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Alterations of lymphocyte subpopulations and TGF-β in children with transient or persistent cow's milk allergy

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ABSTRACT
The aim of the study was to evaluate changes in surface receptor expression of B and T lymphocytes and concentration of TGF-β in children who either developed tolerance to cow's milk protein (CMP) or manifested persistent cow's milk allergy (CMA). The study involved 30 patients with CMA who underwent an open food challenge after 12 months of milk-free diet. After the milk challenge, decreased concentration of $CD19^{+}CD23+$ was observed in children who acquired tolerance to CMP, in comparison with the test before cow's milk (CM) challenge (42.2% vs. 29.1%, $p = .006$). The same group demonstrated lower concentration of TGF-β than patients with persistent allergy (median 37.9 pg/ml vs. 52.8 pg/ml, $p = .003$, respectively). Moreover, before CM challenge, higher percentage of CD3+CD8+CD28+CD152+ cells (median 2.88% vs. 1.2%, $p = .03$) and $CD3+CD4^{+}CD25^{+}CD62L^{+}$ (median 42.3% vs. 13.4%, $p = .032$) was noted in children who acquired tolerance to CMP, in comparison with subjects who remained allergic to CMP.

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Cow's milk allergy; food tolerance; lymphocyte subpopulations; TGF-β

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Introduction

The ability to maintain the internal integrity and adapt to the environment is a phenomenon called immune tolerance (Weiner, 2001). Food tolerance is a part of peripheral tolerance which averts an excessive immune response towards food allergens or microflora of the gastrointestinal (GI) tract (Hughes, 1999). The type of immune response depends on the dose of antigen and the frequency of its administration. High antigen doses promote the development of tolerance by deletion or clonal anergy, whereas low antigen doses usually induce active suppression involving regulatory T lymphocytes (Treg) and immunosuppressive cytokines produced by interleukin-10 (IL-10) and TGF-β (van Wijk & Knippels, 2007).

Cow's milk allergy (CMA) is one of the most common food allergies observed in childhood (Sicherer, 2011). However, the majority of infants with CMA spontaneously develop

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tolerance to cow's milk proteins (CMP) by school age (Savilahti & Savilahti, 2013; Wood et al., 2013). Little is known about the potential existence of a peripheral regulatory mechanism modulating the development of tolerance to CMP. Previously published reports have demonstrated that cow's milk (CM)-specific T cells isolated from children with CMA display a Th2-skewed phenotype (Savilahti et al., 2010). It has been suggested that tolerance to cow's milk allergens is associated with the suppression of the activity of proallergic innate effectors including tissue mast cells, basophils and eosinophils (Jo, Garssen, Knippels, & Sandalova, 2014). Children who outgrew their allergy to CMP had higher levels of circulating $CD4^{+}CD25^{+}$ regulatory T cells and decreased proliferative responses *in vitro* to bovine-lactoglobulin in peripheral blood mononuclear cells compared to children with persistent CMA (Karlsson, Rugtveit, & Brandtzaeg, 2004). In addition, reduction of TGF-β-producing T cells has been observed in the duodenal mucosa of children with food allergy as compared to non-allergic subjects (Pérez-Machado et al., 2003). Low concentrations of specific IgE to CM resulted in tolerance achievement more frequently than high levels (Wood et al., 2013). Other studies have suggested that Th1 responses underlie the immunological mechanism of food allergy resolution (Savilahti & Savilahti, 2013).

The aim of the study was to evaluate changes in the expression of surface receptors of peripheral blood lymphocyte populations (T and B) and the serum concentration of TGF-β in children with CMA who either developed tolerance to cow's milk or manifested persistent allergy. In regard to T cells, we tested the concentrations of T-helper/inducer ($CD3^{+}CD4^{+}$) cells, T-cytotoxic/suppressor lymphocytes ($CD3^{+}CD8^{+}$) cells, regulatory T cells ($CD3^{+}CD4^{+}CD25^{high}$), T cells expressing the receptor of activation ($CD3^{+}CD28^{+}$) and/or inhibitory receptor ($CD3^{+}CD152^{+}$/CTLA-4) and regulatory T cells expressing the central memory receptor ($CD3^{+}CD4^{+}CD25^{+}CD62L^{+}$). We also analysed subpopulations of B cells expressing $CD19^{+}$, $CD19^{+}CD23^{+}$, $CD19^{+}CD40^{+}$ markers. The secondary aim of our study was to find the immunological predictor of CMP tolerance development in children.

