Rhinitis, sinusitis, and ocular allergy

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

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GRAPHICAL ABSTRACT

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

T: CD4+ naïve T cells; Th2: Type 2 follicular helper T cells; Tr: Defective follicular regulatory T cells; B: B cells; PB: Plasmablasts; IgE: Immunoglobulin E; Tr: Follicular regulatory T cells.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication April 29, 2018; revised December 25, 2018; accepted for publication February 8, 2019.
Available online February 21, 2019.
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Supported by National Natural Science Foundation of China (NSFC) grants 81570899, 81630024, and 81325006 (to Z.L.), 81470677 (to X.H.Z.) and 81429003 (to D.Y.); the Natural Science Foundation of Hubei Province of China grant 2017CFA016 (to Z.L.); the “Ten thousand plan”–National High Level Talents Special Support Plan (to Z.L.); Australian National Health and Medical Research Council (NHMRC) fellowship GNT1085509 and the Bellberry-Viertel Senior Medical Research fellowship (to D.Y.); and a China Scholarship Council scholarship 201706160045 (to Y.Y.). Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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Background: The function of follicular regulatory T (TFR) 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 TFR cells in patients with allergic rhinitis (AR).

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

Results: TFR 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 TFR cells in blood were bloodotopically and numerically correlated with tonsillar TFR cells, and a reduction of circulating TFR cells but not total or CXCR5 regulatory T cells was noted in patients with AR compared with healthy control subjects. Moreover, circulating TFR 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 TFR cell frequencies and function with antigen-specific IgE levels or disease severity in patients with AR. After AIT, the frequencies and function of circulating TFR cells were improved, which positively associated with disease remission.

Conclusion: Impairment in TFR cells might contribute to aberrant IgE production in patients with AR, and AIT improves defective TFR cell function. TFR cells might serve as a potential biomarker to monitor clinical response to AIT.

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. Despite numerous studies depicting a strong association between IgE levels and AR, the mechanisms underlying the aberrant IgE production in patients with AR remain elusive. It is now clear that follicular helper T (TFH) 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. Several studies using mouse models demonstrated that TFH cells orchestrated systemic IgE production in patients with allergic asthma. Recently, we have found that increased numbers of peripheral type 2 TFH (TH2) cells positively correlated with upregulated IgE in patients with AR, suggesting a skewed polarization of TH2 cells in promoting IgE production in patients with AR.

Recently, follicular regulatory T (TFR) cells have been identified as a novel effector subset of regulatory T (Treg) cells with a specialized function in limiting TFH cell-mediated B-cell activation and antibody production. TFR cells possess overlapping phenotypic characteristics with both TFH and Treg cells. Similar to TFH cells, TFR cells migrate into B-cell follicles by up-regulating CXCR5 and express Bcl-6. On the other hand, resembling Treg cells, TFR cells express the transcription factor 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). In patients with allergic diseases, the question remains unanswered whether TFR cells regulate TFH 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 TFR cell–like or TFR cell–like populations circulating in human blood. We and others have carefully characterized the phenotype and function of circulating memory TFR cells in human subjects. However, despite a growing body of recent studies that analyzed circulating TFR cells in autoimmune diseases, organ transplantation, and allergic diseases, the phenotypic, functional, and numeric relationship between TFR cells in secondary lymphoid organs and the counterpart circulating TFR 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 TFR cells in blood positively correlated with the frequencies of TFR cells in tonsillar GCs in human subjects. We characterized the phenotypic similarity and difference between circulating TFR cells and GC TFR cells and demonstrated that circulating TFR cells possessed the capacity to suppress TFH cell–mediated antibody production from B cells, distinguishing them from the CXCR5 Treg cells. We found that circulating TFR cells were not only reduced in abundance but also impaired in function in patients with AR. In addition, such TFR cell defects correlated with disease severity and antigen-specific IgE production in patients with AR. Importantly, patients showed increased numbers of TFR cells with improved suppressive function after allergen immunotherapy (AIT).

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
- TH: Follicular helper T
- TFR: Follicular regulatory T
- Treg: Regulatory T
- VAS: Visual analog scale

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.

For the correlation study of circulating and GC TFR 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 TFR 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,12 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.13 The daily combined symptom and medication score (CSMS) was calculated, as previously reported.14 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.12 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.15 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). 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 TFH cells were cultured with autologous B cells with or without T FR cells, as previously described.15 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 Fookes: Laboratorium 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 ± SEMs and analyzed by using the unpaired Student t test, unless specifically stated. For categorical data, χ² 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 TFR cells in tonsils of patients with AR
To examine the function of TF R cells in patients with allergic diseases, we decided to first analyze the TFR 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 TFR cells (Fig 1, A). Importantly, numbers of Foxp3+ cells in GCs (bona fide TFR cells) in patients with AR were dramatically reduced in comparison with values in those without AR (Fig 1, B). The lower frequency of TFR cells in tonsils of patients with AR was further corroborated by using flow cytometric analysis, whereby TFR cells were defined as CD3+CD4+CD45RAlow CXCR5highFoxp3+ cells (Fig 1, C and D).

Circulating TFR cells correspond to TFR cells in secondary lymphoid organs
To investigate the function of TFR cells beyond the small fraction of patients with AR with available tonsillar samples, we first determined whether the altered GC TFR cells in tonsils could be reflected by a change in circulating TFR cells, as defined by CD3+CD4+CD45RAlowCXCR5+CDA25hiCD127lo cells (Fig 2, A).21 In supporting this gating method, we measured Foxp3 expression and validated Foxp3 expression in about 90% of circulating TFR 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 TFR cells within CD4+ T cells in tonsils and those in paired blood samples (Fig 2, B).

