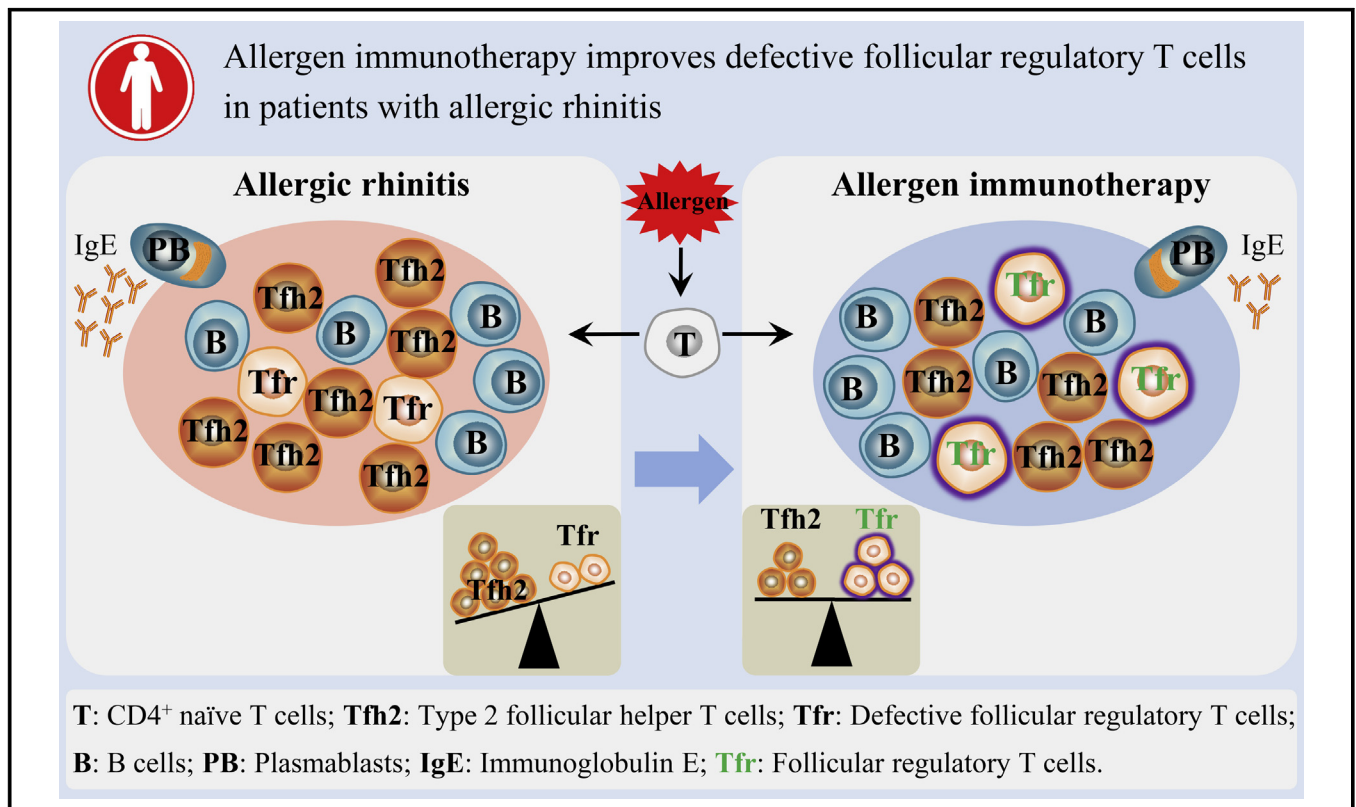


Allergen immunotherapy improves defective follicular regulatory T cells in patients with allergic rhinitis



Yin Yao, MD,^{a,*} Zhi-Chao Wang, MD,^{a,*} Nan Wang, MD, PhD,^a Peng-Cheng Zhou, PhD,^b Cai-Ling Chen, MD,^a Jia Song, MD,^a Li Pan, MD,^a Bo Liao, MD, PhD,^a Xin-Hao Zhang, MD, PhD,^a Yong-Shi Yang, MD,^c Xiao-Yan Xu, MD,^d Rong-Fei Zhu, MD, PhD,^c Di Yu, PhD,^b and Zheng Liu, MD, PhD^a *Wuhan, China, and Canberra, Australia*

GRAPHICAL ABSTRACT



From ^athe Department of Otolaryngology–Head and Neck Surgery and ^cthe Department of Allergy, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan; ^bthe Department of Immunology and Infectious Disease, John Curtin School of Medical Research, Australian National University, Canberra; and ^dthe Department of Otolaryngology–Head and Neck Surgery, China Resources & Wisco General Hospital, Wuhan.

*These authors contributed equally to this work.

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Corresponding author: Zheng Liu, MD, PhD, Department of Otolaryngology–Head and Neck Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, No. 1095 Jiefang Avenue, Wuhan 430030, P.R. China. E-mail: zhengluiant@hotmail.com. Or: Corresponding author: Di Yu, PhD, Department of Immunology and Infectious Disease, John Curtin School of Medical Research, Australian National University, Building 131, Garran Road, Canberra, ACT 2601, Australia. E-mail: di.yu@anu.edu.au.

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Background: The function of follicular regulatory T (T_{FR}) cells, especially in regulating IgE production in patients with allergic diseases, is poorly understood.

Objective: We sought to investigate the phenotype, function, and clinical relevance of T_{FR} cells in patients with allergic rhinitis (AR).

Methods: The phenotype and frequency of tonsillar and circulating T_{FR} cells were characterized by using flow cytometry. T_{FR} cell function was examined in an assay by coculturing with follicular helper T cells and B cells. The associations between T_{FR} cells and the clinical features in patients with AR before and after allergen immunotherapy (AIT) were analyzed.

Results: T_{FR} cells were detected in germinal centers of tonsils, but compared with subjects without AR, the frequencies decreased in patients with AR who were allergic to house dust mites. Circulating T_{FR} cells in blood were phenotypically and numerically correlated with tonsillar T_{FR} cells, and a reduction of circulating T_{FR} cells but not total or CXCR5⁺ regulatory T cells was noted in patients with AR compared with healthy control subjects. Moreover, circulating T_{FR} cells in patients with AR showed a specific defect in suppressing IgE production but were capable of suppressing production of other immunoglobulin types. We identified negative associations of circulating T_{FR} cell frequencies and function with antigen-specific IgE levels or disease severity in patients with AR. After AIT, the frequencies and function of circulating T_{FR} cells were improved, which positively associated with disease remission.

Conclusion: Impairment in T_{FR} cells might contribute to aberrant IgE production in patients with AR, and AIT improves defective T_{FR} cell function. T_{FR} cells might serve as a potential biomarker to monitor clinical response to AIT. (J Allergy Clin Immunol 2019;144:118-28.)

Key words: Allergic rhinitis, follicular regulatory T cells, IgE, allergen immunotherapy

Allergic rhinitis (AR) is an IgE-mediated type I hypersensitivity reaction to airborne allergens.^{1,2} Despite numerous studies depicting a strong association between IgE levels and AR,^{1,2} the mechanisms underlying the aberrant IgE production in patients with AR remain elusive. It is now clear that follicular helper T (T_{FH}) cells, which express CXCR5 to localize to B-cell follicles and rely on the transcription factor B-cell lymphoma 6 (Bcl-6) for differentiation, play a central role in initiating and supporting germinal center (GC) reactions to enable B cells to produce high-affinity antibodies and form humoral memory.³ Several studies using mouse models demonstrated that T_{FH} cells orchestrated systemic IgE production in patients with allergic asthma.^{4,5} Recently, we have found that increased numbers of peripheral type 2 T_{FH} (T_{FH2}) cells positively correlated with upregulated IgE in patients with AR,⁶ suggesting a skewed polarization of T_{FH2} cells in promoting IgE production in patients with AR.

Recently, follicular regulatory T (T_{FR}) cells have been identified as a novel effector subset of regulatory T (Treg) cells with a specialized function in limiting T_{FH} cell-mediated B-cell activation and antibody production.^{7,8} T_{FR} cells possess overlapped phenotypic characteristics with both T_{FH} and Treg cells.⁷⁻⁹ Similar to T_{FH} cells, T_{FR} cells migrate into B-cell follicles by up-regulating CXCR5 and express Bcl-6.^{7,8,10} On the other hand, resembling Treg cells, T_{FR} cells express the transcription factor

Abbreviations used

AIT:	Allergen immunotherapy
AR:	Allergic rhinitis
Bcl-6:	B-cell lymphoma 6
CSMS:	Combined symptom and medication score
CTLA-4:	Cytotoxic T lymphocyte-associated protein 4
Foxp3:	Forkhead box P3
GC:	Germinal center
HC:	Healthy control subject
T _{FH} :	Follicular helper T
T _{FR} :	Follicular regulatory T
Treg:	Regulatory T
VAS:	Visual analog scale

forkhead box P3 (Foxp3) and receptors associated with regulatory function, such as glucocorticoid-induced TNF receptor-related protein and cytotoxic T lymphocyte-associated protein 4 (CTLA-4).¹⁰ In patients with allergic diseases, the question remains unanswered whether T_{FR} cells regulate T_{FH} cell-mediated B-cell activation and antibody production, especially IgE.

