BASIC SCIENCE ARTICLE

Increased frequency of regulatory T cells in pediatric inflammatory bowel disease at diagnosis: a compensative role?

Alessandra Vitale[1], Caterina Strisciuglio[1], Serena Vitale[2], Marianna Santopaolo[3], Dario Bruzzese[4], Teresa Micillo[5], Elena Scarpato[6], Erasmo Miele[6], Annamaria Staiano[6], Riccardo Troncone[6], Giuseppe Matarese[3,7] and Carmen Gianfrani[2,6]

BACKGROUND: Regulatory T cells (Tregs) play a critical role in maintaining immune homeostasis. We investigated two main types of Tregs, the CD4+FOXP3+ and IL-10+ Tr1, in pediatric subjects with inflammatory bowel disease (IBD) both at diagnosis and after the clinical remission.
METHODS: Peripheral blood Tregs were analyzed in 16 children with Crohn's disease (CD), 19 with ulcerative colitis (UC), and 14 healthy controls (HC). Two cocktails of fluoresceinated antibodies were used to discriminate between CD4+FOXP3+ and Tr1.
RESULTS: We observed in both CD and UC groups a higher frequency of Tr1 at diagnosis compared to controls, which decreased at follow-up compared to diagnosis, in particular in UC. Similarly, in UC patients the percentage of CD4+FOXP3+ Tregs markedly decreased at follow-up compared to the same patients at diagnosis and compared to HC. The expression of CTLA-4 in CD4+FOXP3+ Tregs increased in both groups at clinical remission.
CONCLUSION: This study shows that IBD children present at diagnosis an increased frequency of circulating Tregs, probably as a compensative reaction to tissue inflammation. During the clinical remission, the Treg frequency diminishes, and concomitantly, their activation status increases. Notwithstanding, the high Treg density at diagnosis is not sufficient to counteract the inflammation in the childhood IBD.

Pediatric Research (2020) 87:853–861; https://doi.org/10.1038/s41390-019-0662-7

INTRODUCTION

Inflammatory bowel diseases (IBDs) are chronic inflammatory disorders of gastrointestinal (GI) tissues that include Crohn's disease (CD) and ulcerative colitis (UC). IBDs are characterized by a chronic course requiring a lifelong treatment. It has been reported that an overall 25% of cases are diagnosed in childhood.[1] In the pathogenesis of IBD, the adaptive immune system, which is antigen specific and confers long lasting and immunological memory, plays a key role. In contrast, the innate immune system, which is the first line of defense with an immediate protective response against infections, contributes to the initiation of gut inflammatory cascade.[2]

Regulatory T cells (Tregs) are able to prevent excessive inflammation that can lead to the rupture of intestinal homeostasis by modulating cells of both the innate and adaptive immune systems. Indeed, it has been demonstrated that an altered balance between Tregs and effector T cells in the intestinal microenvironment leads to the pathogenesis of IBD.[3] Moreover, in IBD mouse models, Tregs are able to control inflammatory lesions; in particular, their localization in the gut is critical for the suppression of innate immune responses during the colitis.[4] In fact, Powrie et al. demonstrated that Tregs are able not only to prevent but also to cure the murine IBD.[5] In

humans, Tregs have been successfully used in pioneering clinical trials for treatment of hematological malignancies by allogeneic haematopoietic stem cell transplantation, demonstrating that the infusion of Tregs is safe and, possibly, efficacious in some diseases.[6]

Although there are many different subpopulations of Tregs, the most investigated are the type 1 regulatory cells (Tr1) producing interleukin (IL)-10 and transforming growth factor (TGF)-β, the conventional Forkhead box protein 3 (FOXP3)-positive Tregs, and the TGF-β–producing T helper type 3 (Th3) cells, the last two with a role in oral tolerance.[7] The Tr1 and Th3 cells differ from conventional Tregs, as they do not constitutively express FOXP3, and are induced in the periphery by the presence of IL-10 and specialized subsets of antigen-presenting cells.[8] It has been recently demonstrated that CD49b and LAG-3 are markers specific of Tr1 cells, being highly and stably expressed and preserved on mouse and human Tr1 cells. The co-expression of CD49b and LAG-3 allows for the isolation of Tr1 cells and enables the purification of highly suppressive Tregs. Moreover, the use of these markers makes it feasible to track Tr1 cells in different tissue districts and disease conditions.[9]

Recently, Tr1 cells have received increased attention in medical research due to their role in peripheral immune

[1]Department of Woman, Child and General and Specialized Surgery, University of Campania "Luigi Vanvitelli", Naples, Italy; [2]Institute of Biochemistry and Cell Biology, National Research Council (IBBC-CNR), Naples, Italy; [3]Department of Molecular Medicine and Medical Biotechnology, University "Federico II", Naples, Italy; [4]Department of Public Health, University "Federico II", Naples, Italy; [5]Department of Biology, University "Federico II", Naples, Italy; [6]Department of Translational Medical Science (Section of Pediatrics), and European Laboratory for the Investigation of Food-Induced Diseases, University "Federico II", Naples, Italy and [7]Laboratory of Immunology, Endocrinology and Experimental Oncology Institute, National Research Council (IEOS-CNR), Naples, Italy
Correspondence: Carmen Gianfrani (c.gianfrani@ibp.cnr.it)
These authors contributed equally: Alessandra Vitale, Caterina Strisciuglio

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tolerance, as they have been shown to suppress tissue inflammation, including colitis and autoimmune diseases in mouse models and humans.[8,10] While, to our knowledge, Tr1 cells have not yet been investigated in IBD, studies on conventional Tregs in human IBD are more numerous, although with contrasting results, in particular on their frequency and suppressive function.[11,12] Furthermore, the majority of studies on Tregs refers to adult IBD, and very few pediatric studies have been reported.[13–15] An inverse correlation between Treg frequency and disease activity has been demonstrated in UC patients.[16] Indeed, CD4+FOXP3+ Tregs were increased in the peripheral blood during remission but decreased during active disease.[11] In contrast, it has been shown that patients with active IBD have more FOXP3+ Tregs at the site of intestinal inflammation, which retain potent ex vivo inhibitory activity, as documented by the suppression of proliferation and cytokine production of CD4+ T helper cells.[12] Moreover, the FOXP3+ Tregs frequency is reduced by 6-mercaptopurine or azathioprine (AZA) treatment but not by steroids or anti-tumor necrosis factor-α (anti-TNFα) agents.[17] Instead, other studies demonstrated that IBD patients with active disease, while having no differences in circulating CD4+FOXP3+ Tregs levels compared to healthy controls (HC),[18] show a significant increase of this cell type after treatment with anti-TNFα. Interestingly, a negative correlation was found among the Tregs levels and the age of patients or among disease duration and the activity score of CD.[19]

