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1. Introduction

Most egg allergies may have an impression of restricted childhood disease. However, various clinical aspects of egg allergy have been recently identified as follows. First, patients with egg protein allergy occasionally show lethal reactions to egg consumption, such as anaphylaxis to antigens that could potentially threaten ones quality of life. In addition, some patients are not keen on eating eggs even after gaining tolerance to egg proteins owing to a long period of elimination.

The onset of IgE-dependent food allergies (hereinafter referred to as food allergies) is thought to result from sensitization to specific food proteins and the breakdown of oral tolerance. However, the precise mechanism of oral tolerance is unknown in humans.

Animal experiments have shown that Peyer's patches are involved in monoclonal apoptosis and the deletion of antigen-specific regulatory T cells (Treg).[1,2] Unfortunately, it is difficult to observe the immune response in the intestinal mucosa of humans, so functional studies are rare at the cellular level. One approach has been to use endoscopy to collect Peyer's patch samples from adults without cow's milk protein allergy, before reacting the samples in vitro with β-lactoglobulin, a constituent protein of cow's milk. Work by Nagata et al. resulted in the suggestion that oral tolerance may result from the suppression of Th2-type immune induction by inducing $CD4^+$ and $CD8^+CD25^+CD4$ cells.[3]

To answer the research question, we propose observing the in vitro molecular response when reacting the peripheral blood mononuclear cells (PBMCs) of children allergic to egg white with the egg white protein ovalbumin. Previous studies have shown that, when peripheral blood is stimulated with an antigen, T cells are produced that

circulate throughout the body and can be stimulated to proliferate by the antigen.[4] Although it is unclear how accurately immune response can be estimated, because the number of circulating effector T cells may be small, this approach should provide new information about the cellular mechanism of onset of egg white allergy.

The follicular helper T cell (Tfh) may also be a candidate immunocompetent cell that conveys information about the immune response in the peripheral blood to mucosal immunity.[5-12] In recent years, Tfh involved in the production of antigen-specific IgE antibodies in solitary lymphatic nodules, such as Peyer's patches and follicular regulatory T cells (Tfr) that control antibody production, have been detected in the peripheral blood.[10,13] Therefore, it is plausible that these could be used to investigate the involvement of follicular helper or regulatory T cells.[11,13] Providing information about the functional subclass profile of each $CD4^+$ T cell could be achieved by identifying the signal transducer and activator of transcription (STAT) signal and the messenger RNA (mRNA) values of the transcription factors expressed when the circulating effector $CD4^+$ T cells proliferate under ovalbumin stimulation.[14,15] Genes were included as follows: STAT1, STAT4, and T-bet for Th1; STAT6 and GATA3 for Th2; STAT5 and Foxp3 for Treg; STAT3 and RORɤt for Th17; CXCR5 and IL-21 for Tfh; and Foxp3, CXCR5, and CD25 for Tfr.

We aimed to compare and analyze the in vitro response to ovalbumin of PBMCs from children allergic to egg white with that in control and tolerant groups, seeking to

elucidate the mechanism of food allergy onset and remission in humans. The primary research question was “Is Treg in the peripheral blood activated when egg white allergy tolerance occurs?” Secondary research questions were (1) “Is the Th2-type immune response to ovalbumin suppressed in the tolerant group?” and (2) “Is the Th2-type immune response enhanced against ovalbumin in the persistent symptom group compared to the control group?” Finally, we wanted to observe Tfh and Tfr.

2. Materials and Methods

1) Patients

This study was conducted in accordance with the Declaration of Helsinki at the pediatrics department of Tokyo Women’s Medical University Hospital, from December 17, 2019, to September 26, 2020, after receiving the ethics committee approval (no. 5025). The subjects were children under the age of 15 years who were allergic to hen’s eggs, were tolerant to egg whites, and did not have hen’s egg allergy. Children with a history of treatment with steroid, immunosuppressive, anticancer, or biological therapies were excluded.

The group with egg white allergy (hereinafter referred to as the persistent group) was instructed by an allergist to remove all egg white protein from their diets (including heated eggs) because they showed clinical symptoms with positive IgE antibodies specific for egg white proteins. All our patients with egg white allergy showed some clinical reactions to not only heated egg white proteins having serum-specific IgE antibodies but also ovomucoid (data not shown). A tolerant group comprised children who had been treated as having an egg white allergy, but who no longer need to exclude egg white because interviews and oral stress tests indicated that tolerance had developed.

