

Expression of the *Foxp3* Gene in Spleen Mononuclear Cells of a Mouse Model with Allergic Rhinitis

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Key Words

Allergic rhinitis • Forkhead transcription factor 3 • Regulatory T cells • Peripheral blood mononuclear cell • Mouse

Abstract

Objective: There is growing speculation that the impairment in regulatory-T-cell (Treg)-mediated dominant tolerance may play an important role in the pathogenesis of allergic rhinitis (AR). The aim of this study was to investigate whether the changes in the forkhead transcription factor 3 (*Foxp3*) gene expression may aggravate nasal mucosal inflammation in allergic mice, and whether or not these features result from the loss of Tregs. **Methods:** AR was induced by both intraperitoneal injection and intranasal administration of ovalbumin in BALB/c mice, while the control mice were treated with saline. A comparison of the frequency of CD4⁺CD25⁺*Foxp3*⁺ Tregs in peripheral blood mononuclear cells of the AR and control mice was made by flow cytometry. Spleen mononuclear cells were used for RNA extraction and RT-PCR was used to measure *Foxp3* mRNA expression. **Results:** The expression of the *Foxp3* gene was significantly reduced in spleen mononuclear cells in AR mice compared with the control. Moreover, a significant decrease in the percentage of CD4⁺CD25⁺*Foxp3*⁺ Tregs was exhibited in peripheral blood mononuclear cells of AR mice compared with the

control mice. **Conclusion:** The insufficiency of Tregs and the *Foxp3* gene may contribute to the development of AR in mice.

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Introduction

Allergic rhinitis (AR) is a Th2-cell-driven immunological disease, the incidence of which has increased considerably in recent decades in atopic individuals. A number of studies indicate that regulatory T cells (Tregs) play an important role in controlling Th2-biased responses not only in animal models, but in humans as well. CD4⁺CD25⁺ Tregs, a distinct subset of CD4⁺ T cells [1], are regarded as the most important member of the Tregs family, and their essential role in the maintenance of peripheral self-tolerance has been demonstrated. Depletion of Tregs results in the development of organ-specific autoimmune disease [2]. Many studies on Tregs have shown them to control Th1-mediated diseases. However, there is recent evidence that they also control Th2-mediated diseases, such as allergic asthma [3–7].

D.H. and L.X. contributed equally to this paper.

Naturally occurring Tregs specifically express the transcription factor 3 (*Foxp3*), which is a member of the forkhead or winged helix family of transcription factors [8, 9]. The gene was first described as JM2 when its mutation was identified in an X-linked autoimmune and allergic dysregulation syndrome that exhibits early onset diabetes, enteropathy, endocrinopathy syndrome and severe allergic inflammation, including eczema, food allergy, high serum immunoglobulin E (IgE) levels and eosinophil-dominated inflammation [10–12]. *Foxp3* has been identified as a master switch regulatory gene for cell lineage commitment or developmental differentiation of CD4⁺CD25⁺ Tregs [13–15]. Consequently, normal *Foxp3* expression is crucial to maintain the CD4⁺CD25⁺ Tregs function as well as the self-balance of the entire immune system.

Given the evidence presented, we hypothesized that Tregs and the *Foxp3* gene may contribute to the increased inflammation in AR. Therefore, the aim of this study was to investigate whether the changes in *Foxp3* gene expression may aggravate nasal mucosal inflammation in allergic mice and whether these features might result from the loss of Tregs.

Materials and Methods

Animals

BALB/c mice (female, 8 weeks of age, weighing 22–25 g), maintained under international standards of animal welfare guidelines, were used in all experiments. All 30 mice were randomly divided into the ovalbumin (OVA)-sensitized group and the control group (15 mice/group). The experiment was approved by the Animal Experimentation Ethics Committee of the Beijing Institute of Otolaryngology.

Sensitization Procedure

Mice were sensitized by intraperitoneal injection of 50 µg OVA (grade V; Sigma, St. Louis, Mo., USA) in the presence of 5 mg immune adjuvant Al(OH)₃ (Guohua, Beijing, China) dissolved in 1 ml sterile saline on days 0, 2, 4, 6, 8, 10 and 12. Subsequently (14 days later), the mice were challenged by being instilled with 5% OVA intranasally (10 µl/side) once a day for 7 days. Mice in the control group were injected with saline and subsequently challenged with saline intranasally. Twenty-four hours after the last OVA or saline challenge, mice were killed by decapitation and the tissue or peripheral blood was obtained.

