

Prenatal lipopolysaccharide-exposure prevents allergic sensitization and airway inflammation, but not airway responsiveness in a murine model of experimental asthma

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Summary

Background Epidemiological evidence underlines the impact of prenatal environmental factors on the development of postnatal allergies. In this regard an inverse correlation between lipopolysaccharide (LPS) exposure and development of childhood allergy has been found.

Objective To assess the impact of prenatal LPS exposure on the development of postnatal respiratory allergies in a mouse model of experimental asthma.

Methods Female BALB/c mice were exposed to LPS before conception and during pregnancy. Several weeks after birth offspring were sensitized to ovalbumin (OVA) followed by aerosol allergen challenges.

Results Prenatal, maternal LPS-exposure enhanced neonatal IFN- γ , but not IL-4 and IL-2 production. OVA sensitization of prenatally LPS-exposed mice was accompanied by a marked suppression in anti-OVA IgG1 and IgE as well as unchanged IgG2a antibody responses, paralleled by a significant reduction in IL-5 and IL-13 levels following mitogenic stimulation of splenic leucocytes. Assessment of bronchoalveolar lavage fluids following allergen challenges revealed a marked reduction in eosinophils and macrophages in these mice. Surprisingly, development of airway hyper-responsiveness, a hallmark of bronchial asthma, was not affected.

Conclusion This study provides first experimental evidence that LPS may already operate in prenatal life in order to modulate the development of allergies in the offspring.

Keywords airway inflammation, allergy, immune response, lipopolysaccharide, materno-fetal interaction, mice, Th1/Th-2

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Introduction

Expression of T helper type 2 (Th2) immunity against environmental antigens is the hallmark of the allergic phenotype and contrasts the Th 1-like pattern, which is stably expressed in healthy adults throughout life. Recent epidemiological studies indicate that children living on farms under traditional circumstances have a reduced risk to develop an atopic disorder as compared with non-farming children [1–4]. This effect might be attributed to the intensive exposure of farm children to components of microbial pathogens. Currently, of major interest is the endotoxin lipopolysaccharide (LPS), which is a cell wall component of Gram-negative bacteria. Indeed an inverse correlation between LPS exposure and the risk to develop an allergic disease has been described by several investigators, including our consortium [5–7]. These epidemiological studies provide evidence for a close association between LPS exposure

occurring within the first year of life including the exposure during pregnancy and a reduced prevalence of respiratory allergies [8]. This leads to the hypothesis that already the *in utero* development of the child's immune system plays an important role in determining the risk for allergic disease later in life. Several observations support this concept: In cord blood allergen-specific T cells are readily detectable [9]; cord blood T cells show a different cytokine expression profile depending on the environment as well as the allergic state (Th2/Th1) of the mother [10, 11]; cord blood T cells from newborns with atopic family background are characterized by a low Th1 immune response [12]; infants from women used to take probiotic nutrition (*Lactobacillus* GG) during pregnancy and lactation showed a delayed development of atopic dermatitis [13].

Although epidemiological studies reveal a close association between LPS exposure and allergies, it still remains to be shown that prenatal LPS exposure is able to alter the development of respiratory allergies [14]. Experimental data obtained in a previously established animal model support the concept that the prenatal environment affects postnatal allergy development. Offspring from allergic BALB/c mothers showed an impaired Th1 immune response at birth

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that resulted in an accelerated immune response to the same antigen later in life [15, 16]. Recently, Hamada et al. [17] observed that the asthmatic phenotype is transmitted in an allergen-independent fashion from the mother to the offspring. Based on these data it was the aim of this study to explore the effect of prenatal LPS exposure on development of experimental asthma later on in life.

Materials and methods

Animals

Female BALB/c mice aged 6–8 weeks were obtained from Harlaan Winkelmann (Hannover, Germany). They were maintained on an ovalbumin (OVA)-free diet. All of the experimental procedures were approved by the animal ethics committee.

