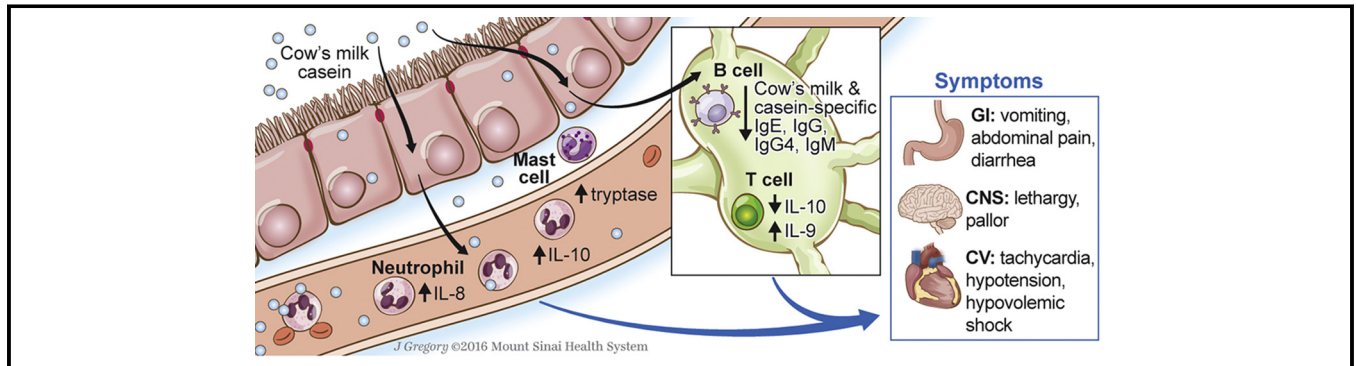


Humoral and cellular responses to casein in patients with food protein-induced enterocolitis to cow's milk

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GRAPHICAL ABSTRACT



Background: Food protein-induced enterocolitis syndrome (FPIES) is a non-IgE-mediated food allergy manifesting within 1 to 4 hours of food ingestion with repetitive emesis and lethargy. **Objective:** We sought to characterize immune responses to casein in children with FPIES caused by cow's milk (CM). **Methods:** Total IgE and IgM, CM-specific IgG, and casein-specific IgE, IgG, IgG₄, and IgM levels, as well as immunoglobulin free light chains, were measured in both patients with active and those with resolved CM-FPIES. Proliferating casein/T-effector cell counts were measured in children with CM-FPIES, children with IgE-mediated CM allergy, and those tolerating CM. Cytokine concentrations in the supernatants were quantified. Serum cytokine and tryptase levels were measured before and after a positive oral food challenge (OFC) result and compared with levels in those with a negative OFC result. **Results:** We found low levels of CM and casein-specific IgG and casein-specific IgG₄ in patients with CM-FPIES versus those tolerating CM ($P < .05$). Although we found both a high CD4⁺ T cell-proliferative response and T_H2 cytokines production after casein stimulation in children with CM-FPIES, results

were similar to those in control subjects. Significantly lower secretion of IL-10 and higher secretion of IL-9 by casein-stimulated T cells were found in patients with CM-FPIES versus those with IgE-mediated CM allergy. Lower baseline serum levels of IL-10 and higher tryptase levels were found in active CM-FPIES versus resolved CM-FPIES. We found a significant increase in serum IL-10 and IL-8 levels after a positive OFC result.

Conclusions: We confirm the paucity of humoral response in patients with CM-FPIES. IL-10 might play a key role in acquisition of tolerance in patients with CM-FPIES. Increased serum IL-8 levels in patients with active FPIES suggest neutrophil involvement. Elevated baseline serum tryptase levels in patients with active FPIES suggest low-grade intestinal mast cell activation or increased mast cell load. (J Allergy Clin Immunol 2016;■■■■:■■■-■■■.)

Key words: Food protein-induced enterocolitis syndrome, non-IgE-mediated, food, allergy, T_H1 response, T_H2 response, humoral response, T cells

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Abbreviations used

CFSE:	Carboxyfluorescein diacetate succinimidyl ester
CM:	Cow's milk
CMA:	Cow's milk allergy
fLC:	Free light chain
FPIES:	Food protein-induced enterocolitis syndrome
IgE-CM:	IgE-mediated cow's milk allergy
Ig-fLC:	Immunoglobulin-free light chain
IP-10:	Interferon-inducible protein 10
IQR:	25% to 75% Interquartile range
OFC:	Oral food challenge

Food protein-induced enterocolitis syndrome (FPIES) is characterized by profuse repetitive vomiting that typically occurs 1 to 4 hours after ingestion of the offending food and can be followed later by diarrhea.¹ Chronic exposure to cow's milk (CM)– or soy-based formula in young infants can result in failure to thrive and hypoalbuminemia.^{2,3} CM and soy proteins are the most common causes, although reactions to other foods, including grains (eg, rice and oat), meats (poultry), fruits, and vegetables, have been reported.^{4,6} Although the true incidence of FPIES is not known, a large population-based cohort study from Israel has reported the cumulative incidence of CM-FPIES to be 0.34% in the first year of life compared with 0.5% for IgE-mediated cow's milk allergy (CMA).⁷ Food allergy guidelines recommend using the medical history and oral food challenge (OFC) result to establish a diagnosis of FPIES.⁸⁻¹⁰

Because FPIES is a diagnosis that can be made clinically, without the need for an intestinal biopsy, there have been few studies reporting histologic findings. The pathophysiology of FPIES has not been clearly defined and requires further characterization. FPIES is considered part of a spectrum of allergic diseases that predominantly affect the gut, representing the more severe end of the spectrum. Results from previous studies suggested a central role of antigen-specific T cells and their production of proinflammatory cytokines. However, the evidence is weak, and these data need to be confirmed.¹¹ The characteristics of the intestinal inflammatory response are largely determined by the cytokine responses triggered by the pathologic mechanism. A deficit in TGF- β response and an overzealous TNF- α response can be important factors in patients with FPIES.^{12,13} Recently, a predominant antigen-specific T_H2 response was highlighted in patients with non-IgE-mediated gastrointestinal food allergies.¹⁴ Humoral immune responses can also be involved in the pathophysiology of FPIES, with an imbalance in specific IgA and IgG₄ antibody responses.^{13,15}

The aim of this study was to characterize the humoral and cellular immune responses to casein in children with FPIES caused by CM. The majority of children with CMA are polysensitized to several milk proteins, with casein being considered the dominant major allergen, followed by whey proteins, α -lactalbumin, and β -lactoglobulin.^{14,16}

METHODS**Study population**

The research protocol was approved by the Icahn School of Medicine at Mount Sinai Institutional Review Board. Written informed consent was obtained before enrollment. Patients aged 0 to 16 years and given a previous

diagnosis of CM-FPIES underwent an OFC in the inpatient clinical research unit to evaluate resolution, usually 18 months after the most recent FPIES reaction. A peripheral intravenous line was inserted before the OFC. During the OFC, CM was administered in 3 equal portions over an hour.^{17,18} The OFC result was considered positive based on the modified diagnostic criteria defined by Powell²: emesis in 1 to 4 hours and an increase in blood polymorphonuclear leukocyte count from baseline, peaking at 6 hours. After a negative OFC result, children were observed for 4 hours, whereas after a positive OFC result, they were treated and observed until stable and usually discharged within 6 hours. Blood samples were obtained immediately before the OFC (CBC with differential, serum samples for tryptase and humoral studies), as well as 4 hours after a negative OFC result and 6 hours after a positive OFC result (CBC with differential, serum tryptase measurement, and T-cell studies).

