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Role of bacteria and mucosal immune system and their interaction in the pathogenesis of inflammatory bowel disease

Ph.D. Thesis

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Statement of originality

I hereby declare that this work has no material which has been previously submitted for a degree or diploma at any other higher education institution or university. To the best of my knowledge this thesis is the product of my own work and contains no material previously published or written by another person, except where due references are made.

Nový Hrádek 9. 3. 2016 MSc. Zhengyu Du

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ABSTRACT

Although the etiology and pathogenesis of inflammatory bowel disease (IBD) is not fully understood, it is generally accepted that the inflammation results from aberrant immune responses to antigens of gut microbiota in genetically susceptible individuals (Sartor et al., 2006). Alteration in intestinal microbiota has been found in IBD patients with increased abundance of certain bacteria and decreased abundance of others. Due to the complexity of the disease, multifaceted interactions between genetic factors, host immune response, gut microbiota and environment factors need to be taken into account.

In this thesis, the pathogenesis of IBD was first reviewed in respect with the four factors mentioned above. Then we concentrated on the interaction between IBD-associated bacteria and mucosal immune system. We investigated the ability of mucosal-associated bacteria (MAB) from IBD patients to induce spontaneous colitis in germ-free (GF) mice and the impact of those bacteria on the development of dextran sulfate sodium (DSS)-colitis. Together with the analysis of the composition of gut microbiota of MAB colonized mice, we demonstrated the potential deleterious microbes were able to increase the susceptibility to DSS-colitis once they found a suitable niche. We revealed the mechanism of an *E.coli* strain which were reported to be more frequently isolated from IBD patients to damage the integrity of the intestinal epithelium by its hemolytic activity. Not only focusing on the “bad guys”, we also elucidated the protective effect of the probiotic strain *Clostridium tyrobutyricum* against acute colitis by promoting the mucosal immune homeostasis and we found butyrate produced by the bacterium as a key component to elicit the anti-inflammatory capacity.

Gut microbiota has a profound impact on immune response with subsequently affecting the total health of a host. This thesis provides us better knowledge of bacteria interacting with the immune system and may bring new insights to treatment of IBD.

ABSTRAKT

Etiologie a patogeneze chronického zánětlivého onemocnění střevního traktu (IBD). není doposud zcela jasná. Je obecně přijato tvrzení, že zánět je důsledkem nepřiměřené imunitní reakce na antigeny rezidentní mikrobioty u geneticky citlivých jedinců. U pacientů s IBD dochází ke změnám ve složení střevní mikrobioty, kdy je většinou zvýšen výskyt jednoho druhu bakterie a naopak dochází ke snížení výskytu ostatních bakteriálních druhů. IBD je velmi komplexní onemocnění a pro pochopení jeho vzniku a rozvoje je nutné brat v úvahu také složité interakce mezi genetickými faktory, imunitním systémem, střevní mikrobiotou a životním prostředím.
V předkládané dizertační práci jsme se zaměřili na interakci mezi střevní mikrobiotou spojenou s IBD a mukózním imunitním systémem. V gnotobiotickém myším modelu jsme zkoumali schopnost bakterií asociovaných s mukózou (MAB), které byly odebrány od pacientů s aktivním IBD, vyvolat spontánní kolitidu. Byl posuzován vliv těchto bakterií na vznik a vývoj experimentálně vyvolané kolitidy dextran sulfátem sodným (DSS). Spolu s analýzou složení střevní mikrobioty, původně bezmikrobních myší kolonizovaných MAB od IBD pacientů, jsme ukázali, že škodlivé bakterie mají schopnost zvyšovat náchylnost k DSS vyvolané kolitidě. Objasnili jsme mechanismus působení *E. coli* p 19 A, která se vyskytuje často u IBD pacientů a která díky své hemolytické aktivitě poškozuje integritu střevního epitelu. Zaměřili jsme svou pozornost i na vliv nepatogenní bakterie s probiotickými účinky *Clostridium tyrobutyricum*. Tato bakterie, produkující butyrát, je klíčovou složkou při udržování homeostázy a zábránila rozvoji DSS kolitidy u myší. Střevní mikrobiota má zásadní vliv na imunitní system a v konečném důsledku i na celkové zdraví jedince. Tato dizertační práce nám umožňuje lépe porozumět bakteriím komunikujícím s imunitním systémem a přináší nové terapeutické možnosti v léčbě IBD.

1. GENERAL INTRODUCTION

1.1. Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is the chronic remittent inflammatory disorder that can affect any part of the gastrointestinal tract and colonic mucosa. There are two major clinically defined forms of IBD, namely Crohn's disease (CD) and Ulcerative colitis (UC). CD is characterized by non-continuous transmural inflammation that can spread through the whole gastrointestinal tract from mouth to anus. Complications such as multiple granulomas, abscesses and fistulas are quite common features in CD patients. On the contrary, UC is typified by superficial inflammation exclusively affect colon and rectum. Inflammation may begin in the rectum, spreads proximally in a continuous fashion and often involves the periappendiceal region (fig.2.1) (Khor et al., 2011; Biasi et al., 2012).

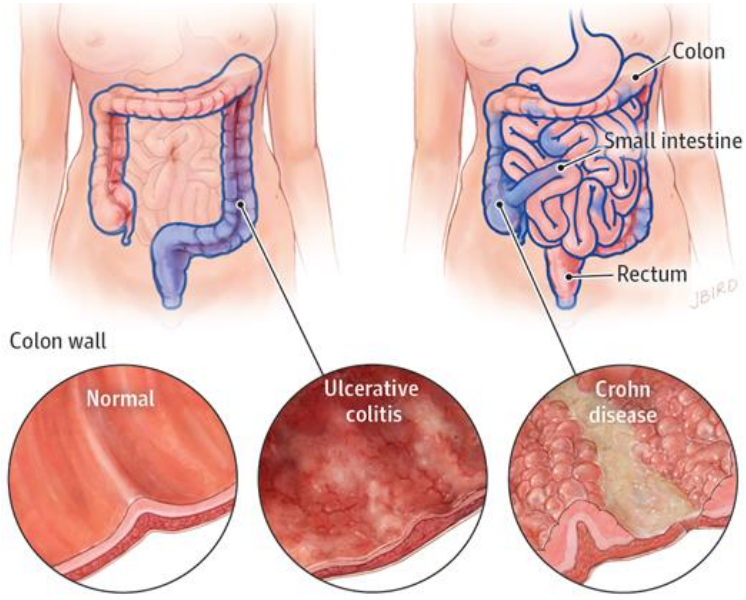

Fig.1.1 Diagram of inflammatory bowel diseases (IBD). Ulcerative colitis (left) affects only the colon with inflammation on the inner layer. Crohn's disease (right) affects any part of the gastrointestinal tract with transmural inflammation. Adopted from Jin (2014)

The rate of incidence of both types of IBD has been climbing rapidly from 6 cases per 100,000 person-years to 19.2 cases for UC in North America since 1981 to 2004, 8.7 cases per 100,000 person-years to 24.3 cases in Europe since 1991 to 2008 and 1.9 cases per 100,000 person-years to 6.3 cases in Asia since 1991 to 2008 (Loftus, 2004; Molodecky

et al., 2011). The highest incidence of CD was found in North America as 20.2 cases per 100,000 person-years, and 12.7 cases in Europe and 5.0 cases in Asia (Loftus, 2004; Molodecky et al., 2011). Till 2011, UC and CD together had affected over 3.7 million people around the world (Abraham and Cho, 2009; Cosnes et al., 2011). Due to the quick rise of incidence over the past several decades, IBD has been one of the extensively studied human conditions, etiology, pathogenesis and treatments of which are investigated intensively. Though the exact mechanisms underlying the pathogenesis of IBD is not clearly uncovered yet, 1) the discordance of IBD among the monozygotic twins (Halme et al., 2006), 2) the increased rate of incidence of IBD in the countries that undergo the process of westernization (Thia et al., 2008) and 3) the strong association between dysfunctions of innate and adaptive immunity and aberrant intestinal inflammatory response (Kuhn et al., 1993; Saitoh et al., 2008; Heller et al., 2002; Kontoyiannis et al., 1999; O'Connor et al., 2009) highlight an important factor that IBD is a multifaceted disorder. Various causative-associated factors may interplay with one another leading to the onset and perpetuation of IBD. Thus, it is widely accepted that IBD results from inappropriate and abnormal inflammatory response to intestinal commensal microorganisms in the genetic predisposed individuals (fig. 2.2).

1.2. Pathogenesis of IBD

1.2.1. Genetic basis

IBD has been considered to have a genetic basis for a long time. According to Halme's study, IBD is familial in 5~10% of individuals and sporadic in the rest of the cases (Halme et al., 2006). Interestingly, compared to the concordance rate of 50~75% in CD in monozygotic twins, phenotypic concordance is much less in UC (10~20%) suggesting heritability is less important in UC compared to CD and other non-genetic factors might play an even crucial role in UC. Nevertheless, meta-analysis of genome-wild association studies (GWAS) has

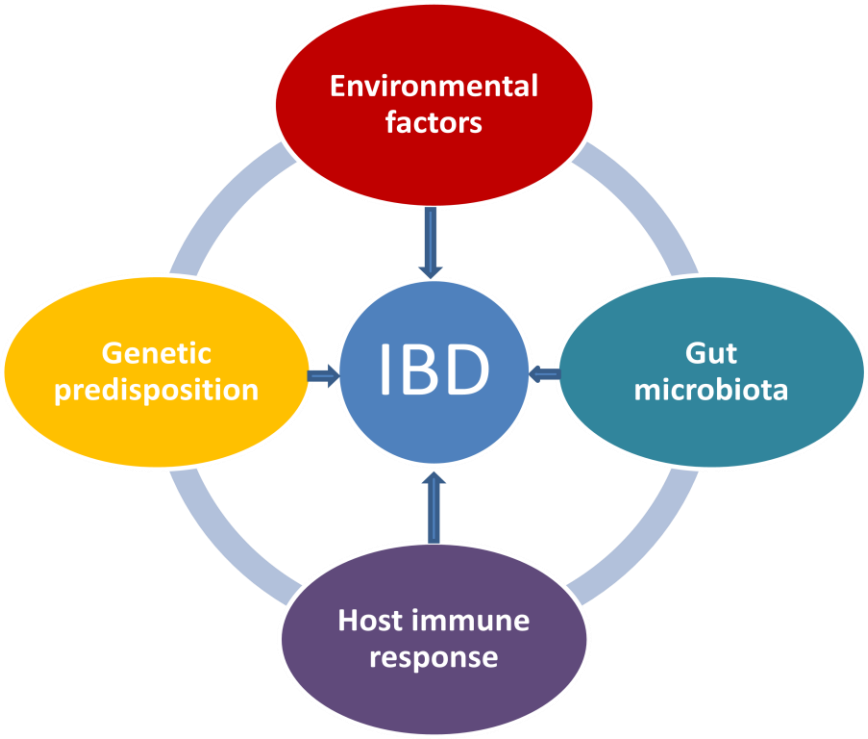

Fig.1.2 Schematic diagram of main factors implicated in the pathogenesis of IBD. Each of mentioned factors interplays with one another, leading to the onset and perpetuation of the inflammation in gut.

identified 163 susceptibility loci for IBD in European population (fig. 2.3) (Jostins et al., 2012). 67% of these IBD risk loci are associated with both CD and UC (e.g. IL23R, IL12B, HLA, NKX2-3 and MST1), 50 of which have an equal risk effect for both phenotypes. 113 out of 163 IBD risk loci are shared with a number of other complex diseases, such as autoimmune diseases (Jostins et al., 2012; Gregersen and Olsson 2009; Zhernakova et al., 2009; Fisher et al., 2008). Among the IBD risk loci, only 23 are classified as UC specific and 30 are CD specific (Jostins et al., 2012). Loci which are involved in regulatory pathways such as interlukin-10, intestinal epithelial cell function such as ECM1 and an E3 ubiquitin ligase such as HERC2 are highly specific to UC, whereas loci implicated in autophagy (e.g. ATG16L1 and IRGM) and antigen recognition (e.g. NOD2 and intelectin-1) appear to be CD specific (Kaser et al., 2010). Interestingly, in contrast to a number of CD- and UC-associated genes and regions that have been identified in European population, there is only one associated gene TNFSF15. This gene expressed

in macrophages inducing Th17 differentiation (Cho and Brant, 2011) was detected to be clearly associated with both CD and UC in East Asian population (Nakagome et al., 2010). Recently, two east Asia-specific IBD susceptibility loci which are common for both CD and UC are first reported by Yuta Fuyuno and his colleagues, namely ATG16L2-FCHSD2 and SLC25A15-ELF1-WBP4 (Fuyuno et al., 2015). The remarkable variation within the IBD risk loci between different populations suggest that substantial genetic heterogeneity exists within and between populations.

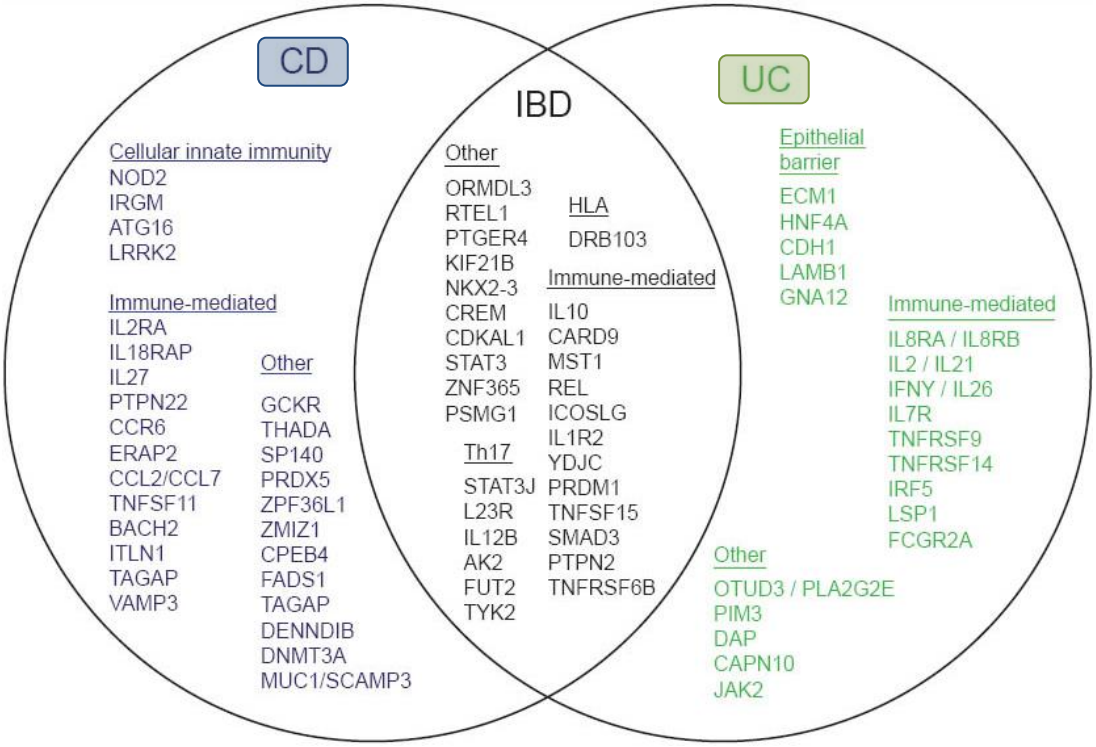

Fig.1.3 IBD susceptibility loci. The loci are represented by lead gene name, according to pathway. The loci attaining genome wide significance ($p < 5x10^{-8}$) are shown for CD in blue, UC in green and IBD in black. Adopted from EK *et al*. (2014)

1.2.2. Environmental factors in IBD

External environment is an important factor that has great impact on mediating the risk of UC and CD by influencing the gut microbiome, host immune response and the epithelial barrier function. Plenty of studies have been

conducted to investigate the effect of a number of environmental factors such as cigarette smoking, diets, vitamin D intake, psychologic and behavioral factor, medication, enteric infection and air pollution on UC and CD (Ananthakrishnan et al., 2014).

Among all these factors, cigarette smoking is the most studied IBD risk factor. It is very interesting that smoking has completely opposite effect in risk of incidence of CD and UC. According to Higuchi's study, active smokers increase the risk of CD by 2-fold compared to non-smokers. But in case of ex-smoker, this risk slightly reduce the effect (Higuchi et al., 2012). On contrary, protection against UC is observed in active smokers with a reduction of the risk by half. However, the risk of incidence UC is significantly increased on smoking cessation (Higuchi et al., 2012; Mahid et al., 2006). It is evident that diet has an inevitable effect on the risk of incidence of both UC and CD. High fiber intake, especially fiber from fruits and vegetables, is reported to be associated with a significant reduction in risk of CD by Ananthakrishnan et al. (2013). While later the same group found that diet contained high long-chain n-3 poly unsaturated fatty acids (PUFA) was associated with a reduction in risk of UC (Ananthakrishnan et al., 2014). Besides fiber and fatty acids, protein is also observed to contribute to the risk of incidence of IBD. A study conducted out in French middle-aged women demonstrated that high consumption of protein from meat and fish is closely associated with an increased risk of IBD (Jantchou et al., 2010). Moreover, long-term diet strongly influences the intestinal microbiota which is considered to remarkably participate in the development of intestinal inflammatory diseases like IBD (Wu et al., 2011; Kostic et al., 2014). Higher intake of vitamin D, another factor believed to be involved in the pathogenesis of UC and CD, appears to reduce the risk of incident of UC and CD (Ananthakrishnan et al., 2012). Other factors such as enteric infection, antibiotic exposure and anti-inflammatory drug treatment are associated with increased risk and relapses of IBDs (Mylonaki et al., 2004;

Ananthakrishnan, 2013; Hashash et al., 2015; Ananthakrishnan et al., 2012). Psychological factors such as stress, emotions and life experiences have been long believed to have a great impact on a person's immune function which is a major player in the development and perpetuation of IBD (Riscalla, 1982; Kiecolt-Glaser et al., 2002; Netea et al., 2004; MacMaster et al., 2003; Heller et al., 2002). Therefore, it is not surprised to see that stress, anxiety and depression as well as rates of relapse and surgery are found to be associated with increased risk of IBD (Camara et al., 2011; Lerebours et al., 2007; Mawdsley and Rampton, 2005; Bitton et al., 2008).

1.2.3. Innate immunity and adaptive immunity in IBD

1.2.3.1. Role of innate immunity in IBD

Innate immunity, the first line of defense against aggregation of external and internal microorganisms, is mediated by a wild range of different cells including various immune cells such as neutrophils, dendritic cells, macrophages etc and non-immune cells such as epithelial cells (Zipfel, 2009). In contrast to adaptive immunity that has been essentially considered to play a critical role in the pathogenesis of IBD, the innate immune response has not been considered as equally important as adaptive immune response for a long time. For this position now owes to numerous outstanding advances in immunology and genetics.

Nucleotide-binding oligomerization domain (NOD)-like receptors NOD2 which is expressed by myeloid cells, intestinal epithelial cells (IECs), Paneth cells and T cells (Gutierrez et al., 2002; Hisamatsu et al., 2003; Shaw et al., 2009) is the first risk gene identified to be associated with increased risk of incident CD (Ogura et al., 2001). NOD2 is a pattern recognition receptor (PRR) that recognizes intracellular pathogen associated molecular patterns (PAMPs) like N-acetyl muramyl dipeptide (MDP) and viral ssRNA (Meylan et al., 2006; Sabbah et al., 2009). Stimulation of NOD2 ultimately results in the activation of

nuclear factor (NF)-κB which induces gene transcription and production of chemokines and cytokines (Abbott et al., 2007). Three uncommon single nuclear polymorphisms (SNPs) in NOD2 that affect the protein structure account for the major CD-associated variants (81%), which are located in the leucine-rich repeated region involving in pathogenic molecule binding (Lesage et al., 2002). Those three major CD-associated NOD2 mutations have been found to be associated with decreased activity of NF-κB by abrogation of receptor-interacting serine-threonine kinase 2 (RIP2) binding and NF-κB essential modulator (NEMO) ubiquitination which subsequently leads to diminished cytokines production in peripheral blood mononuclear cells (PBMCs) (table 2.1) (Inohara et al., 2003; van Heel et al., 2005; Abraham and Cho, 2006). The insufficient secretion of cytokines may result in reduced antibacterial agent production and enhanced pathogenic microbial invasion. As Wehkamp et al. demonstrated that Paneth cells from $NOD2^{3020insC}$ homozygous patients exhibited decreased α-defensin HD4 and HD5 expression (Wehkamp et al., 2005). Interestingly, intact NOD2 signaling inhibits Toll-like receptor (TLR) 2-driven activation of NF-κB. Therefore, mutations in NOD2 could elevate TLR2 mediated NF-κB activity leading to activation of inflammatory pathways caused by excessive Th1 responses (Watanabe et al., 2004). According to Maeda et al. study, increased activity of NF-κB in response to MDP stimulation was observed in $NOD2^{2939insC}$ mice which expressed the mouse homolog of human $NOD2^{3020insC}$. As a result, these mice had an increased susceptibility to dextran sulphate sodium (DSS) colitis (Maeda et al., 2005). Moreover, CD-associated NOD2 variants also exhibit the ability to inhibit the expression of IL-10 produced by monocytes from patients homozygous for $NOD2^{3020insC}$, which suggests NOD2 mutations may result in insufficient immune response to microorganisms leading to intestinal inflammation (Noguchi et al., 2009).

Autophagy is a fundamental highly conserved cellular process in response to

stress and starvation. The cellular process is involved in formation of double-

Table 1.1 features of three main CD-associated NOD2 mutations

NOD2 polymorphism	% of CD-associated variants (Lesage et al., 2002)	allele frequency (Lesage et al., 2002)		consequences
		familial	sporadic	
R702W (SNP8)	32	0.09	0.14	increased risk of clinically significant bacterial infection (Janse et al., 2013); significant reduction in NF-κB signaling and activation of IL-8 promoter (Parkhouse and Monie, 2015); increased IL-8 basal levels (Salucci et al., 2008)
G908R (SNP12)	18	0.07	0.04	increased number of positive antibodies to microbial antigens (Murdoch et al., 2012)
1007fs (SNP13)	31	0.11	0.09	significant reduction in NF-κB signaling and activation of IL-8 promoter (Parkhouse and Monie, 2015); increased number of positive antibodies to microbial antigens (Murdoch et al., 2012); increased intestinal permeability (Buhner et al., 2006); significant reduction in TNF-α, IL-8, IL-12, and IL-10 production (Salucci et al., 2008)

membraned autophagosomes that internalize invading intracellular microbes or cellular constituents to fuse with lysosomes (He and Klionsky, 2009). Therefore, autophagy is a crucial part of innate immune defence against microorganisms. Two genetic risk factors ATG16L1 and IRGM which are implicated in autophagy have been discovered to be associated with CD but not with UC by GWAS (Hampe et al., 2007; Parkes et al., 2007). It was reported that mice lacking ATG16L1 in hematopoietic cells were highly susceptible to DSS-induced acute colitis, which could be ameliorated by IL-1β

and IL-18 blockade. This finding is consistent with the result that stimulation of ATG16L1-deficient microphages by lipopolysaccharide resulted in high amount production of the inflammatory cytokine IL-1β and IL-18 indicating an important role of ATG16L1 in the suppression of intestinal inflammation (Saitoh et al., 2008). In the later work done by Cadwell et al. (2008) mice containing embryonic fibroblasts polymorphic for ATG16L1 exhibited conspicuously abnormalities in Paneth cell morphology, such as lack of mucosal lysozyme staining, reduced and disorganized granules, degenerating mitochondria and absence of apical microvilli. Interestingly, these changes in Paneth cell morphology were also observed in ileocolic resection specimens from CD patients carrying the ATG16L1 risk allele (Cadwell et al., 2008). Furthermore, recent study has uncovered the link between NOD2 and autophagy showing that NOD2 recruited ATG16L1 to the plasma membrane at the bacterial entry site by a mechanism independent of NF-κB (Travassos et al., 2009). The major CD-associated NOD2 mutations were found to be unable to activate autophagy through ATG16L1 and ultimately leaded to severe impairment of clearing invading bacteria which contributed to induction of inflammatory environment (Cooney et al., 2009). Moreover, a bi-directional relationship between commensal intestinal bacteria and NOD2 was discovered (Petnicki-Ocwieja et al., 2009). IRGM was identified as the second autophagy gene that its variants were associated with CD (Parkes et al., 2007). Data from Irgm1 (encoding LRG-47 mouse homolog) knock-out mice showed increased susceptibility to *Toxoplasma gondii, Listeria monocytogenes,* and *Mycobacterium tuberculosis* infection due to decreased ability of Irgm1−/− macrophages to eliminate the bacteria (MacMicking et al., 2003).

Intestinal epithelium, the first physical barrier of defense against intestinal bacteria and food antigens, has been long considered to participate in IBD pathogenesis (Gebbers et al., 1985). Intestinal epithelium is composed of enterocytes and specialized epithelial cells such as goblet cells that secreting

gel-forming mucins and Paneth cells producing antimicrobial peptides and proteins constitutively or due to bacterial stimulation (Birchenough et al., 2015; Uehara et al., 2007). Mucin is the major component of mucus that is organized in an inner dense layer and an outer more loose and permeable layer above the intestinal epithelium (Birchenough et al., 2015). In healthy individuals, gut bacteria are usually separated from the intestinal mucosa by thick layer of mucus (Johansson et al., 2011). However, compared to normal tissues from healthy individuals, there was observed thinner and less sulfated mucin along with increased bacterial colonization of mucosa in colonic tissue taken from patients with UC (Bibiloni et al., 2006; Corfield et al., 1996; Kleessen et al., 2002; Schultsz et al., 1999). This defective mucus layer in IBD patients cannot restrain the microbes resulting in a direct contact of the microbes with the intestinal epithelium, which may have potential pathogenic consequences. This hypothesis is confirmed by studies from MUC2-/- mice which exhibited propensity for colitis and colorectal cancer (an established complication of longstanding UC) due to inadequate prevention of bacterial breach (Van der Sluis et al., 2002; Velcich et al., 2002). However, no human MUC2 gene variants have been announced to be associated with impaired mucosal barrier function (Buisine et al., 1999).

