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Allergen-specific helper T cell response in patients with cow's milk allergy: simultaneous analysis of proliferation and cytokine production by carboxyfluorescein succinimidyl ester dilution assay

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Clinical and Experimental Allergy

Summary

Background The role of antigen-specific T cells in the allergic reaction to cow's milk or in tolerance induction is not yet fully understood.

Objective This study was designed to analyse both cow's milk protein (CMP)-specific T cell proliferation and cytokine production simultaneously in children with cow's milk allergy (CMA) in comparison with subjects with various allergic backgrounds.

Methods Carboxyfluorescein succinimidyl ester was used to detect cow's milk-specific T cells by flow cytometry. The intra-cytoplasmic cytokine production of these antigen-specific T cells was also analysed.

Results Significant differences of both CMP-specific CD4⁺ cell proliferation and cytokine production between CMA and non-allergic children were observed. While the proliferative responses of children who recently outgrew CMA were not significantly different from those of patients, the patterns of cytokine production were similar to those of non-allergic children. **Conclusion** These results suggest that the presence of CMP-specific T cell clones *per se* does not produce CMA, but that the T-helper type 2-skewed pattern of those T cells is associated with adverse reactions. Although it is not possible to distinguish between individual patients with and without CMA on the basis of CFSE assays, these results contribute to the understanding of the pathogenesis and tolerance induction of CMA.

Keywords: CFSE dilution assay, Cow's milk allergy, IFN- γ , IL-10, IL-4, T cell proliferation

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Introduction

Cow's milk allergy (CMA) is defined as an immunologically mediated reaction against cow's milk antigens [1]. It is one of the most common food allergies in childhood and affects 2–3% of infants less than 1 year of age [2, 3]. CMA can present with a wide spectrum of clinical reactions [4], and IgE-mediated or T cell-mediated reactions, operating separately or together, account for these reactions [5]. IgE-mediated reactions cause cutaneous, intestinal or respiratory symptoms and in some cases lead to anaphylactic reactions [6]. T cell-mediated reactions bring about both cutaneous and intestinal symptoms [6]. The spectrum of gastrointestinal syndromes is regarded as a manifestation of IgE-independent delayed-type CMA [4].

The majority of infants with CMA outgrow their allergy before their third year of life, although a small percentage

(15%) of this population has a persistent allergy to cow's milk [7]. It remains to be elucidated why some children outgrow their CMA and others do not. One explanation of tolerance failure is exaggerated immune deviation from the TH2 cytokine pattern and delayed maturation of T-helper type1 cells [8]. An alternative explanation is insufficient active suppression of the Cow's milk protein (CMP)-specific T cell response by various suppressor T cells [9, 10] or by immunosuppressive cytokines [11].

As T cells are responsible for the isotype switching of antigen-specific B cells to IgE, T cell activation is the initial step in determining the outcome of the immune response. Thus, T cells play a major role in the development and maintenance of the IgE-mediated reaction as well as IgE-independent reactions. More insight into the allergen-specific T cell responses of donors with different allergic backgrounds might shed some light on the

pathogenesis and tolerance induction of CMA. However, it is difficult to investigate the functional role of these T cells, as the frequency of allergen-specific T cells in peripheral blood is very low.

Allergen-specific proliferation [12–18] and cytokine production [8, 19] have been studied, but limited and sometimes contradictory information is available [20]. The cloning of antigen-specific T cells has proved to be a powerful tool to investigate the immune response to allergens at the T cell level [11, 21]. Nevertheless, several cycles of T cell stimulation in the presence of antigen, feeder cells and exogenous cytokines are necessary to establish T cell clones [22, 23]. As this is time and labor consuming, only a limited number of clones can be studied. Furthermore, human T cells tend to develop a Th2-skewed cytokine-producing phenotype after repeated stimulation *in vitro* under neutral conditions [24, 25].