In the comparison groups patients with OFC-confirmed IgE-CMA, as well as patients with resolved IgE-CMA, were included. These patients were selected from a larger cohort of patients.¹⁹ Briefly, children were recruited prospectively and challenged with heated milk products according to a standard protocol; heated milk-tolerant subjects were subsequently challenged with nonheated milk. Patients with resolved CM-FPIES, as confirmed by a negative OFC result, were also included in the control group.

Antibody serum measurements

Casein-specific IgE, IgG, and IgG₄. Casein-specific IgE (limit of assay, 0.1–100 kU_A/L), IgG (0.02–2 mg_A/mL), and IgG₄ (0.02–300 mg_A/L) levels were measured with the UniCAP system (Thermo Fisher Scientific, Portage, Mich).

Casein-specific IgM. Serum casein-specific IgM levels were measured by means of ELISA. Briefly, plates were coated with casein in coating buffer. Plates were incubated with serum, and binding was detected with HRP-Anti-Human IgM or IgD (Bethyl Laboratories, Montgomery, Tex).

Free light chains: λ and κ . Total κ and λ immunoglobulin-free light chain (Ig-fLC) serum concentrations were determined by using an ELISA, as published previously.²⁰ Briefly, plates were coated overnight with goat-anti mouse IgG, blocked, and subsequently incubated with mouse-anti human κ or λ Ig-fLC (F κ -C8 or F λ -G9). After incubation with different dilutions of serum samples and standards, plates were incubated with horseradish peroxidase-labeled goat F(ab')₂-anti-human κ or λ Ig-fLC. For each sample, at least 3 data points were used to calculate the concentration.

PBMC casein stimulation and proliferation assay

PBMCs were collected from heparinized blood by means of centrifugation in Ficoll-Paque (GE Healthcare, Uppsala, Sweden) and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; 2.78 μ g/mL; AnaSpec, San Jose, Calif) for 10 minutes. Excessive CFSE was removed by washing with medium (AIM-V; Invitrogen, Grand Island, NY). Lymphocytes were cultured for 7 days with endotoxin-free α , β , and κ caseins (total concentration of 50 μ g/mL) or anti-CD3/CD28 expander beads (Invitrogen Dynal AS, Oslo, Norway) as positive controls, according to the manufacturer's protocol, and medium alone in the presence of IL-2 (20 ng/mL; R&D Systems, Minneapolis, Minn) at 37°C. The phenotypes were characterized by means of flow cytometry by using mAbs for CD3 (Sk7) and CD4 (Sk3). Fluorescence data were acquired on an LSR II running Diva 3.0 software (BD Biosciences, San Jose, Calif). Raw data were analyzed with FlowJo 8.5.3 software (TreeStar, Ashland, Ore).

Cytokines in T-cell culture supernatants

Culture supernatants were collected after incubation for 7 days and frozen at -80°C until use. All supernatants were thawed at the same time and assayed to determine the concentrations of secreted cytokines with FlowCytomix Kits (Bender MedSystems, Vienna, Austria), according to the manufacturer's instructions. These kits allowed the simultaneous quantification of 13 cytokines, including T_H1 cytokines (IFN- γ , IL-2, and TNF- α), T_H2 cytokines (IL-4, IL-5, IL-6, IL-10, and IL-13), T_H17 cytokines (IL-17 and

