

Foxp3⁺ Regulatory T Cells, Th17 Effector Cells, and Cytokine Environment in Inflammatory Bowel Disease

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Abstract

Background Inflammatory bowel disease (IBD) is thought to result from an aberrant immune response. Inflammation in IBD may be caused by the loss of homeostasis between CD4⁺ CD25^{high} Foxp3⁺ regulatory cells (T_{reg}) and proinflammatory Th17 cells. The aim of this study was to investigate T_{reg} and Th17 cells in the peripheral blood and intestinal mucosa of IBD patients and to assess the mucosal cytokine environment.

Adrian Cummins and Simon Barry have contributed equally to this study.

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Methods T_{reg} and Th17 cells were measured in peripheral blood of 63 IBD patients and 28 controls by flow cytometry. Forkhead box p3 (Foxp3), interleukin (IL)-17a, IL-1 β , IL-6, IL-21, IL-23, and transforming growth factor (TGF)- β mRNA were analyzed using real-time reverse transcription polymerase chain reaction in intestinal biopsies of 24 IBD and 18 control subjects.

Results A decrease in T_{reg} and increase in Th17 cells was observed in the peripheral blood of IBD patients. When measured in the same patient and expressed as a ratio, a significant decrease in T_{reg} /Th17 ratio was observed in IBD. Elevated expression of Foxp3, IL-17a, IL-1 β , and IL-6 was observed in the mucosa of IBD patients, while TGF- β was only elevated in ulcerative colitis.

Conclusion IBD is associated with a reduced ratio of T_{reg} to Th17 cells in peripheral blood and is characterized by a proinflammatory cytokine microenvironment, which supports the continued generation of Th17 cells.

Keywords Inflammatory bowel disease · Crohn's disease · ulcerative colitis · regulatory T cells · Th17 effector cells

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the two main forms of inflammatory bowel disease (IBD). The pathogenesis of IBD is thought to originate from an aberrant immune response directed toward resident intestinal bacteria resulting in chronic inflammation [1]. The intestinal mucosa is normally maintained in a state of controlled inflammation in which an equilibrium exists between protective immunity and tolerance to self-antigen and commensal bacteria [2]. This tolerance is maintained by regulatory T cells (T_{reg}), a population of CD4⁺ T cells

that control immune responses in the gut by inhibiting the proliferation and effector functions of other T cells [3]. T_{reg} are identified by expression of the high-affinity interleukin (IL)-2 receptor α -chain (CD25) [3]; however, CD25 is also upregulated on the surface of activated $CD4^+$ T cells, so CD25 is not an exclusive marker for T_{reg} [4]. T_{reg} also express the transcription factor Forkhead box p3 (Foxp3), which is crucial for their development and function [5]. Although the mechanism through which T_{reg} suppress proliferation of other T cells is not clear [6], there is evidence that they play an important role in preventing autoimmunity and controlling colitis and gastritis in vivo [3, 7]. The human disease immunodysregulation polyendocrinopathy and enteropathy X-linked syndrome (IPEX) is associated with loss of immunoregulation due to Foxp3 mutation [8, 9] and provides clear evidence for a role of T_{reg} cells in health and disease in humans.

There is growing evidence that T_{reg} and Th17 cells are linked from a developmental perspective, where the same naive T cell precursor pool that generates T_{reg} cells is capable of generating IL-17a-producing $CD4^+$ T helper (Th)17 cells [10, 11]. In murine models, transforming growth factor (TGF)- β drives the differentiation of naive T cells to a T_{reg} phenotype, whereas Th17 cells are induced in the presence of IL-6 and TGF- β [10, 11]. In humans, the cytokine environment for the generation of Th17 cells is less certain as IL-6 and TGF- β alone are insufficient for the differentiation of human Th17 cells [12]. IL-1 β , IL-6, TGF- β , IL-21, and IL-23 are implicated in promoting human Th17 differentiation, although the exact cytokine combination requires confirmation [13–16].

Th17 cells express the transcription factor retinoic acid-related orphan receptor- γ t (ROR- γ t) and induce a range of proinflammatory mediators that bridge the innate and adaptive immune response enabling the clearance of invading pathogens [17, 18]. Although Th17 cells play a critical biological function in clearing extracellular pathogens [19], the inappropriate production of IL-17a by these cells is thought to contribute to the pathology of a range of inflammatory diseases. IL-17 expression is upregulated in the intestinal mucosa of IBD patients suggesting that a Th17-cell-driven immune response contributes to the pathology of IBD [20, 21]. The balance between T_{reg} and Th17 cells may be essential for maintaining immune homeostasis, but no studies have yet examined this balance in IBD patients.

The aim of this study was to determine whether an imbalance of T_{reg} and Th17 effector cells is characteristic of patients suffering from IBD. We not only investigated each cell type independently but also simultaneously compared the two cell types in the same patient subgroup. We hypothesized that any imbalance seen between these cell types may be driven by the cytokine microenvironment of

the gut. We therefore investigated the expression of IL-1 β , IL-6, IL-21, IL-23, and TGF- β in the intestinal biopsies of IBD and control patients.

