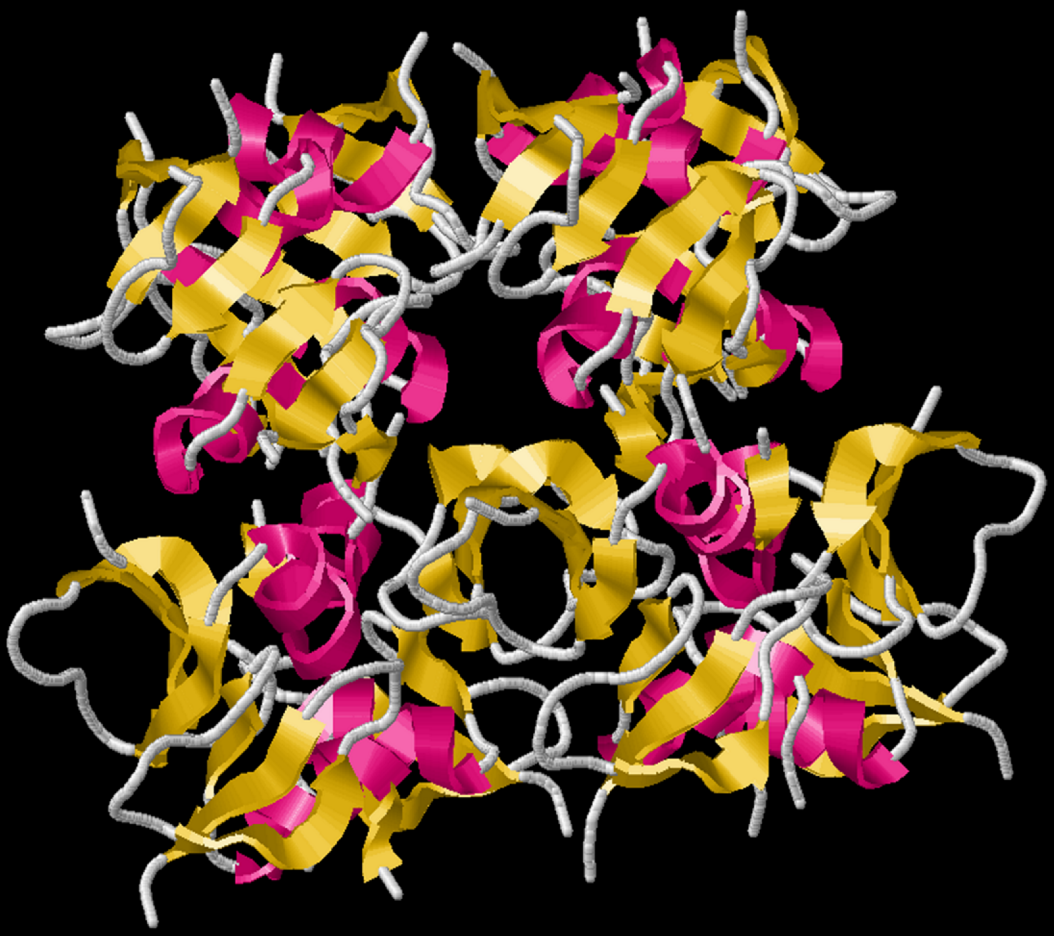
ANTI-MICROBIAL PEPTIDES IN INFLAMMATORY BOWEL DISEASE



Arne Lucas ten Hoeve, BSc
3384004
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Program: Biology of Disease, Utrecht University
Supervisors: dr. E.J.A. Veldhuizen & prof. dr. H.P. Haagsman

Abstract

Research into Crohn's disease and ulcerative colitis has elucidated the multifactorial etiology and pathogenesis of these inflammatory bowel diseases. Anti-microbial peptides are produced by many cell types as part of the innate immunity. Aside from their anti-microbial properties, these peptides also have immunomodulatory effects. Abnormalities in anti-microbial peptide production and function have been discovered in patients suffering from inflammatory bowel disease. Products of a number of genes associated with the risk of developing inflammatory bowel disease apparently influence production and function of anti-microbial peptides. This thesis explores this connection between genetics, inflammatory bowel diseases and abnormal anti-microbial peptide production and function.

Front cover: rendering of human β-defensin 2 using RasWin 2.7 (© Sayle & Bernstein) based on data from Hoover DM, Rajashankar KR, Blumenthal R, Puri A, Oppenheim JJ, Chertov O, Lubkowski J. The structure of human beta-defensin-2 shows evidence of higher order oligomerization. J Biol Chem. 2000; 275(42):32911-8.

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1 Introduction

Inflammatory Bowel Disease (IBD) is an umbrella term for the chronic immunologically mediated disorders ulcerative colitis and Crohn's disease (Sartor, 2006). Both disorders share some features, but ulcerative colitis and Crohn's disease also differ in risk factors and in clinical, endoscopic and histological features (Ordás et al., 2012). Although described by Crohn, Ginzburg and Oppenheimer as "regional ileitis" in 1932, Crohn's disease can according to the modern concept affect the entire gastrointestinal tract, even including the esophagus. The disease presents with abdominal pain, fever, diarrhea and clinical signs of bowel obstruction, caused by stenosis of the intestinal lumen (Baumgart & Sandborn, 2012; Crohn et al., 1932). Ulcerative colitis is clinically featured by symptoms that resemble those of Crohn's disease, but extends generally only from the rectum proximally into colon and is restricted to the mucosal surface (Ordás et al., 2012). The etiology of both diseases is, as in 1932, still unclear, but certain individuals seem to have a dysregulated immune response to commensal gut flora, resulting in bowel inflammation.

Susceptibility seems to be linked to genetic factors and several environmental factors may play a significant role. Prevalence of IBD is traditionally highest in Western populations (Kappelman et al., 2013). In Isreal prevalence is also considerable, while incidence and prevalence is much lower in East-Asian countries (Asakura et al., 2009; Bikenfeld et al., 2009; Zvidi et al., 2009). A comprehensive 2012 review by Molodecky et al., including figures as early as the 1930's, showed the emergence of IBD in regions traditionally low in prevalence, particularly developing countries. IBD occurs more frequently in urban than rural regions. Industrialization and urbanization are associated with changes in lifestyle, sanitation, diet and pollution exposure. These changes are viewed as potential risk factors for IBD and have been suggested to be responsible for this emergence (Molodecky et al., 2012).

Aside from some general aspects of IBD reviewed below, this thesis focusses on the fate of anti-microbial peptides in Crohn's disease and ulcerative colitis. More importantly, a number of important genetic risk factors and their consequences for anti-microbial peptide expression are discussed.

1.1 Intestinal mucosa

Mucosal-lined surfaces separate the host from the environment and as such represent the first line of defense. The mucosa of the gastrointestinal tract is continuously exposed to the gastrointestinal microbial flora, food antigens and potential pathogens. The first cell layer lining the intestinal mucosa

is the intestinal epithelium, which is itself covered by a mucous layer. This mucus layer is dense and firm due to polymerization of gel-forming mucins, which are secreted by goblet cells, closest to the epithelium and becomes more permeable towards the lumen (Geremia et al., 2013). In the colon, the inner dense layer is normally free of bacteria, but can be penetrated by micro-organisms in patients with ulcerative colitis (Ermund et al., 2013; Johansson et al., 2013). The outer layer is populated by commensals that form biofilms to prevent their removal during peristalsis (Muniz et al., 2012).

The epithelium consists predominantly of simple columnar epithelial cells and specialized epithelial cells such as the aforementioned goblet cells and Paneth cells (Geremia et al., 2013). The latter cell type is under normal conditions exclusively found at the base of the crypts in the small intestine. Metaplastic Paneth cells can be found in the stomach and large intestine under certain inflammatory conditions (Sandow & Whitehead, 1979). Intercellular tight junctions, adherens junctions and desmosomes guarantee integrity of the epithelium (Geremia et al., 2013). Both ulcerative colitis and Crohn's disease are characterized by an increased permeability of this barrier. Epithelial cells express pathogen recognition receptors (PPRs), such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs), to sense microbial-associated molecular patterns (MAMPs) from micro-organisms in the intestinal lumen (Muniz et al., 2012). Another type of epithelial cell present in the small intestine is the microfold (M) cell, which, together with follicle-associated epithelial cells, covers the Peyer's patches. These M cells can transport micro-organisms including bacteria, viruses and parasites across the epithelial barrier to leukocytes in the Peyer's patches.

Cellular immunity

Ileal Peyer's patches are one type of the lymphoid organs associated with the intestine collectively called gut-associated lymphoid tissue (GALT; Izcue et al., 2009). Isolated lymphoid follicles, like Peyer's patches located in the mucosa, and mesenteric lymph nodes into which the intestinal lymph drains are also part of the GALT. In the healthy intestine the GALT is populated by several subsets of T cells, immunoglobulin A (IgA) producing B cells, macrophages and dendritic cells (Muniz et al., 2012). Outside the GALT in the lamina propria dendritic cells can be found that are able to open junctions between the epithelial cells and sample bacteria on the luminal side (Rimoldi et al., 2004). Interestingly, these dendritic cells can discriminate between pathogenic and nonpathogenic bacteria and react accordingly. Moreover, epithelial cells can actively modulate the response of these dendritic cells. Typical for both dendritic cells in Peyer's patches and these dendritic cells is the expression of the chemokine (CX3C motif) receptor 1 (CX_3CR1; Coombes & Powrie, 2008). Dendritic cells are common in the lamina propria of the small intestine, but scarcer in the colon lamina propria and there mostly concentrated in isolated lymphoid follicles. Substantial portions of lamina propria dendritic cells are CX_3CR1^-, but express integrin subunit CD103. It is assumed that $CD103^+$ dendritic cells play an important role in maintaining immune homeostasis in the gut (Scott et al., 2011). These dendritic cells induce development of $FoxP3^+$ regulatory T (T_{reg}) cells and imprint gut homing molecules on T and B cells. Next to these peripherally developed inducible T_{reg} (iT_{reg}) cells, natural $FoxP3^+$ T_{reg} (nT_{reg}) cells are produced by the thymus (Lee et al., 2012a). Due to their ability to regulate the immune response T_{reg} cells have received attention in relation to IBD. Before the discovery of T_{reg} cells, IBD was considered to be caused by a classical T_h1/T_h2 disbalance, where Crohn's disease was assumed to be mediated by T_h1 cells and

ulcerative colitis considered to be a T_h2-type inflammation (Brand, 2009). Interestingly, while the number of $Foxp3^+$ T_{reg} cells is significantly decreased in the peripheral blood of Crohn's disease and ulcerative colitis patients, it is increased in the intestinal mucosa (Eastaff-Leung et al., 2010; Saruta et al., 2007; Yu et al., 2007).

T_h17 cells and IL-17

Another cell type that recently caught interest in conjunction with IBD is the T_h17 cell. This T cell subset produces IL-17A, commonly called IL-17, and IL-17F, which exist as homo- and heterodimers and share 60% amino acid sequence identity (Iwakura et al. 2011). IL-17 is also produced by other cell types, including $CD8^+$ T cells, NKT cells, γδ T cells, neutrophils and murine Paneth cells (Cua & Tato, 2010). Although γδ T cells form only a minor population in the peripheral blood this T cell type is well represented in the intestine and may form a significant source of IL-17 in the gut (Holtmeier & Kabelitz, 2005). T_h17 populations at effector sites, such as the lamina propria, can be suppressed by $FoxP3^+$ T_{reg} cells (Lee et al., 2012a). The IL-17 cytokine family comprises a number of other cytokines apart from IL-17A and IL-17F and was extensively reviewed by Iwakura et al. (2011).

Receptors for IL-17 family cytokines are functionally expressed as homo- and heterodimers (Iwakura et al. 2011). IL-17A and IL-17F bind to IL-17 receptor A (IL-17RA) and IL-17RC. Particularly IL-17RA is expressed extensively on epithelial cells, including SW480, HCT116, HT-29, LoVo and LS174T colorectal cell lines, fibroblasts, macrophages, dendritic cells and T cells (Eyerich et al., 2010; Liu et al., 2011). IL-17RC expression is more limited and this receptor can be found on different cell types. Stimulation of IL-17RA/IL-17RC heterodimers leads to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), MAP kinases (MAPKs) and activator protein 1 (AP-1), and CCAAT/enhancer-binding protein alpha (CEBPA; Iwakura et al. 2011). Depending on cell type, IL-17 can therefor trigger expression of many pro-inflammatory cytokines, including tumor necrosis factor α (TNF-α), IL-1, IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and chemokines.

Because of the inflammatory character of the diseases, numerous neutrophils are present in the mucosa of IBD patients outside the circulation (Alzoghaibi, 2005; Boughton-Smith et al., 1993). Macrophage infiltrates are also present in inflamed intestinal tissue of patients with Crohn's disease (Naser et al., 2011). Similarly, lamina propria eosinophil, plasma cell and macrophage infiltration is commonly seen in ulcerative colitis (Di Sabatino et al., 2012).

1.2 Cytokines in IBD

In the peripheral blood of Crohn's disease and ulcerative colitis patients the absolute and relative number of T_h17 cells is increased (Eastaff-Leung et al., 2010). In accordance with this are the raised serum IL-17 levels (Fujino et al., 2003; Mohammadi et al., 2013). Furthermore, while IL-17 expression is undetectable in healthy mucosa, in intestinal mucosa from Crohn's disease and ulcerative colitis patients $IL\text{-}17^+$ cells and IL-17 mRNA are clearly detectable and highest in patients with active disease (Fujino et al., 2003). Interestingly, Hovhannisyan et al. (2011) describe a population of $FoxP3^+$ T cells in the inflamed mucosa of Crohn's disease patients, but not in healthy control and ulcerative colitis

patients, that expresses IL-17 itself. IL-17F mRNA expression is similarly increased in inflamed mucosa of Crohn's disease patients compared to non-inflamed mucosa (Seiderer et al., 2008). In addition, overall mucosal IL-17F mRNA levels are 100 times higher in ulcerative colitis than in Crohn's disease. Expression of other T_h17-associated cytokines is also considerably higher in ulcerative colitis (Bogaert et al., 2010).

Aside from IL-17, IBD is characterized by an increase in pro-inflammatory cytokines, with Crohn's disease and ulcerative colitis in part having a distinct cytokine profile. Traditionally regarded as a T_h1-mediated disease, Crohn's disease is characterized by elevated intestinal IL-12, interferon-γ (IFN-γ) and TNF-α (Műzes et al., 2012). Ulcerative colitis is on the other hand usually described as a T_h2-type disorder by increased expression of IL-5, IL-13 and possibly IL-4. Both disease also share cytokines as serum levels of vascular endothelial growth factor (VEGF) are similarly increased in Crohn's disease and ulcerative colitis (Ferrante et al., 2006). The cytokines referred to above are not limited to either Crohn's disease or ulcerative colitis, TNF-α contributes for example to the pathogenesis of both diseases, as does the basic pro-inflammatory cytokine IL-1 (Műzes et al., 2012). Together the multitude of factors influence a large number of facets in IBD, such as decreased numbers of T_{reg} cells, recruitment of inflammatory cells, initiation of apoptotic and cytotoxic responses, fibrosis and disturbance of the intestinal barrier.

In addition to cytokines, several other factors associated with inflammation are upregulated in IBD. C-reactive protein (CRP) serum levels are known to be elevated in IBD and correlate with extent and severity of the disease (Henriksen et al., 2008; Solem et al., 2005). The acute phase protein orosomucoid 1 (ORM1) is similarly associated with IBD activity (Andre et al., 1981).

