

Inhibition of the IL-4/IL-13 receptor system prevents allergic sensitization without affecting established allergy in a mouse model for allergic asthma

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Background: IL-4 and IL-13 are considered as key regulators for the development of atopic disease.

Objective: This study addresses the therapeutic potential of an IL-4/IL-13 inhibitor on the basis of a mutated IL-4 variant (Q116D, Y119D) during allergic sensitization and in established disease in a murine asthma model with persistent airway pathologic condition.

Methods: BALB/c mice were sensitized with ovalbumin intranasally. Mice were treated with the IL-4/IL-13 inhibitor during the sensitization phase or alternatively after ovalbumin allergy was established. Specific antibodies were measured, and histologic lung sections were examined for goblet cell metaplasia. In addition, bronchoalveolar lavages were performed and checked for airway eosinophilia, IL-5 levels, and the number of IL-4 secreting CD4⁺ T cells. Furthermore, airway responsiveness to inhaled methacholine was assessed.

Results: The inhibition of the IL-4/IL-13 system during allergic sensitization resulted in a dose-dependent reduction of ovalbumin-specific IgEs and inhibition of airway eosinophilia together with decreased IL-5 levels and decreased numbers of IL-4 secreting CD4⁺ T cells. Moreover, goblet cell metaplasia and airway responsiveness to methacholine could be reduced significantly by the IL-4/IL-13 inhibitor. However, the inhibition of the IL-4/IL-13 system at various time points after allergy was established showed only little effect on all measured allergic parameters.

Conclusion: Although the inhibition of the IL-4/IL-13 system can efficiently prevent the development of the allergic pheno-

type, these cytokines seem to play a minor role in established allergy. This is relevant for estimating the therapeutic effects of IL-4/IL-13 inhibitors in patients with allergic asthma. (*J Allergy Clin Immunol* 2003;111:1361-9.)

Key words: Allergy, asthma, in vivo animal models, cytokine inhibitors, therapy

Atopic disorders such as allergic rhinoconjunctivitis, allergic asthma, or atopic eczema are an increasing problem in highly developed countries. Therefore it is important to understand their pathogenesis in order to develop novel therapeutic interventions.

T-helper cells of the T_H2 phenotype and their cytokines IL-4, IL-5, and IL-13 are major players in atopic disorders.¹ IL-4 is considered to be a key cytokine, because it directs differentiation of T_H2 cells and induces the expression of other relevant cytokines, chemokines, or adhesion molecules in allergy such as IL-5, IL-13, Eotaxin, or vascular adhesion molecule-1.^{2,3} In addition, IL-4 together with IL-13 induces class switching to IgE in B cells.⁴

Important lessons about the pathogenesis of allergic asthma have been learned from mouse models.⁵ The airway hyperresponsiveness (AHR) to methacholine, which is a clinical hallmark of asthma, seems to be controlled by at least 3 pathways.⁶ One main effector mechanism is a bronchial eosinophilic inflammation.⁷ Other effector mechanisms include IgE-mediated mast cell degranulation⁸ and IL-13 induced mucin gene expression, accumulation of hyaluronic acid, goblet cell metaplasia, and subepithelial fibrosis.^{9,10}

A variety of studies in mouse models for allergic asthma reported beneficial effects achieved by antagonists of candidate cytokines in allergy.¹¹ Several possibilities to antagonize IL-5 effects have been studied so far. The inhibition of IL-5 by inhibitory IL-5 antibodies¹² or active vaccination to induce an autologous anti-IL-5 antibody response¹³ abolished eosinophilic inflammation and AHR.¹⁴ However, preliminary data of clinical trials with patients with mild asthma showed that a humanized anti-IL-5 antibody was able to reduce eosinophilia in blood and sputum, but the asthmatic late phase reaction after stimulation with allergen and AHR could not be affected.¹⁵

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Abbreviations used

AHR:	Airway hyperresponsiveness
BAL:	Bronchoalveolar lavage
H&E:	Hematoxylin-eosin
IL-4R α ^{-/-} :	IL-4 R alpha knockout
MCh ₅₀ :	Concentration of methacholine that caused 50% reduction of expiratory airflow
MLN:	Mediastinal lymph node
OVA:	Ovalbumin
PAS:	Periodic acid-Schiff
QY:	IL-4/IL-13 inhibitor, double mutated IL-4 molecule (Q116D, Y119D)
RU:	Relative units

Potential selective IL-4 inhibitors are the soluble extracellular domain of the IL-4 receptor alpha chain (sIL-4R α) or antibodies to IL-4.¹⁶⁻¹⁸ Both have been shown to inhibit allergen-specific IgE, airway eosinophilia, and AHR to methacholine, when given during sensitization in mice. In contrast, the eosinophilic inflammation could be reduced only marginally and AHR was not affected when IL-4 antagonists were applied during allergen challenge.¹⁹ However, it was recently reported that inhalation of sIL-4R α ameliorated asthma scores in patients with moderate asthma during phase I/II clinical studies.²⁰

A selective IL-13 inhibitor on the basis of a soluble hIL-13R fusion protein (sIL-13Ra2-FC) or antibodies against IL-13 have been shown to inhibit the development of AHR, goblet cell metaplasia, and eosinophilic inflammation in a murine model for allergic asthma, when applied during allergen challenge.²¹⁻²³

