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Different challenge terms determine disease patterns of antigen-induced pulmonary inflammation in E3 rats

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Antigen induced pulmonary inflammation (AIPI) in rats, a classic animal model for asthma, has greatly contributed to the understanding of the disease pathogenesis, especially for the inflammation process. E3 rats are recently used to induce AIPI model for its susceptibility to pulmonary inflammation, but the features of AIPI with different antigen challenge terms on E3 rats require to be elucidated systemically. The aim of this study was to compare AIPI disease patterns in E3 rats with different challenge terms. E3 rats were immunized and challenged with ovalbumin (OVA) for 1, 4, and 8 weeks. Histological methods were used to determine morphological changes in lungs and cell types in bronchoalveolar lavage fluid. Nitric oxide (NO) concentration was assayed by Griess method. IL-4 and TGF-β expression were detected by real-time PCR. ELISA was used for the determination of serum IgE and OVA-specific IgG1. The results showed that all the sensitized E3 rats had a strong influx of eosinophils into the airway. In 1-week challenge group, the rats showed stronger inflammation, such as elevated levels of NO, delayed type hypersensitivity, IL-4 expression, and inflammatory cell infiltration; while in 8-week challenge group, rats manifested significant tissue destruction, accumulation of collagen and mucus production, and higher levels of antibody production, and TGF-β expression. Hence, the detail characterizations of AIPI model challenged for different terms demonstrated that E3 rats challenged with antigen for 1 week are suitable for studying acute pulmonary inflammation; meanwhile, the model established in the rats challenged for 8 weeks is appropriate for understanding pathogenesis of lung remodelling in chronic inflammation.

Key words: Challenge term; antigen-induced pulmonary inflammation; disease pattern; asthma; E3 rats.

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Given the ethical and logical constraints that severely limit the investigation scope on human being, animal models of asthma represent a useful system for understanding disease pathobiology, developing and testing potential drug therapies. Among animals used as asthma models for over 100 years, the rat has greatly contributed to pinpointing the cellular and molecular mechanisms of human allergic asthma (1, 2). The rat model for asthma has such features of human asthma as the cellular infiltration of lung, antigen-specific IgE production, and a predominant Th2 response (3, 4).

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Inflammation and structural changes are thought to have functional consequences that contribute to asthma symptoms (2).

Notwithstanding some good models simulating specific parts of the disease process, there is currently no animal model that adequately represents all aspects of human asthma. The extrapolation of animal model research to human asthma is highly dependent on various factors, such as the animal species and strains, antigen administration, even the challenge term to experimental animals (5). Because of another reason that rats are easily sensitized and challenged, such rat strains as Brown Norway, Wistar and F344 rats have been widely used for asthmatic animal models (6, 7). Though rat models show several similar features to the human asthma, there are still lots of differences from human asthma pathophysiology. For example, the asthmatic model of Brown Norway rats hardly manifests the airway remodelling process, but can promisingly represent the inflammatory process (3).

Besides rat strains and antigen administration, even the challenge term can decide the features of the animal model. Since no laboratory animal can develop asthma disease spontaneously (8), the antigen induced pulmonary inflammation (AIPI) is extensively used for the study of asthma pathophysiology (8, 9). Through sensitization and challenge to susceptible rat strains with ovalbumin (OVA), the most commonly used antigen, the inflammatory process can be replicated in rat airways. The method of OVA administration, the antigen challenge term and the administration of adjuvant may show different effects on disease development in rats (10, 11). Both in humans and experimental animals, antigen challenge results in a characteristic biphasic pattern of lung damage: the early inflammation response and the late lung remodelling. The antigen challenge term would play a key role in the process of experimental asthma. A majority of protocols to induce AIPI is to immunize and challenge animals for 7 days, because most available rat models are restricted to the acute inflammatory response following a short period of allergen exposure (12, 13). Meanwhile, few experimental studies have been performed to systematically compare effects of different challenge terms on rat AIPI.

The E3 rats were completely resistant to pristane-induced arthritis but highly susceptible to AIPI (14). In this study we observed that E3 rats after antigen challenge with different terms showed various disease patterns. Inflammation is the main initial performance of AIPI, and augmenting airway remodelling, mucus generation and airway smooth muscle hyperplasia appear in the later stage. Meanwhile, eosinophil recruitment in lung tissue is the key response of the disease, no matter in early stage or late stage in the AIPI model.

