

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Prenatal allergen exposures prevent allergen-induced sensitization and airway inflammation in young mice

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Keywords

airway inflammation; allergen sensitization; prenatal immune-modulation; regulatory T cells; tolerance induction.

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Abstract

Background: Immune-modulation such as tolerance induction appears to be an upcoming concept to prevent development of atopic diseases. Pregnancy might present a critical period for preventing allergic sensitization of the progeny. We investigated the effect of maternal allergen exposures during pregnancy on allergen-induced sensitization and airway inflammation in the offspring in a murine model.

Methods: BALB/c mice were exposed to aerosolized ovalbumin (OVA) three times per week from day 7 of pregnancy until delivery (day 0). Offspring were systemically sensitized by six intraperitoneal injections with OVA between postnatal days 21 and 35, prior to airway allergen challenges on days 48, 49, and 50. Analyses were performed on day 52. To examine long-lasting effects of maternal OVA exposures some offspring were sensitized between days 115 and 129; analyses took place on day 147.

Results: Compared to maternal placebo exposures, maternal OVA exposures suppressed OVA-specific IgE serum levels and inhibited development of allergen-induced airway inflammation in the OVA-sensitized offspring on both days 52 and 147. This protective effect was associated with a shift from a predominant Th2 immune response toward a predominant production of the cytokines IFN- γ and IL-10. Further, maternal OVA exposures were associated with development of CD25⁺Foxp3⁺ regulatory T cells (T_{regs}) in the OVA-sensitized offspring. Depletion of T_{regs} or neutralization of IL-10 prior to allergen sensitization re-established OVA-induced sensitization and eosinophilic airway inflammation in the OVA-sensitized offspring.

Conclusions: In our model, maternal allergen exposures during pregnancy prevented later allergen-mediated sensitization and airway inflammation by allergen-specific tolerance induction in the offspring.

It is widely accepted that the development of atopic diseases often originates in early infancy (1–3), and it is intriguing to hypothesize that very early events may provide particularly promising targets for novel concepts of primary prevention against allergy and asthma.

The maternal status of atopy has a strong effect on the development of allergic sensitization of the children (4). Beyond genetic determinants, maternal-fetal interaction at the

placental barrier such as transfer of allergens (5) or antibodies (6) may prime the fetal immune system to develop sensitization or tolerance. In accordance, animal models have shown that maternal predominant T helper 2 (Th2) immune responses enhance allergen-mediated Th2 immune responses in the offspring (7), and that maternal allergen-specific T cells transmit increased asthma risk to the offspring, most likely via placental transfer of cytokines (8). Moreover, we found that prenatal

initiated exposure to an unspecific immune modulator, lipopolysaccharides (LPS), prevented allergen-mediated sensitization and airway inflammation in young mice (9).

The natural mechanism to acquire immune tolerance most likely starts with priming of local dendritic cells by environmental antigens via mucosal barriers of the gastrointestinal or respiratory tract (10, 11). In the present study we therefore investigated whether allergen-specific mucosal tolerance induction by means of repetitive airway allergen exposures during pregnancy prevents allergen-induced sensitization and airway inflammation in the very young offspring in a short-term prevention model. Possible long-lasting effects of maternal allergen exposures during pregnancy on allergen-induced sensitization and inflammatory responses were in addition examined in the older offspring in a long-term prevention model.

Material and methods

Animals

Female BALB/c mice, 10 weeks of age, were purchased from Harlan Winkelmann, Borcheln, Germany; male BALB/c, 4 weeks of age, were kindly provided by Alf Hamann, Charité, Berlin, Germany, and Thy-1.1⁺ male BALB/c, 4 months of age, were kindly provided by Ute Hoffmann, Charité, Berlin, Germany. All mice were kept under specific pathogen-free conditions, and were maintained on an ovalbumin (OVA)-free diet. Breeding was performed in our animal experimental institute. All experimental procedures were approved by the local authorities.

Experimental protocols

Maternal OVA exposures during pregnancy

Pregnant mice were exposed to aerosolized OVA (10 mg/ml, Grade V; Sigma, St. Louis, MO, USA) for 20 min three times per week from day 7 of gestation time (day –14 of the protocol) until delivery (day 0). Pregnant controls were placebo-exposed to aerosolized phosphate buffer saline (PBS; Sigma).

Allergen sensitization and airway challenges of the offspring in the short-term prevention model

The offspring were systemically sensitized by six intraperitoneal (i.p.) injections of 10 µg OVA (Grade VI; Sigma) between days 21 and 35. Respective controls were placebo-sensitized with PBS. On days 48, 49, and 50, mice of all groups were exposed to aerosolized OVA (Grade V, 10 mg/ml, 20 min/day) prior to tests on day 52 (9).

Allergen sensitization and airway challenges of the offspring in the long-term prevention model

Some offspring were systemically sensitized with OVA between postnatal days 115 and 129 prior to OVA airway challenges on days 143, 144, and 145. Analyses were performed on day 147. The same protocol was used as for young animals for direct comparisons between short-term and long-term effects of maternal OVA exposures.

