International Immunopharmacology 69 (2019) 347–357

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Changes among TGF-β1$^+$ Breg cells and helper T cell subsets in a murine model of allergic rhinitis with prolonged OVA challenge

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ARTICLE INFO

Keywords:
TGF-β1$^+$ Breg cells
Treg cells
Th1/Th2 cells
Th17 cells
Allergic rhinitis

ABSTRACT

Background: Allergic rhinitis is a common allergic disease resulting from inappropriate Th2 cell-mediated immune responses to environmental antigens. As such, regulatory B cells and T helper cells play a critical role in the occurrence and development of allergic rhinitis.

Methods: Wild-type mice received ovalbumin (OVA) intranasal challenge for varied lengths of time, then the inflammatory state of their nasal mucosa was analyzed by histology. Changes to the proportion and function of TGF-β1$^+$ Bregs, T helper cells and plasma cells was analyzed by flow cytometry, real-time PCR, ELISA and cytometric bead arrays. Finally, changes in expression of upstream transcription factors related to helper T cells and STAT proteins were detected by western blot.

Results: The most severe inflammatory response was observed in the mucosal tissue, where the percentage of TGF-β1$^+$ Bregs and Tregs decreased, and the percentage and function of Th2 and plasma cells increased significantly. With prolonged OVA challenge, the proportion of TGF-β1$^+$ Bregs and Tregs increased. These factors regulated Th2 cell polarization state and gradually restored balance of the inflammatory state in the nasal mucosa. Moreover, changes to upstream transcription factors and STAT proteins were found to be positively correlated with changes to helper T cells.

Conclusion: TGF-β1$^+$ Bregs cooperated with Treg cells in the development of allergic rhinitis and its recovery process. Reconstitution of nasal mucosal immunity was facilitated via regulation of the proportion and function of helper T cells.

1. Introduction

Allergic rhinitis (AR) is a global health problem which affects 10–20% of the population and is increasing in prevalence [1]. AR is characterized by rhinorrhea, sneezing and nasal congestion due to local inflammation of the nasal mucosa in the upper airway. AR is concomitant with bronchial asthma or allergic conjunctivitis, which together have a much greater effect on quality of life than AR alone. The mechanisms underlying allergic inflammation in the mucosal tissues are characterized by dramatic Th2-driven immunity and elevated allergen-specific IgE, leading to goblet cell hyperplasia and mucus overproduction. In this situation, Th2 cells secrete the cytokines IL-4, IL-5, and IL-13 to promote disadvantageous immune responses by helping plasma B cells produce allergen-specific IgE in the mucosa [2–4]. Recent research has highlighted an alteration in the immune regulatory processes as one crucial factor in the occurrence and development of allergic inflammation [5].

It has been demonstrated in previous studies that an overstimulated immune response in both autoimmune diseases and allergic diseases can drive negative effects on the body utilizing regulatory B cells (Breg) and T-cell (Treg) subsets [6,7]. While the role of regulatory T cells in regulating immune responses has been recognized, a subset of regulatory B cells, among others, has also been characterized. In 1974, it was reported that normal spleen cells inhibited delayed hypersensitivity and B cell-deficient spleen cells did not show such an effect in a guinea pig models of hypersensitive skin, suggesting that B cells played a regulatory role in this sensitivity process [8]. Since then, researchers have conducted intensive studies on regulatory B cells and found that their role is mediated by secreting IL-10 and transforming growth factor beta (TGF-β) [9,10].

Recent investigations have revealed that the levels of IgE can be regulated by regulatory B cells, which produce negative regulatory cytokines [11,12]. Moreover, studies using experimental murine models have suggested that Breg cells broadly control T-cell-mediated immune responses in association with contact hypersensitivity, bronchial asthma, experimental autoimmune encephalomyelitis and

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https://doi.org/10.1016/j.intimp.2019.01.009
Received 12 August 2018; Received in revised form 18 December 2018; Accepted 7 January 2019
Available online 15 February 2019
1567-5769/

collagen-induced arthritis [13–16]. In addition to secreting IL-10, B cells can also secrete TGF-β which promotes regulatory T cell proliferation, downregulates Th2 immune reactions, and inhibits the number of eosinophils in the airway of the mouse model [14]. However, the characteristics and mechanisms of regulatory B cells in disease progression are still unclear.

When the body's immune system is activated, extracellular pro-inflammatory cytokines bind to cytokine receptors, which activate JAK and subsequently the STAT proteins [17]. STATs act as a transcription factor and play a key role in lymphocyte differentiation and activation. Specifically, IL-12 induces STAT1/STAT4 activation thus promoting Th1 cells differentiation; IL-4 activates STAT6 promoting Th2 cells differentiation; IL-6, IL-21 and IL-23 stimulate STAT3-dependent Th17 cells differentiation and IL-2/STAT5 signaling is required for the development of Treg cells [18–21].

In the present study, mouse models of allergic rhinitis (AR) with different challenging time were sequentially established to observe related symptoms of AR, nasal mucosa morphology, eosinophil infiltration, the proportion and function change of B cells and helper T-cell subsets. Changes to specific upstream transcription factors and activation of transcription factors from each subset of helper T cells in nasal mucosa of mice were examined to explore the relationship between such changes in the proportion and function of helper T-cell subsets as well regulatory B cells, development and recovery of allergic rhinitis and potential mechanism involved.

2. Material and methods

2.1. Experimental mice

Experiments were conducted using male Balb/c mice. All mice were 6 to 8 weeks old at the beginning of the experiments. Mice were housed in a controlled environment with a 12/12-h light/dark cycle with free access to food and water; they were treated in accordance with Animal Care and Use Committee approved protocols 2015PS221K under the regulations and guidelines of the National Institutes of Health.

2.2. Murine model of allergic airway inflammation

Mice were divided into four experimental groups; each group consisted of 16 mice. Briefly, mice were sensitized with an intraperitoneal injection of 25 μg of ovalbumin (OVA; grade V; Sigma, St. Louis, MO, USA) and 1 mg of aluminum hydroxide [$Al(OH)_3$; Solarbio, Beijing, China] dissolved in 0.1 mL phosphate buffer saline (PBS) on days 0, 7, and 14. Each group received PBS intraperitoneal injection as a negative control. After general sensitization, mice were locally challenged with 500 μg of OVA dissolved in 10 μL PBS into their nostrils from day 21 [22]. Group A, B, C and D were challenged for 7 days, 14 days, 21 days and 28 days, respectively. Each group received PBS nasal drops as a negative control. There was no statistical difference between the four negative control groups. The procedures for allergen sensitization are summarized in Fig. 1.

2.3. Symptom score and tissue preparation

After final OVA challenge, a blinded observer recorded the frequencies of sneezing and nasal rubbing over a 15-min interval. Mice were then sacrificed 24 h after the last OVA challenge. After perfusion with 4% paraformaldehyde, the heads of first four mice from each group were removed *en bloc* and fixed in 4% paraformaldehyde. After exposing the nasal cavity in the head in the other 12 mice, the nasal and sinus mucosa were taken out meticulously using a small curette. Because the mucosa in nasal cavity and paranasal sinus area of each mouse was too small, these samples could not be used in all experiments. Mucosa of six mice from each group was immediately immersed in liquid nitrogen, until use for real-time polymerase chain reaction (real-time PCR), enzyme linked immunosorbent assay (ELISA), cytometric bead arrays and Western blot. Mucosa of the other six mice from each group was immediately immersed in 1 mL RPMI on ice for the next flow cytometer analysis.

