

Upon Prolonged Allergen Exposure IL-4 and IL-4R α Knockout Mice Produce Specific IgE Leading to Anaphylaxis

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Key Words

IgE · IL-4 · IL-4 receptor · In vivo animal models · Allergy · Anaphylaxis

Abstract

Background: IL-4 and IL-13 are key regulators in atopic disorders and both signal through the receptor chain IL-4R α . IL-4 and IL-13 are also the only cytokines known to induce class switching to IgE. We sought to compare allergen-specific IgE responses and allergic reactivity of wild-type (wt) mice with IL-4^{-/-} and IL-4R α ^{-/-} mice, which lack both IL-4 and IL-13 functions. **Methods:** BALB/c wt, IL-4^{-/-} and IL-4R α ^{-/-} mice were immunized with ovalbumin intranasally or intraperitoneally and specific antibody titers were measured by ELISA. Bronchoalveolar lavage fluids and lung tissue were analyzed cytologically and histologically. Allergic reactivity was determined by active cutaneous anaphylaxis and anaphylactic shock. **Results:** wt mice immunized intranasally or intraperitoneally showed high titers of specific IgE 3 and 6 weeks after primary sensitization, resulting in cutaneous anaphylaxis and anaphylactic shock upon challenge. Intranasal sensitization resulted in airway eosinophilia and

goblet cell metaplasia. In contrast, IL-4^{-/-} and IL-4R α ^{-/-} mice showed no specific IgE after 3 weeks, but produced high titers after 6 weeks. At this time cutaneous anaphylaxis and anaphylactic shock could be induced as in wt mice, but lung pathology was absent. **Conclusions:** We conclude that upon long-term allergen exposure, alternative switch mechanisms independent of IL-4 and IL-4R α may induce IgE but not asthma-like lung pathology. This may be relevant for the development of allergic disease, since long-term allergen exposure is a frequent condition during allergic sensitization.

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Introduction

IL-4 and IL-13, two related cytokines with overlapping functions, are considered to be key players in IgE class switching and in the development of atopic disorders [1]. There are two types of functional IL-4 receptor complexes: type 1 receptor complexes containing the IL-4R α subunit and the common gamma chain (γ c), and type 2 receptor complexes containing IL-4R α and IL-13R α 1. The type 2 IL-4 receptor is identical with the receptor for

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IL-13, so both cytokines share the IL-4R α subunit in their receptor complexes [1, 2]. IL-4R α ^{-/-} mice are strongly impaired in type 2 T-helper cell (Th2)-dependent immune reactions, and cells derived from these mice completely fail to respond to IL-4 or IL-13, which suggests that neither cytokine has functional receptors lacking this chain [3–6].

Class switching to IgE, and to IgG1, in mice is induced when B cells simultaneously receive two stimuli: one is the activation of the shared IL-4/IL-13 receptor by one of its ligands, and the other is the ligation of CD40 by CD40L, which is expressed on activated T cells [7, 8]. Initially, IL-4 was believed to be exclusively responsible for the class switch to IgE in the mouse. The discovery that IL-13 can induce IgE class switching in human B cells [9, 10] suggested a possible role for IL-13 in mice as well. IL-13-induced IgE production was indeed demonstrated in transgenic mice which expressed IL-13 in lymphoid tissues, but lacked a functional IL-4 gene [11].

There have been several reports about IL-4-independent IgE synthesis in infectious diseases. Increased total IgE has been found after infection with parasites like *Plasmodium chabaudi* [12] and *Leishmania major* [13] in IL-4-deficient mice. IL-4-independent IgE synthesis was also demonstrated in IL-4^{-/-} mice upon infection with the MAIDS virus or after stimulation with goat-anti-mouse IgD, which is an unspecific stimulus of IgE synthesis [14]. These observations could be explained by assuming that IL-13 is sufficient for IL-4-independent IgE induction in IL-4^{-/-} mice. This hypothesis would agree with previous findings that IL-4R α ^{-/-} mice are unable to induce IgE upon infection with *Nippostrongylus brasiliensis* [4, 5], *L. major* [6, 15] or allergic sensitization with ovalbumin (OVA) [16]. In contrast to these reports we show that efficient production of OVA-specific IgE occurs in IL-4R α ^{-/-} mice upon prolonged allergen exposure. Since IL-4R α is required for signaling by both cytokines, IgE may be produced in this system independently from IL-4 and IL-13.

