DIFFERENTIAL REGULATION OF INTERLEUKIN-17 AND INTERFERON-γ PRODUCTION IN INFLAMMATORY BOWEL DISEASE

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I declare I have personally conducted and written this work under the supervision of Professor Thomas T. MacDonald during my MPhil studies in London between September 2007 and February 2010.

Laura Rovedatti
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Abstract

Background and Aims. Interleukin (IL)-17 is now known to be involved in a number of chronic inflammatory disorders. However, the mechanisms regulating its production in inflammatory bowel disease (IBD) are still unclear. Methods. Endoscopic biopsies or surgical specimens were taken from inflamed and uninflamed colonic mucosa of 72 IBD patients (38 with Crohn’s disease and 34 with ulcerative colitis), and normal colon of 38 control subjects. IL-17 and interferon (IFN)-γ were detected by ELISA in the supernatants of biopsies cultured ex vivo, and anti-CD3/CD28-stimulated lamina propria mononuclear cells (LPMCs) incubated with IL-12, IL-23, IL-1β plus IL-6, transforming growth factor (TGF)-β1, or anti-IL-21 neutralising antibody. Intracellular flow cytometry was performed to analyse mucosal Th17 and Th1/Th17 cells. Results. IL-17 production by organ culture biopsies was higher in IBD inflamed mucosa than IBD uninflamed mucosa and controls, and was equivalent in amount to IFN-γ. Anti-CD3/CD28-stimulated IBD LPMCs produced higher IL-17 amounts compared to controls. The percentages of Th17 and Th1/Th17 cells were increased in IBD patients than controls. IL-23 and IL-1β plus IL-6 had no effect on IBD LPMC production of IL-17, however IL-12 markedly increased IFN-γ production and decreased IL-17 production. TGF-β1 dose-dependently decreased IFN-γ, but had no significant inhibitory effect on IL-17 production. Blocking IL-21 significantly down-regulated IL-17 production. Conclusions. Our findings support a role for IL-12, TGF-β and IL-21 in modulating IL-17/IFN-γ production in IBD. The abundant IL-17 in inflamed IBD mucosa may help explain the relative lack of efficacy of anti-IFN-γ antibodies in clinical trials of Crohn’s disease.
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## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-ASA</td>
<td>5-Aminosalicylic acid</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CARD15</td>
<td>c-Terminal caspase recruitment domain 15</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine (C-C motif) receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CXCR</td>
<td>Chemokine (CXC motif) receptor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GATA</td>
<td>Trans-acting T-cell-specific transcription factor</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genomic wide association studies</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>LPMC</td>
<td>Lamina propria mononuclear cell</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC II</td>
<td>Major histocompatibility complex type II</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inhibitor protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerisation domain containing</td>
</tr>
<tr>
<td>OCTN</td>
<td>Organic cation transporters</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROR</td>
<td>Retinoic acid receptor-related orphan receptor</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box transcription factor expressed in T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell type 1</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNBS</td>
<td>Trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
</tbody>
</table>
Introduction

1. Inflammatory Bowel Disease

1.1 Definition

Crohn’s disease (CD) and ulcerative colitis (UC) represent the two main forms of inflammatory bowel disease (IBD), an idiopathic relapsing chronic disorder characterized by a swinging course. While CD is a transmural chronic inflammation potentially affecting any gastrointestinal tract from mouth to anus, UC is a non-transmural disease affecting the colon with a caudo-cranial extension without patchiness or skip lesions [1]. Table 1 summarizes clinical and pathological features which help differentiating between CD and UC.

Table 1. Clinical and pathological features for differential diagnosis between Crohn’s disease and ulcerative colitis.

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Crohn’s disease</th>
<th>Ulcerative colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal pain</td>
<td>Severe, mainly in right lower quadrant</td>
<td>Cramping, mainly in left lower quadrant</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>Frequent</td>
<td>Frequent</td>
</tr>
<tr>
<td>Hematochezia</td>
<td>In 20-30% of patients, in distal colonic disease</td>
<td>Always in active disease</td>
</tr>
<tr>
<td>Abdominal mass</td>
<td>Right lower quadrant (inflamed or stenotic ileum)</td>
<td>Left lower quadrant in inflamed sigma or slim patients</td>
</tr>
<tr>
<td>Malnutrition</td>
<td>Frequent</td>
<td>Uncommon</td>
</tr>
<tr>
<td>Abdominal distension</td>
<td>Uncommon</td>
<td>In severe disease only</td>
</tr>
<tr>
<td>Obstructive symptoms</td>
<td>Frequent</td>
<td>No</td>
</tr>
<tr>
<td>Perianal disease/fistulas</td>
<td>Up to 30% of patients</td>
<td>No</td>
</tr>
<tr>
<td>p-ANCA</td>
<td>+ (Crohn’s colitis)</td>
<td>++ (+++ in sclerosing)</td>
</tr>
<tr>
<td>Complications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Abscess</td>
<td>More frequent perianal than intra-abdominal</td>
<td>Rare</td>
</tr>
<tr>
<td>Toxic megacolon</td>
<td>No</td>
<td>Rare</td>
</tr>
<tr>
<td>Stenosis</td>
<td>Common</td>
<td>Rare</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>Rare, most frequent in colonic disease</td>
<td>Relative risk related to disease duration, extension and activity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Extraintestinal manifestations</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythema nodosum</td>
<td>Uncommon</td>
<td>Rare</td>
</tr>
<tr>
<td>Pyoderma gangrenosum</td>
<td>Very rare</td>
<td>Rare</td>
</tr>
<tr>
<td>Sclerosing cholangitis</td>
<td>Rare</td>
<td>Uncommon (5-15%)</td>
</tr>
<tr>
<td>Arthralgia/arthritis</td>
<td>Very frequent</td>
<td>Frequent</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Endoscopic aspects</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution</td>
<td>Any segment of the gastrointestinal tract</td>
<td>Continuous from the rectum</td>
</tr>
<tr>
<td>Ileal involvement</td>
<td>Frequent</td>
<td>Rare (\text{backwash ileitis})</td>
</tr>
<tr>
<td>Rectal involvement</td>
<td>30-50%</td>
<td>Almost always</td>
</tr>
<tr>
<td>Continuous disease</td>
<td>Not frequent</td>
<td>Always</td>
</tr>
<tr>
<td>Linear and serpinguous ulcers</td>
<td>Frequent</td>
<td>No</td>
</tr>
<tr>
<td>Ileal cobblestone appearance</td>
<td>Frequent</td>
<td>No</td>
</tr>
<tr>
<td>Skip lesions</td>
<td>Frequent</td>
<td>No</td>
</tr>
<tr>
<td>Stenosis</td>
<td>Uncommon</td>
<td>Rare, always suspicious for carcinoma</td>
</tr>
<tr>
<td>Mucosal edema</td>
<td>Uncommon</td>
<td>Frequent</td>
</tr>
<tr>
<td>Ulcers</td>
<td>Deep</td>
<td>Often superficial and extensive</td>
</tr>
</tbody>
</table>
### Histological features

<table>
<thead>
<tr>
<th></th>
<th>Rare</th>
<th>Frequent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circumferential inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goblet cell mucin depletion</td>
<td>No</td>
<td>Frequent</td>
</tr>
<tr>
<td>Crypt architectural distortion</td>
<td>No</td>
<td>Frequent</td>
</tr>
<tr>
<td>Crypt abscesses</td>
<td>Rare</td>
<td>Frequent</td>
</tr>
<tr>
<td>Granulomas</td>
<td>Uncommon</td>
<td>No</td>
</tr>
<tr>
<td>Submucosal inflammation</td>
<td>Very frequent</td>
<td>Rare</td>
</tr>
</tbody>
</table>

### Radiological aspects

<table>
<thead>
<tr>
<th></th>
<th>Extensive</th>
<th>Moderate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal wall thickening</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesenteric adenomegalies</td>
<td>Frequent</td>
<td>Not frequent</td>
</tr>
<tr>
<td>Mesenteric fat wrapping</td>
<td>Frequent</td>
<td>No</td>
</tr>
</tbody>
</table>

ASCA, Anti-saccharomyces cerevisiae antibodies; p-ANCA, Anti-neutrophil cytoplasmatic antibodies (perinuclear pattern); +/-, association variably seen; +, associated; ++, frequently associated; ++++, strongly associated.

1.2 Epidemiology

The frequency of IBD is much higher in Northern Europe, United Kingdom, and North America, where incidence rates begin now to stabilize after a progressive increase in the last 50 years (5-6 cases per 100,000 persons/year with a prevalence of 27-106/100,000 for CD, and 6-15 cases per 100,000 persons/year with a prevalence of 80-150/100,000 for UC). On the contrary, incidence and prevalence rates are continuously increasing in low-incidence regions, such as Southern Europe, Asia and developing countries [2,3]. The risk of IBD is related to ethnic and racial factors, as shown by the higher prevalence of CD and UC in North American Ashkenazi Jews [4-6]. However, relevant epidemiological differences between Jewish people living in Israel and
those living elsewhere suggest that environmental factors and lifestyle must be involved [7,8]. CD is slightly more frequent in females (M:F = 1:1.2) and occurs earlier (mean age 26) than UC (M:F = 1.2:1; mean age 34). Another pick of incidence is between 60s and 80s, more frequently for CD than UC [9,10].

1.3 Clinical Features of Inflammatory Bowel Disease

**Crohn’s disease**

Typical features of CD are the common localization in the terminal ileum, the discontinuous involvement of the gastrointestinal tract, and the development of complications such as abscesses, strictures and fistulas. In CD, inflammation involves all layers of gut wall (mucosa, submucosa, muscular layer and serosa) and tends to extend to mesenteric fat and lymph nodes. In early stages lesions typically include cryptic abscess and aphthous ulcers. The chronicization of inflammation leads to the onset of non-caseating granulomas which, although representing a pathognomonic feature of CD, are present in less than 50% of endoscopic biopsies [11] and in 70% of surgical specimens [12], since they localize more often in the submucosa than in the mucosa. Other common features are lymphoid aggregates in the submucosa, abundant lympho-monocytic infiltrate in the lamina propria and mucosal fissurations, which eventually become real penetrating fistulas through the gut wall. These microscopic alterations correspond to a series of macroscopic lesions such as aphthous ulcers which converge and surround areas of unaffected mucosa giving the gut mucosa the typical “cobblestone” appearance. Possible complications of CD are (i) intra-abdominal or intraparietal perianal abscesses, (ii) fistulas, linking gut with another intestinal tract, a contiguous organ (e.g. bladder, ureter, vagina) or the skin, especially at site of surgical scars, in the periumbilical or peri-anal areas, and (iii) strictures, determined by the progressive fibrosis of the intestinal wall.

Patients affected by CD present with variable symptoms depending on site, phenotype and severity of inflammation and the presence of
complications. Typical symptoms are diarrhea, abdominal pain, much more frequent and persistent in comparison to UC, and weight loss. Additional symptoms are asthenia, anorexia, nausea, vomiting and fever, while extraintestinal manifestations (affecting joints, skin, eye, ecc.) are present in at least 1/4 of patients with CD. According to the Vienna classification [13], CD may be classified in three main clinical phenotypes: fistulising (or penetrating), stricturing, and non-penetrating/non-stricturing (or luminal). Even though disease location is usually stable, the behavior tends to change over time [14]. At diagnosis 70% of patients have a luminal disease, but after 25 years of follow-up very few patients have an uncomplicated disease, and virtually all of them have developed either stricturing or penetrating disease. Moreover, after 20 years of disease more than 2/3 of patients have undergone surgical resection of intestinal strictures [15]. Unlike UC, life expectancy in CD is slightly reduced [16].

**Ulcerative colitis**

UC is a relapsing mucosal inflammatory bowel disease affecting the colon with a caudo-cranial extension. Macroscopically, the colon is freckled with superficial ulcers, caused by abnormal matrix metalloproteinase activation with subsequent enterocyte apoptosis [17]. This continuous damage-regeneration process that occurs in inflamed areas, leads to the growth of pseudopolyps, which are a common feature in UC. Histologically, UC appears with microscopic abscesses at the base of the crypts of Lieberkühn, crypt architectural distortion and branching, Paneth cell metaplasia, mucin depletion and lamina propria cell infiltration (especially by neutrophils, eosinophils, plasmacells and macrophages).

UC can be divided into proctitis (rectal involvement only), left-sided colitis (involving the sigmoid colon with or without involvement of the descending colon), or pancolitis (global colonic involvement). Patients with pancolitis can also present inflammation of the terminal ileum, called “backwash ileitis”. In the natural history of UC, disease extent tends to change: more than half of patients with left-sided colitis will
progress proximally, while in more than 2/3 of patients with pancolitis the extent of colonic involvement will decrease [18]. Unlike CD, patients with UC have a normal life expectancy [19].

