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Allergen-specific B cell subset responses in cow's milk allergy of late eczematous reactions in atopic dermatitis

Jae Ho Lee [a], Joonyong Noh [b], Geunwoong Noh [c,*], Hyuk Soon Kim [d], Se Hwan Mun [d], Wahn Soo Choi [d], Sunheui Cho [e], Sangsun Lee [e]

[a] Department of Pediatrics, College of Medicine, Chungnam National University, Daejeon, Republic of Korea
[b] Department of Animal Biotechnology, College of Animal Bioscience and Technology, Konkuk University, Seoul, Republic of Korea
[c] Department of Allergy and Clinical Immunology, Seoul Allergy Clinic, Seoul, Republic of Korea
[d] Department of Immunology, College of Medicine, Konkuk University, Chungju, Republic of Korea
[e] Department of Food and Nutrition, College of Human Ecology, Hanyang University, Seoul, Republic of Korea

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ABSTRACT

B cells have regulatory functions in immune responses. Antigen-specific responses of B cell subsets by allergen stimulation ex vivo were examined in milk allergy of late eczematous reactions. Eight milk allergy subjects and 13 milk tolerant subjects were selected by DBPCFC. PBMCs were stimulated by casein ex vivo and stained for B cell subsets using monoclonal antibodies. CD19+ B cells unchanged from 8.7 ± 3.8% to 8.0 ± 5.1% ($p = 0.504$, $n = 8$) in the milk allergy group and decreased in the milk tolerant group from 8.5 ± 3.2% to 5.0 ± 1.6% ($p = 0.001$, $n = 13$). The fraction of apoptotic B cells in B cells significantly decreased 4.4 ± 3.1% to 1.3 ± 0.4% ($p = 0.027$, $n = 4$) in the allergy group and insignificantly increased from 2.8 ± 0.6% to 5.4 ± 2.6% ($p = 0.059$, $n = 6$) in the milk tolerant group. CD5+ regulatory B1 cell% in B cells decreased in milk allergy subjects from 36.2 ± 5.0% to 31.0 ± 5.7% ($p = 0.010$) and unchanged in milk tolerant subjects from 41.6 ± 10.2% to 43.8 ± 10.0% ($p = 0.413$). IL-10 producing CD19+CD5+ regulatory B cell% in CD19+CD5+ regulatory B cells significantly decreased from 24.9 ± 6.5% to 13.8 ± 5.6% ($p = 0.002$, $n = 5$) by casein stimulation in milk allergy group and unchanged from 44.8 ± 11.3% to 43.9 ± 10.0% ($p = 0.297$, $n = 5$) in the milk tolerant group. B cell subset responses to IL-4 and IL-5 were also similar in both groups. B cell subset changes seemed to have diagnostic value. Exact immunologic roles of regulatory CD5+ B1 cells need further investigation.

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1. Introduction

Food allergies have increased significantly in the past decade and should be classified into IgE, non-IgE, or mixed response categories [1]. Far less is understood regarding non-IgE-mediated food allergies compared to those that are IgE-mediated, and the clinical relevance is likely underestimated in most cases [2,3]. Clinically, non-IgE-mediated food allergies are not well defined due to the lack of easily accessible diagnostic measures.

Food allergy has been known to be modulated by relative imbalances in the Th1/Th2 paradigm [4]. In vitro production of IL-4 in peripheral blood mononuclear cells (PBMC) was enhanced in children with isolated immediate IgE-mediated food hypersensitivity [5]. Serum IL-5 levels were increased in cow milk allergy [6].

Recently negative regulatory mechanisms have been suggested including CD19+CD5+ regulatory B cells [7] in addition to CD4+Foxp3+ regulatory T cells [8] for immune tolerance. CD5 is a pan T cell marker also expressed at various developmental and activation states on human B cells and is a well-established negative regulator of TCR and BCR signaling [9]. CD5 plays a key role in B and T cell selection as well as generation and maintenance of tolerance [10]. A small subset of mouse and human B cells which express CD5 produces much of the serum immunoglobulin, including many common autoreactive antibodies, and accounts for most cases of B cell chronic lymphocytic leukemia [11]. These CD5+ B cells are known to have negative regulatory functions in immune responses [7].

Although, there are many reports concerning the responses of isolated cells to allergen, the changes and resultant responses of PBMCs in which all immunocompetent cells and all possible cytokines may involve the immune responses by ex vivo allergen stimulation may be different. However, PBMCs are the most similar to in vivo conditions and the change of PBMCs may be regarded as important for understanding of integrated in vivo

* Corresponding author. Address: Department of Allergy and Clinical Immunology, Seoul Allergy Clinic, 552-7 2nd Floor, Shinsa-dong, Kangnam-ku, Seoul 135-890, Republic of Korea.
E-mail address: admyth@naver.com (G. Noh).

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responses by allergen stimulation in allergy and tolerance. Also, this understanding is important for diagnostic purposes and for the evaluation of therapeutic effects of tolerance induction. For these reasons, PBMCs were stimulated ex vivo with casein in milk allergy patients and milk tolerant subjects, and the changes of B cell subsets including CD19+CD5+ regulatory B1 cell were compared.