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. BALB/c mice with targeted disruptions of the IL-4 (IL-4^{-/-}) or IL-4R α gene (IL-4R α ^{-/-}) were generated at the Max Planck Institute for Immunobiology (Freiburg, Germany) [6]. All 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.

Allergic Sensitization

Mice were immunized by 2 intraperitoneal injections at day 0 and day 21 with 10 μ g OVA (Sigma, Deisenhofen, Germany; grade V) in a solution containing 140 μ l PBS and 70 μ l Imject Alum (Pierce/KMF, St. Augustin, Germany) as an adjuvant. Alternatively, mice were anesthetized by intraperitoneal injection of ketamine and xylazine, and sensitized by a total of 7 weekly intranasal inoculations with 50 μ g OVA in 50 μ l PBS.

Serum Antibody Titers

Anti-OVA IgE and IgG subclasses were measured by ELISA as described [16]. Briefly, plates were coated with 100 μ g OVA in 100 μ l buffer, blocked, and 100 μ l of serum dilutions were applied overnight at 4°C. Bound antibody was analyzed using HRP-conjugated antibodies against mouse heavy-chain classes (Pharmingen, Hamburg, Germany) [16]. Data were collected in 2 independent *in vivo* tests and are given as relative units. In order to test the specificity of the ELISA, OVA-specific IgE antibodies were measured in sera of representative animals by coating microtiter plates with 1 μ g/ml rat anti-mouse IgE monoclonal antibodies (mAb) (Pharmingen). After blocking with 10% BSA for 1 h at room temperature (RT), serial serum dilutions were added and incubated overnight at 4°C. Appropriate dilutions of OVA-labeled biotin and alkaline phosphatase-labeled streptavidin (Sigma, Deisenhofen, Germany) were added for 1 h at RT [17]. Similar results were obtained with both ELISA methods.

For detection of total IgE, plates were coated with rat anti-mouse IgE mAb 1 μ g/ml (Pharmingen) and subsequently blocked with 10% BSA for 1 h at RT. Serum dilutions were added and incubated for 2 h. Subsequently appropriate dilutions of alkaline phosphatase-labeled rat anti-mouse IgE mAb (Pharmingen) were added. For detection the phosphatase substrate 104 (Sigma) was used.

Active Cutaneous Anaphylaxis

Active cutaneous anaphylaxis was tested after intravenous injection of 200 μ l 0.5% Evans Blue in PBS. The skin of the belly was shaved, and 50 μ l PBS containing 1 mg/ml OVA or PBS alone were injected intradermally in 4 premarked sites of the skin. After 15 min, the mice were killed and the skin was stripped off. Positive reactions towards antigen resulted in mast cell degranulation and fluid extravasation, which led to the formation of a blue patch around the injection site. The intensity of bluing was scored by two independent observers.

Anaphylactic Shock

Mice were injected intravenously with 200 μ l PBS containing 0.5% Evans Blue and 500 μ g OVA. After 15 min, symptoms of an anaphylactic shock were assessed by two independent observers, who were unaware of the sensitization status of each animal. Reactions were scored as indicated according to bluing of ears, nose and footpads, spontaneous activity and responsiveness to external stimuli.

Bronchoalveolar Lavage

After intranasal sensitization, mice were challenged twice intranasally with OVA and killed; the trachea was then cannulated and bronchoalveolar lavages (BALs) were performed by flushing lung and airways 3 times with 1 ml PBS. BAL cells were counted and spun onto glass slides using a cytospin, followed by staining with Dif-Quik (Dade, Marburg, Germany). Total cell counts and number of eosinophils were determined microscopically using standard cytological criteria.

Histological Analysis

Lung tissues were fixed in 10% phosphate-buffered formalin for 24 h and embedded in paraffin wax. Sections (2–3 μ m) were cut and stained with hematoxylin and eosin or periodic acid-Schiff. The stained sections were visualized by light microscopy. Density of goblet cells and inflammatory infiltrate were scored by 2 independent examiners.

