

Allergen-Specific Responses of CD19^{high} and CD19^{low} B Cells in Non-IgE-Mediated Food Allergy of Late Eczematous Reactions in Atopic Dermatitis: Presence of IL-17- and IL-32-Producing Regulatory B Cells (Br17 & Br32)

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Abstract: *Background:* Food allergies are important etiologic factors in atopic dermatitis. CD19 is a B-cell-specific cell-surface molecule, with a critical role in B-cell activation. Recently, B cells showed independent two subpopulations as CD19^{hi} and CD19^{low}. The allergen-specific responses of the CD19^{high} and CD19^{low} B-cell subpopulations were investigated in patients with non-IgE-mediated food allergy.

Methods: Five milk-allergic subjects and eight milk-tolerant subjects were selected by a double-blind placebo-controlled food challenge. Peripheral blood mononuclear cells (PBMCs) were stimulated *in vitro* with casein or ovalbumin and stained with monoclonal antibodies to distinguish the B-cell subsets.

Results: After allergen stimulation, CD19^{high} B cells increased in the number and the fraction in PBMCs in the milk-tolerant group, whereas those remained unchanged in the milk-allergic group. These responses were constant, regardless of the kind of food allergen (milk or egg). The resulting CD19^{high}/CD19^{low} B-cell ratio increased markedly in the milk-tolerant group after allergen stimulation, but was unchanged in the milk-allergic group. IL-10, IL-17, IL-32 and TGF- β -producing regulatory B cells and Foxp3-expressing regulatory B cells were identified predominantly on CD19^{low} and CD5(+) B cells.

Conclusions: The response of the CD19^{high} B-cell subpopulation to allergen stimulation is decisive for immune tolerance of non-IgE-mediated food allergy in atopic dermatitis. CD19^{high} and CD5(+) B cells dominantly produce cytokines and express Foxp3. Especially, IL-17 and IL-32 expressing B cells (Br17 & Br32) are present. The exact immunological role of CD19 and cytokines including IL-17 and IL-32 around B cells in immune tolerance requires further investigation.

Keywords: Atopic dermatitis, B cell, CD5, CD19, Foxp3, IL-10, IL-17, IL-32, milk allergy, TGF- β , tolerance.

INTRODUCTION

Food allergies play an important role in atopic dermatitis (AD). They can be classified as immunoglobulin E (IgE)-mediated food allergies (IFA), non-IgE-mediated food allergies (NFA), or mixed-type allergies [1]. NFA is underdiagnosed and undertreated because, unlike the IFA, there is a lack of appropriate laboratory tests. A Th1/Th2 imbalance is the main immunopathological mechanism underlying NFA [2]. Negative regulatory mechanisms have recently been suggested, including the involvement in immune tolerance of CD19⁺CD5⁺ regulatory B cells and CD4⁺Foxp3⁺ regulatory T cells, but their roles in the immune response remain controversial [3].

The responses of interleukin 10 (IL10)-producing CD19⁺CD5⁺ regulatory B cells (Br1) and transforming growth factor β (TGF β)-producing regulatory B-cell (Br3) are reported to be related to a tolerance for the food allergens that cause late eczematous reactions in humans [4, 5].

Recently, B cells have been divided into two subpopulations, CD19^{high} and CD19^{low} B cells, according to their expression of CD19 [6].

CD19 is a B-cell-specific cell-surface molecule and a member of the immunoglobulin superfamily [7, 8]. It is only expressed on B cells from the earliest stages of pre-B-cell development to B-cell terminal differentiation into plasma cells or follicular dendritic cells. It also plays a critical role in B-cell activation and autoimmunity [9]. CD19 is a signaling molecule known to transduce signals initiated through the B-cell receptor, and positively regulates B-cell responses [10, 11]. Against this background, the changes in the CD19^{high} and CD19^{low} B-cell subpopulations caused by allergen stimulation in subjects allergic to and tolerant of an allergenic food were investigated in this study.

MATERIAL AND METHODS

Subjects and Inclusion Criteria

Subjects who visited the Department of Allergy and Clinical Immunology at the Seoul Allergy Clinic, Seoul, Korea, between March 2010 and October 2010, with a history of recurrent late eczematous reactions or AD

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exacerbation, were screened for this study. They underwent blood tests and skin prick tests, as described below, and met the criteria of Hanifin and Rajka [12]. Informed consent was obtained from either the patient or his/her parent. The study was approved by the Institutional Review Board of Chungnam National University Hospital, Daejeon, Korea.

Double-blind placebo-controlled food challenges (DBPCFCs) were performed with milk and eggs. The diagnostic criteria for food-allergy-related late eczematous reactions have been described fully in previous reports, and include the following: (1) late eczematous reactions, including exacerbation of AD by DBPCFC; (2) exacerbation onset 4 h after the food challenge; and (3) no skin prick test reactions and undetectable food-specific IgE for milk [13].

Blood Tests and Skin Prick Tests

The subjects underwent both blood tests and skin prick tests. The blood tests included a complete blood count, with a differential count for the eosinophil fraction, total serum IgE levels, and blood-specific IgE for milk (measured using UniCAP, Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden). Serum food-specific IgE levels below 0.35 kUA/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 the positive control and physiological saline was used as the negative control. The reactions were assessed after 15 min and classified as negative (0, no reaction; +, reaction greater than the control reaction but less than half that caused by histamine) or positive (++, half the level caused by histamine; +++, equal to the level caused by histamine; or +++++, twice the level caused by histamine).

DBPCFC

The DBPCFC tests were performed to diagnose NFA to milk or egg, as described previously [14]. Briefly, an elimination diet that excluded all possible foods suspected of provoking allergic AD was undertaken by each patient. The DBPCFC was conducted immediately after the completion of the elimination diet. Freeze-dried egg powder or skim milk powder were added to a vehicle of mixed cereal flour, consisting of brown rice flour, glutinous rice flour, and barley flour. Two challenges were performed, separated by five days: in one, only the placebo was given, and in the other, the suspected food allergen was given in vehicle. The challenges were randomized and prepared by dieticians in the clinical research unit of the hospital. All reactions were scored according to type, time of onset, severity, and duration.

