

Inhibition of endotoxin-induced perinatal asthma protection by pollutants in an experimental mouse model

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To cite this article: Reiprich M, Rudzok S, Schütze N, Simon JC, Lehmann I, Trump S, Polte T. Inhibition of endotoxin-induced perinatal asthma protection by pollutants in an experimental mouse model. *Allergy* 2013; DOI: 10.1111/all.12121.

Keywords

asthma; epigenetics; pollutants; pregnancy; primary prevention.

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Accepted for publication 21 December 2012

DOI:10.1111/all.12121

Edited by: Hans-Uwe Simon

Abstract

Background: One of the most promising strategies to face the increasing asthma prevalence and to prevent disease development might be an early contact with microbial compounds. However, little is known about an interaction between an early-life contact to microbial compounds leading to asthma protection in the offspring and a co-exposure to allergy-promoting pollutants.

Methods: Pregnant BALB/c mice were repeatedly exposed to aerosolized endotoxin (lipopolysaccharide, LPS). The offspring was further exposed to aerosolized LPS before allergen sensitization with ovalbumin (OVA). Some of the mice were co-exposed to mycotoxins or diesel exhaust particles (DEP) during pregnancy. The 6-week-old offspring was immunized with OVA and analyzed in a murine asthma model.

Results: While the offspring of naïve mothers developed an asthma-like phenotype, the offspring of mice perinatally exposed to LPS was significantly protected. Co-exposure of mice to mycotoxins or DEP during pregnancy inhibited the LPS-induced protection leading to the development of eosinophilic airway inflammation, airway hyperactivity, and increased antigen-specific IgE levels in the offspring. Furthermore, the asthma-preventive effect of perinatal LPS exposure was IFN-gamma dependent. Additionally, the IFN-gamma promoter of CD4⁺ T cells in the LPS-exposed offspring revealed a significant protection against loss of histone 4 acetylation, which was abolished after prenatal co-exposure to pollutants. Prenatal treatment of mice with the antioxidant N-acetylcysteine reversed the pollutant-induced increased asthma risk in the offspring.

Conclusion: Our results show that exposure to pollutants during pregnancy may cause the development of allergic asthma in the offspring by inhibiting the endotoxin-induced perinatal asthma protection.

The prevalence of asthma, a Th2 cell-mediated immune response to common environmental allergens, has increased substantially in the last two decades (1). Although the development of allergic diseases is strongly familial and has a genetic basis, altered environmental and lifestyle conditions in Western civilization are thought to be responsible for this increase in prevalence. In recent years, a number of studies have shown that environmental exposures in the first 12–24 months of life might indeed determine the development of childhood asthma and allergies (2, 3). Therefore, primary prevention strategies might ideally start in early childhood.

Moreover, it has been shown that maternal exposure to an environment rich in microbial compounds, for example, prevalent in farm settings, might protect against the development of atopic sensitization in children (4). Several microbial compounds were recently identified as protective factors that may modulate the immune system to induce a nonspecific tolerance to allergens (5, 6). In murine models of asthma, an airway exposure to endotoxin (lipopolysaccharide, LPS) or to the gram-negative farm-derived bacteria *Acinetobacter lwoffii* F78 during pregnancy prevented the offspring from developing allergen-induced asthma in response to immunization

(7, 8). Furthermore, in epidemiological studies, a continuous exposure to microbial compounds such as LPS of gram-negative bacteria during pregnancy and early infancy was found to be associated with a lower prevalence of atopy and asthma in children (9, 10). Additionally, it has been shown recently that LPS could be the key molecule in mediating the asthma-protective properties of *Acinetobacter lwoffii* F78 (6). Furthermore, it was indicated that the transmaternal asthma protection induced by *Acinetobacter lwoffii* F78 is achieved by an epigenetic regulation suggesting an early gene–environment interaction (8).

However, an exposure to environmental factors might not only be protective but may also exert asthma-promoting effects. Several environmental pollutants such as environmental tobacco smoke (11), ozone (12), volatile organic compounds (13), diesel exhaust particle (14), or mycotoxins (15) are known to exacerbate allergic immune responses. There is also emerging evidence that prenatal exposure to air pollutants or tobacco smoke can influence epigenetic changes, including DNA methylation and histone acetylation (16, 17). In contrast, little is known about an interaction between an early-life contact to microbial compounds leading to asthma protection in the offspring and a co-exposure to allergy-promoting pollutants. Therefore, in this study, we have investigated in a murine asthma model, whether an exposure to mycotoxins or diesel exhaust particle (DEP) has an impact on the LPS-induced prevention against allergy development in the offspring.

Methods

Mice

Female BALB/cByJ mice (6–8 weeks of age) were obtained from the Elevage Janvier Laboratory (Le Genest St Isle, France). All animal experiments were performed according to institutional and state guidelines. The Committee on Animal Welfare of Saxony approved animal protocols used in this study.

Exposure to LPS, co-exposure to pollutants, and OVA immunization

Balb/c mice were exposed to aerosolized LPS (1 µg/10 ml, *Escherichia coli* O111:B4; Sigma, St Louis, USA) for 10 min 3 times per week from day 7 of gestation time until delivery. Following the prenatal exposure, the offspring was further treated with LPS from the day of birth up to day 28 (perinatal LPS exposure) as described before (7). Gliotoxin (100 ng, i.n.; Sigma-Aldrich), patulin (20 µg, oral, Sigma-Aldrich), or DEP (20 µg i.n.; standard reference material from the National Institute of Standards and Technology, Gaithersburg, MD, USA) was applied during pregnancy 30 min before LPS exposure (Fig. S1). To induce an airway inflammation, 6-week-old offspring was immunized intraperitoneally (i.p.) with OVA (20 µg; Sigma-Aldrich, Steinheim, Germany) adsorbed to 2 mg of an aqueous solution of aluminum hydroxide and magnesium hydroxide (Alum; Perbio

Science, Bonn, Germany) on days 1 and 14 followed by 20-µg OVA in 40-µl normal saline given intranasally (i.n.) on days 14–16 and 21–23. Control mice received Alum i.p. and normal saline i.n. (Fig. S1). To prevent the pollutant-induced effects, the antioxidant N-acetylcysteine (NAC, 300 mg/kg i.p.) was given prenatally to mice 30 min before the pollutants (18, 19). To analyze the effect of pollutants on asthma development after an exposure directly to the offspring, the progeny was exposed to pollutants in weeks 5–6 three times a week and then immunized with OVA. Airway hyperreactivity (AHR) was measured on day 24 (Fig. S1). INF-γ treatment, bronchoalveolar lavage (BAL) analysis, lung histology, IgE and cytokine assays, AHR measurement, and ChIP analysis are described in the Supporting Information.