Methods

Patient selection

The study was performed in The Department of Pediatrics, Gastroenterology and Allergology, Medical University of Bialystok in the years 2010–14. The study design is presented in Figure 1 of Supplementary Material. The investigation involved 30 patients with CMA (18 females/12 males), aged 1–13 years at the beginning of the study. Criteria for CMA diagnosis included clinical manifestations, a positive oral cow's milk challenge test result, a positive skin prick test result and positive cow's milk-specific IgE in serum. Exclusion criteria included a history of an anaphylactic reaction, an allergy to inhalant allergens, known immunodeficiency syndromes and infections of the GI tract. The control group consisted of 20 children (9 females/11 males), aged 2–13 years, without CMA confirmed with sIgE tests. A cow's milk-free diet was introduced in all subjects with CMA 12 months prior to study commencement. A CM open challenge test was performed at the hospital following the recommended protocol during the first appointment (Nowak-Wegrzyn et al., 2009). After a 6-week follow-

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up period, the patients were reassessed during the second appointment. Subjects who manifested allergy symptoms during the CM challenge were included in the group of persistent CMA, while those who did not display allergy symptoms were assigned to the group with CMP tolerance.

The study protocol was approved by the Bioethics Committee of the Medical University in Bialystok. Parental written consent for the children's participation in the follow-up phase of the study was obtained prior to study enrolment. The initial evaluation included an interview with the child's parents, clinical examination and blood sampling. A questionnaire regarding the presence of allergic diseases (i.e. asthma, atopic dermatitis – AD or allergic rhinitis – AR) was completed.

Blood sample collection and blood cell count

Blood samples were collected before the CM challenge (first appointment) and 6 weeks after the CM challenge (second appointment). The samples were obtained by venipuncture, under standard conditions, after a 12-hour overnight fast. The samples of 2 ml whole blood were obtained in EDTA and processed within 4 hours of collection.

Flow cytometry

All samples were processed using the ImmunoPrep whole blood lysis system (ImmunoPrep Work Station, Beckman Coulter). Briefly, 100 µl of whole blood was stained with 10 µl of the following antibodies: anti-CD3 (phycoerythrin-cyanin 5 PECy5-conjugated, UCHT1 clone), anti-CD4 (phycoerythrin-cyanin 7 PECy7-conjugated, SFCI12T4D11 clone), anti-CD8 (phycoerythrin-cyanin 5 PECy5-conjugated, B9.11 clone), anti-CD25 (phycoerythrin-Texas Red ECD-conjugated, B1.49.9 clone), anti-CD69 (phycoerythrin-conjugated, TP1.55.3 clone), anti-CD62L (fluorescein isothiocyanate-conjugated, DREG56 clone), anti-CD28 (fluorescein isothiocyanate, CD28.2 clone), anti-CD152 (phycoerythrin-conjugated, BMI3 clone), anti-CD19 (phycoerythrin-cyanin 5.1-conjugated, clone J3-119), anti-CD23 (phycoerythrin-conjugated, clone 9P25) purchased from Beckman Coulter (Brea, CA, USA), Beckton Dickinson (San Jose, CA, USA) and eBioscience (San Diego, CA, USA). Respective isotype control antibodies were used. The samples were analysed using the five-colour flow cytometer Beckman Cytomics FC 500 MPL (Beckman Coulter). A minimum of 10^5 events were acquired for each analysis.

