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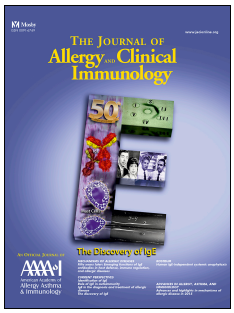

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Correlation of allergen-specific T follicular helper cells with specific IgE and efficacy of allergen immunotherapy

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Running title: Allergen-specific Tfh cells in AR

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Key words: Allergic rhinitis, T follicular helper cells, immunoglobulin E, allergen-specific, immunotherapy

Capsule summary: Allergen-specific IL-4^+ Tfh cells may contribute to allergen-specific IgE production and correlate with clinical efficacy of AIT in AR patients, which may be a promising therapeutic target and biomarker for AIT in AR.

To the editor:

Allergic rhinitis (AR) is caused by an immunoglobulin E (IgE)-mediated hypersensitivity reaction to airborne allergens. Type 2 T helper (Th2) cells have long been considered to regulate B-lymphocyte class-switch recombination to IgE by secreting cytokine IL-4 or IL-13 until a novel $CD4^+$ T cell subset, named follicular helper T (Tfh) cells largely on the basis of their localization in B-cell follicles and germinal centers, has recently been recognized as the central player in regulating B cells to support antibody response.[1] Mouse studies indicate that Tfh cells, but not Th2 cells, regulate IgE production in allergic asthma.[1] We demonstrated that nasal $IL\text{-}4^+$ Tfh cells correlate to local IgE production in eosinophilic nasal polyps.[2] Kamekura *et al.* reported a skewed Tfh phenotype towards type 2 polarization in peripheral blood of AR patients.[3] However, how allergen-specific Tfh cells exactly control the production of allergen-specific IgE (sIgE) in human allergic diseases remains to be defined. In this study, we investigated the frequency and phenotype of circulating Tfh subsets, especially *Dermatophagoides pteronyssinus* group 1 (Der p 1)-specific Tfh cells in patients with house dust mite-induced AR, and examined the associations between Der p 1-specific Tfh cells with sIgE production and their changes accompanying allergen immunotherapy (AIT).

Peripheral blood mononuclear cells (PBMCs) were isolated from 41 subjects with AR and 42 healthy controls (HC) for cytometric and functional analysis of circulating Th cell subsets. Twenty-two AR patients with subcutaneous AIT were recruited to explore the effect of AIT on Th cells, with 20 AR patients without such

89 treatments as control. The combined symptom and medication score (CSMS), Th
90 cell subsets, and Ig were assessed before treatment (baseline) and at 3-, 6- and
91 12-month after treatment. More information regarding study designs is provided in
92 the Online Repository.

93 The gating strategy is shown in Fig E1, A as previously described.[4] Although no
94 difference in the percentages of circulating non-Tfh and Tfh cells in total $CD4^+$ T cells
95 was found between AR and HC (Fig 1, A), we observed a significant increase in the
96 percentages of type 2 Tfh (Tfh2) cells in either Tfh cells or total $CD4^+$ T cells in AR
97 patients, as compared with controls (Fig 1, B and Fig E1, B). Notably, a counterpart
98 type 2 non-Tfh cells (Th2) remained comparable between AR and HC (Fig 1, B and
99 Fig E1, B). As there was no difference in all other examined Th cell subsets between
100 AR and HC (Fig 1, B and Fig E1, B), the difference of Tfh2 cells suggests this Tfh
101 subset might play a prominent role in AR. Different to our findings, a recent study
102 reported that circulating $PD\text{-}1^+CXCR5^+CD4^+$ Tfh cells were reduced in AR patients.[5]
103 This discrepancy might be caused at least partially by different methods to define Tfh
104 cells. Our study included the staining for regulatory T (Treg) cells and excluded this
105 population in the analysis so we carefully avoided the interference of the data
106 interpretation by $CXCR5^+$ Treg cells, which are considered as regulatory follicular T
107 (Tfr) cells. In addition, allergen exposure may affect blood cell dynamics and
108 therefore possibly contributes to the difference in circulating Tfh cells observed in
109 different studies.[6]

110 Circulating Tfh cells have been indicated as a peripheral counterpart of *bona fide*

111 Tfh cells in lymphoid follicles and share similar phenotypes and functions.[4] When
112 co-culturing sorted circulating Th cells with autologous naïve B cells, we found that
113 Tfh cells from AR patients, when compared to those from HC, only enhanced the
114 production of IgE, but not IgM, IgG or IgA (Fig 1, C and Fig E2, A). Consistent with
115 the previous report that Tfh2 cells are the Tfh subset particularly helping B cells to
116 produce IgE,[4] we discovered that Tfh2 cells from AR patients had a markedly
117 enhanced capacity to induce IgE production than those from HC (Fig 1, D and Fig E2,
118 B). Type 2 cytokines promote IgE isotype switching and are secreted from Tfh2 cells,
119 but not from Tfh1 or Tfh17 cells.[4] In T-B cell co-cultures, we found that Tfh2 cells
120 from AR patients secreted higher levels of IL-4, but comparable levels of IFN-γ,
121 IL-17A, and IL-21 in comparison with those from HC (Fig E2, C). We further found
122 that blocking IL-4 diminished IgE production (Fig 1, E). Thus, up-regulated Tfh2
123 response may robustly promote IL-4-mediated IgE induction in AR patients. As a
124 control, we confirmed that non-Tfh cells induced low-level production of Igs and no
125 difference between AR patients and HC was observed (Fig 1, C and D, and Fig E2, A
126 and B).

