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Casein-specific IL-4- and IL-13-secreting T cells: a tool to implement diagnosis of cow's milk allergy

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Abstract

Background: Cow's milk allergy (CMA) is a frequent food allergy in young children. The oral food challenge is the gold standard for diagnosis, and there is currently no reliable biological test. Our aim was to evaluate the diagnostic potential of a functional assay quantifying allergen-specific Th2 cells in CMA children.

Methods: A total of 29 children aged 2.8–10.5 years underwent a double-blind, placebo-controlled food challenge (DBPCFC) to cow's milk. Blood was collected before performing the DBPCFC, and peripheral mononuclear cells were cultured in an 18-h ELISpot assay with casein, α -lactalbumin, or β -lactoglobulin. Numbers of antigen-specific IL-4- and IL-13-secreting lymphocytes and serum-specific IgE, IgG4, and total IgE levels were assessed. Receiver operating characteristic (ROC) curves were generated.

Results: A total of 17 (59%) children reacted to cow's milk and were therefore considered as allergic to cow's milk (CMA). The mean number of casein-specific IL-4- and IL-13-secreting T cells was higher in CMA than in non-CMA children ($P = 0.009$, 0.004 , respectively). Moreover, it was inversely correlated with the cumulative dose of cow's milk tolerated ($P = 0.003$, 0.0009 , respectively). ROC curve of combined IL-4 and IL-13 analysis showed an area under the curve of 0.98 (95% CI 0.90–1.06). For a cutoff of 10 IL-4- and 12 IL-13-secreting T cells, sensitivity and negative predictive value were 100%.

Conclusions: Enumeration of casein-specific IL-4- and IL-13-secreting T cells appears a promising tool to improve diagnosis and, if confirmed in larger studies, could permit less frequent use of the oral food challenge.

The incidence of food allergy has been steadily increasing over the past years especially in children and is associated with a delay of spontaneous regression (1–4). Cow's milk allergy (CMA) is the most common food allergy in children, with rates estimated at 2–3% (2). Between 19% and 78% of subjects outgrow their milk allergy by 5 years of age (3, 5–8). Food allergy can induce from mild itching to life-threatening anaphylaxis. The therapeutic management of severe CMA patients with a risk to develop anaphylaxis is based on strict allergen avoidance and self-injectable epinephrine.

Mechanisms underlying food allergy include a defective immune tolerance to food allergens, leading to abnormal

expansion of allergen-specific type 2 helper (Th2) lymphocytes and deficient immune regulation by regulatory T cells (9–11).

There is currently no biomarker able to reliably confirm the presence of CMA, to predict remission or support that tolerance was induced upon desensitization (12, 13). Larger skin prick test weal size has been associated with the persistence of CMA (6, 14). Low levels of cow's milk-specific Immunoglobulin E (IgE) were associated with recovery, whereas levels higher than 50 kU/l were associated with persistence of allergy (5, 15, 16). Monitoring of casein-specific IgEs was described to have good specificity to predict CMA (17, 18). In such context, oral food challenge is still the gold

standard test to diagnose CMA according to international guidelines in spite of the fact the test is expensive, laborious, requiring a specialized environment and, most importantly, potentially associated with a risk of anaphylaxis (2, 19, 20). Therefore, developing a biological test that may confirm or refute the diagnosis of food allergy and determine thresholds of reactivity to the oral food challenge is of utmost importance. Here, we explored whether quantification of peripheral allergen-specific T cells, using an enzyme-linked immunospot (ELISpot) assay, in CMA children compared with nonallergic children could serve as a reliable functional biological test. We deliberately chose to focus on IL-4- and IL-13-producing Th2 cells whose pathogenic role in allergy, including CMA, is clearly established (21, 22). We analyzed children with CMA undergoing a double-blind, placebo-controlled food challenge (DBPCFC) before starting a tolerance induction protocol.

Methods

Study design

We performed a prospective study in children referred with CMA to our department. The protocol was approved by the Ile de France V ethics committee. Written informed consent was obtained from all parents.

Inclusion criteria

Children presented a diagnosis of IgE-mediated CMA (defined as a history of reaction to the ingestion of cow's milk product and positive skin prick test (wheal ≥ 3 mm with no reaction to the negative control) to cow's milk and specific IgE >0.35 kU/l). Children were older than 3 years. All the patients included had a severe form of CMA allergy. Effectively, a majority of them had experienced severe symptoms after ingestion of milk ($n = 17$) and/or suffered from asthma ($n = 19$) and/or from multiple food allergy ($n = 18$).

Seven sex- and age-matched control subjects without any known allergy/atopic status have been analyzed.

Double-blind, placebo-controlled oral food challenge

The DBPCFC was performed outside of episodes of exacerbation of acute illness. Children were admitted to the allergy clinic on two separate days, and food challenges were performed under the supervision of an experienced allergist. A peripheral intravenous line was inserted in each patient before starting the challenge (23).

Increasing doses of cow's milk were administered 20 min apart. Doses were 1, 2, 5, 10, 20, 40 and 80 ml for a cumulative dose of 158 ml (5.7 g of cow's milk proteins). The vehicle used was mashed potatoes with cow's milk or water for placebo. Challenge was stopped in case of a clinical reaction compatible with an allergic reaction. Antihistamine, methylprednisolone, bronchodilator or epinephrine was administered if necessary. Cumulative dose of cow's milk tolerated defined the reactive threshold in CMA children. No clinical

reaction to a cumulative dose of 158 ml of cow's milk defined nonallergic children (non-CMA).

Biological markers

Measurement of IgE and IgG

Serum levels of total IgE and specific IgE and IgG4 against major cow's milk allergens (casein, α -lactalbumin, and β -lactoglobulin) were determined using the immunoCAP system (Thermo Fischer, Uppsala, Sweden).

