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CLINICAL RESEARCH ARTICLE

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Bifidobacteria modulate immune response in pediatric patients with cow's milk protein allergy

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BACKGROUND: In children with an allergy to cow's milk proteins (CMA), the altered composition of intestinal microbiota influences the immune tolerance to milk proteins (CMP). This study aims to investigate the effect of probiotics on the phenotype and activation status of peripheral basophils and lymphocytes in a pediatric CMA cohort.
METHODS: CMA children underwent 45 days of treatment with Bifidobacteria. The basophil degranulation and the immune phenotype of B cells, T helper cells, and regulatory T cells were analyzed in peripheral blood at diagnosis (T0), after a 45-day probiotic treatment (T1), and 45 days after the probiotic wash-out (T2).
RESULTS: We observed in probiotic-treated CMA patients a decrease in naive T lymphocytes. Among the CD3+ cell subsets, both naive and activated CD4+ cells resulted markedly reduced after taking probiotics, with the lowest percentages at T2. A decreased basophil degranulation was observed in response to all analyzed CMP at T1 compared to T0.
CONCLUSIONS: The probiotic treatment resulted in a decrease of circulating naive and activated CD4+ T cells, as well as degranulating basophils. These data suggest that the Bifidobacteria could have a beneficial effect in the modulation of oral tolerance to CMP.
TRIAL REGISTRATION: ISRCTN69069358. URL of registration: https://www.isrctn.com/ISRCTN69069358.

Pediatric Research (2023) 94:1111–1118; https://doi.org/10.1038/s41390-023-02534-0

IMPACT:

- Probiotic treatment with Bifidobacteria induces a reduction of both naive and activated circulating CD4+ T cells in pediatric patients with cow's milk allergy (CMA).
- The probiotic supplementation induces a decreased basophil degranulation.
- The immunological tolerance persists even after 45 days of the probiotic wash-out.
- Bifidobacteria in vivo supplementation down-modulates the activation of innate and adaptive immunity in pediatric patients with cow's milk allergy.
- Bifidobacteria contribute to the development of immune tolerance in CMA patients.

INTRODUCTION

During early childhood, cow's milk protein allergy (CMA) is very frequent, with an incidence between 2% and 3%. About 80–90% of affected cases acquire tolerance by the age of 5 years.[1,2] However, recent studies demonstrated a change in the natural history of CMA,[3] with an increasing persistence until later ages.[4]

The pathogenesis of CMA is complex and not completely understood, it has been suggested that an altered composition of intestinal microflora results in an unbalanced local and systemic immune response to cow's milk proteins, and to food antigens in general.[5,6] In fact, a different composition of gut microbiota between CMA-affected and healthy infants was reported.[7] These findings suggest that specific beneficial bacteria from the human intestinal microbiota, designated as probiotics, could re-establish intestinal homeostasis. Indeed, some particular probiotics have been successfully used mainly in the prevention, and to a much lesser extent, in the treatment of allergic diseases.[8–11] However, the mechanism by which they modulate the immune system is poorly understood.[12–14]

Further research is necessary to define the specific role of probiotics in inducing immune tolerance to cow's milk allergens in humans, given the limited number of in vivo studies on established CMA.[15] Moreover, since CMA is mainly a childhood disease, specific studies on the pediatric population are needed.

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Received: 9 June 2022 Accepted: 15 January 2023
Published online: 23 March 2023

In particular, in this study we aimed to investigate the anti-allergenic effect of a mixture of 3 Bifidobacterium strains (*B. longum*, *B. breve*, *B. infantis*) in CMA pediatric patients, before and after a 45-day treatment. In particular, we analyzed the process of basophil degranulation through the Basophil Activation Test (BAT), as the BAT has been shown to be a useful technique to define immunological modifications, more accurate than immunoglobulin E (IgE) sensitization tests.[16]

Moreover, we evaluated if the probiotic supplementation induced changes in the frequency of the main immune cell subtypes, including B cells, effector, and regulatory T cells.