Treatment with rat anti-mouse IL-10R or rat anti-mouse CD25 monoclonal antibodies (mAbs)

Offspring were treated i.p. either with 500 µg anti-CD25 (clone PC61.5) or 500 µg anti-IL-10R (clone 1B1.3a) 2 days prior to the first and fourth systemic OVA sensitizations. Respective controls received placebo treatment with polyclonal rat immunoglobulin (Ig)G (Sigma).

Study groups

Study groups are named by two codes according to maternal exposures (PBS_{mat}, OVA_{mat}) and sensitization procedures in offspring (PBS_{offsp}, OVA_{offsp}, OVA_{offsp-late}). If (m)Abs were applied prior to sensitization, groups are named by maternal exposure/(m)Ab treatment/sensitization procedure in offspring.

Measurement procedures

All the following measurements are described in detail in Data S1.

Immunoglobulin (Ig) serum levels

Serum levels of total and OVA-specific Ig were measured by means of ELISA.

Broncho-alveolar lavage (BAL)

In BAL fluids cells were counted and differentiated due to morphological criteria; levels of IL-5 and IL-12 were assessed by means of ELISA.

Allergen-specific cellular reactivity *in vitro*

Allergen-specific cellular reactivity of spleen MNCs (mononuclear cells) cultured with OVA *in vitro* was determined by ³H-thymidine incorporation.

In vitro cytokine production

Levels of IL-5, IL-10, IL-13 and IFN-γ in supernatants of spleen MNCs cultured with OVA *in vitro* were assessed by means of ELISA.

Flow cytometry

Flow cytometry was used to assess frequencies of maternal regulatory T cells (T_{regs}) in spleens on postnatal day 21, frequencies of T_{regs} in bronchial lymph nodes in the offspring on day 52, and frequencies of T_{regs} in spleens, bronchial lymph nodes, and lung tissues in the offspring of Thy-1.1⁺ male and Thy-1.2⁺ female mice on day 52.

Statistical analysis

Statistical analyses were conducted using PASW statistics, version 18 (IBM SPSS® Statistics; SPSS Inc, Chicago, IL, USA). A minimum of three and up to 12 animals per group

were included in each experiment; experiments were repeated up to four times; results of each complete set of experiments are presented herein. Values for all measurements are expressed as median and the 25th and 75th percentile. Pairs of treatment groups were compared using Mann–Whitney-*U*-test and statistical significance was set at $P < 0.05$ (two-sided). For analyses with two comparisons per group (compared with PBS_{mat}/PBS_{offsp} and with PBS_{mat}/OVA_{offsp}), statistical significance was set to $P < 0.025$ due to Bonferroni correction for multiple testing.

Results

Short-term prevention model

First, we investigated whether maternal OVA exposures during pregnancy affected allergen-mediated sensitization and airway inflammation in the offspring on postnatal day 52 (Fig. 1). Of first note is that maternal OVA exposures did not induce any OVA-specific IgE productions in mothers

(data not shown). Further, maternal OVA exposures did not lead to elevated OVA-specific IgE serum levels or significant eosinophilic airway inflammation in non-sensitized OVA_{mat}/PBS_{offsp} offspring (Fig. 1A–C). Compared to PBS_{mat}/PBS_{offsp} mice, PBS_{mat}/OVA_{offsp} offspring developed highly increased IgE serum levels (Fig. 1A), a robust eosinophilic airway inflammation (Fig. 1B,C), and significantly increased IL-5 levels in BAL fluids (Fig. 1D).

In contrast, maternal OVA exposures almost completely suppressed OVA-induced sensitization shown by diminished OVA-specific IgE serum levels (Fig. 1A) and airway inflammatory responses in the offspring (Fig. 1B–D). This preventive effect of maternal OVA exposures was allergen-specific (see Data S1).

Long-term prevention model

In our long-term prevention model maternal OVA exposures prior to late postnatal sensitization (OVA_{mat}/OVA_{offsp-late}) markedly reduced OVA-specific IgE levels, inhibited develop-

