Immunological Differences between Lymphocytic and Collagenous Colitis

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Abstract

Background: Lymphocytic (LC) and collagenous (CC) colitis are the two major forms of microscopic colitis (MC). The aim of this study was to identify similarities and differences in their mucosal immune characteristics.

Methods: Colonic biopsies from 15 CC, 8 LC, and 10 healthy controls were collected. Mucosal lymphocytes were assessed by flow cytometry. Tissue gene expression and protein levels were determined by real-time PCR and ELISA, respectively.

Results: LC patients had lower numbers of CD4\textsuperscript{+} and double-positive CD4\textsuperscript{+}CD8\textsuperscript{+} mucosal T lymphocytes, and higher numbers of CD8\textsuperscript{+} and CD4\textsuperscript{+}TCR\textgammadelta\textsuperscript{+} mucosal T cells, compared with controls and CC patients. Regulatory Treg (CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+}) and double-negative (CD3\textsuperscript{-}CD4\textsuperscript{-}CD8\textsuperscript{-}) T cell percentages were higher in both CC and LC compared with controls, coupled with higher levels of the anti-inflammatory IL-10, both at mRNA and protein levels. By contrast, Th1 and Th17 cells were lower in both CC and LC, although gene expression of Th1/Th17 cytokines was higher in both.

Conclusion: CC and LC share some regulatory and effector mechanisms, but not others. Higher IL-10 levels and higher Treg and double-negative T cell percentages, found in both CC and LC, could be responsible for the lack of progression of structural damage and the blockade of proinflammatory cytokine production. However, CC and LC are revealed as separate, independent entities, as they show clearly different mucosal lymphocyte profiles, which could be caused by different luminal triggers of the two diseases. Hence, CC and LC are two closely related but independent intestinal disorders.

Key Words: Collagenous colitis; lymphocytic colitis; Th1/Th17; IL-10; double-negative T cells

1. Introduction

Microscopic colitis (MC), a common cause of non-bloody watery diarrhoea, is comprised of two main forms: collagenous colitis (CC) and lymphocytic colitis (LC). Although very little is known about their aetiology and pathogenesis, current evidence suggests that luminal agents may induce the development of CC.\textsuperscript{1} Clinical and histological improvement after faecal stream diversion supports this hypothesis.\textsuperscript{2}

It is still a matter of debate whether CC and LC are independent entities or rather two subtypes of the same disease.\textsuperscript{3,4} Cytokine
disturbances, such as increases in IFN-γ, IL-17-A, IL-21, IL-22, IL-6, and IL-15, are described in both CC and LC, without significant differences between them. Increase in T regulatory lymphocytes (Treg) is also observed in both MC subtypes, although their numbers are slightly higher in LC. However, histological differences between CC and LC are evident, and extracellular matrix remodelling elements are differentially expressed, suggesting that the two entities are similar in some immunological pathways but different in others.

Therefore, the aim of the study was to characterize the mucosal immune profile of both CC and LC. To that end, we analysed their colonic lymphocytic profiles (effector and regulatory T cells) by flow cytometry, and their related cytokine patterns by real-time PCR and enzyme-linked immunoabsorbent assay in CC, LC, and healthy control (HC) biopsies. Our results revealed differences in the cytokine milieu and in the intestinal lymphocyte profile in these patients, suggesting that the two entities are mediated by different immunological mechanisms.

2. Material and Methods

2.1. Patients

We enrolled 27 clinically active (≥3 watery stools per day) consecutive MC patients who were not receiving any medication for MC at the time of colonoscopy. Fifteen patients had CC and 8 had LC. After informed consent, colonic biopsies were obtained from a new sigmoidoscopy. Four patients were excluded from the study as they presented normal histology at the time of sample-taking, despite a recent previous diagnosis of MC (initially, 3 CC and 1 LC). As a control group, 10 healthy subjects who underwent colonoscopy for colorectal cancer screening were included. Healthy subjects had no endoscopic or microscopic abnormalities. Demographic features and clinical characteristics of patients and controls are shown in Table 1. All included patients and controls had negative celiac serology (anti-tissue transglutaminase IgA antibodies).

