

IL-9 pathway in asthma: New therapeutic targets for allergic inflammatory disorders

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Background: Asthma is a complex heritable inflammatory disorder of the airways associated with clinical signs of allergic inflammation and bronchial hyperresponsiveness (BHR). The incidence of asthma continues to rise in industrialized countries despite advances in the identification of cellular and molecular mediators that are associated with the disease. Because of its importance in human health, additional research and alternative therapeutic strategies are justified to create more effective treatments for this debilitating disease.

Objective: Studies use recombinant inbred mice to demonstrate that BHR in mouse models of asthma is associated with a genetic alteration at the IL-9 locus, where IL-9 expression in lung is strongly associated with bronchial responsiveness. We have investigated the ability of intratracheal instilled IL-9 to induce asthmatic-like responses in naive C57BL/6 (B6) mice, which express very low levels of IL-9.

Methods: IL-9 or vehicle was intratracheal instilled in naive B6 mice for 10 days. Mice were analyzed for effects on BHR, lung eosinophilia, and serum total IgE levels.

Results: Phenotypic effects of B6 mice instilled with IL-9 were increased eosinophils in the bronchoalveolar lavage and significantly elevated serum total IgE. Moreover, IL-9 was found to induce IL-5R α in vivo and in vitro, suggesting a potential mechanism for the novel actions described for IL-9 on eosinophils.

Conclusion: Increased levels of IL-9 in the airway of naive B6 mice induced lung eosinophilia and serum total IgE levels, which are 2 clinical features of asthma. These data support a central role for the IL-9 pathway in the complex pathogenesis of allergic inflammation. (*J Allergy Clin Immunol* 1999;103:485-91.)

Key words: Asthma, allergy, IL-9, mouse models, IgE, eosinophils

Clinical and physiologic studies of asthma reveal an association with widespread narrowing of the airways caused by edema and the infiltration of numerous inflammatory cells into the wall of the airway lumen. These cells include eosinophils, mast cells, IgE-producing plasma cells, and a specific subset of activated T cells referred to as T_{H2} lymphocytes.^{1,2} Although the precise biologic mechanism underlying these cellular responses

Abbreviations used

B6: C57BL/6J
BAL: Bronchoalveolar lavage
D2: DBA/2J
rmIL-9: Recombinant murine IL-9

is unknown, it is clear that complex interactions between these cells in the airway in response to antigen lead to the elaboration of cytokines and other inflammatory proteins that produce chemical mediators that are critical in this disorder.² Despite these insights, a clear understanding of the pathogenesis of atopic asthma is lacking.

The recognition that specific genetic and environmental factors appear to be important in the cause of atopic asthma has led to an intensive search for those factors that significantly influence susceptibility to this disorder.² Identification of susceptibility genes for asthma is complicated by the complex phenotypes and genetics involved.²⁻⁹ Biologic variability in the allergic and inflammatory response and a clear recognition of the importance of bronchial hyperresponsiveness, IgE, and various cytokines in this response have stimulated analyses of candidate genes for genetic variability in structure and function. Recently, a major gene for asthma was localized to chromosome 5q31-q33 in humans.^{3,6} Several groups have reproduced linkage between asthma or its risk factors with chromosome 5q31-q33.^{2,4,8} Although the human gene(s) predisposing to asthma, bronchial hyperresponsiveness, and atopy have not yet been identified from this chromosomal region, a number of candidate genes for asthma have been proposed, including IL-9.^{2,8,9}

Chromosome 5q31-q33 is known to be syntenic or to share chromosomal architecture with segments of mouse chromosomes 11, 13, and 18.¹⁰ Gene-mapping studies in inbred strains of mice for bronchial hyperresponsiveness have also suggested IL-9 as a candidate gene for asthma. We have shown previously that bronchial hyperresponsiveness is mapped to a qualitative trait locus on mouse chromosome 13, where the IL-9 candidate gene is located.⁹ Moreover, differences between bronchial hyporesponsive C57BL/6J (B6) and hyperresponsive DBA/2J (D2) mice are associated with significant differences in lung IL-9 expression. Hyporesponsive B6 mice express undetectable levels of IL-9 protein at steady state; hyperresponsive D2 mice have robust IL-9 protein levels in