Subjects and Methods

Subjects

IBD patients were recruited from the Department of Gastroenterology and Hepatology at The Queen Elizabeth Hospital (TQEH). Informed consent was obtained from all patients before collection of samples. This study was approved by TQEH Ethics of Human Research Committee and carried out according to the National Statement on Ethical Conduct in Research Involving Humans (1999) of the National Health and Medical Research Council of Australia and was in accord with the Declaration of Helsinki. Thirty-four CD, 29 UC, and 28 control patients were recruited for blood collection. All IBD patients were in clinical remission at the time of blood sampling based on clinical assessment and blood C-reactive protein levels (CRP < 10). Control subjects had non-inflammatory disorders (non-ulcer dyspepsia, reflux, constipation, etc.) or were healthy volunteers. All control subjects were screened for autoimmune disease markers (rheumatoid factor, anti-nuclear antibodies, anti-neutrophil cytoplasmic antibodies, and thyroid peroxidase antibodies). Intestinal biopsies were obtained from an additional subset of IBD patients in various states of disease activity. Biopsies were collected from 11 CD, 14 UC, and 18 control subjects at colonoscopy from non-inflamed tissue. Control subjects had non-inflammatory disorders or were undergoing colon cancer screening. Biopsy samples were collected and stored in RNAlater (Ambion, TX, USA) at -20°C to prevent RNA degradation prior to extraction.

Analysis by Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Marlow, UK). In order to identify T_{reg} cells, 1×10^6 PBMCs were surface labeled with a fluorescein isothiocyanate (FITC) labeled anti-CD4 (BD Biosciences, NSW, Australia) and phycoerythrin (PE)-cyanine (Cy)5 labeled anti-CD25 antibodies (BD Biosciences, NSW, Australia). Surface labeling was followed by permeabilization with the Foxp3 fix/perm solution (eBioscience, CA, USA) and intracellular labeling with a PE conjugated anti-Foxp3 antibody (PCH101, eBioscience, CA, USA), according to the eBioscience Foxp3 staining protocol. PBMCs (2×10^6) used for IL-17a assays were stimulated for 5 h using 50 ng/mL of phorbol myristate acetate, PMA,

(Sigma-Aldrich, MO, USA), and 1 $\mu\text{g}/\text{mL}$ ionomycin (Sigma-Aldrich, St. Louis, MO) in the presence of 5 $\mu\text{g}/\text{mL}$ brefeldin A (Sigma-Aldrich, MO, USA), at 37°C and 5% CO_2 . Cells were washed in PBS and surface-labeled with CD3-PE-Cy5, before fixing with 4% *w/v* paraformaldehyde solution and permeabilization with 0.1% *w/v* saponin solution. After permeabilization, all wash buffers contained 0.1% *w/v* saponin. Cells were blocked with 5% *w/v* non-fat dry milk powder solution in PBS/0.1% *w/v* saponin for 30 min then intracellular labeling performed with anti-IL17a-PE (clone ebio64DEC17, eBioscience, CA, USA). Flow cytometry was carried out using a BD FACScan, in which 300,000–500,000 events were collected, and lymphocytes were gated based on their forward and side light scatter properties. Data were analyzed with the Cell Quest analysis program (BD Biosciences, NSW, Australia). Absolute numbers of T_{reg} cells and Th17 cells were calculated as the product of the total lymphocyte count from the routine complete blood examination (SA Pathology, South Australia, Australia) and target cell frequency of flow cytometric analysis.

Real-time PCR Analysis for FOXP3 and IL-17a Expression

Total RNA was isolated from intestinal biopsies using the RNeasy Lipid Minikit (Qiagen, Victoria, Australia). RNA gel electrophoresis was performed to assess RNA quality, and samples were accepted if 28S ribosomal RNA bands were present with intensity approximately twice that of the 18S RNA band. One microgram of RNA was reverse-transcribed to obtain complimentary DNA using Qiagen Quantitect Reverse transcription kit (Qiagen, Victoria, Australia). Primers were designed to span an intron of the genomic sequence. *Beta actin* forward primer: AAGAGC TACGAG CTGCCTGAC; *beta actin* reverse primer: GTAGTTTCGTGGATGCCACAG *Foxp3* forward Primer: GAAACAGCACATTCCAGAGTTC, *Foxp3* reverse primer: ATGGCCAGCGGATGAG; *IL-17a* forward primer: CAATCCCACGAAAT CCAGGATG, *IL-17a* reverse primer: GGTGGAGATTCCAAGGTGAGG; *IL-6* forward primer: AAATTCGGTACATCCTCGACGG, *IL-6* reverse primer: GGA AGGTTTCAGGTTGTTTTCTGC; *IL-1 β* forward primer: CAGCTACGAATCTCCG ACCAC, *IL-1 β* reverse primer: GGCAGGGAACCAGCATCTTC; *IL-21* forward primer: CATGGAGAGGATTGTCATCTGTC *IL-21* reverse primer: CAGAAATT CAGGGACCAA GTCAT; *IL-23* forward primer: GGACAACAGTCAGTTCTGCTT, *IL-23* reverse primer: CACAGGGCTATCAGGGAGC; *TGF- β* forward primer: CAAGCAGAGTACACACAG CAT, *TGF- β* reverse primer: TGCTCCACTTTTAACT TGAGCC.

