



Maternal exposure to environmental DEHP exacerbated OVA-induced asthmatic responses in rat offspring

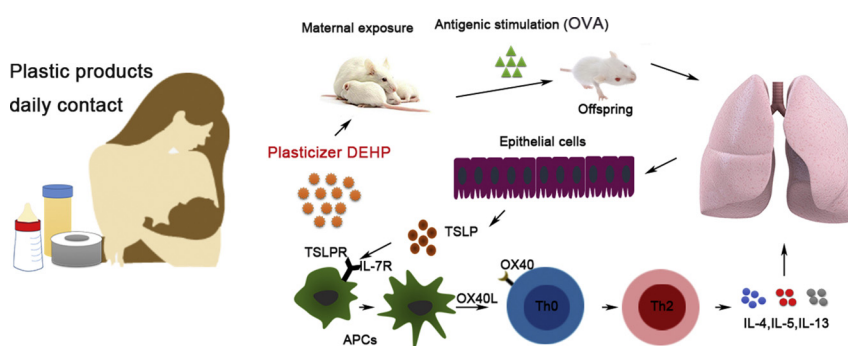
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HIGHLIGHTS

- Maternal exposure to DEHP during pregnancy and lactation aggravates pulmonary inflammation of the rat offspring
- DEHP maternal exposure increased the OVA-induced Th2 immune response the lung of the rat offspring
- DEHP maternal exposure increased OVA-induced Th2 cytokines in lung and promoted the expression of OX40L and GATA3
- These adjuvant effects of DEHP were considered to be closely related to the enhanced TSLP expression and the JAK signals

GRAPHICAL ABSTRACT



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ABSTRACT

Di (ethylhexyl) phthalate (DEHP) is a commonly used phthalates (PAEs) compound as plasticizer and becomes a severe environmental pollutant worldwide. Studies show that DEHP, as an environmental endocrine disruptor, has potential adverse effects on human. Epidemiologic studies indicate that DEHP is positively correlated to allergic diseases. Maternal exposure to DEHP may contribute to the increasing incidence of allergic diseases in offspring. However, the role of DEHP and its detailed mechanism in allergic disease of the offspring are still unclear. The aim of our study is to investigate whether DEHP maternal exposure could aggravate the allergic responses in offspring and its mechanism. Pregnant Wistar rats were randomly divided into three groups and exposed to different doses of DEHP. Half of the offspring were challenged with OVA after birth. All the pups of each group were sacrificed at postnatal day (PND) 14, PND21 and PND28. The number of inflammatory cells in bronchoalveolar lavage was counted, lung pathological changes were observed, Th2 type cytokines expressions were checked, and the expression of TSLP signaling pathway were examined. Our results showed that maternal exposure to DEHP during pregnancy and lactation aggravated the eosinophils accumulation and the pathological inflammatory changes in pups' lung after OVA challenge. And maternal exposure to DEHP during pregnancy and lactation also elevated the levels of typical Th2 cytokines in OVA-challenged rats. What's more, maternal exposure to DEHP during pregnancy and lactation increased the levels of TSLP, TSLPR and IL-7R in the offspring after OVA challenge. Our study suggested that DEHP maternal exposure could aggravate the OVA-induced asthmatic responses in offspring. And this adjuvant effect of DEHP was related with the TSLP/TSLPR/IL-7R and its downstream signal pathways.

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1. Introduction

Di (ethylhexyl) phthalate (DEHP), as the most commonly high molecular-weight (HMW) phthalates (PAEs), is a severe environmental pollutant worldwide, added to plastics to increase the flexibility of the plastic products (Carlstedt et al., 2013; Larsson et al., 2010). Studies showed that DEHP functioned as an environmental endocrine disruptor had potential adverse effects on human. Concentrations of DEHP as great as 110 mg/L have been found in river sediments (Horn et al., 2004). Up to 2700 µg/L DEHP was found in wastewater samples collected in the United States (Jackson and Sutton, 2008). And DEHP has been widely detected in Chinese rivers and lakes, including sources of drinking water (Zhang et al., 2015). Due to polyvinyl chloride (PVC)' wide-spread and growing use, there is an increasing interest and concern about these additives like DEHP have on humans and animals (Wormuth et al., 2006; Schettler, 2006). Since 1990s, a positive correlation was found between the PVC flooring and allergic diseases (Bornehag and Nanberg, 2010; Callesen et al., 2014; Larsson et al., 2010). The immune toxicity of PAEs have been studied for decades. Epidemiology studies indicated that PAEs was positively associated with allergic sensitization (Simmchen et al., 2012; Jaakkola et al., 2004; Kolarik et al., 2008). Animal experiments suggested that whether DEHP could influence the allergic responses might be associated with the exposure route (Dearman et al., 2008; Butala et al., 2004; Larsen et al., 2007; Larsen and Nielsen, 2007; Guo et al., 2012). The studies on phthalate esters' immune adjuvant or immunosuppressive mechanism more limited to in vitro studies (Fischer et al., 1998; Gourlay et al., 2003; Jepsen et al., 2004; Lee et al., 2004; Wang et al., 2012), it is difficult to clarify the mechanism of DEHP in vivo.

With the burden of children's allergic diseases, its relationship with PAEs drawn an increasing attention recently (Bornehag et al., 2004; Hsu et al., 2012; Ku et al., 2015; Lai et al., 2009). The reasons of allergic asthma in children include multiple factors, one of them is prenatal exposure. Prenatal exposure to environmental chemical contaminants might cause asthma or increase the risk of asthma symptoms throughout childhood (Smit et al., 2015; Gascon et al., 2015). Breastfeeding is also an additional means of phthalate exposure for infants. Some studies demonstrated that infants could be exposed to phthalates via breast milk (Bowman and Choudhury, 2016), indicating that exposure to DEHP during pregnancy and lactation could impact on the next generation. Various epidemiological studies of DEHP suggested a positive relation between its exposure and allergic asthma, especially in children with allergic asthma (Ait Bamai et al., 2014; Wang et al., 2014). However, the actual role of DEHP and its detailed mechanism in allergic disease of the offspring were still unclear.

