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Interleukin 33: a locally induced alarmin in the colonic mucosa of ulcerative colitis

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Summary

Ulcerative colitis (UC) is a chronic inflammatory bowel disease involving the mucosal lining of the large bowel (colon and rectum). Common symptoms include bouts of diarrhoea, rectal bleeding and abdominal pain. The pathogenesis of UC is complex and multifactorial.

This thesis investigated interleukin 33 (IL-33), an alarmin of the innate immune system. IL-33 is of interest as it is found to be upregulated in UC during active disease. IL-33 is also found to accumulate beneath mucosal ulcerations in UC suggesting a role in mucosal healing. We aimed to explore mediators associated with IL-33, inflammation and fibrosis in colonic biopsies from patients with acute and quiescent UC disease.

We found mucosal mRNA *IL33* to be raised in biopsies with UC compared to healthy controls. Immunostaining revealed IL-33 to be present in the epithelial barrier during active UC, but not in quiescent disease or healthy controls. Furthermore, a significant number of gene transcripts associated with fibrosis were found to be dysregulated in the UC mucosa and of interest for future research. To investigate IL-33 expression in the colonic epithelium, a human *ex vivo* model based on endoscopic biopsies was developed. Stimulation with hypo-osmotic stress induced a strong IL-33 signal in epithelial cells, linking IL-33 to a cellular stress response.

In conclusion, IL-33 is an inducible nuclear factor in the colonic epithelium. In our studies we found epithelial IL-33 to be associated with active UC disease and novelly induced by hypo-osmotic stress in the colonic epithelium. The use of a human explant model shows promise for further studies of inflammatory mediators in the colon.

Sammendrag

Ulcerøs kolitt (UC) er en kronisk inflammatorisk sykdom som rammer det øverste slimhinnelaget (mukosa) i tykktarmen. Vanlige symptomer inkluderer diare, frisk rektalblødning og magesmerter. Sentralt i patogenesen er tarmens immunforsvar, epitelbarrieren, tarmfloraen, miljø- og genetiske faktorer.

Denne avhandlingen tar for seg en liten del av det inflammatoriske puslespillet ved å se nærmere på signalmolekyler i tarmslimhinnen til pasienter med UC. Sentralt i avhandlingen er signalmolekylet interleukin 33 (IL-33), et signalstoff (cytokin) og alarmin tilhørende det medfødte immunforsvaret. Uttrykket av IL-33 er økt i tykktarmslimhinnen ved aktiv UC, og akkumulerer under sår i slimhinnen i UC. Vi ønsket å undersøke signalstoffer i tarmslimhinnen i både aktiv og tilhelet fase av UC, med særlig fokus på IL-33, inflammasjon og fibrose.

Vi fant at IL-33 er økt i vevsprøver fra tykktarmen til pasienter med UC sammenlignet med friske kontroller. Immunfarging bekreftet at IL-33-positive celler er tilstede i epitelbarrieren ved aktiv betent UC, men ikke i tilhelet UC eller hos friske kontroller. Videre utforsket vi uttrykket av mukosale gener assosiert med fibrose i aktiv og tilhelet UC. Vi fant en signifikant andel med deregulerte gener av interesse for fremtidig forskning. For å utforske uttrykket av IL-33 i epitelceller i kolon utviklet vi en biopsimodell basert på endoskopiske vevsbiopsier tatt fra pasienter med UC og friske kontroller. Vi observerte at et hypo-osmotisk medium ga et sterkt IL-33-signal i epiteliale celler, og dette kan indikere en sammenheng mellom IL-33 og cellulære stressmekanismer fra det medfødte immunforsvaret.

Oppsummert har vi vist at IL-33 er et induserbart cytokin som er lokalisert i cellekjernen i epitelceller i kolon. Hypo-osmotisk stress og aktiv UC betennelse er assosiert med IL-33-uttrykk i tykktarmens epitelbarriere. Vevsmodeller kan være nyttig i fremtidig forskning på signalmolekyler i kolonslimhinnen.

List of papers

1. Loss of interleukin 33 expression in colonic crypts - a potential marker for disease remission in ulcerative colitis**.**
Sci Rep 6, 35403 (2016). PMID: 27748438. DOI:10.1038/srep35403

2. Fibrosis Mediators in the Colonic Mucosa of Acute and Healed Ulcerative Colitis.
Clin Transl Gastroenterol. 2019. PMID: 31584460.
DOI:10.14309/ctg.0000000000000082

3. Hypo-osmotic stress induces the epithelial alarmin IL-33 in the colonic barrier of ulcerative colitis.
(***submitted manuscript***)

Abbreviations

Throughout this text the following abbreviations have been used:

ACTB	Actin beta
ASIB	Advanced study of inflammatory bowel disease
CD	Crohn's disease
CRP	C-reactive protein
CT	Cycle threshold
DAMPs	Damage associated molecular patterns
DSS	Dextran sulphate sodium
GI	Gastrointestinal
GWAS	Genome wide association studies
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
IL	Interleukin
IL-1LR1	Interleukin-1-like-receptor 1
MIQE	Minimum information for publication of quantitative real-time PCR experiments
mRNA	Messenger ribonucleic acid
NFKB	Nuclear factor kappa B
pANCA	Perinuclear anti-neutrophil cytoplasmic antibodies
PAMPs	Pathogen associated molecular patterns
PRR	Pattern recognition receptor
RIN	Ribonucleic acid integrity number
RNA	Ribonucleic acid
RPLP0	Ribosomal protein lateral stalk subunit P0
RT-qPCR	Real-time quantitative polymerase chain reaction
SMAD	Mother against decapentaplegic homolog
STAT	Signal transducers and activators of transcription
TGFB	Transforming growth factor beta
TLR	Toll-like receptor
Th	T-lymphocyte helper cell
TNF	Tumour necrosis factor
T-reg	T-regulatory
UC	Ulcerative colitis

1 Introduction

This introduction gives an overview of ulcerative colitis and the intestinal immune response including alarmins and important cytokines. This is followed by a summary of the cytokine interleukin 33.

1.1 Inflammatory bowel disease

Inflammatory bowel diseases (IBD) are chronic, idiopathic disorders with a relapsing and remitting disease course. Ulcerative colitis (UC) and Crohn´s disease (CD) are the two main entities of IBD. Further, approximately 5-15% show overlapping features and are defined as unclassified IBD (IBD-U)[1-3]. Paediatric onset-IBD is classified separately[4]. This thesis outlines the disease of adult UC, defined as aged 18 years and above.

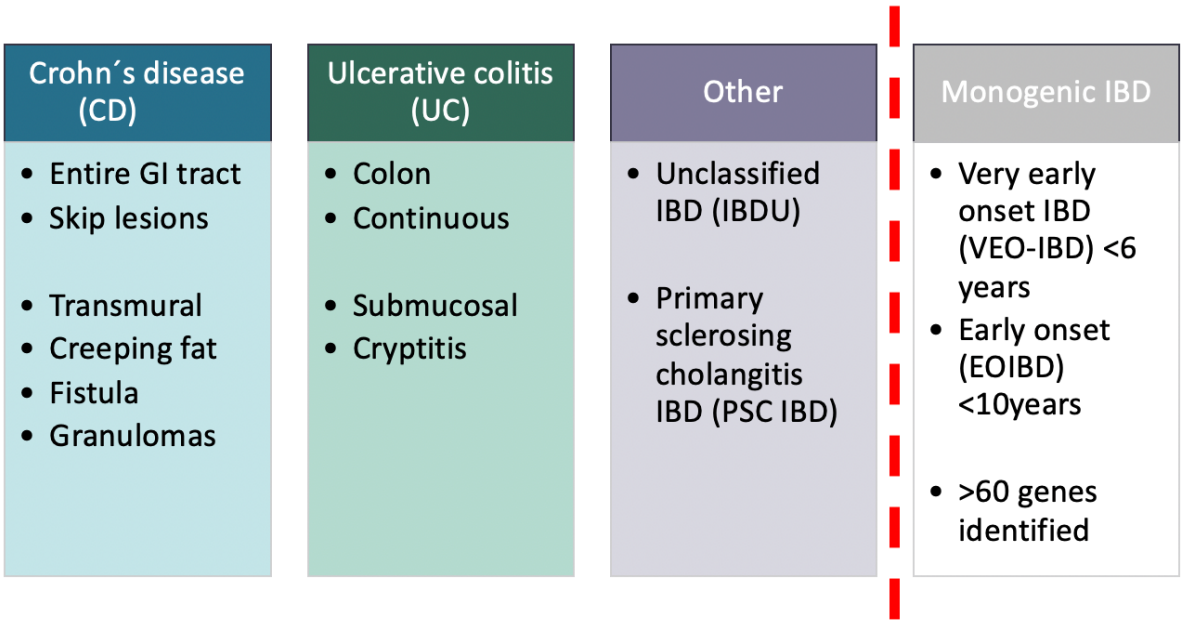

Figure 1. Classification of IBD with common features listed

1.2 Ulcerative colitis

"The disease, in general terms, may be described as an acute inflammation of the large intestine, proceeding to ulceration and sloughing of the mucous membrane" ... "The mucous membrane was ulcerated from end to end; the ulcers were of all sizes...." [S.Wilk 1859][5].

The mucosal inflammation of UC typically affects the upper layers (mucosa and submucosa) of the colonic wall with a continuous distribution starting in the rectum and progressing proximally. Medical therapy is not curative but aims at keeping the colonic mucosa in disease remission. Surgical removal of the large bowel (colectomy) is considered curative[6,7].

1.2.1 Epidemiology

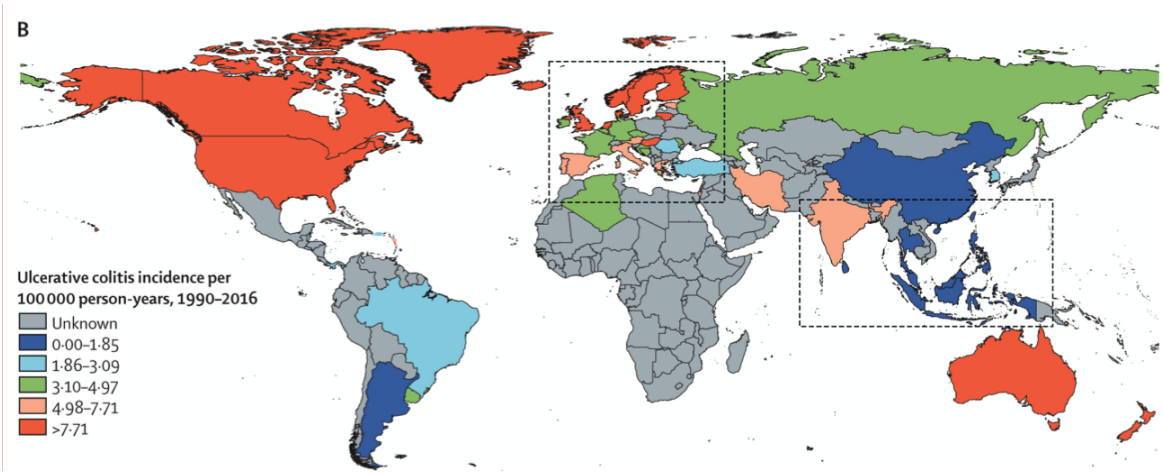

Figure 2. Worldwide incidence of UC
(Reprinted with permission from Elsevier publishing company)[8].

UC is increasingly considered to be a global disease. Northern Europe reports the highest incidence and prevalence rates in the world (incidence of 44/100000 person-years in the Faroe Islands and prevalence rate of 505/100 000 in Norway)[8-10]. In Europe, patterns of a higher north-south and an east-west gradient of disease are also reported[8,11]. Of note, in the last few years a plateauing phase of the incidence of UC and CD has been observed in western-countries, whilst the highest increasing rates are now being reported from newly industrialised countries[12]. Prevalence rates are globally continuing to rise both for UC and CD[8,13]. This has

led to a genuine concern for the future impact of IBD on health care systems in countries with dense populations and low socioeconomic indexes (IBD global consortium)[14,15].

In Norway, the main epidemiological IBD data are reported from a population-based cohort based in the southeast of Norway in 1990-3. The IBSEN study reported an incidence of 13.6 per 100 000 for UC, and 5.8 per 100 000 for CD[16,17]. A follow up study is ongoing (IBSEN III) and preliminary reports show an increasing incidence within the first year of inclusion[18]. Prevalence rates of IBD are newly estimated at 0.77% of the Norwegian population[19]. Studies from the North of Norway are limited, a report from 2000-2002 found an incidence of 26 per 100 000/year in support of a national north-south gradient[20]. In conclusion, the Norwegian population are in the upper tier of incidence and prevalence rates in the world. It is largely unknown what contribution the Sami population play[8].

1.2.2 Clinical features and disease course

Diagnostic criteria for UC are based on a combination of clinical, endoscopic, and histological features. Consensus guidelines are regularly updated, and in our studies we have used clinical guidelines from the European Crohn´s and Colitis organisation (ECCO)[2,21,22].

Clinical features: Patients commonly present with chronic diarrhoea, including passage of bloody stools with pus or mucus. Abdominal pain, tenesmus, faecal urgency and incontinence and fatigue are also disease features. In severe cases a fulminant inflammation affecting the entire colon (extensive/pan colitis) is considered a life-threatening condition if not promptly treated[2,23]. Extraintestinal manifestations including skin rashes, arthritis and uveitis are experienced by 1 in 4 patients with UC during their lifespan[24]. Notably, there is a considerable overlap with other autoimmune disease including ankylosing spondylitis, rheumatoid arthritis, autoimmune liver disease and skin diseases[25].

Endoscopic features: Colonoscopy typically reveals a red, oedematous mucosa with erosions and ulcerations. Findings start from the anal verge and are continuous and circumferential, often with a sharp demarcation between normal and inflamed tissue. Variations with rectal sparing (3%), caecal patches and backwash ileitis (20% of those with extensive disease) are described[2,26]. The Montreal classification is used to define disease extent (see figure 3), whilst several endoscopic grading scales to assess inflammation exist including the Mayo endoscopic score, Baron score, and UC endoscopic severity index score (UCEIS)[1,27,28].

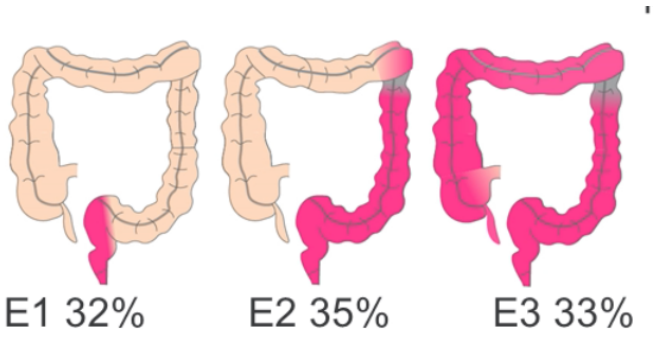

Figure 3. The Montreal classification for UC
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Disease course: Classically UC is described as a relapsing and remitting disease[29]. The group is heterogenous and at the time of diagnosis there are no good markers to predict disease course (a benign disease course with long periods of remission versus a more fulminant disease course). Epidemiological studies have found the most aggressive forms of UC to present within the first year following diagnosis[30]. Young age at onset (<40 years), male gender, extensive inflammation, lack of endoscopic healing and high levels of pANCA in the blood serum are all risk factors for severe disease, though not conclusive [31]. Biomarkers for predicting disease course are currently a hot topic with many international and national ongoing studies (Predict, Pants, ASIB, IBSEN III)[18,32,33,34]

Complications of disease: In the Norwegian IBSEN study a 20-year cumulative colectomy risk of 13% is reported. Extensive disease distribution was associated with an increased risk, whilst mucosal healing within the 1st year of diagnosis reduced this risk[35]. The same group found the cumulative mortality risk after 20 years of UC comparable to a control population[36]. The cumulative risk of colonic cancer was reported in a systematic review between 2.1-7.6% after 30 years of follow up and associated with pancolitis, male gender and presence of primary sclerosing cholangitis as risk factors[37].

Intestinal fibrosis is a likely underreported complication in UC with current figures of fibrostenotic complications in UC given up to 11%[23,38].

1.2.3 Principles of medical management

The main objectives in the management of UC are first to induce disease remission, second to maintain this state and avoid relapse of disease. A healed mucosa is associated with longer relapse free periods[21,39]. Medical management is briefly summarised below according to disease severity using the Mayo clinical score. Two main schools of thought exist termed "top-down" and "bottom-up" strategies[40]. The latter is outlined in national guidelines, increasingly with a treat-to-target strategy[41].

Mild to moderate UC: 5-aminosalicylic acid (5-ASA) are found in systematic reviews to be efficacious for inducing (30-50%) and maintaining disease remission in active disease[42,43]. Combination of oral and topical administration is favourable in an acute bout of UC[44]. 5-ASA acts on epithelial cells, and it's likely that the anti-inflammatory mode of action is through agonism of peroxidase proliferator activated receptor gamma. Although the mechanism is not clear, 5-ASA is also reported to reduce the risk of colorectal cancer in UC[45].

Moderate to severe: Corticosteroids have long been effective for the induction of remission in moderate to severe UC colitis and are indicated in acute severe colitis[44,46]. Side effects are the main reason for short courses. Further, thiopurines (azathioprine and mercaptopurine) have been shown in a metanalysis to be of benefit for maintaining steroid induced remission in UC, with number needed to treat of five patients to prevent one relapse[47].

Failure of response to corticosteroids is a criterion for escalating therapy to include monoclonal antibody therapies. Both anti-TNF (infliximab, adalimumab, golimumab) and anti-mucosal integrin therapy (vedolizumab) have proved efficacious in achieving disease remission in moderate to severe UC[44]. Anti-TNF therapy reported 60-70% response in the initial studies[48]. There is however increasing concern that up to one third of patients do not respond to anti-TNF therapy, suggesting other inflammatory mediators are driving inflammation[49]. Strategies include optimising dose, avoiding anti-drug-antibody development, and advances in the last years have led to options of shifting therapy to target other immune pathways including vedolizumab (anti-integrin), ustekinumab (targets IL-12/IL-23 pathway) and JAK-STAT inhibitors (i.e., tofacitinib)[44].

The intestinal immune response is central in the pathophysiology as well as management of UC. Intestinal immunity is complex, and in the following sections a background of important

immune responses and mucosal architecture is given, before delving into the immunopathogenesis of UC.

1.3 The intestinal immune response

The intestinal immune system in many aspects represents "the epitome of complexity of immune responses" in the human body[50]. It represents an important barrier to the external environment, not only protecting the internal milieu from the external environment (gut lumen) but interacting with the microbes that colonise the intestine and facilitates absorption of required nutrients and fluids. Key properties include pathogen recognition, immune memory and importantly recognition and immune tolerance to self[51,52].

Innate immune defences: Frontline innate immune defences are present from birth and include chemical, physical and cellular defence barriers[53]. Examples include the hostile acidic environment of the stomach **(chemical)**, the protective mucous layer that covers the intestinal epithelium (**physical**), and the tissue resident innate immune cells of the intestinal submucosa that survey for pathogen intrusion or tissue injury (**cellular barrier**)[54,55]. Central for our understanding of innate immunity is the pattern recognition theory[56]. Pattern recognition receptors (PRRs) can be situated on cell membranes or intracellularly where they recognise components from invading microorganisms called pathogen associated molecular patterns (PAMPs). Moreover, PRRs also sense endogenous molecules released following tissue injury or death (termed damage-associated molecular patterns, DAMPs), mechanisms that will be discussed in more detail in section 1.3.1[57]. Once receptor-ligand binding occurs, PRRs effectuate swift and appropriate immune responses based on the instigator involving activation of inflammatory cytokines and chemokines, as well as induction of complement cascades[56-58].

Adaptive immune defences: the adaptive immune system recognises non-self-molecules (antigens) resulting in specific defence mechanisms. Importantly it also "remembers" previous encounters with pathogens enabling a swift and specific response if the same invader is encountered again[53]. The main effector cells are bone marrow dependant (B-) and thymus dependant (T-) lymphocytes. In short, B-lymphocytes are responsible for antibody production, whilst important functions of T-lymphocyte subsets include killing target cells (cytotoxic T-cells, TLCs) and activating other immune cells including B-cells, and regulating the immune response (T-helper cells, Th)[59]. Important reservoirs of B- and T-cells are

distributed along the course of the intestinal barrier in gut-associated lymphoid tissue (GALT) [60].

T helper cells (Th) are further divided into subtypes based on their functions and secreted cytokines[61]. The major subtypes and main cytokines are given in Table 1. Aberrant Th2 and Th17 responses have been associated with UC, whilst Th1 responses have been associated with CD[62,63]. This view is however challenged as several studies report no difference in these subtypes of Th-cell expression between UC and CD.[64,65].

Table 1. Overview of Th-cell subtypes relevant in IBD.

Subtype	Transcription factors	Characteristic cytokines
Th1	TBX21	IFN-gamma, IL-2, TNF
Th2	GATA3	IL-4, IL-5, IL-13
Th9	SPI1	IL-9
Th17	RORC	IL-17A&F, IL-21, IL-22
Treg	FoxP3	TGF-beta, IL-10

1.3.1 Alarmins

Alarmins, also called DAMPs or danger signals, are defined as "endogenous molecules released from damaged/injured or necrotic cells that elicit innate immune responses[66,67]. Good alarmin qualities would include a potent and swift action once danger is sensed, a short duration of action, and a protected location during homeostasis to prevent inappropriate release. Like pathogens, alarmins are promptly and generally recognised by PRRs, therefore initiating common downstream signalling and activation mechanisms of protective immune responses[68].

Alarmins are a heterogenous group and include nucleic acids, ATP, interleukin-1 (IL-1) family cytokines including human mobility box gene 1 (HMGB1), heat shock protein and IL-33. The discovery that alarmins can trigger and shape the resultant inflammatory responses

has led to an interest in alarmins as potential targets and biomarkers of chronic inflammatory diseases[66]. In particular, alarmins located at epithelial barriers to the external environment have gained increased interest as upstream targets of chronic inflammatory disorders including asthma, colitis and atopy[69,70].

The danger theory involves the release of alarmins often as a result of cell injury or death by necrosis. Recently, situations of immense cellular stress have also been recognised to activate alarmins, though interestingly without cell death ensuing or the alarmin having to move cellular compartments[71]. This adds a new dimension with regards to the relationship between cellular stress mechanisms and inflammatory responses and is not fully understood[72].

1.3.2 Cellular stress responses

Cellular homeostasis is a finely tuned and regulated state that includes control of cell volume, osmolality, pH, intracellular ion and electrolyte concentrations within physiological ranges. Any insult or perturbation that challenges the limits of homeostatic control may induce a cellular stress response. The exact mechanisms between cellular stress and inflammatory responses are not fully understood, however their common goal is to restore homeostasis [73,74].

Cellular stress factors include hypoxia, heat shock, irradiation, metabolic stress and osmotic stress[74]. Cytosolic complexes, called inflammasomes, have been found to sense cellular stress and activate caspase-1 (CASP1), which cleaves IL-1 family cytokines classically activating IL-1 beta and IL-18[72]. The NLR family pyrin domain containing 3 (NLRP3) is one of the most studied inflammasomes, and interestingly dysregulations of these complexes are associated with chronic inflammatory diseases including IBD[75].

Osmosis and osmotic stress. The osmolality of the extracellular fluid in humans is tightly regulated and kept a constant 285-295 mOsm/kgH_20[76]. Any change in the intra or extracellular osmolality will affect cell volume and cell water content. Water shifts from low to high osmolality. A cell exposed to a hyperosmotic environment will shrink due to efflux of water across the cell-membrane, whilst hypo-osmosis will cause cell swelling. If not counteracted, cell lysis and -death ensue. Following an osmotic challenge immediate cell counter mechanisms include folding or unfolding of the plasma membrane to regulate cell

volume, in addition to activation of transmembrane transport channels and membrane pumps to re-establish an osmotic equilibrium[76-78].

Perturbations of osmosis are reported to induce inflammatory responses. Hyperosmotic stress is reported to induce pro-inflammatory cytokine IL-8 and IL-1 beta in colonic cancer derived cell cultures[79]. Further, intestinal cells exposed to hyperosmotic media have activated inflammasome nuclear factor of activated T cells 5 (NFAT5), supporting hyperosmotic stress as an instigator of inflammation[76,80]. A recent study in keratinocytes found hypo-osmotic stress to induce the alarmin and cytokine IL-33[81].

1.4 Colonic microanatomy and cellular function

1.4.1 The colonic epithelial barrier

The colonic epithelium is formed by a single layer of columnar enterocytes, organised into crypts. These enterocytes sit on a collagen-based basal membrane and are "glued" together side-by-side by protein complexes that regulate fluid flux across the barrier[82,83]. A thick double glycoprotein mucus layer containing antimicrobial peptides protects the apical surface of the epithelial layer. Epithelial turnover and renewal is rapid and complete in approximately 5-7 days. Cell proliferation occurs at the base of colonic crypts where LGR5+ stem cells are located. They differentiate and proliferate into mature absorptive or secretory colonocytes travelling up the crypt to the surface border. A colonic crypt contains approximately 250 cells. [52,84,85].

Colonocytes are a heterogenous group, and their complex array of tasks were recently affirmed in a large study by Smillie *et al*. where they identified 15 colonic epithelial subtypes including microfold cells, goblet cells and best cells (pH-sensing cells), giving insight into the specialised role of intestinal cells in maintaining homeostasis[86]. Thus, epithelial cells are no longer considered passive cells, but highly interactive and involved in crosstalk between the luminal microbes, adjacent cells and immune cells in the lamina propria[87]. They express TLRs, interestingly on their basolateral surfaces thereby detecting invasion of microbes, and have been found to express cytokines IL-22, IL-33 and TLSP to mount acute immune responses[88].

The intestinal microbiota has had a profound impact on our understanding of the colonic immune system[89]. An unfathomable, estimated 100 trillion microorganisms colonise the colon. Through genomic mapping somewhere between 300-1000 different species are found to colonise the gut[90,91]. The microbiota has been found to modulate nutrient metabolism and absorption, as well as influence the development and function of our gut[92]. Thus, the gastrointestinal (GI) tract is firmly established as a bacterial eco-system, with yet the significance of viral and fungal species contribution to be mapped[89,93].

1.4.2 The lamina propria

The cell-rich lamina propria, also called the stroma or connective tissue, consists of residing immune cells, endothelial cells, mesenchymal cells, and nerve ganglions which are connected and supported by an extracellular matrix (ECM). The ECM is often termed "the scaffolding" of the tissue. It consists of two main parts; the basal lamina layer supporting epithelial cells, and the interstitial matrix that supports cellular movement, motility, tensile strength, and elasticity of the organ. The major components include collagens, glycoproteins such as elastin, laminins, and fibronectins as well as proteoglycans[94-96]. There is a shift in our interpretation of ECM functions— importantly the ECM is not an inert, fixed matter but rather a dynamic matrix, constantly engaged in breakdown and rebuilding to maintain intestinal homeostasis[95-99]. It is also a storage of dormant immune mediators such as TGF-beta, released during remodelling of the ECM[96,99].

ECM remodelling is facilitated by matrix specific metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and ECM matrix protein production by mesenchymal cells (fibroblasts, myofibroblasts). The equilibrium between protease activity and degradation of matrix proteins is tightly regulated[96,97,100]. An imbalance, as seen in IBD, can have detrimental results for the tissue including delayed wound healing and excessive deposition of ECM causing intestinal fibrosis[101,102].

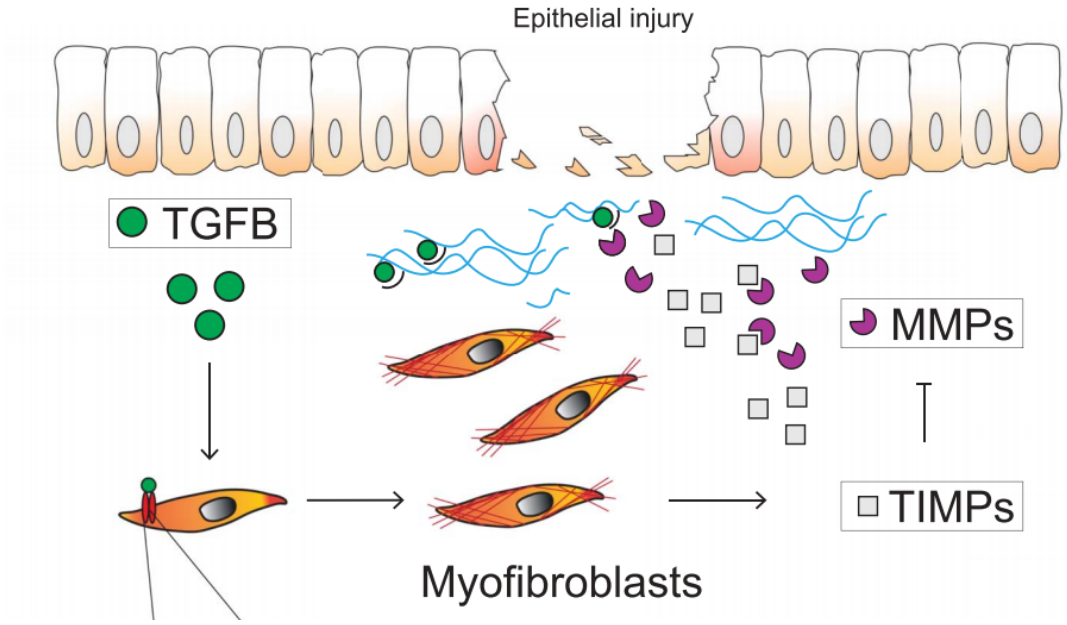

Figure 4. Mediators in the ECM in response to epithelial injury
(Reprint and modification permitted by Wolters Kluwer Health Inc)[103].