Mice were bled from the retrobulbar plexus and 0.8 ml venous blood was drawn into tubes with additive EDTA. Blood was centrifuged at 1,500 rpm for 20 min at 4°C in the presence of Ficoll solution. Peripheral blood mononuclear cells (PBMCs) were obtained from the medial cell suspensions, which were additionally processed by twice washing in 0.9% saline.

Histopathology

The entire partially dissociated nasal cavities from the mice, prepared by fixing in neutral formaldehyde for 24 h and then decalcified with 10% EDTA for 3 weeks, were embedded in paraffin for nasal cavity coronal section (4 µm) and processed for HE staining. Finally, the samples were analyzed using the Olympus BX51 microscope system (Olympus Optical, Tokyo, Japan). All slides were read by an observer who was blind to the 2 groups.

RT-PCR Analysis

RNA samples were extracted from mononuclear cells prepared from fresh spleens of mice. Lobes of the non-lavaged spleens from each mouse were mechanically minced in 0.9% normal saline on ice, and filtered through a sterile piece of nylon gauze to remove cell clumps. Spleen cell suspensions were obtained by the following procedure. Briefly, the cells were centrifuged at 2,000 rpm for 10 min to remove the supernatant, and then incubated with 5 ml red blood cell disruption solution for 30 min in a 4°C refrigerator. Subsequently, another centrifugation at 2,000 rpm for 10 min was performed, and the remaining cell suspensions after removal of the supernatant were the spleen mononuclear cells (SMCs).

Total RNA was isolated from SMC pellets using TRIzol reagent (Invitrogen, Carlsbad, Calif., USA), and thereafter reverse-transcribed into cDNA with oligo dt primers and M-MuLV reverse transcriptase (Fermentas, Burlington, Ont, Canada) according to the manufacturer's instructions. We used specific primers for *Foxp3* (forward primer, AGG AGA AAG CGG ATA CCA; reverse primer, TCA TGA GCC CTT CCA CAATG) and the control house-keeping gene, GAPDH (forward primer, GTG AAG GTC GGT GTG AAC GG; reverse primer, TCA TGA GCC CTT CCA CAATG) (Sangon, Shanghai, China). The reaction was carried out in a volume of 25 µl, containing 2 µl cDNA, 1 µl of each primer, 12.5 µl Taq DNA polymerase and 8.5 µl RNase-free H₂O (Dongsheng, Guangzhou, China). The PCR profile was as follows: *Foxp3*, initial denaturation at 94°C for 15 min, followed by 30 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 10 min; GAPDH, initial denaturation at 94°C for 15 min, followed by 30 cycles of 94°C for 45 s, 61°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. Each PCR product was confirmed by agarose gel electrophoresis and gene expression of *Foxp3* and GAPDH was determined by AAB Software analysis. The relative amount of *Foxp3* mRNA was acquired through the *Foxp3*/GAPDH ratio in terms of the gray scale of each electrophoresis strip.

Flow Cytometry

Cell surface staining was performed in 50 µl PBMC suspension by incubation simultaneously with 10 µl PE-Cy5-conjugated anti-mouse CD4 and 10 µl R-PE-conjugated anti-mouse CD25 mAbs (BD PharMingen, BD Biosciences, San Jose, Calif., USA) for 20 min at room temperature in the dark. After this, the cells were washed with phosphate-buffered solution and centrifuged at 1,000 rpm for 8 min to remove the supernatant. The remaining cell suspensions were incubated with 1 ml *Foxp3* Fix solution for 20 min, and then centrifuged to remove the supernatant. Additional 1 ml *Foxp3* Fix solution was added into the remaining cell suspensions to be incubated for another 20 min. After removing

the supernatant, the cells were suspended twice with 1 ml *Foxp3* Perm medium, and then stained with 5 μ l Alexa Fluor488-conjugated anti-mouse *Foxp3* (Biolegend) for 20 min. Cells were washed once with 1 ml *Foxp3* Perm solution and then resuspended in 0.5 ml *Foxp3* Perm solution before analysis on a FACScan flow cytometer (FACS Calibur, BD Biosciences).