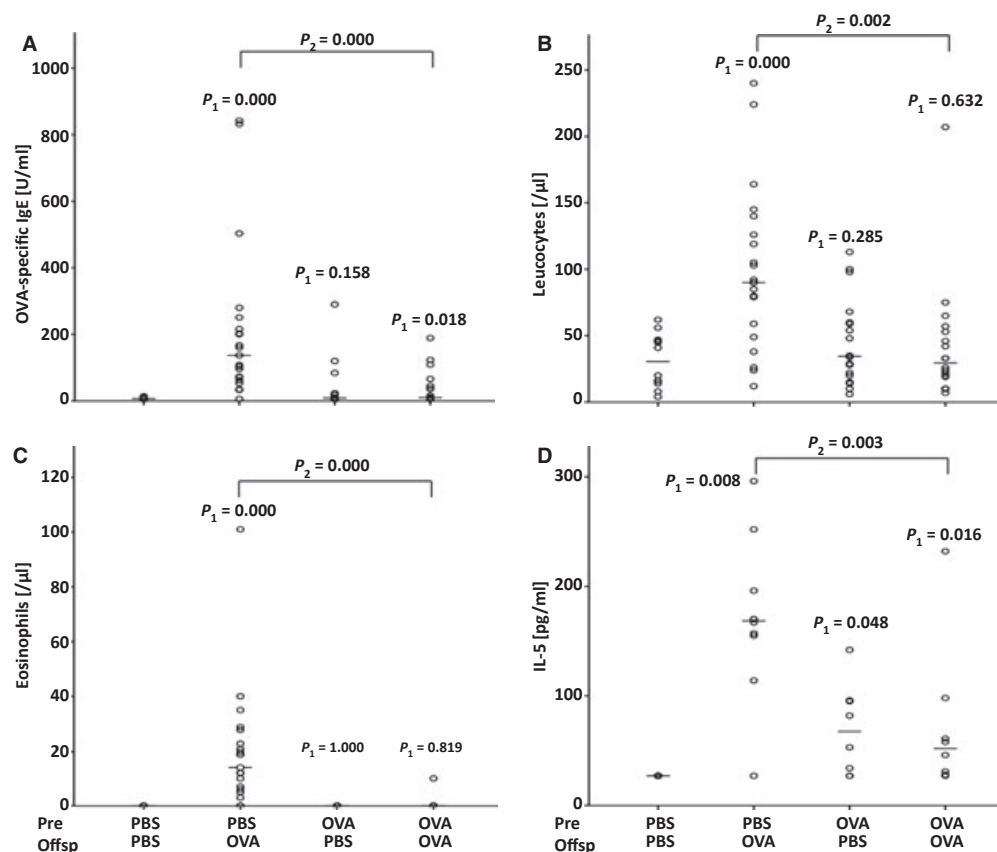


Figure 1 Short-term prevention model. Following ovalbumin (OVA) exposures during pregnancy, offspring were sensitized (days 21–35) and challenged with OVA. On day 52, serum levels of OVA-specific IgE (A), the cellular influx into the airways (B/C), and local IL-5 levels in broncho-alveolar lavage (BAL) fluids (D) were analyzed. Statistical significance was set at $P < 0.025$ (numbers of

animals per group: for OVA-specific IgE and BAL leukocytes and eosinophils n (PBS_{mat}/PBS_{offsp}) = 12, n (PBS_{mat}/OVA_{offsp}) = 21, n (OVA_{mat}/PBS_{offsp}) = 18, n (OVA_{mat}/OVA_{offsp}) = 18; for BAL IL-5 n (PBS_{mat}/PBS_{offsp}) = 6, n (PBS_{mat}/OVA_{offsp}) = 12, n (OVA_{mat}/PBS_{offsp}) = 9, n (OVA_{mat}/OVA_{offsp}) = 8; p_1 vs PBS_{mat}/PBS_{offsp}, p_2 vs PBS_{mat}/OVA_{offsp}, Mann–Whitney-*U* test).

ment of eosinophilic airway inflammation, and diminished levels of IL-5 in BAL fluids, compared to maternal placebo exposures (PBS_{mat}/OVA_{offsp}-late) (Table 1). Thus, maternal OVA exposures during pregnancy exhibited long-lasting preventive effects on allergen-induced sensitization and airway inflammation.

Cytokines in the short-term prevention model

On postnatal day 52 we examined cytokine secretion patterns of splenic MNCs of the offspring, cultured with OVA (Fig. 2A–D). In OVA_{mat}/PBS_{offsp} mice IL-10 production was slightly increased compared to that of PBS_{mat}/PBS_{offsp} mice (Fig. 2C,D). Compared to OVA-sensitized (PBS_{mat}/OVA_{offsp}) mice, maternal OVA exposures during pregnancy suppressed IL-5 and IL-13 productions (Fig. 2A/B) and induced a shift from predominant OVA-specific Th2 cytokines toward predominant productions of IFN- γ (Fig. 2C) and IL-10 (Fig. 2D) in the OVA_{mat}/OVA_{offsp} offspring.

IL-10 and T_{regs} in the short-term prevention model

Since maternal OVA exposures slightly induced IL-10 production in the non-sensitized offspring and suppressed production of Th2 cytokines, but not of IL-10 in the OVA-sensitized offspring, we hypothesized that maternal OVA exposures induced IL-10 producing T_{regs} in the offspring. Indeed, following systemic sensitization and airway allergen challenges numbers of T_{regs} in bronchial lymph nodes of OVA_{mat}/OVA_{offsp} mice were significantly increased compared to numbers of T_{regs} in both, PBS_{mat}/PBS_{offsp} or PBS_{mat}/OVA_{offsp} mice on postnatal day 52 (Fig. 3C).

The T_{regs} in OVA_{mat}/OVA_{offsp} mice might have been transferred from mothers, particularly via the placenta. Thus, we first investigated frequencies of T_{regs} and IL-10 production in OVA-exposed mothers after the breastfeeding period on postnatal day 21. Compared to placebo-exposed mothers, OVA-exposed mothers showed highly increased frequencies of CD4⁺CD25⁺Foxp3⁺ T_{regs} in spleens (Fig. 3A) and

increased IL-10 production by spleen MNCs, cultured with OVA *in vitro* (Fig. 3B).