2. Materials and methods

2.1. Subjects and inclusion criteria

Subjects who visited the Health Guidance Division at the Seodaemun-Gu Health Center for the 'Atopy Safe School Project' and the Department of Allergy and Clinical Immunology at the Seoul Allergy Clinic between March and July 2009 and were suffering from a repeated history of late eczematous reactions or exacerbations of AD, were screened for the current study among the subjects between 8 and 12 years of age. They received blood tests and skin prick tests as described below and fulfilled the criteria of Hanifin and Rajka [12]. Signed consent forms were obtained from either the patient or the parent. The study was approved by the Ethical Committee of the Seoul Allergy and Immunology Association, Seoul, Korea.

Elimination diet was performed as a first step and subsequent double-blind placebo-controlled food challenge (DBPCFC) tests were conducted for milk as described below. Eight positive milk allergy subjects (mean age = 8.4 ± 1.0 yr, M:F = 3:5) were selected according to the diagnostic criteria for milk allergy of late eczematous reactions (a milk allergy group) and 13 subjects (mean age = 9.5 ± 1.4 yr, M:F = 4:9) who did not react to milk were involved as a milk tolerant group (Table 1).

Diagnostic criteria for food allergy of late eczematous reactions were based on the following criteria: (1) late eczematous reactions including exacerbation of AD by DBPCFC, (2) exacerbation onset 4 h after the food challenge, and (3) negative skin prick test and undetectable food-specific IgE level for milk [13].

Table 1
Milk allergy group and milk tolerant group.

	Positive	Negative
n	8	13
M:F	3:5	4:9
Mean age	8.4 ± 1.0	9.5 ± 1.4
Mean WBC counts (counts × 1000/μl)	7.6 ± 2.0	7.6 ± 2.7
Mean eosinophil fractions	7.4 ± 3.1	7.7 ± 4.3
Mean serum total IgE (IU/L)	971 ± 1063	900 ± 1188
Mean blood lymphocyte % in WBC (%)	47.5 ± 10.5	40.9 ± 6.3
Mean milk-specific IgE (kU A/L)	0.04 ± 0.02	0.02 ± 0.02
Mean casein-specific IgE	0.02 ± 0.02	0.02 ± 0.02

2.2. Blood tests and skin prick tests

Subjects who were allergic to milk received blood tests and skin prick tests. Blood tests were performed including a CBC with differential count for the eosinophil fraction and serum total IgE levels. Serum food-specific IgE levels were also measured using the ImmunoCAP System (Phadia AB, Uppsala, Sweden). Serum food-specific IgE levels less than 0.35 kU A/L were classified as undetectable food-specific IgE levels.

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. Physiological saline and glycerol were used as negative controls. 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



Fig. 1. Skin manifestations of milk allergy in atopic dermatitis. Patients showed erythematous popular eruption with severe itching.

by histamine. The minimum size of a positive reaction was 3 mm (+++). Patients were classified as negative when they demonstrated a wheal size less than 3 mm or that of the control.

2.3. DBPCFC

For the diagnosis of milk allergy, DBPCFC was conducted according to the diagnostic methods in the previous reports [14]. Briefly, elimination diet for all possible foods which were regarded as incriminated for the allergy provocation of AD was performed. Patients under elimination diet were instructed to avoid all food stuffs containing foods to be eliminated from their meals.

Conditions for the food challenge procedures were as follows: (1) Obvious clinical improvement was obtained by elimination diet and the patient's clinical status was stable for at least a week; and (2) Foods to be eliminated were restricted from the diet completely.

A double-blind placebo-controlled food challenge (DBPCFC) was conducted just after elimination diet. DBPCFC was performed using the vehicle which was mixed cereal flour, consisting of brown rice flour, glutinous rice flour, and barley flour. Two challenges, separated by 5 days, were performed; one was only the placebo, while the other contained milk in the vehicle. Patients consumed the food twice a day at breakfast and dinner. Randomization 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 NFA appeared as newly developed symptoms or an exacerbation of AD. Because of this, 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 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. Alexa Fluor 488 conjugation with casein

The newly developed Dylight, maleimide sulfhydryl-reactive fluors (Pierce, Rockford, Ill) were used to conjugate to casein (Sigma, St. Louis, Mo). The SNO-derived cysteine residues of casein were labelled with Alexa Fluor 488 by Dylight 488 labelling kit according to the manufacturer's instruction [16].

2.6. Carboxyfluorescein succinimidyl ester (CFSE) labelling

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 α-minimum essential medium (α-MEM; Irvine Scientific, Santa Ana, CA).

For the proliferation assay of B cell subsets, CFSE labelling was performed just before antigen stimulation according to Turcanu et al. [17] with some modifications. Briefly, PBMCs were incubated with CFSE (Labelling concentration of 5 μM), for 10 min. at 37 °C. Labelling was stopped with α-MEM, and excess dye was washed away.

2.7. Antigen, IL-4, and IL-5 stimulation

CFSE-labelled PBMCs were then cultured with or without 50 μg/ml mixture of purified alpha-, beta-, and kappa-caseins (Sigma) in α-MEM. The casein concentration was referred to that in the previous study [18]. To evaluate the IL-4 and IL-5 effects on PBMCs, PBMCs were cultured with 10 ng/ml recombinant IL-4 (Shenandoah Biotechnology, Inc., Warwick, PA, USA) or IL-5 (eBioscience, San Diego, CA, USA) in α-MEM. The IL-4 and IL-5 concentration was also determined according to the previous study [19,20]. Cells were cultured in 24 well plate at a concentration of 1×10^6 cells/ml. As an unstimulated control, another 1×10^6 cells/ml were also cultured without casein, IL-4, or IL-5.