MATERIALS AND METHODS

Rats

The E3 rats, originated from the Section of Medical Inflammation Research, Lund University, Sweden, were bred and maintained at specific pathogen-free animal house with free access to OVA-free food and water. The controlled room temperature was 25 ± 3 °C, and the relative humidity was 40–60%, with artificial lighting 12 h per day. All experiments had been approved by the Institutional Animal Ethics Committee of the University (Xi'an Jiaotong University).

Sensitization and challenge

Rats were immunized by intraperitoneal (i.p.) injection with 1 mL emulsion solution containing 1 mg OVA (Sigma–Aldrich, St Louis, MO, USA) and 50 mg Al $(OH)_3$ (Pierce Biotechnology, Rockford, IL, USA). Two weeks after the sensitization, the rats were subjected to intranasal challenge of OVA (1 mg/mL in PBS). In control group, the rats were sham sensitized and exposed to the same volume of PBS.

The age- and sex-matched rats were divided into five groups and each group contained 10 rats aged from 8 to 10 weeks. In 1-week challenge group, the rats were sensitized and challenged with OVA, once per day for a week. In 4-week challenge group, the rats were sensitized and challenged with OVA, 2–3 times per week for 4 weeks. In 8-week challenge group, the rats were sensitized and challenged with OVA, 2–3 times per week for 8 weeks. In 1-week and 8-week control groups, the rats were sensitized and challenged with PBS. The data from 1-week challenge group were compared with the 1-week control group; and the data from 4-week challenge group and 8-week challenge group were compared with the 8-week control group, respectively.

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Determination of inflammatory cells in bronchoalveolar lavage fluid

Lungs were lavaged by instillation and withdrawal of 2 mL ice cold PBS through the tracheal route and bronchoalveolar lavage fluid (BALF) was collected. After centrifugation (106 *g*), the cellular pellet from BALF was resuspended in 1 mL PBS. Total cell numbers were determined by using a hemocytometer. For determining differential cells, cytocentrifuged preparations were fixed, stained with Giemsa Wright stain and counted according to differentiated morphology.

Lung histology

Lung tissues were stained with haematoxylin and eosin (HE) to observe pathological changes, with periodic acid Schiff (PAS) to detect mucus production, and with Masson to observe the presence of collagen fibres as previously described (15, 16). The scoring of lung histology was performed in a double-blind fashion. A 5-point scoring system to estimate the severity of peribronchial infiltration of inflammatory cells is: 0, no cells; 1, a few cells; 2, a ring of cells and one cell layer deep; 3, a ring of cells and 2–4 cells deep; 4, a ring of cells and higher than four cells deep. To estimate the extent of mucus production, the grading system after PAS staining is: 0, no positive goblet cell secreting mucus; 1, lower than 25%; 2, 26–50%; 3, 51–75%; 4, higher than 76%. Masson staining was used for the detection of collagen fibres stained blue in tissues. The grey scale of the blue colour was determined by IMAGE-PRO PLUS 6.0 software (Media cybernetics, Silver Spring, MD, USA) to estimate the collagen generation. The relative area was calculated as a ratio of collagen area/airway area.

Determination of nitric oxide (NO) in serum

The NO concentration in the serum was measured by the Griess method. Briefly, serum mixed with $ZnSO_4$ and NaOH was centrifuged at 239 *g* for 20 min to remove proteins. The supernatant was obtained and shaken for 1 h after the addition of 400 mg of Cu-(Copper) plated Cd (Cadmium). The obtained supernatant was added to an equal volume of Griess reagent to develop colour. The absorbance was measured at 545 nm wavelength by micro plate reader (Thermo Electron Corporation, Vantaa, Finland). A gradient concentration of sodium nitrite solution was used as a standard to obtain NO concentration in serum.

Delayed type hypersensitivity

Delayed type hypersensitivity (DTH) is a basic test to check the inflammatory response to the antigen *in vivo*. Forty-eight hours before euthanasia, the rats of all groups were injected with 20 μL OVA (1 mg/mL PBS) in the right ear to see inflammatory response and 20 μL PBS in the left ear as control. The weight difference between the two ear pieces represented the magnitude of DTH.

RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from lung and spleen by using TRIzol® method. Total RNA was reversed to cDNA according to the manufacturer's instruction (RevertAid™; Fermentas Life Sciences, Burlington, Ontario, Canada). cDNA was stored at −20 °C. Quantitative real-time PCR was performed by iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with SYBR® Premix *Ex Taq*™ II (TaKaRa, Ohtsu, Shiga, Japan) for determining mRNA expression of IL-4 and TGF-β genes. The information of primers, product size, and annealing temperature is depicted in Table 1. The quantitative real-time PCR system included 2 μL cDNA, 1 μL each primer and 5 μL 2× SYBR® Premix. Each sample was duplicated and GAPDH expression was used to normalize the gene expression.

Determination of OVA-specific IgG1 and total serum IgE

The serum levels of OVA-specific IgG1 and total serum IgE were measured by ELISA. Briefly, the 96-well round-bottomed microtiter plates (Jet Biofil, Guangzhou, China) were coated for overnight reaction at 4 °C with 100 mL of 100 mg/mL OVA (or mouse anti-rat IgE and IgG1 heavy chain) in $NaHCO_3$ buffer (pH 9.6). The plates were washed three times with PBS–0.05% Tween 20, and then

Table 1. Information of quantitative real-time PCR

Gene	Primer sequences	Annealing temperature (°C)	Product size (bp)
IL-4	F 5′ TGCACCGAGATGTTTGTACCAGA 3′ R 5′ TTGCGAAGCACCCTGGAAG 3′	63	92
TGF-β	F 5′ GGACTACTACGCCAAAGAAG 3′ R 5′ TCAAAAGACAGCCACTCAGG3 ′	65	249
GAPDH	F 5′ CGGCAAGTTCAACGGCACAG 3′ R 5′ GAAGACGCCAGTAGACTCCACGAC 3′	65	148

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blocked by incubation with 200 mL 0.05% PBS–Tween and 2% BSA for 1 h at 37 °C. After washing, serum samples diluted 1/1000 were added and the plates were incubated for 1 h at 37 °C. Total serum IgE and OVA-specific IgG1 were measured with horseradish peroxidase-conjugated mouse anti-rat Kappa/Lambda chain. Then, TMB (Tetramethylbenzidine) and H_2O_2 were added as a substrate and incubated at 37 °C for 20 min. The reaction was terminated with 1 M H_2SO_4, and read at 450 nm using an ELISA reader (Thermo Electron Corporation).

Statistical analysis

Data were expressed as mean ± SEM and analyzed by using SPSS software (version 13.0, Chicago, IL, USA). Mann–Whitney test was used for the comparison between control and challenge group. A p-value < 0.05 was considered as statistical significance.

RESULTS

Eosinophil infiltration was a common inflammatory indicator of AIPI in E3 rats in different challenge terms

Total cell numbers in BALF was elevated significantly in the animals after challenge for 1 week but no significant difference after challenge for 4 or 8 weeks was observed (Fig. 1A). Percentages of lymphocytes increased significantly in 1-week challenge group but decreased in 8-week challenge group (Fig. 1B–D). Pulmonary inflammation did not show too much impact on the weight of lung, though the lung/body weight ratio increased in 1-week challenge group (Fig. 1E). A significant increase of eosinophil proportion in BALF was observed in all challenged groups in comparison to control group and the peak eosinophil proportion appeared in 1-week challenge group (Fig. 1B–D).

The rats from 1-week challenge group showed more obvious inflammation compared with the rats from 4- and 8-week challenge groups

The infiltration of lymphocytes occurred in 1-week challenge group and the inflammation score showed significant increase only in 1- and 4-week challenge groups, but not in 8-week challenge group (Fig. 2I). In the 1-week challenge group, a massive peribronchial lymphocyte and eosinophil infiltration were the major features (Fig. 2B). The 1-week challenge rats showed increasing neovascularization and perivascular sleeve-like change in alveoli (Fig. 2F). NO concentration in serum was significantly increased in all antigen challenge groups compared with control group (Fig. 2J). DTH in the 1- and 4-week challenge groups had significant difference but this difference disappeared in 8-week challenge rats (Fig. 2K).