The etiology of allergic asthma is the recognition of allergens or pathogenic microbial antigen by airway epithelial cells. Then a variety of cytokines were secreted, accompanied by reversible airflow obstruction, airway hyperresponsiveness (AHR), and eosinophilic and basophilic airway inflammation. And those symptoms characterized by generation of a predominant helper T cell (Th) 2-based cytokine environment (Lukacs, 2001; Lambrecht and Hammad, 2015). Th2 polarized CD4⁺ T cells secreting type 2 cytokines, including IL-4, IL-5 and IL-13, which stimulate the occurrence of asthma symptoms. The growing and compelling evidence indicated that airway remodeling and chronic airway epithelial injury during the first years of life facilitated the onset of asthma in childhood (Pohunek et al., 2005; Proud and Leigh, 2011). Recently, Studies had shown that the epithelial-derived cytokine thymic stromal lymphopoietin (TSLP) might play a central role in Th2 cell polarization (Gauvreau et al., 2014). TSLP, originally discovered in the supernatant of murine thymic stromal cell in 1994, was broadly defined as an epithelial-derived cytokine (Friend et al., 1994). It is overexpressed in the airways of allergic asthma patients and atopic dermatitis (AD) patients, indicating a key role in allergic inflammation (Shikotra et al., 2012; Soumelis et al., 2002). Since there is a positive correlation between DEHP and allergic diseases, whether DEHP could exacerbate

allergic diseases of the next generation through maternal exposure? And how DEHP exacerbate allergic diseases of the next generation? Is this adjuvant effect of DEHP related with TSLP signal pathway?

In the present study, we investigated the changes of OVA-induced asthmatic responses in rats' offspring after DEHP exposure during pregnancy and lactation period. The regulatory effect of TSLP on OVA-induced Th2 responses was investigated. The mechanism of DEHP aggravated the offspring asthmatic responses through the TSLP pathway was studied. Our results suggested that maternal exposure to DEHP during pregnancy and lactation aggravated the OVA-induced asthmatic responses in offspring of rats. These adjuvant effects of DEHP were considered to be closely related to the enhanced TSLP expression and the changes of JAK signals.

2. Materials and methods

2.1. Animals

Female Wistar rats (130–150 g) were obtained from the Center for Experimental Animals at China Medical University (Shenyang, China) with a National Animal Use License number of SCXK-LN2013-0007. Animal use was approved by the Animal Use and Care Committee at China Medical University with a protocol number of CMU2100014008. All experiments and surgical procedures were approved by the Animal Use and Care Committee at China Medical University, which complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. Rats were housed at a temperature of 25 ± 2 °C with 12-h light/12-h dark cycles. DEHP-free food and water were provided ad libitum. Prior to administration, the rats were quarantined for at least a week.

2.2. DEHP exposure

Female Wistar rats were randomly assigned into three groups: (1) DEHP 0 group (intragastric administration (ig), 0 mg/kg bw/day), (2) DEHP 30 group (ig, 30 mg/kg bw/day), (3) DEHP 300 group (ig, 300 mg/kg bw/day). A total 20 pregnant rats were kept in each group. The male rats were kept under the same conditions as the female rats belonging to the DEHP 0 group. The day of the vaginal plug was taken as gestational day (GD) 0 (female: male = 2:1). During the entire pregnancy, and up to PND21, the dams of all the three groups were received gavage in different dosages.

2.3. Sensitization and challenge

The schematic diagram of the treatment schedule is shown in Fig. 1A. The pups of females received one of the following treatments: (1) At PND7 and 9, the offspring from the dams exposed with 0, 30, 300 mg/kg bw/day DEHP sensitized by intraperitoneal injection (i.p.) of 0.5 ml saline, and then the pups were challenged by intranasal instillation (i.n.) with 100 µl saline for 3 consecutive days from PND11 (DEHP0, 30 and 300 groups). (2) At PND7 and 9, the offspring from the dams exposed with 0, 30, 300 mg/kg bw/day DEHP sensitized by i.p. of 500 µg OVA in 0.5 ml saline, and then the pups were challenged by i.n. with 100 µg OVA in 100 µl saline for 3 consecutive days from PND11 (DEHP0, 30, 300 + OVA group). The same operation would be done again on PND14 and PND21.

2.4. Bronchoalveolar lavage, tissue fixation, and staining

After the pups were euthanized in each group on PND14, PND21 and PND28, lungs were lavaged two times with 1 ml each warm PBS via a tracheal polyethylene catheter. Total cells in bronchoalveolar lavage fluid were counted with a hemocytometer, whereas differential cell counts were performed using cytospin cell preparations stained with