Results

OVA-Specific IgE and IgG1 Synthesis in IL-4^{-/-} and IL-4R α ^{-/-} Mice

Groups of wild-type (wt), IL-4^{-/-} and IL-4R α ^{-/-} BALB/c mice were immunized by 2 intraperitoneal injections of 10 μ g OVA plus alum. In a second experiment, wt and knockout mice were sensitized by 7 weekly intranasal applications of 50 μ g OVA. Mice were bled and OVA-specific serum antibodies were measured by ELISA. Figure 1a, b shows OVA-specific IgE and IgG1 obtained with both sensitization strategies.

Specific IgE was present 21 days after the first intraperitoneal OVA immunization in wt, but not in IL-4^{-/-} or IL-4R α ^{-/-} mice. However, specific IgE synthesis was induced to the same level in all groups of mice by day 42 (fig. 1a). In the intranasal sensitization protocol, IgE titers were very low at day 21. At day 42 all groups of mice had high levels of specific IgE, but the two knockout strains produced less IgE than the wt mice (fig. 1b).

The induction of IgG1 in the various experiments was similar to that of IgE (fig. 1a, b). In contrast, specific IgG2a, IgG2b and IgG3 were consistently upregulated in IL-4^{-/-} and IL-4R α ^{-/-} mice compared to wt animals (not shown).

In addition, total IgE levels in sera of wt and IL-4R α ^{-/-} BALB/c mice were measured before and after 42 days of intranasal OVA sensitization. In wt BALB/c mice total IgE levels increased about 6-fold in the sera during OVA sensitization from 2.12 μ g/ml ($\pm$ 0.64) to 12.62 μ g/ml ($\pm$ 5.69). IL-4R α ^{-/-} BALB/c mice showed markedly lower total IgE levels in their sera than the wt mice, which increased about 2-fold from 0.08 μ g/ml ($\pm$ 0.02) to 0.18 μ g/ml ($\pm$ 0.07) after OVA sensitization.

Fig. 1. OVA-specific antibody levels in the serum. Serum titers for OVA-specific antibodies were measured by ELISA 21 and 42 days after intraperitoneal (a) or intranasal (b) immunization with OVA. Error bars indicate the standard deviation, the number of mice used under each condition is indicated as (n = x).