Experimental design

Maternal lipopolysaccharide exposure Based on previous data female BALB/c mice received three intraperitoneal (i.p.) injections of 10 µg of LPS (*Escherichia coli* serotype 0111:B4; Sigma Chemie, Deisenhofen, Germany) in a volume of 200 µL (reconstituted in PBS; PAA Laboratories, Pasching, Austria) on days 5, 3 and 1 prior to mating (day 1) [18]. During the gestation period mice of all groups were exposed intranasally (i.n.) to 10 µg LPS in a volume of 50 µL every third day. Age matched, sham-exposed control animals received PBS instead of LPS (control group). Numbers of cytokine producing lymphocytes by offspring were assessed on day 2 post-partum (p.p.).

Offspring: exposure to ovalbumin At age of 28 and 42 days offspring were sensitized to OVA by two i.p. injections of 10 µg OVA (grade VI; Sigma Chemie) emulsified in 1.5 mg Al(OH)₃ (Pierce, Rockford, IL, USA) in a total volume of 200 µL (days 1 and 14). Cytokine production and antibody response by OVA-sensitized mice was assessed on day 42.

To assess airway responsiveness a subgroup of animals received an additional i.p. injection of OVA/Al(OH)₃ at day 63. On days 67, 68, 69 and 70 mice were placed in a Plexiglas chamber and exposed to aerosolized OVA (1% w/v diluted in PBS) for 20 min. Airway reactivity was assessed 24 h after the last allergen-aerosol exposure.

Serum levels of lipopolysaccharide-specific immunoglobulin antibodies

Blood samples from maternal mice were taken from lateral tail veins shortly after birth to determine anti-LPS antibody concentrations by means of ELISA as previously described [19]. Briefly, 96-well flat-bottomed plates were coated with 10 µg/mL LPS (*E. coli* serotype 0111:B4; Sigma Chemie) diluted in coating buffer (0.05 M NaHCO₃-buffer (pH 9.6) and magnesium chloride (0.02 M)) at a volume of 100 µL/well. Plates were incubated overnight at room temperature. After washing for five times (PBS/0.05% (v/v) Tween 20), serum dilutions [1:1000 in PBS/0.05% Tween 20, 0.5% BSA and 4% polyethylene glycol 6000 (Fluka, Buchs, Switzerland)]

were added and incubated at 37 °C for 60 min. Plates were washed for five times before adding a peroxidase-labelled goat anti-mouse IgG antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA; 60 min at 37 °C). Detection was performed using the peroxidase substrate Tetramethylbenzidine (TMB; Roche, Mannheim, Germany). Plates were read at 450 nm wavelength.

Cell stimulation and cytokine measurement

Splenic mononuclear cells (MNCs) of the 2 days old neonates and of the OVA-sensitized offspring (2×10^6 /mL) were cultured in 24-well flat-bottomed plates at 37 °C in a 5% CO₂ and 95% humidified air atmosphere. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 10 ng/mL; Sigma Chemie) and Ionomycin (1 µg/mL; Sigma Chemie). After 24 h cell-free supernatants were harvested and levels of IFN-γ, IL-5, IL-4, IL-2 were assessed using the Cytometric Bead Array (CBA)-technique (BD Biosciences, San Diego, CA, USA) accordingly to the manufacturer's instructions. Detection limits were 5 pg/mL for IL-5, IL-2 and IL-4, respectively, and 2.5 pg/mL for IFN-γ. IL-13 production was measured by ELISA technique. Briefly, 96 plates were coated with the monoclonal anti-IL13-antibody (MAB 413) overnight at 4 °C before sample incubation (24 h). A biotinylated anti-mouse-IL13 antibody was added, followed by addition of streptavidin-peroxidase (Sigma Chemie). The OD reading was performed at 450 nm using an ELISA reader (Tecan sunrise, Crailsheim, Germany). Sensitivity of the assay was 7.8 pg/mL. Recombinant IL-13 (413-ML) was used as a standard.

Enumeration of interferon-γ producing cells by the ELISPOT technique

Numbers of cytokine producing neonatal splenic cells were assessed as previously described [16].

Measurement of serum levels of ovalbumin-specific antibodies

Levels of OVA-specific IgG1, IgG2a and IgE antibody titres were determined by ELISA technique as previously described [20].

