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Blood-Circulating Type 2 Follicular Helper T Cells in Pediatric Allergy Patients

[1]Masaaki Hamada, [2]Yoshihiko Sakurai and [3]Tomohiro Takeda

[1]Department of Pediatrics, Yao Municipal Hospital, Yao, Japan
[2]Department of Pediatrics, Matsubara Tokushukai Hospital, Matsubara, Japan
[3]Department of Clinical Laboratory Science, Kansai University of Health Sciences, Kumatori, Japan

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Corresponding Author:
Tomohiro Takeda
Department of Clinical Laboratory Science, Kansai University of Health Sciences, Kumatori, Japan
Email: t-takeda@kansai.ac.jp

Abstract: Pediatric allergic diseases are primarily caused by an IgE-dependent immunological reaction. Despite studies reporting the involvement of T follicular Helper (TfH) cells, especially type 2 TfH cells, in class-switching to IgE production in B cells, TfH subset skewing in peripheral blood in pediatric allergy patients remains to be elucidated. This study aimed to investigate the possible involvement of type 2 TfH cells in the pathogenic mechanism underlying pediatric allergic diseases. We analyzed TfH subsets (type 1, type 2 and type 17) in peripheral blood from pediatric patients with (allergy group, 35 patients) and without (non-allergy group, 26 individuals) allergic diseases via flow cytometry to determine the percentage of each TfH subset in the total TfH cell repertoire. Furthermore, the eosinophil percentage and serum total IgE and Thymus and Activation-Regulated Chemokine (TARC) levels were measured. No significant differences were observed in sex and age between the allergy and non-allergy groups. Since IgE levels were significantly higher in the allergy group than in the non-allergy group, no significant overlap was observed in the number of patients in the allergy and non-allergy groups. Although the total IgE and TARC levels and the eosinophil percentage were significantly higher in the allergy group than in the non-allergy group, the TfH subset analysis did not display a significant skewing of specific TfH subset cells. These results suggest the occurrence of either limited changes in peripheral blood TfH cells or the involvement of the immune cell subtype TfH13 in pediatric allergic diseases.

Keywords: Flow Cytometry, T Follicular Helper Cells, Immunoglobulin E, Peripheral Blood

Introduction

Allergen-specific Immunoglobulin E (IgE) plays a crucial role in allergic reaction and conditions such as food allergy, atopic dermatitis and asthma (Illi *et al*., 2006; Kasperkiewicz *et al*., 2018; Waserman *et al*., 2018). Since IgE levels in patients with allergic diseases remain elevated for a long period, the detailed IgE production-promoting mechanism has been previously investigated. Allergen-reactive type 2 helper T (Th2) cells are considered to be involved in the activation and recruitment of IgE antibody-producing B cells, because class-switching to IgE production in B cells requires Th2 cells to secrete cytokines IL-4 and IL-13 (Lebman *et al*., 1988; Poulsen and Hummelshoj, 2007). However, recent studies revealed that T follicular Helper (TfH) cells-which are one of the CD4 T helper cell subsets in the germinal center of B cell follicles and in circulating blood (Nurieva *et al*., 2009)-play a key role in IgE production induced by T cell-dependent immune responses (Harada *et al*., 2012; Vijayanand *et al*., 2012; Kobayashi *et al*., 2017). TfH cells are a major source of IL-4 and they also secrete IL-2 (Kubo, 2017). Production of IL-4 in TfH cells is regulated through a different mechanism than Th2 cells (Kubo, 2017). Three subsets of TfH cells (type 1, type 2 and type 17 TfH cells; TfH1,

TfH2 and TfH17, respectively) generate type-specific cytokines, regulate antibody production via B cells and are present in circulating blood (Morita *et al*., 2011). Recent studies have reported that circulating TfH cells contain long-lived memory cells, which are also known as blood memory TfH cells (Ueno *et al*., 2015). Although blood memory TfH1 cells cannot support naïve B cells, blood memory TfH2 cells induce IgG and IgE secretion and TfH17 cells stimulate IgG and IgA secretion (Morita *et al*., 2011).

Previous studies have reported a skewing or increase in the number of blood-circulating TfH cells in patients with bronchial asthma, atopic dermatitis and eosinophilic nasal polyps (Gong *et al*., 2014; 2018; Szabo *et al*., 2017; Zhang *et al*., 2016). Furthermore, a recent study reported that polarization toward TfH2 cells in asthma patients (Gong *et al*., 2016) suggests the involvement of TfH2 cells in the pathogenesis of allergic diseases and corresponds to the proposed function of blood memory TfH2 cells.

