



Characterisation of allergen-specific responses of IL-10-producing regulatory B cells (Br1) in Cow Milk Allergy

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ABSTRACT

CD19+CD5+ regulatory B cells regulate immune responses by producing IL-10. IL-10-producing regulatory B cell (Br1) responses by allergen stimulation were investigated in human food allergy. Six milk allergy patients and eight milk-tolerant subjects were selected according to DBPCFC. PBMCs were stimulated by casein *in vitro* and stained for intracellular IL-10 and apoptosis. In response to allergen stimulation, Br1 decreased from 26.2 ± 18.3 to $15.5 \pm 8.9\%$ ($p = 0.031$, $n = 6$) in the milk allergy group and increased from 15.4 ± 9.0 to $23.7 \pm 11.2\%$ ($p = 0.023$, $n = 8$) in the milk-tolerant group. Apoptotic non-IL-10-producing regulatory B cells increased from 21.8 ± 9.3 to $38.0 \pm 16.1\%$ ($p = 0.031$, $n = 6$) in the milk allergy group and unchanged from 28.8 ± 13.8 to $28.0 \pm 15.0\%$ ($p = 0.844$, $n = 8$) in the milk-tolerant group. Br1 may be involved in the immune tolerance of food allergies by producing IL-10 and simultaneously undergoing apoptosis in humans. The exact roles for Br1 in immune tolerance needs to be further investigated.

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1. Introduction

Food allergies are classified as IgE-mediated type and non-IgE-mediated type [1]. Th1/Th2 imbalances are known to be the main immunopathological mechanism of non-IgE-mediated food allergies [2]. CD4+Foxp3+ regulatory T cells have been reported to play an important role in immune tolerance, including allergy tolerance [3], but their role in allergy tolerance is still controversial.

CD5 is a pan-T cell marker that is also expressed in various developmental and activation states on human B cells, and it is a well-established negative regulator of TCR and BCR signalling [4]. CD5+ B cells are a subset of B cells known as regulatory B cells that negatively regulate immune responses [5]. CD5 plays a key role in B and T cell selection, as well as in the generation and maintenance of tolerance [6].

IL-10 is a potent immunosuppressive cytokine that inhibits T-cell activation and cytokine production *in vivo* [7]. A portion of CD5+ regulatory B cells contain IL-10 [8]. In a preliminary study, IL-10-producing regulatory B cells (Br1) decreased in milk allergy patients [9]. IL-10-producing B cell (Br1) responses to casein stimulation were investigated and characterised in this study.

2. Materials and methods

2.1. Subjects and inclusion criteria

Subjects between 10 and 28 years of age who visited the Department of Allergy and Clinical Immunology at the Seoul Allergy Clinic between March 2010 and April 2010 and were suffering from a repeated history of late eczematous reactions or exacerbations of atopic dermatitis (AD) were screened for the current study. They received blood tests and skin prick tests as described below and fulfilled the criteria of Hanifin and Rajka [10]. Signed consent forms were obtained from either the patient or the parent. The study was approved by the Institutional Review Board of Chungnam National University Hospital, Daejeon Korea.

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Double-blind placebo-controlled food challenge (DBPCFC) tests were conducted for milk; diagnostic criteria for food allergy late eczematous reactions were well-described in previous reports and included the following: (1) late eczematous reactions including exacerbation of AD by DBPCFC, (2) exacerbation onset four hours after the food challenge, and (3) negative skin prick test reactions and food-specific IgE levels for milk [11].

2.2. Blood tests and skin prick tests

Subjects received blood tests and skin prick tests. Blood tests included a CBC with differential count for the eosinophil fraction, serum total IgE levels, and blood-specific IgE for milk (which was measured using UniCAP; Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden). Serum food-specific IgE levels less than 0.35 kU A/L were classified as 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 smaller than half that caused by histamine) or positive (+: half the level, ++: equal to, and +++: twice the level caused by histamine).

2.3. DBPCFC

To diagnose milk allergy, DBPCFC was conducted according to the diagnostic methods used in previous reports [12]. Briefly, an elimination diet for all possible foods suspected of provoking allergic AD was performed.

