

Early Life Interventions to Prevent Allergy in the Offspring: The Role of Maternal Immunization and Postnatal Mucosal Allergen Exposure

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Key Words

Allergy · Maternal immunization · Mouse model · Mucosa · Offspring · Prevention · Sensitization · Tolerance

Abstract

Background: Childhood allergy is influenced by maternal factors and allergen exposure in early life, but the factors that determine the development of allergy and tolerance are unknown. Therefore, we compared the effects of two early life interventions, i.e. maternal allergen immunization and postnatal intranasal allergen exposure, as well as a combination of both treatments on allergic responses in the offspring. **Methods:** Female mice were immunized with ovalbumin (OVA) or vehicle during pregnancy. After birth, half the offspring from each group were exposed intranasally to low doses of OVA or vehicle weekly for 5 weeks before intraperitoneal immunization with OVA. **Results:** Maternal immunization reduced OVA-specific IgE and IgG1, but increased IgG2a and T_H2 cytokine responses in the offspring after immunization. Postnatal intranasal OVA exposure similarly reduced both IgE and IgG1, but also spleen cell numbers and cytokine secretion. Following airway challenges of the offspring, IgE and airway inflammation were suppressed only by intranasal exposure, but not by maternal immunization, the effect of which also waned with age. Differential gene

expression in the spleen of offspring supported that IgE suppression by the two interventions was caused by different mechanisms. Despite this, tolerance development after mucosal exposure was obtained in the offspring of immunized dams. **Conclusions:** The study suggests that prevention of allergy is possible if initiated in early life, and early mucosal allergen exposure may play a protective role independent of the maternal immune responses.

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Introduction

Allergic diseases often manifest during early childhood. Apart from genetic determinants, maternal rather than paternal factors are more influential on the risk for atopic disease [1], but the mechanisms responsible are only partly understood [2]. Programming of the immune system may start already in utero and perinatal environmental exposure may shape the ability to respond to challenges later in life [3, 4]. The child's immune system may be influenced by maternal factors (transferred via placenta and/or milk), e.g. maternal allergen-specific IgG [5], leukocytes [6] and cytokines, such as TGF- β [7]. However, it is still unknown how maternal immunity interacts with early life inhalant and dietary allergen expo-

sure of the offspring [8, 9] to affect allergy and tolerance development. Clarification of these processes is necessary for the development of primary prevention strategies.

Prevention of allergy has been investigated in mouse models. First, we and others have shown that manipulation of the maternal immune response by allergen immunization during pregnancy reduces allergen-specific IgE production in offspring after immunization [10–13]. Second, mucosal delivery of allergen to naïve animals induces an active immune response characterized by suppression of allergen-specific IgE and T_H2-mediated inflammatory responses [14, 15], but this has rarely been performed in neonates [16, 17]. Induction of such mucosal tolerance simulates the processes occurring in healthy individuals, who do not develop adverse IgE-mediated reactions. In newborns, where the maturing immune system encounters foreign proteins [18], postnatal mucosal allergen exposure has been proposed as a strategy to induce allergen-specific tolerance [19].

Maternal allergen immunization and mucosal allergen exposure have led to allergen-specific IgE suppression in mice in separate experimental studies. The aim of our study was to compare the two prevention strategies directly and, therefore, elucidate if different mechanisms may cause this IgE suppression. Importantly, by combining the treatments in a factorial design, we investigated how a maternal allergen-specific immune response affected offspring mucosal tolerance development. We evaluated the effects of the interventions in different age groups of offspring both before and after allergen immunization and airway challenge. Serum antibody, airway inflammation and splenocyte cytokine responses were evaluated as well as spleen cell gene expression.

Materials and Methods

Mice

Seven-week-old female and 9-week-old male NIH/OlaHsd mice (Harlan Ltd., UK) were acclimatized for 1 week. Pregnant females were housed individually. Offspring were weaned at 3 weeks of age and housed 3–4 mice per cage. Older males were housed individually to avoid aggressive behavior. Mice were provided the 2018X diet (Harlan Teklad, UK) and tap water ad libitum. The 2018X diet is free of animal protein, including ovalbumin (OVA). Mice were exposed to a 12-hour light/dark cycle (30–60 lx in cages), regulated room temperature ($20 \pm 2^\circ\text{C}$) and 40–60% relative humidity. The experiments were approved by the Institutional Animal Care and Use Committee (Norwegian Institute of Public Health) and performed in conformity with the Norwegian laws and regulations and the European Convention guideline ETS123 on the care and use of experimental animals.

Allergen, Adjuvant and Anesthetics

Chicken egg OVA, grade VII, was obtained from Sigma-Aldrich, USA. The Al(OH)₃ adjuvant (alhydrogel) was from Brenntag Biosector, Denmark. Pentobarbital NAF (Ås Production Lab, Norway) was given intraperitoneally at a dose of 500 mg/kg; 3.5% isoflurane was administered in surgical O₂. The ZRF anesthesia contains 18.7 mg zolazepam, 18.7 mg tiletamine, 0.45 mg xylazine and 2.6 µg fentanyl per milliliter, and mice were administered 0.1 ml/10 g i.p.

Experimental Protocol

Figure 1 shows the layout of the experiment. Synchronization of estrus and mating were performed according to Whitten [20]. The day of observation of a vaginal plug was designated day 1. On day 3, female mice were immunized subcutaneously with 10 µg OVA and 2 mg Al(OH)₃ in Hanks' balanced salt solution (HBSS) in a 0.2-ml bolus. On day 10 of gestation, they were boosted with 1 µg OVA in 0.2 ml HBSS s.c. Control dams were injected 0.2 ml HBSS s.c. A blood sample was taken from the vena saphena lateralis before mating and on day 10 during lactation. After weaning, the chest was opened and blood drawn by heart puncture under ZRF anesthesia.

Ten to 11 days after birth, 12 pups from 9 immunized (IMM) and 10 control (CTRL) dams, respectively, were terminally anesthetized with pentobarbital. Blood was drawn by heart puncture and spleens were excised.

The remaining offspring were allocated to intranasal exposure to either HBSS or 5 µg OVA as described in table 1. The solutions were placed consecutively on both nostrils. The exposure volumes chosen were based on the results published by Southam et al. [21] and on body weight to allow optimal lung deposition. Offspring were exposed weekly from 10 days of age until age 5 weeks, when 1–2 HBSS- or OVA-exposed pups from each of 10 IMM and 11 CTRL dams were anesthetized with the ZRF cocktail. Blood was drawn by heart puncture and spleens were collected.

At age 6 weeks, the remaining HBSS- and OVA-exposed offspring from 10 IMM and 11 CTRL dams were immunized with 10 µg OVA and 2 mg Al(OH)₃ i.p. in HBSS in a 0.4-ml bolus. Two weeks later, offspring were boosted with 10 µg OVA in 0.4 ml HBSS i.p. and euthanized 1 week later. Under ZRF anesthesia, blood was drawn by heart puncture and spleens were collected.

In a separate experiment, offspring from CTRL and IMM dams were treated with OVA or HBSS intranasally and immunized intraperitoneally as described above. At age 9 weeks, they received airway challenges with 10 µg OVA in 35 µl HBSS 1 week after the booster immunization. Challenges were given intranasally on 3 consecutive days under isoflurane anesthesia, and mice were euthanized 3 days later. Blood and bronchoalveolar lavage (BAL) were collected under ZRF anesthesia.

In further experiments, no postnatal intranasal OVA was administered. Offspring of CTRL and IMM dams were intraperitoneally immunized at 1 or 6 weeks of age, booster immunized 2 weeks later as described above and euthanized when 4 or 9 weeks old. Also, naïve mice from CTRL and IMM dams were kept until 20 weeks old, when a blood sample was collected from the vena saphena lateralis. Then, mice were intraperitoneally immunized and euthanized as described above.

Fig. 1. Experimental design: dams were control- or ovalbumin (OVA)-immunized (CTRL/IMM) subcutaneously on days 3 and 10 of gestation. Blood was collected from dams before mating, during lactation and at euthanasia. Offspring from the same litters were allocated to euthanasia, blood and tissue collection at 1, 5 or 9 weeks. 5- and 9-week-old offspring were exposed intranasally to either OVA or HBSS (vehicle). All 9-week-old offspring were immunized intraperitoneally with OVA when 6 and 8 weeks old. In a separate experiment, offspring of CTRL or IMM dams were allocated to OVA immunization beginning at the age of 1 or 6 weeks (early immunization). In another experiment, the offspring were 20 weeks old when receiving the first immunization (late immunization).

