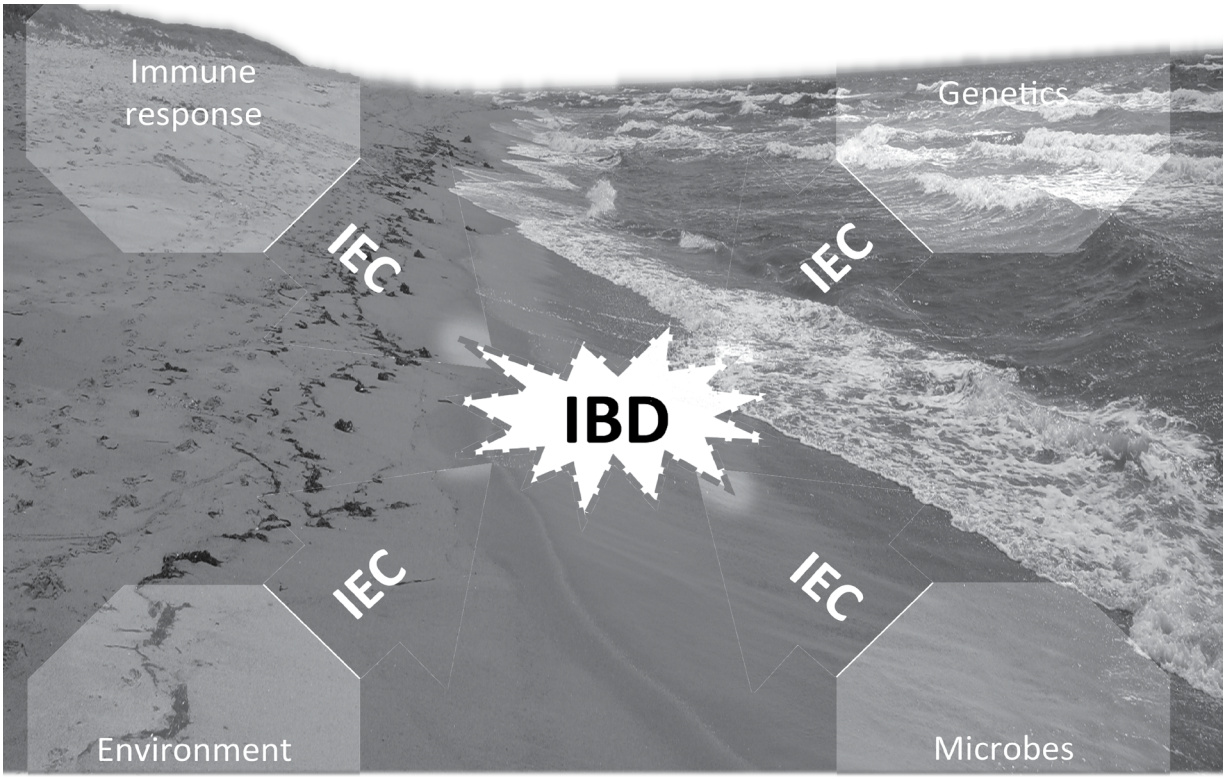
The Role Of Intestinal Epithelium In Inflammatory Bowel Disease And Inflammation Related Intestinal Cancer



J. Jasper Deuring

Stellingen behorende bij het proefschrift:

Intestinal Epithelium in Inflammatory Bowel Disease and Inflammation Related Intestinal Cancer.

1. In het ontstaan en bij de behandeling van patiënten met inflammatoir darmlijden speelt het darmepitheel een belangrijke rol. *(dit proefschrift)*

2. Het verhogen van de PXR expressie in darmepitheelcellen verlaagt de NF-κB activiteit. *(dit proefschrift)*

3. Darmkankercellen hebben een hoge PXR expressie, welke zorgt voor betere overleving bij hoge celdichtheid. *(dit proefschrift)*

4. Tijdens een actieve darmontsteking leidt het agressieve milieu tot verhoogde misvouwing van eiwitten in het endoplasmatisch reticulum. (*dit proefschrift)*

5. Een polymorfisme in het ATG16L1 gen leidt tot verhoogde endoplasmatisch reticulum activiteit specifiek in Paneth cellen. *(dit proefschrift)*

6. Discrepanties in studies naar ROS productie in patiënten met de ziekte van Crohn zijn te wijten aan een polymorfisme in het NCF4 gen. *(dit proefschrift)*

7. Als we wisten wat we deden, heette het geen onderzoek.
(Albert Einstein 1879-1955)

8. It is not clear that intelligence has any long-term survival value.
(Stephen Hawking)

9. A goal is a dream with a deadline. *(Napoleon Hill 1883-1970)*

10. Nothing great is ever accomplished by following standards. *(Geoffroy Birtz)*

11. Het luisteren van instrumentale trance muziek tijdens je werk houd je wakker, scherp en bevordert je prestaties. *(Linda van Putten, Intermediair, 18-09-2012)*

J. Jasper Deuring
Rotterdam, 30 januari 2013

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Colophon

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The Role of Intestinal Epithelium in Inflammatory Bowel Disease and Inflammation Related Intestinal Cancer

De rol van darmepitheel bij chronische darm ontsteking en ontsteking gerelateerde kanker

Proefschrift

ter verkrijging van de graad van doctor aan de
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Content

I

General Introduction, Aim and Thesis Outline

I

Introduction

The intestine is equipped with villi and crypts to harbour the entire 300 square meters of epithelium in just 8 meters of GI tract. Gastro- and intestinal epithelial cells (IEC) form a single cell layer covering the gastrointestinal tract (GI tract). These cells have the important and difficult task to protect the host from harmful luminal content while enabling the uptake of nutrients and water. One way the IEC deal with this dual task is to have IEC witch are specialized for certain task; *e.g.* Goblet cells that produce mucus, Paneth cells producing defensins, enterocytes that absorb water and nutrients, and the enteroendocrine cells that produce hormones. At a molecular level intracellular receptors, membrane bound transporters, and degradation enzymes play an additional but very important role in host-protection mechanisms[1].

Inflammatory bowel disease

Inflammatory bowel disease (IBD) has two major distinct phenotypes: Crohn's disease (CD) and ulcerative colitis (UC). CD is characterized by trans-mural and patchy inflammation that can occur the entire GI tract but mostly involves the terminal ileum and colon, while UC is characterized by superficial inflammation involving the large bowel only. Colorectal cancer (CRC) is a serious and life-threatening complication of longstanding IBD with a poor prognosis compared to sporadic CRC. This is demonstrated by the fact that, although IBD-associated-CRC represents on 1-2% of all CRC, it account for 15% of all CRC-related deaths[2, 3].

IBD is a multifactorial disease in which the onset of disease and also the progression to IBD related CRC is determined by the interplay of multiple factors such as, genetics, the microbiome, immunity, and unfavorable environmental triggers[4]. All these factors partly but often directly affect the IEC function. As such, the IEC play a crucial role in the gut homeostasis and the deregulation as seen in IBD (Chapter II Figure 1B).

Immunity and IEC

In this thesis we focus in particular on the NF-κB signaling pathway, which is one of the key regulators in innate immune responses. Reduced mucus production reduces the IEC-barrier function, allowing pathogens and commensal microbe to directly contact the IEC. Subsequently, bacterial components are able to reach and membrane receptors such as the Toll Like Receptors (TLR's), which activate the NF-κB signaling pathway. Two decades ago NF-κB was discovered as an enhancer-binding protein[5], now known as the classical (canonical) NF-κB signaling pathway. Once activated, NF-κB is able to translocate to the nucleus, where it binds DNA and subsequently activates gene expression[6]. Recently a second NF-κB signaling pathway or so called alternative or non-canonical NF-κB signaling pathway was described[7]. The classical NF-κB pathway is activated by different receptors such as TLR's, TNF-R, Interleukin receptors and even UV light[8]. The triggered receptors are activating the IκB kinase (IKK) complex. The most common form of this complex consists of IKKα and IKKβ catalytic subunits where IKKγ is the regulatory (NEMO) subunit of the IKK complex. Active IKK phosphorylates the NF-κB inhibitory complex IκB. Phosphorylated IκB is detached form the NF-κB complex, ubiquitinated, and proteosomal degraded. Tumour necrosis ligand superfamily members like lymphotoxins, B-cell activating factors (BAFF) and RANKL activate the alternative pathway. Ligand-bound receptors activate a specific IKKα homodimer complex that partly degrades p100 till the P52 subunit is formed. P52 then binds RelB and translocate to the nucleus to induce gene transcription[7] (reviewed in[9]). Both signalling pathways induce transcription of genes involved in IBD i.e. IL-6, IL-8 IL-1β, IL-10, TNFα, ICAM and ELC. The effect of several drugs used for IBD medications may exert their effects via the inhibitory effect on the NF-κB pathway[10].

Xenobiotic pathways in IEC

IEC utilize advanced molecular mechanisms to maintain the host integrity to face toxic compound (xenobiotics) challenges. Appropriate cellular-responses to xenobiotic stress are coordinated through a specific subfamily of the steroid-thyroid superfamily of nuclear hormone receptors (NR). Upon ligand specific binding to the receptor, the NR homo- or heterodimerizes with another NR-family member. Thereafter a pro-transcription factor forms, that by virtue loss of hsp90 binding exposes the nuclear localization signal to the cytoplasmic machinery leading to nuclear translocation[11]. In the nucleus, the complex binds to specific palindromic promoter regions and directs transcription of genes involved in executing the appropriate response to the xenobiotic challenge. A large number of nuclear hormone receptors appears to be involved in orchestrating the transcriptional response to IEC xenobiotic challenges, including the Pregnane X Receptor (PXR/ SXR/ NR2L1), Farnesoid X Receptor (FXR/ NR1H4), the Vitamin D Receptor (VDR/ NR1L1), the Constitutive Androstane Receptor (CAR) and the Retinoid X Receptor (RXR/ NR2B2), and there is clear evidence that PXR is a master xenobiotic sensor[12].

NR activation induces transcription of xenobiotic degradation enzymes such as the Cytochrome p450 (CYP) superfamily and membrane xenobiotic transporters such as the ABC transporters. Degradation enzymes such as CYP3a4 and Sult1a are neutralizing the xenobiotics by oxidation[13]. Whereupon transporters such as, ABCG2 (BCRP, breast cancer resistant protein), MDR1 (multi drug resistant protein) and BSEP (bile salt excretion pump), export the xenobiotics out of the cell and back into the gut lumen[14]. Proper cellular responses to toxic insults go beyond the simple induction of detoxification enzymes[15] and also include the inhibition of pathways associated with further damage (*e.g.* the generation of reactive oxygen species) and the facilitation of regenerative responses to repair tissue damage. A reflection of these processes can be found in the thought that activation of PXR down-regulates the transcriptional activity of NF-κB and thereby reduces intestinal inflammation[16-19]. By contrast, these detoxification pathways are down regulated in patients with active IBD[20-23]. Giving rise to the thought

Back to Index

that this is a mutual interaction between the xenobiotic and NF-κB signalling pathways[16]. To go even further, there is also contradictory literature about the function of PXR in intestinal cancer cell proliferation and apoptosis[24-26]. In conclusion, the function of the xenobiotic pathways goes far beyond their detoxification properties and includes controlling inflammatory reactions, and cell growth and apoptosis.

Genetics and endoplasmic reticulum stress in IEC

In general, membrane-bound and secreted proteins undergo a folding quality check before they are released from the endoplasmic reticulum (ER). Un- or misfolded proteins are extracted from the ER and degraded. This process is called ER-associated degradation (ERAD). Engulfing a part of the ER (autophagy) is an element of the ERAD. However, when unfolded proteins accumulate inside the ER the unfolded protein response (UPR) is activated. Upon UPR three major ER stress pathways are activated, IRE1, PERK and ATF6 (Figure 1). All three proximal UPR effectors start transcription of genes that are involved in reducing the unfolded protein load in the ER, e.g. ERAD. The mechanism of these three pathways were described previously[27]. Intestinal inflammation is associated with difficulties in ER-dependent protein folding (ER stress)[22, 28-31]. However, the question whether uncontrolled ER stress leads to active IBD or whether active IBD leads to ER stress still remains unanswered. A possible explanation for the ER stress in IBD can be found in genome wide associated studies (GWAS)[32]. Identifying single nucleotide polymorphisms (SNPs) associated with an increased risk for developing IBD largely uncovered the genetic basis of IBD. Outcome of this research revealed in more than 99 possibly involved genetic variations in the human genome related to IBD[33-35]. A distinct group of IBD associated SNP is found in genes involved in the ER stress (XBP1[28]) and ERAD (NOD2[36, 37], ATG16L1[38], IRGM[39],) pathways. Functional studies are majorly lacking, but it can be assumed that IBD can originate in an ER associated genetic susceptible host. Other inflammation related factors that can impede the ER dependent protein folding are discussed in this thesis.

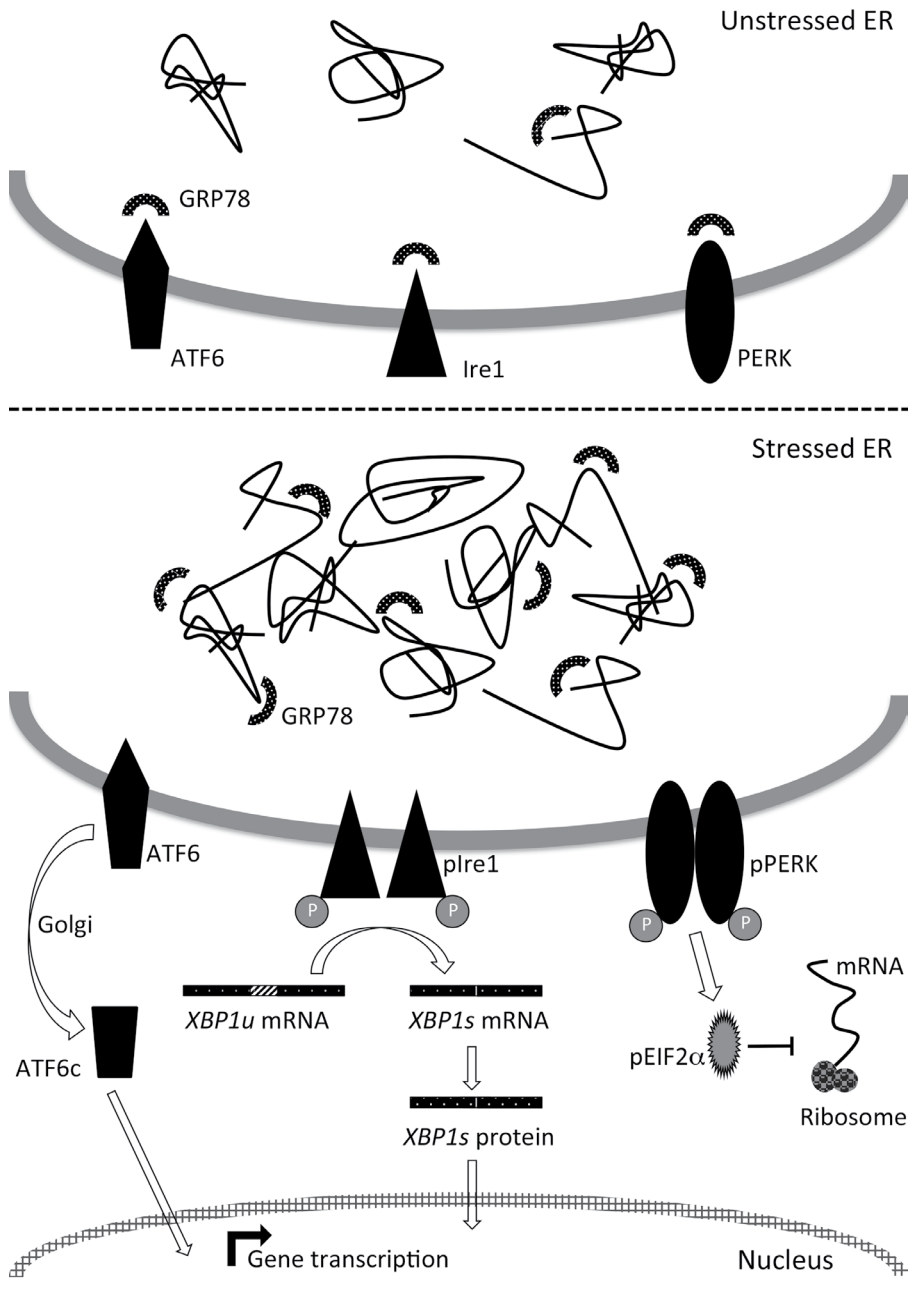

Figure 1: Schematic overview of ER stress pathway. Upon accumulation of unfolded proteins inside the ER the chaperone GRP78 is detached from the signal molecules, e.g. ATF6, Ire1, and PERK, to assist the protein folding. Detachment activates these signal molecules, which initiate ER stress associated pathways to overcome the excessive protein load.

Back to Index

Aim and Thesis outline

The mucosal inflammation in patients with IBD originates from an overactive immune response against the (commensal) luminal bacteria with the IEC in a crucial position between these interactions. Moreover, the question whether dysfunctions in IEC can lead to IBD or whether IBD provokes IEC dysfunction is still unanswered. In this thesis we aim to discuss the function of IEC on the IBD aetiology by investigating multiple molecular pathways, protein folding difficulties, neoplastic progression and genetic associations in IBD.

The importance of IEC in the IBD disease aetiology is pointed out in **Chapter II**. In this chapter we will summarise recent literature on the cellular implication of IEC in the multifactorial IBD pathogenesis. We want to emphasize the importance of IEC on disease-associated mediators and the possibility to improve IEC functioning as therapy for IBD patients.

In **Chapter III** we hypothesised that the activation of PXR inhibits NF-κB activity, to implicate a possible novel therapeutic approach. We tested this hypothesis by activating PXR in freshly isolated human biopsies, and further exploring the mechanism of action using PXR specific knock down cell lines.

In **Chapter IV** we emphasized on the role of PXR in intestinal cancer cells as inhibitor of cell growth and cell survival by measuring the intestinal PXR expression in (non-)IBD related CRC, and investigating the role of PXR expression on the growth of intestinal cancer cells.

In **Chapter V** we will discuss the possible effect of locally produced ROS on ER-depending protein folding, in IBD patients with active inflammation. This concept will be further investigated in **Chapter VI** measuring the ABCG2 expression in biopsies from IBD patients and their functionality using *in vitro* cell line experiments.

Several IBD related SNPs are found in the ER stress or autophagy pathways, however experimental evidence for a possible causal relation is still lacking. In **Chapter VII** we explored the functional effect of these SNPs on high-secretory IEC, such as Paneth cells.

Innate immune activation is accompanied with the production of ROS. As a result of the dramatic effect of ER depending proteins upon excessive ROS production (**Chapter V and VI**) we investigated the consequence of a mutation in the NCF4 gene on the ROS production in our final chapter (**Chapter VIII**).

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Back to Index

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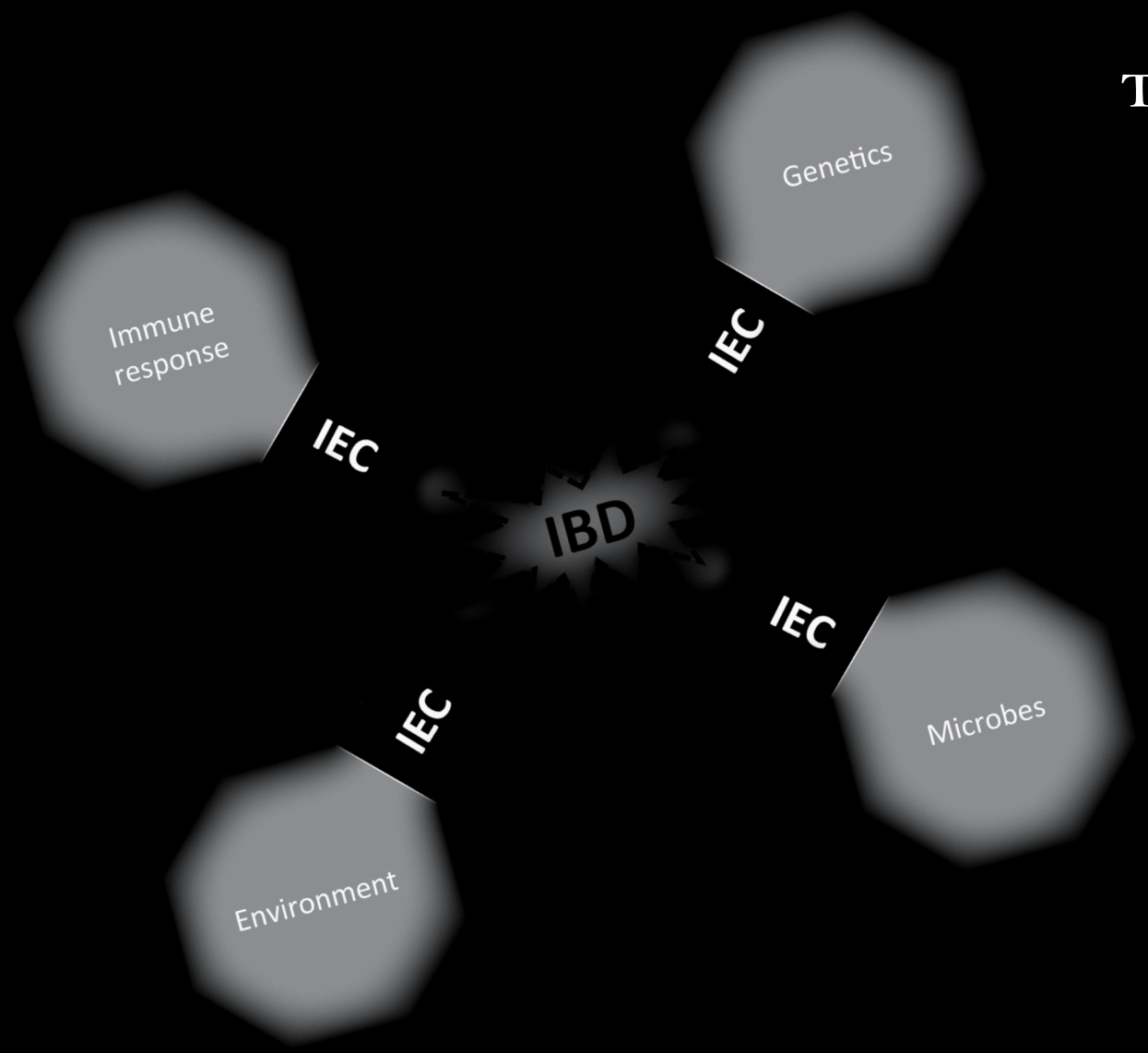
Back to Index

II

The Cell Biology of the Intestinal Epithelium and its Relation to Inflammatory Bowel Disease.



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II

Abstract

The epithelial layer of our intestines must meet two opposing requirements. On one hand it must allow for efficient uptake of nutrients and fluids, on the other hand it is a vital defence barrier between the *milieu interior* and the *milieu exterior*. Different from the lung that by virtue of cilia movement is kept virtually sterile, the gut epithelium is confronted by a stupendous microbiological load and a substantial xenobiotic challenge. The efficiency by which our intestinal epithelium manages to deal with the challenge of efficient nutrient absorption while simultaneously fulfilling its barrier function is testimony to what the forces of evolution can accomplish. Importantly, our understanding as to how our gut epithelial compartment manages this balancing act is now rapidly emerging, answering one of the oldest questions in cell biology. Importantly, when aberrations in this balance occur, for instance as a consequence genetic polymorphisms, increased propensity to develop chronic inflammation and inflammatory bowel disease is the result. Thus the knowledge on intestinal cell biology and biochemistry is not only of academic interest but may also aid design of novel avenues for the rational treatment of mucosal disease.

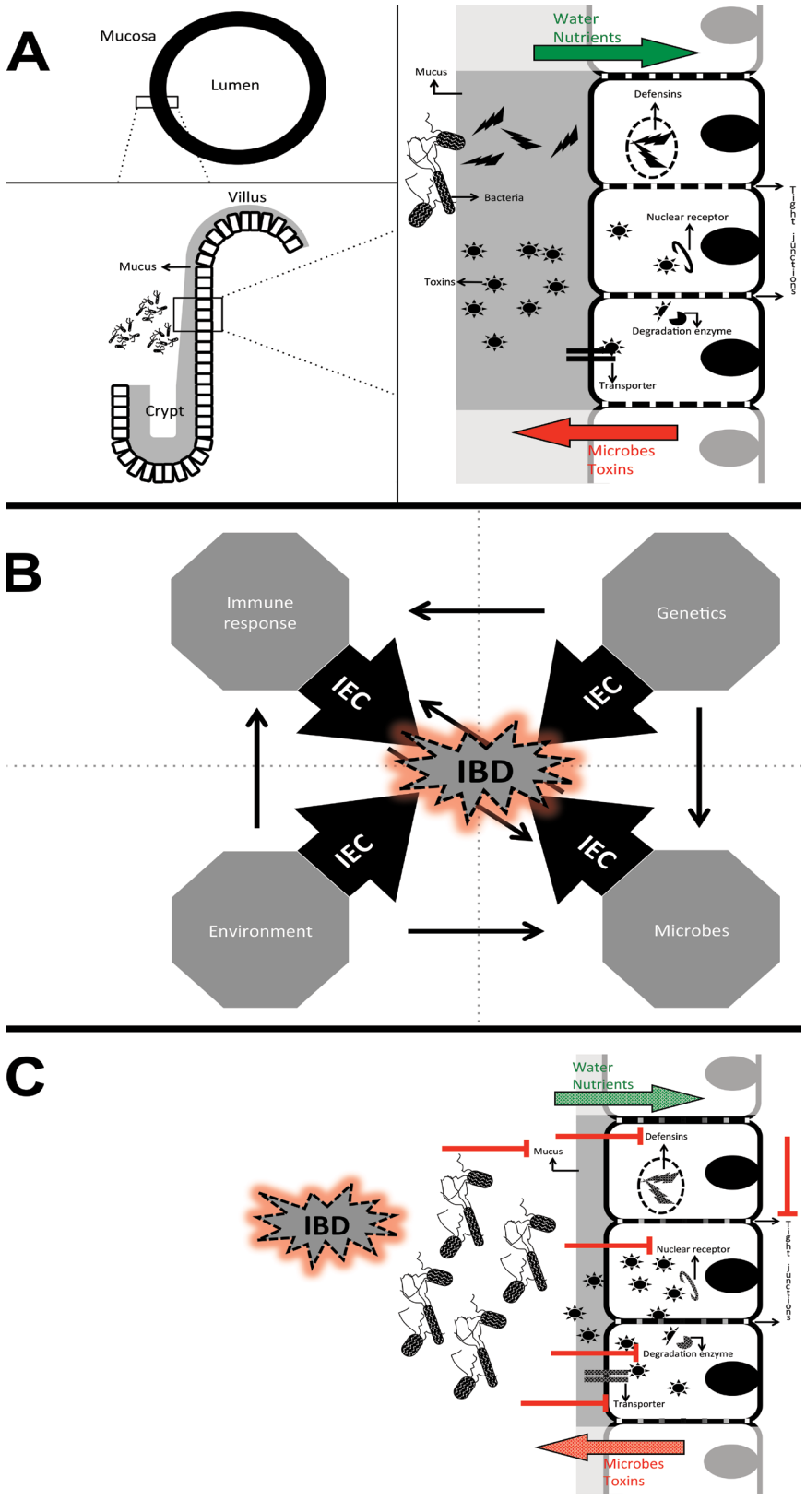
Introduction

A single cell layer of intestinal epithelial cells (IEC) is the first line of defence against the luminal content and the host. IEC are challenged by the complex task of on the one hand to adsorb water and nutrients from the lumen but on the other hand to protect the host from pathogenic microbes and toxic compounds. To deal with this challenge, the cells are equipped with sophisticated protection mechanisms (Figure 1A). Inflammatory bowel disease (IBD) is a chronic inflammatory disorder affecting the gastrointestinal tract with an apparently ever-increasing incidence and tendency to more severe clinical phenotypes. The disease is characterised by an exaggerated immune response to the luminal flora, suggesting that deficiencies in barrier function of intestinal flora may be involved, and recent studies support this notion[1-4]. IBD can be classified into two main groups, Crohn's disease (CD) and ulcerative colitis (UC). CD patients can have inflammatory lesions in their entire gastrointestinal tract, whereas the inflammation in UC patients is restricted to the colon. Apart from the misery inflicted by the life-long chronic intestinal inflammation, patients with long-standing IBD are also substantially at risk for developing colorectal cancer. In the case of UC in combination with primary sclerosing cholangitis the risk for developing colorectal cancer approaches that 100 times as high as the risk for developing colon cancer in the population at large. In addition, IBD related cancers account for 15% of all the intestinal cancer-related deaths[5, 6]. The prevailing view is that defective IEC function in IBD has a multi-factorial background and involves the interplay between genetics, microbiome, immunity and environmental triggers (Figure 1B). In this review we shall attempt to link these factors to IEC cellular physiology.

The single cell layer: intestinal epithelium

Although the IEC compartment constitutes a single layer of cells, a fair number of different specialised cell types can be discerned including the crypt-located stem cells that bear the proliferative burden associated with the constant cell renewal in the intestinal epithelium; goblet cells that produce mucus which has a protective function with respect to the mechanical, xenobiotic and also microbiological load to which this compartment is exposed; crypt-restricted paneth cells that produce anti-microbial defensins and trophic factors and protect and nurture the stem cells; enterocytes that mediate the absorption of water and nutrients and constitute the most abundant cell type in this compartment, M cells that execute specialised tolerogenic antigen-presenting functions to prevent intestinal inflammation and enteroendocrine cells which produce and secrete a variety of hormones and bioactive peptides (schematic overview of small intestinal specialised IEC see Figure 2). The morphogenetic processes, which involve amongst others the concerted action of Wnt, Notch, FGF, Hedgehog and BMP pathways that maintain homeostasis of histology in this organ have now to a large extent be delineated[7]. The resulting epithelial surface forms an impregnable barrier for the in potential harmful luminal intestinal content. The various

Back to Index



components involve from the luminal side, a thick mucus layer made up from heavy glycosylated mucins excreted by the goblet cells which prevents many luminal microbes to IEC, although other bacterial species actually find their ecological habitat in this layer protein and sugar rich layer (*e.g. Akkermansia muciniphila)*[8]. In evolutionary adaptation, goblet cells continuously produce new mucus. The mucus layer also operates as a lubricant so that, together with the peristaltic movement of the bowel, the luminal content including the microbes are transported trough the intestinal tract[9]. Secondly, the Paneth cells produce and secrete defensins and lysozyme to prevent the invasion of the microbes into the mucus layer[2]. However, their spatial restriction to the crypt base suggests that they mainly function to protect the stem cells from bacterium-induced genotoxic damage, a view supported by the production of indispensable trophic factors for the intestinal stem cells by the Paneth cell compartment, suggesting that their main function is in the support of stem cells rather as in the control of microbiological challenge to the epithelial layer[10]. The IEC compartment *in toto* forms a non-penetrable layer of cells by generating cell-to-cell tight-junctions independent of the various cell types present[11]. This barrier is tight enough for active ion-transport to generate an electrolyte potential between the mucosa and the lumen. This subsequent ion flux is needed to absorb water from the intestinal lumen into the mucosa[12]. Deregulation in this system lead to diarrhoea and in the worst cases to dehydration[13]. In addition, the IEC are equipped with pathogen receptors that mediate immune responses once other barriers fail to repel invading bacteria[15, 16]. Many of the risk genes for IBD are involved in IEC defence against the luminal microbiological onslaught, examples will be discussed later in this review, but it has not been demonstrated convincingly demonstrated that the polymorphisms involved are directly linked to reduced intestinal immunity.

Next to the microbes, another major challenge to the IEC are the toxic compounds present in the intestinal lumen, which include secondary bile acids, food derived toxic compounds, and toxins produced by microbes. These

Figure 1: Schematic overview of the intestinal epithelial cells and their function by inflammatory bowel disease.
A) Three at a subsequent simplified schemes that increase in magnification from intestinal organ to intestinal epithelial cell. The upper left corner is a cross section of the intestine with the luminal and mucosal side indicated. The enlarged section beneath schematically indicates a crypt villus structure as can be found in the small intestine. The bacteria and other luminal content are separated from the intestinal epithelial cells by the mucus layer. The intestinal wall is covered with a layer of intestinal epithelial cells, indicated in the enlarged section on the right side.
B) The involvement of intestinal epithelial cells in the inflammatory bowel disease aetiology. Inflammatory bowel disease is a multi factorial disease that includes four main contributors. As such an overaggressive immune response against the commensal bacteria in a genetic susceptible host with favourable environmental factors is needed to give onset to the disease. Important mediators between these four factors are intestinal epithelial cells. The arrows indicate the causal relationship between these factors. The genetic background of a patient can modulate the microbial composition and the outcome of the host immune response. At the same time, the host environment can also modulates these factors, irrespective of genetic background. Whereas the mucosal inflammation and the bacterial composition are counter interactive. IEC = intestinal epithelial cell, IBD = inflammatory bowel disease.
C) Hampered intestinal epithelial cell function in patients with active inflammatory bowel disease. This scheme emphasises the dramatic changes in the intestinal epithelial cell function during active inflammatory bowel disease. Basically all cellular functions are impeded during an inflammatory insult, which also decreases the basic function of the intestine such as nutrient and water absorption.

Back to Index

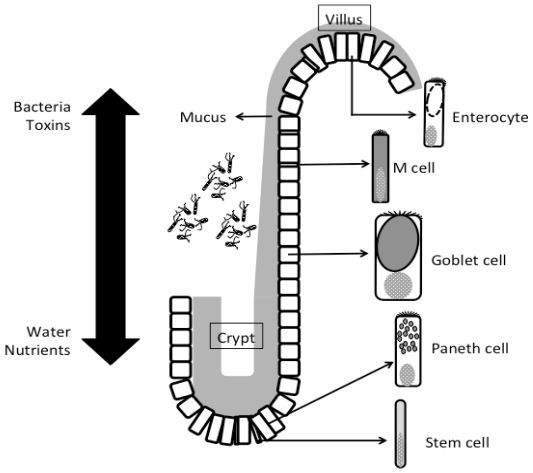


Figure 2: Schematic overview of different specialised IEC in the small intestine
Schematic overview of specialised IEC present in the small intestine. The intestinal epithelium forms an impregnable barrier for bacteria and toxins where water and nutrients are taken up. The small intestine consists of villi that point towards the lumen and crypts that are surrounded by the mucosa. The Paneth cells are located at the crypt base, which produce antimicrobial peptides, lysozyme and indispensable growth factors for its companying stem cells. Stem cells together with the trans amplifying cells are responsible for the regeneration of the entire intestinal epithelium layer every five to six days. Death and redundant cells are shed on the top of the villi where newborn cells arise from the crypt base. Goblet cells, M cells and enterocytes can be found throughout the crypt and villi. Goblet cells continuously produce mucus as lubricant for the bowel movements and to prevent direct contact of the luminal microbes to the epithelium. M cells are the main intestinal hormone producers and important mediators in the adaptive immune system, because these cells can present microbial antigens to activate leukocytes. Enterocytes mainly absorb water and nutrients from the lumen.

non-host produced toxins are called xenobiotics. IEC adapt their physiology to the level of xenobiotic stress to which they are exposed. The first step in this response the recognition of the xenobiotics is their detection by a nuclear receptor (NR) family member[17]. Important NRs in IEC are the Pregnane X receptor (PXR/ SXR/ NR2L1), the Farnesoid X receptor (FXR/ NR1H4), the Vitamin D receptor (VDR/ NR1L1), the constitutive androstane receptor (CAR), the Retinoid X receptor (RXR/ NR2B2) and the Hepatocyte Nuclear Factor 4 α (HNF4α/ NR2A21)[18]. Each receptor has its own specific (xenobiotic) ligands. Once activated, the NR dimerise and translocate to the nucleus and initiate gene expression of degradation enzymes and membrane transporters. Xenobiotic-degrading enzymes such as, the cytochrome P450 family, neutralise xenobiotics via oxidation[19]. Membrane bound transporters such as, ABCG2, MDR1 and BSEP, export the xenobiotics out of the cell back into the lumen[20]. It is known that the xenobiotic clearing system is impaired during active intestinal inflammation[21-23] (Figure 1C). Thus overall, the intestinal epithelium has specialised cells and molecular intracellular mechanisms to protect the host from potential harmful luminal content (Figure 1), but this system dramatically disturbed during active mucosal inflammation.

1: IEC and genetics in IBD

Genome-wide associated studies (GWAS) found more than 163 genomic loci that are associated with IBD, 90 with CD, 73 with UC, and 110 for both diseases[1, 24, 25]. These single nucleotide polymorphisms (SNPs) are located throughout the entire human genome. An important milestone in IBD genetics was the discovery of rare mutations (SNPs) in the NOD2 receptor[26, 27]. By using classical linkage analysis technique, these authors described in 1996 the

first genetic variants that are associated with a higher susceptibility to CD, *in casu* the *NOD2* gene. NOD2 is an intracellular pattern recognition receptor that can detect intracellular microbial compounds and activates an immune response[26, 27]. The discovery that genetic variants of this receptor predispose to IBD thus provided a first rational explanation for the observation that IBD involves an interaction of immunological, microbiological and genetic factors. Functional studies reveal that mutations in NOD2 have functional consequences for Paneth cells[28], reduced Toll like receptor (TLR) signalling[29], and impaired ability to trigger autophagy[30, 31] and thus a link between genetic background and reduced immune functionality in the IEC compartment is now firmly established.

More recent, large GWA studies have and still do identify an increasing number of distinct susceptibility loci in the genome that are associated with IBD and IEC function[1, 32]. Thus far, only a small percentage of all SNPs associated with IBD are linked to a clear functional or clinical phenotype, but those that do manifest their influence on physiology largely in the IEC compartment. For example, a mutation in ATG16L1 (rs22441880) revealed a clear phenotype in Paneth cells of patients with Crohn's disease[33] and (Deuring et al. manuscript in preparation). In addition, a rare variant in the XBP1 gene is associated with increased susceptibility for IBD. IEC specific XBP1 knock out mice have spontaneous ileitis due to malfunctioning endoplasmic reticulum (ER) stress signal pathways[34] in the secretory lineages of the IEC compartment. Also various other genetic variations in or near genes with a link to IEC functionality are associated with IBD such as, IL-10, IL-23R, JAK2, DNMT3a, NDFIP1, and FADS2[24, 25]. Thus the genetics of IBD suggests that the hereditary component of this disease is at least to a certain extent due to innate deficiencies in IEC functionality.

Interestingly, genetics does not only point to a role of IEC microbial immunity in the development of IBD by also to genes involved in IEC defence against xenotoxic challenge are linked to increased propensity to contract this disease. Especially various SNPs in NR genes are linked to IBD, including the FXR[35], HNF4α[36], the PXR[37] and the LXR[38]. However, although these findings further focus attention on the IEC compartment as the structure that manifests hereditary susceptibility to contracting IBD, in the absence of the functional work it is difficult to make definitive statements. A further complicating factor here is that the constant increase in loci mediating susceptibility to IBD makes selecting worthwhile avenues for functional studies more difficult.