It has been postulated that pediatric IBD represents a separate entity with specific clinical peculiarities requiring a specialized medical care.[20] Although pediatric IBD are more severe and aggressive compared to IBD diagnosed in adults,[21] young IBD patients represent ideal population for the study of disease mechanisms because of the different pathogenic origin, natural history, and outcome compared to adults. Such studies are strongly needed to dissect the complex picture of these largely spreading gut disorders.[22] The dysfunction or the deficiency of Tr1 and FOXP3+ Tregs may be involved in the breakdown of immune tolerance to luminal antigens, which is most likely occurring in IBD. In this study, we evaluated the frequency of Tr1 and FOXP3+ Tregs monitored in blood of patients either at disease onset or during pharmacological remission. The density of Tr1 and FOXP3+ Tregs was also correlated with disease activity.

MATERIALS AND METHODS
Study population
The study population included 56 young subjects, enrolled at the Department of Translational Medical Science, University of Naples "Federico II" (Table 1). Nineteen (13.3 years; range 8.4–16.8 years) were affected by CD, 20 (9.9 years; range 2.7–15.1 years) affected by UC, and 17 (11.9 years; range 6.3–15.6 years) were HC. The diagnosis of CD and UC was based upon conventional clinical, radiological, and endoscopic features and was confirmed by histopathological examination of the intestinal biopsies.[23] For each patient, clinical activity of disease was evaluated at the time of diagnosis and at follow-up using the Pediatric Crohn's Disease Activity Index (PCDAI) and the Pediatric Ulcerative Colitis Activity Index (PUCAI). IBD clinical remission was defined as the maintenance of a PUCAI/PCDAI ≤ 10 at follow-up visit.[24,25] Almost all IBD patients were also analyzed after pharmacological therapy (6 months, range 2–9 months). Specifically, in both the CD and UC groups, 11 patients were treated with mesalazine (5-ASA) and 5 with AZA, whereas remaining 4 UC and 3 CD patients were studied only at diagnosis. HC were subjects affected by functional GI disorders, in whom was performed blood analysis to exclude an inflammatory condition.

Table 1. Clinical features of the pediatric subjects enrolled.

Characteristics	CD	UC	HC
Patients	19	20	17
Gender, male/female	10/9	11/9	10/7
Age (year and range)	13.3 (8.4–16.8)	9.9 (2.7–15.11)	11.9 (6.3–15.6)
Age at diagnosis (year and range)	13.3 (8.4–16.8)	9.9 (2.7–15.11)	NA
Disease duration (month and range)	6.4 (3–9)	6 (3–9)	NA
Weight T0 (Kg)	42.12 (21–58.4)	40.61 (13.5–59.2)	37.83 (17.3–71)
Weight T1 (Kg)	47.62 (26–64)	44.51 (14.2–66)	NA
Height T0 (cm)	154.16 (120.5–167)	141.68 (90–174)	140.39 (106–167.5)
Height T1 (cm)	156.09 (121.4–170)	146.09 (94–187)	NA
PCDAI T0	31.40 (15–47.5)	NA	NA
PCDAI T1	1.50 (0–7.5)	NA	NA
PUCAI T0	NA	50.94 (30–65)	NA
PUCAI T1	NA	1.88 (0–15)	NA
Calprotectin T0 (μg/g)	315.88 (153–684.3)	298.06 (149–788)	NA
Calprotectin T1 (μg/g)	169.47 (27–265)	125.88 (15–235)	NA
CRP T0 (mg/dl)	3.18 (1.05–9.4)	3.24 (0.7–8.81)	NA
CRP T1 (mg/dl)	0.42 (0.11–0.99)	0.33 (0.31–0.33)	NA
ESR T0 (mm)	36.13 (14–85)	21.81 (4.9–67)	NA
ESR T1 (mm)	11.51 (0.5–20)	6.44 (2–20)	NA
Paris Classification			
A1b-L1-B1-G0	6	NA	NA
A1b-L2-B1-G0	1	NA	NA
A1a-L3-B1-G0	1	NA	NA
A1b-L3-B1-G0	5	NA	NA
A1b-L3-B1-G1	3	NA	NA
E1-S0	NA	3	NA
E2-S0	NA	3	NA
E2-S1	NA	1	NA
E3-S0	NA	6	NA
E3-S1	NA	1	NA
E4-S0	NA	4	NA
E4-S1	NA	1	NA
Therapy			NA
Aminosalicylates	11	11	
Immunosuppressants	5	5	
Aminosalicylates and immunosuppressants	0	0	
Monoclonal antibodies	0	0	
No therapy	0	0	

CD Crohn's disease, *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate, *HC* healthy controls, *IBD* inflammatory bowel disease, *NA* not applicable, *PCDAI* Pediatric Crohn's Disease Activity Index, *PUCAI* Pediatric Ulcerative Colitis Disease Activity Index, *UC* ulcerative colitis