Colonic biopsy samples were obtained from the sigma-descendent colon using large-capacity biopsy forceps (Radial Jaw 4, Boston Scientific, USA). All patients and controls provided written, informed consent. This study was approved by the ethical committee of Hospital Universitari Mútua de Terrassa.

2.2. Microscopic colitis diagnosis

The diagnosis of both CC and LC was based on both clinical and histopathological criteria, as described elsewhere. The clinical criteria were chronic watery diarrhoea of at least 1 month duration and a grossly normal appearance of colonic mucosa on colonoscopy. The histological criteria were (i) increased chronic inflammatory infiltrate (plasma cells and lymphocytes) in the lamina propria, (ii) increased number of intraepithelial lymphocytes (IELs) (normal ≤7 IELs per 100 epithelial cells), and (iii) damage to surface epithelium, with flattening of epithelial cells and/or epithelial loss and detachment, and minimal crypt architecture distortion. Histological diagnosis of CC required the additional presence of an abnormal surface subepithelial collagen layer with a thickness ≥10 μm, which entraps superficial capillaries and has an irregular lacy appearance of its lower edge. More than 20 IELs per 100 epithelial cells in the absence of a thickened subepithelial collagen layer was necessary to diagnose LC.

2.3. Mucosal lymphocyte isolation

Ten colonic biopsies from the sigma-descendent colon were processed with a smooth enzymatic method as previously described. In a previous study, we analysed various methods of separating mucosal epithelium from lamina propria compartments, demonstrating that none of the methods achieves proper separation and that they also induced a significant cell loss. Thus, we used the whole sample biopsy for lymphocyte isolation purposes. Briefly, biopsies were collected in culture media and immediately processed with 1 mM DTT and 1 mM EDTA (25 min, moderate shaking) for mucus clearance, and then cultured overnight on fresh media (Advanced RPMI supplemented with antibiotics and 2% of FBS, all from GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) with low-dose enzymes (0.5 μg/mL of collagenase, hyaluronidase and deoxyribonuclease) at 37°C with moderate shaking. After 16.5 h, culture supernatant, containing a mixture of intraepithelial and lamina propria lymphocytes, was centrifuged and pelleted. Lymphocytes were counted under a haemocytometer chamber, and cell viability was measured by trypan blue exclusion, being always higher than 90%. Cells were resuspended at a final concentration of 1 × 10⁶ lymphocytes/mL. This protocol has previously demonstrated good viability (≥90%) and good performance in flow cytometry experiments.

2.4. Flow cytometry

2.4.1. Membrane staining

Isolated mucosal lymphocytes were stained with fluorochrome-labelled monoclonal antibodies, including anti-TCRγδ-FITC (clone WT31), anti-CD8-PE (clone SK1), anti-TCRγδ-FITC or PE (clone

Table 1. Demographic features and drug use in patients and controls.

<table>
<thead>
<tr>
<th>Variable</th>
<th>CC (n = 15)</th>
<th>LC (n = 8)</th>
<th>HC (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>1/14</td>
<td>4/4</td>
<td>6/4</td>
</tr>
<tr>
<td>Age (years) (mean ± SEM)</td>
<td>53 ± 3</td>
<td>65 ± 4</td>
<td>50 ± 2</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>4 (3–5)</td>
<td>5 (4–12)</td>
<td>-</td>
</tr>
<tr>
<td>(median, IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bowel movements per day</td>
<td>5 (4–6)</td>
<td>6 (3–9)</td>
<td>-</td>
</tr>
<tr>
<td>(median, IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSAID use at diagnosis</td>
<td>4 (2 ibuprofen, 1 indomethacin, 1 naproxen)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Autoimmune diseases</td>
<td>2 (1 rheumatoid arthritis, 1 thyroiditis)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

CC, collagenous colitis; LC, lymphocytic colitis; HC, Healthy controls; NSAID, non-steroidal anti-inflammatory drug; SEM: standard error of mean; IQR: Interquartile interval, percentile 25 – percentile 75. All comparisons between CC and LC were nonsignificant.
11F2), anti-CD3-PerCP (clone SK7), and anti-CD45-APC (clone 2D1) (all from BD Biosciences, NJ, USA), and anti-CD8-APC (clone 3B5) (from Invitrogen, CA, USA). Surface staining was done at room temperature for 15 min. Treg cells were determined using the Anti-Human-FOXP3 Staining Set PE kit (eBioscience, CA, USA), following the manufacturer's instructions. In all flow cytometry staining procedures, 100 000 lymphocytes were used, except for Treg staining, for which double the amount was used.

