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ORIGINAL ARTICLE

Rhinitis, Sinusitis, and Upper Airway Disease

CD23 expression on switched memory B cells bridges T-B cell interaction in allergic rhinitis

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Abstract

Background: The contribution of B-cell subsets and T-B cell interaction to the pathogenesis of allergic rhinitis (AR) and mechanisms of allergen immunotherapy (AIT) remain poorly understood. This study aimed to outline circulating B-cell signature, the underlying mechanism, and its association with clinical response to AIT in patients with AR.

Methods: IgD/CD27 and CD24/CD38 core gating systems were used to determine frequencies and phenotypes of B cells. Correlations between B cells, T cells, antigen-specific IgE, and disease severity in AR patients were investigated. Switched memory B cells were co-cultured with type 2 follicular helper T (Tfh2) cells and follicular regulatory T (Tfr) cells. Associations between B-cell subsets and clinical benefits of AIT were analyzed.

Results: Frequencies and absolute numbers of circulating memory B cells were increased in AR patients. CD23 expression on $CD19^{+}CD20^{+}CD27^{+}IgD^{-}$ switched memory B cells was significantly enhanced and positively correlated with antigen-specific IgE levels, symptom scores, and Tfh2/Tfr cell ratio in AR patients. Compared with those from healthy controls, Tfh2 cells from AR patients had a greater capacity to induce CD23 expression on switched memory B cells via IL-4, which was unable to be sufficiently suppressed by AR-associated Tfr cells with defective IL-10 expression.

Abbreviations: AIT, allergen immunotherapy; AR, allergic rhinitis; HC, healthy controls; NSM, nonswitched memory; SM, switched memory; Tfh2, type 2 follicular helper T cells; Tfr, follicular regulatory T cells.

Yin Yao and Nan Wang contributed equally to this work.

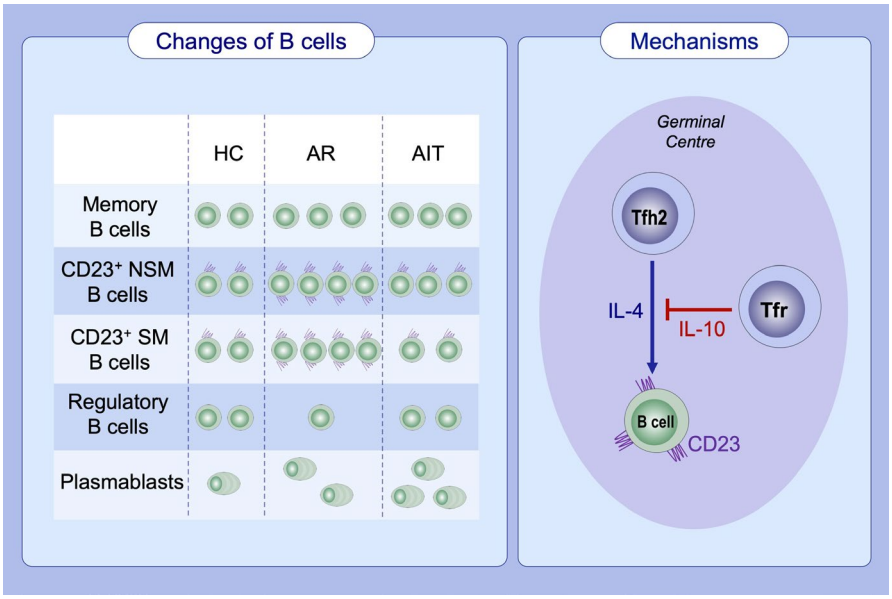
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CD23 expression on switched memory B cells was downregulated after 12-month AIT, which positively associated with disease remission in AR patients.

Conclusion: T-B cell interaction, bridged by CD23 expression particularly on switched memory B cells, may be involved in the disease pathogenesis and mechanism of AIT in patients with AR.

KEYWORDS

allergen immunotherapy, allergic rhinitis, immunoglobulin E, interaction, switched memory B cell



GRAPHICAL ABSTRACT

Circulating memory B cells are increased in AR patients. The enhanced expression of CD23 on switched memory B cells correlates with antigen-specific IgE levels, symptom scores, and allergen immunotherapy efficacy in AR patients. Tfh2 cells from AR patients have a greater capacity to induce CD23 expression on switched memory B cells via IL-4, which is unable to be sufficiently suppressed by AR-associated Tfr cells with defective IL-10 expression.

1 | INTRODUCTION

Allergic rhinitis (AR) is a highly prevalent inflammatory disorder of nasal mucosa caused by immunoglobulin E (IgE)-mediated hyper-reaction against inhaled allergens.[1,2] Although the importance of IgE in the pathogenesis of AR has been clinically validated by the benefit of anti-IgE antibody treatment in patients with AR,[3-5] the mechanisms underlying the aberrant IgE production in AR remain poorly understood. IgE is synthesized by B cells, which require a series of signals provided by dendritic cells and T cells and follow a development path of antigen-specific T-B cell cognate interaction, B-cell immunoglobulin class switching, and B-cell differentiation into memory B cell and IgE-secreting plasma cell.[6,7] In this process, T cells have been discovered to play a critical role.[8-13] Particularly, follicular helper T (Tfh) and follicular regulatory T (Tfr) cells, characterized by their localization in germinal centre (GC), are the major T helper cell subset type that orchestrates B-cell response by regulating isotype switching and affinity maturation as well as the differentiation of B cells to plasma cells or memory B cells.[14,15] Yao et al[12,13] have recently reported that the increased type 2 follicular helper T (Tfh2) cells and defective follicular regulatory T (Tfr) cells participated in the promotion of IgE production from B cells in AR.

B cells are central in determining antibody production; however, their signature and the T-B cell interaction have been limited studied in patients with AR and inconsistent results have been reported.[16-19] Luo et al[16] observed higher percentages of $CD19^+CD27^+$ memory B cells in peripheral blood in AR patients than those in controls. In contrast, Henriques et al[18] demonstrated a significant decrease in $CD19^+CD20^+CD27^+CD38^+$ memory B cells in AR patients. These discrepancies might be caused by, at

least in part, different methods employed to define B-cell subsets. Therefore, it is better to outline B-cell signature with several widely accepted classification schemes simultaneously and comprehensively. In addition, B-cell function may be indicated or regulated by several surface molecules. HLA-DR is an MHC class II cell surface receptor.[20] B cells with CD25 expression display a higher proliferative and antigen-presenting capacity.[21] As a low-affinity receptor for IgE, CD23 expression on B cells is important for facilitated antigen presentation and IgE synthesis.[22] The expression and function of these molecules on distinct B-cell subsets have yet to be defined in patients with AR.

Allergen immunotherapy (AIT) is an effective treatment for AR, which not only has disease-modifying property but also confers long-term clinical benefit after cessation of treatment.[23,24] Several events, including induction of Tfr cells and regulatory B (Breg) cells, and deletion or anergy of type 2 helper T and Tfh2 cells, have been implicated in the development and maintenance of immune tolerance during AIT.[12,13,25-28] However, the change of B-cell subsets other than Breg cells during AIT and their contribution to the action of AIT remain an open question.

In this study, we comprehensively characterized B-cell subsets and phenotypes in adult patients with AR by two widely accepted gating systems, IgD/CD27 and CD24/CD38.[29] We showed that the frequencies and absolute numbers of switched memory B cells were increased in patients with AR with concomitant upregulation of CD23. CD23 expression on switched memory B cells, but not other B-cell subsets, positively correlated with disease severity, antigen-specific IgE levels, and Tfh2/Tfr cell ratio in patients with AR. We showed for the first time that compared with those from healthy controls, Tfh2 cells from AR patients had a greater capacity to induce CD23 expression on switched memory B cells via IL-4, which was unable to be sufficiently suppressed by AR-associated Tfr cells with defective IL-10 expression. Moreover, we found that AIT reduced CD23 expression on switched memory B cells, which positively correlated with clinical efficacy of AIT in patients with AR.

2 | METHODS

2.1 | Subjects

Adult AR patients allergic to house dust mites (HDM) only and healthy controls (HC) were recruited with the approval of the Ethics Committee of Tongji Hospital and China Resources & Wisco General Hospital. The informed consent was obtained from all participants. The diagnosis of AR was made, and symptom was scored based on visual analog scale (VAS) according to the Allergic Rhinitis and its Impact on Asthma guideline.[1] In the study of B-cell profiles in comparison with HC, peripheral blood samples were collected from 31 HC and 38 AR patients. In the study of changes of B-cell profiles during AIT, additional 44 AR patients qualified for AIT were enrolled and divided into two groups: AIT group (patients receiving AIT in combination with standard medication treatment, n = 23) and the group without AIT (patients receiving standard medication treatment only, n = 21) based on patients' preference. Peripheral blood samples were collected at baseline (prior to treatment) and at 3, 6, and 12 months after treatment. The demographic and clinical data for the subjects are shown in Tables S1 and S2 in Appendix S1.

2.2 | Allergen skin prick test

Skin prick test with a panel of 19 inhalant allergens common in our region was performed as we previously described.[12] Complete details on skin prick test can be found in the Appendix S1.

2.3 | AIT and symptoms assessment

Subcutaneous AIT was conducted using the semi-depot HDM allergen extracts (full allergens, 50% *Dermatophagoides pteronyssinus* (Der p) plus 50% *Dermatophagoides farina* (Der f); Allergopharma GmbH). The daily combined symptom and medication score (CSMS) was calculated as previously reported.[13] Additional information is provided in the Appendix S1.

2.4 | Flow cytometry

Peripheral blood mononuclear cells (PBMCs) cells were isolated from whole blood and then stained with fluorochrome-labeled antibodies (Table S3 in Appendix S1) and analyzed by flow cytometry. Circulating B-cell subsets were defined based on CD3, CD19, CD20, IgD, and CD27, or CD3, CD19, CD24, and CD38 expression.[30] T-cell subsets were defined based on CD3, CD4, CD25, CD127, CD45RA, CXCR5, CCR6, and CXCR3 expression as previously described.[13] Complete details on flow cytometry can be found in the Appendix S1.

2.5 | Cell sorting

Peripheral T- and B-cell subsets were sorted from fluorochrome-labeled PBMCs by using a BD FACSAria II cell sorter (BD Biosciences).[13] Switched memory B cells were sorted as $CD3^-CD19^+CD20^+CD27^+IgD^-$ cells, Tfh2 cells were sorted as $CD4^+CD45RA^{low}CXCR5^+CD25^{-/low}CXCR3^-CCR6^-$ cells, and Tfr cells were sorted as $CD4^+CD45RA^{lo\,w}CXCR5^+CD25^{high}CD127^{low}$ cells.[13] The purity of sorted cells was more than 95%. Antibodies used in cell sorting are presented in Table S3 in Appendix S1.

2.6 | Cell culture

Purified switched memory B cells were cultured with autologous or allogenic Tfh2 cells with or without allogenic Tfr cells for 2 days.

Staphylococcal enterotoxin B (SEB, 1 μg/mL, Toxin Technology Inc.) was added to the cultures to mimic the antigen-specific interaction between T and B cells.[31] Complete details on cell culture can be found in the Appendix S1.

2.7 | Cytokine and immunoglobulin measurement

Serum Der p-specific IgE and IgG4 were measured by ImmunoCAP (Phadia) and enzyme-linked immunosorbent assay (ELISA; DR.FOOKE Laboratorien GmbH), respectively.[13]

2.8 | Statistical analysis

Statistical analyses were performed with SPSS 19.0 software (IBM) or GraphPad Prism Software 7.0 (GraphPad Software Inc.). For continuous variables, data are presented in dot plots. Symbols represent individual samples, horizontal bars represent median, and error bars show interquartile range. Mann-Whitney *U* test was used for between-group comparisons. Cell culture data are presented as mean ± SEM and analyzed by unpaired Student's *t* test. For categorical data, chi-squared test or Fisher's exact test was performed. The Spearman test was performed for correlation analyses. Unsupervised hierarchical clustering of variables (Spearman correlation) was performed by using R package "pheatmap" with R software (version 3.6.0) and represented as a dendrogram. Correlation matrix visualization was performed using the R package "corrplot."

3 | RESULTS

3.1 | Increased circulating memory B cells in patients with AR

Based on CD27 and IgD expression, peripheral $CD3^-CD19^+CD20^+$ B lymphocytes cells can be divided into four subsets: $CD27^-IgD^+$ naïve B cells, $CD27^+IgD^+$ nonswitched memory B cells, $CD27^+IgD^-$ switched memory B cells, and $CD27^-IgD^-$ double negative B cells (Figure 1A and Figure S1).[29] Although no significant difference in percentage of $CD19^+CD20^+$ total B cells within lymphocytes was found between patients with AR and HC (Table 1), we observed a significant increase in percentages of nonswitched and switched memory B cells within $CD19^+CD20^+$ B cells in patients with AR compared with those in HC (Figure 1A and Table 1). Coincidently, the frequencies of naive B cells in $CD19^+CD20^+$ B cells were reduced in patients with AR in comparison to HC (Figure 1A and Table 1). There was no difference in double negative B cells between AR patients and controls (Figure 1A and Table 1).

