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

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**Background:** Inflammatory bowel disease (IBD) may result from an aberrant immune response to commensal bacteria. Under normal circumstances, the intestinal immune system is maintained in a state of controlled inflammation, where balance exists between protective immunity mediated by effector cells and tolerance mediated by cells with regulatory function. Hence, the inflammation seen in IBD may be caused by an imbalance between CD4\(^+\) CD25\(^{high}\) Foxp3\(^+\) regulatory cells (T\(_{reg}\)) and pro-inflammatory Th17 cells. The aim of this study was to investigate both T\(_{reg}\) and Th17 cells in the peripheral blood and intestinal mucosa of the same IBD patients and to assess the mucosal cytokine environment. **Methods:** We recruited 63 IBD patients in disease remission and 28 control subjects, and measured T\(_{reg}\) and Th17 cells in peripheral blood by flow cytometry. The expression of Th17 promoting cytokines, IL-1\(\beta\), IL-6, IL-21, IL-23 and TGF-\(\beta\) as well as Foxp3 and IL-17a mRNA was analysed using real-time RT-PCR in the mucosal biopsies of 24 IBD patients in varying disease states and 18 control subjects. **Results:** A decrease in T\(_{reg}\) and increase in Th17 cells was observed in the peripheral blood of IBD patients. The elevated expression of Foxp3, IL-17a, IL-1\(\beta\) and IL-6 was observed in the mucosa of IBD patients, while TGF-\(\beta\) was only elevated in ulcerative colitis. **Conclusion:** IBD is associated with an reduced ratio of T\(_{reg}\) cells to Th17 effector cells in peripheral blood and is characterised by a pro-inflammatory 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 against 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 antigens or commensal bacteria.\(^2\) Tolerance is mediated by regulatory T cells (Tregs), a sub-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\(_{\text{reg}}\) are identified by expression of the high affinity interleukin (IL)-2 receptor \(\alpha\)-chain (CD25),\(^3\) however CD25 is also upregulated on the surface of activated CD4\(^+\) T cells, making this a non exclusive marker.\(^4\) T\(_{\text{reg}}\) also express the transcription factor Forkhead box p3 (Foxp3), which is crucial for their development and function.\(^5\) Although the mechanism through which T\(_{\text{reg}}\) 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 \textit{in vivo}.\(^3,7\)