Below is a brief description of important mediators of the ECM from reviews[95,96,99]:

Mesenchymal cells: Fibroblasts and myofibroblast are important producers of collagen and ECM proteins.

TGF-beta: Pleiotropic cytokine with 3 isoforms (TGF-beta-1, TGF-beta-2, TGF-beta-3). Important key mediator of mesenchymal activation and essential in wound healing. Stored inactive in the ECM and released on degradation of the ECM. Signals via TGF-beta receptor 1 and 2 that induce intracellular SMAD pathways. Increased TGF-beta is associated with the development of fibrosis[104-106].

MMPs: These matrix metalloproteinases degrade ECM proteins and to date 23 different enzymes have been identified, collectively capable of degrading any type of protein. They are subdivided into categories divided by either biological function (collagenases, gelatinases, stromelysins, matrilysin, transmembrane, others), or evolutionary basis (4 groups: MMP-19, MMP-11, -14-15-16-17, MMP-2 and -9, and all other MMPs).

TIMPs: Numbered 1 to 4, inhibitors of MMP activity and the balance between TIMPs and MMPS is crucial for balancing ECM protein degradation and accumulation.

1.4.3 Mucosal wound healing, repair

There is considerable overlap and dependence on both initiation and resolution of inflammatory responses to ensure a proper tissue repair and restoration of function. Classically generic phases of wound healing include **1)** haemostasis and clot formation, **2)** inflammatory phase, **3)** proliferative phase and **4)** restoration and resolution[107]. In the intestine, epithelial restitution starts swiftly (within minutes) following a breach of the epithelial barrier to ensure resealing of the wound[108]. This is followed by cell proliferation, migration, differentiation and finally closure of the wound or ulcer. This requires a finely tuned and regulated collective response to ensure tissue regeneration, matrix remodelling and cessation of inflammation[92,108,109].

1.4.4 Fibrosis – healing gone wrong

Intestinal fibrosis is defined as the "excessive accumulation of extracellular matrix in the intestinal wall"[99]. Instead of restoring the intestinal wall to normal following tissue injury, excessive scar tissue is formed causing harm to the structure and function of the tissue in question. Inflammation is a known trigger of fibrosis, however resolution of inflammation does not necessarily stop fibrosis[23]. TGF-beta is considered one the main cytokines involved in the fibrotic process[100].

Fibrosis is a fairly general process, and well described in all major organs including liver, lungs, heart, kidney and intestine. The dogma that fibrosis is "irreversible" is being challenged, though there is consensus that a "point of no return" exists[110]. Cellular mediators of intestinal fibrosis are comprehensively reviewed by Lawarence et al, of interest they list IL-33 as a pro-fibrotic mediator inducing angiogenesis, fibrosis and myofibroblast proliferation[100].

In the intestine, fibrosis can cause stiffening and narrowing of the lumen, and more often encountered as a complication in the small bowel as the lumen diameter is smaller than in the colon. Stenosis of the bowel wall can cause severe problems with passage of luminal content, often requiring endoscopic or surgical intervention. In addition, stiffening of the bowel wall affects motility and may lead to pain, diarrhoea and incontinence[111]. An increasing awareness of the challenges with intestinal fibrosis is reflected in a ECCO scientific workshop, highlighting the need for increased research focus into intestinal mediators, biomarkers, and potential therapeutic targets[112].

1.5 UC disease pathogenesis

The pathogenesis of UC is currently regarded as multi-factorial. The leading hypothesis can be summed up as follows: *"a dysregulated immune response in a genetically predisposed individual where the gut microbial flora and other environmental factors influence disease development and course"*[6,113].

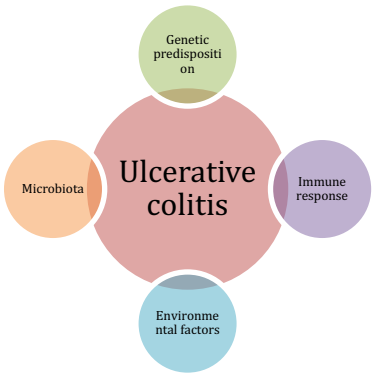

Figure 5. Multifactorial pathogenesis of UC

Genetic factors: Monozygotic twin studies have established a genetic contribution in IBD with concordance rates at 38-63% in CD and 6-27% in UC [114,115]. In adults genetic risk susceptibility has been the focus of large collaborations since the first genome wide association studies (GWAS) in 2005[116]. To date over 240 genetic loci have been identified with a susceptibility for IBD[117,118]. Interestingly, a large proportion (70%) overlap with other autoimmune diseases[25]. Despite the large number of IBD loci identified, they explain less than 30% of disease variability. In summary genetic research has increased our understanding of the immense complexity of IBD pathogenesis, though the current view is that genetics will not give the complete answer[119-121].

Gut microbiota: A reduced diversity of bacterial flora and stability is found in UC[122-124]. Whether this reduction is causative or an epiphenomenon to the disease is not clear[89,125]. Studies of faecal transplantation in UC have yet to show convincing benefit[126].

Environmental factors: Tobacco smoking is one of the most extensively researched environmental factors and although pathogenesis is not clear, it appears to be a protective factor against developing UC. Appendectomy is also found to have a protective effect for UC, most recently seen in a Swedish study[127-129]. Antibiotics in childhood have been associated with an increased risk of IBD.[130]

The immune response in IBD: A dysregulated immune response is evident in UC, with an imbalance between pro and anti-inflammatory cytokines. Deep sequencing of IBD risk loci have identified key immune pathways including those regulating epithelial function in

UC[131,121]. Upregulation of known inflammatory cytokines (TNF, INF-gamma, IL-1 beta, IL-17, TGF-beta-1, IL-10) are reported in UC[6,65]. Initially, a polarized mucosal Th-response with between CD (Th1) and UC (Th2/Th17) was reported, however this is increasingly being challenged in the literature as too simplistic, one metanalysis reports to the contrary similar cytokine profiles between UC and CD[6,64]. A further overview of cytokines in IBD is given in section 1.4.1.

Remarks on IBD/UC hypotheses:

The ”leaky gut” theory describes the increased permeability and paracellular fluid flux as a primary driver of intestinal inflammation. It is not clear whether increased leakiness is a primary or a secondary event[132].

The “hygiene theory” suggests the urbanization and reduced exposure to antigens in childhood causes a dysregulation in the intestinal immune system. This ties in with the “microbial diversity hypothesis” where the diversity of the gut microbiota is central in “educating” the immune system[133].

The presence of a yet unidentified microbe parallel to the history of peptic ulcers and *Helicobacter pylori* has also been proposed, however no such microbe be it bacteria, virus or fungi has been identified as a single causative agent.

1.5.1 Important mucosal mediators in UC

Cytokines are signalling mediators and have multiple cellular sources. Their homeostatic and pathogenic roles are not sufficiently described as solely anti- or pro-inflammatory as they are highly dependent on cellular and tissue context[134]. Biopsies from inflamed UC mucosa have given us a snapshot of the inflammatory state in the colon with the literature reporting an upregulation of proinflammatory mediators including TNF, IFN-gamma, IL-4, IL-5, as well as anti-inflammatory mediators IL-10 and TGF-beta[52,134,135]. Below is a brief description of cytokines important in the context of UC and IBD.

Tumour necrosis factor (TNF): A membrane-bound and a soluble form of TNF exist, both are ligands to the TNF receptors 1 and 2, which activate separate signalling pathways. TNF is crucial in the host-defence against pathogens. Inhibiting TNF has been successful in ameliorating inflammation in IBD in addition to other inflammatory disease including

rheumatic and skin inflammatory conditions[136]. The main source of TNF release is considered to be through macrophages, though many immune cells including monocytes, natural killer cells, T- and B-cells as well as epithelial cells and fibroblasts have been shown to produce TNF[134,135,137].

Interferon gamma (IFN-gamma): A member of type II class interferons and secreted by Th1-cells, cytotoxic T-cells, and natural killer cells. IFN-gamma is critical in innate and adaptive immune responses against viral, bacterial, and protozoal infections. Functions include inhibiting viral replication directly. Binding to its receptor activates JAK/STAT1 pathways which causes phosphorylated STAT1 to translocate to the nucleus[134].

Interleukin 17 (IL-17): Includes six different types ranging from IL-17A-F. Produced by Th17-cells. Antibodies inhibiting IL-17 (secukinumab) are efficacious in the treatment of psoriasis, however exacerbation of IBD has been associated with anti-IL-17 therapy[138]. Studies are still inconclusive to the effect of targeting IL-17 in IBD[139].

Interleukin 23 (IL-23): A member of the IL-12 family of cytokines that promotes Th17 immune responses. IL-23 is considered important in the pathogenesis of UC. Monoclonal antibodies blocking the p40 subunit of IL-23 and IL-12 have emerged as a new target in the treatment of IBD[140].

Interleukin 10 (IL-10): Is classified as an anti-inflammatory cytokine with a central role in IBD pathogenesis. GWAS studies have also linked IL-10 to IBD. Of interest, a recent article has linked anti-TNF therapy mechanisms to modulate macrophage production of IL-10 as a critical step in achieving disease remission[141,142].

Transforming growth factor beta (TGF-beta): See chapter 1.3.4 for summary.

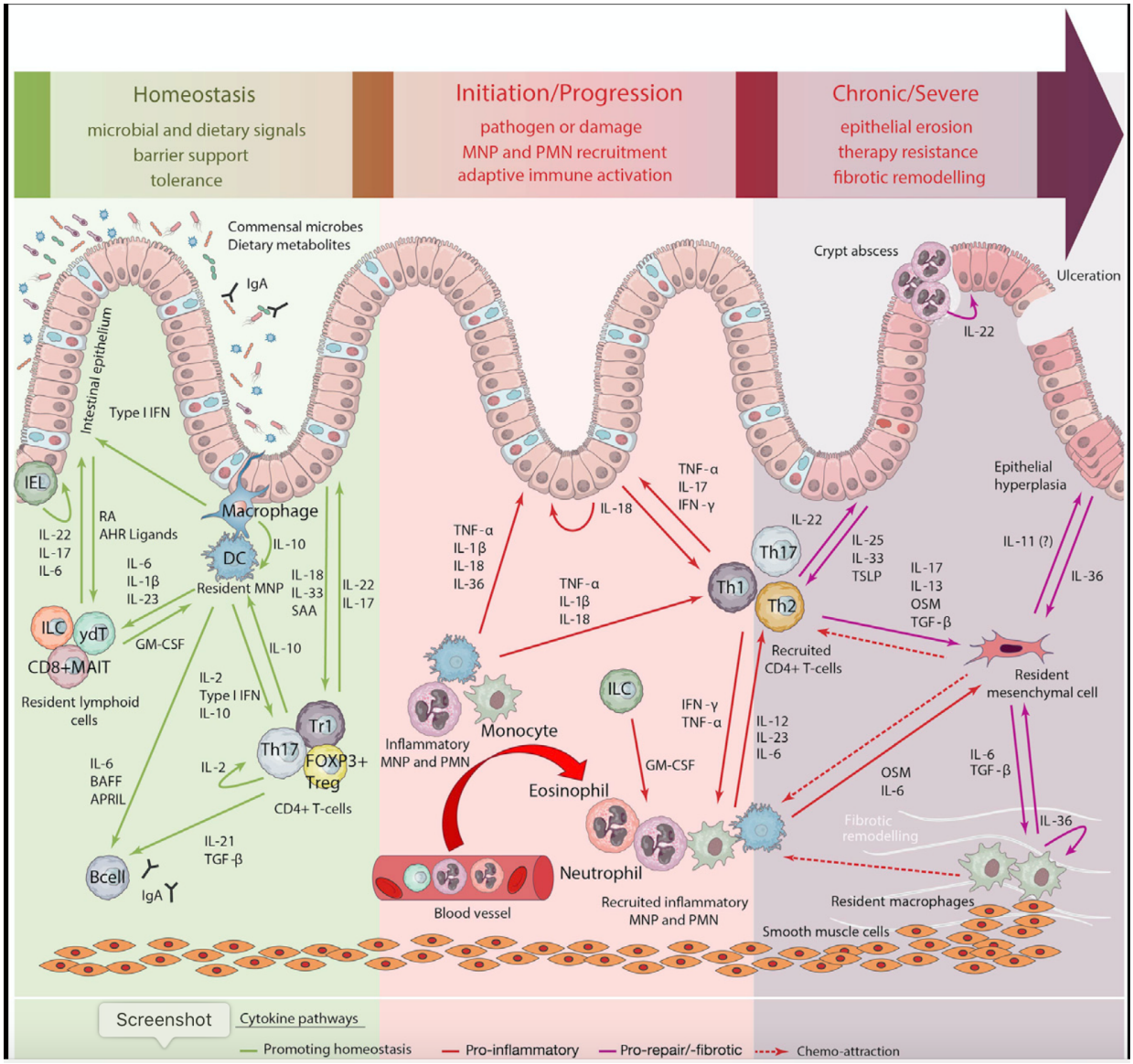

Figure 6. Mucosal immune mediators in IBD
(Reprinted with permission from Elsevier publishing company)[134].

1.6 IL-33 - a pleiotropic alarmin

Interleukin 33 (IL-33) was first discovered in 1999 as protein in vasospastic cerebral arteries of canines with induced subarachnoid haemorrhage[143]. In 2003 the human protein was cloned and visualized as a nuclear factor in high endothelial venules[144]. This led subsequently to IL-33 being recognised as the ligand of the "orphan" receptor interleukin-1-receptor-like-1 (IL-1RL1, alias ST2, IL-33R) and included in the IL-1 family of cytokines[145].

IL-33 was early linked to Th2 immune responses, with increased levels of IL-33 reported in Th2 associated diseases including atopy, asthma, and also UC[146,147]. In addition, IL-33 is recognised as a potent activator of innate lymphoid cells type 2 which produce cytokines IL-13 and IL-5[69]. A large body of evidence has since broadened the view of IL-33 and its receptor, linking them to a wide spectre of immune cells including Th1-cells and T-regulatory cells[148]. IL-33 is noted with >4400 PubMed listings (as of March 2022) linking it to a wide range of diseases including cardiovascular disease, metabolic disease, fibrosis, Alzheimer disease, graft-versus-host disease and infectious disease including helminths.

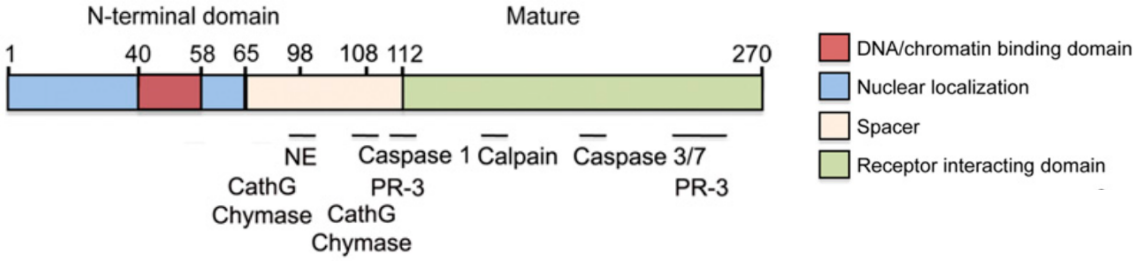

Figure 7. Human gene *IL33*
(Reprinted and modified with permission from Journal of Immunology, The American Association of Immunologists INC)[150].

The distribution of IL-33 in human tissue has revealed IL-33 presence in almost all human organs, with IL-33 found constitutively expressed in endothelial vessels[149]. A summary of IL-33 characteristics including gene, protein, receptor, cellular location, and regulations is given below based on reviews[147,148,150,151].

Gene: The gene *IL33* is located on chromosome 9, in contrast to other IL-1-family members that are present on chromosome 2. Polymorphisms have been linked to asthma susceptibility, and interesting loss of function mutation in *IL33* has been found to protect against asthma development[152]. Gene polymorphisms of *IL33* have also been associated with an increased risk of inflammatory bowel disease and development of a more extensive colitis[153].

Protein: In humans the synthesised full-length IL-33 is a 30kDa protein with 270 amino acids. As other IL-1-family members it adopts a beta trefoil fold structure. Further, the protein consists of a nuclear (N)-terminal, a central activation domain, and a carboxy (C)-terminal region. Importantly, a nuclear localisation domain and a chromatin binding motif are found on the N-terminal, enabling nuclear localisation of IL-33[154]. Only recently has the region responsible for complete nuclear localisation been defined to the amino acid 46-67 segment on the N-terminal[155]. Thus, cleavage after this nuclear localising section results in cytoplasmic IL-33.[155]. Comparison with canine and murine protein structure show 56% and 48% identity, respectively[144].

Receptor: As mentioned, the receptor "serum stimulator -2 (ST2)" alias IL-33 receptor and later renamed IL-1RL1 was discovered 16 years prior to discovery of its ligand and included in the IL-1-like/Toll-like receptor superfamily[156,157]. Located on chromosome 2q12.1 in humans, IL-1RL1 is 40kb long and not to be confused with the suppression of tumorgenicity gene (also dubbed ST2) which is located on chromosome 11p14.3-p12[158]. The gene and protein nomenclature further obfuscate the matter by naming the splice variants of the IL-1RL1 into 4 isoforms named;ST2L (membrane bound); ST2s (soluble form); ST2v (variant form, expressed in the GI-tract) and ST2lv (another variant form that lacks transmembrane domain)[159].The membrane bound ST2L is present in a wide range of immune cells including Th cells, ILC-2, M2 polarized macrophages, innate immune cells[148].

Regulation The soluble ST2´s receptor appears to have a regulative function as a decoy receptor that neutralises IL-33[160]. Interestingly, TNF and IL-1 beta are found to enhance ST2s[158]. Oxidation of cysteine residues shortly after extracellular release are proposed as an important regulator of IL-33. Other mechanisms regulating IL-33 action may include inhibition by the single immunoglobulin domain IL-1R-related molecule (SIGIRR), which interferes with dimerization of the IL-1RL1- IL-1 accessory receptor protein (IL-1RAcP) complex[147]. Post-transcriptional regulation of IL-33 by micro-RNA (miRNA) was examined

in human intestinal biopsies, suggesting members of the miRNA family 387 regulated IL-33 intestinal expression. They proposed that the inflamed mucosa with elevated TNF inhibits miRNA 387a-43p boosts IL-33 expression in intestinal epithelial cells[161].

Secretion: The current view is that IL-33 is released from its nuclear localisation passively following cell injury or necrosis, thus termed an endogenous "alarmin"[67].

1.6.1 Cellular location

Nuclear IL-33: Upon synthesis, IL-33 translocates to its nuclear location and is "hidden" from the immune system, limiting its potent pro-inflammatory effects[67,162]. The nuclear localisation of IL-33 is well supported in the literature, and a nuclear domain (n-terminal) has been identified including a chromatin-binding motif (amino acids 45-53) that binds to histone H2A-H2B-dimers [144,154]. The functional role of nuclear IL-33 remains largely unknown. Travers et al reported that IL-33 binds with high-affinity to chromatin proposed as mechanism to curtail IL-33 release from the cell limiting its potent pro-inflammatory effects[163]. Whether IL-33 affects transcription and chromatin folding, as seen with other epithelial alarmins such as human mobility gene box 1, is unclear[164].

Extracellular IL-33: Once released into the extracellular milieu, full length IL-33 is cleaved by inflammatory proteases (caspase-1) to a more potent form (18KDa), increasing IL-33 activity by 10 to 30- fold. Upon binding with the ST2L receptor, a heterodimeric complex is formed with IL-1RAcP. This activates downstream myeloid differentiation primary response protein MyD88 (MyD88), interleukin-1 receptor-associated kinase (IRAK), TNF receptor-associated factor 6 (TRAF6) signalling and NF-kappa-B activation, inducing pro-inflammatory cytokine synthesis. Effects of extracellular IL-33 have been examined in a murine model where the nuclear terminal was deleted. This resulted in severe multi-organ inflammation and death, emphasising the potent lethality of IL-33 and importance of its nuclear "hidden" localisation[162]. This lethal inflammatory response was not seen in mice who lacked IL-1RL1, thus in mice the potent extracellular effects of IL-33 are linked to its signalling via the IL-33/IL-1RL1(ST2) axis.

1.6.2 IL-33 at epithelial barriers

Epithelial cells at barriers to the external environment are recognised as important sources of alarmins including the skin, airways and intestinal tract[69]. Epithelial alarmins are gaining

increased attention as potential upstream therapeutic targets of inflammation, particularly of interest in atopic disease, asthma, and IBD[165,166].

Several barriers are reported to constitutively express IL-33[149]. The gastrointestinal tract shows a wide variation in cell types, local environment, and function along its course. In the oesophagus IL-33 has been induced by chronic inflammation caused by eosinophilic oesphagatis[167]. In the stomach lining IL-33 is reported to be constitutively expressed in the cell nuclei of gastric glands, and Buzzelli *et al.* found IL-33 to be important for the repulsion of *Helicobacter pylori* in the stomach mucosa[168]. In the colonic epithelium diverging reports of IL-33 expression have been reported, where IL-33 appears to be an inducible factor rather than constitutively expressed[148,169].

In addition to early warning of epithelial injury and loss of epithelial barrier integrity, IL-33 is also put forward as having a seemingly opposite role in wound healing processes through activation of ICL2 and T-reg cells[170].

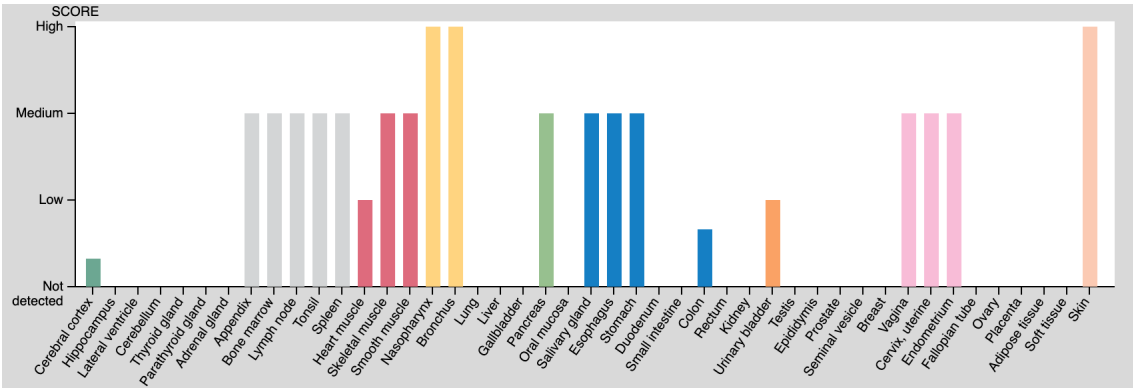

Figure 8. IL-33 protein expression based on organ site
The image is available from v15.proteinatlas.org. Human Protein atlas (www.proteinatlas.org)[171].

1.6.3 IL-33 and IBD

Human studies examining IL-33 in IBD are mainly based on colonic biopsies procured endoscopically or from surgical resections[172]. In addition, a large experimental body of murine models mimicking IBD have been tested[173,174].

The literature supports a marked increase of IL-33 in the human colonic mucosa of active UC compared to both CD and "normal" mucosa[175-179]. With regards to differences in IL-33 expression between UC and CD, studies are limited, yet support that IL-33 is preferentially

expressed in UC[179]. Of interest is the observation by Sponheim *et al*. that IL-33 positive cells accumulate beneath ulcerations of UC but are a lacking feature in CD fissures[175]. Further, a study on paediatric stricturing ileal-CD reported IL-33 expression and eosinophilic accumulation to perpetuate intestinal fibrosis development[180].

Of note from murine studies are models using either IL-33 or ST2 (IL-1RL1) knockout mice. Mice deficient of IL-33 or ST2 appear susceptible to a more severe colitis when induced chemically with DSS[173,174]. Moreover, mice lacking the *IL33* gene (*IL33*-/-) showed a delayed wound healing time following exposure to DSS induced colitis when compared to mice with the *IL33* gene[174]. This has led to a discussion of a protective role of IL-33 in the colonic mucosa of mice. Early studies in mice also observed a boost of intestinal protective features when intraperitoneal injection of IL-33 was administered including epithelial hypertrophy, goblet cell hyperplasia and increased mucous production[145]. Thus, seemingly IL-33 has both an inflammatory (alarmin) role important in the early immune response, and a role in reparative and regulatory role following a study showing that IL-33 promotes T-reg function in the colon[170].

1.6.4 Lack of knowledge

Despite the alarmin signalling functions of IL-33 being widely researched, there are few studies examining factors that induce IL-33 synthesis and accumulation. Further, the homeostatic nuclear function of IL-33 is not fully elucidated. In the time period of this thesis the view of IL-33 being constitutively expressed in the intestinal barrier has been challenged[149,175].

1.7 Models for studying the intestinal immune response

The intestinal immune response is complex and therefore, one argument is that these immune responses can only be truly studied in context from experimental models either from live animal models or culture of human intestinal biopsies[181]. IBD is particularly challenging to model because of its multifactorial pathogenesis and complexity of the intestinal immune system. Below are outlined common models used in IBD research with advantages and disadvantages highlighted.

1.7.1 Cell culture models

Isolated cells in culture are the most widely used experimental models for studying inflammatory responses. The most utilized and robust colonic cell lines have been developed from colonic cancer cells (adenocarcinoma) and include CAC02 and HT29 cell lines. Increasingly, primary intestinal cells lines are being developed. Cell cultures are inexpensive and highly reproducible. Limitations include that the cells are monocultures and poorly reflect *in vivo* physiology following obvious lack of cell diversity, the physiological exposure to substances and cell and tissue interaction observed *in vivo*[182].

In the last decade the development of 3D-cellular in-vitro cell models have evolved; epithelial organoids are based on progenitor stem cells which are isolated from human biopsies and cultured in-vitro. Providing the correct environment and stimulus, the stem cells differentiate and proliferate into 3D circular organoids[183,184]. Epithelial cell diversity is ensured, yet tissue-tissue interaction is missing as well as the enclosed lumen of the organoids that renders its use in luminal drug exposure studies difficult. Organoids are further being developed to be included on organ-on-chip models with the potential of being a tool for higher through-put studies including early drug efficacy[185].

1.7.2 Animal Models

Murine experimental models have been particularly employed in the study of intestinal immune responses. Their main advantage is representing a live environment (*in vivo*) and the possibility of the use of models where genes can be knocked out. Further, they are used as a source for testing new therapeutic targets and toxicity prior to human trials. There are however challenges with the use of animal models; most importantly significant differences between the human and murine immune systems (49-89% differences in genetic analysis)[186].

For the study of IBD, over fifty different experimental murine models have been tested[187]. They are useful in representing parts of the immune response in IBD, though no single model to date encompasses the complexity and full aspects of human IBD. DSS-induced colitis models and other chemically induced models (TNBS and oxazolone) are among the most widely used for studying UC[188,189].

1.7.3 *Ex vivo* tissue culture

Intestinal tissue cultures have existed in many forms since the first successful reported culture by Browning & Tier in 1960[190]. Biopsies can be obtained by endoscopy or surgical resections [191-193]. Different techniques exist including full-biopsy immersion, air-liquid interface or a rocking chamber[194]. Human mucosal explant models have the obvious advantage of avoiding interspecies variation. Compared to single cell cultures they maintain a 3D-structure, cell heterogenicity and in-between cell-cell communication in a limited time-period. General limitations with an explant model include autolysis, contamination, adequate uptake and diffusion through samples, and time limit with regards to tissue integrity[195].