1.3 The genetics of IBD

It has been long suspected that genetics influence susceptibility to Crohn's disease (Crohn, 1934). This suspicion has been confirmed by subsequent research. For example, a study among patients with Crohn's disease in Cardiff showed the risk of siblings of these patients developing inflammatory bowel disease to be 30 times higher than the normal population (Mayberry et al., 1980). A Swedish study reported a 21 times higher prevalence of Crohn's disease among first-degree relatives compared to non-relatives (Monsén et al., 1991). In addition, ulcerative colitis was found to occur six times more often in first-degree relatives. A significantly higher concordance rate of IBD among monozygotic than among dizygotic twin pairs also points to a genetic influence on occurrence of IBD (Orholm et al., 2000). Interestingly, polyarthritis, eczema and allergic rhinitis were found to particularly common in Crohn's disease patients (Hammer et al., 1968). Similar associations between ulcerative colitis and those disease were also noted. As IBD seems to be also associated with other inflammatory diseases, this suggest a common genetic etiology.

Research has also focused on identifying specific loci and genes that influence susceptibility to Crohn's disease and ulcerative colitis. For at least nine loci (IBD1-9) that have been implicated to confer susceptibility to IBD, results have been confirmed and replicated (Gaya et al., 2006; table 1). The number of identified and in this fashion labeled loci to date reaches 28 (Guo et al., 2011). Some loci are linked to both disorders, while other are specifically associated with either Crohn's disease or

ulcerative colitis. For example, IBD1 is considered to be a contributor to susceptibility to Crohn's disease and not ulcerative colitis (Cavanaugh et al., 2001). IBD2 on the other hand appears to have only a relatively minor effect on susceptibility to Crohn's disease.

Locus	Cytogenetic location[1]	Association[1]	Candidate genes
IBD1	16q12	CD	NOD2/CARD15
IBD2	12p13.2-q24.1	Extensive UC (CD)	STAT6?
IBD3	6p21.3	UC/CD	HLA classes I-III genes
IBD4	14q11-q12	CD	EFS
IBD5	5q31	CD/UC	SLC22A4, SLC22A5
IBD6	19p13	CD/UC	CLEC2D, MAST3, IL12RB1, TICAM1, others
IBD7	1p36	UC/CD	CASP9, RUNX3, CD30?
IBD8	16p	UC	?
IBD9	3p26	CD/UC	?

Table 1. IBD1-9. Confirmed and replicated susceptibility loci for IBD.
1. OMIM 266600; Achkar et al., 2006; Wang et al., 2011.

IBD1: NOD2

In 1996 a putative Crohn's disease susceptibility locus was located in the pericentromeric region of chromosome 16 (Hugot et al., 1996). Several genes located in proximity to this locus are involved in the immune system, such as T-cell immunomodulatory protein (TIP), interleukin-4 receptor (IL-4R) and complement receptor 3 (CD11b/CD18). This region is also associated with the immune-mediated diseases sarcoidosis, atopic eczema, asthma and psoriasis (Schreiber et al., 2005). In 2001 nucleotide-binding oligomerization domain-containing protein 2 (NOD2), also known as CARD15, was mapped to the IBD1 locus (Yamazaki et al., 2002). Three NOD2 alleles are associated with Crohn's disease susceptibility and especially ileal involvement: the missense mutations Arg702Trp and Gly908Arg and the frameshift mutation Leu1007fsincC (Gaya et al., 2006). These three genetic alterations are however not found in all patients and may be mainly problematic in Caucasians. No noteworthy mutations in the NOD2 gene were found in a genetic study in 483 Japanese patients with Crohn's disease for example (Yamazaki et al., 2002). Major NOD2 variants associated with increased Crohn's disease risk in Western patients do not increase risk in Chinese, South Korean, Indian and Malaysian populations either (Ng et al., 2012). The Leu1007fsinsC variant was reported to be a risk factor of Crohn's disease in an Italian population, while an association of the Arg702Trp and Gly908Arg mutations and Crohn's disease could not be established (Vavassori et al., 2006). NOD2 is a member of the NLR family, a major subfamily of innate immunity proteins that act as pattern recognition receptors (Correa et al., 2012). The family consists of 22 human members and several orthologues and paralogues exist in other vertebrates and marine invertebrates. Structurally similar proteins are also involved in plant immunity, but are not found in insects or nematodes. A variety of cell types express NOD2, including macrophages, dendritic cells, Paneth cells, keratinocytes, podocytes, eosinophils, basophils and bronchial epithelial cells (Correa et al., 2012; Du et al., 2013; Qiu et al., 2013; Wu et al., 2013).

NOD2 is a receptor for muramyl dipeptide, a constituent of the peptidoglycans present in the bacterial cell wall (Ver Heul et al., 2013). During digestion of bacterial peptidoglycans by intracellular hydrolases

in macrophages and bacterial growth, fragments containing muramyl dipeptide are released which NOD2 can directly bind (Grimes et al., 2012; Inohara et al., 2003). Unlike the well-known transmembrane TLRs, NLRs are cytoplasmic proteins. Characteristic of NLRs is the centrally located nucleotide-binding NACHT domain, which is involved in self-oligomerization and essential for receptor activation (Correa et al., 2012). At the C-terminal region variable numbers of leucine-rich repeat domains responsible for binding of pathogen-associated or danger-associated molecular patterns (PAMPs or DAMPs) are located. NLRs can be further divided into three subgroups on the basis of their N-terminal effector domains, responsible for activating signal transduction pathways and downstream effector proteins. At least five human NLRs, including NOD2, possess the caspase recruitment domain (CARD) as effector domain, a homophilic protein interaction motif which is typically comprised of a bundle of six to seven antiparallel α-helices. Incidentally NOD2 also shares structural similarity with apoptotic protease activating factor-1 (Apaf-1) and is functionally related (Ver Heul et al., 2013).

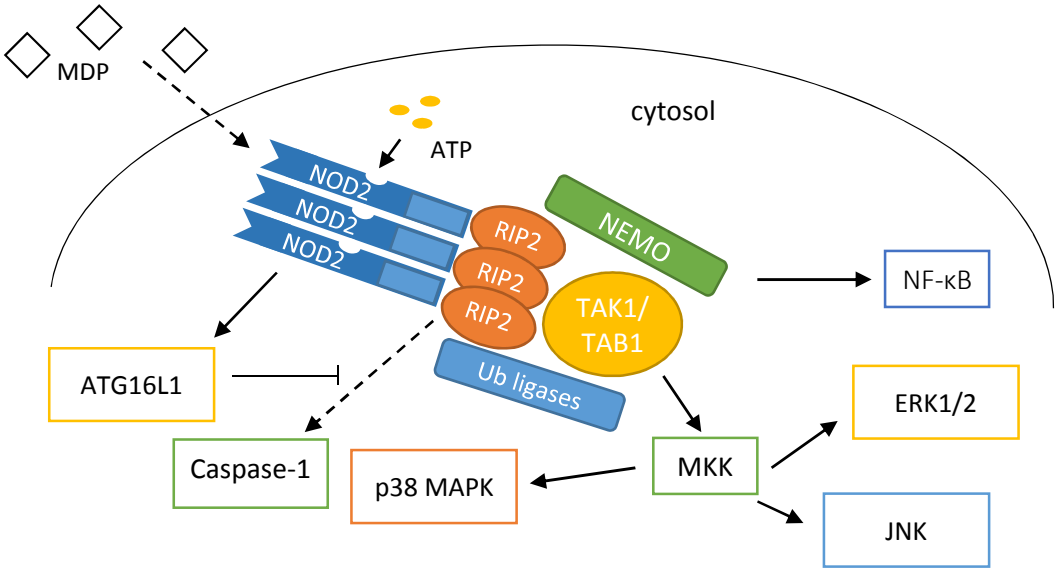

Figure 1. Principal targets of NOD2. Stimulation of NOD2 by its ligand muramyl dipeptide (MDP) results in the activation of a number of downstream targets, including mitogen-activated protein kinases (MAPKs), caspase-1, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and autophagy 16-like 1 (ATG16L1).

The nucleotide-binding domain preferentially binds ATP over other nucleotides (Mo et al., 2012). Binding ATP induces a state which allows NOD2 to bind muramyl dipeptide and to homo-oligomerize through CARD-CARD and NACHT-NACHT interaction (figure 1). Disassembly of the oligomere or hydrolysis of ATP by NOD2 shuts off the signaling complex. Binding of muramyl dipeptide likely promotes formation of an active signaling complex and substantially increases oligomerization. The core components of the signaling complex or 'NODosome' directly interact with NOD2 and recruit other effectors and mediators (Ver Heul et al., 2013). One of the possible core components is the kinase receptor interacting protein 2 (RIP_2), which contains a C-terminal CARD. RIP_2 interacts with ubiquitin ligases such as XIAP, c-IAP1 and c-IAP2, causing it to become conjugated with Lys63-linked polyubiquitin chains at Lys209 (Correa et al., 2012). The polyubiquitinated RIP_2 attracts TAK1 and TAK binding protein 1 (TAB1), which in turn activate MAPK kinases (Jacquet et al., 2008). These can

subsequently induce the p38 MAPK, JNK and ERK signaling pathways (Correa et al., 2012). In addition, RIP_2 interacts with NF-κB essential modulator (NEMO) activating the IκB kinase (IKK) complex. The IKK complex then fosforylates the NF-κB inhibitor IκBα resulting in proteasomal degradation of IκBα. The thereby liberated NF-κB translocates to the nucleus and initiates transcription of its dependent genes. Furthermore, MDP binding to NOD2 elicits a rapid autocrine interleukin-1β (IL-1β) release (Hedl and Abraham, 2011). Not surprisingly, these downstream targets of RIP_2 are also activated in response to other stress and infection-related stimuli, such as LPS, inflammatory cytokines, genotoxic stress, osmotic shock, ultraviolet light, growth factors and ischemia (Feng et al., 2011; Jacquet et al., 2008).

Targets of NOD2: p38 MAPK, JNK and ERK

The p38 MAPK subfamily consists of four members (α-γ), sharing high sequence homology, but expression varies between them in different tissue types and also their substrates differ (Feng et al., 2011). P38δ, also known as MAPK13, is found predominantly in the testes, pancreas and small intestine, while p38α and p38β, alternatively known as MAPK14 and MAPK11 respectively, are ubiquitously expressed. The latter two isoforms both activate MAPK protein kinase 2 (MK2), which stimulation is required for cytokine production and cell migration in inflammation. Furthermore, MK2 influences production of a number of pro-inflammatory cytokines, including IL-1, IL-6, TNF-α and interferon-γ (IFN-γ). Interestingly, while p38α is expressed in relatively high levels in monocytes, macrophages, neutrophils and $CD4^+$ T-cells, p38β protein expression is low in $CD4^+$ T-cells and undetectable in monocytes, macrophages and neutrophils (Hale et al., 1999). Expression of p38δ has been reported in monocytes, neutrophils and $CD4^+$ T-cells. Confusingly Ittner et al. did not detect any p38δ mRNA or protein in peritoneal macrophages, while others have reported expression at both mRNA and protein levels in peripheral blood monocyte-derived and bone marrow-derived macrophages (Ittner et al., 2012; Risco et al., 2012; Hale et al., 1999). A specific function of p38δ in immune cells has remained elusive, but its importance in neutrophil functioning was recently uncovered. Neutrophils from $p38\delta^{-/-}$ mice show a significant reduction in chemotaxis and impaired extravasation (Ittner et al., 2012). Deletion of p38δ diminished recruitment of neutrophils to the peritoneal cavity, while not affecting recruitment of macrophages and lymphocytes, and the lungs. Apparently, p38δ is a strong modulator of chemotaxis by inhibiting protein kinase D1 (PRKD1), which kinase promotes active phosphatase and tensin homologue (PTEN) thereby inhibiting chemotaxis. Recently it has been suggested that p38 MAPKs by themselves actually favor an anti-inflammatory state in macrophages and that autocrine IL-1 is responsible for the up-regulation of pro-inflammatory cytokines (Hedl and Abraham, 2012). Finally, p38 MAPKs also play an important role in the regulation of hematopoiesis (Geest and Coffer, 2009).

The c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs), are encoded by three different genes which are expressed as 10 isoforms (Geest and Coffer, 2009). The loss of individual JNKs does not impair viability of mice, indicating redundancy, while combined JNK1 and JNK2 knock outs exhibit defects in T cell activation and apoptosis of immature thymocytes, confirming the important role of JNKs in the immune system. Purified $JNK1^{-/-}$ T lymphocytes activated with low doses of anti-CD3 and anti-CD28 antibodies produce significantly less IL-2 compared to their wild-type counterparts, resulting in reduced proliferation (Sabapathy et al., 2001). Double stranded

DNA (dsDNA) is usually a potent inducer of IFN production. JNK2$^{-/-}$ mouse embryonic and 3T3 fibroblasts however showed a considerable reduction of IFN-α_1 and IFN-β mRNA synthesis compared to wild type fibroblasts (Chu et al., 1999). A partial reduction was also observed after infection of the fibroblasts with vesicular stomatitis virus. Furthermore, JNK2-deficient cells are also defective in IL-6 and IL-12 mRNA production in response to LPS, IL-1 or dsDNA stimulation. Treatment of CD4+ T-cells with a selective JNK inhibitor potently reduces production of pro-inflammatory IL-2, TNF-α, IFN-γ and anti-inflammatory IL-10 (Bennett et al., 2001). Selective inhibition of JNK also suppresses IL-6 induction by LPS in THP-1 macrophages (de Léséleuc et al., 2013). After JNK inhibition, human peripheral blood mononuclear cells (PBMCs) show a decreased production of cyclooxygenase-2 (COX-2) and TNF-α mRNA and primary human monocytes secrete less TNF-α in response to LPS stimulation (Bennett et al., 2001).

The third major class of MAPKs activated by stimulation of NOD2 are the extracellular signal-regulated kinases (ERKs). The two isoforms ERK1 and ERK2 (often jointly referred to as ERK1/2) are highly identical in sequence, but are not redundant (Roskoski, 2012). The signaling cascade ERK1/2 belongs to, the Ras-Raf-MEK-ERK pathway, participates in an extensive number of cellular processes, including cell adhesion, cell cycle progression, migration, differentiation, and proliferation. In addition, ERK1/2 is involved in the immune function. Selectively inhibiting the direct upstream activators of ERK1/2 considerably reduces CXCL8 (IL-8) mRNA and protein synthesis in airway smooth muscle cells for example (Robins et al., 2011). As CXCL8 is a potent chemo-attractant, it is not surprising that this resulted in diminished recruitment of neutrophils *in vitro*. Proliferation of splenic CD4+ T cells from ERK1 knockout mice in response to anti-CD3 stimulation was significantly reduced (Goplen et al., 2012). Furthermore, the splenic CD4+ T cells produced less IL-4 and IL-5. As is the case with p38 MAPKs, the influence of ERK1/2 on cytokine production may be partially caused by (autocrine) IL-1 (Hedl and Abraham, 2012). ERK1 deficiency also seemed to impair Th2 memory formation and eosinophil function (Goplen et al., 2012). Dendritic cells from ERK1$^{-/-}$ mice show an increased IL-12p70 and reduced IL-10 secretion in response to TLR stimulation (Agrawal et al., 2006). In addition, serum titers of IgG2b, IgG2a and IgG1 antibodies are raised in ERK deficient mice. Also, consistent with a skewing toward Th1 responses, these mice are also more susceptible to experimental autoimmune encephalomyelitis (EAE). Interestingly, the ERK pathway also seems to be of importance in autophagy as an innate defense mechanism, in a process called xenophagy. Infection of bone marrow-derived macrophages with the food-borne pathogen Listeria monocytogenes induces autophagy (Anand et al., 2011). Optimal induction is dependent on NOD1, NOD2 and TLR2. While infection with L. monocytogenes clearly led to increased autophagy levels, treatment of the macrophages with a specific MEK1 inhibitor abolished this increase.

Targets of NOD2: NF-κB

NF-κB is a transcription factor initially identified in activated B cells (Siomek, 2012). Expression of hundreds of genes, involved in cell growth, differentiation and development and immunity, is regulated by NF-κB. Members of the NF-κB family, in Mammalia composed of the five members NF-κB1 (p50), NF-κB2 (p52), RelA, RelB and c-Rel, form the transcription factor as potentially 15 different homo- or heterodimeric complexes. Promoters of the NF-κB regulated genes contain so called κB

elements that allow binding of the activated dimer. While inactive, NF-κB proteins reside in the cytoplasm bound to IκB inhibitory proteins, which cover their nuclear localization sequences. Phosphorylation of IκBs by the IKK complex disrupts this interaction and frees the NF-κB proteins to be activated and translocated to the nucleus.

