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ROLE OF REGULATORY CELLS AND TISSUE CYTOKINES IN PATIENTS WITH ULCERATIVE COLITIS: IMPLICATIONS FOR PATIENT-ORIENTED THERAPY

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ABSTRACT

Ulcerative colitis (UC) is an inflammatory bowel disease that results in a chronic inflammation and ulcers of the mucosa of the colon. In UC, two important issues, specifically related to the prognosis, remain to be clarified. A) Extension of disease greatly influences the prognosis and biological factors involved in the limitation of the disease extent are presently unknown B) Due to increased availability of biological drugs targeting different cytokines, biological therapy need to be optimized on the key cytokine(s) relevant for the inflammatory process. Aim: A) We hypothesized that regulatory T cells might be relevant in limiting the extension of inflammation and that B) UC patients might be stratified according to distinctive cytokine profiles. Methods: In a cross-sectional study, we evaluated the relationship between UC extension and the proportions of CD3+CD4+Foxp3+ and CD3+CD4+LAP+Foxp3-Tregs in the colonic lamina propria (LP) in UC patients undergoing colonoscopy for clinical relapse and controls undergoing colonoscopy for non-inflammatory conditions. The role of these cells in UC extension was also investigated in the murine oxazolone-induced colitis model. B) At the same time, we analyzed tissue cytokine by RTqPCR in endoscopic biopsies from uninvolved and involved tissue of UC patients and controls. Mucosal microbiota analysis was also performed. Results: A) Involved and uninvolved tissue show different and opposite frequency of LP CD3+CD4+Foxp3+ and LP CD3+CD4+LAP+ Tregs with the latter significantly increased in uninvolved *vs* involved tissue. In mice with oxazolone-induced distal colitis, treatment with α-LAP-depleting antibody was associated with the development of extensive colitis; B) We quantified by RTqPCR TNF-α, IFN-γ, IL-10, IL-6, IL-17, and IL-13 and analyzed the results by r square of the k means for cluster solution. IL-17A and IL-13 mRNA tissue content show classificatory power and their distribution points to the existence of discrete clusters of subjects. Six clusters define the optimal partition of data explaining 91% of the total information present in the data set (R-square=0.91). UC patients were distributed in two clusters characterized by high/low IL-13 mRNA tissue content in the context of high IL-17A mRNA tissue content. The two subsets differ in clinical-pathological characteristics. High IL-13mRNA patients are younger at diagnosis and show higher prevalence of extensive colitis than low IL-13mRNA ones. They also show a more frequent use of steroid/immunosuppressant/anti-TNFα therapy during a one-year follow-up. The two subgroups show a differential enrichment of mucosa associated microbiota genera with prevalence of *Prevotella* in patients with

high IL-13mRNA tissue content and *Sutterella* and *Acidaminococcus* in patients with low IL-13mRNA tissue content. Conclusion: A) LP CD3+CD4+LAP+ Tregs efficiently limit the extension of inflammatory lesions. Therapeutic strategy aimed at their expansion may help in containing the spreading of inflammation and possibly in preventing inflammatory relapse. B) Evaluation of IL-13 and IL-17 mRNA content allows the identification of different UC patients' subsets with associated different clinic phenotypes. Therapeutic options might be optimized according to the patients' tissue cytokine profile.

INTRODUCTION

Ulcerative Colitis (UC) is a chronic inflammatory bowel disease (IBD) that results in a chronic inflammation and ulcers of the mucosa of the colon. The inflammation of UC characteristically affects the superficial mucosal layer of the large bowel wall. This inflammation invariably affects the rectal mucosa, and may also affect more proximal colon areas in an uninterrupted fashion

EPIDEMIOLOGY OF UC

Ulcerative colitis can develop at any age but is most commonly diagnosed between the second and third decade of life. The incidence appears to follow a bimodal age distribution with signs and symptoms frequently occurring either in early adulthood or much later in life, at 50-70 years of age. No sex predominance exists in UC (1). The occurrence of UC has been increasing in developed, urbanized countries since the mid-twentieth century, which suggest the presence of environmental risk factor that are unique in this society (2).

Worldwide, UC is more common than Crohn disease. The highest incidences of UC have been reported in northern Europe (24/100000), in Australia (17.4/100000) and North America (19.2/100000) (3). IBD has emerged in countries in which it had rarely been previously reported, including South Korea, China, India, Iran, Lebanon, Thailand, the French West Indies, and North Africa. In these countries, the occurrence of UC preceded that of Crohn's Disease (CD) by about 10 years. In some countries, such as Japan, the incidence of IBD was initially low but has recently increased. A recent systematic review shows different prevalence and incidence of the disease in Eastern, Northern, Southern and Western Europe (Table 1) (4).

Table 1. Prevalence and incidence of Ulcerative Colitis

Ulcerative colitis				
	Incidence per 100 000 person-years		**Prevalence per 100 000**	
	Lowest estimate	**Highest estimate**	**Lowest estimate**	**Highest estimate**
Eastern Europe	0·97	11·9	2·42	340·0
Northern Europe	1·7	57·9	90·8	505·0
Southern Europe	3·3	11·47	14·5	133·9
Western Europe	1·9	17·2	43·1	412·0

In Italy, a study based on the analysis of administrative databases of a region of central Italy (Lazio) provides a population-based prevalence and incidence estimation (5). Prevalence of UC was 177 cases per 100,000 (95% CI: 172–182) in males and 144 cases per 100,000 (95% CI: 140–149) in females, while incidence was 14.5 and 12.2/100000 for male and female respectively. Overall data are similar to the highest estimate for Southern Europe.

Among migrant populations, age at time of migration affects IBD risk; the risk of developing an IBD is highest among children that migrate before the age of 15 years. The observation that geographic variability in incidence parallels a gradient in economic growth and the rise in income that accompanies urbanization, together with the change observed in prevalence in some ethnic groups after migrations to more developed countries suggest that environmental factors have a role in the disease occurrence. Incidence of ulcerative colitis is higher in developed countries than in developing countries, and in urban versus rural areas. These findings could be partly explained by increased access to health care and better medical records in more developed than less developed countries. Furthermore, improved sanitation in industrialized countries might reduce exposure to enteric infections during childhood, thus restricting maturation of the mucosal immune system, which could result in an inappropriate immune response when exposure to infectious microorganisms occurs later in life (6).

RISK FACTORS (GENETIC, ENVIRONMENTAL, MICROBIOTA)

The etiology of ulcerative colitis is unknown but it is generally believed that the disease results from an inappropriate immune response to the enteric microbiota and/or environmental stimuli in a genetically susceptible host (7). Disruption of the intestinal mucosa barrier and perturbations of the intestinal microbiota associated with altered immunological response are believed to be important players in the development of the disease (8, 9).

Genetic factors

The possibility that genetic factors contribute to disease pathogenesis in IBD was first suggested by the observation that IBD cases frequently cluster within families. 8–14% of patients with ulcerative colitis have a

family history of inflammatory bowel disease and first-degree relatives have four times the risk of developing the disease. In addition, such studies showed that in families with several affected members, 75% are concordant for disease type (all affected family members have either exclusively CD or UC), and the remaining 25% are non-concordant (one affected member with CD and another with UC) (10).

Additional insight into the contribution of genetic factors in IBD was provided by concordance studies in twins. The basic notion underlying such studies is that significantly higher concordance rates among monozygotic (MZ) than dizygotic (DZ) twins pairs predict a strong genetic influence, whereas equal concordance rates in these pairs predict a strong environment influence. In UC the MZ twins concordance rate is 18% whereas the DZ twins concordance rate is not significantly suggesting a genetic influence on occurrence of UC although less pronounced when compared with genetic influence observed in Crohn's Disease (MZ twins *vs* DZ twins concordance: 58.3% vs 0%, respectively) (11). Genome-wide association studies have identified > 240 risk loci for inflammatory bowel disease, with most genes contributing to both ulcerative colitis and Crohn's disease phenotypes (12). However, genetics only explain 7.5% of disease variance, have little predictive capacity for phenotype, and currently are of limited clinical use. In UC the presence of association with polymorphisms in loci potentially involved in epithelial barrier function such as loci associated with ECM1, HNF4A, CDH1, and LAMB1 genes provides a way forward for the elucidation of molecular defects of epithelial cells, which could lead to and thus explain barrier defects. An additional set of genetic abnormalities affecting epithelial barrier function involve genes concerned with mucus production and function. One such genetic abnormality results from missense mutations of the Muc2 gene leading to abnormal assembly of Muc2 and accumulation of Muc2 precursors in the endoplasmic reticulum (ER) of goblet cells with consequentenhanced ER stress response and cell death. Risk loci associated with genes affecting mucosal immune cell responses involve the TNFRSF9, FCGR2A, IL7R, IL- 22, and IL-26 genes. Finally, HLA-DQA1 (particularly HLA haplotype DRB1*0103) variants are associated with UC susceptibility suggesting that this disease is related (at least in part) to adaptive immune responses resulting from presentation of a specific antigen to T cells in the colonic environment.

Taken together it appears that the risk polymorphisms specifically associated with UC point to epithelial cell abnormalities, suggesting that a primary epithelial cell defect might be central to disease pathogenesis in UC.

Gut Microbiota

The human intestine constitutes one of the microbial ecosystems with great density of population presents in nature. The human intestinal microbiota, with a quantity that ranges around 10^{13}-10^{14} microorganisms/ g colonic content with a biomass > 1 kg, outnumbers the microbial communities attached to the other surfaces of the human body and they are about 10 times higher than the total number of our somatic and germinal cells (13). The composition of the intestinal microbiota depends on the number and on the type of microorganisms with which the subject comes into contact from the birth and in the early years of life and on the genetic background of the same subject. The variety of environmental interactions and microbial populations with which the subject comes into contact make the microbiota of a single subject exclusive. In the context of a specific intestinal habitat, some microbial components are autochthonous or "residents", while others, so called allochthonous components, originate from the ingested food, from the water or from other elements present in the external environment and these tight interactions make this ecosystem very dynamic. The relationship that is established between the host and components of the microbiota has often been described as commensalism (a partner draws benefit while the other doesn't result influenced), but it is more appropriated to define it as symbiotic or mutual (both the partners increase their fitness). The intestinal microbiota has been demonstrated to be involved in metabolic, trophic and protective functions and contributes to the development and function of immunity. The microbiota in both the colon and small bowel of normal individuals is largely composed of bacteria of six bacterial phyla, Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (the latter two are rarer in humans). Firmicutes and Bacteroidetes comprise nearly 90% of the total bacterial population (14). About half of these dominant phyla were in turn composed largely of bacteria belonging to the Bacteroidales order of the Bacteroidetes and the Clostridiales order (Lachnospiraceae family containing the *Clostridium XIVa* and *IV* groups) of the Firmicutes. In the small intestine increased numbers of bacteria belonging to the *Bacilli* subgroup of Firmicutes (predominately *Lactobacillus*) and to the Actinobacteria and correspondingly decreased numbers of bacteria belonging to the Bacteroidetes and the Lachnospiraceae family of the Firmicutes were found compared with the large intestine. Analysis of the microbiota in colorectum of patients with IBD (both CD and UC) as a whole differ from that in controls in that Bacteroidetes and Firmicutes (Lachnospiraceae family) bacteria were decreased whereas

Proteobacteria (which includes the *E. coli* species) were increased. Alterations of microbiota closely related to the pathogenesis and progression of UC were described (15). In UC it has been reported a decrease of the butyrate-producing species such as *Roseburia hominis* and *Faecalibacterium prausnitzii* (16) which persists also in patients in clinical remission (17). In these patients a positive correlation between *F. prausnitzii* levels and the length of remission and inverse correlation with frequency of clinical relapse was observed (17). Furthermore, *Clostridium coccoides* (also called *Clostridium clusters XIVa*) and *C. leptum* (namely, *Clostridium clusters IV*), were reduced in the UC (18). *Faecalibacterium prausnitzii* with *Clostridium clusters IV* and *XIVa*, are the main producers in healthy mucosa of short-chain fatty acids (SCFAs) that have an anti-inflammatory effect that results from the stabilization and production of regulatory T (Treg) cells. This latter effect is due to the ability of SCFA to directly affect naïve T cells to steer their differentiation into IL-10-producing T cells. Furthermore, SCFA are the main source of energy supply of intestinal epithelial and contribute to condition epithelial cells to produce immune mediators that enhance the gut barrier function. In addition to the two major *Clostridium* species (*Clostridium coccoides*, and *Clostridium leptum*) cited above which have beneficial effect on the mucosa and have been described to be reduced in UC. *C. difficile* infection is increasingly recognized as complicating a significant proportion of patients with UC (prevalence ranging from 5% to 47%) and is associated with an increased risk of hospitalizations, surgery, and even mortality (19).

Environmental factors

Several environmental factors act as triggers or protective factors for ulcerative colitis.

Tobacco Use. A meta-analysis showed that smoking is protective against ulcerative colitis compared with non-smoking (20). Furthermore, who smokes tend to have a more mild disease course than do non-smokers, have a lower risk of colectomy, and disease activity is often increased in those who stop smoking. Smoking cessation in UC is frequently associated with relapse. Smoking modulates both immune responses and the diversity and composition of the gut microbiota through which it may exert its influence on disease risk.

Infections. Episodes of previous gastrointestinal infection (especially *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp.) (21) double the risk of subsequent development of ulcerative colitis, which suggests that acute intestinal infection might lead to changes in gut microbiota, hence triggering the start of a chronic inflammatory process in genetically predisposed individuals (22) .

Appendectomy. Appendectomy before 20 years of age might be protective against ulcerative colitis reducing the risk of development of ulcerative colitis by 69% (23). It has been postulated that the increased CD4/CD8 ratio and infiltration of CD4+ and CD69+ T cells in the appendix of patients with UC may act as a priming site in the pathogenesis of UC (24). However, the effect of appendectomy on the course of UC remains uncertain.

Mode of delivery, breastfeeding, early antibiotic administration. Although it remains unclear whether dysbiosis is causal or a consequence of inflammation in the gut, several environmental factors implicated in the pathogenesis of IBD mediate their effect through altering the gut microbiota (25). Important among these is the exposure to antibiotics. In population-based studies, exposure to antibiotics within the first year of life was associated with an increased risk of IBD, particularly CD (26).

Furthermore the microbiome of an infant is directly correlated with events such as the mode of delivery, intrapartum antibiotics, and breastfeeding. Breastfeeding is protective against subsequent development of UC but only when the duration of breastfeeding is more than 3 months (27).

Diet. Among all the environmental factors, diet is thought to play an important role in the etiopathogenesis of IBD. Diet-induced microbiota dysbiosis, favors the production of proinflammatory products, the disruption of mucus layer, and the increase of intestinal permeability. Fiber intake, if obtained from vegetables or fruits, had the greatest reduction in risk compared to fiber obtained from cereals or whole grains. Higher intake of dietary n-3 polyunsaturated fatty acids (PUFAs; e.g., fish oil), eicosapentaenoic acid, and docosahexaenoic acid was associated with a lower incidence of UC in middle-aged adults; in contrast, a diet rich in n-6 PUFAs (which, in turn, is metabolized to arachidonic acid) was associated with an increased risk of UC due to proinflammatory properties (28, 29). High sulfur– or high sulfate–containing foods were associated with relapse in UC. Sulfur-containing food items, such as preserved meat, alcoholic beverages, milk, dietary supplements such as chondroitin sulfate, or food additives such as carrageenan, are common with the westernization of diet. Sulfate-reducing bacteria produce toxic hydrogen sulfide as a result of oxidative metabolism (24). As opposite some dietary components might have anti-inflammatory properties. Curcumin is a phytochemical derived from the herb turmeric (*Curcuma longa*), a commonly used spice in Asian cuisine and medicine known to have antioxidant and anti-inflammatory properties acting by the inhibition of the TNF pathway as well as in CD4 T-cell proliferation. In a randomized, placebo-controlled trial, curcumin resulted

beneficial to maintain remission in UC (30). A randomized, controlled clinical trial in patients with active mild to moderate UC, showed that high-dose curcumin in combination with mesalamine was superior to mesalamine alone (31). After diagnosis, the majority of patients with IBD believe that diet plays a role in their disease. Certain exclusionary diets such as the Specific Carbohydrate Diet; the low–fermentable oligo-, di-, and monosaccharides and polyol (FODMAP) diet; and the Paleolithic diet are commonly used by patients with IBD. These diets are promoted in the lay literature through anecdotal success stories but to date lack rigorous scientific assessment.

SIGNS AND SYMPTOMS AND NATURAL HISTORY OF ULCERATIVE COLITIS

The signs and symptoms of ulcerative colitis depend on localization and severity of the disease. The hallmarks of the disease include bloody diarrhea with concomitant rectal urgency and/or rectal bleeding. This is usually associated with abdominal pain and loss of weight. The onset of these symptoms is typically gradual, often following by period of spontaneous remission and subsequent relapses. Systemic symptoms such as malaise, fever and cachexia may also occur, but are usually associated with a more severe disease flare. Patients may also progress to a condition known as fulminant colitis, which is characterized by extensive bleeding, associated anemia frequently requiring blood transfusion, elevated temperature, abdominal tenderness, and the colonic dilatation with absence of bowel sounds (known as toxic mega-colon), and which can occur in unusually severe cases. This latter condition implies the presence of loss of neurologic control of colonic function and is potentially fatal (2). Although UC is limited to the colon, up to a third of cases may have extraintestinal manifestations of the disease. There are many extraintestinal manifestations associated with UC, but the most common affect the skin, joints, eyes, and liver. Arthropathies are the most common extraintestinal manifestations.

A detailed knowledge of the natural history of UC is essential to understand disease evolution, evaluate the impact of treatment strategy, identify predictors of outcomes and provide comprehensive information to patients. In population based studies, at presentation, 30–60% of patients with ulcerative colitis have proctitis, 16–45% have left-sided colitis, and 14–35% have extensive colitis and pancolitis (Figure 1) (3). The inflammatory lesions can progress proximally in 10–19% of patients after 5 years, and in up to 28% of patients at 10 years. Rate of progression from proctitis to left-sided colitis was 28–30%, and to extensive

colitis was 14–16%; rate of progression from left-sided colitis to extensive colitis was 21–34%. Overall rates of progression ranged from 12–30%, with cumulative 5-year risk of progression being approximately 13% (32). Most patients with ulcerative colitis have a relapsing and remitting disease course with periodic flares. Age of onset appears to affect the disease course, since patients with disease onset after age 60 years tend to have milder disease compared with younger patients. Risk factors for aggressive or complicated disease include a younger age at onset (<40 years), pancolitis, lack of endoscopic healing while in clinical remission, deep ulcerations, and high concentrations of perinuclear antineutrophil cytoplasmic antibodies (P-ANCA) (33). Patients with UC are at increased risk of colon cancer (CRC) and should undergo a regular endoscopic surveillance to detect dysplasia and early cancer. Despite medical treatments, approximately 15% of patients require surgery for their disease. The most common indications for surgery include toxic megacolon, perforation, uncontrollable hemorrhage, failing medical therapy (or corticosteroid dependence), cancer, or unresectable dysplasia (34).

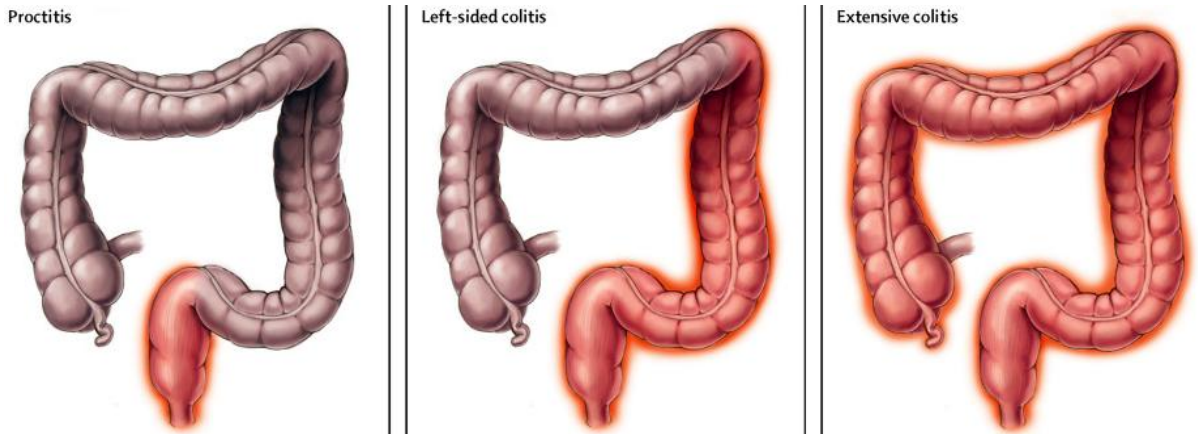


Figure 1. Extension of inflammatory lesion in Ulcerative Colitis patients. (figure modified from Ungaro R, et al. Ulcerative colitis. Lancet. 2017;389(10080):1756–1770).

