

Memory T Cell Proliferation in Cow's Milk Allergy after CD25+ Regulatory T Cell Removal Suggests a Role for Casein-Specific Cellular Immunity in IgE-Mediated but Not in Non-IgE-Mediated Cow's Milk Allergy

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Key Words

Cow's milk allergy • α -Casein • β -Casein • κ -Casein • β -Lactoglobulin • Lipopolysaccharides • Regulatory T cells

Abstract

Background: Previously reported increased lymphocyte proliferative responses in cow's milk allergy (CMA) may have been influenced by the lipopolysaccharides (LPS) which contaminate most commercial cow's milk protein (CMPs). Moreover, peripheral blood mononuclear cells (PBMC) contain both B cells, CD45RA+ naïve T cells, CD25+ regulatory T cells (Tregs) in addition to antigen-specific CD45RA+ memory T cells. **Methods:** PBMC from clinically reactive and tolerated patients with IgE- and non-IgE-mediated CMA were depleted of CD45RA+ T cells and putative CD25+ Tregs. The proliferative index to LPS-depleted α -, β - and κ -casein and β -lactoglobulin was compared in the memory T-cell-enriched, Treg-depleted PBMC and in bulk PBMC. **Results:** Clinically reactive IgE-mediated CMA patients had increased responses to caseins only. Tolerated patients, particularly those with atopic dermatitis, had decreased responses to κ -casein which were restored after Treg depletion. Interleukin-4 and interferon- γ were generally not detected in the culture supernatants. No differences were seen between reactive and

tolerant delayed non-IgE-mediated CMA patients. **Conclusions:** Proliferative responses to α -, β - and κ -caseins (but not β -lactoglobulin) were observed in clinically reactive IgE-mediated CMA patients only. A markedly decreased proliferative response to κ -casein in tolerated IgE-mediated CMA patients with atopic dermatitis, which was abrogated by Treg depletion, suggested a role for κ -casein in tolerance induction. Non-IgE-mediated CMA patients had no increased proliferative response to any milk proteins.

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Introduction

Cow's milk allergy (CMA) is the commonest form of food allergy, affecting 2–5% of children by the age of 3 [1]. CMA reactions have been divided into three major groups according to the elapsed time between challenge and symptoms: (1) an IgE-mediated immediate reaction group with vomiting, urticaria or anaphylaxis within 45 min of milk ingestion, (2) an intermediate reaction group (45 min to 20 h after challenge) with vomiting, diarrhoea and/or eczema, and (3) a slow-reacting group with diarrhoea, asthma or eczematous reactions more than 20 h after milk ingestion [2]. The delayed reactions

are generally not associated with cow's milk protein (CMP)-specific serum IgE. A T-cell-mediated delayed type of hypersensitivity has been suggested in this latter group [3], supported by the positive patch tests described in CMP-induced atopic dermatitis [4] and in CMA with digestive symptoms [5].

The lymphocyte proliferation test (LPT) has been used to investigate T-cell-mediated responses to CMP. However, differences in experimental variables and patient populations have made interpretation of these data difficult [6]. Commercially prepared milk proteins are often contaminated with bacterial endotoxins such as lipopolysaccharides (LPS) [7, 8]. Such endotoxins induce proliferation of peripheral blood mononuclear cells (PBMC) used in LPT [9]. The previous CMP-induced LPT results in CMA patients [6, 10–12] may thus have been influenced by unpredictable amounts of LPS contamination. Therefore, the CMP antigens need to be LPS depleted [8, 13].

Whereas naïve T cells express the 220-kDa CD45RA isoform, memory T cells switch to the 180-kDa CD45RO isoform. Therefore, the CMP-specific T cells responsible for the symptoms in clinically reactive CMA patients should be memory cells (CD45RA⁻) [14]. Antigen-specific T cell proliferation and cytokine production may be inhibited by a CD4⁺ T cell subset, which constitutively express high levels of the interleukin (IL)-2 receptor α -chain, CD25 [15], named CD4⁺CD25⁺ regulatory T cells (Tregs). Functional Tregs may suppress CMP-specific T cell proliferation.

The aim of the study was to compare proliferative activity to LPS-depleted α -, β - and κ -casein fractions and β -lactoglobulin (β -LG), using BSA and tetanus toxoid as control antigens, in PBMC from clinically reactive and tolerised patients with (1) IgE-mediated CMA (with or without milk-sensitive atopic dermatitis), (2) non-IgE-mediated, delayed-onset CMA, and (3) in healthy non-CMA controls. The antigen-specific proliferative index (PI) in bulk PBMC was compared with CD45RA⁺ (naïve T cell and B cell)- and CD25⁺ (Treg)-depleted PBMC.