2: IEC and gut micro flora in IBD

The intestinal tract is loaded with bacteria, which are indispensable for a normal gut homeostasis[39]. Misbalance in the intestinal bacterial composition is associated with IBD[40, 41]. Directly after birth the young sterile intestine is colonised by bacteria. The mucosa has together with the IEC become tolerant against the commensal microbes where possible pathogens need to be eliminated. The establishment of the micro flora is essential for a normal IEC

Back to Index

development and homeostatic gut mucosa[42-44]. In the first few years of life the gut microbiome composition is highly dynamic due to major changes in diet[45, 46]. It is generally assumed that gut microbial ecosystems show wild swings depending on diet and other life style factors[47, 48], although individual-specific core elements of microbial composition are evidently present as well. It has been suggested that a 'Western' micro-flora also resulting from a 'Western' diet might underlie the increasing incidence of IBD in the western countries[47]. Also in the Western world, however, local diets vary wildly and in general IBD appears more correlated with wealth *per se* rather as cultural background and thus it remains uncertain to which extent diet-induced changes in microbiota are implicated in the increasing incidence of IBD. Hampering investigations in this area is that gut microbes are mostly anaerobic bacteria that are challenging to cultivate *in vitro*, making it problematic to profile the entire gut microbiome. Over the past decade, high throughput 16s rRNA sequencing now allows researchers to assess complex microbiome compositions[49]. Much effort is put on finding potential pathogens that may provoke flares or disease onset in IBD patients[50-52]. Until now no distinct pathogen or group of pathogens has been found[52], leading to speculation that the IBD should be considered primarily an overactive immune response against the commensal micro-flora[50]. Indeed, viral infection of the IEC compartment may actually be more important in developing chronic mucosal disease as compared to the microbial challenge: mice with humanised T300A ATG16L1 expression, an important gene in the autophagic process and an important locus in the genetic risk factors for contracting IBD, are more prone to developing CD-like colitic symptoms, but exclusively in the presence of Noroviral infection[53]. As a consequence, next to microbiome profiling, investigation of the virome possibly associated with IBD is now taking off [54].

The first line of defence against microbiological and viral challenge is the mucus layer, as discussed before. This layer is imbued with antimicrobial peptides produced by Paneth (crypts of the small intestine only) and Goblet cells. When microorganisms overcome this barrier, specific transcriptional responses in the IEC compartment help to repel the possible breach of the integrity of the *milieu interior*-protecting barrier. Especially TLR-mediated NF-κB activation is important for mediating transcriptional responses after detection such threats, but other receptors (especially NODs) are important in this respect as well. An integral component of the barrier are the tight junctions between the IECs[11] and diminished tight junctional functionality is a strong trigger for fulminant mucosal immunity. As tight barrier functionality is compromised in IBD patients[55] an genetic or environmental deficiency in tight junctional barrier formation may contribute to IBD, but as inflammation per se is associated with reduced tight junction function cause and effect is difficult to distinguish.

Next to the microbes themselves, these organisms can also produce metabolites that harm the IEC. Microbes digest the food components and primary bile acids, which are present in the gut lumen. The human liver cells originally produce two types of primary bile acids namely cholic acid and

chenodeoxycholic acid from cholesterol. The bile acids are secreted in the intestine where after bacteria dehydroxylate a portion of these two bile acids to deoxycholic acid and lithocholic acid, secondary bile acids. These four bile acids are reabsorbed and directed to the liver. The liver cells can conjugate them to eight different conjugated bile salts and redirected to the intestinal tract[56]. Bile is needed to solubilize lipids and fat-soluble vitamins for a better uptake by IEC. High concentrations of bile acids, however, especially of the secondary monohydroxic bile acid lithocholic acid can be toxic and also carcinogenic to IECs[57]. Next to bile acids, bacteria produce toxins that hamper the function of IEC[58-60]. These intestinal bile acids and toxins are detected by IEC specific NR (FXR, PXR, RXR) and transported by IEC membrane transporters (ABCG2, BSEP, MDR1). Active inflammation in IBD patients is associated with reduced function and expression of these receptors and transporters [21, 61-63]. Hence, the bile acids can build up in the cells and can exert their (geno) toxic damage[57] and devising strategies to limit the deleterious effects of flora-derived metabolites in the IEC compartment represents an important frontier in experimental gastrointestinal biology. Next to these harmful compounds, the microbiota also produces essential vitamins and breakdown indigestible fibers into short-chain fatty acids and digestible carbohydrates for more efficient uptake by IEC. Short chain fatty acids, which include propionate and butyrate, are essential for the energy production in IEC[64, 65] and can protect against colorectal cancer[66]. Dietary components, like fructans favour bacteria associated with beneficial metabolites[67] and are thus called prebiotics and can contribute to healthy life through the microbiome.

In conclusion, direct contact of bacteria to IEC and the toxic metabolites produced by bacteria do harm the intestinal epithelium. This is, under normal circumstances, not necessarily a problem for the IEC. On the other hand, as a result of malfunctioning IEC, bacteria and xenobiotics can almost unchecked enter the gut mucosa and provoke inflammation and intestinal damage. In addition, the micro flora produces essential compounds for a healthy gut. Hence, IEC play an indispensable role in maintaining a healthy gut micro-flora homeostasis, conversely IEC are depending on a homeostatic commensal microflora for optimal functionality.

3: IEC and intestinal immune response

The IEC are constantly exposed to non-human microbial and also food antigens. Receptors on the IEC can detect these antigens and thereafter provoke an immune response. Overactive intestinal immune response is found in many gastrointestinal disorders such as, coeliac disease, IBS, necrotizing enterocolitis, and IBD. In the classical interpretation of immunology, detection of such antigens is limited to professional immune cells, In the past decades, however, it has become clear that also IEC can exert a wide variety of innate immune functions and under certain circumstances even function as antigen-presenting cells (in particular the so-called M cells). The key transcriptional mediator evoking immune relevant gene transcription in IEC is nuclear factor κB (NF-κB). Three decades ago NF-κB was discovered as an enhancer-

Back to Index

binding protein[68]. Once activated, NF-κB translocates to the nucleus, binds DNA and subsequently activates gene expression[69]. The classical, canonical, NF-κB pathway is activated by different receptors, such as TLRs, the TNFα receptors, Interleukin (IL) receptors and importantly, NOD like receptors[70]. When engaged with their cognate ligand, these receptors trigger activation of the IκB kinase (IKK) complex. In its most common form, this complex consists of IKKa en IKKb catalytic subunits where IKKg is the regulatory (NEMO) subunit of the IKK complex. Activation of the IKK complex results in phosphorylation of IkB, thereby targeting it for proteosomal degradation. This in turn releases the inhibitory binding of IκB to the NF-κB complex (p50/p65), allowing NF-κB translocation to the nucleus. Recently a second NF-κB signalling pathway was described[71], now denominated as the non-canonical alternative NF-κB pathway. TNFα-like molecules, except TNFα itself, activate this alternative pathway such as, lymphotoxins and B-cell activating factors (BAFF). The activated receptors subsequently activate a specific IKKa homodimer complex that partly degrade p100 till the P52 subunit is formed binds Rel-b and translocates to the nucleus[71] (reviewed in[72]). Both signalling pathways induce transcription of genes involved in mucosal inflammation including cytokines as IL-6, IL-8 IL-1β, IL-10, TNFα, ICAM and ELC. Several drugs that are commonly used for treatment in IBD, e.g. steroids, are thought to act via the inhibitory effect of the NF-κB pathway, in particular in IEC[73]. But also other *bona-fide* anti-inflammatory processes appear to act through NF-κB inhibition. For instance, the anti-inflammatory action of NR is mainly mediated through NF-κB inhibition, at least in murine liver[22] and intestine[74-76] and thus activation of FXR and PXR is a potential therapeutic target for intestinal inflammation[22, 74] and (Deuring et al. DDW 2012 abstract no. 1291342). Probably, this pathway exists to limit inflammatory responses to xenobiotic agents. This indirectly links the xenobiotic pathways to innate immune responses in IEC. However, caution has to be taken in the complete inhibition of NF-κB, since IEC specific NEMO knock out mice suffer from severe spontaneous colitis[77] and we do not completely understand all interactions involved.

Important innate immune receptors are the family or Toll like Receptors (TLRs), which includes TLR1-9[16, 78-82]. On IEC of healthy individuals TLR4 and TLR2 protein is detected at low levels although, mRNA of all TLRs could be measured in colon and small intestine (reviewed in[79]). However in patients with IBD the IEC decrease the expression of TRLs, except for TLR4 that is significantly increased[16]. It is suggested that the cytokines present during active inflammation up-regulate the expression of TLR4 in IEC[79]. Since activated TLRs initiate the NF-κB pathway, the increased expression may prolong the mucosal inflammation. Next to the innate immune responses, IEC also participate in the adaptive immune responses. As such, IEC express the antigen presenting molecule histocompatibility complex (MHC) class I[83] and class II[84, 85]. The IEC can process and present antigens on these molecules of microbial products or total bacteria. Although, the gut lumen is filled with commensal bacteria the IEC need to distinguish between commensal and

pathogen. The expression of MHC molecules in IEC is regulated by stress signals[86], particular microbial strains[87] and viral infection[88]. In addition patients with IBD have increased T-cell meditated cytotoxicity due to modulated MHC class I and class II expression[84]. That can lead to increased IEC apoptosis. This indicates that IEC also play an important role in the adaptive immune system.

In conclusion, the intestinal epithelium detect, react, engulf, process, and present pathogenic microorganisms and thereby initiate and strong innate and adaptive immune response. Hence, IEC are pronounced mediators in the gut immune response during IBD.

4: IEC and environmental factors in IBD

The gut lumen is a constantly chancing environment, its nature being changed due to food intake, intoxication and even mental conditions. Prandial food intake results in many different antigens to pass the IEC layer and thus it is tempting to relate changes in IBD incidence to dietary differences, especially as other chronic inflammatory conditions of the intestine, *e.g.* celiac disease can be directly related to specific dietary components. Apart from directly supplying antigens, food intake majorly influences gut microfloral composition. However just a few and unpersuasive dietary studies suggest a link between IBD and diet, mostly because they just provide indirect evidence of possible cause-and-effect between these two factors. Excessive refined sugar consumption might be a risk factor for CD but not for UC[89], fat intake has been positively correlated to UC whereas high fiber consumption (*e.g.* fruit and vegetables) were reported to decrease the risk of IBD[90]. In particular children but also adult IBD patients can remain in remission using a well-defined high fiber diet[91]. However, these results should be carefully interpreted, by its small patient number and since diet-studies are an easy subject for experimental bias, due to the difficulties of obtaining the dietary histories. Certain specific food components can modulate the function of IEC, *e.g.* potato glycoalkaloids can permeabilize the cholesterol containing cellular membranes in turn causing disruption of the epithelial barrier[92]. Frying potatoes increases the concentration glycoalkaloids even more which might be consistent with an observation that a high fat diet contributes to risk for IBD[92], at bay with this notion is that observation of very high consumption of potato-based food (as *e.g.* occurs in the former Soviet Union) does not translate in increased epidemiological risk for IBD. Likewise, meat is considered part of the modern 'western diet' and includes a high fatty acid intake with an unfavourable ω-6/ ω-3 ratio[93]. Although increased ω-6/ ω-3 fatty acid ratio has been reported to increase the risk for IBD patients to get a relapse[93, 94]. Thus although changing the diet of IBD patients is suggested to be an additional or even a primary therapeutic strategy targeting IEC physiology[95], the value of such strategies remain largely unproven. A complicating factor might be that certain fatty acids can bind to the IEC NR PPARγ and thus mediate mucosal healing[96] through induction of genes involved in the metabolisation and transport of fatty acids[97].

Back to Index

Smoking has a dichotomous role in both diseases[98]. Whereas smoking is a risk factor for getting CD and for having relapses in CD patients[99]. Can smoking be protective in UC, but smoking cessation increases the risk for UC[100]. However, although the exact function of cigarette-smoke on the IBD aetiology is still largely unknown, smoking augments intestinal macrophage phagocytosis via nicotinic acetylcholine receptor $\alpha4\beta2$[101], which in turn may combat deficiencies in innate immunity common to CD[102]. Moreover, cigarette-smoke in general also modulates genes involved in apoptosis and membrane transporters of heavy metal ions through the IEC layer[103]. It also will be of great interest to investigate the change in micro flora due to cigarette-smoke, since the micro flora play an indispensable role in the normal gut homeostasis.

Another surprising factor that negatively affects the IEC barrier permeability are chronic mental-stress conditions[104, 105]. Intestinal nerve-fibers interact with the immune cells present in the intestinal mucosal. As such mucosal mast cells can get activated upon neural stimulation. Activated mast cell produce high amounts of cytokines and tryptase initially to recruit more mast cells[106-108]. However, elevated cytokine and tryptase expression can also modulate the IEC barrier function. Enhanced IEC macromolecule permeability is observed in conditions with high concentrations of cytokines and tryptase, moreover blocking the tryptase prevents experimental colitis[109]. Many environmental factors can modulate the normal function of IEC. Diet, smoking, and mental stresses play an indispensable role in the function of these cells and can provide clues for designing treatment.

IEC as therapeutic target

Various IEC-mediated treatment modalities for IBD patients exist. An important therapeutic goal in the treatment of IBD patients is fast mucosal healing, in particular in UC patients[110, 111] and reviewed in[112]. Rapid restoration of the intestinal barrier functionality appears beneficial for IBD patients and reduces the change of a relapse. Molecular pathways in IEC and IEC proliferation are key processes in the resealing of the gut mucosa[113] and explain the interest in for instance *Lactococcus*-driven local mucosal trefoil factor (a trophic factor for IECs) application for the treatment of IBD [114] and has been proven successful in rodent models of chronic inflammation[115]. According the European guidelines is 5-aminosalicylates (5-ASA) one of the first given medications for UC patients[116]. Interestingly, IEC have an important function in the metabolism and mechanism of action of 5-ASA, when 5-ASA enters the IEC it is acetylated where after it can bind to PPARγ, which is an important intestinal NR[117]. This binding initiates transcription of genes that reduce the NF-κB activity[118], enhances cyto-protection by reducing production of chemo-attractants[119], and inhibits oxidative stress induced apoptosis[120]. The most likely mechanism of action of 5-ASA on IEC immunity is through phospholipase D and mTOR inhibition[121]. Hence, 5-ASA reduces the overactive innate immune response in IEC and provokes IEC proliferation and thus epithelium resealing.

A second line in the therapeutic strategy for IBD patients is corticoid-steroids, for UC and CD patients[116, 122]. Also these compounds have a pronounced effect on IEC. Steroids reduce the NF-κB activity in IEC and they are involved in the epithelium repair mechanism[123, 124]. However, the working mechanism of steroids is different between CD and UC patients[125-129]. CD patients do benefit from steroid therapy by reducing the disease symptoms, but do not exhibit increased epithelial barrier functionality[128, 129]. Conversely, steroid therapy in UC patients does ameliorate the IEC barrier function and thereby counteracts mucosal inflammation[125-127]. Major drawbacks of long turn steroids use are the severe side effects[130], therefore steroids are mostly used as induction therapy. The popularity of topical steroid therapy for remission maintenance is testimony of the importance of the IEC as a therapeutical target in IBD.

Azathioprine is a strong chemical immunomodulator, which is used for CD and UC patients with moderate to severe inflammation[116, 122]. Azathioprine is a DNA synthesis modulator that impedes the T- and B-cell proliferation to diminish the activated adaptive immune responses[131]. Moreover, azathioprine also has a pronounced function in the IEC homeostasis of IBD patients[132, 133]. Although, classically the action of azathioprine is attributed to its capacity to impede DNA synthesis, the compound provokes fast mucosal healing[132, 133]. The insight that the compound works through Rac inhibition rather as on DNA synthesis provides rational explanations here[131, 134]. Certainly, Rac is abundantly expressed in the IEC compartment and has been identified as a relevant target for anti-inflammatory drugs[135] and thus the notion that the IEC represents a relevant compartment for azathioprine in IBD is not unreasonable.

The last decade the strong anti-inflammatory biological agent anti-TNFα appears to be highly effective for immunological diseases such as IBD[136, 137]. Although the exact working mechanism of anti-TNFα is still unknown, there is a clinical link between anti-TNFα and mucosal healing[138-141]. Besides, anti-TNFα ameliorates the functions of IEC during active intestinal inflammation by reducing p53-mediated apoptosis[142, 143] and reducing IL-33 cytokine expression[144]. By contrast, anti-TNFα also can be detrimental for the tight junctions strength between IEC[145]. This together indicates that IEC have a pronounced function in anti-TNFα therapy but still much research is needed before the precise mechanisms of action are unravelled.

To date there is an established treatment strategy for IBD patients[116, 122]. Since not all patients do respond well to these therapies, there is always room for novel therapeutic approaches. As such, NR can also inhibit the NF-κB activity in IEC. Although this described inhibition needs further investigation, the first results appear to be promising[22, 74] and (Deuring *et al.* DDW 2012 abstract no 1291342). Hence, it is evident that the IEC are important mediators in the current therapeutic strategies for IBD patients, but there is still much research needed to uncover the exact details of IEC physiology and their potential usefulness for therapy.

Back to Index

II

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Back to Index

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Back to Index

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Back to Index

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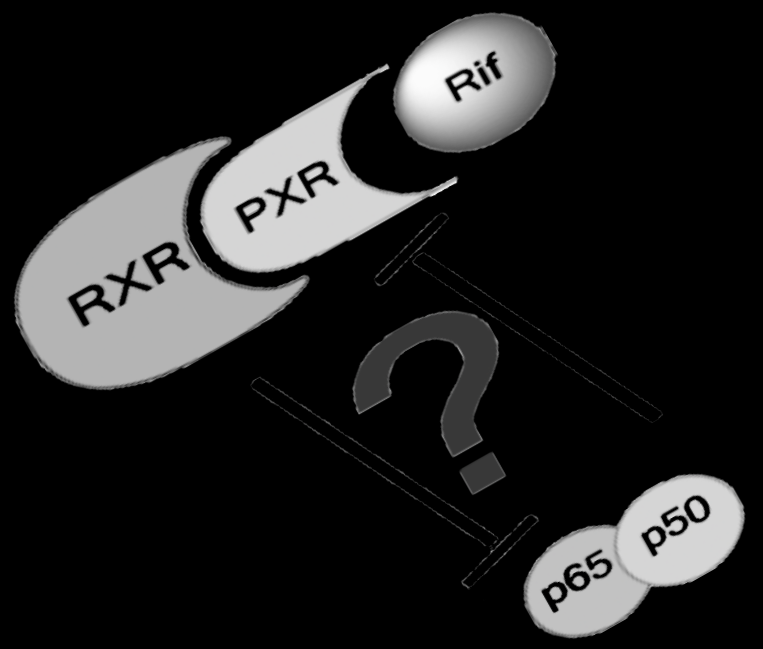
Back to Index

III

Pregnane X Receptor Activation Constrains NF-κB activity during Active Inflammatory Bowel Disease



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Abstract

The Pregnane X Receptor (PXR) is a principal signal transducer in mucosal responses to xenobiotic stress. To facilitate regenerative responses PXR impedes activity of Nuclear Factor-κB (NF-κB), the main transcription factor controlling inflammatory responses. Although it is well-recognised that IBD (inflammatory bowel disease) is accompanied by xenobiotic stress, the importance of PXR in limiting inflammatory responses in IBD has not been investigated.

In total 106 colonic biopsies from 19 CD (Crohn's disease) patients and 36 colonic biopsies from 8 control patients were stimulated with the PXR ligand rifampicin or vehicle. As a readout for NF-κB activity, levels of IL-8 and IL-1β mRNA and protein were employed, whereas PXR levels were assessed using its mRNA expression. *In-vitro* PXR and NF-κB interactions in colon cells were investigated using the PXR knockdown LS174t (siPXR) and its PXR-expressing counterpart LS174t (nt).

PXR levels show a strict inverse correlation with NF-kB target gene expression in biopsies from CD patients with active disease. Furthermore, treatment with rifampicin reduces NF-κB activity showing that PXR activity is a rate-limiting step with respect to CD-associated NF-κB activity. These effects were verified *in-vitro*, showing that LS174t (siPXR) cells have higher NF-κB activity.

Our data suggest that the presence of PXR is the main mechanism counteracting and limiting the epithelial NF-κB activity in patients with active IBD. Modulation of PXR activity thus holds therapeutic promise in the clinical management of CD.

Introduction

Intestinal epithelial cells (IEC) form the physical barrier between the gut luminal content and the mucosa. The function of IEC is multifold: nutrients and water are absorbed, and simultaneously these cells are responsible for the detection[1] and form a first line of defence against both microbial as well as xenobiotic challenges to the body[2, 3]. Appropriate responses to xenobiotic stress are complex but are coordinated through a specific subfamily of the steroid-thyroid superfamily of nuclear hormone receptors (NR). When specific ligands engage such receptors, either homodimerisation or heterodimerisation with other nuclear hormone receptor family members ensues and a pro-transcription factor forms, that by virtue loss of hsp90 binding exposes the nuclear localisation signal to the cytoplasmic machinery leading to nuclear translocation[4]. In the nucleus, the complex binds to specific palindromic promoter regions and directs transcription of genes involved in executing the appropriate response to the xenobiotic challenge. A large number of nuclear hormone receptors appears to be involved in orchestrating the transcriptional response to IEC xenobiotic challenges, including the Pregnane X receptor (PXR/ SXR/ NR2L1), Farnesoid X receptor (FXR/ NR1H4), the Vitamin D receptor (VDR/ NR1L1), the constitutive androstane receptor (CAR) and the Retinoid X receptor (RXR/ NR2B2), but the evidence that PXR is a master xenobiotic sensor is especially strong[5].

Proper cellular responses to toxic insult go beyond the simple induction of detoxification enzymes[6] but also include the inhibition of pathways associated with further damage (*e.g.* the generation of reactive oxygen species) and the facilitation of regenerative responses to repair tissue damage. A reflection of these processes can be found in the association of PXR activity with foetal liver development[7] and cancer cell growth in endodermally-derived tissues[8]. Also inhibition of inflammatory responses plays an important part in fostering a regeneration-permissive environment. Activation of PXR down-regulates the transcriptional activity of NF-κB (the master regulator of genomic inflammatory responses) and thereby reduces intestinal inflammation[9-12]. Conversely, NF-κB activation represses activity of the PXR pathway[9, 13]. Evidence has been provided that activated PXR physically binds the NF-κB subunit p65 and thereby inhibits the NF-κB signalling pathway[9]. The mutual repression of the NF-κB and PXR pathways raises obvious questions as to the importance of these interactions in pathological intestinal inflammation.

Indeed, increasing evidence suggests that PXR is also involved in the pathogenesis of inflammatory bowel disease (IBD) probably via its interaction with NF-κB[9, 12, 14-19]. A single nucleotide polymorphism near the PXR locus is associated with an increased susceptibility to IBD[14, 16]. Furthermore, PXR deficient mice display more severe NF-κB-driven small intestinal inflammation than their non-mutant littermates[9]. Conversely, in patients with active IBD and strong NF-κB activation the transcriptional-activity of PXR is reduced[13], suggesting that the negative reciprocal relationship between PXR and NF-κB is also relevant in the context of human chronic inflammatory intestinal pathology. The importance of PXR activity in human IBD has

Back to Index

not been investigated and rodent results cannot be directly extrapolated to human, as PXR ligands are different for rodents than for humans[20]. Thus the importance of PXR in human IBD prompts urgent clarification.

PXR is also known as a bile acid receptor. High concentrations of bile acids especially of the secondary monohydroxic bile acid lithocholic acid can be toxic and even carcinogenic for the IEC[21]. Next to the toxic effects of bile, it also can have a positive effect on the disease progression. For example, tauroursodeoxicholic acid (TUDCA) can work as a chemical chaperone to diminish endoplasmic reticulum (ER) stress and also may contribute as an immunomodulator[22-25]. This effect was investigated in human intestinal biopsies as described in **Addendum I** (page *53*).

In the present study we show that PXR activity is the major rate-limiting pathway constraining mucosal NF-κB activity in active IBD and thus reveal that PXR signals are much more important in pathology as previously thought. Furthermore, our results imply that modulation of PXR activity holds significant clinical promise in the management of IBD.

Methods

Cell lines and ligands

To study the NF-κB inhibiting potential of PXR activation, the PXR expressing colon cancer cell line LS174t a PXR knockdown LS174t cell line was created to test the specificity of PXR. The LS174t cell line was transduced with a lenti-virus, containing small interference RNA for PXR (siPXR), as described before[21]. The transduced cells were cultured with 100 μM puromycin (Sigma-Aldrich) for three weeks to select for cells that harbour the siPXR. The hepatocyte cancer cell line HepG2 and colon cancer cell lines CACO2 and LS174t were used to investigate the effect of HNF4α on PXR expression. All cell lines needed two passages a week and were cultured according standard culture conditions. NF-κB was in-vitro activated by 2 μL *E. coli* lysate (ELI), a centrifuged 50 mL o/n *E. coli* (DH5α, Invitrogen) culture taken up in 500 μL dH_2O. PXR was activated by 100 mM rifampicin (Rif, Sigma-Aldrich) and HNF4a was activated by 240 μM linoleic acid (LA, Sigma-Aldrich).

Biopsies

This study was conducted with approval of the Institutional Review Board of the Erasmus MC University Medical Centre in Rotterdam. All patients gave written informed consent. During endoscopy biopsies were taken from patients with a known history at least 6 months of CD and from patients referred for colonoscopy but without intestinal abnormalities, further described as control patients. Also patients were asked for additional blood samples. CD was diagnosed according to international guidelines and only the results of the control biopsies were used if there were no abnormalities on pathology, when there was no history of IBD, and no familiar history of IBD. For each patient the biopsies were taken from the ascending colon (n=3), the transversum (n=3) and the descending colon (n=3), if applicable.

Histology

One biopsy from each location was fixed in 4% formalin, dehydrated and embedded in paraffin for histological scoring. Four μM slices from the formalin fixed paraffin embedded (FFPE) tissue specimens were stained with hematoxylin and eosin (Sigma-Aldrich) according standard procedure. Three observers have independently examined each biopsy, in a blinded fashion. Discrepancies were reassessed to reach agreement.

Stimulation of the biopsies

The freshly taken biopsies were immediately placed in ice-cold regular culture medium (DMEM) for transport. Before stimulating the biopsies, they were washed three times with ice-cold PBS containing antibiotics to prevent infection. The biopsies were then stimulated for 18 h at 37°C with 100 mM Rifamcipin (Sigma-Aldrich), 10 mM Linoleic Acid (LA, Sigma-Aldrich) and or solvent. Stimulated biopsies were directly lysed in Tripure (Roche, Switzerland) for RNA and protein extraction, according manufacturer's

Back to Index

protocol. After the Tripure extraction, the RNA samples were purified using the RNA II extract kit from Macherey Nagel (Bioke) according to manufacturer's protocol.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood using Ficoll (Gibco) according standard procedures. The isolated PBMC were stimulated with 100 mM rifampicin for 18 h at 37°C followed by lysing of the PBMC in Tripure (Roche) for RNA isolation.

RT-PCR

Gene expression of GapdH, Ywaz, IL8, IL1b, Cyp3a4, Sult1a, and PXR were measured using quantitative PCR (qPCR, IQ5, Bio-Rad). The primer sequences are shown in Table S1. All genes were analysed using the same qPCR program as described before [22]. Gene expression is plotted as fold change using the ΔCt method[23]. The data from patients with multiple colonic biopsies where averaged. All qPCR reactions where at least performed in duplo.

Protein analysis

The p-p65 (catalogue no. 3037, Cell Signaling Technology) and p-Akt (catalogue no. 11055-2, Signalway Antibody) protein expression was measured using conventional western blot as described before [22]. The IL-8 protein expression in the protein solution isolated from the TriPure fraction was measured using ELISA, Human IL-8 ELISA Ready-SET-Go! (eBioscience).

Statistics and software

All the graphs and the statistical analyses were performed using the Graphpad Prism 5.0 software package for Windows. Data from the paired biopsies were non-parametric statistically analysed using the Wilcoxon matched pairs test. Correlations were determined using the Spearman's rank correlation coefficient. A two-tailed p value <0.05 was accepted as statistically significant. Images were composed using Adobe Photoshop CS5.

Table S1: Primer sequences

Primer name	Sequence from 5' to 3'
GapdH_fw	GCATTGCCCTCAACGACCAC
GapdH_rev	CCACCACCCTGTTGCTGTAG
YWHAS_fw	ACTTTTGGTACATTGTGGCTTCAA
YWHAS_rev	CCGCCAGGACAAACCAGTAT
IL8_fw	CACTGCGCCAACACAGAAATTA
IL8_rev	ACTTCTCCACAACCCTCTGCAC
IL1β_fw	CCCTAAACAGATGAAGTGCTCCTT
IL1β_rev	GTAGCTGGATGCCGCCAT
Cyp3a4_fw	CAGGAGGAAATTGATGCAGTTTT
Cyp3a4_rev	GTCAAGATACTCCATCTGTAGCACAGT
HNF4α_fw	ACATGGACATGGCCGACTAC
HNF4α _rev	TGCCTCAATCTGGCGAGACG
Claudin-15_fw	TGAGGTGGGTGGATTACTTG
Claudin-15_rev	TGTTGAAGGCGTACCAGGAG
Mep1a_fw	TCAAGCCCTATGAAGGAGAG
Mep1a_rev	CCTTATAGGCACATCCTTGG
Sult1a_fw	GCACCCACCCTGTTCTCTAC
Sult1a_rev	ACCACGAAGTCCACGGTCTC
PXR_fw	ATGGCAGTGTCTGGAACTAC
PXR_rev	CAGTTGACACAGCTCGAAAG

Table S2: Baseline characteristics of patients treated with rifampicin

	CD	Control
Total number of patients	19	8
Mean age, yr (SD)	44 (16.6)	62 (3.5)*
Gender (M/F)	5/14	3/4
Mean duration of disease, yr (SD)	13 (8.8)	-
# Smoking (%Yes)	4 (25)	2(33)
# Familiar IBD (%Yes)	5 (36)	-
Concomitant medication:		
- none	1	-
- aminosalicylates	3	-
- corticosteroids	4	-
- immunosuppressives	9	-
- biological	5	-
# Biopsies colon	106	36

* The healthy controls are significantly older than the CD patients. p=0.002

Back to Index

Table S3: Baseline characteristics of patients treated with linoleic acid

	Linoleic acid		Linoleic acid +/- Rifampicin	
	CD	Control	CD	Control
Total number of patients	2	3	2	3
Mean age, yr (SD)	38(7.7)	49(12.9)	50(0.7)	45(6.4)
Gender (M/F)	1/1	2/1	1/1	2/1
Mean duration of disease, yr (SD)	17(7.1)	-	5.5(0.7)	-
# Smoking (%Yes)	0(0)	0(0)	0(0)	1(33)
# with familiar link to IBD (%Yes)	0(0)	-	1(50)	-
Concomitant medication:				
- none	0	-	1	-
- aminosalicylates	1	-	1	-
- corticosteroids	0	-	0	-
- immunosuppressives	0	-	0	-
- biological	1	-	0	-
# Biopsies colon	10	8	10	8

Results

Tissue samples

In total we obtained 106 biopsies from 19 CD patients and 36 biopsies from 8 controls are used for rifampicin stimulation. In total 36 other biopsies are stimulated with linoleic acid (4 CD patients and 6 controls). Demographic patient characteristics are presented in Tables S2 and S3. Five additional patients with quiescent CD and 4 controls agreed to donate blood samples.

Mutual repression of PXR and NF-κB signalling in IBD.

We first determined the expression of NF-κB the target genes IL-8 and IL-1β in non-stimulated biopsies. As expected, the expression of both these cytokines was higher in biopsies of patients with active inflammation compared to biopsies from controls or CD patients with quiescent disease (Figures 1A and B). To clarify the relationship between intestinal PXR expression and the corresponding NF-κB activity both were determined in our patient cohort (Figure 2A). The expression of PXR is similar in control biopsies and in biopsies from quiescent CD patients. However, although not significant ($p=0.15$) the PXR expression levels seemed to be reduced in biopsies from active CD patients (Figure 2A). The reduced PXR expression negatively correlates ($r=-0.6$, $p<0.01$) with increased inflammation and NF-κB activity as judged by IL-8 and IL-1β expression (Figure 2B). The negative correlation between PXR and NF-κB pathway activity suggests that the activation status of xenobiotic and inflammatory pathways is mutually exclusive and constitutes an important regulatory circuit in IBD.

PXR activation is rate limiting for mucosal NF-kB activation in IBD

A prediction, from the notion that the negative regulation of NF-κB signalling through PXR is functionally relevant, is that hyper stimulation of the PXR pathway reduces the inflammatory aspect of gene expression in biopsies of IBD patients. Thus we challenged biopsies with the human PXR ligand rifampicin. Such treatment did not significantly reduce the IL-8 or IL-1β mRNA expression in the biopsies from control and quiescent CD patients (Figures 1A and B), demonstrating that outside the context of active IBD, PXR activity is not a rate-limiting factor with respect to NF-κB-directed gene expression. Importantly, however, rifampicin stimulation also caused a 35 fold reduction of IL-1β mRNA expression in the biopsies with active inflammation ($p<0.01$, Figure 1B). Also at the protein level the IL-8 expression is reduced following rifampicin treatment in CD patients with active disease (-750pg/mL, $p<0.01$), compared to the untreated biopsies (Figure 1C). Thus at least in active IBD it appears that level of PXR activation is the dominant NF-κB pathway activity controlling mechanism.

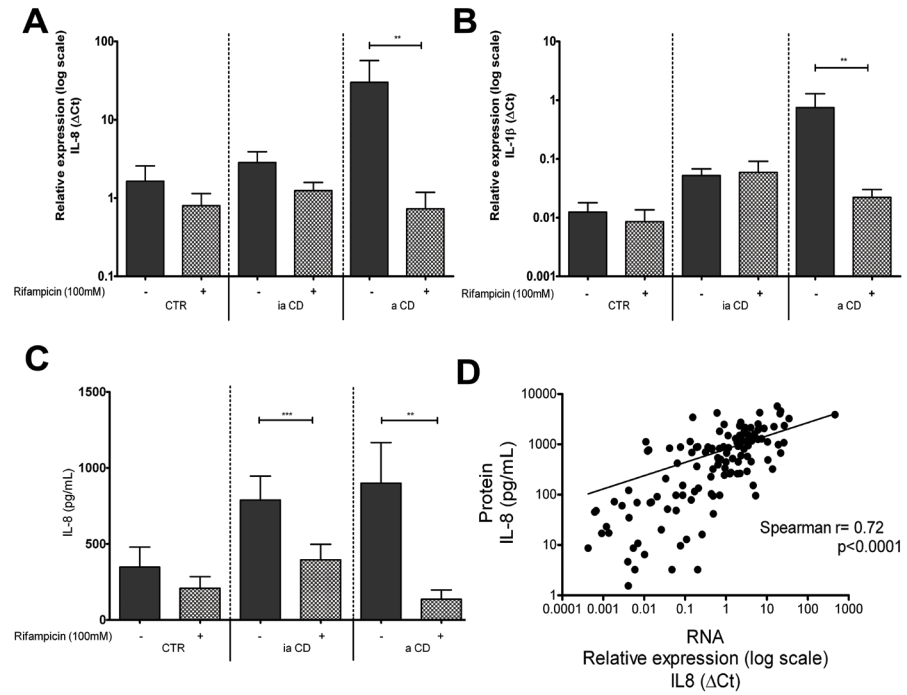


Figure 1: Cytokine expression in human intestinal biopsies with rifampicin treatment.
A) IL-8 mRNA expression in human intestinal biopsies. The graph represents the mean IL-8 mRNA expression on log scale from biopsies stimulated with solvent only (0.1% (v/v) DMSO) or 100 mM rifampicin for 18 h at 37°C. Ctr are the biopsies from control patients (n=36), iaCD are the biopsies from CD patients without active intestinal inflammation (n=66), and aCD are the biopsies from CD patients with active intestinal inflammation (n=40). The error bar is SEM, ** $p<0.01$.
B) IL-1b mRNA expression in human intestinal biopsies. For this graph the same methodology as in A) was used. The error bar is SEM, ** $p<0.01$.
C) IL-8 protein expression in human intestinal biopsies. ELISA was used to measure the IL-8 protein concentration from biopsy homogenates. The same biopsies were used as for the mRNA expression analysis in A) and B). The error bar is SEM, ** $p<0.01$, *** $p<0.001$.
D) IL-8 mRNA and protein expression correlation. The IL-8 mRNA expression is plotted against the IL-8 protein (pg/mL) expression per biopsy. Spearman correlation is calculated, $r=0.72$, $p<0.0001$.

Back to Index

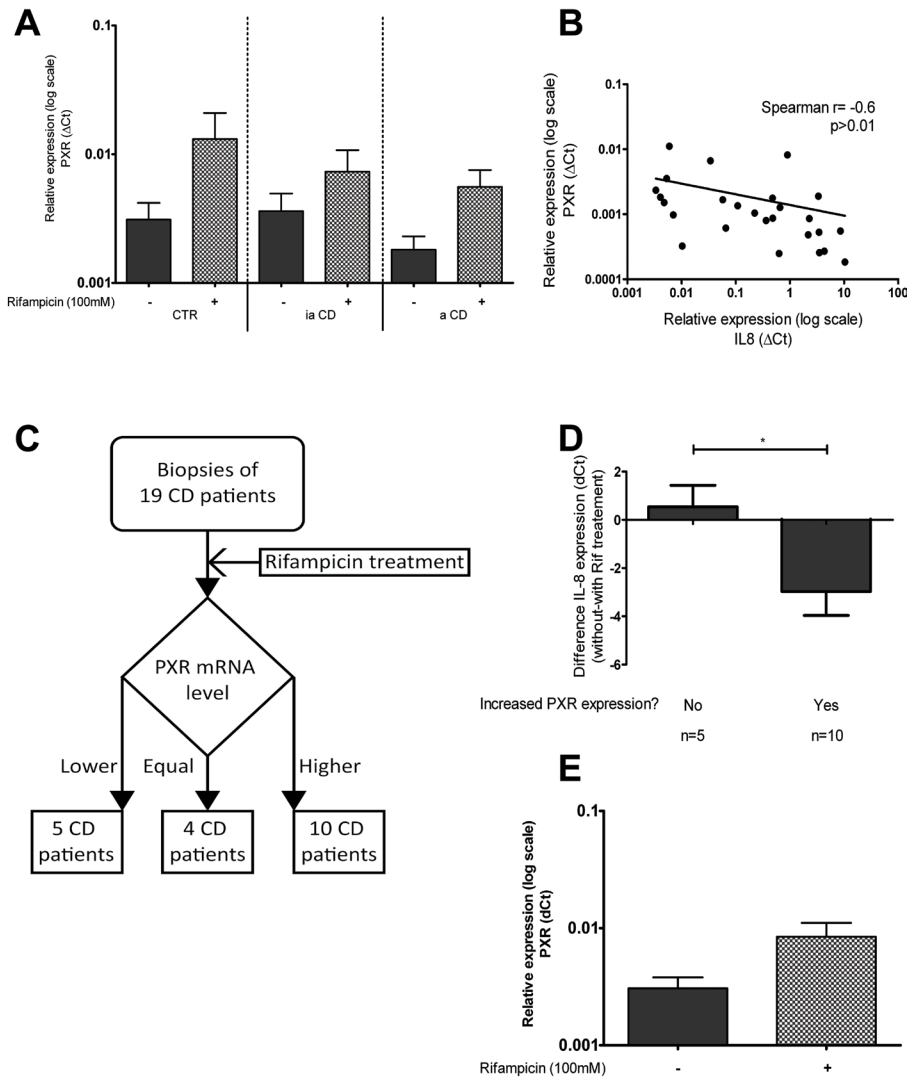


Figure 2: Non-activated PXR and reduced NF-κB activity.
A) PXR mRNA expression in human intestinal biopsies. The same methodology as in Figure 1A was used. The error bar is SEM.
B) Correlation coefficient between IL-8 and PXR from non-stimulated biopsies. The IL-8 and PXR mRNA expression difference with and without rifampicin was calculated and plotted in this graph. For this graph only the non-stimulated biopsies from CD patients were used. Both axes are in log scale. Spearman r = -0.6, p<0.01.
C) Flowchart dividing CD patients into groups based on PXR expression. The CD patients were divided in three groups; group 1 do not have increased PXR expression after rifampicin treatment, group 2 that do have increased PXR expression with rifampicin treatment, and group 3 that have equal PXR expression.
D) The difference in IL-8 mRNA expression with and without rifampicin treatment. For each patient the mean of the difference between non-rifampicin stimulated and rifampicin stimulated biopsies in IL-8 expression is presented in this graph. The error bar is SEM, * p<0.05.
E) PXR expression in biopsies with and without Rifampicin stimulation. The mean PXR mRNA expression of all biopsies, control and CD patients, with or without Rifampicin stimulation is presented in this graph. The error bar is SEM, p=0.056.

