



Tolerogenic effects of interferon-gamma with induction of allergen-specific interleukin-10-producing regulatory B cell (Br1) changes in non-IgE-mediated food allergy

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ABSTRACT

In this study, specific oral tolerance induction using interferon-gamma (IFN- γ) could successfully treat food allergies. Allergen-specific IL-10-producing regulatory B cell (Br1) responses are characteristic in immune tolerance of food allergies. The *in-vivo* effects of IFN- γ on allergen-induced changes in Br1 proportion and numbers in food allergies were investigated. Oral food challenges were conducted and 20 allergic patients to cow's milk were selected. Of these 20 patients, five were treated with IFN- γ and milk (SOTI group), five were treated with only milk, five were treated with only IFN- γ , and five did not receive any treatment. In addition, 10 milk-tolerant subjects were involved in this study. Peripheral blood mononuclear cells (PBMCs) were stimulated using casein and stained for CD5, CD19, annexin V, and IL-10 before and after treatment. Allergy tolerance was induced only in the SOTI group along with induction of allergen-induced Br1 changes. Thus, IFN- γ can show tolerogenic effects *in vivo* when introduced with an allergen, which may be at least partly due to its effect on allergen-induced Br1 responses.

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1. Introduction

Tolerance induction is an important immunologic concern, particularly for allergies and autoimmune diseases. Currently, a specific method for inducing tolerance for food allergies is under investigation, and avoidance is still the only available therapy [1]. Oral immunotherapy for treating food allergies was first proposed in 1996 [2]. Specific oral tolerance induction (SOTI) for treating food allergy was first reported by a study that showed the success of oral immunotherapy using interferon-gamma (IFN- γ) for treating non-IgE-mediated food allergy in atopic dermatitis (AD) [3]. Thereafter, several studies have attempted to treat

IgE-mediated food allergy with SOTI without IFN- γ with limited success [4]. Complete success of SOTI using IFN- γ for treating IgE-mediated food allergy was reported 6 years after the first trial of SOTI using IFN- γ for non-IgE-mediated food allergy [5]. IFN- γ is a representative Th1 cytokine; Th1/Th2 imbalance is the central immune mechanism of allergic diseases, including food allergies [6]. Immunotherapy with IFN- γ was first attempted to desensitize against house dust mites for AD [7], and subsequently applied in SOTI for food allergy [3,5]. However, the precise immunologic mechanisms of tolerance induction by IFN- γ are not clear.

Mechanisms of immune tolerance are complex and involve both cellular and humoral immune components such as cytokines. CD4(+)Foxp3(+) regulatory T cells play a central role in immune tolerance [8]. However, CD4(+)Foxp3(+) regulatory T cells have not been shown to participate in immune tolerance against food allergy. CD5(+) B cells, a subset of B cells, produce IL-10 that plays a role in negative regulation of immune responses [9]. In particular, IL-10-producing regulatory B cells (Br1), a subset of CD5(+) regulatory B cells, prevent and reverse allergic airway inflammation via Foxp3(+) regulatory T cells in a murine model [10]. Recently, Br1

Abbreviations: AD, atopic dermatitis; SOTI, specific oral tolerance induction; OFC, oral food challenge; DBPCFC, double-blind placebo-controlled food challenge; Ag, antigen.

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was implicated in immune tolerance to food allergy [11,12]. In the present case-control study, the therapeutic potential of SOTI using IFN- γ for treating non-IgE-mediated food allergy in AD was assessed, and changes in allergen-specific Br1 changes before and after SOTI with IFN- γ were evaluated.

2. Materials and methods

2.1. Subjects, inclusion criteria, and study design

Subjects who visited the Department of Allergy and Clinical Immunology at the Seoul Allergy Clinic between March 2010 and April 2011 due to repeated history of late eczematous reactions or exacerbations of AD were screened. All prospective subjects fulfilled the criteria of Hanifin and Rajka and received blood tests and skin prick tests as described below [13]. Signed consent forms were obtained from either the patient or a parent. The study was approved by the Institutional Review Board of Eulji University, Daejeon, Korea. Oral food challenges (OFCs) were conducted to test for milk allergy as described below. Diagnostic criteria for late eczematous food allergy reactions have been well-described and include the following: (1) late eczematous reactions, including exacerbation of AD, following OFC (Fig. 1), (2) exacerbation onset 4 h after the food challenge, and (3) negative skin prick test reactions and food-specific IgE levels for milk [14]. The milk allergy group consisted of 20 subjects who were diagnosed to have milk allergies according to the diagnostic criteria for late eczematous reactions, and the milk-tolerant group consisted of 10 subjects who did not react to milk (Table 1). Among the 20 milk allergy subjects, five received SOTI with IFN- γ (SOTI group), five received only milk (Ag group), five received only IFN- γ (IFN group), and five did not receive treatment (untreated) (Fig. 2). Allergen-induced Br1 responses of these four groups were analyzed before and after the treatment.



Fig. 1. Late eczematous skin lesions in response to oral milk challenge. Characteristic eczematous skin lesions that developed in response to milk challenge. Eczematous lesions developed 1 day after milk ingestion. Left, before oral challenge test for milk; right, after oral challenge test for milk; bottom, erythematous eczema and papulomacular eruption.

Table 1

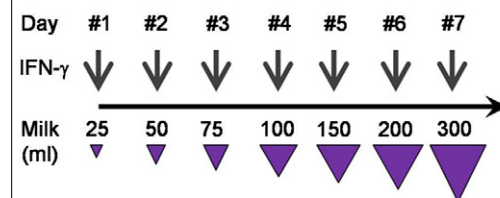
Patients profile.

	Milk allergy	Milk tolerance	Unit
Age (mean $\pm$ SD)	8.1 $\pm$ 14.6	11.5 $\pm$ 16.0	Year
n (M:F)	20(11:9)	10(6:4)	
Eosinophil % in WBC	8.5 $\pm$ 4.9	8.7 $\pm$ 5.1	%
Serum total IgE	2029 $\pm$ 2607	2445 $\pm$ 2947	kU(a)/mL
Serum specific IgE for milk	Undetected	Undetected	
Skin prick test for milk	Negative	Negative	

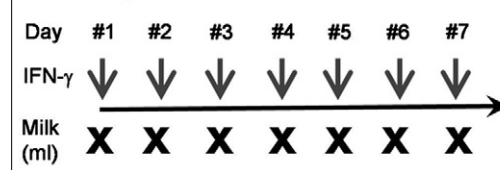
2.2. Blood and skin prick tests

Blood tests comprised of a complete blood count with a differential count for the eosinophil fraction, total serum IgE levels, and blood-specific IgE for milk (UniCAP; Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). Serum food-specific IgE levels <0.35 kU A/L were undetectable. Skin prick tests were conducted on the patient's left forearm using commercial allergen extracts (Bencard, Brentford, England). Histamine hydrochloride (1 mg/mL; Bencard) was used as a positive control, and physiological saline was used as a negative control. Reactions were read after 15 min and classified as negative (0, no reaction; +, reaction greater than the control reaction but lower than half of that caused by histamine), or positive (++, half of the level caused by histamine; +++, equal to the level caused by histamine; and +++, twice the level caused by histamine).