2.8. Cell staining and flow cytometric analysis

PBMCs were stained by two staining sets. Cultured PBMCs with or without casein were stained after 48 h of culture with all-

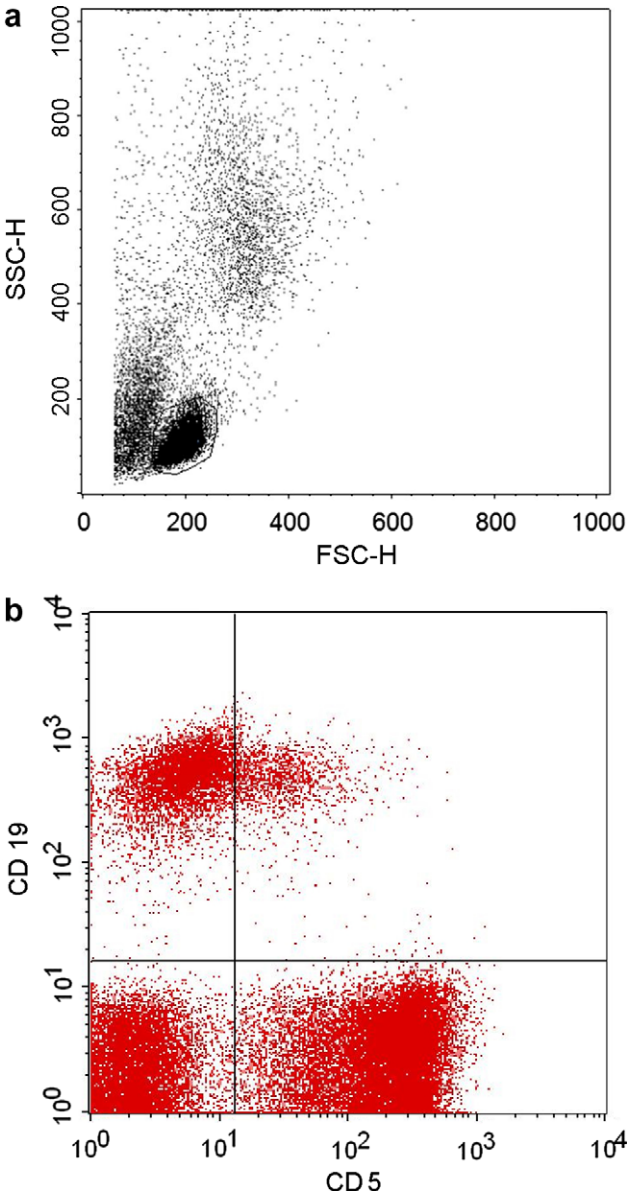


Fig. 2. Flow cytometric analysis of B cell subsets. (a) Lymphocytes were gated from PMBCs, (b) B cells subsets using APC-conjugated anti-CD19 and PE.Cy7-conjugated anti-CD5. There are two groups of B cell subsets by the presence of CD5 molecule on their cell surface.

ophycocyanin(APC)-labelled anti-CD19 mouse IgG1 monoclonal antibody (eBioscience, San Diego, CA, USA), phycoerythrin.Cy7 (PE.Cy7)-labelled anti-CD5 mouse IgG1 monoclonal antibody (eBioscience, San Diego, CA), and phycoerythrin(PE)-labelled anti-CD1d mouse IgG1 monoclonal antibody (eBioscience, San Diego, CA) for B cell subset analysis. To observe the B cell subset proliferation, CFSE labelling of PBMCs was performed first, or to evaluate antigen-specific B cell subset, Alexa Fluor 488-conjugated casein was used together with the above fluorescence-tagged anti-CD19, anti-CD5, and anti-CD1d monoclonal antibodies.

Cells to be stained were prepared at a concentration 1×10^6 in 100 μl of FACS staining buffer (eBioscience, San Diego, CA) in an Eppendorf tube. Monoclonal antibodies were added at a concentration of 1 μg/ml according to the manufacturer's instruction. After incubation for 1 h in a dark room and being washed using FACS staining buffer three times, cells were resuspended into 500 μl of FACS staining buffer just before flow cytometric analysis. Stained cells were acquired with a FACSCaliber (BD, Biosciences, Milpitas, CA, USA), and the data were analyzed with CellQuest software (BD, Biosciences).

As a second set, cell surface staining was done using specific monoclonal antibodies basically with allophycocyanin(APC)-labelled anti-CD19 mouse IgG1 monoclonal monoclonal antibody (eBioscience, San Diego, CA) and phycoerythrin.Cy7 (PE.Cy7)-labelled anti-CD5 mouse IgG1 monoclonal monoclonal antibody (eBioscience, San Diego, CA). Just after finishing cell surface staining for CD5 and CD19, permeabilization/fixation for cells was performed using 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). After staining for intracellular IL-10, the cells were stained with the apoptosis detection kit using FITC-conjugated annexin V (eBioscience, San Diego, CA). Stained cells were also acquired with a FACSCaliber (BD Biosciences, Milpitas, CA, USA), and the data were analyzed with CellQuest software (BD Biosciences).

2.9. Statistical analysis

Statistical analyses were performed using the Wilcoxon Signed Rank Test and Mann–Whitney *U* test with Bonferroni corrections for individual group using SigmaPlot software (Systat Software Inc., San Jose, CA, USA). *P*-values <0.05 were considered significant for all comparisons.