DIAGNOSIS AND MANAGEMENT

Ulcerative colitis is diagnosed on the basis of presenting symptoms consistent with the disease and findings on sigmoidoscopy or colonoscopy showing continuous colonic inflammation starting in the rectum. Biopsies of the colon are confirmatory when specimens show findings consistent with chronic inflammatory changes. Mesalazina, corticosteroids, immunosuppressant and different biologics are used in the treatment of ulcerative colitis. Their use varies according with the activity of disease, the extension of inflammatory lesions (proctitis, left-sided, extensive colitis), and pattern of disease. The disease pattern includes relapse frequency, course of disease, response to previous medications, side-effect profile of medication and extra-intestinal manifestations. The age at onset and disease duration may also be important factors (34, 35).

PATHOPHYSIOLOGY AND IMMUNOPATHOGENESIS OF UC

The prevalent hypothesis for the pathogenesis of UC is that this disease is associated with defect in epithelial cell function where mucous barrier and epithelial barrier defects are strongly compromised (Figure 2) (36). These defects can be either an initiator of the underlying lamina propria inflammation or a secondary factor that greatly amplifies lamina propria inflammation arising from another cause. The epithelial dysfunctions alter intestinal barrier and thus allow entry of commensal microbiota into the lamina propria that come into contact with the mucosal immune system. It is also possible that the contact of the gut microbiota with epithelial cells (in the absence of penetration of bacteria into the lamina propria) could trigger a cytokine response that then initiates and sustains the inflammation.

The mucosa of the gastrointestinal tract is, in the human body, the widest surface of contact with the external environment (Figure 2). A single layer of epithelial cells separates the external environment from that inside, characterized by the presence of cells belonging to the immune system strategically organized below the epithelial barrier to point out the presence of a continuous information interchange among the two environments. The epithelial barrier actively participates in the information interchange among external and internal environment. The integrity of the epithelial barrier is crucial in protecting the host from the microorganisms and from the alimentary antigens.

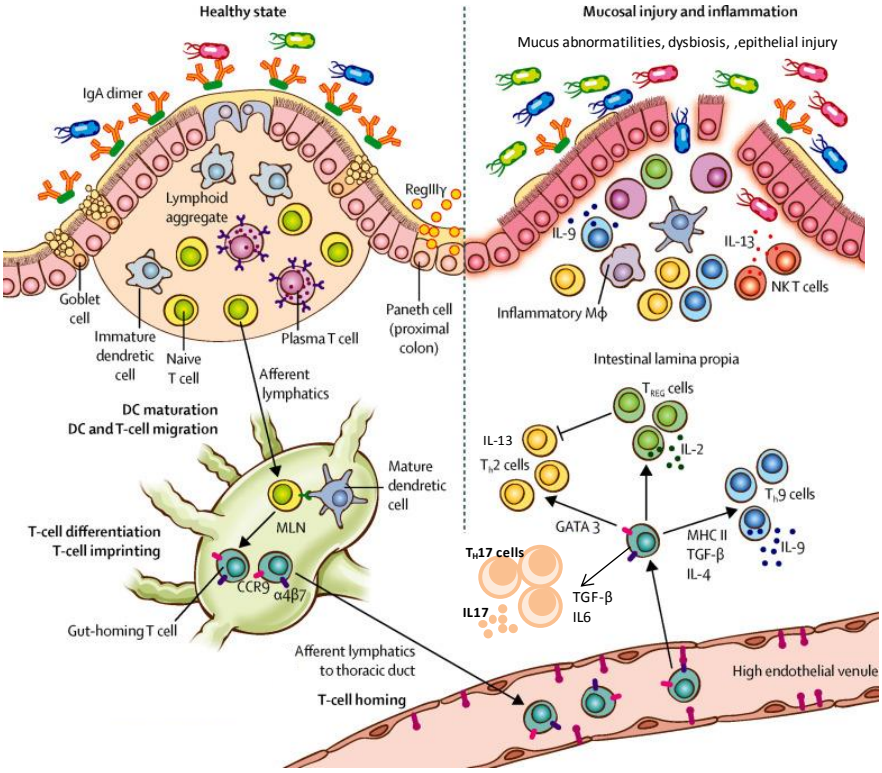


Figure 2. Overview of the intestinal immune system in the healthy state and in ulcerative colitis (figure modified from Ungaro R et al. Ulcerative colitis. Lancet. 2017;389(10080):1756–1770).

The intestinal epithelium includes different types of cells: the enterocytes that have absorptive function, the goblet cells appointed to the production of mucus and trefoil factor peptides (TFF) which has been shown to be important for mucosal restitution together with mucins, the Paneth cells that have the ability to produce antimicrobial peptides and enteroendocrine cells. The mucin (mainly Mucin-2/ Muc2) is a glycoprotein whose glucidic part, being highly absorbent, attracts a lot of water increasing the stringiness. The mucus is organized in a double layer with a denser layer to contact with the epithelial cells and one, less dense, directed toward the intestinal lumen. The microorganisms of the gut microbiota primarily occupy this layer reaching only in small measure the deep layer. At this side it becomes admixed with other secreted molecules such as immunoglobulin (Ig)A antibodies and low molecular weight antimicrobial peptides (37). In the absence of inflammation the mucus layer is about 700μm thick and composed of a dense inner layer in contact with the epithelium and a loose outer layer. Defective mucus layers resulting from lack of MUC2 mucin, mutated Muc2 mucin vWF domains, or from deletion of core mucin glycosyltransferase enzymes in mice result in increased bacterial adhesion to the surface epithelium, increased intestinal permeability, and enhanced susceptibility to colitis (38).

UC is characterized by goblet cell depletion as well as diminished goblet cell thecae; consequently, there is a thinned mucous layer. However, in the case of IL-10 deficiency mouse model, for instance, mild inflammation was associated with normal or even increased mucus thickness and normal-appearing goblet cells, but the mucus produced was more penetrable (39). This is likely related to the fact that IL-10 has been shown to act on goblet cells to promote mucus secretion and prevent mucus misfolding.

Epithelial barrier dysfunction in IBD can also occur as a result of various abnormalities of enterocyte junctions, and indeed there are several reports that dysregulation of various junctional structures such as tight junctions (TJs), adherens junctions, and desmosomes occur in this disease. Structurally, TJs consist of a complex network of strands composed of four different membrane proteins: occludin, claudins, tricellulin, and junctional adhesion protein, which are attached to a perijunctional actin-myosin ring. In IBD TJs exhibit reduced complexity characterized by strand breaks as well as reduced numbers of strands and meshwork depth (40). There is evidence that abnormalities of tricellulin occur that cause increased passage of macromolecules in UC (41). The most obvious hypothesis for the cause of TJ abnormalities is that inflammatory cytokines driving the inflammation have an adverse effect on TJs and increased apoptosis; in UC, IL-13 or IL-9 are likely initial mediators of these effects (42) a process that contributes to barrier dysfunction via incomplete epithelial cell restitution after injury. The mechanism of IL-13-induced epithelial cell apoptosis has been shown to involve IL-13 activation of TWEAK, a TNF-receptor superfamily cytokine, which then interacts with its receptor, Fn14, and thus forms a complex that causes epithelial cell secretion of endogenous TNF-α. The secreted TNF-α then causes caspase activation and apoptosis (43) (Figure 3). Interestingly, IL-13 initiation of TWEAK activation may depend on signaling via the IL-13Rα2 receptor because IL-4 (which signals via IL-13Rα1, but not IL-13Rα2) does not have the same pro-apoptotic effect. The capacity of IL-13 to cause increased epithelial cell apoptosis highlights the fact that the latter process can be a cause of epithelial barrier dysfunction.

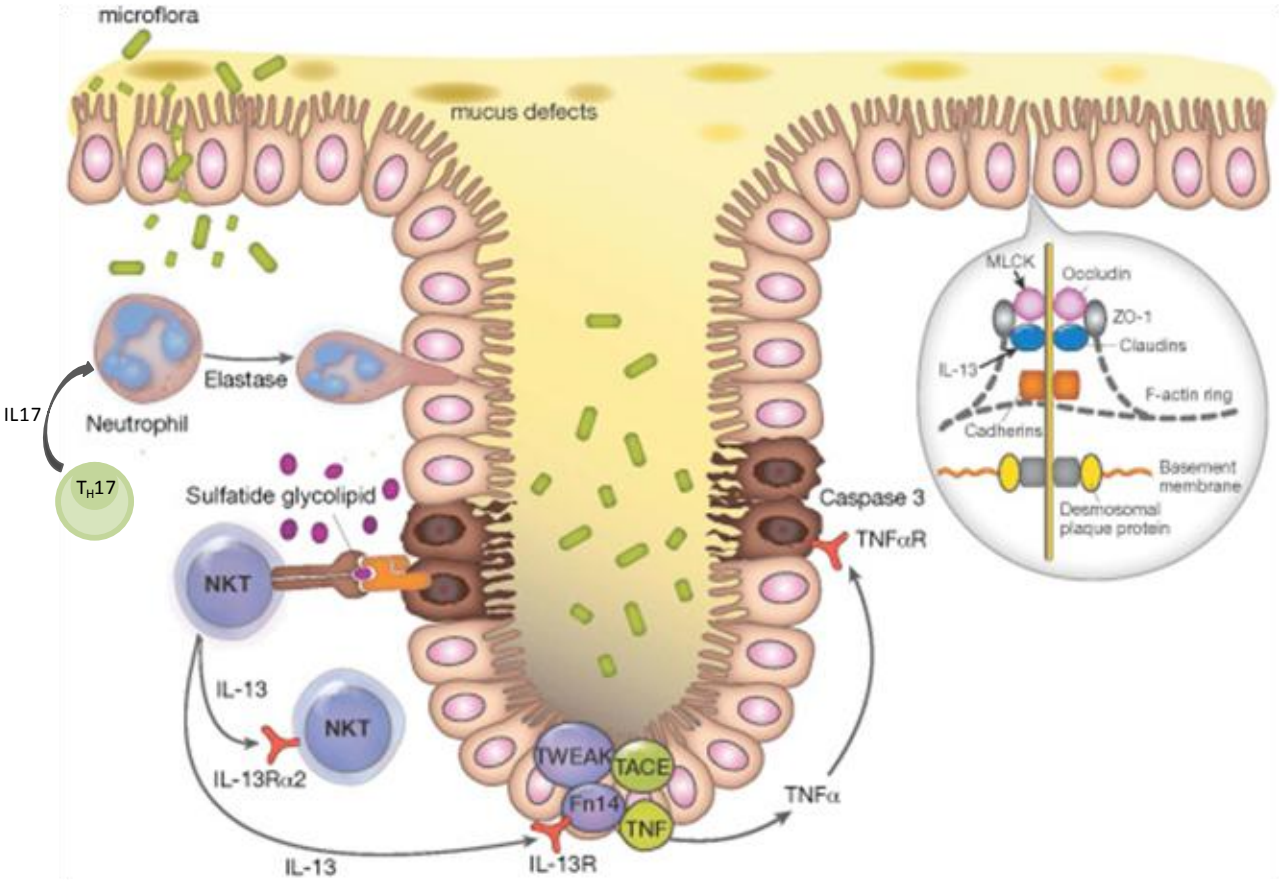


Figure 3. Mucosal barrier abnormalities in ulcerative colitis (Fuss IJ et al. Ulcerative colitis - Mucosal Immunology 2015, 2 (81):1573-1612).

Inside the lymphoid tissue associated to the intestine (Gut Associated Lymphoid Tissue, GALT), the lymphoid cells are either organized in follicles (Peyer's patches in the small intestine, lymphoid follicles in the colon) or dispersed in the lamina propria and in the layer of epithelial cells. The lymphoid structures (Peyer's patches, colon's lymphoid follicles) together with the mesenteric lymph nodes represent the sites of induction of the immune response. The dendritic cells once captured the antigens at the mucosa surface migrate into these sites in order to present the antigens to T lymphocytes. The T lymphocytes, once primed by the dendritic cells acquire the role of effectors of the immune response with different function (lymphocytes CD4+Th-1, lymphocytes CD4+Th-2, lymphocytes CD4+Th-17, lymphocytes T regulatory). They then migrate in the circulatory stream after the acquisition, on the surface, of the expression of integrins (α4β7, αEβ7) that allow their recognition by the lymphoid intestinal tissue where, they migrate back to perform their effector functions in the lamina propria (lymphocytes α4β7+) and in the epithelium (lymphocytes αEβ7+). Regulatory cells are able to inhibit the response and the proliferation of the effector T cells. Two subpopulations of cells can be

identified: natural CD4+ CD25+ (nTreg) regulatory T cells and peripheral regulatory T cells. The natural regulatory cells are originated in the thymus and constitutively express on their cellular membrane the CD25, the CTLA-4 and the GITR. An intracellular marker that is essential for their development and function is the transcription factor Foxp3 (X-linked, forkhead / winged helix transcription factor). The lack of Foxp3 is associated to a morbid condition linked to the chromosome X, called "IPEX", characterized by immune dysregulation, polyendocrinopathies, enteropathies. Foxp3 represses the promoters of the genes involved the inflammation (6). Peripheral regulatory cells are classically divided into 3 groups: inducible Foxp3+ cells (iTreg), regulatory T cells (Tr1) and the Th3 cells. The difference among the different groups of regulatory cells relies on the expression of Foxp3 that seems to characterize the iTregs alone. A forth group of cells has recently been described: the cells that express on their surface the TGF -β linked to its latency peptide called Latency-associated-Peptide (LAP+) T cells (Figure 4).

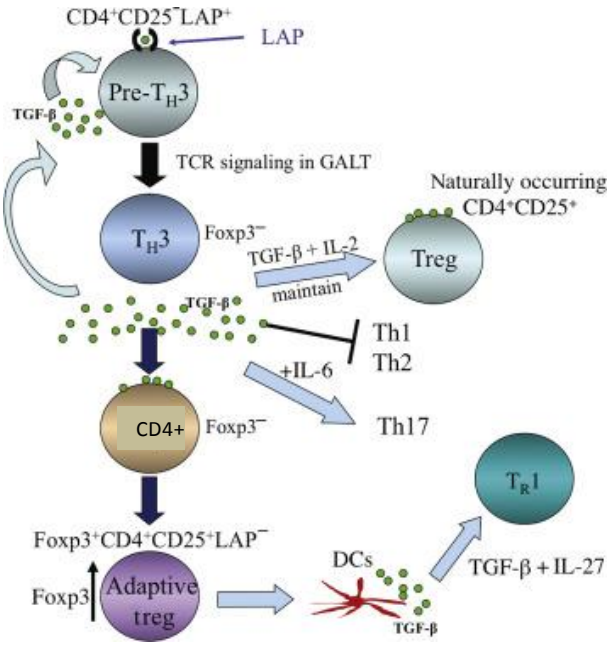


Figure 4. Hypothetical regulatory T cell cascade following induction of TH3 type Tregs (figure modified from Pabst O., et al. Chapter 41 - Mechanisms of Oral Tolerance to Soluble Protein Antigens. Mucosal Immunology (Fourth Edition) 2015;1:831-848

Besides a small % of regulatory Foxp3+ T cells expressing LAP (activated T cells) the vast majority of LAP + T cells do not express Foxp3. It is still under debate whether these cells might be considered as an additional group of regulatory cells or represents Th-3 and/or Tr1 activated cells.

Following triggering in the gut, a Th3 cell secretes TGF-β that acts to maintain naturally occurring CD4+CD25+Foxp3+ Tregs, suppress Th1 and Th2 responses, and, in concert with IL-6, may induce Th17 responses. Secreted TGF-β from Th3 cells can also act on CD4+Foxp3− cells and convert them to iTregs, which are CD4+Foxp3+CD25+LAP−. Depending on the milieu, these cells may become CD4+Foxp3+ CD25+LAP+. These induced Tregs may also condition DCs to secrete IL-27 and, in turn, induce IL-10-secreting Tr1 cells (Figure 4). TGF-β is synthesized in the form of precursor that is cleaved intracellularly by endopeptidase. The proteolysis yields two products that assemble into a dimer. One is latency-associated peptide (LAP), and the other is mature TGF-β. The presence of LAP facilitates the transit to the cellular surface and keeps TGF-β biologically inactive and prevents interactions of TGF-β with receptors. The LAP-associated mature TGF-β is referred to as "latent TGF-β"; it is secreted as precursor and converted in the biologically active form in the extracellular space through the removal of LAP. The mechanisms that lead to the removal of LAP are not fully known, even though it is assumed that the thrombospondin present on the macrophages should be able to affect the LAP-TGF-b complex, releasing the active form of cytokine (44). Recently, integrins have emerged as central players in TGF-β1 activation. CD4+LAP+ cells are increased, in mice, in the presence of increased intestinal permeability and during the homeostatic response to a transient increase of intestinal permeability (45, 46). Their *in vivo* regulatory activity has been proven by the fact that they contribute to the prevention of 2,4,6-Trinitrobenzenesulfonic acid [TNBS] and adoptive transfer colitis in mice. Their contribution was found to be crucial for the protection from TNBS colitis, since transfer of CD4+LAP+ depleted cells enriched with CD4+Foxp3+ cells were not able to prevent TNBS colitis in recipient mice. Notable characteristics of these cells are that in mice they require IL-10 and TLR2 for their in vivo expansion (47). The phenotype and function of Treg cells in the inflamed mucosa or periphery of patients with UC have been described as considerably different from healthy controls (48). In UC patients there is an expansion of FOXP3+CD4+ T cells in mucosal lymphoid tissues. CD4+CD25+ isolated from UC MLN express FOXP3 and display features of TR cells in spite of active mucosal inflammation. These data suggest that their suppressor activity may be abrogated in vivo or they are unable to counterbalance the chronic mucosal inflammation in UC (49). Another study shows similar results: Colonic CD4+ CD25high Tregs are able to suppress colonic effectors T cell activity in vitro, and the Treg frequency in the inflamed intestine increases with disease activity in patients with active UC (50). This suggests that Tregs may be outnumbered

by other inflammatory cells or that their suppressive activity may be influenced by the in vivo inflamed environment.

Recent finding also demonstrated the role of CD4+LAP+Foxp3- regulatory T cells in UC active patients. In a recent study it was shown that in IBD lamina propria (LP) CD4+LAP+ cells are significantly increased exclusively in UC and not in CD (51). These cells are mostly Foxp3- and co-express IL-17, IL-10, and IFN-γ both in control and in UC patients. Notably, in active UC patients LP CD4+LAP+ Foxp3- cells are enriched for IL-17 expression. When isolated from LP of non-inflammatory controls, CD4+LAP+ cells show suppressor activity which is greatly reduced in LP CD4+LAP+ cells isolated from UC patients. The reduced suppressor activity is linked to their IL-17 intracellular expression, since selective depletion of LP CD4+LAP+IL-17+ cells from the CD4+LAP+CD25- population increases the suppressor activity of the latter cell population (51).

As regard as Th effector cells characterization in UC, initial studies established that UC differed from CD in that upon stimulation, cells extracted from UC lesions produced increased amounts of IL-5 and normal amounts of IFN-γ whereas cells from CD lesions produced increased amounts of IFN-γ; these studies suggested that UC was a Th2 T cell-mediated disease whereas CD was a Th1 T cell-mediated disease (52). In later studies, it appeared that UC have a mixed cytokine profile characterized by the presence of Th2 T cells admixed with Th17 T cells producing increased amounts of IL-17. Several studies of IL-17 mRNA production by UC lesional tissues indicated that increased amounts of this cytokine were produced in these tissues (53) and increased IL-17 production was also reported (54, 51) .

IL-13 has a number of characteristics that can account for the inflammation and tissue damage encountered in UC. IL-13 can cause epithelial cell apoptosis via a complex mechanism involving the TNF superfamily member TWEAK and the latter's receptor, Fn14. TWEAK complexed to Fn14 can also lead to up-regulation of TSLP, a cytokine that favors Th2 responses and may therefore reinforce the Th2 orientation observed in UC inflammation. Furthermore it has been reported that exposure of epithelial monolayers to IL-13 causes decreased barrier function by induction of claudin-2, a TJ protein that causes increased epithelial permeability, increased apoptosis, and decreased cellular restitution after intentional disruption (55). Finally a mechanism by which IL-13 can be harmful in UC involves the potential of NKT cells present in the UC lamina propria to attack epithelial cells via their cytotoxic machinery; a well-established feature of NKT cells. This potential is

facilitated by the fact that epithelial cells express an MHC molecule (CD1d) that binds glycolipid antigens, and in this way becomes a target for cytotoxic cells expressing a TCR that recognize these antigens in the context of MHC (56). IL-13 has been shown to bind to NKT cells and augment the latter's cytotoxicity for epithelial target cells bearing CD1 in mice or CD1d in humans with UC (57). The detrimental effects of IL-13 on epithelial cells could explain the prime characteristic of UC, namely its tendency to form ulcerative lesions and breaks in the epithelial lining of the intestine. The physiologic and pathologic activity of IL-13 depends on its signaling function in various cells. This, in turn, depends on the activity of two different cell surface IL-13 receptors, IL-13Rα1 and IL-13Rα2 (Figure 5). IL-13Rα1 is composed of two chains, IL-4Rα and IL-13α1, and binds both IL-4 and IL-13; the latter has a relatively low level of affinity. Binding to this receptor results in activation of signal transducer and activator of transcription (STAT)6 (as well as other downstream signaling molecules including PI3K, STAT3, and MAPK (58). In contrast, IL-13Rα2 is a monomer and binds IL-13 with a high level of affinity that greatly exceeds that of the IL-13Rα1 receptor. Probably this receptor acts as a decoy receptor that binds IL-13 and renders it unavailable for activation of the IL-13Rα1 receptor. However, it has been shown that IL-13Rα2 can also act as a signaling receptor, possibly by binding to an intracellular signaling co-partner. Such signaling results in AP-1 activation (but not STAT6 activation) and has been implicated in the induction of TGF-β and collagen synthesis (59).