Immune function and IgE generation gradually increase in the course of growth. Allergic diseases often develop sequentially in childhood, which is classically known as the atopic march. In this condition, more than one disease often coexists (Aw *et al*., 2020). Furthermore, allergic diseases present distinct clinicopathological characteristics during childhood and adulthood (Brunner *et al*., 2018; Larsen, 2000; Izquierdo-Dominguez *et al*, 2017). Hence, although pediatric allergies have unique characteristics, it remains unclear whether TfH cells, especially TfH2 cells, are involved in pediatric allergies. Therefore, the present study aimed to determine whether blood-circulating TfH subset shift towards IgE production in pediatric patients with allergic diseases.

Materials and Methods

Study Populations

Our study populations were divided into allergy [patients with allergic diseases, n = 35 (male, 24; female, 11), age, 66.89±8.30 (months)] and non-allergy (control) [patients without allergic diseases and healthy children, n = 26 (male, 14; female, 12), age, 58.42±12.77 (months)] groups. The clinical conditions of both groups are summarized in Fig. 1.

Allergic diseases were diagnosed by qualified allergy specialists in accordance with appropriate clinical criteria (Arakawa *et al*., 2017; Ebisawa *et al*., 2017; Saeki *et al*., 2016); however, disease severity was not considered. Written informed consent was obtained before blood collection from all participants included herein. All procedures involving human participants were approved by the Yao Municipal Hospital Ethical Committee, the Tokushukai Ethical Committee and the Kansai University of Health Sciences Ethical Committee and in accordance with the ethical standards of the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Laboratory Tests

Peripheral blood testing and biochemical examination were performed. For eosinophil measurements, eosinophil percentage (Eo%) was used owing to an altered white blood cell count during childhood. To measure serum Thymus and Activation-Regulated Chemokine (TARC) levels, a chemiluminescent enzyme immunoassay was conducted using the HISCL system (Sysmex, Hyogo, Japan) with a TARC assay kit (Shionogi, Osaka, Japan) in accordance with the manufacturer's instructions. TARC, a small cytokine belonging to the CC chemokine family, is associated with the induction of chemotaxis in T cells, particularly Th2 cells, by binding to the chemokine receptor CCR4. Total serum IgE levels were measured using the Phadia ImmunoCAP-System (Phadia, Uppsala, Sweden) in accordance with the manufacturer's instructions.

Flow Cytometric Analysis

Whole peripheral blood cells were stained at 4°C for 30 min using combinations of the following antibodies: Peridinin Chlorophyll protein-Cyanin5.5 (PerCp-Cy5.5)-Conjugated anti-CD3 (BD Biosciences, Tokyo, Japan), Phycoerythrin Cyanine 7 (PE-Cy7)-Conjugated anti-CD4 (BioLegend, Tokyo, Japan), allophycocyanin cyanine 7 (APC-Cy7)-conjugated anti-CD45RA (BioLegend), phycoerythrin-Texas Red-X (ECD)-conjugated anti-CD8 (Beckman Coulter, Tokyo, Japan), Alexa Fluor 488 conjugated CXCR5 (BD Biosciences), Phycoerythrin (PE)-conjugated anti-CCR6 (BD Biosciences), Allophycocyanin (APC)-conjugated CXCR3 (BD Biosciences) and isotype controls (BD Biosciences). Thereafter, red blood cells were lysed using the IOTest 3 Lysing Solution (Beckman Coulter). After incubation for 15 min at 25°C, the cells were washed once with phosphate-buffered saline containing 1% bovine serum albumin. Dead cells were gated after staining using a DRAQ7 far-red fluorescent live cell impermeant DNA dye (Beckman Coulter). The percentages of $CXCR5_{+}CXCR3^{+}CCR6^{-}$ (Tfh1), $CXCR5^{+}CXCR3^{-}CCR6^{+}$ (Tfh17) and $CXCR5^{+}CXCR3^{-}CCR6^{-}$ (Tfh2) cells were enumerated after gating for $CD3^{+}$, $CD4^{+}$, $CD45RA^{-}$ and $CXCR5^{+}$ (Fig. 2). Data were acquired using a Gallios flow cytometer (Beckman Coulter) and Kaluza software (Beckman Coulter) was used for data analysis.