To validate our findings and address immature populations, we further characterized B cells using B-cell lineage marker CD19 and developmental markers CD24 and CD38.[29,30] Within $CD3^-CD19^+$ lymphocytes, peripheral B cells were classified as $CD24^{int}CD38^{int}$ mature naïve, $CD24^-CD38^-$ new memory, $CD24^+CD38^-$ primarily memory, $CD24^-CD38^+$ plasmablasts, and $CD24^{high}CD38^{high}$ transitional B cells (Figure 1B and Figure S1).[29,30] Consistent with the findings of B-cell subsets differentiated by CD27 and IgD, higher frequencies of memory B cells including new memory and primarily memory B cells and lower frequencies of mature naïve B cells

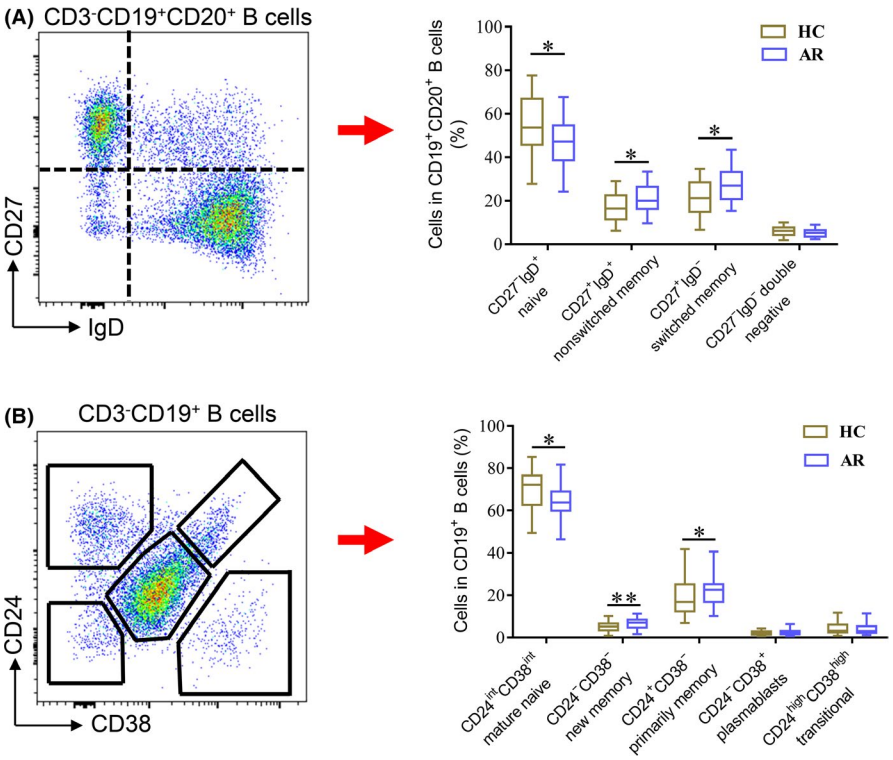


FIGURE 1 Increased circulating memory B cells in patients with AR. (A) Gating strategy and frequencies of $CD27^-IgD^+$ naïve, $CD27^+IgD^+$ nonswitched memory, $CD27^+IgD^-$ switched memory, and $CD27^-IgD^-$ double negative B cells within $CD19^+CD20^+$ B cells in patients with AR and healthy controls (HC). (B) Gating strategy and frequencies of $CD24^{int}CD38^{int}$ mature naïve, $CD24^-CD38^-$ new memory, $CD24^+CD38^-$ primarily memory, $CD24^-CD38^+$ plasmablasts, $CD24^{high}CD38^{high}$ transitional B cells within $CD19^+$ B cells in patients with AR and HC. n = 32 for AR patients and n = 25 for HC. *P < .05 and **P < .01

TABLE 1 Frequencies and absolute numbers of B-cell subsets in peripheral blood of healthy subjects and patients with AR

B-cell subsets	Healthy subjects	AR patients	*P* value
$CD19^+CD20^+$ B cells in lymphocyte (%)	8.7 (5.1, 11.4)	10.6 (7.2, 14.0)	.104
$CD19^+CD20^+$ B cells count (10^7 cells/L)	15.9 (9.7, 27.9)	22.8 (16.7, 29.9)	.093
$CD27^-IgD^+$ naïve B cells in $CD19^+CD20^+$ B cells (%)	53.6 (47.3, 65.4)	47.3 (38.7, 54.3)	**.018**
$CD27^-IgD^+$ naive B cells count (10^7 cells/L)	8.1 (4.8, 15.6)	10.6 (8.2, 13.5)	.495
$CD27^+IgD^+$ nonswitched memory B cells in $CD19^+CD20^+$ B cells (%)	16.4 (11.6, 22.3)	20.1 (16.4, 26.4)	**.039**
$CD27^+IgD^+$ nonswitched memory B cells count (10^7 cells/L)	2.9 (1.4, 4.5)	4.1 (3.0, 7.3)	**.009**
$CD27^+IgD^-$ switched memory B cells in $CD19^+CD20^+$ B cells (%)	21.2 (15.7, 28.2)	27.0 (20.9, 31.7)	**.016**
$CD27^+IgD^-$ switched memory B cells count (10^7 cells/L)	3.3 (1.4, 5.9)	4.4 (3.9, 8.6)	**.011**
$CD27^-IgD^-$ double negative B cells in $CD19^+CD20^+$ B cells (%)	6.2 (4.3, 7.5)	5.3 (3.7, 6.5)	.116
$CD27^-IgD^-$ double negative B cells count (10^7 cells/L)	1.0 (0.4, 1.6)	1.2 (0.6, 1.6)	.460
$CD19^+$ B cells in lymphocyte (%)	8.9 (5.1, 11.7)	10.9 (7.7, 14.4)	.086
$CD19^+$ B cells count (10^7 cells/L)	16.5 (11.1, 28.2)	23.2 (17.3, 30.6)	.152
$CD24^{int}CD38^{int}$ mature naive B cells in $CD19^+$ B cells (%)	72.2 (63.4, 76.4)	63.8 (60.0, 68.6)	**.012**
$CD24^{int}CD38^{int}$ mature naive B cells count (10^7 cells/L)	12.9 (8.0, 21.9)	14.6 (9.6, 21.9)	.399
$CD24^-CD38^-$ new memory B cells in $CD19^+$ B cells (%)	5.1 (3.4, 6.3)	7.0 (5.1, 8.3)	**.009**
$CD24^-CD38^-$ new memory B cells count (10^7 cells/L)	1.0 (0.5, 1.3)	1.6 (0.9, 2.0)	**.006**
$CD24^+CD38^-$ primarily memory B cells in $CD19^+$ B cells (%)	16.8 (12.4, 24.9)	22.7 (17.5, 25.1)	**.038**
$CD24^+CD38^-$ primarily memory B cells count (10^7 cells/L)	2.7 (1.7, 4.5)	5.4 (3.3, 6.9)	**.006**
$CD24^-CD38^+$ plasmablasts in $CD19^+$ B cells (%)	2.1 (1.3, 2.9)	2.4 (1.5, 3.1)	.515
$CD24^-CD38^+CD19^+$ plasmablasts count (10^7 cells/L)	0.3 (0.1, 0.7)	0.5 (0.4, 0.9)	.065
$CD24^{high}CD38^{high}$ transitional B cells in $CD19^+$ B cells (%)	3.2 (2.4, 5.9)	3.1 (2.3, 4.8)	.618
$CD24^{high}CD38^{high}$ transitional B cells count (10^7 cells/L)	0.6 (0.3, 1.3)	0.8 (0.5, 1.2)	.505
$CD20^-CD27^{high}CD38^+$ plasmablasts in $CD19^+$ B cells (%)	0 (0, 0.1)	0.1 (0, 0.1)	**.009**

(Continues)

TABLE 1 (Continued)

B-cell subsets	Healthy subjects	AR patients	*P* value
$CD20^-CD19^+CD27^{high}CD38^+$ plasmablasts count (10^4 cells/L)	0.9 (0.7, 1.4)	1.7 (1.1, 2.2)	**.003**
$CD25^+CD71^+CD73^-$ Breg cells in $CD19^+$ B cells (%)	3.0 (2.5, 4.9)	2.2 (1.3, 3.7)	**.032**
$CD19^+CD25^+CD71^+CD73^-$ Breg cells count (10^7 cells/L)	0.5 (0.3, 0.7)	0.5 (0.3, 0.8)	.865

Note: Data are expressed by medians and interquartile ranges. Numbers in bold indicate the *P* value less than .05.

were observed in patients with AR than those in HC (Figure 1B and Table 1).

Moreover, we found that the absolute numbers of memory B cells, identified by either CD27 and IgD, or CD38 and CD24, were increased in patients with AR in comparison with HC (Table 1). Although there were decreased frequencies of naïve B cells, no significant change of naïve B cell absolute numbers was observed in patients with AR (Table 1).

Although circulating plasmablasts account for the majority of antibody-secreting cells in the blood, it seems to be difficult to define this rare population.[30] In the CD27/IgD plot, plasmablasts were embedded within IgD^-CD27^+ quadrant. In the CD24/CD38 gating systems, plasmablasts were considered as $CD19^+CD24^-CD38^+$ cells. Nevertheless, since CD20 expression is lost at the plasmablastic stage, plasmablasts are more accurately identified as $CD19^+CD20^-CD27^{high}CD38^+$ cells.[31] Based on this gating system, we found increased frequencies and absolute numbers of $CD19^+CD20^-CD27^{high}CD38^+$ plasmablasts in patients with AR in comparison with HC (Table 1 and Figure S2A).

Breg cells represent a small population of B cells participating in suppression of immune responses by secreting IL-10.[32]

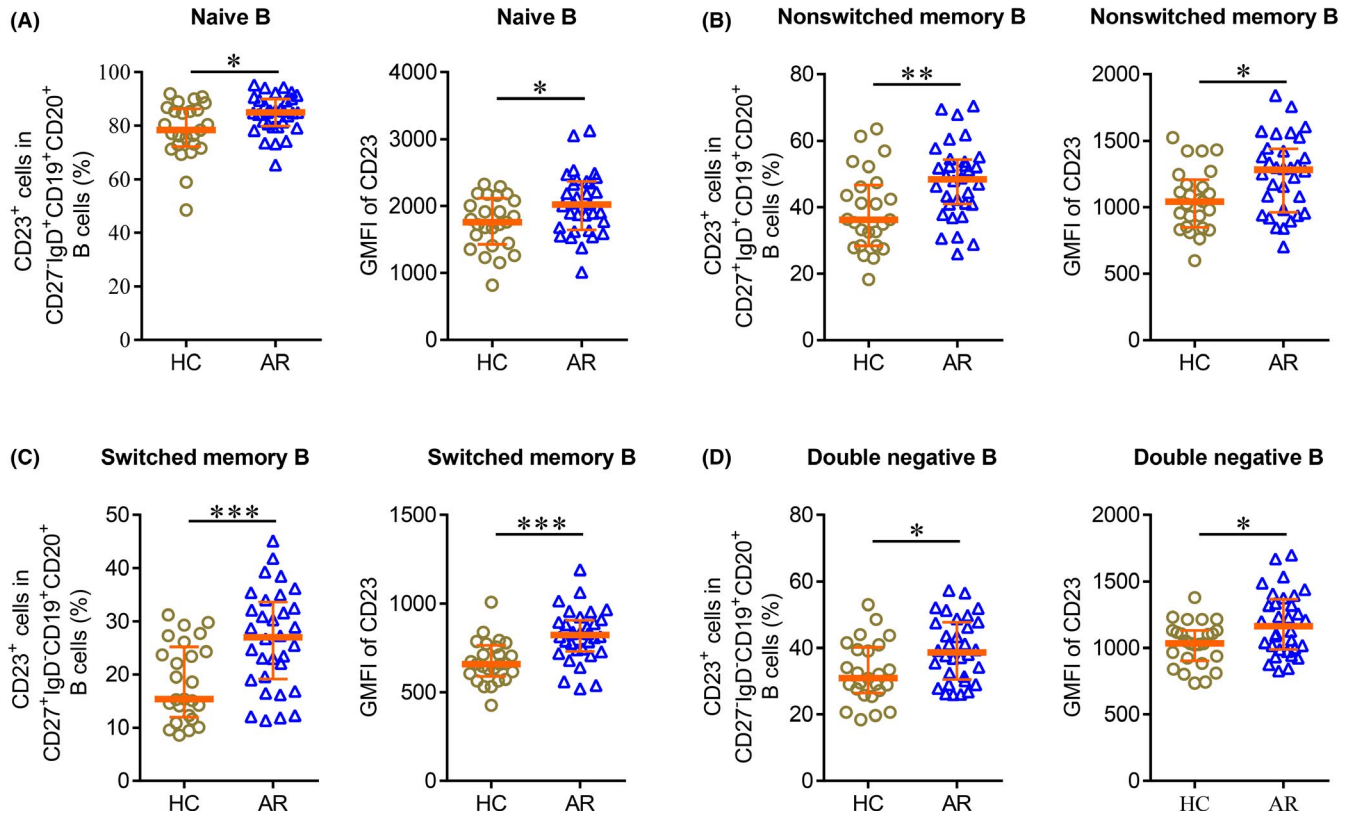


FIGURE 2 Enhanced CD23 expression on B-cell subsets in patients with AR. (A) Frequencies of $CD23^+$ cells within $CD27^-IgD^+$ naïve B cells and geometric mean fluorescence intensity (GMFI) of CD23 of $CD23^+CD27^-IgD^+$ naïve B cells in patients with AR and healthy controls (HC). (B) Frequencies of $CD23^+$ cells within $CD27^+IgD^+$ nonswitched memory B cells and GMFI of CD23 of $CD23^+CD27^+IgD^+$ nonswitched memory B cells. (C) Frequencies of $CD23^+$ cells within $CD27^+IgD^-$ switched memory B cells and GMFI of CD23 of $CD23^+CD27^+IgD^-$ switched memory B cells. (D) Frequencies of $CD23^+$ cells within $CD27^-IgD^-$ double negative B cells and GMFI of CD23 of CD23 positive, $CD27^-IgD^-$ double negative B cells. n = 32 for AR patients and n = 25 for HC. **P* < .05, ***P* < .01, and ****P* < .001

Recent studies have shown that IL-10$^+$ Breg cells are enriched in CD25$^+$CD71$^+$CD73$^-$ B cells.[33] Consistent with previous reports,[17] we found lower percentages of CD19$^+$CD25$^+$CD71$^+$CD73$^-$ Breg cells in patients with AR compared with those in HC (Figure S2B).

3.2 | Enhanced CD23 expression on B cells in patients with AR

Given the significant changes in memory B cells in patients with AR and the better discrimination of memory B cells from naïve subsets by CD27 and IgD classification, we used this classification method for further characterization of B cells in AR.

CD23, CD25, and HLA-DR are the surface markers that reflect the activation and function of B cells. All examined B-cell subsets had marked expression of HLA-DR, whereas CD23 and CD25 were more abundantly expressed on naïve and memory B cells, respectively (Figure S3). We found that the frequencies of CD23 expression were upregulated in all B-cell subsets in patients with AR in comparison with HC; however, such upregulation was more prominent in switched memory B cells (Figure 2 and Figure S4). In order to determine CD23 expression density on B cells, we analyzed the geometric mean fluorescence intensity (GMFI) of CD23 in CD23$^+$ B cells and found that AR patients demonstrated a higher density of CD23 molecules on all examined B-cell subsets than that in HC, and the increase in CD23 density was also more prominent for switched memory B cells (Figure 2 and Figure S4). In contrast, there was no significant difference in frequency or density of CD25 or HLA-DR expression on any B-cell subset between AR patients and HC (Table S4 in Appendix S1).

3.3 | CD23 expression on switched memory B cells correlated with disease severity and Der p-specific IgE level in patients with AR

In order to evaluate possible T-B cell interaction and their association with disease activities, we also studied circulating T-cell subsets simultaneously. Consistent with previous reports,[12,13] a significant increase of Tfh2 cells and reduction of Tfr cells were found in patients with AR as compared to HC (Figure S5). No significant change of other examined T-cell subsets in AR patients in comparison with HC was observed (data not shown). By unsupervised hierarchical cluster analysis, we found that visual analog scale (VAS) scores clustered together with Der p-specific IgE levels, the frequencies of plasmablasts, Tfh2 cells and CD23$^+$ switched memory B cells, and CD23 GMFI of CD23$^+$ switched memory B cells in patients with AR (Figure 3A, red box). However, this clustering pattern was not seen in HC (Figure S6).

