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ORIGINAL ARTICLE

# Intrauterine sensitization of ovalbumin in the third trimester increases the risk of food allergy in progeny



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## KEYWORDS

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**Abstract** Intrauterine sensitization caused by food allergens plays an important role in the food allergy development in progeny. The aim of our study was to determine the critical period of intrauterine sensitization during pregnancy. Female mice were exposed to ovalbumin (OVA) during different trimesters of pregnancy. Lymphocytes from their offspring were isolated and cultured, and proliferation was evaluated by CCK-8 assay. The levels of IFN- $\gamma$  and IL-4 in serum were measured using ELISA. In addition, the expressions of IFN- $\gamma$  and IL-4 mRNAs and proteins were detected by real-time PCR and western blot. The mice were divided into the first trimester pregnancy (FTP1 and FTP2) group, the second trimester pregnancy (STP1 and STP2) group, and the third trimester pregnancy (TTP1 and TTP2) group based on the stages of pregnancy in which their mothers were exposed to OVA and their ages. The OVA-specific lymphocyte proliferation of the TTP1 group was statistically significantly greater than in the FTP1 and STP1 groups. The serum level of IFN- $\gamma$  in the TTP1 group was significantly decreased, and the serum level of IL-4 in the TTP1 group was significantly increased compared with the levels in the FTP1 and STP1 groups. The mRNA and protein expression levels of IFN- $\gamma$  in the TTP1 group were significantly decreased and the mRNA and protein expression levels of IL-4 in this group were significantly increased compared with the levels in

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the FTP1 and STP1 groups. Our results suggest that OVA-induced intrauterine sensitization in the third trimester may increase the risk of food allergy after birth.

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## 1. Introduction

In recent decades, with economic and social development, the incidence of food allergy is rising and causing concerns worldwide. As a widespread health problem from the epidemiology perspective in developed countries, food allergy affects nearly 10 percent of children (Umetsu et al., 2015). Over the past 15 years, the incidence of food allergy has exhibited a rapid growth in the United States, Britain, China and other countries (Prescott et al., 2013; Bunyavanich et al., 2014). Food allergy often occurs in infants and young children within the first three years after birth, and children suffering from food allergy have a higher risk of other allergic diseases compared with non-allergic children, including atopic dermatitis, allergic respiratory diseases, and asthma (Branum and Lukacs, 2009; Peng et al., 2015). Therefore, children suffering from food allergy have an increased risk of developing other allergic diseases at the later stage of life. However, due to the complexity of food allergy pathogenesis, effective prevention and treatment measures are still lacking. The only effective treatment nowadays is to strictly avoid intake of certain food antigens to prevent its occurrence (Patel and Volcheck, 2015). However, this method does not completely cure food allergy and restricting food intake may lead to certain type of malnutrition in children. Therefore, the early prevention of food allergy is particularly important. It is critical to find the initial antigen sensitization period.

Generally speaking, allergen sensitization is the outcome of the allergen and the host. The antigen penetrates through the mucosal epithelial layer of the gastrointestinal tract, skin or respiratory tract and interacts with innate immune receptors, such as Toll-like and protease-activated receptors on epithelial cells, stimulating them to produce cytokines that drive T-helper 2-like adaptive immunity in allergy-prone individuals. While clinical practice finds that infants who were exclusively breastfed do not contact with antigens such as milk, they still will develop early gastrointestinal symptoms indicating milk allergy, which suggests that exposure to food antigens during pregnancy might be one of the mechanisms of early allergen sensitization (Atanaskovic-Markovic, 2014; Liu, 2013). At present, whether exposure to food antigens can cause intrauterine sensitization still remain to be debated. Iván et al. (2003) confirmed that, in mice and other animal models, maternal low-dose peanut exposure during pregnancy and lactation are more effective in alleviating offspring sensitization reactions compared with completely avoiding peanut intake. This study suggested that low-dose exposure of food antigens during pregnancy may facilitate intrauterine tolerance of the fetus and reduce the risk of allergic diseases after birth. However, increasingly more lines of evidence demonstrate that intrauterine sensitization caused by food antigens plays an important role in the allergy development of the progeny. Prokešová et al. (2008) showed that allergen-specific IgE was significantly increased in the blood of mothers who suffered from allergic diseases and fetal cord

blood, and this increase predicted the increased risk that the offspring would suffer from allergic diseases in the future. Desroches (2010) found that pregnant women who were early exposed to food allergens had an increased incidence of fetal food allergy after birth, suggesting that these children had been primed in uterus during pregnancy.