Measurement of serum TGF-β

TGF-β concentrations were determined by ELISA kit (R&D Systems) according to the manufacturer's instructions. Concentration values were presented in pg/ml.

Statistical analysis

Data were analysed using the Statistica10.0 software (StatSoft Inc., USA). The significance of difference was evaluated by the Mann–Whitney *U*-test and $p < .05$ was considered as statistically significant. Cut-off levels, specificity and sensitivity were calculated using the receiver operating characteristic (ROC) analysis.

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Results

Background of study group

Table 1 contains the demographic and clinical characteristics of the study groups at the time of CMA diagnosis. There were no significant differences between the CMA and the control group regarding age and sex. Among the children with CMA, 10 patients with a median age of 2 years (7 females/3 males) demonstrated skin lesions (AD), 10 children with a median age of 4.5 years (5 females/5 males) manifested respiratory tract symptoms (recurrent bronchitis, recurrent rhinitis) and 10 children with a median age of 3.7 years (6 females/4 males) reported GI tract symptoms. No association between the CM tolerance acquisition rate and clinical manifestations of CMA was established (Table 2).

A significantly greater number of patients demonstrated IgE-mediated CMA ($p = .039$). The CM open challenge test result was positive in 13 (43.4%) patients, who re-developed symptoms. The test result was more likely to be positive in the subjects with IgE-mediated CMA (69.2%, $p = .049$) and high-specific IgE levels (4.85–81.2 kU_A/L, class 3–5, respectively) (Table 2). Out of the 10 children with a previous, IgE-mediated allergy who developed tolerance to CMP, the level of specific IgE ranged from 0.46 to 1.19 kU_A/L (class 1–2). No patient presented with severe manifestations such as an anaphylactic shock during the CM open challenge.

Differences in lymphocyte subpopulations in children with persistent CMA, tolerant to CM and controls

In all the study participants, different subpopulations of T ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^+CD28^+$, $CD3^+CD4^+CD152^+$, $CD3^+CD8^+CD28^+$, $CD3^+CD8^+CD152^+$, $CD3^+CD4^+CD25^+CD62L^+$, $CD3^+CD4^+CD25^{high}$, $CD3^+CD4^+CD69^+$, $CD3^+CD8^+CD69^+$) and B ($CD19^+$, $CD19^+CD23^+$, $CD19^+CD40^+$) cells in blood were tested prior to and following the CM challenge. Following the CM challenge, a decrease in the percentage of $CD19^+$ cells co-expressing $CD23^+$, the "low-affinity" receptor for IgE, was observed both in the children with a persistent CMA and those tolerant to CMP. However, a significant post-CM challenge decline was observed in the children who developed tolerance to CMP (42.2% vs. 29.1%, $p = .006$) (Figure 1(A)). Regarding T cell subpopulations, significant differences in $CD3^+CD4^+CD28^+CD152^+$, $CD3^+CD4^+CD28^+$, $CD3^+CD8^+CD28^+CD152^+$ and $CD3^+CD4^+CD25^+CD62L^+$ were noted between the study groups (Figure 1,

Table 1. Background data of paediatric patients with cow's milk allergy (CMA) and control group.

	CMA	Control
Total number	30	20
Girls/boys *N* (%)	18 (60)/12 (40)	9 (45)/11 (55)
Age median years (range)	3.5 (1–13 years)	5 (2–13 years)
CMA *IgE-mediated N* (%)	19 (63.3)	–
CMA *no IgE-mediated N* (%)	11 (36.7)	–
Atopic dermatitis *N* (%)	10 (33.3)	–
Age median years (range)	2 (1–4)	–
Symptoms of respiratory tract *N* (%)	10 (33.3)	–
Age median years (range)	4.5 (2–13)	–
Symptoms from digestive tract *N* (%)	10 (33.3)	–
Age median years (range)	3.75	–

Table 2. Results of the open challenge test with cow's milk (second visit).