The disruption of the physical integrity of the intestinal epithelium is another crucial antecedent factor to the development of IBD since increased intestinal permeability have been observed in patients with both CD and UC for a long time (Salim et al., 2011). The integrity of the intestinal epithelium is mediated by three key compartments of the cell-cell junction namely tight junctions, adherens junctions and desmosomes. Studies have ravealed that altered expression and structural changes of the intestinal tight junction proteins are closely associated with the development of IBD (Zeissig et al., 2006; Schmitz et al., 1999). Moreover, pro-inflammatory cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-α were able to impair tight junction resulting in

increased paracellular permeability and induce apoptosis of IECs, which allowed lumen microbial antigens to across the epithelium and enter the lamina propria, inducing the production of the inflammatory mediators (fig. 2.4) (Marini et al., 2003; Su et al., 2013; Nava et al., 2010). Recently GWAS revealed three new UC genetic risk loci, each of which has at least one

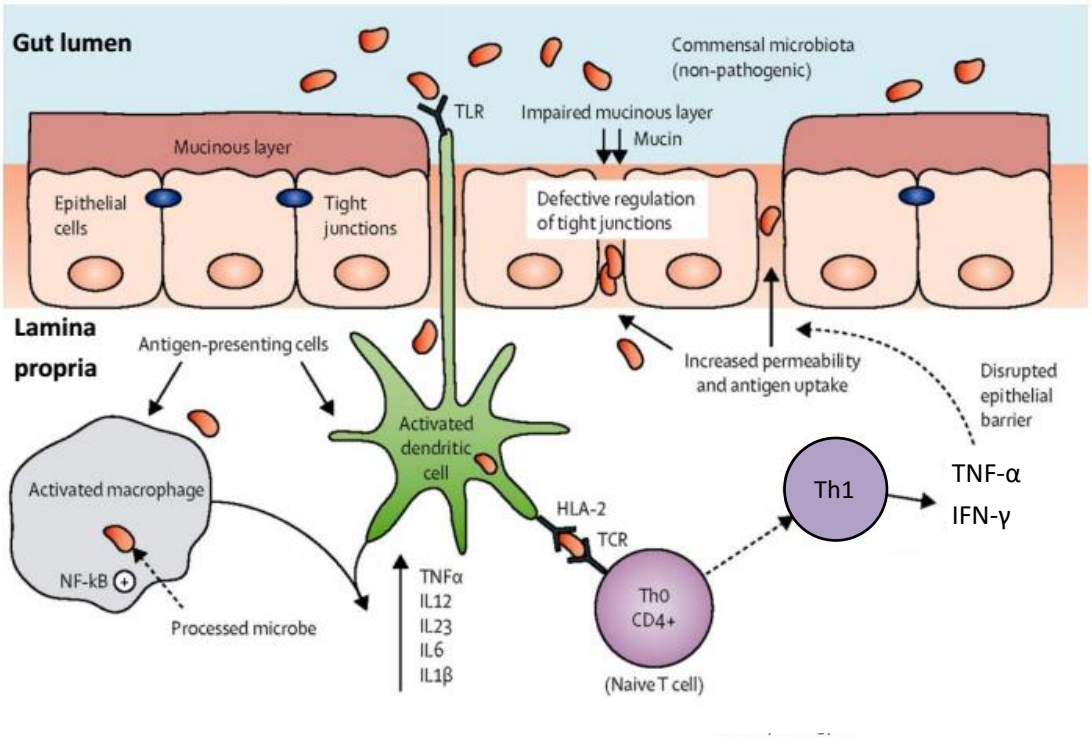

Fig.1.4 Schematic presentation of the tight junction and its regulation by immune system in IBD. Tight junction are composed by claudins, zonula occludens 1 and occludin. These proteins are attached to F-actin and myosin. High levels of TNF-α and IFN-γ can induce the apoptosis of IECs as well as the activation of myosin light chain kinase leads to the phosphorylation of myosin light chain which results in tight junction impair. The defects in epithelium facilitate luminal microbes to cross the epithelium and enter the lamina propria, which induces the production of inflammatory mediators causing further damage of epithelium. The leaky epithelium allows more microbes to get into lamina propria to further stimulate immune cells, which amplifies the cycle of inflammation. Adopted from Ordas *et al*. (2012)

biologically relevant gene candidate involved in the integrity of the intestinal epithelium. HNF4A encodes the transcription factor that regulates the

expression of multiple components that form the intercellular apical junction complex. CDH1, the strongest candidate for UC susceptibility located on chromosome 16q22, encodes E-cadherin which is one of the main components of the adherens junction and a key mediator of intercellular adhesion in the intestinal epithelium. LAMB1 encodes the laminin beta 1 subunit, a component of laminin heterotrimer that is important in anchoring the single-layered epithelium (Barrett et al., 2009; Anderson et al., 2009; Battle et al., 2006; Karayiannakis et al., 1998).

Furthermore, impaired capacity of IEC cells to induce CD8+ suppressor cells was observed in IBD patients suggesting an intrinsic defect in epithelial cells in patients with IBD (Mayer and Eisenhardt, 1990). Reduced expression of peroxisome proliferation-activated receptor (PPAR)-γ was found in colonic epithelial cells from UC patients (Dubuquoy et al., 2003). Due to the fact that PPAR-γ inhibits the expression of pro-inflammatory molecules from myeloid and epithelial cells, its down-regulated expression may contribute to the induction of an inflammatory environment in intestine (Ricote et al., 1998; Jiang et al., 1998). Another defect related to the pathogenesis of IBD is the impaired production of antimicrobial peptides secreted by Paneth cells. The secretion is normally induced by the recognition of bacterial component by PRRs. Samples isolated from patients with ileal CD exhibited a decreased expression of Paneth cells defensins associated with the reduced antibacterial activity. These findings implied innate immune defenses of the ileal mucosa was compromised and might result in onset and perpetuation of the disease (Wehkamp et al., 2005).

1.2.3.2. Role of adaptive immunity in IBD

Distinguish from nonspecific defense of innate immunity which comes into play immediately, adaptive immune response is extremely specific to particular pathogen with long lasting effect exerted by memory cells. To mount an

effective immune response takes adaptive immune system for four to seven days.

T cells, the key players in the adaptive immune system are thought to play a pivotal role in both phenotypes of IBD. Helper T cells (CD4+) and cytotoxic T cells (CD8+) are two forms of naïve T cells which can be activated through a "classical" signal transduction cascade initiated by recognized antigen binding to T cell receptor on the surface of the cells. Naïve CD4+ T cells activated in a particular cytokine milieu can subsequently differentiate into either Th1 or Th2 or Th17 or regulatory T (Treg) cells as defined by their cytokine secretion patterns (Romagnani, 1994). Aberrant Th1 immune responses caused by up-regulated mucosal amount of IL-18 and IL-12 have been reported to be associated with intestinal inflammation in CD (Monteleone et al., 1999; Monteleone et al., 1997). IL-12 is known as a T cell-stimulating factor that induces naïve T cell to differentiate into Th1 cells which produce high amount of IFN-γ (Romagnani, 1994). Many studies have discovered that mucosa T cells from CD patients produced increased level of INF-γ (Breese et al., 1993; Noguchi et al., 1995; Camoglio et al., 1998). Therefore, CD was believed to be a Th1-mediated disease. On the contrary, high levels of IL-5 and IL-13, the principle Th2 cytokines produced by mucosal T cells, were observed in UC patients (Fuss et al., 1996; Heller et al., 2005).Thus, UC was characterized as a Th2 immune response. However, with Th17 cells which produce substantial amount of IL-17, IL-21 and IL-22 came up into the sight, Th1 paradigm in CD had to be reconsidered due to the discovery of IL-17 and IFN-γ production in mucosal lymphocytes in patients with CD and enhanced expression of IL-21 in intestinal tissue from CD patients (Zhou et al., 2007; Annunziato et al., 2007; Monteleone et al, 2005). Additionally, in other studies, high amount of IFN-γ and low level of IL-13 were found in UC biopsy and colonic mucosa of UC patients, which may raise the reconsideration of the notion that UC was a Th2 based disorder (Rovedatti et al., 2009; Kadivar et al., 2004).

Treg cells are essential for maintain immune homeostasis of intestine by mediating immune responses against gut microbes and diet antigen through suppressing naïve T cell proliferation (O'Garra et al., 2004). Elevated number of Treg cells was found in the intestinal mucosa of IBD patients with normal function of repressing Th1-polarized colitis while the number of Treg cells were decreased in peripheral blood of patients with active IBD (Mottet et al., 2003; Maul et al., 2005). Interestingly, an intact signaling of transforming growth factor (TGF)-β which is an anti-inflammatory cytokine produced by Treg is required for the normal function of Treg cell. In IBD patients, despite the high levels of TGF-β, its signaling was found to be impaired in inflamed mucosa due to over expression of the inhibitory molecule Smad7, which led to lamina propria effector T cell resistant to Treg-mediated suppression (Fantini et al., 2009; Fahlen et al., 2005). The reduction of anti-inflammatory activity of Treg may foster intestinal inflammation, as one of the mechanisms implicated in IBD pathogenesis.

Besides the defects of anti-inflammatory processes, abnormal T cell immune response which could be caused by lamina propria T cells resistant to apoptosis appears to drive inflammation in IBD. Usually, antigen-activated T cells are eliminated by apoptosis to prevent potential damage of the host (Strasser and Pellegrini, 2004). However, studies found the mucosal T cells isolated from CD patients to be resistant to multiple apoptotic stimuli (e.g. IL-2 deprivation, Fas Ag ligation, and exposure to TNF-α and nitric oxide) because of the higher ration of Bcl-2/Bax, suggesting that the rate of T cell proliferation exceeded that of cell death (Ina et al., 1999; Sturm et al., 2002). Additional mechanisms involved in mucosal T cell resistant to apoptosis in IBD are 1) mediated by IL-6 trans-signaling (Atreya et al., 2000); 2) associated with increased level of survivin, a member of inhibitors of apoptosis family, prevents cell death (de Souza et al., 2012). The insufficiency in apoptosis could result in the inappropriate accumulation of T cell contributing to chronic inflammation.

1.2.4. Intestinal microbes in IBD

Our body is known to be extensively covered with numerous bacterial cells from skin to mucosal surface. The majority of bacteria reside within our intestine, specifically in large intestine. Total number of bacteria can reach to 9×10^{13} in an individual. This is why the intestinal microbiota is so-called a tissue in the organ (fig.2.5) (Baumgart and Carding, 2007). These bacterial cells make up for the predominant component of microflora which has a great impact on our health and contributes to maintain intestinal homeostasis (Ostaff et al., 2013). The idea that enteric bacteria are implicated in the pathogenesis

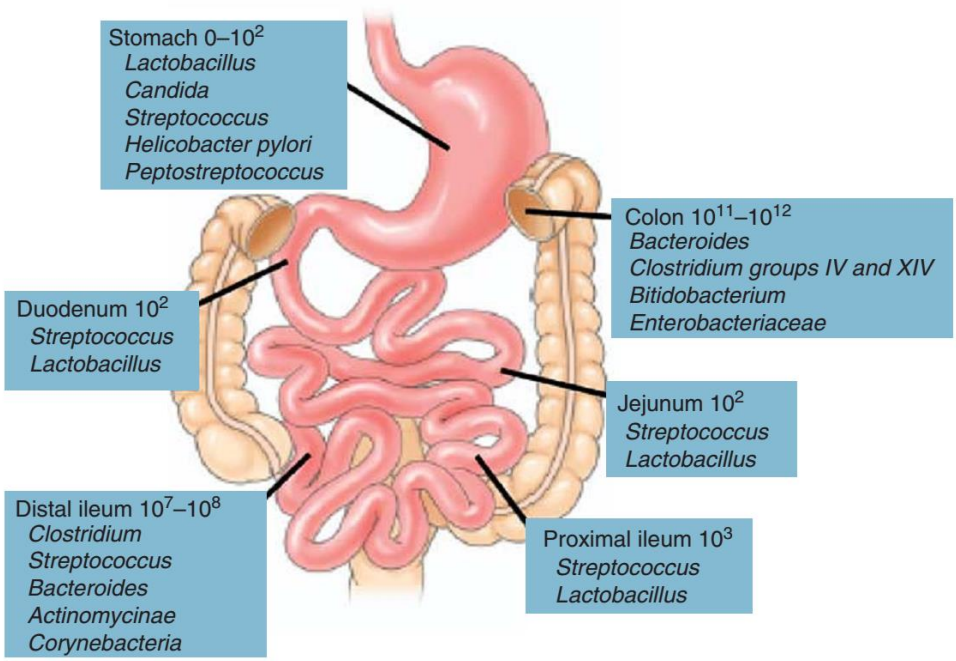

Fig.1.5 Composition and luminal numbers of dominant microbial species in the human gastrointestinal tract. Adopted from Sartor 2008.

of IBD came from studies on gnotobiotic animals. IL-10 knock-out mice in germfree status did not develop spontaneous colitis while severe enterocolitis was observed when the mice were maintained in conventional conditions (Sellon et al., 1998). Additionally, germfree mice were found to be more resistant to DSS colitis than conventional mice (Hudcovic et al., 2001). These

results suggest the participation of gut microbiota in the intestinal inflammation. However, up-to-date, no causative bacterial agent of IBD has been claimed despite the fact that significant differences in both concentration and composition of intestinal bacteria are indeed discovered between healthy individuals and patients of IBD.

Although a variety of bacterial species inhabit in the human gastrointestinal tract, four divisions predominate namely *Firmicutes* which alone account for 64% of the gut microbiota, *Bacteroidetes* account for 23%, *Proteobacteria*, and *Actinobacteria*. In IBD patients, a great reduction in species diversity within *Firmicutes* and *Bacteroidetes* was detected in mucosa associated microbiota whereas *Actinobacteria* and *Proteobacteria* became more abundant (Frank et al., 2007). The composition of intestinal microbiota is unique for each individual. As a matter of fact, it is highly variable between and within individual subjects over time (Human Microbiome Project Consortium, 2012). Nevertheless, decreased biodiversity or species richness which is a measure of the total number of species in a community has been consistently reported in both CD and UC patients compared to healthy subjects (Manichanh et al., 2006; Andoh et al., 2007). Species richness declined in fully inflamed tissue in both CD and UC compared to that in non-inflamed tissue even from the same patients (Sepehri et al., 2007). Intestinal bacteria are ought to be prevented from a direct contact to intestinal epithelium by mucus layers and the production of antimicrobial factors such as defensins in healthy individuals (Swidsinski et al., 2009; Shanahan et al., 2013; Hooper et al., 2012). However, significantly higher concentrations of mucosa associated bacteria were detected in CD and UC patients. Moreover, the mean concentrations were increased in proportion with the severity of the disease, which raises the risk of bacteria bleaching the epithelial barrier and triggers inflammatory responses (Swidsinski et al., 2002). According to Bibiloni's study, the numbers of mucosa associated bacteria from UC patients were approximately double those associated with samples from

CD patients (Bibiloni et al., 2006). This higher number of loaded bacteria might be attributed to the less sulphated mucin in UC while normal colonic mucin is heavily sulphated and this increases its resistance to degradation by bacterial enzymes (Corfield et al., 1996; Pullan et al., 1994).

Particular bacteria species were found either increased or decreased in IBD patients. *Faecalibacterium prausnitzii*, whose culture supernatant exhibits an anti-inflammatory effect by secreting a 15kDa protein which is able to inhibit the NF-κB pathway in intestinal epithelial cells and to prevent colitis in an animal model, was depleted in both CD and UC biopsy samples concomitantly with an increase in *Escherichia coli* abundance (Quévrain et al., 2015; Willing et al., 2009; Lopez-Siles et al., 2016). Additionally, low levels of mucosal *F. prausnitzii* were associated with a high risk of early reactivation of ileal CD while recovery of *F. prausnitzii* after relapse is associated with maintenance of clinical remission of UC (Soko et al., 2008; Varela et al., 2013). Other bacteria like *Bifidobacterium* and *Lactobacillus* which have been demonstrated to have protective effect against mucosal inflammation through several mechanisms such as reinforcement of the epithelial barrier, inhibition of pro-inflammatory cytokines production, or mediating immune responses are reported to be quantitatively decreased in the intestinal microbiota of IBD patients (Kato et al., 2004; Zocco et al., 2006; Zakostelska et al., 2011; von Schillde et al., 2012; Mohamadzadeh et al., 2011; Neish, 2009). Interestingly, there are also studies showing that the diversity and the numbers of *Bifidobacterium* were not diminished in patients with active CD (Scanlan et al., 2006; Seksik et al., 2003). Actually, increased proportions of *Bifidobacterium* and *Lactobacillus* were observed in biopsy samples from both patients with active CD and UC (Wang et al., 2014). Moreover, short-chain fatty acids (SCFAs)-producing bacteria were found to be reduced in IBD patients, for instance, *Odoribacter* and *Leuconostocaceae* were reduced in UC patients and *Phascolarctobacterium* and *Roseburiaare* were reduced in CD patients (Morgan et al., 2012). SCFAs

containing butyrate, acetate and propionate, are the primary energy source for colonic epithelial cells. Recent studies have shown that SCFAs regulate the size and function of the colonic Treg cells and protected against colitis in a Ffar2-dependent manner in mice (Smith et al., 2013). A significant reduction in butyrate-producing bacteria in IBD patients was evident by many studies (Frank et al., 2007; Morgan et al., 2012; Kang et al., 2010; Willing et al., 2009). Butyrate produced in the lumen of the large intestine by bacterial fermentation of dietary fibers (carbohydrates) plays a major role in the physiology of the colonic mucosa. Studies have shown that butyrate was able to ameliorate inflammation in the intestinal cells and mucosa affected by CD by regulation of antioxidant defense machinery and prevent translocation of bacteria across epithelium which subsequently could be benefit for preventing relapse of IBD (Russo et al., 2012; Lewis et al., 2010). On the contrary, concomitant with the decrease in those bacteria which may have protective effect on IBD, several bacteria were found to be either increased or present in IBD patients. A remarkably high abundance of *E.coli* was observed in patients with active CD and increased numbers of the bacteria in the epithelium and within the lamina propria were also detected in patients with active CD compared to patients with inactive CD (Lopez-Siles et al., 2014; Mylonaki et al., 2005). Interestingly, the abundance of *E.coli* in the colonic mucosa of UC patients is quite controversial. There are studies showing increased abundance of *E.coli* in UC patients while there are also other works demonstrating no increase with respect to healthy subjects (Mylonaki et al., 2005; Fujita et al., 2002; Kotlowski et al., 2007; Pilarczyk-Zurek et al., 2013; Martin et al., 2004; Martinez-Medina et al., 2009). Nevertheless, enhanced numbers of *E.coli* were indeed measured in inflamed tissue of UC patients compared to non-inflamed tissue (Pilarczyk-Zurek et al., 2013). It is not until seven years ago, *E.coli* strains of a phylogenetic group B2 came into our sight due to its higher frequency of isolation from IBD patients. And the B2 strains with extra-intestinal pathogenic *E.coli* (ExPEC) genes were found more frequently in patients with active IBD

(Petersen et al., 2009). Analysis of 163 IBD patients (57 UC; 95 CD) revealed that intestinal colonization by phylogenetic group B2 *E. coli* is associated with UC (Peterson et al., 2015). Interestingly, the higher frequency of *E. coli* B2 strains with at least one positive adhesion-related gene was correlated with disease activity in UC patients (Peterson et al., 2009). Moreover, most of B2 strains isolated from IBD patients contained transmembrane protein OmpA which play a role in *E.coli* invasion of IECs (Sepehri et al., 2011; Rolhion et al., 2010). As a matter of fact, adherent and invasive *E.coli* (AIEC) strains, which are characterized by their adhesion and invasion capacity to epithelial cells and are able to survive and replicate in macrophages, are isolated from 38.5% of patients with active ileal CD and 37.5% with colonic CD and also found to be enriched in patients with UC (Baumgart et al., 2007; Sokol et al., 2006). However, the percentage of AIEC within the *E.coli* population in IBD patients is not high. On average, AIEC isolates represented 9.3%, 3.7% and 3.1% of *E. coli* isolates in ileal, ileocolonic and colonic CD patients, respectively (Jensen et al., 2011). Interestingly, AIEC are usually isolated from tissue samples rather than fecal samples though the exact localization in the intestinal mucosa has not been evident yet. AIEC promote intestinal inflammation through its adhesion and invasion capacity by several mechanisms. The binding of AIEC to intestinal epithelial cells induces the secretion of the pro-inflammatory cytokine IL-8 and chemokine CCL20 in the polarized intestinal epithelial cells, which subsequently leads to the high production of IFN-γ and TNF-α by macrophages (Eaves-Pyles et al., 2007). Studies on the prototype of AIEC LF82 reveal that LF82 did not escape into cytoplasm once it was engulfed by macrophages. The AIEC are able to replicate in the phagolysosomes which contain acidic pH, oxidative stress, active proteolytic enzymes, and antimicrobial compounds (Bringer et al., 2006). The continuous replication of LF82 within macrophages ultimately results in the secretion of large amount of TNF-α, which contribute to the mucosa inflammation (Glasser et al., 2001). Another group of adherent and invasive bacteria, *Fusobacteria* have been

found at higher abundance in the colonic mucosa of UC patients compare to control subjects (Ohkusa et al., 2002). However, despite of the discovery of association between specific bacterial strains and IBD patients, it is still not clear that the changes in the composition of the gut microbiota lead to the initiation and progression of the gut chronic inflammation or whether is the gut inflammation results in the shift in the gut microbiota.

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2. SIGNIFICANCE, AIMS AND OUTLINE OF THE THESIS

2.1. Significance of the study

The incidence and prevalence of inflammatory bowel diseases (IBDs) has increased worldwide over the past decades to a remarkably high number. Millions of people are suffered from the chronic intestinal disorders which may last throughout life. People with IBD have reduced quality of life, lower capacity for work and increased disability. However, there is no efficient therapy for this disease. The goals of treatment for IBD are to achieve remission and maintain remission in long term. Up-to-date, there is no cure for IBD due to the less understanding of the etiology of the disease. It was proposed earlier that the cause of IBD could be traced down to a single pathogenic strain. Nevertheless, after years of studies, it is evident that IBD is a multifaceted disorder which involves environmental factor, genetic predisposition, gut microbiota and host immune response. Despite all that, the imbalance of the intestinal microbiota (dysbiosis) is closely associated with the onset and deterioration of the disease, which is evident by alterations in the microbiota of gut in IBD patients uncovered by numerous studies. Therefore, understanding of how intestinal bacterial community interplays with other IBD causative factors will definitely lead to a better knowledge of the pathogenesis of the disease and help to bring new insights and ideas of its treatment.

2.2. Aims and outline of the thesis

The aims of the thesis are to 1) review the pathogenesis of IBD in terms of environmental factor, genetic predisposition, gut microbiota and host immune response up-to-date; 2) enhance our understanding of the role of microbiota playing in IBD and host-microbiota interactions which will be beneficial for finding out the pathogenesis of IBD in microbial factor.

Chapter 1 is review of the current knowledge on pathogenesis of IBD. In chapter 3, we discuss how mucosa-associated bacteria isolated from patients with active ulcerative colitis (UC) could affect the intestinal homeostasis of germ-free mice and the dynamics of gut microbiota after colonization. In chapter 4, we discuss the pathogenicity of an *E.coli* B2 strain isolated from active UC patients and how the bacterium interacts with epithelial cells. In chapter 5, we discuss how *Clostridium tyrobutyricum* could exert a protective effect on dextran sodium sulphate (DSS)-induced colitis in mice. Chapter 6 is a general discussion about how the findings on host-microbe interaction help us to understand the etiology of IBD and bring new insights into the therapy for the disease.

3. DEVELOPMENT OF GUT INFLAMMATION IN MICE COLONIZED WITH MUCOSA-ASSOCIATED BACTERIA FROM PATIENTS WITH ULCERATIVE COLITIS

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3.1. Abstract

Disturbances in the intestinal microbial community (i.e. dysbiosis) or presence of the microbes with deleterious effects on colonic mucosa has been linked to the pathogenesis of inflammatory bowel diseases. However the role of microbiota in induction and progression of ulcerative colitis (UC) has not yet been fully elucidated. Three lines of human microbiota-associated (HMA) mice were established by gavage of colon biopsy from three patients with active UC. The shift in microbial community during its transferring from humans to mice was analyzed by next-generation sequencing using Illumina MiSeq sequencer. Spontaneous or dextran sulfate sodium (DSS)-induced colitis and microbiota composition profiling in germ-free mice and HMA mice over 3–4 generations were assessed to decipher the features of the distinctive and crucial events occurring during microbial colonization and animal reproduction. None of the HMA mice developed colitis spontaneously. When treated with DSS, mice in F4 generation of one line of colonized mice (aHMA) developed colitis. Compared to the DSS-resistant earlier generations of aHMA mice, the F4 generation have increased abundance of *Clostridium difficile* and decrease abundance of *C. symbiosum* in their cecum contents measured by denaturing gradient gel electrophoresis and DNA sequencing. In our study, mucosa-associated microbes of UC patients were not able to induce spontaneous colitis in gnotobiotic BALB/c mice but they were able to increase the susceptibility to DSS-induced colitis, once the potentially deleterious microbes found a suitable niche.

3.2. Introduction

Crohn’s disease (CD) and ulcerative colitis (UC), the two major types of inflammatory bowel disease (IBD), are characterized by chronic relapsing inflammation of the gastrointestinal tract. This inflammation is a result of an aberrant immune response to antigens of resident gut microbiota [1, 2]. In spite

of intensive research, however, the underlying mechanisms are still not fully elucidated. It has been proposed that either imbalances in intestinal microbiota (dysbiosis) or presence of commensal bacteria with increased virulence could both cause excessive immune response to microbiota by penetrating through the mucosal barrier and stimulating local and systemic immunity [3–5].

The gut microbiota ecology in UC patients is significantly different from the microbiota in healthy subjects, with typical reduction of diversity among major anaerobic species [6–8]. Transfer of this luminal dysbiotic microbial community to the germ-free mice renders them more susceptible to experimentally-induced intestinal inflammation than similar transfer from healthy subjects [9]. Due to the close contact with gut mucosa, the adherent microbes may be even more important for disease development. Dominant species of mucosa-associated bacteria are significantly different from those found in feces [10], and patients with UC have more bacteria attached to the epithelial surfaces than healthy individuals [11, 12]. But whether these alterations are cause or a result of the intestinal inflammation is still not entirely clear.

To study these mechanisms and to uncover the participation of bacteria in the development of inflammatory diseases, microbiota analysis is not sufficient and gnotobiology has to be employed. In contrast to established human disease, host-microbe interactions during early stages of the disease development can be studied by using animal models of inflammatory diseases in gnotobiotic conditions (i.e. in germ-free or artificially colonized animals with known microbes). In our previous experiments, acute intestinal inflammation induced by dextran sulfate sodium (DSS) was milder in germ-free (GF) mice compared to normally colonized mice [13], and the mode and timing of the colonization with microbiota modified the future immune phenotype of the host [14].