Recently, Turcanu *et al.* [26] introduced a carboxyfluorescein succinimidyl ester (CFSE)-labelling method to identify food antigen-specific T cells. CFSE is a membrane-permeating dye that binds the amino groups of cytoplasmic proteins with its succinimidyl-reactive group [27, 28]. When cells divide, CFSE-labelled proteins are equally distributed between the daughter cells, thus halving cell fluorescence with each division. Consequently, antigen-specific T cells lose their fluorescence after culture in the presence of the respective antigen (CFSE^{low}) and are distinguishable from other cells in culture (CFSE^{high}).

In this report, we applied this experimental approach, and the frequencies and cytokine-producing phenotypes of these antigen-specific T cells were examined simultaneously. Comparison of the cow's milk-specific T cell response among patients with CMA, children who have recently outgrown their CMA, patients with other food allergies and non-allergic individuals might provide more insight into the regulation of the immune response towards cow's milk.

Materials and methods

Patients

Twenty-three CMA children (median age: 27 months), 18 outgrown children (median age: 36 months), 11 hen's egg

allergy (HEA) children (median age: 30 months) and 17 non-allergic children (median age: 38 months) were included in this study (Table 1), which was approved by the Local Research Ethics Committee. The diagnosis of CMA was based on (1) positive oral challenge test or (2) a clinical history of hypersensitivity, accompanied by cow's milk-specific IgE greater than 3.5 kUa/L. The CMA group was composed of six children with milk-induced urticaria, two children with milk-induced anaphylaxis and 15 children with milk-induced atopic dermatitis. Children who developed delayed-type gastrointestinal symptoms were not included in this study. The children who had outgrown CMA were diagnosed with CMA according to the above criteria, and resolution was confirmed in challenge tests. This group was composed of four children who had outgrown milk-induced urticaria and 14 children who had outgrown milk-induced atopic dermatitis. The non-allergic children had never reacted to cow's milk ingestion and were currently consuming cow's milk as part of their diets. Eleven HEA children were also investigated. The diagnostic criteria of HEA were the same as for CMA, and patients with positive cow's milk-specific IgE (> 0.35 kUa/L) were excluded. There were no significant differences when comparing the ages or sexes of the various groups.

Reagents

Total casein and whey protein were kindly provided by Bean Stalk Snow Co., Ltd (Sapporo, Japan). The lyophilized protein was dissolved in PBS, sterilized by filtration and added to the cell culture at a final concentration of 500 µg/mL. These optimal antigen concentrations were determined by titration in a preliminary experiment. Fluorochrome-labelled monoclonal antibodies (MoAbs) specific for human IFN-γ, IL-4 and CD4 were purchased from Immunotech Coulter (Miami, FL, USA), and MoAb for IL-10 was from Pharmingen (San Diego, CA, USA). CFSE was purchased from Molecular Probes Inc. (Eugene, OR, USA).

Carboxyfluorescein succinimidyl ester labelling and cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood by density-gradient separation

Table 1. Characteristics of subjects

	Number (male/female)	Age (Mo) [†]	Total IgE [†] (kUa/L)	Milk-specific IgE [†] (kUa/L)
Cow's milk allergy (CMA)	23 (15/8)	27 (4–140)	671 (74–3300)	13.3 (0.9– > 100)
Outgrown	18 (12/6)	36 (19–97)	485 (53–6560)	1.34 (< 0.35–39.7)
Hen's egg allergy (HEA)	11 (5/6)	30 (3–118)	52 (4–1620)	< 0.35
Non-allergic	17 (8/9)	38 (1–168)	10 (4–110)	< 0.35

[†]Median value (minimum–maximum).

Statistical significance was measured by Kruskal–Wallis' one-way ANOVA followed by Mann–Whitney *U*-test with Bonferroni corrections for individual groups.

P* < 0.05, *P* < 0.01, ****P* < 0.001.

(Lympho separation medium; ICN Biomedicals, Aurora, OH, USA), and then resuspended at 5×10^7 cells/mL in RPMI1640. CFSE labelling was performed according to Turcanu et al.[26] with some modifications. Briefly, PBMCs were incubated with CFSE (labelling concentration of 5 μ M), for 10 min at 37 °C. Labelling was stopped with RPMI-1640 containing 4% autologous plasma, and excess dye was washed away. CFSE-labelled PBMCs were then cultured with or without 500 μ g/mL of antigens in RPMI-1640 supplemented with 5% autologous plasma. Cells were cultured in culture tubes at a concentration of 1×10^6 cells/mL. LPS contamination of cultures detected by the limulus assay (endotoxin single test Wako; Wako Pure Chemical Industries, Japan) was below 1 pg/mL.