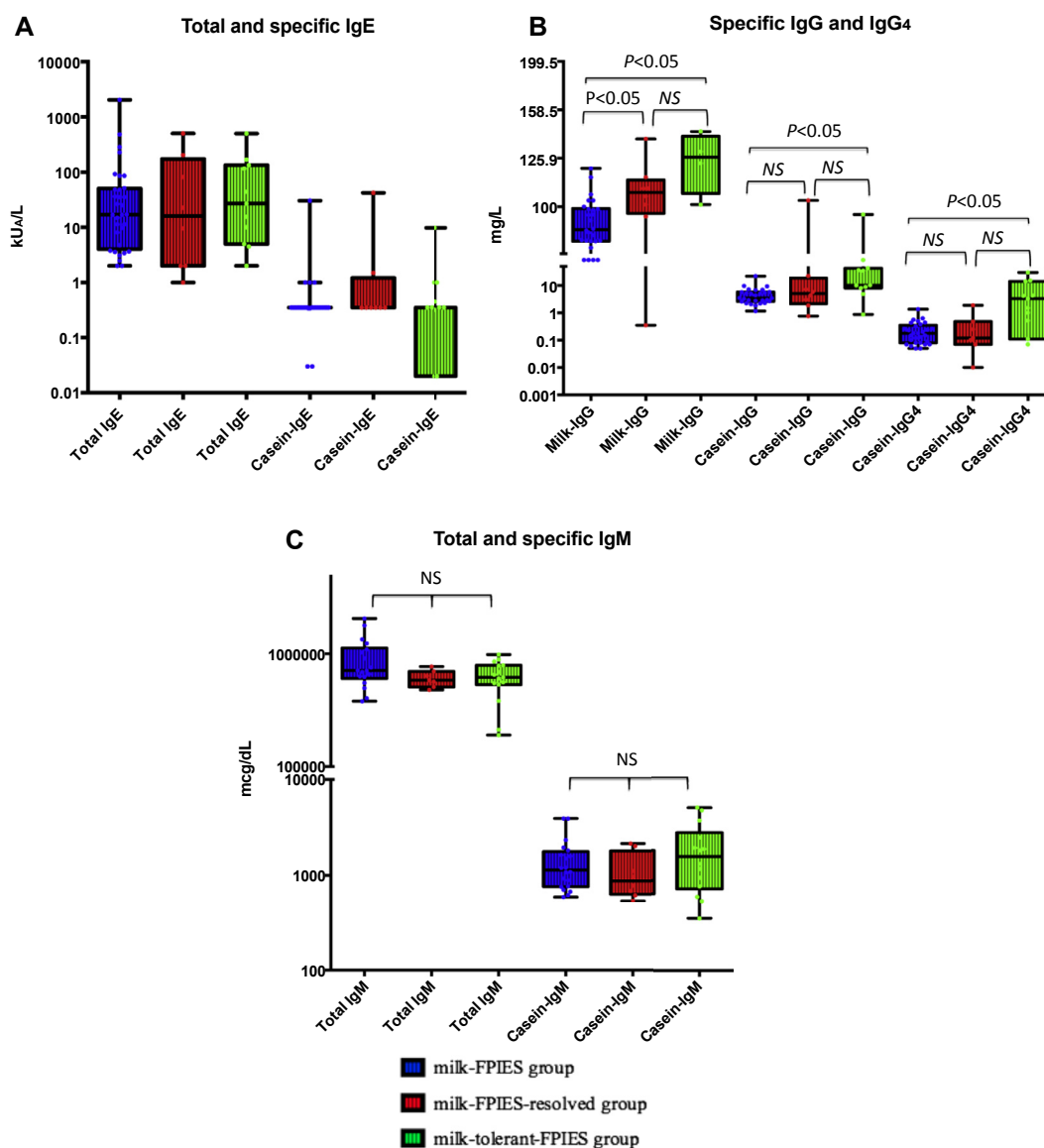


FIG 1. Comparison of humoral responses among children with CM-FPIES, children with resolved CM-FPIES, and those tolerating CM. **A**, Levels of total and casein-specific IgE. **B**, Levels of CM-specific IgG and casein-specific IgG and IgG₄. **C**, Levels of total and casein-specific IgM. Results are presented as a box plot with means and 25th to 75th percentiles, with the limits of whiskers being the smallest and largest values. NS, Not significant.

IL-23), T_H9 cytokines (IL-9), and T_H22 cytokines (IL-12 and IL-22). The test samples were acquired on an LSR II running Diva 3.0 software (BD Biosciences). For each analysis, up to 10,000 events were acquired. The mean concentration of each cytokine was expressed as picograms per milliliter. By using these kits, the minimum detectable concentrations of IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p70, IL-13, IL-17A, IL-22, and TNF- α are 1.6, 4.2, 16.4, 20.8, 1.6, 1.2, 1.5, 1.9, 1.5, 4.5, 2.5, 43.3 and 3.2 pg/mL, respectively.

Serum cytokines

Serum cytokines were measured with a multiplex ELISA kit (Merck Millipore, Billerica, Mass) run on a Luminex 200 platform and analyzed with Millipore Analyst software. The lower limit of detection is 1 pg/mL. Serum

samples were obtained immediately before and 4 to 6 hours after a food challenge.

Serum tryptase

Total serum tryptase levels were measured immediately before and 4 to 6 hours after a food challenge with a commercially available ImmunoCAP Tryptase Assay (Thermo Fisher Scientific). The lower limit of detection is 1 μ g/mL.

Statistical analysis

Analyses were performed with SAS/STAT Version 9.2 software (SAS Institute, Cary, NC). Comparisons of between-group data for continuous variables were assessed with use of a Mann-Whitney *U* test. The Wilcoxon signed-rank test was used for paired analyses. For comparisons between

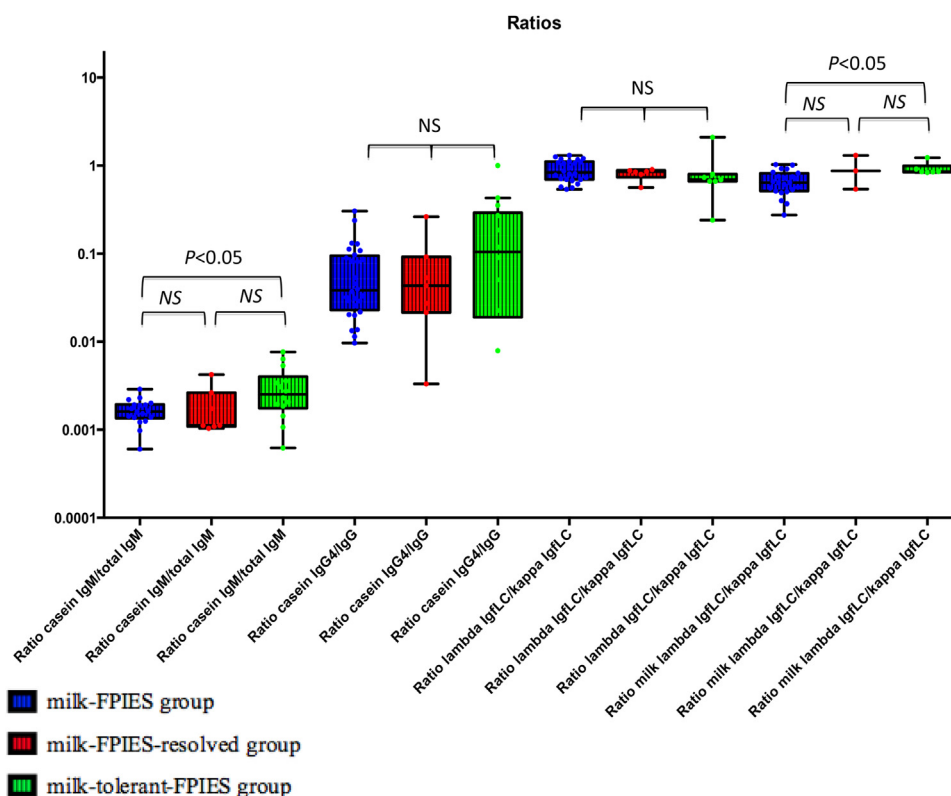


FIG 2. Comparison of the casein IgM/total IgM ratio, casein IgG₄/IgG ratio, and λ Ig-fLC/ κ Ig-fLC and milk λ Ig-fLC/ κ Ig-fLC ratios between groups (ie, CM-FPIES, resolved CM-FPIES, and CM-tolerant FPIES).

more than 2 groups, the Kruskal-Wallis test with Dunn *post hoc* test was used. A probability level of less than 5% was considered significant.

RESULTS

Humoral responses

Because of antigen specificity in patients with CM-FPIES, we chose to characterize humoral antibody responses to casein. We evaluated 38 children with a previous diagnosis of CM-FPIES; 29 reacted during CM OFC (active CM-FPIES group; median age, 4 years; 25% to 75% interquartile range [IQR], 2.3 to 6.9 years), and 9 did not react (resolved milk FPIES group; median age, 2 years; IQR, 1.9-3.4 years; $P = .04$). Fifteen children tolerant to CM but with FPIES to foods other than CM (CM-tolerant FPIES group; median age, 3 years; IQR, 2-4.3 years) were used as negative controls.