MATERIALS AND METHODS

Study population

Children enrolled in the study were referred to a tertiary pediatric allergy center (Paediatric Food Allergy Unit at the Department of Paediatrics at the University of Campania "Luigi Vanvitelli") for a full diagnostic work-up for suspected CMA. The study population included a total of 8 infants (mean age: 9 months; range: 6–12 months) with a confirmed diagnosis of IgE-mediated CMA on a cow's milk protein exclusion diet. Diagnosis of IgE-mediated CMA was based on clinical history, the results of a double-blind placebo-controlled oral food challenge and the level of serum-specific anti-CMP IgE.[17] Patients were excluded from the study if they reported consumption of prebiotic or probiotic products, and/or antibiotics in the previous 4 weeks; a history of cow's milk-induced anaphylaxis; eosinophilic disorders of the gastrointestinal tract; food protein-induced enterocolitic syndrome; concomitant chronic systemic diseases; or other gastrointestinal diseases. Following the initial visit, the children started a commercially available extensively hydrolyzed casein formula (EHCF, Nutramigen, Mead Johnson, Rome, Italy) and treatment with the probiotic mixture for 45 days (5 billion of colony-forming units/day, bnl CFU/die, specifically 3 bnl CFU/die of *Bifidobacterium Longum* BB536; 1 bnl CFU/die of *Bifidobacterium Infantis* M-63; 1 1 bnl CFU/die of *Bifidobacterium breve* M-16V). Blood samples were collected soon before the probiotic treatment (baseline, T0) and at the end of probiotic supplementation (T1). Further, after 45 days from the suspension of the probiotic, while the patients were still on a CMP elimination diet, another blood sample was collected to better understand the specific effects of Bifidobacterium (T2). The demographic and clinical characteristics of enrolled subjects are described in Table 1.

Table 1. Demographic and clinical characteristics of the study population.

Characteristics	CMA patients
Patients, *n*	8
Age, months (±s.d.)	12 (±3)
Male, *n* (%)	3 (37.5)
Weight, kg (±s.d.)	11.3 (±1.5)
Height, cm (±s.d.)	85 (±5)
Prick test, mm (±s.d.)	5 (±1)
Rast, Kua/L (± s.d.)	Milk 15.4 (±5)
Symptoms at CMA onset	
Vomiting, *n* (%)	3 (37.5)
Urticaria, *n* (%)	2 (25)
Diarrhea, *n* (%)	3 (37.5)

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated according to a well-established procedure[18] and freshly used for staining with anti-human fluorochrome-conjugated monoclonal antibodies (MoAbs). All antibodies were purchased at Beckman Coulter and used at the concentration indicated in the manufacturer's instructions. Samples were acquired with FC500 (Beckham), and the analyses were performed with the FlowJo (Tree Star) software and CXP software 2.0.

At least 5000 events were acquired in the gate of live mononuclear cells. Different cocktails (Mix) of MoAbs were used to analyze the main immunological populations. Specifically, the antibody mixes (Mix 1, 2, 3, 4) used are reported in Table 2.

Basophilic activation test (BAT)

The BAT is a method that evaluates the percentage of activation/degranulation of peripheral blood basophils after in vitro exposure to specific allergens through flow cytometric analysis of various surface markers. There are numerous commercial kits to perform the BAT which differ from each other for the specificity of antibodies used and for the cytofluorimetric strategy of basophil identification and their activation/degranulation state. We used "Allergenicity Kit, Cellular Analysis of Allergy, CE, IVD" (Immunotech SAS, to Beckman Coulter Company Marseille, France), which employs the CD203c as a marker of basophilic activation and the combination of CRTH2 and CD3 as negative gating strategy for the definition of the basophilic subset. The samples were processed following the guidelines reported in the manufacturer's datasheet. In particular, the assay was performed with100 µL of whole blood in EDTA, for each tube incubated with the specific allergen to be tested (whole cow's milk proteins, and single casein, lactalbumin and lactoglobulin allergens, 20 µL of 125 µg/mL for each allergen). After an incubation of 15 min at 37 °C in bath sheltered from light, in each tube were added 100 µL of stop solution. The peripheral blood cells were lysed by 10 min of incubation with 2 mL of the "Fix-and-Lyse" buffer prepared extemporaneously according to the kit protocol. Then, the samples were washed 3 times with PBS by centrifuging at 200 × *g* for 5 min. The cell pellet was resuspended in 0.5 mL of PBS 0.1% formaldehyde, before flow cytometry acquisition.

Statistical analysis

For longitudinal data analysis, the variables (T cell subsets and degranulation activity of circulating basophils to the main CMA antigenic proteins variables) were plotted over time, and mixed-effects models were used considering intercept for subjects as random effects and time as a fixed-effect parameter, thus, testing for statistical differences from T0 to T1 and from T0 and T2. It was not possible to test for random slopes and within-subject error autocorrelations because of the limited number of subjects and few observations within each subject.

In a second longitudinal data analysis, we analyzed the relation between the immunophenotypic profiles and basophil degranulation to CMA allergens over time, using mixed-effects models with immunophenotypic

Table 2. Fluorochrome-conjugated monoclonal antibodies used for immune phenotypic analysis.

