Reversible immune abnormality and regulatory T cells in offspring of Der p 1-exposed female mice

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Abstract

Background: Maternal allergic diseases have an important influence on the origin of allergic rhinitis (AR) in offspring, but the mechanism and the duration of the maternal effect are unknown. Previous researches prompted the important roles of Tregs and Foxp3 DNA methylation in the development of allergic diseases.

Objective: To investigate the immune state and Tregs in the offspring of Der p 1-exposed female mice.

Method: BALB/c female mice were exposed to Der p1 to construct the mouse AR model, then mated with normal male mice. Offspring were kept in an allergen-free environment after birth. At postnatal weeks 3, 5 and 8, mice were culled for testing.

Result: Compared with the offspring of PBS-exposed female mice (N-N), the offspring of Der p 1-exposed female mice (A-N) showed increased IL-4 and decreased IL-10 levels in serum at postnatal weeks 3 and 5. Correspondingly, the percentage of Tregs in spleen CD4⁺ cells declined significantly at postnatal week 5 in A-N. Further analysis of the methylation status of spleen lymphocytes revealed hypermethylation of the Foxp3 promoter in A-N mice at postnatal weeks 3 and 5. However, by 8 weeks of age, all abnormalities in cytokines, Treg counts and Foxp3 DNA methylation in A-N mice had returned to normal levels.

Conclusion: Under the influence of maternal AR, offspring have an abnormal immune state at birth. However, without exposure to allergens, the immune state in AR offspring recovered by maturity. Changes in Tregs and Foxp3 DNA methylation may be the mechanism for this reversible immune abnormality in AR offspring.

Keywords: Rhinitis, Allergic; Maternal; Offspring; T-Lymphocytes, Regulatory; DNA methylation

Introduction

The incidence of allergic rhinitis (AR) has been increasing around the world in recent years,¹-³ and the trend in young people has been noted by researchers.⁴ As a complicated allergic inflammatory disorder of the upper respiratory tract, AR is influenced by heredity and the environment. Compared to paternal allergic diseases, maternal ones have more important influence on the origin of offspring allergic diseases.⁵ Genetic inheritance⁶,⁷ and perinatal transfer of maternal mediators (such as allergens, antibodies or cytokines) via the placenta or milk⁸-¹⁰ may be responsible for the maternal effect. Additionally, epigenetic regulation, including DNA methylation, is a promising mechanism contributing to the onset of AR in early life.¹¹-¹⁴

The onset of AR usually starts in childhood.¹⁰ Epidemiological studies have revealed that children and adolescents have higher prevalence of AR than adults,¹⁵-¹⁷ and the prevalence of AR decreases with age.¹⁶,¹⁷ Alexey et al. reported that the offspring of OVA-exposed mice developed airway hyperresponsiveness and inflammation, although the magnitude of the allergic response in offspring declined after 8 weeks of age.¹⁸ This animal experiment enhanced our understanding of the duration of maternally transmitted allergic risk and the
prevalence of allergic diseases in people of different ages. However, the mechanism under this phenomenon was not fully uncovered.

It is widely accepted that AR is the result of Th1/Th2 imbalance, including suppression of the Th1 response and domination of the Th2 response. Additionally, regulatory T cells (Tregs), whose main functions are immune regulation and immune suppression, play a vital role in the balance and maturation of CD4+ T cell polarization.\textsuperscript{19,20} Tregs with a naïve phenotype in the cord blood, which may naturally arise and decline in the fetus, play a potential immunoregulatory role in intrauterine life.\textsuperscript{21} Forkhead box p3 (Foxp3) is the specific transcription factor for CD4+CD25+Treg cells and is critical in the development and function of Tregs.\textsuperscript{22} Reduced expression of Foxp3 leads to the suppression of Tregs and contributes to the onset of AR.\textsuperscript{23,24} The expression of Foxp3 is controlled by epigenetic modifications, such as DNA methylation, which is a relatively stable modification passed on from mother to offspring.\textsuperscript{25} Thus, we hypothesize that Foxp3 DNA methylation and Tregs may well be associated with the maternal transmission of allergic risk.

By using an *Dermatophagoides pteronyssinus* 1 (Der p1) induced AR murine model, we reported the abnormal immune state and suppression of Tregs in neonatal (3 days old) offspring of AR mice.\textsuperscript{26} To elucidate the mechanism and the duration of this maternally transmitted allergic susceptibility, in this study, we investigated the immune state, Tregs and Foxp3 DNA methylation status in the offspring of Der p 1-exposed female mice at postnatal weeks 3, 5 and 8.