Real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out using a Corbett Rotorgene

RG-3000 (Corbett Research, Australia), with two replicates per sample, a non-template control and non-reverse transcription control for each experiment. All reactions were carried out using SYBR green master mix (2 \times) solution (Applied Biosystems, CA, USA). PCR conditions for gene amplification began with a 10 min 95°C enzyme activation step, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Expression of *Foxp3*, *IL-17*, *IL-1 β* , *IL-6*, *IL-21*, and *IL-23* mRNA was normalized to β -actin expression. Relative gene expression was calculated using the ΔCt method. Patient samples were omitted if the target gene was below detection levels. PCR products were purified using Qiaquick PCR purification kit (Qiagen, Vic, Australia), sequenced, and confirmed against the National Center for Biotechnology Information (NCBI) basic local alignment search tool.

Statistical Analysis

The statistical differences between IBD and the control group were evaluated using the two-tailed Mann–Whitney ranked sum test. Comparison of paired samples was carried out utilizing a paired samples *t* test. Data are expressed as mean \pm standard error of the mean (SEM). Statistical significance was achieved when $P < 0.05$. Data were analyzed using Prism 4 software (GraphPad, CA, USA).

Results

Subjects

The average age \pm SEM of CD, UC, and control patients that donated peripheral blood were 37.7 \pm 2.8, 51.4 \pm 4.0, and 46.8 \pm 3.1 years, respectively. Of the total 63 IBD patients that donated blood samples, all were in a state of disease inactivity. Thirty-five received immunosuppressive treatment (azathioprine, 6-mercaptopurine, methotrexate), four were prescribed with corticosteroids, 10 received 5-aminosalicylic acid, six were on a combination of immunosuppressive therapy, corticosteroids, and 5-aminosalicylic acid, and eight were not taking any medication.

Intestinal biopsy samples were collected from 25 IBD patients, and the average age \pm SEM of CD, UC, and control patients were 40 \pm 12.63, 61 \pm 15.1, and 48 \pm 14.8 years, respectively. Of the 25 IBD biopsies collected, 10 were from patients with moderate disease activity, seven had mild disease activity, and eight had inactive disease, based on global colonoscopic appearance and histologic reports (SA Pathology, Adelaide, South Australia, Australia). Eleven patients received immunosuppressive therapy, three received corticosteroids, six were prescribed a

combination of immunosuppressive therapy, corticosteroids, and 5-aminosalicylic acid, and five were not taking any medication.

CD4⁺ CD25^{bright} Foxp3⁺ T_{reg} are Decreased in the Peripheral Blood of IBD Subjects

We defined the phenotype of T_{reg} cells as CD4⁺ CD25^{bright} Foxp3⁺ cells, as only CD4⁺ CD25^{bright} T cells are consistently Foxp3 positive and highly suppressive, while CD4⁺ CD25^{intermediate} T cells also include activated T cells that transiently express Foxp3 and are not suppressive [4]. In order to exclude contaminating CD25^{intermediate} populations, a gate for the CD25^{bright} population was set as the top 0.5% of CD4⁺ cells, capturing the highest expression of CD25 for all patient samples. Representative flow cytometric data demonstrate a high percentage of Foxp3⁺ cells residing with the CD4⁺ CD25^{bright} gate with >85% of these cells shown to be Foxp3⁺ in a control patient, while only 40–60% of CD4⁺ CD25^{bright} cells in representative IBD patients expressed Foxp3 (Fig. 1). The proportion of CD4⁺ CD25^{bright} Foxp3⁺ T_{reg} cells among PBMCs ranged from 0.012% to 0.51% in CD patients, from 0.003% to 0.47% in UC patients, and from 0.121% to 0.55% in the control group. The absolute numbers of CD4⁺ CD25^{bright} T_{reg} (mean ± SEM per ml of whole blood) in the peripheral blood were found to be significantly lower in both CD patients ($5.88 \pm 0.6 \times 10^3/\text{mL}$, $P=0.002$) and UC patients ($5.16 \pm 0.6 \times 10^3/\text{mL}$, $P=0.006$) compared to the control group ($8.08 \pm 0.7 \times 10^3/\text{mL}$; Fig. 2).

Th17 Cells are Elevated in the Peripheral Blood of IBD Patients

Next, we measured the numbers of circulating Th17 cells in the peripheral blood by flow cytometry to determine if they were altered in IBD patients. In order to identify Th17 cells, we utilized their capacity to produce IL-17a upon stimulation [12]. Stimulation with PMA and ionomycin to trigger the production of IL-17a was found to rapidly downregulate CD4 in some patients, confounding the identification of Th17 cells (data not shown). An alternative T lymphocyte marker is CD3, which is also expressed by CD8⁺ T cells. Therefore, the ability of CD8⁺ T cells to produce IL-17a was evaluated, which revealed that less than 0.03% of CD8⁺ lymphocytes could produce IL-17a (data not shown). Therefore, it was possible to identify Th17 cells as CD3⁺ IL17a⁺ PBMCs. The proportion of Th17 cells among PBMCs in control subjects ranged from 0.10% to 0.49%, compared with 0.36–1.25% in CD patients and 0.31–1.66% in UC patients (Fig. 3). Absolute counts of Th17 are given in Fig. 4. Th17 cells were significantly higher in the blood of CD patients ($15.0 \pm 2.8 \times 10^3/\text{mL}$, $P=0.0012$) and UC

patients ($13.4 \pm 2.2 \times 10^3/\text{mL}$, $P=0.0169$) compared with the control group ($7.67 \pm 0.80 \times 10^3/\text{mL}$).

