

CD4⁺CD25⁺CD127^{low} FoxP3⁺ regulatory T cells in Crohn's disease

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Background. Regulatory T (Treg) cell plays a key role in autoimmune diseases. We evaluated the regulatory function and frequency of Treg cells and secreted IL-10, IL-35 concentration in Crohn's disease (CD).

Methods. Twenty-three patients with CD and 25 healthy controls (HC) were included in this study. We analysed the alteration of Tregs frequency using flow cytometry for CD4, CD25, CD127 and FoxP3 markers. Surface expression of CD4, CD25 and CD127 markers were used for isolation of relatively pure Treg cells. Suppressiveness activity of Tregs was determined by measuring their ability to inhibit the proliferation of T responder (Tres) cells. In addition, the amounts of IL-10 and IL-35 cytokines in co-culture supernatants were measured by ELISA assay after stimulation with anti-CD2/CD3/CD28.

Results. CD patients had significantly lower frequency of CD4⁺ CD25⁺ CD127^{low} FoxP3⁺ Treg cells in comparison with controls (2.17 ± 1.04 vs. 2.83 ± 1.07 , $p = 0.0352$). Additionally, Treg cells mediated suppression was not significantly different in CD patients compared to controls. There was a significant difference in IL-10 secretion in response to anti-CD2/CD3/CD28 stimulation compared with HC ($p = 0.0074$).

Conclusion. The frequency of CD4⁺ CD25⁺ CD127^{low} FoxP3⁺ Tregs decreased in active stage of CD but there was no impaired suppressive function of CD4⁺ CD25⁺ CD127^{low} FoxP3⁺ Treg cells. We suggest that an alteration in the balance of Tregs and T effectors may contribute to pathogenesis of CD.

Key words: Crohn's disease, Regulatory T cells, IL-10, IL-35.

INTRODUCTION

Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterized by chronic relapsing immune-mediated inflammation that can affect various sections of the gastrointestinal tract (GIT) [1, 2]. Although the aetiology of CD remains poorly understood, several genetic and immune factors have been implicated in the pathogenesis of inflammatory disorders such as CD. Many studies indicate that the disease is caused by an improper mucosal immune response to microbial flora of GI, which eventually leads to chronic intestinal inflammation. These studies also stated that failure of immunological tolerance toward intestinal antigens plays a key role in the pathogenesis of CD.

Regulatory T cells (Tregs) constitute a permanent lineage of dedicated regulator cells and they are critical for suppression of immune responses and maintenance of homeostasis by promoting peripheral tolerance to self-antigens [3]. Their role in the development of pathology has previously been explored. Thus, impaired tolerance and occurrence of autoimmune diseases may be due to the defective regulatory function of Tregs [4, 5]. Tregs are characterized by expression of CD4⁺, CD25⁺ and low expression of CD127 (IL-7 receptor α). Moreover, FOXP3 is expressed as a specific molecular marker for discrimination between activated regulatory T-cells and other cells like activated CD4⁺ T-cells [6]. There is association between lack of FOXP3 protein expression and scurfy phenotype in mice

and also with immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX) in humans [7]. Interestingly enough, some studies showed that a patient with mutations in expression of the CD25 gene developed an IPEX-like syndrome [8]. The regulatory functions of Tregs are mediated by cell-cell contact and also secretion of inhibitory cytokines such as IL-10, TGF- β , and IL-35. The association of high levels of IL-10 and IL-35 with autoimmune diseases suggests that these cytokines play a special role in their pathogenesis. Therefore, these cytokines act as a crucial modulator in immune regulation in the intestine and the acute inflammatory process of IBD [9, 10].

Given the unknown aetiology of the CD and the role of the Tregs in the regulation of immune responses, we have decided to assess Treg cells contribution to the complex pathogenesis of CD. In addition, we evaluated IL-10 and IL-35 concentrations in the co-culture supernatant.

