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Epithelial IL-1R2 acts as a homeostatic regulator during remission of ulcerative colitis

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Acabar una etapa quan has aconseguit trobar el teu lloc entre gent que t'ha ajudat tant, és molt dur.

CONTENTS

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I- *ABREVIATIONS*

3D	Three dimensions
ACTB	Beta-actin
ADAM17	Metallopeptidase Domain 17
ADF	Advanced DMEM/F12
AMPs	Antimicrobial peptides
ANCA	Anti-neutrophilic cytoplasmic antibodies
ANPEP	Alanyl (membrane) aminopeptidase
APC	Allophycocyanin
APC	Adenomatosis polyposis coli
APCs	Antigen presenting cells
APRIL	Proliferation-inducing ligand
ARTS-1	Aminopeptidase regulator of TNFR1 shedding
ASC	Apoptosis-associated speck-like protein containing a carboxy-terminal CARD
a-TNF	Anti-TNF
ATP	Adenosine triphosphate
AUC	Area under the curve
BAFF	B-cell activating factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CCL	Chemokine (C-C motif) ligand
CD	Cluster differentiation
CD	Crohn's Disease
CDEIS	Crohn's disease index of severity
CDH1	Cadherin 1, E-cadherin
cDNA	complementary DNA
CKIα	Casein kinase 1 alpha
c-Myc 23	V-Myc avian myelocytomatosis viral oncogene homolog
CO_2	Carbon dioxide
CoSC	Colonic stem cells
CRC	Colorectal cancer
Ct	Cycle threshold
CX_3CR1	Chemokine (C-X3-C motif) receptor 1
CXCL	Chemokine (C-X-C motif) ligand
Cy3	Cyanine 3
Cy7	Cyanine 7
DAP	Death-associated protein
DapB	Diaminopimelate B
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic cells
Dkk	Dickkopf
DMSO	Dimethyl sulfoxide
DNAse	Deoxyribonucleic acid deoxyribonucleatease
DPX	Distrene, plasticiser, xilene
Dsh/Dvl	Dishevelled
DSS	Dextran sodium sulphate
DTT	Dithiothreitol
E. Coli	*Escherichia coli*
ECM1	Extracellular matrix protein 1
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked

	immunosorbent assays
Ep-CAM	Epithelial cell adhesion molecule
EphB	Ephrin-B
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FitC	Fluorescein isothiocyanate
fMLP	Formyl Meth-Leu-Phe
Frzb	Frizzled-related protein
FSC-A	Forward Scatter Area
FSC-H	Forward Scatter High
Fz	Frizzled receptor family member
GNA12	Guanine nucleotide binding protein (G protein) alpha 12
GSK3	Glycogen synthase kinase 3
GWAS	Genome-wide association studies
h	Hour(s)
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethil)-1-piperazineethanesulfonic acid
HLA-DRA	Major histocompatibility complex, class II, DR alpha
HLA-DRB1	Major histocompatibility complex, class II, DR beta 1
HNF4A	Hepatocyte nuclear factor 4, alpha
HRP	Horseradish peroxidase
Hs-PPIB	Homo sapiens-peptidylprolyl isomerase B
IBD	Inflammatory bowel disease
IECs	Intestinal epithelial cells
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-18BP	Interleukin 18 binding protein
IL-1R1	Interleukin 1 receptor type 1
IL-1R2	Interleukin 1 receptor type 2
IL-1Ra	Interleukin 1 receptor antagonist
IL-1RAcP	Interleukin 1 receptor accessory protein
IL23R	Interleukin 23 receptor
IL-36Ra	Interleukin 36 receptor antagonist
IL-7R	Interleukin 7 receptor
IL8RA/B	Interleukin 8 receptor A/B
ILCs	Innate lymphoid cells
IPAA	Ileal-pouch anal anastomosis
IRAK4	Interleukin 1 receptor associated kinase 4
IRF5	Interferon regulatory factor 5
JAK2	Janus kinase 2
LAMB1	Laminin, beta 1
Lgr5	Leucine-rich repeat containing G protein-coupled receptor 5
LPS	Lipopolysaccharide
LRP5/6	Low-density-lipoprotein-related protein5/6
LSP1	Lymphocyte-specific protein 1
M	mol/L
M cells	Microfold cells
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex

min	Minute(s)
MLCK	Myosin light chain kinase
MUC2	Mucin 2
MyD88	Myeloid differentiation primary response protein 88
Naked	Dvl antagonist
NF-kB	Nuclear factor kappa B
NK T-cells	Natural killer T-cells
NKX2-3	NK2 homeobox 3
NLR	NOD-like receptor
NLRP3	NLR family, pyrin domain containing 3
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain containing 2
NSAIDs	Non-steroidal anti-inflammatory agents
ºC	Degrees Celsius
P2X7R	Purinergic receptor P2X
pANCA	perinuclear anti-neutrophil cytoplasmic antibody
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
pIgR	Polymeric immunoglobulin receptor
PMA	Phorbol 12-myristate 13-acetate
PP2A9	Protein phosphatase 2A
PRDM1	PR domain containing 1
pro-IL-1α	Interleukin 1 alpha precursor
pro-IL-1β	Interleukin 1 beta precursor
PRR	Pattern-recognition receptors
PTPN	Protein tyrosine phosphatase
REGIIIγ	C-type lectin regenerating islet-derived protein IIIγ
RELMβ	Resistin-like molecule-β
RIN	RNA integrity number
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
ROC	Receiver-operator characteristic
ROI	Reactive oxygen intermediates
RSPO1	R-spondin-1
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
SCs	Stem cells
Ser	Serine
sFRP	Soluble Frizzled-related proteins
SIgA	Soluble immunoglobulin A
SSC	Side-scattered light
STAT3	Signal transducer and activator of transcription 3
sTNFR	Soluble tumor necrosis factor receptor
TA cells	Transit-amplifying cells
TcF/Lef	T-cell factor/Lymphoid enhancing factor
TFF3	Trefoil factor 3
Tg	Transgenic
TGF-β	Transforming growth factor beta

Th cell	T helper cell
Thr	Threonine
TIR	Toll/interleukin-1 receptor
TL1A	Tumor necrosis factor (ligand)
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRAF6	Tumor necrosis factor receptor associated factor 6
T regs	Regulatory T lymphocytes
TSLP	Thymic stromal lymphopoietin
U	Units
UC	Ulcerative colitis
UPR	Unfolded protein response
WIF	Wnt-inhibitor protein
Wnt	Wingless-type MMTV integration site family
Wnt-3a	Wingless-type MMTV integration site family 3A
xg	Relative centrifugal force

II- *INTRODUCTION*

Section I: Ulcerative colitis

Ulcerative colitis (UC) and Crohn's disease (CD) are inflammatory bowel diseases (IBD) that are thought to result from an aberrant immune response to commensal flora. Both UC and CD are intestinal chronic inflammatory disorders characterized by alternating periods of remission and clinical relapse. Nonetheless, they are clearly distinct pathophysiological entities with unique characteristics, risk factors, and clinical, endoscopic, and histopathological features[1]. In brief, in contrast to CD, UC is localized exclusively in the large intestine with inflammation that extends continuously from the rectum and presents superficial inflammation and ulceration.

1. Epidemiology

Both prevalence (7.6 to 246.0 cases per 100,000 per year) and incidence (1.2 to 20.3 cases per 100,000 person-year) in the adult population are greater in UC than CD, with the highest incidence and prevalence rates in North America and northern Europe[2].

2. Diagnosis and clinical course

2.1. Symptomatology

Classical symptoms of UC are bloody diarrhea with or without mucus, urgency or tenesmus, abdominal pain and weight loss. More extensive and severe cases are associated with fever, severe bleeding, and extraintestinal manifestations[3].

2.2. Extension of the disease and endoscopic features

At diagnosis, of patients have mild to moderate symptoms and disease confined to the rectum or the sigmoid colon (distal colitis)[4]. This is often followed by periods of remission and subsequent relapses. The disease in 30% of these patients

evolves with anatomic progressions (e.g., from proctitis to left-sided colitis or pancolitis) (Figure 1). About 50% of patients present disease localized to left-sided colitis or pancolitis at diagnosis[5, 6]. The extension of mucosal inflammation correlates with the severity of the disease, and can be a predictor of colectomy[7] and colorectal cancer[8].

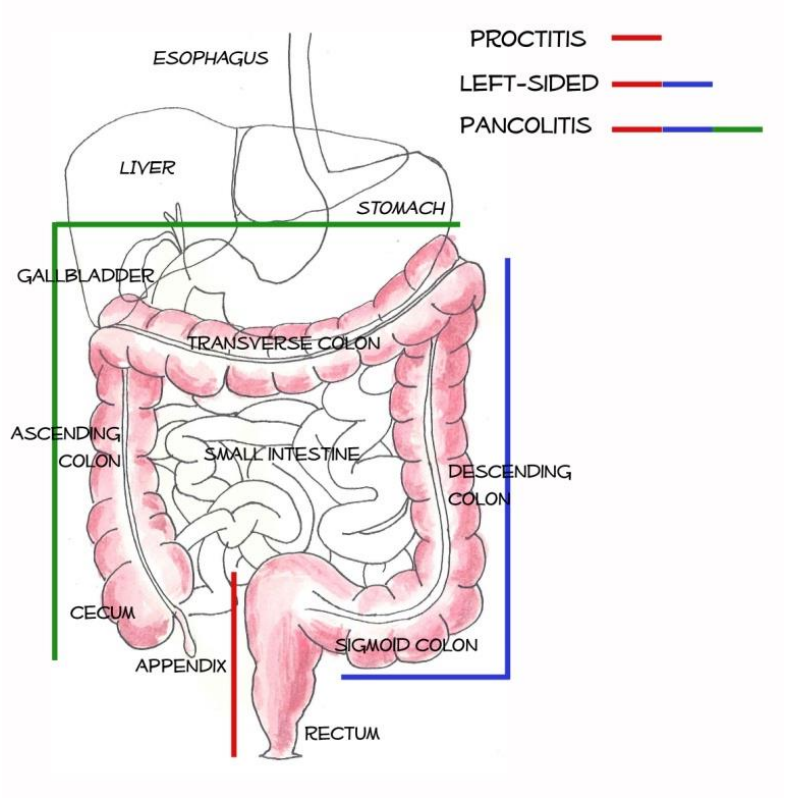


Figure 1. Representative picture of the extension of mucosal inflammation in ulcerative colitis (UC).

Thus far, the most commonly used endoscopic score for categorizing the severity of UC remains the Mayo endoscopic score (Table 1). In mild UC, colonoscopy typically reveals mucosa with an erythematous appearance, granularity, with friability as well as loss of the vascular pattern. Moderate disease is characterized by the presence of erosions or microulcerations, whereas in severe UC, ulcerations with or without spontaneous bleeding are generally observed[9].

Moreover, patients with UC may present extra-intestinal inflammatory manifestations in various organs and systems such as the joints, skin, liver, eye, mouth

and blood (coagulation), and complications such as toxic megacolon, severe bleeding, rupture of the bowel and colon cancer.

MAYO		Characteristics
0	Inactive disease	Normal mucosa
1	Mild disease	Erythema, decreased vascular pattern, mild friability
2	Moderate disease	Marked erythema, absent vascular pattern, friability, erosions
3	Severe disease	Spontaneous bleeding, ulceration

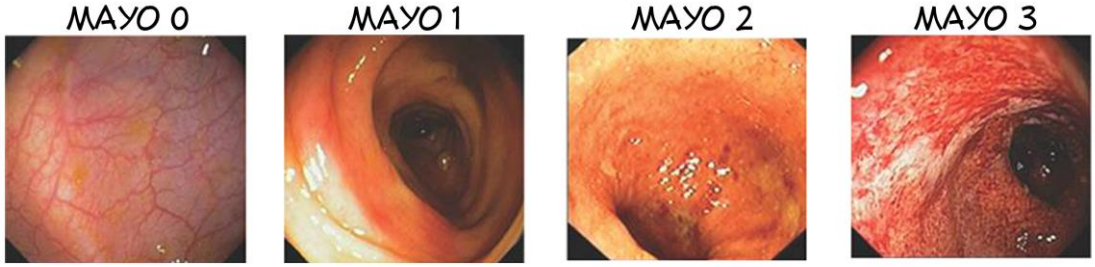


Table 1. Mayo endoscopic scoring of Ulcerative Colitis. Endoscopic images of increasing Mayo scores. Adapted from Pineton de Chambrun G. et al. (2010) Nat. Rev. Gastroenterol. Hepatol[10].

2.3. Histopathological features

Biopsies obtained from UC patients via colonoscopy may help to determine the severity of inflammation and the stage of the disease. In UC, inflammation is characteristically restricted to the mucosal layer. Lamina propria infiltrates of lymphocytes, plasma cells, and granulocytes (present in crypts and crypts abscesses) vary in density and composition during active disease or periods of remission. The epithelial layer characteristically shows goblet-cell depletion, diminished crypt density and distortion of its architecture. In acute flare-ups, erosions or ulcerations result from the complete loss of epithelial cells[11].

3. Pathogenesis

The etiology of UC is complex and incompletely understood, but evidence supports a multifactorial cause, with both genetic susceptibility (genome) and

environment factors (microbiome and exposome) driving a deregulated mucosal immune response to commensal microbiota (immunome) (Figure 2).

3.1. Genome

UC genetic susceptibility is polygenic. Genome-wide association studies (GWAS) have thus far identified 143 associated loci in UC, 110 of which are shared with CD[12]. Genetic variants associated with UC are mostly related to dysfunction of the epithelial barrier. These include risk loci for *ECM1*, *HNF4A*, *CDH1*, *LAMB1,* and *GNA12*, apoptosis and autophagy such as *DAP* whereas *PRDM1, IRF5*, and *NKX2-3* are related to erroneous transcriptional regulation. In addition, UC is associated with several susceptible genes that codify components of the immune response. Among these are *IL23R*, *JAK2*, *STAT3*, *IL12B*, and *PTPN* from the IL-23/Th17 signaling pathway; the major histocompatibility complex class II region near HLA-DRA (α-chain) and different HLA-DRB1 (β-chain) alleles[13]; and cytokine, cytokine receptors and migration-related molecules (e.g., IL (interleukin)-10, IL-7R and IFN-γ, IL1R2, IL8Ra/B, and LSP1)[14].

An imputation-based association analysis using autosomal genotype-level data from 15 GWAS of CD and/or UC revealed the presence of up to 23 specific associated loci in UC. Interestingly, this meta-analysis demonstrated that risk alleles at two CD loci, *PTPN22* and *NOD2*, show significant protective effects in UC, exceptions that emphasize the biological differences between the two forms of IBD[12].

3.2. Exposome

Overtime the incidence of IBD in newly industrialized countries has increased, suggesting the important role of environment and lifestyle in triggering the disease[2].

A range of environmental factors has been shown to confer harmful or protective effects in UC. Two such elements of the latter class are cigarette smoking[15, 16] and appendectomy. Prospective studies have demonstrated that the risk of UC increased within 2-5 years after smoking cessation and remained elevated for 20 years thereafter[17, 18]. Moreover, early life exposure to smoke and passive smoking have

similar effects[19]. Appendectomy in young patients, prior to age 20, has been demonstrated as a protective effect for ulcerative proctitis[20].

Non-steroidal anti-inflammatory agents (NSAIDs) are weakly associated with an increased risk for UC, allegedly due to their effects on prostaglandin production[21, 22]. The role of diet and food antigens[23] and social stress[24, 25] remains ambiguous.

3.3. Microbiome

Many of the genetic and environmental factors that influence UC are related to host-microbe interactions, a fact that strongly support the role of intestinal microbiota in UC pathophysiology. In patients with IBD, microbiota is altered, has less bacterial diversity, and a greater density than in the general population. Nonetheless it is still unclear whether there is a connection between bacterial content and disease specific alterations[26]. The microbial imbalance referred to as **dysbiosis** is gaining more and more interest in IBD studies. Reductions in *clostridium spp*. and increases in *Escherichia coli* (E. Coli) within the microbiota in UC patients has been reported[27]. Although antibiotic therapy is slightly beneficial in luminal CD, it shows no clinical benefit in UC[28]. Studies have confirmed the presence of antibacterial antibodies in serum from CD patients, though they are less common and exist in lower titers in patients with UC[29]. In contrast to the association of CD with reactivity to many bacterial and fungal antigens, thus far only perinuclear anti-neutrophil cytoplasmic antibodies (pANCA), which recognize nuclear antigens and may cross-react with bacterial antigens, have been described in UC[30]. Acute intestinal infection could cause changes in the intestinal microbiota, and it is known that episodes of previous gastrointestinal infection double the risk of developing UC in genetically predisposed individuals[31]. Non-pathogenic enteric bacteria could therefore play a role in the pathogenesis of UC. Indeed, genetically susceptible animals, develop chronic intestinal inflammation after colonization with commensal microbiota, though no inflammation occurs under germ-free conditions[32-36]. Moreover, additional studies suggest that the use of probiotics or beneficial microorganisms can ameliorate IBD[37, 38].

3.4. Immunome

The hallmark of UC is a loss of tolerance to commensal microorganisms that results in chronic, uncontrolled inflammation of the intestinal mucosa. The group of genes and proteins that drive the immune response mediating mucosal inflammation is known as the immunome (Figure 2).

3.4.1. Defects in the innate immune system

The innate immune system represents the first line of defense against infections, providing an immediate protective non-specific response and helping to initiate the adaptive immune response. UC patients present deficiencies in innate barriers of protection.

Alterations in the **intestinal epithelium** (discussed in section III.4), due to disruption of the homeostatic renewal process and loss of barrier integrity, allow free passage of luminal antigens across the epithelial layer. This results in chronic inflammation that may contribute to UC pathogenesis.

Phagocytes (neutrophils, monocytes, macrophages and dendritic cells (DCs)) are able to initiate a response against harmful agents detected by Toll-like receptors (TLRs). Phagocytic cells secrete cytokines and chemokines and maintain homeostasis by removing dead cells, infected cells and microorganisms. Macrophages and DCs are also antigen-presenting cells (APCs) that interact and activate the adaptive immune system. The role of **dendritic cells** (DCs) in the initiation and perpetuation of inflammation in patients with UC has been reported. In brief, there is an increased frequency of activated and mature DCs with higher stimulatory capacity and with altered expression of TLRs in the mucosa of UC patients[39]. In mice, most lamina propria **macrophages** and DCs express the chemokine receptor CX_3CR1. A subpopulation of CX_3CR1^+ cells is located close to the epithelium and extends its processes into the intestinal lumen to gain access to luminal antigens[40]. Genetic deletion of CX_3CR1 results in decreased numbers of lamina propria macrophages and increased translocation of commensal bacteria to mesenteric lymph nodes, with a consequent

increase in the severity of experimental colitis[41]. Increased **neutrophil** trafficking has been implicated in the pathogenesis of many inflammatory mucosal disorders including IBD. Indeed, the accumulation of large amounts of neutrophils in the lamina propria and epithelial crypts of the intestine is a hallmark of active UC. Neutrophil-derived reactive metabolites directly cause damage to the epithelium. In addition, neutrophils function as important pro-inflammatory effector cells that secrete inflammatory mediators influencing other immune cells[42].

Studies in experimental colitis have linked innate lymphoid cells (ILCs) to intestinal inflammatory pathophysiology. **ILCs** are members of the lymphoid linage, but lack an antigen-specific receptor[43]. Population of ILCs that produce IL-17, IL-22, and IFN-γ in response to IL-23 and play a role in innate colitis have been identified in mice[44]. In addition, type 2 ILCs (ILC2) secrete the signature cytokines (IL-5 and IL-13)[45]. IL-13 secreting ILC2s have been reported in the lamina propria during oxazolone colitis, an experimental colitis model dependent on NKT-cells secreting IL-13[11].

3.4.2. Innate cytokines and chemokines

Several studies have documented the role of chemokines and innate cytokine-driven pathways in UC pathophysiology. **CXCL8** expressed by macrophages, neutrophils, and epithelial cells is one of the most abundant chemokines in acutely inflamed tissues, including the intestinal mucosa. Expression of CXCL8 closely correlated with the degree of histological severity in active UC[46]. Other chemokines such as CXCL1, CXCL2 and CCL20, which are mainly produced by the epithelium, also correlate with clinical and endoscopic activity in UC[47-49]. These molecules act as chemoattractants to guide the migration of other leukocytes to the focus of inflammation.

A broadly accepted key cytokine driving IBD pathogenesis is **Tumor necrosis factor alpha (TNF-α)**. TNF-α exists at elevated levels in the blood, stool and mucosa of patients with UC[50-52]. This propensity, together with the efficacy of anti-TNF-α (a-TNF-α) treatment in UC[53], confirms the importance of TNF-α in the pathogenesis of the disease. In the mucosa, there is an increase in the production of both the membrane-bound and soluble form of TNF-α by lamina propria mononuclear cells, in

particular $CD14^+$ macrophages, in patients with IBD[54] TNF-α signaling in colitis drives a range of pro-inflammatory effects, such as augmented angiogenesis, the production of matrix metalloproteinases by myofibroblasts, the activation of macrophages and effector T cells, and the direct damage of intestinal epithelial cells (IECs) via myosin light chain kinase (MLCK) activation[55-58]. **IL-1**, a pleiotropic cytokine has also been linked to UC pathophysiology. A decrease in the ratio of IL-1 receptor antagonist (IL-1Ra) to IL-1 was found in the intestinal mucosa of patients with UC compared with control subjects, which suggests increased activation of the IL-1 system in IBD[59] (discussed in section II.2). Another member of the IL-1 cytokine family, **IL-33**, is associated with UC. Epithelial-derived IL-33 induces Th2 cytokine production and potentiates both Th1 and Th2 immune responses[60-62]. **TL1A** (a TNF superfamily member) is expressed by DCs, and its expression is affected by various bacterial-derived signals in either a stimulatory or inhibitory fashion[63, 64]. Not only has the chronic up-regulation of TL1A in DCs or lymphocytes in TL1A transgenic mice been shown to result in chronic intestinal inflammation[65-67], but TL1A expression has also been found to significantly and selectively increase in the gut tissue of IBD patients[68]. **IL-23** is a key innate cytokine that acts both by driving early responses to microbes as well as by amplifying Th17 responses. The IL-23R gene contains a polymorphism that influences genetic susceptibility in both UC and CD[69].

Expression of **IL-6,** another cytokine highly up-regulated in the inflamed intestinal mucosa, correlates with the Mayo endoscopic score for disease severity and extraintestinal manifestations of the skin[70] .

Moreover, signaling molecules downstream of pro-inflammatory cytokines, such as NF-kB, MAPK and MyD88, have been associated with UC activity[71]. And mutations in genes codifying the anti-inflammatory cytokine IL-10 or IL-10 receptor are associated with severe UC, due to an absence of IL-10 signaling[72].

3.4.3. Defects in the adaptive immune system

Adaptive immunity is carried out by lymphocytes (T and B cells) that upon activation generate effector responses (cytokines and antibodies). These responses are highly specific and provide long-lasting immunity called immune memory. Humoral

(mediated by B cell-derived antibodies) and cellular (mediated by T cells) adaptive immune responses are deregulated in UC. The role of **B cells** in UC pathophysiology is poorly understood. Although increased numbers of infiltrating B cells and plasma cells has been found in active UC disease, depletion of B cells appears to have no beneficial effects[73]. Soluble IgA (SIgA) produced by plasma cells not only forms the first line of defense against pathogens, but also responds to symbiotic bacteria promoting gut homeostasis[74]. In UC lesions an excessive number of IgA^+ and IgG^+ with a disproportionate increase in IgG1 antibodies has been described[75, 76]. Furthermore, the presence of circulating ANCA[77] and anti-colonic epithelial antibodies in patients with UC[78] supports the hypothesis that a deregulation of humoral responses is at work in UC.

T cells may have a central role in the mucosal inflammatory process, based on the expansion of activated $CD4^+$ T cells in the lamina propria, both in mouse and human inflamed intestinal tissues[79, 80]. Some studies have found an association between UC and **atypical Th2** response involving elevated levels of IL-5 and IL-13 (but not the classic Th2 cytokine IL-4) in diseased mucosa, a response mediated by non-classic natural killer T cells[45]. It has been demonstrated that UC patients produce increased amounts of IL-5-secreting Th2 cells, which may contribute to eosinophil recruitment and activation. IL-13 is of particular importance due to its cytotoxic function on epithelial barriers, which can alter the expression of tight-junctions proteins [81]. Nonetheless, two independent studies have recently shown that anti-IL-13 therapy confers no benefits in UC[82, 83].

Although no clear evidence exists concerning the role of natural killer (NK) T cells in human UC, models of experimental colitis have linked this cell population and its cytokine production to inflammation of the colon. **NK T** cells exist at increased levels in the lamina propria of the inflamed colon[84] and produce Th2 cytokines, firstly IL-4 followed by IL-13. These cytokines act in an autocrine manner on NK T cells, thereby amplifying its cytotoxicity in the mucosa. It has been demonstrated that blocking IL-13 and depleting NK T cells prevent the development of colitis in animal models[11, 45].

An overexpression of **Th1 and Th17** products has been detected in UC tissues[85, 86]. Th17 cells respond to pathogenic extracellular bacterial and fungal infections at mucocutaneous surfaces[87]. Th17-secreted cytokines (IL-17, IL-22, IL-26) induce the recruitment of neutrophils, increase the production of pro-inflammatory cytokines and antimicrobial proteins, and stimulate B cells[88]. High transcription levels of *IL17A* and **IL-17A** have been observed in the lamina propria of IBD patients[89]. The involvement of **Th17** cells in the pathogenesis of CD has been widely established. In fact, Th17 cells and their effector cytokines are gaining importance in studies on UC [90]. Indeed, genetic analysis has shown that *IL23R* and other Th17-related genes represent risk loci for UC[14]. Due to ineffectiveness of the anti-IL-17A antibody use in patients with CD[91], some studies have linked the pathogenicity of Th17 cell with the dual production of **IFN-γ** [92, 93]. In fact IFN^+ $IL\text{-}17^+$ double positive T helper cells (**Th1/17**), are specifically enriched in the inflamed intestinal mucosa of UC patients[86]. IFN-γ participates in many pro-inflammatory effects such as the activation and differentiation of T cells, B cells, macrophages and NK cells. Moreover, up-regulation of IFN-γ, together with IL-17, by lamina propria mononuclear cells has been described in UC patients[94].

