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Treatment strategies for intestinal epithelial barrier dysfunction in inflammatory bowel disease

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List of Abbreviations

5-ASA	5-aminosalicylic acid
ACHP	2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3-pyridinecarbonitrile
AIEC	adherent-invasive *Escherichia coli*
AJ	adherens junction
ALPI	alkaline phosphatase, intestinal
ANPEP	alanyl aminopeptidase, membrane
APC	antigen-presenting cell
B. pullicaecorum/BP	*Butyricicoccus pullicaecorum*
BSA	bovine serum albumin
CD	Crohn's disease
CFTR	cystic fibrosis transmembrane conductance regulator
CLDN	claudin
CXCL2	chemokine (C-X-C motif) ligand 2
DC	dendritic cell
DDP4	dipeptidyl peptidase 4
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DSS	dextran sulfate sodium
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FMT	fecal microbiota transplantation
F. prausnitzii	*Faecalibacterium prausnitzii*
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HDACi	histone deacetylase inhibitor
HMBS	hydroxymethyl-bilane synthase
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IFNγ	interferon gamma
i.g.	intragastrically
IL	interleukin
JAM	junctional adhesion molecule
KC/CXCL1	chemokine (C-X-C motif) ligand 1
LCT	lactase
LDH	lactate dehydrogenase
LIMK	Lin11, Isl1 and Mec3 kinase
LPMC	lamina propria mononuclear cell
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase

MCP1	monocyte chemoattractant protein 1
MCT1	monocarboxylate transporter 1
MGAM	maltase-glucoamylase
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MPO	myeloperoxidase
MS4A12	membrane spanning 4-domains A12
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaB	sodium butyrate
NF-κB	nuclear factor κB
NRQs	normalised relative quantities
OCLN	occludin
OPLS	orthogonal partial least squares
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCA	principle component analysis
PDZ	PSD95, Dlg and ZO-1
PG	propylene glycol
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
qRT-PCR	quantitative real-time PCR
ROCK	Rho-associated protein kinase
SCFA	short-chain fatty acid
SDHA	succinate dehydrogenase complex A subunit
SEM	standard error of the mean
SFB	segmented filamentous bacteria
SI	sucrase-isomaltase
TBST	Tris buffered saline with 0.1% Tween-20
TEER	transepithelial electrical resistance
TEM	transmission electron microscopy
TGFβ	transforming growth factor beta
TJ	tight junction
TJP1	tight junction protein 1
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
Treg	regulatory T cell
TUNEL	terminal deoxynucleotidyl transferase dUTP nick-end labeling
UC	ulcerative colitis
VIP	variable importance for the projection
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
ZO	zonula occludens

Summary

Inflammatory bowel disease (IBD) is a chronic, relapsing inflammatory condition of the gastro-intestinal tract comprising two subtypes; Crohn's disease and ulcerative colitis (UC). The aetiology of IBD is not completely understood but the general consensus is that it is a multifactorial disease in which an inappropriate immune response is mounted in genetically susceptible individuals as a result of a complex interplay between unknown environmental triggers, the gut microbiome, and the intestinal immune system. A major hallmark of IBD is increased intestinal permeability caused mainly by tight junction (TJ) deregulation and intestinal epithelial cell (IEC) apoptosis. Directly targeting epithelial dysfunction in IBD would restore intestinal barrier integrity, which forms the structural basis of mucosal healing; a key treatment goal in IBD management that predicts sustained clinical remission and resection-free survival of patients.

In this thesis, we investigated the therapeutic potential of intervening in two pathways directly involved in maintenance and regulation of barrier integrity; increasing colonic butyrate levels through oral administration of *Butyricicoccus pullicaecorum* (*B. pullicaecorum*) 25-3^T, and Rho-associated kinase (ROCK) inhibition using an inhibitor that was specifically designed to have a localised action in the gut.

In the first part of this thesis (**chapter III**), we investigated the barrier-protective properties of *B. pullicaecorum* 25-3^T; a potentially probiotic bacterial strain. This anaerobic butyrate-producer belongs to the clostridial cluster IV, a bacterial community that is particularly depleted in the dysbiotic IBD microbiota. Given the severe butyrate deficiency in IBD patients and the well-known barrier-protective properties of butyrate, *B. pullicaecorum* 25-3^T could be used as a pharmabiotic to promote mucosal healing in IBD. First, *Butyricicoccus* was found to be mucosa-adherent and reduced in UC patients with active disease compared to healthy controls. Next, we found that butyrate-responsive TJ gene expression was severely deregulated in inflamed colonic mucosal biopsies collected from UC patients, in particular claudin-1 (CLDN1) whose mRNA levels were, counter-intuitively, increased. These elevated *CLDN1* levels correlated with a reduced *Butyricicoccus* activity in the colonic mucosa. Administration of butyrate to IEC monolayers of differentiated epithelial Caco-2 cells decreased tumor necrosis factor (TNF)/interferon gamma (IFNγ)-induced CLDN1 levels, which coincided with a restoration of barrier integrity. Interestingly, the conditioned medium of *B. pullicaecorum* 25-3^T reduced *CLDN1* levels in inflamed colonic mucosal biopsies from UC patients.

In the second part of this thesis (**chapter IV**), we investigated the effect of ROCK inhibition on intestinal epithelial barrier dysfunction. The involvement of ROCK in inflammation-induced loss of

barrier integrity and the elevated ROCK activity observed in mucosa of patients with active IBD, has prompted research into the use of ROCK inhibitors to counteract epithelial dysfunction in IBD. In this thesis, we evaluated a locally acting ROCK inhibitor, AMA0825, which does not display the adverse effects of current inhibitors due to its degradation upon systemic exposure. Despite its efficient inhibition of myosin light chain phosphorylation, AMA0825 did not affect TNF/IFNγ-induced loss of barrier integrity or nuclear factor-κB activation in intestinal epithelial cells. AMA0825 did not attenuate the occurrence of epithelial erosions or the associated mucosal inflammation during experimental colitis. Finally, AMA0825 did not prevent TNF/IFNγ-induced epithelial caspase-activation, nor *in vivo* lipopolysaccharide-mediated enterocyte apoptosis or the ensuing intestinal permeability and mucosal pro-inflammatory response. Research from our lab demonstrated that AMA0825 prevents and resolves intestinal fibrosis via effects that combine a reduction in fibroblast activation with an increase in the autophagic response in these cells.

In a the third and final part of this thesis (**chapter V**), we sought to further define the differences between two colonic epithelial cell lines; T84 and Caco-2. Both differentiate spontaneously at confluence into a monolayer of structurally and functionally mature absorptive epithelial cells and are used interchangeably as model systems for human intestinal epithelium, despite evidence suggesting that differentiated Caco-2 cells closely resemble small intestinal enterocytes, while mature T84 cells have been less well described. We demonstrated that differentiated T84 monolayers have - in contrast to differentiated Caco-2 monolayers - shorter microvilli, a lack of brush border-associated enzyme expression, exhibit high levels of colon-specific markers and show a dose-responsive improvement of barrier function towards butyrate. This led us to conclude that differentiated T84 cells do not acquire the signature of mature small intestinal enterocytes like Caco-2 cells, but retain much of their original colonic characteristics throughout differentiation.

Samenvatting

Inflammatoir darmlijden (inflammatory bowel disease, IBD) is een chronische, terugkerende ontsteking van het gastro-intestinaal stelsel met twee subtypes; de ziekte van Crohn en colitis ulcerosa (ulcerative colitis, UC). De etiologie van IBD is niet volledig gekend maar de algemeen aanvaarde definitie is dat het een multifactoriële ziekte is, waarbij een overdreven immuunrespons wordt opgewekt in individuen met een genetische aanleg die het resultaat is van een complexe interactie tussen ongekende omgevingsfactoren, de darmflora, en het intestinale immuunsysteem. Een belangrijk kenmerk van IBD is een verhoogde intestinale permeabiliteit die voornamelijk veroorzaakt wordt door een ontregeling van de zonula occludens (tight junctions, TJ) structuur en door apoptose van intestinale epitheelcellen (IEC). Het direct remediëren van epitheel-dysfunctie in IBD zou het herstel van de intestinale barrière bewerkstelligen, hetgeen de structurele basis vormt voor mucosale heling dat een belangrijk einddoel is in de behandeling van IBD. Mucosale heling voorspelt nl. langdurige klinische remissie van de ziekte en een verminderde nood aan chirurgisch ingrijpen.

Tijdens deze thesis onderzochten we het therapeutisch potentieel van de interventie in twee pathways die rechtstreeks betrokken zijn bij behoud en regulatie van barrière-integriteit; het verhogen van de butyraat-niveaus in het colon door orale toediening van *Butyricicoccus pullicaecorum* (*B. pullicaecorum*) 25-3^T, en inhibitie van Rho-geassocieerde kinasen (ROCK) d.m.v. een inhibitor die specifiek ontwikkeld werd voor een lokale werking in de darm.

In het eerste deel van deze thesis (**hoofdstuk III**) onderzochten we de barrière-protectieve eigenschappen van *B. pullicaecorum* 25-3^T; een potentieel probiotische bacterie. Deze anaerobe boterzuur-produceerder behoort tot de *Clostridium* cluster IV, een bacteriële groep die sterk gereduceerd is in de dysbiotische IBD microbiota. Gezien de uitgesproken boterzuur-deficiëntie in IBD patiënten en de gekende barrière-protectieve eigenschappen van boterzuur, lijkt *B. pullicaecorum* 25-3^T interessant voor gebruik als een zgn. pharmabioticum ter bevordering van mucosale heling in IBD. Ten eerste konden wij aantonen dat *Butyricicoccus* mucosa-adherent was en gereduceerd in patiënten met actieve UC in vergelijking met gezonde controles. Vervolgens demonstreerden we dat boterzuur-responsieve TJ genexpressie sterk ontregeld was in geïnflammeerde mucosale colonbiopten van UC patiënten, in het bijzonder claudine-1 (CLDN1) waarvan de mRNA niveaus verhoogd waren; een tegenstrijdig gegeven gezien de verstoorde barrière-integriteit in IBD. Deze verhoogde *CLDN1* niveaus correleerden met een verlaagde *Butyricicoccus* activiteit in de colon-mucosa. Toediening van boterzuur aan IEC monolagen van gedifferentieerde epitheliale Caco-2 cellen verlaagde de tumor necrosis factor (TNF)/interferon

gamma (IFNγ)-geïnduceerde CLDN1 niveaus, hetgeen samenviel met een herstel van de barrière-integriteit. Interessant was dat het geconditioneerd medium van *B. pullicaecorum* 25-3^T de *CLDN1* niveaus in geïnflammeerde mucosale colonbiopten van UC patiënten deed dalen.

In het tweede deel van deze thesis (**hoofdstuk IV**) onderzochten we het effect van ROCK inhibitie op intestinale epitheliale barrière-dysfunctie. De betrokkenheid van ROCK's bij inflammatie-geïnduceerde intestinale permeabiliteit en de verhoogde ROCK activiteit in de mucosa van patiënten met actieve IBD, heeft het onderzoek gestimuleerd naar het gebruik van ROCK inhibitoren ter behandeling van epitheel-dysfunctie in IBD. In deze thesis evalueerden wij een lokaal werkende ROCK inhibitor, AMA0825, die niet de neveneffecten vertoont van de huidige ROCK inhibitoren wegens zijn degradatie bij systemische blootstelling. Ondanks zijn efficiënte inhibitie van myosin light chain fosforylatie, had AMA0825 geen effect op TNF/IFNγ-geïnduceerd verlies van barrière-integriteit of nuclear factor-κB activatie in intestinale epitheelcellen. Behandeling met AMA0825 gaf geen vermindering van epitheliale erosies of de geassocieerde mucosale inflammatie tijdens experimentele colitis. Tot slot kon AMA0825 de TNF/IFNγ-geïnduceerde epitheliale caspase-activatie niet verhinderen, noch de *in vivo* lipopolysaccharide-gemedieerde enterocyt apoptose of de daaruit volgende intestinale permeabiliteit en mucosale pro-inflammatoire respons. Onderzoek uit ons lab heeft aangetoond dat AMA0825 de ontwikkeling van intestinale fibrose kan verhinderen en omkeren door een combinatie van zowel een reductie van fibroblast-activatie als een verhoging van de autofagische respons in deze cellen.

In het derde en laatste deel van deze thesis (**hoofdstuk V**) hebben we getracht de verschillen tussen twee colonepitheel cellijnen, T84 en Caco-2, verder te definiëren. Deze cellijnen differentiëren spontaan na confluentie tot een monolaag van structureel en functioneel mature, absorptieve IEC'en en worden beide gebruikt als model systemen voor humaan intestinaal epitheel ondanks het feit dat gedifferentieerde Caco-2 cellen eerder lijken op dunne darm enterocyten, terwijl mature T84 cellen minder goed gekarakteriseerd zijn. We konden aantonen dat gedifferentieerde T84 monolagen - in tegenstelling tot gedifferentieerde Caco-2 monolagen - volgende kenmerken hebben: kortere microvilli, een gebrek aan brush border-geassocieerde enzym expressie, hoge concentraties colon-specifieke merkers en een dosis-afhankelijke verbetering van de barrière functie na stimulatie met boterzuur. Hieruit concludeerden we dat gedifferentieerde T84 cellen niet de kenmerken verkrijgen van dunne darm enterocyten zoals Caco-2 cellen, maar dat zij hun originele colon-karakter behouden tijdens differentiatie.

CHAPTER I

GENERAL INTRODUCTION

1.1 INTRODUCTION TO INFLAMMATORY BOWEL DISEASE

In the first part of this introduction, we will focus on the pathology of inflammatory bowel disease (IBD). We will elaborate on the clinical and pathophysiological differences between its subtypes Crohn's disease and ulcerative colitis, its etiology and risk factors, and its clinical management. We will also touch upon future treatment options to address the unmet needs in current disease therapy, in particular those related to our research topic; restoration of intestinal epithelial barrier integrity through butyrate-producing bacteria and Rho kinase inhibition.

1.1.1 Crohn's disease versus ulcerative colitis

Inflammatory bowel disease is the common denominator used to describe two related, chronic and relapsing inflammatory afflictions of the gastro-intestinal tract, i.e. Crohn's disease (CD) and ulcerative colitis (UC). Inflammatory bowel disease occurs worldwide but the incidence and prevalence are the highest in western, developed countries.[1] The main clinical symptoms of IBD are abdominal pain, fever, weight loss and bloody diarrhea.[2] Although both forms of IBD share many similarities, several important clinical and pathophysiological differences do exist. CD can manifest itself along the entire length of the gastro-intestinal tract but the terminal ileum and colon are the most frequently affected areas. UC on the other hand, occurs exclusively in the colon with the occasional case of backwash ileitis in patients with pancolitis. Disease complications of IBD include fistulae and obstruction due to stenosis in CD, and perforation and toxic megacolon in UC.[3] In up to 40% of IBD patients, extra-intestinal disease manifestations in the joints, eyes and skin also occur.[4] On endoscopy, CD presents itself as a patchy inflammation where multiple affected bowel segments are interspersed with unaffected "skip" areas, whereas UC is characterised by a continuous colonic involvement. Histologically, inflammation in CD and UC is characterised by ulceration, infiltration of acute (granulocytes, macrophages) and chronic (lymphocytes, plasma cells) inflammatory cells, and architectural distortions. In UC, inflammation is limited to the mucosal layers and ulcers are superficial, while in CD, inflammation is transmural with granulomas and deep, linear ulcers.[5]

1.1.2 Etiology and risk factors

The etiology of IBD is not completely understood but the general consensus is that it is a multifactorial disease in which an inappropriate immune response is mounted in genetically susceptible individuals as a result of the complex interaction between unknown environmental triggers, the gut microbiome, and the intestinal immune system (Figure 1).[6,7]

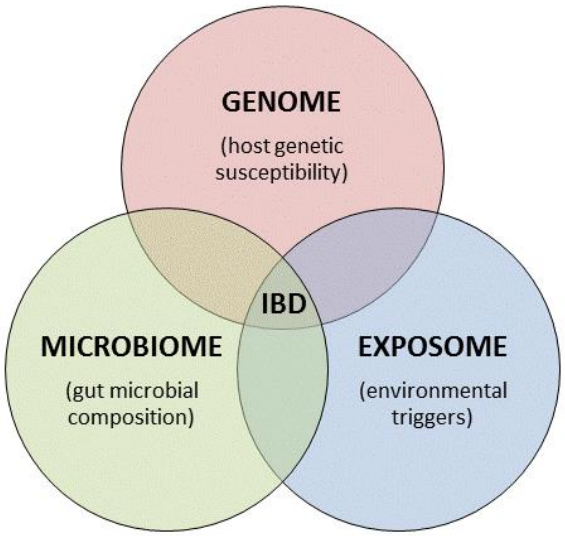


Figure 1. Etiopathogenesis of IBD. *IBD is a multifactorial disease in which an inappropriate intestinal immune response is mounted as a result of the complex interplay between the gut microbiota ("microbiome"), unknown environmental triggers collectively termed the "exposome" (e.g. diet, smoking, drugs, etc.), and the genome of the susceptible host (IBD loci correlating with genes involved in innate immunity, barrier function, etc.).*

Evidence for a genetic contribution to IBD pathogenesis is well-established; 15% of IBD patients have a first-degree relative who also has IBD, and twin studies revealed a 20 to 50% concordance rate for CD in monozygotic twins and a concordance rate of less than 10% in dizygotic twins. The evidence from twin studies in UC showed a concordance rate of 16% and 4% in monozygotic and dizygotic twins, respectively. Collectively, these data suggest an important hereditary component to disease expression, which is stronger in CD than in UC.[8] Genetic association studies have identified over 200 IBD susceptibility loci so far; a meta-analysis combining genome-wide association studies and Immunochip data identified 163 loci associated with IBD, and a more recent trans-ethnic analysis identified an additional 38 new IBD loci.[9,10] Of those 163 loci identified in the first study, 110 conferred risk to both IBD subtypes, whereas 30 and 23 loci were unique to CD and UC, respectively. IBD susceptibility loci share a significant overlap with other immune-mediated disorders (e.g. psoriasis) and, interestingly, also with primary immunodeficiencies like Mendelian susceptibility to mycobacterial disease, which is characterised by a predisposition to clinical disease caused by weakly virulent mycobacteria.[9] Indeed, a substantial amount of IBD loci correlate with genes involved in bacterial response. A textbook example of genetic association in IBD is nucleotide-binding oligomerisation domain-containing protein 2 (*NOD2*), a gene encoding an intracellular pattern recognition receptor for bacterial muramyl dipeptide and the first ever CD susceptibility gene to be discovered.[11] However, the total disease variance explained by these 163 loci amounts to 13.6% in CD and 7.5% in UC.[9] Combined with the IBD concordance rate in identical twins of maximum 50%, these data underscore the importance of the contribution of external factors to IBD pathogenesis.

In the second half of the 20th century, IBD incidence started to rise in Northern America and Europe. Recent data indicate that the incidence rate in these countries may have reached a plateau, whereas incidence has started to increase in newly developing countries in Asia and South America.[12] This distinct geographical distribution pattern of IBD prevalence gave rise to the so-called "hygiene hypothesis", which postulates that the growing frequency in immunological disorders like IBD could be partially attributable to a lack of exposure to infectious agents during early childhood, as is the case in urban areas of industrialised countries, thus impairing the development of immune tolerance to microbial antigens.[13] An array of other environmental risk factors characteristic of a western lifestyle have also been identified as possible contributors to the onset and subsequent flare-ups of IBD, and are collectively termed the "exposome". Examples include dietary factors, air and water pollution, food additives (e.g. aluminium, TiO_2), lifestyle factors (e.g. smoking), stress, drug use (oral contraceptives, non-steroidal anti-inflammatory drugs and antibiotics), infections and gut microbial composition.[14] Unfortunately, population-based studies supporting the involvement of these factors in disease expression often yield inconsistent findings, making causal relationships difficult to establish. The most intensively researched external factor to date is the gut microbiome. There is solid evidence that its composition, which is modified to a great extent by dietary intake, is severely altered in IBD patients, which could modulate immunological responses and will be discussed in more detail in §1.2 of this introduction.

1.1.3 Unmet needs in current management of IBD

1.1.3.1 Current approach to managing active IBD

Given the lack of an obvious causative agent for IBD, the main goal of current IBD management is the induction of remission of clinical symptoms, including cessation of rectal bleeding, normalisation of stool frequency and consistency, absence of abdominal pain and improved general wellbeing. However, evidence suggests that, even during periods of clinical remission, persistent inflammation can be seen, as demonstrated by elevation of serum and fecal biomarkers and/or endoscopic signs of mucosal inflammation. Persistent inflammation is believed to lead to progressive bowel damage, higher relapse risk and complications.[15] Achieving deep remission, encompassing besides the traditional clinical improvement also more objective parameters of inflammation control, such as mucosal healing on endoscopy, histological remission and biomarker normalisation, may therefore greatly improve disease outcomes.[16] Mucosal healing in particular has emerged as a key treatment goal in IBD, with several studies suggesting that the attainment of mucosal healing predicts lower hospitalisation rates, sustained clinical remission and resection-free survival of patients.[17-22]

The conventional strategy for inducing remission in IBD consists of a step-up approach in which corticosteroids are used, alone or in combination with aminosalicylates, as the first treatment option.

In case of a lack of improvement, medication is switched to - in sequence - immunosuppressant drug therapy (e.g. mercaptopurine, azathioprine, cyclosporine), biologicals against tumor necrosis factor (TNF) (e.g. infliximab, adalimumab, certolizumab) or integrin homing receptors (e.g. vedolizumab), and surgical resection of the afflicted bowel segment as the final treatment option. The focus of subsequent maintenance therapy is the withdrawal of corticosteroids and the prevention of symptom recurrence. Several of the drugs described above have been shown to induce mucosal healing but there are substantial differences between CD and UC in terms of the effect of different drugs on mucosal healing. For example, azathioprine and anti-TNF therapy promote mucosal healing in both CD and UC, whereas corticosteroids are able to induce mucosal healing in subsets of patients with UC but have little or no positive effect on the induction of mucosal healing in CD patients.[23-27]

1.1.3.2 Future directions in IBD therapy

Despite available treatment modalities, complete and sustained remission is not guaranteed, even for the most advanced therapies like anti-TNF biologicals. The most important clinical issues with current therapeutics include a lack of response (primary non-response and secondary loss of response), systemic adverse effects related to dose and duration of therapy, opportunistic infections due to the immunosuppressive nature of most drugs, infusion/injection reactions and antibody formation in the case of biologicals, and long-term complications like fibrostenosis.[28-32] The need for a more effective disease control is evident from this, but next-generation IBD therapy is still largely focused on antibody-mediated blocking of effector immune responses.

However, identification of microbial dysbiosis and impaired barrier function as major hallmarks of IBD, have opened up novel therapeutic approaches that do not involve the traditional immunosuppressive mode of action. Targeting these primary defects instead of the underlying inflammatory processes would not only bypass the adverse effects associated with immune suppression but would also directly promote mucosal healing, leading to better clinical outcomes.

In the next three paragraphs, a brief overview will be given of the future directions in IBD management currently under investigation; immunomodulatory strategies in clinical development, microbiota modulation, and identification of targets for mucosal healing that are directly involved in the restoration of epithelial barrier integrity.

1.1.3.2.1 Immunomodulatory strategies

In the following section, a short overview will be given of the next-generation therapeutic strategies focussing on abrogating inflammatory immune responses that are currently being evaluated in clinical trials, as reviewed by D'Haens *et al.*[33]

The introduction of TNF-neutralising agents was a breakthrough in IBD management but primary non-response and secondary loss of response to anti-TNF antibody therapy is common and presents a major clinical problem.[34] Other means of blocking TNF are therefore being investigated, including anti-TNF vaccination, TNF gene silencing and TNF-neutralising nanobodies. For example, vaccination of mice transgenic for human TNF (hTNF) (TTg mice) with a hTNF-kinoid vaccine elicited high titers of antibodies that neutralised hTNF bioactivities but did not result in a cellular response to hTNF. The vaccine was found to be safe and effective in two experimental models of hTNF exposure: immunised TTg mice resisted acute hTNF-driven lethal shock, and were protected from development of chronic, spontaneous arthritis.[35] The safety, immune responses, and clinical effects of active immunisation against TNF using the hTNF-kinoid vaccine has been evaluated in a phase II trial in CD patients.[36] Immunisation with hTNF-kinoid safely induced anti-TNF antibodies with indication of a positive clinical response, suggesting that active immunisation against TNF could represent a new therapeutic strategy in IBD. The idea of reducing TNF levels in IBD patients by selectively silencing the TNF gene using RNA interference is still in preclinical development but McCarthy *et al*. reported on the clinical potential of a cyclodextrin-based TNF siRNA delivery system for the treatment of IBD.[37] They showed that intrarectal administration of TNF siRNA in C57BL/6 mice with dextran sulfate sodium (DSS)-induced colitis resulted in a mild amelioration of clinical signs of colitis as well as a significant reduction in colon weight and colonic *Tnf* levels. Also the use of TNF-neutralising nanobodies (derived from fragments of heavy-chain camelid antibodies that are more stable than conventional antibodies) as therapeutic proteins in IBD is still in the preclinical phases of development, but promising results were obtained with *Lactococcus lactis* bacteria that were engineered to secrete murine (m) TNF-neutralising nanobodies.[38] Oral administration of nanobody-secreting *Lactococcus lactis* resulted in local delivery of anti-mTNF nanobodies at the colon and significantly reduced inflammation in mice with chronic DSS-induced colitis.

Besides TNF, other pro-inflammatory cytokines, such as interleukin (IL)-12, IL-23 and IL-6, have also been reported to play a key role in IBD pathogenesis[39,40] and the therapeutic potential of targeting these cytokines is currently being evaluated. In the case of IL-6, elevated serum levels are considered to be a clinically relevant parameter in patients with active CD and a neutralising antibody against the IL-6 receptor suppressed established experimental colitis in various animal models of CD.[41] Recently, the therapeutic efficacy of a monoclonal antibody (mAb) against IL-6 (PF-04236921) was evaluated in a phase II, randomised, double-blind, placebo-controlled, dose-ranging study in subjects with refractory CD, which revealed statistically significant clinical response and remission rates in this patient population.[42] The heterodimeric cytokines IL-12 and IL-23, which share a common p40 subunit, are both increased in the intestine of patients with CD[43] and anti-inflammatory effects of a

genetic deletion or antibody-mediated neutralisation of IL-12 and IL-23 in mouse models of intestinal inflammation have provided a rationale for the therapeutic targeting of both these cytokines. More specifically, administration of mAbs against IL-12 in a murine model of chronic 2,4,6-trinitrobenzene sulfonic acid-induced colitis led to an improvement of both the clinical and histopathological aspects of disease and frequently abrogated the established colitis completely.[44] Also antibodies against p19, the subunit specific to IL-23, suppressed chronic intestinal inflammation in a T cell-deficient, *Helicobacter hepaticus*-infected RAG knockout mouse model of IBD.[45] The clinical efficacy of ustekinumab, a mAb directed against the p40 subunit of IL-12 and IL-23[46], as an induction and maintenance therapy in refractory CD was recently evaluated in a phase III study, which revealed that patients with moderately to severely active CD receiving intravenous ustekinumab had a significantly higher response rate than those receiving placebo, and that subcutaneous ustekinumab maintained remission in patients who had a clinical response to the intravenous induction therapy.[47]

Another targetable mechanism of action is the homing and endothelial transcytosis of immune cells towards the site of inflammation. Examples of anti-migration agents include mAbs against the leukocyte α4β7 integrin receptor and its endothelial ligand, mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), as well as mAbs against chemokines (e.g. CXCL-10) and selective, small-molecule antagonists of chemokine receptors (e.g. CCR9). The anti-α4β7 mAb vedolizumab has recently been approved for treatment of moderate to severe UC and CD based on the encouraging results from a series of phase III, randomised, placebo-controlled clinical trials performed in patients who have failed TNF antagonist therapy.[48] These studies showed that vedolizumab is effective in the induction of clinical response and remission in moderately to severely active UC and CD patients as well as in the maintenance of remission in patients who achieved an initial response to therapy.[49] Safety and efficacy of an anti-MAdCAM-1 mAb (PF-00547659) was recently evaluated in active, refractory CD and UC patients in two separate randomised, double-blind, placebo-controlled studies. In the UC study, the primary endpoint (remission at week 12) was met[50], whereas in the CD study, the primary endpoint (clinical response at week 8 or 12) was not met but PF-00547659 was pharmacologically active, as shown by a sustained dose-related decrease in MAdCAM-1 levels.[51] A mAb against CXCL-10 (BMS-936557), a chemokine involved in inflammatory cell migration whose expression is elevated in colonic tissue and plasma of UC patients, was evaluated for safety and efficacy during a phase II study in patients with active UC. Endpoints of prespecified rates of clinical response, clinical remission and mucosal healing were not met, but a higher drug exposure correlated with increasing clinical response and histological improvement, warranting further dose-response studies.[52] A pharmacological antagonist of the chemokine receptor CCR9 (CCX282-B), which is selectively expressed on gut-homing T cells and on mucosal lymphocytes in the small intestine,

thus suggesting that CCR9 plays a role in the recruitment and/or retention of lymphocytes to this site[53], was recently evaluated in phase II and III clinical trials in patients with active CD. However, although CCX282-B showed efficacy in inducing and maintaining clinical response in a randomised, placebo-controlled phase II trial[54], these results were not confirmed in the ensuing phase III trial.[55]

Also modulation of the sphingosine-1-phosphate (S1P) signalling axis has recently emerged as a novel therapeutic target for the treatment of IBD. S1P is a bioactive lipid that regulates inflammation via its impact on the trafficking, differentiation, and effector functions of bone marrow-derived immune cells.[56] Targeting S1P signalling in preclinical models of IBD has yielded promising results. For example, functional antagonists of the S1P receptor are able to dampen inflammation in murine models of colitis induced by IL-10 deficiency, DSS treatment, and adoptive T cell transfer by decreasing the number of $CD4^+$ T lymphocytes in the colonic lamina propria, which is associated with a reduction in interferon gamma (IFNγ) production.[56] The S1P receptor modulator RPC-1063 is currently undergoing safety and efficacy evaluation in phase II and phase III trials in patients with moderately to severely active UC, and in a phase II trial in patients with moderately to severely active CD.[57]

A signalling pathway that is also implicated in IBD pathogenesis is the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, which regulates immune functions in response to various cytokines and growth factors.[58] So far, only data from tofacitinib, a novel selective oral JAK inhibitor, are available. Tofacitinib has been shown to inhibit a range of innate and adaptive immune responses, including the acute response to lipopolysaccharide (LPS) as well as T cell activation and differentiation.[58-60] A recent randomised, placebo-controlled, dose-ranging phase II study indicated that patients with moderately to severely active UC treated with tofacitinib over a period of 8 weeks had better clinical as well as endoscopic response and remission rates than those receiving placebo. Tofacitinib thus seems to show promise as a new therapeutic agent for the treatment of UC.[58,61]

Excessive innate immune responses are a major hallmark of IBD and lead to sustained immune activation and tissue damage. For example, NKG2D (KLRK1, killer cell lectin like receptor K1) is an innate immunity receptor expressed predominantly on natural killer (NK) cells, which recognises multiple stress-induced, cell-associated ligands presented by damaged cells, and whose activation potentiates the effector functions of certain T cell populations. The expression pattern of the NKG2D receptor and its ligands on mucosal and circulating innate immunity populations was found to be severely disturbed in IBD.[62] Safety and efficacy of an anti-NKG2D mAb was recently evaluated in a randomised, placebo-controlled phase II trial in subjects with moderately to severely active CD, which revealed that treatment with a subcutaneous dose of anti-NKG2D significantly reduced disease activity versus placebo, supporting further development of anti-NKG2D in IBD.[63] Stimulation of Toll-

like receptor (TLR) 9, also a key activator of innate immunity that recognises unmethylated CpG dinucleotides present in bacterial DNA, has been shown to suppress the severity of experimental colitis[64], and the clinical efficacy of an oral TLR9 agonist (BL-7040) was evaluated in a phase IIa trial in patients with moderately active UC. Results from this study indicate that BL-7040 is safe and effective in treating UC, as demonstrated by the improved clinical response achieved in 50% of the patients enrolled in the study.[65] Deregulated immune behaviour in IBD is also in part due to a lack of suppression by immunomodulatory cell populations such as regulatory T cells (Tregs). Treg suppressor activity of the induction and proliferation of effector s mediated by anti-inflammatory cytokines such as transforming growth factor β (TGFβ), whose inhibitory capacity is abrogated due to Smad7 overexpression.[66] The safety and efficacy of an oral Smad7 antisense oligonucleotide (mongersen) was recently evaluated in a double-blind, placebo-controlled, phase II trial in patients with active CD, which showed that study participants who received mongersen had significantly higher rates of clinical remission and clinical response than those who received placebo.[67] A more drastic way of inducing anergy in the inflammatory environment is through transplantation of bone marrow hematopoietic stem cells (HSC). Myeloablation first eliminates detrimental T cell populations, and subsequent HSC transplantation generates naive cells via *de novo* haematopoiesis. Patients essentially undergo an immune system reboot, which will hopefully bring about long-lasting remission. Trials designed to investigate the potential clinical benefit of autologous HSC transplantation have shown encouraging results in patients with severe, refractory CD. However, existing studies are still inconclusive, so the next few years will be decisive for defining the role of this therapy in the management of IBD.[68]

1.1.3.2.2 Microbiota modulation

IBD patients display substantial intestinal dysbiosis and depletion of key commensal bacteria is thought to be associated with inflammation. The rationale behind therapeutically manipulating the diseased microbiota is the idea that reshaping the gut microbiome to a less pro-inflammatory state would aid in induction and/or maintenance of clinical remission. This can be achieved either through administration of bacterial strains with known anti-inflammatory properties (probiotics), by a diet that selectively promotes the growth of these strains (prebiotics), or by transplanting the fecal microbiota (FMT) from a healthy individual to the patient.

IBD-associated dysbiosis involves various functional groups of commensal bacteria across different taxonomical levels. The most well-defined change that has been noted in patients is the reduced abundance of the phylum *Firmicutes*.[69] Spread across several families within this phylum is a large community of butyrate-producing species, whose numbers are severely depleted during intestinal inflammation. These butyrate-producing bacteria are of specific interest as a new treatment strategy

to promote mucosal healing in IBD patients given the barrier-protective properties of butyrate. The different bacterial communities implicated in IBD-associated dysbiosis, in particular that of the butyrate-producers, the role of butyrate in colonic homeostasis, and the potential application of butyrate-producing commensals as pharmabiotics for IBD, are discussed in more detail in §1.2 of this chapter.

1.1.3.2.3 New targets for the restoration of intestinal epithelial barrier integrity

As mentioned above, several of the currently used IBD therapeutics induce mucosal healing but whether this is due to a direct barrier-protective effect, remains unclear. For example, anti-TNF therapy rapidly induces mucosal healing but several lines of evidence suggest that its key mechanism of action is the induction of T cell apoptosis.[70] Aminosalicylates on the other hand, directly affect barrier integrity *in vitro*; the 5-aminosalicylic acid (5-ASA) precursor, sulfasalazine, is able to prevent TNF-induced barrier dysfunction and morphological tight junction disruption in Caco-2 cells.[71] Up to now, the term mucosal healing refers to endoscopic remission, which includes o.a. the resolution of visible ulcers. However, this definition does not take into account the microscopic and ultrastructural components of the intestinal barrier that are also affected during active disease. Restoration of epithelial barrier integrity following injury involves epithelial restitution (intestinal epithelial cell (IEC) migration into the eroded area), followed by proliferation and differentiation. These processes are regulated by environmental signals like growth factors, cytokines and bacterial products that activate transcription factors such as nuclear factor κB (NF-κB) and STAT3, which mediate anti-apoptotic and proliferative effects in IECs. For example, ameliorating effects on mucosal healing in experimental colitis have been described for targeted modulation of Smad5, protein kinase C (PKC), p53-upregulated modulator of apoptosis (PUMA) and apical TLR9 stimulation.[23] Whether microscopic healing has an added clinical benefit compared to endoscopic resolution of inflammation, remains to be determined but these experimental data demonstrate that targeting signalling pathways involved in epithelial restitution represent possible novel approaches for future therapeutic interventions.

One such a signalling pathway, that of the Rho-associated kinases which are involved in inflammation-induced loss of epithelial barrier integrity, was investigated during this thesis. The role of Rho-associated kinases during intestinal inflammation and their targeting as a potential new treatment option for restoring epithelial barrier integrity, are discussed in more detail in §1.3 of this chapter.