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Cell staining. Peripheral blood mononuclear cells (PBMCs), isolated according to a well-established procedure,[26] were frozen in fetal calf serum and dimethyl sulfoxide (90%v/v) and cryopreserved until the flow cytometric analysis or freshly used. Cells were stained with two different antibody cocktails (Mix) that discriminated between the two main types of Tregs: Mix 1 specific for Tr1: allophycocyanin (APC)-CD45 (HI30; BD-PharMingen): APC-Cy7-CD4 (RPA-T4; BD-PharMingen); phycoerythrin (PE)-Cy7-CD25 (M-A251; BD-PharMingen); fluorescein isothiocyanate (FITC)-CD49b (BMS11; eBio-science); peridinin chlorophyl protein (PerCP)-eFluor71-CD223(LAG-3), (BMS46; eBio-science). Mix 2 specific for FOXP3+ Tregs: APC-Cy7-CD4 (RPA-T4; BD-PharMingen); PE-Cy7-CD25 (M-A251; BD-PharMingen); PE-FOXP3 (259D/C7; BD-PharMingen); Alexa-Fluor488-HELIOS, (22F6; BD-PharMingen); Pacific-Blue-CD279(PD-1) (BV421; BD-PharMingen); APC-CD152(CTLA-4) (BNI3; BD-PharMingen); PerCP-Cy5.5-CD197(CCR7) (150503; BD-PharMingen). After the cell surface marker staining, cells were washed, fixed, and permeabilized (fixation–permeabilization buffer; eBio-science) for intracellular/nuclear staining (CTLA-4, HELIOS, and FOXP3). For the analysis of intracellular IL-10, fresh PBMCs from four young IBD subjects (one CD and three HC) were used. PBMC cells were suspended at 1×10^6 in RPMI-1640 with 5% autologous blood serum and IL-2 (50 U/ml) and incubated for 24 h with medium alone or with anti-CD3/CD28 beads (1.25 μl × 10^6 cells, Thermo Fisher Scientific); brefeldin-A (10 μg/ml) was added for the last 3 h of incubation. Cells were stained with PeCy7-CD45RO (UCHL1, BD-PharMingen), BV510-CD45RA (HI100; BD-PharMingen), APC-Cy7-CD4, FITC-CD49b, PerCP-eFluor-71-LAG3, and PE-IL-10 (JES3-19F1; BD-PharMingen) antibodies. All antibodies were used at the concentration indicated in the manufacturers' instructions. Samples were acquired with FACS-Canto II (BD), and the analyses were performed with the Diva (BD) and FlowJo (Tree Star) softwares. At least 5000 events were acquired in the gate of CD4+ T lymphocytes.

Statistical analysis
All numerical variables of flow cytometric data were summarized using median with range [min–max]. Statistical analysis was performed using the R platform (v3.4.1, http://www.r-project.org/). The cross-sectional between-group comparisons were based on Kruskal–Wallis test followed by pairwise comparison based on Mann–Whitney U test; multiple testing was addressed, in each comparison, using the Holm procedure. Comparisons within the groups were conducted using Wilcoxon signed-rank test. Correlations between variables were assessed using the non-parametric Spearman rank correlation coefficient. In all the analyses, an overall alpha level of 0.05 was used.

Ethical considerations
The Institutional Review Board of the University of Naples "Federico II" approved the study protocol and the questionnaire (registration number 121/15). Written informed consent was obtained from parents of all children. Patients aged >13 years also signed the consent.
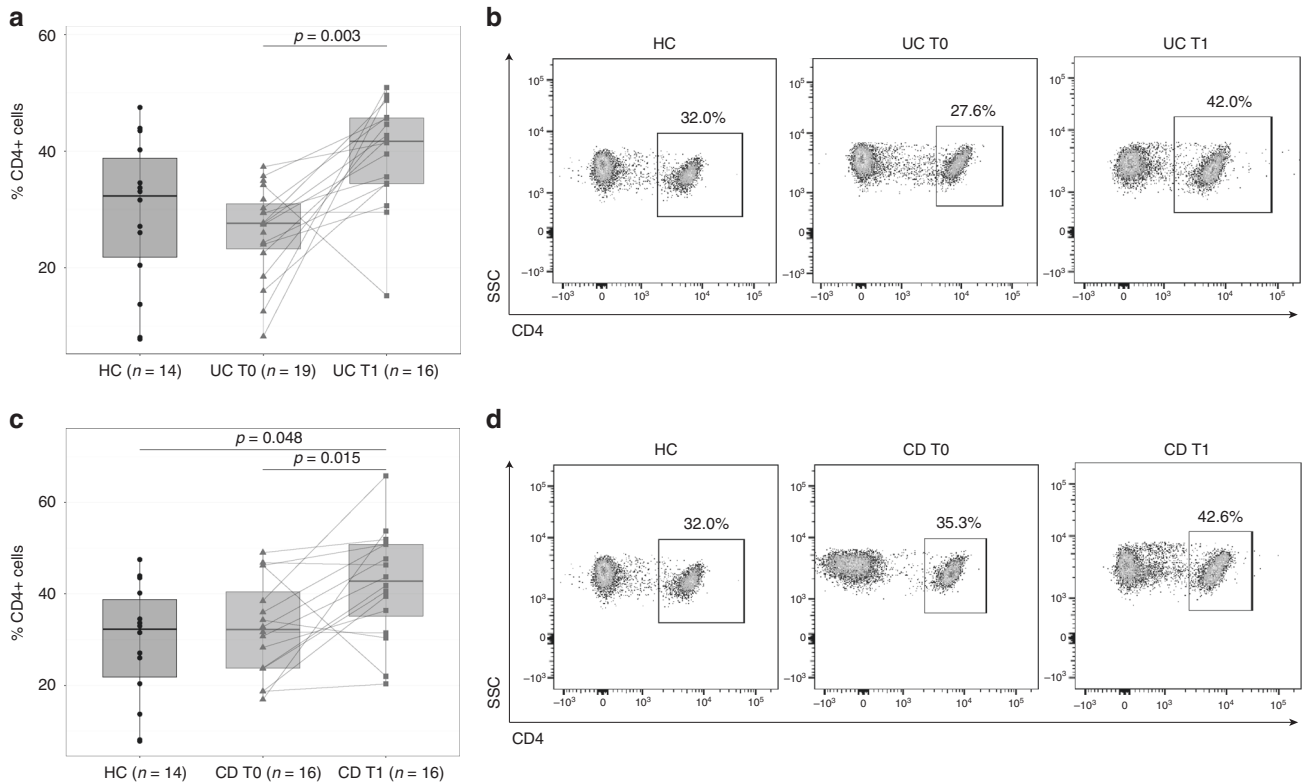

Fig. 1 Increased frequencies of CD4+ cells in the peripheral blood of pediatric patients with IBD in remission after therapy. The frequency of CD4+ cells was analyzed in the gate of total mononuclear cells by multiparametric flow cytometry. The percentage of CD4+ cells was evaluated in peripheral blood of patients at diagnosis (T0) compared to the same patients at follow-up (T1) in 19 UC (**a, b**) and 19 CD subjects (**c, d**), in parallel with 14 HC individuals. Each point represents the percentage of CD4+ cells from a single subject and horizontal bars indicate the median value in each group (**a, c**). Flow cytometric dot plots of CD4+ cells, analyzed before (T0) and after the drug therapy (T1), from representative UC, CD, and HC subject are shown (**b, d**). Kruskal–Wallis test, Mann–Whitney U test, or Wilcoxon signed-rank test were applied, as appropriate, to evaluate differences among the groups, as indicated in Materials and methods. The statistical analysis was performed using the R platform. In all the analyses, a p value <0.05 was considered statistically significant.