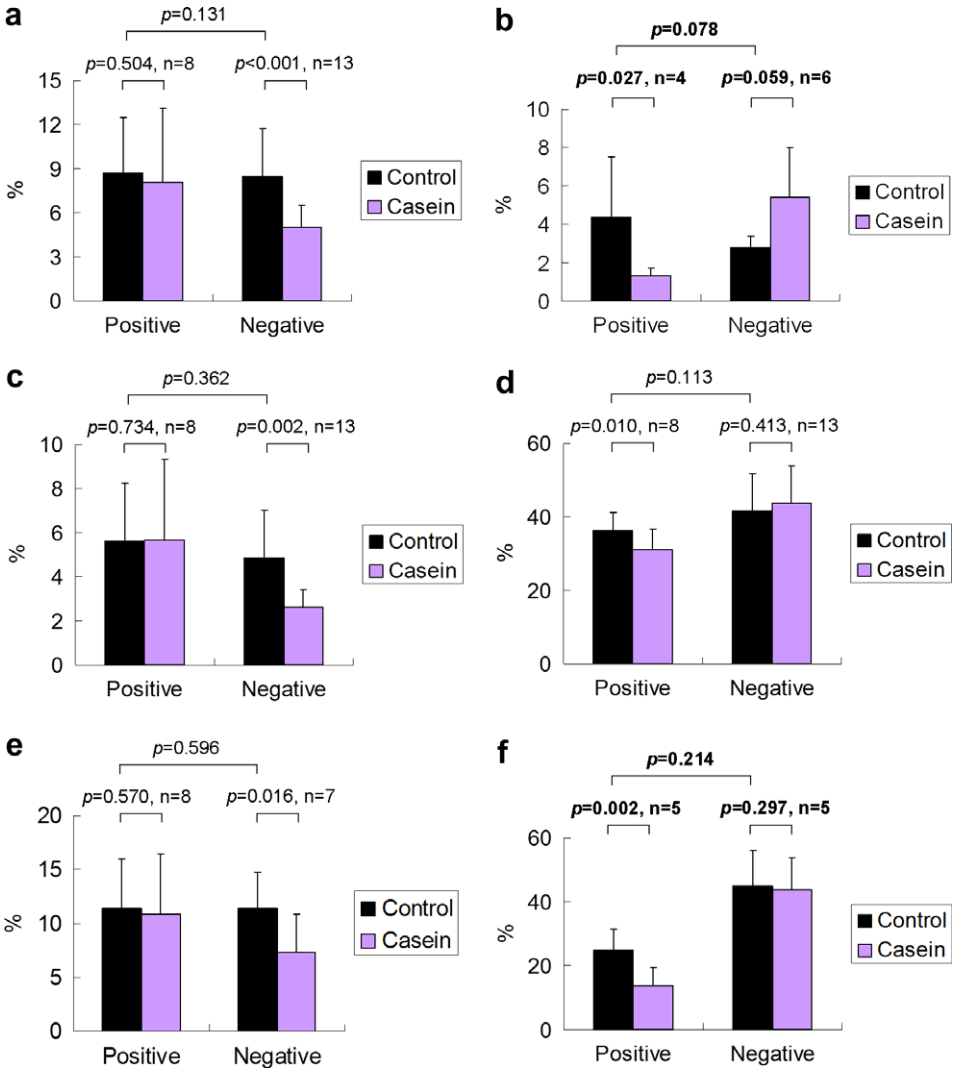


Fig. 3. The changes of cellular composition by casein stimulation in milk allergy group and milk tolerant group. (a) CD19+ B cell% in lymphocytes, (b) Frequency of apoptotic B cells, (c) CD19+CD5− B2 cell% in lymphocytes, (d) CD5+ B1 cell% in CD19+ B cells, (e) CD5+CD1d(hi) B1 cell% in CD5+ B1 cells, (f) IL-10 producing CD5+ B1 cell% in CD5+ B1 cells. Positive, milk allergy group; negative, milk tolerant group; control, non-casein-stimulated cells; casein, casein-stimulated cells.

3. Results

3.1. DBPCFC for milk allergy with late eczematous reactions

Eight milk allergy patients of late eczematous reactions and 13 milk tolerant subjects were selected in atopic dermatitis. By DBPCFC, milk allergy patients showed apparent aggravation of AD or new development of eczematous lesions including erythema and popular eruption with itching (Fig. 1). Blood eosinophil and serum total IgE were high with no significant difference between the milk allergy group and the milk tolerant group (Table 1). One milk allergy patient showed 3+ in skin prick test and the remains of both groups showed negative skin prick tests for milk. Milk- and casein-specific IgE levels were detected in only two patients (case 1, 0.4 for milk and 0.9 casein; case 2, 3.17 for milk and 2.43 for casein) in the milk allergy group.

3.2. CD19+ B cells (B cells)

Basically, lymphocytes were analyzed for CD19+CD5− B2 cells and CD19+CD5− B1 cells using fluorescence-tagged anti-CD19 and anti-CD5 which divided CD19 B cells into CD5− B2 and CD5+ B1 cells (Fig. 2).