Statistical Methods

Results were expressed as means $\pm$ SD. Differences in the 2 groups were analyzed using the unpaired Student t test, as implemented in Stata version 9.1. A value of $p < 0.05$ was considered statistically significant for all analyses.

Results

OVA Sensitization Is Efficient in Establishing the Mouse Model with AR

We employed a classic approach for the establishment of an animal model of AR. Mice were immunized 7 times with OVA and challenged 7 times by being instilled with OVA intranasally. After being sensitized to OVA 2/3 times, the OVA group mice gradually displayed the mild phenomenon of nose scratching. After the procedure entered the challenge stage, the OVA-processed mice showed dramatic symptoms, such as nasal itching, external nasal swelling, discontinuous sneezing, watery rhinorrhea and a degree of excitability and restlessness, while the control group mice remained quiet and displayed no aberrant behavior. Furthermore, when the nasal cavities of the OVA group mice were dissected 24 h after the last challenge, high-power microscopic examination revealed a much more pronounced shedding of nasal mucosa and secretion production, simultaneously accompanied by numerous glandular serosa accretions and significant cellular infiltration containing abundant eosinophils and plasma cells. On the other hand, unimmunized mice (control group) showed a normal nasal mucosal appearance and only very mild inflammation with occasional eosinophils.

Comparison of Foxp3 Expression in Spleen SMCs of OVA and Control Mice

Foxp3 and GAPDH mRNA expression was detected in SMCs prepared from spleens of mice (15 mice/group), and was confirmed by agarose gel electrophoresis (fig. 1). The relative expression of the regulatory marker *Foxp3* (as mRNA) was significantly lower ($p = 0.0206 < 0.05$) in the OVA mice (0.7964 ± 0.1414) compared with that seen in control mice (0.8977 ± 0.0746).

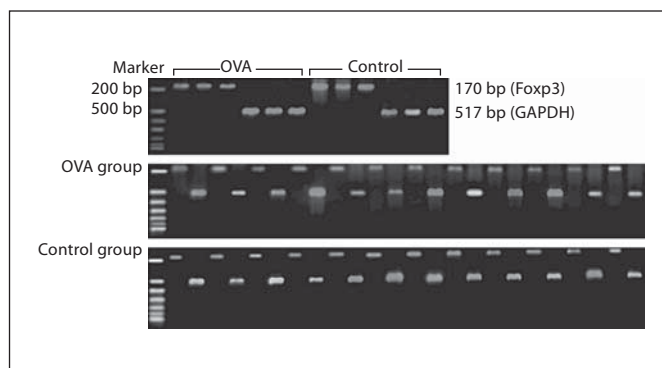


Fig. 1. RT-PCR analysis of *Foxp3* expression of SMCs prepared from OVA mice and control mice. *Foxp3* and GAPDH mRNA expression was detected in SMCs and confirmed by agarose gel electrophoresis. The housekeeping gene, GAPDH, was selected to be the control gene. The amplified products of *Foxp3* and GAPDH were 170 and 517 bp, respectively.

Comparison of the Frequency of CD4⁺CD25⁺Foxp3⁺ Cells in Peripheral Blood of the OVA and Control Mice

Cells were sorted by first gating on their forward-scatter/side-scatter profiles, and then CD4⁺ cells were excluded. Cells were then sorted into 2 populations based on whether they expressed CD25 and their levels of *Foxp3* expression. Specifically, cells were sorted into 2 populations: CD4⁺CD25⁺*Foxp3*⁺ (Tregs) and CD4⁺ effector T cells (CD4⁺CD25⁺*Foxp3*⁺, CD4⁺CD25⁺*Foxp3*⁺, CD4⁺CD25⁺*Foxp3*⁺) (fig. 2). The percentage of CD4⁺CD25⁺*Foxp3*⁺ Tregs as a proportion of total CD4⁺ cells in PBMCs was compared in the AR mice and control mice. A significant decrease in the Tregs percentage was exhibited in the AR mice ($1.662\% \pm 0.5732\%$) compared with the control mice ($3.4887\% \pm 0.9317\%$; $p = 0.0000$; fig. 3).