A double-blind placebo-controlled food challenge (DBPCFC) was conducted just after administration of the elimination diet. DBPCFC was performed using a mixed cereal flour vehicle, consisting of brown rice flour, glutinous rice flour, and barley flour. Two challenges were performed, separated by five days; one was only the placebo, while the other contained the suspected food antigen in the vehicle. Randomisation and preparation of the challenges were performed by dietitians in the clinical research unit. All reactions were scored as to type, time of onset, severity and duration.

2.4. Clinical severity scoring and diagnosis of food allergy

The signs and symptoms of allergy provocation appeared as newly developed symptoms or an exacerbation of AD. Thus, clinical severity was evaluated using the SCORing Atopic Dermatitis (SCORAD) index, a system used worldwide to assess the severity of atopic eczema [13]. A diagnosis of food allergy was made if the clinical severity score increased by greater than 20% in subjects demonstrating a basal score greater than 50 points, or if the score increased by over 10 points for those with a basal score less than or equal to 50 points. Apparent new skin lesions or itching were regarded as positive reactions, which were required for the diagnosis of food allergy by food challenge.

2.5. Casein stimulation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood by density-gradient separation using Ficoll-Hypaque (Biomedicals, Aurora, OH, USA) and then resuspended at 1×10^6 cells/ml in an α -minimum essential medium (alpha-MEM; Irvine Scientific, Santa Ana, CA). PBMCs were then cultured with or without a 50 μ g/ml mixture of purified alpha-, beta-, and kappa-caseins (Sigma, St. Louis, Mo) in α -MEM. The casein concentration was referred to in a previous study [14]. Cells were cultured in 24-well

plates at a concentration of 1×10^6 cells/mL. As an unstimulated control, 1×10^6 cells/mL were also cultured without casein.

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

After eighteen hours of culture, cells were counted, and cultured PBMCs with or without casein were stained with specific monoclonal antibodies with allophycocyanin (APC)-labelled anti-CD19 (eBioscience, San Diego, CA, USA) and phycoerythrin. Cy7(-PE.Cy7)-labelled anti-CD5 (eBioscience, San Diego, CA) was used for B cell subset analysis. With staining for surface CD19 and CD5, the cells were co-stained with an apoptosis detection kit using FITC-conjugated annexin V (eBioscience, San Diego, CA) simultaneously.

Cells to be stained were prepared at a concentration of 1×10^6 in 100 μ l of FACS staining buffer (eBioscience, San Diego, CA) in an eppendorf tube. Monoclonal antibodies were added at a concentration of 1 μ g/ml according to the manufacturer's instructions. After incubation for one hour in a dark room and three washes with FACS staining buffer, cells were resuspended into 500 μ l of FACS staining buffer just before flow cytometric analysis.

Just after finishing cell surface staining, permeabilization/fixation of cells was performed using a permeabilization/fixation kit (eBioscience, San Diego, CA). Intracellular IL-10 was stained using phycoerythrin-labelled anti-IL-10 rat IgG1 monoclonal antibody (eBioscience, San Diego, CA). Stained cells were acquired with a FACSCaliber device (BD, Biosciences, Milpitas, CA, USA), and the data were analysed with CellQuest software (BD, Biosciences).

Table 1

Characteristics of the milk allergy group and the milk-tolerant group.

	Milk allergy group	Milk-tolerant group	Unit
No	6	8	
M:F	3:3	5:3	
Age	19.1 $\pm$ 12.3	18.8 $\pm$ 13.7	Year
Total IgE	1123.3 $\pm$ 1523.5	1411.1 $\pm$ 1563.2	IU/L
Blood eosinophil	7.23 $\pm$ 4.86	8.25 $\pm$ 7.3	%
Blood lymphocyte	35.2 $\pm$ 13.2	34.3 $\pm$ 12.1	%
Milk-specific IgE	Undetectable	Undetectable	kUA/L
Casein-specific IgE	Undetectable	Undetectable	kUA/L
Skin prick test for milk	All negative	All negative	Grade



Fig. 1. Representative skin lesions of late eczematous reactions to cow's milk. Erythematous papular eruptions developed following food challenge.