Clinical Severity Scores and Diagnosis of Food Allergy

The signs and symptoms of allergy provocation appeared as newly developed symptoms or as the exacerbation of AD. Clinical severity was evaluated using the SCORing Atopic Dermatitis (SCORAD) index, a system used worldwide to assess the severity of atopic eczema [15]. A diagnosis of food allergy was made if the clinical severity score increased by more than 20% in subjects showing a basal score of > 50

points, or if the score increased by more than 10 points in subjects with a basal score of ≤ 50 points. Apparently new skin lesions or itching were regarded as positive reactions, required for the diagnosis of food allergy stimulated by the food challenge.

Stimulation of Peripheral Blood Mononuclear Cells (PBMCs) with Casein or Ovalbumin

PBMCs were isolated from venous blood by density-gradient separation on Ficoll-Hypaque (Biomedicals, Aurora, OH) and then resuspended at 1×10^6 cell/mL in α -minimum essential medium (α -MEM; Irvine Scientific, Santa Ana, CA). The PBMCs were then cultured with or without a 50 μ g/mL mixture of purified α -, β -, and κ -casein (Sigma, St Louis, MO) or 50 μ g/mL ovalbumin (Sigma Chemicals, Castle Hill, Australia) in α -MEM. The casein and ovalbumin concentrations have been described in a previous study [16, 17]. The cells were cultured in 24-well plates at a concentration of 1×10^6 cell/mL. As an unstimulated control, 1×10^6 cell/mL were also cultured without casein or ovalbumin for 18 hours.

Cell-Surface Staining, Intracellular Staining, Carboxy-fluorescein Succinimidyl Ester (CFSE) Labeling and Flow-Cytometric Analysis

After 24 hours of culture, PBMCs with or without exposure to casein or ovalbumin were stained with specific monoclonal antibodies. Cells were prepared at a concentration of 1×10^6 cells in 100 μ L of FACS staining buffer (eBioscience, San Diego, CA) in an Eppendorf tube. Allophycocyanin (APC)-labelled anti-CD19 (eBioscience, San Diego, CA, USA) and phycoerythrin-Cy7 (PE-Cy7)-labelled anti-CD5 (eBioscience) were used for analysis of the B-cell subsets. Anti-CD1d (eBioscience) was used for the anti-CD1d expression of the CD19^{high} and CD19^{low} B-cell subsets. The cells were incubated for one hour in the dark and washed three times with FACS staining buffer. After cell-surface staining using the PE-Cy7-labelled anti-CD5 and APC-labelled anti-CD19, the cells were fixed and permeabilised using a fixation/permeabilisation kit (eBioscience, San Diego, CA). After fixation and permeabilization, the cells were stained with a PE-labelled anti-TGF- β (R&D Systems, Minneapolis, MN) or PE-labelled anti-IL-10 (eBioscience) or PE-labelled anti-IL-17 (eBioscience) or PE-labelled anti-IL-32 or PE-labelled anti-Foxp3 antibodies (eBioscience) for 1 hour. To observe the proliferation of the B-cell subsets, the PBMCs were labeled with CFSE just before culture. Briefly, immediately after isolation, the PBMCs were incubated with CFSE (5 μ M) for 10 min at 37 °C. Labeling was stopped with α -MEM and excess dye was removed by washing. Monoclonal antibodies were added at a concentration of 1 μ g/mL according to the manufacturer's instructions. Stained cells were counted using a FACSCaliber device (BD Biosciences, Milpitas, CA, USA) and the data were analyzed using CellQuest software (BD Biosciences).

RESULTS

Clinical Characteristics

Five positively milk-allergic subjects were selected according to the diagnostic criterion of a late eczematous

Table 1. Patient Profiles of the Milk-Allergic Group and the Milk-Tolerant Group

	Milk-Allergic Group	Milk-Tolerant Group	Unit
Number	5	8	
Male:Female	3:2	5:3	
Age	15.3±11.9	13.8±12.5	Year
Total IgE	987.7±12.9	1031.6±1163.8	IU/L
Blood Eosinophil	7.6±4.2	8.5±6.9	%
Blood Lymphocyte	34.9±12.9	33.5±10.9	%
Milk-Specific IgE	Undetectable	Undetectable	kUA/L
Casein-Specific IgE	Undetectable	Undetectable	kUA/L
Skin Prick Test for Milk	All Negative	All Negative	Grade

reaction to milk (the milk-allergic group) and eight subjects who did not react to milk were included as the milk-tolerant group (Table 1). The late eczematous lesions that developed in the milk-allergic subjects were confirmed with DBPCFC. All subjects had negative skin prick tests for milk and undetectable milk-specific IgE.

Three Patterns in the Distribution and Flow-Cytometric Characteristics of CD19^{high} and CD19^{low} B-Cell Subsets

The B cells consisted of CD19^{high} and CD19^{low} B-cell subpopulations (Fig. 1). The CD19^{high} B cells showed high levels of forward light scatter (FSC) on fluorescence-activated cell sorting analysis, whereas the side light scatter (SSC) levels of the CD19^{high} and CD19^{low} B-cell subpopulations were similar. Three patterns of distribution of CD19^{high} and CD19^{low} B cells were observed, according to the degree of their CD19 expression: the CD19^{high}-dominant pattern; the equal CD19^{high} and CD19^{low} pattern; and the CD19^{low}-dominant pattern (Fig. 1).

