Increased levels of circulating Th17 cells in quiescent versus active Crohn's disease

Anders Dige a,⁎, Sidsel Støya, Tue K. Rasmussen b, Jens Kelsen a, c, Christian L. Hvas a, Thomas D. Sandahl a, Jens F. Dahlerup a, Bent Deleuran b, Jørgen Agnholt a

a Gastro-Immuno Research Laboratory (GIRL), Department of Medicine V (Hepatology and Gastroenterology), Aarhus University Hospital, DK-8000 Aarhus C, Denmark
b Department of Medical Microbiology and Immunology, University of Aarhus, DK-8000 Aarhus C, Denmark
c Department of Medicine, Randers Regional Hospital, DK-8930 Randers, Denmark

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KEYWORDS
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Abstract

Background and aims: Th17 cells, a subset of CD4+ T cells that produce interleukin (IL)-17A, IL-17F, IL-21, IL-22, IL-26, and the chemokine CCL20 are critically involved in the mucosal inflammation observed in Crohn's disease (CD). However, their role as mediators of inflammation in CD has been questioned by a recent clinical trial in which anti-IL-17A (secukinumab) treatment was ineffective. Besides being pro-inflammatory, Th17-related cytokines mediate mucosal protective functions. We aimed to investigate the role of Th17 cells in CD inflammation.

Methods: Blood samples from 26 patients with active CD and 10 healthy controls (HC) were analyzed for levels of IL-17A-, IL-21- and IL-22-producing CD45RO+CD4+ T cells using multicolor flow cytometry. Samples were analyzed before and during adalimumab treatment to compare intra-individual changes during active and quiescent disease.

Results: CD patients had statistically significantly higher levels of IL-17A-, IL-21- and IL-22-producing CD45RO+CD4+ T cells in both active and quiescent disease compared with HC. Baseline levels of IL-21 and IL-22 producing CD45RO+CD4+ T cells correlated inversely with mucosal inflammation estimated by fecal calprotectin. Patients who responded to adalimumab treatment demonstrated a 2- to 3-fold increase in levels of IL-17A- and IL-21-producing CD45RO+CD4+ T cells in quiescent disease compared with active disease.

Conclusion: Our data support the involvement of Th17 cells and IL-21- and IL-22-producing CD45RO+CD4+ T cells in CD. Because patients had higher levels in quiescent disease compared with active CD, we question whether Th17 cells are promoters of inflammation. Instead, Th17 cells may counterbalance inflammation and maintain gut homeostasis.

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⁎ Corresponding author. Tel.: +45 7846 2808; fax: +45 7846 2740.
E-mail address: andedige@rm.dk (A. Dige).

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Th17 cells in Crohn’s disease

1. Introduction

In Crohn’s disease (CD), a disturbance of the delicate balance between pro-inflammatory T cell subsets and tolerance-maintaining regulatory T cells (Tregs) contributes to the inflammatory process.1–4 Among the pro-inflammatory T cells, the Th17 subset of CD4+ T cells produces interleukin (IL)-17A, IL-17F, IL-21, IL-22, IL-26, and the chemokine CCL20.5 Th17 cells have been suggested to be of importance in CD.1 At the mucosal level, an increase in IL-17-producing T cells and/or IL-17 mRNA expression6–8 has been described in CD compared with healthy controls (HC)9 or patients with infectious or ischemic colitis.10 In one study, Th17 cells had a low susceptibility to suppression by Tregs.11 Moreover, increased levels of circulating Th17 cells have been reported in CD patients compared with healthy controls.6,9,12 The involvement of Th17 cells in CD pathogenesis is also supported by genome-wide association studies that have identified several IBD susceptibility genes involved in Th17 cell differentiation.13,14

Although Th17 cells are generally considered to be pro-inflammatory, a subset of mucosal IL-17-producing T cells has recently been shown to possess in vitro regulatory functions in CD.15 Additionally, protective barrier functions in the mucosal immune response have been attributed to the Th17-related cytokines IL-17 and IL-22.16–18 Th17 cells may therefore have opposing roles in IC.19 It is possible that the increased level of Th17 cells observed in CD represents a beneficial adaptive response to the mucosal inflammation rather than being the actual cause of the inflammation.20 This hypothesis is supported by a recent clinical trial with the anti-IL17A antibody secukinumab, which was ineffective in patients with CD and even appeared to exacerbate CD in a small subset of patients.21 Animal studies of T cell-mediated colitis have also been ambiguous regarding the importance of Th17 cells in intestinal inflammation. Some studies have reported a central role of Th17 cells,22,23 whereas others have observed that inflammation also develops in mice with deficient IL-17 production in their T cells.24

