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Alteration of circulating type 2 follicular helper T cells and regulatory B cells underlies the comorbid association of allergic rhinitis with bronchial asthma

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Abstract Allergic rhinitis (AR), the most common allergic disorder of the airway, is often accompanied by bronchial asthma. However, little is known about the mechanism by which AR advances to AR comorbid with bronchial asthma (AR + Asthma). To determine the pathophysiologic features of AR and AR + Asthma, we examined subsets of follicular helper T (Tfh) cells and regulatory B (Breg) cells in peripheral blood from AR and AR + Asthma patients. The results showed polarization of Tfh2 cells within Tfh cell subsets in both AR and AR + Asthma cases. Interestingly, the %Breg cells in total B cells were decreased in AR cases and, more extensively, in AR + Asthma cases. Moreover, we found significant correlations of fractional exhaled nitric oxide and blood eosinophil levels with the index %Tfh2 cells per %Breg cells. Our findings

Abbreviations: AR, allergic rhinitis; Tfh, follicular helper T; Breg, regulatory B; Ig, immunoglobulin; Th1, type 1 helper CD4⁺ T; Th2, type 2 helper CD4⁺ T; TSLP, thymic stromal lymphopoietin; IL, interleukin; Tfh1, type 1 Tfh; Tfh2, type 2 Tfh; Tfh17, IL-17-producing Tfh; PBMC, peripheral blood mononuclear cell; FeNO, fractional exhaled nitric oxide; SEM, standard error of the mean; FACS, fluorescence-activated cell sorting.

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indicate that relative decrease in Breg cells under the condition of Tfh2 cell skewing is a putative exaggerating factor of AR to bronchial asthma.

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1. Introduction

Allergic rhinitis (AR) affects 10 to 20% of the population and its increasing worldwide prevalence has made it a global health problem [1]. AR is characterized by rhinorrhea, sneezing and nasal congestion owing to persistent local inflammation of nasal mucosa in the upper airway. It is well known that AR frequently precedes bronchial asthma (in 10 to 40% of cases), and the presence of AR is considered to be a risk factor for new onset of asthma [2]. AR concomitant with bronchial asthma (AR + Asthma) or allergic rhinobronchitis has a much greater effect than AR alone on quality of life because of the severe symptoms of bronchial asthma such as wheeze and dyspnea. Allergic airway disorders are caused by an immunological defect based on type 1 hypersensitivity reactions through activation of eosinophils, basophils and mast cells and increased antigen-specific immunoglobulin E (IgE) [3]. The clinical condition of type I hypersensitivity is attributable to a functional imbalance of type 1 and type 2 helper CD4⁺ T cells (Th1 and Th2 cells, respectively) caused by epithelial cell-derived cytokines including thymic stromal lymphopoietin (TSLP), interleukin (IL)-25 and IL-33. Under this condition, Th2 cells secrete IL-4, IL-5 and IL-13 to promote unfavorable immune responses by helping B cells to produce allergen-specific IgE in the airway [4–6]. However, the mechanism by which AR deteriorates to AR + Asthma, which is a more serious form of allergic airway diseases, is still unclear.

To establish antigen-specific humoral immunity, B cells form germinal centers of lymphoid follicles in concert with follicular helper T (Tfh) cells, which are a class of effector helper T cells [7]. As with conventional helper T cell subsets, circulating Tfh cells (CD3⁺CD4⁺CXCR5⁺) are classified into three distinct subsets based on expression profiles of the chemokine receptors CCR6 and CXCR3, which are shared by Th1 cells and Th17 cells, respectively [8,9]. Such circulating Tfh cell subsets include type 1 Tfh (Tfh1) cells, type 2 Tfh (Tfh2) cells, and IL-17-producing Tfh (Tfh17) cells, which can secrete restricted repertoires of the cytokines Interferon- γ , IL-4 and IL-17, respectively, as can conventional helper T cell subsets. Tfh2 cells, like Th2 cells, have the capacity to help B cells produce IgE, though it is not known whether Tfh2 cells play a role similar to that of Th2 cells in the pathogenesis of allergic disorders [8,10]. Recent investigations have revealed that the levels of IgE can be regulated by regulatory B (Breg) cells, which produce the negative regulatory cytokine IL-10 [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 collagen-induced arthritis [13–16]. Taken together, results indicate that Tfh cell subsets and Breg cells in peripheral blood might have an

unidentified role in the regulation of clinical aspects of AR prone to AR + Asthma.