Statistical analysis

One-way ANOVA and Bonferroni's multiple comparison tests were used to determine the statistical significance of differences between groups. Data were expressed as mean ± SEM. *P* values of less than 0.05 were considered significant.

Results

Prenatal exposure to mycotoxins or DEP inhibits the LPS-induced asthma protection in the offspring

To induce an asthma protection in the offspring, we repeatedly exposed pregnant BALB/c mice to aerosolized LPS followed by a necessary further exposure of the progeny in the first 4 weeks of life as described before (7). To investigate the effect of different pollutants on the LPS-mediated asthma protection, some mice were co-exposed to mycotoxins or DEP during pregnancy at environmentally relevant concentrations (15, 20). The 6-week-old offspring was immunized with OVA and analyzed in our established murine asthma model (15, 21). The OVA-immunized offspring of LPS-exposed mice (perinatally) developed significantly less airway inflammation compared to LPS-untreated mice. In contrast, respiratory exposure of mice to gliotoxin or DEP and exposure to patulin via the intestine during pregnancy increased the inflammatory infiltrates in H&E stained lung sections (Fig. 1A), verified by an objective, investigator-independent computer analysis (Fig. 1B), compared to mice only perinatally exposed to LPS. Both, mycotoxins as well as DEP, enhanced the number of eosinophils in the BAL fluid of OVA-immunized offspring (Fig. 1C) compared to mice that were only perinatally exposed to LPS. These findings are associated with increased serum levels of OVA-specific IgE of sensitized mice prenatally co-exposed to pollutants (Fig. 1D). Co-exposure of OVA-immunized mice during pregnancy to the mycotoxins or DEP reversed the LPS-induced decrease in AHR as measured in a whole-body plethysmograph for mice (Fig. 1E). This was confirmed by assessing AHR using direct invasive assays for lung resistance in intubated mice (Fig. 1F). In parallel, in OVA-sensitized mice prenatally co-exposed to pollutants, we measured increased Th2 cytokine levels and decreased levels of the Th1 cytokine IFN-γ

compared to LPS-exposed offspring in cell culture supernatants of OVA-restimulated splenocytes and mediastinal lymph node cells (Fig. 1G).

Prenatal exposure to pollutants only at the used concentrations did not influence the outcome of the allergic immune response in the offspring (Fig. S2A-D).

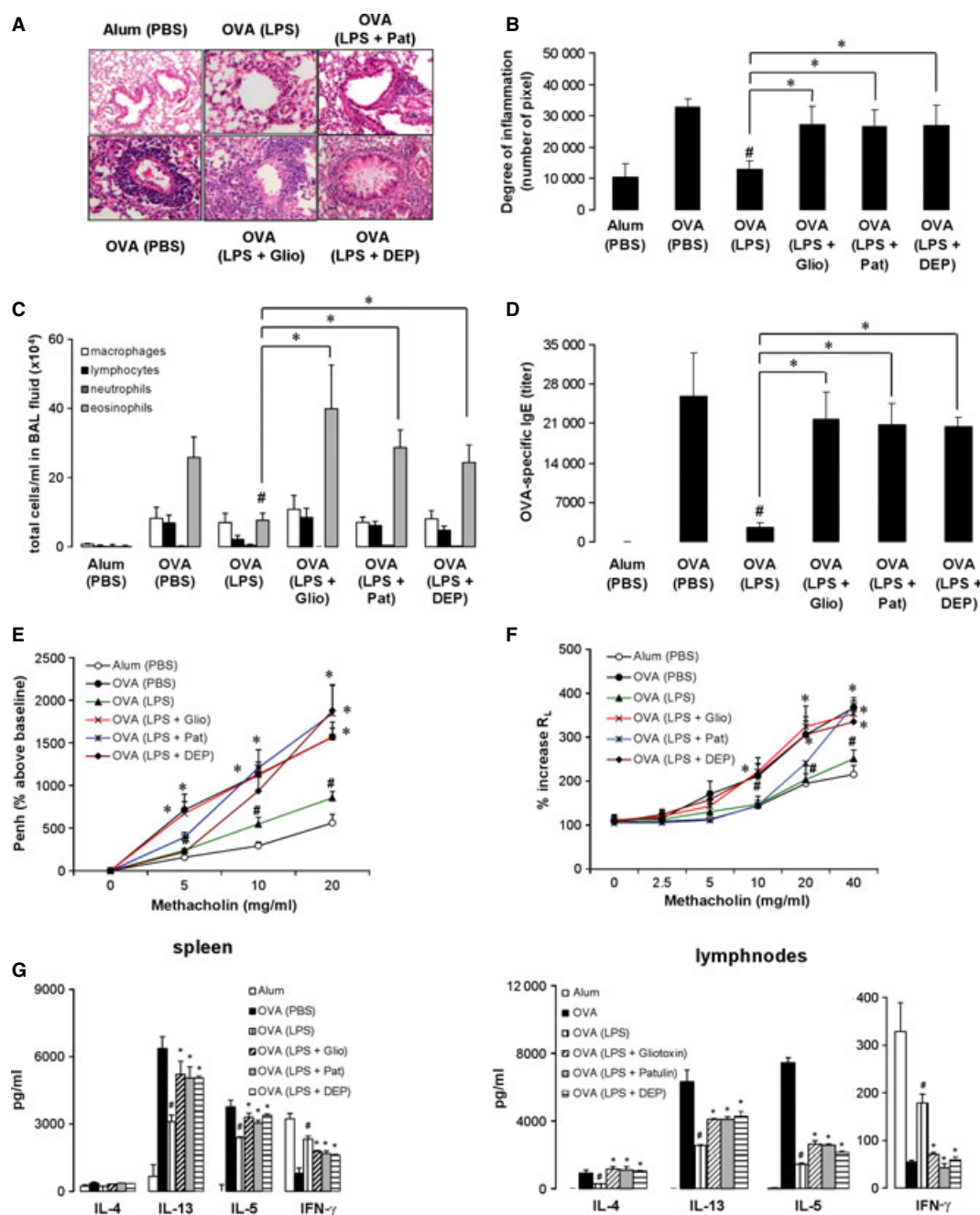


Figure 1 Prenatal exposure to mycotoxins or diesel exhaust particles (DEP) inhibits the lipopolysaccharide (LPS)-induced asthma protection in the offspring. The ovalbumin (OVA)-immunized offspring from mothers co-exposed to mycotoxins or DEP during pregnancy revealed increased airway inflammation in the lung (A), quantified by an investigator-independent computer-based analysis (B), cell numbers in the bronchoalveolar lavage (BAL) fluid (C), OVA-specific IgE levels (D), Airway hyperreactivity (AHR), determined as Penh

(E) and lung resistance (F), elevated Th2 cytokine levels and a reduced IFN-γ production in OVA-restimulated splenocytes and mediastinal lymph nodes (G) compared to OVA-immunized mice perinatally exposed only to lipopolysaccharide (LPS). Data are expressed as mean ± SEM, $n \geq 7$ animals per group; # $P < 0.05$, OVA vs LPS; * $P < 0.05$, LPS vs LPS + pollutants; one-way ANOVA and Bonferroni's multiple comparison test.