To evaluate whether T_{regs} were transferred from mothers or developed most likely in the offspring, we mated Thy-1.1⁺ male with Thy-1.2⁺ female BALB/c mice and measured frequencies of CD4⁺Foxp3⁺ T_{regs} in spleens from offspring by flow cytometry. Only a minority of about 7% up to 12% of T_{regs} were single-positive for Thy-1.2⁺ and therefore of maternal origin without any strong difference between OVA_{mat}/PBS_{offsp} and OVA_{mat}/OVA_{offsp} mice (Table 2). But higher frequencies of fetal T_{regs} of OVA_{mat}/OVA_{offsp} mice were positive for IL-10, compared to fetal T_{regs} of PBS_{mat}/OVA_{offsp} mice (Table 2); these T_{regs} were not double positive for IL-10 and IFN- γ (Fig. 3D). Thus, in OVA_{mat}/OVA_{offsp} mice, most T_{regs} were of fetal, not of maternal origin, and frequencies of IL-10-producing T_{regs} of fetal origin were higher in the OVA_{mat}/OVA_{offsp} than those in the PBS_{mat}/OVA_{offsp} group.

Role of IL-10 and T_{regs} in the short-term prevention model

Next, we investigated whether OVA-sensitized offspring of OVA-exposed mothers developed immunological tolerance, which is known to be mediated by IL-10 and T_{regs} (12) and to result in T cellular non-reactivity. Compared to maternal placebo exposures, maternal OVA exposures were associated with strongly suppressed reactivity of spleen cells to OVA on day 52 confirming a state of immunological tolerance in OVA-sensitized offspring (Fig. 4A).

Furthermore, protective effects of maternal OVA exposures on allergen-induced sensitization (Fig. 4B), and airway eosinophilia (Fig. 4C/D) were abolished after depletion of CD25 in OVA_{mat}/anti-CD25_{offsp}/OVA_{offsp} mice or after blocking of the function of IL-10 in OVA_{mat}/anti-IL10R_{offsp}/OVA_{offsp} mice. In addition, IL-5 levels in BAL fluids of OVA_{mat}/anti-CD25_{offsp}/OVA_{offsp} mice [97.5 pg/ml (68;154 pg/ml), $p_1 = 0.000$, $p_2 = 0.469$, $n = 8$] or of OVA_{mat}/anti-IL10R_{offsp}/OVA_{offsp} mice [131 pg/ml (93;169 pg/ml), $p_1 = 0.000$, $p_2 = 0.165$, $n = 8$] were as high as respective levels of PBS_{mat}/IgG_{offsp}/OVA_{offsp} [78 pg/ml (24;153 pg/ml),

Table 1 Long-lasting preventive effects of maternal ovalbumin (OVA) exposures

Group	n	Serum			BAL fluids								
		OVA-specific IgE			Leukocytes			Eosinophils			IL-5		
		[U/ml]	p ₁	p ₂	[/μl]	p ₁	p ₂	[/μl]	p ₁	p ₂	[pg/ml]	p ₁	p ₂
PBS _{mat} / PBS _{late-offsp}	6	28 (28; 32)			34 (22; 45)			0 (0; 0)			3 (3; 11.5)		
PBS _{mat} / OVA _{late-offsp}	8	287 (132; 473)	0.002		67 (42; 86)	0.020		18 (8; 27)	0.004		20 (11.5; 20.5)	0.009	
OVA _{mat} / OVA _{late-offsp}	7	28 (28; 131)	0.703	0.009	24 (16; 35)	0.252	0.004	0 (0; 1)	0.015	0.011	3 (3; 10)	0.745	0.005

Following OVA exposures during pregnancy, offspring were sensitized (days 115–129) and challenged with OVA prior to analysis on day 147. Medians (25; 75 percentile) for IgE serum levels, cell counts and IL-5 levels in BAL fluids are stated; statistical significance was set at $P < 0.025$ (p_1 vs PBS_{mat}/PBS_{offsp}, p_2 vs PBS_{mat}/OVA_{offsp}, Mann–Whitney–U test).

BAL, broncho-alveolar lavage.

