

Infiltration of Foxp3- and Toll-like Receptor-4–positive Cells in the Intestines of Children With Food Allergy

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ABSTRACT

Objectives: Regulatory T (Treg) cells together with intestinal microflora play a central role in controlling allergic inflammation. We examined the markers related to Treg cells, and bacterial signaling, such as Toll-like receptors (TLR)-2 and -4, in the duodenal mucosa of patients with food allergy (FA).

Patients and Methods: Small intestinal samples were collected from patients with FA on a normal or an elimination diet, from healthy controls and patients with untreated celiac disease. Single and double immunohistochemistry were used to enumerate the densities of Foxp3-positive cells and TLR2- and TLR4-positive cells in the mucosa and evaluate the colocalization of Foxp3 expression in CD4⁺, CD25⁺, and CTLA-4⁺ cells. The mRNA expression of CD25, Foxp3, TLR2, and TLR4 was measured by reverse transcriptase-polymerase chain reaction.

Results: The densities of Foxp3⁺ and TLR4⁺ cells were significantly increased in patients with untreated FA compared with healthy controls ($P = 0.003$, $P = 0.033$), and the Foxp3⁺ cells were higher in untreated than in treated allergic patients ($P < 0.001$). The immense majority of Foxp3⁺ cells were CD4⁺ (median 100%), CTLA-4⁺ (100%), or CD25⁺ (81%). The ratio of Foxp3 mRNA to Foxp3⁺ cells was decreased in patients with FA and in patients with celiac disease compared with controls ($P = 0.036$, $P = 0.035$).

Conclusions: Foxp3⁺ cells are increased in the duodenum of patients with untreated FA, but these cells are not able to suppress the harmful immune response, indicated by the low expression of Foxp3 transcripts. The increase of TLR4⁺ cells and their correlation with TCRγδ⁺ intraepithelial lymphocytes suggest a role for the innate immunity and intestinal microbiota in FA.

Key Words: CD4⁺CD25⁺ regulatory T cells, cytokines, food allergy, Foxp3, Toll-like receptor

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Prevailing immune response to harmless food antigens results in tolerance, a phenomenon known as oral tolerance. Regulatory T (Treg) cells play a primary role in controlling adaptive immune response and maintaining tolerance to self-antigens and harmless non-self-antigens (1). Failure in oral tolerance leads to sensitization to food antigens and food allergy (FA) (2).

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Treg cells can be divided into naturally occurring Treg cells, which are induced against self-antigens expressed in thymus, and adaptive Treg cells, which are induced in the periphery (1,3). Natural Treg cells express CD4, CD25, cytotoxic T lymphocyte antigen 4 (CTLA-4), and Foxp3 transcription factor (4–6). These Treg cells have been shown to control gut inflammation in mouse models of inflammatory bowel disease (7,8) and suppress both T helper 1 (T_H1) and T_H2 immune responses by different mechanisms such as secretion of transforming growth factor-β (TGF-β) (1,9).

Indirect evidence suggests that the development of FA may involve CD4⁺CD25⁺ Treg cells in humans. The X-linked immunodeficiency syndrome IPEX is caused by mutations in Foxp3 and results in the autoimmune disease in multiple endocrine organs, inflammatory disease, and food allergies (10,11). Challenge with food allergens induced CD4⁺CD25^{high} Treg cells in children who had outgrown cow's milk allergy and become tolerant of the allergen (12). Moreover, feeding experiments in mice support a role of CD4⁺CD25⁺ Treg cells in oral tolerance (13). Together, these data suggest that CD4⁺CD25⁺Foxp3⁺ Treg cells are essential in controlling inflammation of the intestinal mucosa in FA, but the expression of Foxp3 has not been addressed in the intestinal mucosa in FA.

The underlying mechanisms of intestinal tolerance versus reactivity toward luminal bacterial ligands are mediated through Toll-like receptors (TLRs) of the innate immune system (14). Mammalian TLRs comprise a family of at least 11 transmembrane receptors, which recognize pathogen-associated patterns (15). TLR2 interacts with peptidoglycan, a component of all bacterial cell walls and TLR4 with lipopolysaccharide, which is a major component of the Gram-negative bacterial outer membrane (16). TLRs are inducible or constitutively expressed by many cell types in the gastrointestinal tract, including intestinal epithelial cells, intestinal macrophages, and dendritic cells (17,18), as well as by CD4⁺CD25⁺ Treg cells (19). In active inflammatory bowel disease (18,20–22) and in celiac disease (CD) (23), TLR2 and TLR4 are significantly increased in intestinal epithelial cells and macrophages.

The role of intestinal Treg cells or TLRs expression is not known in FA. Our aim was to evaluate the balance of Foxp3-positive cells and effector T cells and the expression of TLR2 and TLR4 in FA. For this study we obtained small intestinal biopsy specimens from 19 children with FA, 10 healthy control patients, and 10 pediatric patients with untreated CD. We investigated the protein expression by immunohistochemistry for Foxp3, CD25, CTLA-4, IL-10, and TGF-β as markers for Treg cells, interferon-γ as a marker for T_H1, and interleukin-4 as a marker for T_H2 immune response. The expression of TLR2 and TLR4, and the expression of CD3, CD4, CD8, and T-cell receptor (TCR)γδ cell surface markers were also examined. Double immunostaining of Foxp3 and CD4, CD25, or CTLA-4 were performed, and the specific amounts of Foxp3, CD25, TLR2, and TLR4 mRNA were evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) from the same mucosal samples.

PATIENTS AND METHODS

Patients

Patients included in this study were referred to the Helsinki University Hospital for a gastroenterological consultation because of recurrent abdominal pains, prolonged diarrhea, and/or growth retardation. In the majority, the diagnosis of FA was set according to European Society for Pediatric Gastroenterology, Hepatology, and Nutrition criteria (24). Briefly, diagnosis of FA was based on the disappearance of symptoms on an elimination diet (2 weeks) and their reappearance in an open challenge test. Abdominal pain, diarrhea or loose mucous stools, or exacerbation of dermatitis was each considered a positive result. In the rest, FA was diagnosed on the basis of symptoms, positive effect of elimination diet, and/or positive specific IgE antibodies on skin prick testing or measuring from serum (Table 1).