Specific Responses of CD19^{high} and CD19^{low} B-Cell Subpopulations in the Milk-Allergic and Milk-Tolerant Groups (Intersubject Study)

The changes in the CD19^{high} and CD19^{low} B-cell populations caused by allergen stimulation were compared between the milk-allergic and milk-tolerant groups. CD19^{high} B cells increased in the milk-tolerant subjects but remained unchanged in the milk-allergic group, whereas the CD19^{low} B cells decreased in the milk-tolerant group but also remained unchanged in the milk-allergic group (Fig. 2). An increase in CD19 expression (shift to the right on flow-cytometric analysis by histogram) was observed in the milk-tolerant group after allergen stimulation, whereas there was no change in CD19 expression or in the proportion of CD19^{high} cells in the PBMCs of the milk-allergic group (Fig. 2a). These findings were also clearly observed when CD19 and CD5 expression were analyzed together (Fig. 2b) and in a proliferation study based on CFSE staining (Fig. 2c).

After allergen stimulation, the percentage of proliferating CD19^{high} B cells in the PMBCs increased in the milk-tolerant group from $3.4 \pm 3.2\%$ to $6.8 \pm 3.6\%$ ($P = 0.001$), but remained unchanged in the milk-allergic group ($3.6 \pm 3.7\%$

to $2.8 \pm 2.3\%$, $P = 0.651$) (Fig. 3a), whereas the percentage of proliferating CD19^{low} B cells in the PMBCs decreased in the milk-tolerant group from $5.8 \pm 2.4\%$ to $4.5 \pm 1.7\%$ ($P = 0.016$), but remained unchanged in the milk-allergic group ($4.5 \pm 3.8\%$ to $4.6 \pm 1.3\%$, $P = 0.957$) (Fig. 3b). Consequently, the proliferating CD19^{high}/CD19^{low} B-cell ratio increased markedly from 0.6 ± 1.4 to 1.5 ± 2.1 ($P = 0.000$) in the milk-tolerant group, but remained unchanged (0.8 ± 1.0 to 0.6 ± 1.8 , $P = 0.375$) in the milk-allergic group (Fig. 3c).

Specific Responses of CD19^{high} and CD19^{low} B-Cell Subpopulations to Allergenic and Tolerogenic Food Antigens in the Same Patient (Intrasubject Study)

The CD19^{high} and CD19^{low} cell responses to allergen stimulation were analyzed within individuals who were allergic to either milk or egg but tolerant of the other allergen, to evaluate the food-specific characteristics of these responses and whether these responses can be extrapolated to other foods. The changes in CD19^{high} and CD19^{low} B cells caused by stimulation with the allergenic and tolerogenic food antigens were tested in five patients. The patients were allergic to either milk or egg, and tolerant of the other food. Consistently, the CD19^{high} B-cell subpopulation increased in response to the tolerogenic food antigen, but did not change in response to the allergenic food antigen, as observed in the intrasubject study (Fig. 4). The CD19^{low} B-cell subpopulation decreased in response to the tolerogenic food antigen, but did not change in response to the allergenic food antigen, regardless of the kind of food given. The percentage of CD19^{high} B cells was $1.2 \pm 0.8\%$ without stimulation. This increased to $6.0 \pm 1.4\%$ ($P = 0.041$) after stimulation with the tolerogenic food allergen, but remained unchanged ($2.6 \pm 0.8\%$, $P = 0.188$) following allergenic food antigen stimulation, regardless of whether the allergen was milk or egg. In contrast, the percentage of CD19^{low} cells was $2.3 \pm 0.2\%$ without stimulation, and increased to $5.2 \pm 1.6\%$ ($P = 0.036$) after stimulation with the tolerogenic food antigen, although it remained unchanged ($3.3 \pm 0.3\%$, $P = 0.084$) following allergenic food antigen stimulation. As a result, the increase in the CD19^{high}/CD19^{low} ratio was greater in the antigen-tolerant patients. (From 0.5 ± 0.3 to 0.5 ± 0.1 , $P = 0.903$ in the antigen-allergic patients; and from 0.5 ± 0.3 to 1.8 ± 0.4 , $P = 0.050$ in the antigen-tolerant patients).