	Open challenge test		
CM groups	Negative (CM tolerance), *N* (%)	Positive (CM allergy), *N* (%)	*p*
Total number (%)	17 (56.7)	13 (43.4)	NS
CMA *IgE-mediated N* (%)	10 (58.8)	9 (69.2)	NS
IgE spec. class 1	7 (70%)	0	NS
IgE spec. class 2	3 (30%)	0	NS
IgE spec. class 3	0	6 (66.7)	NS
IgE spec. class 4	0	2 (22.2)	NS
IgE spec. class 5	0	1 (11.1)	NS
CMA *no IgE-mediated N* (%)	7 (41.2)	4 (30.8)	NS
Atopic dermatitis	5 (29.4)	5 (38.5)	NS
Symptoms of respiratory tract	7 (41.2)	3 (23.1)	NS
Symptoms from digestive tract	5 (29.4)	5 (38.5)	NS

Table 3). Only the children with a persistent CMA demonstrated a higher percentage of $CD3^+CD4^+CD28^+CD152^+$T cells before the CM challenge than after this procedure (median 3.1% vs. 1.04%, $p = .038$) (Figure 1B). The same group demonstrated a significantly lower percentage of $CD3^+CD4^+CD28^+$cells, both prior to and following the CM challenge, when compared to the controls (88.8% vs. 94.9%, $p = .01$; and 87.2% vs. 94.9%, $p = .03$, respectively) (Table 3). In turn, children who became tolerant to CMP had a higher percentage of $CD3^+CD8^+CD28^+CD152^+$ cells (median 2.88% vs. 1.2%, $p = .03$; Figure 1(C)) and $CD3^+CD4^+CD25^+CD62L^+$, central memory T cells (median

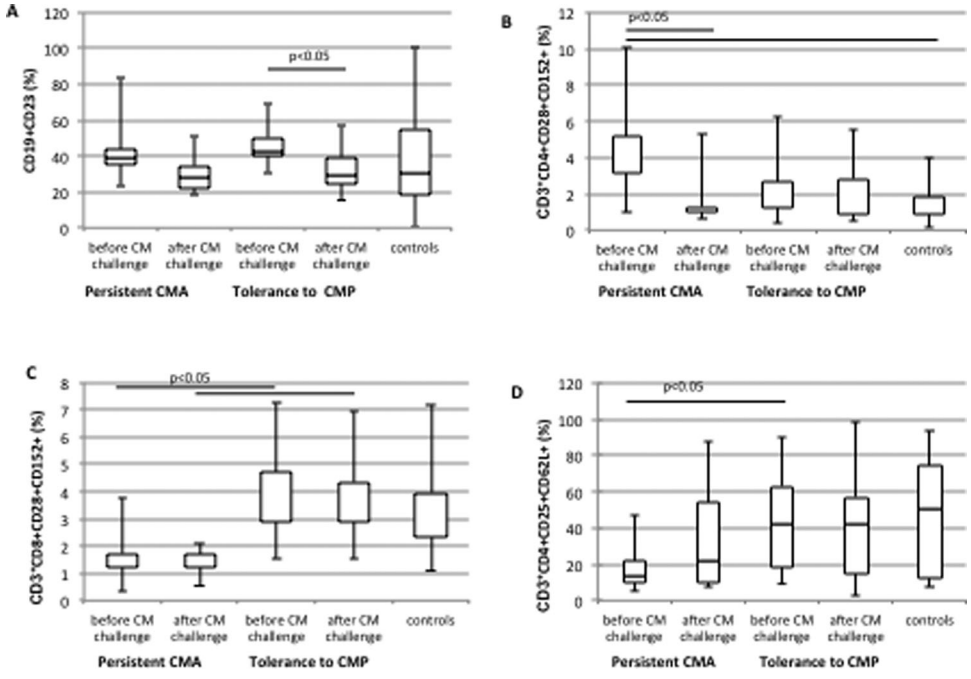

Figure 1. Differences in the expression level of $CD19^+CD23^+$ (A), $CD3^+CD4^+CD28^+CD152^+$ (B), $CD3^+CD8^+CD28^+CD152^+$ (C) and $CD3^+CD4^+CD25^+CD62L^+$ (D) cells in blood obtained from children with a persistent cow's milk allergy (CMA), those who acquired tolerance to cow's milk protein (CMP) and controls before and after CM challenge test.