The control group comprised children with no history of egg white allergy. Clinical data were obtained from the medical records of patients for whom consent was provided.

2) PBMC isolation and ovalbumin stimulation

In total, 4 mL of peripheral blood was taken from each patient. Of this, we used 1 mL and the residual sera for complete blood counts and serological testing (e.g., total IgE antibodies) and antigen-specific IgE antibody values (using ImmunoCAP®). Mononuclear cells were isolated using standard procedures in a Ficoll-Hypaque density gradient. The buffy coat was removed carefully following centrifugation and washed twice in RPMI 1640 medium containing 10% fetal bovine serum. Cells were counted and assessed for viability.

The following PBMC stimulation test was conducted with reference to Nagata et al.'s study.[3] Isolated PBMCs were plated in 96-well flat-bottom plates at 5×10^5 lymphocytes/well in RPMI 1640 medium containing 10% fetal bovine serum (MERCK; endotoxin-free products), before being incubated in the presence or absence of ovalbumin (Sigma; 500 μg/ml), anti-CD3 Ab (Sigma; 0.5μg/ml) for 48 h at 37°C in a 5% CO_2 and 95% O_2 atmosphere saturated with water.

3) Quantification of mRNA by real-time PCR

Cells were harvested and total RNA was extracted with guanidinium thiocyanate (TRIzol, Invitrogen) and chloroform followed by isopropanol precipitation. Complementary DNA was generated using Super Script VILO Master Mix (Thermo Fisher Scientific), according to the manufacturer's instructions. Real-time PCR was set up with TaqMan Fast Advanced Master Mix (Applied Biosystems) and performed on QuantStudio3 (Thermo Fisher Scientific), according to the manufacturer's instructions (denatured at 95°C for 1 s and annealed/extended at 60°C for 20 s; 40 cycles).

Th1, Th2, Treg, and Th17 were taken as representative of circulating effector T cells, and Tfh and Tfr were selected as representative of follicular T cells. We then observed the expression profiles of these transcription factors and cell surface marker mRNAs in our experimental system.

Each gene was amplified by real-time PCR using commercially available primers. In this study I used the following primers. STAT1 (Assay ID : Hs01013996_m1), STAT3 (Assay ID : Hs00374280_m1), STAT4 (Assay ID : Hs01028017_m1), STAT5 (Assay ID : Hs00559637_g1), STAT6 (Assay ID : Hs00598625_m1), T-bet (Assay ID : Hs00894392_m1), Foxp3 (Assay ID : Hs01085834_m1), GATA3 (Assay ID : Hs00231122_m1), RORγt (Assay ID : Hs01076112_m1), CXCR5 (Assay ID : Hs00540548_s1), IL-21 (Assay ID : Hs00222327_m1), CD25 (Assay ID : Hs00158122_m1)

The mRNA expression level is expressed as the number of gene copies for each factor minus the background number of copies in the unstimulated condition (i.e., Δ values) for simple viewing when an expression level of the background copy number was negligibly low.

4) Statistical analysis

Patient demographic and clinical characteristics were compared among the persistent, tolerant, and control groups by chi square and Kruskal–Wallis tests. The Mann–Whitney U test was used to compare the persistent group and tolerant group when

data were not applicable to controls. Analysis of real-time PCR data was performed by Kruskal–Wallis tests when comparing changes among three groups, and significant differences between two groups were evaluated by Mann–Whitney tests. Differences with a p-value <0.05 were considered significant in all analyses.

Abnormal values in real-time PCR were interpreted as being caused by technical error. Outliers for this purpose were defined based on appropriate lower limits (first interquartile range − 1.5 × IQR) and upper limits (third interquartile range + 1.5 × IQR) based on the number of copies detected by PCR, and were therefore excluded.

3. Results

1) Patient profile

We enrolled 24 patients in the persistent group, 12 in the tolerant group, and 22 in the control group. The clinical backgrounds of these children are shown in Table 1. Six in the tolerant group had negative provocation test results against oral egg protein, and the remaining six children in the tolerant group had a history of egg white allergy at the time of consultation by an allergy specialist, but they stopped responding to egg white protein.

There were no significant differences in age, anaphylactic episodes, percentage with other food allergies, history and prevalence of asthma, or median blood parameters for nonspecific IgE antibody levels, eosinophil counts, and basophil counts. Compared with the control group, the persistent group included more boys and children with atopic dermatitis and had higher median IgE antibody levels (each, $p < 0.05$).

