

Segmental allergen challenge in patients with atopic asthma leads to increased IL-9 expression in bronchoalveolar lavage fluid lymphocytes

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Background: IL-9 is a T_H2 cell–derived cytokine that might be involved in the pathophysiology of allergic diseases. Little is known about its expression and release during the allergic response in the human lung.

Objective: The expression of IL-9 was measured in 10 atopic subjects with mild asthma and 5 nonatopic healthy control subjects at baseline and 24 hours after segmental sham and allergen challenge.

Methods: IL-9 protein was measured in bronchoalveolar lavage (BAL) fluid by means of ELISA and detected within the BAL cells by means of immunocytochemistry. Furthermore, IL9 mRNA expression of BAL cells was detected by means of real-time PCR.

Results: Although only low or undetectable amounts of IL9 mRNA and IL-9 protein were present in nonatopic control subjects and atopic asthmatic patients at baseline, there was an increase after segmental allergen challenge in the atopic subjects. Lymphocytes were identified as major cellular sources of IL-9 production by means of immunocytochemistry. Furthermore, IL-9 protein and IL9 mRNA expression correlated with eosinophil numbers in BAL fluid.

Conclusions: These findings demonstrate that IL-9 is specifically upregulated after local allergen challenge in the lungs of atopic asthmatic patients. Lymphocytes are the major cellular source of IL-9. The increased expression and its correlation with eosinophil numbers suggest a potential role for IL-9 in the late phase of the allergic response. (*J Allergy Clin Immunol* 2003;111:1319-27.)

Key words: Atopy, asthma, IL-9, segmental allergen challenge, real-time PCR

Allergic asthma is a chronic inflammatory disorder caused by an inappropriate immune response to allergen. The pathophysiology of this response is characterized by

Abbreviations used

BAL: Bronchoalveolar lavage
PBGD: Porphobilinogen deaminase
RT: Room temperature
TBS: Tris-buffered saline

the involvement of inflammatory cells, including mast cells, eosinophils, and lymphocytes, which produce a broad range of inflammatory mediators. It has been well established that T_H2 lymphocytes and their cytokines play a key role in this inflammatory response.

IL-9 is a member of the T_H2 cytokine family, and the human IL9 gene has been mapped together with the other T_H2-type cytokine genes IL4, IL5, and IL13 in the 5q31-q33 region of chromosome 5.¹

Several in vitro studies have revealed regulatory functions of IL-9 associated with allergic inflammation. It has been shown to stimulate the IL-4–mediated IgE production of B cells and the proliferation of activated T cells and mast cells.²⁻⁴ It induces influx and maturation of eosinophils in synergy with IL-5⁵ and upregulates the cell-surface expression of their IL-5R α , leading to differentiation and increased survival.⁶ Furthermore, IL-9 has been shown to act on airway epithelial cells by inducing T-cell and eosinophil chemotactic factors.^{5,7,8}

In animal models the overexpression of IL-9 in IL9 transgenic mice leads to airway inflammation characterized by eosinophil cell infiltration, production of T_H2 cytokines, and increased airway hyperresponsiveness.^{9,10} In addition, intratracheal instillation of recombinant murine IL-9 induced lung eosinophilia, expression of IL-5R α , and enhanced serum IgE levels in naive C57BL/6 mice.¹¹ Moreover, treatment with an anti-IL-9 antibody inhibits the eosinophilic inflammation and reduces airway hyperreactivity in the ovalbumin asthma mouse model.^{12,13} In contrast to these findings, a recent study has shown that allergen-induced pulmonary inflammation and airway hyperreactivity remained unaffected in IL9 knockout mice.¹⁴ The authors suggest that IL-9 is not obligatory for the development of the allergic inflammation and that other T_H2 cytokines can act in a compensatory fashion. However, one can conclude from the present animal data that at least in established and ongoing allergic inflammation IL-9 clearly plays a role and

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TABLE I. Clinical characteristics of the study subjects

Subjects	Age (y)	Sex	FEV ₁ (% predicted)	PC ₂₀ FEV ₁ methacholine (mg/mL)	IgE (kU/L)	Allergen sensitivity*	Allergen provocation† (SQ-U)
Nonatopic control subjects							
	20	M	115	>8	8	—	Dp 1000
	18	M	114	>8	9	—	Dp 1000
	26	F	123	>8	14	—	gp 1000
	23	M	115	>8	26	—	Dp 1000
	22	F	113	>8	15	—	gp 1000
Median	22	2 F/3 M	115	>8	14	—	
Range	18-26		113-123		8-26		
Atopic asthmatic patients							
	22	M	118	>8	28	gp,ry,tp,mu,ri,Dp,Df,d	Dp 1000
	23	F	105	>8	27	gp,ry,Dp,Df,d,c	Dp 1000
	25	F	105	>8	305	gp,ry,tp,mu,ri,Dp	gp 10
	18	M	88	0.54	249	gp,ry,tp,mu,Dp,Df,d,c,f	gp 1000
	33	M	114	>8	502	gp,ry,Df	gp 1000
	29	F	106	0.15	214	gp,ry,tp,mu,ri,Df,d,c	gp 1000
	25	F	100	2.90	112	Dp,Df,d,c	Dp 1000
	25	M	90	4.18	77	gp,ry,mu,d,c	gp 1000
	23	M	79	0.18	210	gp,ry,Dp,Df,d,c	gp 1000
	33	M	102	0.95	180	gp,ry,tp,Dp,Df,d,c,f	Dp 1000
Median	25	4 F/6 M	103.5‡	3.54‡	195§		
Range	18-33		79-118	0.15->8	27-502		

Dp, *D pteronyssinus*; gp, mixed grass pollen; ry, rye pollen; tp, mixed tree pollen; mu, mugwort pollen; ri, ribwort pollen; Df, *D farinae*; d, dog hair; c, cat fur; f, mixed feather.