Materials and Methods

Study Population

Clinical data and laboratory findings are summarised in table 1. CMA patients with a previous history of clinically controlled milk challenge-induced reactions while on a milk-free diet were included. All, except for 4 highly reactive IgE-mediated CMA children, were retested by a clinically controlled open challenge (n = 27, 10 girls and 17 boys, with a median age of 7 years,

range 4–13) at BKL Voksentoppen, National Hospital, Oslo. The challenge was carried out for 3 days in the clinic, and if no response was noted, the milk diet was continued at home for at least a month. Two additional tolerised patients (males, 21 and 23 years old), with a clinical history of food allergies including milk, referred to the Norwegian Institute of Public Health for re-assessment of their allergies, were included. The patients were either on a milk-free diet (n = 23), consumed milk products (n = 2) or were on unrestricted diets (n = 4).

The patients were divided into those with a clinical history of IgE-mediated CMA with UniCAP levels >2 and/or CMP-sensitive atopic dermatitis (n = 15, median age 8 years, range 4–23), and those with CMP-induced delayed gastrointestinal reactions (n = 14, median age 7 years, range 4–11). A control group (n = 10, median age 38 years, range 1–64), primarily to establish proliferative levels and to function as a reference group for tolerised patients, consisted of 2 non-allergic children and 8 adults (6 females and 4 males), with no history of CMA.

Grouping patients solely on the basis of clinical symptoms or on IgE levels may not address the underlying mechanisms, as CMA-induced slow-onset cutaneous or gastrointestinal reactions may also occur in patients with low CMP-specific IgE levels [16, 17]. Therefore, patients were grouped both on the basis of CMP-specific IgE levels and symptom onset time. The majority of children with CMA (85–90%) have become tolerant by the age of 3 [1], but those with persistent CMA tend to retain it throughout childhood. Previously clinically reactive patients enrolled in the study who were subsequently shown to have become tolerant were included in order to examine lymphocyte proliferative activity in clinical tolerance.

The study was approved by the Regional Committee for Medical Research Ethics, Southern Norway, for the inclusion of CMA patients only, with a lower age limit of 4 years. Ethical approval was denied for the inclusion of younger children (2–4 years) and healthy age-matched non-allergic control subjects, which would have been optimal for the study. The decision of the Ethical Committee thus limited the selection of both patient and control groups. Therefore, the control group consisted mainly of adult subjects and was intended largely for reference purposes and not primarily for direct comparison with patient groups. Enrolment was carried out after informed consent was obtained from the parents. A 1-year-old child, recruited in a separate study and found to be milk tolerant, was additionally included in the control group to demonstrate typical proliferative levels in tolerised children.

Ethical consent was not given for multiple sampling, and therefore, blood samples could only be obtained from the patients prior to challenge, in the course of routine diagnostic sampling. Total and cow's milk-specific IgE serum levels were measured using the UniCAP Total IgE and the UniCAP Specific IgE FEIA systems (Pharmacia Diagnostics AB, Uppsala, Sweden), with skimmed milk as antigen.

Antigens

Commercially prepared milk proteins are often contaminated with bacterial LPS, varying from 200 to 3,500 pg/mg [8, 18]; as little as 10 pg endotoxin/ml may induce lymphocyte proliferation [9]. Thus, at the dose levels used in the present study, the untreated α -, β - and κ -casein subfractions and β -LG (Sigma-Aldrich, Steinheim, Germany) contained sufficient endotoxin to induce

Table 1. Clinical features of individual patients

Patient	Age years	Gender	Tolerant to milk	Reactions to other food	Total IgE kU/l	Specific IgE kU/l	Skin prick test
<i>Group I: IgE-mediated CMA with/without atopic dermatitis (n = 15)</i>							
MA1	4	F	+		598	<0.35	neg.
MA8	13	F	–	egg, fruit	169	<0.35	neg.
MA9	12	F	–	egg, fish	3,342	<0.35	neg.
MA10	8	M	–	egg, fish	1,500	59.4	++++(+)
MA13	23	M	+	fruit/nuts, pollen	183	<0.35	NA
MA14	21	M	+	fruit/nuts, pollen	9.36	<0.35	NA
MA21	13	M	+	egg, fish	707	0.56	++(+)
MA22	4	F	–	fish	81	10.4	+++(+)
MA23	7	M	–	fish	>5,000	78.3	+++
MA25	6	M	+	fish	1,300	13.1	++
MA26	11	M	–		2,017	65.0	++++
MA27	5	M	–		>5,000	>100	+++
MA28	5	M	–		780	47.0	+++
MA30	8	F	+	egg	377	<0.35	neg.
MA31	5	M	–		530	87.0	+++
mean cow's milk-specific IgE 30.86 kU/l (<0.35 to >100)							
<i>Group II: non-IgE-mediated gastrointestinal symptoms (n = 14)</i>							
MA2	6	M	+		18	<0.35	neg.
MA3	8	M	–		<63	<0.35	neg.
MA4	8	M	–		<63	<0.35	neg.
MA5	6	F	+		6	<0.35	neg.
MA7	7	M	–	fish, egg	571	2.0	++
MA11	6	M	+	dust mites	40	<0.35	neg.
MA16	7	F	+		52	<0.35	neg.
MA17	4	F	+		<52	<0.35	neg.
MA18	6	F	+		74	<0.35	neg.
MA19	7	M	+		8	<0.35	neg.
MA24	11	M	–		<70	<0.35	neg.
MA29	10	M	–	egg, fish	571	2.0	++
MA32	9	M	+		580	0.5	neg.
MA33	4	F	+		8	<0.35	neg.
mean cow's milk-specific IgE 0.60 kU/l (<0.35–2)							
<i>Group III: healthy controls (n = 10)</i>							
NC5	4	M	+		353	<0.35	neg.
NC6	58	M	+		544	<0.35	–
MA20	1	F	+		<2.0	<0.35	neg.
NC7	22	F	+		12.8	<0.35	–
NC8	64	F	+		134	<0.35	–
NC9	53	F	+		2.22	<0.35	–
NC10	60	F	+		6.6	<0.35	–
NC11	37	M	+		<2.0	<0.35	–
NC12	31	M	+		7.9	<0.35	–
NC13	39	F	+	wheat, pollen	746	<0.35	–

