

PATHOGENESIS

Enhanced local Foxp3 expression in lung tissue attenuates airway inflammation in a mouse model of asthma

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Abstract

Objective: Bronchial asthma is a chronic inflammatory disease of the airway mediated by a Th2 immune response. A great deal of data has demonstrated that regulatory T cells (Tregs) have the ability to suppress Th2 immune responses and the transcription factor fork-head box protein 3 (Foxp3) is indispensable for the development of CD4+CD25+Tregs. In this study, we hypothesized that enhanced local Foxp3 expression in lung tissue could suppress Th2-mediated allergic asthma. **Methods:** Foxp3/PMX retroviruses containing the mouse *Foxp3* gene were constructed and administered into asthmatic mice through intra-tracheal instillation before ovalbumin challenging. Foxp3 expression, airway hyper-responsiveness (AHR), bronchoalveolar lavage fluid (BALF) and tissue inflammatory cell and cytokine profiles were characterized. **Results:** Foxp3 mRNA and protein were increased in the lung tissue of asthmatic mice. Enhanced expression of Foxp3 locally in the lung tissue reduced the airway AHR, inflammatory cell infiltration and mucus production. It also attenuated Th2 and Th17 immune responses as evidenced by reduced IL-4, IL-13 and IL-17 levels. **Conclusions:** This study demonstrates that enhanced Foxp3 expression in the airway by intra-tracheally instilled Foxp3/PMX retroviruses alleviates allergic airway inflammation by reducing the Th2 immune response.

Keywords

Asthma, Foxp3, regulatory T cells, Th2 cells

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Published online 12 March 2014**Introduction**

Bronchial asthma is a serious global health problem, which affects 300 million individuals and causes 250 000 deaths every year [1]. Asthma is a chronic inflammatory disorder of the airway characterized by the infiltration of lymphocytes, eosinophils, mast cells and macrophages; airway hyper-responsiveness (AHR); and airway remodeling. Among the numerous mechanisms of asthma, the generation of Th2 cytokines, including IL-4, IL-5, IL-13, etc., in response to environmental allergens is considered to be the predominant one [2,3].

Regulatory T cells (Tregs), especially CD4+CD25+Tregs accounting for approximately 5–10% of the peripheral CD4+T cells in normal unimmunized individuals, play an important role in tolerance induction in the mouse model of asthma [4,5]. Although there are other cell types that also participate in these processes and are sometimes named Treg cells, such as IL-10-producing Tr1 cells and TGF- β -producing Th3 cells, our study focused on the most potent regulatory T cell, CD4+CD25+Tregs. The transcription

factor fork-head box protein 3 (Foxp3), a member of the fork-head/winged-helix family of transcriptional regulators, is specifically expressed in CD4+CD25+Tregs and is crucial for the differentiation from naive T cells towards the Tregs phenotype [6–8]. Mutations of *Foxp3* gene have been found in scurfy mice with X-linked lymphoproliferative disease and in humans with immune dysregulation, poly-endocrinopathy, enteropathy and X-linked syndrome (IPEX) [9,10]. Increased exposure to ambient air pollution is associated with hypermethylation of the Foxp3 locus, impairing Tregs function and increasing asthma morbidity [11].

Recruitment of Tregs in the airway or lymph tissues can repress the hallmark features of asthma [12–14]. Many factors, such as Rapamycin combined with TGF- β , IL-2 combined with TGF- β , have been found to induce ectopic Foxp3 expression in CD4+CD25-T cells or NKT Cells, which do not normally have regulatory function, and confer the immune regulatory property. Transferring of these cells to asthmatic mice can reduce the Th2 immune response [15–17]. But all these studies utilized systemic administration methods that may cause potential problems due to their side effects on other organs *in vivo*.

Rudensky found out that overexpression of Foxp3 by retroviruses infection induced suppressor function on peripheral CD4+CD25-T cells [6]. Based on the results *in vitro*, we proposed to enhance Foxp3 expression in the lung tissue locally and supposed that this method would relieve the

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symptoms of asthma. We successfully constructed Foxp3/PMX retroviral vectors containing the mouse *Foxp3* gene and administered these viruses into experimentally induced asthmatic mice by intra-tracheal instillation and characterized lung inflammation, mucus production, AHR and cytokine production.

Materials and methods

Retroviral construction and preparation of viruses

Mouse Foxp3 cDNA was cloned by PCR and subcloned into the retroviral vector PMX-IRES-GFP (Foxp3/PMX). Plasmid DNA was transfected into the Plat-E package cell line using the Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN, catalog No. 11814443001) according to the manufacturer's procedure. Culture supernatant containing Foxp3/PMX retroviruses was harvested 24 h later and used immediately or stored at -80°C . Empty/PMX retroviruses were produced as negative controls. The supernatant had a titer of 6×10^5 TU/ml measured on NIH3T3 cells as previously described [18].