*Allergen sensitivity was determined by means of skin prick testing.

†Allergen provocation was performed with either grass pollen mix (ALK-Scherax) or *D pteronyssinus* mix (ALK-Scherax) in the indicated amount diluted in 10 mL of saline solution.

‡ $P < .05$ compared with nonatopic control subjects.

§ $P < .01$ compared with nonatopic control subjects.

that IL-9 per se can induce an eosinophilic inflammation similar to that of an allergic response.

Despite these convincing data from animal studies, there is only little information whether and to what extent IL-9 is involved in the eosinophilic inflammation of atopic patients, particularly in the lung. It has been shown that the number of IL-9-immunoreactive and *IL9* mRNA-positive cells were increased in bronchial biopsy specimens of atopic patients with stable asthma.^{15,16} Furthermore, it has been demonstrated that the number of *IL9* mRNA-expressing cells was induced in the skin of atopic patients after local allergen challenge.¹⁶ In addition, other investigators have shown that IL-9 is released from peripheral mononuclear cells from sensitized atopic children after stimulation with cat allergen.¹⁷ We have hypothesized that also in the lung the release of IL-9 is induced in sensitized atopic patients after local allergen stimulation and assumed that IL-9 expression correlates with the eosinophilic inflammation. Therefore we examined IL-9 expression in bronchoalveolar lavage (BAL) fluid before and 24 hours after segmental allergen challenge in a group of atopic asthmatic patients in comparison with a group of nonatopic healthy control subjects. IL-9 protein release into the BAL fluid was measured by means of ELISA. The source of IL-9 production in BAL cells was identified by using immunocytochemistry. Furthermore, real-time PCR was used to quantify IL-9 expression in BAL cells at the mRNA level.

METHODS

Study subjects

Ten atopic patients with mild asthma and 5 nonatopic control subjects participated in the study (for patient characteristics, see Table I). All atopic patients had mild intermittent asthma, as defined in the international consensus report.¹⁸ They had a history of episodic wheezing with reversible airflow obstruction, and asthma had previously been diagnosed by an independent physician. Each patient had a positive skin prick test response, defined as a greater than 4-mm-diameter skin wheal response to one or more of 12 common allergens (mixed grass pollen, mixed tree pollen, rye pollen, mugwort pollen, ribwort pollen, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat fur, dog hair, mixed feather, *Alternaria* species, and *Cladosporium* species; ALK-Scherax). The allergen extract that produced the largest wheal response after skin prick testing was used for segmental allergen challenge (mixed grass pollen or *D pteronyssinus*, ALK-Scherax). The chosen concentration was one tenth the dilution in saline that elicited a 3-mm-diameter skin wheal response. The instilled amount of antigen was 10 to 1000 SQ-U diluted in 10 mL of saline solution. Bronchial hyperresponsiveness and PC₂₀ FEV₁ values (methacholine) were determined as previously described.¹⁹ Patients only used salbutamol when relief of symptoms was necessary. None were treated with corticosteroids, sodium cromoglycate, theophylline, or leukotriene antagonists. The healthy control subjects had no history of allergic or other diseases, negative skin prick test responses, normal serum IgE levels (<100 IU/mL), normal lung function test results, and no bronchial hyperresponsiveness (PC₂₀ >8 mg/mL). All study subjects were nonsmokers, and no subject had acute bronchitis 4 weeks before the investigations. All subjects were volunteers and provided their written consent after being fully

informed about the purpose and nature of the studies, which were approved by the Ethical Committee of Hannover Medical School.

Segmental allergen challenge

Segmental allergen challenge was performed as previously described.¹⁹ A commercially available endotoxin detection assay (E-toxate, Limulus ameobocyte lysate; Sigma) was used to demonstrate that the instilled saline and allergen solutions were free of endotoxin. Briefly, all subjects received nebulizer salbutamol (1.25 mg), atropine (0.5 mg administered subcutaneously), and midazolam (mean, 4.8 mg administered intravenously; range, 0–10 mg) before bronchoscopy. Lidocaine was used to achieve local anesthesia of the upper and lower airways. The bronchoscope (BF 160 P, Olympus Optical) was wedged into the anterior bronchus of the left lower lobe, and BAL was performed with 5 × 20 mL of sterile saline solution. The instrument was passed into the anterior lingular bronchus, and 10 mL of saline solution was instilled as a control challenge. Finally, the bronchoscope was passed to the anterior segment of the right upper lobe, and 10 mL of allergen solution of either grass pollen mix or *D pteronyssinus* mix on the basis of previous skin tests was instilled. After 24 hours, subjects underwent rebronchoscopy with the same premedication, and the anterior lingular bronchus and right anterior upper lobe bronchus underwent lavage with 100 mL of saline solution.