By ex vivo casein stimulation of PBMCs, CD19+ B cell percentage in lymphocytes unchanged from 8.7 ± 3.8% to 8.0 ± 5.1% ($p = 0.504$, $n = 8$) in the milk allergy group and significantly decreased from 8.5 ± 3.2% to 5.0 ± 1.6% ($p = 0.001$, $n = 13$) in the milk tolerant group (Fig. 3a). Initial CD19+ B cell percentage in lymphocytes showed no significant difference ($p = 0.131$).

Apoptosis of B cells was analyzed using FITC-conjugated annexin V. By ex vivo casein stimulation of PBMCs, annexin V binding to CD19+ B cells were significantly decreased from 4.4 ± 3.1% to 1.3 ± 0.4% ($p = 0.027$, $n = 4$) in the allergy group and insignificantly increased from 2.8 ± 0.6% to 5.4 ± 2.6% ($p = 0.059$, $n = 6$) in milk tolerant group (Fig. 3b). Initial apoptotic frequency of B cells showed insignificant difference ($p = 0.078$).

3.3. CD19+CD5− conventional B cells (B2 cells) and CD19+CD5+ regulatory B cells (B1 cells)

CD19+CD5− B2 cell percentage in lymphocytes was unchanged from 5.6 ± 2.6% to 5.7 ± 3.7% ($p = 0.734$, $n = 8$) by ex vivo casein stimulation of PBMCs in the milk allergy group and decreased significantly from 4.9 ± 2.2% to 2.6 ± 0.7% ($p = 0.002$, $n = 13$) in the milk tolerant group (Fig. 3c). Initial CD19+CD5− B2 cell percentage in lymphocytes in both groups showed no significant difference ($p = 0.362$).

By ex vivo casein stimulation of PBMCs, CD19+CD5+ B1 cell percentage in lymphocytes decreased significantly from 3.1 ± 1.3% to 2.4 ± 1.5% ($p = 0.016$, $n = 8$) in the milk allergy group. Also, CD19+CD5+ B1 cell percentage in lymphocytes decreased significantly from 3.3 ± 1.3% to 2.2 ± 1.0% ($p = 0.003$, $n = 13$) in the milk tolerant group. Initial CD19+CD5+ B1 cell fraction showed no significant difference ($p = 0.543$). The change of CD19+CD5+ B1 cell percentage in lymphocytes by ex vivo casein stimulation showed no difference between the two groups (0.8 ± 0.8% in the milk allergy group ($n = 8$) and 1.1 ± 0.9% in the milk tolerant group ($n = 13$) ($p = 0.275$)).

B1 cell percentage in B cells decreased significantly in the milk allergy group from 36.2 ± 5.0% to 31.0 ± 5.7% ($p = 0.010$, $n = 8$) and increased insignificantly from 41.6 ± 10.2% to 43.8 ± 10.0% ($p = 0.413$, $n = 13$) in the milk tolerant group (Fig. 3d). Initial B1 cell percentage in B cells showed no difference between the two groups ($p = 0.113$).

3.4. Analysis of regulatory B cell subsets by CD1d expression and Intracellular IL-10 production

CD1d(hi) B1 cell subset among CD5+ B1 cells was unchanged from 11.4 ± 4.6% to 10.8 ± 5.6% ($p = 0.570$, $n = 8$) in the milk allergy group, but decreased significantly from 11.4 ± 3.4% to 7.3 ± 3.6% ($p = 0.016$, $n = 7$) in the milk tolerant group and (Fig. 3e). Initial CD1d(hi) B1 cell subset percentage in CD5+ B1 cells showed no significant difference between the two groups ($p = 0.596$).

IL-10 producing CD19+CD5+ regulatory B cell% in CD19+CD5+ regulatory B cells were significantly decreased from 24.9 ± 6.5% to 13.8 ± 5.6% ($p = 0.002$, $n = 5$) by casein stimulation in milk allergy group and was unchanged from 44.8 ± 11.3% to 43.9 ± 10.0% ($p = 0.297$, $n = 5$) in the milk tolerant group (Fig. 3f).