2.4. Eosinophils infiltration

For evaluation of nasal histology, nasal tissues were decalcified, embedded in paraffin, and sectioned coronally (4 μm thickness) approximately 5 mm from the nasal vestibule. Each section was stained with hematoxylin and eosin, the mean number of eosinophils for each field was calculated (400× magnification) [22] (Fig. 2).

2.5. Real-time PCR for mRNA expression of cytokines and transcription factors

Total RNA was prepared from the nasal and parasinal mucosa with TriZol reagent (Invitrogen, Carlsbad, CA, USA). Using Superscript Reverse Transcriptase kit (Takara, Dalian, China) removed genomic DNA (gDNA) and synthesized Complementary DNA (cDNA). The reaction was performed using a Roche LightCycler 480 II Sequence Detection System (Roche, Basel, Switzerland). The average transcript levels of genes were then normalized to β-actin (Fig. 3A). Primer sequences are listed in Table 1.

2.6. Determination of antigen-specific immunoglobulin levels and cytokine measurements

Samples were taken from liquid nitrogen and weighed, and 19 μL PBS was added to each 1 mg sample. Grinding samples until there is no obvious grain, centrifuge at 4 °C 12000 for 25 min, then remove the supernatant fluid new EP tube, used for ELISA and cytometric bead arrays. OVA-specific IgE levels were determined by using ELISA (BioLegend, San Diego, CA, USA), according to the manufacturer's recommendations. TGF-β1 levels were measured with specific ELISA (BioLegend, San Diego, CA, USA), Levels of IFN-γ, IL-4, IL-5, IL-6, and IL-17a in supernatants were measured with a cytometric bead arrays Flex Set (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions (Fig. 3B).

2.7. Western blot for T-bet, GATA-3, RORγt, Foxp3, STAT3 and STAT5 in the nasal and parasinal mucosa

Protein was obtained from the nasal and parasinal mucosa of each mouse using RIPA lysing buffer (Beyotime, Shanghai, China). Protein concentrations were determined using BCA protein assay reagent (Beyotime, Shanghai, China). Samples (80 μg protein per lane) were separated on 10% Sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred onto PVDF membranes (Beyotime, Shanghai, China). T-bet, GATA-3, RORγt, Foxp3, STAT3, STAT5 and β-actin were immunoblotted with a primary rabbit polyclonal anti-T-bet Ab (Abcam Systems, Cambridge, MA, USA), primary rabbit polyclonal anti-GATA-3 Ab (Abcam Systems, Cambridge, MA, USA), primary rabbit polyclonal anti-RORγt, Ab (Proteintech Systems, Chicago, IL, USA), primary rabbit polyclonal anti-Foxp3 Ab (Proteintech Systems, Chicago, IL, USA), primary rabbit monoclonal anti-STAT3 Ab (Cell Signaling Technology, Danvers, MA, USA), primary rabbit monoclonal anti-STAT5 Ab (Cell Signaling Technology, Danvers, MA, USA) and anti-β-actin Ab (Proteintech Systems, Chicago, IL, USA), respectively. The membrane was then immunoblotted with a secondary anti-rabbit IgG-HRP (ZSGB-Bio, Beijing, China). The blots were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and Luminous instrument (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Quantification of western blots was performed using the ImageJ software from the National Institutes of Health (Fig. 4).

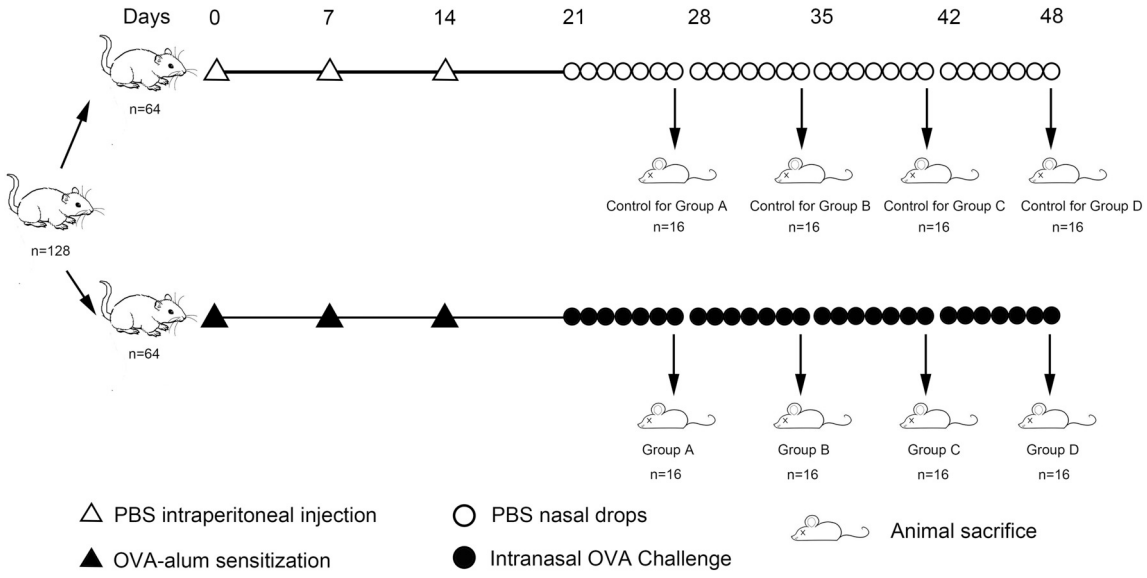

Fig. 1. Experimental protocol.
A total of 128 mice were randomly divided into two groups, an experimental group and control group. Then 64 experimental mice were sensitized with ovalbumin (OVA) and aluminum hydroxide gel on days 0, 7, and 14 (general sensitization). Similarly, 64 control mice received PBS intraperitoneal injection as a negative control. After general sensitization, mice were locally challenged with 500 μg of OVA dissolved in 10 μL PBS into their nostrils from day 21. Group A(n = 16), B (n = 16), C(n = 16) and D(n = 16) were challenged for 7 days, 14 days, 21 days and 28 days. Each group received PBS nasal drops as a negative control. There was no statistical difference between the four negative control groups. Mice were then sacrificed 24 h after the last OVA challenge. Among the 16 mice, four were used for HE staining, six were used for flow cytometry, and six were used for other experiments.