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RESULTS

Increased densities of CD4+ T cells in IBD patients at pharmacological remission

As this study aimed to analyze in childhood IBD the two main CD4+ Treg subsets, the CD25+FOXP3+ and IL-10+ Tr1 cells, we first evaluated the frequency of CD4+ T cells in the peripheral blood of UC ($n = 19$), CD ($n = 16$) patients, and HC ($n = 14$). The great majority of IBD children were also analyzed after pharmacological (5-ASA or AZA) therapy (6 months, range 4–9). The remission was confirmed by the reduction of PUCAI/PCDAI indexes and decreased erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and fecal calprotectin levels. We found a higher frequency of CD4+ T cells at follow-up (T1) compared to at diagnosis (T0) both in UC ($p = 0.003$; Fig. 1a, b) and CD ($p = 0.015$) cohorts (Fig. 1c, d). Furthermore, in CD patients, the frequency of CD4+ T cells at follow-up resulted significantly higher compared to HC ($p = 0.048$; Fig. 1c, d).

Acute IBD are associated with increased level of Tr1 (CD49b+LAG3+) cells

It has been reported that the co-expression of CD49b and LAG3 molecules specifically identified the IL-10+ Tr1.[9] We have further analyzed the density of CD49b+LAG3+ cells in the gate of CD4+ T cells in IBD patients, both at T0 and T1. Tr1 were significant higher at diagnosis in UC patients compared to HC ($p = 0.009$; Fig. 2a, b); while in the CD group, the increment did not reach the statistical significance (Fig. 2c, d). Moreover, the longitudinal analysis showed a marked reduction of the Tr1 cell population at follow-up in comparison to the Tr1 frequency at diagnosis in both UC and CD, although the difference resulted significant only in UC patients ($p = 0.006$; Fig. 2a, c). Furthermore, no significant differences were observed in the frequency of CD49b+LAG3+

cells between the 5-ASA- and AZA-treated patients. We next evaluated whether the Tr1 cells produced IL-10 in additional 4 IBD patients (Supplementary Fig. S1). We observed that CD49b+LAG3+ cells were IL-10 positive even in unstimulated condition, and as expected, the frequency of IL-10+ Tr1 markedly increased after the anti-CD3/CD28 stimulation. However, the low number of patients enrolled for the intracellular IL-10 experiments was not sufficient to observe significant differences between the CD and UC groups (Supplementary Fig. S1).

Clinical remission of UC patients is associated with a reduction of FOXP3+ Tregs

We next examined the frequency of CD4+FOXP3+ Tregs in the peripheral blood of childhood IBD patients. In contrast with Tr1 findings, we did not find an increase in the frequency of CD4FOXP3 double-positive cells at diagnosis compared to non-IBD controls (Fig. 3). Notwithstanding, the longitudinal analysis showed a marked reduction of CD4+FOXP3+ Treg population at clinical remission compared to diagnosis. In UC children, we observed at follow-up a lower frequency of CD4+FOXP3+ cells compared to the density found at diagnosis ($p = 0.001$; Fig. 3a, b). CD4+FOXP3+ Tregs in UC were also less frequent compared to HC ($p = 0.006$; Fig. 3a, b). By contrast, in CD patients, the disease remission was associated with a slight reduction of the circulating FOXP3+ Tregs (Fig. 3c, d). In order to confirm the regulatory phenotype of natural Treg, the analysis was further expanded to the CD4+CD25+ T cell subset at the time of IBD diagnosis (Supplementary Fig. S2) and to the expression of transcription factor HELIOS (Supplementary Fig. S3). We found similar percentages between the density of CD4+CD25+ and CD4+FOXP3+ cells in each group of children, ranging from 1.2% to 12.8%. Notably, the great majority of CD4+FOXP3+ Tregs were HELIOS+
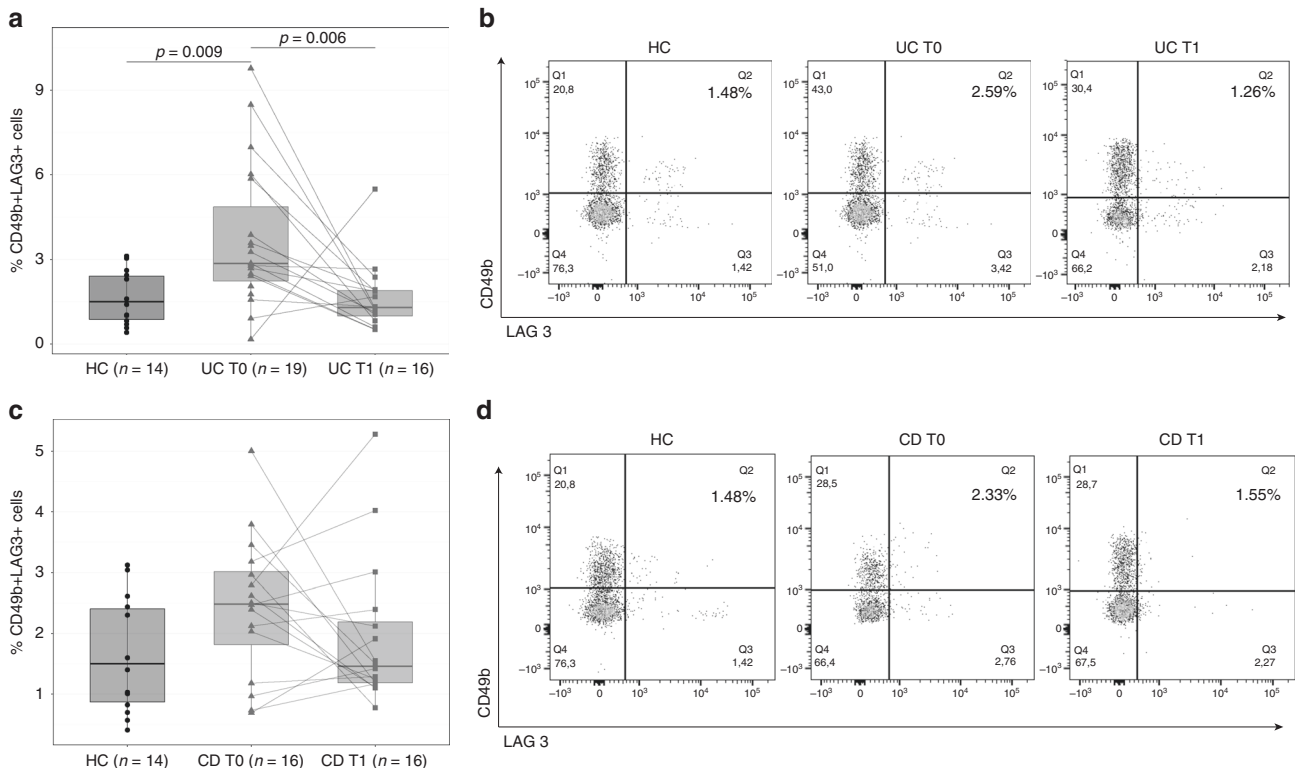