Although there are many studies supporting intrauterine sensitization, the start time of intrauterine sensitization is unclear. Studies have shown that consumption of nuts in the first two months of pregnancy increased the risk of nut allergy in offspring (Hsu et al., 2012, 2013). The offspring of pregnant women who were exposed to birch pollen during the weeks 20–28 of pregnancy had a higher specific response to birch pollen (Duren-Schmidt et al., 1997). There was also a positive correlation between exposure to passive smoking in the third trimester of pregnancy and asthma and other allergic symptoms in offspring (Xepapadaki et al., 2009). The results of our previous study also suggested that pregnant mice exposed to OVA during the period of pregnancy could induce sensitization of fetal mice, and the critical period may be in the late of pregnancy (Hu et al., 2005). Therefore, we hypothesized that a certain stage of pregnancy is a critical period for intrauterine sensitization. Antigen exposure during this period could significantly increase the risk of food allergy in the progeny. This study aimed to determine the critical period of intrauterine sensitization by giving OVA to pregnant mice in the different stages of gestation. Our findings will help to improve the understanding of the underlying mechanisms of food allergy during early childhood.

## 2. Methods

### 2.1. Experimental animals

A total of 20 healthy BALB/c mice (male: 5, female: 15) were obtained from the Chongqing Medical University Experimental Animal Center. According to Keppel's method (Knippels and Spanhaak, 1998), the mice were provided with special purebred feed with successive generations. The third generation of six-to eight-week-old female mice, with an average body weight of  $20 \pm 2$  g, were selected as experimental animals. The institutional guide to the care and use of experimental animals was followed.

Taking the presence of a vaginal plug as the first day of pregnancy, pregnant mice were randomly divided into the following groups: Group FTP (the first trimester of pregnancy), Group STP (the second trimester of pregnancy), Group TTP (the third trimester of pregnancy), and the corresponding Group C (control). All the young born to a mouse at one time were used as a sample, therefore the number of offspring samples in each group corresponded to the parental mice number in each group.

According to the stage of pregnancy in which mice were exposed to OVA, the 5-day-old offspring were divided into the following groups: Group FTP<sub>1</sub>, Group STP<sub>1</sub>, Group

TTP<sub>1</sub> and the corresponding Group C<sub>1</sub>. The 6–8-week-old offspring were divided into these groups: Group FTP<sub>2</sub>, Group STP<sub>2</sub>, Group TTP<sub>2</sub> and the corresponding Group C<sub>2</sub>.

## 2.2. OVA sensitization

The experimental animals were subjected to OVA during pregnancy. Group FTP was immunized on days 1, 4, and 7 by intraperitoneal injections of 10 µg of OVA (Grade V, Sigma–Aldrich, Saint Louis, MI, USA) complemented with 1.0 mg of alum (InjectAlum; Pierce Biotechnologies Inc., Rockford, IL, USA) in a total volume of 0.5 ml saline. Group STP and Group TTP were immunized using the same method on days 8, 11, and 14 and days 15, 18 and 21, respectively. The corresponding control groups were given intraperitoneal injections of 1.0 mg of alum in a total volume of 0.5 ml saline.

The six- to eight-week-old female progeny were sensitized to OVA by intraperitoneal injections of 10 µg of OVA complemented with 1.0 mg of alum in a total volume of 0.5 ml saline. Two weeks later, serum samples were collected to detect OVA-IgE.

## 2.3. Cell isolation and culture

As the 5-day-old mice were quite small, spleens of several mice of littermates were harvested and mixed as a sample. The spleens were prepared for cell suspension by mincing through a 50-µm cell strainer. Lymphocytes were isolated by density gradient centrifugation with EZ-Sep™ Mouse 1× (Dakewei Biotech Company, Beijing, China). A total of  $2 \times 10^6$  cells were cultured in RPMI with 10% FCS and were exposed to OVA (Grade V, Sigma–Aldrich, Saint Louis, MI, USA) for 72 h.