**Materials and methods**

**Animals**

Pathogen-free female and male BALB/c mice (6-8 weeks old) were purchased from the Wuhan Institute of Biological Products Co., Ltd. (No. 42000400002441, Wuhan, China). Female mice were randomly divided into the saline-exposed female mice (N) group or Der p 1-exposed female mice (A) group; the offspring were designated as the N-N group and A-N group, respectively. All animals were kept in a specific pathogen-free biohazard containment facility at the Animal Experiment Center of the Wuhan Third Hospital (permission number: SCXK-2014-0080, Wuhan, China). All procedures were approved by the Institutional Animal Care and Use Committee of Wuhan University.

**Experimental protocol**

Following adaptive feeding for 3 days, Der p 1-exposed female mice (A) were sensitized by initial intraperitoneal (i.p.) injection with 400 μl of phosphate-buffered saline (PBS) containing 1 μg Der p1 (Indoor Biotechnologies, Charlottesville, Virginia) and aluminum hydroxide (4 mg) on day 1 and day 7. From day 21 to day 28, these mice were intranasally challenged with 20 μl of PBS containing Der p 1 (2 μg) on a daily basis. Saline-exposed female mice (N) were sensitized and challenged with the equivalent amount of PBS without aluminum hydroxide in the same way. After the last challenge, all the female mice were placed in cages to mate with normal male mice at a female: male ratio of 2:1 on day 29. The offspring of Der p 1-exposed female mice (A-N) and PBS-exposed female mice (N-N) were kept in an allergen-free environment after birth, and were humanely killed for testing at postnatal weeks 3, 5 and 8 (Figure 1).

**Histopathological analysis of nasal mucosae**

After the mice were culled, their noses were removed and fixed in 4% paraformaldehyde overnight, decalcified in an EDTA decalcifying solution for two weeks, dehydrated in a series of increasing concentrations of ethanol and embedded in paraffin. The paraffin-embedded tissues were sectioned coronally by continuous microtoming at a thickness of 4 μm. The histopathology of nasal mucosae in the mice of both groups was evaluated by hematoxylin-eosin (HE) staining. The cytoplasm of eosinophils in the nasal lamina propria (LP) was stained red by HE and eosinophils were counted in five randomly selected high-power fields at 400× magnification.

**Measurement of cytokines in sera**

Blood was collected from the canthus. After standing for about 2 hours, the blood samples were centrifuged for 20 min at 2000 × rpm to separate the sera. Sera were stored at -80°C until use. The levels of interleukin 4 (IL-4) and IL-10 in the sera were measured by enzyme-linked immunosorbent assay (ELISA) based on the manufacturer’s instructions (eBioscience, San Diego, USA).

![Figure 1. Experimental protocol. Der p1 (Indoor Biotechnologies, Charlottesville, Virginia) was used to sensitize and challenge female mice to construct AR mice models. The offspring of Der p 1-stimulated female mice (A-N) and saline-stimulated female mice (N-N) were not stimulated with Der p 1, and were humanely killed for testing at postnatal week 3, 5 and 8.](image-url)
Reversible immune abnormality and Tregs in AR offspring

Flow cytometric analysis of CD4+CD25+FOXP3+ Treg cells in spleens

Single-cell suspensions of lymphocytes were extracted from fresh spleen tissue using the Mouse Spleen Lymphocyte Separation Medium Kit (TBD Science, Tianjin, China). Single-cell suspensions of lymphocytes were stained for regulatory T cells using the Mouse Regulatory T Cell Staining Kit (eBioscience, San Diego, USA) with anti-CD4-FITC, anti-CD25-PE and anti-FOXP3-APC, according to the manufacturer’s instructions. Flow cytometry was performed and analyzed on a BD FACS-Calibur flow cytometer (BD Biosciences, San Jose, USA).

Methylation analysis of Foxp3 promoter region in spleen lymphocytes

Genomic DNA was extracted from spleen tissue and treated by bisulfite conversion. Bisulfite modified DNA was subjected to PCR under the conditions set as follows: incubation at 98°C for 4 min, followed by 40 cycles of 94°C for 45 s, 66°C for 45 s, 72°C for 60 s with a final extension step at 72°C for 8 min. The up-stream primer for Foxp3 was 5’- TTTTAGATGATTTGTAAGgGTAAG2AG-3’ and its down-stream primer was 5’- ATCAACCTAACTTATAAAAAACTCCAC-3’. DNA isolation, bisulfite conversion of genomic DNA and Foxp3 DNA methylation analysis for 3 CpG sites in the Foxp3 promoter region were performed by Sangon Biotech, Inc. (Shanghai, China).