Fig. 2 Increased frequency of CD49b+LAG3+ (Tr1) Tregs in IBD children at diagnosis. In gated CD4+ cells, the frequency of CD49bLAG3 double-positive cells (Tr1) was analyzed in 19 UC patients at diagnosis (T0), 16 UC patients at pharmacological clinical remission (T1) and in 14 HC (**a**), and in 16 CD patients at both T0 and T1 (**c**). Dot plots of CD49bLAG3 double-positive cells, before and after the drug therapy, are shown from a representative control and disease child (**b**, **d**). The statistical analysis was performed as indicated in Fig. 1. A p value < 0.05 was considered statistically significant.

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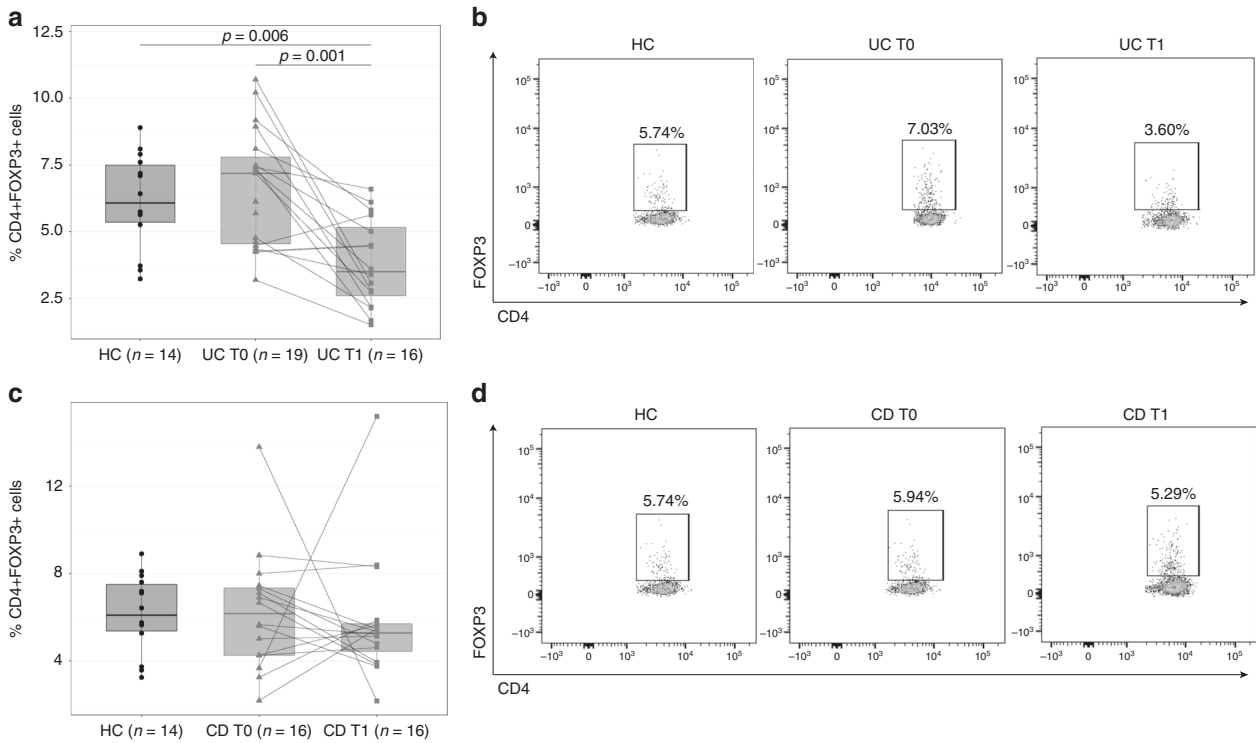A Vitale et al.