Asthma model protocol

Female wild-type BALB/c mice (aged 6–8 weeks) were purchased and maintained in the Animal Research Center of Zhejiang University. All animal protocols were approved by the Animal Subjects Committee, Zhejiang University. These mice were randomly divided into four groups: Saline, OVA, OVA-Foxp3/PMX and OVA-Empty/PMX. All the mice in the last three groups were sensitized by intraperitoneal injection with 80 μg chicken ovalbumin (Sigma-Aldrich, St. Louis, MO, catalog No. A5503) emulsified in 100 μl Imject Alum (Pierce, Rockford, IL, catalog No. 77161) on days 0 and 14, and challenged with an aerosolized ovalbumin (1% ovalbumin in saline) for 40 min at days 25, 26 and 27. The mice in the OVA-Foxp3/PMX group were treated with 50 μl of Foxp3/PMX retroviruses by intra-tracheal instillation at days 22, 23 and 24, while the mice in the OVA-Empty/PMX group were treated with Empty/PMX retroviruses at the same time. The mice in the Saline group were injected with 0.2 ml saline on days 0 and 14 and challenged with saline at days 25, 26 and 27.

Airway hyper-responsiveness

Twenty four hours after the last ovalbumin challenge, the airway hyper-responsiveness (AHR) to inhaled methacholine (MCh) was evaluated using non-invasive flow plethysmography (Buxco, Troy, NY) as previously described [19]. The enhanced pause (Penh) value was plotted as the change from baseline per dose.

Bronchoalveolar lavage fluid cytology

At day 28, the mice were anesthetized and the blood was removed from the right side of the heart. Then the bronchoalveolar lavage fluid (BALF) of the left lung was collected using 0.4 ml phosphate-buffered saline (PBS). Total cell number was determined by placing 10 μl of the BALF for each mouse in a hemocytometer. Then the remainder of the cells was collected by centrifugation at

2500 rpm for 10 min, resuspended in 120 μl PBS and stained with the Wright-Giemsa. Cells were identified as macrophages, eosinophils, neutrophils and lymphocytes. And, a minimum of 300 cells were blindly counted in duplicate for each sample under the microscope.

Lung histopathology

At day 28 after the left lung was lavaged by PBS, it was fixed with 10% buffered formalin followed by paraffin embedding and sectioning to 5 μm thickness. Then the sections were stained with hematoxylin and eosin (H&E) and periodic acid schiff (PAS). Two different 5-point scoring systems were used to quantify the severity of inflammatory cell infiltration and mucus production in lung [20]. Briefly, the scoring system for inflammatory cell infiltration was: 0, no cells; 1, several cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells >4 cells deep. The scoring system for mucus production was: 0, no goblet cells; 1, <25%; 2, 25–50%; 3, 50–75%; 4, >75%. Scoring of inflammatory cells and goblet cells was performed in at least three different fields for every lung section. Mean scores were obtained from five mice. The investigator was blinded to mouse treatment status during this procedure.

Cytokine ELISA

BALF was analyzed for IL-4, IL-13, IL-17A, IL-10 and TGF- β 1 according to the manufacturer's instructions. The kits used were Mouse IL-4, IL-13, IL-17A, IL-10 and TGF- β 1 ELISA Sets (R&D Systems, Minneapolis, MN, catalog No. M4000B, M1300CB, M1700, M1000 and MB100B).

Real-time RT PCR

At day 28, after the left lung was lavaged and removed from the rib cage, the right lung was removed and frozen immediately in -80°C for further RNA or protein extraction. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, catalog No. 15596026) according to the manufacturer's protocol and was reverse-transcribed with oligo(dT) primers (Fermentas, CA, catalog No.#K1622). The cDNA of target genes as well as the housekeeping gene β -actin was amplified by real-time PCR in an ABI PRISM 7500 Sequence Detection System. Relative quantification analysis was performed using the comparative CT ($2^{-\Delta\Delta\text{CT}}$) method. The primer pairs used are as follows: β -actin: 5'-AGAGGGAAATCGTGCGTGAC-3' and 5'-CAATAGTGATG ACCTGGCCGT-3'; Foxp3: 5'-CCCAGGAAAGACAGCAACCTT-3' and 5'-TTCT CACAA CCAGGCCACTTG-3'; IL-4: 5'-GTCATCCTGCTCTTCTTT CT-3' and 5'-CTTCTCCTGTGACCTCGTT-3'; IL-10: 5'-AC ATACTGCTAACCGACTC-3' and 5'-AATGCTCCTTGATT TCTGG-3'; IL-13: 5'-TTGCTTGCCTTGGTGGTC-3' and 5'-AATATCCTCTGGGTCCTGT-3'; IL-17A: 5'-CCTCAGACT ACCTCAACCGT-3' and 5'-CTTTCCCTCCGCATTGACA-3'; IFN- γ : 5'-GCTCTGAGACAATGAACGC T-3' and 5'-AA AGAGATAATCTGGCTCTGC-3'; TGF- β 1: 5'-TCAGACAT TCGGGAAGCAG-3' and 5'-AGCCACTCAGGCGTATCAG-3'; TNF- α : 5'-AATAACGCTGATTTGGTGA-3' and 5'-ACC CGTAGGGCGATTACA-3'.