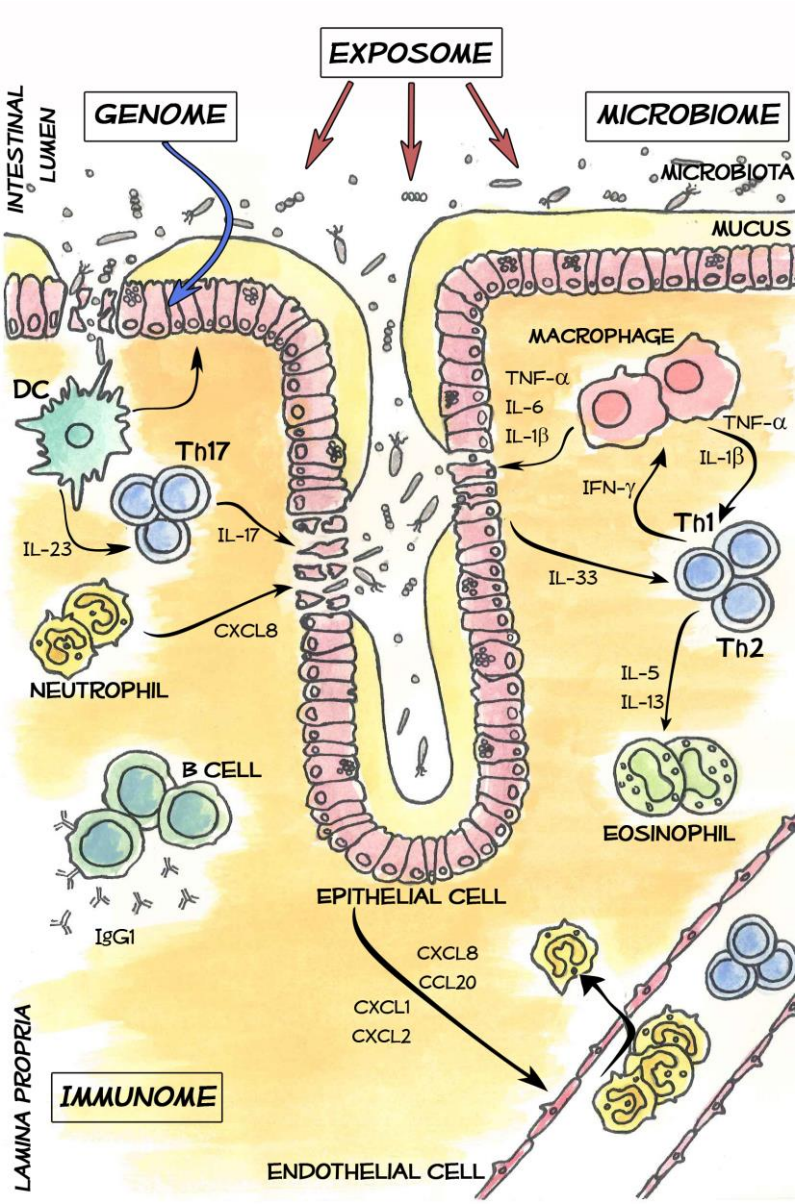


Figure 2. Pathogenesis of ulcerative colitis. CXCL, C-X-C motif ligand; DC, dendritic cell; IL, interleukin; Th, T helper.

4. Treatment

The goal of treatment is to induce remission, defined as absence of clinical symptoms and to prevent relapse. Decisions on the best therapy approach for each patient are made based on clinical activity (mild, moderate, or severe), the extent of colonic involvement (proctitis, left-sided colitis, or pancolitis), the previous course of the disease, and patient preferences[95, 96].

4.1. Induction of remission

The first-line of treatment for mild to moderately active disease, with an expected remission rate of about 50%, is the administration of sulfasalazine and **5-aminosalicylates** (mesalazine, olsalazine, and balsalazide), given orally, rectally, or both [97]. One study reported that the combination of rectal and oral 5-aminosalicylate obtained the best clinical response with higher remission rates [98]. Most patients treated with mesalazine achieve clinical remission within 2-4 weeks[99]. Patients with mild-to-moderate UC that are refractory to rectal therapies and to oral mesalazine, or those with severe disease are candidates for oral or intravenous **glucocorticoids/corticosteroids**[96]. Although almost 70% of patients respond to the first course of corticosteroids, 22% develop steroid dependency during the first year of treatment, and only half maintain corticosteroid-free remission[100]. Patients who continue to require glucocorticoid therapy (corticosteroid-dependent disease), and those who do not respond to it or to optimum doses of mesalazine, can be treated with **immunosuppressants** (mainly azathioprine or 6-mercaptopurine), though variable response rates in UC have been recorded in different studies[101]. Patients refractory to conventional therapy are treated with **biological drugs** as monoclonal antibodies against TNF-α (a-TNF-α) (intravenously infliximab or subcutaneously adalimumab) either alone or in combination with azathioprine[102].

Patients with extensive severe disease are at high risk for colectomy. To avoid this, they must be hospitalized with intravenous corticosteroids treatment, where the overall response rate over two months is almost 70%[103]. The remaining 30% with unresponsive disease are then candidates for colectomy depending on stool frequency, the presence of fecal blood, elevated concentrations of C-reactive protein, albumin or fecal calprotectin, or radiologic and endoscopic findings[104]. To obtain a colectomy-free remission, patients should undergo immediate rescue treatment. Intravenous or oral cyclosporine and oral tacrolimus are highly effective for short-term clinical improvement; however, due to serious adverse events and low effectiveness for maintenance of long-term remission, their use has been limited[105]. Intravenous a-

TNF-α is preferred because it can be used as a maintenance treatment in responding patients, particularly in those for whom immunosuppressants have been ineffective[106].

4.2. Maintenance of remission

Once remission has been achieved, the main goal is to maintain a symptom-free status. Maintenance treatment depends on disease extent and severity during the active phase, the treatment that was used to induce remission, and the failure of previous maintenance treatments. Most patients can remain in remission using oral or rectal mesalazine[107]. Corticosteroids, due to the marked side effects associated with their long-term use, are not advisable for maintenance. Thiopurines (azathioprine or 6-mercaptopurine) or a-TNF-α are recommended for patients who have frequent relapses under mesalazine, steroid dependency, or for those who were previously treated with immunosupressants such as cyclosporine or tacrolimus for a severe flare[108-110]. UC remission induced by a-TNF-α drugs in steroid-refractory patients is effectively maintained by a-TNF-α[106, 111].

4.3. Surgical treatment

Indications for surgical treatment of UC (20-30% of patients)[112] include failure of medical therapy, intractable fulminant colitis, toxic megacolon, perforation, uncontrollable bleeding, intolerable side effects to medications, strictures that are not amenable to endoscopic alleviation, high-grade or multifocal dysplasia that is not amenable to resection, dysplasia-associated lesions or masses, cancer, and/or growth retardation in children. The main surgical procedure is a subtotal colectomy with a temporary ileostomy with no removal of the rectal stump[113]. A restorative operation involving construction of the ileal-pouch anal anastomosis (IPAA) and ileostomy closure is usually done after the patient has fully recovered in order to reduce the risk of complications. Two-stage proctocolectomy with IPAA is currently the procedure of choice for most patients who require elective surgery[114]. Total colectomy with ileorectal anastomosis may be considered for certain carefully selected patients (e.g., elderly individuals). Postsurgical complications include small bowel obstruction,

anastomotic strictures, pouchitis, sexual dysfunction, increased risk of female infertility and pouch failure[115-117].

4.4. Novel therapies

A desirable end point of treatment efficacy is mucosal healing accompanied with a reduced relapse rate, which is not achieved in all patients treated with current therapies. New biological drugs are gaining wider currency in remission induction and maintenance based on established or preliminary evidence of therapeutic efficacy in UC (Table 2).

5. Colorectal cancer: a major complication of UC

Patients with UC or CD are at an increased risk for developing colorectal cancer (CRC), due to chronic inflammation of the gastrointestinal mucosa. The monitoring and detection of dysplasia in patients with long-standing UC remain crucial, given the potential for malignant transformation. Patients with UC, in contrast to non-IBD patients who develop CRC, may develop dysplastic lesions that can be polypoid, flat, localized or multifocal; these are markers of colonic inflammation and increased risk for neoplasia. The molecular and cellular features that may be involved in the development of CRC in UC patients are oxidative stress, pro-inflammatory factors of the innate and adaptive immune systems, and the intestinal microbiota[118, 119]. Although repeated colonoscopies with multiple biopsies is the standard approach, reliable molecular biomarkers are needed to distinguish cases that progress to cancer from those that do not[120].

	DRUG	TARGET	DEVELOPMENT STATUS
Blockade of pro-inflammatory cytokines			
TNF-α	Infliximab	TNF-α	Approved in EU and USA
	Adalimumab	TNF-α	Approved in EU and USA
	Golimumab	TNF-α	Approved in EU and USA
	CT-P13	TNF-α	Approved in EU
IL-13	Tralokinumab	IL-13	Phase II (-)
	Anrukinzumab	IL-13 receptor	Phase II (-)
	Bertilimumab	Eotaxin-1	Ongoing phase II
IL-17	Vidofluidimus	IL-17 release	Phase II (+)
Blockade of the downstream signaling pathways mediated by cytokines			
JAK/STAT pathway	Tofacitinib	Jak1,2 and 3	Ongoing phase III
	Peficitinib	JAK1	Ongoing phase II
IP-10 antagonists	BMS-936557	IP-10	Phase II (±)
Anti-adhesion molecules			
	Vedolizumab	α4β7	Approved in EU and USA
	Etrolizumab	β7	Phase II (+)
	PF-00547659	MadCAM-1	Phase II (+)
	AJM300	α4	Phase II (+)
	Vatelizumab	α2β7 integrin	Ongoing phase II
	GLPG0974	FFA-2	Ongoing phase II
Administration of cytokines			
IL-2	Low dose IL-2	IL-2	Ongoing phase II
Blockade of T-cell stimulation and induction of apoptosis			
	SB012	GATA-3	Ongoing phase I/II
	VB-201	TLR2 dependent innate cell activation	Ongoing phase II
	GSK1399686	Ribosomal 50S subunit	Ongoing phase II
	DIMS0150	TLR9	Ongoing phase III

Table 2. Current and novel biologics used for the treatment of ulcerative colitis. FFA-2, free fatty acid receptor-2; ICAM-1, Intercellular Adhesion Molecule-1; IL, interleukin; IP-10, interferon-γ-*inducible* protein-10; JAK, Janus kinase; MadCAM-1, mucosal address in cell adhesion molecule 1; TNF-α, tumor necrosis factor alpha; TLR, Toll-like receptor.

Section II: IL-1 and Ulcerative Colitis

A significant increase in IL-1 production by lamina propria mononuclear cells, most conspicuously from macrophages has been described in patients with active UC or CD[121, 122].

1. Functions and regulation of IL-1

In the early 1940s, a molecule produced by monocytes/macrophages in studies on fever and infection was described and designated IL-1. Despite the fact that IL-1 was the first cytokine to be identified more than 70 years ago, new biological activities and members of the IL-1 family are still being identified. There are, thus far, 11 ligands of the IL-1 family, including seven molecules with agonist properties (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ), three receptor antagonists (IL-1Ra, IL-36Ra, and IL-38) and an anti-inflammatory cytokine (IL-37)[123].

IL-1 is a pleiotropic cytokine exercising many biological activities on different cell types, as summarized in Table 3.

Target cell		Effect
Epithelial barrier	Epithelial cells	Tight-junction permeability, ↑ chemokine production, bacterial influx, leukocyte homing
Innate immune cells	Dendritic cells	↑ cytokine production, ↑ MHC/co-stimulatory molecules
	Macrophages	↑ cytokine production, phagocytosis
	Neutrophils	Survival, ↑ adhesion, oxidative burst, ↑ protease release
	Basophils	↑ cytokine production, ↑histamine production
	Mast cells	Maturation, ↑cytokine production, survival, ↑adhesion, degranulation
Adaptive immune cells	T naïve cells	Survival and expansion
	T memory cells	Survival and expansion
	Th17 cells	Differentiation
	Tγδ17 cells	↑ cytokine production
	B cells	Proliferation

Table 3: Biological activity of IL-1. MHC, Major histocompatibility complex; Th17, T helper 17 cell. Adapted from John E. Sims and Dirk E. Smith, Nature Reviews, February 2010[124].

The **IL-1α** precursor (pro-IL-1α) is active and constitutively expressed in epithelial layers of many organs. Upon to cell death by necrosis, pro-IL-1α is released. IL-1α rapidly initiates a cascade of inflammatory cytokines and chemokines, which results in sterile inflammation[125, 126].

The primary sources of **IL-1β** are blood monocytes, tissue macrophages, and DCs, although B lymphocytes and NK T-cells can also produce IL-1β. Apart from the generation of microbial products via the binding to toll-like receptors (TLR), IL-1 itself induces its own secretion both *in vivo* and in monocytes *in vitro*[127]. The inactive IL-1β precursor (pro-IL-1β) accumulates in the cytosol; its processing by caspase-1 is triggered by danger signals including uric acid crystals, β-amyloid, cytosolic DNA, cell necrosis, degraded components of the extracellular matrix[128-131], or the most widely

studied adenosine triphosphate (ATP) (Figure 3). ATP activation of the P2X7 receptor (purine ATP-gated receptors family) opens the potassium channels and intracellular potassium levels fall, followed by assembly of inactive procaspase-1 with components of the inflammasome (Figure 3). One limiting step in the processing and secretion of active IL-1β is activation of the inflammasome, a protein complex including NLRP3 (NOD-like receptor family, pyrin domain containing 3) and ASC[132, 133]. The cleavage of the IL-1β precursor by active caspase-1 can take place in the specialized secretory lysosomes or in the cytoplasm. An increase in intracellular calcium is also required for mature IL-1β to exit the cell, which occurs in a phospholipase C-dependent manner[134].

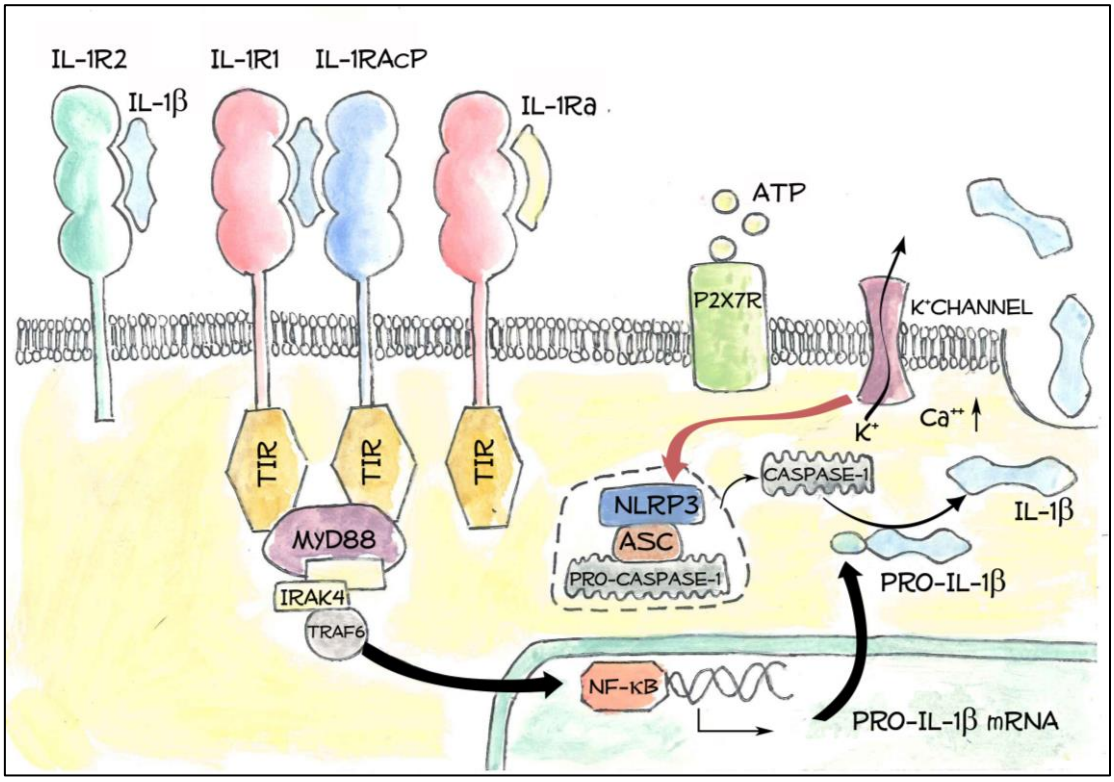


Figure 3. Representation of IL-1 production and signaling regulation. ASC, Apoptosis-associated speck-like protein containing a carboxy-terminal CARD; ATP, Adenosine triphosphate; Ca, calcium; IL-1β, interleukin-1β; IL-1R1, IL-1 receptor type 1; IL-1R2, IL-1 receptor type 2; IL-1RAcP, IL-1 receptor accessory protein; IRAK 4, interleukin-1 receptor-associated kinase 4; K, potassium; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa B; NLRP3, NLR family, pyrin domain containing 3; P2X7R, purinergic receptor P2X; TIR, Toll/interleukin-1 receptor domain; TRAF6, tumor necrosis factor receptor-associated factor 6.

Both IL-1α and IL-1β bind to the extracellular domain of the same receptor, **IL-1 receptor type 1** (IL-1R1), which is ubiquitously expressed (Figure 3). The cytoplasmic domain of IL-1R1 features a homology like that of the *Drosophila* Toll protein, known as the TIR domain, which is similarly found in the cytoplasmic domains of each TLR. For signal transduction, juxtaposition of the IL-1R1 TIR domain with the intracellular TIR domain of its co-receptor, **IL-1 receptor accessory protein** (IL-1RAcP), is required (Figure 3). Formation of the receptor heterodimer enables the recruitment of MyD88, IL-1R-associated kinase 4 (IRAK4), TNFR-associated factor 6 (TRAF6) and other signaling intermediates that activate the NF- κB and mitogen-activated protein kinase (MAPK) pathways[135].

Following the release of active IL-1 from the cell, its actions can be blocked by two physiological mechanisms, one involving the **IL-1 receptor antagonist** (IL-1Ra) and the other mediated by the **IL-1 receptor type 2** (IL-1R2) (Figure 3). IL-1Ra binds tightly to IL-1R1, thereby blocking the binding of IL-1α and IL-1β and preventing the recruitment of IL-1RAP[136]. IL-1Ra expression is induced by IL-1, lipopolysaccharide (LPS) or IgG complexes. Anti-inflammatory cytokines, such as IL-4 and IL-10, further enhance its induction by other signals[137]. Intracellular forms of IL-1Ra are also released following cell death and act extracellularly on IL-1R1, similarly to the secreted form[138].

1.1. IL-1 receptor type 2

IL-1R2, a negative regulator for IL-1 action, acts as a decoy receptor for IL-1β and IL-1α, as reported by Colotta *et al*.[139]. IL-1R2, together with IL-1R1, belongs to the Ig-like superfamily of membrane receptors, with the extracellular portion containing three Ig-like domains. The gene encoding IL-1R2 is highly conserved in evolution, from bony fish to mice and humans, and is found in the genome in a cluster with the gene for IL-1R1 and other members of the IL-1 family (IL-33R, IL-18R, IL-36R)[140]. Moreover IL-1R2 exists as a soluble form, produced by alternative splicing[141] or by the cleavage of the membrane form. Several enzymes such as metalloproteinases (ADAM17)[142], alpha-, beta-, and gamma-secretase[143], or the aminopeptidase regulator of TNFR1 (ARTS-1)[144] can participate in shedding the receptor.

IL-1R2 operates as a negative regulator of IL-1 by different means. First, IL-1R2 binds IL-1β and IL-1α with higher affinity than IL-1R1, whereas the binding with IL-1Ra is 100 times less efficient[145]. Thus, IL-1R2 acts as a molecular trap for IL-1, blocking its binding to IL-1R1[146]. Second, IL-1R2 forms a complex with IL-1 and IL-1RAcP, exerting a dominant-negative effect by sequestering IL-1RAcP[147, 148]. Third, the intracellular form of IL-1R2 binds pro-IL-1β and blocks its processing by the IL-1-converting enzyme caspase-1[145]. In addition, the soluble form of IL-1R2 detected in the cytosol has been shown to interact with active pro-IL1α inside the cell. This interaction prevents cleavage of pro-IL1α by different enzymes (calpain, granzyme B, chymase and elastase), and restrains IL-1α-dependent sterile inflammation during necrosis. In inflammatory or infectious conditions, this blockade would be reverted by caspase-1, which cleaves IL-1R2 restoring IL-1α activity[149]. And fourth, soluble IL-1R2 can interact with ligand-bound soluble IL-1RAcP[150]. This complex enhances the affinity for IL-1α and IL-1β, but does not affect the affinity for IL-1Ra[151].

IL-1R2 is expressed by a limited number of cells types, which also express the ubiquitous IL-1R1. It was first identified by McMahan *et al.* in 1991 cloned from B cells. This was followed by Colotta *et al*. in 1993, who described IL-1R2 as a decoy receptor in myolomonocytic cells. IL-1R2 expression studies have been done largely in myelomonocytic cells, phagocytes or mononuclear cells in different tissues. For instance, monocyte differentiation to M2 macrophages is associated with increased expression of IL-1R2[152, 153]. Several *in vitro* and *in vivo* studies demonstrated that anti-inflammatory signals, such as glucocorticoid hormones, prostaglandins, aspirin, IL-10, IL-4, IL-13 and IL-27, enhance surface and soluble IL-1R2 expression in myelomonocytic cells[154-160]. In contrast, pro-inflammatory molecules inhibit IL-1R2 expression. Bacterial LPS and IFN-γ caused a rapid shedding of surface IL-1R2 and down-regulation of expression in monocytes/myelomonocytic cells[158, 161]. Other stimuli such as formyl Meth-Leu-Phe (fMLP), reactive oxygen intermediates, TNF-α, and Phorbol 12-myristate 13-acetate (PMA) caused rapid shedding of IL-1R2[162, 163]. In addition to studies on IL-1R2 expressed by myeloid cells, the expression of this decoy receptor has been found

in T regulatory cells (Treg), basal epithelial cells of the skin, epithelium of the endometrium, vagina and urethra, and in chondrocytes[164-167].

2. Role of IL-1 in UC intestinal inflammation

Both the *IL1A* and *IL1B* genes are highly up-regulated in the inflamed colonic mucosa of UC[168, 169] and CD patients[170]. A decrease in the ratio of IL-1Ra to IL-1 was found in the intestinal mucosa of patients with UC when compared with control subjects, which indicates increased activation of the IL-1 system in patient mucosa[121]. In animal models of intestinal infection, IL-1β drives innate immune pathology, mediating the recruitment of granulocytes and the activation of innate ILCs[171]. Accordingly, in the T cell transfer colitis model, IL-1R1 signaling in T cells controls the early accumulation and survival of pathogenic $CD4^+$ Tcells in the colon[171].

Interestingly, several α-defensins produced by Paneth cells, including human α-defensin 5, decrease IL-1β secretion, raising the final possibility that elevated IL-1β may result from Paneth cell dysfunction[172]. Genetic studies suggest that inadequate innate IL-1β (and possibly IL-18) at the level of the epithelial barrier activity could be a risk factor for CD and UC[173, 174]. Moreover, there is a close correlation between IL-1 production and the degree of observed mucosal inflammation and necrosis[175].

3. IL-1R2 and ulcerative colitis

Altered expression of IL-1R2 in tissue or bodily fluids has been reported in diverse pathological conditions, such as autoimmune and neuroinflammatory diseases, and tumors. In the context of IBD, existing data demonstrate a decrease in IL-1R2 concentration during active CD and UC compared to healthy controls, both in cultured colonic biopsies[176] and in plasma[177]. In UC, IL-1R2 not only has been identified by GWAS as a candidate gene potentially involved in the disease[178], but also as a blood

biomarker[179]. Gustot *et al.* reported that deficient production of soluble IL-1R2 appeared to be specific to CD, but not UC. In addition, they observed a significant decrease in circulating soluble IL-1R2 in patients with active and remitting CD compared to healthy controls. This report also shows that corticosteroids significantly increased soluble IL-1R2 levels in plasma from active CD patients[176]. Interestingly, our group published a study in 2013 that evaluated the clinical, endoscopic, and histological response to different 5-aminosalicylate treatments compared to placebo in UC patients. We demonstrated an up-regulation of *IL1R2* in the mucosa at week four in patients who showed an endoscopic response regardless of treatment[169].

4. IL-1 blockade as a therapy for IBD

IL-1 blocking agents had shown remarkable efficacy, in severe IL-1-mediated inflammatory diseases. One strategy involves blocking the IL-1R1 by using an analog of the naturally occurring IL-1Ra. Recombinant forms of IL-1Ra (anakinra) have already been shown to have therapeutic benefits in different inflammatory conditions[180], as well as in chronic granulomatous disease[181]. Other IL-1 blocking strategies, such as the IL-1R1/IL-1β recombinant soluble receptor (rilonacept), have also been explored in selected autoinflammatory diseases with little success[180].

In animal models of intestinal inflammation, IL-1Ra administration suppressed acute immune complex-induced colitis in rabbits[182]. In contrast, this was not effective in treating chronic dextran sodium sulphate (DSS)-induced colitis in mice[183]. In humans, the only data available is a case report of a CD patient with worsened disease after receiving the IL-1Ra analog (anakinra)[184]. About a decade ago, Amgen tested for the first time the IL-1R2 as a therapy for arthritis, but no clinical development of this agent has been reported thus far.

Section III: The intestinal epithelium in UC

The epithelium plays a key role in maintaining mucosal homeostasis. Dysfunction of the epithelial barrier represents a potential pathological mechanism underlying the onset of UC. The intestinal epithelial layer constitutes about 400 m^2 of physical and biochemical barrier that separate mammalian hosts from their intestinal lumen, which is highly colonized by commensal bacteria. IECs can sense microbial stimuli and actively participate in appropriate mucosal immune responses, ranging from tolerance to anti-microbial immunity.

1. Physiology of the epithelial layer

The small and large intestine carry out different functions; while the former mainly absorbs nutrients from food, the colon is specialized in water absorption and compacting stool for rapid excretion. The epithelium is composed of diverse structures and cell types along the intestine. The epithelial layer of the **colon** forms multiple invaginations or crypts that are organized to form a flat luminal surface. The epithelial cells within **crypts** are continually renewed by a niche of pluripotent intestinal epithelial stem cells (SCs) that reside at base of the crypt[185] and give rise to the diversity of differentiated IEC lineages specialized in various functions (Figure 4). The life cycle of an individual differentiated epithelial cell is estimated to last less than a week. The majority of epithelial cells in the intestine are absorptive enterocytes with metabolic and digestive functions. Goblet cells, enteroendocrine cells, and Paneth cells secrete mucins, hormone regulators, and antimicrobial proteins, respectively[186, 187]. In contrast to the small intestine, the healthy colonic epithelium has a high numbers of goblet cells but no Paneth cells.

