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Combined T regulatory cell and Th2 expression profile identifies children with cow's milk allergy

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Abstract The role of T regulatory cells in spontaneous recovery from cow's milk allergy (CMA) is unclear. We investigated the mRNA expression of 12 T-cell markers and the protein expression of CD4, CD25, CD127, FoxP3 after *in vitro* β -lactoglobulin stimulation of peripheral blood mononuclear cells from children with persisting CMA ($n=16$), early recovery ($n=20$) or no atopy ($n=21$). Artificial neural networks with exhaustive search for all marker combinations revealed that markers FoxP3, Nfat-C2, IL-16 and GATA-3 distinguished patients with persisting CMA most accurately from other study groups. FoxP3 mRNA expression following β -lactoglobulin stimulation was highest in children with persisting CMA. Also the FoxP3 intensity in CD4⁺ CD25^{high}CD127^{low} cells was higher in children with CMA compared with non-atopic children. The expression profile of both Th2- and T regulatory cell-related genes thus reflects the clinical activity of CMA. Tolerance, in contrast, is not characterized by activation of circulating T regulatory cells.

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Introduction

Cow's milk allergy (CMA) offers a model for natural development of oral tolerance since the majority of patients

with CMA recover by age 3 years [1,2]. Immunological responses to dietary antigens in the gut involve T regulatory cells (Tregs) [3], and the interaction of Th2-cells and Tregs is essential in tolerance development [4,5].

Tregs are characterized by concomitant expression of the surface marker CD25, a high-affinity receptor for IL-2 (IL-2R α) [6], and of forkhead box protein 3 (FoxP3) [7]. Although all T cells express CD25 upon activation [8], high expression of CD25 seems to stabilize forkhead box protein 3 (FoxP3) expression and maintenance of a Treg phenotype [9]. Low

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expression of the surface marker CD127, an IL-7 receptor, furthermore distinguishes T-cells with suppressive function [10–12].

A variant of human immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) caused by a FoxP3 mutation leads to severe food allergies [13]. Furthermore, weaker responses of effector T cells to suppressive Tregs may predispose infants to egg allergy [14]. Clinical recovery from CMA in comparison with active CMA has been reported to associate with heightened numbers of circulating CD4⁺CD25⁺ cells [15] or allergen specific, rather than polyclonal, FoxP3⁺CD25^{hi}CD27⁺, CTLA4⁺, CD45RO⁺CD127⁺ cells [16].

To attain insight into the interplay of Tregs and Th2-cells in allergy and oral tolerance, we investigated the mRNA and protein expression of T-cell markers in β -lactoglobulin-stimulated peripheral blood mononuclear cells (PBMCs) from patients with active CMA at age 5–12 years, patients who had recovered from CMA by age 3 years, and non-atopic subjects. Recent research on Tregs guided us in selecting the expression markers, and artificial neural networks analysis of our data revealed novel expression patterns of T-cells in CMA.

Methods

Study population

All participants and their parents gave an informed and written consent. The Ethics Committee of the Hospital for Children and Adolescents, University of Helsinki, approved the study.

We studied PBMC samples from 57 children aged 5.4–12.3 years (median 8.3 years). At the time of sample withdrawal, 16 had active CMA (median age 8.2; range 5.4–12.3), 20 had recovered from CMA by age 3 years (8.3; 8.0–9.1), and 21 were non-atopic control subjects (8.3; 8.0–9.1). We recruited nine patients with active CMA beyond age 5 years (range 5.4–12.3) in spring 2008 at the Helsinki University Hospital. The other subjects had participated at age 8–9 years to a follow-up of a prospective study on CMA [2]. The non-atopic subjects were randomly selected from the same birth cohort as the patients. They had low total serum IgE, negative skin prick test results in a panel of 18 allergens as described previously [2], and no history of atopic disorders. CMA diagnosis was based on an open CM oral challenge, and the current CMA status on either an oral challenge or regular consumption of CM. In all patients, CMA was or had been IgE-mediated. We ran samples from all 16 patients with persisting CMA on flow cytometry and from 15 on real time polymerase chain reaction (RT-PCR). From some subjects who had recovered or were non-atopic, samples were so scarce that we randomly assigned them to be run on RT-PCR or flow cytometry only.