2.8. Flow cytometer analysis of T and B cell subsets in the nasal and parasinal mucosa

To achieve a single cell suspension, mucosa was crushed in glass grinder in 1 mL of RPMI, then filtered using a cell suspension filter, and counted using a Coulter counter. To stain the T and B cell subsets, cells were transferred to a round bottom 12-well plate (1×10^6 cells/well) and mixed with Leukocyte Activation Cocktail with BD GolgiPlug (BD Biosciences, San Jose, CA, USA), then incubated at 37 °C and 5% CO_2 for 5 h. After centrifugation, cells were resuspended in extracellular antibodies, anti-CD4 (FITC), anti-CD19 (FITC), anti-B220 (FITC), anti-CD25 (PE) from BD Biosciences, anti-CD44 (PE), anti-CD138 (PE/Cy7) from Biolegend, and incubated for 20 min in the dark. After washing and fixation for 20 min in fix-lysing buffer (eBioscience, San Diego, CA, USA) and washing again, cells were incubated with 1 mL permeabilization buffer (eBioscience, San Diego, CA, USA) for 15 min. After centrifuging, cells were resuspended in intracellular antibodies, anti-IL-4 (PE), anti-IFN-γ (APC) from BD Biosciences, anti-IL-17a (PE/Cy7), anti-Foxp3 (APC) from eBioscience, anti-TGFβ1 (APC) from Biolegend, for 30 min in the dark. Cells were washed in PBS three times, and resuspended in 200 μL FACS buffer. Cells were analyzed on a BD Calibur flow cytometer. The corresponding isotype control antibodies, rat-IgG1 (PE) from BD Biosciences, rat-IgG2a (PE/Cy7) from Biolegend, rat-IgG2b (PE), rat-IgG1 (APC) from eBioscience, were used to set positive gates. FlowJo VX software (FlowJo, Ashland, OR, USA) was used to analyze lymphocyte subsets. CD4 was used as a marker for T helper cells. Within the cell population we identified Th1 cells ($CD4^+IFN\text{-}\gamma^+$), Th2 cells ($CD4^+IL\text{-}4^+$), Treg ($CD4^+CD25^+FoxP3^+$) and Th17 cells ($CD4^+IL\text{-}17a^+$) (Fig. 6). CD19 was used as a B cell marker. Within this B cell population, regulatory B cells (Breg) were distinguished $CD19^+CD44^+TGF\text{-}\beta1^+$. $B220^+CD44^+CD138^+$ cells were considered to be plasma cells (Fig. 5).

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (La Jolla, CA, USA) and SPSS software (ver. 23.0; SPSS Inc., Chicago, IL, USA). Depending on data structure, data were assessed by multiple statistical methods. The normal distribution of the obtained data was determined using the Kolmogorov-Smirnov normality testing. Comparisons between two groups of data consistent with the normal distribution were conducted by the Student *t*-test, and two groups of data inconsistent with the normal distribution were done with Mann-Whitney test. Comparisons among multiple groups of data with homogeneous variance were performed using 1-way ANOVA/Tukey, and multiple groups of data with non-homogeneous variance were compared with the Kruskal-Wallis/Dunn testing. A p-value of < 0.05 was considered to indicate a significant difference.

3. Result

3.1. Immune status of the nasal mucosa with the prolonged challenge time

In the present study, mouse models of AR with different sensitization times were established and the related symptoms of AR, nasal mucosa morphology and eosinophilic infiltration were observed. We found that with prolonged allergen exposure time, symptoms of AR were reduced, the disordered and hypertrophic nasal mucosa recovered gradually to a normal morphology, eosinophilic infiltration decreased, and OVA-specific IgE levels reduced gradually. We therefore speculated that factors that regulate the mucosal immune status are involved in the pathogenesis of AR (Fig. 2).

3.2. Regulatory effect of TGF-β1$^+$ Breg and Treg cells on Th2 cells and plasma cells with prolongation of challenging time

Percentages of TGF-$\beta1^+$ Breg and Tregs (Fig. 5A) and the concentration of regulatory cytokine TGF-β1 they secreted were decreased. The percentage of Th2 (Fig. 6A) and plasma cells (Fig. 5A) as well as the levels of IL-4 and IL-5 secreted by these cells all increased significantly when AR occurred (Fig. 3). At that time, the most severe symptoms of AR, the highest accumulation of eosinophils, the most severe

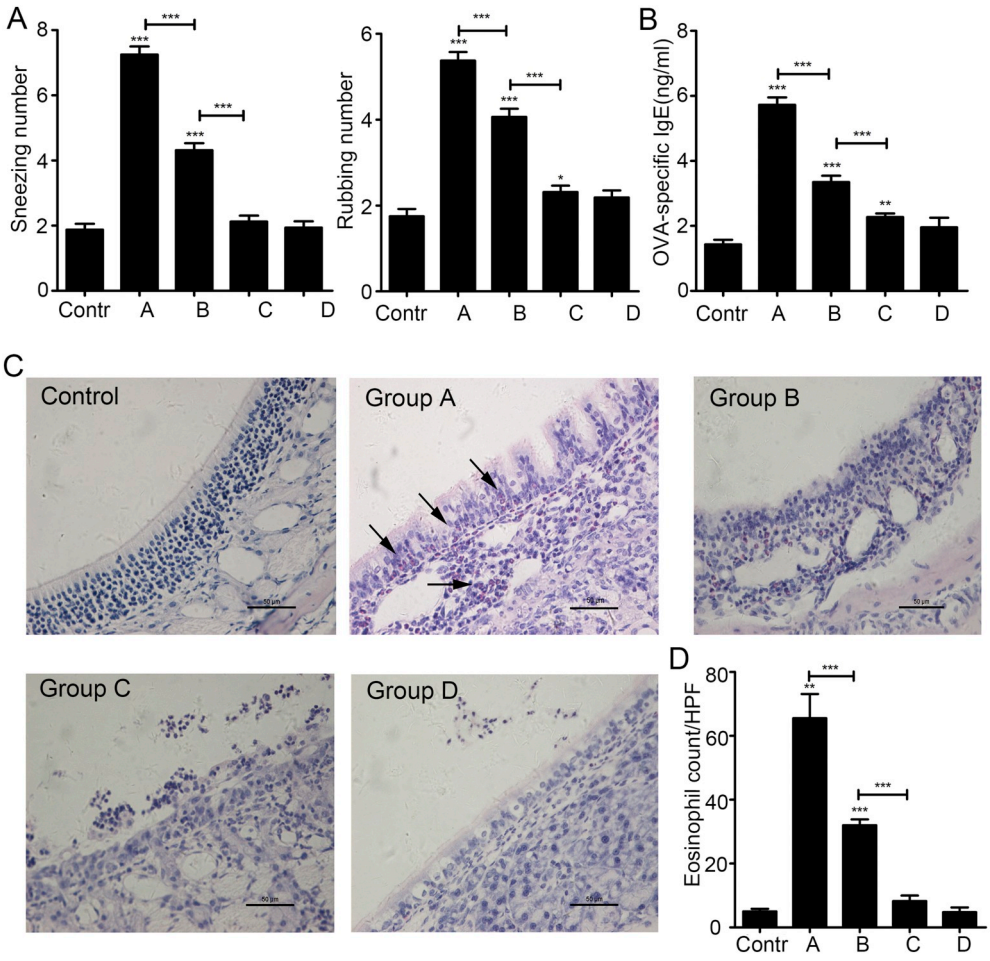

Fig. 2. Symptoms and mucosal histology changed with prolonged sensitization.
(A) The symptom score of AR mice decreased gradually with prolonged OVA challenge. After final OVA challenge, a blinded observer recorded frequency of sneezing and nasal rubbing over a 15-min interval. The change of rubbing symptom score and sneezing symptom score with the prolonged OVA challenge are represented by a histogram. Each of the 128 mice had a symptom score. Comparisons between two groups of data were conducted with a Student *t*-test. Comparisons among multiple groups of data were performed using 1-way ANOVA/Tukey.
(B) The expression of OVA-specific IgE decreased gradually with prolonged OVA challenge. OVA-specific IgE concentration in nasal mucosa was measured by specific ELISA. Data is a representation of six independent experiments of duplicate samples. Comparisons between two groups of data were conducted with a Student *t*-test. Comparisons among multiple groups of data were performed using 1-way ANOVA/Tukey.
(C) Reconstitution of mucosa structure with prolonged OVA challenge. Four mice from each group were removed *en bloc* to observe nasal structure of mice by HE staining. Magnification: 400×.
(D) Eosinophil counts in the nasal mucosa decreased gradually with prolonged OVA challenge. The mean number of eosinophils for each field was calculated in each HE staining section (n = 6). Comparisons between two groups of data were done with Mann-Whitney testing whereas comparisons among multiple groups of data were compared with Kruskal-Wallis/Dunn testing.
Results are mean ± SEM. $*p < .05$, $**p < .01$, and $***p < .001$.