Specific IgE antibody levels to casein were positive in 2 patients with CM-FPIES (6.9%): 1 and 30.2 kU_A/L, respectively (Fig 1, A). Serum casein-specific IgG and IgG₄ levels were significantly higher in the CM-tolerant FPIES group compared with the active CM-FPIES group ($P < .05$, Kruskal-Wallis test with the *post hoc* Dunn test) and the resolved CM-FPIES group, although the difference did not reach statistical significance (Fig 1, B). The casein-specific IgG₄/IgG ratios were lower in patients with active CM-FPIES and patients with resolved CM-FPIES compared with the CM-tolerant FPIES group, although the difference did not reach statistical significance (Fig 2). Although no difference was found regarding levels of casein-specific IgM and total IgM between the groups

(Fig 1, C), the mean ratio of casein-specific IgM/total IgM was significantly lower in patients with active CM-FPIES compared with ratios in those tolerating milk ($P < .05$, Kruskal-Wallis test with the *post hoc* Dunn test; Fig 2).

We also investigated Ig-fLCs because they have been demonstrated to activate mast cells in an antigen-specific manner and fLC concentrations were shown to be increased in patients with inflammatory bowel diseases and those with eosinophilic esophagitis.^{21,22} Sera from 23 patients in the active CM-FPIES group, 6 patients in the resolved CM-FPIES group, and 6 patients in the CM-tolerant FPIES group were tested for total and CM-specific free κ and λ light chains. No significant differences were found in the amount of total κ Ig-fLC, λ Ig-fLC, CM-specific κ fLC, or λ Ig-fLC/ κ Ig-fLC ratio between the groups (Figs 2 and 3). However, CM-specific λ Ig-fLC/ κ Ig-fLC ratios and CM-specific λ fLC concentrations were significantly lower in patients with active CM-FPIES compared with those tolerating CM ($P < .05$, Kruskal-Wallis test with the *post hoc* Dunn test; Figs 2 and 3).

T-cell and cytokine responses after casein stimulation

For cellular studies, PBMCs from 8 children reacting during CM OFC (active CM-FPIES group) were available and compared with 18 patients with IgE-mediated milk allergy (IgE-CMA group) and 7 milk-tolerant patients (resolved IgE-CMA group). The median age of the patients with active CM-FPIES was 8.4 years (IQR, 3.4-12.3 years), that for patients with

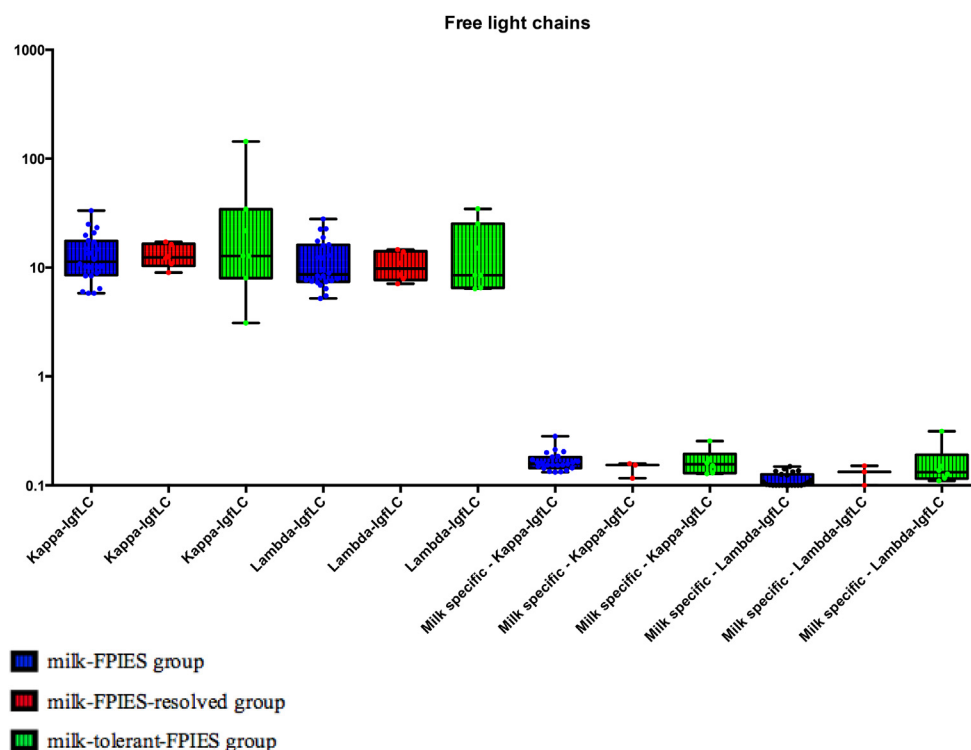


FIG 3. Comparison of concentrations of fLCs (κ Ig-fLC and λ Ig-fLC, as well as milk-specific κ Ig-fLC and milk-specific λ Ig-fLC) among children with CM-FPIES, resolved CM-FPIES, and CM-tolerant FPIES.

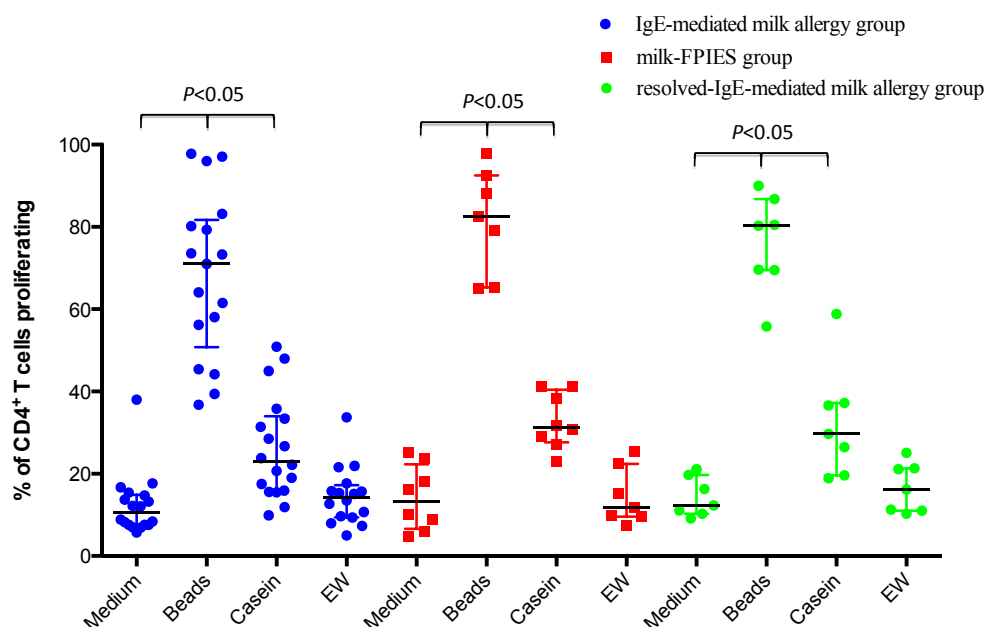


FIG 4. Percentage of proliferating casein T effector cell frequency (CFSE^{low}CD3⁺CD4⁺) by using 3 stimulation conditions (medium, beads, and casein) in children with IgE-mediated CMA, children with CM-FPIES, and those patients with resolved-IgE mediated CMA. EW, Egg white.