Non-invasive measurement of midexpiratory airflow at baseline and of bronchial responsiveness to β-methacholine

The mid-expiratory airflow (EF₅₀) was measured using head-out body plethysmography as described previously [21–23]. Briefly, airflow was measured in the body plethysmograph with a pneumotachograph (PTM 378/1.2, Hugo Sachs Elektronik, March-Hugstetten, Germany) and the amplified analogue signal was digitized via an A/D converter (DAS-16, Keithley, Germering, Germany). Airflow signals were collected over a period of 15 s and processed with the NOTOCORD software (Notocord, Paris, France). Determination of EF₅₀ was carried out 1 h before allergen challenge. The duration of the measurement was 15 min. Measurements of airway responsiveness were performed 24 h after the last challenge. Airflow signals were recorded in response to β-methacholine (Mch) aerosolized with a nebulizer. Con-

secutive β -methacholine concentrations were used for provocation (25, 50, 75, 100, 150 and 200 mg/mL) and the concentration of β -methacholine that caused a 50% reduction in mid-expiratory airflow (MCH_{50} , mg/mL) was determined. Non-invasive measurement of EF_{50} directly correlates with simultaneous invasive measurements of lung mechanics and airway responsiveness [23].

Statistical analysis

Results are presented as mean values $\pm$ SEM (standard error of the mean). Statistical differences between study groups were calculated by the student's *t*- or the Mann-Whitney test. Values were considered as statistically significant when $P < 0.05$.

Results

The effect of maternal lipopolysaccharide exposure on neonatal cytokine production

Female BALB/c mice were LPS primed by repeated i.p. injections before mating followed by consecutive intranasal LPS applications during pregnancy (as described in Materials and Methods). After delivery all of the 'LPS exposed' mice revealed significantly higher ($P = 0.0009$) anti-LPS antibody titres than PBS-control mice (OD 1.1–OD 2.05 vs. OD 0.42–OD 0.8; mean $1.57 \pm SEM 0.13$ vs. mean $0.61 \pm SEM 0.06$), indicating that LPS exposure enhanced the production of anti-LPS antibodies.

To investigate the effect of maternal LPS exposure on the Th1/Th2 balance of the neonatal immune system splenic cells were prepared on day 2 p.p. and the levels of IFN- γ productions well as the number of IFN- γ -producing cells were assessed by ELISA and ELISPOT, respectively (Figs 1a and b). After stimulation of splenic cells with PMA/Ionomycin an increased production of IFN- γ was detected in offspring from mice primed with LPS. In contrast to IFN- γ , the levels of IL-2 and IL-4 production were not altered at birth (data not shown). These data indicate that maternal LPS exposure has a stimulatory effect on neonatal IFN- γ production.

The effect of prenatal lipopolysaccharide exposure on oralbumin sensitization later in life

Offspring from the study groups were i.p. sensitized to OVA later in life. Splenic cells were prepared from OVA sensitized as well as control mice, and stimulated with PMA/Ionomycin to assess cytokine production. As shown in Table 1, the capacity of IL-5 and IL-13 production was significantly ($P = 0.001$; $P = 0.0001$, respectively) reduced in the LPS-exposed group as compared with control mice. In contrast, levels of IL-4 and IFN- γ production were not significantly altered in prenatally LPS-exposed mice.

OVA-specific antibody titer was measured in serum samples. As shown in Table 1 anti-OVA IgE and anti-OVA IgG1 antibody titers were significantly lowered ($P = 0.015$, $P = 0.0001$, respectively) in the LPS-exposed group as compared with controls. The levels of OVA-specific IgG2a anti-

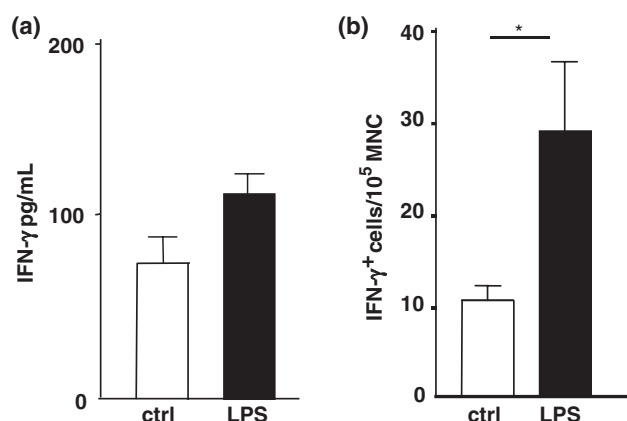


Fig. 1. Effect of prenatal lipopolysaccharide (LPS)-exposure on IFN- γ production of splenic leucocytes at birth. Level of IFN- γ production (a) and frequency of IFN- γ -producing cells (b) were assessed in control (ctrl) and prenatally LPS-exposed (LPS) neonates at an age of 2 days after mitogen stimulation. Offspring were prenatally exposed to LPS (LPS) as described in Materials and methods. Controls were exposed to PBS alone (ctrl). Indicated are mean $\pm$ SEM from 10 animals per study group. Significant differences ($P < 0.05$) are indicated by *.