To further investigate this relationship between PXR expression and NF-κB signalling the CD patients were stratified into two groups (flowchart in Figure 2C); patients that do not have higher PXR expression (n=5) and patients that do have higher PXR expression (n=10) in the biopsies with rifampicin treatment. Four patients had equal or undeterminable PXR levels. Indeed, the IL-8 expression in biopsies from the patients that have high PXR expression following rifampicin is 6 fold lower ($p<0.05$) as compared to biopsies of patients in the patients that do not show high PXR expression following rifampicin treatment (Figure 2D). In the same way, grouping all the biopsies (controls and CD patients) it becomes clear that rifampicin stimulation increases the overall PXR expression by 3 fold ($p=0.056$; Figure 2E), consistent with a scheme in which PXR activity constrains NF-κB activity and *vice versa*. It thus appears that the level of PXR activity is the rate-limiting factor with respect to NF-κB-directed gene expression in active IBD and conversely the amount of NF-κB activity is an important negative regulator for PXR expression.

PXR activity is restricted to the epithelial compartment

Since the biopsies, especially those of patients with active CD, contain a large number of lymphocytes we wanted to determine if these cells contribute to the observed PXR-mediated reduction of NF-kB signalling. Therefore, we investigated the effect of rifampicin on PBMC isolated from blood. No PXR mRNA can be detected in any of the PBMC fractions (data not shown). Rifampicin stimulation does not influence the IL-8 mRNA expression in PBMC from controls and CD patients (Figure S1A). Furthermore the expression of PXR target gene Sult1a is not altered (Figure S1B). Hence, mononuclear cells such as, stromal cells do not seem to interfere in the PXR-mediated inhibition of NF-κB in human intestinal biopsies.

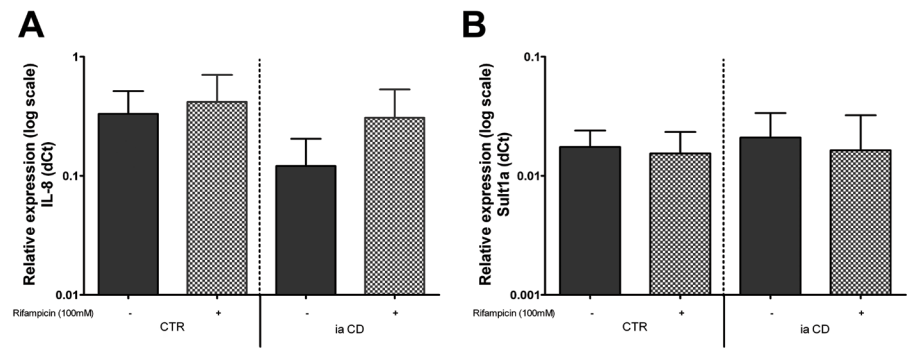
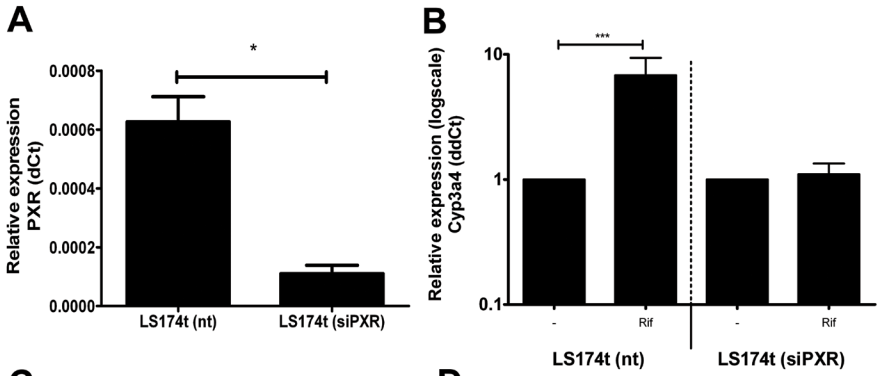


Figure S1: The effect of rifampicin on PBMC.
A) IL-8 mRNA expression in PBMC. PBMC isolated from peripheral blood samples of CD patients and healthy individuals were stimulated for 18 h at 37°C with solvent (0.1% (v/v) DMSO) or 100 mM rifampicin. Ctr is the mean IL-8 expression of the healthy individuals (n=4) and ia CD is the mean IL-8 expression of the inactive CD patients (n=5). The error bar is SD.
B) Sult1a mRNA expression in PBMC. Same methodology as used in A) was used.

Back to Index



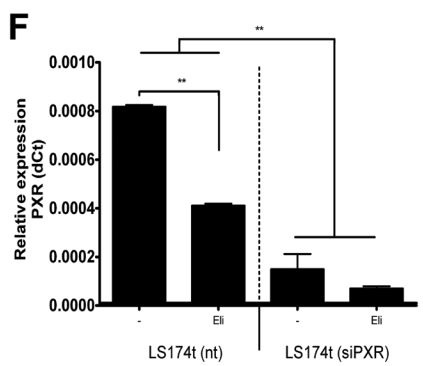


Figure 3: NF-κB signalling in the PXR knock down cell line.
A) LS174t cell line stably transduced with non-target siRNA LS174t (nt) or a siRNA for PXR LS174t (siPXR). The graph represents PXR mRNA expression in LS174t cells from three independent experiments. The error bar is SD, *p<0.05.
B) LS174t (nt) and LS174t (siPXR) cells stimulated with rifampicin. Both cell lines were stimulated with 100 mM rifampicin (Rif) for 16 h at 37°C. The relative mRNA expression of the PXR target gene, Cyp3a4, is presented in this graph. Data is from two in duplo performed independent experiments, the error bar is SD, *** p<0.001.
C) IL-8 mRNA expression in LS174t cells. Both cell lines were stimulated with 2 mL E. Coli lysate (ELI). The graph represents the IL-8 mRNA expression from two in duplo performed independent experiments. The error bar is SD, *p<0.05.
D) Activated NF-kB subunit p65 protein (p-p65) expression in LS174t cells. The same stimulation conditions as in C) were used. Actin protein expression is shown as loading control. This blot is a representative of two independent experiments.
E) Activated Akt (p-Akt) protein expression in LS174t cells. Same methodology as in D) was used.
F) PXR mRNA expression in LS174t cells. Same methodology as in C) was used. The error bar is SD, **p<0.01.

In vitro non-activated PXR mediated NF-κB inhibition

Direct support for the notion that PXR is important for restricting NF-κB activation in the epithelial compartment came from experiments in which we investigated the effect of PXR expression *per se* on NF-κB inhibition. We used the LS174t cells, a generally used model for colonic epithelial cells that recapitulates many aspects of normal enterocyte physiology and generated two derivatives, the LS174t (siPXR) clone that lacks PXR expression and LS174t (nt) as a transfection control (Figure 3A). Consistently, induction of the PXR target gene CYP3a4 is corrupted in the LS174t (siPXR) cells (Figure 3B) but not in the other cells. Following stimulation with *E. Coli* lysate, IL-8 expression is enhanced two fold in the cells lacking PXR ($p<0.05$; Figure 3C). We confirmed this difference by showing a decreased p-p65 and p-Akt protein expression in the LS174t (nt) cell line (Figures 3D and E), demonstrating that also in this model cell line, PXR activity constitutes the rate-limiting step in NF-κB-dependent gene expression. Conversely, stimulation with *E. Coli* lysate and thus activating NF-κB signalling reduces PXR levels in LS174t (nt) cells (Figure 3F). Thus these in vitro experiments show that the negative relationship between NF-κB and PXR signalling is cell-autonomous and provide strong support for the notion that the presence of PXR represents an important mechanism constraining NF-κB signalling in the mucosal epithelial compartment.

HNF4α induced PXR expression

As it becomes increasingly clear from our data that PXR expression per se is already sufficient to inhibit the NF-κB activity, we investigated whether stimulating the HNF4α nuclear receptor could increase the PXR expression as published previously for murine hepatocytes[7]. Linoleic acid stimulation in a hepatocyte cancer cell line, HepG2, increases the expression of HNF4α target gene Claudin-15 ($p<0.001$) but not in colon cancer cell lines, LS174t and CACO2 (Figure S2A). Notwithstanding that, all cell lines have detectable mRNA expression of HNF4α (Figure S2B). Furthermore, the PXR expression is also not increased in any of the cell lines (Figure 4A). Besides, stimulated biopsies do also not have increased PXR mRNA expression after linoleic acid stimulation (Figure 4B). In line with this observation, the expression of IL-8 was also not altered after stimulation of the cells with Linoleic acid (Figure 4C). The biopsies do express the HNF4α receptor and induction of HNF4α target gene (Mep1A) was found in the control biopsies ($p<0.05$, Figures S2C and D). In addition, adding rifampicin to the linoleic acid stimulated biopsies did not further change the mRNA expression of PXR or IL-8 (Figures 4D and E). These data show that linoleic acid is not able to enhance PXR expression or activity in human intestinal biopsies as well as cell lines.

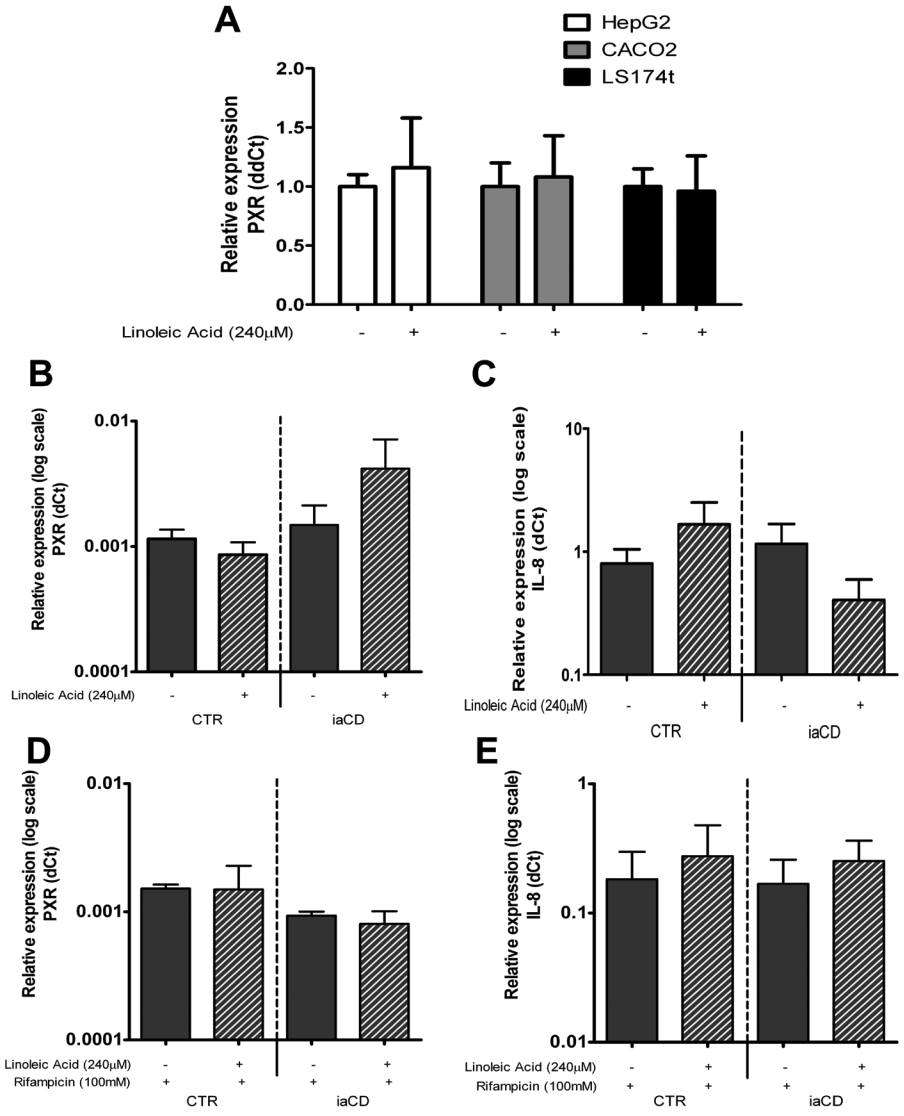


Figure 4: HNF4α stimulation to increase the PXR expression.
A) Cancer cell lines stimulated with HNF4a ligand. Hepatocyte cancer cell line HepG2 and colon cancer cell line CACO2 and LS174t were stimulated with 240 mM linoleic acid for 18 h at 37°C. The graph represents the mean relative PXR expression per cell lines and with and without linoleic acid stimulation line, from two independent in duplo performed experiments. The error bar is SD.
B) PXR mRNA expression in biopsies stimulated with linoleic acid. Biopsies from two CD patients and three healthy individuals were stimulated with solvent (1% (v/v) PBS) or 240 mM Linoleic acid for 18 h at 37°C. The graph represents the mean PXR expression per group. CTR are the controls and iaCD are CD patients with quiescent disease. The error bar is SD.
C) IL-8 mRNA expression in biopsies stimulated with linoleic acid. Same methodology as in B) was used. The graph represents the mean IL-8 expression per group. The error bar is SD.
D) PXR mRNA expression in biopsies stimulated with rifampicin and linoleic acid. Biopsies from two different CD patients and three different healthy individuals were stimulated with 100 mM rifampicin and together with solvent (1% (v/v) PBS) or 240 mM linoleic acid for 18 h at 37°C. CTR are the controls and iaCD are CD patients with quiescent disease. The graph represents the mean PXR expression per group. The error bar is SD.
E) IL-8 mRNA expression in biopsies stimulated with rifampicin and linoleic acid. Same methodology as in D) was used. The graph represents the mean IL-8 expression per group. The error bar is SD.

Discussion

Active IBD is associated with an impaired immune response against microbial activation but concomitantly the increased bacterial load elicits a substantial xenobiotic challenge. As xenobiotic and inflammatory signalling are to a certain extent mutually exclusive[9, 11], this raises important questions as how the relative activities of the two pathways are fine-tuned during fulminate inflammation, especially in IBD. Here we demonstrate that both signalling pathways restrain each other's activity and that in the context of active IBD the PXR pathway is the rate-limiting factor for NF-κB dependent epithelial gene expression. As it is becoming clear that especially ileal IBD might largely be an epithelial disease, our observations have substantial consequences in our thinking of IBD and open the possibility that by targeting PXR signalling therapeutic benefit may be achieved in those patients in epithelial hyper activation of NF-κB signalling is an important factor for pathogenesis.

Earlier studies already demonstrated multiple links between the xenobiotic metabolism and inflammatory pathways[9-12, 15, 24], but did not address the importance of PXR signalling in limiting NF-κB-dependent signalling in IBD. As down modulation of NF-κB-dependent gene expression is probably a secondary response to xenobiotic stress, likely to facilitate regenerative responses. We were surprised to see that PXR emerged as the major rate-limiting step for epithelial NF-κB activation in active CD. Nevertheless, a recent study done in six CD patients with quiescent disease showed that PXR activation can reduce LPS-induced NF-κB activity in human IEC[10]. As such artificial inflammation induced by LPS stimulation may not necessarily relate to NF-κB signalling in active IBD, we assessed PXR effects in active IBD directly. As PXR levels directly negatively correlate with NF-κB, and rifampicin further reduces pro-inflammatory cytokine mRNA and protein expression, we conclude that PXR activity is the rate-limiting step for NF-κB activity in the epithelial compartment in IBD.

Consistent with a critical role for PXR in constraining epithelial inflammatory responses are the genetic studies that link genomic variation in PXR in susceptibility to IBD[14, 16]. Although no functional data is available, Dring et al. and Martinez et al. showed significant associations of two PXR promoter SNPs with Crohn's disease and ulcerative colitis[14, 16]. Two other SNPs were associated with Crohn's disease but not with ulcerative colitis. Although replication studies in different cohorts failed to show these associations[25]. Lack of association of the pregnane X receptor (PXR/NR1I2, based on the data obtained in the present study it is tempting to suggest that in certain patient populations aberrant PXR induction fails to control epithelial NF-κB induction predisposing to disease. Larger studies in other cohorts are essential to clarify the association between PXR polymorphisms and susceptibility to IBD. In addition, with advent of microbiome profiling, it should prove interesting to correlate our findings with certain variations of the microbiome. Understanding the exact interactions between patient genetics, microbiome may however be necessary to exploit our findings for

devising rational therapy for IBD based on modulation of PXR activity. Nevertheless, keeping this caveat in mind, the present study has shown that PXR activity is the major rate-limiting pathway constraining epithelial NF-κB activity in active IBD and implying modulation of PXR activity holds significant clinical promise in the management of IBD.

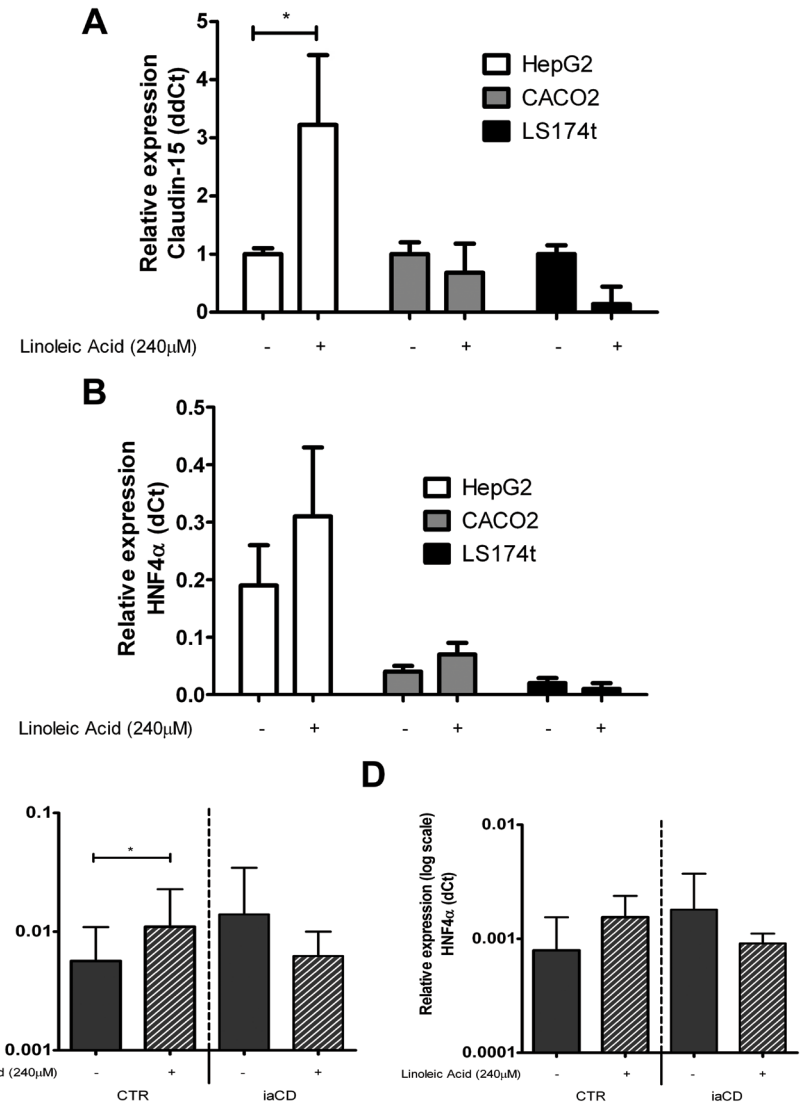


Figure S2: Expression and effect of HNF4α stimulation.
A) HNF4a target gene Claudin-15 expression in cancer cell lines. Hepatocyte cancer cell line HepG2 and colon cancer cell line CACO2 and LS174t were stimulated with 240 mM linoleic acid for 18 h at 37°C. The graph represents the mean relative Claudin-15 expression per cell lines and with and without linoleic acid stimulation line, from two independent in duplo performed experiments. The error bar is SD, * p<0.05.
B) HNF4a expression in cancer cell lines. Same methodology as in A) was used. The graph represents the mean expression of HNF4a per cell line. The error bar is SD.
C) HNF4a target gene Mep1a expression in biopsies. Biopsies from two CD patients and three healthy individuals were stimulated with solvent (1% (v/v) PBS) or 240 mM Linoleic acid for 18 h at 37°C. The graph represents the mean Mep1a expression per group. CTR are the controls and iaCD are CD patients with quiescent disease. The error bas is SD.
D) HNF4a expression in biopsies. Same methodology as in C) was used. The graph represents the mean HNF4a expression per group. The error bas is SD.

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Back to Index

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Addendum I: Bile salt and intestinal immunomodulation

Nuclear receptors such as the Pregnane X receptor (PXR/ SXR/ NR2L1), Farnesoid X receptor (FXR/ NR1H4), and the Retinoid X receptor (RXR/ NR2B2) are responsible for the detection of bile acids in the intestinal tract. High concentrations of bile acids especially of the secondary monohydroxic bile acid lithocholic acid can be toxic and even carcinogenic for the IEC[1]. Bile acid malabsorbtion is a common feature in patients with inflammatory bowel disease (IBD)[2]. Next to the toxic effects of bile, it also can have a positive effect on the disease progression. For example, tauroursodeoxicholic acid (TUDCA) can work as a chemical chaperone to diminish endoplasmic reticulum (ER) stress and also may contribute as an immunomodulator[3-6].

The aim of this additional study was to investigate the immunomodulation and the ER stress reducing capacity of TUDCA on human IEC and whether this effect is mediated by the presence of PXR. Firstly, we chemically induced ER stress in LS174t (siPXR) cells and their PXR expressing counterparts with tunicamycin. TUDCA was added to evaluate its ER stress modulating effect. The GRP78 and CHOP(primer sequences Table Ad S1) expression is 10 fold higher in both cell lines stimulated with tunicamyin, irrespective of the presence of TUDCA (Figure Ad.1A). In addition, the GRP78 and p-EIF2α protein expression is not different with TUDCA stimulation (Figure Ad.1B). Hence, in contrast to previous findings[6], the chemical-induced ER stress could not be reduced by TUDCA in LS174t cells, independent of the presence of PXR. IEC have membrane-bound receptors which are able to detect microbes and initiate an innate immune response[7, 8]. In vitro, a lysate of *E. Coli* (ELY) is able to induce transcription of NF-κB related, innate immunity key regulator, genes such as IL-8 and IL1β (Figure Ad.1C). There is no immune-modulating effect detected with TUDCA co-stimulation (Figure Ad.1C), irrespective of the presence of PXR. Thus, TUDCA has no ER stress- or immune-modulating effect on LS174t cells, without a significant role for PXR. Of note, the expression of IL-8 and IL-1β (NF-κB target genes) is again higher in the LS174t (siPXR) cells (Figure Ad.1C and Figure 3).

Table Ad S1: Primer sequences

Primer name	Sequence from 5' to 3'
GRP78_fw	ACCATCCCGTGGCATAAACC
GRP78_rev	CCTCCCACAGTTTCAATACC
CHOP_fw	GGAGCTGGAAGCCTGGTATG
CHOP_rev	CCTCAGTCAGCCAAGCCAGAG

Back to Index

IEC from IBD patients with active inflammation have an activated innate-immune system[9-11] and have activated ER stress pathways[12-14]. To investigate the immune modulating and ER stress reducing effect of TUDCA on human IEC, we stimulated freshly isolated intestinal biopsies from nine IBD (6 CD and 3 UC) patients and 4 healthy individuals with TUDCA (Table Ad S2). Biopsies from IBD patients with active disease have higher GRP78, IL-8 and IL-1β expression than the biopsies from inactive IBD patients and healthy individuals (Figure Ad.2A and Ad.2B). The GRP78 higher expression is not modulated in the accompanying biopsies stimulated with TUDCA (Figure Ad.2A). However, although not significant, the expression of the cytokines IL-8 and IL-1β is slightly reduced in the TUDCA stimulated biopsies. In conclusion, the ER stress signals are not impeded in the biopsies stimulated with TUDCA, however there is a trend in immune modulation by TUDCA in the freshly isolated biopsies. These findings needs further exploration, but already give new insights in the possible implication of conjugated bile acids in current therapy for IBD patients.

Table Ad S2: Baseline characteristics of patients treated with rifampicin

	CD	Control
Total number of patients	19	8
Mean age, yr (SD)	44 (16.6)	62 (3.5)*
Gender (M/F)	5/14	3/4
Mean duration of disease, yr (SD)	13 (8.8)	-
# Smoking (%Yes)	4 (25)	2(33)
# Familiar IBD (%Yes)	5 (36)	-
Concomitant medication:		
- none	1	-
- aminosalicylates	3	-
- corticosteroids	4	-
- immunosuppressives	9	-
- biological	5	-
# Biopsies colon	106	36

* The healthy controls are significantly older than the CD patients. p=0.002

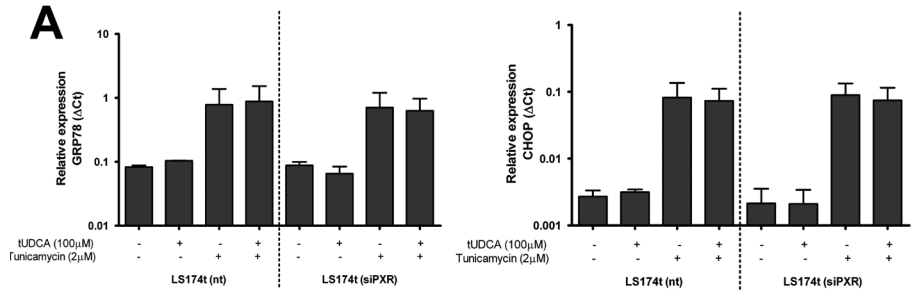

Figure Ad1: Effect of TUDCA on ER stress and innate immune signals in cell lines.
A) ER stress related mRNA expression. LS174t(nt) and LS174t(siPXR) cells where stimulated with tunicamycin (16 h) with or without TUDCA (18 h). The relative mRNA expression of GRP78 and CHOP from two independently in-duplo performed experiments is presented in these graphs. The error bar is SD.
B) ER stress related protein expression. Representative blots of GRP78 and p-EIF2α are presented. Similar methodology as is A) was used.
C) Cytokine mRNA expression. LS174t(nt) and LS174t(siPXR) cells where stimulated with E. Coli lysate (ELY, 16 h) with or without TUDCA (18 h). ELY is derived from a centrifuged 50 mL o/n E. Coli culture, where after the bacteria pallet was lysed in 500 μL dH_2O using five freezing-thawing cycles and sonication. The relative mRNA expression of IL-8 and IL-1β from two independently in-duplo performed experiments is presented in these graphs. The error bar is SD.

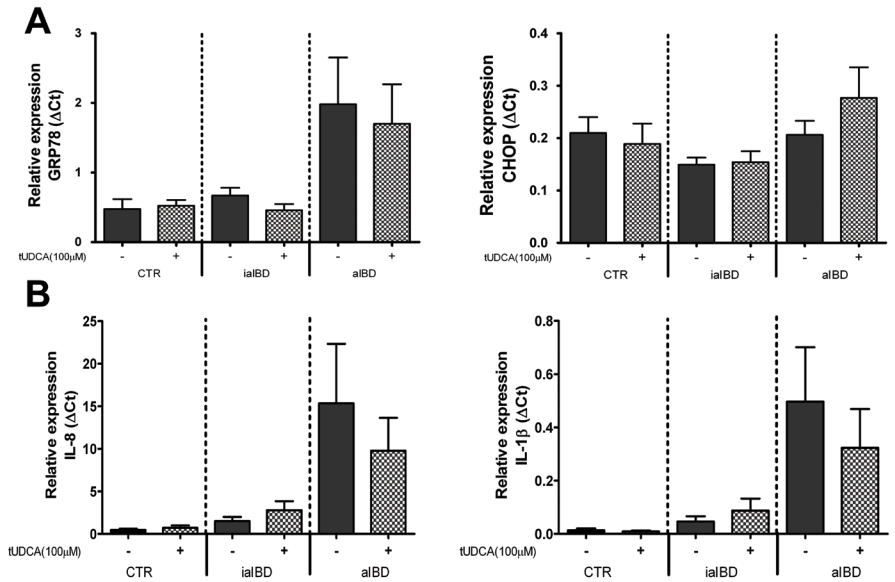

Figure Ad2: Effect of TUDCA in freshly isolated biopsies
A) mRNA ER stress signals in biopsies. Freshly isolated biopsies from healthy individuals (CTR), IBD patients with quiescent disease (iaIBD), and IBD patients with active disease where stimulated with TUDCA or solvent (18 h). The average relative mRNA expression of GRP78 and CHOP is presented in these graphs. The error bar is SD.
B) mRNA cytokine expression in biopsies. Similar methodology as is A) was used. The average relative mRNA expression of IL-8 and IL-1β is presented in these graphs. The error bar is SD.

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IV

High Pregnane X Receptor Expression in Human Intestinal Cancer

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Abstract

Patients with long standing inflammatory bowel disease (IBD) are known with an increased risk for developing neoplastic progression. Nuclear receptors such as, the Pregnane X Receptor (PXR) are involved in detoxification and chemotherapy resistance processes in intestinal epithelial cell (IEC). In addition, PXR expression also mediates cancer cell proliferation and survival. In this study we investigated the expression of PXR during neoplastic progression in patients with IBD, and studied the function of PXR in intestinal cancer cell proliferation and survival. PXR was assessed immunohistochemically (IHC) in colonic tissue samples of; 9 healthy individuals, 61 IBD patients, 10 IBD patients with low-grade dysplasia, 8 IBD patients with high-grade dysplasia, 15 IBD related colorectal cancer (CRC) patients, 5 patient with sporadic polyps, and 14 with sporadic CRC. Similar PXR IHC was performed in small bowel tissue specimens of; 17 healthy individuals, 29 Crohn's disease patients, and 7 patients with Crohn's disease related adenocarcinoma. PXR dependent cell growth and chemotherapy sensitivity was investigated *in vitro* using a PXR knockdown CRC cell line LS174t (siPXR) and its PXR-expressing counterpart LS174t (nt). High PXR expression was found in neoplastic IEC but not in normal or inflamed IEC, which was not related to the presence of IBD or intestinal location. Loss of PXR expression in LS174t (siPXR) cells increases the proliferation and decreases high cell-density survival, but the chemotherapeutic sensitivity was independent of PXR expression. PXR is abundantly present in human intestinal cancers, irrespective of the cancer origin or intestinal location. High PXR expression reduces the cancer cell proliferation, but improves high cell-density survival *in vitro*. As such, PXR expression in cancer cells is sustaining

Introduction

Intestinal epithelial cells (IEC) are well equipped with a variety of protection mechanisms against toxic luminal contents. When toxins enter the cell, they are detected by xenobiotic specific nuclear receptors (NR) such as, the Pregnane X Receptor (PXR)[1, 2]. After binding a specific ligand, PXR hetro-dimerizes with the Retinoid X Receptor and translocate to the nucleus to promote transcription of genes involved in the xenobiotic metabolisation and clearance[2]. Conversely, this efficient process of cellular clearing could also be detrimental i.e. for patients with cancer, where increased PXR activity is related to chemotherapy resistance[3, 4]. Proteins involved in chemotherapeutic agent transport (MDR1, ABCG2) and metabolisation (CYP3a4, Sult1A) are directly regulated by PXR[5, 6].

Deregulation in de PXR mediated xenobiotic metabolism and clearance are described during inflammatory lesions such as in IEC of patients with active inflammatory bowel disease (IBD)[7, 8]. Patients with IBD suffer from chronic intestinal inflammation, which comprises of two main groups; Crohn's disease (CD) and ulcerative colitis (UC). The loss of PXR-mediated detoxification during active inflammation could enhance the cellular sensitivity to, lethal build-up of xenobiotic compounds, which undoubtedly increases change of DNA damage and even neoplastic progression[8]. Moreover, IBD is associated with an increased risk of developing colorectal cancer (CRC). The risk for developing CRC is increasing by 0.5-1% every 8-10 years after IBD diagnosis and is depending on the age of onset[9]. Whereas only 1.5% of all CRC-cases are caused by IBD, the mortality rate of patients with IBD related CRC is dramatically higher than in sporadic CRC[10]. There are clear etiologic differences between sporadic CRC and IBD-CRC. Sporadic CRC arise from earlier formed polyps where IBD-CRC is raised from flat intestinal epithelium. Typical IBD-CRC originates from long standing IBD with normal looking IEC which can transform to low-grade dysplastic (LGD) epithelium, that can further evolve to high-grade dysplasia (HGD) and ultimately in a carcinoma[11].

Recent studies describe that non-activated PXR is also directly involved in other cellular processes such as, proliferation[12, 13], survival[14], and inhibition of inflammatory pathways[15]. However, there are contradictory results about the exact function of PXR in cancer cell proliferation and survival. PXR is described to be anti-apoptotic[16] and increases cancer cell growth[17] in human and experimental colorectal cancers, whereas other studies have found that PXR induces apoptosis in breast cancer cells[14] and is inhibiting the cancer cell proliferation[13]. Since PXR is involved in cancer cell chemotherapy resistance, survival and proliferation, it is important to determine the exact expression and function of PXR in human inflammation-related and non-inflammation related CRC. Since chemotherapy is focused on high proliferative cells, it is also important to examine the function of PXR expression during chemotherapy. In this study we investigated the involvement of PXR in the proliferation and survival of IEC during neoplastic progression in IBD patients. We measured the expression of PXR in human intestinal non-neoplastic and neoplastic

Back to Index

tissue. In addition, we evaluated the cellular involvement of PXR expression in proliferation and chemotherapeutic agent resistance.

Table S1: Baseline characteristics colonic tissue specimens

	Controls	Inactive IBD	Active IBD	LGD	HGD	IBD related CRC	Sporadic Polyp	Sporadic CRC
Total number of patients	9	30	31	10	8	15	5	14
Crohn's disease (n)	-	12	13	1	2	6	-	-
Ulcerative colitis (n)	-	18	18	9	6	9	-	-
Mean age, yr (SD)	41 (16)	53 (16)	43 (16)*	55 (14)	47 (11)	55 (16)	48 (19)	72 (12)**
Sex (M/F)	4/5	12/18	17/14	7/3	5/3	10/5	5/0	7/7
Mean duration of disease, yr (SD)	-	22 (10)	15 (11)*	20 (12)	18 (9)	25 (9)	-	-
Concomitant medication [a]:								
- none	-	6	5	3	1	0	-	-
- aminosalicylates	-	16	18	5	5	6	-	-
- corticosteroids	-	5	11	3	6	9	-	-
- immunosuppressives	-	9	9	1	1	7	-	-
- biologicals	-	2	2	0	0	0	-	-

[a] Data is missing of 2 patients with IBD related CRC

* IBD patients in the group of active colitis were significantly younger and had a shorter duration of disease at the time the biopsies were collected compared to the group of inactive IBD. p=0.001 and p=0.001, respectively

** Patients in the group of sporadic colorectal cancer were significantly older at time of diagnosis compared to the patients with BD related colorectal cancer. p=0.002

Methods

Patient selection

Formalin fixed paraffin embedded (FFPE) colonic tissue specimens were collected from nine healthy controls and 108 patients: 30 with inactive IBD (iaIBD), 31 with active IBD (aIBD), 10 with low grade dysplasia (LGD), 8 with high grade dysplasia (HGD), 15 with IBD related CRC (IBD-CRC), 5 with sporadic polyps, and 14 with sporadic CRC. FFPE small bowel tissue specimens were collected from 17 different healthy individuals and 36 different patients: four with inactive CD, 25 with active CD, and 7 with adenocarcinoma. The demographic patient characteristics are described in Tables S1 and S2.

Histology

An expert pathologist assessed the inflammation and dysplasia score for all selected tissue specimens. The FFPE tissue sections were immunohistological stained for PXR (Biolegend) as described before[18]. The expression of PXR in IEC was determined by the immune reactivity intensity of the staining by two independent observers. Slides with intense staining in >80% of the IEC was scored as positive, staining in 20-80% was scored as mild and <20% positive IEC was scored as negative.

Cell lines and siRNA

Colon carcinoma cell line LS174t was used in all experiments. The cells were cultured using standard culturing conditions and needed two passages a week. To assess PXR involvement in cell growth a PXR knock down cell-line was created using siRNA constructs against PXR (LS174t (siPXR)), as described before[19]. LS174t cells transfected with non-target siRNA (LS174t (nt)) were used as control.

Table S2: Baseline characteristics small bowel tissue specimens

	Controls	Inactive Crohn's disease	Active Crohn's disease	Adeno-carcinoma
Total number of patients	17	4	25	7
Mean age, yr (SD)	45 (15)	41 (19)	42 (17)	61 (9)**
Sex (M/F)	8/9	3/1	10/15	5/2
Mean duration disease, yr (SD)	-	17 (14)	10 (8)*	-
Concomitant medication:				
- none	-	1	5	-
- aminosalicylates	-	-	1	-
- corticosteroids	-	-	7	-
- immunosuppressives	-	1	10	-
- biologicals	-	3	8	-

* CD patients in the group with active disease had a significantly shorter duration of disease at the time the biopsies were collected compared to the group of inactive colitis. p=0.01

** Patients with a sporadic small bowel adenocarcinoma are significantly older that the controls, inactive CD, and active CD patients.