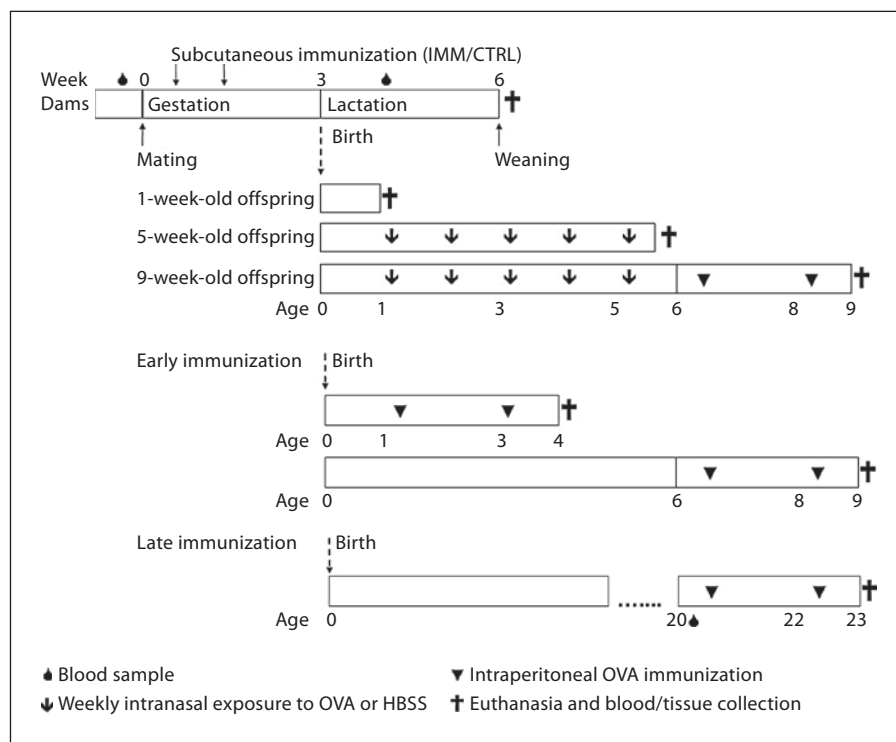


Table 1. Intranasal exposure of offspring

Age of offspring	OVA dose	Intranasal volume	Anesthesia
10 days	5 µg	5 µl	no
2–3 weeks	5 µg	10 µl	3.5% isoflurane
4–5 weeks	5 µg	25 µl	3.5% isoflurane

OVA-Specific Antibodies

OVA-specific IgE antibodies were detected in a capture ELISA as previously described [22], while OVA-specific IgG2a antibodies were determined using indirect ELISA as described by Nygaard et al. [23]. Poly-HRP-streptavidin (Thermo-Scientific, Pierce Biotechnology, Inc., USA) and subsequently stabilized chromogen TBM (Invitrogen, USA) were used for detection in both assays, and stopped with 2 N H₂SO₄ solution. OVA-specific IgG1 was determined in a capture ELISA as previously described [23]. The sera to be tested were analyzed in duplicate following optimal dilution: 1:10 (IgE in dams before mating), 1:50 (IgE and IgG2a in dams and offspring), 1:200 (IgG1 in dams and non-immunized older offspring) or 1:200,000 (IgG1 in newborn and immunized offspring). For all antibody assays, a standard curve was included on each plate. Standard curves were made from duplicates of diluted IgE standard (mouse anti-OVA IgE, AbD Serotec) or serum pools from mice immunized with OVA and Al(OH)₃ for IgG1 or

OVA and CpG oligonucleotides for IgG2a. Since the amount of IgG1 or IgG2a in the standards is unknown, it is given in arbitrary units (AU). The lower detection limits of the IgG assays depend on the dilution factor and are only shown when values below them were found. Optical density was measured at 450 nm on an MRX microplate reader (Dynatech Laboratories, USA) connected to a PC using Revelation software (Thermo Labsystems, USA).

Spleen Cell Preparation and Determination of OVA-Stimulated Cytokine Release

To prepare single-cell suspensions of spleens, the spleen was forced through a 70-µm cell strainer (BD Labware, USA). The cells were washed and counted in a Coulter counter (Z1). After incubation in culture medium (RPMI 1640 with 10% fetal calf serum, 100 U penicillin G and 0.1 mg/ml streptomycin) with or without 1 mg/ml OVA at 37°C and 5% CO₂ for 5 days, the supernatants were removed and the levels of IL-4, IL-5, IL-10 and IFNγ were determined using BD CBA mouse soluble protein flex sets measured on a BD LSR II cytometer and analyzed by the FCAP array software (all from BD Biosciences, USA) according to the manufacturer's protocol. In all analyses, IL-4 was undetectable or measured at only barely detectable levels. IL-4 is therefore not described further in the text.

Real-Time Quantitative PCR

Total RNA was isolated from frozen spleen cells using the Qia-gen Allprep mini kit (Qiagen GmbH, Germany) according to the manufacturer's protocol. Twenty genes were selected based on their involvement in allergy and T_H2/T_H1 commitment [24] and for their association with atopic phenotypes in several indepen-

Table 2. Detection of OVA-specific antibodies in dams

	CTRL	IMM	p value
IgE, ng/ml			
Before mating	0/11 (ND)	0/10 (ND)	NA
Lactation, day 10	0/11 (ND)	10/10 ($569 \pm 1,867$)	<0.001
At weaning	0/11 (ND)	10/10 ($3,672 \pm 2,896$)	<0.001
IgG1, AU			
Before mating	0/11 (ND)	0/10 (ND)	NA
Lactation, day 10	0/11 (ND)	10/10 ($3.57 \times 10^8 \pm 3.69 \times 10^8$)	<0.001
At weaning	0/11 (ND)	10/10 ($2.76 \times 10^8 \pm 3.04 \times 10^8$)	<0.001
IgG2a, AU			
At weaning	$8.68 \times 10^4 \pm 3.60 \times 10^4$	$4.36 \times 10^5 \pm 1.02 \times 10^6$	<0.001

ND = Not detectable as values were below the detection limit; NA = not applicable. The number of responders in each group is given along with median and interquartile ranges. p values from Fisher's exact test on counts are given, except for IgG2a, where the Mann-Whitney U test was used. n = 10–11.

dent studies, making them candidates for susceptibility genes in allergy and asthma [25]. Intron-spanning quantitative PCR assays were designed using the Universal ProbeLibrary Assay Design Center (Roche). Assay efficiencies were calculated using five 4-fold serial dilutions of spleen cDNA. Six genes (*Il4*, *Il5*, *Runx3*, *Cd81*, *Cd28* and *Adam33*) did not pass our efficiency criteria threshold (between 0.90 and 1.1) and were excluded from further analysis.

Real-time quantitative PCR was performed essentially as described by Deutsch et al. [26] and Lyle et al. [27], with minor modifications. Each gene was amplified in 3 replicates per individual. Raw cycle threshold (C_T) values were obtained using SDS 2.4 software (Applied Biosystems), and C_T values were converted to expression values (q) with the formula $q = 2^{-C_T}$. Expression values were normalized by performing a median normalization across all genes, excluding the 75th percentile. Each gene was then median normalized across individuals. Normalized relative expression values thus have a median of 1.

Evaluation of Airway Inflammation

Cells from the BAL fluid were prepared and colored with the Hemacolor rapid staining of blood smear kit (Merck KGaA, Germany) as described previously [23]. Cell differential counting of blinded slides was performed by the same investigator (J.S.H.).