Processing of BAL cells

BAL fluid samples were processed as previously described.¹⁹ Briefly, cells were filtered through a 100- μ m filter and centrifuged, and the supernatant was placed in aliquots and stored at -80°C . The total count of nucleated cells was performed by using a Neubauer hemocytometer. Differential cell counts were performed from cytopsin slides. After Pappenheim staining, 300 cells per slide were counted. For immunocytochemistry staining, cytopsin slides were covered with 40,000 cells and stored at -40°C .

ELISA of BAL fluid

Quantification of IL-9 protein in the BAL fluid was performed by means of ELISA. The goat anti-human IL-9 polyclonal antibody (AF209, R&D Systems) was coated directly onto 96-well ELISA plates (Nunc) at a concentration of 1.25 $\mu\text{g/mL}$ in coating buffer (100 mmol/L $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 9.5) and incubated overnight at room temperature (RT). The plates were blocked with 300 μL of blocking buffer (PBS containing 1% BSA, 5% sucrose, and 0.05% NaN_3) per well for 1 hour at 37°C to eliminate nonspecific binding by the primary antibody. The plates were then washed with PBS-Tween (PBS containing 0.05% Tween 20). A standard curve was generated by using serial dilutions of recombinant human IL-9 (R&D Systems). BAL fluid samples (100 μL) were incubated in duplicate in the 96-well plates at 37°C for 2 hours. After incubation, the plates were washed with PBS-Tween, and 100 μL of biotinylated rabbit anti-human IL-9 polyclonal antibody (50 $\mu\text{g/mL}$, Pepro-Tech) diluted in PBS containing 0.1% BSA was added to each well for 2 hours at RT. The plates were then washed again, and 100 μL of streptavidin-labeled horseradish peroxidase (R&D Systems) diluted 1:200 was added for 20 minutes. After another washing, 100 μL of substrate solution (R&D Systems) containing H_2O_2 and tetramethylbenzidine was added, and the plates were incubated for 20 minutes in the dark. The reaction was terminated by adding 50 μL of stop solution (2 N H_2SO_4). The optical density of each well was determined by using a microplate reader set to 450 nm, and the mean optical density for the highest concentration of the standard curve was 2.110 (range 1.612–2.517). The lower limit of detection for the ELISA was 242 pg/mL. The minimum detectable dose was determined by adding 2 SDs to the mean optical density value of 20

zero standard replicates and calculating the corresponding concentration. The recovery of IL-9 spiked in 2 different concentrations into 4 BAL samples was evaluated. Mean recovery was 98% (range, 82%–116%). Specificity of the antibodies was tested by adding recombinant human IL-5 (R&D Systems) at a concentration of 1500 pg/mL into BAL samples, which yielded in signals at less than the detection limit.

Immunocytochemistry

BAL cells on cytopsin slides stored at -40°C were fixed and permeabilized in methanol/acetone (1:1) at -40°C . After 3 washes in Tris-buffered saline (TBS) at 4°C , nonspecific binding was blocked with Dako Protein Block (Dako) for 15 minutes at RT. Protein block was removed carefully, and cells were incubated with rabbit anti-human IL-9 polyclonal antibody (sc-7923, Santa Cruz Biotechnology) in a dilution of 1:200 overnight at 4°C ; the negative control cells were incubated with rabbit immunoglobulin (X0936, 1:500, Dako). After incubation, all cells were washed 3 times in TBS and incubated with biotinylated swine anti-rabbit (E0353, Dako) in a dilution of 1:200 for another hour at 37°C . Cells were again washed 3 times in TBS before and after 30 minutes of incubation at RT with alkaline phosphatase-conjugated streptavidin (D 0396, 1:300, Dako). Cells were then incubated with Fast Red TR (Sigma) for 15 to 20 minutes at RT. Finally, cells were washed again and stained with Meyers Hämalaun (1:5, Merck), followed by rinsing in water. Slides were mounted with Kaisers glycerol gelatin (Merck). Cells were viewed and photographed through a microscope equipped with a camera (Axiophot, Carl Zeiss GmbH). The immunoreactivity for IL-9 was determined for 400 cells.

Specificity of the staining was controlled by means of preincubation of the polyclonal anti-IL-9 antibody with human recombinant IL-9 at a concentration of 5 $\mu\text{g/mL}$. This preincubation resulted in a complete inhibition of immunoreactivity in BAL cells.

mRNA isolation, reverse transcription, and real-time PCR

Total cellular RNA was extracted from BAL cells by using a commercially available RNA-isolation kit (NucleoSpin RNA II, Macherey-Nagel). Reverse transcription was performed with 0.04 to 0.1 μg of total RNA from each sample in a 40- μL reaction for 60 minutes at 37°C , followed by 5 minutes at 95°C according to the manufacturer's recommendation (Omniscript RT-Kit, Qiagen). Realtime PCR was performed on the Light Cycler Instrument (Roche Molecular Biochemicals) by using the DNA-binding dye SYBR green (Light Cycler-Fast Start DNA Master SYBR Green I, Roche Molecular Biochemicals). PCR reaction mix contained 2 μL of PCR master mix supplemented with 2.5 mmol/L MgCl_2 , custom-synthesized primers (MWG Biotech), and 2 μL of either external standards or cDNA brought to a final volume of 20 μL with water. The specific primers used for PCR were porphobilinogen deaminase (PBGD, M95623, sense: CACACAGCCTACTTTCCAAGC, antisense: TTCAAT-GTTGCCACCACT) and IL-9 (AF361105, sense: CTTCTGGC-CATGGTCCTTAC; antisense: CATGGTCTGGTGCAGTTGTC).