Because of the challenge of obtaining secondary lymphoid organ samples from human subjects, research often focused on study of T_{FH} cell-like or T_{FR} cell-like populations circulating in human blood. We and others have carefully characterized the phenotype and function of circulating memory T_{FH} cells in human subjects.^{11,12} However, despite a growing body of recent studies that analyzed circulating T_{FR} cells in autoimmune diseases, organ transplantation, and allergic diseases,¹³⁻¹⁷ the phenotypic, functional, and numeric relationship between T_{FR} cells in secondary lymphoid organs and the counterpart circulating T_{FR} cell population in blood in both homeostatic and pathologic conditions have not been well characterized.

Here, for the first time, we showed that the frequencies of circulating T_{FR} cells in blood positively correlated with the frequencies of T_{FR} cells in tonsillar GCs in human subjects. We characterized the phenotypic similarity and difference between circulating T_{FR} cells and GC T_{FR} cells and demonstrated that circulating T_{FR} cells possessed the capacity to suppress T_{FH} cell-mediated antibody production from B cells, distinguishing them from the CXCR5⁺ Treg cells. We found that circulating T_{FR} cells were not only reduced in abundance but also impaired in function in patients with AR. In addition, such T_{FR} cell defects correlated with disease severity and antigen-specific IgE production in patients with AR. Importantly, patients showed increased numbers of T_{FR} cells with improved suppressive function after allergen immunotherapy (AIT).

METHODS

Subjects

This study was approved by the Ethics Committee of Tongji Hospital and China Resources & Wisco General Hospital. Informed consent was obtained from all adult participants or parents of minors less than 18 years old, and assent had to be obtained from the minors. The diagnosis of AR was made and disease severity was evaluated based on the Allergic Rhinitis and its Impact on Asthma guidelines.¹

For the correlation study of circulating and GC T_{FR} cells, paired tonsillar and peripheral blood samples were collected from subjects undergoing tonsillectomy because of tonsillar hypertrophy with or without concomitant AR. In the study of phenotype and function of T_{FR} cells, peripheral blood samples

were collected from healthy control subjects (HCs) and patients with AR allergic to house dust mites (HDMs) only, and tonsillar samples were collected from subjects who were healthy other than tonsillar hypertrophy. Additional patients with AR who were allergic to HDM only and qualified for AIT were divided into 2 groups, patients with AIT (receiving AIT and standard medication treatment) and patients without AIT (receiving standard medication treatment only), based on the patient's preference. Peripheral blood samples were collected at baseline (before treatment) and 3, 6, and 12 months after treatment. More information on the subjects is provided in Tables E1 to E4 in this article's Online Repository at www.jacionline.org.

Allergen test

Skin prick tests with a panel of 19 inhalant allergens common in our region was performed, as previously described,⁶ and additional information is provided in the Methods section in this article's Online Repository at www.jacionline.org.

AIT and symptom assessment

Subcutaneous AIT using semidepot HDM allergen extracts (full allergens, 50% *Dermatophagoides pteronyssinus* plus 50% *Dermatophagoides farinae*; Allergopharma GmbH, Reinbek, Germany) was conducted, as previously described.⁶ The daily combined symptom and medication score (CSMS) was calculated, as previously reported.⁶ More information is provided in the Methods section in this article's Online Repository.

Flow cytometry

PBMCs and tonsillar mononuclear cells were isolated, as previously mentioned.¹⁸ Cells were stained with specific primary antibodies (see Table E5 in this article's Online Repository at www.jacionline.org) and analyzed by using flow cytometry.¹⁹ Additional information is provided in the Methods section in this article's Online Repository.

Cell sorting

Peripheral T- and B-cell subsets were sorted from fluorescence-labeled PBMCs by using a BD FACSAria II cell sorter (BD Biosciences, San Jose, Calif).¹¹ The purity of sorted cells was greater than 95%. Antibodies used in cell sorting are described in Table E5, and additional information is provided in the Methods section in this article's Online Repository.

Cell culture

Sorted T_{FH} cells were cultured with autologous B cells with or without T_{FR} cells, as previously described.¹⁸ More information is provided in the Methods section in this article's Online Repository.

Cytokine and immunoglobulin measurement

Levels of serum antigen-specific IgE and IgG₄ to *D pteronyssinus* were measured by using ImmunoCAP (Phadia, Uppsala, Sweden) and ELISA (Dr Fooke; Laboratorien GmbH, Neuss, Germany), respectively. IgG, IgM, IgA, IgE, IL-21, and CXCL13 in culture supernatants were detected with commercial ELISA kits (Thermo Fisher Scientific, Waltham, Mass), as previously described.¹²

Immunohistochemistry

Immunohistochemical staining of paraffin-embedded tissue sections was performed, as previously described.²⁰ Additional information is provided in the Methods section in this article's Online Repository.

Statistical analysis

All statistical analyses were carried out with GraphPad Prism Software 5.0 (GraphPad Software, La Jolla, Calif). Expression data are presented in dot

plots. Symbols represent individual samples, horizontal bars represent medians, and error bars show interquartile ranges. The Mann-Whitney *U* test was used for between-group comparisons. Cell-culture data are expressed as means $\pm$ SEMs and analyzed by using the unpaired Student *t* test, unless specifically stated. For categorical data, χ^2 or Fisher exact tests were performed. Spearman rank correlation analysis was used to analyze the associations. *P* values of less than .05 were considered statistically significant.

RESULTS

Reduced numbers of T_{FR} cells in tonsils of patients with AR

To examine the function of T_{FR} cells in patients with allergic diseases, we decided to first analyze T_{FR} cells in secondary lymphoid organs of patients with AR. In tonsils more than 90% of Foxp3⁺ cells were CD4⁺CD3⁺ T cells (see Fig E1 in this article's Online Repository at www.jacionline.org), suggesting that Foxp3 is a specific marker for Treg cells.

We then investigated localization of Treg cells by labeling Foxp3⁺ cells in tonsils using immunohistochemistry. We revealed the presence of Foxp3⁺ cells within GCs, which correspond to T_{FR} cells (Fig 1, A).⁸ Importantly, numbers of Foxp3⁺ cells in GCs (*bona fide* T_{FR} cells) in patients with AR were dramatically reduced in comparison with values in those without AR (Fig 1, B). The lower frequency of T_{FR} cells in tonsils of patients with AR was further corroborated by using flow cytometric analysis, whereby T_{FR} cells were defined as CD3⁺CD4⁺CD45RA^{low}CXCR5^{high}Foxp3⁺ cells (Fig 1, C and D).

Circulating T_{FR} cells correspond to T_{FR} cells in secondary lymphoid organs

To investigate the function of T_{FR} cells beyond the small fraction of patients with AR with available tonsillar samples, we first determined whether the altered GC T_{FR} cells in tonsils could be reflected by a change in circulating T_{FR} cells, as defined by CD3⁺CD4⁺CD45RA^{low}CXCR5⁺CD25^{high}CD127^{low} cells (Fig 2, A).²¹ In supporting this gating method, we measured Foxp3 expression and validated Foxp3 expression in about 90% of circulating T_{FR} cells (see Fig E2 in this article's Online Repository at www.jacionline.org). Using paired tonsillar and blood samples, we observed a relatively tight correlation between the frequencies of T_{FR} cells within CD4⁺ T cells in tonsils and those in paired blood samples (Fig 2, B).