2.7. Statistical analysis

Statistical analyses were performed using the Mann–Whitney U Test for the initial difference between the milk allergy group and the milk-tolerant group and the Wilcoxon signed rank sum

test for the change of cellular fraction by allergen stimulation in the milk allergy group and in the milk-tolerant group. Signam-Plot Version11 (Systat Software Inc., San Jose, CA, USA) used for all the statistic analysis. $P < 0.05$ was considered significant for all analyses.

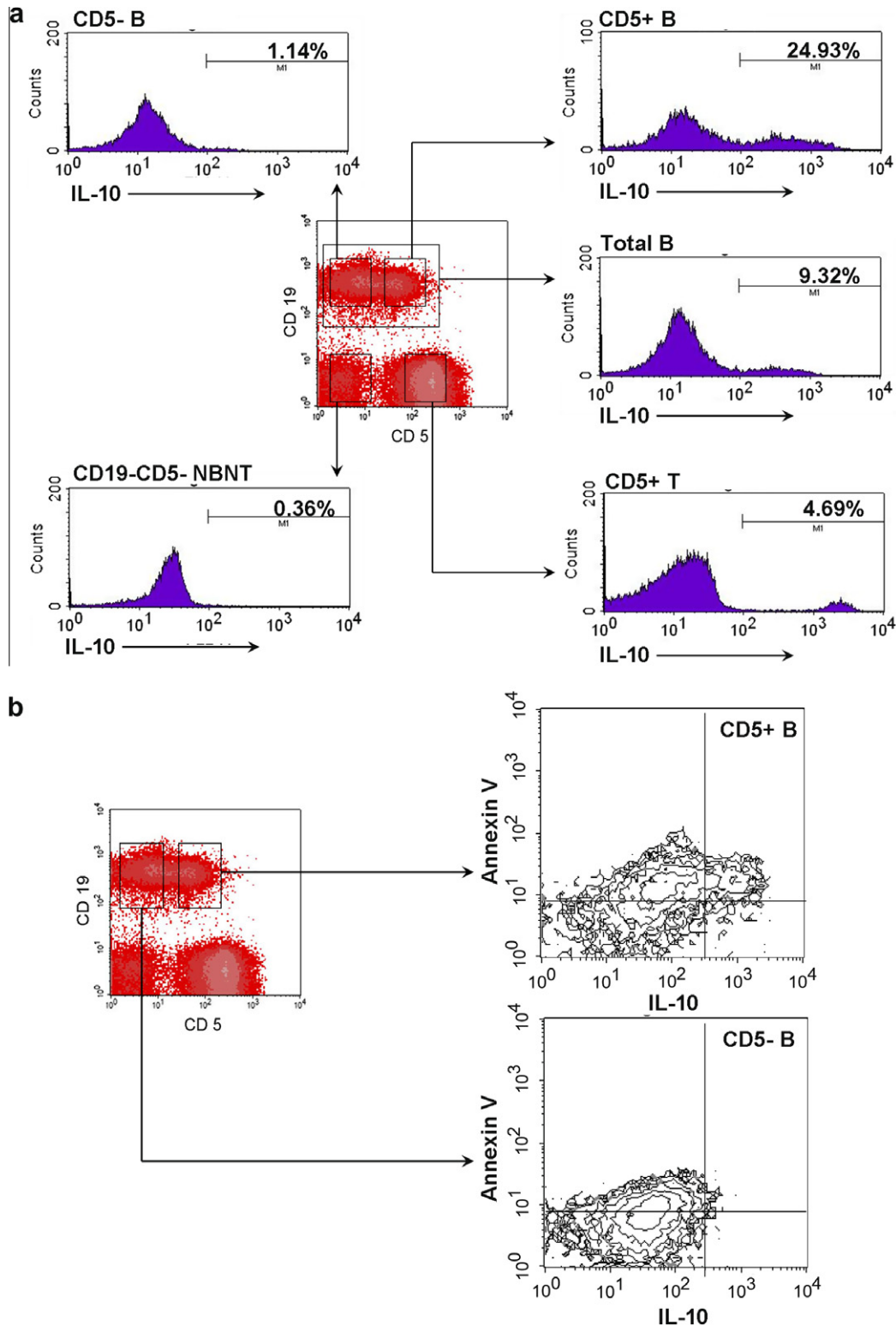


Fig. 2. IL-10 production and apoptosis of CD19+CD5+ regulatory B cells. PBMCs were cultured for eighteen hours and stained using monoclonal antibodies. (a) Representative histograms of IL-10-producing cells, (b) double-stained plots from CD19+CD5+ B cell subpopulations. CD5- B, CD19+CD5- B cells; CD5+ B, CD19+CD5+ B cells; CD5+ T, CD19-CD5+ T cell; NBNT, CD19-CD5- cells.