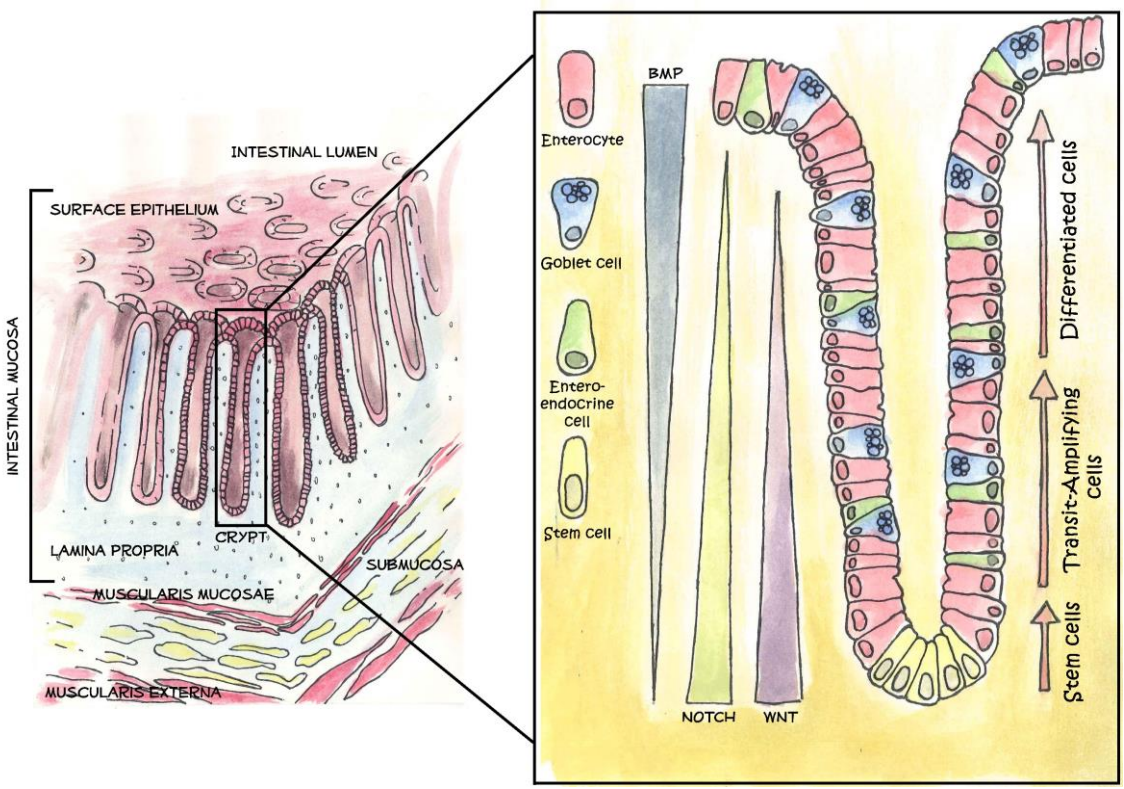


Figure 4. Representation of the human colon mucosa and the colonic crypt. The left side shows a section of the healthy colon wall where the primary layers are represented. On the right, the epithelial crypt cell types and gradients of bone morphogenic protein (BMP), Notch and Wingless-type MMTV integration site family (Wnt) signaling are depicted.

2. Colonic epithelial cell renewal, proliferation, and differentiation.

2.1. Intestinal stem cells

Intestinal SCs exhibit extensive proliferative and self-renewing capabilities, ensuring homeostatic maintenance of the intestinal epithelium and the ability to produce various epithelial differentiated cell lineages. SC proliferation classically occurs by asymmetric mitosis, with one of the daughter cells retaining SC properties and the second becoming a progenitor that continues to divide; the latter is also known as a transit-amplifying (TA) cell. TA cells migrate upward into the crypt to generate terminally differentiated cell lineages[188]. Barker *et al*.[189] identified the leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) as an exclusive SC marker the in mouse small intestine and colon, and demonstrated that Lgr5-positive replicating

columnar SCs occupy the crypt base. *LGR5* is a Wnt (Wingless-type mmtv integration site family) target gene[190], described as a receptor for R-spondin secreted proteins[191]. Canonical Wnt signaling also transcriptionally regulates the expression of ephrin B (EphB) receptors. EphB receptors and their ligands are expressed in counter gradients in the intestinal epithelium, with EphB2 and EphB3 expressed at high levels in intestinal stem cells[192].

2.2. Wnt/β-catenin signaling

Several key regulatory signals, sent out by stromal and epithelial cells, are involved in intestinal SC renewal and differentiation, including the Wnt , BMP (bone morphogenetic protein), and Notch pathways[185]. Increasing evidence has revealed that the Wnt cascade is the strongest signal in controlling cell fate along the crypt, although some studies suggest that there is a close interaction between these three key pathways in directing intestinal epithelial renewal[193, 194] (Figure 4).

2.2.1. Canonical Wnt signaling

Canonical Wnt signaling plays a major role in maintaining the intestinal SC niche and monitoring the proliferation of progenitor cells. Its hallmark is the accumulation and translocation of the adherens junction associated-protein β-catenin into the nucleus. Wnts are glycoproteins secreted by stromal cells and comprise a family of nineteen proteins in humans that bind to a receptor complex, consisting of a Frizzled receptor family member (Fz) and the low-density-lipoprotein-related protein5/6 (LRP5/6)[195]. Wnts act as morphogens whose activity is concentration dependent[196]. There is a diverse number of secreted Wnt antagonists in the extra-cellular matrix; these include Dickkopf (DKK) proteins, the Wnt-inhibitor protein (WIF), soluble Frizzled-related proteins (sFRP), Crebrus, Frzb, and the context-dependent Wnt inhibitor Wise[197]. In addition, proteins such as Norrin and R-spondin2 can bind to the LRP5/6 receptor, and may activate Wnt signaling independently of Wnt ligands[198, 199]. The central player in the canonical Wnt signaling cascade is a cytoplasmic protein named β-catenin. The stability of β-catenin is controlled by a destruction complex regulated within the cytoplasm. In the absence of Wnt signaling, cytoplasmic β-catenin

is degraded by a β-catenin destruction complex that includes Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A9), glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CKIα)[200]. Phosphorylation of conserved Ser and Thr residues by CKIα and GSK3 in the amino terminus of β-catenin occurs within this complex, thus leading to its targeting for ubiquitination and subsequent destruction by the proteosomal machinery. The complex APC/Axin/GSK3 is disrupted by a series of events triggered by the Wnt pathway[201]. After binding of Wnt to the receptor complex, the signal is transduced to the cytoplasmic phosphoprotein Dishevelled (DSH/DVL). At this level the Wnt signal could separate into at least three major cascades: canonical, Planar Cell Polarity and Wnt/Ca^{2+} [202]. Once DSH is activated, it inhibits the activity of the GSK3 enzyme, and activates a complex series of events that prevent the degradation of β-catenin and its consequent stabilization and accumulation in the cytoplasm[203]. Free β-catenin in the cytoplasm can translocate into the nucleus and exert its effect on gene transcription by functioning as a transcriptional co-activator (Figure 5). The best-characterized binding partners for β-catenin in the nucleus are the members of the T-cell factor (TCF) and lymphoid enhancer-bing protein (LEF) DNA-binding transcription factors[204]. In the absence of a Wnt signal, TCF/LEF proteins repress target genes through a direct association with co-repressors such as Groucho. Interaction with β-catenin transiently converts TCF/LEF factors into transcriptional activators[205]. Wnt target genes are diverse and cell- and context-specific[196, 206]. Wnt signaling components - including Fz, LRP5/6, Axin2, TCF/LEF, Naked (a DVL antagonist), DKKL, and R-spondin - are often regulated positively or negatively by β-catenin as Wnt-dependent self-regulatory mechanisms[196, 199, 207, 208].

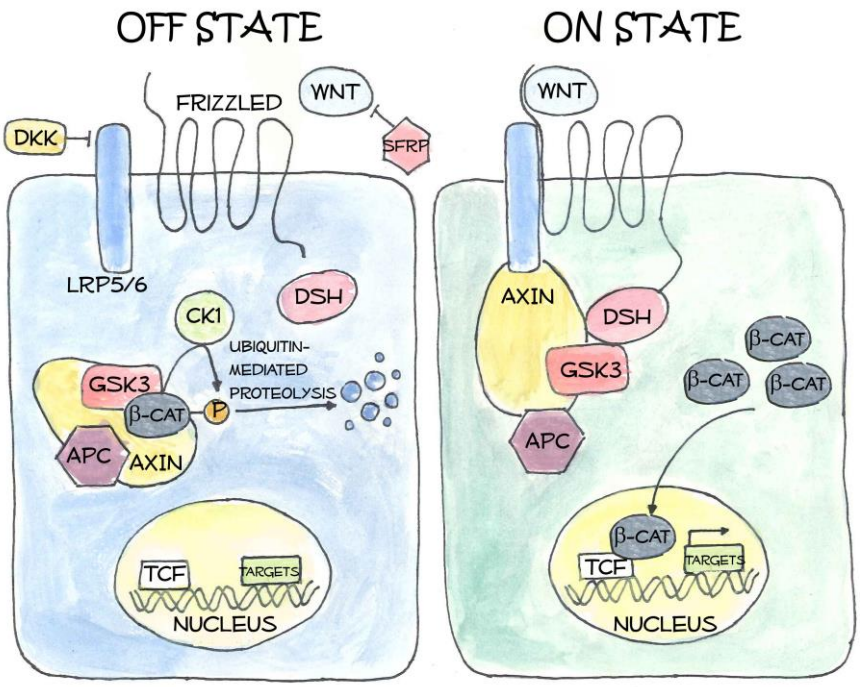


Figure 5. **Canonical Wnt signaling.** β-CAT, β-catenin; DKK, Dickkopf; DSH, Dishevelled. Adapted from Randall T. Moon, Nature Reviews Genetics, 2004[209]

2.3. Role of Wnt/β-catenin in colon regeneration

Wnt signals act over short distances to locally regulate cell behavior, controlling the organization of SCs within the niche and tissue physiology. To keep IEC homeostasis, Wnt/β-catenin gradient delimits the stem cell compartment, thereby preventing uncontrolled SC expansion[210]. Lineage barriers between SCs and progenitors are flexible *in vivo* and can change during times of tissue damage and repair, when progenitor cells could revert to SCs upon crypt loss[211].

Wnt signaling triggers cell proliferation, promoting wound repair in the DSS colitis mice model[212]. These proliferative effects of β-catenin-dependent Wnt signaling must be transient and localized to be beneficial in the wound repair and regeneration process.

Epithelial ulcerations that appear in UC represent highly inflamed wounds caused by local immune responses. Repairing mechanisms to regenerate ulcers, such as canonical and noncanonical Wnt signaling, are turned on during inflammatory processes[193, 213]. Proper wound healing leading to mucosal healing has become a

hallmark of recovery after IBD therapy, as it is associated with durable clinical remission and a reduced risk of surgery[214].

2.4. Role of Wnt/β-catenin in colon cancer

Mutations in different genes encoding those proteins involved in self-renewal or repair mechanisms can drive the development of colon cancer. In the Wnt/β-catenin pathway, inactivation of the *APC* gene results in the destabilization of β-catenin and in the activation of the Wnt cascade[215], and has been linked with sporadic colorectal cancers[216]. Exceptionally, mutants of the scaffolding protein Axin2, a target of β-catenin, or lack of the β-catenin N-terminal Ser/Thr destruction motif have been linked to colorectal cancer[217, 218]. These mutations result in the over-activation of β-catenin, which enters the nucleus and forms stable β-catenin-TCF4 complexes involved in the malignant transformation of epithelial cells. TCF4 target genes such as *MYC23* and *CCND1* are essential components of this machinery in transformed IECs[219]. If the Wnt cascade is mutationally activated, the adenoma cells maintain their progenitor status indefinitely. This allows the adenomas to persist for many years, providing ample opportunity for the acquisition of further mutations.

3. Epithelial cells as regulators of immune homeostasis

3.1. IEC secretory defenses

The first line of defense against microbial invasion is provided by goblet cell secretion of highly glycosylated mucins. The most abundant and important intestinal mucin in the organization of the mucus layer is mucin 2 (MUC2)[220]. MUC2-deficient mice suffer spontaneous colitis and have a predisposition to inflammation-induced colorectal cancer[221]. Goblet cells also produce other regulatory products such as trefoil factors (TFF), which provides structural integrity and promote epithelial repair, migration and resistance to apoptosis, and resistin-like molecule-β (RELMβ), which promotes MUC2 secretion and regulates macrophage and adaptive T cell responses during inflammation[222, 223]. Enterocytes also produce antimicrobial peptides (AMPs)

such as β-defensins and C-type lectin hepatointestinal pancreatic/pancreatitis-associated protein (HIP/PAP) in humans, or its counterpart regenerating islet-derived protein III γ (REGIIIγ) in mice. Paneth cells produce α-defensins, cathelicidins and lysozyme[187]. Dysfunction of these secretory IECs as a result of defects in autophagy or the unfolded protein response (UPR) is associated with human IBD and animal models of intestinal inflammation. In IECs, autophagy acts in an innate immune capacity to limit the propagation of invasive bacteria through the epithelium, and supports the packaging and exocytosis of Paneth cell granules[224-226]. In addition, IECs are responsible for the transport across the epithelial barrier of secretory immunoglobulins, an important component of adaptive immune responses to microbia. Dimeric IgA complexes, produced by plasma cells in the lamina propria, bind to the immunoglobulin receptor (pIgR) on the basolateral membrane of IECs and are actively transcytosed into the intestinal lumen[227].

3.2. Regulation of immune cells by IECs

IECs produce a variety of regulatory signals that promote lamina propria immune cells **tolerance** towards bacteria and that limit inflammation under steady-state conditions. The intestinal microflora acts via pattern-recognition receptor (PRR) signaling to support the production of immune-regulating molecules such as thymic stromal lymphopoietin (TSLP)[228], TGF-β[229], IL-25[230], retinoic acid, and B cell-stimulating factors (proliferation-inducing ligand; APRIL and B cell-activating factor; BAFF)[231, 232].

A continuous crosstalk between the epithelium and antigen-presenting cells within the lamina propria occurs. As a result, intestinal antigen-presenting cells are characterized by their ability to produce IL-10 and retinoic acid[229, 233, 234]. It has been demonstrated in mice that the interaction of $CD103^{+}$ migratory DCs with IECs promotes immune tolerance through the differentiation of regulatory T cells[235]. Furthermore, CX_3CR1^{hi} resident macrophages influenced by TLR signaling in IECs and with the permission of the protein tight junctions between epithelial cells, form trans-epithelial dendrites that penetrate into the intestinal lumen for sampling exogenous antigens[236]. Resident macrophages promote survival and local expansion of primed regulatory T cells, as well as production of IL-10[237]. Similarly, DCs are conditioned by

IEC-derived signals to acquire a gut-homing phenotype through the production of nitric oxide (NO), IL-10 and retinoic acid, in conjunction with TGFβ signalling[238]. In addition, epithelial cell-derived immune-regulatory cytokines, such as IL-25 and IL-7, regulate the ILC response to commensal bacteria[239].

3.3. Sampling of luminal contents by IECs

Specialized IECs in the small intestine, called microfold cells (M cells), mediate the sampling of luminal antigens and microorganisms for presentation to the mucosal immune system. M cells are localized in the follicle-associated epithelium, Peyer's patches and isolated lymphoid follicles. Besides efficient mechanisms and specific antigen-receptor interactions involved in M cell-mediated transport in the small intestine, there also exists a well-established nonspecific uptake and transcytosis of antigens along the intestinal tract[240, 241].

3.4. Microbial recognition by IECs

IECs are able to act as sensors for bacteria, processing their signals into antimicrobial and regulatory responses by PRRs. IECs express members of the TLR, NOD-like receptor (NLR) and RIG-I-like receptor (RLR) families. Although the study of PPR pathways in hematopoietic cells has mostly focused on their pro-inflammatory properties in antigen presentation and effector immune cell populations, it has been shown that PRRs play a crucial role in protecting against intestinal inflammation. Signals through TLRs could also induce the repair of epithelial damage, as demonstrated by animal studies on colitic mice deficient of PRRs, or lacking elements downstream of their pathways[242-244].

4. Epithelial deregulation in UC

The intestinal epithelium is found at the interface between the genetic (genome), environmental (microbiome and exposome) and immunological (immunome) factors

that drive UC pathogenesis. Thus, deregulation of the epithelial compartment can influence disease initiation and maintenance.

IECs from patients with UC respond to chronic inflammation, secreting cytokines and chemokines and initiating an apoptotic process. The epithelium presents permanent changes such as aberrant crypts (defined as crypt branching), the loss of parallel crypt structures and variations in crypt size, as a result of repeated crypt destruction and re-generation in UC[168, 245-248].

Changes in the composition of the epithelial layer and the products produced by epithelial cells are closely related to the course of UC. The first line of defense of the mucosal immune system is the mucus layer, which as previously mentioned is composed primarily of MUC2 produced by goblet cells[249]. A variant in the *MUC2* gene confers susceptibility to IBD in humans, while and *Muc2*-deficient mice develop spontaneous colitis[221]. Additionally, synthesis and alteration of sulphation of MUC2 in UC is decreased[250]. In IBD, defective regulation of tight junctions between epithelial cells has been reported to increase intestinal permeability and dysfunction of the epithelial layer[81]. In addition, expression of selected human β-defensins by epithelial cells is up-regulated in colonic samples of patients with UC. Is still unclear, however, whether the increase in defensins production is induced in response to microorganisms, inflammatory cytokines, or both[251, 252].

To sense microbiota of the intestinal lumen, epithelial cells express pattern-recognition receptors such as TLRs and NOD-like receptors. UC pathobiology has been linked to alterations in TLRs expression. Under physiological conditions, IECs mainly express TLR3 and TLR5, whereas other TLRs are limited. In contrast, TLR2, TLR4 and TLR9 expression is substantially increased in the colonocytes of patients with UC[253-255], the signaling of which triggers pro-inflammatory gene expression and cytokine secretion during recognition of commensal bacteria.

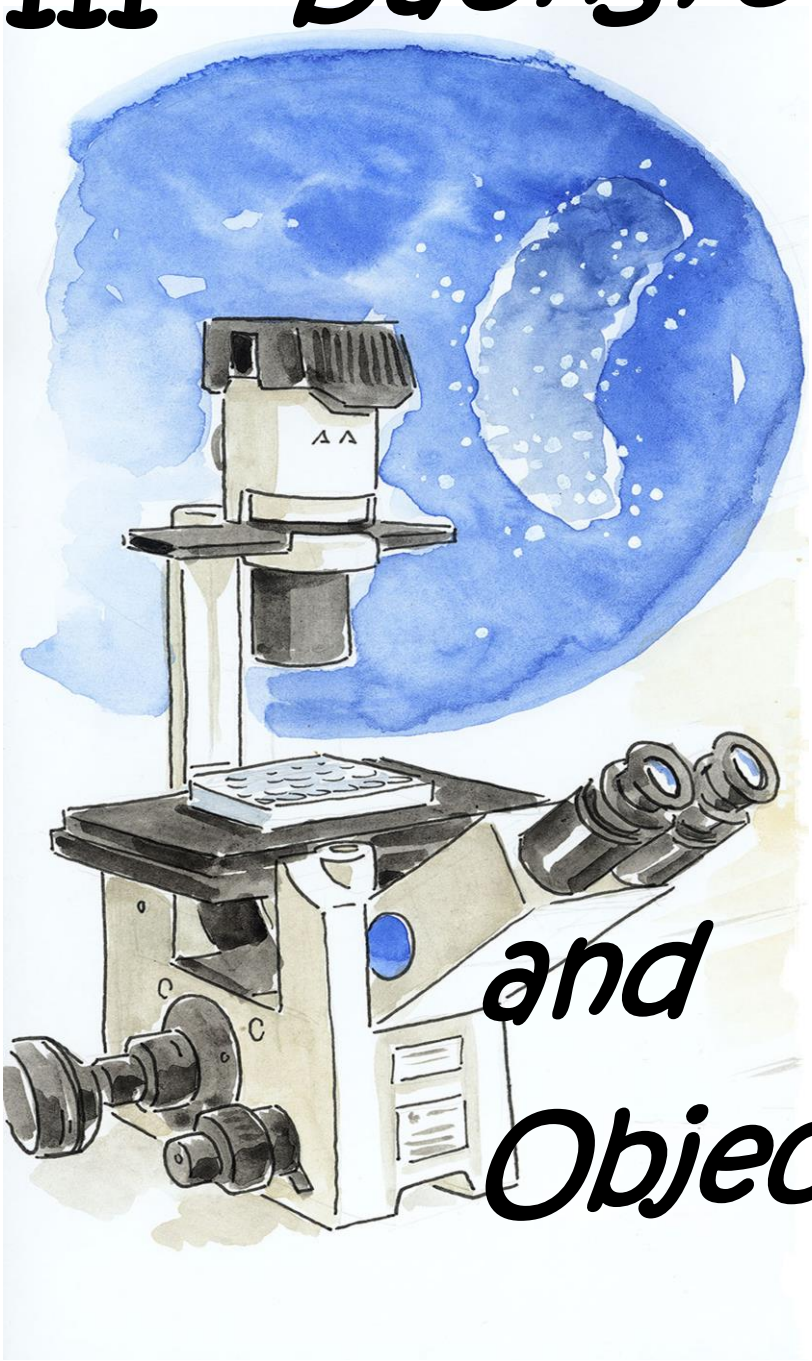
5. The study of the intestinal epithelium

Tissue explant culture was the first approach for studying the physiological activities of intestinal tissues *ex vivo*, although adult intestinal mucosa in organ culture typically degenerates rapidly[256]. This is due to the stringent conditions required to maintain these cells, such as extracellular matrix components and certain growth factors. **Epithelial cell lines** derived from primary tumor tissues have been used extensively to study response to stimuli, migration properties and regenerating processes. IEC lines are useful tools for determining pathways and mechanisms that regulate cell replication and differentiation. Two of the pathways most extensively explored are canonical Wnt/β-catenin and Notch signaling, both of which are involved in development, morphogenesis, and in tissue homeostasis[257].

Nowadays, improvements in tissue dissociation techniques, the availability of a range of components in culture media, growth supplements and appropriate substrates are providing the opportunity to study not only primary intestinal epithelium, but also intestinal epithelial stem cells, which maintain their phenotype and morphology over long periods of time in culture.

One approach for studying primary epithelial cells is culture of **whole isolated crypts**. The protocols that include whole crypts embedded in an extracellular matrix, such as Matrigel, and culture media that contains selective components for maintaining all types of specialized epithelial cells, are useful for investigating *ex vivo* the epithelial layer in a physiological manner[258]. A similar protocol that incorporates Wnt signals in the culture media drives expansion of the crypt's stem compartment. In 2009 Clevers and colleagues generated **gut organoids** from adult intestinal stem cells upon 3D culture in Matrigel[259]. These intestinal stem cells spheroids maintain the ability to proliferate and differentiate in various IEC lineages and represent a useful tool for studying epithelial cell homeostasis and response to injury.

III- Background and Objectives



During the course of acute intestinal inflammation a number of regulatory mechanisms, such as anti-inflammatory cytokine production (IL-10, IFN-α and IFN-β, TGF-β, IL-22, IL-35 and IL-37)[260], endogenous inhibitors of inflammation (IL-1Ra[168], sTNFR[176], IL-18BP[261]) and pro-resolution mediators (lipoxins, resolvins and protectins)[262] are triggered, presumably to limit the inflammatory response and to regain intestinal homeostasis. In fact, some of them have already been explored because of their therapeutic value[262-264].

The main objective of this thesis was to identify potential endogenous homeostatic or anti-inflammatory pathways that may be present during inactive phases of UC and that could be harnessed for the benefit of sustained remission.

In an earlier study our group had shown that compared to active intestinal inflammation, the mucosa of UC patients in remission presented a unique transcriptional signature [168]. In particular, we demonstrated that despite complete healing of mucosal lesions, about half of the genes that are de-regulated during colonic inflammation remain altered in the involved remitting mucosa of UC patients[168].

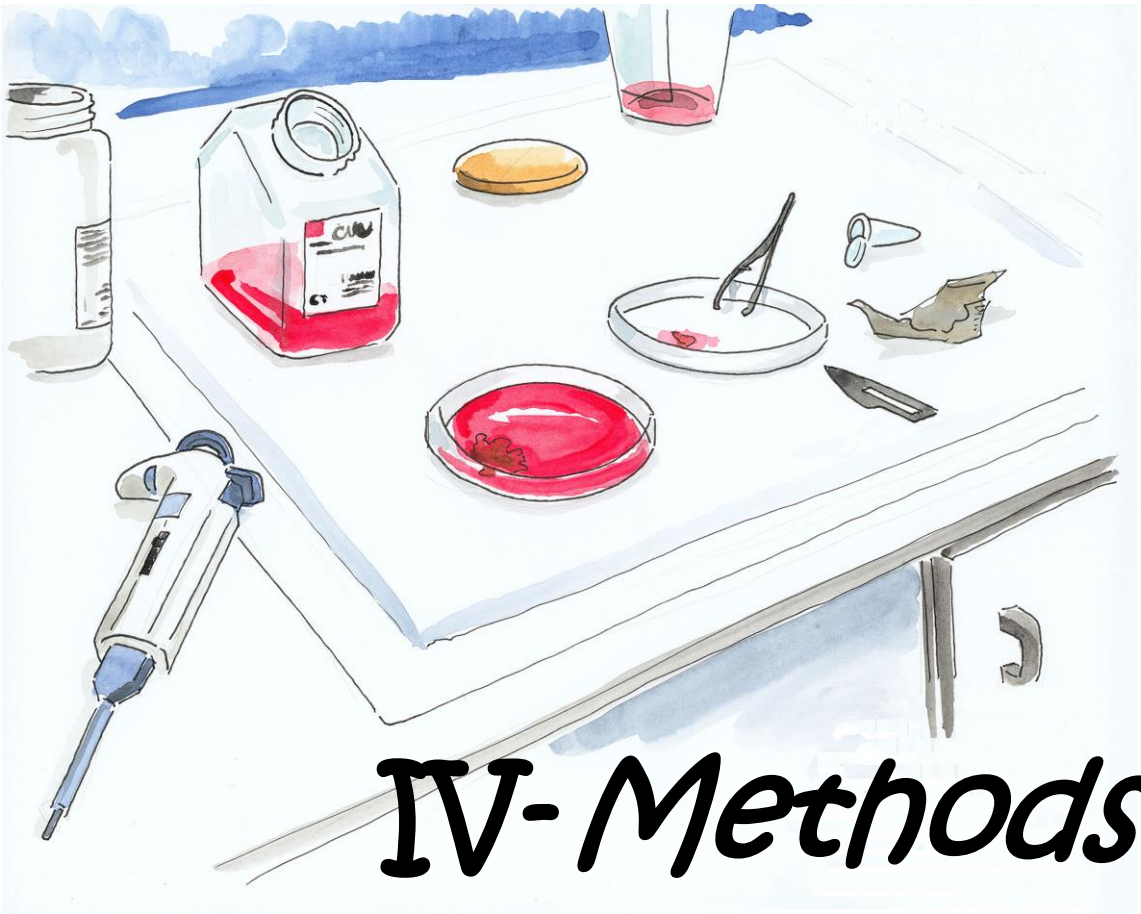
For the purpose of this thesis we further exploited the transcriptional signature of UC in remission and determined that the interleukin-1 receptor type 2 gene (*IL1R2*), a decoy receptor for the pleiotropic cytokine IL-1 (IL-1α and IL-1β), was significantly up-regulated in the mucosa during remission of the disease compared to controls and active inflammation. Based on these observations, **we hypothesize that IL-1R2 represents an endogenous locally acting molecule that may counterbalance low persistent or locally arising IL-1β production in chronic UC patients**. In order to test this hypothesis we set out the following specific aims:

1- To validate the microarray data at the mRNA and protein level.

