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Intestinal T Cell Profiling in Inflammatory Bowel Disease: Linking T Cell Subsets to Disease Activity and Disease Course

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Abstract

Introduction: A dysregulated intestinal T cell response is presumed in patients with inflammatory bowel disease [IBD]. In this longitudinal study, we investigated the changes in intestinal T lymphocyte subsets in IBD at first presentation and over time during endoscopic active or inactive disease, and relate them to disease activity and outcome.

Methods: We included 129 newly diagnosed patients (87 Crohn´s disease [CD], 42 ulcerative colitis [UC]) and 19 healthy controls [HC]. Follow-up biopsy specimens were analysed from 70 IBD patients. Immunophenotyping of specimens was performed by flow cytometry identifying lymphocyte subpopulations.

Results: IBD patients at diagnosis displayed higher percentages of CD4 T^+ cells, Tregs, and central memory T cells [T_{CM}] and with lower percentages of CD8 and CD103 T lymphocytes than HC. Follow-up specimens of patients with endoscopic inactive disease showed T cell subset recovery comparable to HC. Endoscopic active disease at follow-up coincided with T cell subsets similar to those at diagnosis. In UC, lower baseline percentages of CD3 cells was associated with milder disease course without the need of an immunomodulator, whereas in CD, higher baseline percentages of CD4 and Tregs were associated with complicated disease course.

Conclusions: The intestinal T cell infiltrate in IBD patients with active endoscopic disease is composed of increased percentages of $CD4^+$ T cells, Tregs, and T_{CM}, with lower percentages of $CD8^+$ T cells and $CD103^+$ T cells, compared with HC and endoscopic inactive IBD. Baseline percentages of CD3, CD4, and Tregs were associated with disease outcome. Further research is needed to demonstrate the predictive value of these lymphocyte subsets.

Key Words: Intestinal lymphocyte subsets; newly diagnosed; inflammatory bowel disease; T lymphocytes; untreated

1. Introduction

Ulcerative colitis [UC] and Crohn's disease [CD] are immune-mediated, inflammatory bowel diseases [IBD], with a chronic and relapsing character. Diagnosis is based on a combination of internationally accepted clinical, endoscopic, histological, and radiological findings.[1,2] Incidence is increasing, and disease burden is

substantial due to the chronic nature and generally young age at onset of disease.[3] Disease presentation and course are heterogeneous and unpredictable. Approximately 50% of CD patients are expected to develop disease complications over time, requiring aggressive medical therapy such as [biologic] immunomodulators or surgery.[4,5] In CD patients, clinical determinants associated with a complicated disease course are younger age [< 40], perianal or stricturing disease, involvement of the upper gastrointestinal tract, penetrating disease, and smoking.[6,7] Colectomy is considered a solid endpoint for disease severity in UC, and is required in approximately 10% of patients in the first 10 years of disease. Extensive colitis, as well as severe systemic symptoms were shown to be predictors of colectomy.[8–10]

Guidelines for the choice of treatment modality in IBD are still generic and historically based, without much focus on the individual patient. Biomarkers that can predict disease course or response to therapy would help in focusing on the individual patient. Until now, results have been somewhat disappointing. Some efforts have been made in a study describing $CD8^+$ T cell transcriptional signatures associated with frequently relapsing disease in IBD patients.[11] Recently, a list of key research priorities regarding IBD treatment was compiled, including selection of patients for optimal treatment strategies.[12] Identifying biomarkers that relate to disease behaviour and response to therapy would help in this process.

The first step in this process would be to identify potential biomarkers in IBD patients and to analyse the behaviour of these candidate biomarkers in patients over time. A dysregulated T lymphocyte response is often implicated as key mediator of chronic inflammation in IBD.[13,14] This is supported by the growing number of successful therapeutic approaches targeting T cells in IBD patients, such as: thiopurines, inducing T cell apoptosis; anti-α4β7, targeting the traffic of gut homing T cells; and anti-IL12/23 p40, interfering with T helper 1 [Th1] and Th17 lymphocytes.[15]

Most research on T lymphocytes in IBD has focused on activated $CD4^+$ T effector lymphocytes (Th1, Th2, Th17, and regulatory T cells [Tregs]).[13] The research on T lymphocytes in IBD was mainly based on cytokines detected in the mucosa of longstanding IBD patients and animal models. Instead of an overwhelming increase of effector memory T cells [T_{EM}], we previously identified newly diagnosed patients with increased percentages of naïve T lymphocytes [T_N] and central memory T lymphocytes [T_{CM}] in their inflamed gut mucosa.[16] Furthermore, it was shown that patients had higher percentages of Tregs and lower percentages of $CD103^+$ T cells at diagnosis, when compared with healthy controls. Differences in mucosal T cell subsets between IBD patients at diagnosis might have predictive value for response to different therapeutic agents. Our hypothesis is that intestinal mucosal T lymphocyte subsets may be potential biomarkers for disease course in IBD. Therefore, the primary aim of this longitudinal study was to investigate the changes of intestinal mucosal T lymphocyte subsets in IBD patients at diagnosis and during follow-up, in both presence and absence of endoscopic inflammation. A secondary aim was to study these subsets in relation to disease course over time.

2. Methods

2.1. Study population

Patients suspected of IBD were prospectively enrolled at the outpatient clinic of the Crohn and Colitis Centre of Rijnstate Hospital Arnhem, The Netherlands. After standardised work-up including clinical, endoscopic, histopathological and, when applicable, radiological evaluation, all patients met the accepted international diagnostic criteria of CD or UC.[1,2] Patients were recruited before initial diagnostic ileocolonoscopy, enrolled after IBD was diagnosed, and subsequently followed in their clinical course. During the initial ileocolonoscopy, multiple biopsy specimens were taken for histopathological analysis and immunophenotyping by flow cytometry analysis. Lymphocyte subsets at baseline, for part of the IBD patients included in this study and healthy controls [HC], were previously described.[16] These groups were expanded with newly diagnosed patients and HC. Follow-up endoscopy was performed as part of standard care, taking additional biopsy specimens for immunophenotyping.

Healthy controls underwent ileocolonoscopy for polyp surveillance and did not present endoscopic pathology. None of the healthy controls had any signs of IBD or other immune-mediated diseases.