Discussion

We have demonstrated that administration of OVA was followed by increased allergic nasal mucosal inflammation in a mouse model. Features increased by OVA included symptoms of nasal itching and running, sneezing, and eosinophil-dominant cellular infiltration into the nasal mucosa tissue. Meanwhile, a significant decrease in the percentage of CD4⁺CD25⁺*Foxp3*⁺ Tregs was noted in the PBMCs of mice with AR compared with the control mice. Additionally, the expression of *Foxp3* was significantly reduced in SMCs of OVA mice compared

Fig. 2. Flow cytometric analysis of $CD4^+CD25^+Foxp3^+$ T cell subsets in OVA and control mice. Representative FACS analysis of $CD4^+$ -cell-gated PBMCs from an OVA mouse and a control mouse are demonstrated. The upper right quadrant represents the $CD4^+CD25^+Foxp3^+$ T cells. The OVA mouse ($CD4^+CD25^+Foxp3^+/CD4^+ = 1.94\%$) exhibits a pronounced decrease in number of $CD4^+CD25^+Foxp3^+$ T cells compared with the control mouse ($CD4^+CD25^+Foxp3^+/CD4^+ = 3.56\%$).

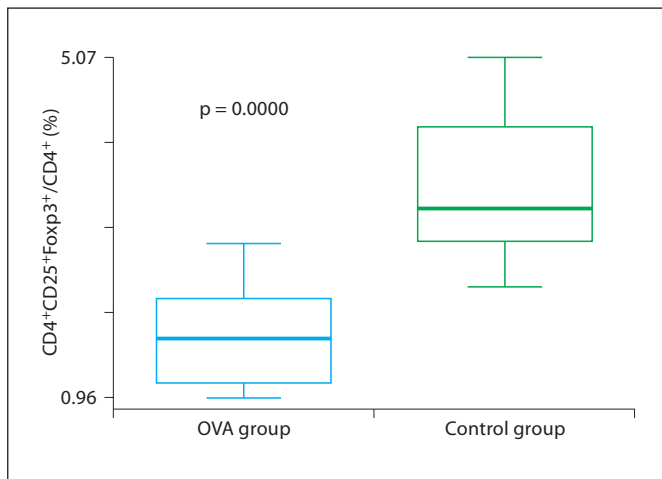
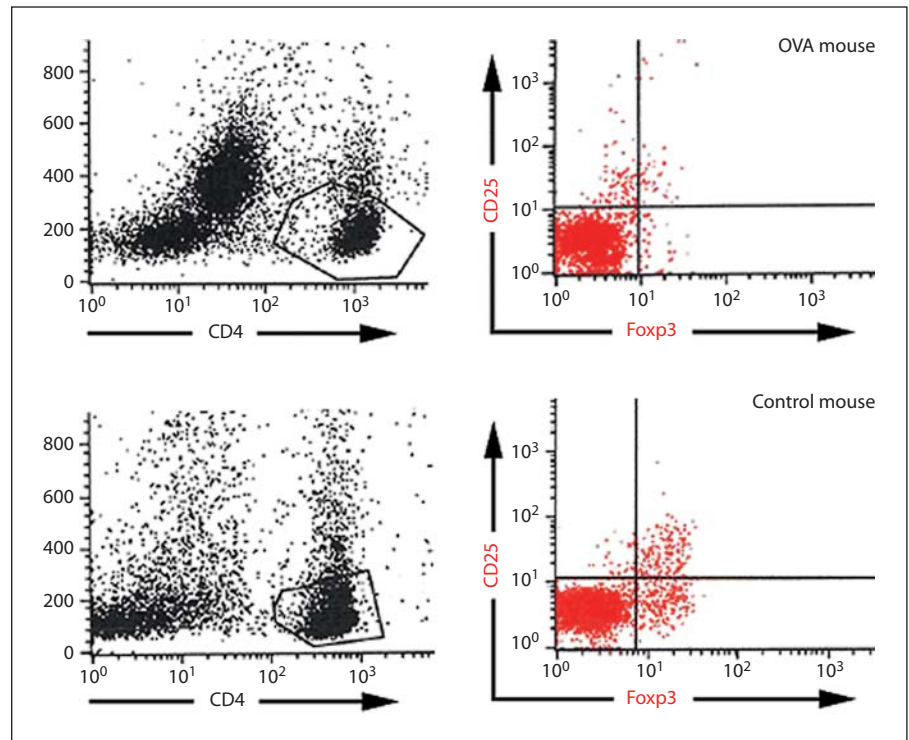