Clinical features are typically bloody diarrhoea, abdominal cramping and rectal tenesmus. Symptoms are less severe in proctitis and left-sided colitis than pancolitis. Extraintestinal manifestations of UC are similar to CD, even if incidence is different. Sclerosing cholangitis, a chronic cholestatic liver disease, characterized by fibrosing inflammatory destruction of the intrahepatic and extrahepatic bile ducts, occurs in about 3% of UC patients [20]. Toxic megacolon and colorectal cancer are the most important complications of UC. Toxic megacolon is a rare and life-threatening complication, characterized by signs and symptoms of peritoneal involvement, occurring in severe and diffuse UC; fortunately its incidence is decreasing, thanks to a better control of the disease by new biological therapies. The prevalence of colorectal cancer in patients with UC is approximately 3.7% overall, and 5.4% for patients with pancolitis. The cumulative probability of colon cancer development is estimated of 2%, 8% and 18% respectively after 10, 20 and 30 years of disease duration [21,22]. Risk factors for colorectal cancer include extent and duration of UC, presence of backwash ileitis, coexisting primary sclerosing cholangitis, a family history of sporadic colorectal cancer and young age at diagnosis [22]. In the last 10 years the prevalence of colorectal cancer in UC patients is decreasing, thanks to surveillance colonoscopy and advancements in treatment, both chemotherapeutic and surgical.

1.4 Diagnosis

The diagnosis of IBD is made on the base of endoscopic, radiologic, histological and blood tests together with the evaluation of clinical conditions and the degree of disease activity [23]. Four steps need to be followed in the diagnosis of IBD: (i) differentiate IBD from other type of colitis and between CD and UC (Table 1); (ii) identify the clinical phenotype in case of CD or evaluate the extent of disease in case of UC,
since therapies can be different; (iii) modulate the therapeutic decision on the base of disease activity; (iv) identify complications that need particular treatments or can be worsen by others.

Ileocolonoscopy with multiple biopsies is fundamental for the diagnosis of IBD. In CD the macroscopic aspect of mucosa is characterized by deep aphthous or longitudinal, polygonal ulcers which alternate with unaffected areas conferring the typical aspect of cobblestone to gut mucosa, especially of terminal ileum. Discontinuity of lesions and spared rectum often make it possible to differentiate CD from UC. Macroscopically, the degree of inflammation can be evaluated on the base of mucosal erythema, bleeding (spontaneous or after the passage of the colonscope), and the presence of ulcers and inflammatory pseudopolyps, the last ones being more common in UC. Microscopically, the presence of granulomas, normality of goblet cells and abundant infiltrate of T lymphocytes and macrophages in the lamina propria, may help making CD diagnosis, while crypt abscesses, crypt architectural distortion with mucin depletion, Paneth cell metaplasia and lamina propria cell infiltrate are typical features of UC.

A standard plain X-ray is useful in patients with severe or fulminant UC to evaluate the degree of intestinal distension, especially in toxic megacolon. Conventional double-contrast barium enema has been largely replaced by colonoscopy, and its role is limited to patients with colonic stenosis. Ultrasound, computed tomography and magnetic resonance imaging (MRI) cannot substitute full colonoscopy, even if their importance in IBD imaging has increased, providing essential information for diagnosis, management, follow-up and detection of potential complications [24]. Radiolabeled leukocyte scan with $^{99m}$Tc and Positron Emission Tomography are reliable and non-invasive techniques in monitoring response to therapy [25,26].

1.5 Therapy

The primary end points of active IBD therapy are: (i) induction of remission; (ii) maintenance of remission; (iii) management of
complications.

*Crohn’s disease*

Therapeutic strategies are different on the base of the site of intestinal lesions (ileo-caecal or colonic disease), the presence of complications (fistula, strictures) and the severity of the disease.

**Induction of remission.** Patients with mild to moderate colonic CD can be treated with budesonide or 5-aminosalicylic acid (5-ASA) orally. Patients who do not respond to mesalazine or budesonide, or with a moderate to severe disease should be treated with systemic corticosteroids. Only 50% of these patients achieve prolonged remission, while the other half requires further strategies. In case of cortico-dependence or -resistance a more aggressive treatment with immunoppressors (azathioprine, 6-mercaptopurine or methotrexate) or biological therapies (monoclonal antibodies) should be considered. The evidence of an association between infliximab, a chimeric murine-human monoclonal antibody, and acute infusion reactions, loss of efficacy, and delayed hypersensitivity reactions [27] has led to the development of less immunogenic, humanized, or fully human antibodies against tumor necrosis factor (TNF)-α, like adalimumab [28]. In USA a third anti-TNF antibody, a pegylated humanized Fab’ fragment called certolizumab pegol, has been approved for CD [29,30]. The involvement of different cytokines in the pathogenesis of CD has led to the development of new selective biological therapies which are currently used in phase II and III trials. Among them etanercept (a fully human dimeric fusion protein) [31], onercept (a recombinant form of the human soluble p55 TNF receptor binding protein) [32], and CDP571 (a humanized IgG1 monoclonal antibody to TNF) [33] have failed to induce and maintain remission in patients with CD. Fontolizumab (a humanized anti-interferon (IFN)-γ antibody) also failed to reach the primary end point in phase II trials but had some efficacy at higher doses [34]. A promising strategy is the blocking of interleukin (IL)-12/23, which acts at the beginning of the Th1 pro-inflammatory cascade. The IL-12/23 p40 subunit is targeted by two human monoclonal antibodies, ABT-874 [35]
and CNTO-1275 ustekinumab [36], both effective in the induction of response in moderate to severe CD in phase II trials. While the blockade of T-cell activation has not shown any efficacy in previous trials [37], ongoing studies are evaluating the safety and efficacy of monoclonal antibodies against IL-6, IL-10, and IL-17, which are involved in the pathogenesis of CD at different levels. Selective adhesion molecule inhibitors, such as natalizumab (a recombinant humanized IgG4 monoclonal antibody to α4-integrin) block adhesion and subsequent leukocyte migration into the gut [38]. Because of the onset of progressive multifocal leukoencephalopathy in patients treated with natalizumab [39], this drug has not been approved for CD in Europe, and a phase III trial with a more selective anti-α4β7-integrin antibody (vedolizumab) has been started.

**Maintenance of remission.** It is achieved mainly with immunosuppressors or with biological therapies; the last ones in particular allow steroid sparing and favour mucosal healing in patients with active CD during treatment with immunosuppressors.

**Management of fistulizing CD.** Antibiotic therapy with ciprofloxacin or metronidazole is the first choice in perianal disease, even though there is no evidence of a sure efficacy of this strategy. As a second-line treatment, azathioprine and 6-mercaptopurine can be considered. Patients with draining fistula, despite treatment with antibiotics and immunosuppressors, can benefit from the association between infliximab or adalimumab (after having excluded the presence of abscesses with an MRI scan) and seton sutures of chronic fistula [40].

**Surgery.** In CD, unlike UC, surgery is not curative. Generally, it is indicated when fibrotic strictures causing an intestinal occlusion or fistula complicated by abdominal abscesses occur. Since recurrences after surgery are quite common, recent studies suggest a possible role for infliximab in combination with azathioprine in preventing them [41].

**Ulcerative colitis**

**Induction of remission.** First-line therapy for patients with mild to moderate UC is 5-ASA, given orally or by topical formulations
depending on lesion distribution [42]. Patients who do not respond to mesalazine [43] should be treated with systemic corticosteroids [44] that still remain the foundation for inducing remission in UC. Patients with severe active UC, without signs of systemic infection, who fail to respond to oral prednisone need intravenous corticosteroids. If intravenous steroid therapy fails, other therapeutic options should be considered, such as immunosuppressors including cyclosporine [45,46] or tacrolimus [46]. Also infliximab has shown a significant benefit in achieving clinical response and remission [47,48]. Open-labeled clinical trials have shown that adalimumab is well-tolerated and appears to be effective in maintaining clinical remission in patients with UC, including those who have previously lost their response to or cannot tolerate infliximab [49,50]. As in CD, vedolizumab showed a higher efficacy than placebo for the induction of clinical and endoscopic remission in patients with active UC [51].

**Maintenance of remission.** First-line therapy for maintenance of remission is oral mesalazine and analogues [52,53]. In addition rectal mesalazine can be used in patients with proctitis or procto-sigmoiditis. Prolonged administration of mesalazine reduces the risk of colorectal cancer, reinducing enterocyte apoptosis [54]. Patients who relapse during maintenance therapy with mesalazine, those who are steroid-dependent, or those who have obtained clinical remission only with cyclosporine or tacrolimus, can be effectively treated with azathioprine and 6-mercaptopurine [52], or infliximab which can be considered a good steroid-sparing drug in maintaining remission [47,48].

**Surgery.** Unlike CD, surgery can be curative in patients with UC. Emergency surgery is indicated in complicated patients (perforation, refractory rectal bleeding, and toxic megacolon not responsive to medical management) [55]. Elective surgery is indicated in patients with dysplasia or frank carcinoma, severe UC refractory to medical management, or when long-term therapies with immunosuppressors or infliximab are contraindicated [56,57].
1.6  Actiopatogenesis

The etiology of IBD is still unknown, but it is clearly the result of a combination of environmental, genetic and immunological factors (Figure 1) [58].

**Figure 1.** Genetic, immunologic and environmental factors involved in the etiopathogenesis of chronic inflammatory bowel diseases. CARD15, caspase activation recruitment domain family-15; CD, Crohn’s disease; NOD-2, nucleotide-binding-oligomerisation domain-2; NSAIDs, non-steroidal anti-inflammatory drugs; OCTN, organic cationic transporter; UC, ulcerative colitis.

**Genetic factors**

*Familiarity.* Genetic studies have shown that in monozygotic twins the concordance rate for CD ranges from 20% to 50%, whereas in dizygotic twins brought up in the same environment is less than 10% [59,60]. The concordance rate of UC is about 16% in monozygotic twins and 4% in dizygotic twins [61]. CD and UC patients have a first-degree relative affected by IBD in 5-22% and 6-16% of cases, respectively [62].

*NOD2/CARD15 gene.* Among the CD susceptibility loci identified by
genome-wide scan, NOD2 (nucleotide-binding-oligomerization domain-2), which is localized on chromosome 16 in the IBD1 locus, has been the most extensively studied [63-67]. It codifies CARD15 (caspase activation recruitment domain family-15), an intracellular protein expressed in the lamina propria by macrophages, neutrophils, dendritic cells, and Paneth cells, which plays a key role in intestinal innate immunity. CARD15 recognizes muramyl dipeptide, a component of both Gram-positive and Gram-negative bacteria, thus inducing the production of pro-inflammatory cytokines which are involved in determining intestinal inflammation. In CD patients NOD2 mutations are associated with an earlier onset of the disease, ileal localization, and presence of extensive strictures and entero-enteric fistulas.

Other genes. Multiple genes are associated with both CD and UC, including IBD5 locus localized on chromosome 5, where genes codify for a family of organic cation transporters, namely OCTN1 and OCTN2 [68], and IBD3 locus on chromosome 6 containing genes which codify for the major histocompatibility complex [66]. In particular, the HLA-DRB*0103 aplotype is associated with a more severe form of UC, while most of the IBD-related extraintestinal manifestations are associated with HLA-B27 or HLA-B35 (arthropaties), or with HLA-B44 or HLA-DRB*0103 (uveitis) [69].

Immunological factors
It is commonly accepted that IBD is a consequence of an inappropriate mucosal immune response to the gut flora (Table 2).

Table 2. Mechanisms of altered innate immune response determining loss of oral tolerance to commensal luminal bacteria in chronic inflammatory bowel diseases.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Cells involved</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced mucin production</td>
<td>Goblet cells</td>
<td>Reduced protective effect of the epithelial glicocalix</td>
</tr>
<tr>
<td>Reduced secretory immunoglobulin A production</td>
<td>Plasmacells</td>
<td>Increased susceptibility to bacterial infections</td>
</tr>
<tr>
<td>Reduced defensin production</td>
<td>Paneth cells expressing altered NOD2 proteins in patients with CARD15 gene mutation</td>
<td>Malfunction of antimicrobial activity of defensins</td>
</tr>
<tr>
<td>Abnormal expression of toll-like receptors</td>
<td>Epithelial cells</td>
<td>Loss of control of epithelial cells-bacteria interactions</td>
</tr>
<tr>
<td>Abnormal activation and maturation of non-tolerogenic dendritic cells</td>
<td>Mucosal dendritic cells (antigen presenting cells with a crucial role in detecting and sampling luminal antigens)</td>
<td>Polarization of T lymphocytes to an inflammatory phenotype (Th1, Th17)</td>
</tr>
</tbody>
</table>

CARD15, caspase activation recruitment domain family-15; NOD2, nucleotide-binding-oligomerisation domain-2; Th, T helper.