2) mRNA expression of effector T cells responded to ovalbumin

The expression level of the background number of copies for mRNA of STAT and

the transcription factors for Th1, Th2, Treg, Th17, Tfh and Tfr was expectedly negligibly low. When PBMCs in the persistent group were stimulated with ovalbumin, the mRNA expression levels of STAT1, STAT4, Foxp3, and STAT3 were significantly lower than in the control group (each $p < 0.05$; Figure 1A, C, D). By contrast, no significant differences in STAT6 and GATA3 mRNA expression were observed between the two groups (Figure 1B). PBMCs in the tolerant group also responded to ovalbumin, with significant increases in the mRNA expression of STAT4 and T-bet (Figure 1A), STAT6 and GATA3 (Figure 1B), STAT5 and Foxp3, (Figure 1C), and STAT3 and RORγt (Figure 1D). These were also significantly increased and enhanced compared with the persistent group. In addition, PBMCs in the tolerant group responded to ovalbumin by showing significant enhancements of T-bet (Figure 1A), GATA3 (Figure 1B), Foxp3 (Figure 1C), and STAT3 (Figure 1D) compared to the control group.

Concerning Tfh and Tfr involvement, PBMCs in the persistent group were not significantly different to those in the control group in terms of CXCR5, IL-21, and CD25 mRNA expression induced in response to ovalbumin. When PBMCs were compared between the tolerant and persistent groups, IL-21 and Foxp3 mRNA expression levels were significantly enhanced beyond that of the control group (Figure 1E, F).

4. Discussion

The prevalence of food allergies is considered to be high among Japanese boys,[16] and in this study, egg white allergy was significantly more prevalent in boys than in girls.[17,18] Atopic dermatitis also had a significantly higher prevalence in the persistent and tolerant groups compared with the control group, suggesting that sensitization to egg white protein could occur through the skin. Although it has been reported that food

allergies increase the risk of bronchial asthma,[19,20] we observed no significant differences between the groups. This could be explained, in part, if we had failed to account for other allergies. The tolerant group was notable for having an IgE level specific to egg white that was significantly lower than in the persistent group, consistent with previous data.[21]

The following observations can be made regarding our research questions. In the tolerant group, we showed that the Treg immune response was significantly enhanced compared with the control group (Figure 1C), and that the Th2-type immune response to ovalbumin was enhanced rather than suppressed (Figure 1B). Foxp3 is expressed not only in Treg but also in activated human CD4+ T cells in low levels.[22] Therefore, Foxp3 positive cells were regarded as representative of Treg in the present study.

As hen's egg allergy improves, it is expected that the immunosuppression of circulating T cells to food antigens gradually improves to match that of normal controls. In the tolerant group, T-bet, STAT3, Foxp3, and IL-21 were significantly higher than in the control group. The period after oral tolerance was achieved also appears to be related, but we could not estimate when oral tolerance was achieved. Thus, a peripheral blood Th1-type response was observed in the tolerant group, but it was unclear in enhanced Th2, Treg, Th17, Tfh, and Tfr-type immune responses suppressed serum egg white-specific IgE antibody production. Finally, the persistent group did not show an enhanced Th2-type immune response to ovalbumin compared with the control group (Figure 1B). It is disadvantageous for the host when a pathogenic antigen invades the peripheral blood, leading to the development of highly responsive anaphylaxis. Therefore, the number of antigen-specific circulating immune cells may be able to reduce to minimize the

aggressive response by peripheral blood cells.

In the present study, the activities of Th1, Treg, and Th17 effector T cells, but not Th2 cells, were suppressed by ovalbumin in the persistent group. Similar reports exist of PBMCs obtained from children with peanut allergy. When stimulated in vitro with peanut antigen and analyzed by flow cytometry, it was reported that the Th1 immune response was significantly suppressed.[4] It has been reported that Treg in the peripheral blood of patients with peanut allergies suppress Th1 and Th17 cell function, but that the suppressive effect of Th2 cells is weak.[23] A study used flow cytometry to analyze the peripheral blood of children with egg allergy that had been stimulated in vivo. This revealed that their peripheral blood had fewer Treg than the control group.[24]

In our experimental system, STAT signals and transcription factors in PBMCs did not show a clear and constant expression in response to stimulation by ovalbumin. This may indicate that only monoclonal cell proliferation did not occur due to antigen stimulation, with an explanation being that $CD4^+$ helper T cells were not the only cells that responded to antigen stimulation. This also suggests an in vivo mechanism in which multiple cells, not just $CD4^+$ helper T cells, react in a balanced manner to become immunosuppressive when the antigen presents in the peripheral blood. Although $CD4^+$ cells should have been isolated before extracting RNA, pure $CD4^+$ cells were too short to be sorted for the real-time PCR analysis. Therefore, there might be a bias due to the reaction of other cells.