In the present study, we aimed to investigate the role of circulating Th17 cells in CD. We used the expression of IL-17A to identify Th17 cells among the CD4+ T cell population. We further examined the expression of IL-21 and IL-22 because IL-21 may be important for the differentiation of Th17 cells in CD,7 and IL-22 is presumed to be a mediator of Th17 functions together with IL-17A.18,25 We focused on the dynamic, patient-specific changes in the levels of IL-17A, IL-21, and IL-22-producing CD4+ T cells and Th17-derived cytokines between active and quiescent CD in patients treated with the anti-tumor necrosis factor (TNF)-α antibody adalimumab (Humira®).

2. Material and methods

2.1. Subjects

Twenty-six patients diagnosed with CD according to clinical, histopathological, and biochemical criteria were included. At the time of inclusion, all patients had moderate to severe disease activity as estimated by a Crohn’s disease activity index (CDAI)26 above 220 (18 patients) or a Harvey–Bradshaw index (HBI)27 above 4 in combination with elevated levels of C-reactive protein (CRP) and fecal calprotectin (8 patients). The patient characteristics are listed in Table 1. Ten healthy controls were also included. Treatment with anti-TNF-α antibodies or corticosteroids or a change in the dose of immunosuppressants (azathioprine or methotrexate) within the 12 weeks prior to the study was not permitted. One patient receiving a stable budesonide treatment (9mg/day) was included. The patients received induction treatment with subcutaneous adalimumab at 160mg on day 0, 80mg two weeks later, followed by 40mg every other week. The patients were monitored for 26 weeks. When evaluating the patient response to adalimumab, predefined criteria were used to discriminate the non-responders from the responders. The non-responders were classified as the patients who experienced a reduction in CDAI of less than 100 in conjunction with a continuously elevated CDAI (above 150) or an HBI reduction of less than 3 points and a continuously elevated HBI (above 4) compared with baseline values. After 1 week, the patients were classified as primary non-responders or primary responders according to these criteria. The treatment response was re-evaluated after 8 and 26 weeks. Patients who did not achieve remission during the first 8 weeks of adalimumab treatment received intensified treatment with 40mg of adalimumab administered weekly. Patients who responded and continued treatment for the entire 26 weeks were classified as having a sustained clinical response. Patients without an adequate response despite intensified treatment were classified as overall non-responders; these patients discontinued adalimumab and left the study.

Venous blood samples were obtained at baseline (day 0), and 1, and 26 weeks after inclusion. Additional samples were obtained at the time of exclusion from the patients who discontinued adalimumab treatment due to an inadequate treatment response.

2.2. Biochemical parameters

The CD activity scores (CDAI and HBI) and biochemical parameters (CRP, total leukocyte count, differential leukocyte count, and fecal calprotectin) were evaluated at the time of blood sampling.

2.3. PBMC isolation and flow cytometric analysis of IL-17A-, IL-21-, and IL-22-producing T cells

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (GE Healthcare Bio-Sciences, Uppsala, Sweden) and stored at −140°C. Before staining for intracellular cytokines, the PBMCs were thawed, washed twice with phosphate-buffered saline (PBS) plus 20% heat-inactivated pooled human AB serum, and adjusted to a final concentration of 2×10^6 PBMCs/ml in culture medium (RPMI 1640 with 10% pooled heat-inactivated human AB serum, 100U/ml penicillin and 100μg/ml streptomycin). Subsequently, 2.5ml of cell suspension was added to flat-bottomed wells (Nunc, Denmark, cat. no. 140675) and cultured for 4h at 37°C in a 5% CO₂ atmosphere. The cells were stimulated with 10μg/ml brefeldin A (Sigma-Aldrich, Denmark, cat. no. B7651), 1μg/ml ionomycin (Sigma-Aldrich, Denmark, cat. no. I0634) and 50μg/ml phorbol
12-myristate 13-acetate (PMA) (Sigma-Aldrich, Denmark, cat. no. P1585). The cells were harvested, and 5×10⁵ cells in 100μl washbuffer (PBS, 2% bovine serum albumin (BSA) and 0.9% azide) were surface-stained with optimized amounts of the antibodies against CD4 (anti-CD4-PerCP, BD Biosciences, cat. no. 345770) and CD45RO (anti-CD45RO-FITC, BD Biosciences, cat. no. 555492). The surface staining was fixed with 1.5ml BD FACS Lysing Solution (BD Biosciences, cat. no. 349202). The cells were then permeabilized with 0.5 ml FACS Permeabilizing Solution 2 (BD Biosciences, cat. no. 340973) and blocked with heat-inactivated mouse serum (Invitrogen, cat. no 10410) before staining with anti-IL17A Alexa-647 (eBiosciences, cat. no. 51-7179-42), anti-IL-21 PE (eBiosciences, cat. no. 12-7219-42) and anti-IL22 PE (R&D, cat. no. IC7821P). Finally, the cells were fixed in 250μl PBS with 1% formaldehyde. Four-color flow cytometry was performed within 24 h using a FACS Canto analyzer (BD Biosciences), and 10⁵ events in the forward-side scatter lymphogate were recorded. The combination of forward-scatter-height and forward-scatter-amplitude was used to exclude the events without single cell appearances. The gating for IL-17A-, IL-21- and IL-22-positive events was based on isotype or fluorescence-minus-one controls. The samples from the individual patients were analyzed en bloc. The data were analyzed using FACS Diva 5.1 software (BD Biosciences).