2 Aims of study

The initial aim of this study was to explore mucosal gene expression of inflammatory and fibrosis associated mediators in UC patients treated with anti-TNF therapy to disease remission. Further, our findings of IL-33 expression prompted questions of what factors contribute to the induction of IL-33 in the colonic epithelium. The following questions were addressed:

- Are there differences between IL-33 mucosal expression in acute and healed states of UC? (**Paper 1)**
- Are fibrosis-associated mucosal mediators differentially expressed in the healed mucosa of UC? **(Paper 2)**
- Does hypo-osmotic stress induce the expression of IL-33 in the colonic epithelium? **(Paper 3)**

3 Material and Methods

3.1.1 Study population

Study participants were recruited from the University Hospital of North Norway (UNN, Tromsø) which is both a local hospital for the municipality of Tromsø and a tertiary referral centre for the north of Norway. For papers 1 and 2 patients were included from the IBD cohort study (2008-2013) with stored biopsies in a biobank. Inclusion and exclusion criteria are given in each article. In paper 3, patients and healthy controls were recruited consecutively at the gastroenterology unit at UNN Tromsø as part of the Advanced Study of Inflammatory Bowel disease (ASIB), a prospective study of newly IBD patients (2013-2020). For papers 1 and 2 the patient population overlaps, and the majority had well-established UC (median duration 52 months in paper 2). The study and storage of biological material was approved by the Regional Committees for Medical and Health Research Ethics, division North (reference number REK 1349/2012) and followed the Declaration of Helsinki principles[196].

A control group was included from patients referred for a colonoscopy at the outpatient clinic, the main reason being colonic cancer screening. Inclusion criteria were a completely normal macroscopic colonoscopy examination with the absence of polyps (rectal hyperplastic polyps less than 5 mm were considered a normal finding as per guidelines), no diarrhoea or concomitant inflammatory disease or cancer in current or past medical history. Pregnancy, lactation, presence of colonic polyps or systemic inflammatory disease were exclusion criteria. All endoscopic biopsies were reviewed by an experienced pathologist and included if reviewed as normal (Geboes score 0).

Paper 1:	Moderate to severe UC n= 29	Control group n= 21
Paper 2:	Moderate to severe UC n= 28	Control group n= 13
Paper 3:	UC n= 23.	Control group n= 14

Definition of UC disease activity: The Mayo score was used to classify disease severity with moderate to severe UC defined as a Mayo score with 6 out of 12 points or more[27].

Disease remission: For UC patients disease remission was defined as according to the full Mayo score of 2 or less, no single score more than 1. This includes an endoscopic sub score of 0 and 1[27].

TNF normalisation: Measurement of mucosal mRNA *TNF* from colonic endoscopic biopsies is performed on a routine basis at the gastroenterology lab UiT, Tromsø. *TNF* is given as absolute values of number of mRNA copies and previously published[32,197,198]. Mucosal gene expression of *TNF* is referred to as "normalised" based on a cut-off determined as the lower 5% percentile of an inhouse validated control cohort.

3.1.2 Tissue handling

Colon biopsies: Standard endoscopic forceps or (specifically for explant models) a jumbo radial raw (@Boston Scientific) with 2,8 mm diameter was used. Biopsies were standardised for healthy controls and taken from the left distal colon, preferably the sigmoid colon. In active UC patients' biopsies were taken from the site assessed by the examining clinician as the most inflamed region, and a consecutive biopsy was taken from within the same region at the time point of disease remission. In all studies these biopsies were taken from the left colon.

Biopsy handling: all biopsies were immediately immersed in either 10% formalin, RNAlater (Ambion, Austin, TX/ Qiagen) or basal transport medium on ice according to study inclusion and use; immunostaining, mucosal gene expression or use for explant biopsy model respectively.

3.1.3 Tissue model

Explant endoscopic biopsy model: An air-liquid, polarized *ex vivo* model was used, modified and based on principles established by Tsingrili, Vadstrup and originally Browning *et al.*[190 191,199]. Biopsies were immediately immersed in transport medium and kept on ice during transport. Time from biopsy to incubator varied between 20 and 120 minutes. Biopsies were orientated under a dissection microscope on a metal grid prior to insertion in a well chamber and added basal medium with or without stimuli up to the metal grid creating an air-liquid interface. Biopsies were incubated for maximum 24 hours at 37 degrees using 95%

atmospheric air and 5% CO2. No supplemental oxygen was used. Four biopsies per individual were obtained, with one biopsy serving as an experiment control incubated in basal medium.

3.1.4 Tissue analysis

A brief description of analysis method and techniques applied in this thesis are outlined below. Detailed descriptions are found in papers 1-3.

Mucosal gene expression

The real time quantitative polymerase chain reaction (RT-qPCR) is a well-established method used for relative or absolute quantification of gene expression. The human genome is encoded within our DNA, located in the cell nuclei tightly packed as 46 chromosomes. For protein synthesis the DNA is transcripted and further translated. On initiation of transcription the DNA unfolds and separates enabling RNA polymerase to read and copy the DNA nucleic acids. A copy with complementary nucleic acids is formed called messenger RNA (mRNA). Translation of mRNA occurs in the cytosol in ribosomes where the mRNA is decoded into specific amino acid chains. In qPCR the amount of mRNA is measured, giving an insight into transcription activity and a cells protein synthesis rate and regulation. An important note is that not every mRNA strand equals protein production[200].

In our laboratory RT-qPCR is performed on a regular basis both for clinical and research purposes and performed by trained bioengineers. Endoscopic biopsies are at the gastroenterology lab immersed directly in RNAlater. RNA extraction is performed consecutively as biopsies are secured and stored at -70 C. In the step of reverse transcription complementary DNA (cDNA) is formed from the mRNA in the sample. The final step of qPCR is performed in a thermal cycler were repeated heating and cooling cycles control activate the polymerase present, resulting in a doubling of the template for every cycle. The results are read by the fluorescence signal released either by the amount of double-stranded DNA present (SYBR-green) or the presence of a specific gene sequence (hydrolysis probes). For papers 1 and 3 hydrolysis probes were used, whilst in paper 2 a pre-defined PCR-array from Qiagen with SYBR-green was used. Further details of RT-qPCR are given in the methods sections of papers 1-3.

A minimal information for publishing qPCR experiments (MIQE) guideline gives a recommendation of elements of the RT-qPCR analysis to reduce error and increase

reproducibility and comparability of results. Quality control steps including negative and positive samples, number of replicates of each sample, inclusion of dilution series, standard curves and melting curves when appropriate[201].

Immunostaining

Immunostaining is a valuable method for visualizing and detecting proteins in a cell or sample. It is based on the use of antibodies to bind to a specific antigen with a secondary antibody attached to a visualizing marker Antibodies are Y-shaped glycoproteins that recognize and bind to specific amino-acids or structural parts of the antigen (called epitope). Immunostaining methods can be immunoenzymatic (where an enzyme, most often peroxidase, or alkaline phosphatase is labelled with a chromogen that precipitates at antigen site) or immunofluorescence (where the antibody is tagged with a fluorescence dye)[202].

We used formalin fixed paraffin-embedded (FFPE) tissue sections in this thesis, with advantages in ease of biopsy handling and preservation of morphology compared to frozen tissue sections[203]. All biopsies were immediately immersed in formalin 10% for 24 hours, further tissue processing including dehydration and paraffin embedding was performed according to clinically validated protocols at the pathology department at the University Hospital of North Norway, Tromsø. Prior to immunostaining 3-4 micrometre sections were cut and mounted on glass slides followed by baking of tissue slides for 1 hour at 60 degrees Celsius and 18 hours at 37 degrees Celsius. All immunostaining was performed manually with included isotype and concentration matched controls.

3.1.5 Statistical methods

In brief, statistical analyses in papers 1-3 were performed with SPSS (version 22, 25 and 28 respectively). In paper 2 analyses were also performed using the R Statistical Environment with Bioconductor R software and the R Stats Package with the lm() function[204].

In paper 1 immunostaining was assessed by two individual raters, both blinded. Interrater agreement was assessed using a weighted Cohen's kappa. In papers 2 and 3 digital software was used for quantification with the Volocity 6.3 software (PerkinElmer, Waltham, MA) and the free resource Qupath respectively[205].

For gene expression studies two main approaches were adopted. For relative quantification in papers 1 and 3 the RT-qPCR results were analysed using delta delta method as outlined by

Livak *et al.*[206,207]. Fold change was expressed as a relative difference between samples and statistically analysed with either paired or independent t-tests or equivalent non-parametric tests. In paper 2 a linear regression model was used for analysis of gene expression between groups following normalisation of data. A paired analysis was used for comparing acute and remission biopsies from the same patient. Differential expression of genes was interpreted and visualised including principal component plot, heatmap, Venn diagrams and bar charts.

4 Summary of Results

4.1 Paper 1

Loss of interleukin 33 expression in colonic crypts – a potential marker for disease remission in ulcerative colitis. Scientific Reports 2016.

In this study we investigated the expression of IL-33 in patients with acute UC treated with infliximab to endoscopic remission (defined as Mayo score of 0 or 1). Mucosal gene transcript levels and immunostaining for IL-33 was used. Biopsies were selected from an IBD biobank including a healthy control group. All UC patients had biopsies from active disease, prior to start infliximab therapy, and at time of endoscopic remission for comparison.

In total fifty study participants (twenty-nine UC patients and twenty-one healthy controls) were included. We observed a significant 3-fold increase ($p<0.001$) in *IL33* mucosal transcript levels when comparing acute UC with healthy controls. Proinflammatory cytokines including *TNF, IL1B, IFNG, IL17A* and *IL23A* were also significantly raised in acute UC. We did not detect a significant difference in mucosal *IL33* transcript levels when comparing acute UC and UC remission. However, when UC remission was stratified into whether *TNF* mucosal levels were normalised (n=10) or not (n=19) a clear reduction in *IL33* was seen in the *TNF* normalised group ($p<0.02$). Immunostaining revealed IL-33 positive cells in epithelial crypts during acute UC in eight out of nine examined biopsies. IL-33 was located to the cell nuclei and not the cytoplasm in epithelial cells. Strikingly, there were no IL-33 positive cells present in the epithelium in disease remission or healthy controls.

Conclusions: *IL33* mucosal gene expression is significantly increased in acute UC. Immunostaining confirmed these findings with IL-33 present in the nuclei of colonic

epithelial cells, vascular endothelial cells and mononuclear cells in the lamina propria. Strikingly, no IL-33 was present in the epithelium of UC in disease remission or in healthy controls. An overall reduction in *IL33* mucosal gene expression was only seen in UC patients when mucosal *TNF* normalised.

4.2 Paper 2

Fibrosis Mediators in the Colonic Mucosa of Acute and Healed Ulcerative Colitis. Clinical and Translational Gastroenterology 2019.

In this study we explored the mucosal gene expression in UC using a qPCR array of eighty-four genes involved in fibrosis pathways. A pairwise study design was used. We included twenty-eight patients with moderate to severe UC who were all treated with infliximab to disease remission defined as a Mayo score ≤2, all with an endoscopic subscore of 0 or 1. In addition a healthy control group was included.

A significant number of differentially expressed genes associated with fibrosis was observed. In total, forty-one upregulated and thirty-eight downregulated genes were observed. Of note, five mucosal genes were found to be independently upregulated in UC remission without overlap in acute UC (actin alpha 2 (*ACTA2*), caveolin-1 (*CAV1*), collagen type III alpha 1 chain (*COL3A1*), lysl oxidase (*LOX*), TIMP metallopeptidase 3 (*TIMP3*) and integrin beta-6 (*ITGB6*). Angiotensinogen (*AGT*) was the only gene with an expression pattern showing upregulation in acute disease and downregulation in a healed mucosa compared to healthy controls. *TGFB* signalling pathways showed an attenuated pattern in acute UC. Pathway analysis also highlighted *TGFB* signalling, extracellular remodelling and profibrotic pathways of interest for further exploration.

Conclusion: A persistent dysregulation of fibrosis-associated mucosal genes was seen in the endoscopically healed mucosa of UC. Of interest, *TGFB* isoforms, *CAV1* and *AGT* are among identified mediators of interest for future research into fibrosis and healing of the UC mucosa.

4.3 Paper 3

Hypo-osmotic stress induces the alarmin IL-33 in the colonic barrier of ulcerative colitis. (Submitted manuscript 2022).

Significant species variation has been reported in IL-33 expression emphasising the need for human models. In this study we established a human *ex vivo* colonic biopsy model to explore factors that could induce IL-33 expression in the colonic epithelium.

We used an air-liquid interface model based on endoscopic biopsies adapted from Vadstrup, Tsilingiri, and Browning& Trier. Biopsies were taken from the sigmoid colon. Stimulants were added to the basal medium. Endoscopic pinch biopsies from healthy controls and patients with UC were cultivated for up to 24 hours. Control biopsies (non-stimulated) were included in every experiment, and all results were repeated minimum x 3 for reproducibility. Biopsy viability was assessed by histological assessment of tissue architecture and immunostaining. Biopsies stimulated with IFN-gamma showed a strong positive epithelial signal for pSTAT1 supporting the viability of the model.

Immunostaining revealed a strong expression of IL-33 in colonic crypts in healthy controls and UC following hypo-osmotic stress for twenty-four hours. No epithelial IL-33 was present in control biopsies. Stimulation with known inflammatory mediators including IFN-gamma and TNF did not induce IL-33 in colonic crypts. A sporadic, but definite presence of a few positive epithelial cells was seen following TGF-beta-1 stimulation for twenty-four hours in healthy controls. RT-qPCR of colonic biopsies also showed an increase in IL-33 following hypo-osmotic stress.

Conclusions: Hypo-osmosis induced a strong IL-33 expression in colonic crypts. The *ex vivo* biopsy model served well as a mechanistic model with potential for future use in IBD research

5 Discussion

5.1 Methodological considerations

5.1.1 Study design

This thesis included two different study designs. In papers 1 and 2 study participants were included from a well-stratified prospective IBD study at the University Hospital of North Norway with biopsies stored in an IBD biobank from 2008-2012. Study participants with moderate to severe UC treated with infliximab were included if they achieved disease remission defined as a full MAYO score of 2 or less. Thus, a fairly homogenous group of patients, all with moderate to severe UC with response to infliximab therapy were included. In addition, a healthy control group was included from the same biobank. The sample size of our studies was limited by available samples in the IBD biobank and not predetermined, this is a limitation and a potential source of type 2 error.

In paper 3 a case-control study was used for developing an *ex vivo* colonic biopsy model. Further, a group of treatment naïve UC patients were included for immunostaining from the prospective advanced study of Inflammatory bowel disease (ASIB) study 2012-2020.

5.1.2 External validity

Study Population: The extent of generalisability of the results of this thesis includes consideration of the selected patient population. In this thesis study participants were included consecutively from a tertiary university hospital clinic. The population of the municipality Tromsø is mainly composed of Caucasians, with a report from 2020 reporting approximately 15% with a Sami ethnicity[208]. Incidence and prevalence rates of UC are reported to have a north south gradient, with higher incidences in the Northern regions. It is not clear whether this is due to genetic, environmental factors and needs to be taken into account when considering the generalisation of study results.

In papers 1 and 2, the exclusion of UC patients that were not currently being treated with infliximab could limit the generalisability of the results as well as a pre-defined selection of moderate to severe UC. Arguable strict inclusion and exclusion criteria limits the external validity.

Selection bias: Patients in paper 1 and 2 represent a select UC group who all received anti-TNF therapy for moderate to severe UC. This is fairly representative for UC patients in Norway where moderate to severe anti-TNF that has failed on steroids is an indication for anti-TNF. In paper 3 patients with acute UC at time of diagnosis were included consecutively. The sample size is small which limits generalisability. The use of a case-control study design includes the potential source of bias through "convenience sampling"[209]. This bias was considered to be minimal as both controls and UC patients were subject to the same examination and follow up as all patients examined at the endoscopy ward and did not warrant any additional examination or follow up.

The question whether our control group was representable for a healthy population was considered. An event (most often screening for colorectal cancer due to rectal bleeding) would be the endoscopy referral reason in this group. The median age in the control groups was higher than the UC group (borderline significant at p= 0.05 in paper 2). This is not unexpected as UC has a peak incidence at 20 years whilst screening for colorectal cancer is reserved for an elderly population often over 50 years of age and needs to be taken into consideration when interpreting the results. Ideally a random sampling of a healthy population would be preferred, however this is not without ethical quandary as colonoscopy is an invasive, uncomfortable procedure.

5.1.3 Internal validity

The extent to which observed results reflect a true observation in the study population is referred to as the internal validity of a study. Information bias (collection, analyses and interpretation of data) and confounding variables are all sources that can influence conclusions being reached.

Confounding variables can be both unmeasured and measured variables that give an alternative explanation to findings or study associations. Efforts to minimize the effect of confounders include adjusting for age and gender. In the linear model in paper 2 age and gender were adjusted for, in addition an analysis adjusting for endoscopic score 0 and 1 was performed. Our study sample size was the main limitation for adjusting for more variables.

Information bias and **measurement errors** are important for the validity and reproducibility of results. Misclassification is an important information bias. Following inclusion of UC

patients all medical records were checked meticulously by a clinician, including endoscopy reports and histology reports to check they met inclusion criteria. Patients in paper 1 and 2 had a longer time of disease duration adding to the certainty of their diagnosis. Population based studies report a small percentage of UC patients whose diagnosis is revised[37,210].

The main methods used throughout this thesis (RT-qPCR and immunostaining) are discussed below with a stepwise approach to potential pitfalls and efforts to reduce this bias outlined below.

5.1.4 Mucosal gene expression (RT-qPCR)

There are many steps in the RT-qPCR procedure, and a standardised approach is important to minimize error, also reflected in the MIQE guidelines by extensive checklists[201]. Correct biopsy handling, fixation and storage, RNA extraction, reverse transcription and the PCR analysis are essential to achieve reliable and repeatable results. Technical variation is reduced by gene normalization including the use of reference genes, whilst biological variability is addressed by sample numbers and replicates. Sources of variance in the lab are day to day variation, batch differences, equipment and changes in laboratory staff[211]. In our laboratory RT-qPCR is performed on a regular basis both for clinical and research purposes and performed by experienced bioengineers.

Pre-analytical factors:

Biopsy sampling is standardised, and colonic biopsies are immediately put in a stabilisation buffer RNAlater (which inhibits enzymatic degradation and reduces the impact of variations in sample handling). The advantage with RNAlater is that once immersed it can be kept in room temperature (25 degrees Celsius), according to manufactures instructions up to 1 week (Qiagen, Hilden, Germany).

RNA extraction: Several methods for RNA extraction exist. We have used membrane and column-based purification and isolation techniques (SV total RNA isolation system, Promega) from 2008 until May 2013 as described in papers 1 and 2. Thereafter a change in manufactures and equipment to optimise the workflow was performed including use of automated spin columns (Qiagen cube), included in paper 3. Advantages with automated column purification is less DNA contamination during RNA isolation, reduced loss of RNA and ease of use.

Storage of nucleic acids: Following RNA extraction biopsies are stored in a -70 degrees Celsius freezer until time of experiment. A neutral pH environment, aliquoting samples to avoid multiple freeze thaw cycles and storage at -20 to -80 degrees Celsius contribute to maintain RNA integrity.

Quality control of RNA: Concentration and purity of RNA was performed using the Agilent bioanalyzer giving a RIN value from a scale of 0-10 where levels of 7 indicates a good integrity. If degraded the cDNA product becomes shorter. In qPCR RIN levels down to 5-6 can be successfully used if assays are designed with short templates. All inhouse assays are designed to be robust and have short templates of 80-120 base pairs. Internal sample controls from our biobank include average RIN of 8.40 with a standard deviation of 1.67.

Analytical considerations:

Reverse transcription: The reverse transcription has shown to have a higher technical variability than qPCR[212]. Factors that improve reproducibility are the same protocol and reaction conditions, optimised for temperature, time and combination of master mix. RNA concentration is measured, and equal concentrations of total RNA is calculated for each tube. In addition, RT-qPCR workflow is kept to few highly expert hands in our lab. To achieve the lowest error more replicates of samples can be included in the reverse transcriptase step, however careful assessment of yield, reproducibility versus considerations to cost, availability of material and experimental design are reasons for not including duplicates in the reverse transcription step.

qPCR: For paper 1 and 3 hydrolysis probes were used, they are designed to only bind to the target sequence. In paper 2 a predefined PCR-array using SYBR-green assays was purchased commercially (Qiagen, Hilden, Germany). Advantages of the PCR-array include the possibility of analysing a high number of genes of interest with small amounts of RNA. An advantage with the hydrolysis probe is that they do not detect primer dimers, an artefact or by-product that can cause inhibition and reduce amplification of the target gene. In papers 1 and 2 biopsies were run in duplicates, whilst in paper 3 this was increased to triplicates due to the small sample size.

The setup of the qPCR plate is also important. We preferred all genes or all samples to be analysed on the same plate, when this was not possible inclusion of an interplate calibrator was used (TATAA interplate calibrator).

Reference genes: The ideal reference gene does not differ between experimental and control groups thus being able to adjust for biological and technical variability. A universal reference gene does not exist, and an individual evaluation is recommended for each given experiment[213]. Several strategies are employed for finding the best suited reference gene – including use of computer software programs such as Normfinder[214]. For RT-qPCR experiments our lab has previously validated the use of beta actin (*ACTB*) in the setting of acute UC mucosal biopsies[215,216]. Further, an inhouse validation of reference genes found 60S acidic ribosomal protein P0 (*RPLP0)* to show good stability and was included in paper 3 as reference genes.

In paper 2, a predefined panel of five reference genes designed by the manufacture was used. These were analysed across all samples and in combination, however significant differences between groups (defined as over 1 cycle threshold) were seen. We thus analysed all genes in the predefined PCR-panel across samples using the Normfinder software selecting endoglin (*ENG)* and transforming growth factor-beta receptor-1 (*TGFBR1)* which showed a stability value of 0.002 across sample groups[103].

DNA contamination: Use of DNase and the inclusion of human genomic DNA control in the qPCR analysis was used to make sure DNA did not contaminate samples. Other measures employed to reduce the risk of sample contamination include physical separation between sample preparation, pre and post amplification areas. Additionally, all probes in our in-house assays span exon splicing points, which precludes detection of genomic DNA in the qPCR step.

Analysis: This thesis was designed to detect relative differences between samples. This is determined by using the qPCR amplification curves. We set an arbitrary threshold manually, well above the background noise in the exponential phase where the curves are parallel to each other, this is best judged in a log scale. The point where the amplification curve crosses the threshold is called the Cq or cycle threshold (CT). The CT values are inversely proportional to the initial copy number - meaning a low CT value represents a relative higher

amount of the gene of interest in a given experiment. Fewer PCR cycles are needed to raise the gene above the set threshold. Limitation of detection was set at 40 cycles in our experiments.

Quality control measures on exported data include check of no template controls, no reverse transcription controls, and positive controls. The raw data is then normalised to a reference gene (as mentioned previously). Assuming 100% qPCR efficiency Livaks equation of delta delta CT can be used to find a ratio between groups, often given as fold change[206].

5.1.5 Challenges with immunostaining

Immunostaining is a valuable method for detecting and visualising of proteins in tissue. Methodological considerations are discussed below with a focus on potential pitfalls and optimisation and choice of staining techniques. In this thesis all immunostainings were performed manually.

Preanalytical factors:

Factors that may influence staining results include tissue handling and fixation methods as well as storage prior to analysis. Both prolonged fixation in formalin and inadequate formalin fixation can influence the immunoreactivity in FFPE sections. Endoscopic pinch biopsies from the colon are small, and penetration of formalin fixative was not considered a problem. Moreover, formalin may mask epitopes which is largely overcome by use of heat induced epitope retrieval and optimal pH in buffers used. In the literature, antigenicity in FFPE sections has been shown to be well preserved in over 60 years[217], though reports of lower antibody affinity have been described compared to freshly cut sections. In this thesis we used freshly cut sections, baked overnight prior to immunostaining.

Analytical factors:

This includes the specificity and affinity of the primary antibodies used. Optimalisation of immunostaining protocols including deparaffinization and rehydration steps, epitope retrieval, antibody concentration, incubation time and temperature, washing steps and blocking steps. The inherent qualities of the tissue also influence choice of detection systems[218].

Antibody selection: Primary antibodies are either monoclonal or polyclonal referring to their generation from a monoclonal B-cell line or from live animals respectfully. Although in

principle a specific antibody should bind only to the targeted antigen on the protein surface, in practice this can be a much more complex and an unpredictable affair[202]. Antibodies are widely available commercially; yet it is increasingly recognised that inconsistencies between suppliers exist. The human protein atlas project is an effort to systematically map human proteins and including antibody imaging[219], however in this work of validating over 5000 specific antibodies for immunohistochemistry Berglund *et al*. found somewhat disconcerting that over half of the commercially available antibodies were not suitable[171]. Thus, antibodies need to be appropriately validated for their application not only for internal validity, but also for the reproducibility and replication of results referring to external validity. There is currently no universal agreement on how to report and validate antibodies. One international working group looking at antibody validation have outlined five pillars for application-specific validation of antibodies to reduce potential pitfalls with antibody reliability and specificity[220].

Antibody validation: In paper 1 we compared two antibodies targeting IL-33 (monoclonal mouse antibody and a polyclonal goat antibody, both previously published for IHC in human colonic tissue). Staining results were also performed in two separate labs supporting reproducibility. The choice of a monoclonal antibody has advantages as more specific for single epitopes, reduce cross reaction with other proteins and inter-batch-variation[221]. For all antibodies we performed dilution series prior to use of new batches, use of different buffers for heat induced epitope retrieval (HIER) as well as inclusion of positive controls (for IL-33 tonsillar tissue was used). Isotype and concentration matched negative controls were routinely used in all experiments to detect any unspecific binding to the tissue. In paper 1 we used two different antibodies targeting IL-33 (mouse and goat origin), using the "independent strategy" outlined in the five pillars of application-specific validation. Gold standard verification of antibody specificity would be to use a knock-out model, for obvious reasons not an option in human studies. In papers 2 and 3 antibodies were selected based on their performance in the Human Protein Atlas database using validated antibodies with a high reliability score[171].

Antigen and epitope retrieval: Formalin modifies the tertiary structure of a protein and its epitopes, thus may hinder antibody binding. High temperature unmasks epitopes, and restores the immunoreactivity despite probable denaturing of the protein[222]. We performed HIER for twenty minutes in a water bath at boiling temperature and twenty minutes of cooling down. A

universal antigen retrieval buffer does not exist, and we tested our antibodies in various buffers for antigen retrieval ranging in pH from 6 -9 prior to experimental use.

Blocking steps: Blocking steps reduce unspecific protein binding, which may lead to false interpretation of results. Colonic tissue has endogenous alkaline phosphatase, thus we chose a immunoenzymatic detection system using horseradish peroxidase[222]. Pre-treatment steps include blocking for endogenous peroxidase prior to immunoenzymatic staining. For immunofluorescence staining a blocking step with serum from the host of the secondary antibody were used. Importantly, we used IgG free BSA as antibody dilutant when using goat-antibodies to avoid unspecific IgG bindings.

Background signals: In our material we experienced that in the FFPE biopsies from the colon a lot of autofluorescence was present particularly when using immunofluorescence at 488 wavelength (green), and could be both due to inherent colon tissue properties or fixation. We increased washing steps in the staining protocol to 5 minutes x 4 which reduced some autofluorescence. Further we observed that shifting the fluorescence emission to higher wavelengths 555 and 647 decreased the presence of autofluorescence. Blocking steps with the serum of the secondary antibody was used routinely (goat serum).

Pitfalls during immunostaining steps include care that the sections at no point dry out which could lead to unspecific background staining and false results. Meticulous care to avoid contamination between different antibodies used in the laboratory is important. Routine inclusion of positive and negative controls in experiments[203].

5.1.6 *Ex vivo* tissue culture

Below is a discussion of general considerations taken during development and choice of the colonic explant model used in this thesis and presented in detail in paper 3. It includes pre-analytical considerations to biopsy sampling, transport and handling as well as analytical remarks with regards to experimental conditions, viability, and biological variance.

Following a review of the literature we found several models based on endoscopic colonic biopsies with an air-liquid-surface. Vadstrup *et al*. used a specifically designed disk with a metal mesh[223]. We adopted a similar model using a triangular mesh placed instead in a 12-well dish, which is similar to a previous successful model used for skin biopsies[224].

Biopsy handling: A jumbo biopsy forceps with 2,8 mm open diameter was used to procure a larger biopsy specimen. They are available with or without a spike in the forceps. They have the advantage of yielding adequate samples (length > 3mm, penetration to the muscularis mucosa and < 20% crush artifact. [225,226]. We chose biopsy forceps without a spike to avoid additional tissue injury as an injury mid-biopsy could induce a wound healing response and act as a confounder. Endoscopic biopsies are routinely taken along the course of the bowel. In general, perforation risks during endoscopy are reported low at 4 per 10 000 colonoscopies[227]. It is also given that the risk is higher in the right colon and caecum compared to the left due to bowel wall thickness. In this thesis all biopsies were taken in the left distal colon. No adverse events were reported.