MATERIALS AND METHODS

Human subjects

Twenty-three patients (12 men and 11 women) aged 37.04 ± 10.44 years (range 19 to 63 years) with CD were studied. The healthy control group (HC) consisted of 25 (13 males and 12 females) with mean age of 35.92 ± 9.72 years (range 20 to 56). All patients had not received any treatment. The diagnosis of this disease was performed according to established criteria including clinical history, physical and laboratory findings, and endoscopic and histo-pathologic features. Crohn's disease activity was actually assessed based on Harvey Bradshaw Index (HBI) [11]. Thus, the mean was 11.6 ± 2.25 (range: 9–16).

None of them had any autoimmune or underlying disorders. This study was approved by the Ethics Committee of Mazandaran University of Medical Sciences, and the written informed consent was obtained from all subjects before the study.

Peripheral blood mononuclear cells (PBMCs) sample collection

Peripheral whole blood (30 mL) was collected into an EDTA anti-coagulated tube. PBMCs were

isolated from fresh whole blood samples using density gradient centrifugation (Biowest, Nuaille, France) based on the manufacturer's instructions. The cells in middle phase were collected and suspended (5×10^6 cells/mL) in RPMI 1640 medium supplemented with 100 mg/mL streptomycin, 100 U/mL penicillin, 10% heat-inactivated fetal calf serum (FCS) and 2mM L-glutamine (Gibco/BRL).

Flow cytometric analysis of Tregs

All antibodies and isotype controls were purchased from BD Biosciences (BD Biosciences, San Diego, CA, USA). To evaluate the frequency of CD4⁺CD25⁺FoxP3⁺CD127^{low/-}Tregs, a portion of the freshly isolated PBMCs (1×10^6 cells/mL) were washed with the staining buffer (Phosphate-buffered saline (PBS) containing 1% heat-inactivated foetal bovine serum FBS, Biowest) and re-suspended in the buffer. Then, PBMCs were fluorescently stained with FITC-conjugated anti-CD127 mAbs (clone HIL-7R-M21), PE-Cy7-conjugated anti-CD25 (clone M-A251), and APC-conjugated anti-CD4 (clone RPA-T4), or with appropriate isotype control Abs (Mouse IgG1, kappa). After cell surface staining, the cells were fixed and permeabilized with Human FoxP3 Buffer Set (BD Biosciences) and subsequently stained with PE-conjugated anti-FoxP3 (clone 259D/C7) or respective isotype control. Compensation controls were also performed for each fluorochrome to ensure that each stain was read in its own channel. The cells were on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA), and the data was evaluated by using Cell Quest (BD Biosciences) and FlowJo (version 7.6.1; Tree Star Inc., Ashland, OR, USA) software. Lymphocytes were gated based on their forward and side scatter (FSC/SSC) properties. CD4⁺ cells and CD4⁺CD25⁺ cells were gated within the lymphocyte gate. Treg cells were defined as CD25⁺Foxp3⁺, CD25⁺CD127^{low/-} and CD25⁺FoxP3⁺CD127^{low/-} cells in gated CD4⁺ T cells. Absolute count of Treg cells was calculated as the product of total lymphocyte ($\times 10^3$ /mL) count from complete blood examination and frequency of target cells determined in the cytometric analysis. Mean fluorescence intensity (MFI) of FoxP3⁺ expressing cells gated on CD4⁺CD25⁺CD127^{low}Tregs was analyzed.

Isolation of T cell subsets

The rest of PBMCs were washed and re-suspended in cold MACS buffer (PBS containing 0.5% BSA with 2 mM EDTA (Biowest, France)). Tregs were isolated from PBMCs in an aseptic condition by using the CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, the cells were incubated with a cocktail of biotin-conjugated mAbs against CD8, CD19, CD123, and CD127 and then with microbeads coated to anti-biotin mAbs; CD127^{low/-}CD4⁺ enriched cells were isolated with negative selection after separation in a magnetic field. Next, the cells were incubated with anti-CD25-coated microbeads and subsequently CD4⁺CD25⁺CD127^{low/-} Treg cells purified by positive selection. To increase the purity, we repeated the second stage by using a new column. The isolation of CD4⁺CD25⁻ responder (Tres) cells was performed with the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (MiltenyiBiotec). Briefly, the cells were incubated with a cocktail of biotin-conjugated mAbs against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ , and CD235a (glycophorin A) and then with anti-biotin microbeads; CD4⁺ enriched cells were separated by negative selection. Afterwards, the cells were incubated with CD25 microbeads and subsequently CD4⁺CD25⁻ Tres cells isolated as the negatively selected cell fraction after separation in a magnetic field. To increase the purity, the isolated cells underwent a second round of CD25 depletion. The purity of the separated CD4⁺CD25⁺CD127^{low/-} and CD4⁺CD25⁻ cells was more than 94% and 90%, respectively.