Therefore it has been suggested that because of independent roles of IL-4 and IL-13 in allergic responses, a single cytokine antagonist might have limited efficacy. Because IL-4 and IL-13 share one common receptor subunit, the IL-4 receptor alpha chain (IL-4R α chain), it seemed to be promising to inhibit both cytokines with 1 molecule by blocking this receptor subunit. Previously, we have developed an IL-4/IL-13 receptor antagonist on the basis of a mutated IL-4 molecule Q116D, Y119D (QY),²⁴ which was shown to inhibit IL-4 and IL-13 mediated reactions completely in vitro. In addition, specific IgE synthesis and the development of immediate-type hypersensitivity could be inhibited completely during primary sensitization with ovalbumin (OVA) in mice.²⁵ Recently, another mutated IL-4 variant (C118 deletion) was shown to inhibit the development of airway eosinophilia, AHR, as well as goblet cell metaplasia in mice, when applied during allergen challenge.²⁶

Although several studies with cytokine inhibitors have shown the pathogenetic relevance of the T_H2 cytokines IL-4 and IL-13 in allergic sensitization and the development of atopic disease when applied during allergen challenge, the role of these cytokines and the therapeutic potential of cytokine antagonists in established and persisting disease are far less understood.

To answer this, IL-4 and IL-13 signaling pathways were inhibited by a double mutated IL-4 molecule (Q116D, Y119D) during allergic sensitization and at different time points in established disease in a murine asthma model with persistent allergic airway pathologic condition.

METHODS**Animals**

Female BALB/c mice between 8 and 12 weeks of age were purchased from Charles River (Sulzfeld, Germany) and were kept under specific pathogen-free conditions. The numbers of animals per group are given in the figures. All animal experiments were performed according to the guidelines for the care and use of experimental animals prepared and published by the Society for Laboratory Animal Sciences (GV-SOLAS), Biberach a. d. Riss, 1988.

Production and purification of the IL-4/IL-13 inhibitor

For inhibition of the IL-4/IL-13 system a murine double mutated IL-4 variant was used, which has been constructed by site directed mutagenesis (Q116D, Y119D) as described previously.²⁴ The cytokine inhibitor was produced in the *Escherichia coli* expression system in a pET-30a vector (Stratagene, Amsterdam, The Netherlands). The protein-containing inclusion bodies were denatured with 8 mol/L guanidinium chloride, 100 mmol/L Tris-HCl pH 8.0, and 2-mercaptoethanol and were renatured by extensive dialysis against 120 mmol/L NaCl, 2 mmol/L KCl, 3 mmol/L NaH₂PO₄, and 7 mmol/L Na₂HPO₄. The refolded protein was purified by ion-exchange chromatography on a CM-Sepharose Fast Flow column (Pharmacia, Freiburg, Germany) equilibrated with 25 mmol/L ammonium acetate and eluted with a 0 to 0.5 mol/L NaCl gradient. Finally, the material was applied on a reverse phase HPLC column and eluted by a gradient of acetonitrile from 30% to 50%. Remaining endotoxins were removed by affinity chromatography by using AffinityPak Detoxi-Gel columns (Pierce/KMF, St Augustin, Germany) according to the manufacturer's instructions. Endotoxin concentrations were determined by using Pyrogen Gel-Clot Limulus Amebocyte Lysate Test (Bio Whittaker, Verviers, Belgium) and were less than 0.06 endotoxin units/mL. Final protein concentrations were determined by the bicinchoninic acid method (Pierce/KMF) by using BSA as standard. Before use the inhibitory function of the inhibitor was assessed in vitro by a receptor binding test in the Biacore 2000 System (Biacore, Freiburg, Germany) and inhibition of cell proliferation in the IL-4 sensitive pre B cell line BAF/3 as described previously.²⁴ The IL-4 variant was lyophilized and stored at -80°C.

Allergic sensitization and application of the IL-4/IL-13 inhibitor

Mice were anesthetized by intraperitoneal injection of ketamine and xylazine, and 50 μ g OVA (Sigma Grade V, Deisenhofen, Germany) in 50 μ L PBS was administered with or without the cytokine inhibitor intranasally during allergic sensitization and after allergy was established as indicated in Table I. Data were collected in 2 to 4 independent animal experiments.

Serum antibody titers

Mice were bled from the tail vein 24 hours after the last challenge. Blood was coagulated at 4°C followed by centrifugation for 20 minutes at 7500 rpm. Circulating anti-OVA IgE and IgG subclasses were measured in the sera by ELISA. Ninety-six-well plates were coated for 6 hours at 37°C with 100 μ L 0.1 mol/L NaHCO₃ containing 100 μ g OVA/mL. Then plates were blocked for 2 hours at 37°C with 200 μ L 3% BSA in PBS. Plates were washed, and 100