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Table 3. Serum TGF-β and peripheral blood lymphocyte subsets of children with persistent cow's milk allergy (CMA) and controls.

	Control, median (25th–75th percentiles)	Persistent CMA, median (25th–75th percentiles)			
		Before CM challenge	*p* compared to control	After CM challenge	*p* compared to control
WBC × 10^3/μl	6.6 (5.7–7.5)	6.9 (5.9–8.5)	*.298*	6.1 (5.6–7.3)	*.763*
Lymphocyte (%)	46.5 (36.9–56.4)	41.7 (40.4–56.1)	*.898*	39.1 (30.8–52.8)	*.728*
CD3 (%)	68.2 (64.1–75.1)	69.5 (65.0–70.6)	*.898*	70.1 (65.7–73.4)	*.640*
CD4 (%)	38.5 (34.1––45.1)	35.4 (21.7–42.8)	*.181*	37.6 (33.1–48.4)	*.919*
CD8 (%)	23.5 (19.3–27.2)	21.6 (19.1–23.0)	*.316*	18.7 (18.1–21.8)	*.169*
CD19 (%)	16.7 (12.5–19.5)	19.2 (14.6–22.8)	*.298*	16.2 (14–19.2)	*.729*
NK (%)	8.6 (4.8–11.2)	10.6 (5.4–13.9)	*.334*	8.7 (5.4–13.9)	*.582*
$CD4^+CD69^+$ (%)	0.2 (0.07–0.5)	0.1 (0.05–0.1)	*.85*	0.5 (0.3–1.2)	*.198*
$CD4^+CD28^+$ (%)	94.9 (92.6–97.5)	88.8 (82.2–92.6)	*.01*	87.2 (84.4–94.2)	*.028*
$CD4^+CD152^+$ (%)	0 (0–0.4)	0.3 (0–2.3)	*.106*	0.1 (0–0.5)	*.528*
$CD8^+CD69^+$ (%)	0.7 (0–1.3)	0.8 (0.4–1.6)	*.681*	2.1 (0.4–6.2)	*.142*
$CD8^+CD28^+$ (%)	59.7 (53.5–67.6)	50.2 (41.5–57.2)	*.223*	57.9 (51.7–66.7)	*.886*
$CD8^+CD152^+$ (%)	0.4 (0–2.8)	1.6 (0–3.0)	*.326*	1.1 (0.4–1.6)	*.669*
$CD4^+CD25^{low}$ (%)	13.4 (12.3–23.9)	9.2 (8.8–15.0)	*.08*	10.4 (7.6–13.2)	*.047*
$CD4^+CD25^{high}$ (%)	3.1 (2.1–5.6)	3.7 (1.8–5.0)	*.478*	2.5 (1.3–3.9)	*.265*
$CD4^+CD62L^+$ (%)	81.9 (76.6–90.1)	80.2 (73.7–95.5)	*.713*	87.9 (73.6–93.5)	*.687*
TGF-β pg/ml	44.2 (36.5–48.3)	51.6 (42.6–54.8)	*.062*	52.8 (44.7–58.2)	*.029*

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42.3% vs. 13.4%, $p = .032$; Figure 1(D)) prior to the CM challenge in comparison with the subjects who remained allergic to CM.

No changes in the percentage of $CD3^+CD4^+CD152^+$, $CD3^+CD8^+CD152^+$ and $CD3^+CD8^+CD28^+$, $CD3^+CD4^+CD25^{high}$ and expression of the activation marker ($CD69^+$) were found in the study groups (Table 3).