### Table 1 Patient characteristics at baseline.

<table>
<thead>
<tr>
<th></th>
<th>Median [IQR]</th>
<th>Sustained clinical response (n=21)</th>
<th>Non-responders (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td>40.0 [29.5–46.2]</td>
<td>22.5 [21.5–35.1]</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td>Male</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>8</td>
<td>4</td>
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<tr>
<td><strong>Smokers</strong></td>
<td></td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td><strong>Disease duration (years)</strong></td>
<td>9.1 [2.8–10.3]</td>
<td>0.7 [0.5–3.1]</td>
<td></td>
</tr>
<tr>
<td><strong>CDAI</strong></td>
<td>234 [194–256]</td>
<td>327 [312–374]</td>
<td></td>
</tr>
<tr>
<td><strong>Localization</strong></td>
<td>Upper GI</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Small intestine</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Colonic</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Perianal</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><strong>Behavior</strong></td>
<td>Stricture</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Non-perianal fistula</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Perianal fistula</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><strong>CRP (nmol/l)</strong></td>
<td>80 [39–144]</td>
<td>132 [104–149]</td>
<td></td>
</tr>
<tr>
<td><strong>Fecal calprotectin (mg/kg)</strong></td>
<td>1250 [646–1250]</td>
<td>1250 [1250–1250]</td>
<td></td>
</tr>
<tr>
<td><strong>Immunosuppressant</strong></td>
<td>None</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Azathioprine</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Methotrexate</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Budesonide</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

**Abbreviations:** CDAI, Crohn’s disease activity index; HBI, Harvey-Bradshaw index; CRP, C-reactive protein (reference <75nmol/l). Fecal calprotectin (reference <50mg/kg feces).

### 2.4. Measurement of IL-17A, IL-21, IL-22 and IL-23 plasma levels

The quantification of IL-17A, IL-21, IL-22, and IL-23 levels in plasma was performed using ELISAs for human IL-17A (eBioscience, San Diego, CA, cat. no. 88-7176-22), IL-21 (eBioscience, cat. no. 88–7216), IL-22 (eBioscience, cat. no. 88-7522-88), and IL-23 (eBioscience, cat. no. 88-7237-88) as previously described. All of the samples were diluted 1:1 with the sample diluent reagent supplied with the kit. The samples were analyzed in duplicate, using the average of the optical density (OD) values to calculate the concentrations. The minimum detection limit (cut-off) was calculated as the average value of the blanks plus 2 standard deviations, giving a cut-off value of 1.1 pg/ml for IL-17A, 20 pg/ml for IL-21, 1.3pg/ml for IL-22, and 4.7pg/ml for IL-23. The values below the cut-off were assigned the same value as the cut-off value.

### 2.5. Statistical analysis

The statistical analyses were performed using Stata 11 (STATA). Non-parametric statistical tests were applied, and p<0.05 was considered statistically significant. The Mann–Whitney U test and the Wilcoxon sign-rank test were used for the unpaired and paired statistical analysis, respectively. Data are presented as medians with interquartile ranges (IQR). The Spearman rank correlation rho was used to examine the associations between the variables.