TABLE I. Sensitization and therapy protocols

	Day									
	0	7	14	21	28	35	42 +43	49	56	62 +63
8 × PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS			
8 × OVA	OVA	OVA	OVA	OVA	OVA	OVA	OVA			
Prophylactic										
8 × OVA + 8 × 1 µg QY	OVA	OVA	OVA	OVA	OVA	OVA	OVA			
	QY	QY	QY	QY	QY	QY	QY			
8 × OVA + 8 × 10 µg QY	OVA	OVA	OVA	OVA	OVA	OVA	OVA			
	QY	QY	QY	QY	QY	QY	QY			
8 × OVA + 8 × 100 µg QY	OVA	OVA	OVA	OVA	OVA	OVA	OVA			
	QY	QY	QY	QY	QY	QY	QY			
Therapeutic										
8 × OVA + 2 × 10 µg QY	OVA	OVA	OVA	OVA	OVA	OVA	OVA			
						QY				
8 × OVA + 4 × 10 µg QY	OVA	OVA	OVA	OVA	OVA	OVA	OVA			
					QY	QY				
6 + 2 × PBS	PBS	PBS	PBS	PBS	PBS	PBS		—Allergen-free interval—		PBS
6 + 2 × OVA	OVA	OVA	OVA	OVA	OVA	OVA		—Allergen-free interval—		OVA
Therapeutic										
6 + 2 × OVA + 2 × 10 µg QY	OVA	OVA	OVA	OVA	OVA	OVA		—Allergen-free interval—		OVA
										QY
6 + 2 × OVA + 5 × 10 µg QY	OVA	OVA	OVA	OVA	OVA	OVA	QY (d42)	QY	QY	OVA
										QY

OVA, Ovalbumin; QY, IL-4/IL-13 inhibitor.

µL of 8 series with 2-fold serum dilutions in PBS containing 1% BSA was applied overnight at 4°C. The amount of bound antibody was analyzed by using horseradish peroxidase conjugated antibodies against mouse heavy chain classes. Rat monoclonal antibodies against mouse IgE and IgG1 were from Pharmingen (Hamburg, Germany); goat antisera against mouse IgG2a and IgG2b were from SBA (Biozol, Eching, Germany). Plates were read in a microplate autoreader (Dynatech MR5000, Denkendorf, Germany) at 405 nm. Quality of ELISAs and detection antibodies was controlled by using a laboratory standard serum pool, which was applied on each ELISA plate and was collected 20 days after sensitization with OVA. Data were evaluated by determination of the serum titer, which was the serum dilution lying 1.5-fold over background optical density of nonimmunized mice.

Bronchoalveolar lavage

After intranasal sensitization, mice were challenged intranasally with OVA twice. Then mice were killed 24 hours after the last challenge, and the trachea was cannulated. Bronchoalveolar lavages (BALs) were performed by flushing lung and airways 3 times with 1 mL PBS.

Determination of differential cell counts in the lavage fluid

BAL cells were counted and spun onto glass slides by using a cytospin, followed by staining with Diff-Quik (Dade, Marburg, Germany). Total cell counts and the number of eosinophils were determined microscopically by using standard cytologic criteria.

IL-5 levels in the BAL fluid

IL-5 levels were determined by ELISA with OptEIA (Pharmingen) according to the manufacturer's instructions. Briefly, plates were coated with anti-mouse IL-5 and blocked with 10% FCS in PBS. Se-

rial dilutions of the standard or the samples were added and incubated 2 hours at room temperature. Bound IL-5 was detected by using biotinylated anti-mouse antibody and horseradish peroxidase conjugated avidin. Plates were read in a microplate autoreader at 405 nm.

Detection of IL-4 producing CD4⁺ T cells

BAL cells were spun down, and single cell suspensions were prepared from the MLNs. The cells were stimulated with phorbol ester (5 ng/mL) and calcium ionophore (0.5 µmol/L) for 6 hours (both reagents from Sigma, St Louis, Mo). Brefeldin A (2 µg/mL; Sigma) was added for the last 2 hours of the in vitro culture period. Two-color stainings with mAbs from Pharmingen (San Diego, Calif) were used to identify IL-4 producing CD4⁺ T cells in the BAL fluid according to the manufacturer's instructions. Briefly, after 6 hours of stimulation, cells were washed and stained with FITC-labeled anti-CD4 mAb (L3T4); then the cells were fixed with 4% formalin in PBS for 20 minutes and later incubated with anti-CD16/CD32 mAb (2.4G2; Fc Block [5 µg/mL; Pharmingen]). After 30 minutes cells were stained with FITC-labeled anti-CD4 and with phycoerythrin-labeled anti-IL-4 mAb (11B11). Specificity of antibody binding was controlled by staining with irrelevant isotype matched control antibodies (rat IgG1-phycoerythrin labeled). The analyses were performed with a FACScan (Becton Dickinson, Mountain View, Calif).

Histologic analysis

Lung tissues were fixed in 10% phosphate-buffered formalin for 24 hours and embedded in paraffin wax. Sections (2 to 3 µm) were cut and stained with hematoxylin-eosin (H&E) or periodic acid-Schiff (PAS). The stained sections were visualized by light microscopy and examined for inflammatory infiltrates and goblet cell metaplasia. The density of goblet cells was scored in relative units (RU) with 0 = no, 1 = weak, 2 = moderate, and 3 = strong goblet cell metaplasia by 2 independent examiners.