3. Results

3.1. Clinical characteristics

Six positive milk-allergic subjects were selected according to the diagnostic criterion of a late eczematous reaction to milk (the milk allergy group), and eight subjects who did not react to milk were included as the milk-tolerant group (Table 1). Milk allergy subjects showed the development of late eczematous lesions by DBPCFC (Fig. 1). All subjects showed negative skin prick test results for milk and undetectable milk-specific IgE.

3.2. IL-10-production of CD19+CD5+ regulatory B cell

IL-10 was produced from B cells (9.32%) as well as from CD5+ T cells (4.69%) in peripheral blood mononuclear cells (Fig. 2a). Among B cells, CD19+CD5+ regulatory B cells predominantly produced IL-10. IL-10-producing cell fraction was higher in CD5+ B cells (24.93%) than that in CD5+ T cells. IL-10 production was also investigated together with apoptotic changes. IL-10 was produced predominantly from CD5+ B cells. Most of these IL-10-producing regulatory B cells (Br1) underwent apoptosis (Fig. 2b). By IL-10 production and apoptosis, CD5+ regulatory B cells were sub-classified into apoptotic non-IL-10-producing cells, apoptotic IL-10-producing cells, non-apoptotic IL-10-producing cells, and non-apoptotic non-IL-10-producing cells.

Each subpopulation of CD5+ regulatory B cells showed different responses to the allergen between the milk allergy patients and the milk-tolerant subjects (Fig. 3). Characteristically, Br1 including apoptotic Br1 subpopulations proliferated in the milk-tolerant group, while the apoptotic non-IL-10-producing subpopulation proliferated in the milk allergy group.

3.3. IL-10-producing regulatory B cell (Br1) responses to allergen

The fraction of IL-10-producing cells among CD5+ regulatory B cells decreased from 26.2 ± 18.3 to $15.5 \pm 8.9\%$ ($p = 0.031$, $n = 6$) in the allergy group and increased from 15.4 ± 9.0 to $23.7 \pm 11.2\%$ ($p = 0.023$, $n = 8$) in the tolerant group (Fig. 4a). There was no difference between the initial fraction of Br1 the milk allergy group and in the milk-tolerant group ($p = 0.936$).

Apoptotic Br1 unchanged from 19.0 ± 16.4 to $12.5 \pm 6.9\%$ ($p = 0.313$, $n = 6$) in the milk allergy group and increased from 11.8 ± 7.5 to $19.5 \pm 9.9\%$ ($p = 0.008$, $n = 8$) (Fig. 4b). Initial apoptotic Br1 fraction showed no difference between the two groups ($p = 0.414$). Non-apoptotic Br1 decreased from 7.3 ± 5.6 to $3.0 \pm 2.3\%$ ($p = 0.031$, $n = 6$) in the milk allergy group and unchanged from 3.6 ± 3.8 to $4.2 \pm 3.0\%$ ($p = 0.461$, $n = 8$) (Fig. 4c). Initial non-apoptotic Br1 fraction showed no difference between the two groups ($p = 0.081$).

3.4. Apoptotic non-IL-10-producing regulatory B cell subset responses to allergen

The fraction of apoptotic non-IL-10-producing regulatory B cells increased from 21.8 ± 9.3 to $38.0 \pm 16.1\%$ ($p = 0.031$, $n = 6$) in the allergy group and was unchanged from 28.8 ± 13.8 to $28.0 \pm 15.0\%$ ($p = 0.844$, $n = 8$) in the tolerant group (Fig. 4d). Initial apoptotic non-IL-10-producing regulatory B cell fraction showed no difference between the two groups ($p = 0.282$).