1.2 MICROBIAL DYSBIOSIS IN IBD: A ROLE FOR BUTYRATE-PRODUCING BACTERIA?

In the second part of this introduction, we will elaborate on the role of the gut microbiome in IBD pathogenesis. We will describe its biogeography and modulating factors, its role in the development of the mucosal immune system as well as in the maintenance of gut immune homeostasis, and its altered composition in IBD patients. We will focus specifically on butyrate-producing bacteria, a community affected by intestinal inflammation, the barrier-protective and anti-inflammatory properties of their butyrate metabolite, and their potential application as pharmabiotics for IBD.

1.2.1 Composition of the human gut microbiome

1.2.1.1 Bacterial colonisation of the gut

In utero, our entire gastro-intestinal tract is completely sterile. Bacterial colonisation of the gut starts during birth with a first inoculation by the vaginal microbiota. Initial bacterial composition is not very diverse and highly dynamic. Once we start consuming solid food, the microbiota composition gains in richness and diversity and by the age of 3, the adult gut microbiome has taken shape with *Bacteroidetes* and *Firmicutes* as the predominant bacterial phyla.[72,73] Throughout life, the microbial composition is fairly robust with major alterations only occurring as a result of antibiotic therapy, infection by gut pathogens or immune-mediated intestinal disorders like IBD. The average bacterial load of the entire gastro-intestinal tract amounts to 10^{14} micro-organisms, outnumbering the human cells 10 to 1. Besides microbes, other micro-organisms like viruses and fungi also abundantly colonise the gut and are collectively referred to as the enteric virome and fungome, respectively. Interestingly, disease-specific alterations in the composition of the enteric virome and fungal microbiota have also been observed in IBD. Norman *et al*. recently demonstrated that the fecal virome in CD and UC patients is characterised by a significant expansion of *Caudovirales* bacteriophages compared to healthy controls. Importantly, it did not appear that the expansion and diversification of the enteric virome in IBD patients was secondary to changes in bacterial populations, suggesting that alterations to the virome composition may contribute to intestinal inflammation and bacterial dysbiosis in IBD.[74] Also the fecal fungal microbiota was shown to be dysbiotic in IBD, displaying an increased *Basidiomycota*/*Ascomycota* ratio, a decreased proportion of *Saccharomyces cerevisiae*, and an increased proportion of *Candida albicans* compared to healthy subjects.[75] Additionally, the observation that correlations between bacterial and fungal components differed between IBD patients and healthy controls implies the existence of disease-specific inter-kingdom alterations, suggesting that, beyond bacteria, fungi might also play a role in IBD pathogenesis.

1.2.1.2 Spatial bacterial variation within the gut

In the gut, the spatial distribution of intestinal microbes occurs along two basic axes; longitudinally from proximal to distal, and radially from the central lumen to the mucosal surface (Figure 2).[76] A general observation for the longitudinal variation is that both load and diversity increase towards the rectum; total load in the small intestine varies between 10^3 and 10^7 with *Lactobacillaceae* and *Enterobacteriaceae* as the predominant families, while the total amount of colonic microorganisms amounts to 10^9-10^{12}. The predominant families represented in the colon are the *Bacteroidaceae*, *Prevotellaceae*, *Rikenellaceae*, *Lachnospiraceae* and *Ruminococcaceae*.[77,78]

Radial bacterial variation is determined by autochthonous (resident) microbes that are closely associated with the intestinal mucosa and the allochthonous (transient) microbes that are located in the central lumen as part of the fecal stream or digesta. The mucosa-associated surface of the colon is predominantly colonised by the *Lachnospiraceae* and *Ruminococcaceae* families of the *Firmicutes* phylum, whereas the *Prevotellaceae*, *Bacteroidaceae* and *Rikenellaceae* families of the *Bacteroidetes* phylum are enriched in the digesta. This difference in composition between both environments suggest that mucosa-associated microbial communities have evolved a symbiotic relationship with their human host, which allowed them to colonise a hazardous niche equipped with numerous endogenous antimicrobial defence mechanisms while at the same time remaining innocuous to their host.[76,77]

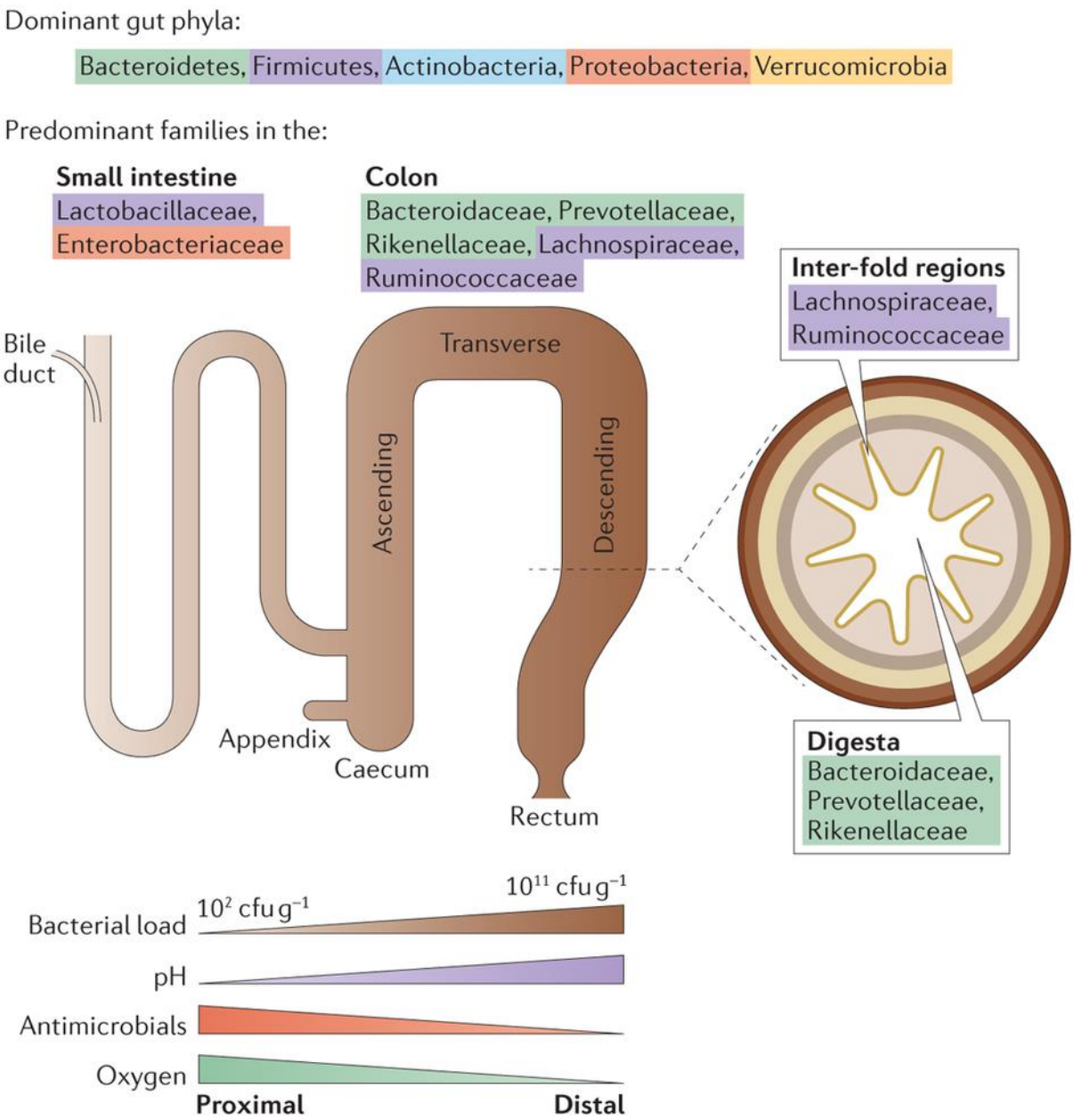


Figure 2. Gut biogeography of the microbiota in the human lower gastro-intestinal tract*. Intestinal microbes are spatially distributed along two basic axes; longitudinal and radial, which is determined by local environmental factors. The dominant bacterial phyla in the gut are Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia. The dominant bacterial families of the small intestine and colon reflect the physiological differences along the length of the gut; longitudinal gradients of oxygen, antimicrobial peptides and pH limit the bacterial density in the small intestinal community, whereas the colon carries high bacterial loads. In the small intestine, the families Lactobacillaceae and Enterobacteriaceae dominate, whereas the colon is characterised by the presence of species from the families Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae and Ruminococcaceae. A radial cross-section of the colon shows the digesta, which is dominated by Bacteroidaceae, Prevotellaceae and Rikenellaceae, and the inter-fold regions of the lumen, which are dominated by Lachnospiraceae and Ruminococcaceae. Colours correspond with the relevant phyla. cfu; colony-forming units. Adapted from Donaldson et al. 2016.*

1.2.1.3 The enterotype concept

Despite this obvious bacterial diversity within the gut, a recent fecal metagenomic, cross-country study revealed that intestinal microbiota variation between individuals is not continuous. It can be stratified into three robust clusters or enterotypes that are not nation- or continent-specific, and are identifiable by the variation in the levels of one of three genera; *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3). These enterotypes do not correlate with host properties like nationality, gender, age or body mass index, although several significant functional correlations with each of the studied host properties were found. Interestingly, despite being mostly based on species composition, these enterotypes also reveal functional differences, e.g. in the metabolic routes used to convert complex carbohydrates in the colon into absorbable substrates. This study thus indicates the existence of a limited number of well-balanced host-microbial symbiotic states that differ both phylogenetically and functionally, and which might respond differently to diet and drug intake.[79]

Enterotypes were also detected in common laboratory mice, enterotype 1 and enterotype 2, whose compositional and community structure properties are strikingly similar to those of the *Ruminococcus* and *Bacteroides* human enterotypes, respectively.[80] An important remark is that this stratified intestinal microbiota variation in mice and humans was observed in caecal content and fecal samples, respectively, which are not representative of the variation within the mucosa-associated microbiome.

1.2.1.4 Major modulators of gut bacterial biogeography

Several internal and external factors influence both longitudinal and radial bacterial variation within the gut. Internal factors, or host factors, include antimicrobial peptides, mucus and the host immune system, which create different physiological environments along both axes. The external factors affecting gut microbial composition are often members of the aforementioned exposome, of which dietary intake is the most important one.

1.2.1.4.1 Host factors

Host factors are the main determinators of radial variation by limiting bacterial access to the gut epithelium. A protective mucus layer serves as the primary barrier to tissue adherence by the microbiota. This mucus layer is made up of different types of mucin proteins (MUC) that are rich in serine, threonine and proline amino acid residues. These residues are extensively glycosylated, giving the mucins their extended and stiff conformation. There are two different types of mucins; transmembrane mucins (e.g. MUC3, MUC12, MUC13, MUC17) and secreted, gel-forming mucins (e.g. MUC2, MUC6).[81] Transmembrane mucins are densely packed on the apical surface of the IECs, forming a first protective layer or glycocalyx. Above this glycocalyx lies a net-like structure of

secreted mucus produced by specialised goblet cells, which consists mainly of MUC2 glycopeptides. The mucus of the small intestine only has one layer, is not attached to the epithelium, and is colonised by adherent bacterial species, including segmented filamentous bacteria (SFB), members of the *Lactobacillaceae* family, and *Helicobacter* species. Colonic mucus, on the other hand, is two-layered; a dense, inner mucus layer that remains attached to the IECs and a loose, outer layer that is similar to the small intestinal mucus layer. The outer mucus layer is colonised by mucin-degrading bacteria, such as *Akkermansia muciniphila*, whereas the inner mucus layer is penetrated at low bacterial density by a more restricted community that includes, for example, the well-characterised symbiont *Bacteroides fragilis*.[77,81] Bacteria penetrate these mucus layers by means of flagella-mediated motility, or secretion of mucus-degrading enzymes or mucinases (e.g., glycosidases, proteases, sulphatases, sialidases).[82] Physical adherence of bacteria to the epithelial surface occurs through appendices like pili, fimbriae and membrane proteins that target ligands on the epithelial surface.[77] Interestingly, interaction with mucus and adherence to IECs seem to be adaptations used both by pathogens during infection and by commensals during persistent colonisation, challenging the dogma that intestinal microorganisms only come into contact with the epithelium during disease. Secondly, in order to persist near the host tissue, bacteria must be able to tolerate the large amounts of immunoglobulin A (IgA) that are secreted into the mucus layer. Secretory IgA physically interacts with the microbiota and this may contribute to mucosal biofilm formation, which prevents bacterial adherence to the epithelium. Finally, bacterial mucosal adhesion is also limited by secretion of antimicrobial peptides (defensins, cathelicidins and histatins)[83] and aerobic host metabolism, which creates a steep, radial oxygen gradient that influences the type of bacteria capable of surviving close to the epithelial surface. Oxygen and antimicrobials concentration also affect longitudinal variation since the gradient of both decreases from proximal to distal as well as from tissue to lumen.[77]

1.2.1.4.2 Dietary intake

Several studies have highlighted the influence of dietary factors on the composition of the intestinal microbiota and have demonstrated that diet-induced alterations result in significant biological changes to the host. A study by Zhang *et al*. aimed at assessing the relative contributions of host genetics and diet in shaping the gut microbiota, revealed that microbial composition strongly correlated with diet.[84] They administered a long-term high-fat (HF) diet to Apoa-I knockout mice, which have impaired glucose tolerance and increased body fat, and their wild-type counterparts. Interestingly, diet changes explained 57% of the total structural variation in the gut microbiota, whereas the genetic mutation accounted for no more than 12%. Most notably, barrier-protecting *Bifidobacterium* species[85] were nearly absent in all animals on the HF diet, regardless of genotype. The dominant role of our diet in shaping the gut microbiome was further emphasised by Carmody *et*

al. who showed that a high-fat, high-sugar (HFHS) diet induced the same alterations to the gut microbiota in different mouse strains despite pre-existing genotype-related differences in gut microbial composition.[86] The perturbing effect of this these HF and HFHS diets on the gut microbiota is particularly interesting since these types of diets represent the modern day dietary regime of the Western world, which is characterised by a high intake of proteins, fat and simple sugars and a reduced intake of complex plant carbohydrates or fiber. The implications of a HFHS diet for the human gut ecosystem was demonstrated by Turnbaugh *et al*. who created humanised gnotobiotic mice by transplanting human fecal microbial communities into germ-free mice. When these transplanted mice were switched from a low-fat, plant polysaccharide-rich diet to a HFHS diet, the microbiota composition shifted within a single day to an overgrowth of *Firmicutes*, including *Clostridium innocuum*, *Eubacterium dolichum*, *Catenibacterium mitsuokai*, and *Enterococcus* species as well as a significant reduction in several *Bacteroides* species.[87] The functional consequence of the alterations in microbial composition induced by these carbohydrate-reduced diets is apparently a shift in the gut microbiome towards a more pro-inflammatory profile in which a decrease in beneficial *Clostridium* cluster XIVa species and a concomitant reduction in fecal butyrate levels are observed as well as an overgrowth of colitogenic mucosa-associated *Escherichia coli* (*E. coli*) bacteria.[88-90] On the other hand, diets specifically enriched for complex carbohydrates show less pathogenic species, such as *Mycobacterium avium* subspecies *paratuberculosis* and *Enterobacteriaceae*, than diets higher in fat or protein as well as increased levels of beneficial *Bifidobacteria* species.[91] In conclusion, these studies indicate that dietary factors have a dominating role in shaping the gut microbiota and that changes in its composition induced by a Western diet may transform a healthy microbiome into a disease-inducing entity.

1.2.2 Mucosal immunoregulation by the gut microbiota

Besides their role in host nutrition as part of the digestive system, the gut microbiota also plays a crucial role in the development of the intestinal immune system as well as in the maintenance of immune homeostasis. The importance of the microbiome in shaping host mucosal immunity was highlighted by several earlier studies using germ-free animals, which have revealed that development of immunological capacity is impaired under germ-free conditions.[92] More specifically, germ-free animals display a decrease in IgA-secreting cells, which reduced the maintenance of oral tolerance to ovalbumin[93,94], a decreased number of intraepithelial lymphocytes[95], and underdeveloped lymphoid tissues characterised by smaller Peyer's patches, isolated lymphoid follicles with fewer germinal centers, reduced lamina propria cellularity, and decreased epithelial production of mucus and antimicrobial peptides.[96,97] Conversely, administration of a sterile, LPS-rich diet to germ-free mice drives the expansion of B and T cells in the Peyer's patches and mesenteric

lymph nodes, with the most prominent expansion being that of the $CD4^+$ T cells, including Tregs.[98] The microbiota thus shapes the host's mucosal immune system and actively regulates the maintenance of gut immune homeostasis under normal conditions by inducing intestinal accumulation of specific immune cell populations necessary for a tolerogenic mucosal response to the commensal microbiota. This accumulation of Tregs in the gut lamina propria is induced by specific immunomodulatory commensal populations.[91,97] For example, the immunomodulatory molecule polysaccharide A associated with the prominent human commensal *Bacteroidetes fragilis* was proposed to activate $CD4^+$ T helper cells and direct the establishment of a proper Th1/Th2 balance.[99] Monocolonisation of germ-free mice with *B. fragilis* promotes Treg development and induces anti-inflammatory cytokine IL-10 production exclusively from these cells, which results in protection from chemically induced colitis.[100] Also adherent SFB have been shown to increase the number of Tregs in both the small intestine and colon[101] as well as induce Th17 responses[102]. Another key commensal group of potent Treg inducers are *Clostridium* species belonging to clusters IV and XIVa.[91,97] Monocolonisation with 46 *Clostridium* species from these clusters in germ-free mice strongly promotes IL-10-producing Tregs.[103] Together, these data demonstrate the vital role of our gut microbiome in the development, maturation and function of the mucosal immune system by promoting the expansion of specific immune cell populations that actively induce mucosal tolerance, thus contributing to intestinal homeostasis. Alterations to the composition of the commensal microbiota may therefore result in a disruption of immune homeostasis between the host and the microbiota, which could contribute to the pathogenesis of chronic, immune-mediated intestinal inflammation.

1.2.3 Characterisation of microbial dysbiosis in IBD

Circumstantial evidence for the pivotal role of gut microbes in the induction and propagation of mucosal inflammation has been substantiated by several observations. For example, surgical diversion of the fecal stream can induce remission in refractory colonic CD and the majority of patients relapse after stoma reversal.[104] Also, antibiotics alleviate both human[105] and murine[106] intestinal inflammation, and mice developing intestinal disease in a conventional or specific-pathogen free environment, generally do not do so in a germ-free one[106-108], with the exception of a few models, including DSS-induced colitis.[109] However, the relationship between bacteria and IBD is more intricate than that; it not simply the presence of a gut microbiome that is associated with disease but rather its particular composition. Indeed, the intestinal microbiotic profile of IBD patients has been shown to be severely altered compared to healthy subjects – a condition termed dysbiosis. Dysbiosis is generally defined as a deviation from the normal microbial community, such as an imbalance in the abundance, membership or localisation of micro-organisms. In the context of IBD,

intestinal dysbiosis entails a decrease in both bacterial load and richness.[77] However, despite the abundant evidence on IBD-associated dysbiosis, the crucial question remains whether changes in gut microbial composition precede, follow, cause or only correlate with the onset of disease.

Numerous studies have described the nature of intestinal dysbiosis in IBD patients and a few general observations can be made. Fecal microbiota composition in both CD and UC patients is characterised by a significant depletion of the *Firmicutes* phylum, particularly members of the *Clostridium* clusters IV and XIVa, while *Enterobacteriaceae* of the *Proteobacteria* phylum are increased in relative abundance, with *Escherichia coli* (*E. coli*) specifically being enriched in feces of CD patients. Data on the *Bacteroidetes* phylum are more ambiguous; inconsistent findings have been reported for their presence in IBD patients compared to healthy individuals.[110-112] The same dysbiotic signatures are also observed when analysing the mucosa-associated microbiota isolated from surgically resected mucosal tissue of IBD patients; abundances of *Bacteroidetes* and *Firmicutes* were reduced compared to non-IBD controls, and the loss of *Firmicutes* was primarily due to a reduction in the abundances of species that belong to the bacterial order *Clostridiales*, particularly members of the *Clostridium* clusters XIVa and IV.[113,114] Also the ileal mucosa of CD patients was found to be selectively enriched for invasive *E. coli*, which correlated with the severity of ileal disease.[115] This observation supports earlier findings of a higher prevalence of adherent-invasive *E. coli* (AIEC) strains cultured from the ileal mucosa of patients with Crohn's ileitis.[116]

Most studies report on dysbiosis during the active phases of disease but the microbiota composition of patients in remission still differs from that of healthy individuals. For example, a dysbiotic signature was shown to persist during clinical remission in UC, which was characterised by a low diversity and temporal instability, and a decrease in bacterial richness prior to relapse.[117,118] A subclinical dysbiosis has also been reported in unaffected relatives of UC and CD patients.[119,120] These observations suggest that an initial abnormal gut microbiome composition might, in combination with other genetic and environmental factors, contribute to the onset and/or flare-ups of intestinal inflammation in IBD.

The intestinal depletion of *Clostridium* clusters XIVa and IV species in IBD is of particular significance since most of the butyrate-producing bacteria cultured so far belong to these clusters, including *Roseburia* and *Faecalibacterium* species, which are two abundant gut colonisers.[121] In accordance with these general findings, a decrease in both *Roseburia hominis* and *Faecalibacterium prausnitzii* (*F. prausnitzii)* has been documented in the stool of UC patients, with both species showing an inverse correlation with disease activity.[122] Other studies have also reported that *F. prausnitzii* was particularly depleted in the ileocolonic mucosa-associated microbiota of IBD patients, and Sokol *et al.* additionally showed that a low proportion of *F. prausnitzii* in resected ileal CD mucosa is associated

with a higher risk of post-operative disease recurrence.[123] Also fecal levels of the butyrate-producing *Butyricicoccus* genus are reduced in both CD and UC patients compared to healthy controls.[124] This depletion of mucosa-associated butyrate producers most likely results in lower butyrate levels near the IECs. Indeed, fecal butyrate concentrations were shown to be significantly decreased in IBD patients compared to healthy subjects.[125-127] The combination of diminished intestinal butyrate levels and the inflammation-induced impaired uptake and metabolism of butyrate[128,129] causes severe epithelial butyrate deficiency, which negatively affects colonic homeostasis.

1.2.4 Contribution of butyrate metabolism to intestinal homeostasis

1.2.4.1 Functions of butyrate within the colon

Butyrate exerts potent effects on a variety of colonic mucosal functions and, although the exact underlying mechanisms of action have not yet been elucidated, butyrate is thought to influence cell function primarily through epigenetic regulation of butyrate-responsive gene expression by acting as a histone deacetylase inhibitor (HDACi).[130] Most importantly, butyrate is the main energy source for colonocytes, stimulates ion absorption, protects against mucosal oxidative stress, stimulates non-specific intestinal defence mechanisms and increases intestinal motility. Butyrate also regulates cell growth by stimulating cell proliferation of normal colonocytes while inducing differentiation and apoptosis in neoplastic cells, a contradictory pattern called the butyrate paradox.[130-133] Immunomodulation by the gut microbiota is also mediated in part by butyrate; commensal-derived butyrate has been described to induce differentiation of colonic Tregs.[134] Especially indigenous *Clostridium* species belonging to clusters XIVa and IV are potent inducers of IL-10-producing Tregs[135], which is a way for commensals to promote their own immunological tolerance. Finally, butyrate also functions as an anti-inflammatory mediator and is involved in maintenance of intestinal epithelial barrier integrity; two functions pertaining to the subject matter of this thesis, which are described in more detail below.

1.2.4.1.1 Anti-inflammatory effects

Butyrate has been shown to ameliorate intestinal inflammation in several murine models of acute colitis. Administration of butyrate enemas during DSS-induced colitis in rats had a clear cytoprotective effect on colonocytes, as evidenced by increased cell viability and reduced permeability, which was mediated in part through a reduction of NF-κB activation.[136] Butyrate enemas also stimulated colonic repair during 2,4,6 trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats, as demonstrated by a clinical recovery, decreased mucosal inflammation and restoration of electrolyte absorption.[137] Also oral butyrate administration improved mucosal lesions and attenuated the inflammatory profile of the intestinal mucosa and local lymph nodes in DSS-induced colitis in mice.[138] NF-κB activation and the concomitant production of pro-inflammatory

cytokines is also increased in the intestinal mucosa of CD patients, and *ex vivo* butyrate administration to intestinal biopsies, lamina propria mononuclear cells (LPMCs) and peripheral blood mononuclear cells (PBMCs) collected from CD patients, resulted in reduced TNF production and pro-inflammatory cytokine mRNA expression in intestinal biopsies and LPMCs. Butyrate also abolished LPS-induced expression of cytokines by PBMCs and nuclear translocation of NF-κB. Butyrate thus dampens mucosal inflammatory responses in part through inhibition of NF-κB activity.[139]

An NF-κB-independent mechanism through which butyrate supresses inflammation in human colonic epithelial cells is by inhibiting IFNγ signalling - one of the main cytokines produced in inflamed IBD mucosa - at the level of STAT1 phosphorylation; the main transcription factor in the INFγ signalling cascade, whose expression and activation is elevated in mucosal samples from IBD patients.[140,141]

1.2.4.1.2 Effect on epithelial tight junction integrity

The anti-inflammatory effect of butyrate indirectly strengthens intestinal barrier integrity by reducing expression and signalling of the pro-inflammatory cytokines that cause barrier dysfunction during intestinal inflammation. But butyrate can also directly improve barrier function by modulating the assembly tight junctions (TJ), the main determinants of intestinal epithelial barrier integrity, and expression of TJ proteins, which is reflected by an increase in transepithelial electrical resistance (TEER).[142] Butyrate facilitates normal TJ assembly; treatment of IEC monolayers with butyrate resulted in an accelerated membrane enrichment of TJ proteins occludin (OCLN) and tight junction protein 1 (TJP1) after calcium switch-induced TJ assembly.[143] Butyrate has also been shown to upregulate protein expression of TJP1 in Rat-1 fibroblasts and of OCLN in HeLa cells, which was attributable to the HDACi activity of butyrate.[144] Butyrate likewise increased transcription of claudin-1 (CLDN1), a pore-sealing TJ protein, in a monolayer of rat small intestinal epithelial cells by facilitating the association between the SP1 transcription factor and the CLDN1 promoter, which resulted in an enhanced intestinal barrier function.[145] Besides increasing epithelial protein levels of pore-sealing claudins, butyrate also reduces those of the pore-forming CLDN2.[146] This butyrate-induced decrease in CLDN2 expression was also confirmed in a microarray study in HT-29 human colonic epithelial cells and is dependent on a reduced binding affinity of transcription factors within the CLDN2 promoter.[147,148]

1.2.4.2 Colonic butyrate production and uptake

Anaerobic microbial communities of the colon produce the three major short-chain fatty acids (SCFA) acetate, proprionate and butyrate as their main non-gaseous fermentation end products.[121] Butyrate is the least abundant SCFA, making up 15% of the total SCFA concentration in the colonic lumen.[131] Fecal butyrate concentrations range from 11 to 25 mM, but this may be an underestimation of the total colonic butyrate production since approximately 95% is rapidly absorbed and metabolised by

the colonocytes.[132] Fermentable substrates with butyrate as end product are mainly indigestible plant fibers like cellulose, starch and other complex polysaccharides.[133]

Bacteria produce butyrate through four known pathways; the glutarate, acetyl-CoA, lysine and 4-aminobutyrate/succinate pathway, with the acetyl-CoA pathway being the predominant one.[149] In this pathway, acetyl-CoA is converted into butyryl-CoA through a series of intermediate metabolites. Acetyl-CoA itself is derived from pyruvate, the end product of glycolysis. Butyryl-CoA is further converted to butyrate via either butyrate kinase or butyryl-CoA:acetate CoA transferase. The latter route is shown to be more dominant in human butyrate-producing bacteria.[133] Indirect butyrate production also occurs through conversion of fermentation products such as acetate and lactate, which are produced by primary substrate degraders, in a process called cross-feeding.[121]

Colonocytes absorb butyrate across their apical membranes through different mechanisms; non-ionic diffusion, SCFA/HCO_3^- exchange, and active transport by SCFA transporters. The transport proteins involved are monocarboxylate transporter isoform 1 (MCT1 or SLC16A1), which is coupled to a transmembrane H^+-gradient and SLC5A8, which is a Na^+-coupled co-transporter.[131,132]

1.2.4.3 Taxonomy of butyrate-producing bacteria within the gut

Culture-independent methods have revealed that the *Firmicutes* phylum dominates the butyrate-producing ecosystem in the gut, but members of nine other phyla, including o.a. *Bacteroidetes* and *Fusobacteria*, were also identified as potential butyrate producers.[149] Within the *Firmicutes*, significant populations of known butyrate-producing species are included in the *Eubacterium* and *Roseburia* genera of the *Lachnospiraceae* family, in the *Clostridium* genus of the *Clostridiaceae* family and in the *Faecalibacterium* genus of the *Ruminococcaceae* family.[121,149] Interestingly, these butyrate-producing species are phylogenetically interspersed with other species that are not known to produce butyrate, making intestinal butyrate producers a functional cohort rather than a monophyletic group.

The butyrate-producing bacteria cultured so far are strictly anaerobic and generally regarded as difficult to grow *in vitro*. They are widely distributed across several clusters that contain species of several genera belonging to the *Clostridiaceae*, *Eubacteriaceae*, *Lachnospiraceae* and *Ruminococcaceae* families within the order *Clostridiales* (Figure 3). These so-called clostridial clusters (I–XIX) form a new nomenclature that rearranges the clostridial species into groups, or clusters, based on the similarity of their 16S rRNA gene sequences.[150] The bulk of butyrate producers belong to the *Clostridium* cluster IV (or *Clostridium leptum* cluster) and the *Clostridium* cluster XIVa (or *Clostridium coccoides* cluster), which include some potentially important butyrogenic species related to *F. prausnitzii* and *Eubacterium rectale*/*Roseburia* species, respectively.[151] In humans, the butyrate-

producing bacteria related to *F. prausnitzii* comprise 5-15% of the total microbiota and 5–10% of the total microbiota species are related to *Eubacterium rectale* and *Roseburia* species.[152,153]

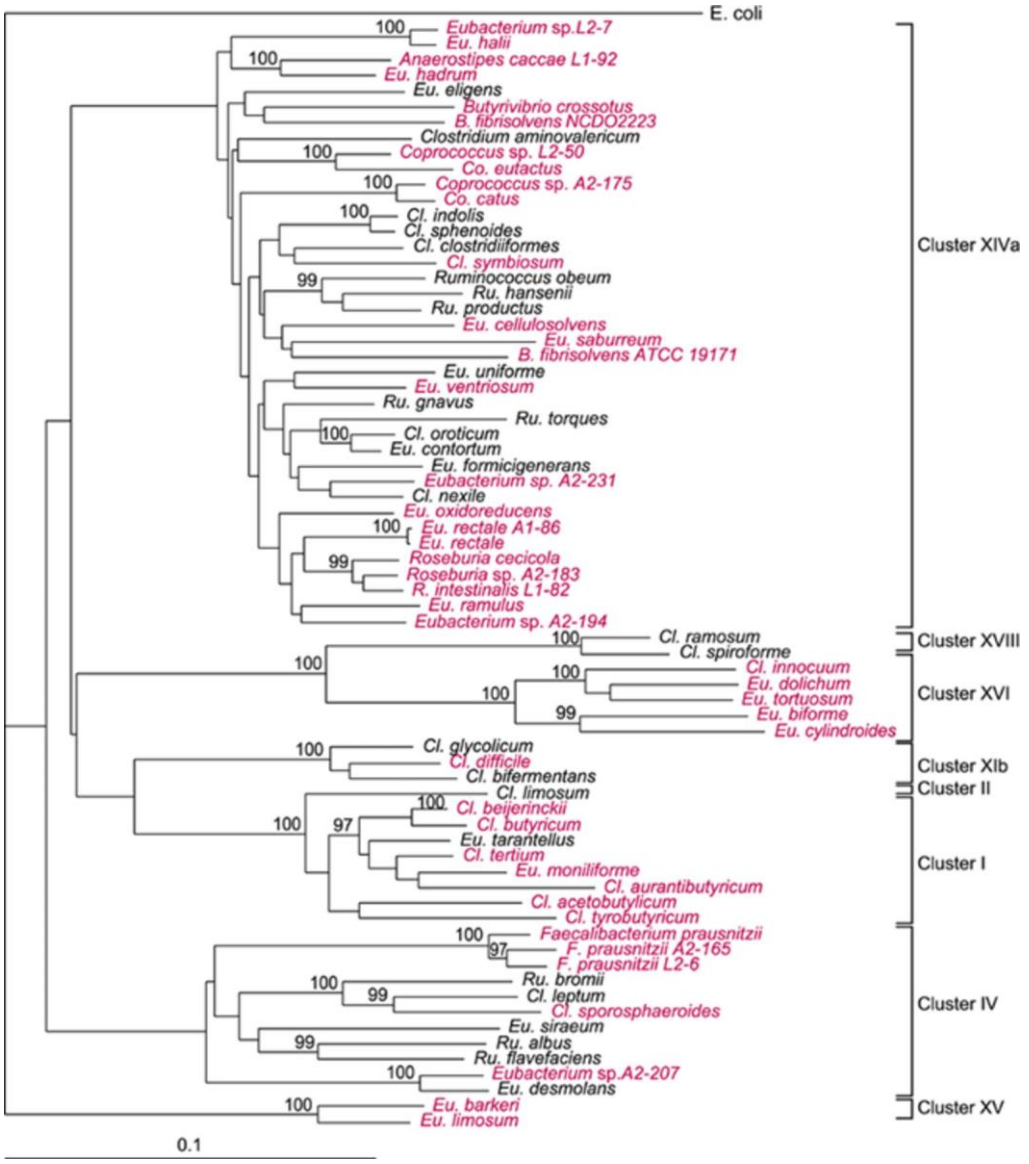


Figure 3. Distribution of butyrate-producing species across different clostridial clusters within the order Clostridiales. *Cultural and molecular studies indicate that most of the butyrate-producing bacteria found in human feces so far, are highly oxygen-sensitive Clostridium-related anaerobes that are widely distributed across several clostridial clusters. This phylogenetic tree shows the inter-relationship of 16S rRNA sequences from butyrate- (red) and non-butyrate-producing (black) bacteria. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. The scale bar represents genetic distance (10 substitutions per 100 nucleotides). Adapted from Pryde et al. 2002.*

One butyrate producer in particular, *Butyricicoccus pullicaecorum* (*B. pullicaecorum*), was extensively researched during this thesis because of its ability to produce high concentrations of butyrate. It was first identified as a novel isolate obtained from the caecal content of a 4-week-old broiler chicken. This isolate was shown to represent a single novel species within a novel genus, for which the name *Butyricicoccus pullicaecorum* gen. nov., sp. nov. was proposed.[154,155] Cells are Gram-positive,

anaerobic, non-motile, coccoid and usually arranged in pairs (occasionally in short chains). The type strain *B. pullicaecorum* 25-3^T utilises acetate and small amounts of proprionate to produce H_2, CO_2 and butyrate. With a secretion of 18.6±1.2 mM butyrate after overnight growth at 42°C in anaerobic M2GSC broth, *B. pullicaecorum* 25-3^T classifies as a high-concentration butyrate producer. Phylogenetic analysis based on 16S rRNA gene sequences demonstrated that *B. pullicaecorum* represents a novel lineage within the *Clostridium* cluster IV in the phylum *Firmicutes*, with *Eubacterium desmolans* as its closest phylogenetic neighbour (about 93% similarity). A human *B. pullicaecorum* 1.20 isolate has since also been isolated. The abundance of the genus *Butyricicoccus* in human fecal samples is low: 0.05% to 0.4% of total fecal microbiota.[156]

1.2.5 Butyrate-producing bacteria as pharmabiotics for IBD

Given its prominent anti-inflammatory and barrier-protective properties, there is increasing interest in using butyrate to restore mucosal homeostasis in IBD. However, its routine clinical application has been impeded by practical issues. Oral butyrate administration in the form of enteric-coated tablets has shown promising results in a small study of patients with mild to moderate CD[157], but the use of tablets does not guarantee controlled release at the intended location and butyrate might not reach the colon in sufficient amounts due to its rapid absorption. Besides, butyrate has a very unpleasant taste and smell. Butyrate enemas have proven to be effective in treating distal UC[158,159], but they are cumbersome for the patient and exposure of the colonic mucosa to the butyrate is brief and discontinuous. The administration of naturally colonising butyrate-producing bacteria that would continuously secrete butyrate into the colonic lumen is an alternative strategy to locally increase butyrate concentrations.