Since the composition and metabolic activities of intestinal microbiota of experimental animals are different from that of human gut microbiota [15],

microbes relevant to the human disease could be missed by using animal models. To overcome this issue, GF animals can be colonized with human microbiota. These humanized or human microbiota-associated (HMA) animals are capable of maintaining the bacterial community of the human gut, thus keeping microbiota composition and its metabolic activities similar to those of the human intestine [16, 17]. Therefore, gnotobiotic animals can be used to cultivate bacteria that are uncultivable by most conventional methods [18].

GF mice with bacteria present in colonic biopsies from three patients with active UC. Our aim was to test whether the mucosa-associated bacteria derived from UC patients could induce spontaneous colitis or render the mice more sensitive to DSS-induced colitis. The composition of bacterial community in cecum content of HMA mice was monitored for several generations to understand its dynamics with respect to colonization at adult age (parental generation) or neonatal mother-to-offspring (filial generations) mode of colonization.

3.3. Materials and methods

3.3.1. Patients and biopsy

Biopsy was taken from inflamed sites of colon descendens from three patients during routine endoscopic examination. First patient (a) was 52-year old male, diagnosed with active UC with shortened colon, caused by a chronic inflammation. Second patient (b) was a 23-year old male, diagnosed with very active UC resistant to both mesalasine (5-acetylosalycilic acid) and azathioprine treatment. Third patient (c) was 28-year old female, diagnosed with very active UC with numerous ulcers. Immediately after extraction, the biopsies were transferred to the laboratory in sterile tubes pre-loaded with Schaedler anaerobe broth (Oxoid Ltd, Cambridge, UK) containing 0.05 %

cysteine-HCl, 10 % glycerol and covered with the layer of paraffin to preserve anaerobes.

3.3.2. Animals

GF BALB/c mice (8–10 week-old) were maintained in isolators under sterile conditions, supplied with sterile water and sterile pellet diet ST-1 (Velaz, Unetice, Czech Republic) ad libitum, to keep them free of live bacteria. The conventional (CV) BALB/c mice on the same diet were regularly checked for the absence of potential pathogens according to an internationally established standard (FELASA).

3.3.3. Human biopsy administration and experimental design

Each human biopsy was homogenized with sterile hand homogenizer, and 2-month old GF mice were colonized with 0.2 ml of this homogenate in a single gavage, which were employed as Parental HMA mice. All biopsies were processed immediately after the transport to the laboratory and under anaerobic conditions until the gavage. Three months later, parental HMA mice were divided into three groups; one group was continuing in breeding for reproduction; one group was used for colitis induction and the other was used as control against colitis induced mice. The offsprings of the parental HMA mice (F1) and the third (F3) and fourth (F4) generations were again divided into three groups as the parental HMA mice did. The microbiota composition of the biopsy homogenate and cecum content of the parental HMA mice (after 3-month colonization of biopsy homogenate) were analyzed by microscopic and cultivation methods and by next-generation sequencing (Fig. 4.1a, b) to analyze the microbiota viability and changes in microbiota diversity during the transfer from humans to mice. Colitis was induced in GF, HMA and CV mice by 7 days lasting exposure to 2.5 % (weight/volume) dextran sulfate sodium (DSS; Mw = 36–50 kDa; ICN Biomedicals, Cleveland, OH, USA) in sterile drinking

water similarly, as described earlier [13, 43]. Controls received sterile drinking water. 8-week old mice were used in all experiments except Parental HMA mice. During the whole duration of these experiments, each line of HMA mice was kept in separate isolator to avoid any contamination with other microbes. The cHMA line of mice did not breed well and it died out shortly after the experiment with parental generation.

3.3.4. Microbiota analysis by cultivation analysis and microscopy

The presence of live microbes in the biopsy lysate and in cecum content of the colonized mice was analyzed by cultivation-dependent methods with subsequent microscopic and enzymatic tests. Before plating, the whole cecum of colonized mice was removed and gently vortex in 5 ml of Schaedler broth containing 0.05 % cysteine-HCl. The samples were cultivated either aerobically using, bovine Blood agar, MRS agar with or without 0.05 % cysteine, Sabouraud agar (all from Oxoid, Hampshire, UK), Endo agar (Merc, Darmstadt, Germany) or anaerobically on VL blood agar (Imuna-Pharm, Slovak Republic). Next, the individual colonies were separated, cultivated and analyzed by microscopy after Gram's staining and by detection of their oxidase (PLIVA-Lachema Diagnostika, Brno, Czech Republic) and katalase activity. Subsequently, their enzymatic activity was determined by oxidative-fermentative test, enterotest, anaerotest, en-coccustest or PYRtest (all from PLIVA-Lachema Diagnostika, Brno, Czech Republic). The software TNW® (PLIVA-Lachema Diagnostika, Brno, Czech Republic) was used to identify the individual species of bacteria (Table 3.2).

3.3.5. Evaluation of acute colitis

Each mouse was examined on day 8 for stool consistency (solid 0 points, loose stool that do not stick to the anus 2 points, and 4 points for liquid stools that stick to the anus) and rectal bleeding (none 0, positive guaiacum

reaction 2 points, and 4 points for gross bleeding), and the clinical colitis score (CCS) was determined as a mean of these two parameters.

The colon was removed and its distal third was fixed in Carnoy's solution for 30 min, and then transferred into 96 % ethanol, embedded in paraffin, sectioned at 5 μm transversal sections and stained with haematoxylin and eosin. Histological grade, ranging from normal (0) through borderline (0.5) to extreme colitis (3), was calculated by evaluating the degree of epithelium ulceration and infiltration of inflammatory cells in each colon segment according to a standardized histological scoring system [43].

3.3.6. Myeloperoxidase (MPO) measurement

The extent of neutrophil infiltration was quantified by measuring MPO activity in the colon tissue homogenate, as described earlier by Krawisz et al. [44] with some modifications. Briefly, 1–2 cm of colon descendens (approximately 50 mg of tissue) was washed in ice-cold phosphate-buffered saline (PBS) and homogenized in 1 ml of potassium buffer (0.05 M KH2PO4, 0.05 M K2HPO4, pH = 6.0). After centrifugation at 12,000*g* for 30 min 4 °C, the pellet was resuspended in 1 ml 0.5 % hexadecyltrimethylammoniumbromide (HETAB) in 50 mM potassium buffer (pH = 6.0). Next, samples were sonificated for 30 s, freeze-thawed three times, sonificated again and centrifuged at 12,000*g* for 30 min. The supernatant was used for the measurement of the MPO activity. All steps of MPO extraction were carried out on ice.

MPO activity was measured by incubating 100 μl of the sample with 2.9 ml prewarmed 50 mM phosphate buffer (pH = 6.0) containing 16.7 % (wt/vol) o-dianisidine and 0.0006 % H2O2 at 37 °C. The reaction kinetics was measured at OD 460 nm for 3 min at 30 s intervals. The MPO activity is expressed in units (U) per 1 gram of the tissue, where 1 U equals the change of OD460 of 1 in 1 min.

3.3.7. Measurement of cytokine production

Single-cell suspensions of spleens were prepared by mashing the tissue and passing the cells through the 70 µm sterile cell strainers (Becton–Dickinson, San Jose, CA, USA). After the lysis of red blood cells with sterile ACK lysing buffer (0.1 mM EDTA, 150 mM NH4Cl, 10 mM KHCO3), and two washes in complete culture medium (RPMI 1640 supplemented with 10 % heat inactivated FCS, 2 mM-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin), the cells were seeded at 5 × 106cells/500 µl of complete medium per well in 48-well flat bottom plates (Corning; Tewksbury, MA, USA) and cultivated for 48 h at 37 °C, 5 % CO2 in humidified incubator. The cell supernatant was then used for determination of IFN-γ, TNF-α and IL-10. The cytokines were measured by ELISA kit (R&D Systems; Mineapolis, MN, USA).

3.3.8. DNA isolation

Bacterial DNA was isolated from cecum contents of HMA mice using ZR fecal Kit™ (Zymo Research, Irvine, CA, USA), according to the manufacturer's protocol. The concentration and quality of isolated DNA was assessed by measuring its absorbance at 260 and 280 nm using spectrophotometer (NanoDrop Technologies, Inc; Wilmington, DE, USA) and its concentration was adjusted to 10 ng/µl.

3.3.9. Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE)

The sequences of bacterial 16S rRNA genes were amplified in the DNA isolated from cecum contents of HMA mice using the universal bacterial primers 338GC and RP534 in a previously described protocol for PCR assays (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCG CCG CCG CAC TCC TAC GGG AGG CAG CAG-3′) and RP534 (5′-ATT ACC GCG GCT

GCT GG-3′) in a previously described protocol for PCR assays [45]. Each PCR mixture contained 2 µl of DNA template, 0.5 µl of each primer (10 µM), 15 µl of ReadyMix™ Taq PCR Reaction Mix (Sigma-Aldrich, Steinheim, Germany), and 12 µl of nuclease-free H2O. Samples were initially denatured at 94 °C for 3 min, followed by 36 cycles of 1 min at 94 °C, 20 s at 61 °C and 40 s at 68 °C with final elongation at 68 °C for 7 min. Products from PCR were then processed by DGGE using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) on 9 % polyacrylamide gel with 35–60 % denaturing gradient, as previously described [46]. Gels were stained in 50 ml of 1× TAE with SYBR Green I dye (0.001 %) for 30 min and visualized by UV light using the Vilber Lourmat System (Marne La Vallée, France). Amplicons of interest were cut from the stained polyacrylamide gel by a sterile scalpel blade. Sterile distilled H2O (100 µl) was added to the excised gel fragment and subject to centrifugation at 10,000 rpm for 10 min to elute DNA. 1 µl of this solution was used for amplification with primers FP341 and RP534 under the same PCR program, as mentioned above. The PCR products were purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced using ABI PRISM® BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc, Foster City, CA, USA) with a PCR thermocycler T-personal Combi (Biometra, GmbH, Goettingen, Germany). Products from sequencing were subsequently purified using BigDye purification kit (Applied Biosystems Inc) and analyzed on 3100 Avant Genetic Analyser (Applied Biosystems Inc) in the Institute of Animal Science sequencing facility (Prague, Czech Republic). The sequences were compared to those in the GenBank database using the BLASTn algorithm [47]. All sequences that did not gave meaningful result were excluded from this search.

3.3.10. Scoring and analysis of bands

Scanned gels were analyzed with BioNumerics (version 7.1, Applied Maths, Sint-Martens-Latem, Beigium). Similarity indices of bands was calculated by using Pearson correlation coefficient and displayed graphically as a dendrogram [48]. The Shannon-Wiener index of diversity was used as a parameter to determine the diversity of taxa present in microbial communities sampled from cecum of HMA mice with and without DSS treatment according to Konstantinov et al. [49].

3.3.11. Next generation sequencing analysis and bioinformatics

For library preparation V3 and V4 region of 16S rRNA was amplified in triplicate PCR reaction using primer pair 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTWTCTAAT) to utilize to the maximum read length of employed 2 × 300 pair-end sequencing at Illumina MiSeq platform (San Diego, CA, USA). Double-indexing was applied to allow for demultiplexing of output reads into original samples. Each PCR reaction was prepared in 25 µl volume using premixed mastermix (AmpliTaq Gold 360 Master Mix, Thermo Fisher Scientific, Waltham, MA, USA) and 0.8 µM of each primer following subsequent cycling conditions: initial denaturation at 95 °C for 3 min following by 35 cycles of 30 s at 94 °C, 1 min at 55 °C and 75 s at 72 °C with final extension for 10 min at 72 °C followed by hold at 4 °C. PCR reactions were checked on agarose gel for presence of expected product in samples and its absence in negative controls. Next, the triplicates from the same template reactions were pooled and cleaned using UltraClean htp 96 well PCR clean-up kit (MoBio, Carlsbad, CA, USA). Concentrations of cleaned samples were measured fluorescently with Quant-iT dsDNA Assay kit (Thermo Fisher Scientific). Sequencing adapters were ligated to the PCR amplicons with the help of TruSeq PCR-Free LT Sample preparation Kit following manufacturer instructions (Illumina, Inc). Next, sample libraries were pooled in equimolar concentration to produce final library, which was sequenced on Illumina MiSeq

instrument at Genomics Core Facility, CEITEC (Brno, Czech Republic). Negative control sample (water) was run through all procedures including DNA extraction, library preparation and sequencing. Sequencing data were processed using QIIME 1.8.0 [50]. Forward and reverse reads were joined to create contigs. Afterwards reads were demultiplexed in parallel with quality filtering allowing minimal Phred quality score of Q20 and maximum number of consecutive low quality base calls of 12 due to the nature of lower quality overlaps of pair-end reads. Resulting reads were clustered to operational taxonomic units (OTUs) using UCLUST with 97 % similarity threshold against bacterial 16S reference database Greengenes gg_13_8 release [51, 52]. Singletons were discarded before producing final dataset. Taxonomic assignment of created OTUs was performed employing RDP classifier [53]. Finally information about read counts for all OTU clusters from all samples together with taxonomic information was output in OTU table. Taxa detected in negative control sample were screened out based on their relative proportional abundance. Resampling to the sequencing depth of 8000 reads per sample was performed to allow comparison of beta diversity measures. The quantitative or qualitative measures of beta diversity of samples were compared using weighted or unweighted UniFrac pairwise dissimilarity matrices, respectively [54]. To measure alpha diversity we calculated Chao1 species richness estimators [55]. Raw demultiplexed sequencing data, with sample annotations, were submitted to the Short Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/) under the study accession number [SRP066136; http://www.ncbi.nlm.nih.gov/sra/SRP066136].

3.3.12. Statistics

The differences between control group and multiple experimental groups (GF vs. all other healthy mice, DSS-treated GF mice vs. all other DSS-treated groups of mice and DSS-treated CV mice vs. all other DSS-treated mice) were

analyzed with one-way analysis of variance (ANOVA) with Dunnet's multiple comparison test. Differences between DSS-treated mice and their healthy littermates or changes in bacteria biodiversity between generations were evaluated using an unpaired two-tailed Student's t test. Data were expressed as mean ± standard deviation (SD). Differences were considered statistically significant at $P < 0.05$. GraphPad Prism statistical software (version 5.03, GraphPad Software, Inc. La Jolla, CA, USA) was used for analyses.

3.4. Results

3.4.1. The inter-individual variability in biopsy samples

To measure the inter-individual differences among biopsy lysates, we estimated the beta diversity metrics using unweighted (qualitative) and weighted (quantitative) UniFrac. This qualitative analysis showed that biopsy b is significantly different from biopsy a and biopsy c and biopsy c was not significantly different from biopsy a. However, there were no differences among samples in quantitative analysis of beta diversity (Table 3.1). This suggests that abundances of major bacterial taxa are similar among all three biopsy samples and low abundance species contributed to the difference between biopsy b and biopsy a or biopsy c.

3.4.2. The diversity of microbiota is decreased after the colonization

GF mice were successfully colonized with bacteria from biopsies of three patients with active UC (Fig. 3.1a, c). Microbial community in samples from human biopsies is characterized by dominance of one or two bacterial orders, Lactobacillales and Enterobacteriales, which comprise more than 80 % of identified reads from each community. After the transfer of microbiota into the mice, composition of communities was shifted, with decrease in abundance of Lactobacillales compensated with an increase in other Firmicutes, namely with

Clostridiales. Moreover, in general abundance distribution of bacterial orders in communities after the transplant was more evenly distributed but total species richness decreased during transfer from humans to mice (Fig. 3.1b). This decrease may be caused either by partial unsuitability of recipient niche for the bacterial community found in the biopsy samples or by dying of less abundant species during the transfer from human to mice. The presence and viability of multiple anaerobic and aerobic bacteria in biopsies and cecum of parental generation of HMA was confirmed by cultivation-based methods (Table 2) [19–21].

Table 3.1 Comparison of microbiota composition in biopsies

Sample 1	Sample 2	P	P (Bonferroni corrected)
Unweighted UniFrac			
Biopsy c	Biopsy a	0.06	0.90
Biopsy c	Biopsy b	0.00*	≤0.01*
Biopsy a	Biopsy b	0.00*	≤0.01*
Weighted UniFrac			
Biopsy c	Biopsy a	0.93	1.00
Biopsy c	Biopsy b	0.42	1.00
Biopsy a	Biopsy b	0.70	1.00

Biopsies show significant inter-individual differences only in presence of low abundance taxa as showed by qualitative (unweighted UniFrac) but not abundance-aware (weighted UniFrac) quantitative analysis. Statistically significant results are marked with asterisk

3.4.3. Colonization of GF mice with mucosa-associated bacteria from IBD patients does not lead to spontaneous colitis

To test if bacteria from the UC biopsies can induce gut inflammation, each mouse was evaluated for colitis. Compared to water-treated GF mice, which remained completely healthy, the clinical colitis score (CCS) and

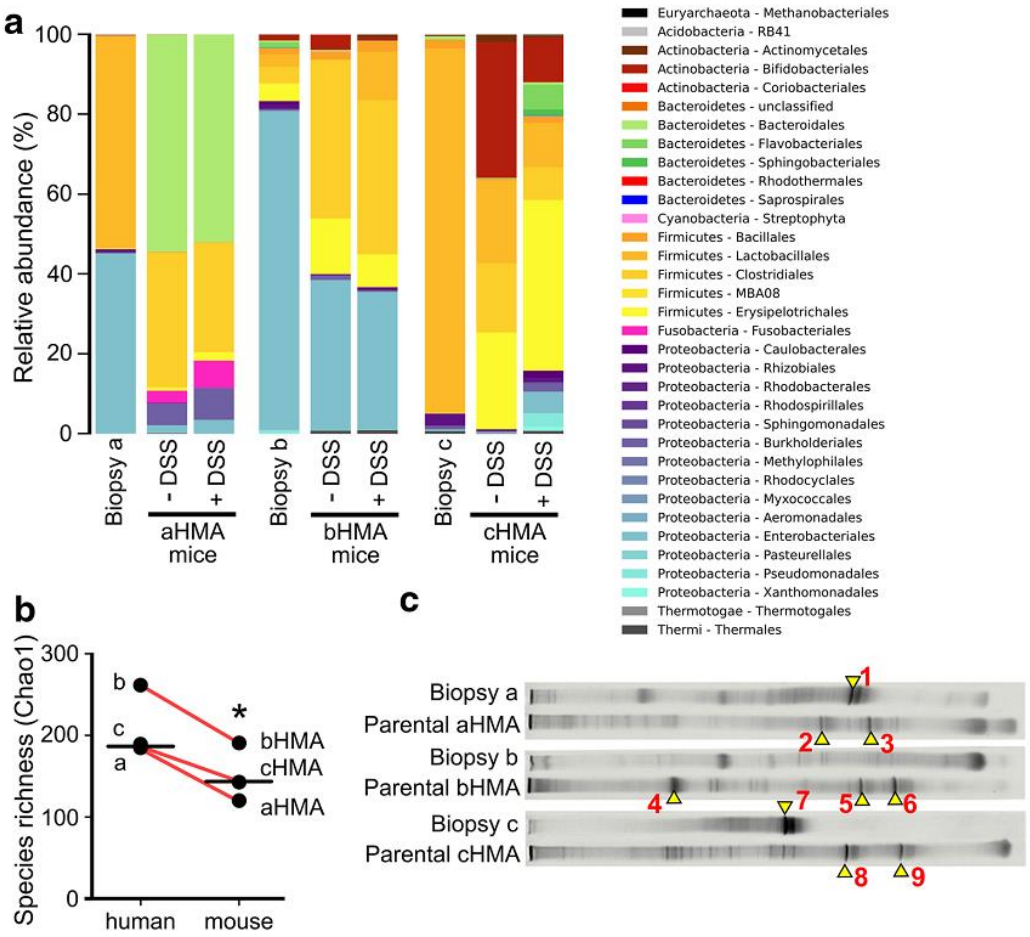

Fig. 3.1 Microbiota composition in colonic biopsies of three patients with active ulcerative colitis and cecum content of parental HMA mice,**a** as measured by 16S sequencing. The composition of each sample is based on the RDP taxonomic assignment of the 16S rDNA sequences. The phylum and the genus level are shown for the most abundant bacterial groups. **b** The Chao1 diversity index of human biopsy samples (*a*, *b* and *c*) was compared with the diversity index of cecum contents of relevant mice (healthy aHMA, bHMA and cHMA) by two-tailed paired Student's *t* test. The black line represent median and the *red lines* connect the related samples. **c** DGGE profiles of 16 s rRNA genes amplified from colonic biopsy lysates and cecum content of biopsy-colonized mice. Excised and successfully sequenced bands are identified with red numbers (*1–9*), see Table 1 for identification

myeloperoxidase (MPO) were significantly higher in water-treated parental and first filial generation (F1) of mice colonized with biopsy a (aHMA) (Fig. 2).

Increase in MPO was also detected in water-treated parental bHMA mice, but no histological signs of colitis were observed in any group of water-treated HMA mice (Fig. 2c). Similar results were found in parental cHMA mice, which did not developed colitis either spontaneously or after DSS-treatment. The cHMA line of mice did not breed beyond parental generation and died out. This suggests that the mucosa-associated microbiota from patients with active UC cannot induce spontaneous colitis in mice, although the process of artificial colonization may induces slight inflammation of colonic mucosa.

3.4.4. aHMA mice exhibited an increase in DSS-colitis sensitivity whereas bHMA mice failed to develop colitis

When treated with DSS, GF mice developed milder colitis than conventional (CV) mice, suggesting that the presence of microbiota increased the susceptibility of mice to colitis. There was a significant increase in CCS and MPO in GF, CV, F1 aHMA, F4 aHMA and F3 bHMA after DSS treatment, compared to their littermates treated with water (Fig. 2a, b). Compared to F1 aHMA mice, CCS and MPO value were higher in F4 aHMA mice though the differences in MPO were not statistically significant. The typical histopathologic picture of DSS-induced colitis was observed only in GF mice (mild to medium), F4 aHMA mice (moderate) and CV mice (very severe) with a characteristic massive loss of goblet cells and crypts, ulceration and inflammatory infiltrate in the lamina propria and submucosa (Fig. 2c). The increase in macro- and microscopic signs of colitis in aHMA mice shows an increase in DSS-colitis sensitivity over the generations. Interestingly both MPO values and CCS in water-treated aHMA mice showed a steady decline tendency over generations. In contrast to aHMA mice, bHMA mice failed to develop colitis in all groups of mice throughout the generations (Fig. 2a–c).

Table 3.2 Bacteria in the parental HMA mice and biopsy lysates used for their colonization, as identified by enzymatic tests and microscopy

	Aerobic bacteria	Anaerobic bacteria
Biopsy a	*Klebsiella oxytoca* *Proteus vulgaris* *Streptococcus parvulus*	*Actinomyces naeslundii* *Fusobacterium necrogenes/ mortiferum*
P aHMA mice	*Enterococcus faecalis* *Enterococcus rafinosus* *Enterococcus faecium* *Streptococcus parvulus* *Klebsiella pneumonie* *Escherichia coli* *Proteus vulgaris*	*Veillonella parvula* *Bifidobacterium breve* *Bifidobaterium sp.* *Bacteroides capillosus* *Actinomyces israelli* Unidentified G+ cocci-rods Unidentified G+ rods
Biopsy b	*Escherichia coli* *Klebsiella pneumonie* *Enterococcus flavescens* *Enterococcus casseliflavus*	*Actinomyces naeslundii* *Veillonella parvula* *Bifidobacterium sp.*
P bHMA mice	*Enterococcus casseliflavus* *Enterococcus faecalis* *Enterococcus sp.* *Klebsiella pneumonie* *Citrobacter amalonaticus* *Escherichia coli*	*Veillonella parvula* *Eubacterium lentum* *Bifidobacterium sp.* *Actinomyces israelli* *Lactobacillus sp.*
Biopsy c	*Streptococcus sp.* *Enterococcus faecium* *Enterococcus raffinossus*	*Lactobacillus jensenii*
P cHMA mice	Yeast *Enteococcus faecium* *Enterococcus raffinossus* *Streptococcus parvulus*	*Clostridium inocuum* *Bifidobacterium sp.* Unidentified G+ spore-forming rods

3.4.5. Production of proinflammatory and regulatory cytokines is increased in colitic F4 aHMA mice

The production of proinflammatory cytokines Tumor necrosis factor (TNF)-α and Interferon (IFN)-γ in spleen cell suspension was higher in DSS-treated F4 aHMA mice than that in their healthy littermates (Fig. 3a, b). Higher production of TNF-α was also found in DSS-treated GF or conventional mice, but the

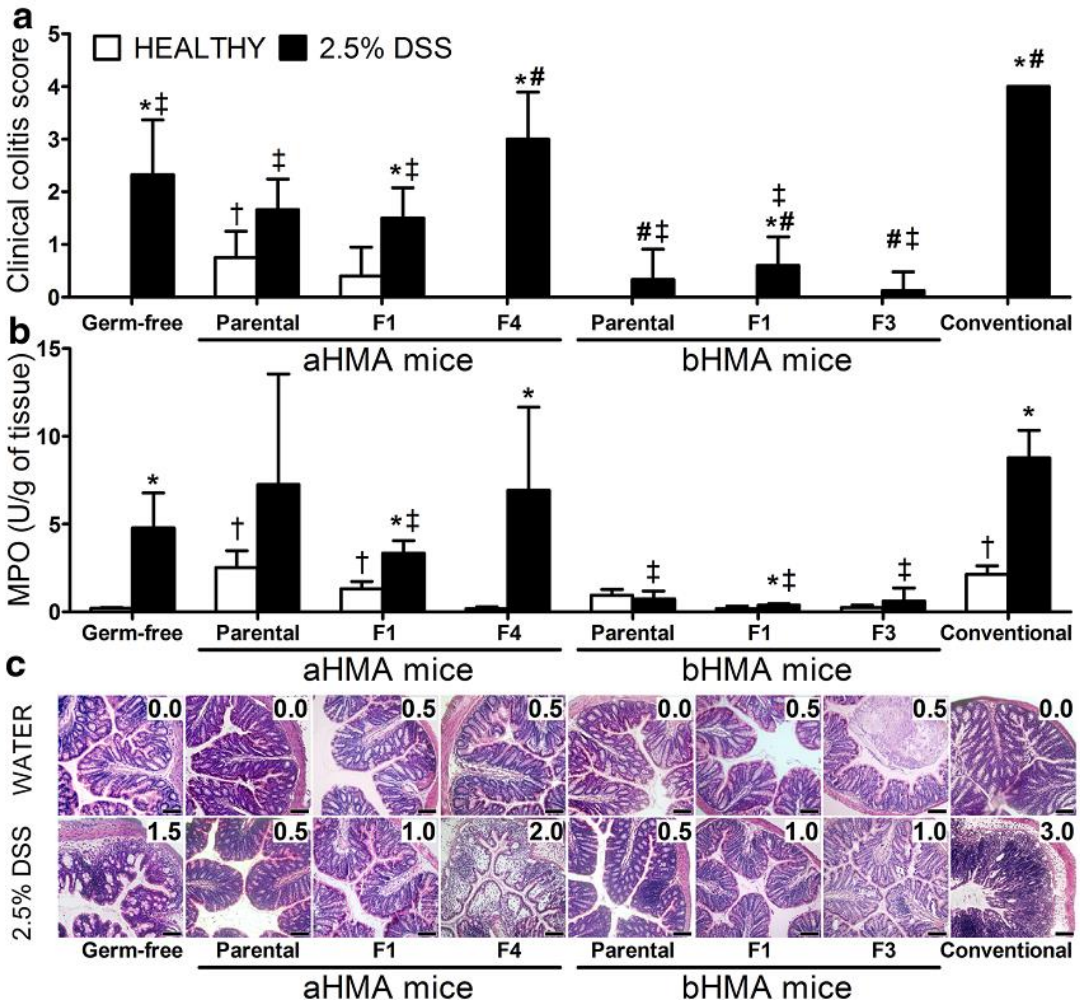

Fig. 3.2 Macro- and microscopic evaluation of DSS induced colitis, as measured by **a** clinical colitis score, **b** colonic MPO activity, and **c** histological analysis of the mucosal damage of the colon descendens. The values are expressed as mean (*bar*) value ± standard deviation (*whisker*). *Each bar* represents 4– mice and histology (paraffin-embedded sections stained with haematoxylin and eosin) is from one mouse showing changes typical for each group. The numbers represent histological grade and the *black bar* is 100 μm. *p ≤ 0.05 vs. non-treated littermates; †p ≤ 0.05, vs. healthy GF mice; #p ≤ 0.05, vs. DSS-treated GF mice; ‡p ≤ 0.05, vs. DSS-treated CV mice; *F1/3/4* 1st/3rd/4th filial generation, *DSS* dextran-sodium sulfate

levels of IFN-γ were not changed. Interleukin (IL)-10, known to regulate immune responses [22], was significantly higher in DSS-treated F4 aHMA mice when compared to their water-treated littermates (Fig. 3c). Interestingly,

contrary to F4 mice, production of IL-10 was significantly higher in water-treated GF mice than in DSS-treated mice. On the other hand, no significant differences in cytokines production were determined between DSS-treated and water-treated bHMA mice. The differences in cytokine production between DSS-treated and untreated mice are, therefore, only apparent in mice with clear phenotype of DSS-induced colitis, such as DSS-treated GF, F4 aHMA and CV mice. These results suggest that changes in the cytokine pattern reflect more the presence of colitis in DSS-treated animals than the differences in microbiota that colonize the mice.