proliferation (α - and κ -casein 600 pg/ml; β -casein 300 pg/ml; β -LG 1,200 pg/ml; Pyrogent Plus LAL gel-clot assay kit, Bio-Whittaker, Walkersville, Md., USA). Therefore, the proteins were purified on Detoxigel columns (Pierce, Rockford, Ill., USA) and sequentially tested to contain no less than the limit of quantification for the endotoxin assay (<12 pg/ml) at the concentrations of antigen used for the cell cultures (50 μ g/ml).

Preparation of PBMC

PBMC were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) and resuspended in RPMI-1640 medium (Bio-Whittaker, Verviers, Belgium), supplemented with 10% heat-inactivated, pre-tested human AB serum pool from 8 healthy donors, with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin (all Bio-Whittaker, Verviers,

Belgium) and 50 μ M mono-thioglycerol (Sigma-Aldrich). PBMC (viability >90% by Trypan blue exclusion) were plated at $0.5-1 \times 10^6$ /ml in 100 μ l medium in round-bottomed cell culture plates (TPP, Trasadingen, Switzerland). Antigens in 50 μ l medium were added in triplicate: α -, β - and κ -casein or β -LG (50 μ g/ml). The recall antigen, tetanus toxoid (10 μ g/ml; Statens Serum Institut, Copenhagen, Denmark) and IgG/endotoxin-free BSA (10 μ g/ml; Sigma, St. Louis, Mo., USA) or medium only were used as positive and negative controls, respectively, since BSA has no LPT effect [8]. Supernatants were harvested after 24 h and frozen at -18°C . Fresh medium, containing IL-2 (25 IU/ml; R&D Systems, Minneapolis, Minn., USA), was added to all wells on days 2 and 5. Additional negative control replicates were incubated without IL-2 to estimate background proliferation.

Preparation of CD45RA- Fractions and Depletion of CD25+ Cells

PBMC from the IgE-mediated ($n = 14$) and delayed ($n = 9$) CMA groups and all controls ($n = 10$) were plated at $2-2.5 \times 10^5$ /well and incubated for 1.5–2 h to allow adherent cells to attach. Non-adherent cells were then harvested by washing with pre-warmed RPMI-1640/5% human AB serum. The adherent cells were incubated overnight with antigen, as described for bulk PBMC. The non-adherent cells were depleted of CD45RA+ cells, using Pan Mouse IgG Dynabeads (Dynal, Oslo, Norway) coated with anti-human CD45RA monoclonal antibody (clone 4KB5, DakoCytomation, Glostrup, Denmark), according to the manufacturer's instructions. They were then depleted of CD25+ cells, using CD25 Dynabeads. The purity of the fractions following removal of CD45RA+ and CD25+ cells (>95% depleted) was confirmed with flow cytometry, using antibodies against CD3, CD4, CD25 (Diatec, Oslo, Norway) and CD45RA (DakoCytomation) (data not shown). The CD25-depleted CD45RA- cells were resuspended in medium and added to the antigen-loaded adherent cells, in triplicate for each antigen. Supernatants harvested after 24 h were frozen at -18°C . Fresh medium, containing IL-2 (25 IU/ml; R&D Systems), was added to all wells on days 2 and 5 to supplement low levels of IL-2 produced in CD25-depleted memory T cells.