4. Discussion

IL-10 was produced by B cells in this study in addition to being produced by T cells. In mice, Ly-1 B (B-1) cells are the main source

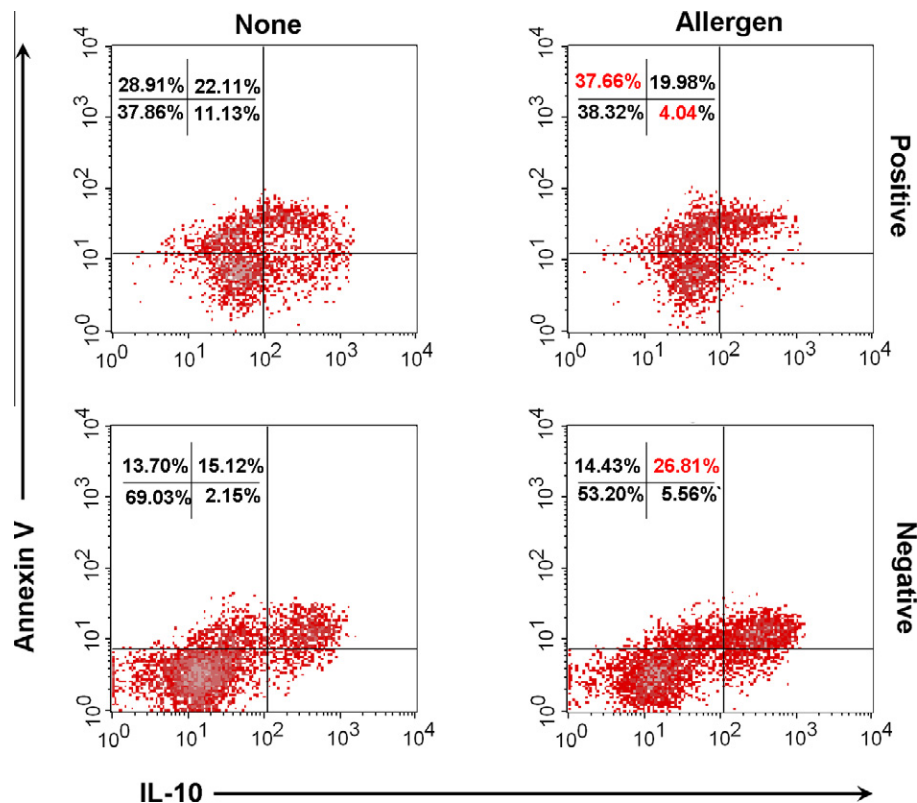


Fig. 3. Representative compositional changes of regulatory B cells in response to allergen stimulation in the milk allergy group and the milk-tolerant group. PBMCs were cultured for eighteen hours and stained using monoclonal antibodies. The percentage of each population is described in the upper left quadrant. X-axis, IL-10 expression; Y-axis, annexin V; None, no stimulation of PBMCs; Allergen, casein-stimulated PBMCs; Positive, milk allergy patient; Negative, milk-tolerant subjects.