Growth assays

The LS174t(nt) and LS174t(siPXR) cells were seeded in a 96 well plate, with identical concentrations, and cultured for indicated time points using normal culture conditions. Cell viability was measured by incubating the cells for 3 hours with 4 mG/mL MTT (Sigma-Aldrich), there after the formed crystals were dissolved in 100 mL DMSO (Sigma-Aldrich). The colour intensity of the DMSO solution was measured using an ELISA plate reader at 490nm and 595nm (BioRad). The soft agar culturing technique was used to assess the colony-forming capacity. Sterile electrophoresis grade agarose was dissolved in warm standard culture medium in a final concentration of 1.5% and 0.5% (w/v). In a 6 well plate, a single cell suspension (3000 cells per well) was taken up in a liquid 0.5% agarose solution and directly poured on the already solidified 1.5% bottom layer. After cooling down to RT 2 mL standard culture medium was added to cover the agarose layers. Every other day the liquid medium was refreshed. Either 100 μM rifampicin or solvent (DMSO 0.1% v/v) was added to the medium for this assay. Starting from day 6 after seeding, images where taken and the colony diameter (pixels) were measured using the Nikon Nis Elements® for windows software.

Chemotherapeutic agent resistance assay

To test chemotherapy resistance the LS174t (nt) and LS174t (siPXR) cells were stimulated with different concentrations 5-Fluoroacil (5-FU). The cells were seeded in a 96 well plate with 10000 cells per well in four different wells per 5-FU concentration. At four time points after adding 5-FU (24, 48, 72 and 96 h) the cell viability was measured using the MTT assay as described before. The mRNA expression of genes involved in PXR mediated detoxification-pathways was measured in both cell lines cells seeded in a 12 well plate stimulated with 15 μM 5-FU for 72 h.

Table S3: Primer sequences

Primer name	Sequence from 5' to 3'
GapdH_fw	GCATTGCCCTCAACGACCAC
GapdH_rev	CCACCACCCTGTTGCTGTAG
Cyp3a4_fw	CAGGAGGAAATTGATGCAGTTTT
Cyp3a4_rev	GTCAAGATACTCCATCTGTAGCACAGT
Sult1a_fw	GCACCCACCCTGTTCTCTAC
Sult1a_rev	ACCACGAAGTCCACGGTCTC
ABCG2_fw	CAGCCGTGGAACTCTTTGTG
ABCG2_rev	CACTCTGACCTGCTGCTATG
MDR1_fw	TTTGGCCATCAGTCCTGTTC
MDR1_rev	CTAATTGCTGCCAAGACCTC
PXR_fw	ATGGCAGTGTCTGGAACTAC
PXR_rev	CAGTTGACACAGCTCGAAAG

RNA isolation and RT-PCR

For mRNA isolation the cultured cells were lysed in TriPure (Roche) and mRNA was isolated as described by manufactures protocol. cDNA was created using the iScript cDNA synthesis kit (BioRad) according manufactures description. Quantitative PCR was performed, as described before[20], for the genes as described in the figure legends. Primer sequences are described in Supplementary Table 3.

Statistics and software

All the graphs and the statistical analyses were performed using the Graphpad Prism 5.0 software package for Windows. Data on different patient groups were compared using the Mann-Witney U test with Kruskal-Wallis post test and the cell viability assays were compared using a students T-test. A two-tailed p value <0.05 was accepted as statistically significant. Images were composed using Adobe Photoshop CS6.

Back to Index

Results

Increased PXR expression in neoplastic intestinal tissue

To evaluate the PXR expression in human intestinal biopsies we stained the tissue samples for PXR expression. The tissue specimens from healthy individuals and patients with inactive IBD, active IBD and LGD do not have detectable PXR protein expression in their IEC. Similarly, no PXR expression was detected in the biopsy samples taken from the small bowel from healthy individuals or CD patients with inactive or active inflammation (Figure 1A). The absence of PXR expression was unrelated to disease phenotype, disease location, duration of disease, or gender. Patients with HGD, IBD-CRC, sporadic polys and sporadic CRC show abundant PXR expression in their IEC, irrespective of intestinal location (Figure 1A). In colonic biopsies 80-90% of the specimens with no dysplasia or LGD where scored as negative for PXR, whereas 60% of the biopsies with HGD or polyp biopsies where scored with mild PXR expression ($p<0.01$) and even 70-90% of the colonic cancer tissue specimens where scores with positive PXR expression ($p<0.001$, Figure 1B). Similar to these results, 90-100% of the non-neoplastic small bowel tissue specimens were graded as negative, whereas 60% of the slides with small bowel located adenocarcinoma were scored as positive for PXR ($p<0.001$, Figure 1C). Thus, PXR expression is undetectable in non-neoplastic intestinal tissue but is detectable in pre-neoplastic and intestinal cancer tissue independent of cancer etiology.

PXR impedes cancer cell proliferation in-vitro

To investigate the effect of PXR expression on the proliferation rate and survival in IEC, a stable PXR knock down (siPXR), was created using the LS174t cell line. From day 11 the number of cells was significantly higher in the LS174t (siPXR) compared to the LS174t (nt) cells ($p<0.001$, Figure 2A), suggesting enhanced proliferation of the siPXR cells. To examine to role of PXR in a high cell-density setting, we seeded the cells at a 16x higher concentration. Although the LS174t (siPXR) cells still grew faster, they also had a strongly reduced survival from day 13 ($p<0.001$, Figure 2B). The LS174t (siPXR) colony size, measured during the soft agar growth assay, was bigger than the LS174t (nt) cells at day 6 and 7 after seeding the cells ($p<0.05$, Figure 2C). This growth advantage was diminished and from day 18 the colonies of the LS174t (nt) cells were bigger than the LS174t (siPXR) cells ($p<0.001$, Figure 2C). An identical growth pattern was found in the cells stimulated with rifampicin (Figure 2D). Only in the PXR expressing cells, LS174t (nt), stimulated with rifampicin form bigger colonies than the non-stimulated cells at day 22 ($p<0.01$, Figure 2E). In total, the LS174t (siPXR) cells formed approximately 100 colonies per well more than the LS174t (nt) cells ($p<0.01$, Figure 2F). Representative images of the formed colonies, per analysed day, are presented in Figure S1.

So, although the expression of PXR is impeding the cancer cell proliferation, it is increasing the high cell-density survival of these cells.

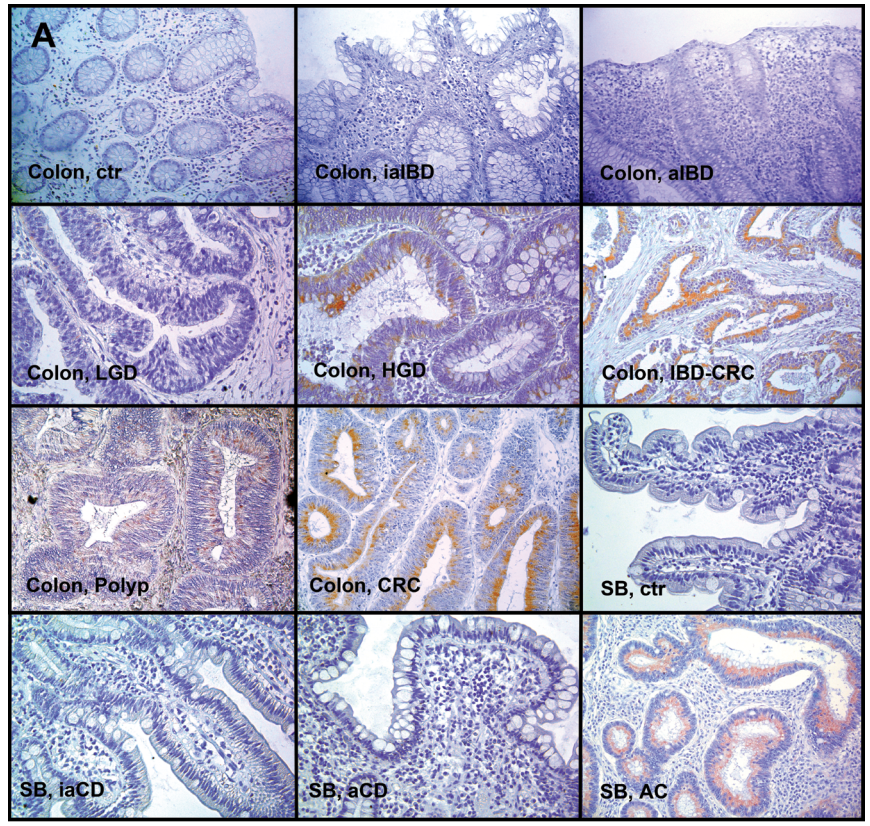

Figure 1: PXR expression in intestinal tissue specimens

A) FFPE Intestinal tissue specimens immunohistochemically stained for PXR. Red-orange immune-precipitate indicates PXR expression. These images are representative images of intestinal tissue specimens from the colon and small bowel. Ctr are controls, iaIBD are quiescent IBD patients, aIBD are IBD patients with active disease, LGD are biopsies with low-grade dysplesia, HGD are biopsies with high-grade dysplasia, IBD-CRC are biopsies with an IBD related colorectal cancer, Polyp are biopsies from sporadic polyps, CRC are biopsies with sporadic colorectal cancer, SB ctr are biopsies from the small bowel of controls, SB iaCD are small bowel biopsies from quiescent CD patients, SB aCD are small bowel biopsies from CD patients with active disease, SB AC are small bowel biopsies from sporadic small bowel adenocarcinomas. Original magnification 200x.

B) PXR expression score per group of colonic tissue specimens. Ctr n=9, iaIBD n=30, aIBD n=31, LGD n=10, HGD n=8, IBD-CRC n=15, polyps n=5, sporadic CRC = 14.

C) PXR expression score per group of small bowel tissue specimens. Ctr n=17, iaCD n=4, aCD n=25, adenocarcinoma n=7.

B) and C) All tissue specimens were scored for PXR expression by two independent observers in a blinded fashion. If 0-20% of the IEC where positive for PXR the slide was scores as negative, 20- 80% was scored as mild and 80-100% was scored as positive. The increase in PXR expression was compared using the organ specific controls. Mann–Whitney U tests was used for statistical analysis. (*$p<0.05$, **$p < 0.01$, ***$p<0.001$).

Back to Index

Chemotherapeutic agent resistance

We measured the cell viability in LS174t (nt) and LS174t (siPXR) cells after 5-FU stimulation to investigate the involvement of PXR on chemo resistance in IEC. In both cell lines the metabolic activity was decreased by 50%, compared with the control 72 h after stimulation with 60 μM 5-FU (Figure 3A). There was no difference in survival between the two cell lines in any of the used concentrations (Figure 3A). Besides, chemotherapeutic agents are known to increase the expression of detoxification genes. As shown in Figure 3B, 5-FU increases the expression of detoxification-associated genes such as Cyp3a4 ($p<0.001$), ABCG2 ($p<0.001$), Sult1a ($p<0.05$) independent of PXR. The only difference that could be detected was the expression of MDR1, which was only upregulated in LS174t (nt) cells ($p<0.01$). Our finding that Cyp3a4 mRNA expression was not increased in the LS174t (siPXR) cells after the stimulation with the specific PXR ligand rifampicin (Figure 3D) further confirms that the 5-FU-mediated upregulation is PXR independent. Hence explaining why there are no detectable differences in chemotherapeutic agent resistance between the PXR expression and non-PXR expressing cells *in vitro*.

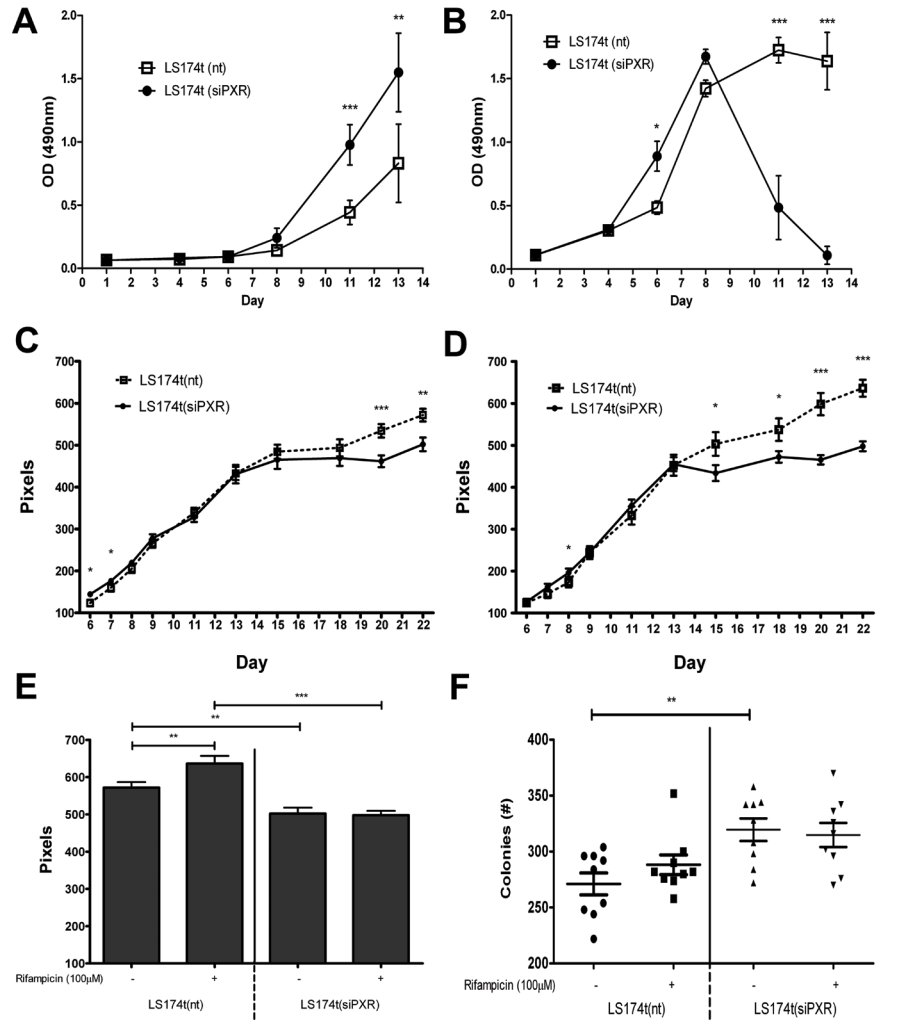

Figure 2: PXR mediated cell proliferation

A) MTT assay on LS174t (nt) and LS174t (siPXR) cells. For both cell lines a 100 μL cell suspension of $1.6E^4$ cells per mL was added in a 96 well plate. At indicated time points the cell viability was measured by incubating the cells for 3 hours with 4 μG/μL MTT. The formed crystals were dissolved in 100 μL DMSO. An ELISA reader using the wavelengths 490nm and 595nm measured the colour intensity of the DMSO solution. This graph represents the average of four wells per experiment of three independent experiments. Error bar is SD, $^{**}p < 0.01$, $^{***}p<0.001$.

B) MTT assay on LS174t (nt) and LS174t (siPXR) cells. Similar methodology was used as in A), only this time 100 mL cell suspension of $2.5E^5$ cells per mL were used. This graph represents the average of four wells per experiment of three independent experiments. Error bar is SD, $^{*}p < 0.05$, $^{***}p<0.001$.

C) Soft agar growth assay. LS174t (nt) and LS174t (siPXR) cells were single cells seeded in a 6 well plate embedded in 0.5% agarose dissolved using standard culture medium with 0.1% (v/v) solvent (DMSO) and incubated for in total 22 days using standard culturing conditions. Each time point, at the exact same spot, one picture per well was taken and the colony diameter was measured (pixels). The graph represents an average diameter of the 4 largest colonies per picture, three wells per cell line per experiment and three independent experiments. Error bar is SEM, $^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

D) Soft agar growth assay with rifampicin. Similar methodology as in C) was used, only now 100μM rifampicin was added. Error bar is SEM, $^{*}p < 0.05$, $^{***}p<0.001$.

E) Soft agar growth colony size after 22 days. The average colony diameter (pixels) per cell line, per condition is presented in this graph. Error bar is SEM, $^{**}p<0.01$, $^{***}p<0.001$.

F) Total amount of formed colonies. For the LS174t (nt) and LS174t (siPXR) cells the total amount of formed colonies per well was counted. The graph represents an average amount of colonies per well, three wells per cell line per experiment and three independent experiments. Error bar is SEM, $^{**}p<0.01$.

Back to Index

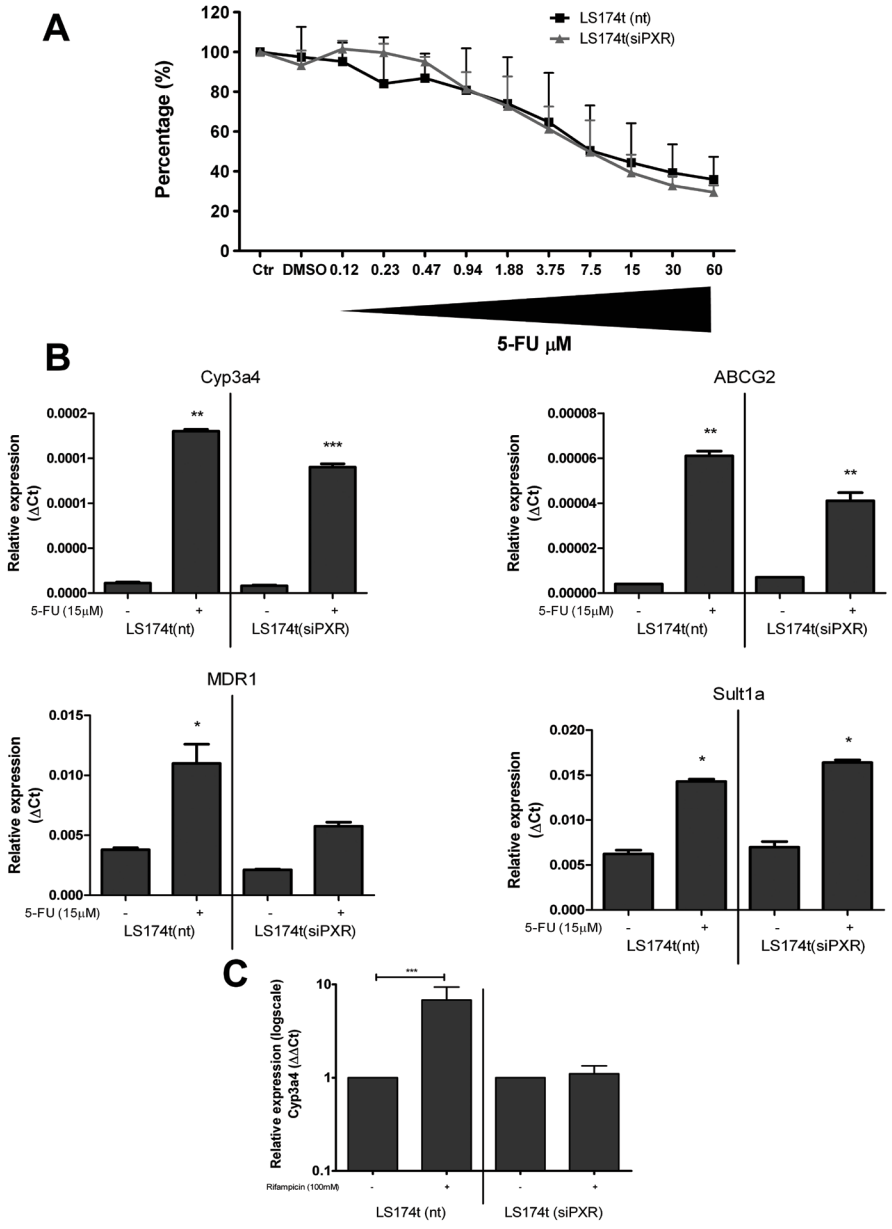

Figure 3: Chemotherapeutic agent resistance

A) MTT assay on 5-FU stimulated LS174t (nt) and LS174t (siPXR) cells. Both cell lines were seeded in a 96 well plate in 10000 cells per well. The cells were stimulated with different concentrations of 5-FU for 72 h. Ctr is the control were nothing was added to these cells, DMSO is the control for the condition with the highest solvent concentration (0.5% v/v). This graph represents the average of six wells per experiment of two independent experiments. Error bar is SD.

B) The expression of multi drug resistant associated genes. The mRNA expression of the indicated genes was measured in the LS174t (nt) cells and the LS174t (siPXR) cells stimulated with 15 μM 5-FU for 72 h. These graphs represent the average of two independent experiments. Error bar is SD. The increase in expression was compared per cell line to the un-stimulated cells using a non-parametric t-test, *p < 0.05, ** p<0.01 and ***p<0.001.

C) PXR specific stimulation by rifampicin. LS174t (nt) and LS174t (siPXR) cells were stimulated with 100 μM rifampicin to test the specificity of the PXR knock down. This graph represents the average, of two independent experiments performed in duplo, mRNA expression of Cyp3a4. Error bar is SD, *** p<0.001.

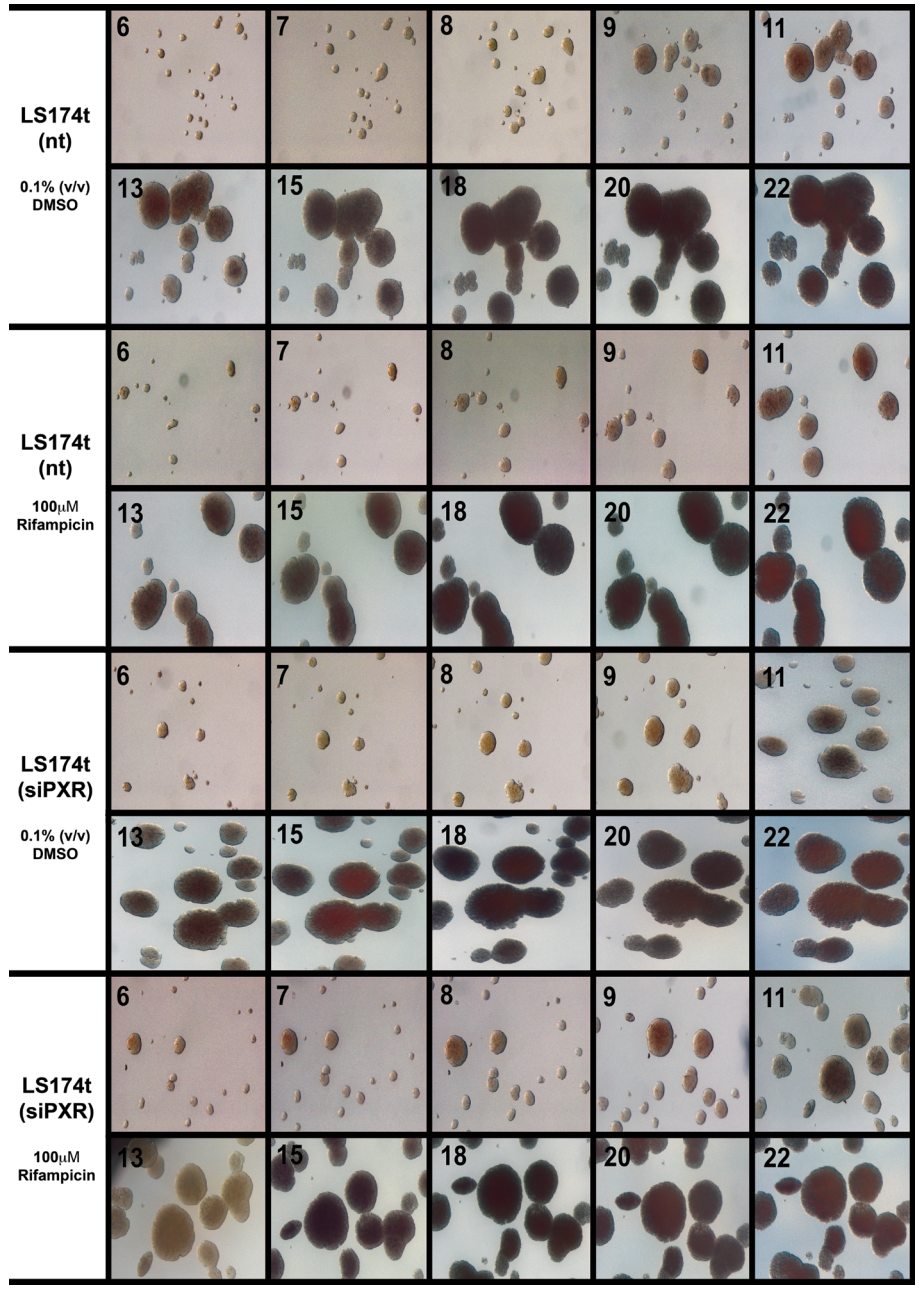

Figure S1: soft agar growth assay images.

LS174t (nt) and LS174t (siPXR) cells (3000 per well) were seeded in a 6 well plate using a 0.5% agarose medium solution on top of a 1.5% agarose in medium base layer. At indicated time points (number left upper-corner each image, days) an image was taken. The medium contains either 0.1% (v/v) DMSO or 100 μM rifampicin.

Discussion

There is no consistent literature concerning the expression and the function of PXR in human cancers[12-14, 16]. Therefore we investigated the expression and function of PXR in IBD related and non-IBD related CRC. In this study we are able to show that the PXR expression is increased in human neoplastic intestinal tissue specimens. In addition we found that although the cells that express PXR grow slower, they had increased high cell density survival. In addition, PXR expression does not play a role in the 5-FU chemotherapy resistance *in vitro*.

In a recent study by Ouyang et al., the authors concluded that the expression of PXR is absent in intestinal cancers and that the presence of PXR is inhibits the cell growth[13]. We also find decreased cell growth in PXR expressing cells in our study, but by contrast we detected high PXR expression in the neoplastic tissue samples. We cannot rule out that this difference is due to different antibodies used for the IHC staining. However, in line with our results another study also showed an abundant PXR mRNA expression in IEC of patients with CRC[3]. Moreover, we used the same antibody as was used for another study in our lab[18] showing that the used antibody is specific for the detection of PXR.

Cancer cells proliferate quickly and unlimited, but even more important is that the cancer cells survive longer than normal IEC, which can lead to tumour progression and metastasis[21]. The MTT and soft agar growth assay show that the LS174t (nt) cells seem to survive longer than the LS174t (siPXR) cells. This indicates that the PXR expression in cancer cells might be beneficial for the tumour. Stimulating PXR expressing cells with rifampicin increases the growth and likely prolongs survival of the cells even further, which is in line with earlier observations[16, 17]. Further research is needed to examine the exact role of PXR in cancer cell survival. Of more interest, actively reducing the PXR expression in cancer can be a promising therapeutic approach for patients with CRC.

PXR is also described to be a biomarker for neoplastic progression in patients with barrettes oesophagus[18]. In the biopsies with HGD, a precursor of IBD-CRC, already a mild increased PXR expression could be detected. Next to that, similar results were found in the polyp tissue-specimens, a precursor for sporadic cancer. We can conclude from this that PXR could also be a marker for neoplastic progression in intestinal tissue specimens. Despite of the nuclear PXR expression that was observed before[18], we could not detect nuclear expression of PXR in IEC. Probably the different luminal ligand composition of the various organs will influence the localisation of PXR. Besides, PXR also has a distinct cytoplasmic function, since that the cytoplasmic presence of PXR is already sufficient to execute PXR mediated cellular effects[15, 16]. In general, PXR expression in IEC is increased in neoplastic intestinal tissues and affects the proliferation and survival of these cells.

A chemotherapeutic agent such as 5-FU is a common therapy in patients with CRC. 5-FU inhibits the DNA replication of proliferating cells[22]. Despite the specificity of 5-FU, the response rate to this compound, if given as first line therapy, is just 10-15% and decreases even further in severe metastatic disease[22]. This lack of treatment response may be contributed by induced expression of multi-drug resistance proteins such as, ABCG2[23] which expression is correlated with PXR expression[24]. From our study we know that neoplastic IEC express PXR, PXR expression inhibits cell growth but prolongs cell survival. Hence, we hypothesised that the cancer cells without PXR expression respond better to the 5-FU treatment, since highly proliferative cells are more sensitive to 5-FU[22]. However, there is no difference in survival between the LS174t (siPXR) and LS174t (nt) cells by using 5-FU stimulation. The expression of genes associated with multi-drug resistance [23] was increased in both cell lines after 5-FU stimulation, suggesting that PXR is not the only NR involved in multi drug resistance, and not involved in 5-FU resistance[25]. Thus, solely reducing the PXR expression, in respect to chemotherapy resistance, might not be sufficient for intestinal cancer patients.

In conclusion, PXR is abundantly expressed in human intestinal cancers irrespective of the cancer origin or intestinal location. High PXR expression reduces the cancer cell proliferation, improves high cell density survival, but PXR expression is not influencing the chemotherapy resistance. In respect to the PXR mediated reduced cell survival, a therapeutically approach to inhibit the PXR expression in intestinal cancer is a promising area for further investigation.

Back to Index

IV

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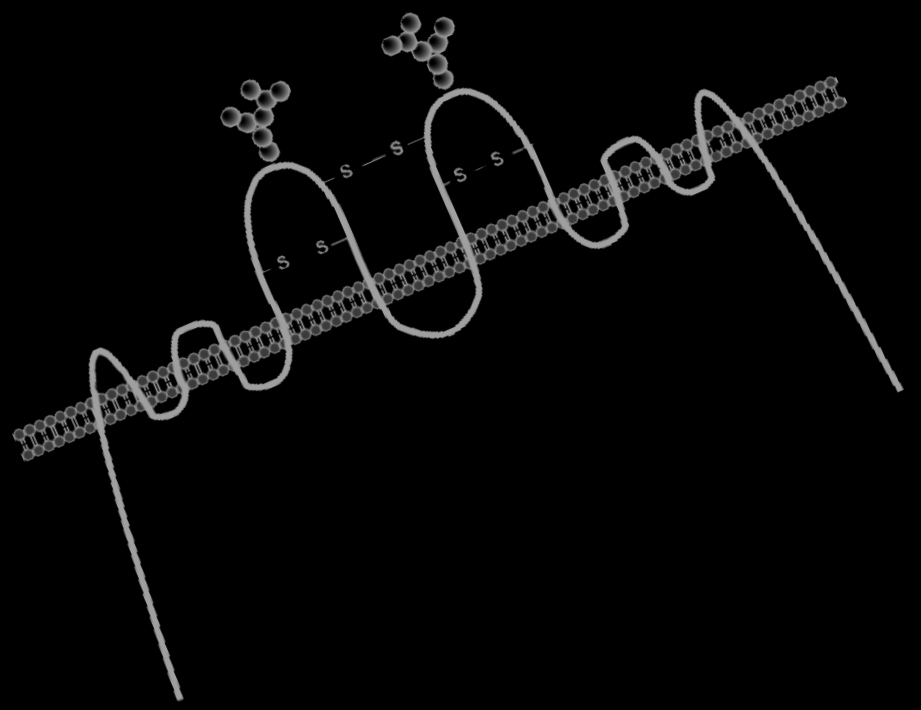
V

Impeded Protein Folding and Function in Active Inflammatory Bowel Disease



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Abstract

An area of 300 square meters of intestinal epithelial cells (IEC) is covering the entire intestinal mucosa. The IEC are equipped with membrane-bound and secreted proteins for protection against luminal xenobiotics, pathogens and commensal microbes. In patients with active inflammatory bowel disease the expression of these proteins, e.g. defensins and ABC-transporters like ABCG2 in IEC is decreased, leaving the underlining tissue less protected against luminal threats.

Correct endoplasmic reticulum (ER)-dependent protein folding is essential for the location and function of secreted and membrane-bound proteins. Inflammatory triggers, like cytokines and nitric oxide can impede protein folding causing unfolded proteins to accumulate inside the ER. As a result, the unfolded protein response is activated which than leads to ER stress. This protein-folding impairment affects several proteins with great importance in cellular protection against xenobiotics. In this review we discuss how inflammation may affect the protein folding leading to IEC malfunction.

V

Introduction

IECs (intestinal epithelial cells) and their products form a physical barrier between the host and the luminal content. They have to deal with the complex situation where they absorb nutrients and water, and also protect the host from commensal microbes, pathogens and toxic compounds. Various specific adaptations, such as tight junctions and microfolds, as well as specialized IECs such as goblet cells and Paneth cells, enable these cells to carry out their complex task[1].

To protect themselves from toxic compounds, such as xenobiotics, IECs are able to sense, degrade and actively transport various toxic compound out of the cell by specific membrane-bound exporters[2]. Mucosal disorders such as IBD (inflammatory bowel disease) are associated with a disturbance of this well-regulated intestinal homoeostasis[3]. One of the cellular processes that seem to be affected by inflammation is ER (endoplasmic reticulum)-dependent protein folding. The present review focuses on how inflammation may affect protein folding and function leading to ER stress followed by IEC malfunction.

ER stress

Secreted and membrane-bound proteins undergo a folding quality check before they are released from the ER. Un- or mis-folded proteins are extracted from the ER and degraded. This process is called ERAD (ER-associated degradation). However, when un- and mis-folded proteins accumulate inside the ER, the UPR (unfolded protein response) is activated. Upon UPR activation, three major intracellular pathways are activated, namely IRE1 (inositol-requiring enzyme 1), PERK PKR (double-stranded-RNA-dependent protein kinase)-like ER kinase and ATF6 (activating transcription factor 6). All three proximal UPR effectors start transcription of genes that are involved in reducing the unfolded protein load in the ER, e.g. ERAD. The mechanism of these three pathways have been described previously[4].

A proportion of proteins, translated in the ER, will always be misfolded due to the complexity of the protein folding process and in the case of high protein production demand. As such, cells with a high secretory activity, e.g. intestinal goblet and Paneth cells, have a constitutively activated ER-stress response. Hence, these cells are extremely sensitive to environmental changes, such as inflammation, which could affect protein synthesis or folding[5]. Interestingly, mutations in genes involved in the ER-stress response are associated with an increased risk for developing IBD[6]. Genetic defects in the ER-stress pathway may therefore contribute to a malfunction of these highly active secretory cells that play a crucial role in intestinal homoeostasis.

Back to Index

Intestinal inflammation, protein folding and ER stress

Patients with IBD, which includes Crohn's disease and ulcerative colitis, are at risk for a chronically inflamed intestinal tract. Active intestinal inflammation is associated with an increased intestinal levels of cytokines, chemokines[7,8] and nitric oxide (NO)[9]. These innate inflammatory responses, such as TNFα (tumour necrosis factor α) and ROS (reactive oxygen species), can directly affect protein folding[10]. At the same time, IEC stimulation is provoked by inflammatory cytokines[11], microbial product recognition by IECs[12] and secreted growth factors[13], which all may increase cellular protein synthesis. These triggers increase the protein production, which can be catastrophic for normal goblet and Paneth cells[14], leading to intestinal inflammation. A large number of high-impact research papers and reviews have been published on the molecular pathogenesis of inflammation provoked by ER stress, genetic predisposition for ER stress and the ability of ER stress to aggravate inflammation[4–6,14–16]. However, the effect of ER stress on protein expression and function in cells is less well known.

Inflammatory triggers that can provoke ER stress

Chaperones and heat-shock proteins mediate protein folding inside the ER. For example, GRP78 (glucose-regulated protein 78) binds and secures unfolded proteins inside the ER until they are properly folded[17]. The PDI (protein disulfide-isomerase) family catalyses the obligatory disulfide bonds within or between proteins[18]. These helper proteins are crucial for the ER to deal with the high protein concentration. A disturbance in the function of these helper proteins can cause unfolded proteins to accumulate and activate ER-stress pathways.

Interestingly, NO inhibits the function of PDI by S- nitrosylation of its catalytic domains, which leads to the accumulation of unfolded proteins inside the ER and subsequently activates ER stress[19,20]. Furthermore, NO is a small and reactive molecule that can affect protein folding through different approaches as well[21]. Activated innate immune cells in active IBD are associated with an increased NO concentration[9]. The inhibitory effect of high concentrations of NO on protein folding is considered to affect protein folding in IECs during IBD. Other inflammatory triggers such as bacterial cytotoxins can also affect protein folding and thereby induce ER stress[22].

As discussed above, protein synthesis in general is stim- ulated by chemokines, cytokines and bacterial compounds[11–13]. It is conceivable that this increased protein synthesis leads to the accumulation of unfolded proteins whereupon ER stress signals are activated.

Other well-described factors that can provoke proteins to misfold are glucose and oxygen depravation. N-glycosylation of proteins inside the ER is an important post-translational protein modification. N-glycosylation increases the stability and solubility of proteins and protects them from proteases. Before ER-dependent proteins pass through the ER quality check, a large group of linked sugar molecules are added to specific amino acids[23]. Active mucosal inflammation increases the need for nutrients and oxygen as activated immune cells are in considerable need of oxygen and nutrients to defend the mucosa from invading microbes[24]. The increased proliferation of IECs[25] also requires a significant amount of energy. As such, inflammation limits the amount of glucose thereby affecting correct protein folding[26].

In addition to this, intestinal inflammation results in oxygen deprivation, e.g. hypoxia and production of ROS such as NO. The epithelium is protected against inflammation- induced hypoxia by activating the transcription factor HIF1 (hypoxia-inducible factor 1)[27]. Nonetheless, in patients with active IBD, HIF1 is abundantly expressed[28], indicating that actively inflamed intestinal lesions are under severe hypoxia. The exact molecular role of hypoxia during inflammation has been described previously[24,29]. Protein folding inside the ER is dependent on an electron-relay system that requires oxygen as electron acceptor. Folding proteins such as PDI are dependent on oxygen as a terminal electron acceptor in the formation of protein disulfide bonds[30]. Interestingly, HIF1 induces the expression of proteins in the relay of protein disulfide bond formation, providing a link between hypoxia and UPR.

In summary, there are several inflammation-associated triggers that provoke impeded protein folding and eventually lead to UPR and ER stress and these can be found in Figure 1.

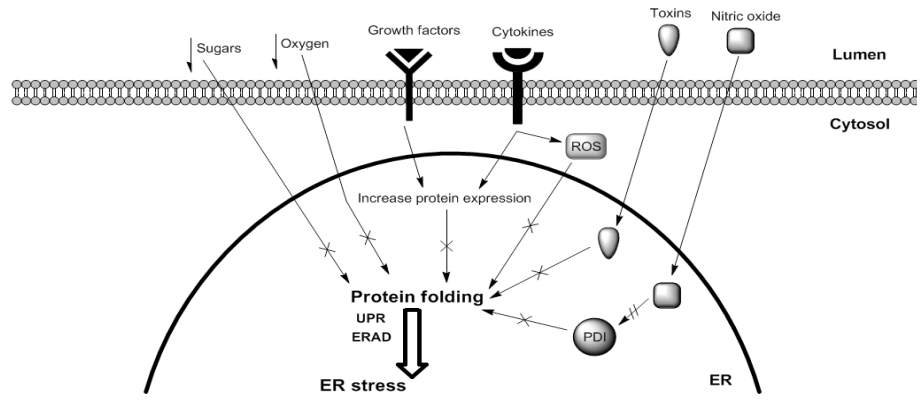

Figure 1. Schematic overview of inflammation-associated triggers which can provoke protein misfolding
ER-dependent protein folding in IECs of patients with active IBD is affected by several inflammatory triggers.

