

Original article

The “time-window” effect of early allergen exposure on a rat asthma model

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Keywords: *asthma; allergen exposure; regulatory T cells; costimulatory molecular*

Background The hygiene hypothesis has been proposed to explain the pathogenesis of asthma. Allergen exposure was shown to inhibit asthma in an animal model. But the optimal timing of allergen exposure remains unclear. This study aims to explore the time effect of allergen exposure and the possible mechanisms.

Methods Neonate Wistar rats were randomly divided into asthma group, control group and day 1, day 3, day 7, and day 14 groups. The day 1, day 3, day 7 and day 14 groups were injected with ovalbumin (OVA) subcutaneously on days 1, 3, 7 and 14 after birth, respectively. Six weeks later, all groups, except the control group, were sensitized and stimulated with OVA to make the asthma model. We observed the pulmonary pathologic changes, detected the regulatory T cells, and CD28 expression level in thymus and spleen by flow cytometry.

Results The asthmatic inflammation in the day 1, day 3 and day 7 groups, but not the day 14 group, was alleviated. The asthma group and day 14 group had lower proportions of regulatory T cells in the thymus compared with the control group, day 1, day 3, and day 7 groups. There was no significant difference in the CD28 expression levels on the regulatory and conventional T cells among groups. But the control group and the day 1, day 3, and day 7 groups had relatively higher proportions of CD28 positive regulatory T cells in the thymus than the day 14 group and the asthma group.

Conclusions There is a “time-window” for early allergen exposure. The impairment of regulatory T cells may promote the development of asthma. Allergen exposure in the “time-window” can make the thymus produce normal quantity of regulatory cells. The CD28 signal on regulatory T cells may participate in the production of regulatory T cells.

Chin Med J 2013;126 (12): 2265-2269

Over the last two decades, the prevalence of asthma has increased extensively worldwide.¹ One intriguing hypothesis, called the “hygiene hypothesis”, has put forth that the reduction of exposure to allergens and microbes in early childhood contributes to the increasing incidence of asthma.² Our previous study in a rat model showed that exposure to ovalbumin (OVA) within the first 24 hours after birth could inhibit the airway inflammation and OVA-specific IgE in adult rats.³ As is well known, however, allergen exposure in mature individual with atopy usually results in allergic hypersensitivity reactions. It seems that the allergen exposure is a double-edged sword. Therefore we speculate that there is an optimal time-window for allergen exposure to induce immune tolerance.

Accumulated studies have demonstrated that allergen stimuli can induce regulatory T (Treg) cells, which can protect against airway inflammation and hyper-reactivity.⁴ However, the mechanisms regulating the generation and development of Treg cells remain unclear. Recent studies indicate that CD28 is one of critical signals that are responsible for the generation of Treg cells in the thymus.⁵ We speculate that the immune tolerance induced by early allergen exposure is associated with the production of Treg cells in the thymus. The present study aims to evaluate how the timing of allergen exposure on infant rats affects the asthmatic airway inflammation and to explore the roles of Treg cells and the costimulatory molecule CD28.

METHODS

Animals

Healthy pregnant Wistar rats were provided by Animal Experiment Center of Peking University First Hospital (Beijing, China), and were bred and maintained under specific pathogen-free conditions. The first 24 hours after birth was called day 1. Newborn rats were randomly divided into day 1 group, day 3 group, day 7 group, day 14 group, asthma group and control group, ten animals in each group. The Peking University Committee on Animal Welfare approved all animal protocols used in this study.

Protocol of early exposure to allergen

The infant rats in the day 1, day 3, day 7, and day 14 groups were injected with 1 mg OVA (Sigma, St. Louis, MO, USA) and aluminum hydroxide gel containing 5 mg aluminum hydroxide (Sigma) in the inguinal region subcutaneously on day 1, day 3, day 7, or day 14 after birth,

DOI: 10.3760/cma.j.issn.0366-6999.20122326

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This research was supported a grant from by the New Teachers' Fund for Doctor Stations of Ministry of Education of China (No. 20110001120044).

respectively.