There is growing evidence in mouse that T\(_{\text{reg}}\) and Th17 cells are linked from a developmental perspective, where the same naïve T cell precursor pool that generates T\(_{\text{reg}}\) cells is capable of generating IL-17a producing CD4\(^+\) T helper (Th)17 cells.\(^8,9\) In murine models, transforming growth factor (TGF)-\(\beta\) drives the differentiation of naïve T cells to a T\(_{\text{reg}}\) phenotype, whereas Th17 cells are induced in the presence of IL-6 and TGF-\(\beta\).\(^8,9\) IL-6 and TGF-\(\beta\) alone however are not sufficient for the differentiation of human Th17 cells.\(^10\) The potential role of IL-1\(\beta\), IL-6, TGF-\(\beta\), IL-21 and IL-23 in promoting human Th17 differentiation has been suggested, although the exact cytokine combination requires confirmation.\(^11\)\(^-\)\(^14\) It is suggested that IL-1\(\beta\) and IL-6 or IL-23 can induce the production of IL-17 from memory T cells, while IL-21 and TGF-\(\beta\) are required for the differentiation of Th17 cells from naïve T cells.\(^14\) Th17 cells express the transcription factor retinoic acid-related orphan receptor-\(\gamma\)t (ROR-\(\gamma\)t) and pattern recognition receptors capable of recognising extracellular bacteria and fungi.\(^15\) In addition to inducing a range of proinflammatory mediators that bridge the innate and adaptive immune response, Th17 cells are potent activators of neutrophils to enable
clearance of invading pathogens. Although Th17 cells are believed to play a critical biological function in clearing extracellular pathogens, the inappropriate production of IL-17a by these cells is thought to contribute to the pathology of a range of inflammatory diseases. Elevated levels of IL-17a have been found in rheumatoid arthritis, multiple sclerosis, asthma and systemic lupus erythematosus. 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. The balance between T_{reg} and Th17 cells may be essential for maintaining immune homeostasis, but no studies have yet examined this balance in individual 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 developed a stringent Flow based staining and gating strategy to accurately enumerate the Treg and Th17 cells using Foxp3 and IL17a as the defining markers. We not only investigated each cell type independently, but also simultaneously compared the two cells 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-1β, IL-6, IL-21, IL-23 and TGF-β in the intestinal biopsies of IBD and control patients. We found that the numbers of T_{reg} circulating in peripheral blood were significantly lower in patients with UC and CD compared to controls, while the numbers of Th17 cells were increased in IBD patients. As we measured both cell types in the same patients we are able to report for the first time the ratio of T_{reg} : T effectors in these cohorts, revealing a reduced Treg ratio in IBD. In contrast, the intestinal mucosa of IBD patients were enriched with both T_{reg} and Th17 marker mRNA, and contained a cytokine environment conducive to the differentiation of Th17 cells.
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, irritable bowel syndrome, reflux) or were healthy volunteers. 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. Control subjects had non-inflammatory disorders or were undergoing colon cancer screening. Biopsy samples were collected and stored in RNA later (Ambion, Austin, TX) 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<sub>reg</sub> cells, 1x10<sup>6</sup> PBMCs were surface labeled with a fluorescein isothiocynate (FITC) labeled anti-CD4 (BD, Sydney, NSW, Australia) and phycoerythrin (PE)–Cyanine (Cy)5 labeled anti-CD25 antibodies (BD, Sydney, NSW, Australia). Surface labeling was followed by permeabilisation with the Foxp3 fix/perm solution (eBioscience, San Diego, CA) and intracellular labeling with a PE conjugated anti-Foxp3 antibody (PCH101, eBioscience, San Diego, CA), according to the eBioscience Foxp3 staining protocol. PBMCs (2x10<sup>6</sup>) used for IL-17a assays were stimulated for 5 h using 50 ng/mL of phorbol myristate acetate (PMA)(Sigma-Aldrich, St. Louis, MO) and ionomycin (1 µg/mL)(Sigma-Aldrich, St. Louis, MO) in the presence of brefeldin A (5 µg/mL)(Sigma-Aldrich, St. Louis, MO), at 37°C and 5% CO<sub>2</sub>. Cells were washed in
PBS and surface labeled with CD3-PE-Cy5, before fixing with 4% w/v paraformaldehyde solution and permeabilisation with 0.1% w/v saponin solution. After permeabilisation, 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, San Diego, CA). 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, Sydney, 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, Valencia, CA). 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 (cDNA) using Qiagen Quantitect Reverse transcription kit (Qiagen, Hilden, Germany). Primers were designed to span an intron of the genomic sequence. *Beta actin* forward primer: AAGAGCTACGAGCTGCTTGAC; *beta actin* reverse primer; GTAGTTTCGTGGATGCCACAG

*Foop3* forward Primer: GAAAACAGCAGATCCAGGGTCCATC, *Foop3* reverse primer: ATGGCCAGCGGATGAG; *IL-17a* forward primer: CAATCCACGAAATCCAGGATG, *IL-17a* reverse primer: GGTGGAGATTCCAAGGTGAGG; *IL-6* forward primer: AAATTCGGTACATCCTCGACGG; *IL-6* reverse primer: GGAAGGTTCAGGTTTCTGC; *IL-1β* forward primer: CAGCTAACGACATCTCGACGG, *IL-1β* reverse primer: GGCAGGGAACCAGCATCTTC; *IL-21* forward primer: CATGGAGGATGTCATCTGTCATCAGGATG; *IL-21* reverse primer: CAGAAATTCCGACGAGGATGAG; *IL-23* forward primer: CAAGCAGAGGATGAGG; *IL-23* reverse primer: CACAGGCTATCAGGGAGC; *TGF-β* forward primer: CAAGCAGAGGATGAGG; *TGF-β* reverse primer: TGCTCCACATTTAATCTCGACGG.
Real time reverse transcription polymerase chain reaction (Real time RT-PCR) was carried out using a Corbett Rotorgene RG-3000 (Corbett Research, Sydney 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 (2x) solution (Applied Biosystems, Carlsbad, CA). PCR conditions for gene amplification began with a 10 min 95°C enzyme activation step, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Expression of Foxp3, IL-17, IL-1β, IL-6, IL-21 and IL-23 mRNA was normalised to β-actin expression. PCR products were purified using Qiaquick PCR purification kit (Qiagen, Hilden, Germany), sequenced and confirmed against refseqs by BLAST.