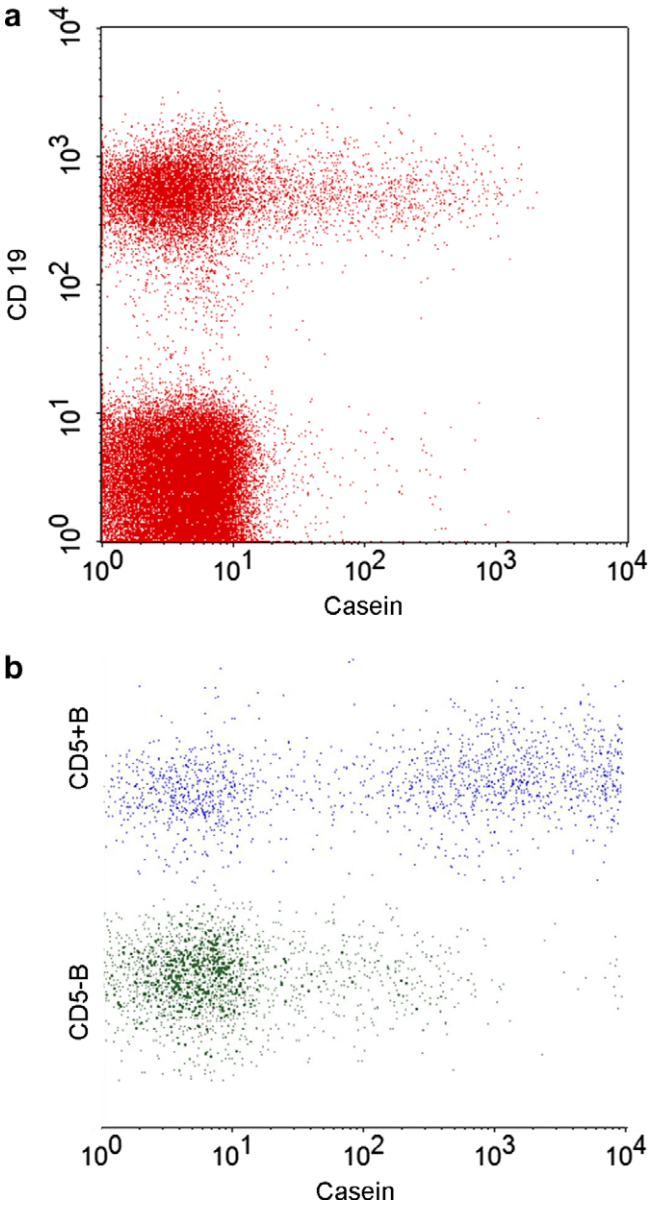


Fig. 4. Casein-specific lymphocytes in milk allergy patients. (a) casein-binding to CD19+ B cells and CD19− cells, (b) casein-binding to CD5− B cells and CD5+ B cells. CD19+ B cells were gated for CD5+ B cells (upper left and right quadrants) and CD5− B cells (lower left and right quadrants). Casein-bound cells are spotted on the right side and casein-unbound cells are spotted on the left side. CD5+ B cell spots (upper right) shifted more right than CD5− B cell spots (lower right).

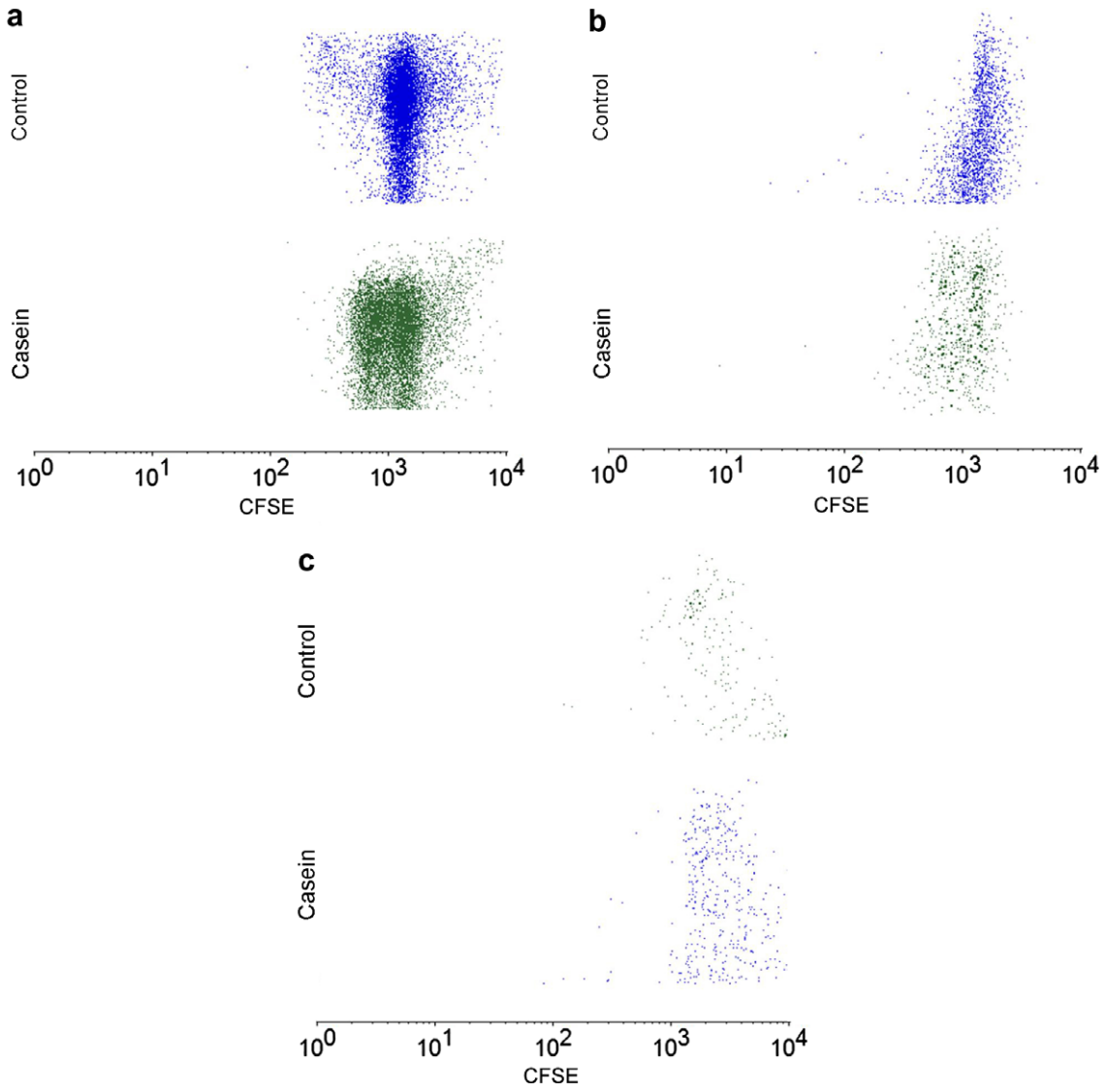


Fig. 5. Proliferation analysis by CFSE staining. (a) CD5+ T cell, (b) CD5− B2 cell, (c) CD5+ B1. Control, no casein-stimulated cells; Casein, casein-stimulated cells.