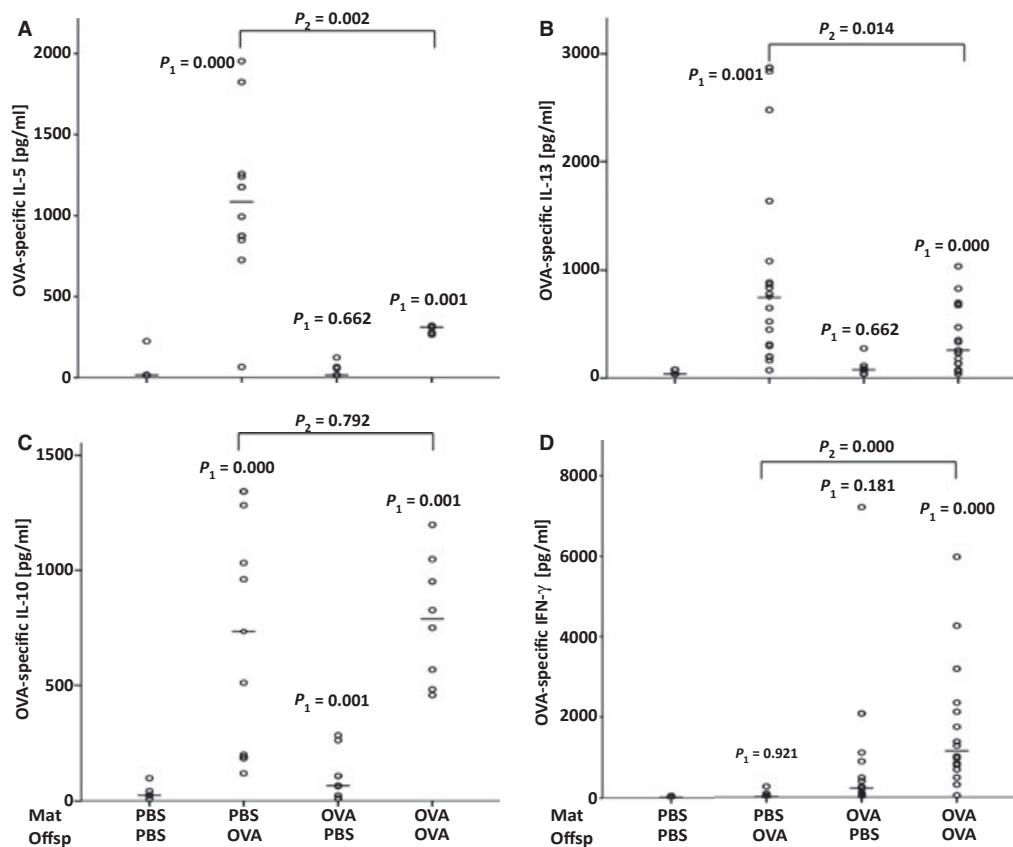


Figure 2 Cytokines in the short-term prevention model. Following maternal ovalbumin (OVA) exposures, offspring were sensitized (days 21–35) and challenged with OVA. On day 52, levels of IL-5 (A), IL-13 (B), IL-10 (C), and IFN-γ (D) were measured in supernatants of spleen cells cultured with OVA *in vitro*. Statistical significance was set at $P < 0.025$ [numbers of animals per group: for

IL-13 and IFN-γ n (PBS_{mat}/PBS_{offsp}) = 12, n (PBS_{mat}/OVA_{offsp}) = 21; n (OVA_{mat}/PBS_{offsp}) = 18, n (OVA_{mat}/OVA_{offsp}) = 18; for IL-5 and IL-10 n (PBS_{mat}/PBS_{offsp}) = 6; n (PBS_{mat}/OVA_{offsp}) = 12, n (OVA_{mat}/PBS_{offsp}) = 9, n (OVA_{mat}/OVA_{offsp}) = 8; p_1 vs PBS_{mat}/PBS_{offsp}, p_2 vs PBS_{mat}/OVA_{offsp}, Mann–Whitney–U test].

$p_1 = 0.002$, $n = 20$] and therefore significantly increased compared to PBS_{mat}/IgG_{offsp}/PBS_{offsp} mice [20 pg/ml (20;21 pg/ml), $p_1 = 0.002$, $n = 10$] or OVA_{mat}/IgG_{offsp}/OVA_{offsp} mice [20 pg/ml (20;65 pg/ml), $p_1 = 0.546$, $p_2 = 0.012$, $n = 14$]. Since either CD25⁺ T_{regs} or IL-10 had to be present during the postnatal sensitization period, we assumed that protective effects of maternal OVA exposures were a major function of immunological tolerance, which, as shown above, developed most likely in the offspring.

Discussion

The need of effective and safe concepts for primary prevention against allergic diseases has opened a controversial discussion about the outcome of prenatal immune-modulation with bacterial components and probiotics (13, 14), dietary factors (15, 16) or allergens (17) on the postnatal development of immune responses. We have shown herein that maternal allergen exposures during pregnancy inhibited two major characteristics of allergic airway disease in the offspring, namely the development of both allergen-induced IgE production and eosinophilic airway inflammation. This

protective effect was allergen-specific and long-lasting and associated with induction of immunological tolerance in the OVA-sensitized offspring. Development of increased airway reactivity in OVA-sensitized and airway-challenged offspring was not affected by maternal OVA exposures during pregnancy (data not shown), showing that it is regulated in complex ways, independently from Th2 cytokines and airway inflammation (18).