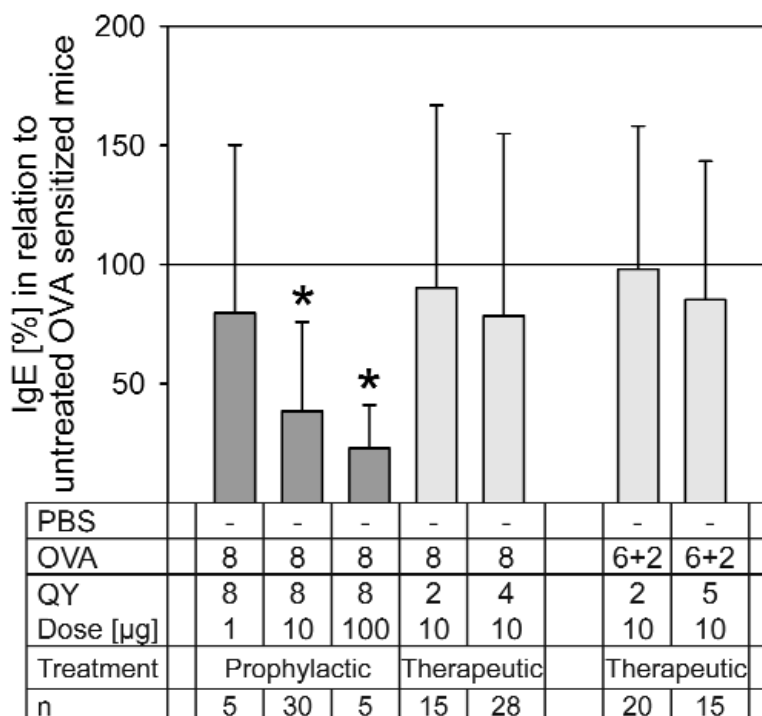


FIG 1. Serum IgE titers. IgE titers were determined by ELISA 6 weeks after completion of the sensitization and therapy courses or 9 weeks with an allergen-free interval (Table I). Data are given as means of 2-4 animal experiments ($n = 5-30$) $\pm$ SD. * $P < .001$, Mann-Whitney rank sum test.

Determination of airway responsiveness

OVA sensitized mice and mice treated with the IL-4/IL-13 inhibitor as indicated in Table I were assessed for airway responsiveness to inhaled methacholine 24 hours after the last OVA challenge in conscious, spontaneously breathing animals by noninvasive head out body plethysmography as previously described.²⁷ Airflow signals were recorded in response to various concentrations of methacholine. Methacholine was aerolyzed in increasing concentrations from 0 to 30 mg/mL in PBS. Each concentration was delivered for 5 minutes with a recovery period of 5 minutes between the administrations. The concentration of methacholine that caused 50% reduction of expiratory airflow was determined (MCh₅₀, mg/mL).

Statistical analyses

Statistical significance for normally distributed samples was analyzed by an unpaired *t* test. Non-normal data or data with unequal variances were tested for significance with the Mann-Whitney rank sum test. Values of *P* less than .05 were considered statistically significant.

RESULTS

Inhibition of the IL-4/IL-13 receptor system prevents OVA-specific IgE induction but does not affect ongoing IgE response

BALB/c mice were weekly sensitized intranasally with OVA for a 6-week period. Subsequently, they were rechallenged with OVA on 2 consecutive days. Alternatively, mice were treated prophylactically with various doses of the IL-4/IL-13 inhibitor, with every OVA application during the sensitization phase or therapeutically at different

time points after OVA allergy was established as indicated in Table I. OVA-specific IgE antibodies were measured by ELISA. IgE levels of each mouse were evaluated by determination of the serum titer, which was the serum dilution lying 1.5-fold over background optical density of nonimmunized mice. To compare data collected in independent animal experiments, the serum IgE titer of each mouse tested in an individual treatment condition was calculated as percentage of the mean IgE titer of untreated OVA-sensitized control mice studied within the same experiment, which was set at 100%. IgE levels of nonimmunized mice or untreated OVA-sensitized control mice are not shown separately because they are integrated in the results by calculation of serum titers and percentages.

OVA-specific IgE synthesis was detectable in OVA-sensitized mice 14 days after the first sensitization and was continuously increasing during the 6 weeks of sensitization (data not shown). Fig 1 shows that the prophylactic inhibition of the IL-4/IL-13 system during allergic sensitization resulted in a dose-dependent reduction of OVA-specific IgE antibodies in mice, which were treated with various doses of the IL-4/IL-13 receptor antagonist. The application of $8 \times 10 \mu\text{g}$ and $8 \times 100 \mu\text{g}$ QY with every OVA sensitization resulted in a significant reduction of OVA-specific IgE antibodies, whereas $8 \times 1 \mu\text{g}$ QY did not show any significant effects. However, although the reduction of OVA-specific IgE antibodies was significant in mice treated with $8 \times 10 \mu\text{g}$ and $8 \times 100 \mu\text{g}$ QY in comparison with nontreated OVA-sensitized mice, specific IgE synthesis could not be inhibited completely (Fig 1). Furthermore, the