The rats from 8-week challenge group showed lung structural destruction, collagen deposition, and high mucus production

In the 8-week challenge group, the infiltration of inflammation cells decreased but the lesion of airways and alveoli was more serious. The airway epithelia were destroyed and the integrity of airway wall disappeared. The airway thickness due to smooth muscle hypertrophy and hyperplasia was obviously observed (Fig. 2D). In addition, extensive pulmonary emphysema emerged in this period, and alveolar ectasia, disintegration, and narrowing of alveolar septum were presented comparing with control lungs (Fig. 2H). In a word, the remoulding of lungs was evident in 8-week challenge group. In the 4-week challenge group, however, the lungs showed the features of the 1- and 8-week challenge groups simultaneously; in other words, they have inflammatory cell infiltration, airway lesion and remodelling (Fig. 2C, G).

Masson staining of lung tissue indicated that the collagenation appeared in the whole disease progression even at the beginning of the disease (Fig. 3B, D). The grey scale of green colour representing the collagen around airways showed a significant difference in the challenge groups. Meanwhile, in the 8-week challenge group, the relative area of collagen was much larger than that in short term challenge groups (Fig. 3I, J). The secretion of mucus by using PAS staining was obvious in OVA challenge rats in comparison to control animals. We scored the lung tissue of individual rat and found that the mucus secretion increased significantly in 1- and 8-week challenge groups (Fig. 3K).

The rats with different challenge terms showed various cytokine productions and humoral immune responses

The mRNA expression of IL-4 in lung elevated in 1-week challenge group rats and returned to

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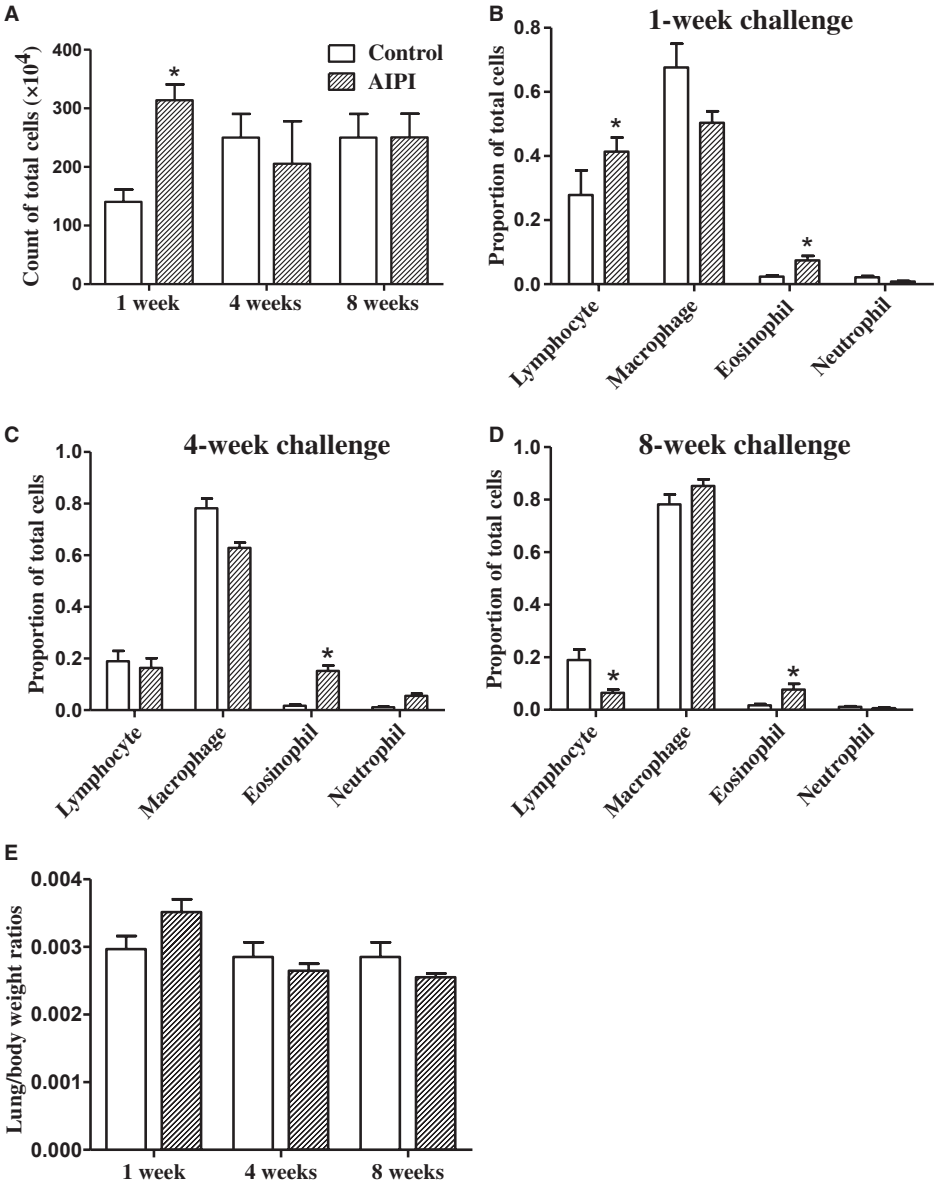

