Aus der
Kinderklinik und Kinderpoliklinik im Dr. von Haunerschen Kinderspital
der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. med. Dr. sci.nat. C. Klein

Expression of Interleukin-37 in paediatric chronic inflammatory bowel disease

Dissertation
zum Erwerb des Doktorgrades der Humanmedizin
an der Medizinischen Fakultät
der Ludwigs-Maximilians-Universität zu München

vorgelegt von
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2017

Mit Genehmigung der Medizinischen Fakultät der Universität München

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Tag der mündlichen Prüfung: 29.06.2017

Dedicated to my grandparents,
Ordinary people who in the aftermath of a nation hit by destruction
With their hands laid a foundation for me to reach this place of education.

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1 Introduction

1.1 Chronic Inflammatory Bowel Disease

Chronic inflammatory bowel disease (IBD) mainly comprises two diseases – Crohn's Disease (CD) and Ulcerative Colitis (UC). The rising incidence of childhood CD and UC underline the need of further investigation of their pathogenesis and treatment options. Males and females are equally affected with an average incidence of 5:100.000 for both diseases. Higher rates of incidence are observed in northern countries (Scandinavia, Great Britain and Germany) than in southern nations (Japan, New Zealand). More than 25% of the patients show first manifestations before their 20th birthday [1].

Patients with CD present with abdominal pain, loss of weight and chronic diarrhoea sometimes associated with bloody stools. Fissures, perianal abscesses und mariskes are likely to be found. Additionally, patients show extraintestinal manifestations such as anorexia, loss of activity, aphthous lesions or relapsing fever as well as involvements of the skin, eyes and joints. Complications include relapsing and stenosing disease, fistula formation and especially in paediatric patients failure to thrive and malabsorption.

UC also presents with abdominal pain, bloody diarrhea and painful tenesms. Extraintestinal manifestations include arthritis, autoimmune hepatitis, sclerosing cholangitis or iridozyklitis. Patients with UC have an increased risk to develop colon cancer [2].

Histology of CD shows discontinuous, transmural inflammation and may involve the upper and lower gastrointestinal tract. Microscopically, epitheloid granuloma can be present. In contrast, UC patients show continuous inflammation starting in the distal colon. Microscopic inflammation is limited to the mucosal layer, whereas deeper layers remain unaffected. Histologically, characteristic crypt abscesses are present [2].

Several factors contribute to the pathogenesis of IBD which can be summarized in three categories: Genes, environment and an altered microbiom as well as interactions of these three lead to an inappropriate inflammatory response[3]. CD and, to a less extent, UC show a familial clustering with genetic involvement. An increasing number of mutations is being identified, such as single nucleotide polymorphisms (SNP) in the genes of nucleotide-binding oligomerisation domain 2 (NOD2) or ATG16L1 (autophagy-related, 16-like) which partake in innate immune responses. Defects of the interleukin-23 receptor (IL-23R) that is part of the interleukin-23 Th-17 pathway[4] or mutations in interleukin-10 (IL-10) or the IL-10 receptor[5] are other factors that could be identified to result in a severe phenotype of early onset inflammatory bowel disease. Secondly, environmental factors are thought to play another role in the pathogenesis of IBD. People in development countries show a lower risk for IBD, but after moving to countries of high IBD incidences, their personal risk also increases[1]. Thirdly, the gut microbiom draws increasing attention to partake in the pathogenesis of

both CD and UC[6]. So far, no single triggering organism has been identified, rather can interactions between the microbiome and the host either be beneficial or can incite inflammation. The epithelium of the gut serves as barrier between gut bacteria and the body. Increased epithelial permeability allows the human immune system to get in contact with exogenous antigens to evocate an aberrant and exaggerated immune response[7,8] that is mainly driven by the innate immune system.

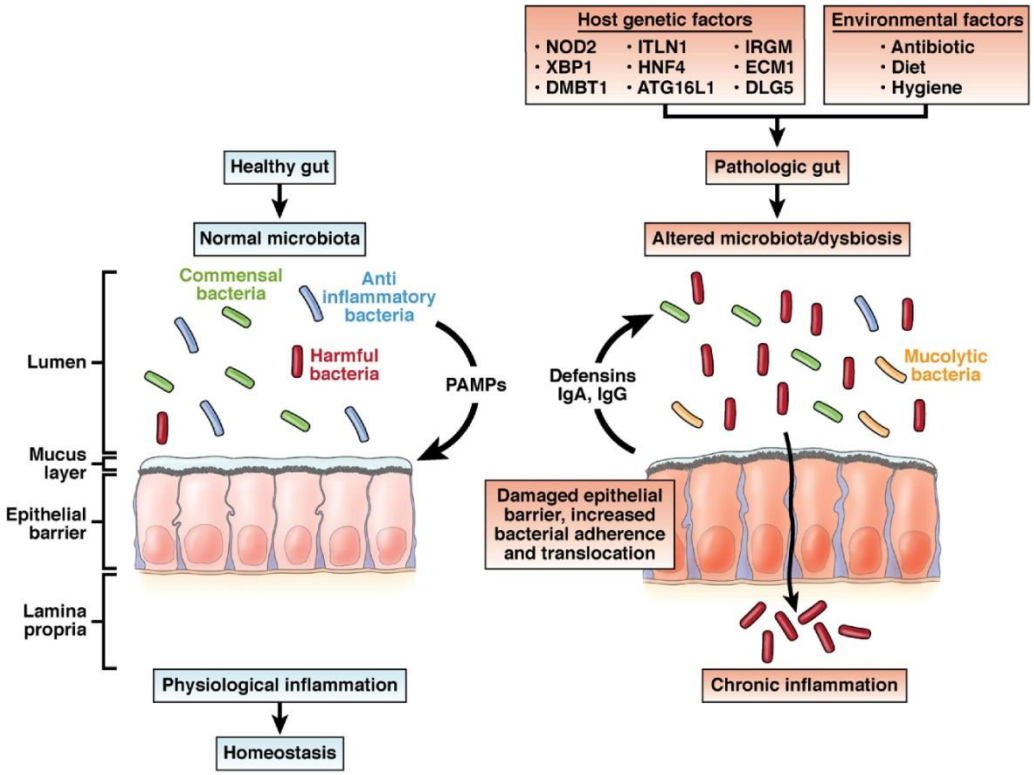

Figure 1.1 Pathogenesis factors for IBD. Left panel: In the healthy gut, commensal and anti-inflammatory bacteria interact with the gastrointestinal mucosa to maintain homeostasis through recognition of PAMPs and a physiologic host response. Right panel: In patients with IBD, inflammation is induced through abnormal interaction between host cells and microbes. Inflammation is due to genetic factors affecting barrier function, innate and adaptive immunity or changes in the quality or quantity of the microbiota. Adapted from Chassaing et al. 2011[6].

1.2 The role of Innate Immune Mechanisms in the pathogenesis of IBD

Innate immune response involves recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors expressed on a variety of immune cells, such as toll-like receptors (TLRs), as well as the production of pro-inflammatory cytokines. TLRs are important sensing molecules for bacterial or viral molecules as lipopolysaccharide (LPS) or double-stranded RNA[9].

LPS stimulates the production of pro-inflammatory cytokines [10]. Cytokines are mainly produced by immune cells and facilitate the communication between cells, stimulate the proliferation of antigen

specific effector cells, and mediate the local and systemic inflammation in an autocrine, paracrine, and endocrine way[11].

For example, activated dendritic cells (DC) and macrophages produce a variety of cytokines, for example interleukin (IL)-1β, IL-6, IL-23, tumour necrosis factor (TNF)-α leading to the differentiation of undifferentiated naïve T-helper (Th0) cells into Th1, Th2, Th17 or Treg (T-regulatory) cells (Figure 1.1)[12]. In response, differentiated T-helper (Th) cells release a variety of pro- or anti-inflammatory cytokines. While CD rather seems to be driven by Th1-cells producing pro-inflammatory interferon (IFN)-γ, TNF-α or IL-18, Th2-cells play a larger role in UC, releasing IL-13 and IL-5. Th17-cells are present in both types of diseases. In contrast, Treg cells produce anti-inflammatory IL-10 and transforming growth factor (TGF)-β. Inflammation is maintained by a dysregulation of pro- and anti-inflammatory factors. This can either happen through an increased activity of effector T-cells or a decreased number or function of Treg-cells (Figure 1.2)[13].

IL-17 has been shown to play a pivotal role in the pathogenesis and maintenance of inflammation in CD and UC. IL-17 is secreted by a distinct T-cell subset that is named after its product - Th17 cells. Th17 cells express the IL-23 receptor on their surface; mutations in genes of IL-23 signalling are associated with susceptibility for both CD and UC, therefore implying a strong involvement of IL-17 in IBD. IL-17 has a strong stimulatory effect on intestinal epithelial cells as well as macrophages by inducing the release of pro-inflammatory cytokines and chemokines, ultimately leading to the perpetuation of intestinal inflammation[14].

The IL-1 family member IL-18 is also linked to auto-immunity in IBD. IL-18 is mainly produced by monocytes and macrophages in response to viral or bacterial stimuli, therefore being a response molecule of innate immunity to host-pathogen interaction. In synergy with other cytokines such as IL-12 or IL-23, extracellular IL-18 in its mature form induces the production of IFN-γ or IL-17[15].

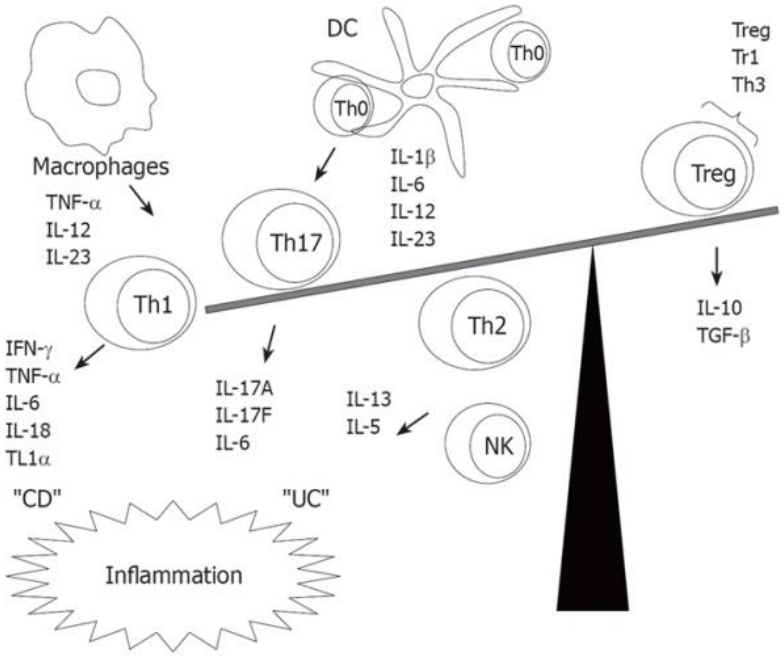

Figure 1.2. An imbalance in T-Cell differentiation induces the production of pro-inflammatory cytokines. These, in turn, uphold tissue inflammation. Adapted from Sanchez-Munoz et al. 2008[12].

1.3 The Interleukin-1 Family of Cytokines and Inflammation

Cytokines of the interleukin-1 family of ligands and receptors play a pivotal role in the modulation of inflammatory and immune responses[16,17]. While IL-1 family members 1 to 4 (IL-1α, IL-1β, IL-Ra and IL-18) have been well investigated, within the last years seven new members have been discovered from expressed sequence tag data base searches[18,19]. There are two nomenclatures used for IL-1 family members (IL-1F): IL-1F1-11 or the "classic" nomenclature as indicated in Table 1.1[20]. Each cytokine exerts its particular function through binding to a specific receptor or receptor complex[21]. Except for the gene of IL-33 and IL-18, the IL-1 family cytokines form a cluster on chromosome 2q13[22] and share a common structure of 12 β-strands[23]. Most of the IL-1 family members show pro-inflammatory activity, while four members including IL-37 have anti-inflammatory effects.

	alternative nomenclature	receptor	function
IL-1F1	IL-1α	IL-1RI, IL-1RAcP	pro-inflammatory
IL-1F2	IL-1β	IL-1RI, IL-1RAcP	pro-inflammatory
IL-1F3	IL-1Ra	IL-1RI	*anti-inflammatory*
IL-1F4	IL-18	IL-18Rα, IL-18Rβ	pro-inflammatory
IL-1F5	IL-36Ra	SIGIRR	*anti-inflammatory*
IL-1F6	IL-36α	IL-1Rrp2, IL-1RAcP	pro-inflammatory
IL-1F7	IL-37	IL-18Rα, IL-1R8	*anti-inflammatory*
IL-1F8	IL-36β	IL-1Rrp2, IL-1RAcP	pro-inflammatory
IL-1F9	IL-36γ	IL-1Rrp2, IL-1RAcP	pro-inflammatory
IL-1F10	IL-38	IL-1RI, IL-36Ra	*anti-inflammatory?*
IL-1F11	IL-33	ST2	pro-inflammatory

Table 1.1. Overview of all IL-1 family members, their receptors and function.

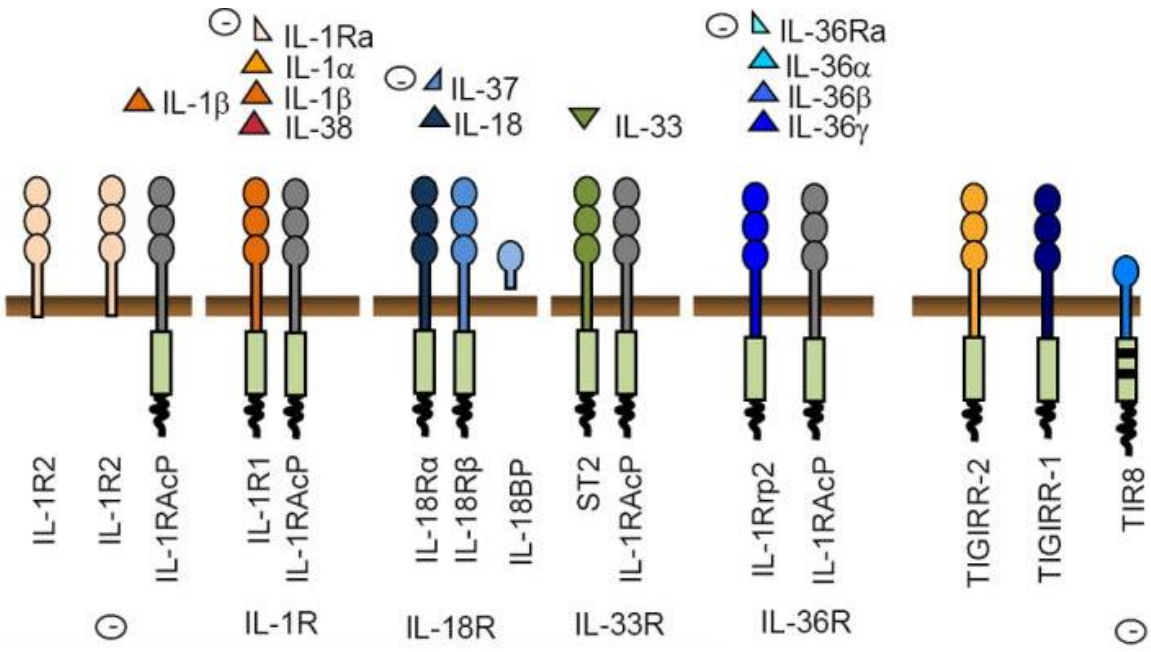

Figure 1.3 IL-1 family ligands and receptor complexes.[24]

The innate immune system enforces pattern recognition receptors (PRR) binding to PAMPs or damage associated molecular patterns (DAMPs). Members of PRR are TLRs, retinoic acid-inducible gene-I (RIG-I)-like receptors and nucleotide-binding oligomerisation domain (NOD)-like receptors NLRs. Activation of NLR proteins (NLRP) induce the formation of multiprotein/protease complexes

that are called the inflammasome of which the NLRP3-inflammasome so far is the most investigated one. This complex mediates the activation of caspase-1. IL-1 family members, such as IL-1β, IL-18 and IL-33 are cleaved from the pro-form into their active forms by this enzyme[25] thus leading to the activation of pro-inflammatory proteins and the maintenance of inflammation. IL-1 family members have been described to trigger inflammation. Mutations in NLR-inflammasome associated proteins are linked with human inflammatory disorders[26]. For example, mutations in NLRP3 cause constitutive maturation of IL-1β leading to recurrent fever and inflammation as described for patients with Muckle-Wells syndrome[27].

1.4 Interleukin-37

IL-37 (interleukin-37, formerly known as IL-1F7) is the seventh member of the IL-1 cytokine family. Until recently, the function of IL-37 was unknown. Now, there is a whole body of evidence that IL-37 plays a pivotal role in downregulating innate and adaptive immune mechanisms[28].