Immunization and airway challenge

Immunization with OVA was performed by subcutaneous injection in the bilateral inguinal regions of 6-week-old rats with 10 mg of OVA adsorbed to 1 ml of aluminum hydroxide gel containing 50 mg aluminum hydroxide. Two weeks later, the rats were nebulized with 20 g/L OVA 20 minutes per day for seven continuous days to provoke airway inflammation. The control group was injected with 1 ml of aluminum hydroxide and exposed to aerosolized phosphate buffered saline (PBS).

Lung histology and periodic acid Schiff (PAS) staining

The same lobes of the lungs from each rat were fixed in 10% formalin for 24 hours. The tissues were embedded in paraffin and 3 μ m sections were cut and stained with H&E and PAS for light microscopy. The histological changes in lungs were reviewed by two experienced pathologists and review of specimens was double-blinded. The histological changes were scored according to eosinophil infiltration around the small airways and blood vessels (from 0=no eosinophil infiltration to 3=most severe infiltration), lymphocyte infiltration around the small airways and blood vessels (from 0=no lymphocyte infiltration to 3=most severe infiltration), hyperplasia of mucin-containing goblet cells (from 0=no hyperplasia to 3=most severe hyperplasia), and loss of epithelial-cell integrity (from 0=epithelium integrity to 3=most severe epithelial shedding). These four scores were added together to obtain a total histological score.

Flow cytometry

Single cell suspensions from spleen and thymus were stained with mouse anti rat CD4-FITC and CD28-APC (eBioscience, San Diego, USA), or mouse IgG1-FITC, IgG2b-APC as the negative control, using 10 μ l per 10^6 cells for 30 minutes at 4°C. The anti-mouse/rat Foxp3-PE staining set was used to stain the intracellular antigens according to the experimental protocol from the manufacturer (eBioscience). Cells were analyzed by flow cytometry (Cell Quest software, FACScalibur, BD Pharmingen, San Diego, CA, USA).

Statistical analysis

The data are expressed as mean $\pm$ standard deviation (SD) and examined for their statistical significance of difference with SPSS (version 11.5 for Windows, SPSS Inc., USA) software. The homogeneity of variance was checked by Levene's test. Comparison of several groups used one-way analysis of variance. Fisher's least significance difference procedure was performed for equal variances, and Tamhane's T2 procedure for unequal variances to determine where the differences occurred. Statistical significance was obtained when $P < 0.05$.

RESULTS

As shown in Figure 1, lung tissues from the asthma group

rats manifested infiltration of numerous eosinophils and inflammatory cells surrounding the small airways, loss of epithelial-cell integrity, and occlusion of the bronchial lumen by mucus. Hyperplasia of mucin-containing goblet cells stained with PAS was also observed. The pathological score of the asthma group was significantly higher than the control group (9.72 ± 1.61 vs. 0.61 ± 0.41 , $P < 0.05$). Exposure to OVA on days 1, 3 and 7 after birth effectively alleviated the airway inflammation and decreased the number of eosinophils around the small airways. The mucin-containing goblet cells in airway epithelium of these groups were also markedly diminished compared with the asthma group. The pathological score of the day 1 group (3.33 ± 0.64), day 3 group (4.06 ± 1.56) and day 7 group (4.11 ± 1.78) was significantly lower than the asthma group (9.72 ± 1.61 , $P < 0.05$, respectively), but still higher than the control group (0.61 ± 0.41 , $P < 0.05$, respectively). The most significant improvement of asthmatic inflammation was observed in the day 1 group with a lower pathological score compared with the day 3 group and the day 7 group. However, the day 14 group presented significant eosinophil and lymphocyte infiltration around small airways and marked hyperplasia of goblet cells, and the pathological score was as high as in the asthma group (8.50 ± 1.07 vs. 9.72 ± 1.61 , $P > 0.05$).