An Imbalance of T_{reg} and Th17 Occurs in IBD

Having shown a decrease in T_{reg} and concomitant increase in Th17 in IBD, this relationship was further explored by investigating the balance of T_{reg} and Th17 within the same IBD patient and control subjects. To do this the numbers of T_{reg} and Th17 cells in the peripheral blood of IBD patients were directly compared in the same individual. The balance of T_{reg} and Th17 cells was assessed in 13 control, 15 CD, and 15 UC patients. While the numbers of T_{reg} and Th17 cells were equivalent in controls, the balance of T_{reg} and Th17 cells in the peripheral blood was disrupted in IBD patients (Fig. 5a). The imbalance observed was characterized by a decrease in T_{reg} and an increase in Th17 cells in the peripheral blood of both CD ($P<0.0001$) and UC ($P=0.004$). For the subset of patients where both T_{reg} and Th17 were enumerated using the same sample, the same defect was observed, and when expressed as a ratio of T_{reg} to Th17 in the same patient (Fig. 5b) we observed near 1:1 T_{reg}/Th17 in controls (0.8 ± 0.04), but lower ratios in both CD (0.55 ± 0.07) and UC (0.35 ± 0.05), $p=0.04$ and 0.0002 , respectively.

Expression of Foxp3 is Increased in the Intestinal Mucosa of IBD Patients

In order to determine if the balance of T_{reg} and Th17 cells was also affected in the intestinal mucosa of IBD patients, mucosal biopsy samples were analyzed by real-time RT-PCR. This was done by measurement of the expression of the T_{reg} specific transcription factor *Foxp3* by real-time RT-PCR. A 10-fold increase in *Foxp3* expression was observed in CD patients ($P=0.0007$) compared to controls, while a 100-fold increase in *Foxp3* expression was observed in UC patients ($P<0.0001$; Fig. 6a). The expression of *Foxp3* in UC patients was highest in those with moderate disease activity, lowest in those with mild disease activity, and variable in those with inactive disease. In contrast, the expression of *Foxp3* was comparable among CD patients with mild, moderate, and inactive disease activity.

Expression of IL-17a is Increased in the Intestinal Mucosa of IBD Patients

In order to determine whether Th17 cell contribute to the maintenance of inflammation in the gut, the expression of *IL-17a* in the intestinal mucosa was measured by real-time RT-PCR. It was found that a 100-fold increase in *IL-17a* expression was observed in CD patients ($P=0.003$) compared to controls, while a 1,000-fold increase in *IL-17a*

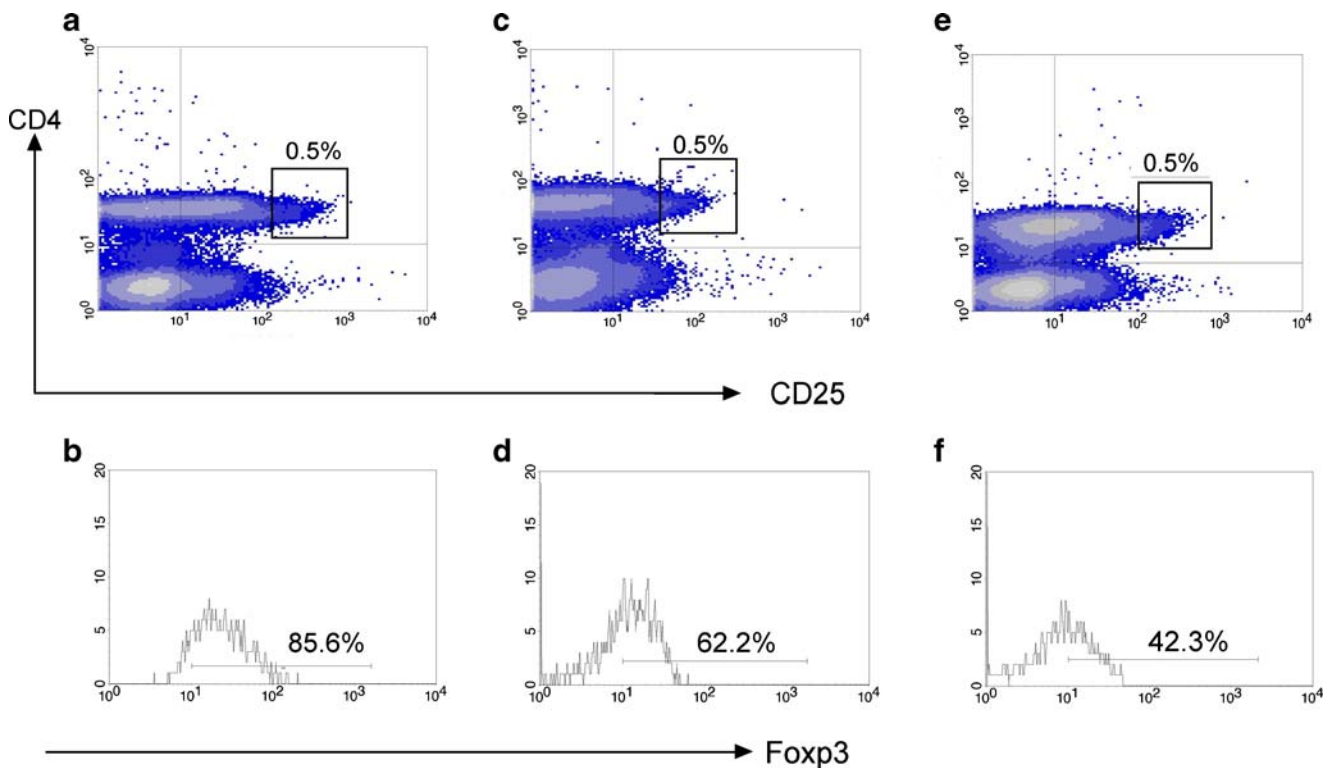


Fig. 1 Detection of human T_{reg} by flow cytometry. PBMC were stained with anti-CD4-FITC, anti-CD25-PECy5, and anti-Foxp3-PE. $CD4^+ CD25^{high}$ gates were set to the highest 0.5% of $CD25^+$, and cells within this gate were analyzed for Foxp3. Analysis of the $CD4^+ CD25^{high}$ population in a representative control patient (a) revealed