After an initial denaturation step at 95°C for 10 minutes, the PCR reaction was initiated at an annealing temperature of 60°C for 10 seconds, followed by an extension phase at 72°C for 9 seconds and a denaturation cycle at 95°C for 1 second. At the end of each extension phase, fluorescence was observed at 72°C , and the PCR reaction was completed after 45 cycles.

The melting point analysis was carried out by heating the amplicon from 65°C to 95°C . This revealed the characteristic melting point for each product. Length and sequence of the product were confirmed by means of electrophoresis and commercial sequencing (GATC-biotech).

TABLE II. Basic BAL fluid data

Subjects	BAL	n	Recovery	Total cells	Eosinophils		Neutrophils		Lymphocytes		Macrophages	
			(mL)	($\times 10^6$)	($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)
Nonatopic control subjects												
	B	5	78 (73-93)	6.0 (3.8-13.0)	0.0 (0.0-0.1)	0.0 (0.0-1.0)	0.2 (0.1-0.3)	3.0 (2.0-3.0)	0.4 (0.3-1.0)	10.0 (5.0-10.0)	5.5 (3.3-11.6)	87.0 (86.0-91.0)
	S	5	82 (80-94)	5.5 (3.8-22.0)	0.1 (0.0-0.2)	1.0 (0.0-1.0)	1.0 (0.2-7.0)	16.0 (5.0-32.0)	0.5 (0.3-1.8)	9.0 (7.0-11.0)	4.0 (3.2-13.0)	76.0 (59.0-85.0)
	A	5	86 (84-88)	8.5 (5.5-38.0)	0.1 (0.1-0.4)	1.0 (0.0-1.0)	2.6 (0.6-25.1)	25.0 (10.0-66.0)	0.9 (0.5-1.6)	8.0 (4.0-11.0)	4.9 (4.3-13.2)	66.0 (30.0-81.0)
Atopic asthmatic patients												
	B	10	73 (58-85)	4.1 (3.0-15.0)	0.0 (0.0-0.1)	1.0 (0.0-2.0)	0.1 (0.0-0.5)	2.5 (1.0-7.0)	0.5 (0.2-3.2)	11.5 (7.0-21.0)	3.6 (2.6-11.4)	83.0 (76.0-90.0)
	S	10	72 (57-85)	7.6* (3.0-58.0)	0.1 (0.0-0.6)	1.0 (0.0-2.0)	0.9† (0.1-33.1)	10.0† (2.0-59.0)	0.8 (0.3-4.6)	9.5* (5.0-14.0)	5.6 (2.4-19.7)	77.5* (26.0-88.0)
	A	10	68* (34-82)	28.8† (6.5-143.0)	19.5† (2.1-91.5)	64.0† (20.0-82.0)	4.6† (0.5-20.0)	12.5† (2.0-40.0)	2.0* (0.8-18.6)	7.5* (4.0-13.0)	6.1 (1.7-12.9)	17.5† (4.0-44.0)

Fluid recovery, cell counts, and percentages of cells in BAL fluid from nonatopic control subjects and atopic asthmatic patients are shown. BAL fluid was obtained at baseline (B) and 24 hours after saline challenge (S) and allergen challenge (A). Values are given as median (range).

* $P < .05$ and † $P < .01$ compared with baseline values.

Preparation of standards and semiquantification of IL-9

Purified PCR products were used to create standards for reference (*PBGD*) and target (*IL9*). Amplicons of *PBGD* and *IL9* were purified with the High Pure PCR Product Purification Kit (Roche Biochemicals). Ten-fold serial dilutions were amplified for 45 cycles to create appropriate standard curves. Target and reference genes were analyzed in all samples (at baseline, after saline, and after allergen challenge) of one patient in duplicate. At the same time, one dilution of the standards, also in duplicate, was analyzed. This was performed in the same Light Cycler run. The imported standard curves were then used by the Light Cycler software to automatically calculate the amount of *IL9* and *PBGD* for each sample with reference to the external standard. The *IL9/PBGD* ratio was calculated, and the mean ratio of the samples from healthy control subjects was set to one. Finally, all values were calculated with reference to this mean.

Statistical analysis

The Mann-Whitney *U* test was used for intergroup comparison between atopic asthmatic patients and nonatopic control subjects. For comparison of paired data within the major study groups (baseline, saline, and allergen), the Wilcoxon signed-rank test was used for the individual comparisons. The Bonferroni correction for multiple comparisons was used throughout. *P* values of less than .05 were considered significant.