Fig. 1. Differential count in bronchoalveolar lavage fluid (BALF) and the lung/body weight ratio in antigen induced pulmonary inflammation (AIPI) model after different challenge terms. Total cell number in BALF was showed in A and the proportions of different cells in BALF were showed in B–D. The weight of lungs did not show too much change after antigen challenge (E). The results in all figures are expressed as mean ± SEM. * and ** represent $p < 0.05$ and $p < 0.01$, respectively, between challenge group and control group after Mann–Whitney test ($n = 10$ for each group).

the control levels in the longer term challenge groups. Simultaneously, IL-4 expression in spleen tissues peaked after the 1-week challenge group (Fig. 4A, B). TGF-β expression increased significantly in lung after the 1-week challenge group. Especially higher expression of TGF-β appeared in the 4- and 8-week challenge groups in spleen (Fig. 4C, D). The levels of total serum IgE and OVA-specific IgG1 in the serum of rats were elevated in all challenge groups compared with the control groups, and the peaks of total serum IgE and OVA-specific IgG1 appeared after 8 week challenge (Fig. 4E, F).

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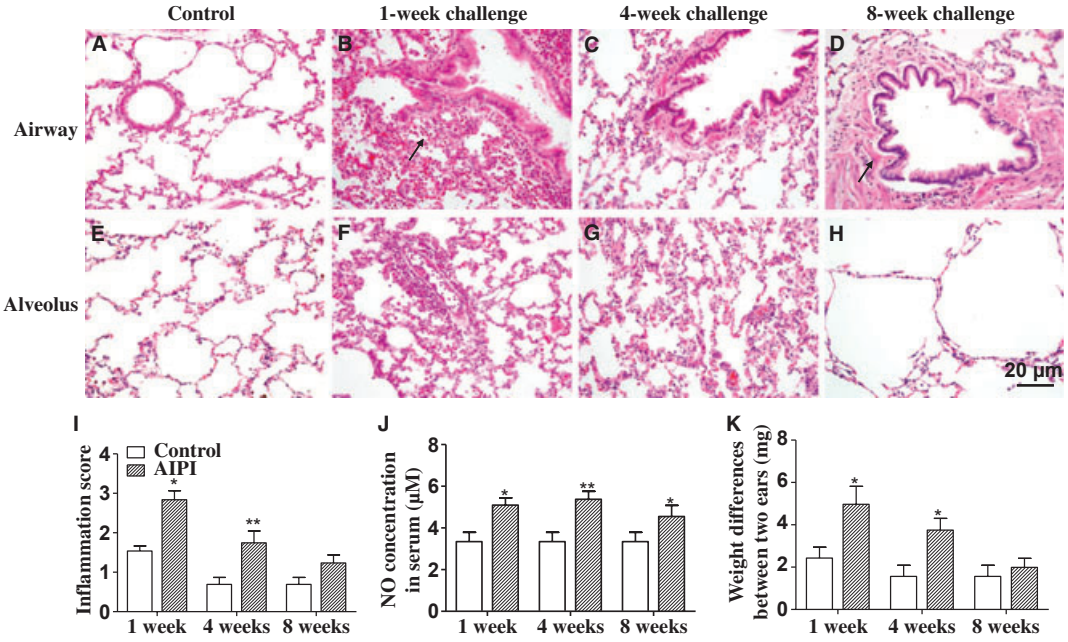

Fig. 2. Histopathological changes and inflammation response in antigen induced pulmonary inflammation (AIPI) model after different challenge terms. Representative histological images of lungs were stained by HE stain. A–D show the changes of bronchus, and E–H show the changes of pulmonary alveoli. The arrow in B indicated the eosinophilia in lung tissue of 1-week challenge group. The arrow in D indicated the smooth muscle proliferation around airway in 8-week challenge group. Emphysema and the destruction of alveolar structure were general in 8-week challenge group (H). Inflammation score elevated in 1 and 4-week challenge groups (I). The change of NO concentration in serum and delayed type hypersensitivity (DTH) showed different changes in different challenge terms (J, K). The results in I–K are expressed as mean ± SEM. * and ** represent $p < 0.05$ and $p < 0.01$, respectively, between challenge group and control group after Mann–Whitney test ($n = 10$ for each group).

