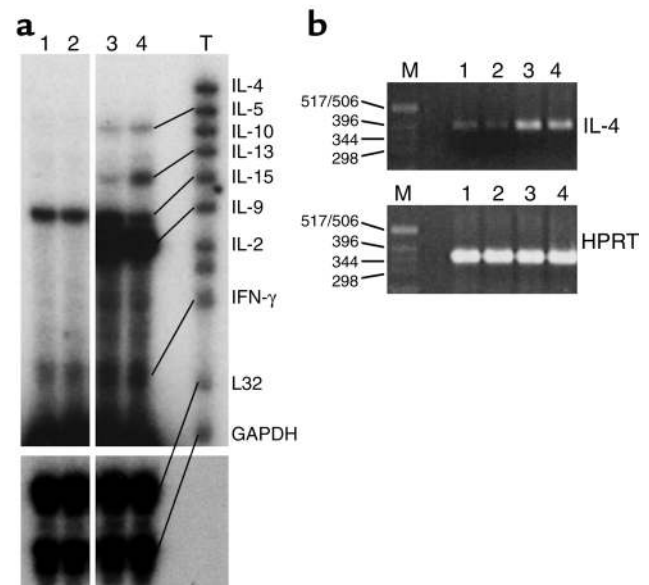


Figure 6

Analysis of cytokine expression in total lung tissue. (a) Levels of various mRNAs encoding predominantly Th2 cytokines from individual IL-9 transgene-negative (lane 1, 2) and -positive (lane 3, 4) mice after transgene induction by doxycycline were compared by RPA. The P³²-labeled multiprobe template set (T) was used as a size marker. Levels of L32 and GAPDH mRNAs encoding housekeeping genes were used to ensure equal loading of samples. (b) Levels of mRNA encoding IL-4 were evaluated by RT-PCR using total lung RNA isolated from transgene-negative (lane 1, 2) and -positive (lane 3, 4) mice 14 days after IL-9 transgene induction by doxycycline. Levels of mRNA encoding HPRT, a housekeeping gene, were used to demonstrate equal loading of samples. Fragment sizes of a molecular-weight marker are indicated in base pairs.