Staining for the detection of cytokine production by flow cytometry

After 7 days of culture in the presence of antigens, PBMCs were restimulated with phorbol myristate acetate (PMA; 50 ng/mL) (Sigma, St Louis, MO, USA) and ionomycin (1 μ g/mL) (Sigma) in the presence of brefeldin A (10 μ g/mL) (Sigma) for 6 h. The cells were stained with PC-5-labelled anti-CD4 antibody, fixed and permeabilized with FIX & PERM cell permeabilization kits (Caltag Laboratories, Burlingame, CA, USA) according to the manufacturer's recommendations and stained with cytokine-specific MoAbs. After seven days of culture with antigens, CD4⁺ CFSE^{low} cells no longer seemed to produce IFN- γ , IL-4 and IL-10 without restimulation with PMA and ionomycin. So, PBMCs cultured in the presence of the same antigens, but not restimulated with PMA and ionomycin were used as negative controls. The results were practically not different when both isotype- and concentration-matched negative control provided by companies were used. Stained cells were acquired with a FACSCalibur (BD Biosciences, Milpitas, CA, USA), and the data were analyzed with CellQuest software (BD Biosciences).

Statistical analysis

Statistical significance was measured by two-tailed Kruskal-Wallis one-way ANOVA for ranks followed by Mann-Whitney' *U*-test with Bonferroni corrections for individual group using SPSS software (SPSS Inc., Chicago, IL, USA). The χ^2 -squared test was used to evaluate the difference among stages.

Results

A significantly high number of carboxyfluorescein succinimidyl ester^{low} CD4⁺ T lymphocytes appeared in

cow's milk allergy patients after cow's milk antigen stimulation

PBMCs from CMA donors were labelled with CFSE and then cultured for seven days in the presence of casein or whey protein. In the cow's milk antigen-treated cultures, a population of CFSE^{low} CD4⁺ T lymphocytes emerged (Fig. 1a); however, there were practically no CFSE^{low} cells when PBMCs were cultured without antigens (data not shown). The proportion of CFSE^{low} CD4⁺ cells in whole CD4⁺ cells after culture with casein was significantly higher in CMA patients than in the non-allergic control (median 34.1%, range 4.8–81.4% vs. median 9.9%, range 0.9–55.0%, $P < 0.01$; Fig. 2a). The proportion of CFSE^{low} CD4⁺ cells emerging in the cultures from outgrown (median 13.9%, range 3.9–58.6%) or HEA (median 17.8%, range 0.6–56.9%) children was between CMA patients and non-allergic controls (Fig. 2a). For cultures with whey proteins (Fig. 2b), the proportion of CFSE^{low} CD4⁺ cells in CMA patients (median 12.9%, range 4.2–84.0%) was significantly higher than in outgrown (median 4.1%, range 0.0–34.1%), in HEA patients (median 1.0%, range 0.3–6.4%) and in the non-allergic control (median 1.3%, range 0.1–12.4%).

Cow's milk-specific T cells display T-helper type 2 polarization in cow's milk allergy children

Figures 1b–d demonstrate the representative results of cytokine production. We expressed the results as a percentage of cytokine-positive cells among CFSE^{low} (antigen-specific) lymphocytes. 8.21%, 11.6% and 7.67% of CFSE^{low} CD4⁺ cells were stained with PE-labelled antibody to IFN- γ , IL-4 and IL-10, respectively.