Asthma-protective effects by perinatal LPS exposure are reduced by a blocking anti-IFN- γ antibody

It has been shown that the transmaternal asthma protection induced by *Acinetobacter lwoffii* F78 was IFN- γ dependent (8). Looking at IFN- γ production after perinatal LPS exposure, we found a comparable increase in this Th1 cytokine in the supernatant of OVA-restimulated splenocytes and lymph node cells (Fig. 1G). The reduction in the elevated IFN- γ levels after prenatal co-exposure to pollutants suggests an involvement of IFN- γ . Therefore, we investigated the role of IFN- γ for asthma protection in offspring perinatally exposed to LPS using a blocking anti-IFN- γ mAb. This treatment reversed the LPS-mediated protective effects, demonstrated by restored airway inflammation, increased number of eosinophils in the BAL fluid, development of airway

hyper-reactivity, and elevated OVA-specific IgE serum levels (Fig. 2A-D). Concurrently, functional blockade of IFN- γ resulted in significantly increased production of the Th2 cytokines IL-13 and IL-5 (Fig. 2E), indicating a regulatory effect of IFN- γ on Th2 cytokine production in this model. The levels of IL-10, a cytokine that can be produced by immune suppressive regulatory T cells, were also reduced after LPS exposure but not significantly affected by anti-IFN- γ treatment.

Prenatal co-exposure to pollutants interacts with epigenetic modifications on histone 4 (H4) acetylation at the IFN- γ promoter by perinatal LPS exposure

Brand and colleagues demonstrate that the decrease in H4ac at the IFN- γ promoter induced by OVA sensitization was

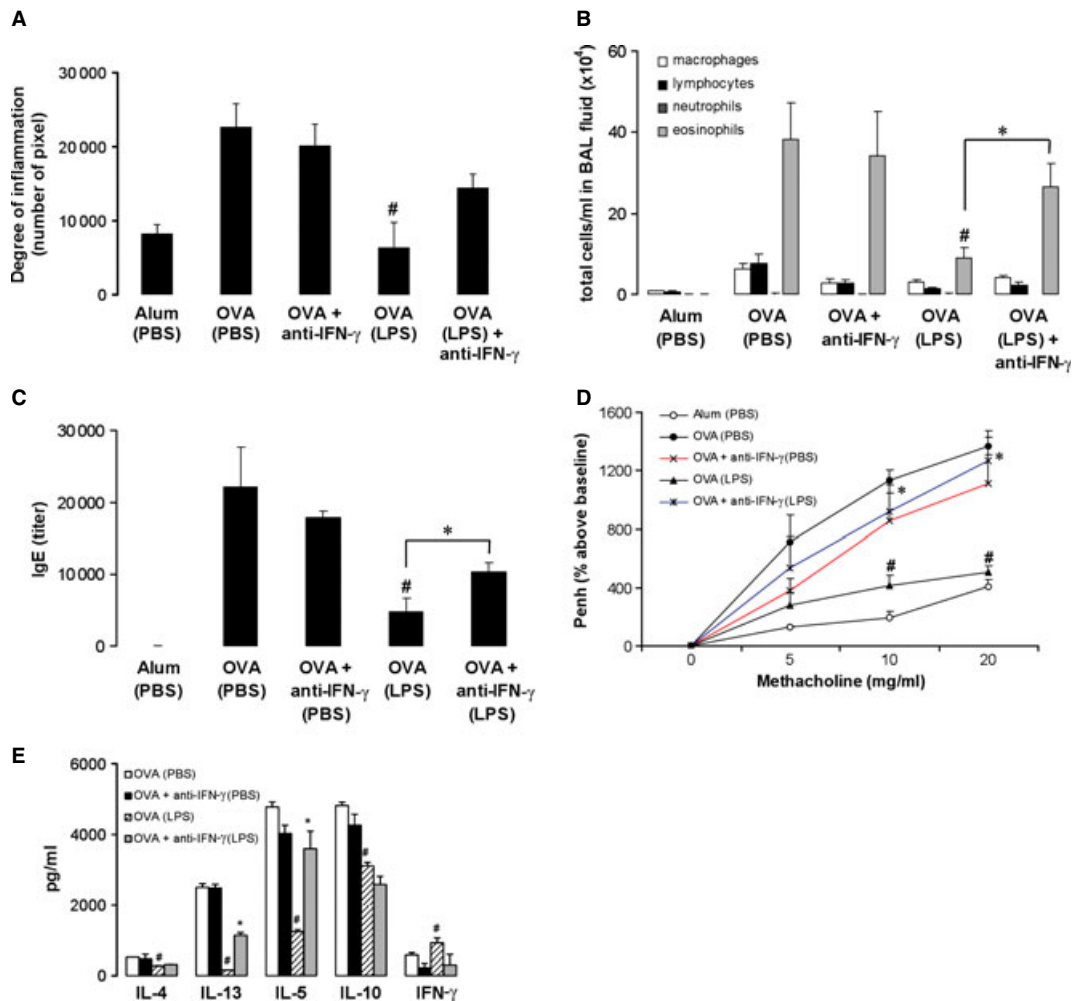


Figure 2 Anti-IFN- γ reverses lipopolysaccharide (LPS)-induced asthma protection in the offspring. The 6-week-old offspring from mice exposed to LPS during pregnancy and in the first 4 weeks of life were immunized with ovalbumin (OVA). Some mice received a blocking anti-IFN- γ mAb on days 14 and 21. Anti-IFN- γ treatment increased the airway inflammation in the lung (A), cell numbers in

the bronchoalveolar lavage (BAL) fluid (B), OVA-specific IgE levels (C), Airway hyperreactivity (AHR) (D), and Th2 cytokine levels (E) compared to OVA-immunized mice exposed only to LPS. Data are expressed as mean $\pm$ SEM, $n \geq 7$ animals per group; [#] $P < 0.05$, OVA vs LPS; * $P < 0.05$, LPS vs LPS + anti-IFN- γ ; one-way ANOVA and Bonferroni's multiple comparison test.