In order to explore the mechanisms by which early allergen exposure affects the development of asthma, we detected the proportion of Treg cells (CD4⁺Foxp3⁺ T cells) among the thymic and splenic CD4⁺ T cells by flow cytometry within 24 hours after the last challenge (Figure 2). The asthma group had a lower proportion of Treg cells in the thymus compared with the control group. After exposure to

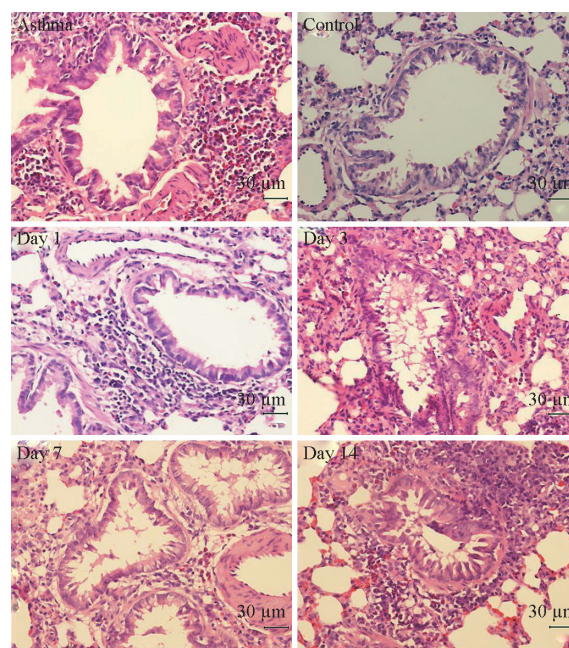


Figure 1. The pulmonary pathological manifestation in different groups (HE, original magnification $\times 200$). The airway inflammation in day 1, day 3 and day 7 groups was obviously alleviated compared with asthma group. But the day 14 group had as marked airway inflammation as severe as asthma group.

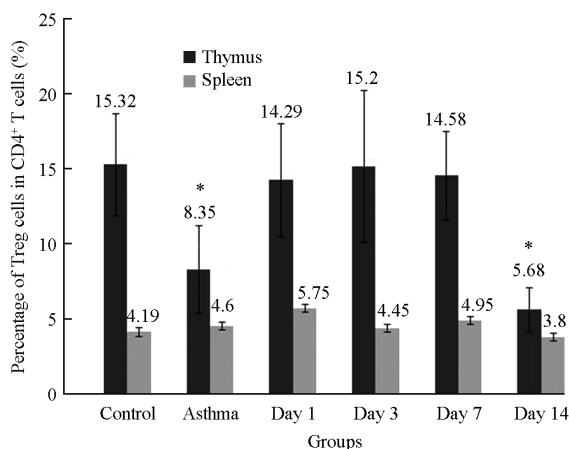


Figure 2. The percentage of regulatory T cells in the CD4⁺ T cells in thymus and spleen. The asthma group and the day 14 group had a low proportion of the regulatory T cells in the thymus, while the day 1, day 3, and day 7 groups and the control group had a relatively high percentage of the regulatory T cells in the thymus. * $P < 0.05$ compared with control group.

OVA on day 1, day 3 and day 7 after birth, the percentage of Treg cells in the thymus could reach the normal level when the mature rat was stimulated with the same allergen. However, the proportion of thymic Treg cells in the rats that were exposed to OVA on day 14 after birth remained at a similar low level to the rats in the asthma group. There were no statistically significant differences among groups in the proportion of Treg cells in the spleen.

Recently, the B7:CD28 interaction has been reported to provide a vital signal for the generation and development of Treg cells and play an important role in the induced immune tolerance.⁶ We detected the CD28 expression on the Treg cells and Foxp3-negative conventional T (Tconv) cells in the thymus and spleen. In spleen almost all of the CD4⁺ T cells expressed CD28, and no significant differences were found among groups. In the thymus about 40% ($39.97 \pm 5.58\%$) of Treg cells and about 30% ($30.99 \pm 2.59\%$) of Tconv cells expressed CD28. The positive expression rate of CD28 on Treg cells and Tconv cells in the thymus did not show any significant differences among groups (Table 1). The percentage of CD28-positive Treg cells among the thymic T cells in the control group was significantly higher than that in the asthma group ($(5.31 \pm 1.71)\%$ vs. $(3.73 \pm 1.10)\%$, $P < 0.05$), although the total quantity of CD28-positive T cells in the thymus did not show any differences between the control group and the asthma group ($(30.94 \pm 5.15)\%$ vs. $(32.35 \pm 5.04)\%$, $P > 0.05$). The day 1, day 3 and day 7 groups had a high percentage of CD28-positive Treg cells and there were no significant differences between them and the control group. While the day 14 group had the lowest percentage of CD28-positive Treg cells and had no significant difference when