2.2. Classification and definitions

Endoscopic severity of the disease at baseline and follow-up was assessed in CD by the simple endoscopy score for CD [SES-CD; ranging from < 4 to> 19] and in UC by the Mayo score [ranging from 0 to 3].[17] Inactive disease was defined as endoscopic remission [Mayo = 0/SES-CD < 4]. Active disease was defined as endoscopic disease activity [Mayo > 0/SES-CD > 3]. Active disease was further subdivided into mild [Mayo 1 or SES-CD 4–10], moderate [Mayo 2 or SES-CD 11–19], or severe [Mayo 3 or SES-CD > 19]. At baseline, all IBD patients were classified with active disease [Mayo > 0/SES-CD > 3]. At follow-up endoscopy, 36% of patients were classified as active and 56% as inactive disease.

Disease phenotype [location and behaviour] at baseline and at latest follow-up was assessed according to the Montreal classification.[18] Clinical disease activity at baseline and follow-up was assessed using the Harvey-Bradshaw index for CD and the Montreal classification for UC.[18,19]

Overall use of and response to medication was recorded. In general, therapeutic management of IBD after diagnosis was done according to the step-up approach, as recommended by the Dutch guidelines.[20] In UC patients, topical and/or oral 5-aminosalicylate [5-ASA] was initiated as induction therapy. In case of a severe pancolitis or when induction with 5-ASA treatment failed, patients were treated with topical or systemic steroids. When UC patients were steroid refractory, anti-tumour necrosis factor [TNF] therapy was started. When patients were steroid dependent or had relapsing disease, a thiopurine was initiated and, in case of therapy failure or relapsing disease, anti-TNF treatment was initiated. Failure in either induction or maintenance of remission could lead to surgical escape therapy, i.e. colectomy. In CD patients, steroids were started as induction therapy. This was usually followed by the initiation of maintenance treatment [thiopurine derivates or methotrexate], especially in case of steroid-dependent disease. Anti-TNF therapy was initiated in case of steroid-refractory or steroid-dependent disease, or in case of extended perianal fistulising disease, usually in combination with a thiopurine derivate. If the disease did not respond to conventional treatment, resective surgery was considered. During relapse, escalation of maintenance treatment was considered.

We determined the highest treatment step in each patient, comparing 5-ASA, steroids, immunomodulator, anti-TNF, and resective surgery as measures of severity of the disease. UC patients only needing 5-ASA treatment, and/or a short initial treatment with steroids, were classified as mild disease. Moderate UC was defined as the use of thiopurines after 5-ASA failure, and severe UC as the need for anti-TNF, vedolizumab or colectomy. CD patients with a mild disease course only needed 5-ASA, steroids, and/or an immunomodulator.

Moderate CD was defined as the need for step-up treatment above an immunomodulator [for instance anti-TNF therapy] without meeting the criteria of severe disease. Severe disease in CD was defined as the occurrence of penetrating or stricturing disease, perianal fistulising disease with the need for anti-TNF initiation and/or surgical intervention, and CD-related resective surgery.

2.3. Tissue samples and cell preparation

Six intestinal biopsy specimens from IBD patients at baseline were obtained from the macroscopically most inflamed areas at primary diagnosis, before the initiation of any medical treatment. At follow-up endoscopy, six biopsy specimens were obtained from the macroscopically most inflamed areas or, when there was no inflammation, from the areas of inflammation at baseline. Regular histopathological analysis was performed at baseline and follow-up, as well as immunophenotyping of the mucosal lymphocyte populations. In healthy controls, six biopsy specimens were randomly obtained from ileal and colonic areas. Regular histopathological examination confirmed the absence of inflammation, and immunophenotyping of the mucosal lymphocyte populations was performed. Biopsy specimens meant for flow cytometry analysis were kept in phosphate-buffered saline solution at 2–8°C and processed within 8 h. Specimens were pooled and finely minced in Hanks'/1% bovine serum albumin using a 70-mm gaze and spatula followed by Ficoll density gradient centrifugation. The homogenate was resuspended, after washing, in 0.5 mL Hanks'/1% bovine serum albumin. The cell concentration of the mononuclear cell susupension was estimated by microscopic counting with a KOVA glasstic slide [Hycor Biomedical, Penicuik, UK].

2.4. Flow cytometry

For flow cytometric analysis, 200 µL of the total cell suspension was used, irrespectively of the absolute cell numbers. Single cell suspensions of the biopsies were analysed using a FACS Canto [BD Biosciences]. Intestinal lymphocyte subpopulations were defined as T cells [$CD3^+$] expressed as percentages of the whole lymphocyte population [CD45+/low side scatter]. Mucosal T cells [$CD3^+CD103^+$], T helper cells [$CD3^+CD4^+$], and cytotoxic T cells [$CD3^+CD8^+$] were expressed as percentages of the T cell population [CD3+/light scatter characteristics]. Regulatory T cells [Tregs, $CD3^+CD4^+CD25^{high}FoxP3^+$] were expressed as percentages of the $CD3^+CD4^+$ population. The maturation state of T cells was assessed by using CD45RA and CD27,[21] defining naive T cells [T_N: $CD45RA^+CD27^+$], central memory T cells [T_{CM}: $CD45RA^-CD27^+$], effector memory T cells [T_{EM}: $CD45RA^-CD27^-$] and T effector memory cells re-expressing CD45RA [T_{EMRA}: $CD45RA^+CD27^-$] and expressed as percentages of the T cell [$CD3^+$] population.

2.5. Statistical analysis

All variables were tested for normality using the Shapiro-Wilk test. Categorical variables were expressed as numbers of patients and percentages and compared with chi-square or Fisher's exact test when necessary. Continuous variables were described as median and interquartile range [IQR] and compared with the Mann-Whitney U test. When more than two groups were compared, we used the Kruskal-Wallis test followed by the Dunn test when applicable. Paired data [baseline and follow-up percentages of lymphocyte subsets] were compared using the Wilcoxon matched-pairs signed-rank test. We performed hierarchical clustering analysis to discriminate the potential signature of inactive and active disease at follow-up, using the software R.[22] Multivariate analyses were performed using binary logistic regression to identify independent predictors of disease course. Comparison of the initiation of thiopurine treatment in UC patients was performed using univariate and multivariate Cox regression analysis, graphically represented by a Kaplan-Meier plot, resulting in a relative risk [RR] with corresponding confidence interval [CI]. SPSS statistics for Windows [version 22.0; IBM Corp., Armonk, NY, USA] and GraphPad Prism [GraphPad Software version 7.0, La Jolla, CA, USA] were used to analyse data. Statistical significance was defined as a p-value lower than 0.05.