### 2.6. Ethical considerations

All participants provided written informed consent, and the study was approved by the Central Denmark Region Committees on Biomedical Research Ethics (journal no. 20080092).
3. Results

3.1. IL-17A, IL-21, and IL-22 productions mainly occur within the CD45RO+ population of CD4+ T cells

In CD4+ T cells, the productions of IL-17A, IL-21, and IL-22 were mainly observed within the CD45RO+ population, i.e. memory or activated T helper cells (median 81% [60%–92%], 76% [65%–85%], and 74% [66%–83%], respectively). Consequently, we focused our analysis of the dynamic changes in levels of IL-17A-, IL-21- and IL-22-producing CD4+ T cells to the CD45RO+ population. In IL-17A-producing CD45RO+CD4+ T cells, we observed only a low level of a concomitant production of IL-21 (median 20% [0%–26%]) and IL-22 (6.7% [2.6%–12%]). For this reason, the analysis of the levels of IL-17A and IL-22 producing CD4+ T cells was expanded to include all CD45RO+CD4+ T cells and not restricted to the IL-17A-producing CD45RO+CD4+ T cells.

3.2. CD patients have elevated levels of IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells compared with healthy controls

At baseline, the CD patients had statistically significantly higher levels of IL-17A-, IL-21- and IL-22-producing CD45RO+CD4+ T cells (0.45% [0.30%–0.75%], 3.0% [1.4%–4.7%] and 2.2% [0.90%–3.3%], for IL-17A, IL-21 and IL-22, respectively) than healthy controls had (Fig. 1). We investigated to what extent the baseline levels of IL-17A-, IL-21- and IL-22-producing CD45RO+CD4+ T cells could be used to identify responders to adalimumab treatment. All patients with a baseline level of IL-22-producing CD45RO+CD4+ T cells above the median level (12 patients) obtained a sustained clinical response. All overall non-responders (5 patients) had levels of IL-22-producing CD45RO+CD4+ T cells below the median.

There was a statistically significant correlation between the levels of IL-17A- and IL-21-producing CD45RO+CD4+ T cells (p=0.002; Spearman’s rank correlation, rho=0.69) and a tendency towards a correlation between the levels of IL-17A- and IL-22-producing CD45RO+CD4+ T cells (p=0.07, rho=0.38) at baseline. The levels of the IL-21- and IL-22-producing CD45RO+CD4+ T cells correlated significantly at baseline (p=0.001, rho=0.62).

Because we aimed to investigate the extent to which IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells reflected mucosal inflammation, we correlated the baseline levels of cytokine-producing CD45RO+CD4+ T cells with the surrogate marker of mucosal inflammation, fecal calprotectin. We observed a statistically significant inverse correlation between the levels of fecal calprotectin and IL-21- and IL-22-producing CD45RO+CD4+ T cells (p=0.01, rho=−0.51 (IL-21) and p=0.001, rho=−0.61 (IL-22)), whereas this inverse correlation with fecal calprotectin was less clear for IL-17A-producing CD45RO+CD4+ T cells (p=0.08, rho=−0.36).

We, furthermore, examined to what extent the disease duration and location of CD influenced the levels of IL-17A, IL-21, and IL-22 producing CD45RO+CD4+ T cells. The analyses revealed a statistically significant correlation between the levels of IL-22-producing CD45RO+CD4+ T cells and the duration of the disease (p=0.02, rho=0.49).

3.3. The levels of circulating IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells increase during remission

In 21 of the 26 patients, adalimumab induced a sustained clinical response that lasted for the entire observation period (Fig. 2). In these 21 patients, the levels of IL-17A- and IL-21-producing CD45RO+CD4+ T cells were increased by 2 to 3-fold when patients were in remission compared with their active disease state (p=0.007 and p=0.006 for IL17A and IL-21, respectively) (Fig. 3). In remission, patients exhibited medians of 1.7% [0.55%–2.7%] IL-17A-producing CD45RO+CD4+ T cells and 6.5% [3.3%–11%] IL-21-producing CD45RO+CD4+ T cells. There was also a minor increase in the percentage of IL-22-producing CD45RO+CD4+ T cells (2.7% [1.6%–4.7%]), but this increase was not statistically significant (p=0.07) (Fig. 3). The observed baseline correlations between IL-17A- and IL-21-producing CD45RO+CD4+ T cells, as well as IL-21- and IL-22-producing CD45RO+CD4+ T cells, were also present 1 and 26 weeks after the initiation of adalimumab treatment (p<0.0001, rho=0.83; p<0.0001, rho=0.81 for IL-17A/IL-21 and p<0.0001, rho=0.74; p=0.005, rho=0.62 for IL-21/IL22 at week one and week 26, respectively). Moreover, we observed a correlation between the levels of IL-17A- and IL-22-producing CD45RO+CD4+ T cells at week 1 (p=0.002, rho=0.68) and 26 (p=0.001, rho=0.76).

If IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells influence inflammation in CD, it would be expected that changes in the levels of the cytokine-producing CD45RO+CD4+ T cells should be detectable before or during the changes in disease activity. Therefore, we investigated the early alterations present one week after the initiation of adalimumab treatment, i.e. the time at which most patients had experienced an amelioration of their symptoms. Although the clinical and paraclinical markers of disease activity fell rapidly during the first week (Fig. 2), we did not observe any significant early changes in the levels of IL-17A- or IL-21-producing CD45RO+CD4+ T cells after 1 week of adalimumab treatment in neither the responders nor the non-responders. The nine patients who had not yet achieved a clinical response at week one showed a decrease in the levels of IL-22-producing CD45RO+CD4+ T cells (p=0.04).

3.4. Plasma levels of IL-17A, IL-21, IL-22, and IL-23 in active and quiescent CD

At baseline, the patients had a median plasma level of 2.2 [1.8–3.5] pg/ml IL-17A, 28 [20–72] pg/ml IL-21, and 3.8 [1.3–12] pg/ml IL-22. The cytokine levels did not correlate with the levels of cytokine-producing CD45RO+CD4+ T cells or fecal calprotectin. Overall, we did not observe significant individual changes in IL-17A, IL-21, and IL-22 plasma levels in neither the responders nor the non-responders during adalimumab treatment (Fig. 4). In general, the IL-23 levels were below the detection level of the assay and were therefore not included in the data analysis.
4. Discussion

This study is the first to investigate dynamic changes in the levels of Th17 cells and Th17-related cytokines in CD. The monitoring of changes during adalimumab treatment allowed us to demonstrate the intra-individual changes in the levels of Th17 cells and Th17-related cytokines in active and quiescent CD.

We observed that the levels of IL-17A-, IL-21, and IL-22-producing CD45RO+CD4+ T cells were higher in CD patients than in HC, which is in agreement with previous reports.6,9,11,12 Besides, we found a 2–3 fold long-term rise in the levels of IL-17A- and IL-21-producing CD45RO+CD4+ T cells and a minor increase in the levels of IL-22-producing CD45RO+CD4+ T cells in quiescent CD compared with active CD. To our knowledge, the levels of circulating IL-21- and IL-22-producing CD45RO+CD4+ T cells in CD have not previously been investigated. The baseline levels of IL-21- and IL-22-producing CD45RO+CD4+ T cells were inversely correlated with the degree of mucosal inflammation as estimated by fecal calprotectin levels. Taken together, our findings support the hypothesis that IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells are involved in CD inflammation. However, their supposed role as drivers of inflammation is brought into question by individual patient levels of IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells being higher in quiescent than in active disease. Instead of maintaining inflammation, IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells may act to restore mucosal homeostasis during chronic inflammation. In this context, the observed increase in the levels of IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells in quiescent CD may represent a compensatory phenomenon.

The exacerbation in a subgroup of CD patients with overt inflammation observed in a clinical trial with the anti-IL17A antibody secukinumab supports the concept that Th17 cells may not play an unequivocally pro-inflammatory role in CD but may also act to counterbalance the inflammation as hypothesized by others.19,29 Furthermore, the finding of an inverse correlation between the levels of IL17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells and the surrogate marker of mucosal inflammation fecal calprotectin is in accordance with a compensatory role of the cytokine producing cells. Such counterbalancing effects of Th17 cells could result from their promotion of epithelial cells

**Figure 1** Levels of cytokine-producing CD45RO+CD4+ T cells in active Crohn's disease (CD) and healthy controls (HC). The flow cytometric analysis revealed statistically significantly increased levels of IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells in active CD compared with healthy controls (HC). The bars indicate the median levels for each group.

**Figure 2** Disease activity during adalimumab treatment. Disease activity was estimated by the Crohn's disease activity index (CDAI). Black triangles represent the patients who demonstrated a sustained clinical response. Open circles represent the overall non-responders who were excluded within the 8 first weeks of the study. A Wilcoxon sign-rank test was applied to compare CDAIs before and during treatment.

Th17 cells and Th17-related cytokines in active and quiescent CD.