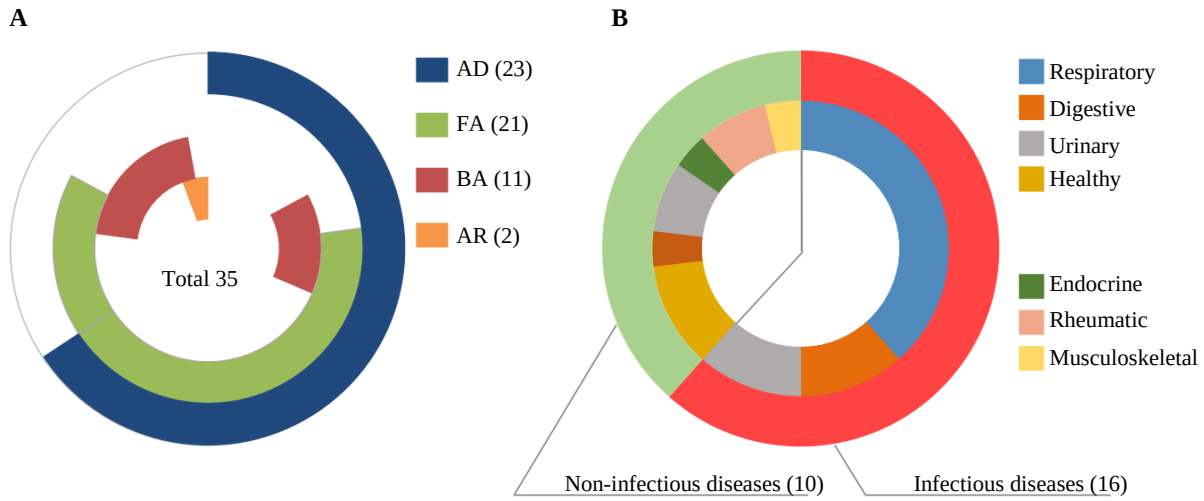

Fig. 1: Clinical conditions of study populations. (A) Allergic diseases include atopic dermatitis (AD; *n* = 23), food allergy (FA; *n* = 21), asthma (BA; *n* = 11), allergic rhinitis (AR; *n* = 2) (each number includes overlaps). (B) Control group includes healthy children (*n* = 3) and patients with non-infectious diseases (*n* = 7) and infectious diseases, such as respiratory tract (*n* = 10) digestive (*n* = 3) and urinary tract infections (*n* = 3).