PI in PBMC and CD25-, CD45RA-Depleted Memory T Cells

The PBMC and the CD25-, CD45RA-depleted memory T cells were pulsed on day 6–7 with 1 μ Ci methyl- ^3H -thymidine (Amersham Pharmacia Biotech, Little Chalfont, UK) in 20 μ l of medium. The cells were harvested after 16–18 h using a Filtermate 196 harvester, and incorporated cellular radioactivity was measured using Microscint O scintillation fluid with a TopCount microplate scintillation counter (all Packard Instrument Company, Meriden, Colo., USA). The LPTs were carried out on separate occasions, and therefore, the CPM data could not be directly compared, due to differences in specific activity of the ^3H -thymidine. For this reason, the PI was used. Mean CPM was calculated for each antigen and corrected for mean background. The PI was calculated as follows: mean stimulated CPM/mean unstimulated CPM. In order to assess the variability of the triplicates, the individual stimulated/unstimulated ratios ($n = 9$) were also calculated for each antigen, and the mean PI and mean SEM for the individual ratios were estimated. The SEM of the PI for α -, β - and κ -caseins was 7.8% (range 1.6–23) and for β -LG 5.7% (range 3.0–8.7) in reactive IgE-mediated CMA patients. In tolerised IgE-me-

diated CMA patients, the SEM for α -, β - and κ -caseins was 8.1% (range 2.1–14) and for β -LG 6.7% (range 3.9–9.5). The corresponding SEMs for reactive non-IgE-mediated CMA patients were 8.8% (range 3.0–13.6) for α -, β - and κ -caseins and 4.9% (range 2.7–7.3) for β -LG. Tolerised non-IgE-mediated CMA patients had an SEM of 9.6% (range 1.9–38.1) for α -, β - and κ -caseins and 9.4% (range 0.1–24.4) for β -LG. The SEMs for controls were 6.4% (range 1.1–15.4) for α -, β - and κ -caseins and 8.1% (range 2.3–13.6) for β -LG. However, these SEM values could not be satisfactorily illustrated due to their small size compared with the symbols used in figure 1.

Cytokine Analyses

IL-4, IL-10 and interferon (IFN)- γ activities were assayed in the frozen 24-hour supernatants, using DuoSet ELISA development kits (R&D Systems), in accordance with the manufacturer's instructions.

Statistical Analysis

The Shapiro-Wilk W test showed non-normally distributed proliferative indices; thus, non-parametric tests were applied. All inter-group comparisons and comparisons of depleted versus non-depleted PI ratios were made using the Wilcoxon rank sum (Mann-Whitney U) test. Adjustment of statistical significance for multiple observations according to Bonferroni may be too stringent in biomedical research when comparing less than 20 values [19] and was therefore not implemented. Statistical calculations were performed using the JMP-5.0.1 software package (SAS Inst., Cary, N.C., USA).

Results

Casein-Induced Proliferation in PBMC Was Greater in Clinically Reactive IgE-Mediated CMA Patients than in Controls

As a group, the IgE-mediated CMA patients ($n = 15$) had a significantly higher median PI to α -casein (1.5; $p = 0.02$) and β -casein (1.7; $p = 0.02$) than controls (1.1 and 1.2, respectively). Splitting this group into clinically reactive and tolerised patients revealed that only the clinically reactive patients ($n = 9$) had a significantly higher median PI to α -casein (1.5; $p < 0.01$) and β -casein (1.8; $p < 0.01$) compared with controls (fig. 1). In addition, these patients had a significantly greater PI to κ -casein (1.6; $p = 0.03$) than tolerised patients ($n = 6$; 0.7) (fig. 1). Tolerised patients did not differ from controls, though individual patients had positive responses.

PBMC from Clinically Reactive IgE-Mediated CMA Patients with or without Atopic Dermatitis Responded Differently to Casein Subfractions

Dividing the IgE-mediated CMA patient group into those with or without CMP-sensitive atopic dermatitis