that approximately 85% of $CD4^+ CD25^{high}$ cells were Foxp3⁺ (b), while in a representative CD patient, the $CD4^+ CD25^{high}$ population (c) was only 62% Foxp3⁺ (d), and in a representative UC patient, the $CD4^+ CD25^{high}$ population (e) was only 42% Foxp3⁺ (f)

expression was observed in UC patients ($P=0.01$; Fig. 6b). Interestingly, UC patients with moderate disease activity were found to have the highest expression of *IL-17a* and were the same patients that had the highest expression of *Foxp3*, while the UC patients with mild disease activity that had previously been shown to express the lowest amounts of *Foxp3* in the intestinal mucosa also expressed the lowest

amounts of *IL-17a*. No difference was observed in the expression of *IL-17a* in CD patients of varying disease activities.

Increased Expression of *IL-1β* and *IL-6* Within the Intestinal Mucosa of IBD Patients

The cytokine environment within the mucosa of patients with IBD may favor the generation of pathological Th17 cells [22]. The expression of the cytokines *IL-1β*, *IL-6*, *IL-21*, *IL-23*, and *TGF-β* within the intestinal mucosa of IBD and control patients was investigated. It was found that *IL-1β* was increased in both CD ($P=0.0032$) and UC ($P=0.0005$), and *IL-6* was expressed at significantly higher levels in the mucosa of CD ($P=0.0007$) and UC ($P=0.0032$) patients (Fig. 7a, b). Expression of *IL-1β* and *IL-6* in UC patients was highest in those with moderate disease activity, lower in those with mild disease activity, and variable in those with inactive disease. No correlation was seen in CD between disease activity and *IL-1β* or *IL-6* expression. However, we found that high levels of *IL-17a* corresponded with high levels of *IL-1β* and *IL-6* for both CD and UC patients.

The expression of *TGF-β* was elevated in UC patients relative to control subjects (Fig. 7c; $P=0.048$), but was unchanged in CD patients regardless of disease activity.

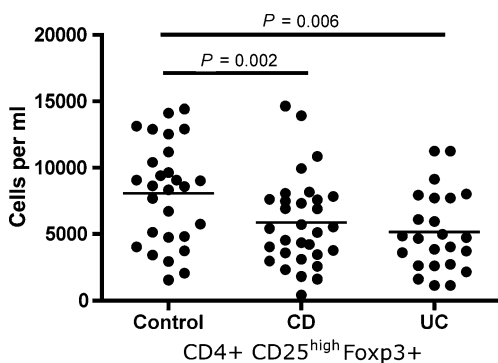


Fig. 2 Quantification of T_{reg} in IBD patients. Absolute numbers of T_{reg} cells were calculated using lymphocyte counts, and the frequency of $CD4^+ CD25^{high} Foxp3^+$ cells was determined by flow cytometry. Each datum point represents an individual patient sample. Median values for each group are represented by the horizontal line

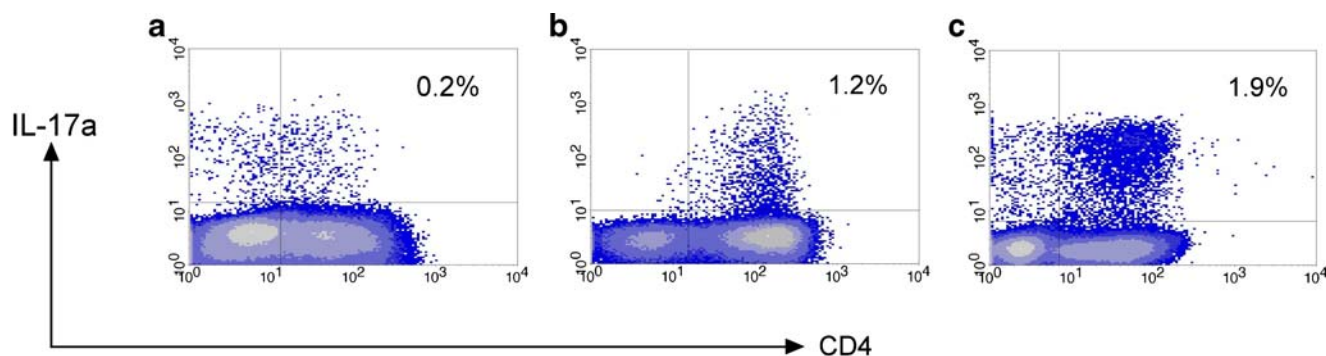


Fig. 3 Detection of human Th17 cells by flow cytometry. PBMCs were stained with anti-CD3-PECy5 and anti-IL-17a-PE. In a representative control subject 0.2% of PBMCs were CD3⁺ IL-17a⁺ cells

(a), while 1.2% of PBMCs in a representative CD patient were CD3⁺ IL-17a⁺ cells (b), and 1.9% of PBMC in a representative UC patient were CD3⁺ IL-17a⁺ cells (c)

Interestingly, a subgroup of UC patients with mild disease activity that had the highest expression of *TGF- β* also had low levels of *IL-1 β* , *IL-6*, and *IL-17a*. However, those patients with moderate disease activity and high levels of *TGF- β* had the highest expression of *IL-1 β* , *IL-6*, and *IL-17*. *IL-21* was expressed at very low levels in both control and IBD patients (data not shown). There was no significant difference in the levels of *IL-23* expressed in the intestinal mucosa of both IBD patients and controls (data not shown).