prevented in offspring of mothers exposed to *Acinetobacter lwoffii* F78 (8). To elucidate whether perinatal LPS exposure similarly influences H4ac at the IFN- γ promoter and whether pollutants may interact with these alterations, we used ChIP to analyze isolated CD4⁺ T cells from offspring of LPS and LPS-/pollutant-exposed mothers. Our results show that perinatal LPS exposure resulted in a protection against loss of H4ac at the IFN- γ promoter after OVA immunization. However, the increased H4ac was abolished in offspring from mothers co-exposed during pregnancy to gliotoxin or DEP (Fig. 3). In contrast, prenatal exposure to patulin had no significant effect on LPS-induced increase in H4ac at the IFN- γ promoter. Analysis of H4ac at the IL-4 or IL-5 promoter revealed no significant changes between the different groups (data not shown).

Effects on LPS-induced asthma protection by pollutants are abrogated by the antioxidant NAC

Effects of pollutants are often mediated via the induction of oxidative stress (15, 22). Therefore, to investigate whether an antioxidant may block the pro-asthmatic interaction between pollutants and LPS during pregnancy, we treated mice with NAC. The pollutant-caused inhibition of the asthma protection in the offspring by perinatal LPS exposure was reversed by pretreatment with NAC as evidenced by decreased airway inflammation, number of eosinophils in the BAL fluid, and OVA-specific IgE levels as well as a reduced Th2 cytokine production in OVA-immunized mice (Fig. 4A–D). Treatment of pregnant mice with NAC alone had no effect on the asthma phenotype in the offspring (data not shown).

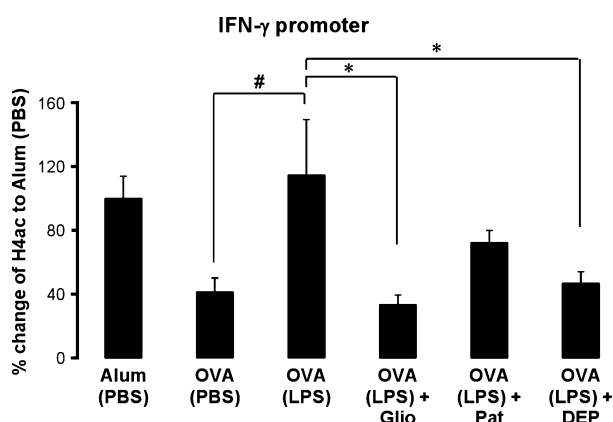


Figure 3 H4 acetylation within the IFN- γ promoter after perinatal lipopolysaccharide (LPS) exposure and prenatal contact to pollutants. CD4⁺ T cells were isolated from spleen, and chromatin immunoprecipitation (ChIP) was performed with antibodies against acetylated H4 (H4ac). Immunoprecipitated and eluted DNA was purified and amplified by means of quantitative PCR. Data are expressed as mean $\pm$ SEM, $n \geq 6$ animals per group. # $P < 0.05$, ovalbumin (OVA) vs LPS; * $P < 0.05$, LPS vs LPS + anti-IFN- γ ; one-way ANOVA and Bonferroni's multiple comparison test.

Pollutants may abolish asthma protection induced by perinatal exposure to LPS

To investigate whether an exposure to pollutants is not only relevant in the prenatal period but also has an effect on the protected offspring, these mice were exposed directly to mycotoxins or DEP in weeks 5–6 of life. Exposure of LPS-treated offspring to gliotoxin, patulin, or DEP increased airway inflammation, OVA-specific IgE levels, and the production of Th2 cytokines compared to mice perinatally exposed to LPS but not to pollutants (Fig. 5A,C,E). Patulin also clearly enhanced eosinophilic inflammation in the BAL but only slightly increased the lung resistance. DEP significantly worsened lung resistance but had only marginal effects on the number of eosinophils, whereas gliotoxin enhanced both parameters (Fig. 5B,D). These data indicate not only an influence of pollutants on the perinatal asthma protection, but underline their additional impact on the asthma phenotype even for an exposure in later life.

Discussion

One of the most promising strategies to face the increasing asthma prevalence could be the induction of a nonspecific tolerance by an application of microbial compounds during pregnancy or early infancy. Already demonstrated in mouse models, a prenatal exposure to farm-derived bacteria or LPS induced an asthma protection in the offspring taking the hygiene hypothesis to a new level. In fact, especially materno–fetal interactions in response to environmental exposure seem to play a crucial role for the determination of the allergy risk of children (23). However, several studies investigating the immune modulatory effects of microbial compounds applying probiotics or bacterial lysates demonstrated a limited use so far (24, 25). Besides other factors that can influence such preventive strategies, there should also be considerate in the possibility of an interaction between microbial-induced effects with immunological responses caused by an exposure to environmental pollutants.

In the current study, we have shown that co-exposure to known allergy-promoting pollutants like mycotoxins or DEP during pregnancy inhibits the development of asthma protection induced by perinatal LPS exposure. Earlier studies demonstrated adjuvant effects or increased asthma susceptibility after exposure to certain pollutants (15, 20). Our data indicate an additional causal and direct role of environmental pollutants in the development of an allergic asthma later in life by disrupting the natural formation of microbial-induced tolerance during pregnancy.

Our data also demonstrate that the LPS-induced asthma protection in the offspring is dependent on IFN- γ , similar to the study showing this for the allergy-preventive effects by *Acinetobacter lwoffii* F78 (8). The anti-IFN- γ treatment in our study did not completely abolish the LPS-induced asthma-preventive effect, and we cannot exclude further involvement of still unknown mechanisms. However, there is strong evidence for an allergy-protective role of IFN- γ in mice as well as in human subjects. Mucosal IFN- γ gene