Study Design and Statistical Analyses

To avoid litter effects, offspring from the same litter were allocated to different treatments, intranasal HBSS or OVA exposure, and euthanized at 1, 5 or 9 weeks of age. Thus, maximally 1 male and 1 female sibling were used at the same time points and individual pups were, therefore, regarded as independent observations. The study was performed as a factorial design and statistical analyses were performed by the general linear model (GLM) procedure with maternal treatment (CTRL/IMM), intranasal exposure (HBSS/OVA) and sex (male/female) as fixed factors. In some analyses, age was used as a fixed factor. Data were logarithmically, square root or rank transformed if necessary in order to obtain

homoscedasticity and normal distribution of the residuals. Statistically significant main and interaction effects are reported in conjunction with the figures. It should be noted that the main effects arise from including data from several subgroups and therefore cannot easily be depicted on figures where all subgroups are shown. A significant main effect of intranasal exposure, for example, implicates that all groups designated HBSS are significantly different from all the groups designated OVA and that the effect is independent of the effects of the other fixed factors. Interaction effects are reported with '×', e.g. 'maternal treatment × sex' implies that the effect of maternal treatment varied between the female and male mice. In case significant interactions were found, these were evaluated statistically by post hoc analysis to enable interpretation of the interaction by identifying which subgroups are significantly different. Post hoc tests were performed by Tukey's method and the differences between subgroups shown (letters a–c or brackets). For the interpretation of significant interactions, it was also investigated if the interaction effects were ordinal or disordinal, but these data are not shown. For parts of the study, Fisher's exact test was used to compare frequencies of non-responder/responders, one-way ANOVA or two-sample t test to compare group means and Mann-Whitney's U test to compare group medians when the data did not conform to the assumptions of parametric statistics. Statistical analyses were performed in Minitab version 15. The level of significance was $p < 0.05$.

Results

Immunization during Gestation Induced Antibody Production in Dams

Pregnant mice were OVA- or sham-immunized during pregnancy (fig. 1). Before mating, no OVA-specific IgE or IgG1 could be detected in serum (table 2). As expected, OVA-specific IgE and IgG1 was detected only in

Table 3. Endpoints in 1-week-old naïve offspring

	CTRL	IMM	p value
IgE, ng/ml	0/12 (ND)	0/12 (ND)	NA
IgG1, AU	0/12 (ND)	12/12 ($1.85 \times 10^{10} \pm 8.2 \times 10^9$)	<0.001
IgG2a, AU	1/12 (ND)	7/12 ($191,500 \pm 94,750$)	0.03
IFN γ , pg/ml			
Unstimulated	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	NA
OVA stimulated	29.4 $\pm$ 37.4	46.3 $\pm$ 61.4	NS ^a
Total spleen cells	$20.15 \times 10^6 \pm 8.14 \times 10^6$	$25.57 \times 10^6 \pm 14.16 \times 10^6$	NS ^b

ND = Not detectable as values were below the detection limit; NA = not applicable; NS = not significant. The number of responders in each group is given along with the median and interquartile range. p values from Fisher's exact test on counts are given, except for IFN γ and number of spleen cells, where the GLM procedure was used to test for effects of sex and maternal treatment (mean $\pm$ SD). n = 12 (6 males and 6 females).

^a Maternal treatment, p = 0.44; sex, p = 0.80, maternal treatment $\times$ sex, p = 0.55.

^b Maternal treatment, p = 0.26; sex, p = 0.85, maternal treatment $\times$ sex, p = 0.13.

IMM and not in sham-immunized (CTRL) dams during lactation and at weaning (table 2). OVA-specific IgG2a was analyzed only at weaning, and the levels were significantly higher in IMM than CTRL dams (table 2).

Effects of Maternal Immunization on 1-Week-Old Naïve Offspring

No OVA-specific IgE was detectable in the serum of 1-week-old naïve offspring (fig. 1, table 3). The number of pups with OVA-specific IgG1 and IgG2a was significantly higher when born from IMM versus CTRL dams (table 3). Offspring spleen cells were stimulated with OVA ex vivo. IL-5 and IL-10 levels were barely detectable (data not shown), and neither IFN γ secretion nor spleen cell numbers were significantly affected (table 3).

Effects of the Interventions on 5-Week-Old Offspring

Offspring of CTRL or IMM dams were exposed intranasally to 5 μ g OVA or HBSS once weekly for 5 weeks starting at age 10 days (fig. 1). To evaluate the effects of postnatal intranasal OVA exposure, serum antibodies were analyzed at 5 weeks of age. No OVA-specific IgE was detectable and OVA-specific IgG2a was not influenced by the maternal treatment, intranasal exposure or sex (data not shown). A significant interaction effect of maternal treatment and intranasal exposure was found for OVA-specific IgG1 (fig. 2). In offspring from CTRL dams, intranasal exposure to OVA increased OVA-specific IgG1 production (fig. 2a vs. b). However, IgG1 levels were still significantly lower than in offspring from IMM dams (fig. 2b vs. c). HBSS- and OVA-exposed offspring from

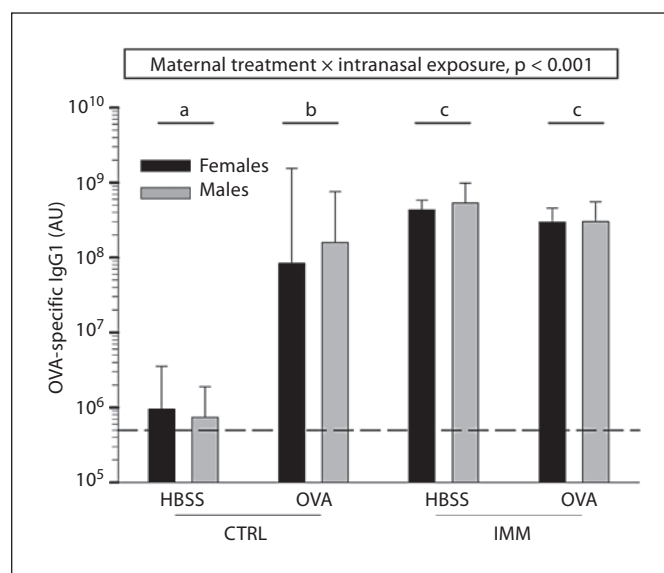


Fig. 2. Effects of maternal immunization and intranasal OVA exposure on OVA-specific IgG1 levels in 5-week-old offspring. Offspring of control- or OVA-immunized (CTRL/IMM) dams were allocated to intranasal exposure to either OVA or HBSS (vehicle) weekly for 5 weeks, where after OVA-specific IgG1 was measured in serum. Medians and interquartile ranges for n = 10 females or males. Broken line indicates the lower detection limit of the ELISA. Statistically significant main and interaction effects are noted above the figure. Significant interactions were subjected to post hoc analyses and significant differences between subgroups are shown on the top, with b being significantly higher than a, and c significantly higher than a and b.

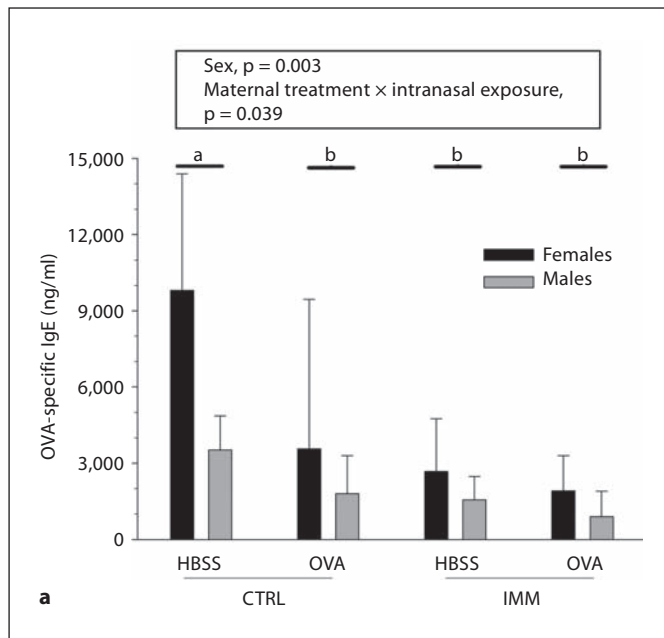
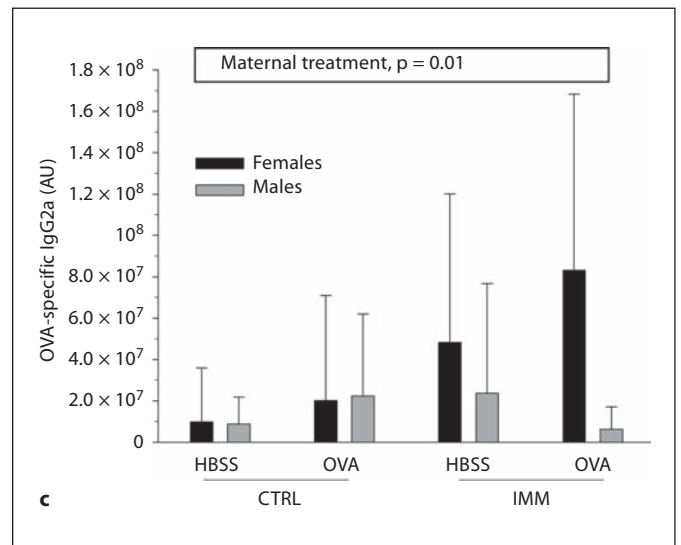
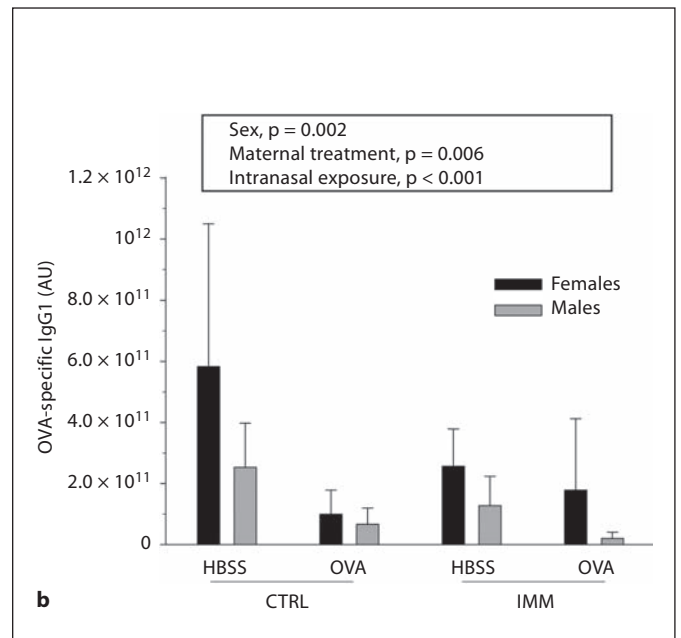