Consistent with previous studies,[13] Der p-specific IgE levels were found to positively and negatively correlate with the frequencies of plasmablasts and CD73$^-$CD25$^+$CD71$^+$ Breg cells in AR patients, respectively (Figure 3A). In addition, we found that frequencies of switched memory B cells, but not other B-cell subsets, positively correlated with VAS symptom scores in AR patients (Figure 3A). Furthermore, the frequencies of CD23$^+$ switched memory B cells and CD23 GMFI of CD23$^+$ switched memory B cells positively correlated not only with VAS symptom scores, but also with Der p-specific IgE levels in AR patients (Figure 3A,B). Compared with the CD23 expression on switched memory B cells, the expression of CD23 on nonswitched memory B cells demonstrated a weaker correlation with VAS symptom scores and no significant correlation with Der p-specific IgE levels in AR patients (Figure 3A).

Interestingly, both frequencies of CD23$^+$ switched memory B cells and GMFI of CD23 of CD23$^+$ switched memory B cells positively correlated with frequencies of Tfh2, and negatively correlated with frequencies of Tfr cells and ratios of Tfr/Th2 cell in AR patients (Figure 3A,B). In contrast, these correlations were not found in HC (Figure S6).

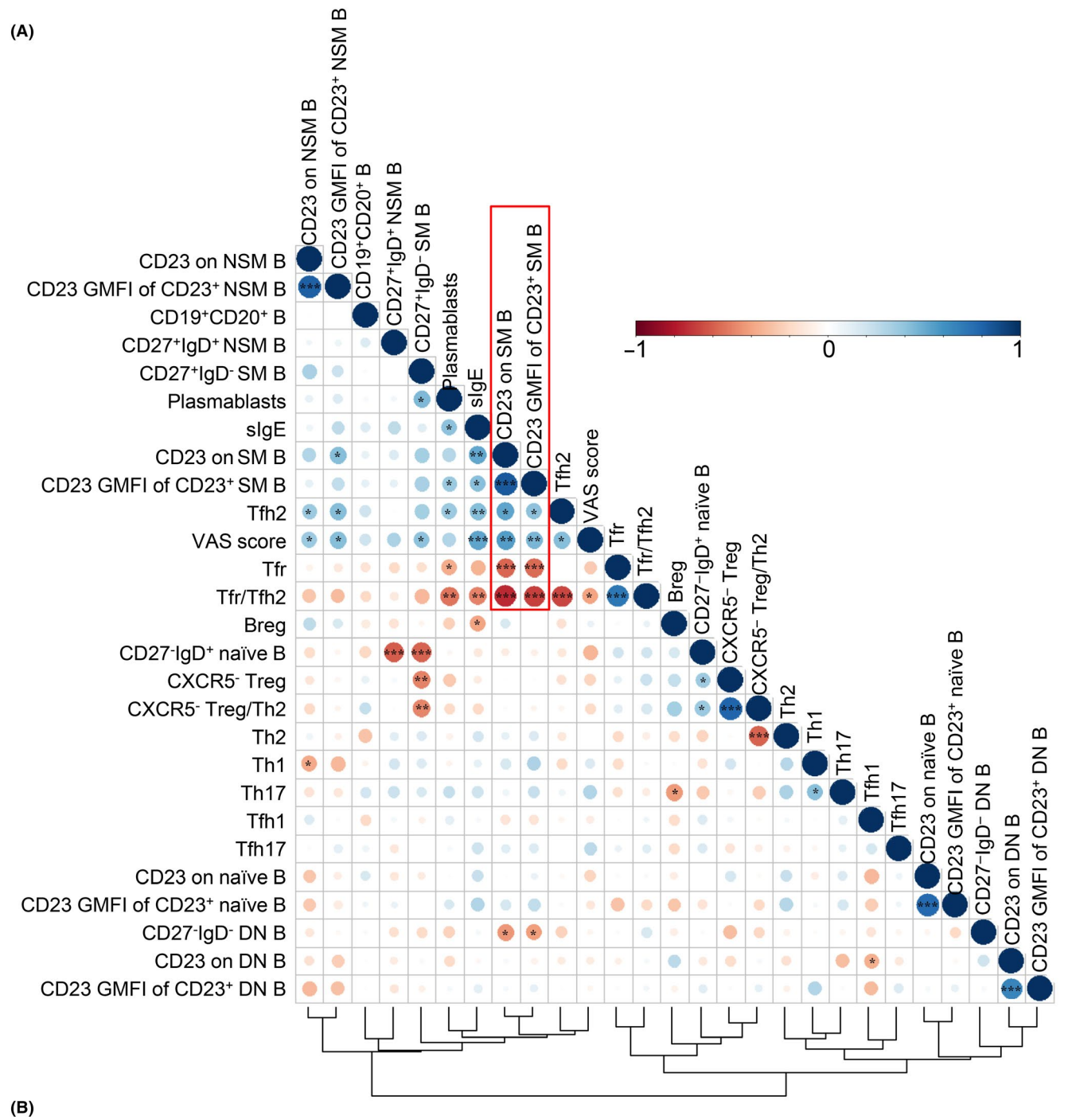
3.4 | Regulation of CD23 expression on switched memory B cells by Tfh2 and Tfr cells

Based on the correlation between CD23 expression and Tfr/Tfh2 cell imbalance, we next sought to examine the regulation of CD23 expression on switched memory B cells by Tfh and Tfr cells in vitro. Adopting a Tfh2-B cell co-culture assay, we found that Tfh2 cells from AR patients exhibited a stronger capacity to induce CD23 expression in both frequency and density on switched memory B cells than those from HC (Figure 4A). Previous studies have shown that IL-4 could induce CD23 expression on B cells.[34] Our previous study showed that Tfh2 cells from AR patients secreted more IL-4 than Tfh2 cells from HC.[12] In line with these reports, we found that anti-IL-4 antibody significantly suppressed CD23 expression on switched memory B cells induce by Tfh2 cells from AR patients in vitro (Figure 4B).

Furthermore, we found that addition of Tfr cells from healthy subjects, but not those from patients with AR, significantly inhibited CD23 expression on switched memory B cells induced by Tfh2 cells from AR patients (Figure 4C), indicating a defective suppressive function of AR-associated Tfr cells. IL-10 has been implicated in the suppressive activity of Tfr cells previously.[35] We found that Tfr cells from HC secreted more IL-10 than those from patients with AR when co-cultured with allogenic Tfh2 and B cells (Figure S7). Moreover, we found that anti-IL-10 antibody treatment led to the loss of suppressive function of Tfr cells from HC on CD23 expression on B cells induced by Tfh2 cells (Figure 4D). Consistently, a reduction of IL-10 expression in Tfr cells was noted in patients with AR compared with HC (Figure 4E).

3.5 | AIT downregulated CD23 expression on memory B cells

To investigate whether AIT will correct B-cell abnormality, we studied the circulating B-cell profiles at baseline and at 3, 6, and


(A)
CD23 on NSM B
CD23 GMFI of CD23+ NSM B
CD19+CD20+ B
CD27+IgD+ NSM B
CD27+IgD- SM B
Plasmablasts
sIgE
CD23 on SM B
CD23 GMFI of CD23+ SM B
Tfh2
VAS score
Tfr
Tfr/Tfh2
Breg
CD27-IgD+ naïve B
CXCR5- Treg
CXCR5- Treg/Th2
Th2
Th1
Th17
Tfh1
Tfh17
CD23 on naïve B
CD23 GMFI of CD23+ naïve B
CD27-IgD- DN B
CD23 on DN B
CD23 GMFI of CD23+ DN B
−1
0
1

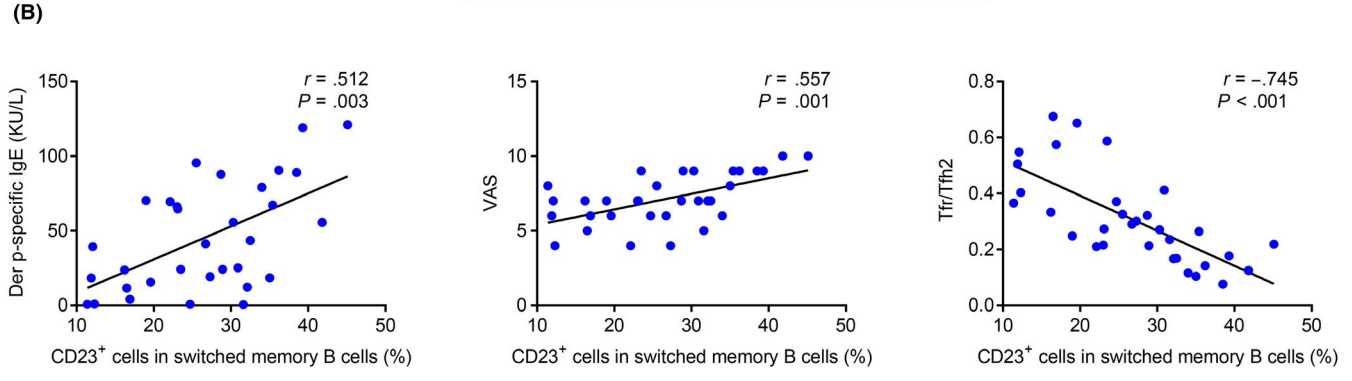

(B)
Der p-specific IgE (KU/L)
r = .512
P = .003
CD23+ cells in switched memory B cells (%)
VAS
r = .557
P = .001
CD23+ cells in switched memory B cells (%)
Tfr/Tfh2
r = −.745
P < .001
CD23+ cells in switched memory B cells (%)

FIGURE 3 Correlation matrix in patients with AR. (A) Spearman's correlation plot demonstrates unsupervised hierarchical clustering of B-cell frequencies, T-cell frequencies, *Dermatophagoides pteronyssinus* (Der p)-specific IgE levels and visual analog scale (VAS) scores in patients with AR. Dendrograms are shown as trees, representing the distance between variables. The correlation matrix shows the positive (blue) or negative (red) correlation of two parameters. Color intensity and circle size reflect correlation strength. VAS scores clustered together with Der p-specific IgE levels, the frequencies of plasmablasts, Tfh2 cells, and $CD23^+$ switched memory B cells, and CD23 GMFI of $CD23^+$ switched memory B cells (red box). (B) Correlations of frequencies of $CD23^+$ switched memory B cells with Der p-specific IgE levels, VAS symptom scores, and ratio of Tfr/Tfh2 cell in patients with AR. n = 32. Breg, $CD19^-CD25^+CD71^+CD73^-$ regulatory B; DN, double negative; GMFI, geometric mean fluorescence intensity; SM, switched memory; NSM, nonswitched memory; Plasmablasts, $CD19^+CD20^-CD27^{high}CD38^+$ plasmablasts; sIgE, Der p-specific IgE; Tfh: follicular helper T; Tfr: follicular regulatory T; Th, helper T; Treg, regulatory T

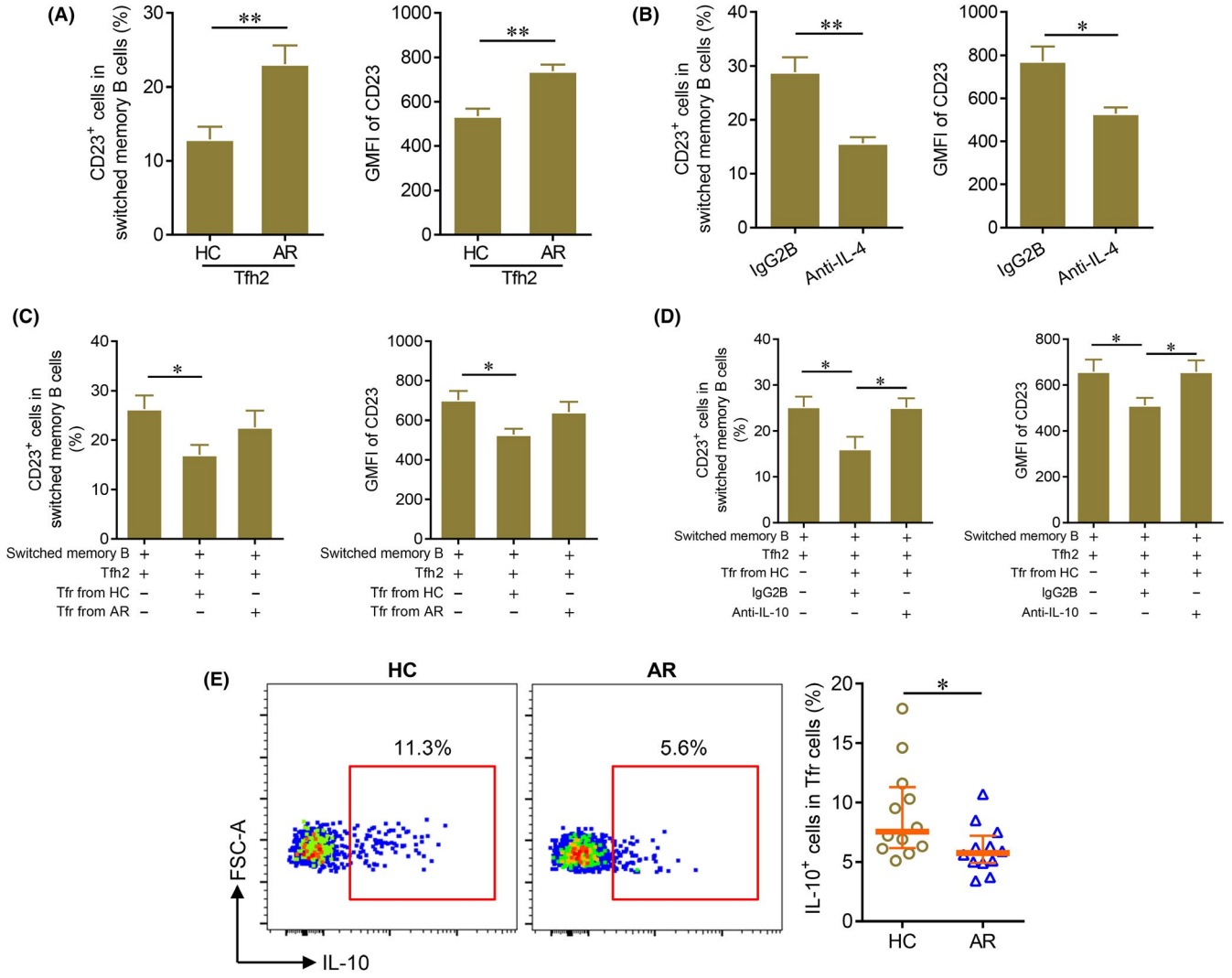


FIGURE 4 Regulation of CD23 expression on switched memory B cells by Tfh2 and Tfr cells. (A) Tfh2 cells (5×10^4/well) from patients with AR and healthy controls (HC) were co-cultured with allogenic switched memory B cells (2.5×10^4/well) from HC (n = 6). (B) Tfh2 cells (5×10^4/well) from patients with AR were co-cultured with autologous switched memory B cells (2.5×10^4/well) in the presence of anti-IL-4 blocking antibody or control IgG2B (n = 5). (C) Tfh2 cells (5×10^4/well) and switched memory B cells (2.5×10^4/well) from same AR patients were co-cultured with or without allogenic Tfr cells (5×10^4/well) from HC or patients with AR (n = 5). (D) Tfh2 cells (5×10^4/well) and switched memory B cells (2.5×10^4/well) from same AR patients were co-cultured with allogenic Tfr cells (5×10^4/well) from HC in the presence of anti-IL-10 blocking antibody or control IgG2B (n = 5). CD23 expression on B cells, which were shown as $CD23^+$ cells and geometric mean fluorescence intensity (GMFI) of $CD23^+$ cells, was detected at day 2 by flow cytometry. In (A), (B), (C), and (D), data are presented as mean ± SEM. (E) Expression of IL-10 in Tfr cells in HC (n = 12) and patients with AR (n = 12). Representative flow cytometric plots are shown. $^*P < .05$ and $^{**}P < .01$