Although the exact immunological mechanisms have not been completely clarified, the co-presence of multiple factors seems to be responsible for the activation of an inflammatory cascade which ultimately leads to the intestinal lesions (Figure 2).
Figure 2. Schematic representation of inflammatory bowel disease immunopathogenesis. The disruption of the epithelial barrier, determined by a loosening of intercellular junctions, is responsible for the paracellular transmigration of bacterial antigens (1). Bacteria can also trigger an inflammatory process by direct interaction with epithelial cells through Toll-like receptors (TLR) (15), or because they are captured by dendritic cells which can open tight junctions and extend their pseudopodes across the epithelium (10). Dendritic cells, which have lost their tolerogenic properties, polarize naïve T lymphocytes in T helper cell type (Th)1, via activation of the intracellular transcription factor T-bet, or in Th17, via interleukin (IL)-23 (11), or in Th2 via IL-4 (14). In Crohn’s disease, bacterial antigens are aberrantly presented by macrophages to naïve T cells, which polarize in Th1 cells and start secreting huge amount of pro-inflammatory cytokines. Among them, interferon (IFN)-γ promotes the macrophage production of IL-12, which in turn sustains the activation of T cells (4). A defective lamina propria lymphocyte apoptosis (3) is responsible for the increased survival of T cells. On the contrary, ulcerative colitis is dominated by Th2 cytokines, such as IL-5 and IL-13, mainly produced by natural killer T cells infiltrating the lamina propria. In both Crohn’s disease and ulcerative colitis, IL-17, produced by Th17 lymphocytes, promotes the recruitment of neutrophils into the inflamed gut (13). Also IL-8, secreted by epithelial cells stimulated by IL-17 (12), or for a direct interaction of epithelial TLR (15) with lumen bacteria, promotes the recruitment of neutrophils from the inflamed mucosa. Crohn’s disease and ulcerative colitis share crucial end-stage pathways, characterized by the release of high amounts of tumour necrosis factor (TNF)-α from activated macrophages, which promotes the recruitment of neutrophils into the inflamed gut by the up-regulation of adhesion molecules on the vascular endothelium of mucosal blood vessels, which interact with integrines expressed on T lymphocytes (9). TNF-α is also responsible for the epithelial damage, determining the mucosal disepithelization and ulceration (5), and promotes the fibroblast production of matrix metalloproteinases (MMPs) which are directly responsible for connective tissue degradation and
ulceration. In Crohn’s disease, TNF-α also leads to the formation of granulomas (8), unbalances fibroblastic secretion toward an excess of metalloproteinase tissue inhibitors (TIMP) with collagen deposition, fibrosis and strictures (7), or MMP with the onset of fistulas (6).

Epithelial barrier defect. A genetically-determined defect in intestinal permeability has been reported in IBD patients and in 10-30% of healthy first-degree relatives of CD patients carrying NOD2 mutations [64,70,71]. In particular, a leaking epithelium is the likely factor responsible for the paracellular passage of bacterial antigens, which, once in the lamina propria, are aberrantly presented by macrophages to T cells which become activated. Furthermore, bacteria can trigger an inflammatory process even if they do not pass through the intestinal barrier, either because they are captured by dendritic cells which extend their pseudopodes through the intercellular spaces, or via Toll-like receptors expressed on epithelial cells [72] (Figure 2).

Defect of lymphocyte apoptosis. A defective lamina propria lymphocyte apoptosis due to an imbalance between pro- and anti-apoptotic genes is supposed to be responsible for the increased survival of T cells, which release higher amount of pro-inflammatory cytokines thus perpetuating the gut mucosal damage [73] (Figure 2).

Increased production of cytokines. In CD both dendritic cells, which have lost their tolerogenic properties, and activated macrophages polarize naïve T lymphocytes in T helper cell type (Th)1 cells [72]. The Th1 cytokine IFN-γ promotes the macrophage production of IL-12 which in turn sustains the activation of T cells [74] (Figure 2). On the contrary, UC is supposed to be dominated by Th2 cytokines, such as IL-5 and IL-13 [74,75]. Higher levels of IL-17, a pro-inflammatory cytokine produced by a newly described lymphocyte population, namely Th17, have been found both in CD and UC [76]. IL-17 might contribute to the pathogenesis of IBD by promoting the recruitment of neutrophils into the inflamed gut through the induction of IL-8 secretion by epithelial cells [77]. Nonetheless, CD and UC share crucial end-stage pathways, as shown by the enhanced production in both conditions of IL-21 [78], IL-
23 [79], and TNF-α which perpetuates the mucosal damage [80] (Figure 2).

Environmental factors

Diet. There is no evidence for the role of food in IBD pathogenesis, although it has been hypothesized that an excessive intake of refined sugar or polyunsaturated fats can increase the risk of IBD, in particular CD [81].

Smoke. Cigarette smoke worsens the course of CD, favours fistula and stricture formation, and increases the number of flares and post-surgical relapses. Recently, also passive smoke has been identified as a risk factor for CD [82]. Conversely, in UC smoke seems to be protective, and it correlates to a less frequent flare of the disease.

Infectious agents. Due to its resemblance to John’s disease, a chronic granulomatous gastroenteritis of ruminants, CD has been thought to be associated with infection by Mycobacterium avium subspecies paratuberculosis [83,84]. Although Mycobacterium avium paratuberculosis has been identified in tissue and blood of CD patients [85], anti-tubercular therapy was not effective in this condition [86]. The demonstration that mice kept in sterile conditions do not develop colitis strongly supports the hypothesis that also normal gut flora can be involved in the pathogenesis of IBD. In particular, research has focused on Bacteroides [87] and Escherichia coli [88] which produce adhesion molecules able to promote intestinal epithelial colonization and cytotoxins that are responsible for epithelium disruption and inflammatory responses. There is very poor evidence about the role of viruses and, up to now, there is no evidence that vaccination with live attenuated vaccines of measles, mumps or rubella viruses can cause IBD [89].

Drugs. There is some evidence supporting an association between non-steroidal anti-inflammatory drug use and flares of IBD [90]. A recent meta-analysis has provided evidence of an association between the use of oral contraceptive agents and development of IBD, in particular CD [91].

Stress. The possible role of psychological stress and depression in
inducing flares in patients with quiescent disease is quite controversial, although recent evidence shows close interaction between nervous and immune system [92].

Appendicectomy. Epidemiological studies suggest that appendicectomy is protective against UC [93]. Conversely, it is associated with a higher risk of developing intestinal strictures in CD [94].

2. Animal Models of Inflammatory Bowel Disease and the Recent Advances in Genetics

2.1 Traditional murine models of CD

Several animal models of intestinal inflammation have been developed using chemical induction, immune cell transfer, or genetic manipulations. The majority of animal models exhibit Th1-dominant immune responses, and they have been used widely for studying CD. Although CD affects both small (ileitis) and large (colitis) intestines, only some CD models develop inflammation in the small intestine. In contrast to Th1-mediated models, mouse models developing Th2-mediated colitis for the study of UC are limited. Animal models cannot fully reflect human diseases, but they have provided important opportunities to examine the mechanisms of IBD more closely. Indeed, during the last decade animal models have not only contributed to our understanding of the mechanisms of IBD but also provided useful interventions for developing novel therapeutic strategies [95].

Chemically induced models. This group of animal models requires administration of an exogenous chemical agent for the induction of colitis. Examples include the trinitrobenzene sulfonic acid (TNBS) [96], dextran sodium sulfate (DSS) [97], and oxazolone [98]. Chemically induced models are useful for studying biochemical pathways of inflammation or for performing antigen-specific studies, such as in the case of hapten-induced gut inflammation (TNBS). These models are also
useful in providing proof of concept for therapeutic interventions in a simple and relatively inexpensive way. In addition, these models are particularly valuable in the dissection of specific aspects or events on the overall background of intestinal inflammation. For example, DSS-induced colitis is characterized by epithelial disruption resulting in luminal bacterial translocation and subsequent infiltration of neutrophils and other acute immune cells. However, although these events might be important in initiating gut inflammation, DSS colitis can be generated in the absence of lymphocytes [99], and does not represent the chronic phases of disease. Therefore, DSS colitis can be considered an appropriate model to investigate epithelial response to injury, neutrophil infiltration or other aspects of the acute phase of colitis pathogenesis, but does not adequately address those events occurring during the chronic phase of gut inflammation.

**Immunological models.** Immunologically mediated models are models of adoptively transferred T cells or bone marrow precursors, which are introduced into immunodeficient recipient mice. Two classical examples are the CD45RB\textsuperscript{high} [100] and bone marrow chimera transfer models[101]. Transfer of hsp60-reactive CD8\textsuperscript{+} T cells is also capable of inducing intestinal inflammation, primarily in the small intestine [102]. Studies in these models have elucidated the role of pathogenic and regulatory T cells in controlling mucosal immunity and intestinal inflammation and offer strong evidence that Th1 polarization plays a key role in CD pathogenesis. However, the profound immune abnormalities in recipient mice probably make these models unsuitable for investigating the innate factors causing human CD.

**Genetic models.** Transgenic and knockout (KO) methodologies have revolutionized the field of animal models of IBD. The majority of the mouse models in this group are gene KO. Examples include the IL-2 [103], TCRa/b [104], IL-10 [105], and Gi2-a [106] KO models. Genetic models greatly contribute to the understanding of the role of immune-related molecules in the pathogenesis of chronic intestinal inflammation. However, it is unlikely that the imposed genetic mutations represent the underlying defect in human IBD, limiting the utility of these models for
understanding causative factors in both ulcerative colitis and CD.

**Spontaneous models.** Spontaneous models represent one of the most attractive model systems for studying intestinal inflammation because, similar to human disease, inflammation occurs without any apparent exogenous manipulations. For example, the C3H/HeJ Bir murine model of colitis is characterized by spontaneous and chronic focal inflammation localized to the right colon and caecal region. Colitis occurs in young mice and tends to resolve with age without recurrence [107]. Similar to severely immunocompromised mice, as well as some of the KO mice, there appears to be a correlation between *Helicobacter* infection and the onset of colitis in the C3H/HeJ Bir model.

### 2.2 Recent Advances in Genetics

A major recent advance in the field of IBD has been made by human gene association studies that have identified many IBD-associated genes specific for either CD or UC or both. In particular, genomic wide association studies (GWAS) have highlighted the importance of genetic susceptibility in IBD. Interestingly, GWAS have revealed a substantial overlap in genetic risk factors between CD and UC [108]. However, some loci are quite unique for CD or UC. For example, autophagy genes (e.g., ATG16L1, IRGM), NOD-like receptors (e.g., NOD2), and intelectins (ITLN1) are highly specific for CD, whereas loci related to regulatory pathways (IL10 and ARPC2), intestinal epithelial cell (IEC) function (e.g., ECM1), and an E3 ubiquitin ligase (e.g., HERC2) appear to be specific for UC [109]. Moreover, GWAS have revealed genetic associations between IBD and many other immune-mediated disorders, which implies that a general inherited propensity to develop autoimmune diseases may exist and that environmental (or epigenetic) factors may determine not only disease phenotype but also the specific immune-mediated disease that develops [110]. A striking but potentially instructive outcome of GWAS is that the vast majority of identified loci individually confer extremely modest risk [odds-ratios (ORs), mostly between 1.11 and 1.29]. Collectively, the loci identified to date represent...
≈10–20% of the overall variance of potential disease risk. Moreover, for most of the confirmed loci the causal gene(s) or variant(s) is not yet known [111].

Besides GWAS (and complementary to them), several KO mouse strains, which are genetically engineered to lack IBD-associated genes, have been developed. Among them, some mouse strains including IL-10 [105], STAT3 [112], XBP1 [113], and GPX1/2 [114] KO mice and human HLAB27 transgenic rats [115] spontaneously develop colitis or ileitis. In contrast, the remaining IBD-associated gene KO mice fail to develop intestinal inflammation spontaneously, suggesting the requirement of additional factors such as immune and environmental factors to fully elicit the development of IBD. Of note, major IBD-associated genes are classified into at least three groups, based on the biological function, including host defence, regulatory immunity, and the IL-23/Th17 axis.