ELISpot assay to cow's milk protein

Blood samples were collected before starting the food challenge test in citrate phosphate dextrose adenine (CPDA)-pretreated tubes processed within 24 h. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation. The ELISpot assay was performed as previously described (21). Briefly, PVDF (Millipore, Guyancourt, France) microplates were permeabilized using 35% ethanol, washed five times with phosphate buffer saline (PBS), and coated overnight at 4°C with antibodies to interleukin (IL-) 4 or IL-13 (U-Cytech, Utrecht, the Netherlands). Following blocking and washing, PBMC suspended in culture medium (RPMI 1640/L-glutamine from Invitrogen supplemented with antibiotics and 10% fetal calf serum) were seeded at a final concentration of 0.3×10^6 cells per well. Casein (α + β + κ , 100 μ g/ml), α -lactalbumin (ALA) (50 μ g/ml), or β -lactoglobulin (BLG) (50 μ g/ml) (all from Sigma-Aldrich, St. Louis, MO, USA) were added to cell triplicates. Cells stimulated with phytohemagglutinin A (PHA, 10 μ g/ml, DIFCO) were used as positive controls; cells cultured in medium alone serve as negative controls. After culture for 18 h at 37°C, the cells were removed, and to visualize spots, a biotin-labeled antibody to human IL-4 or IL-13 (U-Cytech) was added followed by GABA (phi-labeled antibiotin antibody) conjugate and Activator I+II (U-Cytech). Following washing 25 min later, the plates were air-dried and spots were counted using an ELISpot reader (Autoimmun Diagnostika, Strassberg, Germany). Means of triplicate wells were expressed as spot-forming cells per 0.3×10^6 PBMC. Both positive and negative controls were validated: For PHA-positive controls, over 300 IL-4- or IL-13-secreting cells per 0.3×10^6 PBMC had to be detected. The number of spots counted in the negative control wells (culture in medium alone) was deduced from the number of spots counted in the allergen-stimulated wells and in the positive control wells (Fig. S1 shows a representative ELISpot in a CMA and a non-CMA patient). Samples showing a number of spots in the negative control wells ≥ 10 were considered as noninterpretable and were excluded. In our experience of the ELISpot for longitudinal analysis of different types of patients (21, 24) and normal volunteers, this type of 'nonspecific' reaction is sporadic and probably reflects the occurrence of transient inflammatory events.

Statistical analysis

Data are expressed as means and standard deviation (SD); in Table 1, median values are presented. Immunologic

Table 1 Clinical and immunologic parameters in the entire population studied and in CMA vs non-CMA patients (median $\pm$ SD values are presented)

	CMA			Non-CMA			<i>P</i> -value (χ^2)	Entire population		
	<i>n</i>	Median (SD)	IQR	<i>n</i>	Median (SD)	IQR		<i>n</i>	Median (SD)	IQR
Age (years)	17	4.6 (2.3)	3.7	12	3.7 (1.8)	2.9	0.22	29	4.2 (2.11)	3.52
Male/female	11/6			7/5			0.95	18/11		
Age at diagnosis (months)	17	3 (3.2)	4	12	4.5 (4.6)	4.8	0.98	29	4 (3.88)	5
Casein sIgE	12	6.33 (75.1)	37	15	0.17 (18.9)	0.38	0.006	27	3.13 (58.4)	11.3
Casein sIgE/IgE	13	0.054 (0.12)	0.11	11	0.001 (0.005)	0.01	0.02	24	0.008 (0.1)	0.06
ALA sIgE/IgE	13	0.04 (0.04)	0.04	11	0.001 (0.01)	0.006	0.008	24	0.007 (0.04)	0.04
BLG sIgE/IgE	13	0.01 (0.03)	0.03	11	0.001 (0.03)	0.002	0.06	24	0.004 (0.03)	0.02
Casein sIgE/sIgG4	12	0.01 (0.36)	0.04	10	0.0015 (0.004)	0.003	0.001	22	0.006 (0.3)	0.01
ALA sIgE/sIgG4	11	0.01 (0.33)	0.04	8	0.0004 (0.006)	0.0014	0.001	19	0.004 (0.3)	0.04
BLG sIgE/sIgG4	9	0.003 (0.018)	0.01	10	0.001 (0.01)	0.007	0.05	19	0.002 (0.02)	0.01
Casein IL-4 T cells	6	18 (17.86)	21	8	0 (3.57)	2.5	0.009	14	2.5 (16.2)	21
ALA IL-4 T cells	6	3.5 (3.07)	6	8	0 (4.91)	0.5	0.37	14	0 (4.1)	3
BLG IL-4 T cells	6	1.5 (4.78)	9	8	0 (2.48)	0	0.28	14	0 (3.7)	0
Casein IL-13 T cells	12	15.5 (31.68)	29.75	9	0 (4.17)	4.8	0.004	21	5 (26.4)	18
ALA IL-13 T cells	9	8 (28.05)	8	8	4 (11.52)	16	0.28	17	7 (21.7)	15.5
BLG IL-13 T cells	9	8 (20.43)	8	8	2.5 (5.04)	5.5	0.08	17	5 (16.0)	10.5

SD, standard deviation; IQR, interquartile range; IgE, total immunoglobulin E (kU/l); sIgE, specific immunoglobulin E (kU/l); sIgG4, specific immunoglobulin G4 (mg/l); *P*-value for Mann–Whitney *U*-tests (continuous variables) and for Fisher's exact tests (categorical data). Values in bold indicate statistical significance. Casein/ALA/BLG IL-4 T cells: number of casein/ALA/BLG-specific IL-4-secreting T cells/ 0.3×10^6 peripheral blood mononuclear cells. Casein/ALA/BLG IL-13 T cells: number of casein/ALA/BLG-specific IL-13-secreting T cells/ 0.3×10^6 peripheral blood mononuclear cells.

parameters were compared according to two groups (positive vs negative to test results/ <158 vs >158 ml cumulated dose of milk tolerated) using Mann–Whitney *U*-tests for continuous skewed data and Fisher's exact tests for categorical data. To identify whether monotonic relationships existed between the immunologic parameters and the cumulated dose tolerated, Spearman's rank correlation coefficients were calculated. Univariate linear model was fitted to examine the associations between the cumulated dose tolerated and each immunologic parameter. Regression coefficients and confidence intervals allowed estimating the changes in cumulated dose tolerated quantity (ml) for an increase of 1 (UNIT) in each immunologic parameter. Sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) were calculated. The cutoff levels of specific parameters were determined by analysis with the receiver operating characteristic (ROC) curve (25). ROC curves were plotted, and the area under the curve (AUC) was calculated to quantify the accuracy of the number of casein-specific IL-13-secreting T cells, casein-specific IL-4-secreting T cells as well as the ratio of casein-specific IgE/IgG4, each biomarker being considered alone or in association.