PBMC extraction and *in vitro* stimulation

PBMCs were extracted with Ficoll Paque (GE Healthcare Bio Sciences Corp, Piscataway, NJ) centrifugation of heparinized blood samples. They were stored at -135°C in a cell culture medium containing RPMI 1640 with 25 mM Hepes (Gibco

Invitrogen, Carlsbad, CA), 5% human AB serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland), 2 mM L-glutamine (Gibco) and 25 $\mu\text{g}/\text{ml}$ gentamycin (Sigma Aldrich, St. Louis, MO) with 10 % dimethyl sulfoxide ($(\text{CH}_3)_2\text{SO}$, DMSO; Sigma Aldrich). Median cell recovery after thawing in the three study groups ranged from 70 to 77%.

Immediately after thawing, PBMCs were stimulated *in vitro* at $+37^{\circ}\text{C}$, with 5% CO_2 , with 200 $\mu\text{g}/\text{ml}$ bovine β -lactoglobulin (Sigma Aldrich), the purity of which was confirmed with FPLC. Sterile PBS (Biowhittaker Lonza, Basel, Switzerland) served as negative control.

RNA extraction, reverse transcription and quantitative real time polymerase chain reaction (RT-PCR)

We harvested the PBMCs after 72 h of stimulation, extracted RNA with Qiagen RNeasy Minikit (Qiagen, Hilden, Germany) with the additional DNase step, and reverse transcribed with TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA). We ran cDNA samples in triplicates on StepOnePlus RT-PCR system (Applied Biosystems) with TaqMan reagents (Applied Biosystems) using FAM labeled probes and ROX as passive reference. Target specific primers for T-bet, GATA-3, IFN- γ , CTLA4, IL-10, IL-16, TGF- β , FoxP3, Nfat-C2, TIM3, TIM4, STIM-1 and 18 s were purchased from Applied Biosystems. Ribosomal 18 s RNA served as an endogenous control. We programmed the sequence detector for an initial step of 20 s at 95°C , followed by 40 thermal cycles of 1 s at 95°C and 20 s at 60°C . We analyzed the raw data as previously published [17]. We calculated levels of target gene expression with a comparative threshold cycle (Ct) method (as recommended by Applied Biosystems). We calibrated normalized samples with an inter-assay standard: an exogenous cDNA pool generated from PHA-stimulated PBMCs. The relative amount of a target gene in a sample was normalized in relation to both the calibrator and to an endogenous control (18S).

Flow cytometry

We collected PBMCs for flow cytometry after 120 h antigen stimulation. We applied a published protocol [10] combined with manufacturer's instructions of BioLegend (San Diego, CA, USA) Alexa-488-conjugated anti-FoxP3 kit. For surface staining, we used anti-human antibodies PE-conjugated anti-CD127, APC-conjugated anti-CD25, PerCP-conjugated anti-CD4 and appropriate isotype controls from Becton Dickinson (BD Biosciences, San Jose, CA, USA). Cells were first stained with surface markers for 30 min at $+4^{\circ}\text{C}$. After washing once with 0.5% BSA-PBS, cells were fixed for 30 min at RT using 1 $\times$ Fix/Perm buffer provided in the kit. Cells were then washed once with 0.5% BSA-PBS and twice with 1 $\times$ Perm buffer, and blocked with mouse IgG for 15 min at RT. Anti-human Alexa-488-conjugated FoxP3 was added, and cells were stained for 30 min at $+4^{\circ}\text{C}$. After washing twice with 1 $\times$ Perm buffer, cells were suspended in FACS flow solution (BD Biosciences). Stained cell samples were run on FACS Calibur (BD Biosciences), and analyzed with BD FACSDiva software (BD Biosciences).

Table 1 Geometric mean intensity of FoxP3 in CD4⁺ CD25^{high}CD127^{low} cells, percentages of CD25^{high}CD127^{low} in CD4⁺ cells and of FoxP3⁺ in CD4⁺ CD25^{high}CD127^{low} cells, after 120 h *in vitro* β -lactoglobulin stimulation of peripheral blood mononuclear cells in three groups (patients with persisting CMA at 5–12 years, patients who recovered from CMA by 3 years and non-atopic subjects).