In additional experiments to investigate the role of humoral immune responses for prevention of allergen-mediated sensitization and inflammation (see Data S1) we found that maternal OVA exposures enhanced serum levels of OVA-specific IgG and IgG1 in mothers as well as in the offspring, demonstrating that blocking antibodies might carry the protective effect at least in the short-term prevention model. Indeed, high levels of allergen-specific IgG antibodies have been found to prevent allergen-induced sensitization in the offspring (7, 19) and to be associated with less development of atopic symptoms in children at 8 years of age (20). Furthermore, as it was recently demonstrated for infectious antigens (21), allergens might have primed fetal B cells to develop into allergen-specific memory B cells that mediate prevention of

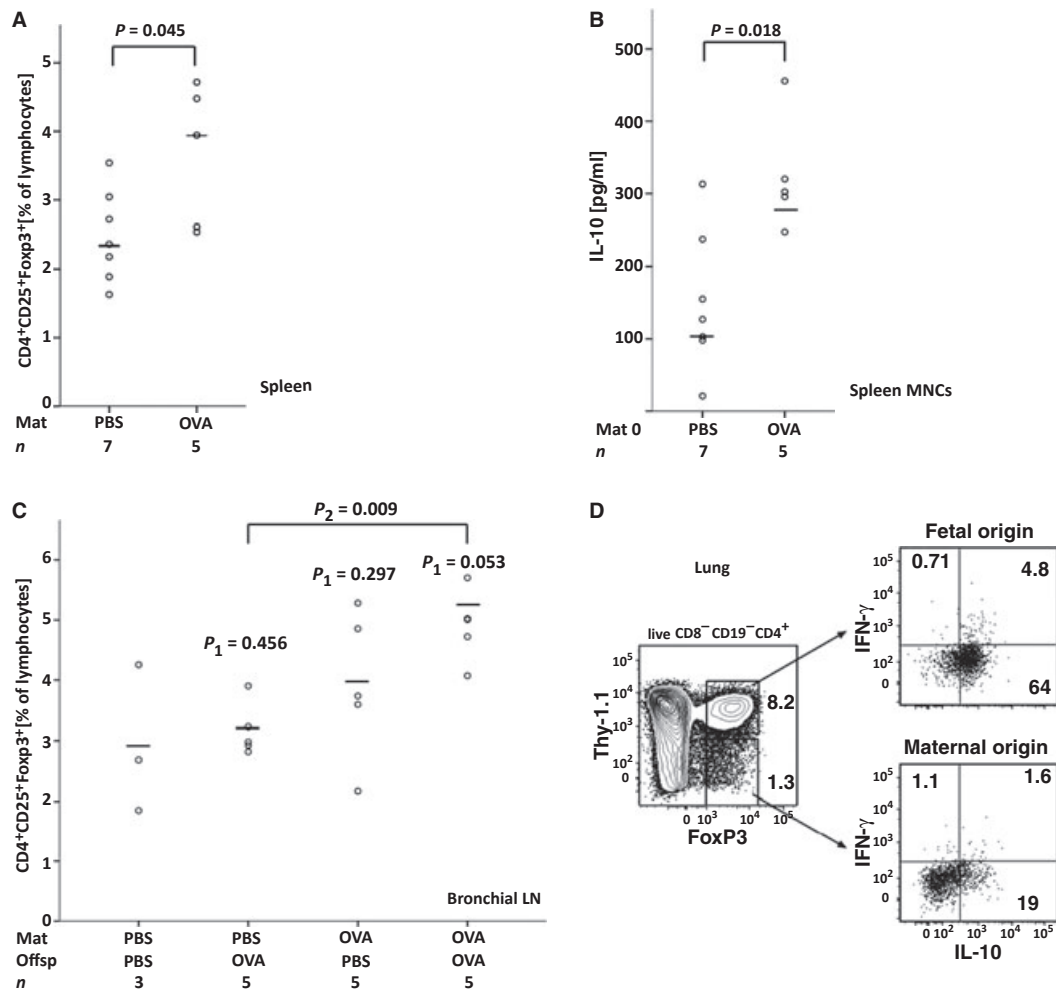


Figure 3 Regulatory T cells (T_{regs}) in ovalbumin (OVA)-exposed mothers splenic T_{regs} (A) and OVA-specific IL-10 production (B) were quantified on day 21; statistical significance was set at $P < 0.05$ (P vs PBS_{mat} , Mann-Whitney- U test). T_{regs} in bronchial

lymph nodes of OVA-sensitized offspring were counted (C), and IL-10- and IFN- γ -producing lung T_{regs} of maternal and fetal origin were analyzed in OVA-sensitized offspring of Thy-1.1 $^{+}$ male and Thy-1.2 $^{+}$ female mice (D).

Table 2 Maternal and fetal origin of T_{regs}

Group	Bronchial lymph node ($n = 3$)		Lung ($n = 6$)		Spleen ($n = 6$)		Spleen ($n = 3$)
	CD4 $^{+}$ Foxp3 $^{+}$ (% of CD4 $^{+}$)	Thy-1.2 $^{+}$ cells (% of CD4 $^{+}$ Foxp3 $^{+}$)	CD4 $^{+}$ Foxp3 $^{+}$ (% of CD4 $^{+}$)	Thy-1.2 $^{+}$ cells (% of CD4 $^{+}$ Foxp3 $^{+}$)	CD4 $^{+}$ Foxp3 $^{+}$ (% of CD4 $^{+}$)	Thy-1.2 $^{+}$ cells (% of CD4 $^{+}$ Foxp3 $^{+}$)	Fetal IL-10 $^{+}$ cells (% of CD4 $^{+}$ FoxP3 $^{+}$)
$\text{PBS}_{\text{mat}}/\text{OVA}_{\text{offsp}}$	12.2 (9.5)	11.5 (9.75)	9.4 (7.2; 11.4)	7.7 (6.5; 8.8)	10.8 (9.2; 12.33)	13 (12.4; 13.2)	7.6 (5.6)
$\text{OVA}_{\text{mat}}/\text{OVA}_{\text{offsp}}$	12.2 (9.45)	10.6 (9.6)	11.7 (10.4; 13.7)	6.3 (5.9; 7.8)	9.4 (7.2; 11.4)	12 (11.5; 12.4)*	10.8 (8.3)*