hypertrophy and hyperplasia of mucosa, and the highest secretion of OVA- specific IgE in nasal mucosa were observed in our mouse model (Fig. 2). These results confirmed the occurrence of AR is induced by the excessive differentiation of Th2 and plasma cells and their enhanced functions. Additionally, the destruction of immune balance may be related to diseased or loss of TGF-β1$^+$ Breg and Tregs in AR [14]. With continuous allergen stimulation, the percentages of TGF-β1$^+$ Breg and Tregs significantly increased, to above normal, and the level of TGF-β1 also increased significantly in mucosa tissue.

Meanwhile, symptoms of the AR mouse model were gradually improved, mucosal structure recovered, secretion of IgE decreased. The percentages of Th2 and plasma cells began to decrease gradually with weakened functions, indicating that the damaged immune balance in mucosa started to recover. These changes may be helped by the increased proportion and function of TGF-β1$^+$ Breg and Tregs. In other words, with continuous stimulation by sensitizing substances, the proportions of TGF-β1$^+$ Breg and Tregs increased, which were regulated by the immune state to polarize Th2 cells and were gradually restored the balance of inflammatory state in nasal mucosa. Interestingly, the proportion of TGF-β1$^+$ Bregs increased and maintained at a level that was slightly higher than normal, unlike Tregs which were present at significantly higher level than the normal level. This finding may be explained by the fact that TGF-β secreted by Bregs could inhibit the proliferation of Bregs via a negative feedback after the peak secretion to avoid excessive immunosuppression [23,24].

3.3. Ratio and function of Th1 cells changed with the prolonged OVA challenging

Th1 cells are also one of the essential helper T-cell subsets in the immune response and the change in the proportion of Th1 and Th2 cells (Fig. 6C) has become the key to the study of the immune responses [25]. In the present study, the proportion of Th1 cells decreased slightly (Fig. 6A) and that of Th2 cells increased significantly in AR, resulting in a significant switch of Th1/Th2 to Th2, the subsequent occurrence of allergic immune response in nasal mucosa. When the stimulation time was prolonged and the proportions of TGF-β1$^+$ Bregs and Tregs and their functions were increased, the proportion and function of Th2 cells gradually decreased under the effect of TGF-β1$^+$ Bregs and Tregs. Meanwhile the Th1/Th2 ratio began to increase and the proportion and function of Th1 cells dominated after 21 days of antigen exposure. With prolonged stimulation time, the proportion of Th1 cells and the immune state gradually returned to normal with the effect of TGF-β1$^+$ Bregs and Tregs.

3.4. Ratio and function of Th17 cells changed with the prolonged OVA challenging

Change in the proportion of Th17 cells was also investigated. The proportion of Th17 cells and the level of the secreted IL-17a were

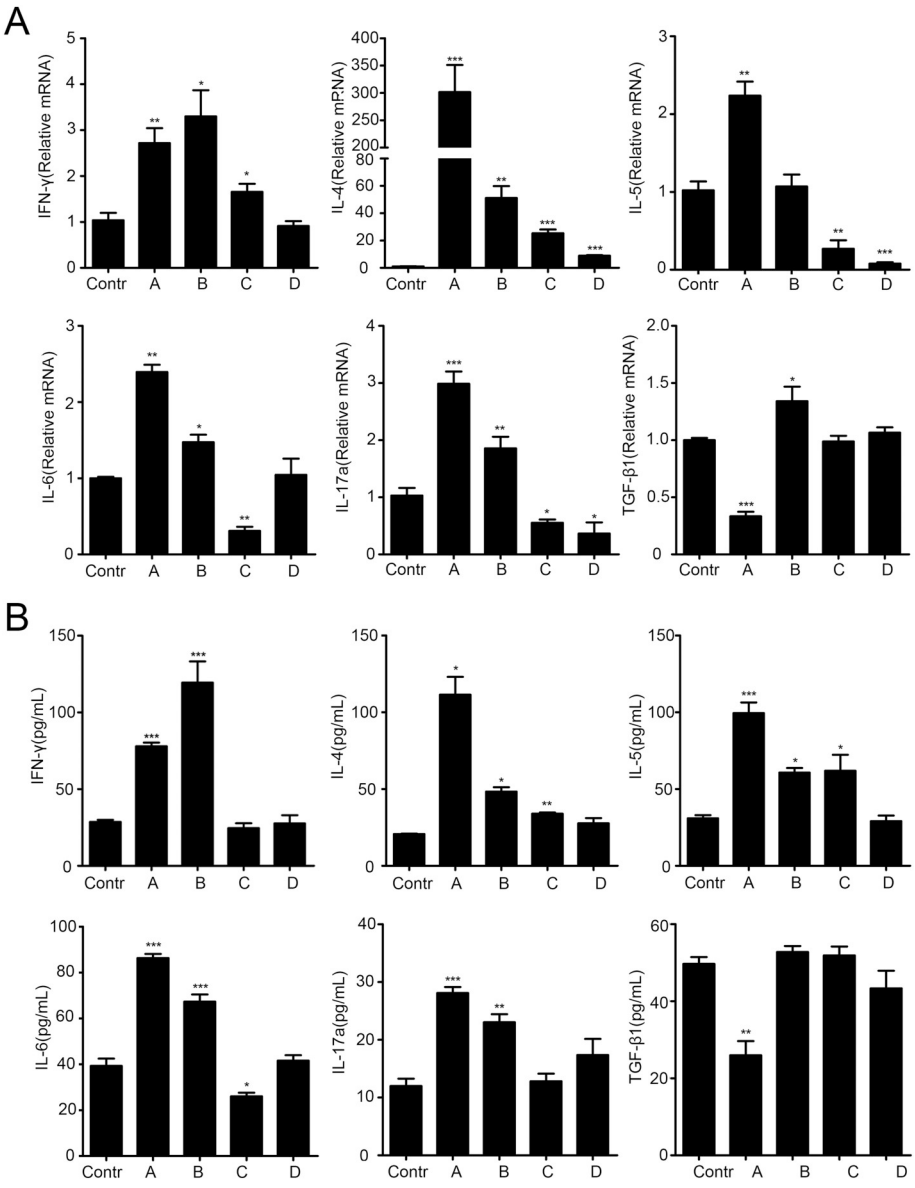

Fig. 3. mRNA and expression of cytokines in mucosa.