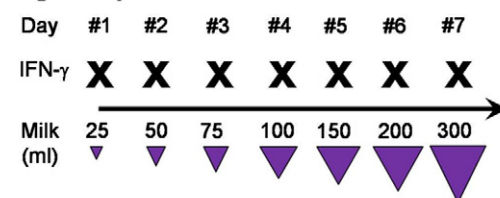
SOTI.



IFN Group



Ag Group



Untreated

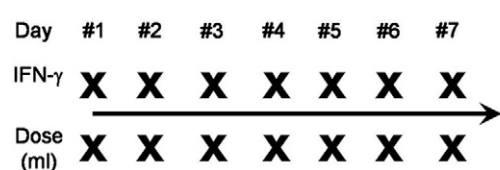


Fig. 2. Study design and protocols for specific oral tolerance induction for non-IgE-mediated food allergy. Schematic protocol of oral immunotherapy using IFN- γ for treating milk allergy. SOTI group, group which received both IFN- γ and milk; IFN group, control group which received only IFN- γ without milk; Ag group, control group which received only milk without IFN- γ ; untreated, untreated control group.

2.3. Oral food challenge (OFC)

To diagnose milk allergies, OFC was conducted by a double-blind placebo-controlled food challenge (DBPCFC) according to previously described diagnostic methods [14]. Briefly, an elimination diet for all possible foods suspected of provoking allergic AD preceded DBPCFC. DBPCFC was conducted just after beginning the elimination diet, and a mixed cereal flour vehicle consisting of brown rice flour, glutinous rice flour, and barley flour was used. Two challenges were performed over 5 days; one challenge included only the placebo, while the other contained milk in the vehicle. Randomization and preparation of the challenges were performed by dietitians in the clinical research unit. All reactions were scored as described below. Confirmatory open challenge tests were also performed according to the open food challenge protocol immediately after completing treatment to confirm whether tolerance to the allergenic foods was acquired [15].

2.4. Scoring clinical severity and diagnosing food allergy

Signs and symptoms of food allergy appeared as newly developed symptoms or exacerbations of AD. Clinical severity was evaluated using the SCORing Atopic Dermatitis (SCORAD) index, a system used worldwide to assess the severity of atopic eczema [16]. A subject was diagnosed to have food allergy if the clinical severity score increased by more than 10% for subjects with a basal score >50 points) or if the score increased by over 10 points for those with a basal score ≤50 points. Apparent new skin lesions and itching were regarded as positive reactions, which were required for a diagnosis of food allergy in response to the food challenge.

2.5. SOTI with IFN- γ for non-IgE-mediated food allergy

Recombinant IFN- γ (Intermax gamma; LGCI, Seoul, Korea) with a specific activity of 2×10^6 IU (50 μ g) was administered by subcutaneous injection at a dosage of 3×10^6 IU/m² (according to body surface area) as previously reported [17]. However, no patients showed any side effects and used acetaminophen in this study. IFN- γ was administered 10 min before allergenic food intake and a scheduled dose of milk was given during 10 min. The standard protocol for treating late eczematous food allergy reactions using SOTI consisted of a 7-day course during which the amount of milk was gradually increased according to a previous protocol [15] (Fig. 2). The Ag group received only milk according to that protocol. The IFN group received only IFN- γ at the same dose, but without milk. An untreated control group received neither IFN- γ nor milk.

2.6. Casein stimulation of PBMCs

PBMCs were isolated from the venous blood by density-gradient separation using Ficoll-Hypaque (ICN Biomedicals Inc., Aurora, OH, USA) and were resuspended at 1×10^6 cells/mL in α -minimum essential medium (Irvine Scientific, Santa Ana, CA, USA). PBMCs were stimulated using casein (Ag) or were unstimulated (none) as a control. For casein stimulation, PBMCs were cultured with or without a 50 μ g/mL mixture of purified alpha-, beta-, and kappa-caseins (Sigma-Aldrich, St. Louis, MO, USA) in 24-well plates. The concentration of casein was the same as that used in a previous study [17].

2.7. Cell surface staining, intracellular staining, and flow cytometric analysis

After 18 h of culture, cells were counted and stained using the specific monoclonal antibodies allophycocyanin-labeled anti-CD19

(eBioscience, San Diego, CA, USA) and phycoerythrin-Cy7-labeled anti-CD5 (eBioscience) was used for B cell subset analysis. The cells that were stained for surface CD19 and CD5 were simultaneously co-stained with fluorescein isothiocyanate-conjugated annexin V by using an apoptosis detection kit (eBioscience). For staining, 1×10^6 cells were suspended in 100 μ L of fluorescence-activated cell sorting (FACS) staining buffer (eBioscience) in an Eppendorf tube. Monoclonal antibodies were added at a concentration of 1 μ g/mL according to manufacturer's instructions. After incubation for 1 h in a dark room and three washes with FACS staining buffer, the cells were resuspended in 500 μ L of FACS staining buffer just before intracellular staining for IL-10. Immediately after cell surface staining, permeabilization/fixation of the cells was performed using a permeabilization/fixation kit (eBioscience). Intracellular IL-10 was stained using a phycoerythrin-labeled anti-IL-10 rat IgG1 monoclonal antibody (eBioscience). Stained cells were acquired using the FACSCalibur device (BD Biosciences, Milpitas, CA, USA), and the data were analyzed using the CellQuest software (BD Biosciences) and FlowJo (Tree Star Inc., Ashland, OR, USA).

2.8. Statistical analysis

For statistical analyses, the Mann-Whitney *U* test was performed for assessing the initial difference between the milk allergy and milk-tolerant group, and the Wilcoxon signed-rank sum test was used to compare changes in the cellular fraction occurring after allergen stimulation in the milk allergy and milk-tolerant group. SigmaPlot Version 11 (Systat Software, San Jose, CA, USA) was used for statistical analyses. $P < 0.05$ was considered significant.