To comprehensively investigate the phenotype of T_{FR} cells, we compared T_{FR}, T_{FH}, and CXCR5⁺ Treg cells in peripheral blood and tonsils from healthy subjects and subjects who were healthy other than tonsillar hypertrophy, respectively (see Fig E3 in this article's Online Repository at www.jacionline.org). Because circulating CXCR5⁺ T cells were mainly CD45RA^{low} memory T cells, we studied and compared CD45RA^{low} memory T cells in gated CXCR5⁺ cells, as previously described (Fig E3).²¹

We first compared phenotypes between human T_{FR} and Treg cells. Both GC T_{FR} and circulating T_{FR} cells, but not T_{FH} cells, expressed the Treg cell markers CTLA-4 and Foxp3 (Fig 2, C, and see Fig E4 in this article's Online Repository at www.jacionline.org). Although *bona fide* T_{FR} and T_{FH} cells in GCs in tonsils had high expression of programmed cell death protein 1, inducible costimulator, and Bcl-6, neither T_{FH} cells¹⁸ nor T_{FR} cells in blood exhibited significant expression of these markers (Fig 2, C, and see Fig E4). We also examined the expression of Ki-67, a marker for

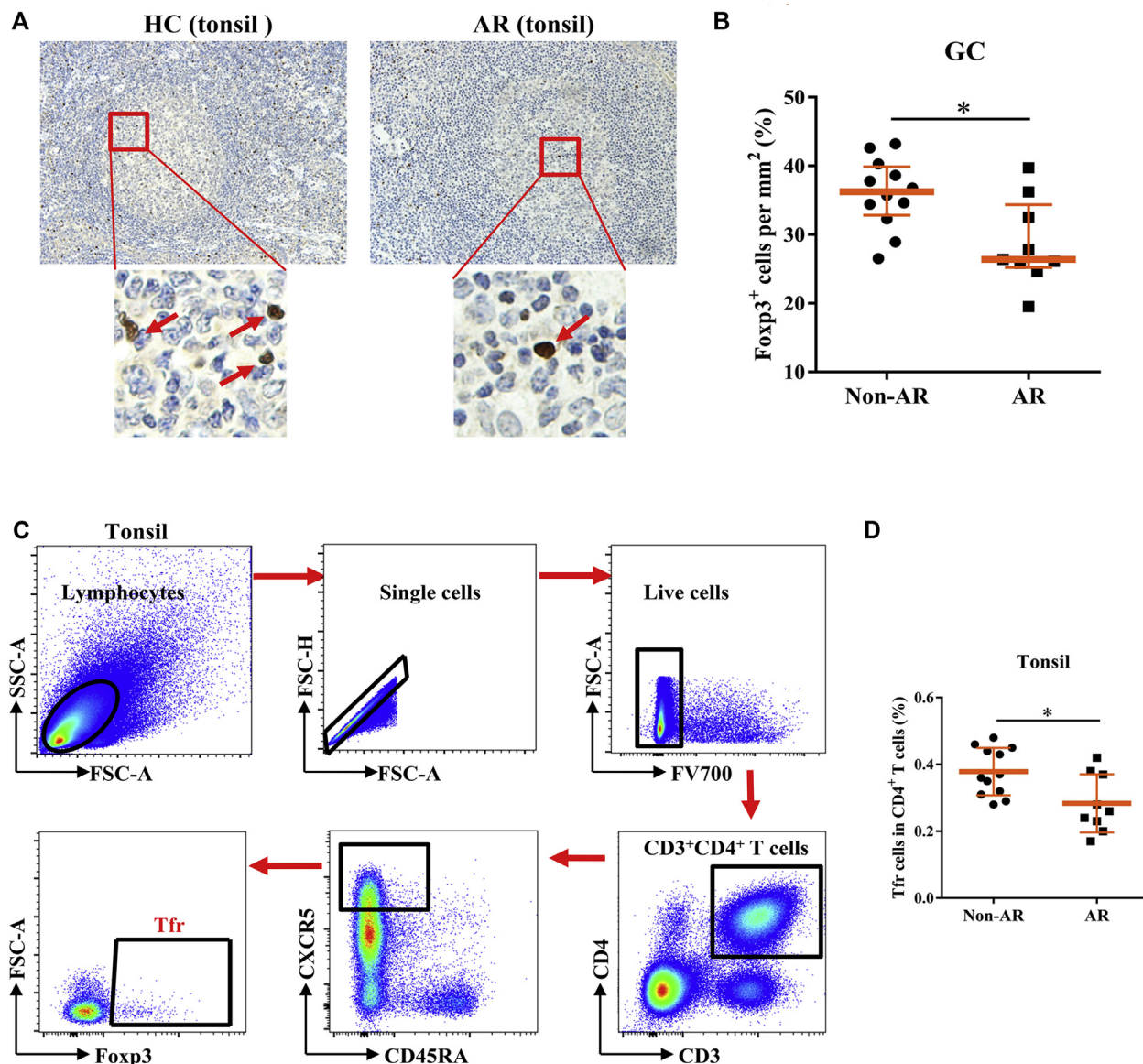


FIG 1. Reduction in numbers of T_{FR} cells in tonsils of patients with AR. **A**, Representative photomicrographs showing immunohistochemical staining of Foxp3 in tonsils (original magnification $\times 200$). Lower images show greater magnification of the outlined areas of the upper images. Arrows indicate Foxp3⁺ cells in GCs. **B**, Quantification of Foxp3⁺ cells in GCs of tonsils from patients with AR ($n = 9$) and subjects without AR ($n = 12$) by means of immunohistochemistry. **C**, Gating strategy used to define T_{FR} cells in tonsils. Within live CD3⁺CD4⁺ T cells, T_{FR} cells were defined as CD45RA^{low}CXCR5^{high}Foxp3⁺ cells. **D**, Quantification of T_{FR} cells in tonsils by using flow cytometry. * $P < .05$. Symbols represent individual samples, horizontal bars represent medians, and error bars show interquartile ranges.

cell proliferation, and found comparable levels of the Ki-67⁺ population in circulating T_{FR} and Treg cells (approximately 25%), which is significantly greater than those of T_{FH} cells in blood and tonsils (Fig 2, C, and see Fig E4). Circulating T_{FH} and T_{FR} cells expressed much greater levels of CCR7 and CD62L, the chemokine receptor and adhesion molecule required by lymphocytes to recirculate to secondary lymphoid organs. In terms of other chemokine receptors, circulating T_{FR} cells, more similar to circulating T_{FH} cells, expressed lower levels of CCR4, CCR5, and CCR6 than those on CXCR5[−] Treg cells (Fig 2, C, and see Fig E4). These indicate that the phenotype of circulating T_{FR} cells was similar to that of GC T_{FR} cells for expression of key Treg cell markers and showed common features of circulating T_{FH} cells for expression of migratory markers.

Reduced frequency of circulating T_{FR} cells in patients with AR

After showing that circulating T_{FR} cells correlated with GC T_{FR} cells in tonsils, we next examined T_{FR} cells in peripheral blood of patients with AR. There was a significant reduction in percentages of T_{FR} cells in CD4⁺ T cells in patients with AR compared with HCs, although frequencies of CD4⁺CD25^{high}CD127^{low} total Treg cells or CXCR5[−] Treg cells in CD4⁺ T cells between patients with AR and HCs were comparable (Fig 3, A). Correspondingly, reduced percentages of T_{FR} cells, but not CXCR5[−] Treg cells, in total Treg cells were observed in patients with AR (Fig 3, B). Absolute numbers also revealed a specific reduction in T_{FR} cells in patients with AR (see Fig E5 in this article's Online Repository at www.jacionline.org). The selective