Effect of inflammation-induced ER stress

As mentioned above, different specialized proteins protect IECs from toxins present in luminal content. The function and expression of the membrane-bound exporters are depending on proper N-glycosylation and disulfide bonds[31,32]. It has been described previously that the expression of xenobiotic and bile acid transporters, such as P- glycoprotein MDR1 (multidrug-resistance 1)/ABCB1 (ATP-binding cassette transporter B1)[33] and ABCG2 (ATP-binding cassette transporter G2) BCRP (breast cancer resistance protein)[34,35], are decreased in mucosal biopsies from patients with active IBD. This process is linked to a decreased mRNA expression. Despite these findings, the reduced mRNA expression is unlikely to be sufficient to explain the complete loss of protein expression as was seen using immunohistological staining[34,35]. Inflammation-induced protein misfolding

Back to Index

(ER stress) provides a possible additional mechanism. As the creation of disulfide bonds is inhibited by NO[20,21] and these disulfide bonds are essential for the function of xenobiotic transporters, such as ABCG2[31,32], it can be assumed that these proteins are not properly folded and therefore reduced in expression during inflammation. A schematic overview of ABCG2 folding and misfolding is shown in Figure 2.

This reduction in transporter protein expression indicates that IECs with inflammation-induced ER stress are impeded in their protection from toxic bile acids and other luminal toxic compounds. As a result, the risk for genomic DNA damages and thus eventually neoplastic progression is increased.

In addition to the inflammation-associated decrease in xenobiotic transporter expression, other proteins are also decreased during inflammation. Two non-intestinal examples of affected protein folding induced by NO have been found. Insulin, a small excreted protein with at least three disulfide bonds, is reduced in expression by an increased NO concentration triggered by inflammation[36]. A similar reduced protein excretion of vascular endothelial growth factor, a protein with at least five disulfide bonds, has been described under the influence of high NO concentrations[37]. Both unrelated excreted proteins are reduced in expression because of impeded protein folding in the presence of a high NO concentration.

Diarrhoea can be a symptom of patients with IBD, and is caused by changes in electrolyte transport during intestinal inflammation. This is underlined by the fact that membrane- bound sodium transporters and their regulating proteins are decreased in expression during active IBD[38]. Moreover, these sodium transporters, e.g. Na^+/H^+ exchangers and epithelial Na^+ channel, require multiple aminoacid modifications such as N-glycosylation. Nutrition deprivation in the intestinal inflammatory lesions apparently reduces the ability to undergo N-glycosylation of ER-dependent proteins, whereupon unfolded proteins accumulate and ER stress is activated[26]. Thus, upon glucose deprivation, these sodium transporters are likely to be misfolded and degraded from the ER. The inflammation-associated decrease in protein expression of Toll-like receptor 5[39] and monocarboxylate transporter 1[40] could be explained by the same mechanism.

Highly active secretory cells, e.g. Paneth cells, are sensitive to changes in protein folding mechanisms because of their constitutive ER stress activation[14]. In addition to the production of mucus-soluble defensins and lysozymes, Paneth cells have important functions in maintaining the stem cell niche in intestinal crypts[41]. Consequently, inflammation-induced protein folding issues inhibit the regular function of Paneth cells and probably diminish intestinal stem cell homoeostasis. This will reduce the ability of a fast regenerative IEC seal in covering the inflamed intestinal mucosa, leaving the lamina propria exposed.

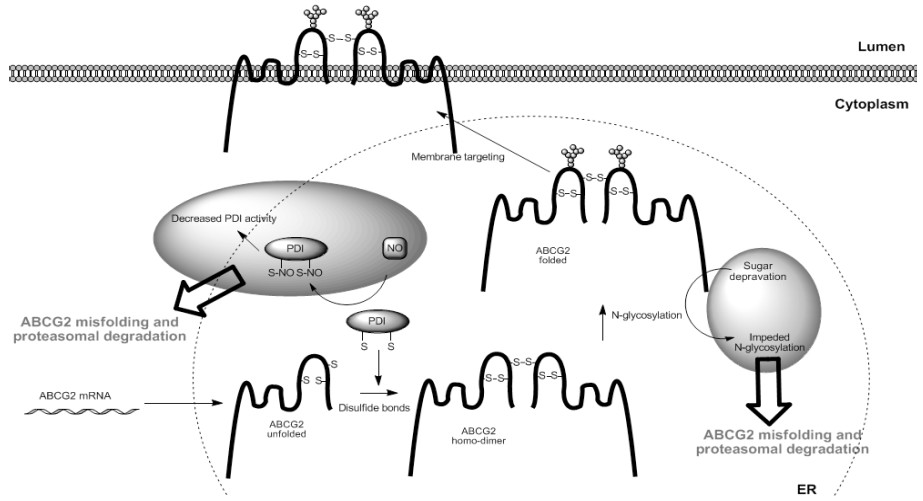

Figure 2. ABCG2 protein folding and misfolding
The function and cellular location of ABCG2 is dependent on proper protein folding. Protein modifications such as inter- and intra-molecular disulfide bonds and N-glycosylation are executed before ABCG2 is localized on the membrane. During active inflammation, the increased NO concentrations and low sugar concentrations affect ABCG2 protein folding whereupon improper folded ABCG2 will be degraded.

ER stress as therapeutic target for intestinal inflammation

Studies in metabolic diseases discuss different approaches to regulate ER stress[42]. In the management of IBD, however, there are almost no ER stress-regulating strategies proposed. From studies in metabolic diseases, chemical chaperones, such as 4-phenylbutyric acid, DMSO and bile acid conjugates of ursodeoxycholic acid, are found to provide protein stability and improve the ER protein folding capacity[43]. Inhibition of phosphorylation of the UPR downstream target eIF2α (eukaryotic initiation factor 2a) by a small molecule named salubrinal protects cells from ER stress- associated apoptosis[44]. Although these approaches are promising, their molecular effect on human disease remains unclear. In contrast, conventional therapy for IBD aims to get IBD patients in inflammatory remission. As such, it can be argued that, as a result of reducing inflammatory activity, fewer proteins will misfold and the stress inside the ER is diminished. Thus the question remains whether it is necessary to investigate the possibilities of reducing protein misfolding in IECs specifically for patients with IBD not suffering from a genetic defect in their ER-stress response.

Conclusions

Current knowledge provides insight into the substantial effects of impeded protein folding in inflammatory intestinal disease. Xenobiotic transporters such as ABCG2, but also excreted proteins such as lysosyme, are reduced in expression in IECs due to impeded protein folding.

Overall, much more research is needed to fully understand the consequence of impeded protein folding during active IBD. Questions such as why are proteins prone to misfold by high concentrations of NO and which other proteins are affected in expression by misfolding are interesting areas of research.

Back to Index

V

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Back to Index

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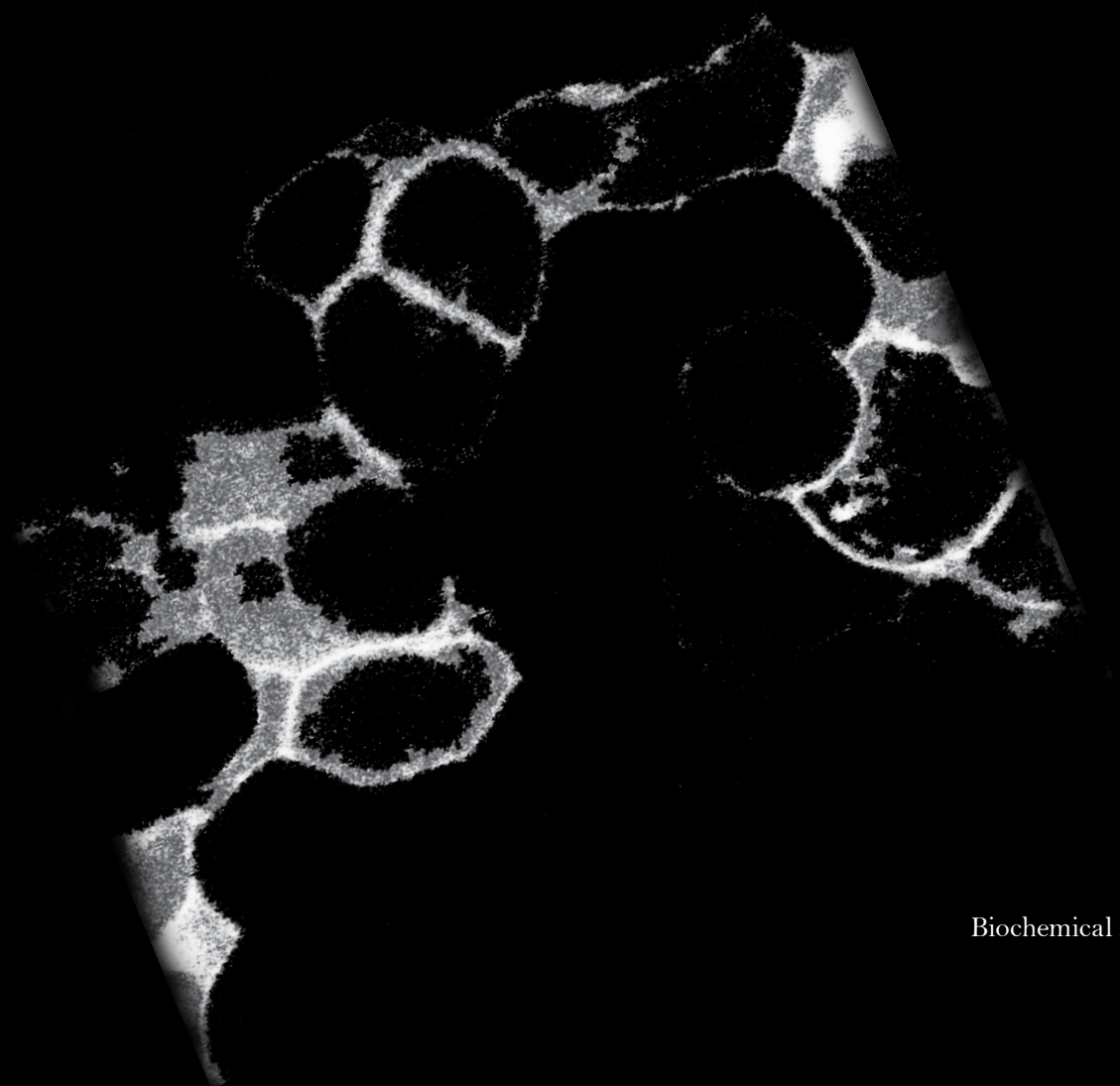
Back to Index

VI

Absence of ABCG2-Mediated Mucosal Detoxification in Patients with Active Inflammatory Bowel Disease is due to Impeded Protein Folding



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Abstract

Xenotoxic damage in inflammatory diseases, including IBD (inflammatory bowel disease), is compounded by reduced activity of the xenobiotic transporter ABCG2 (ATP-binding-cassette G2) during the inflammatory state. An association between the activation of the unfolded protein response pathway and inflammation prompted us to investigate the possibility that reduced ABCG2 activity is causally linked to this response. To this end, we correlated expression of ABCG2 and the unfolded protein response marker GRP78 (glucose-regulated protein of 78 kDa) in colon biopsies from healthy individuals (n = 9) and patients with inactive (n = 67) or active (n = 55) IBD, ischaemic colitis (n = 10) or infectious colitis (n = 14). In addition, tissue specimens throughout the small bowel from healthy individuals (n = 27) and from patients with inactive (n = 9) or active (n = 25) Crohn's disease were co-stained for ABCG2 and GRP78. In all biopsies from patients with active inflammation, irrespective of the underlying disease, an absolute negative correlation was observed between epithelial ABCG2 expression and GRP78 expression, suggesting that inflammation-dependent activation of the unfolded protein response is responsible for suppression of ABCG2 function. The link between the unfolded protein response and functional ABCG2 expression was further corroborated by live imaging of ABCG2-expressing cells, which showed that various inflammatory mediators, including nitric oxide, activate the unfolded protein response and concomitantly reduce plasma membrane localization as well as transport function of ABCG2. Thus a novel mechanism for explaining xenobiotic stress during inflammation emerges in which intestinal inflammation activates the unfolded protein response, in turn abrogating defences against xenobiotic challenge by impairing ABCG2 expression and function.

Introduction

The intestinal epithelium is a single cell physical barrier between the gut luminal content and mucosa, important for detection[1] and defence[2,3] against microbes. IECs (intestinal epithelial cells) are constantly exposed to a large variety of xenobiotic substances, including microbes and their products, as well as exogenous (*e.g.* drugs or food) and endogenous (*e.g.* bile acids) toxic substances present in the luminal content. IECs utilize various mechanisms to maintain epithelial integrity in the face of a xenobiotic challenge. They are protected against exogenous and endogenous toxic compounds by, among others, proteins from the ABC (ATP-binding-cassette) transporter family. This family includes P-glycoprotein/MDR1 (multidrug resistance 1)/ABCB1, MRP1 (multidrug-resistance protein-1)/ABCC1, MRP2/ABCC2 and BCRP (breast cancer resistance protein)/ABCG2[4,5]. In particular, the homodimeric xenobiotic transporter ABCG2, which specifically localizes to the IEC apical membrane, is widely considered to be especially important in combating luminal- derived xenobiotic stress[6,7].

Disruption of IEC integrity leads to inflammatory lesions, which are a hallmark of active inflammation in patients with IBD (inflammatory bowel disease). The appropriate response to such a disruption requires rapid mucosal healing by cell division, differentiation and maturation to restore barrier function[8,9]. Active inflammation in patients with IBD has been associated with a dramatically reduced expression of ABC transporters in IECs and the resulting diminished capacity to deal with luminal xenobiotic challenge is thought to interfere with the efficiency of regenerative responses[7,10–12]. Further compounding this problem is that several medications used in IBD are specific substrates of ABCG2. Hence understanding the mechanisms governing ABCG2 expression is highly relevant for devising improved therapy[13,14]. Nevertheless, the molecular mechanisms driving ABCG2 expression during inflammatory responses remain obscure at best.

Generally, excreted and membrane-bound proteins are translated in the ER (endoplasmic reticulum). If ER function is disrupted, proteins can accumulate inside the ER, and this can initiate a specific ER stress syndrome known as the unfolded protein response. Abnormalities in the ER stress response pathways have been associated with the pathogenesis of IBD[15–17]. Proper function of the ABCG2 protein depends on N-glycosylation and the formation of inter-/intra-molecular disulfide bonds[18,19]. These bonds are formed inside the ER by PDIs (protein disulfide-isomerases). Inflammatory conditions, such as increased nitric oxide production, can dramatically reduce the efficiency of these PDIs, which can initiate misfolding of various proteins, leading to ER stress[20,21]. As correct ABCG2 protein folding is necessary for its functionality, we hypothesized that mucosal inflammation in the setting of IBD alters proper ABCG2 protein folding and thereby reduces its apical expression. In the present study we describe a novel mechanism by which ABCG2 protein expression is decreased during active inflammation.

Back to Index

Methods

Patient samples

The present study was conducted with the approval of the Ethics Committee of Erasmus MC University Medical Centre, Rotterdam. Consent was obtained from all patients participating in the study.

Patient materials

FFPE (formalin-fixed paraffin-embedded) colonic biopsies were collected from nine healthy individuals and from 76 patients: 25 with CD (Crohn's disease), 36 with UC (ulcerative colitis), ten with ischaemic colitis and five with infectious colitis. Biopsies throughout the small intestine were collected from 17 other healthy individuals and 34 CD patients. For mRNA isolation, freshly frozen biopsies were collected from six healthy individuals and from 15 patients: eight with inactive IBD and seven with active IBD. To compare the ABCG2 protein expression throughout the large bowel of IBD patients, two samples per patient were selected from the right and left hemi-colon. To investigate whether the ABCG2 expression pattern was specific for IBD, biopsies from patients with ischaemic and infectious colitis were included. As controls, we obtained colon and small bowel biopsy specimens from patients without IBD or any other underlying disease. The demographic characteristics, number of biopsies per group, duration of disease and medication use are described in Tables S1–S3.

Immunohistochemistry and scoring

FFPE sections were immunohistologically stained using the primary antibodies BXP-21 anti-ABCG2 (1:300, Santa Cruz Biotechnology) and BiP (1:400, immunoglobulin heavy-chain-binding protein) anti-GRP78 (glucose-regulated protein of 78 kDa; Cell Signaling Technology) according to the manufacturers' protocol. The expression of ABCG2 and GRP78 in IECs was scored by the intensity over the whole slide by two independent observers, and discrepancies were re-assessed to come to a final agreement. Individual scores were used for statistical analyses, whereas they were combined into three subgroups for graphic representation. ABCG2-positive staining at the apical membrane of the epithelial cells was defined as positive if >50 % of the IECs were positive, mild if 15–50 % or negative if <15 %. Immunoreactivity for GRP78 was also defined in terms of positive IECs, but now the percentages were: >70 % defined as positive, 20–70 % as mild and <20 % as negative.

mRNA isolation and qPCR (quantitative PCR)

mRNA was isolated from colonic biopsies, which were taken from CD and UC patients, using the NucleoSpin Extract II mRNA isolation kit (Macherey Nagel). Gene expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), IL8 (interleukin 8), IL1β, ABCG2, villin and the ER-stress- associated gene CHOP C/EBP (CCAAT/enhancer binding protein) homologous protein[20] were measured using qPCR (IQ5, Bio-Rad). Villin and ABCG2 are both IEC-specific, thus ABCG2 mRNA was corrected for villin

Table S1: Baseline characteristics of healthy individuals, patients with inactive IBD, active IBD, ischemic- and infectious colitis.

	Controls	Inactive colitis	Active colitis	Ischemic colitis	Infectious colitis
Total number of patients	9	30	31	10	5
Crohn's disease (n)	-	12	13[a]	-	-
Ulcerative colitis (n)	-	18	18[b]	-	-
Mean age, yr (SD)	41 (16)	53 (16)	43 (16)*	61 (23)[†]	59 (18)
Sex (M/F)	4/5	12/18	17/14	3/7	3/2
Mean duration of disease, yr (SD)	-	22 (10)	15 (11)*	-	-
Concomitant medication:					
- none	-	6	5	-	-
- aminosalicylates	-	16	18	-	-
- corticosteroids	-	5	11	-	-
- immunosuppressives	-	9	9	-	-
- biologicals	-	2	2	-	-
Total number of tissue samples	9	67	55	10	14

[a] Includes 3 patients with only right-sided colitis

[b] Includes 4 patients with only left-sided colitis

* IBD patients in the group of active colitis were significantly younger and had a shorter duration of disease at the time the biopsies were collected compared to the group of inactive colitis. p=0.001 and p=0.001, respectively

[†] Ischemic colitis patients are significantly older than all other patients groups. p<0.05

Table S2: Baseline characteristics of small bowel biopsies of healthy individuals, patients with inactive- and active Crohn's disease

	Controls	Inactive Crohn's disease	Active Crohn's disease
Total number of patients	17	4	25
Mean age, yr (SD)	45 (15)	41 (19)	42 (17)
Sex (M/F)	8/9	3/1	10/15
Mean duration of disease, yr (SD)	-	17 (14)	10 (8)*
Concomitant medication:			
- none	-	1	5
- aminosalicylates	-	-	1
- corticosteroids	-	-	7
- immunosuppressives	-	1	10
- biologicals	-	3	8
Total number of tissue samples	27	9	25

* CD patients in the group with active disease had a significantly shorter duration of disease at the time the biopsies were collected compared to the group of inactive colitis . p=0.01

Back to Index

Table S3: Baseline characteristics of healthy individuals, patients with inactive IBD and active IBD, for mRNA isolation.

	Controls	Inactive colitis	Active colitis
Total number of patients	6	8	7
Crohn's disease (n)	-	4	4
Ulcerative colitis (n)	-	4	3
Mean age, yr (SD)	38 (19)	40 (21)	34 (10)
Sex (M/F)	2/4	3/5	5/2
Mean duration of disease, yr (SD)	-	10 (5)	13 (6)
Concomitant medication:			
- none	-	1	3
- aminosalicylates	-	2	3
- corticosteroids	-	4	1
- immunosuppressives	-	1	1
- biologicals	-	2	-
Total number of tissue samples	6	8	7

Table S4: Primer sequences

Primer name	Sequence from 5′ to 3′
ABCG2_Fw(XhoI)	CTCGAGCTGGTGCCGGCGCAGGAGCGGGTGCAGGCGCTG-GAATGTCTTCCAGTAATGTCGAAG
ABCG2_Rev(SalI)	GTCGACTTAAGAATATTTTTTAAGAAATAAC
XBP-1 splicing fw	AAACAGAGTAGCAGCTCAGACTGC
XBP-1 splicing rev	TCCTTCTGGGTAGACCTCTGGGAG
GapdH_fw_qPCR	GCATTGCCCTCAACGACCAC
GapdH_rev_qPCR	CCACCACCCTGTTGCTGTAG
IL-8_fw_qPCR	CACTGCGCCAACACAGAAATTA
IL-8_rev_qPCR	ACTTCTCCACAACCCTCTGCAC
ABCG2_fw_qPCR	CAGCCGTGGAACTCTTTGTG
ABCG2_rev_qPCR	CACTCTGACCTGCTGCTATG
IL-1β_fw_qPCR	CCCTAAACAGATGAAGTGCTCCTT
IL-1β_rev_qPCR	GTAGCTGGATGCCGCCAT
CHOP_fw_qPCR	GGAGCTGGAAGCCTGGTATG
CHOP_rev_qPCR	CCTCAGTCAGCCAAGCCAGAG
Villin_fw_qPCR	GAGCCACCTCATCTTATGTC
Villin_rev_qPCR	CCCGGTCTCCAAGTTGTTAG

to prevent underestimation in biopsies with fewer IECs. From each sample, 1 µg of isolated mRNA was reverse- transcribed to cDNA using the cDNA synthase kit iScript (Bio- Rad). Gene expression was measured using 50 ng of cDNA together with 10 pM gene-specific primer in a 25 µl real-time qPCR mixture (SensiMix; Bioline). Duplicate samples were run three times independently, and the average gene expression was normalized to GAPDH (for IL8, IL1β and CHOP) and villin (for ABCG2) using the ΔΔCt method. The primers used are described in Table S4.

Cell culture

HEK-293T human embryonic kidney-293 cells expressing the large T-antigen of SV40 (simian virus 40); A.T.C.C.; CRL-1573 cells were cultured according to routine procedures. Transient transfection was performed using polyethylenimine (Polysciences). SNAP (S-nitroso-N-acetyl-DL-penicillamine; Sigma–Aldrich) was used as a nitric oxide donor. Tunicamycin from *Streptomyces* sp. (Sigma–Aldrich) was used as a positive control for ER stress induction. The cell culture supernatant nitrate concentrations were measured using Griess reagents (Cayman Chemical), according to the manufacturer's protocol.

XBP1 splicing

ER stress and unfolded protein response activity was assessed by identifying mature XBP1 mRNA and its spliced variant as described previously[16]. In short, mRNA was isolated using TriReagent (Sigma–Aldrich), according to the manufacturer's instructions. cDNA was created using the iScript cDNA synthesis kit (Bio-Rad) using 1 µg of RNA. Standard PCR was performed on diluted cDNA. The PCR conditions were 95°C for 5 min, 30 s at 95°C, 30 s at 63°C and 30 s at 72°C for 30 cycles, and 72°C for 8 min. For primer sequences, see Table S4. We analysed the PCR products by electrophoresis on a 3% agarose gel. Images were created and band intensities were measured with ChemiDoc XRS and accompanying software (Bio-Rad).

Construct cloning

To investigate ABCG2 functionality, we created a GFP–ABCG2 fusion construct by inserting the ABCG2 cDNA sequence into the pEGFP-C1 vector (Clontech). In brief, ABCG2 cDNA was obtained using PCR with primers that flank the cloning sites, XhoI was used for the forward primer and SalI was used for the reverse primer. The forward primer also contains a 6×Ala-Gly repeat as a flexible linker between GFP and ABCG2 (for primer sequences, see Table S4.) We cloned the PCR product directly into a pGEMt-easy cloning vector, after which we used the cloning enzymes as described above, and ABCG2 cDNA was cloned into the pEGFP-C1 vector. Cloning enzymes were used as described by Promega. The GFP–ABCG2 construct was checked for the correct insertion by sequencing. GFP-β-catenin and GFP–H2B were provided by Dr R. Smits.

Fluorescence microscopy

The GFP-tagged proteins were examined using a confocal microscope (LSM510META, Zeiss). For the transport assay, nuclear Hoechst 33342 (4 μM, Sigma-Aldrich) accumulation was determined at 30 s intervals over a total of 12 min. The ER was stained with ER–RFP (red fluorescent protein) using ER-Tracker (Molecular Probes), according to the manufacturer's instructions. We analysed the GFP-tagged proteins with a 488nm laser, Hoechst with a 405 nm laser and the ER–RFP with 543 nm laser. A specific ABCG2 transport inhibitor, Ko143 (2 μM, Sigma–Aldrich), was used to identify ABCG2 transport specificity.

SDS/PAGE and fluorescent immunoblotting

To examine the GFP–ABCG2 fusion protein, whole cell lysates were created using 2× SDS sample buffer containing 120 mM Tris/HCl, pH6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.01% Bromophenol Blue and freshly added 0.1 M dithiothreitol. The proteins were separated by SDS/PAGE (8 % gels; pH 8.8) with a stacking gel (pH6.8). After electrophoresis, proteins were electrophoretically transferred on to a PVDF membrane (Millipore, Immobilon-FL) at 200 mA for 90 min. The membrane was blocked with 1 vol. of Odyssey blocking buffer (LI-COR) and 1 vol. of PBS for 1 h at room temperature (21°C). Then the membrane was incubated with primary antibody: 1:1000 anti- GFP (polyclonal, rabbit IgG, Invitrogen) or 1:100 anti-ABCG2 (monoclonal, mouse IgG2a, BXP-21, Solvo) in blocking buffer overnight at 4°C. The membranes were incubated together with a reference protein antibody, 1:2500 β-actin (monoclonal, mouse IgG1, Santa Cruz Biotechnology) for GFP or 1:5000 tubulin (polyclonal, rabbit IgG, Abcam) for ABCG2. We washed the membrane three times in PBS-T (PBS with 0.05 % Tween 20) at room temperature. Directly thereafter, the secondary antibody GaM IgG IRDye 680 and GaR IgG IRDye 800 CW, both 1:5000 in blocking buffer, were incubated for 1h at room temperature. We washed the membrane twice again with PBS-T and twice with PBS. Then the membrane was scanned for red and green fluorescent bands using an Odyssey scanner (LI-COR).

Statistics and software

Statistical analyses were performed using the SPSS 11.0 software package for Windows. Data on different patient groups were compared using the Mann–Whitney *U* test with a Kruskal–Wallis post-hoc test. Using univariate and multivariate multinomial logistic regression analysis, we examined the association between colitis and GRP78 and ABCG2 expression, adjusting for age and duration of disease. A two-tailed *P* value of <0.05 was accepted as statistically significant. Images were composed using Adobe Photoshop CS3.

Results

Colonic inflammation reduces apical ABCG2 expression in IECs

To investigate ABCG2 expression during human intestinal inflammation, ABCG2 expression in colonic biopsies was qualitatively assessed employing immunohistochemistry. As expected, IECs in biopsies of control subjects as well as of IBD patients in remission (with no active inflammation) showed strong specific staining for ABCG2 at the apical surface (Figure 1A). In contrast, apical IEC ABCG2 staining was dramatically reduced in patients with active IBD, in the colon as well as in the small intestine, and also in biopsies from ischaemic colitis and infectious colitis patients (Figure 1A). Thus diverse colonic inflammatory responses are uniformly characterized by the down-regulation of apical ABCG2 levels, suggesting that processes common to these alternative modes of inflammation underlie the absence of apical ABCG2 during intestinal inflammation.

In apparent agreement, a quantitative study of ABCG2 levels in the human colon revealed significantly less ABCG2 expression in active colitis (24% positive expression, 13 out of 54), ischaemic colitis (10% positive staining, one out of ten) and infectious colitis (0% positive staining, zero out of 14) as compared with either inactive colitis (80 % positive expression, 53 out of 67) or control tissues (100 % positive expression, nine out of nine) (all $P < 0.001$; see Figure 1B). There was no significant difference in ABCG2 levels between active CD (27%, six out of 22) compared with active UC (22%, seven out of 32) and inactive CD (67%, 18 out of 27) compared with inactive UC (88%, 35 out of 40). As a control, we examined left- and right- sided biopsies from IBD patients and controls to investigate a confounding possible differential expression of ABCG2 based on position in the colon, but this analysis demonstrated that only the degree of inflammation is an important factor guiding apical ABCG2 levels. Thus inflammation itself is associated with a marked reduction in apical ABCG2 expression in the human colon.

Small bowel inflammation is associated with down-regulation of apical ABCG2 expression

Mirroring the findings obtained in the human colon, small bowel biopsy specimens obtained from CD patients with active disease by double balloon endoscopy exhibit markedly less ABCG2 staining when compared with biopsies obtained from patients with inactive disease or no intestinal inflammatory phenomena (see Figure 1A). In active CD, there was significantly less small bowel ABCG2 staining (75 % negative staining, 19 out of 25) as compared with inactive CD (11% negative staining, one out of nine) and controls (8% negative staining, two out of 25) (both $P<0.05$; see Figure 1C). We found no significant differences between inactive CD and controls. In all cases, current drug use did not influence the expression of ABCG2. We conclude that human intestinal inflammation is accompanied by reduced expression of the xenobiotic transporter ABCG2 and experiments were initiated to identify the underlying molecular mechanisms.

Back to Index

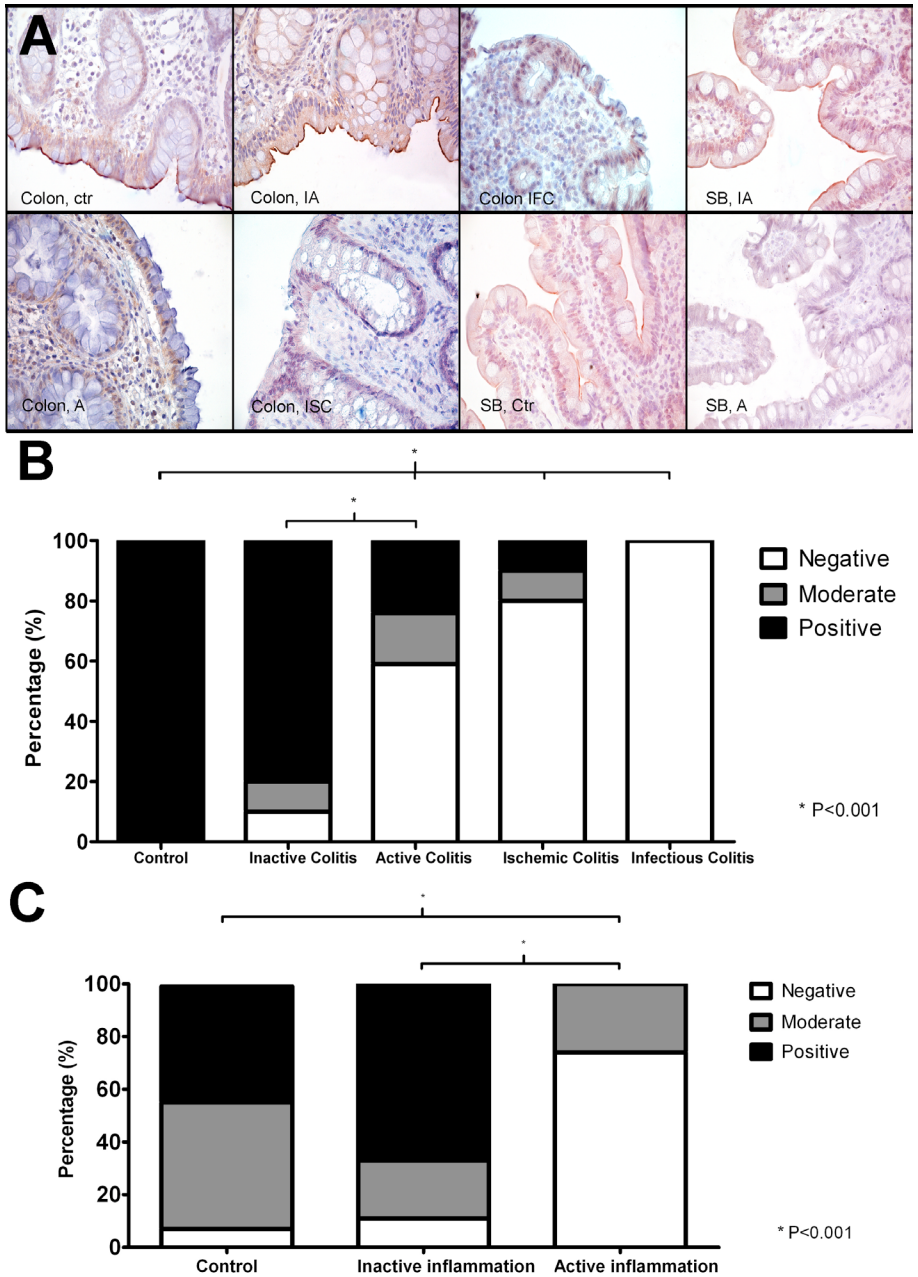

Figure 1. Reduced ABCG2 expression in actively inflamed intestinal biopsies
(A) Representative images of intestinal biopsies stained for ABCG2. Colon, ctr: colon biopsy from healthy individual. Colon, IA: colon biopsy from an IBD patient in remission. Colon, A: colon biopsy from an IBD patient with active inflammation. Colon, ISC: colon biopsy from a patient with ischaemic colitis. Colon, IFC: colon biopsy from a patient with infectious colitis. SB, Ctr: small bowel biopsy from a healthy individual. SB, IA: small bowel biopsy from a CD patient in remission. SB, A: small bowel biopsy from a CD patient with active inflammation. The ABCG2 expression is represented by red/brown staining on the apical membrane of IECs. Original magnification is ×400. **(B)** Decrease in the IEC fraction of colonic biopsies with ABCG2 expression during active inflammation. **(C)** Actively inflamed small bowel displays a reduced expression of ABCG2. **(B and C)** All intestinal biopsies were double blindly examined as negative, moderate or positive for ABCG2 membrane immune reactivity. Mann–Whitney U tests and Kruskal–Wallis tests were used for statistical analysis. (*P < 0.001).

Intestinal inflammation acts to reduce ABCG2 via a post-transcriptional mechanism

To delineate the molecular events reducing the capacity of the intestine to cope with xenobiotic challenge, we first determined whether genomic or post-transcriptional processes are involved in this effect. To this end, we measured ABCG2 mRNA levels in freshly frozen colon biopsies from healthy individuals, and inactive and active IBD patients. Importantly, ABCG2 mRNA levels in IECs did not differ between these three groups (all $P > 0.8$). The expression, however, of the pro-inflammatory cytokines IL8 and IL1β were 10- and 8-fold higher ($P < 0.05$) in biopsies with active mucosal inflammation (Figure 2), and thus technical issues do not underlie the absence of intestinal inflammation-induced effects on apical ABCG2 expression. Hence we are forced to conclude that down-modulation of ABCG2 in human intestinal inflammation is brought about by a post-transcriptional process.

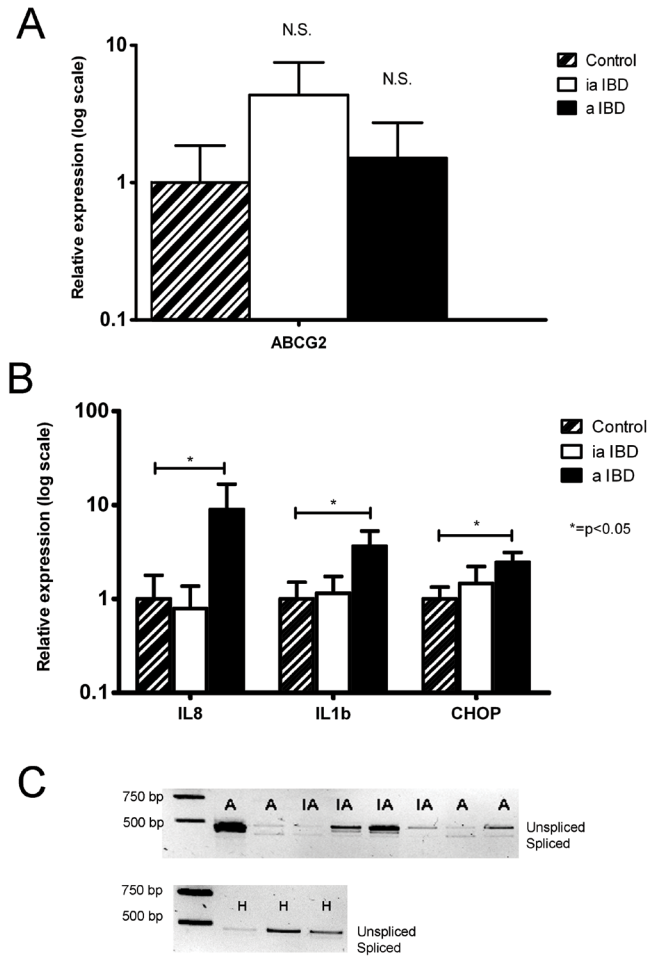

Figure 2. mRNA expression from intestinal biopsies
(A) No difference in ABCG2 mRNA expression in colonic biopsies. Relative mRNA (ΔΔCt) expression corrected for villin. Villin and ABCG2 are both IEC-specific, thus ABCG2 mRNA was corrected for villin to prevent underestimation in biopsies with fewer IECs. N.S., not significant. **(B)** Increased pro-inflammatory cytokine expression in colonic biopsies from patients with active inflammation. Relative mRNA expression (ΔΔCt) corrected for GAPDH. IL8 and IL1β (IL1b) are pro-inflammatory cytokines and CHOP is an ER-stress-related gene. *P < 0.05. **(A and B)** Controls are healthy individuals (n = 6), ia IBD are biopsies with inactive inflammation (n = 8) and a IBD are biopsies with active inflammation (n = 7). Mann–Whitney U tests and Kruskal–Wallis tests were used for statistical analysis. **(C)** XBP1 mRNA splicing. Agarose gel image of XBP1 splicing PCR products. Spliced XBP1 is an indicator of increased ER stress. A is active IBD, IA is inactive IBD and H is healthy control.