	Persisting CMA at 5–12 years, <i>n</i> =16	Tolerant by 3 years, <i>n</i> =15	Non-atopic subjects, <i>n</i> =13	<i>p</i> value Kruskall–Wallis
Geometric mean intensity of FoxP3 in CD4 ⁺ CD25 ^{high} CD127 ^{low} cells median (range)	449 (247–605)	407 (248–734)	355 (175–546)	0.05
CD25 ^{high} CD127 ^{low} % in CD4 ⁺ cells median (range)	0.4 (0.1–1.3)	0.5 (0.1–8)	0.3 (0.1–0.7)	0.7
FoxP3 ⁺ % in CD4 ⁺ CD25 ^{high} CD127 ^{low} cells median (range)	82 (47–96)	78 (9–93)	78 (21–89)	0.7

Statistical analysis

Data of flow cytometry assays and of the RT-PCR markers selected based on neural network analysis (see below) were compared between the three study groups with Kruskal–Wallis and Mann–Whitney non-parametric tests using SPSS 15.0.1.

Neural network analysis

Artificial neural networks are a family of powerful prediction methods that mimic biological neural networks [18,19]. Here, we applied a multi-layer perceptron artificial neural networks algorithm, which is particularly well-suited in elucidating complex patterns in data and has been used in various biomedical applications [20–22].

First, we created all 4095 marker combinations from the 12 markers ($2^{12}-1$) and trained 4095 classifiers using the Δ Ct transformed β -lactoglobulin-stimulated expression marker combinations. Classifiers were built with the MP-algorithm in Weka data mining software version 3.7.0 [23]. The MP-algorithm automatically chooses the number of layers, neurons and connections. After training the classifiers, we evaluated their performances using five-fold cross-validation and with three different statistical methods: confusion matrix, area under the receiver operating characteristic curve (ROC AUC) [19,24] and κ -value [25], which represents the agreement between true and predicted classes.

Results

FoxP3 expression intensity in CD4⁺ CD25^{high}CD127^{low} cells was higher in β -lactoglobulin-stimulated PBMCs from chil-

dren with persisting or recovered CMA compared with non-atopic children (Table 1; $p<0.05$). Values of children with persisting or recovered CMA were comparable (Table 1; $p>0.5$, Mann–Whitney test). No difference between groups was observed in the percentage of CD25^{high}CD127^{low} cells in CD4⁺ cells or the percentage of FoxP3⁺ cells in CD4⁺ CD25^{high}CD127^{low} cells (Table 1).

According to neural network analysis, the combination of T-cell markers FoxP3, IL-16, Nfat-C2 and GATA-3 distinguished most accurately patients with persisting CMA from patients who recovered early and from non-atopic subjects (Table 2). Patients who had recovered early from CMA and non-atopic subjects were less discernable (Table 2). FoxP3 mRNA expression was more substantial in β -lactoglobulin-stimulated PBMCs from children with persisting CMA than from non-atopic children (Table 3; $p<0.005$, Mann–Whitney). The difference between children with recovered CMA or with persisting CMA was suggestive (Table 3; $p<0.07$, Mann–Whitney); children with recovered CMA and non-atopic controls showed no difference (Table 3; $p>0.4$, Mann–Whitney). Expression levels of the other three markers showed no statistically significant differences between groups (Table 3; $p>0.05$, Kruskal–Wallis).

Discussion

Our study shows that after β -lactoglobulin stimulation, the combined mRNA expression profile of a Treg-determining (FoxP3), a multifaceted T cell activation-related (Nfat-C2) and a Th2-determining (GATA-3) transcription factor, and cytokine IL-16 differed between patients with persisting CMA, patients who had recovered from CMA and non-atopic children. FoxP3 up-regulation on both RNA and protein levels

Table 2 Neural network statistics and the confusion matrix of real time PCR markers (FoxP3, IL-16, Nfat-C2, GATA-3) that after 72 h *in vitro* β -lactoglobulin stimulation distinguished most accurately patients with active CMA at 5–12 years from those who recovered from CMA by 3 years, and non-atopic subjects.