3. Results

3.1. Allergen-specific tolerogenic effects of IFN- γ in non-IgE-mediated food allergy

Subjects with milk allergy developed late eczematous lesions after the OFC for milk (Fig. 1). After the OFC, clinical severity scores increased from 28.5 ± 14.6 to 37.5 ± 14.0 ($P < 0.001$) in the milk allergy group, whereas the scores were unchanged (35.0 ± 17.4 to 31.9 ± 16.4 ; $P = 0.362$) in the milk tolerant group (Fig. 3a). Skin prick tests for milk were negative and milk-specific IgE was not detected in subjects belonging to both the groups (Table 1).

Immune tolerance was induced in the SOTI group and not in the other three groups. Clinical severity scores improved after SOTI, the scores increased from 38.6 ± 21.1 to 48.5 ± 18.1 ($P = 0.007$) in response to OFC before SOTI but were unchanged in response to OFC (27.3 ± 11.8 to 27.2 ± 12.1 ; $P = 0.500$) after SOTI due to the acquisition of immune tolerance to allergenic food ($P = 0.049$, between initial scores before and after) (Fig. 3b). There were no adverse reactions (including anaphylaxis) during SOTI. There was no limitation on the quantity of milk that subjects could consume even 19 months after treatment.

In contrast, clinical severity scores increased for the other three groups following OFC both before and after treatment; for the IFN group, the scores increased from 26.2 ± 8.9 to 35.5 ± 7.9 ($P < 0.001$) before and from 19.0 ± 7.4 to 30.5 ± 6.4 ($P < 0.001$) after treatment ($P = 0.014$, between initial scores before and after) (Fig. 3c); for the Ag group, the scores increased from 23.6 ± 10.8 to 30.8 ± 12.0 ($P = 0.002$) before and from 37.0 ± 7.6 to 49.4 ± 8.4 ($P < 0.001$) after treatment ($P = 0.007$, between initial scores before and after) (Fig. 3d), and for the untreated group, the scores increased from 25.8 ± 13.9 to 35.2 ± 13.2 ($P < 0.001$) before and from 26.9 ± 17.2 to 41.2 ± 19.3 ($P < 0.001$) after the same period ($P = 0.290$, between initial scores before and after) (Fig. 3e). The

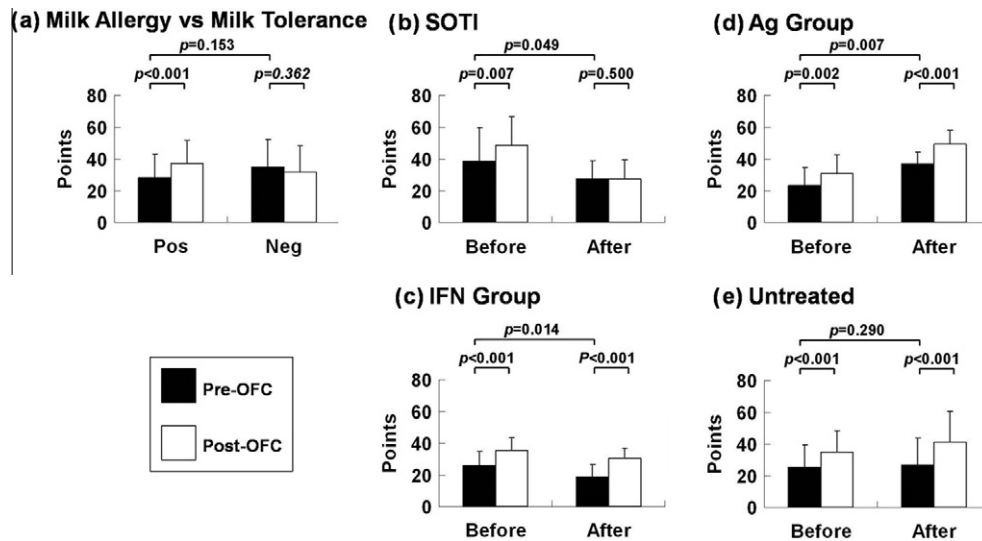


Fig. 3. Allergen-specific tolerogenic effects of SOTI with IFN- γ . (a) Results of oral food challenge (OFC) results in milk allergy group and milk tolerant group. The bars indicate mean clinical severity scores before (pre-OFC) and after (post-OFC) open food challenge. Results of oral food challenge (OFC) results before and after SOTI using IFN- γ (SOTI) (b), before and after only IFN- γ treatment without milk (IFN Group) (c), before and after only milk intake without IFN- γ (Ag group) (d), and untreated group (Untreated) (e). OFC was also conducted before and after the same period of SOTI in the control groups. Mean clinical severity changes in response to OFC before and after treatment are shown. Y-axis, clinical severity score; Pos, milk allergy group; Neg, milk-tolerant group; before, before treatment; after, after treatment; black box (pre-OFC), clinical severity score before OFC; blank box (post-OFC), clinical severity score after OFC with milk.

clinical severity scores improved after IFN- γ treatment in the IFN group, while these scores increased in the Ag group due to repetitive allergy provocation.

3.2. Characteristics of allergen-specific Br1 changes in milk allergy patients and milk-tolerant subjects

CD19(+)CD5(+) B1 cells were isolated following expression of CD5 and CD19 in cultured PBMCs. These B1 cells were subgated according to staining for annexin V and intracellular IL-10 (Fig. 4a and b). The proportion of Br1 cells in B1 cells decreased following allergen stimulation from $31.3 \pm 14.8\%$ to $18.5 \pm 11.1\%$ ($P = 0.002$) in the milk allergy group and increased from $15.3 \pm 9.2\%$ to $21.4 \pm 10.4\%$ ($P = 0.011$) in the milk-tolerant group (Fig. 4c). Total cell numbers increased from $51,630 \pm 15,300$ to $91,492 \pm 21,082$ ($P < 0.001$) in the milk allergy group and from $52,932 \pm 14,933$ to $86,582 \pm 19,087$ ($P < 0.001$) in the milk-tolerant group (data not shown). Taking into account the changes in the total number of cultured cells, Br1 counts decreased following allergen stimulation from 307 ± 236 to 169 ± 169 ($P = 0.010$) in milk allergy subjects and increased from 246 ± 217 to 426 ± 354 ($P = 0.017$) in milk-tolerant subjects (Fig. 4d).