The production of specific IgE antibody is not limited to the conventional activation

of Th2 cells by IL-4.[25] In recent years, Tfh-derived IL-4 has also been identified and noted to interact with B cells in the germinal center of lymphoid tissue, with increasing awareness that it may be involved in IgE antibody production in humans.[5-6] CXCR5 is an important Tfh-specific cell surface molecule, and B cells are thought to be induced to differentiate by the production of IL-21 to become involved in the production of specific IgE.[7,10,26] However, PBMCs in the persistent group of the present study did not enhance ovalbumin-induced CXCR5 expression, so we could not demonstrate the involvement of Tfh and PBMCs in the immune response of children with egg white allergy. Our results were consistent with previous reports using flow.[27]

In the tolerant group, enhancement of IL-21 mRNA expression was considered to be explainable by the observed enhancement of both the Th17 type immune response and the Treg immune response, with the latter leading to enhanced Foxp3 expression. The involvement of peripheral T helper cells in the production of IgE antibody and of IL-21, but not CXCR5 expression, requires further investigation to clarify the aspect of the immune response in children with egg white allergy.[11,28]

We have an important limitation of this in vitro study. In the cell stimulation model of PBMCs, antigen-presenting cells are considered monocytes and the protagonists of mucosal immunity. In this role, they serve as the “control tower” that determines the direction of the immune response through cytokine production. By priming antigens in circulating cells that migrate from effector sites, they may effectively amplify the original functions and thereby faithfully reproduce the mucosal immunity.

Another limitation is that we used intact ovalbumin. Although the digestive juices of children are immature, the egg white antigen absorbed through the gastrointestinal tract is still thought to have been digested to the extent of oligopeptides by the time it reaches

the peripheral blood. However, percutaneously invaded food proteins that are not digested may be close to intact. This lack of comprehensive digestion means that it may retain antigenic properties and may even explain the significantly higher rate of atopic dermatitis in the persistent group compared with the control group.

Additionally, because of a shortage of adequate pure CD4+ cells for the real-time PCR analysis, we cannot prove whether the large number of lymphocytes that responded to ovalbumin stimulation in PBMCs in our control group could contribute peripheral immune tolerance or not in the present study.

Finally, our egg protein allergy patients included a few older children (more than 10 years old; n=3) who may have non-IgE mediated mechanism.

We will perform a similar experiment using ovomucoid in the future, as it is well-known that many patients seem to tolerate this protein at an initial stage.

5. Conclusion

This study is needed to clarify the pathogenic mechanism of oral tolerance and food allergy in humans and to promote safe medical care.[29,30] Additional analyses, including flow cytometry, phosphorylation analysis, and protein quantification, are required to elucidate our findings. We believe that more basic studies are needed in the future.

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TABLE

Table 1. Patient characteristics by study group

	Persistent	Tolerant	Control	*p*-value
Number	24	12	22	
Median age, years (range)	4.2 (1.3–12.5)	4.0 (1.8–14.3)	7.9 (1.7–14.5)	NS
Male sex	17	5	8	$p < 0.05$
History of anaphylaxis	4	2	0	–
Other food allergens	17	8	10	NS
Bronchial asthma	11	7	7	NS
Atopic dermatitis	11	7	3	$p < 0.05$
Median eosinophils [numbers/µL]	230.9 (n = 13)	406.6 (n = 7)	302.8 (n = 15)	NS
Median basophils [numbers/µL]	63.9 (n = 13)	41.6 (n = 7)	48.0 (n = 15)	NS
Median nonspecific IgE [IU/mL] (range)	373 (34.7–2810)	563 (72.6–2750)	447.5 (11.7–4130)	NS
Median egg white-specific IgE [UA/mL] (range)*	16.9 (0.5–119.0)	3.5 (0.25–61.5)	–	$p < 0.05$