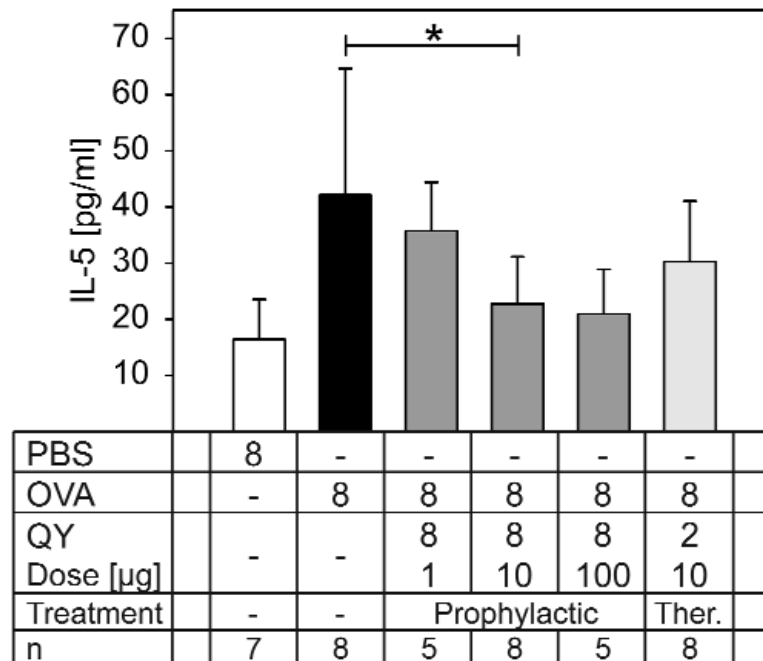


FIG 2. IL-5 levels in the BALs. IL-5 levels are measured by ELISA 6 weeks after completion of the sensitization and therapy courses (Table I). Data are given as means ($n = 5-8$) $\pm$ SD. * $P < .05$, unpaired t test.

therapeutic inhibition of the IL-4/IL-13 system with 2×10 μg inhibitor during OVA rechallenge after 6 weeks or 4×10 μg inhibitor starting at day 28 after primary OVA sensitization, when allergy was already established (Table I), did not lead to any significant reduction of OVA-specific IgE antibodies (Fig 1).

In a separate experiment we wanted to mimic the immunologic situation in patients with seasonal allergy off season in mice and integrated a group of OVA-sensitized mice, in which weekly OVA applications were stopped at day 35, when OVA allergy was established. After a 3-week allergen-free interval, mice were rechallenged with allergen at days 62 and 63 (Table I). After the allergen-free interval OVA-specific IgE levels in untreated OVA-sensitized mice showed constant IgE levels, indicating an established ongoing IgE synthesis. In line with the previous experiments the therapeutic inhibition of the IL-4/IL-13 system with 2×10 μg inhibitor during allergen rechallenge (Fig 1) or even 5×10 μg inhibitor during the allergen-free interval did not result in a significant reduction of specific IgE levels in comparison to untreated OVA rechallenged mice.

Inhibition of the IL-4/IL-13 receptor system reduces the number of IL-4 secreting CD4⁺ T cells and IL-5 levels in the BAL fluid during allergic sensitization but does not affect ongoing cytokine production

After completion of the sensitization and therapy courses (Table I), mice underwent BAL and CD4⁺ T cells were prepared for fluorescence-activated cell sorting

analyses of their intracellular IL-4 content. In addition, IL-5 levels in the lavage fluid were analyzed by ELISA.

OVA-sensitized mice showed an increased number of IL-4 secreting CD4⁺ T cells ($3.0\% \pm 0.9\%$) in comparison to nonsensitized animals ($1.8\% \pm 0.4\%$) combined with increased levels of IL-5 in the BAL fluid (Fig 2). Mice that had been treated prophylactically with 8×10 μg or 8×100 μg IL-4/IL-13 inhibitor during allergic sensitization showed significantly reduced numbers of IL-4 secreting CD4⁺ T cells ($1.2\% \pm 0.3\%$ and $1.1\% \pm 0.4\%$, respectively; $P < .001$). In addition, the inhibition of the IL-4/IL-13 system during allergic sensitization reduced IL-5 levels in the BAL fluid dose dependently (Fig 2). In contrast, the therapeutic application of 2×10 μg IL-4/IL-13 inhibitor during allergen rechallenge after 6 weeks or 4×10 μg inhibitor starting at day 28 after the first OVA sensitization did not lead to any significant reduction of IL-4 secreting CD4⁺ T cells ($2.8\% \pm 0.6\%$ and $3.0\% \pm 1.2\%$, respectively) or IL-5 levels in the BAL fluid (Fig 2). Furthermore, the therapeutic inhibition of IL-4/IL-13 with 5×10 μg inhibitor during an allergen free interval did not significantly reduce the number of IL-4 secreting CD4⁺ T cells ($1.9\% \pm 0.9\%$).