In this study, we analyzed the expression profiles of Tfh cell subsets and Breg cells in peripheral blood mononuclear cells (PBMCs) from AR and AR + Asthma patients. Interestingly, like the Th1/Th2 imbalance, we found a Tfh1/Tfh2 disproportion in both AR and AR + Asthma patients, showing predominance of Tfh2 cells. Thus, polarization of type 2 cells of Th cells and that of type 2 cells of Tfh cells would occur simultaneously in AR and AR + Asthma, by which allergic hyperresponsiveness to external antigens is likely to be heightened. We also obtained evidence that the proportion of Breg cells was decreased during disease progression from AR to AR + Asthma. Moreover, in AR + Asthma cases, the ratio of %Tfh2 cells to %Breg cells was significantly correlated with levels of fractional exhaled nitric oxide (FeNO) and peripheral blood eosinophils, which are biomarkers of allergic airway inflammation. Several mechanisms have been proposed to explain the united airway theory; however, our findings suggest that the pathogenesis underlying disease aggravation from AR to AR + Asthma may be a functional deficit of regulatory B cells under the condition of skewing of type 2 cell species within helper CD4⁺ T cells.

2. Materials and methods

2.1. Study populations

Characteristics of patients with AR (n = 23) or AR comorbid with bronchial asthma (AR + Asthma, n = 19) and healthy volunteers (n = 24) are summarized in Table 1. The diagnosis of AR was established on the basis of medical history and general nasal symptoms for at least 2 years, nasal cytology and specific IgE to four aeroallergens (house dust mite, *Dermatophagoides pteronyssinus*, Japanese white birch and orchard grass). Subjects with bronchial asthma were diagnosed by questionnaires, spirometry, airway hyperreactivity tests in response to methacholine (PC₂₀ methacholine) and measurement of specific IgE. FeNO levels were analyzed in exhaled breath [17]. Levels of specific IgEs to allergens and total IgE levels in serum were measured by ImmunoCAP (Phadia, Uppsala, Sweden). No patient had a history of allergen-specific immunotherapy. Treatment with antihistamines and corticosteroids was stopped at least 4 weeks before the subjects entered the study. All of the healthy volunteers had no abnormal physical and chest X-ray findings and were negative for the CAP test. All subjects were non-smokers. Written informed consent was obtained in all cases according to the Declaration of Helsinki. All protocols were approved by the Institutional Review Boards of Sapporo Medical University Hospital and Sapporo Respiratory Hospital.

Table 1 Subject characteristics.

	Healthy (n = 24)	AR (n = 23)	AR + Asthma (n = 19)
Age (years) mean $\pm$ SD (Range)	40.3 $\pm$ 12.2 (21–62)	44.1 $\pm$ 13.1 (28–69)	44.9 $\pm$ 14.4 (19–63)
Male/female (number)	9/15	9/14	10/9
Total IgE (IU/ml) mean $\pm$ SD (Range)	32.6 $\pm$ 21.9 (2–84)	168.8 $\pm$ 254.5 (20–1243)	907.5 $\pm$ 1104.7 (43–4324)
Specific IgE Number of positive case (%)			
House dust mite	0 (0)	16 (69.6)	16 (84.2)
<i>Dermatophagoides pteronyssinus</i>	0 (0)	16 (69.6)	16 (84.2)
Japanese white birch	0 (0)	14 (60.9)	9 (47.4)
Orchard grass	0 (0)	8 (34.8)	8 (42.1)
Blood eosinophils (number/ μ l) mean $\pm$ SD (Range)	150.1 $\pm$ 115.3 (32–400)	183.7 $\pm$ 140.0 (38–461)	539.7 $\pm$ 389.6 (106–1311)

2.2. Antibodies

Panels of directly conjugated anti-human monoclonal antibodies were used to measure lymphocyte species of Tfh cells (anti-CD3-APC, anti-CD4-APC-Cy7, anti-CXCR5-PerCP-Cy5.5), Tfh cell subsets (anti-CD3-FITC, anti-CD4-APC-Cy7, anti-CXCR5-PerCP-Cy5.5, anti-CCR6-APC, anti-CXCR3-PE-Cy7), and Breg cells (anti-CD19-APC-Cy7, anti-CD24-PerCP-Cy5.5, anti-CD27-FITC) gated by cells negative to anti-CD3-APC. All monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA).