Medium and supplements: Choice of model and medium was based on review of previous successful *ex vivo* colonic explant models in the literature. We adapted features from Vadstrup *et al.* explant model (again based on principles of Tsilingri and originally Browning and Tier) as they included use of IBD derived biopsies as well as controls[190,191,223]. Modifications in our methods is the use of 95% air at sea level atmosphere, and not 95% O2, with the rational that this reflects more the *in vivo* cell oxygen perfusion state. Fletcher, Roberston, Bareiss *et al* have all reported successful explant culture viability with use of 95% air and 5% CO2 during biopsy incubation time[193,194]. Review of the literature revealed both RPMI and DMEM have previously been successful mediums for use of colonic explants[194]. DMEM was utilized and 10% foetal calf serum (FCS) was added. FCS is widely used as a supplement for growth supplements in tissue and cell cultures as abundant micro and macronutrients as well as growth factors are present[228]. Batch to batch variations of FCS were avoided as FCS was aliquoted from the same batch for paper 3 and kept frozen until use. The European Union has a directive on the legislation for protection of animals used for scientific purposes as there are ethical concerns with the use of animal serum[229].

The addition of antibiotics in the basal medium, intended to avoid bacterial overgrowth is however a weakness as the commensal microbial flora and interaction with the epithelial barrier is altered. We did not test biopsies without antibiotics added to the medium.

Explant technique: We chose the metal grid and air-liquid interface model out of practical considerations as simple, quick, and required minimal handling of the biopsy, all considered

as favourable factors. Short proximity between the clinical and research lab was also an advantage with regards to ease of transport and time to incubation.

Biopsy viability: Comparison of histology at time of biopsy (0h) and 24 hours was evaluated. We evaluated tissue morphology and integrity with histological evaluation using hematoxylin and eosin stain. Shedding of epithelial cells was present, however colonic crypts were still preserved. Longer incubation time > 24 hours resulted in increased loss of the epithelium. In biopsies stimulated with hypo-osmotic medium a general increase in shedding and signs of dysmorphological cells was present.

Histological morphology was assessed with hematoxylin and eosin staining. Further we tested the biopsy models with stimulation of IFN-gamma and confirmed a positive pSTAT1 in endothelial and epithelial cells. These findings confirmed the uptake of IFN-gamma throughout the biopsy (from basal side up to the epithelium) and served as a positive control for IFN-gamma. We thus concluded that our biopsy was valid as a mechanistic model in this setting. Further we stained biopsies of the proliferation marker Ki67, again supporting the viability of cells. Variations and quality control of the experimental conditions was confirmed using one of four biopsies as an experimental control without stimulus (only basal medium).

Reproducibility: Inter-subject variation both in UC patients and healthy controls is a concern as the sample size is limited and heterogenous. We were able to reproducibly stimulate IL-33 expression, as well as in patients with UC. Further we found pSTAT1 positive endothelial and epithelial cells in all biopsies stimulated with IFN-gamma.

Other steps that reduce variation include use of a standardised workflow including biopsy sampling and transport method and time.

Analysis: The main advantage of a biopsy explant model is the avoidance of inter-species variation. In addition, the maintained 3D structure complete with cells and structures of the immune system – both known and unknown contributors are present. The lack of, and abrupt removal of tissue from its blood supply is a natural limiting factor which contributes to the limited longevity of biopsies. A potential confounder is the natural tissue denaturation during the time period and local stress responses induced in the tissue.

5.1.7 Statistical considerations

Below is a general discussion of type 1 and type 2 errors relevant to this thesis, in addition independent versus dependant samples and interrater comparison is addressed.

Type 1 error: In papers 1 and 2 the problems with multiple comparisons were addressed. In paper 1 we used the Bonferroni p-value correction to reduce the risk of a type 1 error occurring, i.e., the more times you test you increase your chance of a false positive test (type 1 error) just by chance. The Bonferroni method is a simple adjustment where the p-value is divided by the number of comparison (k) giving an adjusted P-value to controlling the overall type 1 error rate to no more than 5%[230]. Arguably the Bonferroni correction for multiple comparisons may be too strict when the number of comparisons increase. This results in a reduction of the type 1 error at the expense of type 2 error. In paper 1 we had n= 9 gene comparisons and Bonferroni adjustment was employed.

In paper 2 the Bonferroni method was assessed too conservative as a larger set of multiple comparison (n= 84 genes) was used and this was an explorative paper. We used a different approach with the Benjamini Hochberg adjustment and false detection/discovery rate[231].

Type 2 error: All our papers are limited by the small sample size and lack of prior power calculation for the studies. Thus, increasing sample size could reduce the type 2 error. A cost of multiple comparisons, as addressed above, the type 2 error is inflated when strict adjusted p-values are used. For paper 2 we adopted an explorative approach. In this setting it is not agreed that correcting for a type 1 error should be routinely used as this inflates the type 2 error. Rothman *et al*. argues that on the contrary, scientists should not be so reluctant to explore leads that turn out to be false rather than missing potentially important findings[232].

Independent vs dependent samples: Between-subject variation is reduced by using a paired samples design. In papers 1 and 2 biopsies from before and after infliximab therapy were analysed as paired per individual and not as independent samples. In the case of gene expression other variations including biopsy location, time and cellular composition of biopsies can equally drive the variance between samples and gene expression. Correction for gender was used in both comparisons between groups in both dependent sample comparison and in the linear regression model. In paper 2 we also examined the data set for difference in

endoscopic remission score 0 or 1. Our sample size and power limited further corrections for possible confounders.

Missing data: only data with complete observations were included in the final analysis. In paper 2 the assay for gene *IL5* did meet quality control with > 20% missing variables and was thus not included in the final analysis. An imputation algorithm which is a robust model to minimise concerns about selection bias and skewed data set due to missing data was employed in paper 2 in the gene expression analysis based on nearest neighbour averaging method (Impute package R).

Interrater reliability: In paper 1 the presence of IL-33 positive cells were classified on a five-point scale from "negative" to "very high". Misclassification is a potential source of information bias. Cohen´s kappa assesses the agreement beyond chance between two raters or ratings and can be used to assess interrater variability. We chose Cohen´s weighted kappa because there were five ordinal categories, and hence rater disagreement between category one and five is more important than between neighbouring categories. We found a strong agreement (0.83) between raters, to suggest our approach to classification was appropriate[233].

5.2 General discussion

This section is an integrated discussion of results based on all three papers.

The cellular location of IL-33 in the colonic mucosa

In our studies (papers 1 and 3) immunostaining detected IL-33 in the nuclei of epithelial cells in active UC, including both treatment naïve and anti-TNF treated patients[234]. Importantly, in the healthy control groups we did not observe nuclear nor cytoplasmic expression of IL-33 in the colonic epithelium leading to our conclusion that IL-33 is not constitutively expressed in the normal colonic epithelium, rather an inducible factor.

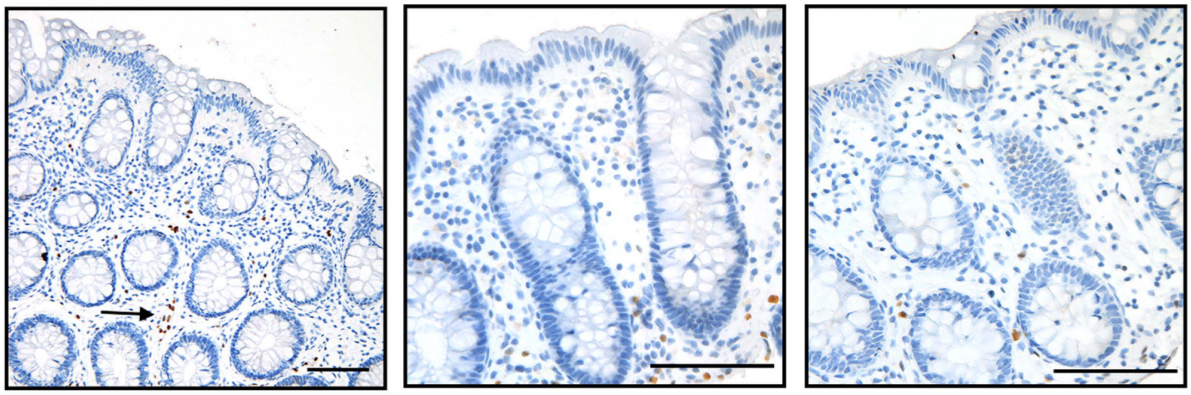

Figure 9. IL-33 expression in colonic biopsies from healthy controls
IL-33 (brown), cell nuclei (blue). Reprinted from https://doi.org/10.1038/srep35403[234].

In the literature there are conflicting data regarding the location of IL-33 in the colonic mucosa. Our findings are in contrast to one of the most cited articles where cytoplasmic and nuclear IL-33 are reported in healthy controls and UC patients[177]. One explanation for these conflicting findings is the use of different antibodies for detecting IL-33. We therefore included both antibodies (polyclonal goat and monoclonal mouse) as part of our study and found good co-localisation. To limit non-specific antibody binding we used tonsillar tissue as positive controls and negative controls to determine working concentrations of the respective antibodies. Differences in results may be due to different staining protocols and working concentrations of antibodies. Further, IL-33 as a nuclear expressed cytokine in the colon is supported by others[173,175].

In concordance with the literature, we found a strong IL-33 signal present in endothelial cells (dual staining performed for IL-33 and CD34). On reflection, it appears useful to compartmentalise the expression of IL-33 in the colonic mucosa, we propose using 1) epithelium 2) endothelium 3) lamina propria as the role of IL-33 is dependent on its cellular and tissue context.

Biopsies in our study were from the left, distal part of the colon. We did not examine the right colon. Differences in mucosal cytokine expressions have been described between the right and the left colon[235]. It is of interest that IL-33 has been found present in the gastric mucosa, an acidic environment that differs greatly from the colonic mucosa[168]. Thus, local environmental factors appear important with regards to the expression of IL-33 in the epithelial border.

IL-33 – an inducible alarmin

Once we had established that IL-33 was not constitutively expressed in the healthy colonic border we were interested in potential inducible factors. We developed a human explant biopsy model to test our hypothesis thus avoiding interspecies variation. Based on our findings in paper 1 that patients with moderate to severe acute UC showed a heterogenous but definite presence of IL-33 in colonic crypts, we looked at inflammatory mediators as inducible factors. The chronic inflammation in UC has classically been thought of as Th2 driven, however our research group and others have questioned this and not found a significant difference between Th1 and Th2 cytokine responses expressed in the mucosa[64,65]. Both proinflammatory cytokines TNF and IFN-gamma were tested as possible inducers, as well as hypo-osmotic stress following reports that both IFN-gamma and hypo-osmotic stress induced IL-33 in the skin[224].

Our most striking observation was the strong IL-33 expression induced in colonic epithelial cells by sustained hypo-osmotic stress (paper 3). This is a novel finding in the colon and relevant in UC considering the role of the colonic barrier in fluid regulation and exposure to luminal content. We did not observe cytoplasmic staining of IL-33 in the epithelial cells and mode of IL-33 secretion was not further determined. One possible mechanism may be that the induction of epithelial IL-33 represents a "fail-safe-mechanism" in cells under immense stress and possibly imminent threat of death. Thus, nuclear IL-33 accumulation may be an effort to protect inadvertent release of IL-33 to the internal milieu where it would potently perpetuate the inflammatory response. Our findings support IL-33 as an inflammatory or cellular stress induced cytokine and not constitutively expressed.

We were able to demonstrate a strong activation of pSTAT1 in the colonic epithelium following IFN-gamma supporting good absorption throughout the biopsy. Moreover, only a negligible response was seen with IFN-gamma alone and TGF-beta-1, whilst TNF did not induce any IL-33 expression in our experiments. This may be due to limitations of an *ex vivo* model, timepoints as cytokines have different half-lives, and stimulus also depends on the cellular environment and cytokine milieu in the biopsy model.

IL-33 in UC

In this thesis we have observed IL-33 as an epithelial located nuclear cytokine in UC during active UC inflammation, irrespective of treatment status and duration of UC disease (paper 1 and paper 3). Interestingly, IL-33 positive epithelial cells in acute UC were associated with neutrophil invasion and adjacent inflammatory infiltrates suggestive of tissue stress (paper 3). We found IL-33 positive cells in epithelial crypts during active disease, in endothelial vessels as well as present in mononuclear cells in the lamina propria. In concordance with others, we found mucosal *IL33* mRNA levels significantly raised in acute UC compared to healthy controls[173,175,176,178], however not significantly changed between acute UC and remission UC. The latter may be due to observed differences in cell populations responsible for *IL33* expression and highlights the value of immunostaining.

It is of interest that differences in IL-33 expression in the lamina propria of inflamed CD and UC have been reported. IL-33 was found to cluster underneath ulcers in UC suggesting of a role in mucosal repair, whilst absent in CD fissures[175]. It would be of future interest to examine whether epithelial IL-33 is present in both entities of IBD.

Experimental models have shown that targeting the IL-33/IL-1RL1 (ST2) pathway ameliorates inflammation. Considering the pleiotropic roles of IL-33, increased knowledge of its nuclear function, secretion mode and role in wound healing in the colon are of interest to further establish its potential as a therapeutic target in UC.

Osmotic stress and UC

Loss of barrier integrity and increased leakiness are central in the pathogenesis of UC. Thus, osmotic stress to the epithelium is of relevance. Small studies from the 1980´s examined osmolality of the faecal fluid in patients with IBD, they reported a higher osmolality content in CD than UC[236,237]. Protective defences of the epithelium are weakened in UC resulting in increased fluid influx and exposure of the internal milieu to the “external” luminal conditions. As proposed by Medzhitov *et al*., the trigger of inflammation likely shapes the resultant inflammatory response, for example the inflammatory response induced by cellular stress of tissue damage would include tissue reparative responses versus a antimicrobial responses following an infectious trigger[74]. Targeting triggers of inflammation are of interest in UC. Our findings in paper 1 and 3 link UC with a potent cytokine (IL-33) of the innate immune system that is induced in colonic crypts following hypo-osmotic stress. A limitation in our studies is

the lack of a hyperosmotic stimulus to establish whether IL-33 responds only to hypo-osmotic perturbations. Osmotic perturbations in experimental models have also shown lasting alternations in the gut microbiota and changes to innate immune response, also of interest in IBD[238]. Following reports in the skin barrier and our findings in paper 3, hypo-osmotic stress may represent a generic homeostatic response of IL-33, inducing nuclear IL-33 expression in both keratinocyte and colonyctes[81].

Mucosal healing

When is the colonic mucosa truly in disease remission, and what constitutes remission in UC? There is no general consensus on the definition of a "healed mucosa in UC". This is reflected in the wide range of definitions including clinical, endoscopic, histological, and immunological remission. In the past few years there has been a shift in the definition of mucosal healing with a trend towards use of stricter definitions of endoscopic remission including use of a Mayo endoscopic subscore of 0 rather than 1, however there is no consensus as yet[2,21,44]. Recent studies also advocate the use of histological remission as associated with improved clinical outcomes[239]. Again, there is no uniform agreement on which histological score to use, three exist including the original Geboes score, and the recently validated Nancy's score and Robarts score[240-242].

Our findings in paper 2 of a continued dysregulation of fibrosis-associated mediators when examining patients in disease remission indicates that there is still an ongoing mucosal response. It is not clear how we are to distinguish between ongoing inflammation, mucosal healing and fibrosis activity, recognising that there is a sliding transition and overlap. Clinical and endoscopic features alone are not sufficient to distinguish between these states.

In papers 1 and 2 we included UC patients defined as disease remission with a Mayo endoscopic subscore of 0 or 1. A score of 1 arguable includes endoscopic appearance of redness and oedema, however there is conflicting data on whether this influences clinical outcome[243].The inter-rater reliability of the Mayo endoscopic subscore also needs to be considered, with a kappa ranging from 0-45 to 0.75 reported in a systematic review[244]. We corrected for endoscopic subscore in the statistical analysis in paper 2, finding no significant impact on our results. We concluded that in a macroscopically defined healed mucosa there is

still ongoing activity beneath the surface. The added use of immunological markers to define disease remission, coined in our research group as *"immunological remission" [Florholmen]*, holds future potential for an individually tailored – therapeutic approach and a more precise reflection of the ongoing pathological mucosal responses.

Fibrosis associated mediators and IL-33

In this thesis we explored snapshots of the mucosal gene expression comparing the UC in the acute inflamed state and disease remission. We found a persistent upregulation of fibrosis associated mediators in the remission mucosa. Of particular interest was the finding of upregulated gene expression of five fibrosis associated mediators specific to the healed mucosa, and not acute disease or controls. These genes are associated with collagen synthesis and degradation. This snapshot of the mucosal dynamics suggest that the restitution of the mucosa is still ongoing. It could represent the fact that endoscopic biopsies in different stages of disease have a heterogenous cell composition[245]. A limitation in paper 2 is that the pre-selected fibrosis panel of genes is neither comprehensive nor specific for intestinal fibrosis, of note *IL33* was not part of the gene panel. There was however overlap in the UC patient material in papers 1 and 2, where in paper 1 *IL33* mRNA was significantly upregulated compared to healthy controls.

The gene *ACTA2* is associated with myofibroblasts, a crucial player both for mucosal wound healing and contraction of the wound, but also central in fibrosis. It is of interest that IL-33 is also linked to the development of fibrosis[175,246]. IL-33 has also been linked to fibrosis and wound healing as well as promoting T-reg function in the intestine[170]. Recently IL-33 has been shown to influence myofibroblasts, essential cells in wound healing and wound contraction that produce collagen[247]. In paper 1 and paper 3 IL-33 positive cells were observed in the lamina propria including mononuclear cells often located subepithelially and with a morphology resembling myofibroblasts, but not further characterised.

With regards to fibrosis there are no current markers to identify early stages of intestinal fibrosis. Trichrome staining of collagen fibres can be performed though no clear guidelines for interpretation. In Crohn's disease over 50% of patients develop fibrostenotic complications, whilst the number most often quoted for UC is 10-11% and believed to be largely underestimated[112,248]. In paper 2 we have identified mediators of fibrosis in UC of

interest both for defining disease remission and as markers of fibrosis. Inflammation is a trigger, but resolution of the inflammatory response does not necessarily stop the pathophysiological response of fibrosis. Myofibroblasts are central in both mucosal healing but also key mediators in fibrosis[100]. Determining what factors drive the development of fibrosis is not fully elucidated.

Human *ex vivo* models for studying IL-33

In this thesis we successfully used a human explant model to investigate IL-33 expression. As a mechanistic model we were able to verify the presence of pSTAT1 signalling following IFN-gamma stimulation, the presence of proliferating cells (Ki67 positive) and maintained morphology of colonic crypts up to twenty-four hours. Thus, we found the explant biopsy model useful as a mechanistic model for studying cytokine expression in the colonic mucosa. The model was simple and inexpensive and may prove useful for testing other hypotheses of the mucosal immune response. Endoscopic pinch biopsies are a common procedure in any colonoscopy clinic, thus access to biopsies is less complicated than biopsies from surgical resected bowel as used by Tsilingiri *et al.*[191]. Our model gives a crude insight into intestinal signalling of IL-33, and importantly firmly establishes IL-33 as an induced nuclear cytokine in the colonic epithelium. Limitations of the model are given in paper 3, in short, the use of antibiotics may interfere with microbial-epithelial interactions and interpretation of results. Further concentration of stimulants, incubations times that may miss important time-dependent mechanisms such as short-lived cytokine signalling and the composition of cells in the biopsy need to be considered. Taken together, these limitations may contribute to our results following TNF stimulation. TNF has different roles according to tissue and cellular context including pro-inflammatory, promotion of apoptosis and in IBD a clear pathophysiological role not fully understood[249]. Moreover, in support of developing a human based model is the increasing concern of discrepancies between experimental animal models and humans. Considerable interspecies variation is reported between murine and humans both in gene and protein expression [150,224,250,251]. This is especially true in the case of IL-33 where significant interspecies variation has been shown in the skin barrier[224].

5.3 Conclusion

In this thesis new knowledge of the expression of IL-33 in the colonic epithelium in Ulcerative colitis and in a healthy control group is given. In addition, our findings give further support to areas where the literature is lacking and show conflicting results.

Specifically;

- IL-33 is expressed in colonic crypts during active UC in a heterogenous manner
- IL-33 is not constitutively expressed in a healthy control population
- Known mediators of fibrosis are present in the mucosa of UC in disease remission (defined as Mayo score ≤2)
- Hypo-osmotic stress is a strong inducer of nuclear IL-33 in epithelial cells in a healthy control population

5.4 Future perspectives

This thesis supports IL-33 as an epithelial alarmin in UC, induced by inflammation and hypo-osmotic exposure. Epithelial alarmins are gaining interest as potential therapeutic targets of chronic inflammation, currently clinical trials where monoclonal antibodies targeting IL-33 and its receptor IL-1RL1 are being tested for chronic asthma[252]. There are however still gaps in our knowledge of the pleiotropic homeostatic functions of IL-33 in the intestine both as an epithelial alarmin and a role in mucosal healing that warrant further investigation.

There is in clinical practice an unmet need for robust markers both for defining when UC patients are in disease remission and to identify early development of complications such as fibrosis. We have identified several mucosal markers associated with fibrosis of interest in UC, however they need further exploration and validation.

The establishment of a simple, effective biopsy model holds potential for future translational projects with a view to a personalised/individualized medical approach. Combined with an omics approach a biopsy model holds potential for hypothesis testing of explorative results generated from transcriptomics, proteomics and metabolomics. Another aspect is the potential of precision medicine – finding the right medication for the patient by identifying and targeting specific immune pathways.

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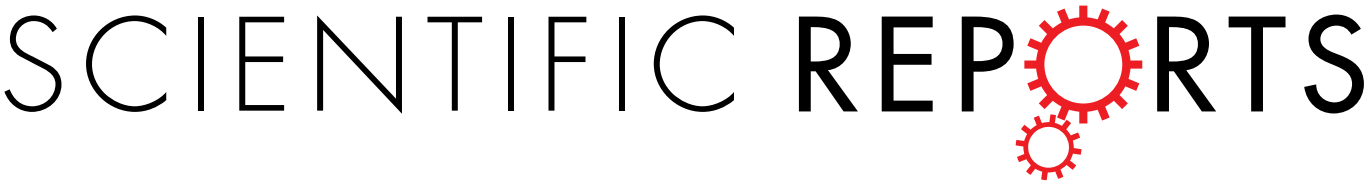
Paper 1



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Loss of interleukin 33 expression in colonic crypts - a potential marker for disease remission in ulcerative colitis

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Interleukin 33 (IL-33) is a cytokine preferentially elevated in acute ulcerative colitis (UC), inferring a role in its pathogenesis. The role of IL-33 in intestinal inflammation is incompletely understood, with both pro-inflammatory and regulatory properties described. There are also conflicting reports on cellular sources and subcellular location of IL-33 in the colonic mucosa, justifying a closer look at IL-33 expression in well-defined clinical stages of UC. A total of 50 study participants (29 UC patients and 21 healthy controls) were included from a prospective cohort of inflammatory bowel disease patients treated to disease remission with infliximab, a tumour necrosis factor alpha (TNF) inhibitor. To our knowledge this is the first study examining mucosal IL-33 expression before and after anti-TNF therapy. In colonic mucosal biopsies we found a 3-fold increase in IL-33 gene expression comparing acute UC to healthy controls ($p < 0.01$). A significant reduction of IL33 between acute UC and disease remission was observed when TNF normalised in the mucosa ($p = 0.02$). Immunostaining revealed IL-33 in the nuclei of epithelial cells of scattered colonic crypts in acute disease, while at disease remission, IL-33 was undetectable, a novel finding suggesting that enterocyte-derived IL-33 is induced and maintained by inflammatory mediators.

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) characterised by a continuous, submucosal inflammation of the colon. The pathogenesis is complex and appears to involve both genetic susceptibility and environmental triggers, that together lead to an imbalance between pro- and anti-inflammatory cytokines in the intestinal mucosa and development of chronic inflammation[1,2]. This assumption is supported by the success of inhibiting the pro-inflammatory cytokine tumour necrosis factor alpha (TNF), which has proven effective in achieving mucosal healing in IBD[3,4].

Interleukin 33 (IL-33), a member of the interleukin-1 family of cytokines, has generated interest in the research field of IBD following reports of its dysregulation[5–9]. Notably, IL-33 is predominantly elevated in acute IBD and most markedly in UC[6–8,10–12]. In addition, genetic polymorphisms of IL-33 or its receptor, IL1RL1 (alias IL-33R, ST2), are associated with an increased risk of IBD and a more extensive colitis[9]. In experimental colitis, IL-33 is found to induce both pro-inflammatory and tissue protective pathways[6,13–18], the latter considering a crucial role for IL-33 in promoting tissue healing and raising the interest of IL-33 as a potential therapeutic target in IBD[16,17].

IL-33 was initially discovered as a nuclear factor of high endothelial venules[19]. A wide range of cells have since been found to express IL-33, including cells at sites protecting the body from the outer environment such as the skin, airways and gastrointestinal tract[13,20]. The term "alarmin" is used to describe the role of IL-33 when released from injured or necrotic cells in response to tissue damage[21,22]. When released, IL-33 acts as a potent activator of the intestinal immune system by binding the transmembrane isoform of the IL-33 receptor IL1RL1. The IL-33/IL1RL1 complex activates NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and MAPK (mitogen-activated protein kinases), including induction of a TH2 cellular response in T cells[5,22,23]. Recent findings

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Baseline characteristics	Ulcerative colitis n = 29	Control group n = 21
Female/male	14/15	8/13
Age study inclusion (years), mean ± SD	39.0 ± 12.7	56.1 ± 13.4
Disease duration, median months, [range]	58 [0–372]	
Newly diagnosed <1month	2	
Disease extent (n)		
- Proctitis	5	
- Left colitis	17	
- Pancolitis	7	
Smoking status (n)		
- Never	15	
- Current	2	
- Ex smoker	11	
- Unknown	1	
UCDAI pre, median,[range]	10 [7–12]	
Endoscopic subscore, median, [range]	2 [2–3]	
Medication (last 3 months) (n)		
- 5ASA	28	
- Steroids (oral)	20	
- Azathioprine or Methotrexate	11	

Table 1. Study population baseline demographics.

also suggest that IL-33 promotes the differentiation of T regulatory cells in the intestine associated with protection against dysregulated inflammatory responses[16]. Murine models with DSS (dextran sulphate sodium) -induced colitis support the hypothesis of IL-33 as a dual function cytokine. Exogenous treatment with IL-33 has been shown by several independent groups to increase the severity of acute colitis[6,13,15,24]. Interestingly, mice deficient of IL-33 have a delayed recovery and resolution after an induced colitis episode[18]. In contrast to IL-33 therapy exacerbating DSS-colitis, a recent report found IL-33 treatment to promote epithelial repair driven by innate lymphoid type 2 cells and regenerative growth factors[17]. Taken together, this implies that the role of IL-33 in the pathogenesis of ulcerative colitis varies according to phases of inflammation and mucosal healing.

The aim of the current study was to characterize the modulation of IL-33 in the intestinal mucosa of patients with acute UC treated with infliximab, a TNF inhibitor, and to unravel the dynamics of IL-33 in the mucosal healing process. To our knowledge this is the first study that examines the mucosal response of IL-33 following anti-TNF therapy in IBD.

Results

Study population. Fifty study participants were included, 29 UC patients and 21 healthy controls. Baseline demographics for the study population are shown in Table 1.

Mucosal levels of IL33 mRNA are increased in acute UC. Quantitative PCR analysis revealed a 3-fold increase of mucosal IL33 transcripts in biopsies from acute UC lesions compared to healthy control samples ($p < 0.001$, Fig. 1a). Expression of the IL33 receptor IL1RL1 was also upregulated, as were several other pro-inflammatory cytokines shown in Fig. 1a. Data from individual patients are shown for IL33 and IL1RL1 (Fig. 1c,d, respectively). We next assessed the modulation of this cytokine profile in each patient before and after infliximab therapy (acute disease versus remission), observing a strong reduction in several cytokines, whilst changes in transcript levels of IL33 or IL1RL1 did not reach statistical significance (Fig. 1b).

IL33 mucosal gene expression decreases when TNF levels normalise. The lack of reduction in IL33 gene expression upon disease remission was somewhat surprising given the association of IL33 expression with a pro-inflammatory cytokine profile. We therefore stratified our cohort according to TNF-normalisation (defined as the upper limit of the 95% confidence interval of a previously published normal control group)[25,26], resulting in two subgroups; those with normalised TNF mucosal levels ($n = 10$) and those with a raised mucosal TNF ($n = 19$). Cytokine expression was compared between these subgroups shown in Fig. 2a. A significant reduction of IL33 mRNA was only seen in the normalised TNF group ($p = 0.02$, see Fig. c), whereas we observed no difference with respect to IL1RL1 (Fig. 2a,c).