In vitro suppression assay

To assess the suppressive capacity of regulatory T cells, freshly purified CD4⁺CD25⁺CD127^{low/-} Treg and CD4⁺CD25⁻ Tres cells were labelled with CFSE dye (carboxyfluorescein diacetate succinimidyl ester) by using the Celltrace CFSE Cell Proliferation Kit (Invitrogen, Life Technologies), which is described in the following: Treg and Tres cells were re-suspended in PBS containing 0.1% BSA and labelled with 3 μ M of CFSE (final concentration, experimentally modified) and incubated at 37°C for

10 min. The reaction was stopped by adding 5 volumes of ice-cold culture media RPMI 1640 (Biowest) to the cells. The cells were incubated 5 min on ice, washed twice, and re-suspended in a fresh medium. Suppression assay was performed in 96-well round-bottom plates (SPL Life Sciences, Gyeonggi-do, South Korea). CFSE-labelled Treg and Tres cells were co-cultured at various ratios in RPMI media (final volume: 250 μ L) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine (Gibco, Life Technologies, CA). We used a ratio of 1:1 to 16:1 Tres to Treg; after optimization, ratios 1:1 and 2:1 (5×10^4 Tres/well to 5 or 2.5×10^4 Treg/well) were used for all patients and controls. For polyclonal T cells stimulation, Macsibead particles coated with anti-CD2, CD3, and CD28 mAbs (Treg Suppression Inspector, MiltenyiBiotec) were added to culture (1 bead/cell). Then, cells were incubated for 5 days at 37°C and 5% CO₂. Cells were fed with additional fresh medium on day 3. As controls, CFSE-labelled Treg and Tres cells were cultured alone with and without stimulus. After 5 days of culture, 70 μ L of supernatants was removed for cytokine detection and then cells were harvested and stained with APC-conjugated anti-CD4 mAbs (BD Biosciences). The proliferation of CD4⁺ T cells was analyzed by flow cytometry based on CFSE signals. In addition, background proliferation of CFSE-labelled Tregs cultured alone in the presence of stimulus (Tres-Treg ratio 0:1) was subtracted from Tres cells proliferation in co-culture. The suppressive capacity (suppression percentage) of Tregs in co-culture was calculated as: $100 \times (1 - \%CFSE^{low}Tres \text{ cells in co-culture} / \%CFSE^{low}Tres \text{ cells alone})$.

Cytokine assays

IL-10 and IL-35 concentrations in the culture supernatant were determined by ELISA following the manufacturer's instructions, IL-10 ELISA Ready-Set-Go kit (eBioscience, San Diego, CA) and IL-35 ELISA Kit (Eastbiopharm, Hangzhou, China) respectively.

Statistical analysis

The data were analyzed by fifth version of GraphPad Prism software for Windows (GraphPad

software, La Jolla, CA). Findings were expressed as mean \pm SD. Comparisons between two groups were done using unpaired *t*-test (unpaired, two-tailed) for normally distributed variables and the non-parametric Mann-Whitney test for non-normally distributed variables. The *p* values were considered significant when < 0.05 .

RESULTS

Clinical characteristics of participants

Clinical and demographic data of CD patients and Subjects are presented in Table 1. White blood cells count ($\times 10^3/\mu\text{L}$) in CD patients (8.64 ± 1.05) was a significantly higher than in healthy controls (7.53 ± 1.36 , $P = 0.0048$). There was significant difference in absolute counts of lymphocytes between the two groups, although the percentage of lymphocytes was lower in CD patients (28.43 ± 4.26) compared to HC (31.04 ± 4.33 , $P = 0.0415$).