Research into the use of probiotics to treat IBD has been ongoing for almost two decades and results from clinical studies have generated considerable excitement, despite a relative lack of rigorously designed, randomised, placebo-controlled trials. Data from these studies show that probiotics are capable of preventing relapse and some can even treat mildly active IBD.[160] Most studies report prolonged maintenance of remission in UC patients[69,105,160] and, although the use of probiotics in prevention and treatment of CD is less substantiated than for UC, some trials in CD patients also show promising results.[105,160,161] The most convincing evidence of the clinical efficacy of probiotics in IBD to date, is a prolonged remission time of chronic pouchitis in post-operative UC patients using the probiotic cocktail VSL#3.[162-164] VSL#3 is a multispecies mix of o.a. lactate-producing lactobacilli and bifidobacteria. In fact, most probiotics tested in these clinical trials are lactate-producing bacteria that may indirectly affect butyrate levels in the gut by cross-feeding butyrate producers that are capable of using lactate.

Clinical trials with butyrate producers in particular are scarce. Efficacy of a *Clostridium butyricum* strain with a proven ameliorating effect in experimental colitis[165], and which is already being used in a clinical setting as a probiotic for patients with functional gastro-intestinal disorders[166], is currently under evaluation in two clinical trials for irritable bowel syndrome. *F. prausnitzii* is currently not being evaluated in clinical trials despite potent anti-inflammatory effects in PBMCs, Caco-2 IECs and two models of acute experimental colitis.[123,167] Finally, *Butyricicoccus* bacteria are also conceptually attractive as probiotics given their natural occurrence within the human gut, their decreased abundance in stool samples of IBD patients, the ability of a specific *Butyricicoccus* strain (*B. pullicaecorum* 25-3^T) to decrease lesion sizes and inflammation in a rat colitis model, and the capacity of *B. pullicaecorum* 25-3^T conditioned medium to prevent cytokine-induced increase in epithelial permeability *in vitro*.[124]

1.3 EPITHELIAL DYSFUNCTION IN IBD: A ROLE FOR RHO KINASE INHIBITION?

In the third part of this introduction, we will elaborate on the morphology and functions of the intestinal epithelium and on the crucial role of tight junctions in maintaining barrier integrity. We will describe deregulated tight junction gene expression and Rho kinase-mediated tight junction protein internalisation as determinants of pathophysiological tight junction deregulation in IBD and discuss the potential of targeting Rho kinases to preserve tight junction integrity.

1.3.1 Morphology of the intestinal epithelium

The epithelium is crucial to intestinal homeostasis by forming both a physical and a functional barrier between the gut lumen and the underlying mucosal compartment (Figure 4). This physical barrier is made up of a single layer of intestinal epithelial cells (IECs) tightly held together through apical junctional complexes, which restricts translocation of luminal antigens and other macromolecules into the underlying interstitium while at the same time allowing selective passage of water, electrolytes and nutrients. Junctional complexes also polarise the IECs by separating the apical from the basolateral membrane compartments.[168]

Within the epithelial layer, four types of IECs can be discriminated, all of which originate from stem cells at the base of the crypts and differentiate upwards along the crypt-villus axis. Absorptive cells, called enterocytes in the small intestine and colonocytes in the colon, are responsible for digestion and absorption of nutrients, water and electrolytes. Enteroendocrine cells secrete peptide hormones that regulate cellular homeostasis, and Paneth cells control bacterial growth by producing antimicrobial peptides. Finally, goblet cells secrete glycosylated mucins that form a protective mucus layer that physically shields the IECs from the luminal environment.[169,170]

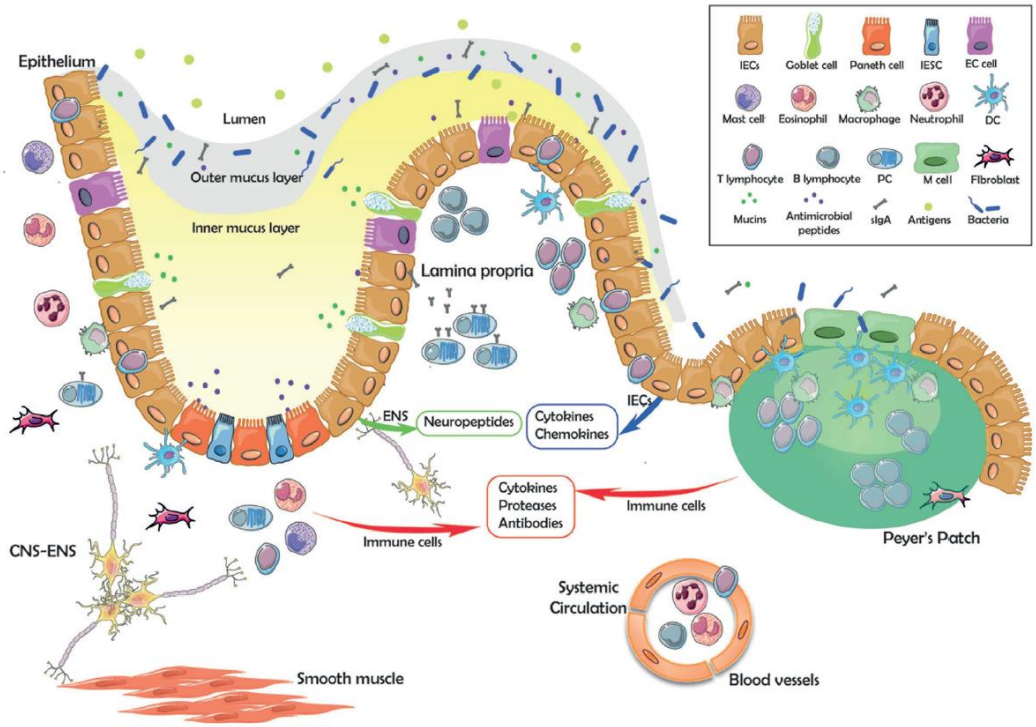


Figure 4. Intestinal epithelial barrier anatomy and components. *The intestinal mucosa comprises a layer of polarised, columnar epithelial cells and a subepithelial region that contains the lamina propria, enteric nervous system, connective tissue, and muscular layers. The epithelium includes enterocytes, goblet cells (which synthesise and release mucin), Paneth cells (which synthesise antimicrobial peptides), enterochromaffin cells (which produce hormones and other substances), and intestinal stem cells. Above the epithelial barrier lies the inner mucus layer; this layer in turn underlies the outer mucus layer, which contains microbiota, secretory IgA, mucins, and antimicrobial peptides. Intraepithelial lymphocytes are above the basement membrane, underlying the tight junctions. The lamina propria includes a diffuse lymphoid tissue made up of macrophages, dendritic cells, plasma cells, lamina propria lymphocytes, and on occasion neutrophils, and a structured lymphoid tissue made up of Peyer's patches, which contain M cells, dendritic cells, and lymphocytes. CNS, central nervous system; ENS, enteric nervous system; IEC, intestinal epithelial cell; ECC, enterochromaffin cell; IESC, intestinal epithelial stem cell; DC, dendritic cell; PC, plasma cell, sIgA, secretory IgA. Adapted from Salvo-Romero et al. 2015.*

1.3.2 Intestinal epithelial cells as sentinels of innate immunity

The intestinal epithelium is not merely a static barrier against intruders but a dynamic, sensory apparatus necessary for host nutrition, defence and immune system development. This is why IECs are equipped with membrane and intracellular receptors that recognise various microbe-associated molecular patterns (MAMPs). Under normal conditions, routine detection of commensal MAMPs does not elicit an acute inflammatory response and serves to educate the mucosal immune system towards oral tolerance. More specialised antigen-presenting cells (APCs) positioned in-between the IECs, such as ileal M cells, intraepithelial lymphocytes, and resident dendritic cells (DCs) and macrophages, also assist in immune surveillance by actively sampling luminal antigens and inducing tolerogenic, regulatory T cell responses.[171] Indirect luminal sampling and antigen presentation to mucosal lymphocytes can also occur via the major histocompatibility complex class II molecules expressed constitutively on the basolateral membranes of the IECs, which allows them to act as non-

professional APCs.[170,172] In contrast, when invasive pathogens are detected, an acute pro-inflammatory response is mounted, involving bacterial clearance and epithelial restitution. One of the ways in which IECs distinguish commensal detection from pathogen invasion, is through spatial expression of TLRs on their membranes. Although the role of epithelial TLRs in innate immunity is beyond the scope of this introduction, we will include TLR5 as a prime example of the physiological relevance of spatial TLR expression on IECs. Under normal circumstances, this receptor for bacterial flagellin is expressed exclusively on the basolateral side of polarised human IECs *in vitro* and native human colonic mucosa. Only flagellin exposure to the basolateral, but not the apical, surface of intact epithelium elicits the secretion of pro-inflammatory cytokines like IL-8, whereas flagellin exposure to an injured colonic mucosa due to DSS administration in mice does result in a TLR5-associated response.[173,174] This divergent response would explain how TLR signalling only elicits a pro-inflammatory response when TJ integrity is compromised and invasive bacteria have actually crossed the epithelial barrier while TLR stimulation by commensals on the apical side of an intact epithelial monolayer induces a homeostatic, anti-inflammatory response.[175]

Besides modulating mucosal microbial responses through induction of soluble mediators for local and systemic immune control, TLR signalling in the gut is also actively involved in maintenance of epithelial barrier homeostasis. Secretion of antimicrobial peptides by Paneth cells, increasing barrier tightness through affecting TJ gene expression by IECs, and production of trefoil factors essential to epithelial restitution by goblet cells are all dependent on bacterial TLR activation. The beneficial role of TLR signalling in maintaining epithelial barrier function is evidenced o.a. by the observation that mice deficient in TLR4 or the TLR adaptor protein MyD88, are both more susceptible to DSS-induced colitis and show increased bacterial translocation.[175-179]

1.3.3 Tight junctions as determinants of intestinal epithelial barrier integrity

There are three types of junctional complexes that link IECs together: tight junctions (TJ), adherens junctions (AJ) and desmosomes (Figure 5). All three are made up of transmembrane protein complexes that interact with their counterparts on the adjacent cell membrane and are linked to cytoskeletal filaments via peripheral adaptor proteins. Adherens junctions and desmosomes are located in the lateral membrane of IECs and are thought to be important in mechanically linking cells at a particular distance (~20 nm apart). The TJs are the apical-most junctional complexes located at the border between the apical and lateral membrane regions. They create an impermeable epithelial barrier by physically sealing off the intercellular space and regulate selective paracellular ionic solute transport.

Within the TJ structure, there are four known families of transmembrane proteins: occludin (OCLN), claudins (CLDN), junctional adhesion molecules (JAM) and tricellulin. Homo- and heterophilic

interactions among these proteins are made between adjacent cells, but also within the same membrane. Usually, several so-called TJ strands are present at the apical membrane, which are interconnected through interlocking junctional complexes. These strands form a circumferential belt-like ring around the IECs. Integral TJ proteins are linked to the cytoskeletal actin filaments through a cytosolic plaque made up of adaptor proteins, such as cingulin and zonula occludens (ZO) proteins. This cytoplasmatic plaque also contains signalling proteins like Rho GTPases that are involved in junctional assembly and barrier regulation.[168,180,181] The three tight junction proteins relevant to this work; zonula occludens 1 (ZO-1), OCLN and claudin-1 (CLDN1) are discussed in more detail below.

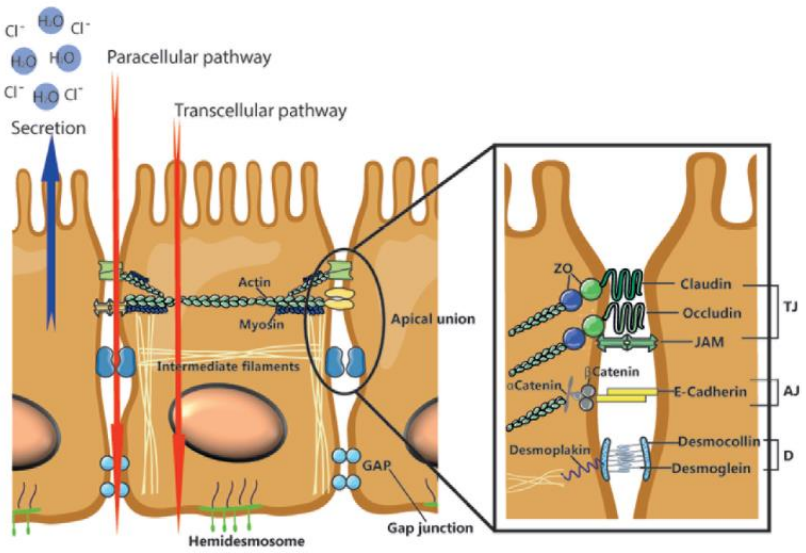


Figure 5. A representation of epithelial intercellular junctions. *Absorptive enterocytes primarily focus on nutrient absorption, and water and chlorine secretion into the intestinal lumen. Substances in the intestinal lumen may move across the epithelium via the transcellular pathway or the paracellular pathway. Intercellular junctional complexes, including tight junctions, adherens junctions, gap junctions and desmosomes, are dynamic structures that restrict the passage of macromolecules. The integrity and structure of epithelial cells are mostly modulated by the cytoskeleton, mainly by actomyosin and intermediate filaments. Cells adhere to the basement membrane through hemidesmosomes. The apical junctional complex is highlighted: tight junctions are primarily made up of claudins, occludins and JAM proteins, which are linked to the actin cytoskeleton via adaptor proteins like zonula occludens. Adherens junctions include cadherins such as E-cadherin, which is linked to the actin cytoskeleton via the catenin adaptor protein. Desmosomes are mainly comprised of desmocollin and desmoglein, which are linked to the intermediate filaments via the desmoplakin protein. TJ; tight junction, AJ; adherens junction; D, desmosome; ZO, zonula occludens; JAM, junctional adhesion molecules. Adapted from Salvo-Romero et al. 2015.*

1.3.3.1 Zonula occludens 1

Zonula occludens 1 or tight junction protein 1 (TJP1) (~220 kDa) contains 3 PDZ (PSD95, Dlg and ZO-1) -containing domains in its N-terminus through which interactions with other TJ proteins occur. The proline-rich C-terminal half binds to the actin filaments. Two different isoforms of ZO-1, alpha-minus and alpha-plus, have been described which result from alternative mRNA splicing. The alpha-plus isoform contains an 80 amino acids motif called alpha which is not present in the alpha-minus isoform. Only the alpha-containing isoform is found in epithelial cell junctions.[182] In epithelial cells,

claudin assembly into TJ strands is dependent on expression of ZO proteins, and cells lacking ZO-1 and ZO-2 fail to form TJs at all. It would seem that interaction between claudins and the PDZ-domain of ZO proteins is necessary for efficient junctional claudin delivery.[183]

1.3.3.2 Occludin

Occludin (60-82 kDa) was the first TJ protein to be identified and is a tetraspanning integral membrane protein with 2 extracellular loops, a short cytoplasmatic N-terminus and a long cytoplasmatic C-terminus. Several OCLN isoforms exist due to alternative mRNA splicing. Subcellular distribution and interaction with other TJ proteins is determined by the type of splice variant.[184] Functional analysis indicated that the transmembrane domains and extracellular loops regulate selective paracellular permeability, while the intracellular C-terminus interacts with the PDZ-containing domains of ZO-1.[168] Despite the fact that OCLN overexpression increases TEER, neither TJ formation nor paracellular barrier function is dependent on OCLN, as demonstrated by the fact that $Ocln^{-/-}$ mice show no changes in TJ appearance or intestinal permeability and that OCLN is not by itself sufficient to form TJ strands. However, occludin function within the TJ can be modulated through phosphorylation. For example, inhibition of PKCη-mediated phosphorylation of OCLN at specific threonine residues in the C-terminal domain disrupted junctional distribution of OCLN, abolished interactions with ZO-1, and compromised paracellular barrier function.[168,181]

1.3.3.3 Claudins

Claudins (20-27 kDa) also contain 4 transmembrane domains, 2 extracellular loops and cytoplasmatic N- and C-terminal domains, but do not show any sequence similarity to occludin.[181] The extracellular loops are critical for homo- and heterophilic interactions with other TJ proteins and for the formation of ion-selective paracellular pores. The C-terminal end anchors the protein to the cytoskeleton through interactions with PDZ domain-containing adaptor proteins. Up to now, 27 members of the claudin family have been identified in humans that are expressed in a tissue-specific manner.[168] Unlike OCLN, the 2 extracellular loops in claudins have a variable number and distribution of several charged amino acid residues, which are crucial to determining paracellular ionic charge and size selectivity of the TJ.[185] Differences in permeability between claudins has led to the identification of pore-forming and pore-sealing claudins, which respectively increase paracellular permeability by creating channels, or decrease it by sealing off the TJ.[186] Claudins forming paracellular channels exhibit 3 types of selectivity; anion, cation and water selectivity. Pore-sealing claudins exhibit strict barrier properties in either an almost charge and size non-selective way, or with higher effectivity for one or the other charge.[187] An example of a cation-selective, pore-forming claudin is CLDN2, which acts as a paracellular water channel and is typically expressed in leaky, water-transporting epithelia like the small intestine.[188,189] CLDN1 on the other hand, is a pore-sealing claudin, which contributes to

barrier tightness by increasing TEER and reducing paracellular flux, and is typically expressed in tight epithelia such as the skin.[190,191] This combination of pore-sealing and pore-forming claudins within the same protein complex allows modulation of the degree of barrier permeability and enables TJs to perform their so-called "gate function", by acting both as a barrier that prevents unlimited passage of water, solutes and luminal antigens while at the same time functioning as a channel that still allows selective transport through the TJ. Like with OCLN, claudin function is further modified through phosphorylation, e.g. CLDN1 phosphorylation by mitogen-activated protein kinases (MAPK) is required to enhance the barrier function of CLDN1-based TJs.[192]

1.3.4 Pathophysiological deregulation of tight junction integrity in IBD

Whether epithelial barrier dysfunction is a consequence of the inflammatory response in IBD, or a primary defect that prompts mucosal inflammation is still a matter of intense debate. However, there is substantial evidence that compromised TJ integrity is an early event in IBD pathogenesis, which would support the suggestion that IBD is an impaired barrier disease with increased intestinal permeability preceding the onset of mucosal inflammation.[169] First, genetic susceptibility to increased intestinal permeability is demonstrated by the fact that several of the IBD candidate genes identified so far are involved in intestinal epithelial barrier regulation, including TJ assembly and regulation, cell adhesion and polarity, mucus and glycoprotein regulation, bacterial sensing, membrane transport, epithelial differentiation and restitution.[193] Secondly, increased intestinal permeability has been documented in a subset of clinically healthy, first-degree relatives of CD patients. Also, CD patients with quiescent disease who display increased intestinal permeability have an elevated risk of relapse. Interestingly, the observed presence of this permeability defect in genetically unrelated relatives of CD patients, e.g. spouses, suggests that this abnormal permeability may be secondary to environmental as well as genetic factors.[169,194-196]

Microscopically, this increased intestinal permeability is characterised by a reduced number of TJ strands as well as increased strand breaks.[197] This loss of TJ integrity is the result of inflammation-induced deregulation of three distinct processes that are responsible for the continuous remodeling of TJ structures under physiological conditions; *de novo* TJ gene expression, proteolytic cleavage of transmembrane TJ proteins, and kinase-mediated internalisation of TJ components.

1.3.4.1 Deregulated tight junction gene expression

Sustained inflammation in IBD is associated with mucosal overproduction of pro-inflammatory cytokines secreted predominantly by various cells of the innate and adaptive immune system, and to some extent by the IECs themselves as well. Prominent Th1/Th17 cytokines produced in affected mucosa of CD patients are TNF, IFNγ, IL-6, IL-17 and IL-23, while Th2 responses in UC patients induce

expression of o.a. IL-5, IL-6, IL-13 and TNF.[198] We will only discuss expressional deregulation of ZO-1, OCLN and CLDN1, the three TJ proteins that were investigated during this research.

1.3.4.1.1 Loss of zonula occludens 1 and occludin expression during intestinal inflammation

Reduced expression of both ZO-1 and OCLN has been described in colonic biopsies from CD and UC patients with active disease.[199-201] *In vitro*, a decrease in ZO-1 and OCLN protein levels is induced by TNF in IECs, which is associated with an increase in TJ permeability.[201,202] Loss of ZO-1 protein expression and concomitant increased permeability preceding the development of significant intestinal inflammation is also observed in acute DSS-induced colitis.[203]

1.3.4.1.2 Ambivalent role of claudin-1 in health and disease

Claudin expression is also severely disturbed in IBD but the change in expression is dependent on the type of claudin. In general, expression of pore-sealing claudins is decreased (e.g. CLDN3, CLDN4, CLDN5 and CLDN8), while expression of pore-forming claudins is increased (e.g. CLDN2).[187,199] Remarkably, increased claudin-1 (CLDN1) protein levels have been documented in IBD and in acute DSS-induced colitis despite it being a pore-sealing claudin.[201,203,204]

Under normal physiological conditions, CLDN1 acts as a key pore-sealing TJ protein crucial to epithelial barrier integrity; its genetic deletion results in rapid postnatal death due to severe epidermal permeability defects.[205] Also, baseline CLDN1 overexpression *in vitro* results in increased barrier tightness while its concomitant knockdown decreases it.[145] Under inflammatory conditions however, the role of CLDN1 is much less straightforward. For example, *in vitro* pro-inflammatory cytokine-induced upregulation of CLDN1 coincides with a redistribution of the protein away from the TJs and a concomitant increase in intestinal permeability.[206,207] Apparently, elevating CLDN1 expression under baseline conditions increases epithelial barrier integrity, whereas under inflammatory conditions, its internalisation causes a weakening of the intestinal barrier despite an increase in overall expression. The *in vivo* relevance of CLDN1 overexpression is demonstrated by the observation that intestinal epithelial overexpression renders mice more susceptible to colitis and impairs their recovery.[208] It appears therefore, that the function of CLDN1 is not restricted to its traditional role of maintaining intestinal barrier function, and that its elevation during mucosal inflammation is detrimental to both epithelial integrity and further downstream pathways responsible for colonic homeostasis.

1.3.4.2 Proteolytic cleavage of transmembrane tight junction proteins

The extra- and intracellular domains of epithelial transmembrane junction proteins are amenable to proteolytic cleavage by extracellular proteases or sheddases (e.g. matrix metalloprotease (MMP), serine protease, bacterial protease) and intracellular proteases (e.g. calpain, capsase, γ-secretase), respectively. Under physiological circumstances these cleavage products are generated as a result of

the continuous turnover of transmembrane junctional proteins and seem to exert distinct biological effects. For example, extracellular cleavage fragments help control normal homeostatic epithelial events like cell proliferation, migration and apoptosis, whereas intracellular cleavage products can translocate to the nucleus to regulate transcriptional activity and cell survival. Recent studies have revealed that in pathological conditions such as inflammation, increased junctional cleavage products are often detected. However, current knowledge on the effect of TJ protein cleavage and their extra- and intracellular cleavage products in inflammation as well as their contribution to intestinal pathogenesis is limited.[209] Most of the results obtained so for concern OCLN and the observation that extra- and intracellular cleavage of this TJ protein is associated with a decrease in epithelial barrier function. Huet *et al*. have reported that the induction of MMP9, a protease whose total levels and activity are increased in the inflamed IBD mucosa[210], results in cleavage of the OCLN extracellular domain and disruption of barrier function associated with pathogenesis of dry eye disease.[211] A perturbation of TJ integrity and decrease in epithelial barrier function can also be induced through extracellular OCLN cleavage by several bacterial proteases, including a heamagglutinin/protease from *Vibrio cholera*[212] and an aerolysin from *Aeromonas hydrophila*[213], two pathogens that cause clinical diarrhea in humans. Occludin is also cleaved intracellularly by a calpain protease activated by the Group A *Streptococcus* virulence factor Streptolysin S, which disrupts barrier function of IECs and keratinocytes.[214] Calpain protease activity is also induced by TLR2 signalling in response to bacterial pathogen recognition in airway epithelial cells, which leads to intracellular cleavage of OCLN in order to a accommodate transmigration of the recruited leukocytes.[215] Finally, Willemsen *et al*. have demonstrated that IFNγ activates serine proteases in IECs *in vitro* that cleave both the intra- and extracellular domains of CLDN2 .[216] Interestingly, serine protease inhibition completely abrogated IFNγ-mediated barrier disruption, which was associated with preservation of CLDN2 expression.

1.3.4.3 Kinase-mediated tight junction internalisation

Besides altering TJ gene expression, pro-inflammatory cytokines also compromise barrier integrity through kinase-mediated internalisation of TJ proteins. A significant body of evidence has accumulated in recent years, identifying TJs as targets of multiple kinases that are activated under diverse (patho)physiological conditions and which enhance or restrict paracellular permeability as well as regulate the formation and disassembly of TJs.[185] In this section, we will only elaborate on the role of Rho-associated kinases (ROCK) and myosin light chain kinases (MLCK) in cytokine-induced TJ internalisation.

1.3.4.3.1 Rho/ROCK and MLCK signalling regulates actomyosin cytoskeletal TJ dynamics

Rho-associated kinases (~160 kDa) are serine/threonine kinases consisting of an N-terminal kinase domain, followed by a potential coiled-coil-forming region containing a Rho-binding domain, and a C-

terminal end containing a pleckstrin homology domain with an internal cysteine-rich domain. Two ROCK isoforms have been identified; ROCK1 and ROCK2, which are ubiquitously expressed in human and mouse tissues. The amino acid sequences of both isoforms show a 65% overall similarity, with the kinase domains showing the highest similarity (92%). In the inactive ROCK, the C-terminal pleckstrin homology domain and Rho-binding domain bind to the N-terminal kinase domain, forming an autoinhibitory loop. The Rho GTPase molecular switch protein binds to the Rho-binding domain of ROCK only in its activated form. Activation of the Rho protein, which entails a conversion from a GDP-bound (inactive) to a GTP-bound (active) conformation, occurs through guanine nucleotide exchange factors, which are themselves activated in response to the binding of extracellular chemical (e.g. growth factors, hormones, cytokines) or physical (e.g. adhesion to the extracellular matrix) stimuli to their respective transmembrane receptors.[217] The interaction between the GTP-bound Rho GTPase and the Rho-binding domain of ROCK is believed to disrupt the negative regulatory interactions between the kinase domain and the C-terminal autoinhibitory region, which frees the ROCK catalytic activity. Activation of MLCK on the other hand, is dependent on a conformational change induced by the binding of a Ca^{2+}-Calmodulin complex, which exposes the catalytic site of the kinase. The main ROCK phosphorylation substrates pertaining to epithelial cytoskeletal dynamics are the non-muscle myosin II light chain (MLC), MLC phosphatase (MLCP) and Lin11, Isl1 and Mec3 kinase (LIMK) (Figure 6). Both ROCK and MLCK phosphorylate MLC at the same serine residue, which enables the binding of myosin to the actin cytoskeletal filaments and allows fiber contraction to begin. ROCK action also increases levels of phosphorylated MLC by inhibiting its dephosphorylation by MLCP. ROCK phosphorylation of LIMKs at specific threonine residues enhances ability of LIMKs to phosphorylate cofilin, an actin-depolymerising protein, which also stabilises actin filaments.[218] Linkage of these actin filaments to the TJs via the cytoplasmatic plaque stabilises the junctional complex at the cell border, but also provide the force for TJ disruption upon contraction of the perijunctional actomyosin ring following MLC phosphorylation. The Rho/ROCK signalling pathway thus participates both in the assembly and disassembly of TJs.

Of note is that ROCK activity can also affect paracellular permeability by directly phosphorylating TJ proteins. For example, phosphorylation of OCLN and CLDN5 by ROCK has been reported in brain endothelial cells and correlates with diminished barrier tightness.[219]

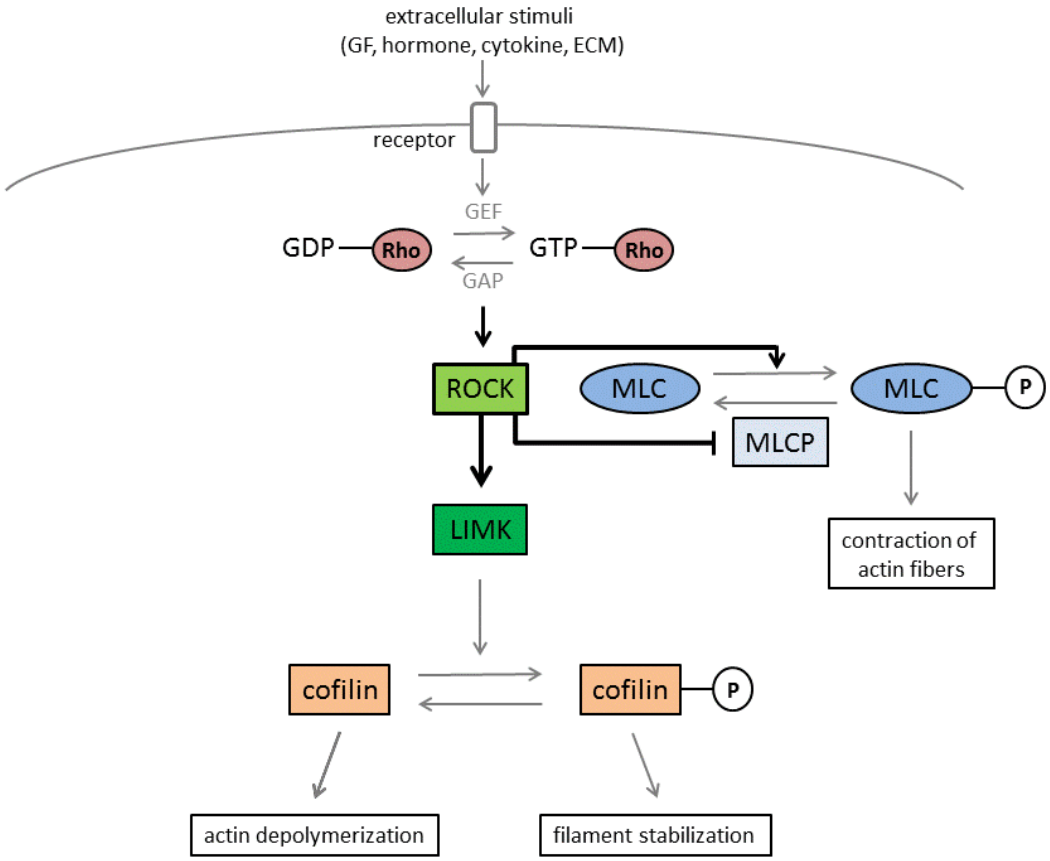


Figure 6. Actin cytoskeletal ROCK targets. *Extracellular stimuli induce activation of the guanine exchange factor (GEF), which in turn actives the Rho GTPase molecular switch protein by stimulating the release of GDP to allow binding of GTP. The Rho protein is cycled back to its inactive form by the GTPase-activating protein (GAP), which enhances the intrinsic GTP-hydrolysis activity of Rho. The GTP-bound Rho activates ROCK, which phosphorylates several substrates pertaining to actin cytoskeletal dynamics. ROCK activity directly enhances contraction of actin fibers by phosphorylating the non-muscle myosin II light chain (MLC). ROCK also indirectly increases phosphorylated MLC levels through phosphorylation of the myosin binding subunit of the myosin light chain phosphatase (MLCP), thereby inhibiting MLCP-mediated dephosphorylation of MLC. ROCK phosphorylation of LIMKs enhances ability of LIMKs to phosphorylate cofilin, an actin-depolymerising protein, which also stabilises actin filaments. GEF, guanine exchange factor; GAP, GTPase-activating protein; ROCK, Rho-associated kinase; MLC, myosin light chain; MLCP, myosin light chain phosphatase; LIMK, Lin11, Isl1 and Mec3 kinase. Adapted from Wikipedia.*

1.3.4.3.2 Cytokine-induced TJ internalisation is mediated by ROCK/MLCK activation

The *in vitro* detrimental effect of pro-inflammatory cytokines like TNF, IFNγ and IL-1β on IEC paracellular permeability has been documented extensively, with altered junctional localisation of OCLN, ZO-1 and CLDN1 being induced through an NF-κB-mediated, apoptosis-independent process.[202,206,220-222] Further research revealed a role for both ROCK and MLCK in this inflammation-induced internalisation process. Utech *et al.* described how IFNγ selectively upregulates expression of ROCK, but not of MLCK, to induce mammalian non-muscle myosin II-dependent endocytosis of OCLN and CLDN1 into actin-coated vacuoles at the apical plasma membrane of T84 monolayers.[223] The involvemement of MLCK in TJ internalisation and concomittant barrier disruption was demonstrated *in vitro* in a study by Wang *et al.*.[71] They showed that IFNγ and TNF synergise to induce

intestinal epithelial barrier dysfunction in Caco-2 monolayers, measured by a drop in TEER, which was associated with an upregulated MLCK expression and an increase in MLC phosphorylation. This TNF/IFNγ-induced loss of barrier function correlated with a morphological disruption of the TJ structure, caused by an evident removal of ZO-1, OCLN and CLDN1 off the TJs into intracellular pools. Also *in vivo*, MLCK activation and subsequent MLC phosphorylation were shown to be essential for cytokine-mediated intestinal barrier dysfunction. In mice, systemic T cell activation following the administration of anti-CD3 antibodies causes acute immune-mediated diarrheal disease associated with barrier dysfunction and induction of IFNγ and TNF, which can be blocked by antagonism of either cytokine.[224] Phosphorylation of MLC increased abruptly after T cell activation and coincided with the development of diarrhea. Genetic knockout of MLCK or treatment of wild-type mice with a highly specific peptide MLCK inhibitor prevented epithelial MLC phosphorylation, TJ disruption characterised most predominantly by OCLN internalisation into intracellular vesicles, and diarrhea following T cell activation. Myosin light chain kinase as also shown to be involved in endotoxemia-induced increase in intestinal epithelial permeability, as demonstrated in a study by Moriez *et al*. who showed that LPS-induced increase in colonic paracellular permeability was associated with changes in TJ morphology and increased MLC phosphorylation.[225] Addition of the selective ML-7 MCLK inhibitor prevented all LPS-induced effects, suggesting that inhibition of MLCK-dependent colonocyte cytoskeletal contraction prevents LPS-induced alterations in paracellular permeability and its subsequent effects. The aforementioned observation that *in vivo* TNF-induced MLC phosphorylation, OCLN internalisation, paracellular barrier loss, and diarrhea are all prevented by genetic or pharmacological MLCK inhibition, suggests that these events are closely linked. The exact mechanism of TNF-induced, MLCK-mediated OCLN internalisation and the fact whether this endocytotic event is required for *in vivo* barrier loss, was further elucidated by Marchiando *et al*.[226] In an *in vivo* mouse model in which barrier disruption and subsequent diarrhea is induced by intraperitoneal TNF injection, OCLN internalisation was identified as the most obvious change in the TJ structure and was shown to precede intestinal fluid secretion. This OCLN removal from the TJs is central to both the barrier defect and diarrhea associated with TNF treatment, as evidenced by the fact that OCLN overexpression as well as chemical and genetic inhibition of caveolin-1-mediated OCLN endocytosis limited barrier loss and prevented diarrhea in this model. Together, these studies demonstrate the essential role of MLCK-triggered, caveolin-1-dependent OCLN endocytosis in TNF-induced loss of barrier integrity.

1.3.5 Therapeutic potential of Rho-associated kinase inhibition in intestinal inflammation

The involvement of ROCK activity in inflammation-induced epithelial barrier function has prompted research into the use of ROCK inhibitors, such as Y-27632 and fasudil, to ameliorate inflammation in

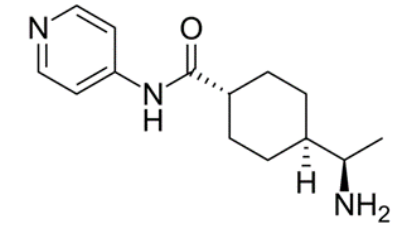
several pathologies. For example, ROCK blockade using Y-27632 in a mouse model of LPS-induced renal failure alleviates inflammation by attenuation of NF-κB signalling.[227] Y-27632 and fasudil also decreased *in vitro* production of TNF, IL-1β and IL-6 by synoviocytes and PBMCs from patients with active rheumatoid arthritis in an NF-κB-dependent manner.[228] The first *in vivo* evidence of a role for RhoA-dependent signalling in IBD was provided by Segain *et al.* who reported an increased activation of RhoA and its effector ROCK in the inflamed intestinal mucosa of CD patients and of rats with TNBS-induced colitis. They showed that blockade of ROCK using Y-27632 inhibited pro-inflammatory cytokine production by LPMCs and PBMCs via inhibition of NF-κB activation, and markedly reduced colonic inflammation in TNBS-colitis rats.[229] An additional barrier-protective effect of ROCK inhibition was observed in an LPS-induced endotoxemia model in rats, in which administration of Y-27632 markedly decreased apoptosis of IECs.[230] Collectively, these data illustrate the potential of specific ROCK inhibition as a novel therapeutic approach to attenuate intestinal inflammation in IBD.