Fig. 3. Effects of maternal immunization and intranasal OVA exposure on antibody responses in 9-week-old offspring after intraperitoneal immunization. Offspring of control- or OVA-immunized (CTRL/IMM) dams were allocated to intranasal exposure to either OVA or HBSS (vehicle) weekly for 5 weeks. All offspring were immunized intraperitoneally, and OVA-specific IgE (**a**), IgG1 (**b**) and IgG2a (**c**) levels were measured in 9-week-old offspring. Statistically significant main and interaction effects are noted on the top of each figure. The significant interaction in **a** was subjected to post hoc analyses, and significant differences between subgroups are shown on the top of the figure, with b being significantly lower than a. Mean and SD of $n = 9$ –10 females or males are presented.



IMM dams did not differ, indicating that in the presence of already high levels of circulating OVA-specific IgG1, IgG1 levels were not further increased by intranasal OVA exposure. Antibody levels did not differ between males and females.

Spleen cell numbers and IL-10 and IFN γ secretion from stimulated spleen cells were not significantly affected (data not shown). Although IL-5 secretion was altered by the treatments, the levels were low and not easily interpretable (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000332963).

Antibody Production in 9-Week-Old Immunized Offspring

Following cessation of intranasal exposure, the offspring were intraperitoneally immunized with OVA at 6 and 8 weeks of age and euthanized at 9 weeks of age (fig. 1). The effects of maternal immunization and post-natal intranasal OVA challenge were investigated on several end points.

The influence of the offspring's sex was clearly reflected by serum levels of OVA-specific IgE and IgG1 as males had significantly lower levels than females (fig. 3a, b). Fe-

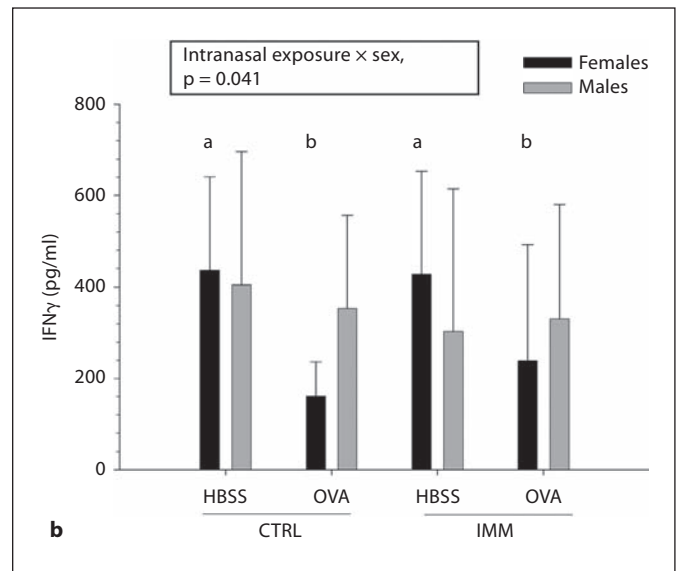
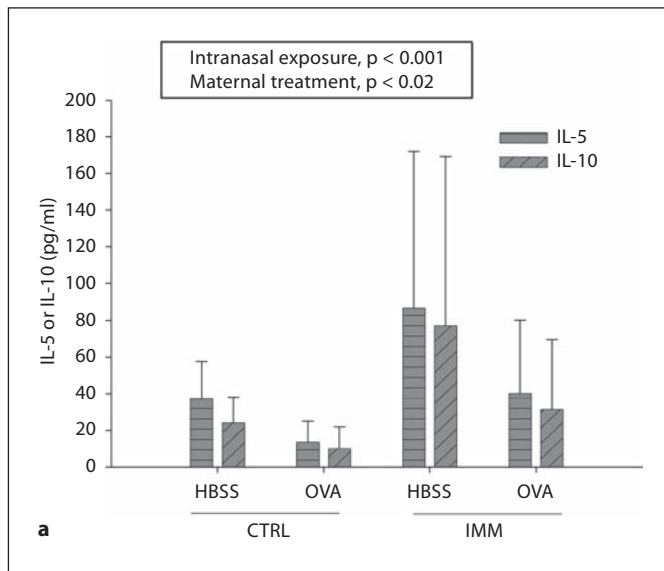
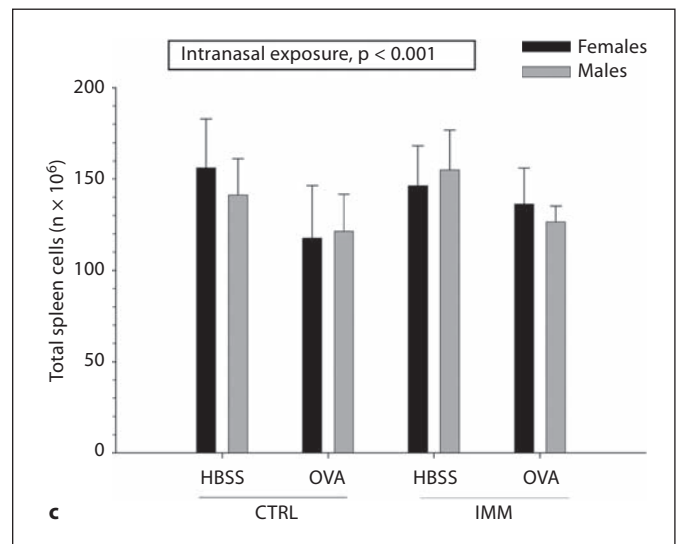


Fig. 4. Effects of maternal immunization and intranasal OVA exposure on spleen cell responses in 9-week-old offspring after intraperitoneal immunization. Offspring of control- or OVA-immunized (CTRL/IMM) dams were allocated to intranasal exposure to either OVA or HBSS (vehicle) weekly for 5 weeks. Then, all offspring were immunized intraperitoneally and spleen cell OVA-stimulated cytokine release and splenocyte numbers assessed. **a** IL-5 and IL-10 secretion ($n = 19$ – 20 mice). No effect of sex was observed, so male and female data are combined. The range of corresponding means for non-stimulated wells was 1.5–3.5 pg/ml for IL-5 and 1.4–2.2 pg/ml for IL-10. **b** IFN γ secretion ($n = 9$ – 10 females or males). The range of corresponding means for non-stimulated wells was 1.5–5.6 pg/ml. **c** Total number of spleen cells ($n = 9$ – 10 females or males). Statistically significant main and interaction effects are noted above the figure. Significant interactions were subjected to post hoc analyses and significant differences between subgroups are shown in the figures, so groups denoted b are significantly lower than groups denoted a. Means and SD are presented.



males had a non-significant tendency to higher IgG2a production than males (main effect of sex, $p = 0.07$, fig. 3c). No sex-treatment interactions were found indicating that the treatments affected antibody production comparably in both sexes.