**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 ± standard error of the mean. Statistical significance was achieved when $P<0.05$. Data were analyzed using GraphPad Prism 4 software (San Diego, CA).
Results

Subjects
The average age ± SEM of CD, UC and control patients that donated peripheral blood were 37.7 ± 2.8, 51.4 ± 4.0 y and 46.8 ± 3.1 y, respectively. Of the total 65 IBD patients that donated blood samples, all were in a state of disease inactivity. Thirty-five received immuno suppressive treatment (azathioprine, 6-mercaptopurine, methotrexate), 4 were prescribed corticosteroids, 10 received 5-aminosalicylic acid, 6 were on a combination of immuno suppressive therapy, corticosteroids and 5-aminosalicylic acid, and 8 were not taking any medication.

Intestinal biopsy samples were collected from 25 IBD patients, and the average age ± SEM of CD, UC and control patients were 40 ± 12.63 y, 61 ± 15.1 y and 48 ± 14.8 y, respectively. Of the total 25 IBD biopsies collected, 6 were from patients with moderate disease activity, 8 had mild disease activity and 11 had inactive disease, based on global colonoscopic appearance and histologic reports (SA Pathology, Adelaide, South Australia). Eleven patients received immuno suppressive therapy, 3 received corticosteroids, 6 were prescribed a combination of immuno suppressive therapy, corticosteroids and 5-aminosalicylic acid and 5 were not taking any medication.

CD4+ CD25bright FOXP3+ T_reg are decreased in the peripheral blood of IBD subjects
We defined the phenotype of T_reg cells as CD4+ CD25bright Foxp3+ cells, as only CD4+ CD25bright T cells are consistently Foxp3 positive and highly suppressive, while CD4+ CD25intermediate T cells also include activated T cells that transiently express Foxp3 and are not suppressive. In order to exclude contaminating CD25intermediate populations, a gate for the CD25bright population was set as the top 0.5% of CD4+ CD25+ 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+ CD25bright gate with >85% of these cells shown to be Foxp3+ in a control patient, while only 40-60% of the same top 0.5% CD4+ CD25bright cells in representative IBD patients expressed Foxp3 (Figure 1). The proportion of CD4+ CD25bright Foxp3+ T_reg cells among PBMCs ranged from 0.012-0.51% in CD patients, from 0.003-0.47% in UC
patients and from 0.121-0.55% in the control group. The absolute numbers of CD4\(^+\) CD25\(^{\text{bright}}\) T\(_{\text{reg}}\) (mean ± SEM per ml of whole blood) in the peripheral blood were found to be significantly lower in both CD patients (5.88 ± 0.6 x10\(^3\)/mL, \(P=0.002\)) and UC patients (5.16 ± 0.6 x10\(^3\)/mL, \(P=0.006\)) compared to the control group (8.08 ± 0.7 x10\(^3\)/mL)(Figure 2).