3.4.6. HMA mice in the later generation exhibited higher biodiversity in intestinal bacterial community

Shannon-Wiener index was used to compare the diversity of microbiota in cecum samples from HMA mice. Significantly higher diversity was measured in F4 aHMA mice and F3 bHMA mice compared to their previous generations (aHMA: F1 = 1.03 ± 0.03 vs F4 = 1.18 ± 0.06, $p < 0.05$; bHMA: F1 = 1.12 ± 0.03 vs F3 = 1.35 ± 0.09, $p < 0.05$). Interestingly, there was no significant difference in diversity between water-treated and DSS-treated HMA mice. Four clusters were roughly generated in cecum samples in each line of HMA mice and the samples from the same generation and treatment clustered well together (Figs. 4b, 5b).

3.4.7. A predominance of colitis-associated *Clostridium sp*. was identified in cecum samples of aHMA mice but not in bHMA mice

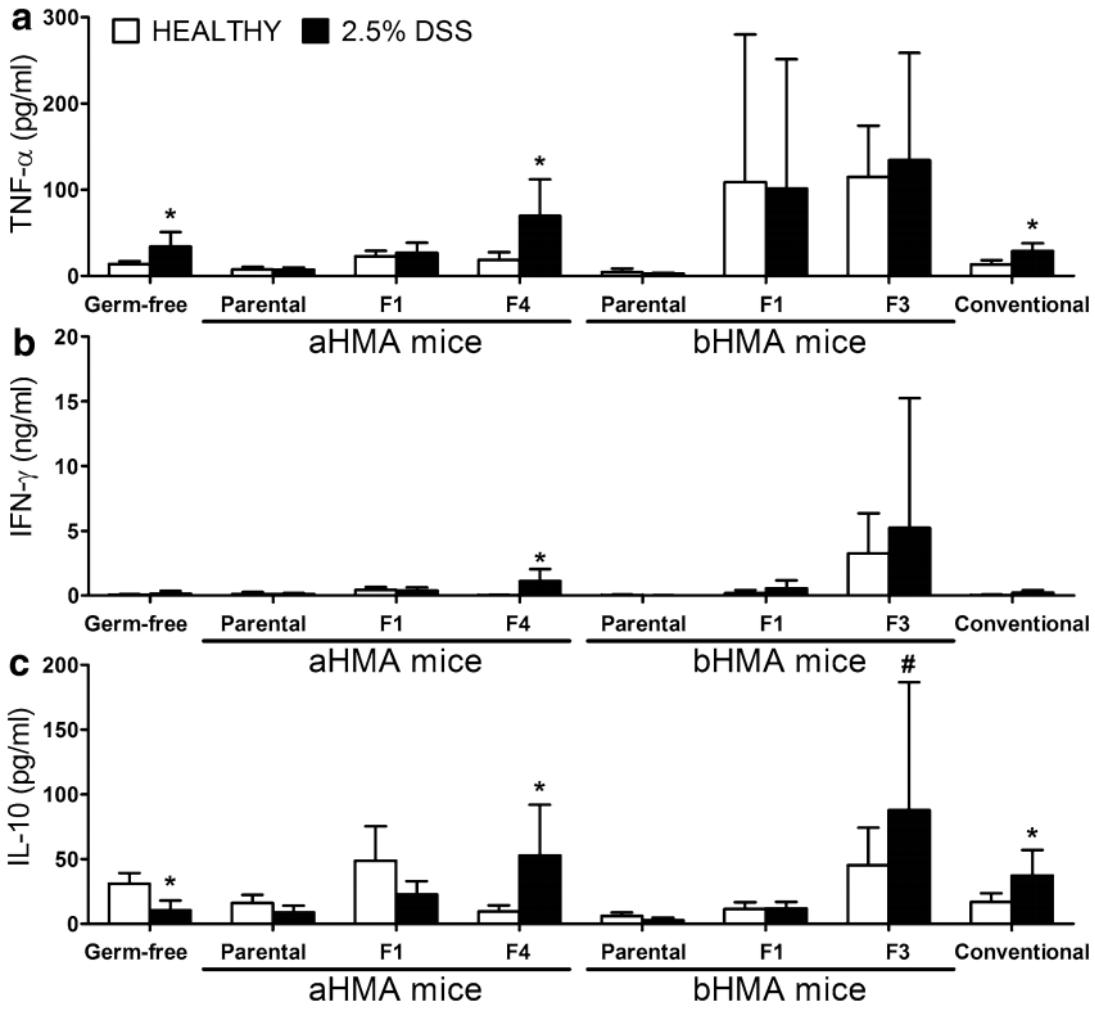

Fig. 3.3 Cytokine production by spleen cells from HMA mice. Each group contained 4–8 mice. **a** TNF-α, **b** IFN-γ, and **c** IL-10 cytokine levels were measured in supernatant from spleen cells. Cytokine values are expressed as mean ± standard deviation, *p ≤ 0.05 vs. non-treated littermates; #p ≤ 0.05, vs. DSS-treated GF mice

Prominent bands from DGGE profiles (Figs. 4, 5) of PCR amplified DNA from cecum content (Additional file 1) were identified as *Clostridium sp*. and *Blautia sp*. in both aHMA and bHMA mice (Table 3). Compared with bHMA mice, in which DSS-induced colitis was not established, aHMA mice conserved higher richness of *Clostridium* species in their cecum samples (Table 1). Substantial amount of *C. difficile* and *C. aurantibutyricum* were identified in F4 aHMA mice, in which DSS-colitis was successfully developed. These mice have substantially lower abundance of *C. symbiosum* compared to healthy F1

aHMA (Fig. 4a), suggesting that this microbe has not been successfully transferred to the later generation of aHMA mice.

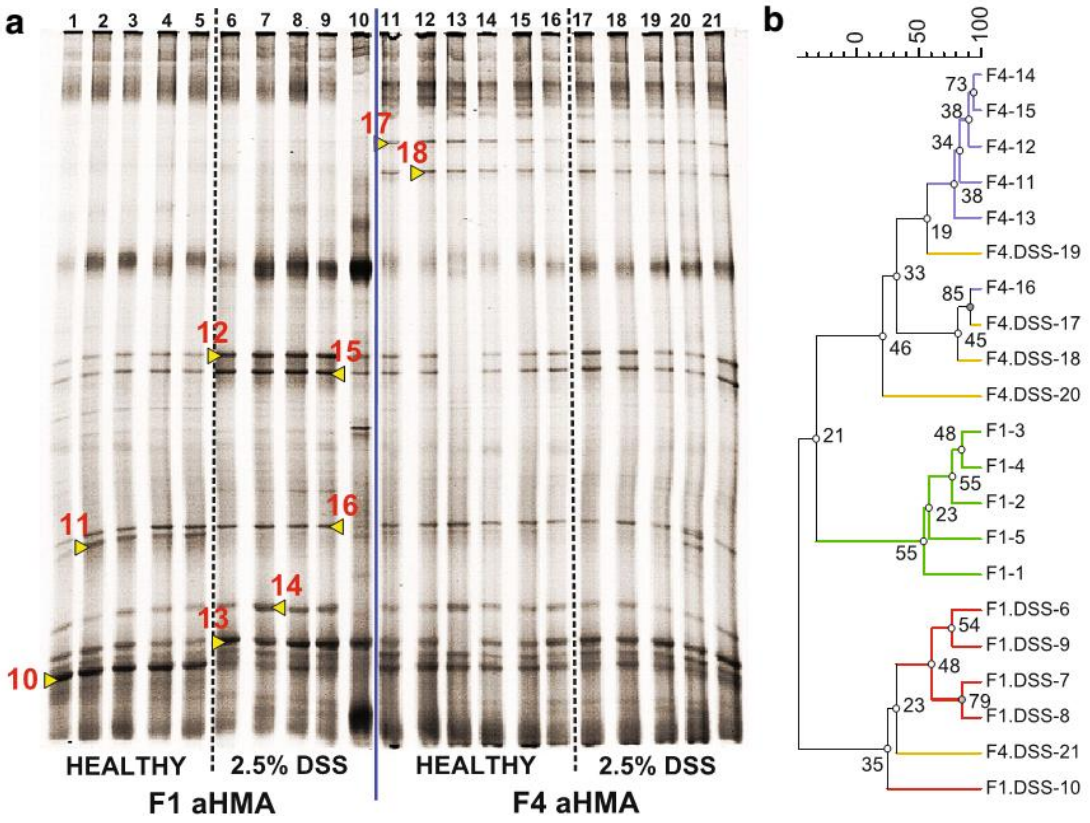

Fig. 3.4 The differences in cecum microbiota of F1 and F4 aHMA mice. **a** DGGE profiles of 16 s rRNA genes amplified from cecum content of human biopsy A-associated mice. Each lane (*1–21*) represents a DNA sample isolated from cecum content of one mouse. Excised and successfully sequenced bands are identified with *red numbers* (*8–16*), see Table 1 for identification. **b** Clustering analysis of DGGE banding profiles of cecum samples. The dendrogram was generated by using the Wards method from a Pearson correlation matrix. The *numbers on the nodes* indicate the bootstrap values expressed as percentage from 1000 replications

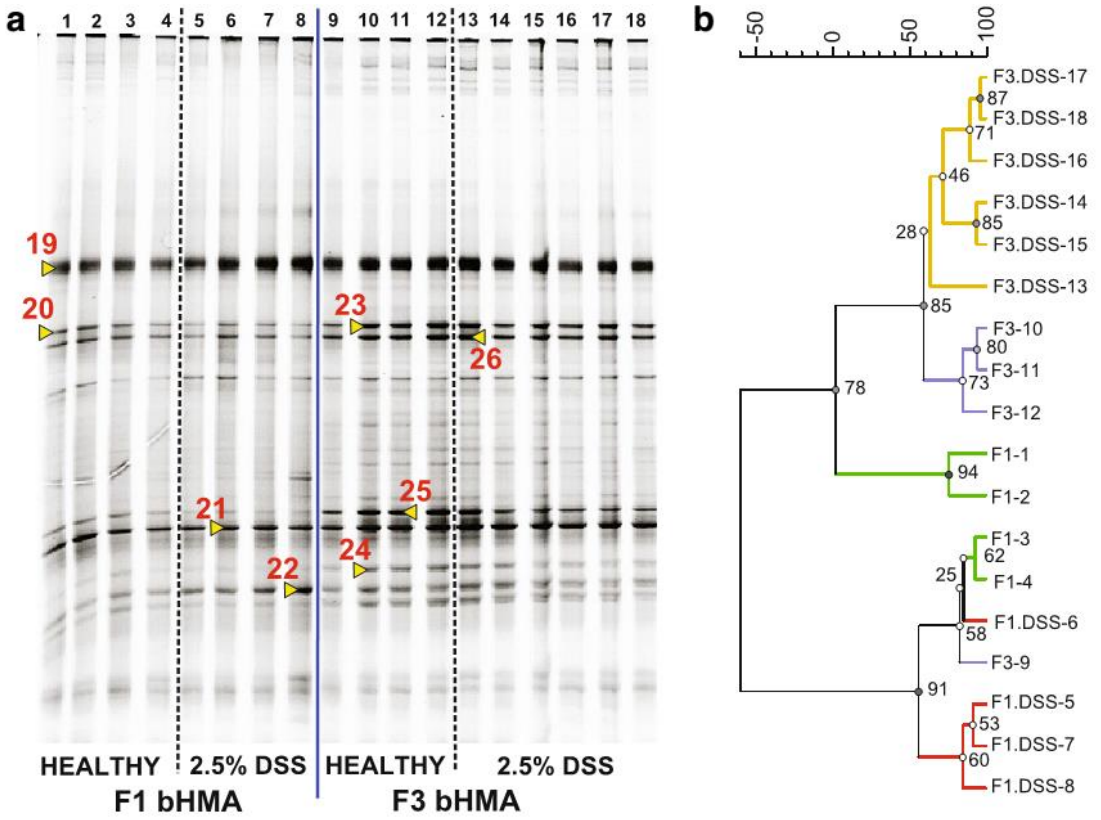

Fig. 3.5 The differences in cecum microbiota of F1 and F3 bHMA mice. **a** DGGE profiles of 16 s rRNA genes amplified from cecum content of human biopsy B-associated mice. Each lane (*1–18*) represents a DNA sample isolated from cecum content of one mouse. Excised and successfully sequenced bands are identified with numbers (*17–24*), see Table 1 for identification. **b** Clustering analysis of DGGE banding profiles of cecum samples. The dendrogram was generated by using the Wards method from a Pearson correlation matrix. The *numbers on the nodes* indicate the bootstrap values expressed as percentage from 1000 replications

Table 3.3 Phylogenetic affiliation of DNA sequences retrieved from DGGE bands

Sample	No	GenBank Accession number	Best match	Identity (% similarity)
Biopsy a	1	NR121743	*Streptococcus lutetiensis*	99
P aHMA	2	NR118699	*Clostridium innocuum*	97
		NR044648	*Eubacterium tortuosum*	91
		NR113409	*Eubacterium dolichum*	90
P aHMA	3	NR119035	*Clostridium sphenoides*	99
P bHMA	4	NR118729	*Clostridium oroticum*	98
P bHMA	5	NR041960	*Blautia luti*	98
P bHMA	6	NR036800	*Ruminococcus gnavus*	100
Biopsy c	7	NC017960	*Enterococcus faecium*	98
P cHMA	8	NR119085	*Clostridium polysaccharolyticum*	97
P cHMA	9	AB971793	*Clostridium innocuum*	98
F1 aHMA	10	NR044715	*Clostridium clostridioforme*	96
		NR036928	*Clostridium hathewayi*	96
		NR118730	*Clostridium symbiosum*	96
F1 aHMA	11	NR118730	*Clostridium symbiosum*	99
F1 aHMA	12	NR119217	*Blautia producta*	99
F1 aHMA	13	NR041960	*Blautia luti*	100
F1 aHMA	14	NR118729	*Clostridium oroticum*	97
		NR117142	*Eubacterium fissicatena*	97
		NR104803	*Eubacterium contortum*	97
F1 aHMA	15	NR118729	*Clostridium oroticum*	96
		NR117147	*Eubacterium contortum*	96
		NR117142	*Eubacterium fissicatena*	96
F4 aHMA	16		No siginificant similarity found	
F4 aHMA	17	NR074454	*Clostridium difficile*	99
F4 aHMA	18	NR044841	*Clostridium aurantibutyricum*	98
F1 bHMA	19	NR118729	*Clostridium oroticum*	98
F1 bHMA	20	NR041960	*Blautia luti*	98
F1 bHMA	21	NR041960	*Blautia luti*	100
F1 bHMA	22	NR036800	*Ruminococcus gnavus*	100
F3 bHMA	23	NR118729	*Clostridium oroticum*	99
F3 bHMA	24		No siginificant similarity found	
F3 bHMA	25	NR118729	*Clostridium oroticum*	98
F3 bHMA	26	NR118729	*Clostridium oroticum*	99

If the identity of the best match was 97 % or less, two other matches were selected. The sequence number correspond to these in Figs. 1, 4 and 5.

3.5. Discussion

Inflammation in patients with UC is usually confined to large intestine, characterized by dysbiosis [23]. When transferred to GF mice, this dysbiotic microbial community in UC patients increase susceptibility to DSS-induced colitis [9]. Luminal microbes forming feces have often only indirect contact with inflamed colon mucosa, so mucosa-associated bacteria are more likely to be involved in UC due to their close proximity to the host epithelium. In healthy individuals, gut bacteria are usually separated from the intestinal mucosa by

thick layers of mucus [24], thus even methods as sensitive as quantitative (q) PCR or Fluorescence in situ hybridization (FISH) is not able to detect any bacteria in most biopsies from healthy subjects [11, 25].

In this study, we found that major bacterial taxa are similar among all three biopsy samples we used for colonization and only low abundance species differ among biopsies from UC patients (Table 1). When the microbial community is transferred from human biopsies to GF mice, the species richness of this community is significantly reduced (Fig. 1b). This may be caused either by partial unsuitability of recipient niche for the bacterial community or by dying of less abundant species during the transfer from human to mice. This methodical difficulty could not be fully excluded even when freshly collected biopsies were used and their contact with oxygen in the air was minimized.

Colonization of GF mice with mucosa-associated microbiota from UC patient a (aHMA mice) increased CCS and MPO activity without damage to colon mucosa. CCS and MPO gradually decreased in subsequent generations, which support the notion that lack of exposure to microorganism in the early life could interfere with the development of immune system and permanently alter important immune functions [14]. Therefore, the increase in MPO and presence of pasty stool in parental aHMA mice appears to be a result of the poorly regulated host-microbe interaction in the ex-GF mice. The absence of mucosal damage in healthy HMA mice suggests that the mucosa-associated microbes from patients with active UC do not induce colitis when transferred to otherwise healthy host. However, this effect cannot be fully excluded, e.g. if some rare and strongly damaging microbial communities are transferred, due to the low number of individual biopsies we tested.

To investigate how the mucosa-associated bacteria increase the sensitivity to colitis, DSS-colitis was induced in GF, HMA and CV mice. Colitis was successfully induced in GF, F4 aHMA and CV mice with varying severity;

mild-moderate in GF mice, moderate in F4 aHMA mice and very severe in CV mice. This is in agreement with our previous study showing that GF mice are more resistant to acute DSS-induced colitis than CV mice [13]. The presence of mild colon inflammation in GF mice suggests that microbiota is not indispensable for colitis development in this model. The absence of colitis in DSS-treated parental, F1 aHMA, F1 bHMA and F3 bHMA mice clearly shows that microbiota might contain certain protective species that actively protected mice from intestinal inflammation. Their presence would explain the failure to induction of DSS-colitis in all bHMA mice and in parental and F1 aHMA mice. The increase in susceptibility to DSS-induced colitis between F1 and F4 aHMA mice suggests that these protective bacteria may be lost or that other, potentially harmful microbes found suitable niche during natural colonization with co-housing. The differences in colitis sensitivity between both lines and different generations also show, that certain specific microbes, and not the presence of any microbe, is the cause of colitis sensitivity in F4 aHMA mice. We did not transfer the microbiota from healthy subjects because we expected that these biopsies will yield inoculums too low for successful colonization with complex microbiota [11, 25]. On the other hand, we cannot exclude that similar effects will be observed also with mucosa-associated bacteria from healthy subjects.

When we sequenced the bands that were different between F1 and F4 aHMA, we found disappearance of *C. Symbiosum* and appearance of *C. difficile. C. symbiosum* (member of the *Clostridium* cluster XIVa) is the most abundant bacterium found in human gut mucins, where it probably protects the mucosa by producing high levels of butyrate [26]. This effect may be responsible for the resistance of the F1 aHMA mice to the DSS-induced colitis. Disappearance of *C. symbiosum* during DSS treatment of F1 aHMA mice could be even partially responsible for the DSS-induced epithelium damage. *C. difficile*, on the other hand, may produce toxins that can damage colon mucosa of infected

patients [27]. Indeed, there is a strong association between UC and colonization with this bacterium [19, 20], and this association is not limited only to toxin-producing *C. difficile* [23]. The close association of *C. difficile* with colitis may be responsible for the marked increase in susceptibility to DSS-colitis between F1 and F4 generations. Since all these microbes could not be introduced in other way than with the original biopsy, their appearance on DGGE of F4 aHMA suggests that they found suitable niche and increased in numbers. PCR-DGGE can detect only more dominant species, because its detection limit is between 10^4 and 10^8 cfu/ml, depending on the selected bacterium [28–30]. Reduced richness of intestinal microbiota is a common feature in UC patients [7, 31–34]. It is interesting that there is no significant reduction in biodiversity of microbiota in cecum samples of DSS-treated HMA mice compared to their healthy littermates. Taking into account that the biopsies were taken from patients with active UC, we can speculate that the bacteria transferred to mice were well adapted to inflammatory environment.

Intestinal inflammation is associated with impaired barrier function, which leads to activation of the systemic immunity and production of pro-inflammatory cytokines [35, 36]. In fact, this activation is less pronounced in the mucosal compartment, including mesenteric lymph nodes, than in systemic one, due to more active inhibitory mechanisms in the gut [37]. This effect is probably caused by the regulatory mechanisms of the mucosal immune system [38]. IL-10 is an important anti-inflammatory cytokine that regulate the colonic inflammation during experimental colitis in the presence of microbiota [39,40]. Therefore, an increased IL-10 production in DSS-treated F4 aHMA mice and DSS-treated CV mice maybe caused by negative-feedback loop, where immune system regulates the inflammation caused by gut barrier breach. The observed decrease in IL-10 production in DSS-treated GF mice may be caused by the immunological immaturity,

indicating that the GF mice do not have fully developed regulatory mechanisms on a level of innate and adaptive immunity [41, 42].

3.6. Conclusions

In summary, we showed that mucosa-associated bacteria from colonic biopsy of the patients with active UC can increase sensitivity to DSS-induced colitis, although not able to induce spontaneous one. The increase in DSS-induced colitis severity between earlier and later generations of aHMA, together with the appearance of *C. difficile* and disappearance of *C symbiosum*, suggests that change in the relationship between these two particular microbes, rather than their presence or absence, is important for the sensitivity to colitis. Production of these “humanized” mice using patient’s biopsy and following the fate of bacteria over generations may bring new insights into host-microbe interaction during intestinal inflammation or in other diseases.

3.7. Acknowledgements

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4. Secretion of alpha-hemolysin by Escherichia coli disrupts tight junctions in ulcerative colitis patients

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4.1. Abstract

The potential of *E. coli* isolated from Inflammatory Bowel Disease (IBD) to damage the integrity of the intestinal epithelium was investigated. *E coli* strains isolated from patients with ulcerative colitis (UC) and healthy controls were tested for virulence capacity by molecular techniques and cytotoxic assays and TER. *E. coli* isolate p19A was selected and deletion mutants were created for Hly clusters and cnf1. Probiotic *E coli* Nissle and pathogenic *E coli* LF82 were used as controls. *E. coli* strains from patients with active UC completely disrupted epithelial cell tight junctions shortly after inoculation. These strains belong to phylogenetic group B2 and are all α-hemolysin (alpha–hemolysin) positive. In contrast, probiotic *E coli* Nissle, pathogenic *E coli* LF82, four *E. coli* from patients with inactive UC and three *E. coli* strains from healthy controls did not disrupt tight junctions. *E. coli* WT as well as cnf1, and single loci of hly mutants from cluster I and II were all able to damage Caco-2 cell tight junctions. However, this phenotype was lost in a mutant with knock out of both Hly loci ($p<0.001$). UC associated *E. coli* producing α-hemolysin can cause rapid loss of tight junction integrity in differentiated Caco-2 cell monolayers. This effect was abolished in a mutant unable to express α-hemolysin. These results suggest that high Hly expression may be a mechanism by which specific strains of *E. coli* pathobionts can contribute to epithelial barrier dysfunction and pathophysiology in IBD.

4.2. Introduction

Crohn's disease (CD) and Ulcerative Colitis (UC) are two different forms of chronic inflammatory bowel disease (IBD), the etiology of which is still unknown. CD and UC are distinguished by their clinical manifestations and inflammatory profiles (1). UC is a chronic inflammatory disorder of the colorectal mucosa while CD is a chronic, segmentally localized granulomatous disease, which can affect any area of the gastro-intestinal tract. CD may even

affect non-intestinal tissue such as lymph nodes and skin. Both UC and CD unforeseeably fluctuate between relapses and remission. IBD can appear at any age, however, most often in the third decade of life (2). The highest reported prevalence values for IBD are in Europe (UC, 505 per 100,000 persons; CD, 322 per 100,000 persons) (3).