The 19 patients with FA were divided into 2 groups: 7 untreated FA patients on a normal diet (3 girls, mean age 2.1 years, range 0.6–5.4 years) and 12 treated FA patients on an elimination diet (5 girls, mean age 4.0 years, range 0.3–6.9 years).

Additionally, 10 healthy control patients (4 girls, mean age 4.0 years, range 1.1–6.7 years) with normal jejunal mucosa and 10 patients with active CD (6 girls, mean age 4.4 years, range 1.9–6.7 years) were included in the study as control groups. The diagnosis of CD was set according to European Society for Pediatric Gastroenterology, Hepatology, and Nutrition criteria (27). All of the CD

patients had high titers of positive IgA antibodies to endomysium (EMA) and tissue transglutaminase (tTGA).

The healthy control patients underwent a jejunal biopsy because of gastrointestinal symptoms, positive anti-gliadin antibodies, growth retardation, or any combination of these. They had no history of CD or dermatitis herpetiformis in the family, they were not taking medication, and they did not have any chronic disease. The morphology of the jejunum was normal, and EMA and tTGA antibodies were negative.

Samples

A Watson biopsy device or a gastroscope was used to take a specimen from the distal duodenum. The specimens were divided for routine histology and immunohistochemical studies. The part for immunohistochemistry was embedded in optimal cutting temperature compound (Miles Laboratories, Elkhart, IN), snap frozen in liquid nitrogen, and stored at -70°C . Frozen tissue samples were cut into 8- μm sections, coded, and evaluated without knowledge of the specimen. The part of the mucosal specimen still left was used for real-time quantitative RT-PCR.

Ethical Considerations

Specimens were taken for diagnostic purposes. Use of biopsy specimens in this study was approved by the ethics committee of the Hospital for Children and Adolescents, University of Helsinki.

TABLE 1. Clinical findings of the patients with FA

Patients with FA and patient no.	Sex	Age, y	Duration of diet, y	Diagnosis	IgE+	Allergen	Morphology	Circulating antibodies			Methods	
								IgA- EMA [*]	IgA- tTGA [†]	Gliad- IgA/IgG [‡]	IHC	RT- PCR
Untreated												
1	M	0.6	—	EDFC	Pos	Milk, egg	Normal	Neg	Neg	Neg/Neg	+	—
2	M	0.6	—	EDFC	Pos	Cereals	Normal	Neg	Neg	Pos/Neg	+	+
3	F	0.9	—	EDFC	Neg	Milk	Normal	NT	NT	NT/NT	+	+
4	M	1.2	—	EDFC	Neg	Milk, cereals	Normal	Neg	Neg	Neg/Neg	+	—
5	F	1.4	—	EDFC	Pos	Milk	Normal	NT	NT	Pos/Pos	+	—
6	F	4.4	—	EDFC	Pos	Milk, cereals	IEL+	50	>300	Neg/Pos	+	+
7	M	5.4	—	FE	Neg	Milk	Normal	NT	Neg	Neg/NT	+	+
Elimination diet												
8	M	0.3		EDFC	Pos	Milk	Normal	NT	NT	NT/NT	+	—
9	M	1.3		EDFC	Pos	Milk, cereals	Normal	NT	NT	NT/NT	+	+
10	F	2.7	1	FE	NT	Milk, cereals	Normal	Neg	Neg	Pos/Pos	+	+
11	M	3.1		FE	Neg	Milk	IEL+	Neg	Neg	Pos/Pos	+	+
12	M	3.7		EDFC	Pos	Milk, egg	Normal	NT	NT	Pos/Pos	+	+
13	F	4.1	3	FE	Pos	Milk	Normal	NT	NT	NT/NT	+	+
14	F	4.8		EDFC	NT	Milk	Normal	Neg	Neg	Neg/Neg	+	—
15	F	4.9	½	EDFC	NT	Milk, egg	Normal	Neg	NT	Pos/Pos	+	+
16	M	5.0		FE	Neg	Milk, cereals	Normal	Neg	Neg	Pos/Pos	+	+
17	F	5.5	3	FE	Pos	Milk	Normal	Neg	Neg	Neg/Neg	+	+
18	M	5.6	4	FE	Pos	Cereals, egg	Normal	Neg	20	Neg/Pos	+	+
19	M	6.9	5	EDFC	NT	Milk, peanuts	Normal	Neg	Neg	Pos/Pos	+	+

EDFC = elimination diet-food challenge; FA = food allergy; FE = favorable response to food elimination; IEL+ = normal villous architecture, increased density of intraepithelial lymphocytes; IgE+ = specific IgE >0.7 IU/L or positive skin prick test; IHC = immunohistochemistry; Neg = negative; NT = not tested; Pos = positive; RT-PCR = real-time quantitative reverse transcriptase-polymerase chain reaction.

*Class IgA endomysium antibodies, upper normal limit 1:5.

†Class IgA tissue transglutaminase antibodies, upper normal limit 8 arbitrary units (25).

‡Class IgA and IgG gliadin antibodies, classified according to Kolho et al (26).

TABLE 2. Monoclonal mouse anti-human antibodies (mAb) used in this study

mAb	Dilution	Concentration, µg/mL	Clone	Company
CD3	1:200	0.03		BD Pharmingen
CD4	1:20	1.56	RPA-T4	BD Pharmingen
CD8	1:400	0.03	DK25	Dakocytomation
CD25	1:10	7.4	ACT-1	Dakocytomatin
γδTCR	1:100	3	5A6.E9	Endogen
CTLA-4	1:20	12.5	BNI3.1	BD Pharmingen
FOXP3	1:40	10	236A/E7	Abcam
IL-4	1:50	20	IL4-II,12.1	Mabtech
IL-10	1:100	5	23738	R&D systems
IFN-γ	1:50	20	1-D1K	Mabtech
TGF-β1	1:200	10	TB21	Serotec
TLR2	1:40	2.5	TL2.3	Abcam
TLR4	1:100	10	HTA125	Abcam
Mouse IgG	1:500	2		Vector Laboratories

Additionally, after an oral explanation of the study plan, written parental consent with a signature was obtained from the parents of all of the children.