Statistical analysis

Data are presented as mean ± SEM. Data analysis was performed using SPSS statistical software, version 22.0 (IBM, Chicago, USA), and GraphPad Prism software, version 6.01 (GraphPad Software, San Diego, USA). Unpaired Student’s t-tests were used to determine differences between two groups at the same time point. Analysis of variance (ANOVA) was used to determine differences between different time points in the same group. P <0.05 was considered statistically significant. n=4–6 for each group.

Results

HE staining of nasal mucosa

Histopathological analysis of the nasal mucosa was conducted by HE staining. In the offspring of saline-exposed female mice (N-N, week 3, 5&8), eosinophils could hardly be found in the nasal lamina propria. The structure of the pseudostratified ciliated columnar epithelium was intact, and the cilia were continuous without impairment or exfoliation. However, the offspring of Der p 1-exposed female mice (A-N, week 3&5) showed infiltration of eosinophils and lymphocytes and tissue edema in the lamina propria, which are the histopathologic characteristics of allergic inflammation. Reduction, exfoliation and discontinuity of cilia were also found in the pseudostratified ciliated columnar epithelium in this group.

Figure 2. HE staining of nasal mucosa (original magnification×400). The cytoplasm of eosinophils (black arrows) in the nasal lamina propria (LP) were stained red by HE. The number of eosinophils was quantified for each group (B). (n=4–6 for each group; **P<0.01, compared with N-N at the same week; #P<0.01, compared with same group at different week). HE, hematoxylin-eosin; N-N, offspring of saline-stimulated female mice; A-N, offspring of Der p 1-stimulated female mice.
group. However, when offspring reached 8 weeks of age, there were only a few eosinophils in the A-N mice, indicating the resolution of allergic inflammation (Figure 2).

**Cytokines levels in sera**

ELISA showed a clear distinction in the levels of IL-4 in the serum between the N-N and A-N groups at postnatal week 3 and 5. Compared with the N-N group, the level of IL-4 in the A-N group was elevated \((P<0.01)\), while the level of IL-10 in the A-N group tended to be lower. This indicated a dominant Th2 immune response in A-N mice from postnatal weeks 3 to 5. However, the ectopic secretion of IL-4 and IL-10 returned to normal level at week 8 (Figure 3).

**CD4\(^+\)CD25\(^+\)FOXP3\(^+\) Tregs as a percentage of CD4\(^+\) lymphocytes in the spleen**

The Treg cell count was performed by flow cytometry and transform into the percentage of CD4\(^+\)CD25\(^+\)FOXP3\(^+\) Tregs in spleen CD4\(^+\) cells (Figure 4A). The Treg percentage in A-N mice was significantly lower compared to the N-N group at postnatal week 5 \((6.29\pm0.714\%\) vs \(9.93\pm0.741\%, P<0.05)\). The difference in Treg numbers was not obvious between the A-N and N-N groups at postnatal weeks 3 and 8 \((P>0.05)\) (Figure 4B).

**Foxp3 DNA methylation level**

We used the bisulfite-assisted genomic sequencing method (BSP) to study three CpG sites in the Foxp3 promoter region.

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**Figure 2.** (Continued) HE staining of nasal mucosae (original magnification×400).

**Figure 3.** Cytokines sections in sera. The concentrations of IL-4 and IL-10 in sera were measured by ELISA. \((n=4–6\) for each group; \(*P<0.05\), compared with N-N at the same week; \#P<0.05, ##P<0.01, compared with same group at different week). IL, interleukin; ELISA, enzyme-linked immunosorbent assay; N-N, offspring of saline-stimulated female mice; A-N, offspring of Der p 1-stimulated female mice.
Figure 4. CD4^+CD25^+FOXP3^+Tregs percentage of CD4^+ lymphocytes in spleens. Tregs count in the spleen was measured by flow cytometry. The analysis of CD4 versus FOXP3 expression was gated in CD4^+CD25^+ cells (red frame, R1). (A). CD4^+CD25^+FOXP3^+ Tregs (green frame) were calculated as the percentage of CD4^+ lymphocytes in each group (B). (n=4–6 for each group; *P<0.05, compared with N-N at the same week; #P<0.05, ##P<0.01, compared with same group at different week). N-N, offspring of saline-stimulated female mice; A-N, offspring of Der p 1-stimulated female mice.
Transcription start was set as +1 bp. These CpG sites were located at -52, -49 and -34 in the sequence and were designated as CpG(-52), CpG(-49) and CpG(-34), respectively. The results show that the methylation level of the Foxp3 promoter was higher in A-N than in N-N mice at postnatal weeks 3 (CpG(-34)) and 5 (CpG(-49) and CpG(-34)). The hypermethylation in A-N recovered spontaneously by week 8 (Figure 5).