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Fig. 3 Decreased frequencies of CD4+FOXP3+ Tregs after therapy in UC patients. In gated CD4+ cells, the frequency of CD4+FOXP3+ Tregs was analyzed in 19 UC patients at T0 and 16 UC at T1 and in 14 HC (**a**). The percentage of CD4+FOXP3+ Tregs was evaluated also in 16 CD at diagnosis compared to the same patients at follow-up and in HC group (**c**). Dot plots of CD4+FOXP3+, before and after the therapy, from a representative HC and from representative UC (**b**) and CD patients (**d**) are shown. The statistical analysis was performed as indicated in Fig. 1. A p value <0.05 was considered statistically significant.
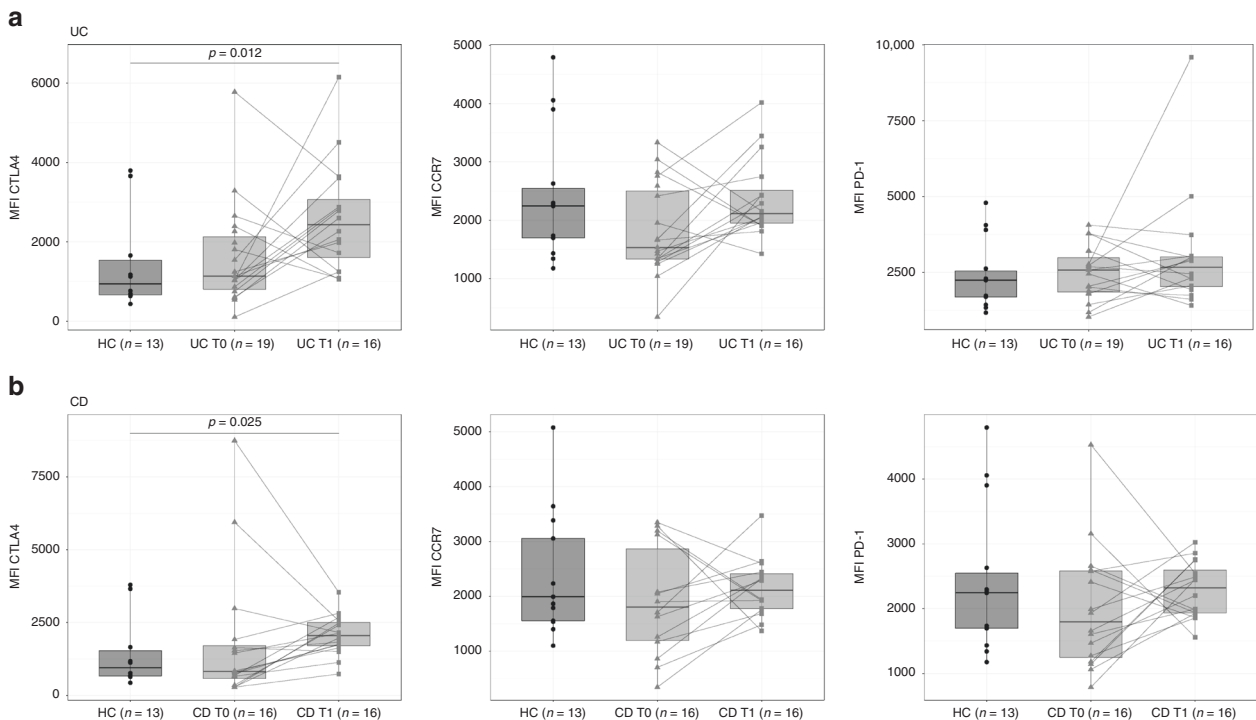
Fig. 4 Impairment of CD4+FOXP3+ Treg activation status in IBD patients at diagnosis. In CD4+FOXP3+ Treg gate, the median fluorescence intensity (MFI) for CTLA-4, CCR7, and PD1 markers was evaluated for UC (**a**) and CD (**b**) patients at both diagnosis (T0) and after the pharmacological clinical remission (T1). Each point represents the MFI of the indicated marker evaluated in each single subject, and horizontal bars indicate the median percentage calculated within each disease group (**a**, **b**). The statistical analysis was performed as indicated in Fig. 1. A p value <0.05 was considered statistically significant.

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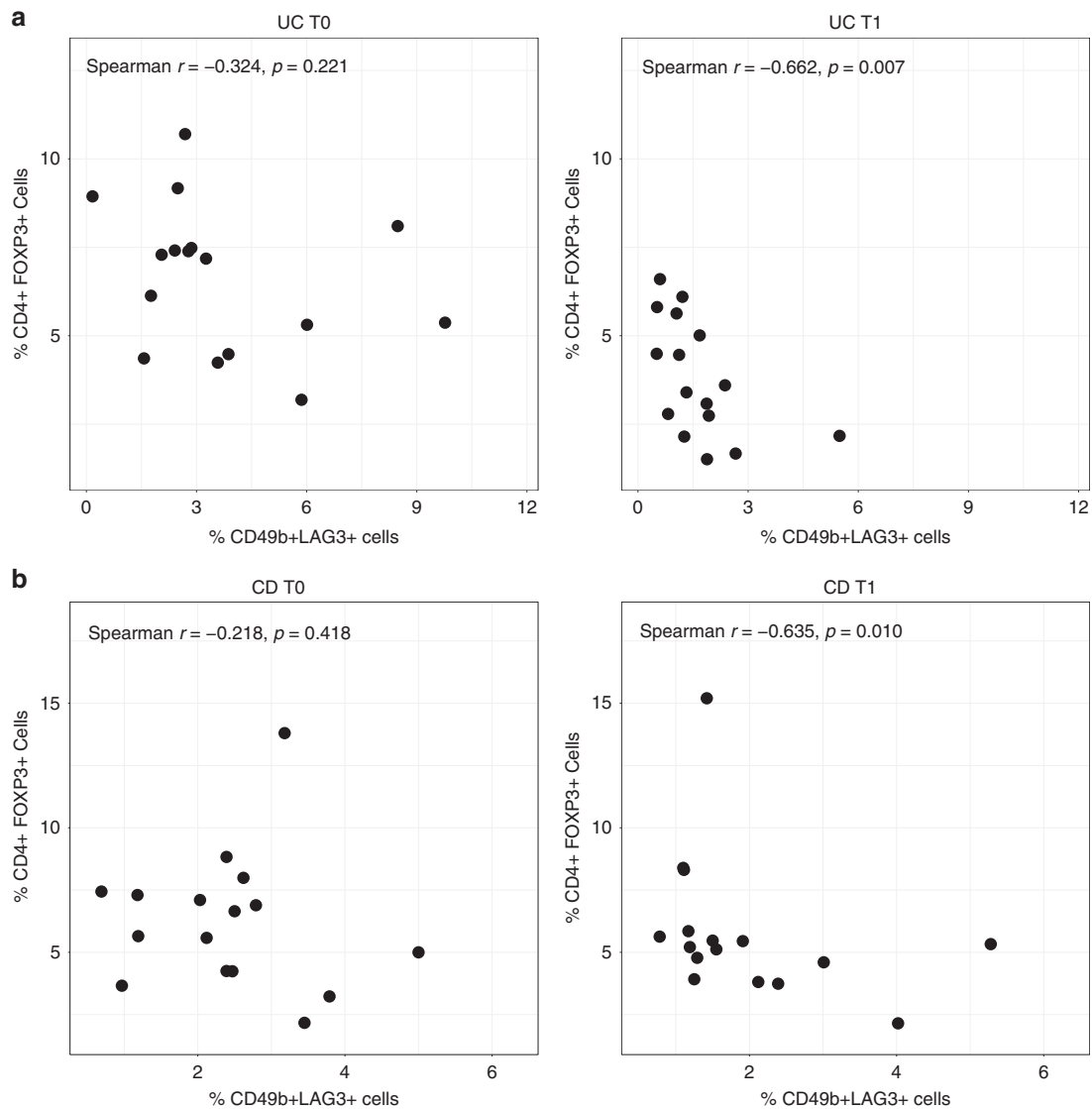A Vitale et al.