Table 1. Expression of CD28 on the Treg cells and Tconv cells ($n=10$, %)

Groups	Thymus		Spleen	
	CD28 expression on Treg cells	CD28 expression on Tconv cells	CD28 expression on Treg cells	CD28 expression on Tconv cells
Control	40.06±4.62	30.76±3.50	91.82±1.85	90.73±2.91
Asthma	42.91±9.25	32.17±6.27	88.09±2.92	89.78±4.85
D1 group	38.85±7.41	31.77±3.54	88.47±2.29	87.99±2.29
D3 group	38.88±4.74	31.82±4.25	90.84±2.45	86.94±3.71
D7 group	43.59±5.17	32.66±2.71	90.35±2.20	88.80±1.73
D14 group	42.51±6.19	31.81±2.82	92.79±1.82	87.88±2.92

D: day.

compared with the asthma group (Figure 3).

DISCUSSION

In the present experiment we showed that exposure to OVA on day 1, day 3 and day 7 after birth could effectively reduce the asthmatic inflammation induced by OVA in adulthood. There were other studies that reported that mice initially exposed to allergen as neonates displayed protective Th1 responses when they were challenged as adults.^{7,8} We also have reported that both the high dose (1 mg) and the low dose (0.2 mg) of OVA exposure in neonatal rats could attenuate the airway inflammation induced by later antigen challenge.² But the high dose of OVA treatment resulted in a more complete immune tolerance with a normalized serum IFN- γ /IL-4 ratio and decreased OVA-specific IgE. On the basis of the previous study, the present experiment paid more attention to the timing of allergen exposure, and showed that only exposure to OVA within one week after birth could reduce the asthmatic inflammation induced by the same allergen in adulthood. Therefore, there exists a “time-window” phenomenon in the effect of allergen exposure in the rat asthma model.

To explain this phenomenon, we hypothesize that in a certain immature period it is easier for the immune system to make a regulatory response to stimulating signals from exogenous allergens, which can limit harmful hypersensitivity. In addition to clonal deletion, T cell anergy

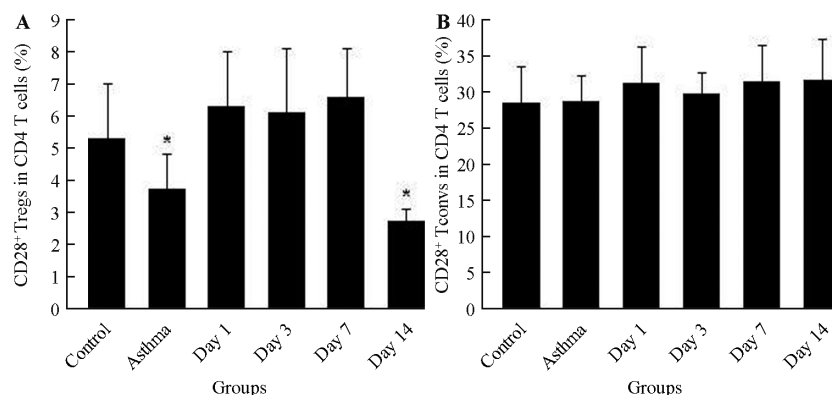


Figure 3. The percentage of CD28-positive Treg cells and CD28-positive Tconv cells in the thymus. **A:** The percentage of CD28-positive Treg cells among the CD4⁺ T cells in the thymus. The control group, day 1, day 3 and day 7 groups had a higher percentage of CD28-positive Treg cells in thymus than the asthma group and day 14 group. **B:** The percentage of CD28-positive Tconv cells among the CD4⁺ T cells in the thymus. There were no significant differences among groups. * $P < 0.05$ compared with control group.