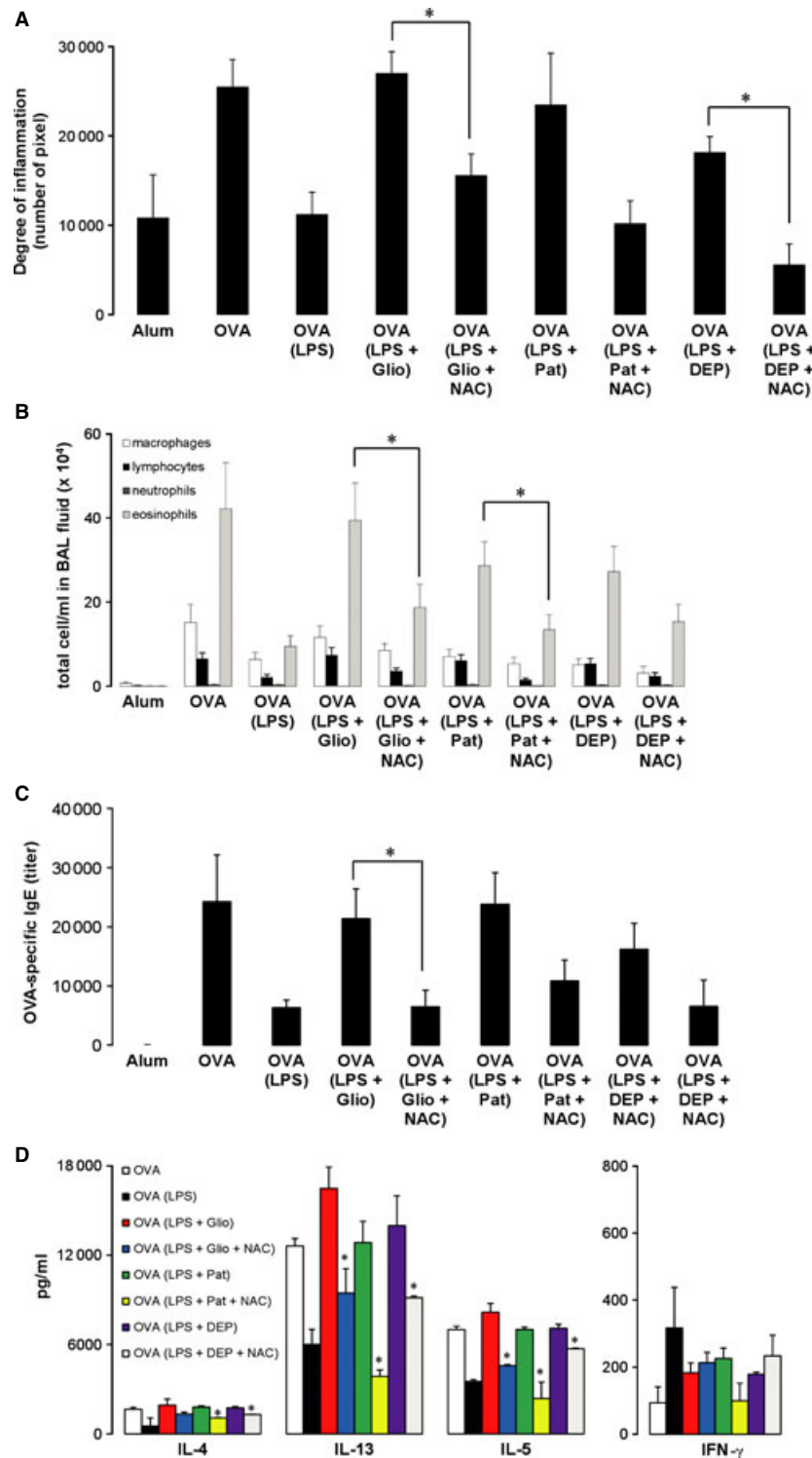


Figure 4 Effects on lipopolysaccharide (LPS)-induced asthma protection by pollutants are abrogated by the antioxidant NAC. Treatment of Balb/c mice with NAC (300 mg/kg i.p.) 30 min before exposure to the pollutants during pregnancy reduced the increased airway inflammation (A), the number of eosinophils in the bronchoalveolar lavage (BAL) fluid (B), the ovalbumin (OVA)-specific IgE

levels in the OVA-immunized offspring (C), and the Th2 cytokine levels in mediastinal lymph nodes compared to the offspring from NAC-untreated mothers (D). Data are expressed as mean $\pm$ SEM, $n \geq 7$ animals per group; * $P < 0.05$, one-way ANOVA and Bonferroni's multiple comparison test.

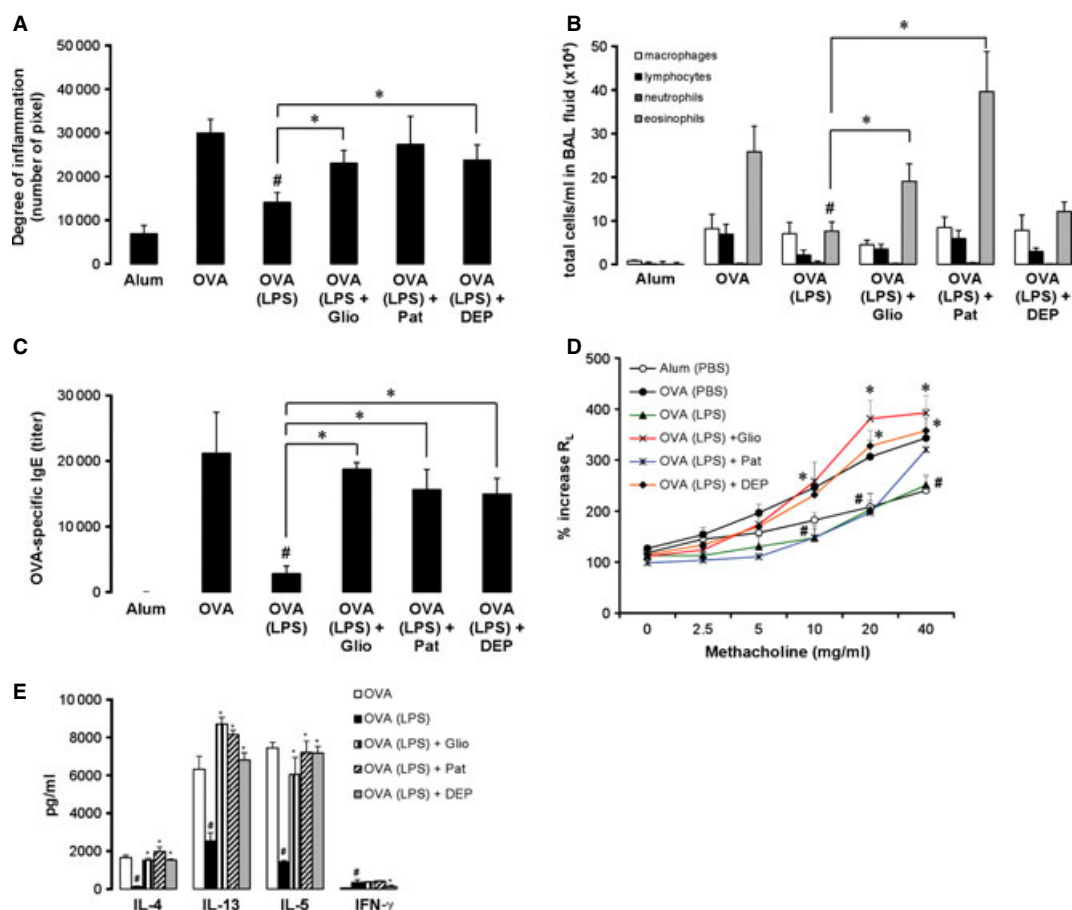


Figure 5 Exposure of 5-week-old offspring to pollutants abolishes asthma protection perinatally induced by lipopolysaccharide (LPS). The offspring from Balb/c mice exposed to LPS during pregnancy was further exposed to LPS in the first 4 weeks of life followed by an exposure to pollutants. The pollutant-exposed ovalbumin (OVA)-immunized offspring revealed increased airway inflammation (A),