127 We next set to examine allergen-specific circulating Tfh cells in AR. We found
128 that Der p 1-pulsed monocytes induced the proliferation of memory Th cells.
129 Although both non-Tfh and Tfh cells from AR patients induced more proliferation
130 than those from HC, Tfh cells displayed a stronger phenotype than non-Tfh cells (Fig
131 E3, A), indicating the significant accumulation of Der p 1-specific Tfh cells in AR
132 patients. Moreover, we measured the expression of IL-4 upon the Der p 1 stimulation

and found that Der p 1-specific IL-4$^+$ non-Tfh and Tfh cells were markedly increased in AR patients with the higher expression of IL-4 detected, again, in Tfh cells than non-Tfh cells (Fig E3, B). The higher expression of IL-4 in Tfh cells indicates that distinct signaling pathways may underlie IL-4 regulation in Tfh and non-Tfh cells. For example, the basic leucine zipper transcription factor ATF-like is important for IL-4 expression in Tfh cells rather than in canonical Th2 cells.[7] There was no significant difference for the expression of IFN-γ, IL-17A and IL-21 in these CD4$^+$ T cell subsets after the stimulation with Der p 1 (data not shown). Interestingly, Der p 1-specific IgE levels in AR patients only positively correlated with the frequency of Der p 1-specific IL-4$^+$ Tfh cells, but not non-Tfh cells (Fig 2, A). We also measured allergen-specific T cells by analyzing the expression of activation marker CD137 and CD154, and found that the frequency of Der p 1-specific IL-4$^+$CD154$^+$CD137$^-$ Tfh cells was increased in AR patients compared with HC (data not shown). Collectively, these data support the notion that allergen-specific Tfh2 cells produce IL-4 and orchestrate the production of sIgE in AR.

Finally, we explored the relationship between Tfh cells and the efficacy of AIT in AR patients. Consistent with previous reports,[8] compared with those without AIT, the AIT group demonstrated significant improvement of symptoms and decrease of Der p-specific IgE levels after the 12-month treatment, which was accompanied by an increase of Der p-specific IgG4 (Fig E4, A and B). Moreover, compared to those without AIT, a significant reduction of both Der p 1-specific IL-4$^+$ Tfh and non-Tfh cells was found in AIT group after treatment with a more prominent reduction for the

155 former (Fig 2, B). In contrast, no difference in other Th cell subsets was recorded (Fig
156 E4, C-E). Importantly, the reduction of Der p 1-specific IL-4^+ Tfh cells positively
157 correlated with the improvement of CSMS only in patients with AIT but not in the
158 non-AIT controls (Fig 2, C). However, we did not observe a significant correlation of
159 sIgE reduction with down-regulation of Der p 1-specific IL-4^+ Tfh cells or
160 improvement of CSMS (Fig E4, F). It suggests that other factors such as Tfr cells and
161 long-lived plasma cells may also be involved in sIgE regulation during AIT. It also
162 reflects the persistence of cell-bound sIgE in nasal mucosa, which is important for
163 symptom expression in AR. Additionally, the mechanisms underlying the change of
164 allergen-specific Tfh cells and its contribution to the rebalance of immune system
165 after AIT warrant further investigations.

166 Our study design didn't focus on patients with asthma given the low frequency of
167 asthma in our cohort. However, it will be interesting to compare the difference
168 between AR patients with and without asthma in future studies. A recent study
169 reported a reduction of total Tfh cells after AIT in patients with grass allergy, however
170 this report didn't examine allergen-specific Tfh cells, nor the association between Tfh
171 cell reduction and the efficacy of AIT.[9] Our findings highlighted an important role of
172 allergen-specific Tfh cells in allergen-specific IgE production and the development of
173 AR. Intriguingly, high expression of CCR2 and CCR5 was detected on
174 allergen-specific non-Tfh cells (Fig E5), suggesting that these cells preferentially
175 migrate to non-lymphoid tissues, such as nasal mucosa to mediate local inflammatory
176 responses through producing type 2 cytokines. On the contrary, high CCR7 expression

on allergen-specific Tfh cells indicates that these cells likely recirculate through secondary lymphoid organs where they encounter antigens and participate in T-dependent antibody responses and support sIgE production by B cells (Fig E5).

In summary, our study provides evidence for the first time that allergen-specific IL-4^+ Tfh cells may contribute to sIgE production and correlate with clinical efficacy of AIT in AR. Tfh2 cells may be a promising therapeutic target and biomarker for AIT in AR. However, our findings here are required to be evaluated and validated in randomized double-blinded placebo control trials.