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Fig. 5 CD4+FOXP3+ and CD49b+LAG3+ Tregs negatively correlated in IBD patients at clinical remission. Correlations between the frequency of CD4+FOXP3+ and CD49b+LAG3+ Tregs were evaluated both at diagnosis (T0) and at follow-up (T1) in UC (**a**) and CD (**b**) children. The densities of CD4+FOXP3+ cells and CD49b+LAG3+ cells were correlated at the different disease condition and between the groups using the non-parametric Spearman rank correlation coefficient.

(from 78.6% to 96%). Finally, when patients were stratified for the pharmacological (aminosalicylates or azathioprine) therapy, no significant differences were observed in the percentage of CD4+FOXP3+ cells.

Higher expression of CTLA-4 in FOXP3+ Tregs in IBD patients in clinical remission
We next looked at the expression of several markers associated with activation and function of FOXP3+ Tregs in pediatric IBD. In particular, we found a more activated status of FOXP3+ Tregs in children with UC and CD at the remission condition (T1) compared to at the time of diagnosis (T0), as demonstrated by a higher expression of CTLA-4 at follow-up in both the IBD groups, with a significant increment compared to healthy children ($p = 0.012$ for UC and $p = 0.025$ for CD, respectively; Fig. 4a, b). No changes were found in the expression of PD-1 receptor and CCR7 in CD4+FOXP3+ cells, independently from the clinical conditions of IBD patients (Fig. 4a, b).

Correlation of Treg frequency with disease activity
Finally, we investigated the correlation between the frequency of circulating Tregs and the score of disease activity. First of all, we found an inverse correlation between the frequency of CD4+FOXP3+ Tregs and Tr1 at follow-up T1 in both the UC ($p = 0.007$) and CD ($p = 0.01$) groups (Fig. 5a, b, respectively). Moreover, we found a positive correlation between the percentage of CD4FOXP3 double-positive cells and CRP in CD at diagnosis ($p = 0.041$) and at clinical remission ($p = 0.0037$) (Fig. 6a, b, respectively). We did not observe a significant correlation of Tr1 and fecal calprotectin at CD diagnosis (Fig. 6c), whereas we found an inverse correlation between the frequency of Tr1 and fecal calprotectin at follow-up ($p = 0.022$) (Fig. 6d). In addition, in UC patients at diagnosis, we evaluated an inverse correlation between CD4+FOXP3+ cells and fecal calprotectin ($p = 0.033$) (Fig. 6e), whereas the frequency of Tr1 correlated in indirect proportion with ESR ($p = 0.047$) (Fig. 6g). No other significant correlations were found between the parameters studied and Treg frequency.
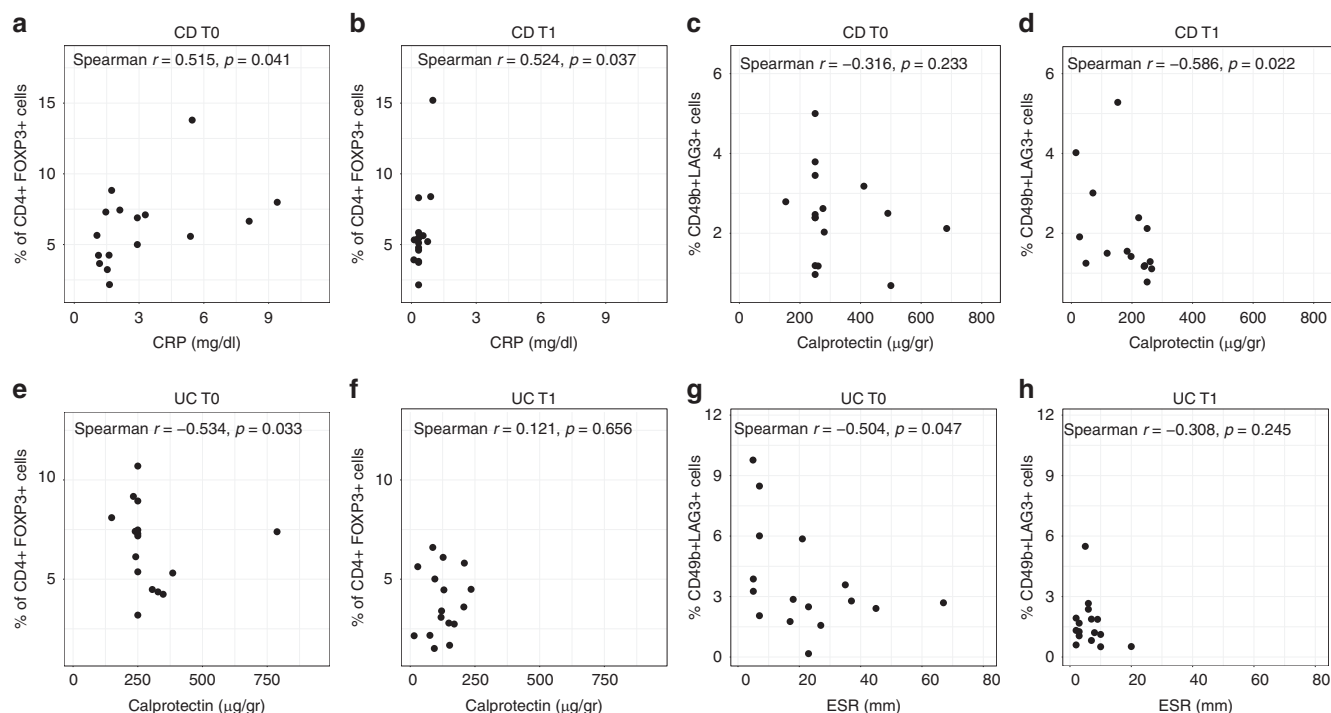
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Fig. 6 Correlation of CD4+FOXP3+ and CD49b+LAG3+ (Tr1) Tregs frequency with inflammatory markers at IBD diagnosis and follow-up. The frequency of CD4+FOXP3+ and CD49b+LAG3+ Tregs was correlated with the indices of disease activity in IBD children. Correlation between CD4+FOXP3+ Tregs and the level of serum C-reactive protein (CRP) in CD at diagnosis (T0) (**a**) and at follow-up (T1) (**b**); Tr1 in CD and fecal calprotectin at T0 (**c**) and T1 (**d**); CD4+FOXP3+ cells and fecal calprotectin in UC at diagnosis T0 (**e**) and T1 (**f**), CD49b+LAG3+ cells and the erythrocyte sedimentation rate (ESR) at T0 (**g**) and T1 (**h**). The correlations were assessed using the non-parametric Spearman rank correlation coefficient.