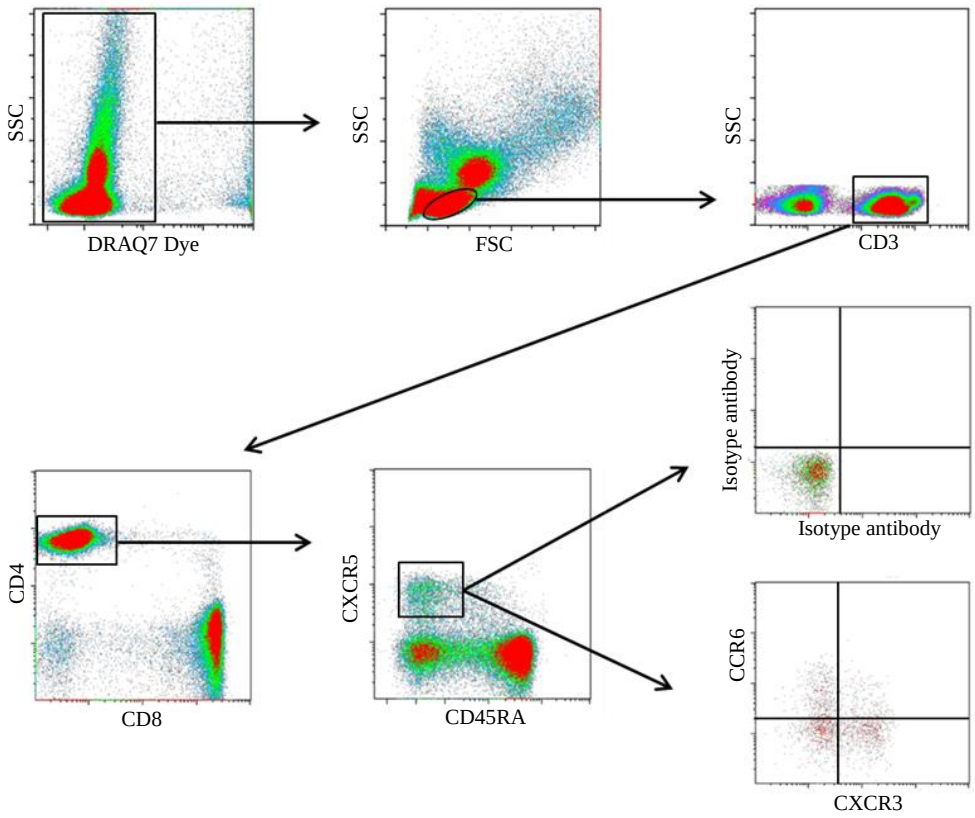

Fig. 2: Peripheral Blood Mononuclear Cells (PBMCs) were analyzed for their uptake of DRAQ7 to determine dead versus live cells. Subsequently, DRAQ7-negative live PBMCs were gated for lymphocytes (SSC vs FSC). The lymphocyte gate was further analyzed for their expression of CD3, CD4 and CD8. Thereafter, $CD3^+$ $CD4^+$ $CD8^-$ helper T cells were gated on CD45RA-$CXCR5^+$ circulating TfH cells. Lastly, CCR6 and CXCR3 surface expression were determined from this gated TfH cells.

Statistical Analysis

All data are presented as mean ± Standard Deviation (SD) values. Statistical analysis was conducted with GraphPad Prism 5 software (San Diego, CA, USA). The Mann-Whitney U-test was used for between-group comparisons of continuous variables and categorical variables between groups [sex (male vs female)] were compared using Fisher's exact test. The Spearman's rank correlation test was performed for correlation analysis. Results were considered significant when $p<0.05$.

Results

Allergy Markers and TfH Subsets in Pediatric Patients with Allergic Disorders

No significant differences were observed between the non-allergy and allergy groups in age and sex ratios. Allergy markers, such as total IgE and serum TARC levels and Eo%, were significantly higher in the allergy group than in the non-allergy group (Fig. 3 upper). Further, no differences were observed in the percentage of TfH2 (TfH2%), TfH1 (TfH1%) and TfH17 (TfH17%) cells in the total TfH cell repertoire between the allergy and non-allergy groups (Fig. 3 lower).

Since IgE levels vary through adolescence, we divided the allergy group into three subgroups: 0-2 years of age (infants), 3-5 years (preschool) and 6 years and more (school-aged children). In the infant subgroup (Fig. 4A), no significant difference was observed in both the allergic markers (total IgE levels, Eo% and TARC levels) and in TfH cell subsets (Tfh1%, Tfh2% and Tfh17%), although IgE levels appeared higher in the allergy group than in the control group. In the preschool group (Fig. 4B), Eo% was significantly higher in the allergy group than in the control group ($p<0.05$). However, IgE and TARC levels did not significantly differ. Furthermore, no significant difference was observed in TfH subsets. Among school-aged children (Fig. 4C), IgE levels and Eo% were significantly higher in the allergy group than in the control group. Furthermore, TARC levels did not significantly differ. Moreover, the TfH2% was significantly lower in the allergy group than in the non-allergy group.

Correlation Between IgE Levels and TfH Subsets

Fig. 5 shows the correlation between IgE levels and TfH subsets. In the non-allergy group, a weak negative correlation was observed between IgE levels and TfH2% (r = 0.522). No correlation was observed between IgE levels and TfH1% or TfH17%. In the allergy group, TfH2% as well as TfH1% and TfH17% was not significantly correlated with IgE levels. Furthermore, no correlations were observed between IgE levels and TfH2/TfH1 cells or (TfH2+TfH17)/TfH1 cells (Fig. 6).