Graphic display and statistical analysis were performed using SAS statistical software version 9.2 (SAS Institute Inc, Cary, NC, USA). *P*-values <0.05 were considered statistically significant.

Results

Twenty-nine patients were included, mean age was 5 ± 2.1 years (2.8–10.5 years), and 62% were boys. Mean age at diagnosis of CMA was 4.5 (± 3.9) years. Seventeen (59%) reacted to the DBPCFC and were considered as children with CMA. Detailed individual patients' information regarding gender, age, age at diagnosis, and oral challenge outcome (cumulated dose of cow's milk tolerated, symptoms, and treatment) is listed in Table S1.

Mean casein-specific IgE was 37.91 and 8.29 kU/l in CMA and non-CMA children, respectively ($P = 0.006$, Table S2). Median values are also shown in Table 1.

Sixty-five percent of the CMA children developed digestive symptoms, 36% cutaneous symptoms (24% localized and 12% generalized), 29% rhinoconjunctivitis, and 18% bronchospasm. 88% required antihistamine, 29% corticosteroids, and 18% salbutamol. None required epinephrine.

A total of 24 blood samples were collected from the 29 patients included.

Quantification of circulating casein-specific IL-4- and IL-13-secreting T cells in CMA and non-CMA children

Mean number of casein-specific IL-4-secreting T cells was 24.33 (± 17.86) in CMA children vs 1.81 (± 3.57) in non-CMA children. Mean number of casein-specific IL-13-secreting T cells was 29.75 (± 31.68) in CMA children vs 2.42 (± 4.17) in non-CMA children (Fig. 1, Table S2). These values for both casein-specific IL-4- and IL-13-secreting T cells were significantly higher in CMA children as compared to non-CMA children ($P = 0.009$ and 0.004 , respectively; Table S2). In contrast, numbers of ALA- and BLG-specific IL-4- and IL-13-secreting T cells did not differ in the two groups of patients (Table 1 and S2).

Furthermore, the mean ratios of serum levels of casein-specific IgE/total IgE and casein-specific IgE/total IgG4 were also significantly higher in the CMA group ($P = 0.02$ and 0.001 , respectively; Table S2). Median values presented in Table 1 show identical results.

In contrast, in the control group (including subjects without any known allergy/atopic status), we never detected any casein-specific T-cell response for IL-4 or IL-13 (Fig. S2).

Association between the number of casein-specific IL-4- and IL-13-secreting T cells and the cumulative reactive dose

The number of casein-specific IL-4- and IL-13-secreting T cells was negatively correlated with the cumulated dose of tolerated cow's milk ($P = 0.003$, $r = -0.58$ and $P = 0.0009$, $r = -0.22$, respectively) (Fig. 2). Similarly, casein-specific IgE/total IgE ratios and casein-specific IgE/total IgG4 ratios were also negatively correlated with the cumulated dose of cow's milk tolerated ($P = 0.008$, $r = -0.53$ and $P = 0.0004$, $r = -0.69$, respectively).

Using a univariate linear regression model, we found that the higher the frequency of casein-specific IL-4- and IL-13-secreting T cells, the lower the cumulated dose of cow's milk tolerated during the food challenge (Table 2).

ROC curves and ELISpot cut points

To define more objectively which test among the IL-4 or the IL-13 ELISpot exhibited the best specificity and sensitivity for the diagnosis of cow's milk allergy, receiver operating characteristic (ROC) curves were generated (25). Area under the curve (AUC) was 0.94 (95% CI, 0.81–1.00) for the frequency of casein-specific IL-13 and 0.87 (95% CI, 0.62–1.00) for casein-specific IL-4-secreting T cells (Fig. 3A). Combining casein-specific IL-13 and casein-specific IL-4-secreting T cells frequencies, we found the highest AUC (0.97, 95% CI, 0.90–1.0) (Fig. 3B). As shown in Table 3, when using a clinical decision point of 10 casein-specific IL-4 and 12 casein-specific IL-13-secreting T cells, a specificity of 80% and a sensitivity of 100% could be achieved, with a predictive positive value of 89% and a predictive negative value approaching 100%.

Furthermore, AUC was 0.81 (95% CI, 0.07–0.1) for casein-specific IgE and 0.86 (95% CI, 0.63–1.0) for casein-specific IgE/IgG4 (Fig. 3A). When using a clinical decision point of 64.5 kU/l, a specificity of 100% and a sensitivity of 27% were observed, with a positive predictive value of 100% and a negative predictive value of 53% (Table 3).