Inhibition of the IL-4/IL-13 receptor system prevents allergic airway disease without affecting ongoing allergy

Mice were sensitized with OVA and treated with the IL-4/IL-13 inhibitor as described above (Table I). After the completion of the sensitization and therapy courses, histologic lung sections were performed and stained with

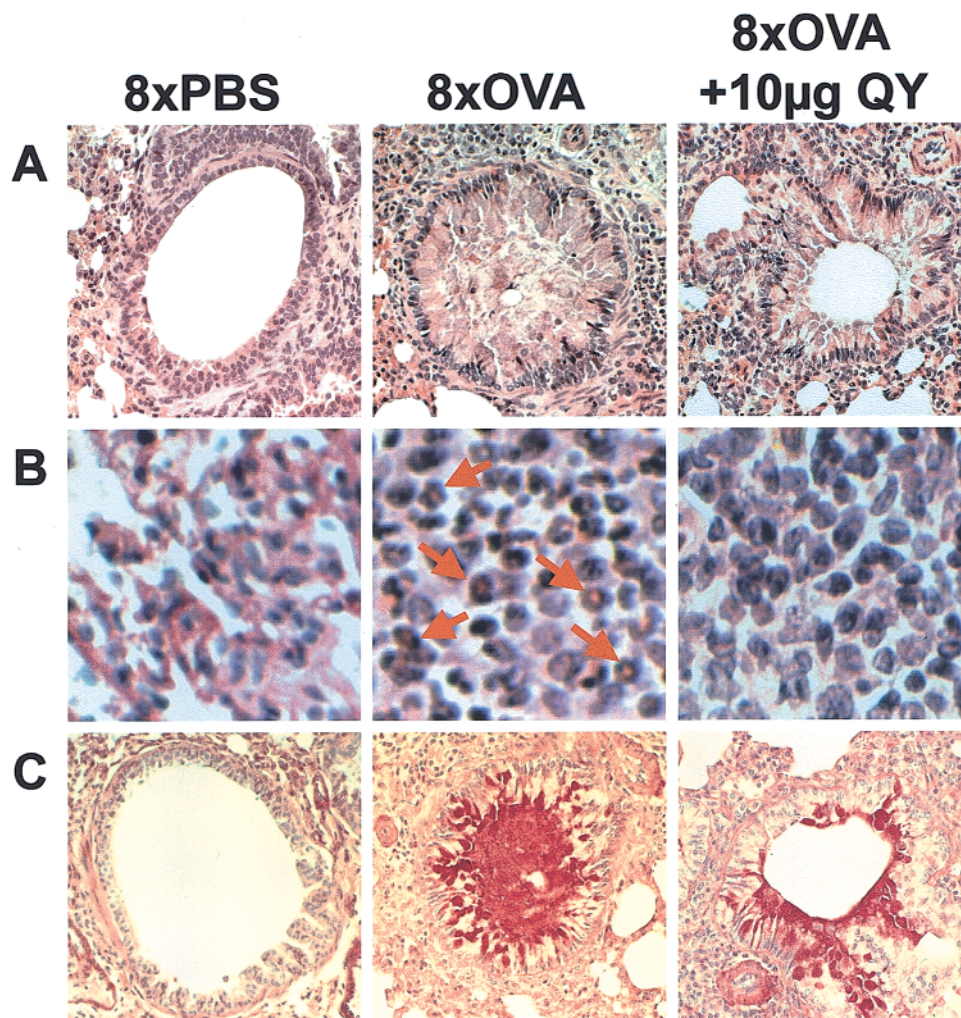


FIG 3. Allergic airway pathologic condition. Lung sections were stained with H&E (**A** and **B**) or PAS (**C**) to detect inflammatory infiltrates and goblet cell metaplasia 6 weeks after completion of the sensitization and therapy courses (Table I).

H&E or PAS for the detection of inflammatory infiltrates or goblet cell metaplasia, respectively. Other groups of mice underwent BAL.

The cells in the BAL fluid were stained with Diff-Quik and were analyzed for their cellular composition.

As demonstrated in Fig 3, OVA-sensitized mice developed extensive allergic airway pathologic condition after 6 weeks of sensitization, with a peribronchial and perivascular airway inflammation, which was dominated by eosinophils and lymphocytes in comparison to non-sensitized mice (Fig 3, *A* and *B*). This was combined with severe goblet cell metaplasia and extensive mucus production in the bronchi (Fig 3, *C*). The airway pathologic condition was continuously increasing with the number of boosts and started to be detectable at day 21 after the first sensitization. During the allergen-free interval the airway eosinophilia declined and remained at increased levels in comparison to nonsensitized mice, indicating established lung pathologic condition (data not shown).

The prophylactic inhibition of the IL-4/IL-13 system with $8 \times 10 \mu\text{g}$ inhibitor during allergic sensitization resulted in a complete inhibition of airway eosinophilia (Fig 4, *A*) and significant reduction of goblet cell metaplasia (1.0 ± 0.9 RU) in comparison to untreated OVA-sensitized mice (2.1 ± 0.7 RU, $P < .001$) (Fig 3, *C*). Although the therapeutic application of $4 \times 10 \mu\text{g}$ of the IL-4/IL-13 inhibitor together with the OVA sensitization starting at day 28 after the first sensitization showed a statistically significant reduction of the goblet cells (1.4 ± 0.4 RU, $P = .042$), no significant inhibitory effects could be seen on the airway eosinophilia in the BAL fluid (Fig 4, *A*). Furthermore, mice that were treated with $2 \times 10 \mu\text{g}$ at OVA rechallenge after 6 weeks of sensitization or with $5 \times 10 \mu\text{g}$ IL-4/IL-13 inhibitor in established allergy during an allergen-free interval did not show significant inhibitory effects on ongoing airway pathologic condition regarding airway eosinophilia (Fig 4, *B*) or goblet cell metaplasia (data not shown).