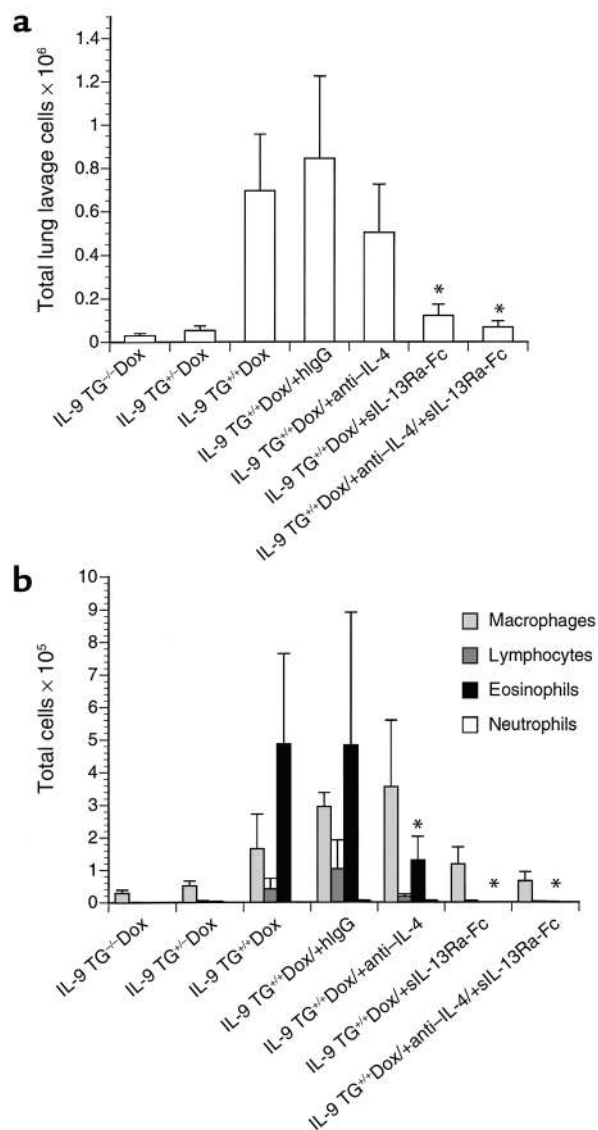


Figure 7 Characterization of inflammatory cells in lung lavage fluid after cytokine blockade. IL-9 transgene-negative (TG⁻) or -positive (TG⁺) mice were housed with (+Dox) or without (-Dox) doxycycline-supplemented food for 14 days. During the entire time of IL-9 transgene induction, selected groups of animals received anti-IL-4 Ab's, sIL-13Rα-Fc, or isotype control Ab's (hlgG) as described in Methods. At day 14, mice were sacrificed and the lung lavaged. (a) Total lung lavage cell counts. (b) Differential cell counts on lung lavage cells. Results are expressed as total cell numbers and were obtained from at least 200 cell counts. Data are expressed as mean ± SD. Significant differences in total cell number (a) or number of eosinophils (b) compared with that of transgene-positive mice that received doxycycline but no Ab's are indicated with an asterisk.

positive mice after 14 days of IL-9 transgene induction by doxycycline. Low levels of IL-4-specific mRNA could also be detected by RT-PCR in total lung RNA from transgene-negative mice (Figure 6b).

The effect of in vivo blockade of selected cytokines during IL-9 transgene expression on lung pathology. To study the contributions of IL-4, IL-13, and IL-5 to the pathology

observed in IL-9 transgenic mice we selectively blocked these cytokines during the entire time of doxycycline-induced transgene expression.

The injection of anti-IL-4 Ab's during transgene expression had only minor effects on lung pathology. Compared with transgene-positive mice that received no Ab's or isotype-control Ab's, the in vivo blocking of IL-4 did not significantly decrease the total number of inflammatory cells retrieved from the lungs by lavage (Figure 7a). However, differential cell counts revealed a significant decrease in the numbers of eosinophils, but not lymphocytes or macrophages (Figure 7b). Histological examination of lung sections from transgene-positive mice that received anti-IL-4 Ab's during transgene expression also did not show major differences when compared with lung sections from transgene-positive mice that received no Ab's or isotype-control Ab's (Figure 8, b-d). There were accumulations of inflammatory cells around airways and blood vessels visible in lung sections of all three groups. Epithelial cells were hypertrophic and showed positive, purple staining for mucin in lung sections stained with AB/PAS (Figure 8, b-d). These pathological changes were not seen in lung sections from transgene-negative mice (Figure 8a). These results indicate that IL-4 is required for airway eosinophilia but not tissue infiltration or mucus production in IL-9-induced lung pathology.

The in vivo blockade of IL-13 by injection of soluble IL-13Rα2-IgG-Fc fusion protein (sIL-13Rα-Fc) during IL-9 transgene expression had a more dramatic effect on the development of lung pathology compared with IL-4 blockade. The number of inflammatory cells retrieved by lavage from transgene-positive mice that received sIL-13Rα-Fc was significantly reduced compared with transgene-positive mice that received no Ab's or control Ab's in the presence of doxycycline and were similar to cell numbers retrieved from transgene-positive and -negative mice that were kept without doxycycline-supplemented food (Figure 7a). Differential cell counts demonstrated that this reduction was due to decreased numbers of macrophages and lymphocytes, but especially due to the total absence of eosinophils in lung lavage fluid from transgene-positive mice after IL-13 blockade (Figure 7b). Furthermore, lung sections stained with AB/PAS from transgene-positive mice that had received sIL-13Rα-Fc during IL-9 transgene expression did not show any pathological changes such as inflammatory cell infiltration or epithelial cell hypertrophy with mucus accumulation (Figure 8e), as seen in lung sections from transgene-positive mice that received no Ab's or control Ab's during IL-9 transgene expression (Figure 8, b-c). Therefore, the development of lung eosinophilia and mucus accumulation in airway epithelial cells during IL-9 transgene expression was dependent on IL-13.