Discussion

Diagnosis of cow's milk allergy is currently based on the performance of oral challenge, the gold standard which is laborious and risky. Thus, the search for biological diagnostic tools has become a major need. Although the dosage of specific IgE together with skin prick tests is a good marker for

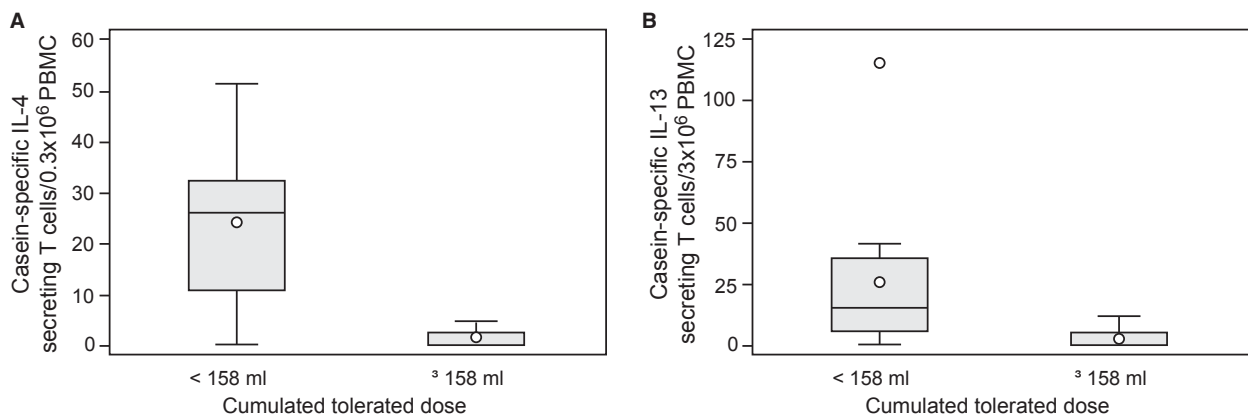


Figure 1 IL-4- and IL-13-secreting cells in CMA and non-CMA patients. CMA and non-CMA patients were defined according to the response in the DBPCFC as described in the methods section. Increasing doses of cow's milk were administered 20 min apart. Cumulative dose of cow's milk tolerated defined the reactive

threshold in CMA children. No clinical reaction to a cumulative dose of 158 ml of cow's milk defined nonallergic children (non-CMA). Numbers of IL-4-producing T cells are presented in the left panel and of IL-13-producing T cells in the right panel.

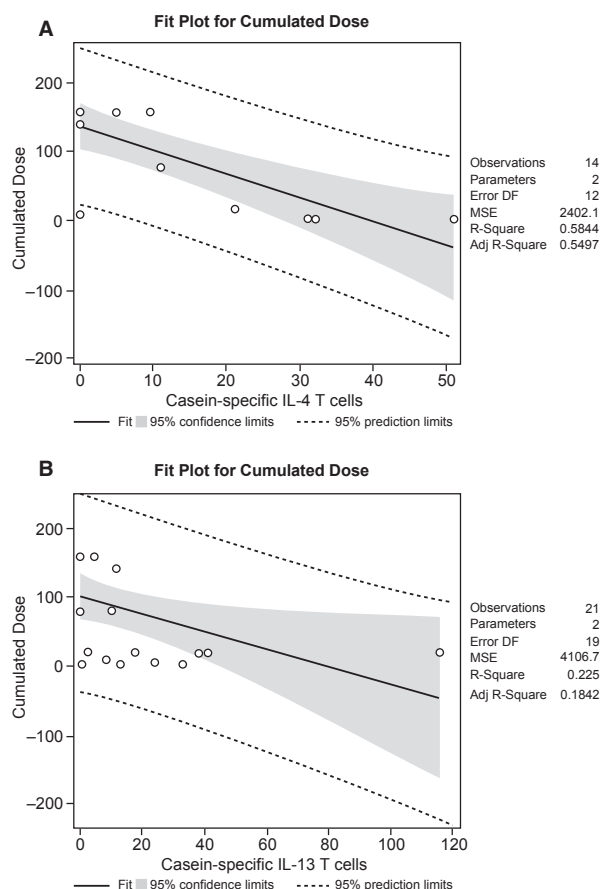


Figure 2 Association between the allergen-tolerated dose and circulating IL-4- or IL-13-secreting cells. Correlation diagram between the cumulative dose of cow's milk tolerated and ELISpot results measuring IL-4-secreting cells (A) or IL-13-secreting cells (B).

sensitization to allergens, they do not correlate with symptomatic allergy, and although they help in deciding whether an oral challenge should be performed, they cannot replace it.

Biological functional tests represent an interesting alternative with studies reporting that levels of allergen-specific Th2 cells showed a good correlation with clinical symptoms in cow's milk allergy and, more recently, in alder pollen and peanut allergy (26–30). Class II tetramers were also used to quantify Th2 lymphocytes specific to some major allergens in respiratory allergy, both at the time of diagnosis and following allergen immunotherapy (22, 27, 28). Similarly, in peanut allergy, circulating CD4⁺ T cells specific to Ara h 1 (a major peanut allergen) were higher in allergic than in nonallergic subjects (26). Other biological tests have been used to approach the distinction between patients with and without resolution of CMA. Effectively, serum concentrations of IgG1- and IgG4-specific antibodies, and the IgE/IgG1 and IgE/IgG4 ratios for milk proteins were found to be significantly lower in patients entering remission of their milk allergy (15). Moreover, patients with persistent milk allergy

Table 2 Association between the cumulated dose of milk tolerated during the provocation test and different biological parameters

	β	95% Confidence limits	P-value
Casein sIgE	−0.39	−0.85 to 0.07	0.1
Casein sIgE/IgE	−406.2	−654 to −158.5	0.001
ALA sIgE/IgE	−1045.42	−1684.55 to −406.3	0.001
BLG sIgE/IgE	−562.07	−1451.15 to 327.01	0.22
Casein sIgE/IgG4	−62.7	−173.9 to 48.55	0.3
ALA sIgE/IgG4	−71.9	−196.2 to 52.5	0.3
BLG sIgE/IgG4	−1771.36	−3793.44 to 250.7	0.09
Casein IL-4 T cells	−3.44	−4.96 to −1.92	<0.001
ALA IL-4 T cells	−1.5	−10.82 to −7.8	0.8
BLG IL-4 T cells	−7.7	−17.3 to 2.00	0.12
Casein IL-13 T cells	−1.4	−2.36 to −0.34	0.009
ALA IL-13 T cells	−0.74	−2.24 to 0.77	0.34
BLG IL-13 T cells	−1.79	−3.71 to 0.14	0.07

IgE, total immunoglobulin E (kU/l); sIgE, specific immunoglobulin E (kU/l); sIgG4, specific immunoglobulin G4 (mg/l). Casein/ALA/BLG IL-4 T cells: number of casein/ALA/BLG-specific IL-4-secreting T cells for 300 000 lymphocytes. Casein/ALA/BLG IL-13 T cells: number of casein/ALA/BLG-specific IL-13-secreting T cells/ 0.3×10^6 peripheral blood mononuclear cells.