DISCUSSION

Despite a large body of literature on the animal models of asthma, there is little information about the effect of antigen challenge term on asthmatic disease patterns (3). The current study therefore attempted to systematically characterize the course of AIPI in E3 rats after different term challenge. The results showed that E3 rats challenged for 1, 4, and 8 weeks manifested the various phenotypes. In the short term challenge, the rats have more severe inflammation, higher NO production and marked DTH response. But in the long term challenge, the rats showed obvious lung tissue destruction and remodelling. Different challenge terms also have different cytokine productions and humoral responses: in the short challenge, IL-4 showed highly expressed, but in long term challenge, TGF-β is a dominant cytokine. In addition, the longer the antigen challenge lasted, the higher the humoral response elevated.

First, we found that all rats from OVA challenge groups showed strong eosinophil infiltration into airways compared with the control groups. Eosinophils have been considered as main players that participate in all aspects of asthma pathogenesis (17). Unremitting aggregation of eosinophils is a feature of the airway inflammation of asthma (18). In human studies, the infiltration of eosinophil at asthmatic attack leads to tissue lesion and more severe clinical manifestation, and asthma remission accompanies the decrease of eosinophil in the lungs (19, 20). Therefore, our results combined with human studies implied that the E3 rat asthma model can simulate the inflammatory character of allergic asthma and is a good model for the pathogenesis research of asthma. Besides the eosinophils, lymphocytes are

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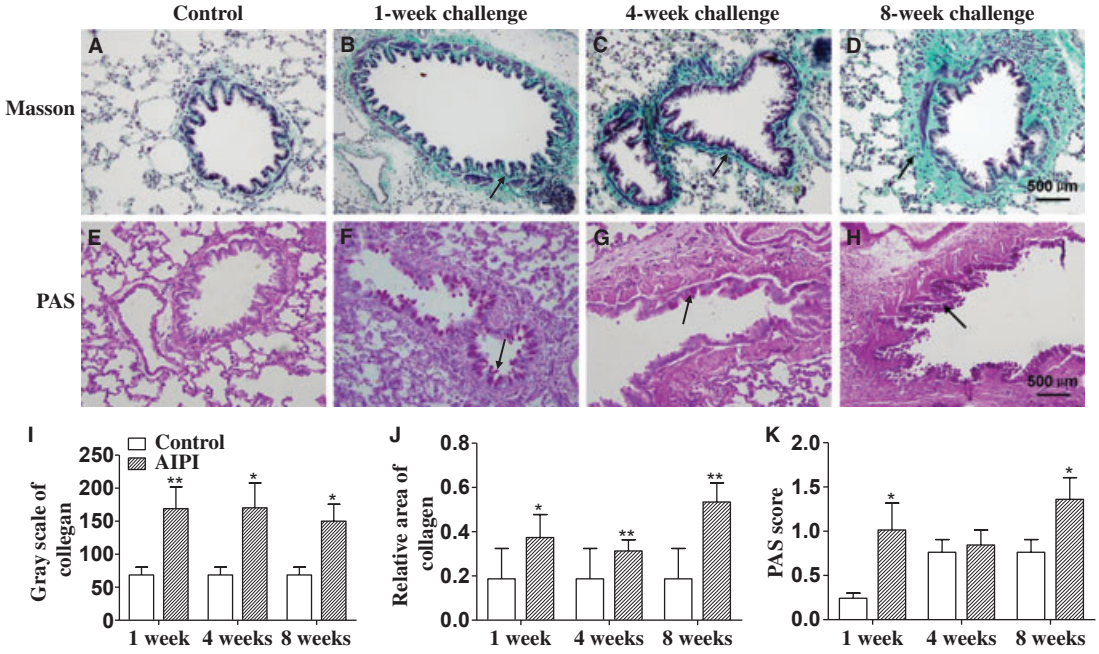