2.4.2. Intracellular cytokine staining and cell culture
For intracellular cytokine staining, 106 cells/ml were placed into 96-well culture plates with or without 10 ng/mL PMA, 100 ng/mL ionomycin, and 10 ng/mL Brefeldin A (Sigma Aldrich, MO, USA) for 6 h, as described elsewhere.15 The FACS & PERM cell fixation and permeabilization kit (CaltagMedsystems, Buckingham, UK) was used, following the manufacturer's instructions. Intracellular antibodies were anti-human-IFN-γ-PE (clone 4S.B3) and anti-human-IL-17A-PE (clone eBio64, both from e-Bioscience, CA, USA). Appropriate isotype-matched controls from the same suppliers were included in intracellular staining to ensure proper compensation and staining specificity.

2.4.3. Gating strategy
At least 10 000 cells in the lymphocyte forward scatter/side scatter (FSC/SSC) gate per tube were acquired in a 4-colour FACSCalibur flow cytometer (BD Biosciences, NJ, USA) and analysis was carried out using Cell-QuestPRO Software. Results were expressed as percentages of the lineage marker/low SSC gate.

2.5. RNA extraction and relative quantification by real-time PCR
Two colonic biopsies from each patient and control were then prepared, and stored at –80°C until analysis. Biopsies were subsequently homogenized with Qiazol reagent (Qiagen, Hilden, Germany) using the QIAshredder (Qiagen, Hilden, Germany) and Qiazol reagent homogenized tissue. Combined primers and probes were purchased from Integrated DNA Technologies (Iowa, USA): hypoxanthine phosphoribosyltransferase 1 (HPRT) (Hs.PT.58v.46521572), IFN-γ (Hs.PT.53a.26729425), IL-10 (Hs.PT.53a.2807216), IL-15 (Hs.PT.58.21299580), IL-17-A (Hs.PT.53a.2545178), TGF-β (Hs.PT.53a.20231516), IL-21 (Hs.PT.58.22750196), IL-23 (Hs.PT.58.38730006.g), TNF-α (Hs.PT.53a.21488839), and FOXP3 (Hs.PT.53a.4225152). Reactions were performed in a 7300 PCR system (Applied Biosystems, CA, USA) and the Premix Ex Taq (TAKARA, Japan). PCR conditions were set as recommended by the manufacturers. All reactions were performed in triplicate. Relative values (expressed as fold change relative to control group) were calculated using the 2−ΔΔCt method.11 Every gene of interest was normalized against an endogenous control (reference gene, HPRT), and values were related to the control group mean.

2.6. Protein level measurement (ELISA)
Two colonic biopsies were snap-frozen in liquid nitrogen and stored at –80°C until use. Tissue homogenates were prepared with a GentleMACS tissue dissociator (protein program, in C tubes, MiltenyiBiotec, Gladbach, Germany), as described elsewhere.16 Biopsies were weighed (ranging 20 to 60 mg) before disruption. In order to use the same concentration of starting material in all experiments, the amount of T-Per reagent (Thermo Fisher Scientific, Waltham, MA, USA) to be added was calculated proportionally for each sample (1000 μL of T-Per for every 25 mg of tissue). Protease inhibitor cocktail (2 mM phenylmethanesulfonylfluoride, 40 μM bestatin, hydrochloride, 15 μM pepstatin A, 14 μM E-64, and 20 μM leupeptin diluted in a 1 mM EDTA solution, Sigma Aldrich, St Louis, MO, USA) was added before freezing. Homogenates were kept at –80°C until further analysis. Culture supernatants were obtained from two biopsies. After collection, a smooth mucus removal treatment (1 mM DTT/1 mM EDTA in HBSS) was done for 25 min and then biopsies were put on culture at 37°C in a 5% CO2 incubator with complete Advanced RPMI media (2% FBS). After 24 h supernatants were collected, centrifuged, and stored at –80°C until use. Human IL-17-A and IFN-γ ELISA kits were purchased from Thermo Fisher Scientific (Waltham, MA, USA), Human IL-15 and IL-10 ELISA kits were purchased from Millipore (Billerica, Massachusetts, USA), and TNF and TGF ELISA kits were from Sigma-Aldrich (St Louis, MO, USA). All assays were performed in duplicate following the manufacturers’ instructions, and results expressed as ng/mL (that is, ng/25 mg homogenated tissue).