The percentages of cytokine-positive cells in CFSE^{low} CD4⁺ T lymphocytes are summarized in Tables 2 and 3. We compared the cytokine production of casein-specific lymphocytes for all study groups and a significant difference in the logarithmic IL-4/IFN- γ ratio between CMA and the non-allergic control (median 0.220, range –0.514 to 1.000 vs. median –0.583, range –1.485 to 0.227; $P < 0.01$) was observed (Fig. 3a). Outgrown children were also significantly different from CMA children (median 0.220, range –0.514 to 1.000 vs. median –0.864, range –1.911 to 0.197; $P < 0.001$). In contrast, no significant difference in the logarithmic IL-10/IFN- γ ratio was seen among the four groups (CMA; median –0.275, range –0.923 to 0.749, Outgrown; median –0.708, range –0.909 to 0.161, HEA; median –0.301, range –1.057 to 0.455, non-allergic control; median –0.226, range –1.158 to 0.828) (Fig. 3b).

For whey protein-specific cytokine production, we also found a significant difference in the logarithmic IL-4/IFN- γ ratio between CMA and non-allergic control (median 0.089, range –1.482 to 1.062 vs. median –0.912,

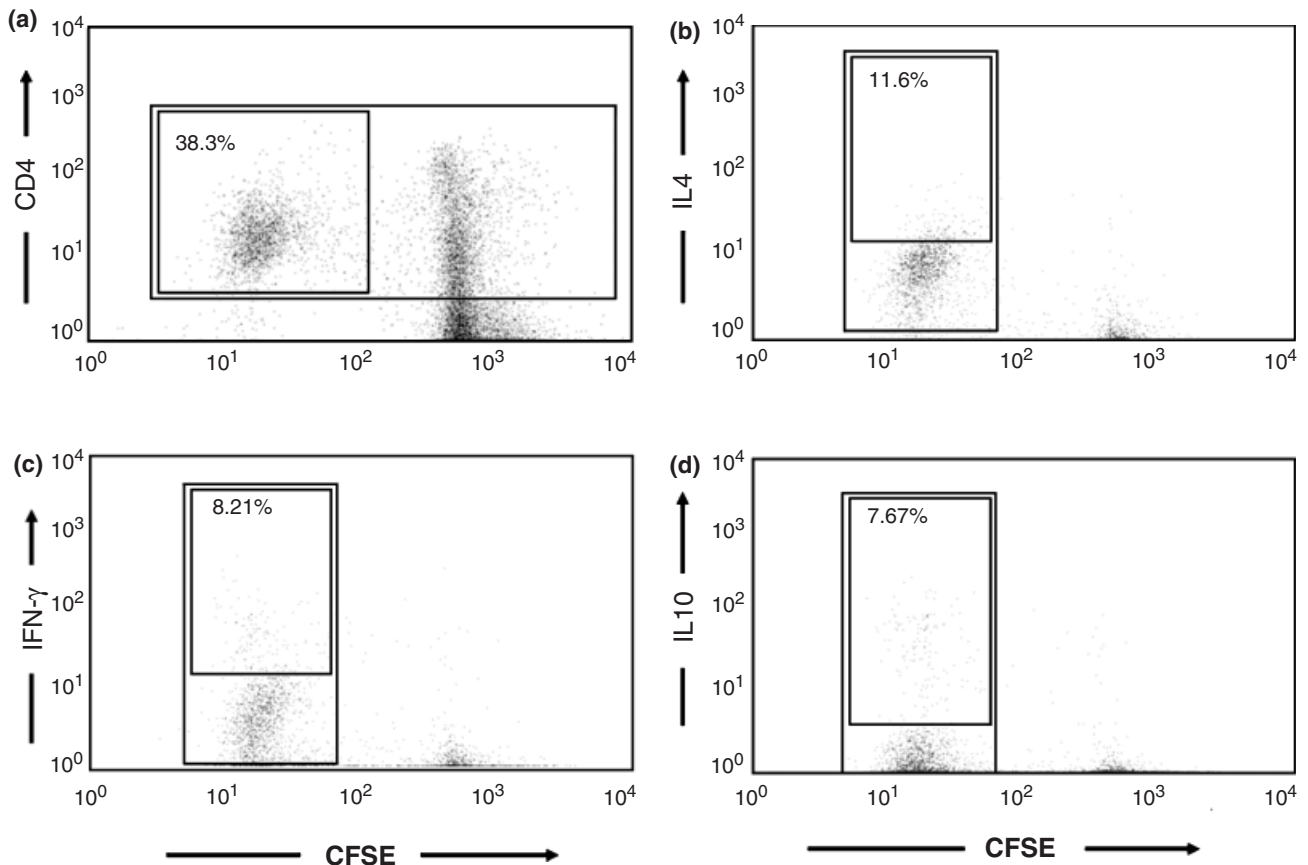


Fig. 1. Peripheral blood mononuclear cells (PBMC) proliferation and cytokine profile were monitored using Carboxyfluorescein succinimidyl ester (CFSE) labelling. PBMCs from a cow's milk allergy patient were isolated, labeled with CFSE, and cultured in the presence of 500 $\mu\text{g/mL}$ casein. On day 7, PBMCs were restimulated with phorbol myristate acetate and ionomycin in the presence of brefeldin A for 6 h, then the cytokine production of PC-5-labelled CD4^+ cells was assessed using intracellular cytokine staining with the PE-labeled MoAbs indicated. Fig. 1a represents the percentage of antigen-specific CFSE^{low} , CD4^+ cells in CD4^+ cells. Fig. 1b–d show the percentage of cytokine-positive cells in antigen-specific CFSE^{low} CD4^+ cells.