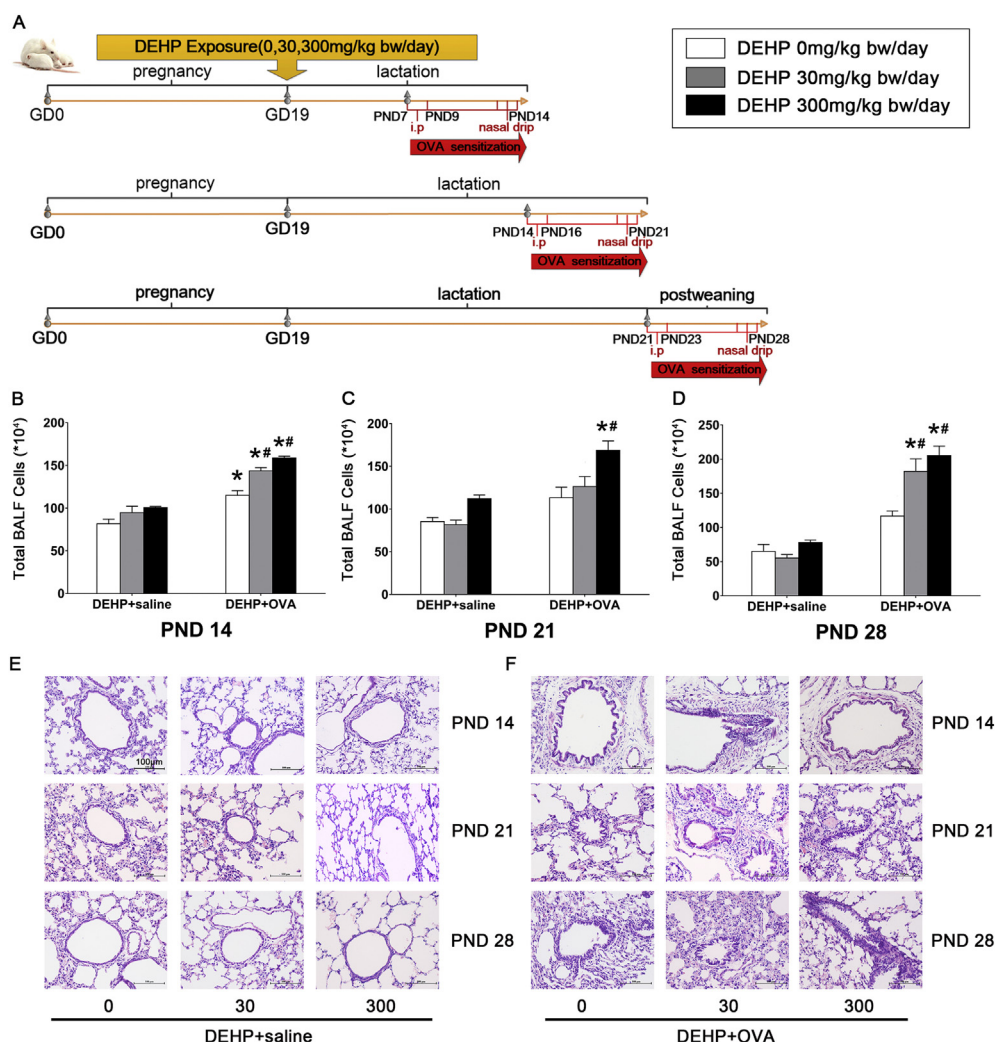


Fig. 1. DEHP exposure during pregnancy and lactation aggravated lung inflammation of the pups. DEHP exposure and Sensitization and challenge protocol (A). Total of inflammatory cells in bronchoalveolar lavage fluid (BALF) of the pups were counted using Giemsa staining after maternal exposure to DEHP during pregnancy and lactation on PND14, 21, 28 (B, C, D) ($n = 3$; “**” means compared between the same dose of DEHP + OVA group and DEHP + saline group $p < 0.05$; “#” means compared with DEHP0 + OVA group $p < 0.05$). Histopathology changes in pups’ lungs of all the DEHP + saline groups and DEHP + OVA groups after maternal exposure to DEHP during pregnancy and lactation observed with H&E staining ($\times 200$) (E, F).

modified Wright-Giemsa stain. After lavage, lungs were excised from thorax cavity. The lungs were inflated with 4% neutral buffered formaldehyde and fixed overnight at room temperature. Tissues were then embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin (H&E) or periodic acid-Schiff (PAS) stain.

2.5. ELISA measurements of cytokines in lung tissue

On PND14, 21 and 28, pups in each group were deeply anesthetized and euthanized by chloral hydrate. The lung tissue were rapidly removed from the thorax cavity and immediately submerged in liquid nitrogen. The tissue sample of each rat was homogenized in 500 μ l of buffered isotonic cocktail containing protease and phosphatase inhibitors. The sample was sonicated and incubated on ice for 30 min and then centrifuged at 13,000 g for 10 min at 4 $^{\circ}C$. The resulting supernatant was re-centrifuged and saved. The protein was estimated by Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Samples were stored at $-80^{\circ}C$ until analyzed. Tissue lysates of each rat sample were diluted to a protein concentration of 1 μ g/ μ l. To test other cytokine and chemokine levels, a rat IL-4 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN, USA), a rat IL-5 enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Biotech Co., Ltd., Wuhan, China), and a rat IL-13 enzyme-linked immunosorbent assay (ELISA) kit

(Cusabio Biotech Co., Ltd., Wuhan, China), were used in this study. All experimental procedures were in accordance with the manufacturer's instructions. Cytokine levels in lung homogenates from 4 different samples were tested three times in independent experiments.

2.6. Real-time PCR

On PND14, 21 and 28, total RNA was isolated from lung and thymus homogenates using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA with PrimeScript RT kit (DRR047A, Takara, Japan). Primers were designed with Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>), and the sequences (supplementary material) were checked using a BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences of specific primer pairs were described in Supplementary Table 1. GAPDH was set as internal control for determining ΔC_T values. Fold increases in expression were normalized to the DEHP0 group by determining $2^{-\Delta\Delta C_T}$ values.

2.7. Western blot analysis

On PND14, 21 and 28, the protein of the lung homogenate of the four pups per group was prepared as previously described. Tissue lysates of each rat sample were diluted to a protein concentration of 3

µg/µl and boiled for 5 min. Then, 10 µl aliquots (i.e., 30 µg protein) of each sample were loaded onto 8% SDS-acrylamide gels. Proteins were separated by application of a constant voltage of 100 V for 90 min and then transferred onto PVDF membranes at a constant voltage of 100 V for 60 min. After blocking nonspecific sites with TBS containing 0.1% Tween20 (TBST) and 5% defatted dried milk, membranes were washed and incubated with primary antibody rabbit anti-TSLP (Cell Signaling Technology, 1:200), rabbit anti-TSLPR (Santa Cruz Biotechnology, 1:200), rabbit anti-IL-7R (Santa Cruz Biotechnology, 1:200), rabbit anti-JAK1 (Abcam, 1:1000), rabbit anti-phospho-JAK1 (Abcam, 1:500), rabbit anti-STAT5 (Abcam, 1:500) and anti-phospho-STAT5a (Abcam, 1:500), rabbit anti-β-actin (Cell Signaling Technology, 1:2000) overnight at 4 °C. Then, membranes incubated with horseradish peroxidase-conjugated secondary antibody (Zhongshan Biotechnology, Beijing, China, goat anti-rabbit, 1:2500). Blots were developed with the high-performance luminol substrate solution (PexBio, Beijing, China). Initial control experiments determined the optimal time for exposing, which was maintained throughout the experimental procedure. Membranes were exposed to film for the optimal time for each antibody and then developed. Protein bands were subsequently quantified with an image analysis program (Gel Image System version 4.00) and the data were recorded, with the net optical density corrected for background chemiluminescence. For each blot, the β-actin lanes were analyzed as a quality control. The signals from target bands on each blot were normalized to the mean signal of the quality control sample bands in order to simplify across the blots and reduce inter-gel variability.