**Th17 cells are elevated in the peripheral blood of IBD patients**

We measured the numbers of circulating Th17 cells in the peripheral blood by flow cytometry to determine if they were increased in IBD patients. In order to identify Th17 cells, we utilised their capacity to produce IL-17a upon stimulation.\(^{10}\) 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 0.10-0.49%, compared with 0.36-1.25% in CD patients and 0.31-1.66% in UC patients (Figure 3). Absolute counts of Th17 are given in Figure 4. Th17 cells were significantly higher in the blood of CD patients (15.0 ± 2.8 x10\(^3\)/mL, \(P = 0.0012\)) and UC patients (13.4 ± 2.2 x10\(^3\)/mL, \(P = 0.0169\)) compared with the control group (7.67 ± 0.80 x10\(^3\)/mL).

**An imbalance of T\(_{\text{reg}}\) and Th17 cells occurs in IBD**

Having shown a decrease in T\(_{\text{reg}}\) and concomitant increase in Th17 in IBD, this relationship was further explored by investigating the balance of T\(_{\text{reg}}\) and Th17 within the same IBD patient and control subjects. To do this the numbers of T\(_{\text{reg}}\) and Th17 cells in the peripheral blood of IBD patients were directly compared by simultaneous measurement. The balance of T\(_{\text{reg}}\) and Th17 cells was assessed in 13 control, 15 CD and 15 UC patients. While the numbers of T\(_{\text{reg}}\) and Th17 cells were equivalent in controls, the balance of T\(_{\text{reg}}\) and Th17 cells in the peripheral blood was disrupted in IBD patients (Figure 5). The imbalance observed was characterised by a decrease in
T\textsubscript{reg} and an increase in Th17 cells in the peripheral blood of both CD ($P < 0.0001$) and UC ($P = 0.004$).

**Expression of Foxp3 is increased in the intestinal mucosa of IBD patients**

In order to determine if the balance of T\textsubscript{reg} and Th17 cells was also affected in the intestinal mucosa of IBD patients, mucosal biopsy samples were analysed by real time RT-PCR. This was done by measurement of the expression of the T\textsubscript{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 ($P < 0.0001$)(Figure 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 1000-fold increase in IL-17a expression was observed in UC patients ($P = 0.01$) (Figure 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-1\beta and IL-6 within the intestinal mucosa of IBD patients**

The cytokine environment within the mucosa of patients with IBD may favour the generation of pathological Th17 cells.\textsuperscript{25} The expression of the cytokines IL-1\beta, IL-6, IL-21, IL-23 and TGF-\beta within the intestinal mucosa of IBD and control patients was investigated. It was found that IL-1\beta 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 (Figure 7a and 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 (Figure 7c) ($P = 0.048$), but was unchanged in CD patients regardless of disease activity. Interestingly, a subgroup of UC patients with mild disease activity that had the highest expression of TGF-β also had low levels of IL-1β, IL-6 and IL-17a. However, those patients with moderate disease activity and high levels of TGF-β had the highest expression of IL-1β, 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 demonstrated that IBD is characterized by a decrease of T<sub>reg</sub> and an increase in Th17 cells in the peripheral blood of patients in disease remission. In varying states of disease activity, an increase in the surrogate markers of T<sub>reg</sub> and Th17 cells, namely Foxp3 and IL-17a, was also observed in the intestinal mucosa. Previous work has shown increased numbers of T<sub>reg</sub> in the lamina propria and mesenteric lymph nodes of IBD patients.\cite{26-28} However, our study found that in the intestinal mucosa of both CD and UC patients, Foxp3 and IL-17a were both highly expressed, suggesting that T<sub>reg</sub> may be actively recruited into the intestinal mucosa in order to suppress pro-inflammatory Th17 immune responses. The expression of Foxp3 in the intestinal mucosa may alternatively be accounted for by activated T cells transiently expressing Foxp3 that do not exhibit suppressive activity,\cite{4} which are hence unable to suppress the Th17 cells. Alternatively, while T<sub>reg</sub> isolated from the gut associated lymphoid tissue and peripheral blood of IBD patients have been demonstrated to be functionally suppressive <em>ex vivo</em>,\cite{28,29} their ability to regulate Th17 proliferation and effector activity may be limited <em>in vivo</em> due to a pro-inflammatory cytokine environment.