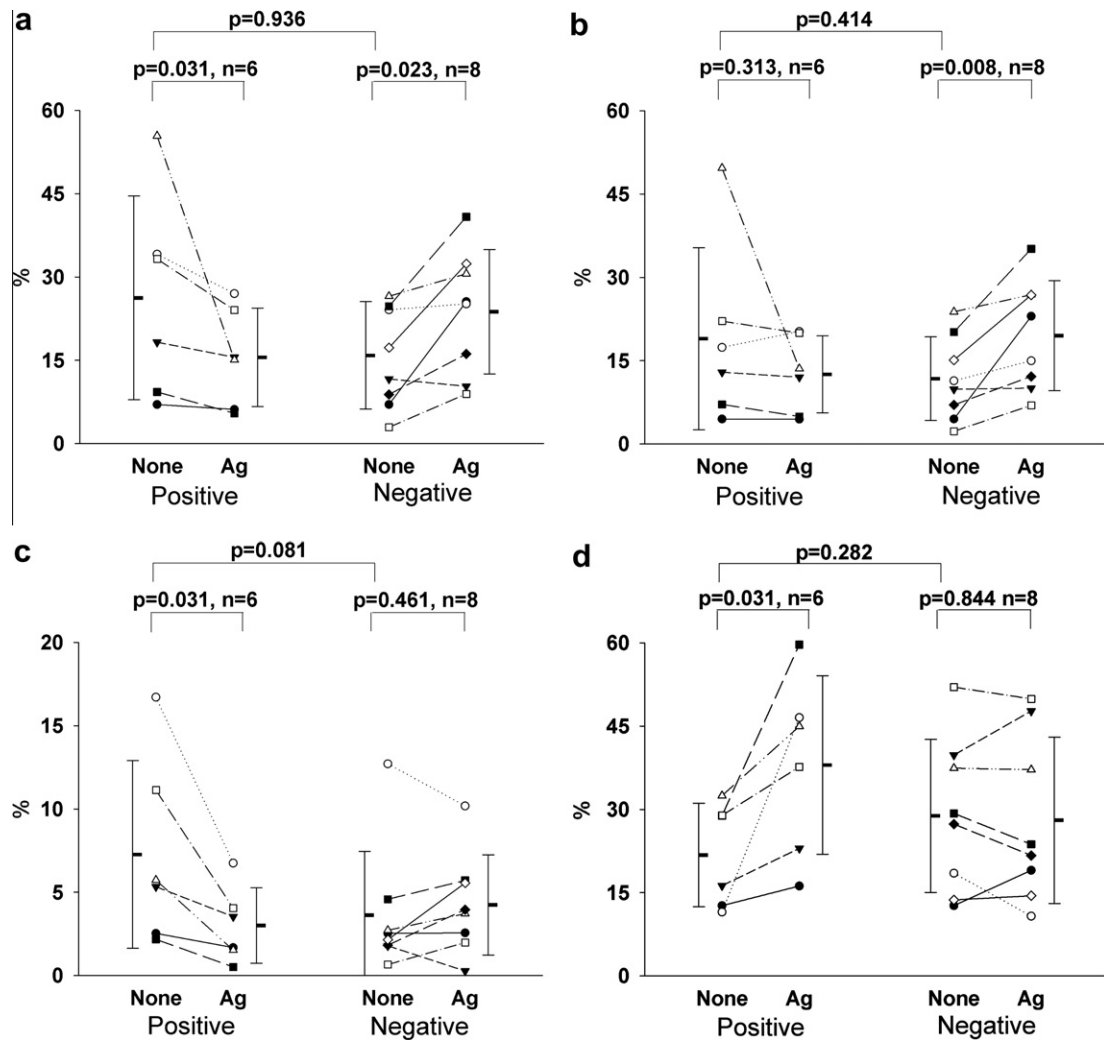


Fig. 4. Individual responses of IL-10-producing regulatory B cell (Br1) in the milk allergy group and the milk-tolerant group following allergen stimulation. The means of each group was presented with standard deviation (mean $\pm$ SD). (a) Individual changes of Br1 proportion by allergen stimulation, (b) individual changes in apoptotic Br1 proportion by allergen stimulation, (c) individual changes in non-apoptotic Br1 proportion by allergen stimulation, (d) individual changes in apoptotic non-IL-10-producing B cell proportion by allergen stimulation. None, no stimulation of PBMCs; Ag, casein-stimulated PBMCs; Positive, milk allergy patients; Negative, milk-tolerant subjects.

of B-cell-derived IL-10 [15], and IL-10 was produced predominantly by CD5⁺ regulatory B cells among B cells in humans (Fig. 2). IL-10-producing cell frequency is higher in CD5⁺ B cells than it is in T cells. IL-10 is produced by mouse CD5⁺ B cells in response to stimulation by LPS, IL-6, and IL-12 [16] as well as by B cells of antigen-primed mice upon antigen re-stimulation in vitro [17]. It is unclear whether IL-10-producing regulatory B cell (Br1) proliferation in the milk-tolerant group was due to allergen stimulation or to the effects of cytokines produced by cells of PBMCs other than Br1 in response to allergen stimulation.