CD4⁺ T cells are presented in Figure 1. The results of this study showed no significant difference in the percentage of CD4⁺ T cells between CD and HC (39.73 ± 6.22 vs. 40.64 ± 6.14 , $p = 0.6129$). The CD4⁺ CD25⁺ subpopulation was divided into two groups on the basis of Foxp3 expression for determining frequencies of CD4⁺ CD25⁺ T effector cells and CD4⁺ CD25⁺ T regulatory cells. This study indicated a significant difference in the percentage of CD4⁺ CD25⁺ T effector cells between CD and HC (5.07 ± 2.15 vs. 3.58 ± 1.36 , $p = 0.0130$). Moreover, it showed a significant difference in the percentage of CD4⁺ CD25⁺ T regulatory cells between CD and HC (2.71 ± 1.06 vs. 3.36 ± 1.08 , $p = 0.0404$) (Table2).

The evaluation of CD4⁺ CD25⁺ CD127^{low} FoxP3⁺ Treg cells in two groups showed that they were significantly lower in CD patients (2.17 ± 1.04) compared to HC (2.83 ± 1.07 , $P = 0.0352$) (Table 2 and Figure 1).

Table 1

Demographics and characteristics of study subjects

Features Number	CD patients (23)	Healthy control (25)	P-values
Age	37.04 \pm 10.45	35.92 \pm 9.72	0.701
Male/Female	12/11	13/12	
Smoking	3	8	
WBC ($\times 10^3/\mu\text{L}$)	83.64 \pm 1.05	7.53 \pm 1.36	0.004*
Lymphocyte % (WBC differential)	28.43 \pm 4.26	31.04 \pm 4.33	0.041*
Harvey Bradshaw Index (HBI)	11.6 \pm 2.25	-	-
Disease distribution	Ileal	4	-
	Colonic	4	-
	Ileo-colonic	12	-
	Upper GI	2	-
	pre-anal	1	-

Data is presented as mean \pm SD.

$P < 0.05$ was considered to be statistically significant*.

Table 2

Frequency of CD4⁺ subpopulations in peripheral blood mononuclear cells of CD and healthy control groups.

CD4⁺ T cell subpopulations are shown as mean \pm SD

CD4 ⁺ T cells subpopulations	CD	HC	P-values
CD4 ⁺ cells (lymphocytes%)	39.73 \pm 6.22	40.64 \pm 6.14	0.612
CD4 ⁺ CD25 ⁺ cells (in CD4 ⁺ T cells)	7.78 \pm 2.84	6.94 \pm 1.78	0.605
CD4 ⁺ CD25 ⁺ FoxP3 ⁻ activated Teff cells (in CD4 ⁺ T cells)	5.07 \pm 2.15	3.58 \pm 1.36	0.013*
CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Treg cells (in CD4 ⁺ T cells)	2.71 \pm 1.06	3.36 \pm 1.08	0.04*
CD4 ⁺ CD25 ⁺ CD127 ^{low/-} Treg cells (in CD4 ⁺ T cells)	4.14 \pm 1.46	4.66 \pm 1.1	0.046*
CD4 ⁺ CD25 ⁺ FoxP3 ⁺ CD127 ^{low/-} Treg cells (in CD4 ⁺ T cells)	2.17 \pm 1.04	2.83 \pm 1.07	0.035*
Ratio of CD4 ⁺ CD25 ⁺ FoxP3 ⁺ CD127 ^{low/-} Treg/ activated Teff cells	0.445 \pm 0.16	0.901 \pm 0.48	< 0.0001*
MFI of FoxP3 in CD4 ⁺ CD25 ⁺ FoxP3 ⁺ CD127 ^{low/-} Treg cells	31.62 \pm 8.07	28.2 \pm 7.38	0.132