1.4.1 **IL-37 gene and protein**

The IL-37 gene contains six exons that are expressed in five isoforms (Figure 1.4). IL-37a holds a unique N-terminus which is only to be found in this isoform encoded on exon 3. Isoforms c, d and e are shorter variants lacking either exon 2, exon 4 or both of these. Isoform 1, also named isoform b (IL-1F7b, IL-37b), is the largest, most investigated and most abundantly expressed isoform with its five exons (1, 2 and 4-6). Exons 1 and 2 hold an N-terminal pro-domain containing a putative caspase-1 cleavage site, which is located in exon 1 at aspartic acid D20[29]. Exons 4-6 are coding for the IL-1 family typical β-strands. Due to an instability element in the coding area for IL-37 mRNA, just small amounts of IL-37 mRNA can be found in vitro in cells being stably transfected with IL-37. Only after stimulation with TLR stimuli, in particular with LPS, expression of IL-37 mRNA is markedly increased. Upregulation of IL-37 mRNA follows rapidly and reaches a maximum 60’ after treatment. This effect can also be seen in human PBMCs[30]. Additionally, two amino acid polymorphisms (V31G and A42T) have been described in IL-37b, based on base pair mutations in exon 2[18,31].

IL-37 transcripts have been detected in human testis, thymus and uterus by real-time PCR[18] whereas IL-37 protein could be detected in tonsils, skin, esophagus, placenta[29], PBMC[32], in some carcinomata such as ductal breast or colon carcinomata[29]. Due to a coding region instability element in the mRNA, IL-37 protein expression is low unless inflammatory signals. In as such stabilisation of IL-37 mRNA is induced by exogenous stimulation in primary human cells or in mouse and human cell lines transfected with IL-37 and followed by an increase of IL-37 protein levels[30]. For example, IL-37 expression is induced in human PBMCs through TLR stimuli as LPS but also anti-inflammatory TGFβ[28]. The molecular size of natural IL-37b in human monocytes is 30 kDa. IL-37b can form homodimers[29].

IL-37 was not discovered in the mouse genome until today.

1.4.2 **IL-37 Function**

Over the last years several properties of IL-37 have been described:

IL-37 is a dual-function cytokine. While most members of the IL-1 family are pro-inflammatory cytokines, IL-37 has anti-inflammatory abilities that have been shown *in vitro*. Mouse RAW macrophages that were transfected with IL-37 plasmid show reduced levels of LPS-induced IL-1α, IL-6, TNF-α and MIP-2 (macrophage inflammatory protein 2)[33]. Intracellular IL-37 translocates to the nucleus after processing by caspase-1 into the mature protein after cell stimulation[33]. Specific mutation of the caspase-1 cleavage site in IL-37 abolishes the nuclear translocation in transfected cells and is therefore required to exert its cytokine-suppressing functions in macrophages[34]. Intracellular IL-37 immuno-precipitates with phosphorylated Smad3. An inhibitor of Smad3 reversed IL-37-mediated inhibition of IL-6 and IL-1α in LPS-stimulated IL-37-RAW-cells[28].

Extracellular IL-37 binds to the IL-18Rα, but not as a direct antagonist to IL-18. IL-37 and IL-18 share similarities such as a similar three-dimensional structure, are both cleaved by caspase-1 and have a similar mRNA stabilisation[35]. The IL-18 receptor consists of the ligand-binding IL-18α chain and a β chain activating different kinases which results in the translocation of nuclear factor κB. IL-37 does not recruit the IL-18 β chain but binds to IL-1R8 to exert anti-inflammatory properties[36]. It has also been shown that IL-37 binds to the IL-18 binding protein (BP). IL-18BP is the natural antagonist of IL-18. IL-37 enhances the antagonistic abilities of IL-18BP, but only at limiting concentrations of IL-18BP and at very high molar excess of IL-37[32]. Whereas secretion of mature IL-37 is linked to cleavage, the IL-37 precursor is active independent of cleavage. Addition of neutralizing IL-37 antibody reverses anti-inflammatory properties if IL-37 in tgIL-37 mice.[34] Thus, IL-37 works as a cytokine with dual function both intra- and extracellular (Figure 1.5).

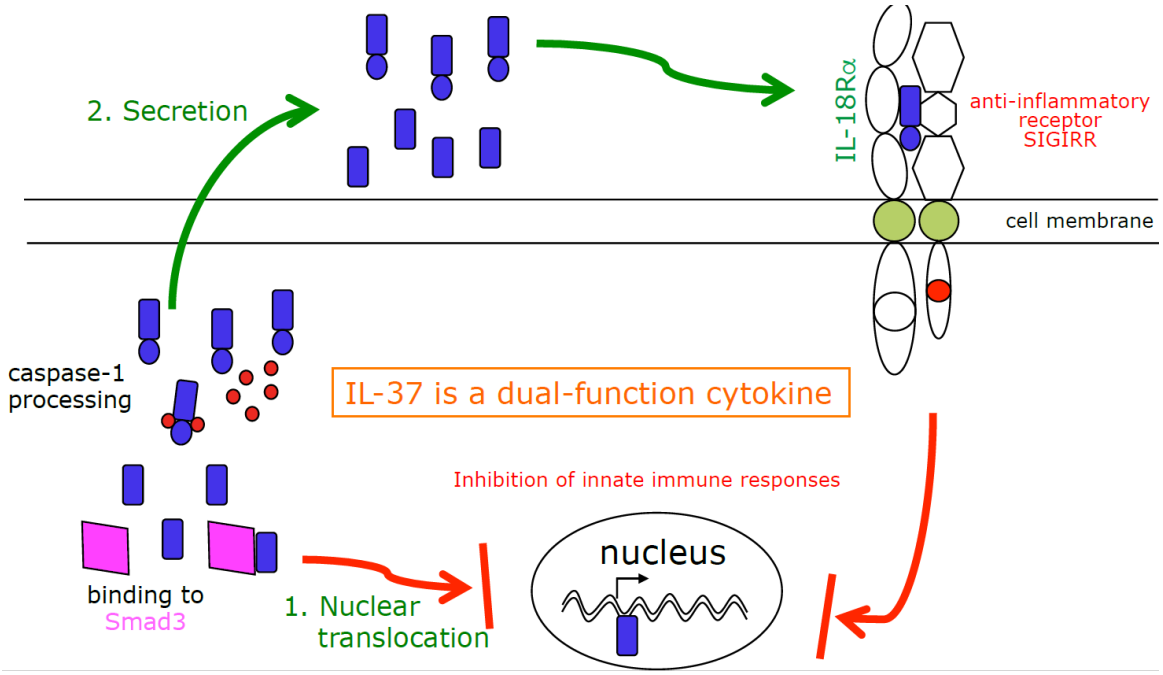

Figure 1.5. Dual function of IL-37. In the intracellular space, after cleavage and activation through caspase-1, IL-37 binds to Smad3 and translocates to the nucleus. There, it exerts anti-inflammatory effects through inhibition of innate immune response. On the other hand, secreted extracellular IL-37 binds to the IL-18 receptor chain α and the IL-1R8, also leading to anti-inflammatory effects.

Since 2010 various disease models were published in IL-37tg mice or wildtype mice treated with human recombinant IL-37 protein[37]. Some of the models are discussed in the following paragraph.

In vivo expression of IL-37 reduces inflammation in a hepatitis mouse model. Serum levels of IL-1α, IL-5, IL-6 and IL-9 were reduced 2 hours after induction of hepatitis with Concavalin A in mice that received injection of IL-37 plasmid DNA in comparison to control mice. However, this effect was not sustained for 24 hours and the histological liver damage was equal in both groups[38].

Transgene mice are protected from LPS-induced shock. As indicated above, IL-37 is naturally not present in mice. Recently, several data from IL-37tg (transgene) mice have been published. Tg-mice are protected from LPS-induced shock and mortality[28]. HCO_3 and pH are higher, pCO_2 lower and liver enzymes less elevated. In homozygous mouse plasma, pro-inflammatory cytokines such as IL-6, IL-1β, IL-17 and IFN-γ are nearly absent. In the spleen of IL-37tg mice, concentration of TGF-β_1 is increased. Mice expressing IL-37 appear to show less LPS-induced DC (dendritic cell) activation. Therefore, lungs, kidneys and liver are protected from endotoxic shock through IL-37b expression.

IL-37 induces anti-tumorous activity. IL-37 in mouse fibrosarcoma, that was infected with AdIL-1H4 (IL-1 homologue 4 recombinant adenovirus) through adenovirus-mediated gene transfer shows anti-tumorous activity. Tumour growth was fully inhibited after multiple injections of AdIL-1H4[39]. In SCID mice and IL-12, IFN-γ or Fas-ligand deficient mice anti-tumour activity could not be observed. Zhao et

al could show that IL-37 expression is decreased in HCC tumour tissue and that high expression of IL-37 correlates with higher survival rate.[40]

IL-37 expression protects mice from acute DSS-induced colitis. hIL-37tg mice that were administered with dextran sulfate sodium (DSS) for 7 days for induction of acute colitis were protected from inflammation in contrast to WT (wild type) mice (Figure 1.5)[41]. DSS is a reagent that induces acute or chronic colitis through epithelial disintegration. hIL-37tg mice lost less weight, have shown lower disease activity scores, had a less reduced colon length and decreases total histological scores than WT mice. It was shown that IL-37 expression correlates with intestinal barrier breakdown. Furthermore, IL-37 suppresses colonic expression of pro-inflammatory cytokines (TNFα, IL-1β) and increases anti-inflammatory IL-10 expression. However, anti-IL-10 receptor blocking did not affect the severity of colitis showing that the protective effect of IL-37 is not dependant on IL-10 induction. As another effect, a reduced recruitment of leucocyte populations to the colonic lamina propria was seen in hIL-37tg mice. WT mice that were transplanted hIL-37tg bone marrow were protected from severe colonic inflammation indicating that hematopoietic IL-37 mediates anti-inflammatory effects rather than stromal IL-37.

1.5 Aim of this study

The rising incidence of Crohn's Disease and Ulcerative Colitis in children and adults is a cumulating individual and socioeconomic burden and underline the need for better understanding of the pathogenesis of IBD in order to develop effective treatment possibilities. Until today, a broad number of new biological treatment options have been applied in IBD, that targeted proinflammatory cytokines as TNFa or IL-12/IL-23, cytokine receptors or adhesion molecules[42]. Some of these therapies proofed to be highly effective. However, not all patients respond to treatment or suffer loss of therapeutic efficacy. Based on its fundamental anti-inflammatory properties IL-37 might be an interesting target molecule to treat human IBD.

The expression pattern of yet unknown proteins may indicate their functional impact in health and disease. We therefore aimed to study the expression level and pattern of IL-37 mRNA and protein in the gut of children with CD and UC. We correlated the results with disease activity in comparison to a healthy cohort. In addition, we analyzed the expression of IL-37 isoforms in different human tissues.

2 Patients, material and methods

2.1 Patients

Paediatric IBD patients were diagnosed according to clinical, endoscopical and pathological criteria. Disease severity was classified using the PUCAI (Paediatric Ulcerative Colitis Activity Index)[43] or the wPCDAI (weighted Paediatric Crohn's Disease Activity Index)[44] for clinical assessment and the Paris Classification for disease location[45].

wPCDAI:

Abdominal pain:

- none (0)
- mild: brief, does not interfere with activities (10)
- moderate/ severe: daily, longer lasting, affects activities, nocturnal (20).

Patient functioning, general well-being:

- no limitation (0)
- occasional difficulty in maintaining age appropriate activities (10)
- frequent limitation of activity, very poor (20).

Stools per day:

- 0-1 (0)
- up to 2 semi-formed with small blood or 2-5 liquid (7.5)
- gross bleeding or >6 liquid or nocturnal bleeding (15)

ESR:

- ESR <20 mm/hour (0)
- ESR = 20-50 mm/hour (7.5)
- ESR > 50 mm/hour (15)

Albumin:

>3.5 g/dL (0)

3.1 – 3.4 g/dL (10)

<3 g/dL (20)

Weight:

- weight gain or voluntary stable (0),
- involuntary stable or loss 1-9% (5)
- weight loss >10% (10)

Perirectal disease:

- none (0)

- 1-2 indolent fistula (7.5)
- active fistula, drainage, tenderness or abscess (15)

Extra-intestinal manifestations (fever >38.5°C for 3 days over past week, definite arthritis, uveitis, E.nodosum, P.gangrensoum)

- none (0)
- one or more (10)

The numbers in brackets show the amount of points to be given for each item. Maximum total sum for wPCDAI is 125 points.

PUCAI:

Abdominal pain:

- no pain (0)
- pain can be ignored (5)
- pain cannot be ignored (10).

Rectal bleeding:

- none (0)
- small amount in less than 50% of stools (10)
- small amount with most stools (20)
- large amount (>50% of the stool content) (30)

Stool consistency of most stools:

- formed (0)
- partially formed (5)
- completely unformed (10)

Number of stools per 24 hours:

- 0-2 (0)
- 3-5 (5)
- 6-8 (10)
- >8 (15)

Nocturnal stools:

- no (0)
- yes (10)

Activity level:

- no limitation of activity (0)
- occasional limitation of activity (5)
- severe restricted activity (10)

The numbers in brackets show the amount of points to be given for each item. Maximum total sum for PUCAI is 85 points.

Paris classification:

Age at diagnosis: A1a: 0 - <10 years; A1b: 10 - <17 years; A2: 17 – 40 years; A3: >40 years.

Location (for CD): L1: distal 1/3 ileum ± limited cecal disease; L2: colonic; L3: ileocolonic; L4a: upper disease proximal to ligament of Treitz; L4b: upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum.

Behaviour (for CD): B1: non-stricturing, non-penetrating; B2: stricturing; B3: penetrating; B2B3 both structuring and penetrating; p: perinanal disease modifier.

Extent (for UC): E1: ulcerative proctitis, E2: left-sided UC (distal to splenic flecture); E3: extensive (hepatic flecture distally); E4: pancolitis (proximal to hepatic flecture).

Severity (for UC): S0: never severe; S1: ever severe (severe defined by PUCAI ≥ 65).

Growth: G0: no evidence of growth delay; G1: growth delay.

Standard serum inflammatory markers (CRP, leucocytes, haematocrit, thrombocytes, ESR [erythrocyte sedimentation rate], fibrinogen and albumin) were protocolled as well as weight, height and BMI at the time of diagnosis with IBD. Units for laboratory markers were used as by the central laboratory of Dr. von Haunersches Childrens' Hospital. BMI (body mass index) is defined as kg/m^2.

Colonic biopsies were taken from macroscopically inflamed sites from 16 children with CD and 12 children with UC through colonoscopy. Control biopsies were taken from 11 paediatric patients with polyps of the colon or Hirschsprung Disease. The diagnosis was made by a pathologist.

For better evaluation, samples were scored for the grade of inflammation (0 – no inflammation; 1 – slight active inflammation; 2 – moderate active inflammation; 3 – intense active inflammation).

Involvement criteria were an age younger than 18 years and no prior treatment with immunomodulatory medication. Therefore, most of the patients were first diagnosed with IBD at the time of colonoscopy. However, three children of the CD group and one child of the UC group had received such medication before colonoscopy but due to active inflammation still were included. Patient data was coded naming first the name of the group followed by the patient number within that group, e.g. CD5 being the fifth patient in the Crohn's Disease group.