Both maternal immunization and intranasal exposure reduced OVA-specific IgE levels following immunization of offspring, and a significant interaction between these treatments was found (fig. 3a). The post hoc test revealed that offspring from CTRL dams exposed intranasally to HBSS had significantly higher IgE levels than those exposed to OVA and all offspring from IMM dams. Apparently, maternal OVA immunization and intrana-

sal OVA exposure reduced IgE production to comparable levels, thus, the effect of intranasal OVA exposure was lower in offspring from IMM dams than from CTRL dams (fig. 3a, a vs. b, and ordinal interaction, data not shown). Both the maternal OVA immunization and intranasal OVA exposure significantly reduced OVA-specific IgG1 levels (fig. 3b). Thus, postnatal intranasal OVA exposure reduced OVA-specific IgG1 in offspring from both CTRL and IMM dams. OVA-specific IgG2a levels were only affected by maternal treatment (fig. 3c); IMM offspring had significantly higher IgG2a levels than CTRL offspring.

Spleen Cell Responses in 9-Week-Old Immunized Offspring

In contrast to the reduced T_H2 -associated antibody production, maternal OVA immunization significantly increased both IL-5 and IL-10 secretion following ex vivo stimulation of spleen cells with OVA. In contrast, intranasal OVA exposure significantly reduced both IL-5 and IL-10 secretion (fig. 4a). These effects were independent of sex. A statistically significant interaction between intranasal exposure and sex on IFN γ secretion was observed (fig. 4b). The post hoc test showed that only in females did the intranasal OVA exposure reduce IFN γ secretion (fig. 4b, a vs. b). Comparable to the inhibitory effect on cytokine production, the total number of spleen cells in the immunized offspring was reduced due to postnatal intranasal OVA exposure (fig. 4c).

mRNA Expression in Spleen Cells from 9-Week-Old Immunized Offspring

To investigate if the effects from the two interventions could be caused by different gene-regulatory pathways, mRNA expression of selected genes in spleen cells from the 9-week-old immunized offspring was investigated. The relative gene expression of *Il-2 α* (*Cd25*), *Ifn γ* , *Cd28*, *Lta*, *Ctla-4*, *Il-10*, *Il-13*, *Stat6*, *Il-4 α* , *T-bet*, *Tnf α* , *Foxp3*, *Tgf β* , *Ifn γ α* and *Gata3* was determined, but no significant differences were observed for the *Il-2 α* , *Ifn γ* and *Cd28* genes. Significant interactions that were difficult to interpret biologically were found for *Foxp3*, *Tgf β* , *Ifn γ α* and *Gata3* and are not discussed (online suppl. fig. 2A). The same applies to significant main and interaction effects associated with the sex of the offspring (online suppl. fig. 2B). Significant main and interaction effects of the treatments were observed on *T-bet*, *Tnf α* , *Il-4 α* , *Il-10*, *Il-13*, *Stat6*, *Lta* and *Ctla-4* gene expression. For clarity, the data in figure 5 are grouped according to significant effects. The relative expression of *T-bet*, *Tnf α* and *Il-4 α* (in females) was significantly increased by maternal immunization (fig. 5a). Intranasal OVA exposure decreased *Il-10*, *Il-13* and *Stat6* and increased *Il-4 α* relative expression (fig. 5b). A significant interaction between maternal immunization and intranasal exposure was found for *Lta* and *Ctla-4* (fig. 5c). The post hoc tests revealed that OVA-exposed offspring from CTRL dams and all offspring from IMM dams had significantly lower expression compared to HBSS-exposed offspring from CTRL dams. The nature of the interaction revealed that *Lta* expression was reduced by intranasal exposure in offspring of both CTRL and IMM dams (as found for OVA-specific IgG1). Intranasal exposure did not reduce *Ctla-4* expression further in offspring from IMM

dams (as found for OVA-specific IgE; ordinal and disordinal interaction, respectively, data not shown).

Airway Inflammation in 9-Week-Old Immunized Offspring

To assess effects on allergic airway inflammation, the airways of 9-week-old offspring treated as above (fig. 1) were subsequently challenged with OVA 1 week after the booster immunization. Three days later, serum antibody levels and airway inflammation were evaluated. After airway challenge, there was no significant effect of maternal immunization on OVA-specific IgE or IgG1, while OVA-specific IgG2a was still increased (fig. 6a–c). In contrast, all antibodies were significantly decreased following intranasal OVA exposure. Macrophage, lymphocyte, neutrophil and epithelial cell numbers in BAL fluid were unaffected by the treatments (data not shown), but eosinophil inflammation was significantly decreased following intranasal OVA exposure only (fig. 6d). Thus, while intranasal exposure still had a suppressive effect, maternal immunization had no significant effect on allergy-related outcome when the airways of the offspring were challenged with OVA.

Suppressive Response Caused by Maternal Immunization Depends on the Age of the Offspring

Several studies [12, 28–31] have demonstrated that maternal allergen-specific IgG, in particular IgG1, may cause suppression of allergen-specific IgE responses in the offspring. If maternal antibodies have this effect, then IgE suppression should wane after weaning when maternal IgG decreases in the offspring's circulation. This was investigated in two separate experiments. In one experiment (early immunization in fig. 1), offspring of IMM and CTRL dams were OVA-immunized intraperitoneally at 1 or 6 weeks of age, booster immunized 2 weeks later and euthanized when 4 or 9 weeks old, respectively. Comparable numbers of females and males were used, but there were too few animals to statistically assess the effect of sex. Although OVA-specific IgE and IgG1 production significantly increased with increasing age (fig. 7a, b), the suppressive effect of maternal OVA immunization was stronger in offspring immunized already during lactation; here, no OVA-specific IgE or IgG1 was detectable. Overall, IgG2a levels did not increase with age, but a significant interaction between maternal treatment and age was found (fig. 7c). The post hoc test showed that while IgG2a was undetectable in young IMM offspring (and not significantly different from CTRL offspring), it was significantly increased in old IMM off-