	Antibody mixtures				
Mix 1	Anti-CD45-FITC[a]	Anti-CD4-RD1[a]	Anti-CD8-R-Phycoerytrin-Texas Red®-x (ECD)[a]	Anti-CD3−PC5[a]	Anti-HLA-DR-PC7, clone Immu-357, cod. B49180
Mix 2	Anti-CD45-FITC[b]	Anti-CD56+CD16-RD1[b]	Anti-CD19-ECD[b]	Anti-CD3-PC5[b]	
Mix 3	Anti-CD45-FITC, clone J33, cod. A07782	Anti-CD4-PE, clone 13B8.2, cod. A07751	Anti-CD45RO-ECD, UCHL1, cod. B49192	Anti-CD3-PC5, clone UCHT1, cod. A07749	Anti-CD25-PC7, clone B1.49.9, cod. A52882
Mix 4	Anti-CD45-FITC, clone J33, cod. A07782	Anti-CD4-PE, clone 13B8.2, cod. A07751	Anti-CD8-ECD, clone B9.11, cod. B08467	Anti-CD28-PC5.5, clone CD28.2, cod. B24027	Anti-CD11b-PC7, clone Bear1, cod. A54822

All antibodies were purchased at Beckman Coulter and used at the concentration indicated in the manufacturers' instructions.
[a]These antibodies are provided within the cocktail AQUIOS Tetra-1 Panel Monoclonal Antibody Reagents (B23533), a four-color monoclonal antibody cocktail consisting of anti-CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5; Clones B3821F4A/SFCI12T4D11/SFCI21Thy2D3/UCHT1.
[b]These antibodies are provided within the cocktail AQUIOS Tetra-2 Panel Monoclonal Antibody Reagents (B23534), a four-color monoclonal antibody cocktail consisting of anti-CD45-FITC/(CD56+CD16)-RD1/CD19-ECD/CD3-PC5; Clones B3821F4A/3G8+N901/J3–119/UCHT1.