## 2.4. OVA-specific lymphocyte proliferation

The cultured cells were used to test antigen specific lymphocyte proliferation with Cell Counting Kit-8 (CCK-8; Dojindo, Shanghai, China) per the manufacturer's instructions.

## 2.5. Cytokine & IgE detection

Supernatants were collected from the cultured cells and analyzed by ELISA (BD Biosciences, Franklin Lakes, NJ, USA) to determine the levels of IFN-γ and IL-4. Sera samples were collected by retro-orbital bleeding in advance of copulation and after delivery. ELISA kits were used according to the manufacturer's instructions. The levels of IgE were measured by ELISA using Rat Anti-Mouse IgE HRP conjugated antibody (Southern Biotechnology Associates Inc., Oxmoor Blvd, Birmingham, USA). IgE levels were analyzed based on the OD values.

## 2.6. Real-time PCR

Total RNA was extracted from the cultured cells using the RNeasy total RNA isolation assay (Takara, Otsu, Japan). Equal amounts of total RNA (800 ng), as determined by a Nanodrop-2000, were used in a 20 µl cDNA synthesis reaction primed with the PrimeScript™ RT Reagent Kit (Takara, Otsu, Japan). Prior to cDNA synthesis, residual genomic DNA was

removed from the total RNA with DNase I treatment (gDNA Eraser Buffer; Takara, Otsu, Japan). Quantitative real time PCR was performed with a CFX96 Fast Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA) using SYBR Green (Takara, Otsu, Japan). PCR conditions consisted of 1 cycle of 95 °C for 30 s; 39 cycles of 95 °C for 5 s, 60 °C for 30 s, 95 °C for 10 s. The relative expression levels were calculated according to the comparative Ct method in which the relative expression equals  $2^{-\Delta\Delta Ct}$ . The PCR primers were designed by TAKARA (Takara, Otsu, Japan). The PCR primers used for each gene were as follows:

IFN-γ forward, 5'-GGCTGTTTCTGGCTGTTACTGC-3';  
 IFN-γ reverse, 5'-CGCTTATGTTGTTGCTGATGG-3';  
 IL-4 forward, 5'-TGAACGAGGTCACAGGAGAAGG-3';  
 IL-4 reverse, 5'-GCACCTTGGAAGCCCTACAGA-3';  
 β-actin forward, 5'-ATATCGCTGCGCTGGTTCGTC-3';  
 and β-actin reverse, 5'-AGGATGGCGTGAGGGA  
 GAGC-3'.

## 2.7. Western blotting

Briefly, total protein extract for each tissue sample or cell line was dissolved in lyses buffer and equal amounts of protein (60 µg) were analyzed by immunoblotting. Rabbit polyclonal antibodies against IFN-γ and IL-4, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit) was obtained from Abgent Corporation.

## 2.8. Statistical analysis

The data are expressed as mean ± standard deviation. First, the normality and homogeneity of variance were assessed for each data set. If the data were normally distributed and of homogenous variance, comparisons between groups were confirmed by a one-way ANOVA. If the data did not conform to a normal distribution and/or homogenous variance, the data were compared by the rank sum test, and pairwise comparisons between the means were performed with the Games-Howell test. Statistical calculations were performed using SPSS 19.0 software (SPSS Inc., Armonk, NY, USA). The values of  $P < 0.05$  were considered statistically significant.

The control groups were given an intraperitoneal injection of saline containing alum. The alum is an inorganic adjuvant without immunogenic. The data of control groups showed no differences, so they were incorporated as one group as Group Control (C) for statistical analysis. The results of the progeny of control groups were also combined as Group C<sub>1</sub>, C<sub>2</sub> to be analyzed.

## 3. Results

### 3.1. The general condition

Due to various reasons such as death or miscarriage within 21 ± 2 d, the final number of mice in all of the groups was summarized in Table 1.