IgE-CMA was 7.6 years (IQR, 6.8-9.7 years), and that for patients with resolved IgE-CMA was 5.9 years (IQR, 4.4-10.6 years; $P = .55$). Proliferating casein-specific CD4⁺ T-cell frequency (CFSE^{low}CD3⁺CD4⁺) was similar among groups: 31.2% (23.1% to 41.2% in children with CM-FPIES), 23% (9.9% to 50.9%) in children with IgE-CMA, and 29.7% (18.9% to

58.8%) in control subjects with resolved IgE-CMA (Fig 4). After antigen-specific stimulation, there was no significant difference among the groups in TH2 cytokine secretion (IL-5, IL-6, and IL-13), as well as in IFN- γ and TNF- α secretion (Fig 5, A-E). However, we observed a significantly lower secretion of IL-10 in patients with active CM-FPIES compared

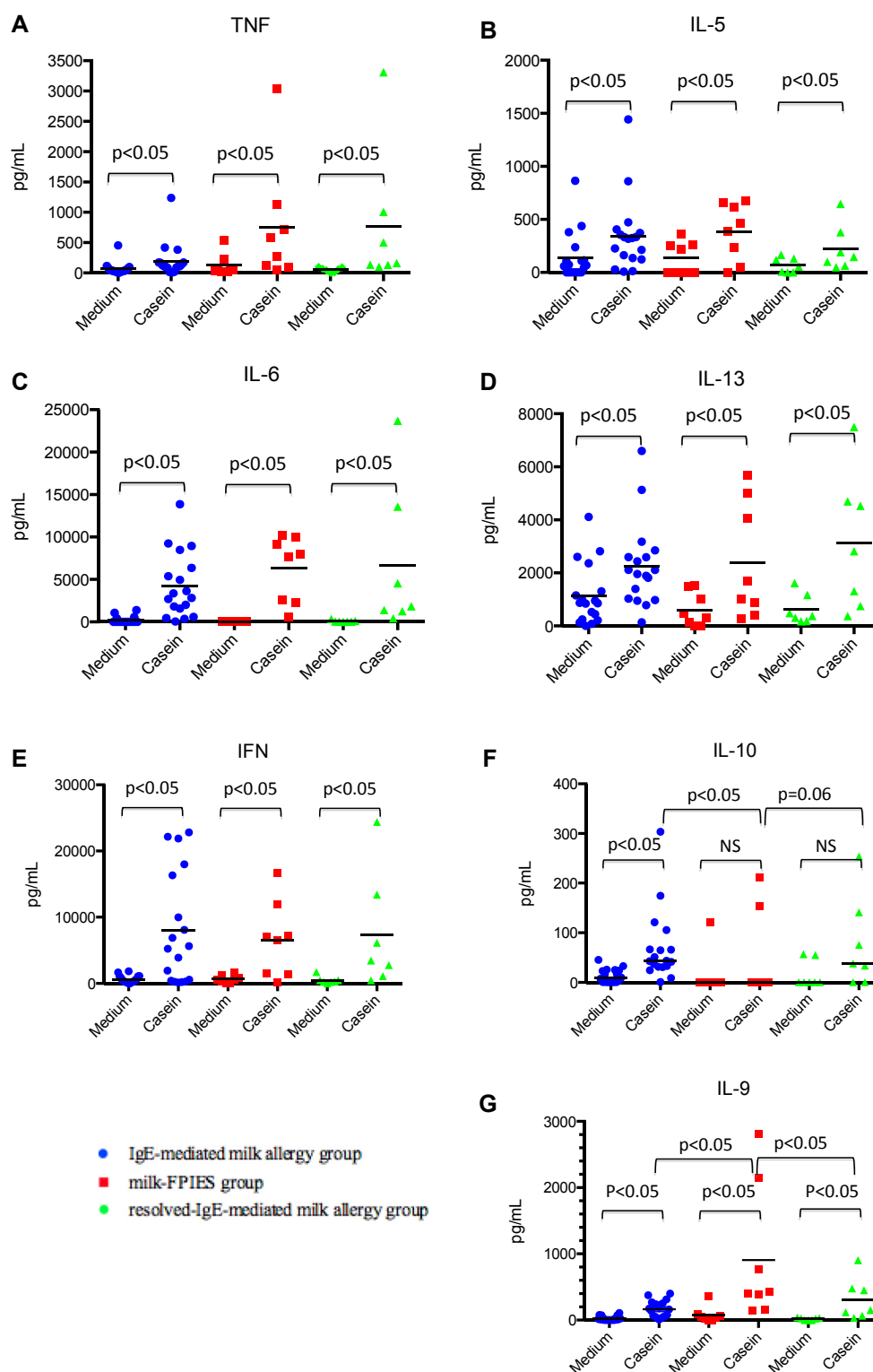


FIG 5. A-G, Cytokine concentrations (in picograms per milliliter) in supernatants (after 7 days of incubation) measured by using a multiplex immunoassay (13plex FlowCytomix Multiplex Kit; eBioscience, San Diego, Calif) in children with IgE-mediated CMA, children with CM-FPIES, and those with resolved-IgE mediated CMA.

with that seen in patients with IgE-CMA (Fig 5, F). In addition, IL-9 secretion was significantly higher in patients with active CM-FPIES compared with those with IgE-CMA ($P < .05$; Fig 5, G).

Serum cytokine levels before and after an OFC

We set out to measure serum cytokine levels because of lethargy, pallor, and hypotension, which are frequently seen in patients with acute FPIES, suggesting a systemic reaction