2.2 Equipment and material

2.2.1 **Equipment**

Dispersing element 5G for Homogenizer T10	IKA (Staufen, Germany)
LaminAir HB2472 cell culture hood	Heraeus Instruments (Hanau, Germany)

Centrifuge 5415D	Eppendorf (Hamburg, Germany)
Big Centrifuge RotantaS	Hettich (Tuttlingen, Germany)
Confocal Microscope FV1000	Olympus (Hamburg, Germany)
7900HT fast real-time PCR system	BD Appl. Biosciences (Carlsbad, CA, USA)
Gel electrophoresis system	Biorad (München, Germany)
Homogenizer T10 basic	IKA (Staufen, Germany)
Crushed ice machine	Ziegra (Isernhagen, Germany)
Hera cell 240 incubator	Thermo Fisher Scientific (Langensebold, Germany)
Royal Bio-Imaging System Intas Gel iX Imager	Intas (Göttingen, Germany)
Mastercycler professional	Eppendorf (Hamburg, Germany)
Microscope Axioskop	Carl Zeiss (Jena, Germany)
Nanodrop spectrophotometer nd-1000	Thermo Fisher Scientific (Langensebold, Germany)
TEC 3000 nitrogen tank	MVT biological systems (Ball Ground, GA, USA)
UV-Visible Spectrophotometer	Amersham Biosciences (Glattbrugg, Switzerland)
PHM 82 standard pH-Meter	Radiometer (Copenhagen, Denmark)
Pipettes Eppendorf professional	Eppendorf (Hamburg, Germany)
Scale 440-47N	Kern and Son GmbH, Germany
Sprout table centrifuge	Sprout/ Biozym (Hessisch Oldendorf, Germany)
Vortexer Reax top	Heidolph (Schwabach, Germany)

2.2.2 **Reagents and material for immunohistochemistry and H.E. staining**

Reagents

Acid Alcohol	see protocol
AEC+	Dako (Hamburg, Germany)
Antibody Diluent	Dako (Hamburg, Germany)
Biotin Blocking System	Dako (Hamburg, Germany)
Ethanol	Merck (Darmstadt, Germany)
Eosin	Leica (Wetzlar, Germany)
DAB+	Dako (Hamburg, Germany)
Hematoxylin	Leica (Wetzlar, Germany)
Hematoxylin Gill's Formula	Vector (Burlingame, CA, USA)
Histoclear	AGTC bioproducts Ltd (Wilmington, MA, USA)
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich (Steinheim, Germany)
ImmPRESS Reagent Kit anti-goat Ig	Vector (Burlingame, CA, USA)
Kaisers Glycerine Gelatine	Merck (Darmstadt, Germany)

DPX Mountant for histology	Sigma-Aldrich (Steinheim, Germany)
PBS (pH 7.5)	see protocol
ProTaqs II Antigen-Enhancer	Quartett Immunodiagnostika & Biotechnologie GmbH (Berlin, Germany)
Target Retrieval Solution Citrate	Dako (Hamburg, Germany)
Target Unmasking Fluid	Pan Path (Budel, Holland)
TRIS-Buffer	Sigma-Aldrich (Steinheim, Germany)
VectaStain ABC-Kit Elite Universal	Vector (Burlingame, CA, USA)
Xylol	AppliChem (Darmstadt, Germany)

Material

Superfrost Plus microscope slides	Menzel (Braunschweig, Germany)
Deckgläser	Menzel (Braunschweig, Germany)
Staining rack with lid	Witeg (Wertheim, Germany)
Staining rack, type Schiefferdecker	Carl Roth (Karlsruhe, Germany)
Staining rack, type Coplin	Carl Roth (Karlsruhe, Germany)

2.2.3 **Reagents and material for PBMC isolation**

Ac-muramyl-Ala-Glu-NH_2 (MDP)	Bachem (Bubendorf, Switzerland)
Dubecco's PBS sterile	PAA (Cölbe, Germany)
Foetal bovine serum	Sigma-Aldrich (Steinheim, Germany)
Ficoll-Paque PLUS	GE healthcare (München, Germany)
Heparin-Natrium 5000 IE	Ratiopharm (Ulm, Germany)
Lipopolysaccharide from E. coli 055:B5	Sigma-Aldrich (Steinheim, Germany)
Neubauer cell counting chamber	Neubauer (Friedrichsdorf, Germany)
Pam3Cys-SKKKK x3 HCl	EMC microcollections (Tübingen, Ger)
Gibco RPMI cell medium 1640 Glutamax	Invitrogen (Carlsbad, CA, USA)
Tryptan blue solution	Sigma-Aldrich (Steinheim, Germany)

2.2.4 **Reagents for RNA/ cDNA preparation**

RNA isolation

Ethanol	Sigma-Aldrich (Steinheim, Germany)
RNase free DNase Kit	Qiagen (Hilden, Germany)
RNeasy Mini Kit	Qiagen (Hilden, Germany)

β-Mercapto-Ethanol	Sigma-Aldrich (Steinheim, Germany)

cDNA transcription

Aqua ad iniectabilia	Braun (Melsungen, Germany)
Desoxyribonucleotides	Invitrogen (Carlsbad, CA, USA)
QIAquick gel extraction kit	Qiagen (Hilden, Germany)
Random Hexamers	Qiagen (Hilden, Germany)
RNase H	Roche (Penzberg, Germany)
SuperScript II Reverse Transcriptase	Invitrogen (Carlsbad, CA, USA)

PCR

iTaq SYBR Green Supermix with ROX	Biorad (München, Germany)
Desoxyribonucleotides	Invitrogen (Carlsbad, CA, USA)
MaximaHotStart Taq DNA Polymerase	Fermentas (St. Leon-Rot, Germany)

Agarose gel electrophoresis

Agarose electrophoresis grade	Invitrogen (Carlsbad, CA, USA)
Gel Red	Biotium (Hayward, CA, USA)
1 kb DNA ladder	Invitrogen (Carlsbad, CA, USA)
Loading Buffer	see protocol
TRIS-Borat-EDTA	Sigma-Aldrich (Steinheim, Germany)

Material

Biosphere tips with filter	Sarstedt (Nürnberg, Germany)
Nalgene Cryogenic Vials 2mL	Sigma-Aldrich (Steinheim, Germany)
DNase zap	Sigma-Aldrich (Steinheim, Germany)
Eppendorf vials	Eppendorf (Hamburg, Germany)
Falcon blue caps	BD (Heidelberg, Germany)
Micro-Amp optical 96-well plate	BD Applied Biosystems (Darmstadt, Ger)
Micro-Amp optical adhesive film	BD Applied Biosystems (Darmstadt, Ger)

IL-37 isoform PCR amplicons were sent to Eurofins MWG Operon, Ebersberg, Germany, for sequencing.

2.2.5 **Reagents and material for Western Blot analysis**

RIPA Buffer	see protocol
Bradford protein assay	BioRad (München, Germany)
SDS polyacrylamide gel (Any kD Resolving Gel)	BioRad (München, Germany)
PVDF membrane (Immun-Blot PVDF Membrane)	BioRad (München, Germany)
Fluka skim milk powder	Sigma-Aldrich (Steinheim, Germany)
Horse-radish conjugated secondary anti-mouse ab	Sigma-Aldrich (Steinheim, Germany)
Amersham ECL Prime WB Detection Reagent	GE Healthcare (München, Germany)

2.2.6 **Buffers and solutions**

TBE-Buffer (TRIS-Borate-EDTA-Buffer)

Tris Base	108 g
Boric Acid	55 g
0.5M EDTA	20 mL (or 9.3 g)
ddH_20	to 1 L

1M TRIS-Buffer

Tris(hydroxymethyl)aminomethane FW	121.4 g/mol, 60.57 g
ddH_20	to 0.5 L
pH = 7.5	

PBS Buffer

Natrium-dihydrogenphosphat-Dihydrat	14.8 g
Kalium- dihydrogenphosphat	2.3 g
Natrium-Chlorid	90.0 g
dd H_20	to 1 L

Acid Alcohol

HCl 37%	1 mL
Ethanol 70%	49 mL

RIPA Buffer

150 mM NaCl

1% Triton

50 mM Tris-Buffer pH=8

0.5 mM PMFS

Loading buffer

30% Glycerol

0.25% Bromophenol Blue

0.25% Xylene cyanol FF

dNTPS 10mM

10 µL each dATP, dGTP, dCTP, dTTP 100 mM

filled up to 100 µL with ddH_20

2.2.7 **Primary antibodies**

IL-37

- IL-1F7/FIL1 zeta antibody, goat IgG, Cat.No. AF1975, LOT-No. KZC01 (R&D- Systems, Abingdom, United Kingdom)
- IL1F7 monoclonal antibody, mouse IgG1, clone 6A6, LOT-No. 11193-6A6 (Abnova, Heidelberg, Germany)
- Anti-IL-37, rabbit polyclonal antibody AG-25A-0111 (AdipoGen, Liestal, Switzerland)
- polyclonal rabbit anti-IL-1F7b[32]
- affinity purified rabbit anti-IL-1F7b (S. Kim, South Korea)
- polyclonal mouse antibody (Ph. Bufler)[30]

IL-17 human affinity purified polyclonal goat IgG, Cat. No. AF-317-NA, LOT-No. ABI03 (R&D- Systems, Abingdom, United Kingdom)

IL-18 human IL-18/IL-1F4 propeptide antibody, polyclonal goat IgG, Cat.No. AF646 (R&D- Systems, Abingdom, United Kingdom)

β-actin sc-7778 monoclonal mouse IgG1 (Santa Cruz Biotechnology, Heidelberg, Germany)

2.2.8 **Recombinant IL-37 Proteins**

- Recombinant human IL-1F7Accession, #NP_775259, Size 18,6 kDa (R&D-Systems)
- IL-1H4 IF-MHB + FXa (Ph. Bufler)[30]

2.2.9 **Primer Sequences**

Name		Sequence	T_m	Amplicon length
IL-37a	fw	5' TGATAGGAGGGAAACAGAAACCA 3'	59°C	100 bp
	rv	5' CATGAATGCTGAATTTCTTCGG 3'	59°C	
IL-37b	fw	5' AGCCTCCCCACCATGAATTT 3'	58°C	90 bp
	rv	5' CTTTGTGATCCTGGTCATGAATG 3'	61°C	
IL-37c	fw	5' CTTAGAAGACCCGGCTGGAA 3'	59°C	100 bp
	rv	5' TCAAGGATGAGGCTAATGCAAAG 3'	59°C	
IL-37d	fw	5' AAGATGAACCCCAGTGCTGC 3'	59°C	50 bp
	rv	5' TCGGGTTTAAGTTCTTCACCTTTG 3'	59°C	
IL-37e	fw	5' AAGATGAACCCCAGTGCTGC 3'	59°C	50 bp
	rv	5' CCTTTAGAGACCCCCAGGAGA 3'	59°C	
IL-8	fw	5' GGACCACACTGCGCCAAC 3'	61°C	90 bp
	rv	5' CCCTCTGCACCCAGTTTTCC 3'	63°C	
IL-10	fw	5' CTTCCCTGTGAAAACAAGAGCA 3'	60°C	90 bp
	rv	5' CACTCATGGCTTTGTAGATGCCT 3'	63°C	
IL-17	fw	5' CCTCAGATTACTACAACCGATCCA 3'	64°C	90 bp
	rv	5' TTTGCCTCCCAGATCACAGAG 3'	61°C	
IL-18	fw	5' TGTAGAGATAATGCACCCCGG 3'	61°C	90 bp
	rv	5' ACTTCACAGAGATAGTTACAGCCATACC 3'	67°C	
TBP	fw	5' GCCCGAAACGCCGAATAT 3'	56°C	75 bp
	rv	5' CCGTGGTTCGTGGCTC TCT 3'	62°C	

The above primers are listed from 5' to 3' end. Primers were designed using PrimerExpress 4.0 and synthesized by Metabion (Martinsried, Germany).

2.3 Methods

2.3.1 **Obtainment, preparation and storage of human material**

2.3.1.1 Obtainment of human tissue

Biopsies of the bowel were obtained through diagnostic ileocolonoscopy. Forceps biopsies were taken from terminal Ileum, Cecum, Colon ascendens, C. transversum, C. descendens, Sigma and Rectum. Formalin-treated tissue samples were embedded in paraffin and stored in the archive of the

institute. Additional biopsies from sites of inflammation were shock frozen in liquid nitrogen at -196°C until preparation.

Healthy liver tissue of one patient was obtained through needle biopsy.

Blood for the isolation of peripheral blood monocyte cells (PBMCs) was taken from a forearm vein of members of the laboratory staff team using a butterfly system. 8 mL blood were collected in a syringe pre-incubated with 5.000 units of Heparin and immediately used for further preparation.

2.3.1.2 Isolation of peripheral blood mononuclear cells

Under the sterile conditions 8 mL of whole blood in Heparin was diluted with 8 mL sterile PBS. Each 8 mL of the mix was carefully added on 4 mL Ficoll (GE Healthcare) in a 15 mL Falcon tube. The blood was centrifuged without brake for 30' at 400x g at room temperature. Human monocyte cells have a lower density than Ficoll solution or granulocytes or erythrocytes that settle down in the tube bottom after separation. The small leucocyte ring was carefully aspirated from the Ficoll-plasma interface layer into a new Falcon tube. The sample was filled up with sterile PBS until 15 mL, centrifuged again for 10' at 400x g and the supernatant was poured of quickly. After this step the cell pellet was taken up in 5 mL of RPMI cell medium (gibco) enriched with 2% FCS (fetal calf serum, Sigma-Aldrich). For cell counting, 10 µL sample were added 90 µL tryptan blue (Sigma-Aldrich, 1:10 dilution). 10 µL were placed into a Neubauer hemocytometer. Intact cells were counted in four quarters of the hemocytometer following the calculation:

Mean cell count/ each large corner square x 10^4 x dilution factor = no of cells/ mL suspension

Cells were resuspended in medium to 10^6/ 0.5 mL. 0,5 mL of stimulant was added. To evoke IL-37 expression, each fraction of one million cells was either not stimulated or challenged with LPS (lipopolysaccharides) (Sigma-Aldrich) 100 ng/µL, Pam3Cys (a synthetic lipopeptide) (EMC microcollections) 100 ng/µL or MDP (muramyl dipeptide) (Bachem) 10 ng/µL. Cells were incubated in a 12-well plate for 4 hours at 37°C in an incubator. After stimulation, the suspension was collected in a 2 mL tube and cells that remained in the 12-well plate were carefully rinsed with PBS. Washing was done twice with ice cold PBS and centrifuging for 5' at 3.000 rpm and 4°C. 350 µL of buffer RLT (Qiagen) and 3.5 µL β-mercaptoethanol (Sigma-Aldrich) were added on the cell pellet for lysis and stored at -20°C until further preparation.

2.3.2 **Immunohistochemical staining**

2.3.2.1 Principle of immunohistochemistry

Immunohistochemistry (IHC) is based on the binding of antibodies (Ab) to a specific antigen (Ag) in tissue sections. Through a secondary Ab and a linked colour reaction the target protein is made visible. First, Ags that have changed their tertiary structure due to fixation have to be retrieved for Abs to bind the specific epitopes. This can be achieved through enzymes or, as in the case of this study, through heat-induced Ag retrieval. Here, for each commercial Ab that was tested prior to serial staining various temperatures and buffers have been used at different pH levels to find the optimal working condition. Since Ag-Ab-reactions are not visible under light microscopy, labels have to be attached to the Ab to allow visualization of the immune reaction. Most commonly, enzymes are used, in this case peroxidase. In presence of substrate and chromogen, a coloured precipitate can be seen. While in the direct method the primary Ab is conjugated to the reporter molecule, the indirect method has a higher sensitivity. Before adding the Abs, endogenous peroxidase has to be blocked with hydrogen peroxide and other epitopes with blocking serum to avoid high background signals. For horseradish-peroxidase it is common to use DAB (3,3' diaminobenzidine tetrachloride), a brown chromogen. When facing high endogenous peroxidase rates red-coloured AEC (3-Amino-9-ethylcarbazole) can be used. For better orientation in microscopy, a counterstain of nuclei is done with haematoxylin.

Most commonly, IgG is used as primary Ab for IHC. Polyclonal Abs detect multiple epitopes of the target protein but have greater likelihood for cross-reactivity with epitopes of other proteins. While polyclonal Abs have higher affinity to the Ag, they are less specific than monoclonal Abs which in contrast have lower Ag binding affinity[46].

2.3.2.2 Protocol for immunohistochemistry

Paraffin-embedded slides were cut in sections of 10µm. For deparaffinisation, slides were incubated in Xylol twice for 15' (15 minutes), following incubation in 100% ethanol, 96% ethanol and 70% ethanol for 5' each step and three times 5' in distilled water. For antigen unmasking slides were boiled for 30' in Target Unmasking Fluid (Pan Path) for IL-37 R&D ab, Pro Taqs II Antigen-Enhancer (Quartett) for IL-37 abnova ab or Target Retrieval Solution Citrate (Dako) for IL-17 and IL-18 ab (R&D) in a 750W microwave, followed by cooling down at room temperature for 20'. Slides were washed twice 5' in TRIS-buffer (pH 7.5). Endogenous peroxidase was first blocked for 10' with 7.5% H_20_2, then rinsed in tap water for 10'. Again, slides were washed twice 5' in TRIS-buffer. Blocking serum from ImmPRESS Reagent Kit (Vector) for R&D anti IL-37 Ab or from Vectastain ABC-Kit (Vector) for Abnova

ab was applied for 20', then the incubation buffer was poured off. For Abnova IL-37 Ab, endogenous biotin was blocked using Biotin Blocking System (Dako). Polyclonal goat anti human primary Ab against IL-37 (original concentration 0.2 mg/mL), IL-17 (original concentration 0,1 mg/mL) and IL-18 (original concentration 0.2 mg/mL) were diluted 1:30, IL-37 (Abnova) was diluted 1:40 in PBS; 100 µL were added to the slides and incubated for 1 hour at room temperature. Following washing 2x 5' with TRIS-buffer (pH 7.5). The secondary ab (Vectastain ABC-Kit, Vector, for Abnova ab, ImmPress, Vector, for R&D Ab) was added for 30' and rinsed 2x 5' in TRIS-buffer (pH 7.5). Slides were stained for 3' with DAB+ for IL-37 ab (R&D and Abnova) or 10' with AEC+ for IL-17 and IL-18 Ab and rinsed in tap water for 10'. Counterstaining was quickly performed for 10'' (10 seconds) with haematoxylin before mounting in Kaisers glyceringelatine (Merck).

2.3.2.3 Protocoll for H.E. staining

H.E. (haematoxylin-eosin) staining is a basic staining method for histology. It is the most common method for medical diagnosis of histologic sections. Here, two different dyes are used: blue haematoxylin binds to nucleophile cell organelles, mainly the nucleus; red eosin binds to basophilic structures, mainly the cytoplasm.

Slides were incubated for 2x 5' in Histoclear, 2x 3' in 100% ethanol, 2' in 95% ethanol, 2' in 80% ethanol and 10' in dH_2O (distilled water) in prepared racks. Staining with haematoxylin was done for 3' and rinsed in running tap water for 2-3'. To remove excess stain and define nuclei, slides were immersed for 10'' in acid alcohol (see buffers and solutions) and rinsed again in running tap water. Eosin was used for exactly 30' followed by another short step of washing with tab water. Slides were quickly incubated in reverse order from dH_2O until Histoclear and left in the last rack for some minutes. Sealing was done with mounting medium and dried overnight at 37°C.