Immunohistochemistry

The avidin-biotin immunoperoxidase system was used on cryostat sections to stain the monoclonal antibodies described in Table 2 as outlined elsewhere (28).

Double immunostaining of CD4, CD25, or CTLA-4 and Foxp3 was performed as described in the previous section, with the following modifications: after staining of CD4, CD25, or CTLA-4 antibody with diaminobenzidine (Sigma, St Louis, MO) the slides were incubated with Foxp3 antibody overnight at 4°C. Bound Foxp3 antibody was visualized with Vector SG (Vector Laboratories, Burlingame, CA) as substrate. Nonimmune mouse IgG1 (Dako, Glostrup, Denmark) was used as negative primary antibody control and incubation with 1% normal horse sera on additional sections as negative control for reagents in the immunoperoxidase system.

Microscopic Evaluation

The slides were investigated blinded to the clinical data. The numbers of positively stained cells were counted under a light microscope through a calibrated graticule at ×1000 magnification, as described previously (29). The positive cells in at least 30 to 50 fields

were counted, either along the surface epithelium or in the lamina propria. Cell densities were expressed as cells per millimeter of surface epithelium and cells per square millimeter in the lamina propria.

Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR analysis of mRNA levels in the small intestinal biopsies was performed as described earlier (28). Total RNA was extracted from homogenized mucosal biopsies with the GenElute Mammalian Total RNA Miniprep kit (Sigma). Reverse transcription was performed by using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA).

Real-time quantitative PCR was performed using TaqMan PDAR (predeveloped assay reagents) or assays-on-demand primers/probes and the ABI-Prism 7700 sequence detection system (Applied Biosystems) in triplicate wells. PDAR Primers/Probes for CD25 (cat. no 4328847F), TLR2 (Hs00610101_m1), and TLR4 (Hs00152939_m1) were used. For Foxp3 assays-on-demand primer/probe (cat. no Hs00203958_m1) was used. Ribosomal 18S RNA was used as an endogenous control (PDAR reactions 4310893E and assays-on-demand reactions Hs99999901_s1).

The quantities of markers were analyzed by comparative threshold cycle (C_T) method. ΔC_T stands for the difference between C_T of the marker gene and C_T of the 18S gene, whereas $\Delta\Delta C_T$ is the difference between ΔC_T of the analyzed sample and ΔC_T of the calibrator. Calculation of $2^{-\Delta\Delta C_T}$, then, gives a relative amount of

TABLE 3. Immunohistochemical staining of lymphocytes in the surface epithelium

Patient group	n	Density of positive cells in the surface epithelium (cells/mm)			
		TCRγδ	CD3	CD4	CD8
FA untreated	7	3.9 (2.9–10.3)	16.5 (11.6–17.6)	0.5 (0–1.3)	12.5 (11.6–16.1)
FA diet	12	1.6 (0.8–2.8)	12.5 (8.0–14.7)	1.1 (0–1.8)	13.5 (6.7–19.6)
Normal controls	10	1.4 (0.9–2.2)	6.1 (5.2–8.7)	0.4 (0–1.5)	12.7 (8.6–13.6)
Celiac controls	10	31.7 (27.2–37.3)	151 (120–214)	3.1 (2.1–4.6)	167 (117–211)

Values for study groups given as medians (interquartile ranges). FA = food allergy.

the analyzed sample compared with the calibrator, both normalized to an endogenous control (*18S*). The relative amounts ($2^{-\Delta\Delta C_T}$) of CD25 were multiplied by 1000, and of Foxp3, TLR2, and TLR4 by 100 to obtain whole numbers for plots.

Statistical Analysis

The nonparametric Kruskal-Wallis and Mann-Whitney *U* tests were used for comparisons between the groups because of the nonlinear distribution of the parameters. The Spearman rank correlation test was applied to analyze correlations between different parameters (SPSS 15.0 for Windows; SPSS Inc, Chicago, IL). A *P* value of less than 0.05 was considered significant. To demonstrate the nature of the responses measured we compared the results of patients with untreated FA with those of controls and treated patients.

RESULTS

CD3⁺-, CD4⁺-, CD8⁺-, and TCR $\gamma\delta$ ⁺-expressing T Cells in the Epithelium

CD3⁺ intraepithelial lymphocytes (IELs) were higher in untreated FA patients and treated FA patients than in healthy controls (*P* = 0.042 and *P* = 0.014). Untreated FA patients showed increased density of TCR $\gamma\delta$ ⁺ IELs when compared with treated FA patients and healthy controls (*P* = 0.035 and *P* = 0.010). Additionally, the density of IELs was higher in patients with CD than in the other study groups, especially CD3⁺, CD8⁺, and TCR $\gamma\delta$ ⁺ IELs (*P* ≤ 0.001 in all comparisons) (Table 3).

Foxp3⁺-, CD25⁺-, and CTLA-4⁺-expressing Cells in Biopsy Samples

Untreated patients with FA had significantly higher density of Foxp3-expressing cells than patients with FA on an elimination diet and healthy controls did. Furthermore, patients with FA showed higher density of Foxp3⁺ cells than untreated patients with CD and controls did (Figs. 1A and 2).

The density of CD25⁺ cells in the lamina propria of patients with untreated FA and treated FA tended to be higher than healthy controls, but differences were not statistically significant. The density of CD25⁺ and CTLA-4⁺ cells were significantly higher in patients with CD than in the other study groups (Figs. 1B, 1C, 2).

CD4⁺Foxp3⁺-, CD25⁺Foxp3⁺-, and CTLA-4⁺Foxp3⁺-expressing Cells in Biopsy Samples

The colocalization of Foxp3 with CD4, CD25, or CTLA-4 was evaluated with double immunohistochemistry. Due to lack of material, double immunohistochemistry was performed only on a subgroup of the investigated subjects from all of the different patient groups. The vast majority of Foxp3-positive cells were CD4⁺ (median 100%, range 100%–100%; *n* = 11 subjects), CTLA-4⁺ (median 100%, range 95%–100%; *n* = 10 subjects), or CD25⁺ (median 81%, range 0%–100%; *n* = 13 subjects), whereas only 16% (mean) of CD4⁺ cells (median 16%, range 4%–38%) and 48% (mean) of CD25⁺ cells were also positive for Foxp3 (median 50%, range 0%–80%). In average 82% of CTLA-4⁺ cells were also Foxp3⁺ (median 82%, range 50%–100%) (Fig. 2).