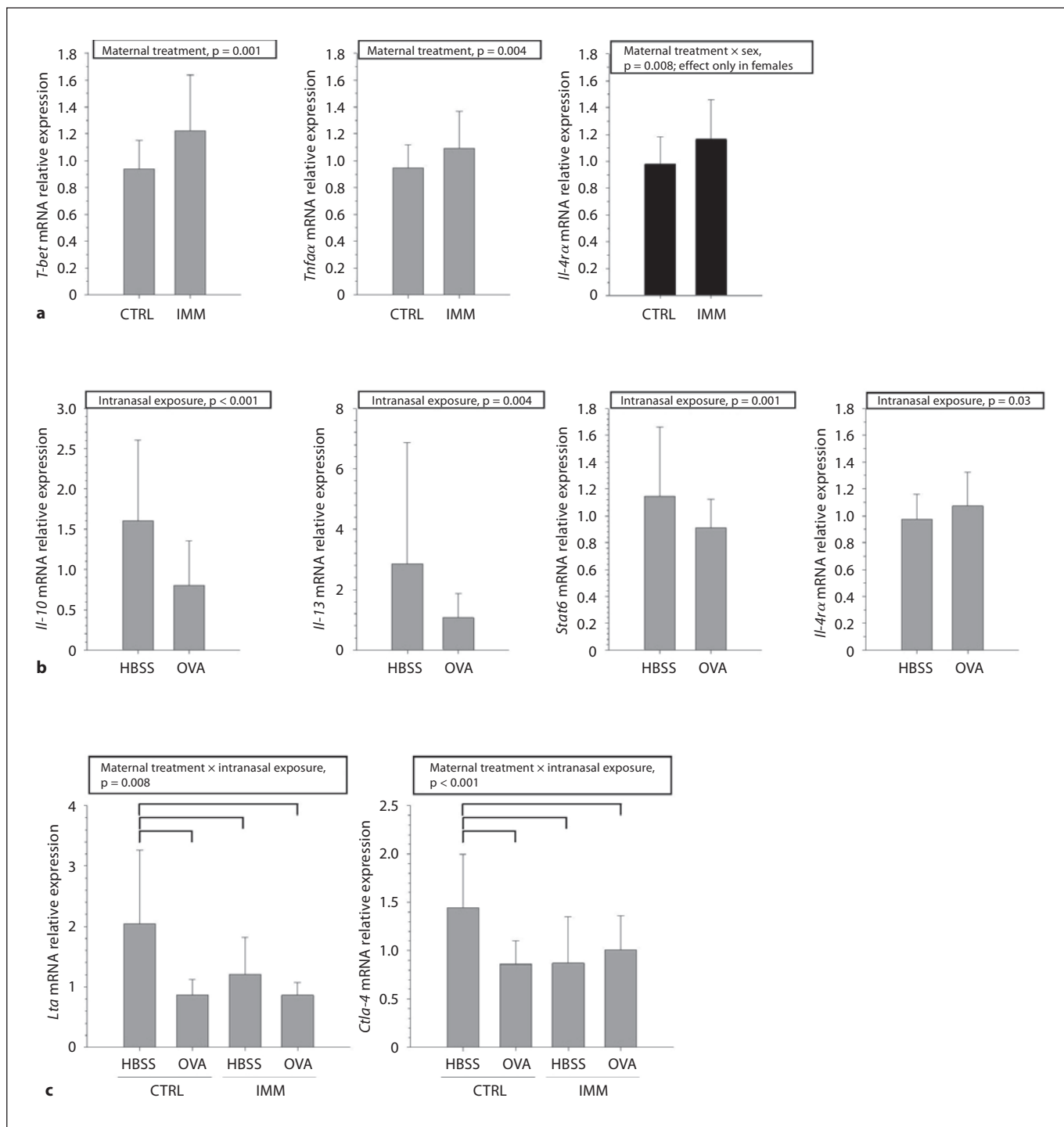
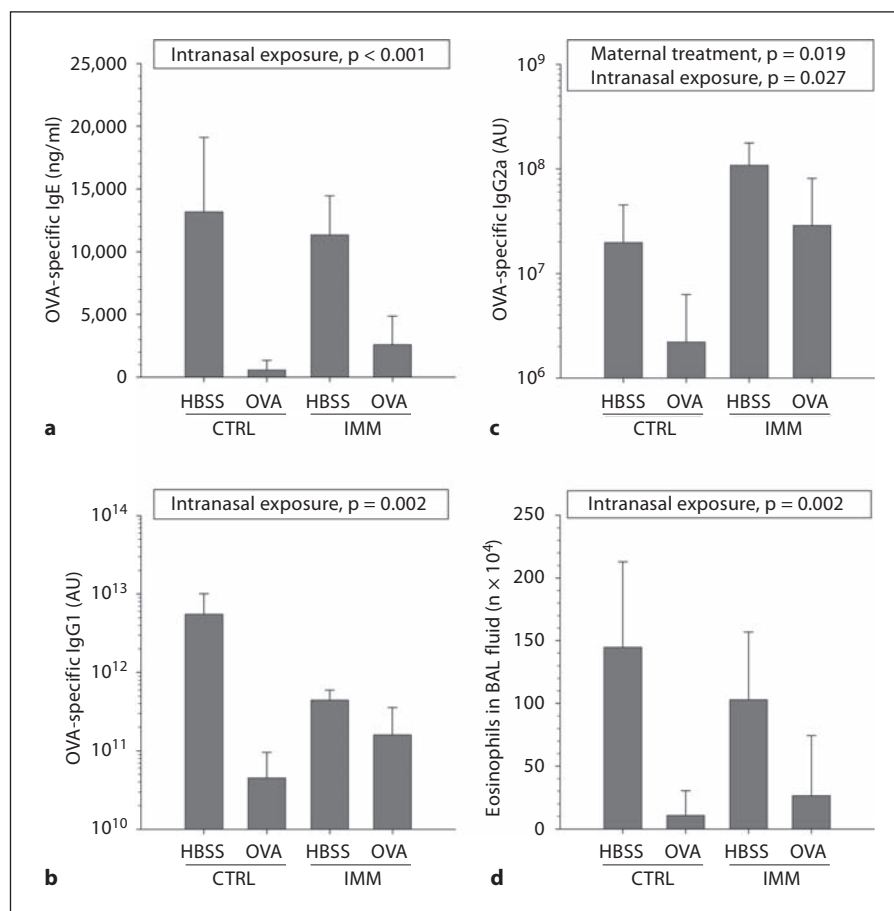


Fig. 5. Effects of maternal immunization and intranasal OVA exposure on mRNA expression in the spleen from intraperitoneally immunized 9-week-old offspring. Offspring from control- or OVA-immunized (CTRL/IMM) dams were allocated to intranasal exposure to either OVA or HBSS (vehicle) weekly for 5 weeks. Then, all offspring were immunized and euthanized at 9 weeks age. The relative mRNA expression in spleen cells was assessed and grouped according to significant effects on expression caused

by maternal treatment (**a**), intranasal exposure (**b**) and a combination of both interventions (**c**). Statistically significant main and interaction effects are noted above each figure. Significant interactions were subjected to post hoc analyses and significant differences between subgroups are indicated by brackets. Mean and SD are presented. For figures with two groups, $n = 33$ –40. For figures with four groups and for *Il4α* (**a**), $n = 18$ –20.

Fig. 6. Effects of maternal immunization and intranasal OVA exposure on airway allergy in offspring of control- or OVA-immunized (CTRL/IMM) dams were allocated to intranasal exposure to either OVA or HBSS (vehicle) weekly for 5 weeks. All offspring were intraperitoneally immunized at 6 and 8 weeks of age and airway-challenged with OVA when 9 weeks old on 3 consecutive days. OVA-specific IgE (**a**), IgG1 (**b**) and IgG2a (**c**) in sera and total eosinophil number in BAL fluid (**d**) were assessed 3 days later. Statistically significant main and interaction effects are noted above the figures. Mean and SD of $n = 3$ –5 mice (both males and females) are presented.



spring, as already observed in figure 3c. Thus, B-cell antibody responses are fully prevented in young animals by maternal immunization. In contrast, only T_H2 -associated antibodies are suppressed in older animals, since IgG2a production is promoted.

In a second experiment (late immunization in fig. 1), blood samples were taken from 20-week-old naïve offspring from IMM and CTRL dams. No OVA-specific IgG1 and IgE (data not shown) were detectable. The offspring were then immunized as described previously and euthanized at age 23 weeks. Subsequently, no statistically significant differences in OVA-specific IgE, IgG1 or IgG2a production were noted in offspring from CTRL and IMM dams (fig. 7d–f).