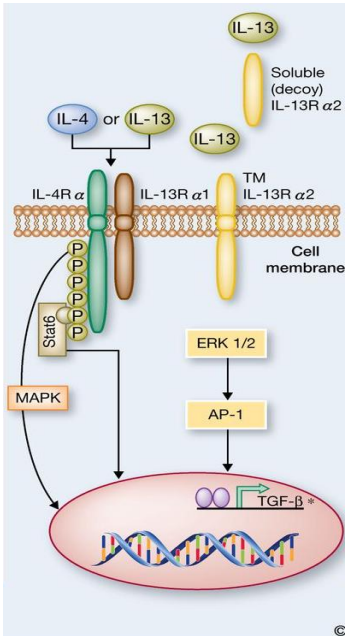


Figure 5. Receptors of IL13 (figure modified from Miranda A. et al. Cytokine Stimulation of Epithelial Cancer Cells: The Similar and Divergent Functions of IL-4 and IL-13. Cancer Res 2012;72:6338–43).

Reinforcing the Th-2 orientation in UC, recent findings (60, 61) show that a novel population of CD4-positive Th cells, which produce IL-9, are identified by the transcription factor PU.1 and contribute to the development of ulcerative colitis. Th9 cells develop after undifferentiated Th (Th0) cells encounter MHC class II-antigen complexes in the presence of the cytokines transforming growth factor-β and IL-4. IL-9 produced by Th9 cells inhibits cellular proliferation and repair, and has a negative effect on intestinal barrier function. Additionally, IL-9 modestly but significantly increases tissue concentrations of tumour necrosis factor-α (TNF-α) (60).

AIM

The primary aim of medical management in UC is to induce and maintain remission with the long-term goals of preventing disability, colectomy, and colorectal cancer. However, in spite of progresses in medical therapy the rate of surgery did not diminish and a group of patients does not respond or loose response to biological therapy. To date therapeutic strategies are founded on clinical criteria including the severity and extension of the disease. The immunological characterization of inflammatory process in the single patient might be useful to tailor therapy to individual patients against the main pathogenic mechanism operating in the patient. Similarly, this approach might result in the discovery and validation of new biomarkers predictive of the course of the disease.

With the aim to better characterize pathogenic mechanisms operating in UC patients we focused on two important issues, specifically related to the prognosis, that remain to be clarified. One of them is related to the extension of disease that greatly influences the prognosis. Biological factors involved in the limitation of the disease extent are presently unknown. The second concerns a better characterization of the key cytokine(s) relevant for the inflammatory process in patients to optimize biological therapy.

SPECIFIC BACKGROUND

Disease Extension

The anatomical extent of mucosal inflammation is one of the most important factors determining disease course and therapeutic strategies. Patients with extensive colitis have a greater risk of colectomy than those with proctitis (62-64), as well as a greater risk of colorectal cancer (65, 66). In patients with distal colitis, inflammation progresses in 21%-34% of patients (32), while regression is observed in about 16% of patients (67, 68). Proximal disease extension is associated with increased disease severity upon diagnosis and greater likelihood of clinical relapse (69, 70). Moreover, extensive colitis is associated with an increased frequency of extraintestinal manifestations, a steroid-refractory disease course, and the need for immunosuppressive and immune-modulating medications and surgery (69, 71).

The biological factors that determine the extents of inflammation in UC are unknown as well as early biomarkers for the prediction of disease extension are not available. Tregs have been identified as a key immunosuppressive population that is critically involved in maintaining intestinal homeostasis. Therefore, we hypothesized that Tregs may be involved in limiting the extension of inflammation. To investigate this hypothesis, we evaluated the frequencies of different Treg types in the lamina propria (LP) of UC patients with varying degrees of disease extension, and examined the contribution of these cells to disease extension in a mouse model of UC. Specifically, we analyzed the frequencies of CD3+CD4+Foxp3+ cells, as well as another type of Treg, CD3+CD4+ latency associated peptide (LAP)+Foxp3- regulatory cells. We previously demonstrated that the latter cell type is present in human LP (72), and is found at a higher frequency in the LP of endoscopically active UC patients, but not in Crohn's disease patients (51). Moreover, LAP+Foxp3- T cells have been described to infiltrate colorectal cancer exhibiting more potent immunosuppressive activity than Foxp3+ regulatory T cells (73).

It is increasingly evident that there is a close relationship between the microbiota composition and gut immune response, which includes both the cellular and the soluble component of the immune response. Commensal bacteria and their metabolites can promote the generation of regulatory T cells (69-72) which, in turn, influence the microbiota composition. In particular, it has been shown that CD3+CD4+LAP+ T

regulatory cells are influenced by microbiota composition in animal models (46) and administration of probiotics is able to increase their % in the lamina propria in patients with ileal pouch-anal anastomosis for ulcerative colitis (72). Therefore, in the same patients, we also analyzed mucosal microbiota composition.

The right treatment for the right patient at the right time: characterization of Ulcerative Colitis patients according with tissue cytokine profile and/or gut microbiota composition

A personalized approach to therapy has great promise to improve disease outcomes. Selection of patients as candidates for the early introduction of highly effective therapy can both maximize treatment efficiency and prevent long-term complications. Although the use of biological therapies (mainly TNF inhibitors) revolutionized the therapy of inflammatory bowel disease (IBD), the increasing availability of agents able to block different cytokines (anti-IL-13 agents, anti- IL-12/23 agents, specific inhibitors of IL-23, anti- IL-6 agents), biologics blocking leukocyte trafficking to the gut (anti-integrin antibodies) highlight the need to choice a pathogenesis-oriented approach in their use, integrating the sequential approach now in use.

Furthermore, modifying the microbiota has potential therapeutic effects on IBD and specific interventions might be tailored on the dysbiosis observed in the patient.

We explored the possibility to detect subsets of ulcerative colitis patients according with the tissue cytokine profile and/or microbiota composition. Exploring this possibility, we also hoped to clarify an ongoing controversy in UC, about the role of IL-13 in the pathogenesis of the disease, and whether IL-13 expression status might identify patients who would benefit more from its targeting. Previous studies reported contrasting evidences on increased IL-13 in UC patients (55, 61, 74-77) and administration of anti- human IL-13 neutralizing monoclonal antibody, did not significantly improve clinical response vs placebo in UC patients (78). However, in the same study, the proportion of patients who achieved clinical remission was statistically significantly higher in the anti-IL-13 treated group compared with the placebo group, suggesting the presence of a UC patient's subgroup responding to neutralization of IL-13. Therefore, we analyzed the mucosal cytokine mRNA content and the mucosa-associated microbiota composition of a cohort of clinically and endoscopic active UC patients.

MATERIALS AND METHODS

HUMAN

Patients

Two cohorts of patient for a total of 183 patients with endoscopically active UC who underwent colonoscopy for clinical flare-up at 2 tertiary centers (IBD, GE Unit, Sandro Pertini Hospital, Rome, and the Department of General Surgery, "P. Stefanini," Sapienza University, Rome) were included in the study. The first cohort was made up of 95 patients and the second cohort of a total of 88 UC patients (Table 2). A control group of 53 subjects (29 participants in the first cohort and 24 in the second cohort) undergoing colonoscopy for colon cancer screening or suspected functional bowel disorders was also included (Table 2). Patients with an endoscopic score ≥1, were considered to have endoscopically active UC and were enrolled in the study. None was on rectal 5-ASA and/or steroids in the last 3 months, Disease activity was assessed using the endoscopic Mayo score (79). In UC patients, disease extension, at the time of endoscopy, was classified using the Montreal classification (80) as follows: proctitis, involvement limited to the rectum; left-sided colitis, involvement limited to a portion of the colorectum distal to the splenic flexure; extensive colitis, involvement extending proximal to the splenic flexure.

Table 2. Clinical-pathological variables of patients at time of endoscopy

	Ulcerative Colitis n.79	**Controls** n. 29	**Ulcerative Colitis** n.77	**Controls** n. 20
	Cohort I		**Cohort II**	
Age at the time of endoscopy years: median (range)	51 (20-82)	56 (26-85)	51 (19-82)	57 (37-86)
Gender M/F (n)/(n)	47/32	15/14	45/32	10/10
Disease Duration from diagnosis years: median (range)	10 (0-32)		9 (0-36)	
Endoscopic Activity (Mayo Score) median (range)	2 (1-3)		2 (1-3)	
Disease Extension				
Proctitis	31		29	
Left Sided Colitis	32		29	
Extensive Colitis	16		19	
Therapy				
CS	2+ 11*		2+ 10*	
5-ASA agents	56		50+ 23*	
Immune modulators	2+7*		1+10*	
Biological Agents	2+1*		2*	
No therapy			4	

*****Patients with combined therapy

Biopsy Specimens

Multiple endoscopic mucosal biopsies were obtained from endoscopically involved and uninvolved areas in UC patients, and from matched areas in controls. Diagnosis of UC was established based on established criteria and the extension of the disease was confirmed by histology. Histopathology was quantified in H&E stained tissue sections using the Robarts histopathology index (RHI) (81). An RHI ≤6 in samples collected from involved tissue was considered indicative of remission, and the sample, together with the paired sample collected from uninvolved tissue, was excluded from the analysis. In these patients, RHI < 3 were recorded for all biopsy samples collected from uninvolved tissue. Of the first cohort the remaining 79 patients were evaluated. For cytokine/microbiota analysis (II cohort), multiple biopsies taken from involved and uninvolved tissue were stored in RNA later for quantification of cytokine profile and identification of mucosa-associated microbiota by metagenomics analysis. Biopsies samples for RNA extraction were available in 81 UC patients and 21 Controls. Twenty controls and 77 patients (77 samples from involved tissue and 50 samples from uninvolved tissue within the same patients) gave rise to a good quality RNA to be amplified. Biopsies for microbiota analysis were available in 24 controls and 88 UC patients. Twenty-one controls and 79 patients (79 samples from involved tissue and 57 samples from uninvolved tissue within the same patients) endowed with a sufficient quality data for sequencing analysis. In 68 patients both RNA and microbiota analysis were available.

Confocal microscopy

Some tissue sections were also analyzed by confocal microscopy. Three micrometer thick paraffin-embedded sections of human colon tissue from controls (CTR) and ulcerative colitis (UC) patients were stained after deparaffinization in xylene (5min, two times), rehydration by sequential washes in 100% ethanol (3min), 95% ethanol (3min), 80% ethanol (3min), 70% ethanol, 50% ethanol, deionized water and antigen retrieval (5min at 95◦C in 10mM sodium citrate, pH 6.0). Slides were saturated with blocking buffer (PBS, 0.05% tween 20, 4% BSA) for 1 h at room temperature. Specimens were stained with a polyclonal rabbit anti-human CD4 at 5µg/ml, followed by donkey anti-rabbit-AlexaFluor-568, and a monoclonal mouse anti-human LAP followed by a goat anti-mouse AlexaFluor 647. After washing, slides were mounted in Prolong Gold antifade medium

containing a DNA dye (DAPI). Confocal laser scanning microscopy (CLSM) observations were performed with a Leica TCS SP2 AOBS apparatus, using a 63x/1.40 NA oil objective. Acquisition of images was performed by Leica confocal software 2.6 (Leica, Germany).

Isolation of Lamina Propria Mononuclear Cells (LPMCs)

LPMCs were isolated from freshly obtained biopsies using a previously described DDT-EDTA collagenase method (51, 72). Briefly, biopsies were washed in HBBS free of calcium and magnesium (HBSS-CMF; Hyclone, Europe LTD, Cramlington, United Kingdom), and then incubated for 5min at room temperature in HBSS-CMF containing 1 mmol/l DTT (Sigma Chemical Co., St. Louis, MO, United States) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 mg/ml; gentamicin, 50 mg/ml; and fungizone, 25 mg/ml). After washing 3 times in HBSS-CMF, the biopsies were cut into smaller pieces and incubated in HBSS-CMF containing 0.75 mmol/l EDTA, 10 mmol/l HEPES buffer, and antibiotics for 15min at 37◦C in a humid 5% CO2 atmosphere to remove epithelial cells. After 2 washes, the tissue was incubated for a total of 2 h (2 × 1-h incubations) at 37◦C in a humid 5% CO2 atmosphere in complete medium (RPMI 1640 plus 10mM HEPES buffer, 2mM/l glutamine, 10% heat-inactivated FCS (Hyclone), and antibiotics) containing 25 U/ml collagenase V (Sigma-Aldrich, Milan, Italy) and 100μg/ml of DNase (Roche Diagnostics, Mannheim, Germany). After incubation, the supernatant containing LMPCs was collected and washed twice in HBSS-CMF + antibiotics.

Antibodies and Reagents

APC-Cy7-labeled anti-CD3, PE-Cy7-labeled anti-IL-10, and isotype-matched control Igs were obtained from Biolegend (San Diego, CA, United States). PE-labeled anti IL-17A, and isotype matched Ig control were obtained from eBioscience (San Diego, CA, United States). FITC-labeled anti-CD4, PE-CF594-labeled anti-CD8 and isotype-matched control Ig were obtained from Becton Dickinson Horizon (San Jose, CA, United States). PerCP labeled anti-LAP (TGF-b1) and isotype-matched control Ig were obtained from R&D Systems (Minneapolis, MN, United States). APC-labeled anti-Foxp3, the Foxp3 staining buffer set, and

isotype-matched control Ig were obtained from eBioscience (San Diego, CA, United States). LIVE/DEAD R Fixable Aqua Dead Cell Stain Kit was obtained from Life Technologies (Carlsbad, CA, United States). Phorbol-12-myristateacetate (PMA) and ionomycin were obtained from Sigma-Aldrich. FITC-labeled anti-CD4, isotype-matched control Igs, and Monensin solution (Golgi Stop) were obtained from BD Pharmingen (San Jose, CA, United States). For confocal microscopy imaging, polyclonal rabbit anti-CD4 (Novus, Colorado, United States), Donkey antirabbit-AlexaFluor-568 (Abcam, Cambridge, United Kingdom), monoclonal mouse anti-human LAP (R&D Systems) and goat anti-mouse AlexaFluor-647 (Abcam) were used.

Immunofluorescence Staining

Isolated LPMCs were incubated for 30min with LIVE/DEAD R Fixable Aqua Dead Cell Stain. Next, cells were washed and stained with anti-human-CD3, anti-human-CD4, and anti-human LAP (TGF-b1). After incubation, cells were washed, fixed, and permeabilized with fixation/permeabilization solution for 40min, and stained with anti-human Foxp3. In previous studies (51), we established that the percentages of LAP+ and Foxp3+ cells remained unchanged following PMA-ionomycin stimulation in the presence of Golgi Stop. Therefore, for evaluation of LAP and Foxp3 expression, together with that of the intracellular cytokine IL-10 and IL-17, LPMCs isolated from biopsies were incubated in X-VIVO15 medium and stimulated for 4 h with PMA(50 ng/ml) and ionomycin (1μg/ml) in the presence of monensin (0.66 μl/ml Golgi Stop). After stimulation, cells were recovered and washed in PBS-1X, incubated for 30min with LIVE/DEADR Fixable Aqua Dead Cell Stain, and washed and labeled. Given the downregulation of CD4 expression that occurs following PMA-ionomycin stimulation, CD8 staining was preferred. Consequently, to assess intracellular cytokine expression, cells were stained with anti-human CD3, anti-human CD8, and anti-human LAP (TGF-β1), and incubated for 30min. After incubation, cells were washed, fixed, and permeabilized with fixation/ permeabilization solution for 40min. Cells were then washed in permeabilization buffer and incubated for 30min with intracellular anti-human IL-10, anti-human IL-17 and anti-human Foxp3 or isotype-matched

control Ig. Next, cells were washed twice with permeabilization buffer solution and fixed with 2% paraformaldehyde. CD4 cells were defined as those with a CD3+CD8-signature. The percentage of viable fluorescent cells was quantified using a GalliosTM Flow Cytometer (Beckman Coulter, Brea, CA, United States).

Cytokine tissue mRNA content quantification

RNA was extracted using RNA mini Kit Plus (Qiagen, Hilden, Germany) and its quality analyzed by Agilent RNA 6000 Nano Kit using Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA). RNA with Refractive Index Detector (RID) ≥ 7 was used for subsequent analysis. cDNA was reverse transcribed from 1μg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™ - Thermo Fisher Scientific, Massachusetts, USA). We quantified by quantitative real-time PCR (RTqPCR) the tissue content of TNF-α, IL-6, IFN-γ, IL-17A, IL-10 and IL-13 using the following primers: TNF-α forward 5-CCCAGGGACCTCTCTCTAATCA, reverse 5-GCTACAGGCTTGTCACTCGG-3; IL-6 forward 5'-CCACTCACCTCTTCAGAACG-3', reverse 5'-GCCTCTTTGCTGCTTTCACAC-3'; IFN-γ - forward: 5'-ATAGGTAACTGACTTGAATGTCCA-3', reverse 5'-GCTCTTCGACCTCGAAACAGC-3'; IL-17A - forward: 5'-ACTACAACCGATCCACCTCAC-3', reverse 5'-ACTTTGCCTCCCAGATCACAG-3'; IL-10-forward 5'-CCCAAGCTGAGAACCAAGACC-3', reverse 5'-TCACAGGGAAGAAATCGATGAC-3'; IL-13 - commercially available primers RT² qPCR Primers Assay (Qiagen) cat.PPH00688FRPS. As housekeeping gene, we utilized RPS9 –forward: 5'-AAGCTGATCGGCGAGTATG-3', reverse 5'-TCTTCAGGCCCAGGATGTAA-3' (82). The gene amplification was performed in duplicate at Tm-60°C using Fast SYBR™ Green Master Mix (Applied Biosystems™ - Thermo Fisher Scientific). Data are expressed as Log2 (Relative Expression) coming from the equation: Relative Expression = 40-DCt where 40 is the maximum number of cycles and DCt is the difference in number of cycles of the gene of interest with the housekeeping gene. We attributed a relative expression of 40 to samples with undetectable expression, (threshold cycle ≥ 40); next, we estimated the relative expression as described above.

Statistical analysis

The presence of a statistically significant difference between controls and UC patients was assessed by Mann-Whitney's U and the two sided Wilcoxon signed-rank test in GraphPad Prism software (GraphPad Software, San Diego, CA, United States). Statistical significance was set at $p < 0.05$.

The optimal 'clustering properties' of IL-17A and IL-13 with respect to the other cytokines were assessed by means of pseudo F statistics (83) bimodality index as well as by the correlation structure among a set of cytokines by means of Pearson correlation and Principal Component Analysis (PCA). The bi-dimensional space spanned by IL-17A and IL-13 was analyzed by K-means (84), the by far most common unsupervised non-hierarchical clustering technique maximizing the Between/Within cluster variance ratio. The choice of the number of clusters (K value) was based upon the maximal distance of the observed R-square (Between cluster variance/Total variance ratio) with the one expected by a Gaussian distribution of data. The search for relevant correlations between cluster composition and clinical-pathological variables was performed by means of Chi-Square statistics and Analysis of Variance for categorical and interval variables respectively.

Mucosal samples collection and bacterial genomic DNA extraction

Endoscopic biopsies were collected in RNAlater and stored at -80°C until extraction of nucleic acids. The bacterial genomic DNA extraction was carried out with DNeasy PowerLyzer PowerSoilKit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA quality was assessed by gel electrophoresis and spectrophotometry, measuring OD 260/280.

16S Ribosomal RNA Gene Amplicons preparation and Illumina MiSeq sequencing

Library of 16S rRNA gene amplicons was prepared by IGA Technology Services (Udine, Italy) through amplification of the V3-V4 hypervariable region by using specific-barcoded primers with overhang adapters. The standard protocol was followed according to the 16S metagenomic sequencing library preparation guide from Illumina (Part # 15044223 Rev. B; https://support.illumina.com/). Pooled V3-V4 amplicon libraries

were sequenced using the Illumina MiSeq platform. Each sample was sequenced at IGA Technology (Udine, Italy) using Illumina MiSeq instrument and a 300-bp paired-end reads protocol.

Sequencing Data analysis

Demultiplexed reads were downloaded and quality checked using FastQC 0.11.5. Reads were further pre-processed by removing Illumina sequencing adaptors with CUTADAPT (85), and by trimming the low quality end of each read with SICKLE (86), with a quality threshold of 20. Trimmed forward and reverse reads were then joined with PEAR (87) and checked for chimera presence with VSEARCH (88). OTUs picking was performed in QIIME 1.8 (89) with uclust algorithm (90) using default values, and assigning the taxonomy using the GreenGenes 13.8 database. Low coverage OTUs were filtered using filter_otus_from_otu_table.py and parameter --min_count_fraction 0.00005.