Patients with CD had significantly higher densities of CD4⁺Foxp3⁺-, CD25⁺Foxp3⁺-, and CTLA-4⁺Foxp3⁺-expressing cells than healthy controls did (*P* = 0.043, *P* = 0.021, and *P* = 0.05, respectively, data not shown).

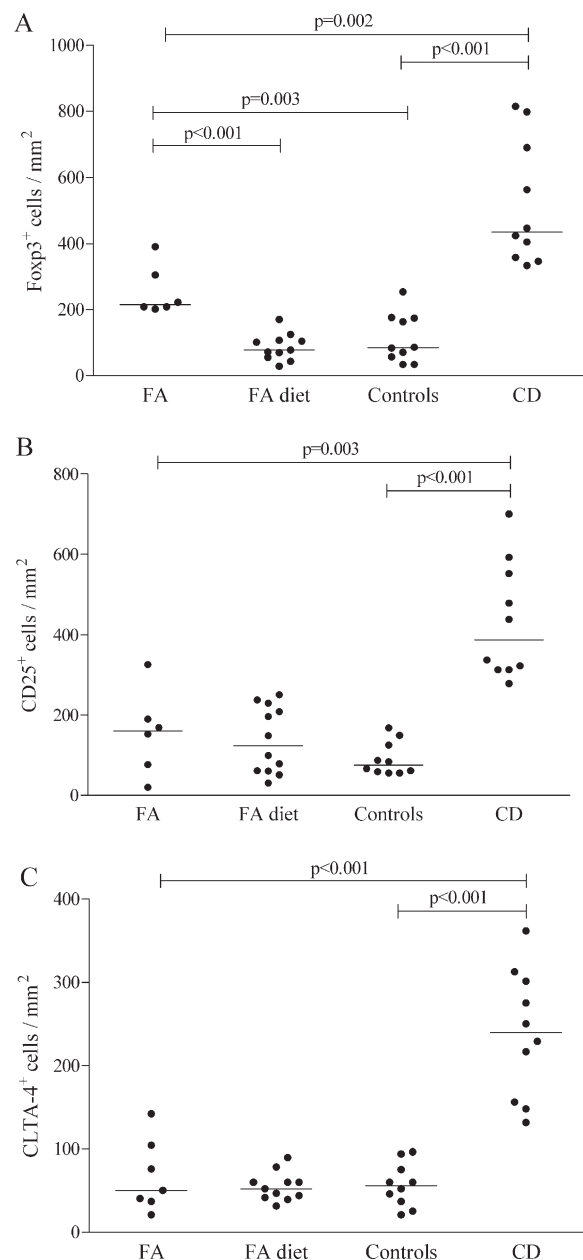


FIGURE 1. Densities of (A) Foxp3⁺, (B) CD25⁺, and (C) CTLA-4⁺ cells detected by single immunohistochemistry in mucosal biopsy specimens of patients with untreated FA, patients with treated FA (FA diet), controls, or patients with CD. The median densities are shown by horizontal lines. Significant *P* values are indicated in the figures (Mann-Whitney *U* test). CD = celiac disease; FA = food allergy.

Expression of FoxP3- and CD25-specific mRNA

The expression of Foxp3 mRNA was significantly higher in the small intestinal mucosa of patients with CD than in untreated patients with FA. The expression of CD25 mRNA did not differ significantly between the study groups (Figs. 3A and B).