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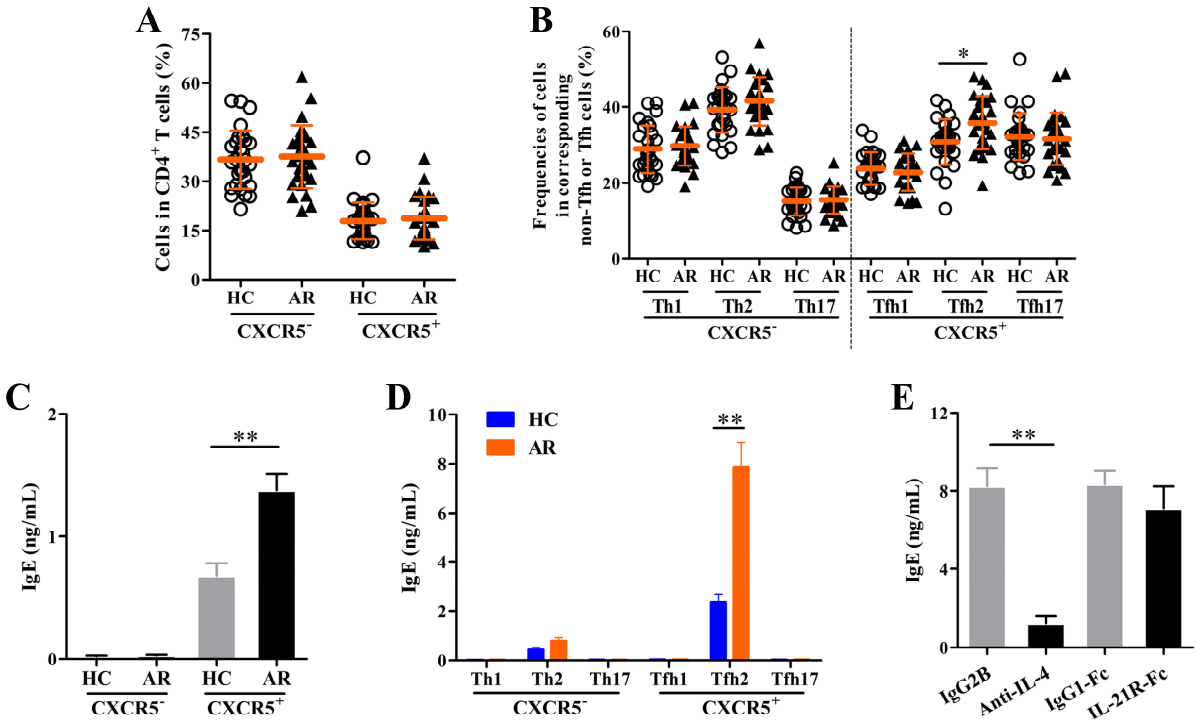
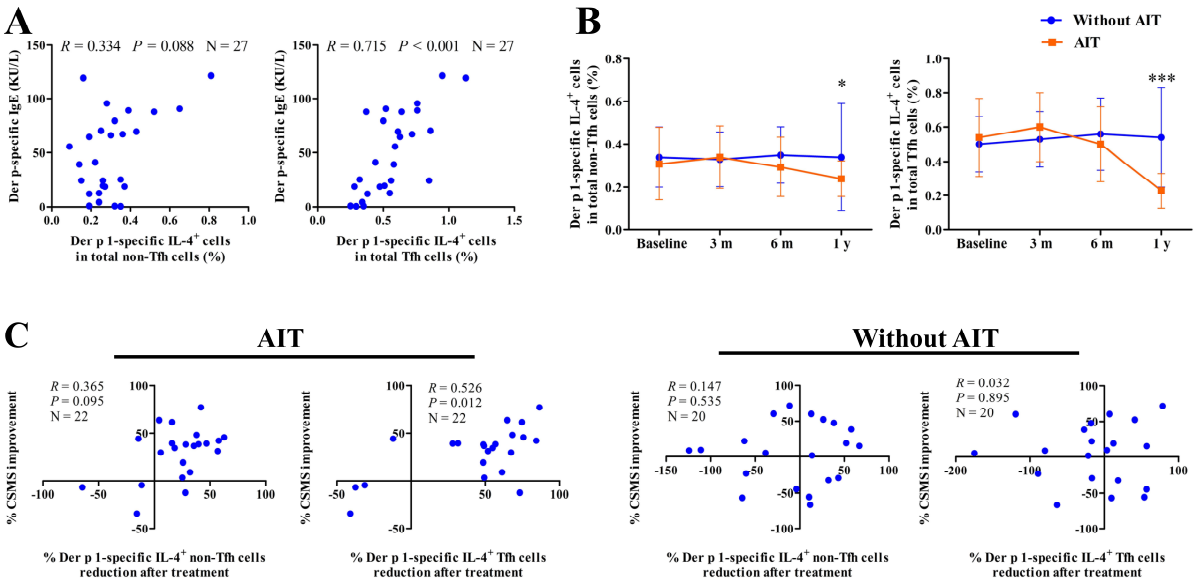
Figure Legends

Fig 1. Increased circulating type 2 follicular helper T (Tfh) cells in patients with allergic rhinitis (AR). (A) Percentages of circulating non-Tfh and Tfh cells within total $CD4^+$ T cells in AR patients (n = 27) and healthy controls (HC, n = 28). **(B)** Percentage of cell subsets in non-Tfh and Tfh cells in AR patients (n = 27) and HC (n = 28). **(C)** Tfh and non-Tfh cells were co-cultured with autologous naive B cells and immunoglobulin (Ig) levels were detected at day 8 (n = 8). **(D)** Non-Tfh and Tfh cell subsets were co-cultured with autologous naïve B cells and IgE levels were detected at day 8 (n = 6). **(E)** Type 2 Tfh cells from AR patients were co-cultured with autologous naïve B cells in the presence of blocking antibody or protein and IgE levels were detected at day 8 (n = 6). In (A) and (B), data are presented as mean ± SD. In (C), (D), and (E), data are presented as mean ± SEM. *P < 0.05; **P < 0.01.

Fig 2. Decrease of circulating allergen-specific IL-4^+CXCR5^+CD4^+ follicular T helper (Tfh) cells after allergen immunotherapy (AIT). (A) Correlations between the frequencies of *Dermatophagoides pteronyssinus* group 1 (Der p 1)-specific IL-4^+ non-Tfh and Tfh cells with Der p-specific IgE levels in AR patients. **(B)** Changes in the percentages of Der p 1-specific IL-4^+ cells within non-Tfh and Tfh cells in AR patients with (n = 22) and without AIT (n = 20) after treatment. Data are presented as mean ± SD. **(C)** Correlations of reduction of Der p 1-specific IL-4^+ non-Tfh and Tfh cells with the combined symptom and medication score (CSMS) improvement in AR patients with and without AIT after treatment. *P < 0.05; ***P < 0.001.





Online Repository

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METHODS

Subjects

This study was approved by the Ethics Committee of Tongji Hospital and the Ethics Committee of China Resources & Wisco General Hospital, and written informed consent was obtained from each patient. Allergic rhinitis (AR) patients and healthy controls (HC) were recruited from these two hospitals during April 2015 to April 2017. Forty-one AR patients and 42 HC participated in the phenotype and function study of circulating $CD4^+$ T helper (Th) cells (Table E1). Additional 22 AR patients with subcutaneous allergen immunotherapy (AIT) were recruited to explore the effect of AIT on Th cells, with 20 AR patients without AIT as control (Table E2).