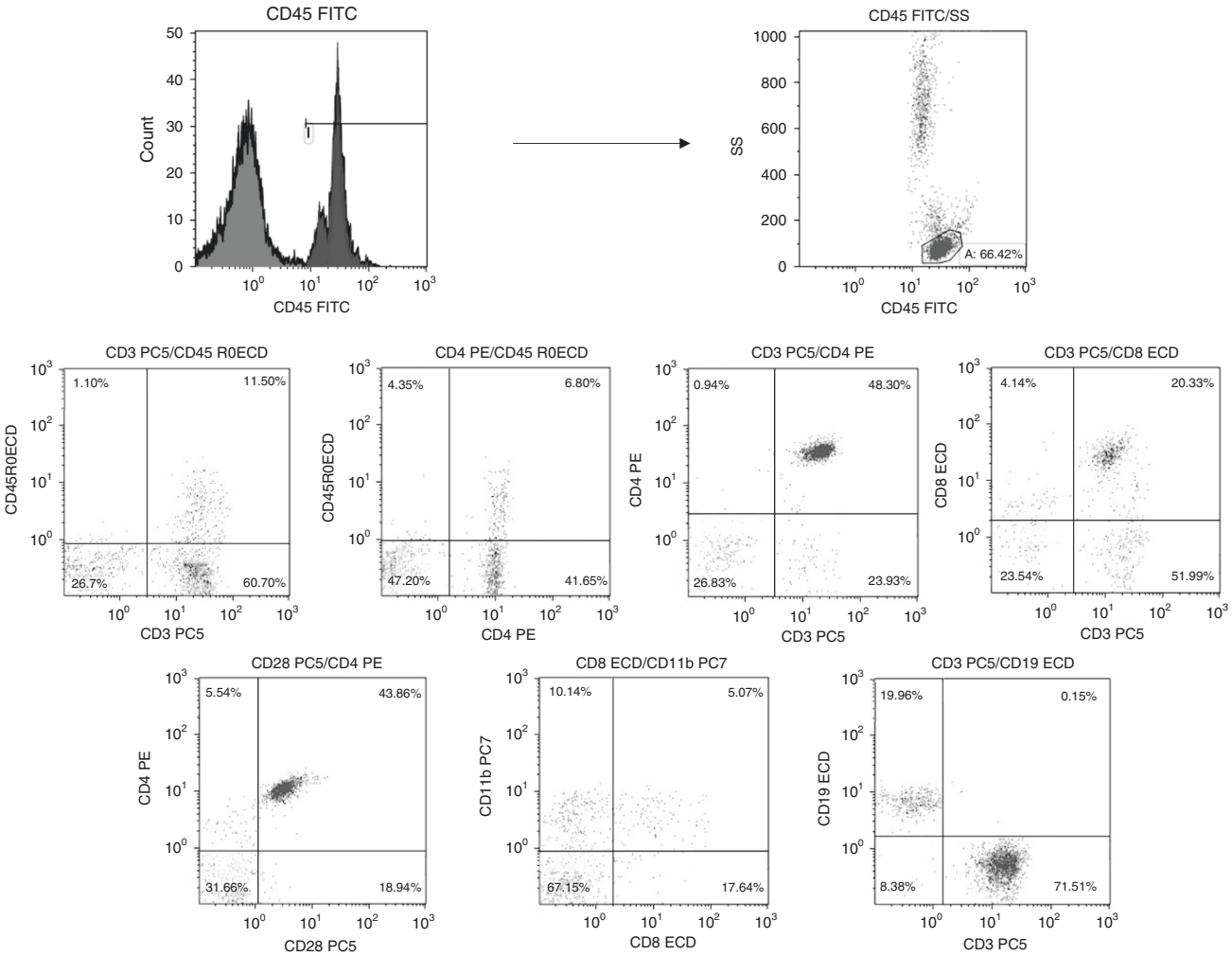

Fig. 1 Cell population percentages analyzed by flow cytometry. Flow cytometry histogram and dotplot panels illustrating the gating strategy used for phenotypic analysis of PBMC from CMA children. Representative dot plots from one CMA patient at T2 (after 45 days of probiotics wash-out) showing in the gated CD45+ mononuclear cells the percentage ofCD3+, CD4+, CD8+, CD19+CD3−, CD4+CD28+, CD11b+CD8+ cells are reported. The percentages of memory CD45RO+CD3+ and CD45RO+CD4+ cells are also reported.

profiles as covariates (Table 2). Mixed-effects models were run separately for each basophil degranulation to CMA allergens (Table 2), using all the available data at each timepoint (T0, T1, T2). All statistical analyses were performed with the R statistical software (version 3.4.4). Mixed-effects models were fitted using the lmer function in the lme4 R package.[19] Statistical significance was defined as a p value <0.05.

RESULTS

Change in the frequency of T and B cells after probiotic treatment

The densities of the main lymphocyte subsets were analyzed in PBMC from CMA patients after the dietary probiotic treatment. In particular, we focused the analysis on T cells (CD3+, both helper CD4+ and cytotoxic CD8+ cells), and on B cells (CD19+) analyzed in CD45-positive cell subset. As shown in Fig. 1, which depicts the gating strategy, the percentages of various cell subsets were further analyzed in naive CD45RO-negative cells. The activation and naive/memory status were also evaluated by detecting the expression of CD28 costimulatory molecule or CD11b adhesion molecule, respectively (Fig. 1). The changes of the immunophenotypic profile were longitudinally monitored in allergic children at the diagnosis (T0), after 45 days of probiotics oral supplementation (T1), and after 45 days of probiotics wash-out (T2).

After 45 days of Bifidobacteria treatment, we observed a significant decrease of circulating naive T lymphocytes (CD3CD45RA-double positive cells), although this reduction reached the statistical significance only at time T2 compared to diagnosis (percentage of positive cells, T0: mean value 64.2%, range 59–79; T2: 58.7%, range 49–73, $p = 0.019$), (Fig. 2a). Among the CD3+ cell subset, the CD4+ cells resulted reduced at T1 compared to the baseline: (T0: 51.75%, 36–67; T1: 43.5%, 30–51, $p = 0.0051$), (Fig. 2b). Interestingly, the reduction of CD4+ T cells persisted until 45 days without the probiotic treatment (T2: 43.5%, 37–51, $p = 0.0051$). Also, the CD4CD45RA- and the CD4CD28-double positive T cells, naive and activated CD4+ T cells, respectively, markedly decreased upon the probiotic consumption, with the lowest percentage reached at T2 (Fig. 2c, d). More in detail, the frequency of CD4+CD45RA+ cells was at T0: 45.5%, 36–63; at T1: 40.3%, 26–49; at T2: 37.1%, 27–48 ($p = 0.010$ between T0-T2); the CD4+CD28+ T cells were at T0: 49.5%, 36–65; at T1: 43.4%, 30–55 ($p = 0.021$); whilst at T2: 42.6%, 36–47 ($p = 0.005$). By contrast, no differences were found in the percentage of CD4+FoxP3+ regulatory T cells at the baseline and at both times of the probiotic treatment, data not shown.