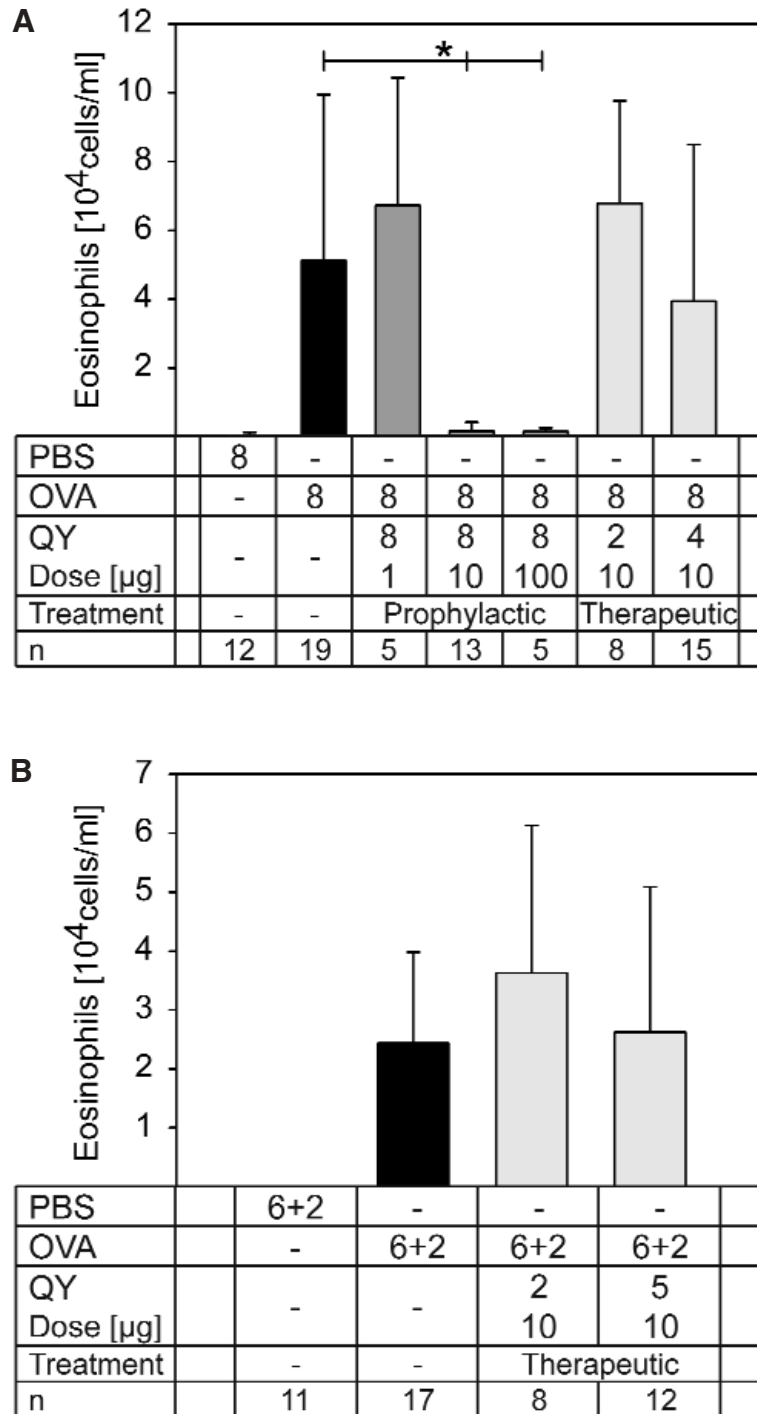


FIG 4. Airway eosinophilia. BAL was performed and the amount of eosinophils was determined 6 weeks (**A**) after completion of the sensitization and therapy courses (Table I) or 9 weeks with an allergen-free interval (**B**). Data are means ($n = 5-19$) $\pm$ SD from 2-4 animal experiments. * $P < .001$, unpaired t test.

In a separate group of mice, airway responsiveness to inhaled methacholine was measured 24 hours after completion of sensitization and therapy courses.

MCh₅₀ was determined. OVA-sensitized mice showed AHR to methacholine with reduced MCh₅₀ (71 ± 17 mg/mL), in comparison to nonsensitized animals ($91 \pm$

14 mg/mL, $P < .05$). In contrast, mice that were treated prophylactically with 8×10 µg of the IL-4/IL-13 inhibitor during allergic sensitization showed a significantly increased MCh₅₀ (113 ± 35 mg/mL, $P < .05$), indicating a normalization of airway responsiveness to inhaled methacholine. However, mice that were treated

therapeutically with $4 \times 10 \mu\text{g}$ IL-4/IL-13 inhibitor when allergy was already established did not show any significant amelioration of the AHR (MCh_{50} , $82 \pm 15 \text{ mg/mL}$).