Western blot

The protein from lung tissue was extracted using RIPA buffer (Sigma-Aldrich, St. Louis, MO, catalog No. R0278) containing protease inhibitor PMSF and the protein concentration was determined by the BCA assay method (Thermo scientific Pierce, Waltham, MA, catalog No. 23225). The mouse anti-Foxp3 primary antibody (Abcam, Cambridge, MA, catalog No. ab36607) was used (1:5000), and the mouse anti-GAPDH primary antibody (Santa Cruz, CA, catalog No. SC-32233) served as an internal control (1:1000). Anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (1:10 000; Santa Cruz, CA, catalog No. sc-2005) was used for visualization using enzyme-linked chemiluminescence (Millipore, Medford, MA, catalog No. WBKLS0050) with the Chemidoc XRS System (Bio-Rad, Berkeley, CA).

Statistical analysis

Data were shown as mean $\pm$ SEM. The results were analyzed using the GraphPad Prism 5 software. p value <0.05 was considered statistically significant using the one-way ANOVA analysis. Two to three experiments were performed per assay, and the representative data were shown.

Figure 1. Expression of *Foxp3* in murine lung tissue. (A) *Foxp3* mRNA in murine lung tissue was quantified by real-time PCR. Data were shown as mean $\pm$ SEM. $**p < 0.01$, $***p < 0.001$; $n = 6-8$ per group. (B) Immunoblot analyses of lysates from murine lung tissue were performed using anti-Foxp3 antibody. Saline, saline sensitized + saline challenged. OVA, ovalbumin sensitized + ovalbumin challenged. OVA-Foxp3/PMX, ovalbumin sensitized + ovalbumin challenged + Foxp3/pMX retroviruses treated. OVA-Empty/PMX, ovalbumin sensitized + ovalbumin challenged + Empty/pMX retroviruses treated.

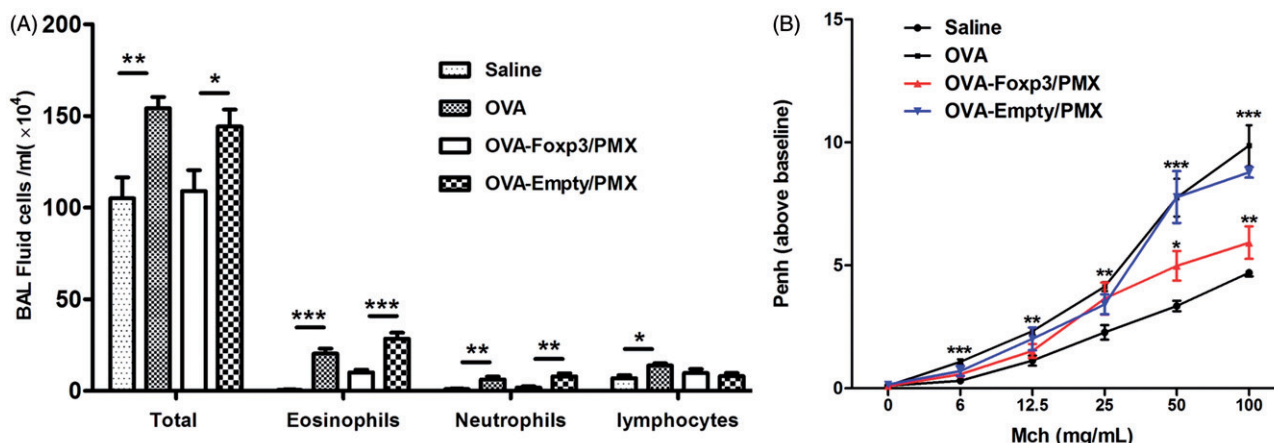
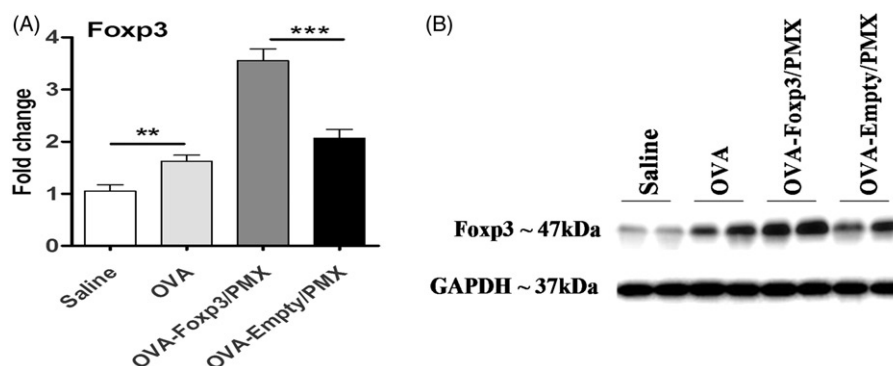


Figure 2. Enhanced *Foxp3* expression ameliorated airway inflammatory cell infiltration and ovalbumin-induced AHR. (A) The total number of cells and cellular profile of BALF. (B) AHR to MCh inhaled in the four groups. Data were shown as mean $\pm$ SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; $n = 6-8$ per group. Saline, saline sensitized + saline challenged. OVA, ovalbumin sensitized + ovalbumin challenged. OVA-Foxp3/PMX, ovalbumin sensitized + ovalbumin challenged + Foxp3/pMX retroviruses treated. OVA-Empty/PMX, ovalbumin sensitized + ovalbumin challenged + Empty/pMX retroviruses treated.