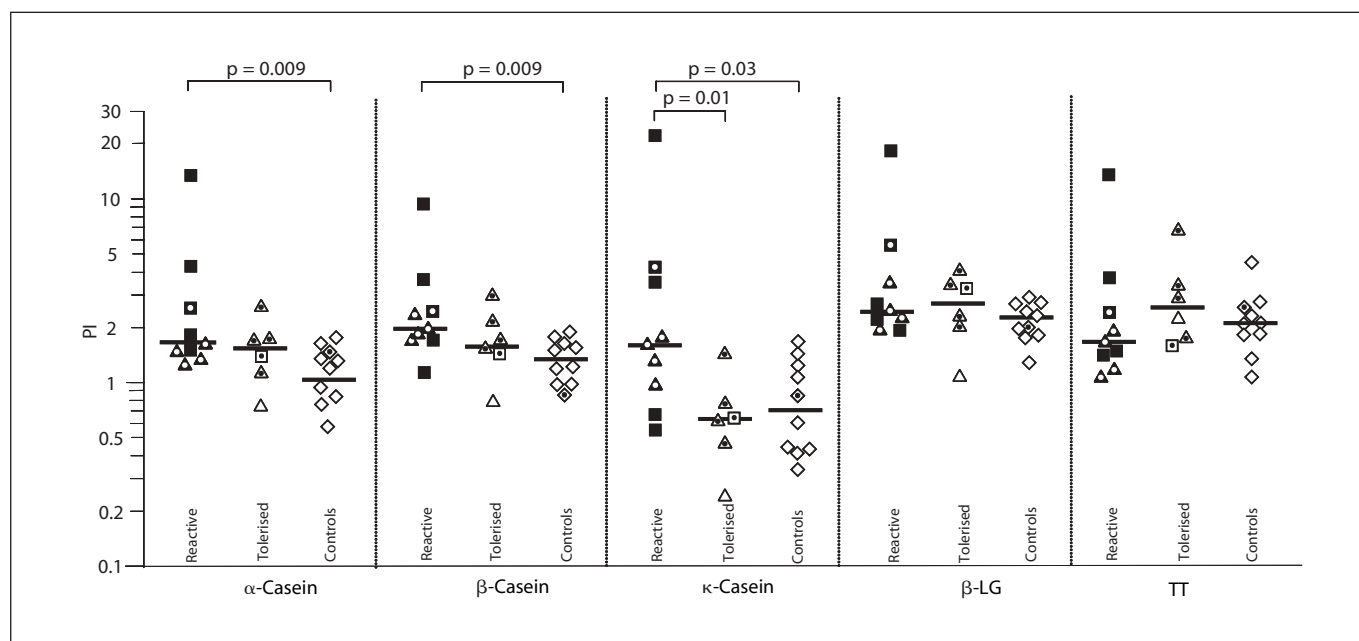


Fig. 1. The PI in response to α -, β - and κ -casein, β -LG and tetanus toxoid (TT) in bulk PBMC from IgE-mediated CMA patients. PBMC were stimulated in vitro with LPS-depleted α -, β -, κ -casein, β -LG (50 μ g/ml) or TT (10 μ g/ml). The PI was defined as ratio of stimulated/unstimulated cells. ■ = IgE-mediated CMA without atopic dermatitis; ▲ = IgE-mediated CMA with atopic dermatitis; ◇ = healthy controls. Reactive patients are represented by closed symbols, tolerised patients by open symbols. Patients reactive to other allergens than cow's milk are marked with a dot within the symbol. Medians are indicated by bars. PIs were compared between patient groups and controls using the Wilcoxon rank sum test. $p < 0.05$ was considered significant. The background proliferation of unstimulated cells was 40.7 cpm. Note that the PI is shown using a logarithmic scale.

revealed that the median PI to κ -casein in clinically reactive milk-sensitive atopic dermatitis patients (1.5, range 1.0–1.7) was significantly greater ($p = 0.05$) than the PI in tolerised, previously CMP-sensitive atopic dermatitis patients (0.7, range 0.2–1.5). The median PI to α -casein in the reactive milk-sensitive atopic dermatitis patients (1.3, range 1.1–1.5) was significantly lower ($p < 0.05$) than in clinically reactive IgE-mediated CMA patients without atopic dermatitis (2.6, range 1.4–13.4).

PBMC from Delayed Non-IgE-Mediated CMA Patients Responded to Caseins

As a group, delayed non-IgE-mediated CMA patients ($n = 13$) responded significantly more often to one or more casein subfractions than controls (46%; $p < 0.05$). However, both clinically reactive ($n = 5$) and tolerised ($n = 8$) patients responded to caseins at similar frequencies (40 and 50%, respectively). There were no significant differences in median PI to α -, β - and κ -casein between non-IgE-mediated patients and controls (data not shown).

Although clinically reactive patients had a slightly increased median PI to α -, β - and κ -casein, it only approached significance for α -casein ($p = 0.06$).

Both Patients and Controls Had Proliferative Responses to β -LG

There were no significant differences between frequency of proliferative responses to β -LG in CMA patients (71% of the patients) and controls (60% of controls) when $PI > 2$ was used to define positive responses. Increasing the cut-off PI to 2.7 excluded 95% of the controls, but there was no significant difference in responder frequency at the higher cut-off, with 39% of the patients having positive proliferative responses compared with 5% of the controls. The median PI in the IgE-mediated CMA patients was 2.4 (range 1.0–19.0) and in non-IgE-mediated CMA 2.3 (range 1.1–10.2), which did not differ significantly from the controls (2.1, range 1.3–2.8) (fig. 1).