In contrast to effector CD4+ T cells, we observed a rise of cytolytic CD8CD11b-double positive cells after 45 days of the probiotic consumption, with an increase statistically significant

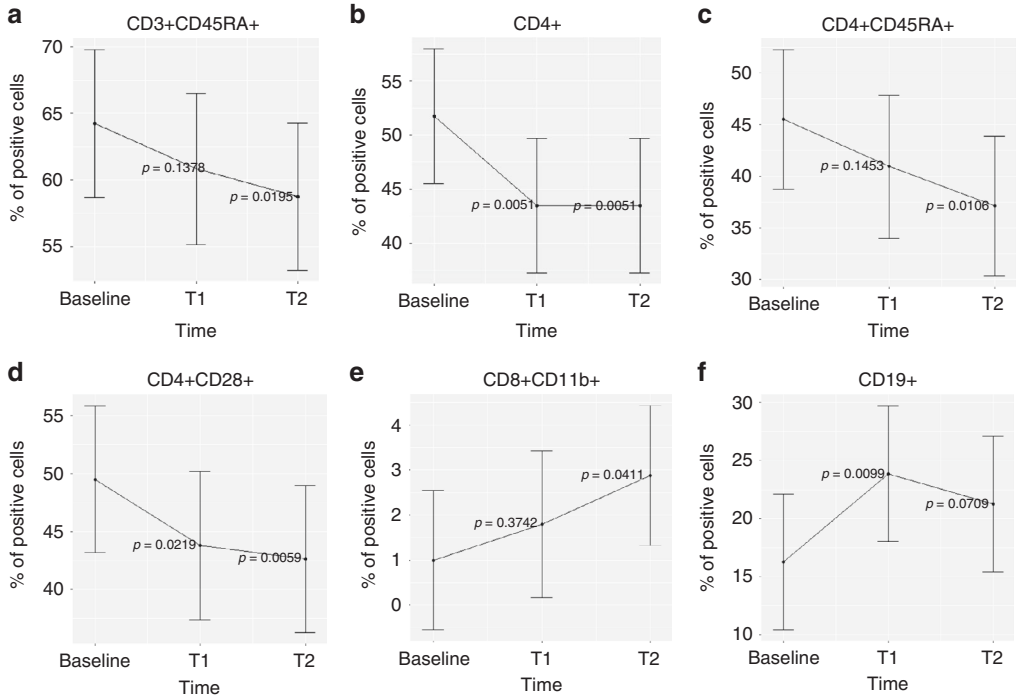

Fig. 2 The effect of probiotic treatment on the frequency of circulating T and B cell subsets. The frequency of various lymphocyte subsets was evaluated in the peripheral blood of 8 young children with CMA at baseline, after 45 days of probiotic treatment (T1), and 45 days from the suspension of the treatment (T2). The percentage of both T and B cell subsets was evaluated by flow cytometry. The percentages of CD3+ and CD4+ naive (CD45RA+) cells were analyzed as CD45RO-negative cell compartment. The average percentage of circulating CD3+CD45RA + (**a**), CD4 + (**b**), CD4+CD45RA+ (**c**), CD4+CD28+ (**d**), CD8+CD11b+ (**e**), and CD19+ (**f**) cells is shown at each time point. Statistical significance was defined by a p value <0.05.

only after 45 days of probiotic interruption compared to the diagnosis (T0: 1%, 0–2; T2: 2.8%, 0–8, $p = 0.041$), (Fig. 2e). Moreover, we found an increased frequency of CD19+ cells at T1 (23.8%, 10–42), compared to T0 (16.2%, 10–25; $p = 0.009$). After the treatment wash-out, the frequency of circulating B cells remained slightly increased compared to the baseline, although the difference was not statistically significant (Fig. 2f).

Basophil degranulation

The percentage of degranulating basophils in peripheral venous blood was evaluated after in vitro exposure to cow's milk allergens, either the whole proteins (CMP) or purified casein, lactalbumin, and lactoglobulin. We found a decreased frequency of degranulation after 45 days of probiotic treatment compared to the degranulation rate found at diagnosis: (CMP T0: 17.2%, range 0–34.4, T1: 13.3%, 0–28.7; casein T0: 18.1%, 0.3–38.2, T1: 12.1%, 0–19.7; lactalbumin T0: 17.4%, 0–36.2, T1: 8.0%, 0–18.9; lactoglobulin T0: 16.0%, 0–36.4, T1: 11.8%, 0–30.3) (Fig. 3a–d).