The NOD2 gene, which has been mentioned above, has the first definitive genetic risk factor identified for CD [63]. Three different mouse models harbouring non-functional NOD2 have been developed. The first mouse model, which carries a NOD22939insC mutation similar to the human CD-associated NOD23020insC frameshift mutation, is susceptible to DSS-induced intestinal injury [116]. Unlike chronic IBD models that do not develop disease under germ-free conditions, DSS-induced acute intestinal injury is exacerbated in the absence of commensal flora [117]. Therefore, the increased susceptibility of NOD2 mutant mice to DSS-induced intestinal injury suggests the involvement of NOD2 in commensal flora mediated protective immunity, particularly during the acute inflammatory phase. The second model is a NOD2 KO mouse strain generated by a deletion of exon 1. This mouse strain is characterized by TLR2 signalling. The production of IL-12p70 by APCs in this model is increased in response to TLR2 stimulation, suggesting an inhibitory role of NOD2 in TLR2-mediated Th1 responses [118]. The third model is another NOD2 KO mouse strain generated by deletion of exon 3. This mouse strain is more susceptible to a Listeria monocytogenes infection because of impaired Paneth cell function and a reduced
production of antibacterial peptides such as cryptidin and defensins [119].

Autophagy is a cellular pathway involved in protein and organelle degradation. Recently accumulating data indicate that autophagy affects different physiological functions ranging from microbial infections to aging. An autophagy-related gene (Atg) 16L1 has been identified as a CD susceptibility gene and a deletion polymorphism upstream of IRGM, a gene essential for autophagy, is also associated with CD, highlighting the significance of autophagy in CD pathogenesis [108]. Two different mouse strains lacking functional Atg16L1 have been developed [120,121]. Both of the Atg16L1 KO mouse strains did not develop intestinal inflammation spontaneously, but one mouse strain with gene trap-mediated disruption of Atg16L1 showed an impaired Paneth cell function particularly the granule exocytosis process. These findings suggest that both NOD2 and Atg16L1 actively participate in epithelial cell defence against commensal flora.

Accumulating evidence from animal models clearly indicates the protective role of IL-10 in IBD. Spontaneous development of colitis in IL-10 KO mice has been known since 1993 [105]. The possible clinical relevance of data from animal models is highlighted by a recent identification of the IL-10 as an IBD susceptibility gene [122]. The expression of Foxp3 in Treg cells is unstable in vivo and potential autoreactive effector T cells can develop from the Foxp3+ Treg cells as a consequence of loss of Foxp3. A recent study shows that myeloid cell-derived IL-10 is required for maintaining Foxp3 expression in Treg cells and preserves the regulatory cell’s ability to suppress colitis [123]. It has been demonstrated that B cells are one of the major sources of IL-10 in the gut associated lymphoid tissue [124]. Although IL-10 KO mice spontaneously develop colitis, specific deletion of IL-10 in B cells does not result in colitis induction, consistent with the ability of IL-10-producing Breg cells to inhibit the progression of established colitis but not the initial induction [125]. Although these findings would suggest IL-10 as a therapeutic strategy for IBD, no apparent beneficial effect of IL-10 therapy has been observed in several human clinical trials. Therefore,
it is possible that the polymorphisms of IL-10 receptors hamper the response of IBD patients to the IL-10 therapy [126]. Alternatively, the instability and short halflife of IL-10 may make the therapy less effective.

In addition to a previously well appreciated Th1/Th2 paradigm, recently accumulating data has unveiled a critical involvement of the IL-23/Th17 pathway in the pathogenesis of IBD [127]. A series of studies using IL-23p19 KO mice or neutralizing anti-p19 mAbs have recently revealed the critical pathogenic role of IL-23, but not IL-12, in different types of experimental colitis, which are mediated by either adaptive or innate immunity. The clinical relevance of data from animal models is well supported by gene association studies in humans. Polymorphisms of the IL-23R are negatively associated with the development of IBD and IL-12p40 has been identified as an IBD-associated gene. These findings highlight IL-23 (p19), rather than IL-12 (p40), as a promising target for treating IBD more effectively and more safely [128]. Although the involvement of the IL-23/IL-17 axis in IBD pathogenesis is a solid concept, recently accumulating studies from animal models suggest that the role of Th17 cells in IBD and the control of IL-17 production may be much more complicated than previously predicted. Paragraph 7 will deal with the evidence of Th17 and Th1/Th17 involvement in IBD, coming from both animal models and the most recent data from humans.

3. Th17 and Th1/Th17 Cells: Beyond the Th1/Th2 Paradigm

Upon activation and expansion, CD4+ T cells develop into different Th cell subsets, with different cytokine profiles and distinct effector functions. The differentiation of naïve CD4+ T cells into effector helper T cells is initiated by the engagement of their T cell receptor (TCR) and costimulatory molecules in the presence of specific cytokines produced by the innate immune system following the encounter of particular
pathogens. On the one hand, IFN-γ and IL-12 initiate the differentiation of Th1 cells that are characterized by high production of IFN-γ and are indispensable for clearing intracellular microorganisms [129]. On the other hand, IL-4 triggers the differentiation of Th2 cells, which produce a series of different cytokines, namely IL-4, IL-13 and IL-25, and organize host defence against extracellular pathogens and help B cells to produce antibodies [130]. Subsequently, the effector cytokines can potentially feed back to amplify Th1 and Th2 and further enhance differentiation of the respective T cell subset. Moreover, IFN-γ and IL-4 antagonize each other at different levels, and thus Th1 and Th2 development is considered mutually exclusive. Therefore, even though the cytokine profile may initially not be entirely polarized with differentiating T cells producing a combination of both Th1 and Th2 cytokines, chronic stimulation leads to unequivocal, terminally differentiated phenotypes.

Over the years, the Th1/Th2 paradigm of T helper cell differentiation, which was first introduced by Mosmann & Coffman about 21 years ago [129], has explained many processes in adaptive immunity and allowed to classify immunological disorders as mainly Th1 (such as CD, multiple sclerosis, and rheumatoid arthritis) or Th2 (like UC, allergic dermatitis and asthma). Recently, this Th1/Th2 paradigm has been updated following the discovery of a third subset of IL-17-producing effector T helper cells, called Th17, which develop via a lineage distinct from the Th1 and Th2 lineages [131]. Th17 cells produce a variety of cytokines (namely IL-17A, IL-17F, IL-22, IL-26, IFN-γ), express the chemokine ligand (CCL)20, and the retinoic acid receptor-related orphan receptor (ROR)γt [132]; they can be characterized by the expression of a combination of chemokine receptor (CCR)4 and CCR6, and IL-23 receptor (IL-23R) [133]. Although the function of this cell subtype is not completely elucidated, emerging data suggest that Th17 cells play an important role in host defence against extracellular pathogens, which are not efficiently cleared by Th1-type and Th2-type immunity. The first pathogen implicated in a Th17 response was observed in human Lyme arthritis caused by *Borrelia burgdorferi*, in which *B. burgdorferi*-derived lipopeptides could
stimulate the production of IL-17A by T cells from synovial fluid, leading to a Th17 lineage differentiation [134].

On the other hand, there is evidence that uncontrolled and persistent Th17 responses are responsible of tissue inflammation by producing IL-17 and other effector cytokines (Figure 3) [135].

Many chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes [136], and IL-17 overexpression has been found in a number of inflammatory disorders, including IBD. In the murine model of psoriasis, evidence has shown that Th17 cells along with their upstream cytokines (e.g. IL-23) and their downstream effector cytokines (e.g. IL-22) might play a critical role in the pathogenesis of psoriasis [137,138]. Increased levels of IL-17 produced by Th17 cells have been observed in murine models of rheumatoid arthritis and correlate with more severe joint damage [139]. IL-17 is significantly high in the serum of patients with systemic lupus erythematosus [140], and contraction of Th17 cells has been associated with improvement in renal disease in mouse models of systemic lupus erythematosus using tolerizing peptides [141]. Elevated levels of IL-17 were detected in the blood and the cerebrospinal fluid of patients affected by multiple sclerosis [142]. Besides, several studies using the experimental autoimmune encephalomyelitis model in mice have suggested that IL-17 may be a critical pathogenetic factor for multiple sclerosis [143, 144].

The role of IL-17 in allergic asthma has been largely studied [145,146], while emerging evidence shows that IL-17-producing cells are also involved in the pathogenesis of allergic dermatitis and contact hypersensitivity [147,148].

Three years ago an heterogeneous population of IL-17 and IFN-γ double-producers belonging to the CCR6+CXCR3+ human memory T cell compartment, has been discovered [149]. This subset of cells show selective expression of IL-23R, CCR6 and the transcription factor RORγt, and they exhibit functional features, similar to those of the Th17 clone, such as the ability to help B cells, low cytotoxicity, and poor susceptibility to regulation by autologous Treg cells. Subsequently, this emerging population of cells have been widely studied in multiple
diseases, and during the last three years experimental works have shown their involvement in plenty of immunological disorders and infections [150-152].

4. **Differentiation of Th17 Cells**

4.1 **Cytokines Involved in Th17 Differentiation**

After T cell activation, the fine balance between the different T cell types is regulated by the cytokine environment. Differentiation of Th1 and Th2 cells follows similar rules in human and mice; Th17 differentiation, in contrast, may not be as conserved. Initial studies in mice suggested that IL-23, a heterodimeric cytokine that shares the p40 subunit with IL-12, induces IL-17 expression [131,135,153]. However other studies demonstrated that IL-23R is only expressed on T cells after activation and therefore IL-23 can up-regulate IL-17 in memory T cells but cannot act on naïve T cells to induce Th17 differentiation [154]. In 2006, three independent research groups found that a combination of the immunoregulatory cytokine TGF-β and the pro-inflammatory and pleiotropic cytokine IL-6 is required to induce Th-17 differentiation in the mouse [155-157]. In addition, TNF-α and IL-1β can further enhance mouse Th17 differentiation, but only in the presence of TGF-β and IL-6 [157-159]. This discovery was quite a surprise, because TGF-β is well known to inhibit most T cell responses as well as to induce differentiation of forkhead box protein 3 (Foxp3)-expressing regulatory T cells (Tregs). These conflicting effects of TGF-β, as an inducer of both anti-inflammatory Tregs and pro-inflammatory Th17 cells, raise a number of interesting questions about the role of TGF-β in physiologic differentiation of T helper cell subtypes, as well as in disease. In mice, Tregs can be a source of TGF-β for naïve Th17 differentiation, and one paper has demonstrated that Tregs themselves can convert into Th17 cells in the presence of IL-6 [160]. Several studies show that TGF-β, a
crucial cytokine for mouse Th17 differentiation, actually inhibits human Th17 development [161,162]. Protocols used in previous human studies soon led to the doubt that TGF-β was not necessary to human Th17 development (Figure 3). In fact, so far human naïve T cells were selected from CD45RA-expressing peripheral blood mononuclear cells. Besides, experiments were performed in the presence of serum and, giving that T cells were isolated from peripheral human blood, they were possibly contaminated with platelet (both source of TGF-β). Eventually, a series of new reports using rigorously sorted naïve T cells proved that TGF-β is indeed required for human Th17 differentiation [163-165]. These differences clearly demonstrate that a direct translation of knowledge on Th17 differentiation from mouse models to humans is impossible.

One of the most effective inducer of IL-17 expression in human naïve T cells is IL-1β which on the contrary seems to play only a supporting role in mouse Th17 development. IL-6 and IL-23 induce a small amount of IL-17 alone and greatly enhance Th17 differentiation in the presence of IL-1β [132,162]. Thus, both IL-23 and IL-1β may play a more important role in humans than in mice (Figure 3). Cosmi et al. [166] demonstrated that all IL-17-producing cells originates from CD161+ naïve CD4+ T cells of umbilical cord blood, as well as postnatal thymus, in response to the combined activity of IL-1β and IL-23. Nevertheless, the potential role of IL-23 in human Th17 differentiation is still unclear, since other groups have found that IL-23 has no effect on naïve human T cells [153,167]. According to data obtained by Langrish et al. [153], IL-23 up-regulates IL-17 production and promotes survival and expansion of activated or memory Th17 cells in vitro, although it is not absolutely necessary. Also IL-21, which is produced by Th17 cells themselves, is known to be involved in Th17 differentiation in the presence of TGF-β. IL-21, acting in a positive feedback loop which amplifies the precursor frequency of Th17 cells, might help sustaining chronic inflammation in Th17-dependent diseases (Figure 3) [168]. Last, but not least, IL-6 can program Th17 differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways (Figure 3). Zhou et. al [169] demonstrated that IL-6 induces the expression of IL-21 that amplifies an autocrine loop to
induce more IL-21 and IL-23 receptor in naïve CD4⁺ T cells.