2.7. Statistics
Number of cells recovered is expressed as mean ± SD, whereas flow cytometry, qPCR, and ELISA results are expressed as median (interquartile range). Nonparametric tests were used for comparison between groups (Kruskal-Wallis and Mann-Whitney tests). Pearson correlation analysis was performed to correlate the degree of diarrhea and all the parameters analyzed (cells, mRNA, and protein levels). Statistical analysis was performed using SPSS software (IBM, NY, USA). Statistical significance was assumed for p values <0.05.

3. Results
3.1. Absolute cell number
The total number of cells recovered from 10 biopsies was significantly higher in CC and LC compared with HCs (CC, 1.52 × 106 ± 0.98 × 106; LC, 1.6 × 106 ± 0.56 × 106; HCs, 0.58 × 106 ± 0.28 × 106; p < 0.001). No differences were found between CC and LC (p = 0.506).

3.2. Mucosal lymphocyte immunophenotype
Mucosal lymphocytes were characterized by flow cytometry. Gating strategy is shown in Figure 1. Main lymphocyte subsets (CD3+, CD3+CD4+, and CD3+CD8+) and their T cell receptor (TCR) expression (TCRβ) or TCRγδ) are shown in Figure 2. Total CD3+ and CD3+CD8+ cells were higher in LC patients compared with HCs and CC (p = 0.001 and p = 0.022, respectively), while CD3+CD4+ were lower in LC patients compared with HCs (p = 0.002). No differences were found in TCR (TCRβ) or TCRγδ expression in CD3+ and CD3+CD8+ T lymphocytes between the three groups.

However, we detected a significant increase in TCRγδ+ percent among the CD4+ subset in LC (p = 0.047), in which total CD4+ cells were reduced. Considering that TCRγδ+ cells are of crucial importance in coeliac disease pathogenesis12 and that some studies suggest that coeliac disease may be associated with MC,13,14 we then determined IL-15 levels, a critical cytokine in coeliac disease pathogenesis,20 for both mRNA and protein expression. IL-15 mRNA expression was slightly higher in LC (2.6-fold, p = 0.002), but not in CC (1.4-fold, p = 0.09) when compared with controls, in accordance with the TCRγδ+ pattern found in LC. However, no significant differences in IL-15 protein levels were noted among the three groups (Figure 3). IL-15 induces an increase in intraepithelial...
TCRγδ+ lymphocytes in cultured duodenal biopsies of coeliac disease.\(^\text{21}\) Thus, our results suggest that a similar mechanism may be acting in LC colonic mucosa.

3.3. Unconventional T cells
Double-positive (DP) (CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>) and double-negative (DN) (CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup>) T cells are two rare subsets whose role in intestinal homeostasis is poorly known. DP T cells were lower in LC compared with CC and HCs (Figure 4B, \(p = 0.047\)), while DN T cells were higher in both LC and CC compared with HCs (Figure 4C, \(p = 0.038\)).

3.4. Regulatory T cell profile
Both CC and LC patients had higher tissue FOXP3 mRNA expression compared with HCs (Figure 5B, \(p < 0.001\)); this was higher in CC patients compared with LC patients (\(p = 0.001\)). To further confirm these results, we also determined the presence of mucosal CD25<sup>+</sup>FOXP3<sup>+</sup> Treg by flow cytometry (Figure 5A), revealing a higher proportion of mucosal Treg cells in both CC and LC patients compared with controls (Figure 5C, \(p = 0.001\)). CD4<sup>+</sup> T cells expressing intracellular FOXP3 but lacking CD25 expression in the membrane were also higher in both CC and LC compared with HCs (Figure 5E, \(p = 0.041\)). Overall intracellular expression of FOXP3 (CD25<sup>+</sup> plus CD25<sup>-</sup>) in mucosal CD4<sup>+</sup> T lymphocytes was higher in both CC and LC compared with controls (Figure 5D, \(p = 0.004\)). However, unlike the gene expression results, no statistical difference was found between CC and LC patients when studied by flow cytometry.