DISCUSSION

In this study we present a murine asthma model in which similar to a sensitization with aeroallergens in humans, repetitive intranasal OVA applications without adjuvant induced AHR and persistent allergic airway pathologic condition, combined with ongoing specific IgE synthesis. In congruence with a previous study we found that the inhibition of the IL-4/IL-13 system could efficiently prevent the development of allergic airway pathologic condition.²⁶ We showed that the development of airway eosinophilia and AHR could be inhibited completely and the development of goblet cell metaplasia could be significantly reduced by the IL-4/IL-13 inhibitor. This underlines the crucial role of both cytokines IL-4 and IL-13 for the development of allergic airway pathologic condition, as published previously.^{17,21,22,28,29}

However, although OVA-specific IgE antibodies could be significantly reduced, specific IgE synthesis was not inhibited completely in mice that were treated with the self-constructed IL-4/IL-13 inhibitor. This is in line with data we published recently, which were collected in IL-4 R alpha knockout (IL-4R $\alpha^{-/-}$) mice.³⁰ After 6 weeks of intranasal OVA sensitization IL-4R $\alpha^{-/-}$ mice developed similar levels of OVA-specific IgE antibodies as wild-type mice treated with the IL-4/IL-13 inhibitor, which acted as IL-4R alpha receptor antagonist (data not shown). In contrast, after primary intraperitoneal OVA sensitization, specific IgE synthesis was not detectable in IL-4R $\alpha^{-/-}$ mice and could be inhibited completely in wild-type mice by the same IL-4/IL-13 antagonist.²⁵ In addition, Tomkinson et al²⁶ found that similar doses of an IL-4/IL-13 receptor antagonist on the basis of a deleted IL-4 molecule (C118 deletion) significantly inhibited specific IgE synthesis, when applied during intranasal challenge after previous intraperitoneal OVA sensitization. However, we showed that an established ongoing IgE response could not be significantly reduced by the IL-4/IL-13 inhibitor in our mouse model. Therefore, alternate, so far unknown mechanisms seem to maintain IgE synthesis independently of the IL-4R α chain on long-term allergen exposure in established allergy, which are different from the induction phase of allergic responses.³⁰

Furthermore, we demonstrate here that the inhibition of the IL-4/IL-13 receptor system at various time points after allergic lung pathologic condition was established did not lead to any significant reduction of asthma-like lung pathologic condition.

In our study the treatment with the cytokine inhibitor was started after sensitization was completed and allergic pulmonary disease was already established. Established lung disease with ongoing allergic lung pathologic condition and allergen specific IgE synthesis was achieved by long-term intranasal sensitization with 8 intranasal

OVA applications during a 6- or 9-week period. However, previous studies with a 2-fold intraperitoneal OVA priming within 2 weeks before short-term intranasal or intratracheal challenge reported inhibitory effects of selective IL-13 antagonists as well as IL-4 receptor antagonists on AHR, airway inflammation, and mucus cell content, when the inhibition occurred during inhaled allergen challenge.^{21,22,26,31}

Because of differences in the protocols used, data cannot be compared directly. However, it could be speculated that intraperitoneal priming before short-term intranasal challenge induces temporary allergic airway pathologic condition, which might efficiently be inhibited by IL-4 or IL-13 inhibitors,^{21-23,26,31} whereas an established immune response induced after long-term intranasal sensitization seems to be regulated differently. In addition, we cannot rule out that there is a difference in effectiveness of the inhibitor given intranasally versus intraperitoneally, possibly as a result of deposition of the inhibitor in the upper airway. Furthermore, the effectiveness might be lower in inflamed than in noninflamed tissue. However, we could show a dose-dependent inhibition of OVA-specific IgE synthesis and other allergic parameters with $10 \mu\text{g}$ of the inhibitor applied intranasally during sensitization. This demonstrates the effectiveness of an intranasal treatment and strongly suggests an efficient systemic absorption.

Although IL-4 and IL-13 are considered as key regulators in allergy, the inhibition of IL-4 and IL-13 effects may be compensated in vivo through other factors, which are relevant for the established allergic phenotype. One candidate cytokine might be IL-5, which is essential for the maturation, differentiation, and survival of eosinophils in allergic responses. Data from IL-5 deficient mice have shown a crucial role of IL-5 for the development of airway eosinophilia and AHR independently from specific IgE synthesis.^{7,12,32} Recent work has shown the potential importance of another T_H2 cytokine, IL-9. IL-9 has been shown to act on many cell types involved in asthma, including T cells, B cells, mast cells, eosinophils, and epithelial cells. The development of transgenic mice overexpressing IL-9 has suggested an important role in the development of the asthmatic phenotype, including eosinophilic inflammation, bronchial hyperresponsiveness, elevated IgE levels, and increased mucus secretion.³³⁻³⁷

In summary, the present study addresses the role of IL-4 and IL-13 in a murine asthma model with ongoing IgE synthesis and persistent allergic airway pathologic condition. We could show that the inhibition of the IL-4/IL-13 system after the development of allergic airway pathologic condition did not show any significant reduction of all measured allergic parameters. This suggests that these cytokines seem to play a minor role in established allergy in our mouse model. These findings are important to estimate the therapeutic potential of allergy therapy with IL-4/IL-13 inhibitors in patients with asthma.

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