2.6. Ethical considerations

Written informed consent was obtained from each participating patient before any study-related procedure was performed. The procedures were performed in accordance with the Declaration of Helsinki. The regional medical ethics committee approved the study protocol [NL28761.091.09].

3. Results

3.1. Patient characteristics

This study included 87 CD patients, 42 UC patients, and 19 HC. Baseline characteristics can be found in Table 1. IBD patients were significantly younger than HC (median 28 years [IQR 21–40] vs 40 years [IQR 32–57], $p = 0.0001$). There were more females in the HC group [89% vs 66%, $p = 0.03$]. The median follow-up duration after initial diagnosis was 32 months [IQR 18–66] for CD patients and was significantly longer compared with UC patients (23 months [IQR 13–38, $p = 0.004$). Endoscopic disease severity, categorised as mild, moderate, or severe disease, was evenly distributed in both CD and UC. CD patients had longer symptom duration before the establishment of primary diagnosis [$p = 0.002$] and a larger proportion were smokers [$p = 0.002$] and had a higher baseline C-reactive protein [CRP] [$p = 0.0001$] than UC patients.

3.2. Lymphocyte distribution at baseline in IBD patients compared with healthy controls

To determine if lymphocyte subsets' distribution in the inflamed gut was influenced by biopsy location, we compared lymphocyte subset percentages from CD patients with ileal location [$n = 30$], biopsy specimens being taken only from the inflamed ileum, with those with colonic location [$n = 17$], biopsy specimens being taken only from the inflamed colon. There were no statistical differences for any of the analysed lymphocyte subsets between ileal and colonic location in CD [Supplementary Table 1, available as Supplementary data at *ECCO-JCC* online]. Therefore, we analysed the ileal and colonic lymphocyte subsets from CD patients together. IBD patients showed a distinctive composition of T lymphocyte subsets in their inflamed intestine when compared with HC [Table 2]. They had higher percentages of $CD4^+$ T cells with lower percentages of $CD8^+$ T cells, whereas HC had higher percentages of mucosal $CD103^+$ T cells and lower percentages of Tregs. When examining T cell maturation in patients and controls, IBD patients had higher percentages of T_{CM} cells. The difference in relative amounts of T lymphocyte subsets compared with HC was most pronounced in UC patients. They had higher percentages of $CD4^+$ T cells and percentages of T_{CM} cells, as well as lower percentages of $CD8^+$ T cells and percentages of $CD103^+$ T cells, than CD patients.

Table 1. Baseline patient characteristics.

	CD [*n* = 87]	UC [*n* = 42]	*p*-Value CD vs UC	HC [*n* = 19]
Patient characteristics				
Median age at diagnosis in years [IQR]	26 [21–41]	28 [22–38]	0.61	40 [32–57]
Female/male	58/29	27/15	0.47	17/2
Duration of symptoms before diagnosis, *n* [%]			0.002	
0–3 months	27 [31%]	23 [55%]		
3–6 months	20 [23%]	13 [31%]		
> 6 months	40 [46%]	6 [14%]		
First-degree relative with IBD, *n* [%]	12 [14%]	11 [26%]	0.12	0 [0%]
Smoking at diagnosis, *n* [%]			0.002	
Current	38 [44%]	5 [12%]		6 [32%]
Former	8 [9%]	6 [14%]		2 [11%]
Never	41 [47%]	31 [74%]		11 [58%]
Laboratory values at diagnosis, *n*, median, [IQR]				
CRP	N = 78, 19 [9–56]	N = 35, 5 [1–17]	0.0001	N = 14, 3 [1–8]
Faecal calprotectin	N = 41, 488 [251–1237]	N = 25, 396 [239–825]	0.54	N = 5, 2 [0–7]
Median follow-up in months [IQR]	32 [18–66]	23 [13–38]	0.004	
Disease characteristics				
Harvey-Bradshaw Index, median [IQR]	8 [6–12]			
Remission [< 5]	8 [9%]			
Mild [5–7]	30 [34%]			
Moderate [8–16]	39 [45%]			
Severe [> 16]	10 [12%]			
SES-CD [0–56], median [IQR]	12 [8–22]			
Remission [< 4]	0 [0%]			
Mild [4–10]	34 [39%]			
Moderate [11–19]	27 [31%]			
Severe [> 19]	26 [30%]			
Disease location [CD] at diagnosis, *n* [%]				
L1 [ileal]	30 [34%]			
L2 [colonic]	17 [20%]			
L3 [ileocolonic]	40 [46%]			
L4 [proximal to ileum]	23 [26%]			
Disease behaviour [CD] at diagnosis, *n* [%]				
B1 [non-stricturing, non-penetrating]	68 [78%]			
B2 [stricturing]	15 [17%]			
B3 [penetrating]	4 [5%]			
P [Perianal disease]	12 [14%]			
Montreal classification of clinical severity at diagnosis, *n* [%]				
S0 [remission]		0 [0%]		
S1 [mild]		13 [31%]		
S2 [moderate]		16 [38%]		
S3 [severe]		13 [31%]		
Endoscopic Mayo score at diagnosis, *n* [%]				
Mayo 0		0 [0%]		
Mayo 1		9 [21%]		
Mayo 2		24 [58%]		
Mayo 3		9 [21%]		
Montreal classification of disease extent at diagnosis, *n* [%]				
E1 [proctitis]		9 [21%]		
E2 [distal colitis]		11 [26%]		
E3 [pancolitis]		22 [53%]		
Highest treatment to reach initial remission			0.0001	
No treatment	2 [2%]	0 [0%]		
5ASA	4 [5%]	23 [55%]		
Steroids	17 [19%]	10 [24%]		
Immunomodulator	46 [53%]	6 [14%]		
Biological	12 [14%]	1 [2%]		
Surgery	6 [7%]	2 [5%]		

CD, Crohn's disease; UC, ulcerative colitis; HC, healthy controls; IQR, interquartile range; IBD, inflammatory bowel disease; CRP, C-reactive protein; SES-CD, Simple Endoscopy Score for CD; 5-ASA, 5-aminosalicylic acid.