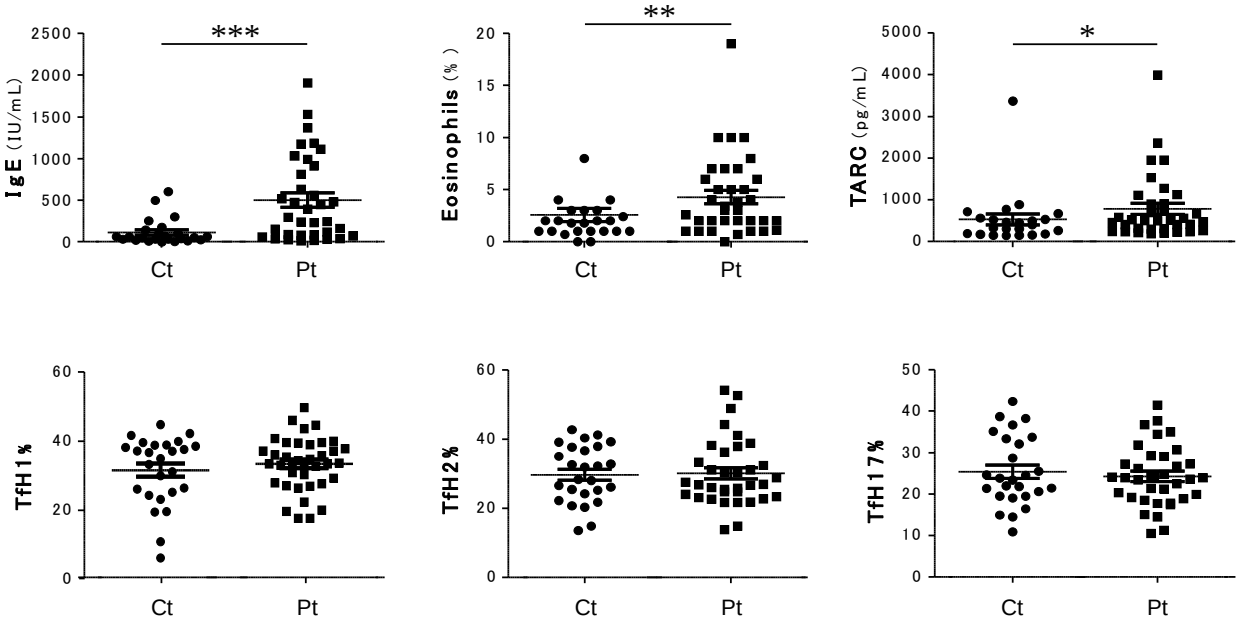

Fig. 3: Allergy markers and TfH subset skewing. Ct: Control (Non-allergy) group, Pt: Patient (Allergy) group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