Results

The expression of *Foxp3* in the lung tissue of asthmatic mice was increased and further enhanced by Foxp3/PMX retroviruses administration

In this study, we detected the levels of *Foxp3* mRNA and protein in the lung tissue of mice. Surprisingly, we found that *Foxp3* mRNA as well as protein was significantly increased relative to healthy control mice. The expression of *Foxp3* mRNA and protein were further enhanced by intra-tracheal instillation of Foxp3/PMX retroviruses in the lung tissue of asthmatic mice as compared with that of the mice treated with Empty/PMX retrovirus administration (Figure 1A and B).

Enhanced *Foxp3* expression ameliorated airway inflammation, ovalbumin-induced AHR and mucus overproduction

As shown in Figure 2(A), the total number of cells in BALF was significantly increased in antigen exposed asthmatic mice relative to saline sensitized and challenged mice, and that was also effectively reduced by forced local expression of *Foxp3*. Analysis of the differential cell count shows that ovalbumin exposure led to high numbers of eosinophils, neutrophils and lymphocytes. In Foxp3/PMX retrovirus treated mice, a significant drop in the numbers of eosinophils and neutrophils

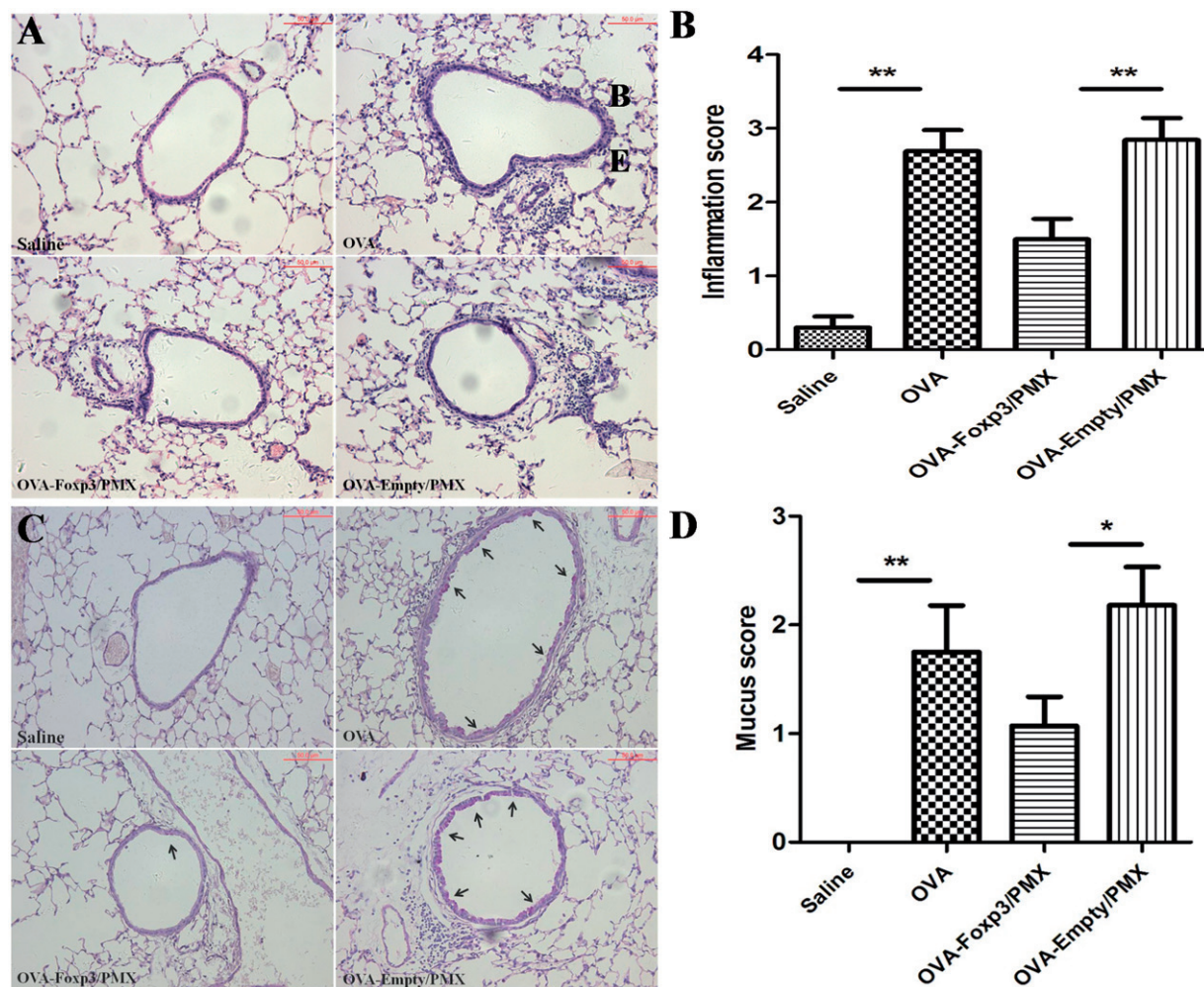


Figure 3. Enhanced Foxp3 expression ameliorated airway inflammation and mucus overproduction. (A) Lung sections were stained with H&E (Magnification 200×). (B) Semi-quantitative analysis of inflammatory cell infiltration in lung sections were performed as described in the “Materials and methods” section. (C) Lung sections were stained with PAS. The arrow represents PAS-positive cells (Magnification 200×). (D) Semi-quantitative analysis of mucus production in lung sections were performed as described in the “Materials and methods” section. Data were shown as mean ± SEM. * $p < 0.05$, ** $p < 0.01$; $n = 6-8$ per group. Saline, saline sensitized + saline challenged. OVA, ovalbumin sensitized + ovalbumin challenged. OVA-Foxp3/PMX, ovalbumin sensitized + ovalbumin challenged + Foxp3/pMX retroviruses treated. OVA-Empty/PMX, ovalbumin sensitized + ovalbumin challenged + Empty/pMX retroviruses treated.