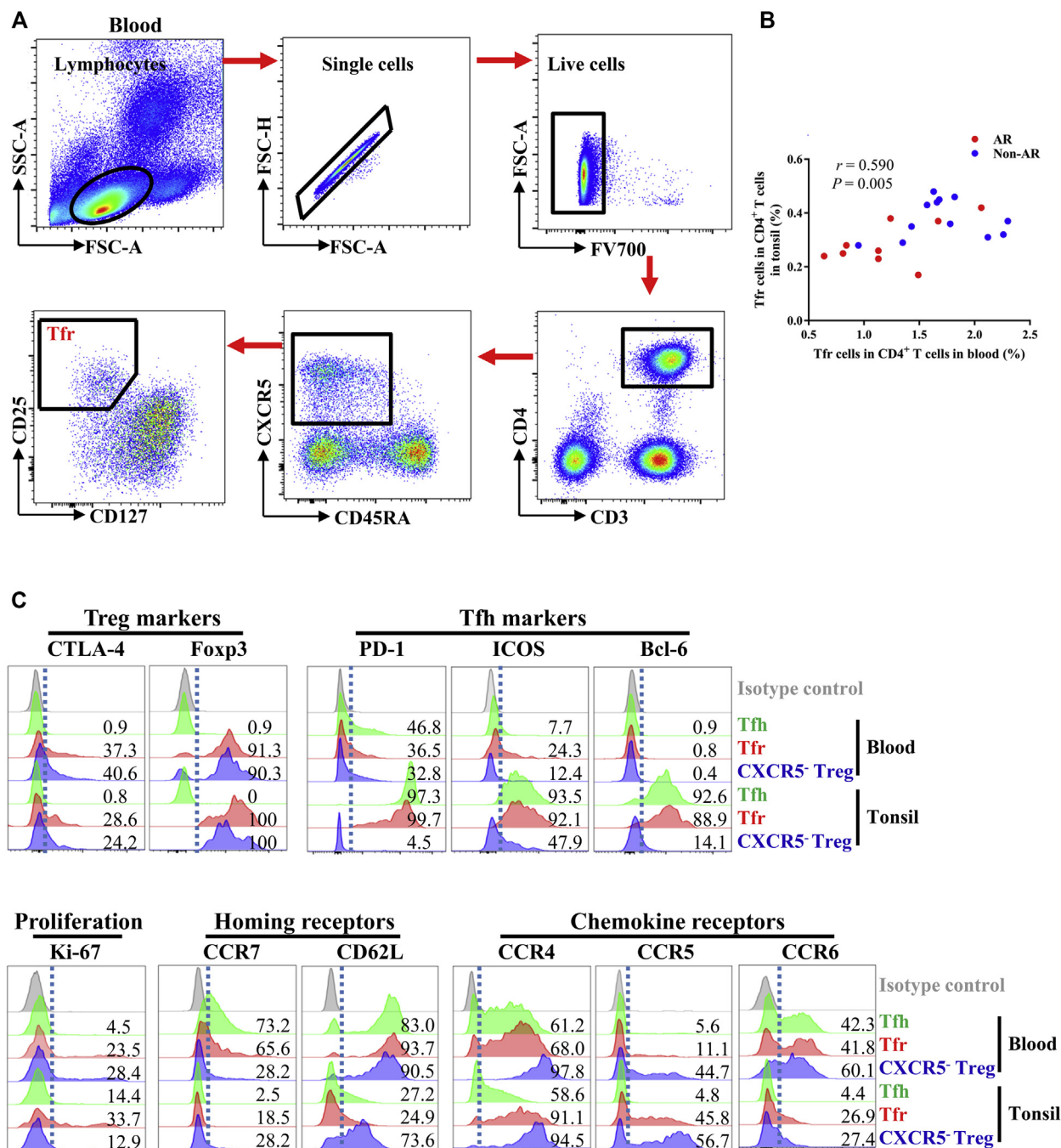


FIG 2. Numeric and phenotypic correlations between T_{FR} cells in peripheral blood and tonsils. **A**, Gating strategy of peripheral T_{FR} cells. Within live CD3⁺CD4⁺ T cells, T_{FR} cells were defined as CD45RA^{low}CXCR5⁺CD25^{high}CD127^{low} cells. **B**, Correlation of tonsillar and paired blood T_{FR} cells from patients with AR (n = 9) and subjects without AR (n = 12) detected by using flow cytometry. **C**, Phenotypic characteristics of T_{FR} cells in peripheral blood and tonsils from healthy subjects and subjects healthy other than tonsillar hypertrophy, respectively. Representative histograms are shown. Numbers in figures refer to percentages of positive cells. ICOS, Inducible costimulator; PD-1, programmed cell death protein 1.

reduction in numbers of T_{FR} cells in patients with AR was also validated by using a different gating method whereby T_{FR} cells were identified as CD4⁺CD45RA^{low}CXCR5⁺CD25^{high}CD127^{low} Foxp3⁺ T cells (see Fig E6 in this article's Online Repository at www.jacionline.org). T_{FR} cells suppress T_{FH} cell-mediated antibody production, whereas T_{FH} cells are the major T_{FH} cell subset that supports B cells for IgE production.¹² We previously reported

increased numbers of circulating T_{FH}2 cells in patients with AR.⁶ Here we observed a lower ratio of T_{FR}/T_{FH}2 cells in patients with AR compared with that in HCs (Fig 3, C), suggesting a loss of balance of negative versus positive regulation for IgE production. The ratio of total Treg/T_{FH}2 cells, total Treg/T_{FR}2 cells, or CXCR5⁺ Treg/T_{FH}2 cells was comparable between patients with AR and HCs (Fig 3, C).

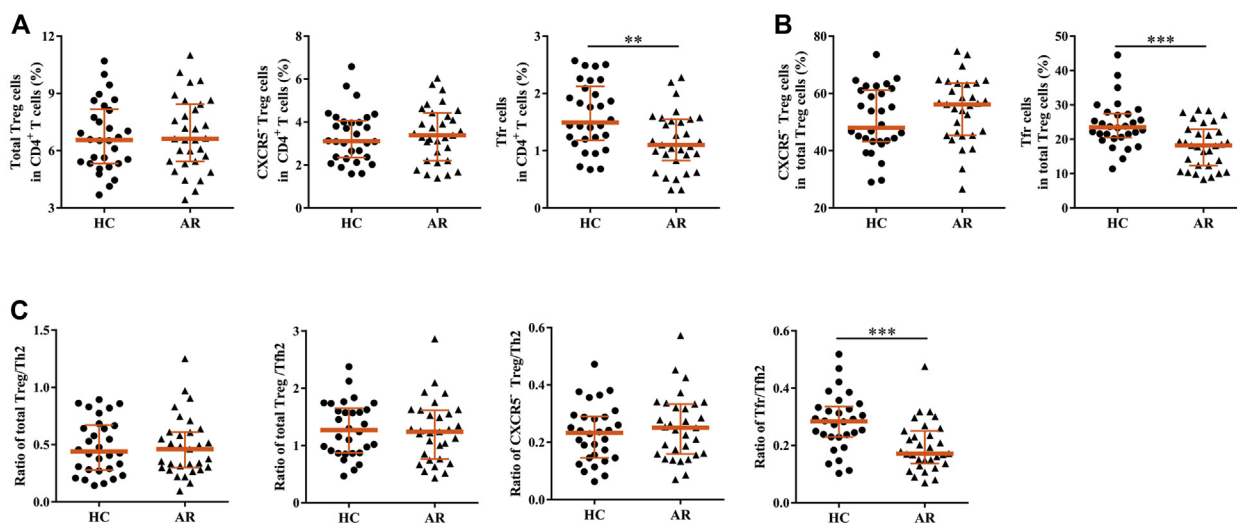


FIG 3. Reduced abundance of T_{FR} cells in peripheral blood of patients with AR. **A**, Frequencies of total Treg, CXCR5⁺ Treg, and T_{FR} cells within CD4⁺ T cells in patients with AR and HCs. **B**, Frequencies of CXCR5⁺ Treg and T_{FR} cells within total Treg cells. **C**, Ratio of total Treg/TH2, total Treg/TFH2, CXCR5⁺ Treg/TFH2, and T_{FR}/TFH2 cells (n = 31 for patients with AR and n = 30 for HCs). **P < .01 and ***P < .001. Symbols represent individual samples, horizontal bars represent medians, and error bars show interquartile ranges.

The decrease in numbers of circulating T_{FR} cells associates with disease severity in patients with AR

To further explore the potential contribution of dysregulated T_{FR} cells in the development of AR, we analyzed correlation between frequencies of T_{FR} cells and visual analog scale (VAS) symptom scores.¹ We found that VAS symptom scores in patients with AR negatively correlated with frequencies of T_{FR} cells in total Treg cells, and a more profound negative correlation was observed between VAS symptom scores and the T_{FR}/T_{FH2} cell ratio (see Fig E7, A-C, in this article's Online Repository at www.jacionline.org). Consistent with the previous report,²² we found that VAS symptom scores positively correlated with serum *D pteronyssinus*-specific IgE levels in patients with AR (see Fig E7, D). In line with this, frequencies of T_{FR} cells within total Treg cells or the T_{FR}/T_{FH2} cell ratio negatively correlated with *D pteronyssinus*-specific IgE levels in patients with AR (see Fig E8 in this article's Online Repository at www.jacionline.org). Notably, frequencies of total Treg cells or CXCR5⁺ Treg cells or ratios of these cells to TH2 or TFH2 cells did not show any significant correlation with VAS symptom scores (see Fig E7) or *D pteronyssinus*-specific IgE levels in patients with AR (see Fig E8).