(A) The cytokine mRNA levels in nasal mucosa were determined by real-time PCR. cDNA was synthesized after reverse transcription of total RNA extracts; β-actin was used as the internal control. The amount of cytokine gene was normalized to the endogenous reference gene β-actin to obtain the relative threshold cycle (ΔCt). Normalized cytokine mRNA expression (ΔCt) of AR and control groups was then related to the ΔCt of controls for their relative expression levels. The relative expression in AR groups compared with control was calculated by ΔΔCt [ΔCt (AR cytokine mRNA expression - β-actin)-ΔCt (Control-β-actin)]. The relative level of mRNA expression was calibrated to obtain the folds changed by $2^{-\Delta\Delta Ct}$ calculation. Data is a representation of six independent experiments of triplicate samples. Comparisons between two groups of data were done with Mann-Whitney testing whereas comparisons among multiple groups of data were compared with Kruskal-Wallis/Dunn testing.

(B) Protein expression level of cytokines in nasal mucosa detected by cytometric bead arrays and ELISA. Data is a representation of six independent experiments of duplicate samples. Comparisons between two groups of data were done with Mann-Whitney testing whereas comparisons among multiple groups of data were compared with Kruskal-Wallis/Dunn testing.

Results are mean ± SEM. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$.

significantly increased in AR. It was found in our previous study that symptoms of AR were significantly recovered, and the immune state was obviously restored in a mouse model when the proliferation and differentiation of Th17 cells were inhibited. Such a finding confirmed that in addition to Th2 cells, proportion and function of Th17 cells serves as an important factor in the occurrence of AR [26]. In the present study, the proportion and function of Th17 cells increased when AR occurred and gradually decreased when the proportions of TGF-β1^{+} Bregs and Tregs significantly increased. Studies have shown that the proportion and function of Th17 cells are negatively correlated with the proportion and function of Th1 cells. In this study, the proportion and function of Th17 cells reached their lowest after 21 days of stimulation, which might be influenced by the dominant effect of Th1 cells. Proportions of TGF-β1^{+} Bregs and Tregs, proportion and function of Th1 cells, and the proportion of Th17 cells gradually returned to normal after 28 days of antigen stimulation.

3.5. Changes by specific transcription factors and activated transcription factors with prolonged sensitization time

We determined the expressions of specific upstream transcription factors of helper T cells and proposed the potential mechanism involved the regulation effect of TGF-β1^{+} Bregs and Tregs on immune state.

We detected the expression of specific transcription factor Foxp3 and transcription activator STAT5 (Fig. 4) in Treg cells and found that the expression of these factors showed a similar pattern to the changes of Tregs. We therefore speculated that the changes in Bregs and Tregs in the body were induced by the activation of the STAT5 pathway in the sustained stimulation of the sensitized substance.

The expression of the specific transcription factor GATA-3 in Th2 cells showed a similar pattern to the proportion of Th2 cells (Fig. 4), indicating that the inhibitory effect of TGF-β1^{+} Bregs and Tregs on the percentage and function of Th2 cells is achieved by inhibiting the upstream transcription factors of Th2 cells. Allergen stimulation induced the loss of TGF-β1^{+} Bregs and Tregs as well as increased the expression of GATA-3. This led to the rapid proliferation of Th2 cells, making the inflammatory response towards Th2 cell polarization. With continuous OVA challenging, the function of TGF-β1^{+} Bregs and Tregs was gradually restored and the expression of GATA-3 as well as the secretion of IL-4 and IL-5 was inhibited (Fig. 3), thereby reducing the number and function of Th2 cells.

The rapidly increased proportion of Th2 cells inhibited the expression of Th1-specific transcription factor T-bet, which resulted in a decreased proportion of Th1 cells and a significantly reduced ratio of Th1/

Table 1
Primers used in this study.

Gene	Primers
IFN-γ-F	5′-AGACAATCAGGCCATCAGCA
IFN-γ-R	5′-TGGACCTGTGGGTTGTTGAC
IL-4-F	5′-TGTACCAGGAGCCATATCCA
IL-4-R	5′-TGTTCTTCGTTGCTGTGAGG
IL-5-F	5′-GACATGCACCATTGCCACTA
IL-5-R	5′-ATGCAACGAAGAGGATGAGG
IL-6-F	5′-GAGCCCACCAAGAACGATAG
IL-6-R	5′-TCAGTCCCAAGAAGGCAACT
IL-17a-F	5′-GGAAAGCTGGACCACCACA
IL-17a-R	5′-CACACCCACCAGCATCTTCTC
TGF-β1-F	5′-ATTCCTGGCGTTACCTTGG
TGF-β1-R	5′-AGCCCTGTATTCCGTCTCCT
T-bet-F	5′-TACAACAGCCAGCCAAACAG
T-bet-R	5′-CACCCTTCAAACCCTTCCTC
GATA-3-F	5′-TACCACCTATCCGCCCTATG
GATA-3-R	5′-GCCTCGACTTACATCCGAAC
RORγt-F	5′-AGCCTTTCCCTTTCTGCACT
RORγt-R	5′-CCATCACTTGCTGCTGTTGT
Foxp3-F	5′-CCAGTGCCCATCCAATAAAC
Foxp3-R	5′-GTATCCGCTTTCTCCTGCTG
β-actin-F	5′-GCAGAAGGAGATTACTGCTCT
β-actin-R	5′-GCTGATCCACATCTGCTGGAA

List of abbreviations used: F, Forward primer; R, Reverse primer.

Th2 and leads to the occurrence of disease [27]. With sustained allergen exposure, the proportion and function of TGF-β1$^+$ Breg and Tregs continued to increase and the immune system was resumed. The proliferation and function of Th2 cells were inhibited and those of Th1 were enhanced by promoting the expression of T-bet as well [27]. Then the ratio of Th1/Th2 shifted towards Th1 cells and fluctuated within the normal range before the immune state returned to normal gradually.

When AR occurred, the number and function of TGF-β1$^+$ Bregs and Tregs was reduced and the expression of the specific transcription factor RORγt increased in Th17 cells by promoting the expression of IL-6 and STAT3. The increased expression of IL-6 could enhance the activation of STAT3 and further increase the expression of RORγt to increase the proportion as well as function of Th17 cells [28]. However, when the number of TGF-β1$^+$ Bregs and Tregs increased, the expressions of IL-6, STAT3 and RORγt decreased and the proportion and function of Th17 cells began to decline. When antigen exposure was prolonged to 21 days, the immune status of the nasal mucosa was slightly switched towards Th1 cells and they dominated the nasal mucosa, ensuring the continuous dropping in the proportion of Th17 cells to a slightly or well below normal levels. With an increase in exposure time, the number and function of TGF-β1$^+$ Bregs and Tregs returned to normal and so did those of Th1 cells. The expressions of IL-6, STAT3 and RORγt as well as the proportion of Th17 cells were gradually restored to normal levels (Fig. 4).