Teff: effector T cell, MFI: Mean fluorescence intensity

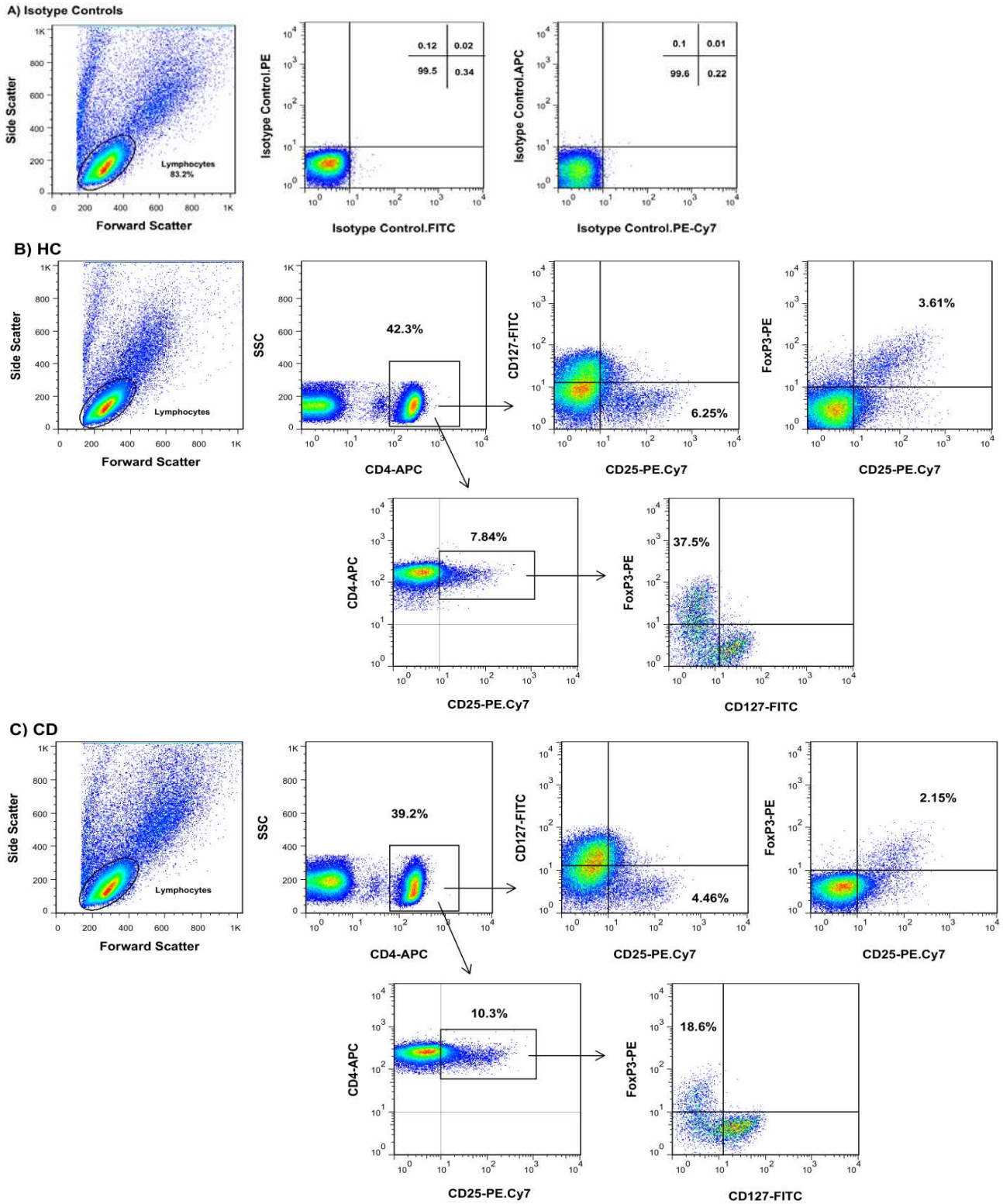


Figure 1. Frequency of Regulatory T cell in a CD patient (C) and single healthy control (B) with isotype control (A) analyzed by flow cytometry. Dot plot analysis of peripheral blood mononuclear cells stained with CD4-APC, Foxp3-PE, CD127-FITC and CD25-PE. Cy7 monoclonal antibodies.

Treg cells and Tres cells co-culture

This study evaluated Treg-Tres co-culture experiment by using CD4⁺ CD25^{high} CD127^{low} cells

as Tregs and CD4⁺ CD25⁻ cells as Tres cells with the CFSE dilution assay for all subjects. After

CFSE-labelled cells' proliferation, the CFSE is equally distributed to division cells. When we calculated the percentage of Treg suppression, there

was no significant difference suppressive capacity of Treg cells in patients compared with controls (Figure 2, Figure 3 and Table 3).