However, there are a few reservations concerning the therapeutic use of current ROCK inhibitors like Y-27632. First, although Y-27632 is classified as a specific ROCK inhibitor and is commonly used to study ROCK functions, it also inhibits other protein kinases, especially at higher concentrations.[218,231] Secondly, important side effects such as cardiovascular hypotension warrant for great caution if these compounds reach systemic circulation.[232]

This is why Amakem Therapeutics developed AMA0825, a potent and selective ROCK1/2 inhibitor specifically designed to have a localised action in the intestine. AMA0825 is structurally related to the reference ROCK inhibitor Y-27632 (Figure 7A). However, due to an international patent application[233], the exact chemical structure of AMA0825 cannot be disclosed. Briefly, the central cyclohexane ring in the ROCK inhibitor scaffold of Y-27632 is replaced by an aromatic phenyl ring in the AMA0825 compound. This phenyl ring is substituted by an aromatic side chain (i.e. an arylamide group), which in itself is further substituted by a cyclic ester group. The ester moiety is amenable to hydrolysis by esterase enzymes present in the blood, which yields a corresponding carboxylic acid metabolite with negligible functional activity compared to the parent compound. This is the so-called soft drug approach in which biologically active compounds are designed to undergo metabolic inactivation by controlled conversion of the parent molecule into a predictable, non-toxic metabolite with a markedly decreased functional activity. As a soft drug, AMA0825 has therefore been optimised as a locally acting compound with sufficient stability in its target organ while being rapidly inactivated once it enters the systemic circulation.[234,235] AMA0825 has been profiled for its *in vitro* on-target potency against ROCK2, revealing an IC_{50} value of less than 0.05 nM, which is approximately a 1000 times lower than the IC_{50} value of Y-27632 in the same assay (Figure 7B). In a second line of testing, cellular activity of AMA0825 was evaluated using an MLC phosphorylation assay in the rat smooth

muscle cell line A7r5, which endogenously expresses ROCK. In this functional assay, AMA0825 displayed an EC_{50} value of 40 nM, which is approximately 25 times lower than the EC_{50} value of Y-27632 in the same assay (Figure 7B). Selectivity of AMA0825 was tested on a panel of more than 340 kinases at a concentration of 100 nM. Kinases whose activity was inhibited by 50% or more are shown as yellow and red dots in the selectivity profile (Figure 7C). The AMA0825 IC_{50} values for some of these kinases are listed in Figure 7D. Off-target inhibition of other kinases besides ROCK is limited; cross-reactivity with PKC δ, ε and θ was observed but with considerably higher IC_{50} values of 5, 20 and 100 nM, respectively. Potent and selective ROCK targeting without the systemic side-effects of traditional inhibitors, therefore represents a promising treatment option for *in vivo* counteraction of inflammation-induced epithelial dysfunction.

A



B

	AMA0825	Y-27632
IC_{50} (nM) – ROCK2	< 0.05	54
EC_{50} (nM) – MLC-PP	40	1000

C

D

Selectivity AMA0825 (100 nM)		
kinase	% activity	IC_{50} (nM)
DMPK2	11	NT
LATS2	45	NT
PKCδ	8	5
PKCε	16	20
PKCθ	69	100
PRK1	9	NT
PRK2	18	NT
ROCK1	2	NT
ROCK2	0	< 0.05

Figure 7. On-target potency and selectivity of AMA0825. *(A) Chemical structure of the traditional ROCK inhibitor Y-27632. (B) AMA0825 and Y-27632 activity in on-target and cell-based assays. IC_{50} values of the compounds for inhibition of ROCK2 activity are listed as well as their EC_{50} values in a cellular assay for ROCK activity, assessed by the measurement of MLC-Thr18/Ser19 phosphorylation (MLC-PP). (C) Kinase selectivity profile of AMA0825 at 100 nM. Data are shown schematically with the color of the dots representing the percentage of inhibition of kinase activity by AMA0825; red: > 90% inhibition, yellow: between 50% and 90% inhibition, and green: < 50% inhibition. (D) AMA0825 IC_{50} values and the percentages of residual kinase activity of ROCK1/2 and some of the off-target kinases that were hit in the selectivity profile (> 50% inhibition, yellow and red dots). DMPK2, dystrophia myotonica protein kinase 2; LATS2, large tumour suppressor kinase 2; PKC, protein kinase C; PRK, protein kinase; ROCK, Rho-associated protein kinase; NT, not tested.*

1.4 EXPERIMENTAL IBD MODELS OF INTESTINAL EPITHELIAL BARRIER DYSFUNCTION

In the fourth and final part of this introduction, a short explanatory overview will be given of the different *in vitro* and *in vivo* models of intestinal epithelial barrier dysfunction that were used during this research to investigate the effect of the *B. pullicaecorum* butyrate-producing bacterium and the AMA0825 local ROCK inhibitor on epithelial protection during IBD-like inflammation.

1.4.1 Mouse models of IBD

Although they are not representative of the complexity of human disease, mouse models are indispensable to both the study of IBD immunology as well as the pre-clinical evaluation of potential therapeutic compounds. Multiple models of mucosal inflammation exist, each with their individual capacities to provide insight into IBD pathogenesis. The IBD mouse models used during this research are all characterised by alterations to the intestinal epithelial barrier, which contribute to disease.

1.4.1.1 Dextran sulfate sodium-induced model of colitis

Dextran sulfate sodium (DSS)-colitis is a chemical mouse model of colitis induced by administration of DSS - a sulphated polysaccharide - through the drinking water. The onset and severity of colitis are controllable by varying the DSS concentration, molecular weight and duration of exposure. Additional factors that further influence response to DSS are host genetic susceptibility (strain, gender) and microbiological composition of the housing conditions and the intestinal microbiota.[107] Clinically, mice develop bloody diarrhea and lose weight progressively. These parameters of disease activity are monitored throughout the course of colitis since they correlate well with the degree of histological inflammation. Histopathologically, DSS-colitis is characterised by epithelial erosion, goblet cell depletion, crypt loss and mucosal and submucosal edema due to infiltration of acute inflammatory cells like neutrophils and macrophages. Other post-mortem analyses that can be performed to further assess the degree of inflammation are; measurement of the colon length, assessment of colonic myeloperoxidase activity to quantify the infiltration of neutrophils, and determination of colonic pro-inflammatory cytokine levels. In this model, epithelial erosion is caused by colonocyte apoptosis which, along with the ensuing increased intestinal permeability, is observed before the onset of any clinical signs of inflammation.[106,236] This would imply that barrier disruption is the initiating event, resulting in translocation of luminal antigens and subsequent influx of inflammatory cells that secrete both Th1 and Th2 pro-inflammatory cytokines, which further propagates the inflammatory response.[237] Given the superficial nature and the exclusively colonic localisation of the inflammation, DSS-colitis best resembles human UC.

The exact pathogenic mechanism of DSS remains unknown. A recent hypothesis states that DSS is metabolised by the intestinal microbiota of the caecum into sulphate ions and a carbohydrate moiety. These sulphate ions are further converted by sulphate reducing bacteria to yield toxic sulfite,

which could directly damage the intestinal epithelium.[238] This hypothesis would also explain the limitation of the inflammation to the most distal part of the colon where the bacterial load is highest. Additionally, DSS is detected in the macrophages of the mesenteric lymph nodes and the colon from the first day after the start of administration. This accumulation of DSS has been reported to lower the bacterial phagocytic ability of the macrophages, which could contribute to inflammation.[239] Exactly how DSS penetrates the mucosal barrier before any microscopic signs of epithelial damage are observed, remains uncertain. However, recent *in vitro* experiments indicated that DSS increases paracellular permeability in intestinal epithelial Caco-2 monolayers, which is not caused by cell damage.[240] Araki *et al.* also described how nuclear translocation of DSS in Caco-2 cells is associated with cell cycle arrest.[241] Given the high turnover rate of the intestinal epithelium, this could lead to loss of barrier integrity and facilitate luminal influx into the underlying tissue.

In conclusion, DSS-colitis is a simple, low cost and reproducible model that allows the study of epithelial response to injury and other aspects of the acute phase of colitis pathogenesis.[242] Furthermore, several currently used IBD therapeutics have shown efficacy in this model, validating DSS-colitis as a relevant model for the translation of mice data to human disease.[243,244]

1.4.1.2 2,4,6-trinitrobenzene sulfonic acid-induced model of colitis

Like the DSS-induced colitis model, 2,4,6-trinitrobenzene sulfonic acid (TNBS)-colitis is chemically induced by intrarectally administrating TNBS combined with ethanol. Severity of colitis depends on the dose of TNBS, percentage of ethanol, and age and genetic background of the mice. The ethanol temporary permeabilises the epithelial barrier, allowing the TNBS to haptenise autologous or microbial proteins, rendering them immunogenic to the host immune system.[245] The colitis induced by a single TNBS application is an acute, local reaction characterised by transmural inflammation with ulceration, infiltration of neutrophils and macrophages into the mucosal and submucosal layers, and production of Th1 cytokines. A specific Th1-cell mediated autoimmune response to the haptenised proteins is only elicited after a second TNBS administration. Several repeated administrations of TNBS results in a chronic colitis, characterised by the development of fibrosis.[246] Given the transmural nature of the inflammation, the presence of epithelial ulcers, the Th1 specific cytokine profile, and the fibrotic lesions in the chronic setting, TNBS-colitis best resembles human colonic CD. The clinical follow-up of disease activity and post-mortem assessment of inflammation in this model are the same as for the DSS-induced colitis model.

1.4.1.3 $TNF^{\Delta ARE/+}$ model of ileitis

This mouse model is the result of a targeted deletion of AU-rich elements in the 3' untranslated region of the TNF gene, which leads to loss of posttranscriptional regulation of the TNF mRNA and increased constitutive and inducible levels of the TNF protein.[247] This chronic overproduction of TNF

leads to two distinct phenotypes in $TNF^{\Delta ARE/+}$ mice from the age of 4 weeks on; polyarthritis and terminal ileitis, which causes reduced weight gain compared to their wild-type littermates. Mucosal inflammation in these mice closely resembles human CD and is characterised by architectural changes in the villi, transmural inflammation, extended infiltration of both acute and chronic inflammatory cells, and granulomata in later stages. The pivotal role of the epithelium in initiating pathology in this model was demonstrated by Roulis *et al.* who showed that IECs as producers, but not as targets, of endogenous TNF suffice to cause intestinal disease.[248] Endogenous overproduction of TNF was shown to be dependent on TLR/Myd88-mediated innate recognition of indigenous microbiota. Although TNF is known to promote loss of barrier integrity, $TNF^{\Delta ARE/+}$ mice do not display any major defects in paracellular permeability or mucus layer formation, and IECs remain largely intact. However, disease progression in these mice is associated with Paneth cell loss and concomitant diminished antimicrobial gene expression. This dampened antimicrobial response results in dysbiosis involving increased abundance of epithelial-adhering SFB, which may further promote disease.[108] The presence of these SFB, which are also abundant colonisers of gut-associated issues in humans, in the lamina propria of the murine small intestine has been shown to induce the appearance of $CD4^+$ T helper cells that produce IL-17 (Th17 cells). Colonisation with SFB was correlated with an increased expression of genes associated with inflammation and antimicrobial defenses, and resulted in an enhanced resistance to the intestinal pathogen *Citrobacter rodentium*. At steady state, these SFB thus contribute to Th17-mediated mucosal protection. However, an inflammation-induced increase in SFB numbers in the case of $TNF^{\Delta ARE/+}$ mice, can aggravate intestinal disease given the pro-inflammatory potential of Th17 cells.[102]

1.4.1.4 Lipopolysaccharide-induced endotoxemia model

In the intestine, epithelial cells are generated in the crypt and migrate along the villus axis before being shed at the villus tip in a highly regulated process of TJ rearrangements, which allow detachment and release of IECs while at the same time preventing gap formation within the epithelium. The small intestine, with an estimated 10^{11} cell being shed per day, therefore has one of the highest cell turnover rates in the body.[249] Under inflammatory conditions however, the rate of shedding from the villus tip greatly exceeds the rate of generation of new cells in the crypt, resulting in gap formation and villus shortening. Indeed, pathological IEC shedding and the ensuing focal permeability defects have been described as an early event in IBD pathogenesis.[250] A mouse model of pathological LPS-induced small intestinal IEC apoptosis and shedding was developed by Williams *et al.* to study this process, since IEC shedding in mice is morphologically analogous to humans.[251] Intraperitoneal administration of a sublethal dose of LPS was found to be a simple, rapid and consistent stimulus for initiating a systemic inflammatory response. Briefly, this model is

characterised by increased IEC apoptosis in the apical 50% of the villus, and shedding at the villus tip, which peaks 1.5 hours after LPS administration. This coincides with villus shortening, fluid exudation into the gut, and the onset of diarrhea. Increased gut-to-circulation permeability, detectable after oral gavage with fluorescein isothiocyanate (FITC)-dextran, is observed at 5 hours post-LPS. Mechanistically, systemically delivered LPS is first detected by resident mononuclear immune cells, which produce TNF in response to TLR4 receptor activation by LPS. TNF subsequently binds to the TNF receptor 1 (TNFR1) expressed on IECs, triggering apoptosis and shedding if NF-κB2 signalling dominates, or cell survival if NF-κB1 signalling is favoured.

1.4.2 *In vitro* models of intestinal barrier dysfunction

For the *in vitro* modelling of human intestinal epithelium we used monolayers of either Caco-2 or T84 IECs. Both cell lines are derived from a colorectal adenocarcinoma and differentiate spontaneously upon reaching confluency into a monolayer of polarised, columnar IECs characterised by the appearance of TJs between adjacent cells, apical microvilli, and the expression of brush border hydrolases.[252] Complete functional and morphological differentiation takes two to three weeks on average. Both cell lines are utilised interchangeably despite evidence that differentiated Caco-2 cells closely resemble small intestinal enterocytes, whereas differentiated T84 cells are less well characterised.[253,254]

Barrier studies are generally performed on Caco-2 or T84 cells cultured on semi-permeable filter supports, since this improves morphological and functional differentiation by allowing free access of nutrients to both the apical and basolateral sides of the monolayer.[252] This system also facilitates measurements of monolayer integrity and permits the separation of apical and basolateral stimulation of the cells. The integrity of the monolayer is determined by the TJs, which restrict paracellular diffusion of ions and larger molecules in a selective manner, based on both size and charge. Ion permeability of the monolayer is generally determined by measuring TEER while paracellular permeability based on size can be monitored using non-ionic, fluorescently labelled hydrophilic tracers like lucifer yellow or FITC-dextran.

Barrier disruption in this model is induced by stimulation of the monolayer with pro-inflammatory cytokines such as TNF[202], INFγ[222,223] and IL-1β[221], or other compounds such as ethanol[255], hydrogen peroxide[256] or bacterial components like LPS[257]. In our experiments, a combination of IFNγ and TNF is used since these cytokines are known to have a synergistic effect on the induction of intestinal epithelial barrier dysfunction.[71,258,259] Barrier-disrupting agents are always added basolaterally given the polarised expression of their receptors on IECs. This is in accordance with the fact that in the gut, activated immune cells such as macrophages, DCs and intraepithelial lymphocytes that secrete these pro-inflammatory mediators, are also located in the mucosa underneath the epithelium. Drugs or

food compounds with a suspected beneficial effect on barrier function are usually added apically to the monolayer. An expansion on this model is the coculture system first described by Satsu *et al.*[260] in which a Caco-2 epithelial monolayer is placed on top of activated macrophage-like THP1 cells. THP1 cells are human, acute monocytic leukemia cells that differentiate into adherent activated macrophages after treatment with phorbol myristrate acetate (PMA) for a minimum of 48 hours. PMA treatment dramatically increases TNF, IL-1β, IL-6 and IL-8 secretion by THP1 cells, which induces a loss of TEER and an increase in lactate dehydrogenase (LDH) release in the Caco-2 monolayer. An indirect coculture system can also be used, in which the epithelial monolayer is stimulated basolaterally with the conditioned medium of PMA-activated THP1 cells, instead of the cultured THP1 cells themselves. Barrier disruption in this coculture model is significantly suppressed by adding TNF neutralising agents, suggesting that TNF is the main damaging factor. Affected monolayers show both apoptotic and necrotic characteristics in which TNFR1-mediated NF-κB signalling seems to be involved. It is well established that pro-inflammatory cytokines like TNF play a key role in IBD pathogenesis, in part by inducing excessive epithelial cell death, and that elevated mucosal TNF levels as well as increased activation of its transcriptional regulator NF-κB, have been reported in IBD patients.[198] The similarity between the *in vivo* inflammation and the nature of the THP1-induced damage to Caco-2 monolayers, validates the use of this coculture system as an *in vitro* model of inflammation-induced epithelial barrier dysfunction, and offers a convenient platform for the evaluation of anti-inflammatory drugs or food compounds as potential treatment options for restoring intestinal epithelial barrier integrity in IBD.

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CHAPTER II

RATIONALE AND AIMS

RATIONALE

Intestinal epithelial barrier dysfunction is a major hallmark of IBD caused mainly by TJ deregulation and IEC apoptosis, leading to an influx of luminal antigens which sustains inflammation. Whether increased intestinal permeability is a pre-existing defect or the consequence of underlying mucosal inflammation remains unclear. Either way, restoration of epithelial barrier integrity is crucial to mucosal healing, a recently emerged key treatment goal in IBD that predicts sustained clinical remission. In current IBD management, mucosal healing is usually achieved as a consequence of administering immunosuppressive or immunomodulating drugs. However, not all patients respond to the current drugs or lose response over time, and considerable side effects often occur (e.g. allergic reactions).

Therefore, the **primary aim** of this thesis was the study of two pathways directly involved in maintenance and regulation of TJ integrity; butyrate production and ROCK function. In the first part, we investigated the butyrate-producing *Butyricicoccus pullicaecorum* 25-3^T strain. Given the butyrate deficiency during intestinal inflammation in IBD and the known barrier-protective properties of butyrate, *B. pullicaecorum* 25-3^T could potentially be used as a pharmabiotic to promote mucosal healing in IBD. Our objective was therefore to specifically analyse the barrier-protective properties of *B. pullicaecorum* 25-3^T (**chapter III**).

The second part focusses on epithelial ROCK function. These kinases regulate actin-dependent TJ dynamics, including cytokine-induced internalisation of TJ proteins. The involvement of ROCK in inflammation-induced loss of epithelial barrier integrity and the elevated ROCK activity observed in the intestinal mucosa of patients with active IBD, has prompted research into the use of ROCK inhibitors to counteract epithelial dysfunction in IBD. Current ROCK inhibitors however, have selectivity issues and display considerable side effects upon systemic exposure. This is why Amakem Therapeutics developed AMA0825; a local ROCK inhibitor specifically designed to target the gut and minimise systemic exposure. In this part of the thesis, we investigated the effect of AMA0825 on inflammation-induced intestinal epithelial barrier dysfunction and the associated mucosal inflammatory response (**chapter IV**).

A **secondary aim** within this thesis was the comparison between differentiated Caco-2 and T84 cells, which were both used frequently as *in vitro* models of epithelial monolayers. During the experiments performed with these cell lines, we observed that they behaved differently in response to various stimuli despite their shared colonic origins. In the third part of this thesis, we therefore further characterised Caco-2 and T84 monolayers (**chapter V**).

SPECIFIC RESEARCH AIMS

1. Investigate the barrier-protective potential of *Butyricicoccus pullicaecorum* 25-3^T

The aims of this study were to:

- determine the expression of butyrate-responsive TJ genes OCLN, TJP1 and CLDN1 in a collection of mucosal biopsies from UC patients and healthy controls and correlate this to the abundance of *Butyricicoccus* in the mucosa of UC patients and healthy controls
- investigate whether butyrate influences *in vitro* expression of OCLN, TJP1 and CLDN1 and evaluate the effect on repair of IEC monolayer integrity
- investigate whether expression of OCLN, TJP1 and CLDN1 is influenced ex *vivo* by *B. pullicaecorum* 25-3^T conditioned medium in mucosal biopsies of UC patients with active disease

The results from this study are described in **chapter III**.

2. Investigate the effect of the new local ROCK inhibitor AMA0825 on inflammation-induced epithelial dysfunction and the associated mucosal response

The aims of this study were to:

- evaluate the barrier-protective properties of AMA0825 on repair of IEC monolayers *in vitro*
- evaluate the anti-inflammatory properties of AMA0825 on IECs *in vitro*
- evaluate the efficacy of AMA0825 in decreasing clinical disease severity, epithelial destruction and the associated inflammatory response in two mouse models of acute colitis induced by a disturbance of epithelial integrity
- evaluate the anti-apoptotic potential of AMA0825 during receptor-mediated apoptosis in IECs *in vitro* and during LPS-induced enterocyte apoptosis *in vivo*

The results from this study are described in **chapter IV**.

3. Compare the differentiation-induced expression profiles of Caco-2 and T84 epithelial cells

The aims of this study were to:

- further characterise differentiated Caco-2 and T84 cell lines through expressional analysis of selected differentiation markers
- compare the functional response to butyrate between both cell lines

The results from this study are described in **chapter V**.

CHAPTER III

REDUCED MUCOSA-ASSOCIATED *BUTYRICICOCCUS* ACTIVITY IN PATIENTS WITH ULCERATIVE COLITIS CORRELATES WITH ABERRANT CLAUDIN-1 EXPRESSION

Taken from

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Reduced mucosa-associated *Butyricicoccus* activity in patients with ulcerative colitis correlates with aberrant claudin-1 expression

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Abbreviations

B. pullicaecorum (BP), *Butyricicoccus pullicaecorum*; BSA, bovine serum albumin; CD, Crohn's disease; CLDN1, claudin-1; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMBS, hydroxymethyl-bilane synthase; IBD, inflammatory bowel disease; IFNγ, interferon gamma; IL-8, interleukin-8; NaB, sodium butyrate; OCLN, occludin; PBS, phosphate buffered saline; qRT-PCR, quantitative real-time PCR; SCFA, short-chain fatty acid; SHDA, succinate dehydrogenase complex A subunit; SEM, standard error of the mean; TBST, Tris buffered saline with 0.1% Tween-20; TEER, transepithelial electrical resistance; TJ, tight junction; TJP1, tight junction protein 1; TNF, tumor necrosis factor; UC, ulcerative colitis; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein.

Abstract

Background and Aims. *Butyricicoccus* is a butyrate-producing clostridial cluster IV genus whose numbers are reduced in stool of ulcerative colitis (UC) patients. Conditioned medium of *Butyricicoccus (B.) pullicaecorum* prevents tumor necrosis factor (TNF)/interferon gamma (IFNγ)-induced increase in epithelial permeability *in vitro*. Since butyrate influences intestinal barrier integrity, we further investigated the relationship between the abundance of mucosa-associated *Butyricicoccus* and the expression of butyrate-regulated tight junction (TJ) genes.

Methods. Tight junction protein 1 (*TJP1*), occludin (*OCLN*), claudin-1 (*CLDN1*) and *Butyricicoccus* 16S rRNA expression was analysed in a collection of colonic biopsies of healthy controls and UC patients with active disease. The effect of butyrate and *B. pullicaecorum* conditioned medium on TJ gene expression was investigated in TNF/IFNγ-stimulated Caco-2 monolayers and inflamed mucosal biopsies of UC patients.

Results. *TJP1* expression was significantly decreased in inflamed UC mucosa, whereas *CLDN1* mRNA levels were increased. *OCLN* did not differ significantly between both groups. Mucosa-associated *Butyricicoccus* 16S rRNA transcripts were reduced in active UC patients compared to healthy controls. Interestingly, *Butyricicoccus* activity negatively correlated with *CLDN1* expression. Butyrate reversed the inflammation-induced increase of CLDN1 protein levels and stimulation of inflamed UC biopsies with *B. pullicaecorum* conditioned medium normalised *CLDN1* mRNA levels.

Conclusions. *Butyricicoccus* is a mucosa-associated bacterial genus underrepresented in colonic mucosa of patients with active UC whose activity inversely correlates with *CLDN1* expression. Butyrate and *B. pullicaecorum* conditioned medium reduce CLDN1 expression, supporting its use as a pharmabiotic preserving epithelial TJ integrity.

Keywords

tight junctions, pharmabiotic, butyrate

Introduction

In the healthy gut, a symbiotic relationship exists between the host and commensal bacteria, which is paramount to our general wellbeing. Commensals are *i.a.* crucial to the maintenance of immunological gut homeostasis. During adult life, the composition of the intestinal microbiota is fairly stable but may fluctuate due to infections or oral antibiotic treatment. This fluctuation generally entails a decrease in both bacterial abundance and diversity - a condition termed dysbiosis.[1] Several chronic diseases, including Inflammatory Bowel Disease (IBD), are associated with intestinal dysbiosis. IBD, comprising Crohn's disease (CD) and ulcerative colitis (UC), is characterised by a chronic, relapsing inflammation of the gastrointestinal tract. The etiology of IBD is unknown but inflammation most likely results from an abnormal mucosal immune response to antigens derived from the commensal microbiota in a genetically susceptible host. One of the most important microbial communities affected by intestinal inflammation are specific butyrate-producing members of the *Firmicutes* phylum like the *Lachnospiraceae* subgroup (which comprises *Clostridium* XIVa and IV groups within the order *Clostridiales*) which are underrepresented in the mucosal microbiota of patients with IBD compared to healthy subjects.[2] Butyrate is a short-chain fatty acid (SCFA) produced during fermentation of dietary fiber in the colon. Besides being the main energy source for colonocytes, butyrate is also responsible for the maintenance of colonic homeostasis by modulating a wide variety of cellular functions including proliferation, differentiation, apoptosis and the control of intestinal epithelial permeability.[3,4] Butyrate is a potent anti-inflammatory mediator given its role in promoting epithelial barrier function,[5] its inhibitory effect on cytokine expression[6] and its ability to induce differentiation of colonic regulatory T cells.[7] Most of the butyrate-producing bacteria cultured so far belong to the clostridial clusters XIVa and IV that include *Roseburia*[8] and *Faecalibacterium*[9] species respectively, two abundant colonisers of the human gut. A decrease in both *Roseburia hominis* and *Faecalibacterium prausnitzii* has been documented in stools of UC patients with both species showing an inverse correlation with disease activity.[10]

Consequently, there is increasing interest in using butyrate to restore homeostasis in IBD. However, its routine clinical application has been impeded by practical issues. When administered orally, butyrate might not reach the colon in sufficient amounts due to its rapid gastric and duodenal absorption. Besides, butyrate has a very unpleasant taste and smell. Rectal butyrate enemas have proven to be effective in treating distal UC[11,12] but are cumbersome for the patient and exposure of the colonic mucosa to the butyrate is brief and discontinuous. The administration of naturally occurring butyrate-producing bacteria that would continuously secrete butyrate into the colonic lumen is an alternative strategy to locally increase butyrate concentrations. One of these high-level butyrate-producing bacteria is *Butyricicoccus pullicaecorum* (*B. pullicaecorum*), an anaerobic Gram-positive clostridial cluster IV species, first isolated from the caecal content of a broiler chicken.[13] The

average number of *Butyricicoccus* is decreased in stool samples of IBD patients and a specific *B. pullicaecorum* strain is able to reduce intestinal inflammation in a rat colitis model. Furthermore, its conditioned medium prevents cytokine-induced increase in epithelial permeability *in vitro*.[14]

A dysfunctional epithelial barrier is one of the key characteristics of IBD.[15] This barrier consists of a single layer of epithelial cells linked together by tight junctions (TJs) which seal off the intercellular space and regulate selective paracellular ionic solute transport. Tight junctions are composed of four different integral membrane proteins: occludin (OCLN), tricellulin, junctional adhesion molecules and claudins which are linked to the actin cytoskeleton through scaffolding proteins like tight junction protein 1 (TJP1).[16] In IBD, expression of most claudins – like other TJ proteins- is reduced.[17] Remarkably, claudin-1 (CLDN1) protein levels are increased in areas of active inflammation.[18] Given the reduction of *Butyricicoccus* numbers in IBD and the ability of its conditioned medium to prevent cytokine-induced epithelial dysfunction, *B. pullicaecorum* bacteria therefore seem conceptually attractive as pharmabiotics to reduce intestinal inflammation or to prevent disease relapse in IBD patients. However, it is currently unknown how *Butyricicoccus* could affect barrier integrity in human IBD. Therefore, the purpose of this study was to detect and quantify *Butyricicoccus* in human colonic mucosa and to further investigate the host response to butyrate and conditioned medium of *B. pullicaecorum* in terms of epithelial barrier function.

Materials and Methods

Ethics statement. The use of patient material was approved by the Ethics Committee of the Ghent University Hospital (permit number UZG 2004/242). Written informed consent was obtained from all participants. Mice were housed in the laboratory animal facility at Ghent University Hospital according to the institutional animal healthcare guidelines. This study was approved by the Institutional Review Board of the Faculty of Medicine and Health Sciences of Ghent University (ECD2014-25).

Patient samples. Biopsies from healthy controls (N=36), UC patients with active disease (N=37) and UC patients in clinical and endoscopic remission (N=16) were obtained during routine colonoscopy. Active disease in UC patients was defined as the presence of endoscopic signs of disease activity (Mayo score of 1 or higher) and biopsies were taken from the inflamed site (sigmoid or rectum). UC patients in remission had no clinical or endoscopic signs of inflammation (Mayo score of 0). Control biopsies were taken from the sigmoid of healthy patients who underwent colonoscopy to screen for cancer. Patient characteristics are provided in Table 1. All biopsies were immediately placed in RNAlater (Ambion, Cambridgeshire, UK) and stored at -80°C.

Mice and experimental protocol. Heterozygous C57BL/6 $TNF^{\Delta ARE/WT}$ mice and $TNF^{WT/WT}$ littermates were conventionally raised in a temperature-controlled room at 20°C with a light/dark cycle of 12/12 hrs. Water and a commercial chow (mice maintenance chow, Carfil Labofood, Pavan Service, Belgium) were provided *ad libitum*. At 24 weeks of age, eight $TNF^{\Delta ARE/WT}$ mice and eight $TNF^{WT/WT}$ littermate controls were sacrificed by cervical dislocation. The terminal ileum was removed, opened longitudinally and washed with phosphate buffered saline (PBS). Tissue samples were cut, snap-frozen and stored at -80°C until analysis.

Bacterial strain and growth conditions. *B. pullicaecorum* 25-3T (LMG 24109^T) was grown overnight at 37°C in an anaerobic (90% N_2, 10% CO_2) workstation (GP-Campus, Jacomex, TCPS NV, Rotselaar, Belgium) in anaerobic modified M2GSC medium at pH 6 prepared as described by Miyazaki *et al*.[19] but without clarified rumen fluid. The bacterial cells were collected by centrifugation (10 min, 5000 g, 37°C) and discarded. The resulting supernatant was sterile-filtered (0.22 µm) and the concentration of short-chain fatty acids (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate and isocaproate) was determined using a gas chromatograph as described previously.[20] Only butyrate was detected in supernatant of *B. pullicaecorum* $25\text{-}3^T$ at a concentration of 4 mM.

Stimulation of colonic mucosal biopsies with *B. pullicaecorum* conditioned medium. Colonic biopsies from patients with active UC (N=25) were obtained as described above and harvested in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100

µg/ml streptomycin, 200 µg/ml gentamycin and 250 ng/ml Fungizone® antimycotic (all Life Technologies, Gent, Belgium). Patient characteristics are provided in Table 1. Biopsies were cultured for 24 hrs with *B. pullicaecorum* conditioned medium diluted to contain 2 mM of butyrate or M2GSC anaerobic medium as control. Biopsies were recovered in RNAlater and stored at -80°C.

Cell culture and induction of barrier dysfunction *in vitro*. Caco-2 cells (HTB-37, ATCC Cell Biology Collection, Manassas, VA, USA) were seeded on 24-well semipermeable inserts (0.4 µm, translucent ThinCerts™, Greiner Bio-One, Vilvoorde, Belgium) at a density of 10^5 cells per well and cultured for a minimum of two weeks in Dulbecco's modified Eagle medium supplemented with 10% FBS (both Life Technologies). After this period, the integrity of the monolayer was evaluated by measuring the transepithelial electrical resistance (TEER) using a Millicell ERS-2 Voltohmmeter (Merck Millipore, Billerica, MA, USA) to ensure that functional polarised epithelial monolayers with absolute TEER-values of more than 3000 Ohm were obtained. The Caco-2 differentiated monolayer was then incubated apically with 8 mM sodium butyrate (NaB) (Sigma-Aldrich, Diegem, Belgium) and basolaterally with recombinant human 100 ng/ml tumor necrosis factor (TNF) and 300 ng/ml interferon gamma (IFNγ) (both Life Technologies) to induce barrier dysfunction measured as a drop in TEER. After 48 hrs, absolute TEER values were normalised to their pre-treatment values and expressed as a percentage of the initial TEER values. After the TEER measurements, Caco-2 inserts were used for CLDN1 detection by either western blotting or immunofluorescence. Medium from the basolateral compartments was used for interleukin-8 (IL-8) detection.

IL-8 measurements. IL-8 secretion into the basolateral medium was measured using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. IL-8 concentration was expressed as picograms of cytokine per milliliter of medium.

Western blot analysis. Caco-2 monolayers were lysed using sonication on ice for 1 min in 200 µl of Radio Immunoprecipitation Assay buffer supplemented with a phosphatase and protease inhibitor cocktail (Sigma-Aldrich). The concentration of protein lysates were determined using the Bio-Rad Protein Assay (Bio-Rad, Temse, Belgium) according to the manufacturer's instructions with bovine serum albumin (BSA) as a protein standard. Thirty µg of each sample was mixed with 1:4 loading buffer (Life Technologies) and 1 mM dithiothreitol (Roche, Vilvoorde, Belgium). Samples were denatured by boiling for 10 min at 95°C, separated on a 4–12% gradient Bis-Tris SDS-PAGE gel and transferred to a nitrocellulose membrane using a wet transfer (all Life Technologies). Afterwards, membranes were blocked with 5% BSA in Tris buffered saline with 0.1% Tween-20 (TBST) (Sigma-Aldrich) and incubated overnight at 4°C with 1:1000 rabbit anti-CLDN1 antibody (Cell Signalling, Leiden, The Netherlands) in 5% BSA in TBST. Next, blots were incubated for 1 h at room temperature with 1:2000 secondary goat anti-rabbit IgG, HRP-conjugated secondary antibody (Cell Signalling).

Bound antibodies were visualised using the BM Chemiluminescence Western Blotting Substrate POD (Roche) according to manufacturer's instructions and membranes were exposed to X-ray films. Equal loading of proteins was confirmed by immunoblotting with 1:5000 anti-tubulin (Abcam, Cambridge, UK) antibody in 5% BSA in TBST.

Immunofluorescence staining. Caco-2 inserts were fixed in cold methanol: acetone (50%: 50%) for 1 min. Blocking step was performed using 10% (v/v) normal goat serum (Sigma-Aldrich) in PBS. Then cells were incubated for 1 h with 1:200 rabbit anti-CLDN1 antibody (Life Technologies) in 2% normal goat serum in PBS. After washing, the cells were stained with 1:200 Alexa Fluor 488 anti-rabbit secondary antibody (Life Technologies) in 2% normal goat serum in PBS for 30 min in the dark at room temperature. Cells were incubated with DAPI (Life Technologies) for 5 minutes to allow nuclear staining. Inserts were mounted using mounting medium containing an anti-fading agent (Dako, Heverlee, Belgium). Cells were examined under a BD Pathway 435 confocal laser microscope (BD Biosciences, San Diego, CA, USA) and images were processed using Adobe Photoshop software 5.5 (Adobe systems, Dublin, Ireland).

RNA extraction. Total RNA from Caco-2, human mucosal biopsies and mice terminal ileum tissue samples was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) with on-column DNase treatment. Concentration and purity was determined using nanodrop technology (BioPhotometer Plus, Eppendorf, Rotselaar, Belgium). All samples exhibited an OD260/OD280 ratio between 1.8 and 2.1.