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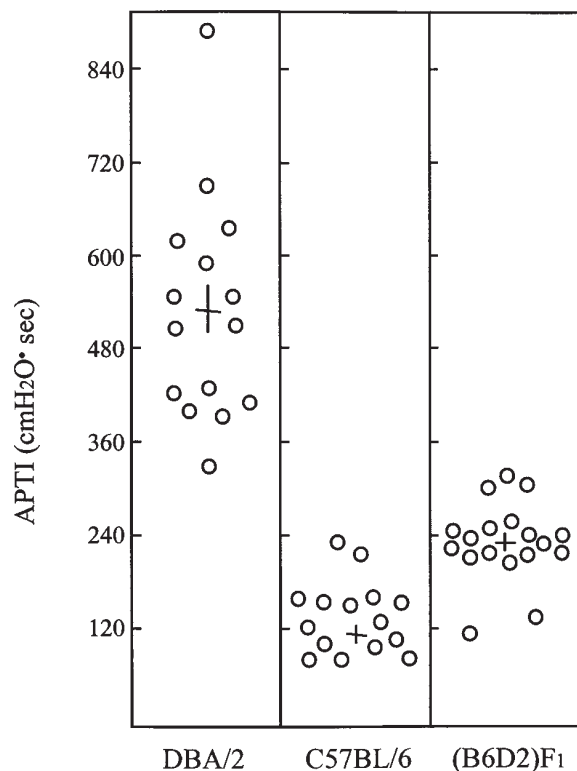


FIG 1. Genetic variability in bronchial responsiveness. Bronchial responsiveness (airway pressure time index [APTI]) to atracurium 20 mg/kg, intravenously, is shown for the hyporesponsive C57BL/6J and hyperresponsive DBA/2J strains and for the (B6D2)F₁ mice that demonstrate an intermediate level of bronchial responsiveness compared with each of the progenitor strains.

lung. Interestingly, the intermediate bronchial responsiveness of the (B6D2)F₁ mice is associated with intermediate levels of IL-9 protein in lung. Thus there appears to be a tight genotype-phenotype correlation.

We show that intratracheal instillation of recombinant murine IL-9 (rmIL-9) to B6 mice genetically deficient in the production of IL-9 increased the concentration of this cytokine in lung. Surprisingly, this lung IL-9 replacement treatment for 10 days significantly increased the serum total IgE and the number of eosinophils in bronchoalveolar lavage (BAL). Moreover, we also observe that IL-9 induces IL-5R α in vitro and in vivo, suggesting a potential mechanism for the actions of IL-9 on eosinophils and a new role for this cytokine in eosinophilic inflammation. These data support a central role for IL-9 as a gene candidate in the complex pathogenesis of bronchial hyperresponsiveness and asthma.

METHODS

Primary cell cultures and cell lines

KG-1 cells were obtained from the American Type Culture Collection and cultured according to the supplier. Mo7e cells were maintained in RPMI-1640 supplemented with 10% FCS and 30 ng/mL of recombinant human GM-CSF. TS1 cells are a murine

T_H2-like cell line that was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 25 ng/mL rmIL-9. One hundred thousand cells were plated in 96-well plates, washed 3 times, and incubated overnight in serum-free media before recombinant IL-9 stimulation.

Mice

The following studies conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and the Department of Health, Education, and Welfare (National Institutes of Health) guidelines for the experimental use of animals and were approved by the Institutional Animal Care and Use Committee. Male and female D2, B6, and (B6D2)F₁ mice 5 to 6 weeks of age were purchased from the Jackson Laboratory, Bar Harbor, Maine. The animals were not maintained in sterile pathogen-free conditions. The animal housing facilities were maintained at 22°C with a light cycle of 10 hours of light and 14 hours of dark. Food and water were present ad libitum.