Circulating T_{FR} cells from patients with AR are defective in suppressing IgE production

We next set out to examine the function of T_{FR} cells. Although T_{FR} cell function has been extensively studied in mice, the function of human T_{FR} cells is yet to be characterized. We adopted a T_{FR}-T_{FH}-B-cell coculture assay^{12,23} and found that T_{FR} cells were effective in suppressing T_{FH} cell-mediated IgM, IgG, IgA, and IgE production from total B cells (Fig 4, A). To our surprise, CXCR5⁺ Treg cells showed little suppressive function in this assay (Fig 4, A), again suggesting that T_{FR} cells are the specialized Treg cell type in inhibiting humoral immunity.

Naive B cells were used in the coculture assay to further determine the role of T_{FR} cells in regulating antibody isotype switching. We observed that circulating T_{FR} cells, but not CXCR5⁺ Treg cells, were efficient to suppress production of both unswitched IgM and IgG, IgA, and IgE, all types of isotype class-switched antibodies (Fig 4, B).

We found that addition of T_{FR} cells, but not CXCR5⁺ Treg cells, in T_{FH}-B-cell coculture significantly reduced levels of the 2 T_{FH} cell effector molecules, CXCL13 and IL-21, in cell-culture supernatants, downregulated the expression of activation-induced cytidine deaminase expression, and led to suppressed B-cell proliferation and/or survival (see Fig E9 in this article's Online Repository at www.jacionline.org). We also discovered that cell-cell interaction was required for T_{FR} cell-mediated suppression. When cultured in one chamber and separated from T_{FH} and B cells in the other chamber in the transwell culture system, T_{FR} cells almost completely lost their suppressive effect on T_{FH} cell-promoted antibody production from naive B cells (Fig 4, C).

After fully establishing an assay to evaluate the suppressive function of circulating T_{FR} cells and generating the finding that T_{FR} cells, but not CXCR5⁺ Treg cells, are a specialized cell type in suppressing T_{FH}-B-cell response, we then compared the function of T_{FR} cells from HCs and patients with AR in this assay. Circulating T_{FR} cells from patients with AR were as effective as counterpart T_{FR} cells from HCs in suppressing IgM, IgG, and IgA production. In contrast, T_{FR} cells from patients with AR were unable to suppress T_{FH} cell-promoted IgE production, whereas T_{FR} cells from HCs could do so (Fig 4, D), suggesting that AR was associated with not only the decrease in T_{FR} numbers but also a specific defect in the ability of T_{FR} cells to suppress IgE production. Moreover, we found that the T_{FR} cell-suppressive capacity negatively associated with *D pteronyssinus*-specific IgE levels in patients with AR (see Fig E10 in this article's Online Repository at www.jacionline.org).

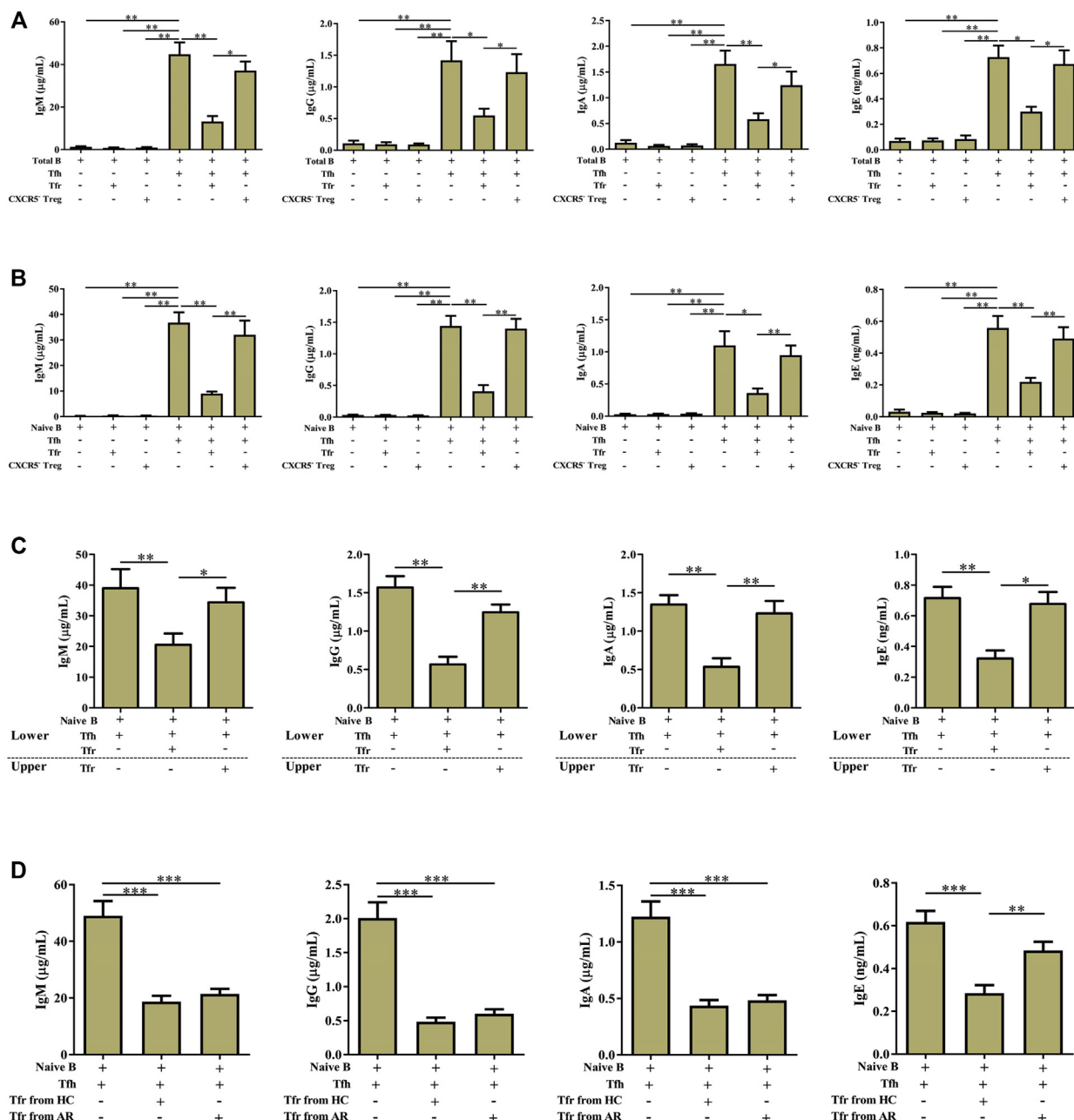


FIG 4. Peripheral T_{FR} cells in patients with AR have impaired function. **A**, T_{FR} cells and total B cells from healthy subjects were cocultured with or without autologous T_{FR} cells or CXCR5⁺ Treg cells. Immunoglobulin levels in culture supernatants were measured by means of ELISA at day 12 (n = 6). **B**, T_{FR} and naive B cells from healthy subjects were cocultured with or without autologous T_{FR} or CXCR5⁺ Treg cells, and immunoglobulin levels were detected at day 12 (n = 6). **C**, T_{FR} and naive B cells from healthy subjects were cultured in the lower chamber, and autologous T_{FR} cells were added in either the upper or lower chamber of transwell culture system. Immunoglobulin levels were measured at day 12 (n = 5). **D**, T_{FR} and naive B cells from healthy subjects were cocultured with allogeneic T_{FR} cells from HCs or patients with AR. Immunoglobulin levels were measured at day 12 (n = 12). *P < .05, **P < .01, and ***P < .001. Data are expressed as mean ± SEM.

Numbers of circulating T_{FR} cells negatively correlate with plasmablasts in patients with AR

Reduced numbers of T_{FR} cells in patients with AR indicated a potential defect in controlling antibody responses. We detected higher frequencies and increased numbers of

CD19⁺CD20⁺CD27^{high}CD38⁺ circulating plasmablasts in patients with AR compared with HCs (Fig 5, A and B). Percentages of circulating plasmablasts in CD19⁺ B cells positively correlated with *D pteronyssinus*-specific IgE levels in patients with AR ($r = 0.438$, $P = .014$).