2.8. Statistics

All analyses were carried out with the SPSS software, version 22.0. Data were expressed as the mean ± SEM. Statistical comparisons were made using a one-way analysis of variance (ANOVA), and for the Post Hoc Multiple Comparisons were using Bonferroni test. The different labels such as “*” meant significant differences between the same DEHP dose with or without OVA challenge groups. And “#” meant significant differences between DEHP0 + OVA and DEHP30, 300 + OVA groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Maternal exposure to DEHP during pregnancy and lactation aggravated pulmonary inflammation of the rat offspring

The inflammatory response was usually observed in allergic disorders. The total inflammatory cells in bronchoalveolar lavage fluid (BALF) showed no obvious difference among three DEHP + saline groups on PND14, PND21 and PND28 (Fig. 1B–D). The total inflammatory cells increased significantly in offspring after OVA challenged compared with that of the saline control groups with the same DEHP dose. What's more, the total inflammatory cells in DEHP30 + OVA group and the DEHP300 + OVA group increased dramatically compared with that of the DEHP0 + OVA group on PND14 ($p < 0.05$) (Fig. 1B). The same trend was observed on PND21 and 28 (Fig. 1C, D). And the tissue section of all the DEHP + saline groups with H&E showed that the alveolar structure was relatively complete, and no obvious infiltration of inflammatory cells in alveolar interval and bronchial (Fig. 1E). But the trachea structures of all the DEHP + OVA groups' showed tracheal stenosis, and the airway remodeling was observed on PND14, 21, and 28 (Fig. 1F). According to these combined results, we could find that DEHP maternal exposure could aggravate the offspring' lung inflammation after OVA challenged.

3.2. Maternal exposure to DEHP during pregnancy and lactation increased the OVA-induced Th2 immune response the lung of the rat offspring

Eosinophilia makes a classical indication to the pathophysiology and pathology of allergic asthma. After the OVA challenged, the DEHP + OVA groups' lung tissue section with PAS could show eosinophil infiltration, bronchial luminal mucus increased, and tracheal thickening with infiltration. With the elevated dose of DEHP, the response increased, symptoms were exacerbated (Fig. 2A, B). The eosinophils increased significantly after OVA challenged compared with that of the saline control groups with the same DEHP dose. The eosinophils in DEHP30 + OVA group and the DEHP300 + OVA group increased dramatically compared with that of the DEHP0 + OVA group on PND14 ($p < 0.05$) (Fig. 2C), the same trend could be observed on PND21 and 28 (Fig. 2D, E).

Studies have shown that eosinophils infiltrating the lung preferentially stimulated Th2 cell responses by OVA. Therefore, Th2 cells are dominant in the bronchial airway epithelium, and Th2 cytokines such as IL-4, IL-5, and IL-13 play a pivotal role in the pathophysiology of asthma. After detection by ELISA, the expressions of IL-4 showed no obvious difference among three DEHP + saline groups on PND14, PND21 and PND28. It elevated much more after OVA challenged compared to the saline control groups with the same DEHP dose, especially the DEHP300 + OVA group (Fig. 1F–H). We also observed another Th2 cytokine IL-13. The difference of IL-13 level between the DEHP + OVA groups and DEHP + saline groups was not significant statistically on PND14, 21, and 28 (S-Fig. 1A–C). These data indicated that OVA could induce the Th2 immune response, and maternal exposure to DEHP aggravated its induction.

3.3. Maternal exposure to DEHP increased the expressions of OVA-induced Th2 cytokines in lung, rather than that in thymus in rat offspring

The relatively expressions of the Th2 cytokine mRNA were assessed in lung and thymus tissue. The expressions of IL-4 in lung showed no obvious difference among three DEHP + saline groups on PND14, PND21 and PND28 (Fig. 3A–C). And it increased significantly in lung after OVA challenged compared with that of the saline control groups with the same DEHP dose on PND21. And the expressions of IL-4 in the DEHP30 + OVA group, the DEHP300 + OVA group on PND21, and the DEHP30 + OVA group on PND28 increased dramatically compared with that of the DEHP0 + OVA group in lung ($p < 0.05$) (Fig. 3B, C), meanwhile the difference of IL-4 expressions between the DEHP + OVA groups and DEHP + saline groups was not significant statistically in thymus.

Another correlative cytokine IL-5 was also examined in lung and thymus tissue. The expressions of IL-5 in lung showed no obvious difference among three DEHP + saline groups on PND14, PND21 and PND28 (Fig. 3D–E). The expressions of IL-5 in lung increased significantly after OVA challenged compared with that of the saline control groups with the same DEHP dose on PND28, and the level of IL-5 in DEHP30 + OVA group increased dramatically compared with that of the DEHP0 + OVA group ($p < 0.05$) (Fig. 3E). The difference of IL-5 expressions between the DEHP + OVA groups and DEHP + saline groups in thymus was not significant statistically on PND14 and PND28. On PND21, the levels of IL-5 in DEHP30 + OVA group was higher than that of the saline control groups with the same DEHP dose.

Besides, IL-13 was another typical Th2 cytokine participated in the development of allergic diseases, without OVA challenged, the expressions of IL-13 of the saline control groups in lung showed no obvious difference with each other on PND14, PND21 and PND28 (Fig. 3G–I). The levels of IL-13 in DEHP300 + OVA group on PND14, DEHP30 + OVA group on PND28 increased significantly in offspring after OVA challenged compared with that of the saline control groups with the same DEHP dose. And also significantly elevated compared with that of the DEHP0 + OVA group ($p < 0.05$) (Fig. 3G, I).