12 months after AIT. Consistent with our previous reports,[12,13] we observed a significant decrease in CSMS and Der p-specific IgE levels, whereas a marked increase in Der p-specific IgG4 levels in the patients with AIT compared with those without AIT after 12-month treatment (Figure S8A,B). The frequencies of switched memory B cells within $CD19^+CD20^+$ B cells, particularly $CD23^+$ switched memory B cells and CD23 density (CD23 GMFI) on switched memory B cells, were significantly reduced in patients with 12-month AIT treatment compared to those without AIT (Figure 5A). AIT also reduced CD23 expression on nonswitched memory B cells; however, the statistical significance was more prominent for switched memory B cells ($P < .05$ vs $P < .01$, Figure 5B). In line with previous reports,[27] compared with those without AIT, a significant increase in frequencies of Breg cells and plasmablasts was found in the AIT group after 12-month treatment (Figure S8C). There was no significant change of any other B-cell subset, or the expression of CD25 or HLA-DR was recorded (data not shown).

3.6 | Reduction of CD23 expression on switched memory B cells correlated with AIT efficacy in patients with AR

To identify potential contribution of B cells to AIT efficacy, we performed correlation analysis of significantly changed B-cell relevant markers during AIT with CSMS improvement. We found that the reduction of frequencies of $CD23^+$ switched memory B cells and CD23 density on switched memory B cells were significantly correlated with the CSMS improvement in AR patients with AIT, whereas no correlation was observed for patients without AIT (Figures 6 and 7). There was no significant correlation between the change of $CD23^+$ nonswitched memory B cells, Breg cells, plasmablasts, Der p-specific IgE or IgG4, and the improvement of CSMS (Figure 6). In addition, significant positive correlations were observed between the increase of plasmablasts frequencies, the increase of Der p-specific IgG4 levels, and the reduction of Der p-specific IgE levels in AR patients with AIT (Figure 6).

4 | DISCUSSION

T-B cell interaction is critical for immunoglobulin production.[36] However, in contrast to T cells, a clear phenotypic and functional profile of B cells beyond Breg cells in humans under AR condition is lacking. Moreover, T-B cell interaction is barely studied in AR.

In this study, we comprehensively analyzed B-cell subsets in patients with AR using two most widely accepted gating strategies of IgD/CD27 and CD24/CD38. In addition to confirming previously reported increase of plasmablasts and decrease of Breg cells in peripheral blood of patients with AR, we discovered a significant skewing of circulating B-cell population toward memory B cells in patients with AR, which was validated by the two different gating methods. Memory B cells including switched and nonswitched type are B-cell subsets, which experienced antigen and react rapidly to generate an accelerated and robust recalled immune response upon

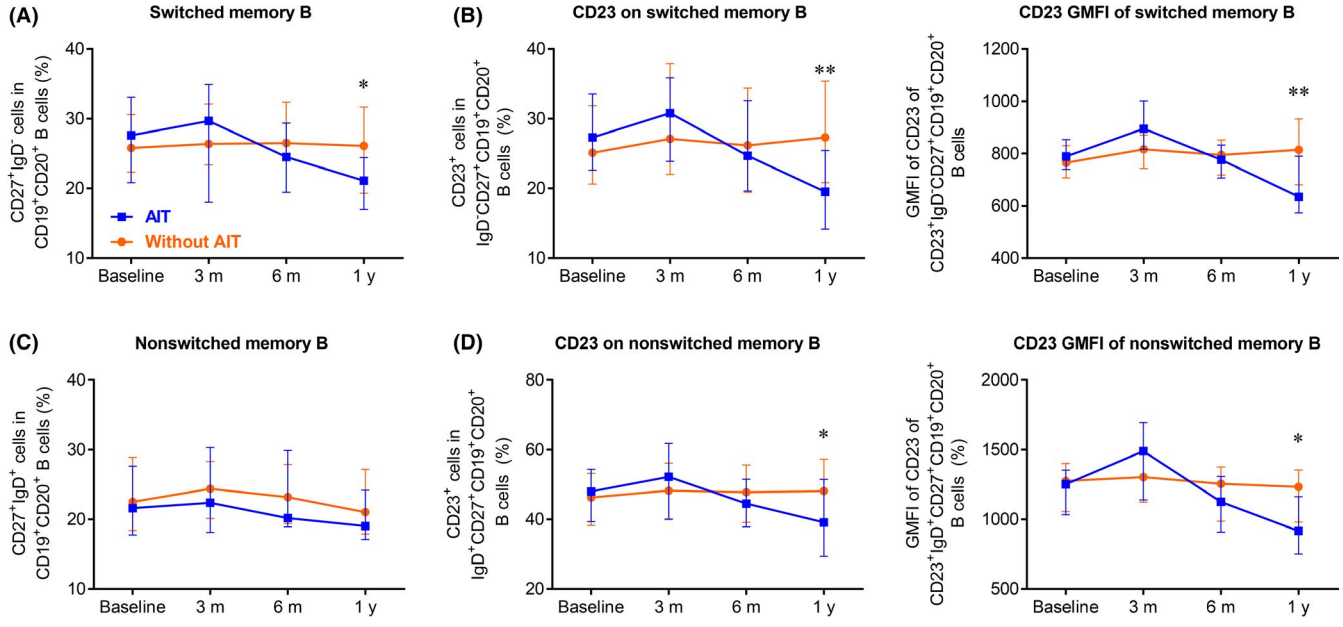


FIGURE 5 AIT reduced CD23 expression on switched memory B cells. (A) Changes of frequencies of $CD27^+IgD^-$ switched memory B cells within $CD19^+CD20^+$ B cells in AR patients with (n = 23) and without AIT (n = 21) after treatment. (B) Changes of frequencies of $CD23^+$ cells within switched memory B cells and CD23 geometric mean fluorescence intensity (GMFI) of $CD23^+$ switched memory B cells after treatment. (C) Changes of frequencies of $CD27^+IgD^+$ nonswitched memory B cells within $CD19^+CD20^+$ B cells in AR patients with (n = 23) and without AIT (n = 21) after treatment. (D) Change of frequencies of $CD23^+$ cells within nonswitched memory B cells and CD23 geometric mean fluorescence intensity (GMFI) of $CD23^+$ nonswitched memory B cells after treatment. *$P < .05$ and **$P < .01$

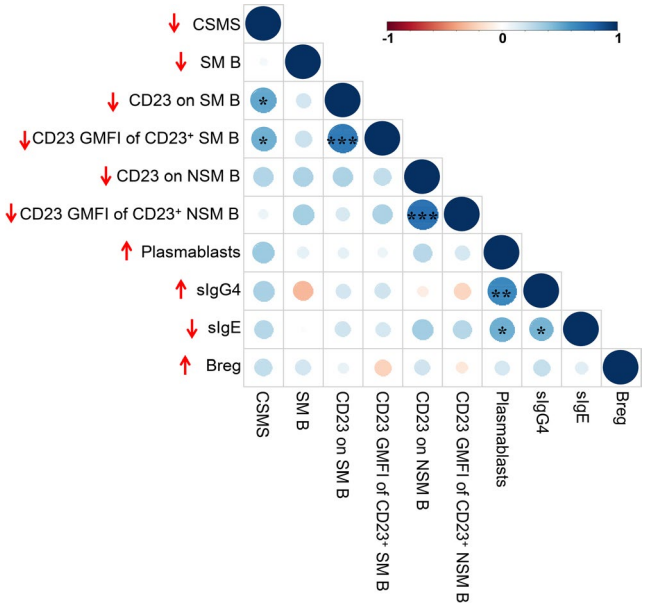


FIGURE 6 Correlations between the B cell relevant markers changed after AIT and the clinical efficacy of AIT at 12-mo follow-up. Data were analyzed using Spearman's test and represented as a correlation matrix. The correlation matrix shows the positive (blue) or negative (red) correlations of every two parameters, with color intensity and circle size reflecting correlation strength. Red arrows up represent increased markers, and red arrows down represent decreased markers after 12-month AIT. n = 23. Breg, $CD19^{-}CD25^{+}CD71^{+}CD73^{-}$ regulatory B; CSMS, combined symptom and medication score; GMFI, geometric mean fluorescence intensity; SM, switched memory; NSM, nonswitched memory; Plasmablasts, $CD19^{+}CD20^{-}CD27^{high}CD38^{+}$ plasmablasts; sIgG4, *Dermatophagoides pteronyssinus* (Der p)-specific IgG4; sIgE, Der p-specific IgE. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$

encountering the same antigen again.[37] Accumulating evidence shows, that switched memory B cells are generated through the classical germinal center (GC) pathway, which is essential for high affinity antibody production,[37] whereas nonswitched memory B cells mainly develop from a less understood GC-independent route.[29,38] Expansions of both types of memory B cells indicate the potential involvement of both GC and extrafollicular response in patients with AR. However, we failed to find any significant correlation of switched or nonswitched B cell frequency with Der p-specific IgE level, suggesting that the functional status of memory B cells may be more important in determining IgE production.

Indeed, for the first time, we demonstrated here that in contrast to comparable levels of HLA-DR and CD25 expression on all examined B-cell subsets between AR patients and healthy subjects, CD23 expression frequency and density was elevated on B-cell subsets in AR patients. CD23 is a key molecule in bridging the T- and B-cell response and is essential for IgE-dependent antigen presentation, T-cell activation, B-cell proliferation, and IgE synthesis.[22] High-density expression of CD23 on B cells promotes allergen uptake and subsequent activation of T cells in allergic patients, which ultimately augment IgE synthesis.[39-41] However, whether CD23 on distinct B-cell subsets has different contributions to the development of allergic diseases is poorly understood. In this study, although we found a high expression level of CD23 on naive B cells, we failed to find its association with antigen-specific IgE and symptom severity in AR patients. On the contrary, we discovered significant correlations of $CD23^{+}$ switched and nonswitched memory B cells with antigen-specific IgE with a stronger correlation revealed for switched memory B cells than for nonswitched memory B cells. Moreover, $CD23^{+}$ switched memory B cells, but not $CD23^{+}$ nonswitched memory B cells, correlated with VAS symptom scores in patients with AR, highlighting a particular role of $CD23^{+}$ switch memory B cells in AR development. The exact role $CD23^{+}$ memory B cells in allergic diseases has received little attention previously. Our findings suggest that the $CD23^{+}$ switched memory B cells may be important in generating robust class-switched IgE in the case of secondary immune response in allergic diseases[6,38] and argue for the further study of $CD23^{+}$ switched memory B cells in allergic diseases in future.

CD23 expression can be regulated by IL-4.[22] However, the cell types responsible for the regulation of CD23 expression are unclear. The antigen-dependent phase of B-cell development occurs in the GC of secondary lymphoid tissues.[42] Tfh and Tfr cells are the specialized T-cell subsets that control B-cell function in lymph organs and reflected by their counterparts in peripheral blood.[13-15] In this study, we found that CD23 expression on switched memory B cells correlated with circulating Tfh2 and Tfr cells. We further found that Tfh2 cells from AR patients had a stronger capacity to induce CD23 expression on switched memory B cells by secreting IL-4, and AR-associated Tfr cells were defective in suppressing CD23 expression on switched memory B cells due to impaired IL-10 production. Therefore, the upregulated IgE production caused by Tfh2 and Tfr cell abnormality may be mediated, at least in part, through modulating $CD23^{+}$ switched memory B cells in AR. Interestingly, we found that VAS symptom scores, Der p-specific IgE levels, the frequencies of Tfh2 cells, and CD23 expression on switched memory B cells were clustered together in patients with AR in unsupervised hierarchical clustering analysis, further supporting an inter-correlation between these elements in AR.

The inhibition of CD23 expression on total B cells has been proposed as one of the mechanisms underlying the benefit of AIT; however, previous studies did not define the contribution of distinct B-cell subsets.[43,44] In this study, we revealed that AIT only significantly downregulated CD23 expression on memory B cells, with a more prominent effect on switched memory B cells, suggesting that disease modification by AIT in AR patients is mainly GC dependent and further highlighting the importance of $CD23^{+}$ switched memory B cells in AR pathogenesis. How AIT decrease the CD23 expression on switched memory B cells? Our previous study reported that AIT reestablished Tfh2 and Tfr cell balance in patients with AR,[12,13] which may contribute to the correction of CD23 on memory B cells by AIT. Nevertheless, our study does not preclude other possible mechanisms underlying the change of CD23 after AIT, such as cleavage of membrane CD23 caused by decreased IgE production.