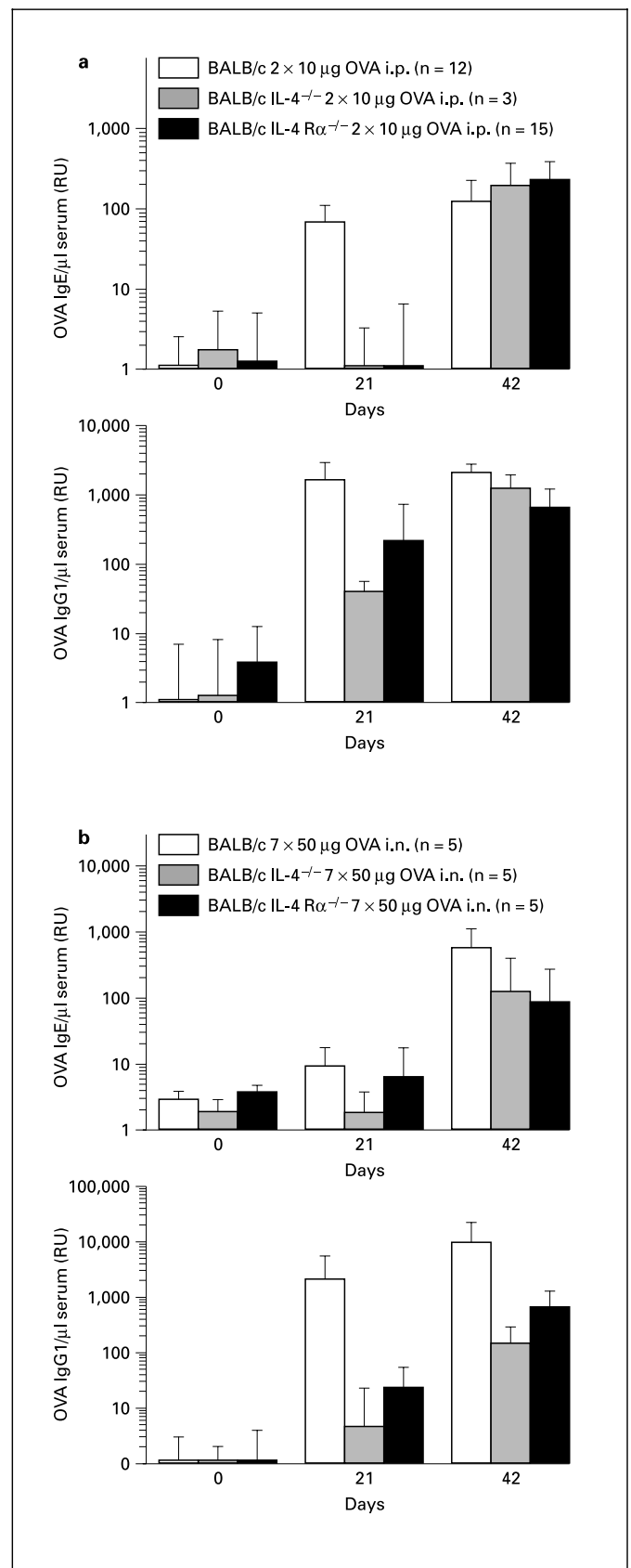


Table 1. Active cutaneous anaphylaxis

Sensitization strategy	OVA/PBS i.p.			OVA/PBS i.n.	
	day 0 (n = 5)	day 21 (n = 4)	day 42 (n = 8)	day 0 (n = 2)	day 42 (n = 2)
BALB/c wt	0/0	2/0	2/0	0/0	2/0
BALB/c IL-4 ^{-/-}	0/0	0.25/0	2/0	0/0	2/0
BALB/c IL-4Rα ^{-/-}	0/0	0.25/0	1.75/0	0/0	2/0

Mice were immunized with OVA. After 21 or 42 days 200 µl Evans Blue was injected intravenously and mice were challenged intradermally with 50 µl PBS with or without OVA. The intensity of bluing, which is the result of mast cell degranulation at the injection sites, was rated as follows: 0 = no blue patch formation; 1 = slight bluing; 2 = strong bluing. Data are given as mean scores per animal group.

Table 2. Systemic anaphylaxis

Sensitization strategy	BALB/c wt			BALB/c IL-4 ^{-/-}			BALB/c IL-4Rα ^{-/-}		
	no OVA (n = 5)	OVA i.p. (n = 6)	OVA i.n. (n = 2)	no OVA (n = 2)	OVA i.p. (n = 2)	OVA i.n. (n = 2)	no OVA (n = 5)	OVA i.p. (n = 8)	OVA i.n. (n = 2)
Bluing of exposed skin (n = no, 1 = yes)	0	1	1	0	1	1	0	1	0.5
Spontaneous activity (0 = running around, 1 = sitting, 2 = lying)	0	2	2	0	2	1.5	0	1	0.5
Responsiveness to stimuli (0 = running away, 1 = slight reaction, 2 = no reaction)	0	1.6	1.75	0	2	1.5	0	1.5	0.5

Mice were immunized with OVA and 42 days later injected intravenously with Evans Blue containing 500 µg OVA. Anaphylactic symptoms were assessed as indicated. The animals were considered to be in a state of shock if alterations in all three of the indicated criteria were observed. Data are given as mean scores per animal group.

OVA-Induced Active Cutaneous Anaphylaxis and Anaphylactic Shock in IL-4^{-/-} and IL-4Rα^{-/-} Mice

To test whether the OVA-specific IgE induced in the knockout strains was functionally relevant to allergen-specific mast cell degranulation, we assessed active cutaneous anaphylaxis in skin responses to OVA challenge at day 21 and day 42. Data are summarized in table 1. At day 21 strong OVA-specific mast cell degranulation and bluing were observed in intraperitoneally immunized wt mice, whereas knockout mice showed only slight bluing. This weak but reproducible reaction is probably due to IgG1-dependent mast cell degranulation [18], since IgG1 was already detectable at that time (fig. 1a). At day 42 all mice displayed strong bluing.