Regression coefficients and confidence intervals evidenced by the univariate linear regression models allowed estimating the changes in cumulated dose tolerated quantity (ml) for an increase of 1 (UNIT) in each immunologic parameter. Boldface values indicate statistical significance.

showed IgE reactivity toward a greater number of sequential milk protein epitopes of major milk allergens such as α s1-, b-, and j-casein as compared to patients who developed clinical tolerance to milk (31). More precisely, in patients showing no remission of CMA, specific IgEs had a greater diversity of the recognized epitopes and a higher affinity (13, 32). Recently, the utility of analyzing some genetic variants has also been tested. Yavuz et al. (33) reported that combination of specific IgE levels and STAT6 gene variants may be relevant to predict longer persistence of CMA.

In the present work, we studied casein-specific Th2 cells using an ELISpot method. Results showed that the number of casein-specific IL-4- and IL-13-secreting T cells was higher in CMA children than in non-CMA children.

Main characteristics of this test making it particularly adapted for this type of study are first, the short-term culture in presence of antigen (i.e., 18 h) that, as well established in the literature, allows detecting with a high sensitivity experienced activated and memory T cells (34). Therefore, the test well mirrors the *in vivo* 'immune activation' status of the patient. Secondly, because of this high sensitivity, there is no need for prior *in vitro* expansion, which is often necessary when using other methods (conventional culture or HLA class II tetramers), which, for obvious reasons, may introduce qualitative and quantitative biases (34). Third, analyzing responses using the entire allergen (casein) allows detecting the reactivity to different antigenic determinants, thereby uncovering the response in a larger number of patients

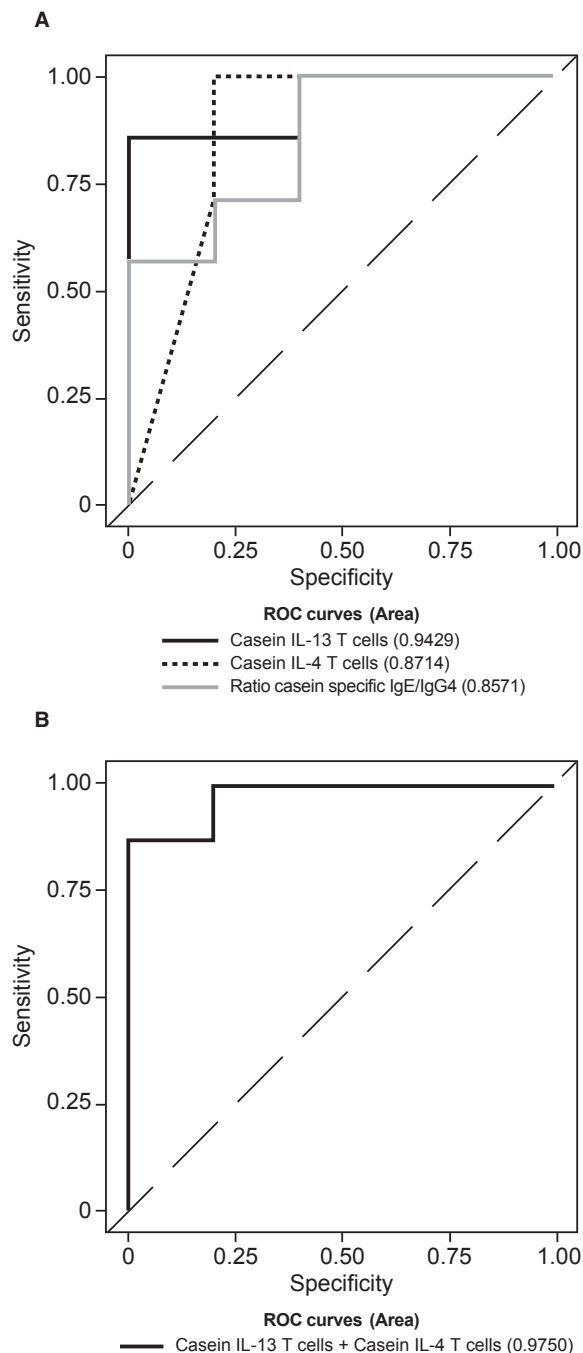


Figure 3 Receiver operating characteristic (ROC) curves showing performance of three tests in predicting clinical reactivity to milk. (A) Casein-specific IL-13-secreting T cells (AUC = 0.94; 95% CI, 0.81–1.00), casein-specific IL-4-secreting T cells (AUC = 0.87; 95% CI, 0.62–1.00), and casein-specific IgE/IgG4 (AUC = 0.86; 95% CI, 0.63–1.00). (B) Casein-specific IL-13-secreting T cells and casein-specific IL-4-secreting T cells combined (AUC = 0.97; 0.90–1.06).

independently from their HLA haplotype. This is at variance with the intrinsic practical problem posed by HLA class II tetramers.