2- To identify the cellular sources of IL-1R2 production.

3- To identify the pathway regulating IL-1R2 expression.

4- To study IL-1R2 functional significance during remission of UC.



IV- Methods

1. Patient population

A total of 241 subjects were recruited. Patients with an established diagnosis of UC or CD and non-IBD controls were included after obtaining written informed consent. Non-IBD controls were those subjects undergoing surgery for colorectal cancer or colonoscopy for mild gastrointestinal symptoms, or colorectal cancer screening and who presented no lesions during examination.

2. Assessment of disease activity

Endoscopic activity at the time of colonoscopy was categorized according to the Mayo endoscopic subscore[265]. Active disease was defined as a Mayo endoscopic subscore of 1-2-3; quiescent disease (remission) was defined as a Mayo score of 0 or 1 with limited erythema in a segment with evidence of active disease in any previous endoscopy; a segment was categorized as uninvolved when no lesions were identified in the current and any previous endoscopy.

3. Intestinal sample collection

Intestinal biopsies from the sigmoid colon were collected during routine colonoscopies from non-IBD controls, UC patients with quiescent disease, and UC patients with endoscopic activity. Samples from uninvolved segments were obtained from both UC patients in remission and from those with active disease. None of the biopsies obtained from the vicinity of samples used for the experiments described below showed evidence of colitis-associated dysplasia or neoplasia. For spheroid culture and Wnt/β-catenin agonist experiments, whole intestinal crypts were isolated from the healthy mucosa of 11 colorectal cancer patients undergoing surgery.

3.1. Biopsy culture

Biopsies (For cytokine production average weight: 6.71 mg, range: 3.2-16; for T cell culture, average weight: 9.63 mg, range 8-13) were washed 3 times in RPMI 1640 medium (Lonza, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biosera, Nuaille, France), 100 U/ml penicillin, 100 U/ml streptomycin and 250 ng/ml amphotericin B (Lonza), 10 µg/ml gentamicin sulfate (Lonza) and 1.5 mM Hepes (complete medium). They were then cultured in 48-well-plates at 37˚C in a humidified atmosphere containing 5% CO_2 incubator for 24h. After overnight culture, the supernatants were harvested, centrifuged (400 xg, 4ºC) and stored at -20˚C until assayed.

3.2. Biopsy cell isolation and flow cytometry

To isolate cells from the intestine, biopsies were collected in Hank's Balanced Salt Solution (HBSS) (Lonza) and washed for 15 min with Dithiothreitol (DTT) 10mM (Sigma, San Louis, MO) solution in RPMI 1640 (Lonza) to remove the mucus. The tissue was washed twice in complete medium and digested in 48-well-plates with collagenase 1.5 mg/ml (Sigma) and DNAse 100 U/ml (Roche, Basel, Switzerland) for 20 min at 37ºC. Digested biopsies were washed in complete medium, filtered through a 70 µm mesh, and stained for flow cytometric analysis. Cells were incubated with the Fc Receptor block (Miltenyi Biotec, Bergisch Gladbach, Germany) and a LIVE/DEAD fixable violet dead cell stain kit (Invitrogen, Carlsbad, CA) prior to addition of the directly labeled antibodies anti-CD45-APC-Cy7 (BD Biosciences, San Jose, CA) and anti-Ep-CAM-FITC (R&D Systems, Minneapolis, MN). Cells were washed with PBS containing 2% FBS and 0.01% sodium azide (Facs Buffer), fixed and permeabilized with Fix and Perm medium (Invitrogen) and incubated with anti-IL1-R2-APC antibody (R&D Systems) or IgG1-APC (BD Biosciences) isotype control. After washing, cells were acquired in a FACS Canto II (BD Bioscience) and analysed with BD FACSDiva Software v6.1.2 (BD Biosciences).

Cells were first gated for singlets (FSC-H vs. FSC-A) and further analyzed for their uptake of the Live/dead stain in order to exclude dead cells. CD45 staining identifies the hematopoietic cell compartment (Figure 6). Within the non-hematopoietic subset ($CD45^-$), epithelial cells were identified by staining with the anti-Ep-CAM antibody. IL-1R2 expression by epithelial cells was determined by intracellular staining.

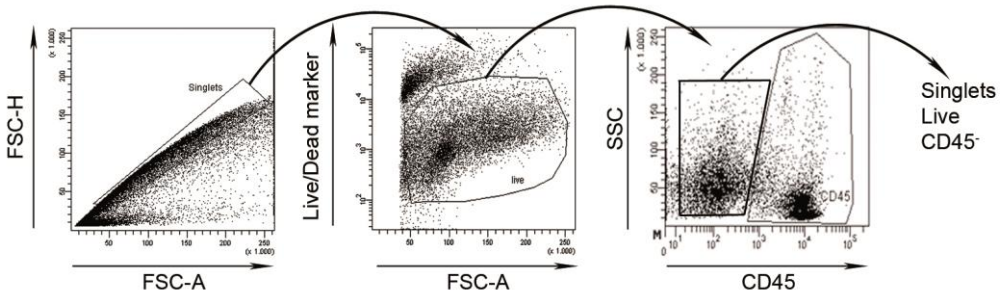


Figure 6. Gating strategy for flow cytometry analysis.

3.3. Colonic crypt isolation

Crypts were isolated from intestinal tissue as previously described[266]. For surgical colectomy samples, the muscle and sub-mucosa layers were carefully removed. The colonic mucosa was cut into small pieces and washed for 20 min at room temperature (RT) in PBS containing a mixture of antibiotics: normocin, gentamycin and fungizone (all from Invitrogen). Next, tissue fragments were washed twice with 10 mM DTT (Sigma) in PBS for 5 min at RT. Samples were transferred to 8 mM EDTA in PBS and incubated under rotation for 40 min at 4ºC. The EDTA-buffer was replaced by fresh cold PBS, and single colonic crypts units were released after 4-6 vigorous shaking washes. FBS was added to a final concentration of 5% and fractions were centrifuged at 150 x*g* for 3 min. A washing procedure was performed with Advanced DMEM/F12 (ADF) (Invitrogen) medium supplemented with 2 mM GlutaMax (Invitrogen), 10 mM HEPES (Sigma), and 5% FBS (Washing buffer: WB)

For endoscopic samples, biopsies were washed in PBS and incubated with the mixture of antibiotics as described above. Next, biopsies were transferred to 8 mM

EDTA, 0.5 mM DTT in PBS and washed for 40 min at 4ºC. The supernatant was replaced by fresh PBS, and single colonic crypts units were released after 6-8 vigorous shaking washes. FBS was added to a final concentration of 5% and fractions were centrifuged 200 x*g* for 3 min. An additional wash with WB was performed as described for the surgical samples. Isolated crypts maintained all the epithelial cell compartments (Figure 7).

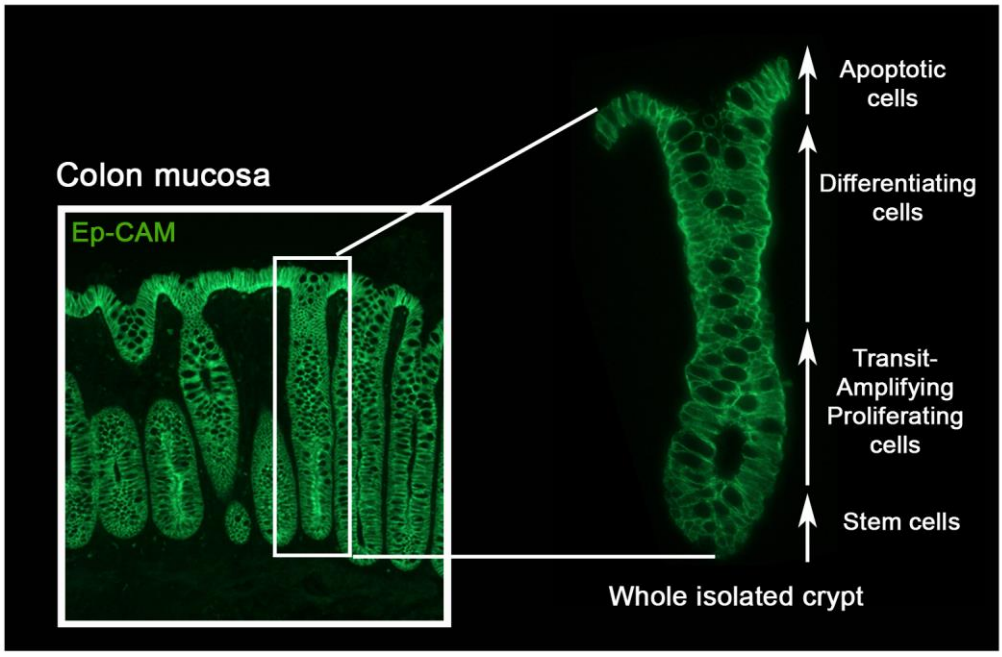


Figure 7. Whole colonic crypts isolation. Representative immune-fluorescent staining of fixed paraffin-embedded colonic tissue (left side) and an isolated colonic crypt (right side). Samples were stained with Ep-CAM (green)

3.3.1. Crypt culture

For short-term crypt culture, 15-30 isolated crypts were embedded in 25 µL of Matrigel (BD Biosciences) and plated on pre-warmed 48-well culture dishes. Crypts were cultured in "complete crypt culture medium": Advanced DMEM/F12 (ADF, Invitrogen), GlutaMax (Invitrogen), 10 mM HEPES (Sigma), N-2 (1x) (Gibco, Grand Island, NY), B-27 without retinoic acid (1x) (Gibco), 1mM N-Acetyl-L-cysteine (Sigma), 500 ng/ml RSPO1 (Sino Biologicals, Beijing, China), 100 ng/ml human Noggin

(Peprotech, Rocky Hill, NJ), 500 nM LY2157299 (Azon MedChem, Groningen, The Netherlands), Normocin 100 µg/ml and 1 mM Valproic acid (Sigma). After overnight culture at 37Cº and 5% CO_2, the supernatants were harvested, centrifuged and stored at -20˚C until assayed. Matrigel-embedded crypts were resuspended in 500 µL Trizol (Ambion, Foster City, CA) and stored at -80ºC until RNA extraction.

Healthy intestinal crypts from surgical samples were cultured with 5, 10 and 20 µM of CHIR-99021 (Selleck Chemicals, Houston, TX) for 18h. DMSO was used as a vehicle control.

When described, crypts from biopsies of UC patients in remission and healthy controls were treated with 0.1 ng/ml IL-1β (CellGenix GmgH, Freiburg im Breisgau, Germany) and 5 µg/ml IL-1R2 blocking antibody (rat anti-hIL1RII-M22; AMGEN, Thousand Oaks, WA) or 5 µg/ml rat IgG2b (eBioscience) for 18h.

3.3.2. Colonic epithelial stem cell culture

In order to obtain 3-D spheroid cultures of colonic stem cells (CoSC), approximately 30 purified human colon crypts/well embedded in Matrigel were overlaid with 250 µl "stem medium" (Wnt3a-conditioned medium and ADF 50:50, Glutamax, 10 mM HEPES, N-2 (1X), B-27 without retinoic acid (1X), 10 mM Nicotinamide, 1 mM N-Acetyl-L cysteine, 500 ng/ml R-spondin-1 (RSPO1), 50 ng/ml human epidermal growth factor (EGF) (Invitrogen), 100 ng/ml human Noggin, 1 µg/ml Gastrin (Tocris Bioscience, Bristol, United Kingdom), 500 nM LY2157299, 10 µM SB202190 (Sigma), and 0.01 µM prostaglandin E2 (PGE2; Sigma)). Medium was replaced with fresh stem medium every other day. For serial passage, Matrigel-embedded spheroids were released using Cell Recovery Solution (BD Biosciences). After re-suspension in HEPES-buffered ADF medium containing GlutaMax and 5% FBS, single cells and debris were removed by centrifugation at 400 ×*g* for 3 min. Spheroids were then incubated in Disaggregation Medium (ADF, Glutamax, 10 mM HEPES, N-2 (1x), B-27 (1x) without retinoic acid, 10 mM Nicotinamide (Sigma), 1 mM N-Acetyl-L-cysteine, 10 µM Y-27632 (Calbiochem, San Diego, CA), 2.5 µM PGE2 (Sigma), 0.1-0.5 mg/ml Dispase (BD Biosciences) for 10-20 min at 37°C in a water bath. Afterwards, the

cell suspension was syringed using a 1.2 mm G20 needle. After re-plating 1:4 in fresh Matrigel, the culture was overlaid with stem medium along with the Rock inhibitor Y-27623 (10 µM) for the first 2-3 days after each passaging step. Medium was changed every other day (Figure 8).

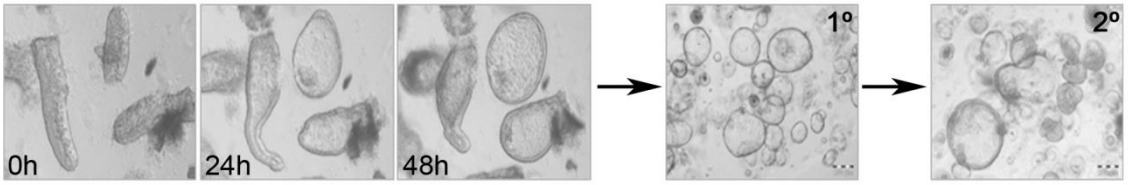


Figure 8. Long-term intestinal stem cell expansion. In both control and UC crypt cultures, "stem culture medium" induces expansion of the stem cell compartment within a few hours of crypt plating, giving rise to 3D "spheroids". Passaging is performed every week at the usual 1:4 ratio and can be stably maintained in culture for at least one month. In the figure the first two passages are shown.

To induce CoSC differentiation, nicotinamide, SB202190 and Wnt3a-conditioned medium were removed from the culture medium. RSPO1 was reduced to 250 ng/ml for the first 4 days and then completely removed thereafter. On the fifth day the supernatants were harvested, centrifuged and stored at -20˚C until assayed. Matrigel-embedded spheroids were washed with PBS and resuspended in 500 µl Trizol (Ambion) for RNA extraction using the Rneasy Kit (Qiagen).

To test the effect of IL-1β on CoSC, spheroids were cultured overnight in stem medium or stem medium without Wnt3a and R-spondin1 in the presence of 5 ng/ml of IL-1β.

4. T cell culture

PBMCs were isolated from healthy donors buffy coats obtained from Banc de Sang i Teixits (BST) by Ficoll (Sigma) gradient centrifugation. $CD4^+$ T cells and $CD14^+$ monocytes were isolated from PBMCs by use of CD4 and CD14 microbeads (Miltenyi Biotec), respectively, according to the supplier's instructions. $1x10^6CD4^+$ T cells were co-cultured with $CD14^+$ monocytes in 48-well-plates at a 20:1 ratio. Heat-killed *Candida*

Albicans (Microbiology Department, IDIBAPS, Barcelona) was used as antigen at 1 UFC: 1 CD4. Cultures with incipient proliferation hr-IL-2 (20UI/ml; eBioscience) were added on day 3 of culture. On day 5-6, T cells were harvest and cultured ($1x10^5$ cells) in 96-well plate with biopsy supernatants (dilution ½ with complete medium). T cells were stimulated with 5µg/ml IL-1R2 blocking antibody (rat anti-hIL1RII-M22; kindly provided by AMGEN) or 5µg/ml rat IgG2b isotype control (eBioscience) overnight.

4.1. Intracellular staining of cytokine production by T cells

For intracellular cytokine staining, cells were restimulated with phorbol myristil acetate (PMA) and ionomycin in the presence of brefeldin A (all from Sigma-Aldrich) at 25ng/ml, 0.5µg/ml, and 10µg/ml, respectively, for the final 4h of culture. Cells were fixed and permeabilized with FIX and PERM (Caltag, Life Technologies) according to the manufacturer's instructions. Cells were stained with a LIVE/DEAD fixable violet dead cell stain kit (Invitrogen) anti-CD4 (BD Biosciences), anti-IL-17 (eBioscience), and anti-IFN-γ (eBioscience) conjugated with different fluorochromes, acquired in a FACS Canto II (BD Bioscience) and analysed with BD FACSDiva Software v6.1.2 (BD Biosciences).

5. RNA extraction

Biopsies were placed in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) and stored at -80°C until RNA extraction. Matrigel-embedded crypts and organoids, and T cells were resuspended in Trizol (Ambion, Foster city, CA) and stored at -80ºC until RNA extraction. Total RNA was isolated using the Rneasy Kit (Qiagen) according to the manufacturer's instructions. RNA was then used for real-time polymerase chain reaction (RT-PCR) as detailed below. Purity and integrity of the total RNA were assessed with the 2100 Bioanalyzer (Agilent, Santa Clara, CA) and quantified using a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Only samples with an RNA integrity number (RIN) greater than 7.0 were used.

5.1. Quantitative real-time RT-PCR (qPCR)

Total RNA (500 ng for biopsies, 250 ng for whole crypts and epithelial spheroids) was transcribed to cDNA using reverse transcriptase (High Capacity cDNA Archive RT kit, Applied Biosystems, Carlsbad, CA). PCR was performed in TaqMan Universal PCR Master Mix and *IL1R2*, *IL1RN, IL1B, IL1RAPC, IL1R1, KI67, AXIN2, LGR5, MUC2, ANPEP, CCL20* and *CXCL8* probes (Applied Biosystems) according to the manufacturer's instructions. ACTB was used as a housekeeping control gene. Fluorescence was detected in an ABI PRISM 7500 Fast RT-PCR System (Applied Biosystems). In order to normalize Ct values, the DeltaCts (ΔCt = Ct mean of reference gene – Ct of the target gene) were calculated using beta-actin (*ACTB)* as an endogenous control gene.

6. Measurement of soluble proteins

Supernatants from spheroid cultures, intestinal crypt cultures and $CD4^+$ cell cultures were centrifuged at 2000 x*g* 4ºC and stored at -20ºC until assayed for soluble IL-1R2, CCL20 and IFN-γ. For serum samples, blood was collected in serum separator tubes (BD Biosciences) with coagulation activators, centrifuged at 1200 xg for 10 min at 4ºC, and serum was stored at -20˚C until assayed for IL-1β and soluble IL-1R2.

Soluble IL-1R2, IL-1Ra, IL-1RAcP, IL-1R1 and CCL20 were detected using commercially available enzyme-linked immunosorbent assays kits (ELISAs) from R&D Systems. IL-1β was detected using eBioscience (San Diego, CA) purified and biotinylated antibodies and human IL-1β recombinant protein as a standard. IFN-γ was detected using commercially available ELISA from BD Biosciences.

7. Immunostaining of intestinal samples

Paraffin-embedded sections (2µm) from mucosa colonic biopsies were pre-treated for deparaffinization, rehydration, and epitope retrieval using Dako EnVision Flex Target Retrieval Solution low pH (50x) in conjunction with PT Link (Dako, Carpinteria, CA), with a warming step of 20 min at 95ºC for immunohistochemical and dual immunofluorescent staining.

For immunohistochemical staining, sections were blocked with 1% BSA for 30 minutes and incubated overnight at 4ºC with commercially available antibodies: Rabbit anti-IL1R2 polyclonal antibody (SIGMA, dilution 1:200), and goat anti-EphB2 (R&D Systems, dilution 1:200). EphB2 signal was amplified using a rabbit anti-goat bridge antibody. Sections were incubated with 3% H_2O_2 for 10 min in order to block peroxidase activity and then incubated for 30 min with a specific secondary antibody (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Immunohistochemical staining was carried out using 3,3'9-diaminobenzidine (DAB) chromogen (Sigma) in the presence of a peroxidase enzyme (avidin/biotinylated enzyme complex, ABC). Sections were counterstained with hematoxylin. Sections were dehydrated through graded ethanol steps and xylene and then mounted with DPX Mountant for histology (Fluka Chemicals, Gillingham, United Kingdom). Staining was examined with an Olympus BX51 microscope.

Dual immunofluorescent staining was performed using anti-IL-1R2 (rabbit polyclonal antibody; SIGMA, dilution 1:100) and anti-CD45 (mouse PE antibody; BD Biosciences, dilution 1:50), anti-IgA (goat biotin antibody; Southern Biotech, AL, USA, dilution 1:200), anti-IgG (mouse V450 antibody; BD Biosciences, dilution 1:50) and anti-Ep-CAM (mouse monoclonal antibody; Dako, dilution 1:100). Sections were blocked with 1% BSA for 30 min and incubated overnight at 4ºC using the following combinations of two primary antibodies: 1) anti-IL1R2 and anti-CD45, 2) anti-IL1R2 and anti-IgA, 3) anti-IL1R2 and anti-IgG or 4) anti-IL1R2 and anti-Ep-CAM. Goat anti-rabbit Cy3, goat anti-rabbit Alexa 488, goat anti-mouse Cy3, goat anti-mouse Alexa 488 and Streptavidin Alexa 488 (all from Jackson Immunoresearch, West Grove, PA) were used as secondary antibodies.

Following immunostaining, all sections were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories) and examined with CellF software using an Olympus BX51 microscope. Negative controls were processed under the same conditions in the absence of the corresponding primary antibodies.

8. RNA chromogenic in situ hybridization

The RNAscope 2.0 assay was performed according to the supplier's instructions (Advanced Cell Diagnostics, Hayward, CA). Briefly, 5 µm paraffin-embedded sections from mucosa colonic samples were deparaffinized via 100% xylene and ethanol washes. Tissues were then treated serially with: Pre-Treatment 1 solution (endogenous hydrogen peroxidase block with Pretreat 1 solution for 10 min at RT); Pre-Treatment 2 (100ºC, 15 min immersion in Pretreat 2 solution); and, Pre-Treatment 3 (protease digestion, 40ºC for 30 min in the HybEZ Oven (Advanced Cell Diagnostics); rinses with distilled water were performed after each Pre-Treatment step. Tissues were then hybridized with IL1R2 probe (Advanced Cell Diagnostics), without a cover slip, at 40ºC for 2h in the HybEZ Oven. An Hs-PPIB probe as a positive control and a DapB probe as a negative control were included. After rinsing with wash buffer, amplification of the hybridized probe signal was obtained by the serial application of Amp 1 (PreAmplifier step), Amp 2 (signal enhancer step), Amp 3 (amplifier step), Amp 4 (Label Probe step), Amp 5 and Amp 6 (signal amplifications steps); rinses with wash buffer were performed after each Amp step. HRP activity was then demonstrated by the application of DAB chromogen for 10 min at RT. Sections were then counterstained with hematoxylin (50% in distilled water), dehydrated through graded ethanol steps (50%, 70% and 100%) and xylene, and then mounted with DPX Mountant for histology (Fluka).

9. Statistical analysis

Numeric data are described as median and range, and categorical variables as absolute frequencies. A Mann-Whitney test was performed to examine statistically different expression patterns between two groups, and a Kruskal-Wallis test was performed to examine statistical significance in multiple group data sets, followed by a Benjamini-Hochberb post-hoc correction test. A Friedman test was performed to examine statistical significance in repeated-measures analysis, followed by the Nemenyi post-hoc test. A Wilcoxon matched-pair test was performed to examine statistical significance in paired data. An adjusted p-value of <.05 was considered statistically significant. Spearman correlation was performed for numeric correlations. The receiver-operator characteristic (ROC) area under the curve (AUC) was calculated to assess the usefulness of *IL1R2* expression for predicting relapse. Data were analyzed using R (version 3.1.0) (Published Online First: 2014. http://www.r-project.org/)

10. Ethical considerations

This study was approved by the Institutional Ethics Committee of the Hospital Clinic of Barcelona (Spain) in March 2006 and was performed in accordance with the principles stated in the Declaration of Helsinki (updated October 1996). All patients signed an informed consent prior to their inclusion in the study.

V- Results

1. Demographic data

We recruited a total of 266 subjects. Table 4 shows the clinical and demographic characteristics from non-IBD controls and UC patients. Samples (biopsies or serum) were distributed into 8 different subgroups based on the different experimental approaches used. Samples from 71 non-IBD controls were distributed in groups 1-7 and 195 samples from patients with UC were included in groups 1-8 (Table 4). Thirty samples from CD patients and their characteristics are described in Table 5.

Tissue samples were obtained from involved areas of patients with inactive disease (UC remission or CD remission) or patients with active inflammation (UC active or CD active). Samples named "UC uninvolved" included in groups 1, 3, and 5 (Table 4) were obtained from non-affected areas of the colon from UC patients with active or inactive disease.

Biopsies from affected areas (active or remission) were from the sigmoid colon and uninvolved areas were from the ascending colon.

No significant differences in age, gender, treatment received, and duration of disease were found between UC or CD patients included into each group, except in the case of UC remission patients included in group 1 who presented significantly longer disease durations compared to the other UC samples (Patient group 1, Table 4). Nonetheless, we did not observe correlations between the expression patterns of any of the genes studied and disease duration.

1. Demographic data

	N	Age (years) Median (range)	Gender M/F	Mayo 0/1/2/3	Treatment	Extension of Disease	Duration of disease (years) Median (range)
Group 1 (q-PCR)							
Control	10	40.5(28-56)	3/7				
UC uninvolved	8	47.5 (25-73)	5/3	0/0/5/3[†]	3/3/1/1/0/0/0/0	1/7/0	2.5 (0-10)[*]
UC remission	12	40 (29-67)	6/6	11/1/0/0	5/2/0/5/0/0/0/0	0/8/4	9 (2-21)
UC active	7	55 (36-73)	5/2	0/0/4/3	2/3/1/1/0/0/0/0	0/7/0	2.5 (0-10)[*]
Group 2 (Serum)							
Control	10	39.5 (22-56)	6/4				
UC remission	12	40 (33-84)	6/6	11/1/0/0	1/6/2/0/1/0/1/1	0/8/4	11.5 (3-24)
UC active	10	42 (22-67)	5/5	0/07/3	1/4/3/1/1/0/0/0	0/7/3	6.758 (0-17)
Group 3 (Biopsy culture)							
Control	10	45.5 (28-68)	7/3				
UC uninvolved	11	34 (27-55)	4/7	2/0/7/2[†]	1/6/0/3/1/0/0/0	1/10/0	5 (1-13)
UC remission	13	50 (27-66)	7/6	9/4/0/0	5/6/1/1/0/0/0/0	0/7/6	13 (1-25)
UC active	17	41 (31-78)	8/9	0/2/12/3	1/10/0/2/2/1/1/0	0/13/4	9 (1-18)
Group 4 (Immunostaining)							
Control	12	45 (22-83)	6/6				
UC remission	10	57.5 (39-76)	6/4	9/1/0/0	1/4/2/1/1/0/1/0	0/7/3	12.25 (3-24)
Group 5 (Biopsy cell isolation and flow cytometry)							
Control	8	53.5 (34-68)	2/6				
UC uninvolved	8	40.5 (33-73)	4/4	1/3/3/1[†]	0/5/0/0/2/0/1/0	1/7/0	11.5 (4-29)
UC remission	10	50.5 (37-73)	6/4	8/2/0/0	0/4/0/1/4/0/0/0	0/5/5	18.5 (6-29)
UC active	10	37.5 (31-54)	5/5	0/3/5/2	1/3/1/1/1/0/4/0	0/7/3	13 (4-18)
Group 6 (Crypts isolation and culture)							
Control	9	62 (48-70)	4/5				
UC remission	10	53 (38-69)	5/5	10/0/0/0	2/5/0/1/0/0/0/2	0/5/5	16.5 (8-39)
Group 7 (T cell culture)							
Control	12	54.5 (33-70)	9/3				
UC remission	12	48,5 (38-58)	4/8	12/0/0/0	2/6/0/0/3/0/1/0	0/6/6	15 (5-24)
Group 8 (Relapse study)							
UC remission:							
No relapse	24	40 (23-71)	10/14	22/2/0/0	5/14/0/4/1/0/0/0	0/19/5	7.5 (1-29)
Relapse	21	43 (33-71)	10/11	20/1/0/0	5/7/0/7/0/0/2/0	0/13/8	13 (0-28)

Table 4. Ulcerative colitis (UC) patients and non-IBD controls.