2.3.2.4 Evaluation of immunohistochemical stainings

Stained tissue sections were evaluated under the microscope using an oil immersion 40x magnification objective. For each patient one sample from the terminal ileum and one from the colon was viewed, counting cells in one field of vision of this magnification containing the epithelium facing the lumen on its edge. IL-17 positive cells could be counted using a cell counter, while evaluation of IL-37 and IL-18 positive cells appeared to be more complex. Therefore, particular scores had to be developed:

<u>Score IL-37:</u>

Epithelium: E1 (weak expression) / E2 (strong expression)	1 / 2	points

Subepithelial Cells: <25%, >25%, >50%, >75% positive staining	0 / 1 / 2 / 3	points
Sum:	*0 – 5*	*points*

Score IL-18:

Epithelium: weak or apical / strong or basal expression	1 / 2	points
Subepithelial Cells: none / few single cells / many single cells or small groups / big areas or strong staining	0 / 1 / 2 / 3	points
Sum:	*0 – 5*	*points*

For further evaluation and documentation of H.E. staining and IL-37, IL-17 and IL-18 immunohistochemistry, slides were digitalized using an Olympus FV1000 Confocal Microscope at 100x, 200x and 400x magnification.

Pathological score:

- No inflammation (0)
- slight active inflammation (1)
- moderate active inflammation (2)
- intense active inflammation (3)

2.3.3 **Polymerase chain reaction**

2.3.3.1 Principle of polymerase chain reaction

Polymerase chain reaction (PCR) is a method used in molecular biology to amplify a piece of DNA generating a great number of copies of that particular fragment. Developed by Kary Mulis in 1983 it has since then become an essential technique. Using specific DNA oligonucleotides (primers) binding complementary to the two DNA strands, a heat-stable DNA polymerase (derived from the bacterium Thermophilus aquaticus) and dNTPS (deoxy-nucleoside-triphosphates), this techniques relies on thermal cycling of three steps: 1) a denaturation step at a temperature of around 94°C to yield two single strands, 2) an annealing step which is typically 4°C below the T_m (melting temperature) of the used primers to let them bind at their complement site of the DNA and 3) an elongation step at the ideal temperature for the polymerase around 70°C. Here, the enzyme synthesizes a new DNA strand complementary to the template strand by adding dNTPs that are complementary to the template in 5' to 3' direction. As soon as the polymerase has started working, the DNA copies are exponentially amplified until a plateau is reached due to a limited availability of dNTPS and primers. In qPCR (or quantitative real-time PCR) fluorescent dyes such as SYBR-green are used. With increasing amount of

double-stranded DNA more dye intercalates and sends a fluorescent signal that can be measured. Fluorescence is plotted against the number of cycles on a logarithmic scale. The number of cycles needed to surmount a threshold set above the background level is called the cycle threshold (C_t). C_t-values of different samples can be evaluated in a quantitative manner that will be discussed below. Official qPCR guidelines (MIQE guidelines = **m**inimum **i**nformation necessary for evaluating **q**PCR **e**xperiments) were considered[47].

2.3.3.2 Isolation of RNA

Shortly, stored biopsied were taken from liquid nitrogen and homogenized for 30'' while still in frozen state. Maintenance of the cold chain is crucial to avoid degradation of the RNA through RNases. Isolation of tissue RNA was performed using RNeasy Mini Kit (Qiagen) according to manufacturer instructions. Lysis was done in a 2 mL collection tube with 350 µL RLT-Buffer, a highly denaturing guanidine-thiocyanate-containing buffer, which inactivates RNases, plus 3.5 µL β-Mercaptoethanol as reducing agent. One volume of 70% ethanol (stored at -20°C) was added to provide appropriate binding conditions. The sample was transferred to an RNeasy Mini spin column, where the RNA binds to a membrane. Column was centrifuged for 15'' at 8000 x g (10000 rounds per minute) to wash the membrane and flow-through was discarded afterwards. 350 µL RW1 washing buffer was added and the column was centrifuged for 15'' at 8000 x g, flow-through was discarded. To eliminate genomic DNA contamination a DNase digestion with RNase free DNase Kit (Qiagen) was performed. 10 µL DNase I stock solution (stored at -20°C) were mixed with 70 µL Buffer RDD, quickly centrifuged and added on the membrane for 15' of incubation at room temperature. Another step with 350 µL of buffer RW1 succeeded. 500 µL Buffer RPE were added, the column being centrifuged at 8000 x g for 15'', and the flow-through discarded again. This step was repeated with a centrifugation time of 2' to wash the membrane. The membrane was placed on a new collection tube for RNA elution with 25 µL of RNase free water and centrifuged for 1' at 8000 x g. To obtain higher RNA gain, this step was repeated on a new collection tube with 20 µL of RNase free water. Eluated RNA was stored at -80°C.

2.3.3.3 Photometric measurement of RNA concentration

The assessment of RNA concentration was performed using a spectrophotometer (NanoDrop Thermo, Thermo Scientific) using 1 µL. RNA absorbs at a wavelength of 260 nm; measurements were made both at 260 and 280 nm. A 260nm/280nm ratio higher than 1.8 indicates pure RNA sample; only those samples were used for further analysis. The biopsies used for this study showed total RNA concentrations between 200 and 600 ng/µL.

2.3.3.4 Agarose gel electrophoresis

To ensure that RNA was not degraded, quality was assessed with agarose gel electrophoresis. Gels made from linear polysaccharide agarose have pores that can be used for the electrophoretic separation of molecules such as proteins or oligonucleotides. Negative charged molecules are transported from the cathode towards the anode and form bands according to their size. Smaller fragments wander faster than larger ones. Nucleic acid stain (GelRed, biotium) intercalating with RNA/DNA shows fluorescence under UV-light and so makes nucleotides visible. The size of the fragments can be read when compared to a RNA ladder that is run simultaneously on the gel.
Agarose gel for RNA assessment was cast using 0.5 g of agarose boiled in 50 mL TBE-Buffer (= one-percent gel) until completely dissolved, then 2 µL of GelRed were solved. After polymerisation 300 ng of RNA (approximately 1 µL mixed with 9 µL 1x loading buffer to keep the sample in the gel) were pipetted into the wells. Gel was run at 120 V for 30' and photographed under UV-light. RNA quality was considered as acceptable if the bands of the large (60S) and the small (40S) ribosomal subunit were detectable without further smear of degraded RNA in the background.

2.3.3.5 Reverse Transcription (RT) reaction

To obtain double-stranded complementary DNA (cDNA) 2 µg of DNase-digested and cleaned-up RNA were used. Considering the concentration of the eluated RNA, samples were filled up to a total volume of 14 µL, being equivalent to a concentration of 142 ng/µL. 10 µL of Random Hexamers (20 ng/µL) (Qiagen) – random oligonucleotides binding to diverse sites of the DNA strand - were added and incubated at 70°C for 10'. Further, 8 µL 5x 1st strand buffer (Invitrogen), 4µL 0.1M DTT (used as reducing agent) (Invitrogen) and 2 µL 10mM dNTPS were added and incubated at 25°C for 10' and heated at 42°C for 2'. With 1 µL of SuperScript II reverse transcriptase (Invitrogen) and incubation at 42°C for one hour and at 70°C for 10' the elongation process started. Before each incubation step samples were mixed by carefully pipetting up and down. With the addition of 1 µL RNase H at 37°C for 20' the remaining RNA was degraded. Finally, the sample was filled up to 100 µL. DNA was stored at -20°C.

2.3.3.6 Designing of primers

All primer (oligonucleotide) design was done using PrimerExpress software. Gene sequences were taken from the website genecards.org. Two successive exon sequences were imported into the designing programme. Amplicon length was limited to a range from 90 to 120 base pairs (bp); melting temperatures were accepted between 58-60°C. The penalty weight, a measure for primer specificity considering product size, primer size, primer T_m, product T_m and primer GC% (proportion of the bases guanidine and cytosine to predict the annealing temperature), was held smaller than 12.

All primers were supplied by Metabion, Martinsried, Germany and dissolved in sterile water under a hood according to data sheets provided by the company. Oligonucleotides were stored at a concentration of 100 pmol/µL at -20°C. Primer sequences, annealing temperature and other technical details for PCR setup are presented in the material section.

2.3.3.7 Quantitative real-time PCR

For quantitative real-time PCR (qPCR) 2 µL of cDNA (prepared as shown above), 1 µL forward primer (fw), 1 µL reverse primer (rv), 6 µL sterile water and 18 µL iTaq SYBR Green Supermix with ROX (Biorad) were used. ROX (6-Carboxyle-X-Rhodamine) is a passive reference dye that is used to normalize non-PCR related fluorescence signal on certain real-time thermocyclers. Final primer concentrations were 0.4 pmol/µL. Due to high viscosity of SYBR Green 10% of reagent volume was added to ensure a final volume of 20 µL in each well. Each sample was run in triplicates. Measurements of each cytokine were made on the same 96-well plate for final evaluation to ensure equal measurement conditions in all patient samples. For standardisation, TBP (TATA-box binding protein) was measured as a reference gene in each PCR run.

The following PCR-programme was used:

Temperature:	95°C	95°C	55°C	68°C	95°C	55°C	95°C
Time:	2’	15’’	15’’	20’’	15’’	15’’	15’’
		40 Cycles				Melting Curve	

Because every double-stranded DNA product has its specific melting temperature, SYBR Green signal decreases with higher levels of single-stranded DNA. This results in a melting curve that shows one peak if one specific PCR product has been amplified. Additionally, PCR products were run on a 2% agarose gel to verify one single band of the expected amplicon size.

Evaluation of qPCR was done with SDS 2.3 programme and Microsoft Excel using the ddCt-method. Ct-value of the target gene was normed to the Ct-value of TBP of the same sample according to the following equation: Expression relative to reference gene = $2^{-[\text{Ct target gene} - \text{Ct TBP}]}$. Then, individual test results were normalized to the collective of all patients of the control group, being equal to the fold change in expression of the gene of interest compared to the control group, according to the equation [48].

$$\text{fold change} = 2^{-[\text{Ct target gene} - \text{Ct TBP}]\ \text{patient} - [\text{Ct target gene} - \text{Ct TBP}]\ \text{control}}$$

2.3.3.8 Endpoint PCR

In contrast to qPCR, endpoint PCR only regards final cDNA concentration, and when applied on agarose gel the size of replicated amplicons can be shown. Amounts of reagents used for one reaction tube were 2 µL of reaction buffer, 0.4 µL of 10 mM dNTPs, each 1 µL fw and rv primers at a concentration of 10 pmol, 1.2 µL $MgCl_2$, 1 µL cDNA (equalling an amount of 20 ng template), 0.2 µL MaximaHotStart Taq polymerase (Fermentas), filled up with water to a final volume of 20 µL. Samples were run in a Mastercycler for 2' at 95°C, followed by 31 cycles of 45'' at 94°C denaturation temperature, 30'' at 56°C annealing temperature and 30'' at 72°C elongation temperature. Final elongation duration was 5' at 72°C to complete replication.

Samples were run in a 2% agarose gel for 40' at 120 V. Each time a negative control using water instead of template was run to exclude contamination of the reagents.

2.3.4 Western Blot analysis

Western blot is an analytical technique in order to detect specific proteins according to their size from a sample of tissue homogenate or extract. Proteins are separated with gel electrophoresis by the length of the polypeptide. After transfer to a membrane, these proteins can be visualized with specific antibodies.

This widely established technique contains several working steps. First, the tissue has to be prepared. Frozen tissue samples were lysated in RIPA-buffer containing phenylmethanesulfonylfluoride (PMSF) (see protocols) on ice for 30'' using a tissue homogenisator (IKA) and centrifuged at 13.000 rpm and 4°C for 5'. PMSF is a serine protease inhibitor preventing the proteins from being degraded by tissue own protease. Protein concentration was measured with Bradford protein assay (Biorad). This technique is based on the absorbance shift Coomassie Brilliant Blue dye: After binding protein the dye turns into blue colour, having an absorbance spectrum maximum at 595 nm, which can be measured with a UV spectrophotometer. The increase of absorbance at 595 nm is proportional to the protein concentration of the lysate. The protein concentration can be determined with a standard solution curve of a protein of known concentration, in this case bovine serum albumin (BSA).

In the next step for western blotting, cell lysates were separated on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Biorad) under reducing conditions. Applying voltage to the gel, proteins migrate through it at different speeds dependent on their size which can be measured in kilodalton (kDa). Then, the protein was transferred onto a polyvinylidene diflouride (PVDF) membrane (Biorad) through electroblotting. The transfer effectiveness can be checked by dying the membrane with Ponceau S. The blots were blocked in 5% non-fat dry milk in PBS + 0.1% Tween 20

for 1 hour at RT to prevent interactions with the membrane and the primary antibody and so reduce background signal of the blot. For detection of IL-37 protein a mouse monoclonal antibody was incubated overnight at 4°C in blocking buffer[30]. After washing with PBS containing 0.1% Tween 20, the membrane was incubated with a secondary horse-radish conjugated secondary anti mouse antibody (Sigma-Aldrich) diluted in blocking buffer at room temperature for one hour. The membranes were developed with enhanced chemiluminescence reagent Amersham (GE healthcare). The chemiluminescence reagent reacts with the reporter enzyme of the secondary antibody producing a light signal which is detected by a photographic film. Membranes were stripped and reprobed with β-actin antibody.

2.4 Statistical Analysis

Statistical analysis was performed with Microsoft Excel for Mac 2011 and Prism GraphPad Version 5.0d for Mac OS X. Statistical differences of patient laboratory markers were compared using the Mann-Whitney test comparing each the CD and the UC group with the control group. Statistical significance was assumed when p-value was <0.05 ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***). Correlation of qPCR data was done using linear regression and is indicated by the coefficient of determination R^2 (range [0; 1]).

2.5 Ethics

This study was approved by the Ethics Committee of the Ludwigs Maximilians University München. Written informed consent was obtained from parents and children themselves if older than 12 years.

3 Results

3.1 Patient Characteristics and Controls

Pediatric IBD patients were diagnosed according to clinical, endoscopic and pathological criteria. 18 children with CD (median age 12.6 years), 14 children with UC (median age 13.3 years) and 11 control patients (median age 9.0 years) were investigated. In all three groups approximately one third of the patients was younger than ten years.

Disease severity was classified using the wPCDAI with a mean score of 43 ± 26 (maximum number 125 points) or the PUCAI with a mean score of 34 ± 12 (maximum number 85 points). For CD, remission was assumed for a total score of less than 12.5 points (11%). Mild disease activity was diagnosed for a score between 12.5 and 40 points (28%), moderate disease activity between 40 and 57.5 points (22%) and severe disease activity for a total score higher than 57.5 (28%). For UC[43], remission of disease was assumed when total score has shown less than 10 points (0%). Mild disease activity was seen as a total score between 10 and 34 points (in 21% of UC patients), moderate disease activity between 35 and 64 points (64%) and severe disease activity when total score was 64 points or greater (none of the included patients).

The Paris classification was used for disease location[45]. Patients with CD had mainly ileocolonic or colonic inflammation and involvement of the upper GI tract was found in 50% of patients. Most patients with UC (61%) had pancolitis (Table 3.1).

Blood samples obtained before colonoscopy (1-3 days in advance) were used to classify the patients of all groups (CD, UC, control). Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and number of leucocytes are standard markers for inflammation in Crohn's Disease and Ulcerative Colitis[25]. Median values for ESR, CRP and leucocyte number were elevated in CD, but only slightly in UC patients compared to the control group. ESR and CRP levels are higher in CD than UC patients (Table 3.1 and Figure 3.1).

During their hospital stay, weight and height of the children were recorded. For both parameters, z-scores were calculated, which is the standard deviation above or below. Z-score ±1 equals the 15th or 85th percentile, ±2 approximately the 3rd and 97th percentile. In both patient groups both parameters were not significantly altered compared to healthy children.

The majority of patients (26/32 were naïve to therapy and biopsies were obtained at time of diagnosis. Six patients were on stable (>6 months) treatment with azathioprine, budenoside, 5-ASA or infliximab (Table 3.1).