Quantitative Real-Time PCR. One microgram of total RNA was converted to single stranded cDNA by reverse transcription using the iScript™ cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. The cDNA was diluted to a concentration of 5 ng/µl and 15 nanogram was used in quantitative real-time PCR (qRT-PCR) with SYBR Green (SensiMix™ SYBR No-ROX kit, GC biotech, Alphen a/d Rijn, The Netherlands) and 250 nM of each primer. A two-step program was performed on the LightCycler 480 (Roche). Cycling conditions were 95°C for 10 min, 45 cycles of 95°C for 10 s and 60°C for 1 min. Melting curve analysis confirmed primer specificities. All reactions were performed in duplicate. Expression of all eukaryotic genes was normalised to the stably-expressed reference gene levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and succinate dehydrogenase complex A subunit (*SDHA*) for the Caco-2 cDNA, to *GAPDH*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (*YWHAZ*) and hydroxymethyl-bilane synthase (*HMBS*) for *B. pullicaecorum*-stimulated active UC biopsy cDNA, to *GAPDH*, *YWHAZ* and *HMBS* for all other human biopsy cDNA and to *Hmbs* and *Gapdh* for mice terminal ileum tissue samples. To enable normalisation to biopsy size, the number of cDNA copies corresponding to the *Butyricicoccus* 16S rRNA gene were normalised to the expression of human *GAPDH*, *HMBS* and

YWHAZ as described previously.[21] The PCR efficiency of each primer pair was calculated using a standard curve of reference cDNA. Amplification efficiency was determined using the formula $10^{-1/slope}$. Sequences of the primer sets are listed in Table 2.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (GraphPad, La Jolla, CA, USA) and SPSS Statistics version 22.0 (IBM SPSS Statistics, Armonk, NY, USA). Values are presented as the mean ± standard error of the mean (SEM). Normality of the data was checked using the Kolmogorov-Smirnoff test. Differences between groups were analysed using an unpaired Student's *t*-test for independent samples in case of normally distributed data (applying the Welch's correction in case of unequal variances) or the Mann-Whitney statistic if the data were not normally distributed. Two-tailed probabilities were calculated and *p*-values less than 0.05 were considered statistically significant. Prevalence of the genus *Butyricicoccus* in healthy controls versus active UC patients was calculated using a Fisher's exact test. The Kolmogorov-Smirnoff test also determined the use of either a parametric (Pearson) or a non-parametric (Spearman) correlation test.

Results

Tight junction gene expression is deregulated during intestinal inflammation

In order to link the presence of *Butyricicoccus* with barrier integrity *in vivo*, mRNA expression levels of three major TJ genes - *CLDN1*, *TJP1* and *OCLN* - were measured in a collection of colonic mucosal biopsies from healthy controls (N=36) and patients with active UC (N=37) using qRT-PCR. Expression of *TJP1* was significantly decreased ($p < 0.001$, Figure 1A) in UC biopsies, whereas *CLDN1* expression was significantly increased ($p < 0.0001$, Figure 1B). Expression of *OCLN* did not differ significantly between active UC patients and healthy controls ($p = 0.091$, Figure 1C). In remission, no significant differences were observed (Figure 1A-C). The inflammation-associated upregulation of *CLDN1* observed in active UC seems counter-intuitive, however increased CLDN1 levels have been reported in experimental models for colitis.[22] Also inflammation of the ileum in $TNF^{\Delta ARE/WT}$ mice[23] exhibits an increased expression of *Cldn1* ($p = 0.0002$, Figure 1D).

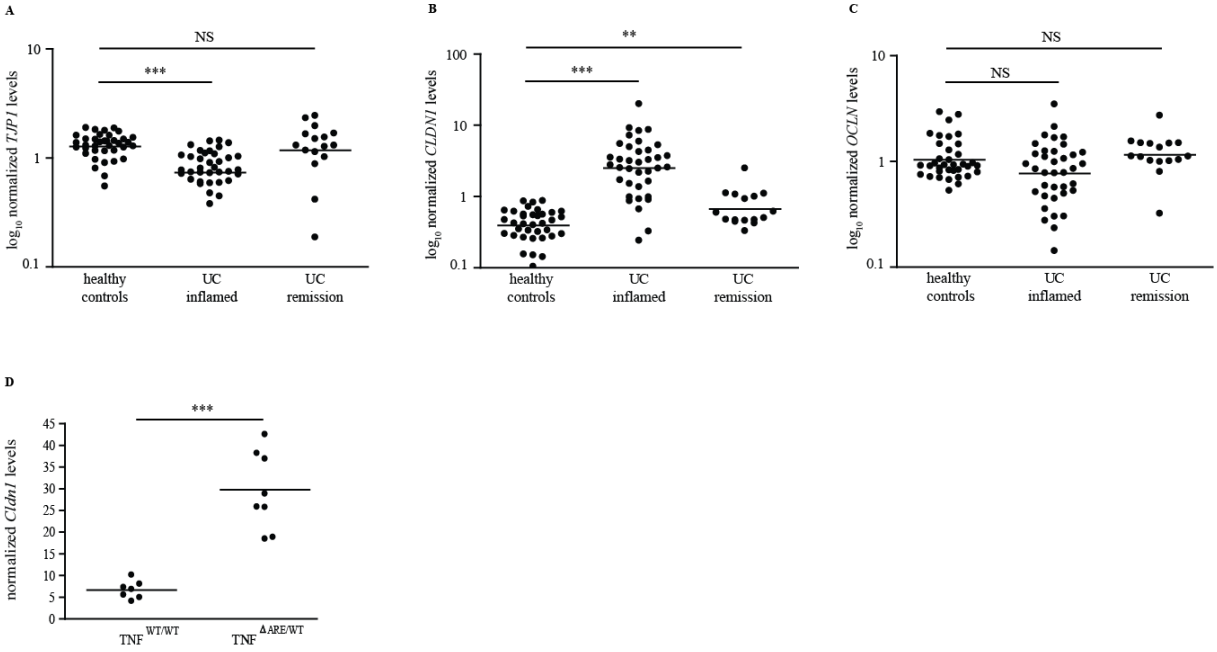

Figure 1. Tight junction gene expression in biopsies of UC patients vs. healthy controls and in $TNF^{\Delta ARE/WT}$ mice vs. $TNF^{WT/WT}$ littermate controls. *(A-C) TJP1, OCLN and CLDN1 mRNA levels in colonic mucosal biopsies of healthy controls (N=36), UC patients with active disease (N=37) and UC patients in remission (N=16). (D) Cldn1 expression in terminal ileum samples of 24-week old $TNF^{\Delta ARE/WT}$ mice (N=8) vs. $TNF^{WT/WT}$ littermate controls (N=8). **p < 0.01, ***p < 0.001. NS, not significant.*

Butyricicoccus activity is decreased in mucosal samples of patients with active UC

Next, we used this biopsy cohort to determine whether *Butyricicoccus* bacteria were present using genus specific 16S rRNA primers. *Butyricicoccus* 16S rRNA levels could be measured adequately, ranging from 0 to 100.000 copies. Functionally active *Butyricicoccus* bacteria were detectable in all healthy control samples but below detection in eight out of 37 active UC samples (Fisher's exact $p = 0.0052$). In UC samples with detectable 16S rRNA transcripts, the absolute numbers were reduced

compared to those of healthy controls (p = 0.046, Figure 2A). Interestingly, the amount of *Butyricicoccus* inversely correlated with *CLDN1* mRNA levels (Pearson R = -0.239, p = 0.024, Figure 2B).

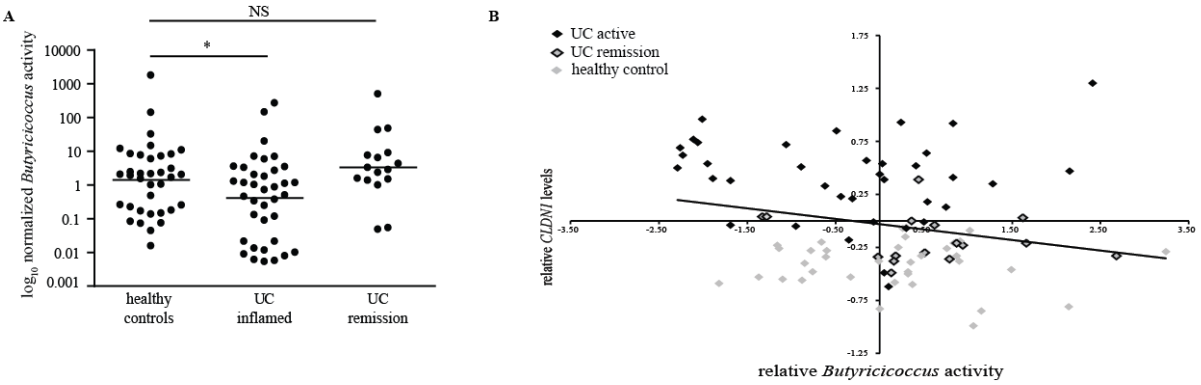

Figure 2. Quantification of Butyricicoccus activity in colonic mucosal biopsies of healthy controls and UC patients. *(A) Butyricicoccus activity in colonic mucosal biopsies of healthy controls (N=36), UC patients with active disease (N=37) and UC patients in remission (N=16). Bacterial activity is expressed as log_{10} copy number of normalised 16S rRNA genes for Butyricicoccus. (B) Correlation plot between CLDN1 levels and Butyricicoccus activity in biopsies of healthy controls (grey), patients with active UC (black) and UC patients in remission (grey with black borders). *p < 0.05. NS, not significant.*

Butyrate counteracts TNF/IFNγ-induced barrier disruption and TJ deregulation

Next, we questioned whether the *in vitro* model for barrier disruption using TNF/IFNγ-stimulated Caco-2 monolayers mimics deregulated TJ gene expression observed in UC biopsies. Forty-eight hrs following the addition of TNF/IFNγ, a significant drop in TEER was observed (p < 0.0001, Figure 3A), which coincided with increased IL-8 production (p = 0.0043, Figure 3B) and a significant reduction of *TJP1* and *OCLN* mRNA levels (p = 0.0065 and 0.0014, respectively, Figure 3C-D), while *CLDN1* mRNA (p = 0.012, Figure 3E) and CLDN1 protein expression were increased (Figure 3F). In addition, CLDN1, which localises to the plasma membrane in functionally intact Caco-2 monolayers, internalised following TNF/IFNγ-stimulation (Figure 3G). The addition of 8 mM butyrate to the transwell culture inhibited the TNF/IFNγ-induced TEER drop (p = 0.001), diminished IL-8 secretion (p > 0.05), and increased the expression of TJ genes *TJP1* (p = 0.0223) and *OCLN* (p = 0.0012) (Figure 3A-D). Although the TNF/IFNγ-induced increase in mRNA expression of *CLDN1* was not influenced by butyrate (Figure 3E), total protein levels of CLDN1 (Figure 3F) were markedly decreased. However, cytoplasmic expression of CLDN1 was still observed (Figure 3G).

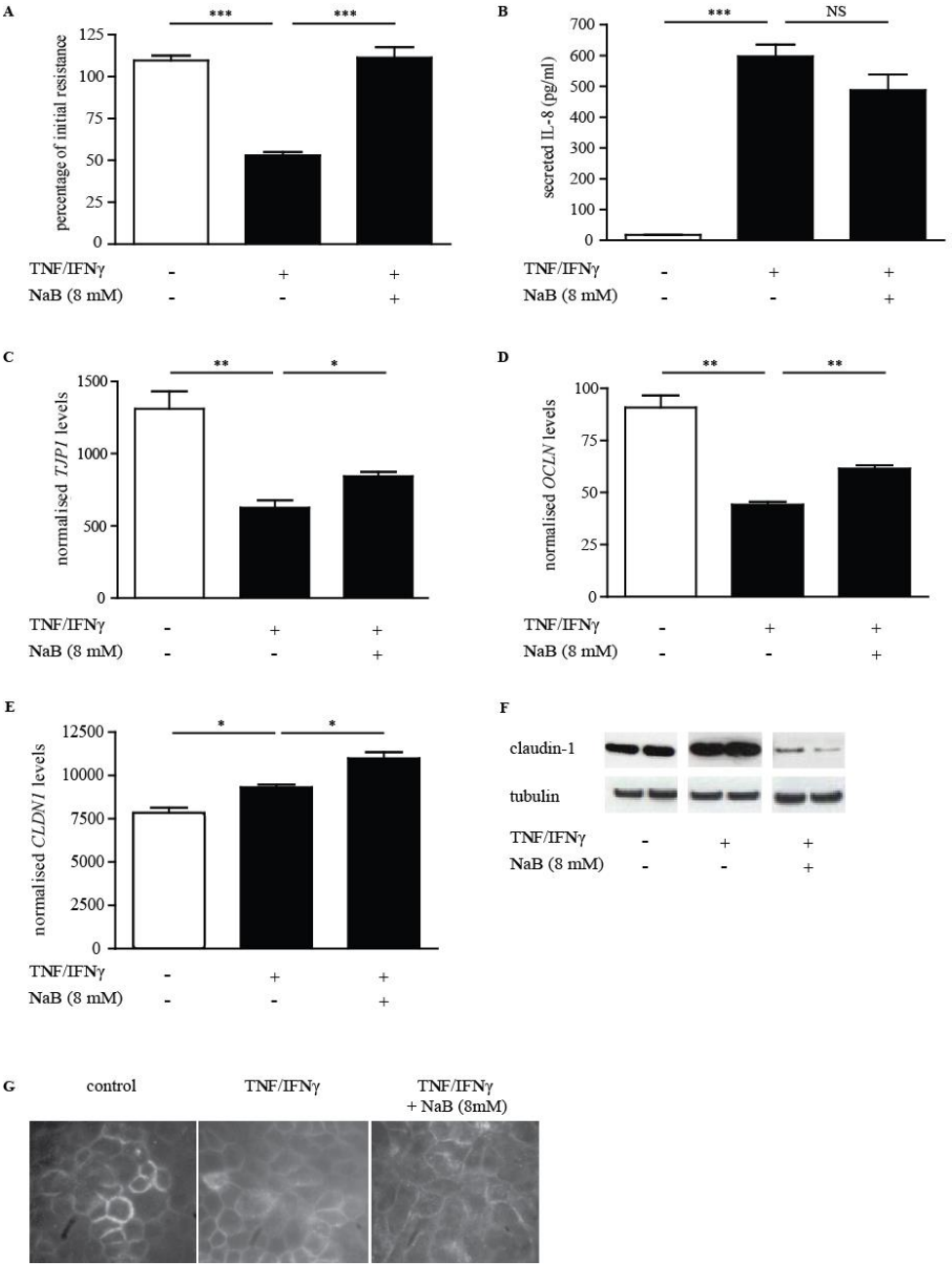

Figure 3. Effect of butyrate on TNF/IFNγ-induced barrier disruption and TJ deregulation in Caco-2 monolayers. *Caco-2 differentiated monolayers were stimulated basolaterally with a combination of TNF and IFNγ and treated apically with 8 mM NaB. After 48 hrs, TEER (A), basolateral IL-8 secretion (B), TJP1 (C), OCLN (D) and CLDN1 (E) mRNA levels and CLDN1 protein expression (F) were determined. (G) Immunofluorescent microscopic images (63x) of CLDN1 internalisation in a Caco-2 monolayer stimulated with TNF/IFNγ and 8 mM NaB. Data in Figures 3A-E represent the mean ± SEM of 3 replicates/group. *p < 0.05, **p < 0.01, ***p < 0.001. NS, not significant.*

B. pullicaecorum conditioned medium reduces CLDN1 expression in mucosa of patients with active UC

Finally, to investigate whether *B. pullicaecorum* affects TJ expression *ex vivo*, mucosal biopsies of UC patients with active disease (N=25) were stimulated with *B. pullicaecorum* conditioned medium diluted to contain 2 mM of butyrate or an equal dilution of the growth medium control. After 24 hrs, a decrease in *CLDN1* mRNA levels compared to medium control (p = 0.001, Figure 4A) was observed, while *TJP1* and *OCLN* expression were not significantly affected by the conditioned medium (Figure 4B-C).

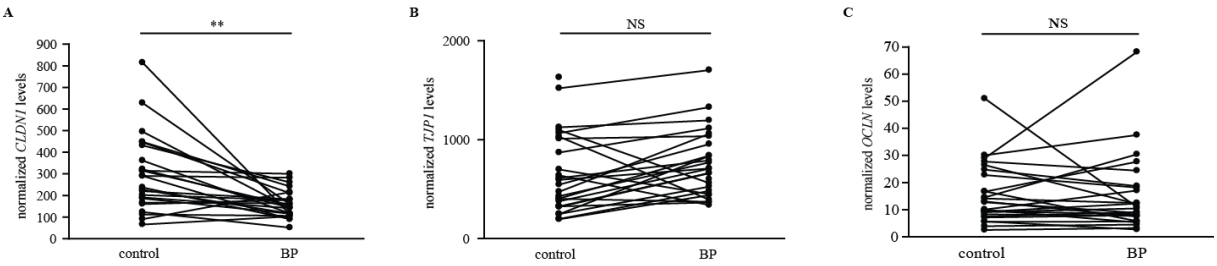

Figure 4. Effect of B. pullicaecorum conditioned medium on TJ expression in colonic mucosal biopsies of active UC patients. *CLDN1 (A), TJP1 (B) and OCLN (C) expression in paired colonic biopsies from the same UC patients (N=25) treated with B. pullicaecorum conditioned medium diluted to contain 2 mM of butyrate or M2GSC anaerobic medium as control for 24 hrs. **p < 0.01. BP, B. pullicaecorum; NS, not significant.*

Discussion

Intestinal epithelial TJ integrity is compromised in IBD. The observed abnormalities include reduced strand numbers, strand discontinuities and reduced depth of the TJ complex. Changes in expression and localisation of specific TJ molecules have also been described.[24,25] In our collection of colonic mucosal biopsies we also observed a severely deregulated expression of two major TJ genes – *TJP1* and *CLDN1* – in samples of patients with active UC compared to healthy controls; *TJP1* is underrepresented in inflamed tissue, whereas *CLDN1* is highly upregulated. A similar increase in *Cldn1* was observed in a model for Crohn's-like ileitis due to constitutive overexpression of TNF; a key pro-inflammatory cytokine in IBD. Likewise, compromised TJ integrity mimicked *in vitro* by stimulating differentiated Caco-2 monolayers with TNF/IFNγ leads to loss of *TJP1* and *OCLN* and an increase in *CLDN1*. In addition, CLDN1, which is localised solely at the plasma membrane in functionally intact Caco-2 monolayers, internalises following TNF/IFNγ-stimulation. Under normal physiological conditions, CLDN1 is a key pore-sealing TJ protein crucial to epithelial barrier integrity; its genetic deletion results in rapid postnatal death due to severe epidermal permeability defects.[26] Also, baseline *CLDN1* overexpression *in vitro* results in increased barrier tightness while its concomitant knockdown decreases it.[5] Under inflammatory conditions however, the role of CLDN1 is much less straightforward. In agreement with upregulated *CLDN1* mRNA levels in our cohort, increased CLDN1 protein levels have been documented in IBD and in acute DSS-induced colitis.[22,27] Also, its intestinal epithelial overexpression renders mice more susceptible to colitis and impairs their recovery.[28] Interestingly, *in vitro* pro-inflammatory cytokine-induced upregulation of CLDN1 coincides with a redistribution of the protein away from the TJs[29,30] and a concomitant increase in intestinal permeability. So it appears that elevating CLDN1 expression under baseline conditions increases epithelial barrier integrity, whereas under inflammatory conditions its internalisation causes a weakening of the intestinal barrier despite an increase in overall expression. Next, we found that this aberrant *CLDN1* expression in colonic mucosa of active UC patients negatively correlates with reduced *Butyricicoccus* activity. The detection of the genus *Butyricicoccus* in mucosal tissue is in agreement with a study published by Nava and Stappenbeck who identified *Butyricicoccus* as an autochthonous microbe predominantly colonising the mucosa-associated surface of the colon.[31] This close proximity of *Butyricicoccus* bacteria to the apical surface of the colonic epithelium facilitates host access to its metabolites, like butyrate, which are essential to cellular homeostasis. Since butyrate is known to promote intestinal epithelial barrier function, we analysed its effect on TNF/IFNγ-induced deregulation of TJ gene expression *in vitro*. Butyrate completely prevented loss of TEER in TNF/IFNγ-stimulated Caco-2 monolayers while increasing *TJP1* and *OCLN* expression. Neither increased *CLDN1* mRNA levels nor CLDN1 internalisation was reduced by butyrate, however a drop in total CLDN1 protein levels was observed. Finally, *ex vivo* stimulation of inflamed UC biopsies showed

that the observed *in vitro* effects of butyrate could be mimicked by *B. pullicaecorum* conditioned medium; *i.e.* a marked reduction in *CLDN1* levels.

In conclusion, this study demonstrates that the functions of CLDN1 are not restricted to its traditional role of maintaining intestinal barrier function and that its elevation during mucosal inflammation is detrimental to both epithelial integrity and further downstream pathways responsible for colonic homeostasis. Given the effect of both butyrate and *B. pullicaecorum* conditioned medium on CLDN1 expression, these results further substantiate the use of *Butyricicoccus* as a pharmabiotic in order to preserve epithelial TJ integrity.

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Conflicts of Interest

VE, RD and FVI are listed as co-inventors on a patent application for use of butyrate-producing bacterial strains related to *B. pullicaecorum* in the prevention and/or treatment of intestinal health problems (International Application Number PCT/EP2010/052184 and International Application Number WO2010/094789 A1). For the remaining authors no conflicts of interest were declared.

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Tables

Table 1. Patient characteristics.

	healthy controls	UC inflamed	UC remission
For baseline expression of Butyricicoccus and tight junction complex genes in colonic mucosa			
N (patients)	36	37	16
Gender (male/female)	16/20	25/12	12/4
Age (years, mean)	52	36	42
Age (years, range)	18-80	6-68	16-74
Disease location (E1/E2/E3)	N/A	8/17/12	N/A
Medication			
None	36	11	1
5-Aminosalicylates	0	15	10
Corticosteroids	0	11	1
Immunomodulators	0	7	6
Biologicals	0	7	4
For stimulation of inflamed UC biopsies with B. pullicaecorum conditioned medium			
N (patients)	N/A	25	N/A
Gender (male/female)	N/A	15/10	N/A
Age (years, mean)	N/A	40	N/A
Age (years, range)	N/A	20-68	N/A
Disease location (E1/E2/E3)	N/A	13/10/2	N/A
Medication			
None	N/A	2	N/A
5-Aminosalicylates	N/A	16	N/A
Corticosteroids	N/A	7	N/A
Immunomodulators	N/A	9	N/A
Biologicals	N/A	10	N/A

E1, proctitis; E2, distal colitis; E3, pancolitis; N/A, not applicable.

Table 2. Primers for qRT-PCR analysis.

Gene symbol	Species	Forward (5'-3')	Reverse (3'-5')	E (%)
N/A	*Butyricicoccus*	ACCTGAAGAATAAGCTCC	GATAACGTTGCTCCCTACGT	74
Gapdh	mouse	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA	88
Hmbs	mouse	AAGGGCTTTTCTGAGGCACC	AGTTGCCCATCTTTCATCACTG	95
Cldn1	mouse	TGCCCCAGTGGAAGATTTACT	CTTTGCGAAACGCAGGACAT	97
CLDN1	human	GGGATGGATCGGCGCCATCG	CGTACATGGCCTGGGCGGTC	104
TJP1	human	CTCACCACAAGCGCAGCCACAA	ACAGCAGAGGTTGATGATGCTGGG	98
OCLN	human	AGACGTCCCCAGCCCAGTCC	CGTACATGGCCTGGGCGGTC	111
GAPDH	human	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	91
SDHA	human	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG	92
YWHAZ	human	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT	93
HMBS	human	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC	101

N/A, not applicable; E, efficiency.

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CHAPTER IV

A NEW LOCAL RHO KINASE INHIBITOR DOES NOT ATTENUATE INFLAMMATION-INDUCED INTESTINAL EPITHELIAL DYSFUNCTION

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A new local Rho kinase inhibitor does not attenuate inflammation-induced intestinal epithelial dysfunction

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Abbreviations

ACHP, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3-pyridinecarbonitrile; CD, Crohn's disease; CXCL2, chemokine (C-X-C motif) ligand 2; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IFNγ, interferon gamma; i.g., intragastrically; IL, interleukin; KC/CXCL1, chemokine (C-X-C motif) ligand 1; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MCP1, monocyte chemoattractant protein 1; MLC, myosin light chain; MPO, myeloperoxidase; MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; PBS, phosphate buffered saline; PG, propylene glycol; qRT-PCR, quantitative real-time PCR; ROCK, rho-associated protein kinase; SDHA, succinate dehydrogenase complex A subunit; SEM, standard error of the mean; TEER, transepithelial electrical resistance; TJ, tight junction; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; UC, ulcerative colitis.

Abstract

Background and Aims. Intestinal epithelial barrier dysfunction is a major hallmark of inflammatory bowel disease, and is caused by enterocyte apoptosis and tight junction (TJ) destabilisation. Both processes are supposed to be mediated in part by Rho-associated kinases (ROCK), and elevated ROCK activity is present in the intestinal mucosa of patients with Crohn's disease. The aim of this study was to evaluate the efficacy of a locally acting ROCK inhibitor, AMA0825, in counteracting inflammation-induced loss of epithelial barrier integrity.

Methods. Barrier-protective effects of AMA0825 were investigated in tumor necrosis factor (TNF)/interferon gamma (IFNγ)-stimulated Caco-2 monolayers. AMA0825 anti-inflammatory potential was evaluated *in vitro* in TNF/IFNγ-stimulated HT-29 epithelial cells through assessment of nuclear factor κB (NF-κB) activation, and in two mouse models of acute colitis induced by the disturbance of epithelial integrity. The effect of AMA0825 on receptor-mediated epithelial apoptosis was examined *in vitro* in HT-29 cells, and *in vivo* in an endotoxemia-induced mouse model of enterocyte apoptosis.

Results. AMA0825 efficiently reduced myosin light chain phosphorylation in intestinal epithelial cells *in vitro*, but did not prevent cytokine-induced intestinal epithelial loss of barrier integrity or NF-κB activation. During dextran sulfate sodium- and 2,4,6-trinitrobenzene sulfonic acid- induced colitis, AMA0825 did not influence disease activity or prevent the development of erosions, and did not alter the ensuing inflammatory response. Finally, AMA0825 was not able to prevent TNF/IFNγ-mediated apoptosis of HT-29 colonocytes, or inhibit *in vivo* lipopolysaccharide-induced enterocyte apoptosis and intestinal permeability.

Conclusions. Despite the previously documented involvement of ROCK in inflammation-induced intestinal epithelial barrier dysfunction, no amelioration was observed using our newly developed selective, local ROCK inhibitor AMA0825.

Comment. Other research from our lab demonstrated a role for ROCK inhibition using AMA0825 in preventing and reversing intestinal fibrosis in two different mouse models. On a cellular level, AMA0825 prevented the transforming growth factor β-induced transition of fibroblasts to myofibroblasts by reducing the activation of the myocardin-related transcription factor and p38 mitogen-activated protein kinase signalling pathways. In addition, AMA0825 potentiated autophagy in these cells.

Keywords

rho kinase inhibitor, intestinal inflammation, apoptosis

Introduction

The intestinal epithelium forms a protective barrier that physically separates the gut lumen from the host's mucosa. This barrier is made up of a single layer of intestinal epithelial cells (IEC) tightly held together through junctional complexes, which restrict the translocation of luminal antigens and other macromolecules into the underlying interstitium while still allowing the selective passage of water, electrolytes and nutrients.[1] Of the three types of junctional complexes that link IECs together, the most apically located tight junctions (TJ) are the ones that actually seal off the intercellular space and regulate selective paracellular ionic solute transport.[2] Transmembrane TJ proteins are anchored to the perijunctional cytoskeletal actomyosin ring through peripheral adaptor proteins that form a so-called cytosolic plaque.[3] This plaque also serves as a docking site for a number of signalling proteins involved in TJ dynamics and barrier regulation.[2,4] One of these is the RhoA GTPase and its associated kinase, the Rho-associated kinase (ROCK), a serine/threonine kinase of which two isoforms, ROCK1 and ROCK2, have been identified. ROCK activity regulates both the assembly and disassembly of TJs, as well as paracellular permeability through phosphorylation of targets like the non-muscle myosin II light chain (MLC), which enables the contraction of the perijunctional actomyosin ring and subsequent disruption of the intercellular junctions, and LIM kinase, which inactivates the actin-depolymerisation factor cofilin and thereby stabilises the junctional actin cytoskeleton. In addition to the regulation of actomyosin contraction and actin polymerisation, ROCK activity can also affect paracellular permeability by directly phosphorylating TJ proteins.[5-7]

One of the hallmarks of inflammatory bowel disease (IBD) - a chronic and relapsing inflammatory condition of the gastro-intestinal tract, comprising Crohn's disease (CD) and ulcerative colitis (UC) - is an impaired intestinal epithelial barrier function[1,8,9], which is caused by deregulated TJ protein expression and their redistribution off the junctional complex, and by increased IEC apoptosis.[10-12] The activity of RhoA and its effector kinase is elevated in the inflamed intestinal mucosa of CD patients, as well as in experimental colitis.[13] Pro-inflammatory cytokines like tumor necrosis factor (TNF) and interferon gamma (IFNγ) are known to increase intestinal epithelial paracellular permeability in IEC monolayers.[14-17] Existing evidence for a protective effect of the Y-27632 ROCK inhibitor against IFNγ-induced, RhoA/ROCK-mediated endocytosis of epithelial TJ proteins[18] and against ethanol-induced increase in Caco-2 barrier permeability[19,20], suggests a rationale for the use of ROCK inhibitors in preventing inflammation-induced loss of TJ integrity. Also membrane blebbing of apoptotic cells has been shown to involve RhoA/ROCK signalling.[21] Evidence for an effect of ROCK inhibition on IEC apoptosis *in vivo* was demonstrated in a study by Ozdemir *et al.*, who showed that Y-27632 reduced endotoxemia-induced enterocyte apoptosis in rats.[22]

Collectively, these data illustrate the potential of ROCK inhibition as a novel therapeutic approach to ameliorate the loss of intestinal epithelial barrier integrity in IBD. However, there are a few reservations concerning the therapeutic use of current ROCK inhibitors like Y-27632. First, although Y-27632 is classified as a selective ROCK inhibitor and is commonly used to study ROCK functions, it also inhibits other protein kinases, depending on the concentration used.[5,23] Secondly, important side effects such as cardiovascular hypotension warrant for great caution if these compounds reach the systemic circulation.[24] To circumvent this problem, we developed AMA0825; a potent and selective ROCK1/2 inhibitor optimised to have a localised action in the intestine due to its degradation upon contact with esterase activity in the blood, which minimises systemic exposure. In the present study, we evaluated the *in vitro* and *in vivo* efficacy of this compound in counteracting inflammation-induced loss of epithelial barrier integrity and the associated inflammatory response.

Materials and Methods

Ethics statements. Mice were housed in the laboratory animal facility at Ghent University Hospital according to the institutional animal healthcare guidelines. This study was approved by the Institutional Review Board of the Faculty of Medicine and Health Sciences of Ghent University (ECD2013-02).

Compounds and reagents. AMA0825 was developed by Amakem Therapeutics (Diepenbeek, Belgium). For the *in vitro* experiments, AMA0825 was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich, Diegem, Belgium). Vehicles used for AMA0825 suspension in *in vivo* experiments; propylene glycol (PG), Labrafil® M1944CS and KLEPTOSE® were bought from Sigma-Aldrich, Mosselman SA (Ghlin, Belgium) and Roquette Pharma (Brussels, Belgium), respectively. Dextran sulfate sodium (DSS, molecular weight 36,000 - 50,000) was bought from MP Biomedicals (Illkirch, France) and 2,4,6-trinitrobenzene sulfonic acid (TNBS, 5% (w/v) in H_2O) from Sigma-Aldrich. Recombinant human IFNγ and TNF were purchased from Life Technologies (Gent, Belgium), butyric acid (sodium butyrate) and lipopolysaccharide (LPS) from Sigma-Aldrich, and ACHP (2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3-pyridinecarbonitrile) from Tocris Bioscience (Abington, UK). ACHP was dissolved in DMSO. The Y-27632 ROCK inhibitor was bought from Cayman Chemical (Anne Arbor, MI, USA) and dissolved in phosphate buffered saline (PBS).

Cell culture. HT-29 (HTB-38) and Caco-2 (HTB-37) intestinal epithelial adherent cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). HT-29 growth medium consists of McCoy's medium supplemented with 10% fetal bovine serum (FBS), Caco-2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. All media and supplements were purchased from Life Technologies. Cells were maintained at 37°C in a humidified atmosphere of air/CO_2 (95:5, v/v) with two medium changes per week.

Induction of epithelial barrier disruption and determination of monolayer integrity. Caco-2 cells were seeded on 24-well semipermeable inserts (0.4 µm, translucent ThinCerts™, Greiner Bio-One, Vilvoorde, Belgium) at a density of 100.000 cells/well. Cells were left to differentiate over the course of 2 to 3 weeks until functional monolayers with absolute transepithelial electrical resistance (TEER) values of more than 6000 Ohm/cm^2 were obtained. Next, monolayers were stimulated apically with AMA0825 (0.1 µM to 10 µM, 10-fold dilution) or butyric acid (5 mM), and basolaterally with a combination of TNF (100 ng/ml) and IFNγ (300 ng/ml) to induce barrier disruption, measured as a drop in TEER. After 48 hrs, absolute TEER values were normalised to their pre-treatment values and expressed as a percentage of the initial TEER values. Each condition was performed in triplicate.

***In vitro* cytotoxicity assays.** HT-29 cells were seeded in 96-well plates (Greiner Bio-One) with each well containing 50.000 cells. The next day, cells were stimulated with a dose range of AMA0825 (1 nM to 10 µM, 10-fold dilution) or an equal concentration of DMSO for 24 hrs. Each condition was performed in triplicate and a positive control of 1% Triton X-100 (Sigma-Aldrich) in PBS was included. Supernatant was collected for lactate dehydrogenase (LDH) quantification according to the manufacturer's instructions (Roche, Vilvoorde, Belgium) and cells were incubated for an additional 3 hrs with 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich). The MTT precipitate was dissolved in DMSO and measured spectrophotometrically at 570 nm (MultiSkan Ascent, VWR International, Leuven, Belgium).

Western blot analysis. HT-29 cells were seeded in 6-well plates (Greiner Bio-One) at a density of 10^6 cells/well. The next day, cells were co-incubated for 24 hrs with a combination of TNF (100 ng/ml) and IFNγ (300 ng/ml), and a dilution series of AMA0825 (0.1, 0.5, 1, 5, 10 µM), a volume of DMSO corresponding to that of the highest AMA0825 concentration or Y-27632 (10 µM). After 24 hrs, cells were lysed using sonication on ice for 1 min in 200 µl of Radio Immunoprecipitation Assay buffer supplemented with phosphatase and protease inhibitors (Sigma-Aldrich). Lysate protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Temse, Belgium) according to the manufacturer's instructions with bovine serum albumin (BSA) as a protein standard. Thirty µg of each sample was mixed with 1:4 loading buffer (Life Technologies) and 1 mM dithiothreitol (Roche). Samples were denatured by boiling for 10 min at 95°C, separated on a 4–12% gradient Bis-Tris SDS-PAGE gel and transferred to a nitrocellulose membrane using a wet transfer (all Life Technologies). Afterwards, membranes were blocked with 5% milk powder in Tris buffered saline with 0.1% Tween-20 (TBST) (Sigma-Aldrich) and incubated overnight at 4°C with 1:1000 rabbit anti-phospho-myosin light chain 2 (Ser19) antibody (Cell Signalling, Leiden, The Netherlands) in 5% BSA in TBST. Next, blots were incubated for 1 h at room temperature with 1:2000 secondary goat anti-rabbit IgG, HRP-conjugated secondary antibody (Cell Signalling). Bound antibodies were visualised using the BM Chemiluminescence Western Blotting Substrate POD (Roche) according to manufacturer's instructions and membranes were exposed to X-ray films. Equal loading of proteins was confirmed by immunoblotting with 1:5000 anti-tubulin (Abcam, Cambridge, UK) antibody in 5% BSA in TBST. Tubulin and phosphorylated MLC protein band intensities were measured using the ImageJ program (Java). Intensity values of the phosphorylated MLC bands were normalised to those of the corresponding tubulin bands.

Measurement of NF-κB p65 phosphorylation. HT-29 cells were seeded in 96-well plates at a density of 25.000 cells/well. The next day, cells were incubated for 24 hrs with a dilution series (0.1 µM to 10 µM, 10-fold dilution) of AMA0825, ACHP (10 µM) or a volume of DMSO corresponding to that of the

highest AMA0825 concentration. After 24 hrs, the supernatant was removed and cells were briefly stimulated with a combination of TNF (100 ng/ml) and IFNγ (300 ng/ml). After 30 minutes, the level of p65 phosphorylation was determined using an enzyme-linked immunosorbent assay (ELISA)-based detection method (NF-κB p65 (pS536) PhospoTracer, Abcam, Cambridge, UK) with a fluorescent read-out signal (λex/λem = 544/590 nm; FLUOstar Omega, BMG Labtech, Germany) according to the manufacturer's instructions. Each condition was performed in triplicate.