Phenotyping

To determine the bronchoconstrictor response, respiratory system pressure was measured at the trachea and recorded before and during exposure to the drug. Mice were anesthetized and instrumented as previously described.¹¹ The bronchoconstrictor response to 20 mg/kg atracurium (Glaxo Wellcome) was assessed by the change in peak inspiratory pressure integrated over time. This parameter, termed the airway pressure time index, is a simple and repeatable measure of the change in peak inspiratory pressure highly correlated with and respiratory system resistance and elastance after a bronchoconstrictor challenge.¹² BAL was carried out as previously described with minor changes.¹³ Briefly, the trachea was cannulated, and the lavage procedure (ie, 3 sequential 0.5 mL PBS washes) was carried out in situ at the conclusion of the physiologic measurements. To determine serum IgE response to the intratracheal administration of rmIL-9, we collected whole blood from venipuncture of the posterior vena cava at the completion of the physiologic measurement of the airway pressure time index. Each blood sample was centrifuged, and the serum was transferred and stored at -20°C until IgE analysis. IgE was measured by ELISA antibody-sandwich assay. Microtiter plates (Corning, Corning, NY) were coated with rat anti-mouse IgE antibody (PharMingen, San Diego, Calif) at a concentration of 2 μ g/mL in coating buffer of sodium carbonate (Sigma, St Louis, Mo). Plates were incubated at 4°C overnight, washed 3 times with 0.05% Tween-20 (Sigma) in PBS for 5 minutes (wash buffer). BSA 1% (Sigma) in PBS was added for 30 minutes at 37°C, and the plate was washed 3 times without incubations. IgE standards (PharMingen) were used to generate a standard curve. Samples and standards were incubated for 2 hours at room temperature. After the samples were washed 3 times, a secondary antibody-biotinylated rat anti-mouse IgE (PharMingen) was added; the plate was covered, incubated 1 hour at room temperature, and washed 3 times. Avidin-peroxidase (Sigma) was added, incubated for 30 minutes at room temperature, and washed 3 times. The substrate (2,2'-azino-di[3]-ethylbenzthiazoline sulfonate; Boehringer-Mannheim, Indianapolis, Ind) was added and incubated for 20 minutes at room temperature in the dark. The absorbance was measured at 405 nm on a plate reader (MR-5000; Dynatech, Chantilly, Va). IgE concentrations were interpolated from a standard curve.

Intratracheal instillation

B6 mice were anesthetized daily by inhalation of 1.5% halothane and placed on a vertical platform. rmIL-9 (R&D Systems, Minneapolis, Minn) was diluted in 0.1% BSA fraction V in sterile phos-