Table 3 Sensitivity, specificity, and positive and negative predictive values for different cutoffs

Positivity cutoff	Se (%)	Spe (%)	PPV (%)	NPV (%)
Casein IL-13 T cells = 5	89	75	73	90
Casein IL-13 T cells = 12	10	58	64	100
Casein IL-4 T cells = 10	100	83	89	100
Casein IL-4 T cells = 10 + Casein IL-13 T cells = 12	100	80	89	100
Casein sIgE = 50.5	27	92	80	50
Casein sIgE = 64.5	27	100	100	53
Casein sIgE/IgG4 = 0.0015	60	100	100	75
Casein sIgE/IgG4 = 0.0032	80	92	89	85
Casein sIgE/IgG4 = 0.0011	100	67	71	100

Se, sensitivity; Spe, specificity; PPV, predictive positive value; NPV, negative predictive value.

Casein IL-4 T cells: number of casein-specific IL-4-secreting T cells/ 0.3×10^6 peripheral blood mononuclear cells. Casein IL-13 T cells: number of casein-specific IL-13-secreting T cells/ 0.3×10^6 peripheral blood mononuclear cells.

To examine further the validity of the ELISpot assay, we confronted the data to the ROC curve analysis used to evaluate the sensitivity and specificity of a diagnostic test (25). Results showed that the AUC was the highest for the diagnosis of CMA (AUC = 0.97) for the analysis of both the number of IL-4- and IL-13-secreting cells. For the sake of comparison, the basophil activation test that has been recently proposed for the diagnosis of cow's milk allergy showed an AUC of 0.866 (81% and 96% of positive and negative predictive values, respectively) (35). In another study, the performance of milk-specific basophil reactivity in differentiating subjects reactive or tolerant to baked milk showed an AUC of 0.69 (36).

With the ELISpot assay used herein, for the first time, a relationship between the number of casein-specific IL-4- and IL-13-secreting T cells and the cumulated dose of cow's milk tolerated in the oral challenge was found, that is, the higher the number of casein-specific Th2 cells, the lower the dose of cow's milk tolerated. Thus, circulating allergen-specific T cells appear to better reflect the pathophysiology of symptomatic food allergy than serum antigen-specific IgE. This may be explained first, by the data in allergic rhinitis, showing that *in situ* levels of specific IgE correlate better with allergic symptoms than circulating specific IgE levels. Thus, in patients presenting grass pollen allergy, specific IgE in the nasal mucosa showed a better correlation with allergen exposure than serum evaluations (37). Similarly, in house dust mite-allergic children presenting rhinitis and asthma, specific IgE in the nasal mucosa significantly increased during allergen exposure while serum levels did not (38). Secondly, the relevance of directly detecting antigen-specific Th2 cells, which play a key role in the development of allergic symptoms, was recently highlighted in patients successfully desensitized to birch in whom a significant reduction in circulating

Bet v 1 (a major allergen from birch)-specific Th2 cells assessed by ELISpot was detected (39).

Another goal of applying the test here described was to assess with as much certainty as possible whether a child is allergic or not to cow's milk to reduce the risk of allergic reaction during oral challenge. Therefore, the threshold values for each of the two associated tests that have the highest negative predictive value were determined. It appeared that the combination of the values of 10 casein-specific IL-4-secreting and 12 casein-specific IL-13-secreting T cells provides a negative predictive value approaching 100%. With this same threshold, a positive predictive value of 89% of cases was obtained. Thus, when the result is below this threshold, the diagnosis of CMA is very unlikely, whereas when it is above this threshold, it seems more reasonable to delay the oral challenge or perform it with all the needed precautions.

If confirmed on a larger series of patients, the casein-specific ELISpot could represent a promising finding with potential application to improved diagnosis of CMA in a field where this is a significant unmet need. It also appears of interest to guide the clinician's attitude toward the oral challenge, namely to identify patients with a potential high risk to develop serious reactions.

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Author contributions

B.M., L.C., J.J. designed the research, interpreted the data, and wrote the manuscript; B.M. performed the experiments and collected and analyzed the data; J.J. and J.A. coordinated the recruitment of patients; R.G.-E. and F.A. contributed to the recruitment of the patients; S.C. and R.C. contributed analytical tools; N.B. performed the statistical analysis; and J.-F.B. supervised the research and provided critical revision of the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. A representative example of the results obtained with the ELISpot assay in a CMA vs a non-CMA patient.

Figure S2. Representative results obtained with the IL-13 ELISpot assay in control subjects without any known allergy/atopic status.

Table S1. Characteristics of patients included in the study.

Table S2. Clinical and immunological parameters in the entire population studied and in CMA vs non-CMA patients (mean $\pm$ SD values are presented).