The involvement of NF-κB in immunity and inflammation is elucidated by deficiencies in NF-κB family members or other proteins in the NF-κB signaling pathway. Mutations in NEMO for example are often accompanied by bacterial and viral infections due to varying immunodeficiency (Hanson et al., 2008). In vitro, NF-κB1$^{-/-}$c-Rel$^{-/-}$ CD4^{+} T cells show a significantly impaired induction of IL-2 expression compared to wild-type CD4^{+} T cells after anti-CD3 and/or anti-CD28 stimulation (Zheng et al., 2003). Furthermore, the number of NF-κB1$^{-/-}$c-Rel$^{-/-}$ CD4^{+} T cells entering the cell cycle in response to anti-CD3 stimulation is considerably lower. In mice, NF-κB1 and c-Rel knockout results in a defective antigen-induced response and reduced numbers of memory and regulatory T cells.

Given the close relation of downstream targets of NOD2 to the immune system, it is not surprising NOD2 deficiency in itself also goes hand in hand with immune system dysfunction. In the absence of NOD2 murine skin wound healing is for example substantially delayed and is accompanied by altered macrophage type polarization and changes in neutrophil recruitment at the injury site (Campbell et al., 2013). NOD2 mutations are also clearly associated with inflammatory gastrointestinal disorders in non-human mammalian species. Genetic association study and gene expression analysis show NOD2 plays an important role in susceptibility for non-specific digestive disorder in rabbits (Zhang et al., 2013).

IBD10: ATG16L1

Aside from ERK, other autophagy related factors are associated with susceptibility to Crohn's disease, like autophagy 16-like 1 (ATG16L1). ATG16L1 forms a complex with the ATG12-ATG5 conjugate, promoting elongation and completion of the autophagosome (Nguyen et al., 2013). ATG16L1, sometimes known as IBD10, was identified as a susceptibility locus on chromosome 2q in 2007 by two separate groups and confirmed by others (Cotterill et al., 2010; Ferguson et al., 2007). A single-nucleotide polymorphism (SNP) resulting in substitution of the threonine at position 300 by an alanine confers the increased risk (Nguyen et al., 2013). The polymorphism is located in the C-terminal WD repeat domain, which is completely lacking in the ATG16L1 yeast homologue and is apparently not essential for autophagy. Noteworthy is that ATG16L1 is recruited to the plasma membrane by NOD2, but this process is independent of RIP2 (Park et al., 2013). Cooney et al. report a considerable reduction in the formation of autophagosomes in MDP-stimulated dendritic cells after RIP2 knock down, indicating RIP2-independent recruitment of ATG16L1 might be of limited importance in autophagy induced by MDP (Cooney et al., 2010). Dendritic cells homozygous for the NOD2 allele 1007fsinsC show significantly impaired autophagosome formation after MDP stimulation. Dendritic cells expressing ATG16L1 Thr300Ala also failed to respond appropriately with autophagosome formation in response to MDP. As autophagy is responsible for effective presentation of loaded MHC class II at the plasma membrane, antigen presentation after MDP stimulation is impaired in dendritic cells expressing the Crohn's disease associated variants of NOD2 and ATG16L1. Consequently, these dendritic cells are considerably less efficient in inducing T cell proliferation. PBMCs homozygous for the ATG16L1 Thr300Ala polymorphism produce and secrete significantly more IL-1β and IL-6 in response to MDP

stimulation (Plantinga et al., 2011). Macrophages deficient in ATG16L1 also secrete more IL-1β after LPS and synthetic lipid A stimulation (Saitoh et al., 2008).

In the supplementary text a more complete overview of susceptibility loci for IBD can be found, IBD1 and IBD10 appear to be of particular interest in elucidating the role of anti-microbial peptides (AMPs) in Crohn's disease and ulcerative colitis.

2 Anti-microbial peptides

AMPs form one of a wide variety of mechanisms multi-cellular organisms deploy against infectious micro-organisms (Guaní-Guerra et al., 2010). This class of cationic peptides is evolutionary conserved and produced by prokaryotes, insects, plants and vertebrates. AMPs produced by bacteria are generally referred to as bacteriocins, which are also synthesized by some species of archaea (Riley and Wertz, 2002). In 1922 Alexander Fleming reported on the bactericidal qualities of nasal mucus isolated from a patient with rhinitis and other secretions (Fleming, 1922). Upon microscopic observation of lytic activity, he attributed this bactericidal effect to 'lysozyme'. Since then over 1500 AMPs of different origin have been reported (Guaní-Guerra et al., 2010). AMPs are highly structurally diverse, but can be categorized based on their amino acid composition, size and conformational structures. In humans, cathelicidins and defensins are the best documented classes.

2.1 Cathelicidins

Cathelicidins consist of a highly conserved N-terminal domain called cathelin and a C-terminal peptide with considerable variation within and between species (Guaní-Guerra et al., 2010). Humans, unlike some other mammals, possess a single cathelicidin gene located on chromosome 3: cathelicidin anti-microbial peptide (CAMP). The full length 170 amino acid inactive precursor encoded by this gene includes the bioactive peptide LL-37 in its C-terminus. CAMP is present in specific granules in neutrophils, NK cells and other lymphocytes, macrophages and monocytes, dendritic cells, mast cells, squamous epithelia and keratinocytes during inflammatory skin diseases (Sørensen et al., 2001; Vandamme et al., 2012). Furthermore, CAMP is expressed in epithelial cells located at the surface and upper crypts of normal human colon and gastric mucosa (Hase et al., 2003; Hase et al., 2002). Activation of TLRs or changes in the cytokine milieu can induce degranulation, releasing CAMP into the extracellular space (Vandamme et al., 2012). Although CAMP itself does not exhibit antimicrobial activity, cleavage by proteinase 3 from neutrophils or kallikrein from keratinocytes yields the broad-spectrum AMP LL-37. Further degradation of LL-37 by serine proteases or microbial proteases, like *Staphylococcus aureus* V8 protease, into smaller fragments, which still have antimicrobial activity, has been reported.

Antimicrobial activity

The smallest peptide that still retains antibacterial activity, KR-12, is formed by residues 18–29 of LL-37 (Vandamme et al., 2012). Like many other cationic AMPs LL-37 does not use the barrel-stave forming model to form pores in bacterial membranes, but a toroidal pore carpet-like mechanism. Essentially, LL-37 oligomers and monomers bind parallel to the surface of the membrane to the head

groups of the phospholipids. The curvature strain caused by the accumulation of membrane bound LL-37 leads to translocation of LL-37 to the periplasm and inner membrane thereby causing formation of small pores and leakage. Electrostatic interaction of LL-37 with protein complexes involved in electron transport for the production of ATP may also contribute to membrane damage. While LL-37 preferentially binds bacterial membranes, possibly due to their LPS or teichoic acid content, LL-37 still interacts with eukaryotic membranes and is, at higher concentrations, cytotoxic. Interestingly, as erythrocyte membranes contain sialic acid higher blood concentrations of LL-37 can cause hemolysis.

In addition to antibacterial activity, LL-37 and fragments thereof also possesses antifungal, antiviral and antiparasitic properties (Rico-Mata et al., 2013; Vandamme et al., 2012). The antifungal activity is based on membrane disruption through direct interaction and induction of ROS production (Vandamme et al., 2012). Disintegration of the membrane into distinct vesicles results in formation of large pores that allow diffusion of protein 40 kDa or smaller. Direct interaction with the viral envelop and capsid is primarily responsible for the antiviral qualities of LL-37. Interestingly, it has been reported that LL-37 can also inhibit HIV-1 reverse transcriptase. Trophozoites of parasitic protozoan *Entamoeba histolytica* trophozoites treated with LL-37 and four LL-37-derived peptides showed decreased integrity and viability (Rico-Mata et al., 2013).

Immunomodulation

Aside from its potent antimicrobial activities, LL-37 has immunomodulatory properties, regulates apoptosis, induces angiogenesis and promotes wound healing (Vandamme et al., 2012). Interaction with a number of receptors has been proposed to account for these effects. Although this is the subject of controversy, several publications indicate LL-37 activates the formyl peptide receptor 2 (FPR2) to induce chemotaxis in neutrophils, monocytes, eosinophils and T cells (Tjabringa et al., 2006; Vandamme et al., 2012). Recently, Subramanian et al. (2011) found immature HMC-1 mast cells not expressing Mas-related G protein-coupled receptor family, member X2 (MrgpRX2) were unresponsive to LL-37. As in mast cell lines expressing MrgpRX2 LL-37 treatment lead to chemotaxis, degranulation and CCL4 production, MrgpRX2 is likely to be another receptor for LL-37. The LL-37 homologue in rats, rCRAMP, appears to induce IL-6 mRNA expression in rat astrocytes and microglia through purinergic receptor P2Y, G protein-coupled, 11 (P2RY11; Brandenburg et al., 2010). Another receptor for LL-37 may be purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7), recently shown to facilitate phagocytosis in the absence of its ligand ATP (Vandamme et al., 2012; Wiley and Gu, 2012). The chemokine receptor CXCR2 has also been suggested to be activated by LL-37 (Zhang et al., 2009). This receptor is present on monocytes, basophils, neutrophils, T cells and epithelial cells and, as a receptor for CXCL8, important in neutrophil chemotaxis.

In addition to the cytokines mentioned above, LL-37 modulates release and production of several others (Vandamme et al., 2012). Primary monocytes produce the chemokines CXCL1, CCL4 and CCL20 and secrete IL-1β, IL-10 and CCL3 in response to LL-37. In mast cells, LL-37 induces IL-2, IL-4, IL-6 and IL-31 release and production and secretion of GM-CSF (Niyonsaba et al., 2010; Vandamme et al., 2012). Furthermore, HUVEC endothelial cells and RAW 264.7 macrophage-like cells release CCL2 after LL-37 stimulation (Vandamme et al., 2012). Similarly, macrophage-like cell lines have been shown to produce and secrete TNF-α in response to LL-37, while keratinocytes, epithelial cells and human gingival

fibroblasts secrete IL-6. 3T3 fibroblasts and keratinocytes release IL-1β and LL-37 induces also CXCL8 and IL-18 secretion in keratinocytes (Chen et al., 2013a; Niyonsaba et al., 2005; Vandamme et al., 2012). The complex immunomodulatory functions become apparent from studies that show that LL-37 can also reduce pro-inflammatory cytokine release and production (Vandamme et al., 2012). Epithelial cells and fibroblast have been shown to secrete less CXCL8 with combined LPS and LL-37 treatment for example. TNF-α secretion by THP-1 macrophages and PBMCs in response to LPS is comparably inhibited by LL-37 (Mookherjee et al., 2006). This could, at least in part, be explained by the ability of LL-37 to sequester LPS and prevent TLR4 activation. However, TLR2 agonist induced inflammatory cytokine production is also inhibited by LL-37 in PBMCs. This may be accomplished by suppressing the translocation of NF-κB to the nucleus. As cytokine production in response to TLR9 or IL-1β is not inhibited in a similar manner, this picture is still far from complete.

Within 8 hours of stimulation with the synthetic TLR3 agonist poly I:C, primary keratinocytes secrete considerable amounts of CXCL8 (Chen et al., 2013a). When combined with LL-37, CXCL8 secretion is increased 2 fold and expression is highly upregulated. Twenty-four hours after poly I:C stimulation substantial amounts of CXCL8, CXCL10, CCL5 and thymic stromal lymphopoietin (TSLP) have been secreted into the medium. Interestingly, when poly I:C is combined with LL-37 these cytokine levels are all significantly lower. Furthermore, while CXCL8 expression is increased after 8 hours treatment with the combination of poly I:C and LL-37, CXCL10, CCL5 and TSLP mRNA levels are considerably lower. In addition, LL-37 stimulation resulted in a profound enhancement of IL-17-induced CXCL8 and IL-6 secretion and expression in keratinocytes.

Noteworthy is also the suppression of apoptosis in some epithelial cell types and neutrophils by LL-37 via P2RX7, FPR2 and ERK1/2 (Vandamme et al., 2012). Conversely, apoptosis can actually be induced by LL-37 in infected airway epithelium. LL-37 fragment FK-16 induces apoptosis in LoVo and HCT116 colonic cells, which is however caused by p53 activation (Ren et al., 2013).

Modulation of cathelicidin expression

Although expression of CAMP is constitutive in neutrophils, NK cells, mast cells and epithelial cells, a number of factors can up- or downregulate its expression (Vandamme et al., 2012). Activation of TLRs induces cathelicidin expression in keratinocytes, but only weakly (Büchau et al., 2008). This is in accordance with the presence of potential NF-κB, AP-1 and CCAAT/enhancer-binding protein-β (CEBPB) binding sites in the CAMP gene (Méndez-Samperio, 2010). However, the transcription elicited by TLR stimulation is greatly enhanced by 1,25-dihydroxyvitamin D_3 (1,25D3). Even 24 hour stimulation with 1,25D3 alone potently induces CAMP mRNA expression in primary monocytes, U937 monocytes, HaCat keratinocytes and normal human keratinocytes (Liu et al., 2006; Schauber et al., 2006a). Similarly, 5 day 1,25D treatment induces CAMP mRNA and LL-37 peptide expression in skin sebocytes (Lee et al., 2012b). The expression of CAMP in U937 monocytes results in secreted LL-37 in the medium (Gombart et al., 2005). Interestingly, activation of TLR2 in monocytes and keratinocytes promotes expression of CYP27B1, which converts 25-hydroxvitamin D_3 (25D) into 1,25D3 (Hata et al., 2008). Incubation for 24 hours with the precursor 25D also results in CAMP and LL-37 expression in keratinocytes at mRNA and protein levels respectively (Schauber et al., 2006a). Bone marrow cells and bone-marrow derived macrophages from healthy individuals also show increased CAMP mRNA

expression after 3 and 5 day 1,25D3 treatment, as do HL-60 promyelocytic leukemia cells (Gombart et al., 2005). Incubation of HT-29 enterocytes with 1,25D3 induces CAMP mRNA expression, but not as robust as in myeloid cells. Like TLR activation, NOD2 stimulation with MDP does not notably induce CAMP expression in keratinocytes (Wang et al., 2010). Pretreatment with 1,25D3 followed by NOD2 activation results on the other hand in considerable expression, higher than 1,25D3 treatment alone. Expression of CAMP can also be induced in LPS-primed THP-1 macrophages with 25D treatment. Interestingly, the vitamin D derivate calcipotriol decreases stimulant-induced cathelicidin mRNA and protein expression (Kim et al., 2009). A small-scale study found oral vitamin D_3 to increase cathelicidin expression in lesional skin of patients with atopic dermatitis (Hata et al., 2008).

The influence of vitamin D_3 on CAMP and LL-37 expression is caused by the presence of a classical DR3-type vitamin D response element (VDRE) in the CAMP promoter (Gombart et al., 2005). The VDR binds to the VDRE in both a ligand-dependent and -independent manner, regulating transcription. For high affinity binding, heterodimerization with related retinoid X receptors (RXRs) is necessary (White, 2010). Curiously, while the VDRE is evolutionary conserved in human and chimpanzee genomes, it is absent in rat, dog and mouse. Incidentally, as a VDR agonist, the secondary bile acid lithocholic acid (LCA) can induce CAMP expression (Termén et al., 2008). The transcription factor spleen focus forming virus proviral integration oncogene (SPI1 or PU.1) appears to be important in this induction as well. Both LCA and 1,25D3 induce another vitamin D responsive gene in HT-29 cells, FET colon carcinoma cells, normal human keratinocytes and HaCat cells that encodes CYP24A1 (24-hydroxylase), an enzyme that degrades 1,25D3 into an inactive form.