Subsequent analyses were performed in R statistical software. The file produced by QIIME was parsed in R with phyloseq package (91) and OTUs count data were normalized by cumulative sum scaling (CSS) as implemented in the metagenomeSeq package (92). Alpha diversity was evaluated using Observed richness (the number of different OTUs in a sample), Shannon's diversity Index (a measure of community diversity, accounting for both abundance and evenness of bacterial OTUs), and Evenness index (a measure of uniformity of relative abundances of the OTUs in a sample) (93). Beta diversity was analyzed by means of principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity index producing an explicit metric space from microbiome profiles dissimilarities. Inferential statistics on the above described metrics was performed by Analysis of Variance and Student's t-test. LEfSe (Linear discriminant analysis Effect Size) (94) on the CSS transformed abundances, was performed to identify plausible bacterial biomarker(s) able to separate different groups. In LEfSe, Kruskal–Wallis rank-sum test (significance threshold 0.05) was firstly used to identify significantly different taxa abundances among classes (in this case, we used patients' clusters as group variable), and then LDA (linear discriminant analysis) is used to estimate the size of the discriminating effect for each feature. A feature (i.e. a Genus-level OTU) is retained as a suitable biomarker of a class if the LDA value (Between/Within Variance ratio) was higher than 2.

Microbiota sequence data are available at http://www.ebi.ac.uk, under the accession number PRJEB31884. The script used for pre-treatment and OTUs picking, as well as the script for data analysis performed in R, are available at the following GitHub repository https://github.com/FrancescoVit/Supplementary-to Butera et-al.-2019

MICE

Induction of Experimental Colitis in Mice

Male BALB/c mice (Charles River Laboratories Italia, Calco, Italy) were housed in the Allevamenti Plaisant (Rome, Italy) animal facility in individually ventilated cages (IVC system) containing enrichment devices. Maintenance of pathogen-free conditions was ensured by monitoring every 6 months, in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) recommendations. Experimental colitis was induced in 6–7 week-old male BALB/c mice by administering 6mg of oxazolone [(4-ethoxymethylene- 2-phenyl-2-oxazolin-5-one) Sigma Chemical Co., St. Louis, MO, United States] dissolved in 50% ethanol (total injection volume,150 μl), via a 3.5-F catheter inserted into the rectum of lightly anesthetized mice, as previously described (73). Control groups consisted of untreated mice and mice treated with 50% ethanol (total injection volume, 150 μl). Body weight was recorded at time zero (moment of intrarectal oxazolone/ethanol or ethanol administration), and on days 1 and 2 post-treatment. Mice were sacrificed on day 2 by cervical dislocation and colons collected for further analysis. In a preliminary experiment, we tested the ability of anti-LAP antibody (TW7-16B4 antibody, kindly donated by Professor HL Weiner, Harvard Medical School, Boston, MA, USA) to deplete the LP CD4+LAP+cells. To this end we treated two different groups of mice for 5 days with daily injection (40 μg, i.p.) of anti-LAP antibody or isotype control mouse IgG1 Clone MOPC-21 (BioXCell; DBA, Segrate, MI). Mice were sacrificed, colons were collected, and isolated LPMC cells were analyzed by immunofluorescence for LAP and Foxp3 expression. In additional experiments, different groups of mice received daily injections (40 μg, i.p.) of anti-LAP antibody (or isotype control), for 5 days before the induction of colitis.

Assessment of Colitis

Collected colons were macroscopically examined to assess the extension of colitis, and histological analysis of colitis was performed in colonic tissue samples that were fixed in 10% neutral buffered formalin solution (Sigma-Aldrich), embedded in paraffin, cut into tissue sections, and stained with hematoxylin and eosin. Multiple serial sections dividing the colon into three sections (proximal, medial and distal) were performed. Stained sections were examined by a pathologist ISTOVET di Luca Crippa and C. S.A.S., Besana in Brianza (MB), Italy] and the extension of inflammatory lesions determined. Histopathologic grading of oxazolone-induced colitis was performed as previously described (95). Briefly, five criteria (hypervascularization, presence of mononuclear cells, epithelial hyperplasia, epithelial damage, presence of granulocytes and mucosal hemorrhages) were scored from 0 to 3 to produce a cumulative histopathologic score (HS) ranging from 0 (no colitis) to 15 (maximal colitis activity).

LPMCs Immunofluorescence Staining

Freshly isolated and washed LPMCs were subjected to Fc block with anti-CD16/CD32 (BD Pharmingen) and then labeled for 30min with LIVE/DEADR Fixable Aqua Dead Cell Stain. After washing, cells were stained by incubation for 30min with APC-Cy7-labeled anti-CD3 (eBioscience), V450-labeled anti-CD4 (eBioscience), PE-labeled anti-LAP (BioLegend), or isotype control PE-labeled mouse IgG1 (R&D Systems). Intracellular Foxp3 expression was evaluated using the APC-anti-mouse/rat Foxp3 staining kit (eBioscience), following the manufacturer's protocol. The cells were then washed twice, and the percentage of fluorescent cells quantified using a GalliosTM Flow Cytometer (Beckman Coulter, Brea, CA, United States). Some tissue sections were also analyzed by confocal microscopy, using the above-described procedure. Briefly, 3 μm-thick paraffin-embedded sections of unlesional and lesional colon tissues from mice treated as described in Induction of experimental colitis in mice paragraph were stained after deparaffinization, rehydration, antigen retrieval and saturation with blocking buffer. Specimens were stained with a monoclonal FITC-conjugated rat anti-mouse CD4 at 5μg/ml (BD Biosciences), and a monoclonal mouse anti-mouse LAP at 5μg/ml (BioLegend) followed by an goat anti-mouse AlexaFluor-633. After washing, slides were mounted in Prolong Gold anti-fade medium containing a DNA dye (DAPI). Confocal

laser scanning microscopy (CLSM) observations were performed with a Leica TCS SP2 AOBS apparatus, using a 63x/1.40 NA oil objective. Acquisition of images was performed by a Leica confocal software 2.6 (Leica, Germany).

Statistical Analysis

Data were analyzed using the two sided Mann-Whitney U-test and the two sided Wilcoxon signed-rank test in GraphPad Prism software (GraphPad Software, San Diego, CA, United States). Statistical significance was set at $p < 0.05$.

Human Study

All participants provided written informed consent prior to inclusion in the study. Ethical approval was provided by the Ethical Committee of the Istituto Superiore di Sanità (Reference: Pre-C-871/14, 25/11/2014).

Animal Studies

This study was carried out in accordance with the recommendations of Decreto Legislativo 4 marzo 2014, n. 26 according with 2010/63/UE (14G00036) direction. The protocol was approved by the Italian Ministry of Health (Reference: 16/2014-PR [DGSAF 12073-A, 05/06/2014], 03/10/2014).

RESULTS

T regulatory cells/ microbiota composition and extension of disease

The frequency of LP CD3+CD4+LAP+ cells is higher in uninvolved vs involved colon tissue from Ulcerative Colitis patients

Preliminary analyses of the percentage of CD3+CD4+LAP+ and CD3+CD4+Foxp3+ Tregs isolated from biopsies from different portions of control colons revealed no differences between colon regions (Figure 6). We next evaluated the frequency of LP CD3+CD4+LAP+ and CD3+CD4+Foxp3+ Tregs in LPMCs isolated from biopsies from controls and from patients with endoscopically and histologically active UC with varying degrees of disease extension. In patients with either proctitis or left-sided colitis, the percentage of CD4+Foxp3+ cells was significantly higher in involved tissue than in uninvolved tissue (Figure 7 A). As opposite % of CD4+LAP+ Tregs was significantly higher in uninvolved vs. involved tissue (Figure 7 B) In agreement with previous observations (51, 49, 50), the percentage of Foxp3+ Tregs in the LP CD3+CD4+ T-cell population was significantly higher in involved tissue, regardless of disease extension, than in controls (Figure 7 A). Similarly, in line with previous reports (51), the percentage of CD4+LAP+ Tregs was significantly higher in involved tissue from patients with extensive colitis and left-sided colitis than in controls (Figure 7 B). In uninvolved tissue, the percentage of CD4+Foxp3+ cells was comparable to that of controls, while the percentage of CD4+LAP+ Tregs tissue was significantly higher. As previously reported (51), the majority of LAP+ cells detected were Foxp3- (Figure 2C). Some biopsy specimens sections were also immunofluorescence stained for tissue assessment of CD4+LAP+ cells by confocal microscopy. As illustrated in Figures 2 D, E, uninvolved tissue showed significantly more CD4+LAP+ double-fluorescent cells when compared with involved and control tissue.

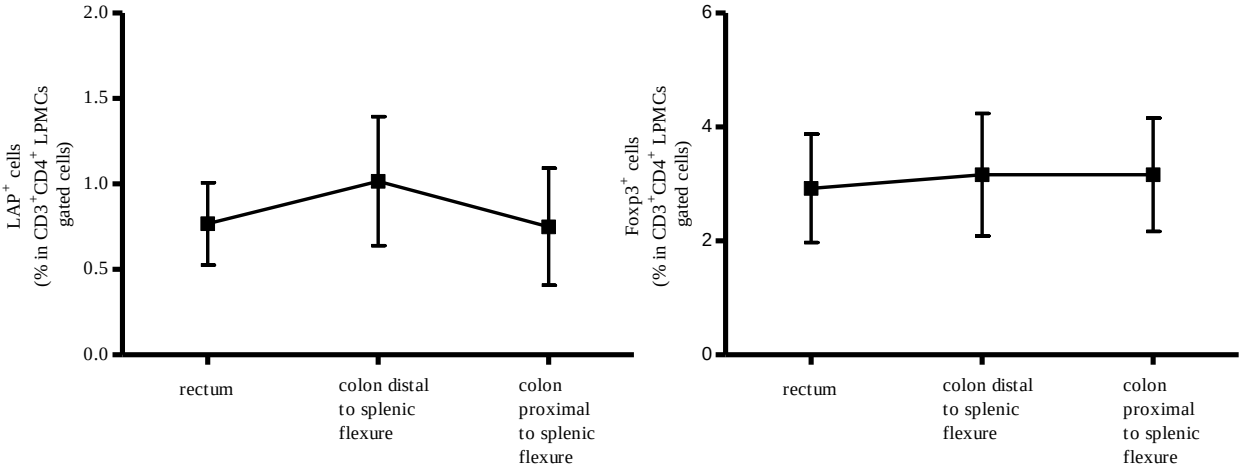
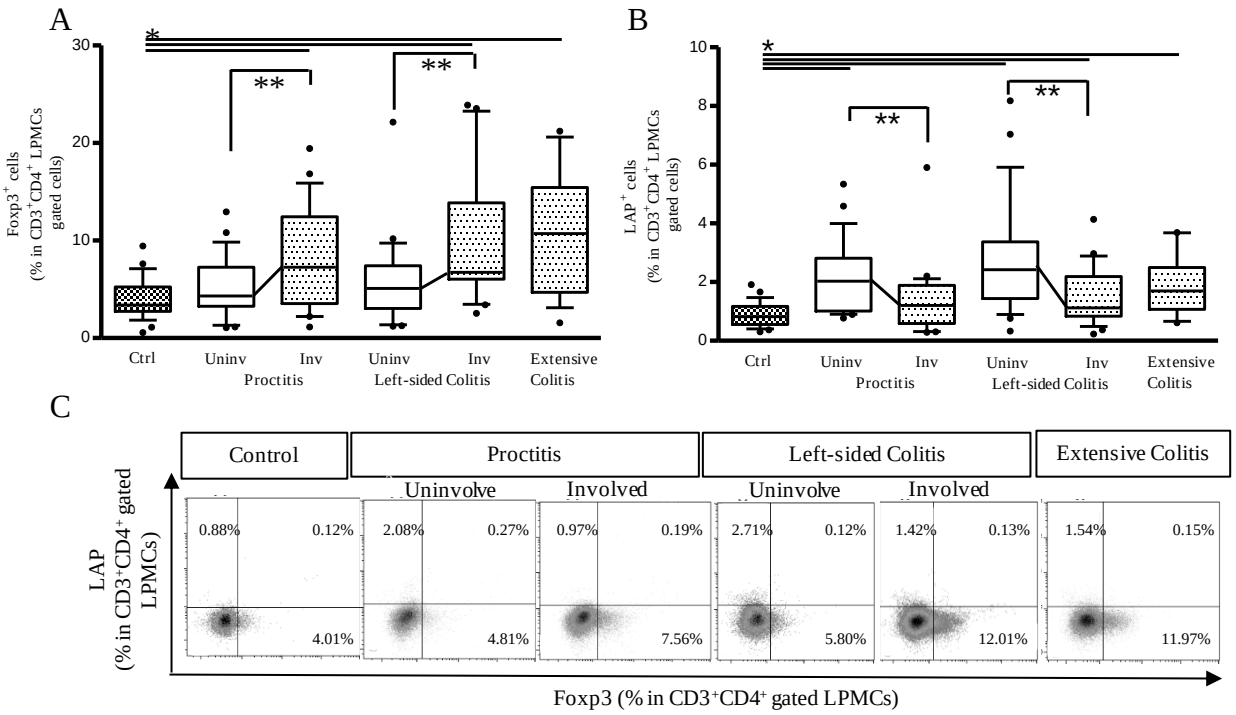
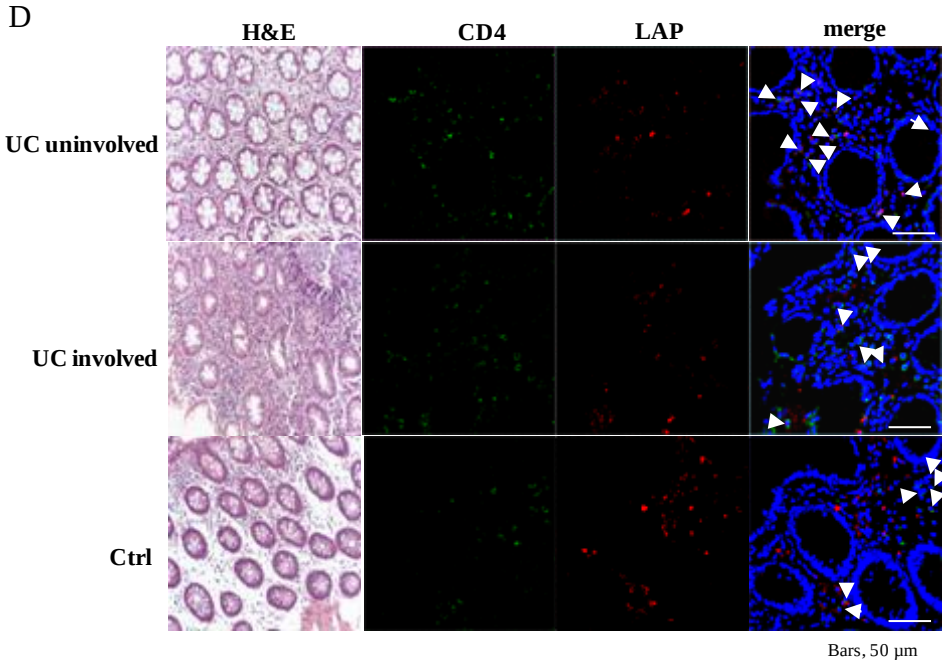


Figure 6. The percentage of LAP+ and Foxp3+ cells in CD3+CD4+-gated LPMCs does not differ among different regions of the colon in human controls.


A
B
Foxp3+ cells (% in CD3+CD4+ LPMCs gated cells)
LAP+ cells (% in CD3+CD4+ LPMCs gated cells)
Ctrl
Uninv
Inv
Proctitis
Left-sided Colitis
Extensive Colitis

C
Control
Proctitis
Left-sided Colitis
Extensive Colitis
Uninvolve
Involved
LAP (% in CD3+CD4+ gated LPMCs)
Foxp3 (% in CD3+CD4+ gated LPMCs)


D
H&E
CD4
LAP
merge
UC uninvolved
UC involved
Ctrl
Bars, 50 μm

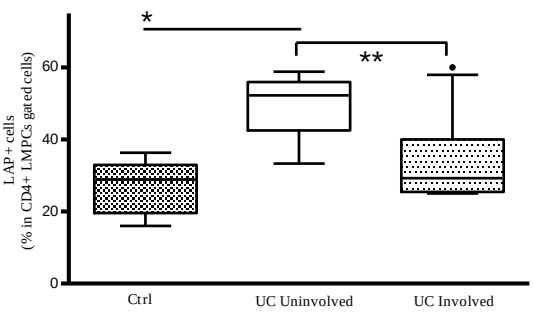

E
LAP+ cells (% in CD4+ LMPCs gated cells)
Ctrl
UC Uninvolved
UC Involved

Figure 7. The frequency of LP CD3+CD4+LAP+ cells is higher in uninvolved (Uninv) versus involved (Inv) colon tissue from UC patients.
A. Frequency of LP Foxp3+ cells in the CD3+CD4+-gated LPMC population: *P<0.05 (Mann-Whitney U-test) for controls (Ctrl) vs. proctitis (Uninv + Inv), left-sided colitis (Inv), and extensive colitis; **P<0.05 (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis and left-sided colitis. Data represent the mean ± SE of 79 UC patients and 29 controls. B. Frequency of LP LAP+ cells in the CD3+CD4+-gated LPMC population: *P<0.05 for Ctrl vs. proctitis (Uninv + Inv) and left-sided colitis (Inv); *P<0.05 (Mann-Whitney U-test) for controls vs. left-sided colitis (Uninv + Inv) and extensive colitis; **P<0.05 (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis and left-sided colitis. Data represent the mean ± SE of 79 UC patients and 29 controls. C. Representative density plots of Foxp3 and LAP expression in LP CD3+CD4+-gated cells. D. Representative CD4+LAP+ T cells in colonic mucosa tissue of UC patients and controls. Confocal microscopy images of CD4 (green), LAP (red) and nuclei (blue) of matched involved and uninvolved colonic mucosa of a patient with UC, and a control subject (original magnification 630x). CD4+LAP+ double positive T cells are indicated by white arrows. For UC, 1 representative staining of 4 patients is shown. For control subjects, 1 representative staining of 2 subjects is shown. H&E stained sections of the corresponding subjects are also illustrated (original magnification 200x).

Ratio between IL-10 and IL-17 Expressing LAP+ Cells is Significantly Higher in Uninvolved vs. Involved Colon Tissue from Ulcerative Colitis Patients

We have previously shown that in active UC patients, LP CD3+CD8- (CD4) LAP+ cells are enriched for IL-17 expression, showing reduced suppressor activity due the intracellular IL-17 expression (51). It has been recently reported that CD4+LAP+ cells expressing IL-10 exhibit regulatory activity (96, 97). We therefore evaluated IL-17 and IL-10 expression in LP CD3+CD8- (CD4) LAP+ cells. As shown in Figure 8 A, the percentage of IL- 17 expressing LP CD3+CD8- (CD4) LAP+ cells was significantly reduced in uninvolved vs. involved tissue while the % of IL-10- expressing LAP+ cells was significantly higher in uninvolved vs. involved tissue (Figure 8 B). As a consequence, the ratio between IL-10 and IL-17 expressing LAP+ cells was significantly higher in uninvolved vs. involved tissue (Figure 8 C). Notably, extensive colitis ratio was significantly lower than the ratio observed both in proctitis and left-sided colitis involved tissue. As previously reported and confirmed in the present study, % of IL-17 expressing LAP+ cells was significantly increased in involved tissues vs. controls (51). The percentage of IL-10 expressing LAP+ cells was significantly increased in uninvolved tissues vs. controls.