Loss of nuclear IL-33 in epithelial crypt cells in healed mucosa. Observing that IL33 transcription was only reduced in patients with normalised TNF levels, we next assessed the expression level of IL-33 protein by means of immunohistochemical analysis. Nine UC patients with mucosal biopsies taken from the same region at both acute inflammation (pre-infliximab) and at disease remission were included. In lesions of acute inflammation, 8 out of 9 biopsies showed a positive, nuclear IL-33 signal in epithelial cells in scattered colonic crypts. We further investigated whether these crypts were associated with crypt abscesses or inflammatory cell clusters, but

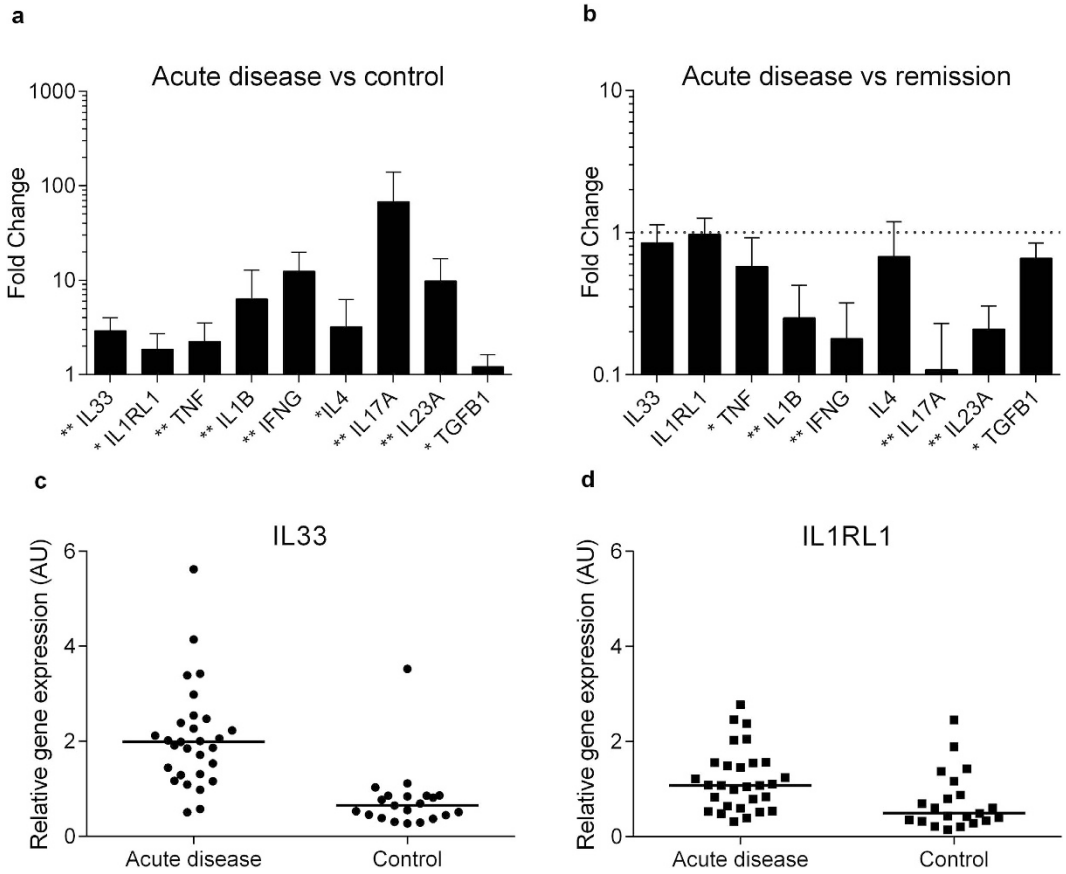

Figure 1. Expression of mucosal IL33 is increased in acute ulcerative colitis. Panel (a) shows an increase in mucosal mRNA expression for selected cytokines from colonic biopsies comparing acute ulcerative colitis (n = 29) with a healthy control group (n = 21). Panel (b) shows the same cytokine profile for patients with ulcerative colitis (n = 29) with a pairwise comparison of biopsies taken during active disease and at disease remission. No change was detected for IL33, IL1RL1 and IL4 ($p > 0.05$). Results for (**a**,**b**) are given as fold change ($2^{-\Delta\Delta CT}$) with mean values, and the upper limit of the 95% confidence interval as error bars. Statistical analysis was performed using Mann U Whitney Test (**a**) and Wilcoxon Signed Ranks Test (**b**) with *p-value < 0.01, **p-value < 0.001 shown in panels. Panels (c) and (d) show individual values of relative gene expression ($2^{-\Delta CT}$) of IL33 and IL1RL1, respectively, for acute disease and normal controls, with the horizontal line representing the median value.

did not detect any distinct pattern. Strikingly, IL-33 was undetectable in all colonic crypts and epithelial cells at the time point defined as disease remission following infliximab therapy (See Fig. 3). Interobserver agreement of quantification of IL-33 positive cells yielded a weighted kappa coefficient of 0.83 (data shown in Supplementary Table S1).

IL-33 in the lamina propria. IL-33 expression in the lamina propria of UC lesions was mainly found in vascular endothelial cells, and in myofibroblast-like cells[7,11]. Quantification of IL-33 positive cells in this compartment showed no change in the number of positive IL-33 cells in the lamina propria comparing acute UC with disease remission (Fig. 3c). In the normal control group (n = 10), only a few IL-33-positive lamina propria cells were observed, most of them morphologically assessed as vascular endothelial cells consistent with previous reports[7,27]. No IL-33 expressing cells were observed in the epithelial border or colonic crypts of the normal control group (Fig. 4).

Discussion

In this study we observed a selective induction of nuclear IL-33 in enterocytes in scattered crypts of acute UC lesions. Strikingly, this expression was lost following successful anti-TNF therapy to disease remission. These are novel findings providing insight into a dynamic expression of IL-33 in well-defined, clinical stages of UC.

Although IL-33 is consistently found to be elevated in acute UC, there is variation in the literature regarding its cellular location[6–8,28,29], particularly in the epithelial intestinal barrier[6–8,29]. In this study we found that IL-33 was present in nuclei of scattered epithelial crypt cells of patients with acute UC, while it was undetectable in disease remission and in healthy controls. These findings suggest that IL-33 may be an inflammation-induced factor in the human intestinal epithelial barrier. In support of this, recent studies at other epithelial barrier sites have described nuclear IL-33 to be associated with inflammation inferring a common pathway of IL-33 regulation,

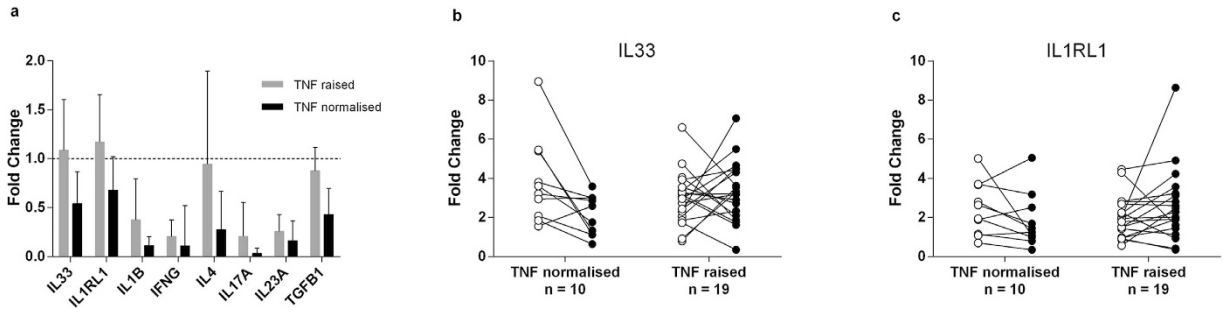

Figure 2. Normalisation of TNF reduces mucosal IL33 gene expression. Pairwise comparison of colonic biopsies from patients with UC pre and post infliximab therapy (acute disease versus disease remission) were analysed with real-time qPCR. Patients are grouped into those who showed normalisation of mucosal TNF at disease remission (n = 10), and those who had a raised mucosal TNF at disease remission (n = 19). Results are given as mean fold change ($2^{-\Delta\Delta CT}$) with error bars marking the upper limit 95% confidence interval. A more significant reduction of inflammatory cytokine transcription was seen in the group with normalisation of mucosal TNF, assessed by Wilcoxon Signed Ranks Test (**a**). Panels (b,c) show individual IL33 and IL1RL1 gene expression for each patient at acute disease (white point) and disease remission (black point) given as $2^{-\Delta CT}$, with ΔCT values generated from the mean of the healthy control group. IL33 showed a significant reduction (p = 0.02) for the TNF normalised group but not when TNF remained elevated (p = 0.8).

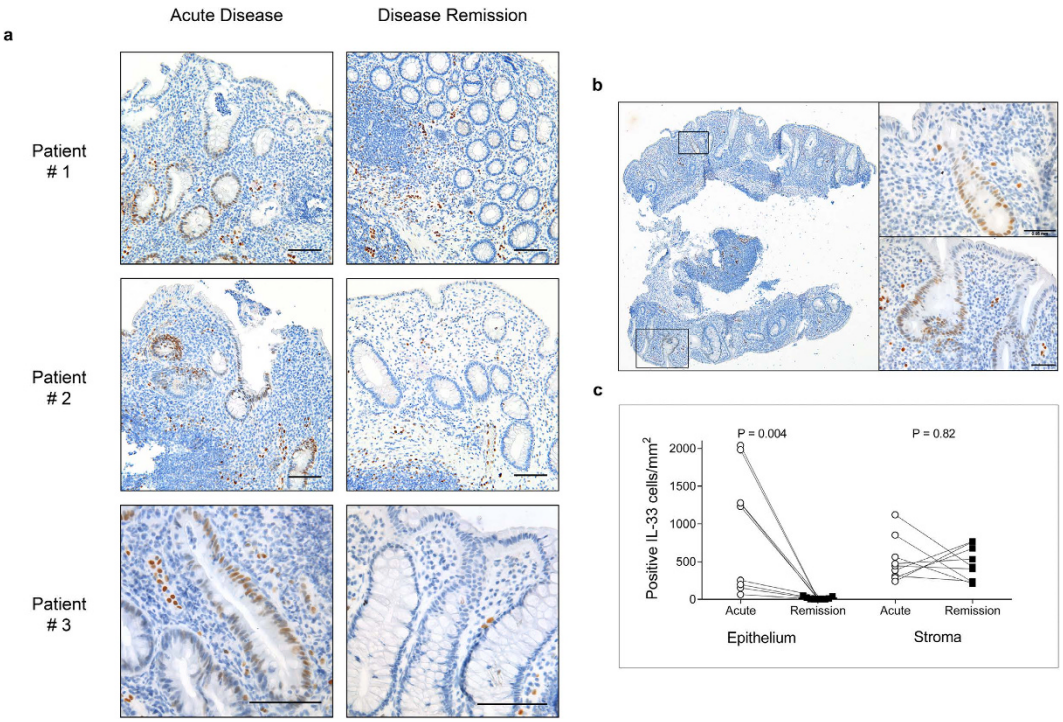

Figure 3. IL-33 is present in epithelial crypts in acute ulcerative colitis. Panel (a) shows colonic biopsies from 3 representative patients with acute UC treated with infliximab (anti-TNF) to disease remission. Endoscopic biopsies were taken from the most inflamed colonic regions pre- infliximab therapy (acute disease), and repeated from the same region at disease remission. Biopsies were formalin fixed, paraffin embedded with immunoenzymatic staining for IL-33 (brown) using monoclonal mouse antibody (Nessy-1). IL-33 positive cells are seen in epithelial crypts during acute disease. At disease remission no epithelial staining for IL-33 was seen. Cell nuclei (blue) counterstained with hematoxylin. Scale bar = 0.1 mm. Panel (b) shows a representative whole biopsy from acute ulcerative colitis (x40 magnification) stained as above, whilst boxes show high-power magnification. Scale bar = 0.05 mm. Panel (c) shows quantification of IL-33 positive cells per mm^2 in individual patients (n = 9) in the epithelium and lamina propria.

including squamous epithelium cells of the oesophagus, and keratinocytes of the skin epithelial barrier[30,31]. We hypothesised that the sporadic distribution of IL-33 positive intestinal crypts might be associated with areas of more severe inflammation such as crypt abscesses, proximity to epithelial injury or aggregates of

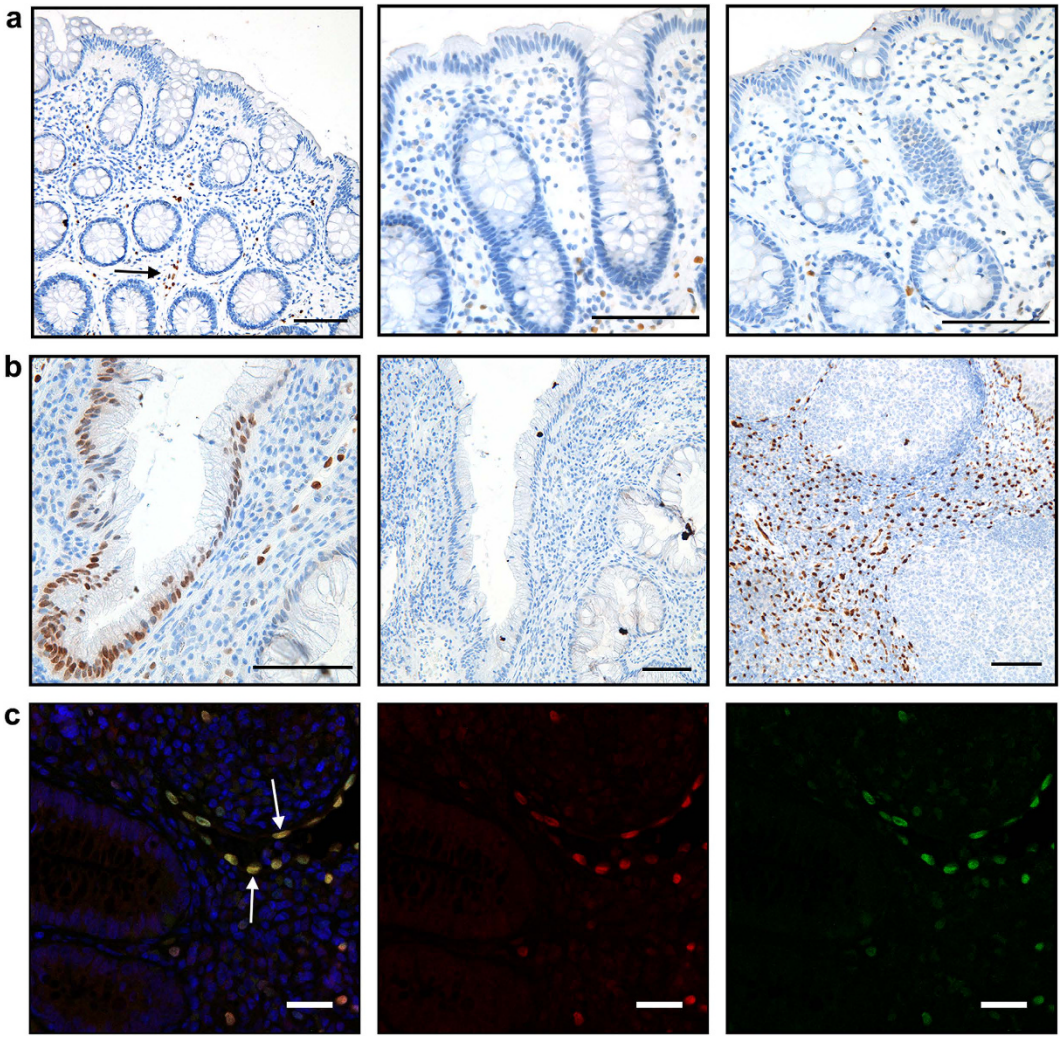

Figure 4. Immunostaining of IL-33 in control groups. Panels (a,b) show formalin fixed, paraffin embedded biopsies with immunoenzymatic detection for IL-33 (brown) using monoclonal mouse antibody (Nessy-1) at [1 μg/ml], with cell nuclei (blue) counterstained with hematoxylin. Panel (a) shows representative sections from colonic biopsies of 3 healthy controls. No IL-33 staining was observed in epithelial cells. IL-33 positive cells are seen in the stroma mainly associated with vascular endothelial cells (black arrow). Scale bar = 0.1 mm. Panel (b) shows from left to right; acute UC with a IL-33 positive colonic crypt, an isotype and concentration matched control from the same biopsy and a positive control using human tonsillar tissue. Scale bar = 0.1 mm. Immunofluorescence images (**c**) of formalin fixed, paraffin embedded colon biopsies from acute UC, with double staining performed for IL-33 polyclonal goat antibody (red) and IL-33 monoclonal mouse antibody (green). Merged image (left) shows good co-localisation of both antibodies (see white arrow), confirming antibody specificity. Cell nuclei are counterstained with Hoechst. Scale bar = 20 um.

inflammatory cells, however we did not observe any distinct pattern. Differences in biopsy orientation must be taken into account and may have underestimated our findings of IL-33 positive cells. Another hypothesis is that enterocyte-derived IL-33 is induced by local environmental factors in the individual lumen of colonic crypts, resulting in the scattered distribution observed.

IL-33 is described as a cytokine of dual functions, referring to its role as an endogenous alarmin, but also to its nuclear, transcriptional repressive functions[21]. We found IL-33 to be present in epithelial cells in association with inflammation, however our data do not imply whether IL-33 may act as an inducer or repressor of the inflammatory response in this setting. Furthermore, the mechanisms regarding its downregulation, degradation or secretion from the nuclei remain unknown. Future research should be designed to understand which inflammatory mediators induce and regulate IL-33 expression in crypt cells, and is an area of focus in our research group.

IL33 mucosal gene expression was increased in acute UC, alongside other known mediators of acute inflammation including TNF. The normalisation of mucosal TNF-expression has previously been associated with long-term disease remission in UC[32]. Interestingly, we found a more pronounced downregulation of inflammatory cytokines in the subgroup of patients (n = 10) whom achieved normalisation of mucosal TNF levels, including a significant reduction of IL-33 ($p = 0.02$). In contrast, IL33 remained upregulated in the patient group

with raised TNF levels at clinical remission (see Fig. 2). These findings may indicate a time-lag for normalisation of IL33 in the intestinal mucosa, possibly dependent on a more complete normalisation of pro-inflammatory cytokines including mucosal TNF. The IL33 receptor IL1RL1 showed a similar tendency, however this must be interpreted with care as our assay did not differentiate between membranous and soluble isoforms of IL1RL1, an issue that should be addressed in future studies. From the above findings we propose that mucosal IL33 expression is only reduced at the stage of remission that allows for normalisation of mucosal TNF levels.

In this study, we have described a cohort of patients with a clinically similar disease course, all of whom were followed from acute UC to disease remission during anti-TNF treatment. Biological variation was reduced by comparing serial biopsies from the same patient. We were also able to examine the mucosal IL-33 response to modulation of TNF *in vivo*. In contrast to the significant reduction in epithelial IL-33 observed in response to anti-TNF treatment, we could not detect any reduction of IL-33 positive lamina propria cells (see Fig. 3). Several factors may be contributing to this result, including different cellular regulation of IL-33 expression. Endothelial cells, observed to be a main source of IL-33 positive cells in disease remission, show constitutive Notch-dependent IL-33 expression and this regulatory mechanism has not been described in other cell types[33]. Other factors include sample size ($n = 9$) and that endoscopic biopsies only sample the superficial layer of the colonic mucosa, excluding deeper stromal layers from our analyses.

Achieving disease remission is associated with a better prognosis in UC, and is the main goal of therapy[34]. Unresolved questions include when therapy can be successfully discontinued following achievement of disease remission. Based on previous findings we propose that clinical and endoscopic criteria are not sufficient for determining disease remission, and that evidence of mucosal normalisation of inflammatory mediators is also needed[26,32].

Conclusion

We have characterized the expression of IL-33 in well-defined clinical stages of ulcerative colitis. IL-33 was present in the nuclei of enterocytes in scattered colonic crypts in acute ulcerative colitis, but was not present in these cells at clinical disease remission. An overall reduction in IL-33 mucosal gene expression was only seen in patients with normalised mucosal TNF levels. Further studies to explore the regulation and nuclear role of IL-33 are warranted, taking cellular location into account. The downregulation of epithelial IL-33 expression may potentially serve as a marker for disease remission in UC together with other biomarkers including mucosal TNF.

Materials and Methods

The study protocol, including storage of biological samples, was approved by the Regional Committee for Medical and Health Research Ethics North (Ref no: 1349/2012), and carried out in accordance with the Declaration of Helsinki Principles. Written and informed consent was obtained from all study participants.

Study population and inclusion criteria. Patients were recruited from a prospective study of IBD patients at the University Hospital of North Norway, aged ≥ 18 years. Mucosal biopsies were obtained from patients with moderate to severe UC defined by a Mayo score ≥ 6 at baseline[35]. Patients were diagnosed according to established clinical, endoscopic and histological criteria[36]. All patients were treated with infliximab (anti-TNF therapy) intravenously 5 mg/kg at 0, 2 and 6 weeks, and every 4th week until disease remission, defined as a Mayo score ≤ 2 with a endoscopic subscore of 0 or 1. Exclusion criteria included anti-TNF therapy within the last 12 weeks, pregnancy, previous cancer or primary sclerosing cholangitis. The control group consisted of patients referred to endoscopy at the outpatient clinic for bowel cancer screening. For this group, exclusion criteria were diarrhoea and presence of colonic polyps. All controls had a normal colonoscopy and a histological normal colonic mucosa assessed by two pathologists.

Colonic biopsies. Endoscopic mucosal biopsies were obtained from the most inflamed bowel region prior to start of infliximab therapy, and a new biopsy obtained from the same region at disease remission. Mucosal biopsies were taken from the rectum or sigmoid colon in healthy controls. A standard endoscopic biopsy forceps was used for mucosal biopsies which were immediately immersed in either 10% formalin or RNA*later* (Ambion Inc., Austin, TX, USA). RNA was isolated using either the Promega method (Promega Corp., Madison, WI, USA) previously published in detail[26,37], or with AllPrep RNA/DNA miniKit using Qiacube (Qiagen, Hilden, Germany), and stored at −70 °C.

Quantitative PCR analysis. Reverse transcriptase was performed with Quantitect 2 step Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Mucosal cytokines were measured in duplicates using hydrolysis probes. Beta actin (ACTB) was used as a reference gene for normalisation. The stability of ACTB in the current setting has previously been tested[26]. Positive and negative controls were included on each plate with standardised cycle thresholds. Assays, apart from interferon gamma (IFNG) were all in-house and listed in Supplementary Table S2. All data was expressed using ΔCT values with fold change expressed as $2^{-\Delta\Delta CT}$. The laboratory investigators were blinded to the clinical data.

Immunostaining. Sections (4 μm) of formalin-fixed, paraffin-embedded biopsies were deparaffinised and rehydrated. Antigen retrieval was performed in citrate buffer pH 6 in a water bath at 100 °C for 20 minutes, followed by 20 minutes cooling. IL-33 monoclonal mouse antibody (Nessy-1, Enzo life Sciences) was used at 1 μg/ml, or a polyclonal goat antibody for IL-33 at 0.1 μg/ml (AF3625, R&D Systems). Primary antibodies were incubated for 60 minutes at room temperature or overnight at 4 °C. Enzymatic detection with 3,3 diaminobenzidine was performed with DAKO envision kit (DAKO, Glostrup, Denmark) following the manufacturer's instructions and counterstained with hematoxylin. Negative controls were performed routinely with the primary antibody being substituted with an isotype- and concentration-matched antibody (mouse IgG1, DAKO).

Tonsillar tissue was used as positive control. For immunofluorescence detection slides were blocked with 10% donkey serum (20 minutes). Secondary antibodies conjugated with Alexa488 or Alexa546 (Life Technologies) were used, with Hoechst (33258, Life Technologies) as counterstain.

Images where obtained using Olympus BX51 microscope with an Olympus U-TVO.5XC camera using AnalySIS 3.2 software (Soft Imaging System). Immunofluorescence images were taken with a Zeiss Axio observer, Z1 LSM780 CLSM system (Carl Zeiss Microscopy GmbH, Jena, Germany) running the Zen 2012 (black edition) software. Images were processed in Adobe Photoshop CS6 (Adobe Systems Software Ireland Ltd, Dublin, Ireland), with adjustments to image histograms applied only for whole images. Two independent observers (SWS, MDG) performed quantification of IL-33 positive cells. 3 representative high field views of x 400 magnification per biopsy where counted for positive and negative epithelial and crypt cells and summarised in the following categories: negative $<1\%$, weak 1–9%, moderate 10–24%, high 25–49% and very high $\geq 50\%$. Positive cells in the stroma were counted per mm^2 based on biopsy area calculations from NIS- elements AR 3.1 software (Nikon Instruments, Europe).

Statistical methods. All statistics are performed using IBM SPSS statistics version 22.0 (IBM corp., Armonk, NY, USA) or GraphPad Prism version 6.03 (La Jolla, CA, USA). Normality plots were assessed with histograms and Shapiro-Wilks test, with appropriate t-test or equivalent non parametric test used. Interobserver variability was assessed with weighted Cohen's kappa for immunohistochemistry analysis.

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Author Contributions

M.D.G. wrote the main manuscript text, with contributions from R.G., J.H., G.H., O.S. and J.F. Tables and figures were prepared by M.D.G., R.G., G.H. and J.H. Quantification analysis was performed by S.W.S. and M.D.G. Patient recruitment, study design and clinical follow up was performed by R.R., T.O., M.D.G., R.G. and J.F. All authors reviewed the manuscript.

Additional Information

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PAPER 2

Fibrosis Mediators in the Colonic Mucosa of Acute and Healed Ulcerative Colitis

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OBJECTIVES: **A healed intestinal mucosa is the aim of therapy in acute ulcerative colitis (UC). Disruption of mucosal wound healing may lead to severe complications including intestinal fibrosis. This study examined mucosal gene expression in the healing process of acute UC with a special focus on known mediators of fibrosis.**

METHODS: **Endoscopic biopsies from patients with acute, moderate to severe UC were analyzed with a quantitative polymerase chain reaction array for 84 genes involved in fibrosis pathways. All patients were treated with infliximab (anti– tumor necrosis factor). Biopsies were taken before therapy and when disease remission was reached, defined as a Mayo score of ≤2, with an endoscopic subscore of 0 or 1. A healthy control group was included. Immunostaining of matrix metallopeptidase 9 and smooth muscle actin was performed.**

RESULTS: **Mucosal biopsies from acute UC (n = 28), remission UC (n = 28), and healthy controls (n = 13) were analyzed. Fibrosis and extracellular matrix-associated genes were upregulated in the endoscopically healed UC mucosa vs controls, with collagen type III alpha 1 chain, actin alpha 2, lysyl oxidase, TIMP metallopeptidase inhibitor 3, and caveolin 1 uniquely showing no overlap with acute disease. Pro- and antifibrotic mediators (interleukin [IL]13 receptor subunit alpha 2, IL1B, IL10, tumor necrosis factor, snail family transcriptional repressor 1, and C-C motif chemokine ligand 2) were upregulated in both acute and healed UC compared with controls. An attenuated pattern of the canonical transforming growth factor beta (TGFB) pathway was observed in acute UC and to a lesser extent in the healed mucosa, except for TGFB2, which was enhanced.**

DISCUSSION: **The endoscopically healed mucosa of UC showed a persisting dysregulation of fibrosis-associated mediators compared with controls, including extracellular matrix remodeling, profibrotic cytokines, and TGFB signaling pathways.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A103

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, relapsing inflammatory disease affecting the colon (1). The pathogenesis is complex involving dysregulated immune responses to mucosal injury, with persistent inflammation and disruption of wound healing (2,3). The proinflammatory cytokine tumor necrosis factor (TNF) plays a pivotal role in mediating inflammation in UC. Antibodies targeting TNF induce mucosal healing in over 60% of patients with inflammatory bowel disease (IBD) (4,5). Achieving mucosal healing is the current goal of treatment in IBD as associated with clinical improvement and longer relapse-free periods (6).

There is an increasing need for knowledge of which mediators are involved in mucosal healing. This is emphasized by the fact that 10%–30% of patients with IBD are unresponsive to anti-TNF therapy, as well the lack of therapies targeting intestinal fibrosis in IBD (7,8). Intestinal fibrosis is a severe complication in IBD, causing excessive scar tissue formation in the bowel wall, with distortion of tissue architecture and intestinal function as sequelae (9,10). In UC, up to 11% experience fibrostenotic complications, vs over 50% in Crohn's disease (9). Recent studies suggested that the complications of intestinal fibrosis may be severely underestimated in UC, indicating that fibrosis is more prominent in the pathogenesis of UC than previously attributed (11,12).