**Table 1** The levels of OVA-specific IgE before and after pregnancy (OD value, mean ± standard deviation).

Group	n	Before mating	After delivery	p values
C	13	1.142 ± 0.402	1.140 ± 0.264	> 0.05
FTP	7	1.145 ± 0.506	1.139 ± 0.407	> 0.05
STP	8	1.130 ± 0.238	1.132 ± 0.168	> 0.05
TTP	7	1.131 ± 0.312	1.130 ± 0.203	> 0.05

3.2. The level of OVA-sIgE

The levels of OVA-sIgE in each group of pregnant mice did not exhibit statistically significant differences between pre- and post-pregnancy (p values > 0.05). The results are summarized in Table 1.

3.3. OVA-specific lymphocyte proliferation

The CCK-8 was used to detect the OVA specific lymphocyte proliferation of splenic lymphocytes harvested from 5-day-old newborn pups. The OD 450 nm value represented the degree of proliferation. Compared with the Group C<sub>1</sub>, the OVA-specific lymphocyte proliferation of the FTP<sub>1</sub> and STP<sub>1</sub> groups did not exhibit significant differences, and the OVA-specific lymphocyte proliferation of the TTP<sub>1</sub> group was statistically significantly increased and greater than the proliferation observed in the FTP<sub>1</sub> and STP<sub>1</sub> groups. The results are summarized in Table 2.

3.4. Immune cytokines

Compared with the control groups, the expression levels of IFN-γ in the FTP<sub>1</sub> and STP<sub>1</sub> groups were slightly reduced but did not exhibit significant differences. The expression level of IFN-γ in the TTP<sub>1</sub> group exhibited a statistically significant reduction. Compared with the expression levels of IFN-γ in the FTP<sub>1</sub> and STP<sub>1</sub> groups, the IFN-γ level in the TTP<sub>1</sub> group was the lowest of the three groups. Compared with the expression levels of IL-4 in the corresponding control groups, there was a significant increase in the levels of the TTP<sub>1</sub> group, while the FTP<sub>1</sub> and STP<sub>1</sub> groups did not exhibit significant differences. The expression level of IL-4 in the TTP<sub>1</sub> group was significantly increased compared with the levels in the FTP<sub>1</sub> and STP<sub>1</sub> groups. The results are summarized in Table 2

3.5. mRNA expression levels of IFN-γ & IL-4

The mRNA expression levels of IFN-γ and IL-4 in the FTP<sub>1</sub> and STP<sub>1</sub> groups were not statistically significantly compared with those in the Group C<sub>1</sub>. The mRNA expression levels of IFN-γ and IL-4 in TTP<sub>1</sub> group were significantly different compared with other groups. The mRNA expression level of IFN-γ in the TTP<sub>1</sub> group was significantly reduced and the mRNA expression level of IL-4 in this group was the most increased out of all the groups (Fig. 1).

Fig. 1 the effect of OVA exposure during different periods of pregnancy on the OVA sensitization of progeny. (A) The mRNA expression of IFN-γ in the TTP<sub>1</sub> group was significantly decreased compared with other groups. (B) The mRNA expression of IL-4 in the TTP<sub>1</sub> group was significantly decreased compared with other groups. (C) The serum OVA-sIgE level in TTP<sub>2</sub> was significantly increased compared with other groups. ★ p < 0.05 compared with other groups.

3.6. Protein expression levels of IFN-γ & IL-4

The protein expression levels of IFN-γ and IL-4 in the FTP<sub>1</sub> and STP<sub>1</sub> groups were not statistically significantly compared with those in the Group C<sub>1</sub>. The protein expression levels of IFN-γ and IL-4 in TTP<sub>1</sub> group were significantly different compared with other groups. The mRNA expression level of IFN-γ in the TTP<sub>1</sub> group was significantly reduced and the mRNA expression level of IL-4 in this group was the most increased out of all the groups (Fig. 2).

Fig. 2 the effect of OVA exposure during different periods of pregnancy on the OVA sensitization of progeny. The protein expression of IFN-γ in the TTP<sub>1</sub> group was significantly decreased compared with other groups, while the protein expression of IL-4 was significantly decreased compared with other groups. The effect of the sensitization of the adult progeny with different periods of antigen exposure during pregnancy.