Discussion

In this study we have investigated T_{reg} and Th17 cells in the peripheral blood of patients in quiescent IBD and also measured cytokine and *Foxp3* mRNA in the intestinal mucosa. We have demonstrated a decrease of T_{reg} and an increase in Th17 cells in the peripheral blood of IBD patients. Additionally, T_{reg} and Th17 cells were measured in

parallel in a subgroup of IBD and control subjects. Control subjects exhibited a near 1:1 ratio of T_{reg} and Th17 cells in the peripheral blood, whereas a reduced ratio was observed in IBD patients indicating elevated Th17 cells concomitant with decreased T_{reg} . Increased expression of *IL-1 β* and *IL-6* was demonstrated in the intestinal mucosa of IBD patients suggesting a proinflammatory microenvironment that may promote the development of Th17 effector cells [13, 16]. The elevated expression of the surrogate markers of T_{reg} and Th17 cells, namely *Foxp3* and *IL-17a*, was also observed in the intestinal mucosa of IBD compared to a control group.

Our study extends previous research on T_{reg} in IBD using a refined approach to measure T_{reg} by combining a stringent gating strategy for CD25^{high} cells with intracellular staining for *Foxp3*. In addition, we measured T_{reg} and Th17 cells from the same patient sample allowing for the first time direct comparison of regulatory and effector cell numbers. By measuring both T_{reg} and Th17 cells, we identified that equilibrium exists between these cells in the control group, with a near 1:1 T_{reg} /Th17 ratio. However, in IBD this balance is disturbed, with subjects demonstrating significantly higher Th17 cells and fewer T_{reg} in the peripheral blood and hence a lower T_{reg} /Th17 ratio. Interestingly, the balance between the numbers of T_{reg} and Th17 cells is similarly perturbed in patients with juvenile arthritis, primary biliary cirrhosis, and coronary heart disease [23–25], suggesting that this may be a characteristic feature of pathologic inflammatory disorders.

The decrease in T_{reg} numbers in IBD is unlikely to be simply the result of immunosuppressive medication as evidenced by other studies. Infliximab has been reported to expand regulatory T cell numbers in children with CD [26]. Regulatory T cell numbers have been reportedly increased in myasthenia gravis patients after immunosuppressive therapy with prednisalone and azathoprine [27]. Treatment of multiple sclerosis relapse with methylprednisolone also demonstrated a rapid increase in T_{reg} number immediately after treatment [28]. Decreased T_{reg} numbers

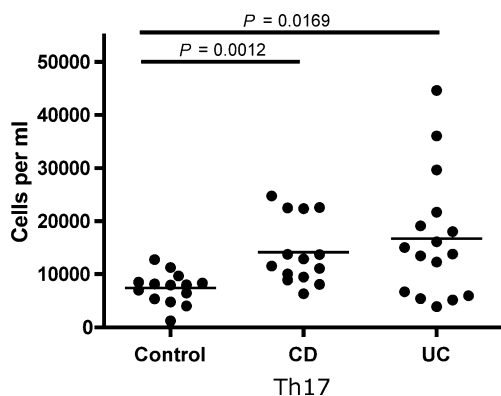


Fig. 4 Quantification of Th17 cells in IBD patients. Absolute numbers of Th17 cells were calculated using patient lymphocyte counts, and the frequency of CD3⁺ IL-17a⁺ cells was determined by flow cytometry. Each datum point represents an individual patient sample. The horizontal line represents median values for each group

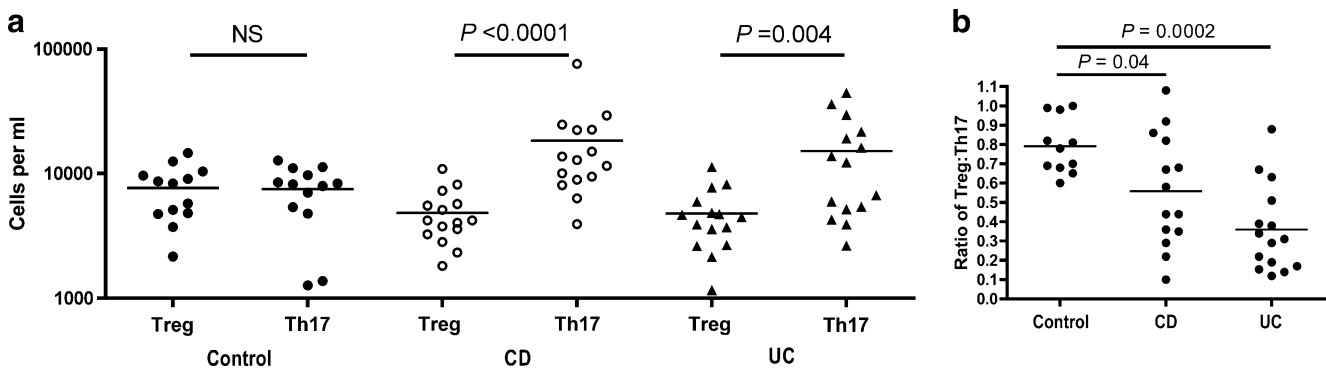


Fig. 5 a The balance of Th17 and regulatory T cell numbers is disrupted in IBD. Absolute numbers of $CD4^+ CD25^{high} Foxp3^+ T_{reg}$ and Th17 cells were determined using the same patient lymphocyte