Instead, the percentage of degranulating basophils increased again after 45 days of probiotic wash-out (T2), but without reaching the levels observed at diagnosis, except for the in vitro lactoglobulin exposure (CMP: 13%, 0–40.4; casein: 13.4%, 0–43.2; lactalbumin: 15.1%, 0.8–38.6; lactoglobulin: 18.3%, 0–44.1), (Fig. 3a–d). However, although the probiotic treatment resulted in a reduction of basophil degranulation in response to the main cow's milk allergens, the differences did not achieve the statistical significances, most likely due the low number of patients analyzed.

Relation between the frequency of circulating lymphocytes and basophil degranulation following probiotic treatment

The frequencies of lymphocyte subsets and the percentage of basophil degranulation were related either before or after the probiotic in vivo treatment. After probiotic wash-out (T2), we found a positive relation between the percentage of the various T cell subsets (CD3+, CD3+CD45RA+, CD4+FoxP3+, CD8+, CD8+CD11b+ cells) and the degranulation activity in response to the main cow's milk antigenic proteins, mostly towards lactalbumin and lactoglobulin, Table 3. By contrast, at T0, CD8CD11b-double positive cells showed an inverse relation, which became significantly positive at T2 after the in vitro incubation with all allergens, except for casein, Table 3. Similarly, CD19+ cells showed an inverse relation at T0 with the basophil degranulation activity to all cow's milk allergens with exception of casein (Table 3).

DISCUSSION

Very few studies have investigated the health benefits of the probiotic Bifidobacteria in a pediatric cohort with CMA. It has been demonstrated that the effect of the probiotics mainly depends on the strain, as specific bacteria induce in the host the production of typical patterns of cytokines that regulate the immune response.[3,4] We chose to study the effect of the commercial probiotic mixture (Tribif) composed by 3 Bifidobacteria species (*B. longum*, *B. breve*, and *B. infantis*), since we have previously shown that Tribif in vitro was able to enhance the compromised ability of antigen sampling and processing by dendritic cells from pediatric Crohn Disease patients.[20]

In this study, instead, we demonstrate that Tribif given in vivo is able to persistently modulate the frequencies of circulating T and B cells, as well as to positively modulate the degranulation rate of basophils.

After Bifidobacteria treatment, we observed a significant decrease of naive and activated T lymphocyte, and interestingly, the reduction of CD4+ T cells persisted after 45 days without the probiotic treatment. On the contrary, for CD4+ T cells, we observed an increased frequency of CD19+ cells, as well as a