Since the in vivo blockade of IL-13 alone during IL-9 transgene expression inhibited the development of lung

pathology in transgene-positive mice, an *in vivo* blockade of IL-4 and IL-13 at the same time during IL-9 transgene expression did not have any further effects. Lung lavage cell counts (Figure 7, a and b) and histological assessment of lung sections (Figure 8, e and f) were comparable to that observed in mice that received sIL-13R α -Fc alone during transgene expression.

The *in vivo* blockade of IL-13 abolished eosinophilia in the lungs of IL-9-expressing mice. To investigate if eosinophils and IL-5 are involved in epithelial cell hypertrophy and mucus production we neutralized *in vivo* IL-5 in transgene-positive mice during IL-9 transgene expression. The injection of anti-IL-5 Ab's reduced the number of total cells retrieved by lung lavage compared with that from transgene-positive mice that did not receive Ab's or isotype control Ab's during IL-9 transgene induction (Figure 9a). Differential counts on lung lavage cells revealed that injection of the anti-IL-5 Ab resulted in reduction of the numbers of eosinophils retrieved from the airways (Figure 9b). In addition, the *in vivo* blockade of IL-5 abrogated inflammation in lung tissue in the presence of IL-9 (Figure 10). There was less accumulation of inflammatory cells detectable around airways and blood vessels. But lung sections stained with AB/PAS still showed epithelial cell hypertrophy and strong positive staining for mucin (Figure 10c). Therefore, IL-5 is necessary for the development of lung eosinophilia but not for mucus production by airway epithelial cells in our system of IL-9 transgene expression.

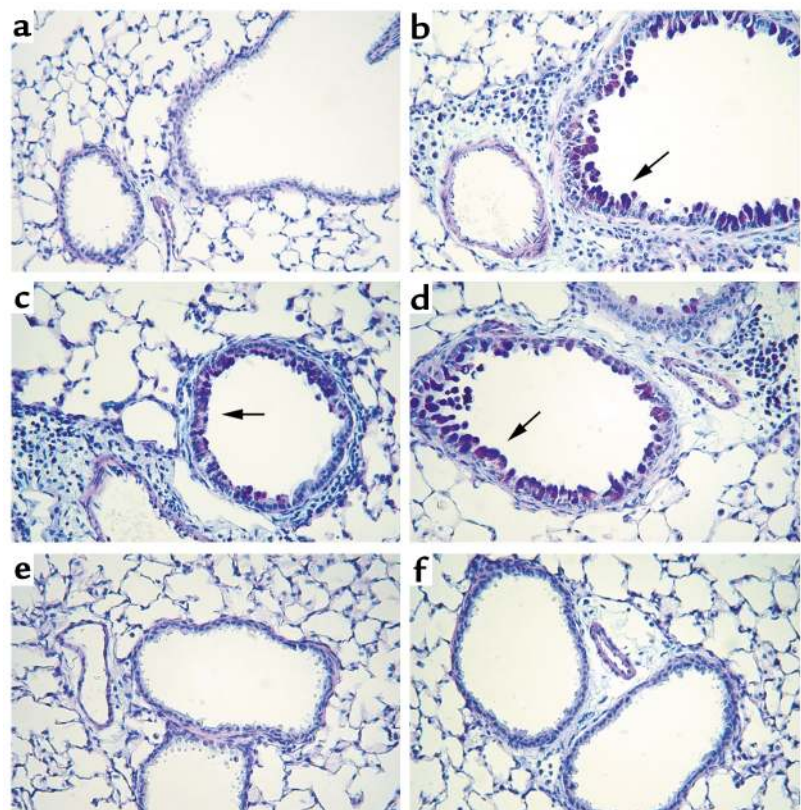
Discussion

Cytokines are important mediators in human asthma, but the role they play in allergic inflammation is still not completely understood. Our study focused on IL-9, a cytokine produced by Th2 cells, mast cells, and eosinophils and its contribution to airway inflammation (32–34). Previously, we had shown that constitutive lung-specific expression of IL-9 in transgenic mice resulted in an asthma-like phenotype with eosinophilic and lymphocytic airway inflammation, mucus hypersecretion, mast cell hyperplasia, and bronchial hyperresponsiveness (21). To dissect the complex phenotype observed in these mice we generated IL-9 transgenic mice in which lung-specific expression of the IL-9 transgene is inducible by doxycycline. This system of regulated transgene expression allowed us to induce the IL-9 phenotype while blocking simultaneously any cytokine of interest. The blockade of selected cytokines in the presence of IL-9 provided us not only with new information about the contributions of these cytokines to airway inflammation in our system but helped us to define more precisely the impact that IL-9 might have in the pathogenesis of asthma.