3.3. Allergen-specific changes of apoptotic Br1 percentages and numbers in milk allergy patients and milk-tolerant subjects

The proportion of apoptotic Br1 cells in B1 cells decreased following allergen stimulation ($24.8 \pm 11.2\%$ to $12.7 \pm 8.2\%$; $P < 0.001$) in the milk allergy group and increased ($13.4 \pm 8.2\%$ to $18.4 \pm 9.8\%$; $P = 0.019$) in the milk-tolerant group (Fig. 4e). Considering the total number of cultured cells, the number of apoptotic Br1 cells decreased following allergen stimulation from 252 ± 208 to 115 ± 141 ($P = 0.003$) in the milk allergy group and increased from 213 ± 188 to 368 ± 311 ($P = 0.019$) in the milk-tolerant group (Fig. 4f).

3.4. Induction of allergen-specific Br1 changes by IFN- γ in SOTI for non-IgE-mediated food allergy in AD

The tolerogenic effects of IFN- γ were accompanied by the induction of allergen-specific Br1 changes. The allergen-specific

changes in proportion of Br1 cells in milk allergy subjects were observed only in the SOTI group which were similar to those observed in milk-tolerant subjects (Figs. 5 and 6). The percentage of Br1 cells among B1 cells was unchanged following OFC (19.4 ± 5.9 – $12.6 \pm 3.8\%$; $P = 0.113$) before SOTI, but increased from $9.2 \pm 4.0\%$ to $19.9 \pm 7.9\%$ ($P = 0.026$) after SOTI ($P = 0.049$, between initial level before and after) (Figs. 5a and 6a). The percentage of Br1 cells after OFC did not significantly change before and after treatment in the other three groups. For the IFN group, the percentage changed from $38.7 \pm 11.9\%$ to $26.2 \pm 5.8\%$ ($P = 0.069$) before and from $26.2 \pm 13.8\%$ to $16.5 \pm 13.8\%$ ($P = 0.023$) after treatment ($P = 0.030$, between initial level before and after) (Figs. 5b and 6b); for the Ag group, the percentage changed from $26.4 \pm 11.8\%$ to $17.2 \pm 14.3\%$ ($P = 0.037$) before and from $26.0 \pm 23.8\%$ to $8.6 \pm 3.5\%$ ($P = 0.014$) after treatment ($P = 0.983$, between initial level before and after) (Figs. 5c and 6c); and in the untreated group, the percentage changed from $21.7 \pm 8.9\%$ to $14.2 \pm 4.3\%$ ($P = 0.032$) before and from $16.0 \pm 6.5\%$ to $8.6 \pm 3.5\%$ ($P = 0.045$) after the same period ($P = 0.425$, between initial level before and after) (Figs. 5d and 6d).

Taking into account the changes in the total number of cultured cells, total numbers of cultured B cells changed from $52,121 \pm 15,012$ to $90,814 \pm 20,185$ ($P < 0.001$) before and from $53,165 \pm 16,095$ to $85,881 \pm 21,155$ ($P < 0.001$) after treatment in the SOTI group, from $51,122 \pm 15,289$ to $90,106 \pm 21,191$ ($P \leq 0.001$) before and from $52,841 \pm 15,002$ to $95,243 \pm 22,188$ ($P \leq 0.001$) after treatment in the IFN group, from $51,921 \pm 15,019$ to $93,285 \pm 22,154$ ($P \leq 0.001$) before and from $49,982 \pm 15,430$ to $85,319 \pm 19,450$ ($P \leq 0.001$) after treatment in the Ag group, and from $51,923 \pm 15,130$ to $91,077 \pm 23,001$ ($P \leq 0.001$) before and from $52,818 \pm 15,311$ to $90,914 \pm 21,119$ after the same period in the untreated controls (data not shown).

Considering the total numbers of cultured cells, Br1 number decreased following allergen stimulation before SOTI from 227 ± 92 to 178 ± 117 ($P = 0.042$), but increased from 105 ± 33 to 812 ± 952 ($P = 0.016$) after SOTI (Fig. 7a). However, Br1 number decreased from 620 ± 80 to 383 ± 218 ($P = 0.046$) and from 156 ± 109 to 68 ± 46 ($P = 0.047$) in the IFN group (Fig. 7b), from 179 ± 42 to 90 ± 45 ($P = 0.016$) and from 135 ± 48 to 101 ± 43 ($P = 0.026$) in the Ag group (Fig. 7c), and from 167.8 ± 101.0 to 132.0 ± 33.4