In this study, there was no significant difference in CD25 or HLA-DR expression in all B-cell subsets between AR patients and HC, or between patients with and without AIT after treatment. Brisslert

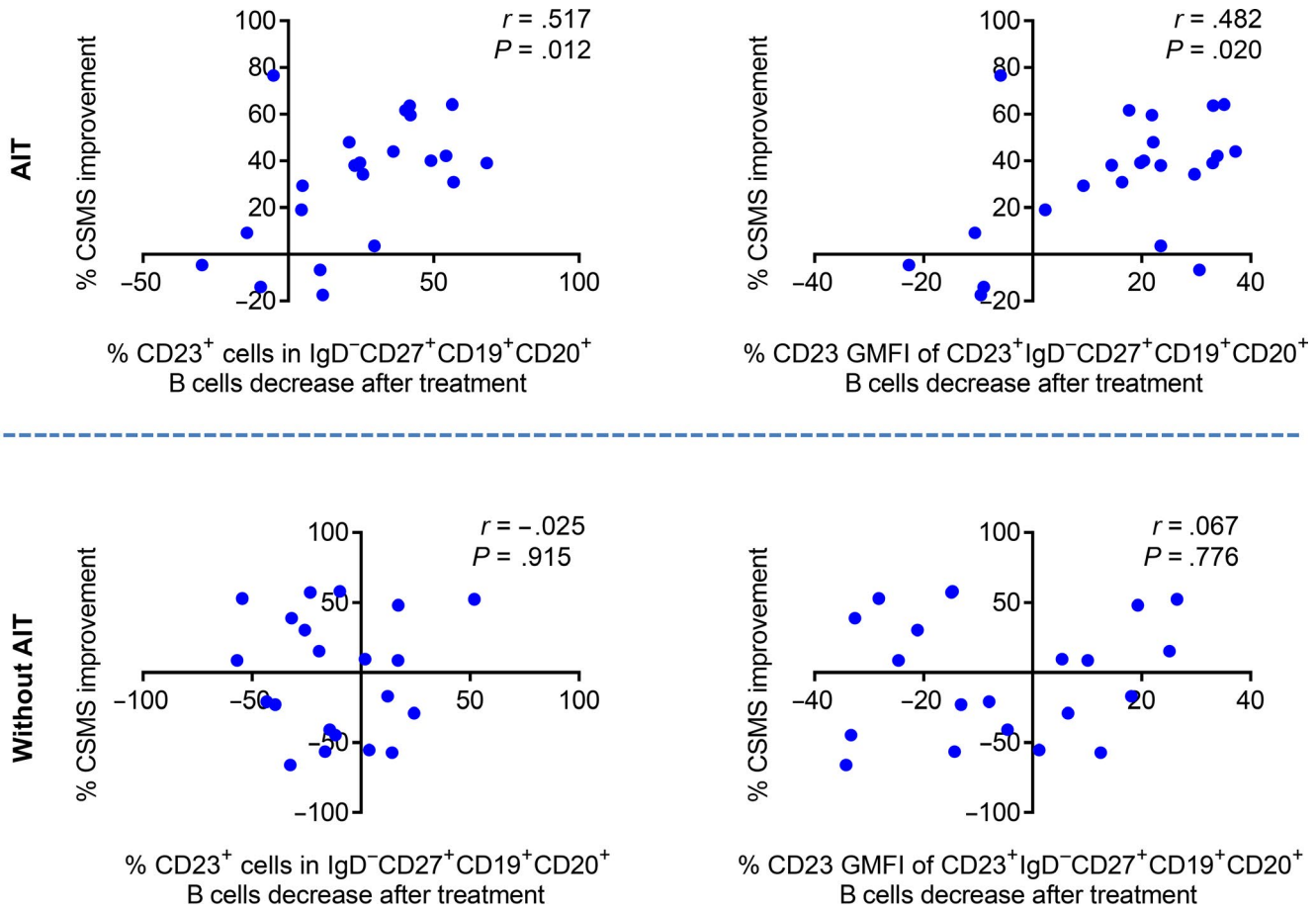


FIGURE 7 Correlations of reduction of frequencies of $CD23^+$ cells within switched memory B cells and geometric mean fluorescence intensity (GMFI) of CD23 of $CD23^+$ switched memory B cells with the improvement of combined symptom and medication score (CSMS) in AR patients with (n = 23) and without AIT (n = 21) after 12-month treatment

et al[21] reported that CD25 expression on B cells was selectively upregulated by Toll-like receptor agonists, suggesting that it is more relevant for infectious than for allergic condition. We found that HLA-DR was abundantly expressed on all types of B-cell subset, which is consistent with several human studies.[45,46] Comparable expression of HLA-DR on B-cell subsets between AR patients and HC reinforces the importance of CD23 in regulating antigen presentation in AR.

There are several limitations in our study. We did not present data on IgE^+ B cells. Owing to their scarcity and the interference by CD23 expression,[7] it is challenging to characterize the true IgE-secreting B cells. We noted a significantly expanded IgE^+ cells defined as $CD3^-CD19^+IgM^-IgD^-IgA^-IgG^-$ cells in patients with AR, as compared to HC (0.034% vs 0.011%, $P < .001$). However, two recent studies demonstrated that few of IgE^+ cells identified by flow cytometric analysis were true IgE^+ expressing cells when analyzed for IgE transcript at the single-cell level.[47,48] In addition, due to the limited amount of blood samples, we did not detect IgE-secreting B cells and antigen-specific B cells simultaneously. Nevertheless, it is critical and interesting to explore these B cells in AR patients in the future.

In conclusion, we demonstrated a potential role of Tfh2, Tfr, and $CD23^+$ switched memory B cell interaction in the regulation of antigen-specific IgE production and disease development in AR. CD23 expression particularly on switched memory B cells might be a candidate therapeutic target for AR.

CONFLICT OF INTEREST

YY has nothing to disclose. NW reports grants from National Natural Science Foundation of China (81800891) during the conduct of the study. CLC has nothing to disclose. LP has nothing to disclose. ZCW has nothing to disclose. JY has nothing to disclose. ZAC has nothing to disclose. YZ has nothing to disclose. STH has nothing to disclose. XYX has nothing to disclose. RFZ has nothing to disclose. DY has nothing to disclose. ZL reports grants from National Natural Science Foundation of China (81630024, 81570899, and 81920108011), grants from Natural Science Foundation of Hubei Province of China (2017CFA016), grants from "Ten thousand plan"—National high level talents special support plan, during the conduct of the study.

AUTHOR CONTRIBUTIONS

ZL designed the study and prepared the manuscript. YY performed flow cytometry, cell culture experiments, data analysis, and manuscript preparation; NW participated in sample collection, data analysis, and manuscript preparation; CLC, ZCW, STH, XYX, and RFZ

participated in sample collection and allergen immunotherapy; LP, ZAC, and YZ participated in data analysis. JY participated in data analysis and discussion and manuscript preparation. DY participated in data discussion. All authors were involved in final approval.

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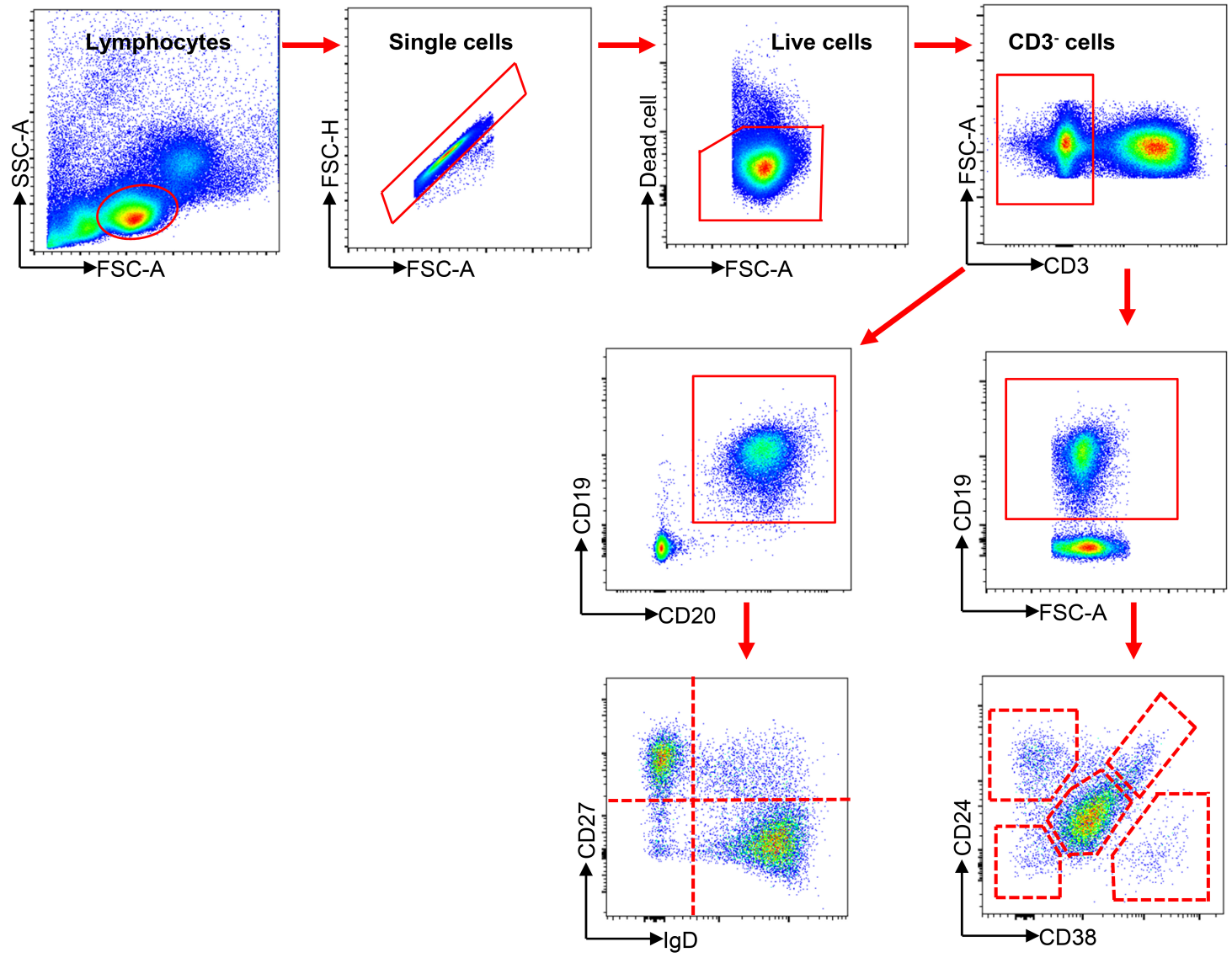
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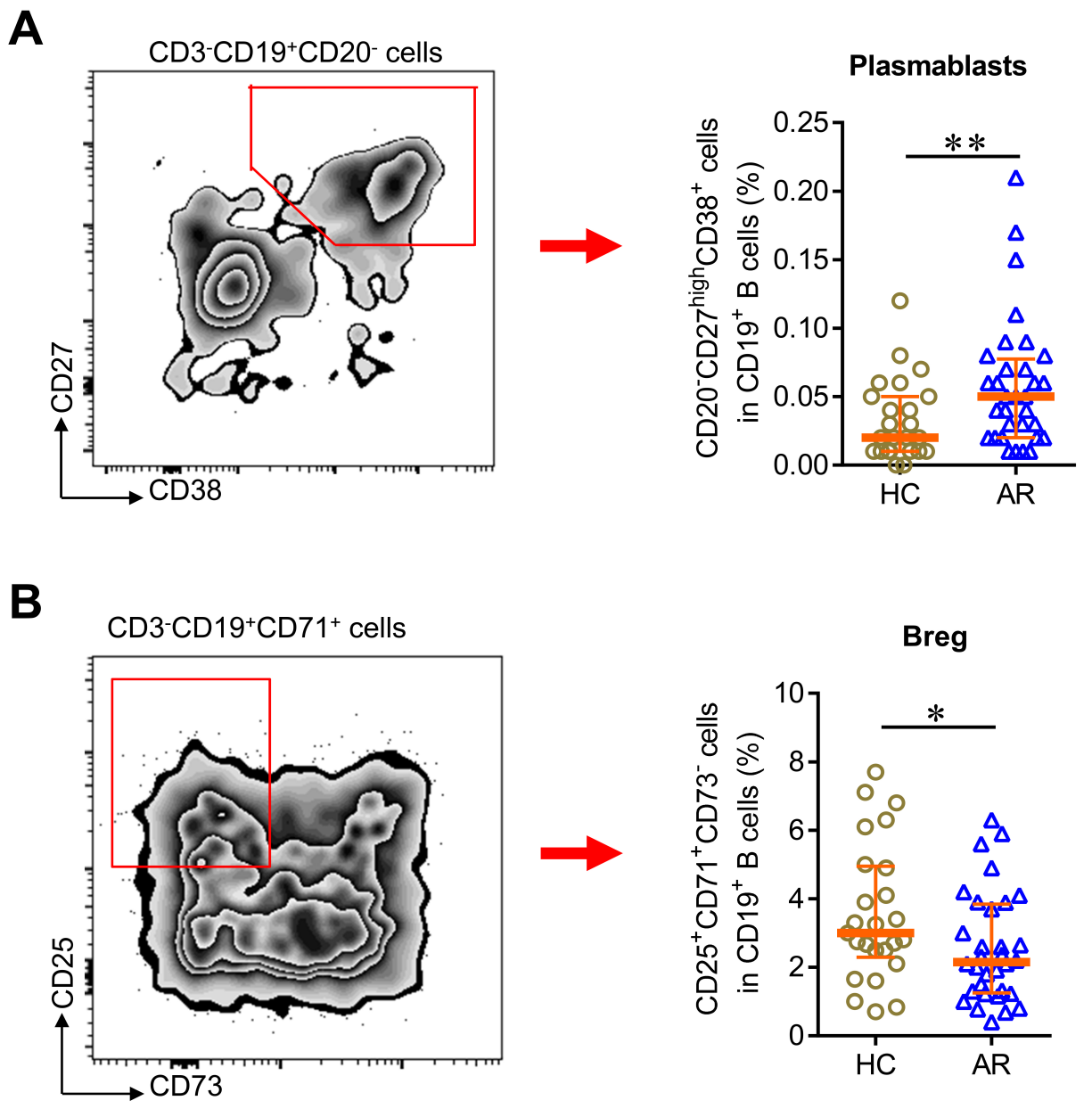
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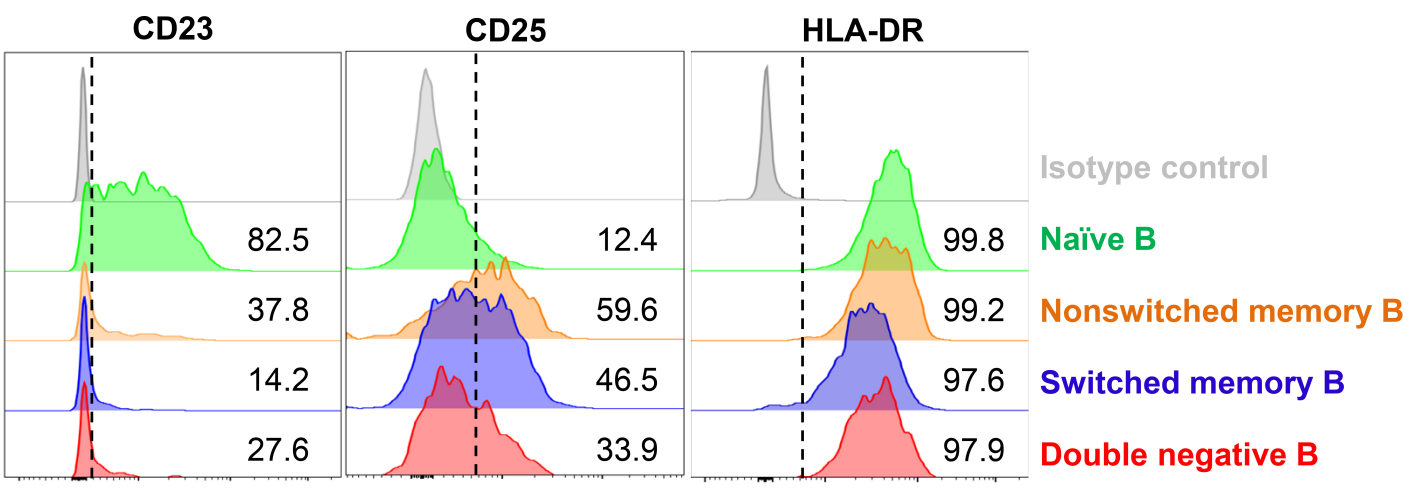
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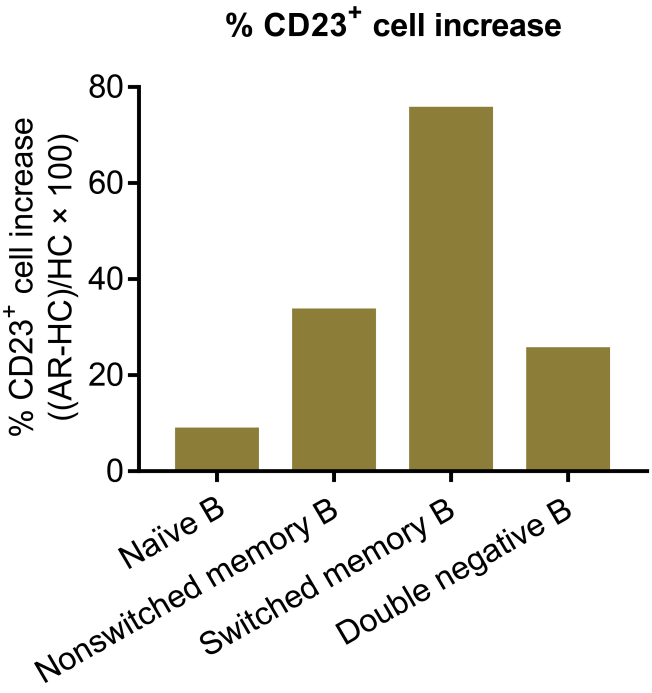
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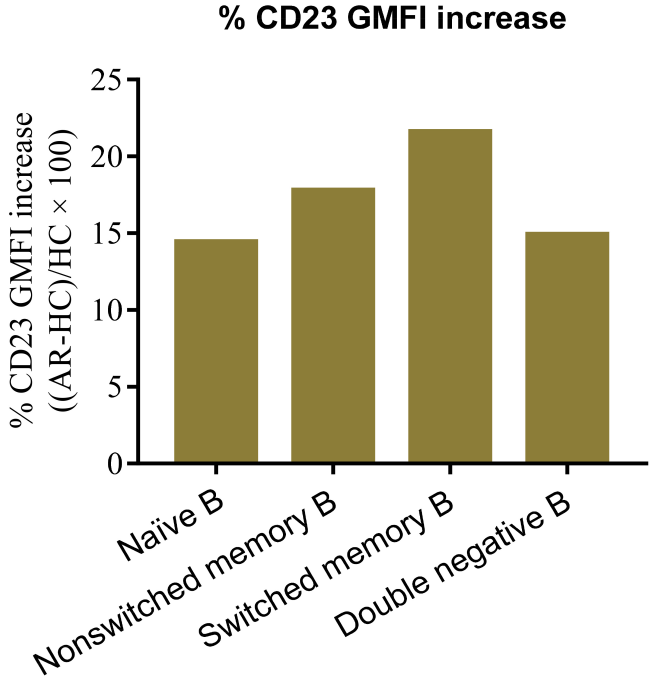
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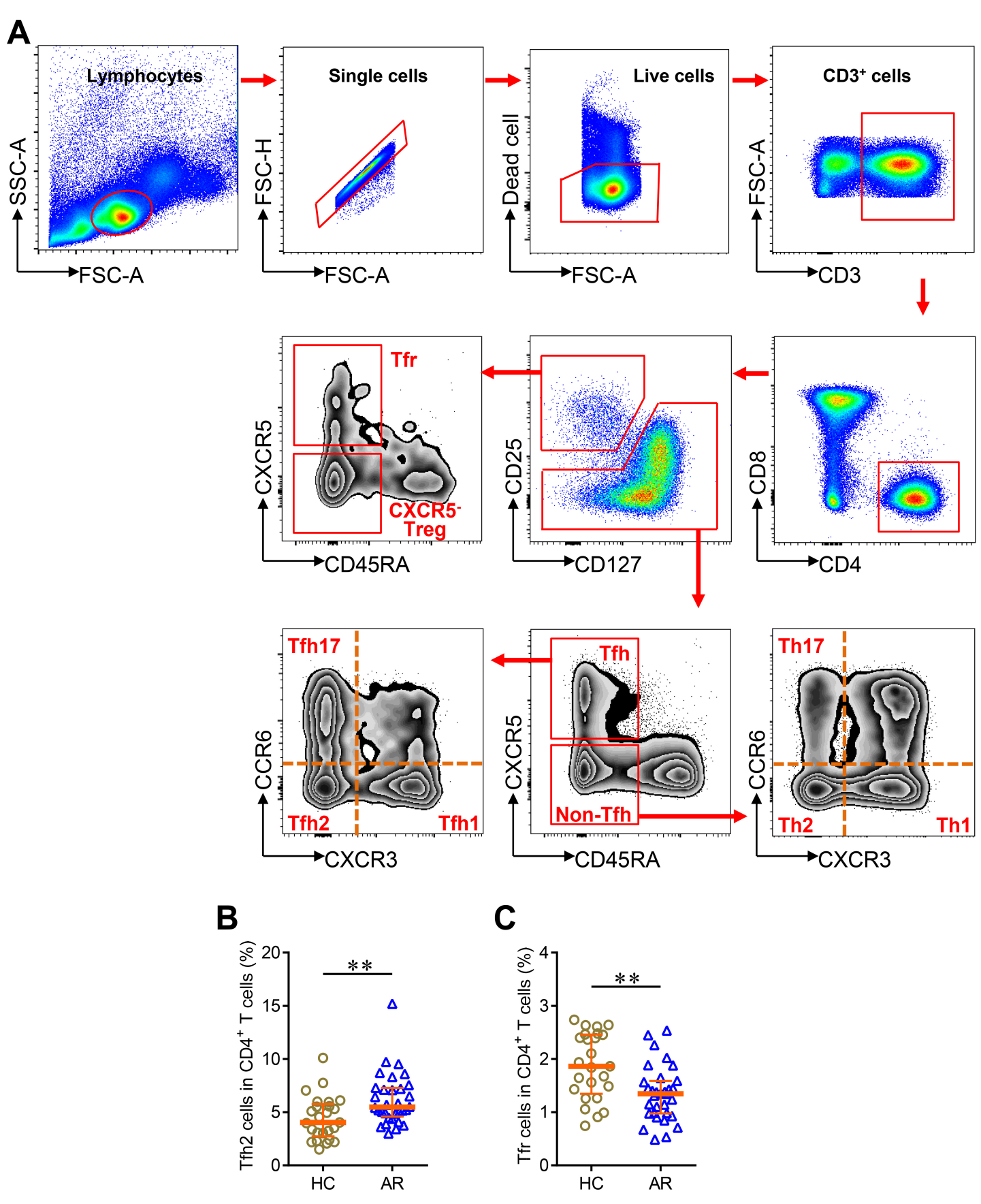