2.3. Flow cytometry

Heparinized PBMCs were isolated from fresh blood specimens by centrifugation over a discontinuous density gradient (Lympholyte®-H; Cedarlane, Burlington, ON, Canada). Cell staining and flow cytometry using FACSCanto™ II and FACS Aria™ II (BD Biosciences) were performed as previously described [18].

2.4. Statistical analysis

All data are shown as means $\pm$ SEM. Significant differences between specimens were determined by using Student's *t* test and the Mann-Whitney *U* test. Correlations were determined by Spearman's ranking. Probability values less than 0.05 were considered significant.

3. Results

3.1. Tfh cell subsets in AR and AR + Asthma cases

As expected, serum levels of total IgE in patients with AR were elevated in comparison to those in healthy controls (Fig. 1A). When AR was combined with bronchial asthma (AR + Asthma), total IgE and blood eosinophil levels were significantly elevated compared to those in AR cases (Figs. 1A,B). This indicates that a possible factor(s) would play a certain role in upregulation of total IgE and eosinophils, causing unfavorable allergic inflammation over the pulmonary parenchyma and in

the upper respiratory tract. Examinations of PBMCs revealed that the ratio of circulating total Tfh cells (CD3⁺CD4⁺CXCR5⁺) to CD4⁺ T cells (CD3⁺CD4⁺) in AR cases was comparable to that in healthy controls and even to that in AR + Asthma cases (Figs. 2A,B). In addition, there was no significant difference in absolute number of total Tfh cells between the control and AR cases or between the control and AR + Asthma cases (Fig. 2B). In contrast to total Tfh cells, it was interesting that both AR and AR + asthma cases had unique polarization of circulating Tfh cell subsets, with Tfh2 cells being clearly overt (Figs. 3A–D). The percentage (in total Tfh cells) and absolute number of Tfh1 cells (CD3⁺CD4⁺CXCR5⁺CXCR3⁺CCR6[−]) in both AR and AR + Asthma cases were significantly decreased to similar levels compared to those in healthy controls (Fig. 3B). Conversely, the percentage and number of Tfh2 cells (CD3⁺CD4⁺CXCR5⁺CXCR3[−]CCR6[−]) in AR and AR + Asthma cases were substantially increased compared to those in controls (Fig. 3C). The percentage and number of Tfh17 cells (CD3⁺CD4⁺CXCR5⁺CXCR3[−]CCR6⁺) in AR and AR + Asthma cases were preserved at levels similar to those in controls (Fig. 3D). Collectively, the results showed that patients with AR and patients with AR + Asthma manifested similar profiles of Tfh1 and Tfh2 cells directing Tfh2 cell skewing, probably underlying the allergic condition of these disorders. This type of skewing of Tfh cell subsets was further supported by analysis of the ratios of percentage of Tfh2 cells to that of Tfh1 cells in AR and AR + Asthma cases (Fig. 3E), like the status of Th1 cells and Th2 cells as seen in allergic respiratory diseases.

3.2. Breg cells in AR and AR + Asthma cases

To further understand cues responsible for the pathogenesis of AR and AR + Asthma, we next assessed circulating Breg cells (CD3[−]CD19⁺CD24^{hi}CD27⁺), as a negative regulator of immunity [11], in AR and AR + Asthma cases (Fig. 4A). The percentage of Breg cells in total B cells (CD3[−]CD19⁺) in AR cases was clearly decreased compared to that in healthy donors (Fig. 4B). Of note, the percentage of Breg cells in AR was more significantly decreased when AR was comorbid with bronchial asthma. Taken together, the results suggest a certain role of Breg cells to limit allergic inflammations probably by controlling allergen tolerance.