Since an increase in Treg cells and mRNA FOXP3 was found in both CC and LC, and given that Treg cells can elicit their immunosuppressive function via IL-10, we next determined mucosal IL-10 production in these patients. The levels of anti-inflammatory IL-10...
mRNA were slightly higher in LC (2-fold, $p = 0.050$) and CC (1.5-fold, $p = 0.008$) compared with HCs (Figure 6A). Protein levels of IL-10 were also consistently higher in both CC ($p = 0.002$) and LC ($p < 0.001$) compared with controls (Figure 6B). These results were further confirmed by measuring the amount of soluble IL-10 secreted by biopsies after 24 h of in vitro culture. Levels of IL-10 released into the culture media were significantly higher in both CC and LC compared with HCs ($p = 0.000$, Figure 6C), confirming that IL-10 synthesis and secretion are clearly activated in both LC and CC (Figure 6).
We also determined the mRNA and protein levels of the anti-inflammatory TGF-β, which is another anti-inflammatory Treg cell-derived cytokine, and we found no differences between LC, CC, and HCs (data not shown), suggesting that Treg cells in MC do not elicit their function by TGF-β production.

3.5. Th1 and Th17 lymphocytes

Microscopic colitis is considered to present a Th1 and Th17 pattern. This assumption is based on gene expression studies that demonstrate overexpression of Th1- and Th17-related cytokines. However, to the best of our knowledge the expansion of IFN-γ and IL-17-A producing T cells in MC mucosa has never been assessed. In order to detect Th1 and Th17 cells, intracellular cytokine production in PMA/Ionomycin-stimulated mucosal lymphocytes culture was assessed with flow cytometry (Figure 7). Contrary to expectations, we found that in both CC and LC, IFN-γ and IL-17-A-producing cells were lower in comparison with healthy controls, in both the stimulated and the unstimulated culture (Figure 7). This phenomenon was observed consistently in total T cells (CD3+), including Th1/Th1 and Th17/Tc17 (Figure 7A) and also in the CD3+CD4+ (Th1/Th17 T cells) fraction (Figure 7B).

Given this apparent contradiction with previous literature, we decided to analyse conventional Th1 and Th17 cytokines at the gene expression (mRNA) and total protein load level. IFN-γ and TNF-α are two pleiotropic cytokines that act as potent immune activators. IFN-γ, considered the main Th1 cytokine, was markedly upregulated in both conditions, LC (49-fold, \( p < 0.001 \)) and CC (13-fold, \( p = 0.000 \)), when compared with HCs. IFN-γ gene expression was significantly higher in LC compared with CC (\( p = 0.001 \)). Protein levels act in the opposite manner, with a significant decrease in CC (\( p = 0.013 \)) and no changes in LC (\( p = 0.798 \)) compared with HCs.

TNF-α is primarily expressed by macrophages, but also by other immune cells, including activated Th1 lymphocytes. TNF-α mRNA levels were increased in both LC (2-fold, \( p = 0.021 \)) and CC (2.5-fold, \( p = 0.005 \)) compared with HCs, although protein levels were equal in the three groups (Figure 8B).

Regarding the Th17 cytokine pattern, we determined the levels of the two main cytokines secreted by Th17 cells: IL-21 and IL-17-A. IL-17-A mRNA was upregulated in both LC (4.8-fold, \( p = 0.001 \)) and CC (2.8-fold, \( p = 0.000 \)) compared with HCs. However, no significant differences in IL-17-A protein levels were noted between the three groups (Figure 8C). IL-21 mRNA levels were increased in both LC (7-fold, \( p < 0.001 \)) and CC (6-fold, \( p < 0.001 \)), compared with HCs (Figure 8D). Interestingly, in none of the Th17 parameters evaluated (gene expression, protein levels, and cells) were differences noted between CC and LC. Taking together gene expression and flow cytometry results, we then assessed gene expression levels of IL-23, a cytokine that is essential for Th17 cell differentiation and expansion, and found that IL-23 mRNA levels were increased in both LC (3-fold, \( p = 0.040 \)) and CC (4-fold, \( p = 0.002 \)) compared with controls (Figure 8E).