Peric et al. (2008) reported over 94% of primary human keratinocytes express the IL-17 receptor A (IL17RA). Stimulation of the keratinocytes with its ligand Th17 cytokine IL-17A resulted in significantly increased expression of IL-6 an IL-8. Remarkably, treatment with 1,25D3 almost completely abolished the increase in IL-6 expression and significantly reduced IL-8 expression. In accordance with literature, 1,25D3 alone resulted in induction of cathelicidin expression. More importantly, while IL-17A alone had no effect, IL-17A and 1,25D3 synergistically increased expression of CAMP in keratinocytes. Other cytokines have been reported to increase cathelicidin expression, such as IL-1α in keratinocytes and IL-13 synergistically with 25D in bronchial epithelial cells (Erdag et al., 2002; Schrumpf et al., 2012). IL-13 and anti-inflammatory cytokine IL-10 on the other hand suppress LL-37 mRNA expression in HaCaT keratinocytes stimulated with anti-CD3-stimulated PBMCs (Howell et al., 2005).

Micro-organisms and microbial products can also influence production of LL-37. The bacterial metabolite butyrate for example stimulates LL-37 expression in HT-29 cells (Schauber et al., 2006a; Termén et al., 2008). Considering butyrate is an end product of fermentation by human faecal microflora, it is not surprising LL-37 expression in monocytes and keratinocytes appears not to be induced by butyrate (Pryde et al., 2002; Schauber et al., 2006a). Infecting monocyte-derived macrophages with *Mycobactrium tuberculosis* resulted in significantly increased cathelicidin expression comparable to LPS stimulation (Rivas-Santiago et al., 2008). Furthermore, exposure of lung epithelial cells and neutrophils to either *M. tuberculosis* or *Salmonella* Thyphi induces LL-37 protein expression in these cells. In contrast, some micro-organisms possess the ability to downregulate LL-37 expression. The bacterial exotoxins cholera toxin and labile toxin, produced by *Vibrio cholerae* and

enterotoxigenic *Escherichia coli* respectively, markedly suppress CAMP expression in HT-29 cells, resulting in less abundant LL-37 presence (Chakraborty et al., 2008). Stimulation of Caco-2 and T84 colonic and INT407 small intestine epithelial cells with cholera toxin similarly reduced CAMP expression. Treatment of mice ileal loops *in situ* with *V. cholerae* or enterotoxigenic *E. coli* diminished expression of mouse CAMP homologue CRAMP at both mRNA and protein levels in the mucosal epithelium. Islam et al. (2001) showed gut biopsies from adults with shigellosis caused by either *Shigella dysenteriae* type I or *S. flexneri* were significantly less often positive for CAMP mRNA than from healthy controls. Even when the patients were considered clinically recovered after 60 days, there was a significant difference between both groups. Interestingly, while the LL-37 peptide was undetectable or present at very low levels in epithelial cells early in the infection, leukocyte infiltrates loaded with LL-37 were visible. *In vitro*, HT-29 enterocytes and U937 monocytes showed diminished cathelicidin mRNA and protein levels upon infection with *S. dysenteriae* type I.

2.2 Defensins

The second well-described class of AMPs in humans comprises the defensins, characterized by six highly conserved cystein residues (Guaní-Guerra et al., 2010). Based on the alignment of the disulfide bridges these cystein residues form, the class is further divided into α-, β- and θ-defensins. Mutation experiments with the mouse α-defensin cryptdin-4 showed the disulfide bridges are not required for antimicrobial activity its activity (Conibear et al., 2013). The mutants lacking disulfide bridges are however particularly sensitive for cleavage by matrix metalloproteinase-7 (MMP-7). Disulfide mutants of β-defensin 3 equally retain their antibacterial activity, but lack the chemo-attractive quality wild-type β-defensin 3 has. θ-Defensins are structurally distinctly different from the other defensins due to their cyclic structure. While disulfide bridges have an important role in θ-defensins stability, again they are not required for antibacterial activity. In contrast, removal of one disulfide bridge from human α-defensin 5 (HD5) attenuates its activity against *S. aureus* (Wanniarachchi et al., 2011). Although α- and β-defensins are expressed at protein level in humans, neutrophils lack θ-defensins peptides (Lehrer et al., 2012). Human bone marrow contains mRNA that resembles the mRNA of rhesus θ-defensin precursors closely, but contains an additional stop codon in its signal sequence. Six α-defensins have been identified in humans, of which 4 are present in neutrophils: human neutrophil peptides (HNPs) 1-4. Since HNP1-3 vary only by one amino acid they are commonly not differentiated. Notwithstanding their designation expression is not limited to neutrophils and HNPs are expressed by Paneth cells, different tissues in the mouth and tracheal epithelium (Jarczak et al., 2013). HD5 and human α-defensin 6 (HD6) are also expressed in neutrophils and constitutively produced by Paneth cells in the small intestine. In addition, HD5 is expressed by 'intermediate cells' in the villi of the small intestine (Cunliffe, 2003). The human genome encodes more than 30 different β-defensins, but to date only 11 β-defensins, namely hBD-1 through -6 and hBD-25 through -29, have been described (Jarczak et al., 2013). Human β-defensin 1 (hBD-1) is considered the most important AMP constitutively produced by epithelial cells at many sites, including the colon, small intestine, female reproductive system, thymus and placenta. Expression of hBD-2 (confusingly also known as β-defensin 4A) is also extensive and includes epithelial cells in the gastrointestinal tract, bone marrow, leukocytes and keratinocytes. A

variety of tissues express hBD-3 (β-defensin 103) and hBD-4 (β-defensin 104), including the esophagus and stomach respectively.

Antimicrobial activity

Like cathelicidins, human defensins have broad spectrum antimicrobial activity. HNP-1 has shown activity against a number of bacterial strains, such as *Enterobacter aerogenes*, *Mycobacterium avium, M. tuberculosis*, *S. aureus*, *E. coli* and *Streptococcus pneumoniae* (Habets et al., 2012; Jarczak et al., 2013). HNP-4 and HD5 are both bactericidal to *E.coli* and HNP-2 and HD5 to *S. aureus* (Jin et al., 2004; Wu et al., 2004). Antibacterial activity of HNP-1, -2 and -3 against *M. tuberculosis* and *S. aureus* has also been reported (Jarczak et al., 2013). Surprisingly, HD6 shows little activity against *E.coli* or *S. aureus* (Wu et al., 2004). Similar to α-defensins, β-defensins have antibacterial activity towards both Gram-negative and Gram-positive strains. hBD-1, -2 and -3 are all bactericidal to *Pseudomonas aeruginosa* and *E. coli* and hBD-3 shows antibacterial activity against *Enterococcus faecium*, *S. aureus*, *Streptococcus pyogenes* and *S. pneumoniae* (Schneider et al., 2005). HBD-4 is strongly active against *P. aeruginosa* and *Staphylococcus carnosus*, but not *E. coli* and *S. pneumonia*.

Defensins exert their antibacterial activity through a multitude of mechanisms, some of which are poorly understood. α-Defensins are thought to generally cause bacterial cell death via membrane disruption (Hadjicharalambous et al., 2008). Experiments on *E. coli* indicate mouse α-defensin cryptdin-4 rapidly permeabilizes the target cell membrane, which is enhanced by cardiolipin, a constituent of bacterial membranes. Transient membrane defects allow cryptdin-4 to insert into the membrane and then translocate to the inner membrane leaflet. Zhang et al. (2010) consider two HNP-1 molecules that line a small central water pore spanning the target membrane to be the most probable configuration. Observations at concentrations close to the minimal inhibitory concentration indicate interference with cell wall synthesis accounts, in part at least, for the antibacterial activity of hBD-3 (Sass et al., 2010). HBD-3 interacts with lipid II, a carrier that facilitates translocation of peptidoglycan components over the cytoplasmic membrane, and inhibits its function. This interference with cell wall biosynthesis resulted in *S. aureus* in protrusions of cytoplasmic contents in some cells. Interestingly, this mechanism of action of hBD-3 may be highly similar to that of oyster defensins (Schmitt et al., 2010).

Although HD6 has little antibacterial activity *in vitro*, HD6 expression in the intestine reduces mortality in mice infected with *Salmonella* Typhimurium (Chu et al., 2012). Six days after intragastric challenge mortality reached 50% in the wild type groups, while survival was 100% in the transgenic mice expressing HD6 in Paneth cells at levels comparable to α-defensin production. While bacterial numbers in the instestinal lumen were similar in both groups, the bacterial burden in the spleen and Peyer's patches of the transgenic mice was significantly lower. Further experimentation indicates HD6 may bind to proteinaceous bacterial surface molecules, initiating self-assembly of 'nanonets' by other HD6 molecules. These nanonets could function to prevent close interaction with bacteria and bacterial invasion, explaining the inhibition of invasion by *Yersinia enterocolitica in vitro*.

Antimicrobial activity of defensins is not restricted to bacteria. HNP-1 and -2 inhibit growth of fungus *Cryptococcus neoformans* at concentrations comparable or lower to those lethal to bacteria (Schneider et al., 2005). Furthermore, the fungi *Candida albicans* and *Malassezia furfur* are both effectively killed

by hBD-1, -2 and -3 (Schneider et al., 2005). Trophozites of the parasitic protozoan *E. histolytica* also appear to be permeabilized by hBD-2 (Ayala-Sumuano et al., 2013). In addition, HNP-1 have been observed to inhibit herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and cytomegalovirus (Gwyer Findlay et al., 2013). HD5 and -6 have also been found to be active against HSV-2. Other viruses inactivated or inhibit by α-defensins include HIV-1, influenza A and several non-enveloped viruses. The mechanism of action varies from interference with endosomal trafficking essential for influenza A to preventing viral entry in case of HSV. Of the β-defensins, only hBD-3 is known to have notable activity against HSV. HBD-2 however was found to inhibit viral entry of respiratory syncytial virus (RSV).

Immunomodulation

Next to antimicrobial properties, like cathelicidins, defensins have immunomodulatory effects. Monocyte-derived dendritic cells treated for 18 hours with HNP-1 or hBD-1 secrete significantly more IL-6, TNF-α and IL-12p70 than untreated, while IL-10 secretion is unaffected (Presicce et al., 2009). In addition, HNP-1 and hBD-1 increase cell-surface expression of co-stimulatory molecules CD80 (B7-1), CD86 (B7-2) and CD40 and HLA-DR. hBD-3 also increases expression of CD80, CD86 and CD40 on dendritic cells and monocytes, which is apparently mediated by interaction with both TLR1 and TLR2 (Funderburg et al., 2007). This is not unlike mouse β-defensin 2 (mBD-2) which has been reported to interact with TLR4 (Röhrl et al., 2010a). Finally, dendritic cells treated with HNP-1 and hBD-1 also induced proliferation of a higher proportion of allogeneic T cells (Presicce et al., 2009).

LPS-primed macrophages release the pro-inflammtory cytokine IL-1β in response to HNP-1 treatment (Chen et al., 2013b). Apparently this activity is achieved through interaction with P2RX7. Expression of IL-1β and CXCL8 mRNA is increased dose-dependently in primary human bronchial epithelial cells by HNP-1, but not hBD-1 (Sakamoto et al., 2004). NF-κB DNA binding and CXCL8 secretion are also enhanced by HNP-1. Likewise, mRNA expression of connective tissue growth factor (CTGF) is increased in human bronchial epithelial cells and lung fibroblasts by HNP-1 stimulation, as is TGF-β1 mRNA and protein expression in lung fibroblasts (Amenomori et al., 2010). Treatment of HT-29 cells with HD5 notably increases CXCL8 secretion (Ishikawa et al., 2010). Treatment of LAD2 mast cells or PBMC-derived mast cells with hBD1-4 increases mRNA and protein expression and release of IL-31 compared to untreated cells (Niyonsaba et al., 2010). Normal human keratinocytes incubated with HBD2-4 show increased IL-18 mRNA expression and secretion (Niyonsaba et al., 2005).

Recently, Tewary et al. (2013) reported on HBD-3's ability to bind human DNA and form large complex DNA nets. While hBD-3 itself appeared to induce some IFN-α secretion by human plasmacytoid dendritic cells and human DNA did not, 24 hour treatment with the hBD-2/DNA or hBD-3/DNA complexes resulted in substantial amounts of IFN-α being released. Furthermore, both hBD-2 and hBD-3 promoted uptake of DNA. Results from experiments with CpG instead of human DNA were comparable. Tewary and collegues further showed the IFN-α release induced by hBD-3/DNA complexes is dependent on TLR9 and treatment of mice with hBD-3/CpG complexes significantly raises serum concentrations of both inflammatory cytokines and IL-10. Of note is also that splenocytes from mice immunized with OVA and hBD-3 when treated with OVA *ex vivo* secreted considerably amounts of IL-5.

Like LL-37, some defensins are reported to have chemo-attractive properties. HBD-2 is chemotactic for $CD45RO^+$ memory T cells and for immature dendritic cells as are mBD-2 and mBD-3 for immature mouse dendritic cells (Röhrl et al., 2010a). In addition, mBD-4:Ig and hBD-2:Ig fusion proteins are chemotactic for human peripheral blood monocytes (PBMs) and mouse resident peritoneal cells (RPCs). Furthermore, hBD-2, hBD-3 and their mouse orthologues were found to be chemotactic for PBMs (Röhrl et al., 2010b). Chemokine (C-C motif) receptors 6 (CCR6) and 2 (CCR2) are thought to mediate this chemo-attraction. Consequently, PBM chemotaxis in response to hBD-2 and hBD-3 could be inhibited by pretreatment with CCR2 agonist CCL2 causing desensitization.

Modulation of defensin expression

While CAMP expression is only weakly under the control of TLRs, activation of these receptors can influence defensing expression considerably. Unlike epithelial cells, peripheral leukocytes do not constitutively express hBD-1 (Fang et al., 2003). Its expression is however strongly induced by LPS in these cells. HNP-1-3 expression is constitutive and is not altered by LPS stimulation. Stimulating SW480 colon epithelial cells with peptidoglycans or LPS increases hBD-2 mRNA and protein expression (Vora et al., 2004). LPS treatment of T84 and Caco-2 cells transfected with TLR4 and MD-2 also induces the hBD-2 gene. Secretion and expression of hBD-2 is also significantly increased by treatment of several epithelial cell lines with TLR2 agonist Pam3CSSNA, TLR3 agonist poly I:C and TLR8 agonist ssPolyU, including SW620 colon epithelial cells (Uehara et al., 2007). NF-κB RelA silencing in HSC-2 oral epithelial cells largely abolished the increase in hBD-2 secretion. HBD-2 expression induced by exposure of Caco-2 cells to enterotoxigenic *E. coli* or *E. histolytica* trophozoites seems to be also mediated through classical TLR2/4-NF-κB signaling (Ayala-Sumuano et al., 2013). Corneal fibroblasts stimulated with TLR9 ligand CpG oligodeoxynucleotides or peptidoglycans showed increased expression of HNP-3 (Rodriguez-Martinez et al., 2006). *Salmonella enteritidis* flagellin induces hBD-2 expression in Caco-2 cells via TLR5 (Ogushi et al., 2004). Stimulation of Coca-2 and HT-29 cells with LPS or protein kinase C activator 12-O-tetradecanoylphorbol-13-acetate (TPA) does not induce mRNA expression of hBD-2 or influence constitutively expressed hBD-1 expression.

Similar to TLR activation, stimulation of NOD1 or NOD2 with iE-DAP or MDP respectively, increases hBD-2 release and expression in SW620 cells and other epithelial cells (Uehara et al., 2007). NOD2 activation also induces HNP-1 mRNA expression in HCT116, SW480 and Caco-2 colonic epithelial cells (Yamamoto-Furusho et al., 2010). Furthermore, HCT116 cells secrete HNP-1 in response to MDP-LD.