was observed, whereas Empty/PMX retrovirus treatment exerted little effect.

Lung sections stained with H&E also showed that enhanced Foxp3 expression attenuated airway inflammation (Figure 3A and B). Compared with mice treated with saline, the mice exposure to ovalbumin exhibited a characteristic peribronchial and perivascular airway inflammation. And there was a significant difference between these two groups according to the five-point scoring system to quantify the severity of inflammatory cell infiltration ($p < 0.01$). Meanwhile, the OVA-Foxp3/PMX group showed less inflammatory cell infiltration as compared with the OVA-Empty/PMX group ($p < 0.01$).

The AHR to inhaled methacholine (MCh) was measured using flow plethysmography to examine the impact on airway reactivity. Compared with the Saline group, the Penh values of the asthmatic mice were significantly increased at all doses of Mch (Figure 2B). Compared with the OVA-Empty/PMX group, the Penh values of the OVA-Foxp3/PMX group were significantly decreased at the 50 mg/ml ($p < 0.05$) and 100 mg/ml ($p < 0.01$) doses.

Lung sections from mice of all groups were stained with PAS to observe the mucus production (Figure 3C and D). Nearly no mucus was observed around the airways of healthy control mice; while many PAS-positive cells were observed in the airways of the OVA group. Foxp3/PMX retrovirus administration inhibited airway mucous production, as the number of PAS-positive cells in the OVA-Foxp3/PMX group was markedly reduced compared with the OVA-Empty/PMX group ($p < 0.05$).

Enhanced Foxp3 expression repressed Th2 and Th17 instead of Th1 inflammatory cytokine production in asthmatic mice

The mRNA expression of Th2 and Th17 cytokines in lung tissue of OVA group was increased as compared with Saline group (Figure 4A–C). Significantly lower expression levels of Th2 and Th17 cytokines were detected in OVA-Foxp3/PMX group as compared with OVA-Empty/PMX group, for IL-4 ($p < 0.05$), IL-13 ($p < 0.01$) and IL-17 ($p < 0.05$). As for the analysis of protein in the BALF (Figure 4D–F), Th2 cytokine production in the OVA group was considerably increased as