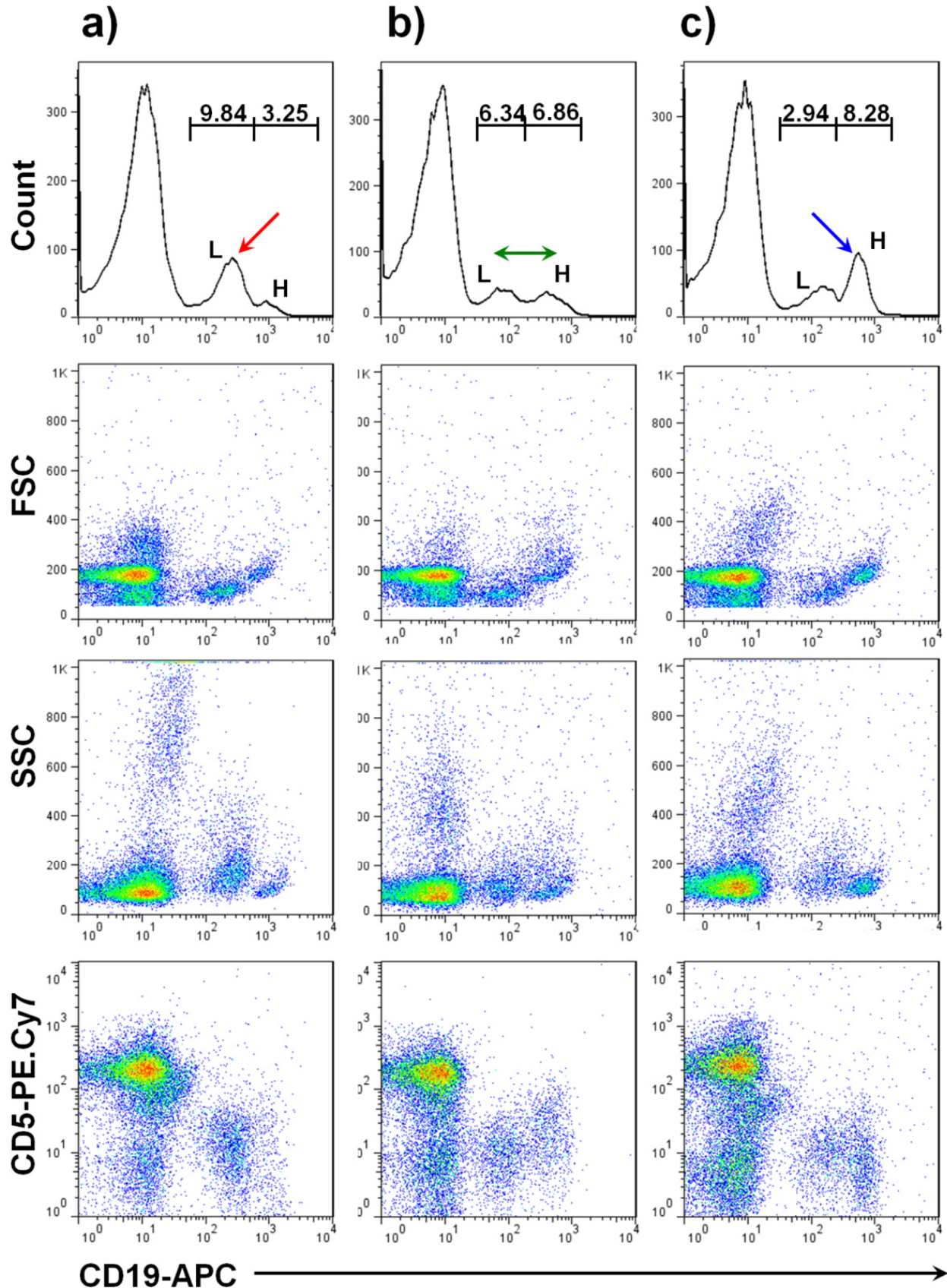


Fig. (1). Representative cases of the three patterns of CD19^{high} and CD19^{low} B-cell composition in PBMCs. L, CD19^{low} subpopulation; H, CD19^{high} subpopulation; FSC, forward light scatter; SSC, side light scatter; PE-Cy7, phycoerythrin-Cy7; APC, allophycocyanin.

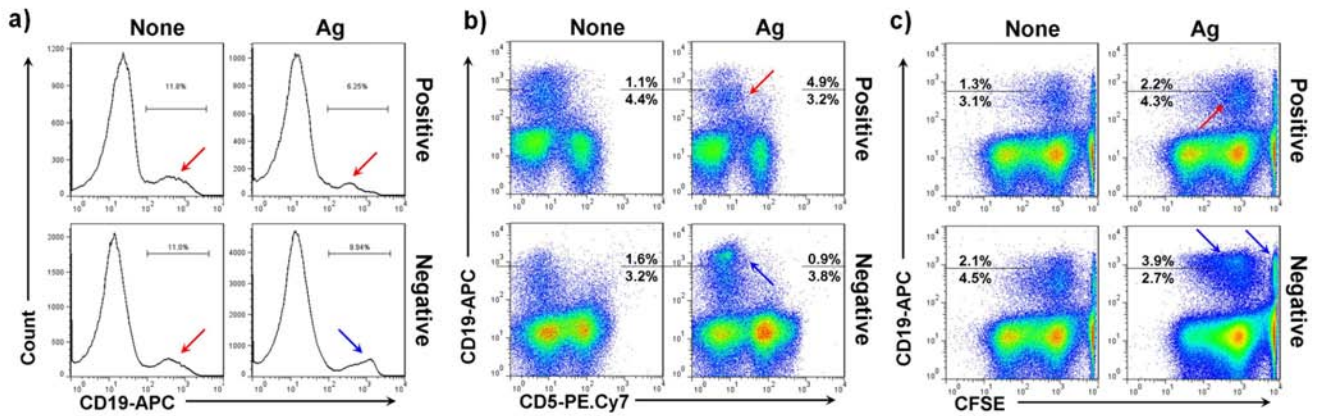


Fig. (2). Representative case for the proportional changes in regulatory B cells in response to allergen stimulation in the milk-allergic group and the milk-tolerant group. **a)** Representative proportional changes in regulatory B cells in response to allergen stimulation in the milk-allergic group and the milk-tolerant group by histogram. **b)** Changes in the CD19^{high} and CD19^{low} B-cell subpopulations caused by allergen stimulation in the milk-allergic group and the milk-tolerant group, using APC-conjugated anti-CD19 antibody and PE-Cy7-conjugated anti-CD5 antibody. **c)** Proliferation analysis using CFSE staining for CD19^{high} and CD19^{low} B-cell subpopulations after allergen stimulation in the milk-allergic group and milk-tolerant group. None, no stimulation of PBMCs; Ag, casein-stimulated PBMCs; Positive, milk-allergic patients; Negative, milk-tolerant subjects; CFSE, carboxyfluorescein succinimidyl ester; PE-Cy7, phycoerythrin-Cy7; APC, allophycocyanin.