Cytokines can modulate defensin expression. Since the hBD-3 promoter lacks NF-κB binding sites hBD-3 expression is generally considered to be NF-κB-independent, but can be induced by IFN-γ (Nomura et al., 2003; Steubesand et al., 2009). Furthermore, in OE21 esophageal epithelial cells *C. albicans* in the presence of neutrophils induces hBD-3 partly through activation of the endothelial growth factor receptor (EGFR) by TGF-α. This induction can be reduced by either inhibiting MEK1, the kinase upstream of ERK1/2, JNK or p38 MAPK. Activation of these kinases apparently results in activation of AP-1 (Jun) transcription factors. TNF-α is a well-known inducer of NF-κB signaling and has been observed to promote hBD-2 and hBD-3 mRNA expression in primary human keratinocytes (Nomura et al., 2003). Considering TNF-α also activates JNK, its effect on hBD-3 expression is likely to be controlled by MAPKs (Littlejohn et al., 2003). HBD-2 and hBD-3 mRNA expression in keratinocytes is similarly

induced by IFN-γ (Nomura et al., 2003). Incidentally, this expression induced by TNF-α and/or IFN-γ is suppressed by IL-4 and/or IL-13. Conversely, IL-13 treatment increases hBD-3 expression in bronchial epithelial cells (Schrumpf et al., 2012). Expression of hBD-2 in primary human keratinocytes is increased after treatment with T_h17 cytokines IL-22, IL-17F and particularly IL-17A (Liang et al., 2006). IL-1α treatment of Caco-2 or HT-29 cells induces strong hBD-2 mRNA expression, while TNF-α or IFN-γ treatment does not (O'Neil et al., 1999). Caco-2 cells stimulated with IL-1α secrete, in contrast to unstimulated cells, also hBD-2 peptide into the medium. Expression of hBD-1 in Caco-2 and HT-29 cells is unaffected by IFN-γ, IL-1α or TNF-α stimulation. In human keratinocytes TNF-α and IL-1β induce hBD-2 expression, while especially IFN-γ increases hBD-3 expression and hBD-4 expression is notably stimulated by TNF-α or IL-1β (Harder et al., 2004).

CAMP expression is especially under control of vitamin D due to VDR binding sites in its promoter. Interestingly, VDREs are also present in the hBD-2 gene promoter (Wang et al., 2004a). It is therefore not surprising 1,25D3 treatment increases hBD-2 mRNA expression in SCC-25 oral and Calu-3 lung epithelial cells and hBD-2 protein expression in SCC-25 cells. Synthesis of hBD-2 mRNA is not inducible in primary monocytes by 1,25D3 alone, but is dependent on VDR activation (Liu et al., 2009). Curiously, stimulant-induced hBD-2 mRNA and peptide expression in keratinocytes is inhibited by vitamin D analogue calcipotriol (Kim et al., 2009). Research by Lagishetty et al. (2010) similarly indicates hBD-2 expression enhanced by LPS stimulation is reduced by 25D. 1,25D3 stimulation was found to significantly enhance hBD-3 expression in primary keratinocytes (Dai et al., 2010). Comparable to inducement of hBD-3 expression by cytokines, this enhancement is significantly reduced by inhibition of ERK1/2, JNK or p38 MAPK. Moreover, peroxisome proliferator-activated receptor γ (PPAR-γ) activity was of notable influence on hBD-3 expression. Noteworthy is that LL-37 also induces release of HNP1-3 from neutrophils via p38 MAPK and ERK1/2 (Zheng et al., 2007). In HL-60 cells HNP-1 expression can be enhanced by retinoic acid (RA; Wang et al., 2004b). In contrast, retinoic acid potently inhibits expression of β-defensins in keratinocytes (Harder et al., 2004).

Of note are the binding sites for transcription factors CCAAT/enhancer-binding protein-β (CEBPB) and transcription factor AP2-α (TFAP2A) present in the proximal 5'-flanking region of both HD5 and HD6 genes (Mallow et al., 1996). Target genes of CEBPB include several cytokines and the CEBPB system can be modulated by cytokines and antimicrobial products (Huber et al., 2012). TFAP2A is apparently an important hub in altered gene expression under inflammatory conditions and is itself a NF-κB target gene (Chen et al., 2008; Schreiber et al., 2006). Futhermore, the HD5 and HD6 promoters contain a high-affinity binding site for transcription factor 4 (TCF4), a Wnt-signaling pathway transcription factor (Wehkamp et al., 2007). The transcription factor SPI1, also important in transcription of CAMP, regulates HNP-1 expression (Termén et al., 2008). Aside from three NF-κB binding sites in the hBD-2 promoter, accounting for its inducibility by activated NF-κB, an AP-1 binding site is also present (Yoon et al., 2010). The hBD-4 promoter region also contains several AP-1 binding sites, but lacks NF-κB or STAT binding sites (García et al., 2001). The hBD-1 gene has multiple binding sites for AP-1, SP1 and CEBPs (Chakraborty et al., 2008; Zhu et al., 2003)

2.3 Lysozyme

Lysozyme catalyzes the β(1-4)glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine residues in bacterial peptidoglycans and between *N*-acetylglucosamine residues in chitodextrins (Rubio, 2011). The spectrum of bacteria susceptible to lysozyme activity is broad and includes *E. coli*, *Staphylococcus spp.* and *Streptococcus spp.* (Vanderwinkel et al., 1995). It is produced by secretory cells in exocrine glands, such as duodenal glands, and various epithelial cells, including intestinal Paneth cells and goblet cells, macrophages and other cells of the myelomonocytic lineage *in vitro* (Fahlgreen et al., 2003; Peters et al., 1989; Rubio, 2011; Wehkamp et al., 2006). Resting murine tissue macrophages, such as lamina propria macrophages, generally do not express lysozyme (Keshav et al., 1991).

Like LL-37 and defensins, lysozyme has immunomodulatory properties. Even concentrations lower than one-tenth of lysozyme serum levels affect the proliferative response of lymphocytes (Yabe et al., 1993). Treatment of lymphocytes stimulated with optimal concentrations of lectins concanavalin A (ConA) or phytohemagglutinin (PHA) with low concentrations of lysozyme retards proliferation, while lysozyme promotes proliferation of lymphocytes cultured with suboptimal IL-2 levels. Furthermore, lysozyme binds the bacterial cell wall components LPS, lipoteichoic acid and peptidoglycan preventing their interaction with PRRs and release of pro-inflammatory cytokines (Cooper et al., 2011). Interestingly, oral administration of human lysozyme has been reported to increase ileal expression of TGF-β1 and the number of intraepithelial lymphocytes.

Expression of lysozyme can be enhanced in chicken HD11 macrophages with LPS (Lefevre et al., 2008). LPS, IFN-γ and TNF-α dose-dependently increase lysozyme secretion by human peritoneal macrophages (Lewis et al., 1990). Similarly, secretion is enhanced by LPS, IFN-γ and TNF-α in peripheral blood monocytes. In HL-60 and U937 monocytes lysozyme protein expression is synergistically increased by treatment with 1,25D3 and TGF-β1, while TGF-β1 alone has practically no effect (Testa et al., 1993). Infection of mice with Bacille Calmette Guerin (BCG) or *Plasmodium yoelii* induces lysozyme expression in macrophages in spleen and/or liver (Keshav et al., 1991).

2.4 Other anti-microbial peptides

Aside from LL-37, defensins and lysozyme, humans express several other AMPs, some of which may be of importantce in IBD. Phospholipase A_2 (PLA2) enzymes cleave membrane phospholipids, resulting in the release of lysophospholipids, arachidonic acid, a precursor of certain pro-inflammatory mediators, and other fatty acids (Balsinde et al., 2002). The PLA2 family includes both cytosolic and secreted members, the latter category participating in the first line of antimicrobial defense, including group IIA secretory phospholipase A2 (sPLA2-IIA; Nevalainen et al., 2008). sPLA2-IIA is highly active against Gram-positive bacteria such as *S. aureus* via hydrolysis of bacterial membrane phospholipids. This PLA2 form is expressed by Paneth cells and expression is inducible by pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6. Neutrophils are not considered to be a source of sPLA2-IIA, other sPLA2 groups are however produced by immune cells.

Lactoferrin is a member of the transferrin family known for its ability to bind iron and other metal ions (Nuijens et al., 1996). The antimicrobial activity against iron-requiring micro-organisms of lactoferrin is partly based on iron sequestration. Some lactoferrin fragments owe their bactericidal effect to interaction with LPS and membrane disruption. Furthermore, lactoferrin degrades proteins required for colonization by enteropathogenic *E. coli*, *S. flexneri* and *Haemophilus influenzae* (Ward et al., 2005). Lactoferrin also has antimicrobial activity against viruses, parasites, including *E. histolytica*, and fungi, such as *C. albicans* (El-Fakharany et al., 2013; León-Sicairos et al., 2012; Viejo-Díaz et al., 2004). Several cell types express lactoferrin, including neutrophils, monocytes, lymphocytes and glandular epithelial cells (Ashida et al., 2004; Viejo-Díaz et al., 2004). In addition to antimicrobial activity lactoferrin has immunomodulatory properties. Several studies suggest lactoferrin inhibits pro-inflammatory cytokine production through direct binding of LPS, bacterial DNA and inhibition of NF-κB activation (Ward et al., 2005). However, Ando et al. (2010) recently showed lactoferrin itself can activate NF-κB, demonstrated by RelA translocation, and JNK in THP-1 macrophages, which was depedent on TLR4. Furthermore, leukocytes have been reported to produce TNF-α, CXCL8 and IL-12 in response to lactoferrin.

Calprotectin is the heterodimer of the S100 family members S100 calcium-binding protein A8 (S100A8) and S100A9 (Ehrchen et al., 2009). Some of the S100 members are recognized as danger-associated molecular patterns, including S100A8 and S100A9. Calprotectin can be expressed by granulocytes, monocytes, macrophages, fibroblasts, keratinocytes and microvascular endothelial cells. Like lactoferrin, calprotectin derives antimicrobial activity from sequestration of metals, such as Zn^{2+} and Mn^{2+} (Damo et al., 2013). Via binding of Mn^{2+} is calprotectin effective against *Staphylococcus* spp., *S. flexneri*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *E. coli* and *Pseudomonas aeruginosa*. In keratinocytes S100A8 expression is synergetically induced by TNF-α and IL-17A through p38 MAPK, independently of NF-κB, JNK and ERK (Mose et al., 2013). Calprotectin acts as an immunomodulator. For example, calprotectin is a potent chemoattractant for neutrophils and treatment of peripheral monocytes with S100A9 or calprotectin dose-dependently induces production of TNF-α, IL-1β, IL-6 and CXCL8 (Ryckman et al., 2003; Sunahori et al., 2006). Interestingly, calprotectin functions as a TLR4 agonist (Ehrchen et al., 2009).

3 Anti-microbial peptides in IBD

The cytokine and immune cell milieu in patients with IBD differs from healthy humans, particularly in the intestine. As extensively summarized in the previous part, expression of at least some AMPs can be altered by PAMPs, DAMPs, cytokines and other inflammation-associated factors. Accordingly, expression and secretion profiles of AMPs also differ, possibly with genetic influence. Expression of CAMP is for example increased in inflamed and non-inflamed colon mucosa from ulcerative colitis patients (Schauber et al., 2006b). The increase is unrelated to NOD2 polymorphisms. In Crohn's disease CAMP expression in both ileum and colon is positively associated with degree of inflammation (Kübler et al., 2009).

3.1 α-Defesin expression in IBD

Substantially more studies have focused on expression of defensins in IBD patients. Neutrophils positive for HNP1-3 are found in healthy intestinal tissue and inflamed IBD samples (Cunliffe et al., 2002; Kanmura et al., 2009). In healthy sections, these neutrophils are however rarely present outside of blood vessels. In the lamina propria and epithelium of inflamed parts of the colon and/or terminal ileum in IBD patients numerous HNP1-3 positive neutrophils are found. It is possible these neutrophils secrete HNP-1-3 into the extracellular space. Furthermore, epithelial cells in inflamed sections of the intestine of IBD patients are highly positive for HNP-1-3 (Cunliffe et al., 2002; Kanmura et al., 2009). Expression in these cells is not ubiquitous however, as HNP-1-3 staining is concentrated near the villous tips in the small intestine and colonic surface epithelium. Conversely, expression of HNP-1-3 in epithelial cells in the non-inflamed colon and terminal ileum is undetectable. HNP-1-3 plasma levels are increased in ulcerative colitis and Crohn's disease patients and are to some extent associated with disease activity (Cunliffe et al., 2002; Kanmura et al., 2009; Yamaguchi et al., 2009). In addition, the HNP1-3 concentration is higher in stool samples from Crohn's disease patients than normal individuals (Zou et al., 2007). The elevated stool concentration is probably due to increased secretion of HNP1-3 into the intestinal lumen by intestinal epithelial cells. Whether raised plasma levels are caused by secretion of HNP-1-3 by neutrophils in the mucosa is doubtful, given the correlation with white blood cell count in Crohn's disease patients and decreased proportion of peripheral (inactivated) spherical neutrophils (McCarthy et al., 1991; Yamaguchi et al., 2009).

Expression of HD5 and HD6 is upregulated in cryptal and luminal epithelial cells isolated from the inflamed colon of ulcerative colitis patients (Fahlgren et al., 2003). Immunohistochemical staining indicates HD5 protein to be predominantly present at the base of crypts. In the normal colon expression of these α-defensins is negligible. While Crohn's disease patients with wild-type NOD2 also show an increased expression of HD5 and HD6 in diseased colonic tissue, this increase is absent in NOD2 mutant carriers, especially in those with the L1007fsinsC SNP (Wehkamp et al., 2004). Interestingly, expression of HD5 differs between Crohn's disease and ulcerative colitis mostly in the sigmoid colon and rectum, where in Crohn's colitis considerably less patients stain positive for HD5 protein in these parts of the intestine (Shen et al., 2003). HD5 expression in the sigmoid colon of ulcerative colitis patients is likely to be associated with Paneth cell metaplasia (Noble et al., 2008). Ileal mRNA expression of HD5 and HD6 in Crohn's disease patients is reduced in inflamed mucosa compared to tissue from healthy controls, where HD5 protein is prominently present in the cryptal base (Arijs et al., 2009; Simms et al., 2008; Wehkamp et al. 2005; Wehkamp et al. 2004). Furthermore, according to Wehkamp et al. (2004) ileal HD5 expression is even lower in patients carrying NOD2 variants associated with an increased IBD risk. The reduced HD5 mRNA expression in wild-type NOD2 and mutant NOD2 groups in the ileum manifested itself at peptide level (Wehkamp et al., 2005). HD5 concentrations in the small intestine are lower in NOD2 mutant carriers, but also in other Crohn's disease patients (Elphick et al., 2008). Ileal expression of HD5 may be highly dependent on degree of inflammation and time since onset of Crohn's disease: Ciccia et al. (2010) found expression to be increased with low-grade inflammation and recent-onset, Wehkamp et al. (2005) however did not find any difference between levels of inflammation. It should be noted that Simms et al. (2008) did not find any difference

in ileal expression between wild-type NOD2 and mutant groups, although they did find a lower expression in inflamed ileal tissue. The lack of any significant difference associated with NOD2 polymorphisms in the latter study may however be caused by forgoing differentiation between NOD2 variants in the latter study: ileal HD5 mRNA levels in Crohn's disease patients carrying the increased-risk variants of Arg702Trp and Gly908Arg do not significantly deviate from the wild-type NOD2 group (Wehkamp et al. 2005). In pediatric ulcerative colitis patients expression of HD5 and HD6 is significantly enhanced in the ascending colon and (non-inflamed) terminal ileum (Zilbauer et al., 2011). Mucosal expression of HD5 and HD6 in pediactric Crohn's disease is also increased in the inflamed ascending colon. Expression of HD5 in the duodenum of pediactric Crohn's disease patients with exclusive colonic involvement, but not ileocolonic disease, was found to be higher than in normal controls.