range -1.083 to -0.348 ; $P < 0.05$) (Fig. 3c). In this case, outgrown children (median -0.426 , range -2.000 to 0.606) were not significantly different to CMA children. Similar to the response to casein, no significant difference in the logarithmic IL-10/IFN- γ ratio induced by whey protein was seen among the four groups (CMA; median -0.067 , range -1.392 to 1.052 , Outgrown; median -0.707 , range -3.420 to 0.617 , HEA; median -0.691 , range -2.468 to 0.065 , non-allergic control; median -0.693 , range -1.290 to 0.108 ; Fig. 3d).

Comparison of staging defined by the results of carboxyfluorescein succinimidyl ester assay with cow's milk allergic status

The relationship of the cow's milk allergic status with a staging defined by the response results to casein is summarized in Table 4. CMA patients tended to belong to stage III, which showed strong proliferation to casein and TH2-deviated cytokine production. In contrast, most non-allergic children belonged to stage I, which responded

weakly to casein. The statistical difference measured by the χ^2 test was significant ($P < 0.01$). Outgrown and HEA children were between CMA and non-allergic children.

Discussion

T cell activation is the initial step in determining the outcome of the immune response and T cells play a major role in maintaining the IgE-mediated reaction as well as T cell-dependent reactions. CMP-specific T cell reactivity in CMA has therefore been examined extensively. In this study, our results showed that the proportion of CFSE^{low} CD4^+ cells in whole CD4^+ cells after culture with CMP was significantly higher in CMA patients than in non-allergic controls, suggesting that the frequency of CMP-specific T cell clones in CMA patients is higher than that of non-allergic children. These results are consistent with previous reports [13–18, 29], although it is generally accepted that it is not possible to distinguish between individual patients with and without food allergy on the basis of proliferation assays with PBMCs. In addition, the