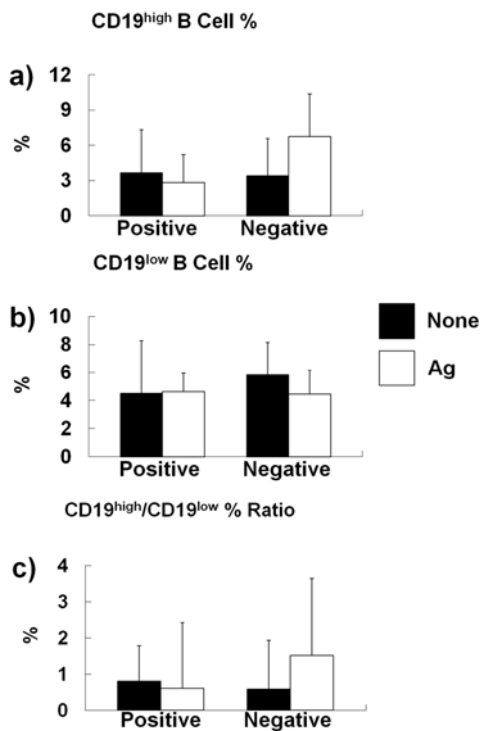


Fig. (3). Specific responses of CD19^{high} and CD19^{low} B cells in the milk-allergic group and milk-tolerant group (intersubject study). **a)** Mean fractional changes in the CD19^{high} B-cell subpopulations in PBMCs after allergen stimulation in the milk-allergic group and the milk-tolerant group, **b)** Mean fractional changes in the CD19^{low} B-cell subpopulations in PBMCs after allergen stimulation in the milk-allergic group and the milk-tolerant group, **c)** Mean ratio changes in the CD19^{high} and CD19^{low} B-cell subpopulations in PBMCs after allergen stimulation in the milk-allergic group and the milk-tolerant group. None, no stimulation of PBMCs; Ag, casein-stimulated PBMCs; Positive, milk-allergic patients; Negative, milk-tolerant subjects.

Effects of Allergen Concentration on the Distribution of CD19^{high} and CD19^{low} B-Cell Subpopulations in Milk-Tolerant Subjects

To evaluate the effects of allergen concentration on the responses of CD19^{high} and CD19^{low} B-cell subpopulations, especially in the milk-tolerant group, casein concentrations of 5 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ were gradually added to the PBMCs. As shown in a representative case (Fig. 5a), the increase in the response of the CD19^{high} B-cell subpopulation was proportional to the allergen concentration. The percentage of CD19^{high} cells increased from $1.3 \pm 0.7\%$ to $3.0 \pm 1.6\%$ ($P = 0.081$) after allergen stimulation at a casein concentration of 5 $\mu\text{g/mL}$ (1/10 Ag) and to $4.6 \pm 2.0\%$ ($P = 0.026$) after allergen stimulation at a concentration of 50 $\mu\text{g/mL}$ (Ag) (Fig. 5b). However, stimulation with neither allergen concentration had a significant effect on the CD19^{low} B-cell fraction: from $2.9 \pm 0.6\%$ to $2.5 \pm 2.3\%$ ($P = 0.208$) at a concentration of 5 $\mu\text{g/mL}$ (1/10 Ag), and from $2.9 \pm 0.6\%$ to $2.3 \pm 1.2\%$ ($P = 0.390$) at a concentration of 50 $\mu\text{g/mL}$ (Ag). The CD19^{high}/CD19^{low} B-cell ratio increased as the concentration of allergen increased: from 0.424 ± 0.187 to 1.627 ± 1.024 , ($P = 0.109$) at 5 $\mu\text{g/mL}$; and from 0.424 ± 0.187 to 3.412 ± 3.533 ($P = 0.049$) at 50 $\mu\text{g/mL}$.

Profile of surface CD1d and CD5 Expression and Intracellular IL-10, IL-17, IL-32, TGF- β and Foxp3 Expression in CD19^{high} and CD19^{low} B-Cell Subpopulations

Profile of CD19^{high} and CD19^{low} B-cell subpopulations was examined. CD5 and CD1d expression was dominant in CD19^{low} subpopulation than in CD19^{high} subpopulation (Table 2, Fig. 6a). Intracellular expression of IL-10, IL-17, IL-32, TGF- β and Foxp3 was predominant in CD19^{low} B cells subpopulation than in CD19^{high} subpopulation and in CD5(+) B cells than CD5(-) B cells (Fig. 6b). Especially, IL-17- and IL-32-producing B cells were present in CD19^{low} and CD5(+) B cells.