Thy-1.1 $^{+}$ male and Thy-1.2 $^{+}$ female BALB/c mice were mated. In OVA-sensitized offspring of OVA-exposed mothers T_{regs} of maternal (Thy-1.2 $^{+}$) and fetal (Thy-1.1 $^{+}$ Thy-1.2 $^{+}$) origin were differentiated on postnatal day 52 (median (25;75 percentile)). *Statistical significance was set at $P < 0.05$ (vs $\text{PBS}_{\text{mat}}/\text{OVA}_{\text{offsp}}$; Mann-Whitney- U test). In $\text{OVA}_{\text{mat}}/\text{OVA}_{\text{offsp}}$ mice 36.6% (32.3) of all T_{regs} were IL-10 $^{+}$, in $\text{PBS}_{\text{mat}}/\text{OVA}_{\text{offsp}}$ mice 32.2% (27.5).

OVA, ovalbumin; T_{regs} , regulatory T cells.

later allergen sensitization in the offspring. Since (i) OVA-specific IgG antibodies declined in the non-sensitized offspring exponentially, (ii) maternal OVA exposures suppressed

allergen sensitization and airway inflammation despite very low levels of OVA-specific IgGs in the offspring in our long-term prevention model, and (iii) OVA-specific IgG1 anti-

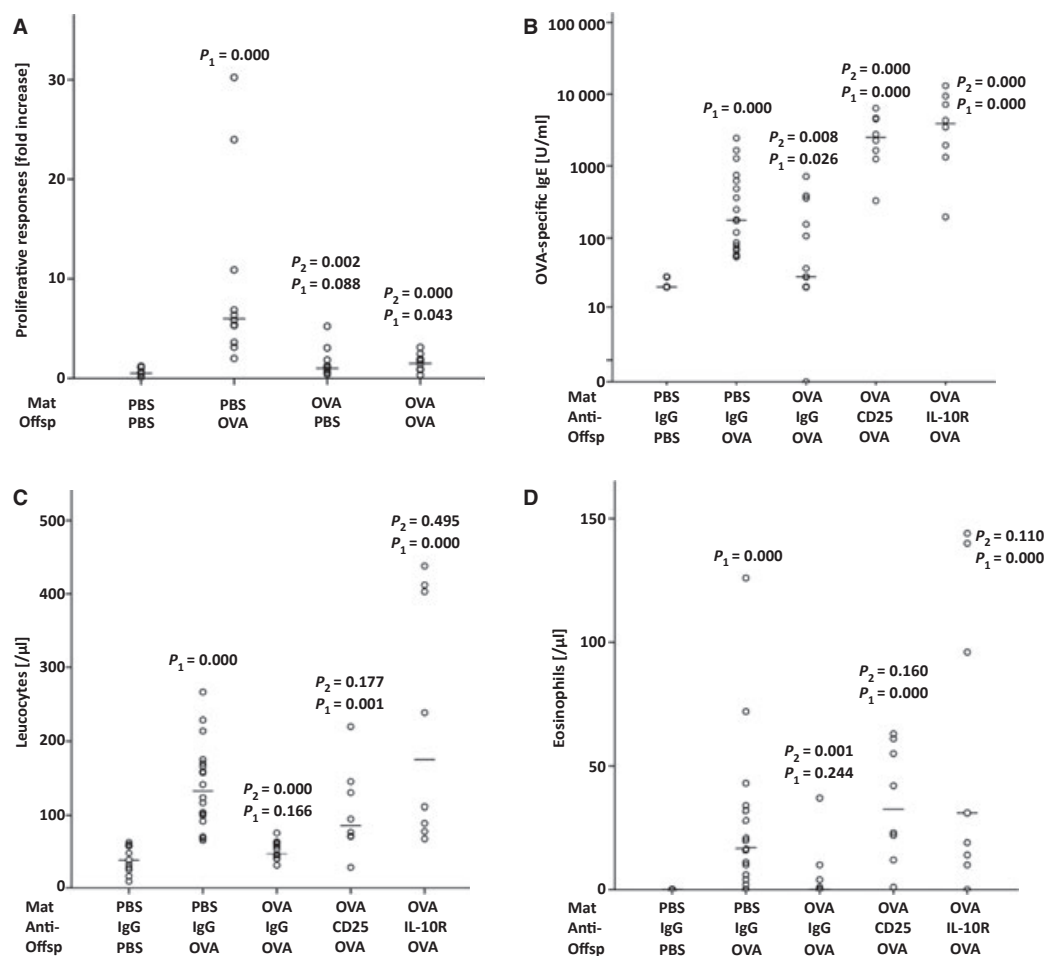


Figure 4 Role of regulatory T cells. Offspring of ovalbumin (OVA)-exposed mothers were sensitized and challenged with OVA. On day 52, OVA-specific cellular reactivity by spleen MNCs was analyzed (A). Following treatment with mAbs against CD25 or IL-10R prior to and during OVA sensitization, OVA-specific IgE serum levels (B) and airway inflammation (C,D) were examined on day 52. Statistical significance was set at $P < 0.025$ [numbers of animals per group: for proliferative responses n (PBS_{mat}/PBS_{offsp}) = 6, n