Lymphocytes
SSC-A
FSC-A
Single cells
FSC-H
FSC-A
Live cells
Dead cell
FSC-A
CD3- cells
FSC-A
CD3
CD19
CD20
CD19
FSC-A
CD27
IgD
CD24
CD38


A
CD3-CD19+CD20- cells
CD27
CD38
Plasmablasts
CD20-CD27highCD38+ cells in CD19+ B cells (%)
0.25
0.20
0.15
0.10
0.05
0.00
**
HC
AR
B
CD3-CD19+CD71+ cells
CD25
CD73
Breg
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HC
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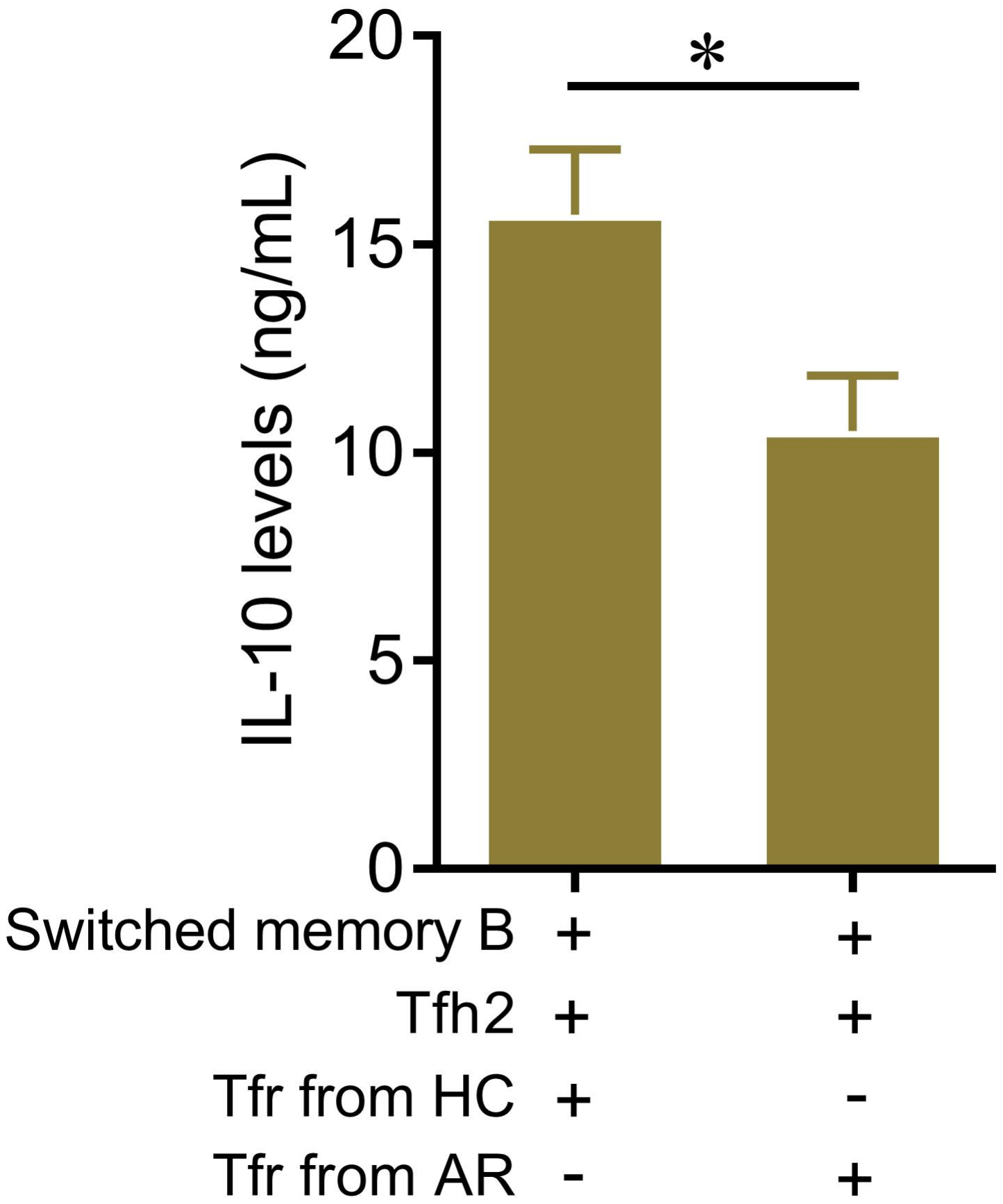

CD23
CD25
HLA-DR
82.5
37.8
14.2
27.6
12.4
59.6
46.5
33.9
99.8
99.2
97.6
97.9
Isotype control
Naïve B
Nonswitched memory B
Switched memory B
Double negative B