Induction of the IL-9 transgene by doxycycline over a period of 14 days resulted in lung inflammation and mucus overproduction similar to that seen in the lungs of transgenic mice that constitutively expressed IL-9 in their lungs (21). Regulated expression of IL-9 caused infiltration of lung tissue and airways with lymphocytes and eosinophils, as well as increased numbers of

Figure 8

Histologic staining for mucin after cytokine blockade. Light micrographs of lung sections stained with AB/PAS from IL-9 transgene-negative (a) or -positive (b–f) mice that were housed for 14 days with doxycycline-supplemented food. During the entire time of IL-9 transgene induction selected groups of animals received Ab's: (c) control Ab's, hIgG; (d) anti-IL-4; (e) sIL-13R α -Fc; (f) anti-IL-4 and sIL-13R α -Fc. Lung sections from transgene-positive mice that received anti-IL-4 Ab's, control Ab's, or no Ab's showed strong positive (magenta) staining for mucin in airway epithelial cells (arrows) and accumulation of inflammatory cells near blood vessels and airways (b–d). Infiltration of lung tissue with inflammatory cells or positive staining for mucin was not observed in sections from transgene-positive mice that received sIL-13R α -Fc alone (e), or in combination with anti-IL-4 (f), or in transgene-negative mice (a). Original magnification $\times 300$.



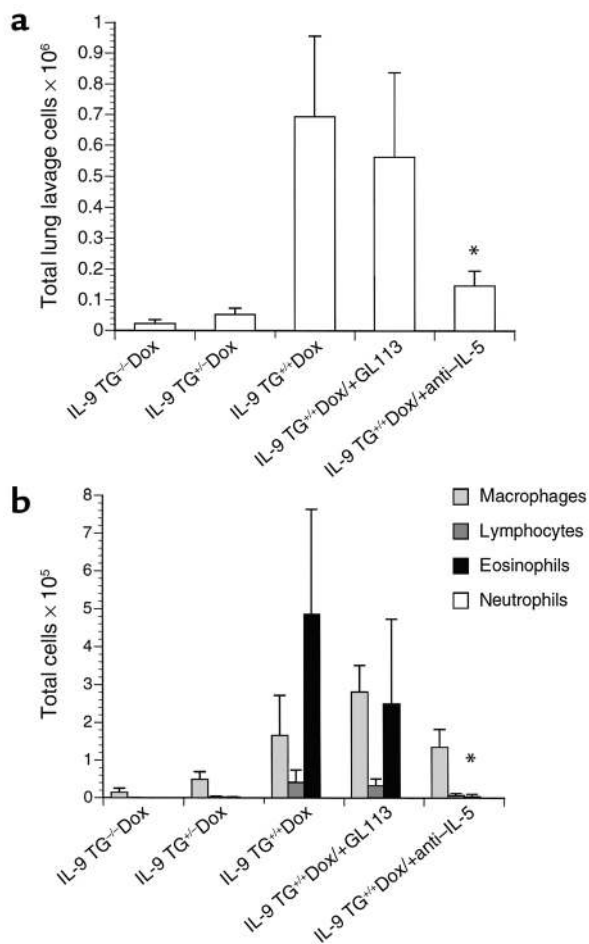


Figure 9 Characterization of inflammatory cells in lung lavage fluid after IL-5 blockade. IL-9 transgene-negative (TG⁻) or -positive (TG⁺) mice were kept with (+Dox) or without (-Dox) doxycycline-supplemented food. After 14 days, mice were sacrificed, lungs lavaged, and cell numbers in lavage fluid evaluated. During the entire time of transgene induction, selected groups of animals received intraperitoneal anti-IL-5 Ab's or isotype control Ab's (GL113). (a) Total cell numbers retrieved by lung lavage. (b) Differential cell counts. Results are expressed as total cell numbers and were derived from at least 200 cell counts. Data are presented as mean ± SD. Significant differences compared with counts from transgene-positive mice that did not receive Ab's during IL-9 transgene expression are indicated with an asterisk.