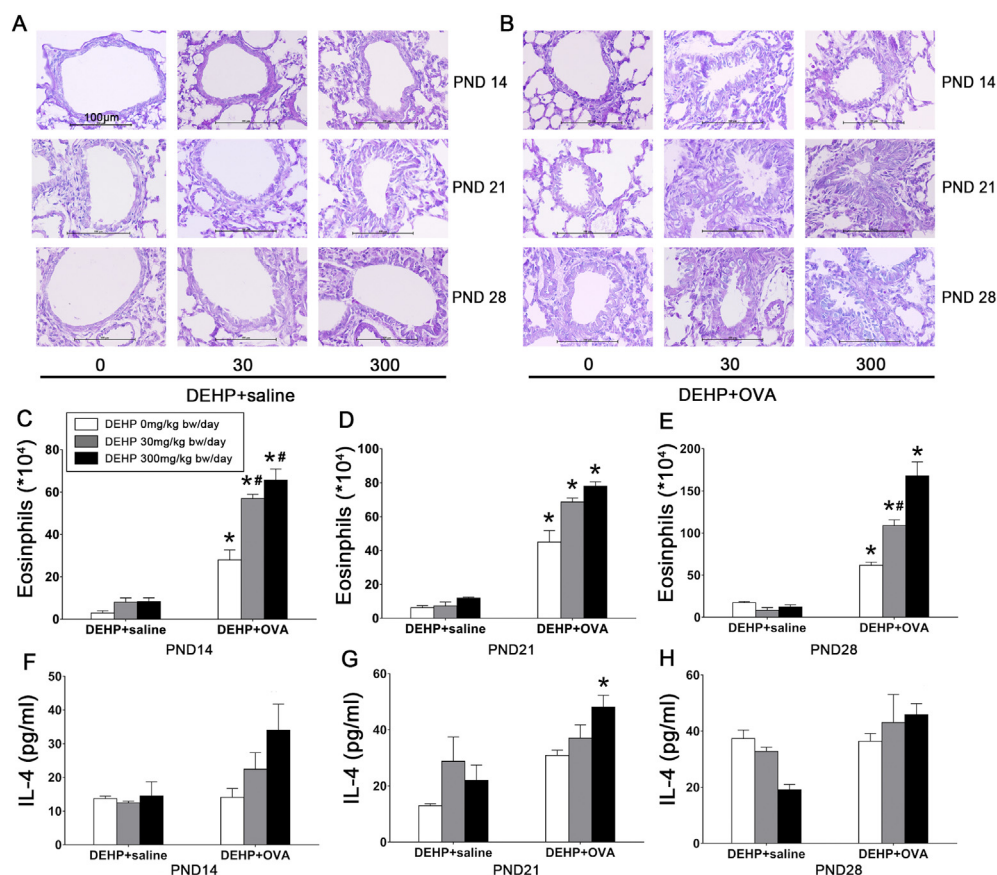


Fig. 2. DEHP exposure during pregnancy and lactation aggravated the symptoms of allergic asthma in the pups. PAS staining was used to determine the mucus production (goblet cells in evidence; $\times 400$) (A, B). Eosinophils in BALF of the pups was counted using Giemsa staining after maternal exposure to DEHP during pregnancy and lactation on PND14, 21, 28 (C, D, E) ($n = 3$). The levels of IL-4 in lung on PND14, 21, 28 (F, G, H) ($n = 4$; “*” means compared between the same dose of DEHP + OVA group and DEHP + saline group $p < 0.05$; “#” means compared with DEHP0 + OVA group $p < 0.05$).

In the result over-view of Th2 cytokines mRNA expressions in lung and thymus tissue, we could observed relatively clear increases of OVA-induced IL-4, IL-5 and IL-13 after maternal DEHP exposure. It was suggested that DEHP could promote the secretion of Th2, thus promoting the occurrence of allergic diseases. And at the same time, thymus Th2 cytokines changed slightly, indicating that DEHP could increase the expressions of OVA-induced Th2 cytokines in lung, rather than that in thymus in rat offspring.

3.4. Maternal exposure to DEHP promoted the expressions of OX40L and GATA3 in OVA-induced asthmatic responses

OX40 on activated T cells with OX40L on antigen presenting cells (APCs) strongly promotes T-cell responses and is critical to the development of Th2-driven airway inflammation (Mehta et al., 2016). In our study, the expressions of OX40/OX40L showed no obvious difference among three DEHP + saline groups on PND28. Both of them in DEHP30 + OVA group and DEHP300 + OVA group were significantly elevated compared with that of the DEHP0 + OVA group (Fig. 4A, B).

The effects of genetic modulation of GATA-3, include Th2 differentiation promotion, directly Th2 cytokine expression upregulation, and consequently classical asthmatic responses enhancement (Ano et al., 2013). Our results demonstrated that the expression of GATA3 mRNA in all the DEHP + saline groups showed no obvious difference on PND14, PND21 and PND28. The GATA3 mRNA levels of DEHP300 + OVA group on PND14 and DEHP30, 300 + OVA groups on PND28 were significantly increased compared with that of the DEHP0 + OVA

group in lung ($p < 0.05$) (Fig. 4C–E). The elevated levels of Th2 transcript factor indicated that maternal exposure to DEHP promoted the OVA-induced asthmatic responses.

3.5. The levels of TSLP and its receptors were elevated during OVA-induced asthmatic responses after maternal exposure to DEHP

Thymic stromal lymphopoietin (TSLP), can trigger dendritic cells (DC)-mediated Th2 inflammatory responses (Wang et al., 2013). TSLP signaling requires a heterodimeric receptor complex composed of interleukin-7 receptor α chain (Gene Symbol, IL-7R) and the TSLP receptor subunit (TSLPR; Gene Symbol, CRLF2) (Zhong et al., 2014). According to the results of western blot, the level of TSLP showed no obvious difference among three DEHP + saline groups, and it was increased significantly in offspring after OVA challenged compared with that of the saline control groups with the same DEHP dose. Especially the level of TSLP in DEHP30 + OVA group increased dramatically compared with that of the DEHP0 + OVA group on PND21 ($p < 0.05$) (Fig. 5A, B). The same tendency could be observed in the expressions of TSLP's receptors TSLPR and IL-7R (Fig. 5C, D). These data suggested that maternal exposure to DEHP enhanced the offspring' TSLP and its receptors to activate the Th2 type asthmatic responses.