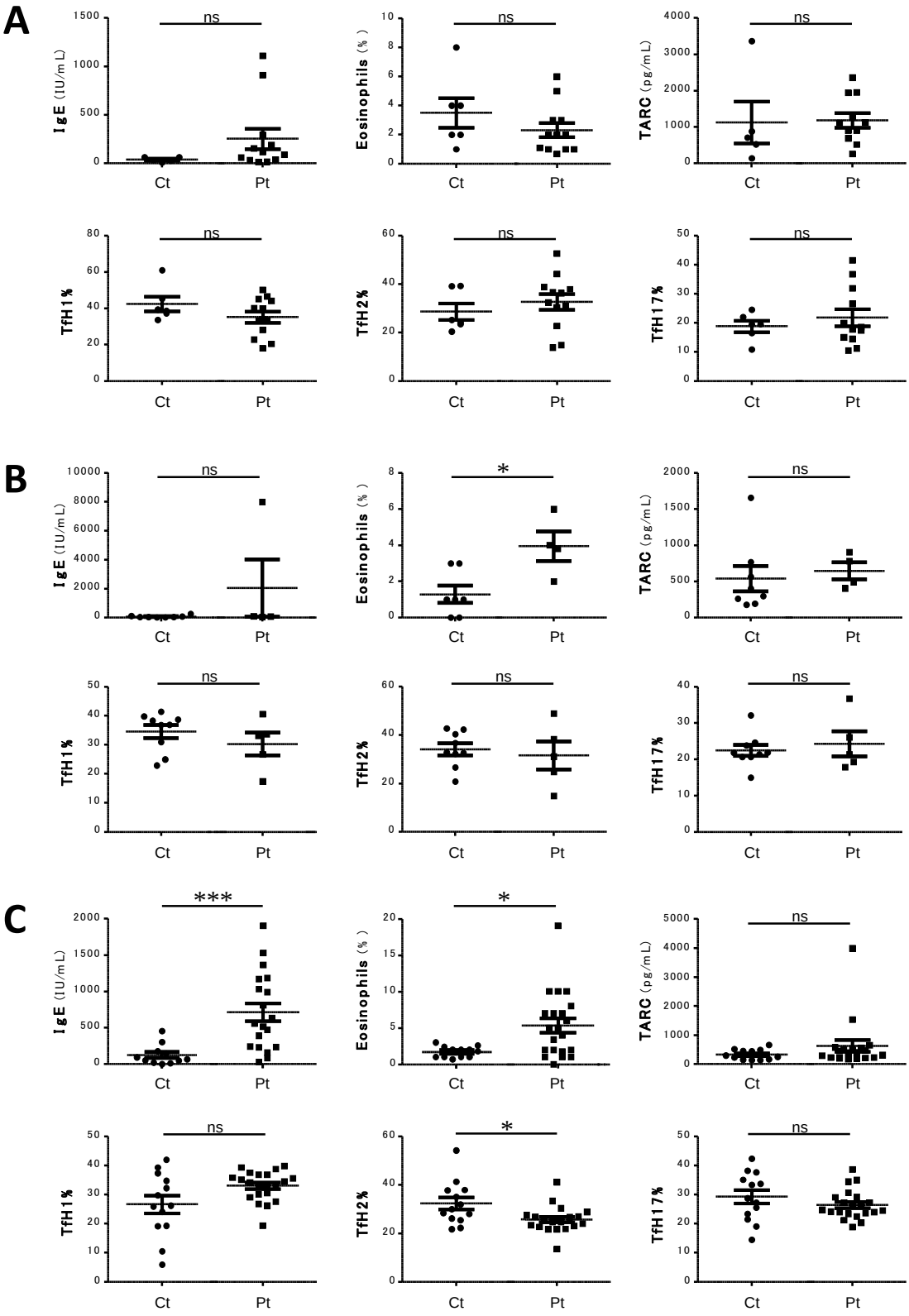

Fig. 4: Allergy markers and TfH subsets skewing by age group. (A) infants, (B) preschool and (C) school-aged children. Ct: Control (Non-allergy) group, Pt: Patient (Allergy) group. ns: Not significant, * $p<0.05$, *** $p<0.001$

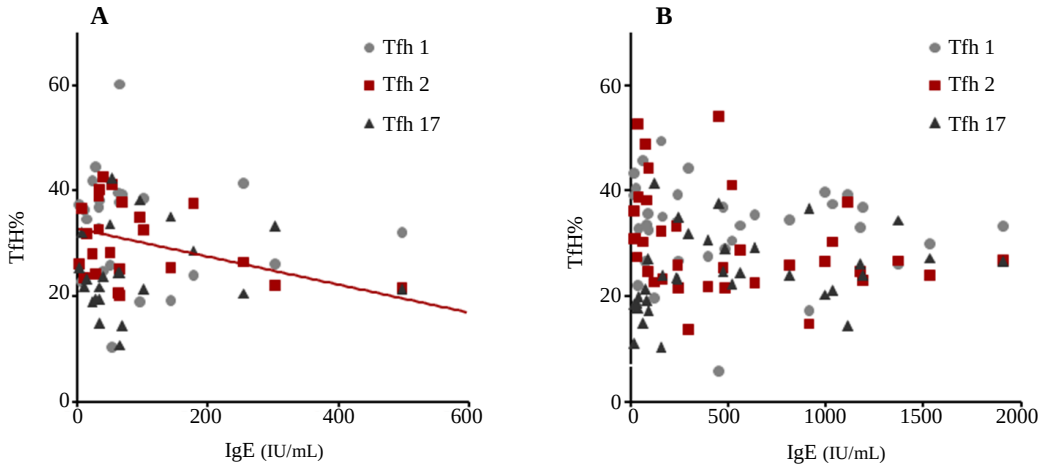

Fig. 5: Correlation between IgE levels and TfH subsets. (A) Control (Non-allergy) group. (B) Patient (Allergy) group