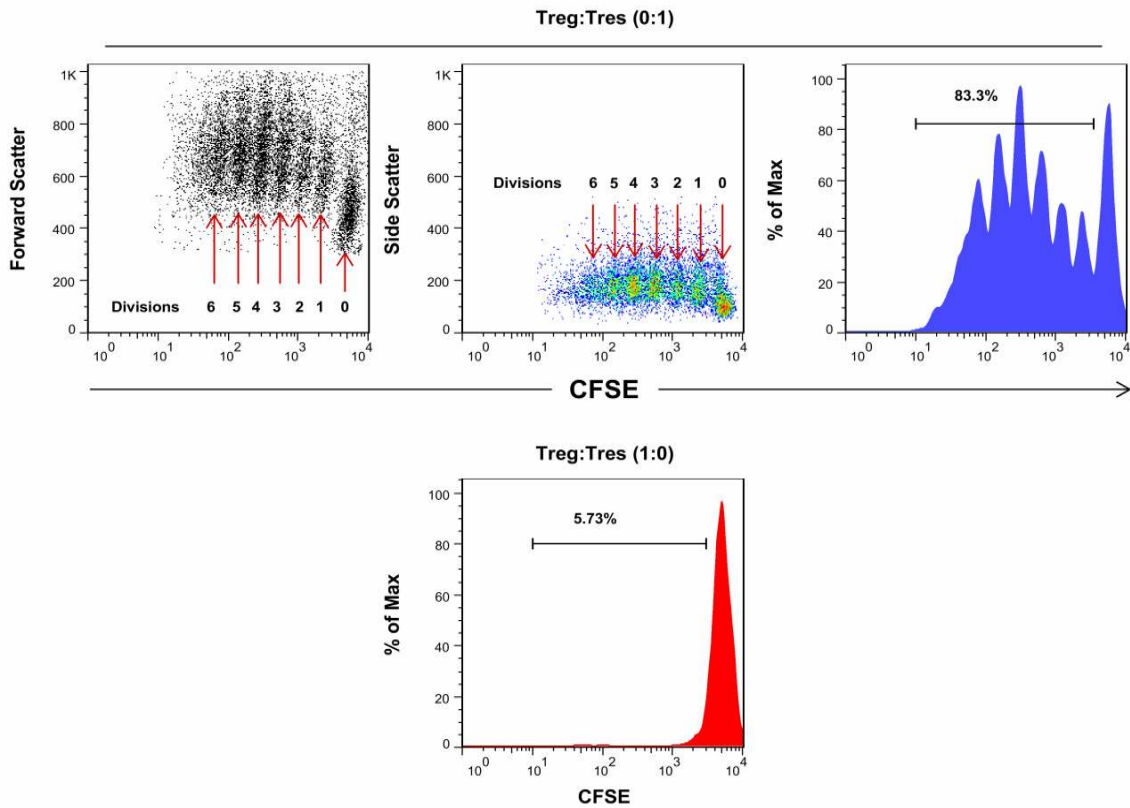


Figure 2. Suppression percentage of Tres in both groups analyzed by CFSE-labelled cells. These results observed by flow cytometry in different ratios.

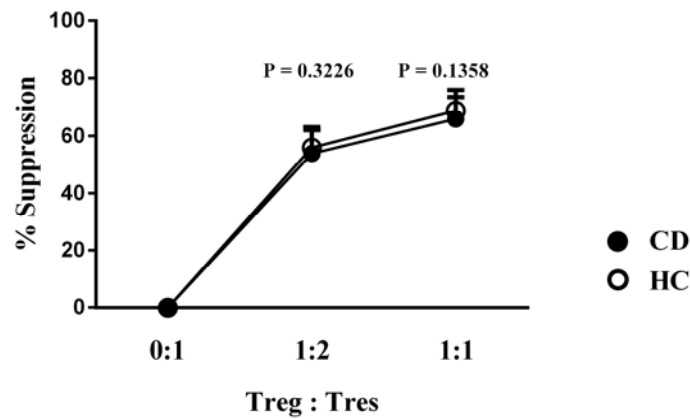


Figure 3. Comparison of proliferation suppressive percentage (Mean ± SD) of Tres cells by Treg cells in different ratios.