bodies did not differ significantly between study groups, although there was a tendency towards higher levels ($P = 0.35$).

The effect of prenatal lipopolysaccharide exposure on development of experimental asthma

To test whether the effect of prenatal LPS exposure influences the development of experimental asthma, OVA-sensitized mice were aerosol allergen challenged for four times. Airway inflammation was investigated by analysing bronchoalveolar lavage (BAL) fluids (Fig. 2). BAL fluids from OVA-sensitized LPS mice contained significantly lower numbers of leucocytes as compared with OVA-sensitized non-LPS animals ($P = 0.0018$). The cellular composition of BAL fluids showed a significant reduction of eosinophils as well as macrophages (eosinophils: $P = 0.0083$; macrophages: $P = 0.04$) whereas lymphocytes only showed a tendency towards lower numbers ($P = 0.08$). Airway responsiveness was determined by head-out body-plethysmography. As compared with non-sensitized

Table 1. Effect of prenatal LPS-exposure on the antibody and cytokine production of OVA-sensitized neonates

	Ctrl	LPS	P-value
Antibody response*			
Anti-OVA IgE ($\times 10^3$ LU/mL)	28.6 $\pm$ 5.8	9.7 $\pm$ 3.5	0.015
Anti-OVA IgG1 ($\times 10^5$ ng/mL)	3.6 $\pm$ 0.6	0.2 $\pm$ 0.1	0.0001
Anti-OVA IgG2a ($\times 10^2$ LU/mL)	5.3 $\pm$ 1.3	7.6 $\pm$ 2.2	0.35
Cytokine production†(pg/mL)			
IL-5	151 $\pm$ 21	38 $\pm$ 7	0.001
IL-13	1958 $\pm$ 133	502 $\pm$ 67	0.0001
IL-4	1250 $\pm$ 55	1046 $\pm$ 97	0.08
IFN- γ	147 $\pm$ 20	108 $\pm$ 36	0.37

*Offspring (28 days of age) were sensitized by intraperitoneal injections of OVA emulsified in Al(OH)₃ at days 1 and 14 as described in Materials and methods. Antibody production was assessed in 6–13 animals at day 42.

†Cytokine production of splenic MNC's was assessed at day 42 after re-stimulation with P/I in 6–13 animals as described in Materials and methods. OVA, ovalbumin; LPS, lipopolysaccharide.