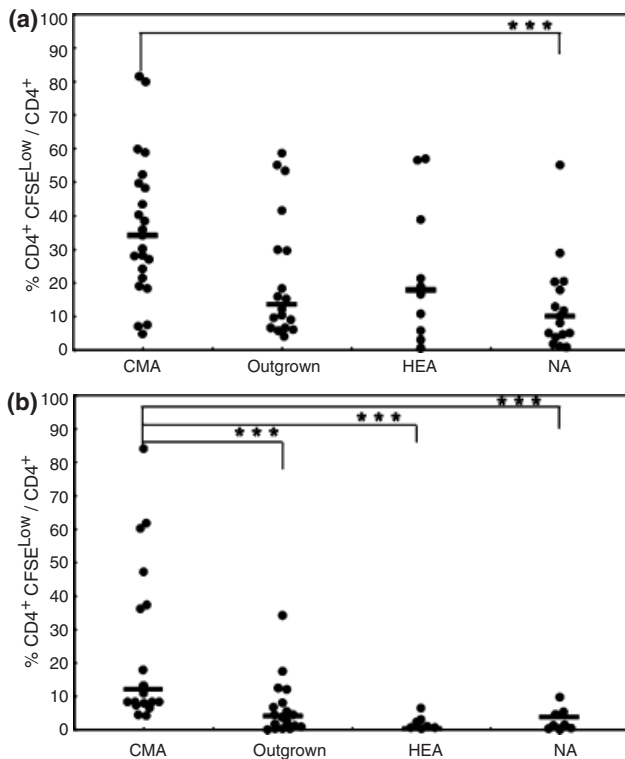


Fig. 2. Proliferation of antigen-specific T lymphocytes. PBMCs were isolated, Carboxyfluorescein succinimidyl ester (CFSE) labelled, and cultured in the presence of casein (a) or whey protein (b) for 7 days, then the percentage of antigen-specific CFSE^{low} CD4⁺ cells in CD4⁺ cells was assessed by flow cytometry. Points represent data from different individuals, while bars show the median values. Statistical significance was measured by Kruskal–Wallis one-way ANOVA for ranks followed by Mann–Whitney U test with Bonferroni corrections for individual groups (****P* < 0.001).

proportion of CFSE^{low} CD4⁺ cells in cultures from outgrown children was intermediate between CMA patients and non-allergic controls, and significant differences from these subjects were not observed, which further emphasizes that the presence of a CMP-specific T cell response *per se* does not cause CMA. It is worth mentioning that because of the restricted sample size and the high variance in the percentage of CFSE^{low} CD4 cells, the power of our analysis at the 5% level of significance was not

sufficient (57.9%). So, the absence of the significant difference did not mean that the frequency of CMP-specific T cell clones in the outgrown group is equal to that of CMA patients.

Although the protein fraction of cow's milk consists of at least 20 different proteins that may provoke an allergic reaction, whey proteins, α -lactalbumin and β -lactoglobulin, together with casein proteins, are proposed as the most important allergenic proteins [30]. In this study, proliferation responses to casein were generally stronger than responses to whey proteins, while the stronger trends of the responses of CMA children than the remaining three groups were essentially not different. These results are consistent with reports indicating that caseins are more immunogenic than whey proteins, and that CMA patients mostly have an allergic reaction against caseins [29, 31]; however, the different proliferative responses between CMA children and the remaining three groups were more remarkable with whey protein stimulation, probably because responses to casein were comparatively strong even in children not allergic to CMPs.

As several groups have already demonstrated for CMA infants [32–34], we found significant skewing to TH2 cytokine production in CMA. In addition, outgrown children also showed significant differences in the logarithmic IL-4/IFN- γ ratio to CMA children, suggesting that the TH2-biased response of CMP-specific T cells, but not the existence of CMP-specific T cells themselves, is associated with the pathogenesis of CMA. It is noteworthy that these TH2 deviations of cytokine production are not solely due to the atopic pre-disposition of the patients, because CMP-specific CD4⁺ T cells from HEA children without CMA did not show statistically significant TH2 deviation.

We also investigated CMP-specific CD4⁺, cytoplasmic IL-10⁺ cells. A subset of regulatory T cells, designated Tr1, has been reported to be induced by IL-10, and also to produce high levels of IL-10, low levels of IL-2 and no IL-4 [35]. It is thought that IL-10 produced by Tr1 is important for the suppression of the allergen-specific T cell response through the inhibition of proliferative responses and cytokine production of these T cells. Unexpectedly, there was no difference in the logarithmic IL-10/IFN- γ ratio among the examined groups irrespective of their atopic

Table 2. Percentages of cytokine-positive cells in CFSE^{low} CD4⁺ T lymphocytes after stimulation with casein

	IFN- γ [†]	IL-4 [†]	IL-10 [†]
Cow's milk allergy (CMA)	5.9 (1.4–22.3)	12.2 (1.0–20.2)	3.0 (0.6–20.3)
Outgrown	16.1 (0.1–52.6)	2.2 (0.0–10.4)	4.3 (0.0–13.0)
Hen's egg allergy (HEA)	10.4 (1.3–20.1)	3.9 (0.3–17.2)	3.7 (0.0–16.8)
Non-allergic	6.2 (0.7–24.5)	1.4 (0.4–10.5)	2.5 (0.0–24.7)

[†]Median value (minimum–maximum).