DISCUSSION

To the best of our knowledge, this is the first study that investigated IL-10-secreting Tr1, identified as CD49b+LAG3+ cells, in pediatric IBD. We observed in peripheral blood of both UC and CD an increased percentage of Tr1 cells at the diagnosis compared to healthy children. This cell population critically decreased upon the pharmacological remission, reaching the same frequencies as found in HC subjects. Instead, we assessed that the frequency of FOXP3+ cells at IBD diagnosis was comparable to that of controls and decreased after therapy.

Few previous studies limited the analysis to the density of CD4+FOXP3+ Tregs in peripheral blood and intestinal mucosa of children with IBD. Sznurkowka et al. reported that at diagnosis the circulating and intestinal CD4+CD25+FOXP3+ cells were significantly higher in the IBD group than in HC.[13] La Scaleia et al. also found that FOXP3+ cells were significantly increased both in peripheral blood and in the intestinal lesions of patients with active IBD.[15] Reikvam et al. instead found an increased mucosal distribution of CD25+FOXP3+ cells in untreated pediatric CD patients compared to controls.[14]

Our observation of increased presence of Tregs in children at the IBD diagnosis is in contrast with most of the literature that find reduced number of Tregs in the peripheral blood of adult IBD patients during active disease compared to inactive disease or in healthy individuals.[16,19] Wang et al. found a decrease of CD4+FOXP3+ Treg cells in the peripheral blood of both CD and UC patients and an accumulation of these cells in inflamed mucosal tissues.[27] Moreover, the percentage of Tregs observed in this research was higher in IBD patients at remission status compared to active disease.[27] By contrast, Di Sabatino et al. did not find a significant difference between the percentage of CD4+FOXP3+ Tregs in IBD patients when compared to HC.[18] However, all the above articles included adult patients with a long follow-up history, whereas our study quantified Tregs in very young patients

at the time of diagnosis and after a short follow-up. Of note, the distinction between adult and pediatric IBD is a very current topic due to the rising incidence of this condition in childhood.[28] Children with IBD seem to be a distinctive population in terms of both pathogenetic mechanisms and clinical management,[15] which, most likely, depend on a different profile of immune reactivity. Indeed, pediatric IBD requires highly skilled and specialized approach for diagnosis and treatment.[29] La Scaleia et al.,[15] who found results similar to ours, suggested that a more pronounced thymopoietic activity in children may explain the maintenance of a higher frequency of circulating Tregs during active IBD, which is different from what occurs in adult patients, in whom the recruitment of Tregs at mucosal sites correlates with a frequency lowering in the systemic compartment.

Moreover, our data suggest that the immunoregulatory potential of IBD may change with the duration of disease. Indeed, it has been demonstrated that there are clear differences in the production and in the response to cytokines by T cells in early and late CD.[30] During the initial disease manifestation, mucosal T cells are strongly polarized toward a Th1 phenotype, with high interferon-γ production and expression of IL-12R. By contrast, once the disease progresses, this pronounced Th1 polarization is lost.[30,31] Our results are also consistent with previous researches in celiac disease,[10] rheumatoid arthritis,[32] and systemic lupus erythematous,[33] suggesting Treg recruitment to counteract the inflammatory stimuli.

It could be envisaged that the reduced frequency of Tregs in IBD patients is due to the action of the immunosuppressant drugs. However, we did not find differences among the two groups treated with 5-ASA or AZA. It has been reported that these drugs may reduce the frequency of Tregs in peripheral blood and stimulate the Treg-suppressive functions through the expression of CTLA-4 and PD-1 inhibitory markers.[34] We found that the CD4+FOXP3+ Tregs expressed higher level of CTLA4 in IBD children at

clinical remission, compared to the early diagnostic state, suggesting a more suppressive ability upon the pharmacological remission. Furthermore, it has been reported that high levels of PD-1 expressed by CD4+FOXP3+ Tregs identify a dysfunctional Treg population.[35] We excluded a functional alteration in IBD subjects, as the expression of PD-1 in Tregs of IBD children was similar at diagnosis and remission, and with controls.

Interestingly, we observed in our cohort of young IBD patients that the frequency of circulating CD4+FOXP3+ Tregs positively correlated with CRP but negatively correlated with fecal calprotectin. The reason for this discrepancy could be due to the fact that CRP is a serum marker of systemic inflammation, whereas fecal calprotectin is a marker of intestinal inflammation, which could directly correlate to the density of intestinal mucosa Tregs. However, limited studies have been conducted on the relationship between Treg frequency and disease activity in IBD, especially in pediatric population. In agreement with our data, Holmen et al. found a positive correlation between Treg percentage and CRP in adult UC patients.[36] The authors concluded that, although Tregs accumulate in the intestinal mucosa of IBD patients, the frequency may not be enough to restrain the intestinal inflammation. Our study demonstrates that, although children with acute IBD show a high number of Tregs, most likely this regulatory cells fail to prevent the inflammation.

Several studies considered the possibility to treat CD patients through a Treg-based cellular therapy.[37,38] Despite the presence of unknown side effect risks, the use of Tregs as medical strategy in IBD may help to reset the adverse intestinal immunity. Current approaches for IBD depend on the use of non-antigen-specific immunosuppressive agents, such as steroids and anticytokine antibodies. However, these pharmacological treatments are not effective in some patients and never provide a complete disease resolution. By contrast, Treg cellular therapy would offer an antigen-specific and potent cure by targeting peculiar antigens at the site of inflammation. In conclusion, the in vitro expansion of Tregs may be an appealing and feasible approach for future autologous cellular therapy for IBD.

AUTHOR CONTRIBUTIONS
A.V., C.S., and S.V. contributed to conception and design of the study, sample collection, analysis and interpretation of data, and drafted the article; M.S., E.S., and E.M. contributed to patient enrolment and analysis, and interpretation of data; D.B. contributed to analysis and interpretation of data; A.S. and R.T. revised the article critically for important intellectual content; G.M. and C.G. contributed to conception, design and intellectual content of the study, revised the data, and drafted the article.

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