Recent research has indicated the plasticity of human T<sub>reg</sub> that express ROR-γt and IL-17 with the loss of suppressive function in the presence of high levels of IL-1β and IL-6.\cite{30,31} Hence, the prolonged exposure of T<sub>reg</sub> to these inflammatory cytokines may not only paralyze their suppressive function, but may culminate in their conversion to Th17 cells.\cite{30,31} This process may account for the deficit in T<sub>reg</sub> numbers that we observed in the peripheral blood of IBD patients, which was accompanied by an increase in the numbers of Th17 cells (Figure 5). The imbalance of T<sub>reg</sub> and Th17 cells circulating in the peripheral blood of IBD patients may account for the relapsing and remitting nature of intestinal inflammation, and approaches that aim to re-establish and maintain this balance may provide an effective therapeutic strategy.

In mice the differentiation of Th17 cells is induced in the presence of IL-6 and TGF-β, while TGF-β alone induces the generation of T<sub>reg</sub>.\cite{8} In contrast, IL-6 and TGF-β are not sufficient for the generation of Th17 cells in humans.\cite{10} A range of additional cytokines are proposed to induce Th17 differentiation in humans, including combinations of IL-
1β, IL-6, IL-21, IL-23 and TGF-β.11-14 While IL-23 is important in the development and maintenance of Th17 cells,32 and has been found to be elevated in CD but not UC,33 we found no difference in the expression of IL-23 in the intestinal mucosa of control and IBD patients. However, signalling through IL-23 may still play a role in disease pathogenesis as genome-wide association studies have identified polymorphisms in the IL-23 receptor (IL-23R) in IBD patients, and increased expression of IL-23R has previously been shown in both CD and UC.32, 34 An alternative pathway of Th17 differentiation involves IL-21 in combination with TGF-β,14 which may be a mechanism that frustrates the resolution of inflammation promoted by TGF-β in IBD. 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. Alternative sources of IL17a in the mucosa could include memory T cells as induction of IL-17a secretion from these cells by IL-1β and IL-6 has been shown.14 The expression of TGF-β was not elevated in CD patients, suggesting that this cytokine does not affect the pro-inflammatory cytokine network associated with Th17 cells in the intestinal mucosa.

Both IL-1β and IL-6 were expressed at significantly higher levels in the intestinal mucosa of IBD patients (Figure 7). IL-1β and IL-6 expression correlated with disease activity in UC, with high expression observed in moderate disease activity and lower expression in mild disease activity. Strikingly, the expression of IL-17a correlated closely with IL-1β and IL-6 expression in both UC and CD patients, suggesting these cytokines are involved in driving Th17 production in IBD patients. This was also true for IBD patients with inactive disease, where Th17 cells may accumulate in the intestinal mucosa and promote relapse into active disease.

The protective role of TGF-β in UC may also be important, as a subset of patients with the highest levels of TGF-β also showed low IL-17a, IL-1β and IL-6 expression (Figures 6 and 7). Elevated TGF-β has been implicated in the differentiation of Treg,35 although these patients concurrently had low Foxp3 expression in the intestinal mucosa suggesting low levels of Treg were present. In patients with moderate disease activity, elevated levels of TGF-β expression were accompanied by high levels of IL-1β, IL-6 and IL-17. TGF-β is a suppressive cytokine with immunoregulatory properties
including inhibition of the differentiation of effector cells and the production of pro-inflammatory cytokines,\textsuperscript{36} hence TGF-\(\beta\) may only be protective when in the absence of pro-inflammatory cytokines and promote Th17 cells in the presence of IL-1\(\beta\) and IL-6.