samples. **b** The ratio of T_{reg} to Th17 is significantly decreased in CD and UC. Each datum point represents an individual patient sample. The horizontal line represents median values for each group

have been observed in patients treated with cyclosporine A [29]; however patients were omitted from the current study if they were prescribed this medication.

Previous work has shown increased numbers of T_{reg} in the lamina propria and mesenteric lymph nodes of IBD patients [30–32]. Our study found that in the intestinal mucosa of both CD and UC patients, *Foxp3* was highly expressed, suggesting that T_{reg} may be actively recruited to the intestinal mucosa in order to suppress proinflammatory immune responses. Activated T cells transiently expressing *Foxp3* that do not exhibit suppressive activity may alternatively account for the high expression of *Foxp3* in the intestinal mucosa [4]. However, previous studies have confirmed that T_{reg} isolated from the gut associated lymphoid tissue of IBD patients are functionally suppressive ex vivo [32, 33], although the ability of T_{reg} to regulate Th17 proliferation and effector activity may be limited in vivo due to a proinflammatory cytokine environment.

Recent research has demonstrated the plasticity of human T_{reg} , with cells identified that express both ROR- γ t and *Foxp3*, with the loss of suppressive function in the presence of high levels of IL-1 β and IL-6 [34, 35]. Hence,

the prolonged exposure of T_{reg} to these inflammatory cytokines may not only paralyze their suppressive function but may also culminate in their conversion to Th17 cells [34, 35]. Our finding of decreased numbers of peripheral T_{reg} cells and increased intestinal *Foxp3* expression indicate a likely sequestration of T_{reg} cells in the intestines of subjects with IBD. However in a cytokine microenvironment high in IL- β and IL-6, these T_{reg} may not retain suppressive functions and if converted to producing IL-17 may even contribute to disease.

Investigation into the cytokine microenvironment in IBD revealed the elevated expression of IL-1 β , IL-6, and IL-17a, which supports previous IBD studies that investigated these factors individually [36–39]. Fujino et al. were the first to identify T cells as a source of IL-17 in IBD, with elevated number of IL-17 $^+$ T cells and serum IL-17 seen in IBD patients compared to controls. Elevated levels of IL-17a have also been reported in rheumatoid arthritis [40], multiple sclerosis [41, 42], asthma [43], and systemic lupus erythematosus [44]. We confirm an increase in Th17 cells in the peripheral blood and elevated IL-17a in the intestinal tissue of IBD patients. However, in conjunction with

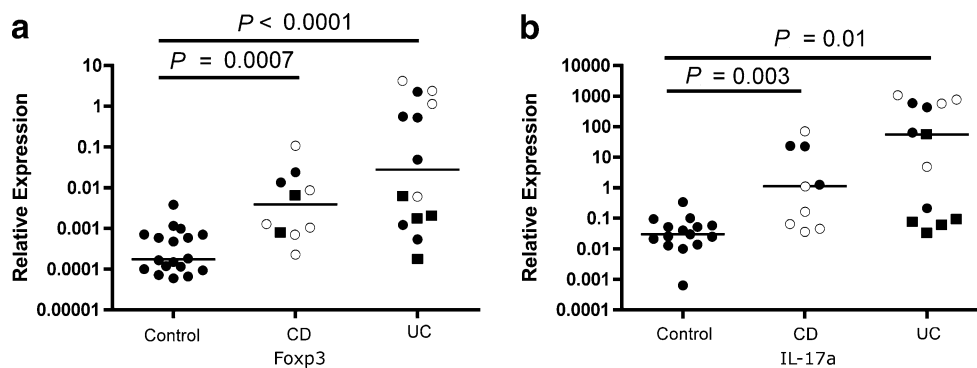


Fig. 6 RNA was extracted from intestinal biopsies with *Foxp3* (a) and *IL-17a* (b) expression quantified by real-time RT-PCR and normalized to β -actin expression. The mucosal disease activity of individual patients is indicated by black circles representing patients with

inactive disease, black squares representing mild disease activity, and white circles indicating patients with moderate disease activity. The horizontal lines represent the median values for each group