Therefore, we demonstrated that despite a clear increase in Th1/Th17-related cytokine gene expression, neither protein levels nor their producing T cells are upregulated in MC mucosa. Hence, it may be concluded that MC, unlike IBD disorders, does not present a Th1/Th17-specific pattern.

3.6. Relation with clinical activity

Finally, we investigated possible correlation between any of the mucosal parameters we assessed and clinical activity of the disease.
There were significant moderate positive correlations between the number of daily stools and mRNA levels of IL-15 ($r = 0.438$, $p = 0.037$), IL-17 ($r = 0.480$, $p = 0.024$), and IFN-γ ($r = 0.626$, $p = 0.001$), as previously shown. By contrast, there were no significant correlations with the other measured parameters (cell and protein levels).

4. Discussion

This is the first time, to our knowledge, in which mucosal T-cells and the associated cytokine milieu have been assessed simultaneously in the same cohort of MC patients. Some differences between LC and CC were found, including a higher proportion of CD4+TCRγδ+ and CD8+, and a lower proportion of CD4+ and DP T cells in LC patients, as previously reported.1,2,3 These changes were coupled with differences in the cytokine milieu, as revealed by higher levels of IL-15, and IFN-γ mRNA in LC that are not seen in CC. In spite of these differences between CC and LC, a number of similarities in immune response in the two forms of MC were found, such as an increase in regulatory and decrease in Th1/Th17 effector T cells. Together, these results suggest that LC and CC are different but related entities sharing some regulatory and effector mechanisms.

Tables 2 and 3 summarize the results for the mucosal lymphocyte and cytokine profile in both CC and LC.
A dramatic increase in cytotoxic T lymphocytes (CD3+CD8+) was found only in LC, confirming previous studies. Further mechanistic studies are needed to elucidate their role in the LC mucosa and to determine whether their functionality is comparable between LC and CC.

We demonstrate for the first time an increment of CD4+ TCRγδ+ cells in LC patients compared with CC and HC participants. The function of intestinal TCRγδ+ lymphocytes has been a matter of intense debate, with some evidence suggesting an immunoregulatory role, as pointed in coeliac disease patients. Indeed, IL-15, a cytokine overexpressed in coeliac disease and related to innate immunity, was higher in LC and remained equal to controls in CC. In contrast, a previous study revealed increased IL-15 mRNA in MC, without differences between CC and LC. Differences in patient selection, including patients in clinical remission and patients under specific treatment in the later study, might explain the differences. Together, the higher levels of mucosal IL-15 mRNA and CD4+ TCRγδ+ T-cells found in the colon of LC patients might suggest the presence of a similar immunological fingerprint to that of coeliac disease, different from that displayed by CC patients.

The decrease in DP T cells observed only in LC patients is in contrast with previous studies showing an increase in this cell type only in CC. It has also been reported by that same group that these DP T cells are increased in post-dysenteric diarrhea-predominant IBS patients. By contrast, a study performed by our group demonstrated that DP T cells have a location-specific pattern in healthy subjects (increased in the ileum compared with the colon) that is maintained even in the presence of IBD. It has been suggested that these cells may play a homeostatic role, being polyvalent in order to develop different phenotypes under different circumstances. In fact, DP T cells have been proposed as effector cells with a memory phenotype capable of producing different and/or higher levels of cytokines in comparison with single positive T cells. At any rate, there is no clear explanation for the differences observed in MC patients, and further studies are required to elucidate this question, since DP cell function is still not clearly understood.

DN T cells were increased in both active CC and LC patients in comparison with HCs, without differences between them. The function and specific phenotype of DN T cells in intestinal mucosa is currently unknown and requires extensive research. However, as DN T cells have demonstrated a potent anti-inflammatory role in other diseases, it may be speculated that DN T cells have a protective anti-inflammatory role in the intestinal mucosa and might protect MC mucosa from undergoing uncontrolled inflammation.