Abbreviations: NS, not significant *: measured by ImmunoCAP®

FIGURE

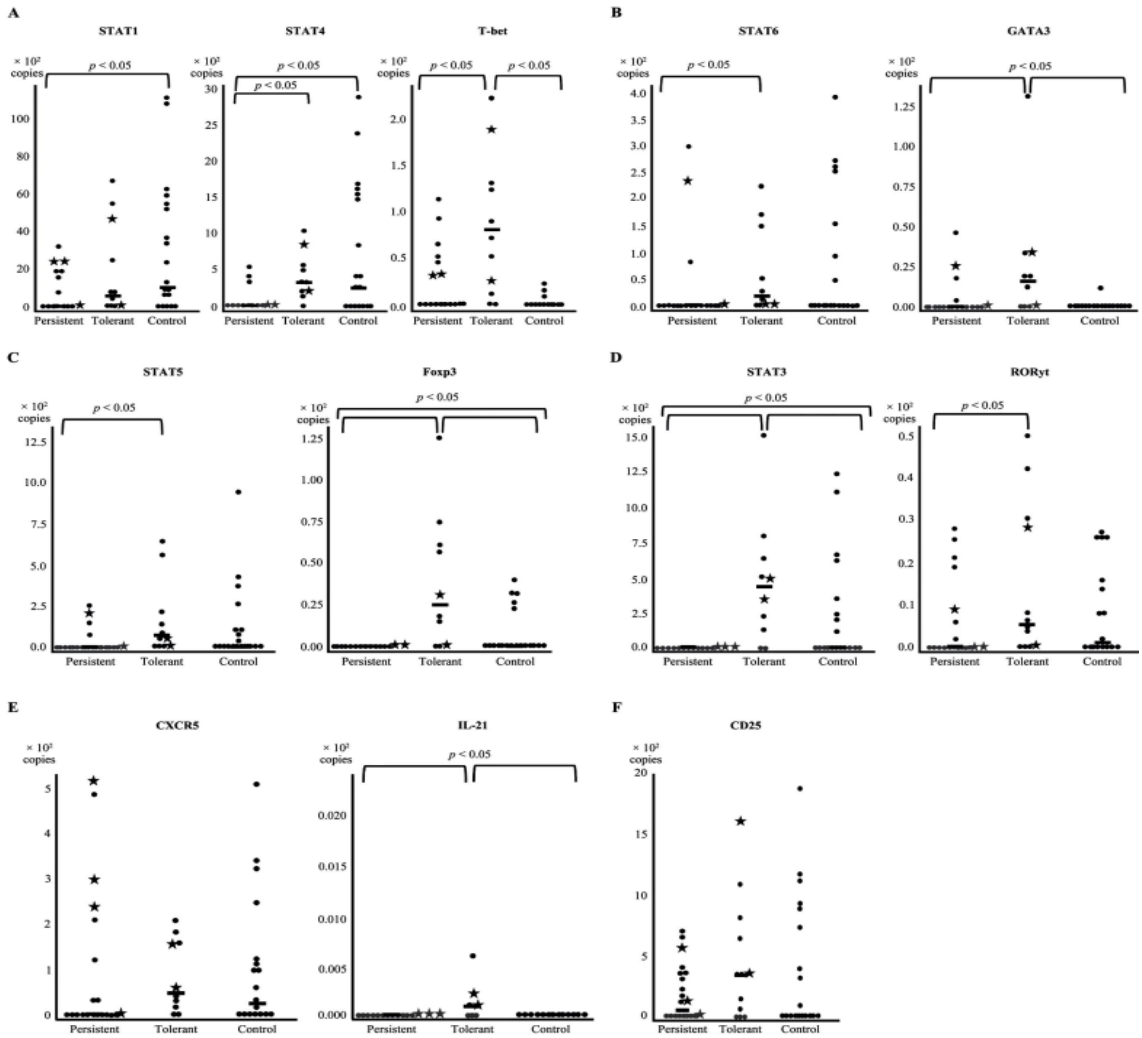

FIGURE LEGEND

Figure 1: mRNA expression of effector T cells responded to ovalbumin

Comparison of PBMC subclasses related to circulating effector $CD4^+$ cells stimulated in vitro by ovalbumin among egg allergy persistent, tolerant, and control groups. The vertical axis represents the number of copies per 50 ng of RNA. The parameters are representative of Th1 (A), Th2 (B), Treg (C), Th17 (D), Tfh (E) and Tfr (F) type immune responses. Foxp3 and CXCR5 are also involved in the Tfr phenotype. The data for patients with a history of resolved anaphylaxis to egg white protein are plotted with a ★.