In summary, we have shown that IBD is characterised by an imbalance between Th17 effector cells and T\(_{\text{reg}}\) cells, with elevated Th17 cells and a cytokine microenvironment exists that promotes Th17 development. The observed deficit of T\(_{\text{reg}}\) in IBD patients may impair the ability of the immune system to limit excessive pathogenic Th17 driven immune responses in the intestinal mucosa. Interestingly, the balance between the numbers of T\(_{\text{reg}}\) and Th17 cells is similarly perturbed in patients with juvenile arthritis, primary biliary cirrhosis and coronary heart disease,\textsuperscript{37-39} suggesting that this may be a characteristic feature of pathologic inflammatory disorders. Therefore, therapeutic approaches that aim to re-establish balance by increasing the number of T\(_{\text{reg}}\) in IBD patients, and specifically targeting Th17 cells, may prove effective in the treatment of IBD. Approaches such as these provide more focussed treatment strategies for the management of IBD than current broad-spectrum immunosuppressive therapies that may leave IBD patients susceptible to cancer or infection. In addition, the imbalance demonstrated in the peripheral blood of IBD patients may provide new options of a non-invasive diagnostic tool.
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Figure Legends

Figure 1: Detection of human T\textsubscript{reg} by flow cytometry. PBMC were stained with anti-CD4-FITC, anti-CD25-PECy5 and anti-Foxp3-PE. CD4\textsuperscript{+} CD25\textsuperscript{bright} gates were set to the highest 0.5% of CD25\textsuperscript{+} and cells within this gate were analyzed for Foxp3. Analysis of the CD4\textsuperscript{+} CD25\textsuperscript{bright} population in a representative control patient (A) revealed that approximately 85% of CD4\textsuperscript{+} CD25\textsuperscript{bright} cells were Foxp3\textsuperscript{+} (B), while in a representative CD patient the CD4\textsuperscript{+} CD25\textsuperscript{bright} population (C) was only 62% Foxp3\textsuperscript{+} (D) and in a representative UC patient the CD4\textsuperscript{+} CD25\textsuperscript{bright} population (E) was only 42% Foxp3\textsuperscript{+} (F).

Figure 2: Quantification of T\textsubscript{reg} in IBD patients. Absolute numbers of T\textsubscript{reg} cells were calculated using lymphocyte counts and the frequency of CD4\textsuperscript{+} CD25\textsuperscript{bright} Foxp3\textsuperscript{+} cells determined by flow cytometry. Each datum point represents an individual patient sample. Median values for each group are represented by the horizontal line.

Figure 3: Detection of human Th17 cells by flow cytometry. PBMC were stained with anti-CD3-PECy5 and anti-IL-17a-PE. In a representative control subject 0.15% of PBMC were CD3\textsuperscript{+} IL-17a\textsuperscript{+} cells (A), while 1.2% of PBMC in a representative CD patient were CD3\textsuperscript{+} IL-17a\textsuperscript{+} cells (B) and 1.87% of PBMC in a representative UC patient were CD3\textsuperscript{+} IL-17a\textsuperscript{+} cells (C).

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

Figure 5: The balance of Th17 and regulatory T cell numbers is disrupted in IBD. Absolute numbers of CD4\textsuperscript{+} CD25\textsuperscript{bright} Foxp3\textsuperscript{+} T\textsubscript{reg} and Th17 cells were determined using the same patient lymphocyte samples. Each datum point represents an individual patient sample. The horizontal line represents median values for each group.

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

Figure 7: RNA was extracted from intestinal biopsies and the expression levels of the cytokines IL-1β (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.
Fig 2

![Graph showing cell counts per ml for Control, CD, and UC groups. The x-axis represents different groups: Control, CD, UC. The y-axis shows cell counts per ml ranging from 0 to 20000. The figure includes two horizontal lines indicating statistical significance: one at $P = 0.006$ and another at $P = 0.002$. The graph indicates $CD4^+ CD25^{bright}$ Foxp3+ cells.](image-url)
Fig 6

Panel A

- Relative Expression
- Control, CD, UC
- Foxp3
- $P < 0.0001$
- $P = 0.0007$

Panel B

- Relative Expression
- Control, CD, UC
- IL-17a
- $P = 0.01$
- $P = 0.003$
Fig 7