Differences in TGF-β in children with persistent CMA and tolerant to CM and controls

Prior to the CM challenge, no differences in TGF-β level were found between the study groups. Following the CM challenge test, significantly higher serum concentrations of TGF-β were noted in the children with a persistent CM allergy in comparison with the subjects tolerant to CMP and the controls (median 52.8 vs. 37.9 pg/ml, $p = .003$ and 52.8 vs. 44.2 pg/ml, $p = .03$, respectively) (Figure 2, Table 3).

$CD3^+CD8^+CD28^+CD152^+$ lymphocyte subpopulation as a predictor of CMP tolerance development

An increased $CD3^+CD8^+CD28^+CD152^+$ lymphocyte subpopulation in serum prior to the CM challenge discriminated children who developed tolerance to CMP with area under the ROC curve (AUC) of 0.779 (95%CI 0.606–0.951, $p = .0015$), a cut-off level of 1.59% and a specificity of 75% (Figure 3(A)). In the children with a previous, IgE-mediated CMA, a cut-off level was 3.12% with a specificity of 88.9% (AUC of 0.827; 95%CI 0.634–1, $p = .0015$) (Figure 3(B)). Similar results were obtained for $CD3^+CD4^+CD25^+CD62L^+$, the marker of central memory on regulatory T cells. Its enhanced value differentiated patients who acquired tolerance to CMP from those with a persistent CMA, at a cut-off level of 21.3% with a 71% specificity (AUC of 0.766; 95% CI 0.571–0.96, $p = .0076$) (Figure 3(C)).

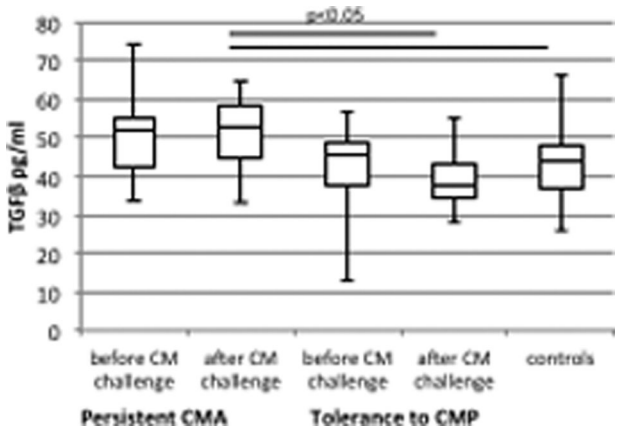

Figure 2. Differences in the serum concentration of TGFβ in children with a persistent cow's milk allergy and acquired tolerance to cow's milk protein (CMP) and controls before and after cow's milk challenge test.

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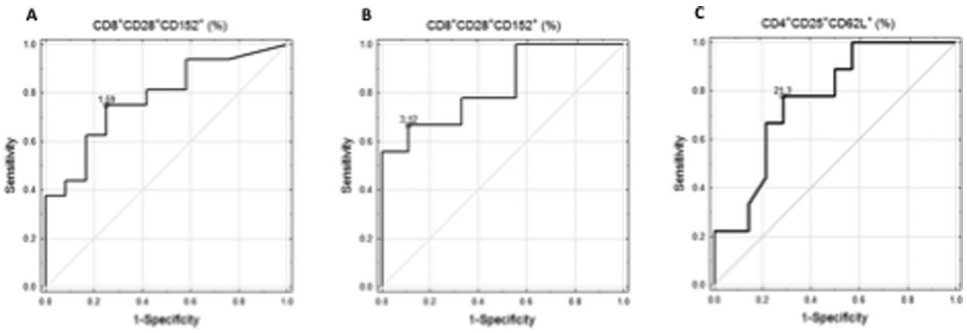

Fig	Marker	AUC (95%CI)	Sensitivity	Specificity	PPV	NPV	p
A	$CD3^+CD8^+CD28^+CD152^+$	0.779 (0.606-0.951)	0.75	0.75	0.8	0.692	0.0015
B	$CD3^+CD8^+CD28^+CD152^+$	0.827 (0.634-1.0)	0.667	0.889	0.857	0.727	0.0009
C	$CD3^+CD4^+CD25^+CD62L^+$	0.766 (0.571-0.961)	0.78	0.71	0.64	0.83	0.0076