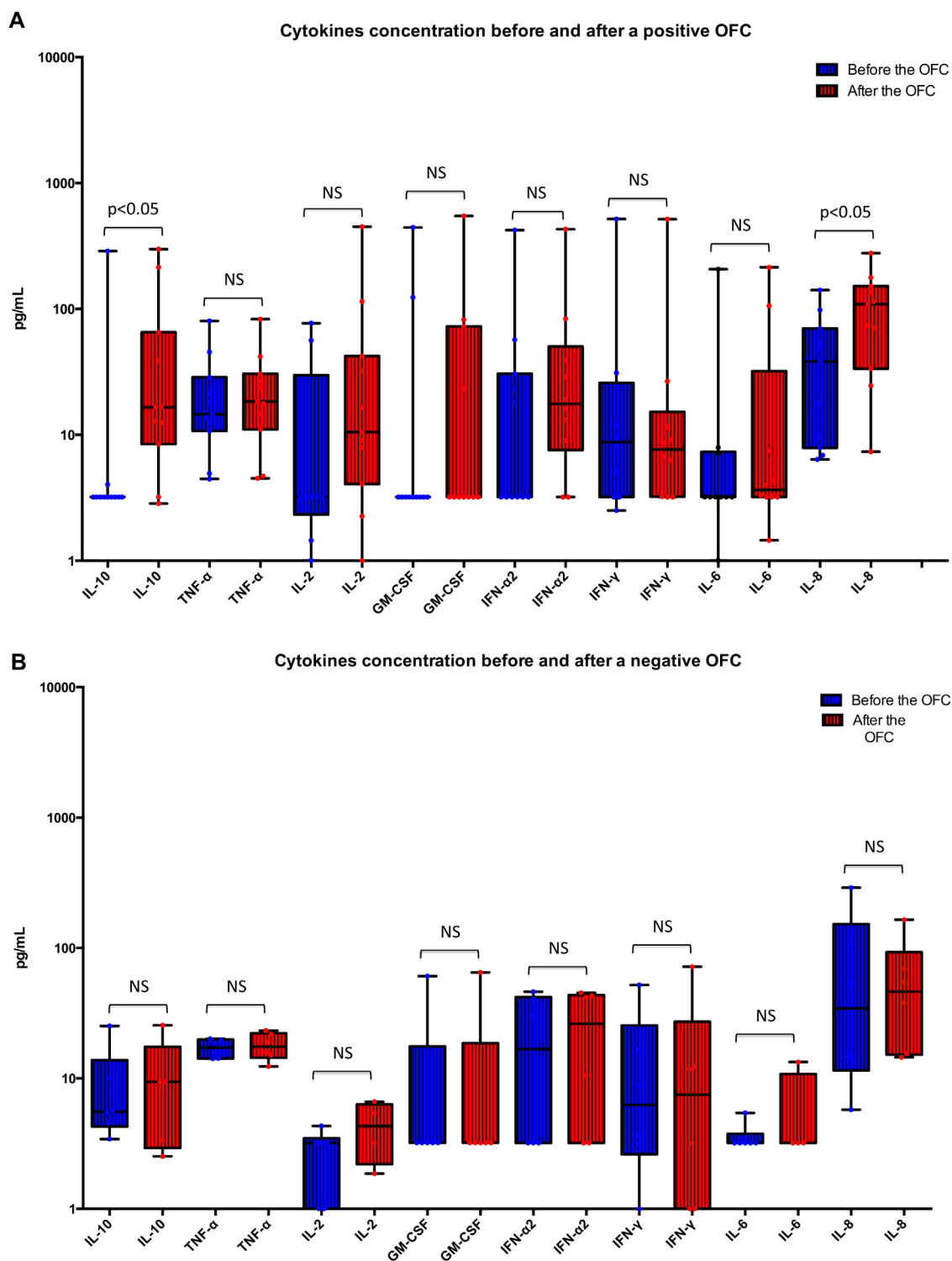


FIG 6. Cytokine concentrations (in picograms per milliliter) in sera of patients with CM-FPIES before and after a positive (**A**) or negative (**B**) OFC result in patients with a previous diagnosis of CM-FPIES. NS, Not significant.

beyond the gastrointestinal tract. Serum cytokine concentrations were measured before and after a positive OFC result in 11 patients with active FPIES to milk ($n = 6$), egg ($n = 1$), rice ($n = 1$), chicken ($n = 1$), beef ($n = 1$), and soy ($n = 1$). These data were compared with those of a group of 6 patients with resolved FPIES and a negative OFC result to rice ($n = 1$), milk

($n = 2$), and soy ($n = 3$). The median age of patients with active FPIES with a positive OFC result was 6.8 years (IQR, 3.4-12.5 years), and that of patients with resolved FPIES was 3.8 years (IQR, 3.4-7.3 years; $P = .1$). We found a significant increase in serum IL-10 concentration after a positive OFC result, whereas no significant change was found in patients

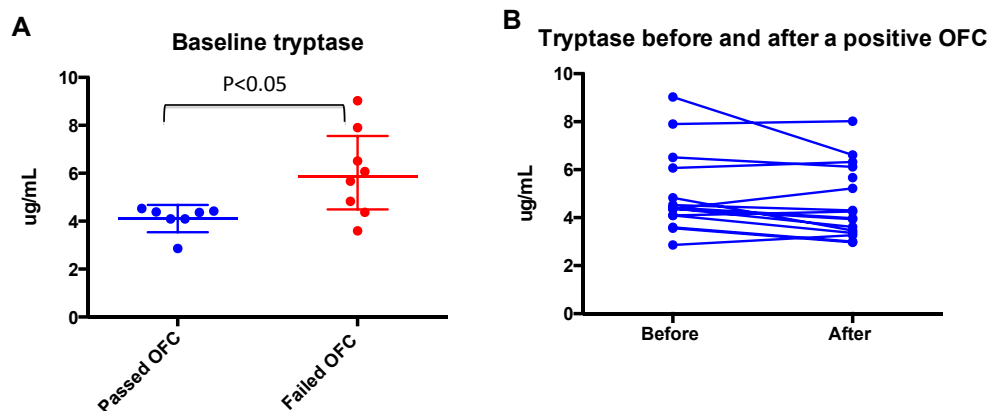


FIG 7. A, Comparison of baseline tryptase levels in patients with negative and positive OFC results. B, Tryptase levels (in micrograms per milliliter) before and after a positive OFC result in children with CM-FPIES.

with a negative OFC result (Fig 6). The baseline median concentration of IL-10 was significantly higher in patients with a negative OFC result compared with those with a positive OFC result ($P < .05$). We found a significant increase in IL-8 secretion after a positive OFC result ($P < .05$), whereas the secretion of monocyte chemoattractant protein 1 and macrophage inflammatory proteins 1 α and 1 β did not change significantly. The baseline serum interferon-inducible protein 10 (IP-10) level was higher in patients with a positive OFC result compared with those with a negative OFC result and increased slightly after a positive OFC result; however, the differences did not reach statistical significance.

Serum tryptase levels before and after OFC

To test for subclinical mast cell activation, we measured serum tryptase levels in a separate group of 9 patients with FPIES with a positive OFC result and 7 patients with a negative OFC result. Baseline serum tryptase levels were significantly higher in patients with a positive OFC result compared with those with a negative OFC result ($P < .02$; Fig 7, A). Serum tryptase levels did not change significantly after a positive OFC result (baseline: median, 4.4 $\mu\text{g/mL}$ [range, 2.9–9 $\mu\text{g/mL}$] vs after the OFC: median, 4.1 $\mu\text{g/mL}$ [range, 2.9–8 $\mu\text{g/mL}$]; Fig 7, B).

DISCUSSION

Although FPIES is relatively common in infants and young children and has been reported in adults, the mechanisms underlying this disorder remain poorly characterized.^{7,11,23} We report a remarkable paucity of humoral immune responses against casein, the major milk allergen, in children with CM-FPIES, indirectly suggesting a lack of T-cell help and lack of involvement of T cells in acute FPIES pathophysiology. Our data suggest that IL-10 might play a central role in tolerance acquisition for this disorder; however, IL-10 is most likely not produced by T cells. The increase in serum IL-8 levels in patients with positive OFC results supports the previously suggested role of neutrophils in the pathogenesis of FPIES. We also found a potential involvement of peripheral antigen-specific T cell–derived proinflammatory

cytokines (ie, IFN- γ , TNF- α , and IL-9) known to regulate the intestinal barrier permeability. All these findings are summarized in Table I.