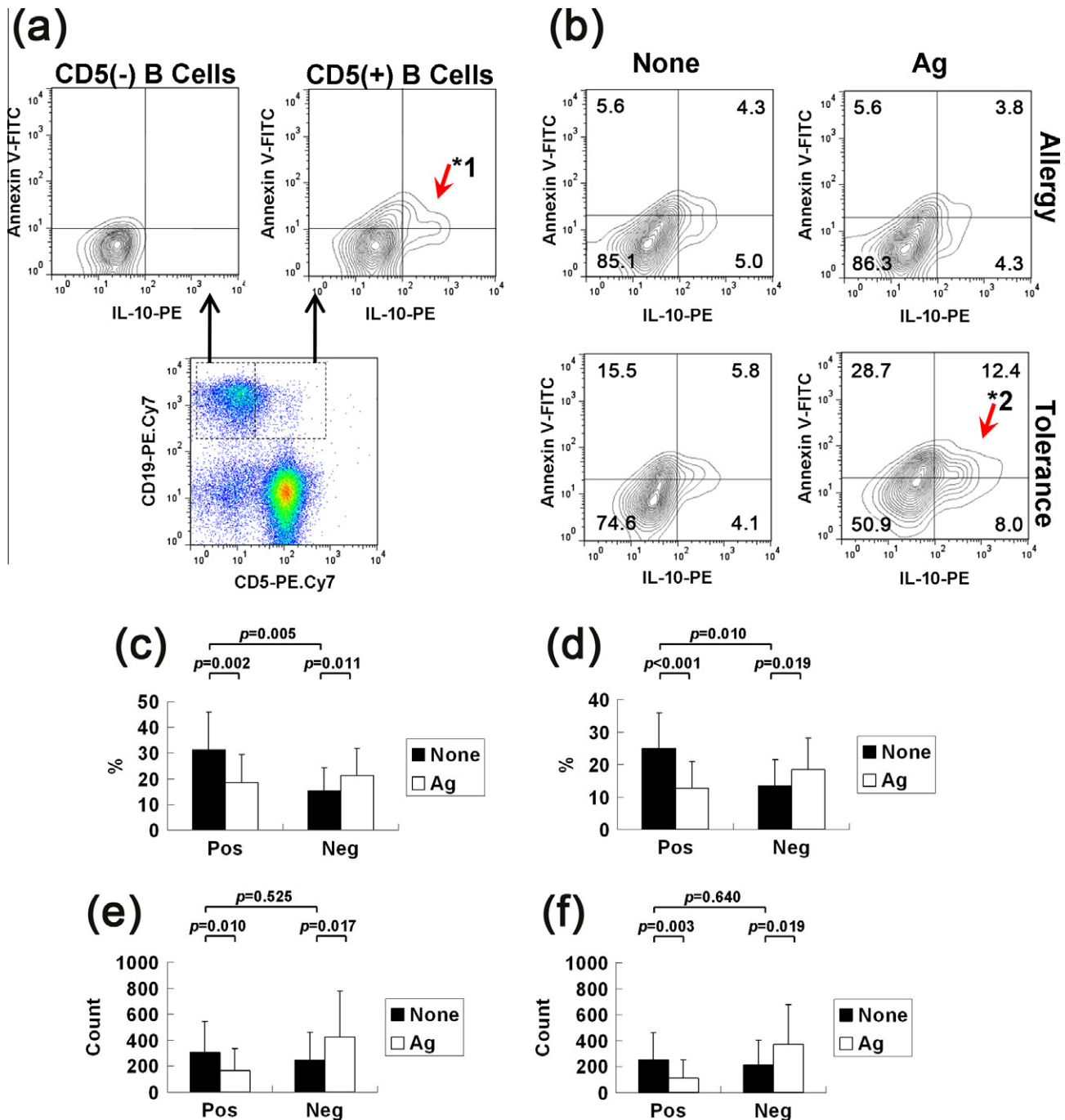


Fig. 4. Flow cytometric analysis of allergen-induced changes in IL-10-producing regulatory B cell proportion and numbers in milk allergy subjects (Allergy) and milk-tolerant subjects (Tolerance). (a) Lymphocytes were gated and analyzed according to CD19 and CD5 expression. Subsequently, CD19(+)CD5(+) B cells were subgated, and cells were analyzed for IL-10 expression and Annexin V binding. Most IL-10-producing B cells (Br1) were observed among CD5(+) B cells (arrow *1). (b) Representative plot of Br1 changes induced by allergen stimulation in milk allergy subjects and milk-tolerant subjects. Br1 fraction increased only in milk-tolerant subjects in response to allergenic challenge (arrow *2). (c) Allergen-specific changes of the Br1 percentage in B1 cells in milk allergy subjects and milk-tolerant subjects. (d) Allergen-specific changes of Br1 counts in cultured PBMCs obtained from milk allergy subjects and milk-tolerant subjects. (e) Allergen-specific changes in the percentage of apoptotic Br1 in B1 cells in milk allergy subjects and milk-tolerant subjects. (f) Allergen-specific changes in the number of apoptotic Br1 in cultured PBMCs obtained from milk allergy subjects and milk-tolerant subjects. Allergy (or AL), milk allergy patient; Tolerance (or TL), milk tolerant-subject; none, unstimulated control; Ag, casein-stimulated control.

($P = 0.279$) and from 139.4 ± 147.8 to 120.2 ± 106.4 ($P = 0.271$) in the untreated controls (Fig. 7d).

3.5. Allergen-specific changes in apoptotic Br1 percentages and numbers by SOTI using IFN- γ

The percentage of apoptotic Br1 in B1 cells decreased following allergen stimulation from $12.1 \pm 4.4\%$ to $7.6 \pm 5.0\%$ ($P = 0.046$)

before SOTI, but increased from $8.2 \pm 3.3\%$ to $17.5 \pm 7.6\%$ ($P = 0.034$) after SOTI ($P = 0.013$, between initial level before and after) (Fig. 8a). This value was unchanged before and after treatment in the other three groups. For the IFN group, the percentage changes for from $34.6 \pm 11.5\%$ to $22.6 \pm 4.6\%$ ($P = 0.043$) before and from $22.9 \pm 12.2\%$ to $10.0 \pm 5.9\%$ ($P = 0.039$) after ($P = 0.043$, between initial level before and after) (Fig. 8b), from $22.3 \pm 9.9\%$ to $10.4 \pm 6.7\%$ ($P = 0.041$) before and from $16.3 \pm 11.2\%$ to $5.0 \pm 3.0\%$