All AR patients fulfilled the following inclusion criteria: (i) over 18 years of age, (ii) with the diagnosis of AR according to Allergic Rhinitis and its Impact on Asthma (ARIA) guideline and presenting clinical symptoms,[E1] (iii) with over 1 year history of AR, (iv) with positive skin prick test to *Dermatophagoides pteronyssinus* (Der p) and the levels of serum specific IgE (sIgE) to Der p $\geq$ 0.7 KU/L (Phadia ImmunoCAP, Uppsala, Sweden). The exclusion criteria were as follows: patients (i) allergic to other inhalant allergens than house dust mites (Der p and *Dermatophagoides farina*) according to skin prick tests, (ii) with asthma or others allergic disease according to ARIA and Global Initiative for Asthma guideline,[E1, 2] (iii) with upper respiratory infection or chronic rhinosinusitis, (iv) with pregnancy or breastfeeding, (v) previous treatment with immunotherapy, (vi) who have had neurological or psychiatric disorders, autoimmune diseases or cancer, and (vii) who used antihistamines or

intranasal steroid in past 1 week, or oral steroids in past 3 months, prior to this study. All of the AR patients in this study had persistent AR according to the ARIA classification.[E1] Healthy subjects having negative skin prick tests and without history of allergy were recruited as HC.[E3] Skin prick tests with a panel of 19 inhalant allergens common in our region were performed as we previously described.[E3]

For those participating in phenotype and function study of circulating Th cell subsets, peripheral bloods were collected after enrollment. For those AR patients participated in treatment study, peripheral bloods were collected at baseline (prior to treatment) and at 3-, 6- and 12- month after treatment.

Allergen immunotherapy and symptom assessment

The semi-depot HDM allergen extracts (50% Der p plus 50% Der f; Allergopharma GmbH, Reinbek, Germany) were used for AIT with subcutaneous injections as previously described.[E4] Over the course of the study, all patients were also managed with standard pharmacotherapy as recommended.[E1]

Participants involved in treatment study with or without AIT were provided with a daily record card for recording the 6 rhinoconjunctivitis symptom scores (RCSS; nasal itching, sneezing, rhinorrhea, nasal obstruction, ocular itching, and ocular tearing) and rescue medication use (oral and/or topical nonsedative H1 antihistamines, intranasal corticosteroids, and oral corticosteroids) during the previous month of scheduled visits (baseline, and 3-, 6- and 12-month).[E5] The daily RCSS and rescue medication score were evaluated and the daily combined symptom and medication

score (CSMS) was calculated at scheduled visits as previously described.[E5] After 12-month of treatment, the improvement of CSMS was calculated as following: (baseline CSMS – CSMS at 12-month)/(baseline CSMS) × 100%.[E6]

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated as previously mentioned.[E7] Cells were stained with fixable viability stain 700 (BD Bioscience, San Jose, CA, USA) to exclude dead cells. For cell surface staining, primary antibodies were incubated with cells for 30 min at 25 °C. For intracellular staining, cells were permeabilized by using Cytofix/Cytoperm (BD Bioscience) for 20 min on ice after surface staining. Antibodies specific for intracellular cytokines were incubated with cells for 30 min at 4 °C. Isotype controls were used as negative controls. The stained cells were analyzed using a BD LSRFortessa™ X-20 (BD Bioscience) instrument and FlowJo software (TreeStar, Ashland, OR, USA).[E8] The gating strategy is shown in Fig E1, A as we previously described.[E9] Antibodies used in flow cytometry are listed in Table E3.

Cell sorting

Monocytes were isolated from PBMCs by negative selection with a Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described.[E10]

Peripheral T cell subsets and naïve B cells were sorted from fluorescence labeled

PBMCs by using a BD FACSAria II cell sorter (BD Biosciences). In $CD4^+$ T cells, non-Tfh and Tfh cells were sorted as $CD4^+CD25^{-/low}CD127^{-/+}CD45RA^{low}CXCR5^-$ and $CD4^+CD25^{-/low}CD127^{-/+}CD45RA^{low}CXCR5^+$ cells, respectively. Tfh and non-Tfh cells were further subgrouped into type 1 ($CXCR3^+CCR6^-$), type 2 ($CXCR3^-CCR6^-$) and type 17 ($CXCR3^-CCR6^+$) cell subset. The type 1, 2, and 17 cell subsets in Tfh and non-Tfh cells were sorted. Naïve B cells were sorted as $CD3^-CD19^+IgD^+CD27^-$ cells. Purity of sorted cells was > 96%. Antibodies used in cell sorting are tabulated in Table E3.

Cell culture

For T-B cell co-culture, sorted T cells (5×10^4/well) were cultured with autologous naïve B cells (2.5×10^4/well) for 8 days in RPMI-1640 (Thermo Fisher Scientific, Waltham, MA, USA) 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 (SEB, 1 μg/mL; Toxin Technology, Inc., Sarasota, FL, USA) in U-bottomed 96-well plates as previously described.[E10] In blocking experiments, anti-IL-4 monoclonal antibody (1 μg/mL; R&D Systems, Minneapolis, MN, USA), IL-21R-Fc (20 μg/mL; R&D Systems), or the corresponding control (mouse IgG2B and IgG1-Fc, R&D Systems) was added as previously described.[E7] Cytokines and Igs in the culture supernatants were measured at day 2 and day 8, respectively.

To examine allergen-specific circulating Th cells, carboxyfluorescein

succinimidyl ester (CFSE)-labeled $CXCR5^-$ non-Tfh and $CXCR5^+$ Tfh $CD4^+$ T cells (1×10^5/well) were cultured with autologous monocytes (1×10^5/well) pre-incubated with Der p 1 (20 μg/mL; endotoxin < 0.03 EU/μg; Indoor Biotechnologies, Manchester, UK) for 15 h.[E10] At day 7, cells were stained with anti-CD4 and anti-CD14, and proliferation of $CD4^+$ T cells were assessed by flow cytometry. To directly illustrate the cytokine production by allergen-specific T cells, PBMCs (5×10^6 cells) were stimulated with 20 μg/mL Der p 1 for 18 h at 37 °C, and 1.7 ng/mL monensine (eBioscience, San Diego, Calif, USA) were added for the last 2 h.[E11, E12] After stimulation, the cells were underwent surface and intracellular staining and subjected to flow cytometry as mentioned above.