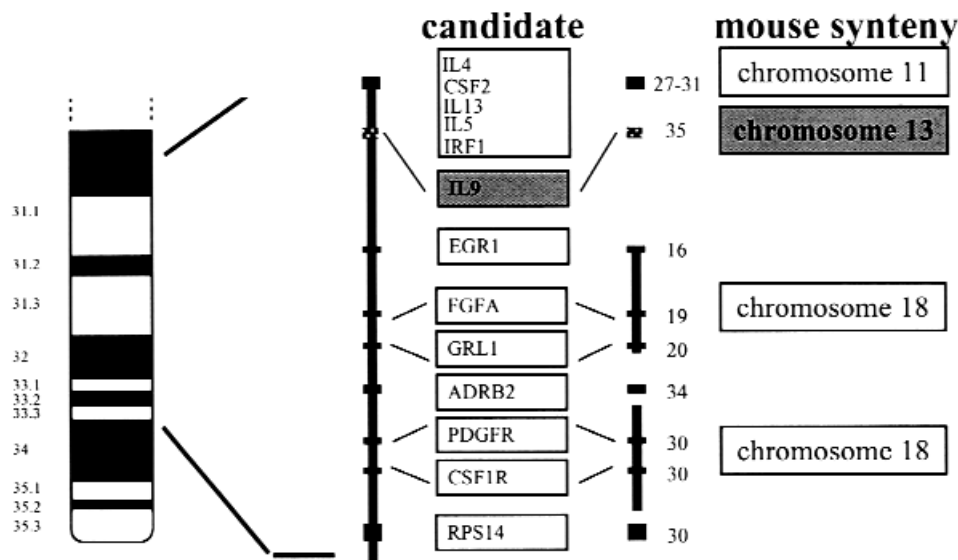


FIG 2. Synteny and linkage homologic features. This is an illustration of the genetic map of human chromosome 5q31-q33 and syntenic regions in the mouse. This region of human 5q31-q33 demonstrates significant evidence for linkage with asthma and bronchial hyperresponsiveness.³ The IL-9 locus on mouse chromosome 13 has been implicated as a candidate gene in bronchial hyperresponsiveness and asthma.⁹ This region is homologous to portions of mouse chromosomes 11, 13, and 18, which contain numerous candidate genes including chromosome 11 (IL-4, IL-5, granulocyte macrophage-stimulating factor [*CSF2*]; IL-13, immune regulatory factor 1 [*IRF1*]), chromosome 13 (IL-9), and chromosome 18 (adrenergic receptor- β 2 [*ADRB2*], glucocorticoid receptor 1 [*GRL1*], fibroblast growth factor acidic [*FGFA*], platelet-derived growth factor receptor [*PDGFR*], colony-stimulating factor receptor 1 [*CSF1R*], early growth response element 1 [*EGR1*], Ribosomal protein S14 [*RPS14*]). Relative map positions in centimorgans are given to the right of each candidate in the mouse (centromere, 0 cM). (From Nicolaides NC, Holroyd KJ, Ewart SL, et al. Interleukin-9: a candidate gene for asthma. *Proc Natl Acad Sci* 1997;94:13175-80. © 1997 National Academy of Sciences, U.S.A.)

phate-buffered saline solution (Gibco/BRL). Five micrograms of mIL-9 in BSA and saline solution (20 μ L), BSA and saline solution (20 μ L), or saline solution (20 μ L) were administered once each day to B6 mice for 10 days with a 50- μ L glass syringe (Hamilton, Reno, Nev) with a 2-inch, 26-gauge blunt-end needle inserted directly into the trachea.

Reverse transcriptase PCR

Total RNA were isolated from cells and lung tissues using the Trizol (Gibco/BRL) method as described by the manufacturer. Lung tissues were carefully dissected from hilar lymph nodes before use. Equal amounts of total RNA were reverse transcribed and amplified with the appropriate primers by PCR as described.¹⁴ Products were electrophoresed on 2% agarose gels and analyzed by Southern blot with a radiolabelled oligonucleotide used as a probe. Primer sets used were: mIL-5R α (accession D90205): 5'-ACC ATG GTG CCT GTG TTA c-3' (centered at nt 310 of the cDNA) and 5'-CCA TCA AAA CGT GGA ATT TCC-3' (centered at position nt 1540 of the cDNA), which produce a 1233-base pair product. The oligonucleotide 5'-ATT CTT GTG GGA GCT TTG GC-3' (centered at position nt 330 of the cDNA) was used as a probe. Primer sets for the hIL-5R α (accession X62156) were: 5'-GGA TAT GAT CAT CGT GGC G-3' (centered at nt 205 of the cDNA) and 5'-TGA AAA GCC TTT GTG GAG G-3' (centered at nt 473 of the cDNA), which produce a 288-base pair product. The oligonucleotide 5'-CAT CCT TTT GGG GGC CAC-3' (centered at nt 232 of the cDNA) was used as a probe. Gels were transferred to nitrocellulose

membranes and probed with a radiolabelled oligonucleotide as previously described.¹⁴

Western blots

Lung tissues were derived from various mice, dissected free of hilar lymph nodes, and snap frozen in liquid nitrogen. Sections (3 mm³) were resuspended in 500 μ L of 2 $\times$ SDS lysis buffer (60 mmol/L TRIS; pH6.8; 2% SDS, 0.1 mol/L 2-mercaptoethanol, 0.1% bromophenol blue) and boiled for 5 minutes. Twenty microliters of each lysate was electrophoresed on 18% TRIS-glycine SDS-PAGE gels and electroblotted onto Immobilon-P (Millipore) membrane in transfer buffer (48 mmol/L TRIS, 40 mmol/L glycine, 0.0375% SDS, 20% methanol). Filters were blocked overnight in blocking buffer (TRIS buffered saline solution 0.05% Tween-20, 5% powdered milk). Filters were probed with polyclonal goat anti-murine IL-9 (R&D Systems) and a secondary horseradish peroxidase conjugated anti-goat (Pierce) and prepared for chemiluminescence.

RESULTS

Genetic variability in bronchial responsiveness

Significant differences in bronchial responsiveness were observed between the hyperresponsive D2 mice and hyporesponsive B6 mice and the inbred (B6D2)F₁ offspring, which were intermediate in phenotype (Fig 1).