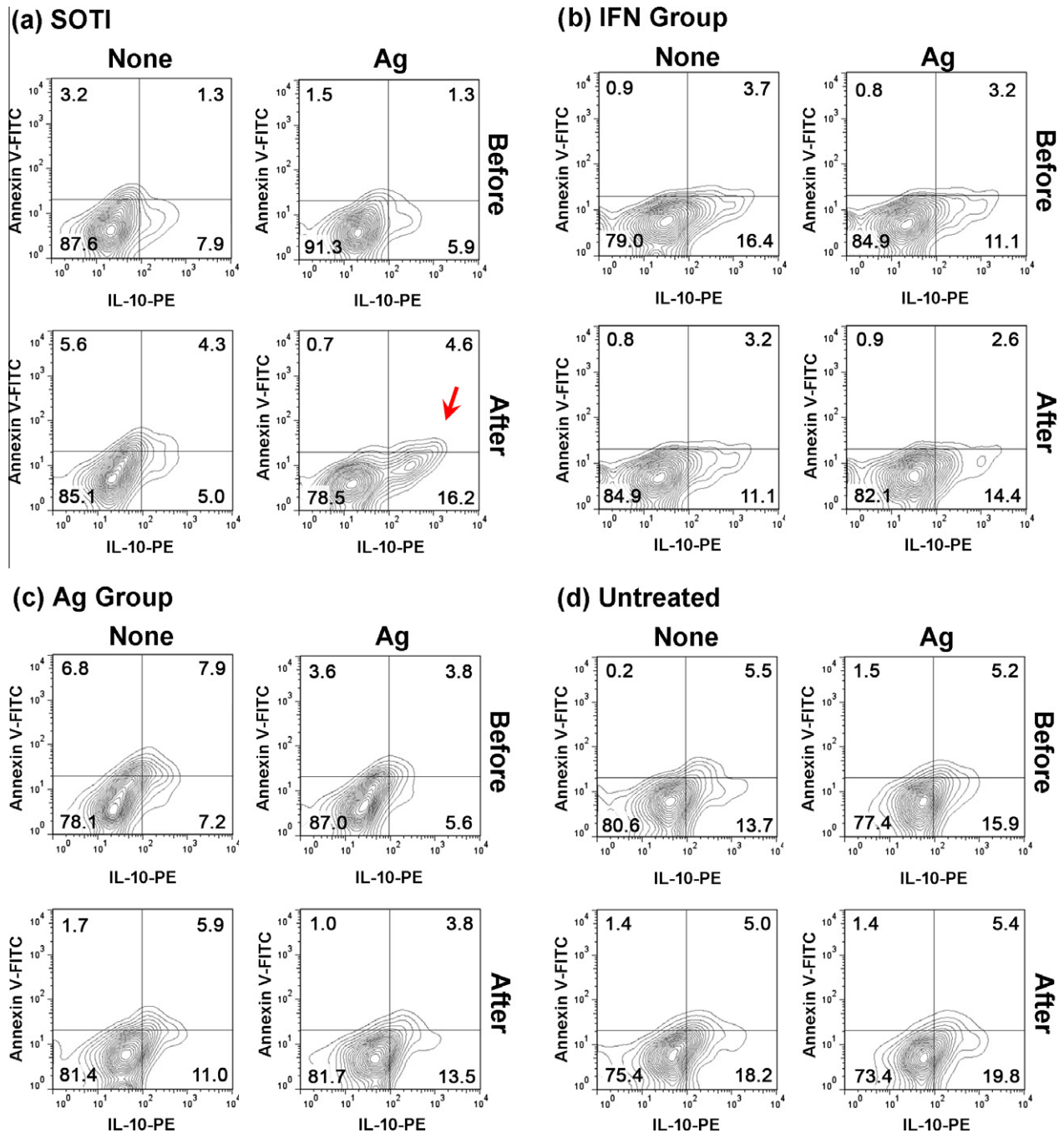


Fig. 5. Effects of SOTI with IFN- γ on allergen-induced changes in Br1 proportion in non-IgE-mediated food allergy. Representative plot before and after treatment. (a) SOTI with IFN- γ (SOTI group), (b) only IFN- γ treatment (IFN group), (c) only allergen administration (Ag group), and (d) no treatment (untreated group). The arrow indicates an increase in proportion of Br1 in B1 cells, which was observed only in the SOTI group and not in other 3 control groups. Before, before treatment; after, after treatment; none, unstimulated control; Ag, casein-stimulated control.

($P = 0.014$) after treatment ($P = 0.271$, between initial level before and after) in the Ag group (Fig. 8c), and from $15.2 \pm 4.4\%$ to $8.9 \pm 3.0\%$ ($P = 0.038$) before and from $11.4 \pm 2.7\%$ to $4.9 \pm 2.1\%$ ($P = 0.033$) after the same period ($P = 0.133$, between initial level before and after) in untreated patients (Fig. 8d).

Taking into account the total numbers of cultured cells, apoptotic Br1 number did not significantly change following allergen stimulation before SOTI from 116 ± 31 to 130 ± 33 ($P = 0.236$), but increased from 97 ± 33 to 737 ± 885 ($P = 0.016$) after SOTI ($P = 0.568$, between initial level before and after) (Fig. 9a).

However, apoptotic Br1 number decreased from 553 ± 72 to 324 ± 185 ($P = 0.045$) before treatment and from 138 ± 98 to 43 ± 23 ($P = 0.018$) after treatment ($P = 0.004$, between initial level before and after) in the IFN group (Fig. 9b), from 152 ± 39 to 60 ± 27 ($P = 0.046$) before treatment and from 119 ± 40 to 94 ± 24 ($P = 0.045$) after treatment ($P = 0.049$, between initial level before and after) in the Ag group (Fig. 9c), and from 114 ± 51 to 85 ± 31 ($P = 0.028$) before treatment and from 93 ± 87 to 64 ± 46 ($P = 0.020$) after the same period ($P = 0.732$, between initial level before and after) in the untreated group (Fig. 9d).

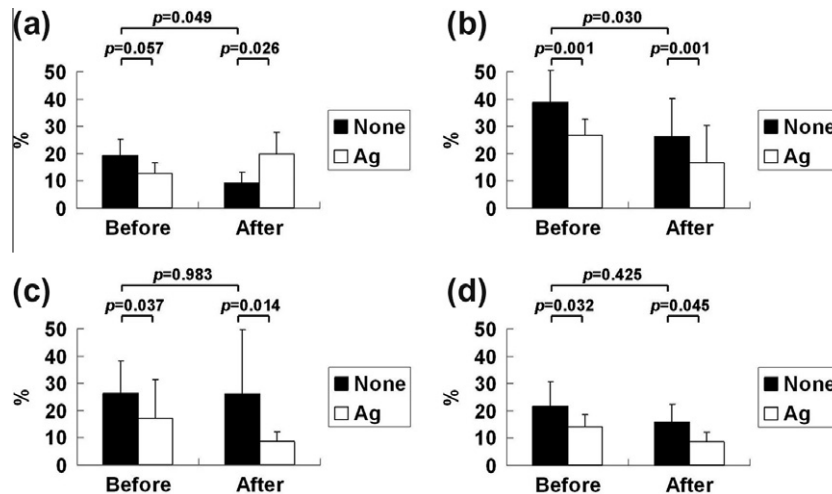


Fig. 6. Effects of SOTI with IFN- γ on allergen-induced changes in Br1 proportion in non-IgE-mediated food allergy. Difference in the allergen-induced changes in percentage of Br1 cells in B1 cells before and after treatment. (a) SOTI group, (b) IFN- γ group, (c) Ag group, and (d) untreated group. Before, before treatment; after, after treatment; none, unstimulated control; Ag, casein-stimulated control.

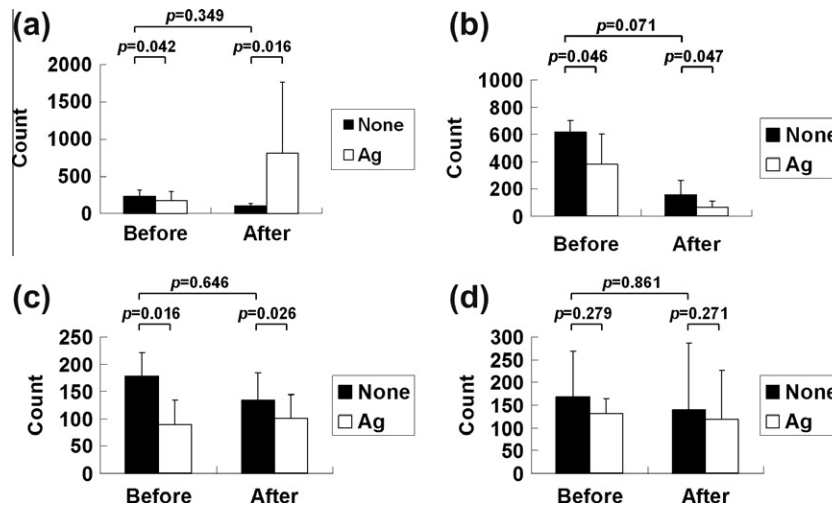


Fig. 7. Effects of SOTI with IFN- γ on allergen-induced changes in number of Br1 cells in PBMCs before and after treatment. (a) SOTI group, (b) IFN- γ group, (c) Ag group, and (d) untreated group. Before, before treatment; after, after treatment; none, unstimulated control; Ag, casein-stimulated control.