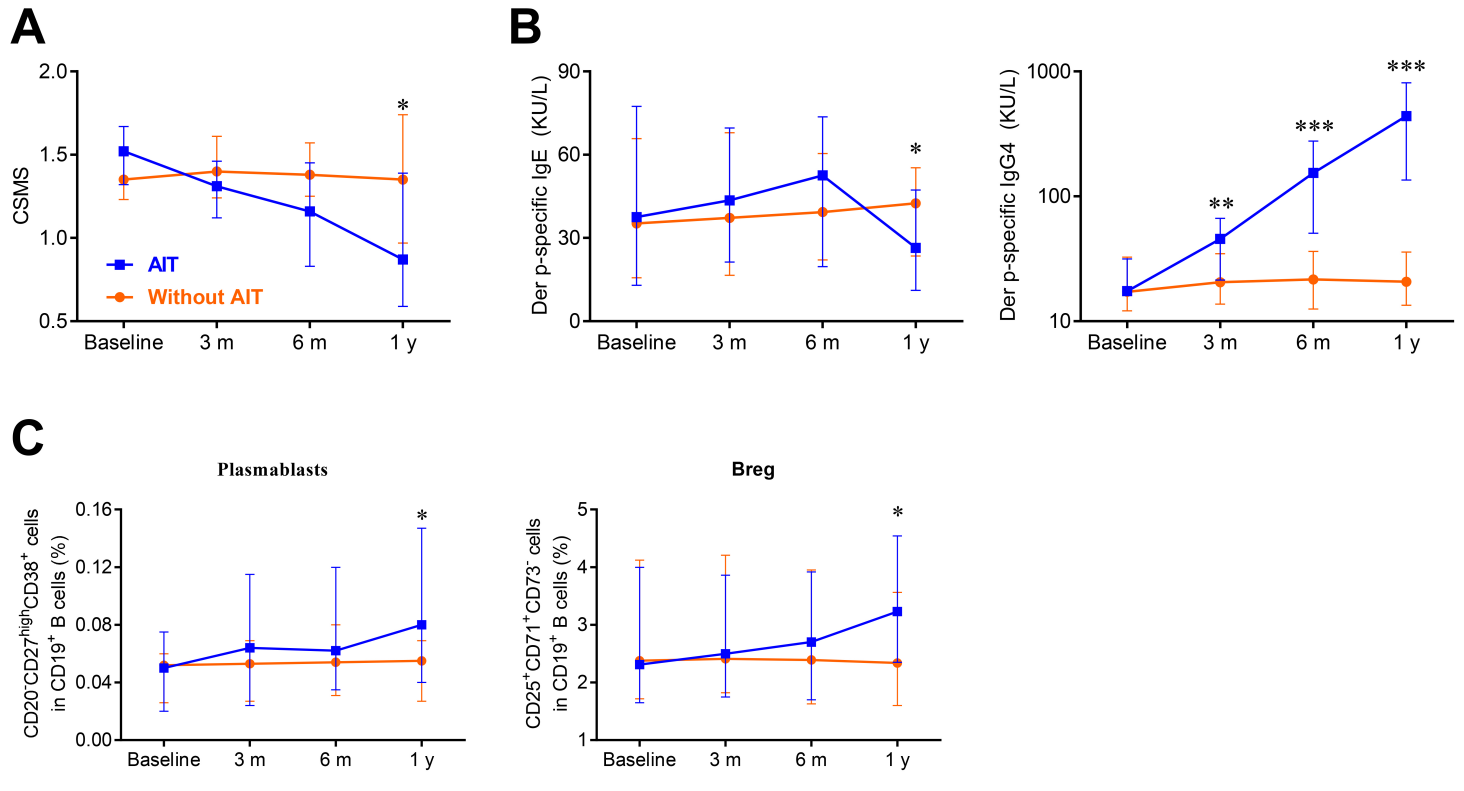

% CD23+ cell increase
% CD23+ cell increase ((AR-HC)/HC × 100)
80
60
40
20
0
Naïve B
Nonswitched memory B
Switched memory B
Double negative B


% CD23 GMFI increase
% CD23 GMFI increase ((AR-HC)/HC × 100)
25
20
15
10
5
0
Naïve B
Nonswitched memory B
Switched memory B
Double negative B


A
Lymphocytes
SSC-A
FSC-A
Single cells
FSC-H
FSC-A
Live cells
Dead cell
FSC-A
CD3+ cells
FSC-A
CD3
CD8
CD4
CD25
CD127
Tfr
CXCR5-
Treg
CXCR5
CD45RA
Tfh17
Tfh2
Tfh1
CCR6
CXCR3
Tfh
Non-Tfh
CXCR5
CD45RA
Th17
Th2
Th1
CCR6
CXCR3
B
Tfh2 cells in CD4+ T cells (%)
20
15
10
5
0
**
HC
AR
C
Tfr cells in CD4+ T cells (%)
4
3
2
1
0
**
HC
AR

CD23 on naïve B
CD23 GMFI of CD23+ naïve B
CD19+CD20+ B
sIgE
Tfr
Tfr/Tfh2
Th17
CD27+IgD+ NSM B
CD27+IgD- SM B
Tfh17
Th1
Th2
CD27-IgD- DN B
Tfh1
Breg
CD27-IgD+ naïve B
CD23 on DN B
CD23 GMFI of CD23+ DN B
Tfh2
VAS score
CD23 on NSM B
CD23 GMFI of CD23+ NSM B
CD23 on SM B
CD23 GMFI of CD23+ SM B
Plasmablasts
CXCR5- Treg
CXCR5- Treg/Th2
-1
0
1


20
15
10
5
0
IL-10 levels (ng/mL)
*
Switched memory B + +
Tfh2 + +
Tfr from HC + -
Tfr from AR - +


A
2.0
1.5
1.0
0.5
CSMS
AIT
Without AIT
*
Baseline
3 m
6 m
1 y
B
90
60
30
0
Der p-specific IgE (KU/L)
*
Baseline
3 m
6 m
1 y
1000
100
10
Der p-specific IgG4 (KU/L)
**
***
***
Baseline
3 m
6 m
1 y
C
Plasmablasts
0.16
0.12
0.08
0.04
0.00
CD20-CD27high CD38+ cells in CD19+ B cells (%)
*
Baseline
3 m
6 m
1 y
Breg
5
4
3
2
1
CD25+CD71+CD73- cells in CD19+ B cells (%)
*
Baseline
3 m
6 m
1 y

Online Data Supplement

CD23 expression on switched memory B cells bridges T-B cell interaction in allergic rhinitis

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METHODS

Subjects

Adult allergic rhinitis (AR) patients allergic to house dust mites (HDM) only and healthy subjects were recruited with the approval of the Ethics Committee of Tongji Hospital and China Resources & Wisco General Hospital. Written informed consents were obtained for all participants before entering the study. Thirty-one healthy controls (HC) and 38 patients with AR participated in the study of abundance and phenotype of B cells in AR (Table S1). Peripheral bloods were obtained from those subjects. In addition, 44 patients with AR were enrolled for study of allergen immunotherapy (AIT). Among them, 23 patients received AIT in combination with standard medication treatment, and 21 patients who refused AIT received standard medication treatment only (Table S2). Peripheral bloods were collected at baseline (prior to treatment) and after 3-, 6- and 12-month treatment of AIT. Peripheral bloods were under centrifuged at 3000 rpm, 20℃ for 10 minutes, and then plasma were collected and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) as previously mentioned.[S1] Plasma were stored at -80 and PBMCs were kept frozen in liquid nitrogen until use. Blood samples were processed and data were analyzed by investigators who were blinded to clinical data of participants.

As previously we described,[S1,2] inclusion criteria for patients with AR included: (i) diagnosis of AR according to the Allergic Rhinitis and its Impact on Asthma (ARIA) guideline[S3] and, (ii) > 1 year history of AR, (iii) allergic to *Dermatophagoides*

pteronyssinus (Der p) identified by skin prick tests and the levels of Der p specific immunoglobulin E in serum ≥ 0.7 KU/L (Phadia ImmunoCAP, Uppsala, Sweden). The exclusion criteria included: (i) sensitization to other allergens than HDM (Der p and *Dermatophagoides farina* (Der f)), such as pollens and fungi, (ii) the presence of other allergic diseases, such as asthma based on ARIA and Global Initiative for Asthma guideline,[S4] (iii) chronic rhinosinusitis or chronic infection, (iv) pregnancy or breastfeeding, (v) previous treatment with immunotherapy, (vi) with severe immunologic disease, tumors, or chronic infection, and (vii) used intranasal steroid or antihistamines in the past 1 week, or oral steroids in the past 3 months, prior to this study.

A visual analog scale (VAS) ranging from 0 (not at all bothersome) to 10 cm (extremely bothersome) was used to quantify the global perception of severity of combined nasal symptoms. HC had no history of allergy and had negative skin prick test to a panel of 19 allergen extracts common in our region.[S1]

Skin prick test (SPT)

SPT was performed as we previously described.[S1] A panel of 19 inhalant allergens, including HDM (Der p and Der f), cockroach, mulberry silk, animal dander (cat, dog, sheep, and horse), tree pollens (*Sabina*, *Platanus*, *Populus*, and *Cryptomeria*), weed pollens (*Artemisia*, *Ambrosia*, and *Humulus*), and fungi (*Alternaria*, *Cladosporium*, *Aspergillus*, and *Paecilomyces*) (Macro-Union Pharmaceutical Co., Beijing, China), were used. A positive histamine (10 mg/mL)

control and negative diluent (saline) control were included. The positive result defined as a wheal with a diameter 3 mm larger than that elicited by the negative control 15 minutes after application.[S1]

Allergen immunotherapy

Subcutaneous AIT was performed with the semi-depot HDM allergen extracts (full allergens, 50% Der p plus 50% Der f; Allergopharma GmbH, Reinbek, Germany) according to manufacturers' recommendation. The treatment consists of build-up phase and maintenance phase. Administration started with the minimum dose, followed by step by step increase to achieve maintenance doses. The injection interval was gradually extended to 4-8 weeks for the maintenance phase. The maintenance doses varied among patients, but the absolute highest dose is 1 mL injection with 5000 TU/mL extracts. The patient was confirmed availability for at least 30 minutes following injections. Over the course of the study, all patients were undergoing standard pharmacotherapy as recommended.[S3]

Symptom and medication use assessment

Symptom and medication use were assessed as we previously mentioned.[S1] Participants involved in AIT study were provided with a daily record card for recording the rhinoconjunctivitis symptom scores (RCSS), including nasal itching, sneezing, rhinorrhea, nasal obstruction, ocular itching, and ocular tearing, and medication use.[S1] Each symptom is scored between 0 and 3. The daily RCSS was the

sum of the 6 symptom scores. The daily rescue medication score (MS) was calculated as follows: 1, oral and/or topical (eyes or nose) nonsedative H1 antihistamines (H1A); 2, intranasal corticosteroids (INS) with/without H1A; 3, oral corticosteroids with/without INS, and with/without H1A. The daily combined symptom and medication score (CSMS) was calculated as follows: RCSS/6 + MS, for the previous month of scheduled visits (baseline, and 3-, 6- and 12-month).[S1] After 12-month of AIT, the improvement of CSMS was calculated as following: (CSMS before treatment - CSMS after 1 year) / (CSMS before treatment) × 100%.[S1]

Flow cytometry

PBMCs were first blocked with 5% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) for 10 min on ice. Then, PBMCs were stained with Zombie Aqua (Biolegend, San Diego, CA, USA) or fixable viability stain 700 (BD Bioscience, San Jose, CA, USA) to exclude dead cells. For surface staining, cells were incubated with fluorochrome-labeled antibodies for 30 min at 25 °C. For IL-10 staining, cells were permeabilized by using Cytofix/Cytoperm (BD Bioscience) for 20 min on ice after surface staining. After that, cells were stained with fluorochrome-labeled anti-IL-10 antibody for 30 min at 4 °C. Isotype controls were used as negative controls. Fluorescence minus one control for each fluorochrome was used to set the gate for positive populations. Data were analyzed using a BD LSRFortessa™ X-20 (BD Bioscience) instrument and FlowJo software (TreeStar, Ashland, OR, USA) as previously described.[S1] Antibodies used in flow cytometry are

listed in Table S3.

Within live $CD3^-CD19^+CD20^+$ lymphocytes cells, peripheral B cells were divided into $CD27^-IgD^+$ naïve B cells, $CD27^+IgD^+$ nonswitched memory B cells, $CD27^+IgD^-$ switched memory B cells, and $CD27^-IgD^-$ double negative B cells.[S5] Within live $CD3^-CD19^+$ lymphocytes, peripheral B cells were classified as $CD24^{int}CD38^{int}$ mature naïve, $CD24^-CD38^-$ new memory, $CD24^+CD38^-$ primarily memory, $CD24^-CD38^+$ plasmablasts, and $CD24^{high}CD38^{high}$ transitional B cells.[S5] The absolute cell numbers of B cells in bloods were calculated based on the complete blood cell counts, lymphocyte frequencies within white blood cells, and B-cell subset frequencies within the lymphocyte population as previously reported.[S6]

Within live $CD3^+CD4^+$ T cells, circulating Tfh, non-Tfh, Tfr, and $CXCR5^-$ Treg cells were defined as $CD45RA^{low}CXCR5^+CD25^{-/low}CD127^{low/high}$, $CD45RA^{low}CXCR5^-CD25^{-/low}CD127^{low/high}$, $CD45RA^{low}CXCR5^+CD25^{high}CD127^{low}$, and $CD45RA^{low}CXCR5^-CD25^{high}CD127^{low}$ cells, respectively, as previously reported.[S1,2] Tfh and non-Tfh cell subsets were identified based on the expression of CCR6 and CXCR3 as previous described.[S5] Type 1 Tfh (Tfh1) cells and type 1 non-Tfh (Th1) cells are $CCR6^-CXCR3^+$ cells, type 2 Tfh (Tfh2) cells and type 2 non-Tfh (Th2) cells are $CCR6^-CXCR3^-$ cells, and type 17 Tfh (Tfh17) cells and type 17 non-Tfh (Th17) cells are $CCR6^+CXCR3^-$ cells. To examine IL-10 expression in Tfr cells, PBMCs (5×10^6 cells) were stimulated with 10 ng/mL PMA and 1 μg/mL ionomycin for 6 hr in the presence of 5 μg/mL brefeldine and 1.7 ng/mL monensin (eBioscience, San Diego, Calif, USA) for the last 4 hr. After stimulation, the cells

were stained for surface and intracellular markers and subjected to flow cytometry as mentioned above.

Cell culture

Autologous or allogeneic cells were used for cell co-culture depending on the purpose of the experiment.[S1,2] To avoid potential immune responses caused by allogeneic cells in co-culture, we prefer autologous cells firstly. However, if we aimed to explore the difference in T cells from AR patients and HC, allogenic B cells from same subjects were used for co-culture with AR- or HC-derived T cells in order to minimize the variation caused by AR- and HC-derived B cells. To compare the function of Tfh2 cells between HC and patients with AR in inducing CD23 expression on switched memory B cells, sorted switched memory B cells (2.5×10^4/well) from healthy subjects were co-cultured with allogenic Tfh2 cells (5×10^4/well) from healthy subjects or Tfh2 cells from patients with AR for 2 days in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% heat inactivated FBS (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Sigma Aldrich, St Louis, Mo, USA) in the presence of *Staphylococcal enterotoxin* B (1 μg/mL; Toxin Technology Inc., Sarasota, FL, USA) in U-bottomed 96-well plates as previously described.[S2] According to previous studies,[S6] 2 days was selected for cell culture to determine cytokines levels in supernatant and CD23 expression on memory B cells. In blocking experiments, sorted switched memory B cells (2.5×10^4/well) from AR patients were co-cultured with autologous Tfh2 cells (5×10^4/well), and anti-IL-4 monoclonal

antibody (1 μg/mL; R&D Systems, Minneapolis, MN, USA), or mouse IgG2B (R&D Systems) control was added as previously described.[S2]

To compare the function of Tfr cells between HC and patients with AR in suppressing CD23 expression on switched memory B cells, sorted switched memory B cells (2.5×10^4/well) and Tfh2 cells (5×10^4/well) from patients with AR were co-cultured with allogenic Tfr cells (5×10^4/well) from healthy subjects or patients with AR for 2 days in the cell culture condition mentioned above. In blocking experiments, Tfh2 cells and switched memory B cells from AR patients were co-cultured with Tfr cells from healthy subjects, and anti-IL-10 monoclonal antibody (1 μg/mL; R&D Systems) or mouse IgG2B control (R&D Systems) was added as previously described.[S2] After culture, cells were collected and marked with anti-CD4 and anti-CD20 antibodies, and CD23 expression on $CD4^-CD20^+$ B cells was detected by flow cytometry. The IL-10 levels in culture supernatants were detected by using commercial ELISA kits (R&D Systems).