cell numbers in the bronchoalveolar lavage (BAL) fluid (B), OVA-specific IgE levels (C), lung resistance (D), and Th2 cytokine levels (E) compared to OVA-immunized mice exposed only to LPS. Data are expressed as mean $\pm$ SEM, $n = 9$ animals per group; # $P < 0.05$, OVA vs LPS; * $P < 0.05$, LPS vs LPS + pollutants; one-way ANOVA and Bonferroni's multiple comparison test.

transfer and nebulized IFN- γ application inhibited eosinophilic inflammation in murine asthma models (26, 27). In addition, maternal farm exposure is significantly associated with a higher capacity of cord blood-derived mononuclear cells to produce IFN- γ , indicating a direct influence of environmental factors on prenatal T-cell maturation (28). On the other hand, it is known that pollutants may interfere with dendritic cell function leading to decreased Th1 differentiation and reduced IFN- γ levels that result in an exacerbated asthma phenotype. Accordingly, we observed a reversal of the asthma-protective epigenetic modification due to prenatal contact to LPS when exposing mice to gliotoxin or DEP, respectively.

Epigenetic processes, heritable changes in gene expression occurring without alterations in the DNA sequences (29), may be pivotal in prenatal immune programming (30). In particular, environmental exposures during pregnancy can alter gene expression via epigenetic mechanisms and are therefore

crucial for developmental processes and cell differentiation, including maturation of Th cells (31). Several human and murine studies provide strong evidence for a critical role of epigenetic processes within the IFN- γ promoter (32, 33). Brand and colleagues showed that reduced H4 acetylation within the IFN- γ promoter of OVA-immunized offspring was abrogated by prenatally exposure to *Acinetobacter lwoffii* F78. Interestingly, they did not detect any changes in DNA methylation at the IFN- γ promoter and the promoters of the Th2-relevant genes IL-4 and IL-5 (8). Therefore, we targeted H4 acetylation of the IFN- γ promoter in OVA-immunized offspring influenced by prenatal LPS exposure and its interaction with pollutants. Similar to *Acinetobacter lwoffii* F78, perinatal LPS exposure prevented against loss of H4 acetylation at the IFN- γ promoter in OVA-immunized offspring. This nicely correlated with LPS being identified as the key molecule of *Acinetobacter lwoffii* F78 inducing protective immune modulation (6). For the first time, we showed here

that also LPS may induce epigenetic alterations at the IFN- γ promoter. However, pulmonary co-exposure to gliotoxin or DEP during pregnancy again clearly reduced H4 acetylation at the IFN- γ promoter. While exposure to gliotoxin or DEP is mainly through inhalation of indoor air (34), we exposed mice to patulin via the intestine because this mycotoxin is primarily found in food (35). Interestingly, patulin exerted no significant change on H4 acetylation suggesting a different, still unknown mode of action.

The mechanisms how pollutants interact with the LPS-induced effects are still unclear. As shown before, several pollutants exert their action via the induction of oxidative stress (15, 22). Therefore, we applied an antioxidant to inhibit pollutant-induced effects. Results of this experiment give clear evidence that oxidative stress is involved in the abolishment of the perinatal asthma protection induced by microbes. Nevertheless, whether pollutants affect maternal tissues or cells leading directly to an interaction with the LPS-induced asthma protection or whether these environmental factors may have an influence on the fetal microenvironment, including independent epigenetic modifications that can counteract the microbial-induced effects, still has to be elucidated. However, the combined application of LPS and antioxidants seems to be a promising strategy to improve the success of an asthma-protective prenatal microbial therapy by avoiding adverse effects by environmental pollutants.

Another important finding of the current study is the abolition of LPS-induced asthma protection in the offspring after an exposure of 5–6-week-old mice to pollutants. These data indicate an increased asthma risk by a reversal of the LPS-induced protection after an exposure to pollutants to a later time point. Therefore, also contact to a microbial environment during pregnancy or early life without a prenatal interaction with other environmental factors may not be a guarantee to be prevented for asthma development in entire life.

In summary, our data show that exposure to pollutants like mycotoxins or DEP during pregnancy can inhibit the development or – in case of an exposure to pollutants later in life – the maintenance of a perinatal LPS-induced asthma

protection in the offspring. Although the use of a murine asthma model reflects human asthma only in some, but not all aspects, our observations emphasize not only the importance of the total maternal environment, but also the complexity of interactions of different environmental factor for the asthma risk of the next generation. Therefore, such interplay has to be considerate in the development of new primary prevention strategies.

Acknowledgments

The study was supported by the Helmholtz Association grant VG-NG-337 and LIFE – Leipzig Research Center for Civilization Diseases as well as by the Helmholtz Alliance on Systems Biology. We thank Michaela Loschinski for excellent technical assistance and Mohini Bhavsar for critical reading of the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

TP involved in conception and design; MR, SR, NS, IL, JS, ST, and TP involved in analysis and interpretation; TP, IL, and ST drafted the manuscript for important intellectual content; MR, SR, NS, IL, JS, ST, and TP gave the final approval of the manuscript.

Supporting Information

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

Data S1. Methods.

Figure S1. Exposure to LPS, co-exposure to pollutants and OVA immunization.

Figure S2. Effect of prenatal exposure to pollutants on the asthma phenotype in the offspring.

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Inhibition of endotoxin-induced perinatal asthma protection by pollutants

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SUPPORTING INFORMATION

METHODS

Mice

Mice were bred and maintained in animal facility at the University of Leipzig (Germany) and housed under conventional conditions with 23°C room temperature, 60% humidity, and 12 h day/night rhythm. The mice received conventional mouse feed (Altromin, Lage, Germany) and water *ad libitum*. All animal experiments involved groups of 4-6 mice/cage and were performed at least 2 times.