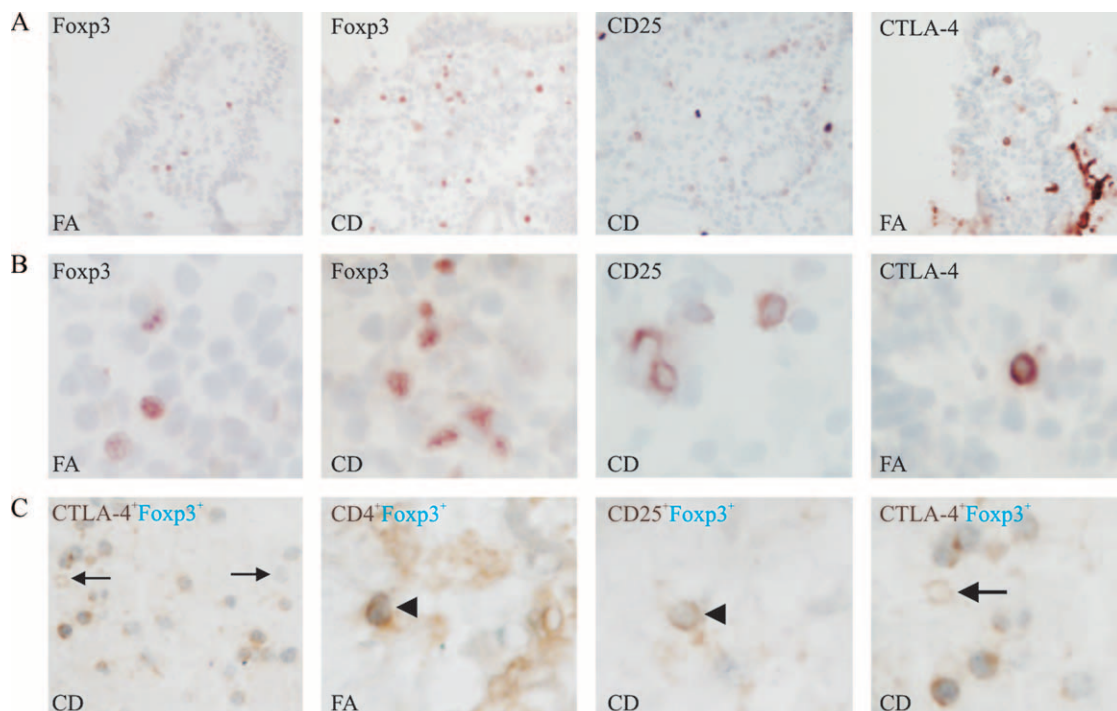


FIGURE 2. (A and B) Representative single immunoperoxidase staining of Foxp3, CD25, and CTLA-4 in mucosal biopsy specimens of a patient with FA (Foxp3 and CTLA-4) and a patient with CD (Foxp3 and CD25). Positive cells are seen with typical nuclear (Foxp3) or membranous (CD25 and CTLA-4) staining. Carbazole-hematoxylin stain, original magnification $\times 200$ (A) and $\times 400$ (B). (C) Representative immunoperoxidase double staining of Foxp3⁺CD4⁺, Foxp3⁺CD25⁺, and Foxp3⁺CTLA-4⁺ in mucosal biopsy specimens of a patient with FA (CD4) and a patient with CD (CD25 and CTLA-4). DAB-Vector SG stain, original magnification $\times 200$ (CTLA-4) and $\times 400$ (CD4, CD25, and CTLA-4). Examples of representative coexpression are indicated by arrowheads and single staining by arrows. CD = celiac disease; FA = food allergy.

To evaluate the degree of activation of the Foxp3⁺ cells we calculated the ratio between Foxp3 mRNA and the number of Foxp3⁺ cells in the biopsy specimens (Fig. 3C). This ratio differed between the groups and was decreased in patients with untreated FA or CD when compared with treated FA and control patients.

Correlations Between Treg Markers

The density of Foxp3⁺ cells correlated positively with the densities of CD25⁺ and CTLA-4⁺ cells ($r = 0.815$, $P < 0.001$ and $r = 0.578$, $P < 0.001$, respectively). The densities of Foxp3⁺, CD25⁺, or CTLA-4⁺ cells in the lamina propria correlated with the quantity of mRNA for Foxp3 ($r = 0.432$, $P = 0.015$, $r = 0.373$, $P = 0.036$, and $r = 0.519$, $P = 0.002$, respectively). The density of CTLA-4⁺ cells also correlated with the quantity of CD25-specific mRNA ($r = 0.394$, $P = 0.026$).

Furthermore, the densities of Foxp3⁺, CD25⁺, and CTLA-4⁺ cells correlated positively with the density of IFN- γ ⁺, IL-4⁺, or IL-10⁺ cells in the lamina propria, as well as with the density of CD3⁺, CD8⁺, or TCR $\gamma\delta$ ⁺ IELs ($P < 0.01$ for all correlations, Spearman correlation, 2-tailed, data not shown).

TLR2⁺- and TLR4⁺-expressing Cells in Biopsy Samples

Immunohistochemical stainings with TLR2 showed positive cells in the lamina propria, especially in the subepithelial compart-

ment. TLR2 also stained positively the surface epithelium and the endothelium of blood and lymphatic vessels. The density of TLR2⁺ cells tended to be higher in untreated patients with FA, but because the individual results were widely spread, the increase was not statistically significant (Fig. 4).

TLR4 stained positively mononuclear cells in the lamina propria as well as IELs in the surface epithelium. The density of TLR4⁺ cells in the lamina propria was higher in untreated patients with FA than in control patients. Patients with CD also showed higher density of TLR4⁺ cells than control patients did. Many of the biopsy specimens of treated patients with FA or control patients lacked TLR4⁺ cells (Fig. 4).

The densities of TLR2⁺ and TLR4⁺ cells correlated positively with the densities of TCR $\gamma\delta$ ⁺ ($r = 0.341$, $P = 0.039$ and $r = 0.484$, $P = 0.002$) and Foxp3⁺ cells ($r = 0.396$, $P = 0.017$ and $r = 0.368$, $P = 0.025$). Additionally, the density of TLR4⁺ cells correlated with the density of TCR $\gamma\delta$ ⁺ IELs ($r = 0.609$, $P < 0.001$). TLR4⁺ cells also correlated with CD25⁺ and CTLA-4⁺ cells, and with the quantity of mRNA for Foxp3 and CD25 ($P \leq 0.03$ for all correlations, data not shown).

Expression of TLR2- and TLR4-specific mRNA

The expression of TLR2 mRNA was significantly increased in the small intestinal mucosa of control patients compared with patients with CD ($P = 0.013$). The expression of TLR4 mRNA did not differ significantly between the study groups (Figs. 4C and D).