4. Discussion

Many studies have found that the allergic disease process is associated with declining proportion and function of Breg and Treg cells. Kim and Kamekura also found that in the peripheral blood of patients with allergic rhinitis, the percentage of Breg cells and T follicular helper cells declined remarkably, confirming allergic rhinitis is related to the declining proportion and functionality of regulatory cells [29,30]. In addition, several studies have confirmed the regulatory role of Breg cells in the airway during allergic disease. In a cockroach allergen-induced asthma model, mice deficient in B10 cells had higher serum IgE levels and more airway inflammation than wildtype mice [31]. In some immunotherapy studies, SIT could induce the production of Breg and Treg, and inhibit almost all aspects of the Th2-mediated hypersensitive reaction [32]. In mouse models of allergic airway inflammation, Breg and Treg cells inhibited and reversed airway hyperresponsiveness (AHR), pulmonary inflammation, and Th2 reactions [33,34]. This may be achieved by secreting negative regulatory factors IL-10 and TGF-β, and inducing the production of protective antibodies IgA and IgG4 [35–39]. Antigen-specific IgA and IgG, especially IgG4, can be induced by SIT and block the interaction between IgE and allergens, thus inhibiting the early response to allergen excitation [40].

Increased Foxp3 expression could directly inhibit the differentiation of Th2 cells. Foxp3 not only interacted with GATA3, but also inhibited the ability of GATA3 to transactivate the promoter of a Th2 target gene [41]. Furthermore, the inhibitory effect of Treg cells on Th1 and Th2 cells has been widely recognized, but the role of Th17 cells has not been determined [42]. In an experimental gastritis mouse model established by Stummvoll et al., Tregs markedly inhibited Th1 and Th2 cell-mediated pathogenesis but had no effect on Th17 cell-mediated pathogenesis. More researchers believe changes to Th17 cells are linked to Treg cells [43]. Th17 cells are the most powerful stimulants of Treg cells, according to studies by Chen and Zhou [44,45]. Therefore, the increase in Breg and Treg cells observed in our study may be stimulated by an increased proportion of Th17 cells.

We hypothesized that the ratio and functional changes to TGF-β1$^+$ Bregs and Treg cells were closely related to the changes in the proportion and function of helper T cells in the nasal mucosal tissues of allergic rhinitis mice. Specifically, there was a change in the immune state of the mucosa which affected the development and recovery from the disease. This regulatory function may be achieved by regulating the ratio and function of helper T cells through the STATs expression.

During the occurrence of disease, proportions of the TGF-β1$^+$ Bregs and Treg cells were reduced and their functions were diminished by the sensitization of allergens in the nasal mucosa. The expression of GATA-3, an upstream specific transcription factor of Th2 cells, was enhanced and the downstream Th2 cells were subsequently over-differentiated, which increased the proportion and function of the Th2 cells; the decreased proportions and functions of the TGF-β1$^+$ Bregs and Tregs down-regulated the expression of T-bet, an upstream transcription factor of Th1 cells, and up-regulated the expressions of IL-6 and STAT3, upstream activation factors, and RORγt, a transcription factor in the Th17 cells. Changes in the proportions and functions of various cell subsets resulted in a disordered nasal mucosal structure, marked accumulation of eosinophils, increased expression of OVA-specific IgE, and consequently clinical symptoms such as nasal itching and sneezing.

When AR progresses, proportions and functions of TGF-β1$^+$ Bregs and Tregs in the nasal mucosa were rebalanced by allergen stimulation. The expression of the upstream transcription factor GATA-3 in Th2 cells as well as the proportion and function of the downstream Th2 cells were reduced; Meanwhile, the expression of the upstream transcription factor T-bet in the Th1 cells also increased significantly, which gradually restored the proportion and function of the Th1 cells; the expression of STAT3 and IL-6, and the transcription factor RORγt gradually decreased, followed by the reduced proportion and function of the Th17 cells, with a minimum shift to Th1 cells in the nasal mucosa observed after 21 days of allergen stimulation. As the proportion and function of the TGF-β1$^+$ Bregs and Tregs were restored, the nasal mucosa tissue structure was rearranged, the infiltration of eosinophils gradually decreased, and the expression of OVA-specific IgE and the onset of clinical symptoms, including nasal itching and sneezing, were finally reduced.

During the recovery stage, TGF-β1$^+$ Bregs and Tregs in the nasal mucosa tissue were functioning and a normal level was restored after prolonged allergen sensitization, and the proportions and functions of each subset of helper T cells and plasma cells were also gradually rebalanced. The nasal mucosa tissue structure, the number of submucosal eosinophils, and the expression of IgE returned to normal levels. At the same time, nasal symptoms gradually resolved in mice and the immune homeostasis was restored in the nasal mucosa.

To date, most studies of Breg cells have focused on the production of the IL-10 B10 cells. Immunological study has confirmed the negative immunomodulatory effect of B10 cells [46,47]. The B10 cell surface