macrophages. Airway epithelial cells were hypertrophic and stained strongly positive for mucin. Also, intraepithelial mast cells could be detected in inducible IL-9 transgenic mice after 14 days of doxycycline treatment. Analysis of transgene-positive mice that did not receive doxycycline also revealed minor infiltration in the airways. These might have resulted from a leakage in the rTA system, as reported before (22), that allows low-level expression of the transgene even in the absence of doxycycline. However, this low-level expression of IL-9 did not interfere with the aim of our study reported here. In fact, the manifestation of mild pathology in our system in response to low level expression of IL-9, especially the early epithelial cell hypertrophy with

mucus overproduction, actually emphasizes the potency of IL-9 to induce lung inflammation.

Induction of IL-9 transgene expression in the lungs by doxycycline caused a complex inflammatory response associated with the expression of various Th2 cytokines, including IL-4, IL-5, and IL-13. The contributions of these cytokines to the observed pathology in the lungs of IL-9 transgenic mice were of particular interest for us since Th2 cell-derived cytokines have been directly linked to the pathology of human asthma (35–38). Various murine models of asthma have been used to study the impact of Th2 cytokines on lung inflammation, eosinophilia, and mucus hypersecretion, but the results are still controversial, emphasizing the complexity of allergic inflammation (4). The exact mechanism(s) by which IL-9 induces the production of Th2 cytokines in the lung is not known at this point. Future studies will have to reveal the direct target cells of IL-9 in the lung as well as the cell source(s) of IL-4, IL-5, and IL-13 expression. Preliminary data that we have obtained with IL-9 transgenic mice backcrossed onto the RAG1-deficient background (39) revealed that these Th2 cytokines were also expressed in the absence of T and B cells (U.A. Temann, unpublished results). This result indicates that not only Th2 cells but also other cell types are likely to contribute to the production of IL-4, IL-5, and IL-13. To analyze the impact that each Th2 cytokine besides IL-9 has on lung pathology in our murine system of lung inflammation, we used neutralizing Ab's to selected cytokines during the entire time of IL-9 transgene expression, over a period of 14 days.

Neutralization of IL-4 during regulated IL-9 transgene expression reduced airway eosinophilia but did not completely abolish lung inflammation. Although the number of eosinophils and lymphocytes were reduced in lung lavage fluid retrieved from the airways of IL-9 transgenic mice that received anti-IL-4 Ab's, there was no obvious effect of the IL-4 blockade on the infiltration of lung tissue with inflammatory cells. Similar results have been presented before using murine models of allergen-induced lung inflammation. The use of IL-4-deficient mice or treatment of mice with anti-IL-4 Ab's in these model systems caused a reduction of inflammatory cells, in particular eosinophils, in the airways but not in lung tissue (40–42). It is established that IL-4 is absolutely necessary for the generation of Th2 cells (43). In the absence of IL-4, Th2 cell differentiation is impaired and so is the production of Th2 cytokines, including IL-5. Since eosinophilia in lung tissue can occur in the absence of IL-4, IL-5 production and eosinophilia in this compartment can be regulated independently of IL-4. But it has been suggested that IL-4 may be required to provide important signals for the recruitment of eosinophils to the airways, since blocking of IL-4 reduces airway eosinophilia (41, 42). Recently, Cohn and coworkers (44) provided evidence using a murine model of lung inflammation involving the adoptive transfer of in vitro-differentiated Th2 cells that IL-4