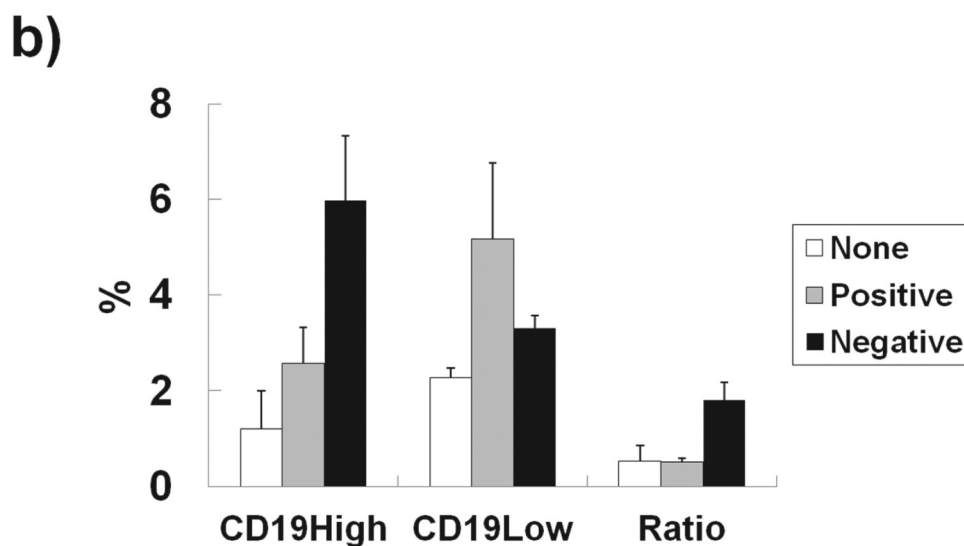
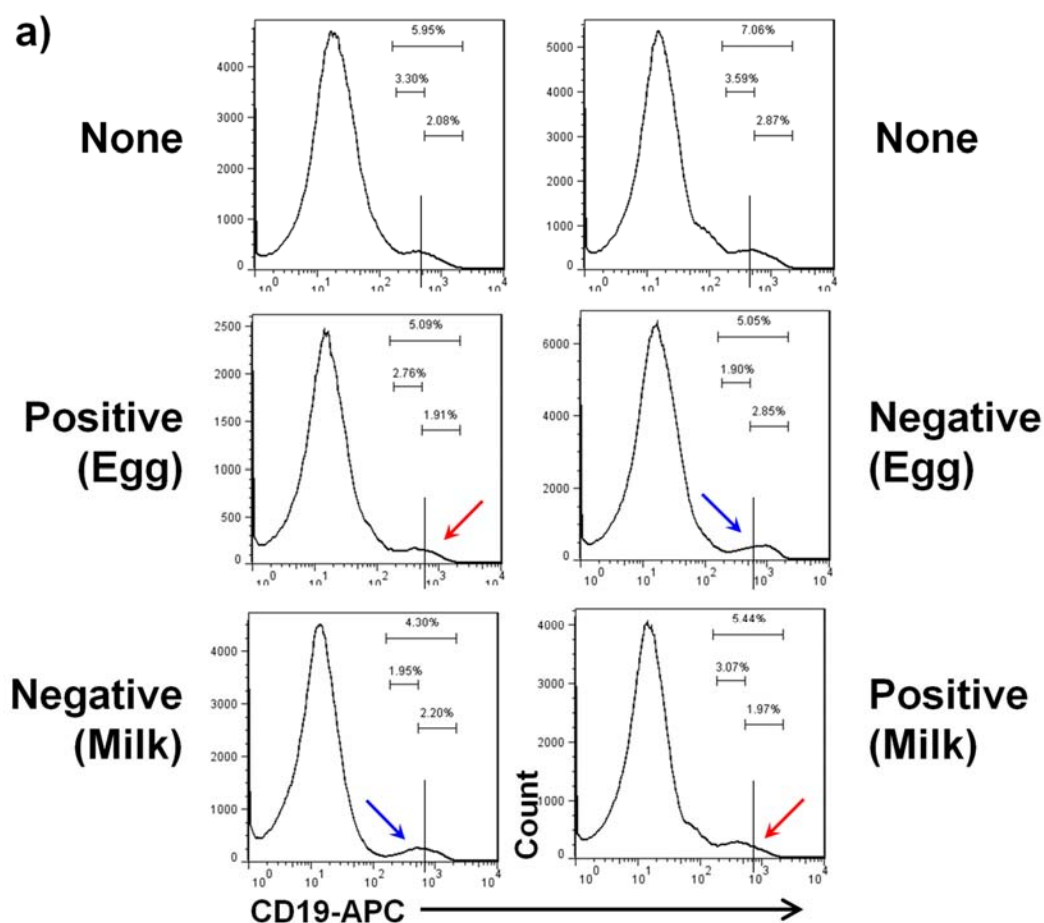


Fig. (4). Specific responses of CD19^{high} and CD19^{low} B-cell subpopulations in PBMCs to allergenic and tolerogenic food antigens in the same patient (intrasubject study). **a)** Representative histograms for two subjects: one was allergic to milk and tolerant of eggs, and the other subject was allergic to eggs and tolerant of milk. **b)** Fractional changes in CD19^{high} and CD19^{low} B cells and changes in the CD19^{high}/CD19^{low} B-cell ratio after allergen stimulation with allergenic food antigen and tolerogenic food antigen ($n = 5$). None, no stimulation of PBMCs; Positive, food (milk or egg)-allergic patients; Negative, food (milk or egg)-tolerant subjects; CD19High, CD19^{high}-expressing B cells; CD19Low, CD19^{low}-expressing B cells; Ratio, CD19^{high}/CD19^{low} B-cell ratio; APC, allophycocyanin.