**Figure 3.** Regulation of Th17 cells and their involvement in inflammation. T helper (Th)17 cells are a newly described subset of CD4⁺ T cells that may have a role in inflammatory disorders. These cells release interleukin-(IL)17A and IL-17F, which act on epithelial cells to release CXC-chemokine ligand 1 (CXCL1) and CXCL8, which attract neutrophils, and IL-6, which enhances the activation of Th17 cells. Th17 cells also release IL-21, which promotes Th17 cell differentiation via a positive autoregulatory loop involving the transcription factor signal transducer and activator of transcription (STAT3) and IL-22, which induces the release of IL-10 and acute-phase proteins. The regulation of Th17 cells is predominantly via IL-23 through the activation of the transcription factor retinoic acid receptor-related orphan receptor (ROR)γt, whereas transforming growth factor (TGF)β may have an inhibitory effect in human cells. TNF, tumour-necrosis factor.
4.2 Transcription Factors Involved in Th17 Differentiation

The differentiation of T helper cells is initiated by the combined signals from the TCR, costimulatory molecules and cytokine receptors. These integrated signals then induce lineage-specific transcription factors. Among them, T-box expressed in T cells (T-bet) or trans-acting T-cell-specific (GATA)-3 transcription factors are specifically and respectively expressed in Th1 and Th2 cells. In particular, T-bet can bind to the IFN-γ promoter and induces the expression of this cytokine. The engagement of IFN-γ leads to the activation of signal transducer and activator of transcription (STAT) 1 and T-bet, responsible for Th1 cell differentiation. In turn, T-bet increases the production of IFN-γ and induces the expression of the inducible IL-12Rβ2 chain of the IL-12R. IL-12, through STAT4, is then essential for the maintenance of Th1 responses. On the other hand, GATA-3 starts a Th2 cell lineage commitment and induces the expression of IL-4. The interaction of IL-4 with its receptor activates STAT6, which together with GATA-3 increases IL-4 production and Th2 commitment.

It has been found that RORγt is the master transcription factor guiding Th17 differentiation in mice [170]. RORγt appears to be required for IL-17 production (Figure 3), as mice reconstituted with the bone marrow of RORγt-deficient mice show an impaired Th17 differentiation. Furthermore, transduction of naïve T cells with a retroviral vector containing RORγt induces the development of IL-17-producing T cells [170]. However, although reduced, IL-17-producing cells are not absent in RORγt-deficient mice. Another member of the retinoid nuclear receptor family, RORα, is also selectively expressed in Th17 cells [171]. RORγt and RORα are both strongly induced by IL-6 or IL-21 in the presence of low amounts of TGF-β. Recent studies reported that other transcription factors cooperate with RORγt in Th17 development. For example, the interferon regulatory factor (IRF)4, which was previously associated with the differentiation of the Th1 and Th2 subsets [172], is required for the differentiation of Th17 cells as well [173]. IRF4 knockout mice failed to mount a Th17 response and were resistant to
experimental encephalomyelitis. Consistently, IRF4-deficient T cells failed to up-regulate RORγt upon stimulation in the presence of TGF-β plus IL-6, and could not be differentiated into Th17 cells. However, overexpression of RORγt in IRF4-deficient T cells failed to fully restore the induction of IL-17, again suggesting that RORγt is not sufficient for full commitment of T cells to the Th17 lineage. Another transcription factor regulating IL-17 production is STAT3 [174] (Figure 3). Mice with conditional deficiency of STAT3 in T cells have impaired Th17 differentiation, and overexpression of a constitutively active form of STAT3 can increase IL-17 production. Thus, STAT3 might affect the expression of IL-17 by increasing the expression of RORγt and RORα, which are upstream of IL-17. However, STAT3 also binds directly to the IL-17 and IL-21 promoters [175]. Therefore, STAT3 and RORγt seem to cooperate, and competent production of IL-17 depends on the presence of both transcription factors. Finally, Th17 are closely linked to Foxp3+ Treg cells, as it has been shown that both cell types require TGF-β for in vitro differentiation and that Foxp3 controls Th17 differentiation by direct interaction with RORγt [161,176]. Just after the discovery of the transcription factors involved in mouse Th17 differentiation, Manel et al. [164] demonstrated that RORc, which corresponds to RORγt in humans, is crucial for Th17 development. Since down-regulation of RORc through transient transfection with short hairpin RNA molecules in memory CCR6+ cells resulted in much less IL-17 expression, it is clear that RORc is required for the maintenance of cytokine production in Th17 cells.

5. Trafficking of Th17 Cells

Th17 cells cause inflammation and immune disorders through both pro-inflammatory cytokines and chemokines with their chemokines receptors, which mediate trafficking of activated T cells into inflamed tissue. Several reports have found that human and mouse Th17 cells
express CCR6. Within this population those cells that coexpress CXCR3 are either Th1 or IFN-γ/IL-17 double-positive, while those that coexpress CCR4 are classical Th17 expressing only IL-17 [133,177]. In addition, the majority of RORc expression was restricted to CCR6+CCR4+ population, with a small amount in the CCR6+CCR3+ population [133]. CCR6 mediates homing to skin and mucosal tissues and has been shown to play an important role in recruitment of pathogenic T cells in many inflammatory diseases now associated to IL-17, including psoriasis, IBD, allergic asthma and rheumatoid arthritis [177-181]. Interestingly, the CCR6 ligand CCL20 is expressed on Th17 cells and is up-regulated on stromal cells by IL-17, thus allowing Th17 cells in inflamed tissue to attract additional Th17 and Th1 cells [132,182].

6. IL-17

IL-17, which has been known for years before Th17 cells were discovered, is the founding member of the IL-17 family of cytokines, which includes IL-17A (also called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F [183]. Whereas all members of the IL-17 family are produced by Th17 cells, IL-17E derives from Th2 cells [130]. IL-17A and IL-17F are syntenic, as they co-localize on the same chromosome (chr.1 in mice; chr.6 in humans); conversely, the other IL-17 cytokines map to different chromosomes. IL-17 shares no sequence homology with other known mammalian proteins and therefore constitutes a distinct cytokine family. IL-17A and IL-17F are effector cytokines produced not only by Th17 cells, but also γδ T cells, natural killer (NK)T cells, neutrophils, eosinophils and NK cells [184]. This suggests that IL-17 cytokines may have a bridging function between innate and adaptive immune responses.

IL-17A binds to IL-17 receptor (IL-17R) with more than ten-fold higher affinity as compared to IL-17F, and acts on both immune and non-immune cells. IL-17R is expressed on a wide range of cells, including
osteoblasts, fibroblasts, epithelial and endothelial cells [185]. IL-17 induces pro-inflammatory cytokine production by macrophages, thus creating a link between innate and adaptive immunity [186], and plays a key role in host defence against bacteria and fungi, particularly at mucosal surfaces (Figure 4), by favouring the local recruitment of immune cells [187]. Consistently, mice knockout for the IL-17R gene have profound defects in host protection [188].

Involvement of IL-17 in inflammation is now well documented: ligation of IL-17 with its receptor promote the synthesis of a broad variety of inflammatory cytokines (e.g. IL-1, IL-6, TNF-α, GM-CSF), chemokines (e.g. IL-8, CXCL1, CXCL8, monocyte chemoattractant protein-1, monocyte inhibitor protein-(MIP)3α), cyclooxygenase-2, and tissue degrading matrix metalloproteinases (MMPs) by several cell types [189]. In particular, IL-17 induces IL-6 and IL-8 production in vitro through mitogen-activated protein kinase (MAPK) pathways, thus favoring the recruitment of neutrophils at sites of inflammation [77], and triggers T cell proliferation and up-regulation of pro-inflammatory molecules, such as inducible nitric oxide synthase and IL-1β [190]. Interestingly, some actions of IL-17 are potentiated by the presence of other pro-inflammatory cytokines, particularly TNF-α and IL-1β.
Figure 4. Role of Th17 cells in the modulation of intestinal inflammation. Th17 cells differentiate from naïve T lymphocytes under the stimulus of TGF-β and IL-6, while the antigen antigen-presenting cells-derived IL-23 and the Th17 cell-derived IL-21 contribute to maintain/expand Th17 cell populations. Th17-derived cytokines, such as IL-17A, IL-21, and IL-22 are involved in the recruitment of inflammatory cells in the intestinal lamina propria, due to their ability to enhance the expression of chemoattractants and adhesion molecules (e.g. ICAM-1) by epithelial and endothelial cells, respectively. IL-17A and IL-21 stimulate fibroblasts to make matrix metalloproteinases, a family of enzymes that could contribute to the tissue damage and remodelling occurring in IBD. IL-17A and IL-22 also stimulate the synthesis of antibacterial proteins, including defensins, by epithelial cells. APC, antigen antigen-presenting cell; TGFβ, transforming growth factor-β; MMPs, matrix metalloproteinases; ICAM-1, intercellular adhesion molecule-1.

7. Th17 and Th1/Th17 Cells in IBD: Beyond Th1 and Th2 Responses

It is well known that CD is characterised by a preponderance of Th1-associated cytokines, including IFN-γ, TNF-α and IL-12, while Th2 cytokines, such as IL-5 and IL-13, are considered to be dominant in UC [75]. Nonetheless, both CD and UC share crucial end-stage pathways, as shown by the enhanced production of IL-21 and IL-23 in both disorders [79,191]. The role of Th1 cells in the pathogenesis of CD is well established, based on a number of observations, including the increased production of IL-12 in macrophages in this condition [192]. Moreover, nuclear extracts of T cells isolated from the inflamed gut areas of CD patients contain high levels of activated STAT4 and T-bet, Th1-associated transcription factors (see above) indicative for IL-12 signalling [193]. Furthermore, in the mucosa of patients affected by CD there is an enhanced production of
IL-18, a cytokine involved in perpetuating Th1 cell responses [194]. In addition, T cells from inflamed gut tissue of CD patients express increased levels of IL-12Rβ2 chain, which is characteristic for Th1 cells, and increased amounts of IFN-γ together with decreased levels of IL-4 compared to controls [74,195]. IFN-γ secreting lamina propria lymphocytes are abundant in the mucosa of patients with CD. Particularly, at onset of the disease mucosal T cells appear to mount a typical Th1 response that resembles an acute infectious process, and is lost with progression to late CD [196]. In addition, a clinical response was observed in a subcohort of CD patients treated with anti-IFN-γ monoclonal antibody [34].

By contrast, T cells from lamina propria of patients affected by UC produce a higher amount of IL-5 in comparison to CD and controls [74]. Moreover, Heller et al. [75] demonstrated that IL-13 is the key effector Th2 cytokine in UC that affects epithelial tight junctions, apoptosis and cell restitution.

In the last decade, several studies indicated that, in addition to Th1 lymphocytes, Th17 cells play a major role in the pathogenesis of CD, and possibly even of UC.

7.1 Th17 in Mouse Models of Gut Inflammation

Studies in IL-17R knockout mice demonstrated that IL-17 is necessary for the development of acute gut inflammation induced by intrarectal administration of TNBS [197], a T cell-mediated colitis showing striking similarities with CD. Consistently, blockade of IL-17 signalling by an IL-17R IgG1 fusion protein significantly attenuated colonic inflammation and prevented weight loss after TNBS administration in mice. Studies in other animal models of colitis such as DSS-induced colitis showed that IL-17F deficiency results in reduced colitis, whereas IL-17A knockout mice develop more severe disease [198], suggesting that IL-17F rather than IL-17A is crucial in sustaining inflammation in chemical-induced colitis. Th17 cells have been also involved in the pathogenesis of colitis induced by transfer of caecal bacterial antigen-
specific C3H/HeJBir (C3Bir) CD4+ T cell line to C3H/HeSnJ SCID mice [199]. In this model, gut inflammation associated with enhanced production of IL-17, and adoptive transfer of IL-17-secreting T cells to SCID recipients resulted in a marked gut inflammation, as compared to that caused by transfer of Th1 cells. Administration of mice with a monoclonal anti-IL23p19 prevented and treated active colitis. By using a novel model of CD8+ T cell-dependent colitis, Tajima et al. [200] have shown that a single adoptive transfer of naïve CD8+ T cells into syngeneic RAG-deficient mice was followed by rapid spontaneous proliferation of these cells in the mesenteric lymph nodes and severe colitis. Analysis of cytokine-secreting CD8+ T cells in the mesenteric lymph nodes showed the existence of IL-17/IFN-γ double-positive cells. Notably, adoptive transfer of naïve CD8+ T cells derived from either IL-17- or IFN-γ-knockout mice associated with a remarkably less severe colitis, raising the intriguing possibility that IL-17 and IFN-γ can cooperate to cause pathology in this model of colitis. In line with these findings, Fina et al. [78] recently demonstrated that IL-21-deficient mice were largely protected against the development of DSS colitis and TNBS-relapsing colitis. This protection was associated with a reduced colonic expression of several Th17-related genes, including IL-17A, IL-17F, and RORγt, supporting the role of IL-21 in Th17 cell differentiation. Additionally, blockade of IL-21 activity with a specific IL-21R fusion protein reduced intestinal inflammation and Th17 response during the course of DSS colitis.