To further understand how the same antigen exposure affected adult progeny exposed to food antigens during pregnancy, the same OVA was given to female adult progeny by intraperitoneal injections. Then, the OVA-sIgE levels in the serum were detected. Compared with the group C<sub>2</sub>, the level of OVA-sIgE in the TTP<sub>2</sub> group exhibited significantly increased levels of OVA-sIgE. The level of OVA-sIgE in the FTP<sub>2</sub> and STP<sub>2</sub> groups did not exhibit significant differences. The results are summarized in Table 3 and Fig. 1.

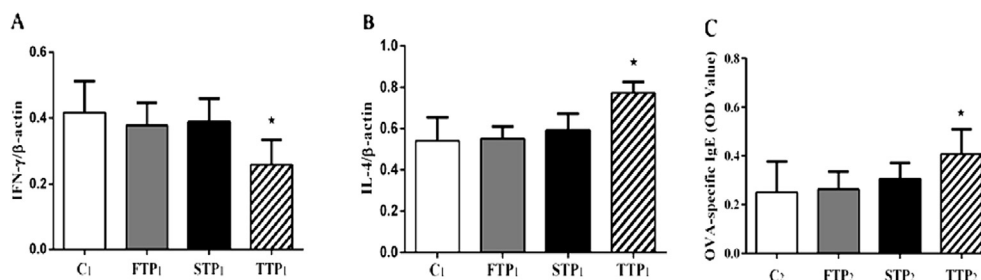
**Table 2** The levels of immune functions of offspring in different periods of OVA exposure during pregnancy (OD value, mean ± standard deviation).

Group	n	OVA specific lymphocyte proliferation	IFN-γ (pg/ml)	IL-4 (pg/ml)
C <sub>1</sub>	13	1.208 ± 0.353 <sup>b</sup>	58.89 ± 16.45	16.79 ± 3.43
FTP <sub>1</sub>	7	1.296 ± 0.124 <sup>b</sup>	53.68 ± 15.46 <sup>a</sup>	16.43 ± 2.04 <sup>b</sup>
STP <sub>1</sub>	8	1.182 ± 0.243 <sup>b</sup>	50.48 ± 10.61 <sup>b</sup>	17.12 ± 2.11 <sup>b</sup>
TTP <sub>1</sub>	7	2.04 ± 0.603	37.64 ± 8.43 <sup>c</sup>	24.72 ± 2.67 <sup>c</sup>

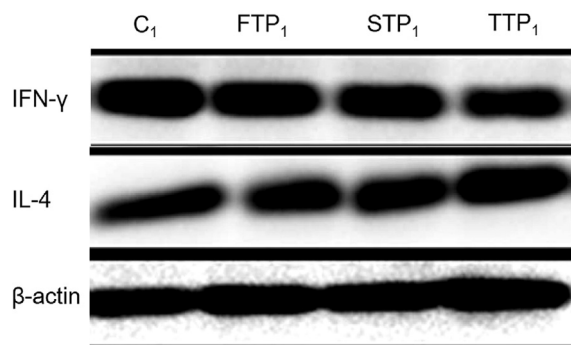
<sup>a</sup> P < 0.05 vs. Group TTP<sub>1</sub>.

<sup>b</sup> P < 0.01 vs. Group TTP<sub>1</sub>.

<sup>c</sup> P < 0.01 vs Group C<sub>1</sub>.



**Figure 1** The effect of OVA exposure during different periods of pregnancy on the OVA sensitization of progeny.



**Figure 2** The effect of OVA exposure during different periods of pregnancy on the OVA sensitization of progeny.

**Table 3** The levels of OVA-specific IgE of adult progeny in different trimesters exposed to OVA.

Group	N	OD value
C <sub>2</sub>	13	0.251 ± 0.126
FTP <sub>2</sub>	7	0.264 ± 0.072 <sup>b</sup>
STP <sub>2</sub>	8	0.307 ± 0.064 <sup>b</sup>
TTP <sub>2</sub>	7	0.408 ± 0.102 <sup>a</sup>

The values are presented as mean ± standard deviation.

<sup>a</sup>  $P < 0.01$  vs. Group C<sub>2</sub>.