3.5. Casein-binding CD5− B2 cells and CD5+ B1 cells

PBMCs were stained with Alexa Fluor 488-conjugated casein to examine the casein-specific cells. Numerous casein-binding CD19+ B cells were observed and there were not many casein-binding T cells (Fig. 4a).

Interestingly, Alexa Fluor 488 intensity of CD5+ B cells was stronger than the intensity of CD5− B cells (Fig. 4b). More casein bound to CD5+ B cells than CD5− B cells and cellular spots of CD5+ B cells were observed more right side than those of CD5− B cells.

3.6. Cellular proliferation by CFSE staining

B cells and T cells were proliferated by casein stimulation in both milk allergy and tolerant groups (Fig. 5). Proliferation of CD19+CD5− B cells by ex vivo casein stimulation was observed and the proliferation of CD19+CD5+ B cells was not observed well by gating for CD5+ and CD5− B cells. Especially, CD19+CD5− B cells diminished simultaneously with proliferation whereas CD19+CD5+ B cells were observed to be diminished without proliferation.

3.7. IL-4 and IL-5 effects on B cell subset responses of PBMC

B cells changed insignificantly by IL-4 from 5.2 ± 3.9% to 5.5 ± 4.1% (p-0.511) and decreased by IL-5 to 4.3 ± 3.6% ($p = 0.005$). CD19+CD5− B2 cells insignificantly increased by IL-4 from 3.1 ± 2.4% to 3.7 ± 2.7% ($p = 0.211$) and were unchanged to 2.9 ± 2.5% by IL-5 ($p = 0.191$) (Fig. 6a). CD19+CD5+ B1 cells decreased from 2.1 ± 1.7% to 1.9 ± 1.5% by IL-4 ($p = 0.036$) and to 1.4 ± 1.2% by IL-5 ($p = 0.002$). CD19+CD5+ B1 cell decrease was more profound by IL-5 than by IL-4 ($p = 0.033$).

CD5+ B1 cell percentage in B cells decreased significantly from 40.3 ± 10.1% to 32.8 ± 7.3% by IL-4 ($p = 0.011$) and to 33.9 ± 12.2 by IL-5 ($p = 0.015$) (Fig. 6b). CD1dhi B1 cell percentage in B1 cells was unchanged from 10.6 ± 6.3% to 9.9 ± 4.1% by IL-4 and significantly decreased to 6.4 ± 4.3% by IL-5.

4. Discussion

B cell subset responses of milk allergy patients to allergen stimulation of PBMCs showed a different pattern from those of milk tolerant patients in atopic dermatitis. B cells decreased significantly

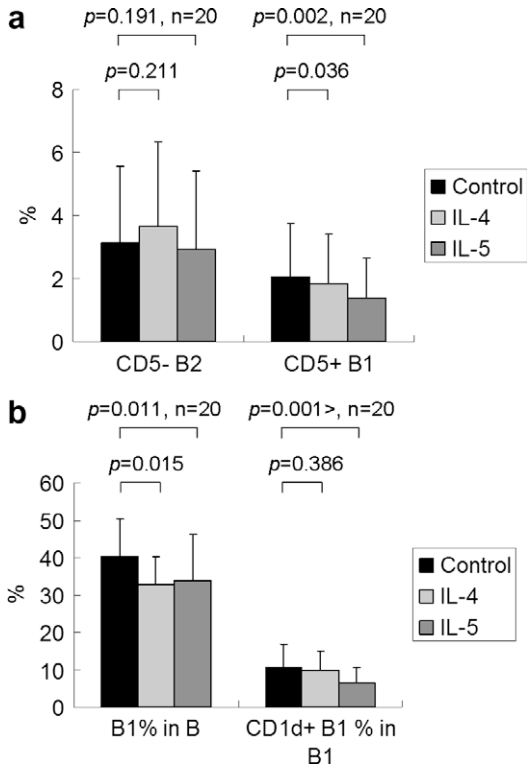


Fig. 6. IL-4 and IL-5 Effects on CD19+CD5+ B1 and CD19+CD5− B2 cell subsets. (a) IL-4 and IL-5 effects on CD5+ and CD5− B cell, (b) IL-4 and IL-5 effects on CD5+ B1 cell% in CD19+ B cells.

only in milk tolerant patients by ex vivo casein stimulation of PBMCs (Fig. 3a). The proliferation of casein-stimulated lymphocytes from children reacting to cow's milk was significantly higher than the proliferation of lymphocytes from children with AD without milk allergy [21]. Proliferating B cells were killed by cytotoxic CD4+CD25+ T cells which are known to be important in immune tolerance [20]. In this study, CD19+ B cells proliferated markedly (Fig. 5a). In milk tolerant status, B cells were decreased due to both allergen-activation-induced apoptosis [22] or killing of proliferation B cell by regulatory T cells [20]. In this study, B cells decreased by casein stimulation in the milk tolerant patients as expected and it seemed to be explained by the results that apoptotic B cells decreased only in the milk allergy group and increased insignificantly ($p = 0.059$) in milk tolerant group (Fig. 3b).