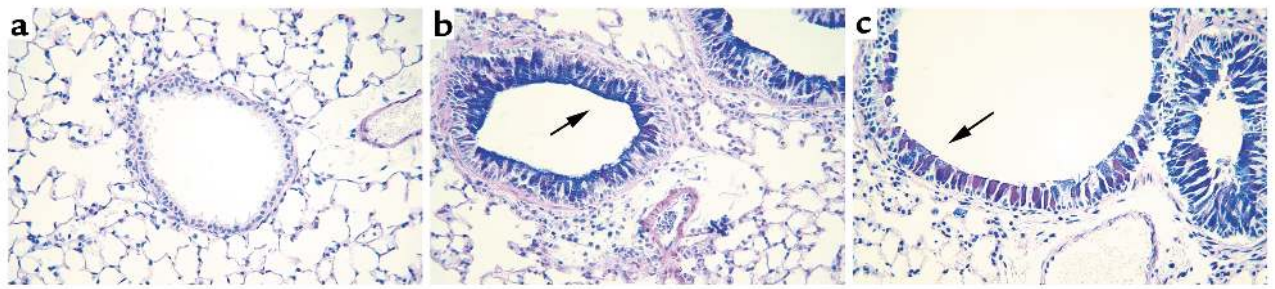


Figure 10

Histologic staining for mucin after IL-5 blockade. Lung sections from IL-9 transgene-negative (a) and -positive (b and c) mice that were kept 14 days on doxycycline-supplemented food, were stained with AB/PAS. Intense, positive (purple) staining for mucin was observed in airway epithelial cells in transgene-positive mice (arrow) (b) and in mice that additionally received anti-IL-5 Ab's during IL-9 transgene expression (arrow) (c). Lung sections from IL-9 transgene-negative mice did not stain for mucin (a). Original magnification $\times 300$.

promotes eosinophilia in the airways by locally suppressing the production of IFN- γ , therefore promoting Th2 cell generation and IL-5 production. The absence of IL-4 during regulated IL-9 transgene expression in our model of lung inflammation also resulted in a reduction of airway eosinophilia. These data demonstrate that the expression of IL-9 in the lung could not compensate for the role of IL-4 in airway eosinophilia.

The blockade of IL-13 during IL-9 transgene expression by using a soluble IL-13R α -Fc fusion protein had a more profound effect on lung inflammation. In contrast to the IL-4 blockade, lymphocytic and eosinophilic infiltration was totally abolished in both compartments, lung tissue as well as airways, from IL-9-expressing mice. These results indicate that IL-13 plays an absolutely essential role in the development of lung inflammation, especially eosinophilia, in our system that is independent of IL-4 and IL-9. The role that IL-13 plays in lung inflammation is still controversial. Overexpression of IL-13 in the lungs of transgenic mice resulted, besides other pathologic changes, in mononuclear and eosinophilic inflammation of the lungs (45). This study suggested that this inflammation had been mediated, at least in part, by the ability of IL-13 to induce locally the production of eotaxin, a major chemoattractant for eosinophils. The function of IL-13 as a potent inducer of eotaxin expression and concomitant eosinophilia was supported by experiments, in which IL-13 had been administered intranasally to naive mice (46). However, in studies using sIL-13R α 2-Fc for blocking IL-13 during lung challenge with antigen, airway eosinophilia was only slightly reduced (47) or not effected at all (48). Similar results were presented from studies using IL-13-deficient mice in a model of asthma (42). Sensitization and challenge of these mice with antigen resulted in the development of pulmonary eosinophilia comparable to that in wild-type mice. These studies show that other factors are able to compensate for the absence of IL-13 in its function in lung eosinophilia. Recently, more evidence has been provided that neither IL-4 nor IL-13 alone, but signaling by either through the IL-4R α chain, is crucial for the development of airway inflammation (47, 49). The IL-4R α

chain is part of both the IL-4 and IL-13 receptor complex and is therefore essential for IL-4 and IL-13 signaling. The use of the same receptor subunit might explain, at least in part, the redundancy of the requirements for IL-4 and IL-13 in lung eosinophilia. Surprisingly, however, in our system of lung-specific IL-9 expression, IL-4 could not compensate at all for the lack of IL-13 and induce, at least in part, lung eosinophilia. This might indicate that lung inflammation might be regulated independently of IL-4 in our system. Furthermore, in the absence of IL-13 eosinophilia also could not develop, even if IL-9 was present. This suggests that there is no direct role for IL-9 in the induction of eosinophilia in the lung. Studies with IL-9-deficient mice using a pulmonary granuloma model also demonstrated no function for IL-9 in eosinophilic inflammation (19). However, all these data do not exclude the potential involvement of IL-9 in an eosinophilic response. IL-9 transgenic mice develop a more severe eosinophilia in their lungs than IL-13 transgenic mice, which might indicate that IL-9 might be involved in intensifying an already ongoing eosinophilic reaction. Studies from systemically expressing IL-9 transgenic mice showed that the overexpression of IL-9 enhanced an eosinophilic inflammation during lung challenge with antigen (50). It was suggested that IL-9 would exert its function by acting directly on eosinophils. It has been shown in vitro and in vivo that IL-9 was able to upregulate IL-5R α expression on human eosinophils that could potentiate differentiation, maturation, and survival of eosinophils through the action of IL-5 (13, 17). From the studies presented here, we conclude that in our system the development of lung inflammation, especially eosinophilia, requires further signals, in addition to IL-9, which can only be provided by IL-13.