We analyzed whether OVA-specific IgE produced in IL-4^{-/-} and IL-4Rα^{-/-} mice could induce systemic aller-

gic symptoms after intravenous OVA challenge. Table 2 shows that at day 42 all OVA-sensitized mice developed anaphylactic symptoms upon challenge, which led to a severe shock 15 min after injection. There was no significant difference in the severity of symptoms between wt and knockout animals. Shock symptoms were absent in unsensitized mice. These results indicate that local and systemic anaphylaxis due to allergen-specific mast cell degranulation can be achieved independently from IL-4 or IL-4Rα.

OVA-Induced Airway Eosinophilia and Goblet Cell Metaplasia Are Absent in IL-4^{-/-} and IL-4Rα^{-/-} Mice

In addition, we investigated whether long-term intranasal OVA stimulation could also induce asthma-like lung pathology. Mice which had been sensitized by weekly

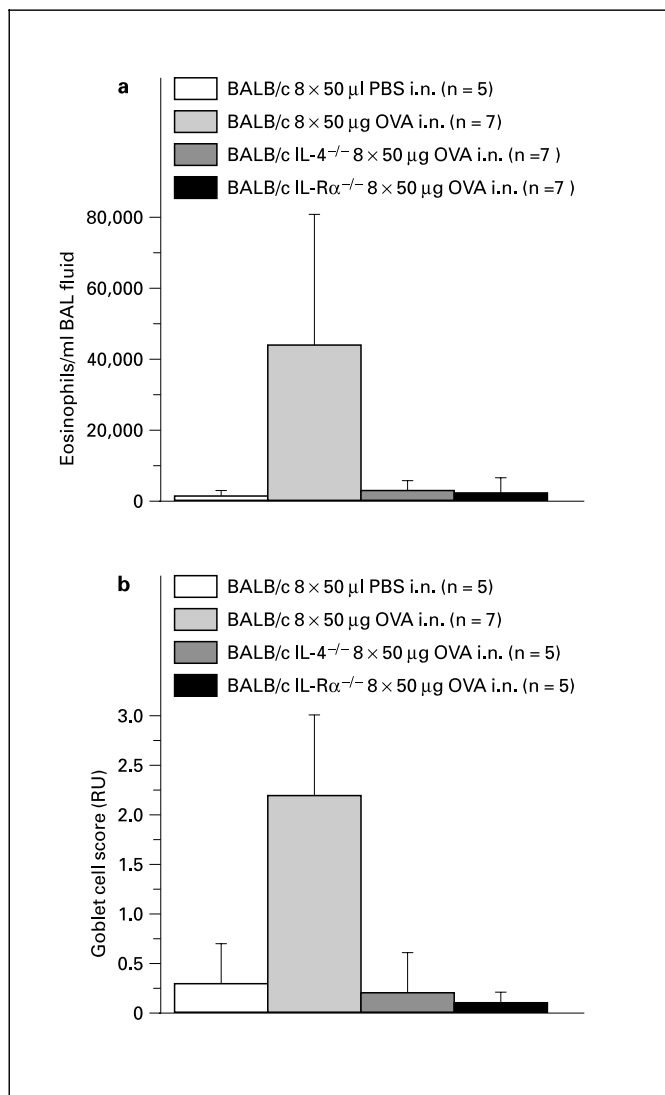


Fig. 2. OVA-induced airway eosinophilia and goblet cell metaplasia. **a** Mice which had been sensitized by weekly intranasal applications of OVA were challenged with OVA intranasally at day 42 and 43 and BALs were performed at day 44. The total amount of eosinophils was determined in the lavage fluid. **b** After intranasal sensitization and OVA challenge lung tissue was removed, fixed in formalin and assessed for goblet cell metaplasia. The density of goblet cells (0–3) is given as relative units (RU).

intranasal applications of OVA were challenged with OVA intranasally at days 42 and 43 and BALs were performed on day 44. In another group of mice lung tissue was removed and assessed for goblet cell metaplasia and inflammatory infiltrates.

wt mice developed strong airway eosinophilia after 6 weeks of OVA application (fig. 2a). In contrast, both IL-4^{-/-} and IL-4Rα^{-/-} mice failed to show eosinophils in the BAL fluid over background levels found in unsensitized mice.