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production of antimicrobial peptides and epithelial healing. In addition, all patients presenting with a baseline level of IL-22-producing CD45RO+CD4+ T cells above the median experienced a sustained clinical response to adalimumab. This suggests that the levels of IL-22-producing CD45RO+CD4+ T cells may serve as a predictor of the response to adalimumab. However, due to the limited number of patients in our study, this hypothesis must be confirmed by larger studies.

Our observation that the levels of circulating IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells are increased in quiescent versus active CD may indicate an influence of anti-TNF-α treatment on the homing pattern of these T cells. In support of an altered homing capability owing to adalimumab treatment, anti-TNF-α treatment was reported to reduce the mucosal expression of the chemokine CCL-20, which is the ligand for CCR6 expressed by Th17 cells and of importance for their homing. In rheumatoid arthritis, the levels of IL-17-producing T cells also increased during long-term adalimumab treatment. The authors speculated that this effect reflects a decreased homing of Th17 cells because the emerging Th17 cells had reduced CCR6 expression.

Figure 3  Levels of IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells in quiescent versus active Crohn’s disease (CD). A: Flow cytometric analysis of IL-17A- and IL-21-producing CD45RO+CD4+ T cells. Data from one representative experiment are shown. Cytokine production was evaluated in CD45RO+CD4+ T cells from patients with active (left) and quiescent CD (middle). The gates were set based on isotype controls (right panel) to define cytokine positive events. A similar gating strategy was used for IL-22 (not shown). B: The individual changes in the levels of IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells during adalimumab treatment (baseline (0), week 1 (w1) and week 26 (w26)) in patients who achieved a sustained clinical response are shown (triangles). A Wilcoxon sign-rank test was applied to compare the levels of cytokine-producing cells in active and quiescent disease. Levels in healthy controls (HC) are also shown (circles).
Our observations are limited to circulating IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells. We are therefore unable to ascertain to what extent the findings reflect mucosal changes. One would expect that changes in the levels of cytokine-producing CD45RO+CD4+ T cells that result from alterations in the homing pattern are present before or at the time when patients experience symptom amelioration. However, we did not observe an early (one-week) change in the levels of the IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells.

Our finding that the IL-17A-producing CD4+ T cells were mainly in the CD45RO+ population is in agreement with previous studies.11,12,33 We observed only a low degree of concomitant production of IL-21 or IL-22 in IL-17A-producing CD45RO+CD4+ T cells. Although there was only a limited co-expression of IL-17A, IL-21, and IL-22, the levels of IL-17A-, IL-21-, and IL-22-producing CD45RO+CD4+ T cells were correlated both in active and quiescent CD, emphasizing that the expression of these cytokines by CD45RO+CD4+ T cells is closely interconnected. A recent study reported that the Th17 cell response in CD is regulated by IL-21 in vitro.7 Our finding of a correlation between IL-17A- and IL-21-producing CD45RO+CD4+ T cells may reflect a regulatory role of IL-21 on the Th17 cell response.

We did observe some deviation in the levels of IL-17A, IL-21 and IL-22-producing CD45RO+CD4+ T cells in the CD patients. This could reflect the known heterogeneity among CD patients. Our study is too small to clarify whether high levels of IL-17A-, IL-21- and IL-22-producing CD45RO+CD4+ T cells are associated with factors such as disease phenotype, disease localization, medical treatment and smoking habits. The levels of IL-22-producing CD45RO+CD4+ T cells correlated with the disease duration. The significance of this finding is unknown but it lends support to the known heterogeneity of CD.

In conclusion, we observed increased levels of IL-17A-producing Th17 cells and IL-21- and IL-22-producing CD45RO+CD4+ T cells in CD. Because patients showed the highest levels in quiescent disease, we question a uniform role of these cells as promoters of inflammation. Instead, these cells may play a compensatory role to counterbalance inflammation and restore gut homeostasis.

**Conflict of interest**

The study was financially supported by an unrestricted grant from Abbott Pharmaceuticals and was also supported by the Danish Colitis-Crohn Foundation, the Beckett Foundation, the Karen Elise Jensen Foundation, and the Desiree and Niels Ydes Foundation. Study sponsors had no involvement in study design, in the collection, analysis and interpretation of data; in the writing of the manuscript or the decision to submit the manuscript for publication.

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AD, carried out the study. AD, SS, and TKR carried out the sample and data analyses. AD performed the statistical analysis and wrote the manuscript. CH, JK, JFD and JA participated in the study design. CH, JK, and BD participated in sample and data analyses. All authors helped draft the manuscript. All authors read and approved the final manuscript.
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