Gender: Male (M) and Female (F); Treatment: None/Mesalazine/Steroids/Immunosuppressant/a-TNF/Mesalazine+Steroids/Mesalazine+Immunosuppressant/Mesalazine+a-TNF; *(Table legend continues)*

Extension of disease: proctitis/left-sided/pancolitis. Data analyzed by Kruskal-Wallis test, followed by Benjamini-Hochberb post-hoc correction test. † Mayo from affected areas.*p< 0.05 *vs.* remission

	N	**Age** (years) Median (range)	**Gender** M/F	**Global CDEIS** Median (range)	**Partial CDEIS** Median (range)	**Treatment**	**Location of the disease**	**Behavior**	**Duration of disease** (years) Median (range)
q-PCR and serum									
CD remission	17	38 (24-58)	3/14	0.25 (0-9)	0	4/1/0/5/4/0/ 1/0/0/1/1	0/11/6/0	15/1/1	8,75 (0-17)
CD active	13	29 (21-56)	2/11	13 (2-36.4)	18 (9-36)	2/0/1/4/2/ 0/0/0/2/0/2	0/7/6/0	11/2/0	5,5 (0-32)

Table 5. Crohn's disease (CD) patients and non-IBD controls.

Gender: Male (M) and Female (F); Partial CDEIS of the area studied (sigmoid or descending colon); Treatment: None/Mesalazine/Steroids/Immunosuppressant/a-TNF/Mesalazine+Steroids/Mesalazine+Immunosuppressant/Mesalazine+a-TNF/Steroids+immunosuppressant/Steroids+a-TNF/Immunosuppressant+a-TNF; Location of the disease: Terminal ileum/Colon/Ileocolon/Upper gastrointestinal; Behaviour: Non-stricturing non-penetrating/Structuring/Penetrating.

2. *IL-1R2 expression in the colonic mucosa of patients with IBD*

2.1. *IL1R2*, the IL-1 decoy receptor, is up-regulated in the intestinal mucosa of UC patients in remission

In a previous transcriptional study, we had shown that despite complete healing of mucosal lesions, about half of those genes de-regulated during colonic inflammation remain altered in the involved remitting mucosa of UC patients[168]. Further analysis of these microarray data* revealed a significant up-regulation of *IL1R2* (>5 fold change) in the mucosa from UC patients in remission compared to those with active disease, and >2-fold overexpression compared to non-IBD controls or with the colonic mucosa of uninvolved segments in patients with UC (Figure 9). In contrast, *IL1B*, *IL1A*, *IL1RAP* and *IL1R1* genes were up-regulated in the inflamed mucosa compared to all the other conditions. Transcription of the IL-1R antagonist gene, *IL1RN*, was significantly up-regulated in all involved mucosa independently of the presence of inflammation (Figure 9).

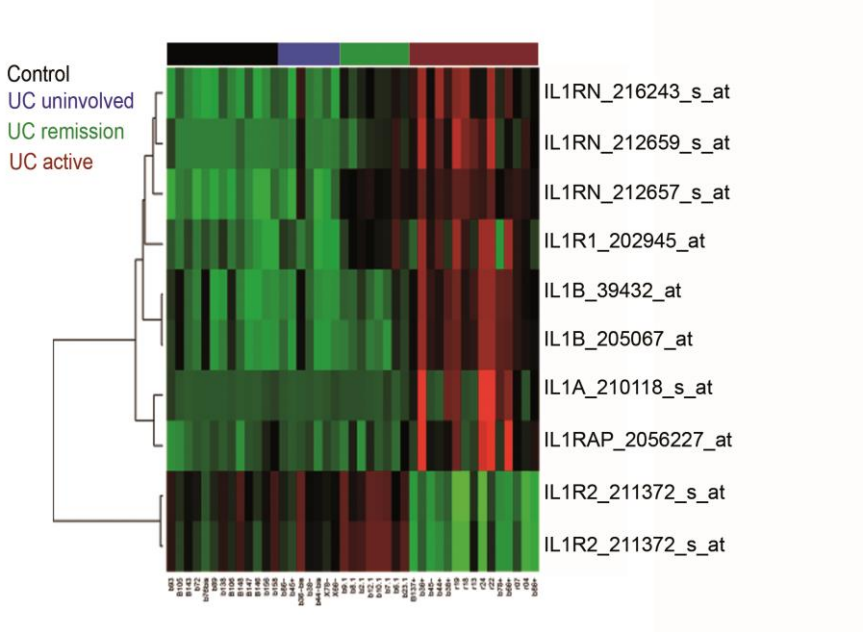


Figure 9. Heatmap representation of microarray expression of IL-1 family genes. *(Figure legend continues)*

* (GSE38713 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38713)

Each row shows one individual probe (representing 6 selected genes, 10 different probes) and each column an experimental sample. High expression levels are shown in red and low expression levels in green. An unsupervised hierarchical cluster method, using a Pearson distance and average linkage method, was applied for each gene classification. Samples belonged to one of the following groups: non-IBD controls (shown in black, n=13), non-involved mucosa segments from patients with active UC (UC uninvolved; in blue, n=7), involved mucosa segments from patients with active UC (UC active; in red n=15) and endoscopically and histologically inactive UC (UC remission; in green, n=8).

In order to validate these findings, we performed RT-PCR in an independent cohort of UC patients and controls (Patient group 1, Table 4). As shown in Figure 10, we confirmed that the highest expression of *IL1B* was observed in the mucosa with active UC compared to all the other groups. Remarkably, expression of *IL1B* in UC remission, although significantly down-regulated compared to active UC, remained overexpressed relative to controls and uninvolved UC mucosa (Figure 10a). In contrast, *IL1R2* was overexpressed in UC patients in remission compared to all the other groups (Figure 10b). Similarly to *IL1B*, *IL1R1*, a positive mediator of the IL-1 signaling cascade, was significantly up-regulated in active UC compared to remission, as well as in uninvolved mucosa and non-IBD controls. (Figure 10c and 10d). Although this did not reach statistical significance, the samples that more highly expressed the co-receptor IL1RAP were from the involved areas from UC patients with active disease (Figure 10d). Indeed, we identified a strong correlation between expression of IL1RAP and IL1R1 ($r=0.68$; $p<0.001$), both required for positive IL-1 signaling (Figure 11). Compared to controls, *IL1RN* was up-regulated in the involved UC mucosa, both in the presence and absence of active inflammation (Figure 10), as shown by microarray analysis (Figure 9).

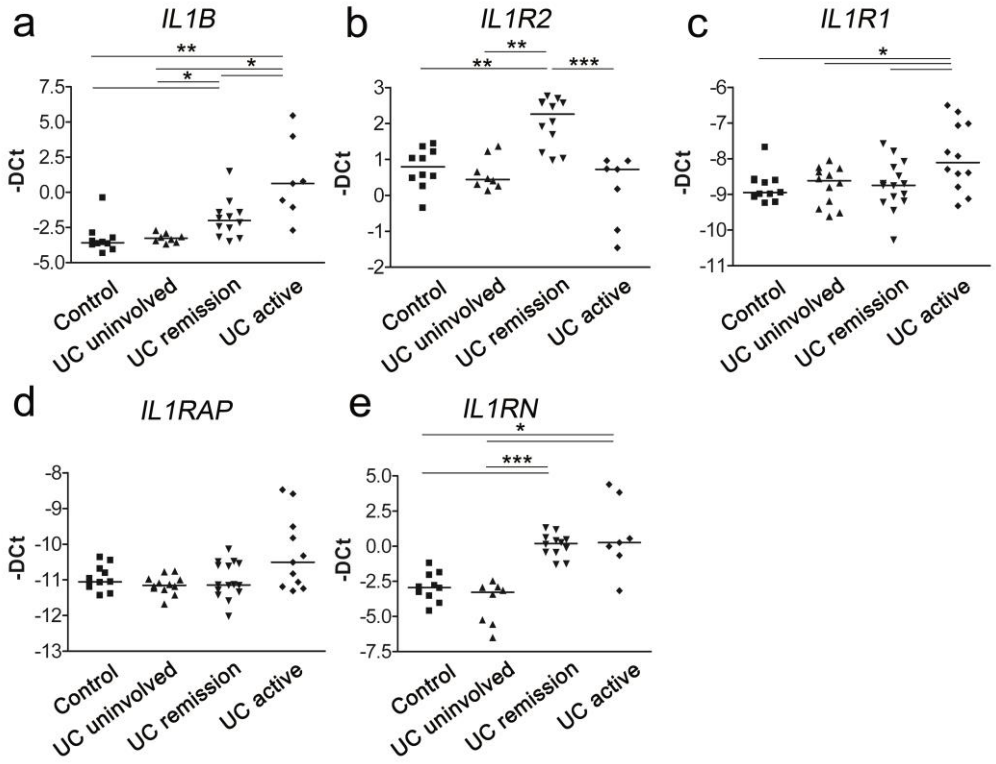


Figure 10. Expression of selected IL-1 family genes in colonic mucosa. (a-e) Dot plot representation (line = median) of mRNA expression of *IL1B*, *IL1R2*, *IL1R1*, *IL1RAP* and *IL1RN* as determined by qPCR (-Delta Ct) in controls (n=10), uninvolved areas from patients with ulcerative colitis (UC; n=8), UC patients in remission (n=12), and patients with active UC (n=7). Gene expression data analyzed by Kruskal-Wallis test, followed by Benjamini-Hochberb post-hoc correction test. $^{*}P<.05$, $^{**}P<.005$, $^{***}P<.0005$.

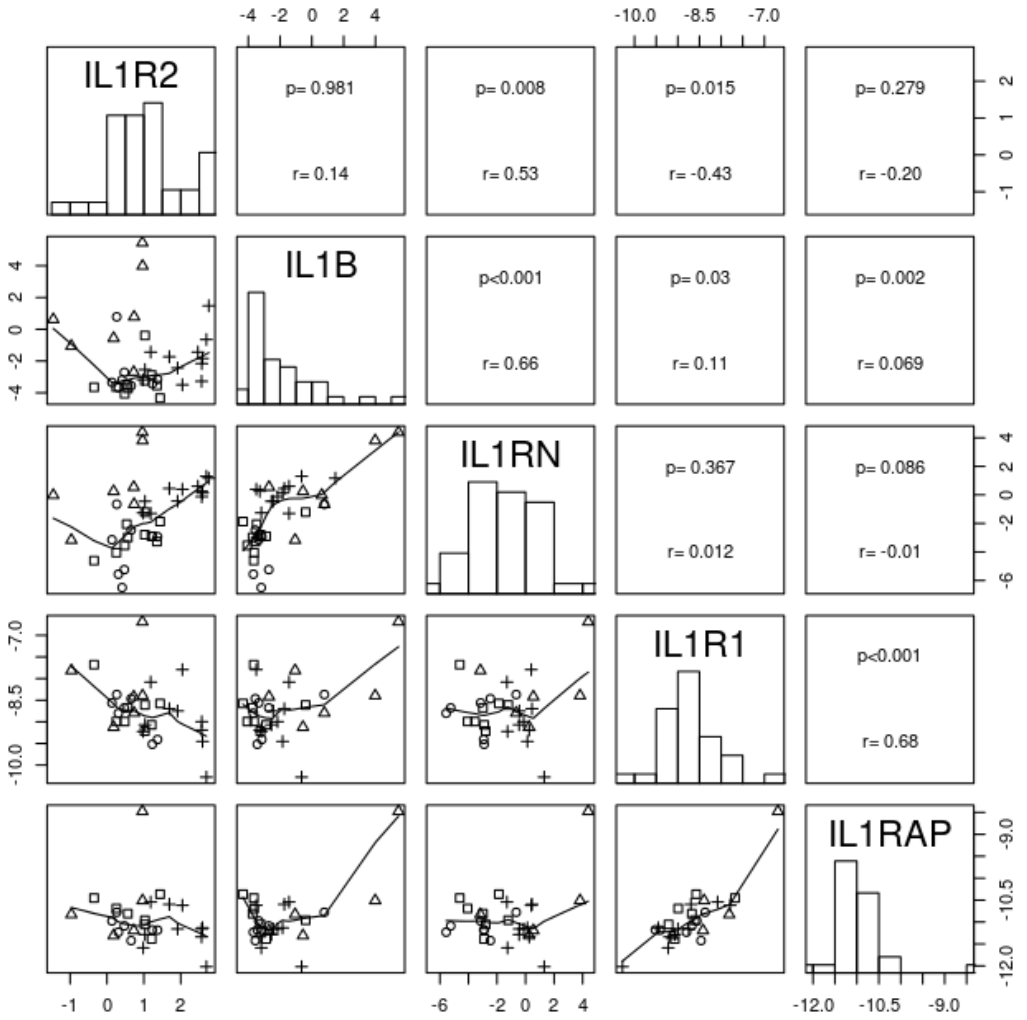


Figure 11. Correlation analysis between IL1-family genes. The distribution of each gene's expression is shown in the diagonal of the matrix. The right side panels show the results of the Spearman correlation test for each combination; the rho and p-values are shown. The left side panels show the dot plot (-Delta Ct) of each combination highlighting the sample; non-IBD control samples are represented with squares, non-involved active UC samples with circles, involved active UC samples with triangles and inactive UC samples with plus symbol (+). The lowess non-parametric regression curve is represented in each case.

Given that CD is also a chronic inflammatory disease of the intestine we tested whether these same alterations in some members of the IL-1 family occurred in the colonic mucosa of CD patients (Table 5). Despite showing increased *IL1B* transcription in the inflamed mucosa, CD patients did not up-regulate expression of *IL1R2* during

remission (Figure 12), suggesting that the changes observed here were exclusive to the remitting UC mucosa.

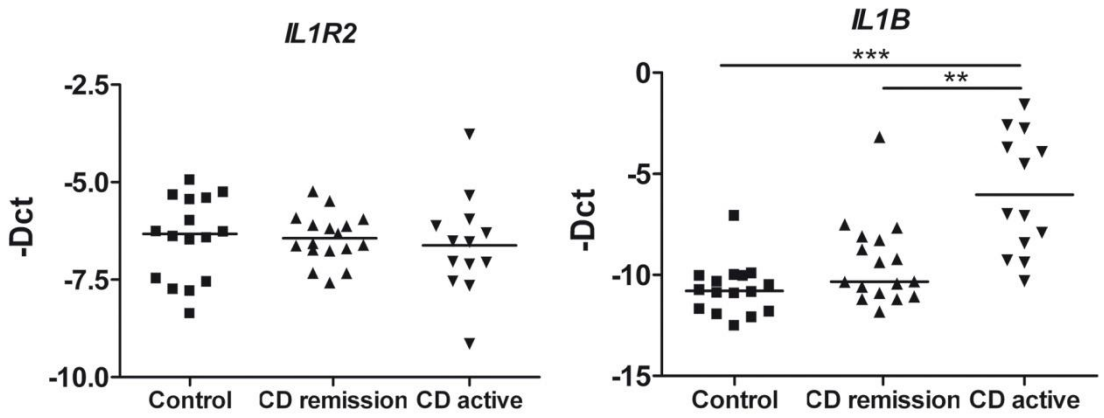


Figure 12. *IL1R2* and *IL1B* expression in colonic mucosa from Crohn's disease (CD) patients. mRNA expression as determined by qPCR (-Delta Ct) in controls (n=16), CD patients in remission (n=16), and CD patients with active disease (n=13). Gene expression data was analyzed by a Kruskal-Wallis test, followed by a Benjamini-Hochberb post-hoc correction test. $^{**}P<.005$, $^{***}P<.0005$.

2.2. Soluble IL-1R2 secretion is elevated in the involved mucosa of UC patients in remission

Next we measured protein secretion of soluble IL-1R2 and other IL-1 family proteins in UC patients. First, we tested serum levels of IL-1β and soluble IL-1R2. Although samples from patients with UC in remission showed a trend towards containing higher concentrations of the soluble receptor, we found no significant differences either in UC or in CD patients (Patient group 2, Table 4; Figure 13).

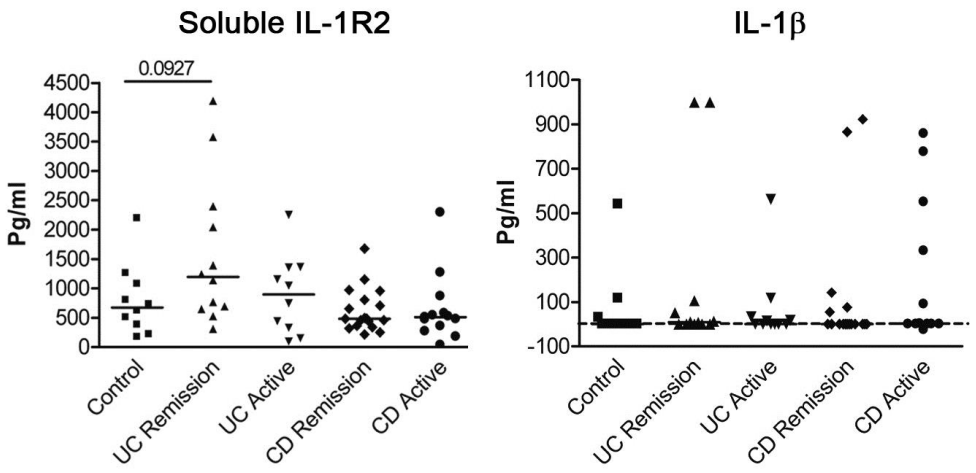


Figure 13. Concentration of serum soluble IL-1 receptor type 2 and IL-1β. Samples from non-IBD control subjects (n=10), patients with ulcerative colitis (UC) disease in remission (n=12), active UC patients (n=10), Crohn's disease (CD) patients in remission (n=18) and patients with active disease (n=13). Mann-Whitney p value from control vs. UC remission is shown.

Second, we measured protein secretion by UC patient tissues. Consistent with the transcriptional profile, secretion of IL-1β, soluble IL-1RAcP and IL-1Ra was significantly higher in cultured biopsies from the involved colonic mucosa of UC patients with active disease compared with all the other conditions (Patient group 3, Table 4; Figures 14a, 14c and 14d). Remarkably, soluble IL-1R2 was found at significantly higher concentrations in the supernatants of cultured mucosal samples from UC in remission compared to active disease, segments uninvolved by disease, and healthy mucosa (Figure 14b). Soluble IL-1R1, however, was not detected in the biopsy culture supernatants of any patient group, suggesting that the concentrations of soluble IL-1R1 were under the limit of detection of the ELISA used or that its expression was limited to the membrane-bound form in colonic mucosa.

Thus, while we measured a significant increase in the transcription levels of both IL-1 blocking genes (*IL1RN* and *IL1R2*) in remission, increased protein secretion was only detected in the case of IL-1R2 (Figure 14d).

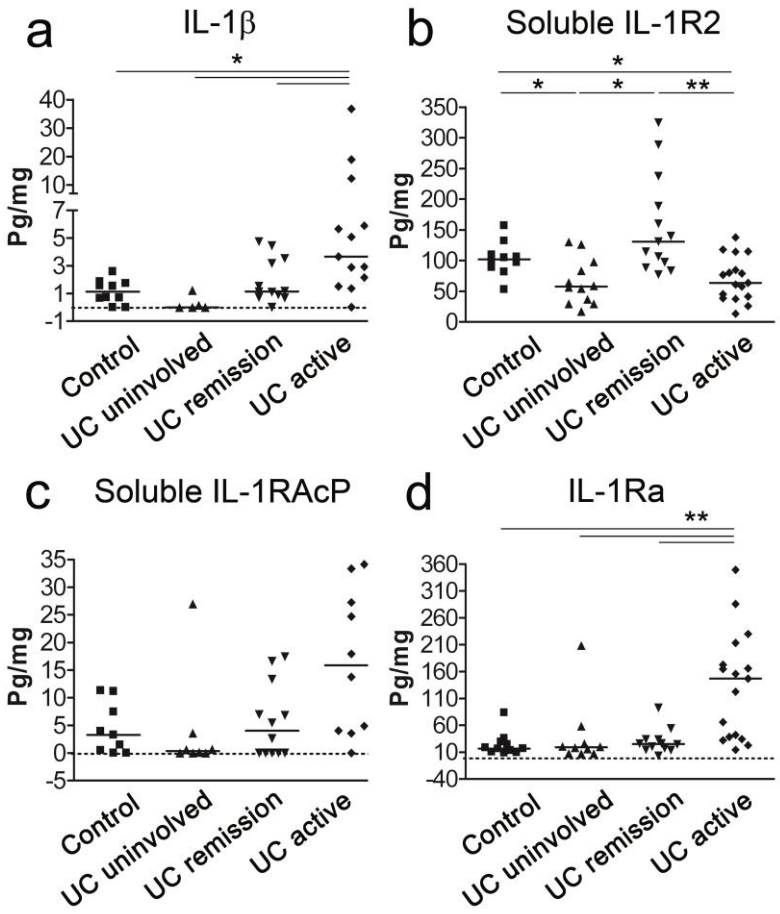


Figure 14. Secretion of selected IL-1 family proteins in colonic mucosa. (a-d) Dot plot representation (line = median) of secretion of IL-1β, soluble IL-1R2, soluble IL-1RAcP and IL-1Ra in 24-hour culture media of colonic biopsies from control (n =10), uninvolved areas from patients with ulcerative colitis (UC) (n = 11), UC patients in remission (n=13) and active UC disease mucosa (n =17). Protein secretion data analyzed by a Kruskal-Wallis test, followed by a Benjamini-Hochberb post-hoc correction test. $^{*}P<.05$, $^{**}P<.005$.

3. IL-1R2 cellular sources in the intestinal mucosa

3.1. Lamina propria plasma cells and intestinal epithelial cells express IL-1R2

As our next goal was to identify the cellular source of IL-1R2 in the intestinal mucosa, we carried out immunofluorescence analysis of colonic tissues.

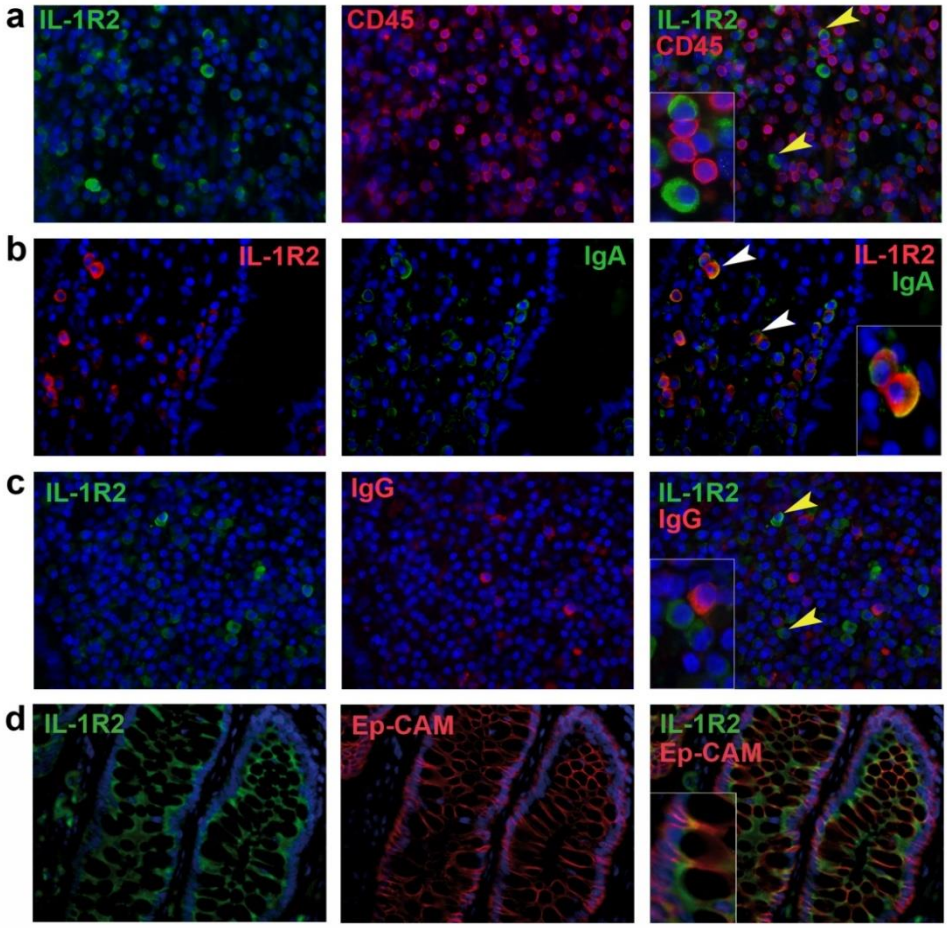


Figure 15. Immunofluorescent staining of intestinal samples shows lamina propria plasma cells and epithelial cells express IL-1R2 in healthy mucosa. Representative two-color immunofluorescent staining of fixed paraffin-embedded healthy colonic tissue. (a) Samples were co-stained with anti-IL-1R2 (green) and anti-CD45 (red). Yellow arrows show IL-1R2 expression by non-$CD45^+$ cells. (b) Samples were co-stained with anti-IL-1R2 (red) and anti-IgA (green). White arrows show IL-1R2 expression by IgA^+ cells. (c) Samples were co-stained with anti-IL-1R2 (green) and anti-IgG (red). Yellow arrows show IL-1R2 expression by non-IgG^+ cells. (d) Samples were co-stained with anti-IL-1R2 (green) and anti-Ep-CAM (red). Sections were counterstained with DAPI (blue). Images were taken using a 40X objective lens.