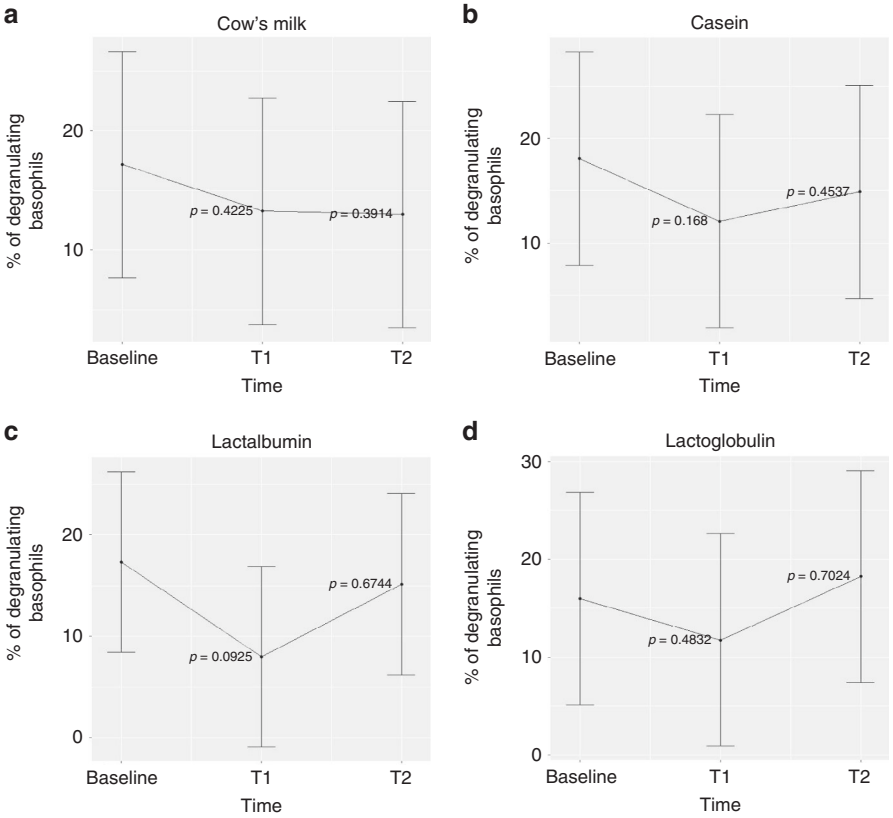

Fig. 3 Effect of probiotic treatment on the percentage of degranulating basophils in response to cow's milk proteins. The percentage of degranulation activity of circulating basophils was evaluated in fresh blood samples of CMA patients ($N = 8$) by basophilic activation test (BAT), after in vitro exposure to cow's milk (**a**), casein (**b**), lactalbumin (**c**), and lactoglobulin (**d**). The basophil degranulation in response to the main cow's milk allergens was reported at baseline, at T1, and T2. Statistical significance was defined by a p value <0.05.

persistent rise of CD8CD11b-double positive cells, where the CD11b expression defines a subset of circulating effector CD8+ T cells,[21] most likely as a compensatory mechanism of CD4+ T cells' reduction.

In previous studies, it has been demonstrated that the percentage of CD8+ T cells is significantly lower in infants with CMA compared to healthy subjects.[22,23] Evidence from both animal and human experiments suggested that an appropriate balance between T cell response mediated by effector CD8+ T cells and helper CD4+ T cells is necessary for the induction of oral tolerance.[24,25] In addition, the activity of CD8+ T cells has also been demonstrated to be lower in children affected by CMA.[23] Recently, a lower absolute number of naive CD8+ T cells was found in CMA children,[23] and the authors speculated that, since CD8+ T cells are one of the main interferon-γ-producing cell populations, the reduced pool of naive CD8+ T cells could be related with the decreased production of this key inflammatory mediator, observed in patients with CMA or other atopic diseases.[23,26,27] However, it is unclear if this T cell subset has a pathogenic or protective function in allergy. Of note, Huber et al. found that the only cellular population increased in the periphery during the early CMA phase is composed by CD4+ Th2 cells secreting IL4, probably as a consequence of the decreased number of circulating regulatory T cells.[26] Actually, it was shown that Treg cells specifically prevent the expansion of CD4+ Th2 cells in the mucosa that could lead to an allergic inflammatory response.[28] Interestingly, we found that Bifidobacteria treatment was able to significantly decrease CD4+ T cells in accordance with the study published by Hol et al.[29] The authors found that after 12 months of probiotic supplementation, based on a combination of *Lactobacillus casei* CRL431 and *Bifidobacterium lactis* Bb-12, there was a significant decrease of circulating CD3+CD4+ T cells compared to placebo treatment. This probiotic effect was most evident in infants with persistent CMA. In our data, the decrease of naive and activated T lymphocytes, and not of regulatory T cells, observed following probiotic treatment, suggested that the Bifidobacteria treatment affects mainly the Th2 cell subpopulation.

To the best of our knowledge, this is the first study that investigates the process of basophil degranulation in children affected by CMA after a probiotic treatment using the BAT. The BAT represents a promising complementary in vitro technique in the work-up of allergy reactions and for the diagnosis of food allergies (FA).[16,30,31] This cellular assay, based on the modulation of CD203c expression on basophils, has the advantage of a higher specificity for the diagnosis of FA compared to the anti-allergen IgE measurement in the serum. In the current study, we investigated the clinical relevance of BAT for monitoring the modulation of immune response to the main CMP induced by probiotic treatment. We observed a reduction of CD203c expression to the main CMP in our patients after 45 days of Bifidobacteria treatment. These results suggest the ability of this mixture of Bifidobacteria to modulate the response of basophils, immune cells known to be among the first cell populations to produce an abundant amount of pro-allergenic Th2 cytokines. In addition, we found a positive relation between the frequency of CD4+ T cells and the degranulation activity in response to CMP, after probiotic wash-out. These data are not surprising as the relation between basophils and lymphocyte subsets performs within the inflammatory phenomena in a cause-and-effect relationship. The effect of Bifidobacteria treatment on the reduction in basophilic reactivity to the allergens may result in a decreased pro-inflammatory response and in a reduction of Th2

Table 3. Relation between the frequency of circulating lymphocytes and basophil degranulation following probiotic treatment.