Genome-wide association studies in IBD have identified genetic polymorphisms contributing to susceptibility to IBD. Many of these gene polymorphisms are associated with pathways involved in intestinal homeostasis, linking host genetics to deregulated host responses to the microbiota (4). The concordance rate among monozygotic twins was 6.3% for UC and 58.3% for CD (5). This clearly indicates a role of genetic factors in CD, but also indicates an important role for environmental factors, particularly in UC. An abnormal microbiota composition and decreased complexity of the gut microbial ecosystem (commonly referred to as dysbiosis) are common features in patients with CD or UC (6). These observations have fuelled efforts to identify opportunistic gut pathogens (or pathobionts) that may play a role in the pathogenesis of IBD.

E. coli pathobionts exhibiting pathogen-like behaviours are more frequently cultured from IBD patients with active disease due to their selective growth advantage in inflammatory conditions (7). Moreover, adherence of the B2 phylotype *E. coli* to human intestinal epithelial cells is mediated through the type 1 pili interaction with mannosylated CECAM6. Interestingly, CECAM6 expression by cultured intestinal cells was shown previously to be up-regulated after treatment with interferon (IFN)-γ and tumor necrosis factor-α (TNF-α) (8). These findings indicate that inflammatory conditions in the gut support *E coli* colonization via increased CECAM6 expression and offer an explanation for their more frequent isolation from patients with active disease.

Among the *Proteobacteria*, adherent invasive variants of the B2 phylogroup *E. coli* (AIEC) have been proposed to play a role in the pathophysiology of IBD (9), owing to their capacity to adhere to intestinal epithelial cells, to invade

intestinal epithelial cells via a macropinocytosis-like process and to survive and replicate intracellularly in epithelial cells and macrophages (10). Others have, likewise, found increased numbers of B2 phylogroup *E. coli* isolated from IBD patients (11). Petersen et al 2009 (12) showed that *E. coli* isolates from fecal samples of primarily UC patients with active disease frequently belong to the B2 phylogenetic group and harbor genes commonly associated with extra-intestinal pathogenic *E. coli* (ExPEC) causing urinary tract infection and meningitis (13).

Recently, a hemolysin (Hly) producing *E. coli* strain was shown to induce localized defects in epithelial integrity colonic cell monolayers and rat colon tissue ex vivo Additionally, wild-type (WT) and colitis susceptible IL-10-/- mice colonized with an HlyA expressing *E. coli* had elevated inflammation scores and an increased epithelial permeability compared to mice colonized with the HlyA-deficient mutant. Furthermore, qPCR analysis revealed that lesions (focal leaks) in mucosal samples from the human colon were associated with 10-fold higher levels of hlyA DNA, suggesting that Hly-expressing *E. coli* play a role in the pathology of intestinal inflammation in IBD. (14).

The aim of this study was to extend the above observations to isolates of B2 phylogroup *E. coli* from IBD patients by testing their effects on permeability, tight junction stability and viability of human intestinal cell epithelial monolayers cultured in vitro. For comparison, we also tested the effect of prototype AIEC strain LF82 and the probiotic *E. coli* Nissle on permeability and viability of polarized human Caco-2 cells. As some strains of B2 phylogroup *E. coli* isolated from UC patients also possess a gene encoding cytotoxic necrotizing factor (cnf1), we investigated the role of cnf1 and hlyA in causing epithelial damage by the construction and testing of genetic mutants in cellular assays.

4.3. Materials and Methods

4.3.1. Study material

Permission for the study was obtained from the Regional Ethics Committee for Copenhagen County Hospitals (Permission no. KA03019), and all participants gave their informed written consents. Healthy controls were recruited among medical students. All controls had a completely normal distal colon as visualized by video sigmoidoscopy (left side colon) at study entry. None of the controls had experienced diarrhea, blood in stools or abdominal pain or any other abdominal discomfort when the stool sample was submitted. Patients with IBD were diagnosed according to standardized criteria (15,16), which included confirmation of inflammation by video sigmoidoscopy and a fresh set of negative stool cultures for common pathogens including Clostridium difficile. Detailed information regarding extent of disease and current medication among the included patients has previously been described (12), neither controls nor patients had received antibiotics within the last 2 months prior to inclusion and all patients had an established diagnosis of IBD prior to inclusion in our study.

Fecal samples were cultured at Statens Serum Institut (SSI): bacteriological analysis, *E. coli* phenotypic characterization, determination of phylogenetic group, and ExPEC virulence gene detection were performed as described previously in Petersen et al 2009 (12). *E. coli* clinical isolates p7, p10, p13, p19A, p22, p25, p26, p27, p32; healthy control *E coli* isolates C2, C4, and C6 were characterized by PCR for virulence genes (data not shown) in this study. The probiotic E coli Nissle 1917 and the pathogenic *E. coli* LF82 (17) were used as a negative and positive control, respectively.

4.3.2. Cell infection assay and measurement of transepithelial electric resistance (TER)

Heterogeneous human epithelial colorectal adenocarcinoma cells (Caco-2 BBE cell line, ATCC CRL 2102) were maintained at 37°C in a humidified with 5% CO2 in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Paisley,

UK) containing Glutamax, 10% heat-inactivated fetal bovine serum (PAA laboratories, Colbe, DE), 100 U/ml penicillin, 100 µg/ml streptomycin ((PenStrep) Sigma, St. Louis, MO), 1% non-essential amino acids (Lonza) and 1% L-glutamine.

Caco-2 cells (between passage 55 and 60) were seeded at a density of 2.6 x 105 cells/cm^2 in a 24-transwell system containing Tissue Culture-treated filters (0.4 µm pore size, BD Biosciences Falcon type # 353494, Erembodegem, B) and grown for 16 days until they differentiated into polarized monolayers. After 14 days, the TER reached 600 to 800 Ohms/cm^2 (Volt/Ohm meter, World precision Instruments, Sarasota, FL).

For bacterial co-incubation experiments, the medium was removed by aspiration from the Transwell filters, and the filters were inserted in the cellZscope apparatus (Nanoanalytics, Münster, DE). Cell culture medium without antibiotics was then added to the upper chambers (450 µl) and lower chambers (800 µl), and the apparatus was placed in a humidified incubator at 37°C containing 5% CO2/95% O2 atmosphere for 2 hours prior to addition of bacteria. Bacteria were grown overnight in LB media at 37°C, centrifuged, re-suspended in DMEM media, and added to the upper chambers (filter) in the cellZscope at a multiplicity of infection (MOI) of 10. TER measurements were recorded continuously for up to 24 hours and TER values were normalized to the initial TER value (100%) and absolute TER is mean of 4 independent measurements. As a control, TER was measured for uninfected Caco-2 cell monolayers (controls in figures). Three independent experiments were performed for p19A WT and its mutant strains.

4.3.3. Detection of occludin by immunofluorescence and western blotting

In order to visualize the effect of bacteria on Caco-2 cells, occludin was detected by immunofluorescence microscopy (18) and western blotting.

Caco-2 cell monolayers were grown as described above and infected with bacteria for up to15 hours at a MOI 100. Caco-2 cell monolayers were either fixed for immunofluorescence or lysed in 100 µl of lysis buffer (Promega) on ice for 5 to 10 min. The cell lysate was centrifuged at 13,000 g for 12 min. to pellet debris, and the supernatant was used for western blotting. 50 µg Caco-2 cell proteins were resolved by 10% SDS-PAGE and transferred to 0.2 µm polyvinylidene fluoride (PVDF) membranes. Membranes were blocked for 1 hour with 3% non-fat milk powder diluted in 0.05 % Tween 20 (TBST), then incubated with primary antibody in 3% non-fat milk powder diluted in TBST overnight at 4°C. Hereafter, membranes were visualized with secondary (SIGMA-ALDRICH, DE) antibody for 1 hour at room temperature. Rabbit polyclonal anti-actin antibody (A2066 SIGMA-ALDRICH, DE) and rabbit anti-Occludin antibody (ABT146 Merck KGaA, Darmstadt, DE,) was used in this study.

4.3.4. Hemolysin assay

The presence of α-hemolysin was demonstrated on 5% sheep blood agar plates (SSI no. 31349 Statens Serum Institut, Diagnostica, DK) after 3 to 4 hours of incubation at 37°C as opposed to enterohemolysin, which was detectable only after overnight incubation at 37°C.

4.3.5. Hemolysis determination by titration assay

Defibrinated horse blood (SSI no. 23699 Statens Serum Institut, Diagnostica,DK) was washed twice in hemolysis buffer (0.0077 M Tris-HCl, 0.137M NaCl and 0.02M CaCl2 pH 7.4) and centrifuged at 300 g for 5 min. Washed red blood cells (RBC) were re-suspended in hemolysis buffer to a final concentration of 2% RBC. Overnight bacterial culture (approx. CFU 2 x108), 5 ml LB, 37°C, was centrifuged and both the bacterial pellet and the bacterial growth supernatant were tested for hemolytic activity. The bacterial pellet was

re-suspended in 5 ml hemolysis buffer. Two-fold serial dilutions (1:2 to 1:1024) in microtiter plates of either 150 µl of bacterial suspension or 150 µl of bacterial supernatant were performed in phosphate buffered saline (PBS) (pH 7.4; Sigma-Aldrich, St. Louis, MO) and finally 150 µl 2% RBC suspension were added and incubated for 2 hours at 37°C. After incubation, the plate was centrifuged for 10 min at 700 g, 150 µl of supernatant was transferred to a new microtiter plate, and the optical density measured at 562 nm. Hemolytic titration assays were performed at least twice with essentially the same results. Hemolysis buffer and PBS were used as negative controls.

4.3.6. Construction of genetic deletion mutants

Isogenic mutants of the *E. coli* clinical isolate p19A were constructed by allelic exchange with antibiotic resistance encoding cassettes using the λ-Red recombinase method as previously described (19). All primers used are shown in Table 1. For deletion of the hly cluster, 289-bp and 422-bp regions flanking the hly gene cluster were amplified by PCR using the primer pairs UphlyC-F/UphlyC-R and DwhlyD/DwhlyD-R and added to a kanamycin cassette. To construct the double hly mutant (hlyI, hlyII), the λ-Red procedure was repeated on the single hly mutant (hlyI) using a tetracycline resistance encoding cassette PCR amplified by primers 379 and 380 containing 50-bp overhangs homologous to up and downstream regions , respectively of the hly gene cluster . The cnf1 cluster was deleted using a tetracycline resistance encoding cassette PCR amplified by use of primers Upcnf-F and Dwcnf-R containing 50-bp overhangs homologous to regions up- and downstream regions the cnf1 gene, respectively. Allelic replacement was mediated via the thermo-sensitive helper plasmid pKOBEGApra, encoding λ-Red recombinase functions. Allelic replacements were verified by PCR. All primers are seen in table 4.1

Table 4.1 Primers used for construction of mutants

Primer name	Sequence 5’ to 3’
UphlyC-F	CGGGCTAACCAATATGCT
UphlyC-R	GAAGCAGCTCCAGCCTACACCCTCCGTGAAATTCTGATACT
DwhlyD-F	GGACCATGGCTAATTCCCATAAGAAAAGAGCAGAGCGA
DwhlyD-R	GTAACAACCCCACCTTCA
379	CACCACGAGTTAATAACTGAAGTAAAAAACAAGACAGATTT-CAATTTTTCATTAACAGGCAAGAATTGCCGGCGGAT
380	CTGTTAGTCTGACTGTAACTGATATAAGTAACTGTATAAACTT-TCTGGTTCGGTATTTCACACCGCATAGC
Upcnf-F	GATTAGGTATTCTGATAAGGTGTAGTAAAATATTAATCTTCACA-GAGGAGCAAGAATTGCCGGCGGAT
Upcnf-R	GCGCTAACAAAACAGCACAAGGGTAACTTATAACAATGGCCAAT-AAATAATTTCCCGGGTATTTCACACCGCATAGCAG
hlyA-F	ACCTTGTCAGGACGGCAGAT
hlyA-R	CCGTGCCATTCTTTTCATCA
RrpoA1	TTGATATCGAGCAAGTGAGTTCG
RrpoA2	GCATCGATGAGAGCAGAATACG

4.3.7. Quantification of hemolysin expression

Total RNA was phenol/chloroform extracted from LB growing cultures at OD600 0.8 (approx. 5 x 106 CFU 7ml) followed by DNase I digestion (# EN0525Thermoscientific). The RNA was then purified using Qiagen column (cat. no 74104) treated with a dsDNase (# EN0771Thermoscientific) and directly used for cDNA preparation using a First Strand cDNA synthesis kit (# K0702, Thermoscientific). For the amplification of hlyA, primers hlyA forward and hlyA reverse were used (20) (Table 4.1). The gene RpoA was used as a housekeeping/reference gene and amplified by primer pair RrpoA1 RrpoA2 (21) (Table 4.1). The Quantitative-PCR assay was performed using Takara SYBR Premix Ex Taq II (RR820A) in a BioRAD CFX96. The PCR was performed using the manufacturer’s recommendations: preheating at 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec for elongation.

4.3.8. Cytotoxicity by Neutral Red assay

Caco-2 BBE cell line (between passage 60 and 68) was maintained at 37°C in a humidified 5% CO2/95% O2 atmosphere, in DMEM containing Glutamax, 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids and 1% L-glutamine for 7 days. After 7 days, the media were removed from the confluent cell layer by aspiration and the monolayer was washed twice with PBS. Trypsinated cell suspension was seeded in 24-transwell plates (seeding density of 0.05 x 10^6/cm2) and incubated overnight before co-incubation with bacteria. Caco-2 cells were infected with an overnight culture of *E. coli* grown in DMEM at an MOI 10 and maintained at 37°C in a humidified 5% CO_2/95% O_2 atmosphere for 4 hours. The monolayer was then washed once with DMEM, and then DMEM containing 50 µg/ml neutral red (N4638, Sigma-Aldrich, Brøndby, DK) was added and incubated at 37°C for 30 min. Hereafter, the cells were washed rapidly with a suspension containing 40% formaldehyde and 10% CaCl2. Neutral red was extracted with 1% acetic acid-50% ethanol and quantified in a spectrometer (OD 450 nm). The amount of extracted neutral red is expressed as a percentage of the amount recovered from uninfected cells.

4.3.9. Statistics

The software "GraphPad Prism 5" was used for statistical analysis. TER and hemolytic titration results were analyzed using the Two-way ANOVA test when compared to blank or negative control. Neutral Red test results were analyzed using the One-way ANOVA test.

4.4. Results

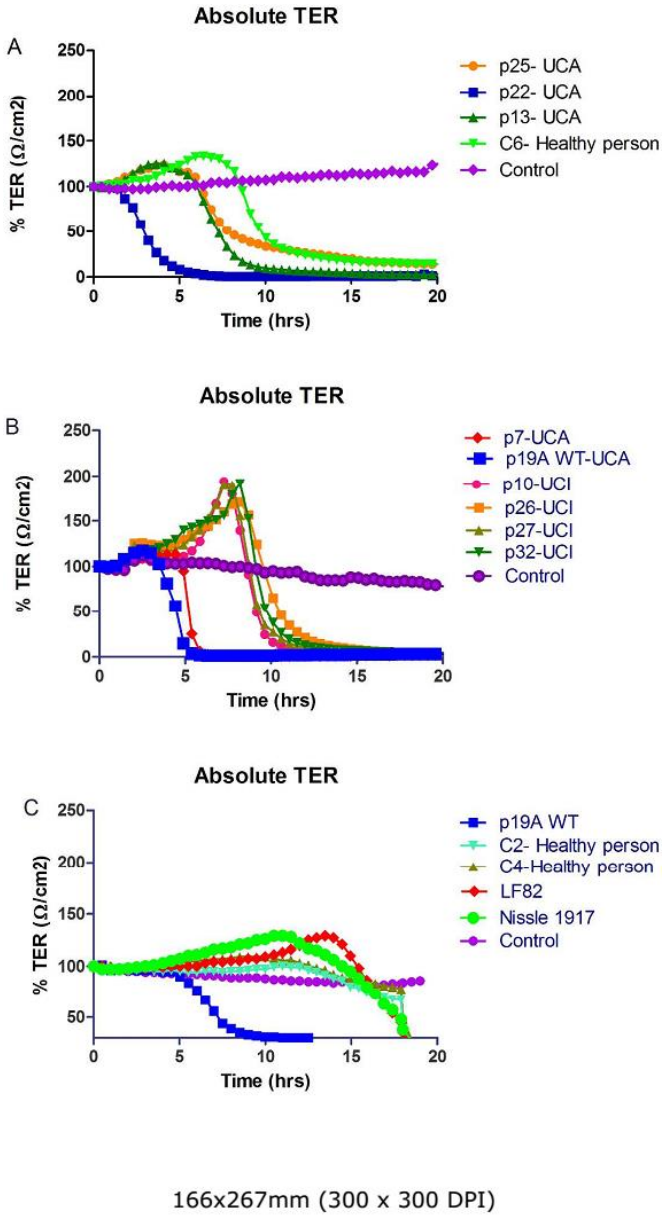
4.4.1. Hemolytic strains of *E. coli* isolated from IBD patients with active disease disrupt the epithelial cell barrier integrity tested

by TER

In this study, we investigated the effect of twelve *E. coli* strains isolated from 9 patients with IBD and 3 control subjects on intestinal epithelial integrity, using TER measurements of Caco-2 cell monolayers grown in Transwells (Table 4.2). Three of the five phylotype B2 *E. coli* strains, p7, p19A and p22, isolated from UC patients with active disease induced a rapid decrease in TER at an MOI 10, starting after about 2 hours and resulting in a complete loss of TER by 6 hours (Figure 4.1 A and B). All four *E. coli* strains isolated from patients with inactive UC or healthy controls decreased the TER after 10 to12 hours, which was similar for the probiotic *E. coli* Nissle and the adhesive and invasive *E. coli* (AIEC) strain LF82 (isolated from an ileal biopsy of a patient with CD (11)) (Figure 4.1 A, B, C). The loss of TER after about 10 to 15 hours is due to the growth of *E. coli* and acidification of the medium. As expected, the TER of untreated Caco-2 cell monolayers was not significantly changed over the 20 hours of incubation. Isolates p7, p19A, and p22 were identified as the only α-hemolytic strains among those tested, therefore implicating α-hemolysin in the disruption of TER (Table 4.2).

4.4.2. IBD-associated strain P19A contains cnf1 and two hly gene clusters

Among the α-hemolysin positive strains from patients with active UC, p19A was chosen for further investigation. In a previous study, we have shown that *E. coli* strain p19A belongs to the phylogenetic group B2, and harbors cnf1 and hly genes (22). To determine the possible role of *E. coli* hly and cnf1 in barrier disruption, deletion mutants of the individual toxin-encoding genes were constructed. The first hly mutant constructed (ΔhlyI) was still hemolytic indicating that P19A contained two hly clusters. The presence of two hly



166x267mm (300 x 300 DPI)

Figure4.1 Effect of *E. coli* clinical isolates from IBD patients and controls on a monolayer of Caco-2 cells measured by transepithelialelectric resistance (TER). A) *E. coli* strains p13, p22, and p25 from patients with Active Ulcerative Colitis (UCA) revealed that p22 disrupted the epithelial cell barrier in less than 6 hours after co-incubation with Caco-2 cells, while strains p13 and p25, and E. coli C6 from a healthy control did not disrupt the epithelial barrier until after 10 hours of co-incubation. B) *E. coli* strains p7 and p19A WT from patients with UCA disrupted the epithelial cell barrier in less than 6 hours after co-incubation with Caco-2 cells. *E. coli* p10, p26, p27, and p32 from patients with Inactive Ulcerative Colitis (UCI) did not disrupt the epithelial barrier until after 10 hours of co-incubation. C) *E. coli* strain p19A WT from UCA patient was compared with the adherent invasive CD associated E. coli LF82, the probiotic *E. coli* Nissle and two *E. coli* strains, C2 and C4, isolated from healthy controls, it is seen that the probiotic and control isolates did not disrupt epithelial barrier until after 15 hours of co-incubation. Simultaneously with all experiments presented (A, B, C) control TER of the media was performed on Caco-2 cells without addition of bacteria. TER values were normalized to the initial TER value (100%). Absolute TER values are mean of 4 measurements.

clusters has been reported for some ExPEC isolates, and in these strains one of the hly clusters is often located upstream of the cnf1 gene (23). Indeed PCR analysis of strain P19A revealed that the intact hly cluster remaining in the ΔhlyI mutant was located upstream of the cnf1 gene. Thus, mutants of P19A lacking the second hly cluster (ΔhlyII) and also both hly clusters were constructed (ΔhlyI, II).

Table 4.2 Origin, molecular and physiological characteristics of *E. coli* strains used in this study

E. coli strain	Phylogenetic group	Disease association	Hemolytic activity	TER reduction	Two-way ANOVA (TER: 5-12 h)
p7	B2	Active UC	Alfa (<4 h)	6h	p<0.05***
p13	B2	Active UC	None	>10 h	ns
p19A	B2	Active UC	Alfa (<4 h)	6 h	p<0.05***
p22	B2	Active UC	Alfa (<4 h)	6 h	p<0.05***
p25	B2	Active UC	Ent (24 h)	>10 h	ns
p10	A	Inactive UC	None	>10 h	ns
p26	A	Inactive UC	Ent (24 h)	>10 h	ns
p27	A	Inactive UC	Ent (24 h)	>10 h	ns
p32	B2	Inactive UC	None	>10 h	ns
C2	A	Healthy	None	>10 h	ns
C4	B1	Healthy	None	>10 h	ns
C6	D	Healthy	None	>10 h	ns
LF82	B2	Crohns Disease	None	>10 h	ns
E. coli Nissle	B2	Probiotic	None	>10 h	ns

Hemolytic titration assays were performed with bacteria and bacterial culture supernatants in order to investigate the hemolytic activity of clinical isolate *E. coli* p19A WT and the hly and cnf1 mutants. The hemolytic activity was only completely abolished in the double mutant lacking hly clusters I and II ($p<0.05$) (Figure 4.2). Deletion of hly cluster II only partially decreased hemolytic activity, compared to the WT suggesting that hly cluster I does contribute to the overall hemolytic activity of the WT strain, despite the fact that no reduction in

hemolysis was observed in the hly cluster I mutant.

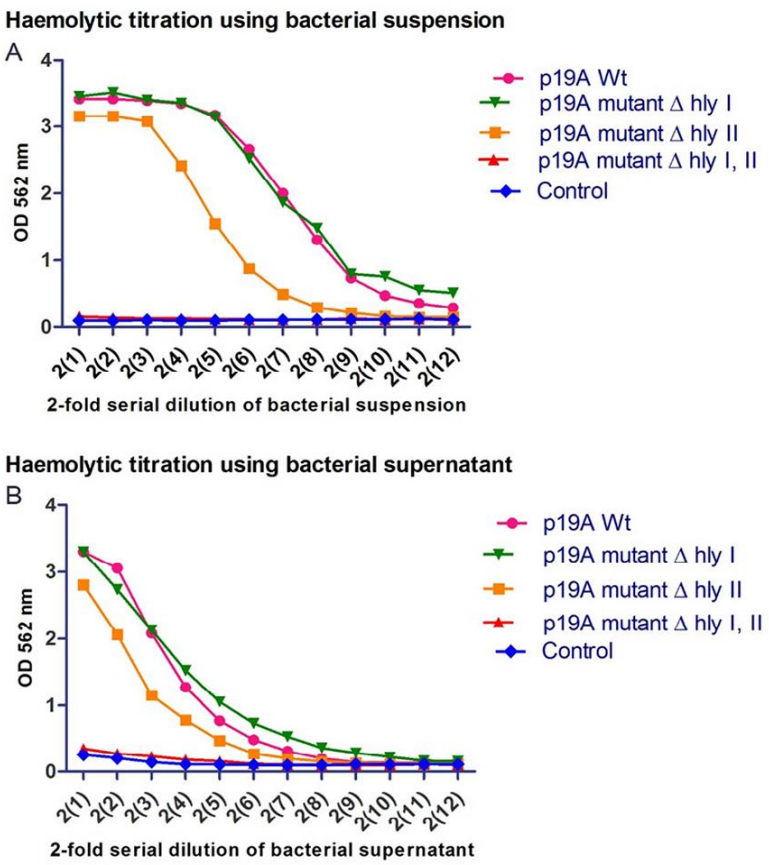


Figure 4.2 Hemolysin activity of clinical isolate p19A WT from UCA patient and its isogenic mutants. Bacterial cell suspension of clinical isolate p19A WT and the two A) single hemolysin mutants (p19A Δhly I, and p19A Δhly II) showed almost strong hemolytic activity, while in p19A hemolysin double mutant (p19A Δhly I and II) the activity was completely hampered. We used buffer as a control. B) Bacterial growth supernatant of the above cultures revealed the same hemolytic activity while the double mutant had abolished activity. We used growth medium as a control.

RT-PCR was used to quantify the relative amounts of the hly transcript in *E. coli* p19A WT and the different hly deletion mutants, using rpoA transcripts as an internal control. The relative expression of hly was two-fold higher in the WT p19A than in the ∆hlyI mutant and four-fold higher than in the ∆hlyII mutant. As expected, only the double ∆hlyI II mutant of p19A lacked hly gene expression (Figure 4.3).

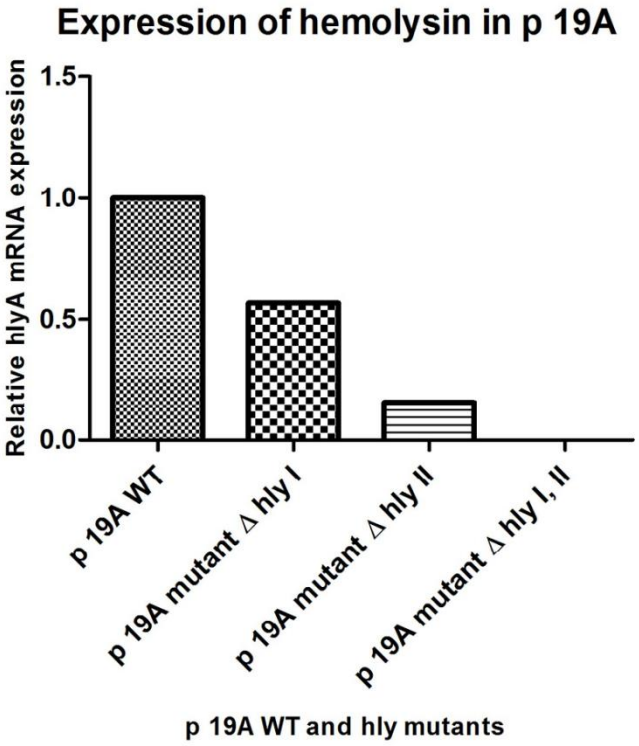


Figure 4.3 Quantification of hemolysin expression in clinical isolate p19A. It is clearly seen that p19A WT expresses more hly compared with the single mutants. Expression of hemolysin is completely abolished in the double mutant p19A Δhly I, II. Relative hlyA mRNA was measured by quantitative RT-PCR. The rpoA mRNA level was used as an internal quantitative control.