Figure 3. Increased percentage of $CD3^+CD8^+CD28^+CD152^+$ (A, B) and $CD3^+CD4^+CD25^+CD62L^+$ in serum before CM challenge discriminates children who developed tolerances to CMP (A, C), particularly those with a previous, IgE-mediated CMA (B); AUC: area under the ROC curve; PPV: positive predicative values; NPV: negative predicative values; CI: confidents interval.

Discussion

The majority of children with CMA develop tolerance to CMP with age and therefore, indications for the continuation of the elimination diet require periodic re-evaluation. Understanding the mechanisms of a persistent CMA might help to establish accurate management guidelines for CMA patients. There is a need to determine a specific marker for CMP tolerance development. The concentration of specific IgE to CM has been suggested as a predictor of the development of CMP tolerance (Wan et al., 2012; Wood et al., 2013). In a study by Wood et al., 72% of children in whom milk allergy resolved had low levels of specific IgE to CM (less than 2 kU_A/L), compared with only 23% of those with the level greater than 10 kU_A/L (Wood et al., 2013). In our study, the level of specific CM IgE was lower than 1.2 kU_A/L in all the children with CMP tolerance whereas the subjects with a persistent CMA demonstrated far higher levels of specific CM IgE (ranging from 4.85 to 81.2 kU_A/L), which is in agreement with previous reports.

It has been reported that T cells displaying a Th2-skewed phenotype are mostly responsible for allergic reactions (Tiemessen et al., 2004). Lymphocyte distribution may change significantly in response to an allergen (Sun, Fu, & Wang, 2015). However, Beyer et al. have suggested that alterations in main lymphocyte populations are not useful in monitoring food challenges, although the authors have not assessed T cell subpopulations (Beyer, Renz, Wahn, & Niggemann, 1998). Our study revealed some changes within T cell subsets in children with a CM allergy. A significantly lower percentage of $CD3^+CD4^+$ cells with $CD152^+$ (CTLA-4) expression, the negative regulator of T cell activation, was found in children with a persistent CMA, both prior to and following the CM challenge in comparison with the control group. Animal model studies have demonstrated that CD152