Table 2. Baseline intestinal lymphocyte populations in healthy controls [HC], Crohn's disease [CD], and ulcerative colitis [UC]. Medians [interquartile range: IQR] are reported

	HC [*n* = 19]	CD [*n* = 87]	UC [*n* = 42]	*p*-Value[a,b]	*p*-Value[a,c]	*p*-Value[d]
$CD3^+$ as % of lymphocytes	66 [56–69]	53 [45–64]	58 [48–69]	**0.021**	0.28	0.17
$CD3^+CD4^+$ as % of T cells	48 [31–76]	67 [53–75]	73 [64–82]	**0.005**	**0.0001**	**0.004**
$CD3^+CD8^+$ as % of T cells	45 [24–58]	28 [22–36]	22 [16–29]	**0.003**	**0.0001**	**0.003**
$CD3^+CD103^+$ as % of T cells	43 [23–66]	17 [9–34]	12 [6–21]	**0.001**	**0.0001**	**0.009**
T_N as % of T cells	9 [3–25]	17 [9–35]	19 [11–30]	0.33	0.27	0.97
T_{CM}as % of T cells	21 [11–27]	33 [24–45]	50 [38–57]	**0.032**	**0.002**	**0.0001**
T_{EM} as % of T cells	41 [24–52]	26 [10–45]	20 [12–29]	0.30	**0.008**	0.23
Treg as % of T cells	4 [2–5]	11 [8–16]	10 [8–16]	**0.001**	**0.004**	0.80

Subsets were defined as described in methods: T_N by $CD3^+CD27^+CD45RA^+$, T_{CM} by $CD3^+CD27^+CD45RA^-$, T_{EM} by $CD3^+CD27^-CD45RA^-$ and Treg by $CD3^+CD4^+CD25^+Foxp3^+$.

[a]Corrected for age.

[b]Comparing CD and HC.

[c]Comparing UC and HC.

[d]Comparing CD and UC.

3.3. Development of lymphocyte subsets during follow-up in IBD patients

Seventy patients [54% of all IBD patients] underwent follow-up endoscopy with consecutive lymphocyte subsets analysis. From these patients, 25 had active endoscopic disease at the time of the follow-up endoscopy [36%] and 39 had inactive endoscopic disease [56%]. Discrepancy of endoscopic disease activity between ileum and colon was observed in six CD patients [8%], with active disease in one bowel segment and inactive disease in the other segment, although these patients had ileocolonic disease at baseline. These patients were left out of the analysis on the change of the lymphocyte subsets between baseline and follow-up.

When compared with baseline, patients with inactive endoscopic disease at follow-up had decreased percentages of $CD4^+$ T cells (fold change [FC] = 0.78, $p = 0.001$) and increased percentages of $CD8^+$ T cells [FC = 1.46, $p = 0.0001$] [Figure 1; and Supplementary Table 2, available as Supplementary data at *ECCO-JCC* online]. They also presented increased percentages of $CD103^+$ T cells [FC = 2.43, $p = 0.0001$], decreased percentages of Tregs [FC = 0.38, $p = 0.004$], and a shift towards more T_{EM} [FC 1.48, $p = 0.0001$] leading to lower percentages of T_N and percentages of T_{CM} [FC = 0.45 and FC = 0.55, $p = 0.002$ and $p = 0.0001$, respectively].

Patients with active endoscopic disease at follow-up showed an even further decrease in percentages of $CD8^+$ cells compared with their baseline percentages [FC = 0.76, $p = 0.016$]. All other T lymphocyte subsets did not differ statistically from baseline in patients with active endoscopic disease. Subdividing UC and CD patients gave similar results to those among the whole group [Figure 1; Supplementary Table 2].

For baseline intestinal lymphocyte populations of the endoscopic inactive and active disease group at follow-up, see Supplementary Tables 5–7 [available as Supplementary data at *ECCO-JCC* online]; percentages at baseline were comparable among both groups.

We additionally performed a hierarchical cluster analysis to compare the lymphocyte subsets signature of patients with active endoscopic disease with those with inactive endoscopic disease at follow-up [Figure 2]. We found that patients with active endoscopic disease had higher percentages of $CD4^+$ T cells, lower percentages of $CD8^+$ T cells, lower percentages of $CD103^+$ T cells, higher percentages of Tregs, higher percentages of T_{CM}, and lower percentages of T_{EM} [all: $p = 0.0001$], and higher percentages of T_N [$p = 0.001$] than patients with inactive endoscopic disease [Figures 3 and 4, Table 3]. Comparing the different grades [mild vs moderate vs severe] of endoscopic disease severity in active disease did not show mutual significant differences. The same results were found when subdividing UC and CD [Supplementary Tables 3 and 4, available as Supplementary data at *ECCO-JCC* online].

The percentages of T lymphocyte subsets in patients without endoscopic disease activity recovered to levels comparable to HC [Figure 3 and 4, Table 3]. Medical treatment at the moment of the follow-up endoscopy was very different between patients. No statistical differences were found for lymphocyte subsets percentages when adjusting for the different regimens. However, no definite conclusions can be drawn about the effect of treatment modality on lymphocyte subsets, due to the small sample size per treatment regimen.

3.4. T cell maturation profiles at baseline and during follow-up

Previously, we distinguished four T cell maturation profiles in the intestinal mucosa of IBD patients [Figure 5].[16] At baseline, 71% of UC patients had a B profile with predominantly T_{CM}, whereas CD patients were more heterogeneous [B in 34%, C in 30%, A in 17%, and D in 16%], and HC had mainly a C or D profile [47% and 37%, respectively, Figure 5]. T cell maturation profiles were not associated with disease phenotypes or course.

In patients with IBD with active endoscopic disease at follow-up endoscopy, 89% of patients with a B profile at baseline retained this profile at follow-up. Among patients with a C or D profile at baseline, 50% changed to an A or B profile when there was disease activity at follow-up endoscopy.

On the other hand, in IBD patients with inactive endoscopic disease at follow-up endoscopy, the A and B profile changed to C or D in 79% of patients. The change of profile from baseline to follow-up in patients with inactive disease at follow-up endoscopy was statistically significant [$p = 0.014$]. Patients with C or D profile at baseline maintained this profile largely when there was inactive endoscopic disease at follow-up [86%].