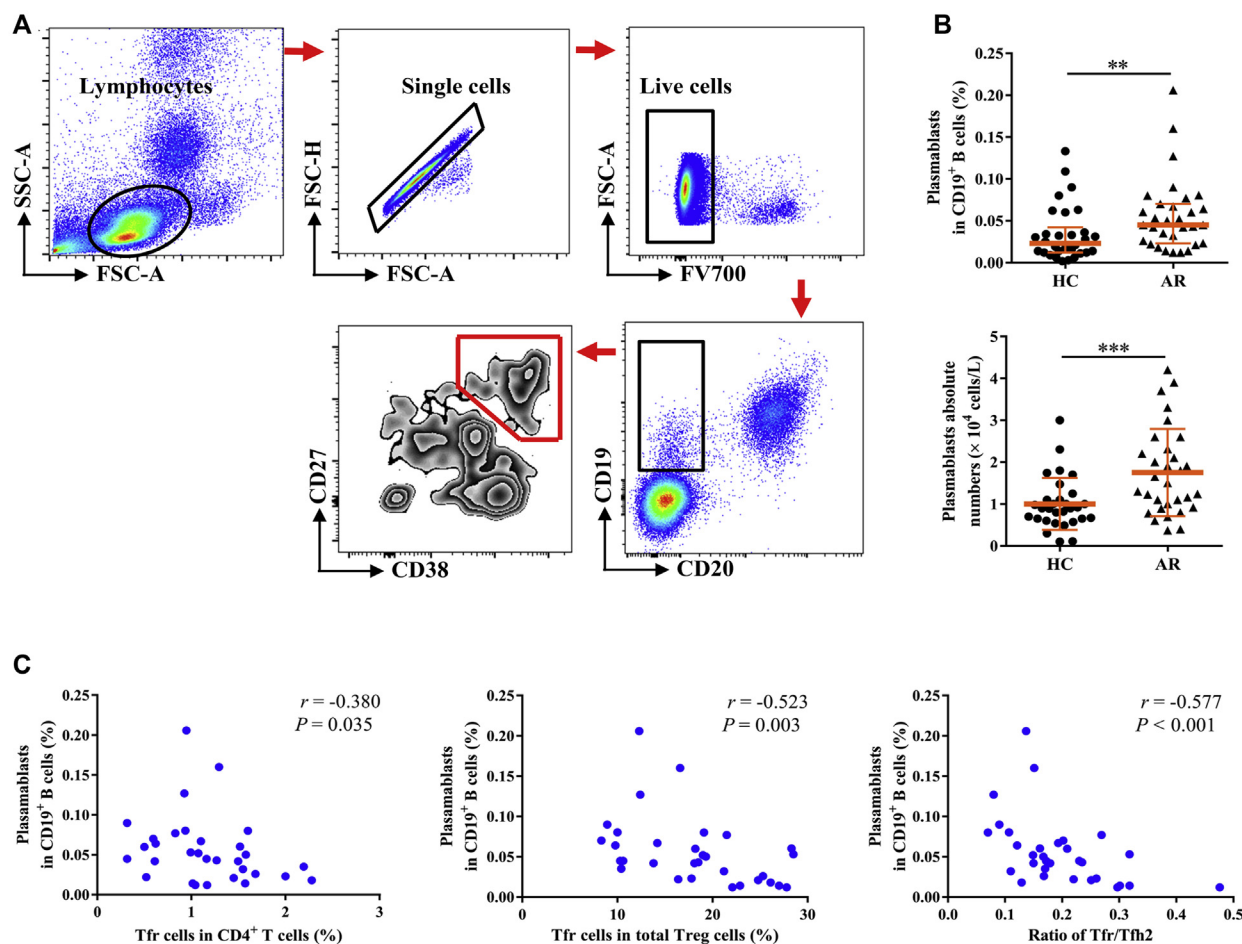


FIG 5. T_{FR} cells correlate with plasmablasts in peripheral blood in patients with AR. **A**, Gating strategy. Plasmablasts were defined as CD19⁺CD20⁺CD27⁺CD38^{high} cells. FSC, Forward scatter; SSC, side scatter. **B**, Frequencies of plasmablasts within CD19⁺ B cells and absolute numbers of plasmablasts in peripheral blood of patients with AR (n = 31) and HCs (n = 30). **C**, Correlations of frequencies of plasmablasts with frequencies of T_{FR} cells in CD4⁺ T and total Treg cells and ratio of T_{FR}/T_{FH2} cells in patients with AR (n = 31). **P < .01 and ***P < .001. Symbols represent individual samples, horizontal bars represent medians, and error bars show interquartile ranges.

We then analyzed the correlation between plasmablasts and T_{FR} cells and found a negative correlation between the frequencies of plasmablasts within CD19⁺ B cells and the frequencies of T_{FR} cells in CD4⁺ T cells or those in total Treg cells (Fig 5, C). An even stronger negative correlation was observed between the frequencies of plasmablasts within CD19⁺ B cells and the ratio of T_{FR}/T_{FH2} cells in patients with AR (Fig 5, C). In contrast, there were no such correlations between the frequencies of plasmablasts within CD19⁺ B cells and either total or CXCR5⁺ Treg cells or the ratio of these cells to T_{H2} or T_{FH2} cells (data not shown).

T_{FR} cells correlate with AIT efficacy in patients with AR

Induction of Treg cells has been proposed as one of the mechanisms underlying the benefit of AIT, but the results were not always consistent.²⁴⁻²⁷ It is of great interest to know how AIT might affect T_{FR} cells. Thus we conducted a prospective study to investigate this question. Consistent with previous studies,^{28,29} we found that CSMS and *D pteronyssinus*-specific IgE levels

decreased significantly, whereas *D pteronyssinus*-specific IgG₄ increased robustly in patients with AIT compared with those without AIT after 12 months of treatment (see Fig E11, A and B, in this article's Online Repository at www.jacionline.org). Frequencies of T_{FR} cells in CD4⁺ T cells or total Treg cells and ratios of T_{FR}/T_{FH2} cells were significantly increased in patients with AR with AIT after 12 months of treatment compared with those without AIT (Fig 6, A). Notably, T_{FR} cells isolated from patients with AR after 12 months of AIT demonstrated a stronger function to inhibit IgE production in the coculture assay than those before treatment (Fig 6, B). There were no significant changes in values of total Treg cells or CXCR5⁺ Treg cells or the ratio of these cells to T_{H2} or T_{FH2} cells accompanying AIT (see Fig E11, C-E).

We also investigated the relationships between changes in T_{FR} cell abundance and the clinical benefits of AIT. We found that the increase of T_{FR} cell percentages in total Treg cells and the ratio of T_{FR}/T_{FH2} cells were significantly correlated with the CSMS improvement in patients with AR with AIT treatment, whereas no correlation was observed in patients without AIT treatment (Fig 6, C). Again, there were no significant correlations between changes in either values total Treg cells or CXCR5⁺ Treg cells or