Regulatory B cells were reported to be involved in the pathogenesis of delayed-type hypersensitivity via mediation of required early T cell recruitment [18] and by mediating airway hyper-reactivity in non-atopic asthma [19]. In another report, regulatory B cells were protective against allergic airway inflammation [20]. Br1 proliferated by allergen stimulation in the milk-tolerant subjects, while apoptotic non-IL-10-producing regulatory B cells increased by allergen stimulation in the milk allergy group (Figs. 3 and 4). CD5⁺ B cells exert their regulatory role through the production of IL-10 [21], and Br1 seemed to be involved in the immune tolerance of human food allergy in this study. However, apoptotic non-IL-10-producing regulatory B cells increased by allergen stimulation in the milk allergy group and the cytokine-producing pro-

file of non-IL-10-producing regulatory B cells, other than IL-10, which is involved in allergy provocation, needs to be investigated further.

IL-10 drives the generation of the CD4⁺ T-cell subset, designated T regulatory cells 1 (Tr1s), which suppress antigen-specific immune responses and actively down-regulate pathological immune responses in vivo [22]. Tr1 cells, in addition to CD4⁺CD25⁺Foxp3⁺ regulatory T cells and TGF-producing regulatory T cells (Th3) [23], represent one pathway by which immune responses are regulated. Br1 cells may play a similar role to that of IL-10-producing Tr1 cells in immune regulation. Also, IL-10-induced Fas ligand-expressing mouse CD5⁺ B-1a cells mediated CD4⁺ T cell apoptosis during parasite infestation [24].

Interestingly, most of the IL-10-producing regulatory B cells (Br1) underwent apoptosis (Fig. 2b). Proliferating B cells are killed by CD4⁺CD25⁺ T cells, which are known to be important for immune tolerance [25]. Also, activation-induced apoptosis was reported in B cells [26]. Both Br1 and non-IL-10-producing regulatory B cells may undergo apoptotic changes by activation-induced apoptosis. However, IL-10 itself protects CD5⁺ B cells from apoptosis [27] and Br1 seemed to increase in the milk-tolerant group and not in the milk allergy group. The only different point was IL-10 production in the proliferation and apoptosis of regula-