3.5. Predicting disease course in UC

We determined the association between baseline lymphocyte subsets and disease course in UC patients with a follow-up duration of longer than one year [*n* = 33, 79% of all UC patients]. Of these

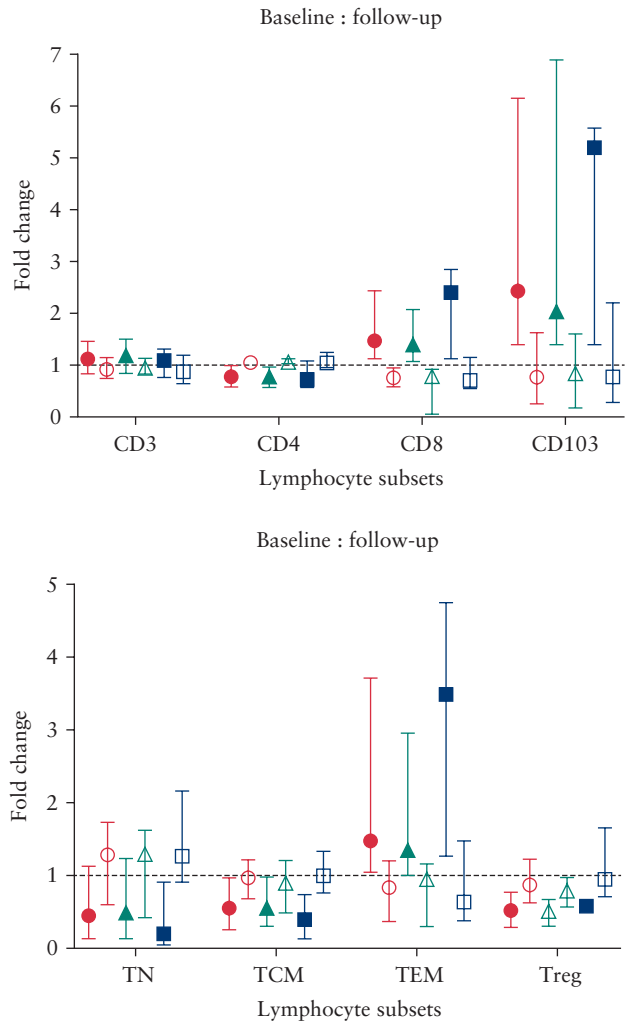

Figure 1. Fold change of different lymphocyte subsets in inflammatory bowel disease [IBD] patients at follow-up endoscopy with either endoscopic active or inactive disease when compared with their baseline values [all with active disease] by Wilcoxon matched-pairs signed-rank test. IBD patients with inactive endoscopic disease at follow-up endoscopy had a statistical significant decrease compared with baseline percentages of CD4 T cells, naïve T cells [T_N], central memory T cells [T_{CM}], and regulatory T cells [Treg], with a significant increase of percentages of CD8 T cells, CD103 T cells, and effector memory T cells [T_{EM}]. In patients with active endoscopic disease at follow-up, T lymphocyte subsets did not differ significantly from baseline percentages, except for an even further decrease of the CD8 percentages. When subdividing ulcerative colitis [UC] and Crohn's disease [CD] patients, the same patterns were found. ● IBD inactive* (n = 39): ○ Active*IBD (n = 25); ▲ CD inactive* (n = 32); △ CD active* (n = 11); ■ UC inactive* (n = 7); □ UC active* (n = 14). *inactive (MAYO=0/SES-CD<4) or active (MAYO>0/SES-CD>3) disease at follow-up endoscopy.

patients, 27 had a disease exacerbation during follow-up and six patients retained remission. Their follow-up duration was comparable (respectively, a median of 26 months [IQR 19–54] and 38 months [20–56], $p = 0.80$). During their follow-up, 13 UC patients used 5-ASA as highest treatment strategy [39%], three patients needed steroids as highest step [9%], 12 patients needed an immunomodulator [36%], two patients used anti-TNF as highest treatment step [6%], and three UC patients ultimately needed resective surgery [9%]. Follow-up duration of these groups was not different [$p = 0.10$]. UC patients with 5-ASA as highest treatment strategy [$n = 13$] had lower baseline percentages of $CD3^+$ cells (median 48% [43–63], $p = 0.03$) than patients in need of step-up treatment ($n = 20$, median 62% [50–71]). Patients with an immunomodulator as highest treatment strategy [$n = 12$] had higher baseline percentages of $CD3^+$ cells (median 65% [57–78], $p = 0.005$) than patients who only needed 5-ASA and/or steroids ($n = 16$, 49% [44–66]).

There were no associations between the percentages of lymphocyte subsets at baseline and the following disease outcomes: surgery, biologics, relapsing disease, or future disease severity.

3.6. Predicting disease course in CD

We determined the association between lymphocyte subsets at baseline and disease course in CD patients with a follow-up duration of longer than one year [$n = 77$, 90% of all CD patients]. Disease exacerbation occurred in 43 CD patients [56%] and 34 patients [44%] retained remission, with comparable follow-up duration between these groups [$p = 0.10$]. From this group, three CD patients [4%] only used 5-ASA during follow-up, nine patients [12%] only needed steroids, 34 patients [44%] used an immunomodulator as highest treatment step, 22 patients [29%] used anti-TNF as highest treatment strategy, and nine CD patients [12%] ultimately needed resective surgery. Follow-up duration of the groups was generally comparable, except between the surgery and steroid groups [$p = 0.03$]. Compared with uncomplicated inflammatory disease [B1, $n = 60$], future stricturing or penetrating disease [B2/B3, $n = 17$] was associated with higher baseline percentages of $CD4^+$ cells (median 74% [IQR 65–79] vs 65% [IQR 52–73], $p = 0.02$) and higher baseline percentages of Tregs (median 15% [IQR 9–23] vs 11% [IQR 7–15], $p = 0.04$). Follow-up duration was comparable between these two groups [$p = 0.49$].

Abdominal surgery was associated with higher baseline percentages of Tregs compared with patients who did not need surgery (median percentages of Tregs 15% [IQR 14–19] vs 10% [IQR 7–15], $p = 0.014$). However, after correction for follow-up duration this was not significant any more [$p = 0.051$]. Steroids as highest treatment modality [$n = 9$] were associated with lower baseline percentages of Tregs (median 8% [IQR 5–10], $p = 0.014$) compared with patients in needing step-up treatment ($n = 65$, median 11% [IQR 8–16]). This remained significant after correction for follow-up duration [$p = 0.033$].

4. Discussion

In the present study, marked differences were demonstrated in intestinal T lymphocyte subset composition of IBD patients with active endoscopic disease when compared with HC and patients in with inactive endoscopic disease. IBD patients at diagnosis displayed higher percentages of CD4, Tregs, and T_{CM}, with lower percentages of CD8 and CD103 T lymphocytes. Patients with active endoscopic disease at follow-up analysis maintained the same compositions of T cell subsets as at the moment of diagnosis, with even lower percentages of CD8 T lymphocytes. This confirms consistency of T cell subset composition in time. Follow-up biopsy specimens of IBD patients with inactive endoscopic disease showed recovery of these subsets to a T lymphocyte composition, comparable to HC. Lymphocyte subset percentages were associated with endoscopic disease activity. Furthermore, in UC, lower baseline percentages of CD3 cells were associated with a milder disease course without the need of step-up to an immunomodulator, whereas in CD, higher baseline percentages of CD4 and higher percentages of Tregs were associated with a more complicated disease course.