(PBS_{mat}/OVA_{offsp}) = 12, n (OVA_{mat}/PBS_{offsp}) = 8, n (OVA_{mat}/OVA_{offsp}) = 8; p_1 vs PBS_{mat}/PBS_{offsp}, p_2 vs PBS_{mat}/OVA_{offsp}, Mann-Whitney- U test; numbers of animals per group for OVA-specific IgE serum levels and for leukocytes and eosinophils in broncho-alveolar lavage fluids n (PBS_{mat}/IgG/PBS_{offsp}) = 10, n (PBS_{mat}/IgG/OVA_{offsp}) = 18, n (OVA_{mat}/IgG/OVA_{offsp}) = 14, n (OVA_{mat}/anti-CD25/OVA_{offsp}) = 8, n (OVA_{mat}/anti-IL-10R/OVA_{offsp}) = 8; p_1 vs PBS_{mat}/IgG/PBS_{offsp}, p_2 vs PBS_{mat}/IgG/OVA_{offsp}, Mann-Whitney- U test].

body-producing plasma cells were not detectable by means of sensitive ELISPOT techniques (22) in the non-sensitized offspring of OVA-exposed mothers (see all results in Data S1), the protective long-lasting effect was most likely not merely a function of transferred maternal protective IgG antibodies or memory B cells.

Here, maternal OVA exposures were associated with suppressed OVA-specific Th2 cytokines, and a predominant production of the Th1 cytokine IFN- γ in the offspring. IFN- γ is well known to suppress Th2-mediated allergen-induced sensitization and airway inflammatory responses (23, 24). On the other hand IFN- γ is also said to act in a pro-inflammatory way in allergen-mediated airway disease (25, 26). IFN- γ -inducing LPS that contaminates commercially available OVA (27), which was used for maternal exposures, did not alone explain the protective effects on allergen-mediated immune

responses in the offspring in our short-term prevention model (see methods and results in detail in the Data S1).

Further, IFN- γ was recently shown to play a pivotal role in the materno-fetal transfer of tolerance (28). But since the protective effect of maternal OVA exposures could only be partly abolished by neutralization of IFN- γ prior to and during sensitization (see methods and results in detail in Data S1), Th1 immune responses played a distinct role in our prevention model.

Allergen-induced airway inflammation was shown to be prevented by CD4⁺ T cells producing both cytokines, IFN- γ and IL-10 (29). Since maternal OVA exposures were associated with induction of the regulatory cytokine IL-10 in the OVA-sensitized young mice, IL-10 might have been a second major mediator, needed for prevention of allergen-induced airway inflammation in our model. Indeed, inhibiting IL-10

function with IL10R-blocking antibodies prior to and during allergen sensitization re-established both allergen-mediated sensitization and eosinophilic airway inflammation in OVA-sensitized offspring of OVA-exposed mothers.

In accordance with data from the literature (30), repeated allergen exposures via the airways induced mucosal immunological tolerance in mothers, characterized by elevated frequencies of T_{regs} and IL-10 production. Thus, transfer of tolerance via the placenta or after delivery via the breast milk could have been a key mechanism for prevention of allergen-mediated sensitization and airway inflammation in the offspring (31–33). Since the protective effect of maternal OVA exposure in our model was allergen-specific, it was most unlikely mediated by simple soluble factors, such as cytokines or chemokines transferred via the placenta. Transfer of T_{regs} from the mother to the fetus via the placenta was conceivable, but T_{regs} in the prenatally OVA-exposed and then OVA-sensitized offspring were mostly of fetal origin. Since IL-10 production by spleen MNCs cultured with OVA *in vitro* was enhanced in prenatal OVA-exposed postnatal non-sensitized mice, and fetal T_{regs} were induced to produce IL-10 in prenatal OVA-exposed postnatal sensitized mice, we assumed that maternal allergen exposures during pregnancy led to direct priming of fetal immune cells to potential T_{regs} in the presence of the maternal cytokine milieu. Thus, with the subsequent allergen contact during postnatal sensitization, immunological tolerance developed in the offspring in the presence of IL-10 and $CD25^{+}$ T cells prior to and during allergen sensitization.

In conclusion, repeated maternal allergen exposures during pregnancy-mediated expansion of T_{regs} in the offspring induc-

ing allergen-specific tolerance and inhibiting the development of allergen-induced sensitization and airway inflammation. Prenatal mucosal tolerance induction might thus provide an innovative concept for primary prevention of atopic diseases, at least in high-risk families.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Processed methods and their results.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

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