After the demonstration that blockade of IL-12/IL-23p40 ameliorated colitis in mice [201], a monoclonal antibody against IL-12p40 was used in a phase II trial to treat patients with active CD leading to good results [202]. Since the p40 subunit is shared by both IL-12 and IL-23 [203], it was not clear if the therapeutic effect of this novel reagent was due to neutralization of IL-12 and/or IL-23. However, studies conducted in various animal models of colitis would seem to indicate that IL-23 is more pathogenic than IL-12 in the gut. For instance, backcrossing IL-10-deficient mice with mice lacking IL-12p35 or IL-23p19, Yen et al. [204] showed that IL-23 was essential for manifestation of chronic intestinal
inflammation, whereas IL-12 was not. CD4+ T cells from IL-10p19 knockout mice still produced large amounts of IFN-γ, thus indicating that Th1 response developed normally in the absence of IL-23 but disease manifestations required the presence of IL-23. Moreover, administration of exogenous IL-23 in RAG mice reconstituted with naïve CD4+ T cells caused a more severe colitis that was associated with enhanced production of IL-6 and IL-17 and preventable by treatment of mice with a blocking IL-6 or IL-17 antibody. Although in this model IL-6 and IL-17 were made by memory T cells, there is no doubt that some of the pathogenic functions of IL-23 in the gut are mediated by a non-T cell population. This was first showed by Powrie and coworkers who analysed the effect of an agonistic anti-CD40 antibody in RAG mice IL-23p19 or IL-12p35 [205]. Administration of anti-CD40 caused a systemic and local inflammation characterized by wasting disease, splenomegaly, increases in serum pro-inflammatory cytokines and colitis. It was shown that the systemic inflammatory response and the elevated concentration of pro-inflammatory cytokines in the serum were derived by IL-12 while the local intestinal inflammation and production of IL-17 in the intestine were controlled by IL-23. More recently using the T cell transfer model of colitis the same group has shown that IL-17-deficient T cells are not impaired in their ability to induce colitis in RAG mice [206]. Protection of colitis seen in RAG mice lacking IL-23 was associated with no decreases in the levels of IL-17, as well as lack of IL-23 did not significantly affected the relative amount of the Th17-specific factor RORγt in the colon. Consistent with this study, Noguchi et al. [207] showed that transfer of naïve CD4+ T cells prepared from IL-17 knockout mice induced severe colitis in RAG mice. Taken together this data indicate that Th17 cell responses are not specifically impaired in the intestine of IL-23-deficient mice and that IL-23-mediated colitis is not strictly dependent on IL-17 production.

A more detailed analysis of the mechanisms underlying the IL-23-dependent pathologies revealed that IL-23 can facilitate colitis not only via direct effect on inflammatory mediators but also indirectly by counteracting regulatory mechanisms. Indeed, protection of colitis seen
after transfer naïve T cells to RAG mice lacking IL-23 was associated with an increase in the frequency of Foxp3-expressing Treg cells in the intestine [206]. Upon naïve T cell transfer administration of mice with either an antagonistic IL-10R antibody or a blocking TGF-β antibody increased colonic inflammation compared to untreated controls. Moreover, naïve T cells isolated from transgenic mice expressing a dominant-negative form of TGF-β receptor II and unable to respond to TGF-β induced significant colitis when transferred to IL-23-deficient RAG mice [206]. High levels of IFN-γ but not IL-17 were seen in colitis mice, thus suggesting that IFN-γ might drive the chronic intestinal inflammation in this setting. Notably, transfer of Foxp3-deficient T cells to IL-23-deficient RAG mice caused severe colitis, thus indicating that IL-23 is not essential to the pathogenesis of intestinal inflammation, if counter regulatory mechanisms are defective or absent [206]. These later findings well fit with the notion that the requirement of IL-23 for the initiation and progress of gut inflammation varies depending on the model. In fact, acute colitis induced by TNBS is driven by IL-12 and negatively regulated by IL-23 [208].

7.2 Th17 in Human Gut Inflammation

The first report on IL-17-producing cells in IBD comes from a study in which it was shown that the inflamed gut of CD and UC patients contained high levels of IL-17-secreting cells in comparison to normal controls or patients with ischemic colitis [76]. By immunohistochemistry, it was shown that in active UC IL-17-expressing cells were located mainly within the lamina propria, while in active CD these cells were scattered throughout the submucosa and muscularis propria. Major sources of IL-17 were CD3+ T cells and CD68+ cells. Moreover, IL-17 was found to be enhanced in the serum of IBD patients. These results were confirmed by the demonstration that RNA transcripts for IL-17A and IL-17F were up-regulated in the inflamed mucosa of UC and CD patients [76,209]. By flow cytometric analysis of mucosal lymphocytes, Annunziato et al. [149] demonstrated that the number of
IL-17-producing T cells is higher in CD than in normal gut mucosa, and that some of these cells produce also IFN-γ. *In vitro* treatment of such cells with IL-12 resulted in enhanced expression of T-bet and IFN-γ, and down-regulation of RORγt and IL-17. Therefore if at one side it seems that Th1 negatively cross-regulates Th17 differentiation, on the other hand the above findings suggest that T cells can coexpress both Th1- and Th17-cytokine signatures, which are equally involved in gut inflammation. Hence, the interactions between Th1 and Th17 cells and the role of IFN-γ on Th17 cells may be more complex than previously assumed and require further analysis to delineate the specific contribution of these cell lines to CD and other autoimmune diseases.

Of note, the inflamed mucosa of IBD patients contains high levels of other Th17-related cytokines. In both CD and UC tissue there is enhanced production of IL-21, a cytokine that is capable of regulating the activity of multiple immune and non-immune cell types [191]. Indeed, IL-21 has been reported to expand the ongoing Th1 cell response in CD [191], to stimulate gut fibroblasts to secrete MMPs [210], and to induce colonic epithelial cells to produce MIP-3α [211], a chemokine involved in the recruitment of activated T cells and dendritic cells in the gut (Figure 4). IL-22 is also highly expressed in mucosal samples of patients with active CD and to a lesser degree of patients with UC [212]. Like other Th17 members, IL-22 stimulates colonic fibroblasts to make inflammatory cytokines (e.g. IL-6, IL-8, IL-11, and leukemia inhibitory factor), chemokines, and MMPs [212]. Moreover, IL-22 enhances the expression of TNF-α, IL-8, and β-defensin [213] (Figure 4). By using an *in vitro* wounding assay Brand et al. [213] showed that IL-22 stimulates the migration of colonic cells by a PI-3 kinase-dependent mechanism, thus suggesting that IL-22 promotes disruption of intestinal barrier integrity. Again, the same group demonstrated that IL-26, the novel Th17 cytokine, is increased in the gut of CD patients and derives from RORγt-expressing cells [214].
Main Project

1. Aim

Even though it is clear that IL-17 is involved in gut inflammation, contrasting data are available on its role and regulation in CD and UC. Kobayashi et al. [215] detected high levels of IL-17 transcripts in both CD and UC mucosa compared to normal gut. This group also found that high IL-23p19 transcript levels correlated with IL-17 levels in UC and IFN-γ in CD, thus concluding that IL-23 differentially regulates the Th1/Th17 balance in UC and CD. In contrast, as mentioned before, Fujino et al. [76] showed by immunohistochemistry that there was higher IL-17 expression in CD than UC mucosa. Nevertheless, the demonstration by Fuss et al. [216] that IL-23 is up-regulated in CD but not UC further confuses the picture, and makes it difficult to understand the control of IL-17 production in UC. As reported before, a number of studies have examined the role of different cytokines in inducing IL-17 by virgin CD4+ T cells. A role for IL-1β, IL-6 and TGF-β in polarizing Th17 cells in man is clear, but the contribution of these cytokines to IL-17 production in the gut is not known. Again, it is well known that IL-12 is up-regulated in IBD and induces an increase of IFN-γ production by Th1 cells [217]. However, its influence on IL-17-producing lamina propria mononuclear cells (LPMCs) has not been investigated. Finally, IL-21 also seems to be implicated in the regulation of IL-17 production by lamina propria lymphocytes according to preliminary work by Fina et al. [78], but its role needs to be further elucidated.

Moreover the novel subset of CD4+ T cells, namely Th1/Th17 cells, which produce both IFN-γ and IL-17, has been shown to originate from a CD161+ CD4+ NK T cell precursors present in the umbilical cord blood, in the presence of IL-1β and IL-23 as polarizing cytokines, but whether this pathway is important in vivo in man is not known.

Main hypothesis. Our hypothesis was that chronic inflammation in IBD is sustained by effector cytokines produced by three different populations
of T cells: Th1, Th17 and Th1/Th17. Moreover, we believed that these T cell subsets were differentially regulated by pro-inflammatory and anti-inflammatory cytokines involved in controlling human gut inflammation. **How hypothesis has been tested.** In order to clarify these issues, we first determined the production of IL-17 and IFN-γ in the supernatants of organ and cell cultures by using intestinal mucosal biopsies of IBD patients (*ex vivo* and *in vitro* experiments). Second, we analyzed lamina propria Th1, Th17 and Th1/Th17 populations in IBD patients through flow cytometry. Third, we investigated the control of IL-17 and IFN-γ production by IBD LPMCs after stimulation with a series of pro-inflammatory (IL-23, IL-1β, IL-6) and anti-inflammatory (TGF-β) cytokines, or blocking monoclonal antibodies such as the anti-human IL-21.

2. **Materials and Methods**

2.1 **Patients and Tissues**

Colonic biopsies or surgical specimens were taken from macroscopically and microscopically inflamed and uninfamed areas of the mucosa of 38 patients affected by CD (Table 3) and 34 patients affected by UC (Table 4). Diagnosis of CD and UC was ascertained according to the usual clinical criteria, and the site and extent of the disease were confirmed by endoscopy and histology. In CD patients, disease activity was assessed by Crohn’s Disease Activity Index. Patients with scores below 150 were classified as being in remission, whereas those with scores over 450 had severe disease [218]. In UC patients, disease activity was assessed according to the Clinical Activity Index of Rachmilewitz [219]. Clinical remission was defined as a score below 4. None of IBD patients had been ever treated with cyclosporine, tacrolimus or anti-TNF antibodies. Mucosal samples were also collected from the colon of 27 subjects who had functional diarrhoea at the end of their diagnostic work-up (mean
age 38.1 years, range 29-68), and from macroscopically and microscopically unaffected colonic areas of 11 patients undergoing colectomy for colon cancer (mean age 51 years, range 39-70). In order to exclude that results were simply due to an unspecific gut inflammation, instead of IBD-related inflammation, we included a positive control group of gut inflammation in the study. Therefore, biopsy specimens taken from the inflamed mucosa of a patient with radiation colitis, two patients with diverticulitis and two patients with ischemic colitis were used as disease controls (non-IBD patients). Some of the mucosal samples were used to isolate LPMCs, some others for organ culture experiments. Each patient who took part in the study was recruited after appropriate local Ethics Committee approval, and informed consent was obtained in all cases.

Table 3. Clinical features of patients with Crohn’s disease (n=38)

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<th>N.</th>
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<tbody>
<tr>
<td>Age (years)</td>
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<td>32.1 (15-63)</td>
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<tr>
<td>Intestinal location</td>
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<tr>
<td>Small bowel and colon</td>
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<td>Colon only</td>
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<tr>
<td>Disease behaviour</td>
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<tr>
<td>Fistulizing</td>
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<tr>
<td>stricturing</td>
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<tr>
<td>Luminal</td>
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<tr>
<td>Duration of disease (months)</td>
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<td>Number of recurrences</td>
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<td>2.2 (0-6)</td>
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<tr>
<td>CDAI</td>
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<td>191 (87-394)</td>
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<tr>
<td>Treatment</td>
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<td>Mesalazine</td>
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<tr>
<td>Mesalazine + topical steroids</td>
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<td>Mesalazine + antibiotics</td>
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### Table 4. Clinical features of patients with ulcerative colitis (n=34)

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<td>6.1 (1-12)</td>
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<tr>
<td>Mesalazine + topical steroids</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Mesalazine + azathioprine/6-MP</td>
<td>5</td>
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### 2.2 Organ Culture

Biopsy specimens were placed on iron grids in the central well of an organ culture dish and the dishes placed in a tight chamber with 95%O₂/5%CO₂ at 37°C [220]. Serum-free HL-1 medium (Cambrex Bio Science, Wokingham, UK), added with 100 U/ml penicillin and 100 μg/ml streptomycin, was used to assess the spontaneous cytokine production by biopsies grown ex vivo. After 24h culture, supernatants were snap frozen and stored at -70°C.