Statistical significance was measured by Kruskal–Wallis' one-way ANOVA for ranks, followed by Mann–Whitney U-test with Bonferroni corrections for individual groups.

P* < 0.05, *P* < 0.01, ****P* < 0.001.

Table 3. Percentages of cytokine-positive cells in CFSE^{low} CD4⁺ T lymphocytes after stimulation with whey protein

	IFN- γ [†]	IL-4 [†]	IL-10 [†]
Cow's milk allergy (CMA)	7.4 (0.3–17.5)	10.2 (0.5–23.9)	4.3 (0.7–16.8)
Outgrown	13.4 (1.1–59.8)	4.2 (0.1–14.3)	4.0 (0.0–6.3)
Hen's egg allergy (HEA)	7.0 (2.9–11.1)	12.5 (0.1–16.7)	1.4 (0.0–12.9)
Non-allergic	13.4 (5.6–19.8)	1.8 (1.5–3.6)	2.7 (0.9–14.7)

[†]Median value (minimum–maximum).

No statistical significance was observed among groups by Kruskal–Wallis' one-way ANOVA for ranks.

state. As the CFSE assay focused solely on clonally expanded IL-10⁺ T cells, we might overlook true Tr1, which proliferates poorly following antigen-specific activation [36]. Further investigation of another important suppressive cytokine, TGF- β , is necessary.

As it was not possible to distinguish between individual patients with and without milk allergy on the basis of either proliferation assays or intra-cytoplasmic cytokine staining separately, a staging system defined by the results of the CFSE assay was attempted. CMA patients tended to belong to stage III, which showed strong proliferation to casein and TH2 deviation. In contrast, most non-allergic children belonged to stage I, which responded weakly to

casein. Outgrown and HEA children belonged between CMA and non-allergic children.

In this report, we showed significant differences of both CMP-specific CD4⁺ cell proliferation and cytokine production between CMA and non-allergic children. While the proliferative responses of children who had recently outgrown CMA were not significantly different from those of the patients, the patterns of cytokine production were similar to those of non-allergic children. These results suggest that the presence of CMP-specific T cell clones *per se* does not produce CMA, but that the TH2-skewed pattern of those T cells is associated with adverse reactions. Although it is not possible to distinguish between