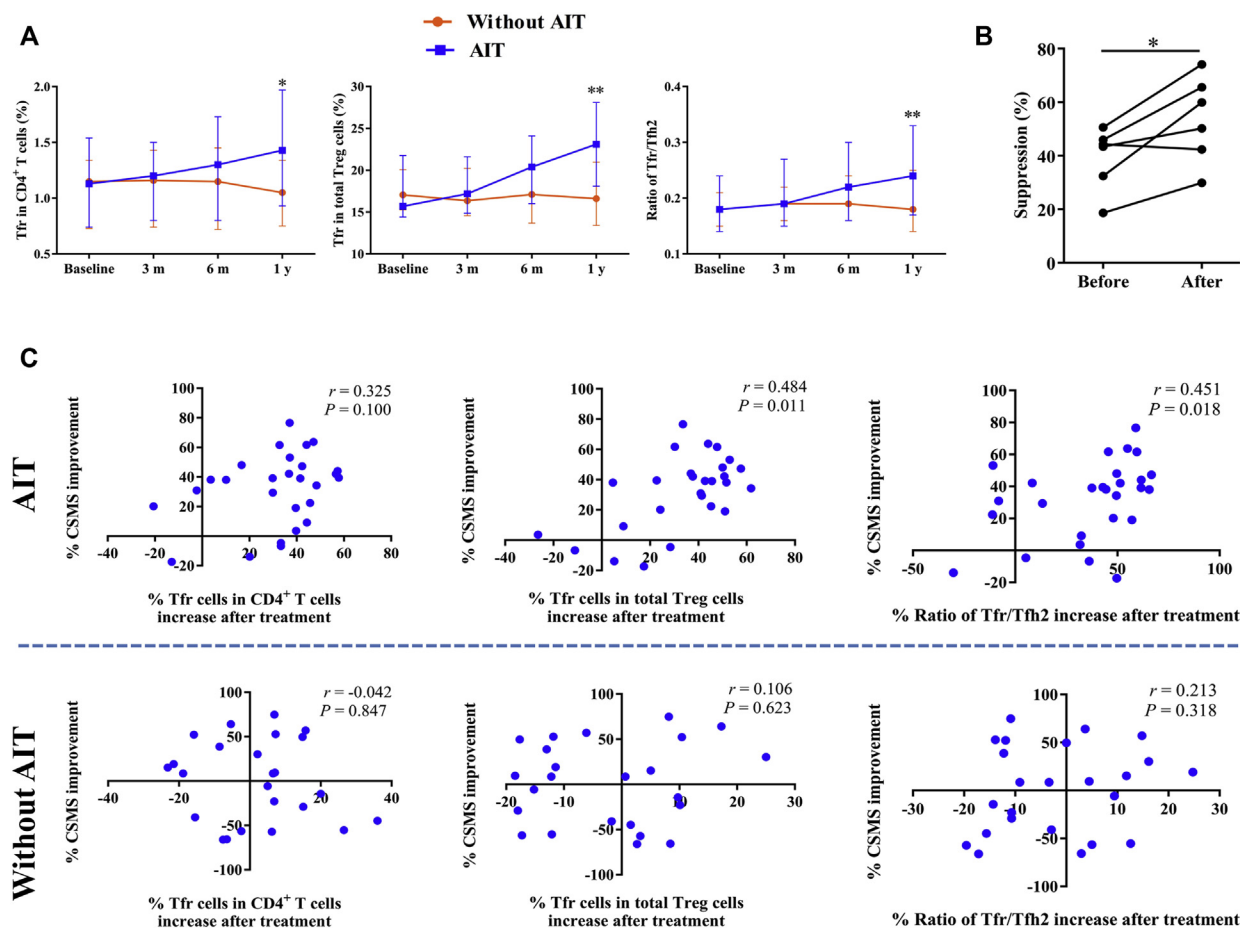


FIG 6. Numeric and functional changes of circulating T_{FR} cells accompanying AIT. **A**, Changes in frequencies of T_{FR} cells within CD4⁺ T cells and total Treg cells and ratios of T_{FR}/T_{FH2} cells in patients with AR with (n = 27) and without (n = 24) AIT after treatment. **B**, T_{FR} cells collected at baseline and after 12 months of treatment from patients with AR were cocultured with allogeneic T_{FH} cells and naive B cells from healthy donors. IgE levels were determined at day 12 (n = 6). The suppressive capacity of T_{FR} cells before and after AIT was normalized to the condition of T_{FH} and B cells only. Percentage suppression = ([IgE production by T_{FH} + B cells] – [IgE production by T_{FH} cells + B cells + T_{FR} cells]) / ([IgE production by T_{FH} cells + B cells] × 100). The paired t test was used for comparison of T_{FR} cells before and after AIT. **C**, Correlations of increased frequencies of T_{FR} cells in CD4⁺ T cells and total Treg cells and ratio of T_{FR}/T_{FH2} cells with CSMS improvement in patients with (n = 27) and without (n = 24) AIT after 12 months of treatment. *P < .05 and **P < .01. Symbols represent individual samples, horizontal bars represent medians, and error bars show interquartile ranges.

the ratio of these cells to T_{H2} or T_{FH2} cells and the improvement in CSMS (see Fig E12 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Efforts are being made to understand the regulation or dysregulation of IgE production in patients with AR. Although our studies have revealed the excessive function of T_{FH2} cells in promoting IgE production in patients with AR,⁶ it remains unclear what role is played by T_{FR} cells, a major force to counteract T_{FH} cell-promoting immunoglobulin production.

In this study we provided several lines of novel evidence for the role of T_{FR} cells in inhibiting IgE production and impairment of such a mechanism in patients with AR. Despite the obvious challenge to analyze *bona fide* T_{FR} cells in GCs of secondary lymphoid organs, for the first time, we demonstrated a reduction

in numbers of GC T_{FR} cells in tonsils from patients with AR compared with those in tonsils from subjects without AR. After establishing the phenotypic and numeric correlation between GC T_{FR} cells and circulating T_{FR} cells, we were able to confirm the AR-associated reduction of T_{FR} cells in a larger patient cohort. Notably, the AR-associated reduction was only observed for T_{FR} cells and not for total Treg cells or CXCR5⁺ Treg cells. We further discovered a second hit of the defective T_{FR} cell function in patients with AR. T_{FR} cells, but not CXCR5⁺ Treg cells, were able to suppress immunoglobulin production in T_{FH}-B-cell coculture. Using this assay, we found circulating T_{FR} cells were incapable of inhibiting IgE production while retaining capability to inhibit other isotypes, including IgM, IgG, and IgA. The relevance of the defect in T_{FR} cells and the development of AR is indicated by positive correlations between the reduction and function deficiency of T_{FR} cells and the disease severity and antigen-specific IgE levels in patients with AR.

A recent study suggested that AIT could modulate the balance of T_{FH} and T_{FR} cells in patients with grass allergy.¹⁶ In this study we revealed that after 12 months of AIT, not only the number but also the suppressive function of T_{FR} cells were significantly improved. Among different methods to quantify the frequencies of T_{FR} cells, it was the ratio of T_{FR}/T_{FH2} cells that demonstrated the strongest indicating power, negatively correlating with the disease severity of AR and positively correlating with the efficacy of AIT treatment. Taken together, our data support a notion that the function of T_{FR} cells is defective but can be improved by AIT in patients with AR. The tilted balance of impaired T_{FR} cell function and skewed T_{FH2} cell polarization significantly contributes excessive IgE production and AR development.

The investigation of T_{FH} and T_{FR} cells in human diseases are limited by the fact that circulating T_{FH} and T_{FR} cells are not *bona fide* populations that execute effector functions in GCs. Therefore it is necessary to understand the relationship between circulating T_{FH} or T_{FR} cells and their counterpart populations in secondary lymphoid organs. We and others have shown the circulating T_{FH} cells reflect the activation and polarization of *bona fide* T_{FH} cells and can be used as a surrogate T_{FH} cell marker.^{11,12} Here we showed that the phenotype and frequency of circulating T_{FR} cells correlated with GC T_{FR} cells.

We also demonstrated the function of T_{FR} cells in inhibiting antibody production of T_{FH} -B-cell coculture. Our findings are in contrast to those of the report by Fonseca et al²³ that peripheral T_{FR} cells were not fully licensed with suppressive function with a naive-like phenotype. The reasons contributing to this discrepancy are not fully understood but could lie in the definition of T_{FR} cells and the conditions used for the T_{FH} -B-cell coculture assay. Nevertheless, our findings are consistent with those of several studies in both human subjects and mice showing that circulating T_{FR} cells more likely had a $CD45RA^{low}$ memory phenotype (data not shown).^{13,15,21,30}

Our study demonstrated different functions for T_{FR} and $CXCR5^{-}$ Treg cells. This is supported by their distinct expression profiles of homing and chemokine receptors, indicating an effector function in GCs and peripheral tissues for T_{FR} and $CXCR5^{-}$ Treg cells, respectively.

Another very intriguing observation from the T_{FR} - T_{FH} -B-cell coculture assay in this study was the specific defect in T_{FR} cells from patients with AR in inhibiting IgE production. There are at least 2 possible explanations. First, there were distinctive mechanisms underlying T_{FR} cell-mediated inhibition for different isotypes of immunoglobulins, whereas the inhibitory mechanism for IgE production was selectively weakened in patients with AR. How T_{FR} cells suppress the T_{FH} -B-cell response is not fully understood. CTLA-4 has been shown to be required for T_{FR} cell-mediated suppression in mouse studies,³¹ but there is no report on isotype-specific mechanisms. In addition to CTLA-4, IL-10 and TGF- β are also implicated in the suppressive activity of Treg cells.³² However, in cell culture TGF- β induces IgA isotype class switching.³³ A second possibility is that although T_{FR} cells in patients with AR retain the general function in suppressing antibody production, they might gradually acquire the activity to enhance IgE isotype class switching. It has been well documented that Treg cells in patients with autoimmune and inflammatory diseases become instable and acquire effector functions. For example, Treg cells also express effector cytokines, such as IFN- γ and IL-17, under inflammatory conditions.³⁴ Future study is required to characterize T_{FR} and $CXCR5^{-}$ Treg cells in patients

with allergic diseases and other pathologic conditions, including infection and autoimmune diseases, by using nonbiased and high-throughput techniques, such as RNA-sequencing for transcriptomic analysis.

A limitation of the present study is the age difference between the donors of blood and tonsillar samples in the phenotyping study of T cells (Fig 2, C). Age might influence the development, maturation, and senescence of the human immune system. Although the function of T_{FH} cells is impaired at the neonatal stage,³⁵ several studies indicate that the immune system is well developed around the age of 12 years.³⁶⁻³⁸ For example, immunoglobulins attain adult levels at that age.^{37,38} In our study the adolescent subjects with tonsillar hypertrophy were older than 12 years. However, even with this fact, we cannot rule out the potential influence of age on our results comparing the phenotypes of T cells in blood and tonsils, and our findings must be considered with caution and would be validated better in paired blood and tonsillar samples from the same subjects.