### 2.3 Cell Isolation

LPMCs were isolated and purified from freshly resected surgical specimens or endoscopic biopsies as previously described [220]. The epithelial layer was removed with 1 mM EDTA (Sigma-Aldrich, Poole, UK). After stirring for 1h at 37°C, the supernatant was removed and the remaining tissue was treated with type 1A collagenase (1 mg/ml; Sigma-Aldrich) for 2h with stirring at 37°C. The resulting crude cell suspension was preferentially enriched for intestinal LPMCs using a Ficoll-Paque Plus gradient (Amersham Pharmacia Biotech, Uppsala, Sweden).
following the manufacturer’s protocol. Cells from the supernatant were washed twice, resuspended in 1 ml RPMI-1640 medium (Sigma-Aldrich) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and kept on ice until used. Cells were not used if viability did not exceed 90%.

2.4 Cell Culture

Freshly isolated LPMCs (2x10^5 cells/well) were stimulated in anti-CD3-coated 96-well plates (BD Biosciences, Oxford, UK) with soluble anti-CD28 antibody (0.5 μg/ml; eBioscience, San Diego, CA), and incubated for 48h with medium containing IL-1β (10 ng/ml) plus IL-6 (20 ng/ml), IL-23 (10 ng/ml), IL-12 (5 ng/ml), TGF-β1 (10, 1, 0.1 and 0.01 ng/ml), a mouse anti-human IL-21 neutralizing antibody (20 μg/ml; kindly provided by Giovanni Monteleone) or its isotype control (mouse IgG). All the above mentioned recombinant human cytokines were from R&D System (Minneapolis, MN). For intracellular cytokine staining, purified LPMCs were stimulated for 4h in RPMI complete medium with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of monensin (2 μM; eBioscience).

2.5 ELISA

Concentrations of IL-17 and IFN-γ in organ culture and LPMC supernatants were measured using specific ELISA kits (R&D System), according to the manufacturer’s instructions.

2.6 Flow Cytometry

Surface-staining of LPMCs was performed at 4°C for 30 min with FITC-conjugated anti-CD3 and APC-conjugated anti-CD161 antibodies (BD Biosciences). After fixation with 100 μl Leucoperm A, and
permeabilization with 100 μl Leucoperm B (Serotec, Oxford, UK), PE-conjugated anti-IL-17 and PE-Cy5-conjugated anti-IFN-γ antibodies (BD Biosciences) were added for 30 min. Appropriate isotype-matched control antibodies were purchased from BD Biosciences and included in all experiments. After washing twice with 250 μl fluorescence-activated cell sorter buffer (phosphate buffer containing 1 mM EDTA and 0.02% sodium azide), cells were fixed with 2% paraformaldehyde and analysed by flow cytometry using a FACSARia II Flow Cytometer (BD Biosciences).

2.7 Statistical Analysis

Data were analyzed in the GraphPad Prism statistical PC program (GraphPad Software, San Diego, CA) using the paired t test for independent samples and the non parametric Mann-Whitney U test (see figure legends). A level of p<0.05 was considered statistically significant.

3. Results

3.1 Ex vivo Production of IL-17 and IFN-γ

First, we aimed to determine the spontaneous production of IL-17 and IFN-γ by organ culture biopsies from the inflamed and uninflamed mucosa of 17 IBD patients (7 with CD and 10 with UC) and normal mucosa of 10 control subjects (Figure 5). The concentration of IL-17 was significantly higher in the supernatants of both CD (mean 188 ± 50 pg/ml, p<0.01) and UC organ culture biopsies (mean 130 ± 47 pg/ml, p<0.05) in comparison to controls (mean 27 ± 5 pg/ml). Uninflamed mucosa from CD and UC patients showed significantly lower levels of IL-17 in comparison to inflamed mucosa (CD: mean 36 ± 11 pg/ml, p<0.01; UC: mean 48 ± 14 pg/ml, p<0.05). Likewise, the concentration
of IFN-γ was significantly higher in the supernatants of both CD (234 ± 53 pg/ml, p<0.01) and UC organ culture biopsies (201 ± 49 pg/ml, p<0.01) in comparison to uninflamed areas (CD: mean 79 ± 24 pg/ml, p<0.01; UC: mean 69 ± 18 pg/ml, p<0.01) and controls (23 ± 6 pg/ml). No significant difference was observed in the production of IL-17 and IFN-γ between CD and UC patients.

![Graphs showing IL-17 and IFN-γ levels](image)

**Figure 5.** Levels of IL-17 and IFN-γ, expressed in pg/ml, in the supernatants of biopsies taken from the inflamed and uninflamed colon of 7 Crohn’s disease (CD) patients and 10 ulcerative colitis (UC) patients, and from the normal colon of 10 healthy controls (HC), and cultured for 24h in the absence of stimuli. Data were analyzed using the Mann-Whitney U test. Results are mean ± SD. *p<0.01 versus HC and uninflamed areas of CD and UC; **p<0.05 versus HC and uninflamed areas of CD and UC.

### 3.2  *In vitro* Production of IL-17 and IFN-γ

We then determined the production of both IL-17 and IFN-γ from unstimulated and anti-CD3/CD28-stimulated LPMCs isolated from the inflamed mucosa of 28 IBD patients (14 with CD and 14 with UC) and the inflamed mucosa of 5 non-IBD patients (one with radiation colitis,
two with diverticulitis and two with ischemic colitis), and the normal mucosa of 14 control subjects (Figure 6). In unstimulated conditions, LPMCs from CD and UC patients produced significantly (p<0.05) higher amounts of IL-17 (mean 862 ± 372 pg/ml and 644 ± 385 pg/ml, respectively) in comparison to control LPMCs (mean 92 ± 11 pg/ml) and LPMCs from non-IBD patients (mean 124 ± 25 pg/ml). No difference was found between control subjects and non-IBD patients, and between CD and UC. Activation with anti-CD3/CD28 antibodies significantly enhanced the LPMC production of IL-17 in comparison to unstimulated conditions in all the four groups, particularly in IBD patients (CD: mean 6421 ± 1869 pg/ml, p<0.001; UC: mean 4696 ± 2049 pg/ml, p<0.001; control subjects: 1795 ± 583 pg/ml, p<0.01; non-IBD patients: 2099 ± 497 pg/ml, p<0.01). No significant difference was found in CD3/CD28-stimulated LPMC production of IL-17 between control subjects and non-IBD patients, and between CD and UC patients. Unstimulated LPMCs from CD patients, UC patients and non-IBD patients produced significantly (p<0.05) higher amounts of IFN-γ (mean 710 ± 212 pg/ml; 544 ± 161 pg/ml; and 503 ± 143 pg/ml, respectively) in comparison to control LPMCs (mean 188 ± 109 pg/ml). After stimulation with anti-CD3/CD28 antibodies, IFN-γ production was significantly enhanced in comparison to unstimulated conditions in all the four groups, particularly in IBD patients and non-IBD patients (CD: mean 7101 ± 2099 pg/ml, p<0.001; UC: mean 5764 ± 2038 pg/ml, p<0.001; non-IBD patients: 5532 ± 2391 pg/ml; p<0.001; control subjects: 2848 ± 851 pg/ml, p<0.01). Of note, if cytokine levels in LPMC supernatants were expressed as the ratio between values in medium only and values after anti-CD3/CD28 stimulation, the increase of IL-17 or IFN-γ production was higher in controls than IBD patients (Figure 6, lower panel). This is not surprising giving that IL-17 and IFN-γ production were already high in unstimulated conditions. Consequently, the ratio is lower in IBD patients than control subjects, whose LPMCs hardly produce both cytokines in unstimulated conditions. As far as the non-IBD patient group is concerned, it seems that stimulation enhances IL-17 more than
IFN-\(\gamma\), which in fact looks already quite upregulated in unstimulated conditions, similarly to the IBD group.

Figure 6. Upper panel. Levels of IL-17 and IFN-\(\gamma\), expressed in pg/ml, in the supernatants of unstimulated and anti-CD3/CD28-stimulated LPMCs isolated from the inflamed colon of 14 Crohn’s disease (CD) patients, 14 ulcerative colitis (UC) patients, and 5 non-IBD patients (1 with radiation colitis, 2 with diverticulitis and 2 with ischemic colitis), and from the normal colon of 14 healthy controls (HC), and cultured for 48h. Data were analyzed using the Student’s \(t\) test. Results are mean  \(\pm\) SD. †\(p<0.05\) versus unstimulated HC LPMCs; §\(p<0.01\) versus unstimulated HC
LPMCs; *p<0.001 versus anti-CD3/CD28-stimulated HC LPMCs and versus all unstimulated LPMCs. Results are mean ± SD. Lower panel. Production of IL-17 and IFN-γ by LPMCs, expressed as ratio between cytokine concentration after anti-CD3/CD28 stimulation and those in unstimulated conditions. Numbers are ratios.

3.3 Analysis of Th17 and Th1/Th17 Cells

To determine the percentage of mucosal T cells producing IL-17 and IFN-γ, we cultured LPMCs isolated from inflamed mucosa of 17 IBD patients (10 with CD and 7 with UC) and from normal mucosa of 9 control subjects for 4h with PMA and ionomycin in the presence of monensin. As shown in Figure 7A, the percentage of CD3+ LPMCs producing IL-17 was significantly (p<0.05) higher in CD patients (mean 5.6 ± 3.3%) and UC patients (mean 6.7 ± 3.1%) in comparison to controls (mean 2.4 ± 1.9%). Likewise, the percentage of CD3+ LPMCs producing IFN-γ was significantly higher (p<0.05) in CD patients (mean 18.8 ± 6.0%) and UC patients (mean 17.5 ± 2.6%) in comparison to controls (mean 11.8 ± 2.7%). Finally, the percentage of CD3+ LPMCs producing both IL-17 and IFN-γ was significantly (p<0.05) higher in CD patients (mean 1.9 ± 1.4%) and UC patients (mean 2.6 ± 1.6%) in comparison to controls (mean 0.7 ± 0.4%). No significant difference was found between CD and UC in terms of production of IFN-γ alone, IL-17 alone, and both IL-17 and IFN-γ. In Figure 7B, representative dot plots show the flow cytometric analysis of gated CD3+ LPMCs producing IL-17 and IFN-γ in a control subject, a CD and a UC patient. A higher proportion of Th1/Th17 cells were observed in the CD3+CD161+ pool than in the CD3+CD161− pool of LPMCs (Figure 7C).
Figure 7. A. Percentages of CD3+ LPMCs producing IFN-γ only (Th1 cells), IL-17 only (Th17 cells), or both (Th1/Th17 cells), assessed by flow cytometry after cytokine intracellular staining in 9 control subjects (HC), 10 Crohn’s disease (CD) patients and 7 ulcerative colitis (UC) patients. Cells were analysed after 4-h stimulation with PMA and ionomycin and the Mann-Whitney U test has been used for statistical analysis. Results are mean ± SD. *p<0.05 versus HC LPMCs. B. Flow cytometric analysis of PMA/ionomycin-stimulated LPMCs producing IL-17 and IFN-γ in gated CD3+ cells. Numbers within the dot plots represent the percentages of Th1 cells (lower-right quadrant), Th17 cells (upper-left quadrant), and Th1/Th17 cells (upper-right quadrant). The example is representative of experiments performed in 9 control subjects, 10 CD patients and 7 UC patients. C. Flow cytometric analysis of PMA/ionomycin-stimulated LPMCs producing IL-17 and IFN-γ in gated CD3+CD161+ and CD3+CD161− cells. Numbers within the dot plots represent the percentages of Th1 cells (lower-right quadrant), Th17 cells (upper-left quadrant), and Th1/Th17 cells (upper-right quadrant). The example is representative of experiments performed in 6 control subjects, 6 CD patients and 6 UC patients.
3.4 Control of IL-17 and IFN-γ Production by Pro-Inflammatory Cytokines

Anti-CD3/CD28-stimulated LPMCs from 11 CD patients and 8 UC patients were cultured for 48h with IL-1β plus IL-6, IL-23 or IL-12. No effect was exerted by IL-1β plus IL-6, or IL-23 alone on the production of IL-17 and IFN-γ both in CD and UC patients (Table 5).