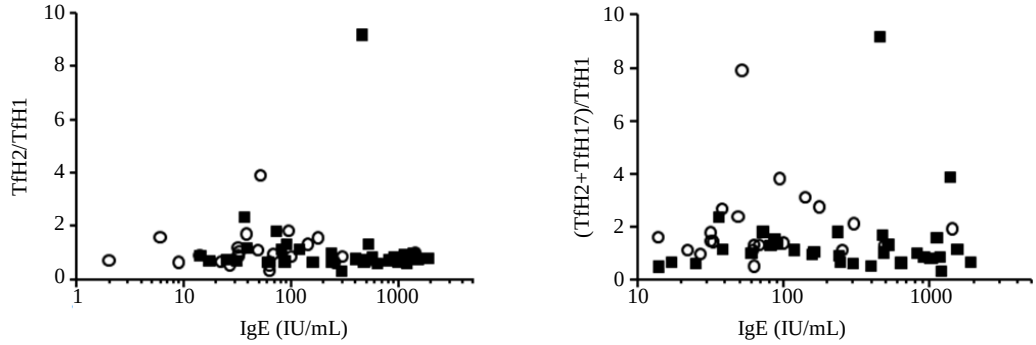

Fig. 6: Correlation between IgE levels and TfH2/TfH1 or (TfH2+TfH17)/TfH1. (A) Correlation between IgE levels and TfH2/TfH1. (B) Correlation between IgE levels and (TfH2+TfH17)/TfH1. Note that the X axis is in log scale. Open circle: Control (Non-allergy) group. Closed square: Patient (Allergy) group

Discussion

In this study, we failed to demonstrate a shift toward TfH2 dominance in peripheral blood in pediatric allergy patients, contrary to our hypothesis of TfH2 polarization within TfH subsets. Furthermore, in the school-aged children, TfH2% was significantly lower in the allergy group than in the non-allergy group; however, the underlying reason remains unclear. These findings suggest the occurrence of limited changes in peripheral blood TfH2 cells in pediatric allergic diseases. Although we observed that the percentage of blood TfH2 cells decreases with an increase in IgE levels, its significance is obscure because this phenomenon was observed only in the non-allergy group wherein IgE levels were almost within normal ranges.

We analyzed three subsets of TfH cells (TfH1, TfH2 and TfH17) in accordance with previous studies. Most recently, Gowthaman *et al*. (2019) reported that Tfh13, which can produce IL-4 and IL-13, is the primary TfH cell subset contributing to high-affinity antigen-specific IgE production, while TfH2 potentially accounts for total IgE production. Furthermore, they reported that the number of circulating TfH13 cells expressing BCL6 and GATA3 is elevated in allergy patients (Gowthaman *et al*., 2019). Our results would not challenge the TfH13 hypothesis (Xie and Dent, 2019). Further studies are required to clarify the role of TfH13 cells in pediatric allergies.

In this study, there were concerns regarding the heterogeneity of individuals in the non-allergic group, which comprised sick children with several disease types and healthy children. However, since the present results displayed a significant difference in IgE levels between the allergy and non-allergy groups, we believe that the composition of the non-allergic group was appropriate for this study; thus, the between-group comparisons yielded valid results. Nevertheless, this study has several limitations, including the small number of participants. Patients in the allergic group had comorbid pleural allergic diseases, which is one of the characteristics of pediatric allergies with atopic march (Aw *et al*., 2020), although atopic dermatitis was dominant. Since atopic

dermatitis is a complex disease involving both innate and adaptive immune responses (Ghosh *et al*., 2018), the role of TfH2 cells might be less important in atopic dermatitis. Furthermore, the allergy group comprised patients with varying degrees of disease severity, which might have influenced our results. Moreover, some allergy patients received antihistamine therapy, which might have also affected the results. Despite these limitations, our study provides insights into the mechanism underlying IgE maintenance in pediatric allergy patients from the viewpoint of TfH cells.

Conclusion

In this study, we hypothesized that a TfH2 shift in peripheral blood maintains elevated IgE levels in pediatric allergy patients; however, we did not observed TfH2 skewing in pediatric allergy patients. Our results raised further questions to be explored, including whether peripheral blood TfH reflects the dynamics of TfH in the lymphoid follicles and whether TfH13 cells are involved in elevating IgE levels in pediatric patients. Further studies are required to elucidate the long-term IgE production-promoting mechanism in pediatric allergy patients.

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Author's Contributions

MH Masaaki Hamada: Collected blood samples, performed laboratory studies, analyzed data and drafted the manuscript.

Yoshihiko Sakurai: Designed the study, interpreted the data and edited the manuscript.

Tomohiro Takeda: Designed the study, performed flow cytometric analysis, analyzed data and edited the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and there are no ethical issues involved.

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