Recently, interest has focused on the roles played by IL-4, IL-5, IL-13, or IL-9 in the regulation of mucus production in the airways. The overexpression of any of these four cytokines in the lungs of transgenic mice was correlated with epithelial cell hypertrophy and mucus overproduction (21, 24, 45, 51). But the complex inflammation present in the lungs of these mice has made it difficult to prove a direct effect of one of these

cytokines on mucin production. IL-9 transgenic mice, who displayed extensive mucin accumulation in airway epithelial cells, also expressed IL-4, IL-5, and IL-13 in their lungs. We therefore used these mice to dissect the contribution of each Th2 cytokine to mucus production in our model system. Blocking of IL-4 or IL-5 by injection of cytokine-specific Ab's during IL-9 transgene expression did not abolish or reduce mucus production in airway epithelial cells. In contrast, neutralization of IL-13 completely abolished mucus production, even in the presence of IL-9 transgene expression. These data indicate that IL-13 is absolutely necessary, directly or indirectly, for the induction of mucus production in our system, independent of IL-4 and IL-5. Furthermore, the expression of IL-9 failed to compensate for the requirement of IL-13 in mucus production. On one hand, these results contribute further evidence that IL-13 is a critical factor for mucin synthesis in airway epithelial cells. Studies using murine models of allergen-induced airway inflammation had shown previously that blocking of IL-13 resulted in complete abrogation of mucin synthesis in airway epithelial cells (47, 48). On the other hand, the inability of IL-9 to induce mucus production in the absence of IL-13 is in conflict with recently published data. It has been demonstrated, *in vitro* and *in vivo*, that IL-9 could directly stimulate mucin gene expression in airway epithelial cells (14, 16), independently from IL-13. These studies have been supported by data from Townsend et al. (19), using IL-9-deficient mice in a pulmonary granuloma model. In this study IL-9 was absolutely necessary for the initiation of mucus production by goblet cells independently of IL-13, since IL-13 expression was not impaired. But a similar role for IL-9 in murine models of antigen-induced airway inflammation has not yet been demonstrated. These conflicting data may reflect the complexity in the regulation of mucus production by several cytokines, including IL-4, IL-9, and IL-13. For IL-4 and IL-9 it has been shown that these cytokines could directly induce mucin gene expression *in vitro* and *in vivo* (14, 16, 52). For IL-13, a direct involvement in mucin gene expression has not yet been demonstrated. It seems to be certain, however, that IL-13 is absolutely necessary for the induction of mucus production. Future studies will have to focus on the interrelationship of these cytokines in the regulation of mucus production. Here, we could show that neither IL-4 nor IL-9 was able to induce mucin expression in the absence of IL-13.

In conclusion, we have shown that regulated IL-9 transgene expression in the lungs induces a strong pulmonary inflammation. We successfully used this murine system to study the contribution of Th2 cytokines to eosinophilia and mucus production, two important features of human asthma. Neutralization of selected cytokines during transgene expression revealed that the inflammatory response needed signals beyond IL-9 to fully develop. The combined action of IL-4, IL-5, and IL-13 are necessary for the develop-

ment of pathology induced by IL-9 in the lung. Although the overexpression of IL-9 could not compensate for the function of important Th2 cytokines in the development of eosinophilia and mucus production, its potency to induce lung inflammation leaves no doubt of an important role of IL-9 in the pathology of asthma. Therefore, future work will have to focus on the mechanism by which IL-9 mediates airway inflammation to fully understand its contribution to lung eosinophilia and mucus production in human asthma.

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