Treatment with anti-IFN- γ mAb

The offspring of LPS-exposed mothers received a neutralizing anti-IFN- γ mAb (XMG1.2, rat IgG1 anti-mouse IFN- γ mAb, 10 μ g/200 μ l, eBioscience, San Diego, California, USA) on day 14 and 21. Control animals received the same amount of IgG from rat serum antibody (1).

Collection of bronchoalveolar lavage (BAL) fluid

Cells in the lavage fluid were counted using a hemocytometer and BAL cell differentials were determined as described previously (1, 2).

Lung histology and computer-based quantification of inflammation. Left lung was fixed in 10% formalin and stained with Haematoxylin & Eosin (H&E, MERCK, Darmstadt, Germany). For quantification and objective evaluation of the degree of histological inflammation, lung sections were scanned with a digital camera (Zeiss, 5 shots per lung) and analyzed with HistoClick-Software (provided by Ingo Hohmann and Wiebke Scherf) based on morphometric image analysis (1, 3). The degree of inflammation is expressed by the number of pixels which correlate to the stained cells of interest.

OVA-specific IgE and IgG₁ assay

OVA-specific IgE and IgG₁ serum levels were measured by sandwich ELISA according to a standard protocol as described previously (1).

Measurement of airway responsiveness

Lung resistance (R_L) and dynamic compliance (C_{dyn}) were measured by invasive plethysmography (EMKA TECHNOLOGIES) in response to inhaled methacholine (Sigma-Aldrich). Therefore, mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine, Bayer, Leverkusen, Germany), intubated, and mechanically ventilated at a tidal volume of 0.2 ml and a frequency of 150 breath/min. Baseline R_L , C_{dyn} and responses to aerosolized saline (0.9% NaCl) were measured first, followed by responses to increasing doses (2.5 to 40 mg/ml) of aerosolized methacholine. To confirm the induction of AHR by OVA sensitization, AHR was assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole body plethysmograph (model PLT UNR MS, EMKA Technologies, Paris, France) as described previously (4).

Cytokine production

Splenocytes or mediastinal lymph node cells (5×10^6 cells/ml per well) were isolated and re-stimulated in vitro with 200 μ g/ml OVA in culture medium (RPMI medium supplemented with 10% FCS, 100 U/ml Penicilin, 100 μ g/ml Streptomycin) one day after airway function test. Cytokines were measured in supernatants from re-stimulated spleen cells after three days or from lung tissues using DuoSet[®] ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Chromatin Immunoprecipitation, ChIP

CD4⁺ cells from spleen were isolated as described earlier (4) and cultured overnight in RPMI containing 10% FCS. For each experiment 5 x 10⁶ cells were cross-linked for 10 min at 37⁰C in complete RPMI containing 1% formaldehyde followed by a quenching step of 10 min with 150 mM glycine. After cross-linking, chromatin DNA was sheared into 200-500 bp fragments by sonication using a Bioruptor®Next Gen (UCD-300, Diagenode SA, Liege, Belgium). Sonicated, soluble chromatin was immune-precipitated with 2.5 ug of an anti-*H4acetyl* antibody (Millipore, Billerica, USA). Control IPs were performed using rabbit IgG (Millipore, Billerica, MA, USA) corresponding to our specific antibody. DNA isolates from immunoprecipitates were used as templates for real-time quantitative PCR amplification using the following primer pair mIFN- γ _ChIP_for CATACCCTTTCCTTGCTTTTC, mIFN- γ _ChIP_rev TTGTGGGATTCTCTGAAAGCA. All ChIP experiments were performed at least five times per treatment condition (biological replicates). The acetylation status of the IFN- γ promotor was determined by calculating $\%Input = 2^{-(C_p[ChIP] - (C_p[Input * DF])}$. The dilution factor (DF) corresponds to the ratio of the chromatin fraction used as input vs. the IP fraction. To allow the comparability between the different animal exposure experiments, %Input of all treatments was normalized to the results of the corresponding ALUM mice.

Figure legend

Figure S1: Exposure to LPS, co-exposure to pollutants and OVA immunization.

Protocol A) Balb/c mice were exposed to aerosolized LPS (1 µg/10 ml) 3 times per week from day 7 of gestation time until delivery. The offspring was further exposed to LPS from day of birth up to day 28. Gliotoxin (100 ng i.n.), patulin (20 µg oral) or DEP (20 µg i.n.) were applied during pregnancy 30 minutes before LPS exposure. To induce an airway inflammation, 6-week old offspring was immunized with OVA i.p. (20 µg) on days 1 and 14 followed by 20 µg OVA i.n. on days 14-16 and 21-23. Airway hyperreactivity (AHR) was measured on day 24.

Protocol B) Balb/c mice were exposed to aerosolized LPS as described under Protocol A. Here, the offspring was exposed to pollutants in weeks 5 to 6 and then immunized with OVA.

Figure S2: Effect of prenatal exposure to pollutants on the asthma phenotype in the offspring.

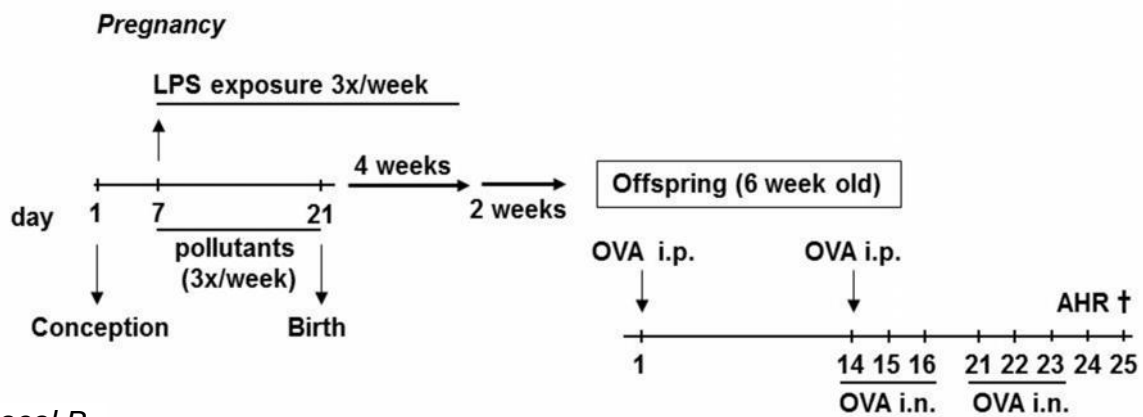
6-week old Balb/c mice prenatally exposed to pollutants were immunized with OVA (day 1 and 14) and then challenged with OVA on days on days 14-16 and 21-23. Effect of gliotoxin, patulin and DEP on airway inflammation (A), cell numbers in the BAL fluid (B), OVA-specific IgE levels (C) and AHR (D) compared to OVA-immunized mice exposed only to PBS.