Fig. 3. Collagen deposition and mucus production in antigen induced pulmonary inflammation (AIPI) model after different challenge terms. Lungs were stained by Masson stain to detect collagen fibres production (arrows) in rat lungs (A–D). Periodic acid Schiff (PAS) stain was used to detect mucus production (E–H). The arrows in F, G and H indicated mucus in airways. Collagen generation (I) and the relative area of collagen area/airway area (J) elevated significantly in challenge groups comparing with control groups. PAS score was significantly higher in 1-week challenge group and 8-week challenge group than control group (K). The results in I, J and K are expressed as mean ± SEM. * and ** represent $p < 0.05$ and $p < 0.01$, respectively, between challenge group and control group after Mann–Whitney test ($n = 10$ for each group).

another important effector in asthma, which are involved in airway hyper-responsiveness (21, 22). We also found that lymphocyte infiltration was extremely serious in 1-week challenge group, but not significant in 4- and 8-week challenge groups.

In addition, NO is an important inflammatory mediator produced by the inflammatory cells and has been considered hallmark of allergic asthma. Several researches have indicated that the NO in expiratory gas of asthmatic patients showed a significant increase compared with healthy subjects, especially after the stimulus of specific antigen (23). In OVA challenge group of this study, significantly increased NO release in serum was observed and this is consistent with our previous study (15, 16).

In chronic asthma, structural destruction of lung tissue is the main pathological feature. From the morphological observation, we found that airway remodelling can be observed not only in small bronchi but also in the lobar bronchi. Various components of the airway showed abnormal changes, such as exuviations and folding of airway epithelium, the increase of matrix and smooth muscle hyperplasia around airways, all of which may lead to the remodelling of the bronchial walls. We also found that the extensive emphysematous alveoli emerged in 8-week challenge group, which demonstrated that the lesion of lung existed not only in airways, but also in pulmonary alveoli. Airway smooth muscle hyperplasia, increased mucus secretion and collagen deposition, as the main reasons for airway remodelling, were also aggravated over time in this study.

The Th2-driven allergic responses have been demonstrated as a central role in the progression of asthma. IL-4 is the self-secreted cytokine of T cells, which facilitates the Th2-type transformation, and induces the occurrence of asthma (24, 25). Our findings showed that IL-4 expression increased significantly when the rats were challenged for 1 week, and this increase

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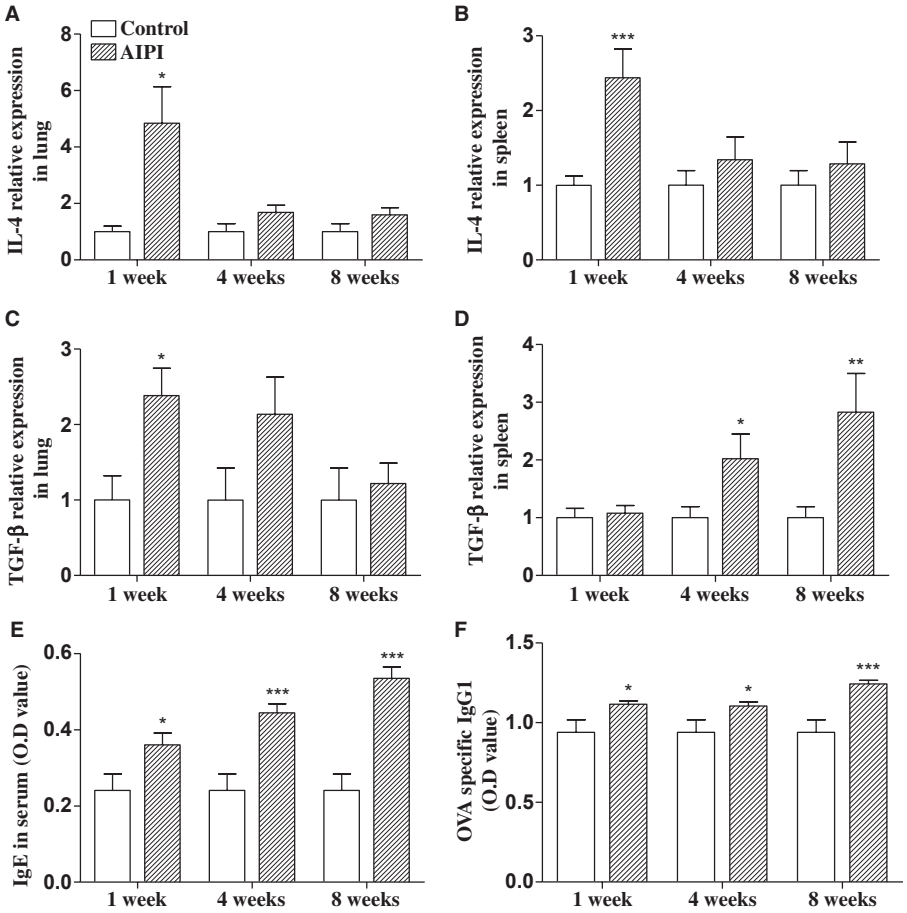