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expression is required for the induction of peripheral T cell tolerance to food (Samoilova et al., 1998; van Wijk et al., 2007). Moreover, in our study, the same group with a persistent CMA demonstrated a significantly higher level of $CD3^+CD4^+CD28^+CD152^+$ T cells before the CM challenge compared to control. Samochocki et al. reported a marked elevation of CD152 expression alone as well as the co-expression of both CD152 and CD28 markers in $CD4^+$ and $CD8^+$ cell populations in individuals with AD as compared to controls (Samochocki et al., 2012). Cells bearing both CD28 and CD152 are the transient subpopulation of T lymphocytes (Samochocki et al., 2012). Little is still known about their role in immune regulation, especially in allergy or immune tolerance to food. Depending on costimulatory signals mediated by the interaction of ligands CD80/CD86, they may transform into active lymphocytes through binding to CD28 or, after binding to CD152, may generate inhibitory signals that "'switch off'" T cell activation, proliferation and IL-2 production (Gardner, Jeffery, & Sansom, 2014). CD28 expression on the T cell surface is required for CD152 upregulation on activated T cells (Walunas & Bluestone, 1998). However, the affinity of CD80/CD86 for CD152 is higher than for CD28 (Linsley et al., 1991). Our results showed a higher percentage of $CD3^+CD8^+CD28^+CD152^+$ cells in children who developed tolerance to CMP, especially with a previous IgE-mediated allergy, in comparison with the subjects with a persistent CMA. To the best of our knowledge, no reports about the co-expression of $CD28^+CD152^+$ on $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells in patients with food allergies have been published to date. Moreover, we found that an increased percentage of $CD3^+CD8^+CD28^+CD152^+$ might discriminate patients who outgrew their allergy from those who remained allergic to CM with a specificity of 75% at a cut-off level of 1.59%. Similar results were obtained in regard to the expression of $CD3^+CD4^+CD25^+CD62L^+$, the marker of central memory on regulatory T cells. Its increased expression characterized patients with tolerance to CMP with a specificity of 71% and a cut-off level of 21.3%. An enhanced number of $CD4^+CD25^+$ T cells with a regulatory function in peripheral blood 1 week after a milk challenge in children who had outgrown their allergy was reported by Karlsson et al. (2004). In humans, more than 95% of $CD4^+CD25^{high}$ Treg have been found to be CD62L-positive (Baecher-Allan & Hafler, 2005). Ermann et al. (2005) showed that $CD4^+CD25^+CD62L^+$ T cells inhibited the expansion of alloreactive $CD4^+CD25^-$ T cells more efficiently than $CD4^+CD25^+CD62L^-$ T cells. Treg cells suppress antigen-specific immune responses not only by direct cell contact with CD152, but also by secreting soluble factors such as IL-10 and transforming growth factor-beta (TGF-β) (Oberg, Juricke, Kabelitz, & Wesch, 2011). Frischmeyer-Guerrerio et al. evaluated allergy development in patients with Loeys-Dietz syndrome, characterized by mutations in the genes encoding receptor subunits for TGF-β. The authors suggested that enhanced TGF-β signalling might predispose individuals to allergic phenotypes through the induction of Th2 cytokine production (Frischmeyer-Guerrerio et al., 2013). Since TGF-β modulates inflammatory response, the increased serum level of this cytokine in children with a persistent CMA found in our study might be associated with its regulatory function. On the other hand, it has been reported previously that the decreased synthesis of TGF-β in duodenal mucosa, a consequence of a decreased number of mucosal Th3 lymphocytes, might be one of the reasons for cow's milk hypersensitivity development (Pérez-Machado et al., 2003).

Immune response in allergic inflammation involves also B lymphocytes producing allergen-specific IgE or IgA, which are important in food tolerance development. Ghosh

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et al. confirmed in an animal model that $CD19^+CD23^+$ B lymphocyte numbers are increased in the allergic lung and suggested a role of $CD19^+CD23^+$ lymphocytes in immunoglobulin secretion in the context of a fungal allergy (Ghosh, Hoselton, & Schuh, 2012). Krogulska et al. analysed $CD19^+CD23^+$ lymphocytes in children with asthma and food allergies, revealing their significant increase 24 hours after an oral food challenge. Moreover, the $CD19^+CD23^+$ increase was greater in individuals with severe reactions than in those with moderate/mild manifestation (Krogulska, Wasowska-Królikowska, Polakowska, & Chrul, 2009). In our analysis, a significant difference in the B cell number was found only in children who developed tolerance to CM, showing a decrease in the percentage of $CD19^+$ cells expressing $CD23^+$, the "low-affinity" receptor for IgE, after the CM challenge.

The limitations of our study were the small number of patients with CMA, various clinical manifestations of CMA and a lack of *in vitro* analysis concerning the functional response of lymphocytes before and after the CMP challenge. However, the prevalence of CMA in the region of our centre is not high, so it would take years to collect an adequate number of patients with single phenotype manifestation of CMA. The *in vitro* experiments require the acquisition of larger blood samples from the studied patients, which is ethically unacceptable in studies on paediatric subjects.

In conclusion, our results confirmed the previously reported involvement of immune cells in the acquisition of CMP tolerance. In particular, low levels of $CD19^+CD23^+$ B cells and high levels of $CD3^+CD8^+CD28^+CD152^+$ and $CD3^+CD4^+CD25^+CD62L^+$ T cells might suggest the stratification of patients who will develop tolerance. More studies are needed to evaluate the usefulness of these markers as predictors of CMP tolerance development.

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