Back to Index

Activation of the unfolded protein response accompanies human intestinal inflammation

As it is becoming increasingly clear that activation of the unfolded protein response accompanies inflammatory processes and that ER stress may interfere with proper production of complexly folded proteins (the 'half' transporter ABCG2 transverses the membrane six times), we examined whether the ER stress path- way is activated in intestinal inflammation and to which extent this activation mirrors the down-regulation of ABCG2. To this end, we employed the expression of the *bona fide* ER stress marker GRP78. Representative images of the GRP78 immunohistochemical analyses of the same biopsies as stained for ABCG2 are shown in Figure 3(A). There was a significant increase in GRP78 expression in active colitis (32% positive expression, 16 out of 54), ischaemic colitis (90 % positive staining, nine out of ten) and infectious colitis (79% positive staining, 11 out of 14), compared with inactive colitis (0 % positive expression, zero out of 67) and control tissues (0 % positive expression, zero out of nine) (all $P < 0.001$; see Figure 3B). There are no significant differences in GRP78 expression between active CD and active UC or between inactive CD and inactive UC. In addition there were no differences in GRP78 expression in left- and right-sided colon biopsies taken from the same patient. We conclude that colonic inflammation is accompanied by the ER stress response, which is absent in the non-inflamed colon.

In the small bowel biopsies of active CD patients, there was increased GRP78 expression (29% positive expression, six out of 21) compared with inactive CD patients (0 % positive staining, zero out of nine) and controls (8% positive expression, two out of 25) (P<0.05; see Figures 3A and 3C). In all actively inflamed tissue samples, elevated GRP78 expression was evident, irrespective of disease type or location. ER stress during intestinal inflammation was further confirmed by measuring the mRNA expression of the ER-stress-associated gene CHOP in colonic biopsies. In biopsies taken from actively inflamed mucosa, a significant increase in CHOP mRNA expression was measured ($P < 0.01$; see Figure 2B). Moreover, an increased ratio of spliced XBP1 mRNA was detected in patients with active intestinal inflammation (Figure 2C). We conclude that activation of the unfolded protein response and intestinal inflammation are highly associated processes and we initiated experiments to investigate whether ER stress explains the down-regulation of ABCG2 in intestinal inflammation.

Impaired ABCG2 function in ER-stressed cells

To investigate the effect of activated ER stress on ABCG2 protein expression and function, we created a GFP–ABCG2 fusion protein. To this end, ABCG2 cDNA was cloned into a pEGFP-C1 vector, which led to the production of GFP– ABCG2 after transfection (Figure 4A). The nitric oxide donor SNAP was used to ascertain whether nitric oxide was able to induce ER stress in 293t-AB (GFP–ABCG2-transfected HEK- 293T) cells. The Griess assay revealed that SNAP application induced robust increases in nitrate concentration, a good proxy measure for nitric oxide production

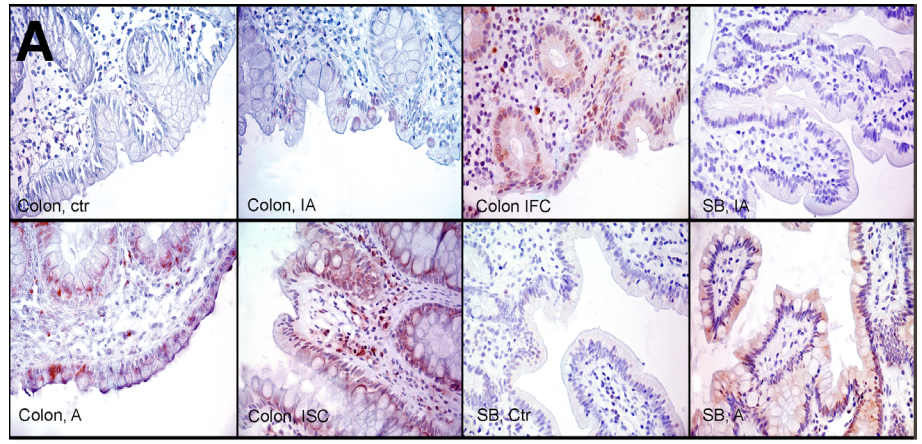

Figure 3. Increased GRP78 expression in actively inflamed intestinal biopsies
(A) Representative images of intestinal biopsies stained for GRP78. Colon, ctr: colon biopsy from healthy individual. Colon, IA: colon biopsy from an IBD patient in remission. Colon, A: colon biopsy from an IBD patient with active inflammation. Colon, ISC: colon biopsy from a patient with ischaemic colitis. Colon, IFC: colon biopsy from a patient with infectious colitis. SB Ctr: small bowel biopsy from a healthy individual. SB, IA: small bowel biopsy from a CD patient in remission. SB, A: small bowel biopsy from a CD patient with active inflammation. Expression of GRP78 is detected as red/brown colour and is present in the cytoplasm of IECs and mononuclear cells in the lamina propria. Original magnification is ×400. **(B)** Increase in the IEC fraction of colonic biopsies with elevated GRP78 expression during active inflammation. *P < 0.001. **(C)** Actively inflamed small bowel displays elevated GRP78 expression. *P < 0.05. **(B and C)** The biopsies were double blindly examined as positive, moderate or negative according the amount of positive GRP78 immune reactivity. Mann–Whitney U tests and Kruskal–Wallis tests were used for statistical analysis.

Back to Index

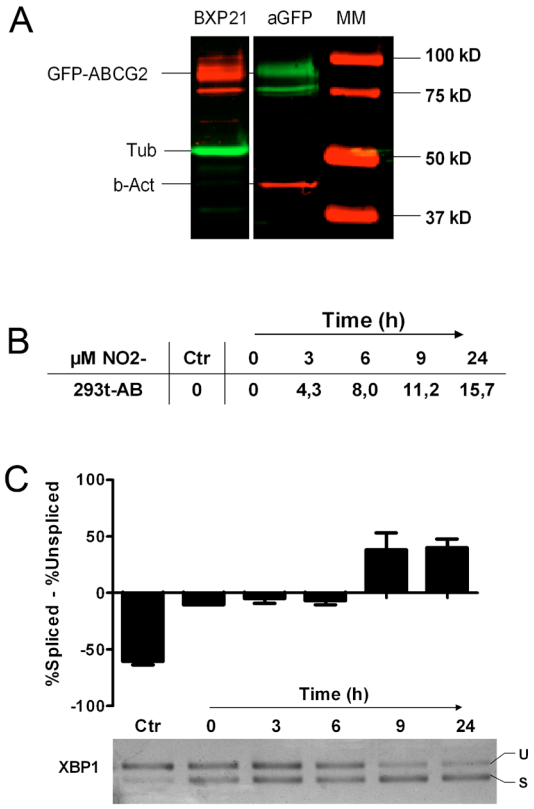

Figure 4. Nitric oxide-induced ER stress reduces ABCG2 protein expression and function

(A) Western blot analysis showing the expression of GFP–ABCG2 protein using the antibodies BXP-21 (anti–ABCG2) and anti–GFP. Tubulin (Tub) and β-actin (b-Act) were used as loading control. MM is the molecular mass marker, with masses in kDa on the right-hand side. **(B)** SNAP induces increasing levels of nitrite, a stable derivative of nitric oxide, over time. Concentrations were measured from supernatants of 293t-AB cells. **(C)** After transfection with GFP–ABCG2, HEK-239T cells were stimulated with 0.625 mM SNAP and XBP1 splicing was determined, and the ratio of un-spliced (U) and spliced (S) XBP1 were plotted. Ctr depicts HEK-239T cells only, 0 are GFP–ABCG2-transfected cells without SNAP stimulation and other time points represents GFP–ABCG2-transfected cells with 0.625 mM SNAP. The gel picture is representative of three independent experiments, and the graph represents the mean+−S.D. of these three individual experiments.

(see Figure 4B). The validity of the assay was demonstrated by experiments showing an increase in the ratio of unspliced XBP1 mRNA over spliced XBP1 mRNA 9 h after the application of 0.625 mM SNAP (see Figure 4C). In agreement with this, administration of tunicamycin, a well-established ER-stress-response inducer, increased the XBP1 splicing ratio to approximately the same level as SNAP application (see Figure S1B). As is consistent with high ABCG2 transport activity, administration of Hoechst 33342 did not stain the nuclei of the 293t-AB cells (Figure 5A), also demonstrating the functionality of the ABCG2–GFP fusion protein in xenobiotic transport. Nevertheless, 16 h after addition of the ER stress inducers SNAP or tunicamycin, the nuclei of 293t-AB cells became Hoechst 33342-positive (Figure 5A and Supplementary Movies S1, S2 and S3 at (http://www.BiochemJ.org/bj/441/bj4410087add.htm). As shown in Figure 5(B), Hoechst 33342 also stained the nucleus blue in 293t-AB cells in the presence of the specific ABCG2 inhibitor Ko143 (see Supplementary Movie S4 at (http://www.BiochemJ.org/bj/441/bj4410087add.htm). No altered GFP–ABCG2 protein function was found when SNAP or tunicamycin was added 48 h after transfection (see Figure S1C). Hence, in this overexpressing model system, ER stress provokes a very strong decrease in ABCG2 function, in agreement with a scheme in which inflammation-dependent induction of ER stress is causatively related to a reduced capacity to deal with xenobiotic challenge.

In support for such a model, all images from 293t-AB cells with ER stress revealed a reduced apical membrane expression of GFP–ABCG2, with intracellular expression remaining. In order to ascertain whether GFP–ABCG2 remained in the ER after stress induction, we stained the ER with ER–RFP. When ER stress was present, some GFP–ABCG2 co-localized with ER–RFP, whereas the majority was present in other cellular compartments (Figure 5C). As shown in Figure S2 the localization and expression of non-ER-dependent GFP-tagged proteins GFP–β-catenin and GFP–H2B (histone 2B) was not changed during ER stress conditions. Thus protein-folding difficulties seems incompatible with functional expression of ABCG2.

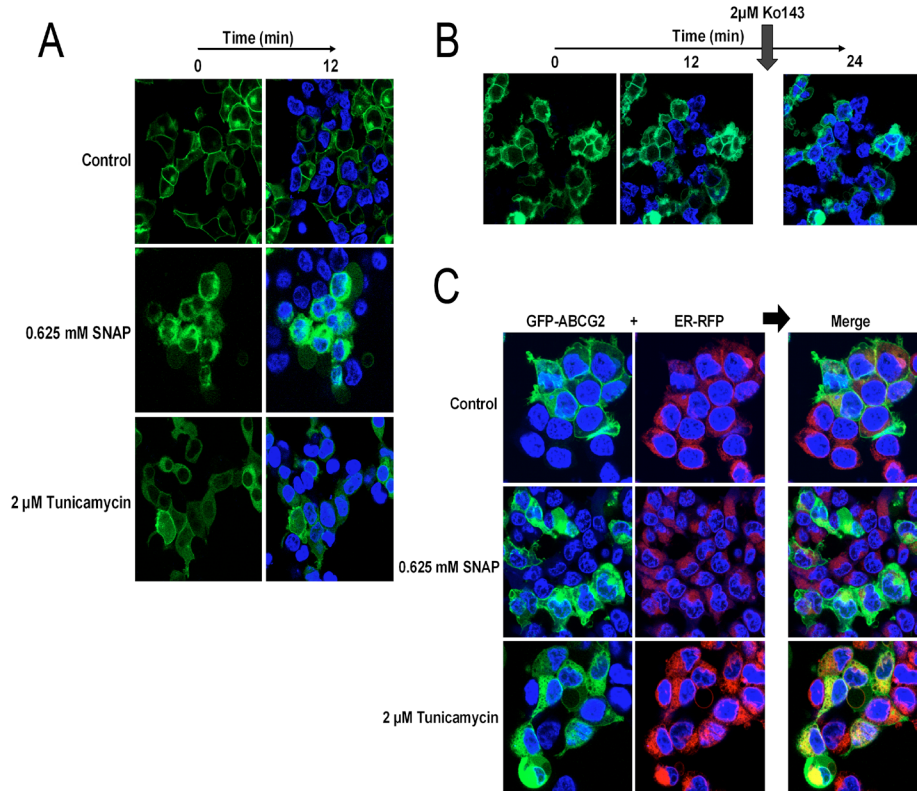

Figure 5. Reduced ABCG2 activity and membrane localization in ER-stressed cells

Hoechst 33342 (4 µM) DNA binding was measured to study the activity of ACBG2. **(A)** ABCG2-transfected cells were exposed to SNAP, tunicamycin or solvent for 16 h, after which Hoechst 33343 binding was studied. See Supplementary Movies S1–S3 (http://www.BiochemJ.org/bj/441/bj4410087add.htm). Original magnification ×400. **(B)** The specific inhibitor of ABCG2, Ko143 (2 µM), was added 12 min after Hoechst 33342 (4 µM), and was used as a positive control for ABCG2 activity. See Supplementary Movie S4. Original magnification ×400. **(C)** 293t-AB cells were stained with ER–RFP. ER stress was induced by addition of SNAP or tunicamycin 16 h before ER–RFP staining. All confocal images are representative of at least three independent experiments. Original magnification 400×.

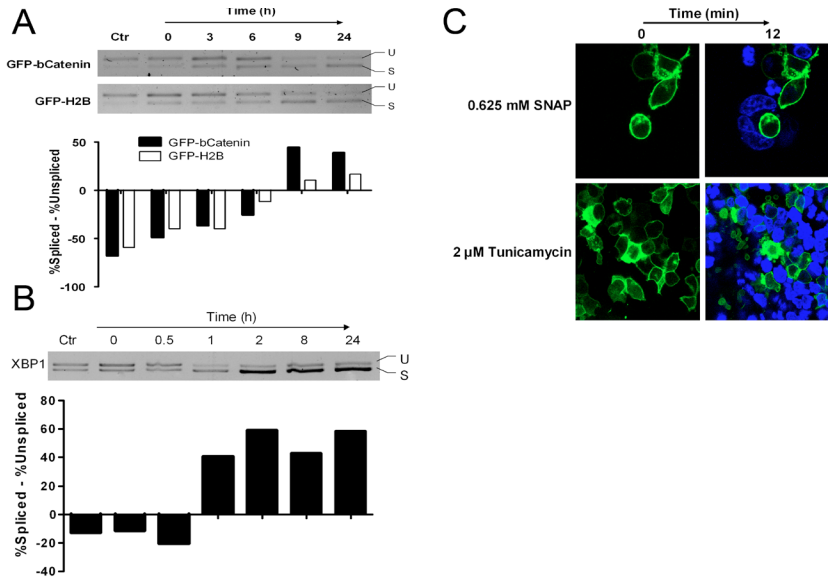

Figure S1. ER stress in control proteins and ER stress do not impede already correctly folded GFP–ABCG2
(A) To investigate whether overexpression of GFP–ABCG2 itself could affect protein folding, two other GFP fusion proteins were used as controls. The same methodology was used as described in Figure 5(B) of the main text. For both GFP–β-catenin and GFP–H2B, a similar XBP1 mRNA splicing pattern was shown. **(B)** XBP1 splicing after tunicamycin stimulation. Tunicamycin is an inhibitor of N-glycosylation and thereby induces ER stress. XBP1 splicing was determined as described in Figure 5 (B) of the main text. An increased amount of spliced XBP1 was measured after addition of 2 μM tunicamycin for indicated time points. **(C)** The 293t-AB cells were cultured for 48 h after transfection before ER stress was introduced. Interestingly, functional ABCG2 was measured in SNAP and tunicamycin ER-stressed cells. This indicates that ABCG2, which was folded in the incubation time without ER stress, was not impeded by the later introduced ER stress. Apparently, only newly formed ABCG2 is affected by ER stress. All confocal images are representative of at least three independent experiments. Original magnification ×400.

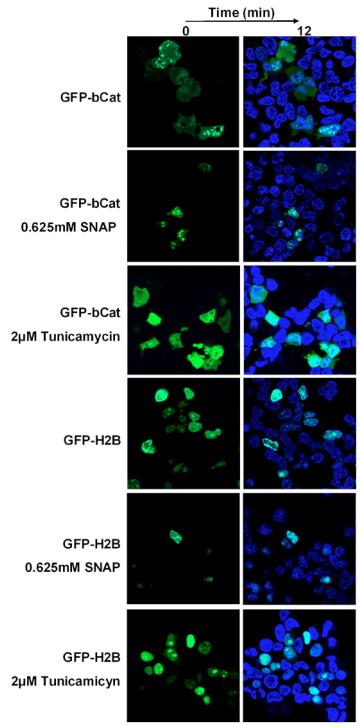

Figure S2. Non-ER-dependent proteins are not affected by ER stress
To investigate whether non-ER-dependent proteins were affected by ER stress, GFP–H2B and GFP–β-catenin expression and cellular location were measured. The same methodology was used as in Figure 5(A) of the main text. SNAP and tunicamycin did not impede the fusion protein localization of GFP–H2B or GFP–β-catenin or affect nuclear Hoechst 33342 accumulation. Original magnification ×400.

Discussion

The results shown in the present study are consistent with a model in which intestinal inflammation drives an epithelial misfolded protein response, which in turn interferes with the ABCG2 expression and function, leading to a diminished capacity of the epithelial compartment to cope with xenobiotic stress. Colonic and small bowel biopsies from IBD patients with active disease and those from patients with ischaemic and infectious colitis all show reduced apical ABCG2 expression in IECs compared with controls and IBD patients in remission. The decline of ABCG2 expression in the presence of active inflammation in IBD patients corresponds with the findings of two studies done exclusively on UC patients[7,22]. In addition, we found that ABCG2 expression is not affected by medication use, patient age or location of the inflammation.

Expression of ABCG2, similar to that of many other proteins, can be regulated at various levels. In addition to a decline in ABCG2 protein expression, others have described a reduction in ABCG2 mRNA expression in actively inflamed intestine[7,22]. We corrected the ABCG2 mRNA expression to villin to prevent an underestimation of ABCG2 expression, since both genes are specific for IECs. Our results show no reduction of ABCG2 mRNA expression in biopsies with active mucosal inflammation. However, since the sample size used in the present study is smaller than those in previous studies, we cannot completely rule out the contribution of reduced mRNA expression to the decline in ABCG2 protein expression. As such, our findings merely imply that the decline in mRNA expression probably does not solely account for the complete loss of ABCG2 protein expression discernable on our immunohistochemistry images and that post-transcriptional mechanisms must be involved. The observation that unfolded protein responses are both general and specific to intestinal inflammation suggest a causal link between ER stress and diminished apical ABCG2 levels. Indeed, ABCG2 is a 'half' transporter, spanning the membrane six times, functionally critically dependent on homodimerization, N-linked glycosylation and various disulfide bonds and thus is probably an early victim of the ER stress responses[18,19]. Disrupted protein-folding mechanisms that can lead to ER stress during active inflammation have been linked to IBD[16,23,24], and may well involve the reduced capacity of the IEC compartment to deal with the xenobiotic stress and thus in turn interfere with demanding regenerative responses that an adequate response to IBD-related intestinal challenges requires.

Our link from inflammation to unfolded protein responses remains unclear, but inflammatory responses are associated with an excessive production of nitric oxide, which in turn is predicted to disturb the function of redox-state sensitive disulfide-bonding chaperones such as PDIs that are necessary for proper protein folding[25]. In all biopsies with active inflammation (i.e. IBD and non-IBD related), the GRP78 expression is significantly higher than in the biopsies of IBD patients in remission and in those of healthy controls. Furthermore, expression of CHOP mRNA (an ER stress marker) is increased in biopsies with active mucosal inflammation. In addition to this, biopsies

Back to Index

with active inflammation reveal an increased amount of spliced XBP1 mRNA expression. Thus factors common to multiple types of inflammation must be involved here and, as nitric oxide production is one of the few cellular responses described to generally accompany inflammation, it is tempting to attribute an important role to excessive nitric oxide generation in inflammation-related induction of ER stress responses[23,25–27]. Indeed, we observed elevated XBP1 splicing[16] following application of the nitric oxide donor, suggesting that nitric oxide can directly affect protein-folding mechanisms[20] and induce ER stress. In the present study we show that the reduction in ABCG2 expression is associated with inflammation, which fits with the observations that ABCG2 expression is reduced in IBD- unrelated inflammatory conditions in other organs[10,12,28]. Other IBD-associated proteins are also reduced in protein expression during active intestinal inflammation[29–31]. Further research is needed to investigate the role of impeded protein folding and the effect of this on cellular processes. Noteably, ABCG2-mediated efflux of toxic components is thought to protect the epithelium of the gut and the underlying tissue to xenobiotic challenges. Importantly, as xenobiotic stress is directly linked to genotoxicity, our results may also help to explain the still poorly understood relationship between intestinal inflammation and colorectal cancer[32]. Decreased ABCG2 expression may have other deleterious consequences as well. Sulfasalazine, a derivate of mesalazine 5-ASA (5-aminosalicylic acid) that is commonly used in the treatment of IBD patients, is actively excluded from the epithelial compartment by ABCG2[33]. Rodents with reduced IEC ABCG2 expression have increased 5-ASA trough levels[34]. In addition to its anti-inflammatory effect, accumulation of sulfasalazine can be toxic for epithelial cells[35,36]. Thus reduced expression and function of ABCG2 may therefore greatly enhance the sensitivity of IECs to sulfasalazine and limit the use of this compound for the treatment of IBD. Hence understanding the mechanisms governing ABCG2 expression are highly relevant for efforts with respect to rational design of novel therapy.

In conclusion, IEC ABCG2 protein expression is decreased in the inflamed bowel, probably as a result of incorrect protein folding due to inflammation-driven ER dysfunction. Thus a novel paradigm explaining xenobiotic stress during inflammation emerges in which intestinal inflammation activates the unfolded protein response, in turn abrogating the defences against xenobiotic challenge by impairing ABCG2 expression and function.

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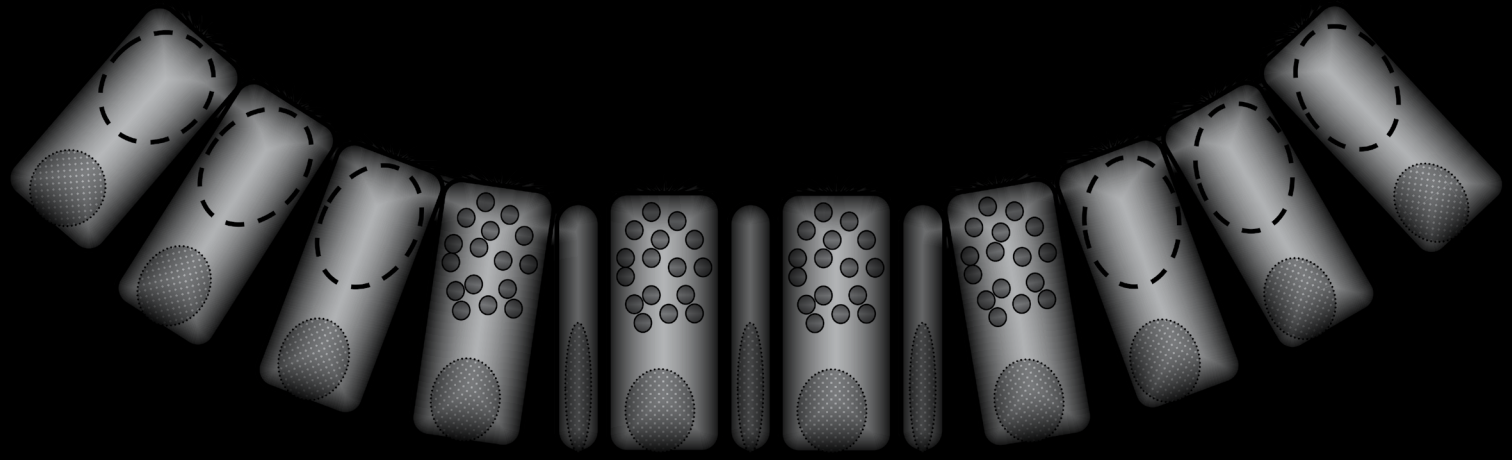
Back to Index

VII

Genomic ATG16L1 Risk Allele-Restricted Panteh Cell ER stress in Quiescent Crohn's Disease



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Abstract

Although genome wide association studies have largely uncovered the genetic basis of Crohn's disease(CD), it remains a challenge to link the underlying genetic polymorphisms to functional intestinal phenotypes. Paneth cells are specialized epithelial cells with topographical restriction to the small-intestinal crypt-base where they have an antimicrobial action. We were interested whether genomic variations in the autophagy-related gene ATG16L1 would affect Paneth cell function.

Genomic variation of ATG16L1(T300A, rs2241880) was determined in DNA isolated from 78 CD patients and 12 healthy controls. Paraffin-embedded ileal biopsies of patients with genotype AA(n=17), GA(n=38) and patients with the GG allele(n=23) were stained for Alcian blue–PAS and immunohistochemically for GRP78 and pEIF2α(ER stress), lysozyme(antimicrobial peptide), cleaved-Caspase 3(apoptosis), pHiston H3(proliferation), p65 and phospho-p38MAPK(active), and PHLDA1(human stem cell marker). Disease phenotype severity and progression was noted.

In patients with quiescent disease but with a mutated ATG16L1 allele, the ER stress markers GRP78 and pEIF2α were highly expressed in the Paneth cell compartment. Other CD risk gene mutations did not correlate with Paneth cell ER stress. Functionally, the patients with ER stressed Paneth cells showed no distinct IEC proliferation or increased apoptosis in their crypt base. Also in these patients the total number of stem or Paneth cells did not differ. Similarly, no nuclear p65 expression or increased phospho-p38 is detected ER stressed Paneth cells. Phenotypically, patients with Paneth cell ER stress have relatively less colonic disease over ileal disease(-21% p=0.04, more fistulas(+21%, p=0.05), and need intestinal surgery more often(+38%, p=0.002).

The ATG16L1 polymorphism defines a specific subtype of CD patients, distinctively characterized by Paneth cell ER stress even during quiescent disease. ATG16L1-linked Paneth cell ER stress is likely to corrupt antimicrobial functionality of this cell type and thus increase vulnerability to chronic bacterial insult.

Introduction

Inflammatory bowel disease (IBD) is the result of a deregulated immune response to gut microbiota. Both genetic and environmental factors play an important role in the development of IBD. The most frequent forms of IBD are Crohn's disease (CD) and ulcerative colitis. By identifying single nucleotide polymorphisms (SNPs) associated with an increased risk for developing CD, genome wide association studies (GWAS) have now largely uncovered the genetic basis of CD, resulting in the identification of more than 70 genetic locations in the human genome apparently linked to this disease[1]. The main challenge now is to link these mutations to altered functionality in SNP carrying individuals explaining their association with disease susceptibility.

The Paneth cells are specialized intestinal epithelial cells that are found at the crypt base of the small intestine. They are characterised by large secretory granules and an extensive ER (endoplasmic reticulum). Initially described as cells specialised with respect to luminal exocytosis cells of antimicrobial products such as; lysosyme and defensins, Paneth cells have recently been suggested to constitute a cardinal component of the intestinal stem cell niche[2]. Thus Paneth cells contribute to controlling the luminal flora as well as repairing the intestinal barrier following an insult. It can be hypothesised that genomic alterations that impede the Paneth cell-compartment functionality would increase the propensity to develop CD.

CD-linked SNPs that might affect Paneth cells include those in NOD2 (CARD15), which have been associated with reduced intracellular microbial sensing, a decrease in Paneth cell-produced α -defensins and reduced antimicrobial defense[3-5]. Also, genetic variation in the XBP1 gene may lead to increased ER stress as a consequence of a defective unfolded protein response (UPR) in highly secretory intestinal epithelial cells (IEC) like goblet and Paneth cells thereby affecting their function[6]. Finally, it is described that a SNP in the ATG16L1 gene (T300A, G is risk allele, rs2241880) may confer its risk for CD development by affecting Paneth cell morphology and function[7]. Overall, Paneth cells seem to be especially affected by a variety of SNPs strongly associated with CD, suggesting that the affected pathways, bacterial/viral sensing (NOD2), ER-stress (XBP-1, ORMDL3) and autophagy (ATG16L1, IRGM) are of crucial importance for normal Paneth cell function in turn affecting. The latter is important for maintaining intestinal homeostasis[2]. This notion is further supported by mouse studies using epithelial-specific deficiencies in these pathways, which show abnormal Paneth cell phenotype and function, and lead in some cases to spontaneous ileitis[5-7]. In contrast, although a recently study described activation of autophagy and crynophagy with concomitant reduced numbers of secretory granules in the Paneth cell compartment of CD patients, these features correlated neither to disease activity or ATG16L1 and IRGM genotype[8]. It is thus fair to say that the link between genotype and Paneth cell functionality in CD remains poorly understood.

An underexplored possible connection between the Paneth cell compartment, CD-linked genetic polymorphisms and intestinal inflammation

Back to Index

is ER stress. ER stress has been shown to induce autophagy as part of the unfolded protein response (UPR), and impairments in the autophagy pathway can lead to ER stress and the induction of the UPR[9]. Activation of NOD2 by its ligand MDP or bacteria induces NF-κB mediated induction of autophagy[3], whereas autophagy has been shown to play an important role in the uptake and delivery of NOD2 signals[4]. We and others have recently shown that elevated ER stress negatively influences the normal IEC function[6, 10, 11]. The possible connection, however, between genotype and Paneth cell ER stress remains unclear.

For these reasons, we investigated whether evidence for the convergence of ER stress and inflammatory pathways could be detected in Paneth cells of CD patients and how these pathways would relate to genetic polymorphisms in CD. In this study we demonstrate that CD patients and healthy controls with at least one risk allele for ATG16L1 (T300A, rs2241880) constitute a specific subset of CD patients with elevated ER stress specifically in their Paneth cells.

Table S1: Baseline characteristics of with Crohn's disease patients and healthy controls by ATG16L1 genotype (rs2241880)

	Allele: AA		Allele: GA		Allele: GG	
	CD	Controls	CD	Controls	CD	Controls
Total number of patients	17	3	38	3	23	6
Mean age, yr (SD)	43 (17)	42 (22)	36 (13)	44 (7)	40 (18)	44 (11)
Sex (M/F)	6/11	1/2	20/18	1/2	8/15	3/3
Mean duration of disease, yr (SD)	11 (13)	-	12 (9)	-	12 (11)	-
Smoking? (%Yes)	41	§	29	§	65	§
Familiar link to IBD? (%Yes)	35	-	32	-	26	-
Concomitant medication:						
- none	6 *	-	4	-	1	-
- aminosalicylates	6	-	22	-	9	-
- corticosteroids	5	-	6	-	7	-
- immunosuppressives	5	-	9	-	9	-
- biologicals	1	-	4	-	1	-

*CD patients with the ATG16L1 (rs2241880) AA allele take significantly less medication than CD patient with the GA of GG allele. p<0.05
§ smoking behaviour unknown

Methods

Patient material

Formalin-fixed paraffin-embedded (FFPE) small intestine biopsies were collected from the pathology archive from in total 78 patients with CD: 17 with ATG16L1 (T300A) allele AA, 38 with the GA allele, and 23 with the GG allele and 12 healthy controls: 3 with ATG16L1 (T300A) allele AA, 3 with the GA allele, and 6 with the GG allele. Healthy individuals have no medical or familiar history in IBD. The CD patients were genotyped using genomic DNA isolated from peripheral blood mononuclear cells using Wizard Genome DNA purification kit (Promega Benelux B.V.) The healthy individuals were genotyped using genomic DNA isolated from FFPE biopsies. Detailed protocol is described below. All obtained biopsies were endoscopic and histologically (Geboes criteria) examined as not actively inflamed. The demographic and disease specific characteristics were subtracted from electronic patient files according the Montreal classification[12] and are presented in Tables S1 and S2.

Table S2, characteristics of healthy controls and CD patients without or with ER-stressed Paneth cells.

	ER stress (-)		ER stress (+)	
	CD	Controls	CD	Controls
Total number of patients	54	8	24	4
Mean age, yr (SD)	39 (15)	45 (15)	38 (15)	40 (6)
Sex (M/F)	26/28	3/5	8/16	3/1
Mean duration of disease, yr (SD)	11 (10)	-	13 (9)	-
Concomitant medication:				
- none	8	-	3	-
- aminosalicylates	25	-	12	-
- corticosteroids	14	-	1	-
- immunosuppressives	17	-	3	-
- biologicals	6	-	0	-

Healthy control genotyping

Genomic DNA from FFPE biopsies was isolated using the Wizard® Genomic DNA purification Kit (Promega), according manufactures description. A 330bp fragment spanning the ATG16L1 (T300A) SNP was amplified using standard PCR (Phusion, Finnzymes, Oslo Finland). The following primers were used: fw-*5'*-TTGGAGTCCACAGGTTAG-*3'* and rev-*5'*-CAGTAGCTGGTACCCTCACTTC-*3'*. The DNA fragment was isolated from a 1% agarose gel using the Invisorb fragment cleanup kit (Invitek, USA). The ATG16L1 T300A SNP was determent two SNP specific q-PCRs, one PXR for the non-risk allele and one PXR for the risk allele. Both PCR reactions were performed using a universal forward primer: fw-*5'*-CTAGAAGGACAGGCTATCAAC-*3'*. The non-risk allele (A) was determent by using the non-risk allele specific revers primer: rev-*5'*-TTACCAGAACCAGGATGAGT-*3'*.

Back to Index

The risk allele (G) was determent by using the risk allele specific revers primer: rev-5'-TTACCAGAACCAGGATGAGG-3'. A standard q-PCR reaction was performed using the purified ATG16L1 fragment (1:10000(v/v) dilution) as template. The Tm was optimized (64.3°C) as only a fragment was created when there was a perfect match with the primer and the template. Thus, an individual with an AA genotype only shows a Ct value in the non-risk allele PCR, an individual with GA shows Ct values in both PCRs, and an individual with GG reveals only a Ct in the risk allele PCR. For each PCR a validated AA, GA and GG patient was included as control.

Genomic DNA analysis

Genomic DNA was isolated from CD patient blood and healthy control FFPE biopsies. The following genetic loci associated with IBD and ER stress were analysed: ATG16L1 (rs10210302)[13], NOD2 (rs2066844, rs2066845, rs2066847)[14], XBP1 (rs35873774)[6] for all the patients using the genomic DNA samples. KBiosciences UK ltd, Hertfordshire, United Kingdom, performed the SNP analysis.

Histology

For this we used serial sections (4 µm) of intestinal FFPE tissue blocks. Paneth cells were stained using PAS - Alcian Blue staining. Alcian blue - PAS staining was performed as follows: slides were deparaffinised, stained with 1% Alcian blue (Sigma-Aldrich) in 3% acetic acid solution for 15 min, washed in tap water, 5 min in 0.5% periodic acid solution, washed dH2O, 10 min in Schiff's Reagent (Merck, Darmstadt, Germany), washed 5 min tap water, 1 min Haematoxylin (Merck), stained in tap water for 5 min, dehydrated and mounted. Paneth cell morphology was scored as described earlier[7].

For immunohistochemistry all the primary antibodies are summarized in Table S3. For IHC the FFPE slides were; deparaffinised, endogenous peroxidises were blocked using 3% H2O2 in methanol, treated for antigen retrieval, incubated o/n at 4°C with primary antibody, incubated for 1 h at RT with DAKO EnvisionTM + System-HRP (DAKO, Glostrup, Denmark) after which the antibody complex was detected by 3-amino-9-ethylcarbazol (DAB, Sigma-Aldrich, St Louis, MO). As a negative control, the primary antibody was replaced by an equivalent IgG (DAKO, Glostrup, Denmark). The slides were examined in a blinded fashion by light microscopy (Zeiss, Axioskop, Oberkochen, Germany). Intestinal biopsies with high numbers of GRP78- and pEIF2α-positive Paneth cells were scored as positive and biopsies with basal levels of GRP78- and pEIF2α- stained Paneth cells as negative. Representative examples of negative and positive score samples are represented in Figure S1. Positive cleaved-Caspase 3 and pHiston 3H cells of at least 20 intestinal crypts per biopsy were counted. Paneth cell specific lysozyme, p65, phospho-p38, and stem cell specific PHLDA1 staining were examined by immune reactivity intensity.

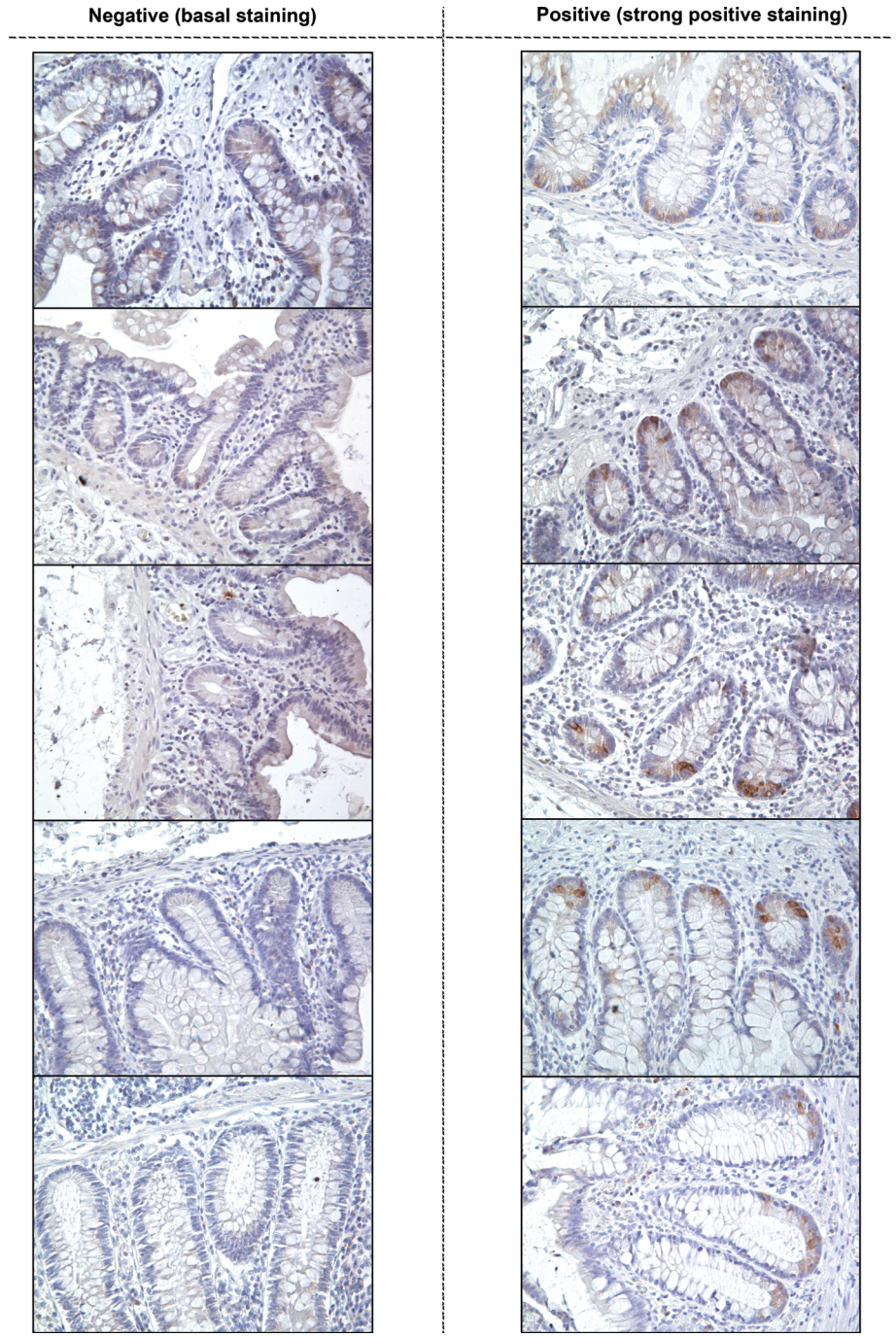


Figure S1: GRP78 score
Representative images of Paneth cells scored as GRP78 positive and GRP78 negative. Original magnification 200x.