4.4.3. Hly expression in IBD-associated strain p19A causes rapid loss of epithelial integrity

To study the effect of hly and cnf1 expression on the intestinal epithelial barrier integrity, TER measurements were performed with p19A WT and its deletion mutants. At a MOI 10, the WT, single mutans ΔhlyI, ΔhlyII and Δcnf1 strains caused loss of TER in Caco-2 cell monolayers in less than 6 hours (Figure 4.4). Deletion of both hly clusters (ΔhlyI, II) in p19A WT abrogated the rapid loss of epithelial integrity, and the effects on TER were comparable to the probiotic Nissle and other E. coli strains not expressing hly (12 hours ($p<0.0001$***).

4.4.4. Hly expression is linked to rapid dissolution of occludin from the tight junctions of epithelial cell monolayers

To investigate the effect of hemolytic strains on tight junctions, Caco-2 cell monolayers were incubated with *E. coli* p19A for 1, 2 or 3 hours and then fixed

and stained for occludin and nuclear DNA (Figure 4.5A). No dissolution of occludin from the tight junction was observed with *E. coli* Nissle or the Hly-negative AIEC strain LF82. A significant reduction in the immunofluorescent staining of occludin was evident, from 2 to 3 hours of co-culture with wild-type p19A ($P<0.001$) as confirmed by western blotting with antibodies to occludin (Figure 4.5). Similar results were obtained with all three Hly-producing E. coli strains p7, p19A or p22, but not with the mutants of p19A ΔhlyI, ΔhlyI II double mutant demonstrating a link between Hly expression and loss of tight junction occludin (data not shown).

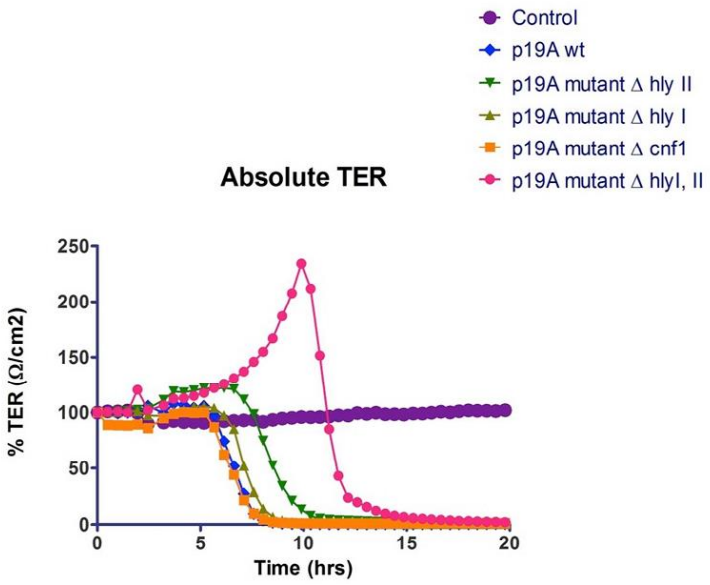


Figure 4.4 Effect of clinical isolate E.coli p19A and its hemolysin deletion mutants on a monolayer of Caco-2 cells measured by transepithelial electric resistance (TER). Wild type p19A and its single hemolysin- and cnf1-deletion mutants all disrupted the epithelial barrier in less than 6 hours, whereas the p19A ΔhlyI, II double deletion mutant did not have any effect on TER. As a control, no bacteria were added to the Caco-2 cells.

4.4.5. Effect of p19A WT on epithelial tight junctions disruption and loss of TER is not due to cytotoxicity

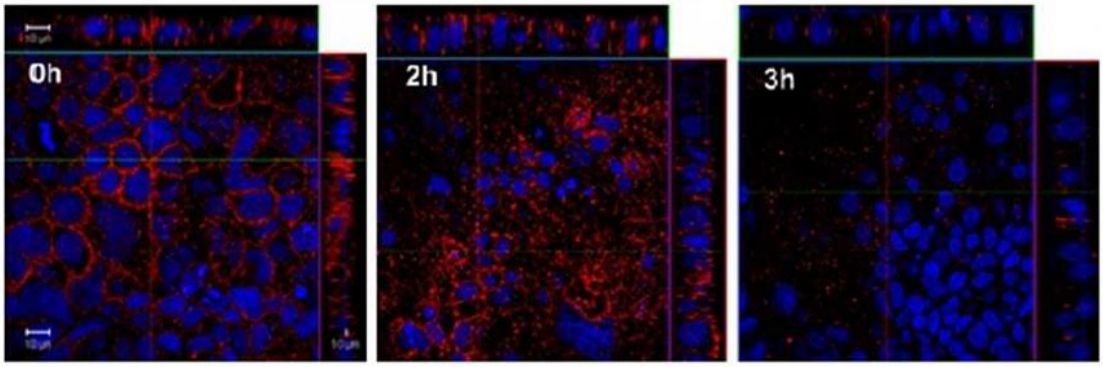


Figure 4.5 Disruption of occluding on Caco-2 cells after incubation with clinical isolate p19A. Confocal images of Caco-2 cell-monolayers stained for occludin (red) and nuclei (blue) after apical incubation with E. coli p19A (MOI: 50) after 2 and 3 hours ($P < 0.01$ and $P < 0.001$, respectively).

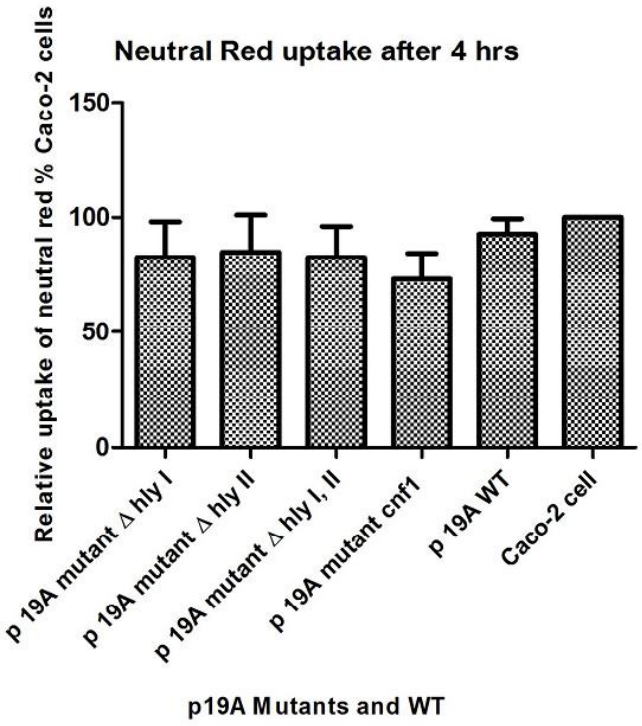


Figure4.6 Cytotoxicity of clinical isolate p19A and its hemolysin mutants on Caco-2 cells. No significant differences in Caco-2 cell viability were found between the p19A wild type and mutants. Neutral Red uptake in Caco-2 cells was measured after co-incubation with E. coli p19A WT and mutants.

To investigate a possible cytotoxic effect of hly and cnf1 genes on epithelial cells in the above assays, Neutral Red uptake by viable Caco-2 cells was measured after 4 hours of co-culture with p19A WT and its mutants at the same MOI. Neutral Red assays were performed 6 times on 3 different days. No significant differences were found between p19A WT and mutants, which rules out the possibility that cytotoxicity of Hly or Cnf1 causes the rapid loss of occludin and decrease in

TER (Figure 4.6).

4.5. Discussion

The epithelial cell layer is an essential constituent of the gut and a highly specialized interface between the host and its environment. Desmosomes, adherence junctions and tight junctions hold the cells of the intestinal epithelial layer together. Tight junctions are important in controlling para-cellular permeability to ions and small molecules and preventing translocation of luminal antigens and bacteria into the lamina propria (24). In this paper, we demonstrated that IBD-associated *E. coli* strains from UC patients that produce α-hemolysin cause disruption of epithelial tight junctions of intestinal cell monolayers, leading to the loss of trans-epithelial resistance. Three of five UC-associated E. coli strains (p7, p19A and p22) isolated from patients with active UC induced a rapid loss of TER at low MOI without any loss of cell viability.

The IBD-associated strains causing loss of epithelial integrity were all of the phylotype B2 and consistent with previous reports showing an increased abundance of the phylotype B2 *E. coli* in UC and CD patients with active disease (11,12,25,26). The role of *E. coli* pathobionts in the pathophysiology in IBD was attributed to their capability to adhere and invade epithelial cells and replicate in macrophages, and the most well studied prototype strain is LF82. In contrast to p19A, strain LF82 does not cause rapid dissolution of epithelial tight junctions, clearly indicating that the phylotype B2 of UC-associated strains differ markedly in pathogenic mechanisms. The type 1 fimbriae of AIEC were shown to bind to carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), which is expressed at higher levels in inflamed intestinal epithelial cells of IBD patients (27). Our UC-associated *E. coli* p19A strain has the same capacity as LF82 to adhere to epithelial cells (data not shown).

All the UC-associated *E. coli* strains that caused loss of tight junctions in epithelial cell monolayers were hemolytic. Four types of hemolysin have been demonstrated in *E. coli*: alpha-hemolysin (Hly A), plasmid- and phage- carried enterohemolysin (EhxA, HlyA) and silent hemolysin (SheA); EhxA and HlyA belong to the RTX (repeat in toxin) related family, which lyse erythrocytes from different mammalian species (28–30). It is known that a number of *E. coli* pathotypes, i.e. urinary tract pathogenic (UPEC), enteropathogenic *E coli* (EPEC) and Enterotoxigenic *E. coli* (ETEC) are all able to produce α-hemolysin (20). The *E. coli* α-hemolysin is known to be able to lyse erythrocytes through binding to the surface protein glycoporin (31-33), but also other cell types including leucocytic cells, bladder and renal tubular cells in a dose dependent manner (25-29). Lysis of immune cells is greatly influenced by the presence of cell receptors CD11a and CD18, which are expressed on B and T cells, as well as neutrophils monocytes and dendritic cells (30, 31).

The role of HlyA in tight junction disruption was further investigated in *E. coli* strain p19A, which possessed two hlyA clusters as previously reported for isolates of uropathogenic *E. coli* belonging to phylotype B2 (34). We showed that both hlyA gene clusters in p19A contributed to the damaging effects on the epithelial integrity, suggesting that intestinal *E. coli* strains possessing more than one hlyA locus may have increased pathological consequences in intestinal inflammation. Although our UC-associated strains did not induce epithelial cell apoptosis, a hly-expressing uropathogenic *E. coli* was previously shown to cause localized regions of apoptosis in HT29/B6 cell monolayers. The difference between these findings and our results may be due to the use of a higher MOI than in our study, the use of different strains or the amount of hlyA expressed (35).

Our demonstration that around 50% of phylotype B2 *E. coli* isolated from UC patients can adhere to epithelial cells and disrupt epithelial tight junctions via an HlyA-dependent mechanism, provides strong evidence that this is an important novel pathogenic mechanism in UC; and distinct of AIEC LF82 in CD.

Lesions in tight junctions of intestinal epithelium from IBD patients with active disease have been associated with a reduction in several tight junction proteins including claudin 1 and 4, occludin and tricellulin (36), and the synthetic octapeptide (AT1001), which prevents the opening of tight junctions, improves colitis in susceptible IL-10-/- mice (37). Further evidence for the importance of HlyA in the epidemiology of IBD comes from a previous study showing that an HlyA-producing strain of *E. coli* but not an HlyA-deficient mutant was a potentiator of inflammatory activity in the colon of susceptible IL-10-/- mice and monocolonized germ-free mice due to its effects on the epithelial barrier function (14). During active UC and high inflammation and increased CECAM6 expression binding of specific *E. coli* is facilitated.

This study is a mandate for further investigation of epithelial barrier disruption in other UC cohorts and geographic locations. Preliminary evidence from genomic sequencing suggest that some strains like *E. coli* p19A carry large conjugative plasmids suggesting lateral gene transfer of hly loci could contribute to the spread of pathogenic traits.

A recent meta-study including 10 randomized trials from CD patients and 9 randomized trials from UC patients yielded an OR of 2.17 (95% CI, 1.54-3.05) in favor of antibiotic therapy (38). These results suggest that antibiotics improve clinical outcomes in patients with IBD. Another meta-study published in 2011 by Khan et al. concluded that antibiotic therapy may induce remission in active CD and UC, although the diverse number of antibiotics tested means the data are difficult to interpret (39). This systematic review proposed further trials of antibiotic therapy in IBD.

Approaches for combating bacteria that adversely affect the barrier function (e.g. HlyA expressing *E. coli*) might provide new treatment options for IBD. This might include antibiotic therapy, vaccination or competition by probiotic bacteria lacking HlyA and other virulence factors that can cause harm to the host.

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5. Protective effect of Clostridium tyrobutyricum in acute dextran sodium sulphate-induced colitis: differential regulation of tumour necrosis factor-α and interleukin-18 in BALB/c and severe combined immunodeficiency mice

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5.1. Abstract

One of the promising approaches in the therapy of ulcerative colitis is administration of butyrate, an energy source for colonocytes, into the lumen of the colon. This study investigates the effect of butyrate producing bacterium *Clostridium tyrobutyricum* on dextran sodium sulphate (DSS)-induced colitis in mice. Immunocompetent BALB/c and immunodeficient severe combined immunodeficiency (SCID) mice reared in specific-pathogen-free (SPF) conditions were treated intrarectally with *C. tyrobutyricum* 1 week prior to the induction of DSS colitis and during oral DSS treatment. Administration of DSS without *C. tyrobutyricum* treatment led to an appearance of clinical symptoms – bleeding, rectal prolapses and colitis-induced increase in the antigen CD11b, a marker of infiltrating inflammatory cells in the lamina propria. The severity of colitis was similar in BALB/c and SCID mice as judged by the histological damage score and colon shortening after 7 days of DSS treatment. Both strains of mice also showed a similar reduction in tight junction (TJ) protein zonula occludens (ZO)-1 expression and of MUC-2 mucin depression. Highly elevated levels of cytokine tumour necrosis factor (TNF)-α in the colon of SCID mice and of interleukin (IL)-18 in BALB/c mice were observed. Intrarectal administration of *C. tyrobutyricum* prevented appearance of clinical symptoms of DSS-colitis, restored normal MUC-2 production, unaltered expression of TJ protein ZO-1 and decreased levels of TNF-α and IL-18 in the descending colon of SCID and BALB/c mice, respectively. Some of these features can be ascribed to the increased production of butyrate in the lumen of the colon and its role in protection of barrier functions and regulation of IL-18 expression.

5.2. Introduction

The enteric mucosa of ulcerative colitis (UC) patients is aberrant due to the abnormal interaction between microbiota and the intestinal mucosal immune

system, which leads to mucosal inflammation [1,2]. In experimental dextran sodium sulphate (DSS) models of colitis, interaction of components from both pathogenic and commensal microorganisms with the host mucosal immune system can trigger inflammatory responses and alter the colonic function [3–5]. Under germ-free conditions mice develop markedly limited incidence of colitis [6]. One of the widely used experimental mouse models of UC involves addition of DSS to the drinking water, which causes rapid alterations in the inner colon mucus layer, making it permeable to bacteria [7,8]. Protection of colonic mucosa requires normal production of mucins, such as secretory MUC-2, synthesized by goblet cells in healthy colon of humans, rats and mice [9]. However, in experimental ulcerative colitis the mucus production is impaired [10]. Butyrate ameliorates inflammation in experimental ulcerative colitis by up-regulating the expression of mucin genes [11,12] and by protecting mucosal surfaces against increased mucosal permeability [13]. Several probiotics have been reported to enhance epithelial permeability and/or protect against barrier disruption by pathogens *in vitro* (reviewed recently [14]). Recently a study was performed in human volunteers which showed that perfusion of *Lactobacillus plantarum* into the duodenum increased the localization (immunofluorescent staining) of occludin and zonula occludens (ZO)-1 in the epithelial tight junctions (TJs) of tissue biopsies [15]. Protection of mice against DSS induced colitis by probiotic *E. coli* Nissle 1917 has been associated with increased expression of TJ protein zonula occludens (ZO-2) expression in epithelial cells [16].

Ulcerative colitis is associated with an elevated production of inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β, leading to increased intestinal permeability and activation of nuclear factor (NF)-κB and c-Jun N-terminal kinase (JNK)/p38 mitogen-activated protein kinase (MAPK) pathways [17]. Decreased production of proinflammatory cytokines constitutes an important mechanism for the partial amelioration of colitis by probiotics [18,19].

Butyrate enemas have been reported to be effective in therapy of UC. Microbially produced butyrate is considered important for colonic health and in the prevention of colorectal cancer, owing to its use as an energy source for colonocytes and as a modulator of oxidative stress and inflammation [20]. Oral administration of *Clostridium butyricum* M 588, characterized by high production of butyrate during fermentative growth, has been shown to protect against DSS colitis in the mouse [21]. Our candidate probiotic *Clostridium tyrobutyricum* (DSM 2637) was isolated from raw cow's milk and is a Gram-positive, rod-shaped, spore-forming obligate anaerobe that can ferment a wide variety of carbohydrates to butyric acid. In a survey of 35 *Clostridium* species and 243 strains, *C. tyrobutyricum* was shown not to produce cytotoxins [22]. To verify further the safety of *C. tyrobutyricum* strain DSM 2637 we assessed its potential to translocate from the gut into the blood and organs of germ-free severe combined immunodeficiency (SCID) mice that were monocolonized for 30 days in a gnotobiotic isolator. All animals colonized with *C. tyrobutyricum* strain DSM 2637 remained healthy and lacked any signs of pathology. As expected, *C. tyrobutyricum* was found in the lumen of the jejunum, ileum, colon ascendens and colon descendens, but could not be detected in the blood, mesenteric lymph nodes (MLNs), liver or spleen.

We have shown previously that immunodeficient SCID (T and B cell-independent) and immunocompetent BALB/c mouse strains reared under specific-pathogen-free (SPF) conditions, but not as germ-free mice, develop colitis after 1 week of DSS treatment [6]. The BALB/c mice survive two rounds of DSS treatment, albeit with clear evidence of colonic damage and T and B cell infiltration in the mucus, whereas SCID mice survive only one round of DSS treatment [6]. The main objectives of this study were to investigate the role of the immune response in regulating colitis in SCID and BALB/c mice and to evaluate the potential protective effect of *C*. *tyrobutyricum* (DSM 2637) on DSS colitis development. Changes in intestinal mucins, barrier function of TJ and production of the inflammatory cytokines IL-18 and TNF-α were measured

during colitis induction. As butyrate enemas and colonic application of faecal bacteria [23] have been shown to be beneficial in clinical studies we also evaluated the therapeutic potential of intrarectal administration of *C*. *tyrobutyricum* in the DSS colitis model.

5.3. Materials and methods

5.3.1. Animals

BALB/c and SCID (background BALB/cJHanHsd-SCID) mice were reared in SPF conditions. The absence of T lymphocytes in SCID mice was proved with fluorescein isothiocyanate (FITC)-labelled monoclonal anti-CD3 antibody (Serotec, Oxford, UK) using fluorescence activated cell sorter (FACS) Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Two-month-old mice were used for these studies and their body weights were measured before and after each experiment.

5.3.2. Bacterial strain and culture conditions

C. tyrobutyricum (DSM 2637) isolated from raw cow's milk was provided from the Food Research Institute, Prague, Czech Republic and cultured in sterile Bryant Burkey bouillon with resazurin and lactate (Merck KGaA, Darmstadt, Germany) at 37°C under anaerobic conditions. Prior to administration to mice a fresh overnight culture of bacteria was adjusted to 109 colony-forming units (CFU)/ml in saline.

5.3.3. Intrarectal administration of C. tyrobutyricum and induction of acute ulcerative colitis by DSS

The experimental groups of five to 10 mice and their respective treatments are shown in Table 5.1. Groups 1, 2, 4 and 5 (Table 5.1) received 2·5% DSS

(molecular weight 40 kDa; ICN Biomedicals, Cleveland, OH, USA) in drinking water *ad libitum* for 1 week. The untreated control groups 3 and 6 received only drinking water. The *C. tyrobutyricum*-treated groups 1 and 4 received intrarectally (via tubing) a daily dose of 2×10^8 CFU of strain DSM 2637 in 0·2 ml saline for 7 days prior to DSS exposure and also during the 7 days exposure to DSS in the drinking water. Control groups 3 and 6 received 0·2 ml saline [phosphate-buffered saline (PBS)]. The following clinical symptoms were measured or assessed: firmness of faeces, rectal prolapses, rectal bleeding and colon length after the mice were killed. The colon descendens was divided into two pieces, one being used for TNF-α determination after 48 h culture and the other for histological assessment. Animal experiments were approved by the Ethical Committee of the Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i.

5.3.4. Histological evaluation of inflammation

The tissue was fixed in Carnoy's fluid for 30 min, transferred into 96% ethanol and embedded in paraffin. Five-µm paraffin-embedded sections were cut and stained with haematoxylin and eosin (H&E) and Alcian Blue and post-stained with Nuclear Fast Red (all from Vector, Burlingame, CA, USA) for mucin production. The samples were viewed under an Olympus BX 40 microscope equipped with an Olympus Camedia DP 70 digital camera, and the images were analysed using Olympus DP-Soft. The degree of damage to the surface epithelium, crypt distortion and mucin production in individual colon segments were evaluated according to Cooper *et al*. [3].

5.3.5. Expression of CD 11b, ZO-1 and MUC-2

Segments of the colon descendens were frozen in liquid nitrogen. Cryosections (5 µm thick) of acetone-fixed colon were used for immunocytochemistry. The membrane marker CD11b was detected directly by

fluorescein-labelled monoclonal antibody anti-CD11b FITC (Serotec, Kidlington, UK). The antigen CD11b is known to be expressed on the surface of polymorphonuclear leucocytes, monocytes and natural killer (NK) cells. Expression of ZO-1 was detected by rabbit anti-mouse polyclonal antibody (Zymed Laboratories, Carlsbad, CA, USA) and secondary antibody Cy3 goat anti-rabbit IgG (Biomeda, Burlingame, CA, USA). Production of MUC-2 was detected by primary, polyclonal rabbit anti-mouse IgG, specific for MUC-2, and secondary antibody Cy3 goat anti-rabbit IgG (all from Biomeda).

Table 5.1 Development of DSS-colitis with association of *Clostridium tyrobutyricum* in conventional BALB/c and severe combined immunodeficiency (SCID) mice: clinical and histological gradings and detection of tumour necrosis factor (TNF)-α

Strain	Treatment	2·5% DSS	Mortality /n	Body weight	Damage score	Mucin secretion	Length of colon	TNF-α
				(g)	(0–4)	(4–0)	(cm)	(pg/10 mg tissue)
1 BALB/c	*C. tyrobutyricum* (i.r.)	+	0/5	18·8 ± 1·2	1·6 ± 0·5ΔΔΔ	2·7 ± 0·4ΔΔΔ	7·6 ± 0·5#	27·9 ± 12·7##
2 BALB/c	saline (i.r.)	+	0/5	17·6 ± 1·9	3·9 ± 0·2ΔΔΔ	0·1 ± 0·2ΔΔΔ	6·9 ± 0·6#	29·3 ± 15·3##
3 BALB/c	saline (i.r.)	–	0/5	19·6 ± 0·5	0	4	9·8 ± 0·7	7·8 ± 5·0
4 SCID	*C. tyrobutyricum* (i.r.)	+	10-Jan	21·0 ± 2·1	0·5 ± 0·4ΔΔΔ	3·5 ± 0·5ΔΔΔ	10·0 ± 1·9*	25·2 ± 5·2**,##
5 SCID	saline (i.r.)	+	10-Feb	20·0 ± 2·4	3·9 ± 0·1ΔΔΔ	0·1 ± 0·2ΔΔΔ	7·8 ± 0·8#	79·3 ± 8·0##
6 SCID	saline (i.r.)	–	0/8	21·5 ± 1·2	0	4	10·9 ± 0·3	11·0 ± 2·2

Values are means ± standard deviation

*$P < 0·05$

**$P < 0·01$, significant difference of group 4 *versus* group 5

ΔΔΔ$P < 0·0001$, significant difference of group 1 *versus* group 2 and group 4 *versus* group 5 of mice

#$P < 0·05$

##$P < 0·01$, significant difference of groups 1 and 2 *versus* control group 3 of BALB/c mice and group 4 and 5 *versus* control group 6 of SCID mice; i.r.: intrarectal administration. DSS: dextran sodium sulphate.

5.3.6. Measurement of colonic TNF-α production

Pre-weighed colonic fragments were cultured in RPMI-1640 medium enriched with 10% bovine serum albumin in 5% CO2 and 95% air at 37°C, in 24-well flat-bottomed plates (Nunc, Roskilde, Denmark) for 48 h. Quantification of TNF-α level was performed by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols using Infinite 200 apparatus (Tecan Group Ltd, Grödig, Austria).

5.3.7. Measurement of IL-18 in tissues by confocal fluorimetry

Expression of IL-18 in acetone-fixed sections of colon descendens was detected by polyclonal rabbit antibody to mouse IL-18 (Acris Antibodies, Hiddenhausen, Germany) and Cy3-labelled goat anti-rabbit IgG (Biomeda). Relative confocal fluorimetry of IL-18 visualized by Cy3 staining was performed by laser scanning confocal microscopy using Leica SPE and Leica SP-2 microscopes with oil immersion objectives ×20, ×40 and ×63 and an excitation line of 532 nm and emission detection at 550–700 nm; a multiple accumulation of weaker signal was used. Fluorescence intensity of Cy3 in epithelial colonocytes was evaluated (expressed in units on a scale of 0–255) using different regions and sections of at least three mice per group. It was possible to distinguish regions with lower and higher amounts of IL-18 (Table 5.2) and evaluates them separately, using correct statistical methods.