References

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Figure S1

Protocol A



Protocol B

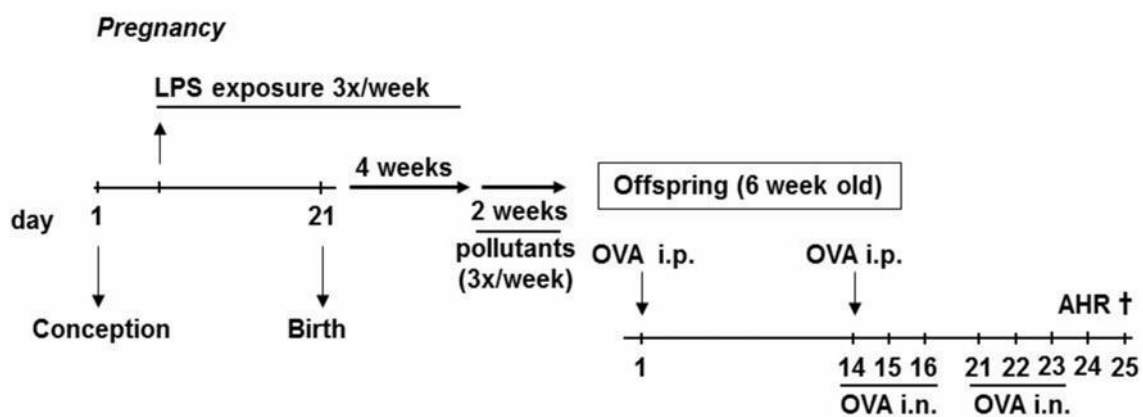


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Protocol B) Balb/c mice were exposed to aerosolized LPS as described under Protocol A. Here, the offspring was exposed to pollutants in weeks 5 to 6 and then immunized with OVA.

Figure S2

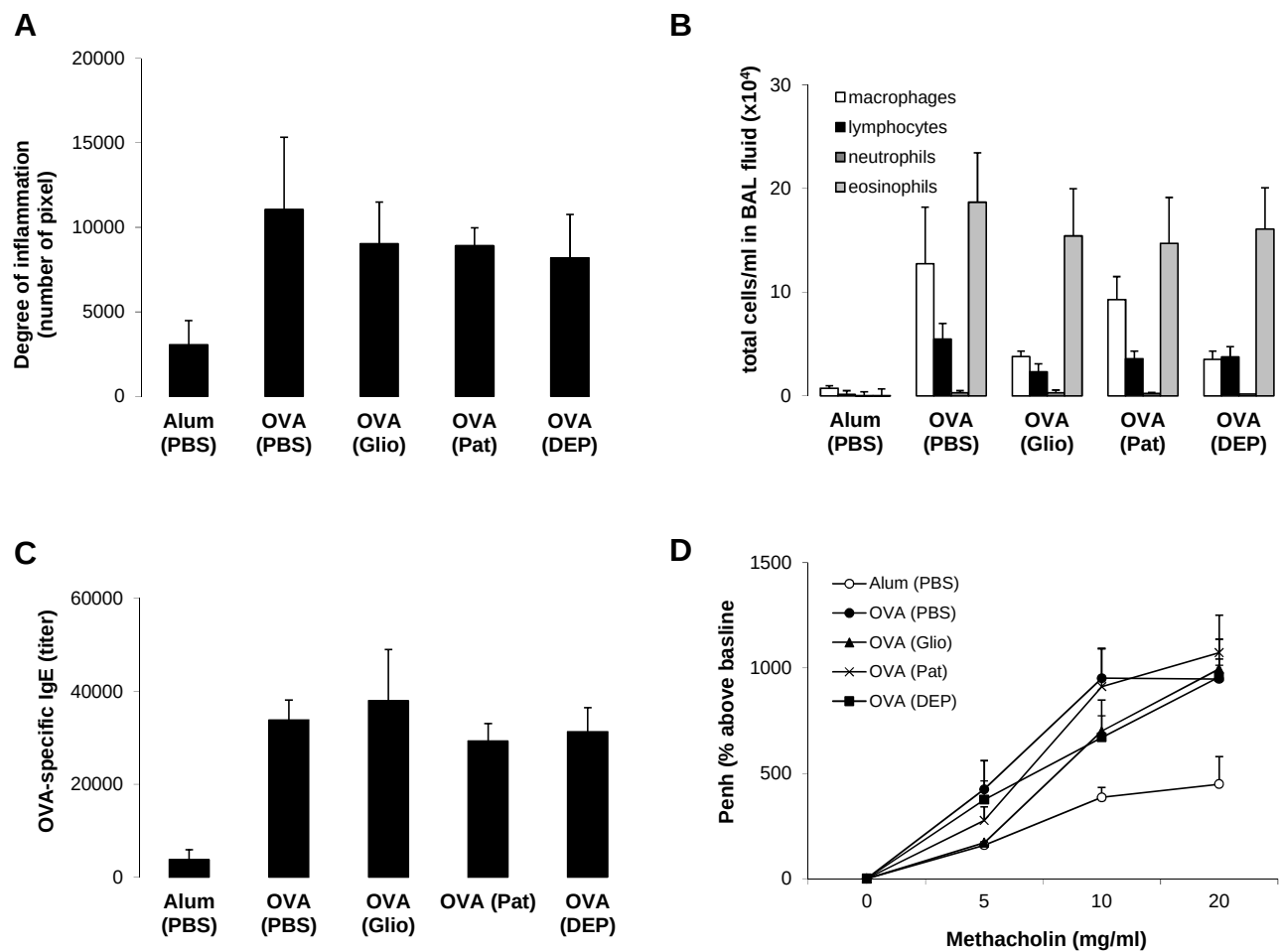


Figure S2: Effect of prenatal exposure to pollutants on the asthma phenotype in the offspring.

6-week old Balb/c mice prenatally exposed to pollutants were immunized with OVA (day 1 and 14) and then challenged with OVA on days on days 14-16 and 21-23. Effect of gliotoxin, patulin and DEP on airway inflammation (A), cell numbers in the BAL fluid (B), OVA-specific IgE levels (C) and AHR (D) compared to OVA-immunized mice exposed only to PBS.