Effect of Maternal Immunization on the Development of Mucosal Tolerance

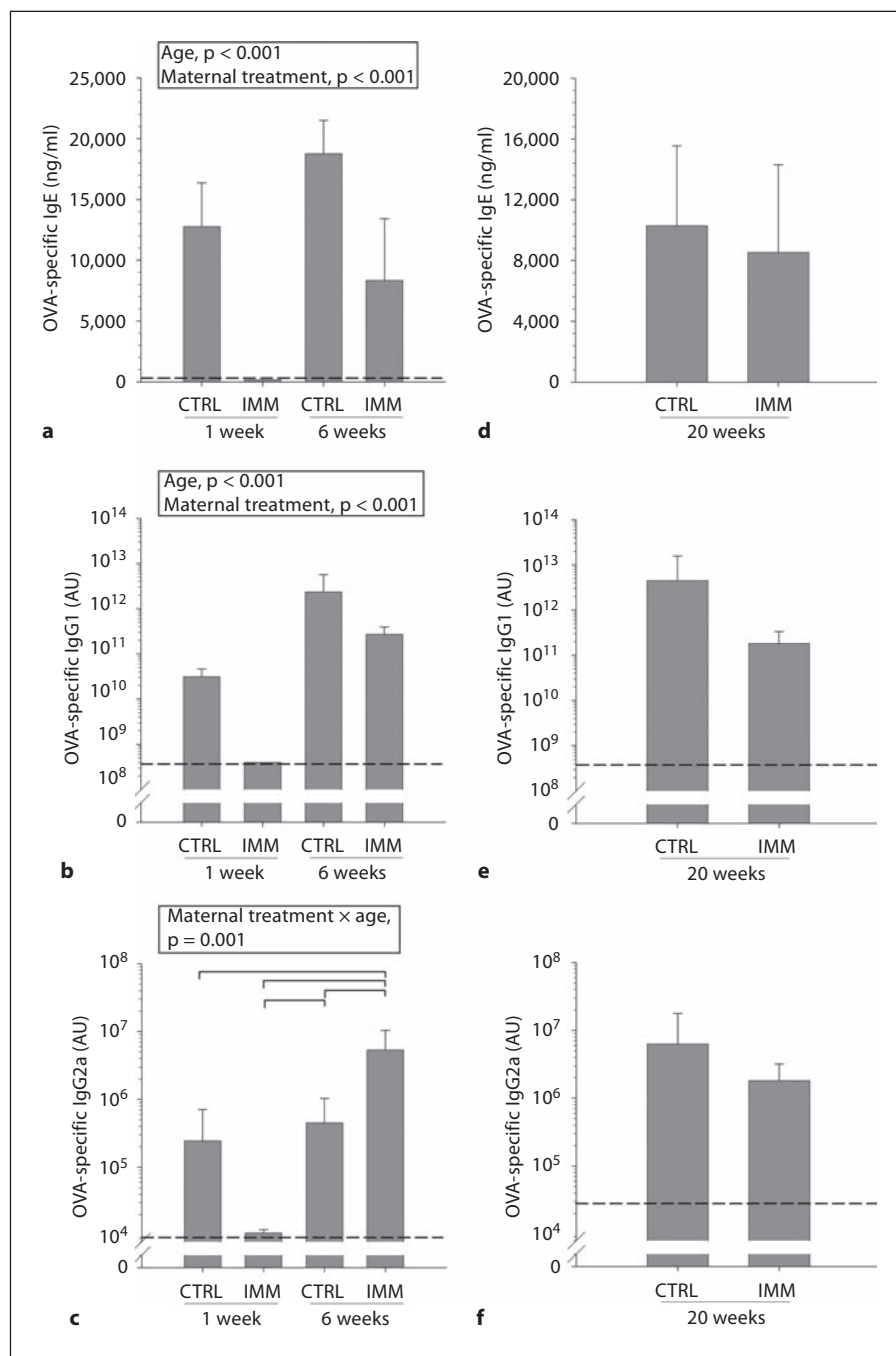
By using a factorial design for the experiment, it was also possible to investigate whether maternal immunization may affect the development of tolerance to muco-

sally encountered antigens. In offspring of IMM dams, intranasal OVA exposure only further reduced IgG1 but not specific IgE (fig. 3a, b). The data in figures 4a and 6 may indicate that tolerance responses in offspring from IMM dams were not as effective as in offspring from CTRL dams. However, no statistically significant interaction effects were detected, which implies that the effect of intranasal OVA exposure did not depend on maternal treatment. Thus, tolerance responses did not significantly differ between offspring from IMM and CTRL dams.

Discussion

The possibility of early life introduction of allergen to prevent allergy is attractive but highly debated [32–34]. To our knowledge, this is the first experimental study to demonstrate that tolerance may be induced in offspring by an early life intervention independently of the maternal immune response. Our study is in keeping with a re-

Fig. 7. Effects of maternal immunization on offspring intraperitoneally immunized at different ages. Offspring of control- or OVA-immunized (CTRL/IMM) dams were immunized when 1 or 6 (a–c) or 20 weeks old (d–f). a–c and d–f were analyzed separately and thus not directly comparable. Serum levels of OVA-specific IgE (a, d), IgG1 (b, e) and IgG2a (c, f) were measured 1 week after a booster immunization. Broken line indicates the lower detection limit of the ELISA. a–c GLM was applied (n = 4–7 mice; both males and females). Statistically significant main and interaction effects of age and maternal treatment are noted above the figures. Significant interactions were subjected to post hoc analyses and significant differences between subgroups are indicated by brackets. d–f The t test was used for statistical analysis (n = 7–8 mice; both males and females, representative of two experiments). Means and SD are presented.



cent paper demonstrating that early introduction of cow's milk protein confers protection against cow's milk allergy independently of parental atopy [35].

Also, we compared the effects of the two early life interventions, maternal allergen immunization and post-natal intranasal allergen exposure in the offspring, on the development of allergy in offspring. After offspring im-

munization, the two interventions reduced OVA-specific IgE and IgG1 to a similar degree. Interestingly, the interaction effects on *Lta* and *Ctla-4* mRNA expression were comparable to the effects on IgE and IgG1, respectively. LTA is necessary for the generation of IgG as well as allergic responses in mice [36, 37] and was suppressed by both interventions. Stimulation through CTLA-4 liga-

tion may inhibit T-cell responses [38]; so, since *Ctla-4* expression was high in mice with high IgE/IgG1 production, this may reflect a requirement for inhibitory signals.

However, the two interventions had different effects on T_H1 and T_H2 responses, and, interestingly, opposite effects on IL-5 and IL-10. *Il-10* mRNA expression and IL-10 and IL-5 protein secretion were significantly reduced in offspring exposed intranasally to OVA, but not affected (*Il-10*) or up-regulated (IL-5 and IL-10) in offspring from IMM dams. In this and previous studies using different mouse models, IL-10 may be associated with T_H2 rather than regulatory responses [39–42], and IL-10 secretion was not associated with either the number or the activation of T_{reg} splenocytes in this study (data not shown). Additionally, intranasal OVA exposure reduced the expression of T_H2 -related *Il-13* and *Stat6* genes. Also, spleen cell numbers were reduced (fig. 4), suggesting an inhibition of splenocyte proliferation. Reduced proliferation could be related to the observed increase in *Il-4 α* mRNA expression, since soluble IL-4R may inhibit IL-4-mediated cell proliferation through neutralizing activity [43, 44]. In accordance with our observations, nasal application of antigen has previously been described to lead to immunological tolerance characterized by induction of IgG1 [14, 45], suppression of IgE, T-cell proliferation, cytokine secretion and allergic inflammation, when the same mice were concurrently sensitized [14, 46]. Accordingly, in juvenile mice, intranasal OVA exposure induced OVA-specific IgG1 production, but restricted OVA-specific IgE and IgG1 production and allergic inflammation after immunization and airway challenge later in life (fig. 6). Importantly, we observed that IL-5 suppression was functionally linked to decreased airway eosinophil inflammation following allergen challenge, which is in line with the role of IL-5 for recruitment, development and survival of eosinophils [47]. A previous study also found IL-5 inhibition following tolerizing antigen inhalation in adult mice [15].

In contrast, maternal immunization stimulated both T_H1 and T_H2 components in the offspring. First, the gene expression of the T_H1 transcription factor T-bet [48] as well as OVA-specific IgG2a were up-regulated in IMM offspring. Second, mRNA expression of the pro-inflammatory cytokine TNF α was up-regulated concurrently with IL-5 and IL-10 secretion. Third, following airway challenge, maternal immunization had no suppressive effect on allergic responses (fig. 6). We are currently investigating if this T_H1/T_H2 profile has implications for the allergy risk in old offspring that were immunized as juveniles.

Overall, this implies that a general T_H2 suppression was induced only by the postnatal intranasal exposure and indicates that different mechanisms underlie the IgE-suppressive effects of the two interventions. Thus, mucosal exposure in early life either directly (our study) or indirectly via breast milk [49] appears to be superior in inducing robust tolerance compared to the effect of maternal immunization alone.

An important observation in our study was that the interventions had comparable outcomes in adult female and male offspring, although OVA-specific IgE and IgG1 production was lower in the male mice. We recently published that the difference in allergic responses between male and female mice in this model is a genuine sex-dependent effect for this age group, and could not be ascribed to the immunization dose [50]. In studies investigating the transgenerational transfer of allergy risk or protection, effects on both sexes are rarely reported despite the fact that allergy prevalences in males and females differ before and after puberty [51].

The complete suppression of antibody responses in offspring immunized at 1 week of age (fig. 7) and the absence of OVA-induced cytokine secretion in neonates from IMM dams (table 3) argue against the possibility of fetal OVA priming due to maternal immunization. Previous studies have shown a direct role for allergen-specific IgG1 transferred from the dam to the offspring in mediating IgE-suppressive effects [10–13]. The presence of OVA-specific IgG1 and IgG2a in naïve 1- and 5-week-old offspring only from IMM dams reflects the transfer of maternal IgG. Victor et al. [52] demonstrated the presence of maternal antibodies up to 9 but not 12 weeks of age, and we could not detect any OVA-specific IgG1 in 13- [unpubl. observ.] and 20-week-old offspring. We did not determine whether maternal IgG had a direct role in the present study; however, IgE and IgG1 suppression due to maternal immunization attenuated and eventually disappeared concurrently with decreasing levels of maternal IgG (fig. 7). This transient suppression was also observed by Fusaro et al. [12] and supports the role of maternal IgG1 on IgE suppression described in other studies.