On the PND14 and PND28, the levels of TSLP and its receptors were slightly up-regulated after OVA challenged (S-Fig. 2A–C, I–K). And at the same time, the mRNA expressions of TSLP were much more clearly increased. The elevated TSLP expressions among all the DEHP + OVA groups were observed on PND14. The DEHP30 + OVA group's TSLP

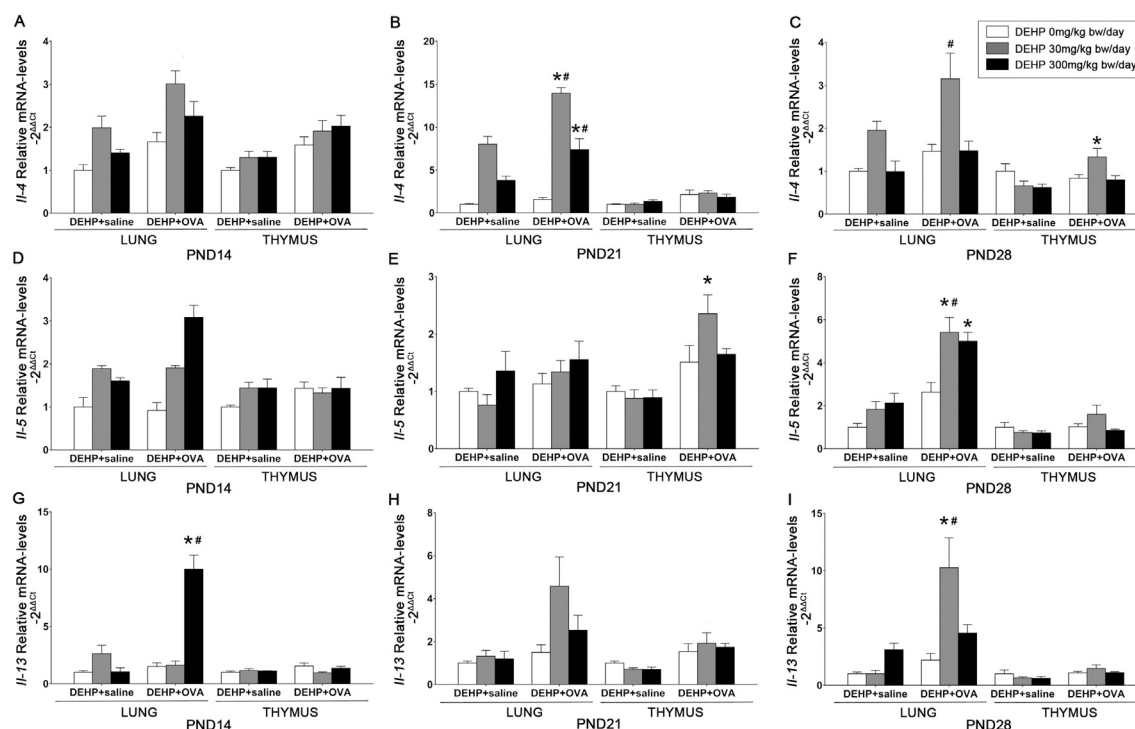


Fig. 3. Compared with the reaction of lung, Th2 type cytokines in the pups' thymus were slightly affected after the OVA sensitization with DEHP exposure during pregnancy and lactation. Relative expressions of typical Th2 cytokine IL-4 (A, B, C), IL-5 (D, E, F), and IL-13 (G, H, I) in lung ($n = 3-6$) and thymus ($n = 3-4$) on PND14, 21, 28 were assayed by realtime PCR. ("**" means compared between the same dose of DEHP + OVA group and DEHP + saline group $p < 0.05$; "#" means compared with DEHP0 + OVA group $p < 0.05$).

mRNA expression increased dramatically compared with that of the DEHP0 + OVA group ($p < 0.05$) (S-Fig. 2E). On PND28, the TSLP and receptors mRNA expressions apparently increased in offspring after OVA challenged compared with that of the saline control groups with the same DEHP dose, and the TSLP mRNA expression in DEHP30 + OVA group, TSLPR mRNA expressions in DEHP30,300 + OVA groups were higher than that of the DEHP0 + OVA group ($p < 0.05$) (S-Fig. 2L–N). All the results demonstrated that maternal exposure to DEHP increased the levels of TSLP and its receptors to promote the asthmatic responses.

3.6. The levels of JAK1-STAT5 pathway were slightly up-regulated after maternal exposure to DEHP

The JAK-STAT pathway is a signal transduction pathway for multiple cytokines, and now it is known that this pathway plays a certain role in T cell immunity (Waldmann and Chen, 2017). In our study, the expressions of JAK1 and STAT5 with their phosphorylated production were assessed on PND21 (Fig. 5H), compared with the control groups, the secretion of JAK1 in DEHP0 + OVA groups increased significantly ($p < 0.05$) (Fig. 5J). Yet the levels of p-JAK1, STAT5, p-STAT5 showed no