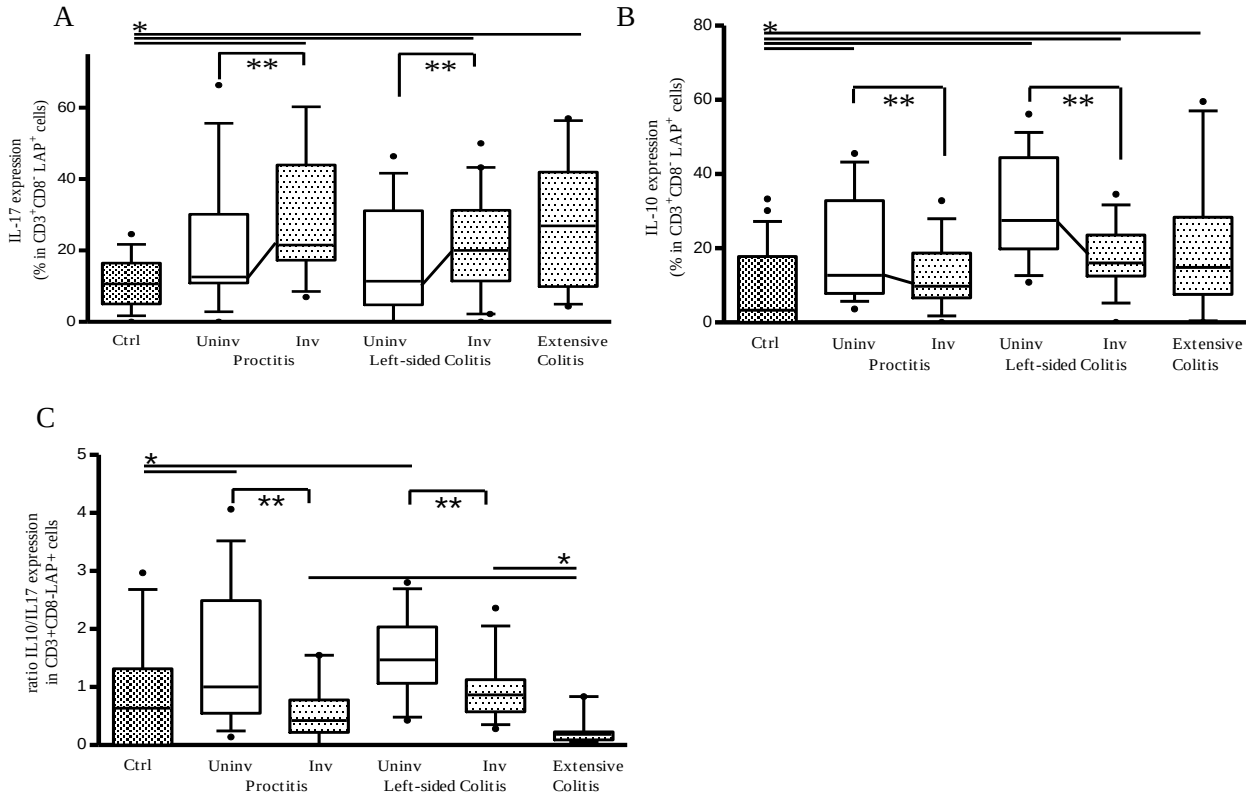


Figure 8. Ratio between IL-10 and IL-17 expressing CD3+CD8-(CD4) LAP+ cells is significantly higher in uninvolved (Uninv) vs. involved (Inv) colon tissue from ulcerative colitis patients.
A. Frequency of LP LAP+ cells expressing intracellular IL-17 *P <0.05 (Mann-Whitney U-test) for controls vs. Involved (Inv) tissue in proctitis, left sided colitis and extensive colitis. **P <0.05 (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in both proctitis and left-sided colitis. B. Frequency of LP LAP+ cells expressing intracellular IL-10: *P <0.05 (Mann-Whitney U-test) for controls vs. proctitis (Uninv), left-sided colitis (Uninv + Inv), and extensive colitis; **P <0.05 (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in both proctitis and left-sided colitis. C. % LP LAP+ cells expressing intracellular IL-10 / % LP LAP+ cells expressing intracellular IL-17 (ratio). **P <0.05 (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis + left sided colitis. Data represent mean ± SE of 43 UC patients (proctitis, 16; left-sided colitis, 17; extensive colitis, 10) and 26 controls.

Reduced Disease Extension is Associated with a Higher Percentage of CD3+CD8–(CD4+) LAP+ Cells in Uninvolved vs. Involved Tissue from Ulcerative Colitis Patients

To determine whether the observed differences in Tregs frequencies were indeed linked to the extension of inflammatory lesions, we analyzed a subgroup of patients with a history of endoscopic assessed extensive colitis in whom inflammatory lesions were endoscopically limited to the distal colon (mainly rectum) at the moment of the entry in the present study. We found that the presence of distal inflammatory lesions was associated with a significantly higher frequency of LAP+ cells in LP cells isolated from uninvolved tissue vs.

both control tissue and involved tissue from UC patients (Figure 9 A). Within this cell population, the percentage of IL-10-expressing CD4+LAP+ cells was significantly higher in uninvolved vs. involved tissue (Figure 9 B). The percentage of CD4+Foxp3+ Tregs was significantly higher in involved vs. uninvolved tissue, in which values were comparable to those detected in controls (Figure 9 C). These findings suggest that the differences in the frequencies of regulatory cells between involved and uninvolved tissue are linked to the extension of inflammatory lesions.

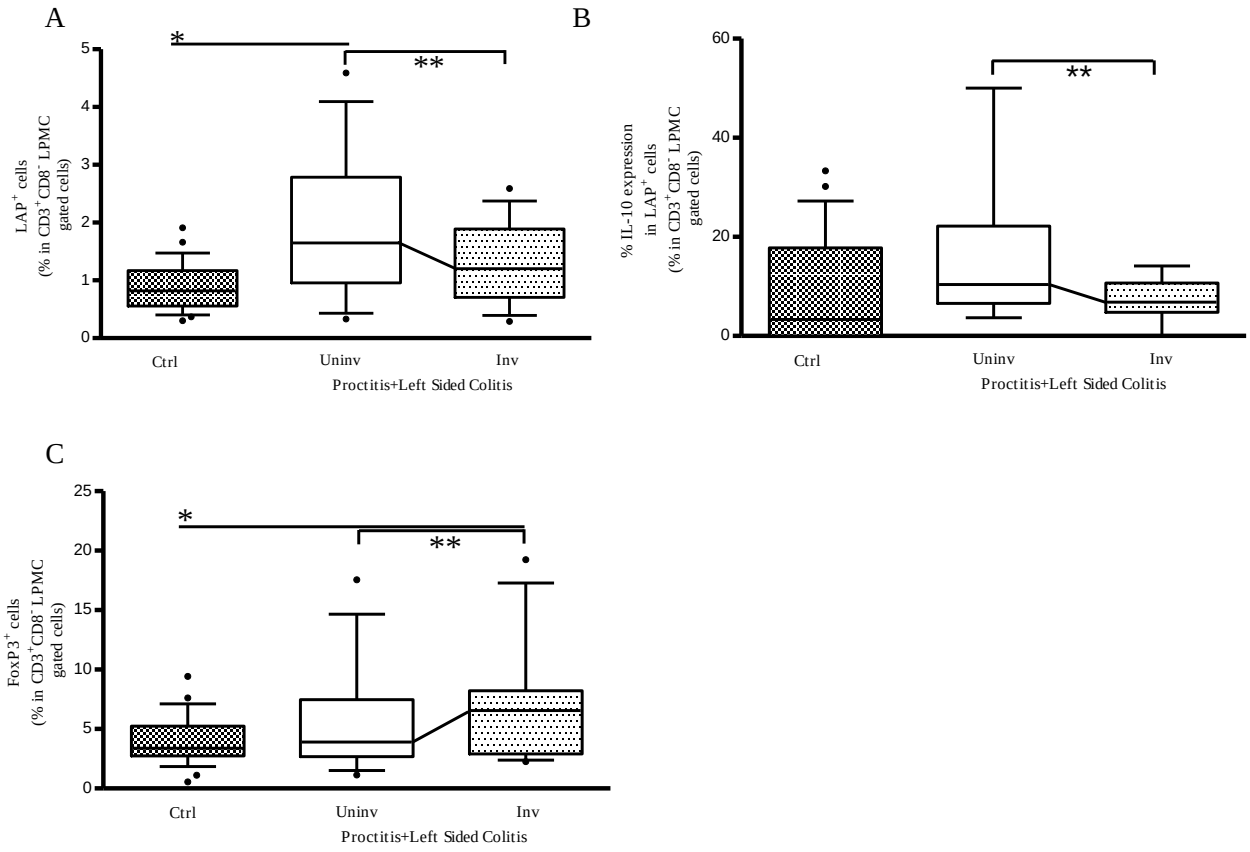


Figure 9. Reduced disease extension is associated with a higher percentage of CD3+CD8-(CD4) LAP+ cells in uninvolved versus involved colon tissue from UC patients.

A. Frequency of LP LAP+ cells in CD3+CD8- (CD4)-gated cells: *P <0.05 (Mann-Whitney U-test) for controls vs. uninvolved tissue in proctitis + left-sided colitis; **P <0.05 (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis + left-sided colitis. B. Frequency of LP LAP+ cells expressing intracellular IL-10: **P <0.05 (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis + left-sided colitis. C. Frequency of LP Foxp3+ cells in CD3+CD8- (CD4+)-gated cells: **P <0.05 (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in proctitis + left-sided colitis. In all cases, data represent the mean ± SE of 26 controls and of 13 UC patients with a history of extensive colitis in whom inflammatory lesions were limited to the rectum or left colon at the moment of endoscopy. Patient clinicopathological variables of this UC subgroup are not different from the whole UC patients population (Age: 53±3 – 53 [29-73] (years) mean ± SE - median [range]; Sex: 7/6 M/F; Disease duration since diagnosis:14±3.2 – 12 [1-32]; (years) mean ± SE - median [range]. Mayo endoscopic score: 2±0.2 – 2 [1-3] mean ± SE - median [range]).

Depletion of LAP+ Cells is Associated with Proximal Extension of Inflammatory Lesions in the Murine Oxazolone-Induced Colitis Model

To gain further insight into the roles of the two aforementioned Tregs subsets in limiting the extension of inflammatory lesions, we evaluated the effect of in vivo administration of an LAP-depleting antibody that has no effect on the frequency of CD4+Foxp3+ cells (98) in mice with oxazolone-induced experimental colitis (99). Intrarectal administration of oxazolone (6mg in 50% ethanol) was associated with weight loss and the onset of distal colitis (Figure 10 A, B), as previously described (99). This colitis was associated with significantly higher percentages of LP CD3+CD4+LAP+ cells (Figure 10 C) in uninvolved tissue as compared with both involved tissue and with tissue from untreated control mice, and a higher percentage of CD3+CD4+Foxp3+ cells in involved tissue as compared with both uninvolved tissue and control untreated mice (Figure 10 D), thus reproducing the observations made in UC patients. In oxazolone colitis, the significant increase % of CD4+LAP+cells in uninvolved vs. involved tissue was confirmed also by confocal microscopy (Figures 10 E, F). After a preliminary validation of the ability of anti-LAP antibody administration to selectively deplete LP CD4+LAP+cell without affecting the % of LP CD4+Foxp3+ cells, we administered the antibody or its isotype control in two groups of mice, before the induction of oxazolone colitis. Administration of anti-LAP antibody had no effect on weight loss, which was comparable to that observed in isotype-treated mice (Figure 11 A), but was associated with more extensive colitis (Figures 11 B, C).

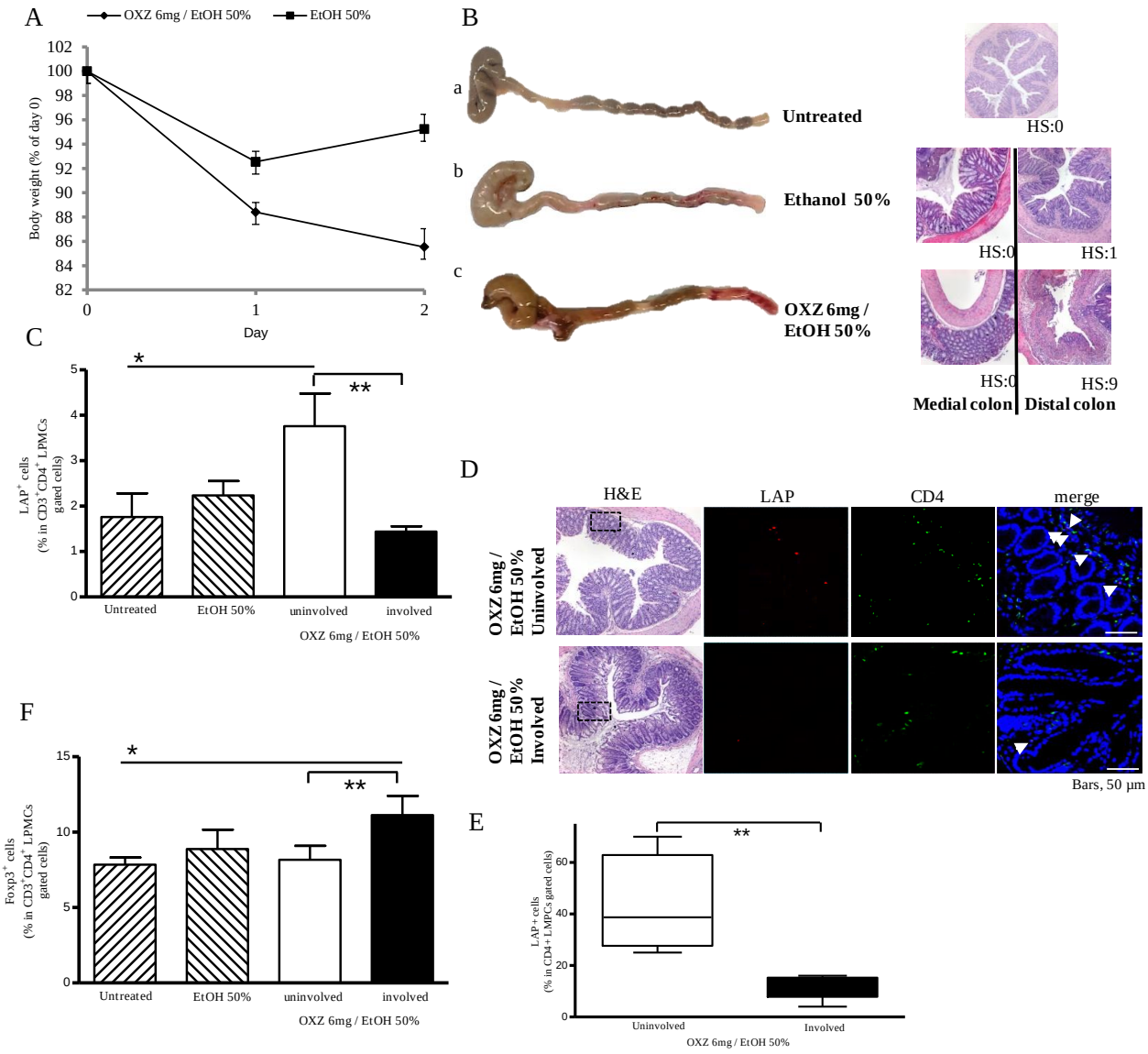


Figure 10. Oxazolone-induced colitis reproduces the observations made in UC patients.

A. Weight changes in mice after intrarectal administration of oxazolone (6 mg/ethanol 50%). Each point represents the cumulative mean (± SE) weight from 3 separate experiments in which 5 mice per group were studied. *P <0.05 (Student's t-test) for EtOH 50% vs. OXZ/EtOH 50%. B. Representative macroscopic and microscopic images of the colons from untreated (a), EtOH 50%-treated (b), and OXZ/EtOH 50%-treated (c) mice, all of which were sacrificed 2 days post-treatment. H&E staining at 40× magnification. HS, histopathologic score (see methods). C. Percentage of LAP+ cells among CD3+CD4+-gated LMPCs isolated from the colon of untreated, ethanol-treated and oxazolone/ethanol-treated mice: *P<0.05 for untreated mice vs. OXZ-treated mice (uninvolved tissue); **P<0.05 for uninvolved vs. involved tissue in OXZ-treated mice. Each point represents mean ± SE of pooled values derived from 3 experiments in which 5 mice per group were evaluated. D. Percentage of Foxp3+ cells among CD3+CD4+-gated LMPCs isolated from the colon of untreated, ethanol-treated, and oxazolone/ethanol-treated mice: *P<0.05 (Mann-Whitney U-test) for untreated mice vs. OXZ-treated mice (involved tissue); **P<0.05 (Wilcoxon signed-rank test) for uninvolved vs. involved tissue in OXZ-treated mice. Each point represents the mean ± SE of pooled values derived from 3 experiments in which 5 mice/group were evaluated.

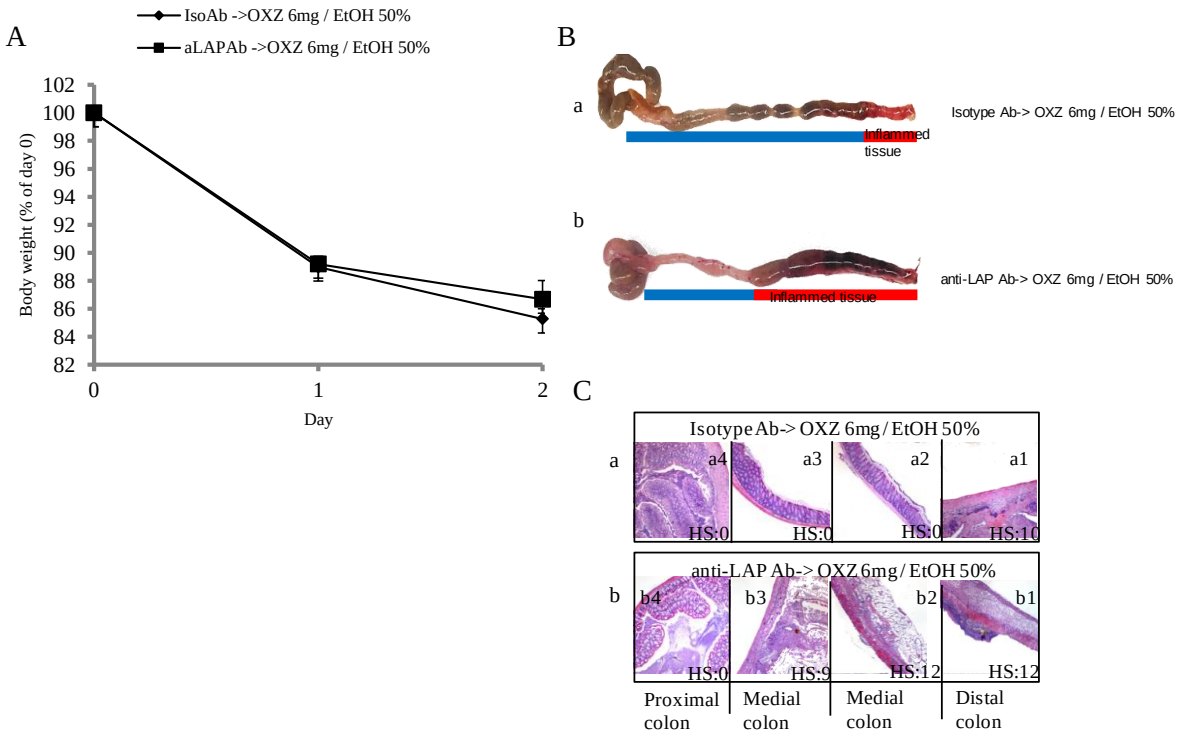


Figure 11. Depletion of LAP+ cells is associated with proximal extension of inflammatory lesions in the mouse oxazolone-induced colitis model.

A. Weight changes in oxazolone-treated mice (6 mg/ethanol 50%) pretreated with either Isotype Ab or anti-LAP Ab. Each point represents the cumulative mean (± SE) weight from 3 separate experiments in which 5 mice per group were studied. B. Representative macroscopic and microscopic images of colons of oxazolone-treated mice pretreated with (a) Isotype Ab or (b) anti-LAP Ab. Panels a1-a4 and b1-b4 show H&E staining (40× magnification) of tissue specimens obtained from different portions of the entire colon. HS, histopathologic score (see methods).

Published article:

Butera A, Sanchez M, Pronio A, Amendola A, De Nitto D, Di Carlo N, Lande R, Frasca L, Borrini F, Pica R, Boirivant M - CD3+CD4+LAP+Foxp3- Regulatory Cells of the Colonic Lamina Propria Limit Disease Extension in Ulcerative Colitis. - Front Immunol. 2018 Oct 30;9:2511. doi: 10.3389/fimmu.2018.02511. eCollection 2018. PMID: 30425718 PMCID: PMC6219428

CONCLUSIONS

We demonstrated that LP CD3+CD4+LAP+ Tregs are responsible for limiting the extension of inflammatory lesions in UC. We observed increases in both the percentage of CD3+CD4+LAP+ cells and the proportion of IL-10-expressing CD3+CD4+LAP+ cells associated with a reduction of IL-17-expressing CD3+CD4+LAP+ cells in uninvolved vs. involved tissue from UC patients. Previous studies have reported increases in the numbers of CD3+CD4+LAP+ cells (51) and CD3+CD4+Foxp3+ cells (49, 50) in involved UC tissue, the former were found to be functionally impaired in vitro, while the latter, although functional in vitro, were ineffective in counteracting inflammation in vivo. Indeed, in the present study, uninvolved tissue is characterized by a significant increase in the ratio between IL-10 and IL-17 expressing LAP+ cells suggesting that the increased % of CD3+CD4+LAP+ cells observed in uninvolved tissue is predominantly represented by functional active regulatory cells. This hypothesis is reinforced by the observations obtained in mice since selective depletion of LAP+ cells in a mouse model of distal colitis was associated with the extension of inflammatory lesions to the proximal colon. These data strongly suggest that CD3+CD4+LAP+ regulatory cells play a key role in limiting the extension of colonic inflammatory lesions in UC and that expansion of LP CD3+CD4+LAP+ cells may constitute a therapeutic strategy to limit and possibly prevent colonic inflammation in UC patients.