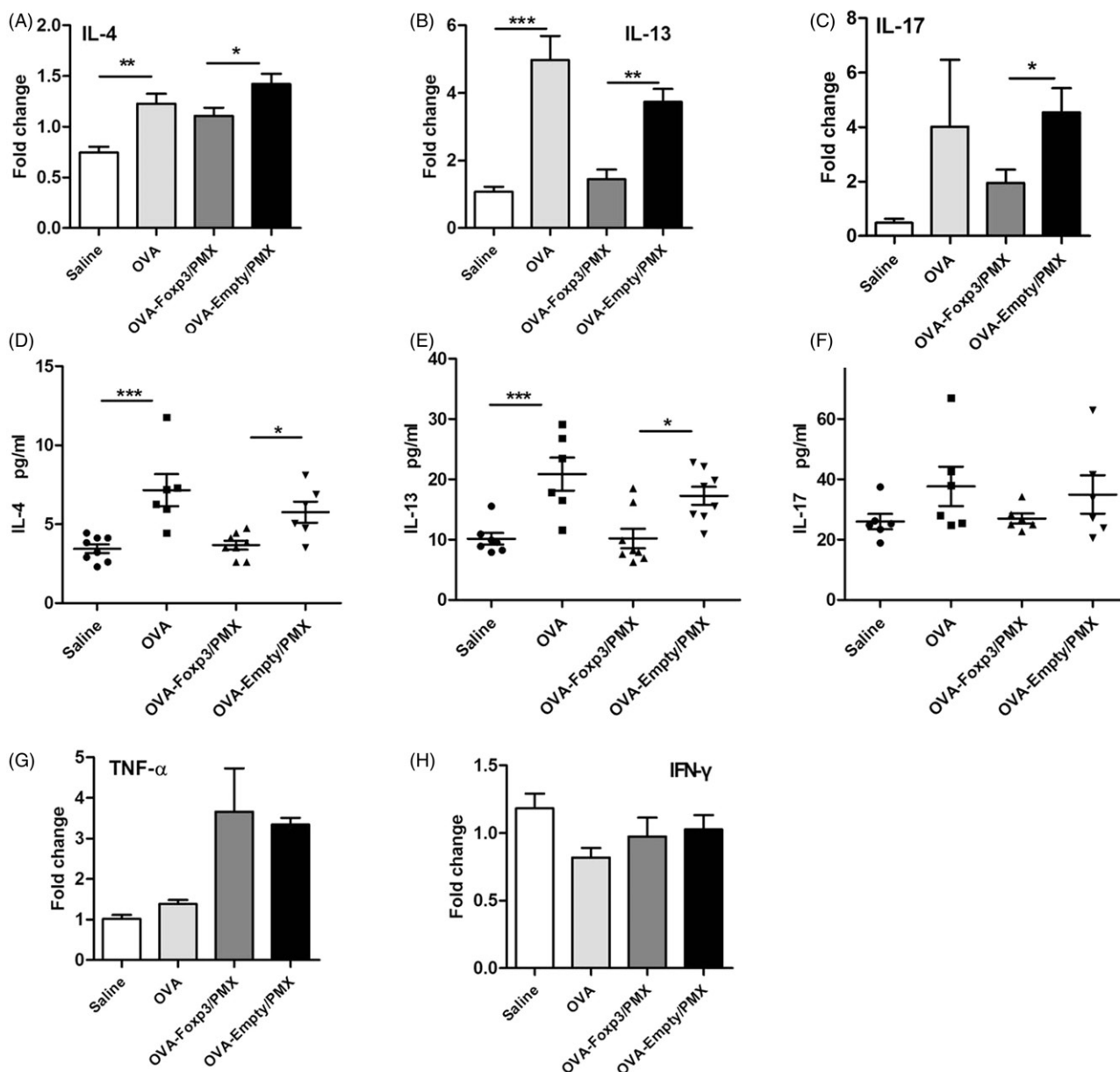


Figure 4. The inflammatory factors in lung tissue and BALF. (A) IL-4 mRNA in lung tissue. (B) IL-13 mRNA in lung tissue. (C) IL-17A mRNA in lung tissue. (D) IL-4 protein in BALF. (E) IL-13 protein in BALF. (F) IL-17 protein in BALF. (G) TNF- α mRNA in lung tissue. (H) IFN- γ mRNA in lung tissue. The mRNA of different inflammatory factors in murine lung tissue was quantified by real-time PCR. The protein of different inflammatory factors in BALF was quantified by ELISA. Data were shown as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001; n = 5–8 per group. Saline, saline sensitized + saline challenged. OVA, ovalbumin sensitized + ovalbumin challenged. OVA-Foxp3/PMX, ovalbumin sensitized + ovalbumin challenged + Foxp3/pMX retroviruses treated. OVA-Empty/PMX, ovalbumin sensitized + ovalbumin challenged + Empty/pMX retroviruses treated.

compared to the Saline group for IL-4 (p < 0.001) and IL-13 (p < 0.001). In contrast, significantly lower levels of Th2 cytokine production were detected in the OVA-Foxp3/PMX group as compared with the OVA-Empty/PMX group for IL-4 (p < 0.05) and IL-13 (p < 0.05). Although there was no statistically significant difference among the four groups for IL-17 protein in the BALF, there seemed to be some differences in the mean values as depicted in Figure 4(F).