Table 5. Levels of IL-17 and IFN-γ (pg/ml) in the supernatants of anti-CD3/CD28-stimulated (αCD3/CD28) LPMCs isolated from the inflamed colon of 11 Crohn’s disease (CD) patients and 8 ulcerative colitis (UC) patients, and cultured for 48h with 10 ng/ml IL-23 or 10 ng/ml IL-1β plus 20 ng/ml IL-6. Data analysis has been performed using the Student’s t test. Results are mean (SE); *p<0.0001.

<table>
<thead>
<tr>
<th></th>
<th>IL-17</th>
<th>IFN-γ</th>
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<tbody>
<tr>
<td></td>
<td>CD</td>
<td>UC</td>
</tr>
<tr>
<td>Medium</td>
<td>67 (37)</td>
<td>27 (11)</td>
</tr>
<tr>
<td>αCD3/CD28</td>
<td>4701 (1183)*</td>
<td>3552 (1517)*</td>
</tr>
<tr>
<td>αCD3/CD28 + IL-23</td>
<td>4771 (1234)*</td>
<td>3277 (1413)*</td>
</tr>
<tr>
<td>αCD3/CD28 + IL-1β + IL-6</td>
<td>4736 (1220)*</td>
<td>3586 (1304)*</td>
</tr>
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In contrast, IL-12 significantly (p<0.05) decreased IL-17 production (from mean 6810 ± 2200 pg/ml to 2201 ± 1069 pg/ml; Figure 8A), while it significantly (p<0.005) up-regulated IFN-γ production (from mean 6979 ± 419 pg/ml to 43341 ± 9086 pg/ml; Figure 8B). We then investigated the effect of IL-21 blockade on IL-17 production by culturing anti-CD3/CD28-stimulated LPMCs from 4 CD patients with an
anti-IL-21 blocking antibody (Figure 8C). The anti-IL-21 antibody significantly (p<0.05) decreased the IL-17 production in comparison to IgG-treated cells (from mean 700 ± 291 pg/ml to 450 ± 105 pg/ml). A significant (p<0.005) difference was also found in the production of IL-17 between anti-CD3/CD28-stimulated cells cultured with IgG and unstimulated conditions (20 ± 8 pg/ml).

Figure 8. Levels (pg/ml) of IL-17 (A) and IFN-γ (B) in the supernatants of anti-CD3/CD28-stimulated LPMCs from the inflamed colon of 3 ulcerative colitis (UC) patients and 3 Crohn’s disease (CD) patient, cultured for 48h with 5 ng/ml IL-12. Data were analyzed using the Student’s t test. Horizontal bars are mean. *p<0.05 versus anti-CD3/CD28-stimulated LPMCs; **p<0.005 versus anti-CD3/CD28-stimulated LPMCs. C. Levels (pg/ml) of IL-17 in the supernatants of LPMCs isolated from the inflamed colon of 4 CD patients stimulated with anti-CD3/CD28 antibodies and cultured for 48h with 20 µg/ml anti-IL-21 blocking antibody or its isotype control (mouse IgG). Results are mean ± SD. *p<0.005 versus unstimulated LPMCs; **p<0.05 versus
3.5 Control of IL-17 and IFN-γ Production by TGF-β1

To investigate the role of TGF-β in modulating IL-17 and IFN-γ production, we cultured anti-CD3/CD28-stimulated LPMCs from 4 CD patients and 3 UC patients with different concentrations of TGF-β1. As shown in Figure 9, at different concentrations of TGF-β1 (10, 1, 0.1 and 0.01 ng/ml) the mean production of IL-17 was respectively 67.7%, 61.2%, 89.6% and 86.2% of that measured in the presence of medium only, while the mean production of IFN-γ was respectively 45.9%, 41.6%, 69.9% and 92.1% of that measured in the presence of medium only. When we compared the inhibitory effects of TGF-β1 on IFN-γ with those on IL-17, we found a significant (p<0.05) greater effect on IFN-γ production in comparison to IL-17 production at the TGF-β1 concentrations of 10 and 1 ng/ml. No significant difference was found at the TGF-β1 concentrations of 0.1 and 0.01 ng/ml.

![Figure 9](image)

**Figure 9.** Production of IL-17 and IFN-γ by anti-CD3/CD28-stimulated LPMCs isolated from the inflamed colon of 4 Crohn’s disease (CD) patients and 3 ulcerative colitis patients (UC), and cultured for 48h with different concentrations of TGF-β1 (10, 1, 0.1 and 0.01 ng/ml). Cytokine levels in the supernatants of LPMCs cultured with TGF-β1 are expressed as percentage of the cytokine production in the presence of medium only.
Data were analyzed using the Student’s t test. Horizontal bars are mean.

4. Discussion

The present study provides evidence that IL-17 production by gut biopsies grown ex vivo and LPMCs cultured in vitro is higher in IBD patients than in control subjects, and is associated with an increased percentage of Th17 and Th1/Th17 cells. Moreover, it seems that a differential regulation is played by pro- and anti-inflammatory cytokines on IL-17 and IFN-γ production by IBD LPMCs, the former down-regulated by IL-12 but not TGF-β, and the latter up-regulated by IL-12 and down-regulated by TGF-β. Finally, the results support a crucial role for IL-21 in controlling IL-17 production by CD LPMCs.

I have first observed that IL-17 production from organ culture biopsies is increased in IBD patients in comparison to controls almost at the same levels of IFN-γ. In contrast with Kobayashi et al. [215], who found a higher in vivo mucosal production of IL-17 in UC than in CD, I did not observed any significant difference between these two disorders. When I measured the concentration of IL-17 in the supernatants of isolated LPMCs, I found a higher production of IL-17 and IFN-γ in IBD patients in comparison to controls, both in unstimulated and stimulated conditions. Again, no difference was found between CD and UC LPMCs. The fact that IL-17 production by organ culture biopsies was not increased in uninflamed areas of IBD patients further strengthens the key role of IL-17 in mediating inflammation. Of note, the absence of IL-17 up-regulation in both unstimulated and stimulated LPMCs from inflamed areas of non-IBD patients suggests the specific contribution of IL-17 to the mucosal damage in CD and UC.

IL-17 had been already investigated in the gut of IBD patients by immunohistochemistry which showed that IL-17 is markedly expressed by CD3+ and CD68+ cells in the inflamed mucosa [76]. Here I have used a dual parameter intracellular flow cytometry to spot IL-17 and IFN-γ
producing cells. Three cellular subsets have been identified, namely IFN-γ-producing T cells (Th1), IL-17-producing T cells (Th17), and IL-17/IFN-γ co-producing T cells (Th1/Th17). All were increased in CD and UC patients, without any difference between UC and CD. Interestingly, I have observed that the majority of Th1/Th17 cells expressed CD161, a well known marker of NK T cells recently identified on IL-17-producing memory T cells [166].

There is good evidence that IL-17 production is differentially regulated in mouse and human. In mice, IL-1β, IL-6 and TGF-β appear to be responsible for Th17 cell differentiation [155,221], while IL-23 is required for their expansion and/or maintenance [154]. In contrast, human studies showed that TGF-β, IL-1β and IL-6 are critical for the priming of Th17 responses [132,155,162]. More recent studies have shown that IL-21, IL-23, TGF-β, and IL-1β plus IL-6 are able to induce IL-17 production from naïve human CD4+ T cells isolated from umbilical cord blood [164,165]. Hence, it seems that IL-1β and IL-6 induce IL-17 secretion by central memory T cells, while TGF-β and IL-21 are required to promote the differentiation of naïve CD4+ T cells into Th17 cells [163]. In my experience, neither IL-1β plus IL-6 nor IL-23 alone were able to induce IL-17 and IFN-γ production by anti-CD3/CD28-stimulated IBD LPMCs. The discrepancy between these and Yang’s [163] data might depend on the different source of cells used - peripheral blood or inflamed mucosa- which could affect the effector-to-memory T cell ratio in the cell sample. In contrast, IL-12 greatly enhanced IFN-γ production, while it reduced IL-17 secreted by anti-CD3/CD28-stimulated IBD LPMCs. Given that it is known that IFN-γ in CD is driven by IL-12 and accessory cytokines such as IL-18 and IL-21, the notion that IL-12 suppresses IL-17 production is interesting because it suggests that perhaps a component of the rather weak efficacy of anti-IL-12/IL-23 p40 antibody in active CD is that it allows Th17 differentiation and a second effector function activated by the therapy itself. However, it must be also noted that IFN-γ, induced by IL-12, appears to play a role in limiting IL-17 production [222].
Interestingly, TGF-β, a cytokine known to have a pleiotropic function in controlling gut inflammation [223], significantly reduced IFN-γ production by anti-CD3/CD28-stimulated IBD LPMCs, while the inhibitory effect was not significant on IL-17 production at the same concentrations. Although experiments have been performed in a small number of patients, this might shed light on the role of IL-17 in IBD as a key cytokine in sustaining chronic gut inflammation, which seems to be less regulated by immunomodulatory factors like TGF-β. It has been previously shown that IFN-γ transcripts are not as well inhibited by active TGF-β in cells from CD patients as they are in cells from controls [224]. I confirmed this here in that TGF-β did not completely shut off IFN-γ production, only reducing it by about 50%. This is in keeping with a couple of studies which demonstrated that Th17 cells appear to be resistant to the suppressive effects of TGF-β-producing Tregs [149,225]. The lack of effect of TGF-β is due to the presence of Smad7, the intracellular inhibitor of TGF-β signalling in cells from IBD patients [226]. Critically however to understand why TGF-β only barely inhibited IL-17 production in IBD is the fact that TGF-β appears to be important for Th17 cell generation. It has been proposed by Zhou et al. [161] that T cells receiving a TGF-β signal can develop into either the T regulatory cell or Th17 lineage. Foxp3 induction may restrain the differentiation of inflammatory Th17 cells in response to TGF-β in the absence of other pro-inflammatory cytokines by inhibiting RORγt activity. In the presence of pro-inflammatory cytokines, the suppression of Foxp3 expression and inhibitory function, together with the concurrent up-regulation or stabilization of RORγt expression, may favour the development into Th17 lineage. Thus, a fine balance between RORγt and Foxp3 might be critical for immune homeostasis in the gut.

IL-21 is another pro-inflammatory cytokine overproduced in the inflamed intestine of patients with CD. CD4+ T cells infiltrating CD mucosa are the main source of IL-21. In CD LPMC cultures, IL-21 blockade reduces the expression of p-Stat4 and T-bet and the production of IFN-γ [191]. In keeping with the findings of Fina et al. [78], these
results show that IL-21 blockade significantly reduces IL-17 secretion by CD LPMCs.

In conclusion, this study demonstrates the importance of Th17 and Th1/Th17 cells in the pathogenesis of IBD, and further clarifies which stimuli can modulate and maintain IL-17 production. The differential effect of TGF-β on IL-17 and IFN-γ production suggests that IL-17 plays a crucial role in sustaining chronic inflammation. These findings also support a role for IL-21, IL-12 but not TGF-β in modulating IL-17 production in IBD.

Treatment strategies in IBD are largely focused on the suppression or modulation of the excessive immune response in the gut. However, due to the involvement of different cytokines and individual variability, the main challenge is to identify inflammatory pathways and develop disease-specific therapies that can be tailored for each patient [74]. The multiple faces of the pathogenesis of IBD will lead soon to develop disease-specific therapies and hopefully to recognize which patients will better respond to a particular therapy. In this context, this study suggests that the abundant IL-17 in inflamed mucosa may also help explain the relative lack of efficacy of anti-IFN-γ antibodies in clinical trials, suggesting the existence of a Th1/Th17 population which may represent an intermediate phenotype between Th1 and Th17 cells.
References


90. Singh S, Graff LA, Bernstein CN. Do NSAIDs, antibiotics, infections, or stress trigger flares in IBD? Am J Gastroenterol 2009;104:1298-313.


140. Wong CK, Ho CY, Li EK, Lam CW. Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. Lupus 2000;9:589-93.


145. Wong CK, Ho CY, Ko FW, et al. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-gamma, IL-4, IL-10 and IL-13) in patients with allergic asthma. Clin Exp Immunol 2001;125:177-83.


151. Murphy AC, Lalor SJ, Lynch MA, Mills KH. Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the


190. Awane M, Andres PG, Li DJ, Reinecker HC. NF-kappa B-inducing kinase is a common mediator of IL-17-, TNF-alpha-, and IL-1 beta-induced chemokine promoter activation in intestinal epithelial cells. J Immunol 1999;162:5337-44.
lamina propria mononuclear cells. Gastroenterology 1997;112:1169-78.


213. Brand S, Beigel F, Olszak T, et al. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression...
and intestinal epithelial cell migration. Am J Physiol Gastrointest Liver Physiol 2006;290:G827-38.