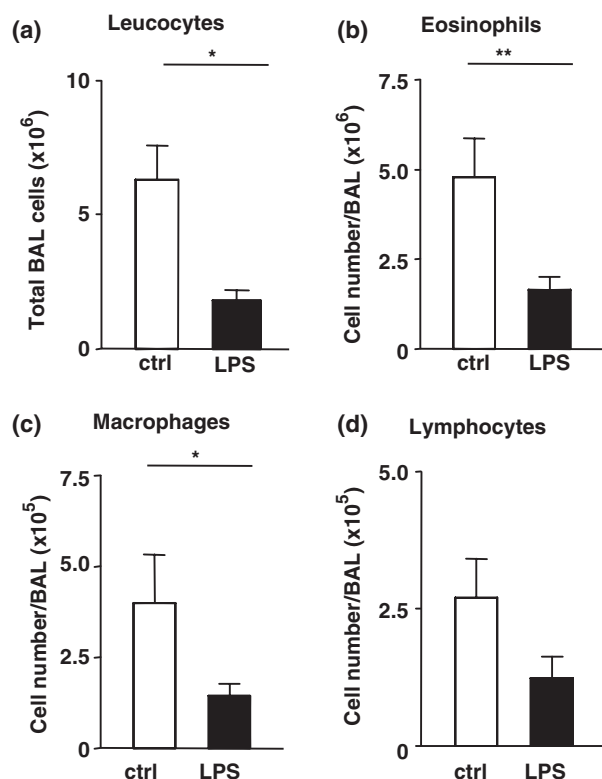


Fig. 2. Effect of prenatal lipopolysaccharide (LPS)-exposure on airway inflammation in ovalbumin (OVA) immunized and allergen-challenged mice. OVA-immunized neonates of the LPS-exposed (LPS) and PBS-exposed (ctrl.) group were OVA-aerosol challenged followed by a lavage of the airways. Cellular composition of BAL fluids was examined. Total number of leucocytes (a), number of eosinophilic cells (b), macrophages (c) and lymphocytes (d) are depicted (* $P < .05$ and ** $P < .01$ vs. ctrl; shown are mean $\pm$ SEM; $n = 4$ ctrl. group; $n = 7$ LPS group).

wild-type animals OVA-sensitized mice from non-LPS mothers showed a significant decrease in MCF₅₀ levels indicating development of airway hyper-responsiveness (AHR) ($P = 0.67$).

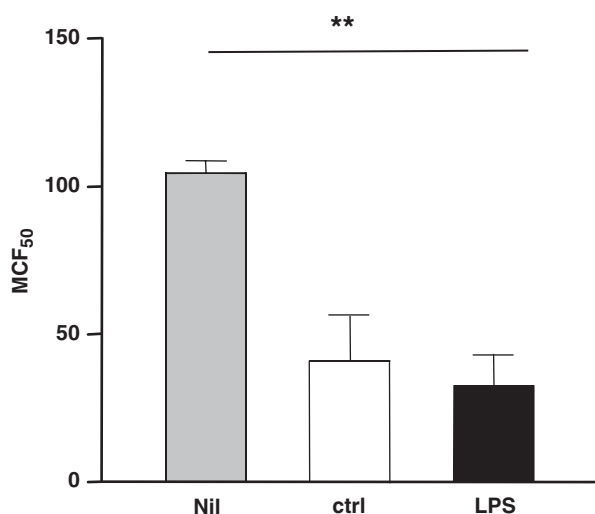


Fig. 3. Effect of prenatal lipopolysaccharide (LPS)-exposure on airway responsiveness to methacholine. Airway responsiveness to methacholine was analysed by head-out body plethysmography. The following groups of mice were analysed: OVA-immunized LPS-exposed ($n = 6$); OVA-immunized control/sham-exposed ($n = 4$); non-immunized, non-exposed controls (Nil; $n = 4$) (depicted are mean $\pm$ SEM; ** $P < .01$ vs. Nil).

An equivalent decrease was detected in OVA-sensitized LPS mice (Fig. 3).

Discussion

It was the aim of our study to assess the effect of chronic LPS exposure as, for example, in the traditional farming environment [6]. Several reports clearly indicate that i.p. application of LPS has strong immuno-modulatory effects [18]. Based on these protocols, we have used this method to induce the initial LPS response. After initiation we have then switched to the intranasal route for chronic LPS exposure in order to mimic the natural way of LPS exposure. For the initial priming via the i.p. route dose – response – experiments have been performed with 1, 10 and 100 μ g per injection. Using the highest LPS dose, we found a none acceptable high frequency of intra-uterine resorptions, whereas the birth rate with 1 and 10 μ g was not significantly different from controls. We, therefore, selected the highest acceptable dose for the experiments in our study to mimic the situation on farms representing an environment with a continuous high exposure of LPS.

This is the first study demonstrating an immuno-suppressive effect of maternal LPS exposure on the development of a Th2 immune response in the offspring later on in life. There is increasing evidence that the development of the fetal immune system is actively and passively controlled by maternal immunity, and that the outcome of this fetomaternal interaction has a strong impact on allergies later in life. Epidemiological data indicate a strong association between environmental exposure of a pregnant mother and particularly respiratory allergies [8]. Furthermore, it has been shown that allergen-specific T cells are already present at birth [11], and that the risk to develop allergies is inversely associated with the capacity to secrete IFN- γ following mitogen stimulation of PBMCs [24]. A further approach to analyse the relationship between maternal and fetal immunity is provided by animal model systems. In this regard we have previously shown that allergen exposure during pregnancy has a strong impact on allergic sensitization by the offspring [16]. We could distinguish between pro-allergic effects of CD4 T cells and immuno-suppressive effects mediated via allergen-specific maternal IgG antibodies.