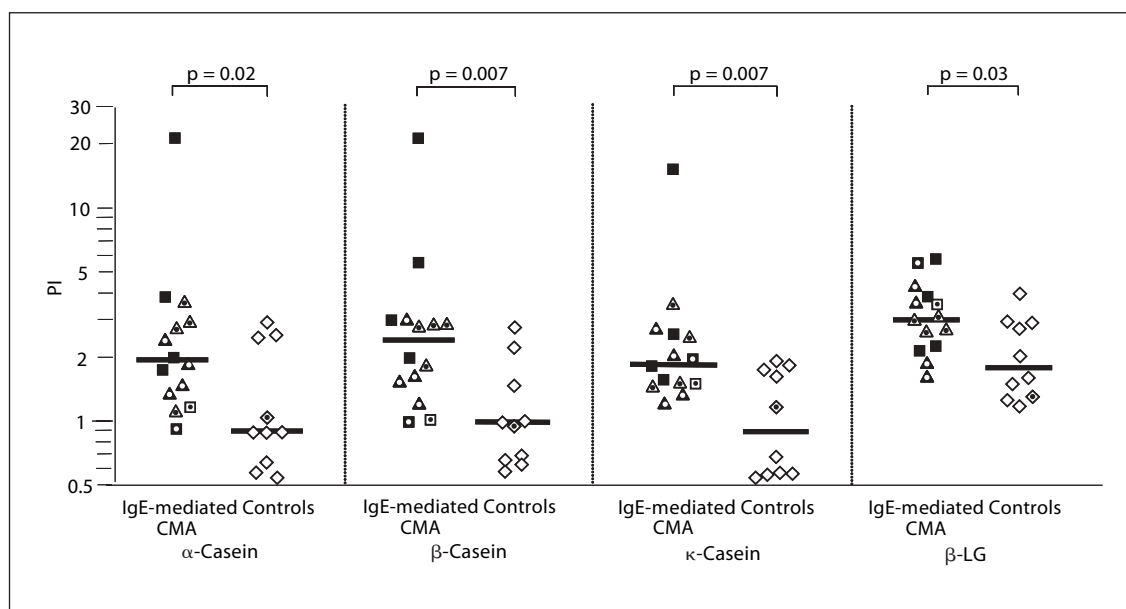


Fig. 2. The PI in response to α -, β - and κ -casein and β -LG in CD45RA-, CD25-depleted PBMC from IgE-mediated CMA patients. CD45RA-, CD25-depleted PBMC were stimulated in vitro with LPS-depleted α -, β - or κ -casein or β -LG (50 μ g/ml). The PI was defined as ratio of stimulated/unstimulated cells. ■ = IgE-mediated CMA without atopic dermatitis; ▲ = IgE-mediated CMA with atopic dermatitis; ◇ = healthy controls. Reactive patients are represented by closed symbols, tolerated patients by open symbols. Patients reactive to other allergens than cow's milk are marked with a dot within the symbol. Medians are indicated by bars. The PIs were compared between patient groups and controls using the Wilcoxon rank sum test. $p < 0.05$ was considered significant. The background proliferation of unstimulated cells was 48.4 cpm. Note that the PI is shown using a logarithmic scale.

CD45RA and CD25 Depletion Increased Proliferation in IgE-Mediated CMA Patients Only

More IgE-mediated CMA patients responded to all the CMPs after the CD45RA+ and CD25+ cells had been removed from PBMC. Also, controls had increased PIs to α - and β -caseins after CD45RA+ and CD25+ cell removal. The median PIs for α -casein ($p = 0.02$), β -casein ($p = 0.007$), κ -casein ($p = 0.007$) and β -LG ($p = 0.03$) were all significantly increased in CD45RA-, CD25-depleted PBMC from IgE-mediated CMA patients compared with controls (fig. 2). Both clinically reactive and tolerated patients had a higher median PI to α -, β - and κ -casein compared with controls, but there were no differences between clinically reactive and tolerated patients. Only clinically reactive patients had a higher PI to β -LG compared with controls.

Tolerated IgE-mediated CMA patients had a significantly increased depleted/bulk PBMC PI ratio to κ -casein ($p = 0.02$) only. Matched-pairs data from the same patients revealed a similar trend in that CD45RA, CD-25

depletion increased κ -casein-specific PI in tolerated IgE-mediated patients ($p = 0.04$) (fig. 3).

Removal of CD45RA+, CD25+ cells did not influence the proliferative response to any CMP in non-IgE-mediated CMA patients. There were no differences between these non-IgE-mediated CMA patients and controls, or between reactive non-IgE-mediated CMA and tolerated patients (data not shown).