RESULTS

BAL fluid recovery and differential cell count

BAL fluid recovery did not differ between baseline, saline-challenged, and allergen-challenged BAL fluid in nonatopic control subjects. In atopic asthmatic patients there was a decreased recovery after allergen challenge ($P < .05$, Table II). The total cell count was increased after saline challenge and allergen challenge in atopic asthmatic subjects, whereas no significant difference was observed in total BAL cells of control subjects. After

saline challenge, the percentages and absolute numbers of neutrophils were increased in atopic asthmatic patients, as well as in nonatopic control subjects, but it did not reach statistical significance in the control group. After allergen challenge, the percentages and absolute numbers of eosinophils, neutrophils, and lymphocytes were increased in the atopic asthmatic patients, whereas the percentages of macrophages was decreased. In the nonatopic control subjects the percentages and absolute numbers of neutrophils were increased after allergen challenge, but this change did not reach statistical significance (Table II).

Concentration of IL-9 protein in BAL fluid

The protein levels of IL-9 in BAL fluid were measured by means of ELISA, as shown in Fig 1, to examine the production of IL-9 in the airways. In the group of nonatopic control subjects, IL-9 protein levels were less than the detection limit. In atopic patients with asthma, IL-9 protein levels were increased at baseline ($P < .05$) and after saline challenge ($P < .01$) compared with those seen in control subjects. After allergen challenge, there was a significant increase of IL-9 in all atopic asthmatic patients ($P < .01$ compared with levels at baseline and after saline challenge).

Detection of IL-9 protein in BAL cells by means of immunocytochemistry

Immunocytochemistry staining was performed by using an IL-9-specific polyclonal antibody to identify the BAL cells expressing IL-9 protein. There was no significant difference of IL-9-positive cells between atopic asthmatic patients and nonatopic control subjects at baseline or after saline challenge. Compared with baseline and saline provocation, there was a significantly increased percentage of lymphocytes that stained pos-

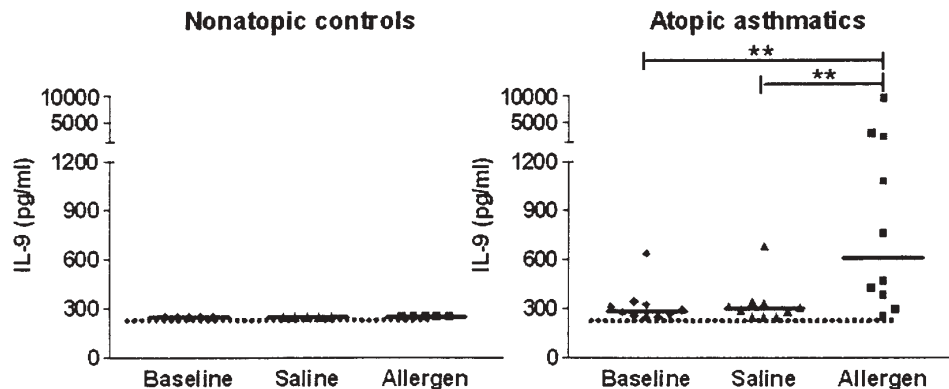


FIG 1. Concentration of IL-9 in BAL fluid measured by means of ELISA before (baseline) and 24 hours after saline and allergen challenge in nonatopic control subjects and atopic asthmatic patients. Detection limit was 242 pg/mL. Bars represent medians. ** $P < .01$

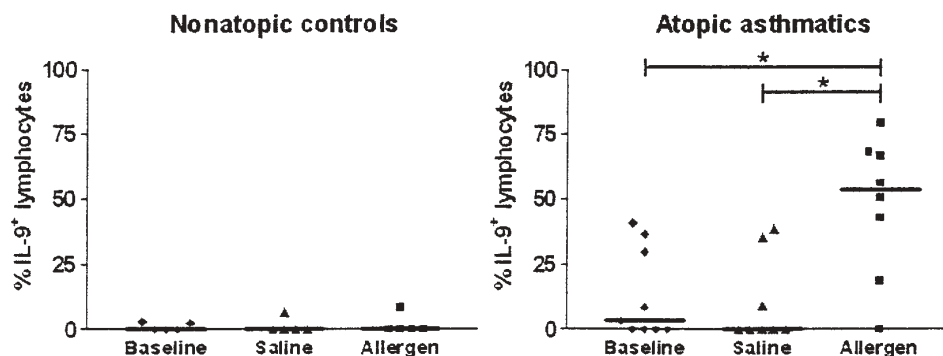


FIG 2. Percentage of IL-9-positive BAL lymphocytes from nonatopic control subjects and atopic asthmatic patients at baseline, after saline challenge, and after allergen challenge. Bars represent medians. * $P < .05$.

itively for IL-9 after allergen provocation in the atopic asthmatic patients (Fig 2). In the control group IL-9-positive lymphocyte numbers were low at baseline and unchanged after saline and allergen challenge. No significant differences in staining for IL-9 were observed in macrophages (baseline, 0% [0%-0%]; saline, 0% [0%-0.3%]; and allergen, 0.8% [0%-26.1%]), neutrophils (baseline, 0% [0%-0%]; saline, 0% [0%-9.7%]; and allergen, 0.0% [0%-0%]), and eosinophils (baseline, 0% [0%-0%]; saline, 0% [0%-0%]; and allergen, 1.3% [0%-6.8%]) of atopic asthmatic patients. In nonatopic control subjects no staining for IL-9 was observed in these 3 cell types. Fig 3, A, shows IL-9-positive lymphocytes from an atopic asthmatic patient after allergen challenge.