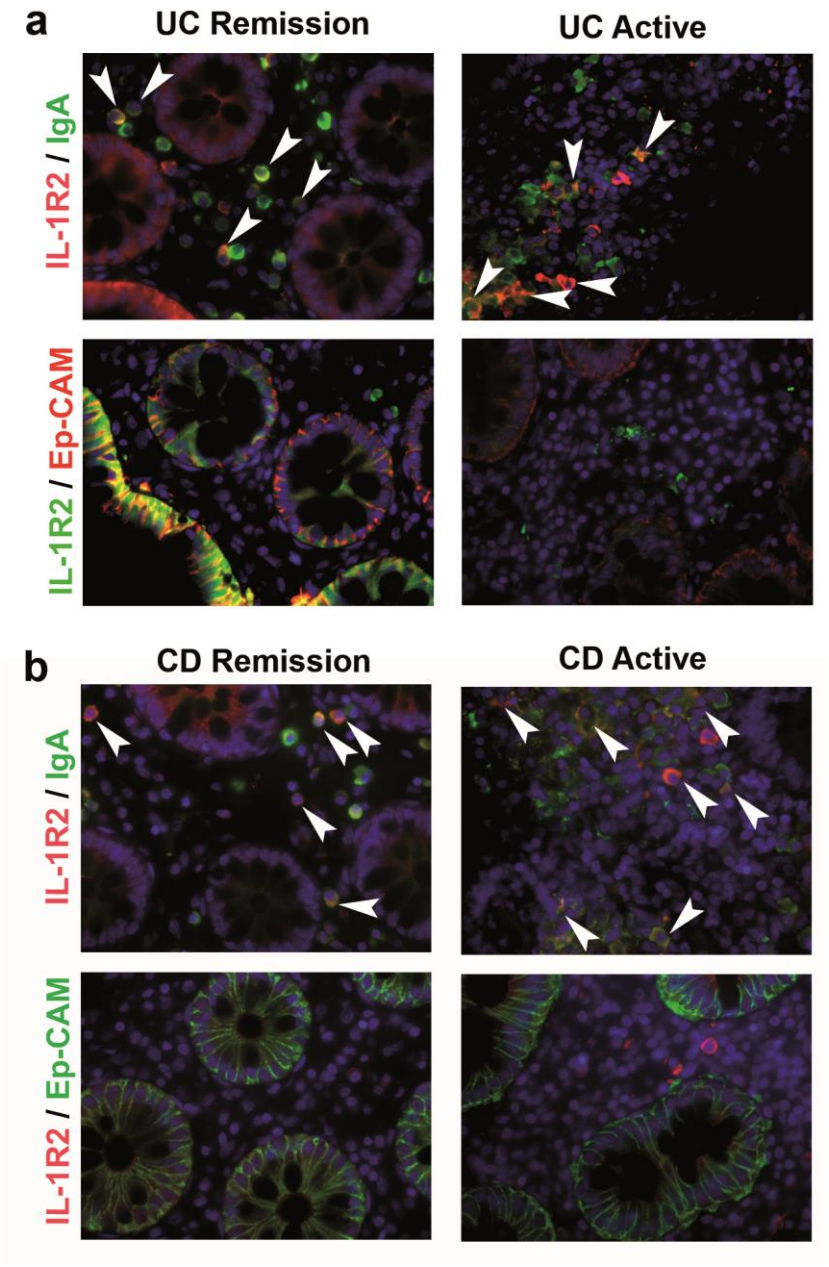


Figure 16. Immunofluorescent staining of intestinal samples shows lamina propria plasma cells and epithelial cells express IL-1R2 in IBD patient mucosa. (a) Fixed paraffin-embedded involved mucosal sections of ulcerative colitis (UC) patients in remission and with active disease. Samples were co-stained with anti-IL-1R2 (red) and anti-IgA (green) and co-stained with anti-IL-1R2 (green) and anti-Ep-CAM (red). (b) Fixed paraffin-embedded mucosal sections of Crohn's disease (CD) patients in remission and with active disease. Samples were co-stained with anti-IL-1R2 (red) and IgA (green) and co-stained with anti-IL-1R2 (red) and anti-Ep-CAM (green). Sections were counterstained with DAPI (blue). White arrows show IL-1R2 expression by IgA^{+}. Images were taken with a 40X objective lens.

Cells marked positively for IL-1R2 could be visualized both within the lamina propria and in the adjacent mucosal Ep-CAM$^+$ epithelium in non-IBD controls, UC and CD patients (Figures 15 and 16). Surprisingly, all IL-1R2$^+$ cells within the lamina propria showed negative (or weak) CD45 staining in healthy mucosa (Figure 15a).

It has been described that terminally differentiated plasma cells down-regulate expression of the hematopoietic marker CD45[267]. Indeed, all IL-1R2$^+$ cells within the lamina propria of healthy (Figure 15b), UC, and CD patients (Figure 16) were identified as immunoglobulin A (IgA)$^+$ plasma cells. No co-localization of IL-1R2 and immunoglobulin G (IgG) was observed (Figure 15c).

3.2. Increased numbers of epithelial cells express IL-1R2 in UC patients in remission

To identify which of the two cellular subsets, plasma cells or epithelial cells, contributes to increased IL-1R2 expression in UC remission, we first quantified the number of IL-1R2$^+$ cells within the IgA$^+$ lamina propria population via immunofluorescence staining of the colon tissue. Due to massive cell infiltration and tissue destruction in the inflamed mucosa of UC active patients, we could not reliably quantify the number of positively stained cells in these patients. While the total number of IgA$^+$ cells was significantly higher in the lamina propria of non-IBD control samples compared to UC in remission, about 50% of the IgA$^+$ population expresses IL-1R2 both in controls and in samples from patients with UC in remission (Patient group 4, Table 4; Figure 17).

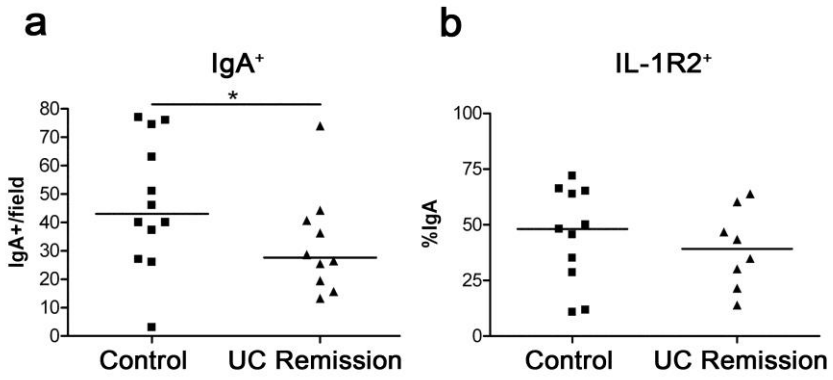


Figure 17. Immunofluorescence quantification. (a) Immunostaining quantification of IgA^+ cells per field under a 20X objective lens. (b) Percentage of $IL\text{-}1R2^+$ among IgA^+ cells in healthy control mucosa (n=12) and in UC in remission mucosa (n=10). Data was analyzed using a Mann-Whitney test. $^*P<.05$.

Alternatively, we quantified IL-1R2 production by the epithelial compartment ($CD45^-Ep\text{-}CAM^+$) in colonic samples using flow cytometry (Patient group 5, Table 4; Figure 18a). Although no surface receptor was detected, an intracellular IL-1R2 could be clearly detected within epithelial cells. Remarkably, samples from the involved mucosa of patients with UC in remission showed a significantly higher percentage of $IL\text{-}1R2^+$ cells among $CD45^-Ep\text{-}CAM^+$ epithelial cells compared to control, uninvolved areas of patients with UC, and active UC samples (Figure 18b).

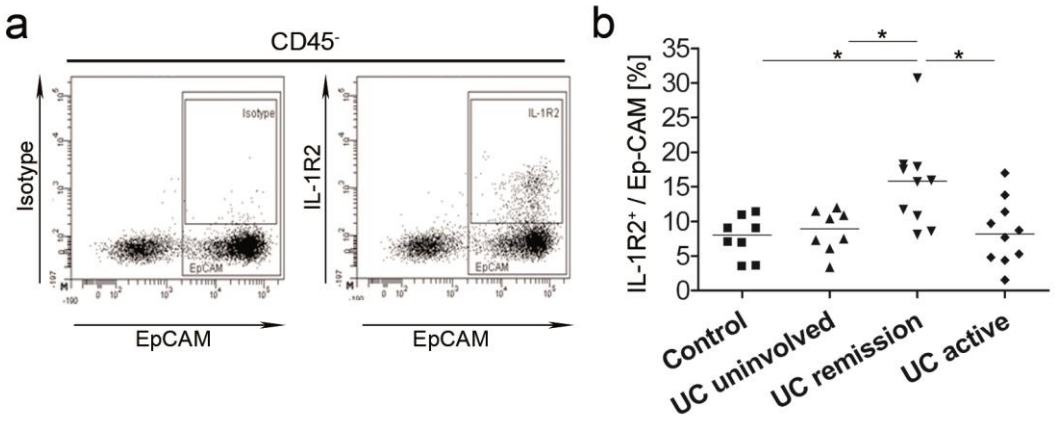


Figure 18. IL-1R2 is overexpressed by epithelial cells from UC patients in remission. (a) Representative flow cytometry dot plots from digested biopsies. (b) Dot plot representation (line = median) of the percentages of intracellular IL-1R2 staining among $Ep\text{-}CAM^+$ from $CD45^-$ cells, from controls (n=8), uninvolved mucosa from ulcerative colitis (UC) patients (n=8), mucosa from patients in UC remission (n=10), and active UC patients (n=10). Data was analyzed by a Kruskal-Wallis test, followed by a Benjamini-Hochberb post-hoc correction test. $^*P<.05$.

Since epithelial cells are responsible for the transport of molecules from the lamina propria to the lumen, such as IgA complexes, we performed an *in situ* hybridization to rule out the possibility that the intracellular IL-1R2 staining detected in epithelial cells was transcytosed protein produced within the lamina propria. *In situ* staining for *IL1R2* transcripts in colonic lamina propria sections confirmed ongoing *IL1R2* transcription by epithelial cells (Figure 19). Based on these findings, we believe that increased expression of IL-1R2 in the involved mucosa of UC patients in remission is, at least in part, due to enhanced production of the decoy receptor by epithelial cells.

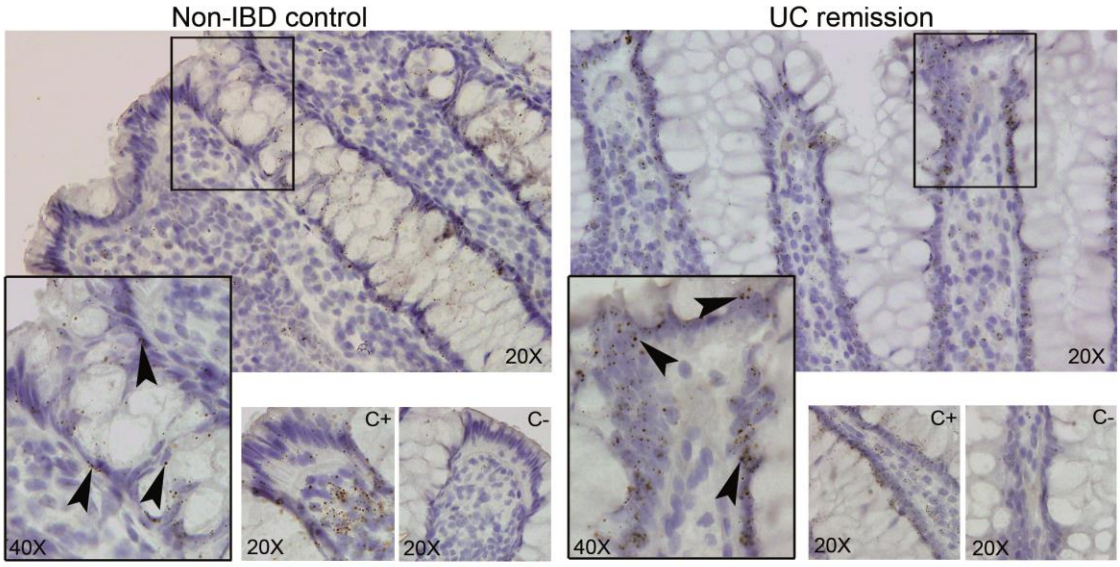


Figure 19. *In situ* hybridization of *IL1R2* transcripts in colonic lamina propria from a control (non-IBD) and an ulcerative colitis (UC) patient in remission. Sections were counterstained with hematoxylin. Images were taken with 20X and 40X objective lenses. Hs-PPIB probe as a positive control (C+) and DapB probe as a negative control (C-).

4. Epithelial IL-1R2 expression regulation

4.1. *IL1R2* is up-regulated upon differentiation of intestinal epithelial cells

Given the increase in colonic epithelial IL-1R2 expression in remission, we sought to determine which pathway drives its expression in this cell population. Immunohistochemical analysis of colonic mucosa revealed a gradient of IL-1R2 expression along the epithelial crypt. Contrary to staining of EphB2, a stem cell marker, more intense IL-1R2 expression was seen at the top of the colonic crypts, where the differentiated epithelial cell compartment (surface epithelium) is localized (Figure 20). This suggests that expression of IL-1R2 may be regulated during epithelial cell differentiation.

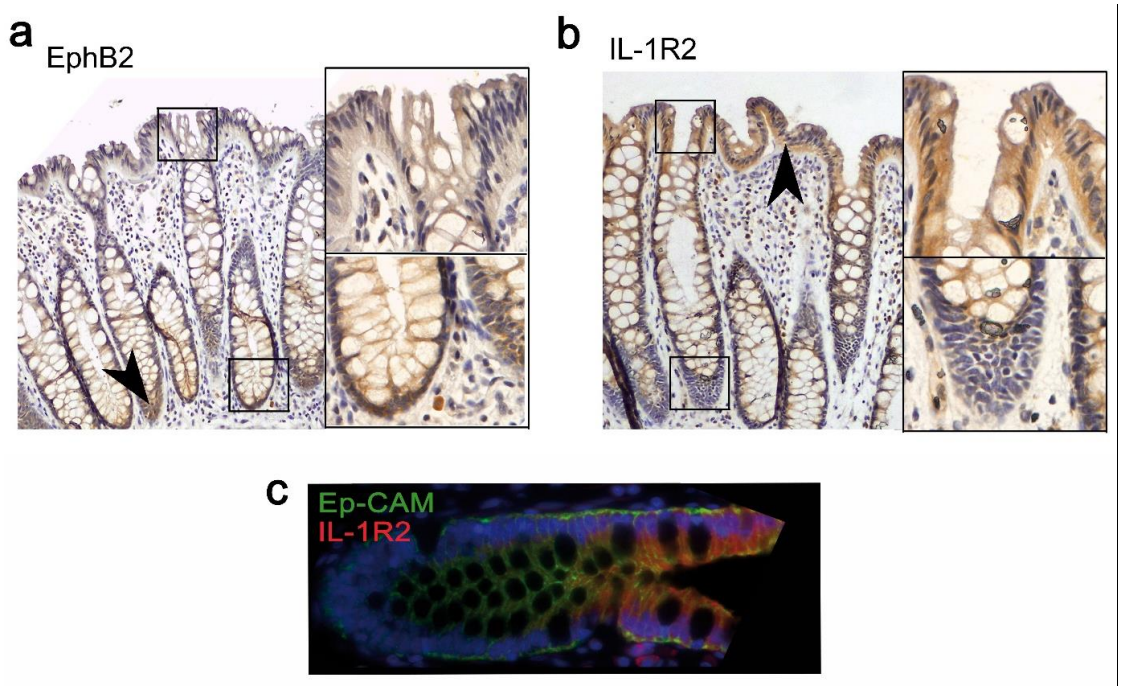


Figure 20. IL-1R2 is overexpressed by differentiated epithelial cells. (a) Representative immunohistochemical staining with anti-EphB2 and (b) anti-IL-1R2 from control colonic tissue. Sections were counterstained with hematoxylin. Images were taken with 20X and 40X objective lenses. Black arrows highlight the more intensely stained areas of the crypt. (c) Representative crypt immunofluorescence staining with anti-Ep-CAM (green) and anti-IL-1R2 (red) taken from a colon sample from a patient with ulcerative colitis in remission. Section was counterstained with DAPI (blue). The picture is taken using a 40X objective lens.

In order to confirm this hypothesis, we used a primary epithelial organoid culture of CoSCs expanded from healthy colonic mucosa. *In vitro* grown CoSCs can be induced to differentiate into epithelial cells by removing Wnt/β-catenin activating signals from the culture media[266].

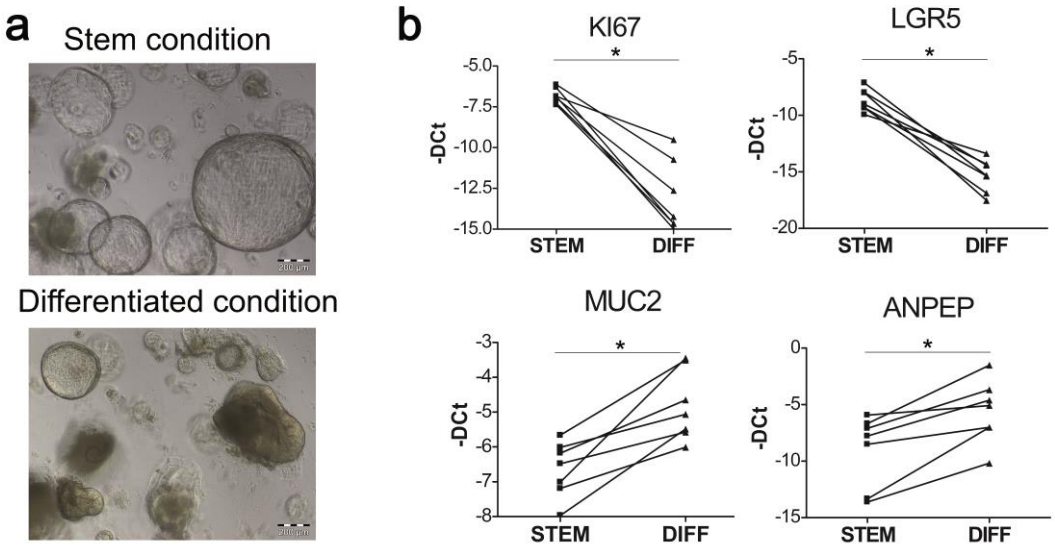


Figure 21. *In vitro* organoid culture of colonic epithelial stem cells. (a) Representative picture of stem and differentiated organoids in culture. (b) *KI67*, *LGR5*, *MUC2* and *ANPEP* gene expression by qPCR (-Delta Ct) of stem and differentiated organoids (n=7). Gene expression data was analyzed using a Wilcoxon matched pair test. $^{*}P<.05$.

As shown in Figure 21, colonic stem cells in culture show changes in morphology (Figure 21a) and down-regulate expression of stem cells markers, such as *LGR5,* and the proliferation marker (*KI67*) in the presence of differentiation medium (Figure 21b). Concomitantly, expression of epithelial differentiation markers, such as *MUC2* highly expressed by goblet cells and the enterocyte-expressed gene *ANPEP,* are markedly up-regulated (Figure 21b). Using this culture system, we show that both transcription of the *IL1R2* gene, as well as secretion of soluble IL-1R2 protein, are significantly increased upon CoSCs differentiation *in vitro* (Figure 22).

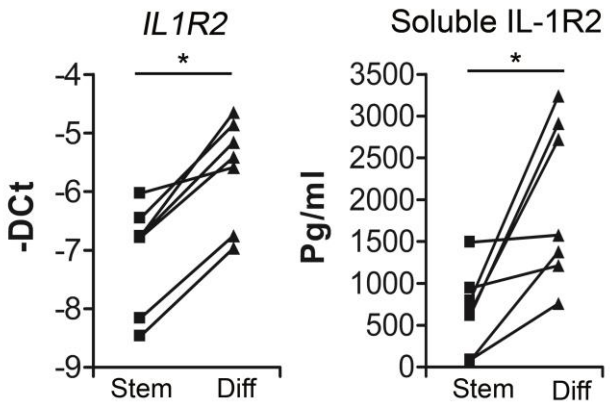


Figure 22. IL-1R2 is overexpressed by differentiated epithelial cells. *IL1R2* gene expression by qPCR (-Delta Ct) and soluble IL-1R2 concentration (determined by ELISA) from cultured stem and differentiated (Diff) epithelial cells (n=7). IL1R2 expression data was analyzed by a Wilcoxon matched paired test. *$P<.05$

4.2. Wnt/β-catenin signaling controls *IL1R2* up-regulation during epithelial cell differentiation

We next tested the action of IL-1β on CoSCs, described as an inducer of IL-1R2 in other cell types[268]. In the intestinal mucosa IL-1β can be produced by immune and stromal cells within the lamina propria, although whether epithelial cells are able to synthesize it remains controversial[269]. Given that we could detect no IL-1β production by colonic stem cells in culture, we added exogenous IL-1β to our system. As previously shown (Figures 21 and 22), removal of the Wnt signaling components from the stem medium (Wnt3a and R-spondin-1) resulted in a marked increase in *IL1R2* expression, as well as a decrease in the expression of the Wnt pathway target gene *AXIN2*. IL-1β stimulation, however, did not induce changes in the decoy receptor transcripts. Additionally, we tested the expression of *IL1R1,* a gene that is neither regulated by Wnt nor by IL-1β. Indeed, we found no changes in its expression under any conditions (Figure 23).

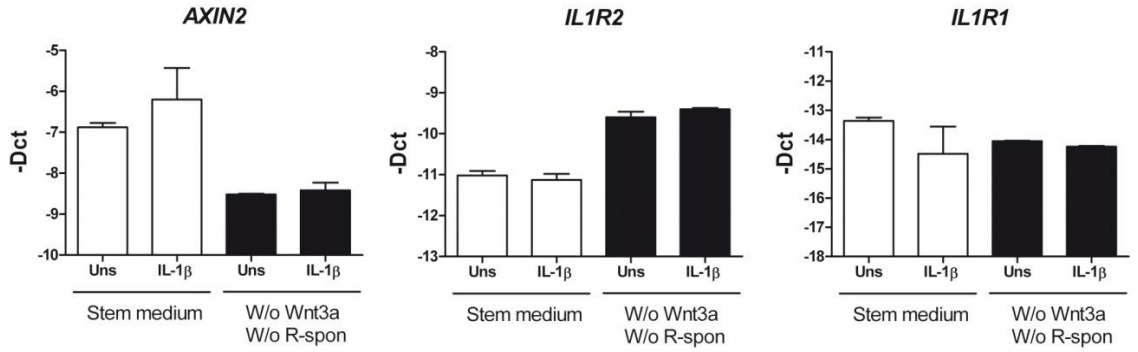


Figure 23. IL-1β did not regulate IL-1R2 expression in an *in vitro* organoid culture of colonic epithelial stem cells. *AXIN*, *IL1R2 and IL1R1* gene expression by qPCR (-Delta Ct) of stem organoids in stem medium or in stem medium without (W/o) Wnt3a and R-spondin-1 (R-spon). Unstimulated (Uns) condition and stimulation with IL-1β 5ng/ml overnight (n=4).

These results suggest that Wnt/β-catenin signals, which are required for the survival and proliferation of the stem cell compartment, could be repressing *IL1R2* transcription and protein secretion. To further test this, we used isolated whole intestinal crypts cultured in the presence of a Wnt/ β-catenin agonist (Chir-99021, an inhibitor of GSK3)[270]. Figure 24a show the increased transcription of the β-catenin target gene *AXIN2* and the proliferation marker *KI67* in intestinal crypts incubated with the Wnt/β-catenin pathway inducer Chir-99021. Concomitantly, transcription of *IL1R2*, as well as protein secretion of IL-1R2, was decreased in a dose-dependent manner (Figure 24b). In contrast, expression of the IL-1R antagonist transcript *IL1RN* was not influenced by Chir-99021 (Figure 24a). Overall, we demonstrate that IL-1R2 is suppressed by Wnt/β-catenin-dependent signals and therefore its expression is up-

regulated upon epithelial stem-cell differentiation.

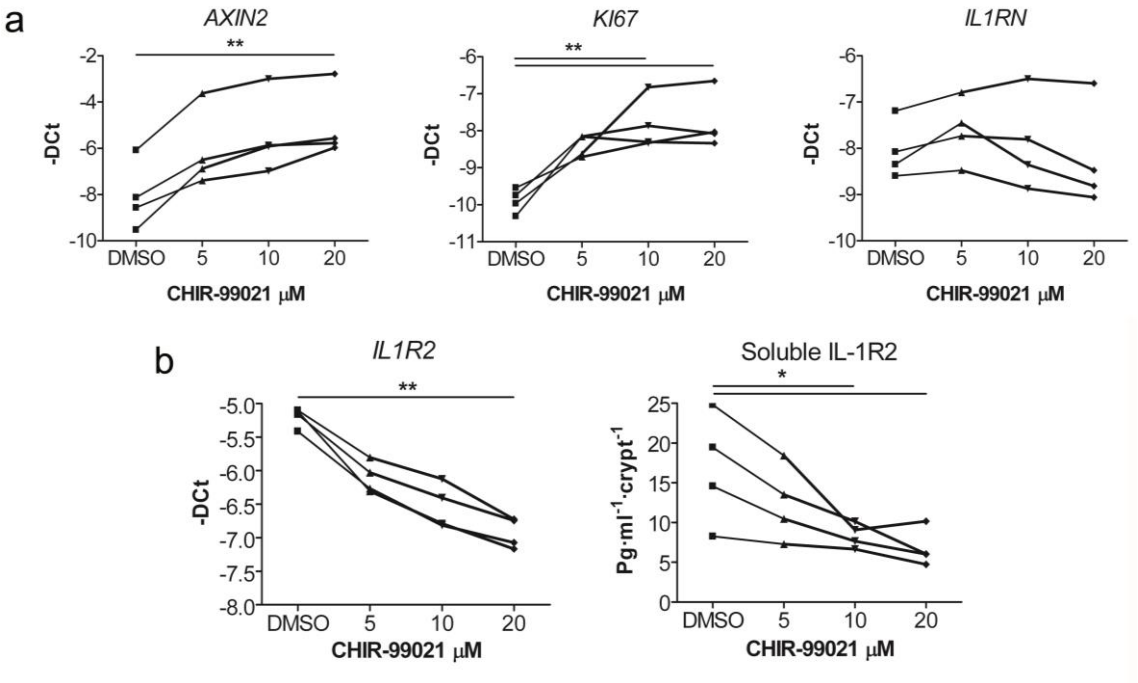


Figure 24. IL-1R2 is suppressed by Wnt/β-catenin-dependent signals. *KI67*, *AXIN2* and *IL1RN* gene expression by qPCR (-Delta Ct) of intestinal crypts stimulated with Chir-99021 (n=4). (d) *IL1R2* gene expression by qPCR (-Delta Ct) and soluble IL-1R2 concentration (determined by ELISA) from intestinal crypts stimulated overnight with Chir-99021. Gene expression data was analyzed by using the Friedman test, followed by the Nemenyi post-hoc test.***P<.05,* ***P<.005.*

Using the whole biopsy transcriptional data[†], we analyzed a set of genes involved in the canonical Wnt/β-catenin pathway in colonic biopsies from UC patients in remission compared to active disease, uninvolved areas of the proximal colon, as well as healthy controls (Figure 25). Overall, analysis of the transcriptional profile showed a deregulation of Wnt-related genes in samples from UC patients compared to controls. Remarkably, some genes altered in UC active patients remained deregulated in UC patients in remission (e.g., up-regulation of *DKK3*, which antagonizes canonical Wnt signaling) (Figure 26). Moreover, we could detect other genes that were up-regulated in patients with UC in remission compared to control samples (e.g., *SFRP1,* a secreted Wnt antagonist) (Figure 26).