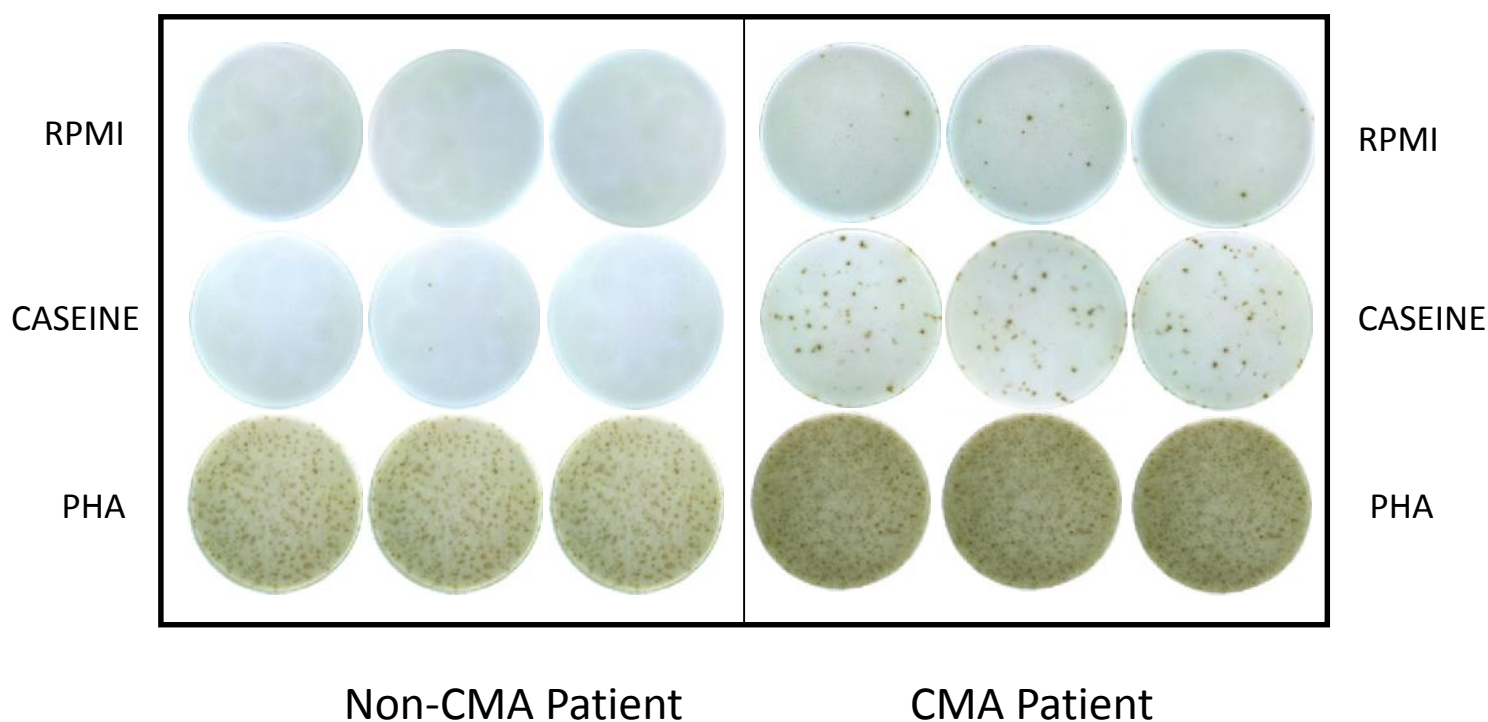
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SUPPORTING INFORMATION

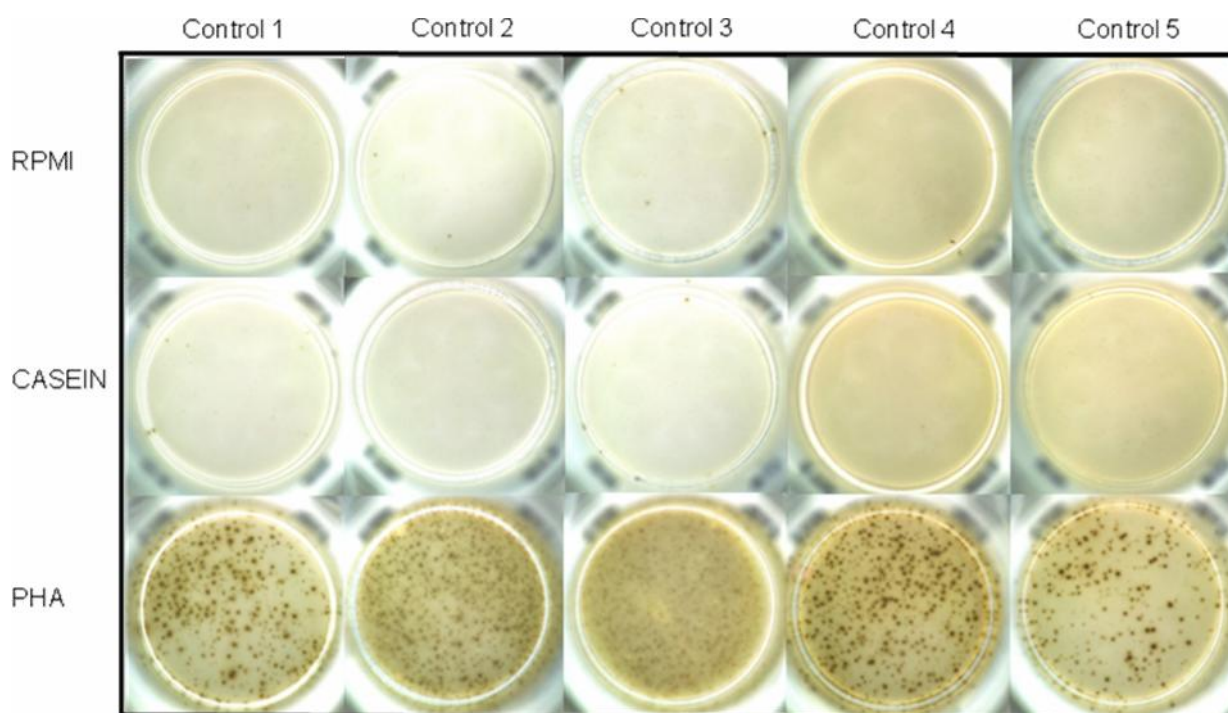
Figure S1: A representative example of the results obtained with the IL-13 ELISpot assay in a CMA versus a non-CMA patient.



As described in the methods section the (22). Peripheral blood mononuclear cells (PBMC) were seeded were plated in wells coated with an antibody to human IL-13 at a final concentration of 0.3×10^6 cells per well. Casein ($\alpha+\beta+\kappa$, 100 $\mu\text{g/ml}$) was added to cell triplicates. Cells stimulated with phytohemagglutinin A (PHA, 10 $\mu\text{g/ml}$, DIFCO) were used as a positive controls; cells cultured in medium alone (RPMI) serve as negative controls. After culture for 18 hours at 37°C, the cells were removed by extensive washing and the spots were visualized using a secondary biotin-labeled antibody to human IL-13, phi-labeled anti-biotin antibodies and Activator I+II. The spots were counted using an ELISpot reader and data were expressed as spot-forming cells per 0.3×10^6 PBMC. Each spot represents a single IL-13 secreting cell. Both positive and negative controls had to be validated. For the

positive control, over 300 IL-13 secreting T-cells per $0.3 \cdot 10^6$ lymphocytes had to be detected in response to PHA. The number of spots counted in the negative control wells (culture in medium alone) were deduced from the number of spots counted in the allergen stimulated wells and in the positive control wells. In these representative cases 33 casein-specific IL-13 secreting T-cells for 0.3×10^6 PBMC were detected in the CMA patient and none in the non-CMA patient.