3.2 β-Defensin expression in IBD

It is clear that, just as expression of α-defensins differs in IBD patients, β-defensin expression is altered as well. According to Wehkamp et al. (2003) mucosal expression of constitutively expressed hBD-1 is significantly decreased in inflamed colonic mucosa of ulcerative colitis and Crohn's disease patients. Arijs et al. (2009) reported a significant 6.7 and 4.8 fold decreased mRNA expression in inflamed colonic mucosa of ulcerative colitis and Crohn's disease patients, respectively. A reduced hBD-1 expression in the Crohn's disease ileum was also reported, but this was not statistically significant. The decreased expression was proposed to be caused by loss of epithelial cell mass and could be partially restored with anti-TNF-α antibody treatment. Interestingly, hBD-1 mRNA expression was found to be significantly increased in the duodenum of pediatric Crohn's disease patients, but not pediatric ulcerative colitis patients (Zilbauer et al., 2010). Furthermore, while expression in the inflamed terminal ileum in pediatric Crohn's disease was comparable to healthy control tissue, expression in non-inflamed tissue of the terminal ileum was 2 fold increased. This difference was even more apparent in the ascending colon of pediatric Crohn's disease patients, but absent in pediatric patients with ulcerative colitis.

Expression of hBD-2 is low in the normal colon, but, due to its inducible nature, enhanced in Crohn's disease and ulcerative colitis (Wehkamp et al. 2002). The increased hBD-2 mRNA levels are most pronounced in inflamed parts of the colon of ulcerative colitis patients and induction takes place in both cryptal and luminal epithelial cells (Fahlgren et al., 2003; Wehkamp et al. 2003). Although elevated in Crohn's disease, the increased colonic hBD-2 mRNA levels are less pronounced than in ulcerative colitis (Arijs et al., 2009). In the inflamed ileum of Crohn's disease patients hBD-2 expression is similarly induced and increases with inflammation (Arijs et al., 2009; Kübler et al., 2009). Aside from epithelial cells, lamina propria plasma cells may contribute to hBD-2 expression in the colonic mucosa (Rahman et al., 2007). In the duodenum and inflamed terminal ileum and ascending colon in pediatric Crohn's disease hBD-2 expression is increased as well (Zilbauer et al., 2010). Expression is even higher in the inflamed ascending colon of pediatric ulcerative colitis patients. In non-inflamed mucosa of the terminal ileum and ascending colon and pediatric ulcerative colitis expression was not significantly different from normal controls. Fecal hBD-2 concentrations are also increased in pediatric IBD and mainly in ulcerative colitis (Kapel et al., 2009).

Like hBD-2, expression of hBD-3 is low in the normal colon (Wehkamp et al. 2003). In Crohn's disease and especially ulcerative colitis in inflamed colon mucosa hBD-3 expression is induced. Increased hBD-3 expression in colonic epithelial cells is restricted to villi and the luminal compartment in Crohn's disease (Fahlgren et al., 2004). In contrast, in ulcerative colitis both cryptal and villous/luminal epithelial cells show an induction of hBD-3. Expression in the ileal epithelium was not found to be significantly different from healthy controls. Plasma cells located in the lamina propria of the ulcerative colitis colon have been shown to express hBD-3 mRNA and may contribute to increased expression as well (Rahman et al., 2007). In pediatric Crohn's disease patients hBD-3 expression is significantly higher in the duodenum and inflamed tissue of the terminal ileum and ascending colon compared to healthy controls (Zilbauer et al., 2010). In the inflamed mucosa of the colon in pediatric ulcerative colitis, hBD-3 is also induced. Expression of hBD-4 was found to be induced in cryptal epithelial cells and more strongly in villous/luminal epithelial cells of the colon in ulcerative colitis (Fahlgren et al., 2004). Arijs and collegues (2009) reported increased hBD-4 expression in the Crohn's disease ileum and IBD colon.

3.3 Expression of lysozyme, sPLA2, calprotectin and lactoferrin in IBD

In cryptal and villous/luminal epithelial cells of the inflamed colon in ulcerative colitis lysozyme expression is increased considerably, while in Crohn's disease only cryptal expression is marginally increased (Fahlgren et al., 2003). Overall lysozyme mRNA is notably increased in the colonic mucosa in ulcerative colitis and Crohn's disease (Arijs et al., 2009). Simms and collegues (2008) found a significant increase in lysozyme expression in non-inflamed ileal mucosa of Crohn's disease patients. In the ileum with a lower degree of inflammation lysozyme expression is also higher than in highly inflamed mucosa and healthy controls (Ciccia et al., 2013). According to Cunliffe et al. (2002) in the healthy terminal ileum only Paneth cells stain positive for lysozyme, while other epithelial cells and colonic epithelial cells are negative. In active IBD mucosa scattered lysozyme-positive epithelial cells, macrophages and neutrophils were found. Fecal lysozyme concentrations are significantly elevated in Crohn's disease and in ulcerative colitis and especially in Crohn's disease patients with diarrhea (Van Der Sluys Veer et al., 1998; Klass & Neale, 1978). In Crohn's disease serum lysozyme levels are positively associated with disease severity (Klass & Neale, 1978).

In patients with active ulcerative colitis sPLA2-II is expressed at mRNA and protein level by metaplastic Paneth cells and columnar epithelial cells in inflamed colonic mucosa, while under normal conditions expression is almost entirely restricted to Paneth cells in the small intestine (Haapamäki et al., 1997). Expression of sPLA2-IIA at mRNA level is increased in the colon of Crohn's disease and ulcerative colitis patients (Arijs et al., 2009). In NOD2 mutant carriers with Crohn's disease, especially in case of the Leu1007fsinsC polymorphism, sPLA2-IIA expression in the inflamed colon is significantly lower compared to wild-type NOD2 carriers (Wehkamp et al., 2004). Activity and protein levels of sPLA2-II are also increased in actively inflamed colonic mucosa of Crohn's disease patients (Minami et al., 1994). According to Simms et al. (2008) mRNA expression is also elevated in the non-inflamed ileal mucosa in Crohn's disease. Ciccia and collegues (2013) found increased sPLA2-IIA expression in low degree inflammation compared to healthy ileal mucosa and a high degree of inflammation. Finally, in Crohn's disease patients sPLA2-II serum concentrations were found to be elevated (Haapamäki et al., 1998).

Fecal lactoferrin and calprotectin concentrations are notably higher compared to healthy controls in IBD patients (Dai et al., 2007; von Roon et al., 2007). In pediatric patients raised fecal calprotectin is also indicative of IBD (Joishy et al., 2009; von Roon et al., 2007). It has been proposed that increased fecal calprotectin is the result of migration of neutrophils into the intestinal lumen, which is possible due to the increased epithelial permeability in IBD (Berstad et al., 2000). Fecal lactoferrin may be directly caused by the presence of leukocytes in the intestinal lumen in IBD as well (Guerrant et al., 1992). Interestingly, cultured inflamed intestinal tissue samples from Crohn's disease and ulcerative colitis patients spontaneously release considerably more calprotectin than non-inflamed or healthy controls (Foell et al., 2008). Expression of S100A8 and S100A9 at mRNA level is substantially upregulated in inflamed ileal and colonic mucosa of Crohn's disease patients (Arijs et al., 2009). In ulcerative colitis mRNA levels are even higher in the colonic mucosa. Whether the production of calprotectin is limited to leukocytes is however unclear, although calprotectin protein containing epithelial cells were found in a Crohn's disease patient (Foell et al., 2008). Lactoferrin mRNA expression is also significantly higher in the colon of IBD patients (Arijs et al., 2009).

3.4 Mechanisms of altered AMP expression

Expression of the AMPs mentioned above is generally increased in the inflamed IBD colon, except for hBD-1 in adult patients, which is actually decreased (figure 2). This is not surprising as a number of them are constitutively expressed by leukocytes or their expression is induced by pro-inflammatory cytokines or other substances. Correlation of expression of several AMPs with cytokine levels supports this notion (Kübler et al., 2009; Wehkamp et al., 2003; Zilbauer et al., 2011). Increased expression may therefor be a direct result of increased inflammation and leukocyte infiltration in the intestinal mucosa. After all, neutrophils, monocytes, macrophages and lymphocytes are known to synthesize LL-37, defensins, sPLA2, lysozyme, calprotectin and/or lactoferrin. On the other hand, the importance of intestinal epithelial cells in AMP expression should not be underestimated. Paneth cells are the primary 'natural producers' of AMPs in the small intestine and their expansion into the colon in principle inevitably results in increased colonic AMP synthesis, as is the case for HD5, which is expressed by metaplastic Paneth cells (Cunliffe, 2003; Tanaka et al., 2001). The significance of Paneth cell development, functioning and NOD2 will be discussed later. Other intestinal epithelial cells are clearly also capable of producing AMPs and do so in the IBD colon. Non-Paneth epithelial cells appear to be primarily responsible for increased expression of hBD-4 in ulcerative colitis, given the larger increase in the villous/luminal compartment (Fahlgren et al., 2004). Increased AMP synthesis by colonic epithelial cells is apparently accompanied by increased apical secretion, resulting in increased fecal concentrations. It seems the effects of inflammation in the mucosa extend beyond the area microscopically classified as inflamed. Expression of hBD-2 is for instance induced in non-inflamed tissue in ulcerative colitis patients (Wehkamp et al. 2003). Considering AMPs possess many pro-inflammatory properties, increased expression may intensify inflammation in the intestine. Especially AMPs released within the mucosa, rather than into the lumen, can function as potent chemoattractants and elicit cytokine release.

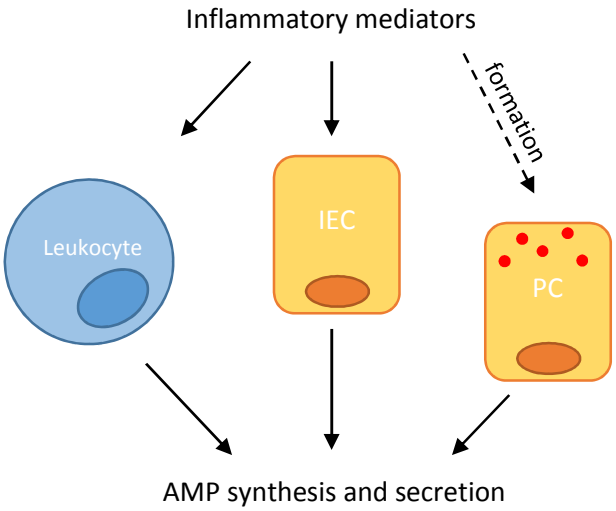

Figure 2. Colonic expression of AMPs in IBD. Inflammatory mediators induce the formation of metaplastic Paneth cells (PC) and stimulate production of AMPs by infiltrated leukocytes, intestinal epithelial cells (IEC) and metaplastic Paneth cells in the IBD colon.

The degree of induction of AMPs in the colon differs between ulcerative colitis and Crohn's disease patients. Expression of hBD-2 and hBD-3 is considerably more upregulated in adult and pediatric ulcerative colitis for example (Wehkamp et al., 2003). Differing cytokine profiles provide a possible explanation. Levels of T_h17-related cytokines are notably higher in colonic mucosa of ulcerative colitis patients (Bogaert et al., 2010). HBD-2 expression is potently induced by IL-17A in human keratinocytes (Liang et al., 2006). It is therefor possible the increased expression is caused by higher T_h17-type activity. However, stimulation with IL-17 does not increase hBD-3 expression in human cells (Kao et al., 2004). The observation of Rahman et al. (2007) regarding the presence of defensin producing plasma cells in greater numbers in the ulcerative colitis colon certainly also provides an interesting addition.

Curious is the reduced colonic expression of hBD-1 in adult IBD patients. This is contrary to the increased expression in pediatric IBD, marking a clear difference between adult and pediatric IBD. As mentioned earlier, epithelial damage has been suggested as causative in the decreased hBD-1 expression (Arijs et al. 2009). In inflamed mucosa of the ascending colon of pediatric Crohn's disease patients hBD-1 expression was comparable to normal controls, while there was a considerable increased expression in non-inflamed mucosa (Zilbauer et al., 2010). Epithelial damage could also account for this difference between inflamed and non-inflamed tissue, it does however not explain the elevated hBD-1 expression in the non-inflamed ascending colon. Moreover, this suggestion does not provide a satisfactory explanation for the lack of a similar increase in the non-inflamed ascending colon of pediatric ulcerative colitis patients. It is also unclear why hBD-1 expression is affected in the adult colon, while expression of other AMPs produced by epithelial cells is not. Furthermore, considering the inducibility of hBD-1 in leukocytes, the presence of these cells in inflamed colonic mucosa should therefor result in additional expression.

Expression in the small intestine and Paneth cells

Expression of many AMPs, including CAMP, HNP1-3, hBD-2, lysozyme, sPLA2-IIA, lactoferrin and calprotectin, is increased in the small intestine of Crohn's disease patients. AMP expression in the small intestine is not invariably upregulated in Crohn's disease however. The α-defensins HD5 and HD6 and the underlying mechanism have been the subjected of some debate in that regard. Although it is clear

expression is significantly reduced in inflamed ileal tissue, whether this is also the case for mildly inflamed mucosa remains undetermined. Research by Wehkamp et al. (2004, 2005) suggests an interesting link between genetic disease susceptibility and functional deficiency. The NOD2 frameshift mutation Leu1007fsinsC in particular seems to be associated with impaired HD5 production. This mutation results in a truncated NOD2 protein lacking the last LRR domain (Philpott et al., 2009). Although the Arg702Trp and Gly908Arg NOD2 variants show reduced NF-κB activation in response to MDP, the impaired function is most dramatic with the Leu1007fsinsC mutation (Abraham et al., 2006). Curiously, mice expressing the corresponding truncated NOD2 have an increased activation of NF-κB. HEK293 epithelial cells transfected with Leu1007fsinsC NOD2 show an impaired induction of hBD-2 expression, which is NF-κB-dependent (Voss et al., 2006). Furthermore, Caco-2 cells overexpressing the truncated NOD2 variant secrete less HNP-1 in response to stimulation with MDP (Yamamoto-Furusho et al., 2010). It also appears NOD2 activation is important in production sPLA2-IIA, given the reduced expression in the colon of mutant NOD2 carrying Crohn's disease patients (Wehkamp et al., 2004).

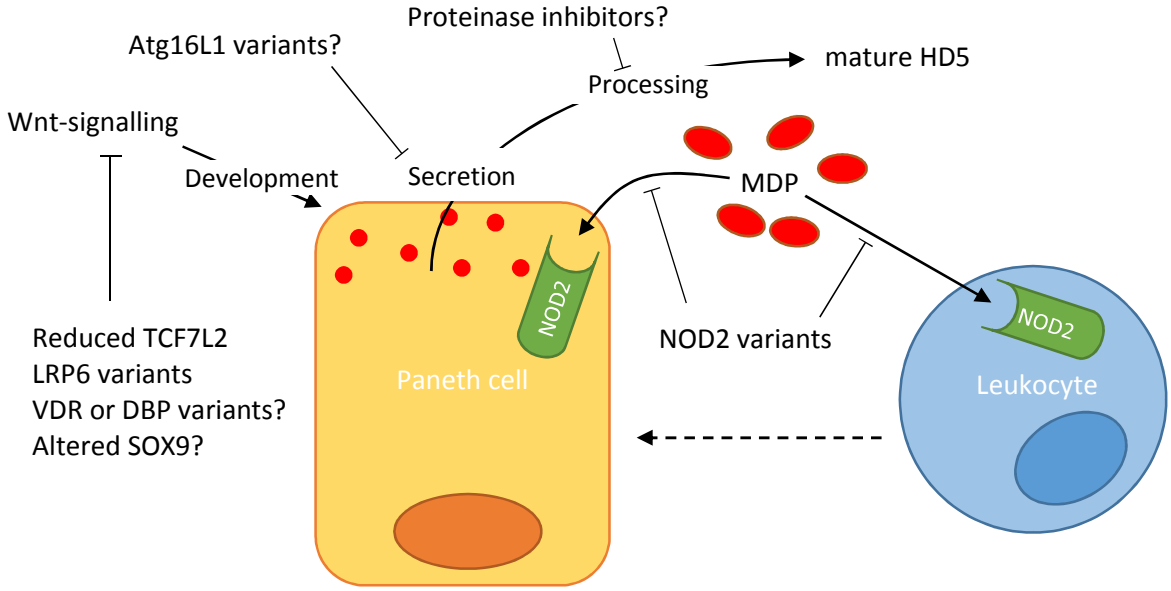

Figure 3. Defective HD5 production in ileal Crohn's disease. Several processes are essential in the production of mature HD5 and secretion into the ileal lumen and may be disturbed by altered gene expression in Paneth cells and known Crohn's disease-associated gene variants. Possible reasons for defective HD5 production are defective Paneth cell development due to reduced Wnt-signalling, insensitivity to MDP caused by NOD2 mutations, impaired formation of exocytotic granules due to Atg16L1 mutations or diminished luminal processing.