[†] (GSE38713 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38713)

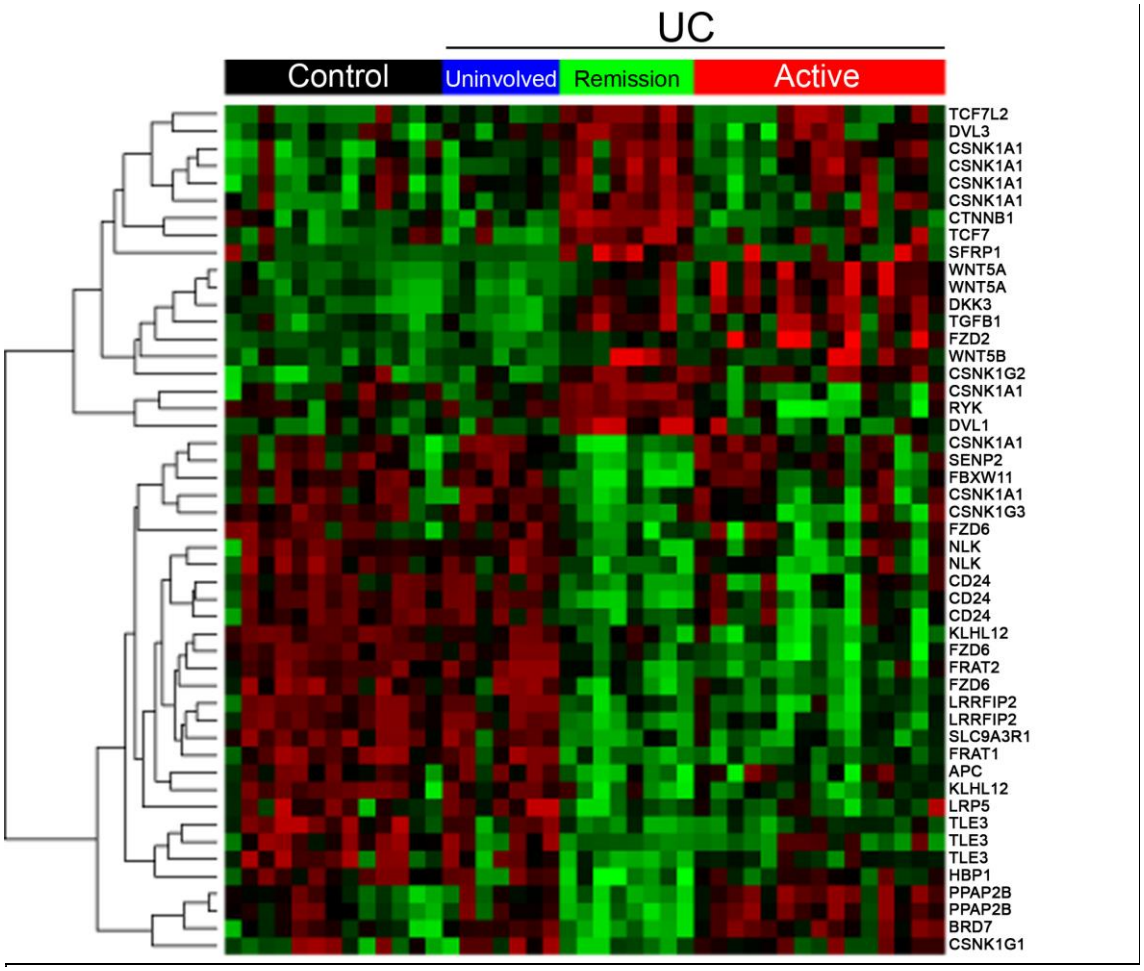


Figure 25. Heatmap representation of microarray expression of Wnt/β-catenin related genes. Each row shows one individual probe (representing 33 selected genes, 50 different probes) and each column an experimental sample. Higher relative expression levels are shown in red and low expression levels in green. An unsupervised hierarchical cluster method, using a Pearson distance and average linkage method, was applied for each gene classification. Samples belonged to one of the following groups: non-IBD controls (shown in black, n=13), non-involved mucosa segments from patients with active UC (UC uninvolved; in blue, n=7), involved mucosa segments from patients with active UC (UC active; in red n=15) and endoscopically and histologically inactive UC (UC remission; in green, n=8).

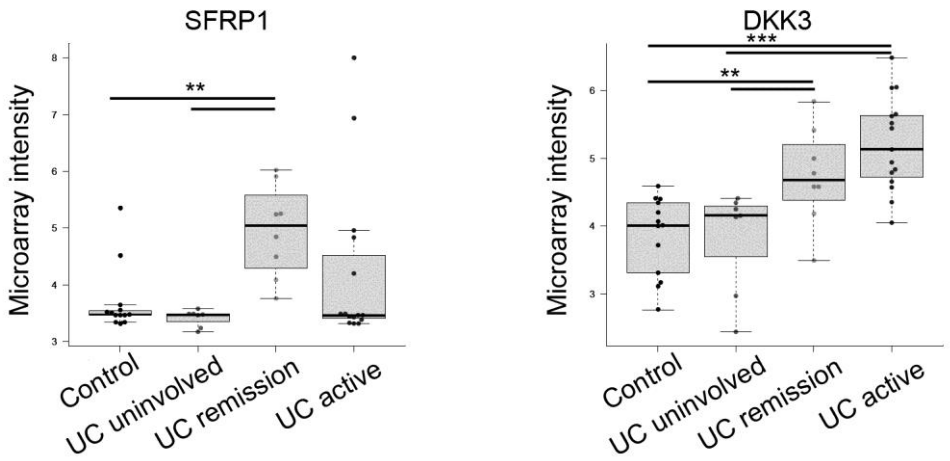


Figure 26. Transcriptional analysis of colon biopsies from microarray data. Microarray intensity representation of *SFRP1* and *DKK3* from non-IBD controls (n=13), non-involved mucosa segments from patients with active UC (UC uninvolved; n=7), involved mucosa segments from patients with active UC (UC active; n=15) and endoscopically and histologically inactive UC (UC remission; n=8). Gene expression data analyzed by a Kruskal-Wallis test, followed by a Benjamini-Hochberb post-hoc correction test. $^{**}P<.005$, $^{***}P<.0005$.

5. IL-1R2 functional significance during remission of UC

5.1. IL-1R2 protein partially prevents chemokine production induced by IL-1β on intestinal crypts

Next, we tested whether epithelial cell-released IL-1R2 could interfere with the effect of IL-1β on the intestinal epithelium. To this end we stimulated intestinal crypts isolated from biopsies (Figure 27a) of healthy colonic mucosa or involved areas of UC patients in remission (Patient group 6, Table 4) with low dose IL-1β (0.1 ng/ml). Using these conditions we assessed the effect of an IL-1R2 blocking antibody on the expression of *CXCL1*, *CXCL2*, CXCL8, *CCL20*, *TNFA*, and *IL6*, pro-inflammatory chemokines highly up-regulated in active UC[47-49]. Transcripts for *IL6* and *TFNA* were not detectable in isolated crypts whether resting or when activated with IL-1β. In contrast, low doses of IL-1β induced enhanced transcription of *CXCL1*, *CXCL2, CCL20*, and *CXCL8* (Figure 27b), and CCL20 production (Figure 27c) in colonic crypts from control or UC samples.

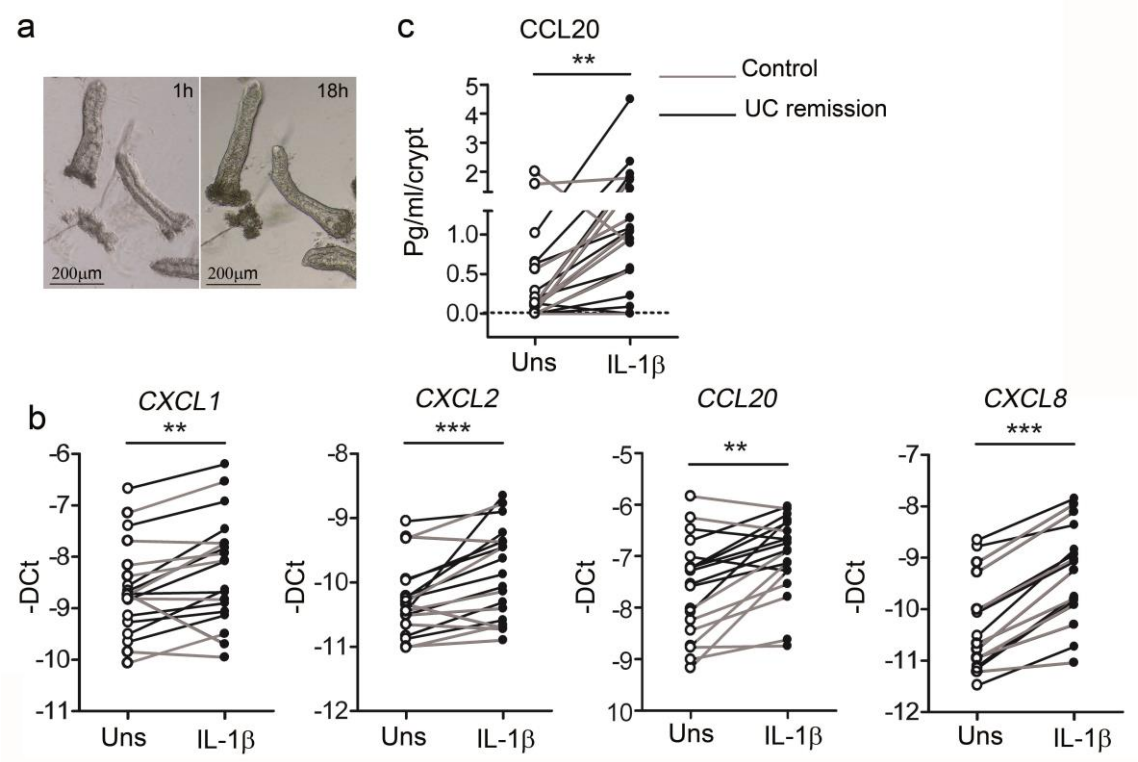


Figure 27. IL-1β induces chemokine production on intestinal isolated crypts in culture. *(Figure legend continues)*

(a) Representative image of matrigel-embedded whole intestinal crypts from colonic biopsies cultured for 18h. (b) *mRNA expression of CXCL1, CXCL2, CCL20,* and *CXCL8* as determined by qPCR (-Delta Ct) in colonic isolated crypts from controls (n=9) and UC patients in remission (n=10), stimulated with IL-1β. (c) CCL20 secretion determined by ELISA in colon-isolated crypts from controls (n=9) and UC patients in remission (n=10) stimulated with IL-1β. Data was analyzed using a Wilcoxon matched paired test. ***P<.005, ****P<.005.*

Importantly, addition of an anti-IL-1R2 blocking antibody significantly increased IL-1β induced transcription of *CXCL1*, *CXCL2* and *CCL20* in samples from UC patients in remission (Figure 28a). CCL20 protein secretion upon IL-1β stimulation by intestinal crypts from UC patients in remission was also significantly increased by blocking IL-1R2 (Figure 28b). Although a similar pattern was observed in intestinal crypts from controls, the differences did not reach statistical significance (Figure 28a and 28b).

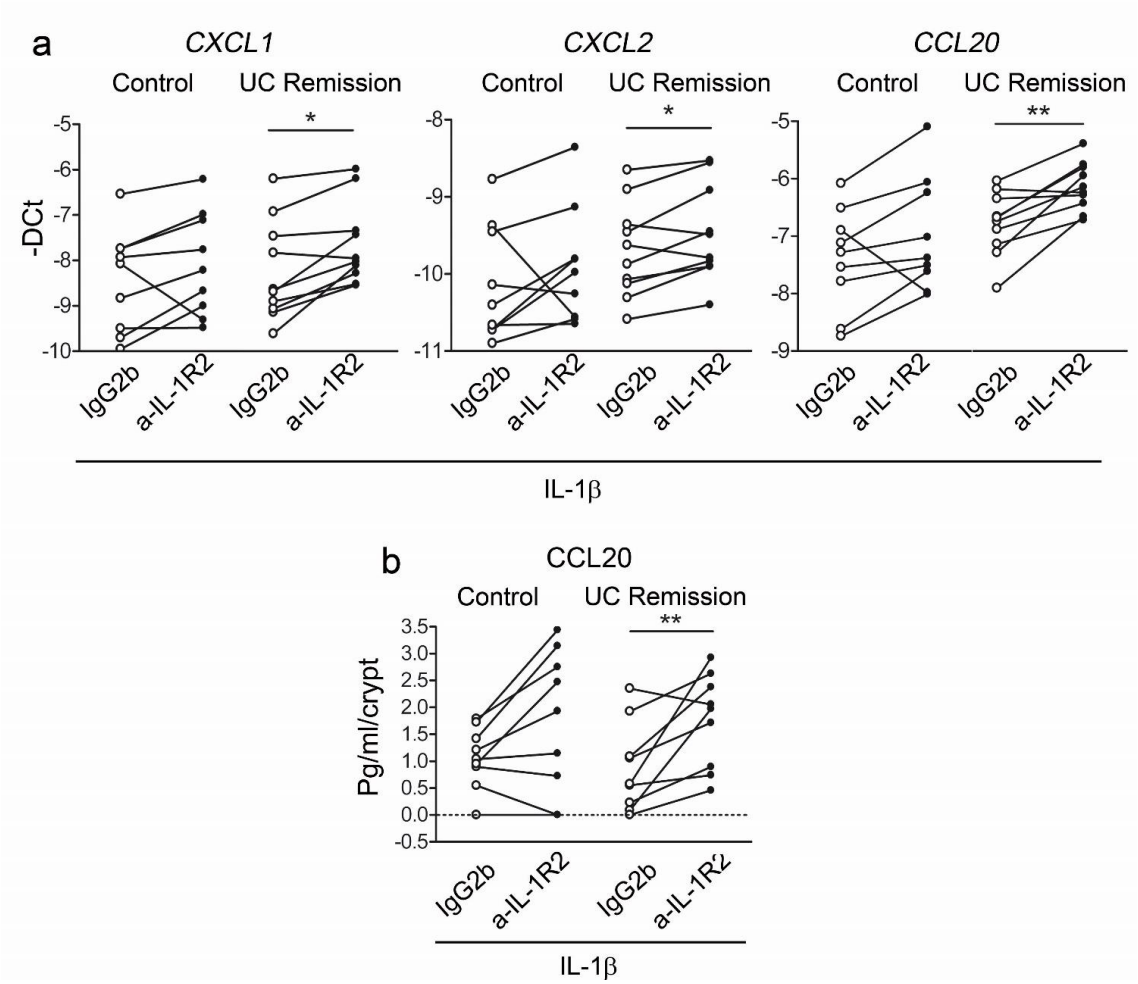


Figure 28. IL-1R2 protein partially prevents chemokine production induced by IL-1β on intestinal crypts. *(Figure legend continues)*

(a) *mRNA expression of CXCL1, CXCL2, CCL20,* and *CXCL8* as determined by qPCR (-Delta Ct) in colon-isolated crypts from controls (n=9) and UC patients in remission (n=10) stimulated with IL-1β and with IL-1R2 blocking antibody (rat anti-hIL1RII-M22) or isotype control (rat IgG2b). (b) CCL20 secretion determined by ELISA in colon-isolated crypts from controls (n=9) and UC patients in remission (n=10) stimulated with IL-1β and with IL-1R2 blocking antibody (rat anti-hIL1RII-M22) or isotype control (rat IgG2b). Data was analyzed using a Wilcoxon matched paired test. **P<.05, **P<.005, ***P<.005.*

5.2. IL-1R2 produced by UC in remission mucosa prevents IFN-γ production by activated T cells

Next, we tested whether IL-1R2 secreted by the intestinal epithelium could act on neighboring immune cells. Th17/Th1 cells have been identified in the mucosa of active UC patients[86]. Recent evidence suggests that IL-1β induces human IL-1R1$^+$ Th17 cells to up-regulate IFN-γ production, thereby giving rise to Th17/Th1 cells with a potentially pathogenic profile[271]. In order to generate cells with that phenotype, we stimulated CD4$^+$ lymphocytes with *Candida albicans.* After expansion with yeast, about 30-40% of CD4$^+$ cells produced IFN-γ (Figure 29a). Expanded T cells were stimulated with biopsy supernatant from controls and UC patients in remission (Patient group 7, Table 4). The effect of an anti-IL-1R2 blocking antibody on IL-17 and IFN-γ secretion was then determined. Blocking IL-1R2 in supernatants from UC patients in remission induced an increase in the secretion of IFN-γ, but not IL-17, which was significantly different to the effect observed when using supernatants from control biopsies (Figure 29b). These results suggest that IL-1R2 endogenously produced by UC patients in remission acts by partially inhibiting the effects of IL-1β on activated T cells.

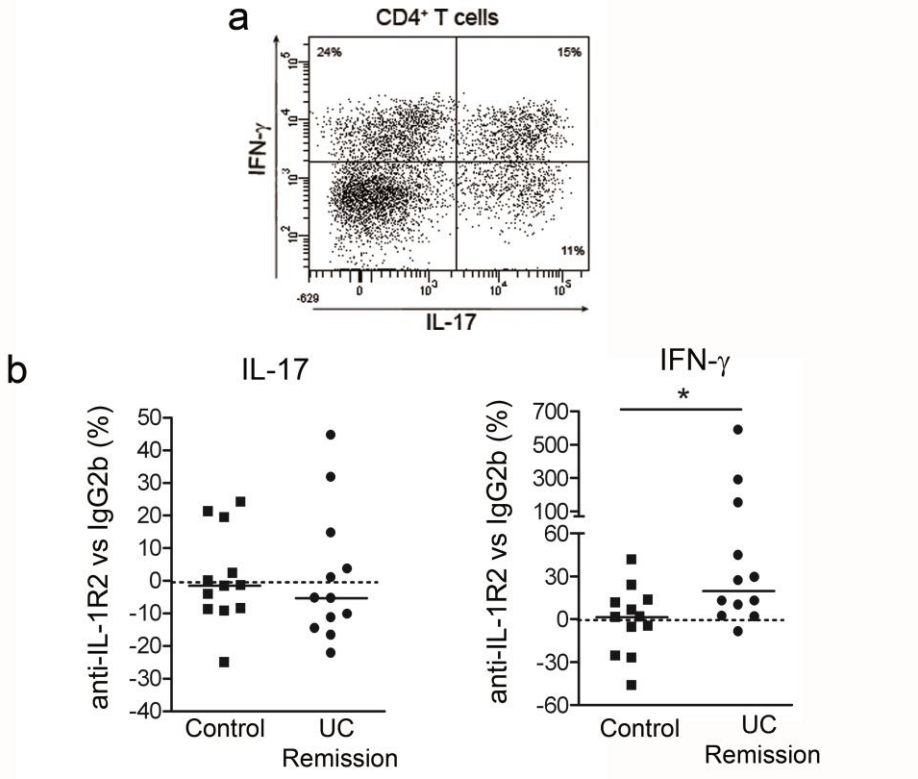


Figure 29. IL-1R2 produced by UC in remission mucosa prevents IFN-γ production by *C. albicans*-expanded CD4$^+$ T cells. (a) Representative flow cytometry dot plot from CD4$^+$ cells expanded with *C. albicans* and autologous CD14$^+$ monocytes. Intracellular staining of IFN-γ and IL-17 is shown (b) *C. albicans*-expanded CD4$^+$ T cells were cultured with supernatants from colonic biopsies (control; n=12 or UC remission; n=12) with IL-1R2 blocking antibody (rat anti-hIL1RII-M22) or the corresponding isotype control (rat IgG2b). IL-17 and IFN-γ secretion was measured in culture supernatants by ELISA. Protein expression is normalized relative (%) to the isotype control condition set at 0 (line = median). Data was analyzed using a Mann-Whitney test. $^*P<.05$.

5.3. Decreased *IL1R2* gene expression is associated with relapse of UC

Our final aim was to address whether IL-1R2 overexpression could be related to disease outcome. In order to test this possibility, we looked at *IL1R2* transcription in a cohort of patients (n=45) in endoscopic and histologic remission that were followed up for one year after having biopsies taken from the distal colon (Patient group 8, Table 4). Patients were classified into two groups, based on whether or not they relapsed during the 12-month follow-up. Relapse was confirmed by endoscopic and histological evaluations in patients presenting symptoms, and remission was assessed by

endoscopy and histology at the end of follow-up. At the time of inclusion, no significant differences existed between the two groups of patients regardless of age, gender, treatment, duration of disease or endoscopic Mayo subscore. In addition, expression of *CXCL8*, *CCL20*, *CXCL1* and *CXCL2* were low and comparable between the two groups (Figure 30a), supporting the complete remission and lack of ongoing subclinical inflammation at the time of inclusion. Remarkably, *IL1R2* transcription was significantly lower in the group of patients that relapsed during the follow-up period of 12 months compared with those patients that remained in endoscopic remission for the same amount of time (Figure 30b). Although the predictive value was low (AUC 0.673; 95% CI: 0.505-0.84), these data suggest that IL-1R2 may play a role in preventing disease relapse.

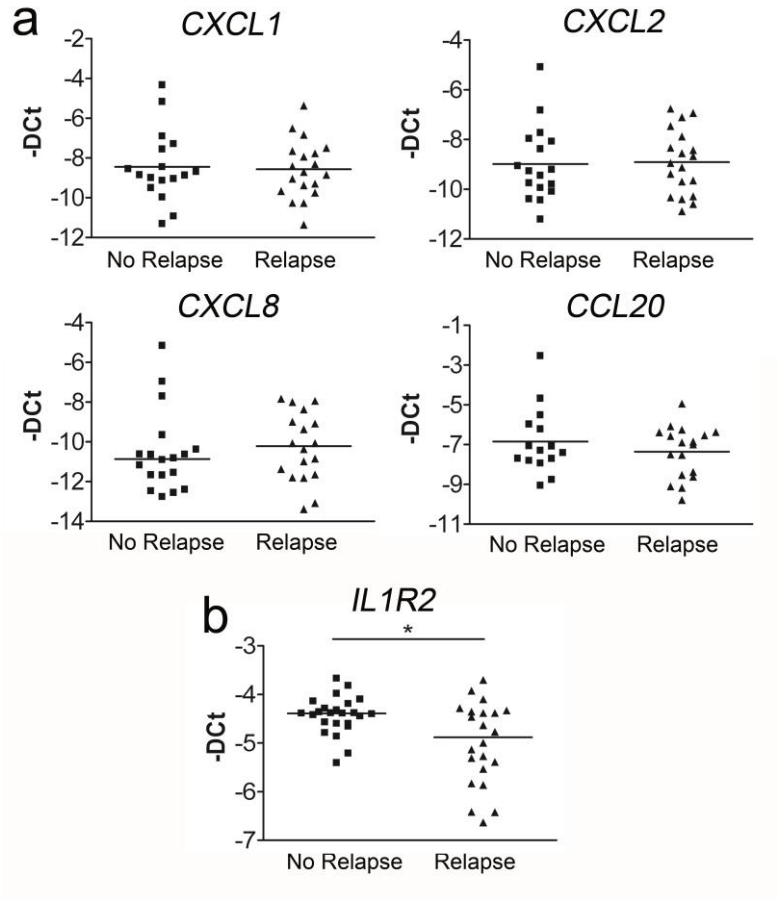


Figure 30. Lower *IL1R2* gene expression in ulcerative colitis patients in remission who relapsed during the following year. Intestinal mucosa (a)*CXCL1, CXCL2, CXCL8, and CCL20;* and (b) *IL1R2* gene expression by qPCR (-Delta Ct) from UC patients in remission (line = median). Patients were classified as Relapse (n=21) or No Relapse (n=24) based on whether or not they had a disease flare (confirmed by the

presence of endoscopic lesions) at some point during their 1-year follow-up. Gene expression data analyzed by a Mann-Whitney test. $^{*}P<.05$.

Remarkably, IL1R2 expression was rather variable among those patients that relapsed, prompting the question of whether or not higher expression of IL1R2 within this group of patients could correlate with lower transcription of IL-1 dependent genes (CXCL1, CXCL2, CXCL8 and CCL20). Interestingly, patients that relapsed and experienced higher expression of IL1R2 showed a significantly lower expression of CXCL8, but not of CXCL1, CXCL2 or CCL20 (Figure 31).

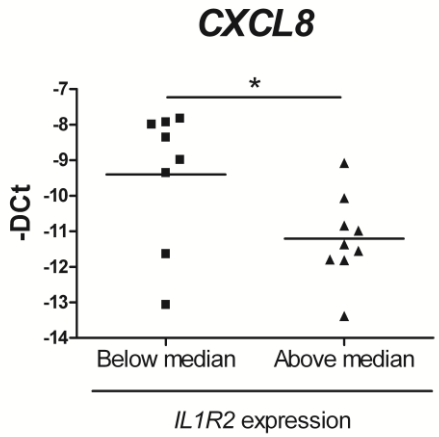


Figure 31. CXCL8 gene expression in UC patients in remission who relapsed during the following year. Intestinal mucosa CXCL8 gene expression by qPCR (-Delta Ct) from UC patients in remission (line = median) who relapsed during the following year. Patients were classified as IL1R2 expression (Figure25b), below (n=8) or above (n=9) the median. Gene expression data was analyzed by a Mann-Whitney test. $^{*}P<.05$.

Based on the previously described results using activated T cells, we measured *IFNG* transcription in this group of patients. *IFNG* expression was also low and similar in UC inactive patients regardless of whether they relapsed or not during follow-up (Figure 32a). Interestingly, in patients that relapsed, *IL1R2* expression negatively correlated with *IFNG* transcription (Figure 32b).

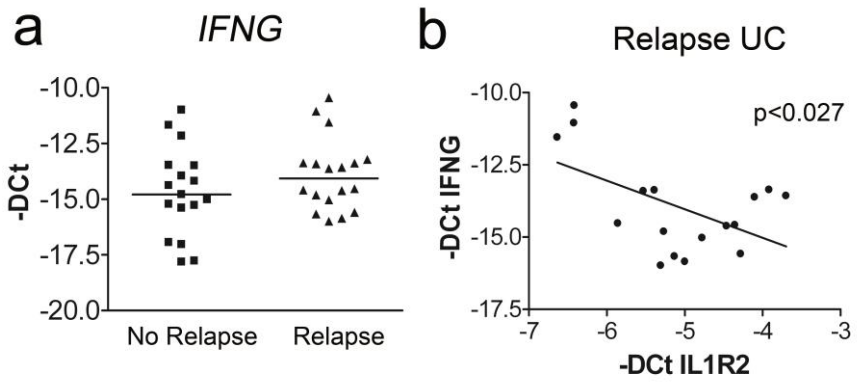


Figure 32. IFNG and IL1R2 correlation in ulcerative colitis (UC) patients in remission who relapsed during the following year. (a) Intestinal mucosa IFNG gene expression by qPCR (-Delta Ct) from UC patients in remission (line = median). (b) *IFNG* and *IL1R2* gene expression correlation in UC patients in remission who relapsed during the following year. A Spearman correlation was performed (Pearson r -0.5336).

Altogether, our results suggest that the IL-1R2 transcriptional profile in patients with inactive disease could be an early predictor of relapse.