Quantification of *IL9* mRNA expression in BAL cells

Quantification of *IL9* mRNA was performed semi-quantitatively with reference to the housekeeping gene *PBGD*. Appropriate standards produced in triplicates showed stable PCR efficiency for both the reference and target gene. All samples of one patient were then measured in duplicates in one run with a duplicate of a single standard probe. Values were found to be highly repro-

ducible. The results are shown as the *IL9/PBGD* ratio (Fig 4). *IL9* mRNA expression of BAL cells was undetectable except in 2 of 5 control subjects at baseline and after allergen challenge and in one subject after saline challenge. None of the atopic asthmatic patients showed *IL9* mRNA expression at baseline. After saline challenge, *IL9* mRNA expression was found at low levels in 5 of 10 patients. Allergen challenge resulted in high levels of *IL9* mRNA expression in all patients ($P < .05$ compared with levels at baseline and after saline challenge).

Relationship between IL-9 expression at the protein and mRNA level and numbers of eosinophils

IL9 mRNA expression in BAL cells significantly correlated with IL-9 protein levels in BAL fluid ($r = 0.8424$, $P = .0037$; Fig 5). Furthermore, *IL9* mRNA and IL-9 protein expression correlated with percentages (protein: $r = 0.6526$, $P = .044$; mRNA: $r = 0.6890$, $P = .031$) and absolute numbers of eosinophils (protein: $r = 0.7939$, $P = .009$; mRNA: $r = 0.7818$, $P = .011$; Fig 6). No correlation was found between IL-9 expression and percentages or absolute numbers of lymphocytes, neutrophils, or macrophages.

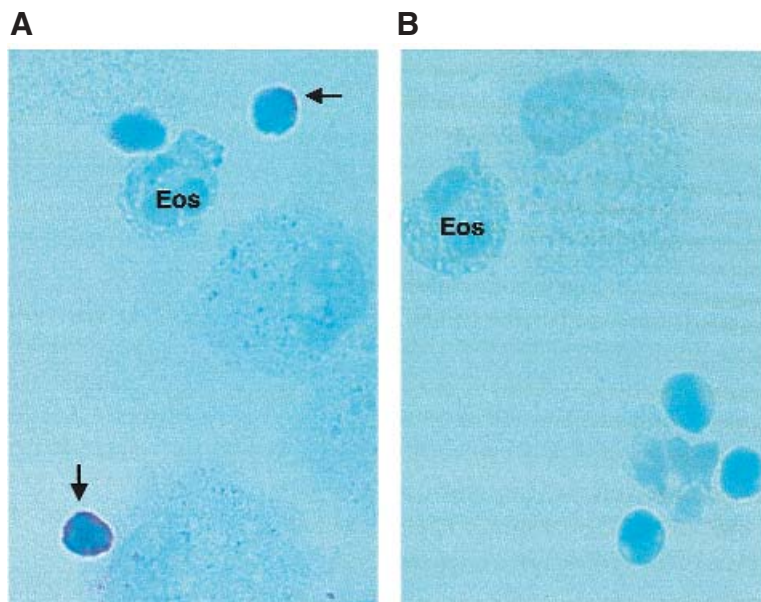


FIG 3. Detection of IL-9 protein in BAL cells from one representative atopic asthmatic patient. Cells were obtained after allergen challenge and stained with rabbit anti-human IL-9 polyclonal antibody (**A**) or rabbit IgG as a negative control (**B**). Arrows indicate lymphocytes that stained positively for IL-9. Eos, Eosinophil.

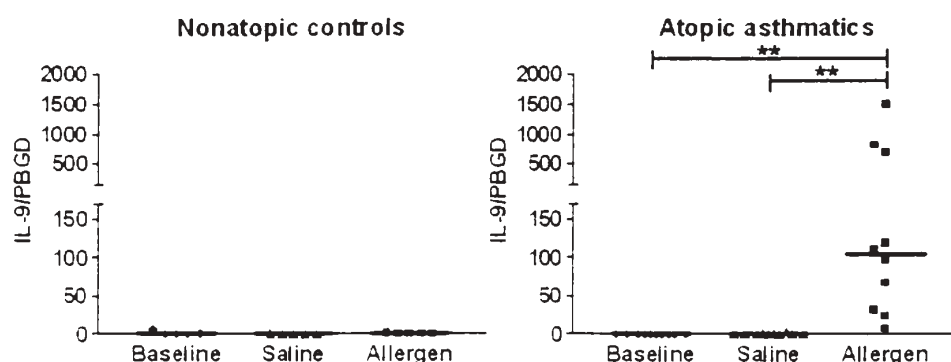


FIG 4. Quantification of *IL9* mRNA in BAL cells before (baseline) and 24 hours after saline and allergen challenge in nonatopic control subjects and atopic asthmatic patients. Data are shown as ratio of *IL9* mRNA to *PBGD* mRNA (housekeeping gene) after calculation, with reference to an external standard. Bars represent medians. ** $P < .01$.