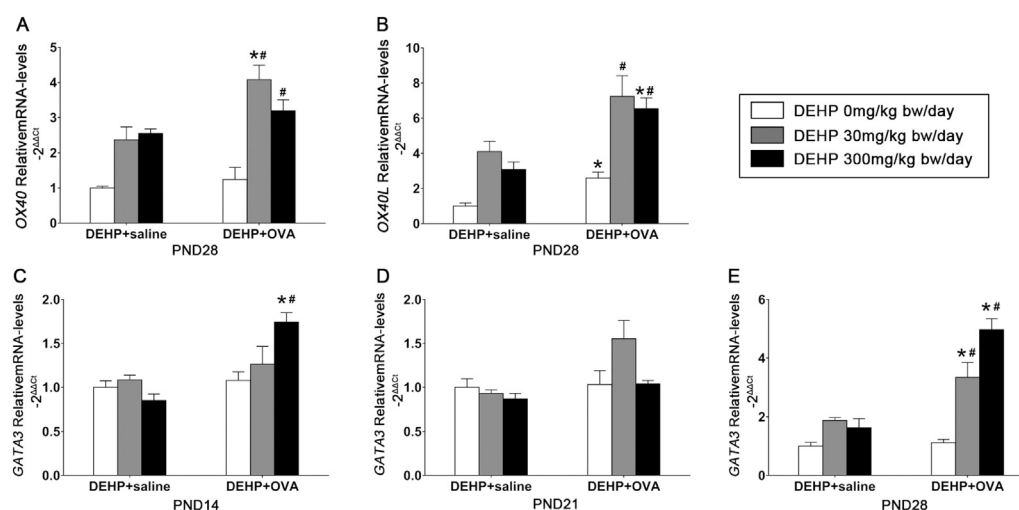


Fig. 4. The relative expression levels of GATA3, OX40/OX40L in the OVA challenged pups' lung tissue were significantly increased after DEHP exposure during pregnancy and lactation. Relative expressions of OX40/OX40L in lung on PND28 (A, B). And Th2 cytokines' typical transcription factor GATA3 in lung on PND14, 21, 28 were assayed by realtime PCR (C, D, E). ($n = 3-6$; "*" means compared between the same dose of DEHP + OVA group and DEHP + saline group $p < 0.05$; "#" means compared with DEHP0 + OVA group $p < 0.05$).

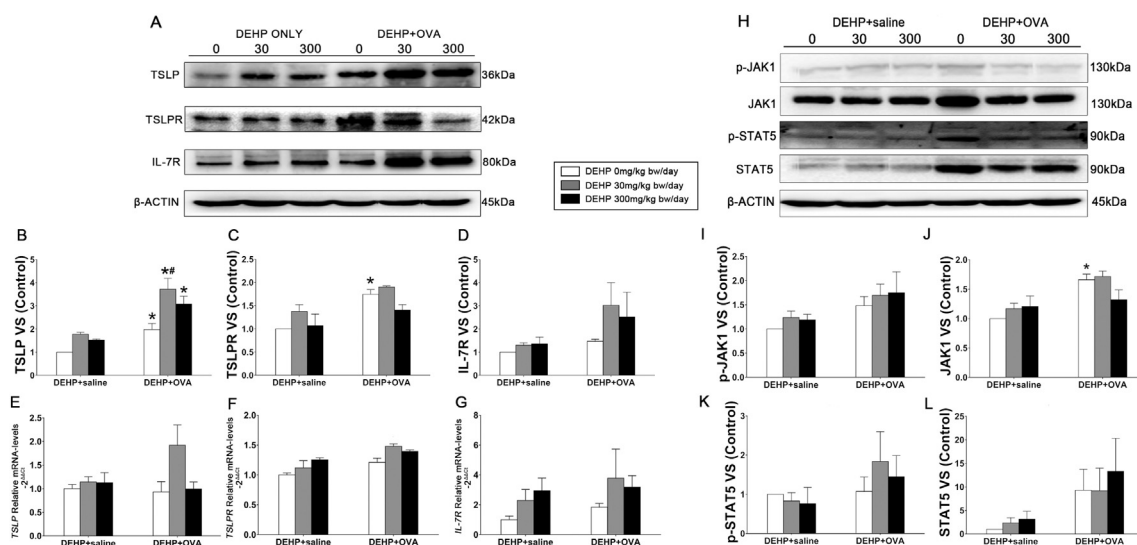


Fig. 5. Sensitization groups' expression of TSLP and its receptors were increased after the DEHP exposure during pregnancy and lactation, with JAK1-STAT5 were slightly changed. Western blot bands of TSLP, TSLPR, IL-7R proteins on PND21(A), Western blot analyses of TSLP(B), TSLPR(C), and IL-7R(D) on PND21 ($n = 3-4$). Proteins expression of JAK1-STAT5 and their phosphorylation product were detected and analysis by Western blot (H, I, J, K, L) on PND21 ($n = 3-4$; “*” means compared between the same dose of DEHP + OVA group and DEHP + saline group $p < 0.05$; “#” means compared with DEHP0 + OVA group $p < 0.05$).

significant difference between groups (Fig. 5I, K, L). And on PND14 and PND28, the expression of JAK1-STAT5 pathway had a slightly up-regulation, although most of the differences were caused by the OVA challenged (S-Fig. 3). In our study, the JAK-STAT pathway was slightly changed after the maternal DEHP exposure.

4. Discussion

In this study, the inflammatory response of the lung was observed, the number of total inflammatory cells and eosinophils in the BALF were increased at multiple time points after birth. This result was consistent with some previous studies, animals injected with DEHP were more susceptible to OVA challenge, which demonstrated the potent adjuvant effect of DEHP (Han et al., 2014; Larsen and Nielsen, 2007; Win-Shwe et al., 2013). Our study further demonstrated that mother exposed to DEHP during pregnancy and lactation, could increase the risk of offspring hypersensitivity, and led to high allergic reactions in offspring even after stopping the breastfeeding. Classical asthma and allergy are inflammatory diseases caused by dysregulated immune responses in the respiratory mucosa. Activated Th2-driven responses promoted the development of asthma. Thus, CD4⁺ T cells producing Th2 cytokines played a prominent role in the lungs of asthmatic subjects (Umetsu and Dekruyff, 2006). Th2 cells express the canonical type 2 cytokines IL-4, IL-5, and IL-13, induced antibody isotype switching in B cells, eosinophilia, and AHR and mucus production respectively (Muehling et al., 2017). In this study, IL-4 significantly statistical increased in the filial rats whose mothers exposed to DEHP. The same tendency was observed in the levels of IL-5 and IL-13 (Izuhara et al., 2002). And we had observed that all the Th2 cytokines acted on epithelial cells to enhance their production of innate cytokines and to augment the secretion of proinflammatory mediators, which caused allergic reaction like eosinophil infiltration, tracheal stenosis and the airway remodeling. However, the specific immune mechanisms still need further explored. Our results suggested that maternal DEHP exposure during pregnancy and lactation aggravated the OVA-induced asthmatic responses in rats' offspring. These adjuvant effects of DEHP were considered to be closely related to the enhanced TSLP expression and the changes of JAK signals.