Analyzing along the time axis, we found that histopathology, cytokines secretion, Treg counts and Foxp3 DNA methylation changed little from postnatal week 3 to 8 in the N-N group. However, in the A-N group, there were dramatic changes from postnatal week 3 to week 8; these mice showed ectopic secretion of cytokines at week 3 and 5, which returned to normal by week 8. Similar changes occurred in the Treg counts and Foxp3 DNA methylation.

**Discussion**

Under the influence of maternal AR, offspring have an abnormal immune state at a young age, suggesting that sensitization of offspring may occur prenatally or early postnatally. This has been observed in animal and clinical studies. Thus, these offspring have potential susceptibility to allergic diseases such as AR. Moreover, this susceptibility of offspring is not only specific to the particular allergen which the mother were exposed to.

Our previous study reported an abnormal immune state, with histopathological characteristics of allergic inflammation in the nasal mucosa, the suppression of Th1 cytokines and the domination of Th2 cytokines in the serum in neonatal (3 days old) offspring of AR mice. This study showed that this abnormal state extended to postnatal weeks 3 and 5, but not week 8. When the mice reached maturity at week 8, the immune state in the offspring of AR mice returned to normal. Note that 8 to 10 weeks is the mature stage in BALB/c mice, while 3 to 5 weeks is the juvenile stage. These results are consistent with the age distribution of AR, i.e. juveniles have the highest incidence of AR, while adults have a lower incidence of AR.

Our research underlines the association between Tregs and the reversible immune abnormality in AR offspring. Tregs play an important role in the regulation of allergic inflammation. Our data show that the Treg counts differed sharply in the offspring of AR mice at postnatal week 5, and recovered to a normal number at postnatal week 8. This finding implicated that the changes in the immune state and allergic susceptibility in offspring could be attributed to Tregs, at least partly.

Epigenetics is a type of gene modification that alters the phenotype without modifying the genetic sequence. There have been heated discussions about the function of epigenetic modifications and DNA methylation in allergic diseases in recent articles. Mikhaylova et al. identified extensive methylation changes in dendritic cells in the neonates of allergic mothers, which were closely related to altered transcriptional responses to allergens and early-life asthma origin. In our study, along with the downregulation of Tregs, hypermethylation of the Foxp3 promoter in spleen cells was noted in AR offspring at a young age, but not in older mice. Foxp3 is the specific transcription factor of CD4(+CD25(+)) Treg cells. DNA methylation of Foxp3 plays an important role in the development of TREMS and the immune state. These dramatic changes may be responsible for the reversible immune abnormality and Treg decline observed in our study.

Why the DNA hypermethylation recovered spontaneously is still unclear. Whole-genome bisulfite sequencing of newborn and centenarian genomes showed that newborn had higher average methylation level of all covered CpG sites than the centenarian sample (80.5% vs. 73.0%); the difference was still unclear. Whole-genome bisulfite sequencing of newborn and centenarian genomes showed that newborn had higher average methylation level of all covered CpG sites than the centenarian sample (80.5% vs. 73.0%); the difference was still unclear.

Figure 5. Foxp3 DNA methylation level. Three CpG islands methylation level in Foxp3 promoter region were measured by Bisulfite sequencing PCR. Transcription start was set as the +1 bp. These CpG sites located -52, -49 and -34 in the sequence and were labeled as CpG(-52), CpG(-49) and CpG(-34), respectively. (n=4—6 for each group; *P<0.05, compared with N-N at the same week; #P<0.05, compared with same group at different week). N-N, offspring of saline-stimulated female mice; A-N, offspring of Der p 1-stimulated female mice.
enzymatic activity for DNA methylation and DNA demethylation, respectively. Targeting them using 5-azacytidine, sulfonpyrazone or vitamin C could regulate Foxp3 DNA methylation and Treg numbers.1

Conclusion
In summary, this study highlights the role of Tregs in AR and the maternal transmission of AR susceptibility. We report an abnormal immune state in the offspring of Der p 1-exposed mice at a young age, which returned to normal when they entered adulthood. This was closely associated with changes in Tregs. Reversible Foxp3 DNA methylation is the probable mechanism for this dramatic phenomenon. Although the offspring of Der p 1-exposed mice were born with a congenital immune abnormality, whether or not they develop AR is also likely to be associated with later allergen exposure and other immune events. More studies are needed to further determine the epigenetic mechanisms involved in the process of maternally transmitted AR susceptibility. This study could increase understanding of the mechanism of AR onset and progression.

Conflict of interest
The authors declare no conflict of interest.

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References