Overall change of B cells and B1 cells lead reduce CD5+ B cell percentage in B cells in the milk allergy group and rather increased in milk tolerant group in this study (Fig. 3d). Although these changes were not so big, the changes of CD5+ B1 cell composition in B cells were consistent and significant. The change of CD5+ B1% in B cells possibly was the secondary results of the change of CD5+ B1 cells and CD5− B2 cells. This change seemed to show the immunologic trend of changes in B cell subsets, but could not explain the immunologic mechanisms for allergy or tolerance.

These changes were consistent and supportive for the report that CD5+ B1 cells are regulatory B cells which regulate the immune system negatively [7]. In some reports, B1 cells were reported to be related with the pathogenesis of delayed-type hypersensitivity by mediating required early T cell recruitment [23] and to mediate airway hyper-reactivity in non-atopic asthma [24]. Although it is still controversial whether CD5+ B cells were harmful or beneficial in allergy, CD5+ B cells seemed to be beneficial for allergy tolerance in this study.

The change of CD5+ B cells subsets was evaluated. CD1d(hi)CD5(+) B cells represent a unique subset of potent regulatory B cell and IL-10 production of B cells was restricted to this CD1d(hi)CD5(+) B cell subset [25]. CD1d(hi) B1 cell% in CD5+ B1 cells decreased by casein stimulation in the milk tolerant group and was unchanged in the milk allergy group, while IL-10 producing CD5+ B cells were decreased by casein stimulation in milk allergy group and unchanged in milk tolerant group in this study (Fig 3f). From these results, IL-10 producing CD5+ B cell subsets seemed to be reactive to allergy provocation as an excessive immune response and not to suppress the immune system primarily or actively as regulatory T cells which increased by antigen stimulation and suppress immunologic effector cells. Exact immune mechanisms need to be investigated further still.

Allergen-specific T cell responses by casein stimulation were different between infants with atopic dermatitis with and without cow's milk allergy concerning Th2 cytokine production [21]. In vitro production of IL-4 and IL-5 in peripheral blood mononuclear cells (PBMC) was enhanced [5,6] in cow's milk allergy. Especially, antigen-specific T cell responses concerning Th2 cytokine production between infants with atopic dermatitis with and without cow's milk allergy was well known [26]. Therefore, allergen stimulation and subsequent IL-4 and IL-5 effects may be expected. Th2 cytokine such as IL-4 and IL-5 are B cell differentiation and growth factors [27]. In this study, IL-4 and IL-5 effects on the change of B cell subsets were evaluated to elucidate that IL-4 and IL-5 were responsible for the changes of B cell subsets by allergen stimulation. IL-4 increased B cell proliferation by ex vivo stimulation. IL-4 is a cytokine of B cell growth and differentiation [28] and this result could also be presumed. Especially, IL-4 increased CD19+CD5− B2 cells, but CD19+CD5+ B1 cells decreased by both IL-4 and IL-5. Overall effects of Th2 cytokine by allergen provocation were expected to decrease CD5+ B cell percentage in B cells. IL-4 and IL-5 which were produced by allergy provocation, seemed to be responsible for the decrease of CD5+ B cell percentage in B cells by casein stimulation in the milk allergy group.

B cells and T cells showed different manners in the immunologic responses by allergen stimulation between in milk allergy and milk tolerant status. Casein-specific CD4+ cell proliferation increased in cow's milk allergy patients [29]. Milk-induced eczema is associated with the expansion of T cells expressing cutaneous lymphocyte antigen [30]. T cell responses were characterized by the proliferation of effector T cells in allergy provocation. Also, allergen-specific regulatory T cells were also associated with tolerance to milk protein and regulatory T cells also increased more by allergen stimulation in tolerant subjects [18]. Both effector T cells or regulatory T cells were known to respond to allergen stimulation by active proliferation both in allergy and tolerance. However, B cells showed the different pattern of responses from T cells. Basically, B cells were decreased by allergen stimulation in milk tolerant patients and B cells unchanged in milk allergy patients. The activation-induced apoptosis was reported in B cells [28]. It was supported from the results of this study in which apoptosis of B cells decreased in milk allergy group and insignificantly increased in milk tolerant group by casein stimulation (Fig. 3b). The response of regulatory T cells was not so predictive to differentiate milk allergy from milk tolerant status [18]. However, the changes of B cells seemed to be more decisive than those of T cells to discriminate allergy and tolerance for food allergy from our study.

It is unexpected result that more casein bound to CD5+ B1 cells than to CD5− B2 cells. Although the quantitative results were not obtained in this study, CD5+ B1 cells seemed to be important in immune responses and the exact roles of allergen-specific CD5+ B1 cells should be elucidated in allergy and tolerance. Interestingly, antigen-binding was more prominent in B cells than in T cells and the antigen-specific proliferation was more extensive in T cells than in B cells. Especially, CD5+ B cells did not show proliferation by allergen stimulation.

Conclusively, the responses of B cells and CD5+ B cell subsets were discriminative for milk allergy patients from milk tolerant patients and seemed to have characteristic diagnostic values. CD5+ B cells seemed to play a role in allergy tolerance and exact immune mechanisms should be elucidated further.

Disclosure statement

No competing financial interests exist.

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