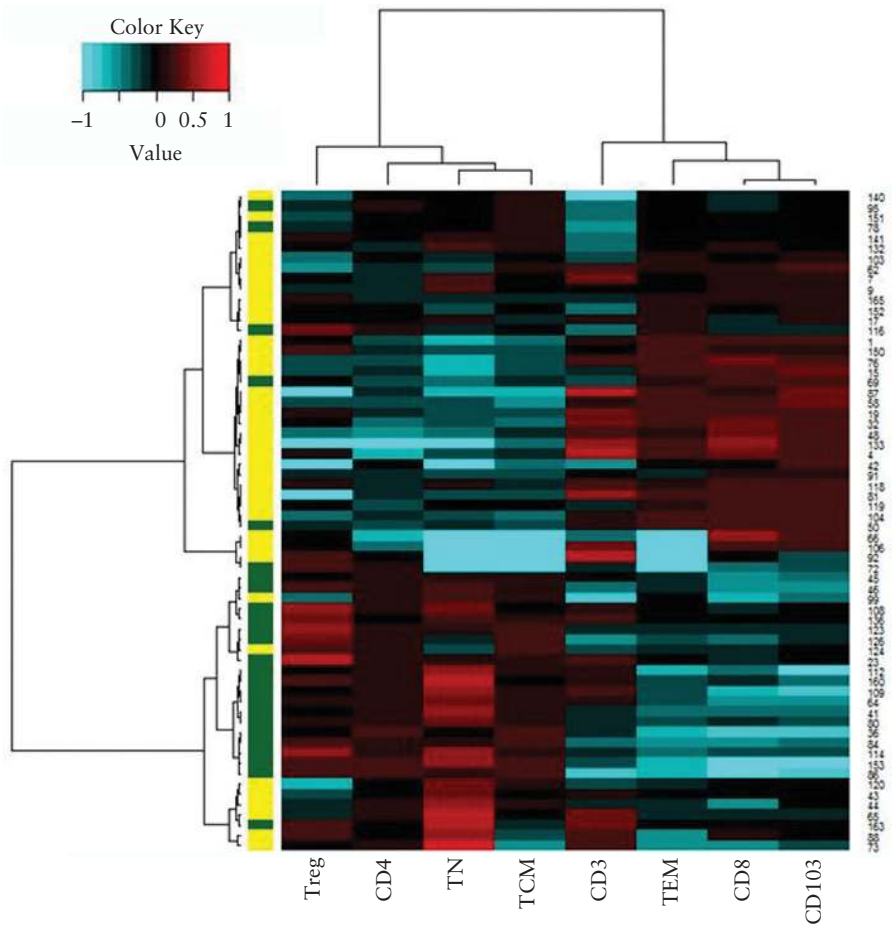

Figure 2. Hierarchical clustering analysis comparing IBD patients at follow-up with active endoscopic [green] and inactive endoscopic disease [yellow]. IBD = inflammatory bowel disease; T_N = naïve T cells; T_{CM} = central memory T cells; T_{EM} = effector memory T cells; Treg = regulatory T cells.

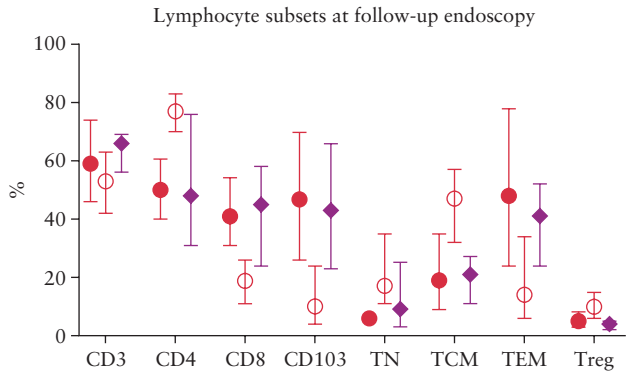

Figure 3. Intestinal lymphocyte subsets at follow-up endoscopy in IBD patients with endoscopic active and inactive disease and healthy controls. The T cell subsets from patients with active endoscopic disease showed a different pattern from healthy controls and patients with inactive endoscopic disease, whereas T cell subsets from patients with inactive endoscopic disease approached those of healthy controls. IBD = inflammatory bowel disease; T_N = naïve T cells; T_{CM} = central memory T cell; T_{EM} = effector memory T cells; Treg = regulatory T cells. ● Inactive*IBD (n = 39); ○ Active*IBD (n = 25); ◆ Healthy controls (n = 19) . *inactive (MAYO=0/SES-CD<4) or active (MAYO>0/SES-CD>3) disease at follow-up endoscopy.

There were no differences found between ileum and colon regarding lymphocyte subsets: the composition of subsets seems more disease related than location related. This is endorsed by the association of the different lymphocyte subsets with endoscopic disease activity. Differences in lymphocyte subsets were more pronounced in UC than in CD patients. This might be explained by the fact that UC is characterised by more continuous inflammation conveyed to the mucosa and submucosa, with high chances that biopsy specimens from these patients are abundantly inflamed, whereas in CD, biopsy samples might have been taken from the edge of an ulcer with partly uninflamed tissue.

Intestinal inflammation in IBD has been attributed to CD4 subsets, based on animal models and cytokine expression in the gut mucosa.[23] However, auto-reactive CD8 cells have also been suggested as instigators of the disease by destruction of epithelial cells, after which exposure to luminal antigens may attract and expand the CD4 lineage, exacerbating inflammation.[24] The imbalance that we found, of increased CD4 with decreased CD8 frequencies in active endoscopic IBD at baseline and follow-up, seems to represent a CD4 expansion over CD8, but cannot provide further information on the real instigators of disease. The imbalance was normalised in the endoscopically inactive mucosa of IBD patients in remission, approaching comparable frequencies to HC. The percentages CD8 and CD4 T cells that we found in the intestines of controls was consistent with a previous study in organ donors.[25] In our study in CD patients, higher baseline percentages of CD4 were associated with the development of