		Study group			κ -statistic	ROC value
		Persisting CMA at 5–12 years, <i>n</i> =15	Tolerant by 3 years, <i>n</i> =15	Non-atopic subjects, <i>n</i> =13		
Group predicted by the neural network analysis	Persisting CMA at 5–14 years	12	1	1	0.4	0.8
	Tolerant by 3 years	2	11	7		
	Non-atopic subjects	1	4	5		

Table 3 The mRNA expression levels in peripheral blood mononuclear cells after 72 h *in vitro* β -lactoglobulin stimulation, as relative units, of real time PCR markers (FoxP3, IL-16, Nfat-C2, GATA-3) that distinguished most accurately patients with active CMA at 5–12 years from those who recovered from CMA by 3 years, and non-atopic subjects.

	Median (range); relative units		p value Kruskal–Wallis	Persisting CMA vs. non-atopic, p value Mann–Whitney		Persisting CMA vs. tolerant, p value Mann–Whitney		Tolerant vs. non-atopic, p value Mann–Whitney	
	Persisting CMA at 5–12 years, n=15	Tolerant by 3 years, n=15							
FoxP3	3.7 (0.5–15)	1.1 (0.7–7.8)	0.02	0.05	0.07	0.07	0.4	0.4	0.4
Nfat-C2	30 (9.4–193)	24 (15–193)	0.9	1.0	0.7	0.7	1.0	1.0	1.0
IL-16	259 (51–1330)	159 (56–1330)	0.3	0.07	0.4	0.4	0.7	0.7	0.7
GATA-3	88 (20–746)	65 (24–746)	0.6	0.3	0.8	0.8	0.6	0.6	0.6

upon CM antigen stimulation suggested activation of regulatory mechanisms in patients with CMA. The methodological strengths were stringent CMA diagnostics, distinctive study groups and the ability of neural network analysis to penetrate complex data.

We chose to investigate CD4⁺CD25^{high}CD127^{low} cell surface markers that identify functionally suppressive Tregs [10–12], since we acknowledged that PBMC samples obtained from children in our study were insufficient for suppression experiments. Treg activation among children with CMA, in contrast to non-atopic subjects, was inferred by strong FoxP3 intensity in circulating CD4⁺CD25^{high}CD127^{low} cells and high FoxP3 mRNA expression in PBMCs in response to *in vitro* β -lactoglobulin stimulation. We observed no difference in the frequencies of circulating Tregs between patients tolerating CM and patients clinically reactive to CM in contrast to a previous study [15]. Our findings are more in line with a more recent study that reported a difference in antigen-specific but not polyclonal Tregs [16].

In addition to FoxP3, the mRNA expression profile distinguishing study groups included the versatile transcription factor Nfat-C2. It may induce T effector cell activation or initiate anergy, or promote Treg function in interaction with FoxP3 [26–28]. Expression of GATA-3 and IL-16 further contributed to discerning study groups. IL-16 may recruit Tregs into tissues [29] and reduce allergen induced Th2 response [30,31]. In contrary, clinical improvement of atopic symptoms has been associated with decreasing serum IL-16 levels in an observational setting [32] as well as during successful allergen specific immunotherapy [33]. GATA-3 is established as inducing the expression of Th2 polarizing cytokines, which appear to inhibit FoxP3⁺ Treg development [34]. Our mRNA expression profiling thus indicates that the balance and interaction between Th2 and Treg activation are crucial in allergy and tolerance development [4,34].

Weak FoxP3 response to *in vitro* β -lactoglobulin stimulation in non-atopic subjects insinuates that anergy rather than up-regulation of FoxP3 in Treg activation is characteristic to non-allergic long-term tolerance. Tolerance to CM in non-atopic individuals may, nevertheless, involve activated Tregs in the gut, which could then be reflected as unresponsiveness in peripheral blood. PBMCs from patients with CMA, in contrast, showed up-regulation of FoxP3 upon CM antigen exposure *in vitro*. This failed, however, to control allergic responses since β -lactoglobulin stimulation activated both Treg and Th2 pathways. Our results thus question the concept that Treg activation in peripheral blood indicates induction of tolerance in food allergy.

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