Table 3.1. Patient characteristics.			CD n=18	UC n=14	control n=11
males (%)			56	36	36
median age ×			12.6	13.3	9.0
range			[6.5; 17.5]	[2.0; 15.3]	[1.3; 15.5]
disease activity index					
	wPCDAI*		43 ± 26		
		remission	2 (11%)		
		mild	5 (28%)		
		moderate	4 (22%)		
		severe	5 (28%)		
	PUCAI*			34 ± 12	
		remission		0 (0%)	
		mild		3 (21%)	
		moderate		9 (64%)	
		severe		0 (0%)	
disease localisation	**Paris Classification**				
	age at diagnosis	A1a	4 (22%)	5 (36%)	3 (33%)
		A1b	14 (78%)	9 (64%)	6 (66%)
	location	L1	3 (19%)		
		L2	7 (44%)		
		L3	6 (38%)		
		L4a	8 (50%)		
		L4b	0 (0%)		
	behaviour	B1	12 (72%)		
		B2	0 (0%)		
		B3	1 (6%)		
		B2B3	0 (0%)		
		p	6 (38%)		
	growth	G0	9 (50%)		
		G1	8 (44%)		
	extent	E1		2 (17%)	
		E2		1 (8%)	
		E3		1 (8%)	
		E4		8 (66%)	
	severity	S0		10 (71%)	
		S1		0 (0%)	
laboratory indices‡	CRP	[mg/dL]	2.71 ± 2.36	0.42 ± 0.49	0.08 ± 0.14
	ESR	[mm/60min]	43.54 ± 15.84***	26.64 ± 18.03	10.83 ± 6.55
	leucocytes	[10^9/L]	8.36 ± 2.47*	9.75 ± 4.46*	7.86 ± 2.99
	hematocrit	[%]	0.33 ± 0.04*	0.33 ± 0.05*	0.39 ± 0.06
	fibrinogen	[mg/dL]	601.7 ± 210.7***	423.3 ± 201.1	303.9 ± 90.9
	albumin	[g/dL]	3.55 ± 0.58***	4.14 ± 0.44	4.64 ± 0.67
associate medical therapy	none		14 (78%)	13 (93%)	11 (100%)
	azathioprine		1 (6%)	1 (7%)	0 (0%)
	budenoside		1 (6%)	0 (0%)	0 (0%)
	5-Aminosalicylic-acid compounds		2 (11%)	1 (7%)	0 (0%)
	infliximab		2 (11%)	0 (0%)	0 (0%)
weight z-score‡			-0.90 ± 1.54 n.s.	-0.13 ± 1.32 n.s.	-0.06 ± 0.88
height z-score‡			-0.59 ± 1.33 n.s.	-0.03 ± 1.81 n.s.	0.24 ± 0.77

‡ values are mean ± s.d.

× differences in age between the groups are n.s.

p-values: *<0.05; **<0.01; ***<0.001 (compared to control group)

n.s. = not significant

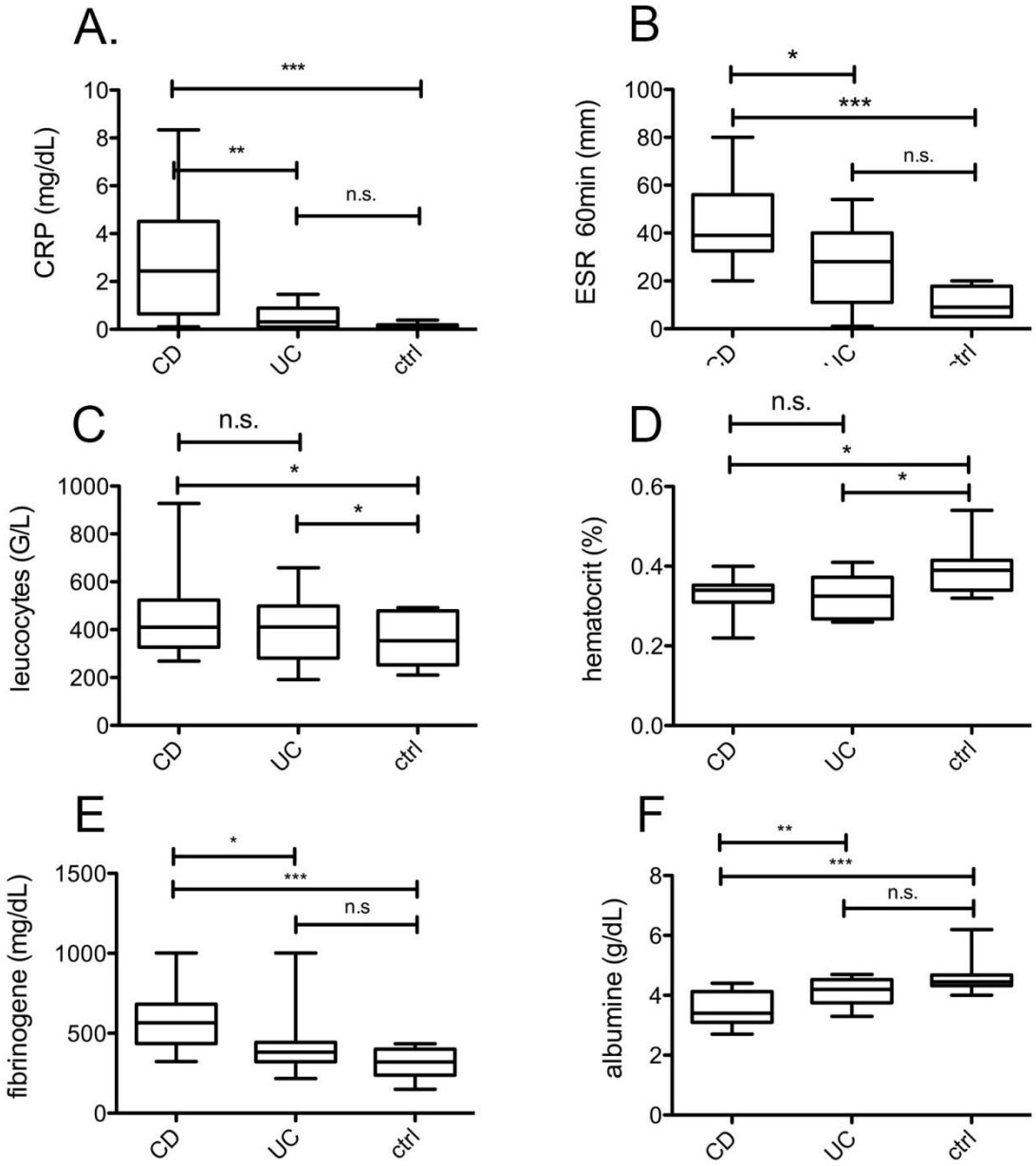

Figure 3.1. Patient laboratory parameters. Inflammatory markers such as CRP (A), erythrocyte sedimentation rate (ESR) (B) and numbers of leucocytes (C) were recorded for CD, UC and healthy children as well as hematocrit (D), fibrinogen (E) and albumin (F). The three groups were compared using the Mann-Whitney test. Statistical significance was assumed with a p-value of <0.05 ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***).

3.2 Immunohistochemical staining against IL-37, IL-18 and IL-17

So far, expression of IL-37 protein in human bowel tissue has not been investigated and we first had to establish immunohistochemical staining against IL-37. We tested five antibodies against IL-37 in various conditions such as variations in antigen retrieval solutions, temperature and chemical conditions. First staining experiments were done on the liver of mice that were injected with IL-37 plasmid-DNA through hydrodynamic tail vein injection as described by Bulau et al.[38] and human tonsils. Later, antibody use was optimized for human bowel tissue. A self-obtained affinity purified rabbit antibody has only shown weak signal and therefore was not used for further staining. A self-obtained polyclonal rabbit antibody as well as a polyclonal rabbit antibody purchased from AdipoGen stained only unspecificly against IL-37, showing a diffuse staining in all cells.

Two different commercial antibodies have shown similar staining for IL-37: A polyclonal goat antibody (R&D-Systems) and a monoclonal mouse antibody (Abnova) (Figure 3.2 A,B). Isotyp control with the same immunoglobulin subclass as the used antibodies did show any signal, also a control staining using all reagents except for the primary antibody was negative (Figure 3.2 C,D). To verify specificity of the staining, the R&D-antibody was pre-incubated overnight with 50 fold molar excess of recombinant IL-37 protein showing a significant decrease of staining intensity (as used in [30]) (Figure 3.2 E,F).

In healthy intestinal tissue, IL-37 is strongly expressed in the ileac und colonic epithelium as well as in local immune cells of the mucosa with a gradient of positive staining from luminal to basal (Figure 3.3 A). The normal gastrointestinal tract contains T-cells and plasma cells with a population of one third of the lamina propria cells[8]. While mostly the cytoplasm of IL-37 expressing cells is strongly stained, some single cells show IL-37 expression in the nucleus (Figure 3.3 B). The majority of lymph follicle cells of the colon express IL-37, although not limited to a specific area in the follicle (Figure 3.3 C).

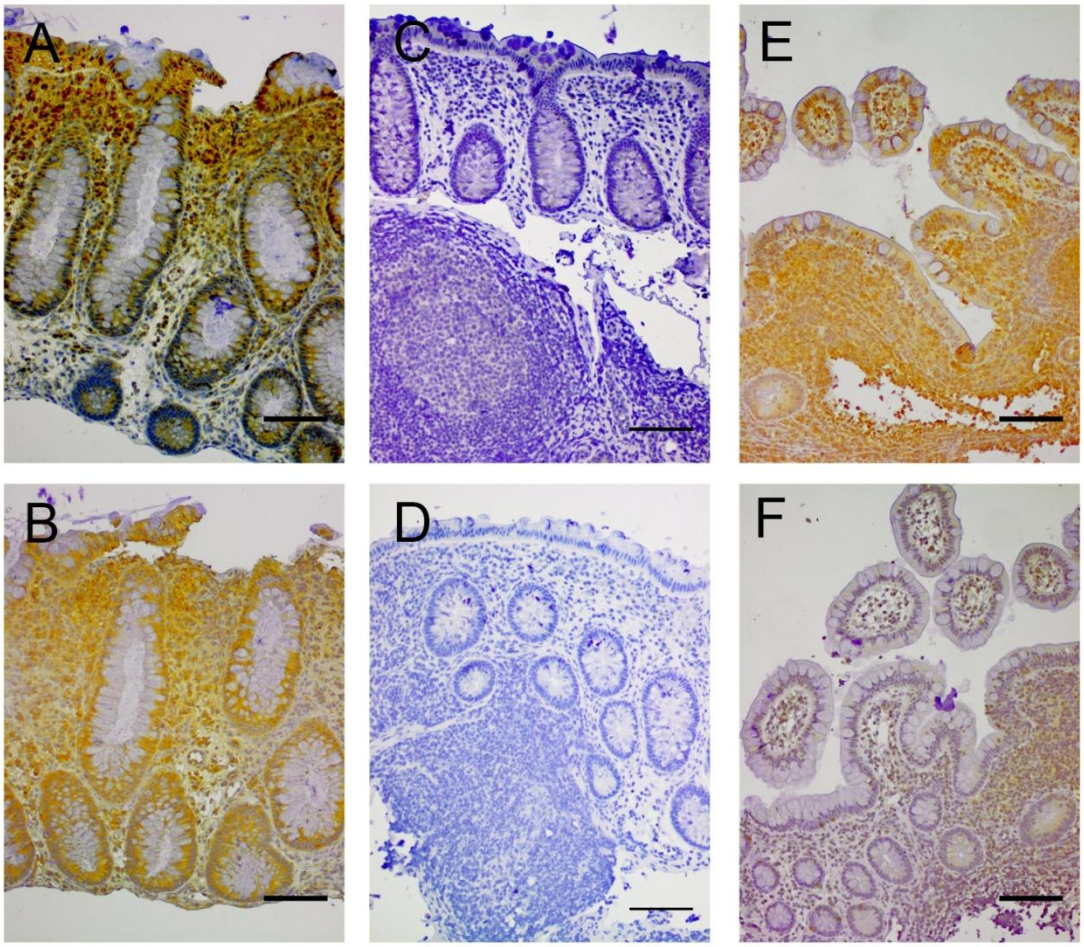

Figure 3.2. Establishment of IL-37 staining on human bowel tissue. (A) A polyclonal goat antibody (R&D-Systems) and (B) a monoclonal mouse antibody (abnova) show a similar staining pattern against IL-37 in healthy human colon. (C) Isotyp control and (D) system control were negative. To test antibody specificy, the R&D antibody was pre-incubated overnight with 50 fold molar excess of recombinant IL-37 protein. (E) Unblocked antibody, (F) pre-incubated antibody on corresponding location.

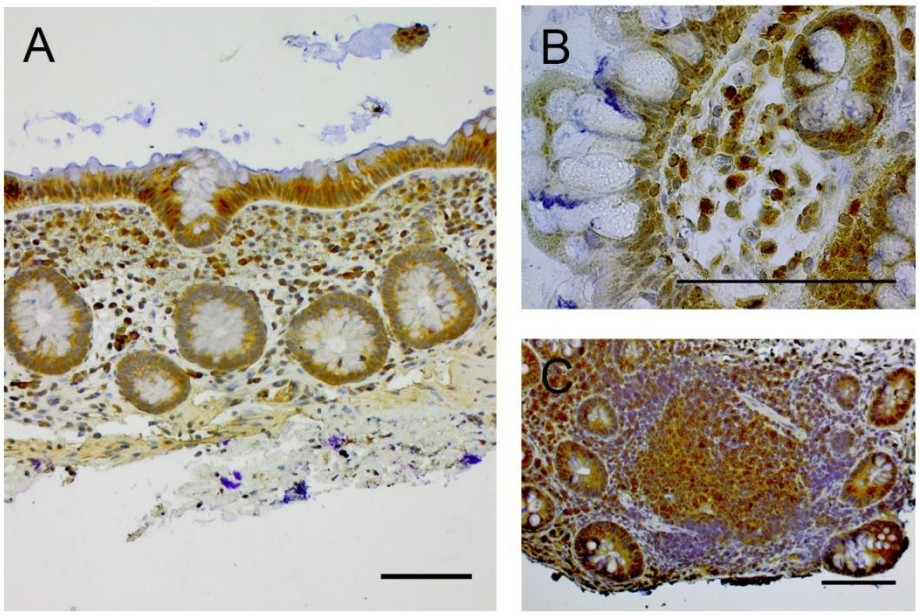

Figure 3.3. Expression of IL-37 in healthy human colon. (A) IL-37 is strongly expressed in the colonic epithelium as well as in infiltrating subepithelial cells with a gradient of positive staining from luminal to basal. (B) Single cells show IL-37 staining of the nucleus. (C) In lymph follicles of the colon a gradient can be seen showing that not all lymphocytes are expressing IL-37. The line on the bottom right indicates a distance of 100 μm. 100-fold magnification.

In patients diagnosed with CD and CU a similar staining pattern against IL-37 is seen in the epithelium, lamina propria cells and lymph follicles (Figure 3.4 A-D). For semiquantitative evaluation of IL-37 staining, a score was developed as described in the methods section. In parallel, we applied a scoring system to quantify the histological severity of inflammation.

IL-37 score plotted against the pathological score (Figure 3.4 E) showed an increase with a higher grade of inflammation: a mean IL-37 score of 3.3 was seen in samples with no inflammation; mean score was 4.4 in samples with slight active inflammation; mean was 4.3 in slides showing moderate inflammation and 4.4 in samples with intensive active inflammation. Even though the mean IL-37 expression score tended to be higher in more inflamed tissue, these findings were not statistically significant.

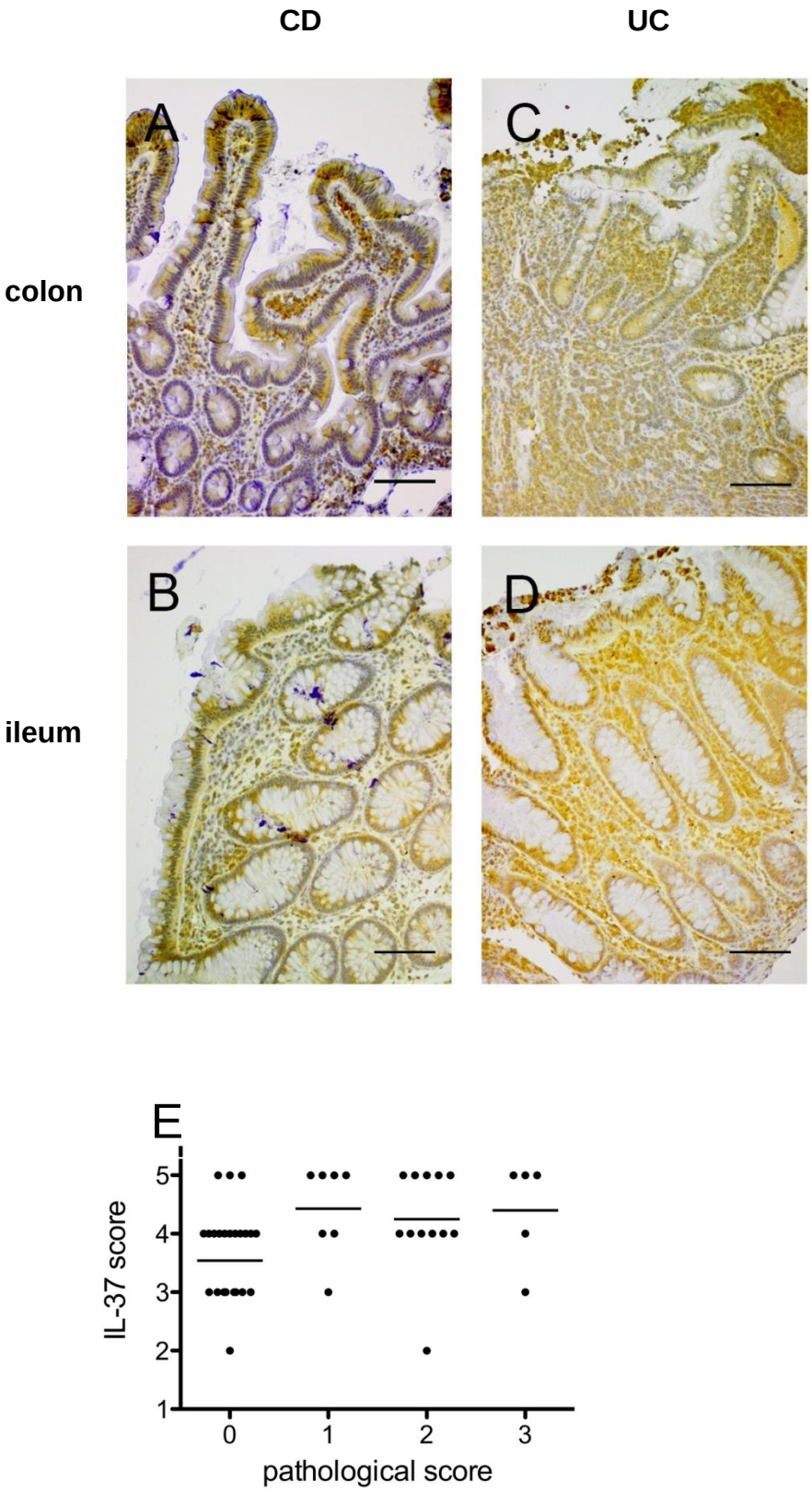

Figure 3.4. Expression of IL-37 in the ileum and colon of CD and UC patients. Expression of IL-37 in the ileum (A) and the colon (B) of a Crohn's Disease patient and in the ileum (C) and colon (D) of an Ulcerative Colitis patient. 100-fold magnification. The line on the bottom right indicates a distance of 100 μm. (E) A score for IL-37 staining was developed and plotted against a score of histologic disease severity (0 – no inflammation; 3 – severe inflammation).