VI- Discussion

Understanding the mechanisms that control intestinal homeostasis and prevent inappropriate inflammatory responses may be especially relevant in patients that suffer from chronic remitting and relapsing inflammatory conditions such as UC. Disease flares in UC are characterized by mucosal inflammatory lesions, and remission is defined as the healing of such lesions and the lack of macroscopic (endoscopic) and microscopic (histologic) signs of inflammation. Our understanding of how mucosal healing can be sustained once remission is attained and relapse prevented remains limited. In a significant percentage of patients, appropriate long-term control of UC with prolonged endoscopic remission is usually achieved by continued immune suppression or biologic therapies, which are associated with certain risks and carry significant costs[272]. Despite the use of the best available care, a proportion of patients will experience repeated flares[273].

In healthy steady-state condition, cells and molecular pathways of the gut are involved in a myriad of processes that promote continuous tolerance towards the commensal flora and food antigens. Moreover, many negative loops and regulatory proteins exist to dampen and control inflammatory responses in the intestinal mucosa. In particular, during an acute inflammatory response there is a temporary up-regulation of regulatory mechanisms to limit damage. Such mechanisms include anti-inflammatory cytokine production (IL-10, IFNα and IFNβ, TGFβ, IL-22, IL-35 and IL-37)[260], endogenous inhibitors of inflammation (IL-1Ra[168], sTNFR[176], IL-18BP[261]) and pro-resolution mediators (lipoxins, resolvins and protectins)[262]. These mechanisms could be exploited to dampen inflammation and, in fact, some have already been explored because of their therapeutic value[262-264].

The aim of this thesis was to reveal, among the transcriptional signatures associated with UC in remission, potential endogenous homeostatic or anti-inflammatory pathways that could be harnessed for the benefit of sustained remission. Data from several groups, including our own[168], strongly support the contention that following resolution of inflammation, a variety of signals remain deregulated in the involved colonic mucosa of UC patients. Many of these signals stem from the intestinal

epithelial compartment, which is permanently altered during the inflammatory response[168, 245-247].

In agreement with our hypothesis, Wallace and collaborators have shown that production of the pro-resolution mediators prostaglandin D2(PGD2), Annexin A1, and Lipoxin A4 is increased during remission of UC[274, 275]. Interestingly, based on transcriptional analysis of intestinal biopsies, we identified IL-1R2 among the molecules up-regulated in the mucosa of patients with UC in remission. IL-1R2, as an IL-1 decoy receptor, can effectively block IL-1 mediated responses. In fact, compared to the signaling receptor IL-1R1, it has a 10-fold higher affinity for IL-1β[276]. In addition to IL-1 binding, IL-1R2 can sequester soluble or membrane-bound IL-1RAcP, an essential component of the signaling IL-1R1 receptor. IL-1R2 is, however, just one of multiple proteins that can affect IL-1 action. Indeed, we show that transcription and protein expression of selected members of the IL-1 family are sequentially orchestrated in active versus inactive inflammation in UC patients, suggesting that tight regulation of IL-1 signaling is crucial to maintaining and/or regaining intestinal homeostasis. Indeed, the IL-1R2 gene and protein were significantly up-regulated in the mucosa from UC patients in remission, despite concomitant down-regulation of IL-1β, IL-1RAcP, and IL-1R1, all positive regulators of IL-1. An opposite profile of these IL-1 family proteins had been found in active disease. This suggests that IL-1R2 may play a role in healed intestinal mucosa homeostasis rather than in repressing ongoing acute inflammation. In contrast, another IL-1 action blocker, the IL-1 receptor antagonist (IL1RN), is transcriptionally up-regulated both in active and in remitting UC. Despite the marked increase in IL1RN transcription, we could only measure augmented soluble IL-1Ra protein in the supernatants of inflamed mucosa, suggesting that IL-1Ra may have a preferential role in active UC. Pre-formed IL1RN transcripts could nonetheless represent a backup mechanism ready to react against arising inflammation. Indeed, two intracellular isoforms of the IL-1 antagonist are released upon cell death [277].

In the context of IBD, existing data demonstrate an overall decrease in IL-1R2 concentration during active CD and UC, both in cultured colonic biopsies [176], as well as in plasma[177]. These studies, however, did not focus on the expression of the decoy

receptor during IBD remission, and there are currently no reports in the literature regarding IL-1R2 regulation in this particular situation. Interestingly, while transcription of *IL1B* was also significantly increased in inflamed CD colonic mucosa, we detected no up-regulation of *IL1R2* in CD patients during remission of the disease. This suggests that the changes we describe regarding IL-1R2 regulation do not constitute a generalized mechanism, but rather are part of a signature specific to the UC-in-remission state. This is especially interesting given the fact that despite the many clinical and phenotypic characteristics that discriminate UC and CD, the number of molecular mechanisms that differentiate both diseases at the intestinal mucosa remains rather limited.

In order to understand the mechanisms driving IL-1R2 expression during UC remission, we first identified the cell type(s) responsible for IL-1R2 production in the intestinal mucosa. Several studies have reported that IL-1R2 can be produced by different cell subsets. Although IL-1R2 was first cloned from B cells[276], it is also expressed in neutrophils, monocytes, M2 macrophages, keratinocytes and endothelial cells[277-279]. However, no information regarding expression in the intestinal mucosa was available.

Here we identify two cellular sources for IL-1R2 production in the human intestine. Immunofluorescent analysis of the colonic mucosa identified IL-1R2 positive cells in the lamina propria that were negative for the hematopoietic marker CD45. Remarkably, IL-1R2^{+} cells co-expressed IgA, but were negative for IgG. Indeed, CD45^{low} plasma cells are abundant in the lamina propria and, as we reveal here, represent the main source of the IL-1 decoy receptor in the intestinal lamina propria. IgA^{+} cells are well-described regulators of mucosal homeostasis by secreting IgA antibodies[74]. IgG^{+} plasma cells, in contrast, have been linked to pathogenic responses[280]. This data paves the way to study a novel regulatory feature of these plasma cells in healthy mucosa. Remarkably, the number of IL-1R2-producing plasma cells in the intestinal mucosa of UC in remission and the non-IBD mucosa was not significantly different, strongly suggesting that the plasma cells were not accounting for the increase in IL-1R2 in the inactive UC mucosa.

Besides identifying IL-1R2 producing IgA^+ lamina propria cells, our analysis showed strong staining of the epithelial layer, revealing epithelial cells along the crypts as an additional source for intestinal IL-1R2 production. Moreover, using flow cytometry analysis of digested intestinal biopsies we detected increased numbers of IL-$1R2^+$ $EpCAM^+$ epithelial cells in samples from patients with UC in remission compared with controls, uninvolved or active UC colon samples. We thus hypothesized that epithelial cells were the most likely cause of IL-1R2 overexpression in UC in remission. Indeed, epithelial cells constitute about 40-50% of cells in endoscopic biopsies and therefore contribute significantly to the transcriptional signature and the total protein secretion observed in biopsy-based experiments.

We detected soluble IL-1R2 secreted by the epithelial cells in culture and we detected intracellular IL-1R2, but not the surface receptor, by flow cytometry. These data support the contention that the IL-1 blocking action of the receptor secreted by the differentiated epithelial component may act not only in autocrine manner, but also may affect other cell types around the crypt in the lamina propria, in a paracrine manner.

After establishing the cellular source responsible for increased IL-1R2 expression within the colonic mucosa of UC patients in remission, we focused on elucidating the molecular mechanisms that regulate IL-1R2 within the epithelial compartment. Previous studies had shown, mainly in myelomonocytic cells, that IL-1R2 is induced by an array of stimuli, including glucocorticoid hormones[281], Th2 cytokines (IL-4 and IL-13), and IL-27[152, 154] among others[152, 160, 277]. In contrast, pro-inflammatory molecules inhibit IL-1R2 expression or cause rapid shedding from the membrane[161, 162]. To our knowledge no information is available on the regulation of the IL-1 decoy receptor in epithelial cells, or within the lamina propria.

Using immunohistochemistry and immunofluorescence we observed that IL-1R2 showed a gradient of expression along the crypt, with low or negative staining at the base of the crypt (where the stem undifferentiated epithelial cells reside) and

increased expression in the upper two thirds of the crypt and surface epithelium. These results suggest that IL-1R2 is up-regulated upon epithelial cell differentiation. In order to test this hypothesis, we used a colonic epithelial stem cell culture system as well as an *ex vivo* whole crypt culture. By utilizing these experimental approaches, we provide novel evidence for the role of Wnt signaling in repressing IL-1R2 transcription and translation. Interestingly, this appears to be a rather unique regulatory mechanism within the IL-1R family, since we detected no connection between the Wnt pathway and IL1R1 or IL1RN transcription.

Canonical Wnt signals activate β-catenin and are critically involved in stem cell proliferation and survival at the base of the intestinal crypts. Conversely, repression of these signals drives epithelial cell differentiation[270]. β-catenin was independently discovered twice, on the basis of its different functions: structural action and signaling. In the absence of a Wnt stimulus, the majority of β-catenin is located at the cytoplasmic side of the membrane as a structural component of cadherin-based cell-cell connections. Upon Wnt ligation, β-catenin is released through the previously described mechanisms. Stem cells at the base of the crypts require continuous β-catenin activation to proliferate and remain undifferentiated. In order for stem cells to give rise to differentiated progenitors, Wnt/β-catenin signaling is inhibited and, according to our data, releases expression of IL-1R2.

Although we herein describe this novel mechanism of IL-1R2 expression by epithelial cells, we do not provide any insight on the molecular or cellular alterations that lead to increased expression of IL-1R2 during remission. We hypothesize that a deregulation of the Wnt/β-catenin pathways could result in such a disrupted expression of the type II IL-1 receptor.

Indeed, results from our microarray data reveal a differential profile of Wnt/β-catenin related genes in UC patients compared to controls. Our results, in agreement with previous data, show that Wnt signaling is up-regulated in acute inflammation, a process that has been described as promoting wound repair[193, 213]. While the transcriptional data provides some evidence of a global deregulation of Wnt/β-catenin

related genes in active and inactive UC mucosa, our results alone can not reveal the real role of the Wnt canonical pathway during the UC-in-remission stage. We have to consider the complexity of the regulatory factors at work in the Wnt/β-catenin pathway, the different β-catenin functions, as well as the heterogeneity in cell composition present in the biopsies.

Other studies had linked β-catenin activity with UC severity[213, 282]. In particular, Brown JB. and colleagues[282] demonstrated that mesalazine, commonly used as a maintenance treatment in UC patients in remission, inhibits epithelial β-catenin activation. In addition, UC patients have an increased risk of developing colorectal cancer[283], which is highly influenced by β-catenin signals as well. These results support the fact that β-catenin is deregulated in chronic UC. Nonetheless, further research is needed in order to fully understand the potential effects on Wnt/β-catenin regulation in UC.

We hypothesize that increased IL-1R2 expression in the involved areas of the colon in UC in remission could reflect changes in the abundance of differentiated epithelial cells, which as we show produced larger amounts of IL-1R2. This would be in agreement with previous data demonstrating that permanent changes in the epithelium, such as crypt cell hyperplasia and crypt branching, result in architectural distortion of the tissue [248, 284, 285].

The final objective of this thesis was to study the biological relevance of increased IL-1R2 expression during remission of UC. While IL-1R2 can bind both IL-1α and IL-1β, the former is pre-formed in most cells and is usually released upon cell death, in particular during necrosis, a phenomenon that may occur downstream from IL-1β secretion in inflammation[260]. IL-1β has been classically described as a pro-inflammatory cytokine that targets many cell populations, promoting cell survival, cytokine and chemokine production, and increasing epithelial cell permeability[124]. IL-1α and IL-1β are both up-regulated in active UC; nonetheless, IL-1β is also secreted at lower concentrations in the non-inflamed intestinal mucosa and, in mice models, has been postulated to promote epithelial cell integrity[183]. This effect may, however, be

dependent upon the available cytokine concentrations, concomitant expression of IL-1 regulatory molecules, and the nature of the targeted cells. Further studies will be required to fully elucidate the complex behavior of IL-1β in mucosal homeostasis.

We hypothesize that in the healthy mucosa IL-1R2 acts as a homeostatic mechanism that can block excessive IL-1 signaling during early phases of the inflammatory response.

To study the decoy receptor function on IL-1β action on the intestinal epithelial component, we stimulated intestinal crypts isolated from biopsies from UC patients in remission and controls with low doses of IL-1β to mimic physiological concentrations. Our data shows that IL-1β can enhance chemokine (CXCL1, CXCL2, CXCL8 and CCL20) expression by isolated intestinal epithelial cells. CXCL1, CXCL2, and CXCL8 bind to CXCR1 and CXCR2 mainly expressed in neutrophils, which are recruited to the intestinal mucosa during acute inflammation[286]. Neutrophil infiltration in the lamina propria and the epithelial crypts (causing cryptitis and crypt abscesses) is characteristic of UC lesions. Interestingly, low concentrations of IL-1β also enhance CCL20 secretion by isolated colonic crypts. Using this experimental system and an IL-1R2 blocking antibody, we demonstrated that the IL-1R2 released by colonic crypts can dampen IL-1β-induced chemokine production. Importantly, this effect was more pronounced in experiments using isolated intestinal crypts from UC-in-remission patients.

CCL20 binds to CCR6, a receptor expressed by dendritic cells, memory B cells and T cell subsets[287]. The CCL20-CCR6 axis has received considerable attention in IBD pathogenesis[288, 289]. Existing research has reported that both Th17 cells and Treg cells express CCR6 and respond to CCL20 chemoattraction, pointing to a potential balancing role of these chemokine[290, 291]. Th17 cells and its product IL-17 have been proposed as therapy targets for IBD. Unfortunately, the first clinical trial targeting IL-17A failed in CD[91]. Th17 cells are well known for their plasticity[292], and novel results demonstrated that an IFN-γ-producing Th17 subset can be derived from Th17 cells and then be specifically involved in intestinal inflammation in CD and UC[86]. Interestingly, a recent study showed that IL-1β not only promotes T cell activation and survival[293] and

participates in differentiation of Th17 cells[294-296], but also acts as an inducer of IFN-γ by Th17 cells[297], thus giving rise to the potentially pathogenic Th17/Th1 subset. In order to test a potential role for intestinal IL-1R2 in interfering with the effects of IL-1β, we generated activated human T cells with a mixed Th17/Th1 phenotype. We primed $CD4^+$ T cells with *Candida albicans*, which is a described method for expanding IL-17 and IFN-γ expressing cells [297]. *Candida albicans* expanded $CD4^+$ T cells were incubated with supernatants from control or UC-in-remission biopsies and the effects of an anti-IL-1R2 blocking antibody on IFN-γ and IL-17 production were measured. Interestingly, we observed that blocking IL-1R2 significantly increased the production of IFN-γ, but not IL-17, by $CD4^+$ T cells incubated with biopsy supernatant from UC patients in remission, but not in controls. Thus, although IL-1β's role in differentiating Th17 cells[296] is widely known, our results support the contention that IL-1β induces IFN-γ once these subsets have already been differentiated.

Overall, our experiments using a blockade of endogenously produced IL-1R2 in isolated colonic crypts or whole biopsies strongly suggest that the IL-1 decoy receptor plays a functional role in controlling relevant pro-inflammatory signals, both in epithelial cells and T cells. Importantly, these effects were significantly different in the context of UC in remission compared to healthy non-IBD mucosa.

Many studies have been designed to test clinical, endoscopic, biological, and histologic parameters as predictors of relapse in patients with quiescent UC. To date, all such studies have focused on the presence of microscopic or molecular inflammatory signals, or they have been based on results from medically refractory UC patients[298-302]. Given the functional role of IL-1R2 in repressing inflammatory signals during UC remission, we asked whether increased expression of the anti-inflammatory receptor, IL-1R2, could be a predictor of relapse. We indirectly approached this question by examining a well-characterized subset of patients[303] that were in endoscopic and histologic remission at the time of endoscopic evaluation and that were followed-up with an endoscopy 12 months later. Expression of *IL1R2*, albeit with a low predictive value, was significantly decreased in patients who suffered a disease flare during the subsequent 12 months. Taking into account the fact that patients who

relapse express a broad range of *IL1R2*, we tested if this also resulted in the different expression of other inflammation-related transcripts within the group experiencing early relapse. Indeed, we showed that patients with lower *IL1R2* expression presented higher amounts of *CXCL8* compared to patients with a relative increase in *IL1R2*. These results suggest that increased *IL1R2* expression could be one, among other markers, of sustained remission in UC.

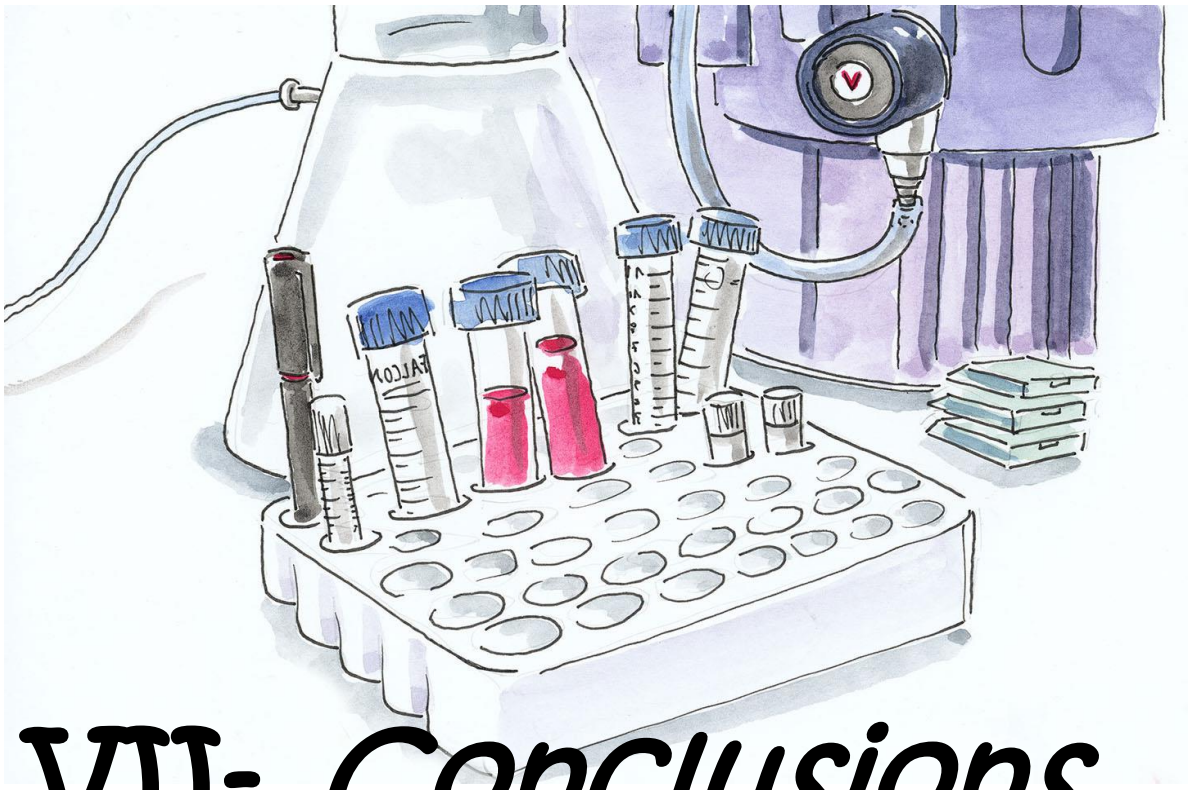
Based on T-cell culture results, we also analyzed the transcriptional levels of *IFNG* relative to *IL1R2* in the cohort of UC-in-remission patients that presented an early relapse. Interestingly, *IFNG* transcript levels negatively correlated with *IL1R2,* supporting the role of IL-1R2 as a regulator of IFN-γ production.

Our data shows that transcription of *IL1B* in UC patients in remission is significantly down-regulated compared to active UC patients, while remaining elevated compared to controls. Nevertheless, we found no differences in the concentrations of secreted IL-1β when comparing UC patients in remission and control samples. Taking into account the different stimuli involved in the production of IL-1β, we cannot rule out the possibility that inactive pro-IL-1β accumulates in the mucosa from patients with UC in remission. We propose that the role of IL-1R2 in inactive UC mucosa may be that of acting as a first-line defense mechanism to neutralize locally produced low doses of IL-1β. Thus, epithelial IL-1R2 may act as a homeostatic regulator, preventing on the one hand an increase in production of chemokines that facilitate tissue infiltration, not only of innate cells as neutrophils, but also memory Th17 cells. On the other hand, IL-1R2 may prevent the production of IFN-γ by these adaptive cells.

Finally, our results using *ex vivo* human cultures together with the available experimental *in vivo* data on IL-1R2[304, 305] show the presence of local anti-inflammatory effects, suggesting that regulation of this receptor could represent a potential therapeutic avenue. Recombinant forms of IL-1Ra (anakinra) have already been shown to have therapeutic benefits under different inflammatory conditions[180], as well as in chronic granulomatous disease[181]. Other IL-1 blocking strategies, such as the IL-1R1/IL-1β recombinant soluble receptor (rilonacept), have been explored with less

success[180]. In contrast to IL-1Ra, IL-1R2 offers the advantage of having a 10-fold higher affinity for IL-1β than the signaling receptor IL-1R1. Moreover, IL-1R2 binds IL-1 while also sequestering IL-1RAcP, thus limiting the signaling capability of IL-1R1. Administration of sIL-1R2, or of signals that promote its expression (e.g., β-catenin antagonists already developed for colorectal cancer), could provide an improved IL-1 blocking tool. Moreover, given the potent colorectal cancer-promoting properties of IL-1β [306], interfering with this cytokine - whether by direct blocking (canakinumab), by sequestering it with IL-1RN (anakinra) or by an as yet to be developed recombinant IL-1R2 - could offer a dual therapeutic benefit: inhibiting/preventing inflammation, while directly interfering with cancer-promoting mechanisms[307, 308].

In summary, we propose that secretion of IL-1R2 by epithelial cells does not play a role in resolving or abrogating the acute inflammatory response, but rather represents a "homeostatic" mechanism that is enhanced in the previously inflamed UC mucosa. We hypothesize that boosting this mechanism could help maintain disease remission.



VII- *Conclusions*

1. The colonic mucosa of UC patients in endoscopic remission is characterized by an increase in transcription and protein secretion of the IL-1 decoy receptor IL-1R2. In contrast, IL-1β, IL-1RAcP and IL-1Ra protein secretion is down-regulated compared to inflamed UC mucosa.

2. Within the control intestinal mucosa, both IgA^+ plasma cells and differentiated crypt epithelial cells can produce IL-1R2.

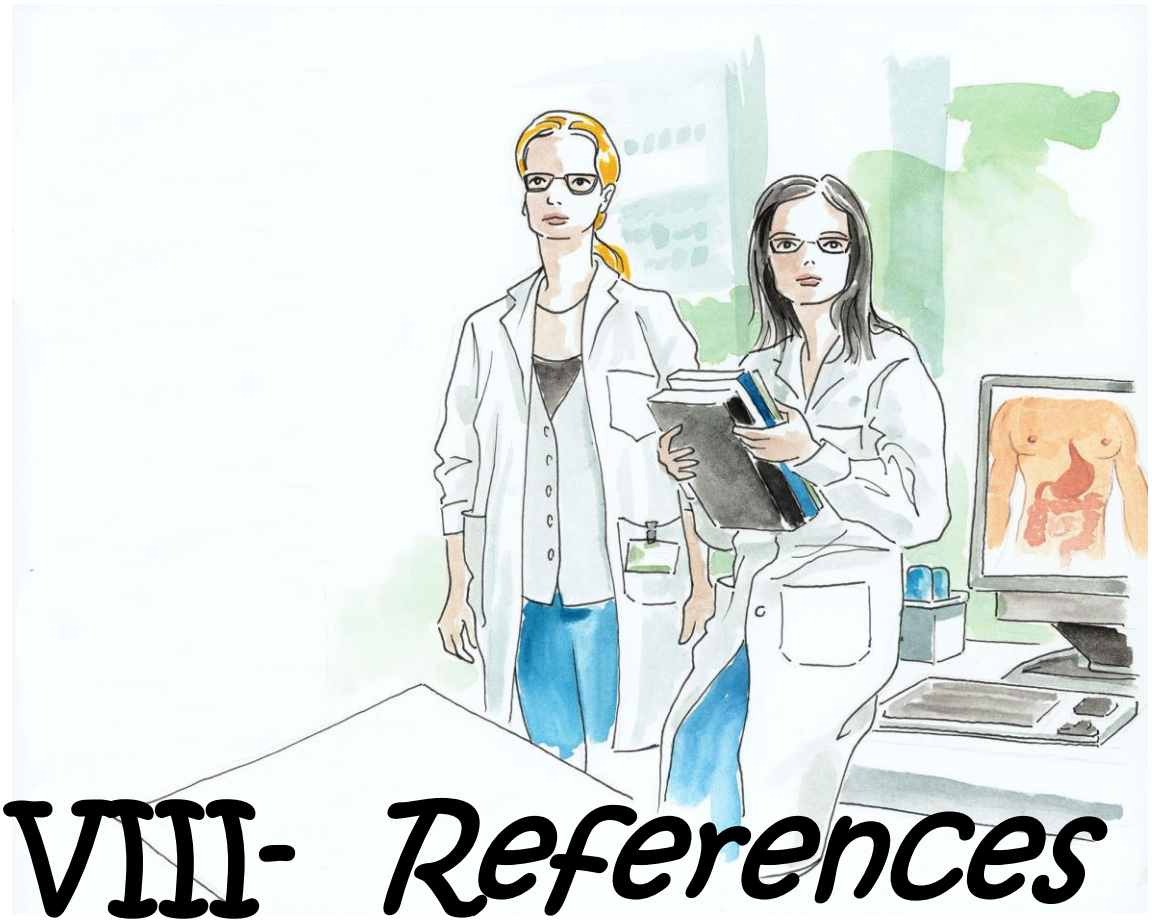
3. The increased numbers of IL-1R2 producing epithelial cells in UC patients in remission could contribute to up-regulation of the decoy receptor in UC inactive mucosa.

4. *IL1R2* is up-regulated upon differentiation of those intestinal epithelial cells negatively controlled by Wnt/β-catenin signaling.

5. IL-1R2 protein partially prevents the pro-inflammatory actions of IL-1β on intestinal crypts and on Th17/Th1 cells in culture.

6. As decreased *IL1R2* gene expression is associated with relapse of UC, these results suggest that increased *IL1R2* expression could be one marker, among others, of sustained remission in UC.

We conclude that enhanced secretion of IL-1R2 by epithelial cells represents a "homeostatic" mechanism in the intestinal mucosa of UC patients in remission. We believe that boosting this mechanism could help maintain disease remission.



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APPENDIX

Publications:

- **Mora-Buch R**, Dotti I, Planell N, Calderón-Gómez E, Jung P, Masamunt M.C., Llach J, Ricart E, Batlle E, Panés J, Salas A. Epithelial IL-1R2 acts as a homeostatic regulator during remission of ulcerative colitis. *Mucosal Immunology* (*In press*) Included in this thesis.

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