Massive goblet cell metaplasia (fig. 2b) and an inflammatory infiltrate dominated by eosinophils (not shown) were found in wt mice. This allergic lung pathology was completely absent in both IL-4^{-/-} and IL-4Rα^{-/-} mice.

We conclude that long-term and repeated sensitization with the protein antigen OVA induces specific IgE and IgG1 synthesis independently from IL-4 and IL-4Rα in BALB/c mice. These antibodies can mediate local and systemic allergic symptoms. In contrast, allergic lung pathology like influx of eosinophils and goblet cell metaplasia seems to depend on IL-4 receptor signaling.

Discussion

In this study we provide the first evidence that allergen-specific IgE synthesis can occur independently of IL-4 and IL-13. We also show that IgE produced independently of IL-4Rα plays a pathophysiological role in allergy, since anaphylaxis could be induced similarly in IL-4Rα-deficient mice and wt animals. Long-term sensitization is required for IL-4- and IL-4Rα-independent IgE production, since 10 days [16] or 21 days (this study) after the first contact with the allergen no specific IgE was detected.

IgE synthesis has been observed previously in the absence of IL-4Rα or STAT6, but with uncertain relevance to atopic disease. Low but significant IgE levels have been found in a study where IL-4Rα^{-/-} mice were infected with *N. brasiliensis* [3]. Infection of STAT6-deficient mice with the MAIDS virus induces levels of total IgE similar to those found in wt mice [19]. Since STAT6 is an important component for intracellular signaling through the shared IL-4 and IL-13 receptor [20], and IL-4 and IL-13 functions are impaired or abolished in STAT6^{-/-} mice [21–23], this finding also points to IL-4- and IL-13-independent IgE production. However, the possibility of IL-4 and IL-13 signaling via the STAT6-independent IL-4 receptor signaling (IRS) pathway [20] could not be excluded in STAT6-deficient mice.

Our results raise the question how IgE can be induced in the absence of stimulation via IL-4Rα. CD40 signaling may be a possible mechanism for IL-4-independent IgE production. Injection of agonistic anti-CD40 antibodies can induce serum IgE in IL-4^{-/-} mice, and purified B cells

isolated from these mice produce sterile I ϵ transcripts when stimulated with anti-CD40 in vitro [24]. A further possibility would be new, unidentified cytokines or receptor subunits in the IL-4/IL-13 system. However, since lymphocytes and monocytes derived from IL-4R $\alpha^{-/-}$ mice are unresponsive to IL-4 or IL-13 [6], we consider it unlikely that productive signaling by these cytokines occurs in the absence of IL-4R α .

In agreement with recent reports we found that mucus overproduction and lung eosinophilia are completely dependent on IL4R α even after long-term OVA stimulation [25, 26]. The finding that allergic lung pathology is lacking even when allergen-specific IgE is present supports previous reports showing that airway hyperresponsiveness and airway eosinophilia occur independently from IgE synthesis in IgE-deficient mice [27]. This is underlined by the fact that blockade of IL-13 affects airway hyperresponsiveness but not IgE levels [25, 28]. This suggests that the asthmatic phenotype – at least in the OVA mouse model – can be mediated by direct effects of IL-4 and IL-13 through IL-4R α signaling in the asthmatic lung parenchyma, also in the absence of specific IgE production.

In conclusion, we found that long-term sensitization with protein antigen could induce specific IgE and IgG1 synthesis independently of IL-4 and IL-13. These IgE antibodies were produced in large quantities and mediated local and systemic allergic symptoms to the same degree as observed in wt animals. Since long-term antigen exposure is a frequent condition during the development of allergy, the observation that allergen-specific IgE can be induced independently of IL-4 and IL-13 may therefore be relevant for atopic disorders in humans.

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