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Following injury to the intestinal barrier, the body is dependent on executing a swift and effective response to prevent pathogen invasion (13). This is a complex process involving hemostasis, followed by fibrogenesis, epithelial regeneration, scar tissue remodeling, and eventually restoration of the intestinal barrier (13,14). Mesenchymal cell activation by transforming growth factor beta (TGFB) is central for production of extracellular matrix (ECM) proteins and wound contraction (ECM) (15). Degradation and turnover of the ECM is tightly regulated by matrix metallopeptidases (MMPs) and their inhibitors (TIMPs) (16). The canonical TGFB pathway is central in fibrosis progression and implicated in IBD (17,18). TGFB binds to the membrane-bound TGFB receptors, which activate intracellular SMAD signaling cascades. Mediators of the TGFB–SMAD pathway are therefore of interest as target for antifibrotic therapy (19–21).

Currently, no methods exist for detecting early stages of intestinal fibrosis (9,10). In this study, we applied a PCR array of fibrosis-associated mediators in a well-stratified cohort of patients with acute UC that have been treated with anti-TNF until disease remission. The differential expression of fibrosis-associated mediators in the healed mucosa of UC may give insights into active pathways and potential therapeutic targets for fibrosis.

MATERIALS AND METHODS

Ethical considerations

The study and storage of biological samples was approved by the Regional Committee for Medical and Health Research Ethics North (Reference no: REK1349/2012) and performed in accordance with the Declaration of Helsinki principles. Written and informed consent was obtained from all study participants.

Patient population

Patients were included from the IBD Biobank at the University Hospital of North Norway; the IBD cohort has previously been described (22). Patients aged 18 years or older, with moderate to severe UC defined as Mayo score ≥6, were included. All patients had been treated with an induction course of infliximab 5 mg/kg intravenously at 0, 2, and 6 weeks followed by maintenance therapy every 4–8 weeks. Only patients who achieved endoscopic remission after infliximab therapy were included. We defined endoscopic remission as a Mayo score ≤2 with no individual subscore >1 (23). Geboes score was assessed on available hematoxylin and eosin-stained slides at endoscopic remission by an experienced pathologist (S.W.S.), with histological remission defined as Geboes score <3.1 (24). Primary sclerosing cholangitis, pregnancy, lactation, and a history of cancer were exclusion criteria.

Tissue samples

Endoscopic biopsies were obtained from the most inflamed region of colonic mucosa before infliximab therapy and a new biopsy obtained from the same region at the time point of disease remission. Biopsies were taken with standard endoscopic forceps and immediately immersed in RNA later (Ambion Austin, TX) or 10% formalin for quantitative polymerase chain reaction (qPCR) or histological analysis, respectively. RNA was extracted using the Promega method (Promega Madison, WI) following manufactures' instructions and stored at −70 °C.

Control group

A healthy control group referred for colonoscopy was included if the following criteria were met: age 18 years or above, a complete colonoscopy examination with normal macroscopic and histological findings, and no diarrhea in the presenting history. Pregnancy or lactation, previous or current cancer, systemic inflammatory disease, and presence or a history of colonic polyps were exclusion criteria. Endoscopic biopsies were taken from the sigmoid or rectal mucosa.

qPCR analysis

RNA concentration was determined with Nanodrop (Thermo Fisher Scientific, Waltham, MA). Reverse transcriptase was performed with Quantitect 2 step Kit (Qiagen, Hilden, Germany). The RT^2 profiler PCR Array Human Fibrosis PAHS-120Z (product nr 330213 Qiagen, Hilden, Germany) was used according to the manufacturer's instructions with RT^{2}SYBER Green mastermix (Qiagen, Hilden, Germany). Real-time qPCR was performed with Bio-Rad CFX96 (Roche Diagnostics, Rotkreuz, Switzerland). A computed tomography (CT) cutoff for lower limit of detection was set at 40 (see Supplementary Information, Supplementary Digital Content 1, http://links.lww.com/CTG/A103).

Immunostaining

Four-μm sections of formalin-fixed paraffin-embedded samples were deparaffinized and rehydrated through a series of xylene to alcohol. DAKO antigen retrieval buffer (pH6) (DAKO, Glostrup Denmark) was used in a waterbath for 20 minutes at 100 °C and 20 minutes of cooling at room temperature. Sections were blocked with 10% goat serum. Primary antibodies were incubated overnight at 4 °C. Secondary goat antibodies conjugated with Alexa 555 (Cell Signaling Technologies, Danvers, MA) or Alexa 647 (Invitrogen, Thermo Scientific, Waltham, MA) were incubated for 90 minutes at room temperature, and cell nuclei stained with Hoechst 33258. Negative isotype- and concentration-matched controls were used (antibody details listed in Supplementary Table 1, see Supplementary Digital Content 1, http://links.lww.com/CTG/A103). Images were obtained with a Zeiss LSM780 CLSM microscope (Carl Zeiss Microscopy, Zena, Germany) using Zen 2012 black edition software. Three nonoverlapping, representative images at ×200 magnification were used for quantification with Volocity 6.3 software (PerkinElmer Waltham, MA) with positive fluorescence area given as a ratio over total nuclei area for each slide. Images were processed in Adobe Photoshop CC 2019 (Adobe Systems Software, Ireland Ltd, Dublin, Ireland), and adjustments to image histograms were applied to whole image only. The Human Protein Atlas database (www.proteinatlas.org) was used to identify validated antibody staining with a high reliability score ("enhanced score").

Statistics

Data were analyzed using R Statistical Environment (https://www.r-project.org) with Bioconductor R software for principal component analysis and impute package for missing values. Normfinder (https://moma.dk/normfinder-software) software was used for evaluating reference genes used for normalization (25).

For comparative gene analysis, we used the linear modeling framework from the R Stats Package with the (lm() function using normalized CT values (delta CT). Independent samples and paired samples were taken into account. Correction for sex and endoscopic remission status was performed. P values were adjusted for multiple testing using the Benjamini Hochberg method, with a significant adjusted $P < 0.05$ set as threshold (26). Comparison of gene expression between groups was given as log2 fold

change. Quantification of immunostaining was performed with *t* tests for independent groups and a paired *t* test when comparing acute and remission disease, all after control of normality with IBM SPSS Statistics version 25.0 (Armonk, NY). Plot and bar charts were visualized with GraphPad prism v. 7 (La, Jolla, CA).

RESULTS

Patient population

Twenty-eight patients with moderate to severe acute UC and 13 healthy controls met the study inclusion criteria. Five patients with UC were newly diagnosed, whereas the majority had well-established disease. Patient demographics are shown in Table 1. At endoscopic remission, 16 patients had a Mayo endoscopic subscore of 0. For endoscopic remission, histology was available for n = 7 patients with UC, of these, n = 6 were in histological remission (Geboes score <3.1) (See Supplementary Table 2, Supplementary Digital Content 1, http://links.lww.com/CTG/A103). Biopsies from all healthy controls (n = 13) showed normal histological findings, with no inflammation.

Unique expression pattern in the healed mucosa of UC

All samples passed qPCR quality control, and interleukin 5 (IL5) was excluded because of >25% of CT values over 40 and not included in further analyses. Principal component analysis showed separation of acute UC, remission UC, and healthy control samples in the first principal component, with 44.5% of the variance explained (Figure 1a). Gene expression analysis revealed a significant number of differently expressed genes in all sample groups with a total of 41 upregulated and 38 downregulated genes. A Venn diagram (Figure 1b) depicts the overlap of differentially expressed genes between the comparisons of groups with active UC (A), remission (R), and control samples (N). We noted that 6 genes were uniquely expressed in the endoscopically healed mucosa of UC vs controls (differentially expressed compared with control samples with no overlap to active UC). These genes actin alpha 2, smooth muscle (ACTA2), caveolin 1 (CAV-1), collagen type III alpha 1 chain (COL3A1), lysyl oxidase (LOX), and TIMP metallopeptidase inhibitor 3 (TIMP3), were all upregulated compared with healthy controls, whereas integrin subunit beta 6 (ITGB6) was significantly downregulated. The latter 4 genes (COL3A1, LOX, TIMP3, and ITGB6) are associated with the ECM remodeling. Angiotensinogen (AGT) was the only gene with an expression pattern showing upregulation in acute disease and downregulation in healed mucosa compared with controls. The entire gene expression pattern is shown in Figure 2a. Gene table and pathway annotations are given in Supplementary Table 3 and Supplementary Figure 1 (see Supplementary Digital Content 1, http://links.lww.com/CTG/A103).

Attenuation of the TGFB canonical pathway in UC

The profibrotic TGFB-SMAD pathway (illustrated in Figure 3) showed a significant dysregulated signaling pattern in acute UC and to a lesser extent in endoscopic remission (Figure 2b).

Table 1. Clinical characteristics at study inclusion time for patients with acute UC and control group

	UC (n = 28)	Controls n = 13	*P*-value[a]
Sex, male/female (n)	18/10	7/6	0.73
Age at diagnosis (median, [range])	51 (18–69)	39 (20–82)	0.05
Smoking status (n) non-/ex-/current smoker	16/1/11	—	NA
Disease duration mo (median, [range])	52.5 (0–372)	—	NA
Disease extent (n)			
Proctitis	6	—	NA
Left sided	15	—	NA
Extensive	7	—	NA
Biopsy location endoscopy (n) (rectum/sigmoid)	13/15	5/8	0.5
Mayo score baseline	10 (7–12)	—	NA
Endoscopic subscore	3 (1–3)[c]	—	NA
Medication at baseline[b]			
5-ASA	19	0	NA
Steroids po/iv	12/0	0	NA
AZA or MTX	11	0	NA
Anti-TNF	0	0	NA

Variables are given as median with range, if not otherwise indicated. No significant differences ($P < 0.05$) were found between the UC and the control group with regard to age, sex, or biopsy location (independent samples *t* test and Fisher exact test for continuous and categorical variables were used, respectively). Disease extent was defined using the Montreal Classification as recommended by ECCO guidelines (6). Patient medication at baseline included no anti-TNF therapy within the last 3 months.
5-ASA, 5-aminosalicylate; AZA, azathioprine; MTX, methotrexate; NA, not applicable; TNF, tumor necrosis factor; UC, ulcerative colitis.
[a]Comparison between UC and healthy controls.
[b]Patients can be on >1 medication at baseline.
[c]Two patients had an endoscopic subscore of 1, however met the inclusion criteria as a full Mayo score was >6, both with high rectal bleeding scores and histologically active disease with cryptitis and crypt abscesses.

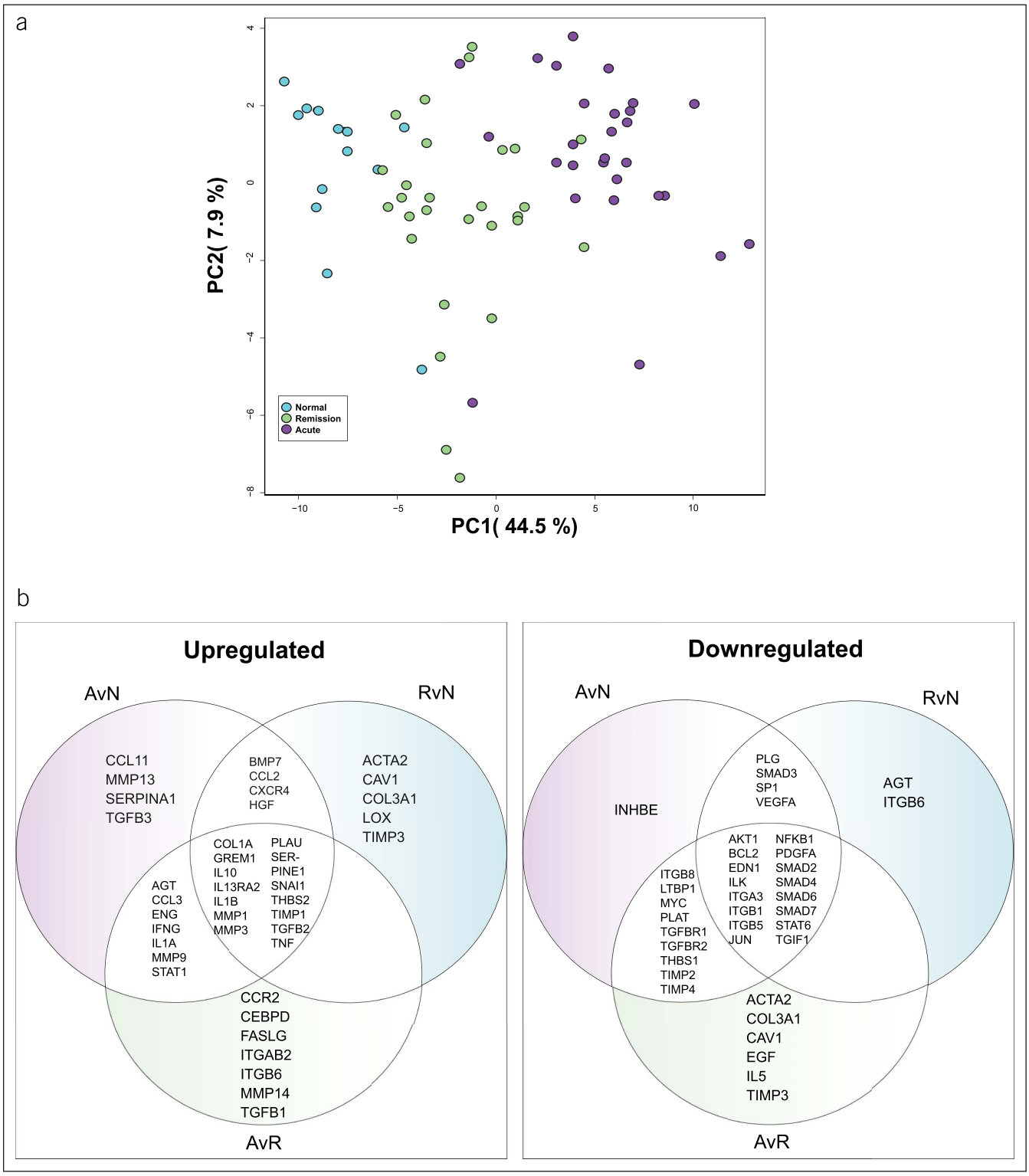

Figure 1. Principal component plot and Venn diagrams. (**a**) PCA with separation of groups seen along the x-axis. Of note, 44.5% of the variance is explained by PCA1. (**b**) Venn diagrams showing overlap of significantly expressed genes (adjusted $P < 0.05$) between groups: acute UC vs healthy control group (AvN), remission UC vs healthy control group (RvN), and acute UC vs remission UC (AvR). PCA, principal component analysis; UC, ulcerative colitis.

Comparison of acute UC and healthy controls revealed an upregulation of TGFB2 and TGFB3, but not TGFB1. Furthermore, the corresponding receptors for these ligands, TGFB receptor 1 (TGFBR1) and TGFB receptor 2 (TGFB2), were downregulated along with intracellular SMAD signaling mediators SMAD2, SMAD3, SMAD4, SMAD6, and SMAD7. Inhibitors of the

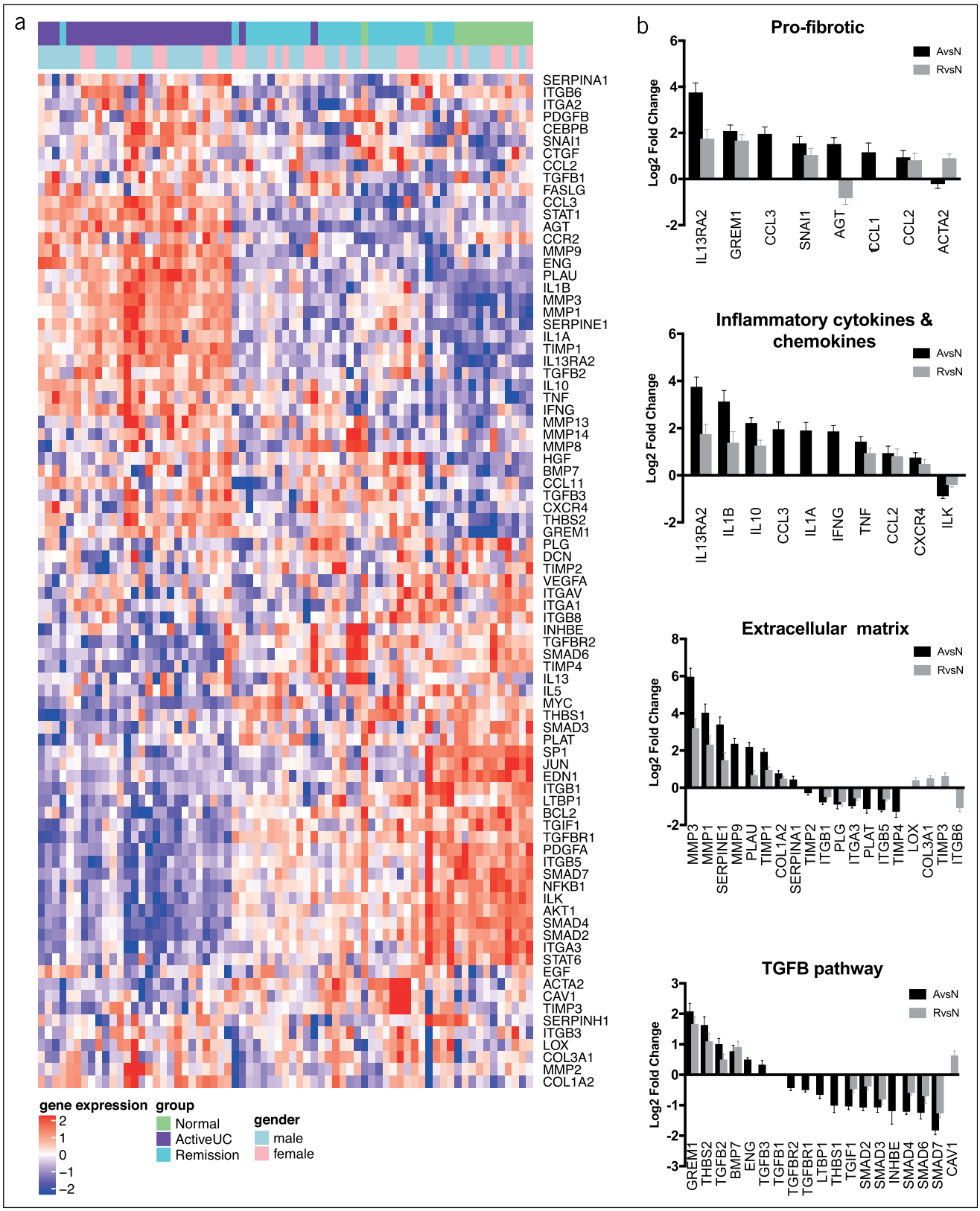

Figure 2. Gene expression analysis. (**a**) Heatmap of all genes examined in the PCR array with an unsupervised cluster analysis, with study groups and sex given. (**b**) Pathway analysis was performed using gene annotations provided by RT^2 Profiler Human Fibrosis array (Qiagen). Selected bar charts show log2 fold changes with SE given for significantly differentially expressed genes (adj. $P < 0.05$). Group comparisons are given as follows: acute UC vs healthy control group (AvsN) and remission UC vs healthy control group (RvsN).UC, ulcerative colitis.

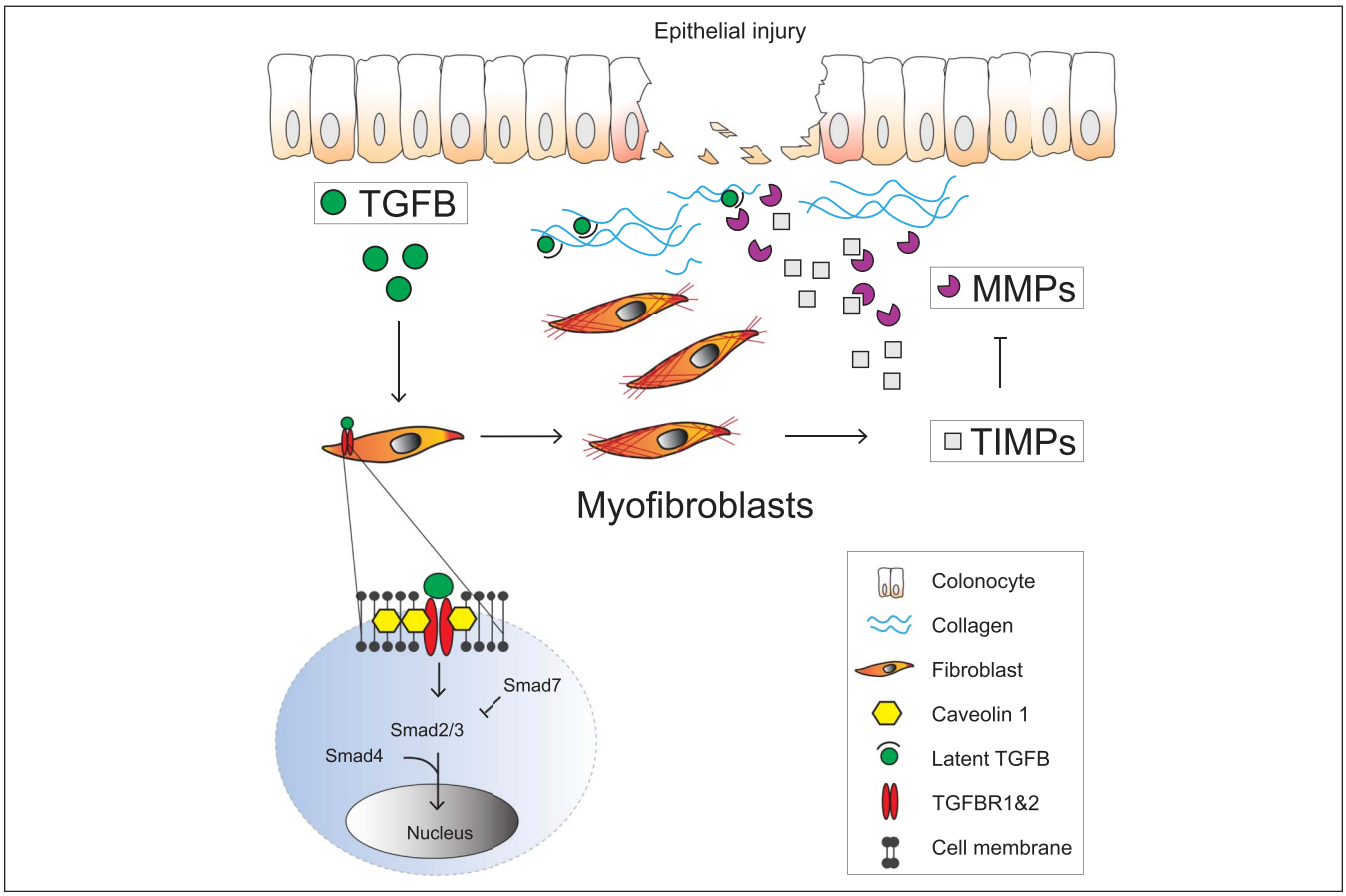

Figure 3. TGFB signaling in the colonic mucosa. Following injury to the epithelial barrier, activity of matrix metallopeptidases (MMPs) is increased. TGFB is bound in a latent form in the extracellular matrix. MMPs degrade the collagen matrix, also releasing and activating TGFB (19). The canonical TGFB pathway involves activation of membrane-bound TGFB receptors 1 and 2, which phosphorylates SMAD 2/3 and together with SMAD4 before translocating to the nucleus (21). TGFB promotes recruitment and differentiation of fibroblasts into myofibroblasts, increasing production of collagen and TIMPs. Caveolin 1, a glycoprotein in the cell membrane, inhibits this signaling by internalizing the TGFB receptors (36). TIMP, tissue inhibitor of metallopeptidase.

TGFB-SMAD pathway, THBS2 and BMP7, were increased in both the acute and endoscopically healed UC mucosa. Furthermore, TGFB3 normalized, whereas a persistent overexpression of TGFB2 was observed at the time of endoscopic remission.

Other relevant fibrotic mediators in UC

ECM remodeling enzymes MMP3 and MMP1 showed the most prominent log2 fold changes in acute and healed UC compared with controls. Proinflammatory cytokines and chemokines were strongly upregulated during acute disease, including IL 1 beta (IL1B), interferon gamma (IFNG), and TNF (Figure 2b). Comparison of the healed UC mucosa with the healthy control group revealed profibrotic genes IL 13 receptor subunit alpha 2 (IL13RA2) and Gremlin-1 (GREM 1) among the top overexpressed genes. Several upregulated inflammatory mediators normalized completely in the healed mucosa including MMP9, IFNG, IL 1alpha (IL1A), and C-C motif chemokine ligand 3 (CCL3). Immunostaining was consistent with the gene expression pattern of MMP9 (shown in Figure 4). In contrast, several fibrosis mediators showed little or no change in gene expression following anti-TNF therapy (BMP7, GREM1, CCL2, and CXCR4). In this cohort, TNF was still found to be upregulated in the mucosa following anti-TNF therapy. Comparison of endoscopic subscore 0 and 1 in disease remission revealed significant differences in 12 genes with log2 fold changes <1. Of these genes, IL10 and MMP14 were upregulated, whereas the remaining genes were downregulated (integrin subunit alpha 1 (ITGA1), integrin subunit beta 1 (ITGB1), integrin subunit beta 6 (ITGB6), SMAD4, signaling transducer and activator of transcription 6 (STAT6), epidermal growth factor, specificity protein 1 (SP1), and latent transforming growth factor–binding protein 1 (LTBP1). The remaining genes ($n = 31$) were not affected by endoscopic remission score 0 or 1. Correction for sex revealed only CCL3 to show a significant sex difference (log2 fold change male vs female: −0, 15).