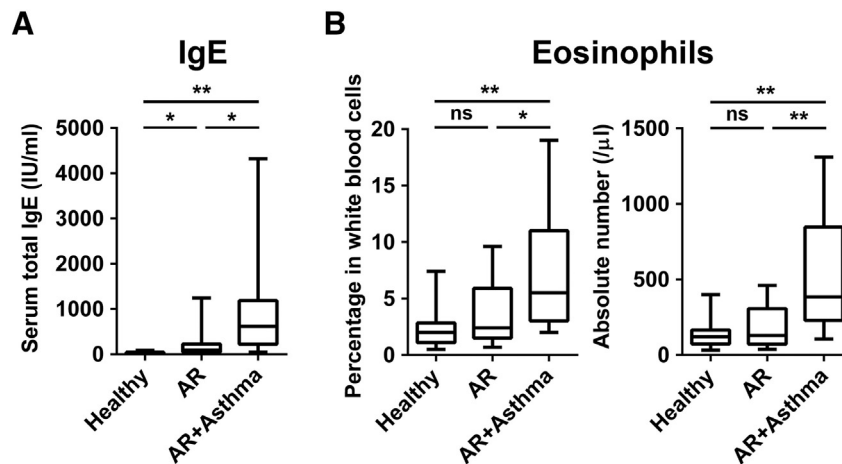


Figure 1 Serum levels of total IgE and blood eosinophils in patients with allergic rhinitis (AR) and patients with AR comorbid with bronchial asthma (AR + Asthma). Serum levels of total IgE and blood eosinophils are shown in (A) and (B), respectively. Levels of blood eosinophils are shown as percentage of white blood cells and absolute number (per μ l blood). * $P < 0.05$, ** $P < 0.01$, ns indicating not significant.

3.3. Relationship between eosinophil activity and relative decrease in Breg cells based on Tfh2 cell skewing

We hypothesized that Tfh2 cell skewing and relative decrease in Breg cells play synergistic roles in the deterioration of AR to AR + Asthma. To prove this hypothesis, we generated a new index calculated from Tfh2 cells (%) and Breg cells (%), which simply means the ratio of Tfh2 cells to Breg cells. The index represents the degree of Tfh2 cell deviation and relative decrease in Breg cells. Interestingly, the index was positively related to the number of eosinophils in peripheral blood from patients with AR + Asthma (Fig. 5A). The index was also correlated with FeNO in AR + Asthma cases (Fig. 5B).

4. Discussion

In this study, we obtained evidence that patients suffering from AR or AR + Asthma of immunological airway diseases

preferentially exhibit Tfh2 cell polarization of peripheral blood lymphocytes. Given that AR and AR + Asthma cases have normal levels of total Tfh cells and Tfh17 cells, such Tfh2 cell skewing is probably attributable to increased levels of Tfh2 cells and decreased levels of Tfh1 cells within circulating Tfh cell populations. To the best of our knowledge, this is the first report of a Tfh1/Tfh2 imbalance in allergic disorders, though it has been shown that patients with autoimmune diseases such as systemic lupus erythematosus and juvenile dermatomyositis have a dominance of Tfh2 and Tfh17 cells [8,9,19]. Allergic inflammatory diseases including AR, bronchial asthma, atopic dermatitis and allergic enterocolitis are primarily based on a disequilibrium of Th1 and Th2 cells, which causes Th2 cell-mediated inflammatory lesions in affected tissues. Interestingly, we found that patients with AR or AR + Asthma show CD4⁺ T cell polarization not only to Th2 cells but also to Tfh2 cells. Th2 cells and Tfh2 cells can produce IgE-related cytokines such as IL-4, IL-5, and IL-13. Indeed Tfh2 cells have the capacity to enable B cells to become IgE-producing cells [8], and the elevated number of Tfh2 cells in AR and

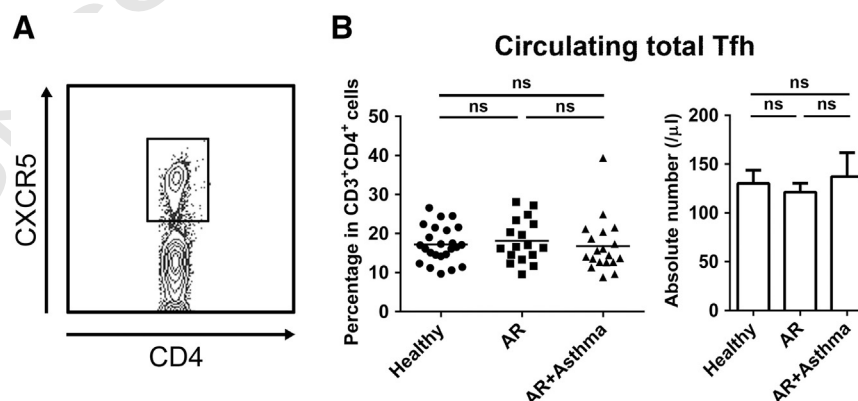


Figure 2 Ratios of circulating total Tfh cells in AR and AR + Asthma cases. (A) Representative fluorescence-activated cell sorting (FACS) profiles indicating total Tfh cells (CD3⁺CD4⁺CXCR5⁺). Plots were pregated on CD3⁺CD4⁺ cells and examined by the levels of CXCR5. (B) Percentage of total Tfh cells in CD3⁺CD4⁺ cells and absolute number of total Tfh cells (per μ l blood) are shown in the left and right panels, respectively. ns indicating not significant.