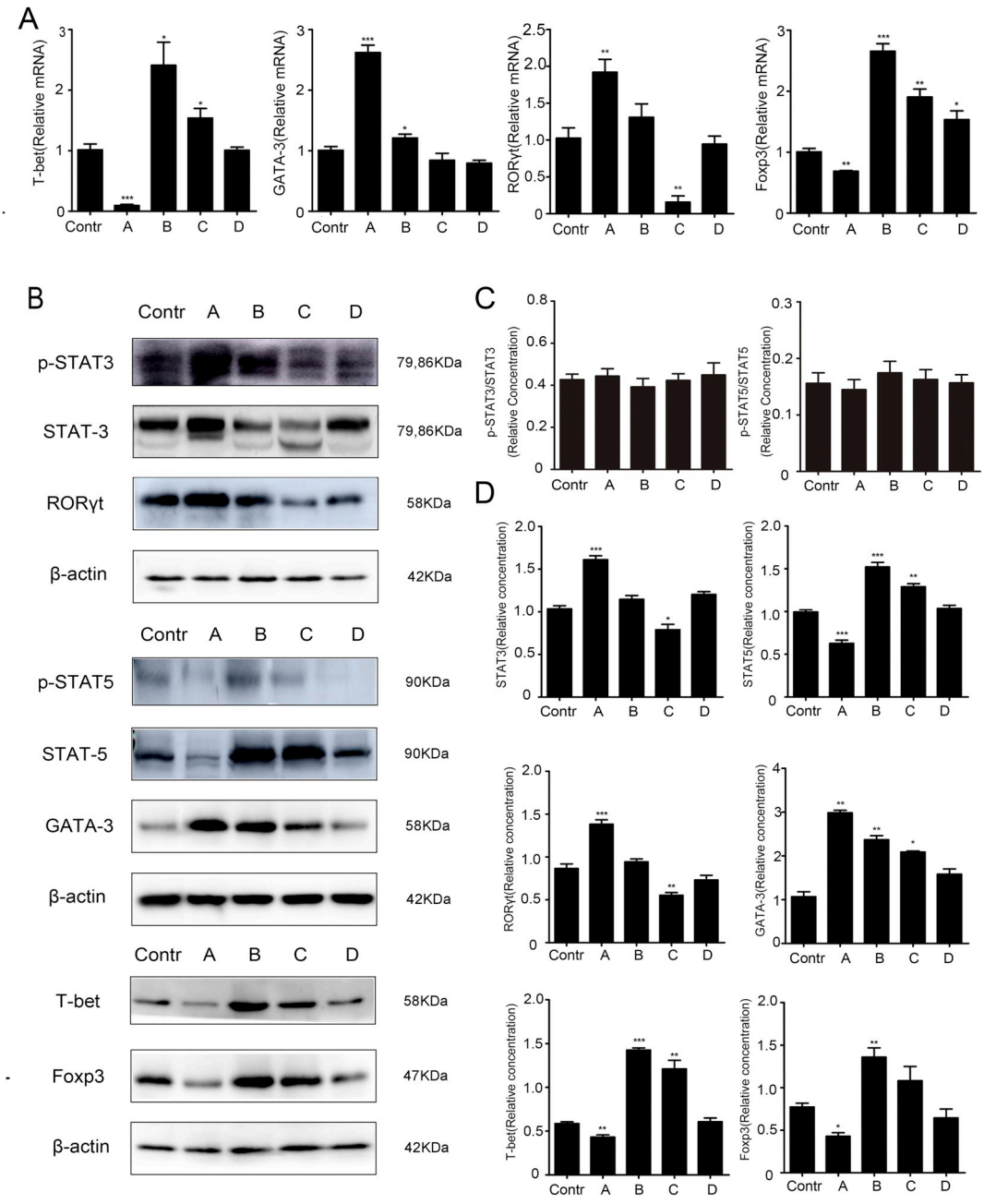

Fig. 4. mRNA and expression of transcription factors in mucosa.

(A) The mRNA levels of transcription factors in nasal mucosa were determined by real-time PCR. The experimental and statistical methods were the same as those for the determination of cytokine mRNA. Data is a representation of six independent experiments of triple samples. Comparisons between two groups of data were done with Mann-Whitney testing whereas comparisons among multiple groups of data were compared with Kruskal-Wallis/Dunn testing.

(B) Western blot analysis for pSTAT3, STAT3, pSTAT5, STAT5, T-bet, GATA-3, RORγt and Foxp3 in nasal and paranasal mucosa. Each lane contains 80 μg of protein Anti-Stat 3 antibody shows both Stat 3α and Stat 3β (86 and 79 kDa, respectively) in all lines. Protein expression was normalized to β-actin. Data is a representation of six independent experiments of triple samples.

(C) Ratio of p STAT3 and STAT3 in nasal and parasinal mucosa. There was no statistically significant change in the ratio between treatment groups. Comparisons between two groups of data were done with Mann-Whitney testing whereas comparisons among multiple groups of data were compared with Kruskal-Wallis/Dunn testing. Data are presented as the mean ± SEM.*$p < .05$, **$p < .01$, and ***$p < .001$.

(D) Quantification of western blots was performed using the ImageJ software. The quantification was normalized to the reference gene β-actin to obtain the relative concentration. Comparisons between two groups of data were done with Mann-Whitney testing whereas comparisons among multiple groups of data were compared with Kruskal-Wallis/Dunn testing. Results are mean ± SEM. *$p < .05$, **$p < .01$, and ***$p < .001$.

expresses $CD19^{hi}CD1d^{hi}CD5^{+}$ and is mainly detected in spleen tissue and peripheral blood [48,49]. However, in a study by Khan et al., $CD19^{+}$ B cells from mesenteric lymph nodes of mice with chronic enteric helminth infections are also capable of suppressing inflammatory response through an IL-10–independent mechanism [50]. In a local inhalational tolerance model of asthma (LIT) established by Natarajan et al, the proportion of $CD5^{+}$ Breg cells and $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells increased significantly [14]. Furthermore, suppressive Bregs arose only in regional hilar lymph nodes (HLNs), and no suppressive B cells were found in spleen or inguinal lymph node tissues. Moreover, LIT

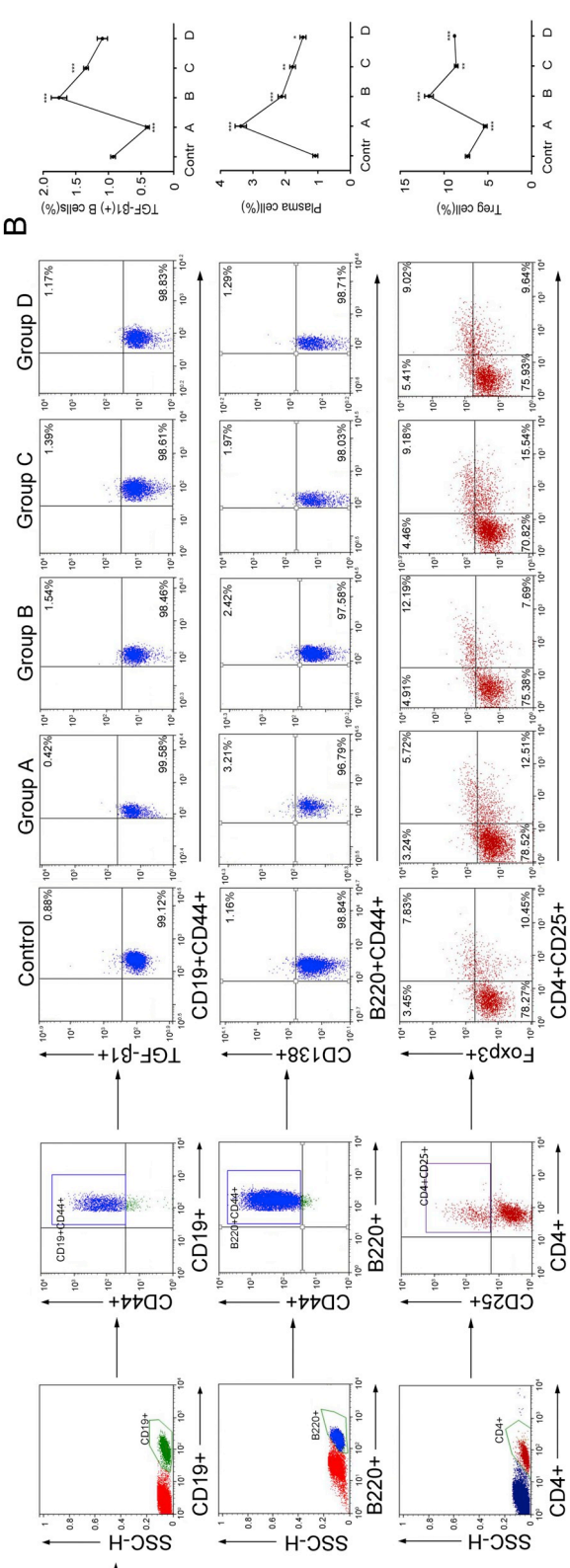

Fig. 5. Flow cytometric analysis of TGF-β1$^+$ Breg cells, Treg cells and plasma cells.
(A) Representative flow cytometry results. Numbers within each quadrant represent the percentages of Breg cells, Treg cells and plasma cells in nasal mucosa. The frequencies of CD19$^+$CD44$^+$TGF-β1$^+$Breg cells, CD4$^+$CD25$^+$FoxP3$^+$Treg cells and B220$^+$CD44$^+$CD138$^+$plasma cells were analyzed by flow cytometry. Data is a representation of six independent experiments.
(B) Change of quantitative flow cytometry results for TGF-β1$^+$ Breg cells, Treg cells and plasma cells in nasal and paranasal mucosa. Comparisons between two groups of data were done with Mann-Whitney testing whereas comparisons among multiple groups of data were compared with Kruskal-Wallis/Dunn testing. Data is a representation of six independent experiments. Results are mean ± SEM. $*p < .05$, $**p < .01$, and $***p < .001$.