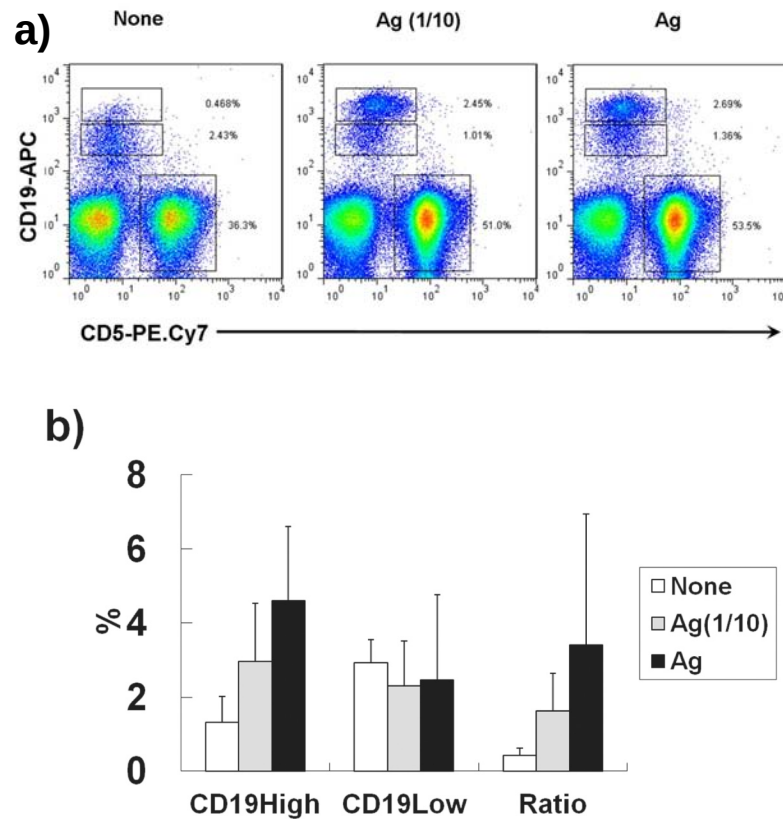


Fig. (5). Specific responses of CD19^{high} and CD19^{low} B-cell subpopulations in PBMCs in the milk-tolerant group after treatment with different concentrations of the stimulating allergen. **a)** A representative case of the concentration effects on CD19^{high} and CD19^{low} B-cell subpopulations after allergen stimulation in a milk-tolerant patient. **b)** Specific fractional changes in CD19^{high} and CD19^{low} B-cell subpopulations in PBMCs and the CD19^{high}/CD19^{low} ratios after stimulation with different concentrations of allergen in the milk-tolerant group (n = 5). None, no stimulation of PBMCs; Positive, food (milk)-allergic patients; Negative, milk-tolerant subjects; CD19High, CD19^{high}-expressing B cells; CD19Low, CD19^{low}-expressing B cells; Ratio, CD19^{high}/CD19^{low} B-cell ratio; Ag, food (milk) allergen; PE-Cy7, phycoerythrin-Cy7; APC, allophycocyanin (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

Table 2. Profile of Surface and Intracellular Expression of CD5, CD1d, Cytokines and Foxp3 in CD19^{high} and CD19^{low} B Cell Subpopulations

n=3	CD19 ^{high}	CD19 ^{low}	p
CD5	1.5±0.3	8.5±2.2	0.001>
CD1d	2.9±0.8	14.2±3.5	0.001>
IL-10	1.0±0.2	5.8±1.2	0.001>
IL-17	1.7±0.3	8.0±2.1	0.001>
IL-32	2.5±0.5	10.3±2.5	0.001>
TGF-β	1.9±0.4	13.7±3.3	0.001>
Foxp3	3.5±0.6	10.8±2.7	0.001>
n=3	CD5(+)	CD5(-)	p
IL-10	2.0±1.1	20.1±5.1	0.001>
IL-17	0.9±0.2	32.5±7.7	0.001>
IL-32	2.0±1.0	52.5±10.1	0.001>
TGF-β	0.6±0.3	43.5±8.9	0.001>
Foxp3	0.5±0.2	35.5±8.1	0.001>

DISCUSSION

CD19⁺ cells showed two CD19^{high} and CD19^{low} peaks on flow-cytometric analysis clearly (Fig. 1), as described in a previous report [6]. The level of CD19 expression regulates B-cell development in humans [18] and the degree of CD19 engagement correlates with B-lymphocyte activation and humoral immunity in mice [19]. Therefore, the degree of CD19 expression may vary according to the immunological status of the subject. As expected, three representative compositional patterns of B cells were observed: CD19^{high} dominant, CD19^{low} dominant, and equal proportions of CD19^{high} and CD19^{low} B cells. These were confirmed in analyses that resolved CD19⁺ cells in several different ways, including by the expression of CD19 and CD5, SSC and CD19, and FSC and CD19. CD19^{high} B cells seem be larger than CD19^{low} B cells, according to FSC levels. The functional relationship between the size and CD19 expression on B cells must be clarified.

Remarkably, CD19^{high} B cells increased in both the number and the proportion in PBMCs after allergen stimulation in the milk-tolerant group, whereas these parameters remained unchanged in the milk-allergic group.