Fig. 4. Th2 cytokine production and humoral immune responses. The expressions of IL-4 and TGF-β of lung (A and C) and spleen (B and D) tissues were detected by quantitative real-time PCR. The levels of total IgE (E) and ovalbumin (OVA)-specific IgG1 (F) in serum were measured by ELISA. The results in all figures are expressed as mean ± SEM. *, ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively between challenge group and control group after Mann–Whitney test ($n = 10$ for each group).

occurred in lung and especially the immune organ, spleen, indicating that IL-4 production has an impact on early inflammation. TGF-β is a pleiotropic mediator and regulates the accumulation, proliferation, and differentiation of fibroblasts, and the synthesis of extracellular matrix (26–28). The mRNA expression of TGF-β in our study increased significantly in lung tissues from AIPI groups and in spleen, especially in 4- and 8-week challenge groups, which indicated that TGF-β strongly influenced on the airway remodelling in chronic asthma. In addition, airway remodelling may be prevented or reversed by using agents which target TGF-β (29). Therefore, the modulation of TGF-β expression or activity represents a potential therapeutic target for asthma, especially in the chronic stage of asthmatic disease.

Humoral immune response definitely participates in the pathogenesis of asthma. IgE contributes to both the early- and late-phase inflammatory cascade in airways. We characterized E3 rat model in greater details and found elevated levels of total IgE and OVA-specific IgG1 in serum in all challenge groups. These findings suggest that AIPI model is sufficient to induce humoral immune response, even in the 1-week challenge group. It has been reported that monoclonal antibody (omalizumab) against IgE in asthmatic patients was efficacious in the treatment of moderate-to-severe and severe persistent allergic asthma,

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significantly reduced asthma exacerbations, improved the life quality and had a steroid-sparing effect (30, 31). The peak of total serum IgE and OVA-specific IgG1 appeared after 8-week challenge in our experiment, which support the view that therapy for IgE is efficient for persistent asthma (32).

In addition, various indicators of asthma, such as eosinophil infiltration, inflammation response (represented by NO), Th2 cytokines and immune response, have some cross links between each other and correlate with asthma progression. NO can block the apoptosis of eosinophils, therefore, NO will enhance the inflammation response in asthma via guanylate cyclase pathway. It has been indicated that eosinophils may activate fibroblasts in the inflamed tissue either directly or by the induction of TGF-β, thereby leading to airway fibrosis (17). IL-4 can also induce lymphocyte adhesion and chemotaxis of eosinophil, and promote the expression of mucin gene and cell metaplasia *in vivo* and *in vitro* (33). High level of IL-4 induces chronic respiratory disease including chronic asthma through the increase of fibroblast proliferation and collagen synthesis (34, 35). Therefore, during the different asthma phases, the key molecules combining with others modulate the disease patterns after different antigen challenge terms as showed in this study.

In conclusion, this study suggested that an antigen-induced pulmonary inflammation model in E3 rats contributes many features in the key ways similar to human asthma, such as eosinophil and lymphocyte infiltration, morphological changes in lung tissue, mucus secretion, lung remodelling, and enhancement of Th2 cytokine expression and IgE production. More importantly, we manifest that the different changes occurred because of different challenge terms, which demonstrated that the AIPI in E3 rats challenged for 1 week is suitable for the establishment of acute asthma model; meanwhile E3 rats challenged for 8-week are appropriate for studying chronic asthma.

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AUTHORS' CONTRIBUTIONS

The following authors QS, YX, and SL conceived and designed the experiments. QS and YX carried out all experiments and data generation and analyzed the data. RMB, FJ, XH, BZ and DL performed part of experiments and data generation. QS, YX and SL had extensive scientific discussion for this study and wrote the paper. All authors read and approved the final manuscript.

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