4. Discussion

As previously reported, IFN- γ shows *in vivo* allergen-specific tolerogenic effects in non-IgE-mediated food allergy in AD (Fig. 3) [3,15]. In our study, allergen-specific tolerance was not induced following IFN- γ therapy without simultaneous oral administration of allergenic foods (IFN group). In addition, allergen-specific tolerance was not induced by oral administration of allergenic food without IFN- γ treatment (Ag group) in non-IgE-mediated food allergy; this is in contrast to the report stating that oral administration of allergenic food can sometimes induce tolerance to allergenic food in IgE-mediated food allergy [4]. Interestingly, clinical severity improved only in the two groups in which IFN- γ was used (SOTI and IFN groups). IFN- γ appeared to improve clinical severity of AD generally as described previously [5], while clinical severity increased following repeated allergenic provocation in the Ag group (Fig. 3c). Thus far, tolerance for allergenic food has been maintained in our subjects for more than 18 months without any limit on the amount of milk that can be consumed. These patients are considered to be cured according to our previous report. In that

study, patients with non-IgE-mediated food allergy received TIFA and showed tolerance for more than 2 years and remain tolerant even after 4.5 years [15]. Further, no recurrence is recorded in patients who were treated in another previous study of ours more than 10 years until now [3].

Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific Th1 and Th2 cells [18]. In particular, production of Th2 cytokines such as including IL-4 [19] and IL-5 [20] in response to allergen stimulation is also closely related to food allergies. In addition to these Th1/Th2 imbalances, unlike milk-tolerant subjects, milk allergy patients are deficient in IFN- γ production in response to allergen stimulation. Allergen-specific IFN- γ production in PBMCs was inhibited following oral challenge in patients who develop AD due to an allergy to cow's milk [21]. IFN- γ generation can be defective in patients with cow's milk allergies [22]. Collective data supports the hypothesis that IFN- γ supplementation is complementary and has been considered for treating the IFN- γ defect to achieve normal tolerant immune response to milk or to correct Th1/Th2 imbalance. Clinically, IFN- γ supplementation successfully

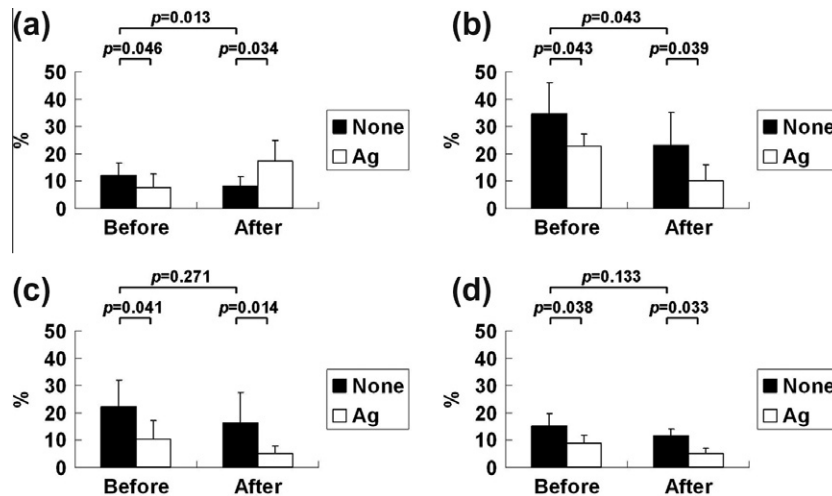


Fig. 8. Effects of SOTI with IFN- γ on allergen-induced changes in proportion of apoptotic Br1 cells in non-IgE-mediated food allergy. Difference in allergen-induced changes in proportion of apoptotic Br1 cells in B1 cells before and after treatment. (a) SOTI group, (b) IFN- γ group, (c) Ag group, and (d) untreated group. Before, before treatment; after, after treatment; none, unstimulated control; Ag, casein-stimulated control.

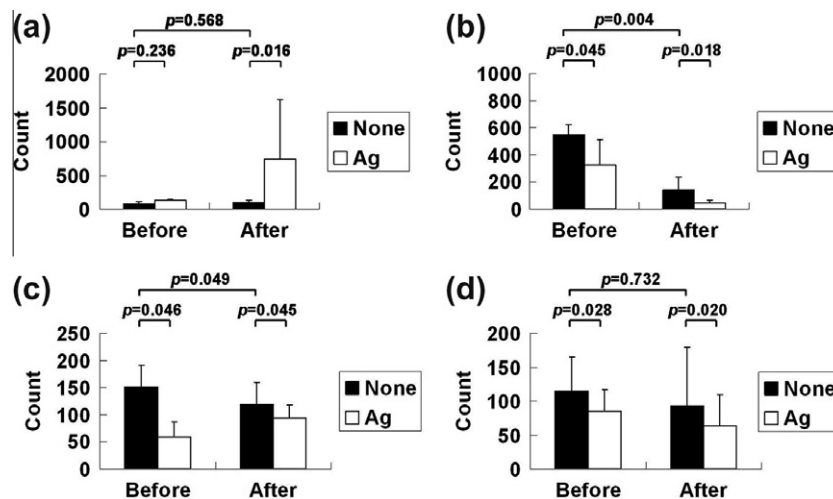


Fig. 9. Effects of SOTI with IFN- γ on allergen-induced changes in number of apoptotic Br1 cells in non-IgE-mediated food allergy. Difference in allergen-induced changes in number of apoptotic Br1 cells in PBMCs before and after treatment. (a) SOTI group, (b) IFN- γ group, (c) Ag group, and (d) untreated group. Before, before treatment; after, after treatment; none, unstimulated control; Ag, casein-stimulated control.

induced immune tolerance to allergenic foods in the present study. During oral tolerance induction, IFN- γ production from T cells can occur [23]. Induction of tolerance to peanut allergens using herbal formula-2 was associated with IFN- γ upregulation [24] by IFN- γ -producing CD8(+) T cells [25]. However, it is not clear whether induction of allergen-specific tolerance in non-IgE-mediated food allergy in AD was achieved by correcting Th1/Th2 imbalance or through the unique tolerogenic effects of IFN- γ . Nonetheless, IFN- γ showed allergen-specific tolerogenic effects for non-IgE-mediated food allergy when it was introduced simultaneously with the allergenic food.