and ignorance, accumulating evidence demonstrates that Treg cells capable of suppressing inappropriate immune responses are an essential part of immune tolerance.^{9,10} It has been demonstrated by *in vitro* and *in vivo* experiments that repeated allergen exposure can stimulate the production of Treg cells and induce immune tolerance.¹¹⁻¹³ Recently, Verhasselt et al¹⁴ reported that mother mice exposed to an allergen during the lactation period, resulted in the transfer of the allergen to their progeny by breast milk and induction of Treg cells and protection from asthma.

Our data show that generation of Treg cells in the thymus was significantly inhibited in the rats in the asthma group compared with the normal rats. We speculate that allergen sensitization and challenge in adult rats have inhibitory roles in the Treg cells in the thymus and result in asthma. However, exposure to allergen within one week after birth was sufficient to eliminate the inhabitation and make the thymus produce normal quantity of Treg cells, even in rats that were sensitized and challenged by the same allergen as adults. But exposure to allergen at 14 days after birth, outside of the “time-window”, did not promote the production of Treg cells, and the rats had a lower proportion of Treg cells in the thymus compared with normal rats. Therefore, early allergen exposure within the “time-window” is critical for maintaining a normal quantity of Treg cells.

Thymus is a critical site for the generation and development of Treg cells.^{15,16} In our study significant changes in the proportion of Treg cells were only observed in the thymus. It seems that the thymus plays a more important role in the effect of early allergen exposure than the spleen. On the other hand, the pathological scores in the day 1, day 3 and day 7 groups were still higher than in the normal group. This means that allergen exposure within the “time-window” can mitigate the asthmatic inflammation but can not eliminate it entirely, although the quantity of Treg cells in thymus remained normal. Therefore, the function of Treg cells in the early allergen exposure groups should be tested in further studies.

Recent studies clearly indicate that CD28 is essential for natural Treg cells development in the thymus and for Treg cells survival and homeostasis in the periphery.¹⁷ Mice deficient in CD28 have a substantially reduced number of Treg cells.¹⁸ Thus, although these mice are lacking a potent costimulation (CD28) for T effector cells, they are also lacking Treg cells, the most effective mediators of self-tolerance, yielding a balanced deficit that results in the preservation of Ag-mediated activation.^{19,20} It has been also shown that CD28 is critical for the generation of CD4⁺CD25⁺Foxp3⁺ Tregs from CD4⁺CD25⁻ T cells both *in vitro* and *in vivo*. The requirement of CD28 in Treg generation is IL-2 dependent.²¹

Our study showed that the proportion of CD28-positive Treg cells in the thymus was higher in normal rats than in asthma rats, although the total positive expression rate of

CD28 in the thymus did not show a significant difference between groups. We speculate that the CD28 expressed on the surface of Treg cells might play an important role in the generation and development of Treg cells. Allergen exposure within the “time-window” increased the percentage of CD28-positive Treg cells in the thymus, while allergen exposure outside the “time-window” did not do that. It suggests that early allergen exposure probably induces the production of Treg cells through the CD28 expression on the surface of the Treg cells.

In summary, our findings revealed that the effect of allergen exposure on asthma existed within a “time-window”. Exposure within the “time-window” could inhibit the development of asthmatic inflammation induced by the same allergen in the adult rats. The protective role seemed to be related to the production of Treg cells in the thymus. The CD28 signaling pathway might participate in the production of Treg cells. Results suggest that the timing of allergen exposure should be well defined and allergen exposure outside the “time-window” might be harmful. Nevertheless, the process and the mechanisms of thymic education of Treg cells remain unclear. Further investigations are necessary to provide better insight of the “time-window” phenomenon of early allergen exposure.

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(Received October 9, 2012)

Edited by WANG Mou-yue and LIU Huan