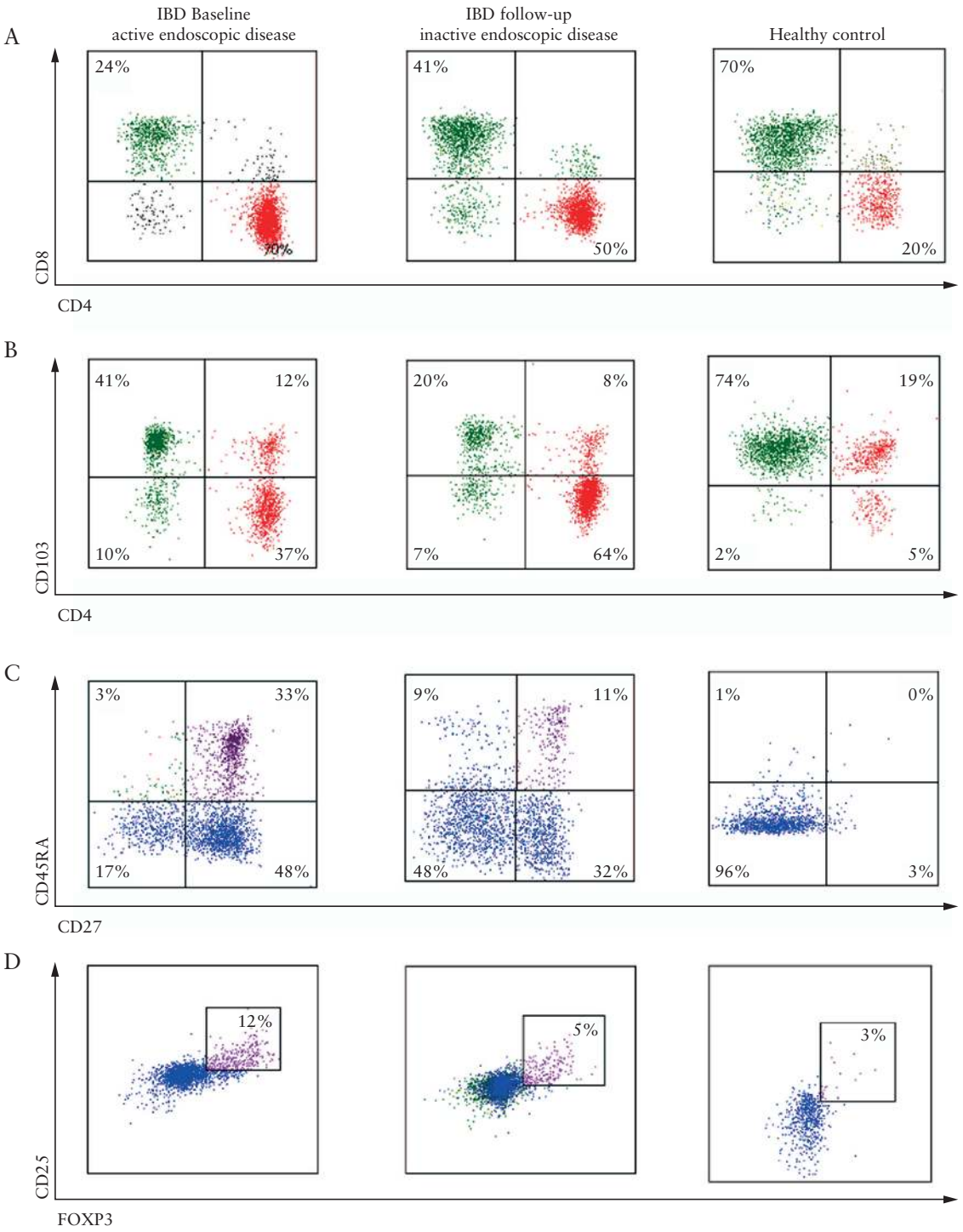

Figure 4. Representative flow cytometric dot plots showing [from left to right] a patient with ulcerative colitis at diagnosis with active endoscopic disease, and the same patient at follow-up endoscopy with inactive endoscopic disease, next to a healthy control. [A] shows CD4/CD8 gated in the CD3$^+$ population, [B] shows CD103$^+$ cells within the CD3$^+$ population, [C] shows T cell maturation based on CD27 and CD45RA within the CD3$^+$ population with naïve T cells [CD27$^+$CD45RA$^+$], central memory T cells [CD27$^+$CD45RA$^-$], effector memory T cells [CD27$^-$CD45RA$^-$], and effector memory T cells re-expressing CD45RA [CD27$^-$CD45RA$^+$], [D] shows regulatory T cells [CD25$^+$FoxP3$^+$] within the CD3$^+$CD4$^+$ population.

strictures or penetrating disease. This could represent increased expansion of [proinflammatory] CD4 lymphocytes accompanied by Treg expansion, in an [unsuccessful] attempt to diminish inflammation, leading to disease complications by unknown mechanisms that need to be investigated further.

Tregs are involved in the maintenance of intestinal homeostasis by suppressing abnormal responses to enteric antigens, by direct interaction with other cells, and through secretion of anti-inflammatory cytokines like TGF-beta and IL-10.[26] In this study, we showed that IBD patients have increased percentages of Tregs at diagnosis

Table 3. Intestinal lymphocyte populations at follow-up endoscopy from IBD patients with either endoscopic active [Mayo > 0/SES-CD > 3] or inactive [Mayo = 0/SES-CD < 4] disease and healthy controls [HC]. Medians [interquartile range; IQR] are reported.

	IBD: endoscopic inactive disease [*n* = 39]	IBD: endoscopic active disease [*n* = 25]	*p*-Value[a]-	HC [*n* = 19]	*p*-Value[b,c]	*p*-Value[c,d]
FU duration from diagnosis until follow-up endoscopy in months, median [IQR]	24 [13–47]	22 [11–39]	0.27	-	-	-
$CD3^+$ as % of lymphocytes	59 [46–74]	53 [42–63]	0.09	66 [56–69]	0.41	0.013
$CD3^+CD4^+$ as % of T cells	50 [40–61]	77 [70–83]	**0.0001**	48 [31–76]	0.86	**0.002**
$CD3^+CD8^+$ as % of T cells	41 [31–54]	19 [11–26]	**0.0001**	45 [24–58]	0.71	**0.002**
$CD3^+CD103^+$ as % of T cells	47 [26–70]	10 [4–24]	**0.0001**	43 [23–66]	0.62	**0.004**
T_N as % of T cells	6 [4–18]	17 [11–35]	**0.001**	9 [3–25]	0.23	0.34
T_{CM} as % of T cells	19 [9–35]	47 [32–57]	**0.0001**	21 [11–27]	0.86	**0.006**
T_{EM} as % of T cells	48 [24–78]	14 [6–34]	**0.0001**	41 [24–52]	0.11	**0.043**
Treg as % of T cells	5 [3–8]	10 [6–15]	**0.0001**	4 [2–5]	0.19	**0.004**

Subsets were defined as described in methods: T_N by $CD3^+CD27^+CD45RA^+$, T_{CM} by $CD3^+CD27^+CD45RA^-$, T_{EM} by $CD3^+CD27^-CD45RA^-$ and Treg by $CD3^+CD4^+CD25^+Foxp3^+$.