Measurement of IL-8 secretion. HT-29 cells were seeded in 96-well plates at a density of 80.000 cells/well. The next day, cells were pre-incubated for 1 hour with an dilution series (0.1 µM to 10 µM, 10-fold dilution) of AMA0825, ACHP (10 µM) or a volume of DMSO corresponding to that of the highest AMA0825 concentration. Following the pre-incubation, a combination of TNF (100 ng/ml) and IFNγ (300 ng/ml) was added and the cells were incubated with TNF/IFNγ and AMA0825, ACHP or DMSO for another 24 hrs. After 24 hrs, IL-8 concentration in the supernatant was determined using an ELISA (R&D Systems, Abington, UK) according to the manufacturer's instructions. Each condition was performed in triplicate.

Induction of apoptosis. HT-29 cells were seeded in 96-well plates at a density of 50.000 cells/well. The next day, cells were co-incubated for 24 hrs with a combination of TNF (100 ng/ml) and IFNγ (300 ng/ml), and a dilution series of AMA0825 (0.1 nM to 10 µM, 10-fold dilution) or an equal volume of DMSO. Each condition was performed in triplicate. After 24 hrs, supernatant was discarded and cells were lysed for quantification of caspase-3/7 activity.

Caspase-3/7 activity assay. Enzymatic activity of effector caspases 3 and 7 was assayed in HT-29 lysates and mouse ileal tissue homogenates using the Caspase-Glo® 3/7 Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. After 1 hour, the release of light following caspase-mediated cleavage of a luminogenic substrate was measured using a luminometer (FLUOstar Omega).

Pharmacokinetic profiling of AMA0825 in C57BL6/J mice. Ten-week old male C57BL/6J mice received a single dose of AMA0825 suspended in PG (20% (v/v) in H_2O) that was administered intragastrically (i.g.). Plasma and colon samples were collected 1, 2, 4, 8 and 24 hrs after AMA0825 administration. Blood was sampled retro-orbitally under anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine) and collected in tubes containing EDTA (5 mM final concentration). Plasma was extracted and samples were stored at -80°C until analysis. Mice were sacrificed by cervical dislocation and their colons were excised, rinsed with ice cold saline, blotted dry, weighed, snap frozen, and stored at -80°C. The colon samples were subsequently homogenised, and the

concentration of AMA0825 in plasma and colon homogenates was determined by liquid chromatography-tandem mass spectrometry (LC/MS-MS) analysis.

Determination of cardiovascular side-effects of AMA0825. Sixteen-week old Spontaneously Hypertensive rats (Charles River Laboratories, Leiden, The Netherlands) were anesthetised using isoflurane and were subcutaneously administered with 7.5 mg/kg carprofen (Rimadyl®). Following a midline incision in the abdomen, a DSI TA11PA-C40 (Data Sciences International, s'Hertogenbosch, The Netherlands) implantable telemetric device was introduced into the peritoneal cavity, and the catheter of the device was inserted facing upstream into the descending aorta at a point below the renal arteries. The abdominal and skin incisions were then closed. The animals were given 100 mg/kg amoxicillin intramuscularly and returned individually to their cages. After 24 hrs, they were given 100 mg/kg amoxicillin subcutaneously. One week later, the animals were placed individually within their home cage on a telemetry receiver (Data Sciences International) to record mean, systolic and diastolic arterial blood pressures (mmHg) as well as heart rate (beats/min, derived from pulse blood pressure). All generated data were acquired and analysed using EMKA Technologies software. AMA0825 was administered i.g., and data were recorded continuously from 30 min before to 4 hrs after the administration of AMA0825. Effects were reported at the following time points: 0, 15, 30, 45, 60, 120, 180 and 240 min after each application. Each animal received both vehicle and the test substance, with a wash-out period of at least 48 hrs between each treatment.

Induction and assessment of colitis. For both the DSS- and TNBS-induced colitis experiments, 8- to 10-week old male C57BL/6 mice were purchased from Harlan Laboratories (Horst, The Netherlands) and conventionally housed in a temperature-controlled room at 20°C with a light/dark cycle of 12/12 h. Mice had free access to water and a commercial chow (mice maintenance chow, Carfil Labofood, Pavan Service, Belgium). In the DSS-induced colitis, mice received 2.5% DSS in their drinking water for 7 days, followed by another 2 days of tap water. Non-DSS control mice were allowed to drink only water. Prior to the start of the experiment, mice were matched for initial body weight. The number of mice used for each treatment group was 8 and 6 healthy control mice were also included. Body weight was recorded daily. Mice were sacrificed by cervical dislocation 2 days after DSS withdrawal. TNBS-colitis was induced by rectally administering 2 mg TNBS in 50% ethanol. The non-TNBS controls received PBS in 50% ethanol. Prior to the start of the experiment, mice were matched for initial body weight. The number of mice used for each treatment group was 7 and 5 healthy control mice were also included. Body weight was recorded daily. Mice were sacrificed by cervical dislocation 4 days after TNBS instillation.

Treatment of colitis. In the DSS-induced colitis, mice were treated prophylactically with AMA0825 (3 and 10 mg/kg) starting 2 days before induction of colitis. AMA0825 was suspended in PG (20% (v/v)

in H_2O) and administered i.g. on a daily basis. Suspensions were prepared fresh every day just before administration and the dose was adjusted to the measured individual body weight on the respective day. Untreated DSS controls and non-DSS controls also received a weight-matched volume of the PG vehicle. In the TNBS-induced colitis, mice were treated prophylactically with AMA0825 (0.3 and 3 mg/kg) starting 2 days before induction of colitis. AMA0825 was suspended in kleptose (5% (w/v) in H_2O) and administered i.g. on a daily basis. Suspensions were prepared fresh every day just before administration and the dose was adjusted to the measured individual body weight on the respective day. Untreated TNBS controls and non-TNBS controls also received a weight-matched volume of the kleptose vehicle.

Tissue sampling of colitis. The colon was excised and its length was measured. The colon was opened longitudinally and feces were removed by flushing with PBS. Distal colon tissue fragments of 5 mm were cut and immersed in formaldehyde (Klinipath, Olen, Belgium), RNAlater (Ambion, Cambridgeshire, UK) or snap-frozen, and stored at -80°C until analysis.

LPS challenge, tissue sampling and determination of intestinal permeability. Thirty-two female, 9-week-old C57BL6/J mice (Janvier Labs, Le Genest-Saint-Isle, France) were i.p. injected with 7.5 mg/kg LPS from *Salmonella enterica serotype abortus equi* or with PBS. LPS-challenged mice were randomised into 3 groups (N=8/group), receiving either 0.3 mg/kg or 3 mg/kg AMA0825 or PG vehicle i.g. 48, 24 and 1 hour prior to LPS administration. One hour after LPS challenge, fluorescein isothiocyanate (FITC)-labelled dextran (4 kDa, Sigma-Aldrich) was administered i.g. to mice at 125 mg/kg. Five hrs later, blood obtained by heart puncture was collected in EDTA-coated tubes (Sarstedt, Essen, Belgium) and plasma was prepared. Ileal tissue fragments were collected in formaldehyde or snap-frozen, and stored at -80°C until analysis. Leakage of FITC-labelled dextran into the circulation was determined by measuring the plasma fluorescence (λex/λem = 488/520 nm; FLUOstar Omega).

Histopathology. Formaldehyde-fixed distal colonic tissue sections of 4 µm were stained with hematoxylin and eosin and scored in a blinded fashion for intestinal inflammation using a validated scoring system.[25] Histological sections were evaluated for epithelial erosions, mucosal and submucosal influx of inflammatory cells, and mucosal and submucosal thickening.

Myeloperoxidase assay. Distal colonic and ileal myeloperoxidase (MPO) activity was determined as described previously.[26] Mucosal MPO values were normalised to the total protein concentration measured using the Bio-Rad Protein Assay according to the manufacturer's instructions.

Luminex. Mouse distal colonic and ileal tissue homogenates were prepared in PBS containing protease and phosphatase inhibitors (Sigma-Aldrich). Total protein concentration was measured

using the Bio-Rad Protein Assay. Pro-inflammatory cytokine levels were determined using the bead-based Bio-Plex Pro™ mouse cytokine platform according to the manufacturer's instructions (Bio-Rad). Measurements were performed using the Bio-Plex MAGPIX Multiplex Reader and data were analysed with the Bio-Plex Manager 6.1 software (Bio-Rad).

TUNEL staining. Apoptotic cells were detected in paraffin-embedded ileal tissue by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) according to the manufacturer's protocol (Promega) and nuclei were counterstained by DAPI (Sigma-Aldrich). The mean number of TUNEL-positive epithelial nuclei were counted from three fields per mouse (×400).

RNA extraction. Total RNA from mouse distal colonic tissue fragments was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) with on-column DNase treatment. Concentration and purity was determined using nanodrop technology (BioPhotometer Plus, Eppendorf, Rotselaar, Belgium). All samples exhibited an OD260/OD280 ratio between 1.8 and 2.1.

Quantitative Real-Time PCR (qRT-PCR). One microgram of total RNA was converted to single stranded cDNA by reverse transcription using the iScript™ cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. The cDNA was diluted to a concentration of 5 ng/µl and 15 nanogram was used in qRT-PCR with SYBR Green (SensiMix™ SYBR No-ROX kit, GC biotech, Alphen a/d Rijn, The Netherlands) and 250 nM of each primer. A two-step program was performed on the LightCycler 480 (Roche). Cycling conditions were 95°C for 10 min, 45 cycles of 95°C for 10 s and 60°C for 1 min. Melting curve analysis confirmed primer specificities. All reactions were performed in duplicate. Expression of mouse cytokine genes was normalised to the stably-expressed reference gene levels of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and succinate dehydrogenase complex A subunit (*Sdha*). The PCR efficiency of each primer pair was calculated using a standard curve of reference cDNA. Amplification efficiency was determined using the formula $10^{-1/slope}$. Sequences of the primer sets are listed in Table 1.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (GraphPad, La Jolla, CA, USA). Values are presented as the mean ± standard error of the mean (SEM). Normality of the data was checked using the Kolmogorov-Smirnoff test. Differences between groups were analysed using an unpaired Student's *t*-test for independent samples in case of normally distributed data (applying the Welch's correction in case of unequal variances) or the Mann-Whitney statistic if the data were not normally distributed. A two-way analysis of variance was performed to determine significances in overall body weight variation between treatment groups throughout the duration of the DSS- and TNBS-induced colitis experiments. Two-tailed probabilities were calculated and *p*-values less than 0.05 were considered statistically significant.

Results

AMA0825 does not prevent TNF/IFNγ-induced *in vitro* intestinal epithelial barrier disruption

We evaluated whether AMA0825 was able to prevent inflammation-induced loss of barrier integrity. Previous work demonstrated that AMA0825 dose-dependently inhibits MLC phosphorylation (quantified via ELISA) within a concentration range of 1 nM to 10 µM in the rat smooth muscle cell line A7r5, which endogenously expresses ROCK (data not shown). TNF/IFNγ-stimulated Caco-2 monolayers were treated apically with a dose range of AMA0825 (0.1 to 10 µM, 10-fold dilution) or butyric acid (5 mM) as a positive control.[27] Forty-eight hrs after stimulation with TNF/IFNγ, a significant drop in TEER was observed (p = 0.0143, Figure 1). The TNF/IFNγ-induced loss of TEER was not counteracted by stimulation with AMA0825 and even significantly increased at a concentration of 10 µM (p = 0.0037, Figure 1).

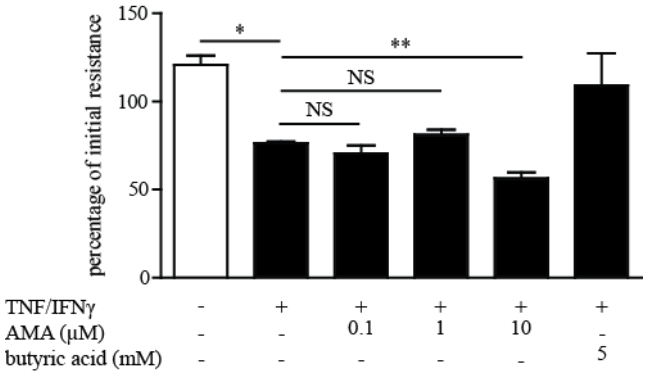

Figure 1. Effect of AMA0825 on TNF/IFNγ-induced barrier disruption in Caco-2 monolayers. *Caco-2 monolayers were stimulated basolaterally with a combination of TNF and IFNγ, and treated apically with a dose range of AMA0825 (0.1 to 10 µM, 10-fold dilution) or butyric acid (5 mM). TEER was measured 48 hrs post-stimulation and expressed as a percentage of the initial resistance prior to stimulation. Data represent the mean ± SEM of 3 replicates/group. *p < 0.05, **p < 0.01. AMA, AMA0825; NS, not significant.*

AMA0825 does not ameliorate *in vitro* TNF/IFNγ-induced NF-κB activation in HT-29 colonocytes

In a preliminary experiment, cytotoxicity of a dose range of AMA0825 (1 nM to 10 µM, 10-fold dilution) on intestinal epithelium were evaluated in HT-29 colonic epithelial cells. AMA0825 was not toxic to HT-29 cells up to 10 µM, i.e. no increased LDH release was observed after 24 hrs (Figure 2A). Cell viability was reduced at 10 µM (p = 0.0023, Figure 2B) but this was not associated with an increased LDH release. Cellular uptake and effectiveness of AMA0825 in HT-29 cells was confirmed by detection of decreased protein levels of phosphorylated MLC after stimulation with TNF/IFNγ, which was added to induce MLC phosphorylation, and AMA0825 at 10, 5, 1, 0.5 and 0.1 µM for 24 hrs (Figure 2C).

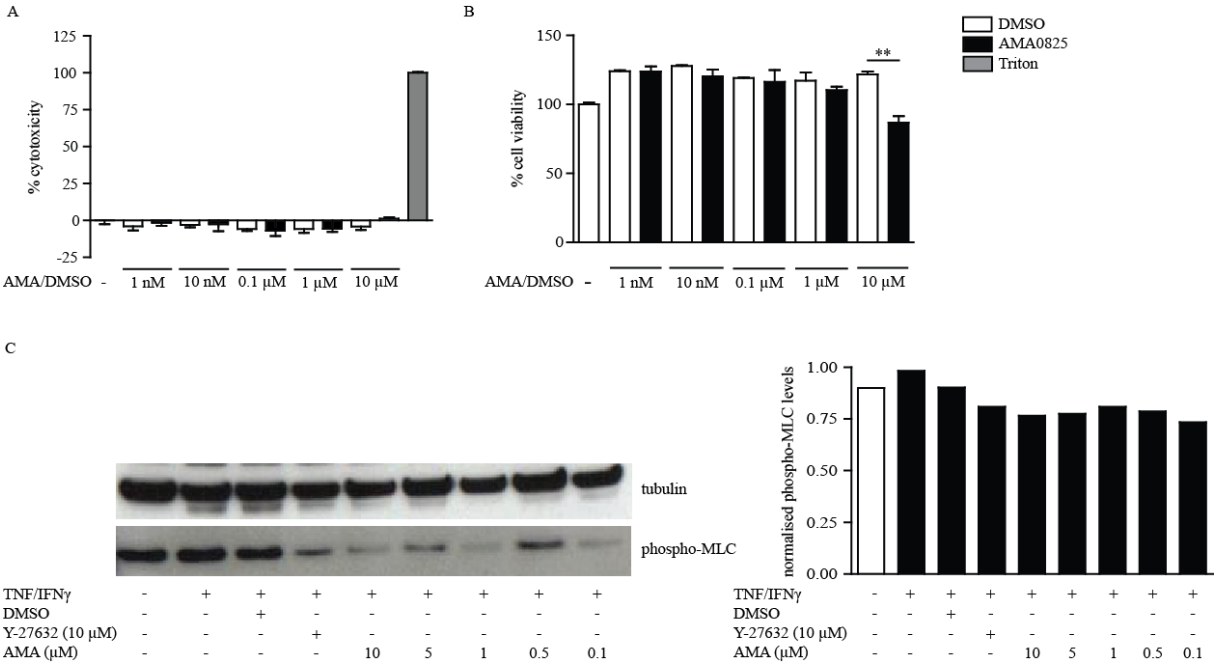

Figure 2. Evaluation of AMA0825 toxicity and effectiveness in HT-29 colonic epithelial cells. *(A) Cytotoxicity and (B) cell viability after stimulation with AMA0825 or DMSO control for 24 hrs. Cytotoxicity is expressed as a percentage of maximal LDH release (i.e. Triton condition), cell viability is expressed as a percentage of control (i.e. unstimulated cells). (C) protein expression of phosphorylated MLC after stimulation with TNF/IFNγ and AMA0825 or Y-27632 for 24 hrs. A condition stimulated with a volume of DMSO corresponding to that of the highest AMA0825 concentration was included to determine if DMSO had any effect on MLC phosphorylation. Levels of phosphorylated MLC were normalised to the corresponding levels of the tubulin loading control. Data in A and B represent the mean ± SEM of 3 replicates/group. **p < 0.01 compared to DMSO control. AMA, AMA0825.*

In the next set of experiments, the anti-inflammatory potential of AMA0825 was evaluated in HT-29 epithelial cells using a combination of TNF and IFNγ to trigger NF-κB activation, which was measured via p65 phosphorylation and the secretion of IL-8. HT-29 cells were treated for 24 hrs with a dose range of AMA0825 (0.1 to 10 µM, 10-fold dilution) or ACHP (10 µM) as a positive control[28], and then stimulated with TNF/IFNγ for 30 min to induce p65 phosphorylation. Phosphorylated p65 levels were significantly increased by TNF/IFNγ (p = 0.0001, Figure 3A) but no reduction was observed in any of the AMA0825-treated conditions. Also in a second set-up, in which HT-29 cells were co-incubated for 24 hrs with TNF/IFNγ and a dose range of AMA0825 (0.1 to 10 µM, 10-fold dilution) or ACHP at 10 µM following a 1 hour pre-incubation with AMA0825 or ACHP alone, TNF/IFNγ-induced IL-8 secretion (p = 0.0258, Figure 3B) was not counteracted by stimulation with AMA0825. Significant results for AMA0825 at 0.1 µM (p = 0.0008, Figure 3B) are apparently due to a DMSO-related effect on inhibition of IL-8 secretion.

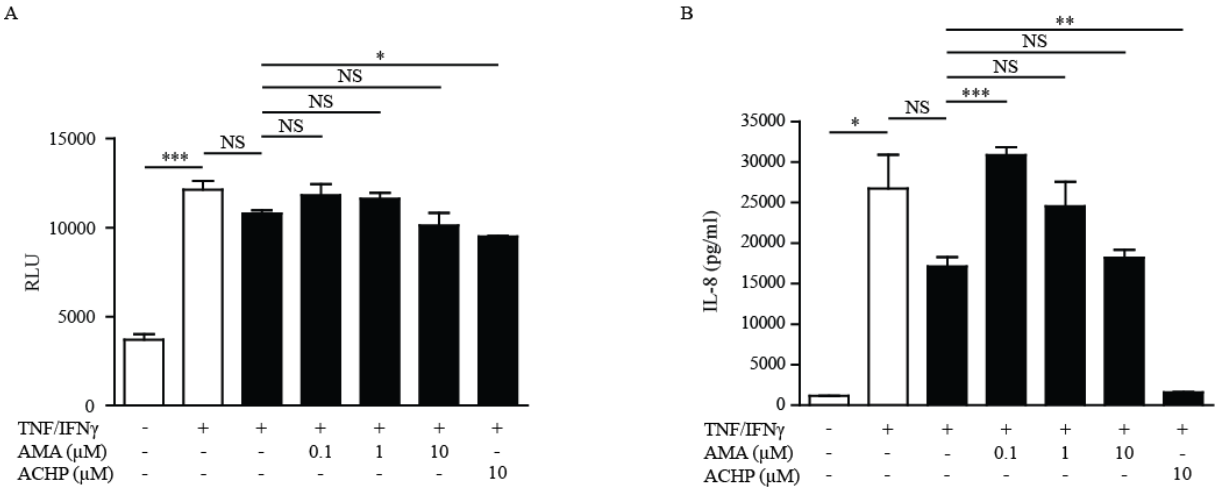

Figure 3. Effect of AMA0825 on TNF/IFNγ-induced NF-κB activation in HT-29 colonocytes. *HT-29 cells were stimulated with a dose range of AMA0825 (0.1 to 10 μM, 10-fold dilution) or ACHP (10 μM) for 24 hrs. A combination of TNF and IFNγ was added (A) for 30 minutes after the 24 hrs stimulation with the compounds, or (B) for 24 hrs together with the compounds following a 1 hour pre-incubation with the compounds alone. Two TNF/IFNγ-conditions were included; one with a volume of DMSO corresponding to that of the highest AMA0825 concentration (black) and one without (white) to determine if DMSO had an effect on NF-κB activation. Phosphorylated p65 levels and secretion of IL-8 in each of the AMA0825-treated conditions are compared to those in the TNF/IFNγ condition treated with DMSO. The amount of phosphorylated p65 is expressed in relative light units (RLU). Data represent the mean ± SEM of 3 replicates/group. *p < 0.05, **p < 0.01, ***p < 0.001. AMA, AMA0825; NS, not significant.*

AMA0825 does not ameliorate intestinal inflammation during acute experimental colitis

In order to further investigate the *in vivo* potential of ROCK inhibition on the development of epithelial erosions, we evaluated the efficacy of AMA0825 in two models of acute intestinal inflammation. In a set of preliminary studies, the pharmacokinetic profile of AMA0825 (3 mg/kg, i.g.) was evaluated in healthy C57BL/6J mice. At each time point (1, 2, 4, 8 and 24 hrs), the plasma concentrations were undetectable (below 1 ng/ml) while the concentration in the colonic tissue was above 150 nM, corresponding to approximately three times the EC_{50} value of AMA0825 in an MLC phosphorylation assay (Figure 4A). This profile supports daily dosing of the compound for *in vivo* application in mice. In line with the pharmacokinetics data in mice, AMA0825 (3 and 10 mg/kg i.g.) did not significantly modify arterial blood pressure and heart rate compared with placebo in spontaneously hypertensive rats (Figure 4B). In conclusion, AMA0825 was shown to be an effective ROCK inhibitor with localised action in the colon that is safe for *in vivo* use.

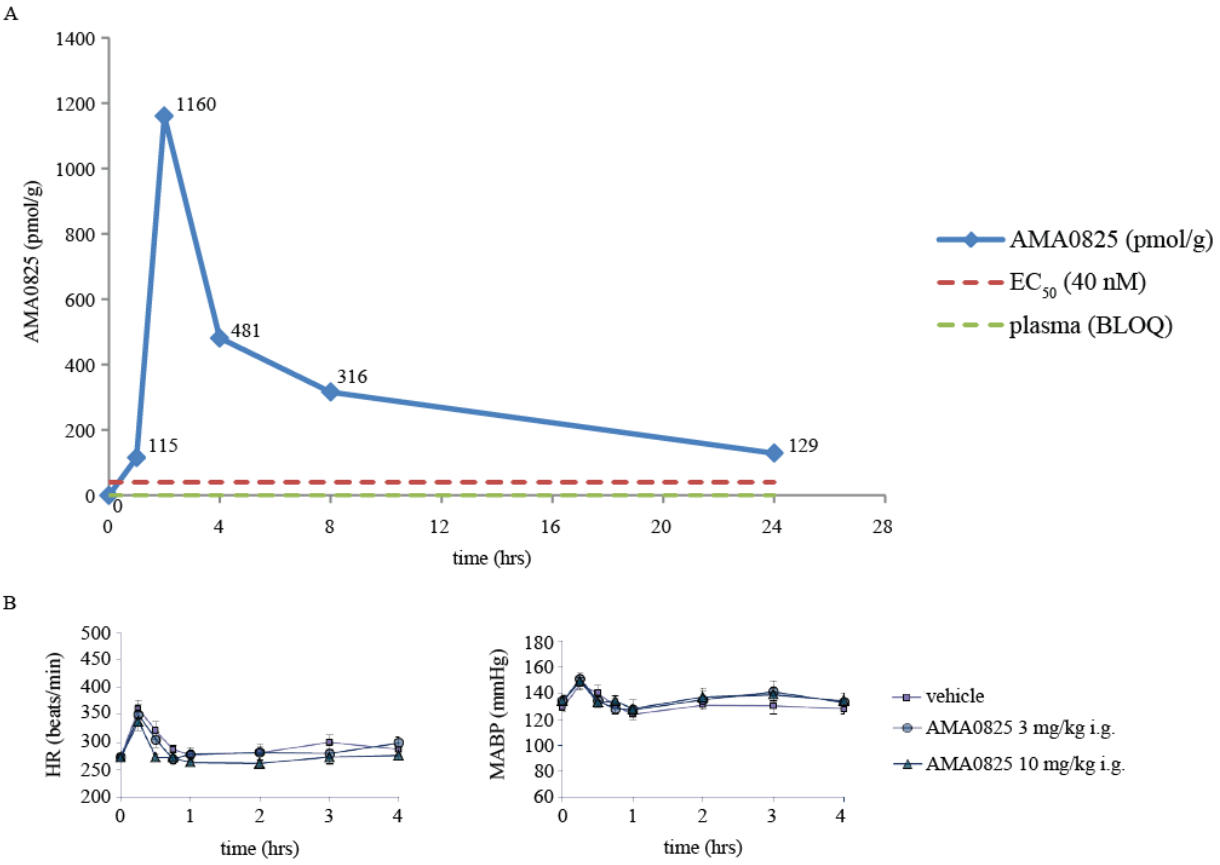

Figure 4. In vivo pharmacokinetic profile and cardiovascular safety assessment of AMA0825 in C57BL6/J mice. *(A) Determination of the AMA0825 concentration by LC-MS/MS analysis in colon homogenates (full blue line, AMA0825 concentration expressed in pmol/ g tissue) and plasma (dotted green line, AMA0825 concentration expressed in ng/ml) collected 0, 1, 2, 4, 8 and 24 hrs after oral dosing at 3 mg/kg. The red dotted line represents the AMA0825 EC_{50} value in the MLC phosphorylation assay. (B) Cardiovascular safety assessment of AMA0825 administered i.g. at 3 and 10 mg/kg in telemetrised Spontaneously Hypertensive rats. Heart rate (HR, beats/min) and mean arterial blood pressure (MABP, mmHg) were recorded up to 4 hrs after dosing. BLOQ, below level of quantification; LC-MS/MS, liquid chromatography-tandem mass spectrometry.*

AMA0825 was first evaluated in acute DSS-induced colitis, a mouse model in which the integrity of the mucosal barrier is directly affected through the toxic effect of DSS on the gut epithelial cells.[29] Prophylactic treatment with AMA0825 at 3 mg/kg decreased overall weight loss compared to the DSS controls ($p = 0.0256$, Figure 5A). Colon length differed significantly between non-DSS and DSS control mice ($p < 0.0001$, Figure 5B), however, AMA0825-treated mice did not show an increase in colon length compared to untreated DSS controls. On histological sections, DSS-induced epithelial erosions and mucosal inflammatory infiltrate ($p = 0.0002$ and $p < 0.0001$, respectively, Figure 5C-D) were not attenuated by treatment with AMA0825. The DSS-induced increase in colonic neutrophil infiltration ($p = 0.0055$, Figure 5E) was not affected by treatment with AMA0825. Levels of the pro-inflammatory cytokines chemokine (C-X-C motif) ligand 1 (KC) and monocyte chemoattractant protein 1 (MCP1) in full-thickness distal colonic lysates were increased upon DSS-administration ($p = 0.0002$ and 0.0055, respectively, Figure 5F) but reduced by treatment with AMA0825.

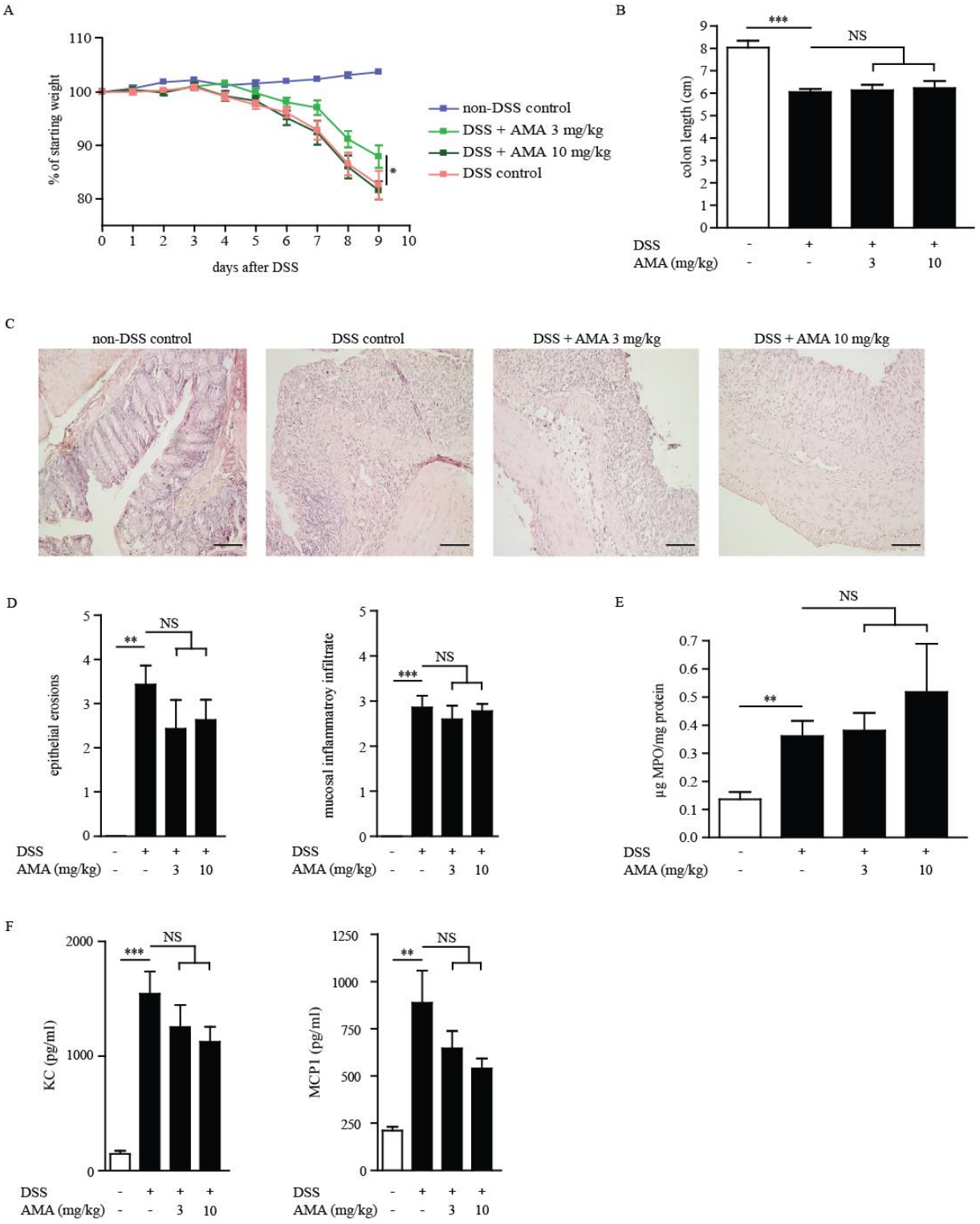

Figure 5. Disease activity parameters and histology following DSS-administration in AMA0825- and control-treated mice. *(A) Weight loss, (B) colon length, (C) representative pictures (200×) of hematoxylin and eosin stained sections of the distal colon of non-DSS control mice and mice treated with DSS and vehicle or AMA0825 at 3 or 10 mg/kg (scale bar represents 100 µm), (D) scores for epithelial erosions and mucosal influx of inflammatory cells in distal colonic histological sections, (E) MPO activity in full-thickness distal colonic lysates, and (F) KC and MCP1 protein levels in full-thickness distal colonic lysates. Cytokine levels are expressed in pg cytokine per ml of lysate and an equal total protein concentration was loaded for each sample. Data represent the mean ± SEM of 8 or 6 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001. AMA, AMA0825; NS, not significant.*

AMA0825 efficacy was evaluated in a second model of mucosal inflammation; the TNBS-induced colitis model in which an initial ethanol-mediated disruption of the mucosal barrier elicits a hapten-induced local colitis.[29,30] In this model, prophylactic treatment with AMA0825 at 3 mg/kg decreased overall weight loss compared to the TNBS controls (p = 0.0237, Figure 6A). However, colon length did not differ significantly between non-TNBS and TNBS controls (Figure 6B), which prevented a fully comprehensive analysis of the effect of AMA0825-treatment on amelioration of this disease parameter compared to placebo. On histological sections, the degree of erosions and mucosal inflammatory infiltration was not significantly different between non-TNBS and TNBS controls (Figure 6C-D). The lack of any significant histological signs of inflammation do not allow for an analysis of the effect of AMA0825 on histological inflammation. Colonic neutrophil infiltration was significantly elevated during TNBS-colitis (p = 0.0145, Figure 6E) but was not reduced by treatment with AMA0825 at 0.3 or 3 mg/kg. Colonic mRNA levels of innate inflammatory (*Tnf*, *Cxcl2*) and Th1 (*Il-12*, *Il-1β*) cytokines were quantified in full-thickness distal colonic lysates but they did not differ significantly between non-TNSB and TNBS controls (data not shown), which prevented a fully comprehensive analysis of the effect of treatment compared to placebo.

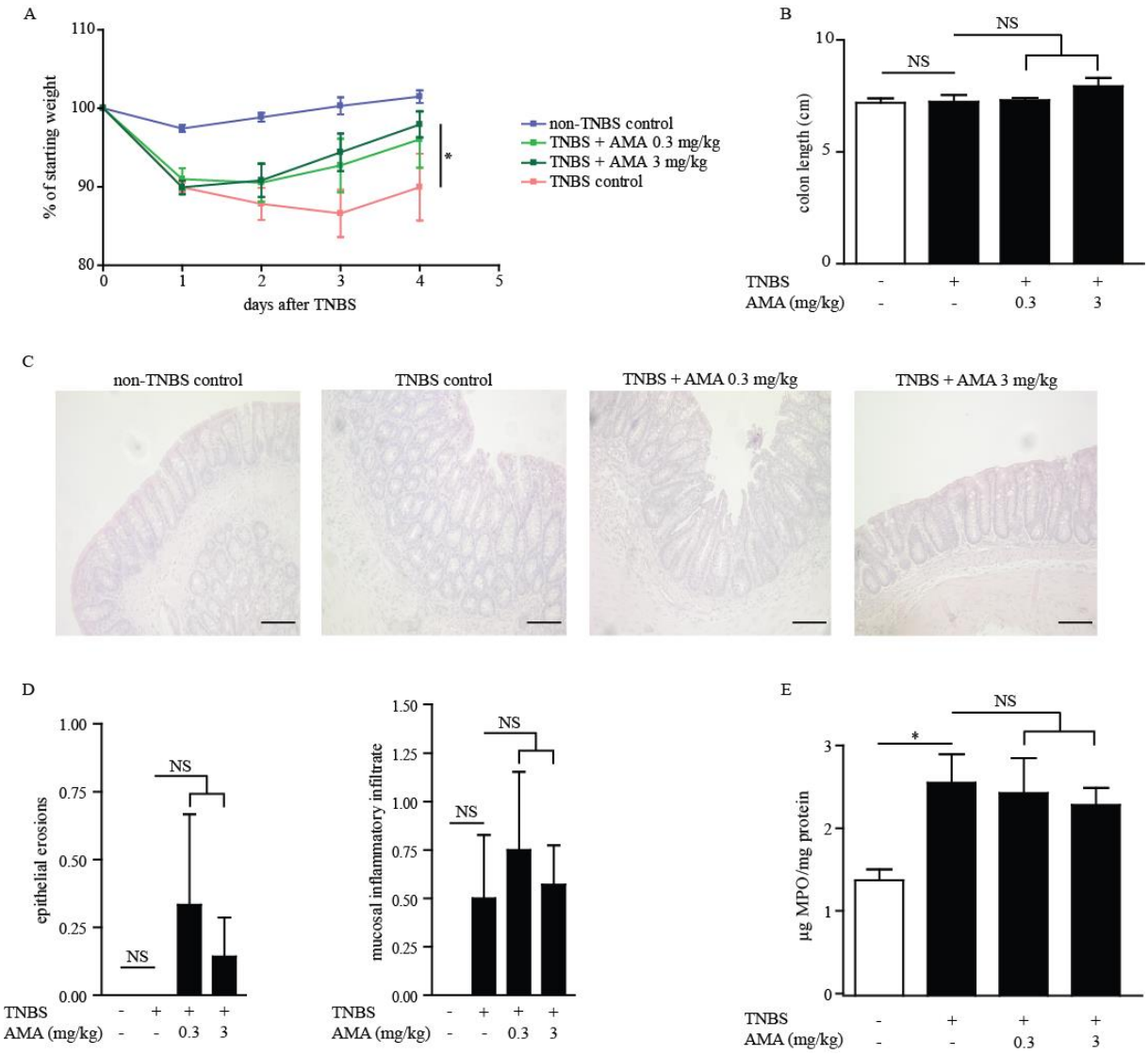

Figure 6. Disease activity parameters and histology following TNBS-administration in AMA0825- and control-treated mice. *(A) Weight loss, (B) colon length, (C) representative pictures (200×) of hematoxylin and eosin stained sections of the distal colon of non-TNBS control mice and mice treated with TNBS and vehicle or AMA0825 at 0.3 or 3 mg/kg (scale bar represents 100 µm), (D) scores for epithelial erosions and mucosal influx of inflammatory cells in distal colonic histological sections, and (E) MPO activity in full-thickness distal colonic lysates. Data represent the mean ± SEM of 7 or 5 mice/group. *p < 0.05. AMA, AMA0825; NS, not significant.*

AMA0825 does not inhibit *in vitro* receptor-mediated caspase activation in HT-29 colonocytes

To evaluate whether AMA0825 exhibits anti-apoptotic properties, HT-29 cells were co-stimulated for 24 hrs with a combination of TNF and IFNγ, two multifunctional cytokines that are highly produced in IBD tissues and which have been implicated in the induction of receptor-mediated IEC apoptosis in IBD[31-33], and a dose range of AMA0825 (0.1 nM to 10 µM, 10-fold dilution). After 24 hrs, TNF/IFNγ significantly induced caspase-3/7 activity (p = 0.0001, Figure 7). However, AMA0825 did not reduce TNF/IFNγ-induced effector caspase-3/7 activation in a dose range of 0.1 nM to 10 µM.