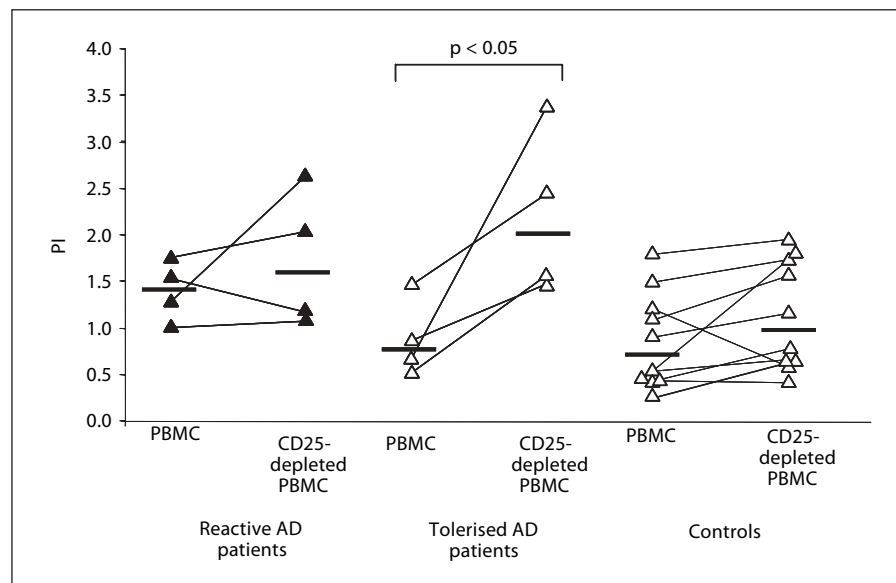
Proliferative Responses to LPS-Depleted BSA

Only 1 highly clinically reactive CMA patient had a very mild reaction to BSA (PI 2.07). Proliferative responses to the recall antigen, tetanus toxoid, were normally low (median PI 2.13, range 1.05–13.84) and not significantly different in any of the groups (fig. 1).

Cytokines in Culture Supernatant

IFN- γ was detected in supernatants from 1 tolerant IgE-mediated CMA patient after α - or β -casein stimulation and from 1 non-IgE-mediated CMA patient after

Fig. 3. Paired data for proliferative activity in response to κ -casein in bulk PBMC and CD45RA-, CD25-depleted PBMC from IgE-mediated CMA patients with atopic dermatitis (AD) and healthy controls, expressed as PI (ratio of stimulated/unstimulated cells). Clinically reactive patients are shown as closed symbols, tolerised patients and controls as open symbols. Medians are indicated by bars. The PI in CD25-, CD45RA-depleted PBMC was significantly increased compared with PBMC in tolerised patients only (Wilcoxon rank sum test, $p < 0.05$).



κ -casein stimulation. Levels in the remaining samples were below quantification limits in all samples (15.6 pg/ml). Both the IL-4 and IL-10 levels were below quantification limits in all samples (31.3 and 46.9 pg/ml, respectively).

Discussion

The present study confirmed that clinically reactive IgE-mediated CMA patients have increased α -, β - and κ -casein-induced PBMC proliferation [17, 18, 20]. Moreover, the significantly increased PI after removal of CD45RA+ naïve T cells and CD25+ Tregs suggested a predominantly CMP-specific memory T cell proliferation. In addition, tolerised CMP-sensitive atopic dermatitis patients had a remarkably low PI to κ -casein, suggesting a particular role for κ -casein, in agreement with Werfel et al. [8], who generated κ -casein-specific T cell clones from such patients. Although these patients also responded to the other casein fractions [13], the distinct decreased lymphocyte proliferation in the tolerised atopic dermatitis patients suggested involvement of κ -casein-inducible Tregs in tolerance development. This was further supported by the significantly increased κ -casein-specific proliferation after Treg (CD25+ cell) removal in tolerised patients. The κ -casein-specific proliferation was also higher in the controls after Treg depletion. This suggested that κ -casein-inducible Tregs are functional in

controls who, by definition, are tolerant to cow's milk. In contrast, IgE-mediated CMA patients without atopic dermatitis had a somewhat more prominent reactivity to α -casein.

Although the number of patients in each subgroup and the PIs were relatively small, nevertheless, the sample size-sensitive non-parametric statistics suggested that T-cell-associated reactivity to casein subfractions may be differently involved in mediating certain CMA symptoms. Although Shek et al. [17] did not observe any differences in PBMC proliferation in response to α -, β - and κ -casein and β -LG between IgE-mediated CMA, milk-induced enterocolitis or eosinophilic gastroenteritis patients with or without urticaria or atopic dermatitis, the results may have been masked by the use of putative LPS-contaminated CMP. As little as 10 pg LPS/ml may induce lymphocyte proliferation [9]. If one uses CMP preparations with similar LPS contamination levels as detected in the study by Werfel et al. [18] as well as in the present study, the cell cultures would contain 2–200 times that amount, as previous studies have used 10–600 μ g β -LG/ml [6, 10, 12, 21], 10–1,000 μ g total casein/ml [6, 10, 12, 21] and 10–500 μ g casein subfractions/ml [12, 20]. Therefore, in these studies, the CMP-induced proliferation may have been influenced by variable amounts of LPS contamination. It has further been demonstrated that even LPS-depleted casein in excess of 200 μ g/ml may induce non-specific PBMC proliferation in healthy subjects [8]. Thus, the moderate proliferative responses seen in the

present study may reflect both the depletion of LPS and the lack of dose-dependent casein-induced mitogenic stimulation. Although other innate immune response-stimulating agents may be involved, further studies would be required to elucidate their potential effects.