DISCUSSION

The aim of the present study was to determine the pulmonary expression of the T_H2 cytokine IL-9 in the allergic response of atopic asthmatic patients. It was hypothesized that the expression of IL-9 in BAL cells of sensitized patients is induced after local endobronchial stimulation with the relevant allergen.

Our data demonstrate that the expression of *IL9* mRNA and IL-9 protein was low or not detectable in BAL cells and BAL fluid of nonatopic healthy control subjects, as well as in atopic subjects with mild asthma. Using the model of segmental allergen challenge, we found a marked upregulation of *IL9* mRNA and IL-9 protein expression in BAL cells of the atopic asthmatic patients 24 hours after the challenge. This was not seen in the nonatopic control

group or after the saline challenge as a control for the bronchoscopic procedure. Therefore we can conclude that IL-9 is specifically upregulated in the lung after stimulation with the relevant allergen in sensitized subjects. This conclusion is true for atopic asthmatic patients. Because we have not included an atopic control group without asthma in our study, we cannot definitively answer the question of whether increased IL-9 expression is related solely to allergy and what role it might play for asthma. Further studies are needed to unravel this question, and the role of IL-9 in nonatopic asthma has to be addressed.

Bhathena et al²⁰ did not detect *IL9* mRNA expression in BAL cells of patients with stable mild asthma. This finding corresponds to our results because we found *IL9* mRNA expression only in a few subjects in very low amounts before allergen challenge. Despite this lack of

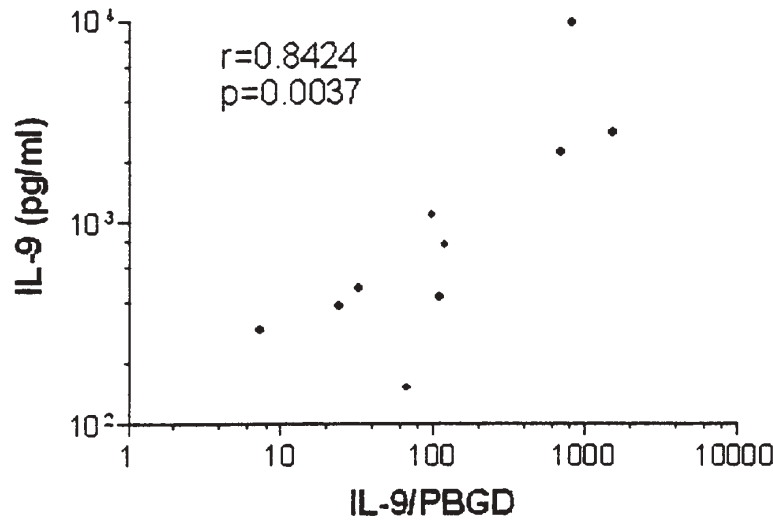


FIG 5. Correlation of IL-9 protein in BAL fluid measured by means of ELISA and *IL9* mRNA detected by means of real-time PCR in BAL cells from atopic asthmatic patients after allergen challenge.

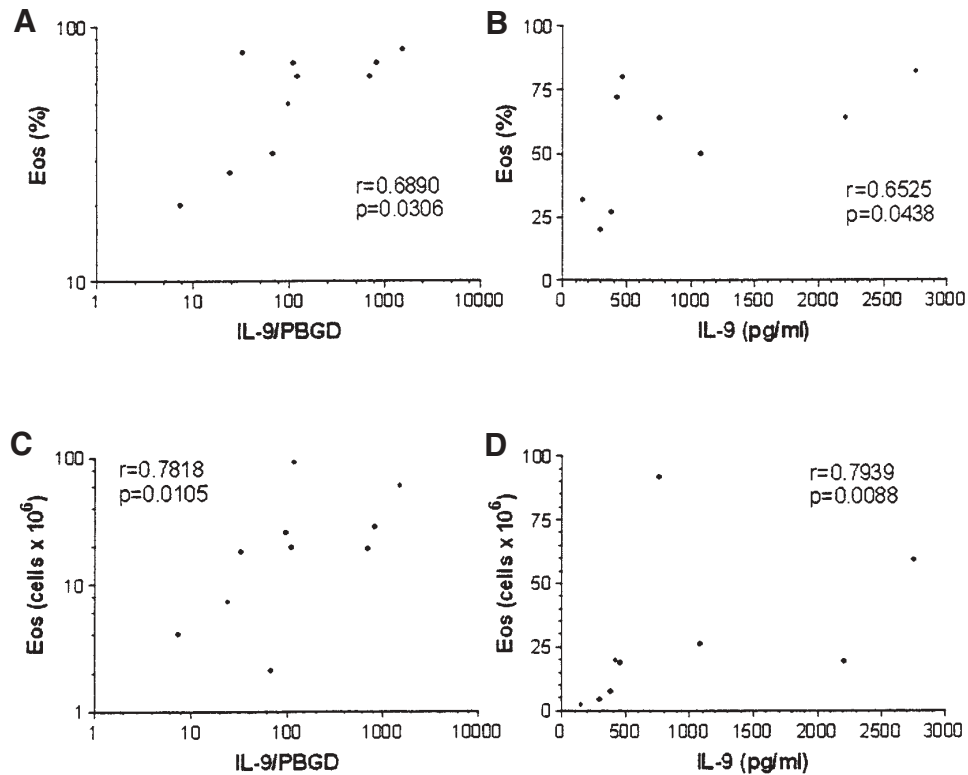


FIG 6. Correlation of percentages of eosinophils in BAL fluid of atopic asthmatic patients after allergen challenge with *IL9* mRNA expressed by BAL cells (A) and with IL-9 protein in BAL fluid (B). Correlation of absolute eosinophil numbers in BAL fluid of atopic asthmatic patients after allergen challenge with *IL9* mRNA expressed by BAL cells (C) and with IL-9 protein in BAL fluid (D).