Humoral responses in patients with FPIES are poorly characterized. Based on the fact that FPIES is rare in exclusively breast-fed children, breast milk IgA is hypothesized to have a protective role.²⁴ Few studies have investigated the potential role of IgA in the pathomechanism of FPIES.^{15,25} Jejunal biopsy specimens of patients with FPIES revealed increased numbers of IgM- and IgA-containing plasma cells,^{26,27} but how this increase affects antibody secretion in the gastrointestinal tract remains unclear. In contrast to Powell's data,² we previously reported that children with CM-FPIES had low serum levels of casein-specific IgG, IgG₄, and IgA antibodies.¹³ Here we provide additional evidence of a paucity of humoral immune responses directed against casein in patients with CM-FPIES. Discrepancies between studies can be explained by different levels of allergen exposure before testing. Indeed, the difference observed between CM-tolerant subjects and patients with active CM-FPIES might be due to the fact that the tolerant subjects are not avoiding milk in their diet. It is well known that IgG antibody synthesis is influenced by antigen exposure. No statistical difference in IgG₄ levels has been found between children with active CM-FPIES and children with resolved CM-FPIES. We measured IgM and fLC concentrations to explore the possibility of their involvement in an alternative mast cell activation pathway but found no evidence of their involvement in FPIES.^{20–22,28} The overall paucity of humoral responses can be caused by lack of T-lymphocyte help, unresponsiveness of B cells, or inadequate antigen processing.

FPIES is often considered a T cell–mediated disorder; however, there are few studies investigating the role of T cells in patients with FPIES with inconclusive results, questioning their role in this disorder.^{15,29–34} Although we found a high CD4⁺ T-cell proliferative response after casein stimulation in children with CM-FPIES, levels were similar to those found in children with IgE-CMA and control subjects who outgrew IgE-CMA (Fig 4). Our results are in line with previous studies showing high T-cell responses to casein stimulation in patients with IgE-mediated and non-IgE-mediated gastrointestinal CMA.^{14,35}

TABLE I. Summary of pathologic findings in patients with FPIES compared with control subjects

	Positive findings	Negative findings
Humoral responses	Lower levels of milk-specific IgG and IgG ₄ in patients with CM-FPIES compared with those in patients tolerating CM (active vs resolved)	No difference in levels of specific and total IgM
	Trend toward lower ratios of casein-specific IgG ₄ /total IgG in patients with active CM-FPIES and patients with resolved CM-FPIES compared with those in patients with CM-tolerant FPIES	No difference in levels of specific IgD to casein, α -lactalbumin, and β -lactoglobulin
	Lower casein-specific IgM/total IgM ratio in patients with active CM-FPIES compared with that in patients tolerating CM	No difference in amounts of total κ Ig-fLC, λ Ig-fLC, CM-specific fLC, or λ Ig-fLC/ κ Ig-fLC ratio
	Significantly lower milk-specific λ Ig-fLC/ κ Ig-fLC ratio in patients with active CM-FPIES compared with that in patients tolerating CM	
T-cell and cytokine responses	Significantly lower secretion of IL-10 in patients with CM-FPIES compared with that in patients with IgE-CMA	Proliferating casein-specific CD4 ⁺ T-cell frequency in patients with CM-FPIES similar to patients with IgE-CMA and control subjects
	Significantly higher IL-9–induced secretion in patients with CM-FPIES compared with that in patients with IgE-CMA	No difference found in T _H 2 cytokine secretion, as well as IFN- γ and TNF- α secretion
Serum cytokines	At baseline:	No change in the secretion of MCP-1, MIP-1 α , and MIP-1 β after a positive OFC result
	1. Higher median concentration of IL-10 in patients with a negative OFC result compared with those with a positive OFC result	
	2. Higher median IP-10 concentration in patients with a positive OFC result	
	Significant increase in IL-10 and IL-8 secretion after a positive OFC result	
Tryptase level before and after an OFC	Baseline serum tryptase levels significantly higher in patients with FPIES with a positive OFC result compared with levels in those with a negative OFC result	Serum tryptase levels not significantly different after a positive OFC result in patients with FPIES

MCP-1, Monocyte chemoattractant protein 1; MIP, macrophage inflammatory protein.

Similar to the data from Morita et al,¹⁴ we detected T_H2 cytokines (IL-5, IL-6, and IL-13) in the supernatants of casein-stimulated PBMCs from children with CM-FPIES and IgE-CMA (Fig 5). However, the absence of a significant difference in comparison with control subjects tolerating CM emphasizes that neither the presence of casein-specific T-cell responses *per se* nor T_H2 skewing of T-cell responses are solely responsible for manifestations of FPIES. Previous studies have shown that nonallergic control subjects had lower T-cell proliferation, as well as absence of T_H2 responses, after stimulation with the incriminated allergen compared with children with IgE-CMA and patients with gastrointestinal CMA.^{14,35} These discrepancies between studies can potentially be explained by differences in the age of the patients included. Indeed, children in the Morita et al¹⁴ study were young (median age, 2 months; range, 1–4 months) with recent symptoms of chronic FPIES, whereas our lymphocyte studies were performed in older children after a prolonged period of dietary milk avoidance (minimum, 12 months). Differences in consumption of CM are important because it has been shown mainly in oral immunotherapy trials that it can influence the reactivity of specific T cells.³⁶ Detection of T_H2 cytokines, which are known to facilitate IgE production in patients with FPIES, could explain why some patients have food-specific IgE either at presentation or during follow-up (referred to as “atypical” FPIES).^{4,6,37} A recent report described a patient with a shift from IgE-CMA to pure CM-FPIES, suggesting that the phenotype of allergic disease is fluid and might progressively evolve in infants and young children.³⁸ The relationship between IgE and

non-IgE mechanisms in patients with FPIES requires further clarification.

Baseline serum IL-10 levels were significantly higher in patients with resolved CM-FPIES (negative OFC result), confirming that increased IL-10 expression is associated with tolerance acquisition in patients with FPIES.³⁹ IL-10 has been mainly highlighted for its role in the induction of oral tolerance, which has been demonstrated in multiple studies of OIT, showing increased IL-10 levels during tolerance acquisition.⁴⁰ The deletion/anergy of reactive T cells, as well as increased regulatory T cells with the production of IL-10, are mechanisms involved in oral tolerance.⁴¹ We also found an increase in serum IL-10 levels after a positive OFC result, whereas no significant difference was found after a negative OFC result. However, the absence of an IL-10 increase in the casein-stimulated PBMC culture supernatants in the patients with FPIES who reacted during milk OFC suggests that circulating T cells are not the source of IL-10. Other cell populations, such as monocytes, can produce compensatory IL-10 in response to antigen exposure, whereas T cells have impaired capacity to secrete IL-10 in patients with active FPIES. In addition, direct comparison of serum and supernatant cytokine results is not possible because different methods were used to detect cytokines (ie, FlowCytomix versus Multiplex ELISA kit), with possible variations between results.