Allergen-specific T cells were also measured based on the expression of activation marker CD137 and CD154 as previously described.[E12] PBMCs (5×10^6 cells) were stimulated with 20 μg/mL Der p 1 (Indoor Biotechnologies) in presence of 1 mg/mL anti-CD40 (Miltenyi Biotec) and 1 mg/mL anti-CD28 antibody (eBioscience) for 7 h at 37 °C. 1.7 ng/mL monensine were added for the last 2 h. $IL\text{-}4^+CD154^+CD137^-$ allergen-specific T cells were determined.

For each stimulation, background enriched from unstimulated control was subtracted. For allergen-specific and cytokine-secreting T cells, a detection limit was defined as > 1.5 fold background cell number (unstimulated control).[E12]

Cytokine and Ig quantification

Serum total IgE and sIgE to Der p were measured by ImmunoCAP (Phadia), and

sIgG4 to Der p was measured by ELISA (DR.FOOKE Laboratorien GmbH, Neuss, Germany). The IgG, IgM, IgA, IgE, IFN-γ, IL-4, IL-17A, and IL-21 levels in culture supernatants were detected by using commercial ELISA kits (Ig, IL-17A, and IL-21 ELISA kits from Thermo Fisher Scientific; IFN-γ and IL-4 ELISA kits from R&D Systems).[E10]

Statistical analysis

All statistical analyses were carried out with GraphPad Prism Software 5.0 (GraphPad Software, La Jolla, Calif, USA) or SPSS 19.0 software (IBM, Armonk, New York, USA). For continuous variables, data are presented as mean ± SD or mean ± SEM, and unpaired two-tailed t-test or Mann-Whitney U test was used to compare paired sets of data. For categorical data, Chi-squared test or Fisher's exact test was performed to determine the difference between groups. Spearman's rank correlation analysis was used to analyze the associations. *P* value less than 0.05 was considered statistically significant.

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Figure Legends

Fig E1. Peripheral follicular T helper (Tfh) and non-Tfh cells in total $CD4^+$ T cells. (A) The gating strategy for $CXCR5^-$ non-Tfh cells and $CXCR5^+$ Tfh and their subsets in peripheral bloods. FV700, fixable viability stain 700. **(B)** Percentages of subsets of Tfh and non-Tfh cells in total $CD4^+$ T cells. $n = 28$ for healthy controls (HC); $n = 27$ for patients with allergic rhinitis (AR). Data are presented as mean ± SD. $^*P < 0.05$.

Fig E2. The immunoglobulin inducing function of circulating follicular T helper (Tfh) and non-Tfh cells. (A) Non-Tfh and Tfh cells from healthy control (HC) or allergic rhinitis (AR) patients were co-cultured with autologous naïve B cells and immunoglobulin (Ig) levels were detected at day 8 ($n = 8$). **(B)** Tfh and non-Tfh cell subsets from HC or AR patients were co-cultured with autologous naïve B cells and Ig levels were detected at day 8 ($n = 6$). **(C)** Type 2 Tfh cells (Tfh2) from AR patients were co-cultured with autologous naïve B cells and cytokine levels were detected at day 2 ($n = 6$). Data are presented as mean ± SEM. $^{**}P < 0.01$.

Fig E3. Allergen-specific circulating follicular T helper (Tfh) cells in patients with allergic rhinitis (AR). **(A)** Carboxyfluorescein succinimidyl ester (CFSE)-labelled circulating $CXCR5^-$ non-Tfh and $CXCR5^+$ Tfh cells were cultured with autologous monocytes, which were pre-treated with *Dermatophagoides pteronyssinus* group 1 (Der p 1) or *Staphylococcal enterotoxin B* (SEB). Cell

proliferation was analyzed by flow cytometry at day 7 (n = 6). Representative histograms are shown. Data are expressed as mean ± SEM. **(B)** The percentages of Der p 1-specific IL-4$^+$ non-Tfh and Tfh cells within corresponding total non-Tfh and Tfh cells. Representative flow cytometric plots are shown. Data are presented as mean ± SD. n = 27 for AR; n =25 for healthy controls (HC). *P < 0.05; **P < 0.01; ***P < 0.001.

Fig E4. Changes of immunoglobulin (Ig) and T helper (Th) cells in allergic rhinitis (AR) patients with or without subcutaneous allergen immunotherapy (AIT) after treatment. (A) Changes in the combined symptom and medication score (CSMS). Data are expressed as mean ± SD. **(B)** Changes of total IgE, *Dermatophagoides pteronyssinus* (Der p)-specific IgE, and Der p-specific IgG4 levels. Data are expressed as mean ± SEM. **(C-E)** Changes of Th cells (C), CXCR5$^-$ non-follicular Th (Tfh) cell subsets (D), and CXCR5$^+$ Tfh cell subsets (E). n = 22 for patients with AIT; n = 20 for patients without AIT. **(F)** Correlations of reduction of Der p-specific IgE with the improvement of CSMS and decrease of Der p 1-specific IL-4$^+$ Tfh cells. Data are expressed as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig E5. Patterns of chemokine receptor expression in *Dermatophagoides pteronyssinus* group 1 (Der p 1)-specific IL-4$^+$ follicular T helper (Tfh) and Der p 1-specific IL-4$^+$ non-Tfh cells in allergic rhinitis (AR) patients. Representative

histograms are shown. The numbers in figures refer to the percentages of positive cells. $n = 2$.

Table E1. Demographic characteristics of subjects in T helper cell phenotype and function study

	Healthy control	Allergic rhinitis patients	*p* value
Total subjects, n	42	41	
Gender, male/female	28/14	25/16	0.651
Age (years), mean ± SD	31.5 ± 10.6	32.7 ± 12.4	0.650
SPT to Der p	Negative	Positive	
Total IgE (KU/L), mean ± SD	20.9 ± 18.2	322.5 ± 250.3	< 0.001
Specific IgE to Der p (KU/L), mean ± SD	0.14 ± 0.1	46.7 ± 33.4	< 0.001

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

Table E2. The baseline characteristics of allergic rhinitis patients in treatment study before treatment.