To comprehensively investigate the phenotype of TFR cells, we compared TFR, TFH, 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 CD45RAlow memory T cells, we studied and compared CD45RAlow memory T cells in gated CXCR5+ cells, as previously described (Fig E3).21

We first compared phenotypes between human TFR and Treg cells. Both GC TFR and circulating TFR cells, but not TFH 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 TFR and TFH cells in GCs in tonsils had high expression of programmed cell death protein 1, inducible costimulator, and Bcl-6, neither TFH cells nor TFR 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
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+CD25highCXCR5low 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

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**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 × 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 CD45RAlowCXCR5highFoxp3+ 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.
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_{FH2} cells are the major T_{FH} cell subset that supports B cells for IgE production. We previously reported increased numbers of circulating T_{FH2} cells in patients with AR.\textsuperscript{6} Here we observed a lower ratio of T_{FR}/T_{FH2} 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/TH2 cells, total Treg/T_{FH2} cells, or CXCR5^{+} Treg/T_{FH2} cells was comparable between patients with AR and HCs (Fig 3, C).
The decrease in numbers of circulating TFR cells associates with disease severity in patients with AR

To further explore the potential contribution of dysregulated TFR cells in the development of AR, we analyzed correlation between frequencies of TFR cells and visual analog scale (VAS) symptom scores. We found that VAS symptom scores in patients with AR negatively correlated with frequencies of TFR cells in total Treg cells, and a more profound negative correlation was observed between VAS symptom scores and the TFR/TFH2 cell ratio (see Fig E7, A-C, in this article’s Online Repository at www.jacionline.org). Consistent with the previous report, 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 TFR cells within total Treg cells or the TFR/TFH2 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 TFR cells from patients with AR are defective in suppressing IgE production

We next set out to examine the function of TFR cells. Although TFR cell function has been extensively studied in mice, the function of human TFR cells is yet to be characterized. We adopted a TFR–TFH–B-cell coculture assay and found that TFR cells were effective in suppressing TFH 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 TFR 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 TFR cells in regulating antibody isotype switching. We observed that circulating TFR 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 TFR cells, but not CXCR5+ Treg cells, to TFH–B-cell coculture significantly reduced levels of the 2 TFH 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 TFR cell–mediated suppression. When cultured in one chamber and separated from TFH and B cells in the other chamber in the transwell culture system, TFR cells almost completely lost their suppressive effect on TFH cell–promoted antibody production from naive B cells (Fig 4, C).

After fully establishing an assay to evaluate the suppressive function of circulating TFR cells and generating the finding that TFR cells, but not CXCR5+ Treg cells, are a specialized cell type in suppressing TFH–B-cell response, we then compared the function of TFR cells from HCs and patients with AR in this assay. Circulating TFR cells from patients with AR were as effective as counterpart TFR cells from HCs in suppressing IgM, IgG, and IgA production. In contrast, TFR cells from patients with AR were unable to suppress TFH cell–promoted IgE production, whereas TFR cells from HCs could do so (Fig 4, D), suggesting that AR was associated with not only the decrease in TFR numbers but also a specific defect in the ability of TFR cells to suppress IgE production. Moreover, we found that the TFR 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).
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 4. Peripheral T_{FR} cells in patients with AR have impaired function. A, T_{FH} 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_{FH} 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_{FH} 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_{FH} 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.
We then analyzed the correlation between plasmablasts and TFR cells and found a negative correlation between the frequencies of plasmablasts within CD19⁺ B cells and the frequencies of TFR 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 TFR/TFH2 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 CXCR5² Treg cells or the ratio of these cells to TH2 or TFH2 cells (data not shown).

TFR 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. It is of great interest to know how AIT might affect TFR cells. Thus we conducted a prospective study to investigate this question. Consistent with previous studies, we found that CSMS and 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 TFR cells in CD4⁺ T cells or total Treg cells and ratios of TFR/TFH2 cells were significantly increased in patients with AR with AIT after 12 months of treatment compared with those without AIT (Fig 6, A). Notably, TFR 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 CXCR5² Treg cells or the ratio of these cells to TH2 or TFH2 cells accompanying AIT (see Fig E11, C-E).

We also investigated the relationships between changes in TFR cell abundance and the clinical benefits of AIT. We found that the increase of TFR cell percentages in total Treg cells and the ratio of TFR/TFH2 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...
the ratio of these cells to T_{FH}2 or T_{FH}2 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_{FH}2 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.

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_{FH}2 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_{FR} 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_{FR} and B cells only. Percentage suppression = \( \frac{[\text{IgE production by T_{FH} cells} + \text{B cells}] - [\text{IgE production by T_{FR} cells} + \text{B cells} + \text{T_{FH} cells}]}{\text{IgE production by T_{FH} cells} + \text{B cells}} \times 100 \). 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_{FH}2 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.
A recent study suggested that AIT could modulate the balance of T FH and T FR cells in patients with grass allergy. 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. 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 al 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 FR–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). 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 underlining T FR cell–mediated inhibition for different iso-types 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, 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. However, in cell culture TGF-β induces IgA isotype class switching. 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. 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 unbiased 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 tonsilar 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, several studies indicate that the immune system is well developed around the age of 12 years. For example, immunoglobulins attain adult levels at that age. In our study the adolescent subjects with tonsilar 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 tonsilar 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. 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.

We thank Xin Gao, Kai-Ming Luo, and Zhi-An Chen in the China-Australia Centre for Personalised Immunology, Shanghai Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China, for their assistance in flow cytometric analysis.

**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**