Regulatory B cells are involved in the pathogenesis of delayed-type hypersensitivity by mediating early T cell recruitment [26]. Regulatory B cells also mediate airway hyper-reactivity in non-atopic asthma [27]. However, regulatory B cells appear to play a negative regulatory role by producing IL-10 and inducing regulatory T cells [10]. IFN- γ also influences the development of regulatory T cells by inducing Foxp3 [28]. Allergen-specific Br1 responses have been linked to tolerance to food allergens that cause

late eczematous reactions [11,12]. IFN- γ may have displayed its allergen-specific tolerogenic effects at least in part by inducing allergen-specific Br1 responses in this study. These results also suggest that tolerance induction for non-IgE-mediated food allergy in AD by IFN- γ appears to be a unique tolerogenic effect of IFN- γ rather than an effect of correcting Th1/Th2 imbalance.

Br1 cells can readily undergo apoptosis. Br1 cells proliferate following allergen stimulation in milk-tolerant subjects but not in milk allergy patients (Fig. 5a–c), as described in a previous report [12]. Proliferating Br1 cells also showed apoptotic characteristics in proportion to their proliferation (Fig. 5d and e). Proliferation and apoptosis are opposite actions. Since IL-10 not only protects CD5(+) B cells from apoptosis, but also acts as an autocrine growth for CD5(+) B cells [28], IL-10 may induce Br1 proliferation. However, activation-induced apoptosis can occur in B cells [29]. In particular, IL-21 simultaneously induces apoptosis of anti-CD40 activated B cells, causing B cell proliferation [30]. In the present study, apoptosis of Br1 cells was observed simultaneously with its proliferation after allergen stimulation. Br1 apoptosis may be

regarded as a counter-regulatory mechanism to prevent excessive suppression.

Allergen-specific tolerance to food allergy was not induced by IFN- γ therapy (IFN group) alone (Figs. 5 and 6), but was induced when IFN- γ was administered simultaneously with allergen stimulation (SOTI group) (Figs. 5a and 6a). These *in vivo* results indicate that IFN- γ can induce allergen-specific Br1 responses in an antigen-dependent manner. The antigen-dependent T cell receptor-mediated pathway is important for peripheral tolerance induction by CD8(+) T cells [31,32]. IL-10 also plays a key role in antigen-specific anergy in immunologic tolerance [33]. IL-10 is produced primarily by Tr1 helper cells [34] as well as by Br1. IL-10 production is related to immunotherapy and allergy remission [35]. Particularly, IL-10 has been linked to late eczematous food allergy reactions [36] and plays a key role in the persistence of milk allergy [37]. Allergen-specific Br1 responses were induced by SOTI in this study. IL-10-producing CD4(+)CD25(+) T cells can be induced by grass pollen immunotherapy [38]. IL-10-producing cells such as CD19(+)CD5(+) Br1s and IL-10-producing CD4(+)CD25(+) regulatory T cells (Tr1s), play important roles in food allergen tolerance. IL-10 appears to be involved in maintaining immune tolerance through allergen-specific anergic mechanisms. Moreover, food allergies can be prevented by administration of either IL-10-secreting *Lactococcus lactis* [39] or an avirulent *Salmonella typhimurium* strain that promotes IL-10 secretion in the gut of mice [40]. The collective data supports involvement of IL-10 in prevention of allergy sensitization in food allergy. Transforming growth factor-beta (TGF- β) as well as IL-10 are the key cytokines responsible for maintaining normal homeostasis in the adult gut by creating a cytokine milieu for appropriate antigen processing and promoting the development of oral tolerance [41]. TGF- β is also involved in oral tolerance by inducing Th3-type TGF- β -secreting cells in autoimmune disease [42]. However, it is unclear whether both IL-10 and TGF- β participate in inducing allergen-specific tolerance for pre-existing allergies to specific food allergens. However, uniquely, IFN- γ exhibited a tolerogenic effect for pre-sensitized food allergens in oral immunotherapy in our *in vivo* study.

IL-10 and TGF- β are thought to be the major cytokines involved in immune tolerance, and these cytokines may be the most beneficial in therapeutic application. However, use of IL-10 and TGF- β can result in adverse effects such as infection and cancer, which occur due to immune surveillance system dysfunction via non-specific. The clinical safety of IFN- γ is well-established, while clinical trials of IL-10 or TGF- β in humans are currently underway. Moreover, IFN- γ has several immunologic advantages over TGF- β and IL-10 in inducing allergen-specific tolerance. First, IFN- γ is generally a pro-inflammatory cytokine that is not associated with risk of infection or cancer development; instead, it prevents or rather improves immune functions differently than the immunosuppressive functions of IL-10 and TGF- β . Second, anti-allergic effects of IFN- γ are mediated by correction of Th1/Th2 imbalance rather than through non-suppression by IL-10 or TGF- β . IFN- γ therapy for tolerance induction to food allergy may improve the clinical and immunological status of AD, as demonstrated in this study and in our previous report [5]. Third, IFN- γ may induce allergen-specific responses in tolerance induction specifically for allergenic foods, in contrast to non-specific Tr1 induction by IL-10. Finally, IFN- γ appears to play a specific role in the tolerance induction process, whereas TGF- β and IL-10 have non-specific immunosuppressive roles such as resultant secondary immunosuppression although IL-10 reportedly plays a tolerogenic role in an allergen-specific manner [33]. Considering its overall immunologic and clinical aspects, IFN- γ and not IL-10 and TGF- β is the most appropriate cytokine for inducing specific tolerance to allergenic food.

In conclusion, IFN- γ exerts *in vivo* tolerogenic effects in food allergy in an antigen-specific manner. This clinical tolerance

induction for non-IgE-mediated food allergy in AD by SOTI is accompanied by induction of allergen-specific Br1 responses. IFN- γ also appears to induce allergen-specific Br1 responses *in vivo*, which may, in part, be the immunologic mechanisms underlying the antigen-specific tolerogenic role of IFN- γ . Further studies are necessary to define the molecular mechanisms of IFN- γ in antigen-specific tolerance induction of food allergies.

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