Although the HD5 and HD6 genes lack a NF-κB binding site and their transcription is not directly affected by reduced NF-κB activation, they do posses CEBPB and TFAP2A binding sites. CEBPB is activated by p38 MAPK, while TFAP2A is a target gene of NF-κB. It is therefor possible expression of HD5 and HD6 is diminished through these transcription factors with defective NOD2 signalling. Interestingly, although Paneth cells and other intestinal epithelial cells express NOD2 and expression of HD5 and HD6 may be directly affected, it is possible leukocytes are involved in reduction of expression. Of Crohn's disease patients receiving small intestine allografts, those with NOD2 mutations

exhibited significantly lower HD5 and hBD-2 peptide and mRNA expression in the grafted tissue (Fishbein et al., 2008). No differences in the number of crypts or Paneth cells were apparent, but recipients with mutant NOD2 had notable decrease in the number and size of Paneth cell secretory granules. Graft failures were also significantly more common in patients with certain NOD2 polymorphisms. These observations provide an interesting putative connection between NOD2 genotype, the cellular immune system and AMP expression in the small intestine. Noteworthy in this respect, is the observation that Crohn's disease patients with NOD2 SNPs associated with increased susceptibility have decreased numbers of lamina propria $Foxp3^+$ T_{reg} cells (Rahman et al., 2010). Furthermore, the mutant NOD2 T_{reg} cells are more susceptible to FasL-induced apoptosis, due to insensitivity to MDP-induced protection.

Quite recently, the transcription factor 7-like 2 (TCF7L2), a Wnt-signaling pathway transcription factor, has been suggested as significant in reduced ileal HD5 and HD6 expression in Crohn's disease (Wehkamp et al., 2007). TCF7L2 is important in progenitor cell maintenance and differentiation and maturation of Paneth cells. Apparently HD5 and HD6 mRNA levels in the ileum are strongly correlated to TCF7L2 expression. In patients without ileal involvement, TCF7L2 expression is comparable to healthy control, while in Crohn's ileitis expression is more than 2 fold reduced. Consequently, in TCF7L2 binding activity is decreased in the small intestine. In $TCF7L2^{+/-}$ mice, expression of cryptdin-1 and -4 is notably lower compared to wild-type mice. In addition, killing of *E. coli* and *S. aureus* is impaired. In pediatric Crohn's disease patients with ileal involvement, expression of TCF7L2 expression was found to be lowered in ileal and colonic mucosa and correlated with HD5 expression (Perminow et al., 2010). TCF7L2 expression in pediatric patients without ileal involvement was not altered. Incidentally, TCF7L2 promoter variants are associated with ileal Crohn's disease (Koslowski et al., 2009). Furthermore, a variant of the Wnt co-receptor low density lipoprotein receptor-related protein 6 (LRP6) is associated with early onset ileal Crohn's disease and lowest HD5 and HD6 expression in ileal disease (Koslowski et al., 2012). Functionally, LRP6 overexpression results in increased HD5 promoter activity.

In breast cancer cells and mammary epithelium, expression of the transcription factor SRY-box 9 (SOX9) was found to regulate LRP6 and TCF7L2 expression and Wnt activation (Wang et al., 2013). In the intestine SOX9 is itself a Wnt/TCF7L2 target gene (Mori-Akiyama et al., 2007). In mice SOX9 activity was found to be essential for Paneth cell formation. Interestingly, Ciccia and collegues (2012) described significantly higher SOX9 and HD5 mRNA levels in Crohn's disease patients with low grade ileal inflammation compared to patients with highly inflamed mucosa and healthy controls. On the other hand, Wehkamp et al. (2007) did not find significant differences in TCF7L2 expression between inflammation severity levels. Infiltrating leukocytes are likely to influence Paneth cell maturation in Crohn's disease, as lamina propria lymphocytes from patients attenuate SOX9 expression at protein level in HT-29 and T84 cells (Roda et al., 2009). Furthermore, the characteristic Crohn's disease cytokine IFN-γ also downregulates SOX9 expression (Roda et al., 2008). However, it has been noted that NF-κB signaling positively regulates expression of SOX9 (Sun et al., 2013). Finally, 1,25D3 regulates TCF7L2 expression *in vitro* in a VDR-dependent manner, indicating a possible role for VDR and DBP polymorphisms in Paneth cell differentiation as well (Beildeck et al., 2009; supplementary text).

Considering the above it is reasonable to implicate Paneth cell dysfunction at least in impaired defensin induction in the Crohn's disease subtype with ileal involvement. As the first and second most produced AMPs in the ileum, defective HD5 and HD6 synthesis may contribute significantly to IBD pathology. Crohn's disease patients with functionally normal NOD2 and complete Paneth cell maturation, may suffer from defects in HD5 processing (Elphick et al., 2008). Moreover, in others secretion of AMPs by Paneth cells could also be affected. Paneth cells of mice deficienct for Atg16L1 (IBD10), Atg5 or Atg7 are morphological abnormal and apparently fail to properly form exocytotic granules (Cadwell et al., 2009). Measurement of mucosal defensin expression at mRNA level is therefor not suitable to assess production of functional defensins. Although expression of other AMPs is generally increased, considering the substantial production of HD5 and HD6 in the normal small intestine this may not compensate for loss of thereof. Accordingly, defective expression is undeniably significant in Crohn's disease pathology. This concept is supported by the lack of association of NOD2 mutations with ulcerative colitis and regular Paneth cell maturation in the ileum of ulcerative colitis patients.

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5 Supplementary text

5.1 IBD2

While IBD1 is primarily a Crohn's disease specific locus, IBD2 is especially associated with ulcerative colitis (Cavenaugh et al., 2001). Achkar et al. (2006) determined that this susceptibility locus on chromosome 12q is specifically linked to the extensive ulcerative colitis phenotype. Located in the IBD2 region are several immune system-related genes, such as Kill cell lectin-like receptor, subfamily K, member 1 (KLRK1), signal transducer and activator of transcription 6 (STAT6) and C-type lectins (Jones et al., 2006; Wolfkamp et al., 2010; Zhu et al., 2006). Although expression of KLRK1 is upregulated in pediatric Crohn's disease and ulcerative colitis patients, known SNPs do not appear to be associated with ulcerative colitis (Jones et al., 2006; La Scaleia et al., 2012). STAT6 is a key transcription factor in IL-4 and IL-13-mediated Th2 responses and therefor an interesting candidate, but only a single study found an association with IBD susceptibility, specifically in Crohn's disease patients not carrying a predisposing NOD2 allele (Klein et al., 2005; Zhu et al., 2006). Also no association was found between C-type lectin domain family 4, member A (CLEC4A) and either ulcerative colitis or Crohn's disease (Wolfkamp et al., 2010).

5.2 IBD3: the HLA complex

Since the early reports in the 1970's such as by Gleeson et al. (1972), many other studies have investigated a link between human leukocyte antigens (HLAs), the human major histocompatibility complex (MHC), and Crohn's disease. The HLA genes are located in a region on chromosome 6p, also known as IBD3, which has strong a linkage with IBD and Crohn's disease in particular (Gaya et al., 2006; Duerr et al., 2000). The HLA gene complex can be divided into three main regions, called class I, II and III (Mangalam et al., 2013).

HLA class I

HLA class I molecules (HLA-A, -B, -C) are composed of the highly polymorphic α heavy chain and the non-polymorphic β2-microglobulin light chain, which is not encoded by a gene in the IBD3 region but on chromosome 15 (Jordanova et al., 2003). By presenting endogenous peptides, the HLA class I molecules play an important role in the detection of virus-infected or tumor cells by the immune system. These peptides are the product of degradation of proteins in the cytosol, which can include viral or otherwise abnormal proteins, and are subsequently transported out of the cytosol into the lumen of the endoplasmatic reticulum (ER) by transporter associated with antigen processing (TAP; Neefjes et al., 2011). The unloaded HLA class I molecules present in the ER lumen are stabilized by the peptide loading complex (PLC), such as calreticulin, ERp57 and tapasin, and bind the antigenic peptides. The stable complex formed by HLA class I and the peptide is transported via the Golgi secretory pathway to the plasma membrane in a process called antigen presentation. At the surface of the cell the loaded HLA class I molecules interact with the T-cell receptor on $CD8^+$ T cells. If the peptide is recognized as foreign or abnormal this results in immune activation and target cell lysis. Furthermore, HLA class I molecules interact with killer immunoglobulin-like receptors (KIRs) which are expressed by natural killer (NK) cells.

Research has shown specific HLA class I genes, alleles and haplotypes to be associated with Crohn's disease. Although some confer increased susceptibility to the disorder, others appear to reduce risk. A Japanese study for example indicates the HLA-Cw*1202 and HLA-B*5201 alleles have a significant protective effect (Okada et al., 2011). The haplotype consisting of these alleles and the HLA class II allele DRB1*1502 accounts for most of the associations in the HLA region with IBD. Surprisingly, while protective against Crohn's disease, this haplotype increases risk of ulcerative colitis. Frequencies of HLA-B41 and -A9 serotypes were significantly higher and antigen A11 frequency lower in Iraqi IBD patients than matched controls (Adhiah et al., 2008). In Japanese ulcerative colitis patients the HLA-B allele B*4002 occurs less frequently, while B*52 is significantly more common than in healthy controls (Aizawa et al., 2009).

Although typically not considered to belong to HLA class I, MHC class I chain-related (MIC) genes are distantly homologous (Bahram et al., 1994). The MICA gene is located in the class I region near HLA-B on chromosome 6. Like the classical class I molecules, MICA is present at the cell surface (Orchard et al., 2001). However, MICA appears to be expressed without the β2-microglobulin light chain and distribution is restricted to epithelium, including the gastrointestinal tract. A study in the United Kingdom indicates the MICA*007 allele to be associated with susceptibility to ulcerative colitis in Caucasians, but not Crohn's disease.

HLA class II

HLA class II serves a similar purpose as class I, the peptides presented are however of exogenous origin (Neefjes et al., 2011). While HLA class I molecules are expressed by all nucleated cells, HLA class II are primarily expressed by professional antigen presenting cells, such as dendritic cells, macrophages and B lymphocytes. Like class I molecules, HLA class II is composed of two polypeptides (Mangalam et al., 2013). Both the α- and β-chain consist of a conserved domain and a highly polymorphic domain, the latter domains forming the peptide binding groove. These components associate with the invariant chain (Ii) in the ER, at which point the complex is transported to the MHC class II compartment (MIIC), a type of late endosomal compartiment (Neefjes et al., 2011). There Ii is partly digested and the remaining fragment, called class II-associated Ii peptide (CLIP), is exchanged for an antigenic peptide while the complex is associated with the chaperone HLA-DM. The loaded MHC class II molecule is subsequently transported to the plasma membrane. At the cell surface the MHC class II molecule presents the antigenic peptide to $CD4^+$ T cells through interaction with the T-cell receptor.

Several studies have identified HLA class II genes and alleles that are associated with IBD and Crohn's disease. Bouzid et al. (2012) for example recently reported on the association of HLA-DRB1/DQB1 alleles with IBD in a small Tunisian sample. The homozygote HLA-DRB1*07 genotype was found to be considerably more frequent in patients with Crohn's disease or ulcerative colitis than healthy controls. Of note are also the profound differences between sexes in the Crohn's disease patient group. The DRB1*07 and DQB1*02 alleles were more frequent in males, while DRB1*13, DRB1*15 and DQB1*06 were more common in female patients. A 2004 study among 507 unrelated Crohn's disease patients also explored the influence of HLA-DRB1 alleles on susceptibility (Newman et al., 2004). Apparently, the risk for Crohn's disease was increased 6.7 times by presence of a DRB1*0103 allele in non-Jewish Caucasians. Interestingly, this allele was also reported to be associated with involvement restricted to

the colon in non-Jewish Caucasians with a familial history of Crohn's disease. The DRB1*04 and DRB1*0701 alleles on the other hand were independently associated with ileal involvement in all patients. In a Japanese sample the allele DRB1*0405 was associated with an increased risk of Crohn's disease (Okada et al., 2011). DRB1*04, DRB1*13 and DRB1*14 were more frequent in Uyghur ulcerative colitis patients, while DRB1*08 showed a lower frequency (Aheman et al., 2013). Conversely, no DRB1 associations with ulcerative colitis were observed in Han Chinese patients. Achkar et al. (2012) determined that the rs9269955 SNP in HLA-DRB1 is most strongly associated with ulcerative colitis. Protection against ulcerative colitis is offered when the polymorphism encodes for valine, glycine or aspartic acid at amino acid 11 in HLA-DRβ1. It was concluded that variation at position 11 is a major determinant of IBD3 association with ulcerative colitis.

HLA class III

The HLA class III region is situated between the class I and II genes on chromosome 6p and contains more than 75 genes (Mehra and Kaur, 2003). The proteins encoded in this gene cluster are structurally unrelated to the other classes and not directly involved in antigen presentation. Several innate immunity-related genes are located in the HLA class III region including 70 kDa heat-shock proteins (Hsp70), TNF-α, TNF-β, natural cytotoxicity triggering receptor 3 (NCR3), complement components 2 and 4 (C2, C4) and complement factor B (CFB; Xie et al., 2003). Another gene of interest in the HLA class III region is the transmembrane receptor Notch4. The Notch signaling pathway plays an essential role in several processes unrelated to the immune system, including the development of the central nervous system and the vascular system (Yamane and Paul, 2013). Notch has also been proposed to influence expression of a number of genes important in T cells differentiation, such as T-box 21 (TBX21) or T-bet, a transcription factor that controls expression of the hallmark T_h1 cytokine interferon γ (IFN-γ). The regulatory T cell master regulator FOXP3 gene is also a target of Notch.

Some studies have focused on the association of the HLA class III genes TNF-α and -β with IBD. Van Heel et al. (2002) note a statistically significant association of the TNF-α_{-857C} promoter polymorphism with IBD in general in the 587 Caucasian families from the United Kingdom tested. Association of this polymorphism with Crohn's disease was also observed in individuals not carrying the known Crohn's disease-associated NOD2 mutations. Interestingly, LPS stimulation of whole blood from individuals homozygous for TNF-α_{-857C} results in a significantly increased TNF-α production compared to TNF-α_{-857T} allele carriers. Another study in the United Kingdom also showed association of the TNF-α promoter polymorphism with Crohn's disease in a large Caucasian cohort (Tremelling et al., 2006). Interestingly, in the Korean population the TNF-α_{-857T} allele was found to confer the increased susceptibility (Yang et al., 2006). Koss et al. (2000) examined frequencies of three biallelic TNF-β polymorphisms and four haplotypes and three other TNF-α biallelic polymorphisms and haplotypes, but detected no differences between Crohn's disease patients and healthy controls. The TNF-α haplotype TNF-2 was however associated with higher TNF-α production by whole blood from Crohn's disease patients stimulated with LPS. The TNF-β allele LTα-2 was also associated with higher TNF-α production by whole blood from patients, while in healthy controls this haplotype resulted in reduced TNF-α production. A recent paper reported no association between the TNF-α promoter polymorphisms at position -308, where alleles TNF-1 (G) and TNF-2 (A) originate, and Crohn's disease and the authors suggested these

polymorphisms do not confer susceptibility to the disease (Santana et al., 2011). On the other hand, the TNF-2 allele is associated with a significant ulcerative colitis risk in East Asians according to a meta-analysis by Lu et al. (2008). Interestingly, TNF-2 does not confer increased susceptibility to ulcerative colitis in European and Asian. A 2011 meta-analysis indicated the -1031C, -863A and -857T TNF-α polymorphisms actually do not increase the risk for Crohn's disease in Caucasians and slightly increase the risk in the Asian population (Han et al., 2010). Apparently, ethnicity is of notable influence on the association of TNF-α variants and IBD. In Koreans, the Thr60Asn substitution in TNF-β seemed to protect against Crohn's disease (Yang et al., 2006).