Fetal and early postnatal life was the critical periods in the development of the immune system during which environmental factors could alter the trajectory of physiological system maturation thereby affect

the risk of chronic diseases in both children and adults (Dietert, 2011). In our study, during the lactation and early weaning, infant rats' thymus was slightly affected after the maternal exposure. The early postnatal rodent immune system was immature and failed to produce antibodies to antigens (Landreth, 2002). And we observed the expressions of Th2 cytokines in progeny' thymus sensitized by OVA after pregnant and lactating exposure to DEHP. On PND14, the sensitized groups and the non-sensitized groups showed no difference. The expressions of Th2 type cytokines in each sensitized groups were higher than that in the non-sensitized groups when sustained breastfeeding on PND21 (i.e., with DEHP exposure). On PND28, the levels of Th2 cytokines between the sensitized groups and the non-sensitized groups showed no obvious difference, which might because the developmental thymus was immature in the early stage of life, so the response to antigens was relatively weak.

As known, in response to an array of environmental triggers, the epithelial cells overproduced multiple cytokines, such as TSLP, IL-25, and IL-33 (Muehling et al., 2017; Mitchell and O'Byrne, 2017). In humans and mice, the functional TSLP receptors complex consisted of a unique common γ (γ c) chain-like TSLP receptor (TSLPR) and the IL-7 receptor α chain (IL-7R α), while the TSLPR chain alone binds to TSLP with low affinity (Pandey et al., 2000; Redhu and Gounni, 2012). Those receptors were expressed on a variety of cell types, including DCs, T cells, B cells, and monocytes (Kitajima et al., 2011). Airway DCs were one of the most important immune cells in the development of asthma bronchiale, because they could activate naïve T cells and polarize primed Th2 cells in the lung (Kuipers and Lambrecht, 2004). TSLP stimulated myeloid DCs, resulted in T cells showing characteristic features of Th2-differentiated inflammatory (Soumelis et al., 2002). Study demonstrated that TSLPR and IL-7R α could associate with JAK2 and JAK1 respectively, followed by the activation of signal transducer and activator of transcription (STAT) proteins in human DCs and murine CD4⁺ T lymphocytes (Salek-Ardakani et al., 2003; Zhou et al., 2005). In human DCs, TSLP has been shown to activate STAT-1, STAT-3, STAT-4, STAT-5 and STAT-6. Mice studies showed that TSLP stimulation led to the activation of STAT5 and the induction of STAT5 responsive genes (Rochman et al., 2010; Tormo and Gauchat, 2013). Activation of its receptors by TSLP was shown to induce STAT5 phosphorylation and expression of the STAT5-inducible gene *Cis* encoding the cytokine inducible SH2-containing protein (Liu et al., 2007). In our study, the levels of TSLP and its receptors were elevated during OVA-induced

asthmatic responses after maternal exposure to DEHP. And the levels of JAK1-STAT5 pathway were slightly up-regulated after maternal exposure to DEHP. Activated DCs migrated to the draining lymph node, where they regulated the differentiation and the proliferation of naïve T cells into effector T cells. Research showed that TSLP induces the expression of OX40L, the ligand for cell survival factor OX40 on DCs; OX40-OX40L interactions were found to be critical for the ability of the mDCs to drive Th2 cell differentiation (Ito et al., 2005). Another expression and production of all these Th2 cytokines have been shown in the process and invertebrates to be controlled by the zinc finger transcription factor GATA3, which is essential for Th2-cell differentiation and activation and is considered to be the master transcription factor of the Th2 pathway of immune activation (Krug et al., 2015). In our study, we observed the increased expression of OX40-OX40L, and the level of GATA3 was also elevated in the sensitized groups.

Allergic diseases were intricate diseases resulting from the interaction of genetic and environmental factors (Hendaus et al., 2016). The bronchial epithelia could be stimulated by allergens, then expressed various pathogen recognition receptors, including TSLP and others. TSLP was demonstrated to activate and instruct DCs by combined its receptors TSLPR and IL-7R α , though JAKs-STATs pathway. Activated DCs produced the OX40L and migrated into the draining lymph nodes, where they triggered the differentiation of allergen-specific naïve CD4+ T cells by binding to the receptor OX40. Then these naïve CD4+ T cells differentiated into inflammatory Th2 cells, producing IL-4, IL-5 and IL-13 (Kaur and Brightling, 2012). These Th2 cytokines recruited a variety of eosinophils around bronchial airway, which could promote mucus secretion, increase airway responsiveness and resistance. In this study, we observed obvious histological and physiological changes in OVA sensitized pups after DEHP exposure during pregnancy and lactation. The dramatic infiltrated inflammatory cells, especially the eosinophils, indicated that the pup's airway inflammation was aggravated by DEHP maternal exposure. The elevated levels of Th2 cytokines suggested that DEHP maternal exposure could increase the risk of asthma in the offspring. These findings indicated that maternal exposure to DEHP facilitate the development of asthma in offspring through the aggravated of Th2 immune response. And these adjuvant effects of DEHP were not affected by weaning, which were consistent with those epidemiologic studies that maternal exposure to chemicals during pregnancy increased the risk of asthma in childhood (Duijts, 2012; Shaheen et al., 2010). What's more, our study also showed an increased levels of TSLP, TSLPR and IL-7R α in OVA-challenged rats' offspring after maternal DEHP exposure. Although the upregulation were not dose-dependent, this study demonstrated that the adjuvant effect of DEHP might be mediated through TSLP pathway. The association between in pregnancy and lactation exposure to DEHP should be noted, and the underlying mechanisms remain largely a matter of speculation. Genetic predisposition and epigenetic changes still need to be explored in future.

5. Conclusion

Our study suggested that maternal exposure to DEHP during pregnancy and lactation might aggravate the OVA-induced asthmatic responses in offspring of rats. These adjuvant effects of DEHP were considered to be closely related to the enhanced TSLP expression and the changes of JAK signals.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2017.09.276>.

Compliance with ethical standards

The manuscript does not contain clinical studies or patient data.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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