There is a clear defect in T_{FR} cell function in patients with AR, but the defect seems to be reversible. Did AIT improve T_{FR} cells directly or indirectly? A recent study demonstrated that there was a slower kinetics of T_{FR} cell generation than that of T_{FH} cells.³⁹ From an immunologic prospective, accumulation of T_{FR} cells after the peak of T_{FH} cell differentiation allows the sequential turn-on of T_{FH} cell-supporting antibody production and turn-off of the program by increasing the T_{FR}/T_{FH} cell ratio. Thus it is conceivable that prolonged antigen stimulation of AIT favors T_{FR} cell generation. Although we demonstrated that improvement in T_{FR} cell frequencies positively associated with disease remission after 12 months of treatment, variations in T_{FR} cell response to AIT among different patients with AR were noted in our study. Our results indicate that the different response of T_{FR} cells might be one mechanism underlying different clinical response to AIT among patients with AR, even with same awareness. However, the mechanisms underlying the change in T_{FR} cells and its contribution to rebalance of the immune system during AIT demand further studies. In addition, the value of using T_{FR} cells as a biomarker for the efficacy of AIT in patients with AR should be formally evaluated in randomized, double-blind, placebo-controlled trials with a large sample size.

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Clinical implications: A better understanding of T_{FR} cells will benefit the optimization of current therapies and the development of novel treatments for allergic disease. Circulating T_{FR} cells represent a potential biomarker to monitor the clinical response to AIT.

REFERENCES

1. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy* 2008; 63(suppl 86):8-160.
2. Wheatley LM, Togias A. Clinical practice. Allergic rhinitis. *N Engl J Med* 2015; 372:456-63.
3. Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T cells. *Annu Rev Immunol* 2016;34:335-68.

4. Ballesteros-Tato A, Randall TD, Lund FE, Spolski R, Leonard WJ, Leon B. T follicular helper cell plasticity shapes pathogenic T helper 2 cell-mediated immunity to inhaled house dust mite. *Immunity* 2016;44:259-73.
5. Kobayashi T, Iijima K, Dent AL, Kita H. Follicular helper T cells mediate IgE antibody response to airborne allergens. *J Allergy Clin Immunol* 2016;139:300-13.
6. Yao Y, Chen CL, Wang N, Wang ZC, Ma J, Zhu RF, et al. Correlation of allergen-specific T follicular helper cells with specific IgE and efficacy of allergen immunotherapy. *J Allergy Clin Immunol* 2018;142:321-4.
7. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3(+) follicular regulatory T cells control the germinal center response. *Nat Med* 2011;17:975-82.
8. Chung Y, Tanaka S, Chu FL, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* 2011;17:983-8.
9. Sage PT, Sharpe AH. T follicular regulatory cells. *Immunol Rev* 2016;271:246-59.
10. Sage PT, Sharpe AH. T follicular regulatory cells in the regulation of B cell responses. *Trends Immunol* 2015;36:410-8.
11. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7(lo)PD-1(hi)CXCR5(+)CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* 2013;39:770-81.
12. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* 2011;34:108-21.
13. Dhazeze T, Peelen E, Hombrouck A, Peeters L, Van Wijmeersch B, Lemkens N, et al. Circulating follicular regulatory T cells are defective in multiple sclerosis. *J Immunol* 2015;195:832-40.
14. Xu BH, Wang S, Zhou MJ, Huang YF, Fu R, Guo CH, et al. The ratio of circulating follicular T helper cell to follicular T regulatory cell is correlated with disease activity in systemic lupus erythematosus. *Clin Immunol* 2017;183:46-53.
15. Chen W, Bai J, Huang H, Bi L, Kong X, Gao Y, et al. Low proportion of follicular regulatory T cell in renal transplant patients with chronic antibody-mediated rejection. *Sci Rep* 2017;7:1322.
16. Schulten V, Tripple V, Seumois G, Qian Y, Scheuermann RH, Fu Z, et al. Allergen-specific immunotherapy modulates the balance of circulating Tfh and Tfr cells. *J Allergy Clin Immunol* 2018;141:775-7.
17. Fonseca VR, Romao VC, Agua-Doce A, Santos M, Lopez-Presa D, Ferreira AC, et al. The ratio of blood T follicular regulatory cells to T follicular helper cells marks ectopic lymphoid structure formation while activated follicular helper T cells indicate disease activity in primary Sjogren's syndrome. *Arthritis Rheumatol* 2018;70:774-84.
18. Zhang YN, Song J, Wang H, Wang H, Zeng M, Zhai GT, et al. Nasal IL-4(+) CXCR5(+)CD4(+) T follicular helper cell counts correlate with local IgE production in eosinophilic nasal polyps. *J Allergy Clin Immunol* 2016;137:462-73.
19. He J, Zhang X, Wei YB, Sun XL, Chen YP, Deng J, et al. Low-dose interleukin-2 treatment selectively modulates CD4(+) T cell subsets in patients with systemic lupus erythematosus. *Nat Med* 2016;22:991-3.
20. Song J, Wang H, Zhang YN, Cao PP, Liao B, Wang ZZ, et al. Ectopic lymphoid tissues support local immunoglobulin production in patients with chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2017;141:927-37.
21. Wei Y, Feng J, Hou Z, Wang XM, Yu D. Flow cytometric analysis of circulating follicular helper T (Tfh) and follicular regulatory T (Tfr) populations in human blood. *Methods Mol Biol* 2015;1291:199-207.
22. Ciprandi G, Comite P, Ferrero F, Fontana V, Bruzzzone M, Mussap M. Serum allergen-specific IgE, allergic rhinitis severity, and age. *Rhinology* 2016;54:231-8.
23. Fonseca VR, Agua-Doce A, Maceiras AR, Pierson W, Ribeiro F, Romao VC, et al. Human blood Tfr cells are indicators of ongoing humoral activity not fully licensed with suppressive function. *Sci Immunol* 2017;2.
24. Ciebiada M, Kasztalska K, Gorska-Ciebiada M, Gorski P. ZAP70 expression in regulatory T cells in allergic rhinitis: effect of immunotherapy. *Clin Exp Allergy* 2013;43:752-61.
25. Mobs C, Slotosch C, Löffler H, Jakob T, Hertl M, Pfützner W. Birch pollen immunotherapy leads to differential induction of regulatory T cells and delayed helper T cell immune deviation. *J Immunol* 2010;184:2194-203.
26. Gomez E, Fernandez TD, Dona I, Rondon C, Campo P, Gomez F, et al. Initial immunological changes as predictors for house dust mite immunotherapy response. *Clin Exp Allergy* 2015;45:1542-53.
27. Pereira-Santos MC, Baptista AP, Melo A, Alves RR, Soares RS, Pedro E, et al. Expansion of circulating Foxp3(+)CD25(bright) CD4+ T cells during specific venom immunotherapy. *Clin Exp Allergy* 2008;38:291-7.
28. Shamji MH, Durham SR. Mechanisms of allergen immunotherapy for inhaled allergens and predictive biomarkers. *J Allergy Clin Immunol* 2017;140:1485-98.
29. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol* 2014;133:621-31.
30. Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest* 2014;124:5191-204.
31. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* 2014;41:1026-39.
32. Noval Rivas M, Chatila TA. Regulatory T cells in allergic diseases. *J Allergy Clin Immunol* 2016;138:639-52.
33. Stavnezer J, Kang J. The Surprising discovery that TGF beta specifically induces the IgA class switch. *J Immunol* 2009;182:5-7.
34. Pandiyan P, Zhu J. Origin and functions of pro-inflammatory cytokine producing Foxp3+ regulatory T cells. *Cytokine* 2015;76:13-24.
35. Debock I, Jaworski K, Chadlaoui H, Delbaume S, Passon N, Twyffels L, et al. Neonatal follicular Th cell responses are impaired and modulated by IL-4. *J Immunol* 2013;191:1231-9.
36. Simon AK, Hollander GA, McMichael A. Evolution of the immune system in humans from infancy to old age. *Proc Biol Sci* 2015;282:20143085.
37. Allansmith M, McClellan BH, Butterworth M, Maloney JR. The development of immunoglobulin levels in man. *J Pediatr* 1968;72:276-90.
38. Butterworth M, McClellan B, Allansmith M. Influence of sex in immunoglobulin levels. *Nature* 1967;214:1224-5.
39. Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat Immunol* 2017;18:1249-60.