HC, healthy controls; IBD, inflammatory bowel disease; SES-CD, Simple Endoscopy Score for Crohn's Disease; FU, follow-up.

[a]Comparing endoscopic active and inactive IBD.

[b]Comparing endoscopic inactive IBD and HC.

[c]Corrected for age.

[d]Comparing endoscopic active IBD and HC.

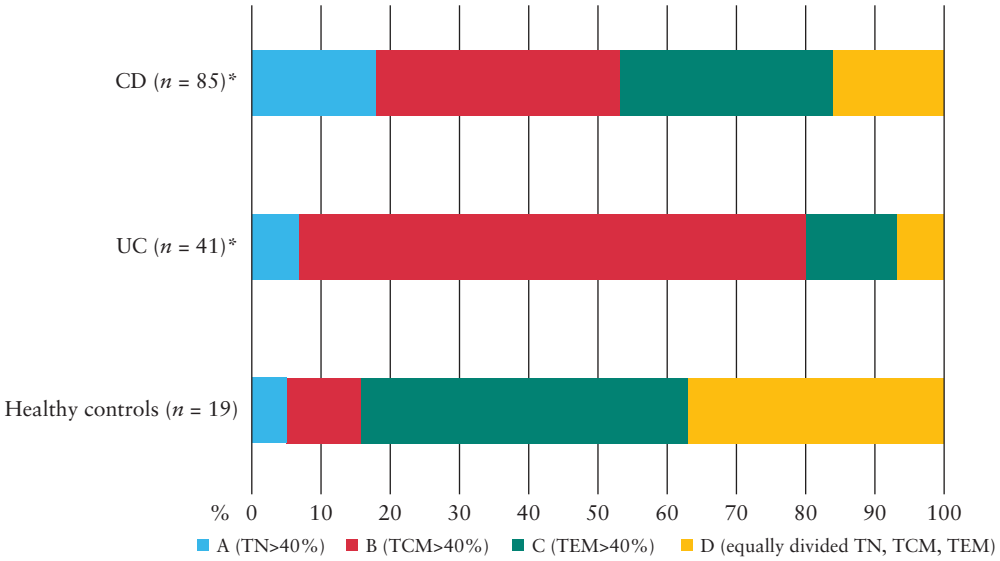

Figure 5. Different intestinal T cell maturation profiles at baseline in healthy controls and inflammatory bowel disease [IBD] patients.
*In two IBD patients (1 UC and 1 CD) the maturation profiles were not determined (technical reasons).

[always with active endoscopic disease] and when there is active endoscopic disease during follow-up, whereas percentages decrease significantly in the same patients at times of inactive endoscopic disease. Furthermore, in CD patients, higher baseline percentages of Tregs were associated with a more complicated disease course with strictures or penetration, and a trend was observed towards more abdominal surgeries. Lower baseline percentages of Tregs in CD patients were associated with a milder course, with steroids as highest treatment step. It is tempting to speculate about a hampered function of Tregs in CD to be compensated by numbers. Increased mucosal Treg frequencies have been shown in active IBD before.[27] This could represent active recruitment and local expansion of these cells, attempting to suppress inflammation. However, this expansion is apparently not sufficient to control inflammation, possible due to the still too small number of cells. Another explanation might be that their function is impaired by upregulation of Smad7 in IBD patients, which inhibits TGF-beta signalling, thereby impairing the suppression of T cell activation and proliferation but possibly also the differentiation of naïve T cells into Tregs.[28,29] Finally, there is evidence that transformation of Tregs into proinflammatory Th17 cells could also contribute to uncontrolled inflammation.[26]

We demonstrated that IBD patients with active endoscopic disease had lower percentages of $CD103^+$ T lymphocytes compared with HC, and a significant increase in percentages of $CD103^+$ T lymphocytes in patients with endoscopic inactive disease at follow-up endoscopy. CD103 is the integrin αE of αEβ7 expressed on several immune cell subsets, and its expression is thought to contribute to the retention of T lymphocytes within the mucosa. Our findings

suggest a less prominent role for CD103+ T cells as pathogenic factor in IBD patients than previously assumed, as it was thought to be upregulated in active endoscopic disease.[30–32] Therefore, it would be very interesting to study the CD103+ cell subpopulations in more detail, with the upcoming development of anti-β7 treatment. The function of the αEβ7+ T lymphocytes needs to be further investigated. αEβ7 is present on tissue-resident memory T cells [T_{RM}, mainly on CD8+ T cells] with an immunosurveillance and protective function.[33] Recently, lung-derived CD103+T_{RM} cells were shown to possess a gene expression programme associated with the inhibition of T cell activation, which might play a role in preventing excessive immunoreactivity.[34] αEβ7+ T lymphocytes might even be necessary to achieve or maintain remission in patients, and a definitive pathogenic role of these cells in IBD has not yet been shown. Thus, from a mechanistic point of view, its blockade remains questionable.[35]

5. Conclusion

The intestinal T cell infiltrate in IBD patients with active endoscopic disease is composed of increased CD4+ T cells, Tregs, and T_{CM}, with lower percentages of CD8+ T cells and CD103+ T cells compared with HC and endoscopically inactive IBD. After patients entered endoscopic remission, the lymphocyte subset composition of the gut recovered to percentages comparable with HC. Baseline percentages of CD3, CD4, and Tregs were associated with disease outcome in this study. Further research is needed to demonstrate the predictive value of these lymphocyte subsets. Knowledge of intestinal immune subsets at baseline and during follow-up could guide the use of new drugs and should be incorporated in the early phases of drug development to identify predictors of response.

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None.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

CS, CH, EvL, PW, and MG participated in the concept and design of the study. CS, CH, EvL, MG, BR, and PW participated in patient recruitment and material collection. CS, CH, EvK, and EvL were responsible for flow cytometric immunophenotyping. CS and JD were responsible for statistical analysis. All authors were members of the writing group and participated in the drafting and revision of the manuscript.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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