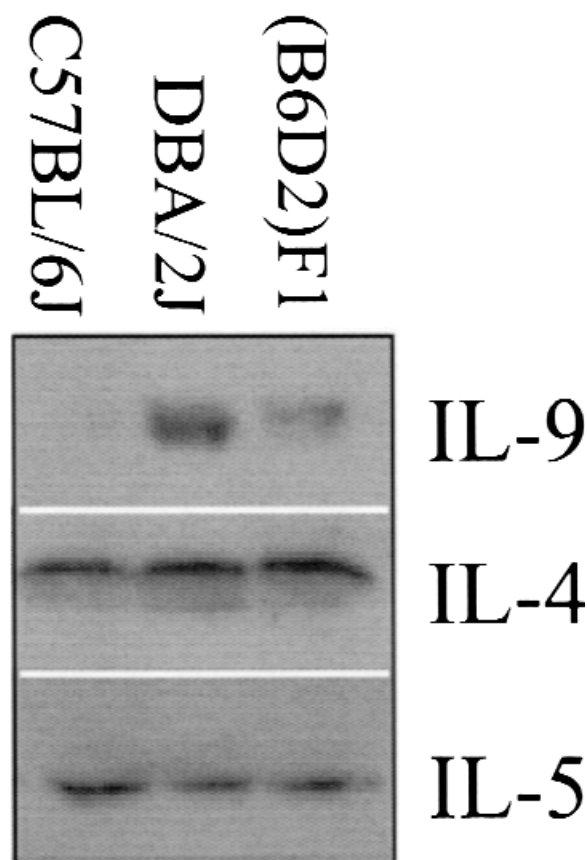


FIG 3. Lung IL-9 protein levels differ significantly among B6, D2, and (B6D2) F_1 mice. Western blot of cytokines from the lungs of hyporesponsive B6, intermediate (B6D2) F_1 , and hyperresponsive D2 mice shows intermediate levels in the (B6D2) F_1 mice and markedly reduced IL-9 levels in B6 mice. (From Nicolaides NC, Holroyd KJ, Ewart SL, et al. Interleukin-9: a candidate gene for asthma. *Proc Natl Acad Sci* 1997;94:13175-80. © 1997 National Academy of Sciences, U.S.A.)

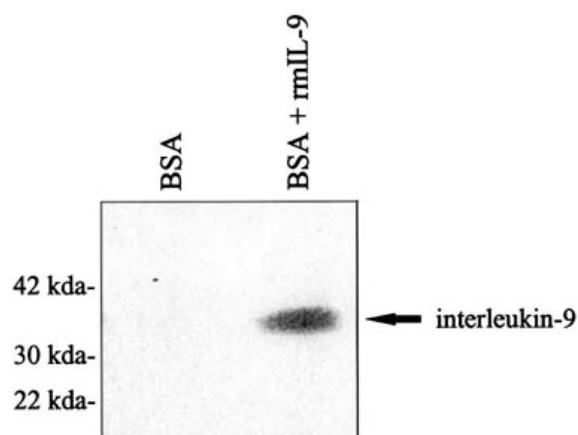


FIG 4. Intratracheal instillation of rmIL-9 significantly increases IL-9 levels. Western blot of IL-9 from lungs of hyporesponsive B6 mice treated with BSA and saline solution or rmIL-9 in BSA and saline solution. Significant levels of lung IL-9 were detected only when rmIL-9 was instilled, demonstrating the effectiveness of the replacement therapy in B6 mice that were genetically deficient in IL-9.

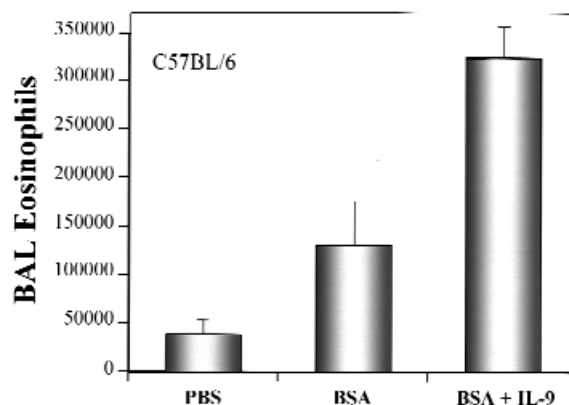


FIG 5. Intratracheal instillation of rmIL-9 increases BAL eosinophils. Eosinophils in the BAL from B6 mice treated with intratracheal saline solution, saline solution and BSA, and rmIL-9 in BSA and saline solution. A significant increase in eosinophils was seen when rmIL-9 was given in comparison with the other 2 groups. Numbers are indicated as total eosinophils from lavaged animals $\pm$ SEM.