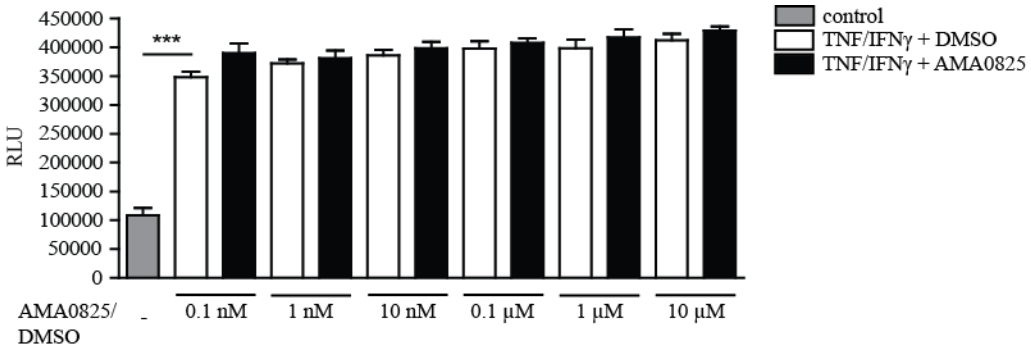

Figure 7. Effect of AMA0825 on TNF/IFNγ-induced effector caspase activation. *Caspase-3/7 activity is expressed in relative light units (RLU). Caspase activity levels in each of the AMA0825-treated conditions are compared to those of their respective DMSO controls. Data represent the mean ± SEM of 3 replicates/group. ***p < 0.001.*

AMA0825 does not prevent *in vivo* LPS-induced intestinal permeability and enterocyte apoptosis

The effect of AMA0825 on *in vivo* intestinal epithelial apoptosis was evaluated in a mouse model of LPS-induced enterocyte apoptosis. In this model, ileal TNF levels peak after one hour and are responsible for increased intestinal permeability, enterocyte apoptosis and luminal shedding, and an inflammatory response.[34,35] The LPS-induced increase in intestinal permeability was measured through detection of FITC-dextran leakage in plasma (p = 0.0047, Figure 8A). Administration of AMA0825 48, 24 and 1 hrs prior to LPS challenge at 3 mg/kg did not reduce plasma levels of FITC-dextran, and even increased it at 0.3 mg/kg (p = 0.0268, Figure 8A). Enterocyte apoptosis was evaluated through determination of effector caspase-3/7 activity and microscopic counting of the number of apoptotic nuclei in the mucosa and intestinal lumen. Both were increased upon exposure to LPS (p = 0.0121, Figure 8B and p = 0.0004, Figure 8C-D, respectively). Treatment with AMA0825 at 3 mg/kg resulted in a significant reduction of caspase-3/7 activity (p = 0.0224, Figure 8B) but this was not associated with an actual decrease in the number of apoptotic enterocytes (Figure 8C-D). Treatment with AMA0825 at 0.3 mg/kg, although displaying increased FITC-dextran leakage, was not associated with elevated caspase-3/7 activity (Figure 8B). For this reason, the effect of this dose on enterocyte apoptosis and luminal shedding was not investigated.

Also the ensuing local inflammation in this model was not attenuated by AMA0825. LPS-induced ileal neutrophil infiltration and production of characteristic pro-inflammatory cytokines (IL-17, IL-1β, IL-6 and MCP1)[36] were not decreased by treatment with AMA0825 at 0.3 or 3 mg/kg (Figure 8D-E).

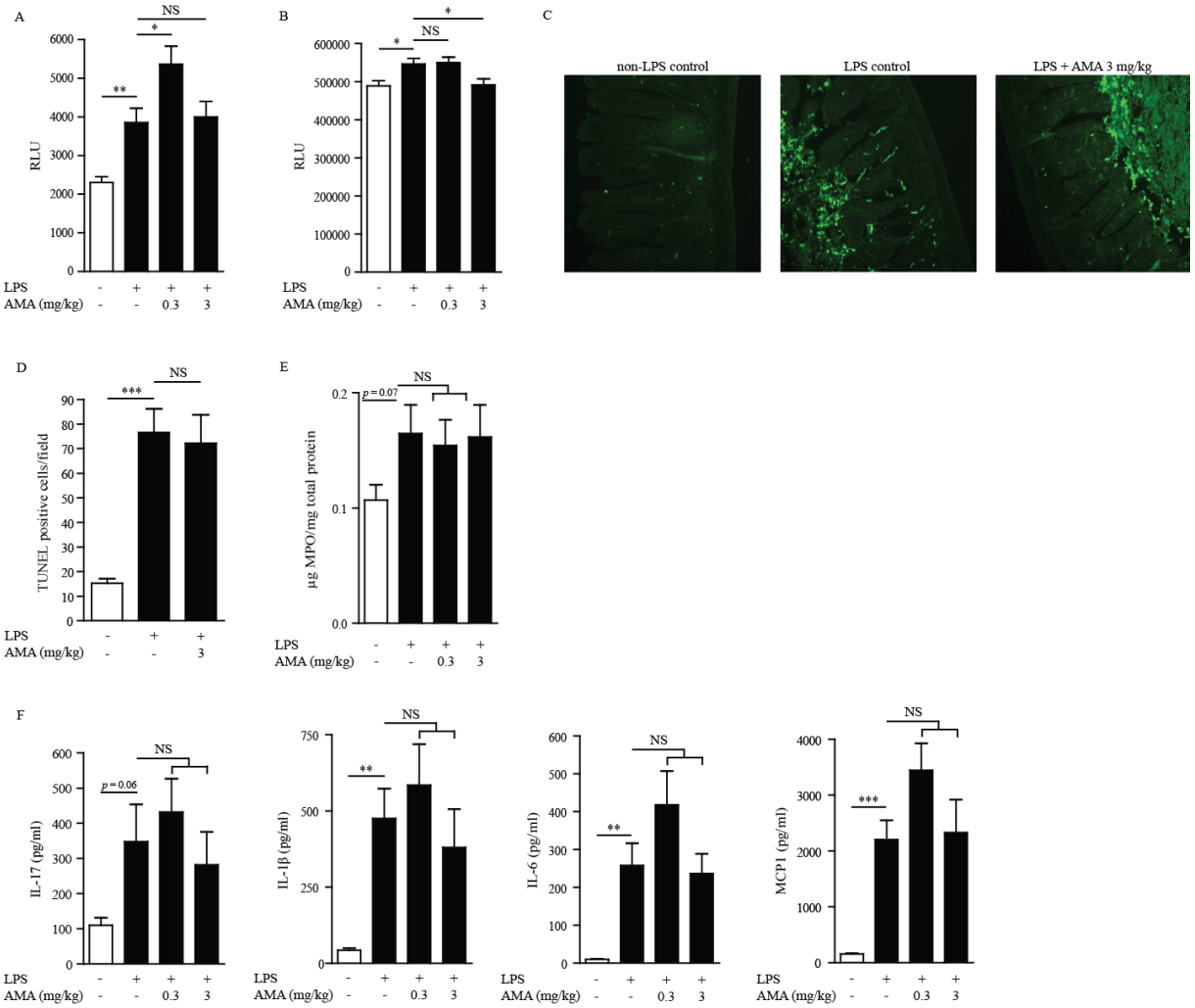

Figure 8. Effect of AMA0825 on LPS-induced intestinal permeability and enterocyte apoptosis. *(A) FITC-dextran fluorescence in plasma, (B) caspase-3/7 activity in full-thickness ileal lysates, (C) representative pictures (400×) of TUNEL-stained sections of the terminal ileum of non-LPS control mice and mice treated with LPS and AMA0825 at 0.3 and 3 mg/kg, (D) TUNEL-positive ileal enterocytes quantified in six fields per mouse (×400), (E) MPO activity in full-thickness ileal lysates, and (F) IL-17, IL-1β, IL-6, and MCP1 protein levels in full-thickness ileal lysates. Cytokine levels are expressed in pg cytokine per ml of lysate and an equal total protein concentration was loaded for each sample. Data represent the mean ± SEM of 8 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001. AMA, AMA0825; RLU, relative light units.*

Discussion

Intestinal epithelial barrier dysfunction in IBD is caused predominantly by disruption of TJ integrity and IEC apoptosis.[37] Both of these processes are partially mediated by the RhoA/ROCK signalling pathway[18,21], whose activity is elevated in mucosa of CD patients.[13] Inhibition of ROCK activity using Y-27632 has proven to be effective at attenuating *in vitro* loss of IEC monolayer integrity[19,20], experimental colitis and mucosal NF-κB signalling[13], and *in vivo* IEC apoptosis.[22] However, the currently available ROCK inhibitors display severe adverse effects upon systemic exposure and harbour selectivity issues.[5,23,24] To circumvent this problem, we developed a very selective, local ROCK1/2 inhibitor - AMA0825 - which is inactivated upon contact with blood esterases. The aim of this study was to evaluate the efficacy of this compound in counteracting inflammation-induced loss of intestinal epithelial barrier integrity and the ensuing inflammatory response.

Although the ROCK-inhibitory capacity of AMA0825 was demonstrated in IECs *in vitro* by means of a reduction in phosphorylated MLC levels, the compound was not able to ameliorate *in vitro* TNF/IFNγ-induced epithelial barrier disruption or NF-κB activation. During acute experimental colitis, prophylactic AMA0825 treatment did not inhibit the development of erosions nor attenuate the downstream mucosal inflammatory response, despite documented local inhibition of ROCK activity.[38] Interestingly, AMA0825 therapy at 3 mg/kg/day resulted in a significant weight loss reduction. The reason for this remains unclear but could not be attributed to an improvement of local inflammation since no effects were observed on infiltration of acute inflammatory cells, the presence of neutrophils, or the secretion of pro-inflammatory cytokines, even though this is the proposed mechanism for the anti-inflammatory effects observed for Y-27632 during TNBS-induced colitis.[13] AMA0825 also did not prevent TNF/IFNγ-induced effector caspase activation in HT-29 IECs. However, mucosal caspase-3/7 activation was reduced in AMA0825-treated mice in a model of LPS-induced intestinal apoptosis, but this was not associated with an actual reduction in the number of apoptotic enterocytes or the ensuing intestinal permeability and mucosal pro-inflammatory response. In conclusion, inhibiting ROCK activity is not an appropriate target for attenuating intestinal epithelial dysfunction and the associated inflammation.

The absence of any effect of the selective AMA0825 ROCK inhibitor on inflammation-induced epithelial dysfunction, suggests that the anti-inflammatory and barrier-protective effects observed for Y-27632 in previous studies are most likely due to the off-target inhibition of other cellular kinases besides ROCK. Davies *et al.* describe how Y-27632 not only inhibits the two ROCK isoforms (IC_{50}= 800 nM) but also several other kinases; i.e. PKC-related protein kinase 2 with a potency similar to that for ROCK2 (IC_{50}= 600 nM), as well as three other protein kinases (mitogen-and stress-activated protein kinase 1, MAPK-activated protein kinase 1b and phosphorylase kinase) with IC_{50}

values 10- to 50-fold higher than those for ROCK2 (IC_{50}= 8.3, 19 and 44 μM, respectively).[23] Other protein kinases like PKCα, PKA and MLCK are also inhibited by Y-27632, albeit at even higher inhibitory concentrations.[39] Like ROCK, all of these protein kinases are known to be involved in TJ regulation and are triggered by diverse pathological conditions.[6] For AMA0825, limited cross-reactivity with PKC δ, ε and θ was observed (data not shown). IC_{50} values for these kinases were respectively 5, 20 and 100 nM, which is substantially higher than the AMA0825 IC_{50} value of less than 0.05 nM for ROCK2. The ROCK-selectivity of AMA0825 compared to Y-27632 therefore lies in its limited cross-reactivity and its extremely low inhibitory concentrations.

Other research performed in our lab focussed on investigating the effects of AMA0825-mediated ROCK inhibition on intestinal fibrosis. Anti-fibrotic effects of ROCK inhibitors have already been described in several non-intestinal pathologies involving pro-fibrotic processes; Y-27632 was shown to prevent interstitial fibrosis in mouse kidneys with unilateral ureteral obstruction, and suppress dimethylnitrosamine-induced hepatic fibrosis in rats, whereas long-term treatment with fasudil improved bleomycin-induced pulmonary fibrosis.[40-42] Structural fibrosis is a common complication in IBD, particularly in patients with ileal CD. Ultimately, up to one-third of CD patients develop an end-stage fibrotic disease, characterised by intestinal strictures, luminal stenosis and organ failure.[43] Although anti-fibrotic agents were recently FDA-approved for idiopathic pulmonary fibrosis, there is currently no treatment available to halt intestinal fibrosis in IBD.[44] We demonstrated that AMA0825 prevents and resolves intestinal fibrosis via effects that combine a reduction in fibroblast activation with an increase in the autophagic response in these cells. These results show that local ROCK inhibition using AMA0825 represents a promising strategy to counteract intestinal fibrosis in CD.

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Conflicts of Interest

KC, SB and AB are former employees of Amakem NV. OD is an independent manager and a shareholder of Amakem NV. DL is a consultant and shareholder of Amakem NV. The other authors do not report any disclosures.

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Tables

Table 1. Primers for qRT-PCR analysis.

Gene symbol	Species	Forward (5'-3')	Reverse (3'-5')	E (%)
Gapdh	mouse	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA	88
Sdha	mouse	CTTGAATGAGGCTGACTGTG	ATCACATAAGCTGGTCCTGT	102
Il-1β	mouse	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA	105
Il-12	mouse	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG	94
Tnf	mouse	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC	93
Cxcl2	mouse	GCGCCCAGACAGAAGTCATAG	AGCCTTGCCTTTGTTCAGTATC	89

E, efficiency.

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CHAPTER V

T84 MONOLAYERS ARE SUPERIOR TO CACO-2 AS A MODEL SYSTEM OF COLONOCYTES

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T84 monolayers are superior to Caco-2 as a model system of colonocytes

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Abbreviations

ALPI, alkaline phosphatase, intestinal; ANPEP, alanyl aminopeptidase, membrane; CFTR, cystic fibrosis transmembrane conductance regulator; DDP4, dipeptidyl peptidase 4; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; HMBS, hydroxymethyl-bilane synthase; LCT, lactase; MCT1, monocarboxylate transporter 1; MGAM, maltase-glucoamylase; MS4A12, membrane spanning 4-domains A12; NaB, sodium butyrate; NRQs, normalised relative quantities; OPLS, orthogonal partial least squares; PCA, principle component analysis; qRT-PCR, quantitative real-time PCR; SEM, standard error of the mean; SI, sucrase-isomaltase; TEER, transepithelial electrical resistance; TEM, transmission electron microscopy; VIP, variable importance for the projection.

Abstract

Colonic adenocarcinoma-derived Caco-2 and T84 epithelial cell lines are frequently used as *in vitro* model systems of functional epithelial barriers. Both are utilised interchangeably despite evidence that differentiated Caco-2 cells are more reminiscent of small intestinal enterocytes than of colonocytes, whereas differentiated T84 cells are less well characterised. The aim of this study was therefore to further characterise and compare differentiated Caco-2 and T84 cells. The objectives were to: (1) compare the brush border morphology, (2) measure the expression of enterocyte- and colonocyte-specific genes and (3) compare their response to butyrate, which is dependent on the monocarboxylate transporter 1 (MCT1), an apical protein expressed primarily in colonocytes. T84 microvilli were significantly shorter than those of Caco-2 cells, which is a characteristic difference between small intestinal enterocytes and colonocytes. Also, enterocyte-associated brush border enzymes expressed in differentiated Caco-2 cells were not increased during T84 maturation, whereas colonic markers such as *MCT1* were more abundant in differentiated T84 cells compared to differentiated Caco-2 cells. Consequently, T84 cells displayed a dose-responsive improvement of barrier function towards butyrate, which was absent in Caco-2 cells. We conclude that differentiated Caco-2 and T84 cells have distinct morphological, biochemical and functional characteristics, suggesting that T84 cells do not acquire the biochemical signature of mature small intestinal enterocytes like Caco-2 cells, but retain much of their original colonic characteristics throughout differentiation. These findings can help investigators select the appropriate intestinal epithelial cell line for specific *in vitro* research purposes.

Keywords

colonocyte, enterocyte, differentiation, epithelial cells, MCT1

Introduction

Both T84 and Caco-2 cells are human intestinal epithelial cell lines derived from a colorectal adenocarcinoma, with the T84 cell line originating from a lung metastasis, whereas the Caco-2 cell line is derived from the primary tumor site. Caco-2 and T84 cells are, when cultured, the only two intestinal epithelial cell lines known to differentiate spontaneously at confluence into a monolayer of structurally and functionally mature absorptive epithelial cells. This maturation is completed within a time-course of two to three weeks and monolayer integrity is usually assessed by measuring the transepithelial electrical resistance (TEER).[1-4] The differentiated phenotype is characterised by a columnar, polarised cell morphology, the formation of tight junctions separating the apical from the basolateral membrane compartments, and the appearance of a microvilli-studded apical brush border.[3] For this reason, differentiated T84 and Caco-2 monolayers are both well-established *in vitro* model systems of human intestinal epithelium and are frequently used to study absorption, metabolism, bioavailability of drugs, electrolyte transport, and the effect of compounds on epithelial barrier integrity.[5-8] Despite the fact that both cell lines are used interchangeably[4], evidence suggests that differentiated Caco-2 cells closely resemble small intestinal enterocytes[9] while mature T84 cells are more reminiscent of undifferentiated crypt cells[10]. However, comparative analysis and a clear characterisation of these cell lines is lacking. Here, we further investigated to what extent mature T84 and Caco-2 cells differ from each another in terms of the genes typically expressed by small intestinal enterocytes and colonocytes.

Materials and Methods

Cell culture. Both Caco-2 (HTB-37) and T84 cells (CCL-248) were obtained from the American Type Culture Collection (Manassas, VA, USA). Caco-2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). T84 cells were grown in DMEM/Nutrient Mixture F-12 supplemented with 5% FBS. All media and supplements were purchased from Life Technologies (Gent, Belgium). Cells were incubated at 37°C in a humidified atmosphere of air/CO_2 (95:5, v/v) with three medium changes per week.

Transmission electron microscopy (TEM) and determination of microvillus length. Caco-2 and T84 cells were seeded on 24-well, 0.4 µm pore diameter, semipermeable inserts (Greiner Bio-One, Vilvoorde, Belgium) at a density of 10^5 cells per well and cultured for two weeks. After this period, the integrity of the monolayer was evaluated by measuring the TEER using a Millicell ERS-2 Voltohmmeter (Merck Millipore, Billerica, MA, USA). Next, the inserts were fixed in glutaraldehyde/paraformaldehyde and rinsed overnight in 0.1 M sodium cacodylate buffer (pH 7.4). Slices were post-fixed in 1% buffered osmium tetroxide (Merck, Summit, NJ, USA), dehydrated in a series of alcohol and embedded in Epon (Aurion, Wageningen, The Netherlands). Semi-thin (1 mm) sections, stained with toluidine blue, facilitated orientation of the tissue. Ultra-thin (90 nm) sections were cut, contrasted with uranyl acetate and lead citrate and examined in a Zeiss transmission electron microscope 900 at 50kV (Carl Zeiss, Oberkochen, Germany). The mean microvillus length was calculated from ten separate measurements of microvilli on TEM images of Caco-2 and T84 apical brush borders (× 12.000).

RNA extraction. Caco-2 and T84 cells were seeded in 12-well plates (Greiner Bio-One) at a density of 10^6 cells/well and allowed to differentiate for 7, 14 or 21 days. For the undifferentiated conditions, 10^6 cells were collected prior to passaging of subconfluent cultures. Each condition was performed in triplicate. Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Concentration and purity was determined using nanodrop technology (BioPhotometer Plus, Eppendorf, Rotselaar, Belgium). All samples exhibited an OD260/OD280 ratio between 1.8 and 2.1.

Quantitative Real-Time PCR (qRT-PCR). One microgram of total RNA was converted to single stranded cDNA by reverse transcription using the iScript™ cDNA synthesis kit (Bio-Rad, Temse, Belgium) according to the manufacturer's instructions. The cDNA was diluted to a concentration of 5 ng/µl and 15 nanogram was used in qRT-PCR with SYBR Green (SensiMix™ SYBR No-ROX kit, GC biotech, Alphen a/d Rijn, The Netherlands) and 250 nM of each primer. A two-step program was performed on the LightCycler 480 (Roche, Vilvoorde, Belgium). Cycling conditions were 95°C for 10 min, 45 cycles of 95°C for 10 s and 60°C for 1 min. All reactions were performed in duplicate. Melting

curve analysis confirmed primer specificities. The PCR efficiency of each primer pair was calculated using a standard curve of reference cDNA. Amplification efficiency was determined using the formula $10^{-1/slope}$ and primer pairs were selected based on an efficiency of 90-110%. Primer pair sequences and their efficiencies are listed in Table 1. Expression data were calculated relative to the mean of the overall expression level and normalised to the expression of the hydroxymethyl-bilane synthase (*HMBS*) reference gene (expressed as normalised relative quantities, NRQs).

Predictive modelling using SIMCA^{TM} software. The qRT-PCR data for Caco-2 and T84 cells were used to construct a prediction model using SIMCA^{TM} 14 software (Umetrics, Malmö, Sweden). Multivariate data analysis was performed by means of unsupervised principle component analysis (PCA), i.e. without a priori knowledge of the data set, in order to visualise the natural interrelationship (including grouping and detection of outliers) among the observed mRNA levels of all enterocyte- and colonocyte-specific genes (*ALPI*, *LCT*, *SI*, *MGAM*, *DDP4*, *ANPEP*, *CFTR*, *SLC16A1*, and *MS4A12*) analysed for Caco-2 and T84 cells at different stages of maturation (subconfluent = day 0 and one, two or three weeks post-confluency; i.e. day 7, 14 and 21, respectively). Using the qRT-PCR data for Caco-2 and T84 cells at day 21 only, a second model, the orthogonal partial least squares (OPLS) model, was computed. This model separates the systematic variation in X (i.e. differentiation markers tested) into two elements: one that is linearly related or predictive to Y (i.e. differentiated Caco-2 or T84 phenotype) and one that is orthogonal or unrelated to Y. The validity of the OPLS model was verified by considering two different quality parameters: the R^2 and Q^2 parameters. R^2 corresponds to the predictive and orthogonal variation in X that is explained by the model and is a measure for the goodness of fit. Q^2 refers to the goodness of prediction calculated by full cross-validation. $R^2 = 1$ indicates perfect description of the data by the model, whereas $Q^2 = 1$ indicates perfect predictability. According to the SIMCA users' guide, $Q^2 > 0.5$ is admitted for good predictability.[11] The obtained R^2 and Q^2 values were 0.998 and 0.993, respectively, indicating a well-fitted, reliable model. A corresponding S-plot was constructed to reveal the significance of each of the selected differentiation markers with respect to the differentiation status of either Caco-2 or T84 cells. Additionally, VIP-values (variable importance for the projection, VIP), summarising the most important X-variables that contribute to Y, were calculated. VIP-values larger than 1 indicate "important", discriminatory X-variables.

Butyrate dose-response. Caco-2 and T84 cells were seeded in semipermeable inserts (24-well, 0.4 µm pore diameter, Greiner Bio-One) at a density of 80.000 cells per well. Starting on day 2 after seeding, developing monolayers were stimulated apically with a dose-range (0.5 mM to 8 mM, 2-fold dilution) of sodium butyrate (NaB) (Sigma-Aldrich, Diegem, Belgium). Evolution of monolayer

integrity was evaluated on day 2 and day 9 post-seeding by measuring the TEER. Cell culture medium was changed three times per week. Each condition was performed in triplicate.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (GraphPad, La Jolla, CA, USA) and SPSS Statistics version 22.0 (IBM SPSS Statistics, Armonk, NY, USA). Normality of the data was determined using the Kolmogorov-Smirnoff test. Differences between groups were analysed using an unpaired Student's *t*-test for independent samples in case of normally distributed data (applying the Welch's correction in case of unequal variances) or the Mann-Whitney statistic if the data were not normally distributed. Two-tailed probabilities were calculated and *p*-values less than 0.05 were considered statistically significant. Values are presented as the mean ± standard error of the mean (SEM).

Results

Differentiated T84 cells have shorter microvilli than differentiated Caco-2 cells

T84 and Caco-2 cells were grown on semi-permeable membranes, which improves morphological differentiation of the monolayers by allowing access of fluids, ions and nutrients to both the apical and basolateral compartments.[3] Two-week old monolayers with TEER values of 700 Ohm.cm^2 or higher were analysed by transmission electron microscope. Both cells lines exhibited a fully differentiated phenotype, i.e. a monolayer of adherent columnar absorptive epithelial cells with an apical brush border (Figure 1A). However, the microvilli on the T84 cells are approximately three times shorter than those on the Caco-2 cells ($p < 0.0001$, Figure 1B), which is in accordance with the *in vivo* observation that the small intestinal brush border is studded with long, densely packed microvilli, whereas the absorptive cells of the colon have short, irregular microvilli.[12]

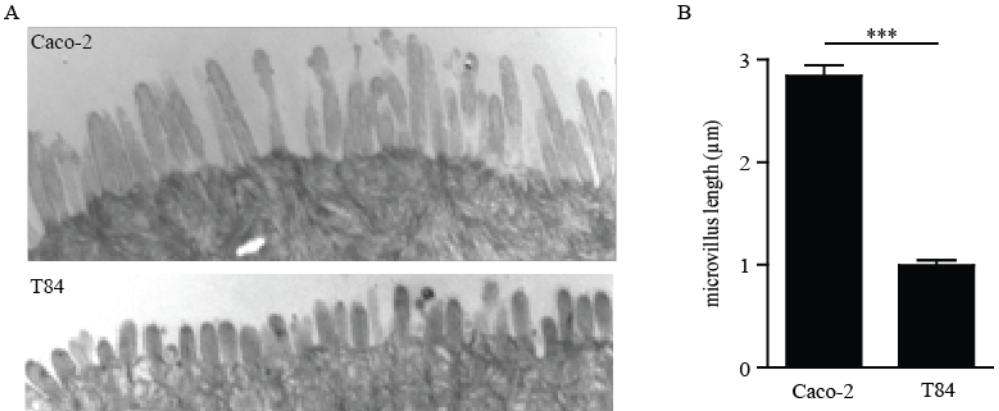

Figure 1. Microvillus length in fully differentiated Caco-2 and T84 monolayers. *(A) Detail TEM images (× 12.000) of the apical brush border of Caco-2 and T84 monolayers. (B) Difference in average microvillus length between Caco-2 and T84 cells. Data represent the mean ± SEM of 10 microvilli/cell type. ***p < 0.001.*

Differentiated Caco-2 cells express enterocyte-specific genes, whereas differentiated T84 cells express colonocyte-specific genes

T84 and Caco-2 cells were grown in standard cell culture plates and left to differentiate for 7, 14 or 21 days. Functional differentiation was assessed through evaluation of the mRNA levels of a set of enterocyte- and colonocyte-specific differentiation markers (Table 1); brush border-associated enzymes[1-3,13,14] (alkaline phosphatase, intestinal (*ALPI*), sucrase-isomaltase (*SI*), lactase (*LCT*), alanyl aminopeptidase, membrane (*ANPEP*), dipeptidyl peptidase 4 (*DDP4*) and maltase-glucoamylase (*MGAM*)), a brush border-associated chloride channel[6,10,15] (cystic fibrosis transmembrane conductance regulator, *CFTR*), and the colonocyte markers membrane spanning 4-domains A12 (*MS4A12*)[16] and monocarboxylate transporter 1 (*MCT1* or *SLC16A1)*[17].

PCA analysis revealed that a distinct differentiation marker expression profile already exists between Caco-2 and T84 cells prior to the start of differentiation, which further persists throughout the different stages of maturation (Figure 2A). The significance of each differentiation marker, with respect to the phenotype of fully differentiated Caco-2 and T84 cells, was demonstrated by the OPLS model using the day 21 mRNA levels for all markers analysed. The resulting S-plot (Figure 2B) demonstrated that all brush border-associated enzymes, except *MGAM*, are highly specific to the differentiated Caco-2 phenotype while on the other hand, *MS4A12* is strongly associated with mature T84 cells. This observation was confirmed by the corresponding VIP-scores of ≥ 1 that identified *LCT*, *SI* and *DDP4* as the most important markers contributing to a differentiated Caco-2 phenotype, and *MS4A12* as the marker most characteristic of the differentiated T84 phenotype.

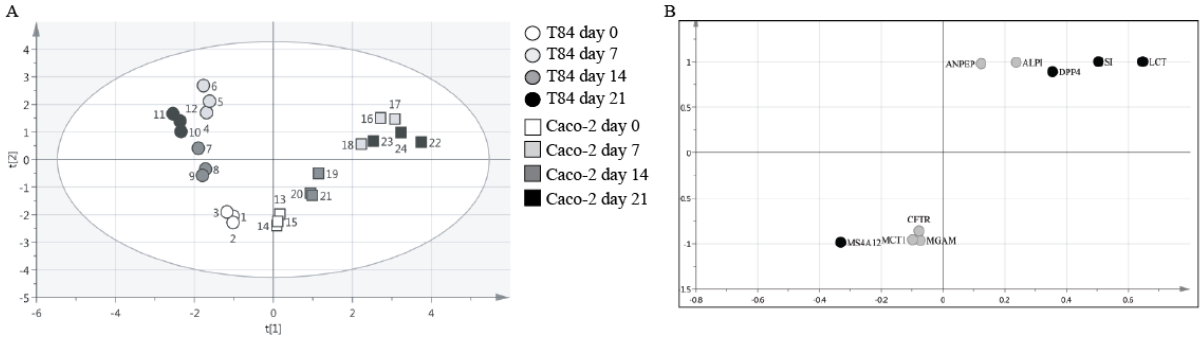

Figure 2. PCA and OPLS plots based on enterocyte and colonocyte-specific gene expression data in Caco-2 and T84 cells. *(A) PCA scatter plot visualising the grouping among Caco-2 and T84 cells before (day 0) and during (days 7 to 21) differentiation, based on the observed mRNA levels for all differentiation markers analysed. (B) The S-plot for the OPLS model revealing the predictiveness of all markers tested in relation to the differentiated (day 21) Caco-2 or T84 phenotype. Caco-2-specific markers are situated in the upper-right corner of the plot and T84-specific markers in the lower-left corner. Markers with a VIP-score of 1 or higher are highlighted in black, markers with a VIP-score below 1 are highlighted in grey.*

qRT-PCR analysis of these selected differentiation markers confirmed the Caco-2-specific, differentiation-induced expression of brush border-associated hydrolases *LCT*, *SI*, *DDP4*, *ALPI* and, to a lesser degree, *ANPEP* (Figure 3A-E). Their mRNA levels at day 0 are already considerably higher in Caco-2 than in T84 cells (except for *ANPEP*), and also the differentiation-induced increase in their expression is substantially greater in Caco-2 than in T84 monolayers. Exceptions are *MGAM* and *CFTR* (Figure 3F-G), whose pre-differentiation levels are similar in both cell lines and whose expression is significantly upregulated during Caco-2 as well as T84 differentiation. For the colonocyte-specific markers, we observed that *MS4A12* is expressed almost exclusively in differentiated T84 monolayers (Figure 3H) and that *MCT1* mRNA levels, although not induced upon differentiation, are also consistently higher in T84 than in Caco-2 cells (Figure 3I).

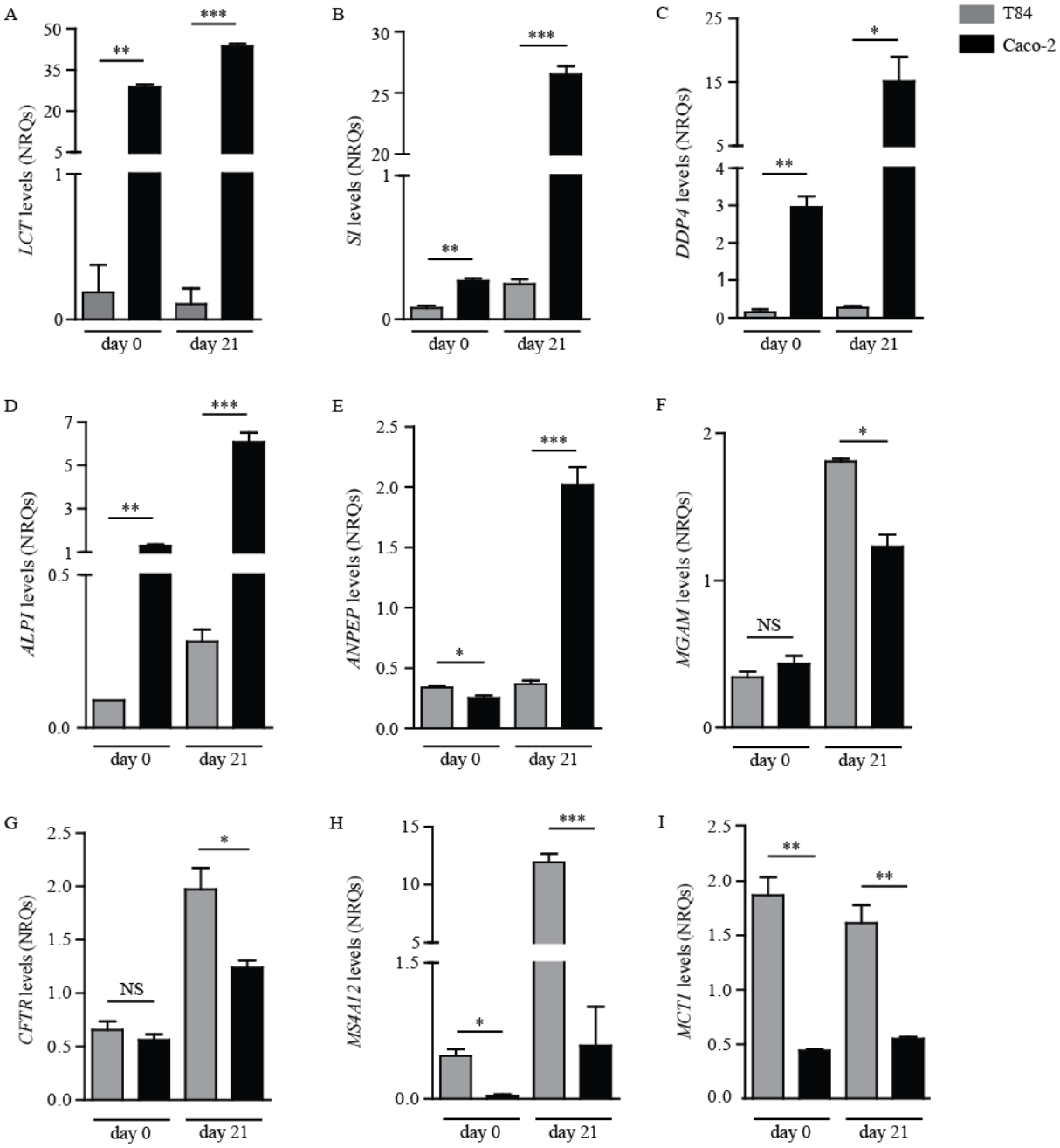

Figure 3. Expression of enterocyte- and colonocyte-specific genes in T84 and Caco-2 cells before and after differentiation. *(A-I) LCT, SI, DDP4, ALPI, ANPEP, MGAM, CFTR, MCT1 and MS4A12 mRNA levels in Caco-2 and T84 cells measured before (day 0) and after (day 21) differentiation. Data represent the mean ± SEM of 3 replicates/group. *p < 0.05, **p < 0.01, ***p < 0.001. NRQs, normalised relative quantities; NS, not significant.*

T84 monolayers are more responsive to butyrate throughout maturation

The observed difference in *MCT1* levels between Caco-2 and T84 cells is of interest since MCT1 is responsible for apical transport of butyrate, a short-chain fatty acid produced during colonic microbial fermentation of dietary fiber.[18] In colonocytes, butyrate functions mainly as an energy source but is also known to promote epithelial differentiation[19], which is reflected by an improved barrier tightness[20-22]. In order to evaluate whether the observed difference in *MCT1* expression between colonocyte-like T84 cells and enterocyte-like Caco-2 cells also translates to a functional

difference, we performed a dose-response experiment in which developing monolayers of T84 and Caco-2 cells were stimulated apically with a dose-range of butyrate. The evolution of monolayer integrity was monitored through intermediate TEER measurements. Only developing T84 monolayers showed a dose-response to butyrate as well as a higher temporal increment in TEER upon stimulation with butyrate compared to Caco-2 cells.