5.3.8. Measurement of short-chain fatty acids (SCFA)

SCFAs were measured in bacterial cultures and in faecal samples of SCID mice. Concentrations of acetic acid, propionic acid, n-butyric acid, iso-butyric acid, valeric acid, iso-valeric acid and 2-methylbutyric acid were measured using gas chromatography on a HP 5890 GC with flame ionization detector (FID) and nitrogen as carrier gas at 1·2 ml/min column flow. The samples were

analysed on an Equity-1 column (30 m × 0·32 mm i.d., 1 µm film thickness; Supelco, Prague, Czech Republic). A 1-µl sample was injected into the gas chromatograph. The split ratio was 1:1. The oven temperature was held at 40°C for 1 min, then raised to 230°C at a rate of 10°C/min, and held at 230°C for 15 min; both injector and detector temperatures were 250°C. Peak identification was confirmed by retention times of commercially obtained standards from Sigma-Aldrich.

5.3.9. Statistical analysis

Statistical analyses were performed using Student's *t*-test. Values of $*P \leq 0{\cdot}05$ were considered significantly different. Levels of Cy3 in colonocytes, expressed in units of fluorescence intensity on a scale of 0–255, were compared in unpaired Student's *t*-test using statistical significance level $*P = 0{\cdot}001$.

Table 5.2 Evaluation of fluorescence for interleukin (IL)-18 in the colon of mice

Mouse strain	**SCID**			**BALB/c**		
DSS	–	+	+	–	+	+
C. tyrobutyricum	–	–	+	–	–	+
IL-18 (lower intensity)	29·1 ± 7·2	19·2 ± 9·0	77·9 ± 15·8	34·8 ± 11·7	82·7 ± 6·6	50·7 ± 6·3
IL-18 (higher intensity)	76·5 ± 4·8	151 ± 25·4	161·9 ± 32·3	72·2 ± 11·1	146·4 ± 15·0	98·2 ± 11·8

Values are expressed as means ± standard deviation (s.d.) in units of fluorescence intensity on scale 0–255. SCID: severe combined immunodeficiency; DSS: dextran sodium sulphate.

5.4. Results

5.4.1. Clinical evaluation of BALB/c and SCID mice under the influence of DSS and *C. tyrobutyricum*

Control BALB/c and SCID mice not exposed to DSS remained healthy during the experiment. In contrast the DSS-treated mice developed clinical signs of colitis, including diarrhoea, rectal prolapses and bleeding. The SCID mice (group 5, Table 5.1) were more susceptible to DSS than BALB/c mice (group 2, Table 5.1), resulting in 20% mortality. No significant changes in body weight were observed in BALB/c and SCID mice as a consequence of exposure to DSS. One of the characteristic signs of DSS-induced colitis is shortening of the colon, which was observed in both saline–DSS-treated BALB/c and SCID mice (groups 2 and 5, Table 5.1) but not in the control mice (groups 3 and 6, Table 5.1). In SCID mice, treatment with *C. tyrobutyricum* prevented shortening of the colon (group 4, Table 5.1), while no significant protection was observed in *C. tyrobutyricum*-treated BALB/c mice (group 1, Table 5.1).

5.4.2. Histological colon damage score in DSS-treated BALB/c and SCID mice

The histological colon damage score after DSS treatment assessed according to the scale (0–4) of Cooper*et al*. [3] is presented in Table 5.1. The damage score was of grade 0 in control BALB/c and SCID mice (groups 3 and 6). The damage score was of grade 3·9 ± 0·2 in BALB/c and 3·9 ± 0·1 in SCID saline-DSS-treated mice (groups 2 and 5; Fig. 5.1a). In *C. tyrobutyricum*-treated BALB/c and SCID mice the damage due to DSS exposure was significantly less, grade 1·6 ± 0·5 (group 1) and grade 0·5 ± 0·4 (group 4, Fig. 5.1b), respectively.

5.4.3. Goblet cell mucins and specific MUC-2 production in the colon

In healthy control mice (groups 3 and 6, Table 5.1), colonic mucin production (Alcian Blue staining) was grade 4 according to the scale (4–0) of Cooper *et al*.

[3]. In both saline–DSS–treated BALB/c and SCID mice (groups 2 and 5, Table 5.1, Fig. 5.1c) it was decreased drastically to grade 0·1 ± 0·2. Treatment with *C. tyrobutyricum* attenuated the loss of mucin due to DSS exposure in both BALB/c mice (group 1 grade 2·7 ± 0·4) and SCID mice (group 4 grade 3·5 ± 0·5, Table 5.1, Fig. 5.1d), with protection being more evident in the SCID mice.

Moreover, changes in the production of the major secreted mucin MUC-2 was verified in SCID mice using immunocytochemistry (Fig. 5.2). In the saline-treated SCID mice (Fig. 5.2a) and BALB/c mice (Fig. 2d), the production of MUC-2 was intact. In the saline–DSS-treated SCID (Fig. 5.2b) and BALB/c mice (Fig. 5.2e) the production of colonic MUC-2 was depressed, whereas in *C. tyrobutyricum*–DSS-treated SCID (Fig. 5.2c) and BALB/c mice (Fig. 5.2f) MUC-2 secretion was preserved.

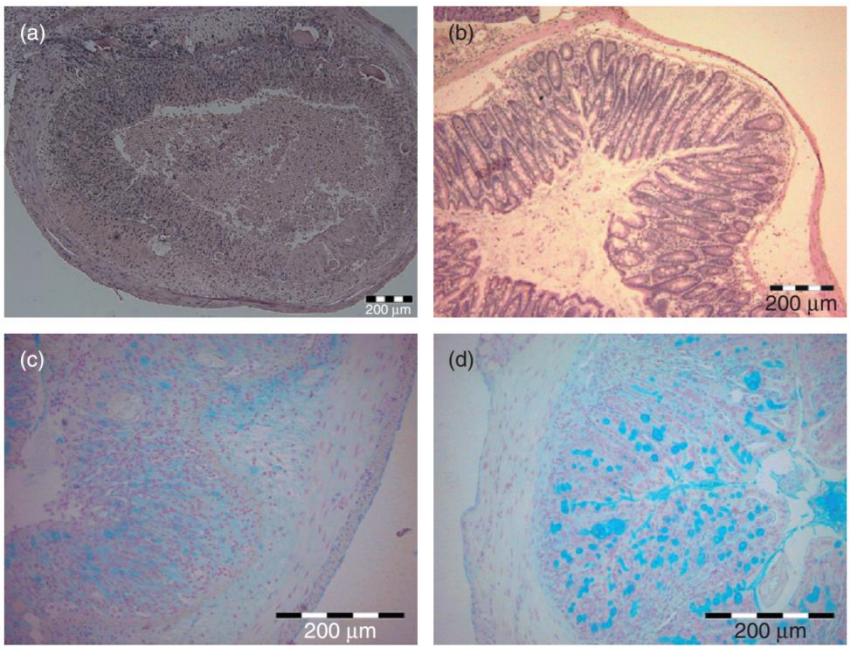

Fig. 5.1 Histological cross-sectional views of colon descendens of severe combined immunodeficiency (SCID) mice in experimental model of acute ulcerative colitis: (a) saline–dextran sodium sulphate (DSS)-treated mice (damage grade 4); (b) *C. tyrobutyricum*–DSS-treated mice (damage grade 0–1) (haematoxylin and eosin staining); (c) saline–DSS-treated mice, mucin production was decreased to grade 0; (d) *C. tyrobutyricum*–DSS-treated mice, grades 3–4, mucin production was preserved (Alcian blue staining).

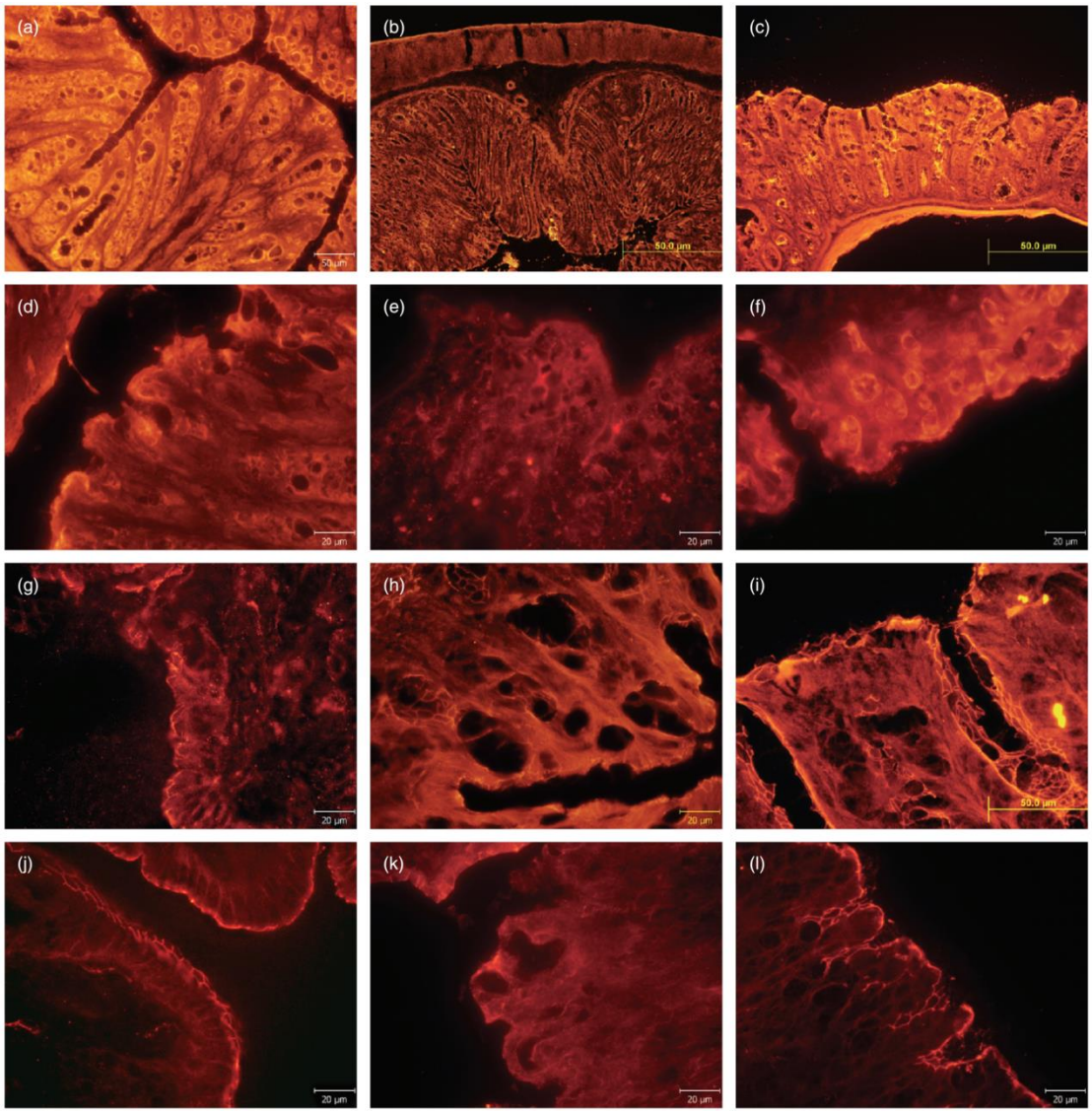

Fig. 5.2 Immunohistochemical evaluation of mucin (MUC)-2 detected with monoclonal antibody MUC-2/CY3 in colon descendens of severe combined immunodeficiency (SCID) and BALB/c mice in experimental model of acute ulcerative colitis: (a) saline-treated SCID mice with intact production of MUC-2; (b) saline–dextran sodium sulphate (DSS)-treated SCID mice with depressed production of MUC-2; (c) *C. tyrobutyricum*–DSS-treated SCID mice where MUC-2 secretion was preserved; (d) saline-treated BALB/c mice with intact production of MUC-2; (e) saline–DSS-treated BALB/c mice with depressed production of MUC-2; (f) *C. tyrobutyricum*–DSS-treated BALB/c mice MUC-2 secretion was preserved. Immunohistochemical evaluation of tight junction protein zonula occludens (ZO)-1 detected using monoclonal antibody ZO-1/CY3; (g) saline-treated SCID mice with intact production of ZO-1; (h) saline–DSS-treated SCID mice with markedly reduced ZO–1 production; (i) *C. tyrobutyricum*–DSS-treated SCID mice with preserved production of ZO-1; (j) saline-treated BALB/c mice with intact production of ZO-1, (k) saline–DSS-treated BALB/c mice with markedly reduced ZO-1 production; (l) *C. tyrobutyricum*–DSS-treated BALB/c mice with preserved production of ZO-1.

5.4.4. Expression of TJ protein ZO-1

Expression of TJ protein ZO-1 is presented in Fig. 5.2. In the saline-treated SCID (Fig. 5.2g) and BALB/c mice (Fig. 5.2j) ZO-1 production was intact. In saline–DSS-treated SCID (Fig. 5.2h) and BALB/c mice (Fig. 5.2k) the production of ZO-1 was markedly reduced, whereas in *C. tyrobutyricum*–DSS-treated SCID (Fig. 5.2i) and BALB/c mice (Fig. 5.2l) the production of ZO-1 was preserved.

5.4.5. Mucosal infiltration of CD11b-positive immune cells

Monoclonal antibody to CD11b, a membrane marker of polymorphonuclear leucocytes, monocytes and natural killer cells, was used (Fig. 5.3). Saline-treated SCID (Fig. 5.3a) and BALB/c mice (Fig. 3d) were without infiltration of immune cells. Massive infiltration of inflammatory cells in lamina propria occurred in saline–DSS-treated SCID (Fig. 5.3b) and BALB/c mice (Fig. 5.3e), while the colon of *C. tyrobutyricum*–DSS-treated SCID mice (Fig. 5.3c) and BALB/c mice (Fig. 5.3f) did not exhibit infiltration of these cells.

5.4.6. Release of proinflammtory cytokine TNF-α in colon organ cultures

Spontaneous release of TNF-α into the medium of cultured sections from colon descendens of control mice was very low (groups 3 and 6, Table 5.1) after 48 h. Colon segments from saline–DSS-treated mice, and especially the SCID mice, released markedly higher amounts of TNF-α (groups 2 and 5, Table 5.1) than the controls. Compared to the saline–DSS-treated SCID mice (group 5, Table 5.1), a significantly lower level of TNF-α was detected in the cultured colon segments from *C. tyrobutyricum*–DSS-treated SCID mice (group 4, Table 5.1). However, the level of TNF-α released from the colon segments from *C. tyrobutyricum*–DSS-treated BALB/c mice (group 1, Table

5.1) did not differ from that of saline–DSS-treated BALB/c mice (group 2, Table 5.1).

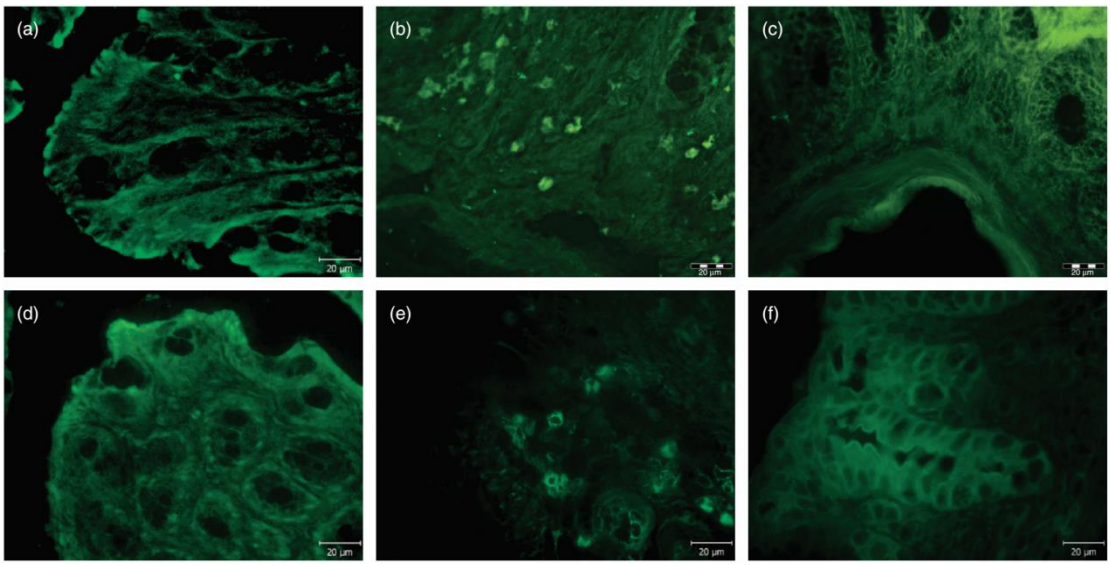

Fig5.3 Immunohistochemical detection of infiltrated proinflammatory cells using fluorescein isothiocyanate (FITC)-labelled monoclonal antibody to CD11b in colon-descendens mucosa of mice: (a) saline-treated severe combined immunodeficiency (SCID) mice without infiltration of immune cells; (b) saline–dextran sodium sulphate (DSS)-treated SCID mice with massive infiltration of proinflammatory cells; (c) *C. tyrobutyricum*–DSS-treated SCID mice, where infiltration of immune cells was not observed; (d) saline-treated BALB/c mice without infiltration of immune cells; (e) saline–DSS-treated BALB/c mice with massive infiltration of proinflammatory cells; (f) *C. tyrobutyricum*–DSS-treated BALB/c mice, where infiltration of immune cells was not observed.

5.4.7. Visualization and quantification of proinflammatory IL-18 in the descending colon

Table 2 shows that DSS-induced colitis in BALB/c mice is associated with increased level of cytokine IL-18 in colon epithelium. *C. tyrobutyricum* treatment reduced intracolonic IL-18 content significantly, although not to the level in non-inflamed mucosa. In SCID mice the severity of DSS colitis was not associated with IL-18 production. *C. tyrobutyricum* enhanced significantly the expression of IL-18 in colon

epithelium of SCID mice. This might be an effect of butyrate on gene and protein production of IL-18.

5.4.8. *C. tyrobutyricum*-increased levels of propionic and butyric acids in SCID mice

More than 95% of the SCFA are absorbed and metabolized rapidly by the host [24]. To see any effect of *C. tyrobutyricum* on SCFA production, we determined the percentage of main short-chain fatty acids (acetic, propionic, isobutyric, n-butyric, isovaleric, 2-methylbutyric and valeric) in bacterial culture and in faecal samples obtained from *C. tyrobutyricum*–DSS-treated SCID mice and saline–DSS-treated SCID mice at the end of the experiment using gas chromatography (Table 5.3). Threefold higher percentage of n-butyric acid and twofold higher percentages of propionic acid were measured in *C. tyrobutyricum*–DSS-treated SCID mice than in the saline–DSS-treated mice. Conversely, a lower percentage of acetic acid was found in the *C. tyrobutyricum*–DSS-treated than in the saline–DSS-treated mice.

5.5. Discussion

In the present study, intrarectal administration of *C. tyrobutyricum* prior to the onset of experimental colitis protected both immunocompetent BALB/c and immunodeficient SCID mice from histological damage, shortening of the colon and decreased mucin production.

Both BALB/c and SCID mice display comparable symptoms of DSS-induced colitis as judged by the histological damage score, colon shortening and weight loss. However, 20% mortality occurred in the DSS-treated SCID mice suggesting that parameters such as loss of body weight cannot be used as a measure of the severity of inflammation in these two mouse strains. This conclusion is supported by several previous studies [25–27] not showing an

apparent body weight loss before day 6 of acute DSS colitis in BALB/c mice. On the basis of the symptoms, the reduction of TJ protein ZO-1 production, depression of MUC-2 mucin production and infiltration of macrophages during inflammation, it appeared that the two mouse strains behave similarly on the qualitative level.

Table 5.3 Production of short-chain fatty acids determined as relative concentrations (%) in the bacterial broth of *C. tyrobutyricum* and in the faeces (taken at the end of the experiments).*Clostridium tyrobutyricum* dextran sodium sulphate (DSS)-treated and saline DSS-treated severe combined immunodeficiency (SCID) mice

	C. tyrobutyricum	*C. tyrobutyricum* (i.r.)	Saline (i.r.)
Acids	**(bacterial culture)**	**(DSS-treated)**	**(DSS-treated)**
Acetic	62·5	59·2	81·4
Propionic	17·9	18·0	7·8
Isobutyric	10·6	9·2	4·8
n-Butyric	9·0	13·6	2·8
Isovaleric	n.d.	n.d.	2·1
2-Methylbutyric	n.d.	n.d.	0·6
Valeric	n.d.	n.d.	0·5

i.r.: intrarectal administration; n.d.: not detected.

Decreased numbers of goblet cells and reduced levels of MUC-2 protein were measured in the inflamed colonic mucosa of DSS-treated BALB/c and SCID mice, a result similar to that observed in humans with active UC [28]. This is in agreement with other experimental models of inflammatory bowel diseases that demonstrate the critical role of MUC-2 in colonic protection [29]. Changes in mucins, particularly in MUC-2 mRNA, in experimental models are considered to contribute to the development of ulcerative colitis [10,30,31].

Recently it has been observed that DSS in drinking water rapidly affects the biophysical structure of the inner mucus layer, making it permeable to bacteria within 12 h [7]. Thus the increased contact of bacteria with the epithelium is probably the trigger for the inflammatory reactions observed in colitis and would explain why DSS does not induce colitis in germ-free mice. Similarly, loss of TJ protein ZO-1 in the intestinal epithelium, as observed in this study, was interpreted as an early event in DSS-induced colitis and is associated with increased permeability and intestinal inflammation [32].

Crucially important in DSS-colitis is an activation of T lymphocytes via macrophages that have been activated directly by DSS [8]. Indeed, direct evidence for the involvement of CD4+ T cells and their proliferation in pathogenesis of DSS-induced colitis has been described previously [33]. Moreover, the introduction of bacterial flagellin-specific CD4+ T cells into naive SCID mice (T and B cell-independent) results in severe colitis [34]. Similarly, transfer of CD4+CD45RBhigh T cell subpopulation from conventional mice into SCID mice also induces severe inflammation [5].

A major difference between BALB/c and SCID mice in development of DSS-colitis relates to the expression of the proinflammatory cytokine IL-18. In agreement with other published studies [35], we showed significantly increased IL-18 colon content in DSS-induced colitis in BALB/c mice. In BALB/c and C57BL/6 mice DSS-induced colitis induces increased expression of IL-18 in the colonic mucosa, where it polarizes CD4+ T cells toward T helper cell type 1 (Th1)-mediated immune response [35] and thus increased production of the proinflammatory cytokine interferon (IFN)-γ. IFN-γ stimulated secretion of IL-18 from enterocyte-like IEC-6 cells has been correlated with IFN-γ-increased expression of caspase-1 activity [36], an enzyme required for cleavage of the precursor form of IL-18 into mature biologically active IL-18. Indeed, inhibition of caspase-1 by the specific inhibitor pralnacasan [26] attenuated DSS-induced colitis, this effect being mediated by suppression of proinflamatory IL-18 and IFN-γ.

Recent observations highlight the role of Nod-like receptors (NLRs) when stimulated by inflammatory mediators to form inflammasomes, multi-protein complexes that serve for activation of caspase-1 essential for maturation and secretion of IL-18. The highest levels of IL-18 were localized in intestinal epithelial cells, then macrophages and dendritic cells of the lamina propria. Interestingly, deletion of macrophages or use of neutralizing antibodies to IL-18 or inhibition of caspase-1 [37] result in prevention of the inflammatory cascade leading to sustained infiltration of macrophages, neutrophils and activation of lamina propria effector T cells. Thus, it seems that inflammasome activation [38] would have a proinflammatory effect, with IL-18 inducing inflammation in lamina propria mononuclear cells. Conversely, in intestinal epithelial cells the inflammasome would have a compensatory proliferative response, the secreted IL-18 mediating protection, proliferation and cell integrity [27,39,40]. This study shows for the first time that in SCID mice (a T and B cell-independent model) the severity of colitis was associated with limited production of biologically active form of IL-18. It seems that only constitutively expressed precursor form of IL-18 is associated with normal and inflamed SCID mouse colon. This finding led us to the assumption that SCID mice lack some components of inflammasome, pro-IL-18 could not be activated, and display an increased susceptibility to DSS-induced colitis associated with increased lethality, especially in the chronic phase of inflammation [6]. In SCID mouse colon we detected increased surface antigen CD11b, associated with an increased number of infiltrating immune cells, possibly macrophages [4]. Lack of IL-18 secretion is compensated by increased secretion of inflammatory TNF-α from the colon found in organ cultures. In comparison with UC patients it was suggested that macrophages migrating into the inflamed mucosa [41,42] secrete high levels of TNF-α. We observed only moderate expression of TNF-α in BALB/c mice with induced inflammation which was not commensurate with the marked infiltration of CD11b-positive polymorphonuclear leucocytes and monocytes into the

mucosa. These mice represent a model of Th1-mediated immune response that contributes to the production of IL-18 and IFN-γ.

Studies of UC in humans have shown a lower availability and diminished capacity to oxidize butyrate [12,43], an energy source for colonocytes and end-product of the fermentation of undigested fibre and complex carbohydrates by the luminal microbiota. Similarly, a decrease in butyrate oxidation was found in the colonocytes of mice with DSS-induced colitis. Recent studies on faecal microflora of UC and IBS (irritable bowel syndrome) patients showed depletion of members of *Bacteroidetes* and *Firmicutes* (comprising some *Clostridium* groups) [44]. In healthy mice synergistic interactions between specific members of these phyla are linked to butyrate formation [45]. Thus, increased production of butyrate in the lumen of the colon was proposed as a treatment to ameliorate the symptoms of UC [46]. However, butyrate enemas in UC patients in remission were shown recently to have only mild effects on inflammation and anti-oxidant status in the colonic mucosa [47]. In our study we expected a higher faecal concentration of butyrate after intrarectal administration of *C. tyrobutyricum* and protection from DSS-induced colitis. In both BALB/c and SCID mice, intrarectal administration of *C. tyrobutyricum* prevented the reduction of MUC-2 protein observed in DSS-induced colitis and led to an almost normal level of MUC-2 secretion, most probably to the reported stimulation of MUC-2 gene expression in mouse colon [12]. We further showed that *C. tyrobutyricum* protects against impairment of the TJs in the colon of BALB/c and SCID mice by preventing dissolution of ZO-1 from the TJ in DSS-treated mice. Bacterial Toll-like receptor (TLR)-2 ligands have also been reported to increase ZO-1 expression and its localization in the TJs [15,48], and cannot be ruled out as a mechanism for the effects of *C. tyrobutyricum* on ZO-1.