We have previously demonstrated that this significant difference in bronchial responsiveness is genetically determined, in part, by a qualitative trait locus located on the midportion of chromosome 13 (Fig 2), which is syntenic with a region on human chromosome 5q31-q33 implicated in asthma.⁹ The IL-9 gene candidate is located within this qualitative trait locus.

Lung IL-9 protein levels differ significantly between B6, D2, and (B6D2) F_1 mice

Distinct differences in steady state lung IL-9 expression were observed by Western analyses between these same 3 strains (Fig 3). Although IL-9 levels differed significantly between these strains, IL-4 and IL-5 levels did not appear to differ in these animals. These protein levels were corroborated by reverse transcriptase-PCR data, which also detected similar steady-state levels of lung IFN- γ in these same strains (data not shown). Interestingly, IL-9 levels of (B6D2) F_1 mouse lungs appeared to be intermediate to B6 and D2 levels, reflecting expression of a single allele, presumably from the D2 strain.⁹ These data further demonstrate the strong relationship between IL-9 lung expression and bronchial responsiveness. No significant differences were observed in transcript abundance or size between the B6 and D2 strains of mice for other loci surrounding the IL-9 locus.⁹ These data support the hypothesis that a genetic alteration at the IL-9 locus is responsible for the differential expression of cytokine and the biologic variability in bronchial responsiveness observed.

Intratracheal instillation of rmIL-9 produces eosinophilia and increased serum IgE in B6 mice

To evaluate whether a significant phenotype would be produced by partially replacing IL-9 in B6 mice that are genetically deficient in IL-9 production, rmIL-9 was

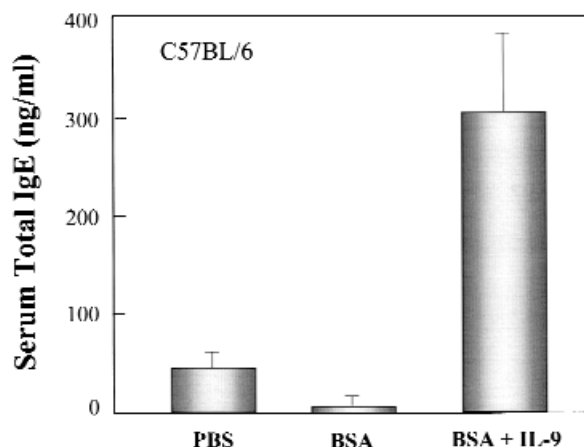


FIG 6. Intratracheal instillation of rmIL-9 increases serum total IgE. Serum total IgE was measured from B6 mice that were treated with intratracheal saline solution, saline solution and BSA, and rmIL-9 in BSA and saline solution. A significant increase in serum total IgE was seen when rmIL-9 was given in comparison with the other 2 groups. Values indicate IgE $\pm$ SEM.

administered directly to the lung for 10 days. Western blot analyses were performed on the lungs from these mice to confirm that significant steady-state levels of exogenous IL-9 were detected (Fig 4). A significant increase in cytokine was observed after direct administration to the lung over this short interval. Surprisingly, in comparison with saline solution, and BSA in saline solution (carrier), rmIL-9 instillation produced a significant increase in BAL eosinophil counts (Fig 5). No significant differences in total cell numbers were observed among the 3 groups of animals (data not shown). In addition, direct administration of rmIL-9 to the lung stimulated significant increases in serum total IgE in comparison with the control animals (Fig 6). A nonspecific effect of the rmIL-9 is not likely because similar changes in BAL eosinophils and serum IgE were not observed in the presence of BSA, which was used as the carrier.

IL-9 induces IL-5 receptor α in vivo and in vitro

To suggest a potential mechanism for the actions of IL-9 on eosinophils, we assayed levels of IL-5R α in the lungs of B6 mice with and without rmIL-9 instillation (Fig 7). IL-5R α was a reasonable candidate to examine because of the established role for this cytokine receptor in the eosinophil biology and because we have previously shown that IL-5 levels do not differ in lungs between B6 and D2 animals despite significant differences in IL-9 levels (Fig 3). Intratracheal administration of IL-9 significantly increased the levels of IL-5R α assayed by reverse transcriptase-PCR in comparison to control animals. This induction was not seen in the absence of reverse transcriptase, ruling out the amplification of this product from genomic DNA by PCR amplification.