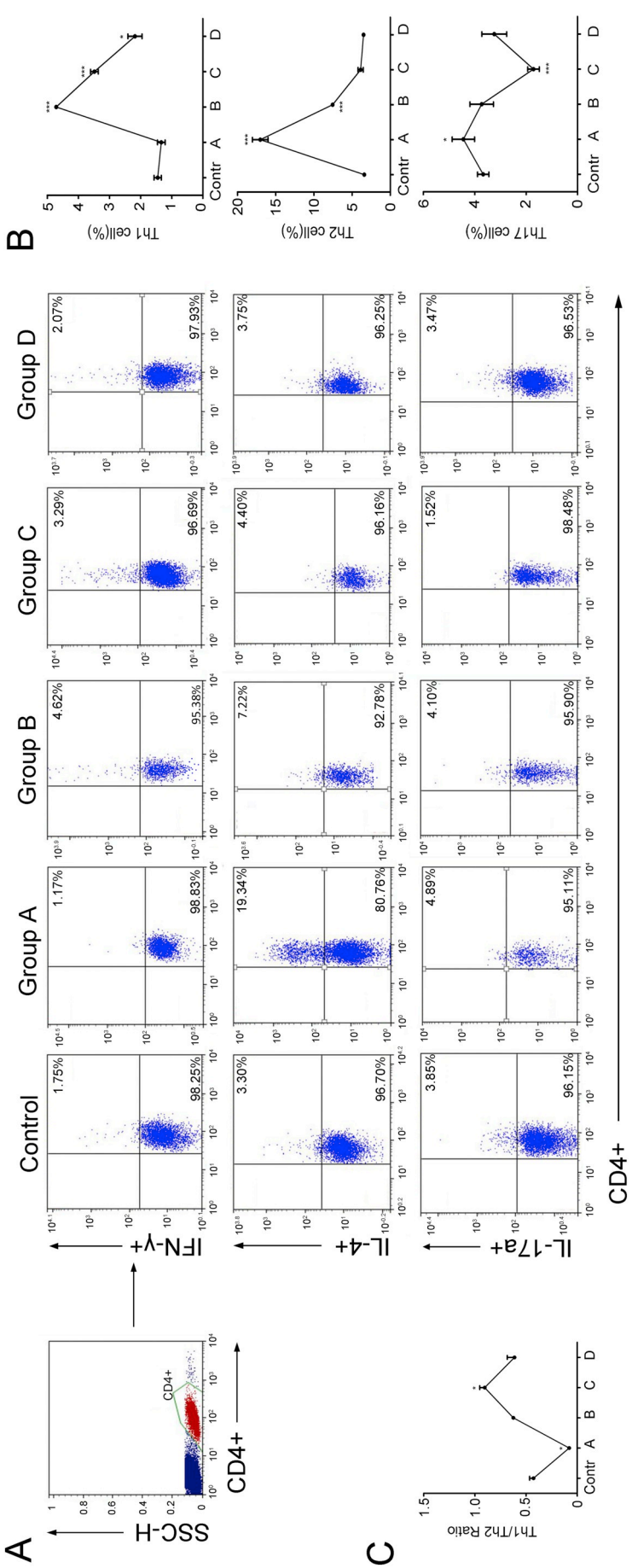

Fig. 6. Flow cytometric analysis of Th1, Th2 and Th17 cells.
(A) Representative flow cytometry results. Numbers within each quadrant represent the percentages of Th1, Th2 and Th17 cells in the nasal mucosa. The frequencies of $CD4^+IFN\text{-}\gamma^+$ Th1 cells, $CD4^+IL\text{-}4^+$ Th2 cells and $CD4^+IL\text{-}17a^+$ Th17 cells were also analyzed by flow cytometry. Data are representative of at least four different experiments.
(B) Change in quantitative flow cytometry results for Th1, Th2 and Th17 cells in nasal and paranasal mucosa. Comparisons between two groups of data were done with Mann-Whitney testing whereas comparisons among multiple groups of data were compared with Kruskal-Wallis/Dunn testing. Data are representative at least four independent experiments. Results are presented as mean ± SEM. $*p < .05$, $**p < .01$, and $***p < .001$.
(C) Ratio of Th1 to Th2 cells in nasal and paranasal mucosa. Comparisons between two groups of data were done with Mann-Whitney testing whereas comparisons among multiple groups of data were compared with Kruskal-Wallis/Dunn testing. Data are presented as mean ± SEM. $*p < .05$, $**p < .01$, and $***p < .001$.

HLN $CD5^+$ Breg cells predominantly expressed TGF-β, the LIT hilar nodes contained 5-fold more TGF-β^+ cells than IL-10^+ cells. In our study, $CD19^+CD5^+$IL-10^+B cells were not detected in the nasal mucosal tissue of the allergic mouse model and the proportion of $CD5^+$ B cells in the spleen tissue did not change throughout the disease process. The role of TGF-β is mainly reflected in the immune tolerance to allergens. Many studies suggest that TGF-β is not only able to inhibit the proliferation and differentiation of effusive B cells while reducing IgE level [51,52], but also promote the further conversion of $CD4^+CD25^+$T cells from the initial $CD4^+$ $CD25^-$T cells [53]. Besides, long-term stable sensitization with allergens can increase the production of TGF-β and reduce the local immune inflammatory state [54]. As with T cells, it is likely that several populations of regulatory B cells exist, and different subsets of Breg cells may be preferentially generated based on the types of stimuli and anatomical sites of antigen presentation [14].

In our study, mouse models of allergic rhinitis (AR) with different challenging durations were sequentially established to represent the process in which mice inhibit this harmful inflammatory response and re-establish a dynamic balance of mucosal immunity. We have confirmed, for the first time, that there is negative regulation of B cells in nasal mucosal tissue, TGF-β1 Bregs, and the occurrence of allergic rhinitis is closely related to the loss of TGF-β1 Bregs and Tregs proportion as well as function. We also tested the number and function of other helper T cell subsets during the occurrence, development and recovery of allergic rhinitis. We observed that the cell proportion and function of helper T cell subsets were associated with the development of disease and concluded that changes in helper T cell subsets may be achieved through the STAT expression during mucosal immune development. These findings support the complex regulatory mechanisms and interactions between Bregs and Tregs in the development of wild animal sensitization [55,56]. The pro-inflammatory and anti-inflammatory effects of subsets of B and T cells may overlap in allergic rhinitis and determine the disease course.

5. Conclusion

In conclusion, our study identified a B cell subgroup with regulatory function in nasal mucosal tissue, which expressed $CD19^+$ $CD44^+$ on the cell surface and generated TGF-β1. TGF-$\beta1^+$ Bregs cooperated with Treg cells in the development of allergic rhinitis and its recovery process as well as, the reconstitution of nasal mucosal immunity via regulation of their proportion and helper T cells functions. Further analysis of the origin and function of such B cells may explain how to implement the adjustment role of allergic immune response in an allergic mouse model and move towards a new strategy for the treatment of allergic rhinitis.

Acknowledgement

This work was supported by the Science and Technology Program, Shenyang, China [grant numbers 17-230-9-51].

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