Reduced serological responses to vaccination have been described in mouse pups [53, 54], calves [55] and children [56] vaccinated under the umbrella of maternal antibodies. This may be caused by epitope masking either directly or sterically due to binding of maternal IgG to the antigen [57]. In a dose-dependent manner, inhibition of offspring antibody production will therefore wane with increasing levels of antigen or decreasing levels of maternal antibodies [56]. Similarly, we observed that antibody

suppression disappeared following airway challenge with high OVA doses and with declining levels of maternal IgG1. Interestingly, effective and even enhanced T-cell responses in offspring were observed in studies where allergen-specific IgE was concurrently suppressed [11, 58] and in the above-mentioned vaccination studies [53–56]. The enhanced IL-5 and IL-10 secretion observed in IgE-suppressed offspring of IMM dams could be due to the uptake of antigen-IgG complexes by antigen-presenting cells and therefore more effective presentation to T cells [56, 59]. Thus, although maternal antibodies effectively prevent primary antibody responses, memory responses may be functional on subsequent antigen exposure [56, 60, 61], which is in agreement with the results following OVA challenge of the airways in immunized offspring.

To be able to extend our findings to future preventive strategies, several questions need to be addressed. It could be explored whether the temporary protection by maternal antibodies is beneficial in terms of allergy prevention in children. Lange et al. [30] demonstrated that continuous allergen stimulation could extend the protective effect beyond the time where maternal antibodies had disappeared. Regarding tolerance induction, the adequate dose differed between young and old mice in a study by Wakabayashi et al. [62]. Our preliminary data showed that continuous mucosal exposure was necessary to maintain inhibition of IgE responses in offspring immunized at 20 weeks of age [unpubl. observations], thus the duration of intervention should be addressed as this may also differ between humans and mice. Even though we used volumes optimized for lung deposition, a large part of the OVA administered may have reached the gut. As continuous oral delivery of antigen also may prevent air-

way responses [63, 64], we cannot distinguish whether primarily nose, lung or gut deposition facilitated tolerance on airway allergy. For primary prevention strategies in children, it will be important to elucidate which mucosal site is optimal with regard to efficacy and safety.

In summary, both maternal allergen immunization and postnatal mucosal exposure of offspring to allergen reduced allergen-specific IgE in offspring following immunization. Different mechanisms may have caused this initial suppression as maternal immunization did not protect against airway inflammation induced by later airway allergen challenge. Within the limitations of the mouse model, we demonstrated that the development of tolerance after mucosal allergen exposure was unaffected by the maternal immune response. Mucosal allergen exposure in early life may become an important tool in the prevention of allergy in children and warrants further basic and clinical studies.

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Disclosure Statement

The authors declare to have no conflicts of interests.

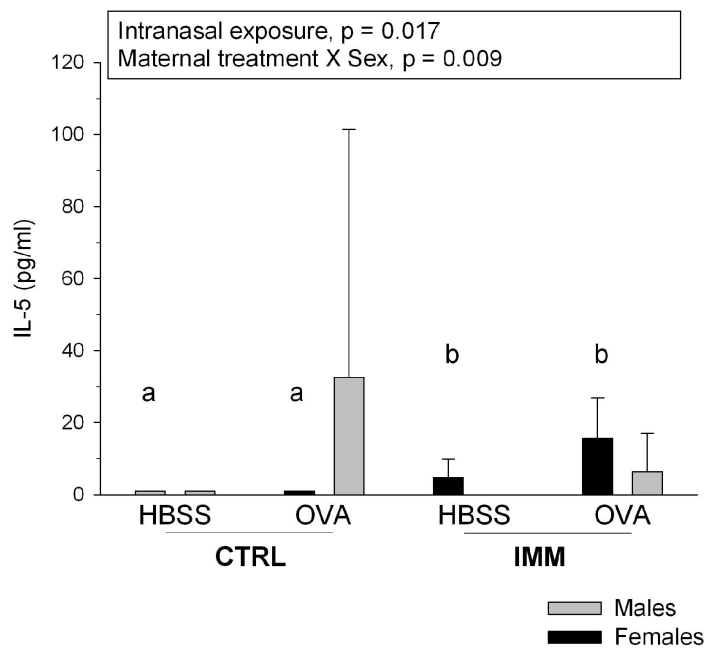
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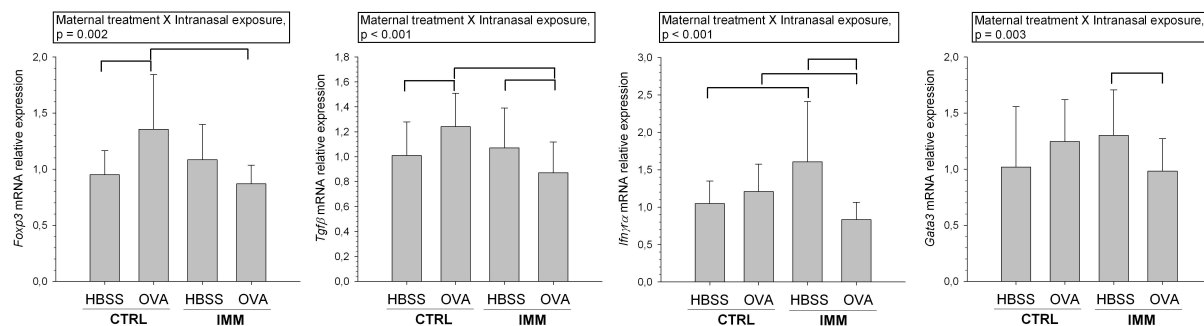
Supplementary Figure 1



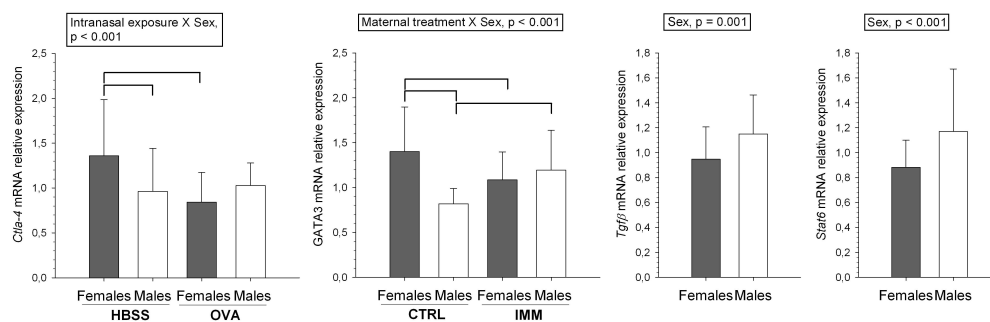
IL-5 secretion from splenocytes in offspring before i.p. immunisation Offspring of control- or ovalbumin (OVA)-immunised (CTRL/IMM) dams were allocated to intranasal exposures to either OVA or HBSS (vehicle) weekly for 5 weeks, whereafter spleen cells were stimulated with OVA *ex vivo* and IL-5 secretion assessed. The range of corresponding means for non-stimulated wells was 0-9 pg/ml. Mean and SD for $n = 3-5$ females or males is shown. Statistically significant main and interaction effects are noted above the figure. Intranasal exposures with OVA significantly increased the IL-5 secretion. The post hoc analyses showed that females from immunised dams (b) had significantly higher IL-5 production than females from control dams (a).

Supplementary Figure 2

A Significant interaction effects of maternal treatment and intranasal exposure



B Significant effects of sex



Effects of maternal immunisation and intranasal OVA exposures on mRNA expression in spleen from immunised offspring

Offspring from control- or ovalbumin (OVA)-immunised (CTRL/IMM) dams were allocated to intranasal exposures to either OVA or HBSS (vehicle) weekly for 5 weeks. Offspring were OVA immunised twice and euthanized at 9 weeks age. The relative mRNA expression in spleen cells was assessed and a selection presented in Figure 5. The remaining data are presented here, grouped according to changes in expression caused by (A) a combination of the two interventions and (B) sex or sex interactions. Statistically significant main and interaction effects are noted above each figure. Significant interactions were subjected to post hoc analyses and significant differences between subgroups are shown on the figures by brackets. Mean and SD are presented. For figures with two groups, $n = 33-40$. For figures with four groups, $n = 18-20$.