DISCUSSION

We have clearly demonstrated that genes involved in ECM remodeling and fibrosis development do not all normalize in the endoscopically healed mucosa of UC (defined as Mayo endoscopic subscore of 0 or 1). Over half of the investigated fibrosis-associated genes were differentially expressed in the healed UC mucosa. This supports the notion that fibrosis is indeed an important component in the pathophysiology of UC. We found an increased TGFB2 and TGFB3 ratio in acute UC, with TGFB3 expression not normalizing in endoscopic

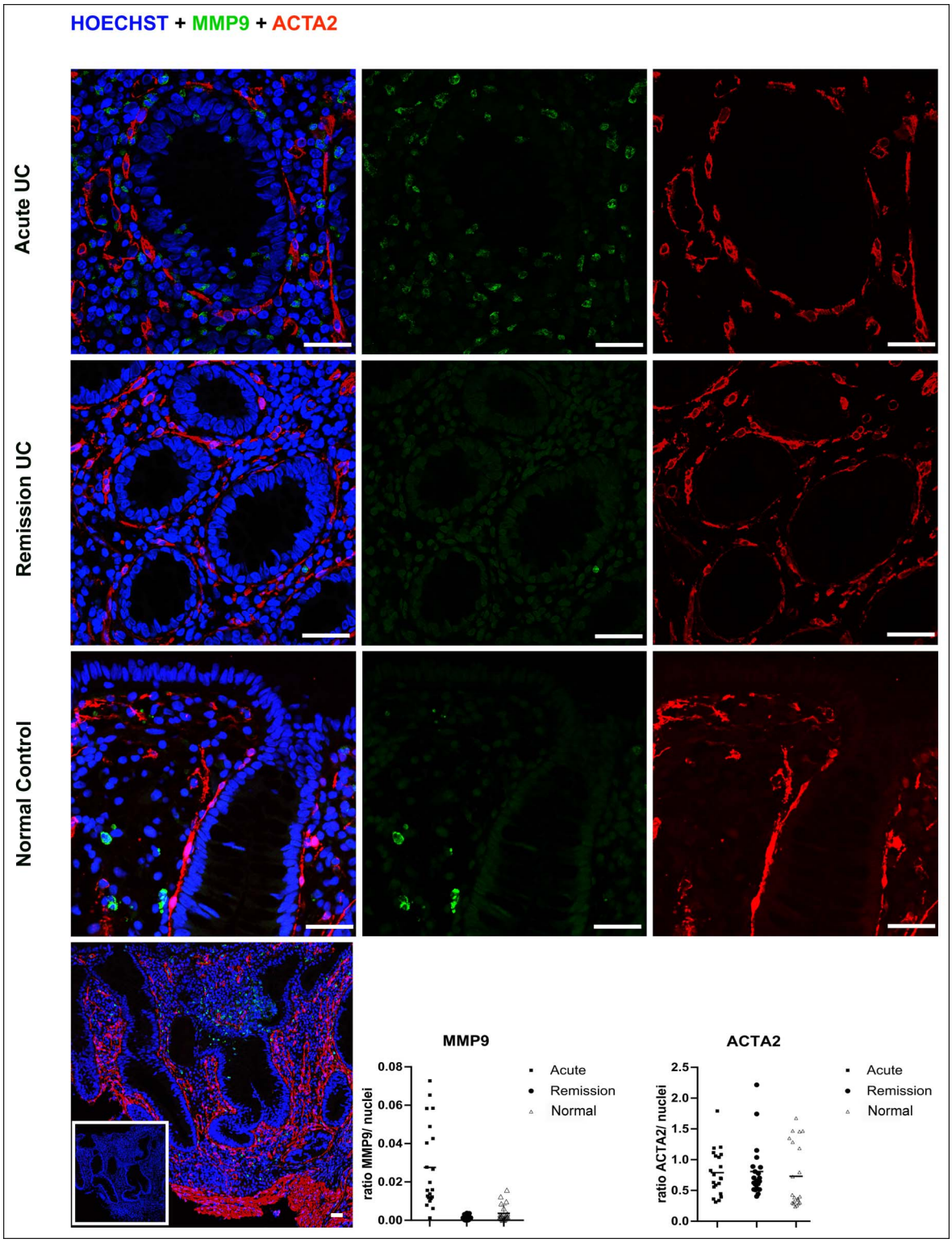

Figure 4. Immunostaining of MMP9 and ACTA2. Antibodies directed at MMP9 and ACTA2 had the highest upregulated and downregulated gene patterns in our study with enhanced antibody scores. Panels show representative immunostaining of formalin-fixed, paraffin-embedded colonic endoscopic biopsies from acute UC, remission UC, and a healthy control group at ×400 magnification. Scale bars = 35 μm. Dual staining with monoclonal rabbit antibody against MMP9 ([1/400], Cell Signaling Technologies) and monoclonal mouse antibody against ACTA2/SMA (DAKO, [0.35 μg/mL]) were used. Cell nuclei are stained with Hoechst (blue). Positive cytosolic staining of stromal cells was observed for MMP9 (green), whereas a membranous/cytoplasmic signal was seen in ACTA2-positive cells (red). A significant decrease ($P < 0.05$) of MMP9 was seen comparing acute UC with remission UC or normal controls, but not for ACTA2. Further antibody details are given in Supplemental Table 1(see Supplementary Digital Content, http://links.lww.com/CTG/A103). A negative control is shown in the white frame for acute UC (bottom left panel). ACTA2, actin alpha 2; UC, ulcerative colitis.

remission. In contrast, the well-known profibrotic TGFB1 was present, but not significantly expressed in the UC mucosa. We believe that the balance of TGFB isoforms in different UC disease phases may be important for achieving mucosal healing and a potential factor to modulate. Indeed, others have reported differences in the TGFB ratio between patients with UC who respond or not to anti-TNF therapy (27). Furthermore, intestinal myofibroblasts isolated from healthy controls and patients with IBD have been shown to secrete different TGFB isoform ratios (28). All 3 TGFB isoforms are present in the intestine; however, their role in UC is unclear (19,21). Looking to other organ systems, TGFB1 and TGFB2 have been found to promote ECM deposition during skin wound healing, whereas TGFB3 reduces scarring (19,29). In sum, knowledge of the TFB isoforms role in UC is incomplete, warranting further research.

Although we found an upregulation of the TGFB2 and TGFB3, the canonical TGFB pathway showed an attenuated response with downregulated TGFB receptors (TGFBR1 and TGFBR2), together with intercellular SMAD signaling mediators. This might be an appropriate response in the acute phase of inflammation, as TGFB is recognized to be released from its latent bound form in the ECM by MMPs and other ECM–degrading factors (19). Indirectly, our findings support this with overexpression of MMPs and extracellular remodeling enzymes in acute UC, suggesting an abundance of released and activated TGFB. However, the canonical TGF pathway does not normalize in the endoscopically healed UC mucosa, suggestive of a persistent dysregulation.

As expected, proinflammatory genes showed a pattern of reduction, and even normalization following anti-TNF therapy in agreement with other studies (27,30–33). In contrast, several fibrosis mediators showed little or no change in gene expression following anti-TNF therapy including BMP7, GREM1, CCL2, and CXCR4. Specific to the endoscopically healed mucosa, a set of genes associated with collagen synthesis and degradation (ACTA2, COL3A1, LOX, and TIMP3) were upregulated compared with controls. In summary, our findings show that fibrosis-associated mediators are still dysregulated in the endoscopically healed UC mucosa compared with healthy controls. This is in keeping with other reports that the healed UC mucosa remains dysregulated (27,32). A recent study by Arijs et al. found a large overlap of mucosal genes that were persistently dysregulated in patients with UC responding to infliximab (anti-TNF) or vedolizumab (anti-$\alpha 4\beta b7$ integrin), suggesting that unidentified pathways are yet to be targeted (34). Furthermore, vedolizumab was found to influence a unique set of mucosal genes independent of anti-TNF therapy. It is exceedingly interesting whether the new biological therapies, including vedolizumab and ustekinumab (inhibitor of p40 subunit of IL12 and IL23), will target additional pathways and influence fibrosis. Our findings highlight the current knowledge gap of fibrosis-associated pathways in UC, also evident in the clinics as no effective antifibrotic therapies exist.

Our finding of an upregulated CAV-1 in the healed UC mucosa may reflect a fibroprotective mechanism, as reported in murine studies (35). The protein Cav-1 acts as the scaffolding in caveolae of the cell membrane, important for endocytosis and cell signaling (36). However, to our knowledge, the role of CAV-1 in IBD is little explored. One study supports the location of CAV-1 and -2 expression in the colonic mucosa (37). Interestingly, the literature reports CAV-1 to exhibit antifibrotic properties by binding and internalizing TGFB receptors on the cell membrane, effectively attenuating TGFB signaling and reducing collagen production (38). A low gene expression of CAV-1 has been reported in other fibrotic disorders including idiopathic pulmonary fibrosis and scleroderma (38). In addition, we found an interesting gene expression pattern for AGT, being upregulated in acute disease and downregulated in the healed UC mucosa. Mediators of the renin-angiotensin-aldosterone system are found in many tissues including the colon (39). Inhibitors of the renin-angiotensin-aldosterone system are well-established therapies for prevention of end-organ damage in cardiac and renal disease, and also of interest in IBD as therapeutic targets for inflammation and fibrosis.

IL13RA2 was among the top DEGs in our study. Interestingly, high mucosal expression of this receptor in IBD has been linked to anti-TNF unresponsiveness and impaired restoration of the intestinal barrier in a murine Dextran sulfate sodium colitis model (40). Both profibrotic and antifibrotic properties have been described in mouse models of colitis and pulmonary fibrosis, respectively; thus, tissue and cellular context is important (41,42). Taken together, IL13RA2 is emerging as an interesting future target both for refractory UC and in modulating fibrosis.

A limitation to the study is the lack of a general consensus on what constitutes a "truly healed" mucosa. The widely used Mayo score includes endoscopic subgrades 0 and 1 in disease remission, with a score of 1 allowing for the presence of mild erythema, decreased vascular pattern, and mild friability. The definitions of mucosal healing are currently being debated, with endoscopic subscore 0 and histological remission being associated with improved clinical outcomes (43). However, histological remission does not necessary imply endoscopic remission and *vice versa*. In a recent study, Magro et al. (44) showed that histological indexes could be associated with endoscopic outcomes with a high sensitivity when the Mayo endoscopic subscore was set at 1. Of the 7 patients in our study with available histological assessment, 1 patient met the criteria for histological remission with an endoscopic subscore of 1. This raises the questions of how do we distinguish between transcription patterns of inflammation, tissue restitution, and those representing the underlying disease? Some overlap is likely. Is the UC mucosa ever truly inactive? Arguably, we could have used stricter remission criteria; on the other hand, most genes did not show any significant difference when corrected for endoscopic score of 0 and 1 in disease remission. Although the paired samples design is a strength reducing interpatient variation, our sample size was limited by available biopsies in the IBD Biobank and at risk of type II error. Another limitation is the use of a preselected gene panels associated with general human fibrosis, thus not comprehensive. The heterogeneity of cell populations across biopsies is also important to take into account when interpreting results (45).

Apart from CCL3, we did not uncover any sex-dimorphic patterns for our gene set. In our study, most patients with UC had well-established UC; however, the role of disease duration on fibrosis development is not clear. A large histopathology study of fibrosis in UC found that the degree of fibrosis was linked to the severity ad chronicity of disease and, interestingly, not to disease duration (46). Thus, intestinal fibrosis is also a complication occurring in short-standing UC. Although different disease entities, we and others have previously found that the mucosal gene expression of inflammatory cytokines between UC and Crohn's disease is remarkably similar (47,48). In view of this, our findings could be relevant for future research in Crohn's disease; however, this remains to be explored. Comparison of mucosal fibrosis mediators in patients with UC with established fibrotic disease was not within the scope of the present

study, but of interest for future work. Combining knowledge of mucosal fibrosis mediators between anti-TNF responders and nonresponders may be useful as therapeutic biomarkers, providing clinicians with tools to personalize therapy.

In conclusion, we have seen a significant modulation of genes associated with both inflammation and fibrosis in patients treated to a clinical and endoscopically verified healed mucosa. The mucosa of UC in disease remission showed a persistent dysregulation of fibrosis-associated genes, with an attenuated pattern of TGFB signaling mediators in UC. We identified mucosal TGFB isoforms, CAV-1 and AGT as potential antifibrotic targets in UC, of interest for future research.

CONFLICTS OF INTEREST

Guarantor of the article: Mona Dixon Gundersen, MD.

Specific author contributions: M.D.G: planning, conducting the study, analysis, interpretation, drafting the manuscript, and approved the final submitted draft. R.G.: planning the study, manuscript review and writing, and approved the final submitted draft. C.G.F: statistical analysis and data interpretation, review of the manuscript, and approved the final submitted draft. E.A: statistical analysis and data interpretation, visualization and editing/writing of the manuscript, and approved the final submitted draft. S.W.S.: analysis of the study material, interpretation, review of the manuscript, and approved the final submitted draft. J.R.F.: planning the study, interpretation of data, manuscript review and editing, and approved final submitted draft. R.H.P: planning and conducting the study, interpretation and data analysis, manuscript review and editing, and approved the final submitted draft.

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Study Highlights

WHAT IS KNOWN

- ✓ Intestinal fibrosis is a severe complication in IBD.
- ✓ No effective antifibrotic therapies exist for intestinal fibrosis.
- ✓ Recent studies report that intestinal fibrosis is underestimated in UC.

WHAT IS NEW HERE

- ✓ Profibrotic mediators are dysregulated in the endoscopically healed mucosa of UC, including transforming growth factor beta 2 (TGFB2).
- ✓ Genes involved in ECM remodeling were uniquely upregulated in the endoscopically healed mucosa of UC compared with healthy controls.

TRANSLATIONAL IMPACT

- ✓ Identification of mucosal markers warranting further exploration as potential antifibrotic targets in UC therapy.

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PAPER 3

Title Page

Hypo-osmotic stress induces the epithelial alarmin IL33 in the colonic barrier of ulcerative colitis

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Abstract

Epithelial alarmins are gaining interest as therapeutic targets for chronic inflammation. The nuclear alarmin interleukin-33 (IL-33) is upregulated in the colonic mucosa of acute ulcerative colitis (UC) and may represent an early instigator of the inflammatory cascade. However, it is not clear what signals drive the expression of IL-33 in the colonic mucosa, nor is the exact role of IL-33 elucidated.

We established an *ex vivo* model using endoscopic colonic biopsies from healthy controls and UC patients. Colonic biopsies exposed to hypo-osmotic medium induced a strong nuclear IL-33 expression in colonic crypts in both healthy controls and UC biopsies. Mucosal IL-33 mRNA was also significantly increased following hypo-osmotic stress in healthy controls compared to non-stimulated biopsies (fold change 3.9, p-value<0.02). We observed a modest induction of IL-33 in response to TGF-beta-1 stimulation, whereas responsiveness to inflammatory cytokines TNF and IFN-gamma was negligible. In conclusion our findings indicate that epithelial IL-33 is induced by hypo-osmotic stress, rather than prototypic proinflammatory cytokines in colonic *ex vivo* biopsies. This is a novel finding, linking a potent cytokine and alarmin of the innate immune system with cellular stress mechanisms and mucosal inflammation.

Background

The alarmin interleukin-33 (IL-33) belongs to the interleukin-1 family of cytokines and is emerging as an important mediator of epithelial immune responses[1,2]. Alarmins are endogenous molecules that instigate and/or perpetuate inflammation upon release from stressed or damaged cells[3-5]. They swiftly alert the immune system of danger and are gaining interest as upstream targets for chronic inflammatory disease. IL-33 is of particular interest in diseases affecting epithelial borders including asthma, atopic dermatitis and inflammatory bowel disease (IBD)[6-8]. IL-33 is characterised as an intrinsic, nuclear cytokine with a chromatin binding motif. Upon extracellular release, it potently activates innate immune cells expressing the surface membrane bound interleukin-1-like receptor (IL1RL1, alias ST2, IL-33R) resulting in a prompt acute inflammatory response[3,9]. Despite a large body of evidence examining the alarmin functions of IL-33, inducers of IL-33 and its nuclear function are not fully explored and warrant further examination.

Defining mechanisms that induce epithelial IL-33 are of particular interest in ulcerative colitis (UC), one of the main entities of IBD[10]. High IL-33 mucosal levels are a feature of active UC, and it is among the top activated cytokines specific to UC in mucosal gene expression studies [11]. In addition, polymorphisms of the IL-33 gene and its ligand receptor IL1RL1 are associated with an increased risk of UC, implying a role for IL-33 in the disease pathogenesis of UC[12-15]. We and others have reported high accumulation of IL-33 in epithelial crypts during active UC, which is strikingly absent in quiescent disease and healthy controls[13,16,17]. This challenges the view of IL-33 being constitutively expressed in the colonic epithelium and prompts questions into which tissue-specific factors induce synthesis and accumulation of IL-33[13,15,17-19]. Recent reports reveal that interferon gamma (IFN-gamma) and hypo-osmotic stress can induce IL-33 expression in the skin barrier while this remains unknown in

gastrointestinal barrier. Osmotic stress is relevant in the pathophysiology of IBD, where loss of intestinal barrier integrity and increased barrier leakiness are well documented[20,21]. Human plasma osmolality is tightly maintained at 277-299 mOsm/kg as fluctuations affect cell volume and function[22]. Exposure to a hypo-osmotic environment results in fluid flux into the cell which will swell[22]. Both hyper- and hypo-osmotic states are described in the colon, influenced by dietary intake, presence of non-absorbable solutes, digestive secretion, (mal)absorption, and intestinal motility[23]. Identifying factors that induce epithelial IL-33 expression may prove beneficial in targeting inflammation in IBD. Moreover, promising clinical trials targeting IL-33 with antibody therapy are reported for chronic inflammatory diseases affecting epithelial borders[8,24]

There is a pressing need to develop simple and robust human models for examining intestinal mucosal responses[25-27]. This is especially true for IL-33 where a strong interspecies variation has been shown[28]. In this study we established a human colonic *ex vivo* model based on endoscopy biopsies of healthy controls and patients with UC. IL-33 epithelial expression was investigated with immunostaining and gene mucosal transcription following exposure to the pathophysiological condition of hypo-osmotic stress; proinflammatory mediators interferon gamma (IFN-gamma) and tumour necrosis factor alpha (TNF); and the immune mediator transforming-growth factor beta-1 (TGF-beta-1)[29].

Results

IL-33 expression in treatment naive acute UC

To determine the distribution of IL-33 in acute UC we examined immunostaining patterns of IL-33 in biopsies from treatment naïve, acute UC patients (n=15). Patient characteristics are given in Table 1. IL-33 showed a sporadic pattern of IL-33 positive epithelial cells

predominately distributed in the mid- and basal parts of colonic crypts in keeping with previously published findings[13,16]. Areas of IL-33 accumulation are shown in Fig.1. Several epithelial crypts with positive IL-33 expression were associated with the presence of intraepithelial inflammatory cells and crypt-abscesses. We confirmed a strong IL-33 immunosignal in the stroma and dual staining the hematopoietic progenitor cell antigen CD34 confirmed the presence of IL-33 in association with small vessel endothelium in the colon as well in (Fig.1H).

Ex vivo model

We established a colonic *ex vivo* model using endoscopic biopsies from healthy controls and patients with UC (Table 2). Biopsies were cultured for up to twenty-four hours with preserved morphological features and architecture (Fig.2A-C). Further, uptake of the pro inflammatory cytokine IFN-gamma from the basal medium to the epithelium was confirmed by a strong signal of phosphorylated signal transducer and activator of transcription 1-alpha/beta (pSTAT1) in the epithelium following IFN-gamma stimulation, but not present in non-stimulated controls after 24 hours (Fig.2D). Additional staining for the cell marker Ki67 confirmed presence of cells in different proliferative states (Fig 2E-F), and no difference when comparing Ki67 immunostaining between available baseline biopsies (n=8) compared with biopsies cultured for 24 hours (Fig.2H). Paired t-test analysis of mucosal gene expression comparing baseline and 24 hours culture showed no significant differences for IL-33 (p-value = 0.3) or TNF (p-value = 0.8) (Fig 2G). RNA integrity was assessed in healthy control biopsies following 24 hours of incubation in basal medium (n=7) with a medium RIN value of 8.6 (range 7.2-9.1) indicating good quality RNA.

Hypo-osmotic stress induces epithelial IL-33 in an *ex vivo* model

The colonic epithelial barrier is constantly exposed to osmotic perturbations. In view of the recent report that hypo-osmosis drives IL-33 expression in the skin barrier[30], we were curious to see whether this also applied to the colonic epithelium. We found that exposure to hypo-osmotic medium (152 mOsm) for 24 hours induced a strong nuclear IL-33 signal in the lower part of epithelial crypts in healthy controls (Fig. 3). In contrast, no IL-33 was present in epithelial crypts following 24 hours when cultured with regular basal medium (~ 300 mOsm) (Fig 3D). Further, the majority of IL-33 cells were found to be Ki67 negative, though not exclusively limited to a set cell-proliferative state.

Next, we included colonic biopsies from patients with UC (n=4) for immunostaining. These patients had an intact mucosal barrier and no presence of ulcers and a Mayo endoscopic score of 0 or 1. We found that hypo-osmotic stress induced epithelial IL-33. The pattern distribution was similar to that observed in mucosal biopsies of acute UC localizing basally in colonic crypts. Mucosal gene expression with qPCR of colonic biopsies showed a significant increase in IL-33 mRNA(fold change 3.9, p value = 0.02) in biopsies exposed to hypo-osmotic stress compared to controls (Fig 3H). An overview of biopsy stimulation experiments is given in Table 3.

IFN-gamma does not drive epithelial IL-33 in a UC *ex vivo* model

The pro-inflammatory cytokine IFN-gamma has been linked to IL-33 expression in oesophagitis, and is also raised in IBD[31]. IFN-gamma is also a known driver of IL-33 in keratinocytes[28]. We therefore investigated the effect of IFN-gamma on the expression of IL-33 in UC. Inactive UC patients (Mayo endoscopic subscore = 0) did not express IL-33 at baseline, nor following 4 hours and 24 hours of IFN-gamma stimulation. IL-33 was present in the epithelial at baseline for one patient with moderate UC disease (Mayo endoscopic subscore = 2) but diminished following 24 hours of IFN-gamma exposure. In healthy controls (n=4) we did not observe any significant induction of IL-33 signal in epithelial cells. IFN-

gamma at 100ng/ml was used following 3 log concentration testing (1-100 ng/ml). To ensure adequate IFN-gamma stimulation and uptake throughout the biopsy, we examined expression of pSTAT1, which confirmed a strong epithelial expression following stimulation (Fig. 4).

TNF did not induce IL-33 in a healthy control ex vivo model

We also tested TNF and TGF-beta-1 in the range of 3 log concentrations. The pro-inflammatory cytokine TNF is central in IBD and a target for therapy during acute inflammation[29]. IL-33 has also been linked to TGF-beta-1 mediated differentiation of regulatory T-cells in the intestine [32]. Interestingly, TNF activation (1ng/ml-100ng/ml) revealed no evidence of epithelial IL-33 expression, whereas endothelial IL-33 was reduced which is in agreement with reports from in vitro and in vivo studies, supporting the activity of our stimulation experiment[33]. Mucosal biopsies from healthy controls stimulated with TGF-beta-1 at 10 ng/ml for 24 hours showed a sporadic but clearly discernible presence of nuclear IL-33 expression in the epithelium and epithelial crypts (see Fig. 5).

Discussion

In this study we have shown that hypo-osmotic stress induces nuclear IL-33 accumulation in the colonic mucosa in UC and in healthy subjects. This is a novel finding in the colonic mucosa, and links osmotic perturbations of the colonic mucosa with a potent alarmin of the innate immune system.

We observed hypo-osmotic stress to consistently induce IL-33 in both UC and healthy controls. This is in keeping with findings from the skin barrier and may represent a generic mechanism of IL-33 induction. The colonic barrier in IBD is exposed to osmotic stress[21]. This is especially relevant in UC where epithelial barrier dysfunction is as a key feature [34]. In this pathophysiological context the intestinal barrier may be more sensitive to osmotic

perturbations. The literature mainly reports on hyperosmotic stress as a potent inducer of inflammation, acting via NAFT5 inflammasomes and stimulating pro-inflammatory cytokines including IL8 and TNF[35-38]. Moreover, a recent study found hyperosmotic stress to affect the function of epithelial cells, reducing cell proliferation, mitochondrial function and also altering the gut microbiota[35,39].Only recently has a hypo-osmotic environment been found to independently instigate inflammation and it remains not well understood[40,41]. It is plausible that disturbance of cell function also occurs following hypo-osmotic stress as seen in reversible cell injury (non-lethal). Mechanisms that respond to cellular swelling include activation of mechanosensitive membrane ion channels such as Piezo1 and osmosensing transient receptor potential vanilloid (TRPV). Aquaporins regulating transcellular fluid flow are also relevant [42-45]. Our findings support the role of hypo-osmotic stress in inflammation following activation of IL-33 in the epithelial barrier and warrants further exploration. Although the initiating cause of IBD is not clear, identifying the trigger(s) of inflammation are important as different stimuli likely shape and give rise to nuances within the inflammatory response and may aid development of specific therapeutic targets.

In concordance with previous studies, we found IL-33 to accumulate in epithelial crypts of acute UC, whilst consistently absent in healthy controls. This firmly establishes IL-33 as an inducible nuclear cytokine in the healthy colonic barrier[29,46]. Surprisingly, our data did not show the pro-inflammatory cytokines IFN-gamma nor TNF to be of relevance for induction of epithelial IL-33. This is in contrast to findings from squamous cells of the oesophagus and keratinocytes of the skin barrier[28,31]. In support of our findings, we demonstrated a strong pSTAT1 expression in our epithelial colonic border following IFN-gamma stimulation, however we did not have equivalent strong controls for TNF or TGF-beta-1 stimulation. The lack of IL-33 positive endothelial vessels observed following TNF stimulation supports our findings. Moreover, the majority of colonic cell culture studies have not shown stimulation

with pro-inflammatory cytokines (TNF, IL1-beta and IFN-gamma) to induce IL-33[14,15,47]. One explanation is that these pro-inflammatory cytokines are unable to induce an epithelial alarmin response alone, perhaps requiring additional mediators activating the inflammasome. This can be driven by mucosal damage driving the generation of reactive oxygen or nitrogen species[48]. Indeed, it remains open if hypo-osmosis activates the expression of enterocyte IL-33 by activation of the inflammasome complex.

By contrast, TGF-beta-1, a pleotropic cytokine with a central role in initiating repair and tissue restoration induced IL-33 in the epithelium albeit less potent than hypo-osmotic stress[49]. IL-33 has been linked to T-cell regulatory mechanisms and proposed a central role in tissue healing following reports from murine models where IL-33 was found important in facilitating wound healing and resolution of inflammation[50]. TGF-beta-1 was also found to induce IL-33 expression in the context of mucosal healing in UC when acting in concert with a TLR3 agonist, like mimicking actions of mRNA released from damaged cells[13]. The idea that alarmins may act both as an inducer and repressor or regulator of the acute inflammatory response is intriguing and underpins the need for further studies to understand the regulation of inflammation to harness therapeutic effects.

The strength of this study includes the use of human colonic biopsies. This is important for any study of IL-33, as strong species differences have been documented[28]. In spite of progress in experimental animal and cell models, important differences may be missed if hypotheses are not tested in human models and correlated to observations in human disease lesions[51]. In this study we show that an *ex vivo* human biopsy model gives reproducible results to study IL-33 mucosal response and expression. Endoscopic biopsies are routine in the gastroenterology departments, and although not true physiological conditions, the confirmation of IFN-gamma absorbed from the basal medium to the epithelial layer with pSTAT1 supports its use as a powerful, yet mechanistic and simple model that is inexpensive and easy to perform.

Advantages to our model include the preserved tissue architecture and cell diversity compared to use of immortal cell lines. The use of antibiotics is arguably a weakness as it inevitably disturbs the gut microbial flora and its interactions with the epithelial lining. Further our experiments are small in sample size, which needs to be taken into consideration when interpreting results. Future perspectives using human *ex vivo* biopsy models include the possibility to individually tailoring therapy for patients based on cytokine expression and responses.

Conclusions

In conclusion, hypo-osmotic stress induces a strong pro-inflammatory cytokine in the epithelial colonic border of both healthy controls and active UC. It represents a link between cellular stress and chronic inflammation and may represent a generic IL-33 response in the human body. The use of human *ex vivo* colonic biopsies performed well as a mechanistic model for studying IL-33.

Material and Methods

Ethical considerations

The study design, protocol and collection and storage of biopsies was approved by the Regional Committee for Medical and Health Research Ethics, Northern Norway (REK-Nord) (ref: No 2012/1349). The study was conducted according to the Helsinki declaration and all study participants were informed and gave written consent.

Study participants

Study participants were included from the prospective Advanced Study of IBD (ASIB) at the University Hospital of Northern Norway[52]. Patients with UC were included if 18 years or

above. The diagnosis of UC was defined by established clinical criteria (ECCO guidelines), determined by the examining gastroenterologist[53]. Disease activity was assessed using the Mayo clinical score which divides acute UC into categories; inactive (0-2), mild (3-5 points), moderate (6-8 points) and severe (9-12 points). Disease remission was defined as a Mayo score of 2 or less with an endoscopic sub score of 0 and no categories with a score above 1[54].

Control group

A normal control group of patients referred for colorectal cancer screening were included if endoscopy was completely normal, confirmed with a normal histology rapport. Exclusion criteria were age <18 years, history of cancer, irritable bowel disease, chronic inflammation or autoimmune disease in their past medical history.

Colonic biopsies

***Ex vivo* colon biopsy model**

An *ex vivo* model was established for endoscopic biopsies using a steel mesh to form an air-liquid interface, adapted from principles based on *ex vivo* models of Brown and Tiering, Vadstrup and Fletcher et al[55-57]. Biopsies were obtained from both healthy controls (as defined above) and patients with UC (including acute and remission states).

Up to four endoscopic biopsies were taken from each patient with a radial jaw forceps (Radial jaw™ 4 3.2mm, Boston Scientific) from the sigmoid colon, and immediately immersed in Dulbeccos modified Eagle's medium (DMEM) high glucose (4500mg/L, L-glutamine, sodium bicarbonate without sodium pyruvate, pH 7,0-7,6. Osmolality 327-361mOs/kg) (Sigma Aldrich) supplemented with 10% fetal bovine serum with 1% penicillin and streptomycin and gentamycin 50ug/ml. This transport medium was kept on ice at all times during transport and during orientation of biopsies under a dissecting microscope. Biopsies were placed apical side up on metal grids and placed in a 12-well plate. Basal medium with

added glutamine 2mmol/L with or without stimulants was added to each well up to the metal grid, giving an air-liquid-interface. The *ex vivo* model was maintained in a humidified atmosphere of 5% C02 and 95% atmospheric air at 37 degrees for maximum 24 hours. At experiment end, biopsies were fixated in 10% formalin for 24 hours prior to fixation and paraffin embedding. Biopsies for qPCR analysis were fixed in RNA*later*® (Qiagen, Holden, Germany). Biopsy morphology was assessed with hematoxylin & eosin staining (Shandon Instant Hematoxylin and Instant Eosin, Thermo Electron Corporation. Cheshire, UK).