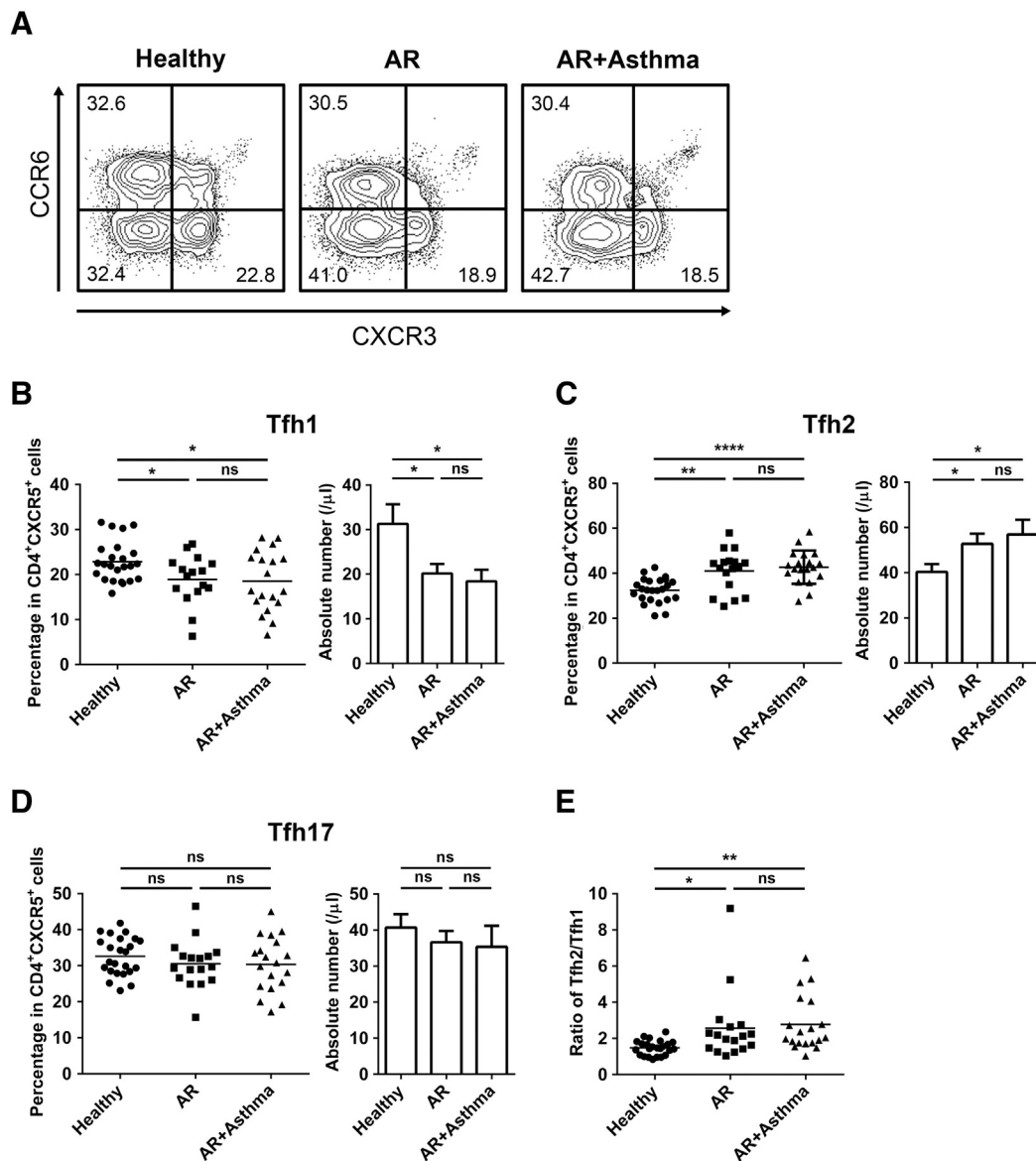


Figure 3 Polarization of circulating Tfh cell subsets in AR and AR + Asthma cases. (A) Representative FACS profiles indicating Tfh1 cells (CXCR3⁺CCR6⁻), Tfh2 cells (CXCR3⁻CCR6⁻) and Tfh17 cells (CXCR3⁺CCR6⁺). Plots were pregated on CD3⁺CD4⁺CXCR5⁺ cells and examined by the levels of CXCR3 and CCR6. Numbers indicate percentage of cells in the gate. (B–D) Percentage of Tfh cell subsets in total Tfh cells and absolute number of Tfh cell subsets (per μ l blood) are shown in the left and right panels, respectively. (B) Tfh1 cells, (C) Tfh2 cells and (D) Tfh17 cells. (E) Ratio of %Tfh2 cells to %Tfh1 cells. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, ns indicating not significant.

AR + Asthma cases can thereby help B cells to produce more antigen-specific IgE, leading to allergic inflammation. A hallmark of Tfh cells is surface expression of CXCR5, which is also advantageously shared by B cells [20,21]. Thus, Tfh2 cells in AR and AR + Asthma cases are thought to easily make contact with B cells for activation through CXCL13, a CXCR5 ligand secreted by various cells including follicular dendritic cells, endothelial cells and also Tfh cells [22]. From this point of view, it is possible that Tfh2 cells, like Th2 cells, contribute to the direct pathogenesis of allergic inflammation mediated by IgE. During the development of tissue-resident Tfh cells from naïve helper CD4⁺ T cells, where a suite of genes including Bcl6, MAF and STAT3 defines core characteristics of Tfh cells,