Regulatory T cells (CD4+CD25+FOXP3+) were similarly increased in both CC and LC, and a consistent increase in Foxp3 gene expression was found in both MC subtypes. Similar results were previously obtained using immunohistochemical techniques in fixed tissue. Non-Treg CD4+CD25+FOXP3+ T cells were also increased in both CC and LC patients. This T cell subtype has been found to be increased in some autoimmune conditions and also seems to have a regulatory profile. Regulatory functional cells with FOXP3+ expression and an immunosuppressive role have been described in IBD. Notwithstanding, functional studies demonstrating the immunosuppressive capacity of FOXP3+ cells in MC colonic mucosa are required. However, it is well established that Treg cells exert their immunosuppressive function in...
part through production of IL-10, the most important immunoregulatory cytokine in the gut. In this regard, in the present study IL-10 was increased in both CC and LC, showing a good correlation between mRNA and protein levels, in both tissue and culture supernatant.

Several studies have investigated cytokine pattern in MC by assessing gene expression levels by real-time PCR. However, these studies are incomplete since a strikingly low correlation between mRNA and protein levels has been described in many scenarios. To our knowledge there is only one previous study in which both colonic gene and protein expression were determined. In our study, despite having found a strong and clear increment in proinflammatory cytokine mRNA levels (IFN-γ, IL-17-A, TNF-α), protein levels were equal or even reduced in MC patients as compared with HCs. This finding is similar to previous studies, in which MC patients demonstrated a mixed Tc1/Th1 and Tc17/Th17 mucosal cytokine profile. We have expanded this observation and found reduced intracellular expression of IFN-γ and IL-17-A in T cells, clearly showing that there is no such response in MC patients. In fact, IL17 and IFN-γ are produced by many other cell types, such as the newly described innate lymphoid cells, which may explain in part the lack of concordance. Hence, assumptions of cellular behaviour based solely on gene expression need to be carefully re-evaluated.

At any rate, there was an increased pro-inflammatory cytokine gene expression, whereas protein levels remained unchanged. It has been suggested that protein levels cannot be detected because of rapid degradation and consumption by cells. However, another possible explanation is that there was suppression at a post-transcriptional level of gene expression, induced either by regulatory cells such as DN T cells or by Treg cells, or by microRNA regulation. This last explanation would be in concordance with the

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**Figure 7.** Box plots for IFN-γ and IL-17-A producing cells over CD3+ cells (A) and CD3+CD4+ cells (B). CC: collagenous colitis (n = 14); LC: lymphocytic colitis (n = 8); HCs: healthy controls (n = 10). *p < 0.05 vs HCs.
absence of macroscopic damage in CM. According to this, IL-10, the most important immunoregulatory cytokine in the gut, is the only one of these that showed a good correlation between mRNA and protein levels, in both tissue and culture supernatant, highlighting the existence of an anti-inflammatory milieu that keeps inflammation at minimum levels in MC mucosa.

Figure 8. Box plots for gene expression measured by real-time PCR and protein levels in biopsy homogenates measured by ELISA Th1/Th17-related cytokines. CC: collagenous colitis (n = 15); LC: lymphocytic colitis (n = 7); HCs: healthy controls (n = 8). ¶p < 0.05 vs HCs. §p < 0.05 vs LC.
In conclusion, LC and CC are different but related entities sharing some regulatory and effector mechanisms. Of note, different types of immunoregulatory mechanisms are highly expressed in both CC and LC, which might be important in maintaining inflammation at a microscopic level in these entities.

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**Conflict of Interest**

None declared.

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**Author Contributions**

AC designed and performed all the laboratory experiments, performed statistical analysis, prepared the figures, and drafted the manuscript. FFB, MR, MA, YZ, and ME recruited patients and did biopsy collection. EP designed and contributed to qPCR experiments. AS performed immunohistochemical studies and microscopic diagnosis. FFB conceived and designed the study, revised, and finalized the manuscript, coordinated the research group, and directed the execution of the study. All authors read and approved the final version of the manuscript.

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