We next tested whether IL-9 can directly induce IL-5R α by first assessing its expression in the T_H2-like lymphocyte cell line TS1 when grown in the presence or

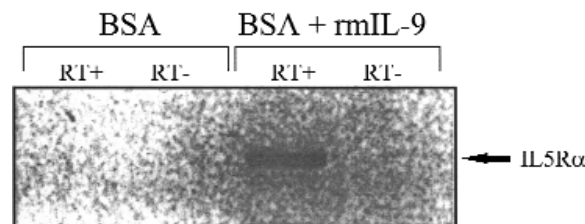


FIG 7. IL-9 induces IL-5R α in vivo. Reverse transcriptase (RT) PCR of IL-5R α from B6 mice that were treated with intratracheal saline solution and BSA and rmIL-9 in BSA and saline solution. A significant increase in IL-5R α message was seen only when rmIL-9 was given in comparison to the BSA and saline solution control group. (RT-, Without reverse transcriptase; RT+, with reverse transcriptase.)

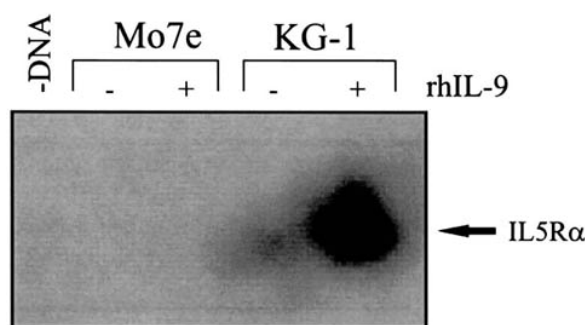


FIG 8. IL-9 induces IL-5R α in vitro. Reverse transcriptase PCR of IL-5R α from Mo7e and KG-1 cells in vitro. A significant increase in IL-5R α message in KG-1 was seen in response to IL-9 (+) in contrast to cells grown without (-) IL-9. No effect expression was observed for Mo7e cells with or without recombinant IL-9.

absence of IL-9. We found that IL-5R α expression was detected in cells grown in the presence of IL-9 (data not shown). In addition, IL-5R α expression was increased in the human promyeloblastic leukemia cell line KG-1 when these cells were growing autonomously and supplemented with recombinant human IL-9. No IL-5R α expression was observed on recombinant human IL-9 treatment of the cytokine-dependent Mo7e cell line, which proliferates in response to this cytokine (Fig 8).

DISCUSSION

We have previously identified the IL-9 gene as a candidate in allergic asthma on the basis of linkage homologic features between humans and mice for bronchial responsiveness.⁹ Moreover, these data demonstrated that allelic variation at the IL-9 locus is responsible, in part, for biologic variability in baseline bronchial responsiveness between inbred strains of mice. The bronchial hyporesponsive B6 strain was found to express very low levels of IL-9 in the lung in comparison with the hyper-responsive D2 strain, although (B6D2)F₁ mice appear to express intermediate levels. Consistent with our hypothesis that IL-9 plays an important role in determining

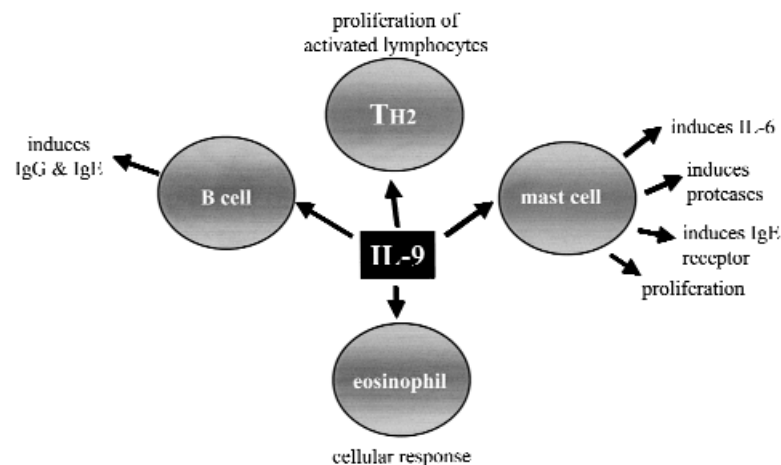


FIG 9. A proposed central role for IL-9 in asthma. A proposed central role for IL-9 in asthma is shown here including the newly described actions of IL-9 on eosinophils along with the many other relevant activities previously demonstrated for this pleiotropic cytokine.

bronchial responsiveness, we show that these F_1 mice also have an intermediate bronchial response, demonstrating a tight genotype-phenotype correlation.