Fig. 3. Comparison of $CD4^+CD25^+Foxp3^+$ cells as a proportion of total $CD4^+$ T cells in OVA and control mice. The percentage of $CD4^+CD25^+Foxp3^+$ Tregs as a proportion of total $CD4^+$ cells in PBMCs is shown for the OVA ($1.662\% \pm 0.5732\%$) and control groups ($3.4887\% \pm 0.9317\%$). The box plots show the average $CD4^+CD25^+Foxp3^+$ ratio among $CD4^+$ cells as gated accompanying the FACS plot from PBMCs within the same mouse (15 mice/group). Error bars indicate SD.

with the control group. The downregulation of the *Foxp3* gene in a mouse model of AR at both the cellular and molecular level in the present study is consistent with the possibility that Tregs and the *Foxp3* gene may contribute to the increased inflammation in AR.

Allergic sensitization is defined by production of IgE against environmental antigens, such as house dust mites, grass pollen and animal proteins and can lead to diseases that include asthma, rhinitis and atopic dermatitis [16]. Activation of Th2 cells is thought to have a major role in allergic sensitization, because such cells can mediate IgE synthesis and eosinophilic inflammation, which contribute to the clinical features of allergic disease [16, 17]. Recent studies have shown that there appears to be a distinct number reduction or functional deficiency of $CD4^+CD25^+$ Tregs in atopic individuals, for example AR patients [18]. It has been revealed that mucosal induction of tolerance against dietary antigens is associated with the development of $CD4^+CD25^+$ Tregs in both animals and humans [19, 20]. Patients with atopic dermatitis have significantly increased numbers of peripheral blood $CD4^+CD25^+$ Tregs with normal immunosuppressive activity [21]. Ling et al. [22] have investigated the ability of $CD4^+CD25^+$ Tregs from allergic donors to suppress al-

lergen-stimulated T-cell proliferation and cytokine production in vitro, and found that CD4⁺CD25⁺ Tregs from nonallergic donors suppressed proliferation and IL-5 production by their own allergen-stimulated CD4⁺CD25⁺ Tregs. Inhibition of proliferation by CD4⁺CD25⁺ Tregs from atopic donors was significantly reduced, and was even more diminished by CD4⁺CD25⁺ Tregs isolated from patients with hay fever, with out-of-season suppression remaining less than that seen by regulatory cells from nonatopic donors.

Current therapy for AR mainly centers on avoiding touching the allergens, use of oral antihistamine and nasal spray glucocorticoid, and specific immunotherapy. Karagiannidis et al. [23] found that glucocorticoid treatment is not only immunosuppressive and anti-inflammatory, but also promotes or initiates differentiation toward Treg cells by a *FOXP3*-dependent mechanism. In this sense, strategies that convert transient glucocorticoid-induced Treg activity into a stable phenotype might improve allergy and AR therapy. Meanwhile Doganci et al. [24] indicated that blockade of mIL-6R induced local expansion of *Foxp3*-positive CD4⁺CD25⁺ Tregs with increased immunosuppressive capacities. In addition, in an in-vivo transfer mouse model of asthma, CD4⁺CD25⁺ T cells isolated from anti-IL-6R antibody-treated mice exhibited marked immunosuppressive and anti-inflammatory functions. Furthermore, another study [25] revealed that the mechanisms underlying peripheral tolerance are linked to the conformation of the antigen, that mucosal tolerance is mediated by separate regulatory cell subsets,

and that the long-term efficacy of immunosuppression is associated with the presence of *Foxp3*⁺ T cells. Altogether, AR might result from an inappropriate balance between allergen activation of regulatory CD4⁺CD25⁺*Foxp3*⁺ Tregs and effector Th2 cells. This imbalance could result from a deficiency in suppression by Tregs or strong activation signals could overcome such regulation.

Taken together, here we have provided the first evidence that OVA-induced allergic nasal inflammation in a mouse model might be due, at least in part, to the reduced number of the subset of CD4⁺CD25⁺*Foxp3*⁺ Tregs as well as downregulation of *Foxp3* expression. This is consistent with the possibility that Tregs and the *Foxp3* gene may contribute to the increased inflammation in allergic diseases. Nevertheless, further studies on humans with AR are still required to increase our knowledge base for Tregs and their regulatory gene *Foxp3*. Treatment to enhance Tregs responses, in concert with reduction of Th2 cell activation, might be useful in prevention and treatment of AR and other allergic diseases.

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