Stimulation with recombinant human IFN-gamma (E.coli derived. Cat.no. 285-IF-100. R&D systems, Minneapolis, MN, USA), rhTNF (E. coli derived.cat.no 210.-TA R&D systems), TGF-beta-11 (HEK293 derived.cat no 7754-BH R&D systems), and hypo-osmotic medium (DMEM basal medium diluted 1:1 with sterile ultrapure H_20). All stimulation experiments were repeated three times as a set minimum. Hypo-osmotic medium was measured with FISKE@ microosmometer. model 2/0. (Fiske associates, Massachutes, USA).

To confirm the viability and usefulness of the *ex vivo* colonic model to our purpose we assessed the expression of pSTAT1 in unstimulated and IFN-gamma-exposed biopsies, observing a strong induction in response to 100ng/ml IFN-gamma in the epithelial lining of biopsies after 4, 12 and 24 hours.

qPCR analysis

Endoscopic biopsies were obtained with standard forceps, immediately immersed RNA later (Qiagen, Holden, Germany). Biopsies were taken from the sigmoid. RNA was extracted using the AllPrep RNA/DNA miniKit with QIAcube instrument (Qiagen, Hilden, Germany, Cat No:80204) according to manufactures instructions. RNA was stored at -70 degrees. Quantity and purity of the extracted RNA was assessed using the Qubit 3 Fluorometer (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). QuantiNova reverse transcription Kit

(Qiagen, Cat no: 205314) and QuantiNova Probe RT-PR kit (Qiagen Cat no: 208352) were used. Real-time qPCR was performed with Biorad XF96 thermal cycler (Bio-Rad Laboratories AB, Hercules, California, US) using hydrolysis probes. Forward and reverse primes for IL-33, TNF, ST2, ACTB, RPLP0 are previously published[58]. Samples were run in triplicates,The average of reference genes ACTB and RPLP0 were used for normalisation. Data were compared using the delta-delta CT-method and given as fold change[59].

Immunostaining

Formalin-fixed, paraffin-embedded samples were cut to 4um sections. Slides were rehydrated through a series of xylene and graded alcohol to phosphate buffered saline. Heat induced epitope retrieval was performed in a waterbath for 20 minutes at boiling temperature with retrieval buffer pH 6 or pH 9 (Dako, Glostrup, Denmark) following 20 minutes cooling at room temperature. DAKO Envision Flex HRP polymer kit for rabbit/mouse detection was used with 3'3 diaminobenzide using the manual protocol (DAKO, Glostrup, Denmark). Primary antibody incubation for 60 minutes at room temperature or overnight at 4 C. DAKO Envision Flex mouse linker was used for primary mouse antibodies (DAKO, Glostrup, Denmark). Counterstain of nuclei with hematoxylin and $NaHCO_3$ for nuclear blueing was performed. Sections were dehydrated from alcohol to xylene prior to mounting with Vectamount permanent mounting medium (Vector Laboratories Inc, Burlingame, CA, USA).

Immunofluorescence staining was performed with blocking of section with 10% goat serum for 30 min (Cell signalling Technologies). Primary antibodies were incubated overnight at 4 degrees. Secondary goat antibodies conjugated to Alexa 555 or Alexa 647 were used (Life Technologies) and counterstained with Hoechst (33258, Life Technologies). Slides were airdried and mounted with aqueous fluoromount (Sigma-Aldrich). Isotype and concentration matched antibody controls were routinely performed (for antibody details see Supplementary Table S1). Images were captured with VS120 slide scanner (Olympus) for both

immunoenzymatic and immunofluorescence staining. Slides were processed using the Olympus OlyVIA 2.9 software. Photos were organised using Adobe photoshop (2020), with adjustment of histograms only made for the whole image.

Statistics

Mucosal transcripts were analysed using the delta delta CT method for relative quantification, and parametric t-test for independent and paired samples as appropriate were calculated with IBM SPSS statistics 24 (IBM Corporation, Armonk, New York, USA). Fischer's exact test was used for categorical data. P-values < 0.05 were considered significant. QuPath quantative pathology and bioimage analysis was used for quantification using positive cell detection for both 3'3 diaminobenzide staining and for immunofluorescence staining. The epithelial and stromal compartments were annotated manually[60].

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Author Contributions

All authors contributed significantly to either concept and design, acquisition, analysis and interpretation. All authors contributed to revision of the manuscript.

MDG: conception and design, acquisition, analysis, interpretation, wrote manuscript.

KBL: image acquisition and analysis, revision of manuscript.

KMJ: acquisition and analysis, revision of manuscript

RG: conception and design, acquisition and analysis, revision of manuscript

JF: conception and design, interpretation of results and revision of manuscript

GH: conception and design, interpretation of results and revision of manuscript

Additional Information

Competing interests

The authors declare no competing interests.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

TABLES

Table 1. Treatment naive acute ulcerative colitis (AUC)

	AUC (n=15)
Gender (male/female)	7/8
Age median, (range)	35.0 (18-68)
Smoking (never/current/ex-smoker)*	5/1/8
Disease distribution (left colitis/extensive)	10/5
Mayo score (median) IQR	7 (6)
Mayo endoscopic subscore (IQR)	2 (0)
Calprotectin (median) (IQR)	975 (1117)
**TNF mRNA copies/ugL Median (range) n=14	11300 (4600-96000)

* 1 unknown. **1 TNF value from baseline characteristics missing

Table 1. Treatment naive acute ulcerative colitis (AUC)

* 1 unknown. **1 TNF value from baseline characteristics missing

Table 2: Baseline characteristics of study participants used in *ex vivo* biopsy model n=22

	Control n= 14	UC n=8
Gender male/female	9/5	3/5
Age, median (range)	63 (22-82)	43.5 (19-70)
Biopsy location	sigmoid	sigmoid
<u>Mayo score</u> Inactive Mild Moderate Severe	0	 5 2 1 0
Calprotectin	NA	25 (25-175)
<u>Medication:</u> 5ASA Prednisolone MTX/AZA Anti-TNF	 0 0 0 0	 3 0 2 6

Table 2. Baseline characteristics of study participants used in *ex vivo* biopsy model n=22

Continuous variables given as median with range.

Table 3. Overview over stimulation experiment setup n= 22

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Control/UC																						
Male/Female																						
Stimulus																						

= UC

= Female

= Hypo-osmotic

= IFN-gamma

= TNF

= TGF-beta-1

Figure 1. IL33 in acute ulcerative colitis (AUC)

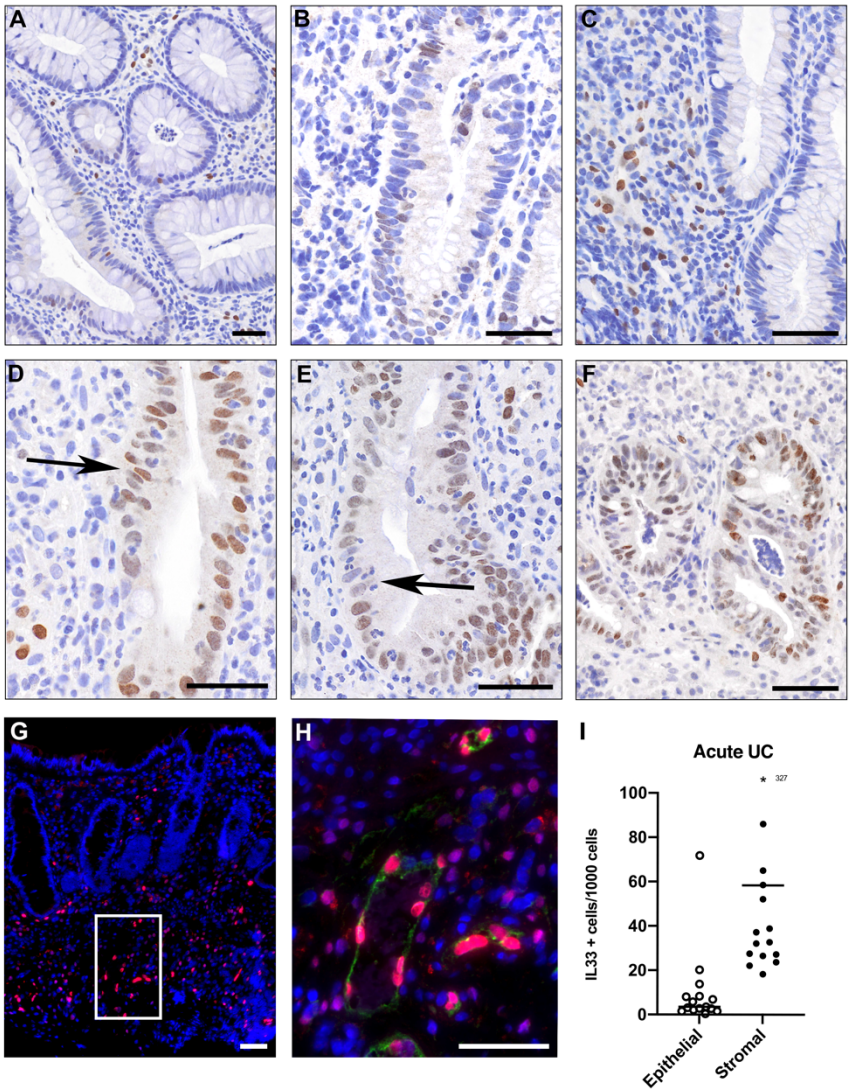

Figure 1. IL-33 in acute ulcerative colitis (AUC)

Image **A-F** show formalin-fixed, paraffin-embedded colonic biopsies stained for IL-33 (brown) and nuclei stained with hematoxylin (blue). Arrows point to inflammatory cells infiltrating the epithelium. Scales bars = 50µm. Image **G** shows dual staining of AUC with IL-33 (red) and endothelial vessels marked by CD34 (green). White frame in image **G** is enlarged as image **H.** Scale bars = 50µm. The graph to the far right (**I**) shows positive IL-33 cells per 1000 cells quantified by Qupath analysis software into epithelial and stromal compartments.* 327 positive cells/1000 cells.

Figure 2. *Ex vivo* colonic biopsy model

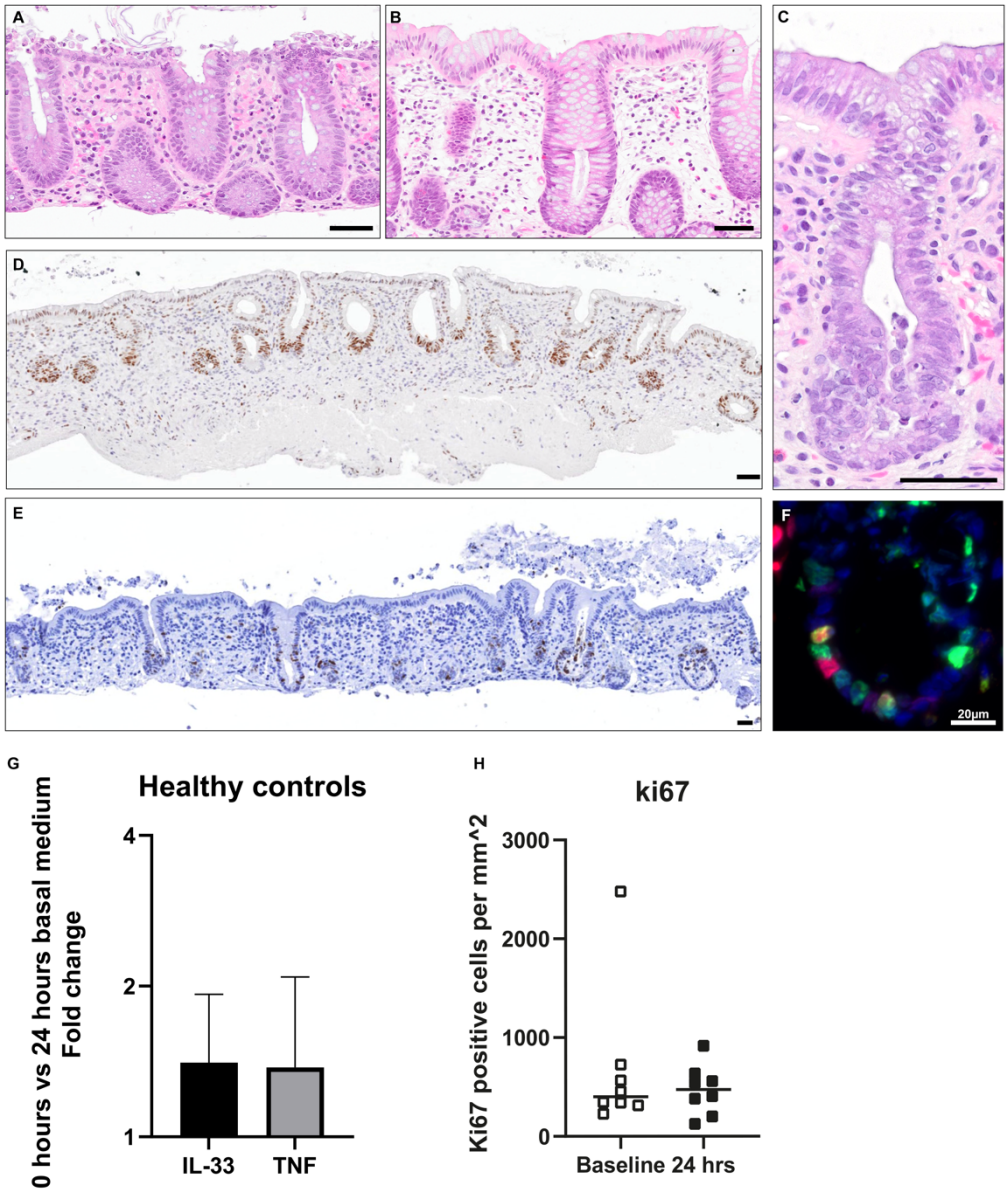

Figure 2. *Ex vivo* colonic biopsy model

Endoscopic pinch biopsies taken from the sigmoid colon were used in an *ex vivo* biopsy model. Images **A-C** show hematoxylin and eosin staining of biopsies from healthy controls following 24 hours in basal medium. Image **D** shows a biopsy from quiescent UC (Mayo score ≤ 2) stimulated with IFN-gamma 100ng/ml for 24 hours in the basal medium.

Immunoenzymatic staining confirms a strong signal of pSTAT1 (brown) in the epithelial border confirming good uptake of IFN-gamma to the epithelium.

In image **E** the proliferative cell marker Ki67 (brown) was detected with immunostaining in healthy controls at baseline and following 24 hours with no significant change in expression shown in graph **H**.. In image **F** dual-staining for IL-33 (red) and Ki67 (green) revealed IL-33 presence also in Ki67 positive cells (yellow). RT-qPCR was performed for IL-33 and TNF in healthy controls comparing baseline (0 hours) with 24 hours incubation in basal medium. No significant differences were seen (IL-33 p-value = 0.3, TNF p-value = 0.8) shown in graph G..

Scale bars = 50µm.

Figure 3. Hypo-osmotic stress induces IL33 in colonic crypts.

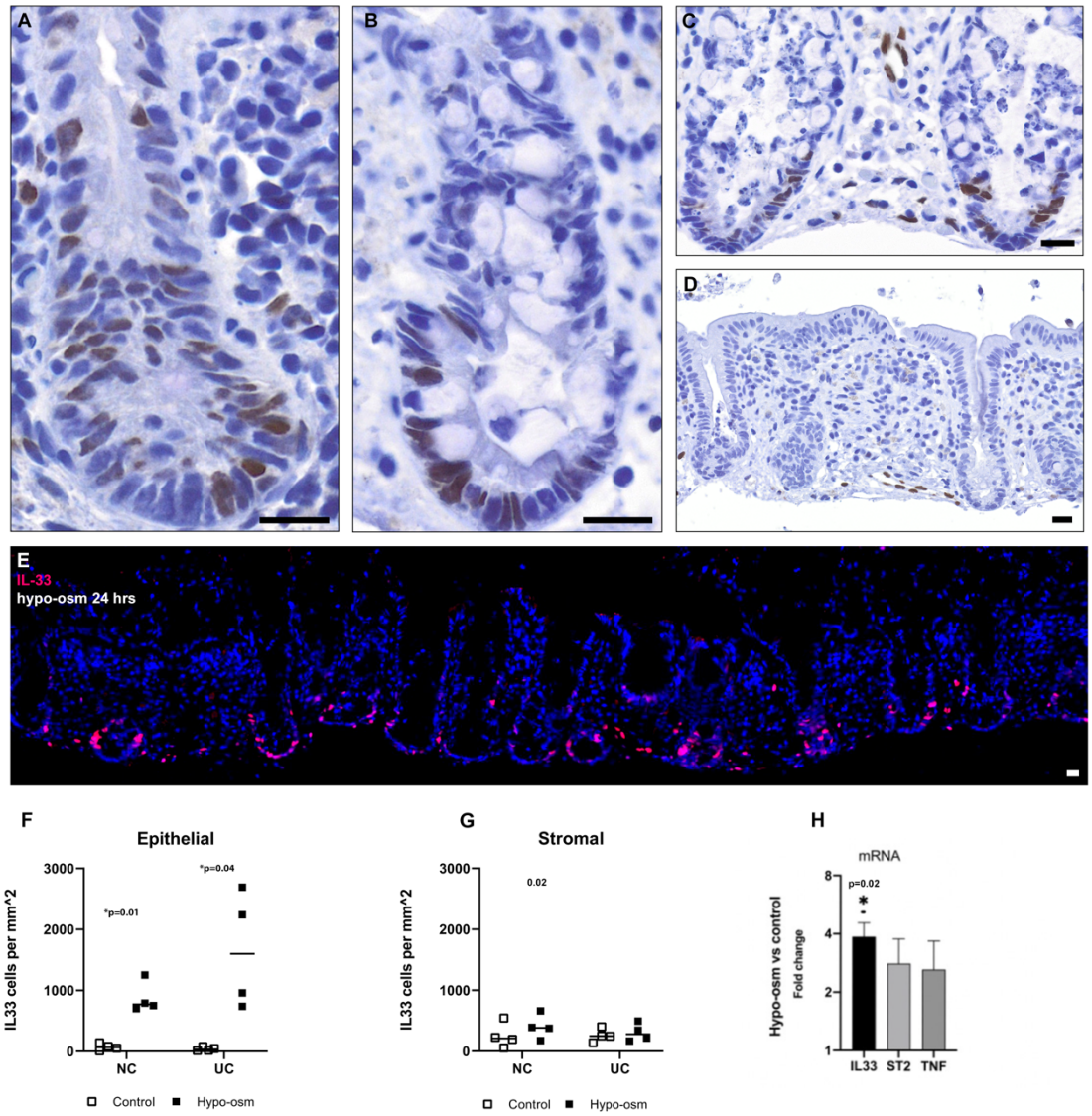

Figure 3. Hypo-osmotic stress induces IL-33 in colonic crypts.

Colonic biopsies from *ex vivo* biopsy cultures including quiescent UC with a a Mayo score ≤ 2 (image **A** and **E**) and healthy controls (image **B-D**). Stimulation with hypo-osmotic medium for 24 hours induces positive IL-33 (brown) cells shown with immunoenzymatic staining in **A-C.** Cell nuclei stained with hematoxylin (blue). Control biopsy cultured for 24 hours in basal medium (image **D)** did not show IL-33 positive cells in the epithelium. Image **E** shows fluorescence staining in UC after 24 hours of hypo-osmotic basal medium, IL-33 (red) is seen in the nucleus of epithelial cells with cell nuclei counterstained with Hoechst (blue).

IL-33 was significantly increased in the epithelium of both healthy controls (NC) with a p-value =0.01 and for UC p-value=0.04 following hypo-osmotic exposure for 24 hours (graph **F**). No significant changes were seen in the stroma (graph **G**) p-values 0.14 and 0.38 for NC and UC. Graph H shows fold change of mucosal gene expression for IL33, ST2 and TNF comparing biopsies stimulated with hypo-osmotic medium or basal medium (controls) for 24 hrs. Scale bars = 20μm.

Figure 4. IFN-gamma stimmualtion

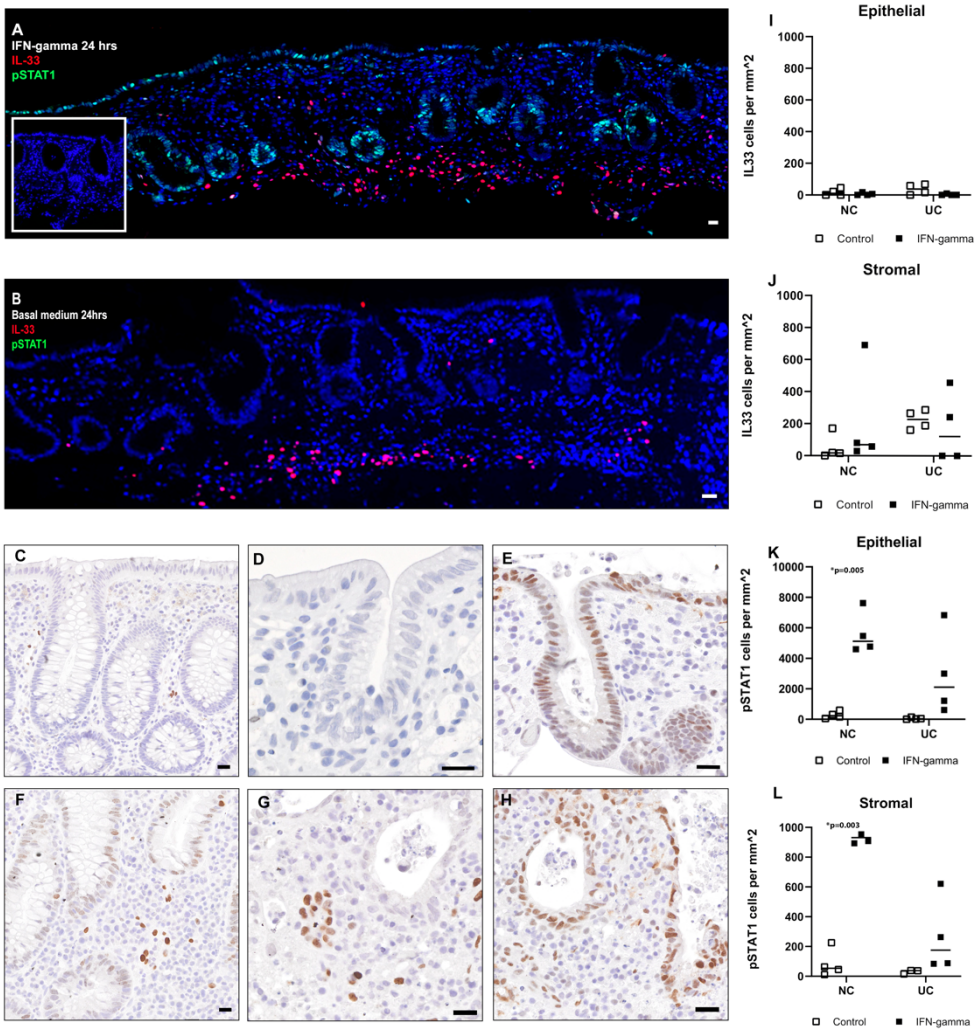

Figure 4. IFN-gamma stimulation

Image **A** and **B** show immunofluorescence staining of quiescent UC (Mayo score ≤2) in colonic biopsies for IL-33 (red) and pSTAT1 (green) and nuclei counterstained with Hoechst (blue). Image **A** shows a strong pSTAT1 signal in epithelial cells following 24 hours of IFN-gamma 100ng/ml stimulation for 24 hours. Infelt to the left is an isotype and concentration

matched control. Image **B** shows the control biopsy cultured in basal medium for 24 hours (non-stimulated) with no pSTAT1 epithelial signal.
Immunoenzymatic staining for IL-33(brown) is shown in a healthy control (**C-D)**, Baseline biopsy at 0 hours (image **C**) and following 24 hours of IFNG stimulation (image **D**) Further pSTAT1 (brown) staining is shown in image E after 24 hours 100ng/ml IFN-gamma stimulation. Acute UC (Mayo score =6) is shown in **F-H**. IL-33(brown) is shown in image **F** (baseline 0 hours) and image **G** (stimulated 24 hours with IFN-gamma). Image **H** shows pSTAT1(brown) following IFNG- stimulation for 24 hours. Quantification of immunostaining is given in graphs **I-L**. Healthy controls = NC. No significant presence of IL-33 was seen in the epithelium following IFN-gamma stimulation for 24 hours. pSTAT1 was significantly increased in the epithelial border and stroma in both healthy controls (NC) and UC.

Scale bars are given at 20s are given at 20μm.

Figure 5. *Ex vivo* biopsies stained for IL-33

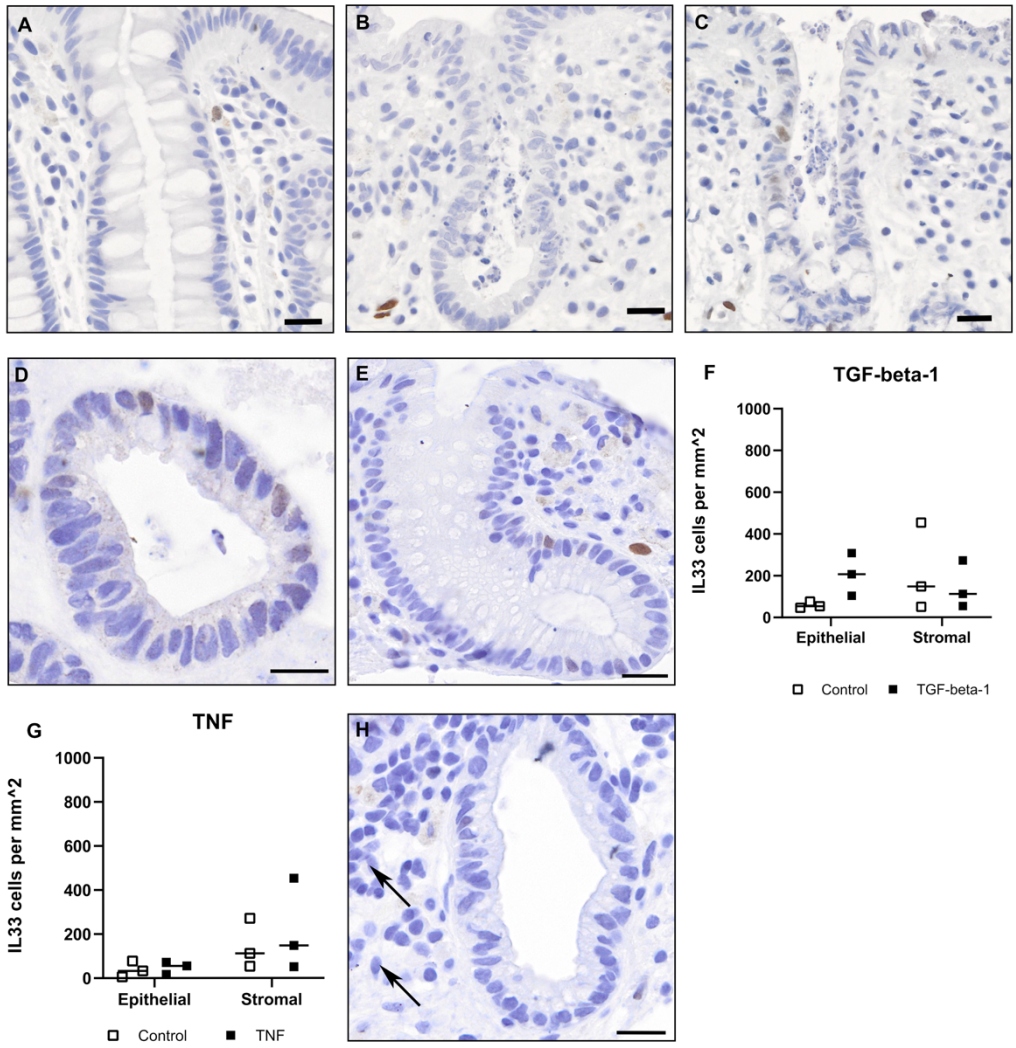

Figure 5. *Ex vivo* biopsies stained for IL-33

Colonic biopsies from healthy controls showing immunoenzymatic staining for IL-33 (brown) and cell nuclei counterstained with hematoxylin (blue). **A-C** shows biopsies at A: baseline o hours (**A**) 12 hours (**B**) and 24 hours (**C**) with stimulation with TGF-beta-1 10ng/ml. Sporadic but positive epithelial cells for IL-33 were seen. Images D and E also show healthy controls after 24 hours of TGF-beta-1 stimulation. Image H shows stimulation with proinflammatory cytokine TNF 10 ng/ml for 24 hours, with no positive epithelial cells for IL-33, also of note IL33 I endothelial vessels was lacking (**arrows)**. Scale bars = 20µm. Graphs F and G shown results from image quantification of positive cells.