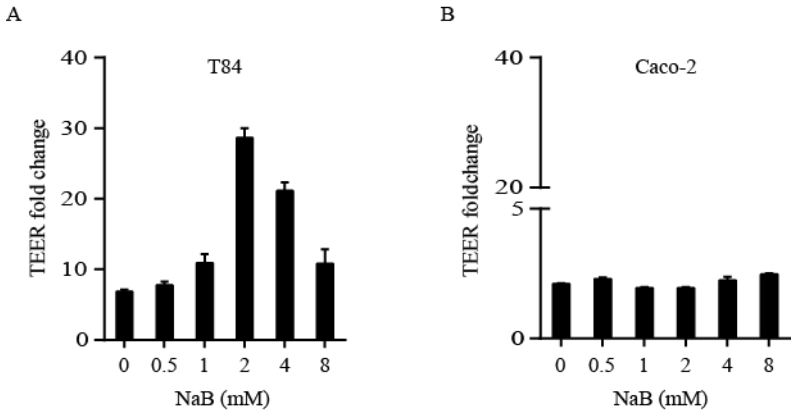

Figure 4. Dose-response effect of butyrate on developing T84 and Caco-2 monolayers. *(A) T84 and (B) Caco-2 developing monolayers were stimulated apically with a dose-range of butyrate (0.5 mM to 8 mM, 2-fold dilution), starting 2 days after seeding. TEER was measured at day 2 and day 9 post-seeding and is expressed as the fold change in TEER on day 9 versus day 2. Data represent the mean ± SEM of 3 replicates/group. NaB, sodium butyrate; TEER, transepithelial electrical resistance.*

Discussion

A differentiated enterocyte is characterised by a columnar cell morphology, interlocking junctional complexes, and the presence of apical microvilli.[3] In this context, an enterocyte is usually defined as a specialised absorptive epithelial cell responsible for nutrient intake, as opposed to other specialised cells like goblet, Paneth and neuroendocrine cells, which are also present in the epithelial lining of the gut. *In vivo*, enterocyte differentiation occurs during migration from the crypts to the surface epithelium in case of colonocytes, or to the villus tip in small intestinal enterocytes.[23] This differentiation is mimicked spontaneously *in vitro* by two cell lines; Caco-2 and T84.[4]

Although both are derived from a colonic tumor, they display different functional and morphological phenotypes upon differentiation. We observed that expression of an array of brush border hydrolases, unique to the small intestine, is upregulated only during Caco-2 differentiation, whereas the expression of typical colonic markers, like *MS4A12* and *MCT1*, are specific to differentiated T84 cells. This is in line with previous observations from Bolte *et al.* who showed that Caco-2 cells express higher amounts of the brush border hydrolases *ALPI*, *SI* and *DDP4* than T84 cells, suggesting a more pronounced small intestinal enterocytic differentiation compared to T84 cells.[4] Although Caco-2 monolayers clearly differentiate to small intestinal-like cells, they still express low but detectable levels of *MS4A12* as well as *MCT1*, albeit it to a lesser degree than T84 monolayers. This means that Caco-2 cells, even after complete differentiation, still retain residues of their colonic origin. The coexistence of enterocyte and colonocyte characteristics in this cell line has also been recognised previously.[24] On the other hand, the characterisation of the differentiated T84 phenotype is less ambiguous. The absence of small intestinal hydrolase expression, their shorter microvilli, their functional response to butyrate in correspondence with the higher *MCT1* levels in this cell line, and their previously documented electrical parameters and ion transport activities – including electrogenic chloride secretion – define mature T84 cells as most akin in structure and function to colonic crypt cells.[6,10]

The reason why differentiated Caco-2 cells display this small intestinal hydrolase expression and differentiated T84 cells do not, is probably due to the fact that Caco-2 cells were derived from the primary colonic tumor site, whereas T84 cells were isolated from a lung metastasis. Cells at the primary tumor site dedifferentiate through activation of the embryonic “epithelial-mesenchymal transition” program to allow cancer cell motility, dissemination and, ultimately, metastasis at a distant site.[25] However, metastases of most common human cancers often show a redifferentiation in the sense of a mesenchymal-epithelial (re-)transition, probably to allow growth and colonisation.[26] Metastatic cells, like T84, are therefore more differentiated than primary tumor cells, like Caco-2, where dedifferentiation is accompanied by re-expression of a fetal-like gene pattern,

which, in the case of the human fetal colon, includes transient expression of small bowel enzymes.[3,24]

Based on both morphological and biochemical characteristics, we conclude that Caco-2 and T84 cells show a distinct pattern of differentiation towards enterocytes and colonocytes, respectively. Researchers should be aware of these distinctive features when selecting the most appropriate intestinal epithelial cell line for a specific *in vitro* experiment.

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Conflicts of Interest

No conflicts of interest were declared.

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Tables

Table 1. Primers for qRT-PCR analysis.

Gene symbol	Forward (5'-3')	Reverse (3'-5')	E (%)
HMBS	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC	101
ALPI	AGTTATCCTGCTCCCCACCTCCGG	GAAGGTCCAACGGCAGGACACCT	92
LCT	CTGACCTCTTCGCCGACTATG	TGGTGGGGAAGCTCCTTGA	108
SI	TCCAGCTACTACTCGTGTGAC	CCCTCTGTTGGGAATTGTTCTG	101
MGAM	ACAGCCCGGTTGAAAAATCTG	CAGCAGCATTTCCACTGAAGG	108
DDP4	CCTTCTACTCTGATGAGTCACTGC	GTGCCACTAAGCAGTTCCATCTTC	100
ANPEP	GACGCCACCTCTACCATCAT	CCCACTTGATGTTGGCTTTC	93
CFTR	CCCACGCTTCAGGCACGAAGG	GCCTGAGGGGCCAGTGACAC	91
SLC16A1	CACTTAAAATGCCACCAGCA	AGAGAAGCCGATGGAAATGA	85
MS4A12	TTGGAGTTCTTCGTAGCTTGTG	CAGGACAGACATATTGGTTGTGG	98

E, efficiency.

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CHAPTER VI

DISCUSSION AND FUTURE PERSPECTIVES

Chronic inflammation in IBD is associated with severe microbial dysbiosis and both conditions affect, directly or indirectly, the integrity of the intestinal epithelial barrier. During this research, we investigated two different pathways involved in the maintenance and regulation of intestinal epithelial barrier function; luminal butyrate production and ROCK function (Figure 1). In this chapter, we will discuss the main research outcomes from chapters III - V and elaborate on the relevance of these results for the future treatment of IBD.

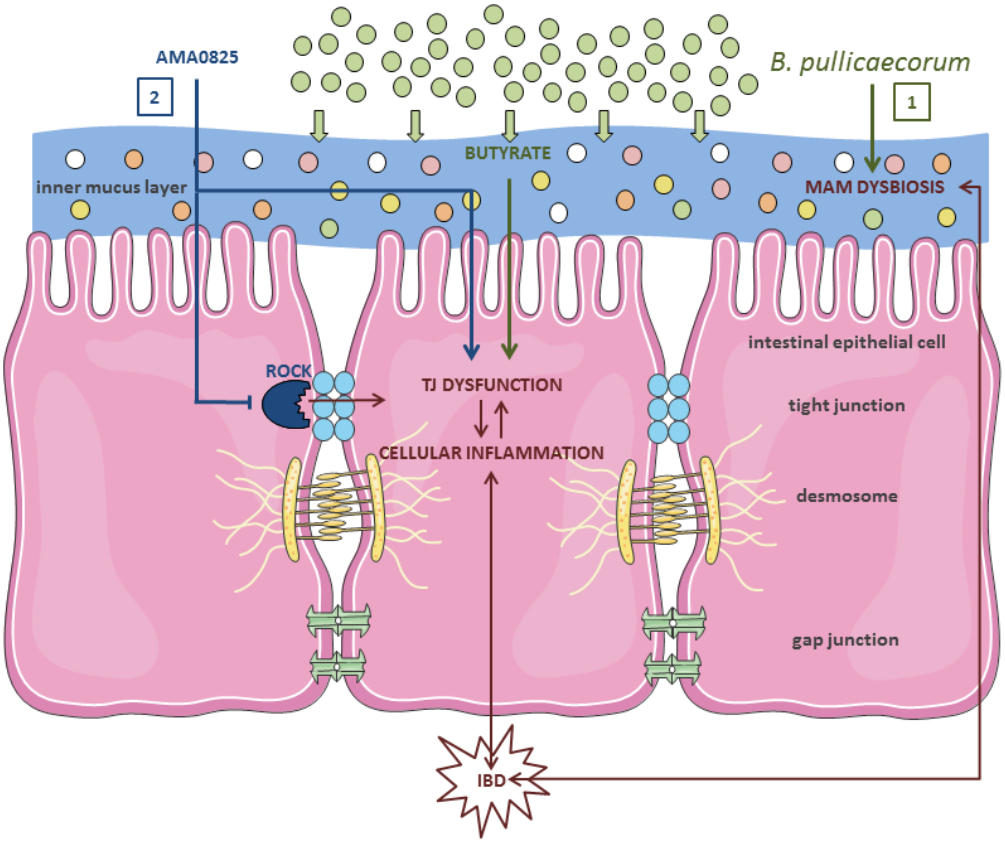

Figure 1. Targeting intestinal epithelial dysfunction in IBD. *Intestinal epithelial dysfunction in IBD is characterised by ROCK-mediated internalisation of TJ constituents, reduced expression of TJ proteins, and activation of pro-inflammatory signalling pathways like NF-κB. The aim of this thesis was to reduce acute cytokine-induced TJ dysfunction and epithelial inflammation by means of two barrier-protective and anti-inflammatory mechanisms; (1) increasing colonic butyrate levels through administration of butyrate-producing Butyricicoccus pullicaecorum 25-3^T bacteria, and (2) local ROCK inhibition using the AMA0825 compound. IBD; inflammatory bowel disease, TJ; tight junction, ROCK; Rho-associated kinase, MAM; mucosa-associated microbiota.*

PROTECTING THE GUT EPITHELIUM FROM INFLAMMATION-INDUCED DYSFUNCTION IN IBD

The resolution of impaired intestinal epithelial barrier integrity is crucial to mucosal healing. Therapeutic strategies aimed at the preservation of an intact epithelial barrier, which could not only prolong remission but also prevent the initial development and subsequent flare-ups of the disease - as opposed to the current immunosuppressive drugs that mainly act by reducing the severity of active episodes - make for a compelling prospect. During this research, we investigated two possible

ways to promote mucosal healing and to preserve intestinal epithelial barrier integrity: butyrate supplementation by means of the *Butyricicoccus pullicaecorum* 25-3^T butyrate-producer, and local ROCK inhibition using the AMA0825 compound.

1. Preserving barrier integrity in IBD through administration of *Butyricicoccus pullicaecorum* 25-3^T

As described in the introduction, various alterations in the composition of the gut microbiome have been reported in IBD patients. The most consistent observation is a reduced bacterial diversity characterised by an increase in bacteria from the *Proteobacteria* phylum and a decrease of *Bacteroidetes* and *Firmicutes*, two dominant phyla within the human gut microbiome.[1,2] The loss of *Firmicutes* is primarily due to the depletion of species belonging to the bacterial order *Clostridiales*, particularly members of clostridial clusters IV and XIVa.[1,3] The exact relevance of dysbiosis in IBD pathogenesis remains obscure; is it simply a consequence of the inflammatory process or is it an active primary driver contributing to the onset and/or perpetuation of inflammation?

Arguments for a causative role of dysbiosis predisposing to active disease is supported by observations such as the persistence of a dysbiotic signature during clinical remission in UC, characterised by a low diversity and a temporal instability, and a decrease in bacterial richness prior to relapse.[4,5] A subclinical dysbiosis has also been reported in unaffected relatives of UC and CD patients[6,7], suggesting that an initial abnormal gut microbial composition might, in combination with genetic factors and unknown environmental triggers, contribute to initiation and progression of IBD. Since the commensal microbiota is critical to induce a tolerogenic environment in the gut mucosa, it seems likely that an imbalance in the healthy microbiota may render the host more susceptible to exaggerated effector immune responses.[8]

The existence of a disease-associated dysbiosis has prompted research into the modulation of the gut microbial composition using probiotics as a treatment option in IBD. Probiotics are preparations of live microorganisms that, when administered in sufficient amounts, can confer a health benefit to the host through competitive inhibition of harmful microbes, production of barrier-protective metabolites like SCFAs, and interaction with lamina propria immune cells to stimulate an anti-inflammatory response.[1,9]

During this research, we focussed on the therapeutic potential of one bacterial strain in particular; *Butyricicoccus pullicaecorum* 25-3^T. This butyrate-producing clostridial cluster IV species was isolated from the caecal content of a broiler chicken[10,11] and is currently being considered as an interesting pre-clinical candidate for probiotic therapy in inflammatory intestinal disorders such as IBD, given the known barrier-protective and anti-inflammatory properties of butyrate.

In **chapter III**, we found that 16S rRNA expression for the genus *Butyricicoccus* was significantly decreased in the colonic mucosa of UC patients with active disease compared to healthy controls, which is in accordance with the previously described reduced abundance of the same genus in stool samples of IBD patients compared to healthy subjects.[12] The mucosa-adherent properties of this genus are in agreement with a study published by Nava and Stappenbeck, which also identified *Butyricicoccus* as an autochthonous microbe predominantly colonising the mucosa-associated surface of the colon.[13] The detection and quantification of *Butyricicoccus* in the mucosal compartment is a physiologically relevant observation. Despite the low abundance of this genus among the autochthonous bacteria associated with the human colonic mucosa (1-2%)[13], the restoration of depleted mucosa-associated bacterial groups that normally reside in close proximity to the apical surface of the colonic epithelium are more likely to have an impact on host metabolism and cellular homeostasis since their biogeographical localisation facilitates host access to their metabolites. Also, mucosa-associated microbial communities have developed a symbiotic relationship with the human mucosa, which has allowed them to colonise this intestinal niche, as opposed to the transient microbiota of the fecal stream. This natural association of mucosa-adherent microbes with the intestinal epithelium might promote a more successful re-colonisation upon oral supplementation as a probiotic. This knowledge also emphasises the relevance of investigating not only the fecal, but also the mucosal disease-associated microbial dysbiosis.

Interestingly, mucosal *Butyricicoccus* activity negatively correlated with the *CLDN1* mRNA levels observed in biopsies, whereas stimulation of Caco-2 monolayers with butyrate reduced CLDN1 protein expression and coincided with a restoration of the TNF/IFNγ-induced loss of barrier integrity. Most importantly, *B. pullicaecorum* 25-3^T conditioned medium decreased *CLDN1* levels in inflamed UC biopsies *ex vivo*. The beneficial effect of reducing CLDN1 expression seems counter-intuitive, however, CLDN1 levels are elevated during active IBD[14,15] as well as in experimental colitis[16], and its intestinal epithelial overexpression renders mice more susceptible to colitis and impairs their recovery.[17] This is explained by the fact that, besides its function as a traditional TJ protein involved in the maintenance of intestinal barrier integrity, CLDN1 also plays a key role in the regulation of intestinal epithelial homeostasis by activating Notch-signalling, which induces colonocyte proliferation and inhibits goblet cell differentiation. Notch activation is also a frequent an early event in Wnt-induced intestinal tumorigenesis. This Notch activation is maintained throughout neoplastic transformation and acts downstream from the hyperactivated Wnt/β-catenin signalling cascade, which in itself also induces a Tcf (T-cell factor)/Lef (lymphoid enhancer factor)-mediated imbalance between cell proliferation and differentiation that further triggers tumorigenesis.[18] Interestingly, CLDN1 has been identified as a target gene modulated by β-catenin/Tcf/Lef signalling and,

conversely, CLDN1 may also regulate β-catenin activity.[19,20] Indeed, increased CLDN1 expression has been reported in primary and metastatic colon carcinomas, with frequent mislocalisation of the protein from the membrane to the cytoplasm and nucleus.[14,20] Also, intestinal epithelial CLDN1 overexpression in the adenomatous polyposis coli $(APC)^{min}$ mouse model of intestinal tumorigenesis significantly increases colon tumor growth as well as frequency while decreasing survival. Tumors in these mice also demonstrate elevated levels of Wnt- and Notch signalling.[21] All this evidence combined supports the notion of CLDN1 as an oncogenic protein that aids in colonic neoplastic progression via upregulation of the Notch- and Wnt/β-catenin signalling pathways. Besides its upregulation in sporadic colon carcinomas, CLDN1 expression is also elevated in IBD-associated dysplasia.[14] The connection between inflammation and tumorigenesis is well-established and IBD is a known risk factor for the development of colon cancer.[22] The pro-inflammatory cytokine TNF is a key mediator of chronic inflammation in IBD but is also involved in epithelial-mesenchymal transition (EMT) and regulation of malignant progression of epithelial tumors by controlling cell migration, invasion and metastasis.[23] Bhat *et al*. recently demonstrated that CLDN1 promotes TNF-induced EMT and migration in HT-29 colorectal adenocarcinoma cells, highlighting the key role of deregulated CLDN1 expression in the progression and metastasis of inflammation-induced colon cancer.[24] The use of *B. pullicaecorum* 25-3^T to reduce CLDN1 levels during mucosal inflammation would therefore not only benefit the maintenance of epithelial integrity but also further downstream pathways responsible for colonic epithelial cell transformation.

Additional arguments for a barrier-protective and anti-inflammatory effect of *B. pullicaecorum* 25-3^T were provided by Eeckhaut *et al*. who reported that *B. pullicaecorum* 25-3^T conditioned medium prevented the loss of transepithelial resistance (as a measure of barrier integrity), and the increase in IL-8 secretion induced by TNF and IFNγ in Caco-2 monolayers.[12] Also, oral administration of live *B. pullicaecorum* 25-3^T bacteria resulted in a significant protective effect in TNBS-colitic rats, which was at least equivalent to the effect of *F. prausnitzii* based on macroscopic and histological criteria, and decreased intestinal MPO, TNF and IL-12 levels.[12]

The future of Butyricicoccus pullicaecorum 25-3^T in probiotic therapy for IBD

Butyrate-producing anaerobic bacteria, in particular those belonging to clostridial clusters IV and XIVa, were previously proposed as novel probiotic candidates for the treatment of IBD.[25] As discussed above, considerable evidence for a barrier-protective effect of the clostridial member *B. pullicaecorum* 25-3^T already exists.[12,26] Below, we will discuss how evidence from other work performed with *B. pullicaecorum* 25-3^T further underscores the suitability of this butyrate-producer as a probiotic candidate for application in IBD therapy.

First of all, *B. pullicaecorum* 25-3^T has been shown to be safe for human consumption. This is of particular importance given the fact that *B. pullicaecorum* 25-3^T is of non-human origin, which is atypical for a probiotic strain, and because it is the first strain of the *Butyricicoccus* genus that is being considered as a probiotic. Steppe *et al.* evaluated the safety of *B. pullicaecorum* 25-3^T for use in humans and found no adverse effects in acute and repeated oral dose toxicity tests performed in rats.[27] The complete *B. pullicaecorum* 25-3^T genome was sequenced and was found to contain no virulence factors, one gene related to harmful metabolites, and 52 sequences with high similarity to antimicrobial and toxic compound resistance genes, which did not correspond with a resistant phenotype. *B. pullicaecorum* 25-3^T did show actual resistance against aminoglycosides and trimethoprim.[27] These results show that *B. pullicaecorum* 25-3^T is a non-pathogenic strain, but carries antibiotic resistance genes, a concern that should be further investigated to determine to what extent these sequences are actually linked with high minimum inhibitory concentrations, acquired resistance, and whether these sequences are horizontally transferable to other gut bacteria. Furthermore, in a placebo-controlled cross-over randomised intervention study, daily oral intake of *B. pullicaecorum* 25-3^T capsules for four weeks was well-tolerated in 30 healthy subjects and did not induce adverse effects. The probiotic intervention induced changes in metabolic activity, although no effect was observed on fecal butyrate levels.[28]

The therapeutic application of *B. pullicaecorum* 25-3^T in patients will occur through oral ingestion. This means that *B. pullicaecorum* 25-3^T, an obligate anaerobe, must pass the environment of the upper gastro-intestinal (GI) tract, which might affect its viability. Geirnaert *et al.* demonstrated that *B. pullicaecorum* 25-3^T bacteria are intrinsically tolerant to the harsh conditions of the stomach and small intestine (pH, bile acids, oxygen).[29] During acidic stomach conditions, *B. pullicaecorum* 25-3^T loses cultivability but remains viable and active. Apparently, this is a sort of survival mechanism to cope with harsh environmental conditions because, once the acid stress factor disappears during small intestinal simulation, *B. pullicaecorum* 25-3^T resuscitates and becomes cultivable again. As for the higher oxygen levels during upper GI passage, *B. pullicaecorum* 25-3^T has been shown to reduce riboflavin to dihydroriboflavin, which in turn reduces O_2 to H_2O.[30] This is a bacterial mechanism that allows strict anaerobes to survive moderate oxygen concentrations in their environment through oxygen reduction via an extracellular redox mediator. Adding riboflavin or another redox mediator to the *B. pullicaecorum* 25-3^T probiotic formulation would be an option to increase the number of viable cells that reach the colon.

Administration of *B. pullicaecorum* 25-3^T to patients will occur through oral intake of capsules containing freeze-dried bacteria. Geirnaert *et al.* have shown that enteric coating of hydroxypropyl methylcellulose capsules with cellulose acetate phthalate better protected bacteria from acid gastric

conditions, resulting in the delivery of more cultivable bacteria and a higher butyrate production during colonic simulation, compared to uncoated capsules.[31] Another way of bypassing the stress-effect of the upper GI environment on the bacteria, as well as improving the shelf life of the probiotic product, would be the use of spores. Sporulation genes were detected in the genome of *B. pullicaecorum* 25-3^{T} [32], however, induction of sporulation has not yet been successful.

A way to increase the success rate of probiotic intervention, is the administration of *B. pullicaecorum* 25-3^{T} as part of a mixture of several probiotic species that would form a stable, co-dependent community ("collaborome"), as opposed to the administration of a single bacterial strain. A multispecies mix of butyrate-producers has been shown to have the highest success rate in terms of increased butyrate production and colonisation capacity in mucus- and lumen-associated CD microbiota.[33]

As a general comment, we would like to point out that *B. pullicaecorum* 25-3^{T} - and by extension other butyrate-producing commensals- do not solely secrete butyrate and that a butyrate-specific effect of treatment with *B. pullicaecorum* 25-3^{T} was not demonstrated explicitly for either the conditioned medium in active UC biopsies and TNF/IFNγ-stimulated Caco-2 monolayers, nor for the live bacteria in the TNBS-induced colitis. Other secreted bacterial metabolites may also be important. For example, a 15 kDa protein named microbial anti-inflammatory molecule (MAM) produced by *F. prausnitzii*, a butyrate-producing *Firmicute* deficient in CD[34], was identified as being able to block the NF-κB pathway in IECs and alleviate experimental colitis in mice.[35]

In addition, *B. pullicaecorum* 25-3^{T} could also exert its effects indirectly by inducing a shift in the gut microbial composition through stimulating the colonisation and proliferation of other beneficial bacteria that could have a protective effect during inflammatory epithelial dysfunction. Future studies will be needed to identify exactly how *B. pullicaecorum* 25-3^{T} exerts its beneficial effects by identifying its targets and mechanism of action.

The last step in exploring the potential application of B*. pullicaecorum* 25-3^{T} as a probiotic treatment for IBD, are clinical trials in which safety, dose range determination, indication, possible side effects, efficacy, and effectiveness are evaluated. If a probiotic effect is confirmed in humans, additional studies would be essential to unravel its mechanism of action and targets.

Finally, the future use of B*. pullicaecorum* 25-3^{T} in IBD patients would most likely be in combination with conventional immunosuppressive medication as an add-on therapy, and probably restricted to periods of remission to prevent relapse, since administration of large quantities of live bacteria to patients in whom intestinal permeability is already increased, is probably ill-advised. Probiotic therapy during quiescent disease in order to maintain remission is also recommended based on

evidence from the limited clinical trials on probiotics in IBD so far, which demonstrate that selected bacterial species can prevent relapse and, in a select few cases only, treat mild to moderately active UC.[36,37] Based on these trials, evidence for the use of probiotics in IBD is also more substantiated for UC than for CD.[9,38]

2. Preventing epithelial dysfunction in IBD through targeted local ROCK inhibition

Besides modulating TJ gene expression using butyrate-producing probiotics, another way of preserving epithelial barrier integrity, is by targeting the inflammation-induced ROCK-mediated TJ destruction and IEC apoptosis.

In **chapter V**, we investigated the effect of ROCK inhibition on intestinal epithelial dysfunction. Previous studies using Y-27632, a compound that is widely used as a selective ROCK inhibitor, reported an ameliorating effect on ethanol-induced increase in Caco-2 barrier permeability[39,40], IFNγ-induced TJ protein endocytosis[41], and endotoxemia-induced IEC apoptosis in rats[42]. In this work, we used the newly developed AMA0825 compound; a ROCK1/2 inhibitor optimised to have a localised action in the intestine due to its degradation upon contact with esterase activity in the blood, which minimises systemic exposure. ROCK-selectivity and potency of AMA0825 was demonstrated by its limited cross-reactivity with other kinases (PKC δ, ε and θ) and its extremely low inhibitory concentrations (< 0.05 nM). AMA0825 efficiently inhibited *in vitro* ROCK activity at nM concentrations, as measured by a reduction in MLC phosphorylation. *In vivo*, oral administration of AMA0825 resulted in colonic drug levels that were above the functionally active concentrations, and local ROCK activity was reduced in mice treated with AMA0825[43], proving that localised ROCK inhibition by AMA0825 is feasible. Surprisingly, AMA0825 did not ameliorate *in vitro* TNF/IFNγ-induced loss of barrier integrity, epithelial NF-κB activation or colonocyte apoptosis, and did not attenuate *in vivo* intestinal permeability or the ensuing mucosal inflammatory response. Apparently, specifically inhibiting ROCK activity is not an appropriate target for attenuating the acute stages of inflammation-induced intestinal epithelial dysfunction. We therefore have to conclude that the barrier-protective and anti-inflammatory effects observed for Y-27632 are off-target effects. Indeed, this was elaborated on in a paper by Davies *et al.*, who demonstrated that Y-27632 also inhibits other kinases.[44] For example, Y-27632 inhibits PKC-related protein kinase 2 with a potency similar to that for ROCK2, as well as three other protein kinases (mitogen-and stress-activated protein kinase 1, MAPK-activated protein kinase 1b and phosphorylase kinase) with IC_{50} values 10- to 50-fold higher than those for ROCK2. Several of these protein kinases are also known to be involved in TJ regulation and are triggered by diverse pathological conditions.[45] The key to unravelling the involvement of a particular kinase in the formation and disassembly of TJs, both under normal and pathological

conditions, therefore lies in the use of inhibitory compounds that are highly selective to the kinase under investigation.

A role for local ROCK inhibition in the treatment of fibrostenotic Crohn's disease

Although beyond the scope of the epithelial dysfunction investigated during this research, our lab recently demonstrated a role for ROCK inhibition using AMA0825 in the prevention and reversal of intestinal fibrosis in two different mouse models. On a cellular level, AMA0825 prevented the transforming growth factor β-induced transition of fibroblasts to myofibroblasts by reducing the activation of the myocardin-related transcription factor and p38 mitogen-activated protein kinase signalling pathways. In addition, AMA0825 potentiated autophagy in these cells.[43] Intestinal fibrosis is a common complication of CD and ultimately results in luminal (sub)obstruction with surgical resection of the afflicted bowel segment as the only treatment option. With up to one third of CD patients developing an end-stage fibrotic disease[46] and the lack of any available drug to halt intestinal fibrosis in IBD[47], there is a clear unmet need for anti-fibrotic therapies. Localised ROCK inhibition using AMA0825 thus represents a promising, novel anti-fibrotic strategy for the prevention and treatment of fibrosis in CD patients.

IMPROVING KNOWLEDGE ON T84 AND CACO-2 MONOLAYERS AS MODEL SYSTEMS OF HUMAN INTESTINAL EPITHELIUM

During the optimisation of the cell culture models of intestinal barrier dysfunction, as well as in the literature, the Caco-2 and T84 colonic epithelial cell lines were used for the *in vitro* modelling of human intestinal epithelium, since they both differentiate spontaneously at confluence into a monolayer of structurally and functionally mature absorptive epithelial cells. Since we found increasing evidence that differentiated Caco-2 cells closely resembled small intestinal enterocytes, while mature T84 cells were less described, we tried to further define the differences between these two intestinal epithelial cell lines. In **chapter V**, we demonstrated that differentiated T84 monolayers have - in contrast to differentiated Caco-2 monolayers - shorter microvilli, a lack of brush border-associated enzyme expression, exhibit high levels of colon-specific markers, and show a dose-responsive improvement of barrier function towards butyrate. This led us to conclude that differentiated Caco-2 and T84 cells each have distinct morphological, biochemical and functional characteristics, suggesting that T84 cells do not acquire the signature of mature small intestinal enterocytes like Caco-2 cells, but retain much of their original colonic characteristics throughout differentiation. These findings can be used by investigators to better select the appropriate intestinal epithelial cell line for specific *in vitro* research purposes.

In the context of the work performed during this thesis, either cell line would suit experiments examining basic cellular processes like kinase-mediated TJ dynamics or experiments with a simple

read-out, such as measuring TEER. This is because both of the examples above pertain to general characteristics of intestinal epithelium, which are met by both Caco-2 and T84 monolayers. Research in which the use of Caco-2 monolayers is more appropriate are, for example, studies investigating the interaction of apical IEC membranes with certain food components, such as proteins and simple carbohydrates, which would require the expression of brush border-associated digestive enzymes as well as transporters for the subsequent uptake of their degradation products. The colonocyte-like T84 cell line would be the model of choice for investigating epithelial responses to probiotic metabolites such as butyrate. Although butyrate-producing commensals are by no means unique to the colonic environment, cfr. the detection of *F. prausnitzii* on resected ileal CD mucosa[34], the bulk of available butyrate in humans is produced during dietary fiber fermentation in the large intestine, which harbours significant populations of butyrate-producing species.[48,49] These bacteria naturally and abundantly reside in the colonic lumen or mucosa and, as such, are in close proximity to colonocytes in particular. Therefore, interactions between the intestinal epithelium and butyrate-producing probiotic candidates would be best approximated *in vitro* by using the T84 cell line. Also epithelial responses to potential barrier-protective and anti-inflammatory metabolites from these probiotic candidates are best studied in T84 cells, since the epithelial expression of receptors and transporters for these metabolites is probably highest in T84 colonocytes.

TAKE-HOME MESSAGES

In this thesis, we have highlighted the intestinal permeability associated with inflammation in IBD, and the importance of restoring epithelial barrier integrity as a key treatment goal in clinical disease management. Restoring an intact epithelial barrier under inflammatory conditions was attempted by modulating two pathways; increasing *in situ* butyrate levels through probiotic treatment with *B. pullicaecorum* 25-3^T, and inhibition of epithelial ROCK activity using the AMA0825 local ROCK inhibitor. We also further characterised T84 and Caco-2 differentiated monolayers, two frequently used models of intestinal epithelium in this thesis.

Our research provided the following insights:

1. *Butyricicoccus pullicaecorum* 25-3^T is a probiotic candidate with barrier-protective potential
    - *Butyricicoccus* is a mucosa-associated microbe and its activity is reduced in UC patients with active disease
    - *Butyricicoccus* activity negatively correlates with *CLDN1* gene expression
    - reduction of CLDN1 expression is associated with restoration of barrier integrity
    - *B. pullicaecorum* 25-3^T conditioned medium reduces *CLDN1* gene expression

2. ROCK inhibition is not a target for attenuating inflammation-induced epithelial dysfunction
    - ROCK inhibition does not prevent the inflammation-induced intestinal epithelial barrier dysfunction or the associated inflammatory response
    - ROCK inhibition prevents and reverses intestinal fibrosis via effects that combine a reduction in fibroblast activation with an increase in the autophagic response in these cells

3. T84 monolayers are superior to Caco-2 as a models system for colonocytes
    - Caco-2 monolayers express enterocyte-specific genes, whereas T84 monolayers express colonocyte-specific genes including *MCT1*, the main transporter for butyrate
    - T84 barrier function improves in response to butyrate in a dose-dependent manner, which is not the case for Caco-2 cells

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Sarah

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2005 - 2010: Master of Sciences in Biochemistry and Biotechnology, Ghent University

Master thesis: Study of the dimerisation between dopamine receptors D2 and D4

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Publications in journals with peer review

Devriese S., Eeckhaut V., Geirnaert A., Van den Bossche L., Hindryckx P., Van de Wiele T., Van Immerseel F., Ducatelle R., De Vos M., Laukens D. Reduced mucosa-associated *Butyricicoccus* activity in patients with ulcerative colitis correlates with aberrant claudin-1 expression. Published in the *Journal of Crohn's and Colitis* (2017).

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Abstracts for oral presentations

Devriese S., Eeckhaut V., Van Immerseel F., Ducatelle R., De Vos M., Laukens D. Reduced *Butyricicoccus pullicaecorum* levels in mucosa of UC patients correlate with aberrant claudin-1 expression. Belgian Week of Gastroenterology, February 12-15, 2014, Terhulpen, BELGIUM.

Devriese S., Eeckhaut V., Van Immerseel F., Ducatelle R., De Vos M., Laukens D. Reduced *Butyricicoccus pullicaecorum* levels in mucosa of UC patients correlate with aberrant claudin-1 expression. Science Day, March 13th, 2014, Ghent, BELGIUM.

Holvoet T., **Devriese S.**, Castermans K., Boland S., Hindryckx P., Bourin A., De Vos M., Defert O., Laukens D. Soft ROCK inhibition prevents intestinal fibrosis in a murine colitis model. Belgian Week of Gastroenterology, February 25-28, 2015, Brussels, BELGIUM.

Holvoet T., **Devriese S.**, Castermans K., Boland S., Hindryckx P., Bourin A., De Vos M., Defert O., Laukens D. Soft ROCK inhibition prevents intestinal fibrosis in a murine colitis model. 10th Congress of ECCO, February 18-21, 2015, Barcelona, SPAIN.

Abstracts for poster presentations

Devriese S., Eeckhaut V., Van Immerseel F., Ducatelle R., De Vos M., Laukens D. Reduced *Butyricicoccus pullicaecorum* levels in mucosa of UC patients correlate with aberrant claudin-1 expression. 9th Congress of ECCO, February 20-22, 2014, Copenhagen, DENMARK.

Devriese S., Eeckhaut V., Van Immerseel F., Ducatelle R., De Vos M., Laukens D. Reduced *Butyricicoccus pullicaecorum* levels in mucosa of UC patients correlate with aberrant claudin-1 expression. Digestive Disease Week, May 3-6, 2014, Chicago, USA.

Holvoet T., **Devriese S.**, Castermans K., Boland S., Hindryckx P., Bourin A., De Vos M., Defert O., Laukens D. Soft ROCK inhibition prevents intestinal fibrosis in a murine colitis model. Digestive Disease Week, May 16-19, 2015, Washington D.C., USA.

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Holvoet T., **Devriese S.**, Castermans K., Boland S., Hindryckx P., Bourin A., De Vos M., Defert O., Laukens D. Soft ROCK inhibition prevents intestinal fibrosis in a murine colitis model. United European Gastroenterology Week, October 24-28, 2015, Barcelona, SPAIN.

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