5.3 IBD4

A linkage study in 1999 identified a novel susceptibility locus for Crohn's disease on chromosome 14q11 (Ma et al., 1999). Later, linkage to this locus was confirmed and it was named IBD4 (Duerr et al., 2000). Genes of RNase A family members are located in this region, as is purine nucleoside phosphorylase (PNP), which deficiency causes a rare primary immunodeficiency with T-cell dysfunction (Dalal et al., 2001). The T-cell receptor α and δ subunits and IL-25 genes are also found on the IBD4 locus. Büning et al. (2003) ruled out the coding region of IL-25 as the source of the linkage to IBD4. Also located in the IBD4 region is the embryonal Fyn-associated substrate (EFS) gene (McGovern et al., 2008). Deficiency of EFS in mice show enhanced cytokine secretion, T-cell-dependent antibody production and intestinal inflammation (Donlin et al., 2005). Characterized by lymphocyte infiltrates in the lamina propria, crypt enlargement and damage of the villi the presentation is not unlike Crohn's disease. Familial-based association testing suggested an EFS polymorphism to be associated with Crohn's disease, but a case-control approach did not confirm this (McGovern et al., 2008). Interesting is however the association of EFS haplotype 3 with a subset of patients with anti-flagellin (CBir1) expression and a more severe Crohn's disease phenotype. As of yet no other genes in this region have been identified as being associated with Crohn's disease.

5.4 IBD5

Rioux and colleagues identified a susceptibility locus for Crohn's disease on chromosome 5q31-33 (Gaya et al., 2006). This region, called IBD5, especially contributes to susceptibility in patients with early-onset disease. The IBD5 haplotype which confers the increased susceptibility occurs in approximately 40% of Europeans, but is uncommon in African and East Asian populations (Huff et al., 2012). Consequently, it has been argued IBD5 plays an important role in ethnic differences on the prevalence of Crohn's disease. The IBD5 locus has also been found to confer increased susceptibility to ulcerative colitis (Achkar et al., 2006). This chromosomal region is also associated with susceptibility to other immune system-related disorders, such as coeliac disease, atopic dermatitis and familial eosinophilia (Babron et al., 2003; Beyer et al., 2000; Rioux et al., 1999). It is therefore not surprising that the cytokine gene cluster is located on the IBD5 locus, which includes the well-known IL-3, -4, -5, -13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) genes (Glén et al., 2012; van Leeuwen et al., 1989). Furthermore, IBD5 also contains five other immunoregulatory genes: interferon regulatory factor 1 (IRF1), solute carrier family 22, member 4 (SLC22A4), solute carrier family 22, member 5 (SLC22A5), PDZ and LIM domain protein 4 (PDLIM4) and prolyl 4-hydroxylase (P4HA2;

Silverberg et al., 2007). Incidentally, Crohn's disease patients have an increased expression of IRF1 and enzymatic activity of P4HA2 and lower expression of SLC22A5 (Huff et al., 2012; Rioux et al., 2001). The exonic SLC22A4 and SLC22A5 promoter variants L503F (C1672T) and G-207C respectively and a number of other SNPs have been considered as causal for the association of IBD5 with Crohn's disease (Silverberg et al., 2007). From their study on a large North American cohort, Silverberg and colleagues concluded one of the latter, the intronic IGR2096a_1, shows the strongest association. A recent meta-analysis indicates that in addition to those polymorphisms the variants IGR2198a_1, IGR2230a_1 and G-207C also carry elevated risk (Wang et al., 2011). The combination of the L503F and G-207C alleles (the TC haplotype) is also significantly associated with Crohn's disease risk. In a recessive model of inheritances the polymorphisms L503F, G-207C, IGR2096a_1, IGR2198a_1 all conferred increased susceptibility to ulcerative colitis, including in the Caucasian and adult subgroups.

SLC22A4 and SLC22A5, previously known as OCTN1 and OCTN2 respectively, are members of the carnitine/organic cation transporter family (Tamai, 2013). SLC22A4 is expressed in a variety of tissues, including the bone marrow and intestine. Although its physiological function is not well established, SLC22A4 transports the potent antioxidant ergothionein to the cytosol, possibly to prevent oxidative stress. Deficiency of SLC22A4 in mice causes significant changes in ergothionein localization and intestinal damage. The amino acid substitution L503F impairs SLC22A4 carnitine transporter activity and the G-207C polymorphism disrupts a heat shock transcription factor (HSF) binding element, impeding HSF-dependent activation of the SLC22A5 gene promoter (Leung et al., 2006). The role of SLC22A5 in Crohn's disease is poorly understood, but the transporter is important in maintaining systemic concentrations of carnitine, which is essential in beta-oxidation. Talián et al. (2009) found no abnormalities in the carnitine metabolism of Hungarian Crohn's disease patients carrying the IGR2230a_1 polymorphism. SLC22A5 deficiency in mice however, leads to gut dysfunction and severe disturbance of the immune system (Sonne et al., 2012). This manifests itself in stunted villous growth and differentiation and considerable spleen and thymus atrophy. In addition, lymphocytic and macrophage infiltrates are present in the gastrointestinal tract. Relative numbers of splenic $CD4^+$ T cells and $CD45R/B220^+$ B cells in the thymus and lymph nodes are elevated. Finally, basal production of pro-inflammatory cytokines IL-2 and IL-6 by lymphocytes is increased in $OCTN2^{-/-}$ mice and IL-2, IL-6, TNF-α and IFN-γ production is higher in response to anti-CD3 stimulation.

5.5 IBD6

A number of linkage studies have revealed the IBD susceptibility region on chromosome 19p also known as IBD6 (Tello-Ruiz et al., 2006). Several both positional and functional candidate genes are located on the locus, including cytokine receptors, such as interleukin-12 receptor, beta-1 (IL12RB1), immune system-related intracellular signaling molecules, like the TLR adaptor TIR domain-containing adaptor molecule 1 (TICAM1 or alternatively TRIF) and killer immunoglobulin-like receptors (KIRs), and ligands and receptors involved in cell homing and migration, C-type lectin CD209 for example (Tello-Ruiz et al., 2006; Jones et al., 2006). Polymorphisms in complement component 3 (C3) and intercellular adhesion molecule 1 (ICAM1) genes, which are also located in the IBD6 region, have been suggested as candidates, but association has not been confirmed (Tello-Ruiz et al., 2006). Research by Wolfkamp et al. (2010) indicates a SNP in C-type lectin domain family 2, member D (CLEC2D or LLT1), resulting in

substitution of the leucine at position 94 for valine, is associated with Crohn's disease. CLEC2D is a ligand for CD161, a C-type lectin receptor expressed by NK cells and subsets of natural killer T (NKT) cells and T cells (Aldemir et al., 2005). Binding of CLEC2D results in reduced NK cell-mediated cytotoxicity and inhibition of IFN-γ production by NK cells. Activation of both CD161 and CD3 in T cells even increases IFN-γ production over CD161 activation alone.

Labbé et al. (2008) observed association of four microtubule-associated serine/threonine kinase 3 (MAST3), another IBD6 gene, coding SNPs with IBD, but did not distinguish between ulcerative colitis and Crohn's disease. MAST3 is expressed by $CD4^+$ and $CD8^+$ T cells and B cells and its knockdown attenuates LPS-induced NF-κB activation. Control of NF-κB activity by MAST3 regulates expression of multiple genes central to mucosal immune responses (Labbé et al., 2012). Expression after LPS stimulation of the potent neutrophil chemoattractant CXCL8 and pro-inflammatory cytokine IL-1β is for example decreased in THP1-derived macrophages with MAST3 knockdown, while expression of the anti-inflammatory cytokine IL-10 is substantially increased.

5.6 IBD7

IBD7 refers to a region on chromosome 1p36, first reported in 1998 as a susceptibility locus for both ulcerative colitis and Crohn's disease, especially in families where only one IBD-type occurs (Cho et al., 2000). Maximal evidence for linkage was observed near marker D1S1597, adjacent to leucine-rich repeat-containing protein 38 (LRRC38). Interestingly, this marker is also located close to the tumor necrosis factor receptor superfamily, member 8 (TNFRSF8 or CD30) gene. CD30 is traditionally known for its overexpression in Hodgkin's lymphoma cells, contributing to a pro-inflammatory tumor micro-environment (Simhadri et al., 2012). Expressed mainly by NK cells and subsets of B and T cells *in vivo* CD30 is activated by interaction with ligand CD30L (TNFSF8). Activation of CD30 leads to secretion of pro-inflammatory cytokines IFN-γ and TNF-α by NK cells. Furthermore, CD30L/CD30 signaling is plays a role in T and B cell differentiation and proliferation and memory $CD8^+$ T cell differentiation (Somada et al., 2012). The extracellular part of CD30 can be cleaved by a zinc metalloprotease, releasing soluble CD30 (sCD30). Serum levels of sCD30 are significantly increased in Crohn's disease patients and correlate with disease severity. Consequently, CD30 should be considered a candidate gene on the IBD7 locus.

Guo et al. (2011) examined the potential of caspase-9 (CASP9) and runt-related transcription factor 3 (RUNX3) genes, also located in the IBD7 regions, as susceptibility genes in IBD. RUNX3 is involved in neurogenesis and thymopoiesis and development and differentiation of NK and T cells and dendritic cell functioning. The importance of RUNX becomes apparent in $RUNX^{-/-}$ mice, which develop a human IBD-like phenotype and possess dendritic cells with a diminished response to TGF-β and enhanced migration. Guo and collegues found a weak association of the CASP9 SNP rs1052571 with ulcerative colitis disease severity. No noteworthy associations were found between RUNX polymorphisms or haplotypes and ulcerative colitis or Crohn's disease, but a number of CASP9 haplotypes were strongly associated with both disorders.

5.7 Vitamin D receptor and vitamin D binding protein

In the past decade vitamin D and its influence on (innate) immunity has emerged as a topic of interest. Consequently, linkage analysis has also been performed on genes related to vitamin D metabolism and function in connection with IBD. Since the vitamin D receptor (VDR) gene is located in close proximity to the IBD2 locus on chromosome 12q, Simmons and collegues considered it a strong positional candidate (Simmons et al., 2000). In their study UK ulcerative colitis and Crohn's disease patients and healthy controls were typed for 3 SNPs previously shown to be associated with susceptibility to infectious disease, osteoporosis and prostate cancer. Homozygosity for the TaqI 't' variant was found to occur significantly more frequent in Crohn's disease patients, while no association with ulcerative colitis was found. This synonymous polymorphism is an A/C substitution in codon 8 and homozygosity is significantly associated with decreased VDR protein levels in PBMCs (Ogunkolade et al., 2002; Simmons et al., 2000). A similar Iranian case-control study showed an association of the FokI polymorphism with Crohn's disease and ulcerative colitis (Naderi et al., 2008). Homozygosity for the 'f' allele was significantly more common in Crohn's disease patients than controls. This SNP results in translation initiation at different sites yielding either a long (f) or short (F) version of VDR (van Etten et al., 2007). Peripheral blood monocytes and dendritic cells with the homozygous short VDR genotype show higher IL-12p35 and IL-12p40 mRNA and IL-12p70 protein expression in response to IFN-γ stimulation. Another VDR polymorphism, BsmI, was found to be associated with susceptibility to ulcerative colitis in Han Chinese, while neither ApaI, TaqI nor FokI was, and Ashkenazi Jews (Pei et al., 2011). In an Irish study none of the SNPs mentioned above were significantly associated with ulcerative colitis and Crohn's disease susceptibility (Hughes et al., 2011).

Vitamin D binding protein (DBP), also known as group-specific component (GC), is a carrier of vitamin D and its metabolites through the circulation to target tissues (Malik et al., 2013). As such, DBP is an important factor in vitamin D metabolism. Interestingly, DBP itself has immunomodulatory qualities as it enhances the chemo-attractive activity of the complement component 5 α chain (C5a). Eloranta et al. (2011) recently examined whether two common SNPs at positions 432 (Asp432Glu) and 436 (Thr436Lys) were associated with IBD. Homozygosity for 436 Lys was negatively associated with both ulcerative colitis and Crohn's disease. Furthermore, while Asp432Glu was not found to be significantly associated with IBD, the DBP_2, which consists of 432 Asp and 436 Lys occurred more frequently in the control group.

5.8 Other loci

As mentioned earlier many other susceptibility loci have been identified, but they are less well described than those above. For some, genes that confer the increased risk have been found, such as interferon regulatory factor 5 (IRF5) located in the IBD14 region on chromosome 7q32 (OMIM 612245, 2008). Activation of pathogen recognition receptors such as TLRs and NOD2 results in the translocation to the nucleus of IRF5, where this transcription factor promotes transcription of pro-inflammatory cytokines (Hedl and Abraham, 2012). A two-marker haplotype in the IRF5 gene was recently found to be associated with IBD (Gathungu et al., 2012). Lymphocytes homozygous for the CGGGG insertion/deletion promoter polymorphism, part of this haplotype, produce more of the cytokine IL-

12p70. Interestingly, also polymorphisms in TLR4 (Asp299Gly and Thr399 Ile) itself may confer IBD susceptibility (Brand et al., 2005). Furthermore, there exists some evidence for a connection between the anti-microbial peptide bactericidal permeability-increasing protein (BPI) polymorphisms and Crohn's disease and ulcerative colitis (Akin et al., 2011).

Noteworthy also are the ATP-binding cassette, subfamily B, member 1 (ABCB1) and interleukin 23 receptor (IL23R) genes, which account for the association with IBD of the IBD13 (chromosome 7q21) and IBD17 (chromosome 1p31) loci respectively (OMIM 171050, 2013; OMIM 607562, 2013). ABCB1 variants are associated with increased risk of ulcerative colitis, but not Crohn's disease (Onnie et al., 2006). Genetic variants of IL23R have been reported to be associated with ulcerative colitis and especially Crohn's disease (Cotterill et al., 2010).

Of interest also is the cytotoxic T lymphocyte-associated 4 (CTLA4) gene located on chromosome 2q33 (Repnik and Potocnik, 2010). CTLA4 is expressed in a membrane-bound form on activated T and B cells and secreted in a soluble form (sCTLA4). It competes with T cell surface receptor CD28 for binding of B7 molecules on antigen-presenting cells, thereby modulating T cell activation. Recent research indicates pregnane X receptor (PXR) and liver X receptor (LXR) polymorphisms may also contribute to IBD risk (Andersen et al., 2011). These nuclear receptors regulate transcription of certain pro-inflammatory genes and inhibit NF-κB activity, clearly indicating their potential influence on IBD-type inflammation.

Also interesting and novel is the association Storr and colleagues describe between the synonymous 1359 G/A SNP in the cannabinoid 1 receptor (CNR1) gene and ulcerative colitis (Storr et al., 2010). The polymorphism did not influence Crohn's disease risk, but homozygous carriers of the G allele were significantly more likely to develop CD before 40 years of age.

This overview of susceptibility loci for Crohn's disease and ulcerative colitis is by no means exhaustive. It shows however the large number of genetic risk factors to be considered in understanding the initiation and continuation of the diseases. Furthermore, considering the variety of pathways and processes of the immune system the gene products are involved in, the pathology is apparently highly complex.

5.9 References

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