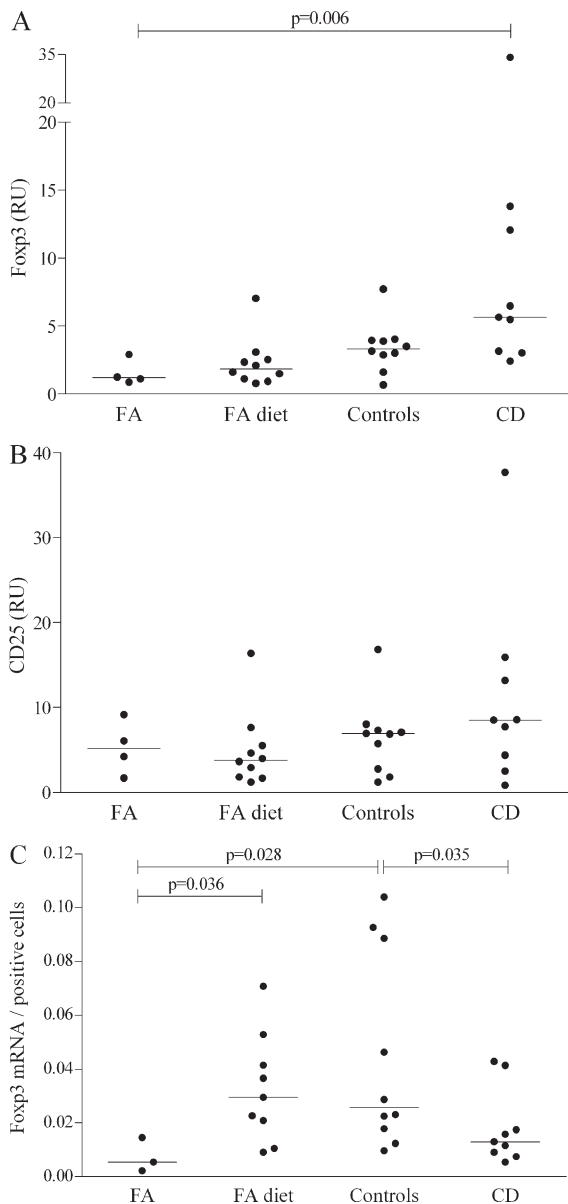


FIGURE 3. Expression of (A) Foxp3- and (B) CD25-specific mRNAs detected by RT-PCR, and the ratio between Foxp3 mRNA and the number of Foxp3⁺ cells (C). The expression level of mRNA is shown as relative units ($2^{-\Delta\Delta C_t}$) multiplied by 100 (Foxp3) or 1000 (CD25) in the small intestinal mucosa compared with the calibrator, both normalized to an endogenous control. Median expressions are marked with horizontal lines. Significant *P* values are indicated in the figures (Mann-Whitney *U* test). CD = celiac disease; FA = food allergy.

Cytokine-expressing Cells in Biopsy Samples

Patients with treated FA showed a higher density of IFN- γ ⁺ IELs than control patients did. Also patients with CD had higher density of IFN- γ ⁺ IELs than the other study groups (Fig. 5B). Additionally, patients with CD showed a higher density of both

IFN- γ ⁺ and IL-4⁺ and IL-10⁺ cells in the lamina propria than untreated patients with FA or control patients, indicating increase of both T_H1 and T_H2 cells (Figs. 5A, C, and D). Patients with untreated FA had the lowest density of TGF- β ⁺ cells in the lamina propria. The differences between the study groups were not statistically significant (Fig. 5E).

DISCUSSION

The present study shows that cells expressing Foxp3 and TLR4 are increased in the small intestinal mucosa of children with untreated FA. Despite the increased numbers of Foxp3-expressing cells, the ratio of Foxp3 mRNA to Foxp3⁺ cells was decreased in patients with FA, suggesting that the activation of Foxp3 transcription was not proportional to the numbers of these cells. This kind of functional defect could explain the presence of inflammation despite the infiltration of Foxp3-positive cells in the inflamed mucosa.

Naturally arising CD4⁺CD25⁺ Treg cells are produced in the thymus (1,3). These Treg cells express Foxp3, which encodes a transcription factor responsible for the development and function of Treg cells (4–6). In humans, it has been shown that Foxp3-expressing CD4⁺CD25⁺ Treg cells can be induced from peripheral CD4⁺CD25⁺ T cells upon stimulation (30). In vitro, naïve T cells can convert into Foxp3⁺ cells by stimulation in the presence of TGF- β and IL-2 (31,32). Foxp3 expression induced upon T-cell stimulation leads to hyporesponsiveness of activated T cells and provides mechanisms of the downregulation of T-cell activation (33). These Foxp3-positive cells do not necessarily mediate suppression of effector T cells and do not represent classical Tregs. In patients with treated FA, the lower density of Foxp3-expressing cells could be a sign of the declined T-cell activation due to the elimination of dietary trigger, as seen by the reduced total number of CD4⁺ cells in the lamina propria of these patients.

In previous studies, patients with FA have presented with low numbers of Treg cells in the peripheral blood (34–38), and their increase has been associated with the recovery from FA (12). In an earlier study performed in a small group of children who had outgrown cow's milk allergy, the frequencies of circulating CD4⁺CD25⁺ T cells among peripheral blood mononuclear cells were higher 1 week after milk was reintroduced in the diet, but the expression of Foxp3 in peripheral blood mononuclear cells was not investigated in this study (12). Moreover, in a recent study, Radulovic et al (39) showed that successful grass pollen immunotherapy in adults with hay fever was associated with markedly higher numbers of CD25⁺, Foxp3⁺, CD25⁺Foxp3⁺, and CD4⁺Foxp3⁺ cells in the nasal mucosa than in untreated adults with hay fever. They saw increases in Foxp3-expressing cells in the nasal mucosa during pollen season in both placebo and treated patients, but increases in CD3⁺CD25⁺Foxp3⁺ cells were observed only in immunotherapy-treated patients. These cells associated with clinical efficacy and suppression of seasonal allergic inflammation, which supports a role for Treg cells in the induction of allergen-specific tolerance in humans as well. Because our study patients did not include patients who had outgrown FA, we do not know whether the density of Treg cells increases locally with the development of tolerance. The decrease of Foxp3-positive cells in our patients with FA receiving elimination diet associates with the suppression of inflammation in general and may not reflect the state of tolerance.

Recently, it was shown that the induction of adaptive Foxp3⁺ Treg cells was essential for establishing mucosal tolerance in the intestinal mucosa in mice (40). Despite the strong correlation between immunological tolerance and allergen-stimulated induction of Foxp3⁺ cells, it is not known whether adaptive Foxp3⁺ Treg cells are essential for mucosal tolerance and FA, especially in humans. Moreover, there are some paradoxes regarding Treg cells