Casein-specific production of IL-9, a cytokine closely associated with T_H2 responses and that has been shown to increase intestinal mast cell numbers, indirectly influencing intestinal permeability,⁴² was significantly greater in children

with CM-FPIES compared with that in children with IgE-CMA ($P < .05$; Fig 5, G). A recent study suggested that mast cell-derived IL-9 is essential in patients with intestinal anaphylaxis.⁴³ Those data raise the possibility of mast cell involvement in patients with FPIES, which could represent a variant of intestinal anaphylaxis. Of note, delayed-onset anaphylaxis has already been described, particularly after ingestion of mammalian food products (eg, beef and pork) in patients with positive specific IgE levels to galactose α -1,3-galactose.⁴⁴ Higher mean baseline tryptase levels were found in patients with FPIES with positive OFC results compared with patients with FPIES with negative OFC results ($P < .05$). Baseline tryptase levels have been shown to be higher in patients with IgE-mediated food allergy compared with those in control subjects.⁴⁵ Higher baseline tryptase levels might reflect a higher mast cell load or low-grade activation of mast cells. However, we did not find a significant increase in serum tryptase levels after positive OFC results, suggesting that mast cells do not degranulate during FPIES reactions. However, lack of a serum tryptase level increase is commonly seen in patients with IgE-mediated food-induced anaphylaxis,^{46,47} which can be explained by slow onset of reaction or because mucosal mast cells contain significantly less tryptase than cutaneous mast cells.^{48,49} We also tested basophil activation by measuring CD63 upregulation on stimulation with casein, as measured by using flow cytometry (data not shown), and found no evidence of basophil degranulation. It remains to be determined whether alternative pathways of mast cell activation without degranulation play a role in FPIES.

TNF- α and IFN- γ were found after stimulation of PBMCs by casein in children with active CM-FPIES (Fig 5, A and E; $P < .05$). Although we found a trend toward higher levels of TNF- α and IFN- γ in children with CM-FPIES compared with those with IgE-CMA, the difference did not reach statistical significance. Release of the proinflammatory cytokines TNF- α and IFN- γ by activated PBMCs has been previously suggested to induce local intestinal inflammation in patients with FPIES.^{31,39} Indeed, it has been hypothesized that a high level of TNF- α release by antigen-specific T cells could act synergistically with IFN- γ to increase intestinal permeability and then increase the amount of antigen uptake into the submucosa with further activation of antigen-specific T cells.³¹ TGF- β has also been incriminated in the pathogenesis of FPIES, particularly a decreased expression of the TGF- β receptors that is known to protect the intestinal barrier from the penetration of foreign antigen, have been found in the intestinal mucosa of patients with FPIES.^{12,27,30,50} We have previously reported that children with active milk FPIES have deficient T cell-mediated TGF- β responses to casein.¹³

An increase in absolute neutrophil count is one of the major criteria to consider a positive OFC result in patients with FPIES.²

We found a significantly higher serum level of IL-8 in patients with FPIES after a positive OFC result compared with those seen in patients with a negative OFC result. IL-8 is a potent chemoattractant of neutrophils from peripheral blood into tissues and is involved in the initiation and amplification of inflammatory processes.⁵¹ IL-8 is also secreted by neutrophils. The functional role of neutrophils in patients with IgE-mediated diseases has been demonstrated.⁵² Antigen-dependent IL-8 release has been suggested to occur through IgE-dependent mechanisms.⁵² The role of neutrophils and particularly IL-8 in the pathogenesis of FPIES requires further investigation.

We also found a trend toward an increase in IP-10 levels in patients with positive OFC results. In response to IFN- γ , IP-10 is secreted by several cell types (eg, monocytes, endothelial cells, and fibroblasts) and has been attributed several roles, particularly as a chemoattractant for activated T cells and monocytes/macrophages.⁵³ Recently, an increase in IP-10 and IL-8 levels was demonstrated in patients with IgE-mediated food allergy, highlighting the importance of these cytokines in patients with these disorders.⁵³ The roles of these cytokines in the pathogenesis of FPIES need to be evaluated more specifically.

The main limitation of our study is the fact that most of our subjects had acute FPIES, whereas previous studies included predominantly patients with chronic FPIES, making direct comparison of the results difficult.^{14,39} Other limitations include a relatively small sample size for cellular studies and lack of age-matched control subjects without food allergy. We chose to investigate casein, which is the dominant major allergen in patients with IgE-CMA, but other CM proteins, such as whey proteins, or nonprotein allergens, such as sphingolipids, might be more important than casein in patients with CM-FPIES.⁵⁴ We did not measure CM or casein-specific IgG₁ antibody levels, which are thought to play a role in the pathophysiology of Heiner syndrome, a very rare non-IgE-mediated food allergic disorder. Therefore there still remains a possibility that food-specific IgG₁ antibodies might be important in patients with FPIES.⁵⁵ Finally, our samples were obtained from patients with acute FPIES confirmed based on a supervised OFC, in which an isolated exposure to an allergen after a prolonged period of dietary avoidance might not be sufficient to produce changes detectable in peripheral blood sampled at a single time point within hours of the challenge. In spite of these limitations, our study provides the most robust characterization of peripheral blood immune biomarkers in patients with FPIES to date.

In conclusion, our data suggest that IL-10 regulatory cytokines might be involved in the achievement of oral tolerance in patients with FPIES and that IL-10 is secreted by cell types other than T lymphocytes. Neutrophils can also play a major role, as suggested by the increase in serum IL-8 levels found in patients with a positive OFC result. Our findings provide support for the potential role of IL-9 in the pathogenesis of FPIES, potentially through its influence on intestinal permeability.¹² Further studies are needed for a better understanding, particularly including measurement of cytokines in stool from patients with FPIES before and after OFCs. Increased baseline serum tryptase levels in patients with active FPIES might indicate low-grade intestinal mast cell activation or an increased mast cell load, although there is no evidence of mast cell degranulation during acute FPIES in the setting of an OFC. We confirm the paucity of humoral responses to casein in patients with CM-FPIES, indirectly providing evidence of a lack of T-cell help in patients with FPIES. Further studies are needed to confirm the role of these cells and their cytokines in the pathogenesis of FPIES and to determine the mechanism of antigen specificity in patients with FPIES. A better understanding of the pathophysiology of FPIES will result in development of noninvasive diagnostic tests and lead to better management of these patients.

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Key messages

- Neutrophils can play a major role in FPIES, as suggested by the increased level of serum IL-8 after a positive OFC result.
- Increased baseline serum tryptase levels in patients with FPIES suggest low-grade intestinal mast cell activation or an increased mast cell load.
- IL-9 might be involved in the pathogenesis of FPIES, potentially through its influence on intestinal permeability.

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