Characterization of Ulcerative colitis patients according with tissue cytokine profile and/or microbiota composition

IL-13 mRNA tissue content allows for a meaningful classification of UC patients

We preliminary analyzed the mRNA tissue content of different cytokines in UC patients and control subjects (see Table 2 cohort II for clinical-pathological variables). As illustrated in Figure 12, in UC patients, all the cytokines evaluated show significantly increased mRNA tissue content in the involved tissue vs uninvolved tissue that in turn show values comparable to controls. However, it is worth noting IL-13 values have a clear bimodal distribution, while IL-17A values in UC involved tissue show the smallest overlap with the controls suggesting discriminatory ability in separating the two groups. Analysis of Variance as applied to controls-patients discrimination resulted statistically significant for all the analyzed cytokines with the only exception of IL-13 (Table 4). Moreover, IL-13 distribution and the related quantile-quantile (Q-Q) distribution (Figure 13, panel A and B, respectively) clearly show a deviation from the normal distribution for both patients and controls, thus not related to "a priori class discrimination" suggesting clustering relevance of IL-13 for the detection of disease sub-groups. This suggestion had a strong proof by the application of two independent computational approaches: a) Bimodality estimation by Pseudo F statistics (83) and b) Pearson correlation and Principal Component Analysis as applied to cytokine profile of UC patients. Pseudo-F statistics corresponds to the ratio of Between/Within cluster variance: the greater this ratio, the greater the relevance of the clusters. In the case of two clusters, the Pseudo-F is an index of bimodality, with high values pointing to the presence of two distinct populations in the same set. We computed the Pseudo-F statistics on the two cluster optimal K-means solution for all the evaluated cytokines independently from the control/patient a priori classification. According with this analysis IL-17A and IL-13 were the two cytokines endowed with the higher values of the index and thus the two optimal variables for classification purposes (Table 5). In the case of IL-17A, Pseudo-F registers the control/patient discriminatory power of this cytokine. On the contrary, the lack of any control/patient discriminant power of IL-13 makes the elevated Pseudo-F values of this cytokine the image in light of a different latent biological categorization. Even more compelling, we found that the differences in mRNA tissue content of the involved vs uninvolved colonic area from the same patient relative to IL-6, TNF-

α, IFN-γ and IL-10 were each other strongly correlated (Pearson correlation coefficient), while IL-17A and IL-13 variations were both independent from all the other cytokine mRNA concentration and independent of each other. This correlation structure gave rise to a three principal components (PCs) solution of the entire cytokine space (Table 6).

The percentage of variance explained by the three-component solution was 48.8%, 19.4% and 16.5% for PC1, PC2 and PC3 respectively for a cumulative 85% of variance explained. This means the relevant information can be summarized into three latent factors shaping the data set variance. Bolded values point to variables most correlated with the different components that in turn are each other independent by construction. The first component (PC1) presents all positive (and very high) loadings with all cytokines with the only exception of IL-13 and IL-17A. This implies PC1 quantifies the 'global amount' (or intensity) of inflammatory response, while PC2 and PC3 correspond to the singular behavior of IL-13 and IL-17A. Components are each other independent by construction, thus the analysis shows that IL-13 and IL-17A play a peculiar role in the disease with respect to the other cytokines. The accompanying figure of table 6 reports the space spanned by aspecific inflammatory response score (PC1) and the IL-13 (left) and IL-17A (right) involved-uninvolved tissue differential response. It is worth noting how both IL-13 and IL-17A are independent from PC1. Keeping in mind IL-17A is the cytokine most specifically linked to the disease and IL13 has the most neat bimodal character, we can focus on these two cytokines for deriving a meaningful sub-typing of the disease independent of the aspecific inflammatory response

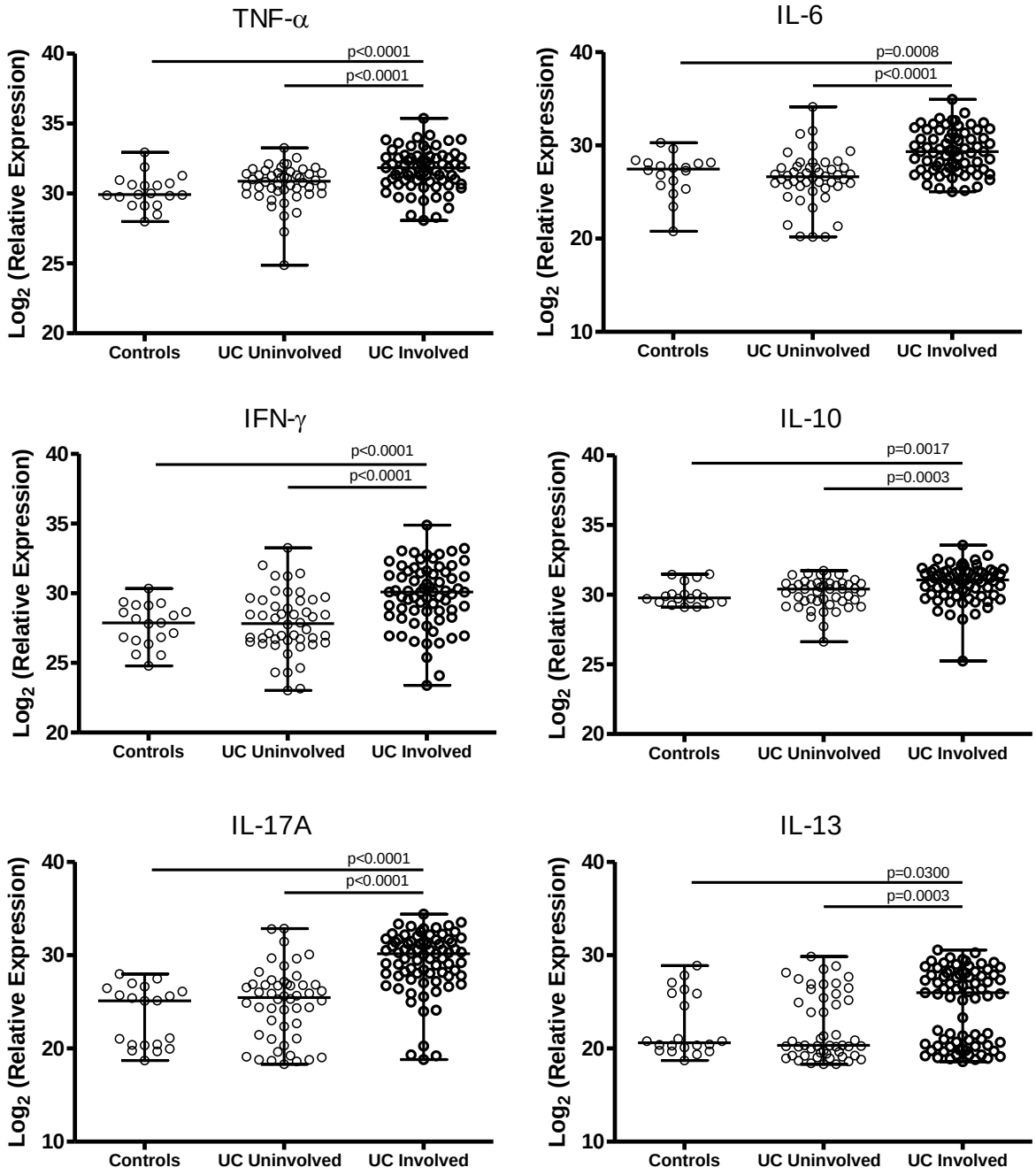


Figure 12: Cytokine mRNA tissue content in controls samples (n=20), in the uninvolved tissue (n=50) and involved tissue (n=77) of UC patients. Lines represent median and range. p= as for Mann-Whitney test.

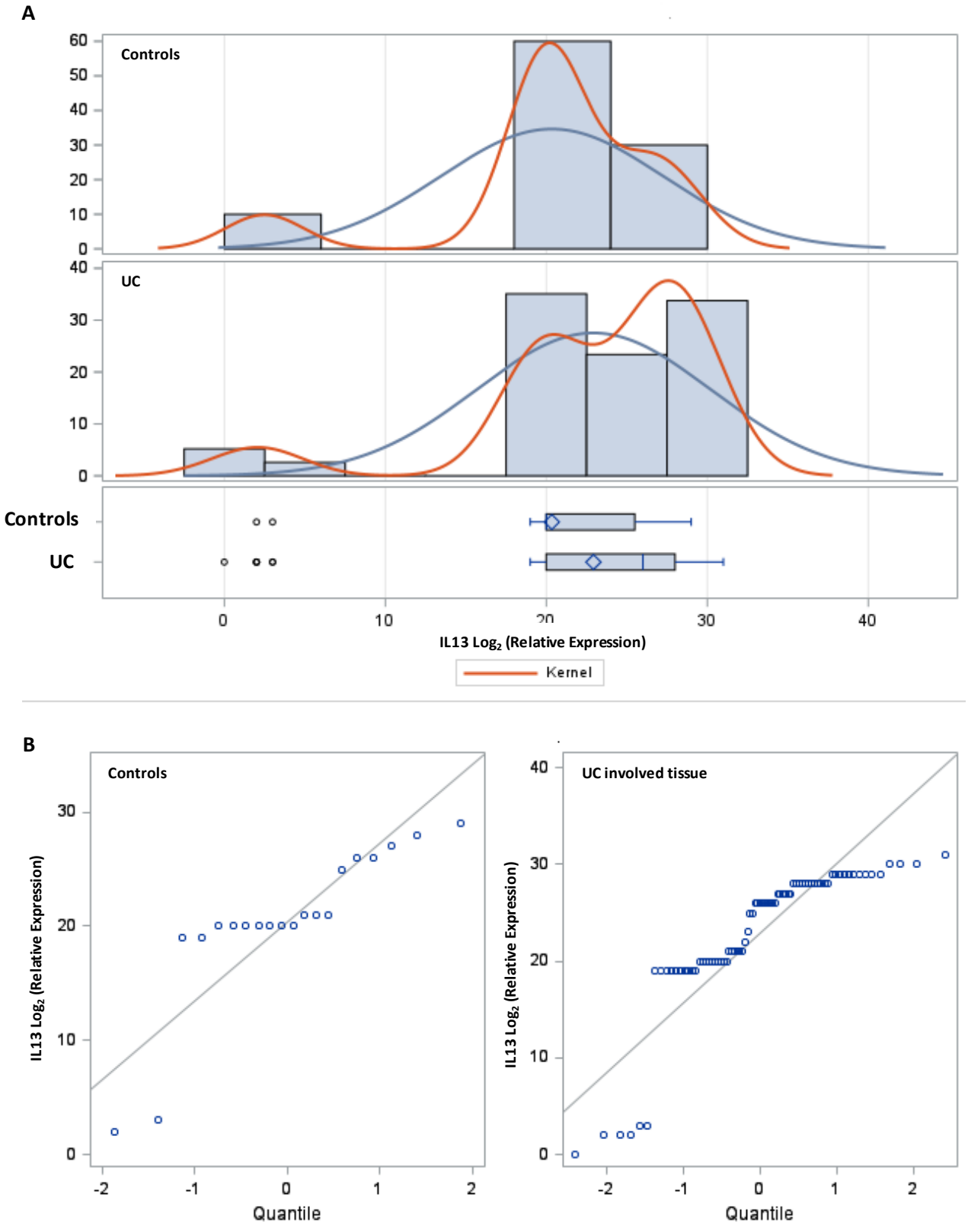


Figure 13. Distribution of patients and controls. (A) IL-13 distribution and (B) the related quantile-quantile (Q-Q) distribution of UC patients and controls.

Table 4. Statistical comparisons between control (healthy) and UC patient group. The comparisons were based on Wilcoxon rank-sum test and statistical significance was assessed by Kruskal-Wallis test.

Variable	Average score (ctr)	Average score (UC)	Kruskal_Wallis p-value
IL-17A	24.40	55.39	0.0001
IL-13	38.12	51.53	0.056
IFN-γ	26.16	52.35	0.0002
TNF-α	24.74	52.72	0.0001
IL-10	28.55	49.74	0.002
IL-6	25.89	50.40	0.0004

Table 5. Bimodality degree

Cytokine	Pseudo F (2 cluster)*
IL-17	**265.78**
IL-13	**241.47**
IL-6	197.21
TNF-α	188.00
IFN-γ	161.21
IL-10	86.14
* ratio Between/Within cluster variance	

Table 5. The Pseudo F statistics is a bimodality index. The higher the value, the higher the separation of the sample into two heterogeneous groups.

Table 6 and Figure.

Table 6. Loadings (Correlation coefficients between variables and components)

Delta (Δ)*	**PC1**	**PC2**	**PC3**
Δ IL-17	0.00270	0.28055	**0.95936**
Δ IL-13	0.02595	**0.94107**	-0.22134
Δ IFN-γ	**0.85625**	0.26554	-0.04993
Δ TNF-α	**0.81067**	-0.34554	0.12704
Δ IL-10	**0.86212**	-0.04289	-0.00842
Δ IL-6	**0.89126**	0.07243	-0.05590
* Δ = Cytokine mRNA tissue content of Involved – cytokine mRNA tissue content of Uninvolved colonic tissue.			

The percentage of variance explained by the three-component solution was 48.8%, 19.4% and 16.5% for PC1-PC3 respectively for a cumulative 85% of variance explained. Bolded values point to variables most correlated with the different components that in turn are each other independent by construction. Principal component solution is separated into 'size' and 'shape' components (100). Size components (here PC1) are characterized by all positive loadings and thus represent the 'global amount' (or intensity) (101) of a given phenomenon, 'Shape' components (here PC2 and PC3) have both positive and negative loadings and describe the 'differential profiles' (shape) of the analyzed data structure.

Figure. Cytokine Response Space

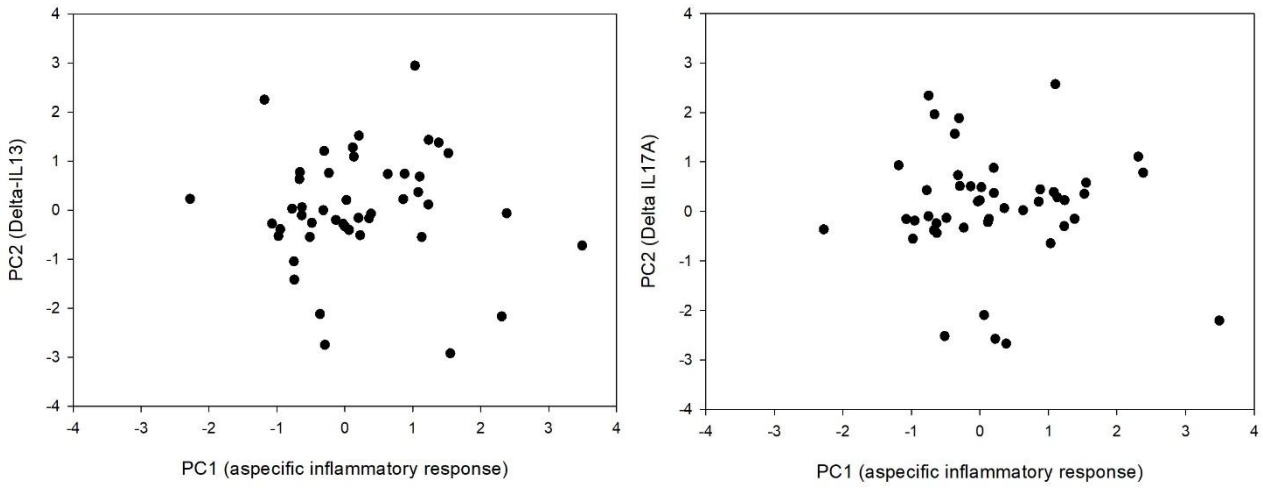


Figure reports the space spanned by aspecific inflammatory response score (PC1) and the IL-13 (left) and IL-17A (right) involved-uninvolved tissue differential response. It is worth noting how both IL-13 and IL-17A are independent from the aspecific inflammation status.

UC patients are distributed in clusters characterized by high and low IL-13 mRNA

Given the above described observations, we concentrated on IL-13 and IL-17A mRNA levels in order to single out a reliable partition of the data. As shown in Figure 14, the combination of the two cytokines indeed clearly separated UC patients from control subjects confirming the significant alteration of the two cytokine expression due to the disease. We then evaluated the distribution of subjects in the IL-17A/IL-13 plane. As shown in Figure 15 A only UC patients have IL-17A values greater than the mean value of IL-17A (right quadrants), while, as far as IL-13 values, the higher than mean values belong to both UC patients and control subjects although the former are prevalent. Thus, distribution of IL-17A and IL-13mRNA values points to the existence of discrete clusters of subjects. A k-means cluster analysis on the IL-17A/IL-13 plane was computed (Figure 15 B). It is worth to stress that k-means is an unsupervised technique and does not take into account the class of the subjects (patients or controls) but only their mutual relations in the IL-17A/IL-13 plane. Data represented in Figure 16 points to a six clusters partition as optimal. The six clusters partition explains 91% of the total information present in the data set (R-square=0.91). With the only exception of cluster 1 (accommodating for two outlier observations), the classes are sufficiently populated to

allow for a reliable profiling of IL-17A and IL-13 tissue content. The IL-17A mRNA tissue content varies smoothly from healthy to diseased patients: the ‘extreme’ cluster 6 and cluster 2 are devoid of any control subject, while the intermediate and ‘left side of the plane’ clusters have a mixed controls/UC populations (Figure 15 B). Cluster 6 is made of patients with ‘high IL-17A/high IL-13’ pattern, while clusters 2 have a ‘high IL-17A/low IL-13’ pattern (Figure 15 B). The above analysis allows us to define a bipartite profile of UC patients according to ‘high IL-13’ (Cluster 6) and ‘low IL-13’ (Cluster 2) mRNA tissue content in the context of high IL-17A mRNA tissue content.

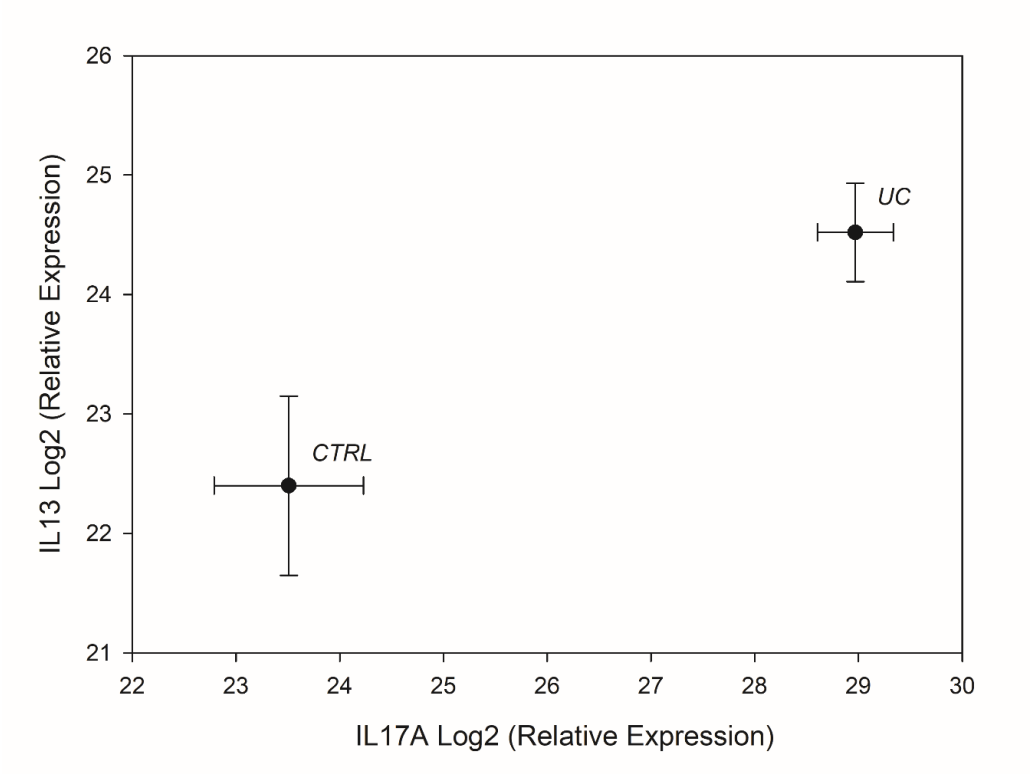


Figure 14: Mean values location in the bi-dimensional IL-17A vs IL-13 plane is different in UC (n=77) and Controls (n=20). Data in a bivariate mean/SE plane for both IL-13 and IL-17A. Points represents mean±SE.