Several *in vitro* and *ex vivo* studies reviewed by Hamer *et al*. [20] assessed the effect of butyrate which, at low concentrations, induces a decrease in permeability associated with increased expression of TJ proteins. However,

overproduction of butyrate might be toxic for maturation of the intestine in premature infants and also in newborn rats [49].

TNF-α at concentrations found in inflamed mucosa may reduce oxidation of butyrate and decrease energy supply to colonocytes, as shown *in vitro* in the mucosa of human colonic biopsies [50]. As intrarectal administration of *C. tyrobutyricum* increased production of butyrate in the colon lumen it could overcome any insufficiencies resulting from the increased production of TNF-α. *C. tyrobutyricum* can be considered as a candidate human probiotic due to its beneficial effects in mouse colitis model. In BALB/c mice the expression of TNF-α in inflamed colon was lower in comparison with TNF-α production in the inflamed colon of SCID mice. Additionally, we found that treatment with *C*. *tyrobutyricum* had no effect on regulation of TNF-α production in BALB/c, but a strongly attenuating effect on TNF-α production in SCID mice.

Our work on IL-18 expression in DSS-induced colitis and its regulation by *C. tyrobutyricum* helped to differentiate between distinct responsiveness of BALB/c and SCID mice. Remarkably, *C. tyrobutyricum*treatment significantly reduced intracolonic IL-18 protein content in the inflamed mucosa of BALB/c mice, although not down to the level in non-inflamed mucosa. In contrast to BALB/c mice, *C. tyrobutyricum* enhanced significantly the expression of IL-18 in colon epithelium of SCID mice which lacked inflammation-associated expression of IL-18. This could be due to the fact that butyrate enhances gene expression and protein production of IL-18 in epithelial cells (HT-29 and Caco-2) *in vitro*, and *in vivo* in butyrate-treated mice [51]. This enhanced effect of butyrate at the transcription level seems to be hidden in BALB/c mice expressing increased IL-18 production in DSS-treatment.

This study demonstrates that in the DSS model, the severity of inflammatory symptoms depends largely but not exclusively on host immune functions. Thus, *C. tyrobutyricum* protection against destruction of mucosal barrier is equally effective in immunodeficient SCID mice and immunocompetent

BALB/c mice. Manifestation of cytokines IL-18 and TNF-α in acute DSS-colitis depends largely on immune cell repertoire of the host mouse. As a typical product of macrophages, TNF-α expression increased significantly in the colon epithelium in SCID mice, while mature IL-18, a Th1 cytokine, important for systemic balance between Th1 and Th2 signalling [52], played a key role in immunocompetent BALB/c mice. The combined effect of *C. tyrobutyricum* in suppressing high levels of both cytokines appears promising in treatment of acute experimental colitis.

5.6. Acknowledgement

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6. GENERAL DISCUSSION

During the past decades, our understanding on the causative factors and development of IBD has been greatly promoted due to a dramatic rise in metagenomic and metabolomic studies of the diseases. Environmental factors, genetic predisposition, host immune response and gut microbiota have been widely accepted as the four main causes that result in the onset and perpetuation of IBD by interacting multiply with one another. For many years, scientists have been trying to track down any individual microbial pathogens that could be causative agents of IBD. So far no such microbe has been claimed to be responsible for triggering the disease. However in IBD, the gut resident bacteria are in the spotlight due to their continuous contact with epithelium (Salim et al., 2011) which leads to a series immune responses which may, to an intensive extent, promote intestinal inflammation.

A recent work done by Hoffmann et al. (2016) revealed that microorganisms had their specific impact on the host. The group of mono-colonized gnotobiotic mice with four IBD-associated microorganisms [*Bacteroides thetaiotaomicron*, adhesive-invasive *Escherichia coli* (AIEC), *Ruminococcus gnavus* and *Roseburia intestinalis*] were used to find out that *B. thetaiotaomicron* had the highest impact on host immune system due to its capacity of notably inducing Treg pathways; *E.coli* AIEC LF82 had an effect on indoleamine 2,3-dioxygenase expression and *R.gnavus* had influence to tryptophan metabolism. On the other hand, bacterial cocktail containing segmented filamentous bacteria and commensals (specific pathogen free mixture) was reported to be able to induce intestinal inflammation in conventional severe combined immunodeficiency (SCID) mice reconstituted with CD45RBhigh CD4+ T cells (Stepankova et al., 2007). These results demonstrated that cooperation between bacteria might be required to induce the inflammation in gut.

The studies carried out and discussed in this thesis provide novel information on potential mechanisms of IBD-associated bacteria damaging the integrity of the intestinal epithelium and increasing the susceptibility to colitis. Moreover, this thesis also reveals the efficacy of a selected probiotic strain *Clostridium tyrobutyricum* on colitis. Since the main points are already discussed in each individual chapter, hereby I will concentrate on role of mucosal immune system of the intestine in IBD and host-microbial interaction based on our results.

6.1. Mucosal immune system of the intestine in IBD

The mucosal immune system of the intestine is composed of various cells, including different subtypes of specialized intestinal epithelial cells which contain absorptive enterocytes, hormone-producing enteroendocrine cells, AMP-producing Paneth cells, mucus-producing Goblet cells and M cells (van der Flier LG and Clevers H, 2009; Noah et al., 2011) as well as immune cells such as neutrophils, monocytes, dendritic cells, B cells and T cells (Sanchez de Medina et al., 2014).

In healthy individuals, the function of epithelium is tightly regulated by the expression of transcription factors and epithelium itself is protected by mucus layers, AMP and the normal gut microbiota. However, in IBD patients, the main function of the intestine is digestion and absorption has been found to be compromised. Intestinal alkaline phosphatase (IAP), a brush border enzyme involved in mediating digestion and transporting nutrients in small intestine (Glodberg et al., 2008), was found to be decreased in inflamed mucosal tissue from IBD patients, which may result from increased bacterial translocation into the mucosa (Molnar et al., 2012).

Indeed, disruption of the integrity of the epithelium is one of the major issues in IBD patients, which leads to the elevated bacterial translocation into the mucosa (Lewis et al., 2010; Johansson et al., 2014). It is known that tight junctions which are involved in holding epithelial cells together are regulated

by myosin light-chain (MLC) phosphorylation by MLC kinase through actomyosin contraction. This contraction has a tendency to open the junctional gap (Cunningham and Turner, 2012). In consistent to the notion, expression and activity of epithelial MLC kinase were observed to be upregulated leading to a significant increase of MLC phosphorylation in colon biopsies from patients with active IBD (Blair et al., 2006), which consequently contributed to the dysfunction of epithelial barrier in the patients. Increased apoptosis of epithelial cells by proinflammatory cytokines TNF-α and IFN-γ is another contributor to the destruction of epithelium integrity (Marini et al., 2003; Nava et al., 2010). We described that an *E.coli* strain belongs to the phylogenetic group B2 which was able to disrupt the epithelium by a rapid loss of tight junction integrity through its hemolytic activity. As a matter of fact, *E.coli* has been long found to be able to impair the barrier function of epithelium by various secreted toxins. The cytotoxic necrotizing factor 1 (CNF1), a protein produced by pathogenic *E.coli* increased the permeability of epithelial cells monolayer by reorganization of the actin cytoskeleton (Falbo et al., 1993; Gerhard et al., 1998). The secreted autotransporter toxin (Sat) whose gene is highly prevalent in urinary tract infection (UTI)-associated *E. coli* was capable of increasing the paracellular permeability by rearrangement of tight junction-associated proteins ZO-1, ZO-3 and occludin (Guignot et al., 2007). By characterization of virulence genes of *E.coli* isolates from IBD patients, Sat was observed to be more prevalent in both CD and UC than CNF1 and hly (Veiborg et al., 2011).

As in healthy mucosa, intestinal epithelium is covered by two layers of mucus, the inner layer of which is supposed to be devoid of bacteria and be sterile. The separation between luminal microorganisms and epithelium by mucus protects epithelial cells from direct contact to any possible pathogens as well as commensal bacteria which may turn into pathobionts under particular circumstances driving the inflammation in gut. However, recent studies have

demonstrated that colon mucus in patients with active UC allowed bacteria to penetrate the inner layer of mucus and reach the epithelium (Johansson et al., 2014). This observation may be explained by the fact of reduced mucosal barrier and reduced number of goblet cells in IBD patients (Fyderek et al., 2009; Johansson et al., 2014). Moreover, containment of microorganisms and bacterial protein from direct contact with epithelium is also exerted by AMPs and sIgA. Thus, defective expression of AMPs and sIgA can greatly influence the mucosal barrier functions and mucosal homeostasis. Indeed, significant decrease in defensins expression was observed in CD patients, which facilitated the bacteria to breach the intestinal epithelium (Arijs et al., 2009; Wehkamp et al., 2005).

Moreover, increased exposure of luminal microbes to IECs enhances the possibility of activation of pro-inflammatory immune response by PRRs reacting to microbial components. Although virulent pathogens and innocent commensal bacteria both contain those conserved microbial motif like lipopolysaccharide (LPS), double stranded (ds) DNA, flagellin and so on, IECs seldomly respond to resident commensals in the gut by inflammation not only due to the intact function of the mucosal barrier which is severely compromised in IBD patients as discussed above but also due to the regulation of PRRs expression. Abreu et al. (2001) discovered that in the stable condition, the expression of TLR4 on the surface membrane of IECs was downregulated and MD-2, an important coreceptor for TLR4 signaling, was not present. This downregulation decreased the recognition of bacterial LPS.

Bacteria or bacterial antigens in intestinal lumen may activate lamina propria CD4+ αβ TCR T cell by three ways. They could enter the Peyer's Patch through microfold (M) cells, then transferred to local dendritic cells, to be presented directly to T cells sitting in the zone between follicles. Alternatively, antigens or antigen loaded dendritic cells may miss the recognition by T cell in

Peyer's Patch and gain access to draining lymph, being distributed to MLNs with subsequent recognized by T cells there. Antigens can also directly enter the MLNs through afferent lymphatics via epithelium covering the lamina propria. Antigen specific T cells go back to mucosa after leaving the MLNs through vessels in the lamina propria to stimulate B cell or innate immune cells resident in tissues to initiate inflammatory response (Mowat, 2003). In healthy individuals, these reactions are tightly regulated by our immune system. Because on one hand, we need the strong inflammatory response to clear those invading microorganisms, prevent them from doing any harm to our body. On the other hand, excessive inflammatory response can destruct bystander normal tissues leading to the persistence of inflammation contributing to the pathogenesis and progression of chronic inflammatory diseases (Lee and Surh, 2012). However, in IBD patients, the regulation of inflammatory response is impaired by many ways. First, antigen-activated T cells are reported to be resistant to apoptosis, the process of which is extremely important to reduce inflammation (Sturm et al., 2002); second, despite the enhanced recruitment of Treg cells in mucosa, elevated apoptosis of these cells was observed in both UC and CD patients so that their anti-inflammatory effect could be limited (Veltkamp et al., 2011); third, the resistance of T effector cells to the immunosuppressive effects of Treg cells also contributed to the development of a deleterious immune response (Fantini et al., 2009); and fourth, some studies showed functional defects of Treg cells which is associated with a mutation in the Foxp3 gene in IBD (Ueno et al., 2013; Okou et al., 2014).

Besides the deficiency in modulation of inflammatory response in IBD patients, decreased phagocytic activity of macrophages due to low level of expression of intracellular TNF was reported in CD patients by Smith et al. (2009). This impaired acute immune response could lead to insufficient bacterial clearance. Interestingly, other study exhibited macrophages produced abundant IL-6, IL-23 and TNF, and promoted the IFN-γ production by local mononuclear cells

(Kamada et al., 2008), which may contribute to the overall intensity of the inflammatory response. The controversial outcomes can be explained by the different sites of macrophage extraction. Macrophages used in Smith et al study were derived from PBMC rather than the gut mucosa.

Compared with normal function of mucosal immune system in healthy individuals, mucosal barrier function is severely compromised in IBD patients as discussed above. The causality between alteration of the normal microbiota in gut and abnormal immune response is still not fully understood and remains to be uncovered.

6.2. The relationship between host and bacteria in gut

It is well-known that the human gut is home to a wild range of highly diverse bacteria, collectively known as microbiota. Those bacteria are deeply involved in maintaining the normal function of IECs and keeping intestinal homeostasis. They not only powerfully influence the host energy balance but also have a great impact on immune regulation. Because intestinal microbiota communicates so closely and profoundly with the host and helps our body in many ways, it was often referred to as the “forgotten organ”.

> *“The rapidly progressing study of the human microbiota is revealing that humans are not individual self-contained beings, but instead hugely complex super-organisms that blur the distinction between where ‘we’ end and ‘they’ begin.” (Institute of Science in Society, 2013)*

Gut microbiota is established after birth and gradually reaches its maturity after 2-5 years (Palmer et al., 2007; Koenig et al., 2011). However, the component of the microbiota is not always stable throughout the life. Way of birth and weaning are the first two important time points that shape the gut microbiota structure. Besides these, an individual genotype, diet habit, lifestyle,

hygiene condition, medical treatment and ingestion of pathogenic and non-pathogenic microbes also affect the composition of gut microbial community (Zoetendal et al., 2001; Mountzouris et al., 2002).

One of the many obstacles which hindered our knowledge of intestinal bacteria was that there are more than 70% of uncultivable bacteria in vitro (Hooper and Gordon, 2001). Thanks to the development of culture-independent methods, how bacteria and which bacterial species interact with the host contributing to the physical health have been progressively revealed.

The importance of bacteria balance in the gut is emphasized by their implication both in aiding of our immune system in protecting us from harmful pathogens and in the etiology and development of many diseases such as diabetes (Cani et al., 2008), atopic dermatitis (Baker, 2006), colon cancer (Guarner and Malagelada, 2003), and cirrhosis (Garcia-Tsao 2004) possibly through weakening the host's immune system. How the bacteria affect the intestinal immune responses has been largely elucidated by comparing the immune responses of GF animals with those reared under conventional conditions (Table 6.1).

Due to the fact that gut microbiota is able to educate our immune cells and shape immune responses, it is reasonable to assume that the presence or absence of certain bacteria could result in diseases or be beneficial for maintaining human health, and be even further advantageous in the treatment of and recovery from inflammatory diseases.

6.3. Probiotics promote homeostasis of our immunity

Probiotics which are good for human health have been used in treating bowel disorders and helping to maintain a stable gut environment for a long time. But not until this century, have their potentially beneficial functions in controlling

inflammation gained so much attention without precedent in the history.

Table 6.1 Intestinal immunological defects in germ-free mice

Immunological defect	Site	Phenotype in germ-free mice compared with conventional housed-mice
Development of small intestine	Peyer's patches	Fewer and less cellular
	Lamina propria	Thinner and less cellular
	Germinal centers	Fewer plasma cells
	ILF	Smaller and less cellular
Development of MLN	Germinal centers	Smaller, less cellular and with fewer plasma cells
CD8+ T cells	Intestinal epithelium	Fewer cells and with reduced cytotoxicity
CD4+ T cells	Lamina propria	Fewer cells; decreased Th17 cells in the small intestine but increased Th17 cells in the colon
CD4+CD25+ T cells	MLN	Reduced expression of Foxp3 and reduced suppressive capacity
Expression of angiogenin 4	Paneth cells	Reduced
Expression of REG3γ	Paneth cells	Reduced
Production of SIgA	B cells	Reduced
Levels of ATP	Intestine	Reduced
Expression of MHC class II molecules	IECs	Reduced
Expression of TLR9	IECs	Reduced
Levels of IL-25	IECs	Reduced

Adopted from Round and Mazmanian (2009)

Probiotics usually exert their beneficial effect by initiating anti-inflammatory immune responses by induction of Treg cells activity, producing increased amount of IL-10. For example, *Bifidobacteria infantis, Lactobacillus rhamnosus* and *Faecalibacterium prausnitzii* have been evident their capacity of ameliorating gut inflammation through 1) increased number of CD4+CD25+

Treg cells; 2) induction of IL-10 expression; 3) down-regulation of TNF-α expression (O'Mahony et al., 2008; Foligne et al., 2007; Sokol et al., 2008; Rossi et al., 2015).

The large intestine the site of which contains the highest CFU of microbes through the whole gastrointestinal tract is well-known to be the place of enzymatic degradation of indigestible fiber by its microbiota that subsequently provides host SCFAs which exert a protective effect on epithelial cells (Scheppach, 1994). However, reduced production of SCFAs resulted from an alteration in the composition of intestinal microbiota has been frequently found in IBD patients (Frank et al., 2007; Morgan et al., 2012; Kang et al., 2010; Willing et al., 2009). We have found that the protective effect of a butyrate-producing strain in acute colitis. *Clostridium tyrobutyricum,* the bacterium we used in the experiment, decreased the production of the proinflammatory cytokines, TNF-α and IL-8, which were the key mediators in mounting pro-inflammatory responses. Additionally, this strain also restored normal MUC-2 and tight junction protein ZO-1 production in DSS model of UC. All together, it benefited experimental mice from reduction of clinical symptoms of DSS-colitis. The positive outcomes inevitably raised up a question if butyrate is the key bacterial molecule to provide this protective effect. Many studies have demonstrated a role of butyrate in modulating inflammation in IBD through several mechanisms, 1) inhibiting release of TNF-α, IL-13 and IL-8; 2) restoring the tight junction barrier by affecting the expression of claudin-2, occludin, ZO-1 and ZO-2; 3) rescuing the redox machinery and controlling the intracellular ROS balance (Russo et al., 2012; Weng et al., 2007; Lewis et al., 2010; Ploger et al., 2012). The results from our studies are highly consistent with what have already showed in the other studies. Higher amount of butyric acid found in DSS-treated mice further confirmed the power of the molecule on attenuating inflammation.

Bacteria help to maintain the immune homeostasis not only by direct

modulating immune responses but also by keeping the growth of bacterial population in balance through their antibiotic properties, such as producing bacteriocins (Boyer and Wisniewsk-Dye, 2009). Bacteriocins can be produced by many probiotics such as lactobacillus (Messaoudi et al., 2013) and Bifidobacterium species (Martinez et al., 2013). *L. salivarius* isolated from chicken intestine have antibacterial activity against *Salmonella* and *Campylobacter jejuni.* This antibiotic property was lost in *L. salivarius* derivative deficient in bacteriocin production (Messaoudi et al., 2013). Collectively, probiotics can modulate intestinal environment by directly functioning on mucosal immune system as well as regulating the composition of the microbiota.

6.4. The role of gnotobiotic models in the development of immunity and the pathogenesis of intestinal inflammation

Gnotobiotic animal models were employed to investigate the significance of IBD-patient derived mucosa-associated bacteria in the initiation of intestinal inflammation. Gnotobiotic animals which are reared in the GF conditions or specific pathogen free conditions have been widely used to elucidate the importance of microbiota in the development of immune system (Tlaskalova-Hogenova H et al., 1981; Cebra et al., 2005; Stepankova et al., 1998). Remarkable differences in immunological features have been revealed by comparing the immune responses of gnotobiotic animals with that of conventional animals as showed in table 6.1 suggesting the essential of the presence of microbiota for developing a complete and mature immune system. On the other hand, gnotobiotic models also contribute to the understanding of the pathogenesis of intestinal inflammation. Mice deficient in IL-2 or IL-10 were found to develop spontaneous intestinal inflammation in conventional condintions (Elson et al., 2005). However, the inflammation failed to develop

when mice were raised in GF conditions (Hudcovic et al., 2001) implying commensals might be involved in the triggering and perpetuating intestinal inflammation. Moreover, gnotobiotic models also provide us opportunities to exam the role of specific bacterial strain(s) in induction of host immune responses which may lead to inflammation by causing excessive immune reactions.

6.5. Future perspectives and conclusion

With the rapid advancements in technology, our understanding towards IBD is speeding up dramatically. Numerous factors in respect of genetics, environment, host immune response and gut microbiota have been revealed in the pathogenesis of the intestinal disorders. Despite the great breakthrough which has been made in uncovering the mechanisms of how these factors are invovled in the initiation and perpetuation of IBD individually, the knowledge of how they interact with each other interact in a multiple way and why it is so is still limited.

In this thesis, we mainly focus on the interaction between host mucosal immune response and IBD-associated bacteria or probiotics. In **Chapter 3**, we demonstrated mucosa-associated bacteria from patients with active UC were unable to induce spontaneous colitis in germ-free mice but increased the susceptibility to DSS-colitis suggesting incompletely developed immune system contributing to the deficiency in responding to bacteria. In **Chapter 4**, a novel mechanism of an IBD-associated *E.coli* damaging intestinal epithelium was discovered. This result brings us new insights on treatment of hemolytic *E.coli* associated intestinal inflammation. In **Chapter 5**, the protective effect of *C. tyrobutyricum* was elucidated suggesting a role of butyrate in mucosal immune response and in immune modulation.

Gut microbiota has a profound influence on our immune system such as educating our immune cells which bacteria to fight off and which to tolerate.

And in turn, our immune system also developed a number of mechanisms to affect the function and the structure of microbial community in gut (Reinoso Webb et al., 2016). It is well-known that our immune reactions have a genetic basis. Hundreds of susceptibility loci have been linked to immune-mediated diseases. On the other hand, genetics could also influence the composition of microbiota evidently indicated by the similarity in the intestinal microbiota between monozygotic twins and between dizygotic twins (Goodrich et al., 2014). Consider the complexity of the pathogenesis of IBD, the interdisciplinary studies between genetics, genomics, immunology and microbiology are the future approaches to advance our understanding of the pathogenesis of IBD. Therefore, in the furture, we will focus on interactions between host immune system of animal models and bacteria isolated from inflamed sites and non-inflamed sites of paitents with active IBD as well as identification and characterization of the microbiota from these patients. The investigation will help to elucidate the mechanisms by which these pathogentical factors interplay with each other.

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Appendix A – List of Abbreviations

AMP - antimicrobial peptide

AIEC – adherent and invasive *E.coli*

aHMA - mice associated with human microbiota from biopsy a

bHMA - mice associated with human microbiota from biopsy b

cHMA - mice associated with human microbiota from biopsy c

Caco-2 cells - heterogeneous human epithelial colorectal adenocarcinoma cells

CCS - clinical colitis score

CD - Crohn's disease

CFU - colony forming unit

Cnf1 - cytotoxic necrotizing factor type 1

CV - conventional (i.e. colonized with normal commensal microbiota)

DGGE - denaturing gradient gel electrophoresis

DSS - dextran sulfate sodium

DMEM - Dulbecco's modified Eagle medium

E. coli - Escherichia coli

ELISA - enzyme-linked immunosorbent assay

ExPEC - Extra Pathogenic E. coli

FACS - fluorenscence activated cell sorter

F1/3/4 - 1st, 3rd and 4th filial generation

FISH - fluorescence in situ hybridization

FID - flame ionization detector

FITC - fluorescein isothiocyanate

GF - germ-free

GWAS - genome-wild association studies

HMA - human microbiota-associated

Hly - hemolysin

IBD - inflammatory bowel diseases

IEC – intestinal epithelial cell

IFN-γ - interferon-γ

IL-10 - interleukin-10

ILF - isolated lymphoid follicles

LB - Luria broth

LPS - lipopolysccharide

MHC - major histocompatibility complex

MLC - myosin light-chain

MLN - mesenteric lymph node

MOI - multiplicity of infection

MPO - myeloperoxidase

NK - natural killer

NLR - Nod-like receptor

NOD - nucleotide-binding oligomerization domain

OD - optical density

OTUs - operational taxonomic units

PBMC - peripheral blood mononuclear cell

PBS - phosphate buffered saline

PCR - polymerase chain reaction

RBC - red blood cell

PVDF - polyvinylidene fluoride

Sat - secreted autotransporter toxin

SCFA - short chain fatty acid

SCID - severe combined immunodeficiency

SPF - specific pathogen free

TER – transepithelial electric resistance

TGF – transforming growth factor

TLR - Toll-like receptor

TNF-α - tumor necrosis factor-α

TJ - tight junction

Treg - T regulatory cell

UC - ulcerative colitis

UCI - inactive Ulcerative Colitis

UCA - active Ulcerative Colitis

UTI - urinary tract infection

ZO - zonula occluden

Δ - knockout

Appendix B – Curriculum Vitae

Born: 15, 12, 1983, Shanghai, China

Eduation and Employment:

- 9.2002 - 6. 2006: Chemistry and Pharmaceutics, East China University of Sciences and Technology (ECUST), Shanghai, China - Bachelor of Science

- 9. 2006 - 10. 2008: Medical Pharmaceutical Sciences, Rijksuniversiteit Groningen (RuG), the Netherlands - Master of Science

- 5. 2009 - NOW: Marie Curie ITN Project, Immunology and Gnotobiology, Institute of Microbiology / Charles University, Czech Republic; Host Microbe Interactomics group, Wageningen University, The Netherlands

AWARDS and HONORS:

Excellent-student academic scholarship – ECUST, 2003

Outstanding student leadership honour – ECUST, 2004

Publications:

Total: 3

Sum of citations: 7

H-index: 1 (WoS)

APPENDIX C – List of Publications

Du Z, Hudcovic T, Mrazek J, Kozakova H, Srutkova D, Schwarzer M, Tlaskalova-Hogenova H, Kostovcik M, Kverka M. *Development of gut inflammation in mice colonized with mucosa-associated bacteria from patients with ulcerative colitis.* Gut Pathog. 2015 Dec 21;7:32. doi:10.1186/s13099-015-0080-2. eCollection 2015

Mirsepasi-Lauridsen HC*, **Du Z***, Struve C, Charbon G, Karczewski J, Angeliki Krogfelt K, Petersen AM , Wells JM. *Secretion of alpha-hemolysin by Escherichia coli disrupts tight junctions in ulcerative colitis patients*. Clin Transl Gastroenterol. 2016 Mar 3;7:e149. doi: 10.1038/ctg.2016.3. (* equally contributed)

Hudcovic T, Kolinska J, Klepetar J, Stepankova R, Rezanka T, Srutkova D, Schwarzer M, Erban V**, Du Z**, Wells JM, Hrncir T, Tlaskalova-Hogenova H, Kozakova H. *Protective effect of Clostridium tyrobutyricum in acute dextran sodium sulphate-induced colitis: differential regulation of tumour necrosis factor-α and interleukin-18 in BALB/c and severe combined immunodeficiency mice*. Clin Exp Immunol. 2012 Feb;167(2):356-65. doi: 10.1111/j.1365-2249.2011.04498.x.