Table 3
Suppressive capacity of Treg cells on Tres cells in the co-culture of Treg with Tres in different ratios. Data is presented as mean ± SD

Treg:Tres	Proliferation% or Suppression%	CD patients	Healthy Control	P-values
0:1	Proliferation%	92.06 ± 7.05	89.14 ± 7.16	0.118
1:2	Suppression%	53.81 ± 8.19	56.22 ± 7.25	0.322
1:1	Suppression%	65.94 ± 7.55	69.16 ± 7.13	0.135

Treg cell culture supernatant and immunosuppressive cytokines

IL-10 and IL-35 cytokines secreted *in vitro* by Treg cells were determined with quantitative ELISA assay after anti-CD2/CD3/CD28-stimulation. In CD patients there was a significant difference in secretion of IL-10 in response to anti-CD2/CD3/

CD28 in comparison with HC ($P = 0.0074$). The concentration of IL-10 in CD patients (78.52 ± 25.18 pg/mL) was lower than that in healthy controls (104.52 ± 37.31 pg/mL). There was no statistically significant difference in the concentration of IL-35 between the two groups (5.75 ± 2.53 ng/mL vs. 6.81 ± 3.34 ng/mL, $p = 0.3027$) (Figure 4).

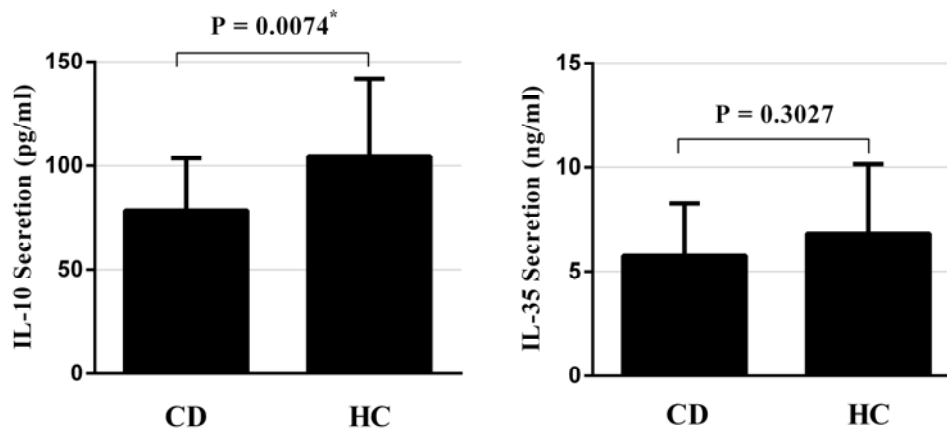


Figure 4. Low concentration of IL-10 and IL-35 in cell culture supernatant of CD patients in comparison with control. After Treg cells' isolation, they were cultured with anti-CD2/CD3/CD28 Mabsibeads for 72 hours. Data is presented as mean \pm SD.

DISCUSSION

Although recent studies in the pathophysiological mechanisms of CD have opened up promising avenues for CD treatment there are still a lot of unsolved issues. Regulatory T cells have a key role for preventing unwanted immunological reactions and are important for continued immune tolerance. FoxP3 is a cytoplasm protein molecule that is currently used for detection of Treg cells. FoxP3 is detected in the nucleus or the cytoplasm being necessary for regulating the anergic state of Tregs. However, there is no single surface-specific marker for isolation of Treg cells. CD4 CD25 in combination with low CD127 are important surface markers for isolation of high FoxP3⁺ Treg cells for co-culture experimental research.

In current study, CD patients have shown a lower frequency of CD4⁺ CD25⁺ CD127^{low} FoxP3⁺ Tregs in comparison with HC. Similar with our data, some studies showed lower frequency of Treg cells in active CD patients [12, 13]. Maul *et al.* described that in peripheral blood of patients with active IBD, CD4⁺CD25^{high}FOXP3⁺T cells were decreased. [14]. Unlike our research, Guidi *et al.*