3.2.1 **Immunhistochemical staining against IL-18 and IL-17**

IL-18, formerly known as IFN-γ inducing factor, has been shown to play a pivotal role in inflammatory bowel disease[15]. This cytokine is mainly produced by monocytes and macrophages in response to stimuli of viral or bacterial origin. Thus, its production is one of the effects of innate immunity initiated by host-pathogen interactions. For this reason and its similarities with IL-37, also immunohistochemical staining against IL-18 were performed on ileac and colonic tissue of CD, UC and healthy patient samples (Figure 3.5 A-C). Immunohistochemistry against IL-18 in bowel tissue has already been described with the antibody used in our study[49].

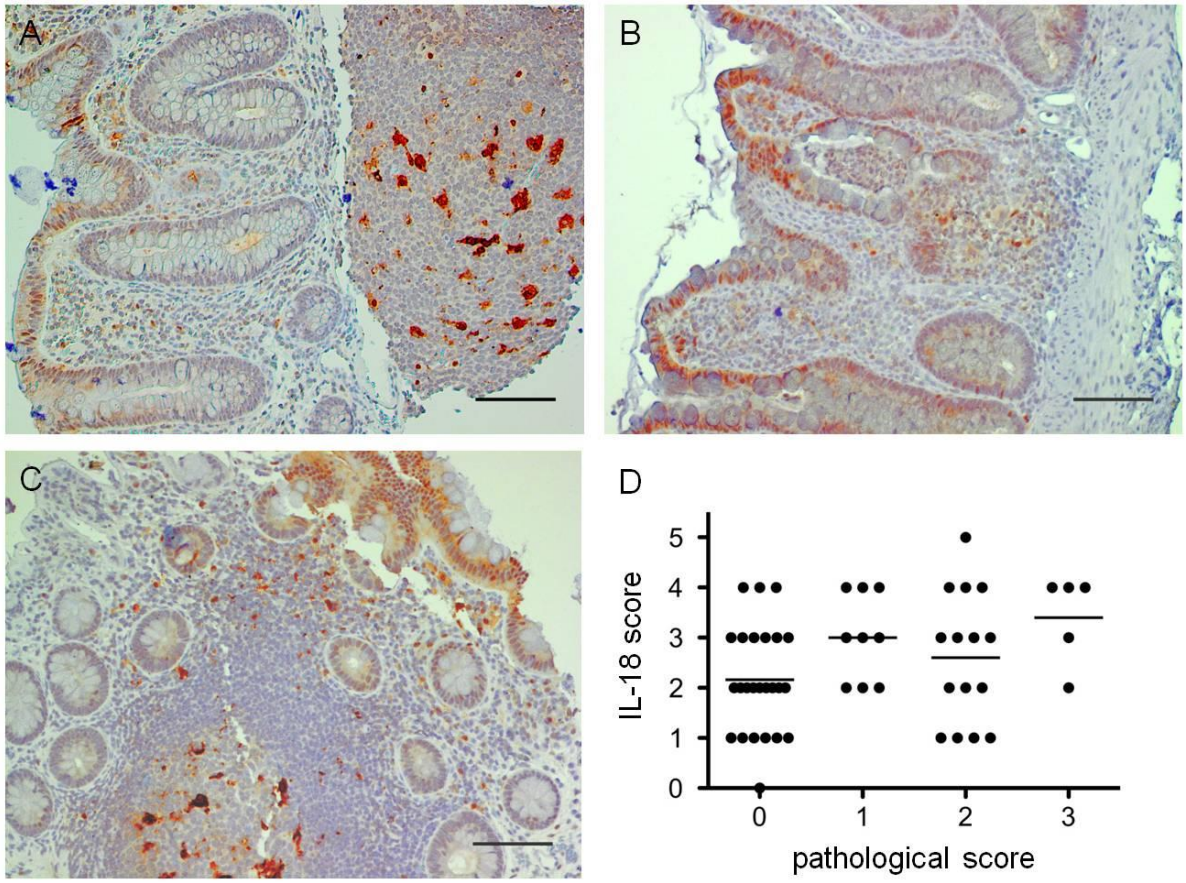

Figure 3.5. Expression of IL-18 in the colon of a CD patient (A), a UC patient (B) and a healthy patient (C). 100-fold magnification. The line on the bottom right indicates a distance of 100 μm. The IL-18 score was plotted against s score describing the histological grade of inflammation (D).

As explained above, a score similar to quantification of IL-37 staining was developed for IL-18. In patients with a pathological score of 0 (no inflammation) IL-18 score is 2.1. With increasing histological score IL-18 staining is slightly higher: 3.0 (1), 2, (2) and 3.2 (3 – severe inflammation) (Figure 3.5 D). As for IL-37, IL-18 staining was positive in the epithelium and in lamina propria cells but more limited to distinct cells. IL-18 staining was weaker in intestinal crypts than in villi. Epithelial IL-37 expression is equal in crypts and villi.

IL-17 is produced by Th17-cells which play an important role in the pathogenesis of inflammatory bowel disease[14]. Therefore, we also performed immunostaining against IL-17 in all biopsies of the ileum and colon. In each slide the total number of IL-17 positive cells was counted in one field of vision that was representative for protein expression. Single cells of the lamina propria were stained positive for IL-17. The mean value of positive cells were 46 cells for CD (p=0.002) and 52 cells for UC (p=0.002), while in the control group 18 positive cells were counted (Figure 3.6).

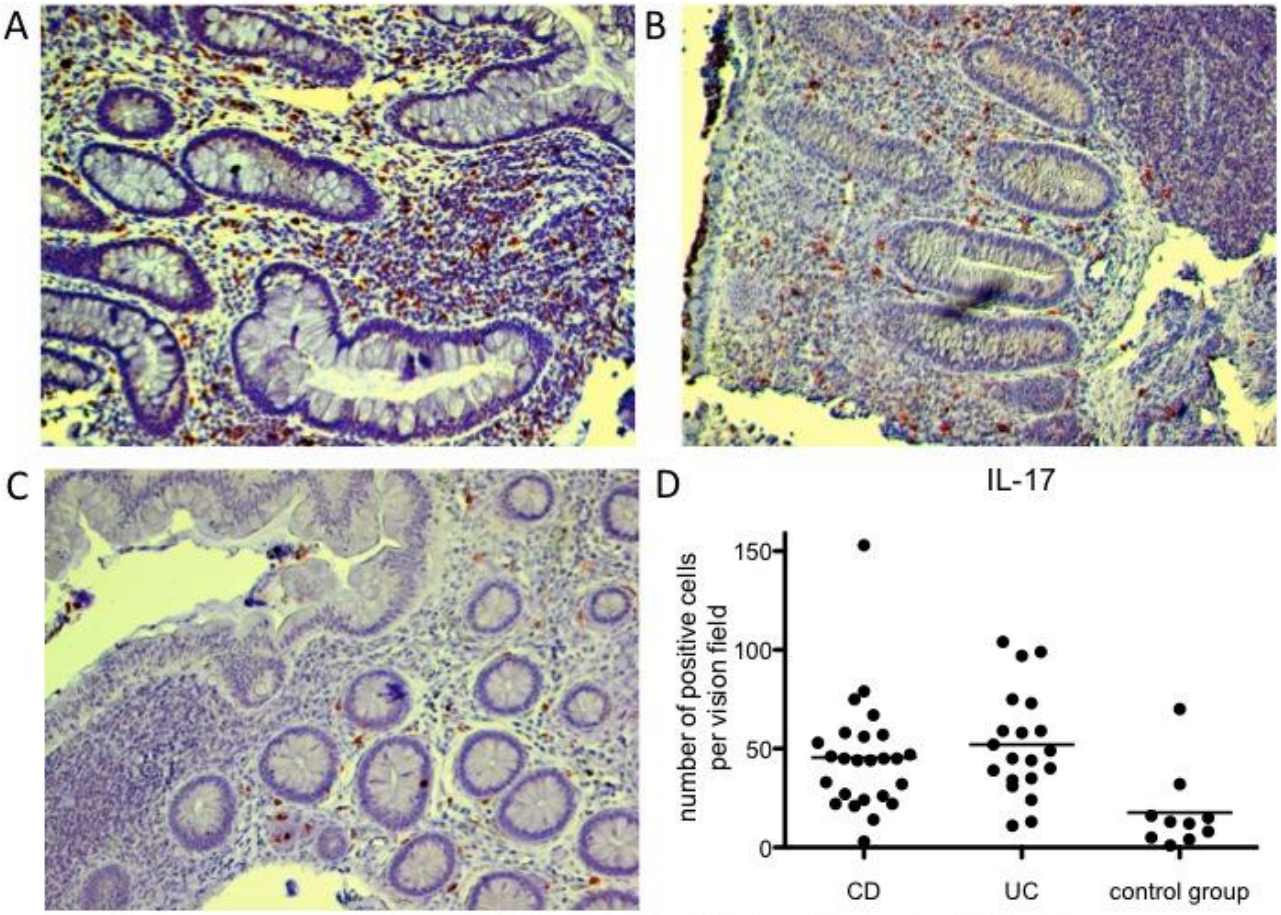

Figure 3.6. Expression of IL-17 in the colon of a CD patient (A), a UC patient (B) and a healthy patient (C). 100-fold magnification. The line on the bottom right indicates a distance of 100 μm. IL-17 positive cells in one representative vision field were counted and plotted for all three groups (D).

We next compared the expression pattern of IL-37 with IL-18. IL-18 is expressed in intestinal epithelial cells and in lamina propria cells that were previously identified as macrophages and dendritic cells[49]. IL-18 and IL-37 positive cells are detected in corresponding tissue areas and both IL-37 and IL-18 are expressed in the epithelium. While IL-18 staining was weaker in intestinal crypts than in villi, epithelial IL-37 expression is equal in crypts and villi. In our stainings, the main source for IL-18 is the epithelium, while IL-37 is more abundant in local and infiltrating immune cells. Staining against IL-17 is not limited to IL-37 or IL-18 positive areas, but rather shows distinct positive cells of the lamina propria.

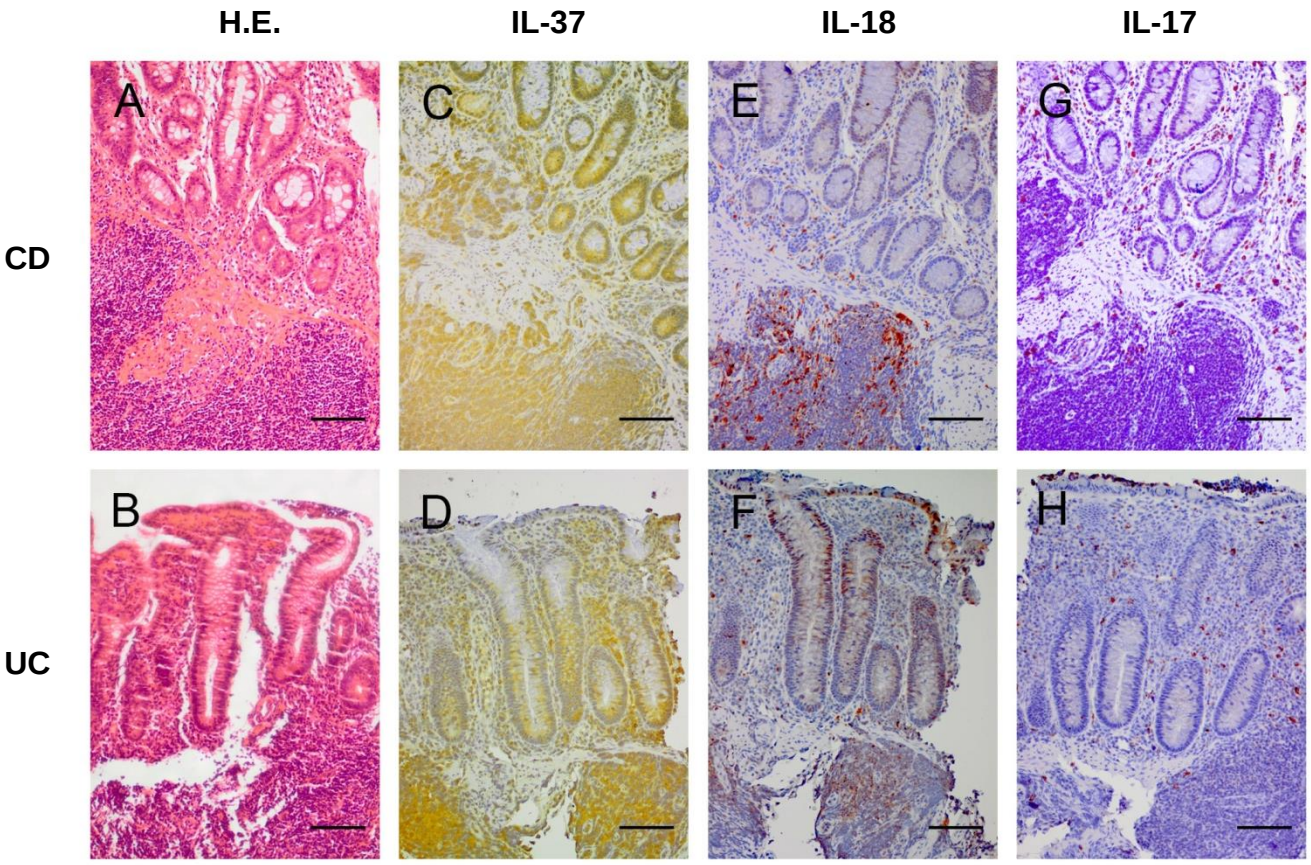

Figure 3.7. Staining pattern of IL-37 in comparison with IL-18 and IL-17 in the colon of patients with CD and UC. (A) H.E. staining and stainings against IL-37 (C), IL-18 (E), IL-17 (G) for one Crohn's Disease patient. Below, staining of a patient's sample with Ulcerative Colitis: H.E. staining (B), IL-37 (C), IL-18 (F), IL-17 (H). The line on the bottom right indicates a distance of 100 μm, 100-fold magnification.

3.3 Detection of IL-37 by Western Blotting

Cell lysate of 3 healthy patient samples, 2 UC patient samples and 8 CD patient samples as well as recombinant IL-37 protein were separated on a SDS polyacrylamide gel and visualized by western blotting. We detected a specific band at 25 kDa and a second band was seen at around 50 kDa (Figure 3.8). The intensity of IL-37 specific signals did not correlate with the pathological score of inflammation.

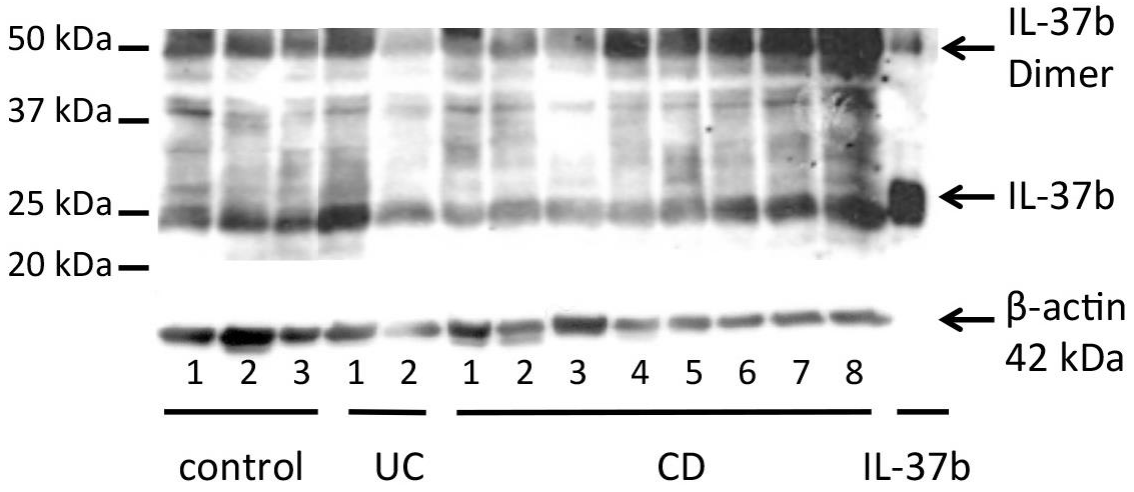

Figure 3.8. Western Blot against IL-37. Proteins of from colonic tissue samples from CD, UC and healthy patients were resolved on a SDS gel and WB was performed using a mouse anti-IL-37 ab.