	With AIT	Without AIT	*p* value
Total subjects, n	22	20	
Gender, male/female	13/9	10/10	0.757
Age (years), mean ± SD	30.5 ± 10.9	34.2 ± 10.6	0.277
SPT to Der p	Positive	Positive	
Total IgE (KU/L), mean ± SD	242.5 ± 194.5	231.2 ± 186.4	0.890
Specific IgE to Der p (KU/L), mean ± SD	46.8 ± 32.6	45.3 ± 31.6	0.871
Specific IgG4 to Der p (KU/L), mean ± SD	26.0 ± 18.6	26.3 ± 21.5	0.835
CSMS, mean ± SD	1.3 ± 0.4	1.3 ± 0.3	0.684

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

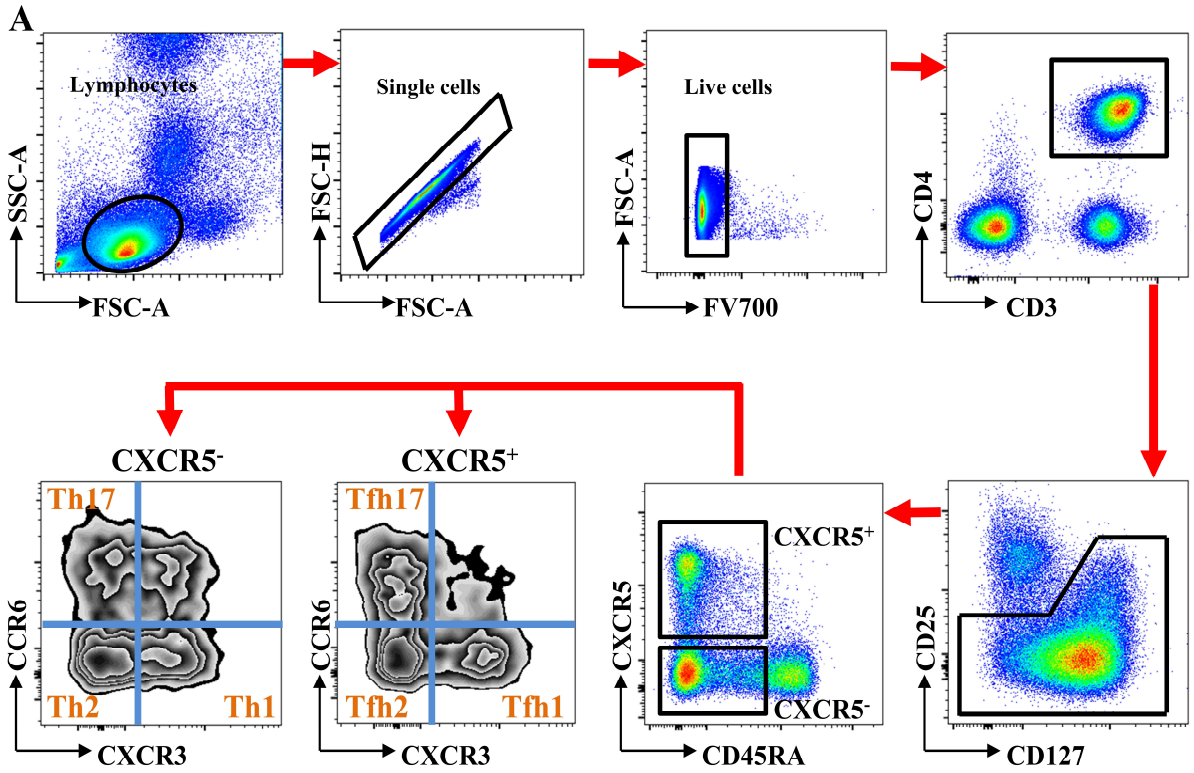
315 **Table E3. Antibodies used in flow cytometry and cell sorting**

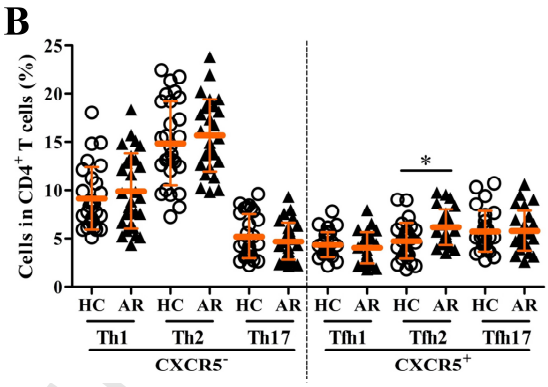
Antibody	Parameter	Clone ID	Source	Isotype	Manufacturer	Dilution
CD3	Brilliant Violet 510	UCHT1	mouse	IgG1, κ	BD Biosciences, San Jose, CA, USA	1:200
CD4	APC-Cy7	RPA-T4	mouse	IgG1, κ	BD Biosciences	1:200
CXCR5	BB515 (FITC)	RF8B2	rat	IgG2b, κ	BD Biosciences	1:100
CD25	PE-Cy7	M-A251	mouse	IgG1, κ	BD Biosciences	1:100
CD127	Brilliant Violet 421	HIL-7R-M21	mouse	IgG1, κ	BD Biosciences	1:100
CD45RA	Brilliant Violet 605	HI100	mouse	IgG2b, κ	BD Biosciences	1:100
CXCR3	APC	IC6/CXCR3	mouse	IgG1, κ	BD Biosciences	1:50
CCR6	PerCP-Cy5.5	11A9	mouse	IgG1, κ	BD Biosciences	1:100
CCR2	Alexa Fluor 647	SA203G11	rat	IgG2b, κ	Biolegend, San Diego, CA, USA	1:100
CCR3	PerCP-Cy5.5	5E8	mouse	IgG2b, κ	Biolegend	1:100
CCR4	PE	L291H4	mouse	IgG1, κ	Biolegend	1:100
CCR5	PE	J418F1	rat	IgG2a, κ	Biolegend	1:100
CCR7	Alexa Fluor 647	3D12	rat	IgG2a, κ	BD Biosciences	1:100
CD62L	PE	DREG-56	mouse	IgG1, κ	BD Biosciences	1:200
IL-4	APC	8D4-8	mouse	IgG1, κ	BD Biosciences	1:100
IL-4	PE-Cy7	8D4-8	mouse	IgG1, κ	BD Biosciences	1:100
CD137	PE	4B4-1	mouse	IgG1, κ	Biolegend	1:100
CD154	APC	TRAP1	mouse	IgG1, κ	BD Biosciences	1:100
CD3	FITC	UCHT1	mouse	IgG1, κ	BD Biosciences	1:200