Back to Index

Cell culture and beta-Catenin reporter assay

Wnt3a producing (Wnt3a) and control cells (L-cells) were kindly provided by Dr. R. Smits (Erasmus MC, Rotterdam, The Netherlands). Cells were stimulated for 18 h with 2μM Tunicamycin to induce ER stress, and the conditioned medium was used in a beta-Catenin reporter assay as previously described[15]. XBP1 mRNA splicing, GRP78 mRNA and CHOP mRNA expression were measured as ER stress indicators[10].

Statistics and software

Statistical analyses were performed using the SPSS 11.0 software package for Windows. Data on different patient groups were compared using the Mann-Witney *U* test, chi-square test, Fisher exact test, and the McNemar's test. Multivariate analyses relating ER-stress to different genotypes are performed using SPSS 11.0. A two-tailed *P* value <0.05 was accepted as statistically significant. Images were composed using Adobe Photoshop CS6.

Table S3: Antibodies used for IHC.

Antibody target	Catalogue number	Antibody type	Manufacturer	Dilution	Antigen Retrieval
BiP(GRP78)	3177	Rabbit monoclonal	Cell Signaling Technology	1:400	10 min boil in 10mM Citrate
Lysozyme	A009902	Rabbit polyclonal	Dako	1:800	10 min RT 10ug/mL ProtK
Phospho-EIF2α	3597	Rabbit polyclonal	Cell Signaling Technology	1:50	10 min boil in 10mM Citrate
PHLDA1 (TDAG51)	SC23866	Mouse monoclonal	Santa Cruz Biotechnology	1:100	20 min boil in 10mM TRIS
Cleaved-Caspase 3	9661	Rabbit monoclonal	Cell Signaling Technology	1:400	10 min boil in 10mM Citrate
Phospho-p38	4511	Rabbit monoclonal	Cell Signaling Technology	1:100	10 min boil in 10mM Citrate
NF-κB p65	4764	Rabbit monoclonal	Cell Signaling Technology	1:100	10 min boil in 10mM Citrate
pHiston 3H	06-570	Rabbit polyclonal	Millipore	1:1000	10 min boil in 10mM Citrate

Results

Increased ER stress in Paneth cells of ATG16L1 mutated patients

To investigate the relationship between genetic polymorphisms and ER stress in Paneth cells, cross-sections of terminal ileum biopsies from patients with quiescent CD, where stained with multiple histological markers. Only in patients with an ATG16L1 risk allele an association with GRP78 positivity (ER stress) in the Paneth cell compartment was detected (Figure 1). GRP78 staining extended to all Paneth cells of the entire biopsy in 37% of the CD patients carrying one risk allele (GA) and to 44% of those carrying two risk alleles (GG), whereas no GRP78+ Paneth cells were observed in patients without any risk allele (AA) (Figures 1A and 1D). Furthermore, in 43% of the ATG16L1 risk allele carriers (GA and GG) also pEIF2α positive Paneth cells were observed and not in the non-risk allele CD patients (AA) (Figure 1B and 1E). No histological differences were detected between the three different ATG16L1 genotypes using the PAS - Alcian Blue and lysozyme staining (Figure 1C). The Paneth cell morphology was not different in respect to ATG16L1 genotype (Figure 2A) or presence of Paneth cell specific ER stress (Figure S2A).

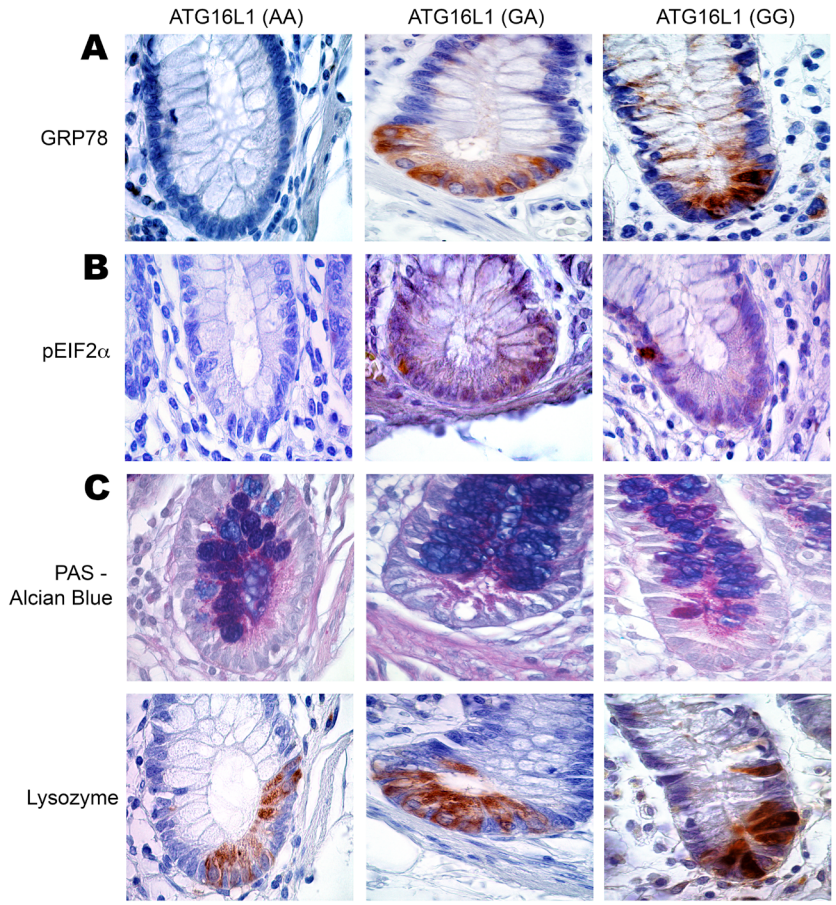
Several CD-associated gene mutations can (in-) directly affect autophagy and Paneth cell function[6, 7]. As such, this might explain the subgroup of patients with ER stress positive Paneth cells in the ATG16L1 mutants. However, excluding the patients with NOD2 (rs2066844, rs2066845), XBP1 (rs11175593) and IRGM (rs13361189) mutations did not affect the percentage of patients with GRP78+ Paneth cells (Figure 2B). In addition, multivariate analysis on the patients with GRP78+ Paneth cells regarding the ER stress associated genes did not reveal significant associations other than ATG16L1 (rs2241880, p<0.002, Table S4).

To establish whether the presence of GRP78+ Paneth cells was a local phenomenon or a constitutive property of the entire small intestine, biopsies from the duodenum and jejunum were investigated as well. CD patients with a terminal ileal GRP78+ Paneth cell phenotype, also presented with a thus affected duodenum and jejunum (Figure 2C).

Table S4, Results multivariate analysis
(ER stressed Paneth cells vs ER stress related SNPs).

SNP number	p-value*
rs2241880 (ATG16L1)	**0.002**
rs2066844 (NOD2)	0.129
rs2066845 (NOD2)	0.229
rs2066847 (NOD2)	0.134
rs13361189 (IRGM)	0.466
rs35873774 (XBP1)	0.802

*Corrected for multiple testing



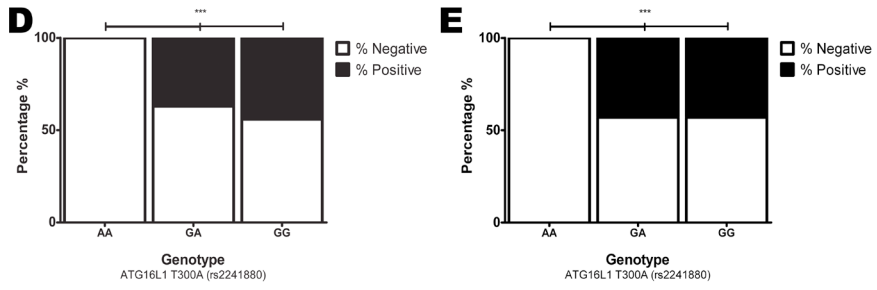


Figure 1: Intestinal crypt characterisation of inactive CD patients with the ATG16L1 (T300A) GA or GG risk allele.
(A) Representative images of small intestinal cross sections of inactive CD patients IHC stained for GRP78. The red-brown colour indicates a positive GRP78 staining. Original magnification 1000x.
(B) Representative images of small intestinal cross sections of inactive CD patients IHC stained for pEIF2α. Red-brown colour indicates positive pEIF2α staining. Original magnification 1000x.
(C) Identical cross sections as in A now stained for PAS - Alcian blue and lysozyme. The blue colour indicates mucus, and the crypt based pink-red colour represents granulomas present in Paneth cells. For the lysozyme staining the immune reactivity in cells at the crypt base indicates the Paneth cells. Original magnification 1000x.
(D) GRP78 immune reactivity scored and presented in graph. The graph represents the percentage biopsies with GRP78 positive and negative Paneth cells per genotype. The tissue specimens where blinded scored as positive or negative according the presence of GRP78 immune-reactivity. *** p<0.001
(E) pEIF2α immune reactivity scored and presented in graph. The graph represents the percentage biopsies with GRP78 positive and negative Paneth cells per genotype. The tissue specimens where blinded scored as positive or negative according the presence of pEIF2α immune-reactivity. ** p<0.01

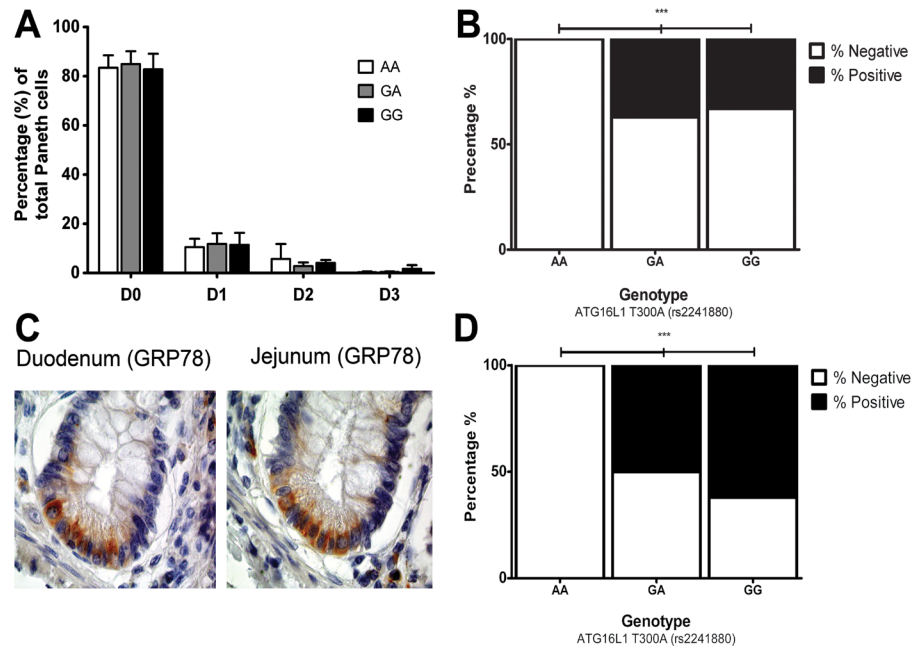


Figure 2: Paneth cell morphology and ATG16L1 specificity
(A) Paneth cell morphology. The small intestinal crypt bases were scored exactly as described before[7]. The percentage per phenotypic group was divided by the ATG16L1 T300A genotype. Error bar is SD.
(B) Other ER stress related genes excluded. Same graph as in (Figure 1D), excluded the patients with mutations in NOD2 (rs2066844, rs2066845), XBP1 (rs11175593) and IRGM (rs13361189). *** P<0.001
(C) GRP78 staining in other part of the small intestine. Tissue specimen stained for GRP78 from the same GG patient as used in (Figure 1A), now originate from the duodenum and jejunum. Original magnification 1000x.
(D) Colonic CD excluded. Same graph as (B), excluded the patients with primary colonic CD. *** P<0.001

The disease location could provide yet another factor that may be related to the GRP78+ Paneth cell phenotype. Stratifying our data based on disease location (small intestine vs. colonic CD), we observed that GRP78+ Paneth cells are a feature that is more associated with small intestine CD (Figure 2D and Table 1).

To determine if the ER-stressed Paneth cells is specific for CD patients, we also stained small intestinal biopsies of healthy controls. Both proteins, GRP78 and pEIF2α, were increased in expression in controls with the ATG16L1 risk allele (Figure 3A). The GRP78 expression is solely increased in 60% of the controls with the GG allele ($p<0.01$, Figure 3B). In 33% of the controls with the GA allele and 50% with the GG allele have increased pEIF2α protein expression ($p<0.05$, Figure 3C).

Overall, a subset of the quiescent CD patients and healthy individuals with the ATG16L1 GA or GG risk allele, have ER stressed Paneth cells in their small intestine. Paneth cells are important in regulating the intestinal stem cell niche[2] and have a clear antimicrobial function. As such, we further investigated the biological effects of the ER stress phenotype in the Paneth cells of CD patients.

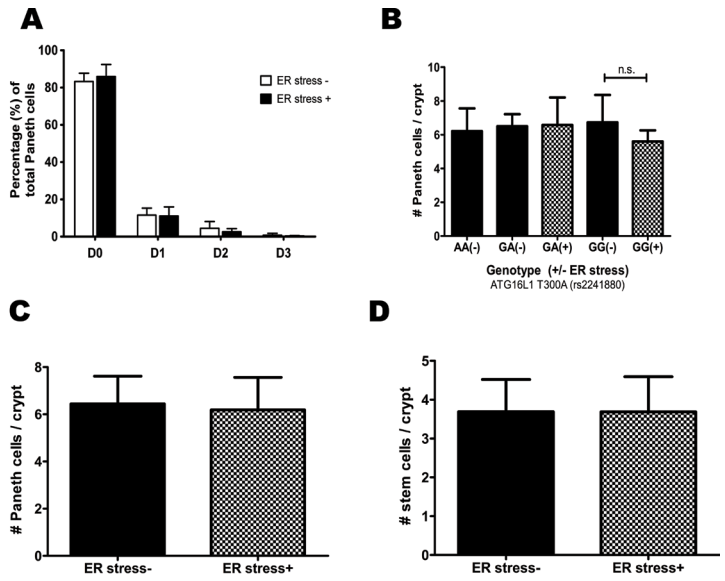


Figure S2: Paneth cell morphology and intestinal stem cell count.
(A) Paneth cell phenotype divided in ER stress (+) and ER stress (-). This graph represents the same scored Paneth cells as in Figure 2A, however now divided in ER stress (+) and ER stress (-).
(B) Number of Paneth cells. The graph represents the number of Paneth cells per crypt. All Paneth cells from at least 20 crypts of each tissue specimen were counted. AA(-): ATG16L1 (T300A) AA allele without ER stressed Paneth cells, GA(-): GA allele without ER stressed Paneth cells, GA(+): GA allele with ER stressed Paneth cells, GG(-): GG allele without ER stressed Paneth cells, GG(+): GG allele with ER stressed Paneth cells. The graphs represent the mean +\- S.D. and n.s. is not significant.
(C) Number of Paneth cells. The graph represents the number of Paneth cells per crypt divided in ER stress (-) and ER stress (+) patients.
(D) Number of Stem cells. The graph represents the number of Stem cells per crypt divided in ER stress (-) and ER stress (+) patients.
(A-D) ER stress (-) indicates biopsies without ER-stressed Paneth cells and ER stress (+) indicates biopsies with ER-stressed Paneth cells. The graphs represent the mean +\- S.D.

Table 1: Disease characteristics

	ER stress (-) N (%)	ER stress (+) N (%)	p-Value
Total number of patients	54 (100)	24 (100)	
Montreal classification: location			
L1; ileal	16 (30)	6 (25)	=0.675
L2; colonic	21 (38)	4 (17)	=0.044
L3; ileocolonic	16 (30)	11 (46)	=0.165
L4; upper disease	1 (2)	3 (12)	=0.084
Montreal classification: behaviour			
B1; no stenosis, no fistula	22 (41)	6 (25)	=0.210
B2; stenosis, No fistula	6 (11)	6 (25)	=0.117
B3; Fistula	11 (20)	10 (41)	=0.050
p; Perianal disease	17 (31)	8 (33)	=0.872
Extra-intestinal manifestations	13 (24)	7 (29)	=0.637
Intestinal surgery	29 (54)	22 (92)	=0.002

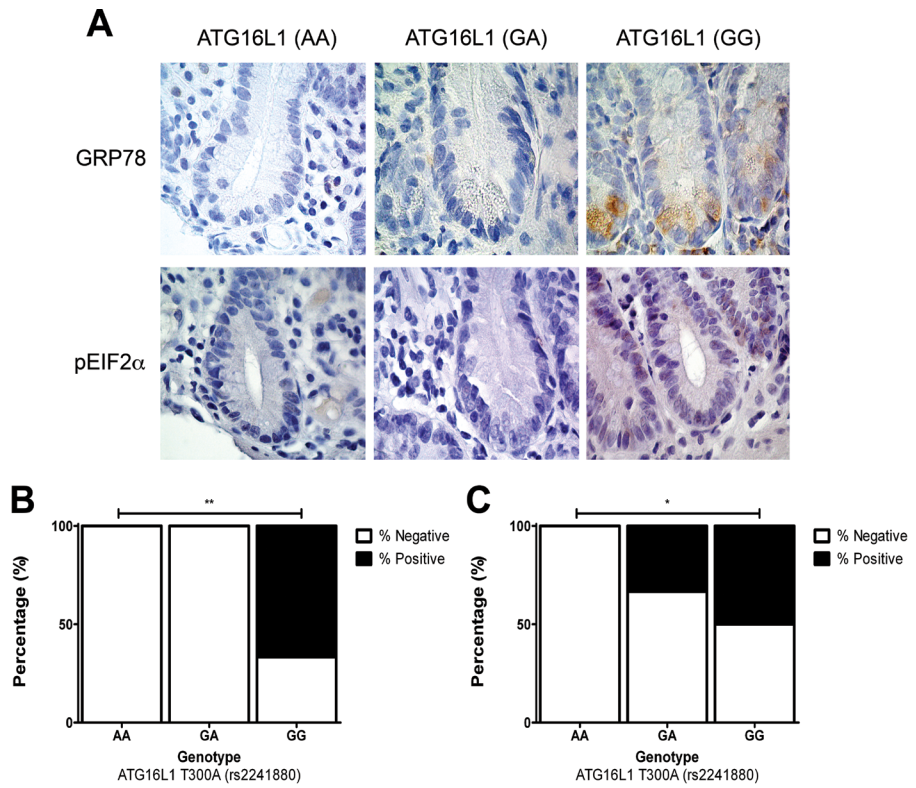


Figure 3: Paneth cell ER stress in healthy controls.
(A) Representative images of small intestinal cross sections of healthy controls IHC stained for GRP78 and pEIF2α. Red-brown participate indicate positive GRP78 staining. Original magnification 1000x.
(B) GRP78 expression score. GRP78 expression was similarly scores as in Figure 1. The GRP78 score is presented per ATG16L1 genotype.
(C) pEIF2α expression score. pEIF2α expression was similarly scores as in Figure 1. The pEIF2α score is presented per ATG16L1 genotype.

ER stress in Paneth cell is not associated with NF-κB activation

A distinctive subset of quiescent CD patients characterised by ATG16L1 risk allele-restricted Paneth cell ER stress, warrants further exploration of the exact characteristics of this phenotype. Activation of Paneth cells by bacterial ligands may enhance the demand of secretory antimicrobial peptides, potentially leading to ER stress[16] thus it is possible that the ER stress in the Paneth cells results from constitutive subclinical inflammation in the affected patients. To investigate this we stained and screened all the biopsies for the sentinel inflammatory transcription factor NF-κB using its nuclear localisation as an activation marker and the general cellular stress regulator phospho-p38MAPK. No nuclear or cytoplasmic p65 expression or increased phospho-p38 levels were found in Paneth cells of either ER-stressed or apparently normal Paneth cells (Figures 4A and 4C), thus the Paneth cell ER stress is not a secondary consequence of an on-going inflammatory reaction, but probably represents an intrinsic property of the Paneth cell compartment in ATG16L1 risk allele patients. Interestingly however, increased cytoplasmic p65 expression is seen in the stem cells and the cells of the trans-amplifying

domain from biopsies with ER stressed Paneth cells ($p<0.05$, Figure 4B), suggesting that the decreased Paneth cell functionality, as a consequence of ER stress, leads to increased inflammation elsewhere in the small intestinal crypt.

ER-stressed Paneth cells do not affect the intestinal stem cell compartment

Recently, it becomes clear that the Paneth cell is an important component of the intestinal stem cell niche through e.g. Wnt3a production. To see if ER stress could affect the expression and excretion of these growth factors, we chemically induced ER stress in Wnt3a producing L-cells. L-cells stimulated for 18 h with 2μM Tunicamycin showed increased XBP1 mRNA splicing (+150%), as well as elevated levels of GRP78 (15 fold) and CHOP mRNA (10 fold), indicating ER stress in these cells (see Figures 5A and 5B). Using the beta-catenin reporter assay, we detected high levels of active Wnt3a in the supernatant of the Wnt3a-expressing L-cells (Figure 5C). However, Tunicamycin-induced ER-stress significantly reduced the levels of Wnt3a in the supernatant by 10 fold ($P<0.001$) (Figure 5C). Hence, we provide mechanistic evidence that ER stress can affect production of growth factors needed for stem cell homeostasis. To investigate the effect of ER-stressed Paneth cells on the intestinal stem cell compartment in patients, the aamount of stem cells (PHDLA1+ cells) and the number of Paneth cells per crypt were measured (Figure 4). The number of stem cells per crypt did not differ between the groups, AA(-) 4.0; GA(-) 3.4; GA(+) 4.0; GG(-) 3.6; GG(+) 3.2; $P=0.79$. (Figure 4D) Furthermore, the amount of Paneth cells per crypt was also not affected by either ATG16L1 mutation, or ER stress+ Paneth cells, AA(-) 6.2; GA(-) 6.5; GA(+) 6.6; GG(-) 6.7; GG(+) 5.6; $P=0.92$ (Figure S2B). In the same way, the amount of Paneth or stem cells is similar when selecting for biopsies without ER stressed Paneth cells or with ER-stressed Paneth cells (Figures S2C and S2D). From these data we conclude that the ER-stressed Paneth cells do not affect the small-intestinal stem cell compartment and thus the effects seen on crypt inflammation most likely can be attributed to diminished antimicrobial activity of the Paneth cell, whereas the effects on stem cell niche function are less important.

No difference in IEC apoptosis or proliferation in patients with ER-stressed Paneth cells

In apparent agreement with this notion were experiments in which we investigated the functional effects of the observed ER-stressed Paneth cells *in vivo*. We first determined the amount of apoptotic (cleaved-caspase 3) and proliferating (pHiston 3H) IEC per crypt. No cleaved-caspase 3 was detected in the crypts of any of the biopsies, irrespective of ER-stressed Paneth cells (Figure 6A). In all the biopsies, again irrespective of the presence of ER-stressed Paneth cells, identical numbers of cleaved-Caspase 3 positive cells were detected at the top of the villi (Figure 6B).

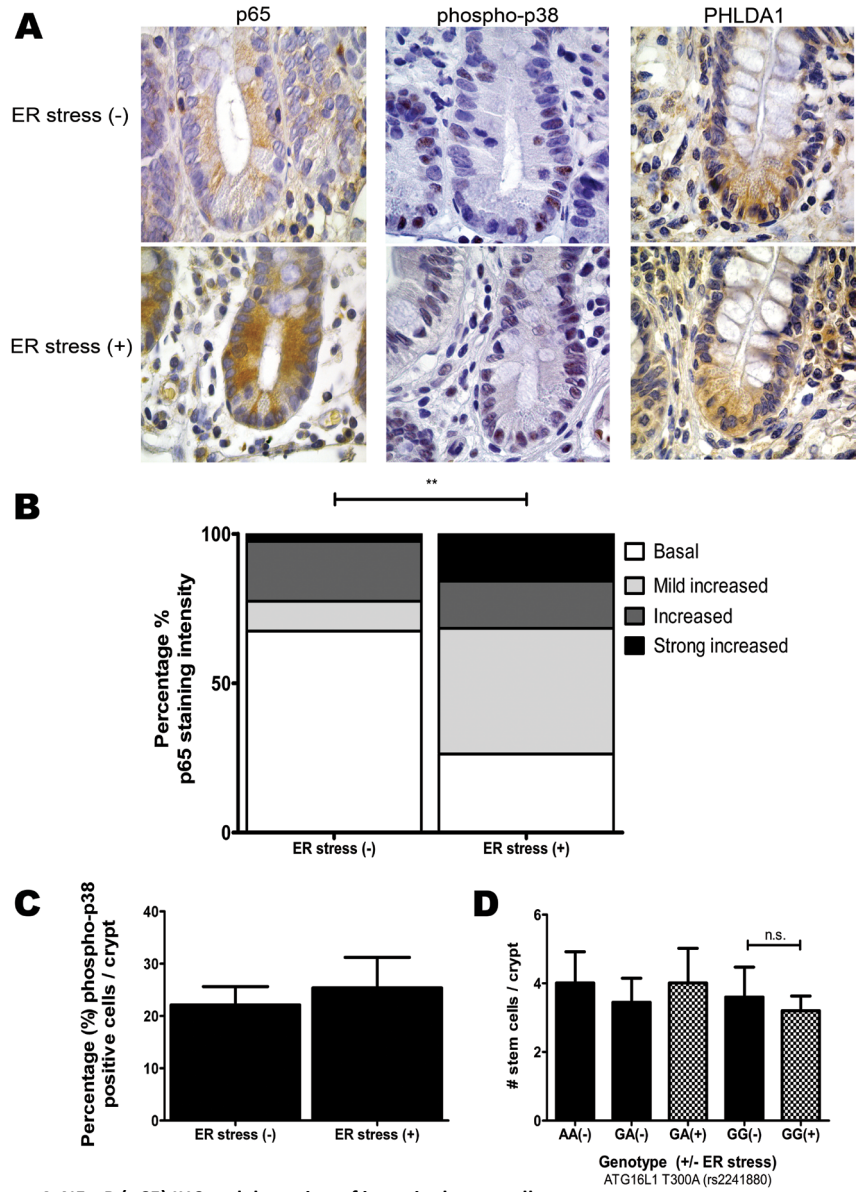


Figure 4: NF-κB (p65) IHC and detection of intestinal stem cells

(A) Cross-sections as used in Figure 1A, now stained for NF-κB subunit p65, phospho-p38MAPK, and PHLDA1. The red-brown immune reactivity represents positive expression. Original magnification 1000x.

(B) Overall p65 immunereactivity presented in a graph. All tissue specimens were scored in a blinded fashion as basal, mild increased, increased, or strong increased according the amount of positive p65 immune reactivity. The graph represents the percentages of tissue samples in with a particular score, subdivided into samples without ER stressed Paneth cells (ER stress (-)) and samples with ER stressed Paneth cells (ER stress (+)). ** $P<0.01$

(C) Phospho-p38 immunereactivity presented in a graph. All tissue specimens were scored as percentage positive cells per crypt. The graph represents the average parentage per group, error bar is SEM. ER stress (-) and samples with ER stressed Paneth cells ER stress (+).

(D) Amount of stem cells per crypt. All crypts base PHLDA1 positive cells from at least 20 crypts of each tissue specimen were counted. AA(-): ATG16L1 (T300A) AA allele without ER stressed Paneth cells, GA(-): GA allele without ER stressed Paneth cells, GA(+): GA allele with ER stressed Paneth cells, GG(-): GG allele without ER stressed Paneth cells, GG(+): GG allele with ER stressed Paneth cells. The graphs represent the mean +\- S.D. and n.s. is not significant.

Back to Index

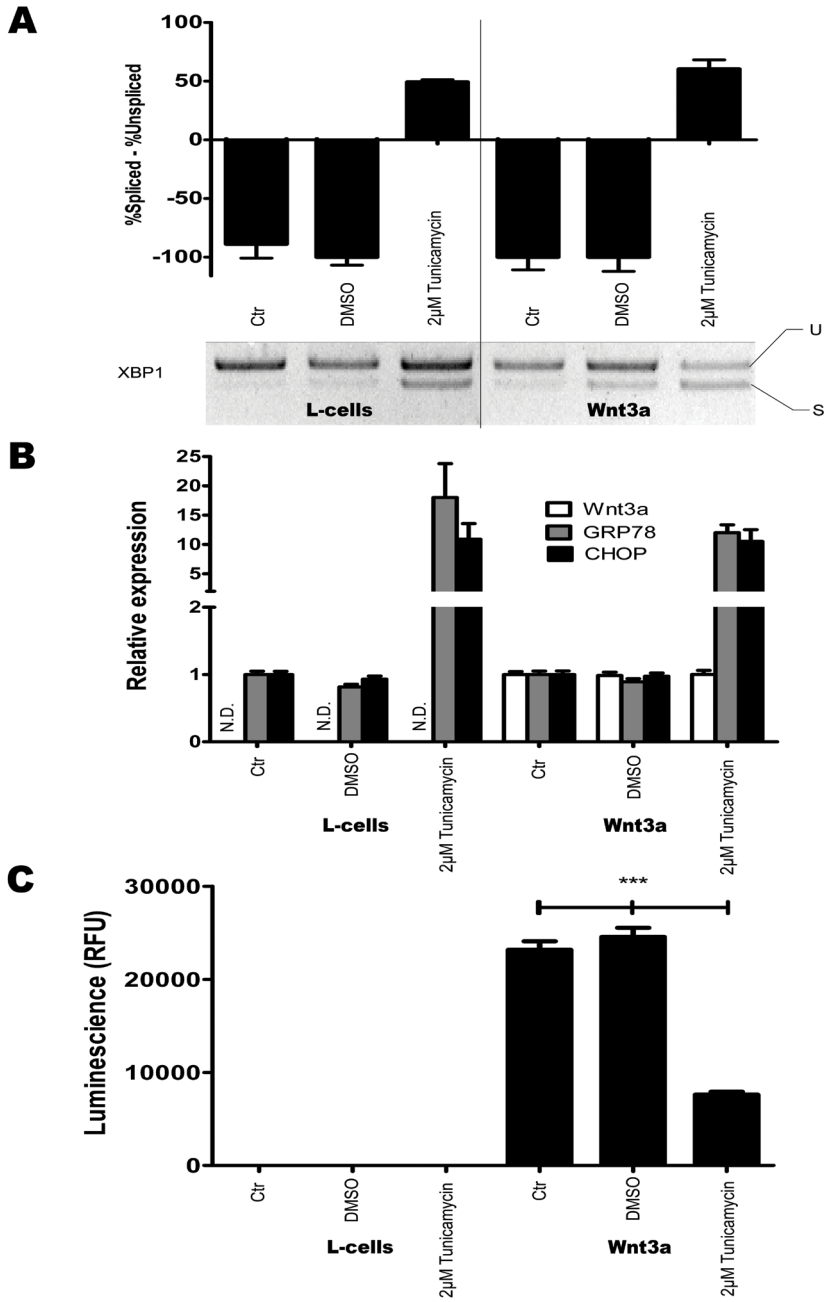


Figure 5: In-vitro Wnt3a production in L-cells with and without ER stress
(A) XBP1 mRNA splicing. From L-cells and Wnt3a producing L-cells, un-stimulated, stimulated with DMSO, or with 2μM Tunicamycin for 18 h, XBP1 mRNA splicing was determined. The ratio un-spliced (U) spliced (S) XBP1 were plotted. Ctr depicts un-stimulated cells, DMSO are cells stimulated with the same concentration DMSO as the Tunicamycin stimulated cells. The gel is a representative image of two independent experiments and the graph represents the mean +/- S.D. band-intensity of the two independent experiments.
(B) GRP78 and CHOP mRNA expression. The same mRNA samples as used in (A) were used to measure the mRNA expression of GRP78, CHOP and Wnt3a by quantitative RT-PCR. N.D. is not determinable.
(C) Wnt3a protein production. Conditioned medium from the cells as used in (A) was used in the β-Catenin reporter assay. β-Catenin reporter activity is measured and presented in a graph as relative fluorescence units (RFU). ***P<0.001

The amount of pHiston 3H positive cells per crypt did not significantly differ between the geno- and phenotypes (AA(-) 1.3; GA(-) 1.1; GA(+) 1.2; GG(-) 1.5; GG(+) 0.9; p=0.84, see Figures 6C and 6D). These results show that the ER-stressed Paneth cells are not apoptotic themselves nor do they seem to affect apoptosis or proliferation of other IEC in the crypt and demonstrate that intrinsic defects in Paneth cell functionality probably underlie the ATG16L1 SNP phenotype.

Clinical differences in patients with ER stressed Paneth cells

Although no obvious cellular differences were detected between the biopsies without ER-stressed Paneth cells (ER stress (-)) and the biopsies with ER-stressed Paneth cells (ER stress (+)), this does not rule out possible enhanced susceptibility for developing CD or disease severity in these patients, due to impeded Paneth cell function. The clinical characteristics of 78 patients with inactive disease were analysed, 54 ER stress (-) CD patients and 24 ER stress (+) CD patients. In general the demographic characteristics were not significantly different between the two patient groups (Table S2). To investigate the clinical disease severity we collected the patient characteristics according to the Montreal classification (Tables 1A and 1B). As mentioned above colonic CD occurred less often in ER stress (+) (17%) than ER stress (-) CD patients (38%, P=0.044) (Figure 1F and Table 1). We also observed a significant difference in the number of fistula between the ER stress (-) (20%) and ER stress (+) (41%) CD patients (P=0.050). In addition, far less ER stress (-) patients underwent intestinal surgery (54%) than the ER stress (+) (92%) CD patients (P=0.002), irrespective of the disease duration (Table S3). Hence, we can concluded that the ER-stressed Paneth cell phenotype is associated with a generally more complicated disease course, occurring predominantly in the small intestine, and requiring surgery more often.

Back to Index

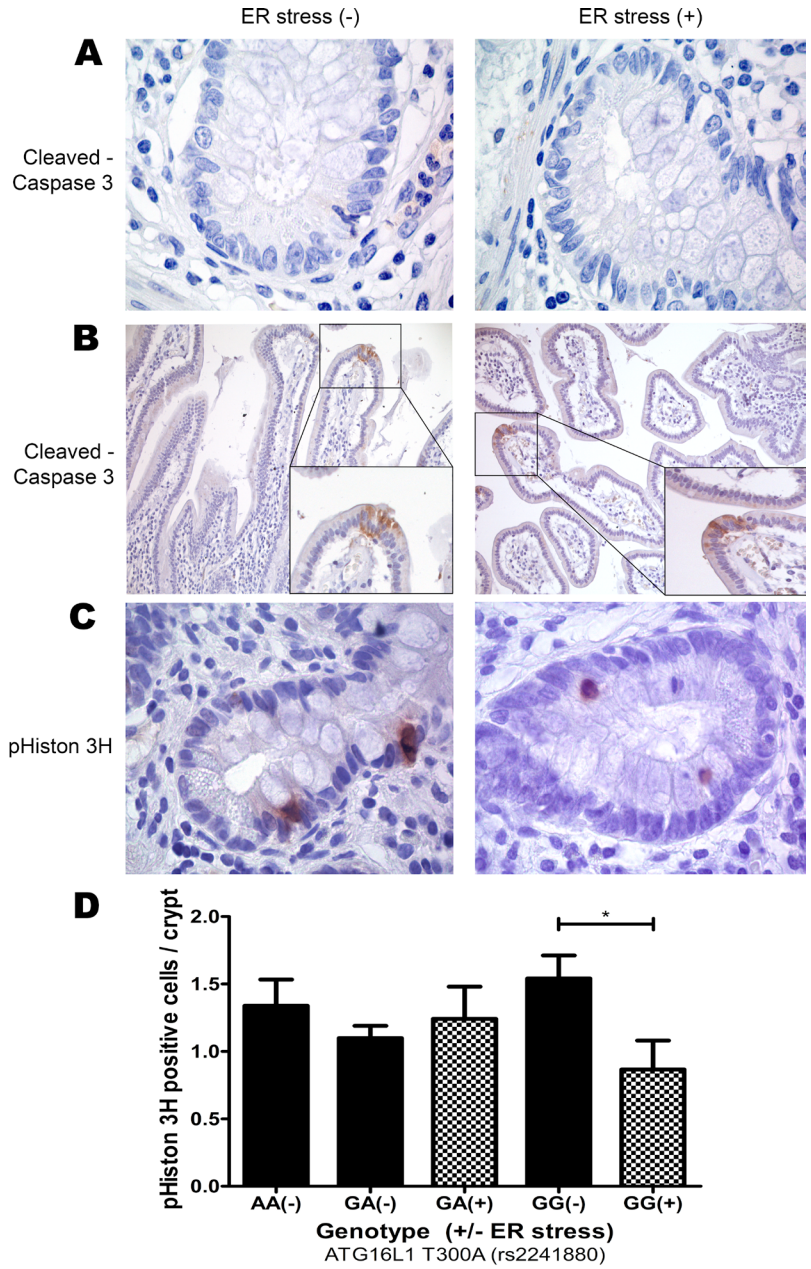


Figure 6: IEC apoptosis and proliferation.
(A) Cleaved-caspase 3 as apoptosis marker. Cross-sections as used in Figure 1A, now stained for cleaved-caspase 3. ER stress (-) represents the CD patient group without ER-stressed Paneth cells and ER stress (+) represents the CD patient group with ER-stressed Paneth cells. Original magnification 1000x.
(B) Apoptosis, only in the villi tips. The same tissue specimens as used in (A) are presented. Immune reactivity represents apoptotic cells, cleaved-caspase 3. Original magnification 200x, magnification of enlarged section 400x.
(C) Proliferation in the trans-amplifying cells. Cross-sections as used in Figure 1A, now stained for pHiston 3H. Original magnification 1000x.
(D) Quantification of crypt cell proliferation. All pHiston 3H positive and negative cells from 20 crypts of each tissue specimen were counted. AA(-): ATG16L1 (T300A) AA allele without ER stressed Paneth cells, GA(-): GA allele without ER stressed Paneth cells, GA(+): GA allele with ER stressed Paneth cells, GG(-): GG allele without ER stressed Paneth cells, GG(+): GG allele with ER stressed Paneth cells. The graph represents the mean +\- S.D. * p<0.05

Discussion

Various SNPs strongly associated with CD seem to affect bacterial/viral sensing (NOD2), ER stress (XBP1, ORMDL3) and autophagy (ATG16L1, IRGM) all of which are of crucial importance to normal Paneth cell function. In this study we provide *in vivo* evidence for the convergence of these different pathways by the detection of ER stress in Paneth cell in a subset of the CD patients carrying the ATG16L1 T300A risk allele (G).