3.4 Expression of interleukin (IL)-37 messenger RNA (mRNA) in comparison with other cytokines

3.4.1 **Analysis of IL-37 isoform expression**

IL-37 isoform b is the mainly expressed isoform. In order to investigate the expression of other isoforms (isoform a, isoforms c-e) in different human tissues, we performed endpoint PCR analysis by isoform specific primers in cDNA samples of human PBMC, liver and bowel tissue. The expected length of the PCR product is 100 base pairs (bp) for isoform a, 90 bp for isoform b, 100 bp for isoform c, and 50 bp each for isoforms d and e (Figure 3.9).

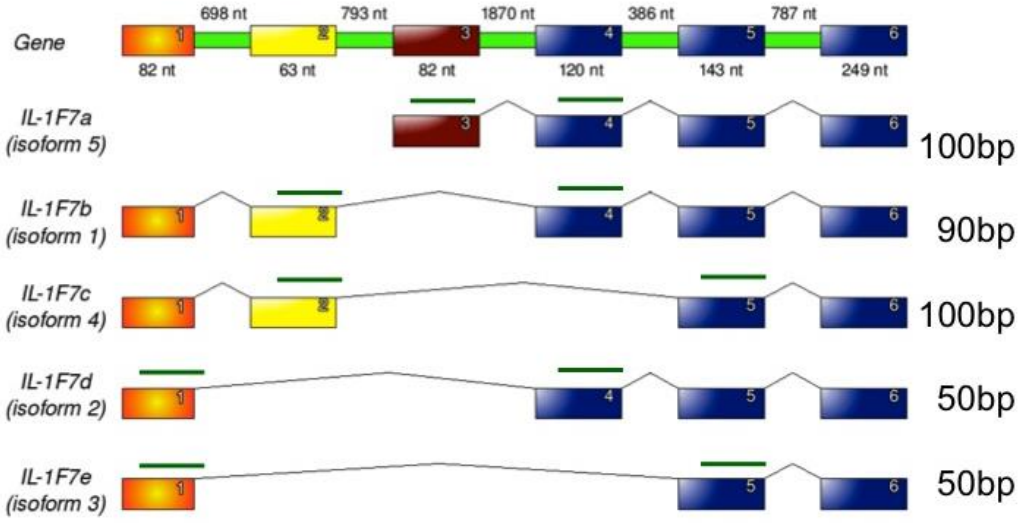

Figure 3.9. IL-37 isoforms (modified according to[35]). Above each exon the position of the used primer is shown. On the right side the expected length of the amplicons specific for each isoform is given.

	IL-37a	IL-37b	IL-37c	IL-37d	IL-37e
primer a	100bp	no amplicon	no amplicon	no amplicon	no amplicon
primer b	no amplicon	90bp	no amplicon	no amplicon	no amplicon
primer c	no amplicon	220bp	100bp	no amplicon	no amplicon
primer d	no amplicon	110bp	no amplicon	50bp	no amplicon
primer e	no amplicon	230bp	no amplicon	170bp	50bp

Table 3.2. IL-37 primers and amplicon lengths.

Both in unstimulated PBMCs and in stimulated PBMCs only isoform b could be seen. IL-37b expression is stronger in stimulated cells. This effect is the strongest upon stimulation with MDP. Also in liver tissue and healthy colon tissue as well as in tissue taken from CD and UC patients only isoform b was detected. IL-37b expression tends to be stronger in IBD patients but could not be quantified by the endpoint PCR as performed (Figure 3.10). Bands detected for isoforms a, c, d and e were not of the expected amplicon length. Instead, these bands are isoform b amplicons detected by the primers for the other isoforms (Figure 3.9, Table 3.2). In some samples two bands were detected for isoform e. After sequencing one was analysed as isoform b, whereas the second band could not be assigned to a gene of the human genome and therefore is no specific product.

3.4.2 Expression of interleukin (IL)-37 messenger RNA (mRNA) in comparison with other cytokines

To examine IL-37 mRNA expression in intestinal tissue quantitative realtime-PCR was chosen. Since IL-37b is the predominant isoform, the experiments using qPCR were limited to isoform b. Quantitative PCR raw data for each measured cytokine was normed to TATA-box binding protein and to the control group pool using the ddC_t-method as described above.

In the CD group, cytokine levels of IL-37 and IL-18 did not significantly differ from the control patients (IL-37: 2.7-fold and p=0.43; IL-18: 1.8-fold and p=0.42). Levels of IL-8, IL-17 and IL-10 showed a significant increase (IL-8: 172.0 and p=0.0002***; IL-17: 31.3-fold and p=0.002**; IL-10: 3.1-fold and p=0.05*).

Similar results could be observed in patients with ulcerative colitis. While no difference could be found in the expression of IL-37 and IL-18 (IL-37: 1.8-fold and p=0.65; IL-18: 1.3-fold and p=0.65), expression of IL-8, IL-17 and IL-10 was increased (IL-8: 182.7-fold and p=0,0.001***; IL-17: 43.5-fold and p=0.0003***; IL-10: 4.2-fold and p=0.03*) (Figure 3.11).

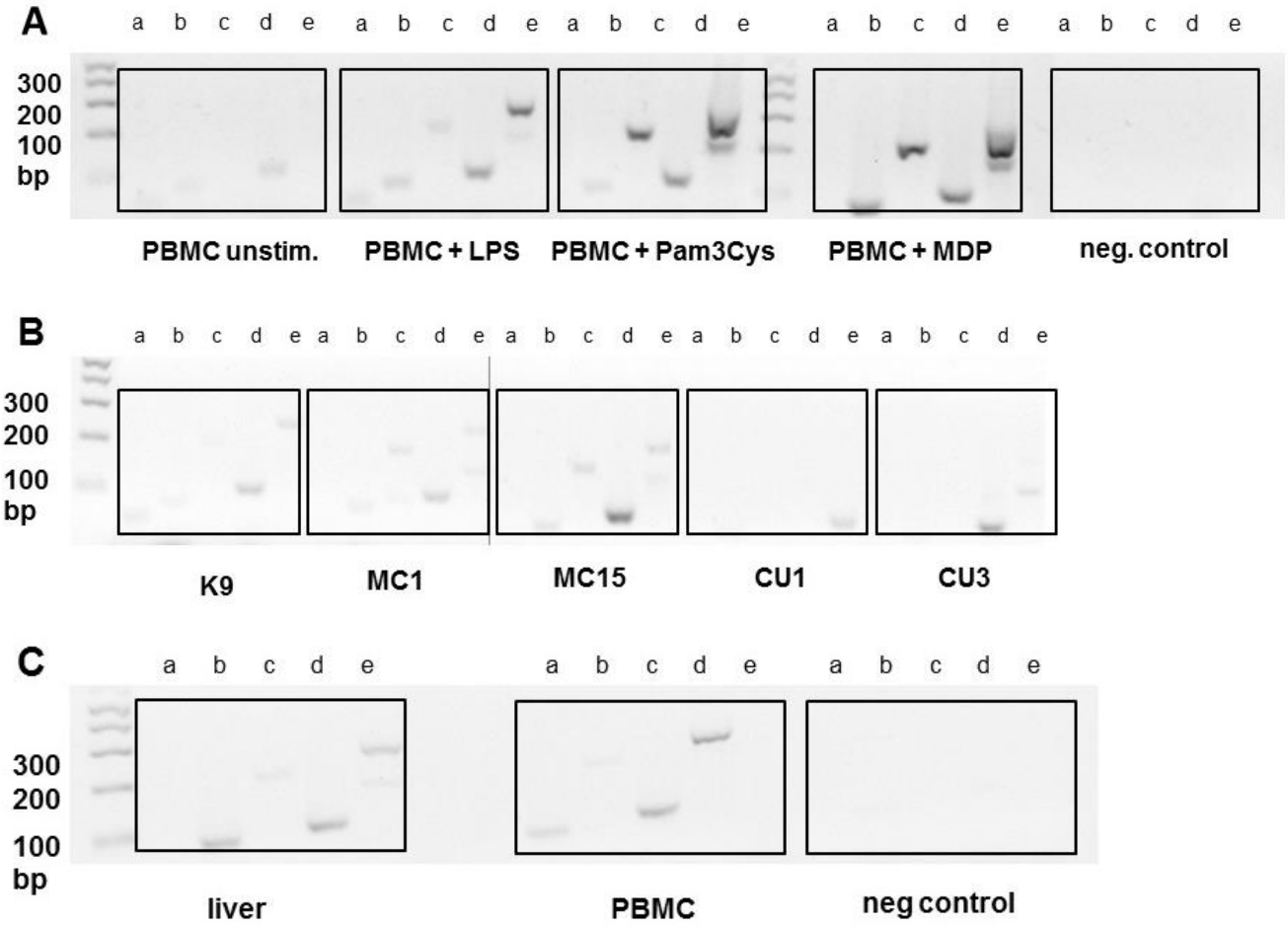

Figure 3.10. Analysis of IL-37 isoforms by endpoint RT-PCR. Gel with PCR product isolated from PBMC stimulated with different agents (A). Gel with cDNA from IBD patients. K9 is a control patient, whereas MC1 and MC15 are CD patients and CU1 and CU3 CU patients (B). Gel with cDNA from liver tissue as well as stimulated PBMCs as positive control and reagents without DNA as negative control(C). Letters a-e prepresent isoforms a-e of IL-37.

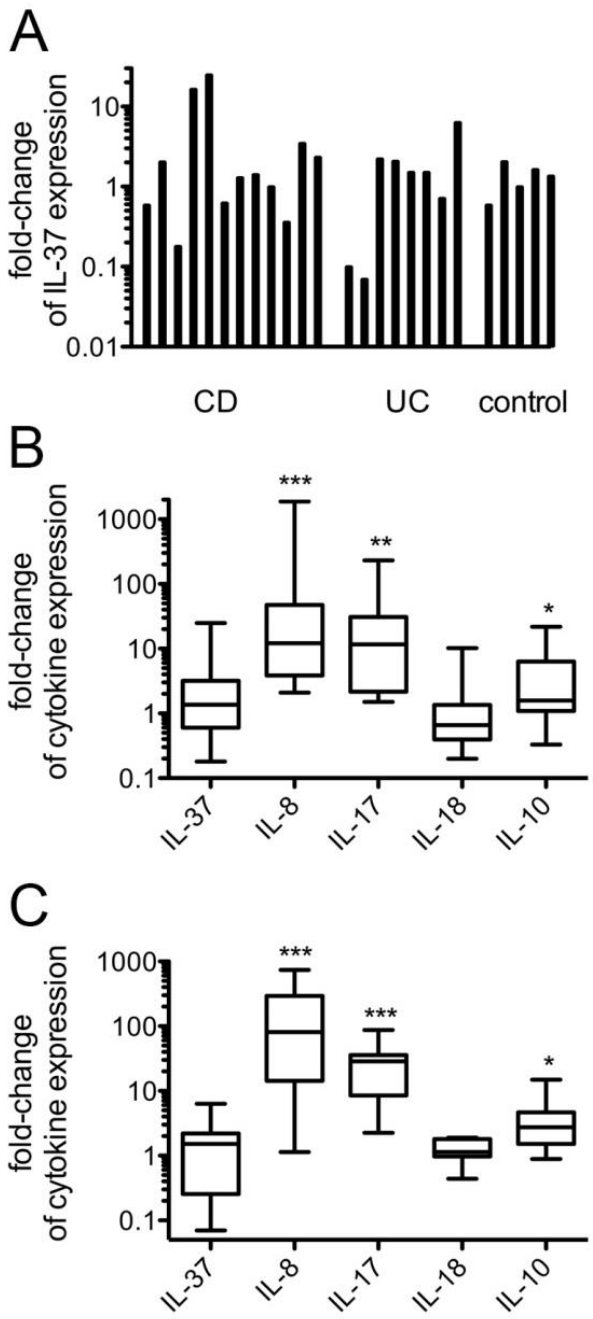

Figure 3.11. Expression of IL-37 mRNA in comparison with other cytokines using qPCR. mRNA was isolated from colonic tissue samples, cDNA was transcribed for qPCR. Logarithmic scale of fold change expression in comparison to a pool of healthy control patients is shown on the Y-axis. (A) Overview of IL-37 expression, each column represents one patient. A fold change of 1 indicates no difference in mRNA expression in comparison to the healthy patients. (B) Fold changes of IL-37, IL-8, IL-17, IL-18 and IL-10 mRNA in the CD group and in (C) the UC group. Significance levels were marked with * ($p<0.05$), **($p<0.01$), or *** ($p<0.001$) and were calculated using the Mann-Whitney-Test.

3.4.3 **Correlations of cytokine mRNA expression**

All fold changes of cytokine expression were compared with each other using linear regression and are indicated by the level of significance (p) or by the coefficient of determination R^2 (range [0; 1]). A high correlation could be seen in the fold-changes of IL-37 and IL-18 mRNA (R^2=0,856). This resulted from a strong correlation of IL-37 and IL-18 fold changes in CD patients (Figure 3.12). Expression of other measured cytokines (IL-8, IL-10 and IL-17) did not show any statistically significant correlation with IL-37 mRNA (Table 3.3).

	CD group	UC group
IL-37/ IL-8	p=0.81	p=0.20
IL-37/ IL-10	p=0.85	p=0.8
IL-37/ IL-18	p=0.0001***	p=0.49
IL-17/ IL-8	p=0.0001***	p=0.79
IL-17/ IL-10	p=0.10	p=0.003**

Table 3.3. Correlation of qPCR cytokine fold-changes.

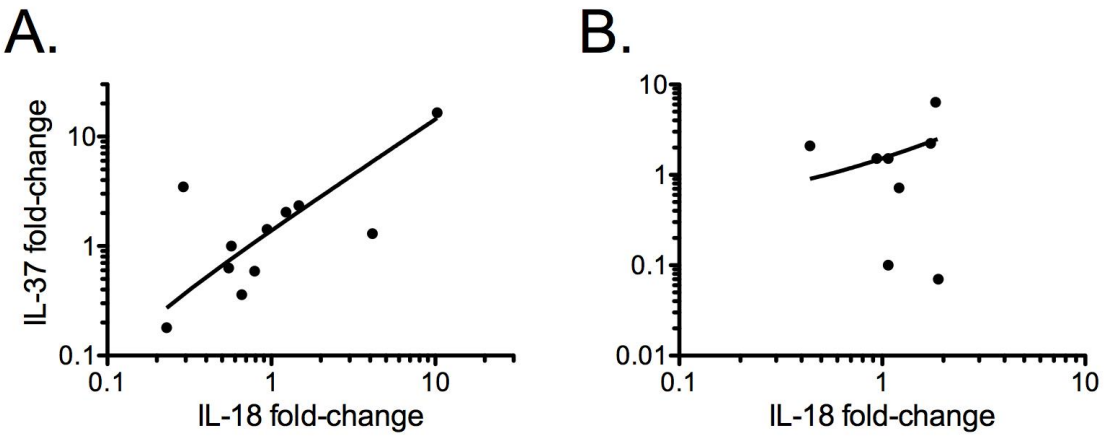

Figure 3.12. Correlation of IL-18 and IL-37 mRNA fold-changes from qPCR. CD patients (A) and UC patients (B).

3.4.4 **Cytokine expression in inflamed versus non-inflamed areas**

Crohn's disease is characterized by a discontinuous inflammation pattern. Therefore, cytokine expressions of one CD patient (CD15) were measured with qPCR in a biopsy from an inflamed section of the bowel and in another biopsy from macroscopically unaffected tissue. No difference of IL-37, IL-18 and IL-10 mRNA expression could be seen between non-inflamed and inflamed tissue, while quantitative levels of IL-8 and IL-17 are highly elevated in inflamed areas of the bowel compared to unaffected tissue (Figure 3.13).

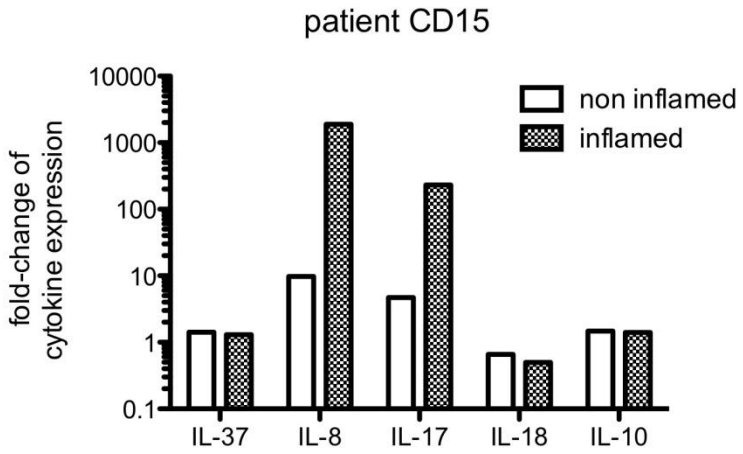

Figure 3.13. Fold-changes in cytokine expression of one representative CD patient in non-inflamed compared to inflamed tissue.