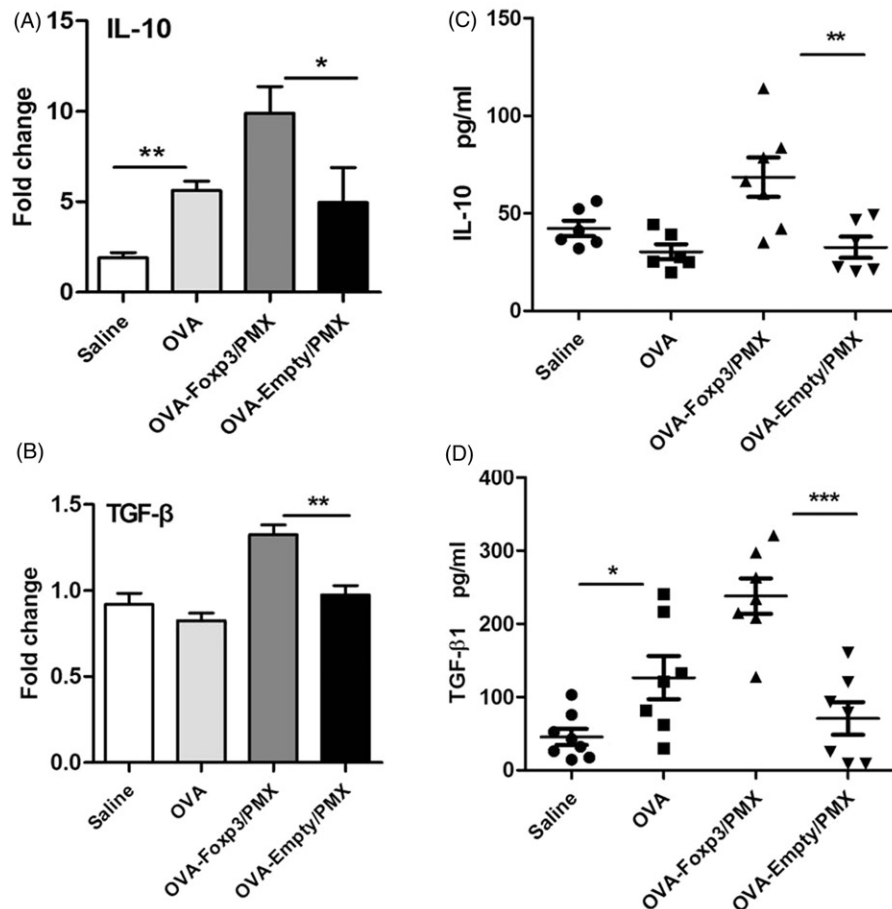
Several literatures showed that CD4+CD25+Tregs reduced the Th2 immune response in asthmatic individuals while the effect on Th1 type immune responses was limited [12,21,22]. We measured the expression levels of IFN- γ and TNF- α mRNA in the lung tissue which was almost produced by Th1 cells (Figure 4G and H). As a result,

there was no significant difference in the levels of IFN- γ and TNF- α mRNA between the OVA-Foxp3/PMX group and OVA-Empty/PMX group, indicating that enhanced Foxp3 expression had no effects on the Th1 type immune response to ovalbumin-induced asthma.

The suppression of the inflammatory response by Foxp3 expression was accompanied by the upregulation of IL-10 and TGF- β

Since Tregs have been shown to require IL-10 as well as TGF- β for full immunosuppressive function *in vivo* [23,24], we analyzed the expression of IL-10 and TGF- β in lung tissue and BALF. We found that the expression of IL-10 mRNA

Figure 5. The levels of IL-10 and TGF- β in lung tissue and BALF. (A) IL-10 mRNA in lung tissue. (B) TGF- β mRNA in lung tissue. (C) IL-10 protein in BALF. (D) TGF- β 1 protein in BALF. The mRNA in murine lung tissue was quantified by real-time PCR. The protein in BALF was quantified by ELISA. Data were shown as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 6-8$ per group. Saline, saline sensitized + saline challenged. OVA, ovalbumin sensitized + ovalbumin challenged. OVA-Foxp3/PMX, ovalbumin sensitized + ovalbumin challenged + Foxp3/pMX retroviruses treated. OVA-Empty/PMX, ovalbumin sensitized + ovalbumin challenged + Empty/pMX retroviruses treated.



in the lung tissue of asthmatic mice was significantly higher than healthy control mice ($p < 0.01$), while there was no difference between the two groups for TGF- β mRNA expression (Figure 5A and B). The OVA-Foxp3/PMX group expressed considerably more IL-10 mRNA ($p < 0.05$) and TGF- β mRNA ($p < 0.01$) than OVA-Empty/PMX group.

We also detected the IL-10 and activated TGF- β 1 protein in the BALF (Figure 5C and D). The level of activated TGF- β 1 in the BALF of asthmatic mice was higher than that of healthy control mice ($p < 0.05$), while there was no difference between the two groups in the level of IL-10. The BALF of OVA-Foxp3/PMX group contained considerably more IL-10 ($p < 0.01$) and TGF- β 1 ($p < 0.001$) than OVA-Empty/PMX group.

Discussion

Previous studies have demonstrated that regulatory cells, including T cells and B cells, are important components of immune system homeostasis, as compromised numbers or function of regulatory cells leads to or aggravates autoimmune diseases and allergy [4,25–28]. Regulatory B cells are a source of inhibitory cytokines such as IL-10 and TGF- β . Regulatory T cells (Tregs) are a developmentally and functionally distinct T-cell subpopulation and the most of the Tregs that have been studied are CD4+CD25+ regulatory T cells. Foxp3 is specifically expressed in these CD4+CD25+ T cells and is required for their development and function [6,29–31]. It has been reported that ectopic expression of Foxp3 in peripheral CD4+CD25-T cells or

invariant NKT (iNKT) cells *ex vivo* leads to immunoregulatory activity [6,16,17].