intermediate cells of Th1/Tfh cells emerge as progenitor cells of Tfh cells [22,23]. However, the mechanisms underlying the development of circulating Tfh cell subsets, especially the mechanism of Tfh2 cell skewing, remain elusive. Recent advances in deep profiling of individual lymphocytes have opened a new paradigm for an understanding of the immune system [24]. Such studies may shed light on the understanding of differentiation and/or survival of Tfh1 and Tfh2 cells and further characterize allergy-prone macrocosm and microcosm mediated by heterogeneous helper CD4⁺ T cells.

In addition to a salient feature of circulating Tfh cell subsets, we showed for the first time a stepwise decline in the percentage of Breg cells from a normal condition to AR

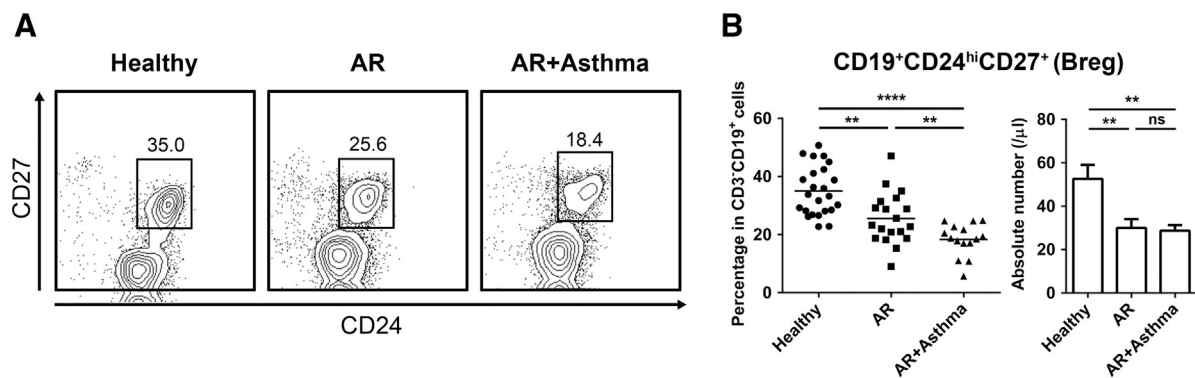


Figure 4 Percentages of circulating Breg cells in AR and AR + Asthma cases. (A) Representative FACS profiles indicating Breg cells (CD24^{hi}CD27⁺). Plots were pregated on CD3⁺CD19⁺ cells and examined by the levels of CD24 and CD27. Numbers indicate percentage of cells in the gate. (B) Percentage of Breg cells in total CD3⁺CD19⁺ B cells and absolute number of Breg cells (per µl blood) are shown in the left and right panels, respectively. ***P* < 0.01, *****P* < 0.0001, ns indicating not significant.