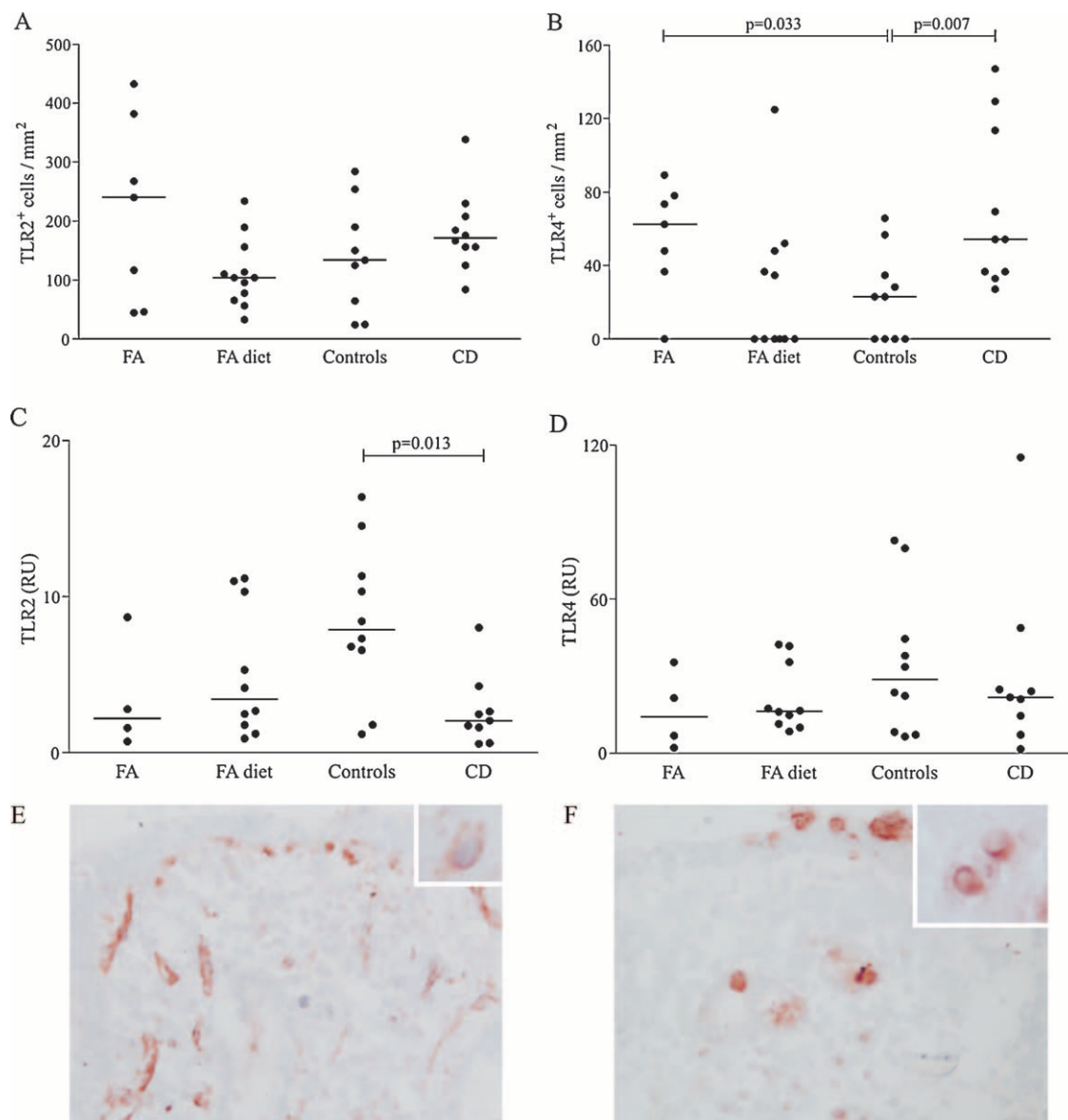


FIGURE 4. Densities of (A) TLR2- and (B) TLR4-positive cells in the lamina propria detected by immunohistochemistry and the expression of (C) TLR2- and (D) TLR4-specific mRNAs detected by RT-PCR in small intestinal mucosa. Median expressions are marked with horizontal lines. Significant *P* values are indicated in the figures (Mann-Whitney *U* test). Representative immunoperoxidase staining for (E) TLR2 and (F) TLR4 in mucosal biopsy specimens from a patient with CD. Positive cells are seen with typical membranous staining (insets original magnification ×400). TLR2 stains additionally endothelium of lymphatic and blood vessels in the lamina propria. Carbazole-hematoxylin stain, original magnification ×200.

and FA. Although several studies on peripheral blood mononuclear cells or cord blood cells suggest deficiencies in allergen-specific Treg cells in allergic diseases (34–38), others have shown the opposite (41,42). This may be due to difficulties in identifying CD4⁺ Treg cells by using regular cell markers. CD25 is also expressed on activated effector T cells, and Foxp3 is transiently expressed on effector T cells during activation (33). In addition, the presence of Foxp3⁺ Treg cells in severe inflammation also remains unexplained. Large numbers of Treg cells are found at sites of active chronic inflammation in the mucosa in inflammatory bowel disease (8,43–47), and in CD (48). The mucosa of patients with CD demonstrated increased densities of Foxp3-positive cells also in

the present study. Our findings suggest that these kinds of Foxp3-positive T cells are unable to carry out their function in chronic inflammation, and it is even possible to raise a question as to whether they contribute to the pathogenesis.

We found equally low densities of TGF-β⁺ cells in all of the study groups. The role of the immunosuppressive cytokine TGF-β in the induction of oral tolerance has been documented in experimental animals (49). The expression of Foxp3 did not correlate with the expression of TGF-β in our study. Actually, reduced production or activity of TGF-β has been documented in 3 studies with children with FA (12,50,51). The reduction of both TGF-β and its type 1 receptor (50), the decreased TGF-β production of T cells