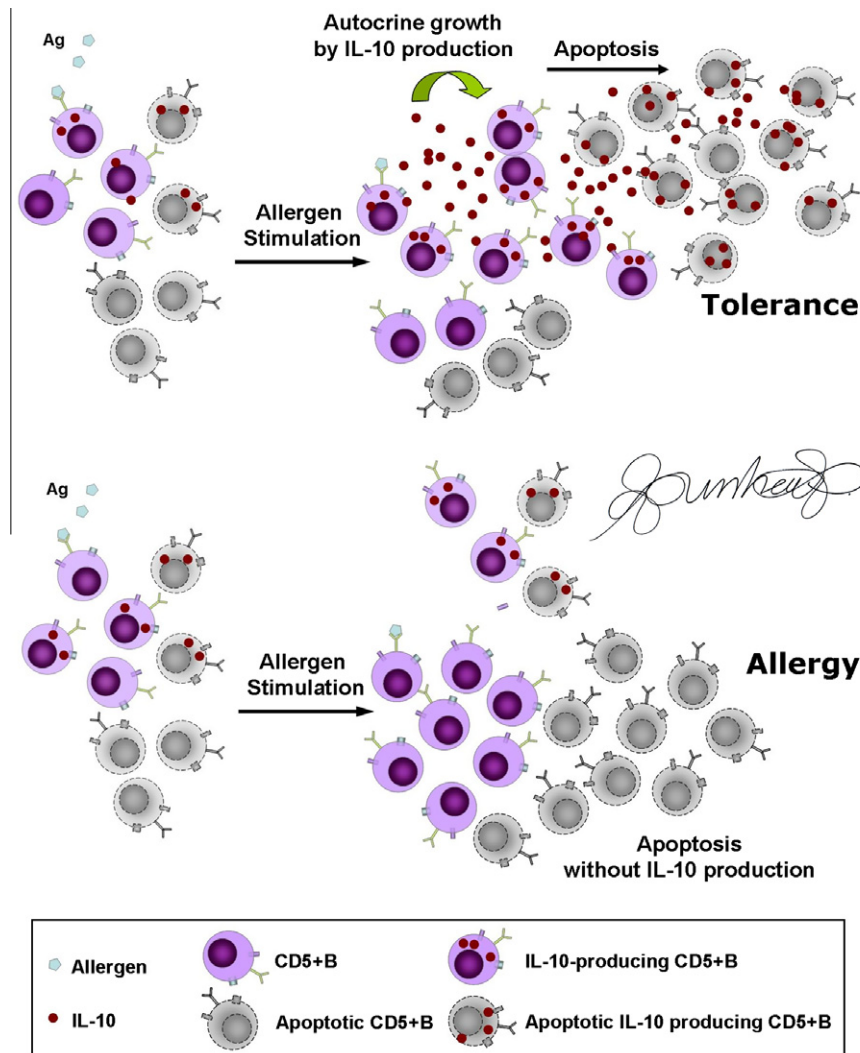


Fig. 5. A schematic diagram of the responses of IL-10-producing regulatory B cells (Br1) in allergy and immune tolerance towards food allergens. IL-10-producing CD19+CD5+ regulatory B cells (Br1) proliferate in response to allergens in milk-tolerant subjects and most of them underwent apoptosis simultaneously, while non-IL-10-producing regulatory B cells proliferated in milk allergy patients and also underwent apoptosis simultaneously. CD5+ B, CD19+CD5+ regulatory B cells; Ag, allergen.

tory B cells. In the milk-tolerant group, apoptosis of Br1 increased along with the increase of Br1, and other mechanisms for the apoptotic change of Br1 seemed to exist. The immune mechanisms for apoptosis of IL-10-producing and non-IL-10-producing regulatory B cells need to be further investigated.

IL-10 production and apoptotic changes of CD5+ regulatory B cells were observed without allergen stimulation (Fig. 3). Regulatory B cells are thought to work continuously to maintain tolerance to foreign materials like foods and self-antigens, as in the milk-tolerance group. We looked at regulatory B cells as negative regulators from a viewpoint of tolerance with production of IL-10. Br1 responses in the milk-tolerant subjects suggest active suppression rather than anergy for immune tolerance mechanisms. PBMCs actively proliferated by allergen stimulation both in the milk allergy group and the milk-tolerant group (data was not shown). However, the kind of responding cells was different between two groups. So, tolerance seemed to be the active immunosuppressive process like immune reactive process in allergy rather than the passive anergic process.

IL-10-producing regulatory B cells (Br1s) seemed to proliferate by antigen stimulation of PBMCs as negative regulatory cells. Mouse CD5+ B cells are long-lived because of autocrine IL-10 production [28], and a positive autocrine regulatory loop for regula-

tory B cells involving IL-10 was suggested [29,30]. IL-10 is an autocrine growth factor for B-1 cells in mice [31]. Br1 may amplify the proliferation of regulatory B cells as well as of Br1 via autocrine stimulation of regulatory B cells by IL-10 [27]. The apoptosis of proliferating Br1 may prevent excessive suppression by the immune control system, which removes activated and proliferating IL-10-producing regulatory B cells. Namely, Br1 may play an important role in the negative regulation of immune tolerance in human food allergies and apoptosis may control this negative regulation for the protection of excessive immunosuppression. IL-10 production of Br1 and its apoptosis may be effective immune mechanisms for negative regulation and its control system.

In conclusion, Br1 seemed to be involved in the immune tolerance of human food allergies by proliferation of apoptotic IL-10-producing regulatory B cells (Br1) in response to allergens in the milk-tolerant group, while apoptotic non-IL-10-producing regulatory B cells proliferated by allergen stimulation in the milk allergy group (Fig. 5). Proliferation of Br1 and its apoptosis may be an effective immune control system for the negative immune regulation in immune tolerance of human food allergies. The B cell system resembles the T cell system in immune regulation, and it is suggested that IL-10-producing regulatory B cells be classified as 'Br1s,' similar to 'Tr1' cells, which are regulatory T cells that pro-

duce IL-10 in the T cell system. Further investigation of the exact immunologic role of Br1s is warranted.

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