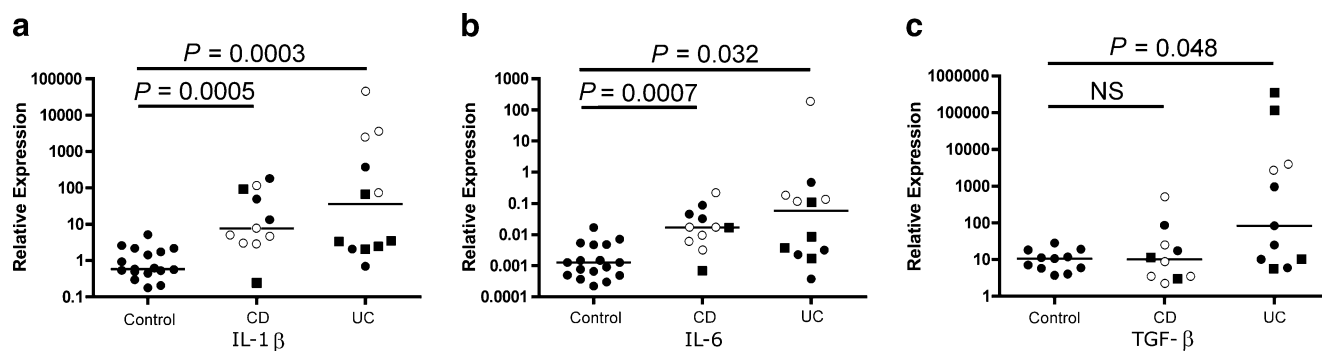


Fig. 7 RNA was extracted from intestinal biopsies, and the expression levels of the cytokines *IL-1 β* (a), *IL-6* (b), and *TGF- β* (c) were determined by real-time RT-PCR and normalized to β -actin expression. The disease activity of individual patients is indicated with *black*

circles representing patients with inactive disease, *black squares* representing mild disease activity, and *white circles* indicating patients with moderate disease activity. *Horizontal lines* indicate median values for each group

increased *IL-17a*, we also showed these patients expressed high levels of *IL-1 β* and *IL-6*. Not only do *IL-1 β* and *IL-6* mediate a range of inflammatory immune responses but they are also linked to the development of Th17 cells [13, 16], and *IL-6* additionally is capable of impeding T_{reg} function [45, 46].

The exact requirements for human Th17 cell differentiation are unclear; however unlike mouse Th17 differentiation, *IL-6* plus *TGF- β* is not sufficient for this process [12]. In humans, *IL-1 β* and *IL-6* are suggested to induce the production of *IL-17* from memory T cells, while *IL-21* and *TGF- β* may be required for the differentiation of Th17 cells from naive T cells [16]. Also *IL-21* in combination with *TGF- β* may be a mechanism that frustrates the resolution of inflammation promoted by *TGF- β* in IBD [16]. However, we were not able to confirm this as the expression of *IL-21* was low or undetectable in the mucosa of IBD patients and those of control patients. Signaling through *IL-21* may still be involved in IBD, with high levels of *IL-21R* found in the intestinal biopsies of IBD patients, suggesting that IBD patients may be sensitive to low levels of *IL-21* in the intestinal mucosa [47]. *IL-23* is believed to be required for the maintenance of Th17 cells [48–50], although we did not detect an increase in *IL-23* in IBD. Signaling through *IL-23* however may also play a role in IBD, as genome-wide association studies have identified polymorphisms in the *IL-23* receptor (*IL-23R*) in both CD and UC, and increased expression of *IL-23R* has previously been shown in both CD and UC [49, 51, 52]. Previous studies have reported elevated *IL-21* and *IL-23* in resected specimens from IBD patients [51, 53]. The inability to detect variations in both *IL-21* and *IL-23* may reflect assay sensitivity as small biopsy samples were used in this study rather than whole resected tissue.

Our investigation into the cytokine microenvironment of IBD also revealed an increase in *TGF- β* expression in UC patients, but not in CD. Defective *TGF- β* signaling has been shown in animal models of IBD [54] and increased

TGF- β 1 seen in UC but not CD [55]. *TGF- β* is involved in both T_{reg} and Th17 development and its function may be context dependant. Animal models have demonstrated that at high levels of *TGF- β* , *Foxp3* expression is upregulated and T_{reg} differentiation is induced, whereas at low levels of *TGF- β* , *IL-6* and *IL-21* synergize to promote the differentiation of Th17 cells [56]. Indeed our findings support this with UC patients exhibiting the highest *TGF- β* levels concomitantly expressing low levels of *IL-17a*, in contrast to those with intermediate *TGF- β* expression that expressed high levels of *IL-17a*.

In summary, we have demonstrated elevated numbers of Th17 cells combined with decreased T_{reg} numbers in the peripheral blood in IBD. In addition, *Foxp3* and *IL-17a* expression was increased in IBD intestinal mucosa. We suggest that this indicates the sequestration of T_{reg} to the gut where a proinflammatory cytokine environment high in *IL-1 β* and *IL-6* restricts T_{reg} activity and promotes the continual differentiation and development of Th17 cells. We propose that T_{reg} and Th17 cells remain in a state of balance within the immune system in health, with changes in the cytokine microenvironment promoting or suppressing the development of T_{reg} and Th17 cells. Current broad-spectrum immunosuppressive therapies potentially leave IBD patients susceptible to cancer or infection. New therapeutic approaches that specifically target Th17 effector cells and/or the cytokines that promote their development, such as *IL-1 β* and *IL-6*, may provide more focused treatment strategies for the management of IBD. Hence, therapeutic approaches that aim to re-establish homeostasis by increasing the number of T_{reg} , while also controlling effector T cell populations, may prove effective in the treatment of IBD. In addition, the imbalance demonstrated in the peripheral blood of IBD patients may provide new options of a non-invasive diagnostic tool. The initiating events that lead to a proinflammatory cytokine environment will now need to be further investigated.

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