Recent studies suggested that asthma was characterized by a relative deficiency in Tregs, as evidenced by the fact that Foxp3 mRNA or protein in peripheral blood mononuclear cells (PBMC) was decreased in patients with asthma than normal individuals [32,33]. But the percentage of CD4+Foxp3+ Tregs was increased in the BALF of patients with moderate to severe asthma compared with both mild asthma patients and healthy subjects [34]. And, in our animal model of asthma, we found that the levels of Foxp3 mRNA and protein in the lung tissue of asthmatic mice were increased relative to healthy control mice. A plausible explanation may be that once the allergen is present in the airway, there need to be numerous Tregs localized to the airway in order to induce immunologic tolerance. In the case of ovalbumin-induced airway inflammation, the number of Tregs appearing in the airway might not be enough to suppress the Th2 immune response. Based on the above-mentioned hypothesis and the pivotal role of Foxp3 in the development of Tregs, we hoped to relieve the characteristics of mouse allergic inflammation by using intra-tracheally instilled Foxp3/PMX retroviruses to raise the number of Tregs in the local airway. In this study, our hypothesis was confirmed, suggesting that increasing the number of Tregs by enhancing local *Foxp3* gene expression in the airway may be a useful therapy for asthma patients in the future.

Until now, many studies focused on how to convert other types of T cells into Tregs and then transfer these cells into

murine models of asthma to reduce the Th2 immune response. All of these studies used systematic administration methods and may cause harmful side effects on other organs *in vivo*. In our study, we successfully constructed *Foxp3*/PMX retroviruses and administered these viruses into asthmatic mice by intra-tracheal instillation. It is believable that the protective effects on asthmatic mice in our study partially through increasing the percentage of *Foxp3*+Tregs. In the most recently published paper by Mays et al. [35], they utilized modified *Foxp3* mRNA to over-express the *Foxp3* protein in murine lung and found out that it could prevent allergic asthma *in vivo*. So the therapeutic method which is realized by locally over-expressing the regulatory T cell transcription factor – *Foxp3*, as an important innovation, may have much greater potential for clinical use in future.

Accumulating data supports the idea that the Th2 arm of the immune system is under tighter control by Tregs than the Th1 arm, indicating that Th2-driven diseases including allergic asthma may be much more responsive to Treg manipulation [12,21,22]. And, in our experiments, we found out that the Th2 cytokines IL-4, IL-13 were markedly decreased when the *Foxp3* expression in the lung tissue was enhanced. The mechanism for this might lie in the increased number of Tregs. We also detected the Th1 cytokines IFN- γ and TNF- α , and found that there was no difference between the group treated by *Foxp3*/PMX retroviruses and the group treated by Empty/PMX retroviruses, indicating that enhanced *Foxp3* expression in the airway reduced inflammation by regulating the Th2 response while exerted no appreciable effects on the Th1 response. As for the level of TNF- α mRNA in both retrovirus treated groups was much higher than OVA group showed in Figure 4, it may due to the response to virus infection according to the paper by Braciale et al. [36]. In their review, they described that virus infection of respiratory epithelial cells leads to the production of TNF by inflammatory cells.

In this study, we found that the expression of IL-17 mRNA in the lung tissue of asthmatic mice was much higher than that of healthy control mice, and the OVA-*Foxp3*/PMX group had much lower IL-17 mRNA than OVA-Empty/PMX group. The protein level of IL-17 in the OVA-*Foxp3*/PMX group was also lower than the OVA-Empty/PMX group, although the difference did not reach statistical significance. Previous studies revealed that *Foxp3* mutation led to increased Th17 cell numbers and regulatory T-cell instability [37]. And our studies demonstrated that Tregs had the potential to inhibit Th17 cells and the production of IL-17 while relieving the physiological characteristics of asthma. Our data also indicated that Th17 cells or IL-17 might be new targets for the treatment of asthma.

It was reported that transferring of Tregs could suppress the Th2 cell-driven response to allergen *in vivo* by an IL-10-dependent mechanism [38]. Although there was extensive evidence *in vivo* suggesting that immunosuppressive cytokines, IL-10 and TGF- β , might be involved in Treg-mediated immunosuppression [23,39,40], contradictory *ex vivo* data also existed [41]. According to our results, IL-10 and TGF- β protein in the BALF of asthmatic mice which received intra-tracheally instilled *Foxp3*/PMX retroviruses before the stage of allergen-challenge were significantly increased,

as well as the IL-10 and TGF- β mRNA in the lung tissue. Our results further demonstrated that IL-10 and TGF- β were important for Tregs to realize their immunosuppressive function *in vivo*.

Conclusions

In summary, Tregs, especially CD4 + CD25 + *Foxp3* + Tregs, play an essential role in regulating airway inflammation. Our major finding was that enhanced local *Foxp3* expression in lung tissue could attenuate the airway inflammation in a mouse model of ovalbumin-induced asthma. The protective effects were due to the inhibition of Th2 responses probably through increasing *Foxp3*+Tregs. The further molecular mechanisms by which the *Foxp3*/PMX retroviruses mediate immunosuppression remain elusive and need to be studied deeply. In all, enhancing *Foxp3* expression in lung tissue offers a potential therapy for asthma.

Declaration of interest

All authors read and approved the final manuscript. All authors declared no conflicts involved. This work was supported by the Natural Science Foundation of China (grant number 81170026), the Natural Science Foundation of Zhejiang province (grant numbers LR12H01001, Y2110146) and the Science and Technology Department of Zhejiang Province (grant number 2011R10062).

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