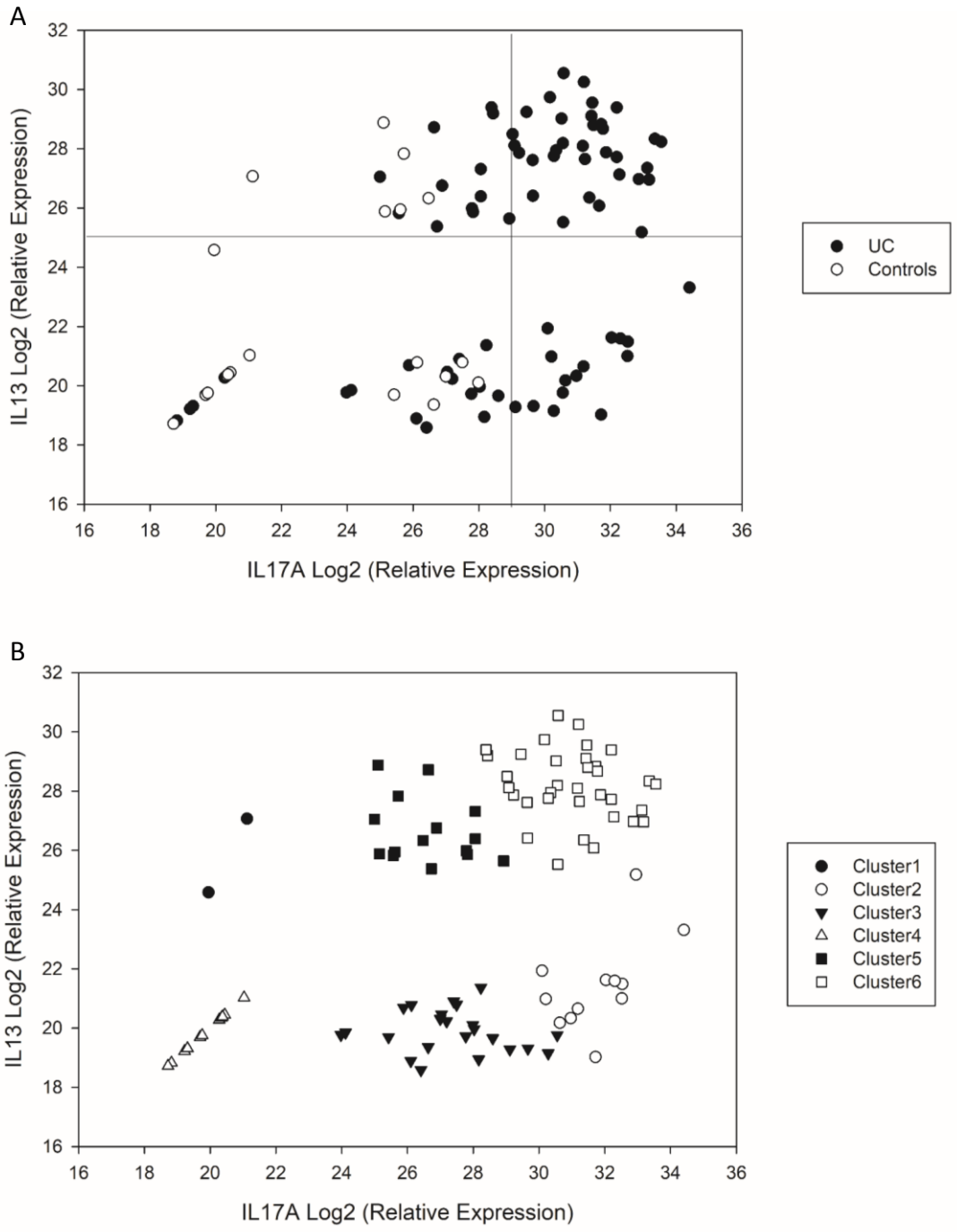


Figure 15: UC patients are distributed in six clusters characterized by high and low IL-13 mRNA and increased IL-17A mRNA tissue content. Panel A: Data distribution of subjects (UC patients and controls) in the IL-17A/IL-13 plane. Each point represents patient or control IL-17A/IL-13 mRNA values. Lines represent mean of IL-17A and IL-13. Panel B: Distribution of clusters in the IL-17A/IL-13 plane. Each point represent patient or control IL-17A/IL-13 mRNA values- associated cluster. Panel A and B: UC patients n=77; Controls n=20.

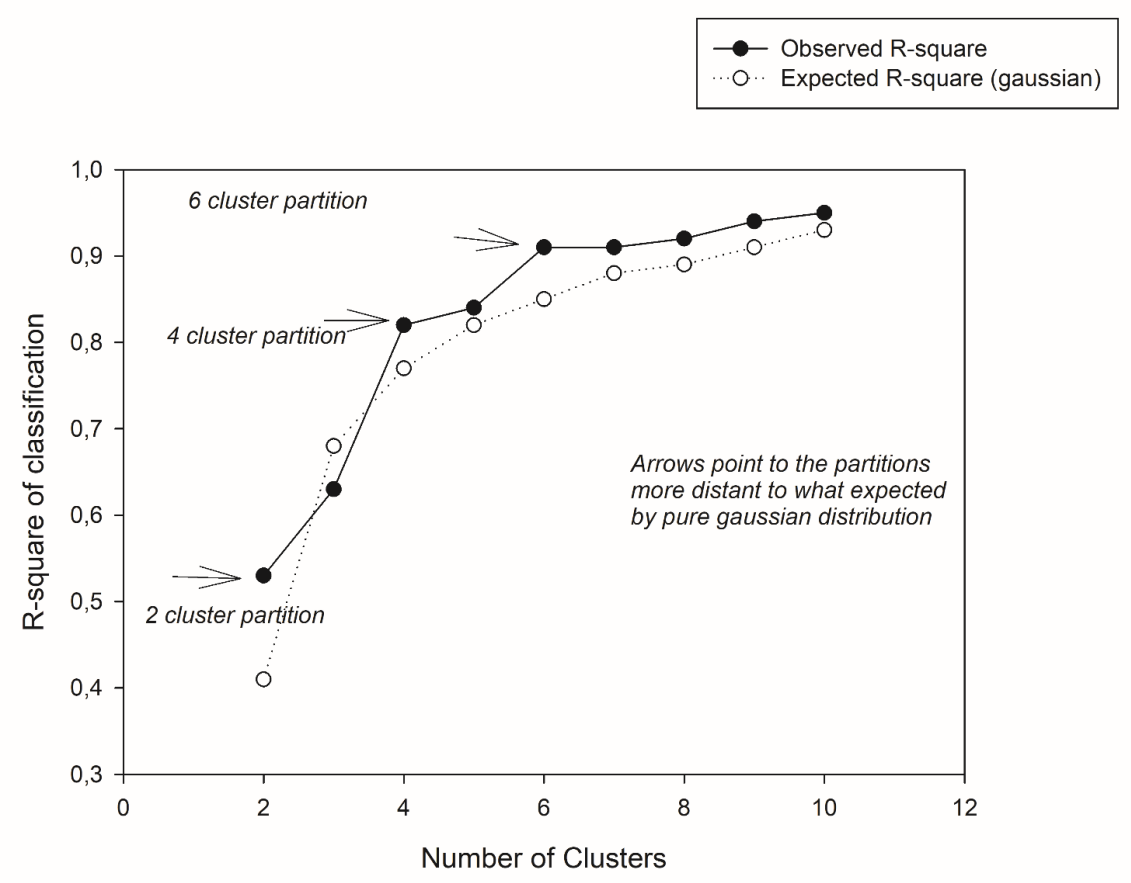


Figure 16. Six clusters partition defines the optimal partition of data. R-square choice of n was computed and compared to what expected by a pure Gaussian distribution. The partition maximizing the distance between actual R-square and expected R-square under Gaussian hypothesis was considered as the partition of choice.

Clusters in the IL-13/IL-17A plane show differences as for sex, age at diagnosis, prevalence of extensive colitis and steroid/ immunosuppressant/anti-TNF-a therapy use

Having proved the existence of patient cluster distribution (further confirmed by bootstrap simulations), we analyzed the observed cytokine-based patient subgroups according to the clinical-pathological variables. We preliminary assessed the presence of differences in the clinical-pathological variable among the clusters 2, 3 and 4 (low IL-13mRNA content) and between the clusters 5 and 6 (high IL-13 mRNA content). Since we did not find any statistical differences we grouped cluster 2, 3 and 4 (low IL-13mRNA content) and cluster 5 and 6 (high IL-13mRNA content) for the analysis. No differential distribution as for disease duration (139±20 vs 123±16 months, mean±SE; p=0.5, low and high IL-13 subset, respectively), therapy at time of

endoscopy (corticosteroids, immunosuppressant, biological therapy at the time of endoscopy: 33% vs 32 % low and high IL-13 subset, respectively) and endoscopic Mayo score (1.9±0.12 vs 2.1±0.11, mean±SE; p=0.38, low and high IL-13 subset, respectively), was observed. A significantly lower prevalence of females was observed in patients belonging to low IL-13 mRNA tissue content (Figure 17 A). Patients with high IL-13 mRNA tissue content show a significant lower age at diagnosis than patients with low IL-13 mRNA tissue content (Figure 17 B). A significant higher prevalence of patients with extensive colitis was observed in patients with high IL-13 mRNA than in patients with low IL-13 mRNA tissue content (Figure 17 C). In 42 out of the 77 patients, information regarding therapy during at least 1 year of follow-up was available. This subset of patients did not differ from the whole group as for clinical-pathological variables at entry. Furthermore, the distribution along different clusters of the 42 patients in which follow up was available did not differ from the whole set distribution (clusters 2+3+4: 41.86% vs 42.86%; clusters 5+6: 58.14% vs 57.14%, respectively). Therefore, we considered the 42 patients subset as an unbiased subsample of the whole set. In this subset of patients, the relative frequency of clinical relapses during the one-year follow-up period did not differ between patients with low and high IL-13mRNA content (27 vs 29 %, proportion of patients with clinical relapses, respectively). However, patients with high IL-13 mRNA showed, during the follow-up, but not at the time of endoscopy (corticosteroids, immunosuppressant, biological therapy: 27.78% vs 20.83% low and high IL-13 subset, respectively), increased steroid/ immunosuppressant/anti-TNF-α therapy use to reach and maintain the remission, when compared with patients with low IL-13mRNA (Figure 17 D). Taken together, the above results suggest that UC patients showing high IL-13 mRNA tissue content have a more severe colitis than patients showing low IL-13 mRNA tissue content. When males and females were separately analyzed for the clinical-pathological variables described above, it appears that the only variable influenced by gender was the age at diagnosis where males contributed to the observed significant lower age at diagnosis in patients belonging to clusters 5+6 (males clusters 5+6: 30 (4-61) vs males clusters 2+3+4: 42 (20-74), median (range); p= 0.0043, by Mann Whitney test).

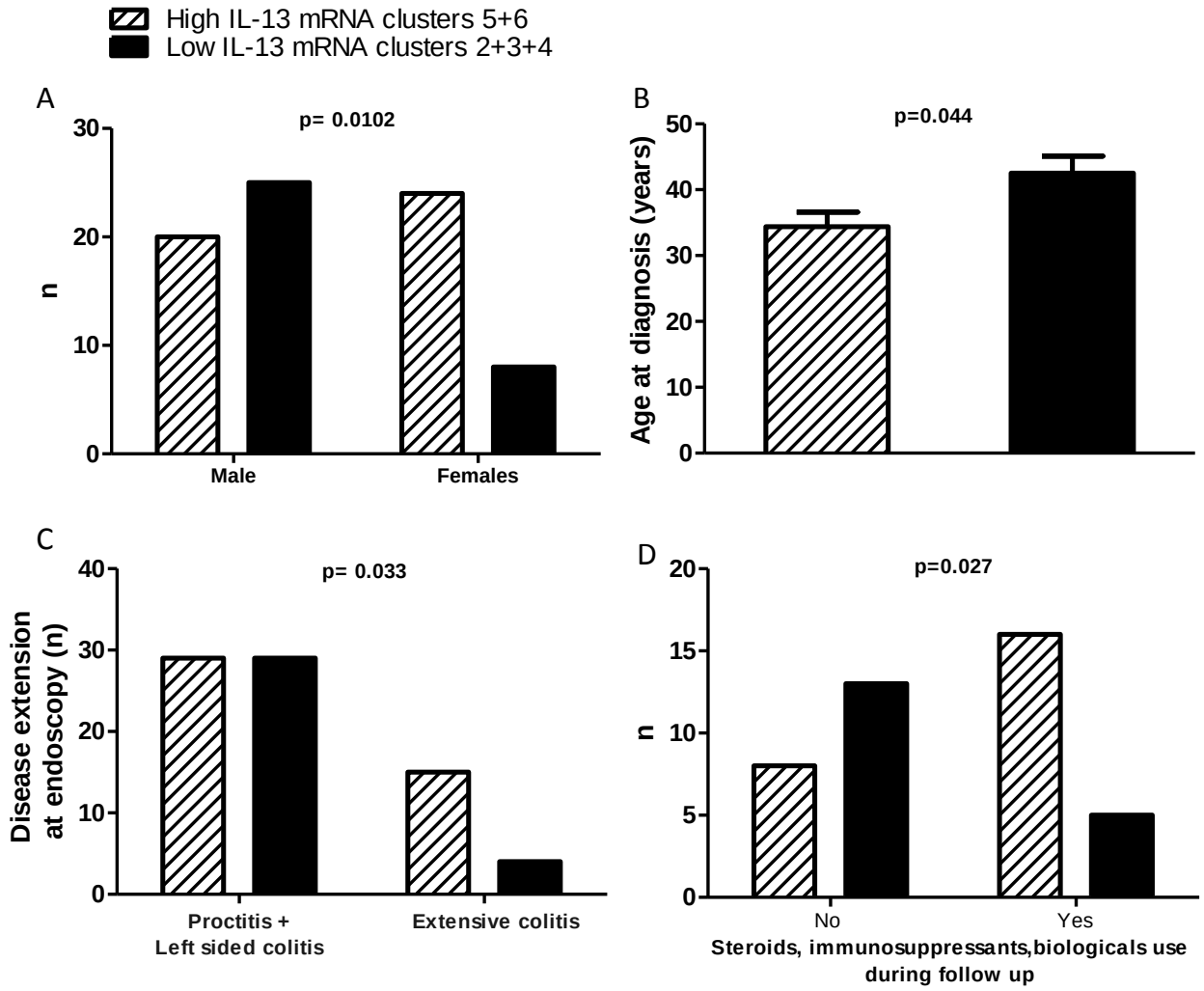


Figure 17. Clusters in the IL-13/IL-17A plane show differences as for sex (A), age at diagnosis (B), prevalence of extensive colitis (C) and steroid/ immunosuppressant/biological therapy use (D). Panel A, B, and C: UC patients n=77. Panel D: UC patients n=42.

Characterization of mucosa-associated microbiota in UC patients and controls

To investigate the ability of microbiota composition to identify different UC patients subsets or to complement cytokine profile information, we preliminary evaluated mucosal microbiota composition in UC patients (both in involved and uninvolved tissue) and in controls, by performing Illumina MiSeq sequencing of 16S rDNA. A summary of microbiota profiles (relative abundances) at family level is reported in Figure 18 A. As for α-diversity (Figure 18 B) and ß-diversity (Figure 18 C), we did not observe major differences in the mucosa-associated microbial profiles between UC and Controls (both involved and uninvolved tissue). Furthermore, we did not observe major differences between involved and uninvolved tissue in UC patients. Interestingly, PCoA performed on Bray-Curtis distances suggests a three group's ordination of

mucosa-associated microbial profiles, varying independently from mucosal tissue type (involved, uninvolved, control mucosa) (Figure 18 C), IL-13 and IL-17 tissue cytokine content (Figure 19), cytokine clusters (Figure 20), as well as clinical-pathological variables (Table 7).

Considering the data-driven characteristic of PCoA, this implies neither disease, nor cytokine profile are relevant order parameters of the microbiota profile. Thus, the global microbiota profile stems from other biological features (e.g. diet, micro-environmental features, genetics) and we can only focus on local microbial features to try to get a 'signature' of the disease and cytokine status. This methodologically implies the shift from a data-driven (PCoA) to a supervised (Discriminant Analysis) approach. When discriminant microbial signatures were investigated by LEfSe analysis (Figure 18 D, E), comparison between controls mucosa-associated microbiota (Controls, gray highlighted node of the tree) and UC mucosa-associated microbiota (both involved and uninvolved, red node of the tree) showed an enrichment of *Roseburia* genus in control mucosa compared to UC (Figure 18 D), as previously reported in several studies (102, 103, 16).

UC samples (involved and uninvolved tissue) showed relative enrichment of Bifidobacteriaceae, Gemellaceae, Enterococcaceae, Eryspelothricaceae, and Lactobacillales, including also *Bifidobacterium*, *Enterococcus* and *Acidaminococcus* genera (Figure 18 D). When we examined differential taxa between involved mucosa and uninvolved mucosa (Figure 18 E), *Bifidobacterium* and *Acidaminococcus* genera and *Gemellaceae* family were enriched in uninvolved mucosa, while, *Lactobacillus*, *Eubacterium* and *Enterococcus* genera were enriched in the involved mucosa. These data are in agreement with the reported increase in UC fecal samples of Enterococcaceae, Lactobacillaceae (104) families and Enterococcus genus (105) suggesting that the contribution to the reported increase in fecal samples is mainly due to the increase observed in inflamed tissue.

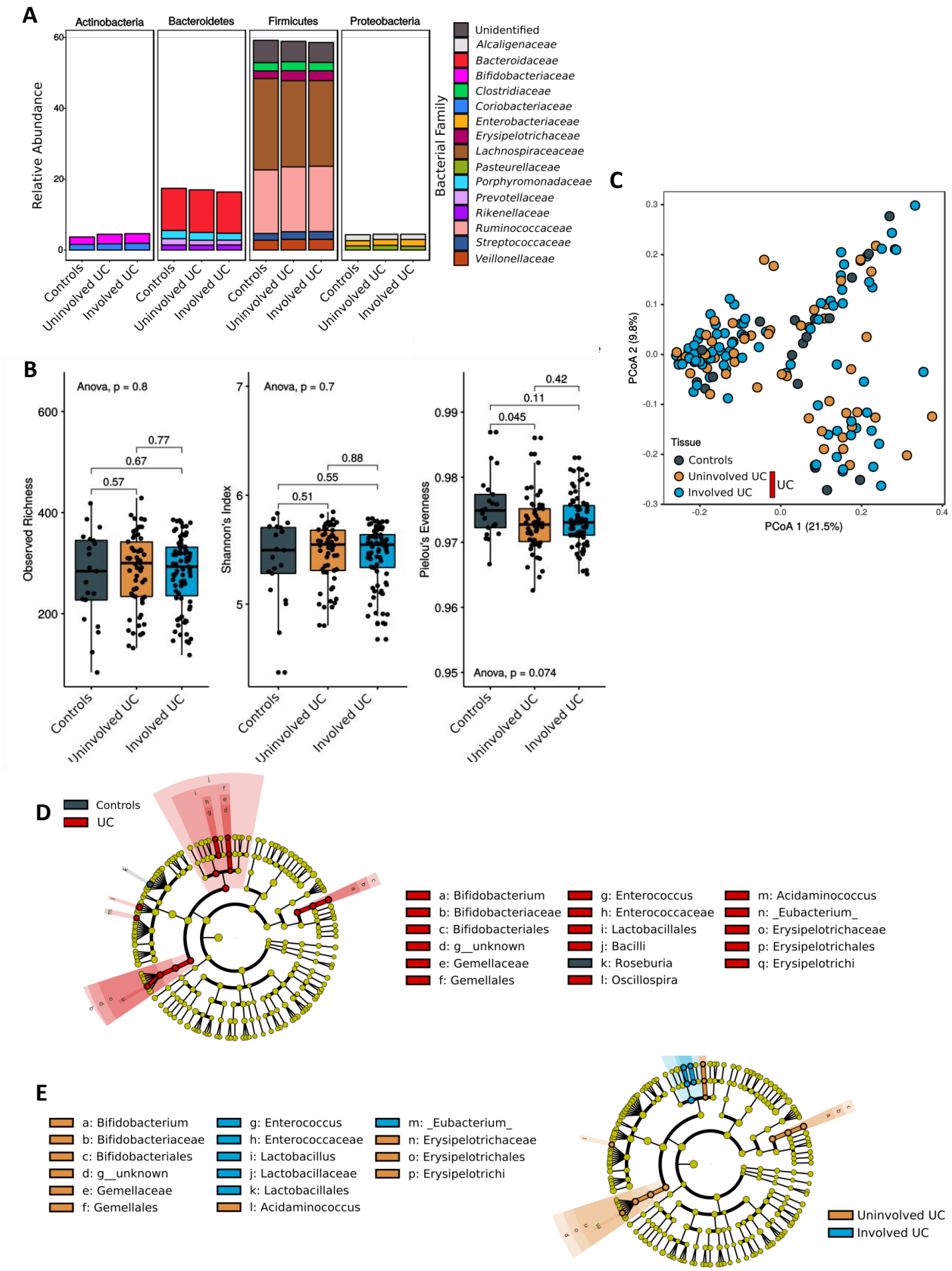


Figure 18: Explorative analyses and comparison of mucosal associated microbiota composition in UC and control subjects. (A) Barplot shows the composition at the Family level, according with the Phylum of belonging. Family with relative abundance < 1% were discarded. (B) Alpha diversity. Box plots represent alpha diversity measured through observed OTUs, Shannon index, Pielou's evenness in controls, UC uninvolved, and UC involved mucosal tissues. (C)

PCoA ordination based on Bray-Curtis dissimilarities of mucosal samples from UC (involved, n=79, and uninvolved, n=57, tissue) and controls (n=21). PERMANOVA using the Adonis function with 999 permutations resulted not significant. (D-E) LEfSe analysis showing significantly biomarker taxa in two different comparisons, based on (D) disease status: UC (both involved and uninvolved tissue, passed as subclasses t LEfSe) compared to control samples, and (E) tissue: controls versus uninvolved versus involved tissue in UC patients. Cladograms show the most discriminative bacterial clades. Coloured regions/branches indicate differences in the bacterial population structure between the different groups. Statistically significant taxa enrichment among groups was obtained with Kruskal-Wallis test among classes (Alpha value = 0.05). The threshold for the logarithmic LDA score was 2.0. Overall statistical significance was calculated by using the ANOVA test, pairwise comparisons were calculated using t-test. P-values are reported in each panel and for each pairwise comparison.