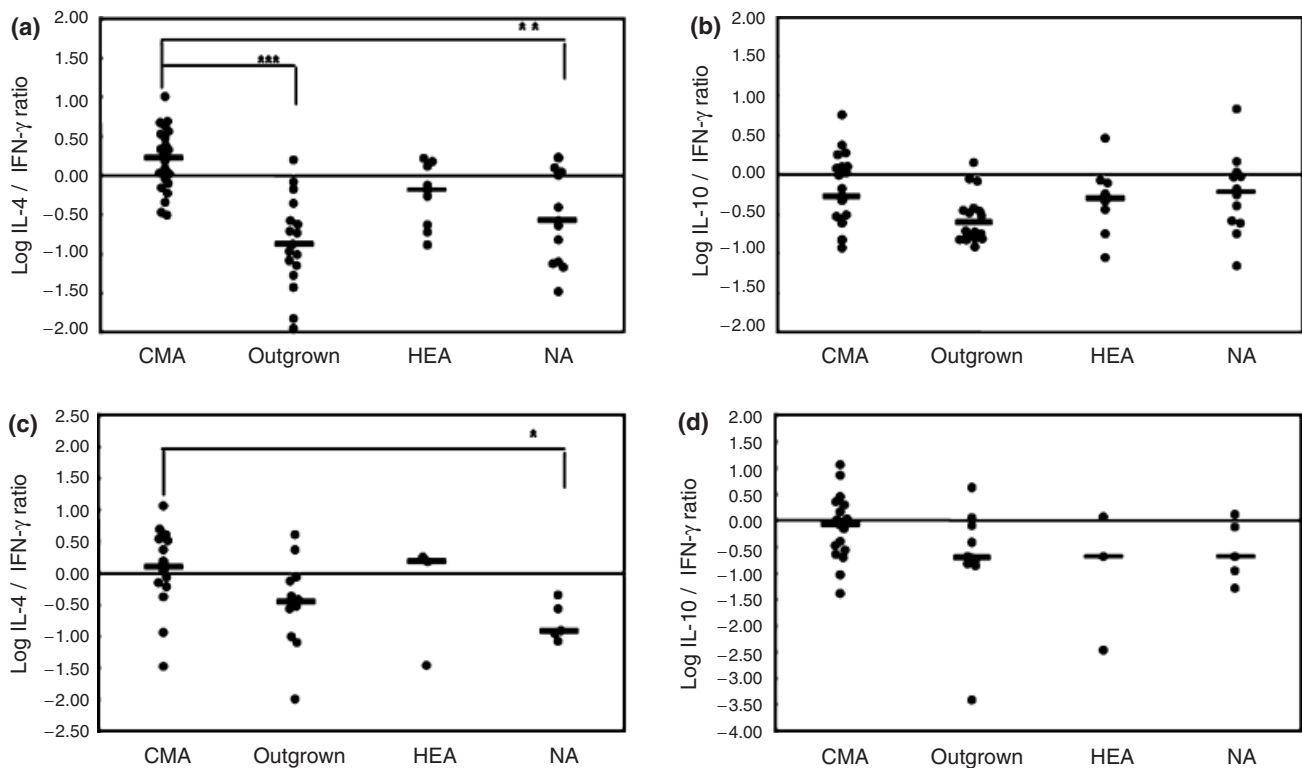


Fig. 3. Intra-cytoplasmic cytokine profiles of antigen-specific T lymphocytes. Peripheral blood mononuclear cells were isolated, Carboxyfluorescein succinimidyl ester (CFSE) labeled, and cultured in the presence of casein (a, b) or whey protein (c, d) in the presence of antigens for 7 days, then the cytokine-producing phenotype of antigen-specific (CFSE^{low}) CD4⁺ cells was assessed by flow cytometry. Intra-cytoplasmic cytokine profiles were presented as logarithmic ratios of CFSE^{low} cell percentages that produce IL-4 or IL-10 to cell percentages that produce IFN- γ . Points represent data from different individuals, while bars show the median values. Statistical significance was measured by Kruskal–Wallis' one-way ANOVA for ranks followed by Mann–Whitney U-test with Bonferroni's corrections for individual groups (* P < 0.05; ** P < 0.01; *** P < 0.001).

Table 4. Comparison of staging defined by the results of CFSE assay with CM-allergic status

	Stage			total
	I	II	III	
CMA	3	5	15	23
Outgrown	7	10	1	18
HEA	3	5	3	11
Non-allergic	8	6	3	17

Stage I: The value of casein-specific CD4⁺ T cell proliferation was less than that of the median value from non-allergic subjects.

Stage II: The value of casein-specific CD4⁺ T cell proliferation was more than that of the *median* value from non-allergic subjects, and the percentage of the IL-4⁺, CD4⁺ cells are less than that of IFN- γ ⁺, CD4⁺ cells.

Stage III: The value of casein-specific CD4⁺ T cell proliferation was more than that of the *median* value from non-allergic subjects, and the percentage of the IL-4⁺, CD4⁺ cells are more than that of IFN- γ ⁺, CD4⁺ cells.

The χ^2 test was used to evaluate the difference among stages ($P < 0.01$). Comparisons between each group were adjusted by Bonferroni's correction.

* $P < 0.05$, *** $P < 0.001$.

individual patients with and without CMA on the basis of CFSE assays with PBMCs, these results contribute to the understanding of both the pathogenesis and the tolerance induction of CMA. Further studies particularly concerning regulatory T cells and suppressor cytokines are necessary to understand the pathogenesis of CMA, and to develop new therapeutic strategies to introduce tolerance to CMP.

Acknowledgements

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