reported no differences in Treg cell frequency between IBD patients and healthy controls [15]. On the other hand, our data showed that the inhibitory effect of Treg cells in CD patients did not decrease during active stage of disease. In agreement with us, other studies showed a higher or even normal inhibitory effect of Treg cells [16-18]. These suppressor cells which migrate to local inflammation lead to this phenomenon. Therefore frequency of functional Treg was decreased in peripheral blood. Saruta *et al.*, showed that there was an expansion of Treg cells in mucosal lymphoid tissues and inflammatory sites of CD patients [19]. Low activity and low frequency of Treg cells could be an important factor for inflammation responses in CD patients [20]. In this study, high disease activity in CD patients resulted in decreased Treg cell frequency and inhibitory capacity. Differences from previous reported data may come from differences in patients recruited, disease duration and methods used for Treg cells evaluation.

IL-10 and IL-35 promote the expansion or survival of regulatory function in Treg cells [21, 22]. IL-10 plays crucial roles in regulation of important biological processes, including cell-cycle

arrest, senescence, and induction of apoptosis. Therefore, low suppressive functions of Treg cells in co-culture with T-res related to low IL-10 and some others in CD patients. In the present study, there was a low concentration of IL-10 in culture media supernatant compared with HC. Franke *et al.* reported that IL-10 is an immunoregulatory cytokine that has been considered to influence IBD pathophysiology [23]. Defective IL-10 function is crucial for IBD pathogenesis [23]. In fact, IL-10 produced by macrophages is important for T-regulatory cell-mediated prevention of IBD [24, 25].

Most of our information about the function of Tregs cells as well as its role in CD comes only from *in vitro* studies. We suggest to expand and modulate Treg cells as a means of controlling unwanted immune reactions in CD patients. Also, assessment of CD4⁺CD25⁺CD127^{low}FoxP3⁺ regulatory T cells in CD patients with active disease

versus non active disease and evaluation of other suppressive cytokines such as TGF- β are recommended. Some limitations of current study were low sample size, lack of evaluation circulatory IL-10 and IL-35 levels and Tregs in inflamed sites of CD patients. In conclusion, we have shown decreased frequency but no impaired suppressive function of CD4⁺CD25⁺CD127^{low}FoxP3⁺ Treg cells in active stage of CD. There was a low concentration of IL-10 in culture media supernatant in comparison with controls. We suggest that an alteration in Tregs and T effectors balance may be necessary in defective suppression observed in these patients thus providing critical role in CD pathogenesis.

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Conflict of interests. All of the authors have declared that no competing interests exist.

Introducere. Limfocitele T reglatorii (Treg) joacă un rol esențial în patogeneza bolilor autoimune. A fost evaluată funcția reglatoare și frecvența limfocitelor Treg și a citokinelor IL-10 și IL-35 la pacienții cu boala Crohn (CD).

Metode. Au fost incluși 23 de pacienți cu CD și 25 de martori sănătoși (HC). S-a analizat frecvența limfocitelor T reglatoare prin citometrie în flux cu markerii CD4, CD25, CD127 și FoxP3. S-au folosit acești markeri și pentru izolarea populației de limfocite Treg. Activitatea supresoare a acestora a fost măsurată prin abilitatea acestora de a inhiba proliferarea limfocitelor T responsive (Tres). Prin metoda ELISA a fost analizată și cantitatea de IL-10 și IL-35 din supernatantul co-culturii după stimularea cu CD2/CD3/CD28.

Rezultate. Pacienții cu CD au avut niveluri semnificativ statistic mai mici ale limfocitelor CD4⁺CD25⁺CD127^{low}FoxP3⁺Treg comparativ cu HC (2.17 ± 1.04 vs. 2.83 ± 1.07, p = 0.0352). În plus supresia mediată de limfocitele Treg nu a fost diferită semnificativ între pacienții cu CD și HC. Nivelul IL-10 sintetizat ca răspuns la anti CD2/CD3/CD28 a fost semnificativ statistic mai mic la pacienții cu CD comparativ cu HC (p = 0.0074).

Concluzii. Frecvența limfocitelor CD4⁺CD25⁺CD127^{low}FoxP3⁺Tregs a fost mai scăzută la pacienții cu CD active, dar nu a fost influențată activitatea inhibitorie a acestora. Astfel, se sugerează că o alterare a balanței Treg/Tefectoare poate contribui la patogeneza CD.

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