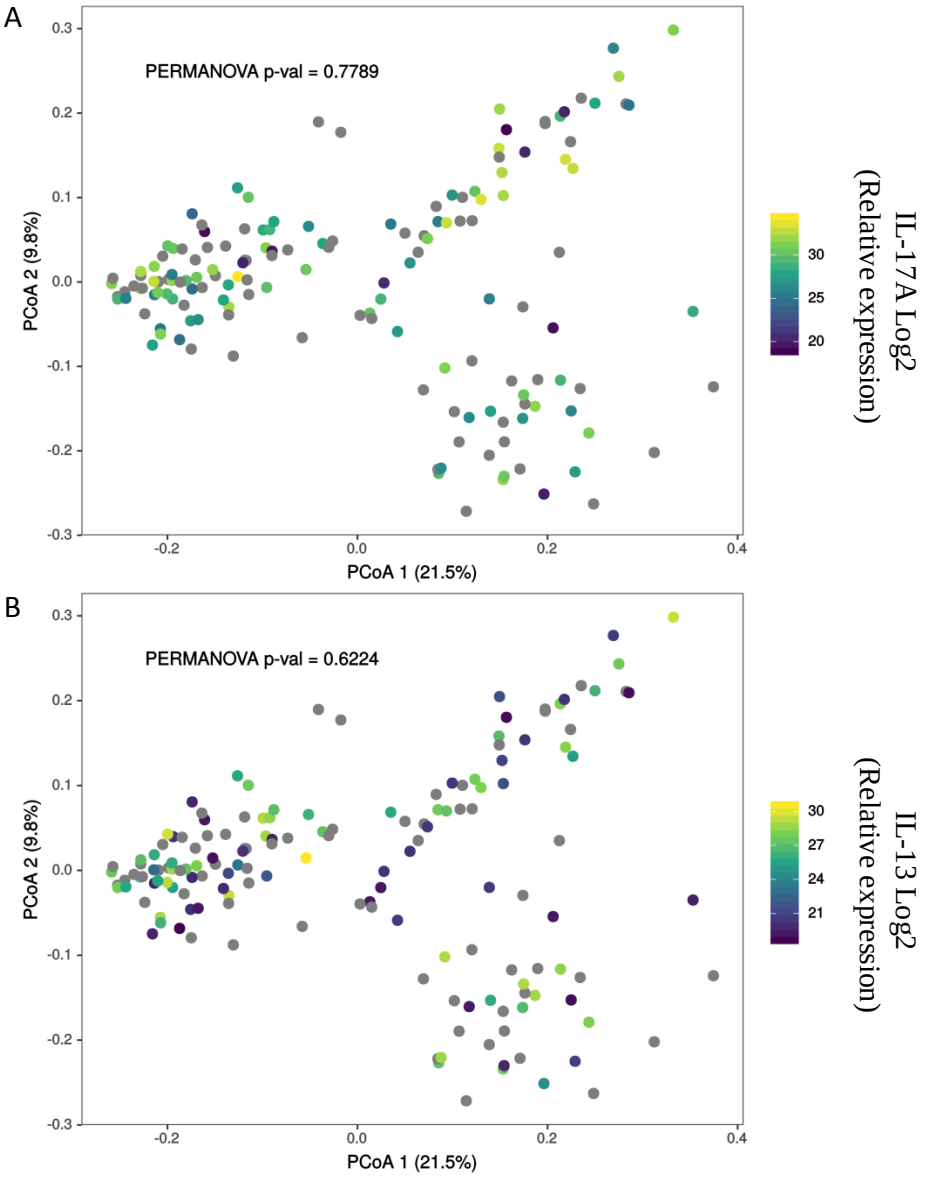


Figure 19. PCoA ordination based on Bray-Curtis distance as reported in Figure 13 B. Points are colored based on IL-17A (A) or IL-13 (B) tissue content.

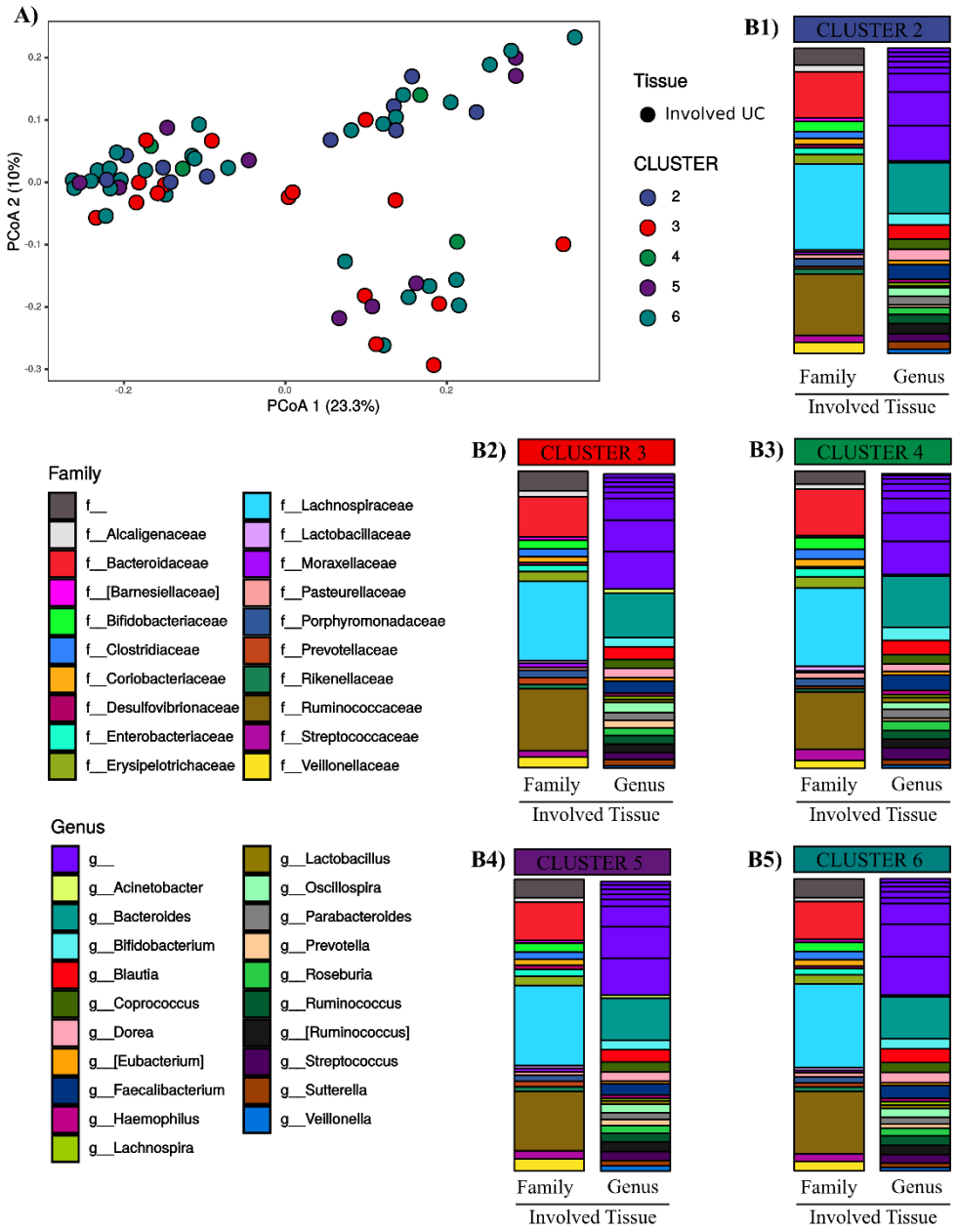


Figure 20. Composition and differences of bacterial community in samples (UC patients, involved mucosa) belonging to different citokynes clusters. (A) PCoA ordination based on Bray-Cutis distance. Points are colored based on the citokynes clusters. (B) For each clusters, a barplot of the attached bacterial community at Family and Genus level is reported. Colored bar at the bases of the barplots indicate which cluster the image refers to; i.e. panel B1 refers to patients classified in cluster 2, panel B2 refers to patient classified in cluster 3, panel B4 refers to patients classified in cluster 4, panel B5 refer to patient classified in cluster 5, and panel B6 refers to patients classified in cluster 6.

Table 7. Permanova analysis results (only R^2 and P-Value are reported) of microbiota composition (Bray-Curtis dissimilarity matrix) in relation to cytokine content and clinical-pathological variables in the involved mucosa of UC patients (n=68). Subset of patients with follow-up information: n=36.

Variable	R^2	P-Value
IL-17	0.016	0.293
IL-13	0.010	0.843
Gender	0.012	0.650
Mayo Score	0.014	0.503
Age at Endoscopy	0.018	0.210
Age at diagnosis	0.021	0.118
Cluster	0.050	0.828
Disease Duration (years)	0.019	0.289
Corticosteroids, immunosuppressant, biological therapy at the time of endoscopy	0.033	0.913
Disease extension (at endoscopy)	0.028	0.552
Corticosteroids, immunosuppressant, biological use during follow-up	0.048	0.060

Comparative analysis of mucosal microbiota composition in CL6 (high IL-13 tissue content) and CL2 (low IL-13 tissue content) patients' clusters

Mucosal microbiota composition was further analyzed focusing on the subset of involved tissues in subjects belonging to cytokine Cluster 6 (CL) and CL 2, the two clusters populated by UC patients only characterized by high and low IL-13 mRNA, respectively. Alpha diversity analysis (i.e. richness, evenness, Shannon's index, and Faith's phylogenetic diversity index) and beta diversity did not highlight differences between involved mucosa-associated microbiota of patients in CL6 and CL2. Interestingly, when we selected only samples belonging to CL6 and CL2 to evaluate their ß-diversity, the spatial arrangement of samples on the ordination space (PCoA based on Bray-Curtis distances) resembled the one observed in the complete samples population (Figure 18 C). This evidence further confirms our previous observation that the global microbiota profile stems from other biological features than cytokine status alone. Nevertheless, when differences between involved mucosa-associated microbiota of patients belonging to the two clusters that shows the lowest (CL2) and the highest (CL6) IL-13 values were evaluated by LEfSe (Figure 21), *Prevotella* genus resulted a microbial signature associated to subjects in CL6, while *Acidaminococcus* and *Sutterella*

genera, were associated to patients in CL2. Interestingly, a more in-depth sequencing analysis at species-level revealed that 2.5% of the total number of the reads (218’557 out of 8’498’053 total number) were assigned to *Prevotella* spp. and *Prevotella copri* was present in more than 50% of the CL6 samples.

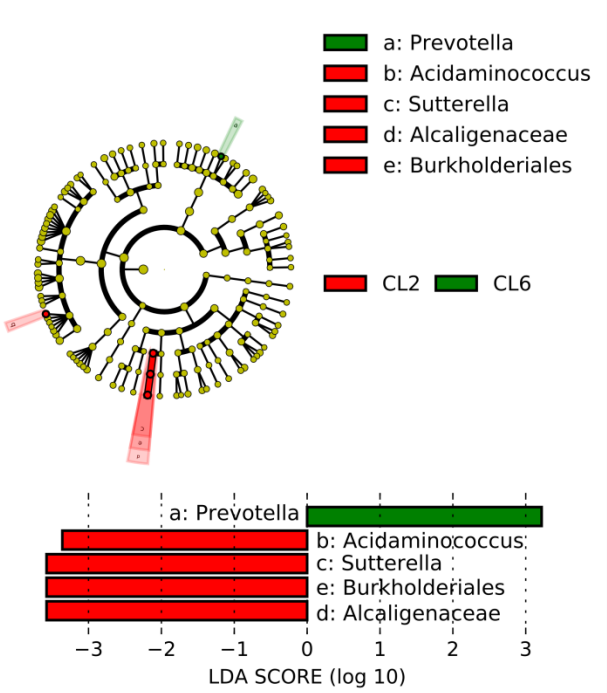


Figure 21. Comparison of mucosal microbial profiles between UC patients belonging to Cluster 2 (CL2, n = 10) and Cluster 6 (CL6, n = 30). LEfSe analysis showing significantly enriched biomarker taxa in CL6 vs CL2 comparisons. Cladogram and barplot show the most discriminative bacterial clades. Colored regions/branches indicate differences in the bacterial population structure between the different CL2 and CL6. Statistically significant taxa enrichment among groups was obtained with Kruskal-Wallis test among classes (Alpha value = 0.05). The threshold for the logarithmic LDA score was 2.0.

CONCLUSIONS

In the present study, we demonstrated the existence of two different subsets of adult endoscopically active UC patients characterized, in the context of a generalized increase of IL-17A tissue mRNA content, by high and low IL-13 tissue mRNA content. Patients in the two subsets show different clinical-pathological characteristics and some local microbial markers.

Among the cytokines explored in the present study, only IL-17A and IL-13 show classificatory ability independent from the global inflammatory status of the patients (PC1, see Results). IL-17A and IL-13 are each other linearly independent, with IL-17A marking the 'presence of disease' and IL-13 the disease sub-typing. Our observation of increased IL-17A expression in UC is in line with previous observations by our and other groups (51, 77, 106, 107), and with a recent report in which a large cohort of pediatric patients was evaluated (75). Notably, in the last reported study, IL-17A and IL-13 mRNA tissue content (with no significant correlation between IL-13 and IL-17A expression) were increased in ulcerative colitis and IL-17A and IL-5 mRNA expression were able to distinguish UC from colon-only Crohn's disease (75). It has been recently shown that the ratio of mucosal IL17A to IL17F expression correlate significantly with endoscopic disease activity in adult UC (107) and we previously demonstrated that dual expression of IL17A by CD4+CD25- regulatory T cells expressing surface transforming growth factor-β in its latent form (LAP) reduces the suppressor activity of these cells in UC (51). In addition to IL-17A, sufficient evidence points to IL-13 as an important pathological factor in adult and pediatric UC patients (55, 4, 75, 76,108), although few reports (109,110) did not show increased level of IL-13 in UC patients. The reason for such discrepancies may reside in both the different experimental techniques (109, 110) and the very low number of patients in which IL-13mRNA was evaluated (109) in the latter mentioned studies. The two subsets of UC patients observed in the present study differ in the prevalence of gender, being females more represented in the high IL-13 subset, as well as in the age at diagnosis that was significantly lower in the high IL-13 patients' subset when compared to the low IL-13 patients' subset. No significant difference between the two subsets in the disease duration from the diagnosis was observed. As regard disease characteristics, the high IL-13 patients' subset show an increased prevalence of extensive colitis. Furthermore, in the group of 42 patients in which clinical information during the follow-up were available, the use of steroids, immunosuppressant and biological agents

to achieve and maintain clinical remission, not significantly different at the time of endoscopy, was higher in the high IL-13 patients' subset than in the low IL-13 patient's subset. Taken together data suggest that UC patients showing high IL-13 mRNA tissue content have a more severe colitis than patients with low IL-13 mRNA tissue content. This finding somehow differs from the observation reported in a treatment-naive UC pediatric patients cohort characterized by concomitant increased mucosal IL-17A and IL-13 mRNA (75). In this cohort of patients, higher IL-13 expression was associated significantly with an increased likelihood of clinical response at 6 and 12 months to first-line therapies (i.e. corticosteroids, mesalamine, and thiopurines) and steroid free remission compared with that of patients characterized by essentially undetectable IL-13 expression. Moreover, during the same period of time, these patients were less exposed to anti-TNF-biologic drugs. In our cohort, patients with high and low IL-13 mRNA did not differ in the clinical response or in the percentage of patients experiencing a clinical relapse during the one year follow-up, however the use of systemic corticosteroids, immunosuppressant and anti-TNF-α biologic drugs was higher in the high IL-13 mRNA patient subset. It is quite possible that in newly diagnosed pediatric patients in which the immune-response is not influenced by the effects of inflammatory relapses and consequent therapy, initial induction of IL-13 is adequately counteracted by the standard therapy. Alternatively, increased production of IL-13 might even have, in the initial phase of the disease, a protective role, as shown in some animal models (111-113). However, with the progression of the disease, inflammatory flares and their treatment might induce changes favoring the occurrence or amplification, in genetically predisposed individuals, of autoimmunity phenomena as trigger of IL-13 production that switch the role of IL-13 production from protective to pathogenic. Indeed, in adult UC patients, it has been recently demonstrated the presence in colonic lamina propria of increased proportion of NKT cells able to produce IL-13 in response to lysosulfatide, a sulfatide glycolipid (56). Sulfatide glycolipids are self-antigens present in cells in many tissues including the epithelial cells in the gastrointestinal tract. A stress response of genetically conditioned epithelial cells to certain type of bacteria in the colonic microbiota may indeed promote the autoimmune-induced IL-13 production**.**

Interesting, in the present study, we observed different mucosal microbial profiles associated to the two patients' subsets. Particularly, while mucosa-associated microbiota in patients with high IL-17A and low IL-13mRNA is enriched in *Sutterella* and *Acidaminococcus*, *Prevotella* (mainly *Prevotella copri*) is enriched in the mucosa-microbiota of patients showing increased IL-13 mRNA content in addition to high IL-17A mRNA

content. It is worth to note that *Sutterella* (114), *Acidaminococcus* (115) and *Prevotella copri* (116) have been reported to be increased in fecal microbiota of UC patients. All of these microorganisms are classically considered commensal bacteria due to their extensive presence in the healthy human body. However, emerging studies have linked increased *Prevotella* abundance and specific strains to inflammatory disorders, suggesting that at least some strains exhibit pathobiontic properties. In particular, it has been recently reported that expansion of *Prevotella copri* in fecal samples strongly correlate with disease in new-onset untreated patients with rheumatoid arthritis, a systemic autoimmune disease (117). In the same study it has been described its ability, after colonization of mice, to increase sensitivity to chemically induced colitis (117). Indeed *Prevotella* might disrupt the mucosal barrier function through production of sulfatases that actively degrade mucus oligosaccharides (118). Thus, it is plausible that the dysbiotic community observed in the high IL-13 mRNA patients subsets (enriched in *Prevotella* genus), might influence the epithelial stress response, favoring the presentation of autoantigens contributing to the pathogenic role of IL-13. On the other hand, the microbial community enriched in both *Sutterella* and *Acidaminococcus*, characterizing the low IL-13 mRNA subset of patients, might contribute to a less severe disease. In fact, although *Sutterella* was described to be associated with lack of remission after fecal microbiota transplantation (119), recent studies, showed the ability of *Acidaminococcus* genus to produce short chain fatty acids (120). Short chain fatty acids are in fact considered important metabolites as they serve as the major energy source for colonocytes, they have anti-inflammatory properties and regulate gene expression, in host cells (121).

The existence of the two subsets of patients observed in the present study introduces some issues about the clinical implications of the finding. The different cytokine profile might be differentially associated to the presence of different serological markers and to their prognostic ability (122, 123), so that it might be useful to combine the assessment of tissue cytokine profile with the assessment of serological markers to obtain an increased prognostic ability.

Finally, the findings described in the present study might have important implications on therapeutic strategies in UC. The observation that IL-6, TNF-α, IFN-γ and IL-10 mRNA tissue content were each other strongly correlated, while IL-17A and IL-13 variations were both independent from all the other cytokines and independent of each other suggests that IL-17A and IL-13 mRNA contents are distinctive features of the pathogenic process of UC. As that, therapy oriented to the neutralization of inflammatory cytokines might

only indirectly and differentially affect the IL-17A and IL-13 expression which in turn might influence the response to the neutralization of inflammatory cytokines. Notably, it has been shown that response to infliximab treatment was significantly associated with higher pretreatment mucosal IL-17A gene expression (124), and fewer Gata3+ T cells were observed in the lamina propria of anti-TNF-α responders, than in non-responders patients (125). Beyond these considerations, an important therapeutic implication of the findings reported in the present study concerns the treatment of patients with high IL-13 mRNA tissue content. As recently highlighted (126, 127) previous results obtained with IL-13 neutralizing treatments might be reconsidered according to the IL-13mRNA colonic content of the patients that have been studied. A treatment approach that specifically target IL-13 might indeed be effective in the patients' subset with high IL-13mRNA tissue content identified in this study.

Published article:

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