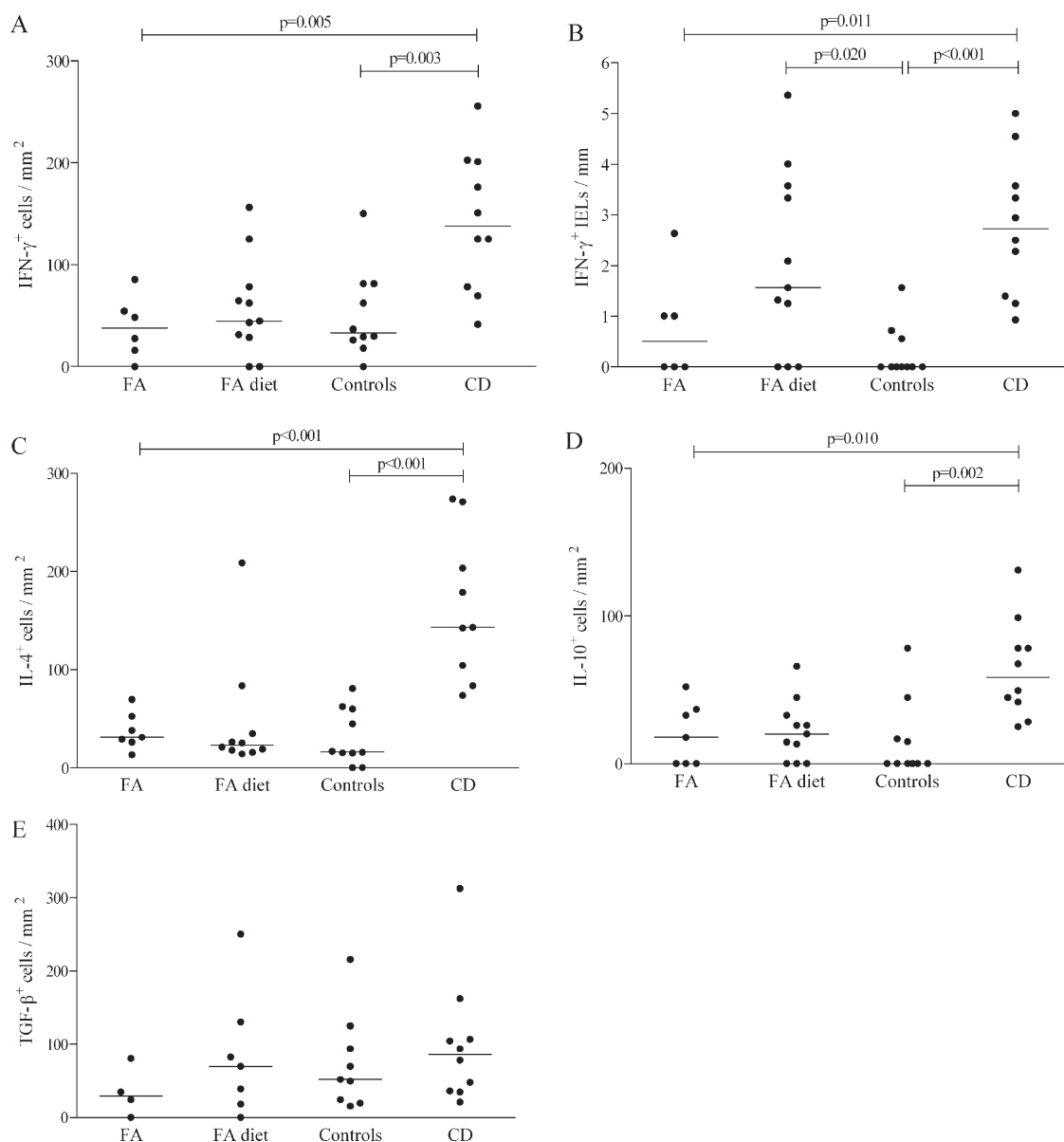


FIGURE 5. Densities of (A) IFN- γ , (C) IL-4, (D) IL-10, and (E) TGF- β -positive cells in the lamina propria and (B) interferon- γ -positive cells in the intraepithelial compartment detected by immunohistochemistry. Median densities are shown by horizontal lines in mucosal biopsy specimens of patients with FA, patients with FA on an elimination diet (FA diet), healthy controls, or patients with CD. Significant *P* values are indicated in the figures (Mann-Whitney *U* test).

from duodenal biopsies of children with FA (51), and the reduced production of TGF- β by stimulated peripheral blood mononuclear cells after milk challenge in cow's milk-allergic children (12) are in line with our finding on low density of TGF- β ⁺ cells in the lamina propria of children with untreated FA.

Interestingly, we found increased protein expression of TLR2 and TLR4 in mucosal samples of patients with untreated FA, although only TLR4 was significantly increased in patients with untreated FA patients as compared with healthy controls. The expression of TLR4⁺ cells was undetectable in many of the patients with treated and in control patients, as previously reported (23).

TLRs play an important role in the activation of innate immunity (15). They protect the intestinal epithelial barrier, provide

tolerance, and promote healing. Under normal conditions, expression of TLRs is low (14), as also shown in our study. Activation of TLRs leads to the upregulation of major histocompatibility complex molecules and costimulatory factors, expression of proinflammatory cytokines, and the activation of adaptive immunity (15). Activation of TLR4-dependent signals inhibits allergic responses to food antigens in experimental animals, and TLR4 knockout mice are highly susceptible to develop FA (52). Induction of tolerance has been shown to be associated with suppression of TLR expression (53,54). It is possible that the breakdown of the epithelial barrier and the massive contact with bacterial antigens in the inflamed mucosa could be responsible for the enhanced expression of TLRs (17,18,22). The increased density of TLR4⁺ cells corre-

lated with the increase in TCR $\gamma\delta$ ⁺ IELs in patients with FA. The activation of TCR $\gamma\delta$ cells is regulated by TLRs (55–57), providing a link to intestinal microbiota. We also found a moderate increase of CD3⁺ and TCR $\gamma\delta$ ⁺ IELs in specimens from patients with untreated FA, which is congruent with earlier studies (58–60).

Our mRNA finding on higher quantities of TLR2 and TLR4 mRNA in controls than in patients indicates that the transcription of TLR is downregulated by feedback mechanisms. The expression of TLR4 by CD4⁺CD25⁺ Treg cells has been reported, and this apparently leaves Treg responsive to bacterial lipopolysaccharide (19). Thus, TLRs expressed in Treg cells generate effector functions and provide a direct link between innate and adaptive immunity. Furthermore, Treg cells may directly respond to proinflammatory microbial products, providing another link between innate and adaptive immunity (19). In our study, we were not able to study the expression of TLRs specifically on Treg cells, but the increased expression of Foxp3 and TLR4 was associated with FA.

In conclusion, our findings of the infiltration of Foxp3- and TLR4-positive cells in lamina propria and increase of TCR $\gamma\delta$ ⁺ IELs in children with untreated FA suggest an interplay between intestinal microbiota, innate immunity, and Foxp3-positive cells in FA. The low number of Foxp3 transcripts in relation to the Foxp3-positive cells may indicate that these Foxp3-positive cells in allergic inflammation are not able to mediate mucosal immune regulation.

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