Among the microbial component that has been of recent interest in this regard is LPS. Several epidemiological studies show an inverse relationship between environmental LPS exposure and the risk of the development of IgE responses and clinical phenotypes such as bronchial asthma [5, 6]. So far, the impact of LPS on the development of allergies has been only studied in adult mice or rats. The phenotypic outcome depends on timing and dosing of LPS. LPS exposure before or early on during sensitization has a protective effect in terms of Th2 immunity and allergies, whereas LPS administered to already sensitized mice has no or an even aggravating effect on respiratory allergies. Furthermore, low-dose LPS augmented Th2 responses whereas high-dose favoured Th 1-dependent inflammation [25, 26]. It was shown that the suppressive effect of LPS was IL-12 dependent and, therefore, a cytokine-based mechanism was postulated.

In our study maternal LPS exposure augmented neonatal IFN- γ , but not IL-2 or IL-4 production levels. This is in line to previous studies showing that high levels of neonatal IFN- γ are associated with allergy protection. Gereda et al. [24] demonstrated that endotoxin directly promotes a differentiation of IFN- γ producing Th1 cells.

The molecular mechanism through which maternal LPS modulates postnatal Th2 immunity is not fully understood yet. A critical aspect in this regard is the feto-maternal interface. The recognition of LPS involves the concerted action of several molecules including the LPS-binding protein (LBP), CD14 and engagement with toll-like receptors (TLRs), TLR-4. It is known that TLR-4 is expressed in the human placenta [27]. It remains to be investigated whether maternal LPS exposure triggers and regulates the level of TLR-4 expression and function in the placental-fetal area. It might be likely that a similar mechanism operates across the placental area as has been identified in LPS-exposed adult mice.

It is important to note that maternal LPS suppressed the development of Th2 immune responses to OVA, even in the context of Al(OH)₃, a potent Th2 favouring adjuvant. Furthermore, there was no indication that Th 1 immunity was augmented. This Th2 inhibitory effect might be related to the increased neonatal IFN- γ response. However, this was not associated with an enhanced Th1 immune response to the allergen. Another mechanism that could be involved in this system could be the development of LPS tolerance. It is well known that LPS induces a state of immunological tolerance, which is transient and can be already observed after initial exposure to LPS. It has been shown that this mechanism is IL-1 β mediated [28].

It was a surprising and unexpected result that the Th2 inhibitory effect was not associated with a normalization of AHR in this system. One reason might be that the remaining IgE/IgG1 antibody responses were sufficient to elicit AHR to methacholine. Alternatively, it might be also possible that the regulation of AHR is distinct from the Th1/Th2 pattern modified by LPS. Indeed, we [29] have recently shown that AHR is the result of neuronal dysfunction in the airways as well, which is not necessarily related to airway inflammation. The elevated IFN- γ expression level at birth indicates a high level of Th1 activity at this time point. It is known that in addition to Th2 cells also Th1 cells can induce AHR accompanied by an influx of eosinophils, lymphocytes and neutrophils [30]. Albeit we could not detect neutrophils in the BAL of the corresponding mice, the development of Th1 cells could be another possible mechanism explaining the AHR of the prenatally LPS-exposed mice.

In previously published cross-sectional studies investigating the farming environment, we have measured endotoxin load in mattresses and dust samples. It has been shown that the natural exposure ranges between 10³ and 10⁵ endotoxin unit (EU) per m² mattress [6]. Since 1 EU equals 0.2 ng of LPS content we can estimate to be in the physiological range of natural LPS exposure. Nevertheless, a potential limitation of our mouse model is that we used an unnatural way of maternal LPS exposure (i.p.) before pregnancy.

A potential role of anti-LPS antibodies has to be taken in consideration. They could affect the immune response of the mother mice and consequently that of the neonate by

neutralization of LPS. An effect of anti-LPS antibodies on the cytokine production profile of macrophages has been previously reported [31]. In conclusion our study provides first experimental evidence for the concept that prenatal exposure to microbial components — LPS in this study — act as potent immuno-modulators for the development of respiratory allergies later in life.

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