The precise mechanism by which IL-9 acts to alter bronchial responsiveness remains unknown. Nevertheless, IL-9 is a T-cell growth factor, and experimental data have shown a correlation between IL-9 production and T_H2 -responses in vitro and in vivo.¹⁵⁻¹⁷ T_H2 cells are well documented to have a crucial role in the development of the allergic response and are presumed to be associated with the pathogenesis of asthma.^{18,19} Moreover, significant biologic variability in airway responsiveness occurs in humans, and baseline bronchial hyperresponsiveness is recognized as a risk factor for asthma.²⁰⁻²² Baseline airway responsiveness also differs between inbred strains of mice and is strongly controlled by genetic factors.^{9,23-26} Recently, T cells were shown to regulate baseline murine airway responsiveness in the absence of an antigen challenge or gross airway inflammation.²⁶ Thus, although factors affecting T-cell growth and differentiation are well known to affect antigen response, they also are likely to be essential in determining the baseline airway response phenotype in these animal models of asthma.

Our findings that IL-9 lung instillation produced a significant increase in BAL eosinophil counts in the B6 animal is the first demonstration that IL-9 may also serve a significant role in eosinophilic inflammation. Eosinophilic inflammation is a central feature of atopic asthma.^{1,2} IL-5 and its receptor are thought to play a critical role in the recruitment of eosinophils to the lung in atopic asthma. The induction of IL-5R α by IL-9 in vivo suggests a direct role of IL-9 on the eosinophils. One potential model describing the link between IL-9 protein expression and the eosinophilic response (ie, growth, differentiation, migration) would be that IL-9 exerts a direct role on eosinophils or their precursors, which in turn, induces IL-5R α expression that enables these cells to

respond to IL-5. This model is supported by the finding that IL-5R α expression is induced by IL-9 at the cellular level in KG-1 and TS1 cells. Interestingly, KG-1 cells, which are a promyelocytic leukemia cell line, are capable of spontaneously differentiating into granulocytes and eosinophils.²⁷ IL-5R α was not induced in the IL-9 responsive megakaryocytic cell line, Mo7e, demonstrating a lineage specificity for this response. These data support a new and important role for IL-9 in eosinophil biologic features and lung pathologic features, which will continue to unfold as we begin to further elucidate the IL-9-induced IL-5R α expression pathway in granulocytes.

A central role for IL-9 in atopic asthma is suggested by several independent studies describing its functions in vitro and in vivo (Fig 9). In addition to its activity on T lymphocytes, IL-9 has also been shown to have direct activity on additional cell types involved in the asthmatic inflammatory response, such as on mast cells, B cells, and eosinophils as suggested in this study (Fig 9). Although mediator release from mast cells by allergen has long been considered a critical initiating event in allergy, IL-9's role as a mast-cell growth factor that upregulates the expression of mast-cell proteases (including mMCP-1, mMCP-2, mMCP-4 and granzyme B) has been well documented.^{28,29} In addition, IL-9 upregulates the expression of the α chain of the high affinity IgE receptor and induces IL-6 secretion in mast cell lines, further suggesting a role for IL-9 in proliferation and differentiation of mast cells.^{29,30} Whereas elevated IgE levels are considered to be a hallmark of atopic allergy and a risk factor for asthma, IL-9 has been shown to potentiate the release of IgE from primed human and murine B cells in vitro and in vivo.³¹⁻³³ Finally, we report here that intratracheal instillation of rmIL-9 causes an increase of eosinophils in BAL and elevated IgE levels in the bronchial hyporesponsive B6 mouse, supporting a role

for this cytokine in coordinating these cellular responses associated with allergic inflammation and asthma.

On the basis of current data, there is substantial support for the IL-9 gene candidate in asthma. These data suggest that further study of IL-9, its receptor, and associated genes in this biologic pathway, including IL-5R α , may provide potentially useful insights into the pathogenesis of asthma. Moreover, novel therapies for allergic inflammatory disorders may be derived by blocking accessible targets in this pathway.

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