3.4.5 **IL-37 mRNA expression according to disease severity**

To validate whether IL-37 mRNA expression changes according to clinical disease severity, the patient groups were divided in subgroups. For both Crohn's disease (wPCDAI) and ulcerative colitis (PUCAI) a score of 40 was chosen as cutting point, dividing between mild and moderate/severe disease activity (Figure 3.14). While in the UC patients no difference in IL-37 mRNA expression was seen between the two subgroups, in the CD patients the mean fold change was elevated, even though not at a statistically significant level. This observation was mainly due to two CD patients with higher IL-37 mRNA expression

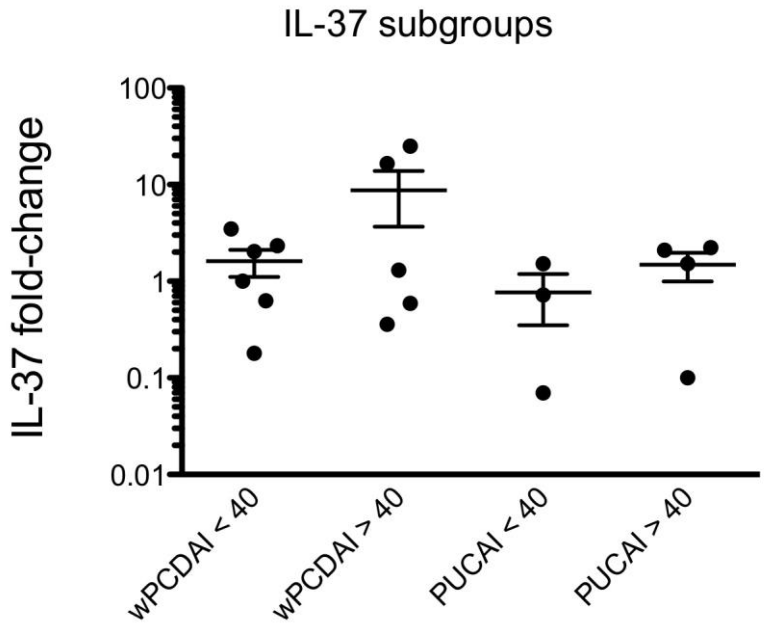

Figure 3.14. Fold-changes in IL-37 mRNA expression according to clinical disease activity. Both CD and UC patient group was divided in two subgroups with a wPCDAI or PUCAI of 40 as cutting point. Each dot represents a patient.

4 Discussion

IL-37 down-regulates innate immune responses[28,33] and suppresses intestinal inflammation in mice[41]. Here, we investigated the expression of IL-37 in the intestine of children with active CD and UC. The expression of IL-37 in epithelial and infiltrating immune cells of ileal and colonic biopsies from control patients as well as pediatric patients with CD and UC in comparison to IL-18 and IL-17 was demonstrated. Expression pattern of IL-37 and IL-18 was similar and differed from IL-17 expression which is restricted to single positive lymphoid cells of the subepithelial layer. IL-37 protein expression was increased with histological severity of inflammation.

The 18 CD and 14 UC patients of this work presented with clinical and inflammatory serum markers suggestive for active IBD[50]. Inflammation was worse in CD compared to UC patients considering transmural inflammation in CD but not UC. Disease localization and clinical assessment match well what has been described for paediatric IBD[51]. Therefore, this cohort of paediatric patients to study intestinal IL-37 expression was characteristic for active CD and UC.

IL-37 shares critical amino acid sequence with IL-18 and binds to the IL-18BP and IL-18Rα[35]. IL-18 expression was detected at increased levels in the colonic epithelium and inflammatory cells of the lamina propria in adult patients with active CD and UC[49]. IL-17 plays a pivotal role in chemokine regulation and IL-17 positive cells are detected at the site of intestinal inflammation[14]. We therefore investigated the expression of IL-37 along with IL-17 and IL-18 in paediatric patients with CD and UC.

Here, the immunohistochemical staining against IL-37 by using various antibodies was established. Two commercially available antibodies revealed the most specific and comparable staining pattern. The specificity of staining was proven by preabsorption of the antibodies by recombinant IL-37b protein which previously was expressed in *E. coli*[32].

IL-37 protein is strongly expressed by intestinal epithelial cells as well as lamina propria lymphoid cells of control patients. In inflamed ileal and colonic tissue of children with CD and UC IL-37 expression was increased with higher numbers of infiltrating IL-37 positive lymphocyte compared to control biopsies. However, epithelial and lymphoid cells express IL-37 even in the absence of inflammation. Constitutive epithelial expression was also described for IL-1α[52]. Our work group has shown that IL-37 is markedly upregulated in human PBMC after stimulation with LPS[32]. In the gut constitutive epithelial expression of both inflammatory and anti-inflammatory immune mediators such as IL-1α[53] and IL-37 might be mandatory to maintain the homeostasis of the local immune response against commensal bacteria.

Single lamina propria cells show strong nuclear expression of IL-37. Previously it has been reported that caspase-1 processing is required for nuclear translocation of IL-37[33]. Since the antibodies against IL-37 used for immunohistochemistry do not differentiate between pro- and mature IL-37b, protein extracts of intestinal biopsies were analyzed by western blotting. However, a minor band

corresponding to mature IL-37b could not be detected, indicating that the amount of mature IL-37 in the gut epithelium is low.

We next compared the expression pattern of IL-37 with IL-18. IL-18 is expressed in intestinal epithelial cells and in lamina propria cells that were previously identified as macrophages and dendritic cells[49]. IL-18 and IL-37 positive cells are detected in corresponding tissue areas but dendritic shape cells of the lamina propria were only positive for IL-18, but not IL-37. Both IL-37 and IL-18 are expressed in the epithelium, but while IL-18 staining was weaker in intestinal crypts than in villi, epithelial IL-37 expression is equal in crypts and villi. In the presented stainings, the main source for IL-18 is the epithelium, while IL-37 is more abundant in local and infiltrating immune cells. Bone marrow transfer from human IL-37tg mice protected wild-type mice from DSS-induced acute colitis[41]. This indicates that IL-37 expression from infiltrating immune cells is more relevant than IL-37 released from epithelial cells to control intestinal inflammation in acute experimental colitis.

In contrast to IL-37 and IL-18 expression, IL-17 staining is limited to single cells located in subepithelial layers or infiltrating epithelial lymphocytes as previously shown[14]. IL-17 positive cells are not linked to areas of strong IL-37 staining.

Different isoforms of IL-37 have been described in various human tissues[35]. Therefore, in this work we tested by endpoint PCR which isoform of IL-37 is most abundantly expressed in human bowel, liver and PBMC. By using isotype specific primers only IL-37b could be detected in human bowel and liver samples as well as PBMC. Hence, in the subsequent real-time PCR experiments only isoform b was measured.

In contrast to IL-8 and IL-17 mRNA[14,54], steady state levels of IL-37b mRNA were not significantly up-regulated in colonic biopsies of children with CD and CU compared to healthy intestinal tissue. This can be explained by the fact that IL-37b mRNA expression is tightly controlled and rapidly degraded under continuing stimulation[30]. Bufler et al. showed that IL-37 mRNA contains coding region instability elements which induce rapid degradation of IL-37 mRNA unless cells undergo stimulation[30]. Therefore, levels of IL-37b mRNA do not parallel IL-37 protein expression in inflamed bowel tissue.

As expected, anti-inflammatory IL-10 mRNA was increased with bowel inflammation. IL-10 acts as a key mediator in the maintenance of gut immune homeostasis[55]. IL-37 does not directly induce IL-10 and vice versa upon LPS-stimulation, IL-10 production in IL-37 expressing RAW macrophages was not altered[33]. In contrast, IL-37tg mice expressed higher levels of IL-10 in DSS-induced colitis. However, the protective role of IL-37 transgene expression was explained by a decreased expression of TNF-α and IL-1β in inflamed colonic tissue since blocking of IL-10R did not alter the severity of colitis[41].

Even if both IL-37 and IL-18 mRNA levels were not up-regulated in colonic tissue of our cohort of children with CD and UC, a positive correlation of quantitative mRNA levels could be observed. This indicates similar regulatory mechanisms to control expression of IL-37 and IL-18 *in vivo*. Indeed, it

was previously demonstrated that IL-18 mRNA, like IL-37, contains coding region instability elements to downregulate steady state levels of specific mRNA[30]. Similar mechanisms to regulate expression of pro- and anti-inflammatory immune mediators such as IL-18 and IL-37 may protect the organism from uncontrolled, potentially deleterious immune responses, either hyperinflammatory or immunosuppressive.

Single nucleotide polymorphisms (SNPs) resulting in a decrease of NLPR3 expression contribute to Crohn's disease susceptibility[56]. This was an unexpected finding since the NLRP3 inflammasome activates caspase-1 and caspase-1 is needed to activate proinflammatory IL-1β and IL-18. In accordance with an increased Crohn's disease susceptibility in humans, *NLPR3*-deficient mice are hyperresponsive to DSS-induced colitis[57]. Since caspase-1 is also needed to process anti-inflammatory IL-37, it might be speculated that the increased susceptibility to Crohn's disease in patients with SNPs within the *NLPR3* gene is in part to be attributed to the impaired activation of anti-inflammatory IL-37.

During the work for this thesis a research group from Japan published the intestinal expression of IL-37 in the inflamed bowel of adult patients with IBD[58]. This group established immunohistochemistry against IL-37 by using the same polyclonal goat anti IL-37 Ab as used for this work and found a similar expression pattern of IL-37. In contrast to the results presented in our study, adult patients express markedly lower basal levels of IL-37 than healthy controls. This indicates that the developing intestinal immune system of children needs higher levels of immunomodulatory cytokines to maintain mucosal homeostasis. However, in this study tissue samples were not compared to biopsies from control patients without colonic disease but to unaffected areas of the same patient undergoing surgery due to therapy-refractory conditions.

A second research group investigated levels of IL-37 in sera and colonic mucosa of adult patients with CD and UC by ELISA, immunohistochemistry and western blotting[59]. The authors used a different IL-37 antibody for immunohistochemistry in their study than we applied (Abcam). The patients were treated with immunomodulating drugs and steroids. IL-37 distribution pattern in the colonic mucosa was similar to our study in children, even though the expression in the control group was weaker than described here. In some contrast IL-37 mRNA expression in qPCR and IL-37 protein quantity was reported to be significantly higher in IBD patients than in controls. This effect could be explained by the treatment with steroids in all patients of their study and 5-ASA derivates as well as the fully developed immune system of adult IBD patients.

A third group from Sweden has shown that IL-37 mRNA was increased in adult patients with UC in remission in comparison to active UC and healthy controls[60]. All patients with active UC and half of the patients with UC in remission were treated with 5-ASA and had been diagnosed with an average course of disease of 14 years. This increase in IL-37 mRNA expression in UC in remission could indicate that a lack of IL-37 participates in the failure to resolve chronic inflammation and that IL-37

plays a role in restitution of colonic integrity. Therefore, IL-37 may be a future therapeutic target to downregulate chronic bowel inflammation by its profound anti-inflammatory properties.

5 Summary

The function of interleukin-37 is not resolved yet. It has been shown that IL-37 suppresses colonic inflammation in mice. To gain more insight into its relevance in human disease, we investigated the expression of IL-37 in the intestine of paediatric patients with chronic inflammatory bowel disease.

Intestinal biopsies were obtained from children with inflammatory bowel disease (18 Crohn's disease (CD), 14 ulcerative colitis (UC) and 11 controls) during endoscopy and analysed for IL-37 expression by immunohistochemistry and real-time PCR. Results were correlated with immunostaining for IL-18 and IL17, mRNA levels of pro- and anti-inflammatory cytokines and clinical parameters.

IL-37 protein was detected in epithelial cells and submucosal lymphoid cells of CD and UC patients as well as healthy controls. IL-37 protein expression tended to be higher with submucosal lymphoid cell infiltration of patients with CD and UC and correlated with histological severity score of inflammation. IL-18 showed a similar staining pattern to IL-37, whereas staining for IL-17 revealed distinct positive cells scattered in the submucosal layer. Messenger RNA-expression of IL-8, IL-17 and IL-10 was up-regulated in CD and UC patients. mRNA-levels of IL-18 and IL-37 were not significantly elevated compared to controls. Levels of IL-37 and IL-18 mRNA showed a positive correlation in the CD group. Furthermore, out of five isoforms, only isoform IL-37b could be detected in IBD tissue.

Future studies are warranted to delineate the specific contribution of IL-37 to modulate chronic bowel inflammation in humans.

6 Zusammenfassung

Die Funktion von Interleukin 37 ist noch nicht völlig geklärt. Es konnte gezeigt werden, dass IL-37 Entzündung im Darm von Mäusen unterdrücken kann. Um mehr Einblicke in dessen Relevanz für Erkrankungen beim Menschen zu erlangen, wurde hier die Expression von IL-37 im Darm von pädiatrischen Patienten mit chronisch entzündlicher Darmerkrankung untersucht.

Es wurden Gewebeproben von Kindern mit chronisch entzündlicher Darmentzündung (18 Patienten mit M.Crohn, 14 Patienten mit Colitis ulcerosa und 11 Kontrollen) während einer Darmspiegelung entnommen und auf die Expression von IL-37 mittels Immunohistochemie und real-time PCR untersucht. Die Ergebnisse wurden mit Färbungen für IL-18 und IL-17, mRNA-Level pro- und anti-entzündlicher Zytokine und klinischen Parametern korreliert.

IL-37 Protein konnte in den Epithelzellen des Darms sowie den submukösen lymphathischen Zellen der Patienten mit M.Crohn und Colitis ulcerosa sowie der Kontrollpatienten nachgewiesen werden. Die Expression von IL-37-Protein war tendenziell höher bei submuköser Infiltration lymphatischer Zellen von Patienten mit M.Crohn und Colitis ulcerosa und korrelierte mit dem histologischen Schweregrad der Entzündung. IL-18 wies ein ähnliches Färbemuster wie IL-37 auf, wohingegen IL-17 nur in einzelnen Zellen in der submukösen Schicht nachzuweisen war. Die messenger-RNA-Expression von IL-8, IL-17 und IL-10 war bei Patienten mit M.Crohn und Colitis ulcerosa hochreguliert. mRNA-Spiegel von IL-18 und IL-37 waren im Vergleich zu den Kontrollen nicht signifikant erhöht. Die mRNA-Expression von IL-18 und IL-37 war im Vergleich zu den Kontrollen nicht signifikant erhöht. Von den fünf bekannten IL-37-Isoformen konnte nur Isoform b im Darm- und Lebergewebe sowie PBMC nachgewiesen werden.

Weitere Studien sind nötig, um die exakte Rolle von IL-37 bei der Modulation der Immunantwort im Rahmen chronisch entzündlicher Darmerkrankungen zu beschreiben.

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8 Acknowledgements

I would like to thank Prof. Dr. med. Philip Bufler for giving me the opportunity to work on this subject and being part of his excellent team. Not only did I learn from him as a researcher but also as an excellent clinician. In particular, I am grateful for continuous support, discussion and new ideas to realize this work.

For assistance, continuous supervision and instruction for scientific I thank Dr. rer. nat. Ana-Maria Bulau. For continuously challenging me in this work, I am grateful to Dr. med. Tobias Schwerd.

Andrea Sendelhofert, Anja Heier and Prof. Dr. Doris Mayr have never ceased to support this work with their excellent knowledge in immunohistochemistry.

I thank Prof. Dr. med. Jürgen Heesemann for allowing me to be part of the “FöFoLe - support for research and teaching” in systems biology.

A massive thank-you to my work group.

One massive thank-you goes to my beloved family – my parents Klaus and Heidrun Weidlich, who have dedicated their lives to get the best out of me right from my first breath. What would I do without my three sisters and friends Johanna, Mirjana and Salome. Life without you would only be half the fun.

9 Index of Abbreviations

5-ASA	5-aminosalicylic acid
Ab	antigen
Ag	antibody
bp	base pairs
CD	Crohn's Disease
cDNA	complementary deoxy-ribonucleic acid
CRP	C-reactive protein
DAMPs	damage associated molecular patterns
DC	dendritic cells
(d)dH_20	(double)distilled water
dNTP	deoxy-nucleotide tri-phosphate
DSS	dextran sulfate sodium
ESR	erythrocyte sedimentation rate
fw	forward
H_20_2	hydrogen peroxide
HE	haematoxylin-eosin
Ig	immunoglobulin
IBD	inflammatory bowel disease
IHC	immunohistochemistry
IL-	interleukin
kDa	kilodalton
LPS	lipopolysaccharide
MDP	muramyl dipeptide
n.s.	not significant
PAMPs	pathogen-associated molecular patterns
Pam3Cys	(S)-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys(4)-OH, trihydrochloride
PBMCs	peripheral blood mononuclear cells
PMSF	phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rpm	rounds per minute
TBP	TATA-box binding protein
TLR	toll-like receptor

rv	reverse
tg	transgene
UC	Ulcerative Colitis
WT	wild type

10 Declaration

Weidlich, Simon

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

'Expression of Interleukin-37 in paedriatic chronic inflammatory bowel disease'

selbstständig verfasst habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung des akademischen Grades eingereicht wurde.

München, den 13.07.2017 Simon Weidlich