<sup>b</sup>  $P < 0.01$  vs Group TTP<sub>2</sub>.

#### 4. Discussion

The immunologic mechanisms of food allergy are difficult to be identified. It has been suggested by numerous studies that the fetal period is a crucial time which determines the occurrence of allergic diseases after birth, which led to the hypothesis that the progeny has been sensitized in uterus during pregnancy (Warner et al., 2000). However, the exact crucial time of intrauterine sensitization remained to be determined (Hsu et al., 2012). According to the experience in our lab, food allergy could be induced by OVA in BALB/c mice (Li and Li, 2005; Hu et al., 2005), the aim of this study was to determine the relationship between OVA exposure in pregnancy and intrauterine sensitization and to identify the crucial time of intrauterine sensitization. In the present study, we demonstrated that OVA exposure during pregnancy caused intrauterine sensitization, the crucial time of OVA-induced intrauterine sensitization was the third trimester, and confirmed the supposition from our previous study (Hu et al., 2005).

In the present study, we found that OVA exposure during pregnancy stimulated OVA-specific lymphocyte proliferation in the newborn progeny, indicating that OVA exposure during pregnancy induced fetal sensitization. Edelbauer et al. (2003) confirmed that the OVA antigen could cross the placenta in mice. While OVA was not detected in the cord blood, the pregnant mice were provided with special purebred feed (excluding eggs). The OVA-specific lymphocyte proliferation in the offspring was significantly increased, which suggested that OVA could directly cause intrauterine sensitization. This finding is consistent with previous studies (Edelbauer et al., 2003; Vance et al., 2005).

Given that OVA could directly induce intrauterine sensitization throughout the pregnancy, we sought to understand whether there is a crucial time for intrauterine sensitization. In the present study, we demonstrated that the progeny exposed to OVA in different trimesters displayed different immune responses accordingly. The progeny exposed to OVA in the third trimester demonstrated significantly increased OVA-specific lymphocyte proliferation and serum OVA-sIgE level, indicating stronger OVA sensitization and an increased risk of food allergy in the progeny. Previous studies have confirmed that T lymphocytes could be detected in the peripheral blood of the fetus at 12 weeks of pregnancy (Durandy, 2001; Campagnoli et al., 2000). However, the lymphocytes of fetus remained naïve with deficient function, therefore the immune response would be delayed when the lymphocytes encountered the antigens for the first time during the first and second trimesters (Durandy, 2001). Numerous studies have shown that the initial sensitization stage of T lymphocytes is in the final stage of pregnancy i.e. the third trimester. In addition, infants with high risk of developing allergic diseases demonstrate a shift in Th1/Th2 balance toward Th2-dominant immunity (Rao and Riggs, 1999; Jones and Warner, 1998; Holt and Sly, 1997).

Th1 cells produce IFN- $\gamma$  and mediate delayed-type hypersensitivity, while Th2 cells produce IL-4 and mediate humoral immune response and immediate hypersensitivity. Many allergic diseases occur because of the Th1/Th2 cytokines imbalance i.e. IFN- $\gamma$ /IL-4 imbalance. Food allergy is mediated by IgE and is characterized by skewing the Th1/Th2 cytokines balance toward increased levels of Th2 cytokine i.e. IL-4. Warner (2009) found that found that the cord blood of children who suffered from eczema after birth had decreased level of IFN- $\gamma$  and increased level of IL-4. The present research also confirmed that the progeny who encountered OVA during the third trimester demonstrated decreased level of IFN- $\gamma$  and increased level of IL-4. In addition, they had higher level of OVA-sIgE, suggesting their susceptibility to OVA was increased as they aged.

## 5. Conclusion

In conclusion, our findings suggested that OVA-induced intrauterine sensitization may be related to food allergy and progeny intrauterine sensitization during the third trimester increased the risk of food allergy after birth. Given the biological differences between murine and human, further studies need to be performed to evaluate the relationship between intrauterine sensitization and food allergy in humans. However, our study suggested a new paradigm for food allergy pathogenesis. Understanding the complex interactions between intrauterine sensitization and food allergy will help develop new measures of prevention and intervention.

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