A previous study performed in seven GG allele CD patients reported earlier that the ATG16L1 mutant genotype coincided with a particular Paneth cells phenotype, comprising of granule abnormalities[7]. Autophagy has been implicated as a crucial part of normal ER function and homeostasis, helping the ER recover from stress[17]. Autophagy can provide an additional degradation pathway for misfolded proteins or degrade damaged parts of the ER itself. This intimate relationship between autophagy and ER stress suggests that defects in autophagy may induce ER stress directly or hamper the recovery from stress. In the current study we show the occurrence of ER stress in Paneth cells in a subset of the patients and healthy controls carrying an ATG16L1 risk allele (G). Of note, we have recently demonstrated that histological assessment of ER stress by using the GRP78 protein marker, is a sustainable methodology to determine modulation in the protein folding machinery[10]. Our ER stressed Paneth cell finding was not linked to SNPs in other possible ER stress-associated genes such as, XBP1, NOD2, and IRGM, nor to active inflammation induced ER stress[10, 18, 19]. Therefore we hypothesised that the ER stress observed in Paneth cells results from a hampered recovery from stress or the increased demand for (antimicrobial) proteins downstream of NF-κB activation[16, 20]. Paneth cells stained negative for nuclear NF-κB subunit p65. Since ER stress pathways can also activate the NF-κB pathway (reviewed in[21]), our data suggest that this pathway is not activated in the ER stressed Paneth cells. This conclusion was further strengthened by the lack of increased nuclear phospho-p38 expression. In addition the ER stressed Paneth cells from the healthy controls also indicate that the ER stress is independent of subclinical inflammation. The overall p65 intensity was significantly higher in the crypts with ER-stressed Paneth cells, despite the fact that all our biopsies were histologically inactive. This can indicate that either the ER stressed Paneth cells excrete more pro-inflammatory cytokines or due to decreased antimicrobial Paneth cells function more microbial ligands reach the crypts[22].

The function and differentiation of highly secretory cells such as Paneth cells and goblet cells depend strongly on a tight basal ER stress balance[6, 7, 11, 23]. Genetic variations (e.g. NOD2, XBP1, and ATG16L1) and environmental conditions such as inflammation and ischemia can easily disturb this balance, what can result in cellular dysfunction and even apoptosis[5-7, 24]. Mutations in the NOD2 gene, one of the other important pathways in Paneth cell function, are associated with decreased defensin production[5]. In addition, in Paneth cells of severely obese individuals lower levels of defensins and lysozyme were found, which was associated with enhanced ER stress[25].

Using immunohistochemistry no obvious difference in lysozyme expression was detected in the ER-stressed Paneth cells. The lack of low levels of inflammation that is associated with severe obesity may be one of several explanations for this difference. If there are differences in the amount of defensins or lysozyme at all, the abundant lysozyme expression could make it difficult to detect the subtle differences. Furthermore, the ER stress response may be part of the high translational activity in the cell and thereby not affecting the protein expression and function. Unfortunately, faecal sampling was not part of this study and we can therefore not report on any potential shift in microbial compositions previously associated with Paneth cells defects and Crohn's disease[26, 27].

Activated ER stress pathways can initiate apoptosis (reviewed in[11, 24, 28]). Moreover, intestinal-ischemia activated ER stress induces Paneth cell specific apoptosis[24]. However, no apoptotic (cleaved-caspase 3) cells could be found in our biopsies with or without ER-stressed Paneth cells, and similar to the results in obese patients with ER-stressed Paneth cells, we also detected no differences in Paneth cell numbers[25]. This suggests that either the ER-stressed Paneth cells are able to cope with certain ER stress levels, or other environmental factors influence whether ER stress can lead to cell death[11, 25].

Recently Paneth cells have been described as important growth factor-producing cells[29], which maintain the intestinal crypt base structure by influencing stem cell homeostasis[2]. As we show that ER stress strongly reduced the Wnt3a production *in vitro* we hypothesised that ER-stressed Paneth cells could affect the intestinal stem cell population. Using a recently described human stem cell marker[30] we could detect slim cylinder-shaped cells at the crypt base. However, no difference in amount of stem cells was detected between the different patient groups. This indicates that the ER-stressed Paneth cells are mildly affected, since severe Paneth cells dysfunction leads to intestinal growth malfunctions[31] and spontaneous enteritis[6]. Biopsies with ER-stressed Paneth do not have a different amount of proliferating cells (pHiston 3H) compared to biopsies without ER-stressed Paneth cells. Since the stem cells divide slowly, we may not have been able to pick up any differences. Furthermore, the majority of the pHiston 3H positive cells are IEC part of the trans-amplifying domain. These cells are not in direct contact with the Paneth cells, and therefore likely not or less affected by the ER-stressed Paneth cells. The ER-stressed Paneth cells do not hamper the intestinal epithelium in inactive CD patients, which corresponds with the normal crypt structures in our non-active patients.

So, although no major histological differences could be detected between the tissue samples with or without ER-stressed Paneth cells, we found distinct clinical differences between the patient groups. The ER stress (+) CD patients have less colonic disease than the ER stress (-) CD patients, which is in line with the observation that XBP1$^{-/-}$ mice develop spontaneous inflammation in their ileum, due to impeded Paneth cell function[6]. The ER stress (+) patients have more fistulas than the ER stress (-) patients. Since microbes seem to be involved in the aetiology of these fistulas[32], our observation reveal an impeded anti-microbial function of the Paneth cells in the ER stress (+) CD patients. Finally, ER stress (+) CD patients more often required intestinal surgery. Interestingly, a resent publication also associates a mutation in IRGM, another autophagy related gene, with an increased need for intestinal surgery in risk allele carrying CD patients[33].

Together our data provide *in vivo* evidence for the interrelationship between autophagy and ER stress in Paneth cells of CD patients. The lack of crypt abnormalities combined with the on average more complicated course of disease suggest that ER stress is probably sensitizing the Paneth cells for further insults and may hamper recovery to normal function after inflammation. As such, ER stress resolution or prevention should carefully be considered as CD therapy. Recent studies with a specific ER stress inhibitor have demonstrated an effect in ER-stressed IEC[34]. However, future research is needed to explore the possibilities of ER stress inhibitors as therapeutic target for CD patients with the ATG16L1 risk allele.

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Back to Index

VIII

Linking Risk Conferring Mutations in NCF4 to Functional Consequeces in Crohn's Disease

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Letter to the Editor

We read with interest the paper from Muise et al. in which they describe a rare variant in the NCF2 gene, which demonstrates a diminished RAC2 binding capacity.[1] The NCF2 encoded protein p67phox is one of the components of the NADPH oxidase complex which drives the production of reactive oxygen species (ROS) during the bactericidal response of innate immune cells. Output of disturbed granulocytic ROS as a result of impaired functioning of this enzyme complex has been shown in a number of diseases, including myelodysplasia (MDS) and chronic granulomatous disease.[2,3] As Muise and colleagues point out, these diseases have been linked to development of a colitis resembling that seen in Crohn's disease (CD), suggesting a potential role for impaired ROS production in CD pathology.

Genome-wide association studies (GWAS) are a promising tool to identify genetic variants of genes linked to an increased risk of developing CD. Amongst the single nucleotide polymorphisms (SNPs) identified so far is a T to C substitution in intron 1 of NCF4, encoding the p40phox NADPH protein. Association of this SNP, rs4821544, with ileal CD was confirmed by Muise et al. However, although in general much is made of the importance of GWAS-identified risk- conferring SNPs in patients, it remains as yet unclear how these SNPs affect patient cell function, as functional studies are mostly lacking. Although Muise and colleagues very clearly show that a non-synonymous mutation in NCF2 affects its interaction with RAC2, their paper provides no direct proof that this affects granulocytic ROS production. Although making a good case, the fact that the c.113 G/A mutation is so rare (they were only able to measure ROS in one patient bearing this mutation), impedes conclusions about its general role in CD occurrence.

To further examine the potential of SNP variants in NADPH oxidase genes to confer a functional consequence in CD, we have investigated granulocyte ROS production in patients with CD bearing either the NCF4 risk allele (C), or patients homozygous for the non-risk allele (T). Patient characteristics (age, treatment, disease location, gender, fistulisation) were identical between the two groups (not shown). We observed no differences in N-formyl-methionyl-leucyl-phenylalanine (fMLP)-triggered intracellular ROS production between carriers and non-carriers of the risk allele. However, fMLP-induced ROS production was significantly lower in granulocyte-macrophage colony-stimulating factor-primed neutrophils from patients with CD with an NCF4 mutation (figure 1). These results are consistent with previous studies, showing that p40phox is important for intracellular ROS production in response to certain triggers such as phagocytosis, but plays a smaller role in phorbol myristate acetate-induced or fMLP-induced ROS production.[3] We observed no differences in granulocyte respiratory burst when patients were stratified according to ATG16L1 (rs10210302, rs2241880), IRGM (rs13361189) or NOD2 (rs2066844, rs2066845, rs2066847) SNP variants (data not shown).

As Muise and colleagues point out, the association between NCF4 and CD is not found in all GWAS studies. However, the rs4821544 SNP may define a subgroup of patients who develop CD in part as consequence of defective granulocyte ROS production. This may also explain why some studies find impaired ROS production in patients with CD, whereas others do not; none of these studies have stratified their patients according to genetic risk factors. Obviously, carrying the rs4821544 cannot be the only factor involved in development of CD, as healthy people also bear this mutation. However, impaired bacterial clearance in patients carrying this risk allele may contribute to the risk of getting CD.

Our results demonstrate for the first time that risk-conferring SNPs within the NADPH oxidase machinery lead to functional alterations in granulocyte ROS production in patients with CD. These data also show that although many of the SNPs found to be linked to CD, including rs4821544, are synonymous, they may nevertheless convey functional consequences.

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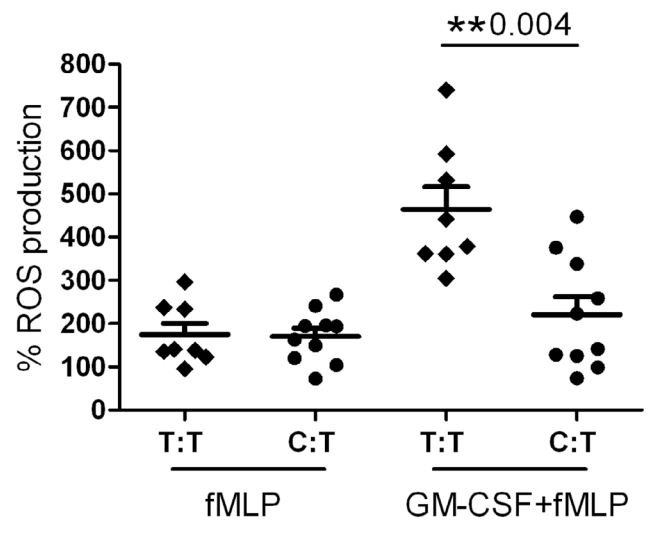

Figure 1. fMLP-stimulated ROS production is decreased in GM-CSF-primed neutrophils from CD patients bearing the NCF4 risk allele (C:T). Freshly isolated granulocytes were pretreated with 5 ng/mL GM-CSF and stimulated with 1 μM fMLP for 30 minutes. Intracellular ROS production was measured by Dihydro-rhodamine 123 assay as described[4] and the increase in intracellular ROS as result of stimulation was presented as a percentage of control (unstimulated cells). The mean ± SEM values of C:T heterozygous (n=10) and T:T homozygous (n=8) CD patients is shown. Mann-Whitney test for unpaired samples was performed. Significant differences were also observed when GM-CSF-primed cells were stimulated with fMLP for 15 minutes (not shown).

Back to Index

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Back to Index

IX

Summary and Conclusions

Summary and Conclusions

The intestinal epithelial cells (IEC) are indispensable factors in the host protection against the harmful luminal content. In this thesis we aimed to gain further insight in the role of IEC in the IBD aetiology, since it is an important mediator between the already known factors that give onset of IBD[1]. The importance of this single cell layer in health and disease is summarised in **Chapter II**. In short, direct links between the innate immune system and IEC are described. Genetic predispositions in genes associated with IBD can have modulatory effects on IEC function. Various environmental factors such as diet, intoxications, drugs and pathogens have a direct effect on the IEC barrier function. These changes are closely related to the microbial composition in the gut, which also modulates the IEC function. We concluded from this review that the function of IEC in the IBD aetiology is of utmost importance and that proper IEC function is needed to prevent disease flares. This latter suggests that manipulation of certain pathways may be interested targets of therapy to get actively inflamed IBD patients in remission.

To investigate the IEC involvement in IBD we stimulated freshly isolated biopsies from CD patients with PXR ligands (rifampicin[2], IEC specific) to inhibit the NF-κB (inflammatory key regulator[3]) signalling (**Chapter III**). Recently it was shown that in CD patients with quiescent disease PXR activation can reduce LPS-induced NF-κB activity in human IEC[4]. As such artificial inflammation induced by LPS stimulation may not necessarily relate to NF-kB signalling in active IBD. To investigate this we assessed the anti-inflammatory effects of activated PXR in active IBD by measuring NF-κB activity in freshly isolated biopsies. The expression of NF-κB regulated cytokines was lower in our rifampicin-stimulated biopsies than in the non-stimulated biopsies. However, further analysis of the data uncovered that the presence of PXR was more important than the actual PXR activation to inhibit the NF-κB activity. This observation was confirmed in *in vitro* experiments using stable PXR knock down cell lines. Complete reduction of NF-κB signalling is fatal for IEC[5], so delegate repression of the NF-κB pathway is required. Hence, compounds that increase the PXR expression to safely inhibit the inflammatory NF-κB activity in IEC could be seen as a novel therapeutic approach.

It becomes increasingly clear that the function of PXR goes beyond its primary function in the xenobiotic detoxification pathways. As such, PXR also has a modulatory function in the proliferation and apoptosis of intestinal cancers. However, there are no conclusive results about the expression of PXR in cancers and its exact role in proliferation and apoptosis[6-9]. To investigate the expression of PXR and its function in cancer cell growth, we measured the expression of PXR in biopsies from IBD and IBD-CRC (**Chapter IV**). We found high PXR expression present exclusively in the neoplastic intestinal epithelium, whereas no PXR protein could be detected in normal or inflamed intestinal tissue. *In vitro* experiments uncovered that this high PXR expression reduces the growth speed of the cells but increases the high cell density survival. Thus, the high PXR expression found in the biopsies could give the cells an advantage in surviving high cell densities. A common given chemotherapy for intestinal cancer is 5-FU. Despite the specificity of 5-FU the response rate to this compound, if given as first line therapy, is only 10-15% and decreases even further in severe metastatic disease[10]. We hypothesised that the cancer cells without PXR expression would respond better to the 5-FU treatment, since highly proliferative cells are more sensitive to 5-FU[10]. However, we found no difference in survival between the cells that express PXR and its PXR knock down counterpart by using 5-FU stimulation. This lack in difference may be a consequence of the induced expression of multi-drug resistance proteins such as, ABCG2[11, 12]. Indeed the expression of multi-drug resistance proteins was increased in these cells, irrespective of PXR activation. In conclusion, PXR is abundantly expressed in human intestinal cancers irrespective of the cancer origin or intestinal location. High PXR expression reduces cancer cell proliferation, improves high cell density survival, but in our in vitro models PXR expression is not influencing the chemotherapy resistance. With respect to the PXR mediated reduced cell survival, a therapeutic approach to inhibit the PXR expression in intestinal cancer is a promising area for further investigation.

Thus, proper cellular responses to xenobiotic insults go beyond the simple induction of detoxification enzymes but also include the modulation of pathways associated with further damage (*e.g.* the generation of cytokines) and the facilitation of regenerative responses to repair tissue damage.

IEC and their protein folding problems

Proper functioning IEC are depending on correctly folded and expressed proteins. Secreted and membrane bound proteins are mainly synthesised and folded in the endoplasmic reticulum (ER). Folding difficulties of these proteins leads to accumulation of unfolded proteins inside the ER, which activates the ER associated degradation (ERAD) pathways. Activation of autophagy, engulfment and degradation of cellular components is part of the ERAD pathway. Several factors can provoke protein misfolding, *e.g.* toxins, reactive oxygen species (ROS), cytokines and genetic mutations. In IBD patients with active intestinal inflammation increased levels of cytokines en ROS are measured. The consequence of these increased levels on ER depending protein folding is discussed in **Chapter V**. In this chapter we postulated a plausible explanation why some ER-dependent proteins, are decreased in expression during active inflammation, with ABCG2 as an example. The ABCG2 protein function and membrane localisation is dependent on post-translational protein modifications inside the ER, such as disulphide bridges[13] and n-glycosylation[14]. The creation of these modifications is sensitive to high ROS levels. Hence, we hypothesised that the decreased expression of ABCG2 during active IBD might be due to the impeded protein folding by the high levels of ROS. This hypothesis was tested in **Chapter VI**. Firstly, we confirmed that the ABCG2 membrane expression is decreased during active intestinal inflammation irrespective of intestinal location and disease type.

This decrease in expression correlated with an increase in GRP78 expression, a histological marker for ER stress, in the IEC. Secondly, we functionally supported our hypothesis by stimulating GFP-ABCG2 expressing cells with nitric oxide (NO), an ER stress inducer and major component of the inflammation produced ROS. These NO-stimulated cells have ER stress and reduced membrane bound ABCG2 expression and function. Thus, ER-dependent proteins are impeded in their expression and function due to elevated ROS production during active inflammation.

Another possible factor that can provoke protein folding problems are genetic mutations in genes involved in (XBP1[15]) and ERAD (NOD2[16, 17], ATG16L1[18], IRGM[19],) pathways. A single nucleotide polymorphism (SNP) in an autophagy related gene (ATG16L1, T300A, rs2241880) is associated with an increased risk for developing CD[18]. A modulated Paneth cell morphology was found in mice, genetically modified to express the human ATG16L1 risk allele in their IEC specifically, and human in IBD patients with the ATG16L1 (T300A) SNP[20]. Autophagy has been implicated as a crucial part of normal ER function and homeostasis, helping the ER recover from stress[21]. Autophagy can provide an additional degradation pathway for misfolded proteins or degrade damaged parts of the ER itself. This intimate relationship between autophagy and ER stress suggests that defects in autophagy may induce ER stress directly or hamper the recovery from stress. In **Chapter VII** we showed the occurrence of ER stress in Paneth cells in a subset of the patients carrying an ATG16L1 risk allele (G). As discussed in Chapter II, Paneth cells are protective cells located in the small intestine at the crypt base. These cells produce anti-microbial substances such as defensins and lysozyme, but also produce growth factors that are indispensable for the intestinal stem cell niche[22-24]. Hence, we hypothesised that these ER stressed Paneth cells are deficient in producing ER depending proteins such as lysozyme and growth factors. Although, we were enable to detect any histological difference in the patients with ER stressed Paneth cells, these patients with ER stressed Paneth cells tend to have a more severe disease course. Together our data provide *in vivo* evidence for the interrelationship between autophagy and ER stress in Paneth cells of CD patients. The lack of crypt abnormalities combined with the on average more complicated course of disease suggest that ER stress is probably sensitizing the Paneth cells for further insults and may hamper recovery to normal function after inflammation. Together, our data provide in vivo evidence for the interrelationship between autophagy and ER stress in Paneth cells of CD patients.

To further explore the role of SNP in CD patients we investigated in our final chapter (**Chapter VIII**) the role of a synonymous SNP in the NCF4 gene (rs4821544[25]) in the ROS production. Excessive mucosal ROS production can lead to impeded ER-dependent protein folding (**Chapter V and VI**), on the other hand diminished ROS production can lead to impaired bacterial clearance resulting in mucosal inflammation[26]. Interestingly, both scenarios are CD-associated, thus proper ROS production is essential for a normal gut homeostasis. As such, we measured the ROS production in granulocytes of CD patients with and without the risk allele. Activated granulocytes from CD patients with the NCF4 risk allele have lower ROS production than the non-risk allele carriers. Our results demonstrate for the first time that risk-conferring SNPs within the NADPH oxidase machinery lead to functional alterations in granulocyte ROS production in patients with CD. These data also show that although many of the SNPs found to be linked to CD, including rs4821544, are synonymous, they may nevertheless convey functional consequences.

Future perspectives

The IEC have an indispensable role in the aetiology of IBD. However, further research is needed to completely understand their exact mechanism of action. Although, we show that the presence of PXR can inhibit the NF-κB activity, it will be of great interest to find other NR family members that also can modulate the NF-κB function. If this process is better understood possible novel therapies could be implicated. However, certain notice should be taken in account since PXR expression also enhances high cell density survival. Thus, better understanding of the function of PXR in IEC is essential for possible further clinical implication.

We have shown in this thesis that inflammatory compounds, such as ROS, can modulate the expression of the membrane bound protein ABCG2. It will be of great value to verify the expression of other ER-depending proteins such as defensins, mucus, growth factors, and bacterial ligand receptors in this respect during active intestinal inflammation. Especially since recent studies already describe a modulated expression pattern of these proteins during active IBD.

GWAS associated many genomic variations to a higher risk for developing IBD, however experimental evidence to show a causal relation is majorly lacking. Thus, future IBD research should and probably will focus on the biological consequence of these SNP linked to IBD. In this thesis we already linked two SNPs to two new specific phenotypes, e.g. ATG16L1 and NCF4. As there was no other histological correlation with the ER stressed Paneth cell in ATG16L1 T300A patients, detailed expression profiles of the Paneth cells and a prospectively followed patient cohort should uncover the biological consequence of the ER stressed Paneth cells.

In conclusion

Overall we conclude that, the intestinal epithelium has a distinct function in the aetiology of IBD patients on many disease-associated factors. This thesis justifies the extensive investigation of IEC driven therapeutic approaches for IBD patients. To even better understand the gut-modulatory function of IEC, further research should focus on the function of IEC during the early stage of the disease.

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X

Nederlandse Samenvatting

Nederlandse samenvatting

De enkelvoudige cellaag tussen de darminhoud en mucosa (intestinale epitheel cellen, IEC) spelen een onmisbare rol in het ontstaan en de behandeling van patiënten met IBD. Het doel van ons onderzoek, zoals beschreven in dit proefschrift, was om een duidelijker beeld te krijgen van de functie van IEC in de etiologie van IBD. Uit eerder onderzoek is gebleken dat IBD kan voortkomen als de volgende factoren samenkomen: genomische variaties, intestinale microbiome, overactief immuunrespons en ongunstige omgevingsfactoren. Een belangrijke tussenspeler in deze factoren zijn de IEC. Het belang van de IEC bij IBD hebben wij samengevat in **Chapter II**. In dit hoofdstuk beschrijven wij o.a. de rol van IEC bij immunologische processen, de consequentie van genetische mutaties op het functioneren van IEC, het effect van dieet en omgevingsveranderingen op IEC en het effect van bacteriën op IEC. Naar aanleiding van dit review kunnen wij concluderen dat gezonde IEC onmisbaar zijn voor een goed werkend maag- en darmkanaal.

In **Chapter III** beschrijven wij een alternatieve functie voor de nucleaire receptor, de Pregnane X Receptor (PXR), in IEC uit verse darmbiopten van Crohn's disease (CD) patiënten. PXR staat bekend als receptor voor de detectie van toxische stoffen. Daarnaast heeft recent onderzoek uitgewezen dat activatie van PXR kan leiden tot verlaging van de activiteit van NF-κB (sleutelregulator bij cellulaire immuunreacties). Echter bleek uit onze experimenten dat niet de activatie van PXR, maar de aanwezigheid van PXR belangrijk is voor de verlaging van de NF-κB activiteit. Hieruit volgt dat het verhogen van de PXR expressie kan worden gezien als een nieuwe therapeutische mogelijkheid. Door onder meer dit hoofdstuk is het duidelijk dat de functie van PXR verder gaat dan zijn primaire detoxificatie taken. Als zodanig is er in de literatuur geen eenduidige conclusie over de functie van PXR bij kanker-celdeling (proliferatie) en celdood (apoptose). Daarom hebben we in **Chapter IV** de expressie en functie van PXR in IEC onderzocht tijdens de vorming van darmkanker, al dan niet gerelateerd aan IBD. In IEC van normaal of ontstoken darmweefsel is geen PXR expressie detecteerbaar in zowel de dunne als de dikke darm. Alleen tijdens de vorming van darmkanker hebben we, al vanaf een vroeg stadium (HGD), een hoge PXR expressie gemeten. Uit daaropvolgende *in vitro* experimenten bleek dat deze hoge PXR expressie de proliferatie iets vertraagd, maar wel de overleving van de cellen bevordert bij hoge celdichtheden. Uit de daaropvolgende chemoresistentie proeven bleek echter dat er geen verschil is in de chemo-overleving van de IEC met hogere PXR expressie. Hieruit kunnen we concluderen dat darmkankercellen een hoge PXR expressie hebben, welke zorgt voor een betere overleving bij hoge celdichtheden. Het verlagen van de PXR expressie in kankercellen zou hieruit volgend een nieuwe therapeutische mogelijkheid kunnen zijn.

IEC en hun eiwit vouwingsproblemen

Goed functionerend darmepitheel hangt of staat bij correct gevouwen eiwitten. Membraan gebonden en uitgescheiden eiwitten worden in het endoplasmatische reticulum (ER) gecreëerd en gevouwen. Bij eiwitvouwingsproblemen (ER stress) stapelen de niet (goed) gevouwen eiwitten op in het ER, waardoor het ER geassocieerde degradatie systeem (ERAD) wordt ingeschakeld. Een belangrijk onderdeel van de ERAD is het opnemen en afbreken van een gedeelte van het ER. Dit proces heet autophagy. Factoren, zoals toxines, zuurstofradicalen (ROS), cytokines, en genetische mutaties, kunnen leiden tot eiwitvouwingsproblemen. Tijdens actieve ontsteking in patiënten met IBD is er een verhoogde expressie van cytokines en ROS gemeten. De consequentie van deze verhoogde aanwezigheid op de eiwitvouwing in IEC is beschreven in **Chapter V**. In dit hoofdstuk beschrijven wij een mogelijk model waarom de expressie van ER afhankelijke eiwitten in IEC verlaagd is tijdens actieve ontsteking, bijvoorbeeld van ABCG2. De activiteit van de membraangebonden transporter, ABCG2, is afhankelijk van modificatie in het ER, zoals de n-glycosilatie en de vorming di-sulfiet bruggen. De vorming van deze eiwitmodificaties kan worden geremd door hoge ROS concentraties. Daardoor kwamen wij op de hypothese dat problemen met de eiwitvouwing zorgt voor de afwezigheid van ABCG2 expressie tijdens actieve ontsteking in IBD patiënten. Deze hypothese hebben wij experimenteel onderbouwd in **Chapter VI**. Eerst hebben wij bevestigd dat er geen detecteerbare ABCG2 expressie is tijdens actieve ontsteking, zowel in de dunne als de dikke darm. Deze verlaging is gecorreleerd aan een verhoogde GRP78 expressie. Hoge GRP78 expressie staat voor ER stress. Daarna is met behulp van *in vitro* experimenten aangetoond dat de ABCG2 expressie en zijn functie kan worden verhinderd door ROS geïnitieerde ER stress. Deze experimenten laten zien dat ER afhankelijke eiwitten gevoelig kunnen zijn voor hoge ROS concentraties met verminderde functie tot gevolg. Hieruit kunnen we concluderen dat de verlaagde ABCG2 eiwit expressie tijdens actieve darmontsteking mogelijk komt door de aanwezige hoge ROS concentraties.

Een andere mogelijke factor die eiwitvouwing kan verhinderen zijn mutaties in genen die betrokken zijn in de ER stress (XBP1) of ERAD (ATG16L1, NOD2, IRGM) signaalroutes. Een enkelvoudige nucleotideverandering (single nucleotide polymorphism, SNP) in een autophagy gerelateerd gen (ATG16L1, T300A, rs2241880) is geassocieerd met een verhoogde kans op het krijgen van IBD. In een eerder onderzoek is een morfologische verandering in Paneth cellen beschreven van CD patiënten met een SNP in het ATG16L1 gen. Omdat er een belangrijke relatie bestaat tussen ER stress en autophagy hebben wij gekeken naar de expressie van ER stress markers (GRP78) in darmweefsel van CD patiënten met en zonder de ATG16L1 mutatie. De GRP78 expressie was verhoogd specifiek in de Paneth cellen van patiënten met de ATG16L1 mutatie. Er zijn geen verdere pathologische verschillen gekoppeld aan de ER gestreste Paneth cellen. Echter zijn er duidelijke aanwijzingen dat patiënten met ER gestreste

Back to Index

Paneth cellen een moeilijker te behandelen ziekte hebben. Het gebrek aan histologische afwijkingen gecombineerd met een moeilijkere te behandelen ziekte wijst erop dat de Paneth cellen gevoeliger zijn voor schade en dat ze daardoor de herstelfase na een ontsteking remmen. Samenvattend hebben wij met dit hoofdstuk experimenteel bewijs geleverd voor de relatie tussen ER stress en autophagy.

In het laatste hoofdstuk (**Chapter VIII**) hebben wij de rol van SNP van CD patiënten verder onderzocht. In dit hoofdstuk onderzoeken wij het effect van mutatie in het NCF4 gen (rs4821544) op de ROS productie. Extreem hoge ROS productie kan leiden tot verminderde eiwitvouwing (**Chapter V and VI**), maar te lage ROS productie kan juist leiden tot verminderde verwijdering van bacteriën wat mogelijk resulteert in een ontsteking. Beide scenario's zijn geassocieerd met CD, waaruit blijkt dat een normale ROS productie essentieel is voor een gezonde darm. Vanwege dit gegeven hebben wij de ROS productie gemeten in granulocyten van CD patiënten met en zonder de NCF4 mutatie. De geactiveerde granulocyten van CD patiënten met de NCF4 mutatie produceren minder ROS dan granulocyten van de niet-gemuteerde CD patiënten. Deze resultaten laten zien dat een SNP in de NADPH oxidatie mechanisme kan leiden tot een vermindering in de ROS productie bij CD patiënten. Daarnaast blijkt hieruit ook dat de synonymous (non-coding) SNP belangrijk kunnen zijn in de functie van een gen.

X

De conclusie

Het wordt steeds duidelijker dat het darmepitheel op veel verschillende raakvlakken een erg belangrijke rol speelt in de etiologie van IBD patiënten. Daarnaast beschrijven wij in dit proefschrift ook enkele nieuwe mogelijke IEC gedreven therapieën voor IBD patiënten. Om waar mogelijk een nog beter beeld te krijgen van de darm modulerende functie van het darmepitheel moet de focus van toekomstig IBD onderzoek liggen bij het onderzoeken van darmweefsel van IBD patiënten vlak voor de klinische diagnose.

Back to Index

Curriculum Vitea

PhD portofolio

List of publications

Aknowledgements

(Dankwoord)

Curriculum vitae of J. Jasper Deuring

J. Jasper Deuring was born in Coevorden, the Netherlands, on the 19th of March 1985. He studied applied life sciences at the Avans Hogeschool from 2002 till 2006. He obtained his bachelor degree in 2006, directly thereafter he started working as a research technician at the Genetics Research and Development department of DSM in Delft. His projects at DSM include genetic and molecular research for microbial-strain development (confidential). In January 2008 he started working as a research technician at the Erasmus MC department Gastroenterology and Hepatology, workgroup Inflammatory Bowel Disease. Under de supervision of Dr. van der Woude and Prof.dr. Kuipers he started up his own research project, which results in a PhD project entitled: *The role of intestinal epithelium in inflammatory bowel disease and inflammation related intestinal cancer*. Under the supervision of promotores Prof.dr. Kuipers and Prof.dr. Peppelenbosch, and co-promotores Dr. van der Woude and Dr. de Haar he will defend his PhD thesis.

PhD portfolio

Certificates

- Molecular Diagnostics course, MolMed, May 2008. (1 ECTS)
- q-PCR course, University Gent, September 2008. (1 ECTS)
- Introduction to Data-Analysis, passed by written exam, NIHES, August - September 2009. (0.9 ECTS)
- Genetics and Genomics course, NIHES, August - September 2009. (1.4 ECTS)
- The Ensemble Workshop, MolMed, September 2010. (0.2 ECTS)
- English Biomedical Writing and Communication, Erasmus MC, August – December 2010. (4 ECTS)
- The Photoshop and Illustrator CS5 Workshop, MolMed, April 2011. (0.3 ECTS)

Supervision student internships

- BSc student, education: biomedical researcher, duration: 8 months, project title: *The role of TLR expression in CRC cell lines.*
- MSc student, education: medical doctor, duration: 12 months, project title: *Pregnane X receptor as therapeutic target for inflammatory bowel disease.*

Attended scientific conferences

- Dutch society for Gastroenterology (NVGE), the Netherlands, 2008, 2009, 2010, 2011, 2012.
- Young Initiative Crohn and Colitis (Y-ICC), the Netherlands, 2010, 2011, 2012.
- European Crohn and Colitis Organization (ECCO), 2009 (Germany), 2012 (Spain).
- Biochemical Society, Inflammatory Bowel Disease meeting, 2011 (UK).
- Digestive Disease Week (DDW), USA, 2011 (Chicago), 2012 (San Diego).
- Biochemical Society, Centenary Celebration, 2011 (UK) (invited speaker).
- Teaching activity of the United Euopean Gastroenterology federation (UEGF), 2011 (Italy) (invited speaker). (11 ECM)

Poster presentations

Breast cancer resistance protein (BCRP/ABCG2) expression is reduced during active colitis and translocated in IBD-related neoplasia, ECCO, February 2009, Hamburg, Germany. Poster awarded as: "Highly Recommended Poster".

ABCG2 Down Regulation In Active Inflammatory Bowel Disease Is Due To Incorrect Protein Folding, ECCO, February 2011, Dublin, Ireland.

Pregnane X Receptor is an unlikely therapeutic target for inflammatory bowel disease, ECCO, February 2011, Dublin, Ireland.
Abstract also presented at DDW, May 2011, Chicago, USA.

Activation of endoplasmic reticulum stress response and ABCG2 protein down regulation in active inflammatory bowel disease, NVGE, March 2011, Veldhoven, The Netherlands.

Pregnane X Receptor as therapeutic target for inflammatory bowel disease, ECCO, February 2012, Barcelona, Spain.
Abstract also presented at DDW, May 2012, San Diego, USA.

ER stress in Paneth cells of inactive Crohn's disease patients with ATG16L1 mutations, ECCO, February 2012, Barcelona, Spain, Poster awarded as: "Highly Recommended Poster".
Abstract also presented at NVGE, March 2012, Veldhoven, The Netherlands.
Abstract also presented at DDW, May 2012, San Diego, USA. Poster awarded as: "Poster of distinction".

Oral presentations
Impeded protein folding and function in active inflammatory bowel disease, UEGF teaching activity on basic science, invited speaker, UEGF, July 2011, Rome, Italy.

Absence of ABCG2-mediated mucosal detoxification in patients with active inflammatory bowel disease is due to impeded protein folding, The molecular biology of inflammatory bowel diseases, Biochemical Society, March 2011, Durham, UK. Presentation awarded as: "Best Presentation Award".
Abstract also presented at Biochemical Society Centenary Celebration, invited speaker, December 2011, London, UK.

Pragnane X receptor as a novel therapeutic target for inflammatory bowel disease, NVGE, March 2012, Veldhoven, The Netherlands.

Fatigue in IBD patients is associated with a change in immune parameters, DDW, May 2012, San Diego, USA.

List of publications

Y. Li, C. de Haar, M. Chen, J.J. Deuring, M.M. Gerrits, R. Smits, B. Xia, E.J. Kuipers, C.J. van der Woude, *Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis*. Gut. 2010;59(2): 227-35. Epub 2009 Nov 18.

J.J. Deuring, M.P. Peppelenbosch, E.J. Kuipers, C.J. van der Woude, and C. de Haar, *Impeded protein folding and function in active inflammatory bowel disease*. Biochemical Society Transaction, 2011. 39(4): p. 1107-11.

R. Somasundaram, J.J. Deuring, C.J. van der Woude, M.P. Peppelenbosch, G.M. Fuhler, *Linking risk conferring mutations in NCF4 to functional consequences in Crohn's disease*, Gut, 2011, doi:10.1136/gutjnl-2011-30134.

J.J. Deuring, C. de Haar, C.L. Koelewijn, E.J. Kuipers, M.P. Peppelenbosch, and C.J. van der Woude, *Absence of ABCG2-mediated mucosal detoxification in patients with active inflammatory bowel disease is due to impeded protein folding*. Biochemical Journal, 2012 441, 87-93 doi:10.1042/BJ20111281.

Y. Li, J.J. Deuring, M.P. Peppelenbosch, E.J. Kuipers, C. de Haar and C.J. van der Woude, *IL-6-induced DNMT1 activity mediates SOCS3 promoter hypermethylation in ulcerative colitis-related colorectal cancer*. Carcinogenesis, 2012, doi: 10.1093/carcin/bgs214.

Y. Li, J.J. Deuring, M.P. Peppelenbosch, E.J. Kuipers, C. de Haar, C.J. van der Woude, *STAT1, STAT6 and cAMP signaling drive SOCS3 expression in inactive ulcerative colitis*, Molecular Medicine, 2012 DOI:10.2119/.2012.00277

J.J. Deuring, E.J. Kuipers, M.P. Peppelenbosch, C. de Haar and C.J. van der Woude, *Genomic ATG16L1 risk allele-restricted Paneth cell ER stress in quiescent Crohn's disease*. Submitted.

J.J. Deuring, T. van den Berg, E.J. Kuipers, M.P. Peppelenbosch, C. de Haar and C.J. van der Woude, *Pregnane X Receptor activation constraints mucosal NF-κB activity during active inflammatory bowel disease*, Submitted.

J.J. Deuring, E.J. Kuipers, M.P. Peppelenbosch, C. de Haar and C.J. van der woude, *High Pregnane X Receptor expression in human intestinal cancer cells inhibits proliferation and prolongs survival*. Submitted.

J.J. Deuring, C. de Haar, E.J. Kuipers, M.P. Peppelenbosch, and C.J. van der woude, *The Cell Biology of the Intestinal Epithelium and its Relation to Inflammatory Bowel Disease*, Submitted.

Back to Index

G.M. Fuhler, R. Somasundaram, J.J. Deuring, C. de Haar, L. Vogelaar, C.J. van der Woude, M.P. Peppelenbosch, W.G. Kerr, *Decreased SHIP1 activity in Crohn's disease*, Submitted.

C.J. van der Woude, E. Bultman, J.J. Deuring, R.L. West, Z. Zelinkova, M.P. Peppelenbosch, *Adalimumab through levels in a prospective cohort of Crohn's disease patients*. Submitted.

L. Vogelaar, B. Aerts, J.J. Deuring, E.J. Kuipers, C. de Haar, C.J. van der Woude, *Fatigue in IBD patients is associated with a change in immune parameters*. Submitted.

K. Parikh, R. Somasundaram, L. Zhou, G.M. Fuhler, J.J. Deuring, T. Blokzijl, A. Regeling, E.J. Kuipers, R. Weersma, V. Nuij, M. Alves, L. Vogelaar, L. Visser, C. de Haar, K.K. Krishnadath, C.J. van der Woude, G. Dijkstra, K.N. Faber, M.P. Peppelenbosch, *Maintaining spontaneous and pharmacologically induced remission in Crohn's disease by active suppression of p21Rac signalling*. Submitted.

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Back to Index

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