mRNA expression, IL-9 protein was detectable in lymphocytes of some subjects at that time. The kinetics of *IL9* mRNA expression and subsequent protein production might account for this discrepancy because IL-9 is expressed only for a limited period after T-cell stimula-

tion.^{21,22} However, 24 hours after allergen challenge, there was a good correlation between *IL9* mRNA and protein expression. Thus at this time point, an ongoing allergen-specific T-cell stimulation might explain the simultaneous gene and protein expression in the atopic subjects.

Immunocytochemical staining showed that almost exclusively BAL lymphocytes were stained positively for IL-9. This is in line with previous investigations, which have demonstrated an increased number of IL-9-immunoreactive and *IL9* mRNA-positive cells in bronchial biopsy specimens of atopic asthmatic patients.¹⁵ In these studies the majority of *IL9* mRNA-positive cells were T cells, followed by *IL9*-positive eosinophils and neutrophils.¹⁶ Although we have not determined T-cell subsets in our study, it is known that BAL lymphocytes, even after allergen challenge,¹⁹ almost exclusively consist of T cells. Therefore we conclude that in the allergic response of the lung, T cells are the major cellular source of IL-9. This could be explained as an allergen-specific T-cell response or more likely through a response of bystander T cells under the local influence of IL-2, IL-4, or transforming growth factor β , which all have been shown to enhance IL-9 expression and release from T cells.^{21,23}

In our study only few of the numerous BAL eosinophils were IL-9 immunoreactive. This observation might be in conflict with a previous study that demonstrated increased *IL9* mRNA and IL-9 protein expression by peripheral eosinophils from atopic asthmatic patients.²⁴ The authors suggested that peripheral eosinophils might be activated in vivo and therefore express higher levels of IL-9. Several reasons might exist for the different expression pattern between peripheral and BAL-derived eosinophils. First, peripheral eosinophils were isolated by means of magnetic cell separation, which might have changed their activation and function,²⁵ whereas BAL cells were used immediately after BAL for cytospin preparations. Second, kinetics of IL-9 production by different cell types might play a role. IL-9 expression in T cells peaks 28 hours after stimulation, which corresponds to the increased expression of IL-9 in BAL lymphocytes 24 hours after segmental allergen challenge.²¹ The kinetics of IL-9 expression in eosinophils might be different and have not been described thus far. Third, eosinophils might release stored IL-9 like they release their basic proteins at the site of allergic inflammation. Therefore they might no longer contain IL-9 at 24 hours after allergen challenge.

Although our data suggest that lymphocytes are a major source for IL-9 production, we cannot exclude that mast cells and, in particular, epithelial cells might further contribute to the increased IL-9 expression in the pulmonary allergic response.²⁶ Because we have not obtained bronchial biopsy specimens in our study, we cannot answer this question.

In atopic patients after allergen challenge, *IL9* mRNA and IL-9 protein expression were closely correlated to the percentages and absolute numbers of BAL eosinophils but not to those of neutrophils, lymphocytes, or macrophages. A similar correlation has been shown by Ying et al,¹⁶ demonstrating a correlation of *IL9* mRNA-positive cells with eosinophils in bronchial biopsy specimens of patients with mild asthma and a correlation of *IL9* mRNA-positive cells and eosinophils in the

skin of atopic patients 72 hours after allergen challenge. Together with the ability of IL-9 to promote the influx and maturation of eosinophils, to enhance the IL-5 receptor expression of eosinophils, to increase eosinophil survival, and to release the eosinophil chemoattractant RANTES from human bronchial epithelial cells,^{5,6,8} these findings suggest a critical role of IL-9 in the induction of the allergic eosinophilic inflammation. Furthermore, IL-9 can influence the allergic inflammation through its direct influence on IL-13 expression, which has been demonstrated in vitro and in vivo.²⁷ Although we have measured increased IL-13 levels in the BAL fluid of our study subjects, we could not detect a significant correlation between IL-9 and IL-13 in either atopic patients or control subjects (data not shown).

In conclusion, we have shown that IL-9 is specifically upregulated in response to an allergic stimulus in the lungs of atopic subjects with mild allergic asthma. It is mainly produced by lymphocytes, and its expression correlates with eosinophilic inflammation. Further studies in human subjects with IL-9 receptor antagonists or antibodies to IL-9 are necessary to elucidate its critical role in the allergic eosinophilic inflammation and its importance for the pathogenesis of allergic diseases, such as asthma.

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