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198

Figure legends

Fig S1. Flow cytometric gating strategies of B-cell subsets in human peripheral bloods. Within live $CD3^-CD19^+CD20^+$ cells, B cells were defined as $CD27^-IgD^+$ naïve, $CD27^+IgD^+$ nonswitched memory, $CD27^+IgD^-$ switched memory, and $CD27^-IgD^-$ double negative B cells. Within live $CD3^-CD19^+$ lymphocytes, B cells were classified as $CD24^{int}CD38^{int}$ mature naïve, $CD24^-CD38^-$ new memory, $CD24^+CD38^-$ primarily memory, $CD24^-CD38^+$ plasmablasts, $CD24^{high}CD38^{high}$ transitional B cells.

Fig S2. Frequencies of $CD19^+CD20^-CD27^{high}CD38^+$ plasmablasts and $CD19^+CD25^+CD71^+CD73^-$ regulatory B (Breg) cells within $CD19^+$ B cells in patients with AR (n = 32) and healthy controls (HC, n = 25). Representative flow cytometric plots are shown.*P < 0.05, **P < 0.01.

Fig S3. The representative histograms of CD23, CD25, and HLA-DR expression on B-cell subsets in peripheral blood of healthy subjects. The numbers in figures refer to the percentage of positive cells.

Fig S4. The increase percentages of CD23 expression on B-cell subsets in AR patients in comparison with that in healthy controls (HC). % $CD23^+$ cell increase = (frequency of $CD23^+$ cells in AR – frequency of $CD23^+$ cells in HC)/(frequency of $CD23^+$ cells in HC) × 100; % CD23 GMFI increase = (CD23 GMFI in AR - CD23 GMFI in HC)/(CD23 GMFI in HC) × 100. n = 32 for AR patients and n = 25 for HC.

Fig S5. Flow cytometric gating strategies of T-cell subsets in human peripheral bloods and changes of type 2 follicular helper T (Tfh2) cells and follicular regulatory T (Tfr) cells in patients with AR. (A) Within live $CD3^{+}CD8^{-}CD4^{+}$ cells, Tfr cells were defined as $CD45RA^{low}CXCR5^{+}CD25^{high}CD127^{low}$ cells, $CXCR5^{-}$ regulatory T (Treg) cells were defined as $CD45RA^{low}CXCR5^{-}CD25^{high}CD127^{low}$ cells, Tfh cells were defined as $CD45RA^{low}CXCR5^{+}CD25^{-/low}CD127^{low/high}$ cells, and non-Tfh cells were defined as $CD45RA^{low}CXCR5^{-}CD25^{-/low}CD127^{low/high}$ cells. Subsets of non-Tfh (also named Th) and Tfh cells were defined based on the expression of CXCR3 and CCR6. Frequencies of Tfh2 **(B)** and Tfr **(C)** cells within total $CD4^{+}$ T cells in AR patients (n = 32) and healthy controls (HC, n = 25). $^{**}P < 0.01$.

Fig S6. Correlation matrix in healthy subjects. Spearman correlation plot demonstrates unsupervised hierarchical clustering of B-cell frequencies, T-cell frequencies, *Dermatophagoides pteronyssinus* (Der p)-specific IgE levels and visual analog scale (VAS) scores in healthy subjects. Dendrograms are shown as a tree, representing the distance between variables. The correlation matrix shows the positive (blue) or negative (red) correlations of all parameters, with color intensity and circle size reflecting correlation strength. n = 25. Breg, $CD19^{-}CD25^{+}CD71^{+}CD73^{-}$ regulatory B; DN, double negative; GMFI, geometric mean fluorescence intensity; SM, switched memory; NSM, nonswitched memory; Plasmablasts,

243 $CD19^{+}CD20^{-}CD27^{high}CD38^{+}$ plasmablasts; sIgE, Der p-specific IgE; Th, helper T;
244 Treg, regulatory T; Tfh: follicular helper T; Tfr: follicular regulatory T. *P < 0.05,
245 **P < 0.01, ***P < 0.001.

246

247 **Fig S7. IL-10 levels in supernatants in Tfr-Tfh2-B cells co-culture system.** Tfh2
248 cells (5×10^4/well) and switched memory B cells (2.5×10^4/well) from same AR
249 patients were co-cultured with allogenic Tfr cells (5×10^4/well) from HC or patients
250 with AR (n = 5) for 2 days. IL-10 levels in supernatant were detected by ELISA after
251 culture. Data are presented as mean ± SEM. *P < 0.05.

252

253 **Fig S8. Change of symptom and medication scores, immunoglobulin levels, and**
254 **B-cell subsets in AR patients with and without AIT after treatment. (A)** Change of
255 the combined symptom and medication score (CSMS) after treatment. **(B)** Change of
256 serum *Dermatophagoides pteronyssinus* (Der p)-specific IgE and Der p-specific IgG4
257 levels after treatment. **(C)** Change of frequencies of $CD19^{+}CD20^{-}CD27^{high}CD38^{+}$
258 plasmablasts and $CD19^{+}CD25^{+}CD71^{+}CD73^{-}$ Breg cells within $CD19^{+}$ B cells after
259 treatment. n = 23 for patients with AIT; n = 21 for patients without AIT. *P < 0.05,
260 **P < 0.01, ***P < 0.001.

261

Table S1. Demographic characteristics of subjects in the study of B-cell signature in patients with AR in comparison to healthy controls

	Healthy subjects	AR patients	*p* value
Total subjects, n	31	38	-
Gender, male/female	17/14	18/20	0.537
Age (years)	27 (24, 41)	29 (25, 40)	0.431
SPT to Der p	Negative	Positive	-
Specific IgE to Der p (KU/L)	0.1 (0.1, 0.2)	40.4 (17.8, 69.7)	< 0.001
VAS symptom score	0 (0, 0)	7.0 (6.0, 9.0)	< 0.001

For continuous variables, data are expressed as medians and interquartile ranges.

SPT, skin prick test; Der p, *Dermatophagoides pteronyssinus*.

Table S2. The baseline characteristics of AR patients in AIT study before treatment

	With AIT	Without AIT	*p* value
Total subjects, n	23	21	-
Gender, male/female	11/12	13/8	0.349
Age (years)	29 (22, 39)	30 (25, 43)	0.398
SPT to Der p	Positive	Positive	-
Specific IgE to Der p (KU/L)	37.5 (12.9, 77.5)	35.2 (15.6, 65.8)	0.829
Specific IgG4 to Der p (KU/L)	17.4 (7.9, 31.6)	17.2 (12.1, 32.6)	0.767
VAS symptom score	7.0 (6.0, 8.0)	7.0 (5.0, 8.0)	0.667
CSMS	1.7 (1.4, 2.1)	1.6 (1.4, 2.1)	0.821

For continuous variables, data are expressed as medians and interquartile ranges.

AIT, allergen immunotherapy; SPT, skin prick test; Der p, *Dermatophagoides pteronyssinus*; GSMS, combined symptom and medication score.

272 **Table S3. Antibodies used for flow cytometry and cell sorting**

Antibody	Parameter	Clone ID	Source	Isotype	Manufacturer	Dilution
CD3	FITC	UCHT1	mouse	IgG1, κ	BD Biosciences (San Jose, CA, USA)	1:200
CD3	Brilliant Violet 650	UCHT1	mouse	IgG1, κ	BD Biosciences	1:200
CD4	APC-Cy7	RPA-T4	mouse	IgG1, κ	BD Biosciences	1:200
CD45RA	Brilliant Violet 605	HI100	mouse	IgG2b, κ	BD Biosciences	1:200
CXCR5	BB515 (FITC)	RF8B2	Rat	IgG2b, κ	BD Biosciences	1:100
CD25	Brilliant Violet 421	BC96	mouse	IgG1, κ	Biolegend (San Diego, CA, USA)	1:200
CD25	PE	M-A251	mouse	IgG1, κ	Biolegend	1:200
CD127	Brilliant Violet 421	HIL-7R-M21	mouse	IgG1, κ	BD Biosciences	1:100
CD127	PE	HIL-7R-M21	mouse	IgG1, κ	BD Biosciences	1:100
CXCR3	APC	G025H7	mouse	IgG1, κ	Biolegend	1:200
CCR6	PerCP-Cy5.5	11A9	mouse	IgG1, κ	BD Biosciences	1:100
CD19	APC-Cy7	SJ25C1	mouse	IgG1, κ	BD Biosciences	1:100
CD20	Brilliant Violet 510	2H7	mouse	IgG2b, κ	BD Biosciences	1:200
CD20	PE/Dazzle™ 594	2H7	mouse	IgG2b, κ	Biolegend	1:200
CD27	PE-Cy7	M-T271	mouse	IgG1, κ	BD Biosciences	1:100
CD38	BB515 (FITC)	HIT2	mouse	IgG1, κ	BD Biosciences	1:100
IgD	PerCP-Cy5.5	IA6-2	mouse	IgG2a, κ	BD Biosciences	1:200
IgD	Alexa Fluor® 647	IA6-2	mouse	IgG2a, κ	Biolegend	1:200
CD24	PE	ML5	mouse	IgG2a, κ	BD Biosciences	1:200

CD23	Brilliant Violet 421	M-L233	mouse	IgG1, κ	BD Biosciences	1:200
HLA-DR	FITC	L243	mouse	IgG2a, κ	Biolegend	1:100
HLA-DR	Brilliant Violet 421	G46-6	mouse	IgG2a, κ	BD Biosciences	1:200
CD71	PE	CY1G4	mouse	IgG2a, κ	Biolegend	1:100
CD73	PerCP-Cy5.5	AD2	mouse	IgG1, κ	Biolegend	1:100

PE, phycoerythrin; APC, allophycocyanin; PerCP, peridinin chlorophyll protein complex; FITC, fluorescein isothiocyanate.

Table S4. Expression of CD23, CD25 and HLA-DR on B-cell subsets in peripheral bloods of healthy subjects and patients with AR

B-cell subsets	Healthy subjects	AR patients	*P* value
$CD23^+$ cells in $CD27^-IgD^+$ naive B cells (%)	78.3 (72.8, 85.8)	85.0 (80.4, 89.7)	**0.017**
CD23 GMFI of $CD23^+CD27^-IgD^+$ naïve B cells	1760.2 (1445.3, 2077.5)	2019.1 (1667.8, 2353.5)	**0.024**
$CD25^+$ cells in $CD27^-IgD^+$ naive B cells (%)	12.5 (10.5, 18.1)	11.5 (8.8, 15.3)	0.316
CD25 GMFI of $CD25^+CD27^-IgD^+$ naive B cells	940.1 (895.5, 967.8)	902.0 (876.9, 947.3)	0.315
$HLA\text{-}DR^+$ cells in $CD27^-IgD^+$ naive B cells (%)	99.8 (99.6, 99.9)	99.9 (99.5, 100.0)	0.612
HLA-DR GMFI of $HLA\text{-}DR^+CD27^-IgD^+$ naive B cells	8393.2 (7469.5, 9991.1)	8556.4 (7354.0, 9215.7)	0.626
$CD23^+$ cells in $CD27^+IgD^+$ nonswitched memory B cells (%)	36.3 (28.4, 46.1)	48.4 (40.9, 53.9)	**0.005**
CD23 GMFI of $CD23^+CD27^+IgD^+$ nonswitched memory B cells	1041.2 (852.3, 1170.3)	1262.5 (977.3, 1428.5)	**0.011**
$CD25^+$ cells in $CD27^+IgD^+$ nonswitched memory B cells (%)	58.0 (55.1, 62.3)	54.9 (49.1, 62.1)	0.261
CD25 GMFI of $CD25^+CD27^+IgD^+$ nonswitched memory B cells	1187.4 (1126.2, 1265.7)	1135.9 (1089.0, 1211.6)	0.155
$HLA\text{-}DR^+$ cells in $CD27^+IgD^+$ nonswitched memory B cells (%)	97.9 (96.8, 98.6)	97.7 (96.2, 99.3)	0.990
HLA-DR GMFI of $HLA\text{-}DR^+CD27^+IgD^+$ nonswitched memory B cells	7453.6 (6250.2, 8744.1)	7847.5 (6589.9, 8913.3)	0.867
$CD23^+$ cells in $CD27^+IgD^-$ switched memory B cells (%)	15.4 (12.3, 24.3)	27.0 (19.5, 32.9)	**<0.001**
CD23 GMFI of $CD23^+CD27^+IgD^-$ switched memory B cells	659.2 (605.3, 755.6)	822.1 (737.2, 898.8)	**<0.001**
$CD25^+$ cells in $CD27^+IgD^-$ switched memory B cells (%)	49.2 (40.3, 54.0)	48.2 (42.2, 53.1)	0.729
CD25 GMFI of $CD25^+CD27^+IgD^-$ switched memory B cells	1120.4 (1048.1, 1166.0)	1108.8 (1064.3, 1163.7)	0.870
$HLA\text{-}DR^+$ cells in $CD27^+IgD^-$ switched memory B cells (%)	97.6 (95.2, 98.5)	97.5 (96.2, 99.2)	0.377
HLA-DR GMFI of $HLA\text{-}DR^+CD27^+IgD^-$ switched memory B cells	6210.5 (5815.4, 7334.8)	6528.3 (5714.2, 7491.6)	0.646
$CD23^+$ cells in $CD27^-IgD^-$ double negative B cells (%)	30.9 (27.2, 39.5)	38.7 (31.1, 47.6)	**0.016**
CD23 GMFI of $CD23^+CD27^-IgD^-$ double negative B cells	1033.2 (923.0, 1131.2)	1162.5 (995.4, 1344.3)	**0.027**
$CD25^+$ cells in $CD27^-IgD^-$ double negative B cells (%)	32.8(27.9, 38.9)	35.6 (25.5, 40.7)	0.808
CD25 GMFI of $CD25^+CD27^-IgD^-$ double negative B cells	1013.6 (969.7, 1077.5)	1043.8 (961.5, 1103.3)	0.445
$HLA\text{-}DR^+$ cells in $CD27^-IgD^-$ double negative B cells (%)	96.9 (94.6, 99.0)	98.1 (96.7, 99.3)	0.223
HLA-DR GMFI of $HLA\text{-}DR^+$ $CD27^-IgD^-$ double negative B cells	7058.0 (6176.6, 8943.7)	8045.5 (6592.3, 9350.1)	0.266

Data are expressed as medians and interquartile ranges. Numbers in red color indicate P value of less than 0.05.