Basophil degranulation												
	Cow's milk						**Casein**					
	T0		**T1**		**T2**		**T0**		**T1**		**T2**	
	β	***p* value**	***β***	***p* value**	***β***	***p* value**	***β***	***p* value**	***β***	***p* value**	***β***	***p* value**
CD3	0.77	0.283	0.39	0.386	0.86	0.135	0.47	0.511	0.17	0.697	0.17	0.761
CD3CD45RA	0.34	0.574	0.03	0.364	1.32	**0.025**	0.41	0.505	0.30	0.619	1.18	**0.043**
CD4Foxp3	0.16	0.936	4.21	0.160	3.69	0.082	−0.07	0.972	0.72	0.805	2.82	0.175
CD8	1.63	0.07	−0.03	0.96	1.03	0.088	1.47	0.094	−0.23	0.715	0.33	0.553
CD8CD11b	−3.02	0.516	1.80	0.356	3.19	**0.024**	−1.49	0.766	−1.24	0.556	2.08	0.155
CD19	−1.71	**0.037**	−0.44	0.299	−1.04	0.078	−1.51	0.065	−0.29	0.501	−0.43	0.455
	Lactoalbumin						**Lactoglobulin**					
	T0		**T1**		**T2**		**T0**		**T1**		**T2**	
	β	***p* value**	***β***	***p* value**	***β***	***p* value**	***β***	***p* value**	***β***	***p* value**	***β***	***p* value**
CD3	0.64	0.323	0.00	0.998	1.40	**0.012**	0.65	0.454	0.57	0.295	0.81	0.244
CD3CD45RA	0.37	0.595	−0.26	0.722	0.54	0.406	0.09	0.910	0.74	0.397	0.11	0.885
CD4Foxp3	1.99	0.300	4.12	0.156	5.24	**0.015**	0.88	0.718	0.59	0.806	5.35	**0.044**
CD8	1.41	0.089	−0.11	0.854	1.67	**0.005**	2.14	**0.046**	0.83	0.273	1.36	**0.057**
CD8CD11b	−2.04	0.707	−1.58	0.478	3.11	**0.048**	−5.92	0.301	3.36	0.157	5.05	**0.044**
CD19	−2.06	**0.001**	−0.06	0.835	−1.77	**<0.001**	2.14	**0.015**	−0.68	0.139	−1.58	**0.015**

Results from mixed-effects longitudinal analysis. Bold *p* values are significant.

lymphocytes that cause the allergic reaction. Indeed, allergen immunotherapy modulates Th2 responses to allergens, and Shreffler et al. demonstrated a marked relationship between clinical tolerance acquired by CMA children and reactive down-regulation of allergen-specific effector cells.[32] Therefore, based on recent data, the investigation of basophil activation seems to be a promising approach for monitoring allergic inflammation, as well as the acquisition of tolerance.

The main pitfall of the present study is the open-label design and the small number of patients. However, the primary outcome of the study was the measurement of laboratory variations that cannot be influenced by the open-label design. Moreover, this is an exploratory analysis that should be validated in larger cohorts and strengthened by a control group, which for ethical reason was not possible to obtain, given the small age of the infants and the necessity of repeated blood draws without an intervention to test. Notwithstanding, the lack of a control group does not rule out the hypothesis that the decrease in basophil degranulation, or the change in cell population densities, may be partly attributed to the start of the diet with hydrolyzed casein formula. However, the most significant effects were found at T1 after probiotic treatment for basophil degranulation, whereas after the washout, during which the children were still on diet with hydrolyzed casein formula, the long-term (T2) effects of probiotics were less pronounced.

In conclusion, the probiotic treatment was able to modulate both innate and adaptive immunity. In particular, we demonstrated that Bifidobacteria treatment leads to a decrease of circulating naive and activated CD4+ T cells, as well as to a reduction of the degranulating basophils. This improvement of immunoregulation persisted long after the interruption of the probiotic oral supplementation. Although further investigation is needed, these data suggest that the Bifidobacteria could have a role in the acquisition of oral tolerance to cow's milk proteins and could provide a significant benefit in the treatment of cow's milk protein allergy.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the study will be available upon request to the authors.

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AUTHOR CONTRIBUTIONS

C.S. and A.V. contributed to the conception and design of the study, sample collection, analysis and interpretation of data, and drafted the article; F.P. performed the BAT experiments; F.G. contributed to sample collection and performed BAT experiments. P.D. performed the statistical and correlation analysis; S.V., T.M., and F.O. contributed to sample collection and analysis; M.M.d.G. contributed to the conception, design, and intellectual content of the study; G.M. and C.G. contributed to the conception, design and intellectual content of the study, revised the data, and drafted the article.

COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Institutional Review Board of the University of Campania "Luigi Vanvitelli" (registration number 07/2016). Written informed consent for participation in the study and publishing of individual patient data were obtained from the parents of all children.

ADDITIONAL INFORMATION

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