Figure S2: Representative results obtained with the IL-13 ELISpot assay in control subjects without any known allergy/atopic status.



The test was carried out as described in the manuscript and in the legend to Figure S1.

Data are shown for 5 of the control subjects tested and are representative of the 7 individual analyzed. As observed in none of the samples was any IL-13 producing cell detected.

Table S1: Characteristics of patients included in the study.

Patient	Gender	Age (years)	Age of diagnosis (months)	Oral challenge outcome		
				Cow's milk cumulated tolerated dose (mL)	Symptoms	Treatment
1	Boy	6.5	8	3.87	Pharyngeal itching	Anti H1
2	Boy	2.8	5	18.87	Abdominal pain, conjunctivitis	Anti H1
3	Boy	8.2	5	158	None	
4	Boy	5.5	2	17	Abdominal pain, rhinitis, conjunctivitis	Anti H1, corticosteroids
5	Boy	2.9	1	158	None	
6	Girl	10.5	6	78	Abdominal pain, vomiting	Anti H1, corticosteroids
7	Girl	5.9	4	158	None	
8	Boy	7.9	8	1.87	Facial urticaria, asthma	Anti H1, corticosteroids, salbutamol
9	Boy	5.9	7	158	None	
10	Girl	4.6	4	18.87	Cough, pharyngeal itching, rhinitis, conjunctivitis	Anti H1
11	Girl	7.9	1	78	Vomiting	Anti H1
12	Boy	8.2	3	0.87	Abdominal pain, facial urticaria, asthma	Anti H1, corticosteroids, salbutamol
13	Girl	3.4	1	78	Abdominal pain, facial urticaria	Spontaneous resolution
14	Boy	3	1	158	None	
15	Boy	3.1	1	18	Facial urticaria	Anti H1
16	Girl	4	2	8	Abdominal pain, pharyngeal itching	Anti H1

17	Boy	3.4	8	158	None	
18	Boy	2.7	4	158	None	
19	Girl	6.6	3	158	None	
20	Boy	4.6	2	8	Abdominal pain, vomiting	Anti H1
21	Girl	4.2	12	158	None	
22	Girl	3.4	5	158	None	
23	Boy	3	1	158	None	
24	Girl	4.2	1	3	Pharyngeal itching, rhinitis, asthma, vomiting	Anti H1, corticosteroids, salbutamol
25	Boy	4.5	4	1.87	Rhinitis, conjunctivitis, urticaria	Anti H1
26	Boy	7.1	5	18	Abdominal pain, pharyngeal itching	Anti H1
27	Boy	3	12	0.87	Diarrhea	Spontaneous resolution
28	Boy	3	0	18.87	Urticaria	Anti H1
29	Girl	4	16	158	None	

Table S2: Clinical and immunological parameters in the entire population studied and in CMA versus non-CMA patients (mean±SD values are presented).

	CMA			Non-CMA			p-value (χ^2)	n	Entire population	
	n	Mean (SD)	IQR	n	Mean (SD)	IQR			Mean (SD)	IQR
Age (years)	17	5,3 (2,3)	3,7	12	4,4 (1,8)	2,9	0.22	29	5.08	3.52
male/female	11/6			7/5			0.95	18/11		
Age at diagnosis (months)	17	3,8 (3,2)	4	12	5,6 (4,6)	4,8	0.98	29	4.55 (3.88)	5
Casein sIgE	12	37.9 (75.1)	37	15	8.29 (18.9)	0.38	0.006	27	24.7 (58.4)	11.3
Casein sIgE/ IgE	13	0.09 (0.12)	0.11	11	0.004 (0.005)	0.01	0.02	24	0.05 (0.1)	0.06
ALA sIgE/ IgE	13	0.04 (0.04)	0.04	11	0.007 (0.01)	0.006	0.008	24	0.02 (0.04)	0.04
BLG sIgE/ IgE	13	0.02 (0.03)	0.03	11	0.01 (0.03)	0.002	0.06	24	0.02 (0.03)	0.02
Casein sIgE/ sIgG4	12	0.12 (0.36)	0.04	10	0.003 (0.004)	0.003	0.001	22	0.07 (0.3)	0.01
ALA sIgE/sIgG4	11	0.13 (0.33)	0.04	8	0.003 (0.006)	0.0014	0.001	19	0.08 (0.3)	0.04
BLG sIgE/sIgG4	9	0.015 (0.018)	0.01	10	0.006 (0.01)	0.007	0.05	19	0.01 (0.02)	0.01
Casein IL-4 T-cells	6	24.33 (17.86)	21	8	1.81 (3.57)	2.5	0.009	14	11.46 (16.2)	21
ALA IL-4 T-cells	6	2.58 (3.07)	6	8	1.88 (4.91)	0.5	0.37	14	2.18 (4.1)	3
BLG IL-4 T-cells	6	3.08 (4.78)	9	8	0.88 (2.48)	0	0.28	14	1.8 (3.7)	0
Casein IL-13 T-cells	12	25.46 (31.68)	29.75	9	2.42 (4.17)	4.8	0.004	21	15.6 (26.4)	18
ALA IL-13 T-cells	9	17.56 (28.05)	8	8	8.85 (11.52)	16	0.28	17	13.5 (21.7)	15.5
BLG IL-13 T-cells	9	15.5 (20.43)	8	8	3.91 (5.04)	5.5	0.08	17	10.05 (16.0)	10.5