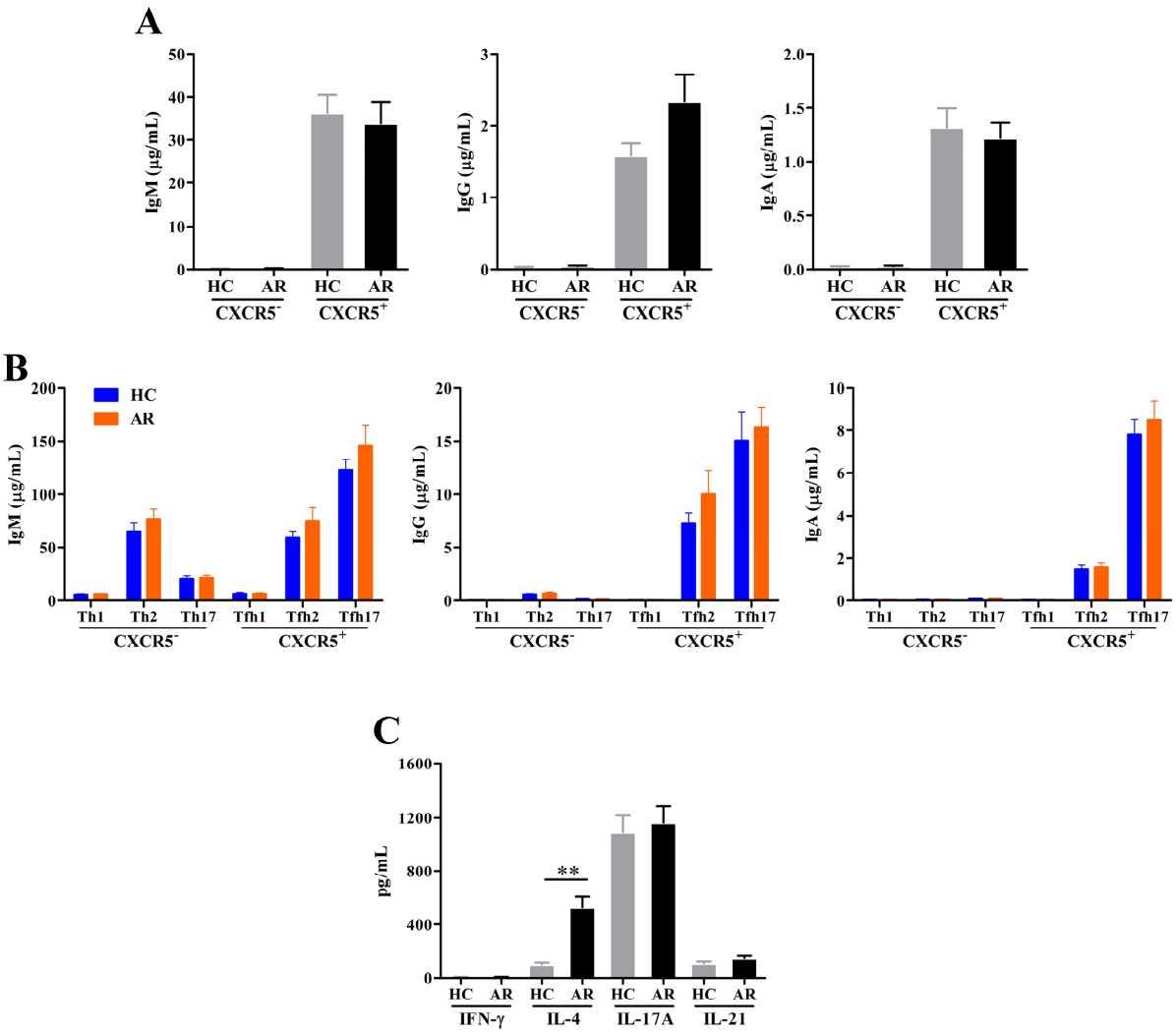
CD19	APC-Cy7	SJ25C1	mouse	IgG1, κ	BD Biosciences	1:100
CD27	PE-Cy7	M-T271	mouse	IgG1, κ	BD Biosciences	1:100
IgD	PerCP-Cy5.5	IA6-2	mouse	IgG2a, κ	BD Biosciences	1:100
CD14	APC	M5E2	mouse	IgG2a, κ	BD Biosciences	1:100