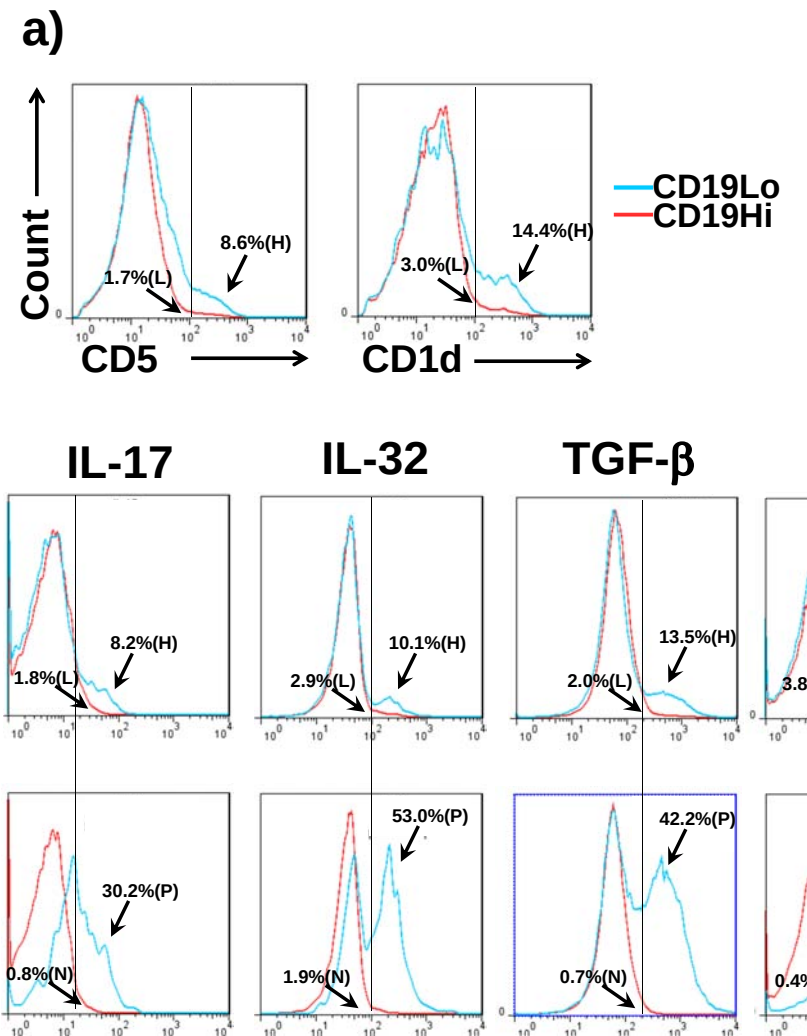


Fig. (6). Representative plotting for surface and intracellular expression of CD5, CD1d, cytokines and Foxp3 in CD19^{high} and CD19^{low} B cell subpopulations. **a)** Surface expression of CD5 and CD1d in CD19^{high} and CD19^{low} B cell subpopulations. **b)** Intracellular expression of IL-10, IL-17, IL-32, TGF-β and Foxp3 in CD19^{high} and CD19^{low} B cell subpopulations. **a)** Blue line (CD19^{Hi}, H), CD19^{high}-expressing B cells; Red line (CD19^{Lo}, L), CD19^{low}-expressing B cells; **b)** Blue line (CD5^{Pos}, P), CD5(+) B cells; Red line (CD5^{Neg}, N), CD5(-) B cells.

Consequently, the CD19^{high}/CD19^{low} ratio increased in response to allergen stimulation in the milk-tolerant group, whereas it remained unchanged in milk-allergic group (Figs. 2, 3). The specific CD19^{high} and CD19^{low} B-cell responses were consistent, regardless of the kind of allergen tested (Fig. 4), and were proportional to the concentration of the allergen (Fig. 5). CD19 is a B-cell marker that positively regulates B-cell responses by controlling intrinsic and stimulus-dependent signaling thresholds [20]. CD19 overexpression also allows anergic B cells to escape immunotolerance by converging with BCR-independent pathways [21]. The degree of CD19 engagement correlates with B-lymphocyte activation and humoral immunity in mice [19]. CD19 amplifies B-lymphocyte signal transduction by regulating Src family protein tyrosine kinases [22]. In Cd19^{-/-} mice, the severity of experimental autoimmune encephalomyelitis is increased, which is associated with polarized Th₁ cytokines and high levels of interferon γ production by B cells [23]. Not only B cells from CD19-deficient mice are hyposensitive to a variety of transmembrane signals, but CD19 loss results in increased

and prolonged reactivity of contact hypersensitivity [24]. However, Contact hypersensitivity responses reduced are in human CD19 transgenic (hCD19Tg) mice overexpressing CD19 [25]. Our results suggest that CD19 expression is related to the functions of B cells, especially those involving immunotolerance of food allergens in atopic dermatitis. How high-level CD19 expression is related to allergen tolerance requires clarification.

The immunoregulatory roles of IL10-producing B cells and regulatory B cells have been described in the contexts of inflammation, autoimmunity, and cancer [26]. IL10-producing regulatory B cells proliferated in response to milk allergen in a milk-tolerant group [4]. In one study, IL10-producing regulatory B cells were reported to have a CD19^{high} phenotype [26], but in another, they were predominantly CD19^{low} B cells [6]. It is unclear whether the increase in CD19^{high} B cells observed in the milk-tolerant group in this study was related to an increase in IL10-producing B cells, as in a previous report [4]. The relationship between the compositional changes in CD19^{high} B cells and IL10-producing B cells must also be clarified.

Profile of CD19^{high} and CD19^{low} B-cell subpopulations is characteristic. CD5 and CD1d were expressed predominantly in CD19^{low} B cell population (Table 2, Fig 1a). In mice, CD1d is expressed in CD19^{low} B cell population [25]. However, in human, CD1d was expressed predominantly in CD19^{low} B cell population in this study. Not only cytokines including IL-10, IL-17, IL-32 and TGF- β were expressed mainly in CD19^{low} B cell population and in CD5(+) B cells, but also, phenotypes of Foxp3-expressing B cells (Breg) were CD5(+)CD19^{low}. In mice IL-10-producing regulatory B cells (Br1) are CD19^{high} B cells [26]. However, IL-10-producing regulatory B cells (Br1) of human are CD19^{high} B cells in the previous report [6] and this report. From the results that regulatory B cells which produce cytokines and express Foxp3 predominantly belong to CD5(+)CD19^{low} B cell population, not only inhibitory roles of CD19 for producing cytokines and expressing Foxp3 may be suspected, but also, by allergen stimulation, regulatory B cells are activated in allergy patients and non-regulatory B cells are activated in tolerant patients.

Especially, in this study, there was an important discovery of IL-17- and IL-32-producing regulatory B cells. Their phenotype is CD5(+)CD19^{low} as other regulatory B cells which produce IL-10 and TGF- β and express Foxp3. Th17 which produces IL-17 which is a cytokine involving in immune regulatory function together with Foxp3-expressing regulatory T cell (Treg) [27]. Recently, Foxp3-expression regulatory B cell (Breg) was discovered [28] and reported to be related with immune tolerance of food allergy [29]. IL-17-producing B cell is named as 'Br17' for the first time in this report. The regulatory functional relationship between Foxp3-expressing regulatory B cell (Breg) and IL-17-producing regulatory B cell (Br17) need to be investigated. In addition to IL-17, IL-32 expression in B cells was also discovered in this study and also named as 'Br32'. B cells should be revised in aspect to regulatory immune responses with producing multiple cytokines.

In conclusion, CD19^{high} B cells proliferated, and increased in number and in their proportional representation in PBMCs after allergen stimulation in the milk-tolerant group, with a consequent increase in the CD19^{high}/CD19^{low} B-cell ratio. Their changes conclusively defined the tolerance for a food allergen in terms of late eczematous reactions. CD19^{high} and CD5(+) B cells dominantly produce cytokines and express Foxp3. Especially, IL-17- and IL-32-producing B cells are present. The immunological role of CD19 expression in the regulation of the immune response in the context of tolerance for a food allergen requires further investigation.

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