The β -LG-induced proliferation did not differ significantly between clinically reactive or tolerised patients and healthy controls in either CMA group, or between reactive and tolerised patients. Although proliferative responses may be influenced by LPS contamination [7], the present study showed that even LPS-depleted β -LG induced proliferative responses in the majority of patients and controls, presumably due to its mitogenic potential [22]. Apparently, this was not a non-specific phenomenon, because no increased proliferation was observed in response to tetanus toxoid or, in disagreement with Kondo et al. [23], to LPS-free BSA.

LPS may also activate Tregs via the Toll-like receptor 4, which increases their suppressor activity by 10-fold [24]. Therefore, using LPS-contaminated CMP may generate rather unpredictable results, as both LPS-induced and CMP-specific memory T cell proliferation may be counteracted by both LPS-stimulated and putative antigen-stimulated Tregs [25]. When putative CD25⁺ Tregs were depleted from PBMC, β -LG-induced proliferative 'memory' T cell responses (CD45RA depleted) increased in clinically reactive IgE-mediated CMA patients only. This may reflect a greater level of T cell reactivity in these reactive patients.

Reactive non-IgE-mediated CMA patients did not have increased proliferation, which is in agreement with a recent study of non-IgE-mediated CMA with gastrointestinal symptoms [25]. However, the β -LG-induced proliferative activity following CD25 depletion was higher in tolerised than in clinically reactive non-IgE-mediated CMA patients 1 week after challenge. The authors speculated that small populations of circulating antigen-specific memory T cells and Tregs reflected a milk-free diet prior to challenge [25]. Antigen-specific memory T cells appear to enter the circulation on antigen avoidance and re-enter the affected tissues upon antigen challenge, as shown for atopic dermatitis patients [26]. However, the frequency of such antigen-specific memory T cells in peripheral blood is presumably rather low [27]. Following challenge and mucosal T cell expansion, activated T cells would later appear in the circulation. This might explain why Treg (CD25⁺) depletion in the present study did not increase either the casein- or the β -LG-induced proliferation in the non-IgE-mediated CMA patients as all were on a milk-free diet. Ethical consent was only given

for blood samples to be taken prior to challenge. Therefore, the expected post-challenge increase could not be investigated, although initially included in the study design.

Moreover, detection of both antigen-specific T cells and Treg functions in the peripheral blood may be limited by regionalisation of CMP-specific T cells to the gastrointestinal tract. This has partly been substantiated by detection of β -LG-induced Treg function in lamina propria lymphocytes which suppressed unstimulated lamina propria lymphocyte proliferative responses in an antigen non-specific manner [22].

Although reactive and tolerised IgE-mediated CMA patient groups had significantly increased median proliferation in response to caseins after Treg removal (CD25⁺), suggesting abrogation of Treg inhibition, paired data showed that not all individuals responded. Clinical allergy may increasingly be counteracted by the Treg system during tolerance induction. Therefore, the increased PI in clinically reactive patients after Treg removal may have reflected an ongoing tolerance induction similar to that reported in adult patients [28].

In general, the non-IgE-mediated CMA patients had a lower proliferative response to caseins than the IgE-mediated CMA patients and, more important, there were no significant differences between patients and controls to any of the LPS-depleted CMP tested. This is in agreement with Beyer et al. [20], but in contrast to Heyman et al. [29]. In fact, the present study observed more proliferative responders in tolerised than in clinically reactive patients, which contradicts the conclusion of Heyman et al. [29] that PBMC are activated during the clinically reactive phase but become insensitive on recovery. The present data also disagree with Suomalainen et al. [30], who reported significantly higher PI to total CMP in non-IgE-mediated CMA patients with gastrointestinal symptoms than in IgE-mediated CMA patients or non-reactive patients. However, the latter study used a high dosage (1,000 μ g/ml) of putatively LPS-contaminated milk powder, which must have influenced the results [7].

The lack of detectable IL-4 and IFN- γ in almost all cell cultures presumably reflected the low numbers of Ag-specific responding T cells, rather than a lack of IL-4/IFN- γ production. Moreover, most lymphocytes express IL-4 and INF- γ receptors which may have consumed any IL-4/IFN- γ produced during the 24-hour culture. Unfortunately, additional time points or cocultures were not feasible due to the ethical constraint on sample size (5 ml blood).

In contrast to the IgE-mediated CMA patients, there was no evidence for casein- or β -LG-specific T cell reactivity in CMA patients with delayed gastrointestinal symptoms, not even after Treg removal. Thus, PBMC from clinically tolerated IgE-mediated CMA patients, in particular those with CMP-sensitive atopic dermatitis, had significantly decreased proliferative responses to LPS-depleted κ -casein, which were ameliorated after Treg removal. Together, this suggested a possible role for

κ -casein in CMP-sensitive atopic dermatitis. Although in general, casein-induced Tregs appeared to be involved in tolerance development in the IgE-mediated CMA patient group, β -LG-induced Tregs were not.

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