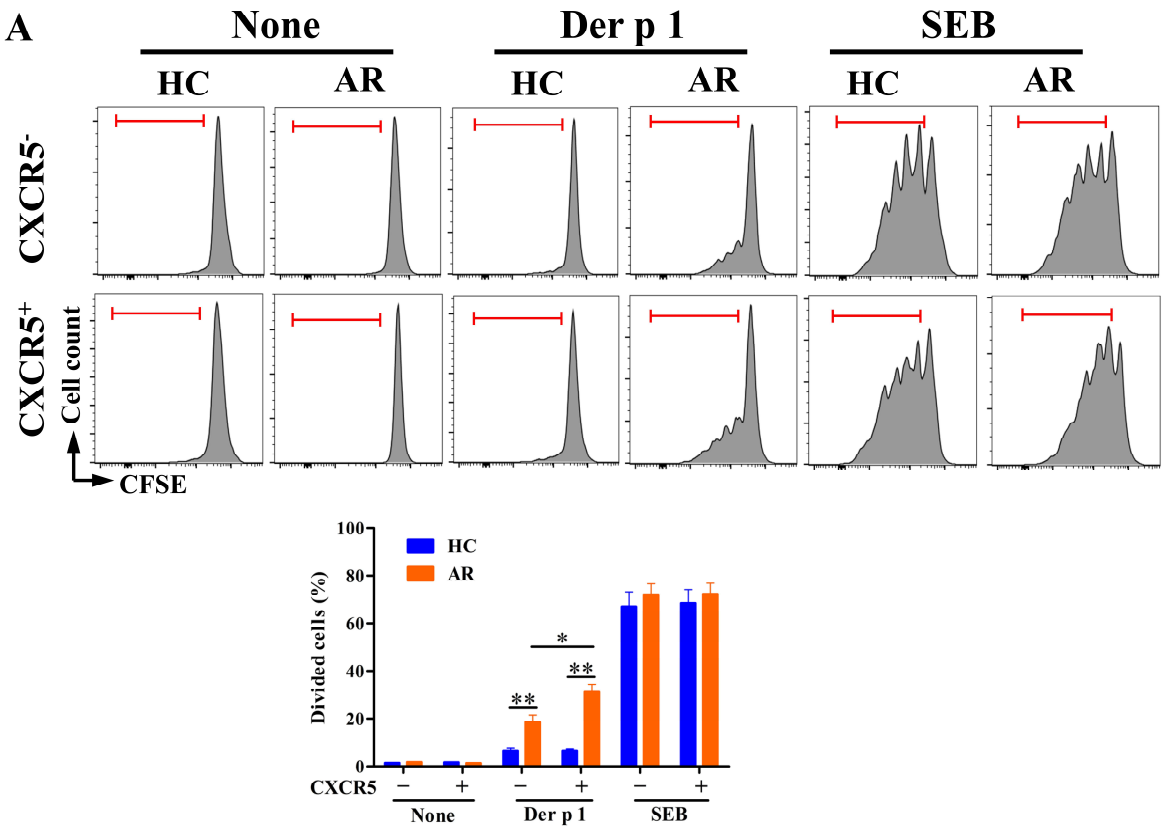
316 PE, phycoerythrin; APC, allophycocyanin; PerCP, peridinin chlorophyll protein

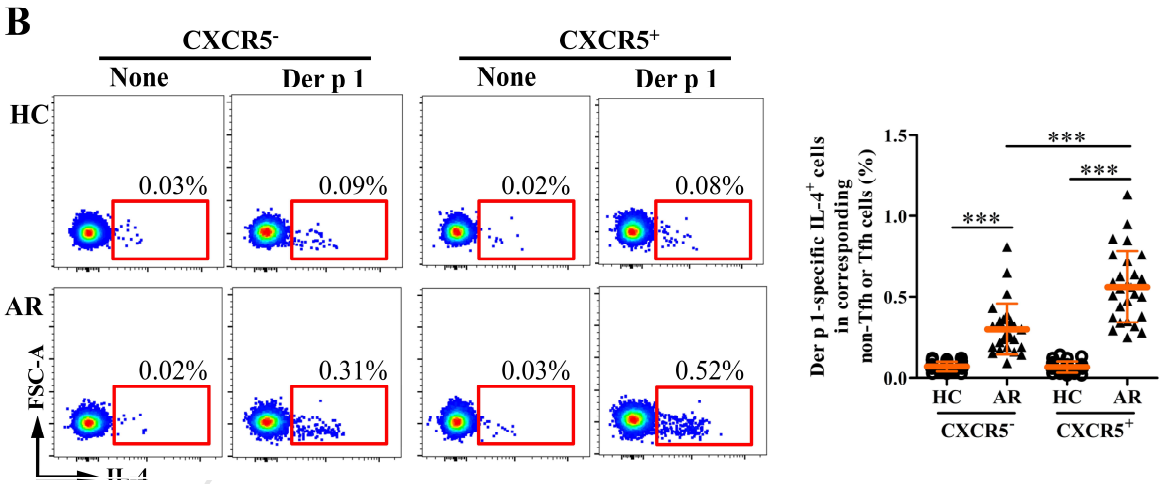
317 complex; FITC, fluorescein isothiocyanate; Ig, immunoglobulin.


A
Lymphocytes
SSC-A
FSC-A
Single cells
FSC-H
FSC-A
Live cells
FSC-A
FV700
CD4
CD3
CXCR5-
CXCR5+
Th17
Th2
Th1
CCR6
CXCR3
Tfh17
Tfh2
Tfh1
CCR6
CXCR3
CXCR5+
CXCR5-
CXCR5
CD45RA
CD25
CD127


B
Cells in CD4+ T cells (%)
25
20
15
10
5
0
*
HC AR HC AR HC AR HC AR HC AR HC AR
Th1 Th2 Th17 Tfh1 Tfh2 Tfh17
CXCR5-
CXCR5+


A
IgM (μg/mL)
IgG (μg/mL)
IgA (μg/mL)
HC AR HC AR
CXCR5-
CXCR5+
B
HC
AR
IgM (μg/mL)
IgG (μg/mL)
IgA (μg/mL)
Th1 Th2 Th17 Tfh1 Tfh2 Tfh17
CXCR5-
CXCR5+
C
pg/mL
**
HC AR HC AR HC AR HC AR
IFN-γ
IL-4
IL-17A
IL-21


A
None
Der p 1
SEB
HC
AR
HC
AR
HC
AR
CXCR5-
CXCR5+
Cell count
CFSE
Divided cells (%)
HC
AR
CXCR5
None
Der p 1
SEB


B
CXCR5-
CXCR5+
None
Der p 1
None
Der p 1
HC
AR
0.03%
0.09%
0.02%
0.08%
0.02%
0.31%
0.03%
0.52%
FSC-A
Der p 1-specific IL-4+ cells in corresponding non-Tfh or Tfh cells (%)
HC
AR
HC
AR
CXCR5-
CXCR5+