and further to AR + Asthma. Such a decline of Breg cells is probably associated with serum levels of total IgE and hyperfunction of eosinophils, in which Breg cells would play a possible role to confine broad allergic inflammation. When immunotherapy using an allergen is performed for reducing symptoms of AR and bronchial asthma, the percentages of Breg cells and regulatory T cells are increased with alteration of serum quantities of antibody subclasses, with the level of IgE being downregulated and levels of other immunoglobulin subclasses including IgG4 being upregulated [25]. Such changes in clinical markers are in part compatible with our observations. In allergic asthma, Breg cells have an impaired capacity to secrete IL-10 under the condition of LPS stimulation [26]. Thus, further functional defects of Breg cells might also occur in combined AR and asthma syndrome. Recent investigations further suggest a possible role of IL-35 produced by Breg cells in the pathogenesis of allergic airway diseases of rodent models [27,28]. Collectively, the results suggest that regulatory B cells play an important part in the transitional process from AR to AR + Asthma.

Although a functional linkage between Breg cells and Tfh2 cells remains to be elucidated, the significant correlation of the index (%Tfh2 cells divided by %Breg cells) and inflammatory activity characteristic to type 1 hypersensitivity indicates that these cell types may contribute to the

spread of allergic inflammatory lesions in the nasal cavity and through the respiratory tract as seen in AR + Asthma cases. CD27⁺ Breg cells are thought to be memory B cells because Breg cells mostly express IgG on their surface (our observation) and have high levels of CD48 and the membrane tyrosine phosphatase CD148, which are expressed on activated B cells and memory B cells, respectively [11]. In this context, functional activation of Breg cells might simultaneously lead to modulation of components of the memory B-cell pool through immunological intervention using an allergen. It is generally assumed that a germinal center reaction helped by Tfh cells results in the formation of antigen-specific B-cell memory, though further experiments are required to determine the role of Tfh2 cells during the development of Breg cells [29]. There are also unanswered questions regarding the possible roles of Tfh2 cells and Breg cells in allergic inflammation with group 2 innate lymphoid cells, in the generation of allergen-specific IgE, and in the maintenance of eosinophilia [30].

In conclusion, Tfh2-dysregulated immunity underlies the pathogenesis of respiratory allergic diseases, and a relative decrease in Breg cells under the condition of Tfh2 cell skewing is a putative exaggerating factor of AR to bronchial asthma. Focusing on a modality to restore the Tfh2 cell shift and increase Breg cells may be a viable strategy to prevent the

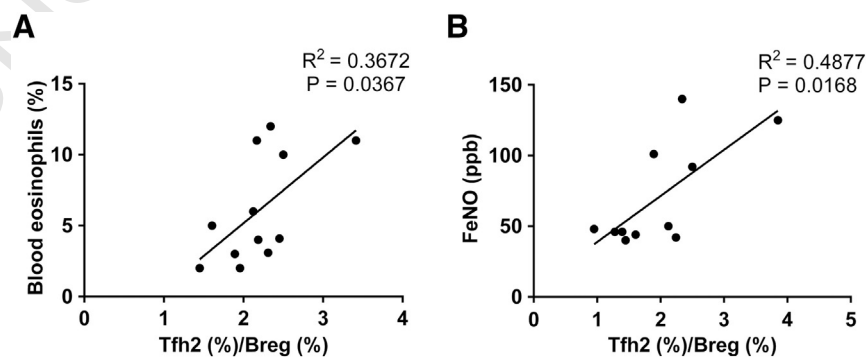


Figure 5 Correlation between eosinophil activity and the value of %Tfh2 cells divided by %Breg cells (Tfh2 (%) / Breg (%)) in patients with AR + Asthma. (A) Graph showing blood eosinophils vs %Tfh2 cells per %Breg cells. (B) Graph showing fractional exhaled nitric oxide (FeNO) vs %Tfh2 cells per %Breg cells.

development of allergic disorders. Since bronchial asthma based on AR is considered to be a global health problem, a strategy to reduce the prevalence of this disease would substantially alleviate the huge socioeconomic burden.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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